

Effect of Cell-Substrate Interactions on Epithelial Cell Mechanics

by

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Abstract

Cell-substrate interactions play a key role in the regulation of epithelial cell mechanics. Through a series of studies, we demonstrate how substrate interactions impact both the response to an oncogene and the cellular contractility and organization of a monolayer. We first examine the effects of oncogenic Ras in cells in adherent and suspended states. To accomplish this, we utilized atomic force microscopy and a microfluidic optical stretcher. We found that adherent cells stiffen and suspended cells soften with the expression of constitutively active Ras. The effect on adherent cells was reversed when contractility was inhibited with the ROCK inhibitor Y-27632, resulting in softer cells. These findings suggest that increased ROCK activity as a result of Ras has opposite effects on suspended and adhered cells. In a subsequent study, we examined the effects of a substrate on contracting and relaxing monolayers. We created a new methodology for measuring the mechanodynamics of epithelial monolayers by culturing cells at an air-liquid interface. These model monolayers were grown in the absence of any supporting structures in hanging drops. We found that the direction of strain in the unsupported monolayers was not correlated to nuclear alignment as observed when the monolayers were grown on soft deformable gels. It was also observed that both gel and glass substrates led to the promotion of long-range cell nuclei alignment not seen in the unsupported monolayers. To further characterize the morphology and mechanics of monolayers clusters observed in our experiments, we created a new computational model based on the vertex model. The energy function used in this model takes into account cell-cell and cell-substrate adhesion as well as anisotropic cellular mechanical properties. The results of these simulations suggest that the promotion of long-range alignment on solid substrates were due to cells having anisotropic elastic moduli with global alignment. They also suggest that the alignment observed in monolayers grown on air water interfaces is due to cells having low substrate adhesion and isotropic moduli. Our findings establish the importance of studying epithelial cell mechanics in different states of attachment.

Contribution Statement

I, Corinne Gullekson, contributed the majority of the work presented in this thesis. In chapters 4 and 5, this work included experimental design, cell culture, building of the microfluidic optical stretcher, experimentation, writing code, data analysis and statistical analysis. In chapter 6, work included developing and writing the code for the computational model as well as performing simulations and analyzing data.

Other researchers have also contributed to this work. Dr. Andrew E. Pelling contributed to the direction and editing of the results presented in chapters 4, 5 and 6. Dr. James Harden contributed to the direction and editing of the results presented in chapters 5 and 6. In a visit to Technische Universität Dresden, guidance on how to set-up an optical stretcher and perform data analysis was provided by Dr. Jochen Guck and his postdoc Gheorghe Cojoc. Dr. Guck's lab's MATLAB code was used to calculate the geometric factors, F_g , used in data analysis. Digital holographic microscopy experiments were performed with his PhD student Mirjam Schürmann using her experimental setup and data processing. They also contributed to the editing of the results in presented in chapter 4. Dr. Benjamin Watts, a technician made the SU-8 holder used in chapter 4. Matt Walker, a PhD student in Andrew Pelling's lab, made the polyacrylamide gels used as a substrate used in chapter 5 and contributed to the editing of the results presented in chapter 5.

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List of Abbreviations and Symbols

Abbreviations

AFM	atomic force microscopy
ATP	adenosine triphosphate
CalA	calyculin A
DAQ	data acquisition
DHM	digital holographic microscopy
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EMT	epithelial to mesenchymal transition
FBS	fetal bovine serum
GDP	guanosine diphosphate
GFP	green florescent protein
GTP	guanosine triphosphate
LSCM	laser scanning confocal microscope
MDCK	Madin-Darby canine kidney
MH	Metropolis-Hastings
MLCK	myosin light-chain kinase
PA	polyacrylamide
PBS	phosphate buffered saline
PDMS	polydimethylsiloxane
PEEK	polyetheretherketone
ROCK	rho associated protein kinase
ROS	reactive oxygen species
SLL	standard linear liquid
WGA	wheat germ agglutinin
WT	wild type

Symbols

A	area
A_0	starting area
c	speed of light
c_1 to c_{10}	fitting constants
E	Young's modulus
E_{xx}	Young's modulus in the x direction
E_{xy}	Young's modulus in the xy direction
E_{yy}	Young's modulus in the y direction
E_l	energy of a ray of light
e_{jk}	2D permutation tensor
F	force
F_G	geometric factor
G	shear modulus
h	height

h_0	starting height
I	intensity
$J(t)$	creep compliance
K	elastic parameter
k	spring constant
k_B	Boltzmann constant
L	perimeter
L_0	initial perimeter
l	side length
l_{ext}	length of an exterior polygon edge
l_{int}	length of an interior polygon edge
n	refractive index
n_1	refractive index of the first medium
n_2	refractive index of the second medium
n_j	normal vector
P	probability
p	momentum
p_i	momentum of the incident ray
p_{i2}	momentum of the incident ray on the second side
p_r	momentum of the reflected ray
p_{r2}	momentum of the reflected ray on the second side
p_t	momentum of the transmitted ray
p_{t2}	momentum of the transmitted ray on the second side
R	refraction coefficient
r	radius
$r_{ }$	parallel fresnel coefficient
$r_{\perp}$	perpendicular fresnel coefficient
S	nematic order parameter
S_{ns}	nuclei-strain order parameter
S_{nl}	nuclei-cluster orientation order parameter
S_{ls}	cluster orientation-strain order parameter
S_{nn}	nuclei-nuclei order parameter
T	temperature
t	time
U	energy
u_{ij}	displacement gradient
u_x	displacement in the x direction
u_y	displacement in the x direction
V	volume
W	strain energy density
w	beam width
w_0	beam width when it leaves the fiber
z	distance travelled in a medium
α	angle of incidence
α_c	opening angle of the cone

β	angle of refraction
Γ	tension parameter
δ	displacement
Δp_{back}	momentum transfer on the back side of the cell
Δp_{front}	momentum transfer on the front side of the cell
ϵ	strain
$\boldsymbol{\epsilon}$	strain tensor
ϵ_1	major principal strain
ϵ_2	minor principal strain
ϵ_M	mean strain
ϵ_{xx}	x axis strain
ϵ_{xy}	shear strain
ϵ_{yy}	y axis strain
η	viscosity
η_s	steady-state viscosity
η_t	transient viscosity
θ	angle between features
Λ	adhesion parameter
Λ_{cell}	cell-cell adhesion parameter
Λ_{ext}	cell-media adhesion parameter
Λ_{sub}	cell-substrate adhesion parameter
λ	wavelength
ν	poisson ratio
σ	stress
$\boldsymbol{\sigma}$	stress tensor
σ_0	peak stress
τ	transition time
ϕ	angle of incidence on the back side of the cell

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Chapter 1

Introduction

Many of the human body's cavities are lined by epithelial monolayers. While they are the simplest tissues in multicellular organisms, they perform crucial roles in normal and diseased physiology. During embryonic development, morphogenesis is driven by the integration of forces generated by epithelial cells into tissue-scale deformations [1, 2].

Cell-substrate interactions play a key role in epithelial cell mechanics. A cell's mechanical properties are largely determined by the actin cytoskeleton [3] which is linked to the substrate by focal adhesions. The focal adhesions are important in cell mechanics because they serve as anchoring points for actin stress fibers. Tension is generated along these fibers with the activation of myosin. In general, softer substrates have less cell-substrate adhesion than stiffer substrates [4, 5, 6, 7]. Fewer stress fibers correlate with softer cells, presumably due to a decrease in prestress in the actin network [8]. For this reason it is critical to consider substrate adhesion in experiments studying cell mechanics.

Many studies are performed on cells cultured on rigid substrates like glass or plastic culture dishes. Measuring cells in complete suspension is on the other end of the scale of adherence, however it only corresponds to the natural state of a few cell types. For most cell types, the natural environment lays somewhere in between these extremes, in a soft, three dimensional environment. These environments result in less focal adhesion points

and fewer, less pronounced stress fibers than harder substrates. Understanding the effects of the substrate is crucial when extrapolating the results of experiments performed on rigid substrates or in suspension to the situation *in-vivo*.

1.1 Motivation and Objectives

Metastatic cancer cells are in different states of attachment during their journey from the primary tumor site to another. During the initial stages, cells are in direct contact with one another and form strong intercellular connections and basement membrane bonds. Following intravasation, the cell travels in the blood stream or lymphatic system in a suspended state. The organization of the actin cytoskeleton is also fundamentally altered during oncogenic transformation. The oncoprotein Ras is of particular interest because its constitutively active form is commonly found in cancer. It sits near the top of a signaling pathway that activates Rho-associated protein kinase (ROCK). Since ROCK regulates actin filament organization and actomyosin contractility, the over-activation of this pathway should have different effects on adherent and suspended cells due to their differences in cytoskeletal structure. An objective of this thesis is to determine how cell-substrate interactions affect the cell mechanics of Ras transformed cells.

Actomyosin contractility is also important in tissue remodeling. Cells use contractility to drive shape changes and movements of cells in development and later on to maintain homeostasis. Understanding how cytoskeletal activity is orchestrated to produce higher order tissue organization is a major challenge in developmental biology [9]. Current methods used to characterize the mechanics and dynamics of monolayers often involve cells grown on solid supports such as glass or gels. However, this is not the situation *in vivo* and results of these studies are fundamentally influenced by these supporting structures. We aim to develop a new methodology to characterize actomyosin mechanodynamics in a multi-cellular system without a mechanically supporting substrate. This would provide a

picture of basal mechanics and dynamics in a simplified system allowing us to infer how the presence of a substrate impacts contractility and long-range multi-cellular organization.

Current computational models of epithelial cell monolayers use isotropic moduli, often using areas of cells [10, 11]. There is a need for a computational model to simulate these monolayers that include both substrate adhesion and anisotropic elastic moduli. Another objective of this thesis is to develop a new computational model to characterize monolayer mechanodynamics. We aim to use this model to explain the remodeling and cellular organization observed in monolayers grown with and without solid supports.

1.2 Outline

In this thesis, we examine the effects of a substrate by characterization and computational modeling of epithelial cells in different states of attachment. In the following, we provide a brief outline of the structure of the thesis.

The purpose of Chapter 2 is to provide sufficient background information to understand the remainder of the thesis. It contains an overview of cytoskeletal structure, including its components, its interactions and how cancerous cells differ from non-pathological epithelial cells. It also describes cell mechanics, including mechanosensing, cell generated stresses and the cellular response to stress and viscoelasticity. The chapter concludes with basic theory of atomic force microscopy, optical stretching and optical microscopy.

Chapter 3 describes the basic experimental details of this thesis. It describes the cell culture protocols used to make our samples. It is followed by a description of the set ups and data analysis used in confocal imaging, atomic force microscopy, and digital holographic microscopy. The design, construction and calibration of an optical stretcher is then detailed with a description of the data analysis used. Lastly, the protocol used to create monolayers in hanging drops and the data analysis techniques used to quantify strain and calculate order parameters are described.

In Chapter 4, we investigate the mechanical properties of cells expressing constitutively active, oncogenic Ras in adherent and suspended states. In order to achieve this, we utilize atomic force microscopy and a microfluidic optical stretcher. The atomic force microscope is used to measure the apparent Young’s modulus of transformed and untransformed epithelial cells grown in a monolayer as well as loosely attached to glass. The optical stretcher is used to measure the compliance of the cells in a suspended state. A ROCK inhibitor is also used to inhibit actomyosin contractility. A modified version of this chapter was published as:

- C. Gullekson, G. Cojoc, M. Schürmann, J. Guck and A. E. Pelling, “Mechanical mismatch between Ras transformed and untransformed epithelial cells” *Soft Matter*, 2017, vol. 13, pp. 8483-8491, 2017.

In Chapter 5, we describe a new methodology for measuring the mechanodynamics of epithelial monolayers by culturing cells at an air-liquid interface. Employing laser scanning confocal microscopy, cell nuclei within the monolayer are used as fiduciary markers to determine the internal strain developed in the clusters. We evaluate the methods potential by observing and quantifying the generation and release of internal stresses upon actomyosin contraction and relaxation in response to chemical treatments. The mechanics and organization of these monolayers are then compared to similar monolayers grown on glass and deformable gel substrates. A modified version of this chapter was published as:

- C. Gullekson, M. Walker, J. L. Harden, and A. E. Pelling, “Measuring mechanodynamics in an unsupported epithelial monolayer grown at an air-water interface,” *Molecular Biology of the Cell*, vol. 28, no. 1, pp. 111-119, 2017.

In Chapter 6, we develop a computational model to study epithelial monolayer clusters. This model includes anisotropic cell mechanical properties and substrate adhesion providing a means to investigate their effects in epithelia. Different types of alignment of elastic

moduli are used, aligning either to the length axes of each cell or to a single axis. We then examine the alignment of cellular features in the monolayer and the strain produced by a change in elastic modulus (simulated to mimic treatment with contractility enhancers and inhibitors). These simulations are then compared to the results of chapter 5 to deduce the type of elastic moduli alignment displayed in unsupported and supported monolayers.

Chapter 2

Background

2.1 Cytoskeletal Structure

Despite the myriad of specific cell types, cells share common design principles that are altered according to their function in the body [12]. The cytoskeleton is largely responsible for the cell's mechanical properties [3]. It is a polymer network that spans the interior of the cell and functions as a dynamic scaffolding and internal transport facilitator[12]. In almost every cell type, the cytoskeleton is constantly changing which results in a very adaptive structure, in particular to extracellular force [13]. Its ability to adapt facilitates the resistance and transmission of stress and the generation of forces within the cell. This allows it to connect to its environment, change its cell shape and migrate [12].

2.1.1 Cytoskeleton Components

The cytoskeleton is made up of three distinct polymer types, flexible to semi flexible intermediate filaments, semiflexible actin filaments and rigid microtubules (Fig. 2.1). Cytoskeletal networks also include cross linking proteins and active elements such as the molecular motor myosin II with transient and non-equilibrium properties [3].

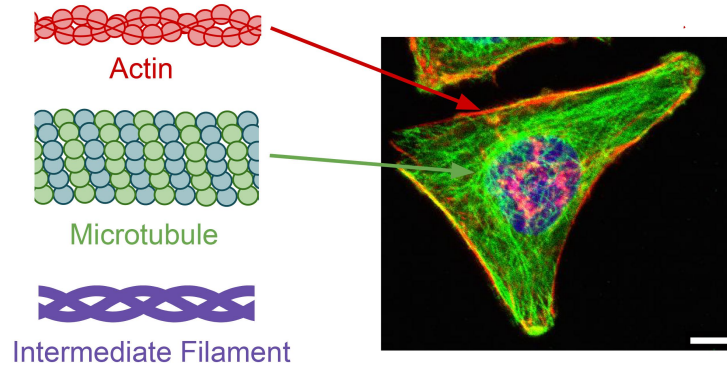


Figure 2.1: Components of the cytoskeleton. The protein structure of actin microtubules and intermediate filaments. Actin (red), microtubules (green) and the nucleus (blue) are shown in a fixed and stained 3T3 fibroblast cell. Scale bar $10\mu\text{m}$.

Microtubules

Microtubules are the stiffest components of the cytoskeleton, spanning the entire cell with its long tubules. They are used in the mitotic spindle during cell division [12]. For the remainder of the cell cycle, microtubules act as intracellular transport highways for motor proteins with vesicles and proteins as their cargo [12].

Intermediate Filaments

Intermediate filaments are the least rigid of the cytoskeleton polymers. A rope-like structure is assembled from filament subunits containing different types of intermediate filament monomers. The intermediate filament network appears to be purely structural. They do not show the polymerization dynamics observed for actin and microtubules and are extremely stable after polymerization. This is fundamental for supporting the structural integrity of whole tissues by the direct connections of the intermediate filaments of one cell to the intermediate filaments of the neighbouring cell [14].

Actin

Actin filaments are less rigid than microtubules but stiffer than intermediate filaments. They are composed of G-actin subunits that form constantly polymerizing and depolymerizing filaments. Since the G-actin subunit is asymmetric, actin filaments have polarity. The two ends of the filaments have different growth rates, with the plus end growing faster than the minus end. Actin filaments can elongate steadily in order to exert forces or disassemble, depending on the amount of monomers available and the need of the cell, which is transmitted by a signaling system [12].

Within the cell, these filaments are organized into different structural arrangements (Fig. 2.2). The two major arrangements are bundles and networks [3]. In bundles, short cross-linking actin filaments align into tight parallel arrays. Bundled actin is used in cell-cell communications or in stress fibers, which connect different focal adhesion points within the cell [12]. In networks, the filaments are cross-linked in a comparatively loosely packed criss-cross arrangement. Networks of actin form a shell like structure underneath the cell membrane called the actin cortex and can be found in the leading edge of motile cells. Besides cross linking proteins there are numerous regulatory proteins that sever, cap, nucleate or polymerize actin filaments and hence control the structural arrangement of the cytoskeleton. Furthermore, motor proteins such as myosin II are able to apply forces to individual proteins. These mechanisms allow the cell to actively modulate its mechanical properties.

2.1.2 Cell Interactions

Cell-Substrate Interactions

The cytoskeleton is linked to the substrate by focal adhesions. The actin cytoskeleton is fundamentally different in adherent cells compared to cells in suspension, mainly re-

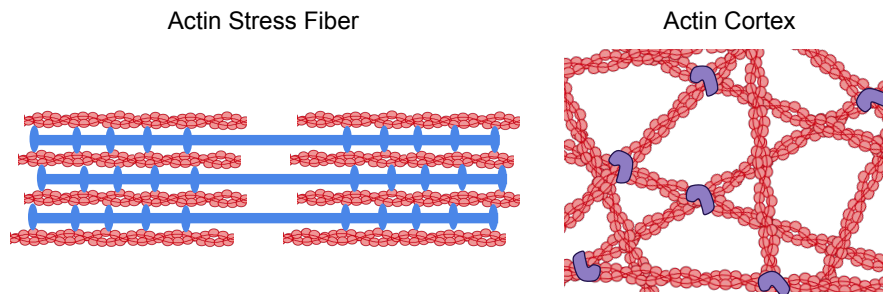


Figure 2.2: Structure of actin in the cytoskeleton. Left: Stress fibers from bundled actin filaments (red), shown here associated with filaments of myosin (blue), and generate tension along one direction. Right: Cortical networks (that is, non-aligned networks), form below the plasma membrane and carry tension loads in multiple directions. Based on [12] .

flected by the absence of stress fibers in suspended cells. Adherent cells change mechanical properties directly after they are detached from substrates and brought into suspension [15]. Therefore, it is not surprising that cell-substrate interactions play a major role in cell mechanics.

Focal adhesions are sites of tight adhesion to the underlying extracellular matrix developed by cells in culture (Fig. 2.3). They provide a structural link between the actin cytoskeleton and the extracellular matrix (ECM) and are regions of signal transduction. They consist of aggregated ECM receptors, called integrins, that span the plasma membrane, interacting with both ECM components and actin stress fibers [16]. Many proteins have been identified in focal adhesions; some of these proteins have a structural role, whereas others are involved in signal transduction [16].

Cell-Cell Interactions

Cell-cell interactions also affect the individual and collective behaviors of cells. Cells form anchoring adhesive junctions involving the cytoskeletons of adjacent cells mediated by cadherins (Fig. 2.4). Cell-cell adhesion is a selective process, such that cells adhere only to other cells of specific types [18]. The role of the cadherins in linking the cytoskeletons of adjacent cells is thus analogous to that of the integrins in forming stable junctions between

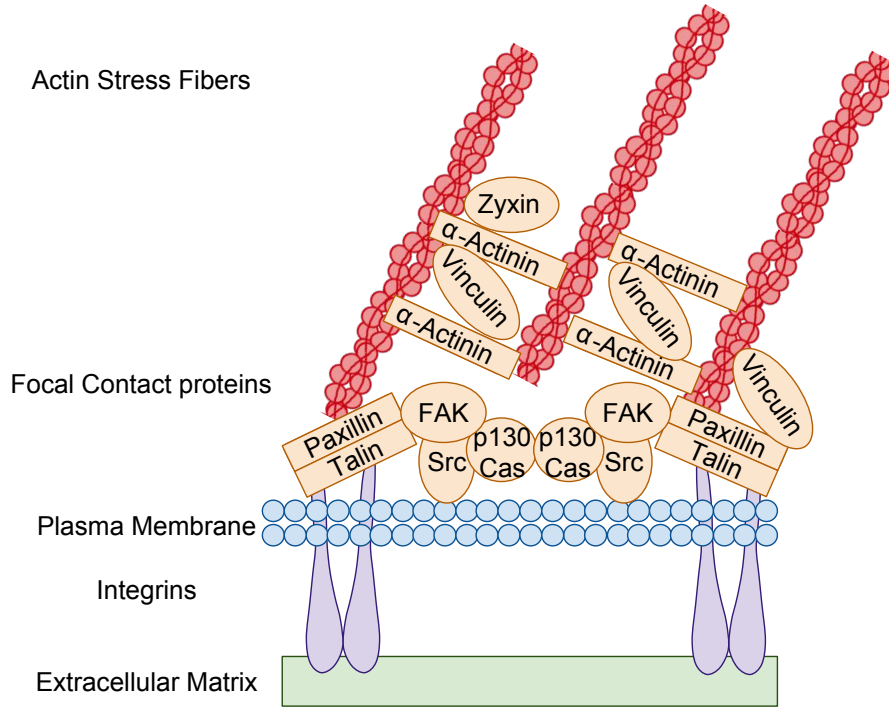


Figure 2.3: Structure of a focal adhesion. The extracellular matrix (green), integrins (purple) and the cell cytoskeleton (red) interact at sites called focal adhesions. Various proteins (orange) crosslink actomyosin stress fibers and tether them to focal contacts. Based on [17].

cells and the extracellular matrix. These cell-cell junctions are of two types: adherens junctions and desmosomes, in which cadherins are linked to actin bundles and intermediate filaments respectively. The type of cadherins found in desmosomes are called desmogleins and desmocollins and the type of cadherin found in adherens junctions between epithelial cells is called E cadherin. Adherens junctions are transmission sites of cell-generated forces [19], connecting stress fibers between adjacent endothelial cells [20]. In epithelia, the adherens junctions link actin bundles into a circumferential belt around the cell [21, 1].

In addition to the adherin junctions mediated by the cadherins, two other types of specialized cell-cell junctions play key roles in tissues. Tight junctions are critically important to the function of epithelial cell sheets as barriers between fluid compartments.

They also separate the apical and basolateral domains of the plasma membrane. There are also other cell-cell interactions mediated by the selectins, integrins, and members of the immunoglobulin superfamily that are transient adhesions, however the cytoskeletons of adjacent cells are not linked to one another.

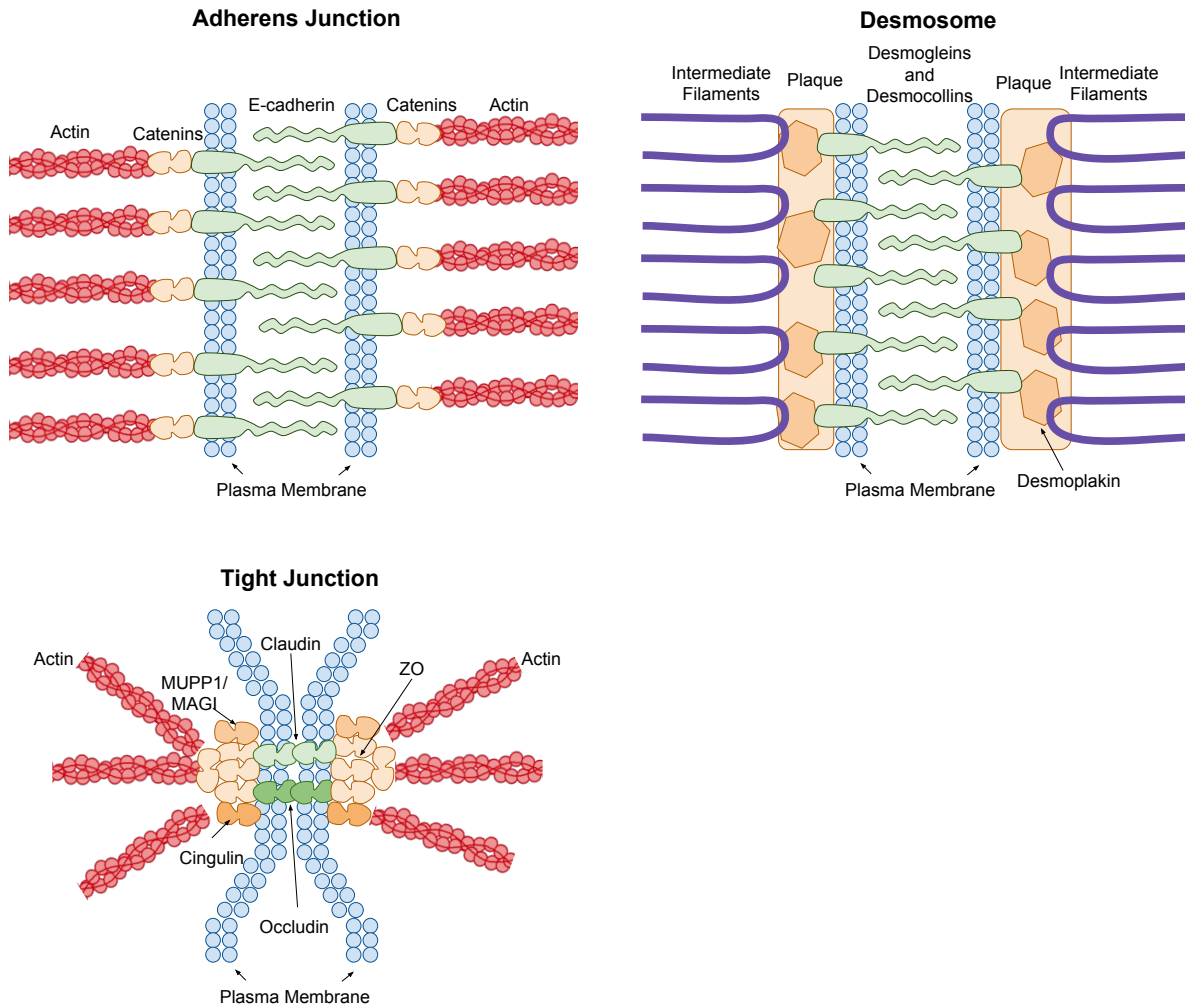


Figure 2.4: Epithelial cell-cell junctions. Homophilic interactions between cadherins mediate two types of stable cell-cell adhesions. In adherens junctions, the E-cadherins are linked to bundles of actin filaments via the catenins. In desmosomes, desmoplakin links members of the cadherin superfamily (desmogleins and desmocolins) to intermediate filaments. Tight junctions are comprised of transmembrane adhesion molecules of various protein families such as the claudins or occludin that are intracellularly linked to the actin cytoskeleton via various adaptor molecules such as members of the zonula occludens (ZO) family. Based on [22].

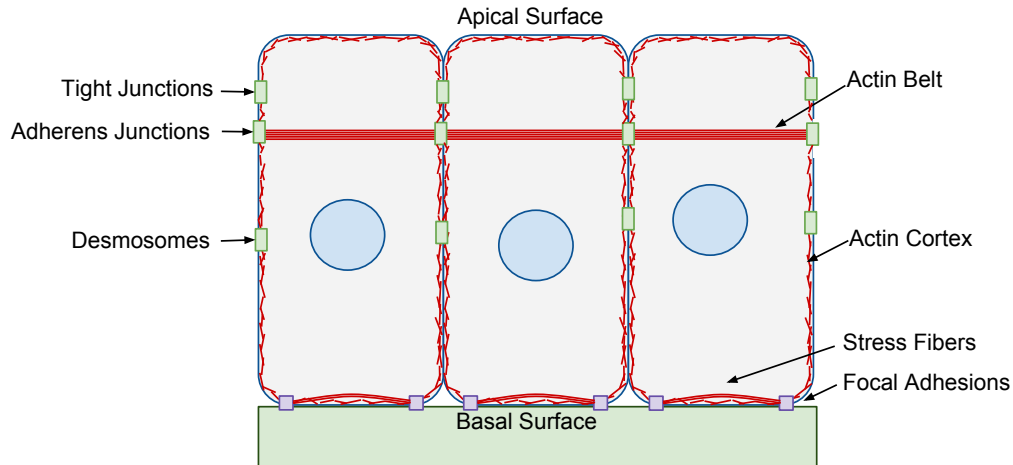


Figure 2.5: Epithelial monolayer adhesion structure. Schematic drawing of the tight junctions as well as the two adhesive epithelial junctions: adherens junctions and desmosomes and focal adhesions. Based on [31, 30].

Cell Monolayers

Many of the human body's cavities are lined by epithelial monolayers. Epithelial organization is highly dynamic as cells move and change shape to dissipate mechanical stress that arises during cell division, intercalation and extrusion and when large scale remodeling and movements take place [23, 24, 25, 26, 27]. Epithelial sheets respond elastically to deformation over shorter timescales but will undergo organizational remodeling in response to sustained stress over long timescales [25, 28]. This is achieved through a complex interplay between contractile actomyosin networks and cell-cell junctions [23, 24, 9, 29]. The formation of a stable, polarized, epithelium requires tight cell-cell and cell-substrate connections [30]. Adherens junctions are located basal to the apical tight junctions and form a belt-like structure which tie neighboring cells together [30] (Fig. 2.5). Tight junctions exist as a continuous band located just below the apical surface between the membranes of neighboring epithelial cells to produce a seal between different tissues and body cavities.

2.1.3 Effects of Cancer

The most common types of cancers are carcinomas (cancers of the epithelium). For this reason epithelial cells are used in all the experiments in this thesis. During the initial stage of carcinogenesis, transformation occurs in a single cell in an epithelial monolayer [32]. Cell transformation can arise from the activation of oncoproteins. A common oncogene is Ras and will be studied in this thesis. This mutation plays havoc with cytoskeleton structure and cell interactions [33]. In short, they disrupt all the dynamics described above.

Epithelial to Mesenchymal Transition

Metastasis is the spread of tumor cells from a primary tumor to a secondary site. It remains one of the most life-threatening aspects of cancer. For most carcinomas, progression toward malignancy is accompanied by loss of epithelial differentiation and a shift towards a mesenchymal phenotype. This process is referred to as epithelial to mesenchymal transition (EMT) (Figure 2.6) [34]. During this transition, the cells undergo multiple changes which include loss of intercellular junctions, increased motility and invasiveness, altered growth control, the ability of cells to grow outside their local environment and greatly increased production of extracellular matrix components [34, 35, 36]. Disruption of actin filaments and a decrease in focal adhesions are also common features following transformation of cells by various oncogenes.

In the next steps of the metastatic process called intravasation, the cancer cell breaks away from their neighbor cells and invades through the basement membrane into a blood or lymph vessel. This allows them to then spread in the blood stream or the lymphatic system and finally travel into a secondary tissue. The cells then leave the vessel, entering the new tissue in a process called extravasation. In each of these steps the cell's adhesive and cytoskeletal properties play essential roles.

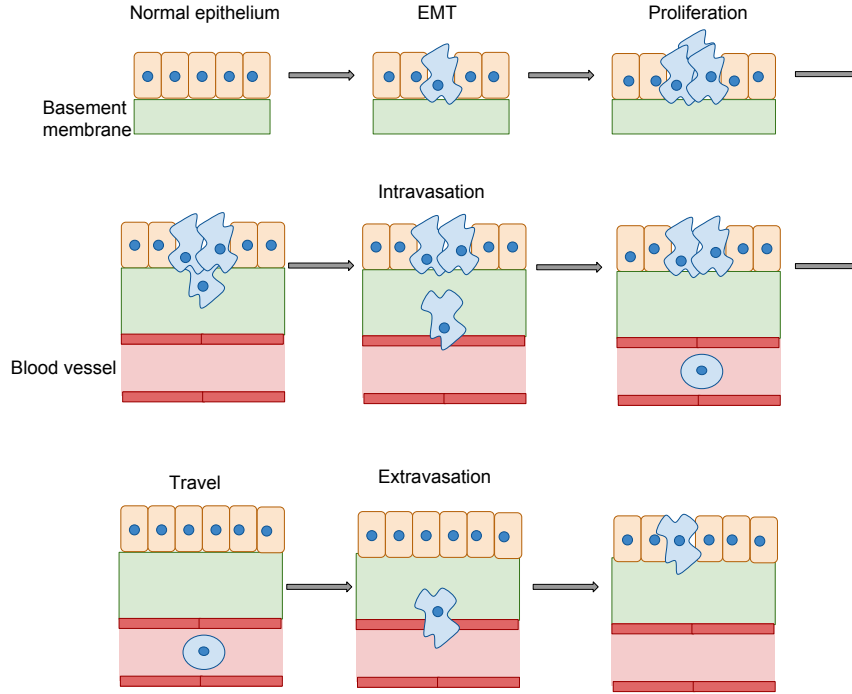


Figure 2.6: Steps in the metastatic process. Metastasis is a multistep process that begins with an epithelial-mesenchymal transition (EMT). The cancer cell breaks away from their neighbor cells and invades through the basement membrane into a blood or lymph vessel (intravasation). The cell then travels to a new site, leaves the vessel, entering the new tissue (extravasation).

Cytoskeleton changes

Typically, it is shown that cancer cells are softer than non-transformed cells and that highly aggressive cancer cells are softer than less aggressive cancer cells [37, 38, 39, 40]. This can be attributed to the cytoskeleton devolving from an ordered and rigid structure to a more irregular and compliant state [38]. It has been shown that the amount of filamentous actin in malignantly transformed fibroblast cells is reduced by $\sim 40\%$ [38]. This is often explained by the need of metastatic cancer cells to squeeze through the surrounding tissue matrix as they make their way to establish distant settlements [41]. Loss of E-cadherin gene expression or of E-cadherin protein is also frequently found during tumor progression

in most epithelial cancers [30]. Since E-cadherin plays a key role in epithelial structure and homeostasis its expression underlies a strict control [30]. Hence, loss of E-cadherin function is a clinical indicator for poor prognosis and metastasis [30].

Extracellular Matrix changes

During cancer progression, the substrate the cells are in contact with, the extracellular matrix, will change as well. Cell behavior has been found to be strongly regulated by biophysical interactions between the cell and the ECM [42]. Multiple regulatory mechanisms exist to ensure that ECM dynamics are normal during organ development and function [43]. Deregulated ECM dynamics disrupt tissue polarity, architecture, integrity and promote an EMT and invasion [44]. Tumors are characterized by an abnormally stiff ECM. Thickening, crosslinking and linearization of collagen fibers are common in tumors [45, 46, 47]. The stiffness of the matrix and degree of orientation of matrix fibers near primary tumor sites strongly correlates with active tissue invasion and worse clinical outcomes [44, 41, 48]. This abnormal ECM affects cancer progression by directly promoting cellular transformation and metastasis [44, 49, 50].

Ras

Ras proteins are signal-transduction GTPases that sit near the top of signaling cascades and coordinate a variety of cellular signaling pathways responsible for growth, migration, adhesion, cytoskeletal integrity, survival and differentiation [33]. Ras GTPases act as molecular switches and timers, alternating between GTP- and GDP-bound states. The GDP-bound state is generally considered inactive, while the GTP-bound state is a prolific signaling molecule that can bind to over twenty different effectors to activate a network of downstream signaling events.

They are also proto-oncogenes that are frequently mutated in human cancer. Muta-

tions in Ras genes have been found in various forms of human cancer including epithelial carcinomas of the lung, colon, and pancreas. Mutations that permanently activate Ras are found in approximately 20% of cancers [51]. Each of these mutations interfere with the catalysis of GTP hydrolysis, causing Ras to remain locked in its active, GTP-bound form [52]. The cells of these cancers, as well as those that have been experimentally transformed by the activated Ras gene, exhibit abnormal growth, morphological changes and alterations of cell adhesions. Ras-transformed fibroblasts often exhibit a loss of stress fibers and focal adhesions [53]. For this reason, there is great interest in developing tumor therapies targeting Ras and Ras effector pathways [51].

Ras-associated Pathways

Ras effectors control a variety of cytoskeleton regulation and cell proliferation pathways (Figure 2.7). The following are important pathways Ras regulates:

- AF6: AF6 has been found to regulate cell-cell adhesion [54].
- Ral: Ral has been found to be involved in survival, anchorage-independent cell growth and cytoskeletal organization [33].
- Rho: The mechanism for Ras induced Rho activation remains unknown, however increased Ras activity is associated with increased Rho activity [55, 56, 57, 58]. Rho activates ROCK (Rho-associated protein kinase) which regulates actin filament organization and contractility [59, 60].
- TIAM: TIAM activates Rac [61], and this activation is involved in actin cytoskeleton reorganization and stress fiber formation [62].
- PI3K: The PI3K activates Akt which performs an important function promoting cell survival through its phosphorylation of multiple proteins that trigger anti-apoptotic signaling pathway [63]. PI3K also activate Rac.

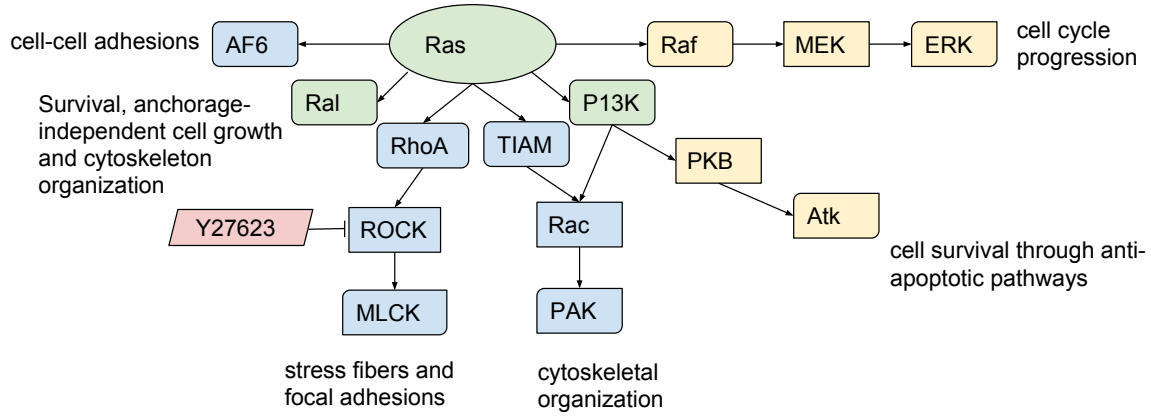


Figure 2.7: Ras Pathways. A few of the effector pathways of Ras controlling cytoskeletal and adhesion structure (blue) and cell proliferation (yellow) are shown. The ROCK inhibitor drug, Y-27632 (red) is also shown.

- Raf: The first Ras effector pathway to be identified was the Raf-MEK-ERK pathway. By altering the levels and activities of transcription factors, this pathway leads to altered transcription of genes that are important for the cell cycle [51, 33]. It plays an important role in cell proliferation and the prevention of apoptosis.

The pathway of particular importance for cell mechanics is the one activating myosin through Rho, Rho-associated protein kinase (ROCK) and myosin light-chain kinase (MLCK). It has been observed that phosphorylation of myosin light chain is enhanced in cells with permanently active Ras^{V12} [26]. The over-activation of this pathway should have different effects on adherent and suspended cells due to their difference in cytoskeletal structure.

Ras^{V12} cells

Previously, a cell line, Ras^{V12}, was created by modifying Madin-Darby canine kidney (MDCK) epithelial cells to express constitutively active Ras in a tetracycline-inducible system [26]. In the absence of tetracycline, they behave like wild type MDCK cells. The addition of tetracycline causes the cells to produce permanently active Ras, transforming

the cell. The cell line was used to study the cellular processes arising at the interface between normal and transformed cells [26]. It was shown that when surrounded by normal cells, Ras^{V12} cells are either apically extruded from the monolayer, or form dynamic basal protrusions and invade the basal matrix. Neither apical extrusion nor basal protrusion formation is observed when Ras^{V12} cells are surrounded by other Ras^{V12} cells. It was shown that ROCK had vital roles in these processes, suppressing apical extrusion. This cell line was used in this thesis.

2.2 Cell Mechanics

The mechanical properties of cells have attracted interest amongst researchers from the very beginning of cell studies [64]. Cells are exposed to, produce and respond to a variety of mechanical stresses *in-vivo* [65, 66]. As material systems, cells are far more complex than engineered materials as they are dynamic and constantly remodeling their structure according to function and external stimuli [67]. Mechanical properties have a large impact on a cell's ability to function. The cell has to be stiff enough to withstand the forces applied to it by its environment, but at the same time it has to be sufficiently flexible to function. For example, if neutrophils trying to transmigrate through the endothelium were too stiff, they would not be able to squeeze through. Or, if the cells making up the heart were too stiff, it would be almost impossible for the heart to beat. Thus, to understand how cells are able to perform whatever their duties are in the organism, we have to understand their mechanical properties and how they are regulated.

2.2.1 Myosin

Myosin is a molecular motor that is a dynamic crosslinker within the cytoskeleton and is necessary to generate migratory and tensile forces within the cell [68]. These molecular

motors can be seen as molecular micro-machines with one or two heads which can walk along F-actin or microtubules using the polarity of the filaments to move in one direction. They can transport cargo within cells along microtubules or slide two actin filaments along each other. Filament sliding is important when the cell needs to change its shape or to exert forces, as it does during cell migration, mitosis, cell contraction, wound closure, and mechanosensing of its environment [68].

In cell mechanics, myosin is mostly associated with actomyosin contractility. The activity of myosin influences the mechanical properties of cells by generating tension along actomyosin stress fibers and by prestressing the actin cortex underlying the plasma membrane. This often leads to a stiffening of the cell. However myosin also affects the fluidity of the cell. Recently there have been findings of networks and filaments under stress quickly fluidizing and re-solidifying afterwards [69, 13]. The explanation of this effect might be an unbinding of crosslinkers under a load and a rebinding to a new position afterwards. The more working active myosin there is, the more filaments can move, and therefore the more compliant and fluid a cell becomes. Such a mechanism has been shown in purified actin gels mixed with inactive myosin [70]. As long as the myosin was inactive, the actin gel did not flow out of an angled cuvette, but as soon as the myosin got activated by ATP addition, the actin gel fluidized and could flow out. This result is similar to actin gliding assays where the speed of single actin filaments *in-vitro* is monitored. Speed decreased significantly after the addition of the myosin inhibitor blebbistatin [71]. It has also been found that increased bending of actin fibers by myosin motors induces actin depolymerization [72, 73, 74].

2.2.2 Mechanosensing

The forces that are sensed by cells lead to specific cell responses. Mechanosensing requires an element that responds to an applied force. Several different structures have been proposed as sensors of force. Classical mechanosensory signaling is based on a transmembrane

system consisting of an ion channel tethered to the cytoskeleton or extracellular matrix [75]. Thus, tension transduced to the channel can modulate local ion transduction, which, in turn, may activate a variety of signaling events [76]. It has been shown that cells probe the external environment locally by applying actomyosin-driven forces to cell-substrate or cell-cell adhesion sites, and these sites respond to that force by changing their size, dynamics, and signaling activity [77].

2.2.3 Cell-Generated Stress

Forces generated within the cell are responsible for the dynamics involved in morphological changes, adhesion processes and motility. The remodeling on cell monolayers is facilitated by cell-generated stress. This can be achieved by activating myosin and placing tension on actin fibers. Contractile forces then are transmitted from the actin cytoskeleton to the ECM via focal adhesions. The magnitude of contractility is coupled with ECM stiffness. Increased substrate stiffness results in increased focal adhesion assembly that drives increased actomyosin contractility [4, 8, 29]. Actin polymerization can also generate forces. The active assembly/disassembly process generates a dynamic linear polymeric chain. In motile cells, forces are generated by branching of dendritic actin networks close to the cell's leading edge [78].

2.2.4 Cellular Response to Stress

Cell signaling results in responses to both intracellular and extracellular forces. For example, compressive forces applied to cell nuclei have been shown to result in altered gene expression [79]. To understand the mechanical properties of the cytoskeleton it is not only necessary to investigate the mechanical properties of the filaments themselves but of the entire cytoskeletal network. Cytoskeletal networks deform differently under the influence of stress. Numerous studies report a stiffening response of networks under stress which can

mostly be explained by a reduction of the possible available configurations of thermally fluctuating filaments within a network when stretched, which is called entropic elasticity [80, 81, 82]. However due to the dynamic nature of the filament structure and crosslinkers, the cytoskeletal response to stress is much more complex. The cumulative effect of the cytoskeletal components control the mechanical properties of the cell. Theoretical models to describe the cytoskeleton as a whole do not exist yet. It is extremely difficult to take every single component into account as well as the connection of the components to the cell membrane and other boundary conditions. An ideal model will have to consider that single filaments might not stay chemically identical during mechanical deformation and the active contribution of molecular motors. However, this level of detail is difficult to work with. Instead, the entire cell is often modeled as a viscoelastic material.

2.2.5 Viscoelasticity

When applying a force to a material, it will respond with deformation. Viscoelastic materials respond in an intermediate form of a purely elastic or a viscous material. The time dependent strain $\epsilon(t) = \frac{\Delta x}{x}$ of a material of linear viscoelastic materials is expressed in terms of stress $\sigma(t) = \frac{F}{A}$ and the creep compliance $J(t)$. In general, for a constant step stress, σ , the creep compliance, $J(t)$, can be given as the ratio of the strain to stress at a given time.

$$J(t) = \frac{\epsilon(t)}{\sigma} \quad (2.1)$$

One way of describing viscoelastic behavior leads to a linear differential equation and is therefore called the differential representation. It uses assemblages of springs and dashpots as models. The spring is used to represent a linear elastic element and a dashpot represents a linear viscous element. These two elements can be arranged in numerous ways to mimic the deformation of the material (Fig 2.8).

For a step stress, a single spring will instantly deform on the onset of force then return

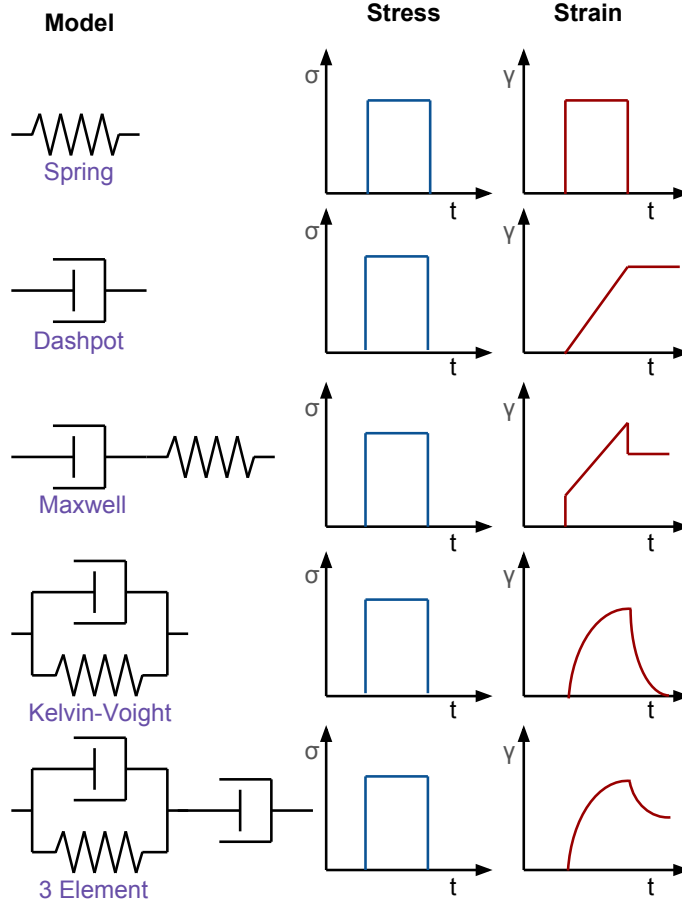


Figure 2.8: Viscoelastic models. A variety of common viscoelastic models and how they respond to a step stress (σ) with different strains (ϵ).

to its original length when the force is removed. A spring can be described with the equation:

$$\sigma = E\epsilon \quad (2.2)$$

where E is the Young's modulus of the spring. A dashpot will deform linearly with the onset of force and remain permanently deformed after the force is removed. A dashpot can be described with the equation:

$$\sigma = \eta \frac{d\epsilon}{dt} \quad (2.3)$$

where η is the viscosity of the dashpot.

A three element model, with a Kelvin-Voigt body in series with a dashpot, is commonly used to model cell deformation. It is also known as the standard linear liquid (SLL) model. The strain in the model can be divided into the strain of the Kelvin-Voigt body, ϵ_{kv} , and the strain of the second dashpot, ϵ_d , with $\epsilon = \epsilon_{kv} + \epsilon_d$. The stresses on the system must balance:

$$\sigma = E\epsilon_{kv} + \eta_1 \frac{d\epsilon_{kv}}{dt} = \eta_2 \frac{d\epsilon_d}{dt} \quad (2.4)$$

where η_1 and η_2 are the viscosities of the two dashpots. Using these differential equations, the strain in each segment stretched with a step stress can be calculated:

$$\sigma = E\epsilon_{kv} + \eta_1 \frac{d\epsilon_{kv}}{dt} \quad (2.5)$$

$$\epsilon_{kv}(t) = \frac{\sigma}{E} \left(1 - e^{-\frac{Et}{\eta_1}} \right) \quad (2.6)$$

$$\sigma = \eta_2 \frac{d\epsilon_d}{dt} \quad (2.7)$$

$$\epsilon_d(t) = \frac{\sigma}{\eta_2} t \quad (2.8)$$

The strain and compliance of the material stretched by a step stress are then:

$$\epsilon(t) = \frac{\sigma}{E} \left(1 - e^{-\frac{Et}{\eta_1}} \right) + \frac{\sigma}{\eta_2} t \quad (2.9)$$

$$J(t) = \frac{\left(1 - e^{-\frac{Et}{\eta_1}} \right)}{E} + \frac{t}{\eta_2} \quad (2.10)$$

After the stress is removed at t_1 , the relaxation of the material can be described as:

$$\epsilon(t) = \frac{\sigma}{E} \left(1 - e^{-\frac{Et_1}{\eta_1}} \right) e^{-\frac{E(t-t_1)}{\eta_1}} + \frac{\sigma}{\eta_2} t_1 \quad (2.11)$$

$$J(t) = \frac{\left(1 - e^{-\frac{Et_1}{\eta_1}} \right)}{E} e^{-\frac{E(t-t_1)}{\eta_1}} + \frac{t_1}{\eta_2} \quad (2.12)$$

η_1 can represent the transient viscosity η_t of the material and η_1 can represent the steady-state viscosity of the material at long timescales η_s . The characteristic timescale at which steady state viscous behavior starts to dominate is $\tau = \eta_t/E$.

2.3 Tools

Estimates of cellular stiffness vary depending on the method used, the type and extent of deformation, and the duration of deformation [64]. One major complication that influences these measurement is inhomogeneity of the cell interior structure. The composition of the cytoskeleton varies spatially and as a function of time. For this reason, mechanical measurements of cells should be interpreted with the method as well as the approximations used in mind.

2.3.1 Atomic Force Microscopy

Atomic force microscopy (AFM) is a local probe technique, designed to measure the interaction forces of a sharp tip with the surface of a sample, making it one of the most popular tools for imaging, measuring and manipulating matter at the nanoscale. The tip is etched out of one end of a thin cantilever, which is fixed at the opposite end to a solid support (Fig. 2.9). As the tip is brought to within interaction-distance of the sample surface, the lever will deflect by an amount proportional to the force experienced by the tip. This deflection is measured by a laser that is focused onto the back of the cantilever. The reflected beam is detected by a four-segment photodiode. The position of the reflected beam on the photodiode is measured to determine the deflection of the cantilever. This is done by comparing the relative intensity of the reflected laser light in each quadrant.

This probe can be used to determine the stiffness of a cell. This is done with a process called force spectroscopy. The tip is brought into contact with the cell and then indents the

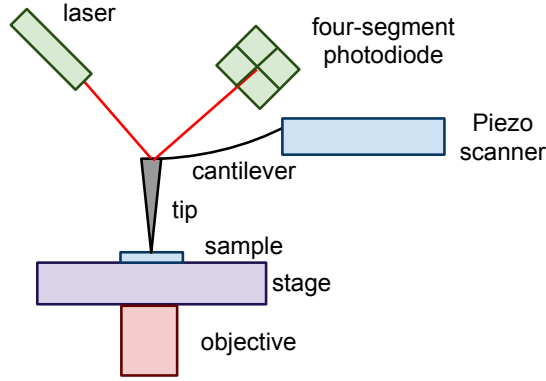


Figure 2.9: Diagram of an atomic force microscope.

cell while recording the force and displacement of the tip. The force versus displacement can be plotted to make force curves that can be fit to calculate an effective Young's modulus.

Force Calibration

When approaching the surface, the interaction force between the tip and the sample is directly proportional to the cantilever deflection. The first stage is to calibrate the distance that the cantilever deflects for a certain measured change in photodetector voltage. The sensitivity of the cantilever is measured by slowly approaching the cantilever tip to a bare hard surface, in order to record the deflection voltage as a function of the piezo height. The slope of the contact portion of the deflection vs distance curve is determined and used as the cantilever's sensitivity. This allows us to determine the deflection (x) in nm by multiplying the voltage recorded by the photodiode by the cantilever's sensitivity.

The spring constant k on the tip is determined with thermal noise. The equipartition theorem states that if a system is in thermal equilibrium every independent quadratic term in its total energy has a mean value equal to $\frac{k_B T}{2}$, where k_B is the Boltzmann constant and T is the temperature. When bending the cantilever by a small amount x its potential energy is $\frac{kx^2}{2}$. The average potential energy would then be $\frac{k\langle x^2 \rangle}{2}$. Making these two

energies equal to each other results in an equation for the spring constant,

$$k = \frac{k_B T}{\langle x^2 \rangle}. \quad (2.13)$$

The mean square amplitude of the cantilever's thermal fluctuations is recorded after the ambient noise is filtered. The area under the remaining peak of the power spectrum represents mean square of thermal fluctuations $\langle x^2 \rangle$ that is used to calculate the spring constant.

Sneddon model

The force curves in this thesis were fit with the Sneddon model [83], a modification of the Hertz model for a conical tip, with an indentation depth of 200nm. This model approximates the sample as an isotropic, elastic solid occupying an infinitely extending half space. The indenter is assumed to be a non-deformable cone. It is also assumed that there are no additional interactions between the tip and the sample. The solution for the equation of force (F) as a function of displacement (δ) is:

$$F = \frac{E}{1 - \nu^2} \frac{2 \tan \alpha_c}{\pi} \delta^2 \quad (2.14)$$

where E is the Young's modulus, ν is the poisson ratio and α_c is the opening angle of the cone. The poisson's ratio is generally set to 0.5. While this method uses a lot of non-valid assumptions, it is the widely excepted model for calculating Young's moduli of cells with atomic force microscopy. It is important to note that it is really an apparent elastic modulus that is being calculated and should only be used to compare moduli of cells calculated with the same technique. Cells are also not homogeneous or purely elastic. The viscosity of the sample, as well as the speed of the indentation of the sample, will effect the measurement. Higher loading rates will produce smaller indentations and will give a higher

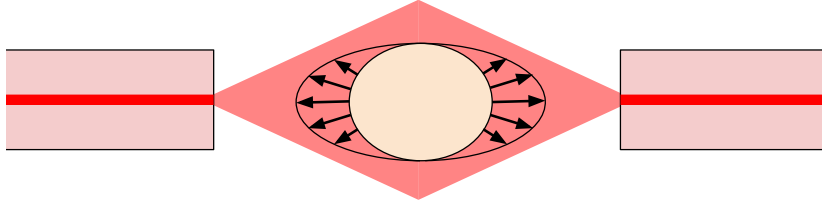


Figure 2.10: Diagram of an optical stretcher. Two opposing divergent laser beams are used to trap and stretch a cell along the beam axis.

apparent stiffness. If the loading rate is too low, the stress can lead to reorganization of the cell (within the timeframe of loading) also providing different moduli. The contact point will also effect the modulus measurement due to the inhomogeneity of the sample. All of these factors make the apparent moduli found in force spectroscopy only valid for comparative studies.

2.3.2 Optical Stretcher

The optical stretcher is a recent development in the evolution of optical traps. In the optical stretcher, two opposing divergent laser beams are used to trap the cell (Fig. 2.10). Increasing the power of the laser light stretches the cell along the laser beam axis.

Ray Optics Model

The stretching of the cell is caused by a momentum transfer of the light onto the cellular membrane and can be quantitatively explained in a ray optics approach (Fig. 2.11). Each ray of light of energy, E_l , carries momentum, p , of the form

$$p = \frac{n_1 E_l}{c} \quad (2.15)$$

where n_1 is the refractive index of the surrounding medium and c is the speed of light in vacuum. When a ray of light with momentum p_i hits the surface of another medium under

an angle α it will be refracted according to Snell's law

$$n_1 \sin(\alpha) = n_2 \sin(\beta) \quad (2.16)$$

where n_2 is the refractive index of the second medium and β is the refracted rays angle.

The refraction coefficient for this interaction would be

$$R(\alpha) = \frac{r_{\parallel}^2(\alpha) + r_{\perp}^2(\alpha)}{2} \quad (2.17)$$

with the fresnel coefficients

$$r_{\parallel}(\alpha) = \frac{n_1 \cos \alpha - n_2 \sqrt{1 - \left(\frac{n_1}{n_2} \sin \alpha\right)^2}}{n_1 \cos \alpha + n_2 \sqrt{1 - \left(\frac{n_1}{n_2} \sin \alpha\right)^2}} \quad (2.18)$$

$$r_{\perp}(\alpha) = \frac{n_2 \cos \alpha - n_1 \sqrt{1 - \left(\frac{n_1}{n_2} \sin \alpha\right)^2}}{n_2 \cos \alpha + n_1 \sqrt{1 - \left(\frac{n_1}{n_2} \sin \alpha\right)^2}} \quad (2.19)$$

When the light hits the front side of the cell, the magnitudes of the momentum of the initial light ray p_i , the transmitted light ray p_t and the reflected light ray p_r are given by

$$p_i = \frac{n_1 E_l}{c} \quad (2.20)$$

$$p_t = \frac{n_2 E_l}{c} (1 - R(\alpha)) \quad (2.21)$$

$$p_r = \frac{n_1 E_l}{c} R(\alpha) \quad (2.22)$$

The transmitted ray is again reflected and transmitted at the back side of the cell (Figure 2.11). For this process, the magnitudes of momentum of initial light ray p_{i2} , the transmitted

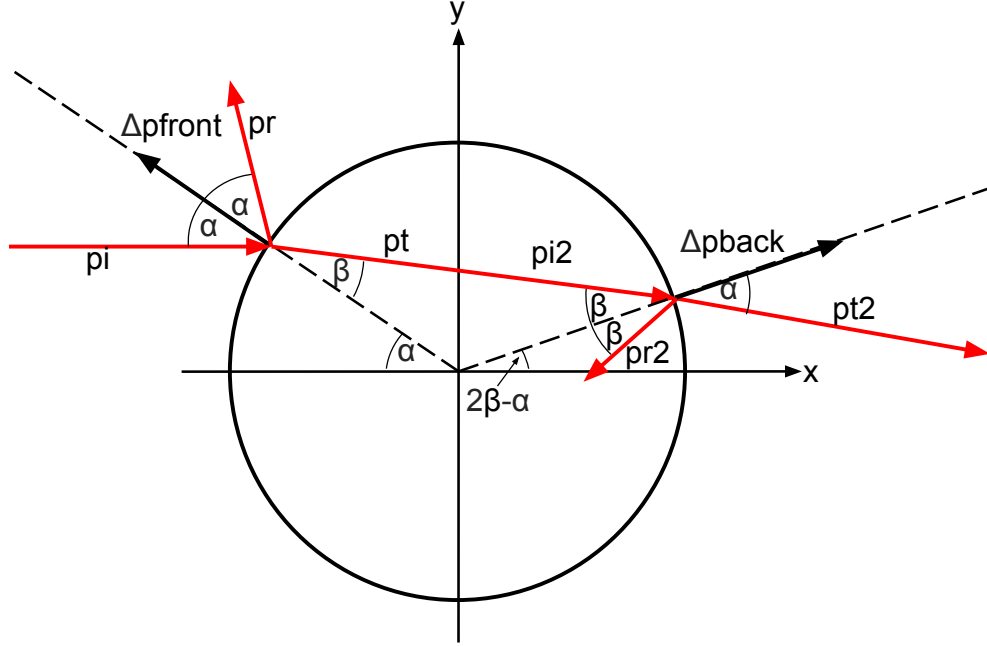


Figure 2.11: Propagation of a ray through a cell. A ray hitting the front of the cell at angle α causes a momentum transfer of $\Delta p_{front}(\alpha)$ on the front side of the cell. After propagating through the cell it causes a momentum transfer of $\Delta p_{back}(\alpha)$.

light ray p_{t2} and the reflected light ray p_{r2} are

$$p_{i2} = p_t \quad (2.23)$$

$$p_{t2} = p_t(1 - R(\beta)) \quad (2.24)$$

$$p_{r2} = p_t R(\beta) \quad (2.25)$$

Conservation of momentum for reflection and transmission of the light on the front and back side of the cell yields

$$\Delta \vec{p} = \vec{p}_i - \vec{p}_r - \vec{p}_t \quad (2.26)$$

with

$$\Delta p_{x,front} = p_i + p_r \cos 2\alpha - p_t \cos(\alpha - \beta) \quad (2.27)$$

$$\Delta p_{y,front} = -p_r \sin 2\alpha + p_t \sin(\alpha - \beta) \quad (2.28)$$

$$\Delta p_{x,back} = p_t \cos(\alpha - \beta) + p_{r2} \cos(3\beta - \alpha) - p_{t2} \cos(2(\alpha - \beta)) \quad (2.29)$$

$$\Delta p_{y,back} = -p_t \sin(\alpha - \beta) + p_{r2} \sin(3\beta - \alpha) + p_{t2} \sin(2(\alpha - \beta)) \quad (2.30)$$

The magnitude of the transferred momentum on the front and back side of the cell are then

$$\Delta p_{front} = \sqrt{\Delta p_{x,front}^2 + \Delta p_{y,front}^2} \quad (2.31)$$

$$\Delta p_{back} = \sqrt{\Delta p_{x,back}^2 + \Delta p_{y,back}^2} \quad (2.32)$$

The direction of momentum transfer is always normal to the surface. Since there are two counter propagating laser beams used in the optical stretcher, a ray hitting the front of the cell at angle α causes a momentum transfer in the same direction as the momentum transfer from the opposite laser beam hitting the back of the cell at incident angle $\phi(\alpha)$ (Fig. 2.12). Due to symmetry of the two beams used in the optical stretcher, the magnitude of the total amount of transferred momentum on either side of the cell is

$$\Delta p(\alpha) = \Delta p_{front}(\alpha) + \Delta p_{back}(\phi(\alpha)) \quad (2.33)$$

The total transferred momentum creates a surface stress $\sigma(\alpha)$ that is expressed by

$$\sigma(\alpha) = \frac{F(\alpha)}{A} = \frac{\Delta p(\alpha)}{\Delta t A} = \frac{\Delta p(\alpha)}{E_l} \frac{E_l}{\Delta t A} = \frac{\Delta p(\alpha)}{E_l} I \quad (2.34)$$

where I is the intensity of light hitting the cell. It has a gaussian profile with the beam width calculated with the distance between the fiber end and the cell. The peak stress on

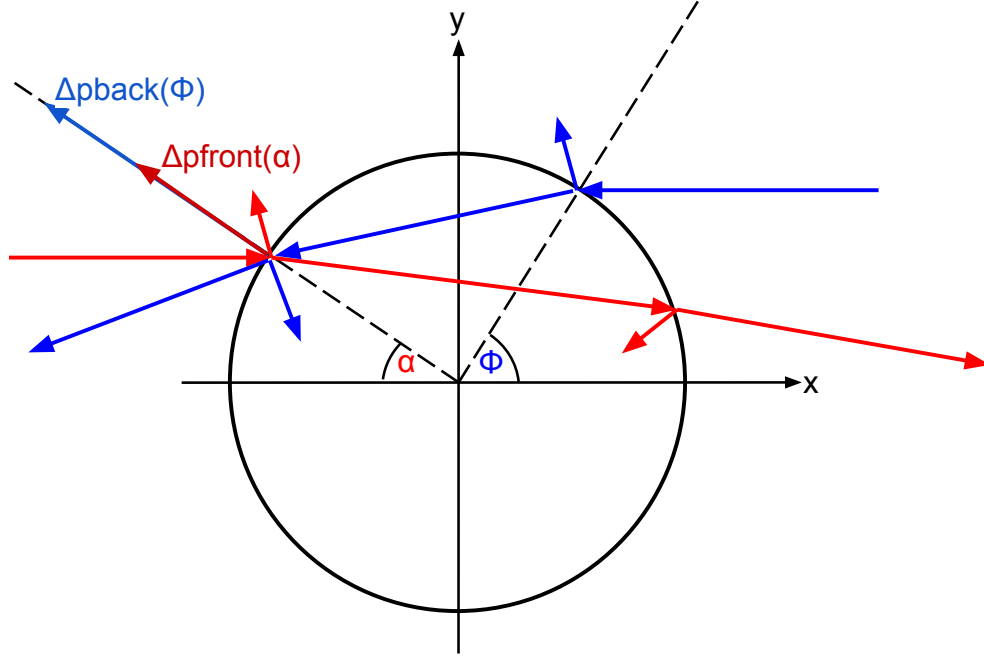


Figure 2.12: Propagation of a two rays through a cell. Two counter propagating laser beams are used in the optical stretcher. A ray hitting the front of the cell (red) at angle α causes a momentum transfer of $\Delta p_{front}(\alpha)$ on the front side of the cell. A ray from the opposite laser beam (blue) hits the back of the cell at incident angle ϕ . After propagating through the cell it causes a momentum transfer of $\Delta p_{back}(\phi(\alpha))$.

the cell, σ_0 the stress at $\alpha = 0$ would be:

$$\begin{aligned}\sigma(0) &= \frac{n_1 I}{c} (R - 2 - R^2) \left(1 - \frac{n_2}{n_1}\right) \\ \sigma_0 &= \frac{n_1 I}{c} \left(\left(\frac{n_1 - n_2}{n_1 + n_2} \right)^2 - 2 - \left(\frac{n_1 - n_2}{n_1 + n_2} \right)^4 \right) \left(1 - \frac{n_2}{n_1}\right)\end{aligned}\quad (2.35)$$

For a set up that involves the light passing through gel, a glass capillary then media, the beam width at the cell would be [84]:

$$w = w_0 \sqrt{1 + \frac{\left(\frac{z_{med}}{n_{med}} + \frac{z_{glass}}{n_{glass}} + \frac{z_{gel}}{n_{gel}} \right)^2 \lambda^2}{\pi^2 w_0^4}} \quad (2.36)$$

where w_0 is the beam width when it leaves the fiber, the n s are the refractive indices

of each medium, the z s are the distance traveled in each medium and λ is the wavelength of the beam.

The peak stress can be calculated using the stress on the x axis ($\alpha = 0$). A geometric factor (F_G) can be calculated taking into account the cell's size and stress distribution in order to get the creep compliance, described in [85].

$$J(t) = \frac{\epsilon(t)}{\sigma_0 F_G} \quad (2.37)$$

Application

For the purpose of this research the optical stretcher has some significant advantages over other cell deformation techniques. The distribution of stress applied to the cell is broad and continuous. This global stress application is in contrast to the local deformations achieved with atomic force microscopy. The optical stretcher is capable of capturing effects that are reflected in the entire state of the cytoskeleton. This makes the optical stretcher a cumulative measure of various mechanical changes that affect the cytoskeleton. A further advantage is that a suspended cell is held in the trap by optical forces and the cell is stretched without any mechanical contact. However, the laser might introduce heating effects that can impact cell mechanics [86]. Measuring cells in the non-adhered state circumvents all possible artifacts of surface attachment, such as focal adhesions and stress fibers. This all makes the optical stretcher a suitable and appealing tool for the investigation of the mechanical properties of cells.

2.3.3 Optical Microscopy

Fluorescence Microscopy

Fluorescence microscopy is a rapidly advancing microscopy technique, and is especially popular for cell imaging. Cellular components can be difficult to view unless they are

labeled with fluorescent components called fluorophores. These fluorophores are widely used by biologists and their protocols are well known. The fluorophores absorb excitation light, and emit light at a longer wavelength. By separating the emission light from the excitation light prior to detection, the locations of the fluorophore-labelled components are revealed.

Fluorescent proteins are normally minimally-invasive natural proteins that can be used at physiological concentrations in the host cell or organism. Genetically-encoded fluorescent proteins allow virtually any non-fluorescent protein of interest to be made to fluoresce by the introduction of the fluorescent protein codon into its own coding sequence. Fluorescent proteins are very stable, being unaffected by fixation or large temperature changes. They are coded for by a single gene, so are easy to transfect into the host organism, require no post-transcriptional modification or exogenous co-factors to work, and are directly visible with any fluorescence microscope.

Scanning Confocal Microscopy

The principal disadvantage of fluorescence microscopy is the out-of-focus signal from samples. Confocal optical microscopy is a technique for increasing the contrast of microscope images. By restricting the observed volume, the technique keeps overlying or nearby scatterers from contributing to the detected signal. The instrument must observe only one point at a time. The term confocal is used because the illumination pinhole, the plane of focus within the specimen and the detector pinhole are all situated at conjugate focal planes (Fig. 2.13). By restricting the illumination to a pinhole, rather than illuminating the entire field of view at once, it becomes possible to build up a sharp image in three dimensions. These sections through the cell or tissue can be collected non-invasively.

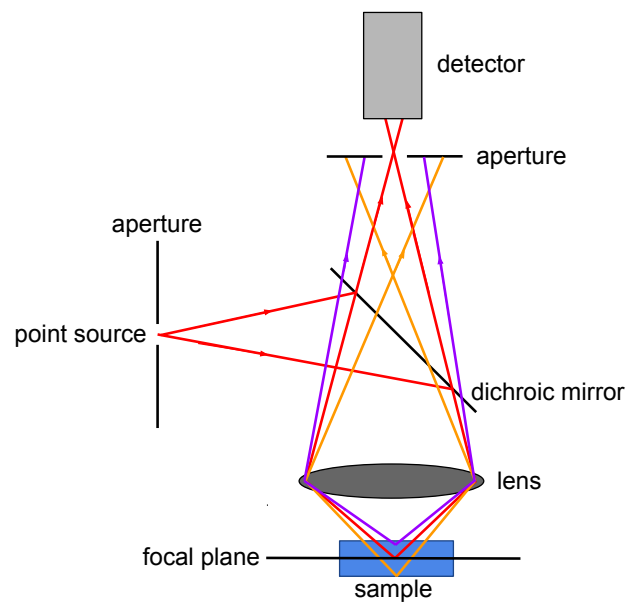


Figure 2.13: Diagram of a confocal microscope. Pinholes are used to collect the desired signal (red) from a single point. It is used to prevent out of focus light (orange and purple), from reaching the detector.

Chapter 3

Experimental Methods

3.1 Cell Culture

In Chapters 4 and 5, cells were cultured according to the following protocol: epithelial cells were cultured in DMEM with 10% fetal bovine serum (FBS), 50mg/ml streptomycin and 50U/ml penicillin antibiotics (all from Hyclone Laboratories Inc.). Cells were cultured at 37°C in a 5% CO₂ incubator on 100mm tissue culture dishes (Corning). Two cell lines were used for this thesis, Madin-Darby Canine Kidney (MDCK) epithelial cells as well as a modified line of MDCK cells, Ras^{V12} [26]. The Ras^{V12} line is a stable MDCK cell line expressing GFP-Ras^{V12}, in a tetracycline-inducible manner. The Ras^{V12} line was a kind gift of Yasuyuki Fujita (Hokkaido Univ) described here [26]. Samples were either grown in mono-culture or in co-culture with a ratio of 1:10 Ras^{V12} to wild type (WT) MDCK cells to create monolayers. The Ras^{V12} cells were activated with tetracycline at a concentration of 2μg/ml and experiments were performed 1 day later.

3.1.1 Drug Treatments

In Chapter 4, the inhibitor Y-27632 was used at a concentration of $10\mu\text{M}$ in a 1 day treatment. In Chapter 5, cells were treated with blebbistatin (Sigma) dissolved in DMSO at a final concentration of $40\mu\text{M}$ or calyculin A (Sigma) dissolved in water at a final concentration of 300nM for 30 minutes in an incubator at 37°C and 5% CO_2 between before and after images.

3.1.2 Solid Substrate Cell Culture

In some experiments in Chapter 5, cells were cultured on solid substrates. In these cases, 5×10^4 cells/ml were cultured in 34mm tissue culture dishes (TPP). The dishes either contained a flexible polyacrylamide hydrogel coating [87, 88, 89] or a bare glass coverslip bottom. In order to immobilize the hydrogels, glass coverslips were coated with (3-aminopropyl)trimethoxysilane to add reactive amino residues. These residues were then coupled with 0.5% glutaraldehyde to the amino groups in the polyacrylamide gel. The hydrogels were polymerized on top of the coverslips with 7.5% acrylamide (Biorad) and 0.053% bisacrylamide (Biorad) to produce a stiffness of 4.8kPa [90]. 1mg/ml ammonium persulfate (Biorad) and 0.15% tetramethylethylenediamine (Biorad) assisted in the polymerization as a source of reactive oxygen species (ROS) and a catalyst for ROS formation, respectively. The hydrogels were functionalized using 0.5mg/mL sulfo-sanpah (Pierce) as a crosslinker between the amino residues on the polyacrylamide surface and $100\mu\text{g/mL}$ collagen (Gibco) in phosphate buffered saline (PBS).

3.2 Confocal Imaging

Confocal imaging was employed in Chapters 4 and 5. Confocal images were acquired on a TiE A1-R laser scanning confocal microscope (LSCM) (Nikon). Images were acquired

with a standard LSCM configuration with appropriate laser lines and filter blocks.

3.2.1 Fixed Staining

Stained images were acquired with a 60X objective. Cells were fixed with 3.5% paraformaldehyde and permeabilized with 0.1% Triton X-100 at 37°C. DNA was labeled with DAPI (Invitrogen). Actin was stained with Phalloidin Alexa Fluor 546 (Invitrogen). In Chapter 4 actin signal intensities at cell boundaries were measured in ImageJ with line segments placed over cell margins in images of MDCK WT:Ras^{V12} co-cultures. The boundaries were either between two Ras^{V12} cells (Ras^{V12}:Ras^{V12}, mean of 11 boundaries per image), a Ras^{V12} cell and a neighboring WT cell (Ras^{V12}:WT, mean of 28 boundaries per image) or the neighboring WT cell and another WT cell (WT:WT, mean of 41 boundaries per image). The average intensity over each boundary type was calculated for each image (5 images per condition) and divided by the average intensity of the WT:WT boarder. In chapter 5 vinculin was also stained with monoclonal anti-vinculin primary antibody (Sigma) and Alexa Fluor 488 rabbit anti-mouse immunoglobulin (Invitrogen) secondary antibody.

3.2.2 Live Staining

In Chapter 5, images of live cells were acquired with a 40X long working distance objective. The hanging drops were imaged with a confocal Microscope. Cells were stained with Hoechst at a final concentration of 1 μ l/ml. In some cases, cells expressing GFP-actin were produced with BacMam reagent (Invotrogen, CellLight Reagent BacMam 2.0) using manufacturer protocols at 20 particles per cell. Cells were plated and incubated for at least 48h before being placed into hanging drops. Cells were also stained with wheat germ agglutinin coupled to Texas Red-X (Invitrogen) to reveal the cell membrane.

3.3 Atomic Force Microscopy

Atomic force microscopy was employed in Chapter 4, to measure the apparent stiffnesses of Ras transformed and untransformed cells.

3.3.1 Set up

A NanoWizard II (JPK Instruments) AFM was used. The AFM was mounted on a TiE A1-R laser scanning confocal microscope (Nikon). All experiments were performed at 37°C, using a temperature-controlled AFM stage (JPK). The thermal fluctuation method was used to calibrate AFM tips, as described in section 2.3.1. The sensitivity and stiffness was measured prior to experiments. All AFM cantilevers had an experimentally determined stiffness (68 ± 1 pN/nm. PNP-TR triangular, Nanoworld). Cells were grown in a monolayer on glass bottomed dishes coated with rat tail collagen. Young's modulus (E) was determined by recording 10 force-curves ($5 \mu\text{m/s}$ indentation rate, 1 nN set point) per cell measured over the center of cell nuclei.

3.3.2 Data Analysis

The force-curves were fit to the Sneddon model, a modification of the Hertz model for a conical tip, with 200 nm indentations in order to derive the local Young's modulus (PUNIAS Software). An example force curve is shown in figure 3.1.

3.4 Digital Holographic Microscope

Digital Holographic Microscopy (DHM) was employed in Chapter 4. Wild type and Ras^{V12} cells were trypsinized and resuspended in PBS. The PBS has a known refractive index of 1.335, determined by a refractometer (2WAJ, Arcarda GmbH). The cells in suspension

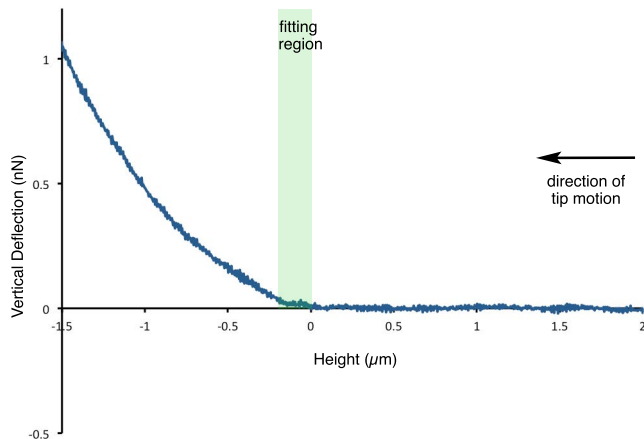


Figure 3.1: Example force-curve showing the approach of cantilever tip. The first 200nm of indentation is highlighted in green.

were put on a glass cover slip coated with poly-l-lysine that was used to produce a charge-based attachment for the cells. Images of the cells were taken with a Digital Holographic Microscope a short time period after settling, so that they retained the spherical shape from the suspended state. The setup and image analysis is described in detail here [91]. In short, the hologram image was used to determine the quantitative phase shift of light induced by the sample. A circle was fit to the contour of the cell and the cell was assumed to be a sphere to determine the height of the sample for each pixel. This was used to get the average refractive index of the cell. An example of a processed image is shown in Figure 3.2.

3.5 Optical Stretcher

Optical stretching was employed in Chapter 4. An optical stretcher is a dual beam laser trap capable of trapping and deforming cells through optically induced stress acting on the cell surface. The microfluidic flow chamber consists of a square glass capillary, aligned perpendicular to two single mode optical fibers, which delivers the cells in suspension. Cells were stretched within one hour of being detached from a culture dish with 0.05% trypsin

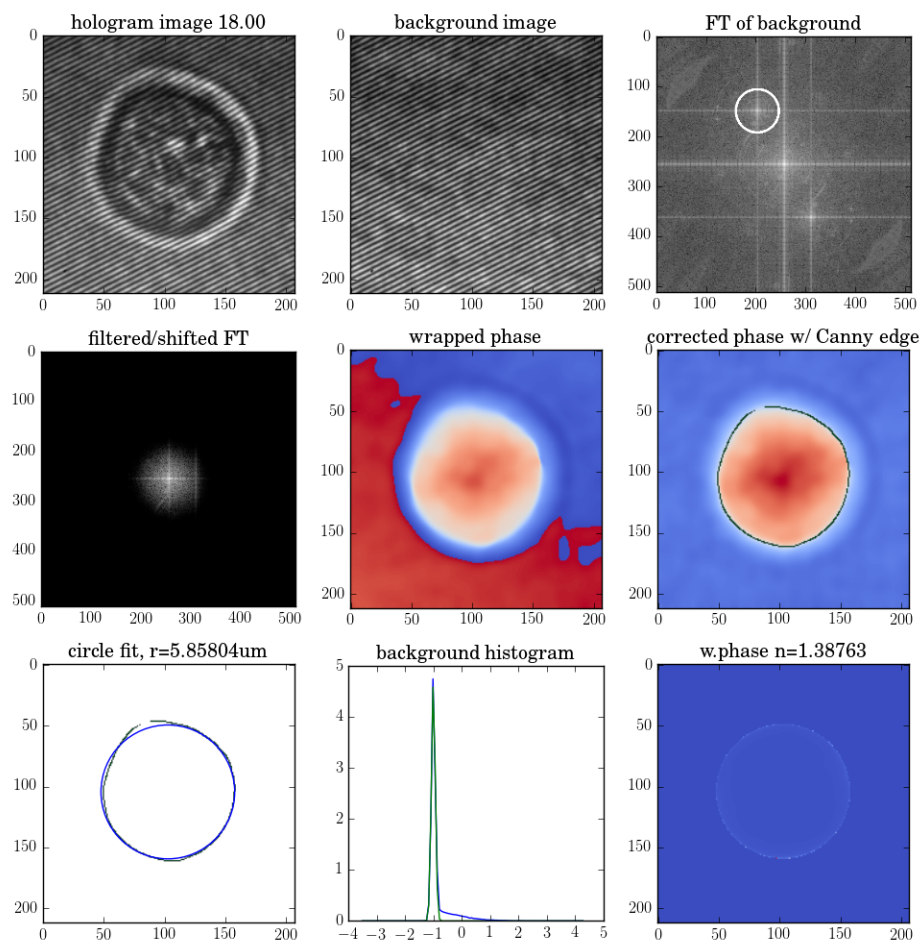


Figure 3.2: Image processing of a digital holographic microscope image.

(enzyme-based dissociative reagent). Single cells were trapped with the laser at low power (0.3W) and held for 2 seconds to stabilize the cell. Subsequently, the cells were stretched at high power (1.5W) for 4 seconds.

3.5.1 Set up

Two optical fibers were embedded into a microfluidic device along with a glass capillary used to flow cells into the laser beams. This was sitting on an optical microscope used to collect images. An image of the full set up can be seen in Figure 3.3.

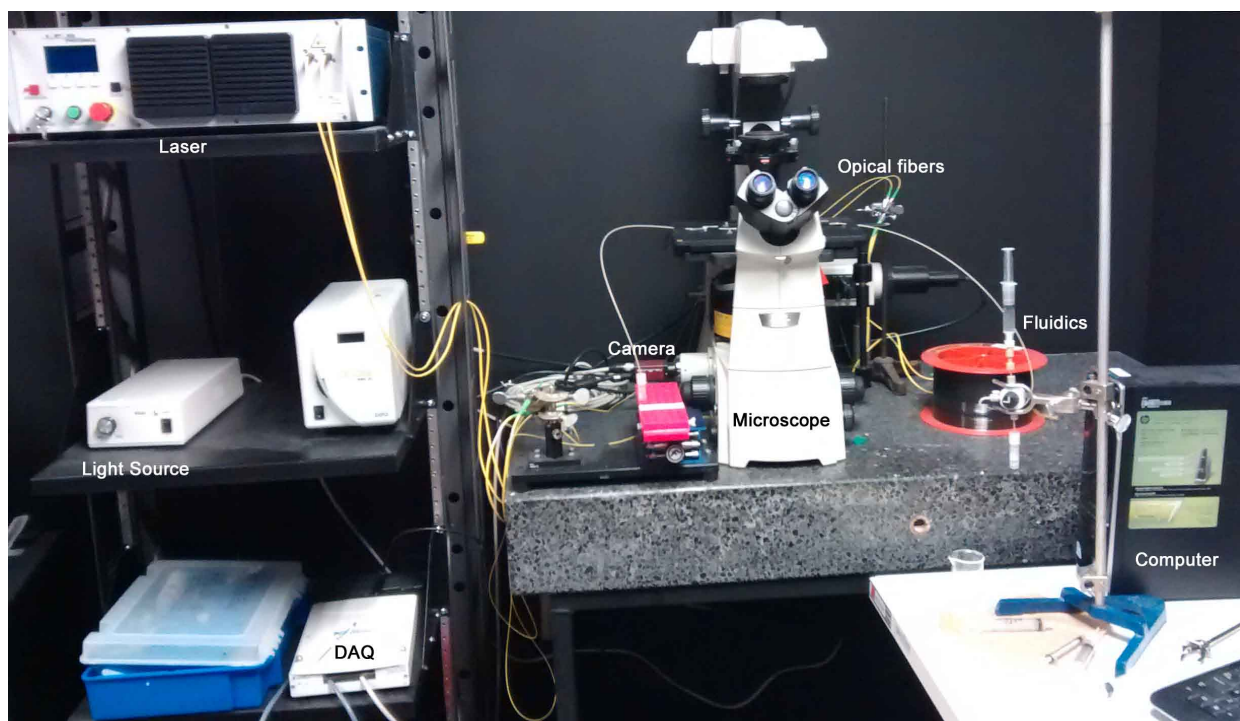


Figure 3.3: Image of the optical stretching set up. The laser, light source, DAQ board, camera (red box), microscope, fluidics (beige tubing) and optical fibers (yellow) and computer are marked.

Laser

The laser used for this process was operating at a wavelength of $\lambda = 1064$ nm. The laser was CW (continuous wave) Yb Fiber Laser Module (YLM-5-1070, IPG Photonics). There was an output of two optical fibers with a max power of 2.5W each. To avoid damage to the laser in the event of a fiber fuse event, the fibers passed through an optical isolator and then a long coil of fiber before reaching the device. The bare ends of the fibers were stripped, cleaved and placed in the optical stretcher holder as described below. The analog voltage control and reference signal of the laser were connected to a data acquisition (DAQ) device (NI-USB-6229, National Instruments). This allowed for control of the laser output power on a computer using custom-built software programmed in LabVIEW (National Instruments). In order to convert the lasers voltage signal into power, the power was measured with a power meter at several voltages and fit linearly (Figure 3.4).

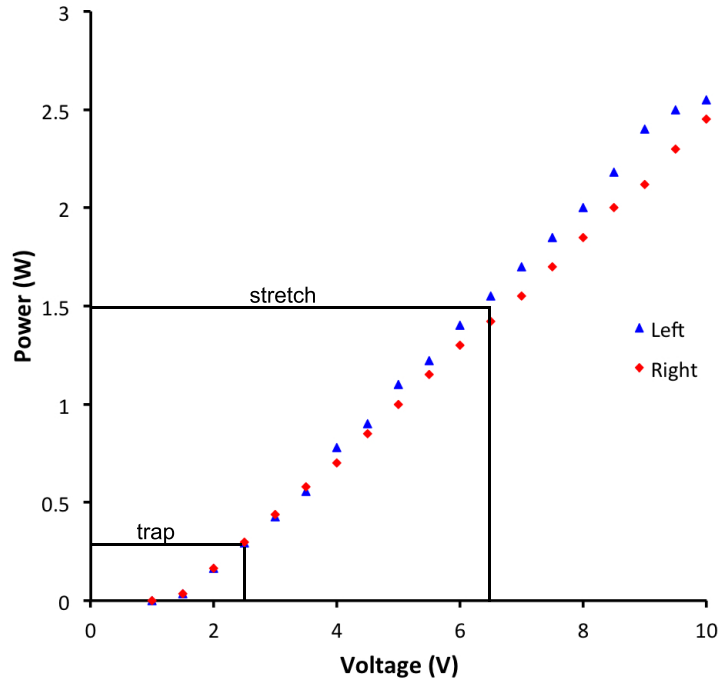


Figure 3.4: Laser power of the left and right fibers versus voltage. The trapping and stretching powers are marked.

Holder and Alignment

Proper alignment of fibers is crucial for a proper force distribution on the cell. In order to precisely align the fibers an SU-8 holder was created. SU-8 was spin-coated on a glass microscopy slide to a thickness of $75\mu\text{m}$. A photomask with 140 and $180\mu\text{m}$ channels was used (Fig. 3.5). This holder is a modified version of design described by Lincoln et al. in [84] where the channels were 110 and $180\mu\text{m}$ wide with a height of $35\mu\text{m}$. With this negative photoresist, only the non-channel parts of the holder are cured and the SU-8 in the channels can be washed away. The $180\mu\text{m}$ channel was used to hold the $160\mu\text{m}$ glass capillary. The photomask was made with wider channels than what was needed to hold the capillary because the photoresist process tended to produce channels smaller than the photoresist. Similarly the $140\mu\text{m}$ channel was used to hold the $125\mu\text{m}$ diameter optical fibers. The optical fibers sat in the channel and were positioned to be about $240\mu\text{m}$ apart (Figure 3.6). This produces a beam width of $9.8\mu\text{m}$ at the cell. The actual distance was measured prior to each experiment to calculate the stress on the cell. A flat sheet of Polydimethylsiloxane (PDMS) with a hole punched out was positioned on top of the glass capillary and optical fibers with the hole in the imaging region. The hole was filled with index matching gel (Thorlabs G608N, $n=1.449$) and topped with a glass coverslip. The index matching gel was chosen for its refractive index similar to glass, to minimize reflection, and its low absorption at 1064nm , to minimize heat absorption. The PDMS was then clamped down. This was to make sure the glass capillary and optical fibers were sitting at the bottom of the channels. It was for this reason that a SU-8 thickness of $75\mu\text{m}$ was chosen, so that it could position the capillary and fibers while allowing for the PDMS to hold clamp them down.

With this set up, both the glass capillary and the optical fibers should be sitting directly on the glass slide (the bottom of the channels). The Lincoln et al. holder was designed with its optical fiber channel ($110\mu\text{m}$) being smaller than the diameter of the fiber. This

kept the optical fibers $2.2\mu\text{m}$ above the glass slide. We chose a wider channel ($140\mu\text{m}$) to lower the trapping area and make the trapping height independent of SU-8 thickness and channel width. The fibers have a diameter of $125\mu\text{m}$, meaning the laser should be centered at $62.5\mu\text{m}$ above the glass slide. The glass capillary has a thickness of $40\mu\text{m}$, so the laser should be centered $22.5\mu\text{m}$ above the bottom of the channel. The average diameter of the cells used was $14\mu\text{m}$ and the beam width of the laser was around $9\mu\text{m}$ at the cell. The cells travelled close to the bottom of the channel and with this set up, were pulled up into the beam at trapping power.

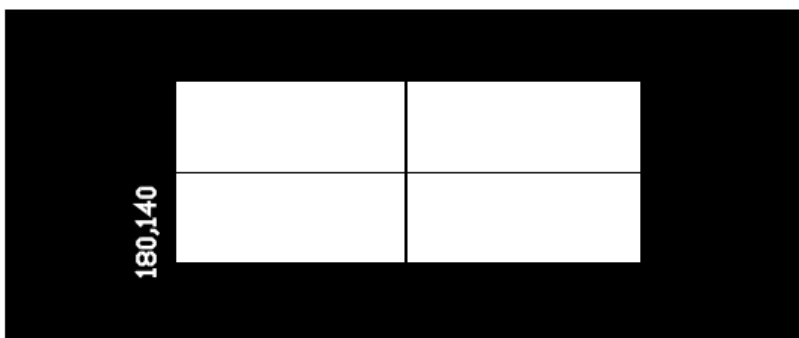


Figure 3.5: Photomask used to make the OS holder. Channels are 140 and $180\mu\text{m}$.

Imaging Optics

The holder was sitting on a inverted microscope (Nikon TiE) equipped for both phase contrast and fluorescence microscopy. A camera (Allied Vision Stingray) attached to the microscope is used to take images at 16fps.

Imaging Media

MDCK and Ras^{V12} cells were dissociated and resuspended in PBS, with a refractive index of 1.335. It has previously been found that cells in media contract above a critical temperature [92]. This contraction is believed to be caused by calcium influx and is not observed when cells are suspended in PBS. For this reason PBS was used instead of media.

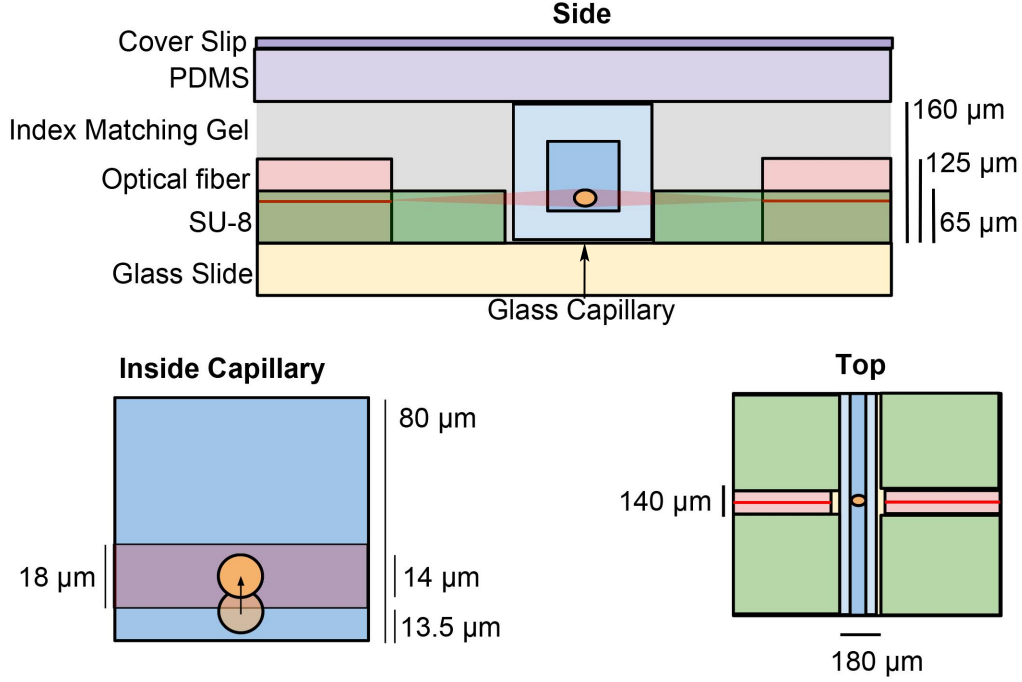


Figure 3.6: Views of the optical stretching chamber. Top: Diagram of a cell trapped by the laser beams. The materials are labeled and the heights of the glass capillary, optical fiber and SU-8 layer are shown on the right. Left: Diagram showing the movement of a cell at the bottom of the capillary being trapped by the laser. The beam waist is shown on the left and the capillary height, cell diameter and height below the laser beam are shown on the right. Right: Diagram of the chamber from above. Widths of the SU-8 channels are shown.

Fluidics

Cells were delivered serially into the trap through a microfluidic channel. The setup used for this thesis consisted of a square glass capillary tube with an $80\ \mu\text{m}$ inner width and a wall thickness of $40\ \mu\text{m}$. The square capillary was connected to Polyetheretherketone (PEEK) tubing (inner diameter $254\ \mu\text{m}$) with PDMS acting as glue (Fig. 3.7A). The PEEK tubing was connected to a reservoir on one side and a T junction with another reservoir and a syringe on the other (Fig. 3.7B). For ease of assembly and cleaning, the PEEK tubing connected to itself with a flexible tubing connector. During the experiment, the T junction was set to reservoir and the cell speed was controlled by changing the relative heights of the two reservoirs. With this method, the flow was stopped when a single cell entered the

trapping area. When loading the cells and during cleaning, the T junction was set to the syringe to flow through at larger speeds. Before each experiment, it was washed with PBS. After an experiment was performed, the tubing was washed with PBS then ethanol.

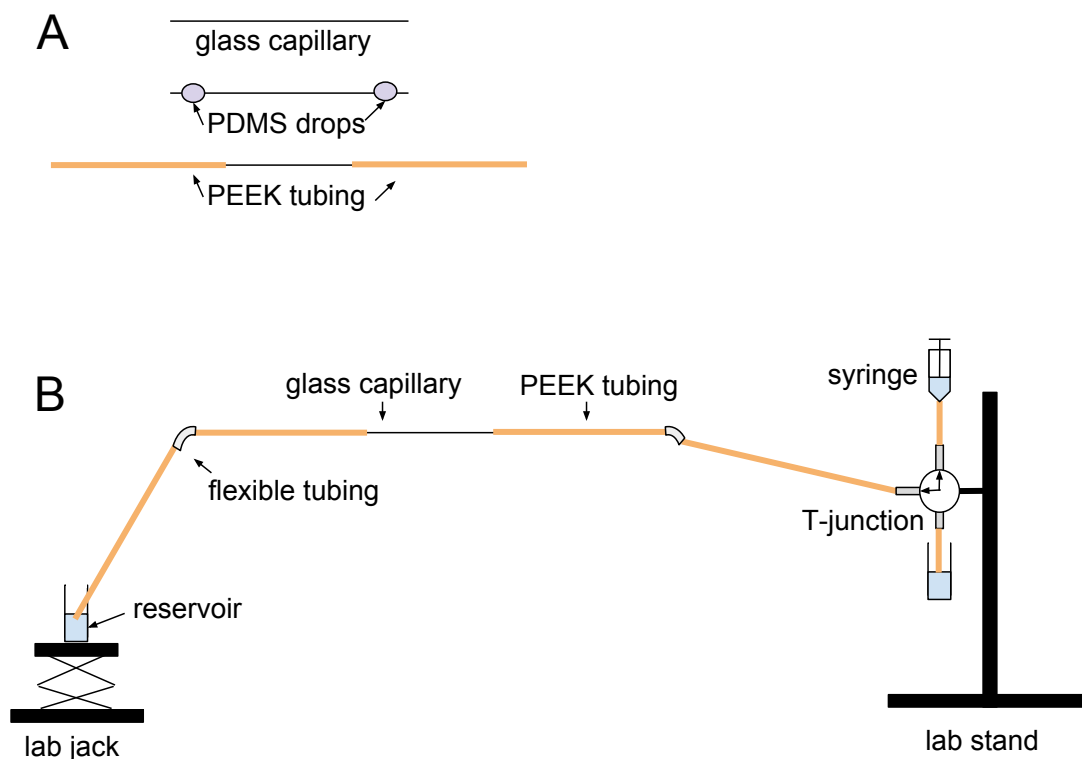


Figure 3.7: Fluidic set-up of the optical stretcher. A) The glass capillary is connected to the PEEK tubing by putting drops of PDMS on the capillary then inserting the capillary into pieces of PEEK tubing. The PDMS is allowed to cure at room temperature before proceeding with assembly. B) The capillary is then connected to the rest of the fluidic system. One side is connected to a reservoir on a lab jack. Its height is used for flow control. The other side is connected to both a syringe and a reservoir by a T-junction. It is connected to the syringe for loading cells and cleaning and the reservoir for flow control.

Labview Program

The power was controlled and images were collected via a custom Labview program. Once a cell was in position and ready to be stretched a stretching program was initiated. The program turned on the trapping power and started saving images. After 2 seconds it

switched to stretching power and returned to trapping power after another 4 seconds. After 5 more seconds it turned off the laser and stops saving images. Each of these images had the laser power in the filename and each run was saved to a separate folder.

3.5.2 Data Analysis

Image Fitting

In order to measure the deformation of the cell, the cell needed to be fit to an ellipse. This was done in MATLAB for each image in a video of the cell being stretched (Fig. 3.8). First the contrast was adjusted using the function *adapthisteq* (Fig. 3.8B). Using a low clip limit, it was used to enhance the contrast of the grayscale image by transforming the values using contrast-limited adaptive histogram equalization. After this, the cell was converted to a binary image using *im2bw* (Fig. 3.8C). The thresholding value was adjusted for each video manually for the best fit. The image was then inverted using *imcomplement* (Fig. 3.8D). The holes in the image were then filled in with *imfill* (Fig. 3.8E). For smoothing, *imopen* was used. It smooths the contour, breaks narrow isthmuses, and eliminates thin protrusions by the process of erosion followed by dilation (Fig. 3.8F). Lastly non-cell objects were removed with *bwareaopen* (Fig. 3.8G). It removes all connected components (objects) that have fewer than 200 pixels. The binary image was then analyzed with *regionprops*. If for some reason multiple regions were detected, regions with areas outside an acceptable range were rejected by the MATLAB program and the region with the smallest eccentricity was used for data analysis.

Strain Calculations

The image fitting process outputs the major axis length, minor axis length and orientation of the fitted cell. From this, the length of the cell in the x direction (parallel to laser propagation) was calculated, however in most cases it was the major axis length. The

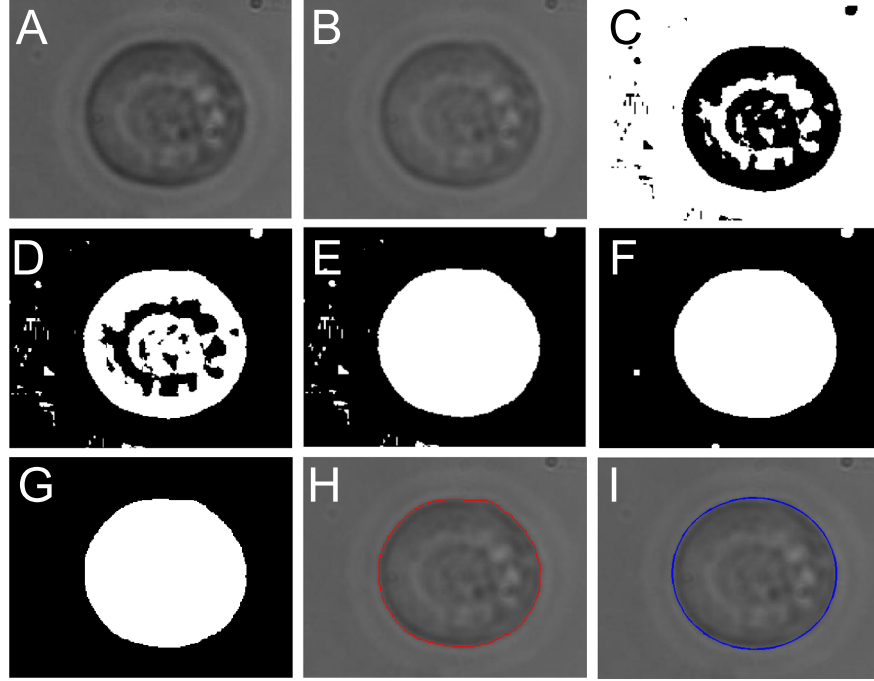


Figure 3.8: Image of a cell in the optical stretcher fitting process. A) Before image B) Image after adapthisteq C) Image after im2bw D) Image after imcomplement E) Image after imfill F) Image after imopen G) bwareaopen H) Image with the cell outline overlaid I) Image with cell fit to an ellipse overlaid.

deformation in the x direction is the most useful, because it should deform roughly twice as much as the y and z directions. In order to convert the length of the cell into a strain, the original length x_0 of the cell was needed. It was calculated using the lengths of the cell in the last 5 frames before stretching started. The strain ($\epsilon(t)$) at each time point was then calculated using change in x length of the cell and used in further data analysis.

$$\epsilon(t) = \frac{(x(t) - x_0)}{x_0} \quad (3.1)$$

An example of an $\epsilon(t)$ graph is displayed in Fig. 3.9.

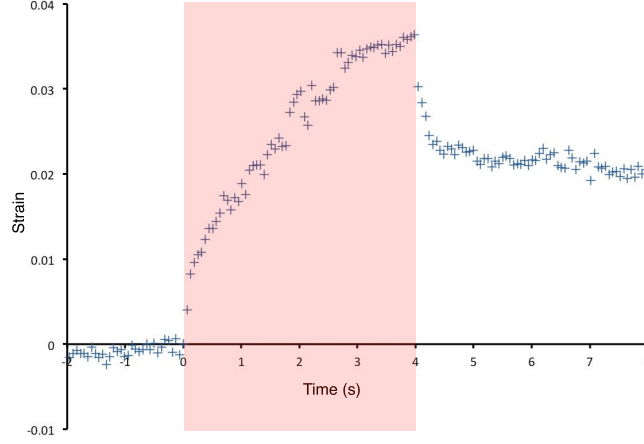


Figure 3.9: Example of a strain vs. time graph for a cell being optically stretched. The time period where the laser is at stretching power is highlighted in red.

Stress Distribution

The amount a cell will stretch is dependent on the stress distribution. As shown in equation 2.35, the stress magnitude is proportional to the intensity of light hitting the cell, which depends on the power of the laser and the distance between the optical fiber and the cell. It also depends on the refractive indices of the cell and surrounding medium. The stress distribution depends on the ratio between the cell radius and the beam width, however the peak stress of the distribution will be the same for any sized cell. As you can see in Figure 3.10, a small cell will experience stresses that are more distributed around the cell, while large cells will experience a more pinched distribution.

In order to compare the strain curves of different cells the strain must be divided by the peak stress of the cell (σ_0) and a shape factor (F_g) in order to get cell compliance ($J(t)$). This shape factor is described in detail here [85].

$$J(t) = \frac{\epsilon(t)}{\sigma_0 F_g} \quad (3.2)$$

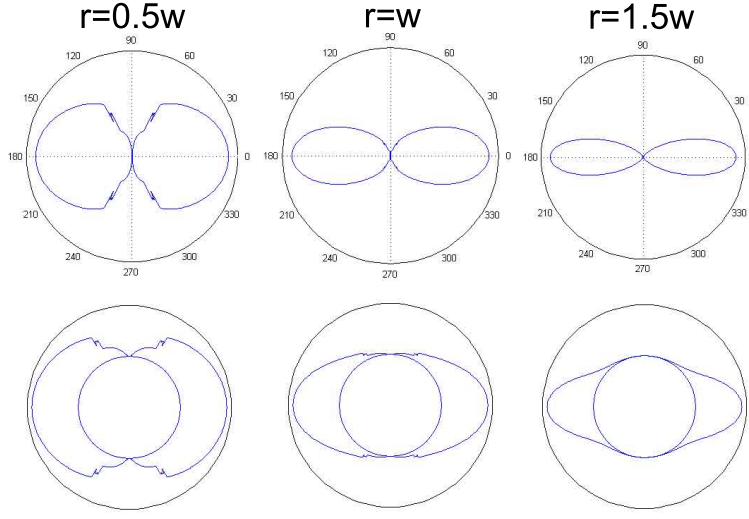


Figure 3.10: Stress distributions on cells in an optical stretcher. Top: The stress on a cell inside the stretcher with radius 0.5, 1 and 1.5 times the beam width. Bottom: The stresses plotted around a cell. These distributions were calculated with the theory discussed in section 2.3.2 in MATLAB.

Compliance Curve Fitting

After dividing the strain by the stress factor to get compliance, the mean compliance is calculated at each point. We fit the creep compliance data to the standard linear liquid (SLL) model, in which a Kelvin-Voigt material in series with a dashpot (described in subsection 2.2.5). This model has been used to successfully fit optical stretching creep compliance curves [93, 86, 92]. The creep compliance during stretching for this model takes the form,

$$J(t) = \frac{1}{E} \left(1 - e^{-\frac{Et}{\eta_t}} \right) + \frac{1}{\eta_s} t \quad (3.3)$$

where E represents the elasticity, η_t represents the transient viscosity and η_s represents the steady-state viscosity at long timescales. The transition time, $\tau = \eta_t/E$, represents the characteristic timescale at which steady state viscous behavior starts to dominate.

For each cell type under different conditions, the number of selected cells per OS exper-

iment was $n \geq 30$. The creep compliance data was displayed as the mean $\pm$ the standard error of the mean. All plotting, statistical analysis, and curve fitting were performed using MATLAB. To compare curves, t-tests were performed between the two conditions compliances at peak deformation.

3.6 Hanging Drops Monolayers

Hanging drop monolayers were utilized in Chapter 5 to study monolayers grown at air-water interfaces.

3.6.1 Set Up

Hanging drop cell culture

MDCK cells were trypsinized and then resuspended in media at a low dilution of 4000 cells/ml. The cells were then placed in hanging drops of $40\mu\text{l}$ on the lower surface of the lids of plastic Petri dishes containing PBS. Hanging drops were imaged after 24h of culture.

3.6.2 Hanging Drop Shape

The shape of the droplet observed in this set up can be approximated as a spherical cap (Fig. 3.11 C). The radius, r , at the droplet's top, where it is attached to the dish lid, was measured optically at $2.9 \pm 0.1\text{mm}$ ($n=7$). The droplet volumes were measured using the mass of the droplets to be 39.49 ± 0.04 , 40.29 ± 0.06 (after Hoechst addition) and $41.18 \pm 0.07\mu\text{l}$ (after drug addition, $n=10$). Using the spherical cap model, the height, h , and radius of curvature, R , of the initial droplet was calculated to be $2.5 \pm 0.1\text{mm}$ and $2.9 \pm 0.1\text{mm}$ respectively. With the addition of $2\mu\text{l}$, the height of the droplet increased

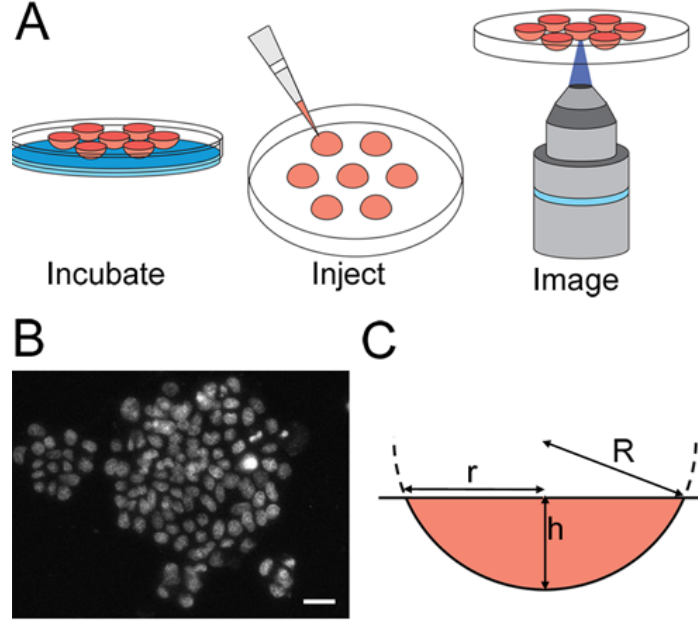


Figure 3.11: Experimental protocol used to create monolayer clusters in hanging drops. A) The process used to grow, stain and image the monolayer clusters before and after the addition of cytoskeletal drugs. $40\mu\text{l}$ droplets were incubated overnight with PBS in the bottom of the dish. The dish lid was flipped and $1\mu\text{l}$ of Hoechst in media was added with a pipette. The droplets were then imaged with a confocal microscope. After this, drugs were added to the droplets, which were incubated for 30minutes and imaged again. B) A typical set of clusters found in a droplet after the addition of Hoechst. C) A diagram describing the shape of the droplet. The droplet shape can be approximated as a spherical cap with a radius of curvature, R . The radius at the droplets top, r , is $2.9\pm 0.1\text{mm}$. The approximate height of the droplet, h , is calculated with the known volume of the droplet, $V = \frac{1}{6}\pi h(3r^2 + h^2)$. Bar, $20\mu\text{m}$.

by $74.4\pm 0.5\mu\text{m}$ and the radius of curvature decreased by $14\pm 8\mu\text{m}$ (0.5% decrease). The average diameter of clusters was approximately $150\mu\text{m}$. With the comparatively large radius of curvature, the center and edge of the cluster has a height difference of only $0.96\pm 0.04\mu\text{m}$. For this reason it was reasonable to approximate the curved bottom of the drop as a flat surface. Due to the small size of the droplet, scans were acquired quickly to avoid evaporation effects. Under the imaging power employed in this study, some evaporation of the droplet did occur, resulting in a height decrease of $0.24\pm 0.02\mu\text{m}$ per second, which corresponds to a change in volume of $0.32\pm 0.02\mu\text{l}$ per minute (Fig. 3.12). For this reason, image collection was always maintained under 45 seconds. The radius of

curvature only decreased by a maximum of $1.5 \pm 0.1 \mu\text{m}$ during imaging, having little effect on the monolayer clusters' curvature (under 0.05%).

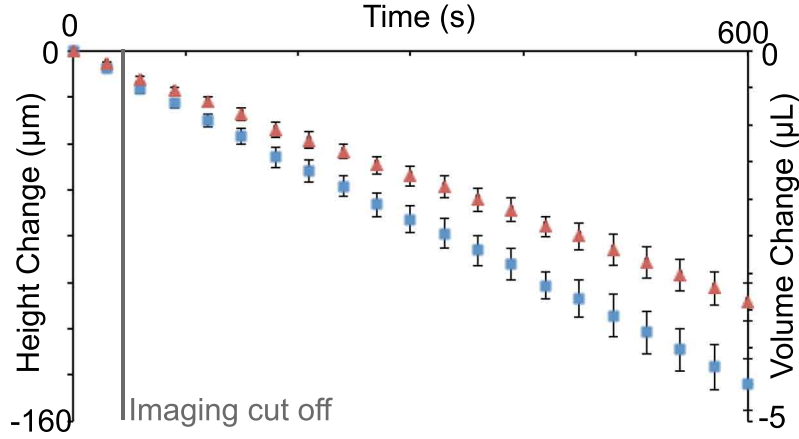


Figure 3.12: Evaporation of the droplet under imaging power. Droplet height change (blue squares) and volume change (red triangles) versus time while imaged under normal imaging power. Imaging cut off time shown with grey line.

3.6.3 Data Analysis

Strain Quantification

The strain in the monolayer was determined with the displacements of the cell nuclei during a 30 minute treatment. This method was validated by tracking the cell membrane boundary of the cluster and by tracking actin in the cell margins. Images were processed and analyzed with ImageJ. Maximum intensity z-projections of 10, 5 μm thick slices were used for strain calculations with no enhancements applied (Figure 3.13 A). The positions of the nuclei in these images were determined using the analyze particles function after thresholding. The positions of the nuclei in the before and after images were manually linked and analyzed using Matlab. A Matlab program was used to account for differences in dish placement in the before and after images, as well as to determine nuclear displacements and calculate the strain tensor of the cluster.

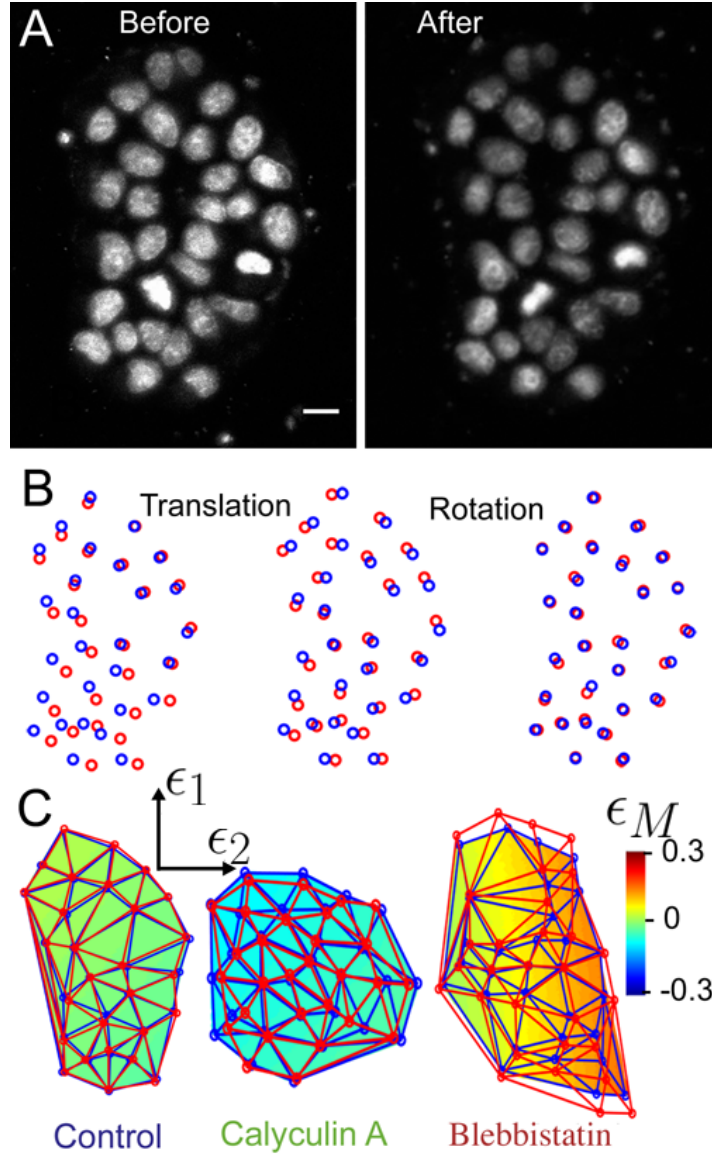


Figure 3.13: The process used to analyze monolayer cluster images. A) Maximum intensity Z-projections of the before and after confocal images of cells in a hanging drop used for a control. B) The positions of the nuclei in the before (blue) and after (red) images determined by thresholding. The raw nuclear positions (left) are shown as well as the positions after the translation (middle) and after the rotation (right) of the after positions. C) Positions of the before (blue) and after (red) nuclei in a mesh over a mean strain map of the cluster. The major and minor principal axes (ϵ_1 and ϵ_2) of the cluster are in the y and x directions respectively. Maps are shown for the control (left) case as well as CalA (middle) and blebbistatin (right) treatments. Bar, 10 μm .

Strain Quantification Program

In this program, nuclei positions were translated in both images so that the center of the nuclei was at the origin. These translated positions were then rotated so that the sum of the nuclei displacements were minimized (Figure 3.13 B). This process was needed so that there were no artificially large displacements from the movement of the dish to and from an incubator between images. The initial positions (x, y) and displacements (u_x, u_y) of the nuclei were then used to fit the cluster with least squares to the displacement equations: $u_x = c_1x + c_2y + c_3xy + c_4x^2 + c_5y^2$ and $u_y = c_6x + c_7y + c_8xy + c_9x^2 + c_{10}y^2$ [94]. Second order terms were included to account for non-constant strain throughout the cluster. These equations were then used to determine the components of Green-Lagrangian strain tensor [95],

$$\epsilon_{xx} = \frac{1}{2} \left(2 \frac{\partial u_x}{\partial x} + \frac{\partial u_x}{\partial x} \frac{\partial u_x}{\partial x} + \frac{\partial u_y}{\partial x} \frac{\partial u_y}{\partial x} \right) \quad (3.4)$$

$$\epsilon_{yy} = \frac{1}{2} \left(2 \frac{\partial u_y}{\partial y} + \frac{\partial u_x}{\partial y} \frac{\partial u_x}{\partial y} + \frac{\partial u_y}{\partial y} \frac{\partial u_y}{\partial y} \right) \quad (3.5)$$

Substitution of our displacement equations resulted in

$$\epsilon_{xx} = \frac{1}{2} \left(2(c_1 + c_3y + 2c_4x) + (c_1 + c_3y + 2c_4x)^2 + (c_6 + c_8y + 2c_9x)^2 \right) \quad (3.6)$$

$$\epsilon_{yy} = \frac{1}{2} \left(2(c_7 + 2c_{10}y + c_8x) + (c_2 + 2c_5y + c_3x)^2 + (c_7 + 2c_{10}y + c_8x)^2 \right) \quad (3.7)$$

These were used along with the shape of the cluster to determine the average x-axis strain, ϵ_{xx} , and average y-axis strain, ϵ_{yy} , in the cluster. The cluster was then rotated to find the principal axes by maximizing the difference between the y-axis strain and the x-axis strain. After this rotation, the y-axis strain was the major principal strain (ϵ_1) and the x-axis strain was the minor principal strain (ϵ_2) (Figure 3.13 C). The mean of the principal strains, $\epsilon_M = (\epsilon_1 + \epsilon_2)/2$, is the deformation due to a change in area [96].

For experiments measuring the deformation of the cluster outline, points along the

cluster outline labeled in the cell membrane stained images were tracked and used in the code above in place of nuclei positions. In some experiments cellular-level strains were determined in cells expressing actin-GFP. Cell deformation was determined by fitting cell boundaries with polygons. The displacements of the vertices were least squares fit to the displacement equations $u_x = c_1x + c_2y$ and $u_y = c_3x + c_4y$ to impose constant strain inside the cell [94]. Only first order terms were required for these calculations because the individual cells were much smaller than the cluster. The strain in each cell was calculated using the principal strain axes of the cluster determined using the nuclei displacements.

Order Parameter Calculations

Orientational order parameters were calculated using the angles between different cluster features (Fig. 3.14). The nematic order parameter was used, which in two dimensions is $S = \langle 2 \cos^2(\theta) - 1 \rangle$ [97]. The order parameter will be 1 if the features are completely aligned, -1 if they are completely antialigned, and 0 if there is no alignment. The nuclei in thresholded before images were fit to ellipses in Matlab to determine the orientations of the major length axes. The orientation of the major length axis of the cluster was determined in Matlab by using the positions of the nuclei and finding the axis that minimized the square of the distances between the axis and the nuclei. Four different types of order parameters were calculated. Nuclei-strain order parameters (S_{ns}) were determined using the angles between the major length axes of the nuclei and the major strain axis of the cluster. Nuclei-cluster orientation order parameters (S_{nl}) were calculated using the angles between the major length axes of the nuclei and the cluster major length axis. Cluster orientation-strain order parameters (S_{ts}) were also calculated with the angles between the major length and major strain axes of clusters. In addition, nuclei-nuclei order parameters (S_{nn}) were calculated using the angles formed between the major axis of each nucleus and the major axis of its nearest neighbor. This was also repeated for next nearest neighbors, third nearest neighbors and fourth nearest neighbors.

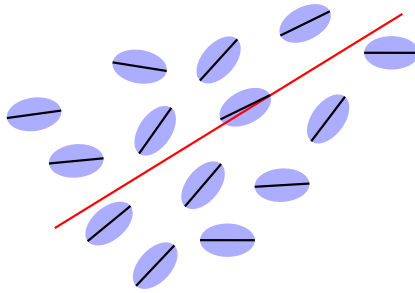


Figure 3.14: Example diagram of the orientation of cluster features. The nuclei (blue) were fit to ellipses to determine the orientations of the major length axes (black lines). The orientation of the major length axis of the cluster (red line) was determined by using the positions of the nuclei and finding the axis that minimized the square of the distances between the axis and the nuclei.

Chapter 4

Mechanical mismatch between Ras transformed and untransformed epithelial cells

A modified version of this chapter was published as:

Mechanical mismatch between Ras transformed and untransformed epithelial cells.

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Motivation

Many studies have demonstrated that the mechanics of cells is altered during oncogenic transformation. Typically, it has been shown that cancerous cells are softer than non-transformed cells. However, little is known about how attachment affects this mechanical mismatch.

Objective

The main objective of this chapter is to determine what role attachment plays in how the oncoprotein Ras affects cell mechanics. We used utilized atomic force microscopy and a microfluidic optical stretcher to measure the mechanical properties of cells expressing, constitutively active, oncogenic Ras, in adherent and suspended states. We postulated that Ras should have different effects on adherent and suspended cells due to their difference in cytoskeletal structure.

4.1 Abstract

The organization of the actin cytoskeleton plays a key role in regulating cell mechanics. It is fundamentally altered during transformation, affecting how cells interact with their environment. We investigated mechanical properties of cells expressing constitutively active, oncogenic Ras (Ras^{V12}) in adherent and suspended states. To do this, we utilized atomic force microscopy and a microfluidic optical stretcher. We found that adherent cells stiffen and suspended cells soften with the expression of constitutively active Ras. The effect on adherent cells was reversed when contractility was inhibited with the ROCK inhibitor Y-27632, resulting in softer Ras^{V12} cells. Our findings suggest that increased ROCK activity as a result of Ras has opposite effects on suspended and adhered cells. Our results also establish the importance of the activation of ROCK by Ras and its effect on cell mechanics.

4.2 Introduction

The actin cytoskeleton is largely responsible for the mechanical properties of the cell. A myriad of actin-binding proteins regulate actin filament assembly and the organization of actin filament networks [99]. Actin filaments are organized into the cortex, a thin cross-linked mesh lying immediately beneath the plasma membrane, and fibers, contractile actin bundles that are anchored by cell adhesions [12, 99, 100]. The actin cytoskeleton is fundamentally different in adherent and suspended cells. It has been shown that the mechanics of cells change after they detach from substrates [15, 101]. Adherent cells have anchoring points to the substrate, called focal adhesions, and actin stress fibers span through the cell from one focal adhesion point to another. Attachment to a substrate is thus a prerequisite for the formation of stress fibers and they are not observed in non-adherent cells. In epithelial monolayers, there is also a band of cell-cell adhesions, called adherens junctions, that encircles the cell [102]. These attachments act as anchors for a

circumferential belt of actin fibers inside the cell [103, 21]. The molecular motor, myosin, can interact and modify the cytoskeleton altering the mechanical properties of the cell. Myosin can generate tension along actin fibers [16] and pre-stress the actin cortex [104]. Cell stiffness increases with the level of tensile stress or pre-stress in the cell [105]. Seemingly due to decreasing pre-stress in the actin network, it has been shown that both the decrease in stress fibers on compliant substrates and the inhibition of myosin on substrates correlate with the softening of cells [106, 8]. However in suspension, the inhibition of myosin correlates to the stiffening of cells [92]. Similarly it has been shown that the formation of adherens junctions coincide with an increase in the apparent stiffness of reforming cell monolayers [107].

Cell transformation can arise from the activation of oncoproteins. A common oncoprotein is Ras, which controls a variety of cellular signaling pathways responsible for growth, migration, adhesion, cytoskeletal integrity, survival and differentiation [33]. Mutations that permanently activate Ras are found in approximately 20% of cancers [108]. For this reason, there is great interest in developing tumor therapies targeting Ras and Ras effector pathways [51]. One pathway of particular importance for cell mechanics is the one activating contraction through Rho, Rho-associated protein kinase (ROCK) and myosin light-chain kinase (MLCK). Ras activity is associated with increased Rho activity [58, 56, 57, 55]. Rho activates ROCK, which regulates actin filament organization and actomyosin contractility [60, 59]. It has been observed that phosphorylation of myosin light chain is enhanced in cells with permanently active Ras^{V12} [26]. The over-activation of this pathway should have different effects on adherent and suspended cells due to their difference in cytoskeletal structure.

During early stages of tumor progression in an epithelial monolayer, cells of different mechanical stiffness are in contact with one another. The mechanical mismatch between transformed and non-transformed cells could be an important driver of cancer progression. Typically, it has been shown that cancerous cells are softer than non-transformed cells

and that highly aggressive cancerous cells are softer than less aggressive cancerous cells [38, 39, 40, 37]. This can be attributed to the cytoskeleton devolving from a rather ordered and rigid structure to a more irregular and compliant state [38]. It has also been shown using a human breast cell line with conditional Src induction, that cells undergo a stiffening state prior to acquiring malignant features [109]. Metastatic cancer cells are also in different states of attachment during their journey from one site to another. During the initial stages the cell is in a monolayer with cell-cell and cell-substrate adhesions. When the cell is in the blood stream or lymphatic system, it is in a suspended state. It is thus important to measure cell mechanics in different degrees of attachment.

A popular method of measuring mechanical properties of adherent cells is atomic force microscopy (AFM), where the surface of the cell is indented by a probe consisting of a cantilever with a sharp tip [67, 110]. This is generally performed on cells adhered to stiff tissue culture dishes made of plastic or glass. An optical stretcher (OS) is an orthogonal method for measuring the mechanical properties of cells in suspension where no adhesion is needed [111]. The OS stretches the cell with optically induced stresses on the cell surface [112]. The distribution of stress applied to the cell is broad and continuous. This global stress application is in contrast to the local deformations achieved with atomic force microscopy that can encounter artifacts due to inhomogeneous cell morphology. The sharp AFM tip can create high local stresses, resulting in higher elastic modulus values than broader stress distributions [113, 114]. However, these techniques both use small deformations to measure the cortical tension of the cell. Moreover, there is a major difference in the attachment states of the cells measured by these two techniques. These two techniques allow us to measure the two extremes of cellular attachment, allowing us to model the different attachment of cells and determine their effect on cell mechanics.

In this study, we investigated the effects of Ras on the mechanical properties of attached and suspended epithelial cells. A cell line that produces constitutively active Ras in MDCK cells was utilized for this purpose [26]. Y-27632 was used to inhibit actomyosin

contractility; it inhibits ROCK, which is downstream of Ras [115]. Our results show that constitutively active Ras increased the stiffness of cells in monolayer and decreased the stiffness of suspended cells. When ROCK was inhibited with Y-27632, the Ras^{V12} cells were softer in monolayer than normal cells. When in suspension, the inhibition of ROCK had an opposite effect and stiffened the Ras^{V12} cells. Our work provides evidence that Ras plays a role in stiffening cells in monolayers on stiff substrates and softening cells in suspension. This demonstrates that Ras has opposite effects on the stiffness of cells in attached and suspended states.

4.3 Results

Ras^{V12} cells were examined in different states of attachment. We created a high state of attachment by seeding Ras^{V12} cells in co-culture with WT cells on collagen-coated glass. The mixture of cells was cultured in the absence of tetracycline until they formed an epithelial monolayer. Tetracycline was then added in the culture medium to induce Ras^{V12} expression, simulating the transformation of a cell in monolayer surrounded by untransformed cells. The cells were examined 24 hours after transformation to examine cell stiffness by AFM as well as basal protrusion, apical extrusion and actin signal. To remove cell-cell interactions single cells loosely attached to glass were also probed by AFM. Suspended cells were allowed to attach for 30min before having their stiffness measured. A fully suspended state with no cell-cell or cell-substrate attachments was also examined with a microfluidic optical stretcher.

4.3.1 Ras Stiffens Cells in Monolayer

To investigate the effects of constitutively active Ras on attached monolayers, co-cultures of Ras^{V12} and WT were studied (Fig. 4.1A). Many Ras^{V12} cells were observed to be

extruding apically out of the monolayer or protruding below the monolayer (Fig. 4.1B). Ras transformation in epithelial cells has been associated with the disruption of E-cadherin mediated cell-cell adhesion [116]. However, this was not observed by Hogen et al when Ras transformed MDCK cells were in co-culture with WT cells [26]. When stained for actin, the signal was more intense in the margins of the Ras^{V12} cells both at the interfaces between two Ras^{V12} cells (Ras^{V12}:Ras^{V12}) and interfaces between Ras^{V12} and WT cells (Ras^{V12}:WT) than the interfaces between two WT cells (WT:WT) ($p < 0.05$) (Fig. 4.2A). The actin signal was also more intense at the Ras^{V12}:Ras^{V12} interfaces than the Ras^{V12}:WT interfaces ($p < 0.05$). More of the Ras^{V12} cells were protruding basal than were apically extruding (Fig 4.2B). This was consistent with the observations by Hogan et al [26]. The cells in monolayer were probed with an AFM to measure stiffness (Fig. 4.2C). Force curves taken over the cell nuclei were fit to determine apparent Young's moduli of the cortical membrane. The Ras^{V12} cells were stiffer than WT cells ($p < 0.05$).

In order to investigate if the stiffer Ras cells were a result of increased ROCK activation/contraction, the inhibitor Y-27632 was employed. The monolayers were inhibited for 24h before testing, simultaneously with the tetracycline treatment. There was still a larger actin signal around the Ras^{V12} cells than at WT: WT interfaces ($p < 0.05$) (Fig 4.2A). Apical extrusion and basal protrusion of Ras^{V12} cells were still observed with this treatment, however extrusion was less frequent and protrusions were more frequent ($p < 0.001$) (Fig 4.2B). This decrease in extrusion was previously observed in MDCK cells overexpressing Ras with the co-expression of dominant-negative ROCK [26] and in MDCK cells overexpressing Src with Y-27632 [117]. As expected, for all samples the Y-27632 treated cells were softer than the untreated sample ($p < 0.001$) (Fig 4.2C). This is consistent with other AFM studies using Y-27632 on fully attached NIH3T3 fibroblast [118], MDCK-II epithelial [107] and MDA-MB-231 metastatic breast cancer [119] cells as well as loosely attached human fore-skin fibroblast [120] cells. The wild type cells were stiffer than the Ras^{V12} cells ($p < 0.001$) when treated with Y-27632, which is the opposite of what was found in the untreated

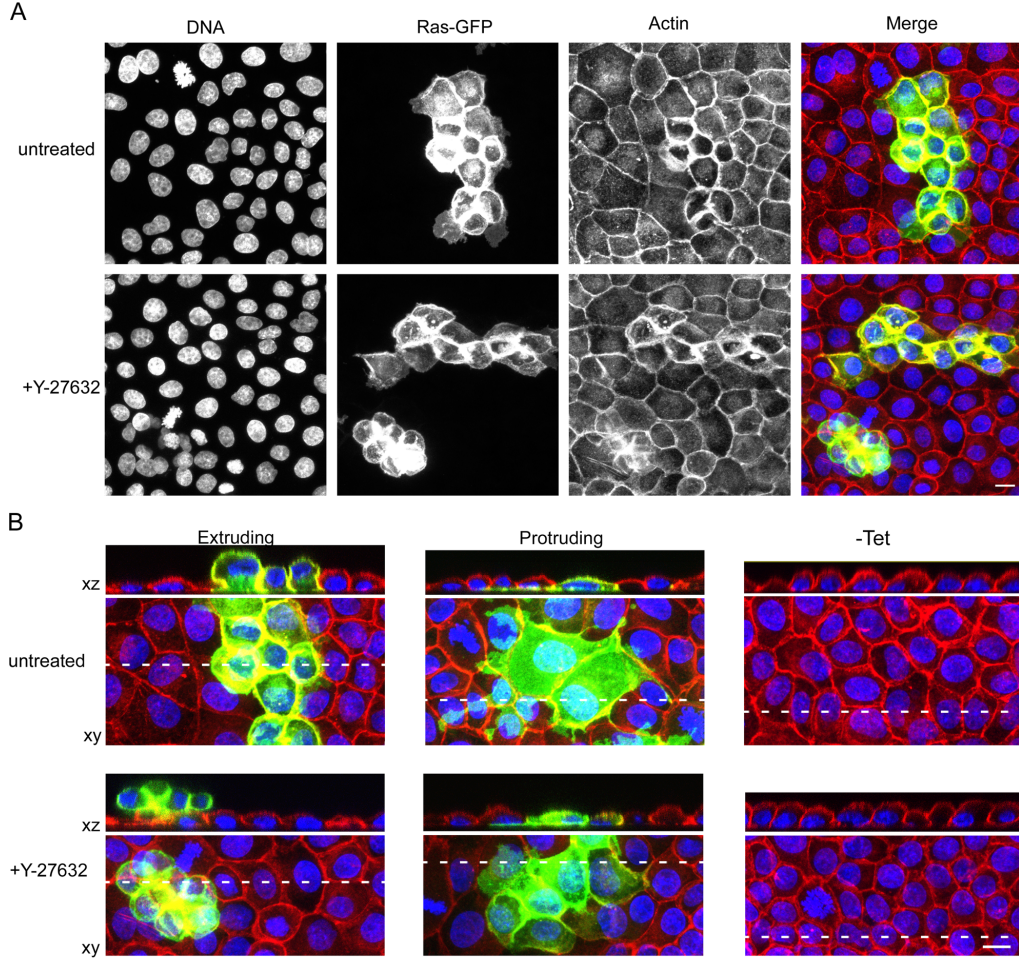


Figure 4.1: Morphology of Ras transformed cells in monolayer with WT cells. A) Maximum projections of confocal images of Ras^{V12} (green) and WT cells in monolayer with tet and with/without Y-27632 stained for DNA (blue) and actin (red) B) Orthogonal views and their corresponding maximum projections of Ras^{V12} and WT in monolayer, with/without tet, with/without Y-27632. White dashed lines indicate the positions of the xz planes used for the orthogonal views. Scale bars=10μm

condition. This demonstrates that the ROCK inhibitor softens the cells in monolayer. The ROCK inhibitor had a greater effect on the Ras^{V12} cells, making the Ras^{V12} cells softer than the WT cells. Since the Ras^{V12} cells were softer than WT cells when ROCK was inhibited, it is likely that there are other Ras pathways affecting cytoskeletal organization, attributing for the softening of the cell. The difference in relative stiffness may account for the differences in extrusion direction in the Y-27632 and untreated cases.

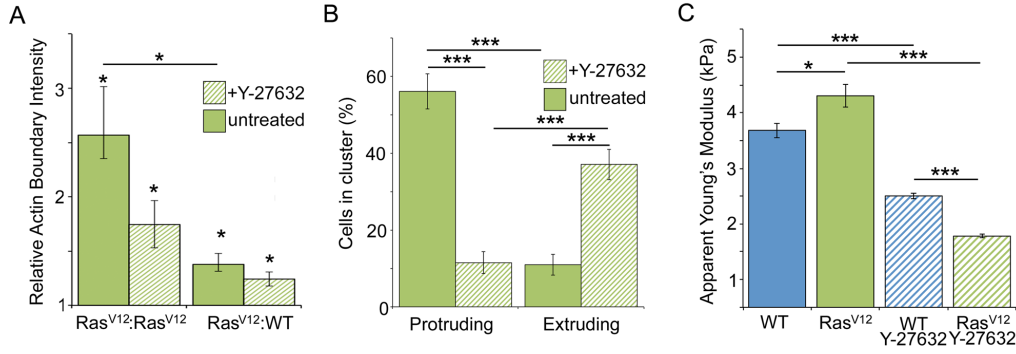


Figure 4.2: Properties of Ras transformed cells in a monolayer with WT cells. A) Average intensity of actin signal around the boundaries of cells either at the interface of Ras^{V12}:Ras^{V12} cells and Ras^{V12}:WT cells with and without Y-27632 relative to the intensity at the WT:WT interface. Each case is significantly greater than 1. B) Percent of Ras^{V12} cells in a cluster that are protruding or extruding with or without Y-27632. C) The AFM apparent Young's moduli of WT cells and Ras^{V12} cells in co-culture, with and without the addition of Y-27632 (n=25 for WT, n=25 Ras^{V12}, n=43 for WT Y-27632 and n=48 for Ras^{V12} Y-27632). Bars represent standard error of the mean, *p<0.05 ***p<0.001.

4.3.2 Ras Softens Loosely Attached Cells

Based on the initial AFM findings we sought to examine the influence of cell-cell adhesion on the physical properties of the cell. Therefore, we carried out the following experiment where single cells were loosely attached to dishes. Cells were removed from a monolayer with trypsin. They were then resuspended in new media and transferred to a fresh glass bottomed dish. After 30min in the new dish, the cells attached to the glass allowing the cells to be probed by AFM (Fig. 4.3A). This allowed us to probe cells by AFM while in a state with no cell-cell adhesions. With this method we can see if cell-cell adhesions are needed for the stiffening effect of Ras. The loosely attached Ras^{V12} cells were softer than the loosely attached WT cells (p<0.001) (Fig. 4.3B). The Hertz model can result in artificially low elastic moduli for loosely attached cells [121]. However, the change in relative stiffness supports the theory that the stiffer Ras^{V12} cells in monolayer were the result of increased actomyosin contractility in the circumferential actin belt. In a previous study, single Ras-activated fibroblasts were also found to be softer than their untransformed

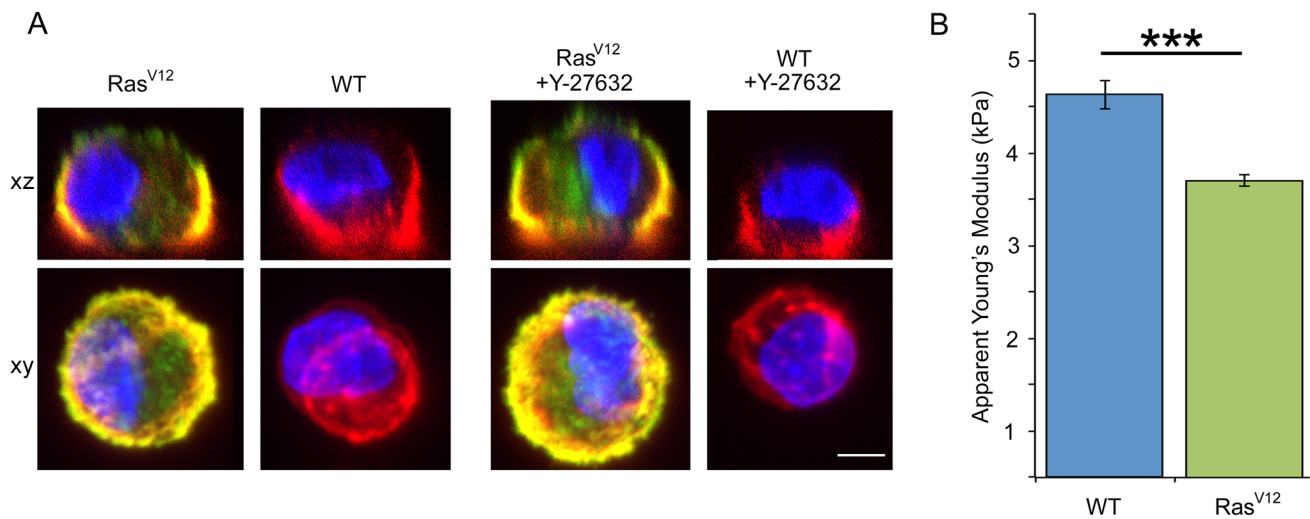


Figure 4.3: Influence of Ras on the apparent stiffness of cells loosely attached to glass probed by AFM. A) Orthogonal views of confocal images of loosely attached Ras^{V12} cells and WT cells with and without the addition of Y-27632. B) The AFM apparent stiffness of WT cells (n=23) and Ras^{V12} cells (n=23) grown in co-culture. Bars represent standard error of the mean, Scale bar=5 μ m, ***p<0.001.

counterparts [122].

4.3.3 Ras Softens Cells in Suspension

Based on the contrasting results found with fully attached and loosely attached cells measured by AFM, we focused on investigating fully suspended cells. Cells detached from Ras^{V12} and WT monolayers were examined in an optical stretcher. In this state there are no cell-cell adhesions or cell-substrate adhesions. This method might also introduce heating effects that can impact cell mechanics [86].

Along with other parameters, the refractive index of the cell is needed in order to calculate the stress it experiences. Refractive index is also related to mass density in biological material [123, 124], and gives some insight into the structure of the cell. By the fitting to the cell contour during the analysis of the DHM images the cell radius is also determined. Cells taken from WT and Ras^{V12} monocultures were examined with this

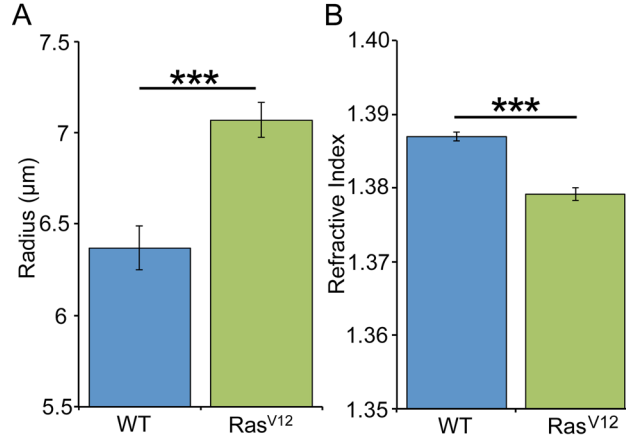


Figure 4.4: Effect of Ras on size and refractive index of cells measured with a digital holographic microscope. A) The radius of WT (n=52) and Ras^{V12} (n=54) cells. B) The refractive index of WT and Ras^{V12} cells. Bars represent standard error of the mean, ***p<0.001.

method. The Ras^{V12} cells were found to be larger than the WT cells (p<0.001) and had a smaller refractive index (p<0.001) (Fig 4.4). It has been found previously that refractive index decreases with increasing size in pancreas tumor cell types [125] and that activation of Ras increases cell size in the drosophila wing [126]. Therefore it is not surprising to observe the same trend in the Ras^{V12} line. The correlation between cell size and refractive index may be explained by increased cell water content [127].

When measured in the optical stretcher, Ras^{V12} cells were found to have a higher compliance at the end of the 4 second stretching than WT (p<0.001) (Fig 4.5). The curves were also fit to the SLL model to extract viscoelastic parameters. The Ras^{V12} cells had a lower elasticity, E , lower steady state viscosity, η_s , and longer transition time, τ , than the WT cells. This data demonstrates that the Ras^{V12} cells were more compliant than WT cells when in suspension and that the increased compliance observed in the Ras^{V12} cells was a result of the Ras^{V12} cells being both elastically softer and less viscous than the WT cells. This is in contrast to what is seen in monolayers where the Ras cells had a higher effective Young's Modulus. When treated with Y-27632 there was no significant difference of compliance at the end of stretching between the WT and Ras^{V12} cells (p>0.05).

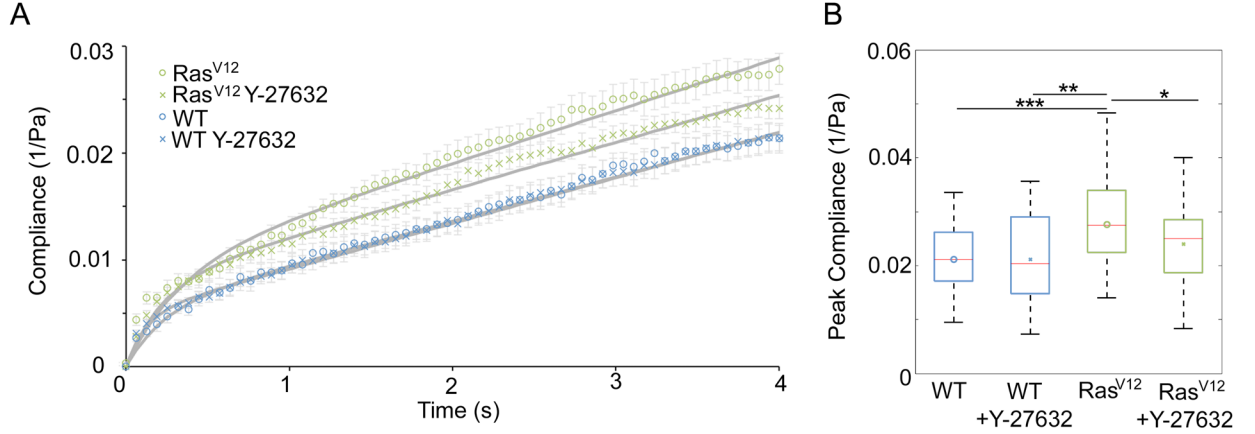


Figure 4.5: Influence of Ras on the compliance of cells in suspension probed by optical stretcher. A) The compliance curves of untreated and Y-27632 treated MDCK and Ras^{V12} cells. Gray lines are SLL fits. Fitted values were $E=109\pm5\text{Pa}$, $\eta_s=202\pm6\text{Pa}\cdot\text{s}$ and $\tau=0.33\pm0.04\text{s}$ for Ras^{V12}, $E=130\pm4\text{Pa}$, $\eta_s=226\pm5\text{Pa}\cdot\text{s}$ and $\tau=0.22\pm0.02\text{s}$ for Ras^{V12} Y-27632, $E=187\pm5\text{Pa}$, $\eta_s=242\pm3\text{Pa}\cdot\text{s}$ and $\tau=0.23\pm0.02\text{s}$ for WT and $E=205\pm4\text{Pa}$, $\eta_s=234\pm2\text{Pa}\cdot\text{s}$ and $\tau=0.12\pm0.01\text{s}$ for WT Y-27632. B) Box plots of the peak compliance (at 4 seconds) of each compliance curve. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

However, the addition of Y-27632 did lower the compliance of the Ras^{V12} ($p<0.05$) while having no significant effect on WT cells ($p>0.05$). The fitting of the curves revealed that when treated with Y-27632, the Ras^{V12} and WT cells both had increased elasticities. The steady state viscosity of the Ras^{V12} cells increased, while having little effect on the WT cells. The Y-27632 treatment decreased the transition time in both the Ras^{V12} and WT cells. This could be due to the increased ROCK/myosin light chain activity in the Ras cells. The ROCK inhibitor had elastic stiffening effect on the cells while increasing the viscosity of Ras^{V12} cells and having a relatively low effect on viscosity the WT cells. This is in contrast to what was seen in monolayers where the ROCK inhibition softened the cells. It has been found previously that inhibition of myosin decreases compliance of adherent cells brought into suspension [92].

4.4 Discussion

Ras has two major effects on the cell cytoskeleton; it alters cytoskeleton organization and contracts it. In cancer, the cytoskeleton is typically intrinsically softer due to its irregular structure [38, 116, 128, 129]. It has been shown that the amount of filamentous actin in malignantly transformed fibroblast cells is reduced by $\sim 40\%$ [38]. However, contraction of cells by the activation of ROCK has a stiffening effect on adhered cells [130, 118, 119]. This was reaffirmed with Y-27632 treated WT and Ras^{V12} cells being softer than their untreated counterparts when probed in monolayer by AFM. We found that Ras^{V12} cells were stiffer than WT cells when in the monolayer, but softer than the WT cells when ROCK activity was inhibited with Y-27632. We also found a greater actin signal in the cell margins of Ras^{V12} cells. The stiffing of Ras^{V12} cells in monolayer co-culture we observed is likely a result of increased pre-stress and cortical tension from the actomyosin contraction. This effect was likely removed with the ROCK inhibitor, showing the softening effects of the altered cytoskeleton organization effect of Ras. Adhesion on compliant substrates leads to less pronounced or even absent stress fibers [131]. The formation of stress fibers could be an artifact of tissue culture, making this imperfect model of the *in vivo* environment.

When cell-cell interactions were removed by loosely attaching the cells, the Ras^{V12} cells were found to be softer than WT cells. In order to compare the moduli of loosely attached cells to fully attached cells a model taking into account cell attachment and cellular brush would be required [121]. When measured in the completely unattached state by applying stress optically, the Ras activated cells were softer highlighting the effect of altered cytoskeleton structure on cell stiffness. This demonstrates that cell-cell adhesion is necessary for the Ras^{V12} cells to be stiffer than WT cells. The suspended Ras^{V12} cells were also stiffer when ROCK was inhibited. Since ROCK increases the activity of myosin, this is similar to another study where the effect of myosin inhibitors were found to have a stiffening effect on suspended cells [92]. It was proposed that myosin-induced actin depolymerization

played a role. However ROCK also inactivates cofilin, through the activation of LIM-kinase, inhibiting actin depolymerization [132] making the cause of the observed effect unclear

Mechanical mismatch between transformed and untransformed cells may be an important factor in metastasis. During early tumor growth cancer cells are in contact with untransformed cells in an epithelial monolayer. It has been shown that the migration speed of single, mechanically soft, cells are enhanced by surrounding stiff cells [133]. The mismatch may also play a role in the extrusion of cancer cells. In healthy epithelia, cells that are destined to die are extruded from the monolayer by the actomyosin contraction of the surrounding cells [134]. Cells in epithelial monolayers undergoing mitosis also round up through increasing their cortical tension by an increase in actomyosin contractility [135, 136, 137]. Cells extruded from monolayers die by anoikis, the programmed cell death of anchorage-dependant cells [138]. Transformed epithelial cells are also extruded from the monolayer [139, 140]. Normally, epithelia extrude cells apically into the external environment, which would essentially eliminate transformed cells [140]. However, oncogenic cells can also extrude basally under the epithelium, which could potentially initiate metastasis [26, 140]. Importantly, these processes of extrusion occur only when wild-type and transformed cells contact one another in an epithelial monolayer not when transformed cells are alone [26, 117]. In our experiments, the Ras^{V12} cells were found to form basal protrusions and apically extrude when grown in co-culture with WT cells. Untreated Ras cells demonstrated basal protrusions more often than apical extrusion. However with the addition of the ROCK inhibitor Y-27632, we observed more apical extrusion and less basal protrusion.

Our finding that Ras^{V12} cells were softer than WT cells in co-culture when treated with a ROCK inhibitor may explain this result. The mechanical mismatch, both with the transformed cells being stiffer or softer than the untransformed cells, was accompanied with apical extrusion and basal protrusions from the monolayer. However, when the transformed cells were softer than the surrounding cells they experienced more protrusion and less

extrusion than when they were stiffer. The monolayer extrusion mechanism may favor basal protrusion for stiffer cells and apical extrusion for softer cells.

The mechanical properties of cells may play a crucial role in cancer progression. After a cell is transformed by a mutation, the cell is either eliminated or it is allowed to proliferate and metastasize by spreading to new sites. The elastic mismatch may play an energetic role in the extrusion of the cells from the monolayer. There may be a link between the softening effect of Y-27632 on Ras^{V12} cells and decreased basal extrusion, suggesting extrusion processes may be dependant on the stiffness of the cells. After the cells enter the blood stream, they must also infiltrate the tissue at the secondary site, where they will form a metastatic tumor [41]. Circulating tumor cells pass through narrow vessels, smaller than their diameters [141]. It is believed that increased cell compliance aids in this process. It has been shown that circulating blood cells differentiate to acquire a compliant phenotype in order to pass through constrictions in blood vessels [93]. Although neither of the attachment states examined in this study is fully equivalent to the situation *in vivo*, it demonstrates that the relative stiffness of oncogenic Ras transformed cells is dependant on the degree of attachment. This may play a distinct role in metastasis process, where attachment levels vary at each step.

4.5 Conclusion

Constitutively active Ras has opposite effects on the relative stiffness of attached and unattached epithelial cells. This was likely a result of the Ras-rho-ROCK pathway having opposite effects on suspended and attached cells. Our results also suggest that Ras transformed cells are inherently softer, as shown when the effects of ROCK were inhibited by Y-27632. This may play a role in cancer cell extrusion, protrusion and migration. This information could be used to further understand the effect of Ras on cell mechanics and potentially aid in the development of treatments that target them.

Chapter 5

Measuring Mechanodynamics using an Unsupported Epithelial Monolayer Grown at an Air-Water Interface

A modified version of this chapter was published as:

Measuring mechanodynamics in an unsupported epithelial monolayer grown at an air-water interface

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Motivation

Actomyosin contraction and relaxation in a monolayer are often studied in cells grown on solid supports such as glass or gels. These supporting substrates influence the results of these studies. Therefore, it remains difficult to assess the intrinsic internal mechanical dynamics and properties of monolayers in the absence of a mechanically supporting surface.

Objective

The objective of this chapter is to develop a new methodology to characterize actomyosin mechanodynamics in a multi-cellular system in the absence of a supportive substrate. This would provide a picture of basal actomyosin mechanodynamics, allowing us to infer how the presence of a substrate impacts contractility and cellular organization.

5.1 Abstract

Actomyosin contraction and relaxation in a monolayer is a fundamental biophysical process in development and homeostasis. Current methods used to characterize the mechanodynamics of monolayers often involve cells grown on solid supports such as glass or gels. The results of these studies are fundamentally influenced by these supporting structures. Here, we describe a new methodology for measuring the mechanodynamics of epithelial monolayers by culturing cells at an air-liquid interface. These model monolayers are grown in the absence of any supporting structures removing cell-substrate effects. This method's potential was evaluated by observing and quantifying the generation and release of internal stresses upon actomyosin contraction ($800\pm 100\text{Pa}$) and relaxation ($600\pm 100\text{Pa}$) in response to chemical treatments. Although unsupported monolayers exhibited clear major and minor strain axes, they were not correlated to nuclear alignment as observed when the monolayers were grown on soft deformable gels. It was also observed that both gels and glass substrates led to the promotion of long-range cell nuclei alignment not seen in the hanging drop model. This new approach provides us with a picture of basal actomyosin mechanodynamics in a simplified system allowing us to infer how the presence of a substrate impacts contractility and long-range multi-cellular organization and dynamics.

5.2 Introduction

Contractility is involved in the remodeling and organization of the cell interior and plays a major role in multi-cellular morphogenesis over long distances and timescales [143, 144, 145]. There is increasing interest in understanding how contractility manifests in multi-cellular systems [146, 9, 143]. Contractile forces generated by the actomyosin cytoskeleton within individual cells collectively generate tissue-level forces [147, 2]. A major challenge in developmental biology is to understand how cytoskeletal activity is orchestrated to pro-

duce higher order tissue organization [9]. Therefore, understanding tissue morphogenesis requires determining how cellular forces are integrated across cells and tissues [2].

Actomyosin contraction is one of the major sources of internal force inside of the cell. The actin cytoskeleton in epithelial monolayers is highly dependant on cell attachment. In epithelia, the cell-cell attachment points, adherens junctions, link a thick circumferential ring of actin and myosin around each cell, aligned with the cell borders [148, 103]. The contractility of these marginal actin bundles is used for morphogenesis facilitating epithelial sheet bending and invagination [149, 23]. The substrate attachment points, focal adhesions, link actin stress fibers. Cell contractility is commonly described and investigated in terms of changes in cortical cell elasticity as well as traction dynamics, the resistance of the substrate to deformation, on flexible substrates [150, 25]. Generally changes in cortical cell elasticity and traction dynamics are linked to sensing of the mechanical microenvironment [151, 152]. Traction dynamics are linked to cortical elasticity, focal adhesion organization and cell morphology [153, 150, 131]. Often fundamental studies of actomyosin contraction and relaxation are examined in situations where cells are grown on, or embedded in, flexible substrates [150, 154, 155, 28]. Currently there is intense interest in understanding the interplay and feedback between the mechanical properties of such substrates and contractility [152]. Although such studies typically employ substrates with systematically altered mechanical properties, the substrate is always present. Therefore, it remains difficult to assess the intrinsic internal mechanical dynamics and properties of cells in the absence of a mechanically supporting surface.

There has been recent interest in intrinsic mechanical properties of substrate-free epithelial sheets [144, 156]. Measurements of the elasticity and failure of monolayers suspended between two flexible rods provided valuable insights into the understanding of epithelial mechanics [144, 156]. Importantly, the measurements yielded mechanical properties of the near substrate-free cell sheet. With tensile testing of the suspended cultured monolayers, it was determined that rupture of intercellular junctions occurs after doubling the monolayer

in length with an average force approximately nine times larger than measured in pairs of isolated cells [157, 144], demonstrating that epithelial organization leads to a strong enhancement in the integrity of the tissue. However this suspended monolayer is still mechanically constrained at suspension points that provide an external form of confinement and pre-stress.

This begs the question, how are cell contractility, the organization of the cell interior and long range structural remodeling altered in the absence of mechanically confining or supporting structures in a multi-cellular system? Therefore, the objective of this study was to develop a new methodology to characterize actomyosin mechanodynamics in a multi-cellular system when no resistance is offered by the surroundings. Understanding these dynamics could provide us with a picture of basal actomyosin mechanodynamics in a simplified model multi-cellular system. This method will also allow us to indirectly infer how the presence of a substrate impacts actomyosin contractility and long-range multi-cellular organization and dynamics.

Here, we employed an *in vitro* hanging drop culture protocol. We created Madine-Darby Canine Kidney (MDCK) epithelial monolayer clusters at an air-water interface. Employing laser scanning confocal microscopy, cell nuclei within the monolayer were used as fiduciary markers to determine the internal strain developed in the clusters during basal and chemically-induced contractile changes. We validated our method using two alternate methods for tracking cell deformation: 1) tracking the cell membrane boundary of the cluster and 2) tracking cell boundaries marked with GFP-actin. Importantly, this new methodology for studying the mechanodynamics of unsupported epithelial sheets opens the door for several future research directions including investigations of the apico-basal polarization, myosin distribution and actin ordering in the unsupported monolayer. This approach allows one to observe the results of forces that are important for many processes that take place in development [1] and may advance the understanding of how those forces are being transmitted to neighbouring cells, and how they are integrated to trigger global

changes in tissue shape. The results of this study might also be relevant to 3D cell culture where hard surfaces are not present.

5.3 Results

5.3.1 Deformation of Monolayer Clusters on Solid Substrates

To examine the influence of a mechanically supporting substrate, monolayer clusters were created on hard glass substrates and soft polyacrylamide gels (4.8 kPa Young's modulus). These clusters naturally occur in MDCK cell culture at low confluence. The clusters on glass were fixed and stained for DNA, actin and vinculin (Figure 5.1A). A strong actin signal was localized in the perimeter of the cluster. Similarly the vinculin also localized around the cluster perimeter. When epithelial cells form a cluster on solid substrates, more cellular adhesions and force generation appear at the cluster perimeter [158, 159, 160]. In order to induce cluster relaxation and contraction in the clusters we employed the agents blebbistatin and CalA. Blebbistatin is a myosin II inhibitor that inhibits contraction and disrupts contractile filament organization [161]. CalA is a phosphatase inhibitor that is well known to induce contraction [9, 161]. Clusters on glass were live stained with Hoechst and the nuclei were tracked with the addition of drugs in order to determine strain in the cluster, as described in section 3.6. There was no significant difference in the mean strains of the clusters on glass with the different drug treatments ($p > 0.05$) (Figure 5.1B). To investigate the deformation on a softer substrate, clusters were cultured on polyacrylamide (PA) gels. Consistent with the glass substrates, there was no significant difference in the mean strains of the clusters on the gels with the different drug treatments ($p > 0.05$) (Figure 5.1C). To more thoroughly examine the intrinsic deformation of a contracting or relaxing monolayer the solid substrate should be removed, for this reason the hanging drop method was introduced.

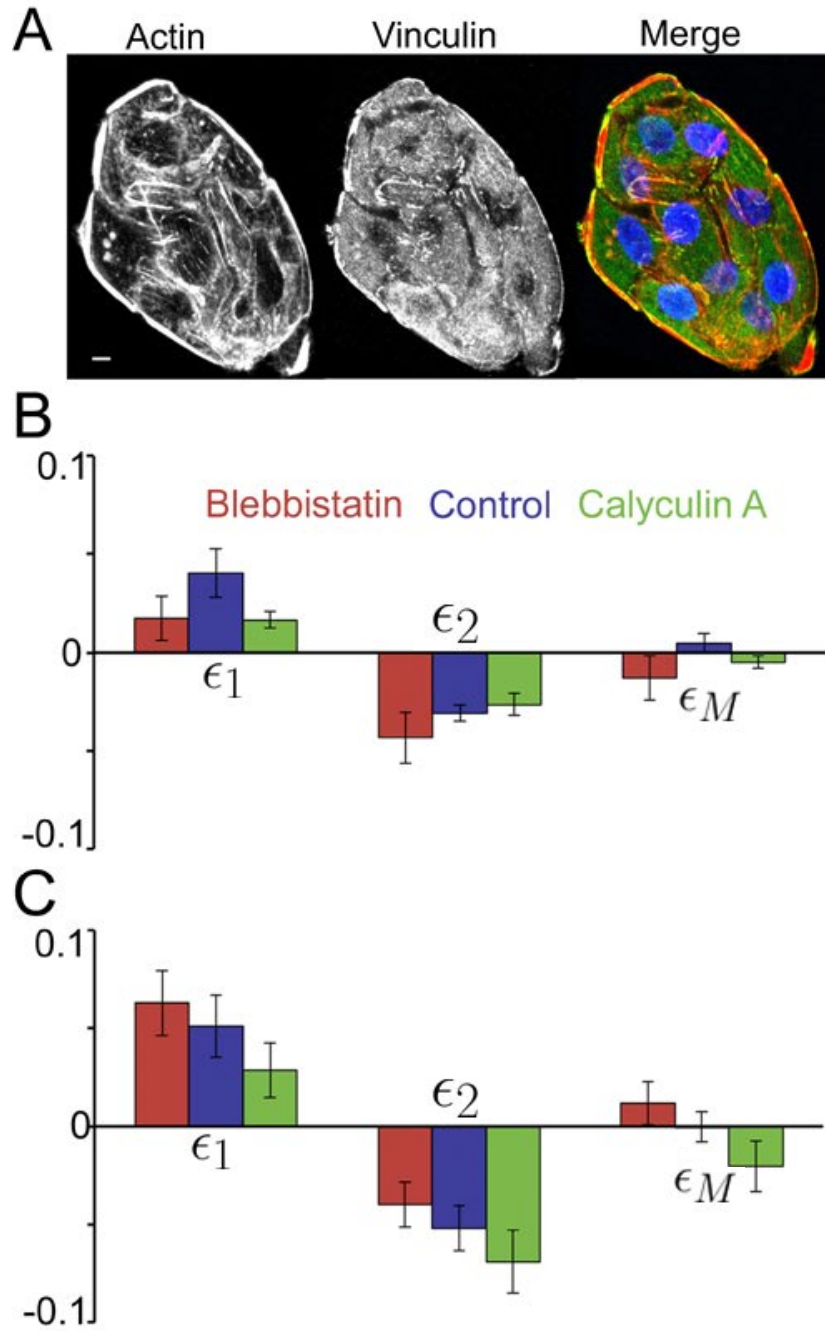


Figure 5.1: Deformation of the cluster on glass and PA. A) Confocal images of the actin (left), vinculin (middle) and merged (right, actin=red, vinculin=green, nucleus=blue) of a cluster of MDCK cells on glass. B) Major ϵ_1 minor ϵ_2 and mean ϵ_M strains of cell clusters on glass after the addition of blebbistatin (red), media (blue) or calyculin A (green). $n=11, 9$ and 12 , $p>0.05$. C) Major ϵ_1 minor ϵ_2 and mean ϵ_M strains of cell clusters on a PA gel after the addition of blebbistatin (red), media (blue) or calyculin A (green). $n=15, 10$ and 15 , $p>0.05$. Bar, $10\ \mu\text{m}$.

5.3.2 Formation of a Cell Monolayer Cluster

As MDCK cells migrate and proliferate in 2D culture, the cells form islands that eventually merge to form a continuous monolayer [162]. In 3D culture, individual MDCK cells plated within an extracellular matrix gel will assemble into a hollow sphere that is lined by a monolayer of polarized epithelial cells [162]. However, after incubation in a hanging drop, MDCK cells will form monolayer clusters of cells [163, 164, 165]. This is in contrast to mesenchymal stem cells, which gradually coalesce into a single central spheroid along the lower surface of the drop [166]. It has been shown that MDCK cells in a hanging drop will form basement membrane-like sheet of cell-secreted proteins providing the matrix for the proliferating cells [163, 165]. Previous immunohistochemical studies demonstrated the presence of type IV collagen, laminin 1, and laminin 5 in the membrane, and enzyme digestion experiments indicated that this membrane was sensitive to collagenase [163]. In previous studies, it has been shown that the tension carried by basement membranes are modest compared to the tension carried by the cellular portion of the epithelium [167] and that the basement membrane is thinner and softer than the cellular component with a Young's modulus of 7.5kPa [168] compared to 20kPa [144]. Moreover, this basement membrane is relatively free to move along the air-water interface. Therefore, this basement membrane-like sheet allows cells to form healthy monolayers while still being relatively unrestrictive to collective cellular deformation.

Epithelial monolayer clusters were grown in these hanging drops and their nuclei were used to track deformations during changes in contractility, as described in section 3.6. The clusters were similar in number of cells (average of 23 ± 2 cells per cluster) to the ones used in the solid substrate experiments (average of 21 ± 2 cells per cluster). To label cell nuclei, we added the common nuclear stain, Hoechst 33342, to the drop with a pipette in the lid's inverted state. In all cases, $1\mu\text{L}$ of a stock solution of blebbistatin, CalA or media (control) was added to the hanging drop for 30 minutes in a cell culture incubator.

The nuclei of suspended clusters were imaged before and after the addition of the drugs or control. On average, only five monolayer clusters formed in each hanging drop and it was straightforward to image the same cluster before and after the $1\mu\text{l}$ addition.

5.3.3 Deformation of Monolayer Clusters in Hanging Drops

The cytoskeletal drugs, blebbistatin and CalA, were used to relax and contract the cell clusters. The displacements of the cell nuclei were fit to determine the major and minor principal strains (ϵ_1 and ϵ_2) of the cluster. As a control, $1\mu\text{l}$ of media was added to the drops followed by incubation for 30 minutes between imaging. We did observe slight movement of nuclei within the cluster. However, the mean strain of the clusters in the control study was not significantly different than zero ($p>0.05$) (Figure 5.2). As expected there was no significant dilation or contraction. The myosin II inhibitor, blebbistatin, caused clusters to dilate appreciably. The mean strain was significantly higher ($p<0.001$) than the control. Clusters also contracted with the addition of CalA resulting in a mean strain that was significantly lower ($p<0.001$) than the control (Figure 5.2).

5.3.4 Cluster Outline Deformation

To determine if the perimeter of the monolayer cluster itself deforms in a manner consistent with the changes in nuclei position, the cluster outline was tracked by fluorescently labeling cell membranes with wheat germ agglutinin (WGA). Tracking points on the cluster perimeter allowed us to determine monolayer strain (Fig. 5.3 A). The major and minor strains in the cluster were calculated from nuclei positions as well as from its outline. The strains calculated using cell nuclei and cluster outlines were not significantly different for the untreated control, blebbistatin and CalA treatments ($p>0.05$) (Fig. 5.3 B). This suggests the contraction and relaxation observed in the nuclear positions is a good indicator of total cluster deformation.

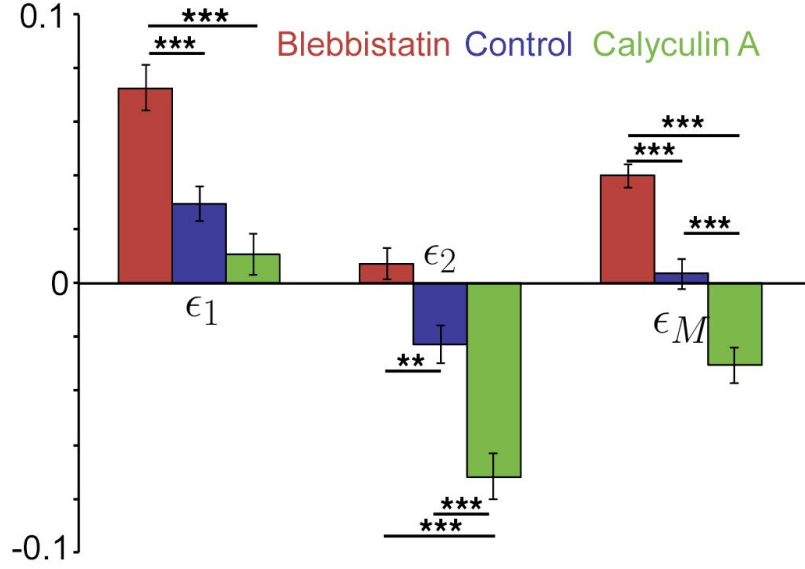


Figure 5.2: Deformation of the clusters with cytoskeletal drugs. Major (ϵ_1), minor (ϵ_2) and mean (ϵ_M) strains of cell clusters after the addition of blebbistatin (red), media (blue) or calyculin A (green). $n=22$, ** $p<0.01$, *** $p<0.001$.

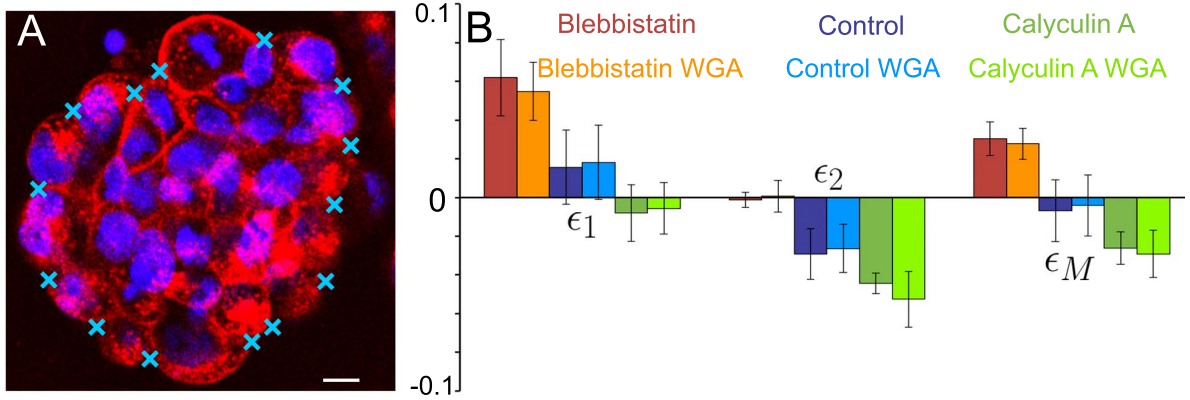


Figure 5.3: Deformation of the cluster outline with cytoskeletal drugs. A) Confocal image of MDCK cells stained with Hoechst and WGA. Cluster outline positions determined with WGA staining are shown with light blue xs (scale bar, 10m). B) Major, minor and mean strains of cell clusters after the addition of blebbistatin, media (control) or calyculin A calculated from nuclear displacements (red, blue and green) and cluster outline displacements determined with WGA (orange, light blue and light green). $n=4$, $p>0.05$.

5.3.5 Individual Cellular Strains

To determine individual cellular strains during cluster contraction and relaxation, cells transiently expressing actin-GFP were used to form suspended clusters. It was found that when a high percentage of cells were expressing actin-GFP, CalA treatments had no effect on contraction. Therefore, we adjusted the transfection efficiency to $\sim 25\%$ of cells in order to create suspended clusters that exhibited the same mechanical dynamics as non-transfected cells. In this case, we felt confident that we would be able to examine individual cellular strains, albeit only on a sub-population of cells within each cluster. Distinct actin stress fibers were not evident but actin was found localized to the cell margins allowing us to easily determine cell boundaries (Figure 5.4 A and B). Experiments with blebbistatin, CalA and control conditions were performed once again and nuclei positions were tracked, as well as the deformation of single cells within the cluster.

In the control, the cluster underwent normal basal remodelling as seen by nuclear displacements. With the addition of CalA and blebbistatin the clusters the nuclear displacements showed contraction and relaxation of the cluster as expected (Figure 5.4 C). The mean of the strains in the individual cells were not significantly different than the cluster strains calculated by nuclear displacements in the control, blebbistatin and CalA treatments ($p > 0.05$). This demonstrates that the monolayer cluster strains determined by nuclei correspond well to the deformation of the individual cells themselves.

5.3.6 Substrate Effects on Strain

Comparing the blebbistatin or CalA induced strains on the glass or PA substrates to the hanging drop (Figure 5.5 A) reveals that they were significantly muted ($p < 0.01$ in both cases on glass, and $p < 0.05$ for blebbistatin on PA) by the substrate. Interestingly however, there was no significant difference in the controls ($p > 0.05$).

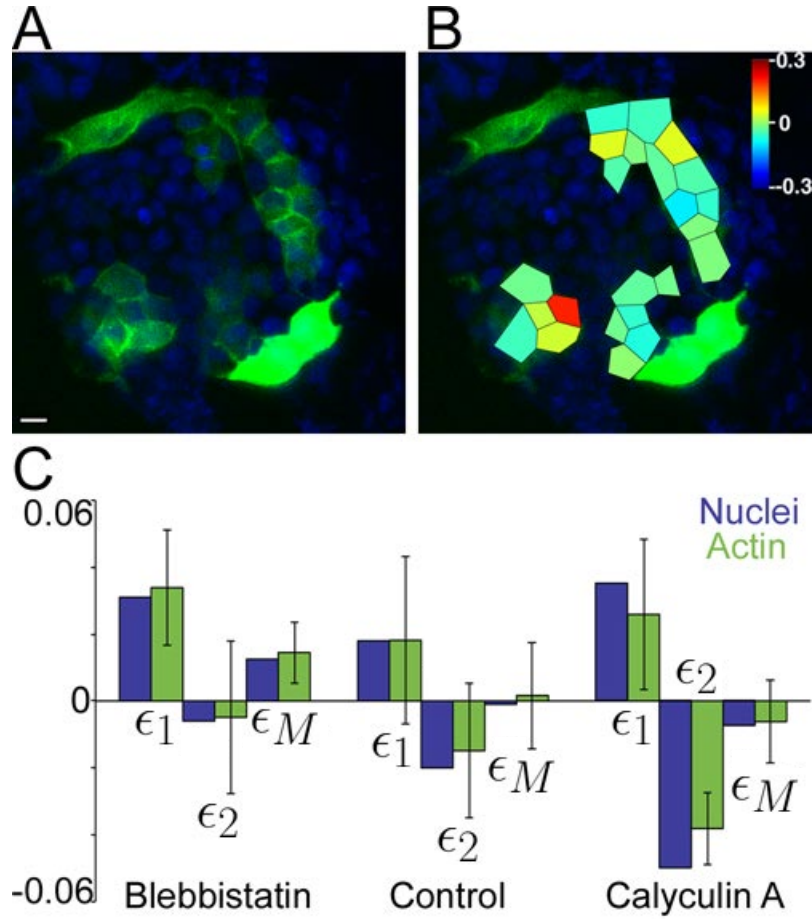


Figure 5.4: Cellular deformations inside a deforming hanging drop cluster. A) The before image of a MDCK cluster stained with Hoechst (blue) partially transduced with GFP-actin (green). B) The mean strain of each cell overlaid on the before image. C) Major, minor and mean strain of cell clusters after the addition of blebbistatin, media, or CalA (blue) compared to the strain in the individual cells as calculated with the actin cell boundaries (green). Bar, 10 μm .

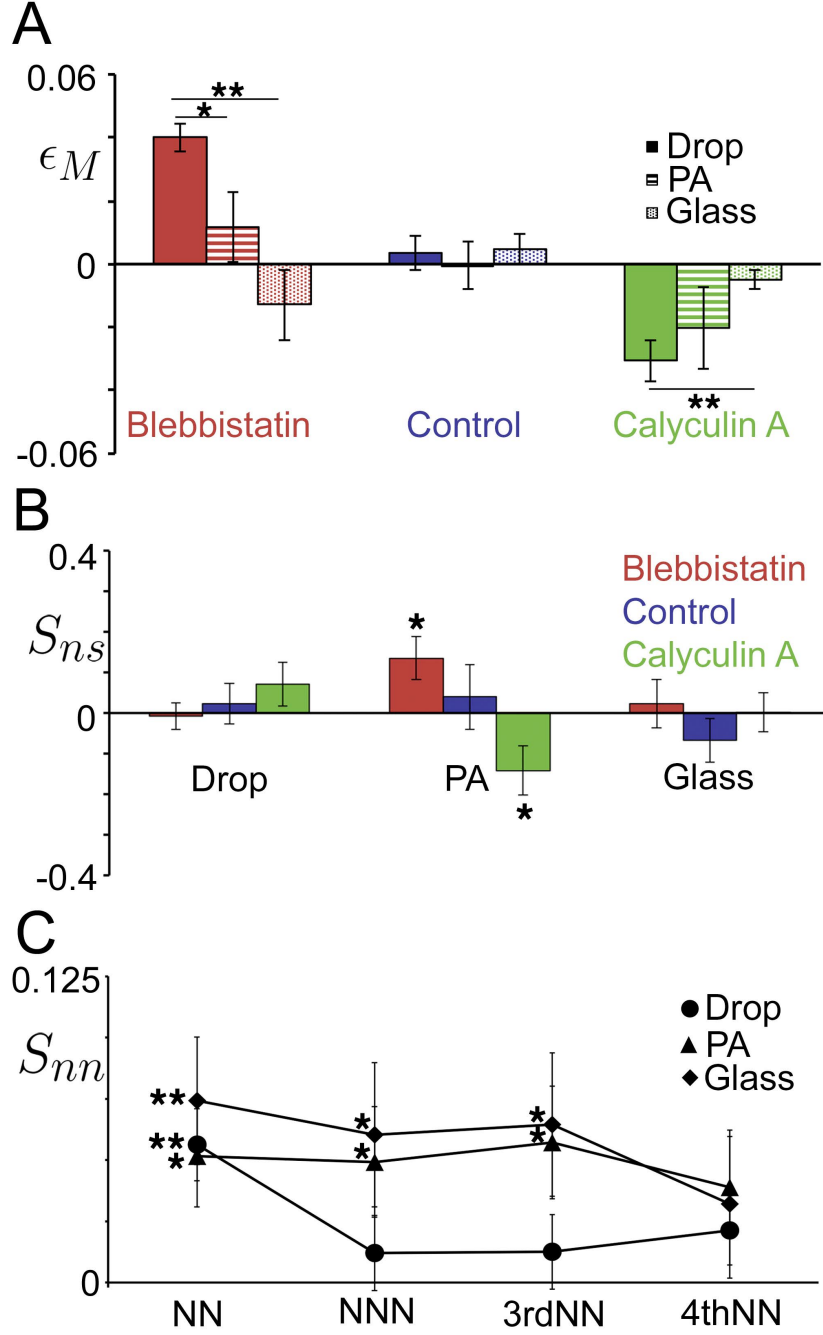


Figure 5.5: Substrate dependence on nuclear deformation. A) Mean ϵ_M strains of cell clusters in the hanging drops (solid, n=22), on PA gels (stripes, n=15, 10 and 15) and glass (dots, n=11, 9 and 12) after the addition of blebbistatin (red), media (blue) or CalA (green). B) Order parameter, S_{ns} , for nuclei (defined by the angle between the major strain axis and the nuclear major axis) for blebbistatin (red), control (blue) and CalA (green) treatments for hanging drops, PA and glass. C) Order parameters, S_{nn} for nuclei (defined by the angle between the nuclear major axis and its neighbours nuclear major axis) in hanging drops (circles), on PA (triangles) and glass (diamonds). The results are shown for nearest, next nearest, third nearest and fourth nearest neighbors. *p < 0.05, **p < 0.01.

5.3.7 Feature Orientation

In order to determine if the deformation of the cluster exhibited a dependence on structural features of the cells, nematic order parameters were calculated as described in 3.6.3. The order parameter for nuclear alignment with strain (S_{ns}) was calculated for all the clusters (Figure 5.5 B). The order parameters in the hanging drops were not significantly different than 0 ($p>0.05$), suggesting no preferential alignment. On glass, the order parameters were also not significantly different than 0 ($p>0.05$), suggesting no preferential alignment. However, since the strains were small on glass, the major strain axis was likely determined by basal remodeling of the cells and not the contraction or relaxation of the actomyosin cytoskeleton. On PA, the control order parameter was not significantly different than 0 ($p>0.05$), however the order parameters of treated clusters nuclei were significantly different than 0. In the blebbistatin treated clusters, the nuclei preferentially aligned with the major strain axis ($p<0.05$). In the CalA treated clusters, the nuclei preferentially aligned with the minor strain axis ($p<0.05$). Therefore, the nuclei tended to be aligned with the relaxation and contraction axes. This demonstrated that there was a correlation between nuclear alignment and cluster strain on PA gels that was not observed in hanging drops or on rigid glass substrates.

The order parameter S_{ls} , calculated with the angle between the major length axis and the major strain axis of the cluster (Figure 5.6 A), was not significantly greater than 0 for any treatment or substrate ($p>0.05$ in all cases). Another order parameter (S_{nl}) examines if the nuclei were aligned with the major length axis on the cluster. Again, this parameter was not significantly greater than 0 for any treatment or substrate ($p>0.05$ in all cases) (Figure 5.6B). The average eccentricities of the nuclei grown on the three different substrates were not significantly different ($p>0.05$). The clusters also had varying major/minor length aspect ratios, however the mean strains observed in the monolayer clusters did not display any correlation with aspect ratio (Figure 5.7).

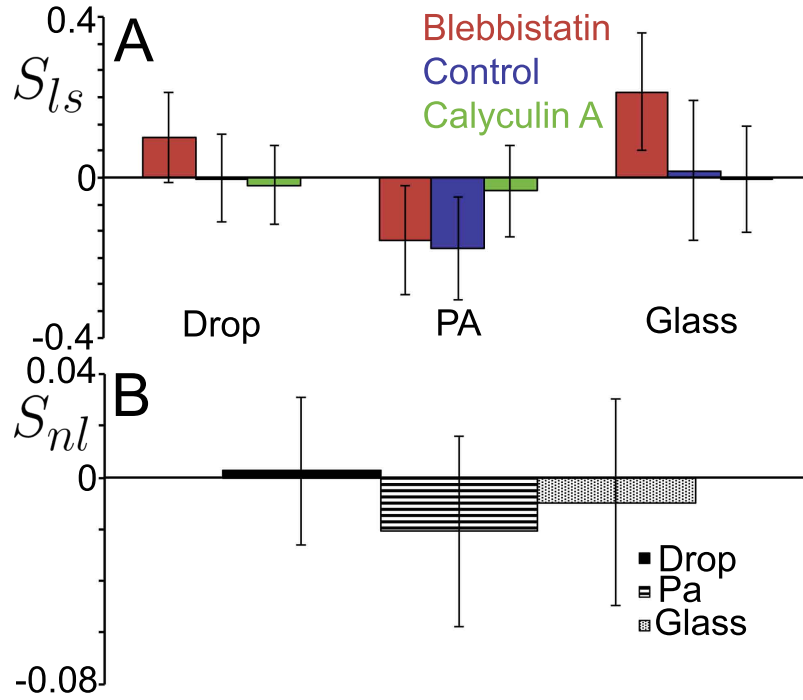


Figure 5.6: Cluster shape order parameters of clusters on glass and PA. A) Order parameter, S_{ls} , for cluster shape and strain (defined by the angle between the major strain axis and the cluster major axis) for blebbistatin (red), control (blue) and CalA (green) treatments on glass and PA. B) Order parameter, S_{nl} for nuclear alignment and cluster shape (defined by the angle between the major length axis of nuclei and the major length axis of the cluster) for cell clusters in drops (solid), on PA (stripes) and on glass (dots). $p > 0.05$

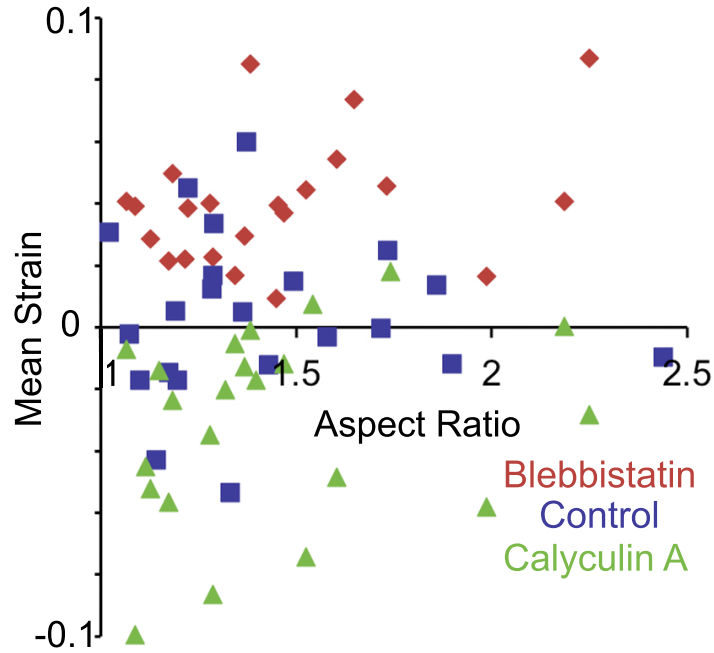


Figure 5.7: Dependence of mean strain on cluster aspect ratio. Cluster mean strain of droplets treated with blebbistatin (red diamonds), media (blue squares) and CalA (green triangles) vs. the aspect ratio of the cluster. There is no significant correlation.

To examine the alignment of cells with their neighbors, the order parameters between nuclei (S_{nn}) were calculated (Figure 5.5C). For all substrates, the order parameters for the nearest neighbors were significantly greater than 0 ($p < 0.01$, 0.05 and 0.01 for hanging drops, PA and glass, respectively) suggesting a local alignment of cells in direct contact. For next nearest neighbors and third nearest neighbors, the order parameter was not significantly greater than 0 for hanging drops ($p > 0.05$), but was significantly greater than 0 for PA and glass ($p < 0.05$). After this, the order parameters were not significantly different than 0 in any case. This suggests that the cells on substrates aligned with cells that were near to them even if they were not in direct contact. Interestingly, this was not seen for cells in hanging drops, suggesting longer-range cell alignment occurs only in monolayer clusters on solid substrates.

5.4 Discussion

Understanding actomyosin contractility is one key aspect of cell mechanics that is required for explaining the dynamics of tissue remodeling. Direct experimental measurements of force generation in monolayer clusters without a rigid surface can aid in our understanding of the remodeling of epithelial monolayers. The objective of this study was to develop a new experimental methodology and analysis framework in order to provide an approach to understanding the mechanodynamics of unsupported epithelial monolayers. The lack of a solid support provided a less complicated system that allowed for remodeling not possible on a solid substrate. This method also provides a rational approach to teasing apart substrate and intercellular connections.

As a validation of the practicality of the hanging drop model, we investigated the effect of cytoskeletal drugs on the deformation of MDCK monolayer clusters in both the absence and presence of mechanically supporting substrates. By comparing cell response in these two support regimes, we were able to identify the intrinsic contractile dynamics of an epithelial monolayer cluster. We did not directly observe significant strain in the monolayer when grown on PA gels or glass. PA gels can deform enough to measure traction forces in monolayers indirectly with the displacements of embedded beads in the substrate [169, 170]. Although it is enticing to relate previous traction measurements on monolayers to our nuclear strain data, it is not immediately clear if there should be a relationship. The methodologies used in these approaches are highly distinct and unlikely to be probing identical aspects. Traction is caused by cytoskeletal dynamics coupled to the substrate [171, 172], whereas nuclear displacements are caused by numerous factors that may not be directly linked to traction dynamics [173, 174]. However, future combined traction force microscopy and nuclear displacement traction experiments could be performed to further probe this phenomenon. The deformation we observed by tracking nuclei was echoed in the deformation of the individual cell and the monolayer cluster outlines. This verified the use

of nuclei as an effective cluster deformation marker. The observed deformations established that the contraction and dilation of monolayer clusters could be directly observed in the absence of a substrate by tracking cell nuclei in hanging drop clusters.

To calculate stress in our droplets we used the mean strains obtained from our data and the Young's modulus reported by Harris et al. for MDCK monolayers of $20 \pm 2 \text{ kPa}$ [144]. This results in an apparent decrease of stress of $800 \pm 100 \text{ Pa}$ for the blebbistatin treatment and an apparent increase of stress of $600 \pm 100 \text{ Pa}$ for the CalA treatment. There was a negligible change in stress for the control case. In other experiments, MDCK cells grown on pillars have been found to have average traction stresses of around 800 Pa in a monolayer [175]. This demonstrates that the hanging drop system can be used to calculate the intrinsic stress produced by actomyosin contraction and relaxation in monolayer clusters.

Furthermore, varying the cluster substrate allowed us to relate contractile dynamics to substrate rigidity. Our hanging drop method allowed us to measure the intrinsic remodeling that can occur in response to actomyosin dynamics. This remodeling was muted by the presence of a solid substrate, demonstrating that cluster contraction is significantly restrained when bound to a rigid surface. In general, stiff substrates increase cell-substratum adhesion [4, 5, 6, 7] and increase traction forces [29]. It has also been shown that compliant substrates promote focal adhesions that are dynamic and irregular in shape while stiff substrates promote the formation of stable arrays of elongated focal adhesions [4]. When the cells are grown on glass, a large portion of the work from the actomyosin contraction was converted into substrate deformation instead of cellular remodeling. On softer substrates (PA gels in particular) cells are still restrained; however, the smaller, more dynamic focal adhesions may allow for more movement. In this case, a smaller portion of the work will go into deforming the substrate. The contraction energy of the hanging drop cells is expected to be smaller than the contraction energy of cells on glass or PA. However, the cells can move more freely in the cluster because they are not attached by focal adhesions to a substrate that is difficult to deform. Only a negligible amount of work should go into

deforming the basement membrane-like sheet below the monolayer cluster, allowing for the larger cellular remodeling events observed in this paper.

Interestingly, when the clusters were grown on PA gels there was a correlation between major strain direction and nuclear alignment. However, for cells grown on at the air-water interface in a hanging drop, the direction of the major strain was independent of nuclear alignment. The nuclei of single epithelial cells grown on flat rigid surfaces have been previously found to align with the actin cytoskeleton and the major length axis of the cell [176, 177]. This suggests that the actin fibers are aligned with the major strain direction when on PA gels. This is congruous with the theory that individual cells are contracting and relaxing preferentially in the direction of their actin cytoskeletons resulting in a global deformation along the cluster axis that many cells were aligned with. It has also been shown that matrix stiffness aids in regulating the polarization and alignment of stress fibers within cells [178]. The lack of correlation between nuclear orientation and strain in the hanging drop may be due to reduced alignment of actin within each cell and the reduction in longer-range correlated cell-cell alignment that was observed on rigid substrates. In the case of the stiff glass substrate, although there should be greater alignment due to the substrate, focal adhesions pinned cells to the substrate leading to null cluster strains, making it arduous to correlate to strain. The softer PA substrate allowed for substrate mediated alignment and limited cell movement permitting a correlation between cluster strain and nuclear alignment to be observed.

Remarkably, a solid substrate appears to be required for longer-range cell-cell alignment. By calculating nuclear order parameters, we found that cells in hanging drops were only aligned with their nearest neighbors, while cells on substrates were aligned with their next nearest and third nearest neighbors as well. This longer-range organization may be a result of substrate cues not present in hanging drops. It suggests also that signalling is necessary to develop long-range stresses. One possibility is that long-range orientational correlations are the result of elastic coupling of cells mediated by deformation of the compli-

ant substrate. This is consistent with work showing that intercellular forces develop within the cluster of cells grown on a gel [159]. This is also consistent with long-range correlations of substrate deformations and cell velocities previously observed in monolayers grown on PA gels [179]. When low-density monolayers were examined on PA gels, they observed velocity correlations up to five cell lengths. They also observed substrate deformation correlations up to four cell lengths. This deformation correlation length is only 25% longer than our nuclear orientation correlation length. This unexpected finding was made evident by comparison with the extreme condition of an air-water interface substrate. It would not have been possible without the novel hanging drop monolayer. Our new methodology has revealed some surprising and novel insights into the mechanodynamics of epithelial monolayers. However, it also raises several new questions that will warrant further study in the future.

For instance, do the cells in the monolayer establish apico-basal polarization? Is this polarization triggered without a solid substrate? This could be determined with more advanced staining techniques. Are the same structures formed on planar non-adhesive substrates or is the curved drop shape needed? Plating cells in dishes with non-adhesive coatings could be a method to investigate this. How do clusters grown on substrates recoil when detached? This could be investigated using trypsin to detach the clusters from the substrate or by using thermo-sensitive polyacrylamide derivatives to allow release of the sheets. Future work will include computational modeling of these scenarios to examine these effects in further detail. These hanging drop monolayers can also be made with epithelial cells stably expressing a fluorescently marked protein of interest and could also be utilized for a broad range of mechanical experiments investigating substrate effects on monolayers. Novel tools and methodologies for understanding the mechanics of monolayers will enable the advancement of the biophysics field.

5.5 Conclusion

There are many studies that systematically alter substrate elasticity to determine how the properties of a solid substrate regulate cell biology and biophysics. There is potential for a great deal more to be learned, elucidated in terms of cell-cell interactions and contractility, if the supporting substrate is removed entirely. This results in the loss of cell substrate interactions, leaving cell-cell interactions to bear a greater role in regulating the system. We found that even in the absence of a solid substrate, there are still controlled mechanical responses to cytoskeletal altering drugs. By comparing the hanging drop monolayers to monolayers on substrates, we found that a solid substrate is needed for higher order structural alignments. This study is the first of its kind, establishing a new methodology for substrate-free strain characterization. Importantly, future utilization of this work may be necessary for determining primary morphogenetic interactions, as seen during development.

Chapter 6

Modeling Mechanodynamics of Epithelial Monolayers

Motivation We demonstrated, in the previous chapter, that the presence of a supporting substrate alters the mechanodynamics, as well as the cellular alignment, of a monolayer. Contracting and relaxing monolayers grown on air-water interfaces lacked the long-range cellular alignment and alignment of cellular features to major and minor strain axes observed in monolayers grown on solid substrates.

Objective The objective of this chapter is to develop a computational model for the monolayer clusters that includes anisotropic cell mechanical properties and substrate adhesion. We aim to use this model to determine what anisotropy of cell mechanical properties is needed to produce the mechanics and alignment observed in the previous chapter. We hypothesize that cellular alignment observed in monolayers on solid substrates is a result of global alignment of cell elastic moduli.

6.1 Abstract

Actomyosin contraction and relaxation of epithelial monolayers plays a central role in development and normal physiology. Computational models offer a useful means by which to investigate these processes. To understand how cellular mechanical properties influence monolayer mechanics, we created a new vertex model. The energy function used in this model takes into account cell-cell and cell-substrate adhesion as well as anisotropic cellular mechanical properties. Simulations using this model were used to characterize the

morphology and mechanics of monolayer clusters observed in experiments. The results of these simulations suggest that the promotion of long-range alignment observed on solid substrates is due to cells having anisotropic elastic moduli with global alignment. They also suggest that the short range alignment observed in monolayers grown on air-water interfaces was due the cells having isotropic moduli. This new computational model is a useful tool which allows one to deduce how potential anisotropic elastic response of cells, the presence of a substrate and mechanical alignment of individual cells each may influence monolayer organization and contractility.

6.2 Introduction

Epithelial monolayers are the simplest tissues in multicellular organisms, but perform crucial roles in normal physiology. The intrinsic mechanical properties of epithelial sheets are central to the mechanical dynamics they produce. In chapter 5, model monolayers were grown at the air-water interface of hanging drops in the absence of supporting structures. They were used to study the generation and release of internal stresses upon actomyosin contraction and relaxation. It was found that the major and minor strain axes in the unsupported monolayers were not correlated to nuclear alignment as observed when the monolayers were grown on soft deformable gels. It was also observed that both gels and glass substrates led to the promotion of long-range cell nuclei alignment not seen in the hanging drop model.

These results raised questions as to what was driving these characteristics. Are the differences observed between the monolayers grown with and without supporting substrates a result of changes in substrate adhesion energy or cytoskeletal mechanics? Do the cells in the unsupported monolayers have isotropic or anisotropic mechanical properties? Can the alignment observed on substrates be a result of cellular level mechanical alignment or is global mechanical alignment needed? The objective of this study was to create a new

computational model to characterize mechanodynamics of epithelial monolayers that takes into account substrate adhesion as well as anisotropic elastic moduli.

We created our new model by modifying a common epithelial monolayer model called the vertex model. We added a substrate adhesion to this model and altered the elastic energy term to include anisotropy. Different types of alignment of the elastic moduli in the monolayer cluster were used. The elastic modulus major axes were either aligned to the length axes of each cell or globally aligned to the major axes of the clusters. We then used this model to study the role of anisotropy of cell mechanical properties and substrate adhesion in determining monolayer mechanics during actomyosin contraction and relaxation. We examined the strain produced by a change in elastic modulus simulating the cytoskeletal drugs, blebbistatin and calyculin A. We also examined the alignment of cellular features in the monolayer and the alignment of cellular features to the strain produced by the change in modulus.

We found that the alignment of nuclei to the strain in relaxing and contracting monolayers and the long-range cellular alignment that we observed in cells grown on substrates was potentially a result of cells having anisotropic elastic moduli with global alignment. The lack of alignment of nuclei to strain and short range cellular alignment that we observed in unsupported monolayers was presumably a result of the cells having isotropic elastic moduli and low substrate adhesion. Importantly, this new model for studying the mechanodynamics of monolayers may facilitate future research of the role of structure of the cytoskeleton and substrate adhesion in monolayers. This model is another tool to advance the understanding of how forces are transmitted between neighboring cells and how they are integrated to produce changes in tissue shape.

6.3 Computational Model

Complementary to experimental approaches, computational models of multi-cellular systems have become extremely useful tools. These models can be used to examine the effects of altering a single variable, a task that is extremely difficult in experimental studies. We wanted a model that is effective in modeling changes in the contractility of a cell monolayer. A common model used to describe epithelial tissues is called the vertex model. We chose this model as our starting point. This model describes epithelia as networks of polygons, where each cell is represented by a single polygon and a height. Hence, the vertex model is most appropriate to represent densely packed epithelial tissue. The vertex model originates from the work introduced by Honda and co-workers [180]. They used Voronoi tessellation to construct polyhedral geometries around cell centers. Extensive reviews can be found in [11, 10]. Early models were evoked in discussions of foam boundaries rather than living tissue. [181, 182]. Vertex models have been used to discuss tissue morphology [183], dynamics [184], and to study detailed problems such as the dynamics of cell sorting [185] and the mechanical regulation of cell division and growth [186]. Similar models have also been used to study wound closure [187] and the geometric structure of ommatidia in the *Drosophila* eye [188]. In these models, the movement of each vertex is determined either by the explicit forces on each vertex or a free energy function. Stationary and stable network configurations satisfy a mechanical force balance. Practically all vertex models assume a cell-cell interfacial tension, and most vertex models assume some kind of area elasticity.

6.3.1 Standard Vertex Model

Farhadifar et al. formulated a model of this type [189] with the energy function:

$$U = \sum_{\text{all cells}} \frac{K}{2} (A - A_0)^2 + \sum_{\text{all edges}} \Lambda l + \sum_{\text{all cells}} \frac{\Gamma}{2} L^2 \quad (6.1)$$

These terms depend on the area of the polygon, A , the preferred area of the polygon, A_0 , the side lengths of the polygon, l , and the perimeter of the polygon, L (Figure 6.1) as well as an elastic parameter, K , a target area A_0 , an adhesion parameter, Λ , and a tension parameter, Γ . This energy function is two-dimensional in nature and any dependence on three-dimensional attributes of the cell must come implicitly in the model parameters K , Λ , and Γ . The first potential energy term comes from the assumption that the cell is elastic, and that the cell wants to return to a target area. The second energy term accounts for cell-cell interactions along the junctional region of cell boundaries. The third term is another elastic energy, accounting for the circumferential actin belts in each cell. We will look into each term in depth to evaluate their applicability to our hanging drop monolayers and how to modify them to study anisotropic monolayer clusters.

6.3.2 Elastic Energy Term

The first term, U_{el} , describes an area elasticity with an elastic coefficient K , for which A is the area of cell and A_0 is the preferred area. While unavailable experimentally, dimensional analysis suggests that K scales with a characteristic 3D modulus of the cell and the mean height of the cell. The preferred area captures the idea that there is a balance between the forces of outside compression on a cell (by its neighbors), and the limited compressibility of the cell's volume. Therefore, a force balance is established at some equilibrium value of cell area. In the simplest version of this model, it is assumed that all cells have the same

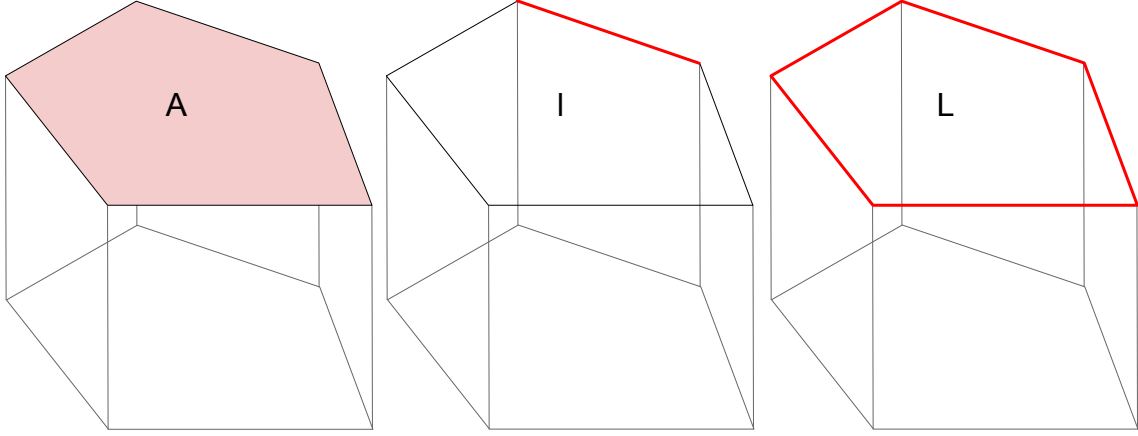


Figure 6.1: The polygon properties used to in the energy equation of the standard vertex model. Polygon area A (left), side length l (middle) and the perimeter L (right) are shown in red.

preferred area.

$$U_{\text{el}} = \sum_{\text{all cells}} \frac{K}{2} (A - A_0)^2 \quad (6.2)$$

Since we are interested in strain in the monolayer, it is advantageous to replace this term with a more complex strain energy term. The strain energy density, W , of a two dimensional network (derivation shown in section A.1) is

$$W = \frac{1}{2} (K_x \epsilon_{xx}^2 + K_y \epsilon_{yy}^2 + 2K_{xy} \epsilon_{xx} \epsilon_{yy} + 4\mu_s \epsilon_{xy}^2) \quad (6.3)$$

where ϵ_{xx} , ϵ_{yy} and ϵ_{xy} are strain terms and K_x , K_y , K_{xy} and μ_s are two dimensional elastic moduli. We calculated the strain terms with the average displacement gradient of a deformed polygon, as determined by the Kruyt-Rothenburg method (described in section A.2)[190]. This makes the two dimensional strain energy of a cell with starting area, A_i :

$$U_{\text{el}} = \frac{A_i}{2} (K_x \epsilon_{xx}^2 + K_y \epsilon_{yy}^2 + 2K_{xy} \epsilon_{xx} \epsilon_{yy} + 4\mu_s \epsilon_{xy}^2) \quad (6.4)$$

The first three constants in this term (K_x , K_y , K_{xy}) should relate to the 2D Young's moduli (E_{xx} and E_{yy}) and 2D Poisson ratios (ν_x and ν_y) of the cells. The fourth constant (μ_s) is a shear modulus. Their units should be in terms of energy per area. Since cells are three-dimensional objects, we have no experimentally available values for these constants. Hence we will use experimentally available parameters, such as cell volumes, heights and characteristic three dimensional moduli, to obtain estimates for these two dimensional parameters as described in section 6.4.1.

6.3.3 Surface Energy Term

The second term, U_s , describes the line tension due to cell-cell adhesion and cell membrane tension at the junction between cells. Here, l denotes the length of the junction and Λ denotes a tension coefficient. This term should correspond to the adhesion energy of the cells to neighboring cells.

$$U_s = \sum_{\text{all edges}} \Lambda l \quad (6.5)$$

In order to make the constants more physical, the edge length of the cell-cell interface, l_{int} , was multiplied by the height of the cell, h , to get the edge area, $l_{int}h$. This was used instead of edge length with a cell-cell surface energy constant, Λ_{cell} . We added cell-substrate interactions by including a term proportional to the basal cell area, A , with a cell-substrate surface energy constant, Λ_{sub} . We also added an interaction energy of the cell with surrounding media by including a term proportional to the area of the cell, A , and the area of the exterior edge of the monolayer cluster, $l_{ext}h$, with an cell-exterior surface energy constant, Λ_{ext} . These are needed because the monolayer clusters do not have a continuum boundary condition. The total area of the cluster will change and the exposed area of the monolayer cluster should have an energy cost. The surface energy term for the

new model would be:

$$U_s = \sum_{\text{all interfaces}} (\Lambda_{\text{cell}} l_{\text{int}} h + \Lambda_{\text{ext}} [l_{\text{ext}} h + A] + \Lambda_{\text{sub}} A) \quad (6.6)$$

6.3.4 Contractile Marginal Band Energy Term

The third term, U_c , describes the contractility of cell perimeter L , quantified by a coefficient Γ , which reflects the mechanics of the actomyosin ring.

$$U_c = \sum_{\text{all cells}} \frac{\Gamma}{2} L^2 \quad (6.7)$$

This term represents junctional contractility. With the finite approximation, if the length of the band is L and the initial length was L_i , the circumferential strain of the band would be $\epsilon = \frac{L-L_i}{L_i}$ and the band energy would be:

$$U_c = \frac{V_{\text{band}} E_{\text{band}}}{2} \epsilon^2$$

$$\Rightarrow U = \frac{A_{\text{band}} E_{\text{band}}}{2} \frac{(L - L_i)^2}{L_i} \quad (6.8)$$

$$\Rightarrow U = \frac{\Gamma}{2} \frac{(L - L_i)^2}{L_i} \quad (6.9)$$

where V_{band} , E_{band} and A_{band} are the volume, elastic modulus and cross sectional area of the contractile band respectively. This makes the energy of the new model:

$$\begin{aligned} U &= U_{\text{el}} + U_s + U_c \\ &= \sum_{\text{all cells}} \frac{A_i}{2} (K_x \epsilon_{xx}^2 + K_y \epsilon_{yy}^2 + 2K_{xy} \epsilon_{xx} \epsilon_{yy} + 4\mu_s \epsilon_{xy}^2) \\ &\quad + \sum_{\text{all interfaces}} (\Lambda_{\text{cell}} l_{\text{int}} h + \Lambda_{\text{ext}} [l_{\text{ext}} h + A] + \Lambda_{\text{sub}} A) + \sum_{\text{all cells}} \frac{\Gamma}{2} \frac{(L - L_i)^2}{L_i} \end{aligned} \quad (6.10)$$

6.4 Implementation

6.4.1 Physical Constants

The coefficients multiplying the geometric quantities in the model are physical parameters that should be guided by experimental values. We will use values collected from experiments discussed earlier in this thesis as well as other papers. We note that precise values of these physical parameters are not necessary for understanding the qualitative features of the cell mechanics we model.

Cell Height

In our model, every cell in the monolayer cluster had the same height and the total volume of the cluster remained constant. The volume of the cluster used was based on cell measurements from Chapter 4 and was $1098\mu\text{m}^3N$, where N is the number of cells in the cluster. In order to keep the cluster volume constant, the height, h , of the monolayer was recalculated at each step to account for the change in area of the cluster.

Surface Energy Constants

If a boundary has surface tension properties, then its surface energy constant should be positive; if adhesion predominates, then it should be negative. Since MDCK cells are spheres when suspended in media, the energy cost of the cell-media interaction should have a positive value. With ultimate strength measurements, the intercellular adhesion energy density per unit area in an MDCK monolayer were previously estimated as 0.07N/m [144]. We used this as the difference between cell-exterior surface energy density (Λ_{ext}) and cell-cell surface energy density (Λ_{cell}). Moreover, MDCK cells form flat sheets on solid substrates and in hanging drops, the condition $\Lambda_{\text{sub}} < \Lambda_{\text{cell}}$ should also be used. Two cell-substrate surface constants, Λ_{sub} were used to simulate the effect of increased substrate

adhesion. The values used in the model were $\Lambda_{\text{cell}}=-0.05\text{N/m}$, $\Lambda_{\text{ext}}=0.02\text{N/m}$ and $\Lambda_{\text{sub}}=-0.07\text{N/m}$ (low substrate adhesion) and $\Lambda_{\text{sub}}=-0.08\text{N/m}$ (high substrate adhesion)

Contractile Band Constant

The contractile band is more difficult to estimate; the volume and modulus of the band would be needed. The elastic modulus of an actin filament is approximately 2.2GPa [191]. The diameter of an actin filament is approximately 7nm. Estimating the band to be equivalent to 12 actin filaments, $\Gamma = A_{\text{band}}E_{\text{band}} \approx 12\pi(3.5\text{nm}^2)(2.2\text{GPa}) \approx 1 \times 10^{-6}\text{N}$.

Elastic Constants

In this study, we looked at isotropic elastic moduli and anisotropic elastic moduli with two types of alignment (Fig. 6.2):

1. To simulate cellular-level alignment, K_y was evaluated along the major length axis of each cell. Actin filaments preferentially align with the length axis on the cell [192]. When the tensile properties of smooth muscle cells isolated from rat thoracic aortas were measured using a micro tensile tester, the cells were found to be stiffer in their major length axes than their minor length axes [193].
2. To simulate cluster-level alignment, K_y was evaluated along a major axis of the cluster. Global alignment of actin in monolayers has been attributed to substrate cues [194, 195, 196] and shear flow [197].

We looked at experimental values of three dimensional elastic moduli to choose the elastic constants. A variety of cell elastic moduli have been reported ranging from 0.1 to 100kPa [198, 199, 200, 201]. Tensile testing of an epithelial monolayers revealed elastic moduli on the order of 10kPa [144]. The two dimensional equivalent of these elastic moduli should be the three dimensional elastic moduli multiplied by a characteristic length scale,

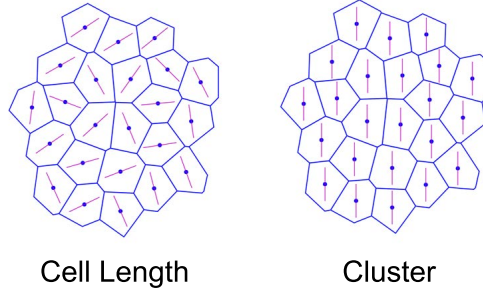


Figure 6.2: Examples of the two types of anisotropic moduli alignment used in this study. Left: cellular-level alignment, where K_y was evaluated along the major length axis of each cell. Right: cluster-level alignment, where K_y was evaluated along a major axis of the cluster. Blue lines and dots represent the outlines and centers of the cells. Magenta lines represent the axes K_y was evaluated along.

which we take to be the initial height of the cell, h_i . In our simulations, we introduced a mean elastic constant, $\bar{K}$, and an elastic moduli deviator, K_{dev} , where $K_x = \bar{K} - K_{dev}$ and $K_y = \bar{K} + K_{dev}$. We used a value of $26.7\text{kPa}h_i$ for $\bar{K}$ and varied K_{dev} for isotropic ($K_{dev} = 0$) and anisotropic ($K_{dev} \neq 0$) conditions. Since h_i depended on the starting configuration of the cluster, the value of $\bar{K}$ varied, but on average was $0.180 \pm 0.002\text{N/m}$. The other constants are difficult to measure experimentally. K_{xy} should be related to K_x and K_y by unknown values of Poisson ratios ($K_{xy} = \nu_y K_x = \nu_x K_y$, as shown in section A.1). We roughly approximated K_{xy} by assuming volume incompressibility ($\frac{\Delta V}{V_0} \approx \epsilon_{xx} + \epsilon_{yy} + \epsilon_{zz} = 0$ for small deformations), that $\epsilon_{yy} \sim \epsilon_{zz}$ for uniaxial stress in the x direction and that $\epsilon_{xx} \sim \epsilon_{zz}$ for uniaxial stress in the y direction. For uniaxial stress in the x direction, this would lead to $\epsilon_{yy} \sim -\frac{1}{2}\epsilon_{xx}$ which corresponds to Poisson ratio of $\nu_x \sim \frac{1}{2}$. Similarly ν_y would also be roughly $\frac{1}{2}$, resulting in $K_{xy} \sim \frac{1}{2}K_x$ and $K_{xy} \sim \frac{1}{2}K_y$. Although this approximation is not entirely valid for anisotropic materials, it gives a rough idea of what values of K_{xy} are acceptable. For this reason we chose to use $K_{xy} = (K_x + K_y)/4 = \bar{K}/2$ in this simulation. Since μ_s is also unavailable experimentally, we chose to use $\mu_s = \bar{K}/4$. However, the precise choices of these values would not change the qualitative nature of the results.

In our experiments, we used the cytoskeletal drugs blebbistatin and CalA that cause

changes in the elastic modulus of the cell. CalA has been found to increase the elastic modulus of cells by roughly 60% [120, 202, 203]. Blebbistatin has been found to reduce the elastic modulus of cells by 30-70% [204, 120, 106, 107, 137]. When a relaxation or contraction treatment was simulated, the elastic constant $\overline{K}$ was rescaled by the factors 0.4 and 1.6 respectively.

6.4.2 Metropolis Monte Carlo

The Metropolis- Hastings Monte Carlo Algorithm was chosen to simulate cell vertex movement. The Metropolis-Hastings (MH) algorithm was first proposed by Metropolis and Rosenbluth [205], where the method was applied in the simulation of molecular systems. It is a type of Monte Carlo simulation that samples from the Boltzmann distribution. Instead of accumulating configurations randomly, and then evaluating their probability-weighted contribution to the desired property, we can accumulate configurations according to their Boltzmann probability. This is accomplished by first making a random move, then evaluating the energy change of such a move and comparing the probability of that move against a random number. If the change of energy is negative, the move is accepted. If the energy change is positive, the Boltzmann probability is calculated and if it is larger than a randomly generated number, the move is accepted; otherwise the system is returned to its original configuration.

$$P(\Delta U) = \begin{cases} 1, & \Delta U \leq 0 \\ e^{-\Delta U/K_B T}, & \Delta U > 0 \end{cases} \quad (6.11)$$

6.4.3 Initial Set up

The initial configuration of the monolayer was determined by randomly placing disks with radius $R = 5\mu m$ in a bounding circle with radius $r = 36\mu m$. If a disk overlapped with a

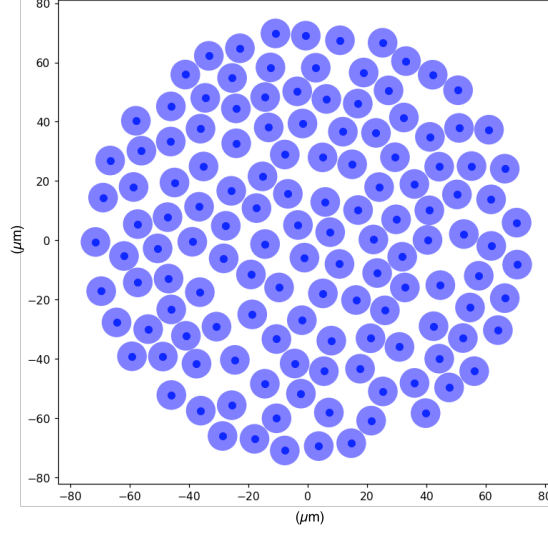


Figure 6.3: Randomly placed disks used to create a model monolayer. The disks were randomly placed in circle and performed random walks in the first step to create a model monolayer cluster.

disk previously put down or the edge of the bounding circle the placement is rejected. After the attempt to lay down a new disk, a random disk was chosen and moved a random step, avoiding other disks and the edge of the bounding circle. The step direction was chosen with a random angle and the step distance chosen with a random value (between 0 and $R/5$). If the step overlapped with another disk or the boundary, the move was rejected. This continued for 100000 steps to equilibrate the random disks (Fig 6.3).

Next, the positions of these disks were used to simulate cells in a monolayer. The positions of these disks were used as generator points to create a Voronoi diagram. Voronoi tessellation used these generator points to create a network of polygons and get the vertex positions needed for the vertex model. A Voronoi diagram is a partitioning of a plane into regions based on distance to the generator points. For each generator point there is a corresponding region consisting of all positions closer to that point than to any other. These regions are called Voronoi cells. The Voronoi cells were used to represent the cells in our monolayer. To avoid the oddly shaped Voronoi cells at the edge of the bounding circle, only the closet N cells to the centre of the circle were used. The number of cells, N ,

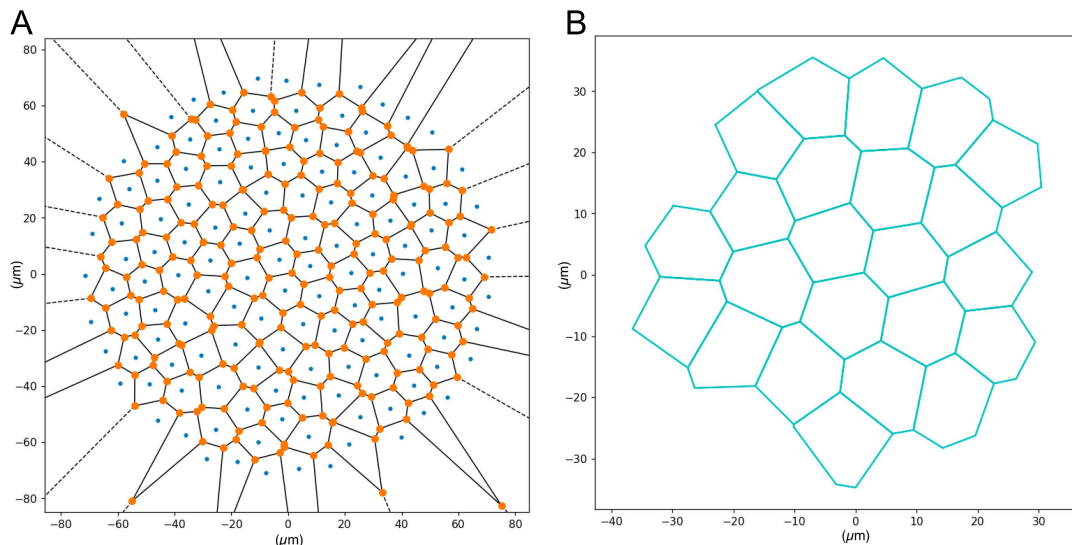


Figure 6.4: Voronoi diagram used to make a model monolayer cluster. A) A Voronoi diagram of made from the positions from the disks in Fig. 6.3. B) The closest 23 cells to the center of the diagram. These cells were used for a model monolayer cluster.

used in the simulation was 23. It was based on the average cells used in the experimental clusters in chapter 5.

6.4.4 Stabilization

Stable configurations of epithelial tissues satisfy a mechanical force balance, which can also be described as the local minima of an energy function. The energy function depends on vertex positions. In order to numerically obtain a force-balanced state, the energy is minimized with respect to the vertex positions. To stabilize the monolayer, the cluster attempted 150K steps. At each step, a vertex was moved a random distance between -0.1 and $0.1\mu\text{m}$ in both the x and y directions. Step acceptance was based on the Metropolis Monte Carlo algorithm. Additionally, if the move resulted in a self overlapping polygon, the move was rejected. These steps allowed the cluster to adopt an energetically stable configuration. To simulate relaxation/contraction of the cytoskeleton, the elastic moduli and contractile band constants scaled for each drug condition. 150K more steps were

attempted with these new parameters, allowing the cluster to stabilize again.

6.4.5 Annealing

When minimizing the energy of the cluster, the system will settle in local minima. While these configurations are stable, stable configurations at lower energies are of greater interest. To avoid getting stuck in a local minima, a simulated annealing process was added. Simulated annealing models the physical process of heating a material and then slowly lowering the temperature to decrease defects, thus minimizing the system energy. Bahrami et al. added annealing in their vertex model by linearly reducing their temperature to values close to zero [206]. The temperature reduction lowers the Metropolis acceptance probability, $e^{-\Delta U/K_B T}$, for steps uphill in energy. Additionally, the mechanical forces that drive the evolution of tissue geometry and topology are molecular in origin, and thus subject to a significant degree of fluctuation. The consequent noise may play a constructive part in the remodeling of tissue by helping to anneal the system, which might otherwise get trapped in local minima of mechanical energy. Farhadifar et al. used fluctuations in line tension to drive remodeling in the simulated annealing of their vertex model [189].

We decided to add noise to our system by adding an annealing event every 5000 steps. Our annealing events were more similar to the method used by Farhadifar et al. than Bahrami et al. in that we did not change the temperature but added a small fluctuation to the system. During this event, all of the vertices moved random distance between -0.001 and 0.001 μm in both the x and y directions. The move was accepted even if it resulted in an increase in system energy. It was only rejected if it caused a polygon to self overlap. This allowed enough noise to get out of a local minimum and move to lower system energies.

6.5 Results

6.5.1 Energy Progression during Monolayer Stabilization

As the cluster stabilizes, it is important to examine the progression of the energy terms. We performed a two step process. In the first step, the simulation is run with an isotropic elastic modulus ($K_{dev} = 0$). In the second step, the simulation is run with a higher or lower elastic modulus to simulate contraction or relaxation respectively. In each step, the simulation must run long enough for the slowest process to stabilize. In figure 6.5, each energy term is plotted per step. In addition, multiple runs from the same initial set-up were used to determine the variation in these energies. If the process is robust enough, each run should stabilize at similar energies. The cell-cell adhesion term stabilized rather quickly, while the cell-substrate and cell-exterior surface energy terms took longer. The energy of the cluster stabilized in each cluster before the elastic moduli was changed. It also stabilized before ending the simulation. The total energy plateau is lower during relaxation and higher during contraction.

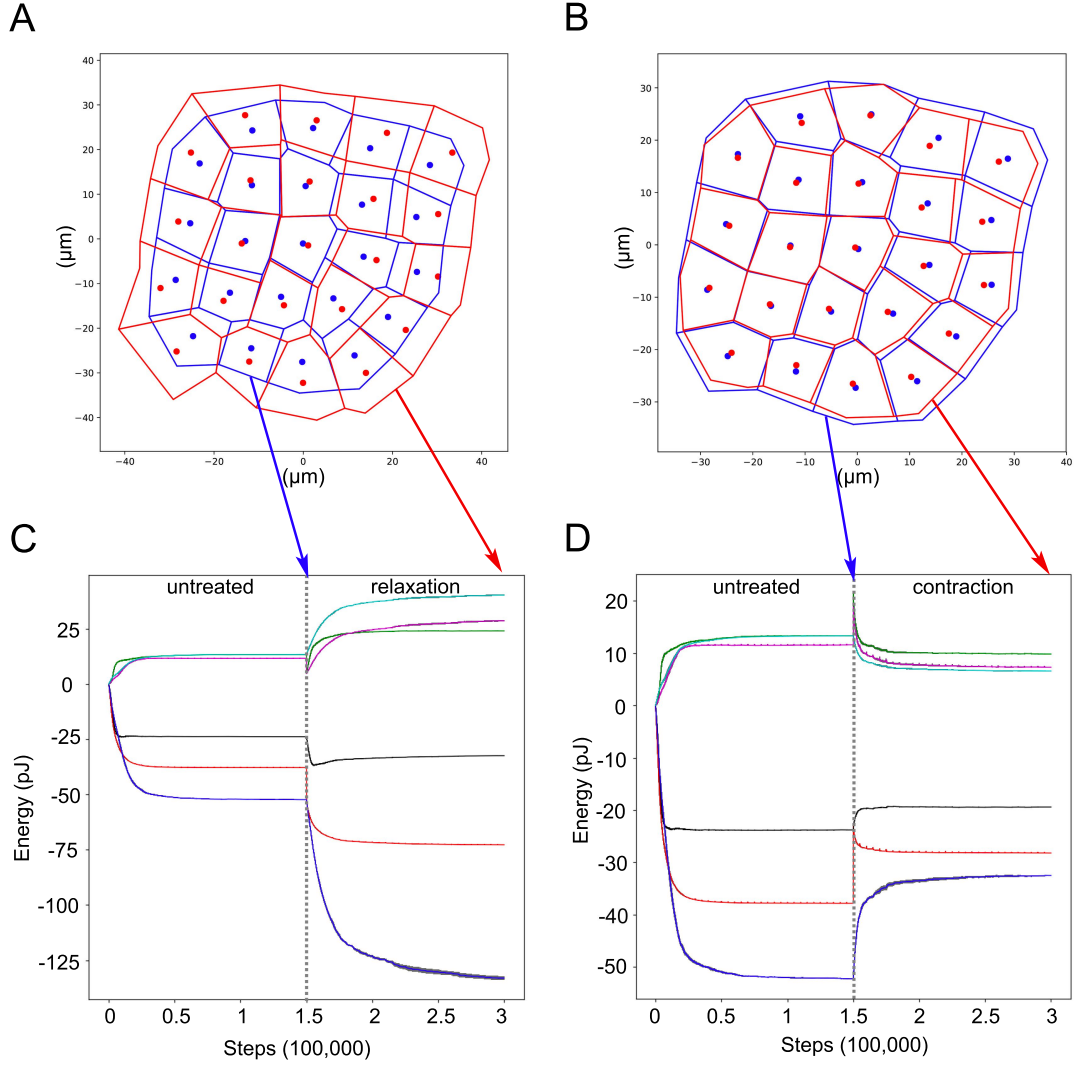


Figure 6.5: Examples of the change in morphology and system energy during relaxation and contraction. The configurations of a cluster after untreated stabilization (blue) and A) relaxation (red) and B) contraction (red). Cell outlines are displayed as polygons and the cell centers are displayed as dots. The total change in cell-cell surface (black), cell-substrate surface (blue), cell-exterior surface (cyan), elastic (magenta), contractile band (green) and total (red) energy changes are displayed as a function of steps for C) relaxation and D) contraction treatments. Standard errors bars are in grey. $\Lambda_{sub} = -0.07 \text{ N/m}$, $n=5$.

6.5.2 Cluster Morphology

The non-continuous nature of the cluster may lead to differences between the cells in the center and the edge of the cluster. The perimeters and areas of cells were examined at different distances from the center of the cluster. Distances from the center were put into $5\mu\text{m}$ bins and their means and standard errors were plotted in Fig. 6.6. The most exterior group of cells had larger perimeters and areas than all of the interior cells during relaxation ($p<0.01$). During contraction, the most exterior group of cells had larger perimeters than the second to sixth most exterior groups ($p<0.01$). They also had larger areas than the second to fourth most exterior groups ($p<0.05$).

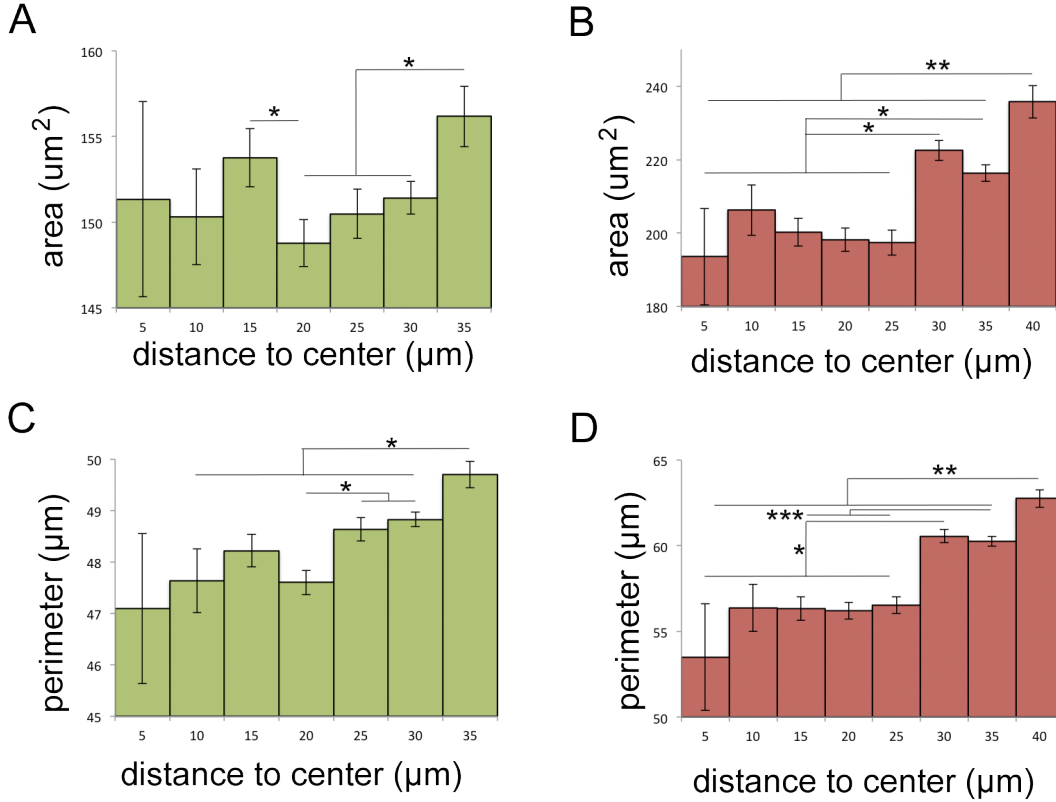


Figure 6.6: Areas and perimeters of cells during contraction and relaxation at various distances to the cluster center. Cell area after A) contraction and B) relaxation treatments. Cell perimeters after C) contraction and D) relaxation treatments. Distances are in $5\mu\text{m}$ bins. $\Lambda_{sub}=-0.07\text{N/m}$, $n=52$. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

6.5.3 Strain in the Monolayer

In chapter 5, we investigated the effect of cytoskeletal drugs on the deformation of MDCK monolayer clusters in the absence of a mechanically supportive substrate. The cytoskeletal drugs, blebbistatin and CalA, were found to relax and contract the cell clusters, respectively (Fig 6.7 A). With our computational model, an isotropic elastic modulus ($K_{dev} = 0$) and cell-substrate surface energy constant, $\Lambda_{sub} = -0.07\text{N/m}$ were used in simulations of low substrate adhesion (Fig. 6.7 B). The relaxation condition produced higher strains than the experimental blebbistatin results ($p < 0.001$). However, the mean strain during contraction was not significantly different of the experimental CalA values ($p > 0.05$).

One major difference between cells on solid substrates and unsupported hanging drops should be the substrate adhesion energy. Experimentally, we did not directly observe significant strain in the monolayers grown on PA gels or glass. To investigate monolayer dependence on substrate adhesion, a second cell-substrate surface energy constant -0.080N/m was used to simulate higher substrate adhesion. Decreasing the cell-substrate surface energy constant increased the strain observed in relaxation and decreased the strain observed in contraction ($p < 0.001$) (Figure 6.7C). However, the mean strain for contraction was still not significantly different than the experimental values for CalA ($p > 0.05$). The strains for relaxation were still significantly larger than the experimental values for blebbistatin ($p < 0.001$). Therefore, when substrate adhesion was increased, it increased the magnitude of the strain observed in the model with both contraction and relaxation.

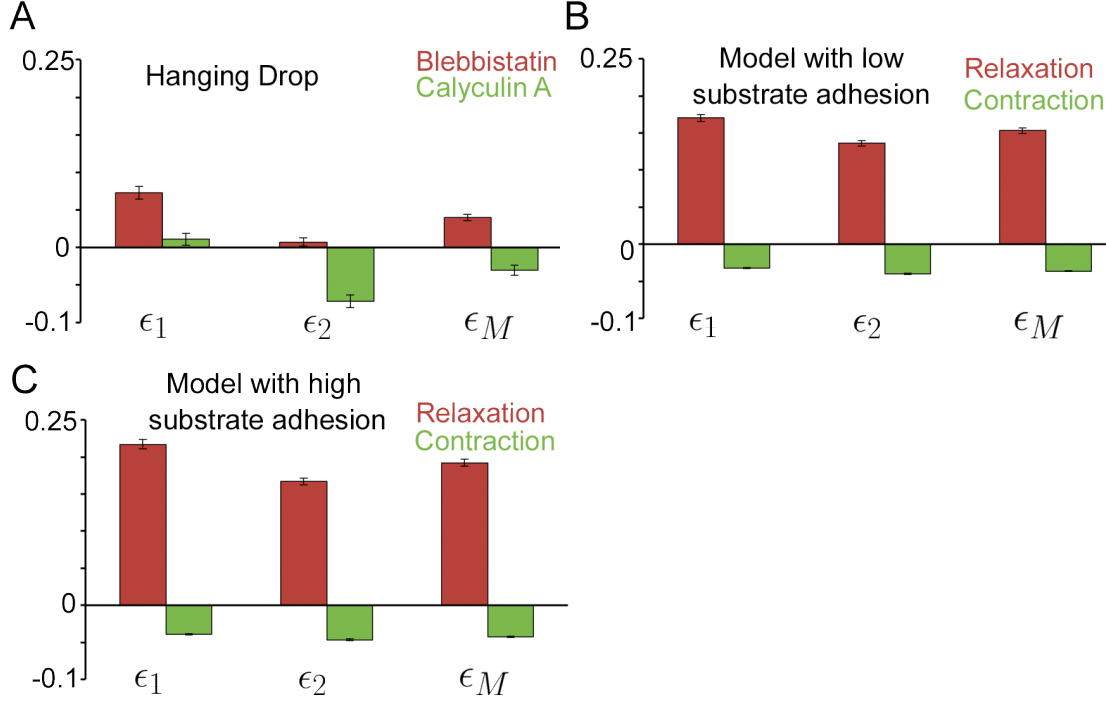


Figure 6.7: Deformation of the experimental and modeled clusters with cytoskeletal drugs. A) Major (ϵ_1), minor (ϵ_2) and mean (ϵ_M) strains of cell clusters in hanging drops after the addition of blebbistatin (red) or calyculin A (green). $n=22$. B) Major (ϵ_1), minor (ϵ_2) and mean (ϵ_M) strains of modeled cell clusters after relaxation (red) or contraction (green) with a cell-substrate surface energy constant, Λ_{sub} , of -0.07N/m . $n=15$. C) Major (ϵ_1), minor (ϵ_2) and mean (ϵ_M) strains of modeled cell clusters after relaxation (red) or contraction (green) with a cell-substrate surface energy constant, Λ_{sub} , of -0.08N/m . $n=15$.

If the cells are anisotropic they should have a higher elastic modulus in one direction. For this reason, an elastic modulus deviator, K_{dev} was introduced where the moduli in the major direction was $K_y = \bar{K} + K_{dev}$ and the moduli in the minor direction was $K_x = \bar{K} - K_{dev}$. The simulations were run with deviators of 0 (isotropic), $0.25\bar{K}$, $0.5\bar{K}$ and $0.75\bar{K}$ (Fig. 6.8). Two methods of assigning the elastic moduli major axis were used in these simulations. In one set of simulations the length axis of the cell was used, the elastic modulus being larger in the major length axis of the cell to simulate cellular level alignment. In the other, a major axis of the cluster was used to simulate cluster level alignment. When the elastic modulus major axis was aligned to the major cell length axis, increasing the deviator decreased the strain in relaxation and increased the strain in contraction. Since

the strain in a contracting cluster is negative, an increase in strain represents a smaller response to the moduli change. When the elastic modulus major axis was aligned to a major axis of the cluster, increasing the deviator increased the mean strain in relaxation and decreased the strain in contraction ($p < 0.001$).

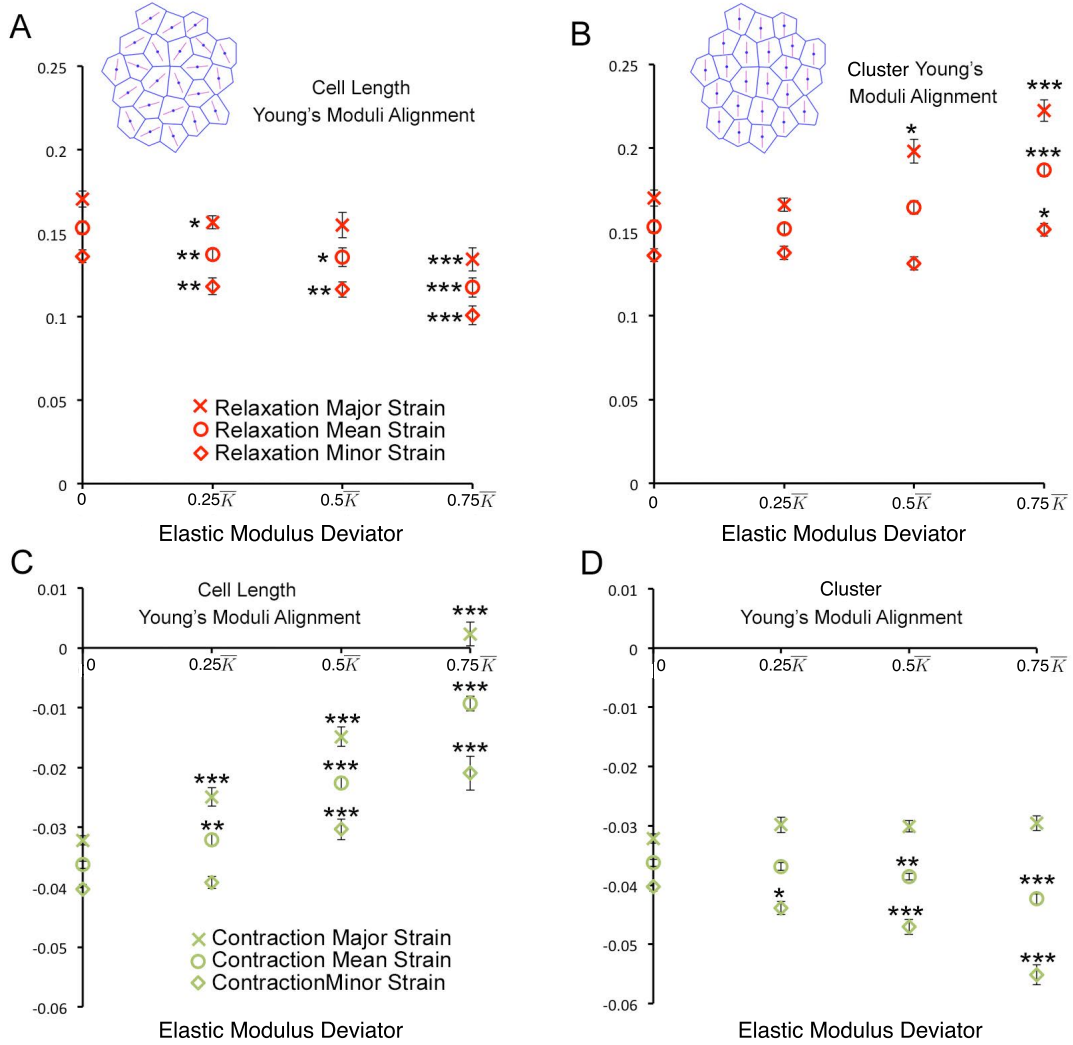


Figure 6.8: Deformation of the clusters with varying elastic modulus deviators. Major (red x), mean (red circle) and minor (red diamond) cell strains of modeled cell clusters after relaxation with A) elastic moduli aligned to each cell's length axis and B) elastic moduli aligned to a major axis of the cluster. Diagrams of the type of alignment are displayed with cells outlines in blue and the elastic modulus major axes in magenta. Major (green x), mean (green circle) and minor (green x) cell strains of modeled cell clusters after contraction with C) elastic moduli aligned to each cell's length axis and D) elastic moduli aligned to a major axis of the cluster. $\Lambda_{sub} = -0.07\text{N/m}$, $n=15$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

6.5.4 Feature Orientation

In order to determine if the deformation of the cluster exhibited a dependence on structural features of the cells, nematic-like order parameters were calculated as described in section 3.6.3. These order parameters were calculated with the structural features of the clusters before they were treated. These features included the major length axis of the nuclei and the major length axis of the cluster. These were compared with the axis of major strain in the cluster. These order parameters were used to determine if there was alignment in the cluster and if it influenced the direction of major strain in the cluster after treatment.

The order parameters for nuclear alignment with strain (S_{ns}) were calculated for all the clusters in our experiments (Fig. 6.9 A). The order parameters in the hanging drops were not significantly different than 0, suggesting no preferential alignment. However on the compliant substrate (PA gels), the order parameters of treated clusters' nuclei were significantly different than 0; the nuclei tended to be aligned with the relaxation and contraction axes. This demonstrated that there was a correlation between nuclear alignment and cluster strain on PA gels that was not observed in hanging drops.

The nuclei of single epithelial cells grown on flat rigid surfaces have been previously found to align with the major length axis of the cell [177]. Since our model does not include nuclei orientation, we will use the length axis of the cells as a substitute. An elastic modulus deviator of $0.5\overline{K}$ was used ($K_y=1.5\overline{K}$, $K_x=0.5\overline{K}$) for the anisotropic conditions, making one modulus three times larger than the other. We started with our higher cell-substrate surface energy constant, Λ_{sub} , of -0.07N/m, to mimic low substrate adhesion.

When the cells were isotropic or the elastic modulus major axis aligned with the length axis of the cell, the order parameters were not significantly different than 0, suggesting no preferential alignment (Fig. 6.9 B). However, when the elastic moduli aligned to the a major axis of the cluster, the S_{ns} order parameters of treated clusters nuclei were significantly different than 0 ($p<0.001$). In the relaxing clusters, the cell length axes preferentially

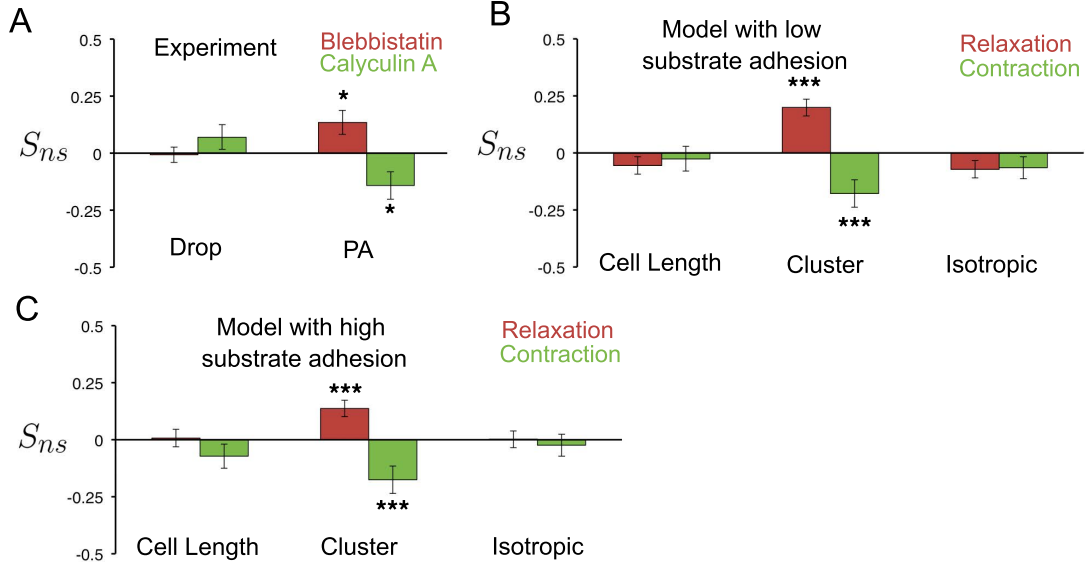


Figure 6.9: Order parameters, S_{ns} , for cell alignment to strain in experimental and modeled clusters. A) Order parameters, S_{ns} (defined by the angle between the major strain axis and the nuclear major axis) from experiments performed in chapter 5. Parameters for clusters grown in drops and on PA with blebbistatin (red) and calyculin A (green) are displayed. Order parameters, S_{ns} , for nuclei (defined by the angle between the major strain axis and the cellular major length axis) for relaxation (red) and contraction (green) in simulations with varying alignment of the elastic modulus axis and a cell-substrate surface energy constant, Λ_{sub} , of B) -0.07N/m and C) -0.08N/m . An elastic modulus deviator of $0.5\bar{K}$ was used for cell length and globally aligned cells, and an elastic modulus deviator of 0 was used for the isotropic conditions. $n=15$, $*p<0.05$, $***p<0.001$.

aligned with the major strain axis. In the contracting clusters, the cell length axes preferentially aligned with the minor strain axis. When we used our lower cell-substrate surface energy constant, Λ_{sub} , of -0.06N/m , to mimic high substrate adhesion, there was no significant difference in the order parameters ($p>0.05$) (Fig. 6.9 C). The nuclear alignment to strain found experimentally in monolayers grown on PA gels most closely resembled the cases where the elastic moduli major axes were aligned to a major axis of the cluster. The alignment of the cells to strain was not present when the mechanical properties of cells were only aligned at a cellular level, suggesting some form of global alignment is needed.

In our experimental investigations, the order parameters between nuclei (S_{nn}) were calculated to examine the alignment of cells with their neighbors (Fig. 6.10 A). These

were calculated using the angles formed between the major axis of each nucleus and the major axis of its neighbor. We found that cells in hanging drops were only aligned with their nearest neighbors, while cells on substrates were aligned with their next nearest and third nearest neighbors as well. This suggested that the cells on substrates aligned with cells that were near to them even if they were not in direct contact. Since this was not seen for cells in hanging drops, it suggested longer range cell alignment occurs only in monolayer clusters on solid substrates.

In the simulations of nearest neighbor alignment, the cell major length axis was again substituted for the nuclei major length axis. The condition where the elastic modulus major axes were aligned to a major axis of the cluster most closely resembled the experimental results for clusters on substrates (Fig. 6.10 B). The order parameter was significantly greater than zero for the nearest neighbors, as well as next nearest and third nearest neighbors ($p < 0.001$). The condition with isotropic elastic moduli resembled the experimental results for clusters in hanging drops. The order parameter was significantly greater than zero for the nearest neighbors ($p < 0.01$) but not for more distant neighbors ($p > 0.05$). This suggests the longer range nuclear alignment observed experimentally on substrates was a result of global cellular elastic moduli alignment and that the nearest neighbor alignment seen on hanging drops was a result of isotropic moduli.

The order parameters between nuclei were also calculated in the modeled clusters with higher substrate adhesion (Fig. 6.10 C). The condition where the elastic modulus major axes were aligned to a major axis of the cluster most closely resembled the experimental results for clusters on substrates. The order parameter was significantly greater than zero for the nearest neighbors, as well as next nearest and third nearest neighbors ($p < 0.01$). This still suggests the long-range nuclear alignment observed experimentally on substrates was a result of global cellular elastic moduli alignment. The experimental results for clusters in hanging drops did not match our model with high substrate adhesion very well. When substrate adhesion was increased the isotropic cells were no longer aligned to their

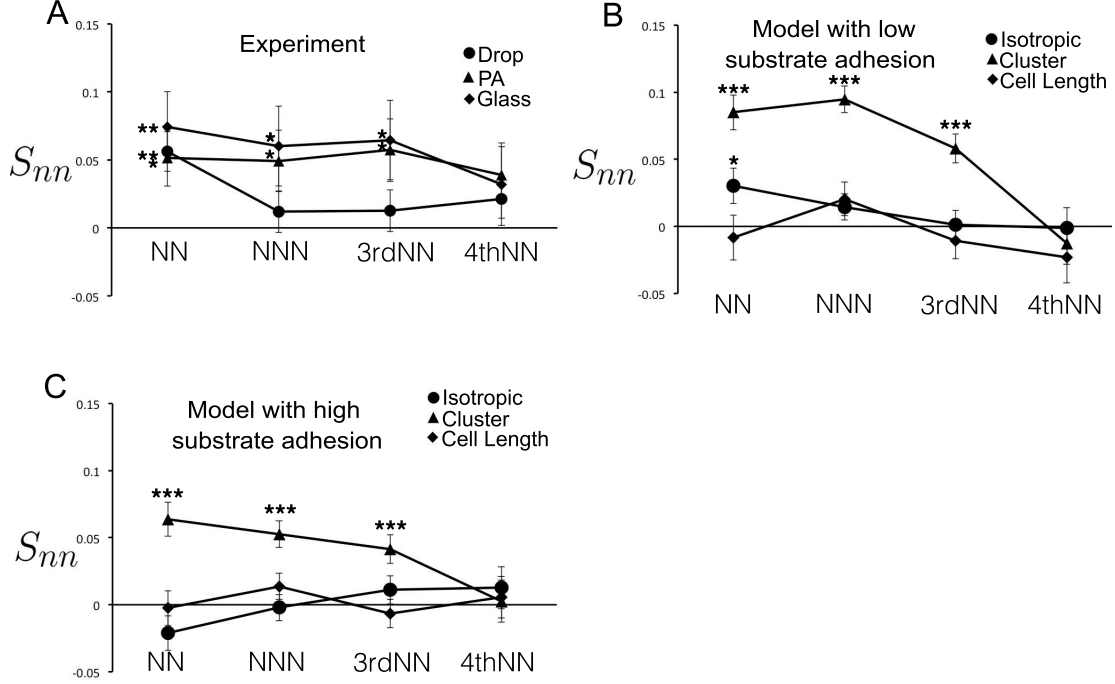


Figure 6.10: Order parameters, S_{nn} for cell alignment in experimental and modeled clusters. A) Order parameters, S_{nn} (defined by the angle between the nuclear major axis and its neighbors nuclear major axis) in hanging drops (circles), on PA (triangles) and glass (diamonds). The results are shown for nearest, next nearest, third nearest and fourth nearest neighbors. Order parameters, S_{nn} (defined by the angle between the cell's major length axis and its neighbors major length axis) in simulations with varying elastic moduli and cell-substrate surface energy constants, Λ_{sub} , of B) -0.07N/m and C) -0.08N/m . An elastic modulus deviator of 0 was used for the circles. An elastic modulus deviator of $0.5\bar{K}$ and an elastic moduli major axis aligned to a major axis of the cluster (triangles) and to the cell's major length axis (diamonds). $n=30$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

nearest neighbors ($p>0.05$). This suggests lower substrate adhesion is needed to model the hanging drop clusters.

6.6 Discussion

An understanding of how the molecular organization of the actin network and its interplay with adhesive complexes contribute to the mechanical properties of cells is required for explaining many phenomena in tissue mechanics. The objective of this study was to develop a model to study epithelial monolayer clusters that incorporates anisotropic cell

mechanical properties and substrate adhesion. Despite the complexity of the molecular mechanics underlying actomyosin contractility, we show that a model with minimal physical assumptions qualitatively captures the remodeling associated with a change in contractility, producing similar strains and cellular alignment to what was observed *in vitro*. In doing so, we have identified the alignment of cellular elastic moduli required to produce the cellular alignment of monolayers grown on supportive and unsupportive substrates.

We found that the mean strain during contraction in our simulations was not significantly different than the experimental values for CalA treated monolayer clusters in hanging drops. Thus, the parameters used in the simulations did a good job at modeling the effect of this cytoskeletal drug. However, the simulated relaxation conditions studied produced higher strains than the experimental blebbistatin results, possibly as a result of too large a decrease in the value of $\overline{K}$ for this case. Experimental studies have shown a large variation in the factor reducing the elastic modulus [204, 120, 106, 107, 137]. Although the strain observed in our model was larger than observed experimentally, it still demonstrated the qualitative effects of a relaxing monolayer. Therefore, it is still useful in illustrating phenomena associated with the effects of relaxation in a monolayer.

When substrate adhesion was increased in our model, it increased the magnitude of strain observed in both contraction and relaxation simulations. Since stiff substrates increase adhesion [4, 5, 6, 7] and traction forces increase with substrate stiffness [207], clusters grown on substrates with higher adhesion energies (like PA or glass) should have higher strain magnitudes than substrates with lower adhesion energies (like hanging drops). However experimentally, we did not directly observe significant strain in the monolayers grown on PA gels or glass. Remodeling was likely muted by the presence of a solid substrate, demonstrating that cluster contraction was significantly restrained when bound to a sufficiently rigid surface.

Our model did not account for the shorter-timescale pinning of the cells to the substrate. The model does not include focal adhesion formation; it only had a term proportional to

adhesion area. It allowed all cells to move freely regardless of the substrate adhesion. A more detailed model would include events simulating the formation and disassembly of focal adhesions. Therefore, what we observed in our model is more like what would be seen on substrates if the cells were not pinned or what would be seen at longer timescales.

Interestingly, there was a correlation between major axis strain direction and cellular alignment for monolayer clusters with elastic moduli aligned to a major axis of the cluster. However, for clusters with isotropic elastic moduli or moduli aligned with length axis of the cell, the direction of the major strain was independent of cellular alignment. In chapter 5, we proposed that the lack of correlation in monolayers grown in hanging drops may be due to reduced alignment of actin within each cell and the reduction in longer-range correlated cell-cell alignment that was observed on rigid substrates. Our simulations rule out the first explanation since our anisotropic cells aligned to the length axis of the cell had the same lack of correlation. Thus, our simulations suggest that global alignment of elastic moduli is needed for cellular alignment to monolayer strain; cells being aligned at the cellular level is insufficient. The clusters grown on the compliant substrate likely possessed some sort of substrate-mediated alignment producing this correlation. Since this was not observed in the clusters grown in hanging drops, they likely lacked this global alignment.

In addition, by calculating cellular alignment order parameters, we found that cells with elastic moduli aligned to a major axis of the cluster were aligned with their nearest, next nearest and third nearest neighbors. This was the same persistence of correlation observed experimentally in nuclear order parameters of cells on substrates. This global alignment may be a result of substrate cues not present in hanging drops. This supports the hypothesis proposed in chapter 5 that long-range orientational correlations are the result of elastic coupling of cells mediated by deformation of the compliant substrate. We also observed that cells with isotropic elastic moduli were aligned with their nearest neighbors, the same correlation that was observed experimentally in cells grown in hanging drops. This did not occur in simulations with high substrate adhesion. Our results suggest that

the alignment observed in monolayers grown in hanging drops was a result of low substrate adhesion and an isotropic elastic moduli in the cells. When the effect of cell substrate interactions is lowered, cell-cell interactions bear a greater role in regulating the system. This may be why the short range alignment was not observed in the monolayers with higher substrate adhesion. It has also been shown that matrix stiffness aids in regulating the polarization and alignment of stress fibers within cells [178]. The lack of a solid substrate in cells grown at an air-water interface likely resulted in weak alignment of stress fibers creating a more isotropic elastic environment.

6.7 Conclusion

Our model proves to be a useful aid in explaining the qualitative features of the mechanodynamics of monolayer clusters grown at air-water interfaces. The results presented highlight the importance of the substrate and its corresponding effect on cytoskeletal structure in monolayer mechanics. We determined that the long-range alignment we observed in monolayer clusters on substrates were likely a result of the elastic moduli of the cells being globally aligned. Cellular alignment of the moduli to the length axes of the cells was not sufficient to create this alignment. The short range alignment observed in clusters grown in hanging drops was likely a result of isotropic elasticity and low substrate adhesion. Substrates affect both adhesion and the structure of the cytoskeleton. Since the model allows for both the alteration of substrate adhesion and modulus of the cell, it should effectively model a variety of experiments where the substrate is altered. Although only two simple types of alignment were investigated in this chapter, more complex alignments could be studied as well. With the ability to vary the anisotropy and alignment of elastic moduli, it will be possible to test how internal forces cooperate to drive the variety of forms and patterns observed during tissue remodeling.

Chapter 7

Conclusion

In this thesis, we have demonstrated the importance of cell-substrate interactions in epithelial monolayers. We found that the presence of a substrate effects the response to oncogenic proteins as well as the basal mechanics and organization of monolayers.

7.1 Summary of Results

Our work reveals that constitutively active Ras has opposite effects on the relative stiffness of attached and unattached epithelial cells (Chapter 4). We found that Ras^{V12} cells were stiffer than WT cells when grown in a monolayer. In suspended cells, the Ras^{V12} cells were found to be softer than WT cells. This demonstrated that the adhesions associated with cells being in monolayer were necessary for the Ras^{V12} cells to be stiffer than WT cells. The stiffening of Ras^{V12} cells in monolayer co-culture was likely a result of increased pre-stress and cortical tension from actomyosin contraction. The effect of Ras on adherent cells was reversed when contractility was inhibited with a ROCK inhibitor, resulting in softer Ras^{V12} cells. The mechanical mismatch observed in monolayer was also accompanied with apical extrusion and basal protrusions. When the transformed cells were stiffer than the surrounding cells, they were more likely to basally protrude than apically extrude. When

the transformed cells were softer than the surrounding cells due to a ROCK inhibitor, they were more likely to apically extrude than basally protrude.

We created a new methodology (Chapter 5) and a new computational model (Chapter 6) to characterize actomyosin mechanodynamics in multicellular systems. The new methodology's potential was validated by observing and quantifying the generation and release of internal stresses upon actomyosin contraction and relaxation in response to chemical treatments. We found that even in the absence of a solid substrate, there are still controlled mechanical responses to cytoskeletal drugs. When similar monolayers were grown on solid supports, we found that the strain was aligned to the nuclei in the monolayers. This effect was not observed in the unsupported monolayers. We also found that the monolayers grown on solid substrates had longer range nuclear alignment than the monolayers grown at an air-water interface. This demonstrated that a solid substrate is needed for higher order cellular alignments. Our computational model was validated by comparing the strains in our simulated monolayers to the strains found experimentally. It demonstrates that the alignment of nuclei to strain and the long range cellular alignment that we observed in cells grown on substrates were likely a result of cells having anisotropic elastic's moduli with global alignment. The lack of alignment of nuclei to strain and short range cellular alignment we observed in unsupported monolayers was presumably a result of the cells having low substrate adhesion and isotropic elastic moduli.

7.2 Significance

Cancer is accompanied by specific changes in the mechanical properties of cells. The mechanical mismatch of Ras transformed cells, observed in chapter 4, probably aids cancer progression. It would seem to play an energetic role in the extrusion of the cells from the monolayer. The monolayer extrusion mechanism presumably favors basal protrusion for stiffer cells and apical extrusion for softer cells. The increased cell compliance, that was

observed with the expression of Ras in unattached cells likely aids in the metastasis of transformed cells when entering the blood stream and traveling to a secondary site.

Substrate adhesions also play a role in cell shaping, tissue patterning and embryonic development [23, 208]. During development, embryonic morphogenesis is in large part due to changes in the organization and mechanics of epithelial monolayers. Our results in chapters 5 and 6 suggested that a supportive substrate are required for long range alignment of cellular features and elastic moduli in monolayers. This could be the result of elastic coupling of cells mediated by the deformation of the substrate or another form of cell signaling.

7.3 Outlook

This work reveals the importance of substrate and intercellular adhesion and provides tools to investigate it further. In chapter 4, our findings demonstrated that the mechanical mismatch between Ras transformed cells and untransformed cells was dependent on both the degree of attachment and the expression of ROCK. This information could be used to further understand the effect of Ras on cell mechanics and potentially aid in the development of treatments that target them. Similar experiments could also be performed to investigate the mechanical effects of the other Ras pathways by inhibiting their downstream targets.

The methodology presented in chapter 5 is a novel tool that can be used to infer how the presence of a substrate impacts contractility and long-range multi-cellular organization. As long as the imaging time is kept low enough to avoid evaporation, this methodology can be utilized for a broad range of experiments. These hanging drop monolayers could be made with epithelial cells stably expressing a fluorescently marked protein of interest. For instance, intermediate filaments could be stained to investigate the dependance of a supporting substrate on their structure. This methodology could also be used to determine how epithelial cells expressing constitutively active Ras in monolayers with untransformed

cells behave without mechanically supporting substrates.

The computational model presented in chapter 6 builds upon existing vertex models by adding more complex elastic moduli to the cells. It has the ability to vary substrate adhesion and the anisotropy and alignment of elastic moduli to effectively model a variety of experiments where the substrate is altered. This model could be applied to predictive pattern formation. For example, it could be used to determine what mechanical alignment would be required to recreate the complex patterns found in epithelial monolayers like the ones found in insect wings. Both these new tools could potentially help characterize many more of the effects of a substrate on monolayer mechanics.

APPENDICES

Appendix A

2D Elastic Theory

A.1 Elastic Energy in Two Dimensions

Here we describe the 2D elastic theory used in Chapter 6. It is based on chapter 5 and appendix D of "Mechanics of the Cell" by David Boal [209], and guidance by Professor Bela Joos from the University of Ottawa.

A.1.1 Strain

The strain tensor, ϵ , quantifies the deformation of an object with the derivatives of the displacement vector $\mathbf{u}$ as a function of position $\mathbf{x}$. It is calculated with the displacement gradient, which can be expressed as $u_{ij} = \frac{\partial u_i}{\partial x_j}$:

$$\epsilon_{ij} = \frac{1}{2} (u_{ij} + u_{ji} + u_{ki}u_{kj}) \quad (\text{A.1})$$

$$\rightarrow \epsilon_{xx} = u_{xx} + \frac{1}{2} (u_{xx}^2 + u_{yx}^2) \quad (\text{A.2})$$

$$\rightarrow \epsilon_{yy} = u_{yy} + \frac{1}{2} (u_{yy}^2 + u_{xy}^2) \quad (\text{A.3})$$

$$\rightarrow \epsilon_{xy} = \frac{1}{2} (u_{xy} + u_{yx} + u_{xx}u_{xy} + u_{yx}u_{yy}) \quad (\text{A.4})$$

The strain tensor is symmetric in indices i and j making $\epsilon_{xy} = \epsilon_{yx}$.

A.1.2 Stress

An object deforms in response to external forces. The forces on the object are represented with the stress tensor, σ . The physical units of the stress tensor depend on the dimensionality of the system. The stress tensor has units of force per length for 2D and force per

area for 3D. For that reason we define the stress tensor in terms of the change in energy density, W .

$$\sigma_{ij} = \frac{\partial W}{\partial \epsilon_{ij}} \quad (\text{A.5})$$

Just as with the strain tensor, the stress tensor is symmetric in indices i and j , making $\sigma_{xy} = \sigma_{yx}$.

A.1.3 Strain Energy

The strain energy of a two dimensional network depends on the stress σ and strain ϵ tensors. For ideal springs in one dimension the restoring force is proportional to the displacement from equilibrium ($F = -kx$) where k is the spring constant. The corresponding relationship for a 2D continuous material is:

$$\sigma_{ij} = \sum_{k,l} C_{ijkl} \epsilon_{kl} \quad (\text{A.6})$$

where material specific constants, C_{ijkl} , are called the elastic stiffness constants or elastic moduli. The strain energy density of a continuous object under deformation is:

$$W = \frac{1}{2} \sum_{i,j,k,l} C_{ijkl} \epsilon_{ij} \epsilon_{kl} \quad (\text{A.7})$$

The elastic constants have dimensions of energy per unit volume for three dimensional materials or energy per unit area for two dimensional systems. Many of these constants are related by symmetry. Because ϵ_{ij} is symmetric under exchange of i and j and ϵ_{kl} is symmetric under exchange of k and l , then $C_{ijkl} = C_{jikl} = C_{ijlk} = C_{jilk}$. Since $\epsilon_{ij}\epsilon_{kl}$ is symmetric under exchange of the pairs of indices ij and kl , $C_{ijkl} = C_{klij}$. In 2D, this leaves 6 independent moduli:

$$\begin{aligned} &C_{xxxx} \\ &C_{yyyy} \\ &C_{xxyy} = C_{yyxx} \\ &C_{xyxy} = C_{yxyx} = C_{xyyx} = C_{yxxy} \\ &C_{xxxy} = C_{xxyx} = C_{xyxx} = C_{yxxx} \\ &C_{yyyx} = C_{yyxy} = C_{yxyy} = C_{xyyy} \end{aligned} \quad (\text{A.8})$$

We will look at materials with two fold symmetry. For materials with 2 fold symmetry (Fig. A.1), the operation $x \leftrightarrow -x$ or $y \leftrightarrow -y$ forces constants with an odd number of x or y indices to vanish (last two independent moduli). Consequently, these systems have the

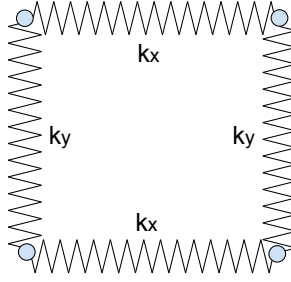


Figure A.1: Two-dimensional network of springs with unequivocal spring constants. k_x and k_y . This is an example of a network with two fold symmetry.

energy density of the form:

$$W = \frac{1}{2} (C_{xxxx}\epsilon_{xx}^2 + C_{yyyy}\epsilon_{yy}^2 + 2C_{xxyy}\epsilon_{xx}\epsilon_{yy} + 4C_{xyxy}\epsilon_{xy}^2) \quad (\text{A.9})$$

We will rename the constants ($K_x = C_{xxxx}$, $K_y = C_{yyyy}$, $K_{xy} = C_{xxyy}$ and $\mu_s = C_{xyxy}$), making the energy density equation:

$$W = \frac{1}{2} (K_x\epsilon_{xx}^2 + K_y\epsilon_{yy}^2 + 2K_{xy}\epsilon_{xx}\epsilon_{yy} + 4\mu_s\epsilon_{xy}^2) \quad (\text{A.10})$$

The stress tensor can be obtained from $\sigma_{ij} = \frac{\partial W}{\partial \epsilon_{ij}}$. If this is applied to the free energy of Eq. A.10, the diagonal components of the stress tensor are:

$$\sigma_{xx} = K_x\epsilon_{xx} + K_{xy}\epsilon_{yy} \quad (\text{A.11})$$

$$\sigma_{yy} = K_y\epsilon_{yy} + K_{xy}\epsilon_{xx} \quad (\text{A.12})$$

Experimentally, one can measure a Young's modulus, E , and Poisson ratio, ν , in each direction by observing the strain arising from a uniaxial stress imposed upon an object. The first three elastic constants K_x , K_y and K_{xy} can be replaced by the combinations (E_x, ν_x) and (E_y, ν_y) , although a complete description of the elastic behavior still requires μ_s . Suppose that the force is only applied along the x-axis ($\sigma_{xx} \neq 0$, $\sigma_{yy} = 0$). Then, the left-hand side of Eq. A.12 vanishes, permitting ϵ_{yy} to be solved in terms of ϵ_{xx} .

$$\epsilon_{yy} = -\frac{K_{xy}}{K_y}\epsilon_{xx} \quad (\text{A.13})$$

Poisson ratio for stress in the x-direction is $\nu_x = -\frac{\epsilon_{yy}}{\epsilon_{xx}}$, making:

$$\nu_x = \frac{K_{xy}}{K_y} \quad (\text{A.14})$$

Young's modulus is defined from the stress-strain relationship under uniaxial stress. For stress in the x direction this is $\sigma_{xx} = E\epsilon_{xx}$. If we sub Eq. A.13 into Eq. A.11, we get:

$$\sigma_{xx} = \left(K_x - \frac{K_{xy}^2}{K_y} \right) \epsilon_{xx} \quad (\text{A.15})$$

and

$$E_x = \frac{K_x K_y - K_{xy}^2}{K_y} \quad (\text{A.16})$$

If we repeat this with uniaxial stress in the y direction ($\sigma_{yy} \neq 0$ and $\sigma_{xx} = 0$), we find:

$$\nu_y = \frac{K_{xy}}{K_x} \quad (\text{A.17})$$

$$E_y = \frac{K_x K_y - K_{xy}^2}{K_x} \quad (\text{A.18})$$

With these equations A.14, A.16, A.17 and A.18 we can write the elastic constants in terms of Young's moduli and poisson ratios.

$$K_x = \frac{E_x}{1 - \nu_y \nu_x} \quad (\text{A.19})$$

$$K_y = \frac{E_y}{1 - \nu_y \nu_x} \quad (\text{A.20})$$

$$K_{xy} = \frac{E_x \nu_y}{1 - \nu_y \nu_x} = \frac{E_y \nu_x}{1 - \nu_y \nu_x} \quad (\text{A.21})$$

A.2 Average Displacement Gradient in a Polygon

When strain in a polygon is calculated by least squares fit at each vertex step, it can take a lot of computation time. A more efficient alternative, the Krut-Rothenburg method of finding the average displacement gradient in the polygon, was used [190] to calculate average displacement gradients in the polygons.

The displacement gradient can be expressed as $u_{ij} = \frac{\partial u_i}{\partial x_j}$. According to Gauss's theorem, the average of displacement gradient in the area, A , can expressed as the surface integral

$$\bar{u}_{ij} = \frac{1}{A} \int_A u_{ij} dA = \frac{1}{A} \int_s u_i n_j ds \quad (\text{A.22})$$

where n_j is the normal vector to the surface which can be rewritten as $n_j = \frac{dx_k}{ds} e_{jk}$, where e_{jk} is the 2D permutation tensor ($e_{xy} = 1, e_{yx} = -1, e_{xx} = e_{yy} = 0$). By substituting the equation for the normal vector and using integration by parts of a closed path, the average

of displacement gradient can now be represented as:

$$\begin{aligned}\bar{u}_{ij} &= \frac{e_{jk}}{A} \int_s u_i \frac{dx_k}{ds} ds \\ \Rightarrow \bar{u}_{ij} &= \frac{-e_{jk}}{A} \int_s \frac{du_i}{ds} x_k ds\end{aligned}\tag{A.23}$$

With the assumption that the displacement gradient is linear on the cell edge, the functions x_k and u_i along the edge length s_0 going from vertex p to vertex q can be written out as:

$$x_k(s) = x_k^p + \frac{(x_k^q - x_k^p)s}{s_0}\tag{A.24}$$

$$u_i(s) = u_i^p + \frac{(u_i^q - u_i^p)s}{s_0}\tag{A.25}$$

Each edge of the polygon would then contribute:

$$\begin{aligned}\frac{-e_{jk}}{A} \int_{s_{\text{edge}}} \frac{du_i}{ds} x_k ds &= \frac{-e_{jk}}{A} \int_{s_{\text{edge}}} \left(\frac{(u_i^q - u_i^p)}{s_0} \right) \left(x_k^p + \frac{(x_k^q - x_k^p)s}{s_0} \right) ds \\ \Rightarrow \frac{-e_{jk}}{A} \int_{s_{\text{edge}}} \frac{du_i}{ds} x_k ds &= \frac{-e_{jk}}{A} (u_i^q - u_i^p) \left(\frac{x_k^p s}{s_0} + \frac{(x_k^q - x_k^p)s^2}{2s_0^2} \right) \Big|_0^{s_0} \\ \Rightarrow \frac{-e_{jk}}{A} \int_{s_{\text{edge}}} \frac{du_i}{ds} x_k ds &= \frac{-e_{jk}}{A} (u_i^q - u_i^p) \left(x_k^p + \frac{(x_k^q - x_k^p)}{2} \right) \\ \Rightarrow \frac{-e_{jk}}{A} \int_{s_{\text{edge}}} \frac{du_i}{ds} x_k ds &= \frac{-e_{jk}}{A} \Delta u_i^c x_k^c\end{aligned}\tag{A.26}$$

where x_k^c is the center of the edge and Δu_i^c is the relative displacement of the vertices. The discrete formulation of average displacement gradient in terms of relative displacements at the edges is then:

$$\bar{u}_{ij} = \frac{-e_{jk}}{A} \sum_{\text{cell edges, c}} \Delta u_i^c x_k^c\tag{A.27}$$

where the displacement gradient was expressed as $u_{ij} = \frac{\partial u_i}{\partial x_j}$, e_{jk} was the 2D permutation tensor ($e_{xy} = 1, e_{yx} = -1, e_{xx} = e_{yy} = 0$), where x_k^c is the position of the center of the edge and Δu_i^c is the relative displacement of the vertices of the edge. The average displacements were then used to calculate the components of the average strain in the cell.

Appendix B

Python Code for Model

```
#!/usr/bin/env python2
# -*- coding: utf-8 -*-
"""
Created on Wed Nov 16 13:51:53 2016
@author: corinnegullekson
"""
def clear_all():
    """Clears all the variables from the workspace of the spyder application."""
    gl = globals().copy()
    for var in gl:
        if var[0] == '_': continue
        if 'func' in str(globals()[var]): continue
        if 'module' in str(globals()[var]): continue

        del globals()[var]
if __name__ == "__main__":
    clear_all()
clear_all()
import random
from copy import deepcopy
import math
from scipy.spatial import Voronoi, voronoi_plot_2d
import matplotlib
import numpy
from scipy.optimize import leastsq
from operator import sub
from shapely.geometry import Polygon
import logging
logger = logging.getLogger()
logger.setLevel(logging.ERROR)
import datetime
```

#####

```

def NearestCelldist(cellposrest,newpos): #Finds the distance to the cell(out of
    cellposrest) closest to newpos
    dists=[]
    for i in range(len(cellposrest)):
        xdiff=newpos[0]-cellposrest[i][0]
        ydiff=newpos[1]-cellposrest[i][1]
        dist=math.sqrt(xdiff**2+ydiff**2)
        dists.append(dist)
    dists.sort()
    output=10000000000
    if len(dists)!=0:
        output=dists[0]
    return output

def PreferredOrientation(cellvert,celloutline):#alignment of elastic moduli
    if alignmenttype==1:
        prefangles=[PolygonOrientation(x,cellvert)+math.pi/2 for x in
            celloutline] #align with length axis
    if alignmenttype==2:
        prefangles=alignedangles #align with axis
    return prefangles

def ClusterGood(cluster,newcellvert):#checks if the cluster has self overlapping
    polygons
    check=1
    for i in range(len(cluster)):
        polygonpoints=[]
        for j in range(len(cluster[i])):
            polygonpoints.append((newcellvert[cluster[i][j]][0],newcellvert[
                cluster[i][j]][1]))
        poly=Polygon(polygonpoints)
        if poly.is_valid==False:
            check=0
    return check

def PolygonCenter(corners,cellvert):#finds the center of a polygon
    xvert=[]
    yvert=[]
    for i in range(len(corners)):
        xvert.append(cellvert[corners[i]][0])
        yvert.append(cellvert[corners[i]][1])
    n = len(xvert) # of corners
    area = 0.0
    for i in range(n):
        j = (i + 1) % n
        area += cellvert[corners[i]][0] * cellvert[corners[j]][1]
        area -= cellvert[corners[j]][0] * cellvert[corners[i]][1]

```

```

    area = area / 2.0
    centerx = 0.0
    centery = 0.0
    for i in range(n-1):
        centerx += (xvert[i]+xvert[i+1])*(xvert[i]*yvert[i+1]-xvert[i+1]*yvert[i])/area/6.0
        centery += (yvert[i]+yvert[i+1])*(xvert[i]*yvert[i+1]-xvert[i+1]*yvert[i])/area/6.0
    centerx += (xvert[n-1]+xvert[0])*(xvert[n-1]*yvert[0]-xvert[0]*yvert[n-1])/area/6.0
    centery += (yvert[n-1]+yvert[0])*(xvert[n-1]*yvert[0]-xvert[0]*yvert[n-1])/area/6.0
    return [centerx,centery]

def PolygonRho(corners): #finds how close a polygon is to the origin
    n = len(corners) # of corners
    centerx = 0.0
    centery = 0.0
    for i in range(n):
        centerx += cellvert[corners[i]][0]
        centery += cellvert[corners[i]][1]
    centerx=centerx/n
    centery=centery/n
    return math.sqrt(centerx**2+centery**2)

def PolygonArea(corners): #finds the area of a polygon
    n = len(corners) # of corners
    area = 0.0
    for i in range(n):
        j = (i + 1) % n
        area += cellvert[corners[i]][0] * cellvert[corners[j]][1]
        area -= cellvert[corners[j]][0] * cellvert[corners[i]][1]
    area = abs(area) / 2.0
    return area

def NewPolygonArea(corners): #finds the area of a polygon in a potential move
    n = len(corners) # of corners
    area = 0.0
    for i in range(n):
        j = (i + 1) % n
        area += newcellvert[corners[i]][0] * newcellvert[corners[j]][1]
        area -= newcellvert[corners[j]][0] * newcellvert[corners[i]][1]
    area = abs(area) / 2.0
    return area

def PolygonOrientation(corners,cellvert): #finds the orientation of the length
    axis of a polygon

```

```

n = len(corners) # of corners
maxlength = 0.0
for i in range(n):
    for j in range(n):
        length = math.sqrt((cellvert[corners[j]][0] - cellvert[corners[i]
            ][0])**2 + (cellvert[corners[j]][1] - cellvert[corners[i]][1])
            **2)
        if length>maxlength:
            maxlength=length
            xpos=cellvert[corners[j]][0] - cellvert[corners[i]][0]
            ypos=cellvert[corners[j]][1] - cellvert[corners[i]][1]
            orientation=math.atan(ypos/xpos)
return orientation

def PolygonNeighbours(cluster): #finds the cells each cell shares sides with
    neighbours=[]
    interior=[]
    exterior=[]
    alledges=[]
    ncells=len(cluster)
    for i in range(ncells): #picks first cell in cluster
        #adds all cell edges to alledges
        celledges=[]
        for o in range(len(cluster[i])-1): #picks a vertex in the cell
            edge=[cluster[i][o],cluster[i][o+1]] #indices of the two vertices in
                an edge
            edge.sort()
            celledges.append(edge)
        edge=[cluster[i][0],cluster[i][len(cluster[i])-1]]
        edge.sort()
        celledges.append(edge) #celledges now has all the cell edges
        alledges.append(celledges) #all the edges organized by cell
    #now to find which ones are interior and exterior
    for i in range(ncells): #picks first cell in cluster
        cellneigh=[]
        cellint=[]
        cellext=[]
        for o in range(len(alledges[i])): #picks and edge
            edge=alledges[i][o]
            for j in range(ncells):
                if i != j:
                    test=edge in alledges[j]
                    if test ==True:
                        cellneigh.append(j)
                        cellint.append(edge)
            test= edge in cellint
            if test == False:

```



```

        cellext.append(edge)
        neighbours.append(cellneigh)
        interior.append(cellint)
        exterior.append(cellext)
    return neighbours, interior, exterior #indices of neighbouring cells, 2
        vertices idices that make up each type of edge

def PolygonNeighbours2(cluster): #finds the cells each cell shares sides with
    alledges=[]
    ncells=len(cluster)
    for i in range(ncells): #picks first cell in cluster
        #adds all cell edges to alledges
        celledges=[]
        for o in range(len(cluster[i])-1): #picks a vertex in the cell
            edge=[cluster[i][o],cluster[i][o+1]] #idices of the two vertices in
                an edge
            celledges.append(edge)
        edge=[cluster[i][len(cluster[i])-1],cluster[i][0]]
        celledges.append(edge) #celledges now has all the cell edges
        alledges.append(celledges) #all the edges organized by cell
    return alledges

def PolygonVector(cluster,neighbours,cellvert,cell): #used to calulate
    displacement gradients in the polygons
    polyvectors=[]
    intedges=neighbours[1][cell]
    extedges=neighbours[2][cell]
    alledges=PolygonNeighbours2(cluster)[cell]
    polyvectorsorder=[[0,0] for i in range(0, len(alledges))]
    nintedges=len(intedges)
    nextedges=len(extedges)
    for i in range(nintedges):
        edgecenterx=(cellvert[intedges[i][0]][0]+cellvert[intedges[i][1]][0])/2
        edgecentery=(cellvert[intedges[i][0]][1]+cellvert[intedges[i][1]][1])/2
        polyvecty=1.0*(edgecenterx)
        polyvectx=-1.0*(edgecentery)
        polyvectors.append([polyvectx,polyvecty])
        if intedges[i] in alledges:
            pos=alledges.index(intedges[i])
        else:
            pos=alledges.index([intedges[i][1],intedges[i][0]])
        polyvectorsorder[pos]=[polyvectx,polyvecty]
    for i in range(nextedges):
        edgecenterx=(cellvert[extedges[i][0]][0]+cellvert[extedges[i][1]][0])/2
        edgecentery=(cellvert[extedges[i][0]][1]+cellvert[extedges[i][1]][1])/2
        polyvecty=1.0*(edgecenterx)
        polyvectx=-1.0*(edgecentery)

```

```

        polyvectors.append([polyvectx,polyvecty])
        if extedges[i] in alledges:
            pos=alledges.index(extedges[i])
        else:
            pos=alledges.index([extedges[i][1],extedges[i][0]])
        polyvectorsorder[pos]=[polyvectx,polyvecty]
    return polyvectorsorder

def DispPolygonVector(cluster,neighbours,cellvert,newcellvert,cell): #calculates
    average displacement gradient of polygon
    pvector=PolygonVector(cluster,neighbours,cellvert,cell)
    alledges=PolygonNeighbours2(cluster)[cell]
    nedges=len(alledges)
    gradxx=0.0
    gradyy=0.0
    gradxy=0.0
    gradyx=0.0
    Area=AreaPolygonVector(cluster,neighbours,cellvert,cell)
    for i in range(nedges):
        deltaedgelengethx=newcellvert[alledges[i][1]][0]-cellvert[alledges[i]
            ][1]][0]-newcellvert[alledges[i][0]][0]+cellvert[alledges[i][0]][0]
        deltaedgelengethy=newcellvert[alledges[i][1]][1]-cellvert[alledges[i]
            ][1]][1]-newcellvert[alledges[i][0]][1]+cellvert[alledges[i][0]][1]
        gradxx+=deltaedgelengethx*pvector[i][0]/Area
        gradyy+=deltaedgelengethy*pvector[i][1]/Area
        gradxy+=deltaedgelengethy*pvector[i][0]/Area
        gradyx+=deltaedgelengethx*pvector[i][1]/Area
    dispgrad=[gradxx,gradyx,gradxy,gradyy]
    return dispgrad

def AreaPolygonVector(cluster,neighbours,cellvert,cell):#used to calulate
    displacement gradients in the polygons
    Area=0
    pvector=PolygonVector(cluster,neighbours,cellvert,cell)
    alledges=PolygonNeighbours2(cluster)[cell]
    nedges=len(alledges)
    for i in range(nedges):
        edgelengethx=cellvert[alledges[i][1]][0]-cellvert[alledges[i][0]][0]
        edgelengethy=cellvert[alledges[i][1]][1]-cellvert[alledges[i][0]][1]
        Area+=(pvector[i][0]*edgelengethx+pvector[i][1]*edgelengethy)/2.0
    return Area

def EdgeCells(cluster,edgeneighbours,cellvert): #finds the cells that have edges
    with no neighbours and returns exposed edge legths of cluster as well as
    interior side lengths
    perimexts=[] #lengths of ext perim of each cell
    perimints=[] #lengths of int perim of each cell

```

```

alledges=[] #lengths of perim of each cell
shortvertices=[] #indices for edges that are short
interior=edgeneighbours[1]
exterior=edgeneighbours[2]
for i in range(len(interior)):
    perimint=0.0
    for j in range(len(interior[i])):
        edge=interior[i][j]
        x1=cellvert[edge[0]][0]
        x2=cellvert[edge[1]][0]
        y1=cellvert[edge[0]][1]
        y2=cellvert[edge[1]][1]
        edglength=math.sqrt((x2-x1)**2+(y2-y1)**2)
        alledges.append(edglength)
        perimint = perimint+edglength
        if edglength<0.0:
            shortvertices.append([edge[0],edge[1]])
    perimints.append(perimint)
for i in range(len(exterior)):
    perimext=0
    for j in range(len(exterior[i])):
        edge=exterior[i][j]
        x1=cellvert[edge[0]][0]
        x2=cellvert[edge[1]][0]
        y1=cellvert[edge[0]][1]
        y2=cellvert[edge[1]][1]
        edglength=math.sqrt((x2-x1)**2+(y2-y1)**2)
        alledges.append(edglength)
        perimext = perimext+edglength
    perimexts.append(perimext)
return perimints,perimexts,alledges

#strain and energy functions
def ClusterEnergy(exteriors,interiors,dareas,strain1diff,strain2diff,
    strainxydiff,areas0,interior0,exterior0,volumes0): #energy of a cluster
    cellcellenergy=ec*(sum(interiors)-sum(interior0))/2*h
    cellextenergy=ee*(sum(exteriors)-sum(exterior0))*h+ee*(sum(dareas))
    cellsubstrateenergy=es*(sum(dareas))
    strainenergy=0
    perimenergy=0
    for i in range(len(interiors)):
        perimenergy+=ep*0.5*((interiors[i]+exteriors[i]-interior0[i]-exterior0[i]
            ))**2/(interior0[i]+exterior0[i])
        strainenergy+=0.5*((ys-ysdev)*strain1diff[i]**2+(ys+ysdev)*strain2diff[i]
            )**2+ys*strain1diff[i]*strain2diff[i]+yssh*strainxydiff[i]**2)*
            volumes0[i]
    clustenergy=(cellcellenergy+cellextenergy+cellsubstrateenergy+strainenergy+

```

```

        perimenergy)
    return clustenergy, cellcellenergy, cellsubstrateenergy, strainenergy,
        perimenergy, cellextenergy

def residualsclust(p, dx, x, y): #used for fitting of cluster strain
    err = dx-p[0]-x*p[1]-y*p[2]-x*x*p[3]-x*y*p[4]-y*y*p[5]
    return err

def fittingxclust(dx,x,y,dxcent,xstrainguess,rotstrainguess): #used for fitting
    of cluster strain
    p0=[dxcent,xstrainguess,rotstrainguess,0,0,0]
    constants=leastsq(residualsclust, p0, args=(dx, x, y))
    return constants[0]

def fittingyclust(dx,x,y,dycent,ystrainguess,rotstrainguess): #used for fitting
    of cluster strain
    p0=[dycent,rotstrainguess,ystrainguess,0,0,0]
    constants=leastsq(residualsclust, p0, args=(dx, x, y))
    return constants[0]

def Cluststrain(newcorners,oldcorners): #calculates strain of cluster
    n = len(newcorners) # of corners
    dx=[]
    dy=[]
    x=[]
    y=[]
    newx=[]
    newy=[]
    for i in range(n):
        dx.append(newcorners[i][0]-oldcorners[i][0])
        dy.append(newcorners[i][1]-oldcorners[i][1])
        x.append(oldcorners[i][0])
        y.append(oldcorners[i][1])
        newx.append(newcorners[i][0])
        newy.append(newcorners[i][1])
    #get first constant value for fitting
    centerx = 0.0
    centery = 0.0
    for i in range(n):
        centerx += x[i]/n
        centery += y[i]/n
    newcenterx = 0.0
    newcentery = 0.0
    for i in range(n):
        newcenterx += newx[i]/n
        newcentery += newy[i]/n
    dxcent=newcenterx-centerx

```

```

dycent=newcentery-centery
#get uxx and uyy constant estimates for fitting
xmaxindex=numpy.argmax(x)
xminindex=numpy.argmin(x)
xlength=x[xmaxindex]-x[xminindex]
newxlength=newx[xmaxindex]-newx[xminindex]
ymaxindex=numpy.argmax(y)
yminindex=numpy.argmin(y)
ylength=y[ymaxindex]-y[yminindex]
newylength=newy[ymaxindex]-newy[yminindex]
xstrainguess=(newxlength-xlength)/xlength
ystrainguess=(newylength-ylength)/ylength
#get uxy and uyx constant estimates for fitting
#rotate vertices 45 degrees
newxrot=[]
xrot=[]
for i in range(n):
    newxrot.append(newx[i]*math.cos(math.pi/4)-newy[i]*math.sin(math.pi/4))
    xrot.append(x[i]*math.cos(math.pi/4)-y[i]*math.sin(math.pi/4))
rotlength=max(xrot)-min(xrot)
newrotlength=max(newxrot)-min(newxrot)
rotstrainguess=(newrotlength-rotlength)/rotlength-(xstrainguess+ystrainguess)/2
#calculate strains
strainx=fittingxclust(numpy.array(dx),numpy.array(x),numpy.array(y),dxcent,
    xstrainguess,rotstrainguess)
strainy=fittingyclust(numpy.array(dy),numpy.array(x),numpy.array(y),dycent,
    ystrainguess,rotstrainguess)
return [strainx,strainy]

def StrainAngle(exx,eyy,exy): #finds orientation of major strain
    if exx==eyy:
        strainorientation=0
    else:
        angle=numpy.arctan(2*exy/(exx-eyy))*0.5
        if exx>eyy:
            strainorientation=angle
        if eyy>exx and angle>0:
            strainorientation=angle-numpy.pi/2
        if eyy>exx and angle<0:
            strainorientation=angle+numpy.pi/2
        if eyy>exx and angle==0:
            strainorientation=numpy.pi/2
        if exx==eyy:
            strainorientation=0
    return strainorientation

```

```
#####

#setting up parameters
filenamestart = 'diagram/'+datetime.datetime.now().strftime("%Y%m%d-%H%M%S")
N=23 #number of points to make cells (23)
R=5.0 #target starting radius of a cell
stepsize=0.1
stepanneal=0.001 #was0.001
A=math.pi*R**2 #target starting area of cell (um^2)
V=4/3*math.pi*6.50**3
rad=numpy.round(math.sqrt(N*A/math.pi))*1.5 #radius used to form cluster (um)
zpos=100000#cell position moves for starting configuration 100K
zstab=150000#iterations to stabilize 150K
zmax=150000#iteratins to stabilize with changed moduli 150K
annealnum=5000
#energies of interactions [in 10(-9)Nm=10(-15)J] in energy calc
ec=-50# #energy of cell and cell
ee=20##energy of cell and exterior
es=-70#-70#energy of cell and substrate
ep=1000 #energy of cell perim
ys=20.0*4/3 #mean elastic modulus KPa
ysdev=10*4/3# #(ys1-ys2)/2
ysshear=20*4/3 #shear modulus
ysfactor=0.4#1.6 or 0.4 factor the moduli change
kt=4.1*10**-6 #should be 4.1*10**-6 boltzmann constant temperature
alignmenttype=1 #1=cell level, 2=y axis
ysnew=ys*ysfactor #mean youngs modulus, 1KPa = 10(-9)N/um2, 20KPa in Harris
paper
ysdevnew=ysdev*ysfactor #(ys1-ys2)/2
ysshearnew=ysshear*ysfactor #shear modulus
epnew=ep*ysfactor
#####

#making starting configuration
mono=0
while mono<1:
    #Making a monolayer with disks
    cellpos=[]
    xpos=0.0
    ypos=0.0
    cellpos.append([xpos,ypos]) #first cell at origin
    n=0
    while n<zpos: #add cells randomly
        theta=random.uniform(-numpy.pi,numpy.pi)
        rho=random.uniform(0,2*rad)
        xpos=rho*numpy.cos(theta)
        ypos=rho*numpy.sin(theta)
```

```

        if NearestCelldist(cellpos,[xpos,ypos])>2*R: #check to see if they
            overlap
            cellpos.append([xpos,ypos])
        theta=random.uniform(-numpy.pi,numpy.pi)
        rho=random.uniform(0,R/5)
        m=random.randint(0,len(cellpos)-1)
        newx=cellpos[m][0]+rho*numpy.cos(theta) #move a cell randomly
        newy=cellpos[m][1]+rho*numpy.sin(theta)
        othercellpos=deepcopy(cellpos)
        othercellpos.remove(cellpos[m])
        radius=math.sqrt(newx**2+newy**2)
        if NearestCelldist(othercellpos,[newx,newy])>2*R:#check to see if they
            overlap
            if radius<(2*rad): #check to see if cell is still in bounding radius
                cellpos[m]=[newx,newy]
        n+=1
# #plot starting disks
matplotlib.pyplot.figure(1)
pointsx=[]
pointsy=[]
r=0
while r<len(cellpos):
    pointsx.append(cellpos[r][0])
    pointsy.append(cellpos[r][1])
    r=r+1
r=0
while r<len(cellpos):
    circ=matplotlib.pyplot.Circle((pointsx[r],pointsy[r]),R, fc='b', alpha
        =0.5)
    ax=matplotlib.pyplot.axes()
    ax.add_patch(circ) #Grab the current axes, add the patch to it
    r=r+1
matplotlib.pyplot.scatter(pointsx,pointsy,c='b')
matplotlib.pyplot.axes().set_aspect('equal', 'datalim')
matplotlib.pyplot.show()
#make cell boundaries
vor = Voronoi(cellpos)
voronoi_plot_2d(vor)
cellvert=vor.vertices
celloutline=vor.regions
celloutline.remove([])
celloutline = [x for x in celloutline if -1 not in x] #removes infinity
regions
#only keep N cells
cellrho = [PolygonRho(x) for x in celloutline]
cellrho.sort()
maxrho=cellrho[N]

```

```

celloutline = [x for x in celloutline if PolygonRho(x)<maxrho]
areas = [PolygonArea(x) for x in celloutline]
#####test if starting monolayer is good, no polygons with
    less than 5 sides
sides = [len(x) for x in celloutline]
monotest=1
if 4 in sides:
    monotest=0
if 3 in sides:
    monotest=0
newcellvert=deepcopy(cellvert)
mono=monotest
#plotting
q=0
matplotlib.pyplot.figure(3)
while q<len(areas):
    pointsx=[]
    pointsy=[]
    r=0
    while r<len(celloutline[q]):
        pointsx.append(cellvert[celloutline[q][r]][0])
        pointsy.append(cellvert[celloutline[q][r]][1])
        r=r+1
    pointsx.append(cellvert[celloutline[q][0]][0])
    pointsy.append(cellvert[celloutline[q][0]][1])
    matplotlib.pyplot.plot(pointsx,pointsy, 'c')
    q=q+1
matplotlib.pyplot.axes().set_aspect('equal', 'datalim')
q=0
matplotlib.pyplot.figure(4)
while q<len(areas):
    pointsx=[]
    pointsy=[]
    r=0
    while r<len(celloutline[q]):
        pointsx.append(cellvert[celloutline[q][r]][0])
        pointsy.append(cellvert[celloutline[q][r]][1])
        r=r+1
    pointsx.append(cellvert[celloutline[q][0]][0])
    pointsy.append(cellvert[celloutline[q][0]][1])
    matplotlib.pyplot.plot(pointsx,pointsy, 'c')
    q=q+1
matplotlib.pyplot.axes().set_aspect('equal', 'datalim')
q=0
areas = [PolygonArea(x) for x in celloutline]
matplotlib.pyplot.figure(4)
while q<len(areas):

```



```

    pointsx=[]
    pointsy=[]
    r=0
    while r<len(celloutline[q]):
        pointsx.append(cellvert[celloutline[q][r]][0])
        pointsy.append(cellvert[celloutline[q][r]][1])
        r=r+1
    pointsx.append(cellvert[celloutline[q][0]][0])
    pointsy.append(cellvert[celloutline[q][0]][1])
    matplotlib.pyplot.plot(pointsx,pointsy, 'c')
    q=q+1
matplotlib.pyplot.axes().set_aspect('equal', 'datalim')
#####

numcells=len(celloutline) # number of cells in the cluster
numvertcells=[len(x) for x in celloutline] # number of vertices in each cell
h=V/sum(areas)*int(numcells)
#Stabilize the Monolayer
icellvert=deepcopy(cellvert) #saving a copy of original vertex positions for
    strain calculations
#calculate starting values for energy calculations
areas = [PolygonArea(x) for x in celloutline]
edgeneighbours=PolygonNeighbours(celloutline) #finds what cells are neighbours
edge=EdgeCells(celloutline,edgeneighbours,cellvert) #used to find what edges are
    cell-cell or cell-media
exterior0=edge[1] #starting lengths of cell exteriors
interior0=edge[0] #starting lengths of cell interiors
exteriorl=sum(exterior0)
interiorl=sum(interior0)
areas0=deepcopy(areas)
dareasl=0
strainl=0
edgeindices=edgeneighbours[2]
volumes0=[h*x for x in areas]
strain1diffs=[0.0 for i in range(0, len(celloutline))]
strain2diffs=[0.0 for i in range(0, len(celloutline))]
strainxydiffs=[0.0 for i in range(0, len(celloutline))]
alignedangles=[math.pi/2 for x in celloutline]#[random.gauss(0,math.pi/2) for x
    in celloutline]
targetangles=PreferredOrientation(cellvert,celloutline)
energyprogression=[]
cellenergyprogression=[]
subenergyprogression=[]
perminenergyprogression=[]
strainenergyprogression=[]
perimextprogression=[]
perimintprogression=[]

```

```

areaprogression=[]
strainprogression=[]
extenergyprogression=[]
endiffprogression=[]
dareas=map(sub,areas,areas0)
edge=EdgeCells(celloutline,edgeneighbours,cellvert)
exteriors=edge[1]
interiors=edge[0]
energies=ClusterEnergy(exteriors,interiors,dareas,strain1diffs,strain2diffs,
    strainxydiffs,areas0,interior0,exterior0,volumes0)
cellenergyprogression.append(energies[1])
subenergyprogression.append(energies[2])
perminenergyprogression.append(energies[4])
strainenergyprogression.append(energies[3])
extenergyprogression.append(energies[5])
#calculate strain in each cell
for i in range(numcells):
    mcell=i
    strain=DispPolygonVector(celloutline,edgeneighbours,icellvert,cellvert,mcell
        )
    uxx=strain[0]
    uyy=strain[3]
    uxy=strain[1]
    uyx=strain[2]
    exx= 0.5*(2*uxx+uxx**2+uyx**2)
    eyy= 0.5*(2*uyy+uyy**2+uxy**2)
    exy= 0.5*(uyx+uxy+uxx*uxy+uyx*uyy)
    e1=exx*numpy.cos(targetangles[mcell])**2+eyy*numpy.sin(targetangles[mcell])
        **2+2*exy*numpy.sin(targetangles[mcell])*numpy.cos(targetangles[mcell])
    e2=exx*numpy.sin(targetangles[mcell])**2+eyy*numpy.cos(targetangles[mcell])
        **2-2*exy*numpy.sin(targetangles[mcell])*numpy.cos(targetangles[mcell])
    e1xy=2*(eyy-exx)*numpy.sin(targetangles[mcell])*numpy.cos(targetangles[mcell]
        )+exy*(numpy.cos(targetangles[mcell])**2-numpy.sin(targetangles[mcell])
        **2)
    strain1diffs[mcell]=e1
    strain2diffs[mcell]=e2
    strainxydiffs[mcell]=e1xy
clustenergy=ClusterEnergy(exteriors,interiors,dareas,strain1diffs,strain2diffs,
    strainxydiffs,areas0,interior0,exterior0,volumes0)
energy=clustenergy[0]
zanneal=0
z=0
while z<zstab:#start stabilization
    zanneal=zanneal+1
    if zanneal>annealnum:
        moveisgood=0
        annealtry=0

```

```

while moveisgood==0 and annealtry<1000:
    annealtry=annealtry+1
    newcellvert=deepcopy(cellvert)
    for i in range(len(cellvert)):
        newcellvert[i]=[cellvert[i][0]+random.uniform(-stepanneal,
            stepanneal),cellvert[i][1]+random.uniform(-stepanneal,
            stepanneal)]
    if ClusterGood(celloutline,newcellvert)==1:
        moveisgood=1
        cellvert=deepcopy(newcellvert)#changed
zanneal=0
edge=EdgeCells(celloutline,edgeneighbours,cellvert)
exteriors=edge[1]
interiors=edge[0]
newareas=[PolygonArea(x) for x in celloutline]
h=V/sum(newareas)*int(numcells) #calculate new height
dareas=map(sub,newareas,areas0)
for i in range(numcells):
    mcell=i
    strain=DispPolygonVector(celloutline,edgeneighbours,icellvert,
        cellvert,mcell)
    uxx=strain[0]
    uyy=strain[3]
    uxy=strain[1]
    uyx=strain[2]
    exx= 0.5*(2*uxx+uxx**2+uyx**2)
    eyy= 0.5*(2*uyy+uyy**2+uxy**2)
    exy= 0.5*(uyx+uxy+uxx*uxy+uyx*uyy)
    e1=exx*numpy.cos(targetangles[mcell])**2+eyy*numpy.sin(targetangles[
        mcell])**2+2*exy*numpy.sin(targetangles[mcell])*numpy.cos(
        targetangles[mcell])
    e2=exx*numpy.sin(targetangles[mcell])**2+eyy*numpy.cos(targetangles[
        mcell])**2-2*exy*numpy.sin(targetangles[mcell])*numpy.cos(
        targetangles[mcell])
    e1xy=2*(eyy-exx)*numpy.sin(targetangles[mcell])*numpy.cos(
        targetangles[mcell])+exy*(numpy.cos(targetangles[mcell])**2-numpy
        .sin(targetangles[mcell])**2)
    strain1diffs[mcell]=e1
    strain2diffs[mcell]=e2
    strainxydiffs[mcell]=e1xy
    clustenergy=ClusterEnergy(exteriors,interiors,dareas,strain1diffs,
        strain2diffs,strainxydiffs,areas0,interior0,exterior0,volumes0)
    energy=clustenergy[0]
    cellenergyprogression.append(clustenergy[1])
    subenergyprogression.append(clustenergy[2])
    perminenergyprogression.append(clustenergy[4])
    strainenergyprogression.append(clustenergy[3])

```

```

extenergyprogression.append(clustenergy[5])
energyprogression.append(energy)
perimextprogression.append(exteriorl)
perimintprogression.append(interiorl)
areaprogression.append(dareasl)
strainprogression.append(strainl)
cell=random.randint(0,numcells-1)
vert=celloutline[cell][random.randint(0,numvertcells[cell]-1)]#choose vertex
#move that vertex
#make sure the move doesn't invert the polygon
newcellvert=deepcopy(cellvert)
newcellvert[vert]=[cellvert[vert][0]+random.uniform(-stepsize,stepsize),
    cellvert[vert][1]+random.uniform(-stepsize,stepsize)]
#calculate properties of move
edge=EdgeCells(celloutline,edgeneighbours,newcellvert)
exteriors=edge[1]
interiors=edge[0]
newareas=[NewPolygonArea(x) for x in celloutline]
h=V/sum(newareas)*int(numcells)
dareas=map(sub,newareas,areas0)
#calculate strain in the cells this move effects
#find the cells it affects
cells=[]
for i in range(numcells):
    if celloutline[i].count(vert)==1:
        cells.append(i)
#calculate strain in each cell
newstrain1diffs=deepcopy(strain1diffs)
newstrain2diffs=deepcopy(strain2diffs)
newstrainxydiffs=deepcopy(strainxydiffs)
for i in range(len(cells)):
    mcell=cells[i]
    strain=DispPolygonVector(celloutline,edgeneighbours,icellvert,
        newcellvert,mcell)
    uxx=strain[0]
    uyy=strain[3]
    uxy=strain[1]
    uyx=strain[2]
    exx= 0.5*(2*uxx+uxx**2+uyx**2)
    eyy= 0.5*(2*uyy+uyy**2+uxy**2)
    exy= 0.5*(uyx+uxy+uxx*uxy+uyx*uyy)
    e1=exx*numpy.cos(targetangles[mcell])**2+eyy*numpy.sin(targetangles[
        mcell])**2+2*exy*numpy.sin(targetangles[mcell])*numpy.cos(
        targetangles[mcell])
    e2=exx*numpy.sin(targetangles[mcell])**2+eyy*numpy.cos(targetangles[
        mcell])**2-2*exy*numpy.sin(targetangles[mcell])*numpy.cos(
        targetangles[mcell])

```

```

        e1xy=2*(eyy-exx)*numpy.sin(targetangles[mcell])*numpy.cos(targetangles[
            mcell])+exy*(numpy.cos(targetangles[mcell])**2-numpy.sin(targetangles
            [mcell])**2)
        newstrain1diffs[mcell]=(e1)
        newstrain2diffs[mcell]=(e2)
        newstrainxydiffs[mcell]=(e1xy)
    newenergy=ClusterEnergy(exterior,interior,dareas,newstrain1diffs,
        newstrain2diffs,newstrainxydiffs,areas0,interior0,exterior0,volumes0)
    energydiff=newenergy[0]-energy
    if ClusterGood(celloutline,newcellvert)==1:
        if energydiff<=0:
            cellvert=deepcopy(newcellvert)
            energy=newenergy[0]
            strain1diffs=newstrain1diffs
            strain2diffs=newstrain2diffs
            strainxydiffs=newstrainxydiffs
            areas=deepcopy(newareas)
            h=V/sum(areas)*int(numcells)
            targetangles=PreferedOrientation(cellvert,celloutline)
            exteriorl=sum(exterior)
            interiorl=sum(interior)
            dareasl=(sum(dareas))
            strainl=(sum(newstrain1diffs)+sum(newstrain2diffs))/2/numcells
            endiffprogression.append(energydiff)
            clustenergy=newenergy
        else:
            if math.exp(-energydiff/kt)>random.uniform(0,1):
                cellvert=deepcopy(newcellvert)
                energy=newenergy[0]
                strain1diffs=newstrain1diffs
                strain2diffs=newstrain2diffs
                strainxydiffs=newstrainxydiffs
                areas=deepcopy(newareas)
                h=V/sum(areas)*int(numcells)
                targetangles=PreferedOrientation(cellvert,celloutline)
                exteriorl=sum(exterior)
                interiorl=sum(interior)
                dareasl=(sum(dareas))
                strainl=(sum(newstrain1diffs)+sum(newstrain2diffs))/2/numcells
                clustenergy=newenergy
                endiffprogression.append(energydiff)

    z=z+1
posbefore=[PolygonCenter(x,cellvert) for x in celloutline]
cellvertbefore=deepcopy(cellvert)
edge=EdgeCells(celloutline,edgeneighbours,cellvert)
exterior=edge[1]
interior=edge[0]

```

```

areas=[PolygonArea(x) for x in celloutline]
h=V/sum(areas)*int(numcells)
#####plotting
matplotlib.pyplot.figure(4)
q=0
while q<len(areas):
    pointsx=[]
    pointsy=[]
    r=0
    while r<len(celloutline[q]):
        pointsx.append(cellvert[celloutline[q][r]][0])
        pointsy.append(cellvert[celloutline[q][r]][1])
        r=r+1
    pointsx.append(cellvert[celloutline[q][0]][0])
    pointsy.append(cellvert[celloutline[q][0]][1])
    matplotlib.pyplot.plot(pointsx,pointsy, 'b')
    q=q+1
matplotlib.pyplot.axes().set_aspect('equal', 'datalim')
matplotlib.pyplot.savefig(filenamestart+"beforepos.pdf")
strains=[]
strainsxx=[]
strainsyy=[]
strainsxy=[]
for i in range(len(celloutline)):
    mcell=i
    gradedgeneighbours=PolygonNeighbours(celloutline)
    strain=DispPolygonVector(celloutline,gradedgeneighbours,icellvert,cellvert,
        mcell)
    uxx=strain[0]
    uyy=strain[3]
    uxy=strain[1]
    uyx=strain[2]
    exx= 0.5*(2*uxx+uxx**2+uyx**2)
    eyy= 0.5*(2*uyy+uyy**2+uxy**2)
    exy= 0.5*(uyx+uxy+uxx*uxy+uyx*uyy)
    e1=0.5*(exx+eyy)+numpy.sqrt(((exx-eyy)/2)**2+exy**2)
    e2=0.5*(exx+eyy)-numpy.sqrt(((exx-eyy)/2)**2+exy**2)
    theta=StrainAngle(exx,eyy,exy) #orientation
    strains.append([e1,e2,theta])
    strainsxx.append(exx)
    strainsyy.append(eyy)
    strainsxy.append(exy)
lengthangles=[PolygonOrientation(x,cellvert) for x in celloutline]
matplotlib.pyplot.figure(5)
q=0
while q<len(areas):
    pointsx=[]

```

```

pointsy=[]
r=0
while r<len(celloutline[q]):
    pointsx.append(cellvert[celloutline[q][r]][0])
    pointsy.append(cellvert[celloutline[q][r]][1])
    r=r+1
pointsx.append(cellvert[celloutline[q][0]][0])
pointsy.append(cellvert[celloutline[q][0]][1])
matplotlib.pyplot.plot(pointsx,pointsy,color='b')
matplotlib.pyplot.arrow(posbefore[q][0],posbefore[q][1],5*numpy.cos(
    lengthangles[q]),5*numpy.sin(lengthangles[q]), color='b')
matplotlib.pyplot.arrow(posbefore[q][0],posbefore[q][1],5*numpy.cos(
    lengthangles[q]+numpy.pi),5*numpy.sin(lengthangles[q]+numpy.pi), color='b
    ')
matplotlib.pyplot.arrow(posbefore[q][0],posbefore[q][1],5*numpy.cos(strains[
    q][2]),5*numpy.sin(strains[q][2]), color='m')
matplotlib.pyplot.arrow(posbefore[q][0],posbefore[q][1],5*numpy.cos(strains[
    q][2]+numpy.pi),5*numpy.sin(strains[q][2]+numpy.pi), color='m')
q=q+1
pointsx=[]
pointsy=[]
r=0
while r<len(posbefore):
    pointsx.append(posbefore[r][0])
    pointsy.append(posbefore[r][1])
    r=r+1
matplotlib.pyplot.scatter(pointsx,pointsy,color='b')
matplotlib.pyplot.axes().set_aspect('equal', 'datalim')
matplotlib.pyplot.savefig(filenamestart+"straintargetsplotbefore.pdf")
matplotlib.pyplot.figure(6)
q=0
while q<len(areas):
    pointsx=[]
    pointsy=[]
    r=0
    while r<len(celloutline[q]):
        pointsx.append(cellvert[celloutline[q][r]][0])
        pointsy.append(cellvert[celloutline[q][r]][1])
        r=r+1
    pointsx.append(cellvert[celloutline[q][0]][0])
    pointsy.append(cellvert[celloutline[q][0]][1])
    matplotlib.pyplot.plot(pointsx,pointsy,color='b')
    q=q+1
pointsx=[]
pointsy=[]
r=0
while r<len(posbefore):

```

```

    pointsx.append(posbefore[r][0])
    pointsy.append(posbefore[r][1])
    r=r+1
matplotlib.pyplot.scatter(pointsx,pointsy,color='b')
matplotlib.pyplot.axes().set_aspect('equal', 'datalim')
angles=[]
for i in range(len(strains)):
    angles.append(strains[i][2])
matplotlib.pyplot.figure(25)
q=0
while q<len(areas):
    pointsx=[]
    pointsy=[]
    r=0
    while r<len(celloutline[q]):
        pointsx.append(cellvert[celloutline[q][r]][0])
        pointsy.append(cellvert[celloutline[q][r]][1])
        r=r+1
    pointsx.append(cellvert[celloutline[q][0]][0])
    pointsy.append(cellvert[celloutline[q][0]][1])
    matplotlib.pyplot.plot(pointsx,pointsy,color='b')
    matplotlib.pyplot.arrow(posbefore[q][0],posbefore[q][1],5*numpy.cos(
        lengthangles[q]),5*numpy.sin(lengthangles[q]), color='m')
    matplotlib.pyplot.arrow(posbefore[q][0],posbefore[q][1],5*numpy.cos(
        lengthangles[q]+numpy.pi),5*numpy.sin(lengthangles[q]+numpy.pi), color='m')
    q=q+1
pointsx=[]
pointsy=[]
r=0
while r<len(posbefore):
    pointsx.append(posbefore[r][0])
    pointsy.append(posbefore[r][1])
    r=r+1
matplotlib.pyplot.scatter(pointsx,pointsy,color='b')
matplotlib.pyplot.axes().set_aspect('equal', 'datalim')
matplotlib.pyplot.savefig(filenamestart+"strainslengthsplotbefore.pdf")
matplotlib.pyplot.figure(26)
q=0
while q<len(areas):
    pointsx=[]
    pointsy=[]
    r=0
    while r<len(celloutline[q]):
        pointsx.append(cellvert[celloutline[q][r]][0])
        pointsy.append(cellvert[celloutline[q][r]][1])
        r=r+1

```



```

    pointsx.append(cellvert[celloutline[q][0]][0])
    pointsy.append(cellvert[celloutline[q][0]][1])
    matplotlib.pyplot.plot(pointsx,pointsy,color='b')
    matplotlib.pyplot.arrow(posbefore[q][0],posbefore[q][1],5*numpy.cos(
        targetangles[q]),5*numpy.sin(targetangles[q]), color='m')
    matplotlib.pyplot.arrow(posbefore[q][0],posbefore[q][1],5*numpy.cos(
        targetangles[q]+numpy.pi),5*numpy.sin(targetangles[q]+numpy.pi), color='m'
    ')
    q=q+1
pointsx=[]
pointsy=[]
r=0
while r<len(posbefore):
    pointsx.append(posbefore[r][0])
    pointsy.append(posbefore[r][1])
    r=r+1
matplotlib.pyplot.scatter(pointsx,pointsy,color='b')
matplotlib.pyplot.axes().set_aspect('equal', 'datalim')
matplotlib.pyplot.savefig(filenamestart+"strainxplotbefore.pdf")
#####

#change moduli of the monolayer
ys=ysnew
ysdev=ysdevnew
ysshear=ysshearnew
ep=epnew
edge=EdgeCells(celloutline,edgeneighbours,cellvert)
exteriors=edge[1]
interiors=edge[0]
areas=[PolygonArea(x) for x in celloutline]
h=V/sum(areas)*int(numcells)
dareas=map(sub,areas,areas0)
#calculate strain in each cell
for i in range(numcells):
    mcell=i
    strain=DispPolygonVector(celloutline,edgeneighbours,icellvert,cellvert,mcell
    )
    uxx=strain[0]
    uyy=strain[3]
    uxy=strain[1]
    uyx=strain[2]
    exx= 0.5*(2*uxx+uxx**2+uyx**2)
    eyy= 0.5*(2*uyy+uyy**2+uxy**2)
    exy= 0.5*(uyx+uxy+uxx*uxy+uyx*uyy)
    e1=exx*numpy.cos(targetangles[mcell])**2+eyy*numpy.sin(targetangles[mcell])
        **2+2*exy*numpy.sin(targetangles[mcell])*numpy.cos(targetangles[mcell])
    e2=exx*numpy.sin(targetangles[mcell])**2+eyy*numpy.cos(targetangles[mcell])

```

```

        **2-2*exy*numpy.sin(targetangles[mcell])*numpy.cos(targetangles[mcell])
    e1xy=2*(eyy-exx)*numpy.sin(targetangles[mcell])*numpy.cos(targetangles[mcell
    ])+exy*(numpy.cos(targetangles[mcell])**2-numpy.sin(targetangles[mcell])
    **2)
    strain1diffs[mcell]=e1
    strain2diffs[mcell]=e2
    strainxydiffs[mcell]=e1xy
    clustenergy=ClusterEnergy(exteriors,interiors,dareas,strain1diffs,strain2diffs,
    strainxydiffs,areas0,interior0,exterior0,volumes0)
    energy=clustenergy[0]
    zanneal=0
    z=0
    while z<zmax:
        zanneal=zanneal+1
        if zanneal>annealnum:
            moveisgood=0
            annealtry=0
            while moveisgood==0 and annealtry<1000:
                annealtry=annealtry+1
                newcellvert=deepcopy(cellvert)
                for i in range(len(cellvert)):
                    newcellvert[i]=[cellvert[i][0]+random.uniform(-stepanneal,
                    stepanneal),cellvert[i][1]+random.uniform(-stepanneal,
                    stepanneal)]
                if ClusterGood(celloutline,newcellvert)==1:
                    moveisgood=1
                    cellvert=deepcopy(newcellvert)#changed
            zanneal=0
            edge=EdgeCells(celloutline,edgeneighbours,cellvert)
            exteriors=edge[1]
            interiors=edge[0]
            areas=[PolygonArea(x) for x in celloutline]
            h=V/sum(areas)*int(numcells)
            dareas=map(sub,areas,areas0)
            for i in range(numcells):
                mcell=i
                strain=DispPolygonVector(celloutline,edgeneighbours,icellvert,
                cellvert,mcell)
                uxx=strain[0]
                uyy=strain[3]
                uxy=strain[1]
                uyx=strain[2]
                exx= 0.5*(2*uxx+uxx**2+uyx**2)
                eyy= 0.5*(2*uyy+uyy**2+uxy**2)
                exy= 0.5*(uyx+uxy+uxx*uxy+uyx*uyy)
                e1=exx*numpy.cos(targetangles[mcell])**2+eyy*numpy.sin(targetangles[
                mcell])**2+2*exy*numpy.sin(targetangles[mcell])*numpy.cos(

```

```

        targetangles[mcell])
    e2=exx*numpy.sin(targetangles[mcell])**2+eyy*numpy.cos(targetangles[
        mcell])**2-2*exy*numpy.sin(targetangles[mcell])*numpy.cos(
        targetangles[mcell])
    e1xy=2*(eyy-exx)*numpy.sin(targetangles[mcell])*numpy.cos(
        targetangles[mcell])+exy*(numpy.cos(targetangles[mcell])**2-numpy
        .sin(targetangles[mcell])**2)
    strain1diffs[mcell]=e1
    strain2diffs[mcell]=e2
    strainxydiffs[mcell]=e1xy
    annenergy=ClusterEnergy(exteriors,interiors,dareas,strain1diffs,
        strain2diffs,strainxydiffs,areas0,interior0,exterior0,volumes0)
    energy=annenergy[0]
    clustenergy=annenergy
    cellenergyprogression.append(clustenergy[1])
    subenergyprogression.append(clustenergy[2])
    perminenergyprogression.append(clustenergy[4])
    strainenergyprogression.append(clustenergy[3])
    extenergyprogression.append(clustenergy[5])
    energyprogression.append(energy)
    perimextprogression.append(exteriorl)
    perimintprogression.append(interiorl)
    areaprogression.append(dareasl)
    strainprogression.append(strainl)
    #pick a cell and vertex
    cell=random.randint(0,numcells-1)
    vert=celloutline[cell][random.randint(0,numvertcells[cell]-1)]#choose vertex
    #move that vertex
    newcellvert=deepcopy(cellvert)
    newcellvert[vert]=[cellvert[vert][0]+random.uniform(-stepsize,stepsize),
        cellvert[vert][1]+random.uniform(-stepsize,stepsize)]
    edge=EdgeCells(celloutline,edgeneighbours,newcellvert)
    exteriors=edge[1]
    interiors=edge[0]
    newareas=[NewPolygonArea(x) for x in celloutline]
    h=V/sum(newareas)*int(numcells)
    dareas=map(sub,newareas,areas0)
    #calculate strain in the cells this move effects
    #find the cells it affects
    cells=[]
    for i in range(numcells):
        if celloutline[i].count(vert)==1:
            cells.append(i)
    #calculate strain in each cell
    newstrain1diffs=deepcopy(strain1diffs)
    newstrain2diffs=deepcopy(strain2diffs)
    newstrainxydiffs=deepcopy(strainxydiffs)

```

```

for i in range(len(cells)):
    mcell=cells[i]
    strain=DispPolygonVector(celloutline,edgeneighbours,icellvert,
        newcellvert,mcell)
    uxx=strain[0]
    uyy=strain[3]
    uxy=strain[1]
    uyx=strain[2]
    exx= 0.5*(2*uxx+uxx**2+uyx**2)
    eyy= 0.5*(2*uyy+uyy**2+uxy**2)
    exy= 0.5*(uyx+uxy+uxx*uxy+uyx*uyy)
    e1=exx*numpy.cos(targetangles[mcell])**2+eyy*numpy.sin(targetangles[
        mcell])**2+2*exy*numpy.sin(targetangles[mcell])*numpy.cos(
        targetangles[mcell])
    e2=exx*numpy.sin(targetangles[mcell])**2+eyy*numpy.cos(targetangles[
        mcell])**2-2*exy*numpy.sin(targetangles[mcell])*numpy.cos(
        targetangles[mcell])
    e1xy=2*(eyy-exx)*numpy.sin(targetangles[mcell])*numpy.cos(targetangles[
        mcell])+exy*(numpy.cos(targetangles[mcell])**2-numpy.sin(targetangles
        [mcell])**2)
    newstrain1diffs[mcell]=(e1)
    newstrain2diffs[mcell]=(e2)
    newstrainxydiffs[mcell]=(e1xy)
newenergy=ClusterEnergy(exterior,interior,dareas,newstrain1diffs,
    newstrain2diffs,newstrainxydiffs,areas0,interior0,exterior0,volumes0)
energydiff=newenergy[0]-energy
if ClusterGood(celloutline,newcellvert)==1:
    if energydiff<=0:
        cellvert=deepcopy(newcellvert)
        energy=newenergy[0]
        strain1diffs=newstrain1diffs
        strain2diffs=newstrain2diffs
        strainxydiffs=newstrainxydiffs
        areas=deepcopy(newareas)
        h=V/sum(areas)*int(numcells)
        targetangles=PreferedOrientation(cellvert,celloutline)
        exteriorl=sum(exterior)
        interiorl=sum(interior)
        dareasl=(sum(dareas))
        strainl=(sum(newstrain1diffs)+sum(newstrain2diffs))/2/numcells
        clustenergy=newenergy
        endiffprogression.append(energydiff)
    else:
        if math.exp(-energydiff/kt)>random.uniform(0,1):
            cellvert=deepcopy(newcellvert)
            energy=newenergy[0]
            strain1diffs=newstrain1diffs

```

```

        strain2diffs=newstrain2diffs
        strainxydiffs=newstrainxydiffs
        areas=deepcopy(newareas)
        h=V/sum(areas)*int(numcells)
        targetangles=PreferredOrientation(cellvert,celloutline)
        exteriorl=sum(exterioriors)
        interiorl=sum(interioriors)
        dareasl=(sum(dareas))
        strainl=(sum(newstrain1diffs)+sum(newstrain2diffs))/2/numcells
        clustenergy=newenergy
        endiffprogression.append(energydiff)

    z=z+1
posafter=[PolygonCenter(x,cellvert) for x in celloutline]
strains=[]
strainsxx=[]
strainsyy=[]
strainsxy=[]
for i in range(len(celloutline)):
    mcell=i
    gradedgeneighbours=PolygonNeighbours(celloutline)
    strain=DispPolygonVector(celloutline,gradedgeneighbours,icellvert,cellvert,
        mcell)
    uxx=strain[0]
    uyy=strain[3]
    uxy=strain[1]
    uyx=strain[2]
    exx= 0.5*(2*uxx+uxx**2+uyx**2)
    eyy= 0.5*(2*uyy+uyy**2+uxy**2)
    exy= 0.5*(uyx+uxy+uxx*uxy+uyx*uyy)
    e1=0.5*(exx+eyy)+numpy.sqrt(((exx-eyy)/2)**2+exy**2)
    e2=0.5*(exx+eyy)-numpy.sqrt(((exx-eyy)/2)**2+exy**2)
    theta=StrainAngle(exx,eyy,exy) #orientation
    strains.append([e1,e2,theta])
    strainsxx.append(exx)
    strainsyy.append(eyy)
    strainsxy.append(exy)
edge=EdgeCells(celloutline,edgeneighbours,cellvert)
exterioriors=edge[1]
interiors=edge[0]
areas=[PolygonArea(x) for x in celloutline]
h=V/sum(areas)*int(numcells)
majstrains=[]
for i in range(len(strains)):
    majstrains.append(strains[i][0])
e1=numpy.mean(majstrains)
minstrains=[]
for i in range(len(strains)):

```

```

    minstrains.append(strains[i][1])
e2=numpy.mean(minstrains)
meanstrains=[]
for i in range(len(strains)):
    meanstrains.append((strains[i][0]+strains[i][1])/2)

#####

#calculate strains of cells from before and after moduli change
cellstrains=[]
for i in range(len(celloutline)):
    mcell=i
    gradedgeneighbours=PolygonNeighbours(celloutline)
    strain=DispPolygonVector(celloutline,gradedgeneighbours,cellvertbefore,
        cellvert,mcell)
    uxx=strain[0]
    uyy=strain[3]
    uxy=strain[1]
    uyx=strain[2]
    exx= 0.5*(2*uxx+uxx**2+uyx**2)
    eyy= 0.5*(2*uyy+uyy**2+uxy**2)
    exy= 0.5*(uyx+uxy+uxx*uxy+uyx*uyy)
    e1=0.5*(exx+eyy)+numpy.sqrt(((exx-eyy)/2)**2+exy**2)
    e2=0.5*(exx+eyy)-numpy.sqrt(((exx-eyy)/2)**2+exy**2)
    theta=StrainAngle(exx,eyy,exy) #orientation
    cellstrains.append([e1,e2,theta])

#Calculate strain of cluster
clusterstrain=Cluststrain(posafter,posbefore)
exxs=[]
eys=[]
exys=[]
for i in range(len(posbefore)):
    uxx=clusterstrain[0][1]+2*posbefore[i][0]*clusterstrain[0][3]+posbefore[i]
        [1]*clusterstrain[0][4]
    uyy=clusterstrain[1][2]+2*posbefore[i][1]*clusterstrain[1][5]+posbefore[i]
        [0]*clusterstrain[1][4]
    uxy=clusterstrain[0][2]+2*posbefore[i][1]*clusterstrain[0][5]+posbefore[i]
        [0]*clusterstrain[0][4]
    uyx=clusterstrain[1][1]+2*posbefore[i][0]*clusterstrain[1][3]+posbefore[i]
        [1]*clusterstrain[1][4]
    exx= 0.5*(2*uxx+uxx**2+uyx**2)
    exxs.append(exx)
    eyy= 0.5*(2*uyy+uyy**2+uxy**2)
    eys.append(eyy)
    exy= 0.5*(uyx+uxy+uxx*uxy+uyx*uyy)
    exys.append(exy)

```

```

exx=numpy.mean(exxs)
eyy=numpy.mean(eyys)
exy=numpy.mean(exys)
clusterel=0.5*(exx+eyy)+numpy.sqrt(((exx-eyy)/2)**2+exy**2)
clusterel2=0.5*(exx+eyy)-numpy.sqrt(((exx-eyy)/2)**2+exy**2)
clustertheta=StrainAngle(exx,eyy,exy) #orientation
#find order parameters
beforelengthangles=[PolygonOrientation(x,cellvertbefore) for x in celloutline]
Sns=[2*math.cos(x-clustertheta)**2-1 for x in beforelengthangles]
Snn=[]
Snnlist=[]
Snnlistn=[]
#finds the distance of a cell to it's nearest neighbour
nneighbours=PolygonNeighbours(celloutline)[0]
rall=[]
for i in range(numcells):
    nn=nneighbours[i]
    x1=posbefore[i][0]
    y1=posbefore[i][1]
    for j in range(len(nn)):
        x2=posbefore[nn[j]][0]
        y2=posbefore[nn[j]][1]
        rall.append(math.sqrt((x2-x1)**2+(y2-y1)**2))
cellcelldist=numpy.mean(rall)
for i in range(numcells):
    x1=posbefore[i][0]
    y1=posbefore[i][1]
    angle1=beforelengthangles[i]
    for j in range(numcells):
        if i !=j:
            x2=posbefore[j][0]
            y2=posbefore[j][1]
            angle2=beforelengthangles[j]
            rtocell=math.sqrt((x2-x1)**2+(y2-y1)**2)
            op=2*math.cos(angle1-angle2)**2-1
            Snn.append(op)
            Snnlist.append(rtocell)
            Snnlistn.append(round(rtocell/cellcelldist))
lengthangles=[PolygonOrientation(x,cellvert) for x in celloutline]
#####
    plotng
matplotlib.pyplot.figure(7)
q=0
while q<len(areas):
    pointsx=[]
    pointsy=[]
    r=0

```

```

while r<len(celloutline[q]):
    pointsx.append(cellvert[celloutline[q][r]][0])
    pointsy.append(cellvert[celloutline[q][r]][1])
    r=r+1
pointsx.append(cellvert[celloutline[q][0]][0])
pointsy.append(cellvert[celloutline[q][0]][1])
matplotlib.pyplot.plot(pointsx,pointsy,'r')
matplotlib.pyplot.arrow(posafter[q][0],posafter[q][1],5*numpy.cos(strains[q]
    [2]),5*numpy.sin(strains[q][2]), color='m')
matplotlib.pyplot.arrow(posafter[q][0],posafter[q][1],5*numpy.cos(strains[q]
    [2]+numpy.pi),5*numpy.sin(strains[q][2]+numpy.pi), color='m')
matplotlib.pyplot.arrow(posafter[q][0],posafter[q][1],5*numpy.cos(
    lengthangles[q]),5*numpy.sin(lengthangles[q]), color='r')
matplotlib.pyplot.arrow(posafter[q][0],posafter[q][1],5*numpy.cos(
    lengthangles[q]+numpy.pi),5*numpy.sin(lengthangles[q]+numpy.pi), color='r'
    ')
q=q+1
matplotlib.pyplot.axes().set_aspect('equal', 'datalim')
matplotlib.pyplot.savefig(filenamestart+"strainstargetsplotafter.pdf")
matplotlib.pyplot.figure(8)
q=0
while q<len(areas):
    pointsx=[]
    pointsy=[]
    r=0
    while r<len(celloutline[q]):
        pointsx.append(cellvert[celloutline[q][r]][0])
        pointsy.append(cellvert[celloutline[q][r]][1])
        r=r+1
    pointsx.append(cellvert[celloutline[q][0]][0])
    pointsy.append(cellvert[celloutline[q][0]][1])
    matplotlib.pyplot.plot(pointsx,pointsy,'r')
    matplotlib.pyplot.arrow(posafter[q][0],posafter[q][1],5*numpy.cos(
        cellstrains[q][2]),5*numpy.sin(cellstrains[q][2]), color='m')
    matplotlib.pyplot.arrow(posafter[q][0],posafter[q][1],5*numpy.cos(
        cellstrains[q][2]+numpy.pi),5*numpy.sin(cellstrains[q][2]+numpy.pi),
        color='m')
    q=q+1
matplotlib.pyplot.axes().set_aspect('equal', 'datalim')
matplotlib.pyplot.savefig(filenamestart+"strainsplotafter.pdf")
matplotlib.pyplot.figure(6)
q=0
while q<len(areas):
    pointsx=[]
    pointsy=[]
    r=0
    while r<len(celloutline[q]):

```



```

        pointsx.append(cellvert[celloutline[q][r]][0])
        pointsy.append(cellvert[celloutline[q][r]][1])
        r=r+1
    pointsx.append(cellvert[celloutline[q][0]][0])
    pointsy.append(cellvert[celloutline[q][0]][1])
    matplotlib.pyplot.plot(pointsx,pointsy,'r')
    q=q+1
pointsx=[]
pointsy=[]
r=0
while r<len(posafter):
    pointsx.append(posafter[r][0])
    pointsy.append(posafter[r][1])
    r=r+1
matplotlib.pyplot.scatter(pointsx,pointsy,color='r')
matplotlib.pyplot.axes().set_aspect('equal', 'datalim')
matplotlib.pyplot.savefig(filenamestart+"strainsplotbeforeafter.pdf")
centerpos=[]
for i in range(len(areas)):
    centerpos.append(math.sqrt(posafter[i][0]**2+posafter[i][1]**2))
import operator
perimeters=map(operator.add,exteriors,interiors)

matplotlib.pyplot.figure(8)
matplotlib.pyplot.plot(energyprogression,color='r')
matplotlib.pyplot.savefig(filenamestart+"energyprogression.pdf")
matplotlib.pyplot.figure(9)
matplotlib.pyplot.plot(perimextprogression,color='r')
matplotlib.pyplot.savefig(filenamestart+"perimextprogression.pdf")
matplotlib.pyplot.figure(10)
matplotlib.pyplot.plot(perimintprogression,color='r')
matplotlib.pyplot.savefig(filenamestart+"perimintprogression.pdf")
matplotlib.pyplot.figure(11)
matplotlib.pyplot.plot(areaprogression,color='r')
matplotlib.pyplot.savefig(filenamestart+"areaprogression.pdf")
matplotlib.pyplot.figure(12)
matplotlib.pyplot.plot(strainprogression,color='r')
matplotlib.pyplot.savefig(filenamestart+"strainprogression.pdf")
matplotlib.pyplot.figure(15)
matplotlib.pyplot.plot(cellenergyprogression,color='b')
matplotlib.pyplot.plot(subenergyprogression,color='g')
matplotlib.pyplot.plot(perminenergyprogression,color='k')
matplotlib.pyplot.plot(strainenergyprogression,color='r')
matplotlib.pyplot.plot(extenergyprogression,color='c')
matplotlib.pyplot.savefig(filenamestart+"allenergies.pdf")
matplotlib.pyplot.figure(19)
matplotlib.pyplot.plot(endiffprogression,color='r')

```

```

matplotlib.pyplot.savefig(filenamestart+"endiffprogression.pdf")
matplotlib.pyplot.figure(20)
matplotlib.pyplot.scatter(centerpos,perimeters,color='b')
matplotlib.pyplot.savefig(filenamestart+"perimetersvscenterpos.pdf")
matplotlib.pyplot.figure(21)
matplotlib.pyplot.scatter(centerpos,areas,color='b')
matplotlib.pyplot.savefig(filenamestart+"areasvscenterpos.pdf")
matplotlib.pyplot.show()
areas==[PolygonArea(x) for x in celloutline]
h=V/sum(areas)*int(numcells)
#
#####
output
filename1=filenamestart
f = open(filename1+"energystab.txt","w")
for i in range(len(energyprogression)):
    f.write("%r" %energyprogression[i])
    f.write('\t')
    f.write("%r" %cellenergyprogression[i])
    f.write('\t')
    f.write("%r" %subenergyprogression[i])
    f.write('\t')
    f.write("%r" %perminenergyprogression[i])
    f.write('\t')
    f.write("%r" %strainenergyprogression[i])
    f.write('\t')
    f.write("%r" %extenergyprogression[i])
    f.write('\n')
f.close()

f = open(filename1+"Sparam.txt","w")
for i in range(len(Sns)):
    f.write("%r" %Sns[i])
    f.write('\t')
    f.write('\n')
f.write("Snn \n")
for i in range(len(Snn)):
    f.write("%r" %Snn[i])
    f.write('\t')
    f.write("%r" %Snnndist[i])
    f.write('\t')
    f.write("%r" %Snnndistn[i])
    f.write('\n')
f.close()

f = open(filename1+"radiusdep.txt","w")
for i in range(len(centerpos)):

```

```

        f.write("%r" %centerpos[i])
        f.write('\t')
        f.write("%r" %perimeters[i])
        f.write('\t')
        f.write("%r" %areas[i])
        f.write('\n')
f.close()

f = open(filename1+"param.txt","w")
f.write("zstab \t %r \t"%zstab)
f.write("\n")
f.write("zmax \t %r \t"%zmax)
f.write("\n")
f.write("ec \t %r \t"%ec)
f.write("\n")
f.write("ee \t %r \t"%ee)
f.write("\n")
f.write("es \t %r \t"%es)
f.write("\n")
f.write("ep \t %r \t"%ep)
f.write("\n")
f.write("ys \t %r \t"%ys)
f.write("\n")
f.write("ysshear \t %r \t"%ysshear)
f.write("\n")
f.write("ysdev \t %r \t"%ysdev)
f.write("\n")
f.write("ysfactor \t %r \t"%ysfactor)
f.write("\n")
f.write("moduli orientation \t %r \t"%alignmenttype)
f.write("\n")
f.close()

f = open(filename1+"posi.txt","w")
for s in icellvert:
    f.write("%r" %s[0])
    f.write('\t')
    f.write("%r" %s[1])
    f.write('\n')
f.close()

f = open(filename1+"posbefore.txt","w")
for s in cellvertbefore:
    f.write("%r" %s[0])
    f.write('\t')
    f.write("%r" %s[1])
    f.write('\n')

```

```

f.close()

f = open(filename1+"posafter.txt","w")
for s in cellvert:
    f.write("%r" %s[0])
    f.write('\t')
    f.write("%r" %s[1])
    f.write('\n')
f.close()

f = open(filename1+"celloutline.txt","w")
f.write("%r" %celloutline)
f.close()

f = open(filename1+"outputparam.txt","w")
f.write("%r \t"%clustere1)
f.write("%r \t"%clustere2)
f.write("%r \n"%clustertheta)
for s in cellstrains:
    for i in range(len(s)):
        f.write("%r" %s[i])
        f.write('\t')
    f.write('\n')
f.close()
matplotlib.pyplot.close("all")

```

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