

The Effect of Confinement on Neutrophil Force Generation

by

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Curriculum Vitæ

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- (8) **Toyjanova, J.**, Bar-Kochba, E., and Franck, C., “Dynamic Cell Behavior under Mechanical Stimulation”, Society of Experimental Mechanics, June 2011

“Tough times never last, but tough people do!”

adapted from Robert H. Schuller

Dedicated to My Family

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Preface

This dissertation is based on the methods that were developed as a result of studying neutrophil-generated forces in unconfined and confined spaces. Neutrophil study was performed in collaboration between School of Engineering, Brown University and the Rhode Island Hospital and the findings are currently under review in *Nature Communications*.

The experimental algorithm, fast iterative digital volume correlation, described in Chapter 2 is in press in *Experimental Mechanics* in co-authorship with Eyal Bar-Kochba. Traction force microscopy for large deformations is published in *PloS One* in co-authorship with Eyal Bar-Kochba. Cell extraction protocols, also described in Chapter 2, were adapted from Reichner's Lab protocol with assistance from Angel Byrd, PhD.

A version of Appendix A is under review in *Soft Matter*.

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Abstract of “The Effect of Confinement on Neutrophil Force Generation”

by Jennet Toyjanova, Ph.D., Brown University, May 2015

Generation of forces is essential for the migration of neutrophils to the sites of infection or injury. The mechanical properties and local confinement of the surrounding microenvironment play a significant role in regulating these forces. However, it is challenging to decouple each of these features experimentally without inherently changing the microstructure of the cell environment. Presented here is an experimental method that is used to isolate confinement and substrate stiffness as single variables in the neutrophil environment. Using a combination of fast iterative digital volume correlation and traction force microscopy techniques, this dissertation demonstrates that neutrophils induce finite displacements on their surroundings. Furthermore, the findings show that neutrophil-generated forces are highly affected by confinement and stiffness of their microenvironment.

CHAPTER 1

Introduction

Engineered 3D cell culture systems open new doors to the study of physiological cell mechanisms *ex vivo*. Since the 1930s, many studies on cellular processes have been performed on 2D planar compliant surfaces [104]. Based on these studies, a lot has been discovered about cell traction force generation as it relates to cell migration and adhesion. However, studies performed on 2D planar surfaces are still too primitive to describe the full effect that physiological conditions have on cell response. In contrast to 2D surfaces, cells in the body are surrounded by an extracellular matrix (ECM) that provides an additional dimension for both cell-ECM interaction and cellular exposure to external cues.

In fact, cells are known to respond to any perturbations in external cues, like stiffness, ECM composition and density, porosity and many other features that describe their natural surroundings (Fig.1.1). Regulating these features is not translatable into studies in 2D systems. To address this issue, many different physiologically relevant cell culture systems have been developed, including 3D collagen or fibrin gels [83], porous scaffolds [103], and gels out of proteoglycans or matricellular proteins [101].

While research is moving towards more physiologically-relevant culture systems, the 3D environment is very topographically complex. To fully regulate and understand biological mechanisms in 3D framework, one has to isolate the specific factors that are operative for each biological mechanism. To comprehend the effect of these fine features on cell migration and adhesion, experimental techniques have to be developed to quantify cell-ECM interaction in fully encapsulated 3D environments.

In this dissertation, experimental techniques are presented to progress in the studies of cell-ECM contact forces in well-controlled confined environment. Specifically, an experimental setup is presented that isolates confinement as a separate variable while

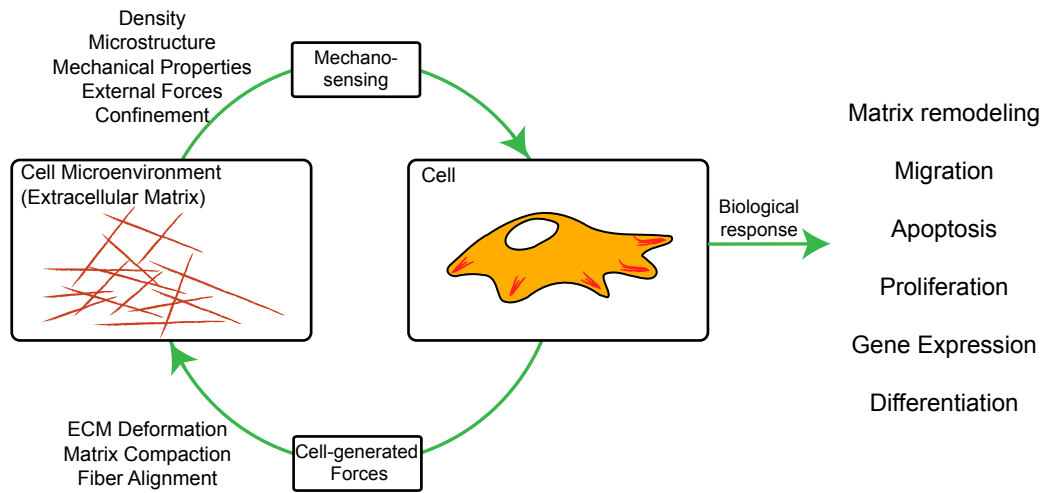


FIGURE 1.1. A schematic of cell feedback mechanism in functional interactions between cells and extracellular matrix. Cellular behavior is governed by ECM topography and composition. As a response to the changes of these fine features of the ECM, cells adapt to their microenvironment by mechanically stabilizing themselves through forces transmitted to the surrounding continuum. Such cell-ECM feedback system is an integral part in organism homeostasis and in biological responses that include but not limited to matrix remodeling, cell migration and cell proliferation.

keeping all of the other experimental factors constant during dynamic cell imaging. Modification and improvement of 3D measurement techniques, like digital volume correlation (DVC) and traction force microscopy (TFM), were developed as a result of this work. This work focuses on the application of the presented methods in the study of migration and traction force generation of neutrophils, the understanding of which is essential for the innate immune system.

1. Role of Mechanics in Cell Biology

As mentioned previously, cell-ECM interactions involve cells sensing and responding to any changes in the surrounding environment. Both of these functions hold not only biochemical but also mechanical character. Since many developmental disorders

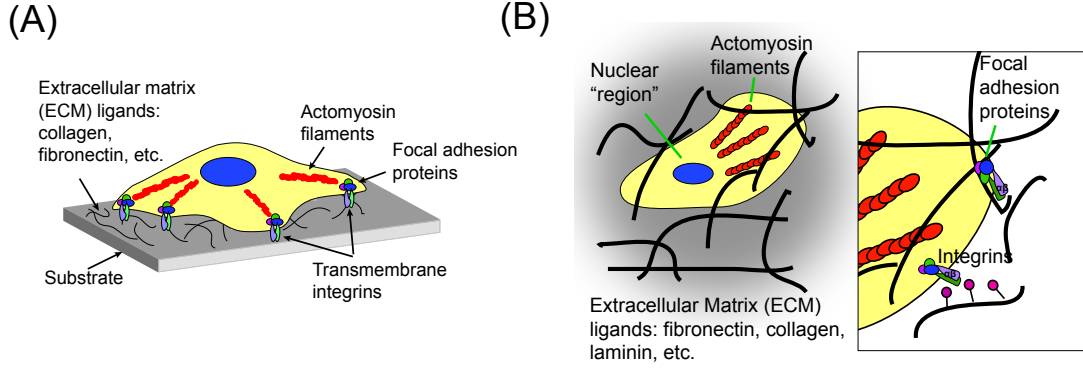


FIGURE 1.2. Schematic of cell cytoskeleton represented (A) on a 2D substrate coated with extracellular matrix protein (ligands) and (B) in the 3D fibrous ECM scaffold.

result from improper cell response to external cues [21], years of interdisciplinary research have been concentrated on the studies of the effect of these mechanical cues on human cells [102, 30, 56].

Naturally, the human body, as a whole, shows visible response to the externally applied mechanical forces. For example, bones fracture from impact [2, 20], or muscles are strained during high-speed sprinting [12].

Similarly, single cells have shown to have an ability to respond to any changes in the surrounding environment [16, 99, 24, 69]. The process of cells sensing and responding to these mechanical cues by altering biochemical signals internally is called mechanosensing and mechanotransduction. If flawed, this can lead to misregulations and mutations that then prompt various developmental diseases [36]. For example, proper hair cell mechanosensing of forces induced by head movement affects deafness in humans [102]; or changes in shear stresses acting on the endothelium impact arterial remodeling and can lead to atherosclerosis [11, 56]. Also, improper response to external cues leads to impairment of immune response and lead to sepsis [13, 39].

Cells are not only force recipients but also donors. To adhere and migrate, cells generate forces that are transmitted to the surrounding environment [8, 42]. These generated forces are a result of intracellular contractile forces of the actomyosin network that are transmitted to the ECM via transmembrane integrins (Fig.1.2).

Studying these cell-generated forces is important in understanding the biochemical behavior of individual cells in the context of tissue formation and repair [58]. Such studies also help to elucidate the driving forces in cell migration [16, 63, 60] and other cellular functions that contribute to the homeostasis of a living organism [8, 76, 59, 28]. These examples show the importance of understanding of how mechanotransduction occurs and motivate the development of tools that allow quantification and advanced analysis of cell-ECM interaction forces.

2. Traction Force Microscopy

Many studies have been performed to understand laws that regulate homeostasis in the living organism from a mechanical equilibrium perspective [40, 47, 90]. Consequently, methods have been developed to quantify cell-generated forces, which play an important role regulating biological processes [52, 63, 8]. From a mechanics point of view, the transmission of cell forces to the ECM surface is a boundary value problem. In this boundary value problem, a substrate – solid – is subjected to cell-generated tractions, $\mathbf{t}_i$, that result in net surface displacement $\mathbf{u}_i$ (Fig. 1.3). The bottom of the solid is fixed, thus u_i is set to zero, and the surface outside of the cell boundary is traction free. Inherently, the substrate is in equilibrium, mainly due to negligible mass of the cell, therefore

$$\frac{\partial \sigma_{ij}}{\partial y_i} = 0 \quad (1.1)$$

Realistically, these forces can result in net deformation of the surface, if the underlying ECM is compliant enough (Fig.1.3). In order to quantify these cell-generated forces, a method was developed that maps surface tractions from the knowledge of cell-induced displacements on the surface of a soft scaffold. This quantitative method is called traction force microscopy, or TFM.

TFM was first pioneered by Dembo and Wang in 1999 [16] by seeding cells on flat, elastic substrates filled with fluorescent particles. As a cell moves and generates forces on the substrate, the fluorescent particles in the substrate are displaced and can be tracked. The response of the substrate is assumed to be linear elastic, hence the

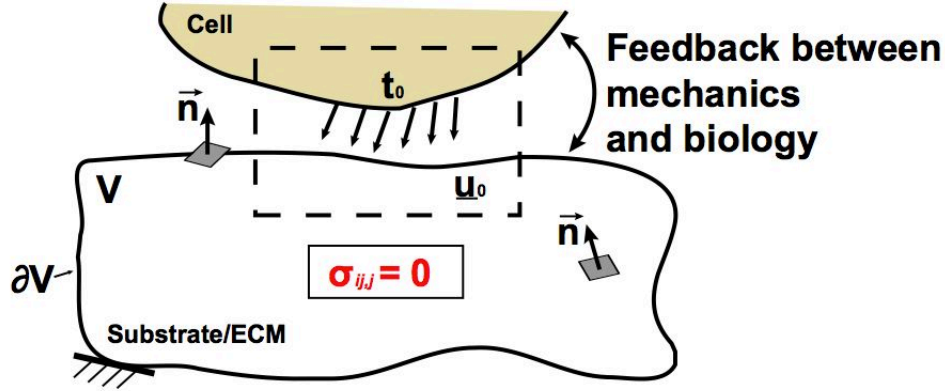


FIGURE 1.3. Schematic of cell-ECM interface as a cell mechanics problem

relationship between planar displacement and traction field is solved via an integral transform presented in Eq.1.2.

$$d_{p,\alpha} = \int \int g_{\beta\alpha}(\mathbf{m}_p - \mathbf{r}) T_{\beta}(\mathbf{r}) dr_1 dr_2. \quad (1.2)$$

Functions $g_{\beta\alpha}$ are coefficients of Green's tensor which are approximated from the Boussinesq theory used for solving the contact problem of an elastic solid with an infinite half-space. Yet, using this inverse formulation is computationally intensive and is subject to assumption that the substrate is linear elastic, infinite half-space.

The TFM method was improved by Butler et al. in 2001 [9], where the calculations are sped up by integrating Green's function via Fourier analysis. Later, Yang et al. in 2006 [106] addressed the limitation of an infinite half-space approximation in the Boussinesq formulation by implementing finite element method,

The development of aforementioned TFM methods showed that cell-generated forces are finely attuned to both the externally applied loads and the stiffness of the surrounding microenvironment [52, 22], and that the magnitude of the forces are highly dependent on the cell type [22, 73]. However, planar TFM methods map surface 2D displacements whereas, as previously mentioned, cell-ECM interaction is 3D in nature.

To address this issue, TFM was improved even further to implement the out-of-plane component in addition to in-plane components in the calculations (3D TFM).

Maskarinec and Franck et al.[60], and Hur et al.[35] utilized confocal microscopy with bead displacement tracking algorithms to capture 3D full-field deformations. Instead of solving the Boussinesq formulation, the stress vector was calculated directly by calculating the deformation gradient tensor [24, 60, 25] or using a finite element approach [35]. Both of the methods showed the importance of quantifying out-of-plane force components within the linear elastic framework, however most of the cell-ECM interactions are non-linear. For example, ECM surrounding cells is viscoelastic, thus ECM deforms in time-dependent manner in response to cell-generated forces [46]. Furthermore, cell-induced displacements fall within the finite regime, rendering a linear material response inappropriate [98], a matter which is elaborated upon in Chapter 2.

3. Neutrophil migration and force generation in confined spaces

TFM method presented in this dissertation allows mapping and quantification of forces that regulate neutrophil migration relevant to host defense. Polymorphonuclear neutrophils (PMNs) play a key role in the innate immune system as they are the first leukocytes to be recruited to a site of injury or infection. An inflammatory response is initiated when circulating neutrophils enter the 3D microenvironment of a damaged tissue or organ (1.4). The consequence of an improperly regulated inflammatory response is medically significant. An inadequate response places the host at risk for the infection to spread. If the response is excessive, hyperactive neutrophils can cause damage to the tissues that they are designed to protect [65, 61]. In order to understand how an optimal inflammatory response occurs, it is important to identify factors within an infected or injured tissue that regulate neutrophil activity.

To date, most *in vitro* studies on the regulation of neutrophil function have been conducted in 2D on surfaces that are rigid and inflexible, unlike bodily tissues. How the physical properties of the inflammatory microenvironment, such as substrate stiffness, ligand composition and spatial confinement, regulate neutrophil function and,

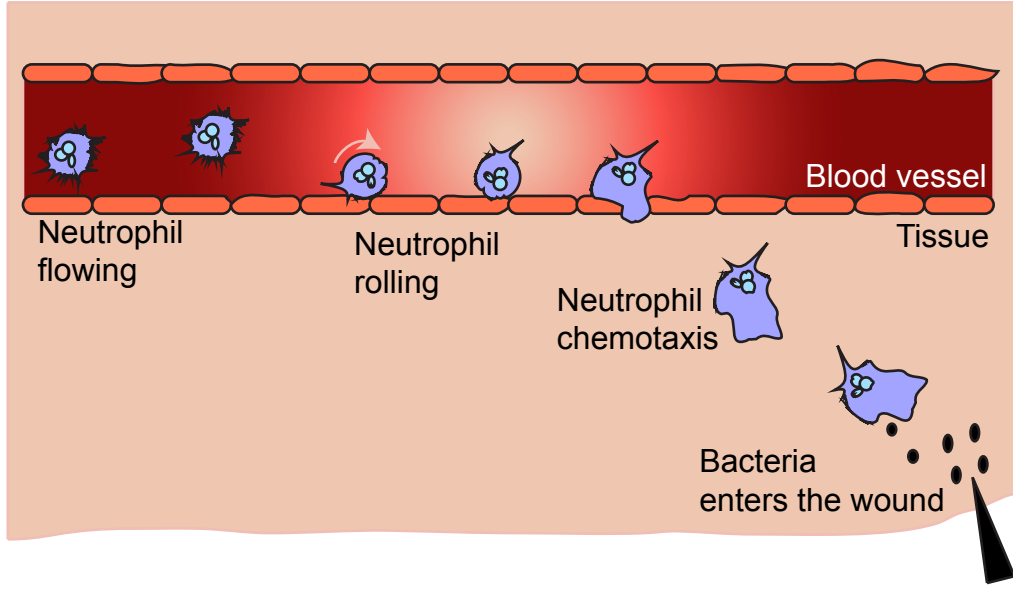


FIGURE 1.4. Schematic of neutrophil migration mechanism in the body. The illustration shows neutrophil invading the 3D interstitial tissue and its migration to the area infected by bacteria.

in particular, motility in 3D is not well understood. This represents a gap in our knowledge of how a well-regulated inflammatory response is achieved.

On 2D substrates, neutrophils, like most other eukaryotic cells, engage ligand-specific integrins to adhere to substrates and generate tractions [91, 69, 38]. However, *in vivo*, as neutrophils pass through the blood vascular wall, they transit from a predominantly 2D to a confined 3D environment, where PMNs reach migration speeds up to $20 \mu\text{m}/\text{min}$ [94, 10].

In contrast to migration on 2D surfaces, in a confined setting, cells can generate tractions through steric contacts with their surrounding environment even when their functional integrins are impaired. This was suggested in a previous study that showed that neutrophils migrate freely when confined between a glass slide and a coverslip $5 \mu\text{m}$ apart in the presence of soluble ethylenediaminetetracetic acid (EDTA), which

suppresses functional integrin engagement [57]. In 2008 Lämmermann et al.[43], reports that leukocytes devoid of integrins migrate with normal kinetics within a draining lymph node and in a confined fibrillar scaffold. This is consistent with an integrin-independent motility mechanism operative in 3D. In contrast, surface contact forces produced by a forward expanding actin network are sufficient to drive cell motility in 2D [43, 44, 79]. It is not known whether confinement alone, regardless of stiffness and ligand density, is sufficient to cause a switch to integrin independence or whether the switch requires complete envelopment within a 3D tissue matrix.

Given the importance of cell tractions in regulating cell motility, in this dissertation, we isolate integrin engagement, matrix stiffness and confinement as distinct variables encountered in a 3D microenvironment to determine the regulatory role of each on traction force generation by human neutrophils.

Chapter 2 presents materials and methods that were developed to perform the study. It includes the development of a double hydrogel system for dynamic imaging of the cell as it switches from unconfined to confined environment. This experimental system overcomes the limitation of more widely used native gels, where changing confinement in the matrix structure coincidentally affects matrix stiffness and ligand density, thereby coupling these physical parameters [78]. Chapter 2 also presents an improved digital volume correlation technique that captures displacements induced by cells on their environment at high resolution and faster speeds. Furthermore, Chapter 2 describes a method used to quantify neutrophil-generated forces that alleviates an assumption of a linear elastic response of the substrate.

Chapter 3 summarizes experimental findings and observations of neutrophil-generated forces in unconfined and confined environments. By decoupling spatial confinement and stiffness as single variables, we show that as the microenvironment changes from a planar system to a confined space, neutrophils significantly increase their cellular tractions on both softer and stiffer substrates. Contrary to most anchorage-dependent cells, we show that over 90% of neutrophil-generated forces are directed against the ECM (out-of-plane direction) on soft and stiff substrates. This

dissertation focuses on quantitative data showing that neutrophils continue to generate tractions while traveling through a confined space, even when ligand-specific integrins are inhibited, which is consistent with the hypothesis of an integrin-independent phenotype.

Lastly, chapter 4 concludes the study and overviews the preliminary results for the future steps of this project.

CHAPTER 2

Materials and Methods

This chapter describes the setup for the double hydrogel system and neutrophil isolation for the experiments. It also describes the development and validation of both full-field displacement measurement technique and previously mentioned TFM.

1. Double Hydrogel System

Substrate preparation and functionalization.

Glass Coverslips and Microscope Slides Surface Modification. Rectangular (75x25 mm², Fisher Scientific) and circular (30 mm in diameter, Esslinger and Company, MN) microscope slides were chemically modified to allow covalent attachment of polyacrylamide substrates using previously developed protocols [60, 25, 72]. Briefly, glass coverslips and slides were rinsed with ethanol and placed in a petri dish containing a solution of 0.5% (v/v) 3-aminopropyltrimethoxysilane (Sigma-Aldrich) in ethanol for 5 minutes. Next, coverslips and microscope slides were washed with ethanol and submersed in a solution of 0.5% glutaraldehyde (Polysciences, Inc.) in deionized (DI) water for 30 minutes. Activated coverslips and slides were washed with DI water and left to dry. To allow easier gel detachment, round glass coverslips of two different sizes (22 mm and 15 mm in diameter) were chemically modified to create hydrophobic surfaces. Briefly, glass coverslips were placed in a petri dish containing 97% (v/v) hexane (Fisher Scientific, MA), 2.5% (v/v) (tridecafluoro-1,1,2,2-tetrahydrooctyl)-triethoxysilane (SIT) (Gelest, Morrisville, PA), and 0.5 % (v/v) acetic acid (Sigma-Aldrich, St. Louis, MO) for approximately one minute. Coverslips were then removed and left to dry.

Preparation of Polyacrylamide Substrates. Polyacrylamide gel substrates were prepared from acrylamide (40% w/v, Bio-Rad Laboratories) and N,N-methylene-bis-acrylamide (BIS, 2% w/v, Bio-Rad Laboratories) stock solutions as described previously [60, 25, 72]. The concentrations of acrylamide and BIS were varied to control the mechanical properties of the substrates and were chosen to be 8%/0.08% for stiffer substrates and 3%/0.2% for softer substrates. The chosen concentrations yielded Young's moduli of 8.3 ± 0.2 kPa and 1.5 ± 0.1 kPa [22]. All substrates contained 14% (w/v) of fluorescent microspheres (0.5 μ m in diameter, carboxylate-modified, Life Technologies). In order to visually differentiate between top and bottom substrates, each substrate was impregnated with either red (top) or green (bottom) fluorescent microspheres (Fig.2.1).

To neutralize any effects due to complex lipopolysaccharides (LPS), 0.01% (v/v) of antibiotic Polymixin B (Life Technologies) was mixed into the gel solution. Crosslinking was initiated through the addition of ammonium persulfate (Sigma-Aldrich) and N,N,N,N-tetramethylethylenediamine (Life Technologies). Polyacrylamide gel solution was vortexed for about 30 seconds, and 30 μ l of polyacrylamide solution with green fluorescent microspheres were pipetted on the surface of an activated microscope slide for bottom substrate and sandwiched with a hydrophobic glass coverslip (22 mm in diameter), yielding a final thickness of approximately 60 μ m. For the top substrate, 20 μ l of polyacrylamide gel solution containing red fluorescent beads were deposited on top of an activated circular coverslide (30 mm in diameter) and sandwiched with a small hydrophobic coverslip (15 mm in diameter), yielding a final thickness of approximately 60 μ m. After polymerization the circular hydrophobic coverslips were removed and the substrates were stored in DI water.

Functionalization of Polyacrylamide Substrates. To promote a physiological surface for cell attachment to polyacrylamide films, top and bottom substrates were functionalized with fibronectin using the bifunctional crosslinker, sulfo-SANPAH (sulfo-succinimidyl 6 (4-azido-2-nitrophenyl-amino)hexonate, Thermo Fisher) [16][60][25].

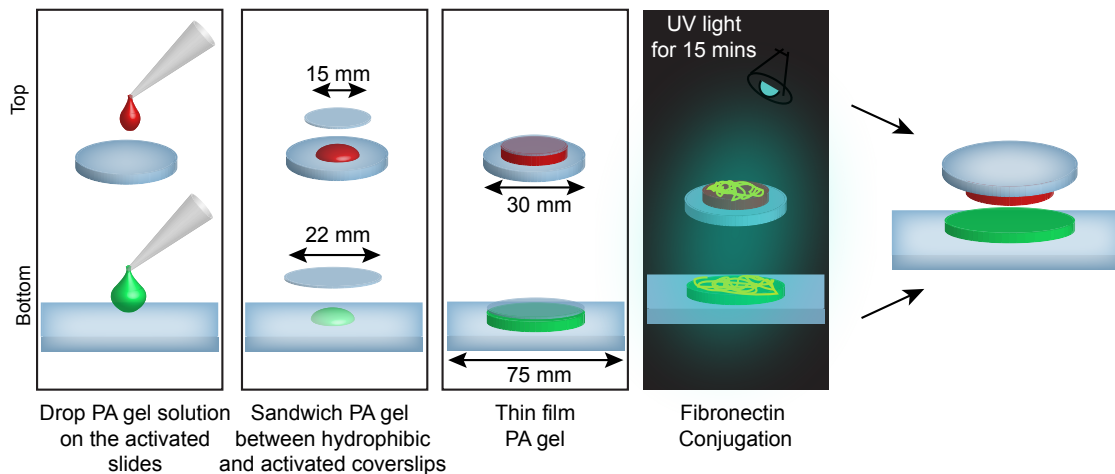


FIGURE 2.1. Schematic of the double-hydrogel system preparation.

Excess water was removed from the substrates prior deposition of 100 μl of sulfo-SANPAH (1mg/ml) onto the surface of each film, followed by a 15 minute exposure to UV light (Fig. 2.1). After UV light exposure, the darkened sulfo-SANPAH solution was aspirated and the procedure was repeated one more time. The samples were thoroughly washed with DI water, and covered with a solution of 0.2 mg/ml fibronectin (Life Technologies) diluted in 50 mM HEPES (pH 8, Sigma-Aldrich) and left undisturbed at 4°C overnight. Following overnight incubation, the substrates were extensively rinsed three times with 1x PBS mixed with Polymixin B (0.01% v/v) and sterilized with UV irradiation before depositing cells.

For control experiments, in place of fibronectin, bifunctional crosslinker was used for coating with polymer poly(L-lysine) with poly(ethylene-glycol) chains (PLL-PEG, 0.2 mg/mL final concentration, SuSoS, Switzerland). PLL-PEG coating was conjugated to the surface following the procedure described above.

Substrate characterization. Briefly, gel samples were cast in circular nylon molds (16 mm in diameter and 10 mm in height) to fabricate cylindrical samples for uniaxial compression testing. Following polymerization, the molds were removed and the samples were submersed in DI water. The custom-built compression device consisted of a centrally positioned linear actuator (Series A, Ultra Motion) equipped

with a built-in linear encoder that provides displacement information with a displacement resolution of $1\ \mu\text{m}$. To measure the compressive forces acting on each sample a 50 gram linear force transducer (LCFA-50F, Omega Engineering Inc) was attached to the end of the linear actuator. To ensure uniaxial compression conditions, a ball point tip-platen top was placed on each gel sample, and all samples were carefully aligned along the central compression axis of the linear actuator [60][25]. Nominal stress, σ , was computed by dividing the measured applied force by the circular contact area. Nominal strain, ϵ , was calculated by measuring the height change of each sample divided by the sample's original height. Original contact area and height of the cylindrical samples were measured by a caliper. All the samples were tested a day after preparation, thus allowing complete swelling and ensuring that material characterization is performed on the same day as cell experiments, i.e. 24 hours after gel preparation. During compression tests – the samples were submerged in DI water.

Each sample was compressed at three different strain rates (10^{-2} , 10^{-1} , and 10^0) to capture any time-dependence in the material behavior. Young's moduli were calculated from the slope of each linear stress-strain curve as $E = \sigma/\epsilon$ and are within the measurements presented by Tse et al. [99]. As shown previously, PA gel can be described as neo-Hookean solid that has the same Young's modulus as the linear stress-strain relationship in the limit of small strains [53]. The Poisson's ratio is taken to be 0.45, which is within the range of the typical values chosen for traction force microscopy studies [62, 96, 22].

2. Neutrophils

Neutrophil isolation and preparation. Neutrophils were isolated from healthy human volunteers into ethylenediaminetetraacetic acid-containing Vacutainer tubes (BD Biosciences, CA) according to Institutional Review Board procedures. Histopaque cell separation was followed by gravity sedimentation through 3% dextran. Hypotonic lysis to remove erythrocytes yielded a neutrophil purity of $\geq 95\%$. Neutrophils were suspended in HBSS (without $\text{Ca}^{++}/\text{Mg}^{++}$) on ice until use. All reagents contained

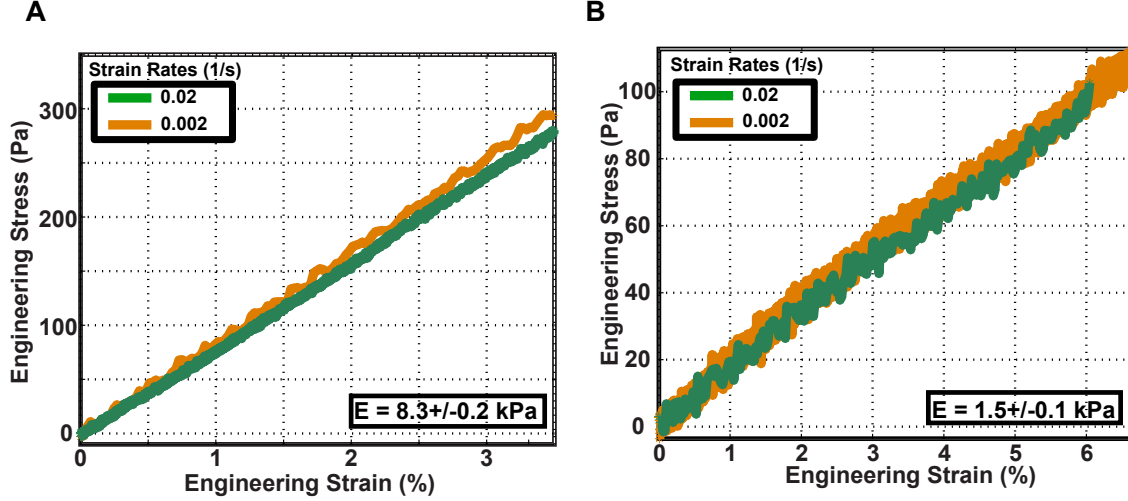


FIGURE 2.2. Stress-strain curve of unconfined compression test of cylindrical hydrated polyacrylamide samples for (A) stiffer substrates ($E = 8.3 \text{ kPa}$) and (B) softer substrates ($E = 1.5 \text{ kPa}$).

$\leq 0.1 \text{ pg/ml}$ endotoxin. For traction measurements, approximately 350,000 cells were resuspended in a vial containing 1mL of phenol free Leibovitz L-15 medium and 2mg/ml glucose. Prior to imaging, formyl-methionyl-leucyl-phenylalanine (fMLF, 100 nM final concentration, Sigma-Aldrich) was added to cell solution to activate and induce migration.

Neutrophil integrin blockade. For integrin blocking experiments, we used a previously described method, where neutrophils were incubated in a $10 \mu\text{g/ml}$ solution of a function-blocking anti-VLA 5 $\alpha_5\beta_1$ integrin antibody (clone P1B5, Abcam Inc) [69]. Anti - β_1 (clone 6S6, EMD Millipore) and anti - β_2 (clone TS1/18, Thermo Fisher Scientific) integrin blockade, neutrophils were incubated in the $10 \mu\text{g/mL}$ concentration of antibody in HBSS(-/-) on ice for 20 minutes. Once incubation is complete, cells were transferred into L15+glucose medium, where the antibody concentration was maintained throughout the experiment. For controls, neutrophils were pretreated with $20 \mu\text{g/mL}$ of IgG1. Similar to blockade experiments, the concentration of $20 \mu\text{g/mL}$ IgG1 was maintained throughout the whole experiment.

Neutrophil actomyosin network inhibition. To inhibit actin polymerization in migrating neutrophils, cells were treated with Latrunculin A (Life Technologies). At high concentrations of Latrunculin A, treated neutrophils neither adhere to nor generate measurable forces on the substrates. Thus, based on a Latrunculin dose curve (500 nM - 10 nM) it was determined that 200 nM concentration is an optimal concentration for force measurements. Before each experiment, cells were incubated in the presence of Latrunculin A on ice for 30 minutes. Once incubation is complete, cells were transferred into L15+glucose medium, where the inhibitor concentration was maintained throughout the experiment.

To inhibit myosin II in migrating neutrophils, cells were pre-treated with Blebbistatin at 50 μ M concentration on ice for 30 minutes. Similarly, cells were then transferred into L15+glucose medium prior to experiment, and the inhibitor concentration was maintained throughout the duration of experiment. Due to sensitivity of Blebbistatin to blue light (488 nm laser), both top and bottom substrates were impregnated with red fluorescent beads and excited with a diode (561 nm) laser for all Blebbistatin-treated cell experiments.

3. Measurement of neutrophil-induced displacements

Note: The work has been performed and a version of this section has been published in Journal of Experimental Mechanics in co-authorship with Bar-Kochba E. [Bar-Kochba E., Toyjanova J., Andrews E., Kim K-S., Franck C., "A fast iterative digital volume correlation algorithm for large deformations", *Experimental Mechanics*, Dec. 2014.] Published data is included in the section with co-author's consent.

Live cell imaging. Three-dimensional image stacks were acquired using a Nikon A-1 confocal system mounted on a TI Eclipse inverted optical microscope controlled by NIS-Elements Nikon Software (Nikon, Tokyo, Japan). A 40x plan fluor air objective

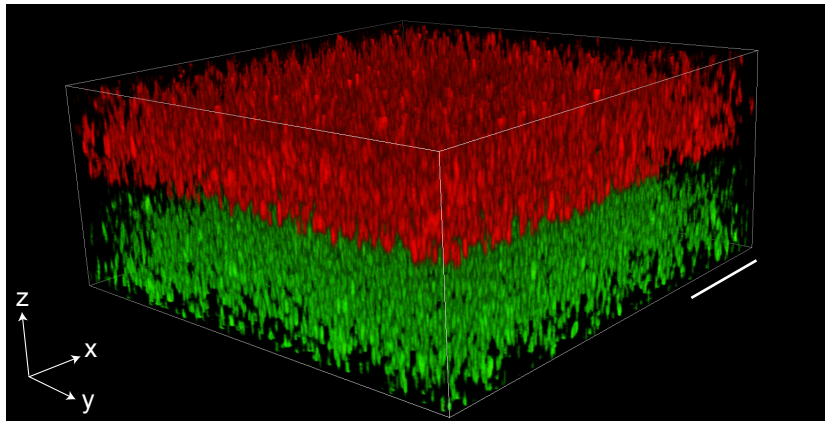


FIGURE 2.3. Volumetric image of the bottom and top substrates captured by the confocal microscope and rendered in NIS Elements software. Scale bar = $20\ \mu\text{m}$

mounted on a piezo objective positioner was used for all the experiments, which allowed to image at speeds up to 30 frames per second. Green fluorescent microspheres were used for the bottom substrate and excited with an Argon ion laser (488 nm). Red $0.5\ \mu\text{m}$ fluorescent microspheres in the top substrate were excited with a diode laser (561 nm) (Fig. 2.3)

$512 \times 512 \times Z$ voxels ($102\ \mu\text{m} \times 102\ \mu\text{m} \times Z$) confocal volume stacks were recorded every 15 seconds with Z ranging from 128 to 250 voxels ($38\ \mu\text{m} - 75\ \mu\text{m}$) depending whether only bottom (2D) or both bottom and top (2.5D) substrates were imaged simultaneously. To ensure physiological imaging conditions within the imaging chamber, temperature was controlled at 37°C using a feedback controlled heater (Air-Therm heater, World Precision Instruments INC., Sarasota, FL). All the samples were attached to the device (Fig.3.4) one hour before the experiment to equilibrate to the temperature in the imaging chamber.

During the experiment, the cell body was imaged by phase contrast (transmitted light) microscopy. To ensure good image quality, prior to experiment, microscope was checked for correct Köhler illumination alignment for 40x air objective. Several attempts of labeling cells with a fluorescent dye were performed, however some deviation in cell behavior was observed during experiments, thus using untreated neutrophils were decided to be the best approach. Phase contrast images did not reveal all the

fine extensions on the spread neutrophils, however it gave a good representation of the location of the cell.

NIS-Elements Nikon Software was used to operate the microscope during imaging. The output files were .nd2 format movies with three separated channels and time stacks collected every $\Delta t = 15$ seconds. Movies were then inputted into Matlab (The MathWorks, Inc., Natick, Ma) by using Bio-Formats [50]. Bio-Formats is a Java library used for reading and converting life sciences imaging formats into a desired format from metadata collected by microscope software.

Following typical experimental approach [24], all simulated volumetric images were deconvolved using the Lucy-Richardson deconvolution prior to calculating the displacement field using FIDVC [81]. Before tracking the motion of the beads, the volumetric images of green and red channels were subtracted from each other - to ensure minimal spectral bleed-through artifacts. In the case of blebbistatin - the top and bottom substrates were visually identified by the gap in between the imaged gels. The top and bottom were cropped at that gap and run through FIDVC separately. This allowed for easier data interpretation, as well as ensured minimal error due to averaging and interpolation of the subvolumes in FIDVC.

Once, three-dimensional image stacks of the motion of the fluorescent beads were converted into Matlab files, cell-generated full field displacements were measured using fast iterative digital volume correlation (FIDVC). This FIDVC technique was developed as a part of the current work, so that it provides significantly higher spatial resolution, signal-to-noise, and faster computation times than previous DVC algorithm [24]. FIDVC formulation is described in more detail in the next section.

Digital Volume Correlation: background and challenges. Digital volume correlation (DVC) is the volumetric extension of the popular surface displacement mapping technique of digital image correlation (DIC) [4]. The mathematical formulation of DVC can be derived from equations used in DIC, where displacement field, $\mathbf{u}$ is found by minimizing a correlation function ϕ_c (Eq. 2.1)

$$\phi_c(\mathbf{x}) = |g(\mathbf{x} + \mathbf{u}(\mathbf{x})) - f(\mathbf{x})|^2. \quad (2.1)$$

Here f and g are gray level intensity functions that represent undeformed and deformed volumes, respectively. Accurately finding displacement field between the two configurations in 3D involves solving for twelve degrees of freedom – displacement, rotation and strains.

$$u_i(X_k + dX_k) \approx u_i(X_k) + \frac{\partial u_i}{\partial X_k} dX_k \quad (2.2)$$

Therefore, application of minimization approach to DVC has suffered from high processing speeds.

In the first development of DVC, large rotations and strains were omitted, thus only displacement vector was taken into account [4]. Later, it was shown that these high-order terms have a significant effect on the accuracy of the calculated displacement fields [92, 24]. In 2011 Gates et al. [27] presented a complete, 12 degree of freedom DVC algorithm derived using minimization approach Eq.(2.1). To handle the issue of computational cost Gates et al. utilized high performance parallel computing producing average run times just under six hour for 39^3 voxel correlation grids with interrogation window sizes 41^3 voxels [27].

Generally, the accuracy of displacement field measured by DVC is highly dependent on the choice of interrogation window size. Interrogation window size determines the spatial resolution and displacement uncertainty of the generated results [45, 70]. However, spatial resolution is generally maximized at the cost of computational speeds [45, 70].

In this section, a method is presented, where these two critical technical hurdles are addressed by modifying a DVC algorithm. The presented DVC is capable of capturing any general non-linear finite deformation that can be implemented on a low cost personal computer with a graphic processing units (GPUs) graphics card. This new algorithm termed fast iterative DVC, or FIDVC, maximizes the spatial resolution

per given deformation field and speckle pattern while providing almost real-time processing speeds even for large 3D volumes. The main principle behind the proposed algorithm lies in an iterative mapping approach similar to schemes widely used in the particle image velocimetry (PIV) community [93, 85, 86]. Similar to the previous DVC algorithm ([24]) the proposed method is based on the standard cross-correlation formulation with the addition of an iterative image deformation method (IDM). This deformation method is applied to both deformed and undeformed volumetric images, which significantly improves the analysis accuracy. One of the main advantages of the method is the multi-step interrogation window refinement during the iteration process allowing for the accurate capture of higher spatial frequencies including highly localized large displacement gradients.

Fast Iterative Digital Volume Correlation: algorithm summary and validation. The FIDVC method is a fast, non-evasive, 3D full-field measurement technique for determining large-deformation internal displacement fields. By utilizing a fast Fourier transform (FFT) based cross-correlation formulation in conjunction with the iterative deformation method (IDM), the FIDVC efficiently computes finite deformation fields generally non-resolvable by conventional DVC algorithms. The flowchart in Fig.2.4 illustrates the basic procedure of how the FIDVC calculates the 3D displacement field, $\mathbf{u} = (u_1, u_2, u_3)$, between two volumetric images, $I(\mathbf{x})$ and $\hat{I}(\mathbf{x})$ (Fig. 2.5), where $\mathbf{x} = (x_1, x_2, x_3)$ is the image coordinate system.

The first step involves capturing volumetric undeformed, $I^0(\mathbf{x})$, and deformed, $\hat{I}^0(\mathbf{x})$, images. For the neutrophil study, the volumetric images are captured by laser scanning confocal microscopy. Intensity distributions in these volumetric images are in the form of micron-sized fluorescent particles that are randomly dispersed throughout the imaging medium [24] (Fig. 2.5). The precision and resolution ultimately depends on this intensity information, including the particle density, size, and distribution.

Calculating Displacements ($\mathbf{u}^k$). Once the set of volumetric images $I^0(\mathbf{x})$ and $\hat{I}^0(\mathbf{x})$ are obtained, each volume is subdivided into interrogation windows of specified

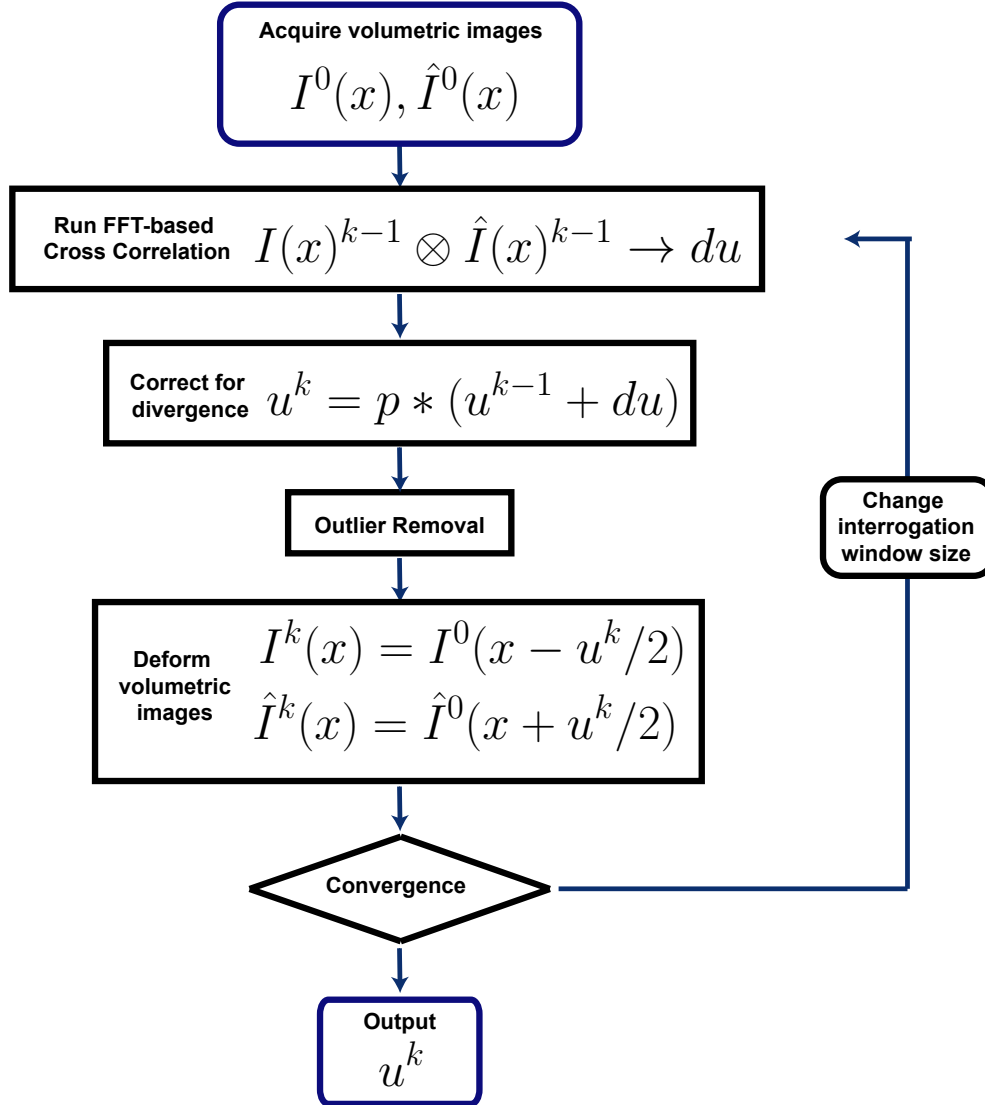


FIGURE 2.4. Flowchart showing the FIDVC method. The final displacement output is equal to the last iteration of the calculated displacement field, u^k .

overlap ratios, i.e., subset spacing. These two factors determine the final resolution of the DVC displacement field, and can be adjusted during each iteration to provide resolution refinement to increase the overall displacement field resolution. During each iteration the incremental displacements, $\mathbf{du} = (du_1, du_2, du_3)$, of corresponding windows in the undeformed and deformed images are estimated using the general

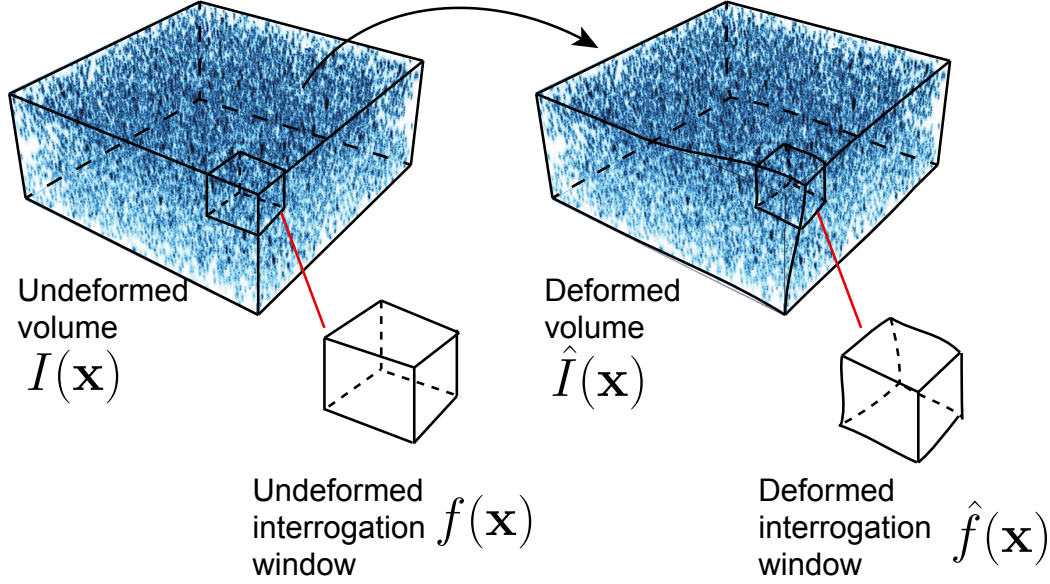


FIGURE 2.5. Schematic of volumetric image with a representation of interrogation windows in undeformed $f(\mathbf{x})$ and deformed configurations $\hat{f}(\mathbf{x})$.

cross-correlation relation

$$I^{k-1}(\mathbf{x}) \otimes \hat{I}^{k-1}(\mathbf{x}) \rightarrow \mathbf{du}, \quad (2.3)$$

with k being the integer iteration counter starting at one until convergence. Equation (2.3) can be rewritten in terms of the cross-correlation coefficient, C , as

$$C(\mathbf{du}) = \sum_{\mathbf{x}=-L/2}^{L/2} f(\mathbf{x}) \cdot \hat{f}(\mathbf{x} + \mathbf{du}). \quad (2.4)$$

Here, $f(\mathbf{x})$ and $\hat{f}(\mathbf{x})$ are the gray level intensity values of the undeformed and deformed interrogation windows (Fig.2.5). Sub-voxel resolution can be achieved by fitting the 3^3 voxel cross-correlation peak to a Gaussian polynomial to yield a more accurate estimate of the true sub-voxel peak location. While the literature has produced a wealth of various sub-voxel algorithms [33], we have previously demonstrated that a 3rd order polynomial Gaussian peak fitting scheme provides both good accuracy and time efficiency [24].

Due to the intrinsic averaging process in the cross-correlation formulation some of its high-frequency content is inevitably lost, which can be related to the Nyquist criterion. Consequently the minimum wavelength that can be resolved is only greater than twice the mean distance between markers within an interrogation window [67]. Therefore, the modular transfer function (MTF) of the classical cross-correlation approach shown in Eq.(2.4) is approximated by the frequency response of a moving average filter [66]. In the case of a 3D moving average filter, the MTF is given by:

$$\text{MTF}(\boldsymbol{\lambda}) = \prod_{i=1}^3 \frac{\sin(\pi L/\lambda_i)}{\pi L/\lambda_i} \quad (2.5)$$

where L is the side length of the interrogation window, and $\boldsymbol{\lambda} = (\lambda_1, \lambda_2, \lambda_3)$ are the wavelengths under study along the $\boldsymbol{x} = (x_1, x_2, x_3)$ directions respectively. It is evident that when $\boldsymbol{\lambda} = nL$, n being a positive integer, the $\text{MTF} = 0$ and thus the frequency response is unstable; the moving average produces irreversible loss of information. Motivated by this, an appropriate choice of a window weighting function can be used to flatten the frequency response of a moving average. While there are many choices of weighting functions that stabilize the frequency information, we choose to adopt the robust and straightforward formulation by Nogueira et al [68] given by

$$\begin{aligned} w(\boldsymbol{x}) = & \prod_{i=1}^3 \left(12 \left| \frac{x_i}{L} \right|^2 - 12 \left| \frac{x_i}{L} \right| + 3 \right. \\ & + 0.15 \cos(4\pi x_i/L) + 0.2 \cos(6\pi x_i/L) \\ & \left. + 0.1 \cos(8\pi x_i/L) + 0.05 \cos(10\pi x_i/L) \right)^{1/2}. \end{aligned} \quad (2.6)$$

By incorporating this particular weighting function into the FIDVC process, the cross-correlation coefficient shown in Eq. (2.4) becomes

$$C(\boldsymbol{du}) = \sum_{\boldsymbol{x}=-L/2}^{L/2} w(\boldsymbol{x}) f(\boldsymbol{x}) \cdot w(\boldsymbol{x} + \boldsymbol{du}) \hat{f}(\boldsymbol{x} + \boldsymbol{du}) \quad (2.7)$$

in which $f(\mathbf{x})$ and $\hat{f}(\mathbf{x})$ are gray level intensity values of the undeformed and deformed interrogation windows respectively, and $w(\mathbf{x})$ is the window weighting function given by Eq. (2.6). In the case for our FIDVC, Eq.(2.7) is implemented in Fourier space as

$$C(\mathbf{du}) = \mathcal{F}^{-1}\{\mathcal{F}\{w(\mathbf{x})f(\mathbf{x})\}^* \cdot \mathcal{F}\{w(\mathbf{x} + \mathbf{du})\hat{f}(\mathbf{x} + \mathbf{du})\}\}. \quad (2.8)$$

As before the integer-voxel displacements are found by finding the location of the global maximum of C . Although the use of the weighting function does not completely eliminate the low-pass behavior of the cross correlation process it significantly improves the reconstruction of the high frequency displacement information per interrogation window.

The Iterative Digital Volume Correlation Method. In order to account for large material deformations but yet remain computationally efficient, the deformation field between images $I(\mathbf{x})$ and $\hat{I}(\mathbf{x})$ is linearized into k -increments for which the cross-correlation algorithm is well suited. As previously mentioned, such an experimental approach has been well documented for particle image velocimetry (PIV) measurements, and is known as the image deformation method (IDM) [34, 37]. Here we extend the IDM method to measure material displacements in three dimensions. Fig. 2.6 illustrates the working principle of the IDM of our FIDVC algorithm. In Fig. 2.6 (A), it shows that the original images $I^0(\mathbf{x})$ and $\hat{I}^0(\mathbf{x})$ are symmetrically warped by the displacement field of the current iteration, $\mathbf{u}^k$, which is equal to the sum of all the previous displacement increments, i.e.,

$$\mathbf{u}^k = \sum_k \mathbf{u}^{k-1} + \mathbf{du}. \quad (2.9)$$

The warping of the two images, i.e.,

$$\begin{aligned} I^k(\mathbf{x}) &= I^0(\mathbf{x} - \mathbf{u}^k/2) \\ \hat{I}^k(\mathbf{x}) &= \hat{I}^0(\mathbf{x} + \mathbf{u}^k/2) \end{aligned} \quad (2.10)$$

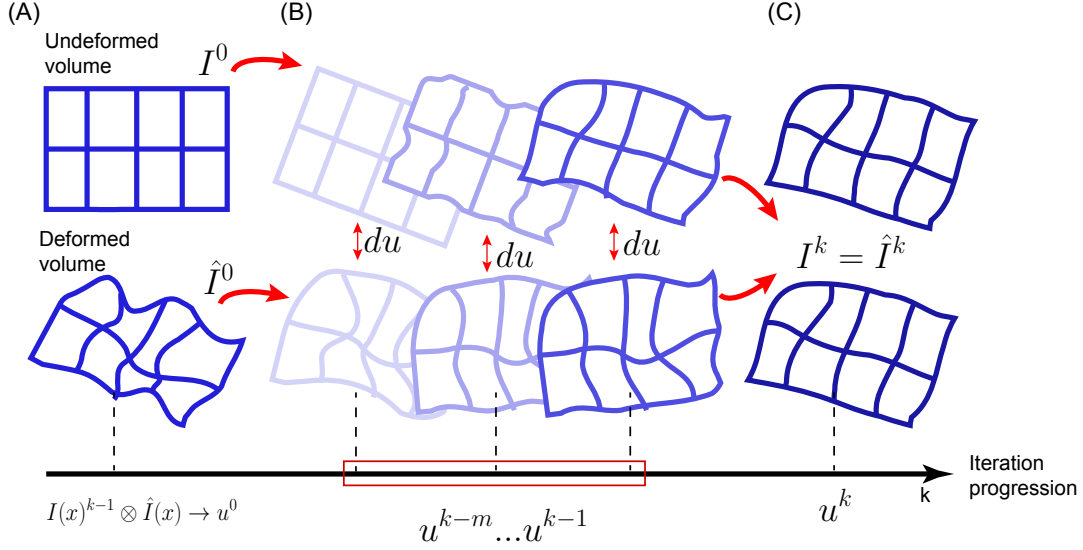


FIGURE 2.6. Schematic showing the IDM. Initially, (A) cross-correlation is used to find displacement field between undeformed and deformed volumes. (B) Next, the undeformed and deformed images are symmetrically warped by the linearized, incremental displacement field until they reach (C) the same final configuration.

is implemented using a trilinear interpolation scheme. Although higher order interpolation schemes have shown to be more accurate [1], efficient calculation usually renders them unpractical for large 3D data sets.

The iterative process in the IDM is intrinsically unstable and unless proper steps are taken, the results may diverge [66, 1, 87]. Furthermore, in multi-frequency displacement fields, the larger spatial wavelengths ($\lambda > L$) tend to diverge later in the iterative process while the smaller spatial wavelengths are still converging. For this reason employing a spatial filter during the iterative process serves as a suitable option to deal with these undesired results. A class of linear low-pass convolution filters have been introduced by Schrijer et al. that are computationally efficient and have the ability to change strength depending on a single input parameter [87]. The one-dimensional formulation of the filter is described by the following equation:

$$p(\xi) = \left(\frac{L}{2}\right)^z - |\xi|^z \quad (2.11)$$

where the kernel window length given by L is equal to the interrogation window side length. The parameter z controls the strength of the low-pass filter. No filtering occurs when $z \rightarrow 0$ since the filter approximates a Dirac delta function and as $z \rightarrow \infty$ the filter becomes a top-hat filter. The filter strength therefore increases with z and provides higher instability damping at the expense of lower spatial resolution. Performing numerical and experimental assessments of different filter strengths show optimal dampening and modulation characteristics over the range of $10^{-3} \leq z \leq 10^{-2}$ [87].

However, the results vary depending on when the filter is applied during the iterative process. For example, the filter can have an indirect influence on the displacement field if it acts on the incremental displacement field, i.e., $p * \mathbf{du}$. It is chosen that the filter acts on the current displacement field, $\mathbf{u}^k$ given by

$$\mathbf{u}^k = p * (\mathbf{u}^{k-1} + \mathbf{du}), \quad (2.12)$$

thus affecting the FIDVC results directly.

In addition to implementing a displacement damping filter prior application of the IDM, any non-physical displacements are also removed by means of a universal median test. This assists in producing smoother gradient fields by ensuring that spurious displacement values are removed. In general, DVC measurements are sensitive to noise in the recorded volumetric images and to the choice of interrogation window size. There is an optimal trade-off between the interrogation window size, the number of spurious displacements and spatial resolution present in the DVC results. For this reason, the results for every iteration are analyzed by the universal median test [105]. In this test, the median value $\mathbf{u}_m$ of a 3^3 voxel neighborhood surrounding a displacement value $\mathbf{u}_0$ is calculated. Using the median value, a residual defined by $\mathbf{r} = |\mathbf{u}_0 - \mathbf{u}_m|$ is determined for each displacement value of the surrounding neighborhood, $[\mathbf{u}_1, \dots, \mathbf{u}_{26}]$. The median residual, $\mathbf{r}_m$ of $[\mathbf{r}_1, \dots, \mathbf{r}_{26}]$ is then used to normalize the residual,

$$\mathbf{r}' = \frac{|\mathbf{u}_0 - \mathbf{u}_m|}{\mathbf{r}_m + \epsilon} \quad (2.13)$$

where ϵ represent the typical fluctuation level of the cross-correlation. This procedure is repeated for every displacement vector and any residual greater than a chosen level is removed.

The final step in the FIDVC process is to check whether the displacement values, $\mathbf{u}^k$ have converged. Having a well defined stopping criterion is important to provide proper balance between accuracy and computation times in the FIDVC algorithm. For example, stopping the algorithm prematurely will produce under predicted displacement fields, while over iterating will be time consuming without significant gain in displacement accuracy. Hence the most natural stopping criterion is at a point in which the images have fully-matching intensity profiles. The image similarity metric of choice is the normalized sum of squared error at the current iteration, e^k , given by

$$e^k = \frac{1}{\bar{\sigma}} \sqrt{\frac{1}{N} \sum_{\mathbf{x}=1}^N \left(I^k(\mathbf{x}) - \hat{I}^k(\mathbf{x}) \right)^2}. \quad (2.14)$$

Here N is the number of voxels, and $\bar{\sigma}$ is the mean standard deviation of the undeformed and deformed images, $I^k(\mathbf{x})$ and $\hat{I}^k(\mathbf{x})$.

After each iteration, the difference in the error between the current and previous iteration is calculated and used as a interrogation window size refinement or stopping criterion. To illustrate how the convergence of the FIDVC works, consider the following example run flowchart in Fig. 2.7. In the first step, the displacement field of the current iteration, k is calculated from Eq. (2.7) and the images are warped according to Eq. (2.10). The normalized sum of squared error is calculated for the current iteration, e^k and is used to calculate the error difference between the current and previous iteration, i.e., $\Delta e = e^k - e^{k-1}$. If $\Delta e^k / \Delta e^0$ is less than or equal to the user defined stopping threshold ϵ_0 than the FIDVC is stopped and said to have converged. If this condition is not met, we test against the interrogation window refinement criterion; if $\Delta e^k / \Delta e^j$ is less than or equal to ϵ_1 the interrogation window length, L is reduced by half and the loop continues. The subscript j is a simple counter denoting the last time point the interrogation window size was changed.

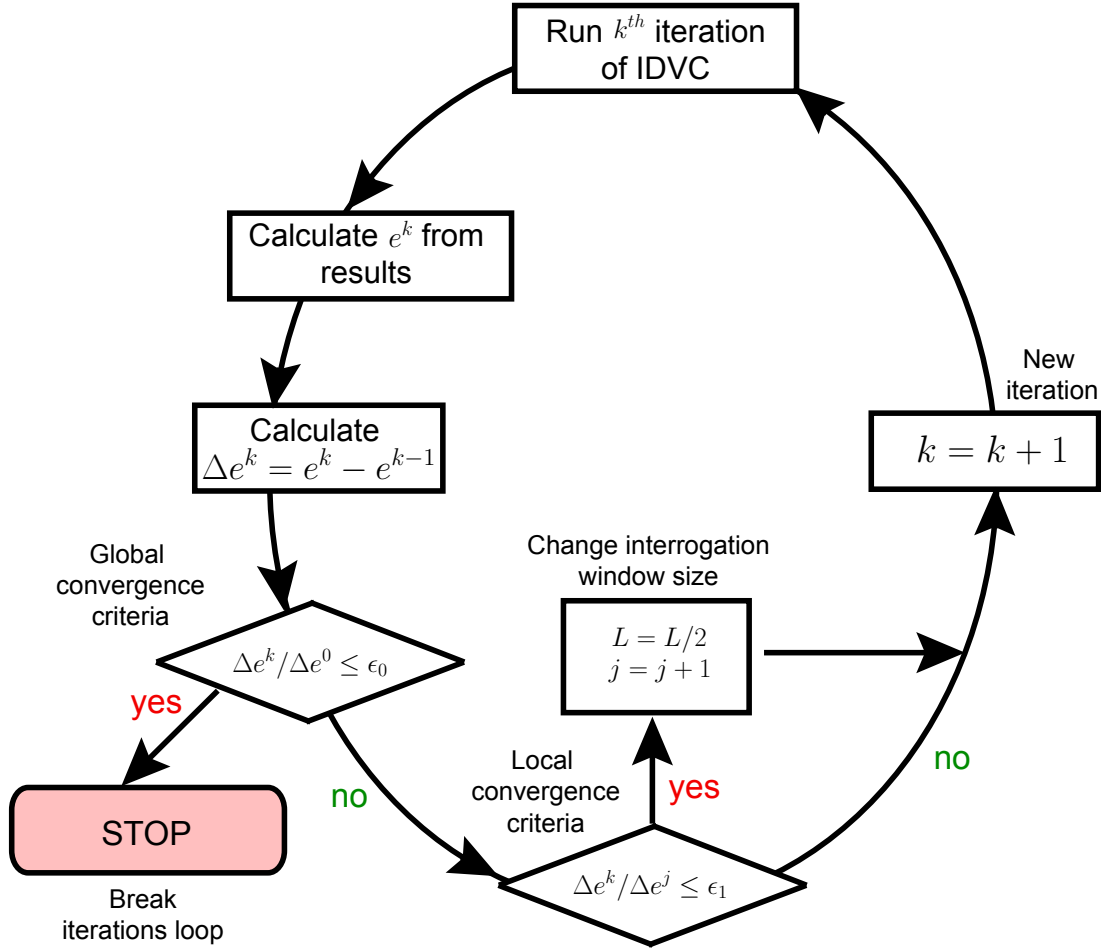


FIGURE 2.7. Run flow chart showing the convergence scheme of the FIDVC.

3D Displacement Gradient Calculations. From elementary continuum mechanics, the strains and stresses due to the displacements are directly related to its gradients. Care must be exercised when differentiating the displacement field in the presence of noise and large displacement gradients. The application of regularization schemes to the displacement values prior to numerical differentiation provides optimal noise mitigation and sample de-aliasing strategies. Such regularization may be accomplished with a low-pass filter designed to match the differentiation kernel to produce an overall optimal gradient operator. In our FIDVC, we identify and implement several suitable filters that are based on the work performed by Farid et al. [23]. Specifically, we formulate the optimal differentiation and interpolation kernel solution as a

minimization problem with the constraints that the filters are linear, separable, and rotationally-invariant. In Fourier space, this set of constraints allows reformulating the problem as a 1D minimization procedure making it fast to calculate large filters with kernel lengths of nine or greater points. The matching pair of filters that make up the gradient operator are called optimal n -tap, where n denotes the length of the filter kernel. Precaution has to be taken when choosing an appropriate filter length: while larger n values reduce errors due to noise, they provide excessive smoothening of the recorded displacements resulting in a poorer gradient estimate. More sophisticated noise mitigation models can be implemented, which are beyond the scope of this work.

Validation of the FIDVC Algorithm. This section presents the quantitative validation of our FIDVC methodology detailing the spatial resolution limits and average processing times. The validation examples presented here are primarily based on simulated deformation fields. To mimic experimental conditions, all of our simulated images are corrupted with experimental noise determined from actual laser scanning confocal microscopy (LSCM) images. The choice in numerical rather than experimental validation allows us to generate analytically exact, non-linear localized material deformations to assess the FIDVC spatial resolution limits quantitatively.

Generation of Simulated Volumetric Images and Displacement Fields. Fig. 2.8 shows three representative volumetric images that were used in the validation of the FIDVC algorithm. The optical system's point spread function (PSF) is determined by least-squares fitting of a 3D Gaussian profile to the intensity values of 1000 randomly sampled micron-sized fluorescent particles within the image [5]. The PSF with amplitude A and standard deviation, $\boldsymbol{\sigma} = (\sigma_1, \sigma_2, \sigma_3)$ is expressed as

$$PSF(\mathbf{x}) = A \exp \left(- \sum_{i=1}^3 \frac{x_i^2}{2\sigma_i^2} \right). \quad (2.15)$$

The following fitting parameters $\boldsymbol{\sigma} = (5, 5, 10)$, and $\rho = 0.004$ markers per voxel are determined from the experimental data and used for the generation of all subsequent

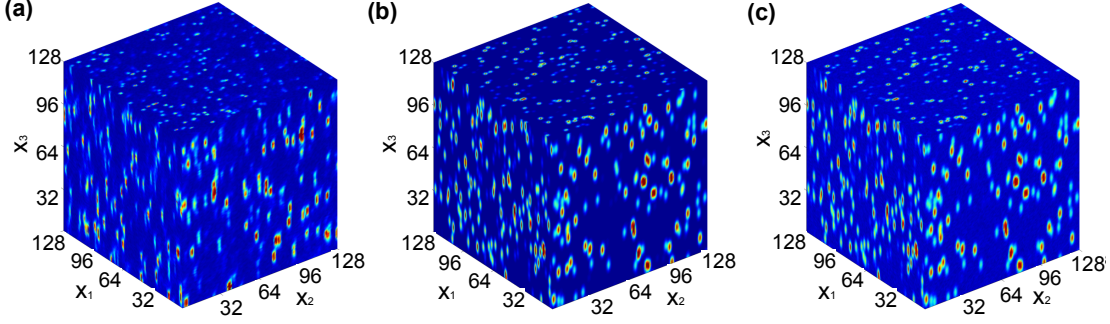


FIGURE 2.8. Slices of three volumetric images used for FIDVC validation. The first image (a) displays an actual experimental image of a transparent polyacrylamide gel embedded with 0.5 micron-sized fluorescent spheres recorded via LSCM. The second image (b) shows a numerically simulated image based on the image properties of (a) without noise. The third image is the numerically simulated image (b) with added Gaussian noise.

synthetic images. Next, a 12-bit grayscale undeformed image $I^0(\mathbf{x})$ is digitally generated by superimposing the intensities of multiple PSFs at random integer locations throughout the image shown in Fig. 2.8(b). This superposition of intensities is given by

$$I(\mathbf{x}) = \sum_{n=1}^N PSF(\mathbf{x} - \mathbf{x}^n) \quad (2.16)$$

where $\mathbf{x}^n = (x_1^n, x_2^n, x_3^n)$ is the center location of the n^{th} micron-sized fluorescent sphere. The total number of beads, N , is calculated by multiplying the marker seeding density, ρ , with the number of voxels of the image, V , i.e., $N = \rho V$. Any image intensity greater than the maximum 12-bit gray scale level, is saturated to simulate true LSCM images. The same procedure is utilized to digitally generate the deformed image, $\hat{I}(\mathbf{x})$, except each marker location, $\mathbf{x}^n$ is translated to its new location, $\mathbf{X}^n = (X_1^n, X_2^n, X_3^n)$ by the prescribed displacement field, $\mathbf{u}^n$, i.e.,

$$\mathbf{X}^n = \mathbf{x}^n + \mathbf{u}^n. \quad (2.17)$$

To rigorously characterize and quantify the spatial resolution of our new FIDVC technique a superposition of Gaussian displacement functions is applied to a set of

volumetric images given by

$$g(\mathbf{x}) = \sum_j A^j \exp \left(- \sum_{i=1}^3 \frac{(x_i - c^j)^2}{2(\sigma_i^j)^2} \right) \quad (2.18)$$

where

- (1) The number of Gaussian functions, j is chosen by the user.
- (2) The Gaussian width is defined as two times the full width at half maximum (FWHM) of the standard deviation, i.e. $w = 4\sqrt{2\ln 2} \sigma$, and is equal in all directions.
- (3) The amplitude, A , is chosen such that the deformation is in the finite deformation regime. The analytic Lagrangian strain is computed from the deformation field given in eq. 2.18. For any user defined width (w), the amplitude (A) is calculated from solving where the peak value of any strain component is equal to 15%.
- (4) The center position of the peak, c for each Gaussian displacement field is chosen not to overlap with any neighboring functions.

First, this approach allows simultaneous application of several well defined spatial deformation wavelengths to assess the FIDVC resolution limits. Second, it provides an analytical benchmark for a quantitative assessment of various finite difference schemes in estimating the Lagrangian strains. Physical examples of such displacement fields can be found in many cell-induced material deformations [16, 52, 25].

The FIDVC algorithm is written in MATLAB (MathWorks, Natick, MA) and executed on a custom built PC with an Intel i7-3820 CPU at 3.6 GHz, 8GB RAM memory, and Nvidia GTX 690 GPU. To significantly reduce computation times, Eq. (2.8) is implemented on the PC's GPU using the commercially available AccelerEyes Jacket library (AccelerEyes, Atlanta, GA).

FIDVC Convergence. Since the FIDVC algorithm is an iterative technique, the convergence behavior of the algorithm needs to be carefully characterized. A simulated image of size $512 \times 512 \times 256$ voxels is deformed according to a displacement

field given by a superposition of four Gaussian functions of widths 32, 64, 96, and 128 voxels (Fig. 2.9(inset)). The total error, e between the undeformed and deformed images given by Eq. (2.14) is used as the convergence metric. Figure 2.9(A) shows the error per iteration along with the corresponding $x_1 - x_2$ contour map of the prescribed displacement in the inset. Figure 2.9(B) presents calculated displacement field magnitudes $|\mathbf{u}|$ in iterations 1, 3, 5 and 7 (2.9(B)). The corresponding interro-

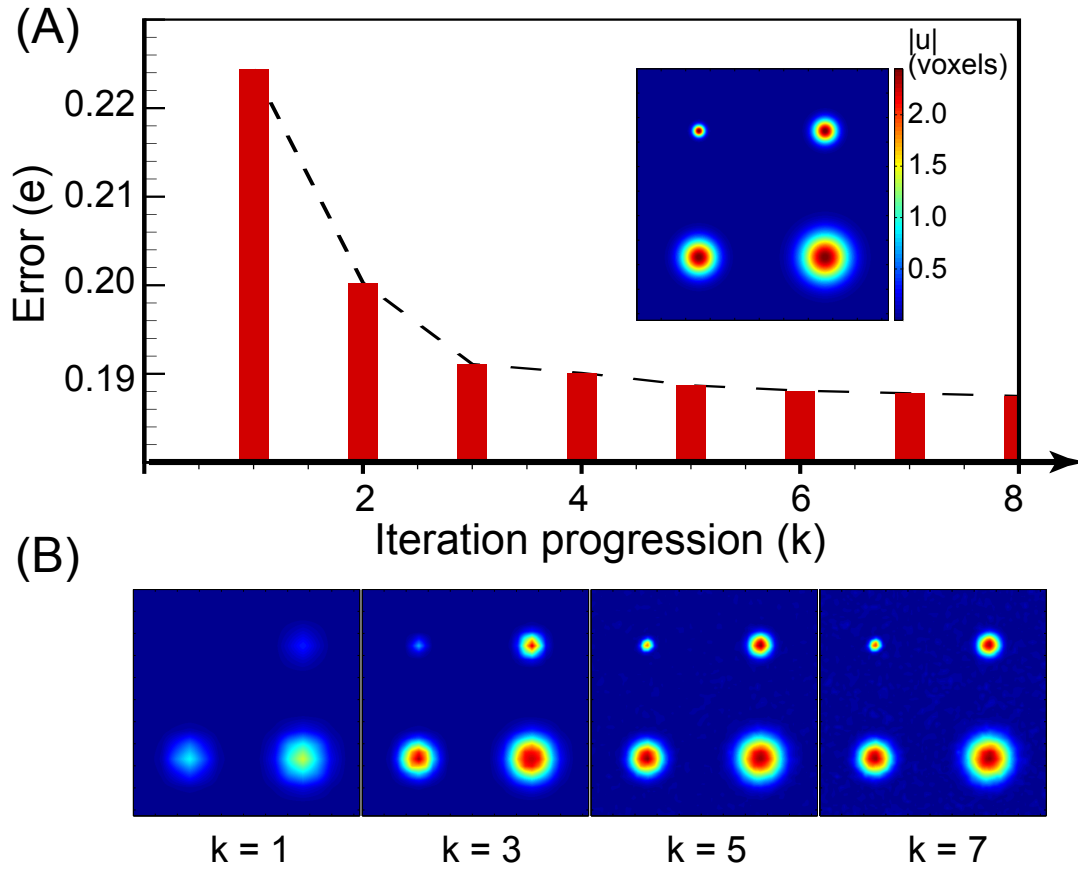


FIGURE 2.9. (A) Normalized residual error, e calculated according to Eq. (2.14). An inset illustrates the prescribed displacement field, $|\mathbf{u}|$. (B) The contour maps represent an $x_1 - x_2$ cross-section of the displacement field magnitude, $|\mathbf{u}|$ for iterations 1, 3, 5 and 7.

gation window lengths for the iterations shown in Fig. 2.9 are 64, 32, and 32 voxels with a window overlap of 50%, 50%, and 75% respectively. The displacement contours in Fig.2.9(B) $k=1$ approximates the typically recovered displacement field using

Franck et al.'s DVC method [24]. Qualitative comparison with the prescribed solution clearly shows that most of the higher frequency content is not properly captured. By utilizing more iterations and smaller interrogation window sizes, the FIDVC successfully captures the higher spatial frequencies and converges towards the prescribed displacement field.

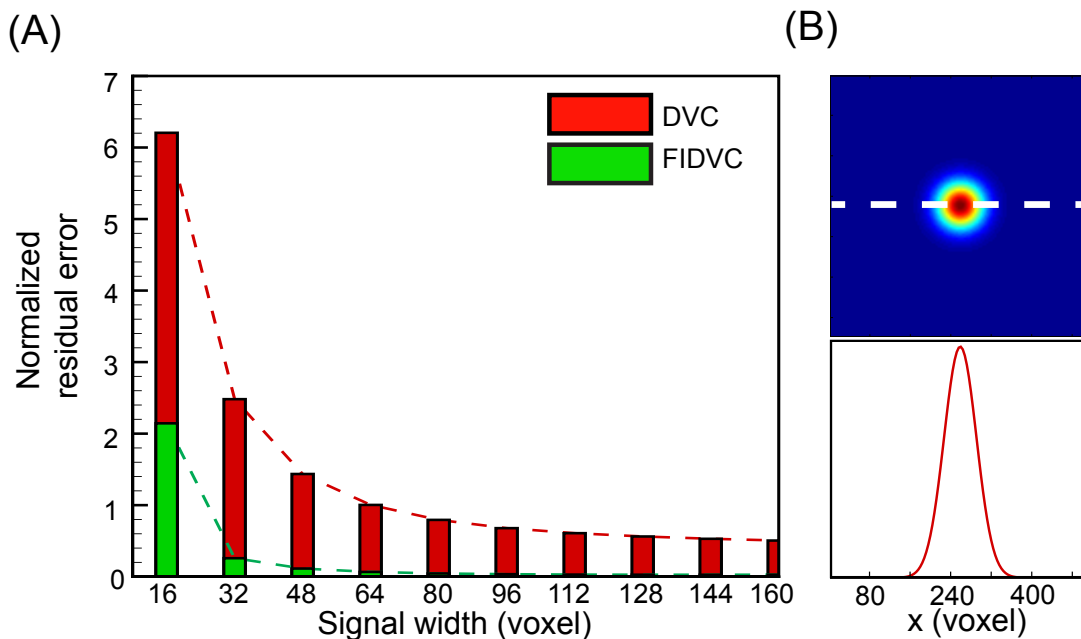


FIGURE 2.10. Comparison of the spatial resolution between the FIDVC and DVC algorithm. (A) Plot of the normalized residual error associated the prescribed Gaussian displacement fields of different signal widths. (B) Contour map of prescribed Gaussian displacement function with width of 128 voxels.

Spatial Resolution of the FIDVC technique. To quantify the resolution limits and errors associated with FIDVC over the previous DVC algorithm more rigorously, an undeformed volumetric image (Fig. 2.5) of size 256^3 voxels is numerically deformed by Gaussian displacement function and processed through both FIDVC and DVC algorithms. The width of the single applied Gaussian displacement function is varied incrementally from 16 to 160 voxels. To quantify the error between the numerically calculated and analytically prescribed displacements, the normalized residual error

for the displacement magnitude, e calculated by Eq. (2.14) for each width is shown in Fig. 2.10(A).

The undeformed and deformed images are processed using the FIDVC algorithm with an initial interrogation window size of 64 voxels and window overlap of 50%. The universal outlier removal filter is performed with a threshold of 2 and a fluctuation level of 0.1 based on the previously published displacement standard deviation values [105, 24, 82]. The polynomial degree of the predictor filter, z is chosen to be 7.5×10^{-3} and the convergence threshold for the interrogation window refinement is $\epsilon_1 = 0.25$. The stopping threshold is set to $\epsilon_0 = 0.0625$ based on empirical evidence of the various validation cases. For all numerical experiments performed using the previous DVC

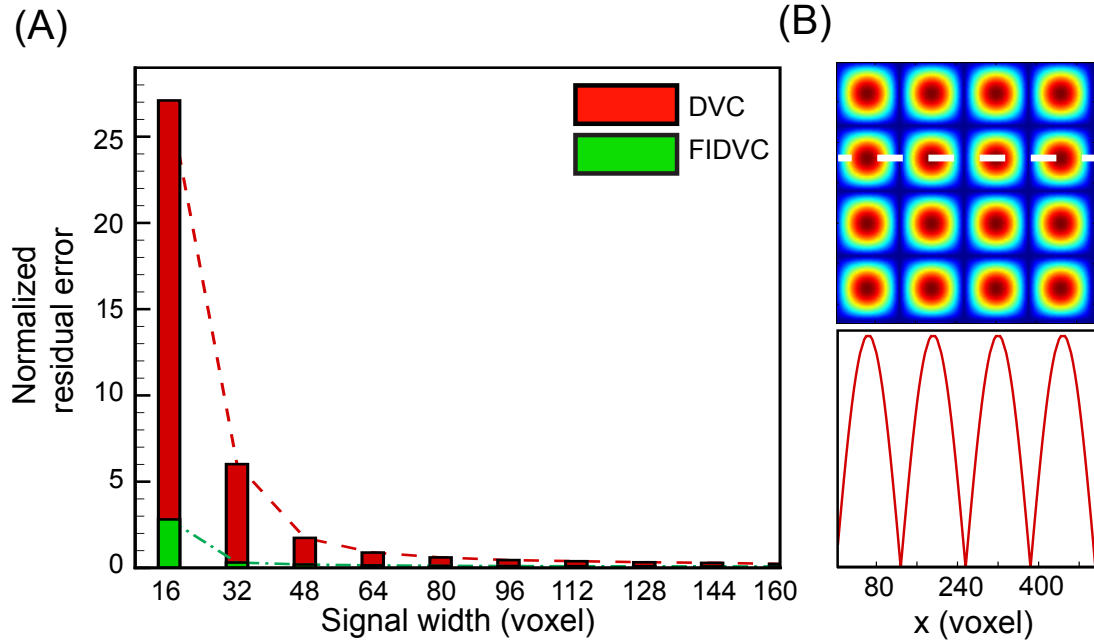


FIGURE 2.11. Comparison of the spatial resolution between the FIDVC and DVC algorithm. (A) Plot of the normalized residual error associated the prescribed sinusoidal displacement fields of different signal widths. (B) Contour map of prescribed sinusoidal displacement function with width of 128 voxels.

algorithm, the interrogation window size and window overlap is fixed at its optimal value of 64^3 voxels with a 75% window overlap. The final spatial resolution for both

the FIDVC and previous DVC algorithms results in an equal grid spacing of 8 voxels, and $49 \times 49 \times 33$ correlation points.

The corresponding normalized error values and DVC/FIDVC error ratios are summarized in Table 2.1.

TABLE 2.1. Normalized residual error of corresponding to the prescribed Gaussian displacement field for the FIDVC and DVC algorithm.

Signal Width (voxel)	Normalized Residual Error (e)		
	DVC	FIDVC	$\frac{\text{DVC}}{\text{FIDVC}}$
16	6.205	2.144	2.894
32	2.481	0.258	9.597
48	1.435	0.113	12.60
64	1.003	0.066	15.11
80	0.794	0.045	17.62
96	0.678	0.035	19.30
112	0.608	0.029	20.65
128	0.562	0.028	20.11
144	0.530	0.027	19.34
160	0.505	0.027	18.11

For both algorithms, the normalized residual error between the prescribed and numerically calculated displacement fields is worse for small signal widths and best for large signal widths as expected. The error from the DVC algorithm is a factor of 3 to 18 larger when compared to the FIDVC, resulting in a poorer overall displacement estimate. This result is not surprising since the regular DVC algorithm does not account for the higher order terms in its estimate of the displacement field.

It is also noted that the FIDVC is capable of detecting spatial widths below the smallest interrogation window size of 32 voxels. This is possible due to the use of the MTF weighting function given by Eq. (2.6). This suggests that the MTF weighting function plays an important role in detecting higher spatial frequencies. With an increase in fiducial marker seeding density, it is possible to decrease the smallest interrogation window size to 16 voxels thereby further improving the spatial detection

capability of the FIDVC algorithm. Similar to prescribed Gaussian displacements,

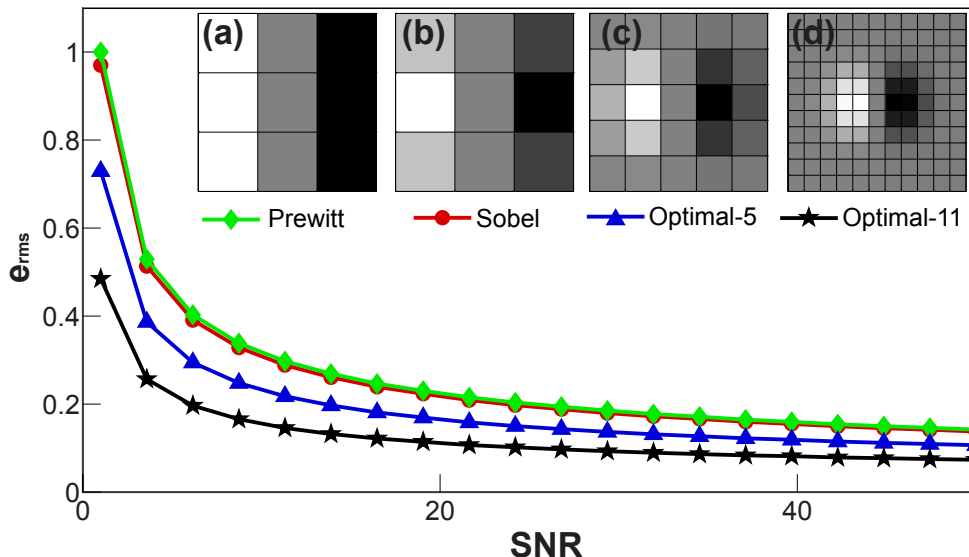


FIGURE 2.12. Root-mean-square error, e_{rms} between the FD kernels (a)-(d) and prescribed analytical displacement gradient magnitude, $|\nabla \mathbf{u}|$ for SNR levels ranging from 1 to 100. The inset shows the (a) Prewitt, (b) Sobel, (c) Optimal-5 tap, (d) Optimal-11 tap finite difference kernels.

the resolution is also validated for the prescribed sinusoidal displacement field to the volumetric images. Figure 2.11 shows higher error for the images deformed by a smaller width compared to the prescribed displacement of higher width. However, at the width size of 32, FIDVC performs at very low error, thus confirming the validity of capturing spatially small and non-linear displacements generated at cell-ECM contact points.

Calculating Displacement Gradients. It is important to assess the performance of different differentiation schemes used to calculate the displacement field gradient in the presence of noise. In this numerical experiment, the previous four-pole Gaussian displacement field with widths 32, 64, 96, and 128 voxels is corrupted with Gaussian white noise corresponding to varying signal-to-noise ratios (SNR). This allows quantification of the performance of various finite difference schemes as a function of SNR. For each displacement field, the gradient is calculated with a Prewitt

[75], Sobel [29], optimal-5 tap, and optimal-11 differentiation kernel and compared to the analytically calculated displacement field gradient. Fig. 2.12 shows the root-mean-square error, e_{rms} value calculated between the FD schemes and the prescribed analytical displacement gradient magnitude, $|\nabla \mathbf{u}|$ for SNR levels ranging from 1 to 100.

Out of the four kernels, the optimal-11 tap shows the best performance in handling noise and recovering the correct displacement gradient. To visualize how the RMS error affects the displacement gradient, Fig. 2.13 shows a contour map of the displacement gradient magnitude, $|\nabla \mathbf{u}|$ along the $x_1 - x_2$ plane for the four differentiation kernels with a SNR of 5.

The Prewitt and Sobel kernels show the most fluctuations in the calculated displacement gradient and thus are the most unreliable predictors of the correct strain field at low SNRs. On the other hand, the optimal-11 tap kernel shows the best performance in recovering the displacement gradient while retaining higher spatial frequencies. In the current work, the full-field deformation gradient tensor is calculated using 11-tap discrete differentiation kernel.

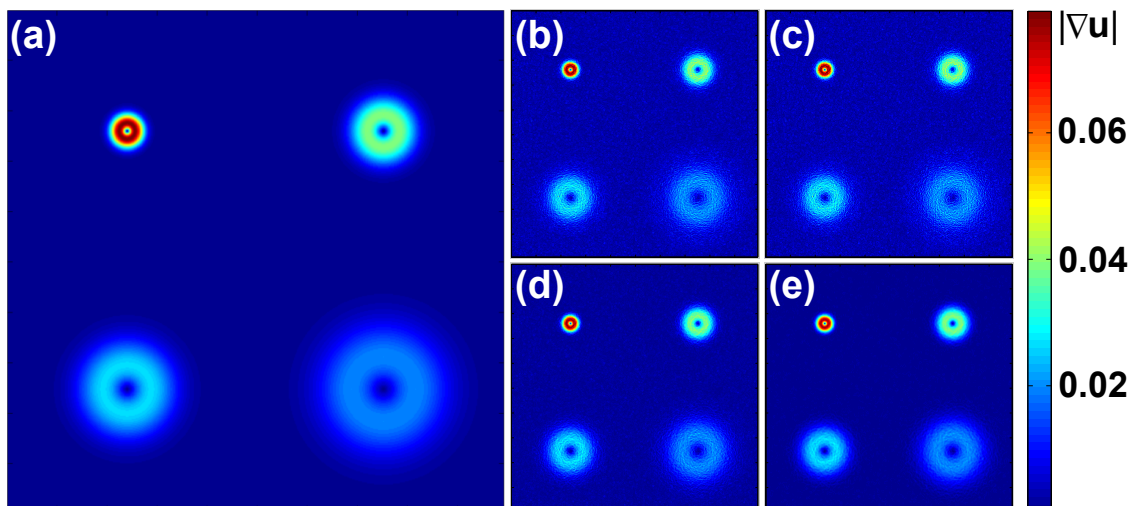


FIGURE 2.13. Comparison of various finite difference schemes applied to a prescribed four-pole Gaussian displacement field with SNR of five. (a) The corresponding analytical gradient magnitude and comparison with the (b) Prewitt, (c) Sobel, (d) optimal-5 tap, (e) optimal-11 tap differentiation kernels.

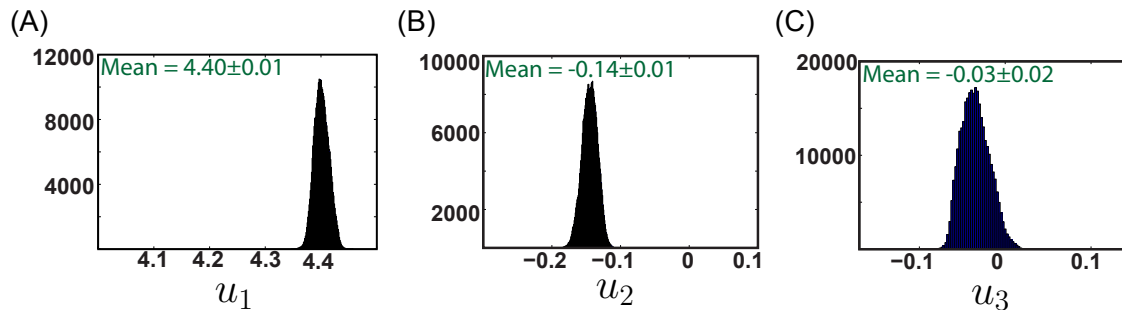


FIGURE 2.14. Histograms of the displacement field corresponding to a experimental rigid translation of 4.4 voxels along the x_1 direction. (A) u_1 , (B) u_2 , (C) u_3 .

Determination of the FIDVC Measurement Precision and Accuracy. To determine the measurement precision of the FIDVC algorithm, a zero-strain deformation is experimentally acquired using LSCM. In this experiment, a polyacrylamide hydrogel embedded with 0.5 micron-sized fluorescent particles is rigidly translated by 4.4 voxels along the x_1 direction. Images before and after translation are processed through the FIDVC to calculate the displacement field. The distribution along with the mean and standard deviation values are summarized in the histogram shown in Fig. 2.14. The mean and standard deviation of the corresponding Lagrangian strain field is computed using an optimal-11 tap gradient kernel and is summarized in Table 2.2.

The values of the standard deviation of the displacements are an order of magnitude smaller than those previously reported [82, 24]. This large increase in precision

TABLE 2.2. Mean and standard deviation values corresponding to the experimental zero-strain deformation

Component	Stationary		Translation	
	Mean (%)	SD (%)	Mean (%)	SD (%)
E_{11}	1.78×10^{-4}	0.003	1.39×10^{-3}	0.018
E_{22}	8.06×10^{-4}	0.006	4.67×10^{-4}	0.009
E_{33}	6.60×10^{-3}	0.005	7.47×10^{-3}	0.015
E_{23}	4.86×10^{-3}	0.003	4.13×10^{-3}	0.005
E_{13}	3.42×10^{-3}	0.003	8.30×10^{-3}	0.008
E_{12}	2.53×10^{-4}	0.002	6.62×10^{-4}	0.006

is likely due to the universal outlier removal process that is performed during each iteration. The axial precision of the displacements and strains in the x_3 direction is approximately 3-4 times lower than the transverse precision in the x_1, x_2 directions, caused by an axially elongated PSF seen in Fig. 3. The values of all strain components are close to zero and smaller than their corresponding standard deviation values confirming a zero-strain deformation.

Spherical Inclusion Problem (Validation). The full capability of the FIDVC algorithm is demonstrated by measuring a ubiquitous non-uniform 3D deformation, the classical solution of a spherical inclusion in an infinite linear elastic solid subjected to a remote stress along the x_3 direction (Eq.2.19) [6].

$$u_i = \frac{(1 + \nu)\sigma_0}{2E} \left(\left[2 + \frac{5(5 - 4\nu)}{(7 - 5\nu)} \frac{a^3}{R^3} + \frac{6}{(7 - 5\nu)} \frac{a^5}{R^5} \right] x_3 \delta_{i3} + \left[\frac{-2\nu}{(1 + \nu)} + \frac{(5\nu - 6)}{(7 - 5\nu)} \frac{a^3}{R^3} + \frac{3}{(7 - 5\nu)} \frac{a^5}{R^5} \left(1 - \frac{5x_3^2}{R^2} \right) \right] x_i \right) \quad (2.19)$$

In the presence of the inclusion under stress, the material both deforms non-uniformly around the cavity perimeter and homogeneously due to compression. To perform the numerical experiment, the analytical displacement field of an inclusion of radius $a = 48$ pixels under a remote stress of $\hat{\sigma}_\infty = \frac{\sigma_\infty}{E} = 0.04$ is applied to a 256^3 voxel undeformed image (Eq.2.19). The generated undeformed and deformed images are processed through the FIDVC algorithm, and the calculated full-field deformation fields are compared against the prescribed analytical solution. Fig. 2.15(a)-(b) compares the displacement field, u_3 of the calculated FIDVC results to the prescribed inclusion solution along the $x_2 - x_3$ inclusion center plane. The corresponding contour map of the deformation gradient's first invariant, $tr(\mathbf{F})$ is shown in Fig. 2.15(c)-(d). For visualization purposes, the spherical inclusion is shown in black and the region around the cavity is cropped to 128^3 voxels to better visualize the high gradients close to the inclusion. Qualitative comparison of the contour maps of the displacement fields between the FIDVC computed results and the prescribed solution show

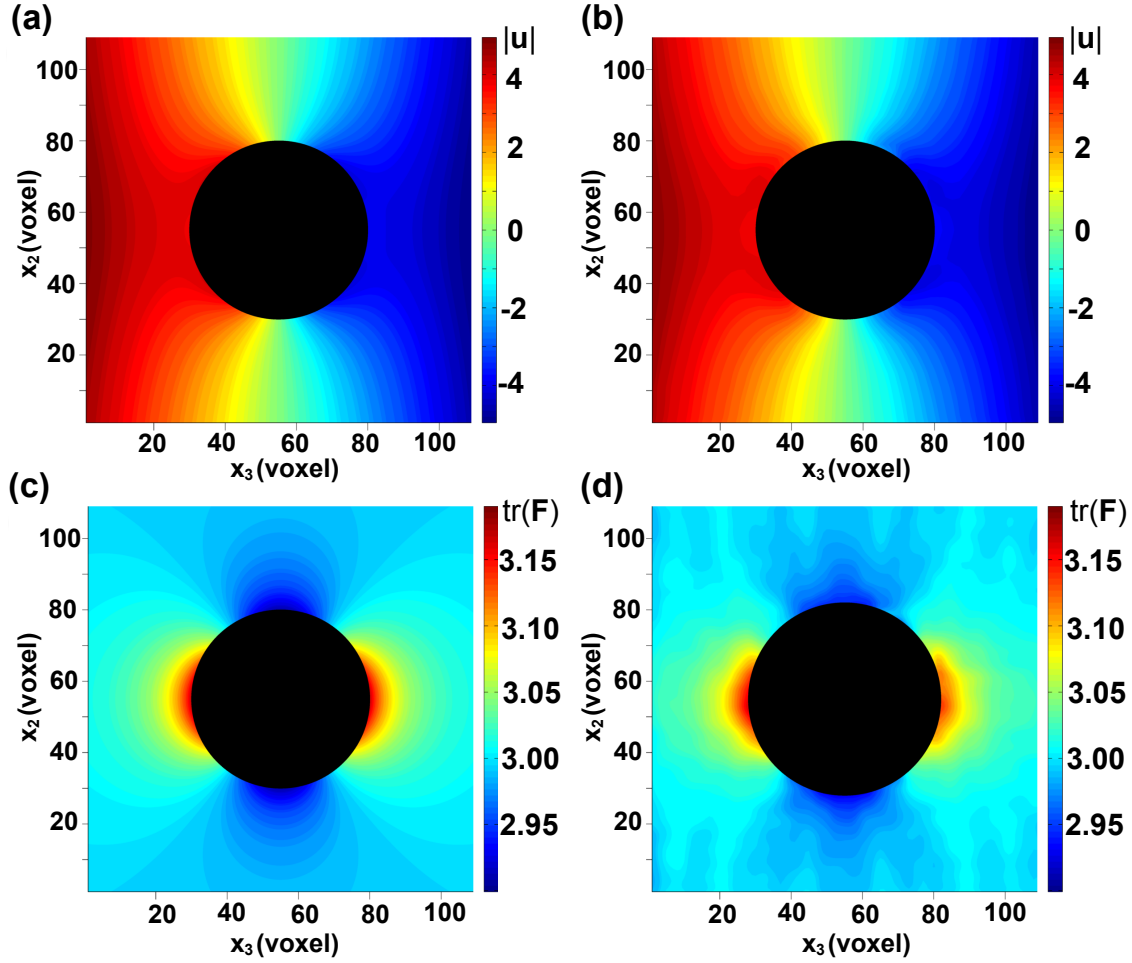


FIGURE 2.15. $x_2 - x_3$ slice comparing the (a) prescribed displacement field, u_3 , near a spherical inclusion under uniaxial compression with the (b) FIDVC results. The corresponding (c) analytical and (d) numerical calculation of the deformation gradient's first invariant, $tr(\mathbf{F})$.

excellent agreement in both the spatial distribution and magnitude of the color contour levels. When examining the first invariant, there is some deviation between the analytical solution and the FIDVC results due to slight numerical error associated with the FIDVC method.

Efficiency. The computational cost of DVC computations is expected to be significantly higher than that of regular 2D DIC due to the cubic interrogation window size. Consider two similar calculations, one with a DIC displacement grid of m^2 points with a square interrogation window size of L^2 pixels, and one with a DVC

displacement grid of m^3 points with a cubic interrogation window size of L^3 voxels. The computational cost of the DVC calculations will be $m \times L$ times greater than that for the DIC. The computational cost will be even higher at the usual DVC grid sizes of m equals 50 to 100 points with an interrogation window size of 64^3 voxels. For DVC applications to be competitive and widely applicable, fast computation speeds without compromising high-accuracy measurements are imperative.

The computational efficiency of our FIDVC algorithm is investigated by using the prescribed displacement field in Section 3 with sizes of $512 \times 512 \times M$ voxels where M is varied from 32 to 256 voxels in increments of 32 voxels. A given simulated image is processed using the FIDVC algorithm on both the PC's CPU and GPU using an initial interrogation window size of 64^3 voxels. The computation times of the result are then compared to the time it takes the original DVC algorithm to compute the same displacement field using a fixed interrogation window size of 64^3 voxels. To ensure a fair comparison between algorithms, the final interrogation window spacing is eight voxels for both algorithms. Table 2.3 shows the average computation times and speed up factors for an image of size $512 \times 512 \times 256$ voxels that has a final displacement grid of $65 \times 65 \times 33$ voxels or 1,081,665 computation points.

TABLE 2.3. Processing times and speed up ratios for an image of size $512 \times 512 \times 256$ with $65 \times 65 \times 33$ computation points. The image is analyzed with the FIDVC algorithm executed on a CPU (FIDVC-CPU) and a GPU (FIDVC-GPU), and compared to our original DVC algorithm executed on a CPU (DVC-CPU). The FIDVC converged after seven iterations.

Algorithm	Total Time (min)	Time per Iteration (min)	Speed Up Factor
DVC-CPU	35.5	—	—
FIDVC-CPU	12.2	1.75	2.90×
FIDVC-GPU	2.14	0.31	16.6×

The results clearly show that the FIDVC algorithm outperforms our original DVC code on both the CPU and GPU. This is in part due to the lower overlap factor throughout the early FIDVC iterations. It is also important to note the substantial

speedup the GPU provides; approximately $5.7\times$ when compared to executing the algorithm on the CPU. This is especially important since the purchasing cost of GPUs are far less than both supercomputing time and high end CPUs. In fact, the same GPU processing times can be maintained within a very simple computer architecture of a low to medium range personal computer. We hope that this will allow many end-users to take advantage of the FIDVC formulation for their studies.

4. Measurement of neutrophil-generated tractions

Note: The work has been performed and a version of this section has been published in Plos One in co-authorship with Bar-Kochba E. [Toyjanova J., Bar-Kochba E., Lopez-Fagundo C., Reichner J., Hoffman-Kim D., and Franck C., "High Resolution, Large Deformation 3D Traction Force Microscopy", *Plot one* (2014), 9]. All the published data included here with co-author's content.

Section 3 described FIDVC algorithm used to track the motions of embedded submicron-diameter fluorescent particles in PA substrates. Fig. 2.16 presents a side by side comparison of neutrophil induced displacements that were measured with the original DVC algorithm [24] and new FIDVC algorithm [3]. The displacement results are different in magnitude by $\approx 50\%$. There is also an obvious difference in the spatial resolution that is more localized with new FIDVC than what is found by original DVC.

As a result of this improvement in spatial resolution of DVC algorithm, it is found that cells can indeed generate non-linear material strains. This finding does not only have implication for traditional TFM methodologies, but also for cell traction assays that utilize very compliant micropillars [64]. Most micropillar systems calculate the applied post tractions according to the Euler-Bernoulli beam theory, which applies only to small deformations (SD) and strains. This may be entirely adequate if the

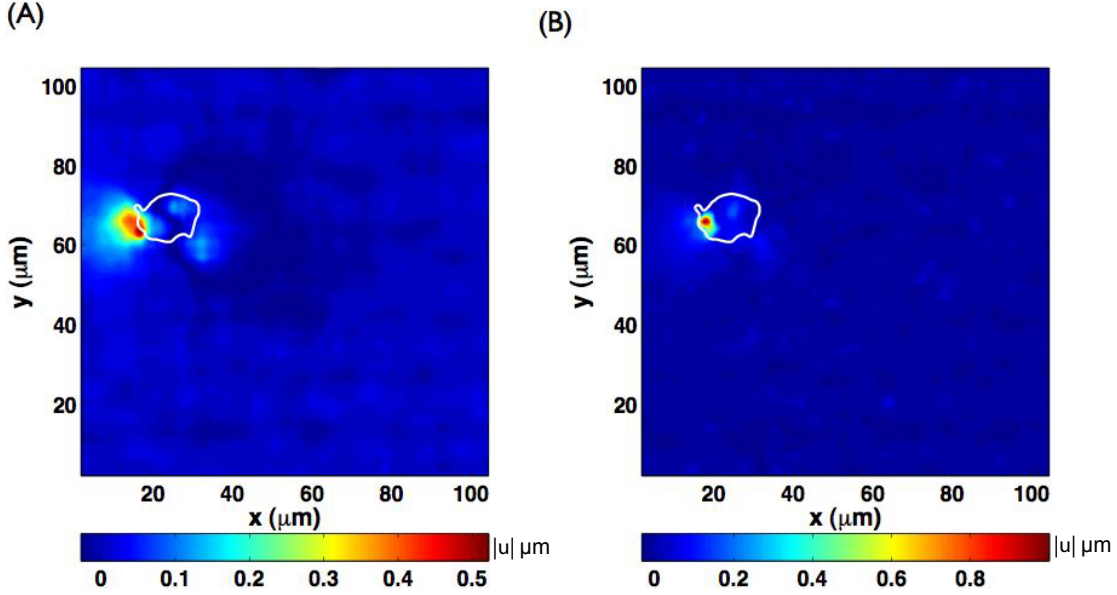


FIGURE 2.16. Side-by-side presentation of displacement field induced by a migrating neutrophil on 2D substrate measured by (A) original DVC algorithm and (B) new FIDVC algorithm

strains are indeed small, however in the presence of large strains may warrant additional large deformation (LD) analysis. This section describes implementation of LD analysis into the TFM methodology.

Finite deformations of migrating cells. In continuum mechanics, a material point undergoing a deformation from location $\mathbf{x}$ to location $\mathbf{y}$ can be represented by its material displacement, $\mathbf{u}(\mathbf{x})$ by

$$\mathbf{y} = \mathbf{x} + \mathbf{u}(\mathbf{x}). \quad (2.20)$$

Differentiating both sides with respect to $\mathbf{x}$ yields

$$\nabla \mathbf{y} = \nabla(\mathbf{x} + \mathbf{u}(\mathbf{x})) = \mathbf{I} + \nabla \mathbf{u}(\mathbf{x}) = \mathbf{F}, \quad (2.21)$$

where $\mathbf{I}$ is the identity matrix and $\mathbf{F}$ is the material deformation gradient tensor. The gradient tensor physically describes the shearing and stretching motion that a small line segment $d\mathbf{x}$ undergoes as it moves from configuration $\mathbf{x}$ to configuration $\mathbf{y}$.

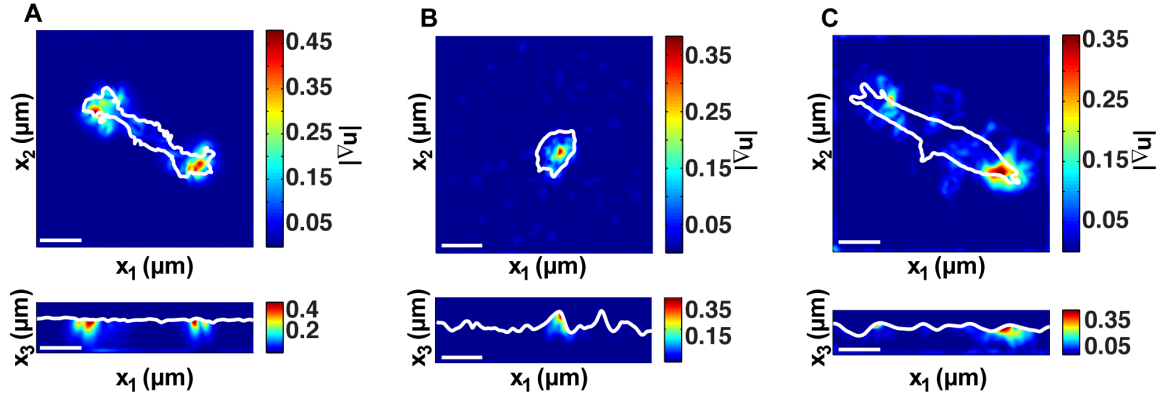


FIGURE 2.17. $x_1 - x_2$ and $x_1 - x_3$ cross-sections of the calculated 3D displacement gradient magnitude for (A) a Schwann cell (scale bar = $40 \mu\text{m}$), (B) a polymorphonuclear leukocyte (scale bar = $20 \mu\text{m}$) and (C) a NIH 3T3 fibroblast (scale bar = $20 \mu\text{m}$).

To calculate the material strains associated with that deformation, we compute the Lagrangian strain tensor as

$$\mathbf{E} = \frac{1}{2} (\mathbf{F}^T \cdot \mathbf{F} - \mathbf{I}). \quad (2.22)$$

Equation 2.22 can be rewritten in terms of the material displacement as

$$\mathbf{E} = \frac{1}{2} (\nabla \mathbf{u} + (\nabla \mathbf{u})^T + \nabla \mathbf{u} \cdot (\nabla \mathbf{u})^T), \quad (2.23)$$

where the term $\nabla \mathbf{u}$ is known as the displacement gradient.

Equations 2.20 - 2.23 are valid for any non-linear finite (large) homogeneous deformation. In small strain and linear elasticity, which is the fundamental framework of almost all traction force microscopy (TFM) methodologies [15], the displacement gradient term is assumed to be small, i.e., $\nabla \mathbf{u} \ll 1$, which simplifies Eq. 2.22 to

$$\boldsymbol{\epsilon} = \frac{1}{2} (\nabla \mathbf{u} + (\nabla \mathbf{u})^T). \quad (2.24)$$

The quantity $\boldsymbol{\epsilon}$ is known as the infinitesimal strain tensor. Most of the previous TFM studies justified this assumption by showing that the measured cellular strains were within 10% of their nominal or principal values [22, 19, 14].

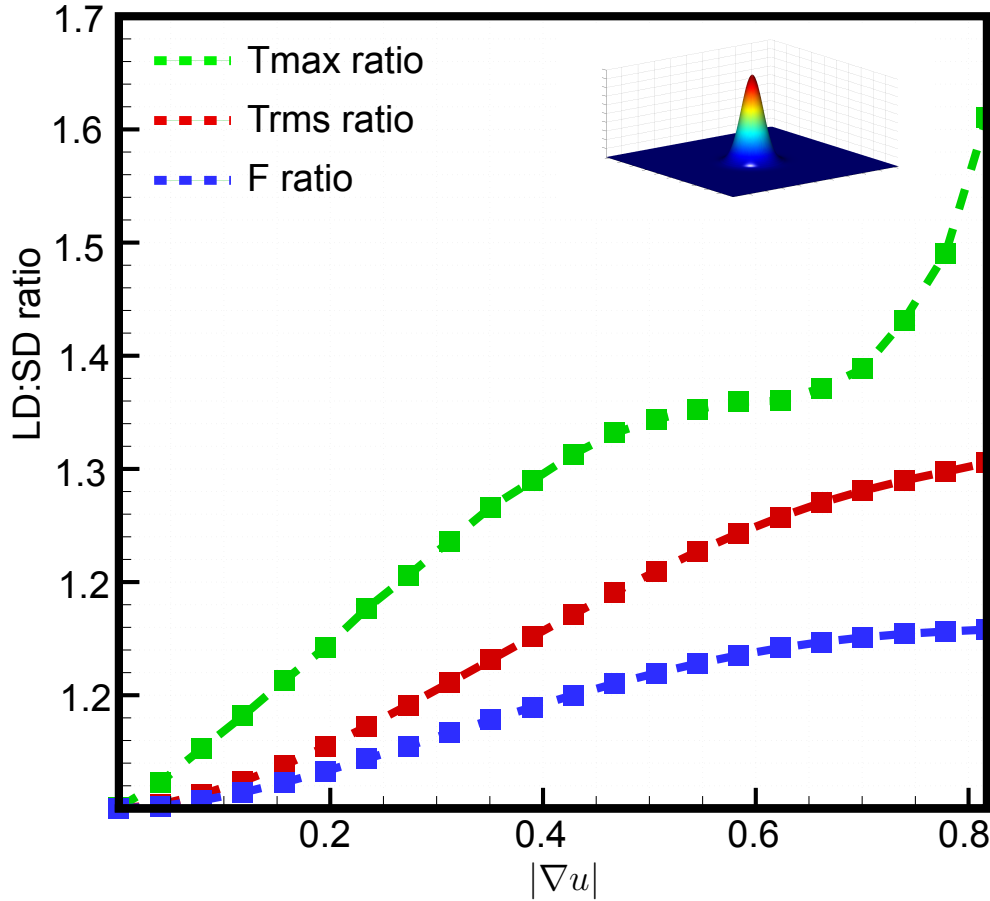


FIGURE 2.18. Total force (F), root mean squared tractions (T_{rms}) and maximum traction (T_{max}) ratios plotted against the displacement gradient under the application of a 3D Gaussian-shaped displacement field (inset). The numerator in the ratios is calculated using the new large deformation approach whereas the denominator features the results from the traditional linear elastic, small deformation material approximation.

Due to the improvement in spatial resolution of digital volume correlation algorithm presented in Section 3 of this chapter, it was found that cells, including neutrophils, are indeed capable of producing large deformations. Figure 2.17 presents color contour plots of three different cells seeded on polyacrylamide gels: (A) a Schwann cell, (B) a polymorphonuclear neutrophil (PMN), and (C) an NIH 3T3 fibroblast all generating large displacement gradients, or material strains. The choice

of plotting the magnitude of the displacement gradient rather than a particular strain component is to show with specific numbers that the non-linear component can no longer be neglected, and that the finite deformation behavior of the material and its associated Lagrangian strains ($\mathbf{E}$) need to be accounted for in Eq. 2.23. This is further illustrated in Fig. 2.18 showing significant deviations in the traction estimates between the small strain, linear and finite deformation theory as the displacement gradient term increases. Figure 2.18 quantifies the deviation in surface tractions and total force between the LD and SD analyses as a function of the displacement gradient magnitude. This is not to say that linear theory is always inappropriate, but rather to point out that for high cellular displacement gradients the use of small strain theory can produce significant error in reporting accurate cell tractions.

Estimating 3D LD Cellular Traction. Figure 2.19 illustrates the steps that have to be taken to use 3D TFM technique for large deformations (LD 3D TFM). Analogous to the previous 3D TFM technique [25], 3D volumetric image stacks are acquired using laser scanning confocal microscopy (LSCM) (described in Section 3). The overall dimensions of each stack are user-dependent, but are generally within the range of $512 \times 512 \times P$ voxels ($x_1 \times x_2 \times x_3$), where P ranges from 64 to 256 pixels. Using a resonant Galvano mirror and Piezo objective nosepiece, 3D volumes can be acquired within time lapse intervals as small as ten seconds, which allows following fast cells like neutrophils.

After the cellular displacements, $\mathbf{u}$, are determined via FIDVC, the deformation gradient tensor, $\mathbf{F}$, is calculated via finite differences. As shown in Eq. 2.21, the displacement gradient, $\nabla \mathbf{u}$, and the deformation gradient tensor, $\mathbf{F}$, are directly related to the gradients of the measured displacement field. The current differentiation kernel featured in the here-presented LD 3D TFM technique employs an optimal-11 tab filter, which is described in detail by Farid et al. [23] and in section 3 of this chapter.

Similar to the SD 3D TFM approach, calculating cellular surface tractions first requires determination of the material's true stress tensor, or Cauchy stress $\boldsymbol{\sigma}$. Given

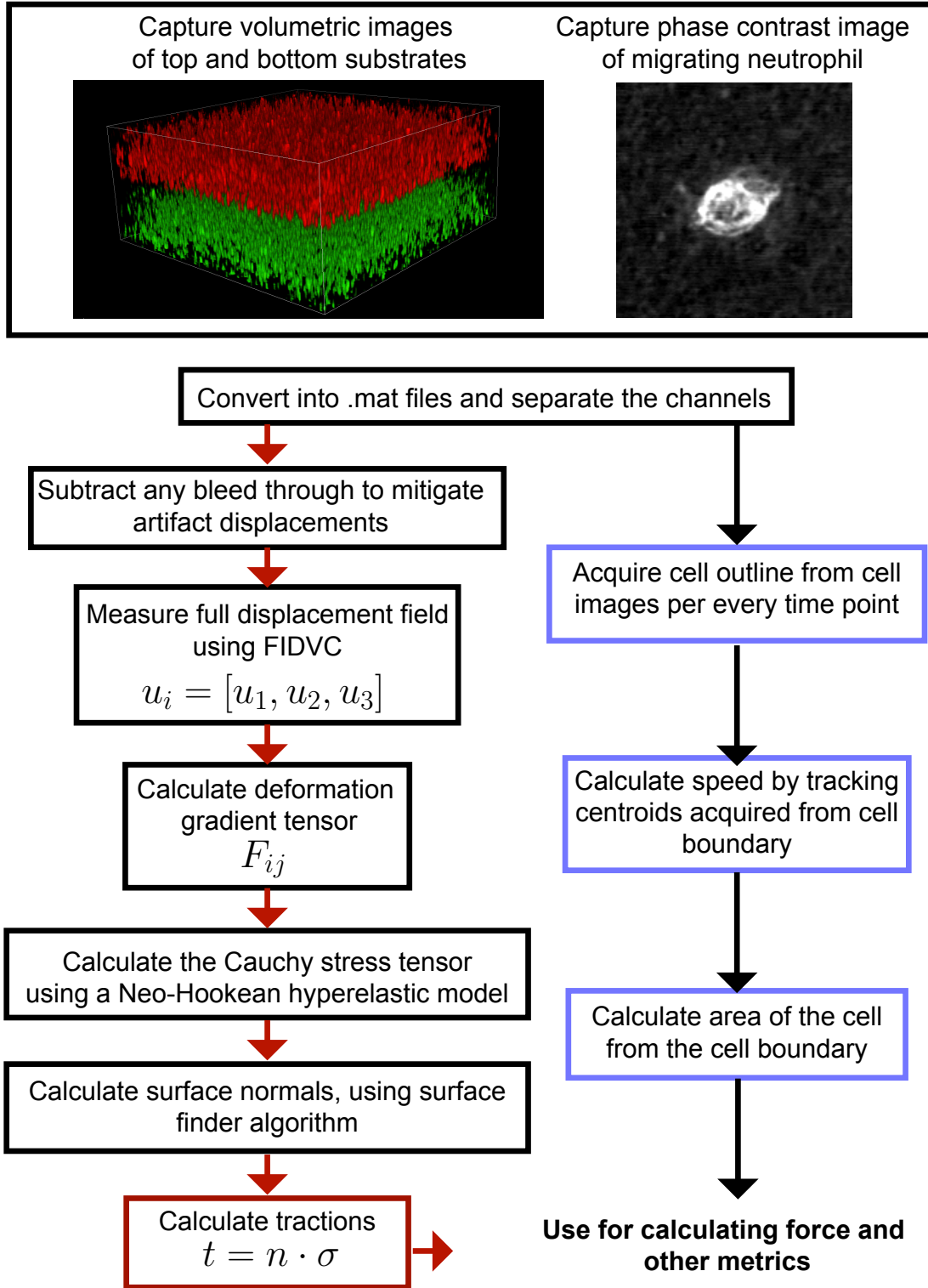


FIGURE 2.19. Flowchart of the large deformation high-resolution 3D TFM technique illustrating how cell surface tractions are being calculated.

that for the LD, the reference and deformed configurations are no longer identical, computation of the Cauchy stress tensor involves the deformation gradient tensor. While this technique is general enough to allow numerical implementation of any constitutive material law (Appendix A presents implementation of viscoelastic model), this section presents the formulation of the stresses in polyacrylamide (PA) gel, which can be approximated by a Neo-Hookean material model [53]. Thus its Cauchy stress, $\boldsymbol{\sigma}$, can be expressed [6] as

$$\boldsymbol{\sigma} = \frac{\mu}{J^{5/3}} \left(\mathbf{B} - \frac{1}{3} \text{trace}(\mathbf{B}) \cdot \mathbf{I} \right) + K(J - 1)\mathbf{I} \quad (2.25)$$

where the parameters μ , and K are the material's shear and bulk modulus. Both of these quantities can be related to the Young's modulus of a material by

$$K = \frac{E}{3(1 - 2\nu)}, \quad \mu = \frac{E}{2(1 + \nu)}. \quad (2.26)$$

The quantities, J , and $\mathbf{B}$ are the Jacobian of $\mathbf{F}$, and the left Cauchy Green's tensor, respectively. They are mathematically defined as

$$J = \det(\mathbf{F}) \quad (2.27)$$

and

$$\mathbf{B} = \mathbf{F} \cdot \mathbf{F}^T. \quad (2.28)$$

Finally, the surface tractions are calculated via the Cauchy relations, i.e.,

$$\mathbf{t} = \mathbf{n} \cdot \boldsymbol{\sigma}. \quad (2.29)$$

The out-of-plane and in-plane tractions on the deformed substrate can be calculated respectively by

$$\mathbf{t}_\perp = (\mathbf{t} \cdot \mathbf{n})\mathbf{n} \quad (2.30)$$

$$\mathbf{t}_\parallel = \mathbf{t} - \mathbf{t}_\perp \quad (2.31)$$

Before Eqs. 2.29 - 2.31 can be evaluated, the surface normals, $\mathbf{n}$, need to be determined. There are two important details to notice: First, the coordinate system in the deformed state is no longer equal to the coordinate system in the undeformed state, i.e., $\mathbf{x} \neq \mathbf{y}$. Second, whereas in the undeformed state the $x_1 - x_2$ traction components are planar (2D), in the deformed configuration the $y_1 - y_2$ traction components are fully 3D with respect to the original $x_1 - x_2 - x_3$ coordinate system. Another way to visualize this is by looking at the angle change between the surface normals of the deformed and reference configuration. If the reference and deformed surfaces remain the same, as assumed in linear elasticity, then the two normal vectors are identical and their vector dot product, or cosine between the normals, is equal to one. Figure 2.20 presents the simulated results of prescribed Gaussian displacement (Eq. 2.32) to the volumetric images.

$$f(x_1, x_2, x_3) = A \left(e^{-\frac{1}{2k^2}(x_1-x_0)^2+(x_2-x_0)^2+(x_3-x_0)^2} \right). \quad (2.32)$$

Magnitudes of different displacements were prescribed to the surface of the volume with A ranging from 0.2 to 16, using MATLAB (Mathworks, Natick, MA). The displacement gradient was calculated, as well as the normal vector to the deformed volume and its deviation from $[0, 0, 1]$ vector in the reference volume. As shown in Fig. 2.20, in the presence of a non-negligible displacement gradient, the surface normals can deviate significantly, causing an initially planar surface to deform out-of-plane. Most of the deviation in the $\mathbf{n} \cdot \hat{\mathbf{n}}$ term in Fig. 2.20 is caused by significant material rotations at the free surface.

4.1. Estimating surface normals. Contrary to small deformation theory that intrinsically assumes that any deformed configuration is the same as the undeformed reference configuration, finite deformation theory features large topographical surface changes as those experimentally observed in Fig. 2.17(A)–(C). Fig.2.20(inset) schematically illustrates how the local $x_1 - x_2 - x_3$ coordinate system of the undeformed (reference) configuration maps into a deformed configuration $y_1 - y_2 - y_3$ under the application of a large deformation. Although it is theoretically possible

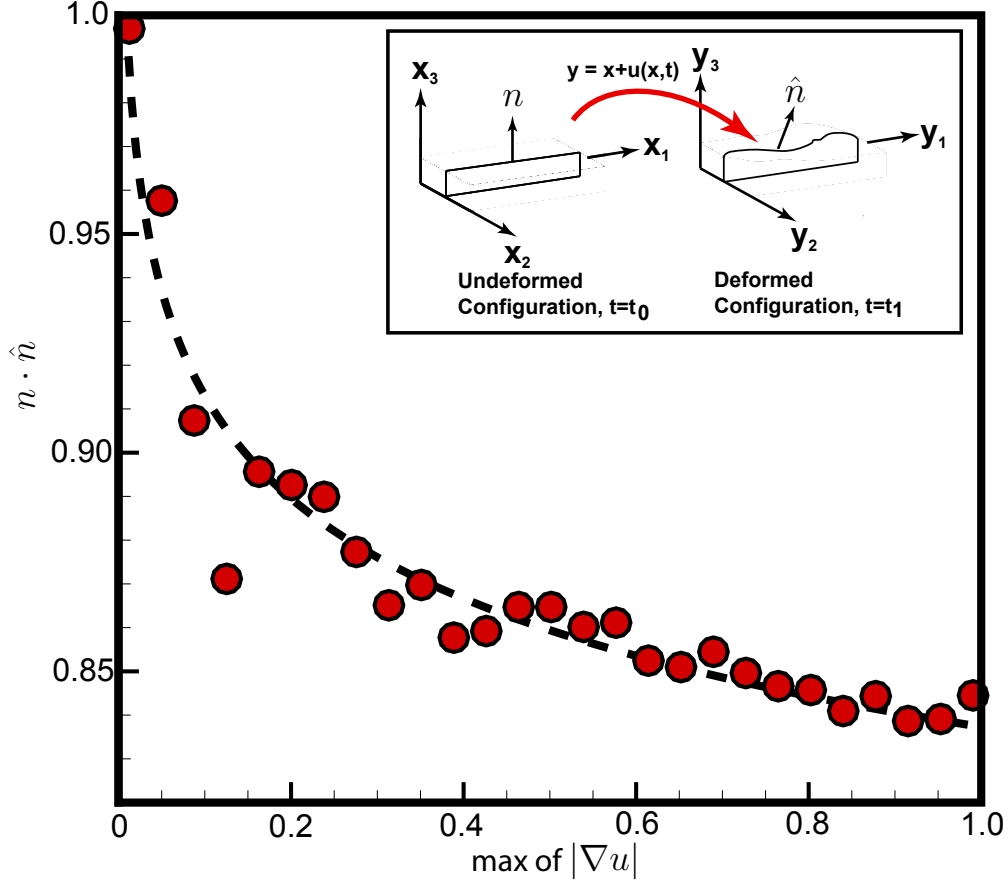


FIGURE 2.20. Angle change between the undeformed and deformed surface normals in $\mathbf{n}$ and $\hat{\mathbf{n}}$, under the application of a cell-simulated Gaussian displacement field profile (Eq.(2.32)). The x-axis denotes the maximum value of the full-field 3D displacement gradient magnitude. The dot product represents the cosine of the angle between the two surface vectors. The inset illustrates a schematic of how a material deforms from a reference configuration, $\mathbf{x}$, at time t_0 into a deformed configuration, $\mathbf{y}$, at time t_1 .

to estimate the deformed surface and surface normals in the deformed state, via the deformation gradient tensor and the reference surface, this procedure is practically challenging, since the reference surfaces and normals are often not known. To address this issue, this work used surface finding algorithm that uses the LSCM images to determine the true surface plane, i.e., $\mathbf{n}(\mathbf{x})$.

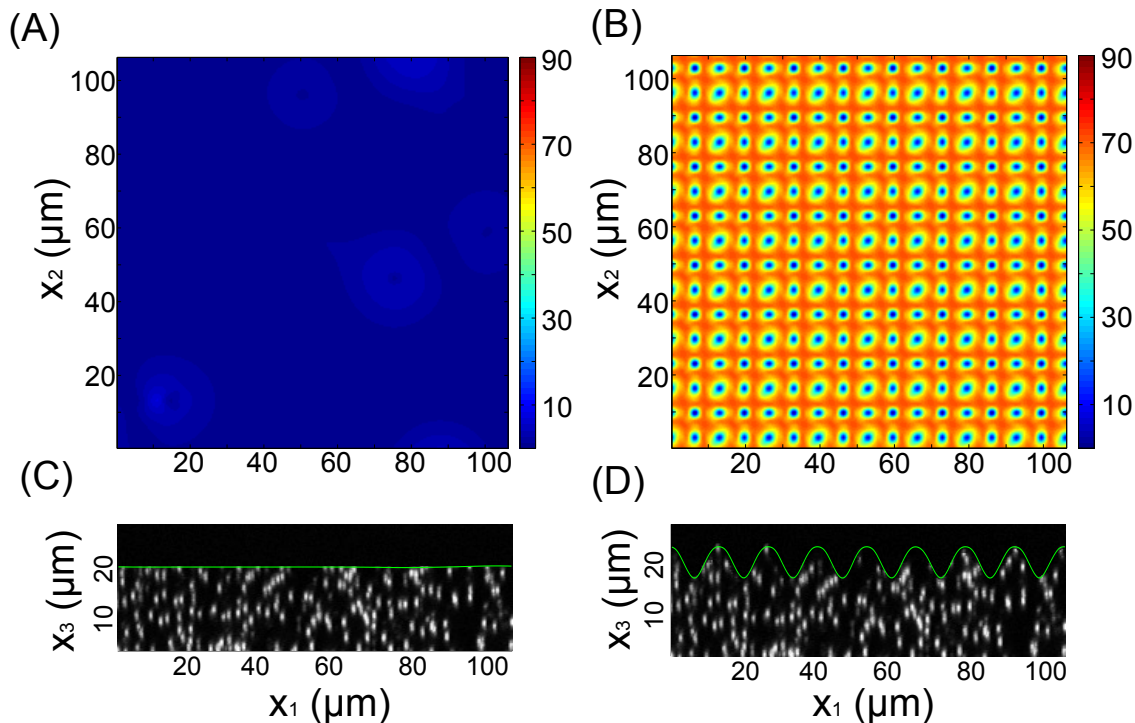


FIGURE 2.21. Analytical benchmark validation examples of the free surface finder algorithm. (A) shows the results of the surface finder given a perfectly flat surface, whereas in (B) the surface has regular imposed wave surface undulations described by Eq.2.33. (C) and (D) show the corresponding $x_1 - x_3$ views of the simulated volumetric images.

In the current work, each deformed surface is determined by utilizing the spatial locations of the fluorescent beads in the raw LSCM images. A surface is built from scattered grid data that is calculated using the local maxima of sliding windows along the $x_1 - x_2$ plane. The final surface is generated by a least-squares fitting procedure of the scattered data, ensuring the surface gradient is as smooth as possible [18]. The surface normals, $\mathbf{n}(\mathbf{x})$, are calculated using the Delauney triangulation representation of the surface. Figure 2.21 shows two validation test cases of the surface finding algorithm for two surfaces that are numerically simulated from experimentally acquired volumes. In the first example, Fig. 2.21(A) shows the angle deviation between the calculated surface normal and the analytically imposed surface normal, $\mathbf{n} = (0, 0, 1)$. The corresponding cross section of the volumetric image is shown in Fig. 2.21 (C) with the superimposed outline (green) that is found by the surface

finding algorithm. Similar validation was performed on a surface that is defined by

$$f(x_1, x_2) = A \left(\sin \left(\frac{2\pi}{\lambda} x_1 \right) + \cos \left(\frac{2\pi}{\lambda} x_2 \right) \right), \quad (2.33)$$

where A is the amplitude and λ is the wavelength of the surface profile, which were set to $3.6 \mu\text{m}$ and $13 \mu\text{m}$. The root mean squared difference between the calculated and analytically exact values of the surface wave is within 1%. Using this procedure, the full-field surface normals for each volumetric image are calculated, and the surface tractions along that particular plane are computed according to Eq. 2.29.

The material shown in Fig.2.21(C-D) is a polyacrylamide substrate with embedded $0.5 \mu\text{m}$ fluorescent particles. These substrates are prepared following the recipe described in Section 1 of this chapter. The green line represents the free surface of the sample. Consequently, if only a 2D representation of the cell-induced tractions is sought, the occurrence of large deformations requires a 3D imaging modality, e.g., confocal microscopy, to resolve those surface shear tractions properly. If instead, only a 2D image is acquired of the embedded fluorescent fiducial markers before and after finite deformation, the recovered displacements can only provide optical projections of the actual 3D material displacements, which can yield significant measurement error.

4.2. Other metric: Force calculations. Various metrics of interest can be calculated from known stress tensor, $\boldsymbol{\sigma}$ and traction vector, $\boldsymbol{t}$. In recently published work of López-Fagundo and Bar-Kochba et al., the authors used tractions to calculate the work done by the cell to deform the substrate [54].

In this document, the total force generated by neutrophils is of interest, specifically, decomposed total force into in-plane and out-of-plane force components.

$$F_{\parallel} = \oint |t_{\parallel}| dS \quad (2.34)$$

$$F_{\perp} = \oint |t_{\perp}| dS \quad (2.35)$$

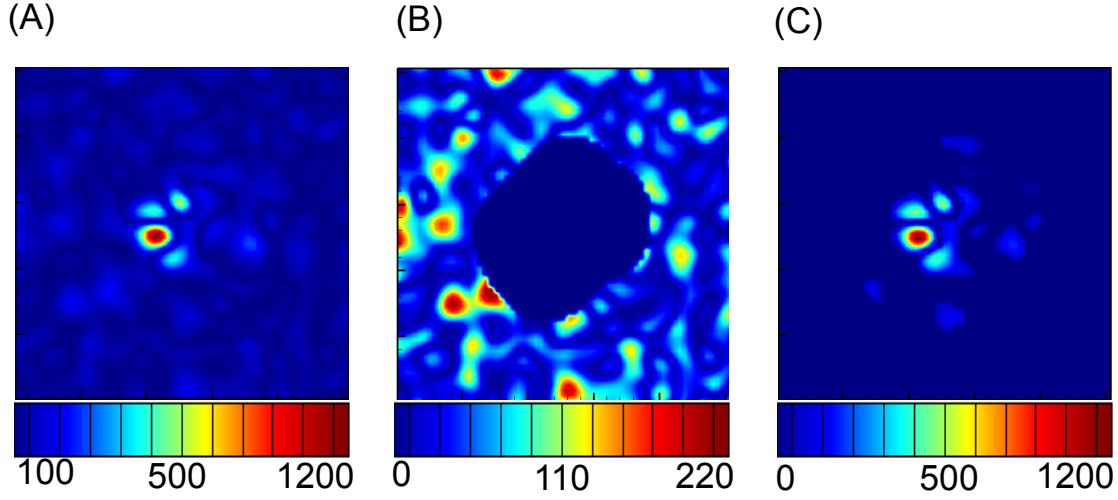


FIGURE 2.22. Traction magnitude contour plot represented as (A) raw data, (B) noise outside the cell boundary, and (C) signal with thresholded noise outside the cell boundary. Colorbar is in Pascals (Pa)

The surface integral is calculated using summation of the area within and outside of the cell outline using MATLAB (MathWorks, Natick, MA).

4.3. Resolution and Measurement Sensitivity. The noise floor for tractions and total forces were calculated from the experimental data. The tractions are calculated following the flow chart Fig.2.19. The outline of the cell was found from phase contrast images taken during the experiment. To minimize any ambiguity of calculating noise from control data (data run without cells) – the actual experimental data was used for noise calculations collected outside of cell boundary. To identify appropriate data, in this work, the signal-to-noise ratio was calculated for each time point, the following way:

- Calculate T_{RMS} for each time point for the top and bottom substrates within and outside the cell area (Eq. 2.36).

$$t_{RMS} = \sqrt{\frac{1}{N} \sum_{i=1}^N t_i^2} \quad (2.36)$$

where t_i is the traction vector located at every i th point along the surface of the boundary, enclosing N points.

- Calculate signal-to-noise ratio (SNR) (Eq.2.37)

$$SNR = \frac{t_{RMS}(\text{inside})}{t_{RMS}(\text{outside})} \quad (2.37)$$

Once acceptable data was chosen for force calculations, the noise floor was calculated by collecting all of the calculated tractions outside of the cell boundary Fig.2.22 for each substrate stiffness separately. The noise floor presented in Table 2.4 is the median with two standard deviations. The noise floor for the force was calculated similar way but integrated over the area of the cell.

Global Force and Moment Balance. We compute the sum of all forces and moments acting on any given control volume inside each polyacrylamide gel at every time point to verify static force and moment equilibrium are satisfied. The overall procedure is identical to our previously published methodology [25]. Under all experimental conditions and imaging time points we found static equilibrium satisfied with force and moment magnitudes on the order of $10^{-9} - 10^{-10}$ N (forces), and $10^{-15} - 10^{-14}$ N·m (moments), respectively. These numbers are consistent with our noise floor measurements.

Neutrophil Speed Calculations. To calculate PMN speed during migration in both 2D and 3D systems, cell boundaries from captured phase images were recorded and cell centroids were computed during each imaging increment using a custom-written Matlab algorithm (MathWorks, Natick, MA). Using this information, cell speed per time increment can be calculated as

$$v = \sqrt{v_x^2 + v_y^2} \quad (2.38)$$

It should be noted since the cell speed information based on the centroid calculations derived from phase images, only the in-plane (x,y) velocity components could be detected.

4.4. Statistical Analysis. Data were subjected to the chi-square goodness-of-fit test to determine if a data sample comes from normal distribution. Kruskal-Wallis non-parametric test was used to compare the medians of the groups of data to determine if the samples come from the same population. Wilcoxon ranksum test was used for comparison of the medians of the data and their interactions.

TABLE 2.4. Noise floor for measurable tractions and forces on the bottom and top substrates of the double-hydrogel system.

	Bottom substrate (E = 1.5 kPa)	Top substrate (E = 1.5 kPa)	Bottom substrate (E = 8.3 kPa)	Top substrate (E = 8.3 kPa)
In-plane tractions, t (Pa)	40	50	200	250
Out-of-plane tractions, t (Pa)	200	200	700	800
In-plane total force, F (nN)	0.4	0.45	1.7	2.2
Out-of-plane total force, F (nN)	1.7	1.7	6.2	7.1

CHAPTER 3

Results and Discussion

For proper innate immune responses inflammatory cells, neutrophils, have to use fast and efficient technique for migration and infiltration of any tissue. Neutrophils accomplish this by undergoing a multi-step migration process characterized as amoeboid migration, which involves rapid cytoskeletal shape changes and contact exchanges with the surrounding ECM [26]. In contrast to amoeboid migration, mesenchymal cells form stable focal contacts with the ECM, thus perform much slower migration. Besides speed and adhesive capability, another difference between the cells is their response to the dimensionality of the surrounding matrix.

For stable contact and prolonged interaction with a 2D planar surface, both amoeboid and mesenchymal cells adhere to the substrate. Through the contacts with the surface, cells transmit forces to its surroundings to propel forward [26, 43]. In 3D encapsulated environments, mesenchymal cells, such as fibroblasts or smooth muscle cells, also establish adhesion to ECM via receptors to migrate through the matrix. Thus, blocking these receptors diminishes cell migration in 3D [55, 26, 44]. However, amoeboid cells, like neutrophils, still maintain high migration speeds in the absence of integrins in 3D environment [7, 43, 79]. It is not well understood whether it is confinement alone or a combination of other factors that allow this integrin-independent migration in 3D. In this chapter, the results show the effect of confinement on neutrophil force generation and migration. Section 1 presents forces and migration speeds on neutrophils on planar 2D surface. In Section 2 the forces on planar substrates are directly compared to those generated by migrating neutrophils in confined environment. Section 3 and 4 discusses the effects of integrins and actomyosin network on neutrophil force generation.

1. Neutrophil traction force generation in unconfined environment

Traction and force calculations were carried out using LD TFM approach in combination with FIDVC technique described in Chapter 2. The results shown in this section are calculated for migrating neutrophils on planar polyacrylamide substrates of two stiffnesses: $E = 8.3$ kPa (stiff) and $E = 1.5$ kPa (soft). The confocal volume stacks of deformed substrates for each cell were captured every 15 seconds for 10-15 minutes total. The tractions presented here are calculated incrementally, meaning between reference configuration at time, t , and deformed configuration at time, $t+1$. Fluorescent microspheres were captured simultaneously with the cell movement (details in Chapter 2).

Figure 3.1 presents 3D traction vector of a migrating neutrophil on soft and stiff substrates. The superimposed outline in Fig. 3.1 was estimated from the cell boundary captured via phase contrast microscopy. During experiments, neutrophils were exposed to the uniform concentration of chemoattractant, fmlF, thus the character of migration is not directional towards a point source. The tractions are spatially local and distributed randomly throughout the neutrophil body relative to migration direction. Similar to fibroblasts, neutrophil average magnitude of displacements in the gel is independent of stiffness [60, 69]. This results in traction magnitude scaling with the stiffness – lower traction values on softer substrates (Fig.3.1(A)) compared to those on stiffer substrates (Fig.3.1(B)). From traction distributions of the single cell presented in Fig.3.1, it is obvious that neutrophil generated tractions in directions 1 and 2 (parallel to the surface, or in-plane) are much lower than those generated in the 3rd direction (normal to the surface, out-of-plane). This type of traction force generation was previously observed by Lòpez-Fagundo and Bar-Kochba et al. in the study with schwanz cells [54], however is not common for the mesenchymal cells [60, 35, 25].

Figure 3.2 presents in-plane and out-of-plane total forces on soft and stiff substrates. The in-plane and out-of-plane forces are calculated integrating tractions over the neutrophil surface area. Only the data with the background noise at least twice

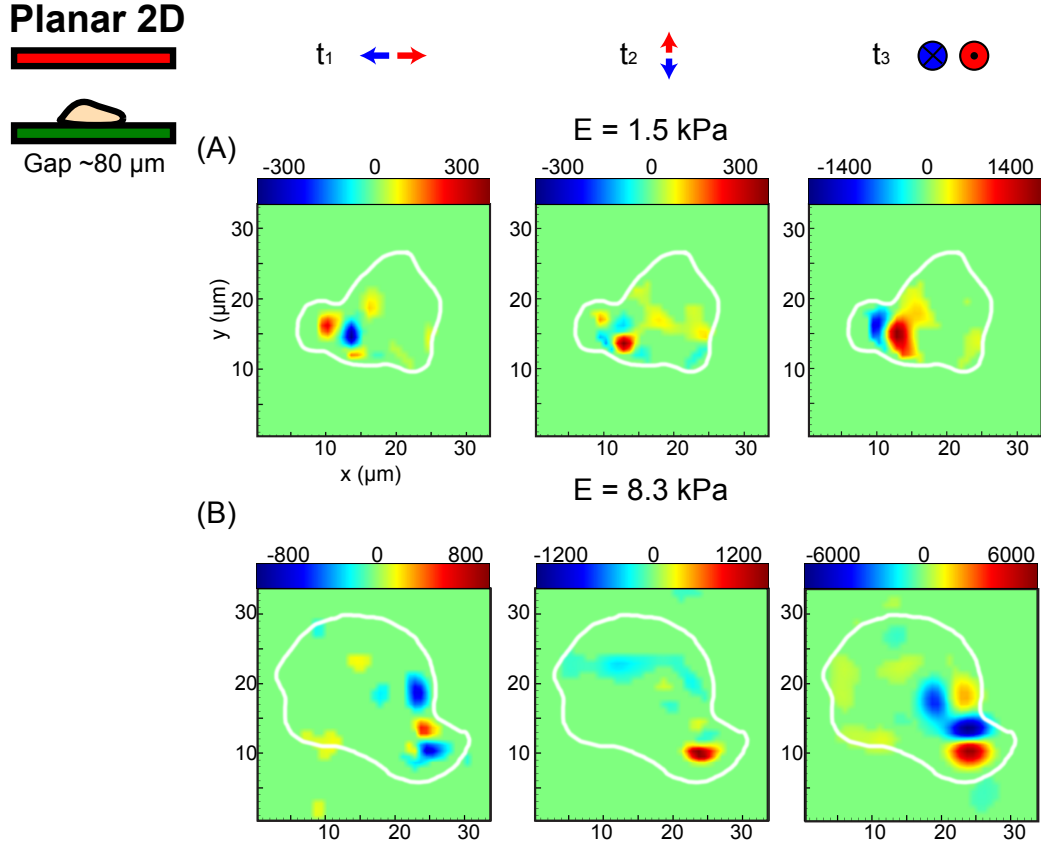


FIGURE 3.1. Cell traction contours on 2D unconfined environment on (A) soft and (B) stiff substrates.

lower than the signal traction values are used for the calculations. Even though not quantified as a scope of this work, neutrophils are known to have higher spreading area on stiffer substrates than on softer substrates [69, 94]. Similar is observed qualitatively in the experiments performed during this study. Therefore, it is not surprising that integrating higher tractions values over higher areas lead to higher total in-plane and out-of-plane forces on stiffer substrates compared to those generated on softer ones.

Figure 3.3 presents neutrophil migration speeds on the planar substrates. Similar to the observations in other studies, neutrophils perform slower migration on stiff substrates compared to those on soft substrates [69, 94]. It should be noted that this migration and force generation on planar surface is possible only due to the transmembrane integrins neutrophils use to attach to the substrate. In the event of

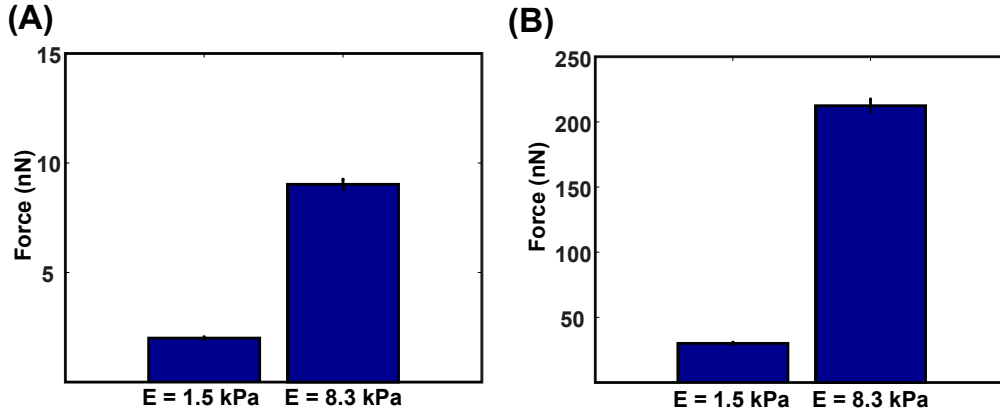


FIGURE 3.2. Total neutrophil-generated (A) in-plane and (B) out-of-plane forces on soft ($E = 1.5$ kPa) and stiff ($E = 8.3$ kPa) 2D substrates

integrin blockade performed during this work – neutrophils stopped performing both of the functions in 2D space.

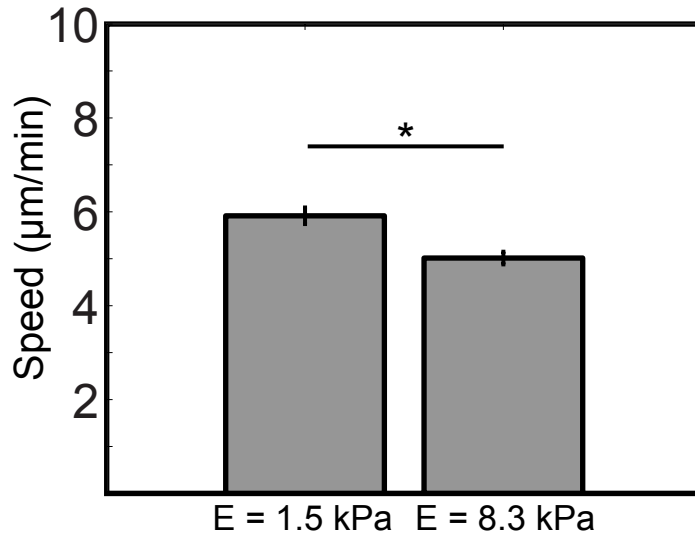


FIGURE 3.3. Neutrophil migratory speed on the planar substrate on soft ($E = 1.5$ kPa) and stiff ($E = 8.3$ kPa) substrates.

2. Neutrophil traction force generation in confined environment

To investigate how confinement affects the traction signatures of neutrophils during migration in 2D and under confinement, surface curvature, along with the mechanical and biochemical properties of the substrate and extracellular matrix (ECM)

are kept spatially constant. This is achieved by seeding freshly isolated PMNs between two compliant fibronectin-coated polyacrylamide substrates separated by an adjustable gap distance to switch from a 2D to a highly confined (2.5D) microenvironment. In order to control confinement of the neutrophil-gel system, a custom-designed micromanipulator system is built. Figure 1 presents a schematic of the micromanipulator and neutrophil-gel system (Fig.3.4A), as well as the orientation of the two surface normal vectors used in the 3D traction calculations (Fig.3.4B).

To determine dynamic cell tractions, neutrophil-generated substrate deformation fields are recorded and analyzed using our recently developed high-resolution 3D TFM technique [98]. Although the basic working principle is similar to our previous 3D TFM technique [60, 25], this new technique features significantly improved spatial resolution, signal-to-noise, and computational efficiency. Based on laser scanning confocal microscopy this technique yields a displacement noise floor of $0.08 \mu\text{m}$ in x and y (in-plane), and $0.15 \mu\text{m}$ in the z (out-of-plane) directions. To determine the influence of substrate stiffness on neutrophil force generation and migration speed as the matrix changes from an unconfined 2D to a confined 2.5D system, two substrate

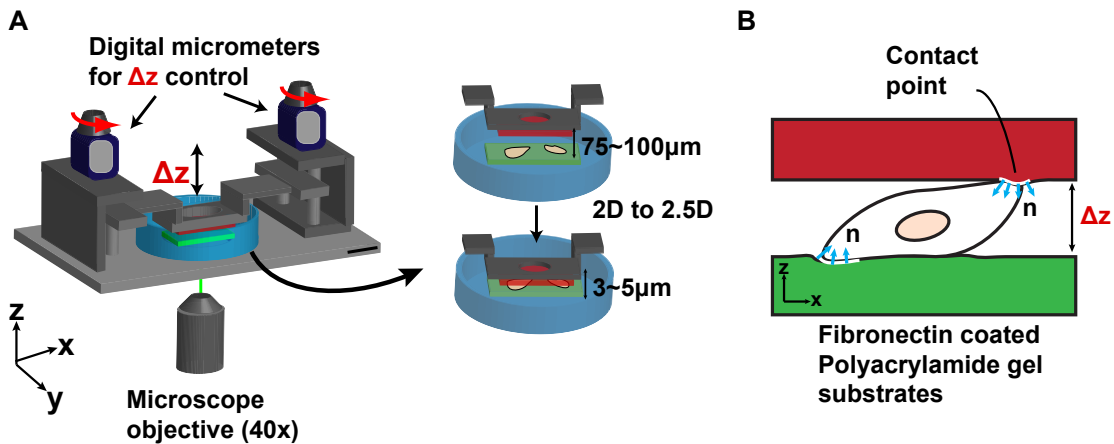


Figure 1 (Franck)

FIGURE 3.4. (A) Schematic overview of a custom-built micromanipulator to switch from a 2D to a confined 2.5D neutrophil gel-system. (B) A schematic of the cell-surface contact points and definition of the surface normal on the top (red) and bottom (green) substrates.

stiffnesses are chosen: $E = 1.5$ kPa to mimic soft tissues, such as endothelial or lung tissue, and $E = 8.3$ kPa to mimic stiffer tissues, like muscle.

Initially, PMNs are seeded on the bottom gel (Fig.1A) while maintaining a gap height of approximately $80\text{ }\mu\text{m}$, which is significantly larger than the average spherical cell diameter ($8\text{ }\mu\text{m}$ [97]) thus simulating a 2D environment. Once adhered to the bottom gel, time-lapse video of cellular motion and cell-generated substrate deformations are recorded every 15 seconds for a total of 10 minutes via confocal microscopy. This time period is sufficient to recapitulate cellular morphologies and trajectories when compared to previous 2D studies [91, 69, 38]. Figure 3.5 shows a representative map of cellular tractions as a single neutrophil switches from a 2D to a confined environment (gel modulus: $E = 1.5$ kPa). Figure 3.5A depicts a snapshot of the neutrophil morphology and a color contour map of the magnitude of the 3D traction vector during chemokinesis in 2D (gap height $80\text{ }\mu\text{m}$). The magnitude of the 3D traction vector peaks around 1300 Pa at the rear of the cell relative to its direction of motion. The observation of asymmetric traction profiles is consistent with previously reported 2D studies on neutrophil motility [91, 38].

Following the initial 2D acquisition period ($t = t^*$), the top substrate is lowered to an effective substrate-to-substrate gap size of approximately $3\text{--}5\text{ }\mu\text{m}$, which is slightly less than the average spherical neutrophil cell diameter [97]. This change in gap size generates a confined 2.5D space, allowing for neutrophil attachment to either, or both, top and bottom surfaces. Cell position and substrate deformations are recorded for another 10 minutes at 15 second intervals. Figure 3.5B is representative of the typically observed neutrophil morphology and the magnitude of the 3D traction vectors for both bottom and top substrates. The bottom contour plot shows cellular traction generation with magnitudes similar to that shown in Fig. 3.5A, whereas the top substrate shows roughly a doubling of tractions when compared to the 2D case. The asymmetric distribution of tractions seen in 2D is maintained in confinement in both the upper and lower gels. Although these data represent only single time points

during time-lapse, they represent the typical behavior observed for most neutrophils in 2D and confined 2.5D environments.

Even more striking is the breakdown of the total force into its in-plane and out-of-plane components during the transition to confinement. Figure 3.5C illustrates strong upregulation of the out-of-plane force component as the neutrophil switches from a 2D to a confined 2.5D environment with little attenuation in its in-plane component. Similarly striking is the observation that the average neutrophil speed remains unattenuated despite the significant increase in total traction force. Dynamic oscillations in force and speed magnitudes observed in Fig.3.5C are similar to the force undulations observed in previous 2D studies, where these oscillations are generated by variations in the amoeboid cell protrusion, contraction, adhesion, and relaxation force transmission cycle [89, 62].

To account for the donor dependence and variance among individual neutrophils, we compute the total in-plane and out-of-plane forces for all recorded cells summarized in Fig. 3.5D, represented by their median and standard error. For both substrate stiffnesses, the in-plane and out-of-plane force components are approximately three times larger in the confined system as compared to 2D, although force magnitudes scale with substrate stiffness. The ratios of out-of-plane to in-plane force components are significantly higher than previous measurements on other anchorage-dependent cells [35, 25]. Higher out-of-plane force measurements support previous theories of a fast peeling mechanism during cellular detachment for amoeboid-type locomoting cells [95, 49, 74].

3. Neutrophil integrin-independent migration

On 2D substrates neutrophils engage ligand-specific integrins to adhere and generate forces [69, 38]. However, it has been reported that integrins may be dispensable for migration in 3D spaces [7, 79]. A recently proposed 3D leukocyte migration model suggests that there might be two different modes of force generation. The dominant

mechanism in 2D relies on forming adhesive ligand-integrin contacts and is, therefore, adhesion-based [44, 79]. Migration in 3D is characterized by pressure-induced substrate deformations which does not rely on the use of integrins, and is integrin-independent [44, 79]. Our studies have been designed to determine if variation in spatial confinement is sufficient for the acquisition of integrin independent neutrophil motility. Measures of cell speed and force generation of a given cell is determined on fibronectin-conjugated hydrogels of different stiffnesses beginning in 2D with repeated measures taken over time as confinement (2.5D) is imposed. Speed and force measures are determined in the presence or absence of function-blocking integrin antibodies. Blocking the $\alpha_5\beta_1$ integrin in 2D, has abrogated migration speed and force generation on fibronectin-coated gels as expected, since $\alpha_5\beta_1$ is the canonical fibronectin receptor [107]. In contrast, once a cell is subjected to spatial confinement, $\alpha_5\beta_1$ blockade does not diminish speed or generation of in-plane and out-of plane forces (Fig.3.6). Cells treated with nonspecific IgG at identical concentrations are not different than untreated control cells (Fig.3.7). Integrin independent speed and force generation is corroborated in additional experiments in which antibody blockade is extended to cover all β_1 and β_2 integrins (such as CD11b/CD18) that may also recognize fibronectin and regulate function. Integrin independent speed and force generation are also shown by confined cells moving on PEGylated hydrogels in the absence of matrix ligands. Although in some cases speed and/or force generation is increased in the absence of integrin engagement (Fig. 3.6), our results demonstrate that upon imposition of confinement, neutrophils do not require integrins for movement or force generation.

Neutrophil migration speed and force generation have been shown to be highly sensitive to substrate stiffness on planar 2D, integrin-dependent fibronectin-conjugated hydrogels [69, 94, 38], therefore experiments were conducted to determine if substrate stiffness plays a regulatory role in cells migrating under confinement. The double hydrogel system has the decided advantage over fibrillar gels in that substrate stiffness

can be varied without altering ligand density or confinement (porosity) and this property is used to compare speed and traction of cells migrating on 1.5 kPa and 8.3 kPa fibronectin-conjugated matrices. Confined cells migrated and generated forces under conditions of integrin blockade on both soft and stiff matrices suggesting that acquisition of integrin independence by confinement is not regulated by substrate stiffness. Greater in-plane and out-of-plane forces are generated on the stiffer matrix as compared to the softer matrix as expected, but blocking the $\alpha_5\beta_1$ integrin does not reduce speed or traction force generation. Figures 3.6B and 3.6C show a minor ($\leq 10\%$), but statistically significant, reduction in force generation by blocking β_2 integrins alone on 8.3 kPa but not on 1.5 kPa gels as compared to control cells. This diminution is obviated by simultaneous β_1 blockade, suggesting that in the absence of β_1 ligation any vestigial role for β_2 is lost.

4. Neutrophil contractile and protrusive forces

To determine the effect of confinement on the cytoskeletal force-migration machinery of the neutrophil, actin and myosin are differentially blocked with Latrunculin A and Blebbistatin. In confined 2.5D environments, we find cell speed depends on contributions from both actin and myosin-based machineries and is not a function of matrix stiffness (Fig.4). The role of myosin on cell speed and force generation, other than a modest effect of Blebbistatin on soft substrates, is significant regardless of confinement and substrate stiffness (Fig. 4A). Like myosin, actin plays an essential role in generating cell speed under confinement and irrespective of stiffness as noted by sensitivity to Latrunculin A (Fig. 4B). In contrast to myosin, the role of actin in generation of force under confinement is highly mechanosensitive being dispensable on soft substrates but essential on stiff substrates.

5. Conclusion

Neutrophils are the most abundant white blood cell and the first leukocytes mobilized in response to infection or injury. Coordinated migration within the confined

space of a 3D tissue is essential for neutrophils to properly execute a controlled inflammatory response limiting infection and initiating healing. Irregularities in the neutrophils motility machinery can lead to deleterious effects on the host including excessive tissue damage which, when severe, can lead to multiorgan system failure [61]. In order to fully understand how an optimal inflammatory response occurs, it is important not only to identify biochemical mediators and receptors that first promote and subsequently dampen neutrophil activity, but also physical factors with regulatory influence such as tissue stiffness and spatial confinement. In this study we utilize a tunable, double-hydrogel system that allows neutrophils to be studied under conditions that more closely model a tissue microenvironment. Using 3D traction force microscopy, both in-plane (x,y) and out-of-plane (z) traction force measurements are obtained for neutrophils in 2D, and under highly confined 2.5D conditions. We find that their traction force signature is highly 3D with the strongest traction force contribution stemming from the out-of-plane traction component regardless of confinement or substrate stiffness. This suggests the predominance of out-of-plane traction is a generalized mechanism by which neutrophils exert forces upon their substratum in both planar, integrin-dependent and confined, integrin-independent conditions. Whereas inhibition of ligand-specific integrins prohibits cell attachment and force generation in 2D, we show that integrin blockade does not impede force generation or cell speed in a highly confined system. On the contrary, neutrophil speed in 2.5D is greater when integrins are blocked, suggesting that integrins in a highly confined space may act as a regulatory brake. In addition to the well-defined role for $\alpha_5\beta_1$ in regulating adhesive-based functions of neutrophils on fibronectin, the β_2 integrin CD11b/CD18 can also play a regulatory role [31, 32]. Furthermore, cross-talk between β_1 and β_2 integrins has been shown to heighten the role of CD11b/CD18 in some reports [100, 51]. For example, antibody-specific activation of $\alpha_5\beta_1$ on human neutrophils led to the recognition of fibronectin-coated beads by a mechanism entirely dependent on the β_2 integrin CD11b/CD1830. Figure 3.6 shows a small but statistically significant reduction in speed and force upon β_2 blockade alone, so we

cannot entirely rule out a vestigial contribution of β_2 integrin to migration speed and generation of total force when confined cells are studied on stiff but not soft matrices (Fig.3.6). However this differs significantly from the dominant role for β_2 integrin on adhesion to fibronectin after β_2 activation. The biological relevance of the small difference we find upon β_2 inhibition under the more rigid of the substrates remains to be determined. We investigate the role of actin and myosin II activity during force-dependent motility and find that neutrophil speed and force generation in 2.5D is decreased upon inhibition of myosin II and actin polymerization, but not by the absence of integrin-engagement (Figs.3.6 and 3.8). To our knowledge, these are the first quantitative 3D force measurements for human neutrophils across different substrate dimensions and stiffnesses following inhibition of its cellular force machinery. Similar observations have been previously documented for dendritic cells confined in a 3D collagen network, where the inhibition of actin polymerization and myosin II contraction reduces cell migration speeds but integrin blockade was without effect [44]. Taken together, our results demonstrate the pivotal roles the physical characteristics of matrix confinement and stiffness each play in regulating neutrophil-generated tractions, speed and integrin utilization. Furthermore, we demonstrate that the double-hydrogel system is a biomimetic of leukocyte migration in vivo since traction and motility are integrin-dependent while cells migrate on a 2D surface, and then become integrin independent when cells are in a confined 2.5D space. This sensitivity to both geometric and mechanical cues should be considered in the future development of anti-inflammatory drugs.

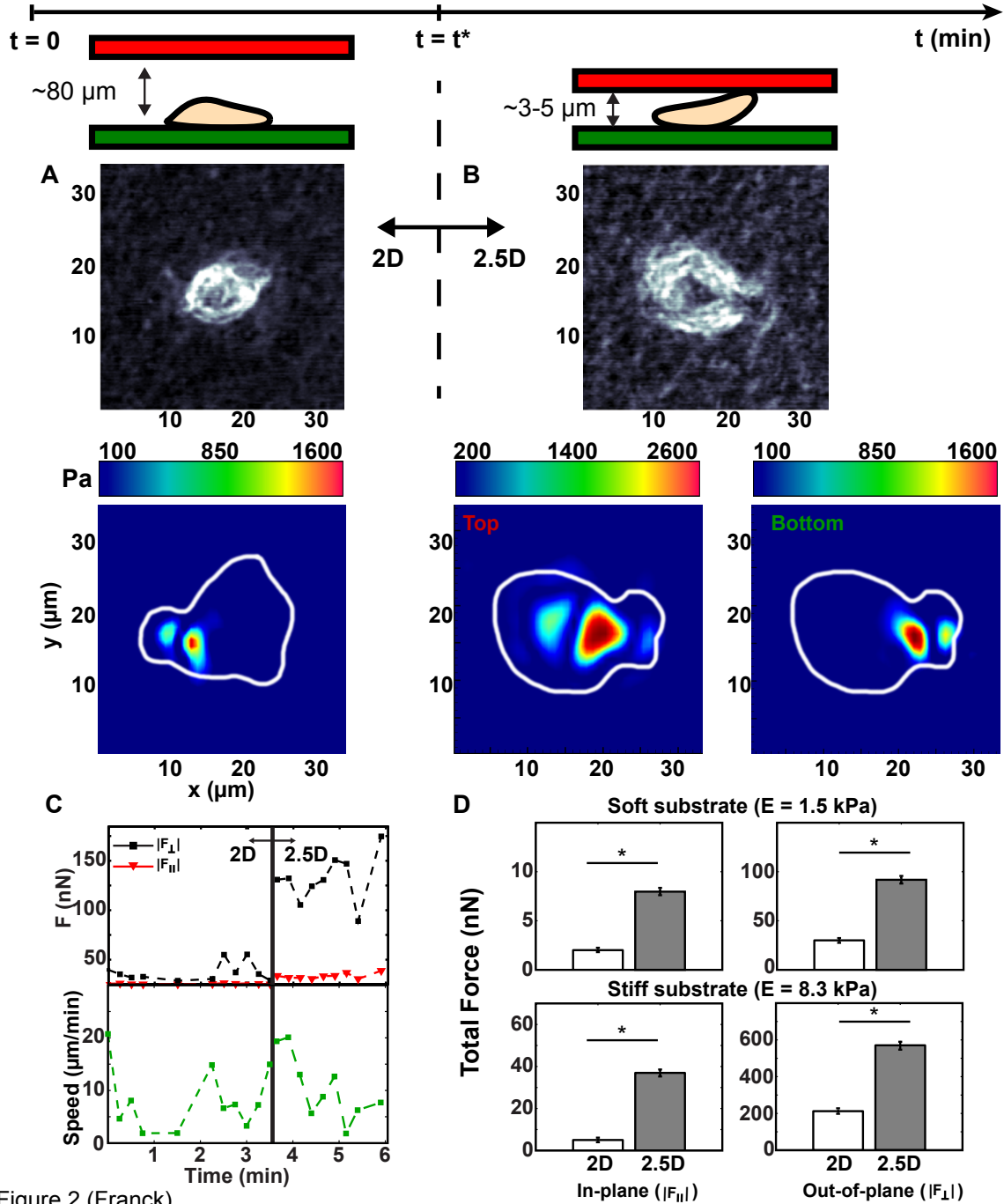


Figure 2 (Franck)

FIGURE 3.5. Phase contrast images of a neutrophil and its associated cell traction profiles in (A) a 2D system; and (B) a 2.5D system. The color contour map displays the magnitude of the 3D traction vector. (C) Dynamic out-of-plane (black) and in-plane (red) total forces, and cell speed (green) for a neutrophil during dimensional switch from a 2D to a 2.5D system. (D) Total out-of-plane (right column) and in-plane (left column) force components in our 2D (white) and 2.5D (gray) systems on soft and stiff substrates (top and bottom rows). Quantitative data reflects the median and standard error (* $P < 0.01$ vs. 2D, Kruskal-Wallis test. $N > 100$).

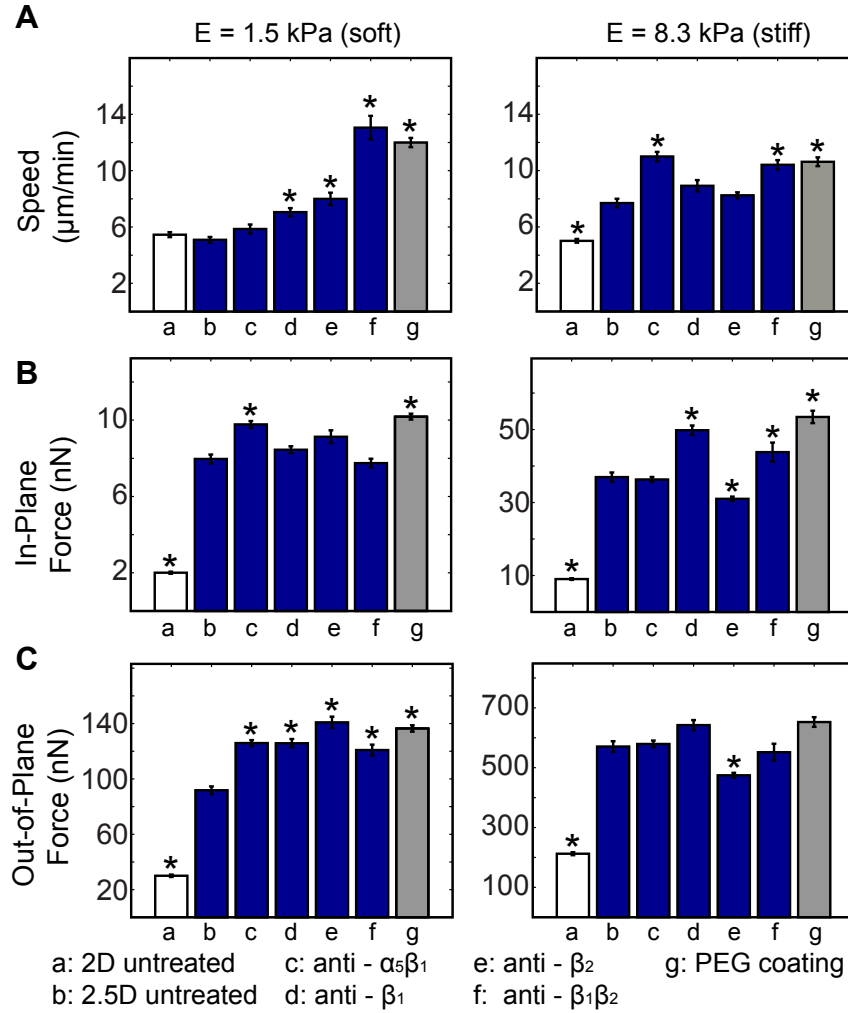


Figure 3 (Franck)

FIGURE 3.6. Neutrophils migrate and generate forces in the absence of integrin-engaged adhesions. Bar plots present (A) the average cell speed, (B) in-plane and (C) out-of-plane forces on soft (left) and stiff (right) substrates in our 2D and 2.5D systems. All of the data is presented for untreated and treated (anti- $\alpha_5\beta_1$ (10 μ g/mL), anti- β_1 (10 μ g/mL), anti- β_2 (10 μ g/mL) and anti- $\beta_1\beta_2$ (10 μ g/mL each)) neutrophils on fibronectin and untreated neutrophils on PEG coated substrates. All quantitative data are represented by their medians and standard error. Statistical significance is shown against the untreated cells in 2.5D (* $P < 0.01$ vs. 2.5D, untreated; Kruskal-Wallis test. $N > 100$).

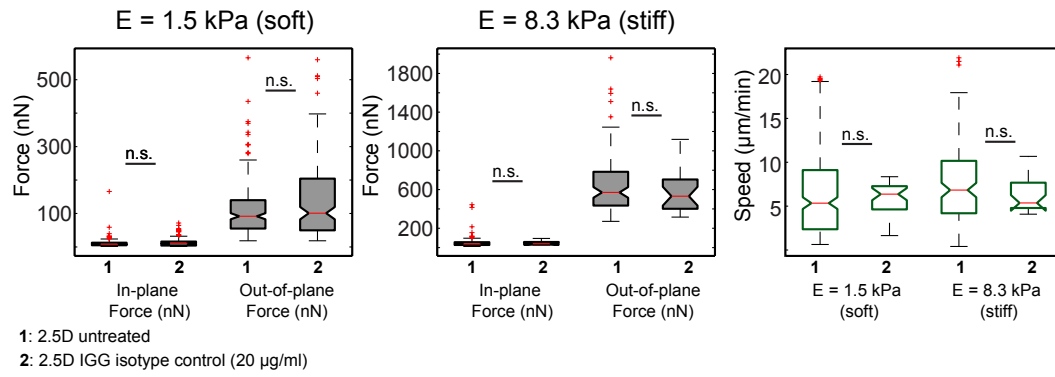


FIGURE 3.7. Comparison of force and speed data for untreated neutrophils against neutrophils treated with unspecific IgG (20 µg/ml).

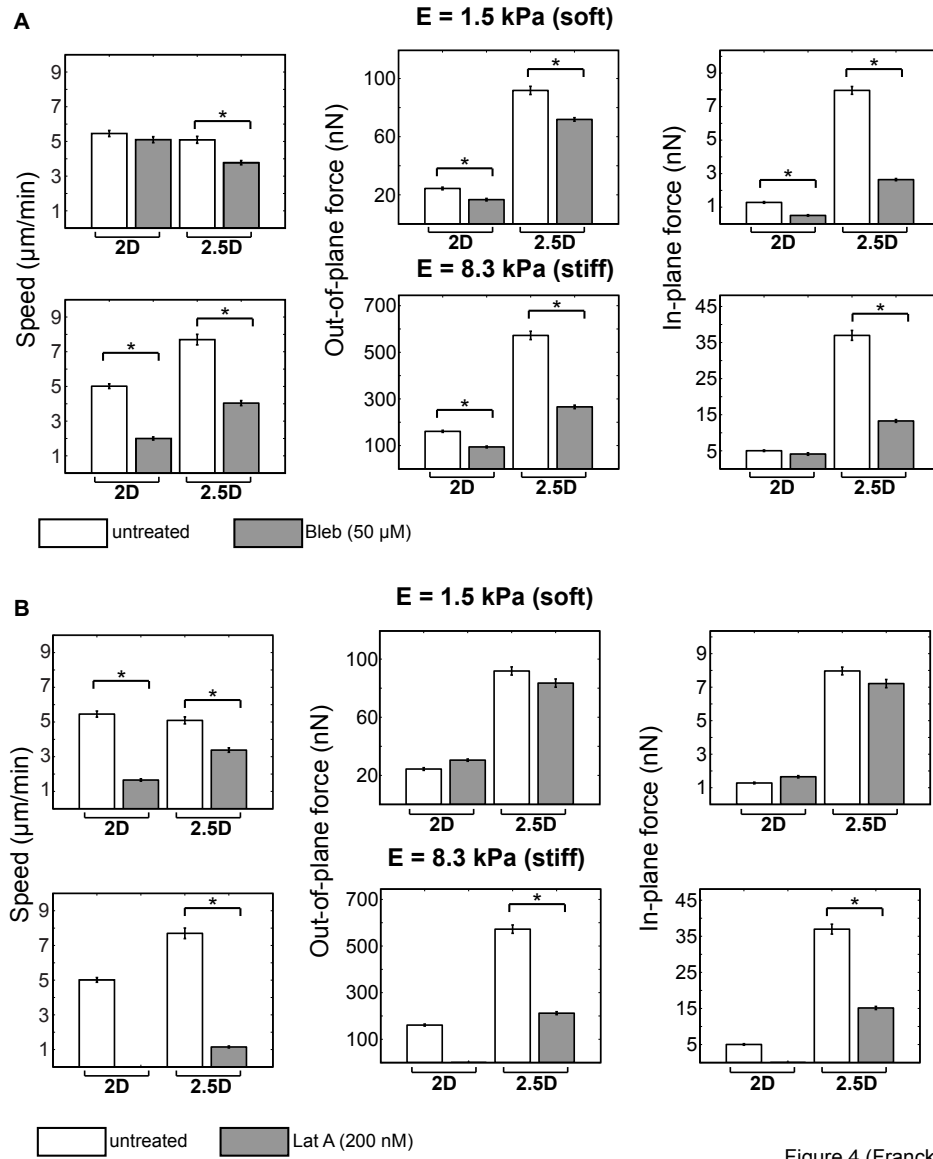


Figure 4 (Franck)

FIGURE 3.8. Neutrophil migration and force generation decrease with inhibition of (A) myosin-based contractile and (B) actin-based protrusive forces. Bar plots present the average cell speed, in-plane and out-of-plane forces on soft (top) and stiff (bottom) substrates in our 2D and 2.5D systems. All of the data is presented for untreated and treated (50M Blebbistatin and 200 nM Latrunculin A respectively) neutrophils on fibronectin coated substrates. All quantitative data are represented by their medians and standard error. Statistical significance is shown against the untreated cells (* $P < 0.01$ vs. untreated; Kruskal-Wallis test. $N > 100$).

CHAPTER 4

Conclusion and Future Work

In the current work FIDVC and LD TFM were used to study neutrophil migration and force generation in a confined environment. The TFM approach is general and could be used for any material constitutive model. In Appendix A, for example, TFM is implemented for viscoelastic material undergoing large deformation (hyperelastic spring instead of linear). The double hydrogel system, described in this work, is a reductionist system of confined environment that does not encapsulate all of the finer features of physiological conditions. Using more complex 3D scaffolds for studies of cell-generated forces is challenging due to the absence of well defined constitutive models. The development of a constitutive model for 3D fibrous materials would progress studies of cell-generated forces in more natural physiological materials.

1. Micro-scale characterization device: preliminary results

This section provides design and development of a system that in the future could be used to characterize complex scaffolds like collagen and fibrin gels. Calculations and preliminary validation results are also presented for the magnetic system.

1.1. Design and experimental setup. In the last 20 years, a lot of advances have been seen in developing more physiologically relevant 3D ECM scaffolds [83, 101, 103]. It has been shown that ordinary cellular processes change once the cell is encapsulated in 3D environment compared to the studies performed on 2D planar systems [43, 71, 80]. In contrast to planar *in-vitro* studies, little quantitative information about the cell-material interactions is identified in 3D scaffolds. This is so due to the lack of the quantitative constitutive model for complex 3D scaffolds at the scale relevant to cells. 3D cell culture scaffolds are complex materials that include intricate internal structures with fibers and pores down to the nanometer scale [83].

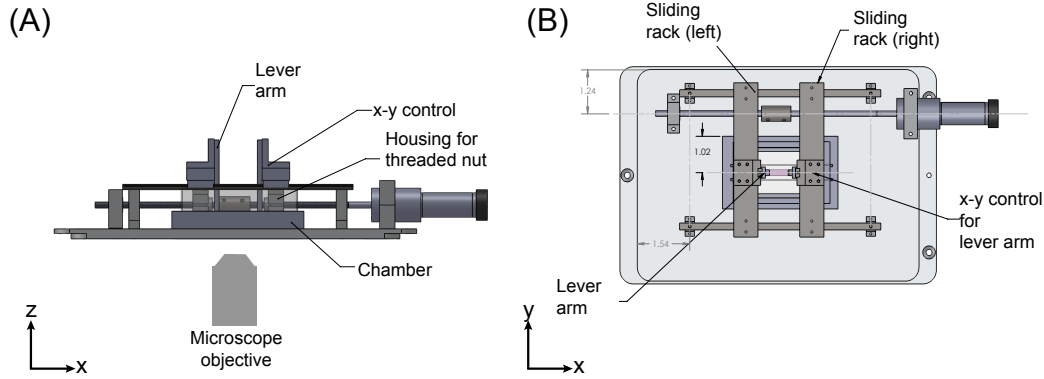


FIGURE 4.1. Illustration of (A) side and (B) top view of microscope-mounted magnetic system

The macro-scale mechanical characterization of these materials does not accurately predict the effects of porosity, reinforcement with fibers, and internal heterogeneity on the stiffness.

To address this obstacle, a micro-mechanical magnetic system was designed. The system is based on the application of a known magnetic force on a scaffold impregnated with ferromagnetic particles. The application of this force is done with precise control of location and strain rates. If the magnetic force is strong enough, the ferromagnetic particles encapsulated in soft material would displace towards the magnets. Mapping full-field 3D deformations of magnetic beads under the load at both micro- and macro- scale in both quasi-static, and dynamic loading conditions would assist in developing constitutive model.

An illustration of microscope mounted magnetic system is shown in Fig.4.1. Briefly, the magnetic system considered in this study consists of external permanent magnet attached to one or both of the lever arms, and a motor to control the location of the magnet. The position of both lever arms is controlled by a brushless DC motor (Maxon Motors, Serial Number: 283333). Two lead screws, one with right-handed thread and one with left-handed thread are attached to the motor to translate the rotation into linear motion. The lead screw diameter is 4 mm (5/32 inches) and lead is 0.84 mm (0.033 inches). Having two lead screws of different threads allows

backward-forward movement of both lever arms simultaneously, thus the system can also be used to perform uniaxial stretching on soft materials.

1.1.1. *Calculations and simulation.* Any magnet shape can be chosen for the application, however the magnet used in this setup is of a shape of cylindrical rod made of sintered NdFeB. Specifically, the cylindrical magnet has diameter, $2a$, of $300\text{ }\mu\text{m}$ and length, L , of 2.54 mm . The remanent induction of the magnet is along the long axis, B_r of 1.43 T .

Biot-Savart law is used to describe magnetic field of wire-wound ideal solenoid at point $\mathbf{x}$ (Eq.4.1) and serves as a good model to describe the permanent cylindrical magnet [17]. In Eq.4.1, the solenoid of current nI is assumed to be divided into strips of width, dz' and $\mathbf{R} = \mathbf{x} - \mathbf{x}'$.

$$\mathbf{B}(\mathbf{x}) = \int_{-L}^L \frac{\mu_0}{4\pi} \oint (nI dz') \frac{d\mathbf{x}' \times \mathbf{R}}{|\mathbf{R}^3|} \quad (4.1)$$

Analytically solving the integrals in Biot-Savart law in 3D space requires evaluating elliptic integrals, which is not straight-forward. Some elliptic integral evaluation methods have been presented using numerical or semi-analytical approaches [77, 17]. In this work, COMSOL Multiphysics (version 4.3) was used to simulate the magnetic field around the cylindrical magnet. COMSOL Multiphysics is a finite element based numerical solver. The physics module named Magnetic Fields (*mf*) was used in the simulations. The cylindrical magnet and the surrounding environment were modeled using 3D geometry. The space around the magnet is infinite, thus to minimize unnecessary mesh elements and reduce simulation time and memory, *mapped infinite elements* in AC/DC module was used. Spherical shape of the external boundary was chosen to implement *infinite element*, in consequence the coordinates at the external boundary of the model are scaled toward infinity and the inner boundary coincides with local domain (Fig.4.2). The model is not complex, thus stationary solver configuration and fine mesh were appropriate to converge to the solution.

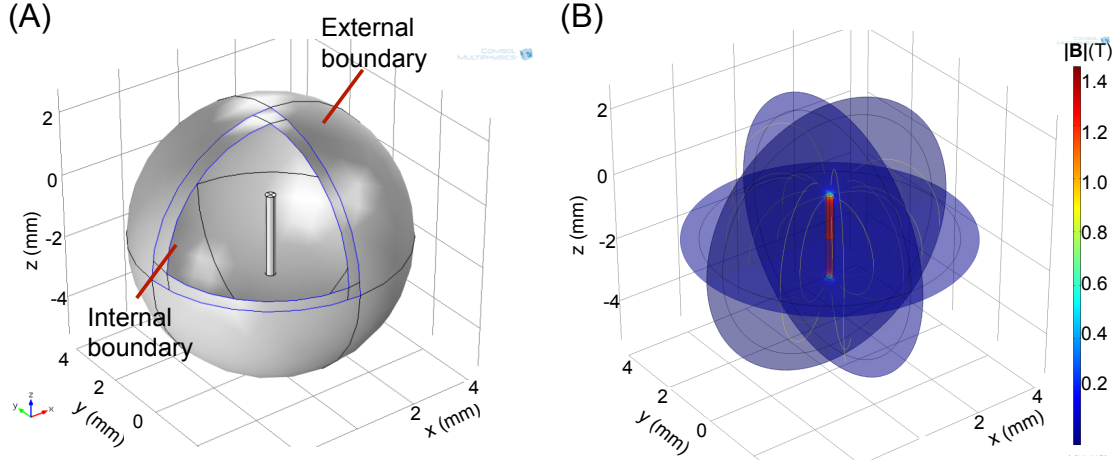


FIGURE 4.2. Snapshot from COMSOL Multiphysics of a (A) 3D geometry setup. The external boundary of the setup is scaled towards infinity enforcing infinite boundary condition. Internal boundary is part of the local domain. (B) Magnetic field is calculated using Magnetic Field *mf* module for cylindrical magnet axially magnetized in *z* direction.

The force acting on a magnetic particle inside the applied magnetic field, B (T) is given by

$$\vec{F} = \rho V \nabla (\vec{M}_0 \cdot \vec{B}) + \frac{V \chi_{bead}}{\mu_0} \vec{B} \quad (4.2)$$

where V and ρ are the volume (m^3) and density (kg/m^3) of the magnetic particle, respectively [88]. χ_{bead} is the difference of magnetic susceptibilities between the particle and the surrounding medium (dimensionless). $\mu_0 = 4\pi \times 10^{-7}$ is the magnetic permeability constant of the vacuum (Tm/A).

Magnetic susceptibility, χ_b , is calculated from the slope of the beginning part of the magnetization curve (Fig.4.3) and is given by Eq.4.3 [88]:

$$\chi_b = \rho \frac{\Delta M}{\Delta H}, \quad (4.3)$$

where H is the applied field on the magnetic beads in Amperes/meter (A/m) and is related to B -field by $H = \frac{B}{\mu_0} - M$.

Spatial gradients of a magnetic field are necessary to solve for the force acting on magnetic particles. These gradients could be solved for apart from COMSOL

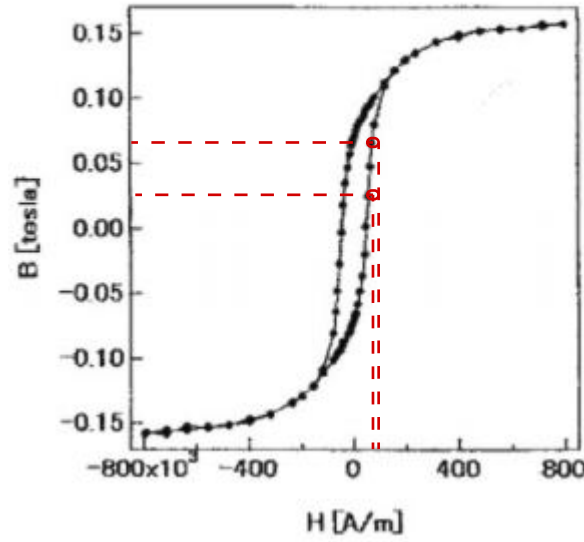


FIGURE 4.3. Plot provided by Spherotech Inc. showing ferromagnetic bead characteristics under applied magnetic field, H

Multiphysics by exporting the output of the magnetic field. The advantage of this approach is in the speed of the calculations. Specifically, in MATLAB, the gradients could be solved via convolution with finite difference kernel in a short time. The disadvantage is in the sampling of the exported data and the memory required to output the oversampled magnetic field vector. In this work the speed was sacrificed, and spatial gradients of B were solved for in COMSOL Multiphysics by mapping the results of calculated magnetic field on *Lagrange elements* via *coefficient form*, *PDE* interface.

TABLE 4.1. Parameters used in the experiments and models of magnetic system. The values were collected from the manufacturer

Variable	Name	Value	Units
Radius of the magnet	a	150	μm
Length of the magnet	L	2.54	mm
Surface magnetization of the magnet	B_0	1.43	T
Radius of the bead	R	2.2	μm
Susceptibility of the bead	χ_b	250	—
Density of the bead	ρ	1.810	g/cm^3
Chromium dioxide layer	—	20 %	—

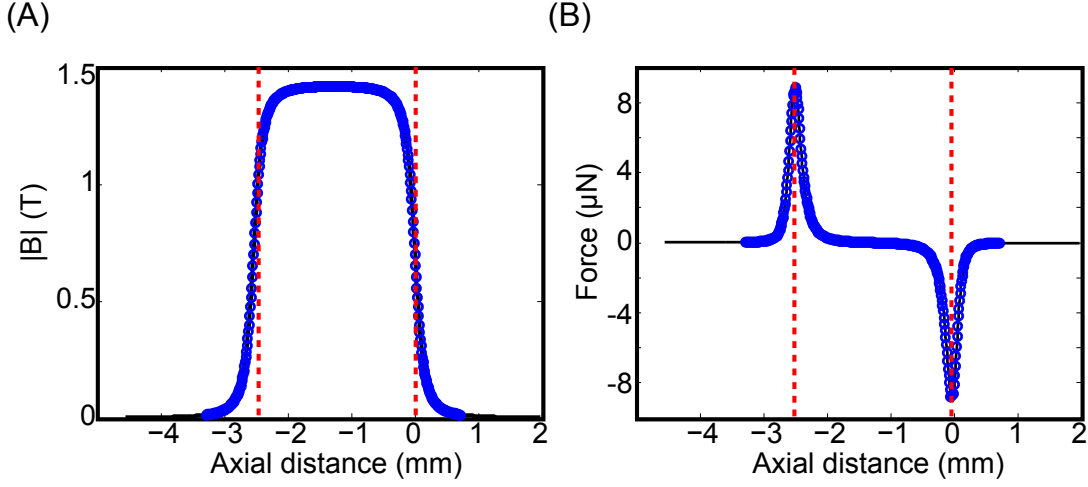


FIGURE 4.4. Plot of analytical formulation for (A) magnetic field and (B) magnetic force acting on a spherical particle at the central axis of the cylindrical magnet. Analytical formulation is plotted in black line superimposed with COMSOL Multiphysics solution (blue circles). Red dashed lines illustrate the axial location of the magnet.

To confirm the validity of the model, the known analytical expression of magnetic field and force at the central axis of cylindrical magnet were chosen. Equation 4.4 is an analytical formulation of the magnetic field on central axis of the cylindrical magnet at any point z away from the magnet cross-section. B_0 in Eq.4.4 is the surface magnetization of the magnet that is measured by Gaussmeter or provided by the manufacturer; L and a are the axial length and the radius of the cylindrical magnet. The values used for the calculations are listed in Table 4.1. Analytical formulations for both magnetic field and force match perfectly with the superimposed values acquired from COMSOL Multiphysics solver. The red dashed lines in Fig.4.4 illustrate the edges of the cylindrical magnet in the axial direction. As shown in Fig.4.4, magnetic field and force decay fast as the distance progresses away from the edge. Thus, for higher gradient or force, the edge of the magnet has to be close to the substrate of interest.

$$\mathbf{B}(z) = \frac{1}{2}B_0 \left[\frac{(L/2 - z)}{\sqrt{a^2 + (L/2 - z)^2}} + \frac{(L/2 + z)}{\sqrt{a^2 + (L/2 + z)^2}} \right] \quad (4.4)$$

1.2. Experimental Validation of the Magnetic System. As mentioned previously, the magnetic system is mounted to a microscope. The tested material is

attached to a clear petridish that could be imaged with an inverted microscope from the bottom (Fig. 4.1). Similar to the neutrophil studies, a Nikon Confocal microscope is used for the validation. Consequently, the locations of ferromagnetic beads are acquired while capturing time-lapse movies by NIS-Elements (Nikon Software).

The speed of the motor and x position of the lever arm are controlled through an NI LabView (National Instruments, Austin, TX) interface. The nominal linear speed of the motor is computed using a simple relation: $V_{\text{linear}} = p \times n/60$, where p is the pitch of the screw (0.84 mm) and n is the output speed of the motor (8000 rpm). A 14:1 gearbox is used for the motor in this system, therefore the output linear speed of the lever arms is ≈ 8 mm/sec and the maximum speed is ≈ 20 mm/sec.

The magnetic force is calibrated by measuring the velocity of ferromagnetic beads through a viscous, uncured polydimethylsiloxane silicone (PDMS, Sylgard Silicone Elastomer Kit, Dow Corning). Two different PDMS grades were chosen with two different viscosities: $\eta = 4.5$ Pa·s and $\eta = 66.7$ Pa·s. The cylindrical magnet tip was immersed into the PDMS solution and imaged at different locations using the NIS-Elements microscope software. The force acting on the bead was computed using Stokes' formula:

$$\mathbf{F} = 6\pi \times \eta \times r \times \mathbf{v} \quad (4.5)$$

where r and $\mathbf{v}$ are the bead radius and velocity.

Care must be taken in choosing and treating ferromagnetic beads for these experiments. Ferromagnetic beads used in the current work are prepared by coating a layer of chromium dioxide ($\approx 20\%$) onto a polystyrene core particle (data from Spherotech). Therefore, the force-to-volume of the beads is not as high as one would expect. Additionally, smaller particles are more susceptible to the random thermal forces in the system (drift). Before every experiment, a PDMS solution with beads was imaged without applied force to get the bead drift information (noise floor). Only 2D images were captured during the validation process due to the fast speed of the magnetic beads. Calculated force magnitudes are highly affected by choosing the

proper characterization properties describing ferromagnetic beads. These parameters are not easily found in the literature. Some of the data is taken from the product information sheet from the manufacturer (Spherotech Inc. Lake Forest, IL), but it is known that susceptibility of the beads may vary considerably even within the same batch [88]. In Kollmannsberger et al. [41], ferromagnetic beads with the best force-to-volume ratio were custom made, which is highly recommended for future progress of the project.

Positions of beads under applied magnetic force were tracked from captured images with particle tracking MOSAIC plugin in ImageJ developed by ETH Zurich [84]. Table 4.2 summarizes the parameters chosen for the images. During validation in PDMS 184 the images were captured at the estimated bottom of the cylindrical magnet every 5 seconds and at the center of the cylindrical magnet every 2 seconds (Fig.4.5). The bottom plane of the outer surface of the cylinder was estimated by immersing the magnet in fluorescent dye prior to the experiment. Once the bottom of the magnet is found, the center was estimated by changing the focal plane of the microscope by $150\text{ }\mu\text{m}$ (radius of the magnet). Figure 4.5 shows the captured microscope images. It also presents the trajectories of beads tracked by particle tracking plugin. Only trajectories that lasted more than 10 timestacks are shown in the figure. The maximum speed of tracked particles of the magnet is approximately $4.5\text{ }\mu\text{m}/\text{sec}$ at the bottom plane and $12\text{ }\mu\text{m}/\text{sec}$ at the central plane.

Figure 4.6 shows force vector maps calculated from the speeds of tracked beads at the bottom plane of the magnet outer surface. Although, qualitative assessment

TABLE 4.2. Parameters used in particle-tracking tool MOSAIC plugin in ImageJ.

Parameter	Value
Kernel Radius	4
Cutoff Radius	5
Percentile	0.1
Displacement	10
Linkage	1

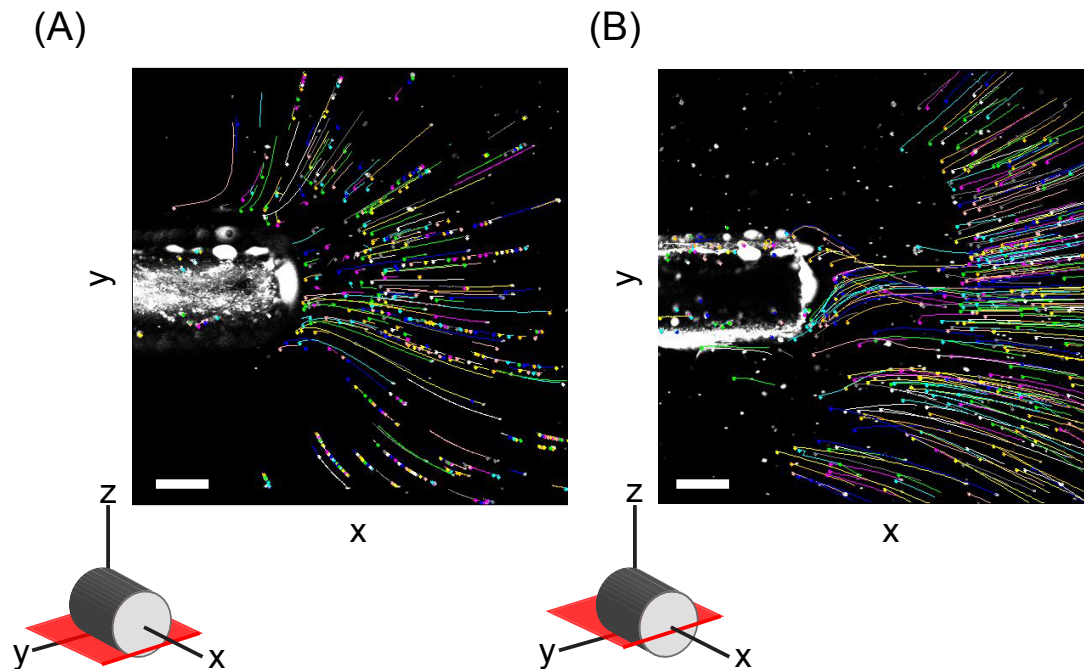


FIGURE 4.5. Images captured by confocal microscope of the beads focused (A) at the bottom and (B) at the central plane of the cylindrical magnet. Both of the images are superimposed with position vectors tracked by MOSAIC plugin in ImageJ. Scale bar = $150 \mu\text{m}$

of force direction vectors shows a good match between experimental and simulated results, Fig.4.6 shows a significant difference in force magnitudes. This is not surprising due to the poorly characterized bead properties. Specifically, susceptibility value and density of the beads play a significant role in the values. Additionally, since the beads are ferromagnetic, there is a particle-particle interaction that should be taken into account.

Validating the system in PDMS 186 with dynamic viscosity of $\eta = 66.7 \text{ Pa}\cdot\text{s}$, only the particles close to the magnet moved at the time interval of 50 seconds. Naturally, due to the higher viscous drag around the spherical particle, the maximum speed of the particles was only $0.5 \mu\text{m}/\text{sec}$. The calculated maximum force was calculated to be $\approx 2.0 \text{ nN}$, showing that the force that particles experience is about the same in both of the silicone solutions. Overall, these experiments demonstrated that PDMS 184 and 186 are appropriate for force calibration of the magnetic system.

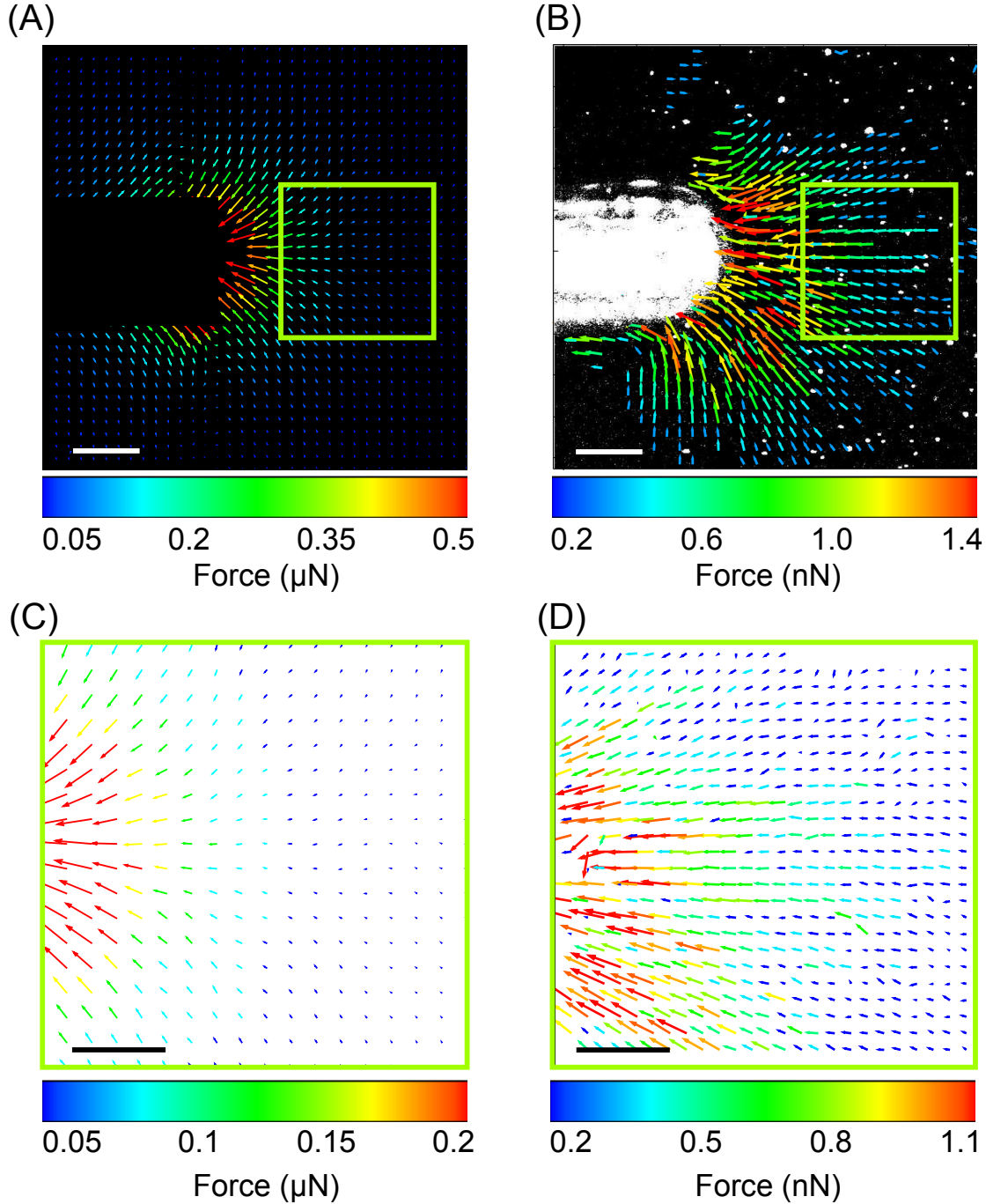


FIGURE 4.6. Illustration of the (A) COMSOL Multiphysics force calculations in comparison with (B) experimentally calculated force results collected from validation of the magnetic system using PDMS 184 solution. Results are presented as a vector plot that are colored according to the force magnitudes. Scale bars = $200\ \mu\text{m}$. Zoomed in results from (C) COMSOL Multiphysics and (B) experimental data are concentrated on the area $\approx 200\ \mu\text{m}$ away from the edge of the magnet shown as green box in (A) and (B). Scale bar in (C) and (D) = $100\ \mu\text{m}$.

For validation experiments where the particles can freely rotate in the suspension of PDMS, the $\vec{M}_0$ of the magnetic particle was aligned with the applied magnetic field. Therefore, for experiments in gels (e.g. collagen gel), the beads should be aligned manually with the a large magnet prior to completion of gelation.

2. Recommendation for Future Work

The magnetic system as described herein is promising for characterizing collagen at the microscale, but several improvements are suggested for the future. Specifically, a magnet with a smaller radius would allow for higher gradients, resulting in higher forces. With smaller magnet, it would also be easier to find the central axis of the magnet. Furthermore, better characterized magnetic particles would allow for better analytical and computational approximation of true force.

Currently the magnet is attached permanently to the lever arm, however a new attachment of the magnet to the lever arm would be ideal to allow for easy replacement of the magnet, and it would force the magnet to be parallel to the imaging plane. Additionally, using a glass coverslip bottom petridish would also allow for both better imaging quality and reflective imaging to capture actual fiber distribution during material characterization testing. Currently used plastic petri dishes are too thick to allow this type of imaging.

FIDVC described in this dissertation could be used to measure displacement fields of the collagen gel under the magnetic quasi-static loading. In this case, undeformed and deformed volumetric images could be captured three-dimensionally before and after the magnet has moved to the appropriate distance away from the gel edge. For dynamic loading, the displacement might be too quick for capturing 3D images, thus a 2D method of particle tracking would need to be used.

Accurate quantification of non-linear, full-field deformations described in this dissertation is not limited to the field of cell mechanics. By combining FIDVC technique with advanced imaging techniques, such as X-ray computed tomography or magnetic resonance imaging, measuring full-field deformations of any hierarchical materials

other than soft polymers would be possible. This would allow for a deeper understanding of the response of composite materials, specifically quantifying the effect of geometrical arrangement and size of internal microstructure on deformations induced by loading. For example, applying FIDVC on hierarchical nanotruss systems developed in the Greer group at California Institute of Technology could lead to a better understanding of the relationship between micro-architectures and overall strength of the materials.

Another natural extension of the application of a deformation measurement method like FIDVC is in quantifying 3D damage response of materials at different length scales. Most natural materials have different crack growth and stopping mechanisms at various hierarchical levels. Developing new experimental techniques that utilize the FIDVC method could assist in studying non-linear deformation fields in materials undergoing localized strain concentrations with high damage, such as crack nucleation and growth.

APPENDIX A

3D Viscoelastic Traction Force Microscopy

Note: The work has been performed and a version of this section is under review in *Soft Matter* [Toyjanova, J., Hannen, E., Bar-Kochba, E., Darling, E., Henann, D., and Franck, C., “3D Viscoelastic Traction Force Microscopy”, *Soft Matter*].

Native cell-material interactions occur on materials differing in their structural composition, chemistry, and physical compliance. While the last two decades have shown the importance of traction forces during cell-material interactions, they have been almost exclusively presented on purely elastic in-vitro materials. Yet, most bodily tissue materials exhibit some level of viscoelasticity, which could play an important role in how cells sense and transduce tractions. To expand the realm of cell traction measurements and to encompass all materials from elastic to viscoelastic, this paper presents a general, and comprehensive approach for quantifying 3D cell tractions in viscoelastic materials.

This methodology includes the experimental characterization of the time-dependent material properties for any viscoelastic material with the subsequent mathematical implementation of the determined material model into a 3D traction force microscopy (3D TFM) framework. Utilizing this new 3D viscoelastic TFM (3D VTFM) approach, we quantify the influence of viscosity on the overall material traction calculations and quantify the error associated with omitting time-dependent material effects, as is the case for all other TFM formulations. We anticipate that the 3D VTFM technique will open up new avenues of cell-material investigations on even more physiologically relevant time-dependent materials including collagen and fibrin gels.

1. Introduction

Over the past two decades Traction Force Microscopy (TFM) has emerged as a powerful, quantitative approach for characterizing the physical interactions between cells and their surroundings [1, 2, 3, 4]. TFM has provided a deeper understanding on the role of physical forces during cellular homeostasis, disease progression, and many other cellular processes. For example in 2005, Paszek et al. showed, using TFM that tensional homeostasis, i.e., the regulation of intracellular tension, plays a significant role in determining the ultimate outcome in cancer cell malignancy [5].

With the availability of improved spatial imaging techniques, TFM has evolved to deliver two-dimensional and three-dimensional (3D) descriptions of cell-surface traction measurements of single cells and cell sheets [6, 7, 9, 8, 10, 11]. It has been shown that most of the tissues in the body are characterized as time-dependent viscoelastic materials [12, 13, 14]. Yet, until recently, almost every TFM approach featured a linear elastic continuum mechanics framework [15, 16, 17]. Through a recent significant advancement in our displacement detection scheme we showed that cells on soft gels are capable of generating large material deformations exceeding the traditional linear formulation limits [18]. We addressed these observations by presenting a new, reformulated hyperelastic 3D TFM approach, capable of accurately capturing finite elastic material deformations [18].

Still, there is currently no documented approach of incorporating material viscosity into a TFM framework. Such formulation may be of significant importance given that the literature has shown that most tissues, including more sophisticated in-vitro culture systems, possess some level of viscosity as part of their microstructure [19, 20, 21, 22]. Since cells possess the ability to sense and probe their microenvironment dynamically, incorporating viscoelastic material properties into a TFM framework is important to provide the most accurate measurements of cell-material interactions [23, 24, 25, 26].

In this paper, we address this challenge by presenting an integrated material characterization and 3D TFM approach to perform cell traction force measurements on

materials with time-dependent physical properties. This technique, named 3D Viscoelastic Traction Force Microscopy (3D VTFFM) is general enough to allow the incorporation of any linear or non-linear elastic and viscous material properties. Specifically, we present a combined experimental and numerical approach on determining the viscoelastic material properties of soft agarose substrates, and their subsequent use in determining cell-induced material tractions. To examine differences in the spatial distribution and magnitude of the time-dependent surface traction fields due to the viscosity of the substrate material, we construct an analytical test case. Finally, we present actual experimental cell surface traction data using our 3D VTFFM technique of breast cancer cells deforming collagen-functionalized agarose substrates.

2. Materials and Methods

Glass Coverslips and Microscope Slides Surface Modification. Circular glass coverslips (25 mm, diameter, Fisher Scientific, Waltham, MA) were chemically modified to allow covalent attachment of polyacrylamide substrates using previously developed protocols [1, 6, 7]. Briefly, glass coverslips and slides were rinsed with ethanol and placed in a petri dish containing a solution of 0.5% (v/v) 3-aminopropyltrimethoxysilane (Sigma-Aldrich, St. Louis, MO) in ethanol for 5 minutes. Next, coverslips were washed with ethanol and submersed in a solution of 0.5% glutaraldehyde (Polysciences, Inc., Warrington, PA) in deionized (DI) water for 30 minutes. Activated coverslips were washed with DI water and left to dry.

To allow easier gel detachment, rectangular glass microscope slides (75x25 mm) were chemically modified to create hydrophobic surface. Briefly, glass coverslips were placed in a petri dish containing 97% (v/v) hexane (Fisher Scientific, Waltham, MA), 2.5% (v/v) (tridecafluoro-1,1,2,2-tetrahydrooctyl)-triethoxysilane (SIT) (Gelest, Morrisville, PA), and 0.5% (v/v) acetic acid (Sigma-Aldrich, St. Louis, MO) for approximately 1 minute. Coverslips were then removed and left to dry.

Preparation of Polyacrylamide-covered coverslips. Polyacrylamide (PA) gels were prepared from acrylamide (40% w/v, Bio-Rad Laboratories, Hercules, CA)

and N,N-methylene-bis-acrylamide (BIS, 2% w/v, Bio-Rad Laboratories, Hercules, CA) stock solutions as described previously [1, 6, 7]. The concentrations of acrylamide and BIS were chosen to be 8%/0.1%. Crosslinking was initiated through the addition of ammonium persulfate (Sigma-Aldrich, St. Louis, MO) and N,N,N,N-tetramethylethylenediamine (Life Technologies, Grand Island, NY). The PA solution was vortexed for about 30 seconds, and 12 μL of the PA solution were pipetted on the surface of an activated coverslip and sandwiched with a hydrophobic glass slide. PA gel thickness was measured to be $\sim 5 \mu\text{m}$. PA gel substrates were then submerged in distilled water and allowed to polymerize and hydrate for approximately an hour. Once the coverslip was removed, PA gel substrates were left uncovered at room temperature to dry out. Exploiting the strong cohesion between agarose and PA, the thin PA layer was used to firmly attach each agarose substrate to the underlying glass coverslip (Fig.A.1(a)).

Preparation of Agarose Substrates. For agarose substrate preparation, 0.5 % (w/v) agarose powder (Benchmark Scientific, Edison, NJ) was dissolved in 1x PBS (Invitrogen, Carlsbad, CA). Each agarose solution was heated in an oven to approximately 90 °C for complete dissolution. It should be noted, that the vial with the solution was loosely covered, yet boiling was avoided at all times. While heating, the solution was stirred occasionally, until the agarose powder had completely dissolved. Next, the agarose solution was combined with 10% (v/v) of 0.5 μm yellow-green fluorescent microspheres (Invitrogen, Carlsbad, CA). All agarose samples were thoroughly vortexed, and 35 μL of the final agarose solution were sandwiched between a dried PA gel-covered coverslip and a plain glass slide (75x25 mm). Upon complete gelation of the agarose solution, the assembly was immediately immersed in distilled water for approximately one hour. Finally, agarose coverslips were carefully peeled off the plain glass cover slide (Fig.A.1(a)). The final thickness of agarose gel layer after swelling was measured to be $\sim 40 \mu\text{m}$.

Collagen-Functionalization of Agarose Substrates. To promote cell attachment, agarose substrates were functionalized with type I collagen using the bifunctional crosslinker, sulfo-SANPAH (Thermo Fisher Scientific, Waltham, MA) [2, 6, 7]. Figure A.1 presents a complete overview of the functionalized agarose substrate preparation setup. Excess water was removed prior to deposition of 100 μL of sulfo-SANPAH (1mg/mL) onto the surface of each film, followed by a 15 minute exposure to UV light. The darkened sulfo-SANPAH solution was aspirated and the procedure was repeated. The samples were thoroughly washed with DI water, and covered with a solution of 0.2 mg/mL bovine type I collagen (BD Bioscience, Franklin Lakes, New Jersey) and left undisturbed at 4 °C overnight. Following overnight incubation, the substrates were rinsed three times with 1x PBS and sterilized with UV irradiation before depositing cells.

Cell Culture and Seeding. MDA-MB-231 metastatic breast adenocarcinoma cells were chosen as a representative contractile cell phenotype. Cells were cultured in medium containing 10% fetal bovine serum and 1% penicillin-streptomycin in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA). Cells were maintained in the incubator at 37 °C and 5% CO_2 . Seeding density was 30 cells/ mm^2 . Once seeded, cells were allowed to spread for approximately 5 hours in the incubator prior to imaging.

Live Cell Imaging. Three-dimensional image stacks were acquired using a Nikon A-1 confocal system mounted on a TI Eclipse inverted optical microscope controlled by NI-Elements Nikon Software. A 40x plan fluor air objective mounted on a piezo objective positioner was used for all the experiments, which allowed imaging at speeds up to 30 frames per second. Green fluorescent microspheres were used for the substrate and excited with an Argon (488 nm) laser. 512 x 512 x Z voxels ($102\mu\text{m} \times 102\mu\text{m} \times Z$) confocal volume stacks were recorded every 100 seconds with $Z \sim 128$ voxels ($38\mu\text{m}$), illustrated in Fig.A.1(b). To ensure physiological imaging conditions within the imaging chamber, temperature and pH were strictly maintained at 37 °C and $\sim$

7.4 during time lapse recording as previously described [6, 7]. The outline of the cell was estimated from phase contrast microscopy images.

Calculation of cell-generated displacements. Cell-generated full field displacements were determined following a similar methodology described in Toyjanova et al. [18]. First, three-dimensional time-lapse volumetric images of fluorescent beads embedded in agarose substrates were recorded using laser scanning confocal microscopy (LSCM). Then, the motion of embedded fluorescent beads was tracked in all three dimensions by using a new fast iterative digital volume correlation (FIDVC) algorithm [27]. Bar-Kochba et al. showed that the newly developed FIDVC technique provides significantly higher spatial resolution, signal-to-noise, and faster computation times than our previous DVC algorithm [28].

Nanoindentation Measurements. Force-indentation measurements of agarose gels were collected using an Asylum MFP-3D-BIO atomic force microscope (AFM) (Asylum Research, Santa Barbara, CA) following a similar procedure as previously described [29, 30]. The indenter geometry consisted of individual borosilicate glass spheres (5 μm diameter) attached to the tip of AFM cantilevers (Novascan Technologies, Inc., Ames, IA). Figure A.2(a) provides a schematic overview of the indentation

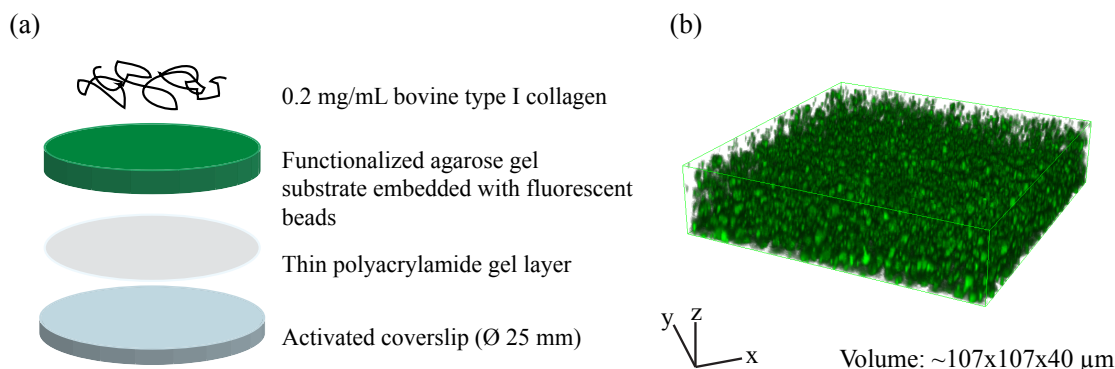


FIGURE A.1. Schematic of (a) the agarose substrate preparation and (b) a representative volumetric image of the agarose substrate embedded with green fluorescent microspheres recorded by laser scanning confocal microscopy.

experiments. The cantilever spring constant was determined to be 0.02 N/m using the power spectral density of the inherent thermal noise fluctuations of the cantilever [31].

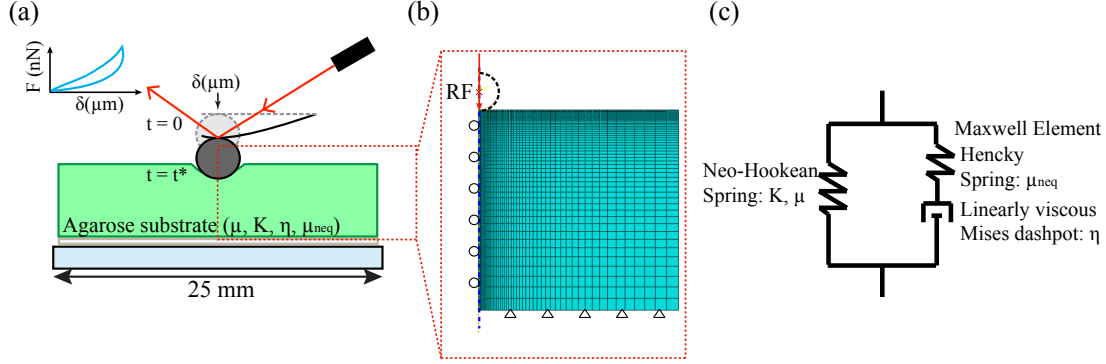


FIGURE A.2. Schematic of (a) the experimental setup of AFM indentation of the agarose substrate with a spherical cantilever tip radius of $2.5 \mu\text{m}$. (b) Modeling setup mimicking the indentation experiment. The reference point (RF) to control the indentation is set at the center of the sphere. (c) Schematic of the finite-deformation viscoelastic agarose constitutive model.

To probe the viscoelastic material properties of our agarose substrates, loading-unloading indentation experiments were performed at three different cantilever approach velocities: $0.1 \mu\text{m}/\text{sec}$, $1 \mu\text{m}/\text{sec}$, and $10 \mu\text{m}/\text{sec}$. Additionally, 30 second force relaxation tests were performed using an initial loading velocity of $10 \mu\text{m}/\text{sec}$ and an indentation depth of 450 nm.

Data were sampled at 5 kHz for loading-unloading measurements and at 200 Hz for 30 s for viscoelastic relaxation measurements. A 2-3 nN force trigger, resulting in 400-600 nm indentation depths, was used to prescribe the point at which the cantilever approach was stopped and either retracted for loading-unloading tests, or held constant for relaxation tests. To account for any day-to-day variability among the agarose substrates, all AFM indentation tests were performed on the same batch of agarose, and on the same day as the cell experiments. All experiments were carried

out at room temperature in a fluid environment. The AFM was allowed to equilibrate before each measurement period to minimize deflection laser and/or piezo drift.

Finite Element Simulation. Simulations of the nanoindentation process were conducted using the commercial finite element software, ABAQUS 6.12 (Dassault Systemes Simulia Corporation, Providence, RI). The geometry of the agarose substrate was discretized by using 2D axisymmetric elements (CAX4H). The indenting spherical tip was modeled as analytical rigid solid with a radius of $R = 2.5 \mu\text{m}$. The agarose substrate boundary conditions were set to mimic those of the indentation experiments (Fig. A.2(b)). The indentation simulation was executed using the same displacement control loading condition as in the AFM experiments with the same loading rates. The material behavior of agarose used for fitting the experimental force-indentation curves is a Neo-Hookean rubber elasticity model with a finite-deformation Maxwell element in parallel (Fig. A.2(c)).

The bulk modulus was assumed to be $K \geq 100\mu$ for a nearly incompressible solid, and the remaining three material parameters were determined by fitting the simulated force-indentation curves to the experimentally obtained ones in a least-squares sense. The actual values of the fitted material constants are reported in Table 1.

3. Agarose Material Characterization

The objective of this investigation is to present the reader with a complete approach of determining the viscoelastic material properties of a given substrate material, and provide a straightforward approach of implementing the determined material model into a 3D TFM framework for cell traction measurements. To illustrate this process in detail, we chose an agarose gel as a model viscoelastic substrate material [32, 33]. Typical material characterization techniques for determining the material properties of soft hydrogels and tissue-mimicking materials have involved uniaxial compression, tension, AFM indentation and shear rheometry experiments. AFM indentation characterizations are attractive for determining TFM-substrate properties,

since they have the ability to measure the local mechanical properties directly at the surface where the cell is applying its tractions.

While elastic material AFM-based characterization approaches are relatively straightforward, the analysis of time-dependent material properties usually requires additional experimental measurements, such as creep or stress (force) relaxation tests [30]. If a large strain constitutive relation is sought, numerical simulation of the experimental procedure, followed by least-square fitting of the numerical to the experimental data provides a robust overall material characterization approach.

Figure A.2(a) presents a schematic of the AFM indentation experiments that was utilized to collect force-indentation curves of agarose substrates at different loading rates, as well as force relaxation tests (see technical details in Materials and Methods). Next, we constructed a simple, axisymmetric FEM model (Fig.A.2(b)) mimicking the experimental indentation process using the non-linear viscoelastic material model shown in Fig.A.2(c). The constitutive model is defined by the equilibrium shear modulus (μ) and bulk modulus (K) of the Neo-Hookean spring (Fig.A.2(c), left); the Maxwell element is described by a Hencky (logarithmic) spring with a non-equilibrium shear modulus (μ_{neq}), and a Maxwell element viscosity (η) (Fig.A.2(c), right). The time-dependency in this model is characterized by the deviatoric shear response of the Maxwell element. The emphasis behind the model was to use a minimal set of fitting parameters to appropriately describe the viscoelastic behavior of agarose under finite deformations. Given the high water content of agarose, the bulk modulus of the Neo-Hookean spring was set to $K \approx 100\mu$ assuming near incompressibility, with the condition that $K \gg \mu$. To determine the remaining three material parameters, μ , μ_{neq} , and η , the numerical FEM simulation was iteratively adjusted to fit the experimental force relaxation data in a least-squares sense. The resulting best-fit material parameters describing the finite viscoelastic behavior of agarose are presented in Table 1.

Figure A.3 (a) and (b) present the experimental and best-fit numerical indentation results for a typical force-relaxation experiment. The agarose sample is first indented

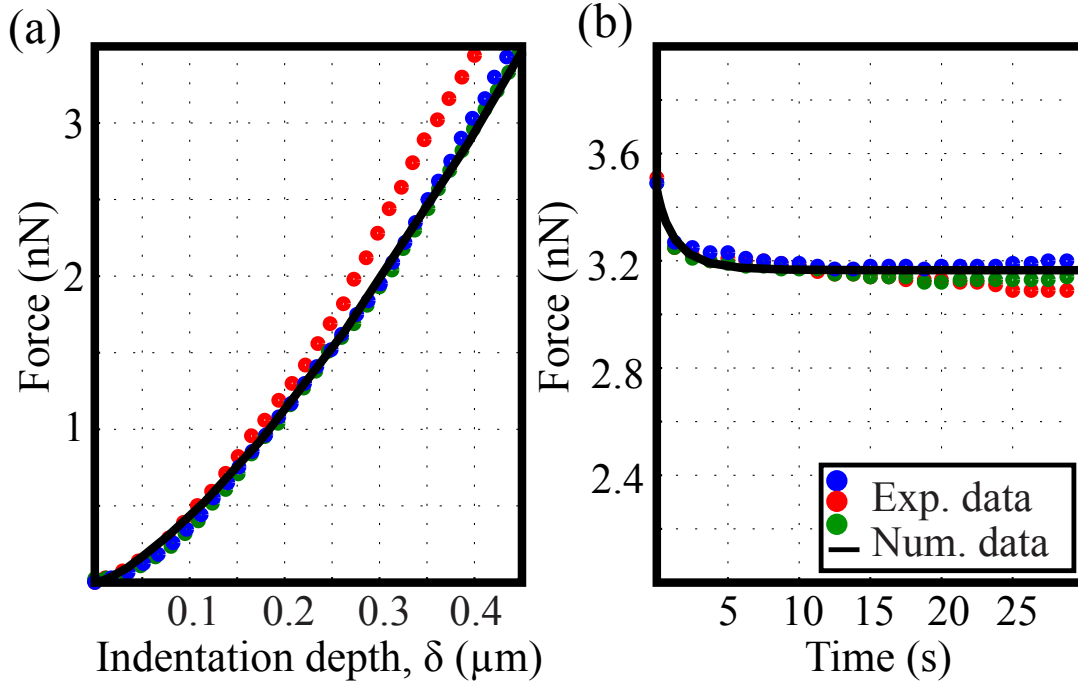


FIGURE A.3. Representative experimental indentation results for agarose substrates (dots) and corresponding numerical results (solid line) as computed from fitted material constants. (a) Force (nN) vs. indentation depth, δ (μm) at a loading rate of $10 \mu/\text{msec}$. (b) A representative force relaxation plot for agarose substrates.

to a depth of approximately 450 nm using a constant loading rate of $10 \mu\text{m}/\text{sec}$ (Fig. A.3(a)). Then, while maintaining the current indentation depth, the force is allowed to relax over a total period of 30 seconds (Fig. A.3(b)). Comparison between the experimental and numerical force-indentation curves shows good agreement. Given the relatively simple nature of the viscoelastic material model presented in Fig. A.3(b), some differences especially at larger indentation depths (Fig. A.3(a)) can be expected. Figure A.4 compares the experimental indentation loading-unloading force curves to the numerical indentation results based on the same material parameter set (given in Table 1) used to describe the force relaxation curves in Fig. A.3. As Fig. A.4 shows, the FEM simulations are in good agreement with the experimental results for all three different loading rates. Indenting the agarose gel at different loading rates allows for the examination of the time-dependent, viscous material behavior, which the model

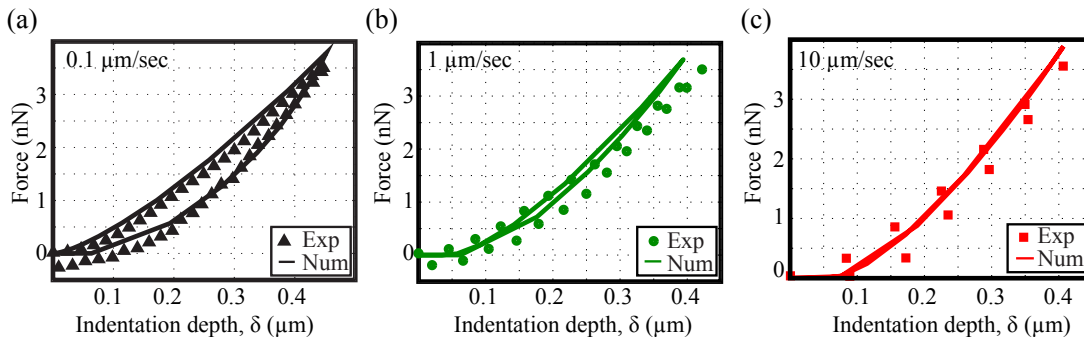


FIGURE A.4. Representative loading-unloading indentation results for loading rates of (a) $0.1 \mu\text{m/sec}$, (b) $1 \mu\text{m/sec}$, and (c) $10 \mu\text{m/sec}$. Dotted curve denotes experimental results, continuous solid lines are best fits based on the fitted agarose material constants.

seems to capture adequately. When comparing Figs. A.4(a) - (c), only the slowest loading rate of $0.1 \mu\text{m/sec}$ leads to significant loading-unloading hysteresis, which is indicative of viscoelastic relaxation during the indentation cycle. The indentation force curves shown in Fig. A.4 are representative of 5 indentation measurements at each loading rate.

4. 3D Viscoelastic Traction Force Microscopy (3D VTfM)

Cell-generated full-field displacements and tractions can be determined in a variety of different ways, with two of the most common approaches being image correlation-based measurements and single particle tracking algorithms. We previously presented a large-deformation 3D image correlation-based approach for determining cellular displacement fields with high resolution [27, 18]. Regardless of the chosen displacement detection scheme, once the 3D displacement field is obtained, its spatial derivative, i.e. the deformation gradient, is used within the framework of the previously described viscoelastic material model (Fig. A.2(c)) to determine the cell surface tractions. Given that most tissue materials and more complex hydrogels exhibit some time-dependent and viscous behavior [19, 20, 21], and the observation that cells can generate finite deformations [18], accurate cell traction analysis requires the utilization of a finite-deformation viscoelastic material model. Although our example is

specific to agarose, we purposefully set up the methodology such that the same steps can be utilized to incorporate other, experiment-specific, viscoelastic material models into the same overall 3D VTfM framework.

To model the observed viscoelastic response of agarose under finite-deformations, we utilize a simple generalization of a standard linear solid (Fig. A.2(c)). The left branch represents the equilibrium response of the material, which we model as a nearly-incompressible Neo-Hookean material. The Cauchy stress in the equilibrium branch is denoted as $\boldsymbol{\sigma}^{\text{eq}}$. To model time-dependent, non-equilibrium effects, we include a finite-deformation Maxwell element in parallel with the left Neo-Hookean spring, and denote the Cauchy stress in this branch as $\boldsymbol{\sigma}^{\text{neq}}$. Thus, the total Cauchy stress in the substrate is given by $\boldsymbol{\sigma} = \boldsymbol{\sigma}^{\text{eq}} + \boldsymbol{\sigma}^{\text{neq}}$ [35, 36].

Finite deformations are described by a displacement function, $\mathbf{y} = \mathbf{x} + \mathbf{u}(\mathbf{x}, t)$, which represents a one-to-one mapping of points $\mathbf{x}$ in the reference configuration to points $\mathbf{y}$ in the deformed configuration at each time t . The deformation gradient is then the gradient of the displacement function with respect to $\mathbf{x}$, that is $\mathbf{F} = \nabla \mathbf{y} = \mathbf{I} + \nabla \mathbf{u}(\mathbf{x})$, with the volume ratio given by the Jacobian of $\mathbf{F}$, $J = \det \mathbf{F} > 0$. Experimentally, the deformation gradient tensor, $\mathbf{F}$, can be directly calculated from the full-field displacements using various discrete differentiation kernels. Farid et al., and Bar-Kochba et al. provide a description of several robust differentiation kernels [34, 27].

To specify the mechanical response of the equilibrium branch of the material model in Fig. A.2(c), we assume that the spring is described by a Neo-Hookean free energy of the form

$$\psi^{\text{eq}}(\mathbf{F}) = \frac{\mu}{2} \left(\frac{(\text{tr } \mathbf{B})}{J^{2/3}} - 3 \right) + \frac{1}{2} K (J - 1)^2, \quad (\text{A.1})$$

where μ and K are the ground-state, equilibrium shear and bulk moduli, respectively, and $\mathbf{B} = \mathbf{F}\mathbf{F}^\top$ is the left Cauchy-Green tensor. The first term represents the free energy due to distortional (constant volume) deformations, and the second term represents purely volumetric deformations. Assuming near-incompressible material

behavior, we set $K \geq 100\mu$. The associated Cauchy stress is then

$$\boldsymbol{\sigma}^{\text{eq}} = J^{-1} \frac{\partial \psi^{\text{eq}}(\mathbf{F})}{\partial \mathbf{F}} \mathbf{F}^\top = \frac{\mu}{J^{5/3}} \left(\mathbf{B} - \frac{1}{3}(\text{tr } \mathbf{B})\mathbf{I} \right) + K(J - 1)\mathbf{I}. \quad (\text{A.2})$$

The time-dependent, viscous material response is described by the Maxwell element (Fig. A.2(c), right branch). For this branch, the deformation gradient, $\mathbf{F}$, is decomposed into its elastic and viscous parts: $\mathbf{F} = \mathbf{F}^e \mathbf{F}^v$, where $\mathbf{F}^e$ is the elastic deformation gradient, representing the deformation of the spring, and $\mathbf{F}^v$ is the viscous deformation gradient, representing the deformation of the dashpot. To specify the mechanical response of the non-equilibrium branch, we take the spring to be described by a Hencky (logarithmic) spring with shear modulus μ_{neq} and the dashpot to be in a linearly viscous Mises form with viscosity η . The details of the constitutive equations describing this branch are given in the Appendix. Here, we give the recipe for handling the Maxwell element in time-discrete form.

The approach is summarized in Fig. A.5. At a given time $t = t_n$, the deformation gradient $\mathbf{F}_n$ and viscous deformation gradient $\mathbf{F}_n^v$ (and hence the non-equilibrium elastic deformation gradient $\mathbf{F}_n^e$) are known. (Note that if t_n corresponds to the initial time, $\mathbf{F}_n = \mathbf{F}_n^v = \mathbf{I}$.) Then at time $t = t_{n+1} = t_n + \Delta t$, the deformation gradient $\mathbf{F}_{n+1}$ is given. In an experiment, this is simply the deformation gradient measured at the subsequent time point. To proceed forward in time, we need to calculate the Cauchy stress due to the Maxwell element, $\boldsymbol{\sigma}_{n+1}^{\text{neq}}$, and viscous deformation gradient, $\mathbf{F}_{n+1}^v$, at time t_{n+1} . The steps are given below:

- (1) First, we consider a trial state, in which viscous flow is frozen, see Fig. A.5, and calculate a trial non-equilibrium elastic deformation gradient, $\mathbf{F}_{\text{tr}}^e$, based on the old value of the viscous deformation gradient $\mathbf{F}_n^v$:

$$\mathbf{F}_{\text{tr}}^e = \mathbf{F}_{n+1} (\mathbf{F}_n^v)^{-1}. \quad (\text{A.3})$$

In this intermediate state, all deformation between times t_n and t_{n+1} is assumed to be accommodated by the Hencky spring. We then perform the

polar decomposition of $\mathbf{F}_{\text{tr}}^e$ and calculate the trial strain:

$$\begin{aligned}\mathbf{F}_{\text{tr}}^e &= \mathbf{R}_{\text{tr}}^e \mathbf{U}_{\text{tr}}^e, \\ \mathbf{E}_{\text{tr}}^e &= \ln \mathbf{U}_{\text{tr}}^e,\end{aligned}\tag{A.4}$$

where $\mathbf{R}_{\text{tr}}^e$ is the trial rotation, $\mathbf{U}_{\text{tr}}^e$ the trial right stretch tensor, and $\mathbf{E}_{\text{tr}}^e$ the trial Hencky (logarithmic) strain.

- (2) Next we use the trial strain to calculate a trial stress

$$\mathbf{M}_{\text{tr}}^e = 2\mu_{\text{neq}} \text{dev}(\mathbf{E}_{\text{tr}}^e),\tag{A.5}$$

where μ_{neq} is the non-equilibrium shear modulus. This particular stress measure is work conjugate to the Hencky strain and is referred to as the Mandel stress, and the specific stress-strain relation reflected in (A.5) arises due to a free energy function ψ^{neq} associated with the non-equilibrium spring (see the Appendix for details). Note that we only consider the contribution to the stress due to the deviatoric, or shear, strain. In this manner, the non-equilibrium Maxwell element only adds viscoelasticity to the shear deformation and leaves the volumetric response of (A.2) unaffected.

- (3) Next, we calculate the viscous stretching – that is, the strain rate in the dashpot – at time t_{n+1} :

$$\mathbf{D}_{n+1}^v = \frac{\mathbf{M}_{\text{tr}}^e}{2(\eta + \Delta t \mu_{\text{neq}})},\tag{A.6}$$

where η is the constant viscosity of the dashpot. See the Appendix for the derivation of this important step.

- (4) Calculation of $\mathbf{D}_{n+1}^v$ allows for the update of $\mathbf{F}^v$ by means of the exponential map:

$$\mathbf{F}_{n+1}^v = \exp(\Delta t \mathbf{D}_{n+1}^v) \mathbf{F}_n^v.\tag{A.7}$$

This brings us to the corrected, updated state in Fig. A.5.

(5) Finally, we may update the Cauchy stress due to the Maxwell element $\boldsymbol{\sigma}^{\text{neq}}$:

$$\begin{aligned}\mathbf{M}_{n+1}^e &= 2\eta \mathbf{D}_{n+1}^v, \\ \boldsymbol{\sigma}_{n+1}^{\text{neq}} &= (\det \mathbf{F}_{\text{tr}}^e)^{-1} \mathbf{R}_{\text{tr}}^e \mathbf{M}_{n+1}^e \mathbf{R}_{\text{tr}}^{e\top},\end{aligned}\tag{A.8}$$

where $\mathbf{M}_{n+1}^e$ is the Mandel stress at time t_{n+1} .

The total Cauchy stress due to both the equilibrium and non-equilibrium branches (Fig. A.2(c), left and right branch) may then be calculated by

$$\boldsymbol{\sigma}_{n+1} = \boldsymbol{\sigma}_{n+1}^{\text{eq}} + \boldsymbol{\sigma}_{n+1}^{\text{neq}},\tag{A.9}$$

where $\boldsymbol{\sigma}_{n+1}^{\text{eq}}$ is calculated by A.2 using $\mathbf{F}_{n+1}$. One may then repeat these steps, marching forward in time, to calculate the stress history corresponding to a measured deformation history. We encourage readers who are interested in the details of the model as well as the derivation of the time-discrete version to refer to the Appendix.

Once the Cauchy stress is calculated, the cell surface tractions can be found using the well-known Cauchy relation

$$\mathbf{t} = \boldsymbol{\sigma} \mathbf{n},\tag{A.10}$$

where $\mathbf{n}$ is the surface normal, and we have dropped the $(n+1)$ subscript for simplicity of notation. The surface normal vectors can be determined directly from the laser scanning confocal images. Toyjanova et al. previously described such a method in-detail [18]. The magnitude of the three-dimensional traction vector is then calculated as

$$|\mathbf{t}| = \sqrt{t_x^2 + t_y^2 + t_z^2},\tag{A.11}$$

where t_x and t_y are the in-plane traction force components under the cell and t_z corresponded to the out-of-plane component. The total cell force, a common metric used in cell TFM studies, is calculated by integrating the magnitude of the substrate surface tractions over the total cell area S , i.e.,

$$\mathbf{F} = \int_S |\mathbf{t}| d\mathbf{S}.\tag{A.12}$$

Further details on how to calculate cell forces from 3D traction data can be found elsewhere [18].

5. Analytical and Experimental 3D VTfM Examples - Influence of Material Viscosity on Surface Traction

Given the slightly higher complexity of a viscoelastic over a purely elastic 3D TFM formulation, our intent is to provide the reader with a quantitative metric to determine the penalty associated with ignoring the viscous effects for a given substrate material. Figure A.6 plots the ratio between the total traction magnitude that includes the time-dependent contribution, $|t|$, and the purely elastic traction magnitude, $|t_e|$, in the absence of the viscous dashpot in Fig. A.2(c) as a function of the viscoelastic material properties of the substrate. Depending on the intrinsic relaxation time, $\tau = \eta/\mu_{neq}$, and the viscosity, η , of the material at hand, the error associated with neglecting the viscoelastic nature of the substrate can be significant. Since any 3D and 2D TFM framework requires careful material characterization of its substrate material, Fig.A.6 can be utilized to determine whether a viscoelastic 3D TFM framework is necessary or whether a simpler, purely elastic formulation will suffice to estimate the cell-generated surface tractions.

Analytically Simulated Viscoelastic Cell Deformation Fields. While Fig. A.6 provides a quantitative estimate on the penalty associate with neglecting the viscous stresses in a material as a function of the characteristic relaxation time, τ , it is important to understand the relationship between τ and the actual imaging intervals utilized during live cell imaging. If τ is much less than the minimal time-lapse imaging interval, i.e., $\tau \ll \Delta t$, then only the elastic contributions of the material behavior can be estimated. This concept is illustrated in Fig. A.7 using an analytically generated Gaussian displacement dipole on a viscoelastic material obeying the constitutive model shown in Fig. A.2(c). The material constants were chosen such that it matches the properties of embryonic liver and heart tissues[19]. The characteristic relaxation time, τ , is set to 10 seconds. The analytical displacement

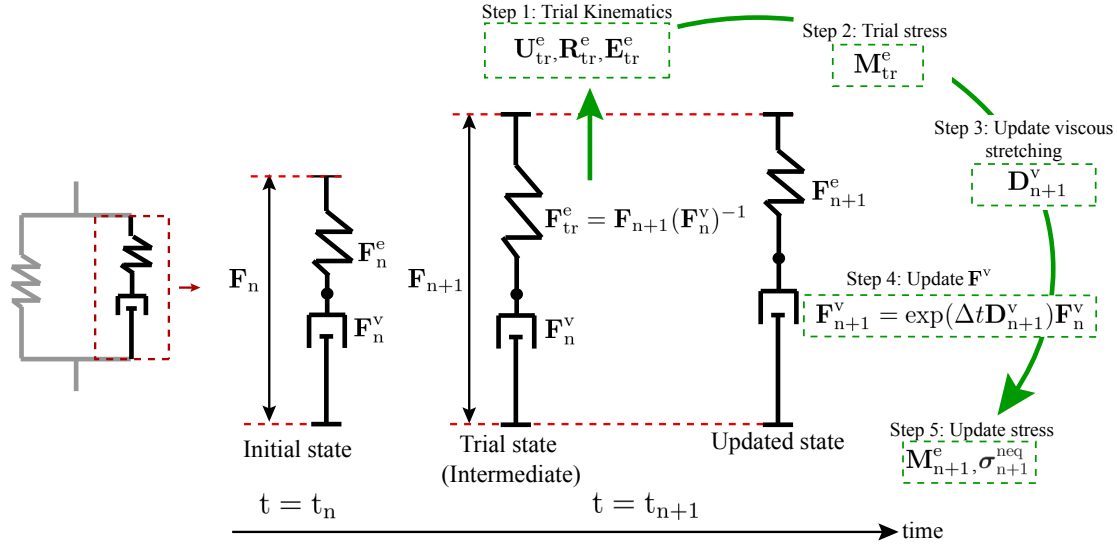


FIGURE A.5. Schematic of the time-discrete algorithm for the non-equilibrium, Maxwell branch of the constitutive model.

dipole is applied along the free surface of the $x_1 - x_2$ plane in the form of

$$\mathbf{u}(\mathbf{x}) = A \exp \left(-\frac{(x_1 - b_1)^2 + (x_2 - b_2)^2 + (x_3 - b_3)^2}{2\sigma^2} \right) \quad (\text{A.13})$$

Fig. A.7(a)-(b) depict the magnitude of the applied Gaussian displacement dipole and its associated displacement gradient. Using the displacement data, the viscoelastic cell surface tractions are calculated and are plotted in Fig. A.7(d) for different time-lapse imaging intervals. Figure A.7(d) presents a panel series of the magnitude of the 3D surface traction vector for the analytical displacement dipole shown in Fig. A.7(a) for different imaging intervals, Δt . Given a material relaxation time, τ of 10 seconds, Fig. A.7(c) quantitatively describes the effect different time-lapse imaging intervals have accurately estimating the applied traction magnitude. For fast acquisition times, i.e., $\Delta t \leq 1$ second, most of the viscoelastic traction magnitudes are sufficiently captured. If the imaging interval is increased to ~ 10 seconds, the peak tractions will be underestimated by approximately 17%, and with imaging times on the order of 100 seconds most of the viscoelastic material response has reached the equilibrium state, resulting in a traction estimation error of $\sim 30\%$. The shape of

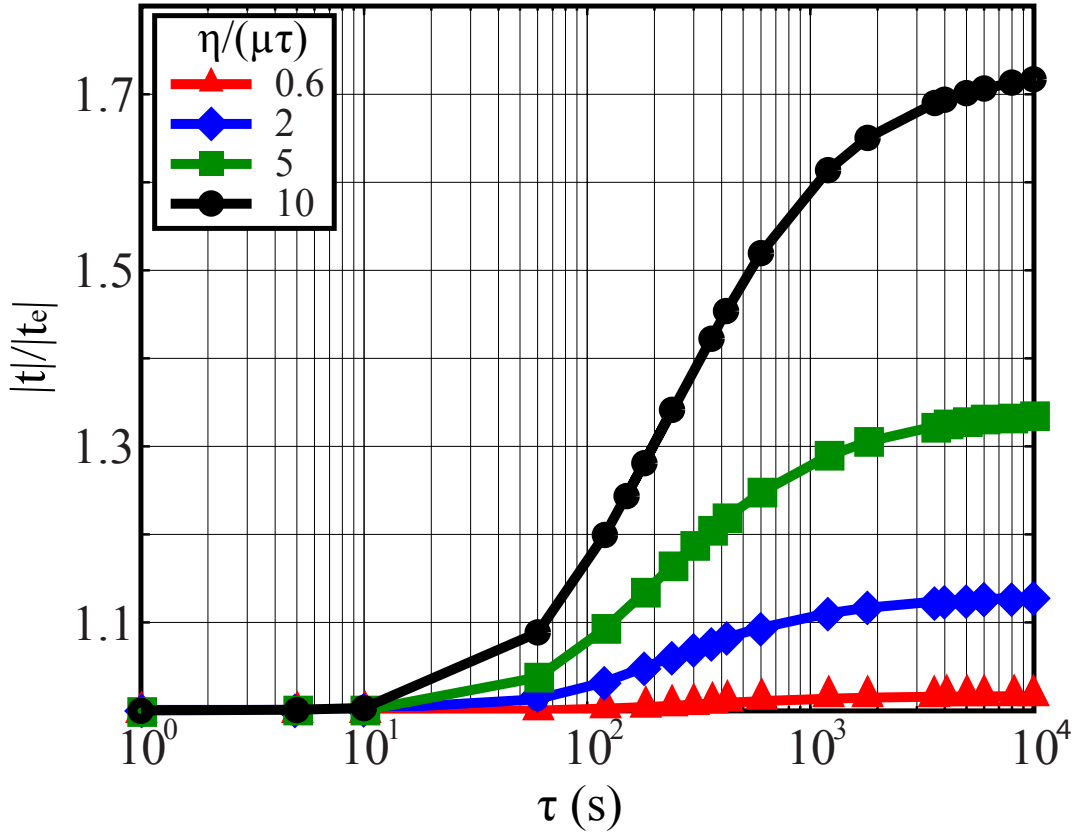


FIGURE A.6. Plot of the ratio of the total traction magnitude to the purely elastic traction contribution vs. relaxation time, $\tau = \eta/\mu_{\text{neq}}$. The calculations are shown for $(\eta)/(\mu\tau)$ of 0.6 (triangle), 2 (diamond), 5 (square) and 10 (circle).

the curve and the absolute numbers will vary depending on the particular viscoelastic material model employed, but can be generated via means of Eqs. A.1 - A.12.

While Fig. A.7 shows that the particular time-lapse imaging interval, Δt , can have a profound effect on accurately representing the spatial distribution and magnitude of the traction pattern in a viscoelastic medium, practical considerations, such as low signal to imaging noise, phototoxicity, and photobleaching might limit the range of available imaging intervals. Thus, once the material of interest has been characterized and evaluated, the error associated with practical imaging intervals can be evaluated by generating the type of plot shown in Fig. A.7(c). This should allow the user to

directly evaluate whether a viscoelastic TFM framework is necessary for the desired TFM measurements or whether a simpler, purely elastic formulation suffices.

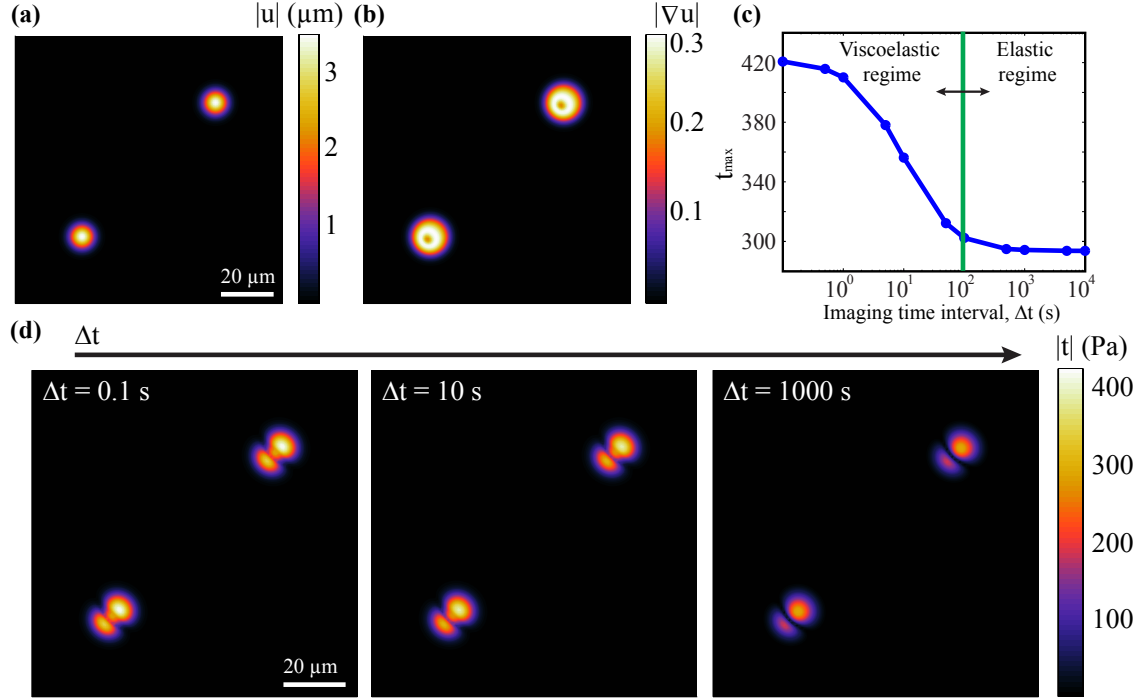


FIGURE A.7. Analytical traction example of (a) prescribed Gaussian displacement dipole and its (b) associated displacement gradient magnitude. (c) Plot of the magnitude of the maximum total surface tractions, $|t_{\max}|$ (Pa), vs. imaging time interval, Δt (seconds). (d) Contour maps of the calculated surface tractions at imaging time intervals of $\Delta t = 0.1$ seconds, $\Delta t = 10$ seconds, and $\Delta t = 1000$ seconds.

Experimentally Observed Viscoelastic Cell Deformation Fields. To illustrate the resolution and applicability of the 3D VTfM technique to capture actual cell-induced traction data, Fig. A.8 shows snapshots of a MDA-MB-231 cell deforming an agarose substrate at two different time points. The left panel shows phase contrast images of the cell on top of the agarose substrate, followed by the in-plane and out-of-plane tractions. The traction resolution sensitivity was 500 Pa for in-plane and 1000 Pa for out-of-plane tractions. The observed traction patterns in Fig. A.8 are similar in shape and magnitude to previously observed traction patterns for mesenchymal cells [37, 38, 39, 15, 6]. As can be seen from Fig. A.8, the utilization of

the 3D VTFM methodology produces high-resolution traction maps with similar resolution and sensitivity capabilities to our previously published 3D large deformation elastic TFM approach [18].

It should be noted that the characteristic relaxation time for the agarose gels used in this study was ~ 1.1 seconds, and that time-lapse images were acquired every 100 seconds. Any shorter time-lapse intervals yielded negligible material deformations indistinguishable from noise.

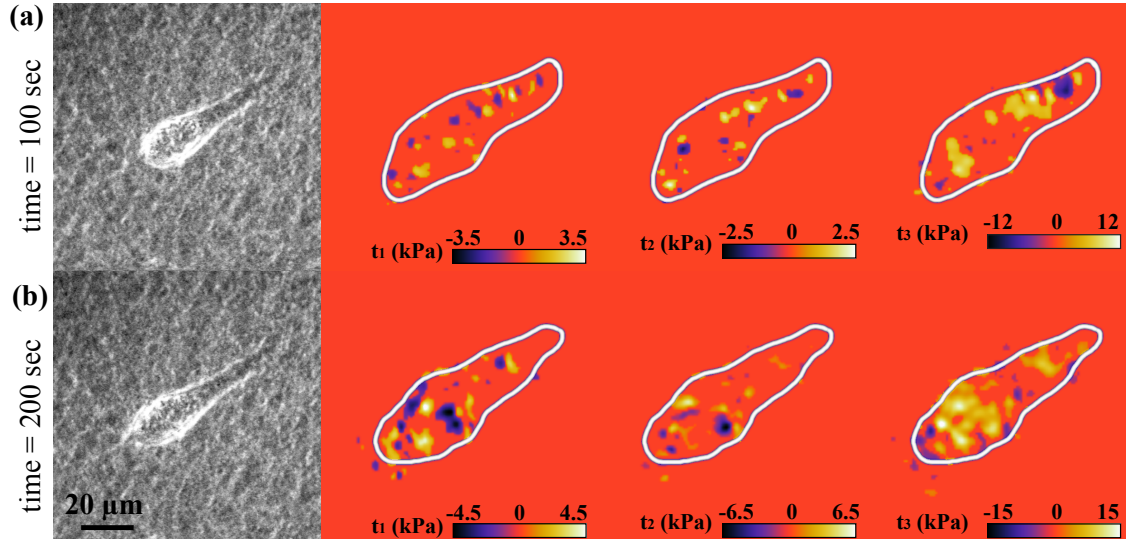


FIGURE A.8. Experimental example of a migrating MDA-MB-231 cells on the surface of an agarose substrate. The phase contrast image (left panel) presents the outline of the cell, followed by contour plots of surface tractions, t_1 , t_2 , and t_3 in kPa.

6. Conclusion

This paper presents a 3D Viscoelastic Traction Force Microscopy (3D VTFM) technique that allows the computation of cell-generated material tractions on viscoelastic materials. Using a combined experimental and numerical AFM indentation approach, our study details how to determine the material properties of the viscoelastic substrate material of interest, which in our simple example is an agarose gel. We would like to note, that the extraction of the viscoelastic material properties of the

substrate material at hand can also be achieved by various other mechanical testing approaches besides indentation, including stress relaxation, creep and frequency-dependent tests in rheometer or tension/compression setups. As long as the material stress-strain state is recorded as a function of time, the viscoelastic material properties of the particular substrate material can be determined. Next, utilizing the obtained material properties we provide a general, finite deformation framework for integrating the viscoelasticity of the substrate material into a 3D TFM methodology. The same general framework we described can be simply modified by the reader to allow the incorporation of his/her own substrate specific viscoelastic material properties into our 3D VTfM code structure to perform 3D traction measurements. Finally, we provide the reader with a quantitative estimate on the penalty associated with neglecting the viscous effects for the given viscoelastic material, and what the minimum TFM time-lapse interval is required to adequately capture the viscous contribution of the material response. We conclude by showing traction contour plots of breast cancer cells migrating on collagen-functionalized agarose substrates. The 3D VTfM code package can be downloaded from our website (<http://franck.engin.brown.edu>).

In conclusion, this study provides a new 3D TFM technique that is capable of incorporating time-dependent material characteristics for studies that involve more realistic tissue-mimicking substrate materials. While the last two decades have provided a wealth of cell-material interactions on elastic substrates, we anticipate that the 3D VTfM technique will open up new avenues of cell-material investigations on even more physiologically relevant time-dependent materials including native tissue derivatives such as collagen and fibrin gels.

Appendix: Viscoelastic model details

In this appendix, we give a detailed account of the finite-deformation viscoelastic model used to describe experimental data, as well as a derivation of the time-discrete version summarized in Section 4. Mathematically, the viscoelastic model involves the following definitions: $\mathbf{u}(\mathbf{x}, t)$, displacement; $\mathbf{y} = \mathbf{x} + \mathbf{u}(\mathbf{x}, t)$, motion; $\mathbf{F} = \nabla \mathbf{y}$,

$J = \det \mathbf{F} > 0$, deformation gradient; $\mathbf{B} = \mathbf{F}\mathbf{F}^\top$, left Cauchy-Green tensor. We take the free energy due to the equilibrium branch to be given in Neo-Hookean form

$$\psi^{\text{eq}}(\mathbf{F}) = \frac{1}{2}\mu \left(\frac{(\text{tr } \mathbf{B})}{J^{2/3}} - 3 \right) + \frac{1}{2}K(J - 1)^2, \quad (\text{A.14})$$

where μ and K are ground-state, equilibrium shear and bulk moduli, respectively. The first term represents free energy due to distortional (constant volume) deformation, and the second term arises due to purely volumetric deformation. To model nearly-incompressible behavior, we take $K \approx 100\mu$. The associated Cauchy stress is then

$$\boldsymbol{\sigma}^{\text{eq}} = J^{-1} \frac{\partial \psi^{\text{eq}}(\mathbf{F})}{\partial \mathbf{F}} \mathbf{F}^\top = \mu J^{-5/3} \text{dev}(\mathbf{B}) + K(J - 1)\mathbf{I}, \quad (\text{A.15})$$

where dev denotes the deviatoric part of a tensor.

The Maxwell element in parallel with the Neo-Hookean spring then allows for modeling non-equilibrium time-dependent behavior. To describe deformation in this element, the deformation gradient, $\mathbf{F}$, is multiplicatively decomposed into elastic and viscous parts: $\mathbf{F} = \mathbf{F}^e \mathbf{F}^v$; with $\mathbf{F}^v$, $J^v = \det \mathbf{F}^v = 1$, denoting the (constant-volume) viscous distortion and $\mathbf{F}^e$, $J^e = \det \mathbf{F}^e > 0$, denoting the non-equilibrium elastic distortion. The free energy due to the non-equilibrium spring is based on the Hencky (logarithmic) strain, utilizing the following definitions: $\mathbf{F}^e = \mathbf{R}^e \mathbf{U}^e$, polar decomposition of $\mathbf{F}^e$; $\mathbf{U}^e = \sum_{\alpha=1}^3 \lambda_\alpha^e \mathbf{r}_\alpha^e \otimes \mathbf{r}_\alpha^e$, spectral decomposition of $\mathbf{U}^e$; and $\mathbf{E}^e = \sum_{\alpha=1}^3 (\ln \lambda_\alpha^e) \mathbf{r}_\alpha^e \otimes \mathbf{r}_\alpha^e$, the logarithmic elastic strain. We take the free energy due to the non-equilibrium spring as

$$\psi^{\text{neq}}(\mathbf{E}^e) = \mu_{\text{neq}} |\text{dev}(\mathbf{E}^e)|^2, \quad (\text{A.16})$$

where μ_{neq} is the non-equilibrium shear modulus. The stress conjugate to the elastic strain, $\mathbf{E}^e$, is referred to the Mandel stress:

$$\mathbf{M}^e = \frac{\partial \psi^{\text{neq}}(\mathbf{E}^e)}{\partial \mathbf{E}^e} = 2\mu_{\text{neq}} \text{dev}(\mathbf{E}^e), \quad (\text{A.17})$$

and the contribution to the Cauchy stress due to the Maxwell element is then given through

$$\boldsymbol{\sigma}^{\text{neq}} = J^{\text{e}-1} \mathbf{R}^{\text{e}} \mathbf{M}^{\text{e}} \mathbf{R}^{\text{e}\top}. \quad (\text{A.18})$$

Finally, the evolution of $\mathbf{F}^{\text{v}}$ is given by

$$\dot{\mathbf{F}}^{\text{v}} = \mathbf{D}^{\text{v}} \mathbf{F}^{\text{v}}, \quad \mathbf{F}^{\text{v}}(\mathbf{X}, t = 0) = \mathbf{I}, \quad (\text{A.19})$$

with the viscous stretching, $\mathbf{D}^{\text{v}}$, given by

$$\mathbf{D}^{\text{v}} = \frac{1}{2\eta} \mathbf{M}^{\text{e}}, \quad (\text{A.20})$$

where η is the Maxwell element viscosity. (We refer to the dashpot as a Mises dashpot since this choice of constitutive equation asserts that the viscous stretching and Mandel stress tensors are codirectional, a hallmark of Mises plasticity.) Note that since ψ^{neq} is taken to not depend upon J^{e} , the contribution of the Maxwell element to the stress is purely deviatoric. In this way, we have only added viscoelasticity to the shear deformation and left the volumetric response of (A.15) unaffected. The four material parameters appearing in the model are the equilibrium shear modulus μ , the non-equilibrium shear modulus μ_{neq} , the Maxwell element viscosity η , and the bulk modulus K . The three parameters $\{\mu, \mu_{\text{neq}}, \eta\}$ are selected to best fit the experimental behavior, and the K bulk modulus is chosen to be high enough ($K \approx 100\mu$) so as to approximate incompressible behavior.

Since experimental measurements are made at discrete time-points, we next derive the time-discrete version of the model used both to analyze the experimental data as well as the one implemented in the Abaqus UMAT used in the simulations. At a time t_{n} , the deformation gradient, $\mathbf{F}_{\text{n}}$, and the viscous deformation gradient, $\mathbf{F}_{\text{n}}^{\text{v}}$, are known. Then, given the deformation gradient, $\mathbf{F}_{\text{n}+1}$, at a time $t_{\text{n}+1} = t_{\text{n}} + \Delta t$, we are to determine the Cauchy stress, $\boldsymbol{\sigma}_{\text{n}+1}$, and viscous deformation gradient, $\mathbf{F}_{\text{n}+1}^{\text{v}}$, at time $t_{\text{n}+1}$. In the experiments, Δt is the time increment between the two successive images. The evolution equation for $\mathbf{F}^{\text{v}}$ is implicitly integrated by means of an exponential map

[40]:

$$\mathbf{F}_{n+1}^v = \exp(\Delta t \mathbf{D}_{n+1}^v) \mathbf{F}_n^v \quad \text{with} \quad \mathbf{D}_{n+1}^v = \frac{1}{2\eta} \mathbf{M}_{n+1}^e. \quad (\text{A.21})$$

Using (A.21)₁, we have that the elastic deformation gradient at the end of the step is

$$\mathbf{F}_{n+1}^e = \mathbf{F}_{n+1} \mathbf{F}_{n+1}^{v-1} = \underbrace{\mathbf{F}_{n+1} \mathbf{F}_n^{v-1}}_{=\mathbf{F}_{\text{tr}}^e} \exp(-\Delta t \mathbf{D}_{n+1}^v) = \mathbf{F}_{\text{tr}}^e \exp(-\Delta t \mathbf{D}_{n+1}^v), \quad (\text{A.22})$$

where $\mathbf{F}_{\text{tr}}^e$ is the trial value of the elastic deformation gradient. A trial quantity represents the value of that quantity evaluated when viscous flow is frozen. With (i) the polar decompositions $\mathbf{F}_{n+1}^e = \mathbf{R}_{n+1}^e \mathbf{U}_{n+1}^e$ and $\mathbf{F}_{\text{tr}}^e = \mathbf{R}_{\text{tr}}^e \mathbf{U}_{\text{tr}}^e$; (ii) the knowledge that $\mathbf{D}_{n+1}^v$, $\mathbf{M}_{n+1}^e$, and $\mathbf{U}_{n+1}^e$ share principal directions and therefore commute; and (iii) the uniqueness of the polar decomposition; we have that

$$\begin{aligned} \mathbf{R}_{n+1}^e &= \mathbf{R}_{\text{tr}}^e, \\ \mathbf{U}_{n+1}^e &= \mathbf{U}_{\text{tr}}^e \exp(-\Delta t \mathbf{D}_{n+1}^v). \end{aligned} \quad (\text{A.23})$$

Taking the logarithm of (A.23)₂, we have

$$\mathbf{E}_{n+1} = \mathbf{E}_{\text{tr}} - \Delta t \mathbf{D}_{n+1}^v \quad \text{with} \quad \mathbf{E}_{\text{tr}} = \ln \mathbf{U}_{\text{tr}}. \quad (\text{A.24})$$

Multiplying the deviatoric part of (A.24) by $2\mu_{\text{neq}}$, by the stress relation (A.17), we have

$$\mathbf{M}_{n+1}^e = \mathbf{M}_{\text{tr}}^e - 2\mu_{\text{neq}} \Delta t \mathbf{D}_{n+1}^v \quad \text{with} \quad \mathbf{M}_{\text{tr}} = 2\mu_{\text{neq}} \text{dev}(\mathbf{E}_{\text{tr}}), \quad (\text{A.25})$$

which upon combining with (A.21)₂ yields

$$\mathbf{D}_{n+1}^v = \frac{\mathbf{M}_{\text{tr}}^e}{2(\eta + \Delta t \mu_{\text{neq}})}. \quad (\text{A.26})$$

Finally, the viscous deformation gradient is updated by (A.21)₁, and the Mandel and Cauchy stresses due to the Maxwell element are updated by

$$\begin{aligned} \mathbf{M}_{n+1}^e &= 2\eta \mathbf{D}_{n+1}^v, \\ \boldsymbol{\sigma}^{\text{neq}} &= (\det \mathbf{F}_{\text{tr}}^e)^{-1} \mathbf{R}_{\text{tr}}^e \mathbf{M}_{n+1}^e \mathbf{R}_{\text{tr}}^{e\top}. \end{aligned} \quad (\text{A.27})$$

The sequence of time-integration steps for the Maxwell element, with reference to the steps shown in Fig. A.5, is outlined below.

- (1) Compute the trial elastic deformation gradient, $\mathbf{F}_{\text{tr}}^e$, and its associated kinematic quantities using the viscous deformation gradient from the previous time increment:

$$\begin{aligned}\mathbf{F}_{\text{tr}}^e &= \mathbf{F}_{n+1} \mathbf{F}_n^{v-1}, \\ \mathbf{F}_{\text{tr}}^e &= \mathbf{R}_{\text{tr}}^e \mathbf{U}_{\text{tr}}^e, \\ \mathbf{E}_{\text{tr}}^e &= \ln(\mathbf{U}_{\text{tr}}^e).\end{aligned}\tag{A.28}$$

- (2) Compute the trial Mandel stress:

$$\mathbf{M}_{\text{tr}}^e = 2\mu_{\text{neq}} \text{dev}(\mathbf{E}_{\text{tr}}^e).\tag{A.29}$$

- (3) Calculate the viscous stretching

$$\mathbf{D}_{n+1}^v = \frac{\mathbf{M}_{\text{tr}}^e}{2(\eta + \Delta t \mu_{\text{neq}})}.\tag{A.30}$$

- (4) Update the viscous deformation gradient:

$$\mathbf{F}_{n+1}^v = \exp(\Delta t \mathbf{D}_{n+1}^v) \mathbf{F}_n^v.\tag{A.31}$$

- (5) Update the Mandel stress and Cauchy stress due to the Maxwell element:

$$\begin{aligned}\mathbf{M}_{n+1}^e &= 2\eta \mathbf{D}_{n+1}^v, \\ \boldsymbol{\sigma}_{n+1}^{\text{neq}} &= (\det \mathbf{F}_{\text{tr}}^e)^{-1} \mathbf{R}_{\text{tr}}^e \mathbf{M}_{n+1}^e \mathbf{R}_{\text{tr}}^{e\top}.\end{aligned}\tag{A.32}$$

This completes the calculation of the contribution to the Cauchy stress due to the Maxwell element. The contribution due to the equilibrium Neo-Hookean spring is simply computed from the deformation gradient at the end of the step $\mathbf{F}_{n+1}$:

$$\begin{aligned}\mathbf{B}_{n+1} &= \mathbf{F}_{n+1} \mathbf{F}_{n+1}^\top, \\ \boldsymbol{\sigma}_{n+1}^{\text{eq}} &= \mu (\det \mathbf{F}_{n+1})^{-5/3} \text{dev}(\mathbf{B}_{n+1}) + K[(\det \mathbf{F}_{n+1}) - 1] \mathbf{I}.\end{aligned}\tag{A.33}$$

allowing for the calculation of the total Cauchy stress:

$$\boldsymbol{\sigma}_{n+1} = \boldsymbol{\sigma}_{n+1}^{\text{eq}} + \boldsymbol{\sigma}_{n+1}^{\text{neq}}. \quad (\text{A.34})$$

μ	Shear modulus of hyperelastic spring	1270 Pa
K	Bulk modulus of hyperelastic spring	130,000 Pa
μ_{neq}	non-equilibrium shear modulus	800 Pa
η	Maxwell element viscosity	900 Pa·s

TABLE A.1. Table of the viscoelastic material constants for agarose substrate

Bibliography

- [1] Pelham R. J. Jr and Wang Y-L. 1997 Cell locomotion and focal adhesions are regulated by substrate flexibility. *Proc Natl Acad Sci USA* **94** 13661–13665.
- [2] Dembo M. and Wang Y-L. 1999 Stresses at the cell-to-substrate interface during locomotion of fibroblasts. *Biophys J* **76** 2308–2016 (DOI 10.1016/S0006-3495(99)77386-8)
- [3] Munevar S., Wang Y-L., and Dembo M. 2001 Traction Force Microscopy of Migrating Normal and H-ras Transformed 3T3 Fibroblasts. *Biophys J* **80** 1-14 (DOI 10.1016/S0006-3495(01)76145-0)
- [4] Wang J.H-C, and Lin J-S 2007 Cell traction force and measurement methods. *Biomech Model Mechan* **6** 361-371 (DOI 10.1007/s10237-006-0068-4)
- [5] Paszek M.J. et al. 2005 Tensional homeostasis and the malignant phenotype. *Cancer cell* **8** 241–254 (DOI 10.1016/j.ccr.2005.08.010)
- [6] Maskarinec S.A., Franck C., Tirrell D.A., and Ravichandran G. 200) Quantifying cellular traction forces in three dimensions. *Proc Natl Acad Sci USA* **106** 22108-22113 (DOI 10.1073/pnas.0904565106).
- [7] Franck C., Maskarinec S.A. , Tirrell D.A., and Ravichandran G. 2011 Three-dimensional traction force microscopy: a new tool for quantifying cell-matrix interactions. *PLoS ONE* **6** 1–15 (DOI 10.1371/journal.pone.0017833).
- [8] Jannat R.A., Dembo M., and Hammer D.A. 2011 Traction forces of neutrophils migrating on compliant substrates. *Biophys J* **101** 575-584 (DOI 10.1016/j.bpj.2011.05.040.)
- [9] Kraning-Rush C.M., Carey S.P., Califano J.P., Smith B.N., Reinhart-King C.A. 2011 The role of the cytoskeleton in cellular force generation in 2D and 3D environments. *Phys Biol* **8** 015009 (DOI 10.1088/1478-3975/8/1/015009.).

-
- [10] Kim J.H. et al. 2013 Propulsion and navigation within the advancing monolayer sheet. *Nat Mater* **12** 856-863 (DOI 10.1038/nmat3689).
 - [11] del Alamo J.C. et al. 2013 Three-Dimensional Quantification of Cellular Traction Forces and Mechanosensing of Thin Substrata by Fourier Traction Force Microscopy. *PloS one* 8:e69850 (DOI 10.1371/journal.pone.0069850).
 - [12] Fung Y.C., 1993, *Biomechanics: Mechanical Properties of Living Tissues*, Springer-Verlag, New York
 - [13] Katsuda S., and Kaji T., 2003, Atherosclerosis and extracellular matrix, *J Atheroscler Thromb* **10** 267–274.
 - [14] Iyo T., Sasaki N., and Nakata M., 2004, Anisotropic viscoelastic properties of cortical bone. *J Biomech* **37** 1433–1437.
 - [15] Butler J.P., Tolić-Nørrelykke I.M., Fabry B., and Fredberg J.J. 2002 Traction fields, moments, and strain energy that cells exert on their surroundings. *Am J Physiol-Cell Ph* **282** C595-C605. (DOI 10.1152/ajpcell.00270.2001)
 - [16] Sabass B., Gardel M.L., Waterman C.M., and Schwarz U.S. 2008 High resolution traction force microscopy based on experimental and computational advances. *Biophys J* **94** 207–220. (DOI 10.1529/biophysj.107.113670)
 - [17] Polio S.R., Rothenberg K.E., Stamenovic D., and Smith M.L. 2012 A micropatterning and image processing approach to simplify measurement of cellular traction forces, *Acta Biomater.* **8** 82–88 (DOI 10.1016/j.actbio.2011.08.013)
 - [18] Toyjanova, J., Bar-Kochba, E., Lòpez-Fagundo, C., Reichner, J., Hoffman-Kim, D., and Franck, C., “High Resolution, Large Deformation 3D Traction Force Microscopy”, *Plos One*, Accepted.
 - [19] Forgacs G., Foty R.A., Shafrir Y., and Stinberg M.S. 1998 Viscoelastic Properties of Living Embryonic Tissues: a Quantitative Study. *Biophys J* **74** 2227-2234 (DOI 10.1016/S0006-3495(98)77932-9)
 - [20] Ahearne M., Yang Y., Haj El A.J., Then K.Y., and Liu K-K 2005 Characterizing the viscoelastic properties of thin hydrogel-based constructs for tissue engineering applications. *J R Soc Interface* **2** 455–463 (DOI 10.1098/rsif.2005.0065)

-
- [21] Storm C., Pastore J.J., MacKintosh F.C., Lubensky T.C., and Janmey P.A. 2005 Nonlinear elasticity in biological gels. *Nature* **435** 191–194 (DOI 10.1038/nature03521)
 - [22] Levental I., Georges P.C., and Janmey PA 2007 Soft biological materials and their impact on cell function. *Soft Matter* **3** 299–306 (DOI 10.1039/B610522J)
 - [23] Balgude A.P., Yu X., Szymanski A., and Bellamkonda R.V. 2001 Agarose gel stiffness determines rate of DRG neurite extension in 3D cultures. *Biomaterials* **22** 1077-1084 (DOI 10.1016/S0142-9612(00)00350-1)
 - [24] Raz-Ben Aroush D., Zaidel-Bar R., Bershadsky A.D., and Wagner H.D. 2008 Temporal evolution of cell focal adhesions: experimental observations and shear stress profiles. *Soft Matter* **4** 2410–2417 (DOI 10.1039/B804643N)
 - [25] Sasaki N., Imai T., Hashimoto A., and Yasuda H. 2010 Effect of pericellular matrix formation by chondrocytes cultured in agarose gel on the viscoelastic properties of agarose gel matrix. *J Biorheol* **23** 95-101.
 - [26] Hanazaki Y, Ito D, Furusawa K, Fukui A, Sasaki N 2011 Change in the viscoelastic properties of agarose gel by HAp precipitation by osteoblasts cultured in an agarose gel matrix. *J Biorheol* **26** 21-28.
 - [27] Bar-Kochba E., Toyjanova J., Andrews E., Kim K-S., and Franck C., A fast iterative digital volume correlation algorithm for large deformations, *Exp Mech*, Submitted
 - [28] Franck C., Hong S., Maskarinec S. A., Tirrell D. A., and Ravichandran G. 2007 Three-dimensional full-field measurements of large deformations in soft materials using confocal microscopy and digital volume correlation, *Exp Mech* **47** 427-438 (DOI 10.1007/s11340-007-9037-9)
 - [29] Darling E.M., and Zauscher S., and Guilak F. 2006 Viscoelastic properties of zonal articular chondrocytes measured by atomic force microscopy. *Osteoarthritis and cartilage* **14** 571–579.
 - [30] Darling E.M., and Zauscher S., and Block J.A., and Guilak F. 2007 A thin-layer model for viscoelastic, stress-relaxation testing of cells using atomic force

- microscopy: do cell properties reflect metastatic potential? *Biophys J* **92** 1784–1791.
- [31] Hutter J.L., and Bechhoefer, J. 1993 Calibration of atomic-force microscope tips. *Rev Sci Instrum* **64** 1868–1873 (DOI 10.1063/1.1143970)
- [32] Normand V., and Lootens D. L., and Amici E., and Plucknett K. P. 2000 New Insight into Agarose Gel Mechanical Properties - Biomacromolecules, *Biomacromolecules* **1** 730–738
- [33] Roberts, J. J., Earnshaw, A., Ferguson, V. L. and Bryant, S. J. 2011 Comparative study of the viscoelastic mechanical behavior of agarose and poly(ethylene glycol) hydrogels. *J. Biomed. Mater. Res.* **99B** 158169. (DOI: 10.1002/jbm.b.31883)
- [34] Farid H. and Simoncelli E. P. 2004 Differentiation of discrete multi-dimensional Signals. *IEEE Trans Image Process* **13** 496–508 (DOI 10.1109/TIP.2004.823819)
- [35] Anand L., Ames N. M., Srivastava V., and Chester S. A. 2009 A thermo-mechanically coupled theory for large deformations of amorphous polymers. Part1: Formulation, *Int J Plasticity*, **25** 1474–1494 (DOI 10.1016/j.ijplas.2008.11.005)
- [36] Henann D. L. and Bertoldi K. 2014 Modeling of elasto-capillary phenomena, *Soft Matter*, **10** 709–717 (DOI 10.1039/C3SM52583J)
- [37] Aratyn-Schaus Y., and Gardel M. L. 2008 Biophysics Clutch Dynamics, *Science*, **322** 1646–1647
- [38] Maruthamuthu V., and Sabass B., and Schwarz U. S., and Gardel M. L. 2011 Cell-ECM traction force modulates endogenous tension at cell-cell contacts, *Proc Natl Acad Sci USA*, **108**, 4708–4713
- [39] Legant W. R., and Choi, C.K., and Miller J.S., and Shao L., and Gao L., and Betzig E., and Chen C.S. 2013 Multidimensional traction force microscopy reveals out-of-plane rotational moments about focal adhesions, *Proc Natl Acad Sci USA*, **110**, 881–886
- [40] Weber G. and Anand L. 1990 Finite deformation constitutive equations and a time integration procedure for isotropic, hyperelastic-viscoplastic solids, *Comput*

Method Appl M, **79** 173–202 (DOI 10.1016/0045-7825(90)90131-5)

Bibliography

- [1] T. Astarita. Analysis of interpolation schemes for image deformation methods in PIV: effect of noise on the accuracy and spatial resolution. *Exp. Fluids*, 40(6):977–987, May 2006.
- [2] P. Augat, U. Simon, A. Liedert, and L. Claes. Mechanics and mechano-biology of fracture healing in normal and osteoporotic bone. *Osteoporosis International*, 16(S02):S36–S43, September 2004.
- [3] E. Bar-Kochba, J. Toyjanova, E. Andrews, K-S. Kim, and C. Franck. A fast iterative digital volume correlation algorithm for large deformations. *Exp. Mech.*, December 2014.
- [4] B.K. Bay, T.S. Smith, D.P. Fyhrie, and M. Saad. Digital Volume Correlation: Three-dimensional Strain Mapping Using X-ray Tomography. *Exp. Mech.*, 39:217–226, August 1999.
- [5] Z. Bo, Z. Josiane, and J.-C. Olivo-Marin. Gaussian approximations of fluorescence microscope point-spread function models. *Applied Optics*, 46:1–11, March 2007.
- [6] A. Bower. *Applied Mechanics of Solids*. CRC Press, Boca Raton, Florida, May 2009.
- [7] A.F. Brown. Neutrophil Granulocytes: Adhesion and Locomotion on Collagen Substrata and in Collagen Matrices. *J. Cell Sci*, 58:455–467, August 1982.
- [8] D.T. Butcher, T. Alliston, and V.M. Weaver. A tense situation: forcing tumour progression. *Nature Reviews*, 9:108–122, February 2009.
- [9] J.P. Butler, I.M. Tolic-Norrelykke, B. Fabry, and J.J. Fredberg. Traction fields, moments, and strain energy that cells exert on their surroundings. *Am J Physiol Cell Physiol*, 282(3):C595–C605, October 2001.

-
- [10] K.L. Butler, V. Ambravaneswaran, N. Agrawal, M. Bilodeau, M. Toner, R.G. Tompkins, S. Fagan, and D. Irimia. Burn Injury Reduces Neutrophil Directional Migration Speed in Microfluidic Devices. *PloS one*, 5(7):e11921, July 2010.
 - [11] J-J Chiu, S. Usami, and S. Chien. Vascular endothelial responses to altered shear stress: Pathologic implications for atherosclerosis. *Annals of Medicine*, 41(1):19–28, January 2009.
 - [12] E.S. Chumanov, B.C. Heiderscheit, and D.G. Thelen. Hamstring Musculo-tendon Dynamics during Stance and Swing Phases of High-Speed Running. *Medicine & Science in Sports & Exercise*, 43(3):525–532, March 2011.
 - [13] M.F. Coughlin, D.D. Sohn, and G.W. Schmid-Schönbein. Recoil and Stiffening by Adherent Leukocytes in Response to Fluid Shear. *Biophys J*, 94(3):1046–1051, February 2008.
 - [14] J.C. del Alamo, R. Meili, B. Alonso-latorre, J. Rodriguez, A. Aliseda, R.A. Firtel, and J.C. Lasheras. Spatio-temporal analysis of eukaryotic cell motility by improved force cytometry. *PNAS*, 104(33):13343–13348, 2007.
 - [15] J.C. del Álamo, R. Meili, Á-G. Begoña, A-L. Baldomero, 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*, 8.
 - [16] M. Dembo. Stresses at the Cell-to-Substrate Interface during Locomotion of Fibroblasts. *Biophys J*, 76(4):2307–2316, 1999.
 - [17] N Derby and S Olbert. Cylindrical Magnets and Ideal Solenoids. *Am. J. Phys*, 78:229, September 2010.
 - [18] J. D’Errico. Surface fitting using gridfit. *MATLAB Central File Exchange*, 2005.
 - [19] D.E. Discher, P. Janmey, and Y.-l. Wang. Tissue cells feel and respond to the stiffness of their substrate. *Science (New York, N.Y.)*, 310(5751):1139–1143, 2005.

-
- [20] M. Doblaré, J.M. Garca, and M.J. Gómez. Modelling bone tissue fracture and healing: a review. *Eng. Fract. Mech.*, 71(13-14):1809–1840, September 2004.
 - [21] C.C. DuFort, M.J. Paszek, and V.M. Weaver. Balancing forces architectural control of mechanotransduction. *Nature Reviews Molecular Cell Biology*, 12(5):308–319, May 2011.
 - [22] A. Engler, L. Bacakova, C. Newman, A. Hategan, M. Griffin, and D. Discher. Substrate Compliance versus Ligand Density in Cell on Gel Responses. *Biophys. J.*, 86:617–628, December 2004.
 - [23] H. Farid and E.P. Simoncelli. Differentiation of Discrete Multi-Dimensional Signals. *IEEE Trans. Image Process.*, pages 1–16, June 2004.
 - [24] C. Franck, S. Hong, S.A. Maskarinec, D.A. Tirrell, and G. Ravichandran. Three-dimensional Full-field Measurements of Large Deformations in Soft Materials Using Confocal Microscopy and Digital Volume Correlation. *Exp. Mech.*, 47(3):427–438, March 2007.
 - [25] 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*, 6(3), 2011.
 - [26] P. Friedl. Prespecification and plasticity: shifting mechanisms of cell migration. *Curr. Opin. Cell Biol.*, 16(1):14–23, February 2004.
 - [27] M. Gates, J. Lambros, and M.T. Heath. Towards High Performance Digital Volume Correlation. *Exp. Mech.*, 51(4):491–507, December 2010.
 - [28] G.A. Gomez, R.W. McLachlan, and A.S. Yap. Productive tension: force-sensing and homeostasis of cellâ“cell junctions. *Trends in Cell Biology*, 21(9):499–505, September 2011.
 - [29] R.C. Gonzalez and R.S. Woods. *Digital Image Processing*. Prentice Hall, Upper Saddle River, NJ, May 2008.
 - [30] J.H. Haga, Y-S.J. Li, and S. Chien. Molecular basis of the effects of mechanical stretch on vascular smooth muscle cells. *J Biomech*, 40(5):947–960, January 2007.

-
- [31] 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. *J Immunol*, 162, 1999.
- [32] S.J. Henry, J.C. Crocker, and D.A. Hammer. Ligand density elicits a phenotypic switch in human neutrophils. *Integrative Biology*, 2014.
- [33] Z. Hu, H. Xie, Lu. J., T. Hua, and J. Zhu. Study of the performance of different subpixel image correlation methods in 3D digital image correlation. *Applied Optics*, 49(21):4044–4051, 2010.
- [34] H.T. Huang, H.E. Fiedler, and J.J. Wang. Limitation and Improvement of Piv .2. Particle Image Distortion, a Novel Technique. *Exp. Fluids*, 15(4-5):263–273, 1993.
- [35] S.S. Hur, Y. Zhao, Y-S. Li, E. Botvinick, and S. Chien. Live Cells Exert 3-Dimensional Traction Forces on Their Substrata. *Cell. Mol. Bioeng.*, 2(3):425–436, August 2009.
- [36] D.E. Jaalouk and J. Lammerding. Mechanotransduction gone awry. *Nat. Rev. Mol. Cell Biol.*, 10(1):63–73, January 2009.
- [37] K. Jambunathan, X. Y. Ju, B Dobbins, and S Ashforth-Frost. An improved cross correlation technique for particle image velocimetry. *Meas. Sci. Technol.*, 6:507–514, October 1995.
- [38] R.A. Jannat, M. Dembo, and D.A. Hammer. Traction forces of neutrophils migrating on compliant substrates. *Biophys J*, 101(3):575–584, August 2011.
- [39] J.Y. Ji, H. Jing, and S.L. Diamond. Hemodynamic Regulation of Inflammation at the Endothelial–Neutrophil Interface . *Ann. Biomed. Eng.*, 36(4), 2008.
- [40] S. Jungbauer, H. Gao, J.P. Spatz, and R. Kemkemer. Two characteristic regimes in frequency-dependent dynamic reorientation of fibroblasts on cyclically stretched substrates. *Biophys J*, 95(7):3470–3478, 2008.
- [41] P. Kollmannsberger and B. Fabry. High-force magnetic tweezers with force feedback for biological applications. *Rev. Sci. Instrum.*, 78(11):114301, 2007.

-
- [42] C.M. Kraning-Rush, J.P. Califano, and C.A. Reinhart-King. Cellular traction stresses increase with increasing metastatic potential. *PloS one*, 2012.
- [43] 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*, 453(7191):51–55, May 2008.
- [44] T. Lämmermann and M. Sixt. Mechanical modes of ‘amoeboid’ cell migration. *Curr. Opin. Cell Biol.*, 21(5):636–644, October 2009.
- [45] H. Leclerc, J.N. Périé, S. Roux, and F. Hild. Voxel-Scale Digital Volume Correlation. *Exp. Mech.*, 51(4):479–490, September 2010.
- [46] I. Levental, P.C. Georges, and P.A. Janmey. Soft biological materials and their impact on cell function. *Soft Matter*, 3(3):299, 2007.
- [47] B. Li and J. H-C. Wang. Application of Sensing Techniques to Cellular Force Measurement. *Sensors*, 10(11):9948–9962, 2010.
- [48] M. Li, M.J. Mondrinos, X. Chen, M.R. Gandhi, F.K. Ko, and P.I. Leikes. Co-electrospun poly(lactide-co-glycolide), gelatin, and elastin blends for tissue engineering scaffolds. *J. Biomed. Mater. Res., Part A*, 79A(4):963–973, August 2006.
- [49] Y. Lin. A model of cell motility leading to biphasic dependence of transport speed on adhesive strength. *J. Mech. Phys. Solids*, 58(4):502–514, March 2010.
- [50] M. Linkert, C.T. Rueden, C. Allan, J.-M. Burel, W. Moore, A. Patterson, B. Lorange, J. Moore, C. Neves, D. MacDonald, A. Tarkowska, C. Sticco, E. Hill, M. Rossner, K.W. Eliceiri, and J.R. Swedlow. Metadata matters: access to image data in the real world. *J. Cell Biol.*, 189:5777–5782, June 2010.
- [51] V.K. Lishko, V.P. Yakubenko, and T.P. Ugarova. The interplay between integrins $\alpha_5\beta_1$ and $\alpha_2\beta_1$ during cell migration to fibronectin. *Exp. Cell. Res.*, 283, 2003.
- [52] C-M. Lo, H-B. Wang, M. Dembo, and Y-l Wang. Cell Movement is guided by the rigidity of the substrate. *Biophys J*, 79:144–152, June 2000.

-
- [53] R. Long, M. S. Hall, M. Wu, and C.-Y. Hui. Effects of Gel Thickness on Microscopic Indentation Measurements of Gel Modulus. *Biophys. J.*, 101(3):643–650, August 2011.
- [54] C. Lopez-Fagundo, E. Bar-Kochba, L.L. Livi, D. Hoffman-Kim, and C. Franck. Three-dimensional traction forces of Schwann cells on compliant substrates. *J. R. Soc. Interface*, 11(97):20140247–20140247, May 2014.
- [55] K. Maaser, K. Wolf, K.C. Eberhard, B. Niggemann, K.S. Zänker, E.-B. Brocker, and P. Friedl. Functional hierarchy of simultaneously expressed adhesion receptors: integrin $\alpha 2\beta 1$ but Not CD44 mediates MV3 melanoma cell migration and matrix reorganization within three-dimensional hyaluronan-containing collagen matrices. *Mol. Biol. Cell*, 10:3067–3079, September 1999.
- [56] P.J. Mack, Y. Zhang, S. Chung, V. Vickerman, R.D. Kamm, and G. Garcia-Cardena. Biomechanical Regulation of Endothelium-dependent Events Critical for Adaptive Remodeling. *J. Biol. Chem.*, 284(13):8412–8420, March 2009.
- [57] S.E. Malawista and C.A. de Boisleury. Random locomotion and chemotaxis of human blood polymorphonuclear leukocytes (PMN) in the presence of EDTA: PMN in close quarters require neither leukocyte integrins nor external divalent cations. *PNAS*, 94(21):11577–11582, October 1997.
- [58] T. Mammoto and D.E. Ingber. Mechanical control of tissue and organ development. *Development*, 137(9):1407–1420, April 2010.
- [59] V. Maruthamuthu, B. Sabass, U.S. Schwarz, and M.L. Gardel. Cell-ECM traction force modulates endogenous tension at cell-cell contacts. *PNAS*, 108(12):4708–4713, March 2011.
- [60] S.A. Maskarinec, C. Franck, D.A. Tirrell, and G. Ravichandran. Quantifying cellular traction forces in three dimensions. *PNAS*, 106(52):22108–22113, December 2009.
- [61] T.N. Mayadas and X. Cullere. The Multifaceted Functions of Neutrophils. *Annu Rev Pathol.*, 2013.

-
- [62] R. Meili, B. Alonso-latorre, J. C. del Alamo, R.A. Firtel, and J.C. Lasheras. Myosin II is Essential for the Spatiotemporal Organization of Traction Forces during Cell Motility. *Mol. Biol. Cell*, 21:405–417, January 2010.
- [63] S. Munevar, Y.L. Wang, and M. Dembo. Distinct roles of frontal and rear cell-substrate adhesions in fibroblast migration. *Mol. Biol. Cell*, 12(12):3947–3954, 2001.
- [64] K. Nagayama, A. Adachi, and T. Matsumoto. Heterogeneous response of traction force at focal adhesions of vascular smooth muscle cells subjected to macroscopic stretch on a micropillar substrate. *J Biomech*, pages 1–7, August 2011.
- [65] C. Nathan. Neutrophils and immunity: challenges and opportunities. *Nat. Rev. Immunol.*, 6(C):173–182, March 2006.
- [66] J. Nogueira, A. Lecuona, and P.A. Rodriguez. Local Field Correction PIV: on the Increase of Accuracy of Digital PIV Systems. *Exp. Fluids*, 27:107–116, June 1999.
- [67] J. Nogueira, A. Lecuona, and P.A. Rodriguez. Local Field Correction PIV, Implemented by Means of Simple Algorithms, and Multigrid Versions. *Meas. Sci. Technol.*, 12:1911–1921, October 2001.
- [68] J. Nogueira, A. Lecuona, and P.A. Rodriguez. Limits on the resolution of correlation PIV iterative methods. Fundamentals. *Exp. Fluids*, 39(2):305–313, July 2005.
- [69] P.W. Oakes, D.C. Patel, N.A. Morin, D.P. Zitterbart, B. Fabry, J.S. Reichner, and J.X. Tang. Neutrophil morphology and migration are affected by substrate elasticity. *Blood*, 114(7):1387–1395, August 2009.
- [70] B. Pan, D. Wu, and Z. Wang. Internal displacement and strain measurement using digital volume correlation: a least-squares framework. *Meas. Sci. Technol.*, 23(4):045002, February 2012.
- [71] A. Pathak and S. Kumar. Independent Regulation of Tumor Cell Migration by Matrix Stiffness and Confinement. *PNAS Early Edition*, pages 1–6, August 2012.

-
- [72] R J Jr Pelham and Y-l Wang. Cell locomotion and focal adhesions are regulated by substrate flexibility. *PNAS*, 94(December):13661–13665, November 1997.
- [73] S.R. Peyton and A.J. Putnam. Extracellular matrix rigidity governs smooth muscle cell motility in a biphasic fashion. *J. Cell. Physiol.*, 204(1):198–209, 2005.
- [74] M.K. Pospieszalska, I. Lasiecka, and K. Ley. Cell protrusions and tethers: a unified approach. *Biophys J*, 100(7):1697–1707, April 2011.
- [75] J.M.S. Prewitt. Object enhancement and extraction. *Picture processing and Psychopictorics*, 1970.
- [76] P.P. Provenzano and P.J. Keely. Mechanical signaling through the cytoskeleton regulates cell proliferation by coordinated focal adhesion and Rho GTPase signaling. *J. Cell Sci.*, 124(8):1195–1205, March 2011.
- [77] R Ravaut, G Lemarquand, S Babic, V Lemarquand, and Akyel C. Cylindrical Magnets and Coils: Fields, Forces, and Inductances. *IEEE Transactions on Magnetics*, 46(9):3585–3590, 2010.
- [78] C.A. Reinhart-King, M. Dembo, and Hammer D.A. The dynamics and mechanics of endothelial cell spreading. *Biophys J*, 89, 2005.
- [79] J. Renkawitz and M. Sixt. Mechanisms of force generation and force transmission during interstitial leukocyte migration. *EMBO reports*, 11(10):744–750, September 2010.
- [80] A. Ribeiro, S. Balasubramanian, D. Hughes, S. Vargo, E.M. Powell, and J.B. Leach. β -Integrin cytoskeletal signaling regulates sensory neuron response to matrix dimensionality. *Neuroscience*, 248(C):67–78, September 2013.
- [81] W H Richardson. Bayesian-Based Iterative Method of Image Restoration. *J. Opt. Soc. Am. A*, 62:55–59, February 1972.
- [82] B.A. Roeder, K. Kokini, J.P. Robinson, and S. L. Voytik-Harbin. Local, Three-Dimensional Strain Measurements Within Largely Deformed Extracellular Matrix Constructs. *J. Biomech. Eng.*, 126(6):699, February 2005.

-
- [83] 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. *J. Biomech. Eng.*, 124(2):214, 2002.
- [84] I.F. Sbalzarini and P. Koumoutsakos. Feature point tracking and trajectory analysis for video imaging in cell biology. . *J. Struct. Biol.*, 151(2):182–195, 2005.
- [85] F. Scarano and M.L. Riethmuller. Advances in iterative multigrid PIV image processing. *Exp. Fluids*, 29(7):S051–S060, 2000.
- [86] F.F.J. Schrijer and F. Scarano. On the stabilization and spatial resolution of iterative PIV interrogation. *13th Int Symp on Applications of Laser Techniques to Fluid Mechanics*, (1999):26–29, May 2006.
- [87] F.F.J. Schrijer and F. Scarano. Effect of predictor–corrector filtering on the stability and spatial resolution of iterative PIV interrogation. *Exp. Fluids*, 45(5):927–941, May 2008.
- [88] S.S. Shevkoplyas, A. C Siegel, R.M. Westervelt, M. G. Prentiss, and G.M. Whitesides. The force acting on a superparamagnetic bead due to an applied magnetic field. *Lab on a Chip*, 7(10):1294, 2007.
- [89] M.E. Shin, Y. He, D. Li, S. Na, F. Chowdhury, O. Poh, Y.-C. Collin, P. Su, P. de Lanerolle, M. Schwartz, N. Wang, and F. Wang. Spatiotemporal organization, regulation, and functions of tractions during neutrophil chemotaxis. *Blood*, 116(17):3297–3310, October 2010.
- [90] N. Slomka and A. Gefen. Confocal microscopy-based three-dimensional cell-specific modeling for large deformation analyses in cellular mechanics. *J. Biomech.*, 43(9):1806–1816, June 2010.
- [91] 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. *Biophys J*, 92(7):L58–L60, April 2007.

-
- [92] T.S. Smith, B.K. Bay, and M.M. Rashid. Digital volume correlation including rotational degrees of freedom during minimization. *Experimental Mechanics*, 42:1–7, June 2002.
- [93] J. Soria. An Investigation of the Near Wake of a Circular Cylinder using a Video-based Digital Cross-Correlation Particle Image Velocimetry Technique. *Exp. Therm Fluid Sci.*, 12:221–233, March 1996.
- [94] K.M. Stroka and H. Aranda-Espinoza. Neutrophils display biphasic relationship between migration and substrate stiffness. *Cell motility and the cytoskeleton*, 66(6):328–341, June 2009.
- [95] P. Sundd, E. Gutierrez, M.K. Pospieszalska, H. Zhang, A. Groisman, and K. Ley. Quantitative dynamic footprinting microscopy reveals mechanisms of neutrophil rolling. *Nature*, 7(10):821–824, September 2010.
- [96] T. Takigawa, Y. Morino, K. Urayama, and T. Masuda. Poisson’s ratio of polyacrylamide (PAAm) gels. *Polymer Gels and Networks*, 4(1):1–5, August 1996.
- [97] H.P. Ting-Beall, D. Needham, and R.M. Hochmuth. Volume and osmotic properties of human neutrophils. *Blood*, 81(10):2774–2780, May 2003.
- [98] López-Fagundo C. Reichner J Hoffman-Kim D. Toyjanova J., Bar-Kochba E. and Franck C. High Resolution, Large Deformation 3D Traction Force Microscopy. *PloS One*, 9(4):e90976, April 2014.
- [99] J.R. Tse and A.J. Engler. *Preparation of Hydrogel Substrates with Tunable Mechanical Properties*. John Wiley & Sons, Inc., Hoboken, NJ, USA, May 2001.
- [100] J. M. van den Berg, F.P.J. Mul, and E. Schippers. β 1 integrin activation on human neutrophils promotes β 2 integrinmediated adhesion to fibronectin. *Eur J Immunol.*, 2001.
- [101] S. Vauthey, S. Santoso, H. Gong, N. Watson, and Zhang S. Molecular self-assembly of surfactant-like peptides to form nanotubes and nanovesicles. *PNAS*, 99:5355–5360, April 2002.

- [102] M.A. Vollrath, K.Y. Kwan, and D.P. Corey. The Micromachinery of Mechanotransduction in Hair Cells. *Annu Rev Neurosci.*, 30(1):339–365, July 2007.
- [103] G. Wei and P. X. Ma. Macroporous and nanofibrous polymer scaffolds and polymer/bone-like apatite composite scaffolds generated by sugar spheres. *J. Biomed. Mater. Res., Part A*, 78A(2):306–315, 2006.
- [104] P Weiss. In vitro experiments on the factors determining the course of the outgrowing nerve fiber. *J Exp Zool*, 68:393–446, May 1934.
- [105] J. Westerweel and F. Scarano. Universal outlier detection for PIV data. *Exp, Fluids*, 39(6):1096–1100, August 2005.
- [106] Z. Yang, J-S Lin, J. Chen, and J. H-C Wang. Determining substrate displacement and cell traction fields—a new approach. *J. Theor. Biol.*, 242(3):607–616, October 2006.
- [107] Z. Zhang, K. Vuori, J.C. Reed, and E. Ruoslahti. The alpha 5 beta 1 integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression. *PNAS*, 92(13):6161–6165, 1995.