Chapter 1

Introduction

1.1 Mechanics in the Context of Cell Biology

Mechanical forces play an important role in the activities of our daily lives from sitting to lifting objects to running. During all of these activities our bones and ligaments experience cycles of different mechanical loads, and need to adjust accordingly. For example, a runner's foot constantly experiences physical forces as it periodically impacts the ground. These forces are balanced by the runner's joints and bones, where the bone carries some of the compressive loading. If we examine the structure of the runner's bone in more detail, we would find a fairly porous structure made up of mostly calcium phosphate and collagen fibers, much resembling a light-weight composite structure. Intertwined with this structure are cells, some of which are constantly remodeling and restructuring the bone adjusting to the external loading.

In fact, one can think of the bone as an active mechanical system in which a particular type of cells called osteocytes act as mechanosensors. These "sensor" cells can communicate with other cells through an intricate feedback system to ensure the health or homeostasis of the bone itself. In particular, two of the cells with which osteocytes communicate are osteoblasts and osteoclasts. Osteoblasts are responsible for bone remodeling or remineralization. Osteoclasts, on the other hand, are in charge of removing or resorbing bone. So, in order to maintain a healthy bone structure, the activities of osteoblasts and osteoclasts need to be balanced.

However, since this system is based upon an active feedback loop, if the external mechanical

loading were to be removed, as in the case of an astronaut in space (zero-gravity environment), then the osteoblasts will cease to create more healthy bone structure due to the absence of the mechanical stimulus. Another type of imbalance is created in the case of osteoporosis, where insufficient osteoblasts exist to counteract the bone removal created by the more numerous osteoclasts. Of course this is a simplification of a delicate biochemical and mechanical system, which needs to be fully understood in order to maintain healthy, long-lasting bones.

Another example of the importance mechanical forces play in cellular behavior, is when tumor cells metastasize, or spread to the surrounding tissue in the body. In this case, as the tumor cells spread from their primary site they must venture through a jungle of extracellular proteins, such as proteoglycans, collagen, and elastin fibers collectively known as the extracellular matrix (ECM). For these malignant cells to successfully reach the arterial walls they need to push, squeeze, and cleave their way through the ECM, which involves exerting mechanical forces. Yet unfortunately, this migration mechanism, in particular the three-dimensional cell-matrix force exchange is relatively poorly understood.

As suggested by the above examples, there is an established connection between mechanical forces and cellular function [45]. In fact, these physical forces are today viewed equally as important as biochemical stimuli in directing cellular response. The translation of mechanical forces into biochemical signals that are responsible for determining cellular behavior is coined *mechanotransduction* [23]. Since the purpose of this investigation focuses on the physical interactions of cells and their ECM, the following overview presents a rather simplified version of the intricate interplay between mechanical forces and biochemical signaling, which can be found in more detail elsewhere [7, 24, 29, 36].

There are at least three ways in which mechanical forces can introduce biochemical signals [53]. The first one involves the opening and closing of cell membrane ion channels, which are activated through physical stresses. The second consists of the unraveling of protein molecules by mechanical forces, thus opening up previously hidden or cryptic binding sites. In the third, the application of physical forces can stabilize chemical bonds that are otherwise unstable or short-lived. However, for

these forces to take place, the cell needs to anchor itself to the surrounding ECM. These particular anchor points that connect the cell to the ECM are called focal adhesion sites. They serve as a gateway for the cell to transmit its internal forces to the ECM and vice versa.

Internal cellular forces are created through the interaction of actin with myosin II. Actin is one of the three structural elements found inside the cell, beside microtubules and intermediate filaments. Actin filaments can form long bundles also known as stress fibers that form a collaborate intracellular network that connects the focal adhesion sites (e.g., α and β integrins), much like truss structures support a steel bridge. Myosin II is a molecular motor protein that hydrolizes adenosine-5′-triphosphate (ATP) for energy, and induces contraction of the actin framework. The cell membrane has receptors or integrins that connect to the ECM proteins or ligands at the focal adhesion site. Cell contraction is first felt by actin, then transmitted to the membrane receptors across a multitude of focal adhesion proteins and finally to the ECM ligands. In order for the cell to move, all it needs to do is to detach from a particular focal adhesion site, thus creating a force imbalance. Not only do these cellular forces or cell traction forces play an important role in cell migration, they are also significant to many other physiological and pathological processes, both at the tissue and organ level. Some of these include wound healing, inflammation, angiogenesis, and embryogenesis [54]. Hence, quantification and understanding of their nature and regulation become an important part for the development of new implant material and drug treatments.

1.2   Previous Work on Quantifying Cell-ECM Interactions

Within the last few decades, studies began to quantify surface traction forces that are developed by migrating cells through a variety of techniques. In 1980 Harris et al. demonstrated that cellular forces could be visualized by creating thin elastic silicone rubber substrates and tracking the wrinkle formation response to the cell's applied stresses [20]. However, since wrinkle formation or buckling is an intrinsically nonlinear and unstable process, the quantitative characterization using this technique is difficult. In 1995 Oliver et al. and Dembo et al. developed a quantitative, non-wrinkling technique called *traction force microscopy (TFM)* to study fibroblast migration on two-dimensional

substrate surfaces [13, 34, 27]. This method utilizes optical phase and wide-field microscopy to track substrate surface displacements due to cellular traction forces via fluorescent particles embedded in a polyacrylamide substrate. This technique is currently one of the most widely used methods in determining cellular traction forces that are part of the mechanotransduction process. It will be described in more detail within the next section.

Another method to determine cell-induced traction forces was proposed by Tan et al. in 2003 where, instead of using fluorescent beads, thin cantilevers (micro needle-like posts) are fabricated out of poly-dimethylsiloxane (PDMS) substrates, from which traction forces can be calculated according to linear beam theory [49]. This method produces similar spatial resolution as the TFM technique on the micrometer scale with resolved forces of the order of nano-Newtons. Yet another approach demonstrated by Balaban *et al.* relies on displacement measurements of submicron patterned dots on PDMS susbtrate. However, both this technique and TFM employ an inverse Bousinessq formulation to convert the observed displacements into traction forces as will be explained later. Although these reports have contributed much to describing cell behaviors in two-dimensional environments, many physiological processes are three dimensional in nature and recent studies have not only shown morphological differences in cells cultured on two-dimensional substrates versus three-dimensional matrices, but their intrinsic ECM interactions and migration behavior also appears different [12, 16, 55]. Yet few advances have been made to address the need for three-dimensional quantitative imaging techniques [32]. Thus, a new class of experimental tools capable of quantitatively capturing such interactions is needed for more in-depth studies. This thesis presents a three-dimensional imaging technique capable of quantitatively measuring cell traction forces in all three spatial dimensions.

1.2.1   Traction Force Microscopy (TFM)

As previously mentioned probably the most wide-spread technique for investigating cellular traction forces utilizes traction force microscopy [7, 41, 14, 38]. This method measures surface displacement using either a single-particle or digital image correlation (DIC) based approach between two successive images. In most studies submicron fluorescent tracker particles are embedded in poly-

acrylamide or similar polymer based substrates with typical Young's moduli ranging from $\sim$ 1 - 30 kPa [14, 7, 38]. Balaban et al. used a geometrical pattern stamped into PDMS to track cell-induced surface displacements. To record cell surface deformations, cells are initially seeded on the substrate material and allowed to spread and migrate. After some time, a first image is recorded via an optical microscope, where typically both the cell and the tracker particles are recorded simultaneously. Then, the cells are chemically detached from the surface involving cleaving proteases such as trypsin to disrupt all cell substrate attachments. Without moving the microscope objective another image is captured serving as the undeformed or reference configuration. The two-dimensional full-field displacements are then determined from the set of the two images by either using a single particle tracking or digital image correlation algorithm.

In order to determine the cell-induced surface traction forces from the recorded displacement data, the Boussinesq formulation is utilized. The Boussinesq theory describes the displacement equilibrium solutions inside a semi-infinite elastic half-space with applied forces at its free boundary (surface) via the governing equation

$$\nabla(\nabla \cdot \mathbf{u}(\mathbf{x})) + (1 - 2\nu)\nabla^2\mathbf{u}(\mathbf{x}) = 0 \tag{1.1}$$

where $\mathbf{u}(\mathbf{x})$ is the three-dimensional displacement vector, and ν is the Poisson's ratio of the linearly elastic material. The details of the derivation in formulating the solution is given by Landau and Lifshitz [25], so only the end result is presented here. The displacement field $\mathbf{u}(\mathbf{x})$ in the semi-infinite half space can be written as a convolution integral with the Green's tensor $\mathbf{G}(\mathbf{x})$ and the applied surface traction forces given by

$$u_i = \int\int G_{ik}(\mathbf{x}_1 - \mathbf{x}_1', \mathbf{x}_2 - \mathbf{x}_2', \mathbf{x}_3 - \mathbf{x}_3')P_k(\mathbf{x}_1', \mathbf{x}_2')d\mathbf{x}_1'd\mathbf{x}_2', \quad (i, j, k = 1, 2, 3) \tag{1.2}$$

where the surface traction forces $P_k(\mathbf{x}_1', \mathbf{x}_2')$ at a particular location $(\mathbf{x}_1', \mathbf{x}_2')$ are expressed as

$$P_k = F\delta(\mathbf{x}_1')\delta(\mathbf{x}_2'), \tag{1.3}$$

where F is a concentrated surface force. In the TFM calculations, Eq. 1.2 is simplified by setting ν = 0.5, and assuming only two-dimensional, in-plane displacements resulting in,

$$G_{\alpha\beta}(\mathbf{r}) = \frac{3}{4\pi E r}(\delta_{\alpha\beta} + \frac{x_\alpha x_\beta}{\mathbf{r}^2}), \quad \mathbf{r} = \sqrt{\mathbf{x}_1^2 + \mathbf{x}_2^2}. \tag{1.4}$$

Finally, in order to determine the surface traction forces Eq. 1.2 needs to be discretized and inverted. However, the inversion of this problem is ill posed, especially in the presence of noise in the displacement data, and thus requires regularization schemes to calculate the traction forces accurately [14, 42]. Butler et al. addressed this issue and developed an approach of performing the inverse calculations in Fourier space and noted that the Fourier transform of the Green's tensor is diagonal and invertible without regularization or other modifications to the problem [7].

While the Boussinesq solution provides an approach to determine surface tractions and traction forces directly, it depends on the assumption of a semi-infinite elastic half-space or an elastic substrate of finite thickness. However, the criterion of when a substrate can be treated as infinitely thick is difficult to assess without any direct information about the deformation extending in the third spatial dimension (i.e. the thickness direction). It has been shown that the Boussinesq solution underestimates the forces when cells are seeded on gels ranging from 5 - 60 μm thick, and that finite height corrections are necessary [31]. To apply the Boussinesq solution, one must ensure that the displacement data is indeed recorded at the free surface, which is difficult to determine without depth information.

Another limitation to the conventional TFM formulation is that extreme care must be exercised when recording two-dimensional image data to ensure no focal (out-of-plane) drift occurs during imaging. This is especially challenging when imaging biological systems that require constant physiological temperature ($37°$C), where thermal drift is likely to occur.

Although these reports have contributed much to describing cell behaviors in two-dimensional environments, it has been recently demonstrated that many cell types show distinct three-dimensional morphologies and interactions, as expected *in vivo* [12, 16, 55]. Therefore the extension of TFM into the third spatial dimension has the capability to provide a more complete picture of cell-ECM

interactions.

1.3 Previous Digital Volume Correlation (DVC) Techniques

To extend the existing two-dimensional traction force microscopy techniques into three-dimensional, a different set of instruments and algorithms needs to be utilized. A few recent studies have begun to develop quantitative three-dimensional imaging techniques using X-ray tomography and laser scanning confocal microsopy in conjunction with digital volume correlation. In 1998 Bay *et al.* first proposed an extension of the DIC method into three-dimensional and named it digital volume correlation (DVC) [4]. In their study, volumetric images were acquired using X-ray tomography of trabecular bone, where the microstructure of the porous bone is used as a tracking pattern. Analagous to DIC, DVC algorithms divide the entire volume image into smaller sized subsets. Bay *et al.* based their correlation algorithm on the translation of the center of these subsets only, where terms in the deformation gradient are ignored. In 2002 Smith *et al.* refined the existing DVC algorithm developed by Bay *et al.* by accounting for finite rotations, presenting an improvement in the overall accuracy of the technique when rotations exceed 0.05 radians [46, 9]. Their study utilized a micro computer tomography scanner and aluminum foam samples, where again the material microstructure was used as the tracking pattern. In 2004 Roeder *et al.* combined a translation-based only DVC algorithm similar to Bay *et al.* with laser scanning confocal microscopy to measure the three-dimensional deformations of a collagen based gel under uniaxial tension. To track material displacements they utilized the native material microstructure under autofluorescent illumination [39]. In 2005 Pizzo *et al.* applied the translation-based DVC and LSCM method developed by Roeder *et al.* to measure volumetric strain and cell area in human dermal fibroblast cells encapsulated in collagen I extracellular matrices. The purpose of this study was to investigate the role of collagen fibril density in regulating local cell-ECM behavior. In particular, the connection between cell shape and proliferation as a function of fibril density was examined. Again, the microstructure of the collagen-based ECM was used to track cell mediated displacements [37]. Throughout these studies

it was found that DVC subsets of the size 64 x 64 x 64 voxels[1] gave optimal correlation results, while the subset spacing or grid resolution was typically between 64 - 16 voxels due to computational constraints.

1.4   Accomplishments

This thesis presents a quantitative imaging technique using laser scanning confocal microscopy (LSCM) and digital volume correlation (DVC). This technique has the capability of determining the three-dimensional full-field displacements inside transparent materials, in particular, soft biomaterials. This technique was validated by comparing experimentally measured displacement and strain field data obtained from uniaxial compression and spherical inclusion tests, and obtained excellent agreement with the predicted analytical solution [18]. Next, this method was applied to migrating single cells on the surface of polyacrylamide substrates and successfully captured the three-dimensional deformation fields induced by these cells. This demonstrates that the newly developed LSCM and DVC method has the capability of quantitatively capturing the three-dimensional mechanical interaction of cells and their extracellular matrix.

One of the advantages of the developed three-dimensional quantitative imaging technique over those reported previously [4, 46, 39] is that the method presented here takes into consideration the stretch deformation of each volume subset. This allows for a more accurate strain estimate, especially when local strains are large and subset deformation is significant. Another advantage over previous studies is the addition of a deconvolution algorithm, which minimizes the blurring of confocal images. Thus, the overall resolution of the images, in particular along the optical axis, is significantly improved leading to an increase in the accuracy of the subsequent DVC calculations. Also advantageous over previous studies is that the presented results do not depend on the local sample feature size to achieve high correlation resolution, but rather can be tailored to the relevant length scale of interest. This is achieved by utilizing commercially available fluorescent markers rather than relying on autofluorescence of the sample, which can limit the field of view. The method

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[1]A voxel is generally defined as a three-dimensional pixel.

presented here allows the user to choose virtually any field of view provided the appropriate markers are available. Using this method, locally varying displacement and strain fields around motile cells are determined in all three spatial dimensions. Also, this technique can be used to experimentally determine the material properties of soft materials, especially where conventional characterization techniques fail due to the compliant nature of the material.

In the second part of this thesis, an application of the new LSCM and DVC technique to live cell systems is described. In particular, it presents the time-dependent measurements of the three-dimensional deformations induced by single migrating fibroblasts on polyacrylamide substrates. Results are presented for cell-induced displacements and cellular traction forces information during different stages of cell movement in all three dimensions for the first time. While the focus in this study was to track single cell material deformations, this technique is capable of measuring three-dimensional deformation fields of cell clusters and cell sheets of many different cell types.

Chapter 1 has described the motivation of this project and presented a literature review of the crossroads between biochemistry and mechanics in cell-matrix interactions followed by the currently employed two-dimensional (two-dimensional) traction force methodologies. It also reviewed previous developments in digital volume correlation, and concluded by stating the accomplishments of this thesis. The remainder of this the thesis is organized as follows:

Chapter 2 presents the development of the quantitative full-field three-dimensional imaging technique based on laser scanning confocal microscopy and digital volume correlation, and its validation. Chapter 3 describes the experimental setup of the cell system, which has been chosen as an application of the developed quantitative imaging method. Chapter 4 presents the experimental findings and observations of applying the LSCM-DVC method to migrating fibroblast cells on polyacrylamide substrates. Lastly, Chapter 5 concludes this study by providing a summary of findings, including preliminary experimental observations on cells interacting with artificial extracellular matrix (aECM) proteins, and provides recommendations for future work.