The Cellular Mechanoresponse:
Single-Cell Studies by Atomic Force Microscopy

by

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Abstract

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Cells in their native environment are bombarded by mechanical signals ranging from strains within a developing embryo to stiffening of diseased tissue. How these extracellular mechanical signals are converted to biological activity on the cellular scale is a complex and unresolved problem in biology with implications in development and disease. This dissertation focuses on development and implementation of Atomic Force Microscopy (AFM)-based techniques to probe the interactions of cells with the mechanical microenvironment and use of these techniques towards characterizing and explaining the cellular mechanoresponse.

We began by integrating a DNA-based adhesion technology with AFM that enables the manipulation of cells by a cantilever without influencing cell viability or signaling. This technique surpasses existing approaches both in the tunability and magnitude of adhesion strength to allow single-cell de-adhesion experiments that measure cell-ligand bonds without cell-surface rupture.

The first step in the cellular mechanoresponse is the translation of an extracellular mechanical signal to an intracellular mechanical signal. We used high-resolution three-dimensional multi-particle tracking to measure how local stress applied by an AFM cantilever is propagated through an adherent cell. We observe a distance-dependent propagation on the timescale of seconds that requires an intact cytoskeletal network. This slow stress propagation is consistent with a poroelastic description of the cell that defines the timescales and lengthscales over which external stresses can be transmitted through cells.

Recent studies have demonstrated that cells exhibit stiffness-dependent behaviors over long timescales, but the mechanism of how cells sense stiffness over short timescales remains particularly elusive. To study early events in stiffness sensing, we developed a feedback algorithm that enables dynamic and reversible control of the stiffness exposed to a single cell. We employ this stiffness clamp technique to
study the contractile response of cells to sudden changes in extracellular stiffness. We find that the cell contraction velocity and tensile rate adapt by accelerating upon a step decrease in stiffness or decelerating upon a step increase, all on a timescale of seconds. This seconds-timescale adaptation is independent of focal adhesion signaling, but depends strongly on cell contractility suggesting that extracellular stiffness signals are filtered by the viscoelastic cytoskeleton.

Together, the techniques described here provide novel and tunable control of the mechanical signals presented to and measured from a single cell with AFM precision. The results obtained using these techniques describe important timescales and considerations towards understanding the cellular mechanoresponse.
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Chapter 1. Introduction
1.1 Overview

Any student of cell biology can note the importance of mechanics in vivo: pushing and pulling of cells in a developing embryo, stretching of cells in an expanding lung, contracting of cells in a beating heart, and fluid shear on endothelial cells exposed to blood flow. Nearly a century ago D’Arcy Wentworth Thompson (zoologist, mathematical biologist, and classics scholar) applied the laws of physics and mechanics to explain biological form and morphology in his 1917 treatise “On Growth and Form” as a challenge to the all-encompassing evolutionary theory of his day (Thompson, 1917). Indeed, cells in their native environment are constantly bombarded by mechanical signals and changes in the mechanical environment are often linked to disease. For example disturbed or turbulent blood flow leads to atherosclerosis, improper loading results in bone loss and osteoporosis, and cancerous tumors are often identified by palpation of stiff tissue.

During D’Arcy Thompson’s era, the precise role and importance of these mechanical signals was difficult to study due to the lack of tools and techniques to both quantify and control the mechanical inputs presented to cells. In the last 50 years an impressive number of interdisciplinary studies have emerged applying concepts of physics and engineering to understand how these mechanical inputs are transduced to biological responses.

It is precisely this conversion from mechanics to biological activity that has been a continuous challenge to characterize and explain. Many studies have reported mechanically dependent cell behaviors including morphology (Peyton and Putnam, 2005), motility (Lo et al., 2000), proliferation (Ghosh et al., 2007), differentiation (Engler et al., 2006), tumorigenesis (Paszek et al., 2005), and gene expression (Li et al., 1987). However understanding the mechanism of the mechanoresponse – the conversion of extracellular mechanics to biological cell activity - requires further investigation. Early work in the field describes the mechanoresponse as a two-step process: first the extracellular mechanical input must be transmitted to an intracellular mechanical signal, that is then transduced to a biochemical signal. The second step requires a mechanosensor or transducer while the first step involves material properties or a mechanical filter (Grigg, 1986; Hsu, 1968). These two classifications are still relevant today and a number of relevant studies have focused on different pieces of the mechanoresponse puzzle as I will describe below.

This dissertation describes the development of Atomic Force Microscopy (AFM)-based techniques to probe the interaction of cells with the mechanical microenvironment. The approaches described allow precise control of the mechanical boundary conditions presented to single cells and precise measurement of the cellular mechanoresponse.

In the following introduction I first present examples of mechanical signals in vivo and in each case discuss the techniques used to reproduce and study the cellular
response to these signals in vitro. In sections 1.3 and 1.4 I classify cellular mechanoresponses into two sequential categories: mechanotransmission and mechanotransduction, in keeping with the early classifications of Hsu and Grigg. Finally in section 1.5 I describe Atomic Force Microscopy – the main tool used in my graduate work.

1.2 Mechanical Signals In Vivo and the In Vitro Techniques to Study Them

As described earlier, simple observation and reasoning clearly indicate the existence of mechanical inputs on the cellular level, yet it is far harder to quantitatively measure these signals. Therefore in order to study the cellular mechanoresponse, a number of in vitro techniques have been developed to simulate in vivo mechanical signals. Below I will give examples of in vivo measurements where possible and will then describe the techniques used to simulate these mechanical signals in vitro.

1.2.1 Force and deformation, stress and strain

The most obvious and most intuitive mechanical inputs are force and deformation (aka stress and strain in engineering terms). These are in fact two ways of describing the same external signal from the cell’s perspective and I will therefore address them simultaneously. According to Hook’s Law: $F = kx$, the force, $F$, associated with a given deformation, $x$, is determined by the material properties of the cell – in this case the spring constant, $k$. (The equivalent equation in engineering terms is: $\sigma = E\varepsilon$. This will be further discussed in sections 1.2.3 and 1.3.1) Extreme changes in cell shape during embryogenesis, indicative of large deformations and strong forces, have fascinated developmental biologists since D'Arcy Thompson (Paluch and Heisenberg, 2009). While in vivo measurements of strain are still incredibly difficult and rare, advances in microscopy have enabled optical measurement within developing embryos and implanted strain gauges allow direct measurement of strain within bone tissue as described below. These in vivo measurements provide motivation and context for in vitro studies. Imaging of amnioserosa cells during closure reveal a 14-27% decrease in projected cell surface area due to purse-string contraction and apoptosis of neighboring cells (Toyama et al., 2008) and 50% strain in the epidermis of Caenorhabditis Elegans embryos due to contraction of surrounding muscle (Zhang et al., 2011). In addition, laser ablation has been used to study the balance of forces required for dorsal closure (Hutson et al., 2003). Another intuitive case of strain in vivo is lung expansion where epithelial basement membrane surface area increases up to 40% in rats according to digitally analyzed electron micrographs of fixed and isolated Sprague-Dawley rat lungs (Tschumperlin and Margulies, 1999). Measured strains within bone are much smaller. Implanted strain gauges measure ~0.05% strain within human tibiae under
resting conditions, and up to 0.2% strain during rigorous activity (Burr et al., 1996). Failure strain for bone tissue is reported at 3% strain (Jacobs et al., 2010). Finally, while I will not discuss them here, primary cilia and mechanosensory hair cells in the inner ear are one of the best-studied examples of mechanotransduction due to deformation, in this case due to acoustic vibrations. Please see the following reviews for treatment of these topics (Resnick and Hopfer, 2008; Schwander et al., 2010).

Stretched Substrates
Cells may be easily deformed in vitro by simply stretching the substrate to which cells are adhered. This method was first implemented to stretch Xenopus laevis embryos revealing the importance of tension for organ orientation and the development of intercellular contacts (Belousoff et al., 1988). Stretch may be applied uni-axially, bi-axially, or axisymmetrically over a wide range of magnitudes and frequencies to best mimic physiological conditions (Trep at et al., 2007) and the popularity of this technique has even led to commercially-available systems by FlexCell® and other companies. Local deformations may also be applied by pulling or pushing on the flexible substrate near a cell.

Probes: microneedles, microplates, microposts, and cantilevers
On a whole-cell scale microplates or cantilevers may be used to compress or stretch cells, the latter requiring adhesion to two surfaces. This approach has been used to simulate pressure applied to Caenorhabditis Elegans epithelium by surrounding contractile muscle (Zhang et al., 2011) and to determine the mechanical properties of single cells (Thoumine and Ott, 1997). Locally, microneedles were initially used to poke and prod cells (Maniotis et al., 1997; Heidemann et al., 1999) to determine the mechanical linkage of intracellular components. More controlled forces have since been applied using AFM cantilevers (Mathur et al., 2000; Rosenbluth et al., 2008; Watanabe-Nakayama et al., 2011) or magnetically-controlled microposts (Sniadecki et al., 2007) to determine the propagation of a local mechanical perturbation across the cell either mechanically or biochemically. These two approaches are complementary in that AFM can apply controlled forces perpendicular to the spread axis of the cell whereas magnetic microposts can apply controlled forces parallel to the spread axis. In both cases geometry of the tip or post and ligand coating are chosen to determine the nature of the interaction.

Optically trapped beads
For very precise control of local deformations, nanoparticles or beads may be coated in ligand, attached to the cell surface, and controlled by an optical trap or laser tweezers. Briefly, a dielectric particle will be attracted to the point of highest electric field, in this case the focal point of a laser beam, allowing for manipulation of the particle by adjusting the beam. Forces up to 100 pN may be applied, beyond which the required laser power is damaging to cells. For further information on the capabilities of optical trapping please see (Sheetz, 1998; Moffitt et al., 2008) and associated references. The Sheetz lab has conducted several seminal studies using laser tweezers to demonstrate that if resistance is applied at a focal adhesion, the adhesion will be reinforced (Choquet et al., 1997; Galbraith et al., 2002). In addition,
laser tweezer manipulation of a bead was used to demonstrate a novel FRET reporter to track intracellular Src signaling upon local mechanical perturbation (Wang et al., 2005).

**Magnetic pulling & twisting cytometry**

Similar to optical trapping, magnetic beads may be ligand-coated and precisely controlled using magnetic fields. A magnetic microneedle can apply tensional forces to superparamagnetic beads for magnetic pulling cytometry or small ferrimagnetic particles may be manipulated using a sinusoidal magnetic field to oscillate and twist the sites of adhesion to cells. These methods have been used to determine the propagation of a local mechanical perturbation across the cell either mechanically or biochemically (Deng et al., 2006; Glogauer et al., 1995; Hu et al., 2003; Matthews et al., 2006).

### 1.2.2 Fluid Shear

Fluid shear is another intuitive and long-studied source of mechanical signaling. Vasodilation due to increased arterial blood flow was observed as early as 1933 (Schretzenmayr, 1933) and since then blood flow has been shown to influence a number of signaling pathways, cytoskeletal organization, and gene expression (Davies et al., 1995; Davies et al., 2005). Shear stress magnitudes in vivo have been estimated using geometries and flow rates of blood vessels and are reported to be 20-40 dyn/cm² in large vessels with relatively laminar flow, 40-50 dyn/cm² in more turbulent sections, and up to 100 dyn/cm² in cases of hypertension (Davies, 1995). Endothelial cells are subjected to both cyclic strain and fluid shear stress and are therefore a good model to study simultaneous mechanosensory mechanisms. Fluid shear stress by nodal flow is involved in establishing left-right asymmetry in vertebrates (McGrath et al., 2003) and it has been proposed that urine flow triggers polycystins that modulate kidney tubule growth (Delmas, 2004; Praetorius and Spring, 2005).

**Flow chambers and microfluidics**

Fluid shear is well-studied thanks to the relatively easy in vitro techniques available to study the effect of fluid flow on cells. Cell culture flow systems – whether macroscopic or on the microfluidic scale - allow control of the flow rates and turbulence exposed to cells by specifying flow rates and chamber geometry and are easily integrated with imaging systems (Dewey et al., 1981; Young and Simmons, 2010). Fluid shear may also be applied locally with a micropipette as done by Bohnet et al. to study the forces necessary to stall protrusion of the keratocyte lamellipodium (Bohnet et al., 2006).

### 1.2.3 Stiffness, Elasticity

Stiffness sensing is distinct from the sensing of force, deformation, and fluid shear in that it requires active probing of the cell. Specifically, a cell only senses extracellular
stiffness if it applies a force to the external environment and “senses” the resulting displacement: $\Delta F = k \Delta x$. The elasticity of explanted tissues have been measured by atomic force microscopy ranging from ~1kPa for brain to ~10kPa for muscle to ~100kPa for bone (Engler et al., 2006). Furthermore developing tissues have been shown to stiffen over time with myocardial elasticity increasing from ~1 to 10kPa from 50-400 hours post-fertilization (Young and Engler, 2011). And diseased tissue is often measured as stiffer than normal tissue. For example normal mammary tissue is ~170 Pa whereas diseased tissue is ~4kPa (Paszek et al., 2005). Stiffness influences can be indirect. For example, the structural interactions and large discrepancy in stiffness between individual chondrocytes and the surrounding tissue results in particular distributions of stress and strain within the cell, and this has implications for the bulk mechanical properties of bone (Guilak and Mow, 2000).

It is worth noting briefly that stiffness, elasticity, and rigidity are all used in the field. Comparison between studies often requires converting from stiffness to elasticity or vice versa, which depends on the geometry of the system: $E = \frac{\sigma}{\varepsilon} = \frac{3F}{A l} = k \frac{l}{A}$, where $A$ is the contact area and $l$ is a characteristic length of the system. Appropriate values for $l$ and $A$ can be difficult to define and are often changing over the course of the experiment. This conversion can therefore only be used for rough calculations except in very well-defined scenarios.

**Hydrogels**

Hydrogels made of polyacrylamide allow tuning of stiffness via crosslinking and attachment of any ligand via surface chemistry making them a popular method for studying the role of stiffness in cell morphology (Peyton and Putnam, 2005), motility (Lo et al., 2000), development (Engler et al., 2006), cancer progression (Munevar et al., 2001; Wang et al., 2000), and many other cellular processes. For a more physiologically-relevant geometry, cells are embedded in gels such as collagen or matrigel (Paszek et al., 2005); however varying the stiffness in this case requires varying collagen concentration, which may have unwanted side effects. A number of other biocompatible materials of tunable stiffness have been developed for research purposes and for tissue engineering (Banta et al., 2010; Boontheekul et al., 2007).

**Micropillars**

Micropillars, developed in the Chen lab at the University of Pennsylvania, also provide 2D surfaces of tunable stiffness by varying the geometry of microfabricated pillars or columns to which cells may adhere (Fu et al., 2010). The force, $F$, exerted by a cell on the micropillar is calculated as: $F = \frac{3EI}{L^3} x$ where $E$ is the elasticity of the pillar material (often PDMS), $I$ is the area moment of inertia (calculated based on geometry of the pillar), $L$ is the height of the pillar, and $x$ is the resulting shift of the top of the pillar. This technique is limited to 2D, but has been used extensively by
the Chen lab and others to study cellular rigidity sensing (Ghibaudo et al., 2008; Yang et al., 2011).

Atomic force microscopy and microplates
Finally, both the Fletcher Lab and the Asnacios group at the Centre National de la Recherche Scientifique and Université Paris-Diderot have used flexible levers – either an AFM cantilever or a flexible microplate – to expose single contracting cells to a stiffness signal defined by the spring constant of the flexible lever (Lam et al., 2011; Mitrossilis et al., 2009; Mitrossilis et al., 2010). In these systems, the force, \( F \), exerted by the cell is calculated as \( F = k_{\text{lever}} \times x \) where \( x \) is the deflection of the lever (measured by video microscopy for the microplate system and an optical lever system in the case of AFM as described in section 1.5) and \( k_{\text{lever}} \) is the spring constant of the flexible lever, determined empirically. Chapter 4 of this dissertation explores a novel AFM-based technique to dynamically vary the stiffness signal presented to a single cell and Chapter 5 makes use of this technique to explore the timescales of stiffness sensing.

1.2.4 Geometry
In vivo cells are rarely presented with smooth flat surfaces, but are often embedded in fibrous networks or confined to a defined space or geometry. The concept of cellular contact guidance describes the behavior of cells moving along a topographical feature. While geometrical cues presented to single cells have not been characterized in vivo to my knowledge, the effects of geometrical constraints and features are well-studied in vitro and therefore deserve mentioning.

Microfabrication and nanopatterning
Patterned ligand has been used to restrict cell adhesion and spreading. For example ligand carefully spaced with gold nanoparticles indicates the minimum RGD-spacing for stable integrin-based adhesions (Cavalcanti-Adam et al., 2007) and patterning sub-cell sized areas of ligand determines the threshold contact area for growth and apoptosis (Chen et al., 1997). Furthermore microfabrication enables control of surface topography. Sea urchin embryos have been cultured in microwells to determine the effect of confined 3D geometry on cell division (Minc et al., 2011). Microfabricated surfaces have demonstrated contact guidance of features as small as 11 nm (Curtis and Wilkinson, 1999) and fibroblasts have been shown to crawl in a specific fashion using a specific myosin isoform on a single collagen fiber (Meshel et al., 2005). For further details on the use of microfabrication and nanopatterning for studies of mechanosensing please see the following reviews (Geiger et al., 2009; Vogel and Sheetz, 2006).
1.3  Mechanotransmission: The cell as a material

Before any biochemical signaling can occur, an extracellular mechanical signal must first be transmitted into the cell to become an intracellular mechanical signal, and this transmission depends directly on the material properties of the cell. Will an external force elastically deform the cell? Viscoelastically deform it? Will the deformation persist or relax over defined timescales? Will the deformation remain local or propagate across the cell? Fortunately numerous studies have been conducted to describe the cell’s material properties; however each technique yields a different answer and there are several strongly supported, but competing theories. I will outline each briefly below in the interest of understanding the timescales and lengthscales over which mechanical signals may be propagated or mitigated within the cell. It should be noted that there are entire books and lengthy reviews devoted to this topic. For reading beyond the cursory information presented here please see the following (Boal, 2003; Fung, 1993).

All of the models of cell mechanics described below have been useful in describing different aspects of the cellular mechanoresponse. Pure elasticity is an overly simple view of the cell in most contexts, but is an important component of viscoelasticity and poroelasticity, which are explored and compared in detail in Chapters 3 and 5. While the specific model of tensegrity is not as widely used, the importance of pre-stress and intracellular tension is a recurring theme in studying the cellular mechanoresponse and is directly employed in Chapter 3. Finally, the specifics of the soft glassy rheology and power law models have not been as applicable for the experiments described here; however the concept of no single distinct timescale for the cellular mechanoresponse is important when considering the myriad of biological processes acting simultaneously within the cell at any given time to produce an evolving mechanoresponse.

1.3.1  Elasticity

Applying a force to a cell will result in a deformation that varies greatly by cell type and context. Section 1.2.3 described the elasticities of different tissues and cells have been shown to mimic the rigidity of their environment (Alcaraz et al., 2008; Solon et al., 2007). Cell elasticity has been studied in a number of different contexts including relative measurements of leukemia cells (Rosenbluth et al., 2006). Perhaps more informative than whole-cell stiffness are the stiffnesses of intracellular components that may transmit or absorb forces. Several publications have suggested that a rigid cytoskeleton may directly propagate forces allowing for mechanical coupling or “action-at-a-distance” across the length of a cell (Janmey, 1998; Wang and Suo, 2005); whereas proposed mechanosensors are often flexible proteins that will measure deformation upon applying a specific force, as will be discussed in sections 1.4.2 and 1.4.3.
<table>
<thead>
<tr>
<th>Model</th>
<th>Cartoon</th>
<th>Governing equation</th>
<th>Characteristic Timescale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elasticity</td>
<td><img src="image1.png" alt="Image" /></td>
<td>$F = kx \quad (\sigma = E\varepsilon)$</td>
<td>Immediate</td>
</tr>
<tr>
<td>Viscoelasticity (Voigt)</td>
<td><img src="image2.png" alt="Image" /></td>
<td>$F = kx + \gamma x$</td>
<td>$\tau_\sigma \frac{\gamma}{k}$</td>
</tr>
<tr>
<td>Viscoelasticity (Kelvin or Standard Linear Solid)</td>
<td><img src="image3.png" alt="Image" /></td>
<td>$F = k_1 x + i\gamma(1 + \frac{k_1}{k_2}) - \frac{\gamma}{k_2} \dot{F} + \gamma \frac{\gamma}{k_2} (1 + \frac{k_1}{k_2})$</td>
<td>$\tau_\sigma = \frac{\gamma}{k_1} (1 + \frac{k_1}{k_2})$</td>
</tr>
<tr>
<td>Poroelasticity</td>
<td><img src="image4.png" alt="Image" /></td>
<td>$F = \partial_x (\phi p - E \partial_x u)$</td>
<td>$\tau_{eq} = \frac{x^2 \phi}{4KE}$</td>
</tr>
<tr>
<td>Tensegrity &amp; pre-stress</td>
<td><img src="image5.png" alt="Image" /></td>
<td>NA</td>
<td>Immediate</td>
</tr>
<tr>
<td>Soft glassy and power law rheology</td>
<td><img src="image6.png" alt="Image" /></td>
<td>$E' \propto f^a$</td>
<td>None</td>
</tr>
</tbody>
</table>

**Table 1.1 Comparison of models of cell mechanics.** $F$ is force. $x$ is deformation. Viscoelasticity: $\tau_\sigma$ is the relaxation time constant under constant stress. $\tau_e$ is the relaxation time constant under constant strain. (Fung, 1993) Poroelasticity: $\phi$ is the fluid fraction. $p$ is the fluid pressure. $E$ is the elastic modulus of the network. $u$ is displacement of the network. $\phi$ is the local fluid volume fraction. $v$ is the fluid velocity. $K$ is the hydraulic permeability. $\tau_{eq}$ is the timescale for the pressure gradient to equilibrate over distance $x$. (Charras et al., 2005; Rosenbluth et al., 2008) Power law: $E'$ is the elastic modulus. $f$ is the frequency of perturbation. $a$ is the scaling exponent.
1.3.2 Viscoelasticity

A viscoelastic material behaves elastically over short timescales and displays a viscous relaxation response over longer timescales. Any combination of springs (the elastic element) and dashpots (the viscous element) may be assembled to represent a viscoelastic response and two examples – the Voigt model and Standard Linear Solid (or Kelvin) model are highlighted in table 1.1. While measurement of the elastic properties of a cell are fairly simple, measuring the viscous nature of the intracellular environment has proven quite difficult yielding viscosity values that span several orders of magnitude depending on the technique used (Kreis et al., 1982; Mastro et al., 1984; Paine et al., 1975). Consequently many studies model the cell or certain components of the cell as viscoelastic to obtain fitted values for each spring and dashpot, instead of measuring viscosity directly (Thoumine and Ott, 1997; Bausch et al., 1998; Trickey et al., 2000). This approach is used in Chapter 5. In summary, a viscoelastic model describes both an immediate elastic response and a slower viscous relaxation for a homogeneous material.

1.3.3 Poroelasticity

Several studies have applied poroelasticity – a theory developed to describe sand or soil (Biot, 1941) – to cells. Parallel to porous soil, cells are described as a dense cytoskeletal network with interstitial cytosolic fluid (Charras et al., 2008; Charras et al., 2005; Mitchison et al., 2008). Upon deformation, a poroelastic material first deforms elastically creating a local buildup of pressure, which is then released through flow of interstitial cytosol to areas of lower pressure. The equilibration of pressure away from the point of deformation, described in table 1.1, introduces a spatial dependence that is not described by viscoelasticity. These two models will be directly compared in Chapter 3.

1.3.4 Tensegrity and pre-stress

Donald Ingber has championed yet another model of cell mechanics that builds on an old idea of Buckminster Fuller describing structures as having opposing compressive and tensile elements. Ingber’s theory of tensegrity applied to cells proposes that actin stress fibers are in a constant state of “prestress” while microtubules are under compression and further supported by intermediate filaments. Under this assumption, perpendicular forces cause actin filaments to respond like the taut strings of a violin, and longitudinal forces may be propagated over long distances along both actin bundles and microtubules. (Ingber, 1998; Ingber, 2006; Matthews et al., 2006; Wang et al., 1993) While the exact theory of tensegrity has been supported predominantly by Ingber himself, the concept of pre-stress and the cell being constantly under tension is strongly supported by inward-directed traction forces and the relaxation of laser-cut stress fibers (Kumar et al., 2006).
1.3.5 Soft Glassy rheology and power law rheology

The models described thus far can all be characterized by specific timescales of relaxation; however a final class of models is specifically defined by the lack of a defined relaxation timescale. The intracellular environment is incredibly crowded and therefore potentially comparable to a category of materials represented by soft glasses, ketchup, and toothpaste. Consider sand in an hourglass or coffee beans in a dispenser in which particles are so crowded as to be trapped in a metastable configuration. Perturbing the system may jostle the components just enough to rearrange or remodel them, as is sometimes necessary to get the coffee beans out! Fredberg and others argue that the cellular cytoskeleton similarly fluidizes and remodels when subjected to mechanical perturbation. The timescales of these rearrangements are generally longer than those described in other models and scale-free. (Bursac et al., 2005; Deng et al., 2006; Trepat et al., 2007) It was the scale-free nature of mechanical measurements that first indicated the potential similarity between the intracellular environment and soft glasses. A number of studies found elastic stress to be related to the frequency of perturbation by a power law (Alcaraz et al., 2003; Fabry et al., 2003; Hoffman et al., 2006) as described in table 1.1, indicating an uncommon overlap of condensed matter physics and biology.

1.4 Mechanotransduction: The cell as a biochemical machine

As discussed in section 1.3, the effect of transmitting extracellular mechanical inputs to intracellular mechanical signals is to change the arrangement of components and force balance within the cell. How then is altered structure or force balance transduced to a biochemical signal? This question remains unresolved, but an impressive amount of progress has been made and is well-described in the literature (Chen, 2008; Moore et al., 2010). This complex topic requires a more in-depth treatment than is possible within the confines of this introductory section. I therefore will highlight the important points relevant to this dissertation and recommend reviews on specific topics wherever possible.

1.4.1 Key players

The number of proteins proposed to be involved in integrin-mediated mechanosensing has grown so large that they have collectively been referred to as their own organ – the mechanosome (Bidwell and Pavalko, 2010) or adhesome (Zaidel-Bar and Geiger, 2010)! I will first introduce the key players before describing proposed mechanisms of mechanosensing.
Focal adhesions

An obvious suspect for the transmission of mechanical signals into the cell is the point of contact with the extracellular environment – a cell’s adhesions. These are complex and evolving structures, but the basic architecture was recently imaged by Clare Waterman using high-resolution microscopy, as shown in Figure 1.1 (Kanchanawong et al., 2010). Briefly, integrins - a family of heterodimer transmembrane proteins - span the cell membrane anchoring to extracellular ligands outside the cell and providing the base for the adhesion inside the cell (Hynes, 2002). Instead of a simple linkage between the integrin anchors and the cytoskeleton, focal adhesions are complex, dynamic (protein exchange rates ranging from seconds to minutes (Giannone and Sheetz, 2006)), and highly responsive to the extracellular environment. Nascent adhesions develop by incorporating additional proteins – paxillin, FAK, Src, talin, zyxin, vinculin - as the force sustained by the adhesion increases (Gardel et al., 2010). Intriguingly, many of the proteins composing focal adhesions have multiple binding partners – both structural and signaling-related, as illustrated in Figure 1.2 (Zamir and Geiger, 2001). Vinculin, for example, is often used as an indicator of focal adhesion maturity and is known to bind to paxillin, talin, the lipid bilayer, F-actin, VASP, vinexin, and PtdIns(4,5)P2 among other proteins. In the closed, inactive state vinculin’s head and tail domains are bound causing the protein to fold over on itself and mask most binding sites. Upon binding of signaling protein PtdIns(4,5)P2, the molecule unfolds revealing the inner binding sites for structural proteins enabling growth and reinforcement of the adhesion via vinculin. This is just one example of the interplay between multiple signaling and structural focal adhesion proteins demonstrating the potential complexity and diversity of focal adhesion architecture and wiring. For further details I recommend the following reviews (Balaban et al., 2001; Geiger and Bershadsky, 2001; Geiger et al., 2009; Giannone and Sheetz, 2006; Riveline et al., 2001; Zamir and Geiger, 2001).

Focal adhesion size has been shown repeatedly to correlate with substrate stiffness (Balaban et al., 2001; Peyton and Putnam, 2005) and local mechanical perturbations elicit a recruitment of focal adhesion proteins and strengthening of adhesions both locally and distally (Riveline et al., 2001; Galbraith et al., 2002; Mathur et al., 2000). In short, the literature strongly indicates that integrin-based adhesions are one avenue for mechanosensing.
**Figure 1.1 Architecture of a focal adhesion as determined by high-resolution microscopy.** This illustration highlights the main structural components of the focal adhesion. (Figure provided courtesy of Nature Publishing Group from (Kanchanawong et al., 2010).)

**Actomyosin**

As shown in Figure 1.1, focal adhesions ultimately attach to filamentous actin. The intracellular-extracellular force balance is maintained by the activity of myosin II motors creating tense actin cables in a cytoskeletal network responsible for the shape and force production of the cell. An impressive literature describes the function of actomyosin contraction within the cell (Howard, 2001; Martin, 2010); however for the sake of this dissertation I will focus on two specific aspects of actomyosin: force production and the force-velocity relationship. A single myosin II head generates between 1.3-3.7 pN (Finer et al., 1994; Guilford et al., 1997) at rates that are both ATP and load-dependent. Myosin contraction within the context of the cell is predominantly directed inward and allows retrograde actin flow at a rate of 50-210 nm/s, or 10-20 nm/s in the presence of focal adhesions, and centripetal integrin movement at a rate of 80-230 nm/s within the lamellipodium of a cell spread on a 2D substrate (Aratyn-Schaus et al., 2011; Moore et al., 2010). Muscle cells are known to exhibit an inverse force-velocity relationship first characterized by Hill (Hill, 1938) and then explained by load-dependent myosin-binding kinetics by Huxley (Huxley, 1957). This aspect of actomyosin interactions is now being applied to non-muscle cells as a potential mechanism for stiffness sensing (Mitrossilis et al., 2009) as will be described in section 1.4.3.

Notably, reduced actomyosin contractility can eliminate cellular sensitivity to rigidity and a response expected on a rigid substrate can be induced on a soft substrate by increasing contractility (Engler et al., 2006; Paszek and Weaver, 2004; Wozniak et al., 2003). The interplay between intracellular and extracellular forces has been described as inside-out and outside-in signaling. This concept is highlighted in Chris Chen’s review of mechanotransduction where he highlights the
importance of force balance by noting that a 1pN imbalance would be enough to break known single-receptor bonds and accelerate an entire cell at 1m/s² (Chen, 2008)! Furthermore, well-formed stress fibers and strong traction forces are considered hallmarks of the cellular response to a rigid substrate (Discher et al., 2005; Peyton and Putnam, 2005). In summary, the literature is full of evidence that actomyosin contractility plays an important role in the cellular mechanoresponse both in maintaining cell mechanics (as described above) and in modulating intracellular tension for mechanotransduction.

**Biochemical Signaling**
A mechanotransduction event is accompanied by changes in the enzymatic activity of a number of enzyme proteins to initiate downstream signaling towards longer timescale responses such as reinforced adhesions, increased contractility, or altered gene expression. Given the complexity of regulating phosphorylation levels for each protein, it is difficult to discern whether phosphorylation levels are directly affected by mechanical stimuli or are simply downstream and indirect effects of other mechanosensors; however a relationship between phosphorylation levels in adhesions and substrate rigidity has been confirmed (Pelham and Wang, 1997). Phosphotyrosine signaling as a result of force application has been studied in several specific cases: including focal adhesion kinase (FAK) (Tomar & Schlaepfer 2009), Src family kinases (Sawada et al., 2006), and Receptor-like Protein Tyrosine Phosphatase alpha (RPTPα) (von Wichert et al., 2003). Phosphomimetic mutations are now being used to further explore these complex phosphorylation networks (Pasapera et al., 2010). Specific regulators of cell contractility control myosin phosphatase activity via myosin light chain kinase (MLCK) and Rho family GTPases such as Rho, Rac, and cdc42. This contractility pathway deserves further reading and I recommend the following reviews to the reader (Kumar and Weaver, 2009; Schmitz et al., 2000).

**Calcium**
The elephant in the room of mechanotransduction research is calcium. Given that calcium is involved in a diverse set of biological processes, it is difficult to tease out a specific role for calcium in the case of mechanosensing. Calcium flashes in response to local forces and deformation have been documented on sub-second timescales (Hayakawa et al., 2008) and inhibition of stretch-activated ion channels by gadolinium or removal of extracellular calcium by EGTA has been shown to mitigate responses to stretch-activation or local perturbation (Beningo et al., 2004; Glogauer et al., 1995; Matthews et al., 2010; Munevar et al., 2004). These results suggest an important, if complex, role for calcium in mechanotransduction.
1.4.2 Where is the mechanosensor?

A pleasingly simple mechanism for mechanosensing is the existence of mechanosensing proteins. Integrins themselves have been proposed to be mechanosensory in that integrin-ligand cooperativity can drive integrin clustering on rigid substrates (Paszek et al., 2009). Furthermore, integrins may operate as a catch bond – one that strengthens under force – giving it an inherently mechanosensitive behavior (Kong et al., 2009). Intriguingly, the actin-myosin bond

**Figure 1.2 The integrin “adhesive”**. Illustration of the many players and connections involved in focal adhesion signaling emphasizes the complexity of the network. (Figure provided courtesy of Nature Publishing Group from (Geiger et al., 2009).)
has also been described as a catch bond (Guo and Guilford, 2006), though this relation is more often invoked when explaining stiffness sensing specifically, as will be described in the next section.

A more commonly proposed mechanism is that the application force or deformation can unfold or stretch a protein to reveal phosphorylation sites or other binding sites. Talin, for example, has been demonstrated to have an extended conformation allowing for binding of vinculin – a protein known to reinforce adhesions as they mature. (del Rio et al., 2009; Kanchanawong et al., 2010) Stretch-induced conformational changes of Cas enable phosphorylation of the substrate domain of p130Cas by Src kinase, which in turn opens further binding sites (Moore et al., 2010; Sawada et al., 2006). Cys shotgun labeling of adherent cells in a tense versus drug-relaxed state reveals shielded cysteines within vimentin and myosin IIa (Johnson et al., 2007). Furthermore, the range of forces required to unfold focal adhesion proteins to reveal cryptic binding sites are indeed physiological (Moore et al., 2010; Vogel, 2006). This theory is also supported by the fact that mechanosensing requires intracellular tension and contractility, as described above.

I would be remiss not to mention stretch-activated ion channels. The major evidence for calcium ion channels as mechanosensors was discussed above and in fact the mechanism is essentially equivalent to other mechanosensors described. The balance of external forces and cell-generated actomyosin forces or membrane tension regulates the opening of ion channels and the resulting influx or outflux of calcium and other ions feeds directly into the biochemical pathways described.

### 1.4.3 Is there a stiffness sensor?

Stiffness sensing may be explained by the same mechanisms as all other mechanoreponses, but it requires closer examination as stiffness is not detected unless the cell applies a probing force or deformation.

*Stress gauge or strain gauge?*

To measure stiffness requires either exerting a force and measuring the resulting deformation (as does a strain gauge) or measuring the force required to stretch a known displacement (as does a stress gauge). Therefore as Janmey stated in his 2009 review, stiffness-sensitive cells can be either stress-controlled or strain-controlled (Janmey et al., 2009). Stress-control would require cells to exert a constant force (for example constant actomyosin contraction) until a given protein was deformed or stretched enough to reveal cryptic binding sites and initiate downstream signaling. Evidence of stress control lies mainly in the fact that focal adhesion size grows with force yielding a constant focal adhesion stress measured by some studies (Balaban et al., 2001; Tan et al., 2003). Evidence for strain control lies in traction force and contraction studies measuring force increasing proportional to stiffness (Allioux-Guérin et al., 2009; Ghibaudo et al., 2008; Lam et al., 2011; Mitrossilis et al., 2009) suggesting a constant internal strain. In his recent
review Sheetz even proposed a 100 nm displacement sensor (Moore et al., 2010). In the case of a soft substrate, extension to 100 nm happens quickly and the bond to retrograde actin flow is lost whereas on a rigid substrate, extension to 100 nm is slow allowing time for reinforcement. Either mechanism requires actomyosin contraction to yield either a controlled stress or strain, consistent with the loss of rigidity sensing capabilities when contractility is reduced (Engler et al., 2006; Paszek et al., 2005).

**Theoretical models**

A number of systems-based mechanical models have developed to explain rigidity sensing. Instead of specific proteins acting as stress or strain gauges, in these models mechanosensitive binding kinetics or load-dependent rates yield stiffness sensitive behaviors such as orientation in an anisotropic stiffness environment or stiffness-dependent motility.

Recently Chan and Odde described a model where molecular clutch adhesions link F-actin to the substrate and mechanically resist myosin-driven F-actin retrograde flow. This “motor clutch” system yields a biphasic relation of traction forces to substrate stiffness and retrograde flow rate to substrate stiffness as observed experimentally. The model further suggests that durotaxis cells have a stiff optimal stiffness such that traction force decreases with stiffness and velocity increases with stiffness within the physiological range. (Chan and Odde, 2008) This model nicely describes cell behavior on 2D substrates, but is potentially less applicable to physiological 3D environments since actomyosin forces are not in general applied parallel to a flat substrate.

Several other models choose to focus on binding kinetics. Schwarz, Erdman, and Bischofs propose a 2-spring model combining application of the Bell model to adhesion rupture dynamics with a linearized force-velocity relationship of actomyosin. In this model forces act perpendicular to the adhesion cluster and therefore rebinding occurs under load. This model predicts an increasing force rate with increasing stiffness, as observed experimentally. (Schwarz et al., 2006)

A third recent model by Walcott and Sun proposes biological friction for both adhesion complexes sliding across their substrate and for actomyosin sliding. A combination of Kramer’s Theory and the Hill force-velocity relationship is used to predict adhesions moving under the influence of myosin. The model predicts that adhesions will move faster and experience less tension on soft substrates and that stress fibers will develop more extensively on more rigid substrates, as observed experimentally. (Walcott and Sun, 2010)

It should be noted that the Hill force-velocity relationship was proposed by the Asnacios group to be responsible alone for rigidity sensing. This is based on the relation between contraction velocity and load at a specific extent of contraction under different environmental stiffness conditions. The theory argues that stiffness-dependence originates in internal friction due to cross-linkers resisting myosin contraction. (Mitrossilis et al., 2009)
1.5 Atomic Force Microscopy

The Atomic Force Microscope (AFM) was first proposed in 1986 by Binnig, Quate, and Gerber as an application of a Scanning Tunneling Microscope (STM) to detect small deflections of a flexible cantilever beam sensitive enough to detect atomic-scale forces. (Binnig et al., 1986) While the STM was soon traded for an optical lever detection system, the use of a flexible lever acting as a Hookean spring remains. Small deflections of the lever are amplified and recorded by a laser beam bouncing off the top of the cantilever and hitting a photo-sensitive detector, as shown in Figure 1.3. The voltage signal may be converted to a displacement using an experimentally-determined lookup table. Measured displacement, $x$, may be converted to force, $F$, by the spring constant, $k$ as with a Hookean spring: $F = kx$, where the cantilever spring constant, $k$, is defined by the material properties and geometry of the lever. According to the equipartition theorem, the resolution of AFM measurements are thermally-limited and depend on the cantilever spring constant; however given environmental conditions such as thermal drift, acoustic vibrations, and wind currents, the sub-nm limit is only reached in very carefully controlled environments. While the AFM was originally developed as an imaging tool, the ability to measure forces at high resolution makes AFM a convenient tool for mechanical measurements as described below.

![Figure 1.3 Schematic of an Atomic Force Microscope setup.](image-url)
1.5.1 Unfolding and de-adhesion measurements

AFM lends itself perfectly to the measurement of forces required for protein unfolding or de-adhesion. A sample attached to the AFM cantilever is allowed to interact with a sample on an underlying substrate. The two samples are then separated by ramping of the cantilever base away from the substrate at a constant rate. The deflection of the lever away from resting position indicates the force required to rupture the interaction and small steps in the retraction trace indicate unfolding events or individual rupture events. This type of measurement has been used to measure single molecule unfolding, single-protein interactions, cell-surface interactions, and cell-cell interactions (Chaudhuri et al., 2009; Dupres et al., 2005; Fisher et al., 2000; Florin et al., 1994; Sun et al., 2005; Zhang et al., 2004).

Cantilevers with suitable spring constants must be chosen to probe the regime of forces desired. It should also be noted that adhesive forces are rate-dependent as described by Bell (Bell, 1978). Finally, the appropriate attachment ligands should be used such that the measurement reflects rupture of the two samples and not rupture of the sample from the cantilever or substrate. Chapter 2 describes a technique to strongly adhere cell samples for de-adhesion measurements and cell manipulation.

1.5.2 Elasticity measurements

Indentation measurements to determine the elasticity of a sample are another common use of AFM. Deflection of a cantilever as it contacts the surface qualitatively indicates how stiff or soft the sample is with respect to the cantilever. The elasticity may be quantitatively determined given the geometry of the indenter and spring constant of the cantilever. In 1882 Hertz described the interaction of a conical tip with a semi-infinite plane. The contact area increases with indentation such that the force, \( F \), required to obtain a deflection, \( \delta \), into a semi-infinite plane of elasticity \( E \) and Poisson’s ratio \( \nu \) is given as

\[
F = \frac{\delta^2 \pi E}{2 (1 - \nu^2)} \tan(\alpha)
\]

where \( \alpha \) is the half angle of the indenter (Hertz, 1882). This formula was generalized for non-conical indenters by Sneddon in 1965 (Sneddon, 1965). Hertzian analysis of indentation curves is now commonly used, described in several reviews and books (Fung, 1993; Radmacher, 1997), and has been used to measure the elasticities of cells ranging from yeast (Touhami et al., 2003) to leukemia cells (Rosenbluth et al., 2006). Recently, a new method has been developed for the analysis of indentation measurements as an alternative to the Hertz model that does not require the assumptions of a homogeneous and linearly elastic sample (Costa et al., 2006). This technique has been cited 31 times since publication in 2006 (based on a Web of Science search) suggesting a significant adoption of this alternative model.
1.5.3 Novel techniques: AFM as a tool for probing single-cell mechanics and mechanoresponses

The Fletcher lab has developed several AFM-based techniques that go beyond the conventional approaches described thus far. As described in section 1.2.1, the AFM cantilever has been used as a simple probe to exert known local deformations and observe the cellular mechanoresponse. Chapter 3 of this dissertation describes the use of AFM to apply a known mechanical perturbation to the cell surface towards understanding the propagation of stress through the cell. Recently the Fletcher lab has used AFM cantilevers to control the mechanical boundary conditions exposed to cells by allowing a cell to adhere to both a ligand-coated cantilever and ligand-coated substrate (Chaudhuri et al., 2009; Lam et al., 2011; Webster et al., 2011). The cell then contracts under stiffness conditions defined by the cantilever. Chapters 4 and 5 of this dissertation outline the use of a new feedback technique allowing for the dynamic control of stiffness.

1.6 Scope of Dissertation

The remaining chapters of this dissertation describe in-depth the projects completed during my graduate career at Berkeley developing and utilizing a toolbox to study the cellular mechanoresponse. The literature suggests that the weakest link in the mechanoresponse machinery is not any internal component, but the extracellular adhesions. I therefore begin the experimental section of this dissertation describing a DNA-mediated adhesion technique developed in collaboration with the Francis lab to attach cells to AFM cantilevers to allow experimental manipulations without rupture of cell-surface attachments. This technique enables tunable adhesion strength up to values far beyond measured cell-ligand bond strengths without influencing cell viability or signaling. In chapter 3 I describe how stress due to a local mechanical perturbation is propagated across a cell. This required the development of a three-dimensional multi-particle tracking technique to track cell surface movements in z. Chapter 4 describes a novel AFM-based technique to dynamically control the stiffness exposed to a single cell and Chapter 5 employs this technique to reveal a seconds-timescale adaptation of a contracting cell to step changes in extracellular stiffness. Finally in Chapter 6 I offer concluding remarks on the future directions of these projects and understanding the cellular mechanoresponse. Enjoy.
Chapter 2. DNA-coated AFM cantilevers for the investigation of cell adhesion and the patterning of live cells


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### 2.1 Main Text

The forces governing cell-cell adhesion are vitally important to many biological processes, including cell differentiation, tissue growth (Discher et al., 2005; Krieg et al., 2008), tumorigenesis (Discher et al., 2005; Paszek and Weaver, 2004), and proper functioning of the vertebrate immune response (Simon and Green, 2005; van Kooyk and Figdor, 2000). The strengths of these interactions are typically characterized through the attachment of single living cells to probes that are capable of force measurement. For example, individual cells have been grasped by suction using micropipettes (Chu et al., 2004; Shao and Hochmuth, 1996) to quantify the strength of lymphocyte interactions. However, this manipulation process can easily damage the cell membrane, and it does not adequately decouple the adhesion forces of interest from technique artifacts. More recently, optical tweezers (Dufresne and Grier, 1998; Thoumine et al., 2000) have been applied to capture single cells and measure these forces with added accuracy, but this technique is limited to applying forces in the piconewton range (Clausen-Schaumann et al., 2000). Atomic force microscopy (AFM) (Binnig et al., 1986) provides an attractive alternative to these methods because it is capable of quantifying forces in the piconewton to nanonewton range, and this technique has been used to measure the mechanical properties of live single cells (Radmacher, 2002) and study adhesion forces at the single cell level (Benoit et al., 2000; Franz et al., 2007; Puech et al., 2006; Zhang et al., 2004; Zhang et al., 2002; Zhang et al., 2006). In these studies, the AFM cantilevers were first coated with lectins that bind to carbohydrate moieties on the cell surface. Several fundamental adhesion measurements have been achieved using this method (Benoit et al., 2000; Franz et al., 2007; Zhang et al., 2004; Zhang et al., 2006), but the cell binding molecules themselves have been reported to have a degree of cytotoxicity that can influence the cellular properties being evaluated (Ballerstadt et al., 2006; Franz et al., 2007; Ohba et al., 2003; Palacios, 1982). Thus, while these studies highlight the utility of AFM for the measurement of cell receptor-ligand interactions, an expanded set of cantilever attachment methods will be needed to realize the full potential of this technique, especially for the study of cell-cell interactions over widely varying time scales.

To address this need we have compared three biomolecule-mediated methods for the attachment of live cells to AFM cantilevers, with an emphasis on the cell viability, adhesion strength, and probe reuse that each technique can achieve. These studies have indicated that cell attachment through the use of complementary DNA strands has the least influence on viability and does not appear to activate cell signaling pathways. This method also offers superior adhesion strength, but this parameter can be attenuated to allow cells to be transferred from one surface to another. We were able to demonstrate this concept by picking up free cells and placing them in exact positions on a substrate bearing DNA strands with longer complementary regions. This “Dip-Pen” live cell patterning demonstrates the reusability of the DNA-mediated cell adhesion method, and could prove useful for the construction of complex mixtures of cells with well-defined spatial characteristics.
To allow the comparison of several attachment strategies, three different biomolecules (including DNA, concanavalin A, and an antibody) were attached to AFM cantilevers for cell anchoring. For all attachment methods, the thin layer of silicon oxide on the working surface of the silicon nitride AFM cantilever was first cleaned using oxygen plasma to maximize the number of hydroxyl functional groups. Trimethoxysilylpropanal was then coupled to the cantilever by chemical vapor deposition (CVD) to yield a surface covered with aldehydes (Figure 2.1a). The surfaces produced using these steps were characterized by contact angle measurement (Figure 2.5). In more than 50 separate experiments, this two-step modification process has provided a reproducible way to introduce aldehyde functional groups onto cantilever surfaces.

Figure 2.1. Covalent attachment of biomolecules to cantilevers and cell surfaces. (a) After surface oxidation using an oxygen plasma, aldehyde functional groups were introduced onto silicon nitride cantilevers using chemical vapor deposition (CVD). (b) Solutions of anti-CD3 IgG or ConA containing sodium borohydride were introduced onto aldehyde-coated cantilever surfaces in a humid chamber. DNA modification was achieved by immersing cantilevers in an amine functionalized ssDNA solution at 100 °C for 30 min, followed by exposure to a sodium borohydride solution. (c) Metabolic engineering was used to introduce azide groups onto cell surfaces via treatment with peracetylated-N azidoacetylmannosamine (Ac4ManNAz). Phosphine functionalized ssDNAs were synthesized and covalently attached to the exterior of cells via the Staudinger ligation.
Amine functionalized DNA was attached to the aldehyde groups through reductive amination (Figure 2.1b) (Schena et al., 1995; Zammatteo et al., 2000). First, the aldehyde-coated cantilever was immersed in an amine functionalized single-strand DNA (ssDNA) solution and then heated to drive imine formation. After cooling to room temperature, an aqueous solution of sodium borohydride was used to reduce the imines to non-hydrolyzable amine linkages. This step also served to reduce any unreacted aldehyde functional groups to yield alcohols. By coupling 5'-amine functionalized DNA strands bearing fluorescein at the 3'-end, the presence of the strands could be verified by fluorescence imaging (Figure 2.6).

In previous efforts, proteins have been attached to AFM tips through non-specific adsorption and through glutaraldehyde crosslinking to amine groups introduced on the tip surface (de Odrowaz Piramowicz et al., 2006). To afford more well-defined linkages (and thus realize more homogeneous cell attachment), we chose instead to use the simple reductive amination strategy that was used for the amino- DNA strands. Surface lysine residues on ConA and anti-human CD3 antibodies (anti-CD3) were reacted with the aldehyde functional groups on the cantilever surfaces (Figure 2.1b) (Zheng et al., 2005), but a lower concentration of reducing agent (66 μM) was used to minimize the reduction of disulfide bonds that are required to maintain protein tertiary structure. As described above for DNA, FITC-labeled ConA and anti-CD3 samples were used in some experiments to verify biomolecule attachment using fluorescence microscopy (Figure 2.7).

To prepare live cells bearing ssDNA on their surfaces, we first introduced azide functional groups into glycoproteins embedded in the plasma membrane, as previously described (Chandra et al., 2006). Peracetylated N-α-azidoacetylmannosamine (Ac4ManNAz) was added to cells, which then metabolized and displayed the azide on their surfaces (Figure 2.1c) (Dube and Bertozzi, 2003). Triarylphosphine-modified ssDNA was prepared through the reaction of 5'-amine-modified ssDNA with a phosphine pentafluorophenyl (PFP) ester. This reagent was then used to label the cell-surface azides through a Staudinger ligation (Saxon and Bertozzi, 2000), yielding stable amide linkages. Flow cytometry experiments have previously verified the ability of phosphine-DNA conjugates to undergo ligation to azide-modified cell surfaces (Chandra et al., 2006). Although many cell types would be expected to be compatible with this system (and have been explored previously using the DNA-based adhesion method) (Chandra et al., 2006), non-adherent Jurkat cells were chosen for these studies because they do not secrete their own extracellular matrix. Thus, all cell adhesion events arise solely from the biomolecules on their surfaces.

Comparison of the cell capture efficiencies of these three cell adhesion methods was achieved by first coating commercially available aldehyde-coated glass slides with the same set of biomolecules using the reductive amination procedure outlined above. Cells bearing or lacking (in the case of ConA or anti-CD3) DNA were exposed to these surfaces and the number of cells per unit area were counted (Figure 2.2b).
Figure 2.2. Comparison of biomolecule based adhesion methods. (a) The morphology of immobilized cells on DNA, ConA, and anti-CD3 IgG coated slides was determined after 10 min and 48 hours. ConA and antibody immobilized Jurkat cells exhibited significant morphological changes during the incubation time. (b) To evaluate cell capture efficiency, solutions of 20 μM FITC-labeled ssDNA, 20 μM FITC-labeled ConA, and 1 mg/mL FITC-labeled anti-CD3 IgG were applied to aldehyde-coated glass slides and the biomolecules were attached via reductive amination. Solutions containing 1x10^7 Jurkat cell/mL were then introduced onto the resulting slides. The samples were incubated for 10 min at room temperature, and then washed with two portions of PBS before evaluation. (c) Cell growth analysis was also determined in the presence of the adhesion molecules. A suspension of Jurkat cells was combined with ConA or anti-CD3 IgG, and a solution of DNA coated cells was combined with the complementary DNA strands. At various time points the total number of cells was counted. The control sample was grown in the absence of any adhesion molecules. (d) To evaluate cell viability, cells were immobilized on DNA, ConA, and anti-CD3 IgG coated aldehyde slides. After immobilization for 24 h and 48 h, the cells were incubated with a solution of annexin V-FITC (orange bars) and PI (blue bars). The cells were evaluated within 1 h by fluorescence microscopy. *ConA and antibody immobilized cells that were partially stained by annexin were counted as cells undergoing apoptosis. NB represents control samples that were not bound to the surfaces.

All three surfaces were able to achieve efficient cell binding, with the DNA-based system showing the highest coverage for cell capture (cell density = 1200 cells/mm²). Cells did not adhere to slides lacking the biomolecules (Figure 2.8). The DNA-conjugated cells appeared morphologically unchanged when observed after 48 hours of binding, whereas the ConA- and anti-CD3- immobilized cells exhibited
significant changes during this time period (Figure 2.2a). The aggregation and physiological changes of the free cells after adding ConA or anti-CD3 are likely due to crosslinking of surface receptors (Freywald et al., 2006; MacDermott et al., 1978). In other studies, both ConA and anti-CD3 have been shown to bind to CD3 receptors on the cell surface and activate the immune response of human T cells (Phillips and Lanier, 1986). In contrast, the DNA molecules appear only to hybridize with their complementary partners, and do not activate any signaling pathways that would disturb the physiology of the cells.

The effects of the adhesion molecules on the viability of the cells were assessed using two different methods. First, suspensions of unmodified Jurkat cells were supplemented with ConA or anti-CD3 antibodies, and the solutions of DNA-coated cells were supplemented with the complementary sequence. Figure 2.2c shows the growth curves of the resulting cells over a three day period. The growth curve of DNA modified cells was the same as that of unmodified cells, but the anti-CD3 treated cells showed delayed growth. ConA coated cells aggregated and were no longer alive after 12 hours. The morphologies of cells after the addition of the reagents are shown in Figure 2.9.

The viability of surface-immobilized cells was also investigated after 24 and 48 h using annexin V and propidium iodide (PI) staining (Chen et al., 2007). For the DNA-immobilized cells, the low percentage of apoptotic and necrotic cells was similar to that of unmodified cells (Figure 2.2d). However, the ConA and anti-CD3 immobilized cells show significantly higher numbers of apoptotic cells compared to the control samples.

Live cells were captured by AFM tips bearing all three of the biomolecules. This was accomplished simply by touching the cell membrane with the cantilevers, with contact times as short as 5 seconds resulting in the transfer of the cells to the AFM tips. No cells were captured by tips lacking the appropriate biomolecules. The strength of the interaction between the cell and the cantilever fundamentally limits the range of forces that can be measured when other surfaces are brought into contact with the receptors. Our assay to measure the strength of this interaction was designed such that cell-cantilever adhesions were fewer in number, and therefore weaker overall, than DNA-based adhesions between a cell and the complementarily functionalized glass slide. Due to this arrangement the cell-cantilever interaction would be expected to rupture first, yielding the strength of the interaction that a relatively low concentration of biomolecules can achieve. Rupture of the cell-cantilever interaction before the cell-surface interaction was verified by visual observation during experiments. The force of de-adhesion was measured for each attachment method using two different retraction rates and two different contact forces (Figure 2.3a). The measured force of deadhesion increased with contact force and retraction rate across all attachment methods, as predicted by the Bell model (Bell, 1978). The ConA attachment method yielded zero-force attachment events in 12% of the de-adhesion measurements. Such events were not observed in the DNA and antibody cases.
A significant spread of forces was observed for all three attachment methods; however under all experimental parameters, the DNA method displayed the strongest average adhesion, followed by antibody attachment, then ConA (Figure 2.3b). As a control experiment, we have also demonstrated that the capture efficiency of ConA and anti-CD3 is not affected by the presence of DNA strands introduced on the cell surface (Figure 2.8). It should be noted that de-adhesion forces determined for each attachment strategy will depend on the details of the preparation conditions, and therefore should not be taken as absolute measurements. Rather, the trends demonstrate that under typical preparation conditions the DNA hybridization method will lead to the most robust attachment.

**Figure 2.3. AFM measurement of de-adhesion force.** (a) Six sample traces for a single cell are shown in lighter colors, with the average trace shown in black. At zero distance, the cell is in full contact with the cantilever that is applying a positive force. As distance increases, the cantilever is pulled away from the glass slide surface, causing the cell-cantilever linkage to rupture and result in the zero-force, no-contact region. The force of de-adhesion was calculated as the difference between the curve minimum and the horizontal no-contact region. (b) Adhesion forces were measured under different retraction rates (15.7 μm/s and 8.2 μm/s) and contact forces (400 pN and 200 pN) for the DNA, ConA, and antibody systems. Data were obtained by measuring six de-adhesion events on more than four different cells.

The strength of the cell-cantilever interaction can be tuned by varying the number of interacting strands and the length of the complementary regions, and the reversibility of DNA hybridization also allows the tips to be used many times. Both of these advantages allowed us to use AFM tips to arrange cells one at a time into patterns. In a recent report (Bell, 1978), it was shown that individual DNA strands could be moved from one location to another on a printed substrate, allowing small molecule dyes to be printed in a similar fashion.

To do this, a 5 μM solution of a shorter DNA strand (13 bases) was applied to the cantilever and an 80 μM solution of a longer strand (20 bases) was coupled to the glass slide. DNA-coated Jurkat cells were incubated in CO₂ independent media and applied to the non-coated side of glass slide under an AFM. To attach a cell to the modified cantilever, the cantilever was lowered into contact with the cell for 10
seconds with a contact force of 400 pN. The cantilever was then retracted, and cell attachment to the cantilever was confirmed visually. The attached cells were then moved to the DNA coated side with maximum rate of 1 mm/s. The cantilever was lowered into contact with the slide, and the cell was allowed to interact with the substrate for 10 s with a 400 pN contact force. The cantilever was then retracted, whereupon the cell remained attached to the glass slide. By applying this printing method, cells can be given an (x,y) coordinate to position them precisely on a 2D substrate (Figure. 2.4). The cells were found to remain viable after patterning, as shown in Figure 2.11.

**Figure 2.4. Dip-Pen patterning of live cells.** (a) By attaching a shorter DNA strand (13 bases) to a cantilever and a longer strand (20 bases) to the glass slide, a single living cell can be transported by the AFM and directly printed at a desired location on the glass slide. This process is shown stepwise for the formation of a single pattern of cells in b-d.

In summary, we have described the development of a versatile DNA-based adhesion method for the study of cell-cell interactions by AFM. The key advantages of this platform include the reusability of the tip, the tunability of the interaction strength, and the use of well-defined chemical linkages. Of the three biomolecule-based attachment strategies that were used, the DNA method proved superior in terms of cell viability after attachment. The use of AFM to form accurate and programmable patterns of individual cells provides a useful tool that can be used to understand the influence of neighboring interactions on cell differentiation and regulation. In a previous report, we have shown that complex patterns can be prepared through the self-assembly of DNA-coated cells on surfaces printed with complementary oligonucleotides (Douglas et al., 2007). The AFM dip-pen method described here provides a useful complement to this technique that can achieve the higher resolution that would be needed to create and interrogate clusters consisting of multiple cell types. We are currently using this method to elucidate fundamental adhesion mechanisms involved in cancer metastasis, immune synapse formation, and cell-cell communication.
2.2 Supporting Information

2.2.1 General Experimental Procedures

All cell culture reagents were obtained from Gibco/Invitrogen Corp (Carlsbad, CA) unless otherwise noted. Cell culture was conducted using standard techniques. Jurkat cells were grown in T-25 culture flasks (Corning, USA) in RPMI Medium 1640 supplemented with 10% (v/v) fetal bovine serum (FBS, HyClone) and 1% penicillin/streptomycin (P/S, Sigma).

All oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). Concanavalin A and FITC-labeled concanavalin A were purchased from Sigma (St. Louis, MO). Anti-human CD3 IgG (UCHT1) and FITC-anti-human CD3 IgG (UCHT1) were obtained from eBioscience (San Diego, CA).

Fluorescence micrographs were acquired with an Axiovert 200M inverted microscope (ZEISS) with fluorescence filter sets for DAPI/Hoechst, fluorescein/fluo-3, and rhodamine. Ultraviolet absorption of the different oligonucleotides was determined at 260 nm on a UVIKON 933 double beam UV/Vis spectrophotometer (Kontron Instruments, United Kingdom).

2.2.2 AFM instrumentation and cantilever calibration.

Force microscopy measurements were obtained on a modified commercial AFM. Briefly, a Bioscope AFM (Veeco, Santa Barbara, CA) mounted on an epifluorescent microscope (Carl Zeiss, Germany) held the fluid-cell-mounted cantilevers (Veeco, Santa Barbara, CA). V-shaped silicon nitride cantilevers (Microlevers, Veeco) were used in the case of DNA coating and V-shaped gold-coated silicon nitride cantilevers (Microlevers, Veeco) were used for ConA and antibody attachment methods. All cantilevers had a spring constant in the range of 9-18 pN/nm, as measured by the thermal noise method (Hutter, 1993). A closed-loop single-axis 50 μm range, 0.7 nm accuracy piezoelectric positioning platform (“piezo”, Mad City Labs, Madison, WI) was used to control cantilever-slide separation for all de-adhesion measurements.

2.2.3 Introduction of aldehyde functionality onto AFM cantilevers.

To enhance reflection of the laser used to quantify force, we used silicon nitride cantilevers coated with gold on one side (opposite the attached cell) for the protein attachment experiments. Non-gold-coated silicon nitride cantilevers were used for DNA attachment because the heating step involved in the modification process caused bending when the metal layer was present. A silicon nitride AFM probe (gold coated probes: MLCT-AUNM; unmodified probes: MLCT-NONM, Veeco Instruments, Sunnyvale, CA) was washed with acetone and placed into a glow discharge plasma.
panel for 2 min under 140 mtorr and 18 Watt to introduce a uniform layer of SiOx groups onto the silicon nitride surface. A small container charged with 0.4 mL of trimethoxysilylpropanal (TMSP, United Chemical Technologies) was placed on a 60 °C heat block in a bell jar desiccator that was subsequently purged with N2 for 1 min. The freshly cleaned AFM probe was placed in the jar, which was then evacuated and sealed to the atmosphere for 1 h. The heat block and the TMSP were then removed, and the desiccator was purged with N2 gas for another 1 min. The resulting probe was stored in this environment until use. The cantilever surface was characterized by contact angle measurement (Figure 2.5) and fluorescence imaging before and after biomolecule attachment (Figure 2.6).

![Figure 2.5](image)

**Figure 2.5.** Contact angle measurements of silicon nitride cantilever surfaces. From left to right, the three pictures depict contact angle measurements of the cantilevers before modification, after glow discharge, and after CVD modification. The table lists the contact angles of the coated cantilevers and analogous glass slides.

<table>
<thead>
<tr>
<th></th>
<th>Before Modification</th>
<th>Glow Discharged</th>
<th>CVD (Aldehyde)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cantilevers</td>
<td>80.8 ± 3.5°</td>
<td>14.7 ± 3.7°</td>
<td>52.4 ± 3.5°</td>
</tr>
<tr>
<td>Glass Slides</td>
<td>0.3 ± 0.4°</td>
<td>Unmodified SiO₂</td>
<td>Aldehyde Coated</td>
</tr>
</tbody>
</table>

The contact angle is the angle at the interface of a drop of pure water and a planar substrate, and provides a measure of surface hydrophobicity. The steeper the angle, the greater the hydrophobicity. After glow discharge, the contact angle of cantilever surface dropped from 80.8° to 14.7°, which is consistent with the production of bare silicon oxide. By applying the CVD method to introduce aldehyde functionality onto the surface, the contact angle increased back to 52.4°, which was similar to that measured for commercially available aldehyde-coated glass slides.

The contact angles were measured with a semi-automatic contact angle meter (Contact angle measuring system G10, KRUSS). This includes a video camera that records a live picture of a water droplet. For measurements on the cantilever and glass surfaces, a drop volume of 5 μL was chosen. Each contact angle measurement was repeated three times, and the contact angles were calculated using DTAcquire and ImageJ software.
2.2.4 Characterization of Modified AFM Cantilevers using Fluorescence Microscopy

![Image of fluorescence characterization of modified cantilevers]

**Figure 2.6. Fluorescence characterization of modified cantilevers.** To confirm the success of the reductive amination procedure, 3’-FITC-labeled 5’-amino ssDNA was coupled as a fluorescent probe. All cantilevers were oxidized by glow discharge before use. A) Maximum fluorescence was observed only for the aldehyde-coated substrate. Samples lacking DNA (B) or the aldehyde groups (C) showed significantly less fluorescence intensity. D) A gold-coated silicon cantilever after the reductive amination procedure with FITC-labeled DNA. The reflective metal surface increased the fluorescence intensity significantly.

2.2.5 Covalent attachment of DNA, lectins, and antibodies to cantilevers.

For DNA-mediated cell adhesion studies, a complementary oligonucleotide sequence pair (A/A’) was designed. The sequence identities were as follows:

A: 5’-TCA TAC GAC TCA CTC TAG GG-3’
A’: 5’-CCC TAG AGT GAG TCG TAT GA-3’

An aldehyde-coated cantilever (MLCT-NONM) was immersed into a 20 μM solution of 5’-amine functionalized ssDNA in 3X saline/sodium citrate buffer (45 mM sodium citrate, 450 mM NaCl, pH 7.0) for 15 min, heated in an oven at 100 ºC for 30 min, and then washed with 0.2% SDS solution and distilled water (1 min each). The resulting cantilever was soaked in a fresh solution of 0.1 g of NaBH₄ in 10 mL of ethanol and 30 mL of PBS solution for 15 min, and then it was washed with 0.2% SDS solution and water (1 min each). The cantilever was dried under N₂ and stored in a low moisture environment until use. The ssDNA coated cantilever was characterized by coupling 3’-FITC-labeled 5’-amino ssDNA (A strand) to the aldehyde-coated cantilever surface, followed by imaging with a fluorescence microscope.

Concanavalin A and anti-CD3 IgG monoclonal antibodies were also coupled to an aldehyde-coated cantilever (MLCT-AUNM) surface by a reductive animation procedure. An aldehyde-coated cantilever was exposed to a 20μM (Con A) or 1 mg/mL (Anti-CD3) solution of the protein in pH 7.0 PBS buffer solution containing...
66 µM NaBH₄ in a humid chamber for 2 h. The cantilever was then washed with excess PBS and water, and stored in pure PBS solution at 4 °C until use.

2.2.6 Cell-surface DNA modification.

A modified version of a previously published protocol was used (Chandra et al., 2006). To prepare the DNA-Staudinger ligation reagent, a solution of 5'-amine functionalized ssDNA (0.69 mg in 27.6 µL of water) was reacted with phosphine-PFP (1.7 mg in 96.6 µL of DMF) and N,N-diisopropylethylamine (27.6 µL) at rt for 20 h. Both phosphine-PFP and N,N-diisopropylethylamine were obtained from Sigma (St. Louis, MO). The solution was then diluted with 697 µL of water, eluted through a NAP 5 SEC column (GE Biosciences), and purified by semi-prep HPLC using an Agilent 1100 system (Agilent Technologies, USA). Analyte detection for all HPLC analyses was achieved using an in-line diode array detector (DAD). Preparative reversed-phase HPLC was accomplished using an Agilent Eclipse XDB-C18 column (Agilent Technologies, USA) and an acetonitrile/aqueous 0.1 M triethylammonium acetate (TEAA, buffered at pH 7) gradient. The eluent was lyophilized, and the residue was redissolved in degassed phosphate-buffered saline (PBS, pH 7.4) and quantified by UV-Vis spectroscopy. The phosphine-DNA solution was stored at -20 °C under an atmosphere of N₂ until use.

Acetylated ManNAz (Ac₄ManNAz) was synthesized according to previously published procedures (Dube and Bertozzi, 2003). A 10 mM ethanolic stock solution of the sugar was sterilized using 0.2 µm mesh Acrodisc® 13 mm filters (Pall Life Sciences, USA). The appropriate volume of Ac₄ManNAz stock solution was pipetted using sterile technique into a culture flask and the solvent was allowed to evaporate. Jurkat cells were grown in culture media that was 25 µM in Ac₄ManNAz for 3 d under the conditions described in General Experimental Methods section. The cells were then centrifuged, washed twice with 5 mL of PBS containing 1% FBS, and reacted with 125 µM phosphine-DNA in 1% FBS/PBS (total volume of 100 µL) for 1 h at 37 °C. The cells were then rinsed with two 5 mL portions of 1% FBS/PBS solution at rt, and then used in cell-adhesion assays within 1 h after preparation.
2.2.7 Confirmation of Cell Binding to AFM Cantilevers and Glass Slides

Figure 2.7. Capture of living cells by DNA, ConA, and Anti-CD3 coated cantilevers. FITC-labeled DNA (A), ConA (B), and Anti-CD3 (C) were coated onto aldehyde-functionalized silicon nitride cantilevers using the reductive amination procedure. Jurkat cells bearing complementary DNA sequences were captured by the DNA-coated cantilevers (D), and unmodified Jurkat cells were captured by the ConA (E), and Anti-CD3 (F) coated AFM cantilevers.

Figure 2.8. Binding of Jurkat cells to glass slides printed with biomolecules. FITC-labeled DNA (A), ConA (B), and Anti-CD3 (C) were coated onto glass slides using the reductive amination procedure. Jurkat cells bearing complementary DNA sequences were captured by the DNA spots (D), and unmodified Jurkat cells were captured by the ConA (E), and Anti-CD3 (F) coated areas of the slides. Dramatically lower cell binding was observed in the uncoated regions.
2.2.8 Evaluation of cell viability.

The unmodified Jurkat cells were seeded in three separate Petri dishes with initial cell densities of 5x10^5 cells/mL in 0.9 mL of cell media. A 0.1 mL solutions of ConA (20 μM) or a 0.1 mL solution of anti-CD3 IgG (1 mg/mL) were added to two of them. The A' strand-coated cells were seeded in another 1 mL Petri dish, and A strand DNA was added into the solution to a concentration of 2 μM. The number of cells was counted in each of the four samples using a hemocytometer after 12, 24, 48, and 72 hours. Cell viability was monitored by adding Trypin Blue. An annexin V-FITC/propidium iodide apoptosis detection kit was obtained from BD Biosciences. After immobilization on to slides by DNA, ConA, or antibody adhesion, the cells were incubated in the media at 37 °C for 24 or 48 hours, as indicated. A solution consisting of of 900 μL of 1X binding buffer, 30 μL of the annexin V-FITC stock solution, and 30 μL of the PI stock solution was prepared. After 24 or 48 hours, 100 μL of the solution was applied to each slide for 15 min at rt. The cells were imaged within 1 hour using a fluorescence microscope.

Figure 2.9. Cell morphology changes induced by soluble biomolecules. Jurkat cells were grown in normal media (Control), DNA-modified Jurkat cells were grown in the presence of 2 μM DNA, and unmodified Jurkat cells were grown in the presence of 2 μM ConA or 0.1 mg/mL Anti-CD3. The resulting cells were examined under a light microscope after a period of 12 h. The appearance of the cells was largely unchanged in the presence of DNA, but cells grown in the presence of ConA and Anti-CD3 exhibited aggregation and other morphological changes.
2.2.9 Cell capture efficiency.

Solutions of 20 μM FITC-labeled ssDNA, 20μM FITC-labeled ConA, and 1 mg/mL FITC-labeled anti-CD3 IgG were spotted onto aldehyde-coated glass slides (SCHOTT Nexterion, Louisville, KY), and the resulting imines were reduced with NaBH₄ using the same protocols described above for modification of cantilever surfaces. All spots were imaged with a fluorescence microscope to confirm the presence of the desired biomolecules. Jurkat cells coated with ssDNA (A’ strand) were prepared and diluted to a concentration of 1x10⁷ cells/mL. The cells were then applied to a glass slide coated with complementary ssDNA (A strand) for 10 min at rt. The slide was then washed twice with PBS. For the ConA and anti-CD3 IgG systems, unmodified Jurkat cell solutions were concentrated to 1x10⁷ cells/mL and applied directly to the appropriately coated glass slides. Cells were incubated on the slides at rt for 10 min, and then the slides were rinsed twice with PBS. Following the rinses, each slide was imaged and photographed under a fluorescence microscope. Each experiment was repeated in triplicate, and the number of bound cells was counted and plotted for an area corresponding to 0.1 mm².

![Figure 2.10. Cell capture efficiency of DNA-modified cells and unmodified cells on ConA and Anti-CD3 coated surfaces.](image)

No statistical differences in binding were observed, as indicated by a T-test. This suggests that the attachment of DNA molecules to the cell surface does not block receptor access.

2.2.10 Force measurements.

A 3x10⁶ cells/mL solution of Jurkat cells coated with ssDNA (A’ strand) in CO₂ independent media was first applied to a glass slide modified with a 80 μM solution of ssDNA (A strand). A cantilever (MLCT, Veeco, spring constant 0.01 N/m) modified with a 20 μM ssDNA (A strand), 20 μM ConA, or 1 mg/mL anti-CD3 IgG solution was prepared as described above and mounted onto the fluid cell. Jurkat cells 20-25 μm in diameter were chosen for de-adhesion force measurements to control for cell-cantilever contact area. The cantilever was then lowered to the point of contact with the cell. The cell was sandwiched between the cantilever and the glass slide for 10 sec at 200 pN or 400 pN of contact force. The glass slide substrate and cantilever were then separated at a rate of 15.7 μm/s or 8.2 μm/s, causing the cell-cantilever
adhesion to rupture and thereby allowing the force of de-adhesion to be measured. Visual observation confirmed that the rupture event occurred between the cell and the cantilever.

**Figure 2.11. Post-patterning analysis of cells using annexin V-FITC and propidium iodide (PI) assays for apoptosis.** Seven cells were positioned by Dip-Pen patterning and then incubated at rt for 3 h before staining. (A) DIC image of the cells after this procedure. (B) Fluorescence image (fluorescein filter set) of the cells after exposure to annexin V-FITC. (C) Fluorescence image (rhodamine filter set) of the cells after PI staining. No signal was observed in either (B) or (C), indicating that the Dip Pen process did not induce apoptosis. (D-F) As positive controls, the apoptosis was induced by incubation with 30% ethanol for 5 min. (E) was taken with a fluorescein filter, and (F) was taken using a rhodamine filter.

### 2.2.11 Direct Cell Patterning

Glass slides were prepared by coating one half with an 80 μM solution of ssDNA (A strand), leaving the other half unexposed. Treatment with NaBH₄ solution was then carried out as described above to reduce all of the aldehydes and imines on the slides. A silicon nitride cantilever was modified with a 5 μM solution of a 13-mer ssDNA as described above. This strand was a truncated version of the full A strand sequence, with the exact sequence of 5’-TCA TAC GAC TCA C-3’ A 50,000 cells/mL solution of ssDNA (A’ strand) coated Jurkat cells was then applied to the unmodified side of the glass slide. The cantilever was lowered into contact with cells and held in place for 10 s with 400 pN of force to allow DNA hybridization. The cell was then lifted from the glass slide with the cantilever. After moving the cantilever to the side of the slide that was coated with 20-mer ssDNA, the cell was lowered into contact with the substrate for 10 s with a 400 pN of pushing force, as monitored by a photodiode detector. The cantilever was then retracted. The transferred cell was then observed and photographed under the microscope.
2.3 Acknowledgements

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Chapter 3. Slow stress propagation in adherent cells


* Both authors contributed to this work equally.

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3.1 Abstract

Mechanical cues influence a wide range of cell behaviors including motility, differentiation, and tumorigenesis. While previous studies have elucidated the role of specific players such as ion channels and focal adhesions as local mechanosensors, investigation of how mechanical perturbations propagate across the cell is necessary to understand the spatial coordination of cellular processes. Here we quantify the magnitude and timing of intracellular stress propagation using atomic force microscopy (AFM) and particle tracking by defocused fluorescence microscopy. The apical cell surface is locally perturbed by AFM cantilever indentation and distal displacements are measured in 3D by tracking integrin-bound fluorescent particles. We observe an immediate response and a slower equilibration, which occurs over times that increase with distance from perturbation. This distance-dependent equilibration occurs over several seconds and can be eliminated by disruption of the actin cytoskeleton. Our experimental results are not explained by traditional viscoelastic models of cell mechanics, but are consistent with predictions from poroelastic models that include both cytoskeletal deformation and flow of the cytoplasm. Our combined AFM-particle tracking measurements provide direct evidence of slow, distance-dependent dissipative stress propagation in response to external mechanical cues and offer new insight into mechanical models and physiological behaviors of adherent cells.

3.2 Introduction

Treatment of the cell as a material has provided a foundation for understanding mechanical responses of cells and modeling coordinated cell behaviors (Kasza et al., 2007). Recent studies investigating the relaxation behavior of cells have demonstrated a power-law dependence of material properties on the frequency of perturbation (Fabry et al., 2001; Hoffman et al., 2006), suggesting that cells behave as a soft glassy material (Bursac et al., 2005; Trepat et al., 2007). These cell rheology studies have provided insight into localized cellular relaxation, but they have not investigated how a cell spatially equilibrates in response to an applied stress. Other studies investigating the structural organization of cells have mechanically perturbed the cell by a variety of techniques and observed displacement of focal adhesions or intracellular fiduciary markers away from the stimulus (Bausch et al., 1998; Helmke et al., 2003; Hu et al., 2003; Mack et al., 2004; Maniotis et al., 1997; Mathur et al., 2000; Paul et al., 2008; Ragan et al., 2006; Wang and Suo, 2005). These studies demonstrate elastic coupling to be heterogeneous, propagating applied stresses between specific points within the cell, but they do not systematically address the timescales of the relaxation behavior, which are critical to understanding cell dynamics.
Several mechanisms have been proposed to describe the spatial and temporal aspects of mechanical coupling in cells. Constitutive viscoelastic theory describes the elastic response and viscous relaxation of the whole cell as a single homogeneous material, whereas tensegrity or “action-at-a-distance” models describe the cytoskeleton as a conduit for stress propagation (Wang and Suo, 2005). Recent work has proposed that cells behave like a poroelastic material (Charras et al., 2008; Charras et al., 2005; Mitchison et al., 2008). Poroelastic models provide a prediction of the spatiotemporal connections within a cell by treating it as a biphasic material with a tightly meshed elastic network infiltrated by a viscous cytosol (Charras et al., 2005; Dembo and Harlow, 1986). Devised by Biot to predict settling of porous soil (Biot, 1941), poroelasticity theory has been used to explain the mechanical behavior of biological materials such as bone, soft tissues, and collagen gels (Cederbaum, 2000; Chandran and Barocas, 2004). When a poroelastic material is locally stressed, the elastic phase deforms, creating a localized pressure increase in the interstitial fluid, whose flow is impeded by the dense network. Over time, the pressure equilibrates radially away from the site of perturbation. Charras et al. applied poroelasticity theory to explain various cell behaviors - including motility, morphology, division, and blebbing - and predicted a diffusive response to a local change in the network, with an equilibration time that increases with distance from perturbation (Charras et al., 2005; Mitchison et al., 2008).

In order to measure the distance-dependent mechanical response of cells to localized stresses and evaluate specific mechanical models of the cell, a new technique is needed to quantify mechanical responses with high resolution at multiple locations and set distances from the perturbation. Typically, the cellular response at one point, such as displacement of the cell surface, is measured by visually tracking a fiduciary marker in two dimensions (Bausch et al., 1998; Bursac et al., 2005; Fabry et al., 2001; Hoffman et al., 2006; Hu et al., 2003; Panorchan et al., 2007; Trepat et al., 2007). However, out of plane (z) motion often comprises a significant component of the response and cannot be neglected. Recently, several strategies have been developed for tracking particles in 3D in a single image plane by using defocused epifluorescence microscopy (Speidel et al., 2003; Toprak et al., 2007; Wu et al., 2006). These techniques are hindered, however, by long computation times or by the inability to track closely-spaced particles, effectively limiting probe density.

Here we present an atomic force microscopy (AFM) based method to apply local stresses and simultaneously measure the mechanical response away from the perturbation (Figure 3.1), and we use it to quantify stress propagation in adherent cells. This method uses the AFM as a mechanical input, rather than its traditional uses as an imaging tool or a local material property probe. To quantify cell surface displacement in response to the AFM-induced mechanical perturbation, we track the motion of 500 nm integrin-bound fluorescent particles in three dimensions using defocused fluorescence microscopy. This combination of AFM and defocused microscopy enables exploration of distance-dependent cellular responses in 3D to spatially localized external perturbations, something previously unattainable. Using
this technique, we observe a biphasic response of adherent cells to an applied stress - an immediate propagation followed by a distance-dependent equilibration, which cannot be explained by traditional viscoelastic models.

Figure 3.1. Combined AFM and defocused microscopy. (A) An AFM cantilever is used to locally indent the cell, and the displacement of the cell surface is tracked in 3D by defocused epifluorescent microscopy of 500 nm fibronectin-coated fluorescent particles bound to the cell. Stage motion is controlled by a single-axis piezo-electric platform. Arrow in (A) indicates the perspective of the objective. A typical field of view is shown in (B) with the AFM cantilever outlined in white. This endothelial cell has been fixed and stained to show the nucleus (blue) and actin cytoskeleton (red), in addition to the fluorescent particles (green). Scalebar is 15 µm.

3.3 Materials and Methods

3.3.1 Cell culture and sample preparation

Bovine Aortic Endothelial Cells (BAECs) were cultured in endothelial growth media supplemented with 0.1% hEGF, 0.1% hydrocortisone, 0.1% GA-1000, 0.4% BBE, 2% FBS (Lonza). Cells were plated on acrylic-reinforced glass coverslips coated with fibronectin (BD Biosciences) and incubated overnight. The morning of experiments, the sample media was replaced with a 0.009% solids fibronectin-coated particle solution (500 nm YG Fluoresbrite™ carboxylate microspheres, Polysciences, Inc.) in CO2 independent media (GIBCO). Cells were incubated in the particle solution for 45 minutes and then rinsed thoroughly in CO2 independent media (GIBCO). The sample remained in CO2 independent media with 3µL/mL Hoescht stain (to visualize the nucleus) for the remainder of the experiment. Not all cells showed significant particle displacement upon cantilever indentation. For this analysis, only well spread cells with at least one instance of particle displacement greater than 500 nm are included. Particles that did not show significant displacement, even when the cantilever was a few microns away, were most likely endocytosed by the cell. The dynamics of particle endocytosis were examined using confocal microscopy to determine experimental guidelines to exclude endocytosed particles from analysis (data not shown).
3.3.2 Multi-point 3D particle tracking

By focusing several microns above or below the particle plane, each particle appears as a set of concentric rings in the image plane, with the image determined by the diffraction pattern of the particle and point spread function of the imaging system (Figure 3.2A-C) (Speidel et al., 2003). The radius of the outer ring is predictably related to the distance from the particle to the image plane and can be used to determine relative z displacements (Figure 3.2D). For every frame of the acquired image stack, a modified Hough transform was used to determine the (x, y) position and radius of each ring corresponding to the particle of interest. A Hough transform is an image processing technique used to find arbitrary shapes within an image (Ballard, 1981). Using Matlab 7 (Mathworks), the ring image was inserted into the middle of a 3D matrix of zeros. The Fourier transform of this image matrix was convolved with the Fourier transform of a thin-walled cone with radius spanning the range of the particle ring radii. The inverse Fourier transform of the convolution yielded a 3D matrix in (x, y, radius) space resembling a point spread function with center coordinates corresponding to the (x, y) position and radius, respectively, of the original ring image. We fit the center of this object to a 3D Gaussian to determine the position and radius of the ring, which was then translated to particle position using a lookup table created after each experiment (Fig. 3.2).

![Figure 3.2. Defocused multi-point 3D particle tracking in a single image plane. (A) A defocused fluorescent particle appears as concentric rings in the image plane (B,C). The outer ring radius increases predictably with distance from the object plane. (D) A particle is stepped in 250 nm increments away from the object plane and the outer ring is fit with our modified Hough transform numerical technique. Using this method, we can track the z-position of the particle over a range of at least 5 µm. (E) A particle is subjected to 10 nm steps on a piezo-controlled platform, demonstrating the resolution of our technique. (F) Our modified Hough transform method allows for tracking of multiple, overlapping rings with up to 4 nm and 80 ms resolution. This analysis enables multi-point tracking of particles as close as 1.5 µm to each other in (x, y), which is essential for tracking multiple points on the same cell. Scalebars are 2 µm.](image-url)
3.3.3 Atomic Force Microscopy

Indentation of cells was performed with a modified Bioscope AFM (Veeco Metrology Inc.) atop an epifluorescent microscope (Zeiss Axiovert 25, Carl Zeiss, Inc.) at 23° C (as described in (Rosenbluth et al., 2006)). A cell was approached with a pyramid-tipped AFM cantilever (3 μm tip height, 35° half-angle, 30 pN/nm stiffness; Microlevers, Veeco). Cells were indented with a sub-nanometer accuracy closed-loop piezo platform (Mad City Labs) controlled with a software-based data acquisition system (LabVIEW, National Instruments).

3.3.4 Stress propagation experiment

In our experiments, we dropped the focus below the sample plane in epifluorescence to form rings from the particles in the image plane. Images of the particles were acquired at 80-100 ms/frame using a 100x 1.3 NA oil immersion objective (Zeiss NeoFluar) and a Retiga-SRV camera (QImaging) as the cantilever was stepped vertically in and out of the cell with 2 μm steps of the stage. This step cycle was repeated for several cantilever positions on a single cell, with each particle returning to its original position upon each cantilever retraction. The repeatability of both particle displacement (see Figure 3 for example) and cantilever deflection (data not shown) for a series of step cycles suggests the indentation did not significantly damage the cell. The average cantilever indentation depth for experiments presented was 1703±182 nm with 93±3% (mean ± s.d., n = 37) of the total indentation occurring in an immediate elastic step (less than 100 ms). These indentations are larger than those in traditional AFM elasticity experiments, and as such the displacements are likely to be influenced by the substrate elasticity. Stress propagation through cells adhered to compliant substrates would be an interesting subject for future study. To determine if position on the cell affected the particle response, cells were indented at multiple positions in both the nuclear and lamellar regions. After stress propagation experiments, the cantilever was removed and z-positions of the particles were calibrated by raising the piezoelectric stage in 1 μm steps and capturing images at each stage height. This created a lookup table for each particle, which was used to convert ring radius into z-position.

To account for drift of the system during experiments, particle position was referenced with respect to a particle fixed to the glass surface in the same field of view as the cell-bound particles. This reference particle was also used to determine relative heights of particles associated with cells as a precaution against using endocytosed particles. After some experiments, cells were fixed and stained to visualize the actin cytoskeleton (Alexa Fluor 488 phalloidin conjugate, Molecular Probes) using standard immunofluorescence protocols.
3.3.5 Mechanical modeling

To determine if a traditional viscoelastic model explains the observed cellular response, we modeled the cell as a Voigt-Kelvin viscoelastic material (Brinson, 2008; Shaw, 2005) undergoing a step compression strain. To account for spatial variation of viscoelastic properties, the model uses a series of viscoelastic elements, with the first being a simple elastic (modeled as a spring) and the second two being viscoelastic (modeled as a spring and dashpot in parallel) (Figure 3.8A). To simulate the experiment, a step compression is applied and the relaxation of points at increasing distances from the compression point is observed. We then vary the viscosity of the viscous elements and observe the motion within the material to determine if the relaxation response matches the observed experimental behavior (Figure 3.8B-D).

We also compare the observed cellular response to predictions of the poroelasticity model based on theory from Charras et al. (Charras et al., 2005). After a deformation, fluid propagates through a cell due to a local pressure increase in a 2D diffusion-based manner in time \( t = \frac{x^2}{4D} \) where \( x \) is distance and the diffusion constant, \( D = kE/\phi \), where \( k \) is the hydraulic permeability, \( E \) is the Young’s modulus of the elastic phase of the material, and \( \phi \) is the fluid fraction. For a fluid-filled porous material with a small fluid fraction, hydraulic permeability is \( k \approx \frac{\xi^2}{(\mu \phi^2)} \), where \( \xi \) is the pore size and \( \mu \) the cytosolic viscosity. The Young’s modulus, \( E \), of endothelial cells has been measured at 1-9 kPa (Costa et al., 2006) with AFM and the fluid fraction has been measured to be 29-34% by volume change after exposure to hyperosmotic conditions (Alexopoulos et al., 2002; Charras et al., 2008). Pore size \( \xi \) can be estimated to be 13-26 nm from hindered tracer particle diffusion experiments and interstitial cytosolic viscosity \( \mu \) has been measured to be between 0.004-0.18 Pa-s based on nanometer-sized particle and actin diffusion experiments (Kreis et al., 1982; Mastro et al., 1984; Paine et al., 1975).

3.4 Results

3.4.1 Stress propagation observed with combined AFM and multi-point 3D particle tracking

We measured intracellular stress propagation by generating a local mechanical perturbation using AFM and observing the distal surface displacements of the cell by tracking fluorescent particles bound to integrins. We followed 3D motion of closely-spaced particles by acquiring defocused images of the particles (Figure 3.2A-C), which appear as rings on the image plane. Using a custom modified Hough transform method, we were able to track 3D particle position with up to 4 nm and 80 ms resolution by determining \((x,y)\) position of the particle from the \((x,y)\) position
of the ring and z-position from the ring radius (Figure 3.2D-F, see Materials and Methods for details).

We then tested our experimental system by indenting a thin polyacrylamide gel with an AFM cantilever at increasing distances from a single 500 nm fluorescent particle bound to the surface. We observed a decay of equilibrium particle displacement with distance from indentation (Figure 3.7) which closely follows the expected displacement of an elastic half-space (Hertz, 1882; Sneddon, 1965) or thin film (Dimitriadis et al., 2002).

To characterize stress propagation through adherent cells, we tracked the motion of fibronectin-coated 500 nm fluorescent particles bound to cultured BAECs as an AFM cantilever tip was repeatedly stepped into the cell (Figure 3.3). Particle displacement occurred primarily in the z direction. When the cantilever was stepped into the cell, the particle was significantly displaced within the first frame (100 ms), which we call the initial fast response. We refer to the particle's subsequent creep towards an equilibrium displacement position as the slow response.

**Figure 3.3. Cantilever indentation retraction cycles induce particle displacement.** An indentation and retraction of the AFM cantilever into the cell is repeated by moving the stage in 2 µm steps towards and away from the cantilever, with stage position held constant for 10-15 seconds after each step. A particle 2.5 µm away from the cantilever is displaced due to this indentation. Z displacement (red) accounts for the majority of total particle displacement (blue). Upon each cantilever step, an immediate fast response is observed, followed by a slower equilibration approaching a final displacement. Upon cantilever retraction, the particle again displaces elastically and then relaxes towards the original particle position.

When we indented an endothelial cell at increasing distances from a single particle, a distance-dependent decay in the displacement magnitude was observed (Figure 3.4A-D), similar to results from the polyacrylamide gel control. The binned average of n=71 coupling instances clearly displayed distance-dependent decay (Figure 3.4E) with particles farther from the indentation point displacing upwards, as might be expected for indentation of a constrained-volume material. Clear heterogeneity
was observed, with some particle displacements behaving substantially differently from the expected response of an elastic material.

**Figure 3.4. Particle displacement magnitude decays in a distance-dependent manner.** The displacement of a single particle on a cell was quantified as an AFM tip was stepped into the cell at distances ranging between 0-7 µm from the particle (A-C, taken from D). As distance from the tip increased, particle movement decayed towards zero, sometimes rising up at the farthest distances (D). Displacement magnitude was measured after particle relaxation. Error bars represent the fitting error of a flat line to equilibrated particle position as recorded over several seconds. The dark grey curve is the predicted surface for a semi-infinite elastic halfspace (Sneddon, 1965). The light grey shaded area represents the cantilever tip. (E) When pooled, average displacement from n=71 coupling instances (16 particles on 7 cells) show a similar distance-dependent decay that closely follows the elastic model, though single points clearly exhibit heterogeneity. Error bars represent standard error of the mean (SEM). Each color point represents a different cell and each shape represents a different particle.
3.4.2 Equilibration time increases with distance from perturbation

To better understand the equilibration behavior of the endothelial cells, we measured the timescale over which stresses propagated through the cells by tracking particle movement immediately after the mechanical perturbation by the AFM cantilever. Equilibration time was quantified by fitting a single exponential to the z component of the slow response of the particle, beginning 100 ms after indentation (Figure 3.5A-C). In Figure 3.5D, we show the response of 4 particles to indentations at 3 different points on a single cell. Notably, equilibration time increased with distance for the 4 particles tracked. This distance-dependent increase in equilibration time was further seen in the population response of n=57 coupling points (16 particles on 7 cells) (Table 3.1, Figure 3.5E).

To quantify the correlation between distance and equilibration time, the Pearson correlation coefficient was used. A coefficient value of 0 indicates no correlation and a value of 1 or -1 indicates perfect positive or negative correlation, respectively. The Pearson correlation coefficient of all 57 coupling points was \( r=0.68 \) (\( p<0.001 \)), indicating that a distance-dependent equilibration response was common and occurred in a majority of cases. This correlation was even higher in an independent analysis of 4 of the 5 cells with enough coupling points to perform statistics (Table 3.1). An additional analysis showed this increase in equilibration time with distance was seen in 93% of the particles with equilibration time values for at least two distinct distances (13 of 14 particles).

<table>
<thead>
<tr>
<th>Cell</th>
<th>n</th>
<th>r</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell 1</td>
<td>9</td>
<td>0.86</td>
<td>0.003</td>
</tr>
<tr>
<td>Cell 2</td>
<td>8</td>
<td>0.93</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cell 3</td>
<td>11</td>
<td>0.95</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cell 4</td>
<td>13</td>
<td>0.51</td>
<td>0.072</td>
</tr>
<tr>
<td>Cell 5</td>
<td>14</td>
<td>0.79</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>All particles</td>
<td>57</td>
<td>0.68</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Table 3.1. Particle equilibration time increases with distance from indentation. For each cell individually as well as all observed particles, equilibration time was positively correlated with distance from indentation (with each relationship being statistically significant except for Cell 4). n is the number of particles and r is the Pearson’s correlation coefficient.

No significant variations in response were observed when either particle location or cantilever perturbation was above the nuclear or lamellar regions, indicating that the observed trend was not dominated by proximity to the nucleus. To further ensure that the observed trend was not due to position on the cell, the depth of cantilever indentation was measured for each indentation. There was no significant
correlation between either equilibration time or particle displacement and cantilever indentation depth ($r = -0.18 \ (p = 0.39)$ and $r = -0.15 \ (p = 0.51)$, respectively).

Figure 3.5. Particle equilibration time increases with cantilever-particle distance. As distance between the indentation point and a particle increases, the particle takes a longer time to relax to equilibrium (A-C). Blue traces represent raw data and red displays the exponential fit to the data. (D) Four particles on a single cell showed increasing equilibration time with distance from perturbation when indented at three different positions. Data from each particle is represented by a different color and shape. Error bars represent the fitting error to a single exponential decay function. (E) Equilibration response of n=57 coupling instances (16 particles on 7 cells). Each cell is represented by a different color. Each particle on each cell is represented by a different shape. Average equilibration time is shown by the black points with error bars representing the SEM.
3.4.3 The actin cytoskeleton is required to maintain distance-dependent equilibration

To determine the effect of the actin cytoskeleton on the measured distance-dependent equilibration response, we exposed cells to cytochalasin D, an inhibitor of actin polymerization. We then periodically indented the cells at a fixed point over the course of 1 hour and observed the response of particles away from the indentation. While the fast response was still observed, the equilibration time of the particles decreased significantly within the first 30 minutes after cytochalasin D exposure and after one hour, equilibration time dropped to a small fraction of the initial equilibration time. This illustrates a clear decrease in equilibration time over the course of cytochalasin D action. (Figure 3.6) The Pearson correlation coefficient for time after exposure to cytochalasin D and equilibration time was -0.66 (p<0.001). Particles on cells not exposed to cytochalasin D had no observed decrease in equilibration time over the same time period with no significant correlation of equilibration time to time after exposure to cytochalasin D.

![Figure 3.6](image)

**Figure 3.6. Particle equilibration time decreases after cell exposure to cytochalasin D.** In two separate experiments, a cell with at least one particle located several microns from the cantilever tip was exposed to cytochalasin D at time=0 min. Indentation retraction experiments were repeated at the same location every 5 minutes for over 1 hour. Equilibration time of all 3 particles (3 particles on 2 cells) decreased over time after exposure to cytochalasin D. Equilibration time for each particle is normalized by the maximum observed equilibration time (the first time point in all 3 cases) and represented by a different color and shape. Average normalized equilibration time is shown by the black points with error bars representing the SEM.

3.5 Discussion

Here we present a new method for directly quantifying mechanical coupling in cells and observe an unexpectedly slow stress propagation and distance-dependent
equilibration. Distance-dependent relaxation behaviors are outside the scope of most existing cell mechanics models, due in part to the lack of experimental data and techniques to address this phenomenon.

We compared our results to two material models: viscoelasticity and poroelasticity. A single-phase homogeneous viscoelastic material, such as the traditional spring and dashpot standard linear solid model, cannot explain the observed behavior, as it assumes that the material will simultaneously relax in response to a local perturbation with a single time-constant. To determine if a heterogeneous viscoelastic model can explain this behavior, we modeled the experiment as a step-strain of a series of parallel spring-dashpot pairs (Voigt-Kelvin material (Brinson, 2008; Shaw, 2005)), which could account for varying viscoelastic properties within a cell. (See Materials and Methods and Supplementary Figure 3.8 for details.) By increasing the viscosity of the dashpots furthest from the step strain, a distance-dependent increase in equilibration time can be observed. However, placing the more viscous elements closer to indentation results in a distance-dependent decrease in equilibration time. As our findings were spatially invariant - wherever the cell was indented, a distance-dependent increase in equilibration time was observed - this model of a series of spring-dashpot pairs cannot explain the observed behavior.

The poroelastic model can account for the observed slow distance-dependent equilibration across the cell. The biphasic nature of a poroelastic material results in both a fast propagation of stress through the solid phase (cytoskeleton) as well as a much slower diffusive equilibration of hydrostatic pressure of the fluid phase (cytosol), resulting in an increasing equilibration time with distance (Cederbaum, 2000). Using cytoplasm viscosity, cell fluid fraction and porosity, and cytoskeletal elasticity measurements from the literature (Charras et al., 2005; Alexopoulos et al., 2002; Costa et al., 2006; Kreis et al., 1982; Luby-Phelps et al., 1987; Mastro et al., 1984; Paine et al., 1975), we found our experimental results are consistent with and on the same scale as the diffusive equilibration predicted by poroelasticity theory (see Materials and Methods for calculation details). While this suggests a poroelastic behavior, such predictions are highly dependent on the values of parameters put into the model, and some parameters, such as cytosolic viscosity, have been measured previously to range by an order of magnitude or more.

Poroelasticity theory predicts that equilibration time depends quadratically on distance from perturbation (Charras et al., 2008; Charras et al., 2005; Mitchison et al., 2008). When the averaged equilibration data (Figure 3.5E) was fit to a linear \( t = C_0 + kx \) versus quadratic \( t = C_0 + kx^2 \) model, the resulting Chi-square values were 2.070 and 0.289, respectively. The significantly smaller Chi-square value for the fit to a quadratic model indicates that the experimental data is more in line with the quadratic trend predicted by the poroelastic model. However, equilibration times of each individual cell exhibited a more linear dependence on distance, indicating that a quadratic poroelastic model does not fully account for all observations and additional mechanical behaviors may be important. (See Figure 3.5D for an
example.) In summary, the observed increase in equilibration time with distance from perturbation cannot be explained by conventional viscoelastic models of cell mechanics, but is in agreement with the poroelastic model.

The observed decrease of equilibration time after exposure to cytochalasin D is also consistent with the poroelastic model. The actin cytoskeleton serves as both the source of elasticity and the barrier to flow of the cytosol. As the actin cytoskeleton begins to depolymerize due to cytochalasin D, pore size increases, resulting in less impeded flow of the cytosol and reduced time to equilibrium. Eventually, the cytoskeleton is disrupted to the point that it no longer serves as an effective barrier to the cytosol, significantly reducing equilibration times across the cell.

As described by Mitchison et al. (Mitchison et al., 2008), it is not entirely surprising that a cell would not behave like a simple viscoelastic material, since viscoelasticity assumes the material is a single homogeneous phase. As cells contain a dense and crowded cytoplasm consisting of water, soluble and non-soluble proteins, organelles, cytoskeleton, and a complex lipid bilayer membrane, it seems unlikely that these components do not move relative to each other, as viscoelasticity theory implicitly assumes. Modeling the cell as a two-phase material consisting of fluid and solid portions which can move with respect to one another allows for the investigation of a more dynamic cytoplasm.

Our measurements of stress propagation in single cells using a combination of AFM and defocused microscopy raise three major points. First, our data shows that a distance-dependent equilibration can occur over several seconds, demonstrating temporary storage of mechanical energy by the cell. The potential existence of long-lived pressure gradients has significant implications for cell motility since localized pressure gradients created by acto-myosin contraction of crawling cells could induce fluid flow that contributes to the coordination of cell protrusions. Second, our results have important implications for mechanical signal transduction. Pressure gradients and cytosolic flow may result in convective biochemical transport, thereby speeding up signaling pathways. The fact that mechanical equilibration takes longer at greater distances from the perturbation could serve as a distance-dependent low-pass filter with only lower frequency deformations transmitted across the whole cell. Finally, our results suggest that a thorough material model of the cell must go beyond the traditional viscoelastic representation. Experiments explained in the context of soft glassy rheology have shown that a single timescale cannot be applied to cell relaxation (Bursac et al., 2005). We add an additional dimension to this by showing that the observed timescales of equilibration are also dependent on distance from the perturbation location. Our combined AFM and high resolution 3D multi-particle tracking method offers a powerful approach to further probe these theories, as well as to directly quantify mechanical coupling in cells in response to mechanical stimuli and during highly coordinated mechanical processes including motility, shape change, cytokinesis, and cell-cell and cell-extracellular matrix interactions.
3.6 Supplementary Information

3.6.1 Distance-dependent decay of displacement magnitude on a polyacrylamide gel

As a proof of concept of our system, a 10 µm thick polyacrylamide film was indented with an AFM cantilever at several locations and the surface displacement was quantified by tracking a 500 nm fluorescent particle bound to the surface. Surface displacement decayed as cantilever-particle distance increased, as expected for elastic half-space or thin film models (Figure 3.7).

Acrylamide thin films were made by coating (3-Aminopropyl)trimethylsiloxane for 5 minutes onto glass coverslips, washing, incubating with 0.5% glutaraldehyde for 30 minutes, and then washing again. 8% polyacrylamide solution with dilute 10 µm polystyrene beads (Polysciences) was sandwiched between the coated coverslip and a clean coverslip to ensure a 10 µm thickness. Once gelled, the coverslip was removed.

![Figure 3.7](image.png)

Figure 3.7. Particle displacement magnitude decays in a distance-dependent manner on a thin acrylamide gel. The out of plane displacement of a single particle on a cell was quantified as a function of cantilever-particle distance as an AFM tip was stepped into the cell at distances ranging between 0-13 µm from the particle. Particle displacement decayed towards zero as distance increased. Displacement magnitude was measured after particle relaxation. Error bars represent the fitting error. The dark grey curve is the predicted surface for a semi-infinite elastic halfspace. The light grey shaded area represents the cantilever tip.
3.6.2 Voigt-Kelvin model of the cell

Figure 3.8. Voigt-Kelvin model of the cell. (A) To test if a simple viscoelastic model of the cell could explain the observed distance-dependent equilibration, we modeled the cell as a series of viscoelastic materials, with the first material being a simple elastic component and the second two materials being viscoelastic. All elastic constants (represented by springs) are assumed to be equal. To simulate the cantilever indenting the cell, a step compression of the material is induced at time = 0 s, and the relaxation of the different nodes (a-c) is observed over time to evaluate how points away from the indentation will move. Each material in this model is assumed to be of equal length. (B) In the first case, the viscosity of dashpots 1 and 2 are set to be equal and a step compression strain of 20% is induced in the material at point (a). Point (a) immediately moves 20% of the total material length and remains there. Points (b) and (c) compress to their final positions with the same equilibration time, as expected, since both dashpots are the same viscosity. (C) In the second case, dashpot 2 is set to a viscosity three times that of dashpot 1. As a result, the equilibration time of point (b) is much faster than that of point (c), demonstrating that placing viscous elements of increasing viscosities further away from the step indentation could result in an increasing equilibration time at distances further from indentation. (D) In this example, the viscosity of dashpot 1 is set to three times that of dashpot 2, and the reverse situation from (C) is observed—point (c), the point furthest away from the indentation, equilibrates in a shorter time than point (b). This example demonstrates that a Voigt-Kelvin model of the cell cannot explain the experimental observations observed, even when material properties are spatially varied. While it can produce different equilibration times at different points away from indentation, it cannot produce a consistently increasing equilibration time as distance from indentation increases, unless viscous elements are very specifically rearranged.
3.7 Acknowledgements

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Chapter 4. An AFM-based stiffness clamp for dynamic control of rigidity

4.1 Abstract

Atomic force microscopy (AFM) has become a powerful tool for measuring material properties in biology and imposing mechanical boundary conditions on samples from single molecules to cells and tissues. Constant force or constant height can be maintained in an AFM experiment through feedback control of cantilever deflection, known respectively as a ‘force clamp’ or ‘position clamp’. However, stiffness, the third variable in the Hookean relation $F = kx$ that describes AFM cantilever deflection, has not been dynamically controllable in the same way. Here we present and demonstrate a ‘stiffness clamp’ that can vary the apparent stiffness of an AFM cantilever. This method, employable on any AFM system by modifying feedback control of the cantilever, allows rapid and reversible tuning of the stiffness exposed to the sample in a way that can decouple the role of stiffness from force and deformation. We demonstrated the AFM stiffness clamp on two different samples: a contracting fibroblast cell and an expanding polyacrylamide hydrogel. We found that the fibroblast, a cell type that secretes and organizes the extracellular matrix, exhibited a rapid, sub-second change in traction rate ($dF/dt$) and contraction velocity ($dx/dt$) in response to step changes in stiffness between 1-100 nN/μm. This response was independent of the absolute contractile force and cell height, demonstrating that cells can react directly to changes in stiffness alone. In contrast, the hydrogel used in our experiment maintained a constant expansion velocity ($dx/dt$) over this range of stiffness, while the traction rate ($dF/dt$) changed with stiffness, showing that passive materials can also behave differently in different stiffness environments. The AFM stiffness clamp presented here, which is applicable to mechanical measurements on both biological and non-biological samples, may be used to investigate cellular mechanotransduction under a wide range of controlled mechanical boundary conditions.

4.2 Introduction

Atomic force microscopy (AFM), initially developed as a topographical imaging modality, has become an important tool for investigating the mechanical properties and dynamic behavior of biological molecules, materials, cells, and tissues (Jena, 2002). AFM-based techniques in cell and molecular biology leverage the high resolution of AFM in space, time, and force to study properties such as cell adhesion mechanics (Helenius et al., 2008), polymer network dynamics (Chaudhuri et al., 2007), and protein folding (Puchner and Gaub, 2009). Here we present the development of a method for dynamically varying AFM cantilever stiffness that takes advantage of precise AFM feedback control to create changes in the external rigidity felt by active samples. We use this method, which we call a ‘stiffness clamp’ by analogy to the existing ‘force clamp’ and ‘position clamp’, to investigate the cellular response to rigidity.

The rigidity of the cellular microenvironment has been shown to be an important input signal that influences a range of biological processes (Janmey et al., 2009). The
resistance to deformation of tissues in vivo, characterized by an elastic modulus, varies from near 100 pascals for soft tissues such as the brain to tens of thousands of pascals for muscle tissue and up to millions of pascals for cartilage. This tissue rigidity, or stiffness, serves as an important in vivo cue in processes such as embryogenesis (Jacot et al., 2010), cell proliferation (Klein et al., 2009), and angiogenesis (Mammoto et al., 2009). Notably, numerous experiments have demonstrated the influence of microenvironmental rigidity in vitro on cellular morphology (Yeung et al., 2005), motility (Saez et al., 2007) and differentiation (Engler et al., 2006). While the importance of stiffness has been well-documented, the dynamics of rigidity sensing are poorly understood.

The predominant methods for studying the effects of microenvironmental rigidity on cellular behaviors involve culturing cells on deformable substrates (e.g. thin rubber films (Keese and Giaever, 1991), polyacrylamide hydrogels (Pelham and Wang, 1997), and microfabricated posts (Tan et al., 2003)). These studies, while instrumental in establishing the effect of substrate rigidity on cellular behaviors, are limited to a single static rigidity for each experiment. Similarly, the spring-like behavior of optical traps, AFMs, and microplates has also been used to expose single cells to different microenvironmental rigidities but these usually use only a single rigidity per experiment (Lam et al., 2011; Mitrossilis et al., 2009; Mizuno et al., 2009). To expose a given cell to multiple rigidities, some studies have employed static rigidity gradients (Allioux-Guérin et al., 2009; Lo et al., 2000) or substrates of anisotropic rigidity (Ghibaudo et al., 2008). Recent studies have demonstrated hydrogels with dynamic rigidities that utilize UV exposure (Frey and Wang, 2009) or DNA crosslinking (Jiang et al., 2010) to change rigidity mid-experiment, though the stiffness changes are relatively slow, not reversible, and can only sample a narrow range of elastic moduli. Furthermore, none of these techniques distinguish between the cell’s response to force, deformation, and stiffness. Recently, a custom-built parallel microplate system was used in combination with double-feedback to change the effective stiffness experienced by a single cell spread between the microplates (Mitrossilis et al., 2010). While AFMs have the advantage of high resolution in space, time, and force, and cells can spread between a microfabricated cantilever and a surface (Chaudhuri et al., 2009; Lam et al., 2011), AFM systems are currently limited to a single stiffness per experiment given by the native cantilever stiffness.

We have developed an AFM feedback algorithm to reversibly and rapidly change the stiffness presented to the sample while accurately measuring force and deformation. We apply this AFM stiffness clamp to study the dynamics of an expanding hydrogel and a single cell in response to step changes in stiffness.
4.3 Results

4.3.1 Stiffness clamp concept

The mechanical interaction of contractile cells with their microenvironment, which is composed of polymeric extracellular matrix (ECM) proteins and other cells, can be modeled most simply as a cell pulling on a spring (Figure 4.1a). Setting aside the nonlinear behavior of the ECM temporarily, a cell that deforms a Hookean spring experiences a resistance force given by the spring constant and the amount of deformation. The goal of the stiffness clamp is to tune the apparent stiffness a cell experiences by controlling how much force the cell must exert to change its height a given amount through feedback control of the spring deflection (Figure 4.1b).

![Diagram of stiffness clamp concept](image)

**4.1. Feedback control can change the apparent stiffness a cell experiences.** (a) A contracting cell in a soft extracellular matrix (ECM) experiences little resistance to its contraction and can be modeled with a soft spring. (b) A contracting cell in a stiff ECM experiences a large resistance to its contraction and can be modeled with a stiff spring. Using the AFM stiffness clamp, a soft spring can be made to appear stiff (or vice-versa) by controlling the spring’s extension as a function of the cell’s contraction. This approach can be broadly applied to make springs appear stiffer or softer than their actual value.

In theory, a wide range of apparent stiffnesses may be achieved using only a single spring together with feedback control (Figure 4.2). If the spring base is moved away from the cell as it contracts, the spring will appear stiffer to the contracting cell than it actually is (Figure 4.2a). If the spring base is moved upwards, away from the cell by the same amount that the cell deflects the spring downward, then the cell height, $x_{cell}$, will remain constant. Given this constraint, regardless of the force the cell exerts on the spring, the cell’s height does not change, thereby exposing the cell to an infinitely stiff microenvironment $k_{apparent} = \frac{\Delta F}{\Delta x_{cell}} = \frac{\Delta F}{0} \to \infty$. By moving the spring base toward the cell as it contracts, the spring will appear softer than it actually is (Figure 2B). If the feedback routine moves the spring base such that the spring does not change in length, the force exerted on the spring remains constant, and the stiffness of the microenvironment appears to be infinitely soft.
The two limits of constant height and constant force have been used elsewhere and are known as the position and force clamp, respectively (Sheetz, 1998). Force and position clamps are based on a simple PID-feedback routine that uses the error between a given setpoint force or position and the current force or position to adjust the sample position. In contrast, stiffness is defined as the change in force over the change in displacement and therefore cannot be controlled using conventional feedback routines. The AFM stiffness clamp presented here is able to dynamically tune apparent stiffness between the extremes of infinitely soft and stiff.

4.2. Conceptual design of the AFM stiffness clamp. (a) A stiff spring can be simulated using a spring of a smaller stiffness. A cell applying a given force against a stiff spring achieves a smaller change in height than a softer spring. Moving the spring base up as the cell contracts makes a softer spring appear stiffer to the contracting cell. Plotting contractile traction force \( F \) versus cell height \( x_{cell} \) produces a trace whose steep slope is the apparent stiffness, \( k_{apparent} \) (dotted line) and is greater than the native spring stiffness, \( k_{spring} \) (solid line). (b) A soft spring can be simulated using a spring of a greater stiffness. A cell applying a given force against a soft spring achieves a greater change in height than a stiffer spring. Moving the spring base down as the cell contracts makes a stiffer spring appear softer to the contracting cell. Plotting traction force \( F \) versus cell height \( x_{cell} \) produces a trace whose gradual slope is the apparent stiffness, \( k_{apparent} \) (dotted line) and is less than the native spring stiffness, \( k_{spring} \) (solid line).

4.3.2 Stiffness clamp applied to an expanding hydrogel

We tested the ability of the AFM stiffness clamp algorithm to produce a range of apparent stiffnesses with an expanding hydrogel, and we characterized the material’s response to step changes in stiffness. Addition of phosphate buffered saline (PBS) to a dehydrated \( \sim 1 \) kPa polyacrylamide hydrogel caused it to gradually expand. As the gel expanded and increased in height, it pushed against the cantilever applying an increasing force (Figure 4.3.a and b). Without the stiffness
clamp feedback loop, the spring constant of the cantilever defined how much force the gel applied to increase its height. When we changed the apparent stiffness of the cantilever using the stiffness clamp between 1-100 nN/μm, there was an immediate change in the force rate due to the modified feedback control of the cantilever position, while the gel expansion rate remained essentially constant (Figure 4.3c). This behavior was observed for N = 5 gels.

4.3. Response of expanding hydrogel to step changes in stiffness. (a) The AFM stiffness clamp was applied to a rehydrated hydrogel that deflected an AFM cantilever as it expanded. Cantilever position is precisely measured using an optical lever system. Feedback was implemented by moving a piezo-controlled substrate. (b) A typical trace of how force and gel height (x\text{gel}) changed over time as the cantilever deflected in response to the expansion of the hydrogel against apparent stiffnesses of 1, 10, and 100 nN/μm. Separate experiments conducted on 5 different gels all exhibited the same stiffness-dependent behavior shown above. Note that the slope of the force trace clearly changes when the
apparent stiffness changes, while the slope of the height trace remains basically constant over this range of stiffness. (c) Categorical plot of the force rate and velocity of gel expansion under three different apparent stiffnesses from the trace depicted in (b). The rates are determined from a linear regression fit where the 95% confidence interval for each slope is within $\pm 0.25 \, \text{nN/min}$ and $\pm 5 \, \text{nm/min}$ for the force and height, respectively. Force rate changes with stiffness while expansion rate does not over this range of stiffness. (d) Plot of force $(F)$ versus gel height $(x_{\text{gel}})$ as the gel expanded under a wide range of apparent stiffnesses. Each trace represents a different apparent stiffness listed in the table and applied using the stiffness clamp algorithm. The traces were translated to begin at the origin for comparison. The horizontal and vertical traces represent desired stiffnesses approaching 0 and $\infty$, corresponding to a force and position clamp with standard deviations of $15 \, \text{pN}$ and $0.34 \, \text{nm}$. Inset depicts the discrete but highly linear nature of the data. The * marks the trace without any feedback loop and whose slope is the spring constant of the cantilever, $42 \, \text{nN/\mu m}$. With a single AFM cantilever with spring constant $k_{\text{cantilever}}$, we used the stiffness clamp to apply 11 different stiffnesses ranging from 0 to infinity as the gel expanded. By plotting the cantilever force versus the gel height we obtained a series of traces where the slopes define the achieved apparent stiffness (Figure 4.3d). The apparent stiffness measured from the slope of the traces in Figure 4.3d was less than 0.1% different from the desired value for a range of stiffnesses spanning two orders of magnitude from $1/16$ to $16 \, k_{\text{cantilever}}$. The most extreme apparent stiffnesses (force clamp and position clamp) produced traces with Gaussian noise around a constant force and height with standard deviations of $15 \, \text{pN}$ and $0.34 \, \text{nm}$, respectively. (See supporting information section 4.6 for further information.) Figure 4.3d demonstrates that we can accurately apply a wide range of apparent stiffnesses on an expanding hydrogel, all with a single cantilever, using the AFM stiffness clamp.

4.3.3 Stiffness clamp applied to a contracting cell

Fibroblast cells are used extensively as a model system to investigate the effect of substrate rigidity (Janmey et al., 2009; Lo et al., 2000; Pelham and Wang, 1997; Tan et al., 2003; Yeung et al., 2005). After demonstrating the range and precision of the stiffness clamp algorithm with a hydrogel, we used NIH 3T3 fibroblast cells to investigate how cellular rigidity sensing responds to a reversible step change in stiffness. Figure 4 shows the results of a typical experiment. Cells in suspension were flowed into a chamber and within minutes were brought into contact with both a fibronectin-coated glass surface and a fibronectin-coated tipless AFM cantilever ($k_{\text{cantilever}} = 18 \, \text{nN/\mu m}$). After a small compressive force ($4 \, \text{nN}$) established contact, adhesions formed on both surfaces, and the cell contracted (Figure 4.4a). Once contraction started we cycled between stiffnesses of $1/5, 1, \text{ and } 5 \, k_{\text{cantilever}}$ ($3.6, 18, 90 \, \text{nN/\mu m}$) every 30 s. We chose a cycle period of 30 s to allow for exchange of cytoskeletal and focal adhesion components (timescale of seconds) but not full
reorganization of adhesions or the cytoskeleton (timescale of minutes) (Giannone and Sheetz, 2006). A typical resulting traction force and cell height trace is shown in Figure 4.4b.

4.4. Cell contraction rapidly responds to stiffness changes. (a) An AFM was used to expose a single fibroblast cell to dynamically changeable apparent stiffness values with the AFM stiffness clamp. The piezo-controlled substrate was moved in response to deflections of the cantilever, which were precisely measured with an optical lever system. (b) Force and cell height as the cell contracts under different apparent stiffnesses from a typical experiment. A total of 30 cells were tested, all exhibiting the same stiffness-dependent behavior shown above. Each interval is under an apparent stiffness of 3.6, 18, or 90 nN/μm as indicated at the top of the graph. The traction rate and contraction velocity rapidly change with a step change in stiffness. A segmented linear regression fit is plotted highlighting the change in traction rate (inset). Data displayed in (c) and (d) are compiled from this trace. (c) Traction rate increases with apparent stiffness while corresponding contraction velocity decreases. The rates are determined from a linear regression fit where
the 95% confidence interval for each slope is within ± 0.4 nN/min and ± 20 nm/min for the force and height, respectively. (d) Plot of force versus cell height. The three linear, distinct traces each have slopes that indicate that the desired apparent stiffnesses were achieved. The * marks the trace without any feedback loop. Each interval was translated to begin at the origin for comparison.

We found that when the apparent stiffness changed to a larger value, the cell’s traction rate \( \frac{dF}{dt} \) rapidly increased while the corresponding contraction velocity \( \frac{dx}{dt} \) decreased (Figure 4.4.d). Notably, this change in traction rate and contraction velocity happens nearly instantaneously (within 0.5 s) (Figure 4.4b inset), indicating that cells can reversibly respond to a stiffness cue on a whole cell level on a timescale of seconds. The stiffness-dependent traction rate and velocity were found to be reversible and consistent for a given cell, despite changes in absolute cell height and contractile force. Even though the absolute cell tension was greater later during contraction, the traction rate was dependent only on the instantaneously applied stiffness (and similarly for cell height and contraction velocity). Importantly, this indicates that the response of contraction rate is specifically due to a change in stiffness and not the cell tension or height. This behavior was observed for N=30 cells.

4.4 Discussion

The AFM stiffness clamp provides a high-resolution method for varying apparent stiffness and evaluating cellular responses including contraction behavior. Using the AFM stiffness clamp, we show that cells rapidly change their traction rate and contraction velocity in response to step changes in apparent stiffness. Importantly, the stiffness clamp algorithm dynamically changes the apparent stiffness while the force and height are unchanged in the instant before and after the stiffness change. Therefore, any cellular response is a function of the step change in stiffness and not force or height. This decoupling of stiffness from force and height unambiguously shows that stiffness changes alone caused the change in contraction.

Our observation of stiffness dependent contraction of single cells is consistent with several previous studies. We recently used the high-resolution of AFM to characterize the contraction dynamics of single human platelet cells (Lam et al., 2011) and found that the force generation of platelets was dependent on microenvironmental stiffness, though each platelet was exposed to only a single stiffness. Other techniques, using systems limited to a single stiffness per experiment, have also observed a dependence of contraction on stiffness with a variety of cell types (Allioux-Guéran et al., 2009; Mitrossilis et al., 2009; Saez et al., 2007). Our results with the AFM stiffness clamp are consistent with a recent study by Mitrossilis et al. that used a custom-built parallel microplate system to change
the stiffness experienced by a single myoblast cell and found that traction rate was higher for larger stiffnesses and did not depend on absolute force (Mitrossilis et al., 2010).

It is worthwhile to note that the AFM stiffness clamp presented here only alters stiffness in one axis, though as demonstrated above, this appears to be sufficient to elicit a response from the contracting cell. Due to the fact that stiffness can only be measured by displacing a sample, the apparent stiffness can only be applied when cell height is actively changing, for example during fibroblast contraction, cardiomyocyte beating, neutrophil shape change in response to chemoattractants, and cell rounding during mitosis.

This AFM-based approach to dynamically tuning microenvironmental rigidity is broadly applicable to both biological and non-biological experimental situations. In essence, the algorithm we present can be applied to any system with a spring where there is precise knowledge of the force and a single means of adjusting the position of the spring base (as illustrated in Figure 4.2). This stiffness clamp algorithm has the advantage of requiring only one actuator and therefore can be used with existing commercial AFMs. Furthermore, the algorithm can be adjusted to emulate nonlinear elastic properties, such as those of specific ECM networks.

In the case of single molecule experiments on mechanosensitive molecules, which typically employ an AFM or optical trap (Wen et al., 2007), the AFM stiffness clamp could be implemented to sample a wide range of apparent stiffness values. The stiffness clamp can also be integrated with cell rheology measurements and fluorescence microscopy to characterize the viscoelastic properties of the cell and protein localization under various apparent stiffnesses. At the multicellular scale, tissue stiffness has been shown to affect the cancerous phenotype of cell colonies (Paszek et al., 2005), and the AFM stiffness clamp could be used to study the responses of tissues in microenvironments of changing stiffness. Importantly, our system allows for the use of apparent stiffness values outside of those that can be achieved by standard cantilever fabrication methods.

In this study, we have presented an AFM-based method for dynamically changing the apparent stiffness of the microenvironment surrounding a cell. We demonstrated the high temporal and spatial resolution of the AFM stiffness clamp using an expanding hydrogel and contracting cell, finding that the cell contraction rate reversibly changes nearly instantaneously with stiffness and does not depend on absolute force or cell height. Both cellular traction rate and contraction velocity were stiffness-dependent, whereas the expansion velocity of the hydrogel used in our experiments remained constant for stiffnesses ranging 1-100 nN/μm. The AFM stiffness clamp provides a powerful tool for investigating the role of mechanical boundary conditions on cellular behavior.
4.5 Materials & Methods

4.5.1 Stiffness clamp algorithm

The AFM stiffness clamp is implemented using a feedback algorithm based on the extension of a Hookean spring \( \Delta F = k_{spring} \Delta x_{spring} \), though this analysis can be extended to nonlinear springs. The microenvironmental stiffness a cell experiences is given by the amount of force it must apply to change its height, \( \Delta F = k_{\text{apparent}} \Delta x_{cell} \). If the base of the spring can move by an amount \( \Delta x_{\text{base}} \), the change in cell height is given by the difference between spring extension and movement of the spring base, \( \Delta x_{cell} = \Delta x_{spring} - \Delta x_{\text{base}} \). The force resisting the change in cell height is provided solely by the extension of the spring. Therefore, equating the expressions for \( \Delta F \) and solving for the movement of the spring base gives

\[
\Delta x_{\text{base}} = \frac{k_{\text{apparent}} - k_{spring}}{k_{\text{apparent}}} \Delta x_{spring} \quad (4.1)
\]

which defines how much the base must be moved to achieve the desired apparent stiffness, \( k_{\text{apparent}} \), for a given deformation of the spring. Note that the position clamp can be obtained from Eq. (4.1) when \( k_{\text{apparent}} = \infty \), in which case the base moves the same amount as the spring deforms, and the cell height remains constant. Similarly, the force clamp results when \( k_{\text{apparent}} = 0 \) and \( \Delta x_{\text{base}} \) cancels out the movement of the spring, such that \( \Delta x_{spring} = 0 \).

The AFM stiffness clamp feedback algorithm uses the desired apparent stiffness \( (k_{\text{apparent}}) \), the spring stiffness \( (k_{spring}) \), and Eq. (4.1), together with a measure of how much the cell deforms the spring, to determine how far to move the base. Equation (1) is directly used in the feedback algorithm for \( k_{\text{apparent}} > k_{spring} \), but for \( k_{\text{apparent}} < k_{spring} \) Eq. (4.1) grows out of bounds as \( k_{\text{apparent}} \) approaches zero. For \( k_{\text{apparent}} < k_{spring} \), we alter Eq. (4.1) so that it iteratively converges to the same ratio \( \frac{\Delta x_{\text{base}}}{\Delta x_{spring}} \) without growing out of bounds according to

\[
\Delta x_{\text{base,}i} = \frac{k_{\text{apparent}} - k_{spring}}{k_{spring}} (\Delta x_{spring} - \Delta x_{\text{base,i-1}}) \quad (4.2)
\]

where \( i \) is the index for each cycle of the iteration and \( \Delta x_{\text{base,i-1}} \) is the amount the base was moved in the previous iteration (see supporting information section 4.6 for a detailed derivation).
4.5.2 Atomic force microscope

Atomic force microscope (AFM) experiments were conducted using a modified Veeco Bioscope I mounted on a Zeiss Axiovert 25 inverted microscope. The Bioscope I z-axis piezo in our system has a range of only 4 \(\mu\)m. Since a larger z range is more convenient for working with cells, the substrate was moved instead of the cantilever base with a feedback-controlled Mad City Labs piezo-actuator stage and controller with a range of 50 \(\mu\)m and a resolution of 0.1 nm. Cantilever deflection and substrate position was controlled with a National Instruments 16-bit, 250 kS/s PCI-6229 digital I/O card and a custom LabVIEW program to implement the stiffness clamp algorithm running at 100 Hz. The substrate was mounted on a heated stage and maintained at 37°C for cell experiments. Tipless silicon nitride MLCT (30-50 \(nN/\mu m\), Veeco) and Arrow cantilevers (10-20 \(nN/\mu m\), Nanoworld) were used for the gel and cell experiments, respectively. Calibration of the optical lever was conducted before each experiment by ramping a glass coverslip substrate up and down while in contact with the cantilever. The surface was ramped 450 nm and the average of 15 cycles was used to determine the volts to meters conversion factor. See supporting file 1 for a discussion on the effect of calibration errors on the apparent stiffness applied by the stiffness clamp. We then determined the cantilever spring constant before each experiment by recording the thermal fluctuations of the cantilever out of contact in air and fitting the first resonance peak of the power spectra with a Lorentzian function using the equipartition theorem (Hutter et al., 1993). This indicates that the resolution of the detection of the cantilever position was thermally limited.

To monitor drift in both the cell and gel experiments, we placed the cantilever in contact with the glass substrate in force clamp mode, immediately before each experiment. Experiments were not started until the system had equilibrated, such that a force clamp could be maintained with no significant change in stage position (generally 10-60 minutes). Drift over the course of the experiment was measured in two ways. First, the zero deflection point of the cantilever was compared before and after each experiment to measure any cantilever drift. Second, for cell experiments, the contact point between the surface and cantilever was measured before and after each experiment. These measurements confirmed that the drift over the course of the experiment was negligible compared to the active contraction of the cell and expansion of the gel. Drift accounted for <10% of the total deflection for all experiments used.

4.5.3 Polyacrylamide hydrogels

The ~1 kPa polyacrylamide hydrogel was dehydrated at 4°C overnight and was rehydrated immediately before the AFM experiment with a standard phosphate buffered saline (PBS) solution. The cantilever was brought into contact as the gel rehydrated and expanded.
4.5.4 Cell culture

NIH 3T3 fibroblast cells were cultured in DMEM (GIBCO) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained in an incubator at 37°C with a humid, 5% CO₂ atmosphere. A trypsin solution was used to detach cells at which point trypsin neutralizer was added and cells were then centrifuged at 300 g for 5 minutes. The resulting supernatant was discarded and cells were resuspended in their culture medium (DMEM plus supplements). KOH cleaned glass substrates and cantilevers were immersed for 30 min in a 50 μg/ml fibronectin solution (F0895, Sigma). The fibronectin solution was then washed off and cells were added and the cantilever was brought on top of a cell as it settled on the substrate.

4.5.5 Statistical analysis

The inset of the FIG. 4B demonstrates the rapid change in traction rate upon a change in apparent stiffness. We found that this change occurred within 0.5 s. This response time was calculated by comparing two models with an F test with P values < 0.01. First, a 30 s window was applied centered on the timepoint when $k_{\text{apparent}}$ was changed. Then a simple linear regression was compared with a segmented linear regression where the timepoint of the intersection of the two segments must be determined from the data. This 30 s window was then moved earlier in time and the two models were again compared. The point at which the preferred model shifted to the simple linear regression is defined as the point when the traction rate has statistically changed.

4.6 Supporting Information

4.6.1 Derivation

In the text the movement of the base to maintain the stiffness clamp is defined as:

$$\Delta x_{\text{base}} = \frac{k_{\text{apparent}} - k_{\text{spring}}}{k_{\text{apparent}}} \Delta x_{\text{spring}} \quad (4.3)$$

Noting that for $k_{\text{apparent}} \ll k_{\text{spring}}$ this equation becomes unstable for $\Delta x_{\text{base}}$, we use a modified iterative equation that achieves the same ratio of $\frac{\Delta x_{\text{base}}}{\Delta x_{\text{spring}}}$ as Eq. (4.3) but
does not result in $\Delta x_{\text{base}}$ growing out of bounds for $k_{\text{apparent}} \to 0$:

$$
\Delta x_{\text{base},i} = \frac{k_{\text{apparent}} - k_{\text{spring}}}{k_{\text{spring}}}(\Delta x_{\text{spring}} - \Delta x_{\text{base},i-1})
$$  \quad (4.4)

This equation converges to Eq. (4.3). Assume the deflection of the spring in each iteration is defined as: $\Delta x_{\text{spring},i} = R + \epsilon \Delta x_{\text{base},i-1}$, where $R$ is the distance the cell contracts in a single time step and $\epsilon$ is the absorption coefficient of the spring, defined as the ratio of the combined spring constant of the cell and spring in series and the spring's stiffness, where $\epsilon = \frac{k_{\text{cell}}}{k_{\text{spring}} + k_{\text{cell}}}$. $\epsilon = 1$ corresponds to the spring absorbing all the change in height and $\epsilon = 0$ corresponds to the cell absorbing all the change in base height with no spring deflection.

Using the iterative model we derive an equation that relates how rapidly Eqs. (4.3) and (4.4) converge as a function of the relevant experimental parameters. We define the error as the ratio of the difference between Eqs. (4.4) and (4.3) over Eq. (4.3) as a function of $i$.

$$
\text{error}(i) = \frac{\Delta x_{\text{base},i} - \Delta x_{\text{base}}}{\Delta x_{\text{spring}}}
$$  \quad (4.5)

Using the iterative definitions, we derive Eqs. (4.7) and (4.8) for $i = 0, 1, \ldots, \infty$.

$$
\Delta x_{\text{base},i} = R\left(\frac{k_{\text{apparent}}}{k_{\text{spring}}} - 1\right)\sum_{n=0}^{i-1}\left(\frac{k_{\text{apparent}}}{k_{\text{spring}}} - 1\right)(\epsilon - 1)^n
$$  \quad (4.6)

$$
\Delta x_{\text{spring},i} = R + \epsilon R\left(\frac{k_{\text{apparent}}}{k_{\text{spring}}} - 1\right)\sum_{n=0}^{i-2}\left(\frac{k_{\text{apparent}}}{k_{\text{spring}}} - 1\right)(\epsilon - 1)^n
$$  \quad (4.7)

Substituting Eqs. (4.6) and (4.7) into Eq. (4.5) we find that $R$ cancels out and we are left with a function for the error that, after substituting for a finite geometric series, tells us how rapidly Eq. (4.4) converges to Eq. (4.3) as a function of $\epsilon$ and the ratio $\frac{k_{\text{spring}}}{k_{\text{apparent}}}$.

$$
\text{error}(i) = \frac{\left(\frac{k_{\text{apparent}}}{k_{\text{spring}}} - 1\right)(\epsilon^{-1} - 1) - \frac{k_{\text{spring}}}{k_{\text{apparent}}} + 1}{\left(\frac{k_{\text{apparent}}}{k_{\text{spring}}} - 1\right)(\epsilon - 1)^i(\epsilon^{-1} - 1) + \frac{k_{\text{spring}}}{k_{\text{apparent}}} - 1}
$$  \quad (4.8)
Figure 4.5 demonstrates how the error is affected by varying the experimental parameters. For the experiments described in the text, our $\epsilon$ was approximately 0.5.

Even under the extreme conditions of low $\epsilon$ and high $\frac{k_{\text{spring}}}{k_{\text{apparent}}}$, Eq. (4.5) converges in just a few iterations. This error function, Eq. (4.8), can be used to select the required experimental parameters such as the spring constant or algorithm frequency for a desired experiment.

![Figure 4.5. Graph of the error function, Eq. (4.8), as a function of its parameters.](image)

(a) A contour plot of the error function for a constant $\epsilon = 0.5$. (b) A contour plot of the error function depicting the required number of iterations for Eq. (4.5) to converge within 10% of Eq. (4.4).

4.6.2 Experimental Conditions

Atomic force microscope (AFM) experiments were conducted using a modified Veeco Bioscope I mounted on a Zeiss Axiovert 25 inverted microscope. The substrate position was controlled with a Mad City Labs piezo-actuator and controller with a range of 50 $\mu$m and a resolution of 0.1 nm. Cantilever deflection and substrate position was controlled with a National Instruments 16-bit, 250 kS/s PCI-6229 digital I/O card and a custom LabVIEW program to implement the stiffness clamp algorithm running at 100 Hz. The substrate was mounted on a heated stage and maintained at 37 °C for cell experiments. Tipless silicon nitride MLCT (30-50 nN/µm, Veeco) and Arrow cantilevers (10-20 nN/µm, Nanoworld) were used for the gel and cell experiments, respectively. Calibration of the optical lever was conducted before each experiment by ramping the substrate up and down while in contact with the cantilever. The surface was ramped 450 nm and the average of 15 cycles was used to determine the volts to meters conversion factor. We then determined the cantilever spring constant before each experiment by recording the thermal fluctuations of the cantilever out of contact and fitting the first resonance peak of the power spectra with a Lorentzian function using the equipartition theorem (Hutter et al., 1993). This indicates that the resolution of the detection of the cantilever position was thermally limited.
The polyacrylamide hydrogels were generously provided by An-Chi Tsou. The hydrogel was dehydrated at 4 °C overnight and was rehydrated immediately before the AFM experiment with a standard phosphate buffered saline (PBS) solution. The cantilever was brought into contact as the gel rehydrated and expanded.

NIH 3T3 fibroblast cells were cultured in DMEM (GIBCO) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained in an incubator at 37 °C with a 95% humidity, 5% CO₂ atmosphere. A trypsin solution was used to detach cells at which point trypsin neutralizer was added and cells were then centrifuged at 300 g for 5 minutes. The resulting supernatant was discarded and cells were resuspended in their culture medium (DMEM plus supplements). Clean glass substrates and cantilevers were immersed for 30 min in a 50 µg/ml fibronectin solution (F0895, Sigma). The fibronectin was then washed off and cells were added and the cantilever was brought on top of a cell as it settled on the substrate.

4.6.3 Range of apparent stiffnesses

The stiffness clamp was very accurately applied over a large range of stiffnesses on an expanding hydrogel (Figure 4.3). The extreme clamped stiffnesses (4.2 x 10⁹ and 4.2 x 10¹¹ nN/µm) produced slopes in Figure 4.3 of essentially 0 and ∞, respectively. This is demonstrated by showing that the force and height respectively remain constant with only a small degree of Gaussian noise (Figure 4.6).

**Figure 4.6** (a) Histogram of the variance in force under a stiffness clamp of 4.2 x 10⁹ nN/µm. The histogram is fit with a Gaussian with a standard deviation of 15 pN. (b) Histogram of the variance in cell height under a stiffness clamp of 4.2 x 10¹¹ nN/µm. The histogram is fit with a Gaussian with a standard deviation of 0.34 nm.
4.6.4 Calculation of response time

The inset of the Figure 4.4b demonstrates the rapid change in traction rate upon a change in apparent stiffness. We found that this change occurred within 0.5 s. This response time was calculated by comparing two models with an F test with P values \( \leq 0.01 \). First, a 30 s window was applied centered on the timepoint when \( k_{\text{apparent}} \) was changed. Then a simple linear regression was compared with a segmented linear regression where the timepoint of the intersection of the two segments must be determined from the data. This 30 s window was then moved earlier in time and the two models were again compared. The point at which the preferred model shifted to the simple linear regression is defined as the point when the traction rate has statistically changed.

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Chapter 5. Adaptation of single cells to sudden changes in extracellular stiffness
5.1 Abstract

Extracellular stiffness has been clearly shown to alter long timescale cell behaviors such as growth and differentiation, but the cellular response to changes in stiffness on short timescales is not understood. Characterization of the timescales involved in stiffness sensing would both inform and test current stiffness sensing models. By studying the contractile response of cells to dynamic stiffness conditions we observe a seconds-timescale adaptation to step changes in extracellular stiffness not accounted for by current models of stiffness sensing. Specifically, we observe acceleration in contraction velocity and tensile rate upon a step decrease in stiffness and deceleration upon a step increase in stiffness. Surprisingly, this seconds-timescale adaptation to a change in extracellular stiffness is not explained by focal adhesion signaling or stretch-activated ion channels, but depends exclusively on cytoskeletal mechanics and is well-described by a simple mechanical model of an independent actuator pulling against a viscoelastic element or network. This model accurately predicts the behavior of both a contracting cell and an expanding hydrogel to sudden changes in extracellular stiffness and enables the calculation of viscoelastic parameters of the actomyosin network in the context of the whole-cell exposed to different mechanical boundary conditions. Exposure of v-Src transformed fibroblasts, which have altered contractility and motility behaviors, to sudden changes in stiffness shows that the stiffness response of these cells is indistinguishable from control on the timescale of seconds. We propose an alternative model of stiffness sensing in which extracellular stiffness signals are filtered through the viscoelastic cytoskeleton such that cellular responses to stiffness are dictated by both intracellular and extracellular mechanics.

5.2 Introduction

Numerous studies have reported stiffness-sensitive cellular behaviors including morphology (Peyton and Putnam, 2005), motility (Lo et al., 2000), proliferation, differentiation (Engler et al., 2006), and tumorigenesis (Munevar et al., 2001; Wang et al., 2000), and further studies have implicated over 150 signaling and structural proteins involved in stiffness sensitivity (Geiger et al., 2009; Moore et al., 2010). Mechanosensing signaling pathways are known to involve feedback and redundancy such that it is difficult to tease out the essential components and the timeline describing the initial signaling events and subsequent signaling cascades and structural rearrangements. Actomyosin contraction is known to play an important role in stiffness sensing as bundled actin stress fibers only appear on stiff substrates (Peyton and Putnam, 2005) and actomyosin activity is required for stiffness-directed stem cell differentiation (Engler et al., 2006), cytoskeletal coherence (Cai et al., 2010), and vinculin recruitment and reinforcement via FAK-mediated paxillin phosphorylation (Pasapera et al., 2010). The cell is often represented in a state of tensional equilibrium such that extracellular stiffness is opposed by intracellular
elastic components and myosin-generated contractility (Chen, 2008; Paszek et al., 2005; Schwarz et al., 2006). The cell is then perfectly poised to respond to changes in extracellular stiffness by the immediate adjustment of this equilibrium, which changes the tension across mechanosensory proteins such as talin or p130cas to expose phosphorylation sites or binding sites initializing a cascade of signaling events (del Rio et al., 2009; Moore et al., 2010; Sawada et al., 2006). Following this model, timescales of the cellular response to changes in extracellular stiffness are defined by the immediate shift in mechanical equilibrium and the subsequent phosphorylation and signaling that lead to structural rearrangements and long timescale behaviors involving gene expression. Recent studies of the cellular response to a local force perturbation demonstrate that signaling events such as Src activation and calcium spikes can occur on a sub-second timescale (Hayakawa et al., 2008; Na et al., 2008), but the dynamic sequence of a stiffness-specific response and the relevant timescales remain uncharacterized.

Several recent studies have presented platforms to vary the stiffness cues exposed to a single cell over time to better understand the mechanism and relevant timescales of cellular stiffness sensing. Novel gels have been produced to enable changes in stiffness over the course of minutes to hours by photo-exposure (Frey and Wang, 2009), DNA cross-linking (Jiang et al., 2010), or polymer cross-linking dynamics (Young and Engler, 2011). In addition, feedback algorithms have been employed on microplate or AFM systems to reversibly control stiffness signals exposed to a single-cell extended between two substrates in real-time (Mitrossilis et al., 2010; Webster et al., 2011). The geometry of these experimental setups is ideal for specifically measuring the cellular contractile response and material properties of the cell as described previously (Thoumine and Ott, 1997). The studies by Mitrossilis et al. and Webster et al. highlighted a sub-second contractile response to changes in stiffness suggesting a key role of actomyosin on fast timescales.

Here we focus on the whole-cell scale contractile response to sudden changes in extracellular stiffness to better understand the mechanism and timescales of stiffness sensing. This study builds on previous work demonstrating stiffness-dependent contraction of single-cells (Lam et al., 2011; Mitrossilis et al., 2009; Mitrossilis et al., 2010; Webster et al., 2011) to focus on the underlying mechanism of the fast-timescale stiffness response. Upon a step change in stiffness we observe a near-immediate change in both contraction velocity and tensile rate, followed by a slower change in rate on a timescale of seconds, which we call adaptation. The seconds-timescale adaptation to a step change in extracellular stiffness is well-described by a simple mechanical model and depends on cytoskeletal mechanics, but not focal adhesion signaling. We tested our system on v-Src transformed fibroblasts, known to have altered motility and Rho signaling, and found no significant difference in the stiffness adaptation response. We therefore propose an alternative model of stiffness sensing whereby extracellular stiffness is filtered through cytoskeletal mechanics.
5.3 Materials and Methods

5.3.1 Cell culture and sample preparation

NIH 3T3 fibroblasts were cultured in DMEM (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (Lonza, Walkersville, MD), and 1% Penicillin/Streptomycin (Sigma, St Louis, MO). Prior to experiments, cells were trypsinized and resuspended in CO₂-independent media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, and 1% Penicillin/Streptomycin. For inhibition experiments, cells were resuspended in CO₂-independent media containing the appropriate drug concentration and incubated for 30 minutes prior to experiments. Drugs used include pp2 (Calbiochem, Gibbstown, NJ), FAK inhibitor (Tocris, Ellisville, MO), gadolinium chloride (Sigma, St Louis, MO), cytochalasin D (Sigma, St Louis, MO), nocodazole (Sigma, St Louis, MO), and blebbistatin (Sigma, St Louis, MO). Control experiments conducted in the presence of 0.33% DMSO, the maximum required for any drug experiments, showed no distinct behavior from CO₂-independent media without DMSO or drug additions.

5.3.2 Experimental setup

Experiments were conducted with a modified Bioscope AFM (Veeco Metrology, Santa Barbara, CA) with closed-loop piezo platform stage (Mad City Labs, Madison, WI) and temperature control (Warner Instrument Corporation, Hamden, CT) atop an inverted microscope (Zeiss Axiovert 25, Carl Zeiss, Thornwood, NY) allowing brightfield imaging and alignment of the cantilever and cell (see Figure 5.1b). Data acquisition and the stiffness clamp feedback algorithm are controlled by custom software (LabVIEW, National Instruments, Austin TX). For further details on the feedback algorithm please see (Webster et al., 2011). Tipless PNP cantilevers from Nanoworld (Neuchatel, Switzerland) were used in all experiments with spring constants ranging from 50-800 nN/µm as determined by fitting thermal fluctuations of each cantilever.

5.3.3 Stiffness cycling experiment

Prior to adding cells, the AFM cantilever and glass substrate were incubated with 50 µg/mL fibronectin (Sigma, St Louis, MO) for at least 30 minutes and then rinsed. Concanavalin A (Sigma, St Louis, MO) and poly-L-Lysine (MW>300,000, Sigma, St Louis, MO) were also used as alternatives to fibronectin for experiments where indicated. All experiments were performed at 37°C with perfusion of media exchanging the chamber volume every hour to compensate for evaporation. The point of contact between the glass substrate and AFM cantilever was recorded as zero height and cell height was measured with respect to this point. Prepared cells in suspension were flowed into the system and within minutes of settling, a single
cell was brought into contact with both the AFM cantilever and glass substrate with a 4nN contact force. The cell was then allowed to adhere and contract a minimum of several nN before stiffness cycling began. Step changes in stiffness were imposed every 20 seconds. Multiple time intervals were tested to confirm that a terminal contraction velocity and tensile rate are reached within 20 seconds.

5.3.4 Osmotic swelling of polyacrylamide hydrogel

A polyacrylamide hydrogel of stiffness \( \approx 100 \) Pa was osmotically swelled by replacing the 10x PBS surrounding the gel with water. As the gel swelled against the AFM cantilever, the stiffness clamp was applied and the change in gel height and gel expansion force were measured in the same manner as for the contracting cells. Step changes in stiffness were imposed every 40 seconds to allow ample time for adaptation. Multiple time intervals were tested to confirm that a terminal contraction velocity and tensile rate are reached within 40 seconds.

5.3.5 Ratio analysis

The nonlinearity of the contractile response to step changes in stiffness was initially established by measuring the slope over the first quarter of the interval and the last quarter of the interval. A ratio greater than 1 indicates acceleration, while a ratio less than 1 indicates deceleration. The p-value was determined using a binomial test with a null hypothesis of equal probability of acceleration or deceleration.

5.3.6 Curve fitting

The adaptation timescale was determined by fitting a linear-plus-exponential equation to each stiffness interval:

\[
\hat{f}(t) = c_0 + c_1 t + c_2 e^{-t/\tau}
\]

where \( c_0 \) is a constant offset, \( c_1 \) is the slope after adaptation, \( c_2 \) is the multiplier of the exponential term, and \( \tau \) is the adaptation timescale. In order to compare adaptation timescales, an F-test comparing the nested linear model to the linear-plus-exponential was used with only traces with \( p<0.1 \) used. This was done to exclude data whose noise precluded an accurate measurement of the adaptation timescale. Given the shorter adaptation timescale for step increases in stiffness, the f-value criteria yields fewer usable values for inhibition experiments where data is not as clean. Therefore comparison of adaptation timescales for inhibition experiments is based on step decreases in stiffness and analysis of the height trace.
The maximum contraction velocity occurs during a force clamp and is measured as the slope during the last 5 seconds of a force clamp interval where the velocity is constant (equivalent to $c_1$ from the equation above).

5.3.7 Statistical analysis

The results of each experimental condition were compared to the control using a non-parametric rank sum test (Mann-Whitney U test) because the underlying data is not normally distributed. Levels of significance are reported for each condition in the text. P-values are calculated for all ratios by comparing to the null hypothesis of equal probability of the ratio being greater than or less than 1. Percentile values are calculated based on transitions (indicated by n in each box plot figure), which have been shown to be independent by statistical test.

5.4 Results

5.4.1 Contracting cells adapt to sudden changes in extracellular stiffness on a timescale of seconds

We determined the cellular contractile response to changes in mechanical boundary conditions by measuring the contraction of a single cell while changing the extracellular stiffness exposed to the cell. Briefly, a single fibroblast is simultaneously brought into contact with a fibronectin-coated tipless AFM cantilever and fibronectin-coated glass substrate. As the cell adheres to the two surfaces and contracts, we record the cell-generated forces and decrease in cell height with AFM precision. Without feedback, cell contraction is resisted by a single extracellular stiffness in the vertical direction defined by the spring constant of the cantilever. (We note that the cell also experiences the lateral stiffness of the cantilever and glass substrate.) In this case, contractile rates increase to a constant contraction velocity ($\mu$m/min) and tensile rate (nN/min) and then slow. Contraction occurs predominantly during the middle linear region, as shown in Figure 5.1c.

To dynamically change the resistance felt by the contracting cell, we employ a stiffness clamp feedback algorithm that has been described previously (Webster et al., 2011). Briefly, the stiffness clamp allows a rapid and reversible tuning of extracellular stiffness, independent of cell-generated force or contraction, by adjustment of the substrate position. For example, in the extreme case of infinite stiffness: $k_{ci} = \frac{\Delta F}{\Delta X} \to \infty$, no change in height is achieved ($\Delta X = 0$) regardless of the force applied by the cell. To achieve this, every incremental deflection of the
Figure 5.1. Contracting cells adapt to sudden changes in extracellular stiffness on a timescale of seconds. (a) Setup of single-cell contraction experiments. Cell-generated forces and cell height are measured by deflection of the AFM cantilever while extracellular stiffness is controlled in real time using the stiffness clamp feedback algorithm (Webster et al. Plos One 2011). (b) Top-down view of cell adhered to AFM cantilever and substrate. Cell is outlined in red. (c) Typical trace of cell height and tensile force of a single cell contracting under a constant extracellular stiffness as illustrated by cartoons. Once contact with both surfaces is established, the cell contracts at a constant rate for several minutes before slowing. (d) Step changes in extracellular stiffness between 10 and 100 nN/µm every 20 seconds yield changes in both contraction velocity and tensile rate. (e) Maximum contraction velocity depends directly on extracellular stiffness. (f) The ratio of the slope over the last quarter of the interval to the slope over the first quarter of the interval is calculated for each 20 second stiffness interval for the height traces. n represents number of stiffness transitions, N represents number of cells, and box plot presents median, 25th and 75th percentile and 10 and 90th percentile outliers.
cantilever is accompanied by an identical step of the substrate such that cell height remains constant. This feedback technique, referred to as a position clamp, is commonly used in force microscopy. By a similar argument, a force clamp \((\Delta F = 0)\) yields an extracellular stiffness of 0 and any intermediate stiffness is obtained by appropriate adjustment of the substrate based on cell-generated cantilever deflection. For further details please see (Webster et al., 2011).

We expose single contracting cells to a series of step changes in extracellular stiffness every 20 seconds. Figure 5.1d shows a typical cellular response to a series of step changes between 10 and 100 nN/µm. Both contraction velocity and tensile rate are stiffness-dependent and upon a step change in stiffness, we immediately observe a change in both tensile rate and contraction velocity, as described previously (Mitrossilis et al., 2010; Webster et al., 2011). However, careful examination of the traces reveals a further rate change on a timescale of seconds. Specifically, the cell accelerates to a constant rate upon a step decrease in stiffness and decelerates to a constant rate upon a step increase in stiffness. To quantify the observed curvature, we take the ratio of the slope during the last quarter of the stiffness interval to the slope during the first quarter of the stiffness interval. For a stiffness transition from 10 to 100 nN/µm, the ratio is <1 indicating a deceleration. For a stiffness transition from 100 to 10 nN/µm, the ratio is >1 indicating an acceleration. These ratios, illustrated in Figure 5.1f, are consistently distinct from 1 with \(p<0.001\). The identical trend is observed in the force trace as in the height trace (see supplemental Figure 5.7), indicating proper functioning of the stiffness clamp.

To further explore the observed stiffness adaptation behavior, we cycled stiffness between the extremes of \(k_{ex}=0\) (force clamp) and \(k_{ex} = \infty\) (position clamp) every 20 seconds. As shown in Figure 5.2a, we observe a more pronounced acceleration upon a step decrease in stiffness and deceleration upon a step increase in stiffness. This stiffness adaptation behavior is observed repeatedly for every stiffness change over the entire cell contraction period as illustrated by the normalized and overlaid stiffness intervals shown in Figure 5.2b and 5.2c. Again, the ratio of the slope over the last quarter of the stiffness interval to the slope over the first quarter of the stiffness interval is <1 for a step increase in stiffness and >1 for a step decrease in stiffness with \(p<0.001\), as illustrated in Figure 5.2d.

To quantify the timescale of the stiffness adaptation we fit a linear-plus-exponential to each stiffness interval, as shown in Figures 5.2e and 5.2f, which yields distinct adaptation timescales for distinct stiffness transitions. Specifically, the adaptation timescale is longer for a step decrease in stiffness than a step increase in stiffness as shown in Figure 5.2g and also depends on the magnitude of the stiffness change as shown in Figure 5.2h. Furthermore, the adaptation timescale is independent of the tensile force, cell height, tensile rate, and contraction velocity (see supplemental Figure 5.8). The consistent and specific nature of the adaptation timescale is suggestive of a specific mechanism beyond a simple elastic model that predicts an immediate adjustment. We therefore chose to explore whether focal adhesion signaling was involved in this seconds-timescale behavior.
Figure 5.2. Adaptation timescale depends on both magnitude and direction of extracellular stiffness change. (a) Extreme step changes between $k_{ex}=0$ and $k_{ex}=\infty$ every 20 seconds yield changes in both contraction velocity and tensile rate. (b) For a single cell, the height trace for every interval from a decrease in stiffness is normalized and overlaid, revealing an acceleration to constant contraction velocity. The dark line represents the average of all traces shown. (c) For a single cell, the force trace for every interval from an increase in stiffness is normalized and overlaid, revealing a deceleration to constant tensile rate. The dark line represents the average of all traces shown. (d) The ratio of the slope over the last quarter of the interval to the slope over the first quarter of the interval is calculated for each 20-second stiffness interval. Ratio for a decrease in stiffness calculated from height traces and ratio for an increase in stiffness calculated from force traces. $n$ represents number of stiffness transitions, $N$ represents number of cells, and box plot presents median, 25th and 75th percentile and 10 and 90th percentile outliers. (e) Linear plus exponential fit to typical height trace. (f) Linear plus exponential fit to typical force trace. (g) Adaptation timescale for a decrease vs increase in $k_{ex}$ * indicates $p<0.0001$. $n$ represents number of stiffness transitions and $N$ represents number of cells. Median timescales are shown below each box plot while the plot presents median, 25th and 75th percentile and 10 and 90th percentile outliers. (h) Adaptation timescale for a 10x vs infinite decrease in $k_{ex}$ * indicates $p<0.0001$. Box plot description same as (g).
5.4.2 Focal adhesion signaling affects contraction velocity, but not the seconds-timescale adaptation to a step change in extracellular stiffness

A number of studies have directly linked cellular stiffness sensing with focal adhesion activity. Specifically, the kinase activity of focal adhesion kinase (FAK) is regulated by mechanical stretching and likely plays a role in stiffness-sensitive adhesion turnover (Moore et al., 2010). Src family kinases are proposed to play an early role in the rigidity sensing cycle and are activated within 300 ms of mechanical perturbation via fibronectin linkages (Moore et al., 2010; Na et al., 2008). We therefore expect FAK and Src family kinases to be involved in the seconds-timescale contractile response to a step change in extracellular stiffness.

Focal adhesion activity was inhibited using either a FAK inhibitor or pp2, a Src family kinase inhibitor. Pre-incubation with either drug decreased the maximum contraction velocity in a dose-dependent manner, as shown in Figure 5.3a. At 50 µM of either drug, cells are able to adhere, as indicated by significant forces of de-adhesion required to detach the cell from either surface. This indicates successful integrin-fibronectin binding; however these cells were unable to contract, indicative of the finely-tuned nature of the contractile machinery. The ability to adhere without contraction is consistent with the reported ability to form nascent adhesions in the absence of myosin II (Choi et al., 2008). Similarly, attachment by either poly-L-lysine or concanavalin A yields no contraction (data not shown), illustrating the need for integrin-mediated adhesion to generate contractile force in this system.

Surprisingly, neither the FAK inhibitor nor pp2 had a significant effect on the adaptation timescale at concentrations that decreased the maximum contraction velocity. As shown in Figure 5.3b, the adaptation timescale was not statistically distinct from the control. These results indicate that while focal adhesion signaling is known to be involved in long timescale stiffness-dependent behaviors, the seconds-timescale adaptation to a step change in extracellular stiffness is independent of focal adhesion activity.

5.4.3 Stretch-activated ion channels are not involved in seconds-timescale contraction or adaptation to step changes in extracellular stiffness

Stretch-activated ion channels are another proposed mechanosensor and calcium signaling is expected to operate within the seconds timescale observed here (Beningo et al., 2004; Munevar et al., 2004; Matthews et al., 2010). Gadolinium chloride has been used previously to inhibit stretch-activated ion channels resulting in decreased traction forces and migration (Munevar et al., 2004). However blocking of stretch-activated ion channels by gadolinium chloride did not significantly affect the maximum contraction velocity or the adaptation timescale compared to control, as shown in Figure 5.3, indicating that stretch-activated ion channels do not play a significant role in the seconds-timescale stiffness adaptation observed here.
Figure 5.3. Focal adhesion signaling affects maximum contraction velocity, but not adaptation timescale. For all box plots, n represents number of transitions or intervals and N represents number of cells. Median values are shown below each box plot while the plot presents median, 25th and 75th percentile and 10 and 90th percentile outliers. (a) Maximum contraction velocity compared for control, FAK inhibitor, Src family kinase inhibitor pp2, and gadolinium chloride at different concentrations. (b) Adaptation timescale for $k_{ex} = \infty$ to $k_{ex} = 0$ transition under pp2, FAK inhibitor and gadolinium are not statistically distinct from control.

5.4.4 A simple mechanical model predicts the seconds-timescale adaptation to sudden changes in extracellular stiffness

The lack of involvement of focal adhesion signaling or stretch-activated ion channels combined with the consistency of behavior between cells and at different timepoints over the course of contraction suggested a mechanical explanation for the seconds-timescale adaptation behavior. We therefore sought a simple mechanical model to describe the observed behavior. Since the cell responds almost instantly to a change in stiffness, independent of the tensile force, we hypothesized that the passive viscoelasticity of the whole cell was influencing the coupling of the AFM cantilever to an underlying contractile process that is stiffness and force independent. The simplest viscoelastic model that has a transient response for both step increases and decreases in stiffness is the standard linear solid viscoelastic model, which is a spring in parallel with a spring and dashpot in series, as shown in Figure 5.4a. We found that a constant actuator representing a simple spring with a reference length changing at a fixed rate captures most of the response of the cell to step changes in stiffness, suggesting that remodeling and changes in the underlying contractile behavior are dwarfed by passive whole-cell viscoelasticity on the seconds timescale. The specific damping parameter of the dashpot and spring constants of the viscoelastic model may not directly map to cellular structures and could arise from
any of a number of physical mechanisms, e.g. poroelasticity or polymer viscoelasticity of the cytoplasm or actomyosin network specifically. Moreover, the connection between such a microscopic model and the parameters of the transducer could change with the configuration of the cell. The actuator is independent of the current state of the transducer, and a fortiori also independent of the instantaneously applied extracellular stiffness.

Figure 5.4. Simple mechanical model predicts adaptation upon a step change in stiffness. (a) Cartoon illustrating the independent actuator moving at rate $\alpha$ in series with the standard linear solid (SLS) element consisting of a spring $k_2$ and dashpot $\gamma$ in parallel with a spring $k_1$. As extracellular stiffness conditions change, different elements of the SLS absorb the sudden change in stiffness as illustrated. (b) Predictions of the model perfectly simulate the observed adaptation behavior for a step increase and step decrease in stiffness, for both the height and force behavior, as shown by the solid lines labeled “Total” (indicating whole-cell behavior). The activity of individual elements is indicated by dashed lines. For the height trace, the change in height of the actuator is constant as indicated by the linearity of the trace marked $\alpha$. The standard linear solid element (SLS), however, adapts to the step change in extracellular stiffness. For the force channel, the individual activity of the two sides of the SLS model are shown: the lone spring $k_1$ and the spring and dashpot in series: $k_2+\gamma$. The sum of these two curves yields the total force exerted by the whole-cell. The roman numerals indicate corresponding time points in (a) and (b).

As shown in Figure 5.4, the model perfectly predicts the observed deceleration upon a step increase in stiffness and acceleration upon a step decrease in stiffness, in
addition to the rate-independence of the adaptation timescale and the dependence on the magnitude and direction of the stiffness change. Derivation of the response of the model to step changes in stiffness is described in the supplemental text section 5.6.2. The model predicts the adaptation timescale for any step change to extracellular stiffness $k_{ex}$:

$$\tau_{k_{ex}} = \gamma \left( \frac{1}{k_1 + k_{ex}} + \frac{1}{k_2} \right) \quad (5.1)$$

The extreme cases of the force clamp ($k_{ex}=0$) and height clamp ($k_{ex}=\infty$) follow:

$$\tau_{k_{ex}=0} = \gamma \left( \frac{1}{k_1} + \frac{1}{k_2} \right) \quad (5.2)$$

$$\tau_{k_{ex}=\infty} = \gamma \frac{1}{k_2} \quad (5.3)$$

5.4.5 Swelling hydrogel exhibits seconds-timescale adaptation to a step change in extracellular stiffness

Our simple mechanical model predicts the described adaptation to step changes in extracellular stiffness for any system with an independent actuator and viscoelastic material properties. A hydrogel is a standard viscoelastic material that can be driven to expand by changes in osmotic pressure. We therefore expect a swelling hydrogel to mimic the observed cellular behavior. A polyacrylamide hydrogel was subjected to a change in osmotic pressure and exposed to the same stiffness cycling between $k_{ex}=0$ and $k_{ex}=\infty$, but at 40 second intervals to accommodate longer adaptation times. As shown in Figure 5.5, we indeed observe the same acceleration upon a step decrease in stiffness and deceleration upon a step increase in stiffness with adaptation timescales longer than those observed for cells, but still dependent on the direction of the stiffness change. The ratio of the slope over the last quarter of the stiffness interval to the slope over the first quarter of the stiffness interval is $<1$ for a step increase in stiffness with $p=0.0005$ and $>1$ for a step decrease in stiffness with $p=0.001$, as illustrated in Figure 5.5d. This supports both the mechanical nature of the cellular response and the ability of the model to accurately describe adaptation to a step change in extracellular stiffness.
Figure 5.5. Expanding hydrogel exposed to step changes in extracellular stiffness also yields seconds-timescale adaptation. (a) Extreme step changes between \( k_{\text{ex}} = 0 \) and \( k_{\text{ex}} = \infty \) applied to an expanding hydrogel every 40 seconds yield changes in both expansion velocity and expansion rate. (b) Cartoon schematic of setup for osmotically-swelling hydrogel experiments. (c) Force and height traces for each stiffness interval are normalized and overlaid, revealing a deceleration for a step increase in stiffness (force trace) and an acceleration for a step decrease in stiffness (height trace). Force and height traces are displayed on the same plot to emphasize curvature. (d) The ratio of the slope over the last quarter of the interval to the slope over the first quarter of the interval is calculated for each 40-second stiffness interval. Ratio for a decrease in stiffness calculated from height traces and ratio for an increase in stiffness calculated from force traces. \( n \) represents number of stiffness transitions and box plot presents median, 25th and 75th percentile and 10 and 90th percentile outliers. (e) Gel adaptation timescale for an extreme step increase versus a decrease in stiffness. * indicates \( p < 0.001 \). \( n \) represents number of stiffness transitions and box plot presents median, 25th and 75th percentile and 10 and 90th percentile outliers.

5.4.6 Perturbation of myosin confirms the dependence of the adaptation timescale on cytoskeletal mechanics

The model predicts that the adaptation timescale is determined by intracellular mechanical parameters. We therefore expect that disrupting the mechanical integrity of the cell, using cytochalasin D, nocodazole, or blebbistatin will affect the adaptation timescale. As expected, all three drugs decreased the maximum contractile velocity in a dose-dependent manner, as shown in Figure 5.6a and supplemental Figure 5.9a.
Figure 5.6. Intracellular mechanics determine the adaptation timescale.
For all box plots, n represents number of transitions or intervals and N represents number of cells. Median values are shown below each box plot while the plot presents median, 25th and 75th percentile and 10th and 90th percentile outliers. (a) Maximum contraction velocity compared for control, cytochalasin D, and blebbistatin at different concentrations illustrates dose-dependence. (b) Adaptation timescale for $k_{ex} = \infty$ to $k_{ex} = 0$ transition under cytochalasin D and blebbistatin compared to control. * indicates $p<0.0001$. (c) Calculation of model parameters based on percentile data, as described in supplementary section 5.6.2, reveals a decrease in viscoelastic parameters for both 500 nM cytochalasin D and 30 µM blebbistatin compared to control. Each cell presented as median (25th percentile, 75th percentile). (d) Contraction velocity of v-Src transformed fibroblasts compared to control. (e) Adaptation timescale of v-Src transformed fibroblasts is not significantly different from control.
Surprisingly, the adaptation timescale was not significantly affected by disruption of the actin cytoskeleton or microtubules at concentrations that decreased the maximum contraction velocity as long as minimal contraction occurred. Disruption of myosin ATPase activity by blebbistatin, however, showed a significant change in the adaptation timescale, indicating the importance of myosin as a cross-linker in addition to its motor activity. At first glance, it is quite surprising that disruption of myosin ATPase changes the adaptation timescale compared to control while disruption of the actin network does not. However, calculation of the model parameters using the reported percentile values, as described in the supplementary text section 5.6.2, provides a potential explanation. As described in the table in Figure 5.6c, both 500 nM cytochalasin D and 30 µM blebbistatin cause a significant decrease in all viscoelastic parameters compared to control. However, the decrease in the viscous component is greater for cytochalasin D than for blebbistatin and as seen in equation (5.2), the elastic and viscous components have opposing effects on the adaptation timescale. Therefore, in the case of cytochalasin D, the decrease in viscous component cancels the decrease in elastic components resulting in an adaptation timescale consistent with control. As discussed in the supplemental text, we calculate an increase in elastic and viscous components in the case of microtubule inhibition with nocodazole that similarly cancel to yield an adaptation timescale consistent with control. This surprising effect emphasizes the complexity of the role of intracellular mechanics such that changes in distinct mechanical components may not affect some processes on the whole-cell scale.

5.4.7 v-Src transformed fibroblasts exhibit the same adaptation timescale to a step change in extracellular stiffness as normal fibroblasts

Given the contractility-specific nature of the observed response, we next tested the ability of a cell type with altered contractility and motility to adapt to step changes in extracellular stiffness. Specifically, we chose a cancerous phenotype since previous studies have linked tumorigenicity with stiffness insensitivity (Wang et al., 2000). Overexpression and hyperactivation of Src correlates with an invasive and cancerous phenotype both in vitro and in vivo. (Frame, 2004; Irby and Yeatman, 2000; Kim et al., 2009) Fibroblasts transformed by the Rous sarcoma virus Src gene (v-Src, the first oncogene identified (Hunter and Sefton, 1980)) have been used as a model cancer system resulting in altered Rho-stimulated contractility (Frame, 2004), and ERK5 signaling (Schramp et al., 2008), disrupted actomyosin network (Boschek et al., 1981), and increased motility and invasiveness (Frame, 2004; Martin, 2001). A normal phenotype is recovered by drug-induced compensation of contractility (Schramp et al., 2008; Zhong et al., 1997). Therefore, to test if the transformed phenotype of v-Src transformed fibroblasts is reflected in the seconds-timescale stiffness-sensitivity, we exposed them to step changes in extracellular stiffness between k_{ex}=0 and k_{ex} = infinity every 20 seconds. The experimental procedure was identical to that performed for normal fibroblasts as described above. Maximum contraction velocity is not statistically distinct in v-Src
transformed fibroblasts compared to normal fibroblasts nor is the adaptation timescale significantly changed, as shown in Figure 5.6d and e. This suggests that for the seconds-timescale regime, v-Src transformed and normal fibroblasts are indistinguishable.

5.5 Discussion

Our measurements of cellular contraction upon a step change in extracellular stiffness reveals a seconds-timescale adaptation dependent on intracellular mechanics. We observe acceleration to a constant contraction velocity (and tensile rate) upon a step decrease in stiffness and deceleration upon a step increase in stiffness. The adaptation timescale depends on both the magnitude and direction of the stiffness change as predicted by a simple mechanical model of an independent driving motor pulling constantly against both the extracellular stiffness and intracellular mechanics. By fitting the model predictions to control data we find a median actuator rate: $\alpha = 13 \text{ nm/s}$ ($25^{\text{th}}, 75^{\text{th}}$ percentile: 8.3, 20 nm/s), which is consistent with reported velocities of retrograde actin flow within the lamellipodium in the presence of focal adhesions: 10-20 nm/s (Aratyn-Schaus et al., 2011). As expected, disruption of either the actin network with cytochalasin D or myosin ATPase activity with blebbistatin decreases $\alpha$ as shown in Figure 5.6.

Furthermore calculated elastic and viscous components of the actomyosin network agree with previously published values of $E_1$ and $E_2 \sim 0.5$-20 kPa (Solon et al., 2007; Thoumine and Ott, 1997) and $\gamma \sim$1-100 kPa*s (Bausch et al., 1998; Thoumine and Ott, 1997). Finally, a study by Humphrey et al. showed that actomyosin networks of a similar ratio of actin to myosin as in vivo environments relieve macroscopic stress over an average relaxation time of $\sim$8 seconds – remarkably similar to the adaptation timescale observed here. (Humphrey et al., 2002) We therefore expect that the physiological equivalent of the viscoelastic component of our model is dominated by the actomyosin network, which may include contributions from the cortex, stress fibers, or developing internal structures. Furthermore, our experimental system and model present a platform to study the combined role of extracellular and intracellular mechanics in determining the whole-cell response to mechanical signals.

According to the model, adaptation timescale can by explained by viscoelastic parameters and does not require incorporation of force-dependent motor activity. Indeed we observe the adaptation timescale does not depend on contraction velocity. (See supplemental Figure 5.8.) Furthermore, the increase in adaptation timescale at 30 µM blebbistatin indicates the importance of the cross-linking role of myosin in determining intracellular mechanics since blebbistatin blocks myosin II in an actin-detached state (Kovács et al., 2004). We were initially surprised that disruption of the actin cytoskeleton by cytochalasin D did not increase the adaptation timescale similar to blebbistatin. However close examination of the
viscoelastic parameters reveals a robustness of the adaptation timescale to coordinated variation in both the viscous and elastic components that agrees with experimental observations. The combined experimental system and model therefore present a platform for studying actomyosin mechanics within the context of the whole-cell and under different mechanical boundary conditions.

Certain models have predicted the ability of actomyosin interactions to produce a stiffness-dependent contractile response based on the force-velocity relationship characterized for skeletal muscle. Some models combine the force-velocity relationship with binding and unbinding kinetics of adhesions to predict stiffness-dependent motility and stress fiber development (Schwarz et al., 2006; Walcott and Sun, 2010). It has also been proposed that actomyosin contraction itself may be stiffness-dependent either due to catch bond behavior (Guo and Guilford, 2006; Moore et al., 2010) or load-dependent resistance from internal friction due to cross-linkers (Mitrossilis et al., 2009). While the inverse force-velocity relationship has been well-characterized for skeletal myosin and on the whole-cell scale for muscle cells where load is directly and efficiently applied to myosin networks (Hill, 1938; Mitrossilis et al., 2009; Piazzesi et al., 2007), non-muscle cells remain an unknown. Indeed recent studies of non-muscle myosins IIa and IIb suggest the load dependent kinetics are complex (Kovács et al., 2007; Norstrom et al., 2010) and further complicated in the context of the whole cell and the actomyosin networks of non-muscle cells which are less organized and constantly remodeling. Indeed our model proposes a constant rate motor, potentially due to activity in a regime of minimal force sensitivity and the lack of organized stress fiber structures resulting in roughly constant myosin activity.

Here we propose an alternative simple model of a constant rate motor and viscoelastic relaxation of cytoskeletal networks to describe stiffness-dependent behavior. This model elaborates on the simple elastic equilibrium model proposed to explain stiffness-sensitive behaviors. Specifically, extracellular stiffness is coupled through the viscoelastic cytoskeleton such that stretching of mechanosensory proteins and subsequent intracellular signaling result from a combination of extracellular stiffness and cytoskeletal mechanics. We therefore establish a seconds-timescale step to the cellular stiffness response, independent of focal adhesion signaling and dependent only on actomyosin mechanics. Examination of subsequent timescales will undoubtedly reveal the sequential involvement of mechanosensory proteins, focal adhesion signaling, and ultimately longer behaviors of gene expression, differentiation, and tumorigenesis.
5.6 Supporting Information

5.6.1. Supplemental Figures

![Graph showing ratio of end slope to initial slope over different k_{ex} transitions.]

5.7. Same trend observed for force trace as for height trace upon a step change in stiffness. The ratio of the slope over the last quarter of the interval to the slope over the first quarter of the interval is calculated for each 20-second stiffness interval for the force trace. At a given stiffness, the force and height traces are directly related by the extracellular stiffness. Therefore by definition of the system we observe the same trend as seen for the height trace in Figure 5.1f. n represents number of stiffness transitions, N represents number of cells, and box plot presents median, 25th and 75th percentile and 10 and 90th percentile outliers.

![Scatter plots showing adaptation timescale versus cell height, tensile force, contraction velocity, and tensile rate.]

5.8. Adaptation timescale is independent of tensile force, cell height, tensile rate, and contraction velocity.
5.9. **Microtubule disruption by nocodazole affects maximum contraction velocity, but not adaptation timescale.** For all box plots, n represents number of transitions or intervals and N represents number of cells. Median values are shown below each box plot while the plot presents median, 25th and 75th percentile and 10 and 90th percentile outliers. (a) Maximum contraction velocity compared for 0, 5, & 30 µM Nocodazole. (b) Adaptation timescale for 30 µM Nocodazole not statistically significant from control. Cytochalasin D and Blebbistatin shown for comparison.

5.6.2 **Data-based calculation of model parameters**

The model predictions may be retroactively applied to the data to get median and percentile values for the independent actuator rate, \( \alpha \), and viscoelastic parameters as follows:

\[
\alpha = \left( \frac{dX}{dt} \right)_{k_x=0}
\]

\[
k_1 = \left( \frac{dF}{dt} \right)_{k_x=\infty} / \left( \frac{dX}{dt} \right)_{k_x=0}
\]

\[
k_2 = k_1 \left( \frac{\tau_{k_x=0}}{\tau_{k_x=\infty}} - 1 \right)
\]

\[
\gamma = k_1 \tau_{k_x=\infty}
\]
where \( \tau \) is the adaptation timescale. Calculations based on the median, 25\(^{th}\) and 75\(^{th}\) percentile values reported yield the following presented as median (25\(^{th}\) percentile, 75\(^{th}\) percentile):

<table>
<thead>
<tr>
<th></th>
<th>( \alpha ) (nm/s)</th>
<th>( k_1 ) (kPa)</th>
<th>( k_2 ) (kPa)</th>
<th>( \gamma ) (kPa*(s))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-13 (-8.3, -20)</td>
<td>3.6 (3.5, 3.7)</td>
<td>5.3 (2.7, 7.5)</td>
<td>14 (7.8, 24)</td>
</tr>
<tr>
<td>30 ( \mu )M Blebbistatin</td>
<td>-4.8 (-3.5, -6.7)</td>
<td>1.6 (1.2, 2.3)</td>
<td>2.5 (1.4, 3.4)</td>
<td>8.9 (8.2, 9.0)</td>
</tr>
<tr>
<td>500 nM Cyto D</td>
<td>-6.7 (-3.8, -10)</td>
<td>1.5 (0.70, 2.3)</td>
<td>1.2 (0.31, 4.6)</td>
<td>6.4 (5.1, 8.3)</td>
</tr>
<tr>
<td>30 ( \mu )M Nocodazole</td>
<td>-4.7 (-2.5, -6.3)</td>
<td>9.3 (7.3, 9.7)</td>
<td>14 (4.8, 19)</td>
<td>34 (21, 60)</td>
</tr>
<tr>
<td>25 ( \mu )M pp2</td>
<td>-14 (-8.2, -17)</td>
<td>4.0 (3.4, 5.8)</td>
<td>8.5 (6.1, 11)</td>
<td>14 (9.2, 15)</td>
</tr>
<tr>
<td>30 ( \mu )M FAK inhibitor</td>
<td>-6.7 (-3.7, -9.5)</td>
<td>4.2 (1.1, 5.3)</td>
<td>5.2 (1.2, 6.7)</td>
<td>17 (6.0, 18)</td>
</tr>
<tr>
<td>200 ( \mu )M Gadolinium</td>
<td>-9.2 (-5.9, -16)</td>
<td>5.1 (4.3, 5.5)</td>
<td>11 (3.8, 12)</td>
<td>17 (9.2, 24)</td>
</tr>
</tbody>
</table>

Given that our data is recorded in units of nN and \( \mu \)m, we must convert to stress and strain units to calculate elasticity values for comparison to the literature. We use a basic conversion assuming cell height is on the order of 10 \( \mu \)m and contact area on the order of 100 \( \mu \)m\(^2\), then \( E_{cell} = \frac{k_{cell}H_{cell}}{A_{contact}} = 0.1k_{cell} \). This conversion is used in calculation of the values listed above.

The calculated viscoelastic parameters reveal interesting inhibition-based trends that are not visible by simply studying the adaptation timescale. Specifically, both elastic and viscous parameters decrease for blebbistatin and cytochalasin D treated cells while elastic and viscous parameters increase for nocodazole-treated cells. These changes in mechanical properties are not observed in the adaptation timescale because elastic and viscous properties have opposing effects:

\[
\tau_{k_{2=0}} = \gamma \left( \frac{1}{k_1} + \frac{1}{k_2} \right).
\]

Therefore corresponding increases or decreases in both parameters may cancel resulting in an adaptation timescale consistent with control. The decrease in elastic components in the cases of blebbistatin and cytochalasin D and the increase in elastic components in the case of nocodazole are consistent with previously published results (Sen and Kumar, 2009). Inhibition of FAK, Src family kinases, or stretch-activated ion channels did not yield any major difference in \( k_1 \) or \( \gamma \) components compared to control in our system. We note that internal spring parameter \( k_2 \) shows the greatest inhibition-induced changes compared to control in
all cases, including focal adhesion signaling. We therefore expect we may see a difference in adaptation timescale for extreme step increases in stiffness:

$$\tau_{k_x = \infty} = \frac{\gamma}{k_2}.$$ While we do anecdotally observe the expected trends, we are unable to show statistical significance with the limited number of force trace intervals that pass our f-value criteria. We therefore leave investigation of this intriguing phenomenon to a subsequent study.

### 5.6.3 Derivation of the response of the model to step changes in stiffness

The equations for the model are:

$$f(t) = k_1 (x_1 - \alpha t) + f_2$$

$$f_2 = \gamma \dot{x}_2 = k_3 x_3$$

$$x_1 = x_2 + x_3$$

where $f(t)$ is the tensile force applied at the cell-cantilever interface, $\alpha$ is velocity at which the reference length of the spring $x_1$ is changing (i.e. this is the actuator), and $x_2$ and $x_3$ describe the state of the internal spring and dashpot.

In the experiment, the effective stiffness jumps at the set of times $\{t_j\}_{j=1}^N$. This can be described within the model by setting

$$f(t) = f(t_j) - k_{cE}(t_j)(x_1(t_j) - x_1(t))$$

for $t \in [t_j, t_{j+1})$ where $k_{cE}(t)$ is the applied stiffness at time $t$. When the intervals between jumps in stiffness are very long, we can analyze the model by considering its behavior for single jump. First we define: $t' = t - t_j$ and

$$x_1'(t') = x_1(t' + t_j) - x_1(t_j)$$

$$x_2'(t') = x_2(t' + t_j)$$

$$x_3'(t') = x_3(t' + t_j) - x_1(t_j)$$

so that the equations take the form

$$f(t_j) - k_{cE}(t_j)x_1' = k_1 (x_1' - \alpha t') + k_1 (x_1(t_j) - \alpha t_j) + f_2.$$
\[ f_2 = \gamma x_2' = k_3 x_3' \]
\[ x_1' = x_2' + x_3'. \]

This can be rearranged to
\[ f' = k_1' (x_1' - \alpha' t') + f_2 \]
\[ f_2 = \gamma x_2' = k_3 x_3' \]
\[ x_1' = x_2' + x_3'. \]

with
\[ k_1' = k_1 + \alpha_k(t_j) \]
\[ \alpha' = \frac{k_1'}{k_1} \alpha \]
\[ f' = f(t_j) - k_1(x_1(t_j) - \alpha(t_j)). \]

The solution of this model is
\[ x_2'(t') = x_2'(0) \exp \left( -\frac{t'}{\tau} + \frac{f'}{k_1} \left( 1 - \exp \left( -\frac{t'}{\tau} \right) \right) + \alpha \left( t' - \tau \left( 1 - \exp \left( -\frac{t'}{\tau} \right) \right) \right) \]
\[ x_1'(t') = \frac{f'}{k_1} - \left( \frac{f'}{k_1' - x_2'(0)} \right) \left( \frac{k_2}{k_2 + k_1} \right) \exp \left( -\frac{t'}{\tau} \right) \]
\[ + \alpha \left( t' - \tau \left( 1 - \exp \left( -\frac{t'}{\tau} \right) \right) + \frac{\gamma}{k_2} \left( 1 - \exp \left( -\frac{t'}{\tau} \right) \right) \right) \]
\[ \tau = \gamma \left( \frac{1}{k_1'} + \frac{1}{k_2} \right) \]

and at long times we find
\[ x_2'(t') \sim \alpha' t' + \frac{f'}{k_1'} - \alpha' \tau \]
\[ x_1'(t') \sim \alpha' t' - \alpha' \tau + \frac{\gamma \alpha'}{k_2'} + \frac{f'}{k_1'}. \]
Using these asymptotic relations we can deduce the values of $x_2'(0)$ and $f'$ for the immediately following stiffness jump at $t = t_{j+1}$, let us call these $x_2'(0)$ and $f''$. Assuming $t' \gg \tau$ so that we can use the asymptotic formulas, with the recurrence formula for $f(t)$ and the formula for $\tau$ we find $f'' = \gamma \alpha'$. We can also easily find that $x_2''(0) = -\frac{\gamma}{k_2} \alpha'$. These formulas can be used to give the final complete solution for widely spaced stiffness jumps as

$$x_i(t) - x_i(t_{j+1}) = \alpha \frac{k_1}{k_1 + k_{ex}(t_{j+1})} \left(t - t_{j+1}\right)$$

$$+ \alpha \frac{k_1 k_\gamma}{k_1 + k_{ex}(t_{j+1})} \left(\frac{1}{k_1 + k_{ex}(t_j)} - \frac{1}{k_1 + k_{ex}(t_{j+1})}\right) \left[1 - \exp\left(-\frac{t - t_{j+1}}{\tau_{j+1}}\right)\right]$$

where

$$\tau_{j+1} = \gamma \left(\frac{1}{k_1 + k_{ex}(t_{j+1})} + \frac{1}{k_2}\right).$$

The increment to the measured force during the interval $[t_{j+1}, t_{j+2})$ can be derived from this relation by multiplying by $k_{ex}(t_{j+1})$, and in the case of a displacement clamp, taking the limit $k_{ex}(t_{j+1}) \to \infty$.

5.7 Acknowledgements

I would like to thank Kevin Webster, Evan Hohlfeld, and Win Pin Ng for their hard work on this project. I would also like to thank Daniel Fletcher, Ross Rounsevell, Lina Nilson, Ben Ricca, Gautham Venugopalan and other members of the Fletcher Lab for helpful discussions, and Luke Cassereau for donation of polyacrylamide material.
Chapter 6. Concluding Remarks
6.1 Summary

In this dissertation I have described four projects that developed and employed novel techniques to explore the interactions of single cells with the surrounding mechanical environment. I will here outline the remaining questions and future directions of each project individually and for the field as a whole.

In chapter 2 I describe a technique enabling the attachment of individual cells to AFM cantilevers or glass substrates with the strength of DNA hybridization. This approach exceeds current methods in strength of adhesion, tunability, and long-term cell viability. DNA scaffolds are now being used for 3D patterning of cells into microtissues (Koyfman et al., 2009) for both research and tissue engineering applications and co-author Sonny Hsiao has founded Adheren – a company employing DNA-based live-cell patterning. Given the biocompatibility of DNA and impressive control we now have over DNA structures, I am confident the use of DNA for precise spatial control and manipulation of cells will prove useful in a wide range of applications.

Chapter 3 combines the AFM cantilever as a mechanical probe with novel three-dimensional particle tracking of fluorescent nanoparticles as fiduciary markers to explore how stress is propagated across a cell. The results demonstrate that a viscoelastic treatment of the cell could not describe the spatial inhomogeneities observed; whereas a poroelastic model is consistent with slow stress propagation across the cell. In the context of cell mechanics, poroelasticity has been used predominantly to describe bone cell mechanics (Cowin, 1999) and amoeboid motility (Charras et al., 2005). More generally, the field of mechanosensing has long been interested in how stresses are propagated across the cell. “Action-at-a-distance” for fast propagation through solid elements of the intracellular environment and slower equilibration provide multiple timescales for mechanical signaling within the cell. These timescales are consistent with both the power law rheology concept of cell mechanics and the complex feedback and signaling cascades of mechanotransduction. Much work remains to determine how the mechanics of stress propagation integrate with cell signaling to create an evolving and self-amplifying whole-cell mechanoresponse. Specific proposed experiments are discussed at the end of this section.

Chapter 4 describes a novel AFM-based technique to dynamically and reversibly control the environmental stiffness cues presented to a single cell. This technique is then immediately applied in Chapter 5 where we reveal a myosin II based contractile response to step changes in stiffness on a timescale of seconds. Together these studies present important fast timescales of stiffness sensing that likely provide the initial signal of changes in intracellular tension that ultimately result in long-timescale stiffness-dependent behaviors such as altered focal adhesion and cytoskeletal architecture and even gene expression. Furthermore these studies present a platform for future exploration of actomyosin contraction in a whole-cell context and the timescales for involvement of specific cytoskeletal and focal adhesion components during an evolving rigidity response.
6.2 Future Outlook

These studies will be continued by Kevin Webster, Win Pin Ng, and Ben Ricca in the Fletcher Lab by combining the AFM-based technique for controlling mechanical boundary conditions with live-cell fluorescent labeling of specific components such as vinculin, paxillin, zyxin, and myosin II to determine the timescales and order of involvement of each component in response to a specific mechanical signal. The combination of precise control and measurement of deformation, force, stiffness and geometry with high resolution TIRF (Total Internal Reflection Fluorescence) imaging of any labeled intracellular component will finally allow the mechanisms behind mechanosensing to be studied with the spatiotemporal control required. These experiments will be integrated with longer timescale gel-based experiments to determine how the integration and amplification of the fast timescale rigidity response results in longer timescale behaviors such as gene expression. Finally, through collaborations and developing expertise in the lab these studies will be expanded from the model system of the fibroblast to physiological contexts of cancer progression, development, and the epithelial-mesenchymal transition by using appropriate cell lines and even primary cells.

Two additional emerging areas of interest and potential study include the study of podosomes – invasive adhesive structures observed in cancerous cells and osteoclasts, shown to be mechanosensitive (Collin et al., 2008) – and the mechanosensory abilities of cell-cell adhesions informing studies of mechanoresponses on the multicellular scale (Chopra et al., 2011; Maruthamuthu et al., 2010). A variety of studies are beginning to reveal parallels between differentiation during development and “de-differentiation” towards tumorigenesis warranting comparison and study of these two physiological contexts within the same experimental systems. Both podosomes and cell-cell adhesions contain many of the same structural components and signaling associated with focal adhesions and preliminary studies have demonstrated mechanosensitivity in both contexts. Simple imaging studies as described above could investigate the role of vinculin and talin in the response to different mechanical boundary conditions within the adhesions of normal fibroblasts compared to the podosomes of v-Src transformed fibroblasts. How does the organization and expression of actin and protrusion-associated proteins such as alpha-actinin vary between focal adhesions, podosomes, and cell-cell junctions? Are the dynamics of formation, dissolution, and mechanically stimulated reinforcement the same in all three cases? Are common mechanosensory proteins such as talin or p130cas active in all three contexts? Furthermore the extracellular interface may be varied to present single-cells with different ligands (collagen, laminin...) or binding sites of a neighboring “artificial cell” using commercially available recombinant N-cadherin Fc chimeras or other ligands mimicking cell junction binding or supported lipid bilayer techniques in collaboration with the Groves lab.
The work presented in this dissertation is a small fraction of the impressive body of work contributing to incredible progress over the last century from the observation-based theories of Thompson to the precise measurement and control of intracellular and extracellular mechanical signals. New tools and techniques are constantly being developed to further explore the cellular mechanoresponse. Imaging of thermal fluctuations provides a noninvasive approach to measure the tension of intracellular polymers and proteins. FRET sensors have been recently developed for Src and vinculin (Grashoff et al., 2010; Wang et al., 2005) allowing pN resolution and dynamic measurement of the tension across specific mechanosensory proteins. Further control by light-activated proteins and temperature-sensitive mutants allows an on-off switch for specific proteins or activity. These specific manipulations and imaging techniques may be implemented in a multicellular context and combined with precise control of mechanical boundary conditions to reveal different pieces of the mechanoresponse puzzle. I look forward to the evolution of our understanding of the mechanisms and dynamics of the cellular mechanoresponse as new techniques are developed and interdisciplinary collaborations enable new and creative experiments.
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