

Methods for Multi-Dimensional Analysis of Neutrophil Mechanosensing

by

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**Abstract of “Methods for Multi-Dimensional Analysis of Neutrophil
Mechanosensing”**

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The mechanical interaction of neutrophils with the extracellular matrix (ECM) is essential in regulating neutrophil migration to sites of inflammation. The vital chemotaxis response of the neutrophil becomes dysregulated in many disease states, including sepsis and certain autoimmune diseases, which can lead to increased risk of infection, organ failure, and death. Therefore, understanding the neutrophil chemotaxis response and its subsequent dysregulation in disease models from a mechanical perspective is of utmost clinical importance. Many experimental and computational challenges prevent neutrophil biologists from studying three-dimensional neutrophil force generation, including prohibitively expensive imaging equipment requirements and difficulties characterizing and modeling fibrous materials. To this end, presented here are two new methods for quantifying three-dimensional traction forces produced by neutrophils. The first is for cells crawling on planar substrates imaged using epifluorescence microscopy, an inexpensive imaging modality that historically limited users to two-dimensional traction force reconstruction. The second method is for measuring tractions from neutrophils fully embedded in fibrous ECM using a material model that can be experimentally validated and fit to fibrous hydrogel systems using the described three-dimensional material characterization technique. This technique is then applied to measure surface tractions produced by neutrophils undergoing chemotaxis in a novel chemokine gradient-producing device. These developments provide the fundamental experimental and analytical techniques required for future work probing neutrophil force generation and dysregulation.

Curriculum Vitæ

Lauren was born and raised in Bordentown, N.J., where she graduated from high school in 2010. She earned her Bachelor of Science in Biomedical Engineering from The College of New Jersey in 2014. In the fall of 2014, she started her graduate studies as a Ph.D. student in Biomedical Engineering at Brown University, performing research under Dr. Christian Franck and Dr. Jonathan Reichner. While at Brown, Lauren acted as a teaching assistant for two semesters, as an instructor for Summer at Brown classes for two years, and as an instructor for the Lincoln School's Introduction to Engineering course for two years. The final two years of her graduate career were spent as a visiting scholar at the University of Wisconsin-Madison in the Mechanical Engineering department, where she began a collaboration with Dr. Jeniel Nett.

Peer Reviewed Publications

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DEDICATED TO MY FAMILY
IN LOVING MEMORY OF REV. EARL W. ZELLERS

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Chapter 1

Introduction

1.1 Role of Mechanics in Cell Biology

Cells *in vivo* reside within complex tissue microenvironments. These dynamic networks of cells and extracellular matrix (ECM) materials regulate cell behavior through a combination of biophysical and biochemical signals [1–4]. Living cells convert physical stimuli into biological responses in a process known as mechanotransduction; the ability of cells to sense mechanical cues is called mechanosensing. It is widely accepted that cells are mechanosensitive [5–12], capable of probing and responding to not only direct interactions with the ECM [2,13,14], but also to applied forces and fluid shear flow [15–17], and to neighboring cells [18–20]. Force production and sensing by cells within their microenvironment has been shown to regulate such vital cellular processes as spreading, migration, differentiation, morphogenesis, embryogenesis, cellular homeostasis, and the disease and trauma response [4,7–9,9,11,21–29]. Understanding the mechanisms with which cells respond to mechanical forces is fundamental to advancing clinical knowledge of diseases like sepsis [17,30], cancer (and in particular cancer metastasis) [27,31–34], atherosclerosis [35,36], and traumatic brain injury [37,38].

Understanding the exact mechanisms by which cells are able to sense and transmit forces within their complex and heterogeneous microenvironments is an important step in the field

of cellular biomechanics. The primary site of force transmission for the cell is the focal adhesion complex: discrete assemblies of proteins directly under the cell membrane which form the primary mechanism by which cells bind to extracellular matrix molecules. Focal adhesions (FA) facilitate the reception and transmission of cell-matrix force interactions [2]. Cells have been shown to transmit forces and rotational moments about focal adhesion complexes [39, 40], regardless of whether these FAs are nascent or mature and in both two- and three-dimensional matrix environments [2, 3, 41, 42]. Focal adhesion proteins such as vinculin, talin, and paxillin interact to transmit forces effectively from the external ECM to the cellular cytoskeleton, which mediates cellular force sensing and transmission internally [2, 43, 44]. The cytoskeleton is a dynamic protein structure that provides mechanical stability and controls cellular contractility. Actin fibers, namely F-actin, along with the motor protein myosin II form stress fibers, which function to transmit forces between the cell and the ECM through focal adhesion complexes. During mechanotransduction, the interaction between the FA and the stress fiber result in downstream signalling to the nucleus, which controls gene expression and the cellular response to forces [2]. Despite this progress toward understanding the processes by which cells are able to experience and transmit forces, the mechanisms by which this occurs depend on many variables, including cell type, migration mode (amoeboid vs. mesenchymal), and the extracellular microenvironment [1, 2]. Therefore, developing techniques to probe the physical interactions between the cell and its microenvironment is an important step toward advancing our understanding of cellular mechanotransduction.

1.2 Measuring cellular force production

Measuring cellular forces has long been of interest to cellular biomechanicians. Harris et. al. were the first to observe substrate distortions caused by cell-exerted forces: cells cultured on thin polymeric silicone sheets caused measurable wrinkling of the underlying substrate [45]. Others sought to measure cellular force generation using raised micropillar structures: changing the dimensions of the pillar structure alters the pillar stiffness and therefore the force required to manipulate the pillars [46, 47]. Cells cultured on a bed of micropillars or tissues grown around pillar formations will deform the pillars during migration or other

cellular processes, and the force required is computed by treating the pillars as cantilever beams using the known pillar dimensions, stiffness, and amount of pillar deflection [46, 48–53]. Micropillar assays have been used to probe cell-cell force interactions in addition to single cell force production [54–56]. Dembo and Wang were able to show that the traction forces exerted by a cell on a passive flexible substrate could be measured and mapped based on the displacements of beads embedded within the substrate [6]. This technique has become known as traction force microscopy (TFM) and is one of the most common methods for computing cell-generated traction stresses in two- and three-dimensional systems [6, 57–59]. Traction force microscopy is performed by culturing or seeding cells onto an elastic deformable substrate embedded with fluorescent microbeads that serve as fiducial markers. The deformation field is reconstructed by tracking the motion of the embedded fiducial markers, and the cell-produced surface tractions are computed using the deformation field and the matrix material properties [53, 60–63]. The general workflow for TFM, including options for computation and considerations for various methods, is shown in Fig. 1.1.

Traction force microscopy was initially limited to two-dimensional information from two-dimensional systems: in-plane or shear forces were computed from cells cultured on planar substrates [6, 57]. This was due partially to the available imaging modalities, since reconstructing three-dimensional information from two-dimensional imaging systems is challenging (see Chapter 2), and partially to the mathematical and computational complexity of computing traction forces in three dimensions. Three-dimensional traction forces have since been computed from cells on planar substrates [58, 59, 65, 66] and from cells fully embedded in three-dimensional fibrous matrices [31, 51, 67, 68].

Generally, TFM requires fluorescence images of fiducial particles embedded in the cell substrate [6, 53, 62] or micro-patterned onto the substrate surface [69–71]. To reconstruct full-field, three-dimensional displacement and traction information, three-dimensional image stacks are required. A traction-free, cell-free reference image is also required, except in cases where the reference condition is known [69–71]. Typically, displacement fields are reconstructed using either particle image velocimetry or digital image correlation for two-dimensional reconstruction, or the three-dimensional correlates of these techniques (i.e.

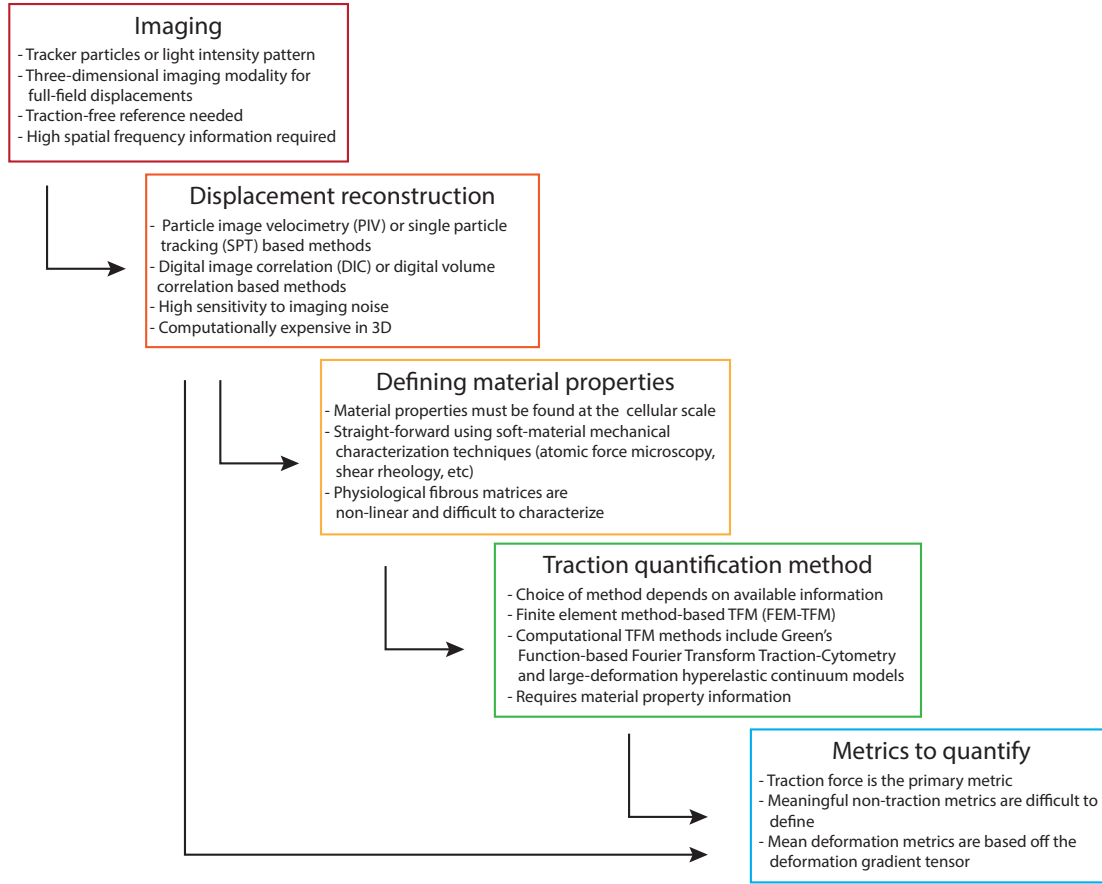


Figure 1.1: **Breakdown of the steps and processes required for traction force microscopy.** First, images must be acquired: usually these are three-dimensional (3D) images of fluorescent tracker particles or other fluorescent patterns. Second, the displacement field must be reconstructed, which is usually done using PIV or DIC-based methods. Then, material properties of the underlying or surrounding matrix must be found, usually using standard material characterization techniques, though more complex material models are required for non-linear, fibrous extracellular matrix materials. Next, the stress and traction fields are computed, usually using either FEM-TFM or computational methods. Finally, useful metrics are pulled from this data to describe cellular behavior: typically, TFM studies seek to compute tractions specifically, though without known matrix material properties, mean deformation metrics [64] or other displacement-based metrics [34] can also be used.

single particle tracking or digital volume correlation) [62, 63, 72–75]. Displacement field reconstruction relies on high spatial frequency information, and can be highly sensitive to image noise, therefore care must be taken for proper microscope alignment and selection of imaging parameters such as microscope objective and size and density of fluorescent features for tracking relative to the desired spatial frequency of the reconstructed displacement field.

Computing cellular tractions from the displacement field requires knowledge of the

boundary geometry between the cell and the extracellular matrix or substrate and of the matrix material properties. The boundary geometries need to be supplied either as part of the inverse formulation or through explicit microscopy image segmentation [53, 60–63]. Material properties can be determined using a variety of soft-material mechanical characterization techniques, including atomic force microscopy (AFM) and shear rheometry. For linear isotropic materials, characterizing either Young’s modulus and Poisson’s ratio or shear and bulk moduli is sufficient to describe the material response; for non-linear and particularly for fibrous materials, more complex constitutive equations describe material behavior and thus additional material properties need to be defined and measured or computed. In the case that the matrix material properties or constitutive relations are unknown or have not been measured, metrics that rely on the computation of the deformation gradient tensor or displacement field rather than the stress field can be used to describe cellular behavior [34, 64]. Recent work in material modeling and characterization has produced models and methods which aim to characterize three-dimensional fibrous materials with the intent of computing tractions produced by cells embedded in *in vitro* fibrous material systems in ways that account for the highly-nonlinear and non-affine response of biological matrices [12, 67, 68, 76–79].

Using the matrix material properties and displacement field information, tractions are computed using a number of methods, which generally fall into one of two categories: finite element method traction force microscopy (FEM-TFM) or continuum mechanics-based computational models [6, 57–59]. Early TFM computations relied upon the inverse formulation of the classic linear elasticity solution for a point force applied to an elastic half-space, i.e., the Boussinesq solution. The Boussinesq solution is solvable both in real space, using the boundary element method (BEM), a pre-cursor to more modern finite element method TFM (FEM-TFM) [6], and in Fourier space using Fourier Transform Traction Cytometry (FTTC) [57, 80]. The BEM and FTTC are useful metrics, but rely on an assumption of infinite gel thickness. FEM-TFM has been used to improve upon these methods by accounting for finite gel thickness [58] and even to compute tractions from cells fully embedded in a 3D matrix [31, 68, 81]. FEM-TFM is used to compute stresses by applying the computed

displacement field onto a discretized finite element mesh representing the substrate and applying a system of equations based on the material properties to numerically compute the solution to the boundary value problem [58,62,81–83]. Meanwhile, computational-based methods have been developed that involve solving for tractions by direct solution of the governing constitutive relations, which include purely linear elastic material models and large deformation material models to more accurately reconstruct cell-produced tractions [59,84]. Despite the progress in the field of TFM, there are still major gaps that exist. For example, expensive three-dimensional imaging modalities are required for computing three-dimensional traction fields, and fibrous materials are difficult to characterize and model effectively for reconstructing accurate traction forces. The field is constantly evolving to include new developments which are necessary to increase the accuracy of reported tractions and to draw conclusions about cellular behavior.

1.3 The biological and mechanical neutrophil response

Neutrophils, or polymorphonuclear leukocytes (PMNs), are the most abundant circulating white blood cells in the human body and a vital part of the innate immune system. These cells are bone marrow-derived and terminally differentiated upon release into circulation, with a distinct multi-lobed nuclear phenotype [85–87]. The primary function of neutrophils is to seek out and eliminate infectious agents within the body, including bacterial and fungal pathogens. Neutrophils circulating in the bloodstream are in a resting or inactive state, but undergo significant phenotypic changes in the presence of a variety of biochemical signaling molecules, or cytokines. These changes are the beginning of the neutrophil recruitment cascade [86–88], which can be seen in more detail in Fig. 1.2. In a typical protective immune response, the neutrophil recruitment cascade is mediated by ligand-receptor interactions with the inflamed endothelium as neutrophils undergo initial attachment (i.e., capture), followed by rolling adhesion along the endothelium and firm arrest, at which point the neutrophils are able to crawl along the endothelial layer and eventually transmigrate into the interstitial tissue [86,87]. At this stage, fully activated neutrophils are able to migrate toward the invading microbes or injured tissue following a gradient of cytokines produced

by the invading microbes, injured tissues, or other leukocytes, among other sources. Once a neutrophil has reached the infection site, it attempts to eliminate the infection and releases its own signalling molecules to recruit monocytes, macrophages, and other neutrophils to the area. There are three primary mechanisms by which neutrophils can induce pathogen death: degranulation, phagocytosis, and release of nuclear material as neutrophil extracellular traps (NETs) [86, 87, 89]. Eventually, neutrophils in tissue undergo apoptosis and are cleared by resident phagocytes such as macrophages or dendritic cells [88, 90].

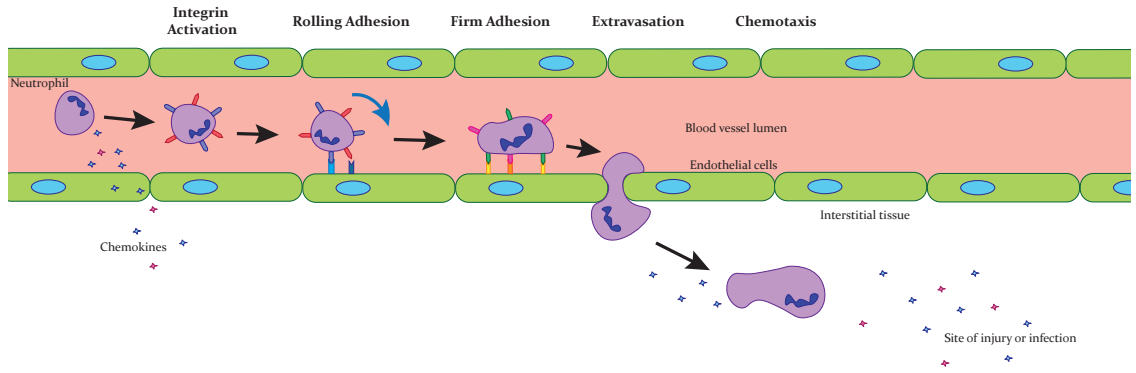


Figure 1.2: **Schematic of the neutrophil recruitment cascade.** Neutrophils circulate through the bloodstream until activated by the presence of chemokine signalling molecules, at which point they are able to adhere to the inflamed endothelium (first through rolling and then firm adhesion). At this point, neutrophils exit the blood vessel and migrate along a chemokine gradient to the site of inflammation, where they work to clear the infection and release their own signalling molecules to control the inflammatory response from other immune cells.

Neutrophils are part of a subgroup of leukocytes known as granulocytes because they contain cytoplasmic granules. Neutrophils specifically contain three distinct types of granules that contain a variety of antimicrobial and signalling molecules: primary or azurophilic, secondary or specific, and tertiary or gelatinase, named for the order in which they develop during neutrophil formation and their contents [86]. Neutrophils also contain secretory vesicles, which are sometimes considered to be a fourth type of granule [86, 87] because they have similar contents and purpose. In response to microbial infection, neutrophils undergo degranulation, where the membranes of the granules fuse with the plasma membrane and release the contents of the granules into the extracellular space, where they disrupt microbe activity [86, 87, 90]. In addition, neutrophils produce reactive oxygen species (ROS) through

membrane-bound NADPH oxidase complexes that reduce molecular oxygen to superoxide and hydrogen peroxide, two potent molecules which give rise to other ROS [86,91,92]. ROS production is increased following neutrophil activation, which leads to ROS release in a process known as respiratory or oxidative burst [91,92]. Importantly, neutrophils are also phagocytic; they are capable of recognizing non-self, pathogenic compounds through toll-like receptors (TLRs), binding to, and phagocytosing these pathogens. Following phagocytosis, granules can fuse with the phagosome and release their contents within, and NADPH oxidase complexes can assemble on the phagosome membrane, releasing ROS into the phagosome and eliminating the microbes. Finally, neutrophils have the ability to trap and kill larger microbial invaders through the release of decondensed chromatin through the release of NETs, or NETosis. NETs contain antimicrobial proteins like histones and high concentrations of ROS, which help them disrupt microbial activity [86,89]. It is worth noting that NETs have been shown to have detrimental effects when dysregulated: they are prevalent in chronic inflammatory diseases such as atherosclerosis [93], have been implicated in the systemic autoimmune disease lupus erythematosus [94], can trap platelets and lead to venous thrombosis [95], and have been found in the sputum of cystic fibrosis patients, where they may contribute to mucosal thickening [96].

For many years, it was assumed that the neutrophil population was fairly homogeneous, with phenotypic or functional differences between individual cells arising primarily due to circumstance of activation, known as neutrophil priming. Recent work has led to the belief that there is in fact a large degree of heterogeneity in the neutrophil population, with subpopulations of neutrophils presenting distinct phenotypes and functional plasticity, though whether this is due to distinct neutrophil lineages or plastic neutrophil precursors has yet to be determined [88,90,97]. Low-density neutrophils, thus named because this subset of neutrophils separates out in the low-density fraction of whole blood in a Ficoll density gradient rather than the high-density fraction as is typical for neutrophils, are associated with cancer, sepsis, autoimmune disease, HIV infection, and asthma [88,97]. Neutrophils have also been shown to undergo phenotypic changes during cancer: cells exhibit a distinct polarity within

the tumor microenvironment, showing phenotypes that are either pro-inflammatory and anti-tumorigenic, capable of killing cancer cells, or anti-inflammatory and pro-tumorigenic [98]. Even the traditional recruitment cascade (i.e., circulating neutrophil activation and extravasation, chemotaxis and microbe elimination followed by apoptosis and clearance by macrophages) does not fully describe the variety of behaviors and functions of the human neutrophil that have been observed. For example, neutrophils in a rat glomerular capillary injury were shown to largely clear the injury site and re-enter circulation, rather than undergo apoptosis locally [99]. Later, similar findings were shown to extend to neutrophils from zebrafish, mouse, and *in vitro* human neutrophils; neutrophils were observed leaving the site of inflammation in a process called neutrophil reverse migration, and later entering the vasculature following a response to injury in a process termed reverse transendothelial migration [97, 100–102]. The exact mechanisms for neutrophil reverse migration and reverse transendothelial migration are unknown, though these behaviors represent an interesting target for clearing neutrophils from tissue in diseases characterized by excessive neutrophil activation or accumulation in tissue [97].

Among the most important and more unique properties of neutrophils is their ability to sense and respond to signalling molecules known as chemoattractants or chemokines. Signalling molecules regulate many neutrophil behaviors, including extravasation, phagocytosis, and leukocyte recruitment, by inducing phenotypic changes in a process known as neutrophil activation. Importantly, the presence of chemoattractants induces neutrophil chemotaxis: a process by which neutrophils sense a chemokine gradient, polarize, and undergo directed migration along the gradient toward the chemokine source [90, 103, 104]. Chemokines present in uniform concentrations can induce random motion, or chemokinesis. Chemokinesis is often used as a positive control for neutrophil activation assays, since chemokines induce many of the phenotypic and behavioral changes characteristic of cell activation [103, 104]. During chemotaxis, neutrophils respond to chemokine gradients in a hierarchical manner, with chemokines originating from the immediate vicinity of the infection, such as bacterially-derived formylated peptides (e.g., *N*-formyl-methionyl-leucyl-phenylalanine, or fMLP) or

complement components, taking higher priority over signals originating from intermediary sites, such as the endothelium or other leukocytes, including Leukotriene B₄ (LTB₄) and interleukin 8 (IL-8): thus chemokines can be categorized as ‘end target’ or ‘intermediary’ [90,104,105]. Most chemokines bind to G-protein coupled receptors (GPCRs) expressed on the neutrophil cell surface, and primarily induce movement through the PI3K and MAPK signalling pathways [86,90,104].

Chemotaxis is a vital part of the immune response as neutrophils must navigate the interstitial tissue to the site of inflammation in order to respond to and control an infection. However, in many disease states, neutrophil chemotaxis and other functions become dysregulated as the cells become hyperactivated. Many of the antimicrobial and ROS molecules that neutrophils contain and release to target invading microbes can also cause necrosis of native tissue. Systemic neutrophil activation, which can be seen in patients following severe traumatic injury, with systemic inflammatory response syndrome (SIRS), sepsis, or autoimmune disease can result in serious tissue damage; organs such as the liver, lung, kidney and brain are at risk for failure, which can lead to death [106–109]. In patients with SIRS and sepsis, neutrophil migration is dysregulated, leading both to an accumulation of neutrophils in vital organs, putting patients at risk for organ failure, and insufficient neutrophil recruitment to sites of microbial invasion, putting patients at risk of systemic infections [108–111]. The exact mechanisms by which SIRS and sepsis alter the ability of neutrophils to respond to chemokine signals is unknown: numerous studies have failed to pinpoint any specific signalling pathway dysregulations or to produce effective treatments [111–114]. This leads to the conclusion that neutrophil chemotaxis and subsequent dysregulation occurs as a function of both chemical and mechanical signals. Studying the mechanical chemotaxis response in both healthy and disease states could reveal important information about neutrophil hyperactivation and dysregulation which could translate clinically to treatment modalities that target mechanical signaling mechanisms.

Research into neutrophil mechanosensing and the response of these cells to biomechanical cues in combination with the more readily studied chemical signalling mechanisms is therefore of utmost clinical importance. Researchers have already started studying traction force

generation from neutrophils in two- and three-dimensional systems. Biomechanics studies in two-dimensional systems primarily entail seeding neutrophils onto planar substrates to record traction forces. These studies have shown that neutrophil behavior differs based on the stiffness of the underlying substrate: neutrophils on stiffer substrates spread significantly more, generate larger tractions, and undergo more direct migration under both chemotaxis and chemokinesis conditions [11,115]. Neutrophils have also been shown to migrate on planar substrates with traction patterns displayed in pairs and focused primarily in the uropod, or rear, of the cell [115–117]. Other studies have focused on studying the mechanisms for neutrophil migration under confinement or in fully three-dimensional systems. These studies have shown that neutrophils do not require integrin interactions to migrate [118–120], and rely on expansion of the actin and myosin networks with characteristic leading edge protusive flowing and squeezing contraction of the trailing edge, which propels the nucleus forward [119]. Other research into neutrophil biomechanics focuses on the neutrophil interaction with the endothelium [121,122], mechanisms behind phagocytosis [123], and mechanical responses under fluid shear [17].

However, there is very little research into the biomechanical neutrophil response in disease states, particularly sepsis. To effectively study changes in neutrophil mechanosensing in disease states, cells from septic patients need to be collected and compared with healthy donor samples. Studying healthy human neutrophils presents a myriad of challenges: neutrophils are among the smallest (8-10 μm in diameter), fastest moving (capable of moving upwards of 19 μm per minute during chemotaxis [124]) cells in the human body, which makes them difficult to image, particularly in three-dimensional systems. They are also incredibly sensitive to a number of stimuli, and the exact activation state of a neutrophil after isolation from whole blood is nearly impossible to discern. These cells are terminally differentiated when they enter circulation, preventing growth in tissue culture, and attempts at immortalizing cell lines have failed to reproduce the functional heterogeneity of primary cells [86]. Neutrophils survive in the blood stream for only a few hours to a few days [90], and in most culture systems are very short-lived. In addition to all of these challenges, retrieving patient samples, particularly from severely ill septic patients, can be deemed an unnecessary burden

for the patient. The work presented in this thesis provides new methods for studying the neutrophil biomechanical response in healthy and disease states, providing researchers with straight-forward, repeatable experimental methods and freely available code packages that address some of these challenges and aid in increasing research productivity in this field.

This thesis presents two novel techniques for use in studying neutrophil mechanosensing: one a three-dimensional analysis of neutrophil behavior on a planar 2D surface, and the other a three-dimensional analysis of neutrophils embedded in a 3D hydrogel system with an established chemokine gradient. The first system, described in Chapter 2, is a purposefully low-threshold system, designed for any neutrophil biologist with access to an epifluorescence microscope to be able to probe displacements and traction forces produced by cells as they move on a linear elastic planar substrate. Traction forces from neutrophils in disease states, neutrophil NETosis, cell clustering, frustrated phagocytosis, or cell interaction with various surface coatings are just some of the many open questions that could be probed using this system. The second system is described in Chapters 3 and 4, and is intended for more advanced cell mechanicians with access to 3D imaging modalities such as confocal or multiphoton microscopes. By embedding cells in 3D fibrous hydrogels meant to model the tissue microenvironment and optionally introducing chemokine gradients to direct cell behavior, this system increases the physiological relevance for research questions being probed. Analysis of changes in neutrophil behavior in different disease states or models, in different stiffness, density, or material microenvironments, in the presence of multiple or varying strength chemokine gradients, or even in the presence of bacteria or fungal pathogens could all be performed in this system.

1.4 Accomplishments

This thesis presents two new methods for quantifying three-dimensional traction forces produced by neutrophils: one for cells crawling on a planar substrate, and one from cells fully embedded in a fibrous hydrogel, which is then applied to compute traction forces from neutrophils in a novel chemotaxis device.

Chapter 2 presents a novel analytical technique for reconstructing a fully three-dimensional displacement field and 3D planar surface traction field from epifluorescence microscopy images. Despite the importance of out-of-plane displacement and traction components to fully understanding cell behavior, epifluorescence images are generally not used for 3D traction force microscopy (TFM) experiments due to limitations in spatial resolution and reconstructing out-of-plane motion. To extend epifluorescence-based TFM into the third dimension, the technique outlined in this chapter involves embedding a single layer of fluorescent tracker particles at the cell-substrate surface interface, deconvolving the epifluorescence images of these particles to improve z -localization, and employing a topology-based single particle tracking algorithm to compute the 3D displacement of each particle. Finally, finite element analysis is used to compute the fully three dimensional displacement field and corresponding stress field, from which the 3D tractions at the substrate surface are computed. This technique is validated using a combination of synthetic and experimental images to confirm the accuracy of the displacement measurement, and to test the ability of the procedure to evaluate tractions at high spatial frequencies and large amplitudes. The technique is also applied to measure displacement and traction outputs from Madin-Darby canine kidney cell clusters and primary human neutrophils.

Chapter 3 presents a method for measuring fibrous material properties using an embedded contracting particle with known mechanical properties, which produces radial strains with known quantities. This method is unique in that it provides measurements from within the hydrogel in a manner that mimics the native contractile behavior of a cell. Collagen hydrogels with embedded fluorescent tracker particles and poly(N-isopropylacrylamide) (PNIPAAm) particles, a linear isotropic material which undergoes a phase transition at a temperature of around 35°C, are imaged and analyzed to measure the resultant displacement fields. The technique for fitting an existing material model, presented by Steinwachs *et. al.* [67], to any fibrous hydrogel using this method by computing the necessary fibrous material coefficients is presented. To demonstrate this method and validated material model can be applied to compute cellular tractions, primary human neutrophils were embedded in collagen hydrogels and activated with N-Formylmethionyl-leucyl-phenylalanine (fMLP) and

the cell-produced displacement and traction fields were reconstructed.

Chapter 4 describes the development of a novel device for producing stable chemokine gradients across a 3D hydrogel microenvironment. This device allows for live-cell imaging on an inverted microscope, addition of up to two chemokines for investigating competing chemokine gradients, is fully enclosed to prevent hydrogel dehydration, and completely embeds cells within the hydrogel microenvironment to allow for probing of cellular displacements and tractions following the protocol outlined in Chapter 3 of this thesis. The device is inexpensive and simple to produce using a 3D-printed mold, poly-dimethylsiloxane (PDMS), and 2 glass coverslips. The planar chemokine gradient produced within the device hydrogel is modelled analytically, and the efficacy of the established gradients were shown by introducing neutrophils within the device to fMLP and LTB_4 in collagen type-1 hydrogels. The neutrophil response was quantified by examining cell motion, including overall cell displacement, velocity, and path straightness in response to the gradient compared to cells in the same device under chemokinesis or no activation conditions. Additionally, displacements and tractions produced by a single neutrophil embedded in a collagen matrix in response to an fMLP gradient are computed to demonstrate the device allows for live cell imaging of sufficient quality for traction reconstruction. The device is demonstrated to produce differential responses during chemokinesis, chemotaxis, and no stimulation from primary human neutrophils embedded in a collagen-1 hydrogel. These findings demonstrate that the device was successful at introducing a chemokine gradient in a device that permitted live cell imaging and measurement of cell-generated displacements and tractions. This chapter details the development, validation, and testing of the device.

Chapter 5 summarizes the findings presented and details suggestions for future research.

Chapter 2

Three-Dimensional Traction Force Reconstruction from Epifluorescence Images

Note: A version of this chapter has been submitted for publication. All data and figures included are done with co-authors' consent.

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Epifluorescence-based three-dimensional traction force microscopy. Under review.

2.1 Introduction

The production of cellular forces has been shown to regulate many important cellular processes including embryogenesis, cellular homeostasis, and disease and trauma response [7, 9, 27, 28]. To investigate cellular and inter-cellular force generation, traction force microscopy (TFM) has become a well-established technique in mechanobiology that quantifies tractions produced during cell-cell or cell-matrix interactions, i.e., cell-imposed forces acting on interfaces between a cell or cells and the microenvironment [61–63]. TFM has been cast in both two-dimensional (2D) and three-dimensional (3D) variants [6, 53, 58, 59], from

single cells [6, 57–59] to multicellular clusters and sheets [20, 125–127]. While the original development of TFM started with 2D images acquired from phase contrast and epifluorescence microscopy and accounted for only small material deformations, most recent TFM approaches have the capability to fully reconstruct 3D motion and traction fields in a variety of linear, non-linear, and viscoelastic material systems [53, 62, 64, 81, 128] using confocal, multiphoton, or superresolution techniques [65, 81, 84, 129]. Despite the significant variations and evolution of TFM, the technique itself consists of three basic steps: reconstructing a cell-induced displacement field from microscopy images using either single particle or image correlation based techniques, inverting a constitutive relation to connect material displacements to stresses, and computing and visualizing traction vector fields [61–63].

Almost all cellular traction field reconstructions rely on accurate measurements of the cell-induced material deformation fields, which are typically computed from microscope images of tracker particles, either embedded in the cell substrate [6, 53, 62] or micro-patterned onto the substrate surface [69–71], and analyzed with either a single particle tracking or image correlation-based method. Digital image correlation (DIC) and particle image velocimetry (PIV) are popular correlation-based techniques in 2D; while digital volume correlation and single particle tracking algorithms are commonly found in 3D investigations [62, 63, 72, 74, 75]. The choice of algorithm often comes down to the type of images acquired (e.g., 2D vs. 3D, fluorescent vs. phase contrast, etc.), image content being tracked (e.g., individual embedded particles vs. intensity patterns), and the spatial resolution required.

Once the cell-induced material displacement fields have been determined, the cell-applied stresses, and thus tractions, can be computed in a number of ways. In the early days of TFM, most traction reconstructions relied on the inverse formulation of the classic linear elasticity solution of a point force applied to an elastic half-space. The Boussinesq solution was used to compute tractions initially in real space using the boundary element method [6] and later in Fourier space (so called Fourier transform traction cytometry, FTTC) [57]. Methods for computing tractions have evolved from these early two-dimensional reconstructions with advancements in imaging modalities and high throughput computing; currently,

three-dimensional traction forces can be computed in various ways, including direct solution of the governing constitutive equations [59, 84], using FTTC [80] or the finite element method [58, 81–83]. Finite element method traction force microscopy (FEM-TFM) traditionally imposes the measured displacement field onto a pre-defined grid, or mesh, of small elements representing the substrate, and then solves for tractions using a system of equations defined by the mesh discretization of the solution domain [62]. FEM-TFM has greatly expanded the capabilities of 3D-TFM measurements by accounting for complications such as finite gel thickness [58] and cells fully embedded in a 3D matrix [31, 68, 81]. It is noteworthy to point out that reconstruction of cellular traction fields using any of these techniques invariably relies upon knowledge of matrix material properties (e.g., Young’s modulus and Poisson’s ratio or shear and bulk moduli) and of the boundary geometry between the cell and the extracellular material (e.g., the hydrogel). Material properties can be determined using a variety of soft-material mechanical characterization techniques while the boundary geometries need to be supplied by the end user either as part of the inverse formulation or through explicit microscopy image segmentation. Several reviews exist that compare the various displacement measurement and traction reconstruction techniques, including their specific applications and advantages and disadvantages [53, 61–63].

Advances in 3D-TFM techniques have revealed that cells produce large deformations into or out of the plane of the substrate [84], as well as rotational moments about focal adhesions [39, 40]. This combination of shear and normal force information reveals substantial new insight into cellular behavior, including how force production is tied to intercellular machinery, how this machinery interfaces with the cell substrate, and how cells turn force production into motion: information that would not be readily available from 2D-TFM. Unfortunately, most 3D-TFM techniques rely upon full-field 3D displacements, which in turn rely upon fully three-dimensional imaging modalities such as confocal or multiphoton microscopy. The availability of these more advanced imaging systems is often limited by prohibitively high costs, which constrains many TFM users to epifluorescence or phase contrast imaging. Due to the native limitations of these traditionally 2D imaging systems, namely

the increase in out-of-plane light scattering, those seeking to use these systems for TFM often use a single layer of fluorescent microbeads as fiducial markers to limit out-of-plane light from particles randomly dispersed throughout a gel [130, 131]. This adaptation results in planar displacement data, which makes reconstructing 3D stresses for computing tractions challenging. Del Alamo demonstrated that using a thin top layer of beaded substrate to limit out-of-plane light scattering in spinning disk confocal images was sufficient for accurately capturing 3D displacements and, in conjunction with 3D Fourier TFM methods, fully 3D traction fields [80]. Until recently, epifluorescence or phase contrast images limited users to in-plane, or two-dimensional, traction information. Makarchuk *et al.* used holographic traction force microscopy to localize and track particles embedded within the substrate from phase contrast images [132]. In a similar technique, Hall *et al.* used epifluorescence along with a three-dimensional defocused particle tracking method to reconstruct displacement fields from fluorescent particles embedded in a substrate [12]. These techniques were shown to be effective at localizing 3D bead displacements with high resolution at low spatial frequencies; however, due to the high degree of light scattering native to phase contrast and epifluorescence images, are not able to resolve particles at higher densities and thus are unable to acquire high spatial frequency three-dimensional information.

Here we introduce a technique for computing a fully three dimensional displacement field and both in-plane and out-of-plane traction components from epifluorescence images of a single layer of beads using a combination of deconvolution, single particle tracking, and finite element analysis. A single layer of tracker particles limits out-of-plane light from the fluorescent tracker particles, and enables a high in-plane particle density, allowing us to obtain high spatial frequency information. Our topology-based single particle tracking (TPT) algorithm [72] allows us to accurately reconstruct high spatial frequency, three-dimensional displacements from the dense layer of fluorescent particles. The single-plane 3D displacement measurements from the particle tracking algorithm are directly used in a finite element analysis to solve for the full-field, 3D volumetric displacement and stress field quantities, from which surface tractions are computed. By relaxing imaging requirements for 3D-TFM to epifluorescence microscopy while maintaining three dimensional measurements and high

spatial frequency capabilities of traditional 3D-TFM, we aim to enable a broad segment of cell biologists to investigate mechanobiology.

2.2 Methods

This section outlines the method for validation of the technique using synthetic and experimental data, along with the protocols for experimental setup and image acquisition for both validation experiments and two cell test cases. Data acquisition and analysis parameters are presented in Table 2.1.

2.2.1 Synthetic validations

To vet the accuracy of the algorithm with known imposed fields, synthetic images were generated with Gaussian indentation-like traction distributions. In this way, we test the algorithm while independently and self-consistently varying the spatial frequency and amplitude content of the displacement signal over a range of synthesized experiments.

A previously described bead seeding algorithm [72,74] that places bead images into an image volume at subpixel locations and with minimal interpolation was employed. Undeformed images were generated in a four step process. First, a representative real image of a bead was extracted from an experimental image (see the Cell Preparation and Imaging section). Second, bead center positions were seeded in the volume with random non-overlapping coordinates at the approximate density and spatial distribution observed in experiments. Third, bead images were added to the image volume at the predefined locations, and, fourth, the final images were corrupted with a Gaussian white noise profile and dynamic range adjusted to match measurements from our experimental test cases. To then generate the deformed images the displacements for each seed location were sampled from the analytical solution for Gaussian z -traction indentation of an elastic half-space with material properties corresponding to typical experimental parameters ($E = 1500$ Pa and $\nu = 0.45$) for a series of increasing spatial frequencies, controlled via the full-width half-magnitude (FWHM) of the signal ranging from $W = 7.68\mu\text{m}$ to $W = 76.8\mu\text{m}$ in steps of $7.68\mu\text{m}$ at a fixed 0.32

Table 2.1: Process parameters used for the complete workflow with experimental rigid displacement and synthetic traction validations, and neutrophil and MDCK cell datasets.

<i>Parameter</i>	<i>Rigid displacement validation</i>	<i>Synthetic validation</i>	<i>Neutrophil cell experiment</i>	<i>MDCK cell experiment</i>
Image set up parameters				
Magnification	40X (0.6 NA)	40X (0.6 NA)	40X (0.6 NA), 1.5X optical zoom	40X (0.6 NA)
Full image size [vx]	2560×2156×101	1025×1025×42	2560×2156×101	2560×2156×41
Cropped image size [vx]	1024×1024×101	1024×1024×42	1024×1024×44	1024×1024×41
μm-per-pixel conversion (<i>x,y</i>)	0.16 $\frac{\mu m}{px}$	0.16 $\frac{\mu m}{px}$	0.11 $\frac{\mu m}{px}$	0.16 $\frac{\mu m}{px}$
z-step size (μm)	1.0	0.5	0.3	1.0
Bead size (μm)	0.5	0.5	0.5	0.5
PSF cube half size [vx]	10	10	10	10
Deconvolution	Lucy-Richardson, 10 iterations			
Localization parameters				
Threshold	0.125	0.125	0.125	0.125
Minimum size (vx)	2	2	2	2
Maximum size (vx)	200	200	200	200
For loop steps	1	1	1	1
Tracking parameters				
KNN Feature similarity	10	10	10	10
KNN Feature descriptor	16	16	16	16
Matching threshold	2	2	2	2
Outlier threshold	5	7	7	7
Number of spheres	2	2	2	2
Planarization parameters				
Number of standard deviations	1.2	1	0.8	1.8
regularization smoothness x-y	0.00001	0.00001	0.00001	0.00001
regularization smoothness z	0.00001	0.00001	0.00001	0.00001
Finite element parameters				
Elastic modulus (Pa)	—	1500	1500	8300
Poisson’s ratio	—	0.45	0.45	0.45
Gel thickness (μm)	—	70	70	70
Number of steps	—	10	10	10
Quadrature degree	—	7	7	7

μm peak displacement amplitude and force amplitudes from $F = 40\text{ nN}$ to $F = 400\text{ nN}$ in steps of 40 nN at $W = 10.24\mu\text{m}$. The frequency and amplitude ranges were selected to encompass typical experimental observations, for example those found in Toyjanova *et al.* [118]. For each case, the analytical solution for displacements and tractions are based on a convolutional Fourier-space Boussinesq solution that are simultaneously computed at each measurement point.

The synthetic volume images were propagated through the complete analysis workflow of Fig. 2.1, and uncertainties were computed for displacements and tractions. Figure 2.6 shows the mean uncertainty magnitude (see Eq. 2.1) of TPT displacement, post-finite element solved displacements, and traction magnitude. The finite element mesh with color contours of measured displacement in the deformed configuration (10x warping factor) is shown in Fig. 2.6. The uncertainty quantification between the analytically solved and measured synthetic fields is computed using the mean of the pointwise error between reconstructed and imposed ground truth via

$$|\text{Uncertainty}| = \frac{\sqrt{\sum_n (\chi_{\text{measured}} - \chi_{\text{imposed}})^2}}{N} \quad (2.1)$$

where n are the measurement points (e.g., quadrature points or mesh vertices on the top surface), χ is a field quantity (e.g., displacement or traction), and N is the total number of measurement points for the quantity. The results from this uncertainty formulation, which describes an approximate non-parametric confidence interval-type uncertainty for a given measurement point in its base units, are plotted independently as functions of signal magnitude or spatial frequency in Fig. 2.6b and c with the standard error of the measurement shown as shaded area.

2.2.2 Polyacrylamide gel preparation

Polyacrylamide gels with a single layer of fluorescent micro-beads were created using a modified version of the protocol previously described by Knoll *et al.* [130], and is shown in Fig. 2.2.

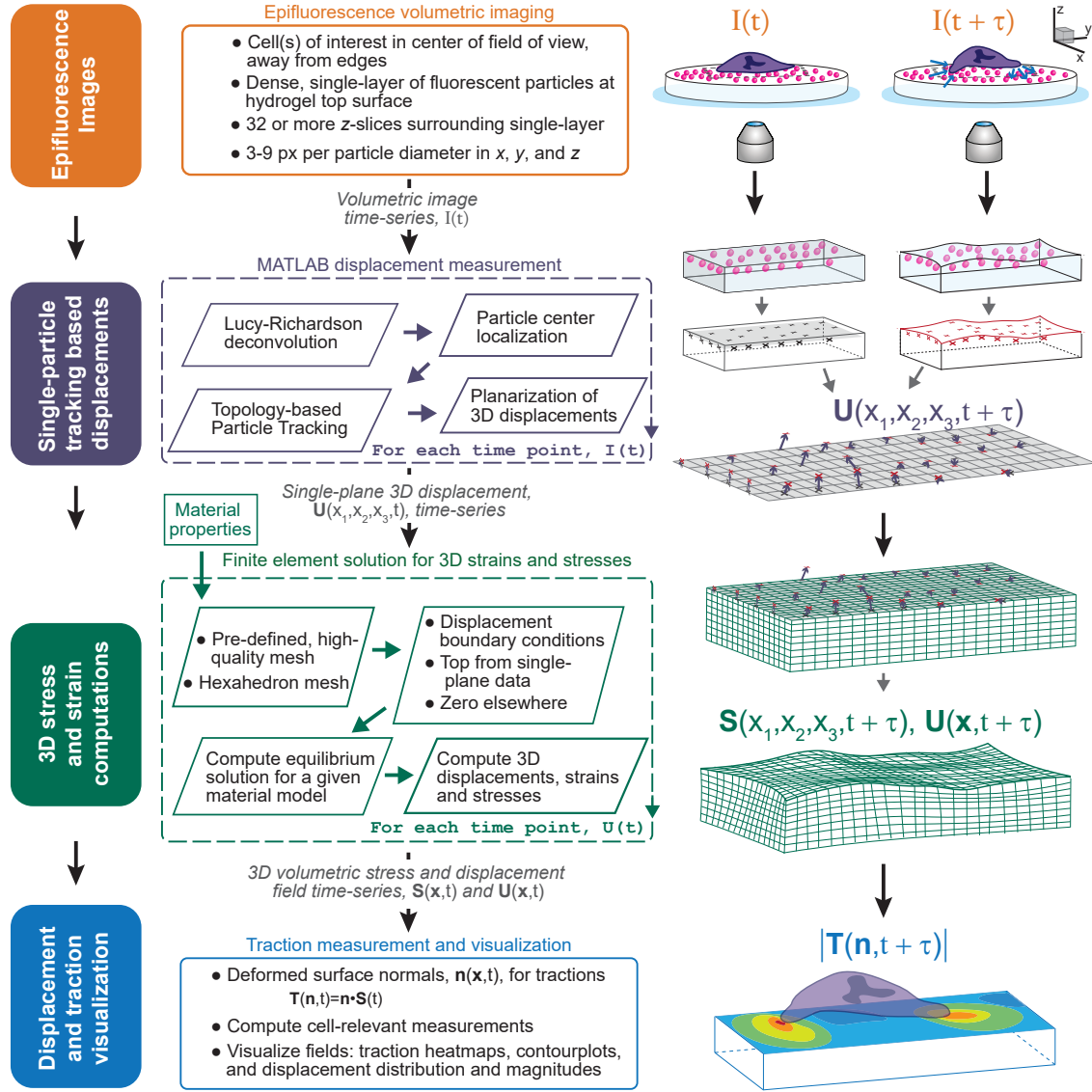


Figure 2.1: **Flow-chart detailing the procedure for calculating tractions from epifluorescence images.** Volumetric epifluorescent images of spherical fiducial markers (i.e., fluorescent microbeads) embedded at the surface of a cellular substrate medium are imported into Matlab where they are deconvolved and a particle tracking algorithm is employed to calculate 3D displacements. The nearly-planar scattered displacements are mapped onto a planar grid with a second order lookup-table regularization. These 3D surface displacements are then input into a finite-element based solution to the boundary value problem defined by the known and measured displacement fields and material properties. From this computation, the stresses and a full-field 3D displacement field in the substrate domain are extracted and used to compute in-plane and out-of-plate traction components. The right-hand column provides a simplified illustration of the process for images taken at time, t , and after some time, τ .

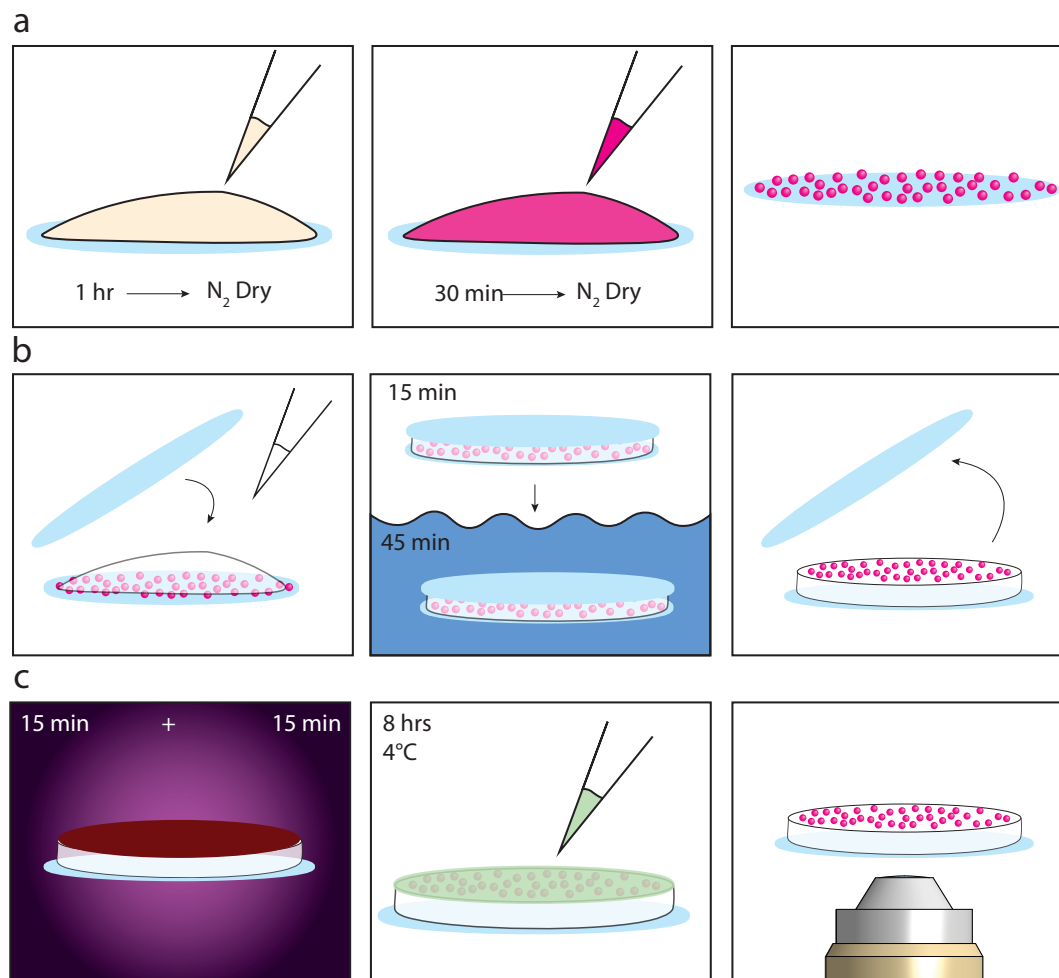


Figure 2.2: **Procedure for making polyacrylamide gels with a surface layer of fluorescent beads.** (a) A clean coverslip is coated with Poly-L-Lysine for 1 hour, dried with lab air, then coated with fluorescent microbead solution for 30 minutes before being blown dry again. (b) This bead-coated coverslip is used with a hydrophilically-coated glass coverslip to sandwich polyacrylamide gel solution, which polymerizes for 15 minutes in air before being allowed to swell in water for 45 minutes. The previously bead-coated coverslip is removed using tweezers, leaving the beads embedded in the top surface of the polymerized gel. (c) This PA gel is then coated with 1 mg/mL Sulfo-SANPAH solution and placed under UV light for 15 minutes, the Sulfo-SANPAH is replaced and the UV exposure is repeated a second time. The treated gel is coated with protein and left in a 4°C refrigerator overnight. Finally, the gel is ready to be seeded with cells and imaged.

Briefly, glass coverslips were cleaned using ethanol and dried. 0.1% w/v Poly-L-Lysine (PLL) was coated onto the surface of the coverslips and allowed to sit for 1 hour. Suspended 0.5 μ m carboxylate modified polystyrene beads (Thermo Fisher Scientific) were vortexed for 30 s to 1 min prior to dilution to a final ratio of 1:100 in deionized water. Poly-L-Lysine was

blown off of the surface of the glass coverslips using air, and the treated surfaces were then coated with the 1:100 bead solution and allowed to sit for 30 minutes before being blown dry again, as shown in Fig. 2.2a. Glass coverslips were hydrophilically treated using amino 0.5% (v/v) 3-aminopropyltrimethoxysilane in ethanol, followed by 0.5% glutaraldehyde in deionized water. Polyacrylamide gels were fabricated using the method originally described by Pelham and Wang [5], where polyacrylamide gels are polymerized between two glass coverslips; in this case a hydrophilic coverslip on the bottom and a bead coated coverslip on the top, as shown in Fig. 2.2b. Gels of two stiffnesses were fabricated by varying the relative concentrations of acrylamide and bis-acrylamide (Bio-Rad), to be 8% /0.08% bis and 3%/0.2%, to produce gels with Young’s moduli of 8.3 ± 0.2 kPa and 1.5 ± 0.1 kPa, respectively, as measured by Toyjanova *et. al.* [118]. Crosslinking was initiated with the addition of ammonium persulfate (Sigma-Aldrich) and N,N,N,N-tetramethylethylenediamine (Sigma-Aldrich). For MDCK cell experiments, 8.3 kPa gels were used, and for neutrophil and all other experiments, gels of 1.5 kPa were used. Gels were given 15 minutes to polymerize before being immersed in deionized water, and allowed to swell for 45 minutes. The PLL-coated coverslip was peeled off, leaving the layer of beads embedded in the gel, as seen in Fig. 2.2c. PA gel stiffness for MDCK and neutrophil experiments were selected to promote cell adhesion and to permit cells to produce measurable matrix deformations, using previously published experimental data for each given cell type [11, 115, 118, 133].

2.2.3 Single-layer gel imaging parameters

Polyacrylamide gels with a single layer of fluorescent beads were imaged on a Nikon TI-2 epifluorescence microscope using a 40X/0.6 NA air objective with an aligned correction collar. A three-dimensional stack of epifluorescence images was taken around the beads, with a μm -per-pixel ratio of 0.16 and a z -step size of $0.3 \mu\text{m}$ (or $1.0 \mu\text{m}$ for MDCK cells), per the recommended Nyquist sampling rate for the chosen objective and system. Raw images of $2560 \text{ voxels} \times 2156 \text{ voxels} \times 101 \text{ voxels}$ are typical for our imaging system (pco.edge 5.5, PCO AG) and can be used at full resolution in the algorithm on a workstation computer with 64GB RAM, but are cropped to $1024 \text{ voxels} \times 1024 \text{ voxels} \times 101 \text{ voxels}$ to reduce

computational resources required when the cells of interest reside in a subset of the image, as was the case for our neutrophil and MDCK cell test data shown in Figs. 2.7 and 2.8.

2.2.4 Rigid body displacement validation

To assess the ability of our algorithm to accurately localize and track displacements from epifluorescence images and to create a planar displacement field that matches a known displacement condition, we performed a series of rigid body displacement experiments. This experimental image set was captured by taking an initial image stack with the single layer of beads centered in z and imposing a known incremental stage motion in x -, y -, or z -directions before acquiring a new image stack. The stage motion setpoints were 0.0 μm , 1.0 μm , 2.0 μm , 3.0 μm , 5.0 μm , 10.0 μm , and 15.0 μm , and images were captured of the microbeads after the prescribed stage motion in each of the three orthogonal directions. We then interrogate the accuracy and precision with which our method resolves known displacements based upon the read out from the stage encoder (Nikon Instruments, positioning accuracy $\pm 0.1 \mu\text{m}$). We plot the distribution of the reconstructed displacements for a zero rigid body motion case in Fig. 2.5a, as well as the measured versus applied displacement, for all of the rigid body motion cases, as shown in Fig. 2.5b. The positional accuracy of the stage encoder is much smaller than imposed rigid motions, ($\pm 0.1 \mu\text{m}$ vs. minimum 1 μm), and does not appear to be a significant error source based on this displacement analysis.

2.2.5 Cell preparation and imaging

For cellular experiments, polyacrylamide (PA) gels were treated with a 1 mg/mL concentration of the crosslinker sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate) (Sulfo-SANPAH, Thermo-Fisher Scientific) for two, fifteen minute increments under UV light, washed with 1X PBS, and then coated overnight with either 0.2 mg/mL fibronectin (neutrophils) or 0.2 mg/mL collagen-I (MDCK cells), following established protocols [84]. Excess protein solution was washed off using PBS before the PA gels were incubated at 37 °C in imaging media prior to the addition of cells.

Blood was taken from healthy volunteers with written informed consent through a protocol approved by the University of Wisconsin Internal Review Board. Primary human neutrophils were isolated by negative antibody selection using the MACSxpress Neutrophil Isolation and MACSxpress Erythrocyte Depletion kits (Miltenyi Biotec, Inc.). Cells were labeled by 8 minute incubation in 2 μ M Calcein AM in Hank's Balanced Salt Solution, without calcium, magnesium, or phenol red. Cells were seeded onto fibronectin-coated PA gels in Leibowitz's L-15 media with 2 mg/mL glucose added, and allowed to adhere for 20 minutes at 37°C prior to imaging. Neutrophil images were taken with the same 40X/0.6 NA air objective as all other images, but with the Nikon TI-2 1.5X optical zoom lens in place to help resolve the smaller cells.

Madin-Darby canine kidney type II cells (Sigma Aldrich) were cultured in low glucose Dulbecco's modified eagle medium (DMEM, Fisher Scientific) and fetal bovine serum until nearly confluent. MDCK cells were removed from culture dishes by incubation with Trypsin-EDTA (Thermo Fisher Scientific) and then seeded onto PA gels coated with collagen-I. The cells were allowed to adhere and proliferate for 24 hours, until nearly confluent, prior to staining with 8 μ M Calcein AM for 20 minutes in media, and then imaging.

All live-cell experiments were conducted in a custom-built temperature control chamber at 37°C. Three-dimensional z -stacks of fluorescent microbeads in the substrate and fluorescently labeled cells on the substrate surface were taken simultaneously to capture displacement information. Fluorescence images of a single plane of the neutrophil and MDCK cell cluster z -stacks can be seen in Fig. 2.3, and a maximum intensity projection of the bead images can be seen in Fig. 2.4. Following acquisition of images of both types of cells, cells were removed from the PA gels using 5% sodium dodecyl sulfate (SDS). A final volumetric image stack for the reference condition was acquired 15 minutes after the addition of the SDS, when all of the cells had detached from the gels.

2.2.6 Computational requirements

Cell image stacks were taken at 2560 px $\times$ 2156 px by 101 slices and cropped to 1024 voxels $\times$ 1024 voxels $\times$ 40 voxels around the cell border for analysis on a desktop PC with

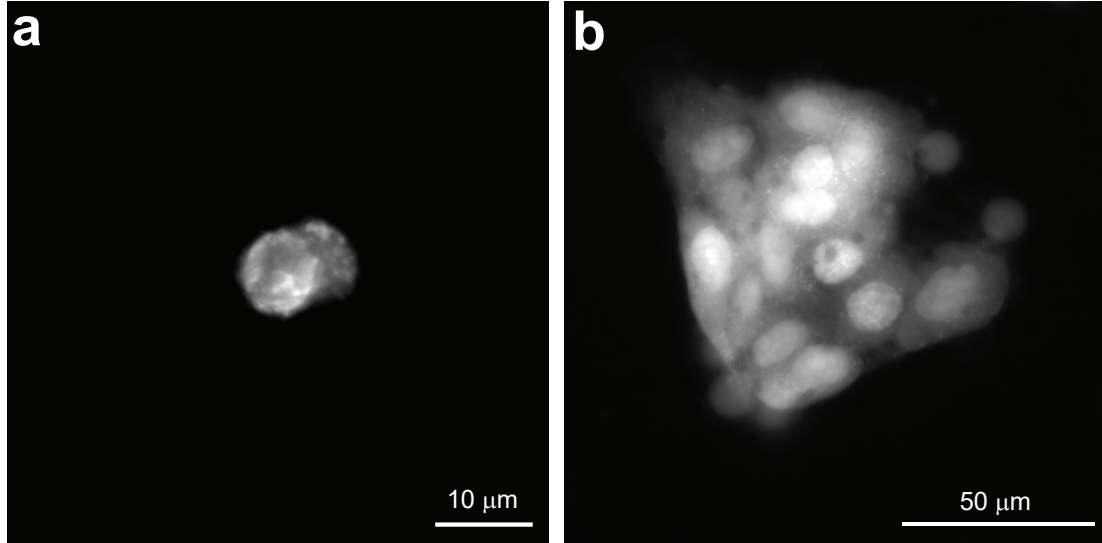


Figure 2.3: **Fluorescence images of a single neutrophil and MDCK cell cluster.** A single plane of the full image is shown, chosen at approximately the cell-surface interface, though full three-dimensional image stacks were acquired for 3D cell segmentation. (a) Epifluorescence image of a Calcein-AM labeled primary human neutrophil which has spread on a fibronectin-coated polyacrylamide gel into an irregular morphology. (b) Epifluorescence image of Calcein-AM dyed MDCK cells that were allowed to adhere and form clusters on collagen-i coated polyacrylamide for 24 hours.

32 GB of RAM. Deconvolution, the most memory-intensive step of the process, requires approximately 64 GB RAM for full-size images, which may take several hours per image, but is significantly reduced for the cropped images. Regarding processing time (Intel i7-4790 at 4 GHz, 32 GB DDR3 at 1600 MHz, NVME M.2 solid state storage drive, synthetic validation test case), deconvolution of the cropped images takes approximately 90 s per image stack. Timing for bead localization and computing displacements closely follows the timing results in Patel et al. [72] – approximately 20 s on 3 parallel threads. Regularization is completed in approximately 1 min, and the following plotting and output steps are accomplished in approximately 20 s. By far the most time consuming stage of the computational process is the finite element solver, which is computed with 16 GB and 2 CPUs allocated for Docker in approximately ten minutes per time point image pair. In future, more extensive multi-processor capability could be straightforwardly added if needed. Post-processing to compute surface tractions take only a few tenths of a second. Thus total runtime (neglecting time for user interaction) may be budgeted at approximately 15 min per time point image pair.

2.3 Results

We present a new method to obtain 3D displacement and surface 3D traction fields from epifluorescence images. The complete procedure is outlined in Fig. 2.1 and the description of the method is broken down in more detail in the remainder of this section. To summarize these steps, 3D volumetric image stacks of cells on a planar substrate with a single layer of fluorescent microbeads at the surface are taken on an epifluorescence microscope. These are imported to Matlab, where the images are deconvolved, and particles localized and tracked using topology-based particle tracking (TPT). The resulting nearly planar layer of measurement locations are used to project the displacement onto a planar grid to define a displacement boundary condition before being output to the free and open source finite element analysis (FEA) program FEniCS [134, 135], where the solution for the full-field displacement and stress information is computed. This full field information is then imported into Matlab for final traction computation and visualization.

2.3.1 Single-Layer bead polyacrylamide substrate fabrication

Polyacrylamide gels with a single layer of fluorescent microbeads were created using a modified version of the protocol previously described by Knoll *et al.* [130]. Briefly, beads were adhered to a poly-L-Lysine coated glass coverslip, which was then used to sandwich a droplet of polyacrylamide gel solution during polymerization, as originally described by Pelham and Wang [5]. The bead-coated coverslip was removed, leaving the layer of beads embedded in the gel surface. For more information on polyacrylamide gel preparation, see the Methods section. Representative images demonstrating limited z -spread of the bead positions, with beads randomly distributed with uniform density in x - y (i.e., limited bead clumping, empty regions, or other inhomogeneities) are shown in maximum intensity projections in Fig. 2.4.

2.3.2 Epifluorescence volume image deconvolution

We measure high spatial resolution displacements using our single particle tracking algorithm. This technique utilizes densely seeded microbeads in our volumetric images, and requires each bead to be fully resolved at high resolution with Gaussian intensity and a characteristic diameter of approximately three to nine voxels in each direction. To obtain volumetric images of microbeads with these characteristics from epifluorescence microscopy, we deconvolve the experimental images using the point spread function (PSF) collected from a single microbead directly from the experimental images. To do this, we choose an isolated fluorescent microbead and select a sufficiently large region of interest surrounding the bead in all three dimensions to capture the light spread from only the isolated bead. An example of a microbead PSF extracted from an experimental image can be seen in the single-bead maximum intensity projection in Fig. 2.4. Further imaging details are given in the Methods section. Under the assumption that each spherical microbead is a point-like light source, the single bead image thus describes the transfer function of light from a point source at the imaging plane to the detector. Under these assumptions, we have found a straightforward application of the maximum-likelihood estimator-based Lucy-Richardson deconvolution scheme [136,137] sufficiently repeatable to obtain bead images that admit accurate localization in x -, y -, and z -directions; see Fig. 2.5a and b. It is, however, noteworthy to mention that images should be taken with care to avoid skewness and severe imaging artifacts, which substantially degrade reconstruction and subsequent microbead localization and tracking performance.

To compute the deconvolution, we use a the Lucy-Richardson type scheme that assumes the transfer function of the optical system is applied to the true bead image in the presence of shot noise and background fluorescence (i.e., additive Poisson and Gaussian noise). The deconvolution proceeds by solving for the pristine image estimate via an iterative inversion approximation of the transfer function plus noise [138]. We have employed the more sophisticated implementation of this algorithm built into the Matlab Image Processing Toolbox. Pre- and post-deconvolution volumetric image reconstructions are given in Fig. 2.4, which show the reduction of apparent particle size in the x - y plane, which aids precision, and

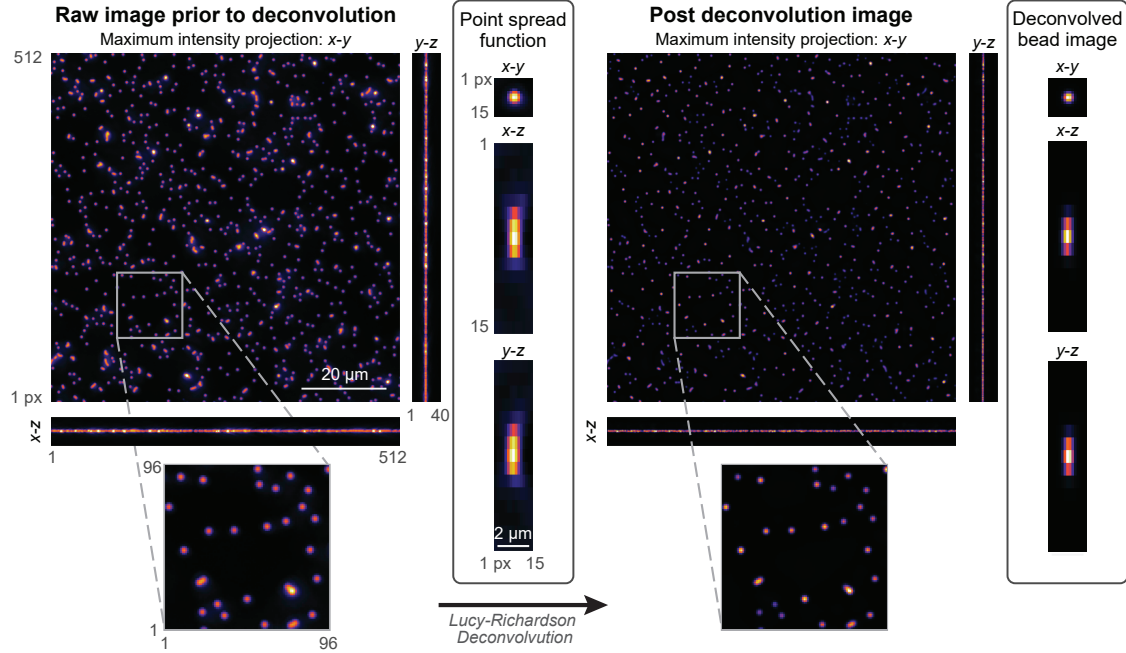


Figure 2.4: **Typical single-layer beaded gel images from epifluorescence microscopy and deconvolution results.** Maximum intensity projections from both pre- and post-deconvolution results for an experimental image using the experimentally-gathered point spread function shown in the center column. The deconvolved point spread function is shown in the column on the right. Insets show a closer view of the maximum intensity z -projections of experimental images pre- and post-deconvolution.

results in a dramatic reduction in z -spread, which enables sufficient precision and accuracy in the z -direction localization for effective out-of-plane deformation measurement.

2.3.3 3D displacement field from single-particle tracking

Our Topology-based Particle Tracking (TPT) algorithm is used to reconstruct high-spatial frequency, large deformation displacement information from the deconvolved images. To begin, the bead centers from deconvolved bead images are localized to determine bead positions with subpixel accuracy via the radial symmetry method, described by Liu *et al.* [139], as implemented in TPT. Then, for each image time point the regional particle location data are used to build a feature vector describing each bead in the image. For each bead, TPT links these feature vectors between reference (undeformed, $t = 0$) and deformed ($t = t + \tau$) images to determine a 3D displacement vector. By employing the iterative deformation method,

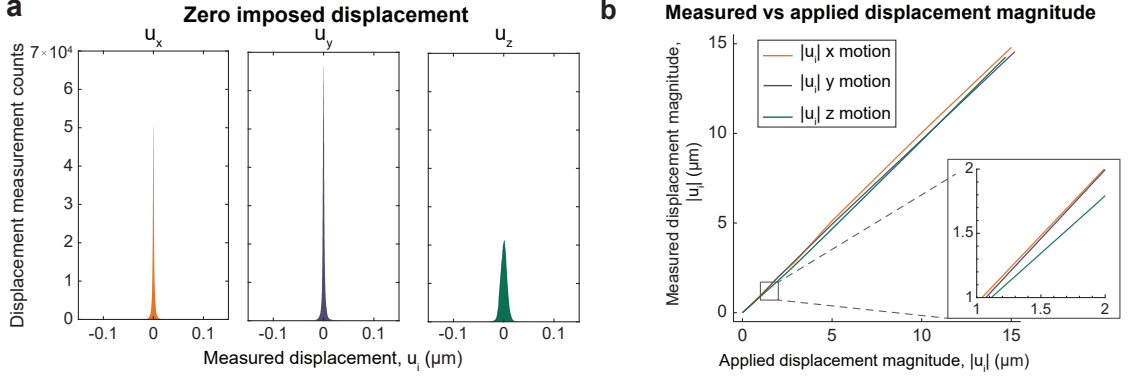


Figure 2.5: **Rigid-body motion experiments for validation of displacement reconstruction accuracy.**(a) Histogram showing the distributions of the reconstructed planarized displacement magnitudes in x , y and z from a nominally zero-displacement experimental image pair. (b) Line plot showing the measured mean displacement magnitude versus the applied experimental displacement magnitude from three individual rigid body displacement experiments, each imposing rigid motion in one of the three principal directions with sequentially increasing displacements along the indicated axis. Standard deviation is plotted as a shaded error bar (very small); inset shows the shaded error bars in more detail.

link verification, and displacement outlier removal schemes, a relatively simple feature vector is used to track large, high spatial frequency displacements and provides a computationally efficient means to fully resolve 3D displacements at single particle resolution. For our nominally single-layer gels all beads found and successfully linked within the image volume are tracked, including spurious out-of-plane beads, resulting in a scattered loci of measured displacement vectors. The subsequent planarization step both selects displacement data only on the dominant top single layer of beads and resamples the displacements onto a regular grid, see below. In the future, globally-aware tracking and linking, e.g., via an augmented Lagrangian-type regularizer to guarantee the global kinematic compatibility [75], a mesh-free type interpolant [12], or more computationally demanding feature vector could further improve the quality of tracked 3D displacement fields.

2.3.4 Planarization of 3D TPT data

Displacement data recovered using TPT are located at bead coordinates within the volume, and thus are scattered. Outlier beads are rejected from the tracking result using a user-tuned

parameter for the number of standard deviations that an individual bead lies from the overall mean bead z -height. Outliers are uncommon using our single layer bead technique and thus are typically isolated and suffer from increased noise. In addition, to impose the top-surface Dirichlet-type boundary condition on the initially flat computational volume, displacements must reside on a single z -plane. To address this, displacements are resampled onto a regular grid located on the best-fit plane of bead locations; this grid is the top surface mesh that will be used in subsequent 3D finite element simulations. The displacement resampling is performed by a curvature regularization scheme of the scattered bead displacements, with user-adjustable smoothing coefficients for the regularizer implemented independently between $(x-y)$ and z -displacements. This leads to less noisy and more robust displacements ($[u_x, u_y, u_z] \in \text{Hilbert space } H^2$) for use in determining volumetric stresses and strains via a forward solution of the boundary value problem with finite elements. Figures 2.5a and b show the distribution and accuracy of planarized displacement information from a zero-displacement rigid body motion experiment and the measured displacements plotted against the applied rigid displacement for three rigid body translation experiments (one in each of x -, y -, and z -directions), respectively. These demonstrate that this technique provides a level of accuracy and precision necessary to compute stress fields for small, low force-producing cells such as the human neutrophil.

2.3.5 3D finite element calculations for strain, stress, and traction

We directly solve the volumetric boundary value problem defined by known, measured displacements and substrate material properties using the finite element method as implemented in FEniCS. A baseline, convergent finite element mesh (tested via a synthetic 6σ -width Gaussian indentation displacement with amplitude approximately $0.27\text{ }\mu\text{m}$), consisting of 25,992 hexahedron mesh elements (i.e., first order Lagrangian bricks [135]) is a baseline for all experiments, see Fig. 2.6a. The mesh is automatically pre-refined in the region of the cell according to an adjustable Gaussian decay in element density. This initial mesh provides a consistent, high resolution, and high quality starting point on which to apply the TPT-based planarized displacement and solve the volumetric boundary value

problem. Linear hexahedron elements with center nodes are used to avoid locking, given the relatively large Poisson’s ratio of typical hydrogels used for TFM. To accommodate varying experimental configurations, the base mesh is uniformly warped to fill the image volume, as defined in the output from the Matlab-based pre-processing steps detailed above. Since the base mesh is a regular rectangular solid and most imaging domains are similar in shape and aspect ratio to this mesh, distortions from this reshaping are typically negligible. As with planarization, more sophisticated meshing (e.g., adaptive remeshing) is certainly possible, but we have obtained efficient and robust solutions with acceptable accuracy using the straightforward approach described above. Regarding displacement boundary conditions, the left face of the computational volume is fixed in the x -direction (i.e., $u_x = 0$), the back face is held fixed in the y -direction (i.e., $u_y = 0$), and the bottom face held fixed in the x , y , and z -directions (i.e., $u_i = 0$), thus fully constraining the solution domain. The top surface displacement boundary condition is given by our planarized 3D cell-imposed displacement vectors output from the preceding analysis steps. The validation of this technique (see the Synthetic Validation section in the Methods and Fig. 2.6) demonstrates that small, subpixel displacements (i.e., those generated by tens of nanonewton cellular forces) and high spatial frequencies (i.e., micrometer-scale full-width half-magnitude (FWHM) frequency) are observable with signal to noise ($\text{SNR} = \frac{|\text{Uncertainty}|}{|\text{Signal}_{\max}|}$) ratios of approximately 300 and 100 for displacement and traction, respectively.

The stored strain energy density function, Ψ , used to define element-wise constitutive relations is given by the compressible form for the neo-Hookean hyperelastic solid, i.e.,

$$\Psi = \frac{\mu}{2} (\text{Tr } \mathbf{C} - 3) - \ln J + \frac{\lambda}{2} (\ln J)^2, \quad (2.2)$$

where material properties μ and λ are the Lamé constants computed from user-input elastic modulus (E) and Poisson’s ratio (ν) of the hydrogel. The tensor-valued $\mathbf{C}$ and scalar-valued J terms represent right Cauchy-Green deformation tensor and volume change ratio of a generic finite-strain deformation of the element from a reference (undeformed) to deformed configuration and $\text{Tr}(\cdot)$ is the trace operator (see, for example, the textbook of Bower [140] for a complete discussion of large deformations in continua). In short, we take $\mathbf{C} = \mathbf{F}^T \mathbf{F}$,

where $\mathbf{F}$ is the spatial deformation gradient of the displacement field and $J = \det(\mathbf{F}) > 0$ where $\det(\cdot)$ is the determinant operator. Stresses are projected on each element in the volume via second-order quadrature of the hexahedron elements.

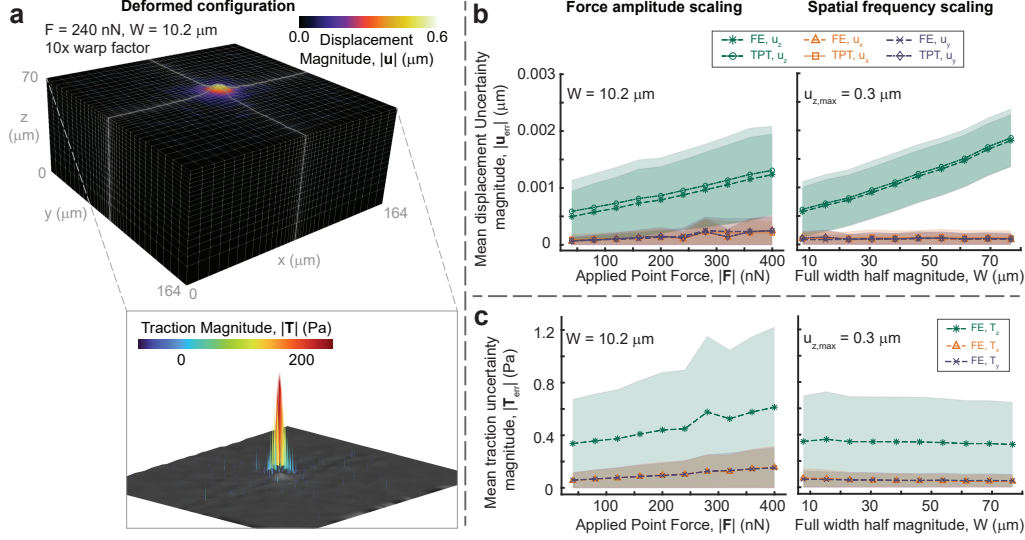


Figure 2.6: **Finite-element set-up and uncertainty analysis for synthetically applied traction with independently varied magnitude and width.** (a) The final converged hexahedron mesh in the deformed configuration with refinement at the center of the applied Gaussian traction field. Displacement magnitude contours are shown on the the deformed mesh, to which a 10x deformation amplification has been applied to aid visualization. (*Inset*) Measured surface traction vector field for this example case plotted on the deformed configuration top surface. (b) Displacement uncertainty magnitude for synthetically generated Gaussian-profile tractions with varying applied force amplitude and spatial frequency (full-width half-magnitude of the Gaussian traction profile, with fixed peak resultant displacements). (c) Traction reconstruction uncertainty projected onto the undeformed surface (i.e., normal and two in-plane traction vectors) for the same test cases. The shaded regions reflect the pointwise standard error for each case.

2.3.6 Computing 3D cell traction fields

We apply this procedure to compute 3D traction fields from two cells types to demonstrate its effectiveness in practice. Images of neutrophils and MDCK cells labeled with Calcein AM fluorescent dye were acquired using epifluorescence and are shown in 2.3. 3D boundaries of the cells were segmented using built-in Matlab functions for image thresholding and binarization. Phase contrast images of cells could also be segmented and binarized to be used for plotting planar information but are not typically sufficient for segmenting and

plotting three-dimensional cell boundaries, such as those shown in Figs. 2.7 and 2.8a-d(iii). First, we examine a single primary human neutrophil. Neutrophils have been shown to exhibit various traction patterns using TFM, including concentrated forces in the uropod of the cell, differential tractions on different substrate stiffnesses, and large out-of-plane deformations, among others [11, 84, 116]. In addition, neutrophils are both small, typically 8-10 μm in diameter, and fast-moving, thus demonstrating the ability of our technique to quantify high temporal and spatial frequency displacement and traction information. An example of a three-dimensional displacement field from a human neutrophil can be seen in Fig. 2.7a. The 3D displacement magnitude is shown in Fig. 2.7a(i), the x - y displacement of a cropped region is shown in (ii), and the displacement projected onto an x' - z plane perpendicular to the surface is shown in (iii). The corresponding traction field is shown in Fig. 2.7b, with the magnitude, x - y , and x' - z tractions shown in (i), (ii), and (iii), respectively. Second, we demonstrate that our technique can be used for not only single cells, but also multicellular monolayers or two dimensional cell clusters using an epithelial cell type. For this case, we chose Madin-Darby canine kidney cells, which are commercially available and have been shown to produce quantifiable traction forces during collective migration, and as individual cells or in groups or clusters [125, 133, 141]. An example of the three-dimensional displacement and traction fields produced by a cluster of approximately 15 MDCK cells can be seen in Fig. 2.8a and b, respectively, in a layout identical to that described for the neutrophil figure.

2.4 Discussion and Conclusion

The procedure described in this chapter represents a new technique for calculating three-dimensional displacement, stress and surface traction fields from epifluorescence images of fluorescent particles embedded in a two-dimensional surface layer and acted upon by an external force, such as a cell or cell cluster. Using deconvolution with an experimentally determined point spread function, the particle centers are accurately localized in the z -direction. The deformation is reconstructed with a single particle tracking algorithm (TPT)

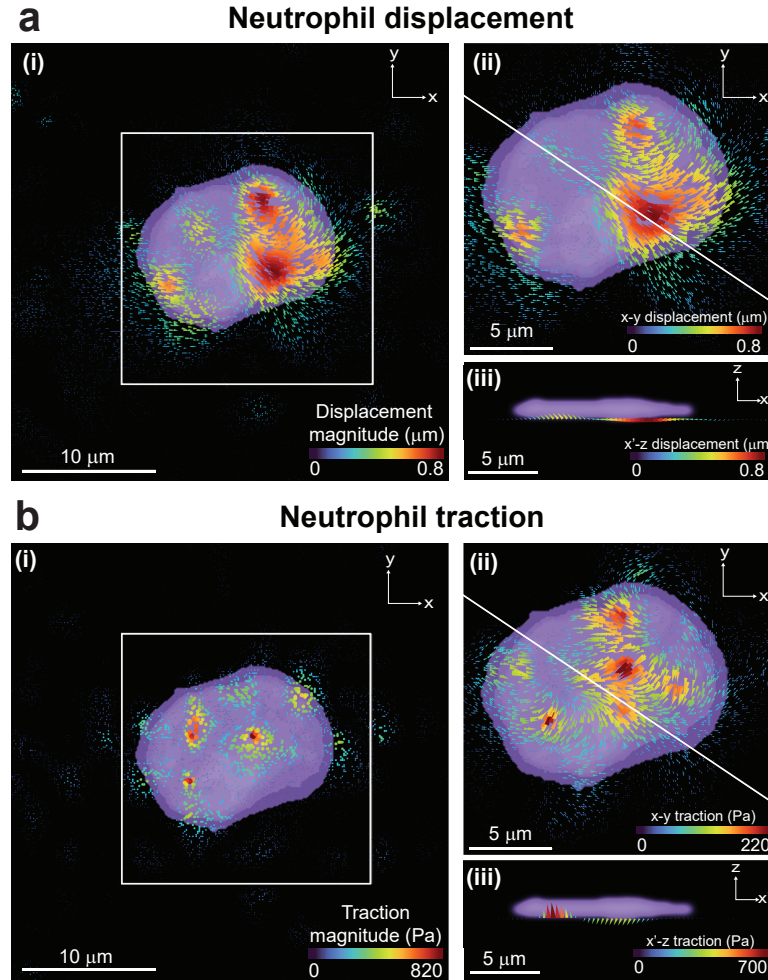


Figure 2.7: **Neutrophil-produced 3D displacement and traction fields on the top surface of the hydrogel.** Maximum intensity projections of the cell bodies are shown in purple. Cone size and color imply displacement or traction magnitude, while direction is given by the cone points. **(a)** The displacement field visualization for an activated neutrophil migrating on a fibronectin coated polyacrylamide hydrogel showing localized peak displacements toward the cell center and downward into the gel. **(b)** The resultant surface traction vector field for the neutrophil case computed on the deformed top surface reconstructed in the finite element analysis. The traction distribution exhibits a push-pull pattern often associated with amoeboid cell migration. For each case, (i) shows the xyz -magnitude, (ii) shows a magnified view of the xy -magnitude of the area within the white box shown in (i), and (iii) shows the projection of the xz -vector field onto the x' - z plane, i.e., a view orthogonal to the white line shown in (ii).

that excels at reconstructing high-spatial frequency motions in high particle density environments. The tracked particle displacements are then resampled onto the best-fit plane of the initial bead locations. This planarized single-layer displacement information is then applied as a surface Dirichlet-type boundary condition in the FEniCS finite element solver.

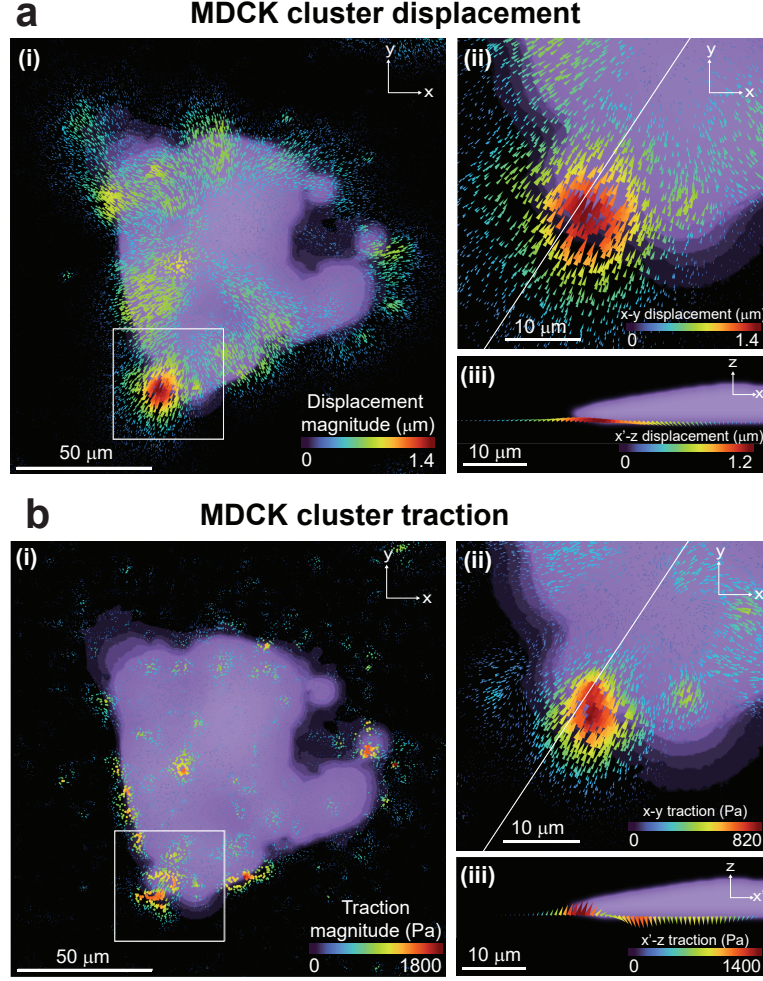


Figure 2.8: **MDCK cell cluster-produced 3D displacement and traction fields on the hydrogel surface.** Maximum intensity projections of the cell bodies are shown in purple. Cone size and color imply displacement or traction magnitude, while direction is given by the cone points. **(a)** The displacement field visualized for a multicellular cluster of MDCK cells during collective migration along a collagen-I coated polyacrylamide hydrogel displaying contractile displacements around the entire cell cluster. **(b)** The traction field for our MDCK cell cluster. The traction patterns show distinct rotational forces around the edge of the cell cluster. For each case, (i) shows the xyz -magnitude, (ii) shows a magnified view of the xy -magnitude of the area within the white box shown in (i), and (iii) shows the projection of the xz -vector field onto the x' - z plane, i.e., a view orthogonal to the white line shown in (ii).

With user input material properties (elastic modulus and Poisson's ratio) given for the substrate, the full 3D displacement and state of stress of the substrate volume is directly solved assuming a hyperelastic neo-Hookean material model. The resultant top-surface tractions are computed using finite deformation continuum principles [84] and, finally, complete

visualizations of displacement and traction fields are rendered.

We validated and tested this protocol using a number of synthetic and experimental cases to probe the accuracy and precision of the reconstructed displacement and traction fields, and to examine its capacity to reconstruct high spatial frequency motion fields. We also demonstrate the technique on two cellular cases. To assess the accuracy and precision of the process for measuring displacements in real experiments, rigid body motions with displacements ranging from 1 μm to 15 μm were imposed and reconstructed. Spatial frequency, amplitude, and traction field reconstruction was validated using synthetic Gaussian tractions, which demonstrates the ability of our algorithm to accurately resolve tractions at cell-relevant scales. Finally, we compute traction fields from two cellular examples to represent two different types of data that are commonly analyzed using traction force microscopy: a single cell and a multicellular cluster. This suit of tests demonstrates the ability of our algorithm to reconstruct displacements and quantify tractions from a variety of cell-produced substrate deformations. We chose to compute traction from a cell cluster in our example case, rather than tractions from fully confluent monolayers, to preserve a zero-displacement edge condition [20, 125, 126, 133, 142], though the technique could be readily extended to compute intercellular stresses of a confluent monolayer of cells, e.g., Serrano *et al.* [126].

Our displacement and traction example cases showcase some of the information that is only accessible with three-dimensional TFM analysis. A visual comparison between Figs. 2.7 and 2.8a-d (ii) with (i) and (iii) shows that the out-of-plane displacement and traction produced often match or exceed the in-plane values. In addition, the three-dimensional analysis highlights the rotational behavior of cell displacement and traction forces concentrated at the edges of the cell or cell cluster initially described by Legant *et al.* [39]. These observations carry across our two very different cell examples, and highlight the importance of using three-dimensional traction force microscopy techniques to make complete observations about cell behavior.

In principle, epifluorescence imaging can be used for three-dimensional traction force microscopy without the need to use a single layer of beads and hence finite element methods for analysis. However, the spatial resolution of the resulting displacement and traction

information will be limited by the ability to resolve and adequately localize bead spread in the z -direction [12]. That determination will ultimately depend on the reconstructed 3D image quality and the quality of the optical system. Here, a single layer of microbeads allows us to use a high seeding density, and thus resolve higher spatial frequency information without having to directly contend with light scattering from epifluorescence inhibiting our ability to accurately localize out-of-plane microbeads. As in the fully 3D volumetric case, mean deformation metrics [64] of the cell, e.g. mean contractility in the principal directions, could be extracted from reconstructed motion estimates if cell-scale constitutive relations for a substrate material are unavailable. Considerations for viscoelastic or alternative non-linear material model formulations could be straightforwardly incorporated via implementation of the strain energy density function in FEniCS, further increasing the potential applications of this protocol.

The complete Matlab and FEniCS-based implementation of the workflow are packaged with a user manual and are available for download as free and open-source software (see <https://github.com/FranckLab>). By enabling users to implement an inexpensive and readily available imaging modality to acquire the necessary data, and by releasing a straightforward, documented protocol with example data and all algorithms required for computation and plotting included, we seek to lower the threshold for users to probe three dimensional cellular mechanics.

Chapter 3

Three-Dimensional Fibrous Collagen Characterization for Measuring Cell-Produced Traction

Note: The work presented in this chapter is being prepared for publication. All data and figures included are done with co-authors' consent.

Hazlett, L., Proestaki, M., Yang, J., Notbohm, J., Franck, C. *Three-dimensional fibrous collagen material fitting for measuring cell-produced traction forces*. In preparation.

3.1 Introduction

It has long been accepted that cells are capable of sensing and responding to forces they experience within their microenvironment [5–12]. The cellular microenvironment contains a combination of cellular and non-cellular components, and the cells are capable of sensing and responding to direct interactions with the extracellular matrix (ECM) [2, 13, 14], in addition to applied mechanical forces and fluid shear flow [15–17] and to neighboring cells [18–20]. Cellular mechanosensing and mechanotransduction, the ability of the cell to sense and respond to forces from within the extracellular microenvironment, have been shown

to regulate important cellular processes, including cell spreading, migration, differentiation, morphogenesis, embryogenesis, cellular homeostasis, and the disease and trauma response [4, 7–9, 11, 21–29]. Gaining a deeper understanding of cellular mechanosensing has clinical relevance for diseases like sepsis [17, 30], cancer (and in particular cancer metastasis) [27, 31–34], atherosclerosis [35, 36], and traumatic brain injury [37, 38].

Traction force microscopy (TFM) is an established biomechanics technique that is used to quantify cellular tractions during cell-cell or cell-matrix interactions [61–63]. Cell-generated stresses are reconstructed from experimentally-observed displacement fields: traditionally, cells are cultured on top of a planar substrate or embedded within a three-dimensional ECM construct. Position changes of fluorescent particles contained in the substrate are tracked over time in three-dimensions using digital volume correlation (DVC) or single particle tracking (SPT)-based methods [62, 63, 72, 73, 75]. Using knowledge of the matrix material properties (e.g., Young’s modulus and Poisson’s ratio or shear and bulk moduli) and of the boundary geometry between the cell and the extracellular material, tractions are computed from the displacement field [53, 61–63]. This is done using continuum mechanics-based computational models or finite element method-based TFM (FEM-TFM) [6, 57–59]. FEM-TFM computes tractions by imposing the measured displacement field onto a pre-defined grid, called a finite element mesh, of small elements to model the known cell-matrix interface, solving for tractions numerically by computing solutions to the mechanical boundary value problems [62]. Cells *in vivo* exist within complex, heterogeneous, and dynamic three-dimensional tissue microenvironments that contain ECM proteins (primarily fibrous proteins such as collagen and laminin), cells, cytokines, and other signalling molecules.

Cell mechanobiologists seek to study cellular force generation in three-dimensional *in vitro* systems in order to reproduce some of this physiological complexity: such systems involve embedding cells within three-dimensional fibrous hydrogels which are notoriously difficult to characterize mechanically, a key step toward reconstructing tractions. Some studies have used deformation or displacement field metrics which do not rely on material characterizations rather than tractions to explicitly characterize mechanical interactions between the cell and fibrous ECM [34, 64]. FEM-TFM models have already been implemented

to compute tractions produced by cells fully embedded in three-dimensional matrices as shown by Legant *et. al.*, Koch *et. al.*, and Song *et. al.* [31, 68, 81]; though each of these examples either neglect to account for fibrous material behavior or instead encapsulate cells in synthetic matrices that can be treated as linear elastic. It is theoretically straightforward to implement more complex fibrous material models in FEM-TFM and even to model and measure cellular force generation within these systems [13, 67, 143, 144], though measuring the fibrous matrix material properties and implementing an appropriate material model is a remaining challenge for cell mechanobiologists.

At the length scale of the cell, fibrous material properties are dominated by the behavior and interactions of a random dispersion of individual fibers, which behave as a network of beams that buckle, stretch, and bend. This leads to non-linear constitutive relationships and the introduction of new material parameters to describe the observed material behaviors. In general, fibrous materials tend to exhibit weakening under compression, a small linear regime under small extensions, and stiffening under large extensions, as shown in Fig. 3.2 [13, 79, 143, 145–150]. Traditional methods for characterizing soft materials at the macroscale involve introducing uniform compression or tension, simple shear, or some combination of these, and measuring the material response [149, 151–154]. However, the observed differences between the macro- and microscale material behaviors for fibrous materials, such as those described by Owens *et. al.* [155], make it important to characterize the material at the length and force scales of the cell (i.e., tens of microns and hundreds of Pascals). Methods like nanoindentation (e.g., using atomic force microscopy) can be used to quantify material properties using applied forces similar in magnitude to cell-applied forces, but the quantification is at the material surface rather than the internal fiber structure experienced by the cell and the analysis typically assumes material linearity [149, 150]. Active microrheology techniques (e.g., optical tweezers) can be used to produce local forces to quantify stiffness from within the fibrous hydrogel, but fail to match cellular displacement magnitudes or reproduce the complexity of cellular contractile forces [156–160]. Burkel *et. al.* introduced a novel experimental method for simulating cellular contractile forces within fibrous networks in order to measure the material response; spherical particles made of an

active hydrogel, poly(*N*-isopropylacrylamide) (PNIPAAm), which decreases in volume by up to 50% when raised above their phase transition temperature, produce local contractile displacements when embedded within a fibrous network [150]. This technique uniquely establishes a displacement and resultant stress field within the fibrous matrix that mimic the self-equilibrated local forces produced by cells.

Using these measured material properties to create an accurate mathematical model or constitutive relation is the final step toward computing tractions from within fibrous hydrogels. Non-linear, non-affine fibrous material behavior could easily lead to incredibly complex mathematical models that are too computationally expensive to be of practical use. Thus, most early models prioritized the specific properties of fibrous hydrogels that are believed to be most influential to cellular mechanosensing. For example, cells have been shown to transmit forces over longer distances in fibrous materials compared with linear elastic substrates; a method cells use to communicate mechanically with each other over long distances and which facilitates mechanical feedback between cells and the tissue microenvironment [14, 18, 142, 143, 161]. Some studies concluded that strain-stiffening was key in explaining long-range force transmission along individual fibers [145–147], while others attributed this force transmission to fiber cross-linking [13], or compression weakening due to fiber microbuckling [79, 148]. More complex material models begin to consider the bulk material behavior separately from the behavior of the fibers: such fiber-based constitutive models treat the strain energy density function as the superposition of the contributions from a hyperelastic (i.e., isotropic Neo-hookean) matrix and the nonlinear elastic fiber network [14, 143, 162]. Unfortunately, these complex models rely on numerous experimentally-determined material properties and can be difficult to fit to experimental data, which could deter potential users that do not have the necessary equipment or knowledge. Steinwachs *et. al.* introduced a more simplistic model that considers the non-affine behavior of local fibers along with the experimentally measured macroscopic bulk material behavior. These bulk material measurements included a cone-plate rheometer for simple shear, a uniaxial stretch experiment to determine the Poisson’s ratio, an extensional rheometer, and magnetic tweezers [67]. These techniques are limited to surface responses, but provide excellent

data for fitting the material model to a specific hydrogel without necessarily needing to account for these bulk behaviors using a hyperelastic material model. The model proposed by Steinwachs *et. al.* utilizes a statistical average of the stress contributions of an isotropic and homogeneously distributed randomly oriented fiber network to derive a constitutive equation fitted to the mechanical behavior of the bulk material. Other fiber-based models use more sophisticated simulations of discrete fibrous networks, rather than statistical averaging over fiber networks [163], though these models have not been experimentally validated and remain computationally involved.

The goal of the proposed project is to implement the material model proposed by Steinwachs *et. al.* using Matlab finite element software to compute cellular tractions. The model proposed by Steinwachs *et. al.* was selected in part because of its simplicity to implement: it has already undergone significant fitting to experimental bulk material properties and accounts for such non-linearities as fiber buckling and strain stiffening. An experimental technique for validating this fibrous material model is presented based upon the experimental technique developed by Burkel *et. al.* [150] and expanded by Proestaki *et. al.* [76]. This technique is applied at the cellular scale from within the hydrogel to produce cell-like forces of known quantities, making it possible to characterize material properties experienced by embedded cells. The methods for fully validating the applied material model, adapting the model to account for thermal effects, and fitting the model to the experimental PNIPAAm displacement results are presented as future works. Surface tractions are computed from a single neutrophil embedded within a collagen hydrogel using a linear elastic finite element model as proof of principle to demonstrate that the described techniques are capable of resolving cellular-scale displacements and tractions from within fibrous material matrices.

3.2 Methods

3.2.1 PNIPAAm particle preparation

Spherical poly(N-isopropylacrylamide) (PNIPAAm) particles were made using a previously described oil/water immersion protocol [76, 150], which is illustrated in Fig. 3.1 a. The oil

solvent, comprised of Kerosene with 3.5% Span 80, a nonionic surfactant, (Tokyo Chemical Industries), was de-gassed for 1 hour under vacuum. The solvent was maintained in nitrogen for 10 minutes, and then placed on a magnetic stir plate at 450 rpm at 22°C for an additional 5 minutes. The aqueous solution, containing 0.25 g *N*-isopropylacrylamide (Sigma Aldrich), 0.05 g ammonium persulfate (APS, Bio-Rad), 1.6 mL of 2% bis-acrylamide (Bio-Rad), 1.5 mL of 1X tris-buffered saline, and deionized water to a final volume of 10 mL, was also de-gassed under vacuum and maintained under nitrogen. Tetramethylethylenediamine (TEMED, Bio-Rad) was added and mixed with the aqueous solution immediately prior to mixing with the oil solvent. The emulsion was stirred at 450 rpm at 22°C under nitrogen for 1 hour until polymerized particles formed. These particles were allowed to settle overnight and were then washed twice with hexane, followed by isopropyl alcohol, ethanol, deionized water, and 1X PBS. Finally, the particles were treated with 1 mg/mL Sulfo-SANPAH (Proteochem) and exposed to UV for 10 minutes, before being washed again with 0.05 M HEPES buffer and 1X PBS. These particles were immediately polymerized into collagen gels to facilitate Sulfo-SANPAH adhesion to collagen fibers.

3.2.2 Collagen hydrogel preparation

Collagen hydrogels are prepared in a manner similar to that described by Scimone *et al.* [164], with minor adjustments to account for the addition of the PNIPAAm particles and to ensure even distribution of fluorescent tracker particles. The collagen gels were fabricated in two layers to keep the PNIPAAm particle suspended in the hydrogel rather than sunk to the bottom. To prepare the collagen gels, 1.0 μm diameter carboxylate-modified red and green fluorescent microspheres (Fluospheres, Thermo Fisher Scientific) to total 10% and 2% v/v, respectfully, of the final gel volume were centrifuged down and resuspended in a volume of 10X phosphate buffered saline solution (PBS) to total 10% of the final gel volume. For the top layer of collagen, PNIPAAm particles at a concentration of 1000 particles/ μL in 1X PBS were added to the PBS/microsphere solution, totalling an additional 10% of the final gel volume. Leibovitz's L-15 media (Gibco) without phenol red and with 2 mg/mL glucose was added to the PBS solution for both layers and pipetted to ensure thorough mixing. The

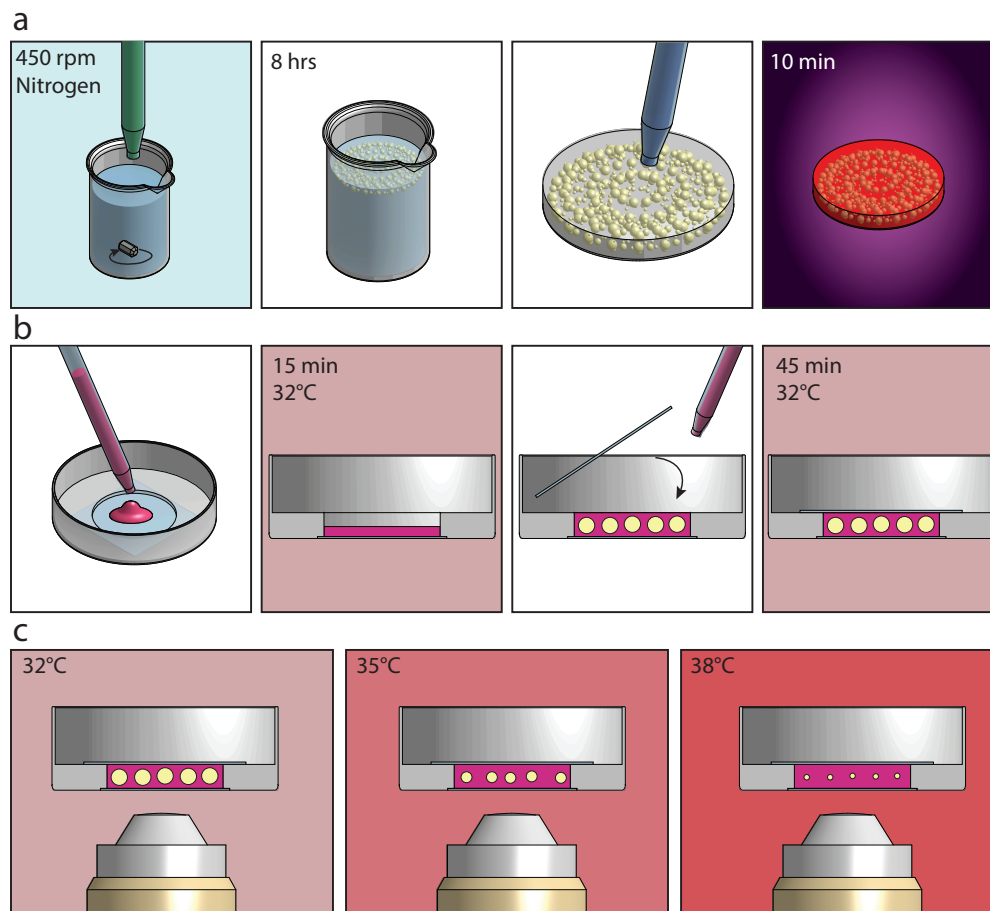


Figure 3.1: Methods for producing PNIPAAm particles, embedding them in collagen hydrogels, and the imaging setup. (a) Panels show how PNIPAAm particles are made: first, a water-oil immersion is de-gassed and placed on a magnetic stir plate in a Nitrogen environment where the polymerizing agents are added and then the solution is allowed to sit overnight while the particles settle out of solution, allowing the particles to form. Next, the particles are rinsed with a series of solvents, and treated with Sulfo-SANPAH under UV light for 10 minutes before being rinsed again with 0.05 M HEPES buffer in 1X PBS. (b) Panels show the procedure for making collagen gels with embedded PNIPAAm particles: a single layer of collagen without PNIPAAm particles is pipetted into the bottom of a 35mm glass-bottom dish and allowed to partially polymerize at 32°C before a second layer of collagen solution with PNIPAAm particles is added on top of the first layer and a glass coverslip is placed on top. The entire gel is allowed to polymerize fully at 32°C for 45 minutes. (c) The imaging setup involves tuning the temperature of the microscope enclosure to 32°C, 35°C, and then 38°C and imaging full volumetric image stacks of the PNIPAAm particles and surrounding matrix behavior.

total volume of L-15 media was computed to bring the final concentration of collagen to 2 mg/mL. Rat tail collagen-I (Corning) was added to the media solution and pipetted gently to ensure thorough mixing without introducing air bubbles. Finally, 1M NaOH totaling

1.3% of the total volume was added to the collagen gel to neutralize the acetic acid solution that the collagen is shipped in, bringing the pH up to 8.5 and facilitating collagen gel polymerization. Approximately 230 μL of collagen gel solution without PNIPAAm particles was pipetted onto the glass surface of a 35mm glass bottom dish with a 20mm micro-well (Cellvis), and allowed to polymerize for 15 minutes in an incubator set to 32°C, after which time an additional 230 μL of collagen solution with PNIPAAm particles was pipetted onto the first layer, an 18mm glass coverslip was gently placed on top, and the gel was allowed to polymerize fully at 32°C for an additional 45 minutes, before being fully submerged in 1X PBS. Table 3.1 shows the collagen hydrogel recipe used for the top layer gels with PNIPAAm particles suspended, and Table 3.2 shows the recipe for collagen hydrogels used for the bottom layer.

	<i>Volume (μL)</i>	<i>Percentage (%)</i>
Collagen-I	292	58.4
Microbeads in 10X PBS	50	10
Media	101.5	20.3
PNIPAAm particle suspension in 1X PBS	50	10
1M NaOH	6.5	1.3
Total Volume	500	100

Table 3.1: Relative volumes of collagen hydrogel mixture components for a stock collagen concentration of 3.77 mg/mL and a desired final collagen concentration of 2.2 mg/mL. Example volumes are also shown for a 500 μL total volume hydrogel solution.

3.2.3 Microscopy

Collagen gels were imaged on a Nikon TI2 multiphoton microscope with a 40X/1.15NA water immersion objective, in a custom-built heating enclosure set to 32°C. 547 z-plane images were taken of the fluorescent beads surrounding the particles, for a total imaging volume of 317 $\mu\text{m} \times 317 \mu\text{m} \times 150 \mu\text{m}$. The temperature of the microscope enclosure was raised to 35°C and then 38°C, with full image stacks taken at each temperature. The temperature of the samples was verified using a thermometer placed inside a second 35mm glass bottom dish containing 1X PBS on the microscope stage, and imaging at each temperature did not commence until this second dish of PBS had stabilized at the set temperature.

3.2.4 Live cell imaging experiments

Primary human neutrophils were isolated from whole blood taken from healthy volunteers with written informed consent, as per an Internal Review Board protocol approved by the University of Wisconsin, using the MACSxpres Neutrophil Isolation and MACSxpres Erythrocyte Depletion kits (Miltenyi Biotec, Inc.). Cells were fluorescently labeled by incubation in 2 μ M Calcein AM in Hank's Balanced Salt Solution, without calcium, magnesium, or phenol red for 8 minutes. Following labeling, cells were centrifuged at $100\times g$ for 2 minutes to form a loose pellet and resuspended in Leibowitz's L-15 media. Collagen gels containing neutrophils were prepared in a similar manner to that above, except: 10% v/v 0.5 μ m diameter red fluorescent microsphere solution was added to 10X PBS, no PNIPAAm particles were added to the cell solution, and the L-15 media was added in two stages to avoid placing cells directly into a hypertonic 10X PBS solution. Half of the L-15 was added prior to adding the collagen, as above, and the other half, with cells suspended at a concentration of $\sim 1 \times 10^6$ cells/mL, was added after the addition of the collagen. After the addition of the cells in media, the entire collagen solution was pipetted gently to mix. Collagen gels were polymerized using 1.3% 1M NaOH for 45 minutes at 32°C, as above. Table 3.2 shows the recipe for collagen hydrogels with cellular suspension, similar to that described by Scimone *et. al.* [164].

Collagen gels with embedded neutrophils were polymerized in the PDMS-cast chemotaxis device described in detail in Ch. 4 and seen in 4.1, which polymerizes hydrogels between two hydrophilically-treated glass coverslips and introduces a planar chemokine gradient from one side well of the device. In this case, a 10 nM concentration of N-formylmethionyl-leucyl-phenylalanine (fMLP) in L-15 media was added to one side port to induce neutrophil chemotaxis. Images of neutrophils were acquired on a Nikon TI2 multiphoton microscope with a 40X/1.15NA water immersion objective every 2-3 minutes, and a reference image of the fluorescent beads was acquired after the cells had moved out of the field of view.

	<i>Volume (μL)</i>	<i>Percentage (%)</i>
Collagen-I	292	58.4
Microbeads in 10X PBS	50	10
Media (optional: cell suspension)	151.5	30.3
1M NaOH	6.5	1.3
Total Volume	500	100

Table 3.2: Relative volumes of collagen hydrogel mixture components for a stock collagen concentration of 3.77 mg/mL and a desired final collagen concentration of 2.2 mg/mL. Example volumes are also shown. Optional: including cells suspended in the media. Cells should be added to half of the media volume, with half of the media getting mixed into the collagen and PBS solution prior to addition of the cells.

3.2.5 Displacement field reconstruction

The displacement fields from both the PNIPAAm particle experiments and the cell experiments were computed using the topology-based single particle (TPT) tracking algorithm developed by Patel *et. al.* [72]. The TPT algorithm excels at tracking large numbers of particles seeded at high spatial frequencies quickly, and is capable of tracking particles with different attributes (i.e., size or color) seeded at different densities to reconstruct high and low spatial frequency information.

3.2.6 Image segmentation and FE mesh generation

Images of PNIPAAm particles contain a 10% concentration of green fluorescent beads, and a 2% concentration of red fluorescent beads. The PNIPAAm particles were not fluorescently labeled, and thus the location and size of the PNIPAAm particle within the image was segmented by first combining the fluorescence intensity from the red and green bead images and performing an image dilation to approximate the particle void space. Next, an ellipsoid fitting of the void space was performed. Image dilation resulted in a underfitting of the particle void space, which was corrected by eroding the ellipsoid particle surfaces. The finite element mesh for the regular ellipsoidal particle surface and surrounding matrix material was generated from this ellipsoid surface using a previously published mesh generation Matlab code from Persson *et. al.* [165]. Images of neutrophils were segmented using direct image thresholding from the captured Calcein AM fluorescence. Finite element mesh generation

from the irregular cell shapes and the surrounding collagen matrix was done using the Matlab/Octave-based mesh generation toolbox published by Fang *et. al.* [166].

3.2.7 Mechanical characterization of PNIPAAm particles

Proestaki *et al.* [76] previously performed extensive analysis of the PNIPAAm particle contractile strength and bulk modulus. This was accomplished by first quantifying the material properties of a linear elastic polyacrylamide (PA) gel (made from bis-acrylamide, acrylamide, APS, TEMED, and deionized water) using a rheometer: the PA gel used had a shear modulus of 9.6 Pa. Next, the contractile radial strain of the PNIPAAm particles when heated past their phase transition temperature when in aqueous solution (intended as a ‘no matrix’ reference condition) and embedded within the 9.6 Pa polyacrylamide gel was computed at a range of temperatures between 30° and 38°C. The radial contractile strain in aqueous solution gives the thermal strain ε^T for each temperature, which was shown to vary between 10% and 70%. The total strain ε is given by the superposition of the thermal strain ε^T with mechanical strain of particles contracting in the PA gels, ε^M , i.e., $\varepsilon = \varepsilon^T + \varepsilon^M$. Because both the PNIPAAm particles and polyacrylamide matrix are isotropic, linear elastic materials and assuming small material deformations are produced, the linear spherical inclusion problem [167] can be solved straightforwardly using the known PA shear modulus μ^M to compute the bulk modulus of the PNIPAAm particles K^P per given temperature as follows:

$$K^P = \frac{4\mu^M}{3\left(\frac{\varepsilon^T}{\varepsilon} - 1\right)} \quad (3.1)$$

The bulk modulus of PNIPAAm particles was found to be on the order of 10 Pa across the range of measured temperatures [76].

3.2.8 Material characterization of fibrous collagen hydrogels

Given the non-linear behavior of collagen, the linear inclusion relation described in in Eq. 3.1 above cannot be used for computing the shear modulus of collagen given the bulk modulus of PNIPAAm. We turn instead to the material model introduced by Steinwachs *et. al.*, which

introduces four new material parameters for describing a nonlinear potential function $w(\lambda)$ with three distinct regimes [67]. First, fibers are known to buckle in compression, leading to an exponential decay of stiffness described by the buckling parameter, d_0 . Second, under small tensile forces, fibers exhibit a constant stiffness κ_0 , which is related to the Young's modulus, E as follows: $E = \frac{\kappa_0}{6}$. Third, when fibers are stretched above the linear stretch range, characterized by λ_s , the fiber stiffness increases exponentially scaled by the strain stiffening coefficient, d_s . Taken together, the differential fiber stiffness $w''(\lambda)$ is described as follows:

$$w''(\lambda) = \kappa_0 \begin{cases} e^{\lambda/d_0} & \forall \lambda < 0 \\ 1 & \forall 0 < \lambda < \lambda_s \\ e^{(\lambda-\lambda_s)/d_s} & \forall \lambda_s < \lambda \end{cases} \quad (3.2)$$

Using a combination of uniaxial stretch and shear and extensional rheometer experiments, Steinwachs *et. al.* determined the linear stretch range, λ_s , buckling parameter, d_0 , and strain stiffening coefficient, d_s , do not vary with collagen concentration [67]. Because our material validation technique does not include material compression, we take the measured fiber buckling parameter d_0 measured by Steinwachs *et. al.* to be true. If we assume that the Young's modulus of the collagen matrix, E^M , can be defined on the local scale using linear elasticity theory, this can be computed using the bulk modulus of the PNIPAAm particle, K^P as shown by Proestaki *et. al.* [76] using the following relation:

$$E^M = \frac{3K^P(1+\nu)}{2} \left(\frac{\varepsilon^T}{\varepsilon} - 1 \right). \quad (3.3)$$

This linear estimate of the Young's modulus of collagen at 2.2 mg/mL can be validated by comparing with the Young's moduli of various density (i.e., 0.6, 1.2, and 2.4 mg/mL) collagen hydrogels measured by Steinwachs *et. al.* by interpolating between the measured density values, which is a value of 750 Pa. As previously stated, the constant stiffness parameter is related to the validated Young's modulus through $\kappa_0 = 6E^M$. Steinwachs *et. al.* experimentally determine the Poisson's ratio of fibrous materials including collagen,

fibrin, and Matrigel to be uniformly 0.25, so we use this value for Poisson’s ratio in our experiments as well.

Finally, in order to validate the final material parameters presented by Steinwachs *et. al.*, the strain stiffening coefficient d_s and linear strain range λ_s can be determined from our PNIPAAm contraction experiments. The finite element model will be used to compute full-field displacements, which will be iterated against the experimental full-field displacement results measured directly using TPT particle tracking using a least-squared minimization scheme. Using this technique, the experimentally determined collagen material coefficients E , d_s , and λ_s , will be validated using our embedded contractile-based measurement technique, and the existing material model will be fit to our collagen hydrogels such that tractions from cells embedded in these collagen hydrogels can be correctly measured. An outline of the techniques performed and measurements used to validate and fit the nonlinear material model is shown in Fig. 3.2.

3.2.9 Finite element material model implementation for traction computations

The material model described by Steinwachs *et. al.* was originally implemented in Python and is freely available for download (see <https://github.com/Tschaal/SAENO>). To make the code more user-friendly and in order to make future adjustments easier to execute, the fibrous material model was implemented in Matlab. To improve convergence, the linear tetrahedral mesh employed by Steinwachs was replaced with a second quadratic tetrahedral element structure. Scattered displacement results from TPT were regularized and then interpolated onto the PNIPAAm particle and neutrophil FE meshes. The computed displacements on the particle and cell surfaces are applied as a Dirichlet-type boundary condition in the finite element framework. A stress free boundary condition is applied to the outer edges of the collagen gel meshes. We then directly solve the volumetric boundary value problem defined by the measured displacements and experimentally validated substrate material properties using the finite element method in Matlab.

For the sake of comparison to the fibrous finite element model, a linear elastic material

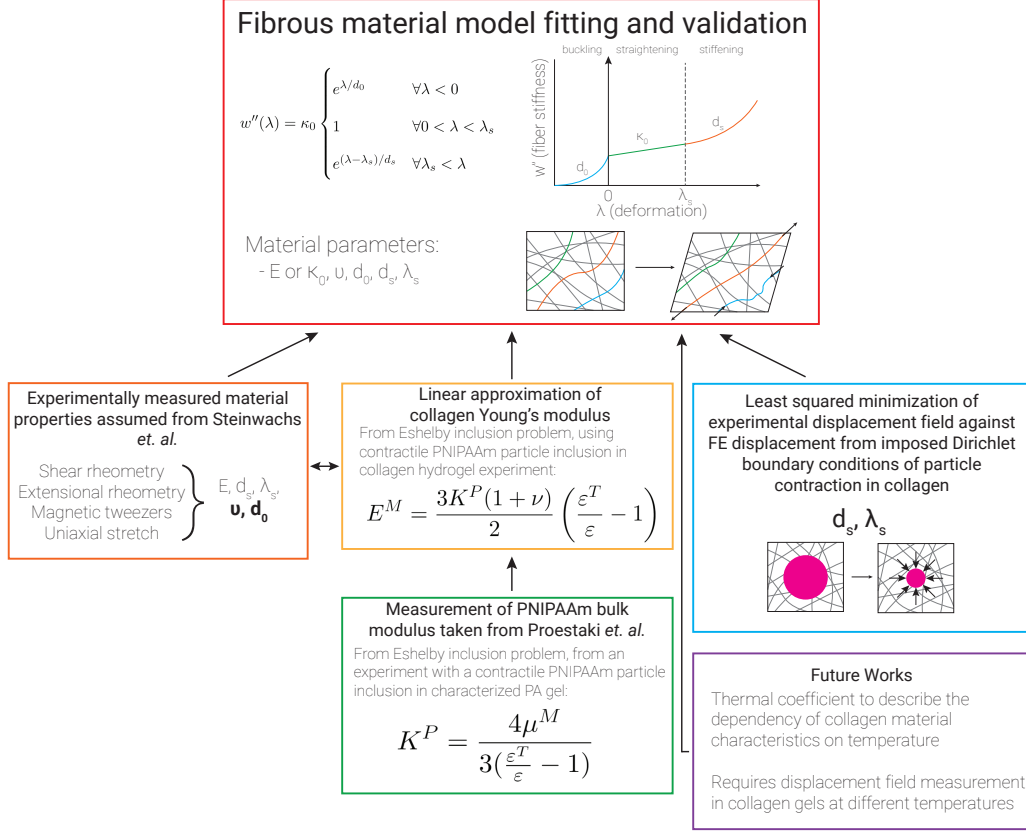


Figure 3.2: Methods for fibrous material model fitting and validation. The fibrous material model requires the definition of E or κ_0 , ν , d_s , d_0 , and λ_s to describe the relationship of the fiber stiffness to the applied deformation field. These material parameters describe the fibrous material behavior, including compression weakening caused by buckling and fiber straightening and stiffening under tension. All of these parameters were measured experimentally or computed by Steinwachs *et. al.* [67] for collagen gels made from 0.6, 1.2, and 2.4 mg/mL concentrations of collagen and can be used to estimate the values for our 2.2 mg/mL collagen hydrogels. PNIPAAm inclusion experiments in polyacrylamide performed by Proestaki *et. al.* [76] are used to measure the bulk modulus of the PNIPAAm, which is in turn used to estimate the Young's modulus of collagen using a linear approximation from an experiment with a PNIPAAm inclusion in a collagen hydrogel. This Young's modulus estimation is used to validate the measured Young's modulus from Steinwachs *et. al.* [67]. ν and d_0 are taken directly from Steinwachs' experiments. Experimental displacement data from PNIPAAm inclusion experiments in collagen is used to determine d_s and λ_s using a least squares minimization of a finite element simulation compared to experimental results. Future experiments will determine temperature effects on collagen material behavior.

framework was implemented in Matlab and used to compute cell-produced tractions. For this thesis, these results will represent a temporary stand-in for the fibrous model results, which requires additional experiments to characterize the temperature-dependence of the collagen response before it can be properly fitted and implemented as described above.

The finite element model computes the force vector at each node, which are the coordinate locations of the finite element mesh where displacements are computed. This nodal force can be used to compute the surface traction of a given finite element mesh face by averaging the nodal forces at each corner of the face and dividing by the surface area of the face. However, nodal forces are often used in place of traction when reporting finite-element computed force fields, as they are directly measured using the finite element framework and therefore not subject to computation error or noise.

3.3 Results and Discussion

Preliminary results from the PNIPAAm inclusion experiments designed for fitting the existing fibrous material model to the 2.2 mg/mL collagen hydrogels used in our experiments are presented in this section. The finite element mesh generated for both the PNIPAAm particle data and an experimental data case of displacements produced by a single human neutrophil are shown. A linear material model was applied to compute the tractions from the neutrophil-produced displacements as a temporary stand-in for the fibrous material model, which has yet to be fully validated. This linear data will eventually serve as a baseline of comparison for the final implemented fibrous model, and currently demonstrates that the finite element mesh generation and measured experimental displacements are of sufficient quality to reconstruct surface tractions.

3.3.1 Contracting PNIPAAm particle displacement fields

The displacement fields produced by PNIPAAm particles embedded in collagen matrices when heated above their phase transition temperature were computed by tracking the positions of embedded fluorescent microbeads from volumetric image stacks taken at 32°C (below phase transition temperature), 35°C (around phase transition temperature), and 38°C (above phase transition temperature) using TPT [72]. Nominal additional displacements were observed for most particles when heated from 35°C to 38°C, so the displacement field of a single particle computed at the 35°C timepoint is shown in Fig. 3.3. The full-field displacement

shown in Fig. 3.3a shows an unexpected finding from all of our imaged particles: displacements in the far field (far away from the particle border) were higher than the displacements recorded at the particle surface. At first, we attributed this to poor microbead tracking at the particle boundary due to large localized displacements, or long-range transmission of forces by the particles through the fibrous material. However, when visually examining the TPT results we found the microbead tracking near the particle surface to be sufficient, and therefore the transmission of forces would be expected to decay in the far-field rather than increase, as predicted by many fiber models [13, 142–144]. Therefore, we concluded there may be some thermal effects on the collagen behavior or structure that has to our knowledge not been described in any existing material model. Section 4.2.3 details our planned methodology for including thermal effects into our collagen material model in the future.

3.3.2 Finite element mesh generation

Finite element meshes were generated for the PNIPAAm particles, neutrophils, and surrounding collagen matrices using previously published and freely available Matlab scripts, as shown in Fig. 3.4. The finite element mesh generation tool used for the spherical PNIPAAm particle and surrounding collagen matrix (Fig.3.4a) was unable to capture the small shape irregularities of a neutrophil. To preserve the integrity of the neutrophil boundaries within the finite element framework, a different mesh generation tool was used for the neutrophil and surrounding matrix (Fig.3.4b and c). In both cases, the measured displacement results interpolated onto the finite element mesh surface were imposed as Dirichlet-type boundary condition within the finite element framework. The outer edges of both collagen meshes were treated as stress-free Neumann-type boundary conditions given the distance of these boundaries from the particle or neutrophil is sufficient to assume transmitted forces have decayed to zero.

3.3.3 Cell induced displacements and tractions

Next, the displacement field measured from a single neutrophil in a 10 nM gradient of the chemokine fMLP is shown. Displacements were computed using topology-based single

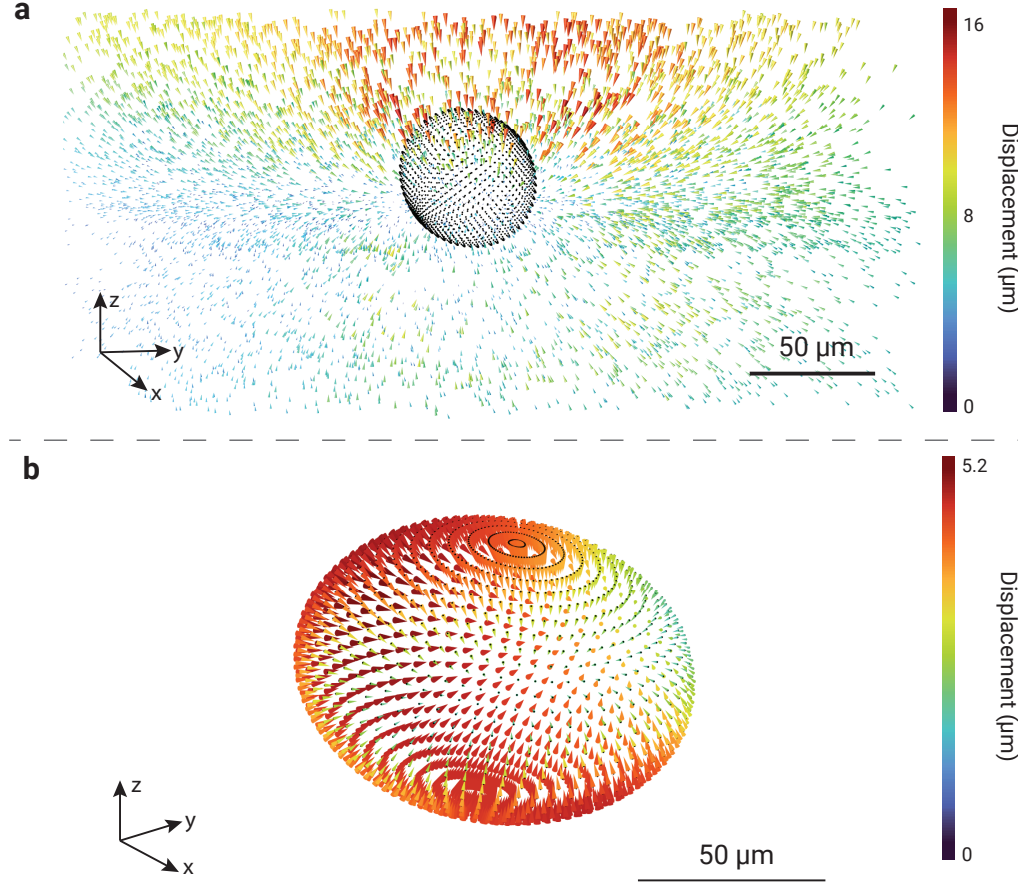


Figure 3.3: **Example displacement field generated by contracting PNIPAAm particle in collagen hydrogel.** (a) The full-field, three-dimensional displacements produced by a PNIPAAm particle (black) when heated from 32°C to 35°C. Nominal additional displacement were observed when the particle was heated past 35°C. (b) Displacement magnitude mapped onto the ellipsoid fit for the particle surface. For each case, cone color and size show magnitude of displacement and cone direction indicates displacement direction, and vertices of the ellipsoid fit particle are shown as black dots.

particle tracking [72], and interpolated onto the segmented neutrophil surface, see Fig. 3.5a. The magnitude of the displacement is shown mapped onto the surface as a contour plot, with the inset image showing a magnified view of the displacement directions displayed using cones plotted on the cell surface. The interpolated displacement magnitudes and directions can be compared to the full-field displacement results shown in Fig. 3.5b to reveal a close match between the interpolated and measured displacements.

The displacement field was then regularized onto the finite element mesh of the neutrophil, shown in Fig. 3.6a. The regularization scheme chosen involves a smoothing step,

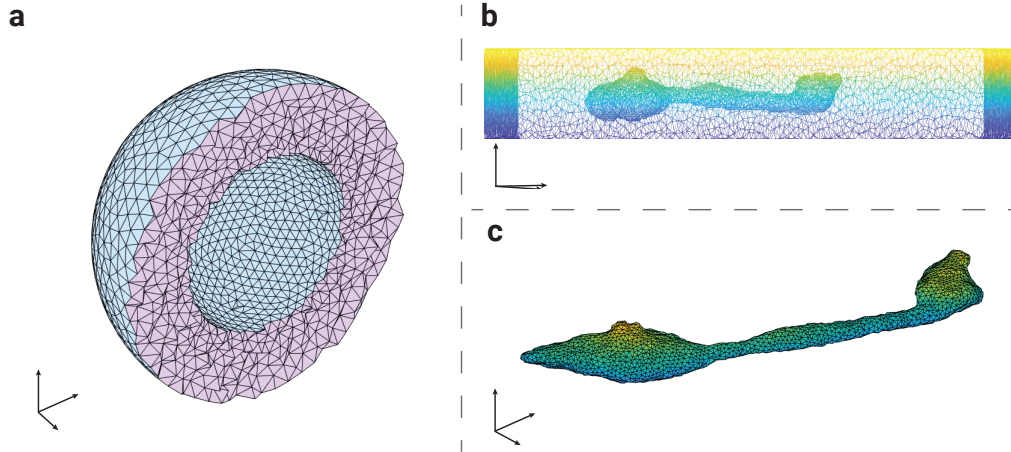


Figure 3.4: **Finite element mesh generated for PNIPAAm particle, neutrophil, and surrounding collagen matrices.** (a) Finite element mesh generated for a PNIPAAm particle (inner blue sphere) and surrounding collagen matrix (outside, purple). (b) Finite element mesh generated for a single neutrophil, shown within the surrounding collagen hydrogel mesh and (c) isolated from the collagen mesh, showing the complex mesh geometry. Color correlates to the height of the mesh.

which accounts for the differences in displacements interpolated to the cell surface directly from the TPT results (shown in Fig. 3.5a) and those interpolated from the regularized FE mesh framework. The cell-generated forces computed using the linear material finite element framework at each nodal point on the FE cell mesh are shown in Fig. 3.6b. As expected, the areas on the cell surface with high nodal forces are shown to align with areas of high displacement in Fig. 3.6a. Theoretically, the nodal forces or surface tractions could be resolved at higher spatial frequencies by using a finer finite element mesh, but this also increases the computation time for the analysis and can decrease the chances of convergence on a solution, particularly for more complex (i.e., fibrous) material models.

3.4 Future Work and Conclusion

Further experiments are necessary to characterize the effects of the temperature changes on the collagen material characteristics. Specifically, separate collagen gels without PNIPAAm particles will be polymerized using the recipe shown in Table 3.2 in two layers to reproduce the gel polymerization technique used to prevent PNIPAAm particles from sinking and in

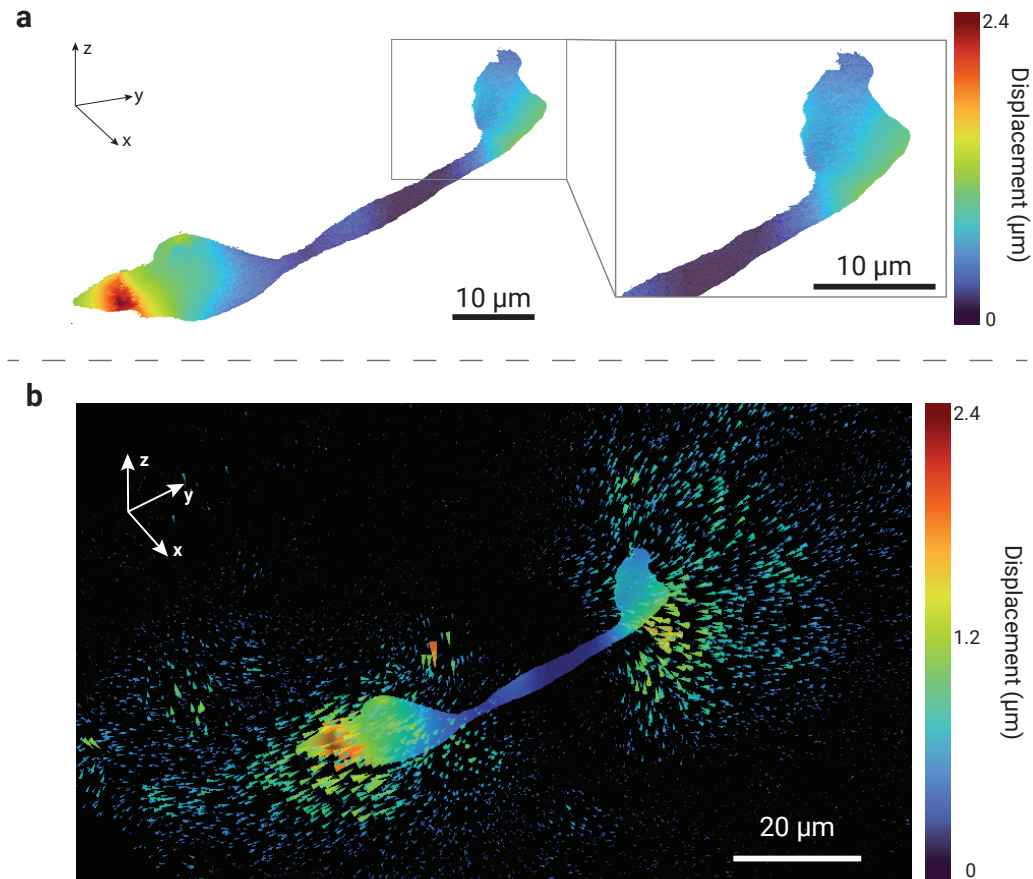


Figure 3.5: **Displacement field generated by single neutrophils in a 10nM gradient of fMLP.** (a) Displacement magnitude mapped onto the cell surface. A magnified view of the cell area within the gray box on the right shows the displacement direction at the cell surface using cones: the direction of the cone shows the direction of displacement, the color and size indicate the displacement magnitude. (b) The full-field, three-dimensional displacements produced by a neutrophil embedded in collagen in the presence of an fMLP gradient. Cone size and color indicate displacement magnitude, cones direction indicates displacement direction.

one single layer to control for the possibility that the observed material behavior (e.g., large far-field displacements) are caused by poor integration of the collagen layers using this collagen fabrication technique. These collagen gels will be imaged using the same technique as the PNIPAAm-embedded gels, raising the temperature from 32°C to 35°C to 38°C, and the resulting displacement fields will be computed using TPT. The full-field temperature-induced strains will be computed from the displacements, and compared against the finite element

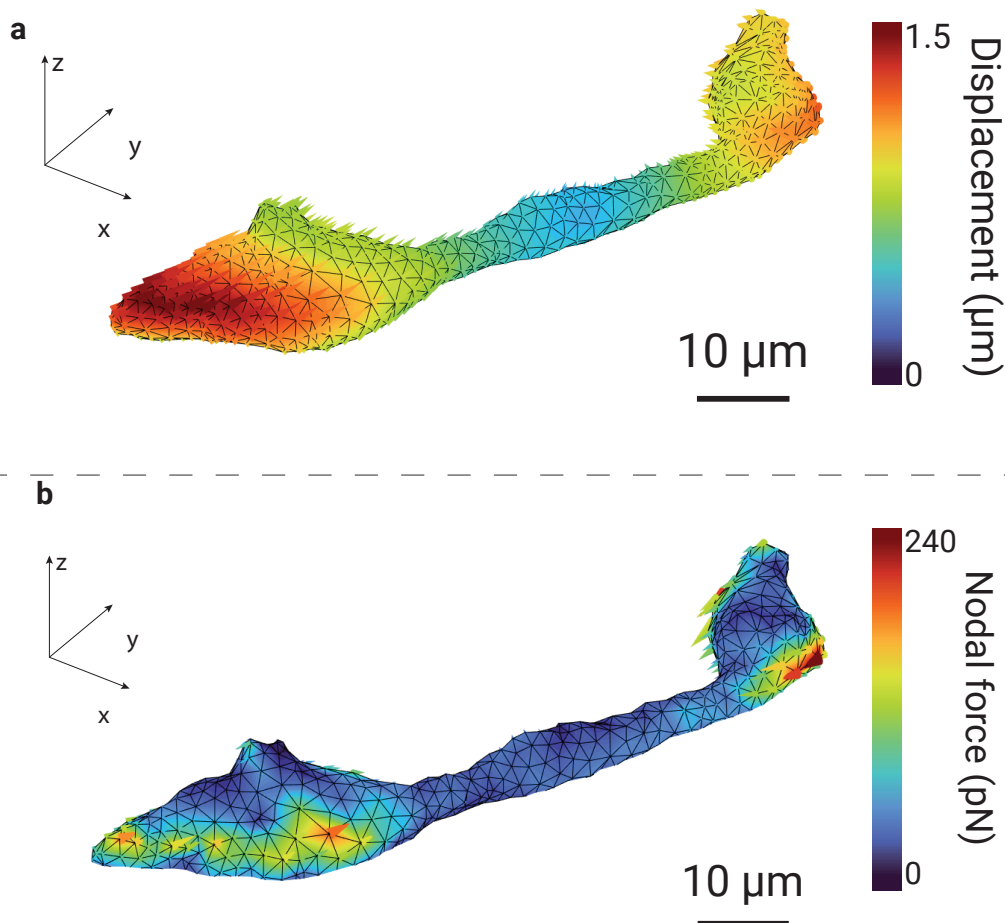


Figure 3.6: **Finite element generated displacements and nodal forces produced by a single neutrophil in an fMLP gradient within a collagen hydrogel.** (a) Cell-produced displacement magnitude mapped onto the finite element cell surface mesh, with coneplot direction indicating direction of generated displacement and coneplot size and color indicating displacement magnitude.. (b) Nodal force magnitude computed using FEA mapped onto the finite element cell surface mesh, with coneplot direction indicating direction of generated forces and coneplot size and color indicating force magnitude.

model predicted strain field using the fibrous material model (i.e., zero strain). If appropriate, a new, temperature-dependent material parameter will be introduced to the fibrous material model to account for thermally-induced strains. This in itself will represent a novel finding for fibrous material characterizations, which normally neglect to account for thermal effects. The response of collagen to temperature will be included in the material model; an important factor for studies modeling physiological systems which undergo variations in temperature, such as the immune inflammatory response. The measured PNIPAAm-induced

strain field will then be treated as the superposition of the collagen thermal strain with the particle contraction-induced strain field, which will enable us to isolate the PNIPAAm induced strains, and give a proper estimation of the Poisson's ratio of the collagen gel matrix using Equation 3.3. These measurements will be used to validate the material model originally presented by Steinwachs *et. al.* [67], as detailed in Fig. 3.2. Taken with the methods presented in this chapter, these future directions outline a plan for measuring and modeling the temperature effects on the fibrous material response, and to fit the fibrous model to the material properties that were obtained from experiments performed on a cellular scale from within the hydrogel. This method could be applied to measure material properties and perform model fitting for any number of fibrous hydrogel systems, including fibrin and collagen gels of different densities or polymerization conditions.

Overall, this chapter presents a method for measuring the material properties of fibrous materials, using these measurements and experimental results to validate an existing material model, and for fitting the model to the measured material. This method represents a fully three-dimensional, cell-scale measurement technique which will account for thermal effects on fibrous material behavior, though results from the direct implementation of this method is left to future works. The experimental methods developed for this validation are presented and have been fully implemented, with the measured displacement fields from a contractile PNIPAAm particle in collagen type-I hydrogel presented. A preliminary linear material model is implemented in Matlab to compute cell-generated displacements, forces, and surface tractions from a single neutrophil, demonstrating that such reconstructions are possible using the experimental methods described. In the future, this linear material model will be replaced with the fibrous material model adapted from Steinwachs *et. al.*.

Chapter 4

Development and Validation of a Live-Cell Imaging Chemotaxis Device

4.1 Introduction

Chemotaxis is the process by which cellular migration is directed by the presence of a soluble extracellular chemical gradient. Chemotaxis has been shown to regulate many cellular processes, including angiogenesis, wound healing, reproduction, and cancer metastasis [168–171]. Leukocyte recruitment to sites of inflammation is perhaps one of the most well-known examples of chemotaxis migration [86, 90, 104]. During an immune response to tissue injury or infection, neutrophils are the first responders to the site of injury: quiescent neutrophils in circulation are activated by soluble cytokine signals, which prompt the neutrophil to extravasate through the blood vessel endothelial layer. Neutrophils then migrate through the interstitial tissue to the precise site of injury or infection following the chemokine gradient. Once there, the neutrophil will attempt to eliminate the infection through one of the three primary mechanisms by which neutrophils can induce pathogen death: degranulation, phagocytosis, and release of nuclear material as neutrophil extracellular traps (NETs) [86, 87, 89]. Neutrophils respond to signalling molecules from a number of sources, including injured or inflamed tissue, other leukocytes (including other neutrophils), and invading pathogens [90, 104]. The neutrophil response to different chemokine

signals is varied, and specifically hierarchical to prioritize 'end target' chemokines, usually those released from the source of infection, such as complement components or bacterially-derived formylated peptides like *N*-formyl-methionyl-leucyl-phenylalanine (fMLP). Other chemokines are released from more 'intermediate' sites, such as the inflamed endothelium or other leukocytes, including Leukotriene B₄ (LTB₄) and interleukin 8 (IL-8) [90,104,105].

Neutrophil chemotaxis is a vital part of the innate immune response, as evidenced by the fact that dysregulation in the chemotaxis response, as seen in severe trauma, autoimmune disease, systemic inflammatory response syndrome (SIRS), and sepsis [106–109]. Neutrophils produce and store a number of highly toxic components, including the reactive oxygen species (ROS) superoxide and hydrogen peroxide, which serve to eliminate microbial invaders in a well-regulated immune response, but can also lead to necrosis of native tissue when neutrophils are hyperactivated [86,91,92]. In patients with SIRS and sepsis, neutrophil migration is dysregulated, leading both to an accumulation of neutrophils in vital organs and insufficient neutrophil recruitment to sites of microbial invasion [108–110]. Unregulated accumulation of neutrophils in vital organs can result in serious tissue damage; organs such as the liver, lung, kidney and brain are at risk for failure, which can lead to death [106–109]. Inability of neutrophils to migrate to sites of infection puts patients at increased risk of severe, systemic infections. The exact mechanisms by which SIRS and sepsis alter the ability of neutrophils to respond to chemokine signals is unknown: numerous studies and clinical trials related to treatments for sepsis have failed to locate a specific mechanism for dysregulation within cellular signalling pathways, or indeed an effective treatment modality [111–114]. This leads to the conclusion that neutrophil chemotaxis is regulated by both chemical and mechanical signals, which highlights the importance of developing experimental systems for probing the mechanics of neutrophil chemotaxis.

By far one of the most popular methods for studying cellular mechanosensing and force generation is traction force microscopy (TFM). TFM is a biomechanical technique that reconstructs cell-generated stresses from experimentally observed displacements [6]. Generally, cells are either cultured on top of an elastic substrate or embedded within a fibrous

hydrogel; in either case, fluorescent particles contained within the substrate are used as fiducial markers for reconstructing displacements. For three-dimensional analysis, displacement field reconstruction is typically done with either digital volume correlation (DVC) or single particle tracking (SPT)-based methods [62,63,72,73,75]. Stress fields are reconstructed from the displacement field using finite element method-based TFM (FEM-TFM) or continuum mechanics-based computational models [6,57–59]. In either case, knowledge of the matrix material properties (e.g., Young’s modulus and Poisson’s ratio or shear and bulk moduli) and of the boundary geometry between the cell and the extracellular material are required.

Studies have examined the biomechanical responses of neutrophils using TFM [11,115–120], but much of this research has been limited to studies of neutrophils migrating along planar substrate surfaces. Neutrophil chemotaxis *in vivo* typically occurs through complex tissue microenvironments: to increase the physiological relevance of chemotaxis research, many studies have moved into three-dimensional systems [64,172,173]. Limitations with characterizing the matrix material properties of these physiological, non-linear, fibrous ECM materials (e.g., fibrin and collagen) have prevented neutrophil biomechanics work from similarly moving into three-dimensions. Stout *et al.* developed a method for computing deformation-based metrics for characterizing neutrophil chemotaxis behavior in collagen, which does not rely upon material properties [64], however traction force remains the most well-known and universal metric for reporting cellular biomechanics behavior.

Research into characterizing fibrous material behavior for the purpose of reconstructing cell-produced tractions has long been of interest in the field of traction force microscopy. Different models attempt to characterize the unique, non-linear, and non-affine behavior of fibrous materials in different ways: some models focus on strain-hardening to explain long-range force transmission along fibers [145–147], others postulate that fiber cross-linking is key to force transmission [13], and still others have observed microbuckling of individual fibers and have modeled this behavior as compression weakening of the material [79,148]. Steinwachs *et al.* considered the non-affine behavior of local fibers along with the macroscopic bulk material behavior to introduce a continuum model which accounts for fiber buckling in compression, constant stiffness under small extensions, and strain stiffening for large

tensile forces, averaging the stress contributions of many fibers to derive the constitutive equations [67]. Other models have been introduced which describe fiber-based constitutive models that consider the bulk hyperelastic matrix behavior and the nonlinear elastic fiber behavior to be coupled [14, 143, 162]. These more complex models seek to account for contributions of the bulk material in addition to the fibrous material characteristics that influence long-range force transmission, including fiber alignment and response to applied force. However, these complex models can be very difficult to validate and fit to experimental data, often requiring complex measurements of material properties which could deter interested users. Chapter 3 of this thesis presents the experimental method for validating the material model presented by Steinwachs *et. al.* [67] in collagen using a radially contracting particle in a method similar to one described by Burkel *et. al.* [150], and provides a method for computing the surface tractions from individual cells. This model was selected because it was straightforward to implement, had already undergone fairly significant fitting to experimental material measurements, and accounted for the most frequently described non-linearities seen in fibrous materials (i.e., fiber buckling in compression, constant stiffness under small extensions, and strain stiffening for large tensile forces). Many experimental systems have been developed for studying cellular chemotaxis, including collagen or fibrin hydrogel systems [174], the Boyden chamber [175], under agarose systems [176], and the Dunn chamber [177] among others [178]. Some of these systems are limited to two-dimensional analysis [179–181], do not allow for the high quality live-cell imaging necessary for reconstructing a displacement field [172, 182–184], or are open to air and liable to dry out [64]. Many chemotaxis systems are microfluidic devices, and rely upon expensive manufacturing techniques such as soft lithography for device production and fluid flow control devices (e.g., syringe pumps, peristaltic pumps, or pressure controllers) for a stable chemokine gradient, limiting their reproducibility for new users [185, 186]. Additionally, many devices, particularly microfluidics devices, do not have appropriate geometries for reconstructing tractions from fibrous hydrogels: the channels are simply too small [187, 188]. This is because cells are able to transmit forces for longer distances in fibrous materials compared to linear elastic substrates, which is generally thought to be a mechanical signalling mechanism by which cells communicate over long distances and which enables mechanical feedback between the

cell and ECM [14, 18, 142, 143, 161]. In one study by Notbohm *et al.* stresses in fibrin were predicted to decay proportionally to r^{-2} , rather than with r^{-3} , as is predicted by linear elasticity models [142]. Other studies have shown that force transmission can occur over distances that are 10 to 20 times the diameter of the cell [13, 143, 144]. This presents a challenge for computing traction forces from cells in three-dimensional fibrous *in vitro* systems: the cells need to be fairly far away from the hydrogel-glass interface to ensure the local mechanical properties experienced by the cell are not influenced by the underlying glass. Not only would sensing the glass influence cell behavior, which is known to be affected by substrate rigidity [7, 8, 10, 11], but would also render the characterization of the hydrogel material properties and subsequent stress computations incorrect. This limitation renders many microfluidics devices unusable for measuring cell-generated tractions.

An appropriate device for measuring cell-generated forces from neutrophils undergoing chemotaxis in a three-dimensional system needs to provide a stable chemokine gradient, allow for live cell imaging, have a geometry that is large enough to account for cellular force transmission and sensing in fibrous materials, and ideally be easy and inexpensive to manufacture and use. To this end, we present a polydimethylsiloxane (PDMS) device that is cast from a 3D-printed mold. The geometry of the device is simple to customize using computed aided design (CAD) software, and is inexpensive to print using a 3D printer. The device uses standard glass coverslips to assemble, and allows for live-cell imaging. We validate the efficacy of the device in creating a stable chemokine gradient using computational models, and demonstrate its ability to induce neutrophil chemotaxis using two different chemokines, LTB₄ and fMLP. Finally, we compute the displacement field and traction stresses from individual cells in an fMLP chemotaxis gradient.

4.2 Methods

4.2.1 Device design and production

An easily reproducible chemotaxis device was designed using a free CAD program (OnShape, Inc). To create the devices, mold negatives are made from clear resin using a stereolithography (SLA) 3D printer (FormLabs). Two-part polydimethylsiloxane (Sylgard 184, Dow, Inc.) elastomer is mixed in a 10:1 ratio per the manufacturer’s instructions and de-gassed under vacuum for 15 minutes. The 3D-printed molds are placed in the bottom of a plastic petri dish, and the PDMS solution is poured over top. The petri dish is placed under vacuum for an additional 15 minutes and then placed in an oven set to 60°C overnight. The cured PDMS is removed from the petri dish, the PDMS is cut into approximately 25mm by 50mm rectangles around the molds, and then the molds are removed, as shown in Fig. 4.1a. The final PDMS devices are washed with soap and water and sanitized using ethanol. The exact dimensions of the molds can be found in Fig. 4.2.

4.2.2 Device assembly

18mm square and 25mm by 50mm rectangular No. 1 glass coverslips are hydrophilically treated by washing coverslips in ethanol, then placing them in 0.5% (v/v) 3-aminopropyltrimethoxysilane in ethanol for 5 minutes, followed by another rinse with ethanol and submersion in 0.5% glutaraldehyde in deionized water for 30 minutes. The coverslips are then washed with deionized water and placed into a 37°C oven for at least 30 minutes or until dry. The device is turned upside down for assembly, and the exposed PDMS surfaces are coated with vacuum grease to create a leak-proof seal. A hydrophilically-treated 18mm square coverslip is placed on the lower ledge surface with tweezers and gently pressed down to ensure the vacuum grease seal is airtight.

Collagen hydrogel solution is prepared using an adapted version of the method described by Scimone *et al.* [164]. 0.5 μm red fluorescent microspheres (Fluospheres, Thermo Fisher Scientific) were vortexed for 1 minute and then a volume totaling 6% of the final gel volume

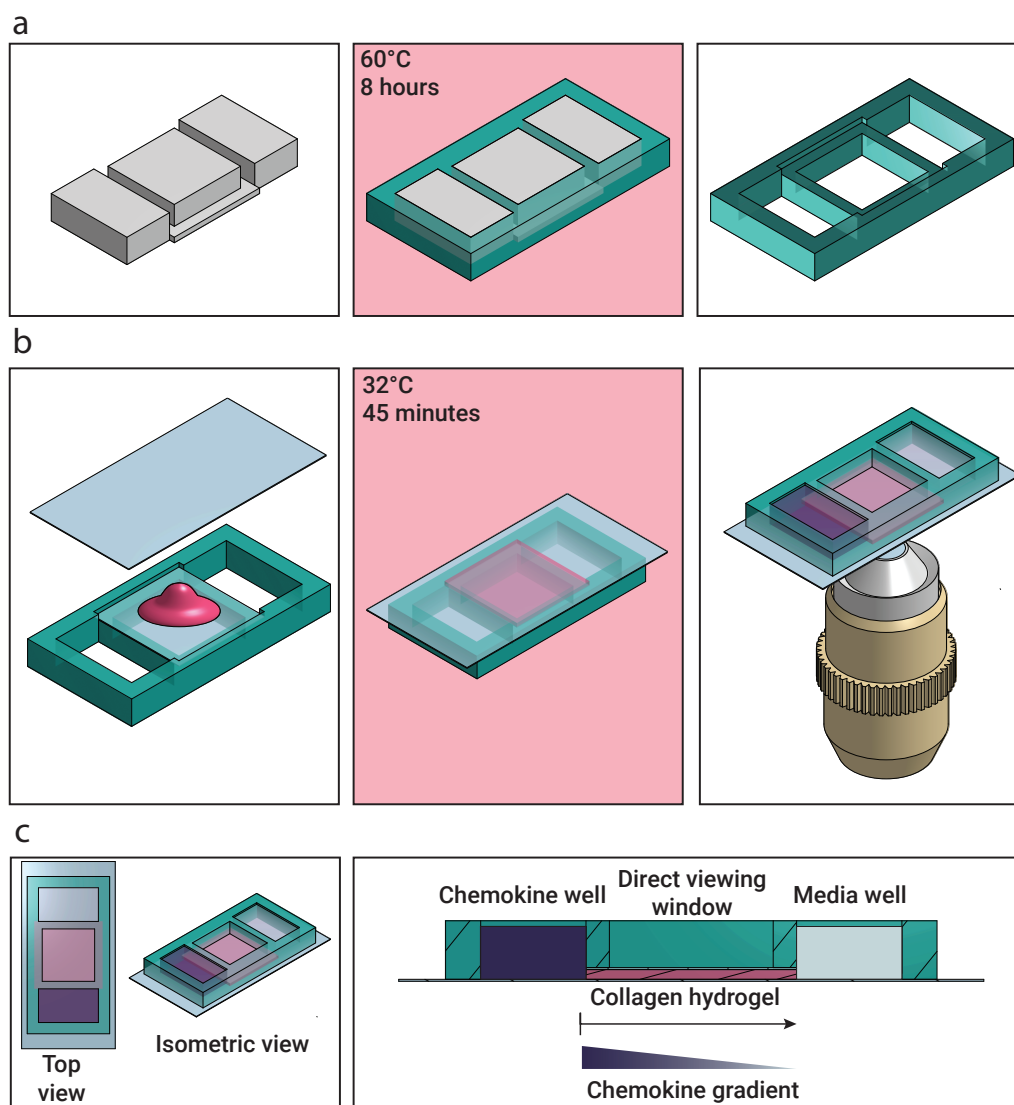


Figure 4.1: **Methods for fabricating a PDMS chemotaxis device for live-cell imaging.** (a) A negative mold of the device is 3D printed using an SLA 3D printer. The molds are used to cast PDMS devices by mixing PDMS components per manufacturer's instructions, degassing, and allowing to cure at 60 °C overnight before cutting the PDMS to size and removing the mold. (b) The PDMS device surfaces are coated in vacuum grease, and a hydrophilic 18mm square glass coverslip is pressed down onto the lower surface. Collagen hydrogel solution is pipetted onto the coverslip and covered with a 25mm by 50mm hydrophilic glass coverslip. The collagen gel is allowed to polymerize in an incubator for 45 minutes before media and/or chemoattractants are added to the side wells of the device and images are acquired. (c) Alternative views of the PDMS device (right panel), and (left panel) the cross-section of the device, including two side wells holding chemokine and media solution, the collagen hydrogel contained between the two glass coverslips, the open space above the collagen hydrogel to permit direct viewing using transmitted light or bright field microscopy, and the direction of the chemokine gradient through the collagen hydrogel.

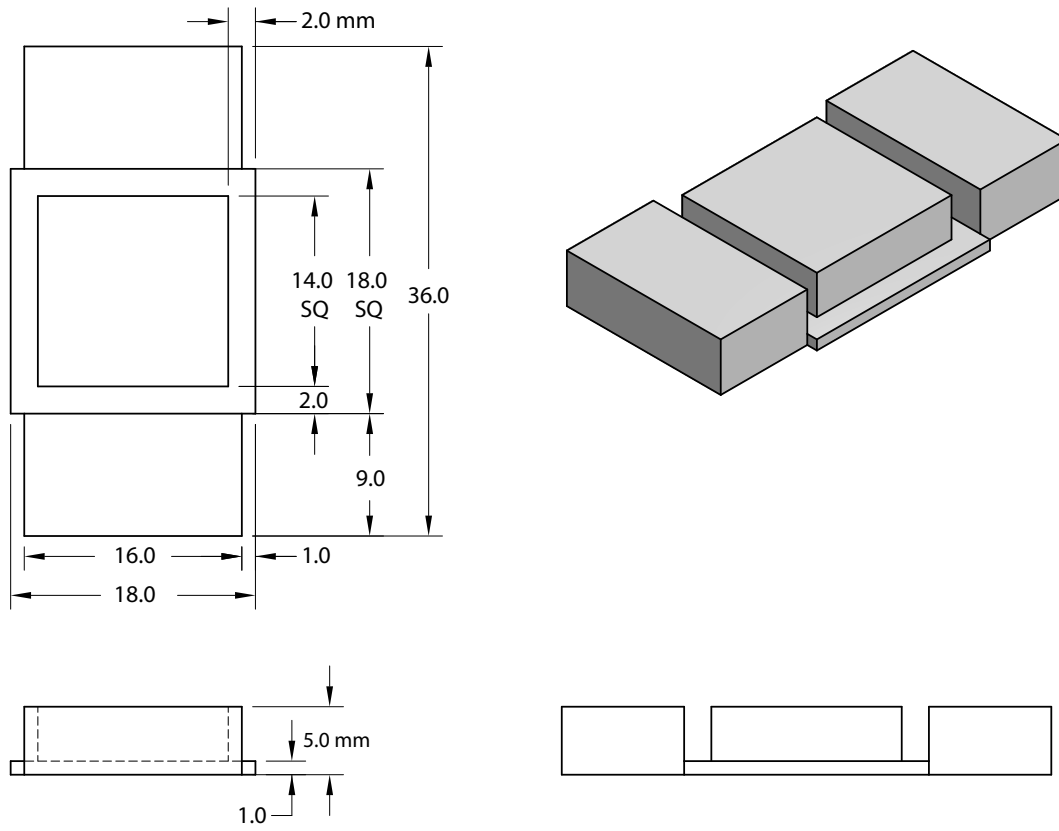


Figure 4.2: **Engineering drawing of chemotaxis device mold**, showing the dimensions (in mm) which are easily customized for different applications, cell types, or to alter the profile of the chemokine gradient.

was centrifuged to remove the beads from the shipping solution. Beads were resuspended in a volume of 10X phosphate buffered saline solution (PBS) to total 10% of the final gel volume. A volume of Leibovitz's L-15 media (Thermo Fisher Scientific) is computed to bring the final collagen concentration in the hydrogels to 2.2 mg/mL. Half of the computed volume of L-15 with 2 mg/mL glucose added is put into the PBS/bead solution. Next, rat tail collagen-I (Corning) was added to the PBS/bead/media solution and pipetted gently to mix without introducing air bubbles. The remainder of the media, containing live cells, (see Section 4.2.3 for more information) is added into the collagen gel mixture and pipetted gently but thoroughly, avoiding introducing air bubbles into the mixture. Finally, a small volume (about 1% of the total volume), of 1M NaOH was added to the solution and mixed into the solution. NaOH is needed to neutralize the acetic acid solution that the collagen

is shipped in, raising the pH and allowing for collagen gel polymerization. Approximately 250 μ L of collagen gel solution is pipetted onto the 18mm glass coverslip in the device and spread around using the tip of the pipette to ensure the solution reaches both PDMS edges. A 25mm by 50mm hydrophilic glass coverslip is placed down on top of the device and pressed down gently to form a seal with the vacuum grease without introducing air bubbles to the collagen solution. The device is placed into an incubator set to 32°C for 45 minutes to allow the gels to polymerize prior to the addition of chemokine and media to the side wells of the device, and finally imaging, as shown in Fig. 4.1b. Alternative views of the device, including the top and isometric views and a cross-section of the assembled device are shown in Fig. 4.1c.

4.2.3 Live cell imaging experiments

Neutrophils are isolated from whole blood taken from healthy human volunteers with written informed consent through a protocol approved by the University of Wisconsin-Madison Internal Review Board. Primary human neutrophils were isolated by negative antibody selection using the MACSxpress Neutrophil Isolation and MACSxpress Erythrocyte Depletion kits (Miltenyi Biotec, Inc.). Cells were labeled by 8 minute incubation in 2 μ M Calcein AM in Hank's Balanced Salt Solution, without calcium, magnesium, or phenol red. Following labeling, neutrophils are centrifuged down at 100 $\times$ g for 2 minutes and resuspended in L-15 media to a final concentration of 6,000 cells/ μ L.

Large field-of-view image stacks (i.e., 635 μ m by 635 μ m by 125 μ m) containing approximately 60-150 cells each were acquired over a period of time to analyze cellular behavior in each of three conditions: no-activation, chemotaxis, and chemokinesis. No-activation controls were run in collagen gels with L-15 media added to both wells of the device, and capturing three-dimensional image stacks every 2 minutes for 15 minutes. Following the imaging of the no-activation condition, one of two different chemotaxis gradients were established in the device by replacing media in one well with either 10 nM N-Formylmethionine-leucyl-phenylalanine (fMLP) or 10 nM Leukotriene B₄ (LTB₄). Image stacks of the chemotaxis

condition were acquired every 2 minutes for 1 hour. Chemokinesis conditions were established by activating cells with 10 nM fMLP or LTB₄ prior to forming the gels by adding the chemokine directly into the L-15 media containing the cells prior to polymerization. Following polymerization, 10 nM fMLP or 10 nM LTB₄ were added in equal volumes to both ports of the devices, and volumetric image stacks were acquired of the cells every 2 minutes for 1 hour. All images were acquired on a Nikon inverted multiphoton microscope with a 20X/0.75NA air objective, with a custom-built heat enclosure set to 37°C. For each case, 125 μm z-stacks were acquired with a μm -to-voxel ratio of $0.62 \times 0.62 \times 1.5$ in x, y, and z-directions.

Individual cells were also imaged under chemotaxis conditions with 10 nM fMLP in order to compute cell-generated displacements and stresses. Images of individual cells were acquired with a 40X/1.15NA water immersion objective on a Nikon A1 multiphoton inverted microscope with the heat enclosure set to 37°C.

For all live cell images, volumetric image stacks were acquired at a location within the hydrogel approximately 1 mm away from the edge of the device closest to the chemokine well, to account for any edge effects from the diffusion at the perimeter of the gel. In addition, all image stacks were acquired at a minimum distance of 50 μm from the bottom glass coverslip. This was done to permit measurement of tractions from a location sufficiently far from the glass coverslip to prevent cells from sensing the glass stiffness without being so far into the gel that image quality deteriorated significantly and prevented particle localization and displacement measurement.

4.2.4 Computational gradient validation

Chemokine gradient formation in the device was validated analytically using a mathematical diffusion model. The collagen hydrogel within the device is taken to be a rectangular prism with each end kept at a constant concentration, C_0 and C_L . The planar interfaces between the collagen and the side wells allow us to assume a planar diffusion profile that is uniform in z . The model is derived from the diffusion equation, $q = -D \frac{\partial C}{\partial x}$, where D is the diffusivity coefficient, C is concentration, x is distance, and q is the flux. Knowing that $\frac{\partial C}{\partial t} = -\frac{\partial q}{\partial x}$,

allows us to substitute such that: $\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2}$. We treat this as a partial differential equation (PDE), with the following boundary conditions:

$$C(0, t) = C_0, \quad 0 < t < \inf \quad (4.1)$$

$$C(L, t) = C_L, \quad 0 < t < \inf \quad (4.2)$$

and initial condition:

$$C(x, 0) = 0, \quad 0 < x < L \quad (4.3)$$

Where L represents the total length of the collagen hydrogel, which is 18 mm. This system has non-zero boundary conditions, and thus the solution will contain some equilibrium condition, or steady-state solution, in addition to the transient solution. Essentially, $C(x, t) = \{\text{steady-state solution}\} + \{\text{transient solution}\}$. We begin with the steady state solution, where we consider $t \rightarrow \inf$, at which point there will be some equilibrium concentration $C_E(x)$. From the diffusion equation and our boundary conditions, we know:

$$0 = \frac{\partial^2 C_E}{\partial x^2}, \quad \text{and} \quad C_E(0) = C_0, \quad C_E(L) = C_L, \quad (4.4)$$

and integrating gives us $C_E(x) = Ax + B$. When we apply our boundary conditions, this leaves:

$$C_E(x) = C_0 + \frac{x}{L}(C_L - C_0), \quad (4.5)$$

or, when $C_L = 0$,

$$C_E(x) = C_0 - \frac{C_0 x}{L} \quad (4.6)$$

$$C_E(x) = C_0 \left[1 - \frac{x}{L} \right]. \quad (4.7)$$

We know: $C(x, t) = C_E(x) + w(x, t)$, and next, we solve for the transient solution,

$w(x, t)$. Due to the behavior of the steady-state solution, we know that $w(x, t)$ and $C(x, t)$ must satisfy the same PDEs, so the boundary conditions and initial conditions apply. Thus, our new PDE is $\frac{\partial v}{\partial t} = D \frac{\partial^2 v}{\partial x^2}$, where $w(0, t) = 0$, $w(L, t) = 0$, and $w(x, 0) = f(x) - C_E(x)$, with $f(x)$ describing the initial condition $C(x, 0)$. Solving gives:

$$C(x, t) = C_0 \left[1 - \frac{x}{L} \right] + \sum_{n=1}^{\infty} A_n \sin\left(\frac{n\pi x}{L}\right) e^{-Dt\left(\frac{n\pi}{L}\right)^2} \quad (4.8)$$

where:

$$A_n = \frac{2}{L} + \int_0^L (f(x) - C_E(x)) \sin\left(\frac{n\pi x}{L}\right) dx \quad \text{for } n = 1 \rightarrow \infty. \quad (4.9)$$

The final solution thus becomes:

$$C(x, t) = C_0 \left[1 - \frac{x}{L} \right] - \sum_{n=1}^{\infty} \frac{2C_0}{n\pi} \sin\left(\frac{n\pi x}{L}\right) e^{-Dt\left(\frac{n\pi}{L}\right)^2}. \quad (4.10)$$

A non-dimensionalized version of this equation was modeled in Matlab using the diffusivity coefficient reported by Stout *et al.* [64] based on experimental analysis of diffusion of fMLP through a 2.2 mg/mL collagen hydrogel, which was found to be $1.28 \pm 0.35 \times 10^{-6}$ cm²/s. We assume a similar diffusion profile applies to LTB₄ because the size of fMLP and LTB₄ are similar relative to the size of the collagen pores.

4.2.5 Cell motion analysis and traction computation

The large-field, multi-cell chemotaxis experiments were analyzed using Imaris cell tracking software (Imaris, Inc) to track the three-dimensional cell center positions over time. Mean cell velocity and path directness were quantified in Matlab using the Imaris tracking results. Mean velocity was computed as the change in position over change in time for a given cell at each timepoint. The mean path directness was computed as the net displacement of a cell, or the position of a given cell at the first timepoint taken from the position at the final timepoint, divided by the total distance travelled by that cell. Path directness is used as a

metric for straightness, or persistence, of neutrophil migration.

For individual neutrophils within collagen gels surface tractions (see Ch. 3 for methods) were computed from displacement fields reconstructed using topology-based single particle tracking (TPT) results (see Ref. [72]) in Matlab. Briefly, images of the fluorescent microbeads in the gel were tracked over time compared to a cell-free reference imaged using TPT, and the changes in the bead positions were used to reconstruct the three-dimensional volumetric deformation field. Fluorescence images of cell bodies were segmented, and a finite element mesh of the surface was generated using an existing mesh generation toolbox [166]. The displacement field was plotted directly from the TPT results, and reconstructed onto the cell surface for visualization purposes. The scattered TPT displacement results were regularized onto a finite element mesh representing the surrounding collagen hydrogel as the imposed displacement condition. The finite element solution was generated in Matlab using a linear material model with second quadratic tetrahedral elements.

4.2.6 Statistical analysis

All statistical analysis was performed using SigmaPlot (Systat Software Inc.). Analysis of variance (ANOVA) on ranks test was used to analyze differences between groups. No data sets for velocity or path straightness passed the Kolmogorov-Smirnov test for normality, and the Kruskal-Wallis test was rejected so Dunn's test was used to calculate p-values. All statistical comparisons were performed between activation conditions, on a between-population basis.

Statistical analysis was not performed on any displacement or force results: this data was acquired from a single cell and represents too small of a sample size for statistical analysis to be meaningful. These results are intended to demonstrate the effectiveness of the device and showcase general trends in experimental results that can be obtained rather than to show any findings of biological significance.

4.3 Results

A novel device for establishing chemokine gradients through three-dimensional hydrogels is introduced. The device is cast from PDMS using a 3D printed mold. The diffusion of chemoattractant through the device over time is validated computationally using the diffusion equation and known boundary conditions. Neutrophils embedded within collagen hydrogels in the device are exposed to three different activation conditions (i.e., no chemokine, uniform chemokine concentration, and chemokine gradient) and the response is recorded and analyzed. Cellular tractions from neutrophils responding to a chemokine gradient are computed. The chemokine concentration and gradient validation along with the cellular responses are presented in this section.

4.3.1 Chemokine gradient validation

Chemotaxis is the cellular response to the presence of a chemical gradient; therefore characterizing the chemical gradient within the collagen hydrogel was imperative to understanding the response of cells embedded in the device. The hydrogel forms a planar interface with the two side wells where media and chemokine are introduced, as shown in Fig. 4.1c, thus the diffusion profile is assumed to be planar and uniform in the z -direction. The diffusivity of fMLP through collagen is taken from the experimental analysis performed by Stout *et al.*, and the diffusivity of LTB₄ through collagen is assumed to be similar due to the relative sizes of fMLP and LTB₄ compared with typical collagen pore sizes. The concentration of the chemokine at given position x and time t are given in Eq. 4.10. Figure 4.3a presents the chemokine concentration within the collagen hydrogel, $C(x, t)$, normalized by the concentration at the inlet well, C_0 , along with the normalized concentration gradient, $\nabla C/C_0$, as a function of the normalized length of the gel, L/L_0 . The planar diffusion profile is modeled in Fig. 4.3b as the normalized chemokine concentration through the normalized length of the gel, L/L_0 , at three specific timepoints, $t = 0$ hours, 4 hours, and 50 hours to showcase the start ($t = 0$ hr) and equilibrium ($t = 50$ hr) concentrations, along with an example concentration seen shortly after chemokine addition, as might be seen during neutrophil

experiments ($t = 4$ hr). The diffusion profiles within the live cell experimental field of view, at a location approximately 1 mm into the gel, is indicated by a lavender region in Fig. 4.3a. The location and area of $635 \mu\text{m}$ by $635 \mu\text{m}$, is shown in 4.3b by a white square.

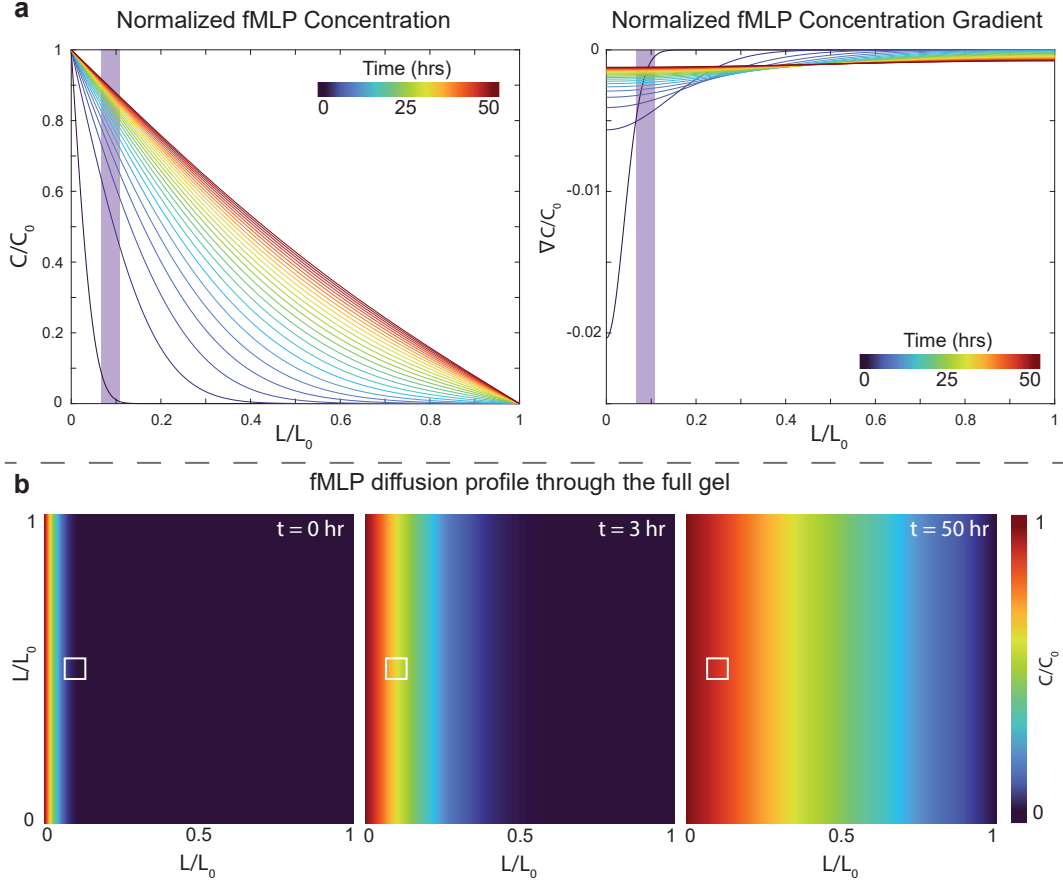


Figure 4.3: **Computational validation of the fMLP chemokine gradient.** (a) Normalized chemoattractant concentration C/C_0 and chemoattractant concentration gradient shown over time as a function of the normalized device length, L/L_0 . The location and width of the experimental field of view relative to the full length of the device, L is given by the lavender rectangle. (b) The normalized planar diffusion profile across the normalized device length, L/L_0 , shown at the 0, 4, and 50 hour timepoints as a function of the normalized chemokine concentration C/C_0 . The location and size of an experimental field of view relative to the entire gel is shown by the white square.

4.3.2 Neutrophil migration metrics

Individual neutrophil migration from large field image stacks of hundreds of cells were traced over time using Imaris software and plotted using Matlab. Individual cell migration paths

were traced over time, projected onto the x - y plane and plotted from the same origin, as shown in Fig. 4.4a. Neutrophils with no added chemokine, a uniform 10 nM fMLP concentration through the gel, and a 0 to 10 nM fMLP gradient are shown. Neutrophil migration paths in three-dimensions are shown in 4.4b. Figure 4.5 shows identical plots for neutrophils in identical activation conditions with LTB_4 in place of fMLP as the chemokine.

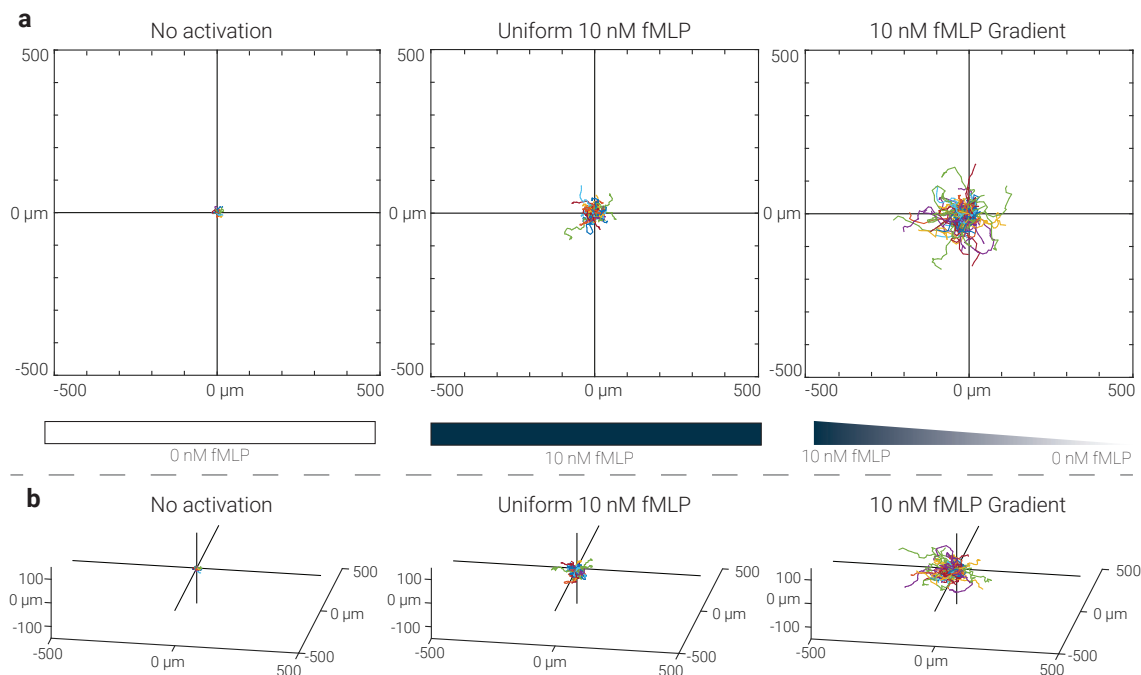


Figure 4.4: **Migration plots showing the motion of neutrophils in the device in response to fMLP.** (a) Two- and (b) three-dimensional migration paths of neutrophil in collagen hydrogels with no added chemoattractant (left column), with a universal concentration of 10 nM fMLP (center column), and with a 10 nM fMLP planar gradient introduced from the left (right column).

The mean neutrophil migration path directness, defined mathematically as $\frac{\text{net displacement}}{\text{total displacement}}$, was calculated for neutrophils under all three activation conditions for both fMLP and LTB_4 , as shown in Fig. 4.6a. Cell displacements were computed in three-dimensions, and the net and total displacement represent three-dimensional displacement magnitudes. Path directness is used here as a metric for the straightness of cellular migration in response to the chemokinesis or chemotaxis conditions: cells that are migrating along a chemokine gradient are expected to migrate in more direct paths. Figure 4.6b presents the mean neutrophil migration velocity, $\frac{\text{total displacement}}{\text{time}}$, under the same conditions, another metric for quantifying

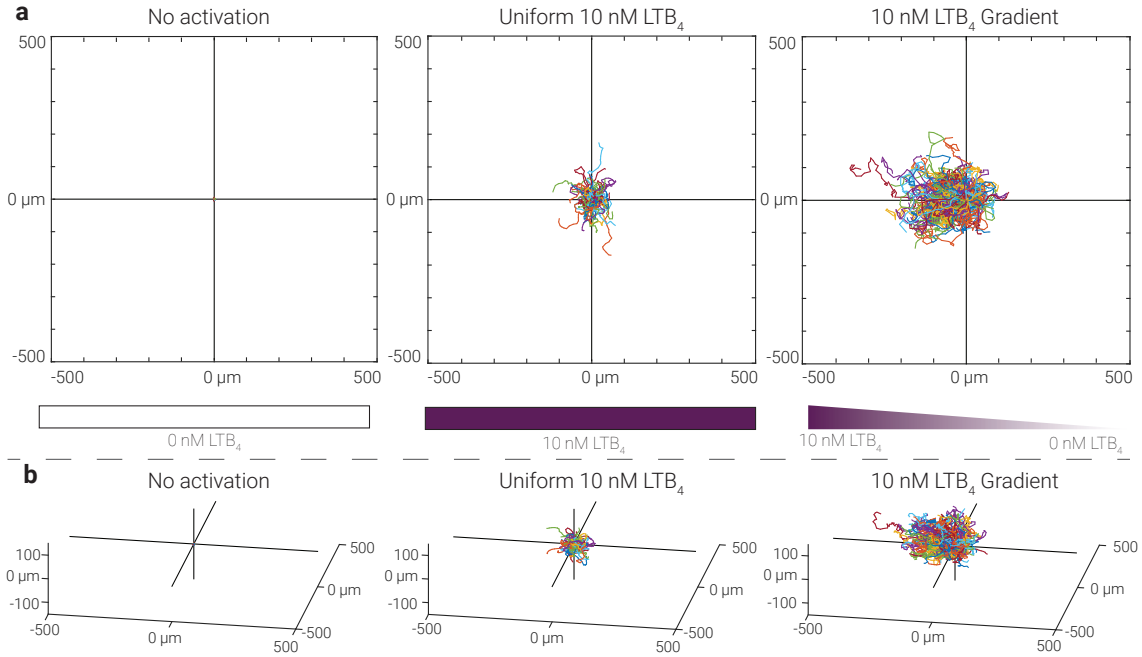


Figure 4.5: **Migration plots showing the motion of neutrophils in the device in response to LTB₄.** (a) Two- and (b) three-dimensional migration paths of neutrophil in collagen hydrogels with no added chemoattractant (left column), with a universal concentration of 10 nM LTB₄ (center column), and with a 10 nM LTB₄ planar gradient introduced from the left (right column).

neutrophil activation.

4.3.3 Neutrophil-produced three-dimensional displacement and traction fields

Volumetric images of individual neutrophils in the presence of a 10 nM fMLP gradient in the device were acquired. Cell-produced displacements were computed using TPT, and fluorescently-labeled cell borders were segmented using image thresholding. The TPT-computed displacement field was interpolated onto the cell surface, as shown in Fig. 4.7a. The full-field, three-dimensional displacements are shown in Fig. 4.7b. Next, the scattered displacement data was regularized and interpolated onto a finite element mesh generated from the segmented cell image, as shown in Fig. 4.8a. The linear finite element model was applied to compute cell-generated forces localized to the nodes of the finite element mesh at the cell surface, shown in Fig. 4.8b.

4.4 Discussion and Conclusion

The novel chemotaxis device described in this chapter represents an easily and reproducibly manufactured device, which can be straightforwardly modified or customized for any number of biological systems. The device addresses a gap in the field of chemotaxis devices by providing an affordable method to measure cell-generated tractions from within fibrous materials. Most available or published devices tend to be expensive to manufacture (e.g., using standard soft lithography techniques to build microfluidics devices) or to operate (e.g., using fluid flow control systems to establish a stable chemical gradient), or do not allow for live-cell TFM experiments. TFM experiments in fibrous hydrogels require high spatial resolution live-cell imaging, with cells conservatively 10-20 cell body lengths away from the hydrogel-device interface to account for cellular force transmission and sensing of the stiff device surfaces [13, 143, 144].

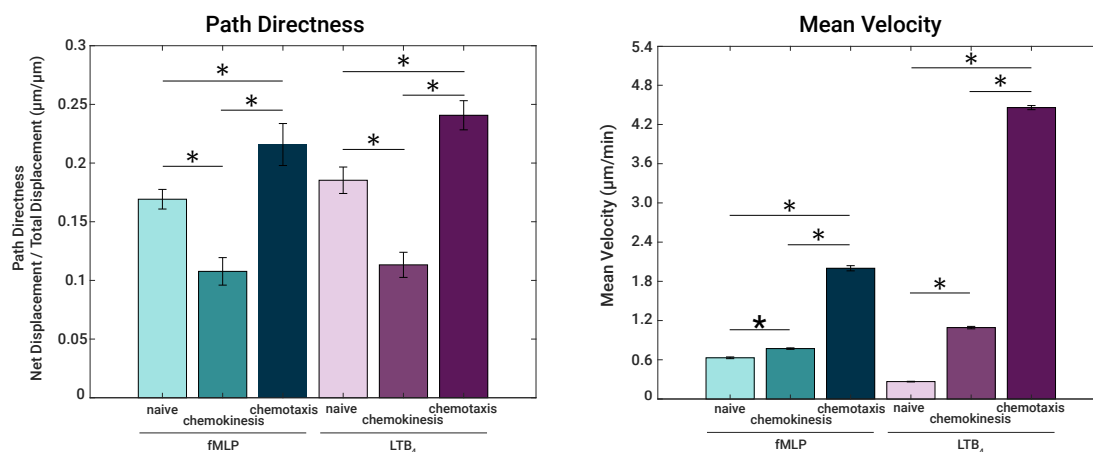


Figure 4.6: **Bar plots of the mean velocity and path directness of neutrophils in response to fMLP and LTB₄ gradients.** (a) Mean path directness, defined as the net neutrophil displacement per total displaced distance, and (b) mean velocity of neutrophils under naive or no activation conditions, universal concentration of chemokine, and exposed to planar chemokine gradient. All plots shown are means $\pm$ standard error of the mean, * indicates significance between groups of $p < 0.05$.

The device introduced here is made from PDMS cast from a 3D printed mold and requires standard, commercially available glass coverslips, making it inexpensive and simple to manufacture. The assembly of the device for TFM experiments requires polymerization

of an 18 mm by 18 mm by 870 μm thick collagen hydrogel embedded with fluorescent microbeads and fluorescently-labeled cells, and introduction of a chemokine from one or both side wells, for chemokinesis or chemotaxis experiments, respectively. The chemokine diffusion profile and concentration gradients were successfully validated using a mathematical model of the planar chemattractant diffusion through the collagen hydrogel. Neutrophil chemotaxis experiments were also run to validate that the cellular responsiveness to the established gradient, with the chemotaxis, chemokinesis, and no activation migration, velocity, and displacement path directness evaluated. Finally, the device was used to image single neutrophils to measure the cell-generated tractions produced under chemotaxis conditions.

The normalized concentration and concentration gradient profiles shown in Fig. 4.3 demonstrate that the steepest concentration gradients exist in the first few hours immediately following addition of the chemokine to the device, from approximately 0 to 4 hours. The location and width of the imaging field of view used in neutrophil live cell experiments is highlighted in a lavender rectangle in Fig. 4.3a and within a white square in Fig. 4.3b. These plots show that the imaging location captures some of the steepest concentration gradients within the gel at any given time, but particularly within the first 2 hours, when images are acquired, and highlights that the concentration and gradient do not vary appreciably within the experimental field of view during the experimental time course. This is important because there is evidence that cells, including neutrophils and neurons, respond differentially to gradients of varied steepness [189–192], and thus the cellular response within the device may be varied as well. Capturing the cellular behavior in a location with a steep gradient, but without significant variance across the field of view ensures the cells will respond to the gradient dependably but without significant variation in behavior across the given field of view. The chemokine gradient stabilizes over time; after approximately 50 hours, the gradient is no longer significantly changing with time and the device can be considered to have a steady-state chemokine concentration. Important to note as well is that this device contains two side wells through which competing chemoattractant gradient, with identical, opposing gradient profiles, could be introduced. Neutrophils *in vivo* are exposed to chemokine gradients from multiple sources (e.g., from injured tissue, from invading microbes, and from

other cells), and determining the neutrophil responsiveness to competing gradients *ex vivo* may help researchers parse out how neutrophils prioritize their response to these different signals.

The mathematical model only considered the chemokine diffusion of fMLP through collagen, neglecting to consider the diffusion of LTB₄ individually. Though the neutrophil response to an LTB₄ gradient in the device illustrated the presence of a chemokine gradient similar to that seen for fMLP validated our assumption that the diffusion profiles of LTB₄ and fMLP would be similar, further analysis could be done. The diffusion originally performed by Stout *et. al.* experimentally modelled the diffusion of fMLP through a collagen hydrogel using a fluorescent dye (rhodamine) with a similar molecular weight could be repeated for LTB₄ using fluorescein, another fluorescent molecule of similar size to LTB₄. In these experiments, the diffusion of the dye through a gel was imaged over time, and the results of the diffusion experiment were used to determine the diffusion coefficient for the given molecule and hydrogel.

The cell migration response was characterized for neutrophils in no activation, uniform chemoattractant concentration, and chemoattractant gradient conditions for fMLP and LTB₄, and the migration paths for each condition are shown in Figs. 4.4 and 4.5, respectively. These plots show a differential migration response between these three conditions, with cells migrating longer distances in the uniform concentration conditions compared to the no activation condition, and cells in the chemokine gradient condition migrating still further than cells in the uniform concentration condition. Cells in the gradient condition underwent noticeable chemotaxis toward the chemokine source. Additionally, cell migration in the no activation and uniform concentration conditions was similar in the x , y , and z directions, as shown in Figs. 4.4b and 4.5b, but in the chemokine gradient conditions, cells migrated longer distances in the x - y plane. This shows that random cell motion, as is seen with unactivated cells or cells in uniform field of chemokines (i.e., chemokine conditions) occurs uniformly in all directions. It also demonstrates that the established chemokine gradient was planar and uniform in z , as modelled. The neutrophil response under naive (no activation) conditions, chemokinesis (uniform concentration) conditions, and chemotaxis

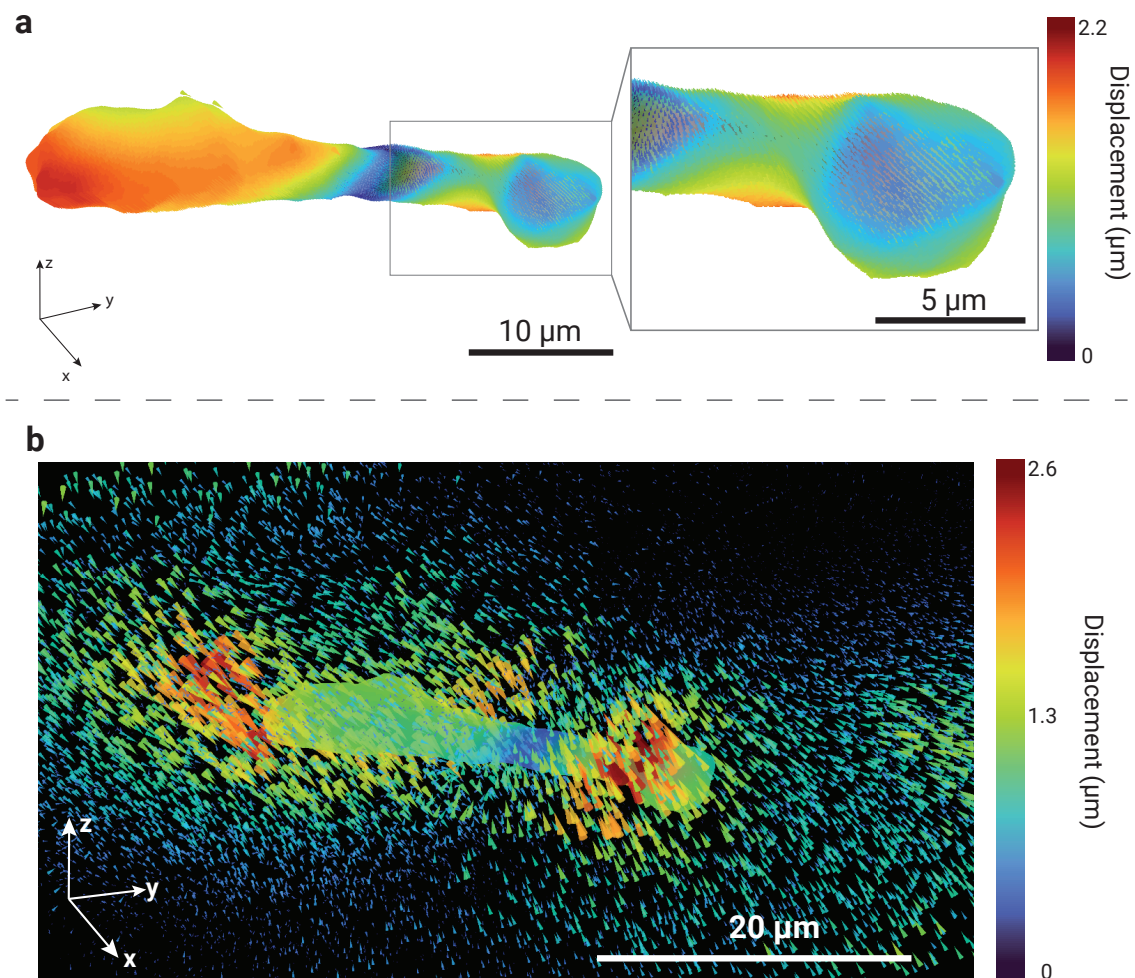


Figure 4.7: Displacement field generated by single neutrophils in a 10nM gradient of fMLP. (a) Displacement magnitude mapped onto the cell surface. A magnified view of the cell area within the gray box on the right shows the displacement direction at the cell surface using cones: the direction of the cone shows the direction of displacement, the color and size indicate the displacement magnitude. (b) The full-field, three-dimensional displacements produced by a neutrophil embedded in collagen in the presence of an fMLP gradient. Cone size and color indicate displacement magnitude, cones direction indicates displacement direction.

(chemokine gradient) conditions was further analyzed by computing the directness of the path (i.e., the path straightness) and mean velocity, shown in Fig. 4.6. Both metrics are considered to be hallmarks of neutrophil chemotaxis: cells that migrate quickly along a straight line toward the chemokine gradient. Though no statistical analysis was done, the general trends from the analysis show that the cells in chemotaxis conditions did migrate faster and along straighter paths than cells in the chemokinesis or naive states.

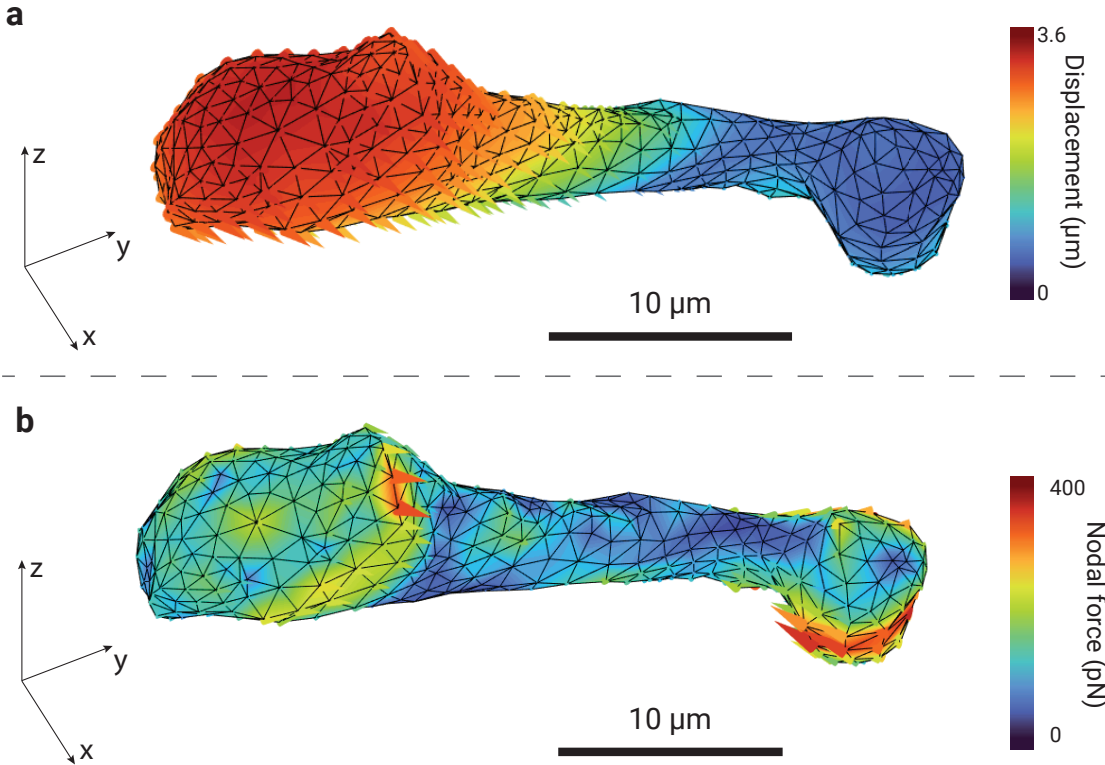


Figure 4.8: **Finite element generated displacements and nodal forces produced by single neutrophil in an fMLP gradient within a collagen hydrogel.** (a) Cell-produced displacement magnitude mapped onto the finite element cell surface mesh, with coneplot direction indicating direction of generated displacement and coneplot size and color indicating displacement magnitude. (b) Nodal force magnitude computed using FEA mapped onto the finite element cell surface mesh, with coneplot direction indicating force direction and coneplot size and color indicating force magnitude.

Cell-produced displacements were computed using topology-based single particle tracking and are shown in Fig. 4.7 as a full volumetric displacement field and interpolated onto the segmented cell surface. The neutrophil shown is fairly polarized and is producing contractile forces toward the cell body. This measured displacement field was regularized, a process which includes a native smoothing step, and then interpolated onto the finite element mesh of the cell body. These interpolated displacements are seen in Fig. 4.8a. There is some mismatch in displacement magnitude observed between the interpolated TPT results onto the cell surface (Fig. 4.7a) and these regularized results, which can be attributed to the smoothing that occurs with the regularization. Given the scattered nature of the TPT displacement results, some amount of smoothing for regularization or interpolation

onto a regularized grid or mesh (as FEA requires) is necessary and we do not consider this to be a significant source of error in our computations. The cell-produced forces, computed at the nodes of the cellular finite element mesh are shown in Fig. 4.8b. Taken together, these results demonstrate that cell-produced displacement and force fields can be straightforwardly computed from images of neutrophils under chemotaxis conditions within a collagen hydrogel.

The device introduced in this chapter has a mathematically validated chemokine gradient, is inexpensive and simple to produce, and is capable of recording live-cell imaging experiments from which tractions can be measured. Neutrophils have already been shown to respond to established chemokine gradients and produce tractions within the device, which opens the door for future neutrophil mechanosensing studies. Aside from altering the chemokines in the device or introducing competing gradients, neutrophils can be primed with various factors to study the effects on chemotaxis and traction generation, including lipopolysaccharide, which is derived from the membranes of gram-negative bacteria and has been shown to dysregulate neutrophil chemotaxis [193] or β -glucan, which is derived from fungal cell walls and has been shown to influence neutrophil chemotaxis to fMLP in two-dimensional systems [194]. Further, chemokine signals could be introduced in the form of yeast or bacteria added to the polymerizing hydrogel or to one of the wells of the device to study the direct interaction of the neutrophil with these microbes.

This device could easily be used to study other migratory cell types, including invading cancer cells, which could be added into the device well instead of directly added to the polymerizing hydrogel to probe tractions during cell invasion of hydrogels in response to a gradient introduced from the opposite well. Due in part to the simplicity of the device, modifications allowing for three-dimensional cell culture within the hydrogel would also be possible. The biggest challenge for this adaptation would be enabling sufficient nutrient diffusion, which could be done by changing the device dimensions (i.e., by creating a thinner or smaller gel). This would allow for live-cell imaging or traction force experiments to be performed within the device in the presence of growth factor or other chemical gradients. A larger degree of control over the chemokine gradient is also possible: the height of the side

wells of the device can be modified such that the hydrostatic pressure from a larger volume of fluid or from an attached, raised chemokine source on one side could be used to drive diffusion through the device. This pressure-driven diffusion could also be useful for ensuring sufficient delivery of nutrients through the device for long-term cell culture applications. These experimental suggestions barely scratch the surface of potential future applications for this device, which is easily adaptable for multiple applications and provides a simple method for measuring tractions from cells in three-dimensional matrices under chemotaxis conditions.

Chapter 5

Conclusion

5.1 Summary

The aim of this thesis is to introduce new experimental methods to aid the examination of three-dimensional neutrophil traction force generation. In Chapter 2, a means for resolving high spatial frequency three-dimensional displacements and tractions from cells using epifluorescence microscopy was introduced. This technique was tested to verify the accuracy of the displacement and traction field reconstruction over a range of experimentally applied rigid displacement conditions and synthetically applied forces ranging in spatial frequency and amplitude. The technique was used to test displacement and traction production by an amoeboid neutrophil, demonstrating its ability to capture high spatial and temporal frequency data, as well as a cluster of epithelial Madin-Darby canine kidney cells, demonstrating the technique could also be applied to study collective migration, and expanded to study cell-cell interactions. In Chapter 3, a method for measuring traction forces from neutrophils embedded in a three-dimensional fibrous collagen hydrogel was developed by adapting an existing fibrous material model to fit experimentally applied strain fields. The validation and fitting of the fibrous material model was performed at the scale of the cell from within the collagen hydrogel using embedded spherical particles that undergo a phase transition with temperature increase, contracting with a known radial force. These results showed a possible temperature dependence on collagen material behavior, which will require

additional experiments to characterize. A temporary linear material model was applied to compute surface tractions from neutrophils embedded in collagen hydrogels, though the methods for fully implementing a nonlinear fibrous model with a new thermal coefficient for describing the changes in collagen behavior with temperature, are introduced in Section 3.4. The material model was applied to compute surface tractions from neutrophils embedded within the collagen hydrogels. In Chapter 4, an experimental platform was developed for generating predictable planar chemokine gradients through three-dimensional collagen hydrogels. This new device allows for live-cell imaging and is specifically intended for use measuring cell-generated tractions. The device was made to be easily and inexpensively reproduced and modified. The functionality of the device was analyzed by producing two separate chemokine gradients and quantifying the neutrophil response, using metrics like cell migration path, directness of migration path, velocity, and cell-produced displacements and tractions.

5.2 Recommendations for Future Work

This thesis presents two methods for probing three-dimensional traction forces produced by cells. These methods could theoretically be applied to any number of cell types and any number of mechanobiology questions. In the field of neutrophil mechanobiology alone, there are many questions that could be explored using the experimental protocols and methods described. Limitations, future directions, and possible modifications to the methods developed in this thesis are presented in this section.

In Chapter 2, a method for measuring tractions from a cluster of epithelial cells is presented. One limitation of the study presented is that the cell-cell forces from this epithelial sheet were not computed. An extension of the technique presented in this chapter could include modifications for measuring cell-cell forces produced by integrating monolayer stress microscopy [126]. This technique is especially interesting to those studying wound healing, tissue growth, cancers, or any number of other epithelial cell types. For those looking to study the effects of substrate material on cell behavior, non-linear material models could

be applied using FEniCS to allow for studies to be performed on fibrous or non-isotropic materials, so long as the assumption that the cell remains on the surface of the planar substrate remains true.

In planar two-dimensional systems, neutrophil chemotaxis can be induced using simple diffusion of a chemokine from a glass micropipette tip placed near the imaging location: in this way, the neutrophil chemotaxis response could be studied in response to various chemokine gradients, on gels of a variety of stiffnesses, or with different surface coatings. Neutrophils from patients with various autoimmune disease, recovering from traumatic injuries, or affected with sepsis could also be studied to probe changes in the biomechanical response with disease states and potentially test treatment and therapy options for future clinical use. Various neutrophil activation and treatment conditions have been studied in planar two-dimensional assays using glass or plastic substrates which can be extended using our method to probe the question of the influence of substrate stiffness or coatings; these include introduction of bacteria or fungal pathogens to cell media, NETosis, clustering, and frustrated phagocytosis. It is noteworthy to point out that in order to probe some of these behaviors, modifications to the finite element framework would need to be made to account for stresses produced by numerous individual cells or cell clusters within the field of view.

The methods for the material model validation presented in Chapter 3 will be applied to one specific type of collagen hydrogel: that polymerized at 2.2 mg/mL at 32° using our exact polymerization conditions. This is a significant limitation of our work given that polymerization temperature, specific conditions (e.g., pH at polymerization or type of media used), and collagen density are known to affect collagen polymerization and ECM architecture [156, 195]. The methods presented could be applied to measure the material response of any number of collagen formulations, or for different fibrous materials (e.g., fibrin), given the user has access to a three-dimensional imaging modality like confocal or multiphoton. As detailed in Chapter 3, the change in temperature used to initiate the phase change in PNIPAAm particles during the validation experiments also affected the material behavior of the collagen: additional experiments are needed to determine thermal effects on collagen

material behavior. This will represent an interesting advancement in the current understanding of fibrous hydrogel systems, and will result in a more complete material model for future computations of cell-produced tractions. A temporary linear material model was applied to measure traction forces from individual neutrophils in collagen to demonstrate the methods presented are capable of reconstructing displacement fields and surface tractions from small, highly motile cells.

The device presented in Chapter 4 is designed to probe neutrophil mechanical responses to various chemokine signals, and could be used for studying cells from patients of various disease states, including SIRS or sepsis. In addition, many modifications to the device can be made to probe different biological questions. Competing chemokine gradients can easily be established using this device by introducing one chemokine into each of the side wells: this can be used to help inform our understanding of the hierarchy of chemokine signalling in a three-dimensional system [105]. Different bacteria or fungal pathogens could also be introduced into one well of the device, providing their own complex biochemical signalling mechanisms and invading the collagen gel. Such a system would provide a method for live cell imaging of the three-dimensional neutrophil response and associated force production during such cellular processes as phagocytosis or NETosis. Through the method introduced in the chapter, neutrophils are polymerized into the collagen gels, but interesting analysis of neutrophil force production during invasion could be observed by adding cells into one of the device wells after the collagen has been polymerized. Further, the device can easily be customized using CAD to fit different size or shape glass coverslips. Modifying the shape of the gel (e.g., by using circular coverslips to create a radial diffusion profile), could be of interest for observing migration of cells out of spheroids, or other radially symmetrical systems [34, 196–198]. In fact, creating shorter, thinner gels could also allow for different cell types to be cultured within the device for several days or weeks, provided nutrient diffusion through the gel is sufficient. The device could then be used to introduce growth factors from one or both wells to create aligned cell growth of cells like neurons or cardiomyocytes, which typically have an aligned architecture *in vivo*, or for studying the biomechanical effects of such factors or signalling molecules on cellular behavior.

Bibliography

- [1] F. Martino, A. R. Perestrelo, V. Vinarský, S. Pagliari, and G. Forte, “Cellular Mechanotransduction: From Tension to Function,” *Frontiers in Physiology*, vol. 9, p. 824, 2018.
- [2] K. Jansen, P. Atherton, and C. Ballestrem, “Mechanotransduction at the cell-matrix interface,” *Seminars in Cell & Developmental Biology*, vol. 71, pp. 75–83, 2017.
- [3] B. M. Baker and C. S. Chen, “Deconstructing the third dimension – how 3D culture microenvironments alter cellular cues,” *Journal of Cell Science*, vol. 125, no. 13, pp. 3015–3024, 2012.
- [4] B. Cheng, M. Lin, G. Huang, Y. Li, B. Ji, G. M. Genin, V. S. Deshpande, T. J. Lu, and F. Xu, “Cellular mechanosensing of the biophysical microenvironment: A review of mathematical models of biophysical regulation of cell responses,” *Physics of Life Reviews*, vol. 22, pp. 88–119, 2017.
- [5] R. J. Pelham and Y.-l. Wang, “Cell locomotion and focal adhesions are regulated by substrate flexibility,” *Proceedings of the National Academy of Sciences*, vol. 94, pp. 13661–13665, 1997.
- [6] M. Dembo and Y.-L. Wang, “Stresses at the Cell-to-Substrate Interface during Locomotion of Fibroblasts,” *Biophysical Journal*, vol. 76, pp. 2307–2316, 1999.
- [7] C.-M. Lo, H.-B. Wang, M. Dembo, and Y.-l. Wang, “Cell Movement Is Guided by the Rigidity of the Substrate,” *Biophysical Journal*, vol. 79, pp. 144–152, 2000.

- [8] D. E. Discher, P. Janmey, and Y.-l. Wang, "Tissue Cells Feel and Respond to the Stiffness of Their Substrate," *Science*, vol. 310, no. 5751, pp. 1139–1143, 2005.
- [9] A. J. Engler, S. Sen, H. L. Sweeney, and D. E. Discher, "Matrix Elasticity Directs Stem Cell Lineage Specification," *Cell*, vol. 126, pp. 677–689, 2006.
- [10] V. Vogel and M. Sheetz, "Local force and geometry sensing regulate cell functions," *Nature Reviews Molecular Cell Biology*, vol. 7, no. 4, pp. 265–275, 2006.
- [11] P. Oakes, D. Patel, N. Morin, D. Zitterbart, B. Fabry, J. Reichner, and J. Tang, "Neutrophil morphology and migration are affected by substrate elasticity," *Blood*, vol. 114, pp. 1387–1395, 2009.
- [12] M. S. Hall, R. Long, C.-Y. Hui, and M. Wu, "Mapping three-dimensional stress and strain fields within a soft hydrogel using a fluorescence microscope," *Biophysical journal*, vol. 102, no. 10, pp. 2241–2250, 2012.
- [13] X. Ma, M. E. Schickel, M. Stevenson, A. Sarang-Sieminski, K. Gooch, S. Ghadiali, and R. Hart, "Fibers in the Extracellular Matrix Enable Long-Range Stress Transmission between Cells," *Biophysical Journal*, vol. 104, no. 7, pp. 1410–1418, 2013.
- [14] M. S. Hall, F. Alisafaei, E. Ban, X. Feng, C.-Y. Hui, V. B. Shenoy, and M. Wu, "Fibrous nonlinear elasticity enables positive mechanical feedback between cells and ECMs," *Proceedings of the National Academy of Sciences*, vol. 113, no. 49, pp. 14043–14048, 2016.
- [15] J. Wolff, "Das Gesetz der Transformation der Knochen, (berlin: Verlag von august hirschwald)," 1892.
- [16] P. F. Davies, A. Remuzzi, E. J. Gordon, C. F. Dewey, and M. A. Gimbrone, "Turbulent fluid shear stress induces vascular endothelial cell turnover in vitro," *Proceedings of the National Academy of Sciences*, vol. 83, no. 7, pp. 2114–2117, 1986.
- [17] M. F. Coughlin, D. D. Sohn, and G. W. Schmid-Schönbein, "Recoil and Stiffening by Adherent Leukocytes in Response to Fluid Shear," *Biophysical Journal*, vol. 94, no. 3, pp. 1046–1051, 2008.

- [18] C. A. Reinhart-King, M. Dembo, and D. A. Hammer, “Cell-cell mechanical communication through compliant substrates,” *Biophysical journal*, vol. 95, no. 12, pp. 6044–51, 2008.
- [19] X. Trepap, M. R. Wasserman, T. E. Angelini, E. Millet, D. A. Weitz, J. P. Butler, and J. J. Fredberg, “Physical forces during collective cell migration (SUPPLEMENTAL),” *Nature Physics*, vol. 5, no. 6, pp. 426–430, 2009.
- [20] D. T. Tambe, C. C. Hardin, T. E. Angelini, K. Rajendran, C. Y. Park, X. Serra-Picamal, E. H. Zhou, M. H. Zaman, J. P. Butler, D. A. Weitz, J. J. Fredberg, and X. Trepap, “Collective cell guidance by cooperative intercellular forces,” *Nature Materials*, vol. 10, pp. 469–475, 2011.
- [21] M. P. Sheetz, D. P. Felsenfeld, and C. G. Galbraith, “Cell migration: regulation of force on extracellular-matrix-integrin complexes,” *Trends in Cell Biology*, vol. 8, no. 2, pp. 51–54, 1998.
- [22] G. C. Reilly and A. J. Engler, “Intrinsic extracellular matrix properties regulate stem cell differentiation,” *Journal of Biomechanics*, vol. 43, no. 1, pp. 55–62, 2010.
- [23] G. E. Davis and C. W. Camarillo, “Regulation of Endothelial Cell Morphogenesis by Integrins, Mechanical Forces, and Matrix Guidance Pathways,” *Experimental Cell Research*, vol. 216, no. 1, pp. 113–123, 1995.
- [24] C. Okimura, Y. Sakumura, K. Shimabukuro, and Y. Iwadate, “Sensing of substratum rigidity and directional migration by fast-crawling cells,” *Physical Review E*, vol. 97, no. 5, p. 052401, 2018.
- [25] W. J. Hadden, J. L. Young, A. W. Holle, M. L. McFetridge, D. Y. Kim, P. Wijesinghe, H. Taylor-Weiner, J. H. Wen, A. R. Lee, K. Bieback, B.-N. Vo, D. D. Sampson, B. F. Kennedy, J. P. Spatz, A. J. Engler, and Y. S. Choi, “Stem cell migration and mechanotransduction on linear stiffness gradient hydrogels,” *Proceedings of the National Academy of Sciences*, vol. 114, no. 22, pp. 5647–5652, 2017.

- [26] C.-P. Heisenberg and Y. Bellaïche, “Forces in tissue morphogenesis and patterning,” *Cell*, vol. 153, no. 5, pp. 948–62, 2013.
- [27] I. Indra, V. Undyala, C. Kadow, U. Thirumurthi, M. Dembo, and K. A. Beningo, “An in vitro correlation of mechanical forces and metastatic capacity,” *Physical Biology*, vol. 8, p. 015015, 2011.
- [28] J. T. Yang, H. Rayburn, and R. O. Hynes, “Cell adhesion events mediated by alpha 4 integrins are essential in placental and cardiac development,” *Development (Cambridge, England)*, vol. 121, pp. 549–60, 1995.
- [29] A. J. Engler, C. Carag-Krieger, C. P. Johnson, M. Raab, H.-Y. Tang, D. W. Speicher, J. W. Sanger, J. M. Sanger, and D. E. Discher, “Embryonic cardiomyocytes beat best on a matrix with heart-like elasticity: scar-like rigidity inhibits beating,” *Journal of Cell Science*, vol. 121, no. 22, pp. 3794–3802, 2008.
- [30] J. Y. Ji, H. Jing, and S. L. Diamond, “Hemodynamic Regulation of Inflammation at the Endothelial–Neutrophil Interface,” *Annals of Biomedical Engineering*, vol. 36, no. 4, pp. 586–595, 2008.
- [31] T. M. Koch, S. Münster, N. Bonakdar, J. P. Butler, and B. Fabry, “3D Traction Forces in Cancer Cell Invasion,” *PLoS ONE*, vol. 7, p. e33476, 2012.
- [32] M. Plodinec and C.-A. Schoenenberger, “Spatial organization acts on cell signaling: how physical force contributes to the development of cancer,” *Breast cancer research : BCR*, vol. 12, no. 4, p. 308, 2010.
- [33] S. Kumar and V. M. Weaver, “Mechanics, malignancy, and metastasis: The force journey of a tumor cell,” *Cancer and Metastasis Reviews*, vol. 28, no. 1-2, pp. 113–127, 2009.
- [34] S. E. Leggett, M. Patel, T. M. Valentin, L. Gamboa, A. S. Khoo, E. K. Williams, C. Franck, and I. Y. Wong, “Mechanophenotyping of 3D multicellular clusters using displacement arrays of rendered tractions,” *Proceedings of the National Academy of Sciences*, vol. 117, no. 11, pp. 5655–5663, 2020.

- [35] C. Hahn and M. A. Schwartz, “The Role of Cellular Adaptation to Mechanical Forces in Atherosclerosis,” *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 28, no. 12, pp. 2101–2107, 2008.
- [36] J.-J. Chiu, S. Usami, and S. Chien, “Vascular endothelial responses to altered shear stress: Pathologic implications for atherosclerosis,” *Annals of Medicine*, vol. 41, no. 1, pp. 19–28, 2009.
- [37] B. J. Pfister, T. P. Weihs, M. Betenbaugh, and G. Bao, “An In Vitro Uniaxial Stretch Model for Axonal Injury,” *Annals of Biomedical Engineering*, vol. 31, no. 5, pp. 589–598, 2003.
- [38] E. Bar-Kochba, M. T. Scimone, J. B. Estrada, and C. Franck, “Strain and rate-dependent neuronal injury in a 3D in vitro compression model of traumatic brain injury,” *Scientific Reports*, vol. 6, no. 1, p. 30550, 2016.
- [39] W. R. Legant, C. K. Choi, J. S. Miller, L. Shao, L. Gao, E. Betzig, and C. S. Chen, “Multidimensional traction force microscopy reveals out-of-plane rotational moments about focal adhesions,” *Proceedings of the National Academy of Sciences*, vol. 110, no. 3, pp. 881–886, 2013.
- [40] C. López-Fagundo, E. Bar-Kochba, L. L. Livi, D. Hoffman-Kim, and C. Franck, “Three-dimensional traction forces of Schwann cells on compliant substrates,” *Journal of The Royal Society Interface*, vol. 11, no. 97, p. 20140247, 2014.
- [41] A. D. Doyle, M. L. Kutys, M. A. Conti, K. Matsumoto, R. S. Adelstein, and K. M. Yamada, “Micro-environmental control of cell migration – myosin IIA is required for efficient migration in fibrillar environments through control of cell adhesion dynamics,” *Journal of Cell Science*, vol. 125, no. 9, pp. 2244–2256, 2012.
- [42] S. I. Fraley, Y. Feng, R. Krishnamurthy, D.-H. Kim, A. Celedon, G. D. Longmore, and D. Wirtz, “A distinctive role for focal adhesion proteins in three-dimensional cell motility,” *Nature Cell Biology*, vol. 12, no. 6, pp. 598–604, 2010.

- [43] C. Grashoff, B. D. Hoffman, M. D. Brenner, R. Zhou, M. Parsons, M. T. Yang, M. A. McLean, S. G. Sligar, C. S. Chen, T. Ha, and M. A. Schwartz, “Measuring mechanical tension across vinculin reveals regulation of focal adhesion dynamics,” *Nature*, vol. 466, no. 7303, pp. 263–266, 2010.
- [44] Z. S. Wilson, H. Witt, L. Hazlett, M. Harman, B. M. Neumann, A. Whitman, M. Patel, R. S. Ross, C. Franck, J. S. Reichner, and C. T. Lefort, “Context-Dependent Role of Vinculin in Neutrophil Adhesion, Motility and Trafficking,” *Scientific Reports*, vol. 10, no. 1, p. 2142, 2020.
- [45] A. Harris, P. Wild, and D. Stopak, “Silicone rubber substrata: a new wrinkle in the study of cell locomotion,” *Science*, vol. 208, no. 4440, pp. 177–179, 1980.
- [46] J. Fu, Y.-K. Wang, M. T. Yang, R. A. Desai, X. Yu, Z. Liu, and C. S. Chen, “Mechanical regulation of cell function with geometrically modulated elastomeric substrates,” *Nature Methods*, vol. 7, no. 9, pp. 733–736, 2010.
- [47] L. Trichet, J. L. Digabel, R. J. Hawkins, S. R. K. Vedula, M. Gupta, C. Ribault, P. Hersen, R. Voituriez, and B. Ladoux, “Evidence of a large-scale mechanosensing mechanism for cellular adaptation to substrate stiffness,” *Proceedings of the National Academy of Sciences*, vol. 109, no. 18, pp. 6933–6938, 2012.
- [48] W. H. Zimmermann, C. Fink, D. Kralisch, U. Remmers, J. Weil, and T. Eschenhagen, “Three-dimensional engineered heart tissue from neonatal rat cardiac myocytes,” *Biotechnology and bioengineering*, vol. 68, no. 1, pp. 106–114, 2000.
- [49] J. L. Tan, J. Tien, D. M. Pirone, D. S. Gray, K. Bhadriraju, and C. S. Chen, “Cells lying on a bed of microneedles: An approach to isolate mechanical force,” *Proceedings of the National Academy of Sciences*, vol. 100, no. 4, pp. 1484–1489, 2003.
- [50] H. Vandenburgh, J. Shansky, F. Benesch-Lee, V. Barbata, J. Reid, L. Thorrez, R. Valentini, and G. Crawford, “Drug-screening platform based on the contractility of tissue-engineered muscle,” *Muscle & Nerve*, vol. 37, no. 4, pp. 438–447, 2008.

- [51] W. R. Legant, A. Pathak, M. T. Yang, V. S. Deshpande, R. M. McMeeking, and C. S. Chen, “Microfabricated tissue gauges to measure and manipulate forces from 3D microtissues,” *Proceedings of the National Academy of Sciences*, vol. 106, no. 25, pp. 10097–10102, 2009.
- [52] S. Ghassemi, G. Meacci, S. Liu, A. A. Gondarenko, A. Mathur, P. Roca-Cusachs, M. P. Sheetz, and J. Hone, “Cells test substrate rigidity by local contractions on submicrometer pillars,” *Proceedings of the National Academy of Sciences*, vol. 109, no. 14, pp. 5328–5333, 2012.
- [53] W. J. Polacheck and C. S. Chen, “Measuring cell-generated forces: a guide to the available tools,” *Nature Methods*, vol. 13, no. 5, pp. 415–423, 2016.
- [54] A. Ganz, M. Lambert, A. Saez, P. Silberzan, A. Buguin, R. M. Mège, and B. Ladoux, “Traction forces exerted through N-cadherin contacts,” *Biology of the Cell*, vol. 98, no. 12, pp. 721–730, 2006.
- [55] Z. Liu, J. L. Tan, D. M. Cohen, M. T. Yang, N. J. Sniadecki, S. A. Ruiz, C. M. Nelson, and C. S. Chen, “Mechanical tugging force regulates the size of cell-cell junctions,” *Proceedings of the National Academy of Sciences*, vol. 107, no. 22, pp. 9944–9949, 2010.
- [56] V. Maruthamuthu, B. Sabass, U. S. Schwarz, and M. L. Gardel, “Cell-ECM traction force modulates endogenous tension at cell–cell contacts,” *Proceedings of the National Academy of Sciences*, vol. 108, no. 12, pp. 4708–4713, 2011.
- [57] J. P. Butler, I. Tolić-Nørrelykke, B. Fabry, and J. J. Fredberg, “Traction fields, moments, and strain energy that cells exert on their surroundings,” *American Journal of Physiology - Cell Physiology*, vol. 282, pp. C595–C605, 2002.
- [58] S. Hur, Y. Zhao, Y.-S. Li, E. Botvinick, and S. Chien, “Live Cells Exert 3-Dimensional Traction Forces on Their Substrata,” *Cellular and Molecular Bioengineering*, vol. 2, pp. 425–436, 2009.

- [59] C. Franck, S. A. Maskarinec, D. A. Tirrell, and G. Ravichandran, “Three-Dimensional Traction Force Microscopy: A New Tool for Quantifying Cell-Matrix Interactions,” *PLoS ONE*, vol. 6, no. 3, p. e17833, 2011.
- [60] M. Zündel, A. E. Ehret, and E. Mazza, “Factors influencing the determination of cell traction forces,” *PLOS ONE*, vol. 12, no. 2, p. e0172927, 2017.
- [61] T. Ando, S. P. Bhamidimarri, N. Brending, H. Colin-York, L. Collinson, N. De Jonge, P. De Pablo, E. Debroye, C. Eggeling, C. Franck, *et al.*, “The 2018 correlative microscopy techniques roadmap,” *Journal of physics D: Applied physics*, vol. 51, no. 44, p. 443001, 2018.
- [62] U. S. Schwarz and J. R. Soiné, “Traction force microscopy on soft elastic substrates: A guide to recent computational advances,” *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, vol. 1853, no. 11, pp. 3095–3104, 2015.
- [63] R. W. Style, R. Boltianskiy, G. K. German, C. Hyland, C. W. MacMinn, A. F. Mertz, L. A. Wilen, Y. Xu, and E. R. Dufresne, “Traction force microscopy in physics and biology,” *Soft Matter*, vol. 10, no. 23, pp. 4047–4055, 2014.
- [64] D. A. Stout, E. Bar-Kochba, J. B. Estrada, J. Toyjanova, H. Kesari, J. S. Reichner, and C. Franck, “Mean deformation metrics for quantifying 3d cell–matrix interactions without requiring information about matrix material properties,” *Proceedings of the National Academy of Sciences*, vol. 113, no. 11, pp. 2898–2903, 2016.
- [65] C. Franck, S. Hong, S. Maskarinec, D. Tirrell, and G. Ravichandran, “Three-dimensional Full-field Measurements of Large Deformations in Soft Materials Using Confocal Microscopy and Digital Volume Correlation,” *Experimental Mechanics*, vol. 47, pp. 427–438, 2007.
- [66] S. A. Maskarinec, C. Franck, D. A. Tirrell, and G. Ravichandran, “Quantifying cellular traction forces in three dimensions,” *Proceedings of the National Academy of Sciences*, vol. 106, pp. 22108–22113, 2009.

- [67] J. Steinwachs, C. Metzner, K. Skodzek, N. Lang, I. Thievessen, C. Mark, S. Münster, K. E. Aifantis, and B. Fabry, “Three-dimensional force microscopy of cells in biopolymer networks,” *Nature Methods*, vol. 13, no. 2, pp. 171–176, 2016.
- [68] D. Song, L. Dong, M. Gupta, L. Li, O. Klaas, A. Loghin, M. Beall, C. Chen, and A. A. Oberai, “Recovery of Traction Exerted by Single Cells in 3D Nonlinear Matrices,” *Journal of Biomechanical Engineering*, 2020.
- [69] N. Q. Balaban, U. S. Schwarz, D. Riveline, P. Goichberg, G. Tzur, I. Sabanay, D. Mahalu, S. Safran, A. Bershadsky, L. Addadi, and B. Geiger, “Force and focal adhesion assembly: a close relationship studied using elastic micropatterned substrates,” *Nature Cell Biology*, vol. 3, pp. 466–472, 2001.
- [70] M. Bergert, T. Lendenmann, M. Zündel, A. E. Ehret, D. Panozzo, P. Richner, D. K. Kim, S. J. P. Kress, D. J. Norris, O. Sorkine-Hornung, E. Mazza, D. Poulikakos, and A. Ferrari, “Confocal reference free traction force microscopy,” *Nature Communications*, vol. 7, p. 12814, 2016.
- [71] O. A. Banda, C. R. Sabanayagam, and J. H. Slater, “Reference-Free Traction Force Microscopy Platform Fabricated via Two-Photon Laser Scanning Lithography Enables Facile Measurement of Cell-Generated Forces,” *ACS Applied Materials & Interfaces*, vol. 11, no. 20, pp. 18233–18241, 2019.
- [72] M. Patel, S. Leggett, A. Landauer, I. Wong, and C. Franck, “Rapid, topology-based particle tracking for high-resolution measurements of large complex 3d motion fields,” *Scientific Reports*, vol. 8, no. 1, p. 5581, 2018.
- [73] E. Bar-Kochba, J. Toyjanova, E. Andrews, K.-S. Kim, and C. Franck, “A Fast Iterative Digital Volume Correlation Algorithm for Large Deformations,” *Experimental Mechanics*, vol. 55, pp. 261–274, 2015.
- [74] A. K. Landauer, M. Patel, D. L. Henann, and C. Franck, “A q-factor-based digital image correlation algorithm (qdic) for resolving finite deformations with degenerate speckle patterns,” *Experimental Mechanics*, vol. 58, pp. 815–830, Jun 2018.

- [75] J. Yang, L. Hazlett, A. K. Landauer, and C. Franck, “Augmented Lagrangian digital volume correlation,” *Experimental Mechanics*, pp. 1–17, 2020.
- [76] M. Proestaki, A. Ogren, B. Burkel, and J. Notbohm, “Modulus of Fibrous Collagen at the Length Scale of a Cell,” *Experimental Mechanics*, vol. 59, no. 9, pp. 1323–1334, 2019.
- [77] L. Dong and A. A. Oberai, “Recovery of cellular traction in three-dimensional nonlinear hyperelastic matrices,” *Computer Methods in Applied Mechanics and Engineering*, vol. 314, pp. 296–313, 2017.
- [78] M. C ndor, J. Steinwachs, C. Mark, J. Garc a-Aznar, and B. Fabry, “Traction Force Microscopy in 3-Dimensional Extracellular Matrix Networks,” *Current Protocols in Cell Biology*, vol. 75, no. 1, pp. 10.22.1–10.22.20, 2017.
- [79] P. Rosakis, J. Notbohm, and G. Ravichandran, “A model for compression-weakening materials and the elastic fields due to contractile cells,” *Journal of the Mechanics and Physics of Solids*, vol. 85, pp. 16–32, 2015.
- [80] J. C. d.  lamo, R. Meili, B.  lvarez Gonz lez, B. Alonso-Latorre, E. Bastounis, R. Firtel, and J. C. Lasheras, “Three-Dimensional Quantification of Cellular Traction Forces and Mechanosensing of Thin Substrata by Fourier Traction Force Microscopy,” *PLoS ONE*, vol. 8, p. e69850, 2013.
- [81] W. R. Legant, J. S. Miller, B. L. Blakely, D. M. Cohen, G. M. Genin, and C. S. Chen, “Measurement of mechanical tractions exerted by cells in three-dimensional matrices,” *Nature Methods*, vol. 7, pp. 969–971, 2010.
- [82] Z. Yang, J.-S. Lin, J. Chen, and J. Wang, “Determining substrate displacement and cell traction fields—a new approach,” *Journal of Theoretical Biology*, vol. 242, pp. 607–616, 2006.
- [83] D. Ambrosi, “Cellular Traction as an Inverse Problem,” *SIAM Journal on Applied Mathematics*, vol. 66, pp. 2049–2060, 2006.

- [84] J. Toyjanova, E. Bar-Kochba, C. López-Fagundo, J. Reichner, D. Hoffman-Kim, and C. Franck, “High Resolution, Large Deformation 3D Traction Force Microscopy,” *PLoS ONE*, vol. 9, p. e90976, 2014.
- [85] L. O. Carvalho, E. N. Aquino, A. C. D. Neves, and W. Fontes, “The Neutrophil Nucleus and Its Role in Neutrophilic Function,” *Journal of cellular biochemistry*, vol. 116, no. 9, pp. 1831–6, 2015.
- [86] B. Amulic, C. Cazalet, G. L. Hayes, K. D. Metzler, and A. Zychlinsky, “Neutrophil Function: From Mechanisms to Disease,” *Annual Review of Immunology*, vol. 30, no. 1, pp. 459–489, 2012.
- [87] T. N. Mayadas, X. Cullere, and C. A. Lowell, “The Multifaceted Functions of Neutrophils,” *Annual Review of Pathology: Mechanisms of Disease*, vol. 9, no. 1, pp. 181–218, 2014.
- [88] C. Rosales, “Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types?,” *Frontiers in Physiology*, vol. 9, p. 113, 2018.
- [89] V. Brinkmann, U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D. S. Weiss, Y. Weinrauch, and A. Zychlinsky, “Neutrophil Extracellular Traps Kill Bacteria,” *Science*, vol. 303, no. 5663, pp. 1532–1535, 2004.
- [90] E. Kolaczowska and P. Kubes, “Neutrophil recruitment and function in health and inflammation,” *Nature Reviews Immunology*, vol. 13, no. 3, p. 159, 2013.
- [91] G. T. Nguyen, E. R. Green, and J. Mecsas, “Neutrophils to the ROScUE: Mechanisms of NADPH Oxidase Activation and Bacterial Resistance,” *Frontiers in Cellular and Infection Microbiology*, vol. 7, p. 373, 2017.
- [92] C. Dahlgren and A. Karlsson, “Respiratory burst in human neutrophils,” *Journal of Immunological Methods*, vol. 232, no. 1-2, pp. 3–14, 1999.
- [93] R. T. A. Megens, S. Vijayan, D. Lievens, Y. Döring, M. A. M. J. v. Zandvoort, J. Grommes, C. Weber, and O. Soehnlein, “Presence of luminal neutrophil extracellular

- traps in atherosclerosis,” *Thrombosis and Haemostasis*, vol. 107, no. 03, pp. 597–598, 2012.
- [94] J. S. Knight and M. J. Kaplan, “Lupus neutrophils: ‘NET’ gain in understanding lupus pathogenesis,” *Current Opinion in Rheumatology*, vol. 24, no. 5, pp. 441–450, 2012.
- [95] T. A. Fuchs, A. Brill, D. Duerschmied, D. Schatzberg, M. Monestier, D. D. Myers, S. K. Wroblewski, T. W. Wakefield, J. H. Hartwig, and D. D. Wagner, “Extracellular DNA traps promote thrombosis,” *Proceedings of the National Academy of Sciences*, vol. 107, no. 36, pp. 15880–15885, 2010.
- [96] V. Marcos, Z. Zhou, A. O. Yildirim, A. Bohla, A. Hector, L. Vitkov, E.-M. Wiedenbauer, W. D. Krautgartner, W. Stoiber, B. H. Belohradsky, N. Rieber, M. Kormann, B. Koller, A. Roscher, D. Roos, M. Griese, O. Eickelberg, G. Döring, M. A. Mall, and D. Hartl, “CXCR2 mediates NADPH oxidase-independent neutrophil extracellular trap formation in cystic fibrosis airway inflammation,” *Nature Medicine*, vol. 16, no. 9, pp. 1018–1023, 2010.
- [97] S. d. Oliveira, E. E. Rosowski, and A. Huttenlocher, “Neutrophil migration in infection and wound repair: going forward in reverse,” *Nature Reviews Immunology*, vol. 16, no. 6, pp. 378–391, 2016.
- [98] Z. G. Fridlender, J. Sun, S. Kim, V. Kapoor, G. Cheng, L. Ling, G. S. Worthen, and S. M. Albelda, “Polarization of Tumor-Associated Neutrophil Phenotype by TGF- β : “N1” versus “N2” TAN,” *Cancer Cell*, vol. 16, no. 3, pp. 183–194, 2009.
- [99] J. Hughes, R. J. Johnson, A. Mooney, C. Hugo, K. Gordon, and J. Savill, “Neutrophil Fate in Experimental Glomerular Capillary Injury in the Rat. Emigration Exceeds in Situ Clearance by Apoptosis,” *American Journal of Pathology*, vol. 150, no. 1, pp. 223–234, 1997.

- [100] J. R. Mathias, B. J. Perrin, T. Liu, J. Kanki, A. T. Look, and A. Huttenlocher, "Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish," *Journal of Leukocyte Biology*, vol. 80, no. 6, pp. 1281–1288, 2006.
- [101] J. Wang, M. Hossain, A. Thanabalasuriar, M. Gunzer, C. Meininger, and P. Kubes, "Visualizing the function and fate of neutrophils in sterile injury and repair," *Science*, vol. 358, no. 6359, pp. 111–116, 2017.
- [102] B. Hamza and D. Irimia, "Whole blood human neutrophil trafficking in a microfluidic model of infection and inflammation," *Lab on a Chip*, vol. 15, no. 12, pp. 2625–2633, 2015.
- [103] L. Gambardella and S. Vermeren, "Molecular players in neutrophil chemotaxis—focus on PI3K and small GTPases," *Journal of Leukocyte Biology*, vol. 94, no. 4, pp. 603–612, 2013.
- [104] B. Petri and M.-J. Sanz, "Neutrophil chemotaxis," *Cell and Tissue Research*, vol. 371, no. 3, pp. 425–436, 2018.
- [105] D. Kim and C. L. Haynes, "Neutrophil chemotaxis within a competing gradient of chemoattractants.," *Analytical chemistry*, vol. 84, no. 14, pp. 6070–8, 2012.
- [106] K. Brown, S. Brain, J. Pearson, J. Edgeworth, Lewis, and D. Treacher, "Neutrophils in development of multiple organ failure in sepsis," *The Lancet*, vol. 368, no. 9530, pp. 157–169, 2006.
- [107] C. Brun-Buisson, "The epidemiology of the systemic inflammatory response," *Intensive Care Medicine*, vol. 26, no. S1, pp. S064–S074, 2000.
- [108] G. Drifte, I. Dunn-Siegrist, P. Tissières, and J. Pugin, "Innate Immune Functions of Immature Neutrophils in Patients With Sepsis and Severe Systemic Inflammatory Response Syndrome," *Critical Care Medicine*, vol. 41, no. 3, pp. 820–832, 2013.
- [109] R. C. Reddy and T. J. Standiford, "Effects of sepsis on neutrophil chemotaxis," *Current Opinion in Hematology*, vol. 17, no. 1, pp. 18–24, 2010.

- [110] C. N. Jones, M. Moore, L. Dimisko, A. Alexander, A. Ibrahim, B. A. Hassell, H. S. Warren, R. G. Tompkins, S. P. Fagan, and D. Irimia, “Spontaneous Neutrophil Migration Patterns during Sepsis after Major Burns,” *PLoS ONE*, vol. 9, no. 12, p. e114509, 2014.
- [111] T. Butler and J. Levin, *Sepsis and Septic Shock: A Review of Definitions, Pathogenesis, and Treatment*, pp. 807–835. Cham: Springer International Publishing, 2019.
- [112] D. Annane, E. Bellissant, P. E. Bollaert, J. Briegel, D. Keh, Y. Kupfer, R. Pirracchio, and B. Rochwerg, “Corticosteroids for treating sepsis in children and adults,” *Cochrane Database of Systematic Reviews*, vol. 12, no. 12, p. CD002243, 2019.
- [113] G. U. Meduri, “An Historical Review of Glucocorticoid Treatment in Sepsis. Disease Pathophysiology and the Design of Treatment Investigation,” *Sepsis*, vol. 3, no. 1, pp. 21–38, 1999.
- [114] M. Paul, V. Shani, E. Muchtar, G. Kariv, E. Robenshtok, and L. Leibovici, “Systematic Review and Meta-Analysis of the Efficacy of Appropriate Empiric Antibiotic Therapy for Sepsis,” *Antimicrobial Agents and Chemotherapy*, vol. 54, no. 11, pp. 4851–4863, 2010.
- [115] R. A. Jannat, G. P. Robbins, B. G. Ricart, M. Dembo, and D. A. Hammer, “Neutrophil adhesion and chemotaxis depend on substrate mechanics,” *Journal of Physics: Condensed Matter*, vol. 22, no. 19, p. 194117, 2010.
- [116] L. A. Smith, H. Aranda-Espinoza, J. B. Haun, M. Dembo, and D. A. Hammer, “Neutrophil Traction Stresses are Concentrated in the Uropod during Migration,” *Biophysical Journal*, vol. 92, pp. L58–L60, 2007.
- [117] R. Jannat, M. Dembo, and D. Hammer, “Traction Forces of Neutrophils Migrating on Compliant Substrates,” *Biophysical Journal*, vol. 101, no. 3, pp. 575–584, 2011.
- [118] J. Toyjanova, E. Flores-Cortez, J. S. Reichner, and C. Franck, “Matrix confinement plays a pivotal role in regulating neutrophil-generated tractions, speed, and integrin utilization,” *Journal of Biological Chemistry*, vol. 290, no. 6, pp. 3752–3763, 2015.

- [119] T. Lämmermann, B. L. Bader, S. J. Monkley, T. Worbs, R. Wedlich-Söldner, K. Hirsch, M. Keller, R. Förster, D. R. Critchley, R. Fässler, and M. Sixt, “Rapid leukocyte migration by integrin-independent flowing and squeezing,” *Nature*, vol. 453, no. 7191, pp. 51–55, 2008.
- [120] T. Lämmermann and M. Sixt, “Mechanical modes of ‘amoeboid’ cell migration,” *Current Opinion in Cell Biology*, vol. 21, no. 5, pp. 636–644, 2009.
- [121] K. M. Stroka, H. N. Hayenga, and H. Aranda-Espinoza, “Human Neutrophil Cytoskeletal Dynamics and Contractility Actively Contribute to Trans-Endothelial Migration,” *PLoS ONE*, vol. 8, no. 4, p. e61377, 2013.
- [122] V. A. Morikis and S. I. Simon, “Neutrophil Mechanosignaling Promotes Integrin Engagement With Endothelial Cells and Motility Within Inflamed Vessels,” *Frontiers in immunology*, vol. 9, 2018.
- [123] S. A. Freeman and S. Grinstein, “Phagocytosis: Mechanosensing, Traction Forces, and a Molecular Clutch,” *Current Biology*, vol. 30, no. 1, pp. R24–R26, 2020.
- [124] A. N. Hoang, C. N. Jones, L. Dimisko, B. Hamza, J. Martel, N. Kojic, and D. Irimia, “Measuring neutrophil speed and directionality during chemotaxis, directly from a droplet of whole blood,” *TECHNOLOGY*, vol. 01, no. 01, pp. 49–57, 2013.
- [125] V. Maruthamuthu, B. Sabass, U. S. Schwarz, and M. L. Gardel, “Cell-ECM traction force modulates endogenous tension at cell–cell contacts,” *Proceedings of the National Academy of Sciences*, vol. 108, pp. 4708–4713, 2011.
- [126] R. Serrano, A. Aung, Y.-T. Yeh, S. Varghese, J. C. Lasheras, and J. C. d. Álamo, “Three-dimensional Monolayer Stress Microscopy,” *Biophysical Journal*, 2019.
- [127] S. E. Leggett, M. Patel, T. M. Valentin, L. Gamboa, A. S. Khoo, E. K. Williams, C. Franck, and I. Y. Wong, “Mechanophenotyping of 3D multicellular clusters using displacement arrays of rendered tractions,” *Proceedings of the National Academy of Sciences*, vol. 117, no. 11, pp. 5655–5663, 2020.

- [128] J. Toyjanova, E. Hennen, E. Bar-Kochba, E. M. Darling, D. L. Henann, and C. Franck, “3d viscoelastic traction force microscopy,” *Soft matter*, vol. 10, no. 40, pp. 8095–8106, 2014.
- [129] H. Colin-York, D. Shrestha, J. H. Felce, D. Waithe, E. Moeendarbary, S. J. Davis, C. Eggeling, and M. Fritzsche, “Super-resolved traction force microscopy (stfm),” *Nano letters*, vol. 16, no. 4, pp. 2633–2638, 2016.
- [130] S. G. Knoll, M. Y. Ali, and M. T. A. Saif, “A novel method for localizing reporter fluorescent beads near the cell culture surface for traction force microscopy,” *Journal of Visualized Experiments : JoVE*, p. 51873, 2014.
- [131] H. Colin-York, C. Eggeling, and M. Fritzsche, “Dissection of mechanical force in living cells by super-resolved traction force microscopy,” *Nature Protocols*, vol. 12, pp. 783–796, 2017.
- [132] S. Makarchuk, N. Beyer, C. Gaiddon, W. Grange, and P. Hébraud, “Holographic Traction Force Microscopy,” *Scientific Reports*, vol. 8, p. 3038, 2018.
- [133] A. Saraswathibhatla and J. Notbohm, “Traction and Stress Fibers Control Cell Shape and Rearrangements in Collective Cell Migration,” *Physical Review X*, vol. 10, no. 1, p. 011016, 2020.
- [134] M. Alnæs, J. Blechta, J. Hake, A. Johansson, B. Kehlet, A. Logg, C. Richardson, J. Ring, M. E. Rognes, and G. N. Wells, “The FEniCS project version 1.5,” *Archive of Numerical Software*, vol. 3, no. 100, 2015.
- [135] A. Logg, K.-A. Mardal, and G. Wells, *Automated solution of differential equations by the finite element method: The FEniCS book*, vol. 84. Springer Science & Business Media, 2012.
- [136] L. B. Lucy, “An iterative technique for the rectification of observed distributions,” *The astronomical journal*, vol. 79, p. 745, 1974.
- [137] W. H. Richardson, “Bayesian-based iterative method of image restoration,” *JoSA*, vol. 62, no. 1, pp. 55–59, 1972.

- [138] D. S. C. Biggs and M. Andrews, “Acceleration of iterative image restoration algorithms,” *Appl. Opt.*, vol. 36, no. 8, pp. 1766–1775, 1997.
- [139] S.-L. Liu, J. Li, Z.-L. Zhang, Z.-G. Wang, Z.-Q. Tian, G.-P. Wang, and D.-W. Pang, “Fast and high-accuracy localization for three-dimensional single-particle tracking,” *Scientific Reports*, vol. 3, p. 2462, 2013.
- [140] A. F. Bower, *Applied mechanics of solids*. CRC press, 2009.
- [141] H. Jang, J. Notbohm, B. Gweon, Y. Cho, C. Y. Park, S.-H. Kee, J. J. Fredberg, J. H. Shin, and Y. Park, “Homogenizing cellular tension by hepatocyte growth factor in expanding epithelial monolayer,” *Scientific Reports*, vol. 7, 2017.
- [142] J. Notbohm, J.-H. Kim, A. Asthagiri, and G. Ravichandran, “Three-Dimensional Analysis of the Effect of Epidermal Growth Factor on Cell-Cell Adhesion in Epithelial Cell Clusters,” *Biophysical Journal*, vol. 102, pp. 1323–1330, 2012.
- [143] H. Wang, A. S. Abhilash, C. S. Chen, R. G. Wells, and V. B. Shenoy, “Long-Range Force Transmission in Fibrous Matrices Enabled by Tension-Driven Alignment of Fibers,” *Biophysical Journal*, vol. 107, no. 11, pp. 2592–2603, 2014.
- [144] M. S. Rudnicki, H. A. Cirka, M. Aghvami, E. Sander, Q. Wen, and K. Billiar, “Non-linear Strain Stiffening Is Not Sufficient to Explain How Far Cells Can Feel on Fibrous Protein Gels,” *Biophysical Journal*, vol. 105, no. 1, pp. 11–20, 2013.
- [145] D. Vader, A. Kabla, D. Weitz, and L. Mahadevan, “Strain-Induced Alignment in Collagen Gels,” *PLoS ONE*, vol. 4, no. 6, p. e5902, 2009.
- [146] J. P. Winer, S. Oake, and P. A. Janmey, “Non-Linear Elasticity of Extracellular Matrices Enables Contractile Cells to Communicate Local Position and Orientation,” *PLoS ONE*, vol. 4, no. 7, p. e6382, 2009.
- [147] S. Motte and L. J. Kaufman, “Strain stiffening in collagen I networks,” *Biopolymers*, vol. 99, no. 1, pp. 35–46, 2012.

- [148] J. Notbohm, A. Lesman, P. Rosakis, D. A. Tirrell, and G. Ravichandran, “Microbuckling of fibrin provides a mechanism for cell mechanosensing,” *Journal of The Royal Society Interface*, vol. 12, no. 108, p. 20150320, 2015.
- [149] A. S. G. v. Oosten, M. Vahabi, A. J. Licup, A. Sharma, P. A. Galie, F. C. MacKintosh, and P. A. Janmey, “Uncoupling shear and uniaxial elastic moduli of semiflexible biopolymer networks: compression-softening and stretch-stiffening,” *Scientific Reports*, vol. 6, no. 1, p. 19270, 2016.
- [150] B. Burkel and J. Notbohm, “Mechanical response of collagen networks to nonuniform microscale loads,” *Soft Matter*, vol. 13, no. 34, pp. 5749–5758, 2017.
- [151] O. V. Kim, R. I. Litvinov, J. W. Weisel, and M. S. Alber, “Structural basis for the nonlinear mechanics of fibrin networks under compression,” *Biomaterials*, vol. 35, no. 25, pp. 6739–6749, 2014.
- [152] A. M. Stein, D. A. Vader, D. A. Weitz, and L. M. Sander, “The micromechanics of three-dimensional collagen-I gels,” *Complexity*, vol. 16, no. 4, pp. 22–28, 2010.
- [153] B. A. Roeder, K. Kokini, J. E. Sturgis, J. P. Robinson, and S. L. Voytik-Harbin, “Tensile Mechanical Properties of Three-Dimensional Type I Collagen Extracellular Matrices With Varied Microstructure,” *Journal of Biomechanical Engineering*, vol. 124, no. 2, pp. 214–222, 2002.
- [154] P. A. Janmey, M. E. McCormick, S. Rammensee, J. L. Leight, P. C. Georges, and F. C. MacKintosh, “Negative normal stress in semiflexible biopolymer gels,” *Nature Materials*, vol. 6, no. 1, pp. 48–51, 2007.
- [155] L. M. Owen, A. S. Adhikari, M. Patel, P. Grimmer, N. Leijnse, M. C. Kim, J. Notbohm, C. Franck, and A. R. Dunn, “A cytoskeletal clutch mediates cellular force transmission in a soft, three-dimensional extracellular matrix,” *Molecular Biology of the Cell*, vol. 28, no. 14, pp. 1959–1974, 2017.

- [156] C. A. R. Jones, M. Cibula, J. Feng, E. A. Krnacik, D. H. McIntyre, H. Levine, and B. Sun, “Micromechanics of cellularized biopolymer networks,” *Proceedings of the National Academy of Sciences*, vol. 112, no. 37, pp. E5117–E5122, 2015.
- [157] T. T. Falzone and R. M. Robertson-Anderson, “Active Entanglement-Tracking Microrheology Directly Couples Macromolecular Deformations to Nonlinear Microscale Force Response of Entangled Actin,” *ACS Macro Letters*, vol. 4, no. 11, pp. 1194–1199, 2015.
- [158] F. G. Schmidt, B. Hinner, and E. Sackmann, “Microrheometry underestimates the values of the viscoelastic moduli in measurements on F -actin solutions compared to macrorheometry,” *Physical Review E*, vol. 61, no. 5, pp. 5646–5653, 2000.
- [159] D. Velegol and F. Lanni, “Cell Traction Forces on Soft Biomaterials. I. Microrheology of Type I Collagen Gels,” *Biophysical Journal*, vol. 81, no. 3, pp. 1786–1792, 2001.
- [160] M. Shayegan and N. R. Forde, “Microrheological Characterization of Collagen Systems: From Molecular Solutions to Fibrillar Gels,” *PLoS ONE*, vol. 8, no. 8, p. e70590, 2013.
- [161] A. Buxboim, K. Rajagopal, A. E. X. Brown, and D. E. Discher, “How deeply cells feel: methods for thin gels,” *Journal of Physics: Condensed Matter*, vol. 22, no. 19, p. 194116, 2010.
- [162] D. Song, N. Hugenberg, and A. A. Oberai, “Three-dimensional traction microscopy with a fiber-based constitutive model,” *Computer Methods in Applied Mechanics and Engineering*, vol. 357, p. 112579, 2019.
- [163] A. S. Abhilash, B. Baker, B. Trappmann, C. Chen, and V. Shenoy, “Remodeling of Fibrous Extracellular Matrices by Contractile Cells: Predictions from Discrete Fiber Network Simulations,” *Biophysical Journal*, vol. 107, no. 8, pp. 1829–1840, 2014.
- [164] M. T. Scimone, H. C. Cramer III, E. Bar-Kochba, R. Amezcua, J. B. Estrada, and C. Franck, “Modular approach for resolving and mapping complex neural and other cellular structures and their associated deformation fields in three dimensions,” *Nature Protocols*, vol. 13, no. 12, pp. 3042–3064, 2018.

- [165] P.-O. Persson and G. Strang, “A Simple Mesh Generator in MATLAB,” *SIAM Review*, vol. 46, no. 2, pp. 329–345, 2004.
- [166] Q. Fang and D. A. Boas, “Tetrahedral mesh generation from volumetric binary and grayscale images,” *2009 IEEE International Symposium on Biomedical Imaging: From Nano to Macro*, pp. 1142–1145, 2009.
- [167] J. D. Eshelby, “The elastic field outside an ellipsoidal inclusion,” *Proceedings of the Royal Society of London. Series A. Mathematical and Physical Sciences*, vol. 252, no. 1271, pp. 561–569, 1959.
- [168] S. Werner and R. Grose, “Regulation of Wound Healing by Growth Factors and Cytokines,” *Physiological Reviews*, vol. 83, no. 3, pp. 835–870, 2003.
- [169] C. L. Stokes, M. A. Rupnick, S. K. Williams, and D. A. Lauffenburger, “Chemotaxis of human microvessel endothelial cells in response to acidic fibroblast growth factor.,” *Laboratory investigation; a journal of technical methods and pathology*, vol. 63, no. 5, pp. 657–68, 1990.
- [170] M. Eisenbach and L. C. Giojalas, “Sperm guidance in mammals — an unpaved road to the egg,” *Nature Reviews Molecular Cell Biology*, vol. 7, no. 4, pp. 276–285, 2006.
- [171] E. T. Roussos, J. S. Condeelis, and A. Patsialou, “Chemotaxis in cancer,” *Nature Reviews Cancer*, vol. 11, no. 8, pp. 573–587, 2011.
- [172] L. E. Hind, P. N. Ingram, D. J. Beebe, and A. Huttenlocher, “Interaction with an endothelial lumen increases neutrophil lifetime and motility in response to *P aeruginosa*,” *Blood*, vol. 132, no. 17, pp. 1818–1828, 2018.
- [173] P. H. McMinn, L. E. Hind, A. Huttenlocher, and D. J. Beebe, “Neutrophil trafficking on-a-chip: an in vitro , organotypic model for investigating neutrophil priming, extravasation, and migration with spatiotemporal control,” *Lab on a Chip*, vol. 19, no. 21, pp. 3697–3705, 2019.
- [174] A. F. Brown, “Neutrophil granulocytes: adhesion and locomotion on collagen substrata and in collagen matrices,” *Journal of cell science*, vol. 58, pp. 455–67, 1982.

- [175] S. Boyden, “The Chemotactic Effect of Mixtures of Antibody and Antigen on Polymorphonuclear Leukoctyes,” *Journal of Experimental Medicine*, vol. 115, pp. 453–466, 03 1962.
- [176] R. D. Nelson, P. G. Quie, and R. L. Simmons, “Chemotaxis under agarose: a new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes.,” *Journal of immunology (Baltimore, Md. : 1950)*, vol. 115, no. 6, pp. 1650–6, 1975.
- [177] D. Zicha, G. A. Dunn, and A. F. Brown, “A new direct-viewing chemotaxis chamber.,” *Journal of cell science*, vol. 99 (Pt 4), pp. 769–75, 1991.
- [178] W. Zhao, H. Zhao, M. Li, and C. Huang, “Microfluidic devices for neutrophil chemotaxis studies,” *Journal of Translational Medicine*, vol. 18, no. 1, p. 168, 2020.
- [179] G. L. Manderino, A. F. Suarez, A. A. Hirata, and P. A. Ward, “Chemotaxis under agarose utilizing human serum depleted of C-5 derived peptides,” *Journal of Immunological Methods*, vol. 45, no. 3, pp. 283–299, 1981.
- [180] D. Irimia, G. Charras, N. Agrawal, T. Mitchison, and M. Toner, “Polar stimulation and constrained cell migration in microfluidic channels,” *Lab on a Chip*, vol. 7, no. 12, pp. 1783–1790, 2007.
- [181] V. V. Abhyankar, M. A. Lokuta, A. Huttenlocher, and D. J. Beebe, “Characterization of a membrane-based gradient generator for use in cell-signaling studies,” *Lab on a Chip*, vol. 6, no. 3, p. 389, 2006.
- [182] J. L. Haddox, R. R. Pfister, and C. I. Sommers, “A visual assay for quantitating neutrophil chemotaxis in a collagen gel matrix,” *Journal of Immunological Methods*, vol. 141, no. 1, pp. 41–52, 1991.
- [183] E. K. Sackmann, E. Berthier, E. W. K. Young, M. A. Shelef, S. A. Wernimont, A. Huttenlocher, and D. J. Beebe, “Microfluidic kit-on-a-lid: a versatile platform for neutrophil chemotaxis assays,” *Blood*, vol. 120, no. 14, pp. e45–e53, 2012.

- [184] U. Haessler, Y. Kalinin, M. A. Swartz, and M. Wu, “An agarose-based microfluidic platform with a gradient buffer for 3D chemotaxis studies,” *Biomedical Microdevices*, vol. 11, no. 4, pp. 827–835, 2009.
- [185] N. Jeon, H. Baskaran, S. K. Dertinger, G. M. Whitesides, L. Water, and M. Toner, “Neutrophil chemotaxis in linear and complex gradients of interleukin-8 formed in a microfabricated device,” *Nature Biotechnology*, vol. 20, no. 8, pp. 826–830, 2002.
- [186] D. Kim and C. L. Haynes, “On-Chip Evaluation of Neutrophil Activation and Neutrophil–Endothelial Cell Interaction during Neutrophil Chemotaxis,” *Analytical Chemistry*, vol. 85, no. 22, pp. 10787–10796, 2013.
- [187] W. Saadi, S. W. Rhee, F. Lin, B. Vahidi, B. G. Chung, and N. L. Jeon, “Generation of stable concentration gradients in 2D and 3D environments using a microfluidic ladder chamber,” *Biomedical Microdevices*, vol. 9, no. 5, pp. 627–635, 2007.
- [188] S. Chung, R. Sudo, P. J. Mack, C.-R. Wan, V. Vickerman, and R. D. Kamm, “Cell migration into scaffolds under co-culture conditions in a microfluidic platform,” *Lab Chip*, vol. 9, no. 2, pp. 269–275, 2009.
- [189] R. R. Kay, P. Langridge, D. Traynor, and O. Hoeller, “Changing directions in the study of chemotaxis,” *Nature Reviews Molecular Cell Biology*, vol. 9, no. 6, pp. 455–463, 2008.
- [190] Z. Xu, P. Fang, B. Xu, Y. Lu, J. Xiong, F. Gao, X. Wang, J. Fan, and P. Shi, “High-throughput three-dimensional chemotactic assays reveal steepness-dependent complexity in neuronal sensation to molecular gradients,” *Nature Communications*, vol. 9, no. 1, p. 4745, 2018.
- [191] B. A. Bicknell, Z. Pujic, J. Feldner, I. Vetter, and G. J. Goodhill, “Chemotactic responses of growing neurites to precisely controlled gradients of nerve growth factor,” *Scientific Data*, vol. 5, no. 1, p. 180183, 2018.
- [192] S. H. Zigmond, “Ability of polymorphonuclear leukocytes to orient in gradients of chemotactic factors.,” *Journal of Cell Biology*, vol. 75, no. 2 Pt 1, pp. 606–616, 1977.

- [193] B. P. Boribong, M. J. Lenzi, L. Li, and C. N. Jones, “Super-Low Dose Lipopolysaccharide Dysregulates Neutrophil Migratory Decision-Making,” *Frontiers in Immunology*, vol. 10, p. 359, 2019.
- [194] M. B. Harler, E. Wakshull, E. J. Filardo, J. E. Albina, and J. S. Reichner, “Promotion of neutrophil chemotaxis through differential regulation of beta 1 and beta 2 integrins,” *Journal of immunology (Baltimore, Md. : 1950)*, vol. 162, no. 11, pp. 6792–9, 1999.
- [195] A. D. Doyle, “Generation of 3D collagen gels with controlled, diverse architectures,” *Current Protocols in Cell Biology*, pp. 10.20.1–10.20.16, 2016.
- [196] Y.-T. L. Dingle, M. E. Boutin, A. M. Chirila, L. L. Livi, N. R. Labriola, L. M. Jakubek, J. R. Morgan, E. M. Darling, J. A. Kauer, and D. Hoffman-Kim, “Three-Dimensional Neural Spheroid Culture: An In Vitro Model for Cortical Studies,” *Tissue engineering. Part C, Methods*, vol. 21, no. 12, pp. 1274–83, 2015.
- [197] M. Vinci, C. Box, and S. A. Eccles, “Three-Dimensional (3D) Tumor Spheroid Invasion Assay,” *Journal of Visualized Experiments*, vol. 99, no. e52686, 2015.
- [198] G. Cheng, J. Tse, R. K. Jain, and L. L. Munn, “Micro-Environmental Mechanical Stress Controls Tumor Spheroid Size and Morphology by Suppressing Proliferation and Inducing Apoptosis in Cancer Cells,” *PLoS ONE*, vol. 4, no. 2, p. e4632, 2009.