Characterization of Endothelial Elastic Properties and Gap Closure under Barrier-Regulatory Agonists

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THESIS
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This thesis is dedicated to my parents for being my pillar of support at all times.
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<tr>
<td>S1P</td>
<td>Sphingosine-1-phosphate</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<td>EC</td>
<td>Endothelial cell</td>
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<tr>
<td>HPAEC</td>
<td>Human pulmonary artery endothelial cell</td>
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<tr>
<td>HLMVEC</td>
<td>Human lung microvascular endothelial cell</td>
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<td>AFM</td>
<td>Atomic Force Microscopy</td>
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<td>MLCK</td>
<td>Myosin light chain kinase</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>PEG</td>
<td>Polyethyleneglycol</td>
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<td>ALI</td>
<td>Acute lung injury</td>
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<td>Acute respiratory distress syndrome</td>
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<td>SphK1</td>
<td>Sphingosine kinase 1</td>
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<td>LPP</td>
<td>Lipid-phosphate phosphatase</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>FTY720</td>
<td>(2-amino-2-(2-[4-octylphenyl] ethyl)-1, 3-propanediol)</td>
</tr>
<tr>
<td>SF</td>
<td>Scatter factor</td>
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<tr>
<td>PAR</td>
<td>Protease activated receptor</td>
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<td>skMLCK</td>
<td>Skeletal MLCK</td>
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<tr>
<td>cMLCK</td>
<td>Cardiac MLCK</td>
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<tr>
<td>smMLCK</td>
<td>Smooth muscle MLCK</td>
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<td>MLCK1</td>
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SUMMARY

Vascular integrity is primarily determined by endothelial cell (EC) cytoskeletal structure that is differentially regulated by natural barrier-promoting agents such as sphingosine 1-phosphate (S1P) and edemagenic agents such as thrombin. Direct quantification of cytoskeletal remodeling requires a reliable methodology for assessing cytoskeletal-driven force generation. In this study, we further explored mechanistically agonist-induced regulation of EC barrier function by measuring cellular elasticity with atomic force microscopy (AFM). Here atomic force microscopy is used to characterize structural and mechanical properties in the cytoskeleton of cultured EC in response to various stimuli. The measured Young’s modulus provides valuable insights about the cell elasticity variations under baseline, diseased and treatment conditions. I employed AFM to characterize Young’s modulus in cultured human pulmonary artery EC (HPAEC) and human lung microvascular EC (HLMVEC) in response to barrier protective agents S1P and HGF (hepatocyte growth factor) or the barrier disruptive antagonist thrombin. S1P structural analogue FTY720 since this compound is currently in clinical use as FDA-approved therapy. In studies presented here, HPAEC’s demonstrated a much higher Young’s modulus overall as compared to HLMVEC for each treatment condition. Results indicate a value of about 2.9 KPa for HPA control cells and 1.8 KPa for HLMV control cells with subsequent increases 10 and 30 minutes after S1P stimulation. S1P induced the highest Young’s modulus increase (6.1KPa) compared to the other barrier enhancing stimuli tested, HGF (5.8KPa) and FTY720 (4.1KPa), for the 30 minute treatment time. In contrast, the barrier disruptive agent thrombin decreased values from 2.5 KPa down to 0.7 KPa depending on the cell type and treatment time. AFM images also support the quantitative biophysical data regarding EC stiffness since there
was cytoskeleton rearrangement observed toward the periphery by barrier enhancing agonists and away from the periphery by the barrier disruptive agent thrombin.

We also developed a wound healing assay to examine the EC physiological response to physical injury. Injury by physical wounds, severe inflammation or mechanical ventilation, causes formation of paracellular gaps within the endothelium. EC migrate as a sheet into the injured area to reform a protective barrier. The wound healing assay, in which a scratch from a blade separates a cultured EC intact monolayer, enables quantification of cell migration rates that can be modulated by barrier enhancement agonists like S1P and HGF, or ectopic expression of the barrier regulatory cytoskeletal protein MLCK (myosin light chain kinase) adenovirus constructs (with and without stimulation). Cell migration begins with successive, broad protrusions of the cell membrane (called lamellipodia) that are powered by local polymerization of the underlying cytoskeleton. The same barrier agonists that increase cell stiffness in an intact monolayer may also promote lamellipodial extensions that lead to cell migration into paracellular gaps providing insights into biomechanical properties of EC that regulate vascular barrier function and have applicability to vascular leak syndromes and acute lung injury. We have compared gap closure rates in unstimulated and stimulated EC to evaluate the influence of barrier protective agonists on lamellipodial extension and cell migration. We also investigated the role myosin light chain kinase (MLCK) may have on cell migration through over-expression of MLCK by adenovirus constructs. Sealing of paracellular gaps can then restore cell stiffness and barrier integrity. The gap sizes ranged from 37 µm to 357 µm with average migration rates of 150 µm²/min for control cells under absence and presence of S1P, 1930 µm²/min and 4970 µm²/min for MLCK infected cells under the absence and presence of a S1P respectively. These
standardized assays can be used to model lamellipodia dynamics to enhance its tissue engineering application.
1. INTRODUCTION

1.1 Atomic force microscope (AFM)

AFM is a form of scanning probe microscopy and an important tool in modern day nanotechnology. Though its initial application was in materials science, AFM application has expanded and is being used in a wide range of technologies affecting electronics, materials, energy, chemical and biological industries. Its unique capabilities make it a useful tool for the study of biomechanical parameters for cells and tissues. There are various kinds of cantilever tips available, and the choice of appropriate tip is an important factor in AFM applications. Most tips used for force microscopy are microfabricated from Si or Si<sub>3</sub>N<sub>4</sub>. The force measured is usually less than 10<sup>-9</sup> N (minimum of 10<sup>-6</sup> N) between a probe (<10nm) and a surface at a short distance (0.2-10nm probe-sample separation). The probe is supported by a flexible cantilever and hence microcantilever deflection in measured instead of direct force measurement. Probes are manufactured in different lengths, materials, spring constants and coatings for specific applications like chemical force microscopy (CFM) and magnetic force microscopy (MFM). The AFM operates in three different modes which are chosen based on output requirements for each sample. [1]

Biological AFM requires the use of fluid cells for maintaining living cells under media and this development has expanded its application in the biological world. Fluids can be used with imaging and force measurement modalities which provides structural, mechanical and functional information. It is the only instrument that can non-destructively image living cells and molecules with a resolution that is at par with electron microscope. AFM is becoming a more popular imaging modality in the biological world because:

1. Data acquisition does not require light
2. Live cells are maintained and investigated under their physiological conditions, providing more accurate results.

3. Topography can be studied simultaneously while acquiring functional data.

1.1.1 Principle

The tip on the cantilever interacts with the sample producing forces between the sample atoms and the atoms in the probe material which are recorded with sub-nanometer precision. It provides extremely high-precision tip-sample positioning. Cantilever motion is detected most commonly by the optical system which has a semiconductor diode laser reflected (“beam bounce” method) from the cantilever and detected by a position sensitive photodiode as illustrated in Figure 1. The instrumentation includes a feedback loop and piezoelectric scanners. The deflections are recorded digitally and observed real-time on a computer screen for all AFM’s. There is no light involved and when the tip touches the surface of the sample, the system records the topography and other material characteristics (mechanical properties are the most common). Resolution of the image is affected by the radius of the tip, its shape and the spring constant of the cantilever.

Force between the probe and sample are recorded based on the spring constant of the cantilever and the distance between the tip and the sample surface. The best representation would be through Hooke’s Law:

\[ F = -k \cdot x \]

- \( F \) = Force
- \( k \) = Spring constant
- \( x \) = cantilever deflection [1]
Spring constants for cantilevers in the market are in the range of 0.1-1 N/m and when this is lesser than that of the surface, the cantilever bends which facilitates detection of the force. Van der Waal’s interactions are the most dominant at the small distance between the tip and the sample. [1]

![Figure 1: Schematic of AFM showing beam bounce method of detection using a laser and position sensitive photodiode detector](image)

When the probe comes in contact with the surface initially, it predominately experiences repulsive Van der Waals forces (contact mode) and causes tip deflection. When the tip moves further away from the surface attractive Van der Waals forces are dominant (non-contact mode). This is depicted in Figure 2 and constitutes an important basis for measurement of mechanical properties like elasticity. As will be seen in the pages to come, contact mode and tapping mode operate with strong repulsive forces whereas non-contact mode operates with weak attractive forces. [4]
1.1.2 Modes of operation

1.1.2a Contact Mode

This is the most common form of imaging in which the tip is brought in contact with the sample surface at a separation distance of less than 0.5nm, scanning is performed in the x-y raster pattern. The cantilever bends when its spring constant is lesser than that of the surface and the tip experiences a repulsive force when it is close to the surface of the sample. The feedback loops maintain a constant cantilever deflection and force which produces a topographic image of the surface. [3]

This mode is the simplest of all three and allows fast scanning. It also uses controlled load forces and hence more robust cantilevers. The biggest issues faced while imaging is when the probe encounters a bump or a trough. In the initial case, the feedback system elevates the cantilever holder to adjust the value and then returns to the original position. In the case of a
trough, the cantilever holder is lowered and this allows adjustment to the original value and then the tip position. In some cases, the tip tends to carry poorly attached parts with it while scanning the surface and the tip will have to be removed and cleaned before reusing the AFM. The mode works best only with fixed cells and structures, it may not be very suitable to image live cells. It uses soft cantilevers with spring constants of 0.001-1 N/m. [3]

![Figure 3: Contact mode height images of a smooth muscle cell at its periphery and center. [5]](image)

1.1.2b Non-contact mode

This is also a type of surface topography imaging without contacting the surface and based on a vibration technique. The separation range is from 0.5 to about 2 nm between the probe and the surface. Since the probe is at a higher distance from the surface, attractive Van der Waal’s forces dominate in the adsorbed fluid layer that is present just above the surface. The low force exerted does not cause any scratching or indentations on the surface. It requires ultra high vacuum for best operation but has a low resolution generally. [3]

It can be operated in force mode and force-volume mode. In contrast to contact method, the tip does not hold a lateral movement but the scanner has an up-down movement which moves the cantilever in the vertical plane. The principle behind the force mode is that force exerted depends on the distance of the tip from the sample, when far from the sample, there is no force exerted. When the z position of the sample is raised by the scanner, it comes closer to the tip and then
touches the surface and moves in the direction of the sample and this region of the force curve is called region of constant compliance. The tip can be controlled and retracted whenever required by the operator; the force curve up to retraction is called the approaching curve and after retraction is called the retracting curve. The hysteresis between the approaching and retracting curves causes hysteresis and used to determine the viscous and elastic properties. The choice of tip is important in measuring surface elasticity; the spring constant of the tip has to be in the same order of magnitude as the surface stiffness. This method has ambiguity in the definition of the tip-sample contact and hence the force-volume method was adopted to overcome this issue. [3]

The force-volume method can be used to make multiple measurements of topography and force simultaneously. The cantilever itself moves towards the surface until it touches the surface and the cantilever deflection reaches the trigger level after which the cantilever motion is reversed. The cantilever is then pulled back up to the initial cantilever distance from the surface called the ramp size. This process records the approaching and retracting curves as mentioned under force mode. At the ramp distance, the tip advances laterally recording force curves in a square raster pattern and scanning the topography simultaneously. The only disadvantage is that only few data points are collected over the long operation time as compared to force mode. [3]

Figure 4: The three modes of AFM operation (a) contact mode, (b) non-contact mode, (c) tapping or intermittent mode [6]
1.1.2c Tapping mode

Tapping mode is similar to contact mode except that the tip is oscillated at a resonant frequency. [3] Since the cantilever is a sensitive force transducer, the probe is used to push the sample and extract useful elastic mechanical properties. The force-displacement relation is collected and is analyzed with a contact mechanics approach, with the Hertz model being the most common. The Hertz model describes the indentation of an infinitely hard spherical structure which is the probe on the elastic sample surface with normal force. The force would have a normal and lateral component because of the angle at which the tip interacts with the sample but the lateral component is disregarded due to its small value. The elastic modulus would be the slope of the withdrawal portion of the force-displacement curve that was plotted based on this equation:

\[ F_{\text{Hertz}} = \frac{4}{3} \frac{E_{\text{surface}}}{(1-\nu^2)} \sqrt{R_{\text{tip}}} (s_0 - s)^{3/2} \]  

In the above equation:
F is the applied load
\( \nu \) is Poisson's ratio for the sample
r is the radius of curvature of the AFM probe
\( \delta = (s_0 - s) \) is the indentation depth
\( E_{\text{surface}} \) - local Young’s elastic modulus

![Diagram](image)

Figure 5: Left: The hard sphere on soft surface known as the Hertz model. Right: The rigid cone on soft surface model by Sneddon. [7, 8]
Adhesion force is taken to be zero in the Hertz model. All indentations are in the nano range but there are chances of scratching the surface and leading to permanent indents in the sample as well. Parameters that can be determined from the curve (Figure 5) are elastic modulus, maximum (peak) force, deformation, adhesion force and energy dissipation. [7]

Figure 6: Representation of $\delta$ and $z$ values for comparison of force curve mentioned in the figure below [9]

Figure 7: (a) AFM Indentation Force Curve [4], (b) Force curve and probe-sample interactions [10]
The Hertz model makes the following assumptions:

1. Isotropic and homogenous contacting materials.

2. Static loads are applied to the surface and the seismic dissipation of energy between the two objects due to collisions is neglected.

3. Linearly elastic material and Hooke’s law becomes applicable.

4. The radii of curvature of the contacting bodies are much larger than the contact radius.

5. Dimensions of the bodies are larger than the dimensions of the contact surface. Contact surface produces maximum stress on the sample.

6. Smooth contacting surfaces which neglects friction effects at the area of contact.

7. Small deformations which assumes infinitesimal strains. [8]

Older models that were not based on experiments may not have a significant effect on AFM measurements. Force-indentation relations proposed by Miyazaki and Hayashi (1999) are:

\[ F = a \exp(b\delta) - 1 \]

In the above equation:

a and b are constants

The slope of the above curve is represented as:

\[ \frac{dF}{d\delta} = bF + ab = bF + c \]

a is an index associated with the shape of force-indentation curves and spatial change of inhomogeneous structure inside a cell

b is the rate of modulus change by stress and explains structural homogeneity

c = ab is the initial modulus of cell stiffness [11]

Both these equations are empirical and provide measurements for structural stiffness. [11]
Another model that is in practice is the DMT model (Derjagin, Muller, Toropov – 1975). It is applied to tips which have a small radius of curvature and high stiffness. Van der Waals forces are considered to act along the contact area perimeter resulting in additional attraction and reducing the elastic forces of repulsion.

\[ a = \sqrt[3]{\frac{R}{E_{\text{red}}}} \left( F + 2\pi RW \right) \]  \hfill (2)

\[ \delta = \frac{a^2}{R} = \left( F + 2\pi RW \right)^{2/3} \sqrt[3]{\frac{2E^2}{RE_{\text{red}}}} \]  \hfill (3)

\[ F_{\text{adh}} = -2\pi RW \]  \hfill (4) [12]

In the above equations,
- F is the force (applied load)
- \( E_{\text{red}} \) is reduced elastic modulus,
- R is the radius of curvature of the AFM probe
- \( \delta \) is indentation depth
- a is the contact radius between the AFM probe and sample
- W is the work of adhesion

The reduced elastic modulus (\( E_{\text{red}} \)) is determined from the above two equations without the usage of the sample’s Poisson ratio (\( \nu_s \)), an unknown parameter. Sample’s Young’s modulus, \( E_s \) has to be less than that of the tip, \( E_t \) for indentation to occur and the equation can be simplified.

\[ \frac{1}{E_{\text{red}}} = \left( \frac{1 - \nu_s^2}{E_s} + \frac{1 - \nu_t^2}{E_t} \right) \]  \hfill (5)

\[ \frac{1}{E_{\text{red}}} \approx \left( \frac{1 - \nu_t^2}{E_s} \right) \]  \hfill (6) [12]
In the above equations,

- $E_s$ is the Young’s modulus of the sample
- $E_t$ is the Young’s modulus of the tip material
- $\nu_s$ is the Poisson’s ratio of the sample
- $\nu_t$ is the Poisson’s ratio of the tip material

Another model that can be used with consideration of adhesion forces between the tip and the sample is the JKR model (Johnson, Kendall, Roberts – 1964-1971). The JKR theory is applicable for large probes with large radius of curvature and soft samples (low stiffness) whereas the DMT theory applies to probes with a small radius and stiffer samples. JKR applies to strongly adhesive systems and accounts for Van der Waal’s forces within the contact region.

[13]

![Figure 8: Plot of force vs penetration depth][13]

1.1.3 Tips used in AFM

A wide variety of tips can be used to image and measure parameters for biological molecules, cells, tissues and biomaterials. The most common probe materials are made of silicon or silicon nitride but the selection of the tip depends on the application and the nature of the sample. Living cells are usually imaged in liquid and the pH and electrolytes in the solution should not affect the tip material. The tip material can develop positive or negative charges depending on the physiological conditions and this can develop attractive and repulsive forces. Avoiding these charges is a requirement for accurate imaging and measurement of samples. For biological
samples, special tips with surface charges or varied hydration properties are being produced as well. These are applied in cases of antibody or DNA imaging. The latter case of increasing the tip’s hydrophobicity tends to reduce the applied and capillary forces for imaging in air. [14]

1.1.3.1 Treatments performed on tips

**Plasma Treatment**

This treatment changes the hydration properties of the tip. Hexafluoro - propene discharge is carried out on the tip to produce a Teflon-like coating which has the hydrophobic quality. Hydrophilic quality is produced by a one minute glow discharge in air instead of hexafluoro – propene. [14]

**Silanization**

This method also aids in changing the wetting properties and surface charge as explained initially. The modification of tip surface helps to support biological samples especially for binding of DNA samples to a positively charged surface. Chemical methods are used to bind organochloro- and organoalkoxysilanes to the probe surface and the non –reactive surface groups are responsible for the hydrophobicity. An example of chemical that renders positive charge and hydrophobicity to silicon oxide tips is trimethoxysilylpropyldiethylenetriamine (DETA) and N-3(3-trithoxysilylpropyl) perfluoro (polyisoproxy 2-methylacethyl) amide can be used for silicon nitride hydrophobicity. [14]

1.1.3.2 Functionalization of tips

Molecular interactions can be studied by coating tips with specific functional groups that interact with the samples.

**Chemical coating**

This is performed as silanization or with thiol groups which is followed by Biological coating.
**Biological coating**

The mapping of binding or interacting groups can be performed with biological coating and followed by force measurements as well. The kinds of forces that are investigated are:

- forces between a receptor and a ligand like antigen-antibody-pairs
- forces between molecules and cells
- forces between different cells
- intramolecular forces

Proteins can be attached directly to the surface or with the help of spacers to orient the protein to expose maximum binding sites to the sample. Polyethyleneglycol (PEG) is the most common spacer used even in biological applications. For binding to the silicon nitride tip, a thiol group will be required and an amine for binding the protein. Biotin is coated after the tip is coated with bovine serum albumin (BSA). [14]

**1.1.4 Probe Parameters**

The main two parameters required for choosing tips are tip shape and mechanical parameters like cantilever spring constant, resonance frequency and quality factor (Q). [14]

Tip shape is the radius of curvature of the tip and is also called its sharpness. It helps to determine the lateral resolution as is explained in the advantages section. The topography of the sample is depicted correctly only when the right choice of tip size and shape are made. The most common effect is called the tip broadening effect and the narrower tips provides a more clean image as compared to a wide topography from a dull tip. For biomolecules, the optimal resolution is enhanced by smaller tip radius and this provides a topographic image with greater resolution. Cells and biomolecules require a soft tip for their imaging than a sharper tip because it can damage the cell. The tip shape can be studied with electron microscopy or with field emission microscopy. Another method to rate the tip would be to scan a standard sample like
colloidal gold particles, latex beads or known biomolecules which have a known topography. Most scope’s have a calibration standard in the software which can estimate the shape of the tip. The most common artifact of tip production is generation of double tips due to etching and this produces overlapping repeated topography. [14]

Spring constant of the cantilever is an important factor to choose the right tip for the corresponding application. It is the ratio of force (F) to the cantilever deflection (Δd), which is called Hooke’s law. Force measurement accuracy is decided by the errors in the spring constant in the detection system. There are four methods used to determine the tip’s spring constant:

- The equipartition theorem: spring constant is deduced from the thermal vibration spectrum
- Resonant frequency changes can be measured by loading the cantilever with small weights
- The resonant frequency can be measured and combined with the geometrical dimensions or the quality factor to determine the spring constant
- Loading with known weights or exerting force with another cantilever with known force constant can be used to determine the spring constant

Resonance frequency and quality factor are used to determine spring constant. Resonance frequency of the cantilever is used for operating the tip in tapping mode and represents the first natural mode of vibration. The cantilever geometry, material and environment conditions affect the resonant frequencies and hence the applications. For the most commonly used materials, silicon nitride it is 900 Hz to 88 kHz and for silicon cantilevers it is 60 kHz to 400 kHz. Tuning the frequency close to that of the surface is important because there is a possibility of a frequency shift as the tip approaches the surface. [14]
Quality factor Q affects scan speed and sensitivity; it is a measure of the dissipation mechanisms that damp the oscillation of the cantilever. \( Q \) is defined as:

\[
Q = \frac{2\pi m F_0}{b} \quad \text{or} \quad Q = \frac{F_0}{\Delta F'}
\]

In the above equation:
- \( m \) = cantilever mass
- \( F_0 \) = resonance frequency
- \( \Delta F' \) = width of the resonance peak
- \( b \) = damping factor

A higher value of \( Q \) is better when operating in tapping mode (100 to 300 in air and around 1 in water). [14]

1.1.5 Advantage of using AFM in biological studies

As mentioned earlier, the major advantage of AFM is the ability to measure cell properties in their natural environment like in buffer solutions, \( \text{in situ} \), and \( \text{in vitro} \), if not \( \text{in vivo} \). Other advantages include:

- There are no restrictions on the sample preparation, the temperature conditions, buffer composition (requirement to be transparent).
- Biological sample measurements have a lateral resolution (molecular) of about 1 nm whereas the vertical resolution can be higher in the range of 0.01 nm.
- Data provides true topographical information of cells and biological structures
- Force ranges of zero, single-molecule and large destructive forces can be studied by the AFM
Various biomechanical properties like viscosity, elasticity, adhesion, friction, etc can be studies from the force curves. [3]

1.1.6 AFM Limitations

Tip convolution:

The probe used in AFM imaging of biological samples is not strictly sharp because living molecules would not respond well to sharp interactions and this may cause variations. As a consequence, the image represents the interaction of the probe with the sample and not the real topography. This variation due to the radius of curvature of the tip is called the tip convolution.

High-aspect ratio probes are required to obtain the best images and these are becoming more prominent with the use of carbon nanotubes or tungsten spikes, but cost remains a reason that is holding back its wide usage. [1]

![Image of ideal and low aspect ratio tips](image.png)

**Figure 9: Ideal tip with high-aspect ratio and good lateral resolution and a low-aspect ratio [1]**

Tip contamination is the biggest problem faced by using the tip for imaging biological samples. The protein molecules or parts of live cells may detach and move with the tip when imaging or measuring force. This hampers the sensitivity and resonance frequency of the tip causing distorted images and irregular recording of force. The lateral resolution is greatly reduced and double images can also be produced apart from distorted images. Electron microscopy can be used to view the contaminated tip and the tip should be cleaned before continuing imaging with
the AFM. Alcohol and compressed air are used in common to clean the tip. The tip should be cleaned before and after every use to maintain a clean tip without and deposition of salts from the buffer. UV-light treatment is the most common for removing organic debris. In cases to heavy debris deposition, the tip can be incubated in piranha solution (mixture of sulfuric acid and hydrogen peroxide) for 30 minutes. For tip functionalization, they are exposed to argon plasma for about 30 seconds to clean the tip surface. Generally a cleaned probe is hydrophilic in nature because the organic contaminants are removed from its surface. The chemical and physical parameters of the probe affect AFM measurements and also reduce resolution. [14, 15, 16]

An example of the list of probes from Veeco Company with their applications is described below:

<table>
<thead>
<tr>
<th>SAMPLE TYPE</th>
<th>PROBE FAMILY/MODEL</th>
<th>EXPERIMENT</th>
<th>AFM MODE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomolecules (Nucleic Acids, Proteins, Lipids, Carbohydrates, etc.)</td>
<td>Silicon Nitride</td>
<td>OTESPA</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RTESP</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TESP</td>
<td>—</td>
</tr>
<tr>
<td>Biomolecules (Nucleic Acids, Proteins, Lipids, Carbohydrates, etc.)</td>
<td>Silicon Nitride</td>
<td>(D)NP-S</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NP-STT</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OTR4</td>
<td>X</td>
</tr>
<tr>
<td>Cells</td>
<td>Silicon Nitride</td>
<td>(D)NP</td>
<td>X</td>
</tr>
<tr>
<td>Tissues</td>
<td>Silicon Nitride</td>
<td>TESP</td>
<td>—</td>
</tr>
<tr>
<td>Tissues</td>
<td>Silicon Nitride</td>
<td>(D)NP</td>
<td>X</td>
</tr>
<tr>
<td>Biomaterials</td>
<td>Silicon Nitride</td>
<td>FESP</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OTESPA</td>
<td>—</td>
</tr>
<tr>
<td>Biomaterials</td>
<td>Silicon Nitride</td>
<td>TESP</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(D)NP-S</td>
<td>X</td>
</tr>
<tr>
<td>Force Measurements</td>
<td>Silicon Nitride</td>
<td>(D)NP</td>
<td>X</td>
</tr>
<tr>
<td>Force Measurements</td>
<td>Silicon Nitride</td>
<td>MSCT</td>
<td>X</td>
</tr>
</tbody>
</table>

Table 1: Probe types available for biological samples and imaging modes at Veeco [17]
1.2 Cells, agonists and adenovirus

1.2.1 HLMVEC

Human lung microvascular endothelial cells maintain low pressure pulmonary circulation in the smaller vessels and allow effective diffusion in the airspaces of the lungs. Most research related to pulmonary endothelial cell biology is carried out by culturing pure populations of primary pulmonary arterial endothelial cells and microvascular endothelial cells as a comparative study. Lung tissue possesses metabolically active and functionally responsive cells that are capable of interacting with substrates that circulate in the blood stream. These endothelial cells (EC) also interact with elements that regulate the systemic arterial blood flow rate and target organ functions. Lung EC also act as a semipermeable barrier between the vascular contents and pulmonary airspaces. This in turn plays a critical role in regulating tissue fluid homeostasis and the inflammatory response. Intracellular contractile forces, cell-cell and cell-matrix forces need to be at a balance to maintain the paracellular pathway which determines pulmonary endothelial permeability. Acute inflammation, especially acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) produce conditions of increased vascular permeability leading to flooding of the alveolar airspaces and respiratory failure. Effective therapies for preserving the endothelial barrier are not available. Examination of the elastic properties of these critical lung EC is the primary focus of this thesis.

The use of AFM and wide-field epifluorescence provides information about the biomechanical properties and rate of wound healing. Pulmonary endothelium plays a role in vascular homeostasis and in ventilation perfusion matching which enables efficient gas exchange in the lungs. [19, 20] The differential properties and functions of pulmonary artery endothelial cells and lung microvascular endothelial cells makes it important to compare and contrast the biomechanical properties of each type of lung EC. Venous endothelial cells are the easiest to
isolate but do not represent an accurate model of the pulmonary EC most involved in vascular leak. [21]

Figure 10: A. Human lung microvascular endothelial cells, B. HLMVEC immunofluorescently stained with anti-CD31 antibody and nucleus stained with PI, C.&D. HLMVEC transfected with GFP plasmid DNA[18]

Elevated chemotactant cytokine expression produces migration of leukocytes across HLMVEC and into the lung tissue at the onset of acute lung injury (ALI). Studying HLMVEC provides a useful in vitro model of the pulmonary microvasculature.

1.2.2 HPAEC

Pulmonary arteries are divided into three types based on their histology: elastic, muscular and non-muscular. The main pulmonary artery branches with a diameter of 500 µm are elastic, arteries with diameter of 70-500 µm are muscular and those smaller than 70 µm in diameter are non-muscular. Muscular arteries have continuous muscle with internal and external elastic lamina. Non-muscular arteries extend into the alveoli and form the smallest cross-section regions. Primary pulmonary artery endothelial cells are isolated from the elastic and muscular arteries whereas the microvascular endothelial cells are isolated from the non-muscular arterioles and the alveolar capillaries. [19, 22, 23]
HPAEC demonstrate a large number of enzymatic activities, respond to large range of vasoactive substances and hence have been used to study vascular permeability and inflammatory responses. Permeability can also be studied as a measure of the arrangement of endothelial fibers at the cell periphery which is approximated by the elasticity modulus.

Figure 11: A. Human Pulmonary Artery Endothelial Cells, B. HPAEC immunofluorescently stained with anti-CD31 antibody and nucleus stained with PI, C. &D. HPAEC transfected with GFP using plasmid DNA [24]

1.2.3 Cell structure and their biomechanical relation

Cytoskeletal structure is intimately linked to cell functioning especially barrier creation and modification of permeability, and these functions are a reflection of the cells’ mechanical properties. More specifically, elastic properties can be studied in the form of force-deflection curves and the corresponding Young’s modulus or Modulus of elasticity as a measure of the cells elasticity or stiffness. Prior studies demonstrate large differences in local stiffness among cells, and uneven distribution of stiffness within a single cell under normal conditions. Under diseased conditions, there are large variations in local stiffness from the baseline values. Cells change their shape, structure and mechanical properties in response to stimulants and mechanical stress. Mechanical properties such as stiffness can be determined by monitoring the cytoskeletal
structures of cells. Biomechanical properties are essential for determining the mechanisms of tissue and organ physiology under normal conditions and when affected with disease.

Methods used to measure mechanical properties of cells apart from AFM are:

(a) Micropipette aspiration of a whole cell- mostly blood cells and muscle cells
(b) Microplates by compressing and stretching the whole cell
(c) Microplates to stretch a whole cell
(d) Cell poking
(e) Micropipette aspiration of local cell surface layer
(f) Twisting of embedded or surface attached magnetic particles with external magnetic field
(g) Bending of an extended portion of adherent cell with micro needles
(h) Scanning acoustic microscopy [11]

Measurements to determine the mechanical properties of cells are made using these methods:
(a) measurement of single cell properties as a whole, (b) measurement of the local properties within the cell, (c) measurement of properties of a confluent layer as the best replica of the normal physiological condition, (d) measurement of properties in a sub confluent layer of cells to mimic cells under a diseased condition with treatment of the respective barrier regulatory agonists.

Microvascular endothelial barriers have physical forces, chemical factors and circulating cells as challenges to maintaining fluid homeostasis and organ function. Disruption of endothelial integrity is clinically difficult to correct as a part of microvascular diseases and disorders.

1.2.4 **Sphingosine-1-phosphate**

Sphingosine-1-phosphate is a bioactive lipid compound that regulates diverse cellular functions by binding to extracellular S1P receptors and acts as a secondary messenger. HPAEC
utilize extracellular S1P to generate intracellular S1P, which is catalyzed by enzymes such as lipid phosphate phosphatase (LPP) and sphingosine kinase 1 (SphK1). S1P formed inside the cells is not released to the external medium. Recent work has identified a novel pathway for intracellular S1P generation through the conversion of extracellular S1P to sphingosine (Sph) by LPP-1 followed by Sph uptake and intracellular conversion of Sph to S1P by SphK1. [25]

S1P plays an important role in regulating intracellular mobilization of Ca\(^{2+}\), cytoskeletal reorganization, cell growth, differentiation, motility, angiogenesis, and survival. S1P is generated by phosphorylation of sphingosine by sphingosine kinase 1 and 2 present in most mammalian cells. S1P lyase, S1P phosphatases and lipid phosphate phosphatases maintain the balance of S1P by degrading it to maintain a low basal level. As an extracellular ligand, it can bind to five G-protein coupled receptors S1P-1 (Edg-1), S1P-2 (Edg-5), S1P-3 (Edg-3), S1P-4 (Edg-6), and S1P-5 (Edg-8). Extracellular S1P is also known to stimulate angiogenesis and acts as a chemotactic factor for endothelial cells. S1P and its analogue the immunosuppressive drug FTY720 can regulate lymphocyte homing and immunoregulation. S1P acts as a secondary messenger for Ca\(^{2+}\) mobilization and suppression of apoptosis. Endothelial cells secrete less S1P after stimulation by agonists like TNF-\(\alpha\) or thrombin. In human lung endothelial cells, exogenous S1P is rapidly converted into intracellular S1P. Intracellular S1P can then affect angiogenesis, endothelial cell motility and survival. [25, 30]

Stimulation of monolayers with S1P improves barrier function in a dose dependent fashion. In vivo, S1P infusion attenuates lipopolysaccharide (LPS)-induced lung edema and inflammation in some animal models. Activation of the G protein coupled S1P receptor 1 causes S1P induction of Rac-dependent peripheral translocation and colocalization of cortactin and nonmuscle myosin light chain kinase (MLCK), and cortical actin ring formation to improve barrier function. [26]
S1P also improves barrier function by tyrosine phosphorylation of focal adhesion kinase causing its redistribution to the cell periphery, adherens junction and tight junction assembly.

1.2.4a FTY720

FTY720 (2-amino-2-(2-[4-octylphenyl] ethyl)-1, 3-propanediol) is a synthetic structural analogue of S1P. FTY720 is an immunosuppressant that is currently FDA approved for the treatment of multiple sclerosis. FTY720 impairs lymphocyte recirculation by sequestering lymphocytes in secondary lymphoid organs and preventing their action at peripheral inflammation sites and tissue grafts. Recirculation is effected by phosphorylated FTY720 by enhancing entry into lymphoid organs or other mechanisms. Acute respiratory distress syndrome disrupts pulmonary endothelial barrier function without any specific pharmacologic treatment. FTY720 reduces vascular permeability and promotes barrier-promoting effects on intracellularsignaling and junctional assembly formation in human pulmonary endothelium in a manner similar to S1P but the mechanism has not been completely characterized yet. [27, 28]

FTY720 has been shown to decrease LPS induced pulmonary leak and inflammation in a mouse model with ALI. In human lung endothelial barrier models in vitro, delayed enhancement is observed with FTY720 as compared to S1P. FTY720 does not induce MLC phosphorylation or cortical actin formation and does not cause activation of the S1P 1 receptor. Because it is now an FDA-approved therapy for multiple sclerosis, FTY720 has the potential for faster translation than S1P into intensive care unit treatment of ALI/ARDS. Improved understanding of its barrier enhancement mechanism would also help in identifying novel targets for development of ALI/ARDS therapies. [29]
1.2.5 Hepatocyte growth factor (HGF)

Hepatocyte growth factor is a well known angiogenic factor and endothelial cell chemoattractant. Angiogenesis involves stabilization of the endothelial cell barrier within newly formed capillaries, and HGF elevates transendothelial electrical resistance of EC monolayers (indicative of improved barrier function), which is dose dependent. HGF causes dissolution of cytoplasmic actin stress fibers and augmentation of the cortical actin ring. HGF mediated EC cytoskeletal rearrangement and barrier enhancement depend critically on activation of cytoskeletal proteins like MLCK. HGF induces endothelial separation, regulates expression of cell adhesion and is found in increased amounts in the vitreous fluid of patients who have proliferative retinopathy, and in this condition is also called a scatter factor (SF). [32, 33, 34]

1.2.6 Thrombin

Thrombin is a multifunctional serine protease which has the following functions:

- decreases barrier function and hence a related increase in permeability
- effector enzyme of coagulation and activates fibrin cross-linking and hence coagulation process and homeostasis
- interacts with a membrane protein on the surface of endothelial cells called thrombomodulin and acts as an anticoagulant enzyme to generate activated protein C
- has a proinflammatory effect on vascular cells and acts as an edemagenic agonist of conduit vessel endothelial cells inducing activation, contraction, paracellular gap formation and barrier disruption with an increase in vascular permeability

Thrombin interacts with fibrinogen and seven G protein coupled receptors called the protease activated receptor (PAR) family. Since PAR interacts with proteases like thrombin, it is shown to be associated with cellular functions that respond to vascular injury. Activation of endothelial
PAR-1 receptor recruits and activates various heteromeric G proteins whose subunits mediate subsequent endothelial cell signaling. Permeability is increased by formation of paracellular gaps in confluent layers to allow solutes and fluid to extravasate into the surrounding perivascular space through the activation of vascular endothelium in response to an inflammatory stimulus. Increased vascular permeability is a cardinal feature of acute lung injury and similar inflammatory diseases. Thrombin increases vascular permeability and also exacerbates LPS-induced microvascular dysfunction. [35, 36, 37]

1.2.7 MLCK adenovirus

Myosin light chain kinase (MLCK) maintains equilibrium by phosphorylating myosin light chains (MLC) and inducing actomyosin rearrangement to cortical actin filaments which align around the cytoplasm and stabilize endothelial cell morphology under the influence of barrier enhancing agents such as S1P. There is simultaneous promotion of lamellipodia formation and strengthening of intercellular contacts. This is important in muscle contraction, cell migration and endocytic or exocytic processes apart from cell signaling and barrier function. Activation of MLCK occurs through physiological factors and inflammatory and angiogenic mediators which causes vascular hyperpermeability. In vitro, endothelial monolayers treated with MLCK inhibitors, or when transfected with inhibiting peptides, demonstrate that MLCK activity is necessary for hyperpermeability and permeability regulation and plays a major role in vascular barrier dysfunction. [39] Stimulation with thrombin causes the actin cytoskeleton to rearrange into thick, elongated stress fibers that cross the cell. MLCK is activated causing the long stress fibers to contract and pulling cell-to-cell contact apart, causing gap formations, increased permeability and disruption of the endothelial barrier and ultimately inflammation.

Muscle MLCK has three types: skeletal (skMLCK), cardiac MLCK (cMLCK) and smooth muscle MLCK (smMLCK). Calcium released from intracellular stores binds to calmodulin and
induces MLCK to phosphorylate MLC which increases the actomyosin contraction. In skMLCK and cMLCK contractile strength increases MLCK activation, and in smMLCK actin-myosin contraction is induced. MLCK1 is the major non-muscle isoforms and is the predominant form expressed in endothelial cells, gut epithelium, and neutrophils. Microvascular cells which are cultured may not retain all physiological barrier properties as seen in the microvasculature. Hyperpermeability responses vary depending on the type of agonist. Thrombin induces MLC phosphorylation, cellular contraction and formation of intercellular gaps and has a primary stimulatory effect on MLCK1 activity. Inability of the MLC to dephosphorylate due to the presence of an inhibitor will cause increased formation of cellular gaps and leakage of fluids from the cells and increases the hyperpermeability elicited by the agonists. An extensive body of literature indicates that activated MLCK is a major determinant of microvascular barrier dysfunction and occurs in response to multiple inflammatory mediators and pathophysiological processes. S1P protects the barrier through activation of S1P receptor and this enhances the activity of nmMLCK and suppresses vascular hyperpermeability due to treatment of edemagenic agents like thrombin. [38, 40]

1.3 Wound healing and Migration assays

Wound healing assays are used to study directional cell migration in vitro as a model to mimic the cell migration involved in wound healing in vivo. This assay involves artificial wound creation in a cell monolayer and imaging the cells at regular intervals during the cell migration as the gap closes. Comparison of these cell migration movies determines the relative migration rates of the cells and the rates of wound closure. Effects related to cell-matrix properties and cell-cell interaction can be determined from the migration rates. For some experiments, migration of
individual cells is tracked by monitoring the movement of the leading edge of the wound to
determine localized effects. [41, 42]

Tissue wounds undergo an ordered series of events to repair the local injury. These events
include the following: infiltration of inflammatory immune cells, increased vascularization by
angiogenic factors, increased cell proliferation and extracellular matrix deposition. Proliferation
rates can be determined by in vitro migration assays in addition to migration rates in response to
various culture conditions. These studies require a confluent cell monolayer to best mimic the
physiological conditions that occur in vivo. This monolayer is then disrupted by scratching a line
which destroys or displaces a group of cells in the region. The healing process which is
monitored by cell migration into the gap may take several hours to a day depending on the cell
type, condition and width of the wound or gap. The best results are obtained with >90%
confluency and by maintaining aseptic conditions. Tips or sharp objects used for gap creation
should be sterilized to avoid any external contamination. For studying individual cells in more
detail, a resolution of 40x can be used so changes in morphology at the leading and trailing edges
of cells can be acquired. For more information about the rate of wound closure, lower
magnification of 10x or 20x can be used to observe movement of a group of cells as they close
the wound. [43]

These assays have been used for many years to study cell migration in vitro. Cell migration
into the wound varies with the cell type. The cells can be loosely connected populations or sheets
of cells which mimic cell behavior during migration in vivo. This technique is used for
directional cell migration and regulation by cell interaction with extracellular matrix (ECM) and
cell–cell interactions as an enhancement to time-lapse microscopy and Boyden chamber assays.
Cell migration is regulated by the extracellular membrane (ECM) and soluble factors and hence
maintaining conditions similar to in vivo cell movement are required. Migration assays can be
combined with transfection to study the effect of expression of exogenous genes on individual cell migration by monitoring the leading edge and thereby regulating directional migration. The supplies required to carry out a migration assay are simple, inexpensive and are mostly a part of standard cell culturing materials. [41]

Wound creation is performed by various techniques depending on the width of the gap that is to be studied. The most common comparison method involves the creation of images in time-lapse format to study the rate of migration. Microscopy combined with image analysis software differs from conventional time-lapse microscopy only in measurement of directional migration instead of random movement of sub confluent cells. [41]

The parameter that is measured is the change in cell covered area over time or the rate of change of area. Proliferation assays can demonstrate cell division rates like mitosis over time and per the total cell numbers for investigating cell growth of differentiation into stem cells. Common experimental comparisons made with migration assays:

- Wound healing and migration behavior between cells and mutant types
- Wound healing potential of drugs or agonists
- Wound healing through action of inhibitors and enhancers

Figure 12: Example of a wound healing migration assay [44]
Use of high-resolution fluorescence microscopy to visualize molecular mechanisms

Collection of information about cytoskeletal changes or signal transduction

Chemotaxis

Some companies produce culture inserts that can be purchased for seeding cells between the two gaps as shown in the figure. Once the insert is removed before imaging, the gap is created and cell migration into the gap can be observed. The major disadvantages of this method may be:

- Standardized gap width can only be created and it is difficult to produce smaller gaps for effective migration assays
- Dispersion of cells in directions away from the gap since the chamber width is much smaller than the size of the dish in which it is placed

The same method also has the following advantages:

- Co-cultivation of two or more types of cells or tissue placed in the same medium
- Ease of barrier removal while creating the gap [44]

1.4 ImageJ/Fiji - Image analysis

Fiji and ImageJ are two important software programs used for image analysis of different frames of a movie. The migration assays are usually recorded at various positions on a dish, and each position results in a movie that is analyzed using ImageJ. ImageJ can display, edit, analyze, process, save and print 8-bit, 16-bit and 32-bit images. The application used in this thesis is primarily for analysis of area and pixel measurements, and to lesser extent for density histograms and profile plots.
Figure 13: Wound outline as viewed through ImageJ [45]

Standard image processing for viewing under required brightness and sharpness helps in improving manual adaptation to perform the best possible analysis using the plugin and macro tools. The outline described above is an extension of analyzing the area of one frame and this is further used to determine rate of migration and wound healing. Data values obtained from the software can be further analyzed using Excel to come to calculate various migration rates.
II. MATERIALS AND METHODS

1. Cell culture and plating

1.1 Cell culturing and plating for AFM experiments

Human lung microvascular endothelial cells (HLMVEC) (#CC-2527), human pulmonary artery endothelial cells (HPAEC) (#CC-2530) and EGM2-MV media (#CC-3202) were all purchased from Lonza (Walkersville, MD) for AFM and migration assay studies. HLMVEC and HPAEC are purchased as frozen primary cells in their third passage, stored in liquid nitrogen thawed before each use and cultured in EGM2-MV media with - 5% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) in a 5% CO₂ humidified 37° C incubator.

The cells were cultured in T75 flasks and were propagated further once they reached 90% confluence. For AFM experiments, cells in their fifth and sixth passage were plated on 22 x 22 microscope cover glasses (#12-541B, Fisherbrand, Pittsburgh, PA), which were placed in a six well plate. Cover glasses were prepared for seeding by following a series of steps- initial sterilization with ethanol, followed by a coating with 0.02% gelatin (Sigma-Aldrich, St. Louis, MO) which was removed after 5 minutes. For cell preparation, each T75 flask was washed thrice with DPBS 1X- without calcium and magnesium (#21-031-CV, Gibco, Carlsbad, CA) followed by addition of 1 mL of 0.05% Trypsin EDTA (#25300, Gibco, Carlsbad, CA) and placed in the incubator for 2 minutes.

Following cell detachment, medium was added to raise the total volume to 12 mL, from which 2 mL was transferred to each well in the six-well plate to attain 100% confluency in 24 hours. Sub-confluent cells were also examined in the AFM and for achieving sub-confluence; volume of cells seeded was reduced to 0.5mL from 2 mL.
1.2 Cell culturing and plating for Migration Assays

Human lung microvascular endothelial cells (HLMVEC) and EGM2-MV basal medium were obtained from Lonza (Walkersville, MD). Fetal bovine serum (FBS) and sphingosine-1-phosphate were purchased from Sigma-Aldrich (St. Louis, MO). Trypsin (0.05%) was purchased from Life Technologies (Grand Island, NY) and phosphate-buffered saline from Mediatech (Manassas, VA). Non-muscle MLCK1 and control vector adenoviruses were generous gifts from Dr. Peter Usatyuk and Dr. Viswanathan Natarajan (University of Illinois at Chicago) who had originally obtained the adenoviruses from the Gene Transfer Vector Core at the University of Iowa, Iowa City, IA. Feather surgical stainless steel scalpel blades (no. 11) were acquired from Fisher Scientific (Pittsburgh, PA).

HLMVEC were cultured in complete medium containing 5% FBS/EGM2-MV from passages 5-7 in a 37°C and 5% CO₂ incubator. Cells were harvested with 0.05% trypsin/0.5 mM EDTA, resuspended in complete medium, and replated at a density that formed a monolayer in sterile 35-mm dishes.

1.3 Adenovirus Infection and Wound Formation

HLMVEC were infected at a MOI of 7 pfu with vector control or MLCK1 adenoviruses [46] in complete cell culture medium and incubated for 24-48 hours. Before the experiment, cells were washed thrice with sterile D-PBS and returned to 3 mL cell culture medium.

Initial trials at wound creation with photolithographically developed substrate on a glass slide did not produce effective results. Barrier widths of 20 μm, 30 μm, 50 μm and 70 μm were evaluated. The paraylene polymer used for lithography did not seem to provide a conducive environment for cell migration. Micropipettes were also employed at a failed attempt in wound
creation similar to a scratch assay. Successful gaps in the HLMVEC monolayer were created by manually scratching with a scalpel blade that created gaps approximately 82 to 151 µm in width.

2. AFM operation

Two AFM’s that were employed for imaging and force measurement in this study are: Nanoscope III SPM (Plainview, NY) and Novascan Synergy ESPM 3D (Ames, IA). The appropriate tip for operation of the AFM was decided before use and was purchased from AppNano (Santa Clara, CA) for imaging and Novascan (Ames, IA) for force measurements. Colloidal HYDRA2R-50N probes were purchased from AppNano which are designed for imaging and force-distance measurements. They have rectangular narrow cantilevers which can be used for contact mode and tapping mode applications. The tip is primarily made of Silicon with a Silicon nitride cantilever of height of 4-6µm to allow for the tip to travel over the heightened nucleus of the cells. Spring constant is the most important factor for tip usage and has to be greater than that of the surface on which it is being operated. The colloidal tip has a spring constant of 0.084N/m which was ideal for imaging samples. Novascan specific PT.GS tips were purchased which have a 5.0 µm borosilicate glass particle attached as the tip to the edge of the cantilever with a spring constant of 0.12N/m. [47, 48] This spring constant has been tested to be the best applicable for endothelial cells without any surface modifications.

Nanoscope III was mainly used to obtain images of control and agonist treated cells which provided a visual evidence of variation in cytoskeletal fibers. Samples could be fixed or analyzed as living cells present in a liquid environment depending on the chamber that is used for recording images. Fixed cells were placed on a circular cover slip that was attached to a magnetic holder with an adhesive. The flat metallic surface served as the sample chamber and
was placed in the AFM over which was positioned the tip holder with the appropriate tip installed within the head. This is followed by adjustments to the laser, detector and photodiode; followed by obtaining a signal in the required range. Laser adjustment over the tip in the x-y direction needs to be in conjunction with the lateral movements of the head within which the tip is located over the sample. Control parameters like scan size, scan rate and z-range can be set to user requirements. Image quality may be affected by dirty tips or presence of loose surface material on the sample. Cantilevers with more than one tip attached should be used more carefully to avoid effect of the second tip on the image quality. Images obtained were converted to readable JPEG format and can be viewed in the results section.

Novascan was used mainly for determining Young’s moduli of various cells after treatment with agonists. Cells were plated on 22 x 22 mm microscope cover glasses in order to be secured well into the AFM sample chamber. Calibration of plain glass coverslips before force measurements on the sample is necessary to standardize the curve. A sharp curve with plain glass suggests good tip functionality and sensitivity. Calibration involves a combined effort of tip positioning from the surface, laser targeted on the tip, user precision with the detector and effective interaction with the surface. Hank’s buffered salt solution 1X (HBSS) (# 14175079 Gibco, Carlsbad, CA) is used to keep the cells in their physiological state. To avoid drying of the tip or salt deposition, regular monitoring of the buffer in the recording chamber is crucial. Force curves were recorded by the system at three locations on each cell of the sample and this procedure was repeated for up to 40 cells in a single sample to allow reproducible results. In the current study we compared three barrier enhancing and one barrier disrupting agonist versus control cells: - control, S1P (Sigma-Aldrich, Saint Louis, MO), HGF (PeproTech, Rocky Hill, NJ), FTY720 (generously provided by Novartis, Basel, Switzerland), and thrombin (Sigma-
Aldrich, Saint Louis, MO). Each agonist was measured beginning at a 10 minute or 30 minute treatment time after stimulation. The total volume of buffer in the sample holding chamber was 2 mL and the agonists were added correspondingly. S1P and FTY720 were used at a concentration of 1µM, HGF was used at a concentration of 25 ng/mL and thrombin was used at a concentration of 10 mg/mL. Both cell types (HLMVEC and HPAEC) were exposed to each of the agonists in order to assess their responses. Results were analyzed with a software program developed from Visual Basic. The final result is the Young’s modulus which is a numerical value obtained from the Hertz model. The model has made assumptions for analyzing all force-deflection curves and converts the deflection and detector signal data to obtain the slope as the Young’s modulus.

![Figure 14: a. Tip holder in Novascan III ESPM 3D AFM, b. Representation of AFM measurements](image)

3. Microscopy and Image Analysis

Gap closure was tracked on either of two apparatuses, an Olympus VivaView or a UIC epifluorescence microscope. Wound healing was initially tracked in a Olympus VivaView wide-field microscope equipped with a 37°C humified, 5% CO₂ incubator and controlled with
MetaMorph (v. 7.6.2.0) image acquisition software (Molecular Devices, Sunnyvale, CA). Twelve-bit grayscale differential interference contrast images with a pixel resolution of 161.2 nm/pixel were acquired every six minutes with an exposure of 100 ms and a UPLSAPO40X objective lens (NA 0.95). Metamorph software was used to acquire and analyze images to obtain a value for rate of change of area and hence the rate of wound healing under control and treatment conditions.

When the Olympus VivaView microscope was unavailable, migration assays were conducted in wide-field mode on a Zeiss Axio Observer.Z1 TIRF microscope equipped with a Pecon XL TIRF S incubation system providing the necessary gaseous and temperature controls which were best conducive to normal cell function. Within the incubation chamber surrounding the microscope stage and a heated stage insert 5% carbon dioxide levels, humidity and 37°C were also stably modulated during overnight assays. Sixteen-bit grayscale phase contrast images (322.5 nm/pixel) were acquired every six to ten minutes with Axiovision 4.7 software (Carl Zeiss MicroImaging GmbH, Jena, Germany), a Hamamatsu C4742-98-24ERG CCD camera, and an EC Plan-Neofluar 20X 0.5 NA objective lens. Single dishes were scanned at different lateral and axial positions with the aid of the motorized stage, thus making time-lapse imaging possible at different XYZ positions and for cell migration studies. [49, 50]

Migration assays were performed with control adenovirus and MLCK1 adenovirus infected cells under control conditions and after stimulation with 1 µM S1P.
Post-acquisition image analysis was done with Fiji software [51]. The various tools that were used in Fiji are similar to those from ImageJ (Wayne Rasband, National Institutes of Health, Bethesda, MD) and include plugins specific for area determination apart from image processing. Movies were tracked manually with free hand outlines in some cases and with a Manual Tracking plugin (Dan White, Max-Planck Institute of Cell Biology and Genetics, Dresden, Germany). Both cases produced numerical values of the area within the wound, its width, perimeter and time interval between each frame and such metrics were taken for every frame until complete gap closure. Wound healing rates were calculated as area (µm²) per minute. Individual rates of wound healing for the positions were combined and averaged to compare various test conditions. Comparisons are represented with the aid of bar graphs. [51]
III. RESULTS

AFM results are divided into two parts- a) comparison of images of the endothelial cells as recorded from the Nanoscope III; and b) a discussion of the cell elasticities in terms of their Young’s modulus. The control cells were compared with four barrier modifying agents and the results are summarized:

1. S1P
2. HGF
3. Thrombin
4. FTY720

3.1 Imaging mode

3.1.1 Control cells

Control cells initially showed height ranges from 1000 nm at the center of the cell (nucleus) to approximately 500 nm towards the periphery. The image shows a normal distribution of cytoskeletal fibers as expected. Previous AFM studies have reported height measurements ranging from 2500 nm to 650 nm from the nucleus to the cytoplasm. [52]

![Control HPAEC as viewed under Nanoscope with the corresponding height information](image)

Figure 16: Control HPAEC as viewed under Nanoscope with the corresponding height information
Endothelial cells are highly plastic, and stable junctions can be maintained with the help of the cortical actin belt. Actin stress fibers generate certain centripetal tension that weakens junctions. The cortical actin rim is known to interact with cell-cell and cell-matrix adhesion complexes to maintain a functional endothelial cell barrier. [55] The scan size was maintained at a square area of 100 μm x 100 μm and a scan rate of 1.001 Hz with an average time of less than 5 minutes per image (4.3 minutes). One hypothesis concerning why height measurements here were lower than earlier publications may be because of different cell fixation methods. Usually endothelial cells can range from 5-14 μm in width and up to 25 μm in length. [56] Height of some cells tends to be at around 4-6 μm, but fixed cells show a lower height range as compared to live ones.

### 3.1.2 S1P treated cells

S1P was added to the media before imaging, and the acquired image is displayed below.

![Image](image_url)

Figure 17: S1P treated HPAEC as viewed under Nanoscope with the corresponding height information
Cells show an altered distribution of height towards the cell periphery as compared to control cells. S1P is a barrier enhancing agent that induces rearrangement of the actin cytoskeletal fibers towards the periphery that serves to strengthen the cortical ring and prevent fluid leakage. The image is magnified to a 50 μm x 50 μm scale with the same height ranges as the control. Stress fibers show interaction among neighboring cells with the creation of paracellular gaps. S1P has a dose dependent effect on increasing the barrier function in endothelial cells.

3.1.3 HGF treated cells

HGF treatment induced a similar response by rearrangement of fibers towards the periphery. The magnified 50 μm x 50 μm shows a clear picture of possible formation of paracellular gaps along with actin stress fiber arrangement along the cell periphery. With a scan rate of 1.001 Hz, images were obtained every 4.3 minutes. As seen from the image, interaction of neighboring cells displays barrier enhancing activity.

Figure 18: HGF treated HPAEC as viewed under Nanoscope with the corresponding height information
The peripheral regions are consistent with the observation that HGF treated endothelial cells display a strong augmentation of the cortical actin ring and cytoplasmic actin stress fiber dissolution. Height scales up to 1 µm were observed similar to the previous images.

3.1.4 Thrombin treated cells

Thrombin is a barrier disruptive agonist, and its effects were expected to be the opposite of S1P and HGF. Thrombin effects produce vascular leak conditions. Thrombin induces formation of F-actin stress fibers in the cytoplasm of the EC, and this can be seen by the heightened response displayed by the centers of the cells. The height scale has been increased to 2 µm because of the increased rearrangement of most filaments towards the nucleus, causing a barrier disruptive effect on the endothelial cells. Cells also display an obvious rounding and this is consistent with earlier studies as will be discussed. Certain samples when treated with S1P following a thrombin treatment displayed the therapeutic effect of S1P in negating the barrier disruption caused by thrombin.

Figure 19: Thrombin treated HPAEC as viewed under Nanoscope with the corresponding height information
Tip issues due to increased height of cells were of consideration while imaging. Certain regions could not be imaged due to lack of resolution at heights higher than 3-4 µm. Images have been recorded on a 50 µm x 50 µm scan size and at an average rate of 4.3 minutes per image.

3.2 AFM Force curves and Young’s modulus

Control cells were compared to S1P, HGF, FT720 and thrombin treated cells to study variations in Young’s modulus. Treated cells under all conditions were tested in two time intervals. Agonists were treated for 10 minutes and 30 minutes, rinsed off and measurements were made from that time point. Regions of the cells between the edge and the nucleus (the periphery) were recorded expecting the cells to show maximum variations when treated with these agonists. Considering action of S1P after the treatment time as well, effective values obtained are at the 10 minute to 50 minute time gap and these are compared with the 30 minute to 70 minute treatment times (with an average of 1 minute per cell and measurements made in sets of either 20 cells or 40 cells). The comparison is based out on visible differences that were observed beyond the 30th/50th minute which also corresponds to the 20th cell that is measured. There has been significant differences in agonist actions after the 50th minute (20th cell) measured to the 70th minute (40th cell) resulting in two comparison levels. All time points and agonists have been compared in HPAEC and HLMVEC to characterize the differential effects of these agonists on these subpopulations of lung EC.

Comparison of control and S1P treated HPAEC and HLMVEC are shown in Figures 20, 21 and 22. The Standard error of means for Figures 20-22 are displayed in Table 2.

Young’s moduli displayed by HLMVEC are numerically lower under all conditions as compared to that of HPAEC. Figure 20- S1P treated HPAEC show a steady increase in modulus values and there is a difference of almost 1000 Pa between the two treatment conditions. This demonstrates that there is a steady increase in the cytoskeletal fibers arrangement along the
periphery with an increase exposure to S1P. HLMVEC exhibited an increase that is not as significant as the HPAEC, with numerical values three times lower in comparison. There is significant difference (p < 0.05) observed between control and HPAEC and between HPAEC and HLMVEC treatments as indicated by the green stars but not between the control and HLMVEC treatments.

Figure 21- HGF treated HPAEC demonstrate Young’s moduli variations similar to that produced by S1P treatment with a lower magnitude of values. HLMVEC demonstrate a different trend by displaying higher Young’s modulus for the 10 minute treatment time as compared to the 30 minute treatment time. This suggests that increased HGF exposure (over 30 minutes) has a less pronounced effect on EC barrier function than at earlier time points. Significant differences are observed between HPAEC and HLMVEC treatments but the trends of values are different for HLMVEC as compared to HPAEC.

Figure 22- Thrombin treatment shows a similar trend in both HPAEC and HLMVEC. Both show a decreased peripheral elastic modulus as compared to the control. These results are expected because of a disrupted cortical actin ring at the periphery, which is also supported by the image explained earlier. The 10 minute treatment time of HPAEC did not show a significant decrease in modulus as compared to the 30 minute treatment time. Numerically elasticities are much lower than those observed in S1P and HGF treatments, supporting a barrier disruption phenotype. Though HPAEC and HLMVEC show significant differences within their groups, the differences between HPAEC and HLMVEC groups are not consistently significant.

<table>
<thead>
<tr>
<th>Table 2-10' to 70' (Pa)</th>
<th>HPAEC SEM</th>
<th>HLMVEC SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>207</td>
<td>153</td>
</tr>
<tr>
<td>S1P 10min</td>
<td>293</td>
<td>76</td>
</tr>
<tr>
<td>S1P 30min</td>
<td>290</td>
<td>140</td>
</tr>
<tr>
<td>HGF 10min</td>
<td>251</td>
<td>147</td>
</tr>
<tr>
<td>HGF 30min</td>
<td>299</td>
<td>407</td>
</tr>
<tr>
<td>Thrombin 10min</td>
<td>146</td>
<td>162</td>
</tr>
<tr>
<td>Thrombin 30min</td>
<td>168</td>
<td>297</td>
</tr>
</tbody>
</table>

Table 2: Standard error of means for Figures 20, 21 and 22
Figure 20: Comparison of Control and S1P treatments from 10' to 70'  

Figure 21: Comparison of Control and HGF treatments from 10' to 70'  

Figure 22: Comparison of Control and Thrombin treatments from 10' to 70'
The cells were compared in two sets and an explanation for these comparisons is displayed in the diagram below for better understanding of the groups that will be represented in the following pages:

The first twenty cells will be represented as the 10 minute to 50 minute interval combining both the treatment times. The second twenty cells will be represented as the 30 minute to 70 minute time group. Comparison of each set demonstrates the reproducibility of results and justifies that the total data can be analyzed by combining into one bigger set. Figure 23-25 represent the first 20 cells followed by Figure 26-28 which represent the second 20 cells.

Figures 23, 24 and 25 display data recorded for the first 20 cells measured immediately after treatment. The Standard Error of Means for Figure 23-25 are displayed in Table 3.

Figure 23- Comparing the moduli for only the first 20 cells made from the time of treatment completion shows a trend similar to that of the complete data set as observed in the earlier three
graphs. HPAEC demonstrate a consistent increase in values, but the 10 minute and 30 minute treated HLMVEC do not exhibit a significant increase in values. There is significant difference (p < 0.05) observed between control and HPAEC and between HPAEC and HLMVEC treatments as indicated by the green stars but not between the control and HLMVEC treatments.

Figure 24- HPAEC demonstrate a similar trend as mentioned for Figure 20. HLMVEC also exhibit a trend similar to that of Figure 21, the only difference being a higher elasticity value for the 30 minute treatment compared to the control as opposed to lack of any significant difference in the previous case. Significant differences are similar to Figure 23 and varied significance is observed between the HLMVEC 10 and 30 minute groups compared to the HPAEC group.

Figure 25- Thrombin data demonstrates a similar decreasing trend from control to the 30 minute treatment. Significant difference is observed between the 10 minute and 30 minute treatment with a difference of about 890 Pa for HPAEC. HPAEC demonstrate a much larger range of variations within the first 20 cells as compared to the overall data, and this may also reflect a more potent activity of the agonist within the first 30 minute. Though HPAEC and HLMVEC show significant differences within their groups, the differences between HPAEC and HLMVEC groups are not consistently significant.

<table>
<thead>
<tr>
<th>Table 3- 10' to 50' (Pa)</th>
<th>HPAEC SEM</th>
<th>HLMVEC SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>326</td>
<td>299</td>
</tr>
<tr>
<td>S1P10min</td>
<td>346</td>
<td>173</td>
</tr>
<tr>
<td>S1P30min</td>
<td>469</td>
<td>402</td>
</tr>
<tr>
<td>HGF 10min</td>
<td>269</td>
<td>527</td>
</tr>
<tr>
<td>HGF 30min</td>
<td>380</td>
<td>138</td>
</tr>
<tr>
<td>Thrombin 10</td>
<td>194</td>
<td>101</td>
</tr>
<tr>
<td>Thrombin 30</td>
<td>162</td>
<td>185</td>
</tr>
</tbody>
</table>

Table 3: Standard error of means for Figures 23, 24 and 25
Figure 23: Comparison of Control and S1P treatments from 10' to 50'

Figure 24: Comparison of Control and HGF treatments from 10' to 50'

Figure 25: Comparison of Control and Thrombin treatments from 10' to 50'

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Figures 26, 27 and 28 display data recorded for the second 20 cells measured immediately after treatment of the total 40 cells. The Standard Error of Means for Figure 26-28 are displayed in Table 4.

Figure 26- The last 20 cells of HLMVEC did not demonstrate an increase in the Young’s modulus for the 10 minute treatment as compared to the 30 minute treatment. There seems to be a dip in the graph from control to 10 minute treatment followed by an increase in the 30 minute treatment level, and this would have affected the overall average of the S1P treatments for HLMVEC. HPAEC follow the same pattern with decreased numerical Young’s modulus values. There is significant difference (p < 0.05) observed between control and HPAEC and between HPAEC and HLMVEC treatments as indicated by the green stars but not between the control and HLMVEC treatments.

Figure 27- HGF treatment for HLMVEC demonstrates a decrease in the 10 minute treatment level compared to the 30 minute treatment. It is an accurate representation of the overall average and confirms that an increased time of treatment (over 30 minutes) does lead to changes in HGF barrier regulation on HLMVEC. HPAEC demonstrate an increase in values, but the magnitude of individual values is lower as compared to the first 20 cells and displays a decrease in HGF activity over time. Though both cell types display a decrease in agonist activity with time, the nature of the variations are different. A common conclusion would be an increase in treatment time leads to a decrease in HGF effect on cells but to varied degrees on specific endothelial cell types, and each cell type is time dependent. Significant differences are observed between HPAEC and HLMVEC treatments but the trends of values are different for HLMVEC.

Figure 28- HPAEC demonstrate a varied trend on the second half of the 20 cells. The 30 minute treatment group exhibits an increase in values as compared to the first 20 cells. HLMVEC demonstrate a regular decrease from control to 30 minute treatment consistent with the previous two thrombin treatments bars. The overlapping time points occur in the two treatment groups, and these data sets demonstrate a similar trend. Both ranges have modulus values below 2 KPa. HLMVEC show significant differences within their group, but the differences in the HPAEC group treatment are not significant.
Figure 26: Comparison of Control and S1P treatments from 30' to 70'

Figure 27: Comparison of Control and HGF treatments from 30' to 70'

Figure 28: Comparison of Control and Thrombin treatments from 30' to 70'
Table 4-30' TO 70'

<table>
<thead>
<tr>
<th></th>
<th>HPAEC SEM</th>
<th>HLMVEC SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>326</td>
<td>154</td>
</tr>
<tr>
<td>S1P 10min</td>
<td>605</td>
<td>104</td>
</tr>
<tr>
<td>S1P 30min</td>
<td>410</td>
<td>181</td>
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<tr>
<td>HGF 10min</td>
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<td>645</td>
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<tr>
<td>HGF 30min</td>
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<tr>
<td>Thrombin 10</td>
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<td>342</td>
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<tr>
<td>Thrombin 30</td>
<td>399</td>
<td>205</td>
</tr>
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</table>

Table 4: Standard error of means for Figures 26, 27 and 28

Control and FTY720

Control cells were compared to a 10 minute and 30 minute treatment of FTY720 to determine if its effects were consistent with those of S1P. Figure 28- the 10 minute treatment did not show an increase in elasticity from the control, but the 30 minute treatment shows an increase of about 1.7 KPa from the control and the 10 minute treatment level. This indicates that FTY720 has an increased effect on HPAEC after 30 minutes of treatment. Since it is an analogue of S1P, this increase in values was expected and suggests its potential use as a therapeutic agent in ALI syndromes. Significant difference (p < 0.05) was observed between control and 30 minute FTY720 treatments.

Figure 29: Comparison of Control and FTY720 treatments from 10' to 70'
3.3 Migration assays

Rates of change of area were compared for control adenovirus infected cells and MLCK adenovirus infected cells under the presence and absence of S1P as an agonist. The results are displayed in Figure 29. All assays were standardized beginning with cell culturing, and the assay has been developed to suit cell migrations over a gap created manually with the aid of scalpel blades. Initial trials with micropipettes for gap creation proved unsuccessful because of wider gaps, presence of shredded glass pieces over the imaging region, highly irregular gap sizes and inability to establish a concrete scratch creation procedure. Use of photolithographic techniques to create petri dishes with inlaid polymer patterns which were followed by plating cells and final removal of polymer to create gaps proved inefficient because cell growth was not compatible within the region of polymer layers. In addition, they displayed motion over the gaps to the opposite side or lack of any migration from their initial positions. These techniques were followed up with scalpel blade scratches for wound creation in a HLMVEC monolayer. Table 5 displays the standard errors of the means of control and MLCK1 adenovirus under the two conditions.

<table>
<thead>
<tr>
<th>(Pa)</th>
<th>HPAEC SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>207</td>
</tr>
<tr>
<td>F2Y720 10 min</td>
<td>304</td>
</tr>
<tr>
<td>F2Y720 30 min</td>
<td>503</td>
</tr>
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</table>

Table 5: Standard error of means for Figures 29

<table>
<thead>
<tr>
<th></th>
<th>SEM $\mu m^2$/min</th>
<th>Control Adenovirus</th>
<th>MLCK Adenovirus</th>
</tr>
</thead>
<tbody>
<tr>
<td>No stimulation</td>
<td>12</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>S1P stimulation</td>
<td>10</td>
<td>152</td>
<td></td>
</tr>
</tbody>
</table>

Table 6: Standard error of means for control adenovirus and MLCK1 adenovirus infected samples with relevant treatment condition
The results did not display a significant difference between unstimulated and stimulated control cells. In contrast, the MLCK1 adenovirus infected cells showed a significant difference with an increase in rates ~ 850 µm²/min when treated with S1P as compared to absence of any treatment. The average gaps created while measuring the movies under each condition were 79 µm for control (no stimulation), 37 µm for control (S1P stimulation), 130 µm for MLCK (no stimulation), and 357 µm for MLCK (S1P stimulation). Two different microscopic systems were used to monitor migration progress, and although each system had different functionalities, the same end result was achieved and allowed comparable data. It was hypothesized that enhanced expression of the contractile protein MLCK1 would show a higher rate of migration over non-stimulated control adenovirus-infected cells. S1P treated control cells would have been expected to display a faster rate of migration than unstimulated MLCK1 adenovirus infected cells as well. Significant difference (p < 0.05) in values was observed between the MLCK1 no stimulation and S1P stimulation; but there was no significant difference between the two control groups.

Figure 30: Rate of change of area observed from the migration assays.
Each stimulation condition was analyzed through normalization, and the complete data set was represented in the form of bell curves. Figure 30 represents the Gaussian distribution for control cells under the absence of S1P treatment. Considering a total area of 1.00, the range of values is represented with the respective color coding. The presence of 95% of the total values within the defined zones (orange lines) displays the consistent average rates of migration observed from different imaging positions. Most rates had a range from 120\(\mu\text{m}^2/\text{min}\) to 150\(\mu\text{m}^2/\text{min}\). These data indicate that for control cells which are not stimulated, rate of migration does not change with time and gap size. The average gap lengths obtained for control cells were 79 \(\mu\text{m}\) with movies created at 6 minutes per frame and averaged over ten befitting migration positions.

![Control adenovirus with no stimulation](image)

**Figure 31: Normalized data curve for Control and no stimulation data set**

Figure 31 represents the normal distribution for control cells treated with S1P and show 95% values in the range of 125 \(\mu\text{m}^2/\text{min}\) to 150 \(\mu\text{m}^2/\text{min}\). This would have expected to be higher since it overlaps with the range displayed by control cells which were not stimulated. The advent of
such an outcome could be related to various uncertainties involved in creation of this assay and will be discussed in detail in the section that follows. It could also be due to a smaller (n=6) sample size as compared to the control and unstimulated population (n=10).

Figure 32: Normalized data curve for Control and S1P stimulation data set

Figure 32 and 33 represent a similar curve for MLCK1 untreated cells and MLCK1 S1P stimulated cells respectively. The range of rates of cell migration for the former condition was between 1430 µm²/min and 1930 µm²/min, which is more than a ten times increase in values as compared to control cells. The MLCK1 S1P treated cells displayed maximum number of rates from 3700 µm²/min to 4970 µm²/min, which demonstrates a robust effect of S1P on endothelial cell migration under this condition. Both ranges display 95% of the total data that were collected. Though we hypothesized that the rates of S1P stimulated control cells would be higher than that of untreated MLCK, the results do not fit these expectations. Possible explanations for this observation are outlined in the discussion section. The sample size compared for the two movies are different, n=11 for unstimulated MLCK1 infected cells and n=7 for stimulated cells and may explain why the standard error of mean displays a higher value for the latter case.
Figure 33: Normalized data curve for MLCK and no stimulation data set

Figure 34: Normalized data curve for MLCK and S1P stimulation data set
IV. DISCUSSION

AFM studies were focused on determining the effect of elasticity in terms of Young’s modulus in the absence and presence of barrier-modifying agonists on two types of lung endothelial cells. S1P and HGF were tested to see their positive effects on cells by enhancing barrier function visually and through numerical values. Thrombin was tested to characterize the biophysical effects of its barrier disruptive activity through monitoring the elastic modulus. Previous AFM studies with HPAEC indicate an average unstimulated cell height of 2.5 µm at the nucleus and down to 0.65 µm in the cytoplasmic region for live cells. [52, 53] For fixed cells a maximum height of 1 µm was observed, which is consistent with the fact that fixed cells also displayed higher elastic modulus values in the same study. Previous studies also indicate elastic modulus for untreated control cells to range from 0.1-1 MPa, [52, 54] which is a broad range and values represented in the graphs above are encompassed within this range. Migration assays served as a basis for comparing the rates of wound healing and compare them with the elasticities giving a final combination of results. The differential effects of S1P, HGF and thrombin as agonists on cytoskeletal elements are shown to correlate with elastic modulus and their respective therapeutic effect in terms of rate of wound healing.

S1P:

Our results demonstrate that S1P increases the peripheral elastic modulus in lung EC at treatment times of 10 minutes and 30 minutes. While HPAEC demonstrate an increase in elastic modulus of 3.2 KPa and 4.2 KPa over the control values of 2.6 KPa recorded on an average for control cells, HLMVEC exhibit lesser increases of 0.6 KPa and 1 KPa only. These differential effects on macro- compared with microvascular lung EC are not surprising given the multiple
phenotypic differences between these cell types reported in earlier studies. Contractile forces within the cell are regulated by tethering forces between different cells and between each cell and its underlying matrix. [57] S1P induces a decrease in height and effective cell flattening thus measuring a maximum height of 1 mm at the nucleus in the AFM images recorded. Initially, S1P stimulation causes rapid cortical actomyosin redistribution in the EC cytoskeleton which explains the well spread out height response from the AFM images. [57, 58] Rac protein has been determined to be an important factor mediating the downstream action of S1P in translocating cortactin (F-actin binding protein) towards the cell periphery followed by formation of stable adherens junction and creation of focal adhesion complexes on the cell membrane after S1P stimulation. For this reason, AFM elasticity measurements were made towards the cell periphery in this study and thus produced results of increased elastic modulus which is concurrent with the actin arrangement in the periphery regions. [57, 59] The cortical actin ring tightens the barrier causing decreased permeability and preventing movement of paracellular fluid and solute translocation. The image obtained displays shortened heights of the cell structure due to continuous arrangement of fibers along the periphery following formation of the cortical ring. As studied earlier, disruption of peripheral actin filaments causes height increases [60, 61] and regular arrangement would cause a decrease in height, which is supported by our observations. S1P treatment of endothelial cells induces rearrangement of actin fibers at the periphery, and our elastic modulus measurements support the data. The differences in responses elicited within HPAEC and HLMVEC are not completely understood and may relate to a longer response time of the agonist in HLMVEC due to slightly larger cell sizes (or lower ratio of actin filament rearrangement required for barrier protection), changes in stimulation pathway or variations in cell culturing for the two types of cells.
Actin-myosin interaction is regulated by MLCK phosphorylation of MLC, which in turn affects the actomyosin-based contractile forces appearing in the vascular endothelium. [38] Endothelial paracellular permeability and hyperpermeability may be controlled by MLCK dependent or independent mechanisms. [39] Considering the MLCK dependent pathway, phosphorylated MLC co-localizes with cortactin in the cortical actin ring in S1P stimulated EC, allowing MLCK to translocate to the region. The phosphorylation of MLC causes myosin to interact with actin and stabilizing the actin cytoskeleton which in turn stabilizes the cortical interactions with the adhesive elements. [57] Inhibition of MLCK activity can cause intracellular gap formation in a monolayer, loss of peripheral catenin and contractile cytoarchitecture. [38] It has been demonstrated that EC MLCK and cortactin interaction is a necessary condition for maximal S1P induced MLC peripheral phosphorylation. [62, 66] In wound closure studies reported here, a lower rate of wound closure is observed in unstimulated MLCK infected cells compared with S1P treated MLCK infected cells. It is important to note that permeability responses in endothelial cell lines may not resemble the microvascular exchange processes in vivo because they are derived mainly from non-exchange vessels. The confluence or monolayer requirement may also be a factor affecting varied rates in case of control cells.

FTY720:

The results obtained with FTY720 are consistent with prior studies of this agent. It is an analogue of S1P but does not produce a barrier enhancing response in cells within 5 minutes of treatment as S1P does. It takes a minimum of 30 minutes to begin to elicit barrier enhancement [68]. This has been supported by its corresponding Young’s modulus values. The 10 minute treatment group did not display any change in elasticity from the control cells but the 30 minute treatment group has a 1.7 KPa hike in Young’s modulus. The delayed response is concurrent with previous results of decreased cortical actin formation towards the periphery as well.
FTY720 is an FDA-approved medication for multiple sclerosis [31], so this drug could rapidly be tested in trials of patients with ALI as an alternative to S1P.

Figure 35: Phalloidin staining in HPAEC displaying F-actin filaments under control, 5 minute S1P treatment and 5 minute and 30 minute FTY720 treatments [26]

HGF:

HGF has a barrier enhancing effect similar to S1P but has been shown to elicit the effect to a lower extent than S1P. HGF also augments the cortical actin ring and leads to the dissolution of cytoplasmic actin stress fibers. Increased actin rearrangement and improved adherens junction integrity are due to the cadherin/β-catenin association with the cytoskeleton along with increase in phosphorylated MLC chains near the periphery similar to S1P. The results for HLMVEC elasticity measurements demonstrate a different trend as compared to S1P. The 10 minute treatment time demonstrates a higher elasticity value as compared to the 30 minute treatment time. The HPAEC demonstrate an increase in elasticity with increased treatment time, and this is consistent with that of S1P. In HLMVEC, longer treatment times (greater than 30 minutes) with HGF may lead to a weaker effect as was observed with some of the enantiomeric forms of FTY720. [31] This could be related to a condition where increased treatment with HGF failed to reverse the negative effect created by an agonist (cytochalasin B – disrupts F-actin fibers), but
S1P is capable of reversing the barrier disruptive effects of thrombin, and HGF also shows the same effects on thrombin activity [64].

![Figure 36: Phalloidin staining in HPAEC displaying F-actin filaments under control, 5 minute and 15 minute HGF treatments [33]](image)

Barrier function increases with HGF are due to increased focal adhesions or cell-cell tethering produced by cadherin linkage via the catenin to the actin cytoskeleton. [63] HGF has not been as widely studied as S1P probably because of the lower significant effects that is possesses in comparison to S1P for the same treatment conditions.

**Thrombin:**

The AFM images display variations in deposition of fibers as observed from the color changes on a scale that has a maximum height of 2 µm as opposed to 1 µm seen for the previous two conditions. The maximum limit of the scanner was surpassed at some locations making it impossible to obtain a clear image. Prior research has measured nucleus heights of up to 5 µm with thrombin treatment. [52] There is visible rounding of cells and actin rearrangement in thrombin treatment conditions. Previous studies have established that thrombin induces cell rounding and retraction of EC with widening of interendothelial junctions. AFM studies of endothelial cells displayed an increase in cell height with actin depolymerization, and this is an immediate effect of thrombin and other barrier disrupting agonists. Studies reveal that disruption of F-actin fibers not only increase cell height but also decrease the elastic modulus, this being
consistent with the results obtained for the imaging and measurement of Young’s modulus.\[61\] Thrombin has been shown to balance the contractile and tethering forces in the formation of a network of actin stress fibers and cellular contraction, increased actomyosin interaction, cellular contraction along these cables, disruption of adhesion complexes and paracellular gap formation. Thrombin has also been shown to activate a pathway that causes MLCK independent stress fiber formation. \[52, 57\] Research related to elastic modulus mapping has demonstrated that thrombin treatment causes higher elastic modulus at the nucleus site with decreased values towards the cytoplasm. \[52\] Our data add to those results through recording of the numerical values of elastic modulus at the periphery which have minimal cytoskeletal elements and higher gap creations. Thrombin stimulation causes actin stress fiber formation, increased intracellular tension, paracellular gap formation and reduced transendothelial monolayer resistance indicating increased permeability. \[57, 59\] Thrombin also induces a decrease in cortactin at the periphery (HPAEC) as opposed to S1P activity \[52, 65\]. This finding contributes to the altered periphery structure after thrombin leading to lower elastic modulus values.

![Figure 37: Phalloidin staining in HPAEC displaying F-actin filaments under control and 5 minute Thrombin treatment \[35\]](image-url)

HLMVEC also demonstrate a similar trend with significantly decreased values of elastic modulus with increasing thrombin treatment time. Thrombin appears to affect cytoskeletal
elements through similar pathways at equal treatment times in HPAEC and HLMVEC but produces a lower degree of F-actin disruption and gap creation in HLMVEC than HPAEC. These findings suggest that a reduced level of barrier dysfunction and vascular leak are induced in HLMVEC compared to HPAEC by thrombin under these conditions.

All treatment groups have displayed lower numerical values for HLMVEC elastic modulus than HPAEC. This likely is an indication of varied mechanical properties of the two types of endothelial cells. There could be many reasons for the varied response in case of biological parameters- number of actin filaments involved in barrier regulation are lower for HLMVEC, increased drug dosage may be required to elicit a higher response with S1P and thrombin, receptors may be expressed at different levels, or existence of slight variations in the stimulation pathways at the molecular scale. [67, 69] Reasons on a more materialistic scale due to human error are discussed below.

Important potential limitations and/or sources of error in this work are as follows:

- Effects of agonists could be affected by sample preparation technique and AFM measurement procedure
- Individual cells were measured on each sample for AFM and these may have been in different growth stages
- Earlier studies with multiple tips used for AFM studies on the same sample have produced varied results in spite of accurate calibration
- HPAEC are known to have poor adhesion making live cell imaging complicated
- It is difficult to monitor the exact time scales of agonist/antagonist action on the cells under the AFM [52]
- Actin rearrangement and improved adherens junction integrity are due to the cadherin/β-catenin association with the cytoskeleton along with increase in phosphorylated MLC chains near the periphery similar to S1P.

- **S1P**
  - INCREASED ELASTIC MODULUS - PERIPHERY
  - Cytoskeletal rearrangement
  - Adherens junction assembly
  - Focal adhesion assembly
  - Increases rate of wound healing
  - FTY720 increases modulus for treatments longer than 30 minutes

- **Thrombin**
  - DECREASED ELASTIC MODULUS - PERIPHERY
  - Actin stress fibers formed
  - Increased actomyosin interaction
  - Cellular contraction
  - Disruption of adhesion complexes
  - Paracellular gap formation

- **HGF**
  - INCREASED ELASTIC MODULUS - PERIPHERY
  - Actin rearrangement - periphery
  - Adherens junction assembly
  - Assembly of focal adhesion complexes

Reverses the effect of thrombin
V. CONCLUSION

AFM has proven to be an important tool in the biological world with more specific applications, especially in determining biomechanical properties of cells. Elasticity maps of HPAEC have been compared in previous studies with accurate height range measurements. This thesis functions as an extension which provides specific Young’s modulus values for each cell type and its treatment condition. S1P, FTY720, and HGF display their barrier enhancing effects, and thrombin displays barrier disruptive effects which are confirmed through elasticity measurements. The variations observed among two types of endothelial cells provide additional insights into the mechanisms regulating vascular barrier function.

Migration assays have been standardized to a level of understanding the basic parameters involved. Creating a scratch of acceptable width with minimized irregularities, imaging cells with required confluence, regulating the cell migration environment (like maintaining temperature and CO₂ content) and using various analysis tools have been resolved. This allows more room for further development and reduces the number of limiting parameters. MLCK adenovirus produced results as would have been expected, but the control cells did not display expected increase in migration rate under S1P treatment. Both studies are concurrent with reduction of endothelial permeability and decreased fluid edema in animal models of ALI.

Future research can be targeted at specifically measuring elasticities after S1P and HGF treatments of longer time intervals in HLMVEC to see if the response eventually equals that of HPAEC. In addition, earlier time points can be examined for the S1P response in HLMVEC to see if it is particularly rapid and peaks before 10 minutes. Other barrier altering agonists can be examined in these assays to compare their biophysical effects on lung EC with those of the agonists described here. Specific labeling of actin filaments before AFM measurements to
determine the elasticities of the actin filaments would be a more specific approach. Migration assays can be further developed by fluorescently labeling cells and observing individual cells migrating over a barrier in unison with the surrounding cells.
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