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The Effect of Polydimethylsiloxane Substrate Modification on A549 Human Epithelial Lung Cancer Cell Morphology and Biomechanics

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THE EFFECT OF POLYDIMETHYLSILOXANE SUBSTRATE MODIFICATION
ON A549 HUMAN EPITHELIAL LUNG CANCER CELL
MORPHOLOGY AND BIOMECHANICS

by

Sherissa A. Ward

A dissertation submitted in partial fulfillment
of the requirements for the degree of

of

MASTER OF SCIENCE

in

Biological Engineering

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2015
ABSTRACT

The Effect of Polydimethylsiloxane Substrate Modification on A549 Human Epithelial Lung Cancer Cell Morphology and Biomechanics

by

Sherissa A. Ward, Master of Science
Utah State University, 2015

Major Professor: Dr. Anhong Zhou
Department: Biological Engineering

In this thesis the effect of mechanical stimuli on A549 lung cancer cells is studied. Modifications of polydimethylsiloxane (PDMS) surfaces are employed to alter the mechanical stimuli applied to the cells. Flat substrates are first studied and then micro-pillared substrates are designed, fabricated, and tested as a method to alter the mechanical properties of the PDMS surfaces.

Molds with micro-pillars are designed then fabricated from silicon using deep reactive ion etching. From these molds, a negative then a positive replicate is made using PDMS. The pillared PDMS substrates are fabricated in 10 geometries and used for experiments. A549 cells are cultured on these surfaces then analyzed using fluorescence microscopy and atomic force microscopy (AFM). Fluorescence microscopy images processed by ImageJ software measure the cell spreading area (\( \mu \text{m}^2 \)) while AFM quantifies the cell stiffness (kPa).
For flat substrates, the cell stiffness and spreading area increase with increasing substrate stiffness. Further, results on pillared substrates show a similar trend based on pillar geometry changes. For pillared substrates, the A549 cell stiffness and spreading area increase as the height decreases, yet there is decreased cell stiffness and spreading area as the diameter and spacing decreases. The experiments show that changes in surface properties and only mechanical stimuli alter cellular morphology and biomechanics.
PUBLIC ABSTRACT

The Effect of Polydimethylsiloxane Substrate Modification on A549 Human Epithelial Lung Cancer Cell Morphology and Biomechanics

The function of cells is dependent on factors including chemical, physical, and mechanical stimuli. A compilation of these factors make up the cellular niche. Though the impact of chemical stimuli has been a known factor for many years, the impact of mechanical and physical stimuli are areas of recent interest now being studied. Previous studies on mechanical and physical stimuli have shown a large impact on various aspects of cellular function.

The effects of cancer are widespread throughout the entire population. The majority of cancer research and treatment is regarding intracellular chemical pathways. Yet, many forms of cancer are often diagnosed based on differences in the rigidity of the cancer tissue and surrounding healthy tissues. Types of treatment regarding only chemical pathways neglect the usefulness of other stimuli in treating cancerous cells.

The substrates that cells are cultured on provide rigidity and topography signals. By modifying the substrate, it is possible to modify the types of signals received by cells on particular substrates. Polydimethylsiloxane is a biocompatible thermoset polymer that is easily molded and modified. These characteristics make it an optimal substrate for cell culture and fabrication of modified substrates.

The changes in cell morphology and biomechanics provide a way to quantify the effects of the mechanical stimuli on cancer cells. These changes are monitored using instruments to probe the elasticity of the cells as well as measure the size of the cells.

A better understanding of cellular function based on all types of stimuli can help determine the best possible way to treat cancers. By determining the effect of certain mechanical stimuli on these cells we will be closer to finding more effective cancer treatments.

Sherissa A. Ward
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Sherissa A. Ward
CONTENTS

| ABSTRACT .................................................................................................................................................. iii |
|------------------------------------------------------------------------------------------------------------|--------------------------------------------------|
| PUBLIC ABSTRACT .................................................................................................................................... v |
| ACKNOWLEDGMENTS .................................................................................................................................. vi |
| LIST OF TABLES ........................................................................................................................................ ix |
| LIST OF FIGURES ..................................................................................................................................... x |
| 1. INTRODUCTION .................................................................................................................................... 1 |
| 1.1 Background and motivation .......................................................................................................... 1 |
| 1.2 Working principle and applications of atomic force microscopy in cell studies ............................................ 4 |
| 1.3 Quantitative fluorescence image analysis .................................................................................... 5 |
| 1.4 Aims .................................................................................................................................................. 6 |
| 1.5 Outline of technical contents ...................................................................................................... 7 |
| 2. MATERIALS AND METHODS ............................................................................................................. 8 |
| 2.1 Fabrication of flat PDMS substrates .......................................................................................... 8 |
| 2.2 Fabrication and preparation of silicon master molds .................................................................. 8 |
| 2.3 Fabrication and preparation of pillared substrates .................................................................. 10 |
| 2.4 Fibronectin coating of all substrates ....................................................................................... 11 |
| 2.5 Cell culture on all substrates ................................................................................................. 12 |
| 2.6 Fluorescence microscopy imaging and cell area calculations ................................................. 13 |
| 2.7 Atomic force microscopy measurements and biomechanics calculations ...................................... 15 |
| 2.8 Live/Dead viability tests ............................................................................................................. 18 |
| 2.9 SEM imaging of micro-pillars and cells on micro-pillars ........................................................ 18 |
| 2.10 Statistical analysis .................................................................................................................... 20 |
| 3. RESULTS AND DISCUSSION FOR FLAT SUBSTRATES ................................................................ 21 |
3.1 Cell area measurements .................................................................21
3.2 Cell stiffness measurements ..........................................................26
3.3 Live/Dead viability of A549 cells ....................................................31

4. RESULTS AND DISCUSSION FOR MICRO-PILLARED SUBSTRATES ........32
   4.1 Fabrication of micro-pillared substrates .......................................32
   4.2 Cell area measurements ...............................................................35
   4.3 Cell stiffness measurements ........................................................42
   4.4 SEM imaging ..............................................................................49
   4.5 Live/Dead viability of A549 cells ...................................................52

5. CONCLUSIONS AND ENGINEERING SIGNIFICANCE ......................55

6. FUTURE WORK ..............................................................................56

REFERENCES .......................................................................................58

APPENDIX ..........................................................................................63
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. AFM parameters to obtain force-distance curves of cells cultured on flat and pillared substrates</td>
<td>17</td>
</tr>
<tr>
<td>2. Nominal and actual measurements of micro-pillar array dimensions based on SEM images</td>
<td>34</td>
</tr>
<tr>
<td>3. Average A549 cell spreading area measurements based on geometry differences of micro-pillar arrays</td>
<td>42</td>
</tr>
<tr>
<td>4. Average A549 cell stiffness measurements based on geometry differences of micro-pillar arrays</td>
<td>44</td>
</tr>
<tr>
<td>A.1. ANOVA table for cell area measurements on ibidi substrates.</td>
<td>66</td>
</tr>
<tr>
<td>A.2. Post-hoc REGWQ test for cell area measurements on ibidi substrates.</td>
<td>68</td>
</tr>
<tr>
<td>A.3. ANOVA table for cell area measurements on flat hard substrates</td>
<td>69</td>
</tr>
<tr>
<td>A.4. Post-hoc REGWQ test for cell area measurements on flat hard substrates</td>
<td>71</td>
</tr>
<tr>
<td>A.5. ANOVA table for cell stiffness measurements on ibidi substrates</td>
<td>72</td>
</tr>
<tr>
<td>A.6. Post-hoc REGWQ test for cell stiffness measurements on ibidi substrates</td>
<td>74</td>
</tr>
<tr>
<td>A.7. ANOVA table for cell stiffness measurements on flat hard substrates</td>
<td>75</td>
</tr>
<tr>
<td>A.8. Post-hoc REGWQ test for cell stiffness measurements on flat hard substrates</td>
<td>77</td>
</tr>
<tr>
<td>A.9. ANOVA table for cell area measurements on pillared substrates</td>
<td>78</td>
</tr>
<tr>
<td>A.10. Post-hoc REGWQ test for cell stiffness measurements on pillared substrates</td>
<td>81</td>
</tr>
<tr>
<td>A.11. ANOVA table for cell stiffness measurements on pillared substrates</td>
<td>82</td>
</tr>
<tr>
<td>A.12. Post-hoc REGWQ test for cell stiffness measurements on pillared substrates</td>
<td>85</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>1. AutoCad drawing of micro-pillar arrays in a hexagonal arrangement</td>
<td>9</td>
</tr>
<tr>
<td>2. 35 mm diameter polystyrene Petri dish containing fabricated PDMS micro-pillars</td>
<td>11</td>
</tr>
<tr>
<td>3. 10x magnification of fluorescently stained A549 cells on 1.5 kPa flat PDMS substrate (ibidi)</td>
<td>21</td>
</tr>
<tr>
<td>4. 40× magnification of fluorescently stained A549 cells on a glass substrate</td>
<td>22</td>
</tr>
<tr>
<td>5. Histograms of A549 cell spreading area data gathered on flat substrates</td>
<td>23</td>
</tr>
<tr>
<td>6. Average spreading areas of A549 cells on flat fibronectin coated ibidi PDMS substrates</td>
<td>24</td>
</tr>
<tr>
<td>7. Average spreading areas of A549 cells on flat hard substrates</td>
<td>25</td>
</tr>
<tr>
<td>8. A typical force-distance curve collected of an A549 cell cultured on a flat substrate via our AFM setup</td>
<td>26</td>
</tr>
<tr>
<td>9. Average cell stiffness’s of A549 cells on flat fibronectin coated substrates</td>
<td>27</td>
</tr>
<tr>
<td>10. Mean cell stiffness’s of A549 cells on flat hard substrates</td>
<td>28</td>
</tr>
<tr>
<td>11. Histograms of A549 cell stiffness data gathered on flat fibronectin coated substrates. Histograms represent 150 curves measured on cells from a single substrate</td>
<td>29</td>
</tr>
<tr>
<td>12. Qualitative and quantitative images of A549 cell topography and stiffness on PDMS substrates</td>
<td>30</td>
</tr>
<tr>
<td>13. Live/Dead fluorescence viability of A549 cells on 1.5, 15, and 28 kPa substrates, respectively (a-c). Green color indicates the living cells, red spots are dead cells</td>
<td>31</td>
</tr>
<tr>
<td>14. Images of silicon master mold after fabrication of micro-pillars using DRIE (a-b). Scale bars represent 1 mm</td>
<td>32</td>
</tr>
<tr>
<td>15. SEM images of fabricated PDMS micro-pillars</td>
<td>33</td>
</tr>
<tr>
<td>16. 40× magnification of PDMS micro-pillars prior to cell seeding</td>
<td>35</td>
</tr>
<tr>
<td>17. 10× magnification bright field image of A549 cells on micro-pillars of height 4, diameter 2, and spacing 2 μm</td>
<td>36</td>
</tr>
<tr>
<td>18. 10× magnification fluorescent image of A549 cells on micro-pillars of height 4, diameter 2, and spacing 2 μm</td>
<td>37</td>
</tr>
</tbody>
</table>
19. 40x magnification bright field image of A549 cells on micro-pillars of height 4, diameter 4, and spacing 2 µm .................................................................38

20. 40x magnification fluorescent image of A549 cells on micro-pillars of height 4, diameter 4, and spacing 2 µm .................................................................38

21. Histograms of A549 cell spreading area data gathered on PDMS pillared fibronectin coated substrates. Histograms represent at least 50 areas measured on cells from a single substrate .................................................................39

22. Average A549 cell spreading area measurements based on geometry differences of micro-pillar arrays .................................................................................41

23. A typical force-distance curve collected of an A549 cell cultured on a PDMS micro-pillared substrate via AFM .................................................................43

24. Average A549 cell stiffness measurements based on geometry differences of micro-pillar arrays .................................................................................44

25. Histograms of A549 cell area data gathered on PDMS pillared fibronectin coated substrates. Histograms represent at least 50 areas measured on cells from a single substrate .................................................................45

26. AFM topography (a) and 3D deflection (b) image of an A549 cell on a micro-pillared surface of dimensions H(2) D(2) S(4) .................................................................47

27. AFM topography (a) and 3D deflection (b) image of an A549 cell on a micro-pillared surface of dimensions H(4) D(2) S(4) .................................................................48

28. AFM topography (a) and 3D deflection (b) image of an A549 cell on a micro-pillared surface of dimensions H(4) D(4) S(2) .................................................................48

29. AFM topography (a) and 3D deflection (b) image of an A549 cell on a micro-pillared surface of dimensions H(2) D(4) S(2) .................................................................49

30. SEM image of an A549 cell on micro-pillar arrays of dimensions height 4, diameter 4, and spacing 2 µm at a 45° angle .................................................................50

31. Top view SEM image of an A549 cell on micro-pillar arrays of dimensions height 4, diameter 4, and spacing 2 µm .................................................................50

32. SEM image of an A549 cell on micro-pillar arrays of dimensions height 8, diameter 4, and spacing 2 µm at a 45° angle .................................................................51

33. Top view SEM image of an A549 cell on micro-pillar arrays of dimensions height 8, diameter 4, and spacing 2 µm .................................................................51
34. Live/Dead fluorescent viability studies of A549 cells on micro-pillared arrays of heights 2, 4, and 8 μm respectively (a-c). Scale bars represent 10 μm.................52

35. Correlation between A549 cell spreading area and stiffness on micro-pillared substrates. Points represent overall mean values on each micro-pillar geometry.......53

A.1. Steps to obtaining cell spreading area measurements in ImageJ .........................64

A.2. Steps to obtaining cell stiffness measurements..........................................................65

A.3. Fit diagnostics plots of cell area measurements on ibidi substrates.........................67

A.4. Box-Cox analysis of cell area measurements on ibidi substrates .........................68

A.5. Fit diagnostics plots of cell area measurements on flat hard substrates...............70

A.6. Box-Cox analysis of cell area measurements on flat hard substrates ...............71

A.7. Fit diagnostics plots of cell stiffness measurements on ibidi substrates .............73

A.8. Box-Cox analysis of cell stiffness measurements on ibidi substrates ..............74

A.9. Fit diagnostics plots of cell stiffness measurements on flat hard substrates...........76

A.10. Box-Cox analysis of cell stiffness measurements on flat hard substrates........77

A.11. Fit diagnostics plots of cell area measurements on pillared substrates..........79

A.12. Box-Cox analysis of cell area measurements on pillared substrates .............80

A.13. Fit diagnostics plots of cell stiffness measurements on pillared substrates.......83

A.14. Box-Cox analysis of cell stiffness measurements on pillared substrates.......84
CHAPTER 1
INTRODUCTION

1.1 Background and motivation

Cellular function is dependent on factors including chemical, physical, and mechanical stimuli. Combinations of these factors make up the niches for different cell types (Li et al., 2013). The impact of chemical stimuli on cell function has been a known factor for many years and chemical stimuli is the main method used for treating disease, but the impact of mechanical and physical stimuli factors are areas of recent interest. Some studies have shown that mechanical stimuli has the possibility of having as large of an impact as chemical stimuli (Janson and Putnam, 2014). For example, many diseases and cancers result in physical changes of cells such as decreased stiffness (Cross et al., 2007, 2008; Wu et al., 2010). By mechanotransduction, mechanical signals from diseased cells are then propagated through surrounding cells to tissues and finally to entire organs (Ingber, 2003). Further, mechanical stimuli from a stiffer extracellular matrix can cause a stiffer solid tumor (Suresh, 2007). This knowledge has lead researchers to look more closely at the importance of mechanical properties of cells and interactions between cells and cellular substrates.

The majority of cancer research is regarding intracellular chemical pathways. Still, many types of cancer are often diagnosed based on differences in the rigidity of the cancer tissue and surrounding healthy tissues (Huang and Ingber, 2005). By studying the effect of mechanical cues on cancerous and healthy cells we may be able to better understand cancer and possible treatments. We may also be able to determine what type of mechanical signals or pathways trigger malignancy (Ingber, 2008). Ingber even
speculates that cancer cells can be reverted back to healthy cells by recreating the physical, mechanical and chemical stimuli of the tumor microenvironment (Ingber, 2008).

Additionally, this area of research is important for future cellular research purposes. The elastic properties of typical culturing materials are far outside the range of native tissues. These elasticity changes then alter the morphology of the cells from their native environment. By softening the tissue culture materials used, there may be changes seen in experiments involving chemical stimuli. Changes in the substrate can allow for tuning of cellular conditions.

Modification of polydimethylsiloxane (PDMS) and other elastic substrates that serve as the extracellular matrix (ECM) for cells in vitro is a beginning to understanding mechanically triggered pathways. PDMS is often used because it is an optimal substrate for cell culture. PDMS is an inherently biocompatible thermoset polymer that is fabricated using a liquid base and curing agent. This fabrication method makes the polymer to be used easily with molds. Further, adjusting the ratio of base to curing agent, curing time, or curing temperature can alter the stiffness of PDMS. These properties make PDMS an ideal choice for these studies.

The simplest way to alter the stiffness of PDMS substrates is to alter the base to curing agent concentration during fabrication. With consistent curing temperatures and times, this method leads to specific and consistent substrate stiffness’s. Yet, because the change in concentrations of the base and curing agent can lead to changes in surface chemistry and the surface nano-structure, this method is not the ideal choice for these types of experiments. Curing time and temperature are more complicated and difficult to
replicate so a different method has been developed to alter the stiffness of substrates
(Yang et al., 2011). Arrays of micro-pillars on a surface alter the mechanical properties of
that surface based on their individual geometries. The fabrication of micro-pillar surfaces
provides a way to alter the mechanical properties of the surface without altering the
surface chemistry of the substrate.

Similar to the heart, lung tissues experience a cycling of expansion and
compression during respiration (Hecht et al., 2012). The act of breathing subjects all lung
tissues and cells to mechanical loading. Thus, A549 lung epithelial cells are a good
candidate to determine the effects of mechanical stimuli on cellular function. These cells
already experience and change due to large changes in mechanical stimuli. Further,
because these cells are cancerous these experiments allow the study of mechanical stimuli
on cancerous cells. The lung cells will be responsive to the mechanical stimuli and
provide insight into malignancy triggered by mechanical pathways.

Previous work has shown that cell morphology, motility, and traction force
change due to bulk and surface modification of PDMS substrates (Musah et al., 2012;
Rodriguez et al., 2014; Tzvetkova-Chevolleau et al., 2008). This research has been
conducted with respect to substrate stiffness and topography (Cheng et al., 2009; Nelson
et al., 2005; Tzvetkova-Chevolleau et al., 2008; Wang et al., 2012; Zhao and Zhang,
2006). The results have indicated that the extracellular matrix (ECM) or substrate
provides adhesive, structural, and mechanical signals to the cell (Musah et al., 2012).
Despite the morphology, motility, and traction force experiments, these studies do not
measure the change in cellular biomechanics, which is important to truly understand the
interaction between cells and tunable geometries of the substrates. Though morphology
changes are sometimes indicative of mechanical changes, these changes have not been quantified or modeled. We hypothesize that there would be a detectable change in cell biomechanics with a change in substrate mechanical stimuli.

1.2 Working principle and applications of atomic force microscopy in cell studies

Atomic force microscopy (AFM) is a micro- and nanotechnology that is used to qualify and quantify mechanical, electrical, and magnetic characteristics of samples (Kuznetsova et al., 2007). This instrument is capable of determining local and global mechanical properties using probes of various shapes and sizes (Liu et al., 2013). The mechanical properties are determined by indenting a flexible cantilever a short distance into the cells (Alcaraz et al., 2003). This method quantifies the cell stiffness on modified substrates (Kuznetsova et al., 2007). AFM determines the cell stiffness based on the deflection of the probe when it is pressed into a sample. The deflection is recorded based on the movement of a laser that is directed to the end of the probe tip. Using the parameters of the tip as well as the deflection of the probe, a contact mechanics equation is then used to determine the stiffness of the sample. The contact mechanics equation applied in this case is the Hertz model with a cone geometry. This model is the simplest case used for the indentation of a cone into an elastic sample. The model assumes the probe and sample are two perfectly homogeneous smooth bodies, the indenter has a parabolic shape, and the indentation is much smaller than the sample so the sample is considered extremely thick (Kuznetsova et al., 2007; Rico et al., 2005). The Hertz model for the cone tip geometry is given by:
\[ F = \frac{\pi E}{2(1-v^2)} a^2 \tan \theta \]

where, \( F \) is the total force applied to the sample, \( E \) is the Young’s modulus, \( v \) is Poisson’s ratio of the sample, and \( a \) is the contact radius. The radius of contact and indentation depth are related by the following:

\[ d = \frac{\pi}{2} a \tan \theta \]

where, \( d \) is the total indentation depth. Therefore, the following equation relates the indentation depth and the force:

\[ F = \frac{2E}{\pi(1-v^2)} \frac{d^2}{\tan \theta} \]

These equations allow the extraction of the Young’s modulus from curves obtained by the AFM (Poon et al., 2008) AFM has been successfully used to determine the stiffness of a variety of cells, such as bacterial cells (Wu and Zhou, 2009, 2010), cancer cells (Hecht et al., 2012; Rico et al., 2005), and stem cells (Nikolaev et al., 2014; Pillarisetti et al., 2011).

1.3 Quantitative fluorescence image analysis

Fluorescence microscopy is used to determine the cell area and the localization of cytoskeletal organelles in the cells. To obtain images via fluorescence microscopy, the cells are stained with fluorescent dyes. A dye that is selective for filamentous actin (F-actin) as well as a dye that is selective for DNA is used. These dyes bind to their specific
cellular organelles and fluoresce specific colors when excited by a laser. Hence, the dyes allow the visualization of cellular structure and further the analysis of cell morphology. The morphology of the cells is quantified using free NIH ImageJ software to calculate the area of the cells (Hecht et al., 2012). The software uses a pixel brightness analysis to select for the cell area defined by the F-actin.

The ultimate goal of these studies regarding cell-substrate interaction is to determine the types of substrates or scaffolds that cause changes in diseased tissues. To do this, substrates will be designed and fabricated to provide different mechanical stimuli to cancer cells. The effect will be quantified by measuring cell spreading areas as well as cell stiffness. These studies provide a clearer picture of the impact of mechanical stimuli on cancer cells.

1.4 Aims

The overall objective of this thesis project is to show that cells react by becoming more or less spread and stiff when receiving different mechanical stimuli from substrates. The first step is to verify past results using A549 lung cancer cells on commercially obtained flat PDMS substrates. Fluorescence imaging to calculate cell area will be used to verify previous results on similar substrates. Additionally, AFM will be used to measure the stiffness of the cells on the flat substrates. After past trends are verified on flat substrates with this particular cell line, pillared substrates will be used. This study will exclude the possibility of the change in surface properties from the flat substrates. The molds for pillared substrates are designed then fabricated. The pillar substrates are then fabricated from the molds and used as substrates. These samples are then analyzed
using fluorescence microscopy imaging and AFM. In these experiments, it is important to measure a significant number of cells for all analyses because there is large variation between cancer cells. Large measurement units as well as replication of the experiments will provide the statistical power needed to draw statistically significant conclusions from these studies.

1.5 Outline of technical contents

The remaining chapters of this thesis are focused on the design, fabrication, analysis, and future work regarding modified PDMS substrates and the subsequent cellular effects of those changes. The information presented first is the experimental methods used for all experiments with flat and micro-pillared PDMS substrates (chapter 2). Next the results and discussion for the experiments on all substrates will follow in subsequent chapters (chapters 3 and 4, respectively). Finally, conclusions will be stated and related to engineering significance of this work (chapter 5) and future research to be performed (chapter 6).
CHAPTER 2
MATERIALS AND METHODS

2.1 Fabrication of flat PDMS substrates

First, the glass substrate was cleaned to reduce the chances of contamination. 22 mm × 22 mm microscope cover glass (Fisher Scientific) were sonicated in 3:1 piranha solution (vol/vol 96% sulfuric acid/30% hydrogen peroxide) for 10 minutes. The cover glass was then rinsed with deionized (DI) water and allowed to dry. The glass substrates were then anchored to the base of a polystyrene 35mm diameter Petri dish (Thermo Scientific) using PDMS. The PDMS (Sylgard® 184 Silicone Elastomer Base and Curing Agent Dow Corning) was weighed at a 10:1 base to curing agent concentration and mixed vigorously. After degassing the PDMS in a vacuum for 30 minutes, 0.1 mL of the uncured PDMS was added to a Petri dish. The cover glass was then added to the top of the PDMS droplet. Manual light pressure was then applied to ensure a secure hold and an even coating of PDMS between the cover glass and polystyrene. The PDMS was then cured on a hot plate set at 65°C for 30 minutes.

Cell culture grade Petri dishes with a flat PDMS bottom were obtained from ibidi (Verona, WI, USA) in three different stiffness, which were 1.5, 15, and 28 kPa. Non-treated cell culture grade polystyrene Petri dishes were obtained from Corning.

2.2 Fabrication and preparation of silicon master molds

Three silicon master molds with pillars of various dimensions were fabricated by the Nanofab Laboratory at the University of Utah using the Bosch method of deep reactive ion etching (DRIE). The molds were fabricated based on masks designed in
AutoCAD 19.0.55.0 as can be seen in Fig. 1. Each master mold had four replicates of a 2 mm x 2 mm grid containing 4 sections. Each grid had the following pillar diameter and spacing measurements respectively D(4 µm), S(2 µm); D(4 µm), S(4 µm); D(2 µm), S(2 µm); and D(2 µm), S(4 µm). Further, each mold was fabricated to a specific height that was 2 µm, 4 µm, and 8 µm. The small pillar diameter and spacing measurements were decided based on the small size of A549 cells. Further, the literature was considered when deciding pillar heights and aspect ratios (Cheng et al., 2013; Yang et al., 2007, 2011).

![AutoCad drawing of micro-pillar arrays in a hexagonal arrangement. Geometries as follows; upper left D(4) S(2), upper right D(4) S(4), lower left D(2) S(2), lower right D(2) S(4). Each square section is 1 mm x 1 mm in total size. Scale bar represents 50 um.](image)

**Fig. 1.** AutoCad drawing of micro-pillar arrays in a hexagonal arrangement. Geometries as follows; upper left D(4) S(2), upper right D(4) S(4), lower left D(2) S(2), lower right D(2) S(4). Each square section is 1 mm x 1 mm in total size. Scale bar represents 50 um.

After receiving the molds, they were cleaned with piranha solution for 15 minutes, rinsed with DI water, dried on a hotplate for 1 hour, and then allowed to cool to
room temperature. The molds were then cleaned with oxygen plasma for 45 seconds at 75 W and approximately 1000-500 mTorr. To allow easy release from the molds, the master molds were treated with silane by vapor deposition. To do this, 3-5 drops of (Tridecafluoro-1,1,2,2-Tetrahydrooctyl)-1-Trichlorosilane on a glass slide were placed in a desiccator with the silicon master molds and subjected to vacuum for 4 hours.

2.3 Fabrication and preparation of pillared substrates

After treating the master molds with silane, negative PDMS molds were made. To fabricate the PDMS negative molds, base and curing agent of PDMS at a 10:1 ratio was mixed vigorously then degassed under vacuum for 30 minutes. Glass rings were placed around the molds, PDMS was poured onto the molds, and the molds were set aside for 10 min. The molds were moved to a hot plate at 110°C for 15 minutes to cure the PDMS. The molds were then removed from the hot plate and after cooling to room temperature, the PDMS negative mold was carefully peeled away from the master molds.

To fabricate the pillared substrates, the PDMS negative molds were treated with oxygen plasma for 30-45 seconds at 20 W and approximately 1000-500 mTorr. Once oxidized the molds were then silanized by vapor deposition for 30 minutes. PDMS at a 10:1 concentration was mixed and degassed again as stated previously and 0.1 ml were added to the PDMS negative molds. After resting for 5 minutes, the negative molds and uncured PDMS were inverted onto cleaned glass coverslips. The PDMS pillars were then cured on a hot plate at 110°C for 1 hour. The substrates were then allowed to cool to room temperature and carefully peeled away from the PDMS negative molds. Once fabricated, the pillared substrates were secured to a Petri dish as can be seen in Fig. 2. To
secure the pillared substrates, 0.1 mL of PDMS was added to a Petri dish. The pillared array was then placed on top of the drop of PDMS and manual light pressure was then applied to ensure an even coating and a secure hold. The PDMS was then cured at 65°C for 30 minutes.

Fig. 2. 35 mm diameter polystyrene Petri dish containing fabricated PDMS micro-pillars.

2.4 Fibronectin coating of all substrates

All substrates were treated with oxygen plasma for 30-45 seconds at 20 W and approximately 1000-500 mTorr. This oxidizes the substrates making them more hydrophilic. The substrates were then coated with human plasma fibronectin protein (Sigma Aldrich). A 30 µg/mL solution of the protein was prepared and mixed thoroughly. 800 µL of the fibronectin solution was added to each of the substrates and they were allowed to incubate at 37°C for 1 hour. The fibronectin solution was then removed from the Petri dishes and they were each rinsed twice with 1 mL of phosphate buffer solution (PBS).
2.5 Cell culture on all substrates

Human lung carcinoma A549 cells (ATCC, USA) were cultured in F-12k medium supplemented with 10% fetal bovine serum and 5% Pen Strep (all three from Gibco). An incubator at 37°C with a humid atmosphere and 5% CO₂ was used for culture conditions. Cells were passaged at 80-90% confluency and used for experiments.

To passage cells in a T25 flask, the spent media was removed and the cells were rinsed once with 2 mL Dulbecco’s Phosphate-Buffered Saline (DPBS) (Thermo Scientific). After rinsed, 1 mL of 0.5% Trypsin-EDTA (10X) (Gibco) was added to the cells and incubated at 37°C for 5 minutes. Then 5 mL of stop media was added to the flask and mixed with the suspended cells. The cell solution was transferred to a 15 mL centrifuge tube and 20 µL was removed for cell viability using Trypan Blue assay. The cell solution was then centrifuged at 1500 RPM for 6 minutes in the CL 2 Centrifuge from Thermo Scientific. The supernatant was removed and the cells were resuspended in 3 mL of cell culture media. The reserved portion of the cell solution was then mixed with 20 µL of Trypan blue solution (0.4%) (Sigma). Then 10 µL of the Trypan blue cell solution was added to each side of a hemocytometer and the cells were counted. With the Trypan blue, it was observed that the cells were at least 90% viable before using in experiments.

After coating the substrates with fibronectin, 3 mL of the cell culture media were added to the Petri dishes. The Petri dishes were seeded with approximately 1.5-4 × 10^4 cells depending on the topography of the substrate and intended analysis. Flat substrates analyzed by fluorescence microscopy were seeded with 3 × 10^4 cells while flat substrates analyzed by AFM had 1.5 × 10^4 cells. All pillared substrates were seeded
with 4 x 10^4 cells. These cell seeding numbers allowed sufficient space between the cells to take individual cell measurements. The seeded Petri dishes were then moved to a 37°C incubator to allow the cells to attach to the substrates overnight.

2.6 Fluorescence microscopy imaging and cell area calculations

Fluorescence microscopy was used to determine the spreading area of cells on each of the various substrates. The filamentous actin (F-actin) and nuclear architecture were stained with selective dyes Alexa Fluor® 488 phalloidin and DAPI (both from Invitrogen) using the following steps. The cells were washed twice with 1 mL of PBS (Thermo Scientific). Then 500 µL of 4% paraformaldehyde (Electron Microscopy Sciences) was then added to the Petri dishes and allowed to incubate for 10 minutes at room temperature (RT). The cells were washed twice with 1 mL of PBS. Then 500 µL of 0.1% Triton X-100 (Sigma Aldrich) was added to the Petri dishes and incubated at RT for 5 minutes. The cells were washed twice with 1 mL of PBS. Then 500 µL of 1% BSA (Invitrogen) were added to the Petri dishes and allowed to incubate for 30 minutes at RT. The cells were washed twice with 1 mL of PBS. The Alexa Fluor® 488 phalloidin methanolic stock solution mixed with PBS at a concentration of 1:40. Then 200 µL of the mixture was added to the Petri dishes for 20 minutes. The cells were washed twice with 1 mL of PBS. Then the DAPI stock solution mixed with PBS at a concentration of 1:3000 was added to the Petri dishes at a volume of 200 µL for 3 minutes. The cells were then washed twice for the final time with 1 mL of PBS. Samples were filled with 3 mL of PBS prior to imaging.
Cell samples were imaged at a 10x magnification on an Olympus 1X71 inverted fluorescence microscope equipped with an Olympus DP30BW CCD camera. The microscope was equipped with filters corresponding to the fluorescent dyes. A blue filter for DAPI with an excitation wavelength of 345 nm and emission wavelength of 455 nm as well as a green filter for Alexa Fluor® 488 phalloidin with an excitation wavelength of 495 nm and emission wavelength of 519 nm. Olympus DP-BSW Controller and Manager Software were used to take the images.

From the images obtained, the average cell areas were determined on each substrate using NIH ImageJ 1.48 software (http://imagej.nih.gov/). This free software uses brightness thresholds from black and white images to select the fluorescently labeled cells. To achieve the most accurate measurements possible, individual cells with only one nucleus were chosen for measurements. Images of the cytoskeleton were opened in ImageJ and were converted to 8-bit type images. A scale bar on the photo was then used to calibrate the software to the magnification of the image by measuring the length of the line of known distance on the image. Once calibrated, the threshold function on the software was modified to allow selection as closely as possible to the edge of each cell. The wand tool in ImageJ was then used to individually select cells and the individual cell areas were measured. Figures presenting the process of determining spreading area are given in Fig. A.1. A minimum of 100 cells was measured on each flat substrate and 50 on the pillared substrates because of the smaller substrate size. The average of the individual measurements was then taken for each Petri dish. Three replicates were performed for better statistical analysis.
2.7 Atomic force microscopy measurements and biomechanics calculations

AFM experiments were carried out using an Agilent 5500 PicoPlus AFM microscope (Agilent Technologies, Inc.). PicoView 1.18 software with the ElasticityPyramid plug-in (Christian’s Super Plug-In Package; http://www.picocafe.com) was used to operate and process the data obtained from the AFM.

To set up the AFM, an AFM probe was mounted on the AFM scanner and the laser was aligned to the tip. DNP-10 AFM probes (Bruker) that are V-shaped silicon nitride probes with a pyramidal tip were used. The AFM probes have a nominal spring constant \( k = 0.06 \text{ N/m} \) and front angle (FA) = \( 15 \pm 2.5^\circ \). To set up the AFM software with the proper parameters, the spring constant, deflection sensitivity, and software plug-in parameters were determined. The spring constant of the tip was determined using the thermal K feature in the Pico View software, which is derived from the thermal noise method. The thermal noise method relies on a simple energy balance to determine the stiffness of the cantilever. The cantilever is modeled as a simple harmonic oscillator using the equipartition theorem. This model uses the Boltzmann constant and absolute temperature to determine the spring constant of the probe cantilever based on thermal fluctuations of the cantilever (Lubbe et al., 2013). Thermal K acquired thermal fluctuations in air at room temperature for ten seconds before calculating the cantilever spring constant. Spring constant values between 0.03-0.13 N/m were determined using the thermal noise method. The deflection sensitivity on silicon was then determined. To do this, force-distance curves were acquired on a piece of silicon in double DI water at room temperature. Prior to acquiring force-distance curves, the laser was realigned to the tip of the probe because when placed in water the laser is deflected slightly off the tip. A
z-scanner range of 0.375 µm, rate of 1.875 µm/s, and duration of 0.4 seconds was used to obtain these curves. Figures showing the process of selecting and obtaining a curve from a cell are presented in Fig. A.2. Curves collected had 2000 data points. Each curve contains a branch for the approach of the probe as well as a branch for the retraction of the probe. With the force-distance curves, the deflection sensitivity was determined based on the slope of the retracting line. The final step to setting up the AFM was to adjust the plug-in parameters in the software based on the geometry of the AFM probe. This software plug-in requires the front angle of the pyramidal tip as well as the Poisson ratio of the sample. The nominal value was used for the front angle of the AFM probe and 0.5 was used because of the softness of the samples (Rico et al., 2005). The topography/deflection images, stiffness mapping, and point elasticity measurements were determined using low force contact mode.

Samples were removed from the incubator and allowed to cool for approximately 10-20 minutes prior to taking measurements in the cell culture media. This reduced the deflection of the AFM probe based on temperature changes. Once cooled the samples were loaded onto the sample holder and the tip was approached to the surface of the sample. The average stiffness of A549 cells on various substrates was determined using point elasticity measurements. To obtain these point elasticity measurements, force-distance curves were collected in 10 distinct locations over the peri-nuclei region of each cell. This region is the most homogeneous and yields the most consistent stiffness measurements (Thomas et al., 2013). The parameters used to obtain the curves can be seen in Table 1.
Table 1
AFM parameters to obtain force-distance curves of cells cultured on flat and pillared substrates.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Flat Substrates</th>
<th>Pillared Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-scanner range</td>
<td>4.5 µm</td>
<td>3.5 µm</td>
</tr>
<tr>
<td>Rate</td>
<td>1.5 µm/s</td>
<td>1.167 µm/s</td>
</tr>
<tr>
<td>Duration</td>
<td>6 s</td>
<td>6 s</td>
</tr>
<tr>
<td>Force Limit</td>
<td>2.0 V (6 nN)</td>
<td>2.0 V (6 nN)</td>
</tr>
</tbody>
</table>

Once obtained, each curve was processed using the ElasticityPyramid plug-in available with the PicoView software. This plug-in uses the Sneddon variation of the Hertz model as described in section 1.1. This is a simplification generally used for pyramidal tips. For flat and pillared substrates, 10 point elasticity measurements were obtained from each individual cell and 15 cells were measured on each substrate yielding 150 measurements per substrate. Experiments on each substrate were replicated three times. The average of cells on each substrate were averaged and compared.

AFM topography/deflection images were gathered using a scan rate of 0.5 lines/second for flat substrates and 0.25 lines/second for pillared substrates. The slower scanning rate on pillared substrates caused less pillar deflection and thus better images. Topography/deflection images could not be obtained for cells on 8 µm height pillars because the maximum range of the piezoelectric scanner is 6.66 µm. For 3D images, WSxM 5.0 Develop 5.0 software was used. To convert into a 3D image, the deflection images were imported into the software and a 3D representation was made.
To gather elasticity mapping images, the AFM probe was positioned over the center of a cell on an ibidi substrate. The square image frame was set between $50 \times 50$-70 $\times 70$ µm based on cell size and gathered using a $16 \times 16$ pixel grid.

2.8 Live/Dead viability tests

For viability tests on PDMS substrates, LIVE/DEAD® Viability/Cytotoxicity Kit (Life Technologies) was used according to the manufacturer’s protocol. Samples used in AFM tests were used for viability tests after AFM tests and a subsequent overnight incubation. The first step was to mix the dye solution. In one 15 mL centrifuge tube, 5 mL of DPBS, 10 µL of component B, then 2.5 µL of component A and the tube was vortexed. The tube was then covered in foil. Cell samples were removed from the incubator, spent media was removed, and washed twice with 1 mL of DPBS. The dye solution was added to the cell samples and allowed to incubate at RT for 30 minutes. The samples were then imaged immediately on the Olympus 1X71 inverted fluorescence microscope.

2.9 SEM imaging of micro-pillars and cells on micro-pillars

To obtain scanning electron microscope (SEM) images of pillared samples with cells under high vacuum, it was necessary to do a fixation and chemical drying. The fixation and chemical drying is to preserve the shape of the cells. Cells were seeded on pillared substrates as described previously and allowed to attach overnight. A 2% buffered glutaraldehyde and HEPES solution was prepared with 5.96 grams HEPES in 10 mL 50% glutaraldehyde. The solution was then brought to 250 mL with DI water. Also
prepared was a 0.1M HEPES buffer solution with 5.96 grams HEPES in 250 mL of DI water. After cells were attached they were removed from the incubator and washed twice with 1 mL of the HEPES solution. Then 1 mL of the 2% glutaraldehyde solution was then added to the cell samples and allowed to incubate 24 hours at RT. The spent glutaraldehyde solution was then removed and the samples were washed with 0.1 M HEPES three times for 5 minutes each with gentle agitation. A series of alcohol dehydration steps were then performed with 50, 70, 95, and 100% ethanol solutions (Pharmco-Aaper, Fisher Scientific). 1 mL 50% ethanol was added to the samples 2 times for 10 minutes each with agitation. These steps were repeated for 70 and 95% ethanol. 1 mL 100% ethanol was added to the samples three times for 15 minutes each with agitation. The samples were then chemically dried using solutions of ethanol and Hexamethyldisilazane (HMDS) (Electron Microscopy Sciences). For the first step 2 parts 100% ethanol mixed with 1 part HMDS was added to the samples for 15 minutes then removed. For the second step 1 part 100% ethanol mixed with 1 part HMDS was added to the samples for 15 minutes then removed. For the third step 1 part 100% ethanol mixed with 2 part HMDS was added to the samples for 15 minutes then removed. For the final step HMDS alone was added to the samples for 15 minutes and allowed to evaporate in a fume hood overnight.

The fixed and chemically dried samples were gold coated to decrease the charging of the substrate while imaging. To coat with gold, the samples were placed in the coating chamber (Kurt J. Lesker) and the chamber was vacuumed to 500 mTorr. The chamber was then purged with argon gas and activated to start the coating process. The thickness
of the gold was monitored using a Cressington thickness monitor. The samples were coated with 10 nm of gold and removed from the chamber.

Samples were then imaged at high vacuum in a Quanta FEG 650 SEM (FEI). The samples were loaded into the sample holder and subjected to vacuum at approximately 1x10⁻⁵ Torr. xT Microscope Control v6.2.7 was used to operate the SEM. The samples were imaged using a beam spot size of 3, voltage of 15 kV, 1536 × 1024 resolution, and 4× line integration.

From the SEM images of the pillars, the exact dimensions of the pillars after fabrication and replication were determined using NIH ImageJ software. The calibration was done as described previously for cell area measurements. Once calibrated, simple line measurements were used to determine the sizes of the pillars.

2.10 Statistical analysis

Data were analyzed from statistical significance using SAS® OnDemand for Academics software 9.4. To perform the analysis two-way analysis of variance tests were used with REGWQ post hoc tests for significance (p<0.05 or p<0.10) between conditions. Full code and results from statistical tests can be seen in Appendix sections A.3-A.8. The preliminary results in these sections show that the underlying assumptions of independent and identically distributed data are met.
CHAPTER 3

RESULTS AND DISCUSSION FOR FLAT SUBSTRATES

3.1 Cell Area Measurements

An example of the 10x magnification fluorescent images of A549 cells on an ibidi 1.5 kPa stiffness flat substrate can be seen in Fig. 3. In general most differences in the spreading of A549 cells on the different flat substrates cannot be seen. Most cells were well spread on every surface, so a simple visual check was not possible. In the image it can be seen that there is large size variation between the cancer cells used in this study. The variation in cell size is the reason so many cells were measured on each substrate. Other researchers used up to 30 cells on substrates for cell area measurements (Mizutani et al., 2004).

Fig. 3. 10x magnification of fluorescently stained A549 cells on 1.5 kPa flat PDMS substrate (ibidi).
A 40× magnification fluorescent image of A549 cells on cover glass can be seen in Fig. 4. This image shows well spread cells on flat substrates. Cells that are not well attached show a large increase in concentration of F-actin on the edges of the cells. This can be differentiated from well-attached cells by the brightness of the fluorescent images. The larger concentration of F-actin leads to a much brighter fluorescence at the edge of the cells. Individual cells that were well attached were used in cell area measurements. In the image it is also apparent that there are large amounts of F-actin throughout the entire cell. There are not large concentrations of F-actin in certain areas of well-attached cells.

Fig. 4. 40× magnification of fluorescently stained A549 cells on a glass substrate.
After cell area measurements were made, histograms were plotted of the cell area measurements on each Petri dish in order to determine whether the median or the mean of the measurements would be a more appropriate representation of the data (Fig. 5). From the histograms it is easy to see that all histograms have a right skew that is characteristic of biological measurements. It was decided to use the mean of the cell area measurements because the size of the right tail varies in each histogram. This way the peak as well as the tail is represented by the mean.

Fig. 5. Histograms of A549 cell spreading area data gathered on flat substrates.
The average spreading area for cells on the flat 1.5, 15, and 28 kPa PDMS substrates were 1157.471, 1173.601, and 1332.707 µm², respectively. Fig. 6 shows the graphical representation of the cell area measurements. As shown in the graph the spreading of the cells increased as the stiffness of the substrate increased. This is consistent with results on similar substrates from previous groups (Wang et al., 2012).

![Graph showing cell spreading areas](image)

**Fig. 6.** Average spreading areas of A549 cells on flat fibronectin coated ibidi PDMS substrates. Error bars represent one standard deviation of n=3 on each substrate of stiffness 1.5, 15, and 28 kPa. The * shows statistical significance of p < 0.10.

The results from the polystyrene and glass substrates can be seen in Fig. 7. The average spreading areas for the fibronectin coated polystyrene, non-coated polystyrene, fibronectin coated cover glass, and non-coated cover glass were 2325.193, 1785.382, 1289.581, and 1181.420 µm², respectively. Polystyrene has a stiffness of 1 GPa while cover glass has a stiffness of approximately 70 GPa (Kolahi et al., 2012). These results are different from the previous set of results because they show a decrease in cell
spreading with an increase of substrate stiffness. Though these substrates are used for cell culture without any coating, they were coated to aid in cell adhesion. This would allow more easy comparison of the hard substrates to the soft PDMS substrates. There is a notable increase in the cell spreading area when the polystyrene was coated with fibronectin, but only a slight increase in the cell spreading area when the cover glass was coated with fibronectin. The change in trend on these hard substrates could be due to two possibilities. First, there could be an inconsistent coating of fibronectin on the surfaces, which would lead to inconsistent attachment of cells to the substrates. Second, these results could also be due to an interaction between the cells and the specific surface properties of the substrates. The cells could be interacting differently with the nanotopography or the surface chemistry on the hard substrates. The second reason shows why it is necessary to alter the stiffness of the substrates while keeping the surface properties consistent.

Fig. 7. Average spreading areas of A549 cells on flat hard substrates. Error bars represent one standard deviation of n=2 on each substrate.
3.2 Cell stiffness measurements

An example of the force-distance curves obtained from A549 cells on flat substrates can be seen in Fig. 8. The graphic shows the shape of the curves obtained on the AFM. It is apparent from the Fig. 8 that the curves obtained are normal and consistent with other curves obtained on soft biological samples. Curves similar to this were obtained for all substrates. After these curves were individually processed with the PicoView ElasticityPyramid plug-in, the Young’s modulus of each curve was extracted.

![Fig. 8. A typical force-distance curve collected of an A549 cell cultured on a flat substrate via our AFM setup. The red curve shows the approaching branch while the blue curves shows the retraction branch. AFM probe begins approaching sample (a), probe comes in contact with sample (b), probe indents into sample (c), probe begins retracting from sample (d), probe releases and fully retracts from sample.](image)
The average A549 cell stiffness measurements for flat 1.5, 15, and 28 kPa PDMs substrates were 9.856, 13.947, and 15.721 kPa, respectively. These results are from 10 curves of each cell on 15 cells per substrate with 3 replicates, as shown in Fig. 9. These results show that the cells become stiffer as the substrate stiffness increases. These results are consistent with those from a previous study (Liu et al., 2013) and they show that, as cells become more spread, cell Young’s modulus increases.

![Graph](image)

**Fig. 9.** Average cell stiffness’s of A549 cells on flat fibronectin coated substrates. Error bars represent one standard deviation of n=3 on each substrate. The * represents statistical significance of p < 0.05.

The results on the other flat substrates are also consistent with the data achieved from the fluorescence experiments. These results are opposite of those expected on hard substrates as stated previously. These results can be seen in Fig. 10. The average cell stiffness values for the fibronectin coated polystyrene, non-coated polystyrene, fibronectin coated cover glass, and non-coated cover glass were 17.434, 14.470, 13.825, and 12.809 kPa, respectively. It is also suggested that the coating of fibronectin increases
cell adhesion, which in turn increases the cell stiffness. There is also a similar effect of the fibronectin on the cell spreading area (Fig. 7).

![Bar graph showing mean cell stiffness of A549 cells on flat hard substrates.](image)

**Fig. 10.** Mean cell stiffness’s of A549 cells on flat hard substrates. Error bars represent one standard deviation of n=3 on each substrate.

Histograms of the cell stiffness data achieved via AFM are presented in Fig. 11. These histograms also show a right skew of the data, which is a similar shape with those from the fluorescent study.

Topography images of single cells obtained from the AFM on each of the flat PDMS substrates can be seen in Fig. 12 (a-c). Also in Fig. 12 (d-f) are 3D deflection images obtained from the AFM. Topography and 3D deflection images show the shape of the living cells on the PDMS substrates. Elasticity mapping images in Fig. 12 (g-h) are very descriptive as to the elastic properties throughout the cells. From these images we see that the central region above the nucleus is the most homogenous region of the cell and the cell stiffness increases as you move away from the nucleus. 3D surface plots of
the elasticity can be seen in Fig. 12 (j-l). The 3D surface plots give a height visualization of the change in the cell elasticity magnitude between the cells and the soft PDMS substrates.

Fig. 11. Histograms of A549 cell stiffness data gathered on flat fibronectin coated substrates. Histograms represent 150 curves measured on cells from a single substrate.
Fig. 12. Qualitative and quantitative images of A549 cell topography and stiffness on PDMS substrates. (a-c) topography of A549 cells on 1.5, 15, and 28 kPa flat PDMS substrates respectively; (d-f) 3D deflection images of the same A549 cells; (g-i) elasticity mapping images of the same A549 cells; (j-l) 3D surface plots of elasticity values of the same A549 cells. AFM data was collected in cell medium. Scale bars represent 10 um.
3.3 Live/Dead viability of A549 cells

To confirm the viability of A549 cells on flat PDMS substrates a live/dead fluorescence viability kit was used on a set of samples after AFM experiments, as shown in Fig. 13. From this test we see that the cells have a high viability on all of the substrates. Those few red spots in the images may have even been due to the indentation of the AFM probe.

Fig. 13. Live/Dead fluorescence viability of A549 cells on 1.5, 15, and 28 kPa substrates, respectively (a-c). Green color indicates the living cells, red spots are dead cells. Scale bars represent 10 um.
CHAPTER 4

RESULTS AND DISCUSSION FOR MICRO-PILLARED SUBSTRATES

4.1 Fabrication of micro-pillared substrates

The fabricated silicon master molds are given in images (a) and (b) in Fig. 14. Each mold contained 4 replicates of the same 2x2 grid with dimensions as stated previously. From this image it can be seen that pillared molds reflect color. This reflection of color provides a quick and easy visual check for the integrity of the molds. Dirty or broken molds reflect little or no color.

![Fig. 14. Images of silicon master mold after fabrication of micro-pillars using DRIE (a-b). Scale bars represent 1 mm.](image)

SEM images of the PDMS micro-pillar fabrication can be seen in Fig. 15. In these images it can be seen that the dimensions of the 4 µm diameter pillars are more consistent between molds than the 2 µm diameter pillars. It can also be seen that even within a single mold the dimensions of the molds differ. This is because the density of the array determines the etching speed. Those arrays with more densely packed features etch...
Fig. 15. SEM images of fabricated PDMS micro-pillars. The images show a top view of each of the micro-pillar dimensions (a-j) and a 45-degree angle view of each of the micro-pillar dimensions (k-t). H: height; D: diameter; S: spacing, in µm. Au particles were deposited in SEM imaging to enhance contrast. Scale bars represent 10 µm.
slower so the dimensions of the pillars may not be exactly as designed. These changes are not an issue in this project because specific sizes were not necessary; rather a range of pillar dimensions was the goal. The actual diameter and spacing of the pillars were measured from the SEM images and are given in Table 2. The height of the micro-pillars was not measured because the walled design of the micro-pillared arrays made it too difficult to obtain quality SEM images of the cross section.

Table 2
Nominal and actual measurements of micro-pillar array dimensions based on SEM images.

<table>
<thead>
<tr>
<th>Nominal</th>
<th>Actual</th>
<th>Percent Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height</td>
<td>Diameter</td>
<td>Spacing</td>
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<tr>
<td>2</td>
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</tr>
<tr>
<td>2</td>
<td>4</td>
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</tr>
<tr>
<td>8</td>
<td>4</td>
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</tr>
</tbody>
</table>

A bright field image of the center of a micro-pillar array is presented in Fig. 16. In this image there are no defects from the fabrication process and the micro-pillar array is suitable for cell culture. Micro-pillar arrays with defects or missing pillars were avoided in experiments.
4.2 Cell area measurements

Once seeded with cells the fluorescent experiments were used to determine the cell area. Examples of the bright field and fluorescent images of A549 cells used for cell spreading area measurements on micro-pillared arrays can be seen in Figs. 17 and 18, respectively.

A 40× magnification images of A549 cells on micro-pillars can be seen in Figs. 19 and 20. The bright field image in Fig. 19 shows simply the cells on the micro-pillars, but the fluorescent image in Fig. 20 shows the distribution of the F-actin in the cells. The cells are well attached to the pillared surfaces based on the roughly even distribution of the F-actin in the cells. Yet in images there is a slight increase in the concentration of F-actin are right around the edges of each of the micro-pillars. This increase in F-actin around the pillars shows that the cells are most strongly attached at those points.
Fig. 17. 10× magnification bright field image of A549 cells on micro-pillars of height 4, diameter 2, and spacing 2 µm.
Fig. 18. 10× magnification fluorescent image of A549 cells on micro-pillars of height 4, diameter 2, and spacing 2 μm.
**Fig. 19.** 40× magnification bright field image of A549 cells on micro-pillars of height 4, diameter 4, and spacing 2 μm.

**Fig. 20.** 40x magnification fluorescent image of A549 cells on micro-pillars of height 4, diameter 4, and spacing 2 μm.
Again, histograms were plotted of the cell area measurements per Petri dish in order to determine whether the data shared the same characteristic curves as those on flat substrates (Fig. 21). The histograms show a similar shape to the previous measurements on flat surfaces despite the decrease in measured cells.

Fig. 21. Histograms of A549 cell spreading area data gathered on PDMS pillared fibronectin coated substrates. Histograms represent at least 50 areas measured on cells from a single substrate.
Fig. 21 Continued.
The summary of the fluorescence area measurements on pillared substrates is shown in Fig. 22. In these results there is a decreasing trend with each change in geometry feature. The A549 cells increase in cell area as the height decreases, yet there is decreased cell spreading as the diameter and spacing decreases. The specific average area measurements of the A549 cells on the substrates are given in Table 3.

**Fig. 22.** Average A549 cell spreading area measurements based on geometry differences of micro-pillar arrays. Error bars represent one standard deviation of n=3 on each pillar dimension. The * shows statistical significance of p < 0.05.
Table 3
Average A549 cell spreading area measurements based on geometry differences of micro-pillar arrays.

<table>
<thead>
<tr>
<th>Pillar Dimensions</th>
<th>Average A549 Cell Spreading Area (µm²)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height DiameterSpacing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 4 4</td>
<td>1218.804</td>
<td>91.865</td>
</tr>
<tr>
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<td>1143.828</td>
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</tr>
<tr>
<td>2 4 4</td>
<td>868.982</td>
<td>125.658</td>
</tr>
<tr>
<td>2 4 4</td>
<td>900.658</td>
<td>85.020</td>
</tr>
<tr>
<td>2 4 4</td>
<td>826.084</td>
<td>141.488</td>
</tr>
<tr>
<td>8 4 4</td>
<td>689.531</td>
<td>81.594</td>
</tr>
<tr>
<td>8 4 2</td>
<td>607.125</td>
<td>69.843</td>
</tr>
</tbody>
</table>

4.3 Cell stiffness measurements

A typical force-distance curve attained on pillared substrates is presented in Fig. 23. The shape of this curve is very similar to those curves collected on flat substrates. Yet, in this image there is a larger decrease in force towards the end of the retract curve. This is most likely due to an increase in the adhesion at the particular position of the cell being measured. Small variations like this do not affect the cell stiffness measurements because the cell stiffness is calculated based on the approach curve.

The A549 cell stiffness experiments on pillared substrates are summarized in Fig. 24. These results show a similar trend to those achieved in the fluorescence experiments (Fig. 22). The results show there is an overall decreasing trend with each change in geometry feature. The A549 cells increase in cell stiffness as the height decreases, but there is decreased cell stiffness as the diameter and spacing decreases. The specific
average cell stiffness measurements of the A549 cells on the micro-pillared substrates are given in Table 4.

**Fig. 23.** A typical force-distance curve collected of an A549 cell cultured on a PDMS micro-pillared substrate via AFM. The red curve shows the approaching branch while the blue curves shows the retraction branch. AFM probe begins approaching sample (a), probe comes in contact with sample (b), probe indents into sample (c), probe begins retracting from sample (d), probe releases and fully retracts from sample.
Fig. 24. Average A549 cell stiffness measurements based on geometry differences of micro-pillar arrays. Error bars represent one standard deviation of n=3 on each pillar dimension. The * shows statistical significance of p < 0.10.

Table 4
Average A549 cell stiffness measurements based on geometry differences of micro-pillar arrays.

<table>
<thead>
<tr>
<th>Pillar Dimensions</th>
<th>Average A549 Cell Stiffness (kPa)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height Diameter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 4 4</td>
<td>16.534</td>
<td>1.363</td>
</tr>
<tr>
<td>2 2</td>
<td>15.703</td>
<td>2.588</td>
</tr>
<tr>
<td>4 4 4</td>
<td>15.601</td>
<td>2.194</td>
</tr>
<tr>
<td>2 4 2</td>
<td>15.742</td>
<td>1.310</td>
</tr>
<tr>
<td>4 4 2</td>
<td>14.538</td>
<td>1.167</td>
</tr>
<tr>
<td>2 4 2</td>
<td>13.781</td>
<td>2.288</td>
</tr>
<tr>
<td>8 4 4</td>
<td>13.382</td>
<td>1.452</td>
</tr>
<tr>
<td>8 2</td>
<td>13.139</td>
<td>1.912</td>
</tr>
<tr>
<td>8 4 2</td>
<td>12.412</td>
<td>5.084</td>
</tr>
<tr>
<td>8 2</td>
<td>8.646</td>
<td>4.749</td>
</tr>
</tbody>
</table>
The histograms plotted below (Fig. 25) show a consistent trend with those measured on flat substrates. These histograms represent 10 force-distance curves measured on 15 cells per substrate for a total of 150 curves. The histograms show a right skew, which is standard for measurements on biological samples. These histograms are slightly less normal than those for the cell areas because of the variation inherent in AFM measurements.

Fig. 25. Histograms of A549 cell stiffness data gathered on PDMS pillared fibronectin coated substrates. Histograms represent at least 50 areas measured on cells from a single substrate.
Fig. 25 Continued.
Topography and 3D deflection images collected using AFM can be seen in Figs. 26-29. The images show the topography of living cells on the micro-pillar arrays. There is no visible deflection of the pillars in these images and they show an even spreading of the cells. The images also show that the cell membrane attaches and shapes to the geometry of the pillars at the edges of the cells.

Fig. 26. AFM topography (a) and 3D deflection (b) image of an A549 cell on a micro-pillared surface of dimensions H(2) D(2) S(4). Image is of a non-fixed living cell taken in cell media.
Fig. 27. AFM topography (a) and 3D deflection (b) image of an A549 cell on a micro-pillared surface of dimensions H(4) D(2) S(4). Image is of a non-fixed living cell taken in cell media.

Fig. 28. AFM topography (a) and 3D deflection (b) image of an A549 cell on a micro-pillared surface of dimensions H(4) D(4) S(2). Image is of a non-fixed living cell taken in cell media.
**Fig. 29.** AFM topography (a) and 3D deflection (b) image of an A549 cell on a micro-pillared surface of dimensions H(2) D(4) S(2). Image is of a non-fixed living cell taken in cell media.

4.4 **SEM imaging**

SEM images that were taken of the cells on micro-pillar arrays, as presented in Figs. 30-33. These images show the morphology of the cells after they were fixed and chemically dried. The images show that there is some deflection inward of the pillars from the cells. This contraction is most likely due to the chemical drying process because there is very little deflection of the pillars when compared to the bright field images such as Fig. 19. The images also show that the cells grow part of the way down the pillars, but the majority of the cell is on top of the pillars. This can be prevented on micro-pillars of a single dimension by using a fibronectin stamp only on the tops of the micro-pillars instead of coating the entire surface (Yang et al., 2011).
Fig. 30. SEM image of an A549 cell on micro-pillar arrays of dimensions height 4, diameter 4, and spacing 2 µm at a 45° angle. Samples were fixed, chemically dried, and gold coated prior to imaging under high vacuum.

Fig. 31. Top view SEM image of an A549 cell on micro-pillar arrays of dimensions height 4, diameter 4, and spacing 2 µm. Samples were fixed, chemically dried, and gold coated prior to imaging under high vacuum.
Fig. 32. SEM image of an A549 cell on micro-pillar arrays of dimensions height 8, diameter 4, and spacing 2 µm at a 45° angle. Samples were fixed, chemically dried, and gold coated prior to imaging under high vacuum.

Fig. 33. Top view SEM image of an A549 cell on micro-pillar arrays of dimensions height 8, diameter 4, and spacing 2 µm. Samples were fixed, chemically dried, and gold coated prior to imaging under high vacuum.
4.5 Live/Dead viability test

To confirm that cells remained viable after being cultured on the micro-pillar arrays, a live/dead fluorescent viability kit was used on a set of samples after AFM experiments. The results from this test can be seen in Fig. 34. From this test we see that the cells continued to have a high viability on all of the substrates. The number of red dead cells in these images is similar to that of the previous viability experiments on the flat PDMS substrates.

Fig. 34. Live/Dead fluorescent viability studies of A549 cells on micro-pillared arrays of heights 2, 4, and 8 µm, respectively (a-c). Scale bars represent 10 µm.
Fig. 35. Correlation between A549 cell spreading area and stiffness on micro-pillared substrates. Points represent overall mean values on each micro-pillar geometry.

From the results on flat substrates we see that the cells followed the trend expected. There was an increase in cell spreading area and stiffness with an increase in substrate stiffness. Yet with the pillared substrates there were similar but slightly different results. The geometries of the individual micro-pillared arrays contribute to the overall surface stiffness. Taller micro-pillars are less stiff than shorter micro-pillars, larger diameter micro-pillars are stiffer than smaller diameter micro-pillars, and more closely packed pillars lead to a stiffer substrate than less packed pillars. According to the results there was a decrease in cell spreading and stiffness on taller micro-pillars and there was a decrease in cell spreading and stiffness on smaller diameter micro-pillars, but there was in decrease in cell spreading and stiffness on more closely packed micro-
pillars. These results are different than the expected outcome, and there is not a mechanical explanation for this change. Still, the results on the micro-pillared surfaces yielded a positive correlation between the cell spreading area and stiffness (Fig. 35).

Cancerous cell behavior is difficult to predict with the knowledge that we have of the signaling pathways triggered by mechanical stimuli. Therefore, anomalies such as the change discussed above shed light on possible methods of treatments. There is also room for understanding of cancerous cell behavior with further experimentation into the reason for the unexpected outcomes.

These results assist in solidifying theories that cancerous cells can be altered due to mechanical stimuli and may even be able to be reverted back to healthy cells. This thesis research applied AFM to quantitatively analyze cancer cells on pillared substrates for the first time. The fluorescence imaging verified those results obtained from the AFM. Further, proper statistical significance using analysis of variance was established. These could greatly impact cancer research.
CHAPTER 5
CONCLUSION AND ENGINEERING SIGNIFICANCE

In this work, fluorescence microscopy and atomic force microscopy were used to detect the change in cell spreading area and stiffness of A549 human epithelial lung cancer cells cultured on substrates of varying stiffness. These cells were cultured on flat and micro-pillared substrates to provide various degrees of mechanical stimuli to the cells. Cells on the flat substrates increased in cell spreading area and stiffness as the substrate increased in stiffness. The cells also increased in cell spreading area and stiffness as the geometries of the pillars changed. Statistical significance was established for these trends. Though the changes were not entirely as expected, these abnormalities can be used in studies of cancer cells and treatments.

This project provided experience using scientific knowledge and developing engineering skills. The knowledge of biological responses due to stimuli was studied. Designing of pillared substrates was used to study various mechanical stimuli. These surfaces were fabricated to achieve surfaces that provide mechanical stimuli to cells. The effect of those different mechanical stimuli was then detected using fluorescence microscopy and AFM. These studies provided greater knowledge as to the impact of mechanical stimuli as well as the pathways through which the stimuli act.
CHAPTER 6
FUTURE WORK

The results from this thesis research could continue with many future experiments. Some of those experiments could include using an inhibitor to determine the main cytoskeletal component related to cell spreading area and/or cell biomechanics. This experiment would include choosing an inhibitor that inhibits a certain portion of the cytoskeleton. It would most likely be an inhibitor of F-actin. This inhibitor would be used with cells cultured on micro-pillared substrates. AFM and fluorescence microscopy could then be used to determine the effect of the inhibitor on the cells. The results from this experiment would contribute to determining the pathways activated by mechanical stimuli.

Another experiment could be to treat the cells with an anti-cancer drug. The results from the anti-cancer drug could be compared to the results from the inhibitor to determine if there is any sort of change in effectiveness of the anti-cancer drug on the different surfaces.

Quantitative PCR would be a very useful analysis in determining the gene expression changes on flat and pillared substrates. It could also be useful in determining the specific cellular effect of geometry changes on cellular gene expression. This would provide further knowledge to the use of mechanical stimuli pathways.

The final use of this information presented in this thesis could include the use of micro-pillar arrays in microfluidic chambers with various cell types. This type of application would significantly expand our understanding of the synergetic effects of
surface modifications with fluidic conditions within a microfluidic device. This knowledge could be applied to microfluidics involving concentration gradients as well as the delivery of specific compounds to different cells within a single microfluidic device.
REFERENCES


APPENDIX
**A.1. Process to obtain cell area measurements on A549 cells using ImageJ**

Fig. A.1. Steps to obtaining cell spreading area measurements in ImageJ. The cytoskeleton portion (b) of the fluorescent image (a) is opened in ImageJ. A pixel brightness threshold is applied (c) and a single cell is selected (d). That cell is then measured (e).
A.2. Process to obtain point elasticity measurements on A549 cells using AFM

Fig. A.2. Steps to obtaining cell stiffness measurements. The cell to be measured is chosen and the probe is brought close to that cell (a). The probe is moved toward the intended cell (b-c) being careful to place tip of the probe directly over the nucleus of the cell (d). The laser is turned on, the tip is approached to the cell, and measurements are made (e).

A.3. SAS code and statistical results for cell area experiments on ibidi substrates
/*Cell Morphology Ibidi Substrates*/
data Area;
  input Substrate Cell @@;
datalines;
  1.5 1088.5129 15 1126.8123 28 1236.4374
  1.5 1198.9977 15 1275.1983 28 1391.3606
  1.5 1184.9016 15 1118.7909 28 1380.3223
; run;

proc glm data=Area plots=diagnostics;
  class Substrate;
  model Cell = Substrate ;
  means Substrate / REGWQ alpha=0.1;
  title1 'Comparison of Substrates Stiffness to Cell Area;
run;

proc transreg data=Area;
  model boxcox(Cell / lambda=-3 to 3 by 0.05)
     =class(Substrate);
  title1 'Box-Cox on response';
run;

Table A.1
ANOVA table for cell area measurements on ibidi substrates.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
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<tbody>
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<td>58533.81173</td>
<td>29266.90587</td>
<td>4.66</td>
<td>0.0601</td>
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<tr>
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<td>37689.43387</td>
<td>6281.57231</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>8</td>
<td>96223.24560</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Fig. A.3.** Fit diagnostics plots of cell area measurements on ibidi substrates.
**Fig. A.4.** Box-Cox analysis of cell area measurements on ibidi substrates.

**Table A.2**
Post-hoc REGWQ test for cell area measurements on ibidi substrates.

<table>
<thead>
<tr>
<th>REGWQ Grouping</th>
<th>Mean</th>
<th>N</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1336.04</td>
<td>3</td>
<td>28</td>
</tr>
<tr>
<td>B</td>
<td>1173.60</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>B</td>
<td>1157.47</td>
<td>3</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Means with the same letter are not significantly different.
A.4. SAS code and statistical results for cell area on flat hard substrates

/*Cell Morphology Flat Hard Substrates*/
data Area;
  input Substrate $ Cell @@;
datalines;
  FnP 2245.1967 P 1702.2513 FnG 1375.7463 G 1447.2409
  FnP 2405.1892 P 1868.5130 FnG 1203.4152 G 915.5984;
run;

proc glm data=Area plots=diagnostics;
  class Substrate;
  model Cell = Substrate;
  means Substrate / REGWQ alpha=0.05;
  title1 'Comparison of Substrates Stiffness to Cell Area;
run;

proc transreg data=Area;
  model boxcox(Cell / lambda=-3 to 3 by 0.05)
    =class(Substrate);
  title1 'Box-Cox on response';
run;

Table A.3
ANOVA table for cell area measurements on flat hard substrates.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
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<td>1647197.103</td>
<td>549065.701</td>
<td>12.02</td>
<td>0.0181</td>
</tr>
<tr>
<td>Error</td>
<td>4</td>
<td>182791.144</td>
<td>45697.786</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>7</td>
<td>1829988.247</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. A.5. Fit diagnostics plots of cell area measurements on flat hard substrates.
Fig. A.6. Box-Cox analysis of cell area measurements on flat hard substrates.

Table A.4
Post-hoc REGWQ test for cell area measurements on flat hard substrates.

<table>
<thead>
<tr>
<th>REGWQ Grouping</th>
<th>Mean</th>
<th>N</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2325.2</td>
<td>2</td>
<td>FnP</td>
</tr>
<tr>
<td>B</td>
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<td>2</td>
<td>P</td>
</tr>
<tr>
<td>B</td>
<td>1289.6</td>
<td>2</td>
<td>FnG</td>
</tr>
<tr>
<td>B</td>
<td>1181.4</td>
<td>2</td>
<td>G</td>
</tr>
</tbody>
</table>
A.5. SAS code and statistical results for cell stiffness experiments on ibidi substrates

/*Biomechanics Ibidi Substrates*/
data Stiffness;
   input Substrate Cell @@;
datalines;
    1.5  7.5752  15 11.081  28 17.151
    1.5 11.754  15 16.739  28 14.818
    1.5 10.238  15 14.022  28 15.194
;run;

proc glm data=Stiffness plots=diagnostics;
   class Substrate;
   model Cell = Substrate ;
   means Substrate / REGWQ;
   title1 'Comparison of Substrates Stiffness to Cell Stiffness';
run;

proc transreg data=Stiffness;
   model boxcox(Cell / lambda=-3 to 3 by 0.05) =class(Substrate);
   title1 'Box-Cox on response';
run;

Table A.5
ANOVA table for cell stiffness measurements on ibidi substrates.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
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</thead>
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<tr>
<td>Model</td>
<td>2</td>
<td>54.28976467</td>
<td>27.14488233</td>
<td>5.80</td>
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<tr>
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<td>28.10417133</td>
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<td></td>
<td></td>
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<tr>
<td>Corrected Total</td>
<td>8</td>
<td>82.39393600</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. A.7. Fit diagnostics plots of cell stiffness measurements on ibidi substrates.
Fig. A.8. Box-Cox analysis of cell stiffness measurements on ibidi substrates.

Table A.6
Post-hoc REGWQ test for cell stiffness measurements on ibidi substrates.

<table>
<thead>
<tr>
<th>REGWQ Grouping</th>
<th>Mean</th>
<th>N</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>15.721</td>
<td>3</td>
<td>28</td>
</tr>
<tr>
<td>B</td>
<td>13.947</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>B</td>
<td>9.856</td>
<td>3</td>
<td>1.5</td>
</tr>
</tbody>
</table>
A.6. SAS code and statistical results for cell stiffness on flat hard substrates

/*Biomechanics Flat Hard Substrates*/
data Stiffness;
   input Substrate $ Cell @@;
datalines;
run;

proc glm data=Stiffness plots=diagnostics;
   class Substrate;
   model Cell = Substrate;
   means Substrate / REGWQ alpha=0.05;
   title1 'Comparison of Substrates Stiffness to Cell Stiffness';
run;

proc transreg data=Stiffness;
   model boxcox(Cell / lambda=-3 to 3 by 0.05) =class(Substrate);
   title1 'Box-Cox on response';
run;

Table A.7
ANOVA table for cell stiffness measurements on flat hard substrates.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
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<tbody>
<tr>
<td>Model</td>
<td>3</td>
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<td>11.85323510</td>
<td>16.40</td>
<td>0.0009</td>
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<td>Error</td>
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<td>5.78240648</td>
<td>0.72280081</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>11</td>
<td>41.34211179</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. A.9. Fit diagnostics plots of cell stiffness measurements on flat hard substrates.
Fig. A.10. Box-Cox analysis of cell stiffness measurements on flat hard substrates.

Table A.8
Post-hoc REGWQ test for cell stiffness measurements on flat hard substrates.

<table>
<thead>
<tr>
<th>REGWQ Grouping</th>
<th>Mean</th>
<th>N</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>17.4343</td>
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<td>FnP</td>
</tr>
<tr>
<td>B</td>
<td>14.4698</td>
<td>3</td>
<td>P</td>
</tr>
<tr>
<td>B</td>
<td>13.8249</td>
<td>3</td>
<td>FnG</td>
</tr>
<tr>
<td>B</td>
<td>12.8092</td>
<td>3</td>
<td>G</td>
</tr>
</tbody>
</table>
A.7. SAS code and statistical results for cell area on pillared substrates

/*Cell Morphology on Pillared Substrates*/
data Area;
  input Substrate Cell @@;
datalines;
244 1271.842 242 992.9519 224 1154.010 444 856.8043 442 729.1056 424 829.827 422 668.6213 424 877.203 422 942.5367 844 972.3251 424 994.944 422 987.203 422 942.5367
run;

proc glm data=Area plots=diagnostics;
  class Substrate;
  model Cell = Substrate ;
  means Substrate / REGWQ;
  title1 'Comparison of Substrates Pillar Geometry to Cell Area';
run;

proc transreg data=Stiffness;
  model boxcox(Cell / lambda=-3 to 3 by 0.05) =class(Substrate);
  title1 'Box-Cox on response';
run;

Table A.9
ANOVA table for cell area measurements on pillared substrates.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
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<th>F Value</th>
<th>Pr &gt; F</th>
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<tbody>
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<td>7.68</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
<td>295466.604</td>
<td>14773.330</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>29</td>
<td>1317177.810</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. A.11. Fit diagnostics plots of cell area measurements on pillared substrates.
Fig. A.12. Box-Cox analysis of cell area measurements on pillared substrates.
Table A.10
Post-hoc REGWQ test for cell stiffness measurements on pillared substrates.

<table>
<thead>
<tr>
<th>REGWQ Grouping</th>
<th>Mean</th>
<th>N</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
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<td>244</td>
</tr>
<tr>
<td>B A</td>
<td>1143.83</td>
<td>3</td>
<td>242</td>
</tr>
<tr>
<td>B A</td>
<td>1066.96</td>
<td>3</td>
<td>224</td>
</tr>
<tr>
<td>B A</td>
<td>1051.45</td>
<td>3</td>
<td>222</td>
</tr>
<tr>
<td>B A C</td>
<td>953.38</td>
<td>3</td>
<td>444</td>
</tr>
<tr>
<td>B D A C</td>
<td>900.66</td>
<td>3</td>
<td>424</td>
</tr>
<tr>
<td>B D C</td>
<td>868.98</td>
<td>3</td>
<td>442</td>
</tr>
<tr>
<td>B D C</td>
<td>826.08</td>
<td>3</td>
<td>422</td>
</tr>
<tr>
<td>D C</td>
<td>689.53</td>
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<td>844</td>
</tr>
<tr>
<td>D</td>
<td>607.13</td>
<td>3</td>
<td>842</td>
</tr>
</tbody>
</table>
A.8. SAS code and statistical results for cell stiffness on pillared substrates

/*Cell Biomechanics on Pillared Substrates*/
data Stiffness;
    input Substrate Cell @@;
datalines;
;
run;

proc glm data=Stiffness plots=diagnostics;
    class Substrate;
    model Cell = Substrate ;
    means Substrate / REGWQ alpha=0.10;
    title1 'Comparison of Substrates Stiffness to Cell Stiffness';
run;

proc transreg data=Stiffness;
    model boxcox(Cell / lambda=-3 to 3 by 0.05) =class(Substrate);
    title1 'Box-Cox on response';
run;

Table A.11
ANOVA table for cell stiffness measurements on pillared substrates.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>9</td>
<td>139.5240792</td>
<td>15.5026755</td>
<td>1.98</td>
<td>0.0976</td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
<td>156.6342299</td>
<td>7.8317115</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>29</td>
<td>296.1583091</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. A.13. Fit diagnostics plots of cell stiffness measurements on pillared substrates.
Fig. A.14. Box-Cox analysis of cell stiffness measurements on pillared substrates.
Table A.12
Post-hoc REGWQ test for cell stiffness measurements on pillared substrates.

<table>
<thead>
<tr>
<th>REGWQ Grouping</th>
<th>Mean</th>
<th>N</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>16.534</td>
<td>3</td>
<td>244</td>
</tr>
<tr>
<td>B A</td>
<td>15.742</td>
<td>3</td>
<td>222</td>
</tr>
<tr>
<td>B A</td>
<td>15.601</td>
<td>3</td>
<td>224</td>
</tr>
<tr>
<td>B A</td>
<td>15.411</td>
<td>3</td>
<td>242</td>
</tr>
<tr>
<td>B A</td>
<td>14.538</td>
<td>3</td>
<td>444</td>
</tr>
<tr>
<td>B A</td>
<td>13.781</td>
<td>3</td>
<td>442</td>
</tr>
<tr>
<td>B A</td>
<td>13.383</td>
<td>3</td>
<td>424</td>
</tr>
<tr>
<td>B A</td>
<td>13.140</td>
<td>3</td>
<td>422</td>
</tr>
<tr>
<td>B A</td>
<td>12.405</td>
<td>3</td>
<td>844</td>
</tr>
<tr>
<td>B</td>
<td>8.656</td>
<td>3</td>
<td>842</td>
</tr>
</tbody>
</table>