

**Abstract of “Three-Dimensional Kinematic Measurements of Nonlinear  
Deformations in Cellular Biology”**

by Eyal Bar-Kochba, Ph.D., Brown University, May 2015

The mechanical interaction between cells and their microenvironments is a significant factor affecting biological and physiological processes. Cell-matrix interactions have historically been measured by computing cell-induced forces using a well-established technique, traction force microscopy (TFM). TFM assumes that the surrounding microenvironment has well characterized linear elastic mechanical properties, only applicable for small deformations. However, the linear elastic assumption is invalid for many synthetic and native tissues since the underlying microstructure is heterogeneous and exhibits a nonlinear deformation response to loading. Presented here are new experimental analysis techniques that measure the kinematic signatures of nonlinear deformations without relying on the material properties of the matrix.

An algorithm called fast iterative digital volume correlation (FIDVC) is developed to noninvasively track the nonlinear motion of materials from three-dimensional (3D) images within pragmatic computation times. FIDVC is applied to measure the nonlinear surface deformations generated by neutrophils migrating through a fibrillar material that is not mechanically characterized. Visualization techniques are presented that make it easier to interpret the spatially complex 3D patterns of the deformation field. A new mathematical framework is developed to convert these complex surface measurements to simple yet powerful metrics that provide information about the overall expansion/contraction and rotational behavior of the cell. These metrics may be used by researchers to investigate the cellular migration mechanisms in more complex, physiologically relevant materials.

Another application of kinematic analysis is presented to study the cellular injury response to externally applied deformations. An electromechanical device is built to apply uniaxial compression to neurons embedded in a heterogeneous microenvironment. New kinematic analysis techniques are developed to show that neuron death is correlated with the orientation-dependent mean strain experienced by the cell during deformation.





# Three-Dimensional Kinematic Measurements of Nonlinear Deformations in Cellular Biology

by

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May 2015

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This dissertation by Eyal Bar-Kochba is accepted in its present form by  
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# Curriculum Vitæ

## Education

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## Patents

- [1] Franck C. and Bar-Kochba, E. (2013). *Device and system for mechanical measurement of Biomaterial*. WO/2013/090738. Geneva, Switzerland: World Intellectual Property Organization.

## Peer Reviewed Publications

- [1] Bar-Kochba, E., Toyjanova, J., Andrews, E., Kim, K.S., & Franck, C. (2014). A Fast Iterative Digital Volume Correlation Algorithm for Large Deformations. *Journal of Experimental Mechanics*, 55(1):261–274. doi: 10.1007/s11340-014-9874-2
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ילש אבאהו אמאל

## Acknowledgments

I would like to begin by expressing my sincerest gratitude to all of the incredible faculty at Brown whom I have had the pleasure of learning from. Inside and outside of the classroom, your guidance and discussions have made my experience truly enriching and special. First, I wish to thank my thesis readers, Professors Diane Hoffman-Kim and Gabriel Taubin. I know that this thesis is not a compelling novel, but your careful review and comments are truly appreciated. I would like to thank Professors Allan Bower, K-S. Kim, and Haneesh Kesari for their advice regarding research and life in general. I also want to thank Professors Janet Blume, Tom Powers, and L.B. Freund for their academic support through this process. Lastly, I wish to thank my advisor Professor Christian Franck for his excellent guidance and immeasurable patience. Throughout this journey, I have been incredibly fortunate to have an advisor who has given me the freedom to explore while helping me recover if I falter. It is sad to think that the first generation of students are leaving the Franck lab, but I know that with your leadership the future is bright.

Many friends have helped me stay sane through these years, and their support has helped me overcome many personal and professional setbacks. I will always remember the wonderful times all of us have shared. Thank you to my immediate and extended lab family Jon Estrada, Mark Scimone, Mohak Patel, Dan Keenan, David Stout, Kyle McNamara, Erin Hannen, Molly Boutin, and Cristina López Fagundo. I want to especially thank one of the most caring people I have been fortunate enough to meet, my friend Jennet Toyjanova. We met our first year at Brown and have been through thick-and-thin together. Lastly, I would like to thank my great friend Johanna Sisalima that I met over a decade ago as an eager freshman at Stony Brook University.

I am forever grateful to my family for their unconditional love and support throughout my life. To my mother, Rachel Bar-Kochba, for her big heart that has shown me persistent care and compassion. To my father, Dan Bar-Kochba, who has always been a role model of dedication and hard work while being fun-loving at heart. To my four siblings, for providing endless amounts of fun and laughter. I want to especially thank my younger brother Ronnie Bar-Kochba. I will always reminisce all of the adventures we have shared. Even though he will never read this, thank you to Barf for being my best friend, my trusty companion, and above all else, my dog. Finally, a very special acknowledgment goes out to Hillary O'Brien. The last few years have been the toughest in my life and without her love and support I do not think I would have made it. Hillary, I am truly blessed to have you in my life.

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# 1. Introduction

## 1.1 Mechanics in Cell Biology

Cells are constantly sensing and responding to mechanical changes in their surrounding microenvironment by triggering biochemical signaling cascades that influence their behavior [205, 58, 159, 61, 67, 195]. Understanding these mechanotransduction events is important in elucidating mechanisms of cell adhesion [304, 49, 89, 66], migration [205, 58, 159, 188] and differentiation [93, 67], which are all essential for healthy cellular function. Dysregulation in mechanotransduction are correlated with a wide array of diseases (see [116] for review) such as atherosclerosis [99, 44, 167], sepsis [48, 124], and cancer [212, 135]. Focusing on the nervous system, mechanical forces regulate a wide array of mechanisms that are important for normal brain function. Neurons, the fundamental unit of the nervous system, require internally generated mechanical tension to modulate neurotransmission [253, 219] and active vesicle transport [3]. During development, cortical folding in the brain is hypothesized to result from axonal tension [70], whereby neurons pull on interconnected cortical regions, or from differential growth, whereby differences in growth rate between cortical layers cause surface buckling or folding [301, 31]. Mechanical forces, when externally applied at severe loading conditions can disrupt normal brain function. In traumatic brain injury, the underlying tissue deformation elicits a cellular response that leads to a host of acute and long term neurological deficits and disorders, e.g. coma, memory and motor skill loss, Alzheimer’s disease, and chronic traumatic encephalopathy [6, 254, 43, 117, 244, 179, 125, 126, 283]. It is necessary to develop new experimental tools that allow researchers to accurately quantify this mechanical interaction, particularly in complex heterogeneous tissues and emerging biomaterials.

## 1.2 Traction Force Microscopy

Efforts at measuring cellular forces can be traced back to 1980 when Harris et al. observed the wrinkling of thin polymeric silicone substrates during cell migration [101]. Unfortunately,

the inherent nonlinearity of the silicon deformation made it difficult to quantify the cell generated forces. From 1994 to 1996, a number of groups developed traction force microscopy (TFM), a technique that estimates cell induced surface tractions by tracking the motion of fluorescent microspheres embedded in the underlying substrate from microscopy images [150, 199, 57]. In their formulation, a linear elastic, isotropic, and homogeneous material behavior was assumed where the stresses  $\boldsymbol{\sigma}(\mathbf{x})$  are related to the displacement field  $\mathbf{u}(\mathbf{x})$  through Hooke's law, i.e.,

$$\boldsymbol{\sigma} = \frac{E}{2(1+\nu)} \left( \boldsymbol{\nabla} \mathbf{u} + (\boldsymbol{\nabla} \mathbf{u})^T + \frac{2\nu}{(1-2\nu)} (\boldsymbol{\nabla} \cdot \mathbf{u}) \mathbf{I} \right), \quad (1.2.1)$$

where  $\mathbf{I}$  is the identity tensor,  $E$  is Young's modulus,  $\nu$  is Poisson's ratio, and  $\boldsymbol{\nabla}$  and  $\boldsymbol{\nabla} \cdot$  denote the vector gradient and divergence operator respectively. The tractions  $\mathbf{t}(\mathbf{x})$  and therefore the cellular forces are related to the substrate normal vector  $\mathbf{n}(\mathbf{x})$  and the stress by

$$\mathbf{t} = \mathbf{n} \cdot \boldsymbol{\sigma}. \quad (1.2.2)$$

Static mechanical equilibrium in the absence of body forces requires that

$$\boldsymbol{\nabla} \cdot \boldsymbol{\sigma} = 0 \quad (1.2.3)$$

or incorporating equation (1.2.1),

$$(1-2\nu) \boldsymbol{\nabla}^2 \mathbf{u} + \boldsymbol{\nabla} (\boldsymbol{\nabla} \cdot \mathbf{u}) = 0. \quad (1.2.4)$$

Equations (1.2.1) – (1.2.4) were solved by inverting the discrete form of the Boussinesq solution for an infinite half-space [58]. Unfortunately, this inversion problem requires a regularization scheme to accurately back-calculate the tractions since it is ill-posed in the presence of noise in the displacements [58, 248]. By reformulating the problem in Fourier space, Butler et al. in 2002 circumvented this issue, while gaining computational efficiency [37]. In 2006, Yang et al. addressed the limitation of an infinite half-space approximation in the Boussinesq formulation and thus accounted for finite thickness substrates by implementing

it into the finite element method (FEM) framework [305, 54]. These traditional TFM methodologies have contributed a wealth of information to understanding the relationship between cellular biology and mechanics, e.g. the dependence between force magnitude and cell type [65, 208], and that traction generation is regulated by the stiffness of the surrounding microenvironment [159, 65]. However, they ignore two inherent features of native tissues that can significantly alter cell morphology and function [49, 71, 307], i.e. the heterogeneous three-dimensional (3D) and nonlinear deformation behavior of the microenvironment [

By utilizing confocal microscopy and more advanced particle tracking algorithms, Maskarinec and Franck et al. [175] in 2009 captured the 3D full-field displacement field. Instead of inverting the Boussinesq solution, the tractions were directly calculated using the discrete form of the deformation gradient tensor in equation (1.2.1). Alternatively, FEM can be utilized to compute cell tractions on 2D substrates [114, 55, 268] and for cells fully encapsulated in 3D materials [152]. Although these 3D techniques have shown that higher dimensions cannot be ignored, the underlying assumption of a linear elastic framework is not appropriate for most physical interactions between cells and the surrounding extracellular matrix (ECM) [276]. Additionally, many 3D TFM formulations suffer from high computational cost, making them not practical for high-throughput screening [172].

In naturally-derived and synthetic ECM materials such as fibrin, collagen, laminin, agarose, matrigel, and polyethylene glycol [93, 168, 209, 213], the microstructure at the cellular length scales is fibrous and porous [285, 7]. These complexities are especially true for native tissues [82], where the underlying ECM is mainly comprised of various proteins such as collagen, elastin, fibronectin, and laminin [4], each having their distinct material properties [231, 263]. The heterogeneous nature of the ECM makes the material response to loading generally nonlinear, and dependent on length and time scale [231, 230, 263]. Additionally, on polyacrylamide substrates commonly used in 2D TFM studies, cells were observed exerting large finite material deformations, rendering the traditional linear elastic framework inappropriate [276, 161]. Regardless of these complexities, all traction and force measurements rely on the kinematic tracking of material displacements and strains.



### 1.3 Kinematic tracking in cellular biology

Ever since the 1950's [177], quantitative kinematic analysis has successfully improved our understanding of cellular processes at multiple length and time scales [181]. Cell tracking algorithms have uncovered migration mechanisms [222, 206, 214, 186] that are fundamental to cell development [173, 181, 45], wound healing [173, 155], and invasion [298, 81, 299] in both 3D environments [299, 181] and populations of cells [154, 181]. With increasing computational power, these kinematic analysis techniques have translated to high-throughput screening using conventional fluorescence microscopy [207]. On the subcellular scale, kinematic analysis has been used to track intracellular organelle and vesicle dynamics [140, 220], and the cytoskeleton [214, 190, 258, 204]. Instead of noninvasively tracking cellular kinematics, others have studied the cellular response to kinematic deformation fields, such as strain. By applying kinematic deformations, studies have successfully uncovered mechanisms of cellular injury [146, 156, 143], cellular remodeling [156, 239], and mechanosensing [286, 142, 284].

### 1.4 Accomplishments

This thesis presents new experimental techniques capable of measuring the kinematics of nonlinear deformation fields, which are measured in investigations of cell migration and injury in 3D.

Chapter 2 will review previous developments in a commonly used displacement tracking algorithm, i.e. digital volume correlation (DVC). A new algorithm is presented that is capable of capturing large nonlinear material deformations from 3D images, while providing tractable computation times. This algorithm, called fast iterative DVC (FIDVC) builds on the previous digital volume correlation algorithm developed by Franck et al. [78] and incorporates powerful methods commonly implemented in tracking nonlinear fluid flows by means of particle image velocimetry. As with other image correlation methods, FIDVC can be extended to multiple length and time scales since it is independent of the imaging platform.

Chapter 3 provides a new mathematical framework that converts the FIDVC deformation measurements to simple kinematic metrics that describe the overall shape change of the

cell without the need for mechanical properties. These mean kinematic quantities can provide information about the mean expansion/contraction and rotational behavior of the cell as it migrates through heterogeneous microenvironments. New visualization techniques are presented that make it easier to interpret the spatially complex 3D patterns of the deformation field. The capability of this technique is demonstrated measuring the nonlinear surface deformations generated by neutrophils migrating through a heterogeneous collagen matrix that is not mechanically characterized.

As another application of nonlinear deformation study, Chapter 4 is dedicated to understanding how externally applied deformations to collagen, a naturally heterogeneous and nonlinear material, gives rise to cellular injury. Specifically, a new 3D *in vitro* model of traumatic brain injury (TBI) is developed to investigate the secondary effects of compression on neurons embedded in fibrillar 3D collagen matrices. Methods to compute the local neuron geometry are presented and used to establish local and global injury metrics. These injury metrics are correlated with traditional TBI micropathologies of diffuse axonal injury, i.e. axonal blebbing and membrane permeability, which are also characterized as a function of loading rate.

Lastly, chapter 5 provides a summary of the findings and recommendations for future directions. For completeness, Appendix A describes a 3D large deformation TFM (LDTFM) method that no longer requires the assumption of a linear elastic material response. Appendix B applies LDTFM to investigate the traction fields of Schwann cells spreading on planar polyacrylamide hydrogels.

## 2. Measuring Nonlinear Deformations in 3D with Fast Iterative Digital Volume Correlation

**Note:** A version of this chapter is published in *Journal of Experimental Mechanics* with Jennet Toyjanova as a first co-author. Published data has been included in the section with co-author's consent.

Bar-Kochba, E., Toyjanova, J., Andrews, E., Kim, K.-S., & Franck, C. (2014). A Fast Iterative Digital Volume Correlation Algorithm for Large Deformations. *Exp. Mech.* 55(1):261–274. doi: 10.1007/s11340-014-9874-2

### 2.1 Introduction

Advances in three-dimensional (3D) imaging modalities have increased the need for accurate full-field displacement measurement techniques in studying classical mechanics problems and in quantifying the interaction of cells with soft biomaterials [175]. Digital volume correlation (DVC), the volumetric extension of digital image correlation (DIC) (for review see [265]) has begun to address this need [17]. The mathematical formulation of DVC can be derived using the same fundamental mathematical formulations as DIC where a displacement field  $\mathbf{u}(\mathbf{x})$  is computed by minimizing a correlation or similarity function  $\chi(\mathbf{x})$  between an undeformed  $I(\mathbf{x})$  and deformed image  $\hat{I}(\mathbf{x})$ . e.g.

$$\chi(\mathbf{x}) = \|\hat{I}(\mathbf{x} + \mathbf{u}(\mathbf{x})) - I(\mathbf{x})\|^2. \quad (2.1.1)$$

For proper image image correlation, images  $I(\mathbf{x})$  and  $\hat{I}(\mathbf{x})$  require a distinct traceable pattern such as randomly scattered tracker particles [78], or inherent material textures such as fibers [230, 77] and pores [17]. Accurately computing  $\mathbf{u}(\mathbf{x})$  in 3D involves solving up to the first order of the series expansion of  $\mathbf{u}(\mathbf{x})$ ,

$$\mathbf{u}(\mathbf{x}_0 + \Delta\mathbf{x}) \approx \mathbf{u}(\mathbf{x}_0) + \nabla\mathbf{u}(\mathbf{x}_0) \cdot \Delta\mathbf{x} \quad (2.1.2)$$

where  $\nabla \cdot$  is the vector gradient operator. The first term in equation (2.1.2) is the rigid translation, and the second term is the homogeneous deformation, i.e. stretch and rotation, between the configurations. For 3D deformations, these terms create twelve degrees of freedom during minimization and consequently produced high computational cost.

Another common displacement measurement technique is based on single particle tracking (SPT) using a Lagrangian frame of motion. SPT has been successfully used to analyze fluid flow [166], study cell membrane dynamics [240], and characterize new nanoparticles [300]. All of these applications benefit from low to medium particle seeding densities which SPT is well suited for. However, SPT suffers from two main drawbacks; it is error-prone for higher bead densities, and more importantly requires the use of tracker particles. The later drawback makes it unsuitable for many biological applications where tracker particles cannot be seeded, whereas DVC can take advantage of the inherent textures of the material, e.g. fibers in collagen,[230] or pores in bone [17].

Early developments of DVC only considered rigid displacements, and did not account for large rotations and stretches [17]. Later studies showed that ignoring these higher-order terms can have a significant effect on the accuracy of the computed displacement field [259, 78]. To overcome these limitations, Gates et al. in 2011 presented a full 12 degrees of freedom DVC algorithm using an iterative minimization approach [85]. High computational cost was addressed by utilizing parallel computing, producing run times of 6 hours for  $39 \times 39 \times 39$  voxel correlation grids and  $41 \times 41 \times 41$  voxel interrogation window sizes [85]. In general, the choice of interrogation windows size will determine the spatial resolution and uncertainty of the displacement field [200, 58]. With these improvements, computing dynamically evolving 3D displacements with high resolution from large time-lapse volumetric images would be unpractical.

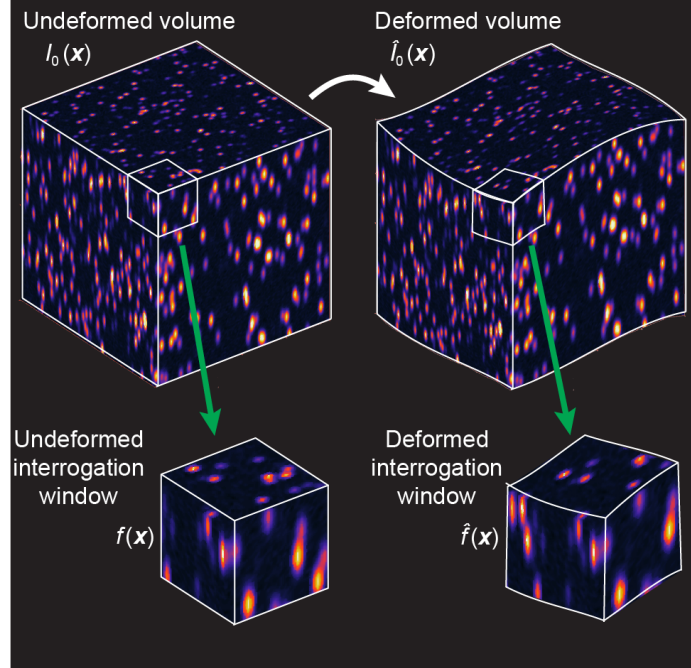
These challenges, namely the computational cost and spatial resolution, are addressed with the development of the fast iterative digital volume (FIDVC) algorithm capable of capturing any general nonlinear finite deformation at tractable computation times. The proposed algorithm is based on the standard cross-correlation formulation similar to the previous

DVC algorithm [78] with the addition of an iterative image deformation method (IDM) recently used in particle image velocimetry (PIV) algorithms [261, 243, 245]. IDM computes the displacement field by iteratively deforming or warping the undeformed and deformed images by the displacement field estimate. During the iterative procedure, the interrogation window size is refined, allowing for the accurate capture of high spatial frequencies including large displacement gradients.

## 2.2 Methods

### 2.2.1 The Fast Iterative Digital Volume Correlation (FIDVC) Algorithm

FIDVC is a fast, non-invasive, full-field measurement technique for computing a nonlinear displacement field,  $\mathbf{u}(\mathbf{x}) = (u_1(\mathbf{x}), u_2(\mathbf{x}), u_3(\mathbf{x}))$  between two volumetric images,  $I_0(\mathbf{x})$  and  $\hat{I}_0(\mathbf{x})$ , with image coordinate system  $\mathbf{x} = (x_1, x_2, x_3)$  (Algorithm 1). These volumetric images may be acquired using a variety of 3D imaging modalities such as computed tomography (CT), magnetic resonance imaging (MRI) or, for the purpose of this study, laser scanning confocal microscopy (LSCM) [17, 259, 19]. In general, these images should contain unique intensity variations, or speckle patterns, to produce accurate displacement estimation [266].



**Figure 2.1:** Schematic of undeformed  $I_0(\mathbf{x})$  (left) and deformed  $\hat{I}_0(\mathbf{x})$  (right) volumetric images subdivided to respective interrogation windows,  $f(\mathbf{x})$  and  $\hat{f}(\mathbf{x})$ .

Intensity distributions in these volumetric images are in the form of micron-sized fluorescent particles that are randomly dispersed throughout the imaging medium [110] (**Fig. 2.1**). The precision and resolution ultimately depends on this intensity information, including the particle density, size, and distribution.

---

**Algorithm 1** The fast iterative digital volume correlation algorithm.

---

**Input:** Volumetric Images,  $I_0(\mathbf{x})$  and  $\hat{I}_0(\mathbf{x})$

**Output:** Displacement field,  $\mathbf{u}(\mathbf{x})$

---

```

1: int  $i = 0$                                  $\triangleright$  Initialize interrogation window size resizing counter
2: do
3:    $\chi(\mathbf{x}) = I_{i-1}(\mathbf{x}) \otimes \hat{I}_{i-1}(\mathbf{x})$        $\triangleright$  Compute FFT-based cross-correlation function
4:    $\mathbf{du}(\mathbf{x}) = \underset{\mathbf{du}(\mathbf{x})}{\operatorname{argmax}} \chi(\mathbf{x})$        $\triangleright$  Compute incremental displacement
5:   if  $i = 0$  then
6:      $\mathbf{u}_0(\mathbf{x}) = \mathbf{du}(\mathbf{x})$                                  $\triangleright$  Initialize displacement field
7:   else
8:      $\mathbf{u}_i(\mathbf{x}) = \mathbf{u}_{i-1}(\mathbf{x}) + \mathbf{du}(\mathbf{x})$        $\triangleright$  Add displacement from previous iteration
9:   end if
10:   $\mathbf{u}_i(\mathbf{x}) = p * \mathbf{u}_i(\mathbf{x})$                                  $\triangleright$  Correct for divergence
11:   $\mathbf{u}_i(\mathbf{x}) = \operatorname{RemoveOutliers}(\mathbf{u}_i(\mathbf{x}))$        $\triangleright$  Remove outliers with universal median test
12:   $I_i(\mathbf{x}) = I_0(\mathbf{x} - \mathbf{u}_i(\mathbf{x})/2)$                                  $\triangleright$  Symmetrically deform images
     $\hat{I}_i(\mathbf{x}) = \hat{I}_0(\mathbf{x} + \mathbf{u}_i(\mathbf{x})/2)$ 
13:   $L = \operatorname{RefineWindow}(L)$                                  $\triangleright$  Refine interrogation window size
14:   $i = i + 1$ 
15: while  $(I_i(\mathbf{x}) \approx \hat{I}_i(\mathbf{x}))$                                  $\triangleright$  Check for convergence
16: return  $\mathbf{u}_i(\mathbf{x})$ 

```

---

## 2.2.2 Calculating Displacements

### 2.2.2.1 Estimating Incremental Displacement Field

Volumetric images  $I_0(\mathbf{x})$  and  $\hat{I}_0(\mathbf{x})$ , are subdivided into their respective interrogation windows,  $f(\mathbf{x})$  and  $\hat{f}(\mathbf{x})$  (**Fig. 2.1**). The interrogation window side length,  $L$ , and overlap ratio determines the final resolution of  $\mathbf{u}(\mathbf{x})$ , and can be adjusted during each iteration to increase the overall spatial resolution. The incremental displacement field  $\mathbf{du}(\mathbf{x})$  is estimated by first computing the basic cross correlation coefficient of  $f(\mathbf{x})$  and  $\hat{f}(\mathbf{x})$ , i.e.

$$\chi(\mathbf{x}) = \sum_{\mathbf{x}=-L/2}^{L/2} f(\mathbf{x}) \cdot \hat{f}(\mathbf{x} + \mathbf{du}(\mathbf{x})). \quad (2.2.1)$$

Equation (2.2.1) is expressed using the general cross-correlation relation,

$$\chi(\mathbf{x}) = I_{i-1}(\mathbf{x}) \otimes \hat{I}_{i-1}(\mathbf{x}), \quad (2.2.2)$$

where  $i$  is the integer iteration counter starting at one until convergence. The displacement field is computed by finding the location that maximizes  $\chi(\mathbf{x})$ , expressed mathematically as

$$\mathbf{du}(\mathbf{x}) = \underset{\mathbf{du}(\mathbf{x})}{\operatorname{argmax}} \chi(\mathbf{x}) \quad (2.2.3)$$

Sub-voxel resolution can be achieved by fitting the  $3 \times 3 \times 3$  voxel cross-correlation peak to a polynomial to yield a more accurate estimate of the true sub-voxel peak location. There exists many sub-voxel algorithms [281, 109, 110]; however a 3rd order polynomial Gaussian peak fitting scheme provides both good accuracy while being computationally efficient.

#### 2.2.2.2 Increasing Spatial Resolution with a Weighting Function

Due to the intrinsic averaging process in the cross-correlation formulation some of its high-frequency content is inevitably lost [236, 111]. The Nyquist criterion sets the minimum wavelength that can be resolved as twice the mean distance between markers within interrogation windows [193]. The modular transfer function ( $MTF$ ) of the cross-correlation in equation (2.2.1) is best approximated by a 3D moving average (or box) filter [241, 242], expressed mathematically as

$$\text{MTF}(\boldsymbol{\lambda}) = \prod_{i=1}^3 \frac{\sin(\pi L/\lambda_i)}{\pi L/\lambda_i} \quad (2.2.4)$$

where  $\boldsymbol{\lambda} = (\lambda_1, \lambda_2, \lambda_3)$  are the wavelengths along each dimension. The frequency response of the cross-correlation is unstable and produces irreversible loss of information when  $MTF(\boldsymbol{\lambda}) = 0$  at integer multiples of  $L$ . To overcome this instability, a weighting function  $w(\mathbf{x})$  may be designed to flatten the frequency response of a moving average filter. There exists many choices of  $w(\mathbf{x})$  that stabilize the frequency. Nogueira et al. presented a robust

$w(\mathbf{x})$ , given as

$$w(\mathbf{x}) = \prod_{i=1}^3 \left( 12 \left| \frac{x_i}{L} \right|^2 - 12 \left| \frac{x_i}{L} \right| + 3 + 0.15 \cos(4\pi x_i/L) + 0.2 \cos(6\pi x_i/L) \right. \\ \left. + 0.1 \cos(8\pi x_i/L) + 0.05 \cos(10\pi x_i/L) \right) \quad (2.2.5)$$

[192], which was implemented in FIDVC. By incorporating  $w(\mathbf{x})$  into equation (2.2.1), the cross-correlation coefficient becomes

$$\chi(\mathbf{x}) = \sum_{\mathbf{x}=-L/2}^{L/2} w(\mathbf{x}) f(\mathbf{x}) \cdot w(\mathbf{x} + \mathbf{du}(\mathbf{x})) \hat{f}(\mathbf{x} + \mathbf{du}(\mathbf{x})) \quad (2.2.6)$$

To improve the computation speed of  $\chi(\mathbf{x})$ , equation (2.2.6) can be implemented in Fourier space as

$$\chi(\mathbf{x}) = \mathcal{F}^{-1} \{ \mathcal{F} \{ w(\mathbf{x}) f(\mathbf{x}) \}^* \cdot \mathcal{F} \{ w(\mathbf{x} + \mathbf{du}(\mathbf{x})) \hat{f}(\mathbf{x} + \mathbf{du}(\mathbf{x})) \} \}. \quad (2.2.7)$$

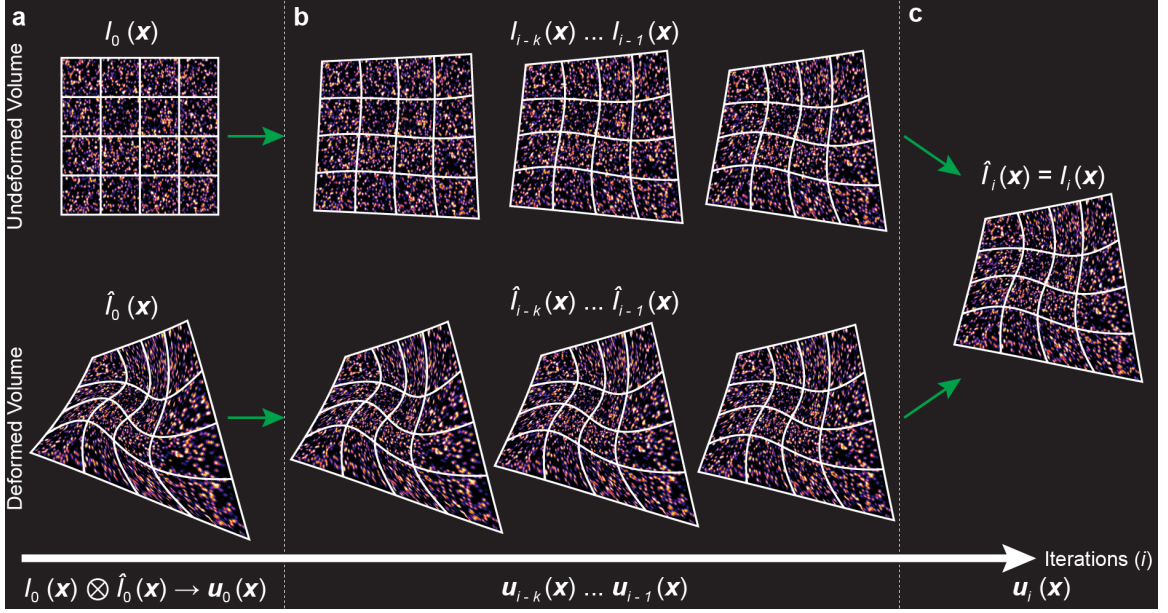
where  $\mathcal{F}\{\cdot\}$  is the Fourier transform and  $\mathcal{F}^{-1}\{\cdot\}$  is the inverse Fourier transform in 3D. It is important to note that the weighting function does not completely eliminate the low-pass behavior of the cross correlation since rigid translation is still assumed between the windows. However, it significantly improves the reconstruction of the high frequency displacement information.

### 2.2.3 Applying the Iterative Image Deformation Method to FIDVC

The Image deformation method (IDM) has been well documented for measuring complex fluid flows using particle image velocimetry (PIV). In this study, IDM is extended to measure nonlinear 3D material displacements in a computationally efficient manner. The displacement field between  $I(\mathbf{x})$  and  $\hat{I}(\mathbf{x})$  is expanded into multiple incremental linear deformations that can be computed accurately with cross-correlation. At each iteration  $i$ , the images are symmetrically warped by the current displacement field ( $\mathbf{u}_i(\mathbf{x})$ ) estimate (**Fig. 2.2**), which is equal to the sum of the previous displacement increments, i.e.

$$\mathbf{u}_i(\mathbf{x}) = \sum_i \mathbf{u}_{i-1}(\mathbf{x}) + \mathbf{du}(\mathbf{x}). \quad (2.2.8)$$





**Figure 2.2:** Schematic of the image deformation method. The (a) undeformed and deformed image are symmetrically deformed by the (b) linearized, incremental displacement field until they reach the (c) same final configuration.

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

$$\begin{aligned} I_i(\mathbf{x}) &= I_0(\mathbf{x} - \mathbf{u}_i(\mathbf{x})/2) \\ \hat{I}_i(\mathbf{x}) &= \hat{I}_0(\mathbf{x} + \mathbf{u}_i(\mathbf{x})/2) \end{aligned} \tag{2.2.9}$$

is implemented using a trilinear interpolation scheme. Generally, higher order interpolation schemes can be implemented at the expense of increased computational cost [13, 85]; however symmetric image warping and trilinear interpolation achieves second order accuracy at reasonable computational cost [13, 85]. Although not presented in this work, GPU based interpolation can also help to decrease computation times [234].

### 2.2.3.1 Correcting for Displacement Divergence

The iterative process in the IDM is intrinsically unstable and the results may diverge at specific wavelengths  $\lambda$  [191, 13, 246], e.g. larger spatial wavelengths ( $\lambda > L$ ) converge faster than smaller wavelengths. 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 has been introduced by Schrijer et al. that are computationally efficient and change

strength based on a single input parameter [246]. The one-dimensional formulation of the filter is given as

$$p(\xi) = \left(\frac{L}{2}\right)^z - |\xi|^z \quad (2.2.10)$$

where the kernel window length  $L$  is equal to the interrogation window side length. The parameter  $z$  controls the strength of the low-pass filter. As  $z \rightarrow 0$  no filtering occurs 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  $10^{-3} \leq z \leq 10^{-2}$  [246]. The results vary depending on when the filter is applied during the iterative process. It is chosen that the filter directly influences the FIDVC results by acting on the current displacement field ( $\mathbf{u}_i(\mathbf{x})$ ), i.e.

$$\mathbf{u}_i = p * (\mathbf{u}_{i-1} + d\mathbf{u}). \quad (2.2.11)$$

as oppose to the incremental displacement field, i.e.  $p * d\mathbf{u}(\mathbf{x})$ .

### 2.2.3.2 Removing Outliers with Normalized Universal Median Test

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 the desired spatial resolution in the DVC results. These spurious displacements must be removed by means of a normalized universal median test [295] to produce smoother displacement gradient fields.

The normalized universal median test is as follows. Let  $\mathcal{U}$  be a  $3 \times 3 \times 3$  voxel displacement field surrounding a location  $\mathbf{x}_n$ , i.e.  $\mathcal{U} = \{\mathbf{u}(\mathbf{x}_n) | n = 1, \dots, 27\}$ . A residual  $r_n$  at  $\mathbf{x}_n$  is first calculated using

$$r_n = |\mathbf{u}(\mathbf{x}_n) - med(\mathcal{U})|, \quad (2.2.12)$$

where  $med(\cdot)$  denotes the median of a set of numbers. The median of these 27 residuals is

computed and used to normalize the standard median test, i.e.

$$\frac{|\mathbf{u}(\mathbf{x}_n) - \text{med}(\mathcal{U})|}{\text{med}(r_n) + \epsilon} < \epsilon_{\text{thresh}}, \quad (2.2.13)$$

where  $\epsilon$  represent the typical fluctuation level of the cross-correlation and  $\epsilon_{\text{thresh}}$  is a threshold for spurious displacement removal.

### 2.2.3.3 Checking Convergence

The final step in FIDVC is to check whether the displacement values,  $\mathbf{u}_i$  have converged. Having a well defined stopping criterion is important to provide proper balance between accuracy and computation times in FIDVC. Stopping the algorithm prematurely will produce under predicted displacement fields, while over iterating will be time consuming without significant gain in displacement accuracy. The most natural stopping criterion is at a point in which the images have matching intensity profiles, i.e.  $I_i(\mathbf{x}) \approx \hat{I}(\mathbf{x})$ . The image similarity metric of choice is the normalized sum of squared error ( $e_i$ ) given as

$$e_i = \frac{1}{\bar{\sigma}} \sqrt{\frac{1}{N} \sum_{\mathbf{x}=1}^N \left( I_i(\mathbf{x}) - \hat{I}_i(\mathbf{x}) \right)^2}. \quad (2.2.14)$$

where  $N$  is the number of voxels, and  $\bar{\sigma}$  is the mean standard deviation of  $I_i(\mathbf{x})$  and  $\hat{I}_i(\mathbf{x})$ . 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

---

#### Algorithm 2 FIDVC convergence algorithm

---

```

1: int  $j = 0$  ▷ Initialize iteration counter
2: int  $i = 0$  ▷ Initialize interrogation window size resizing counter
3: do
4:   Compute  $\mathbf{u}_i(\mathbf{x})$  using FIDVC
5:   Compute  $e_i$  ▷ Equation (2.2.14)
6:    $\Delta e_i = e_i - e_{i-1}$  ▷ Compute error difference
7:   if  $(\Delta e_i / \Delta e_j \geq \epsilon_1)$  then
8:      $L = L/2$  ▷ Refine interrogation window size
9:      $j = j + 1$ 
10:  end if
11:   $i = i + 1$ 
12: while  $(\Delta e_i / \Delta e_0 \geq \epsilon_0)$  ▷ Check for FIDVC convergence
13: return  $\mathbf{u}_i(\mathbf{x})$ 

```

---

(Algorithm 2). In the first step, the displacement field of the current iteration,  $k$  is calculated from equation (2.2.6) and the images are warped according to equation (2.2.9). The sum of squared error is calculated for the current iteration,  $e_i$  and is used to calculate the error difference between the current and previous iteration, i.e.,  $\Delta e = e_i - e_{i-1}$ . If  $\Delta e_i / \Delta e^0$  is less than or equal to the user defined stopping threshold  $\epsilon_0$  then FIDVC is stopped and said to have converged. If this condition is not met, we test against the interrogation window refinement criterion; if  $\Delta e_i / \Delta e^j$  is less than or equal to  $\epsilon_1$  the interrogation window length,  $L$  is reduced by half and the loop continues. The subscript  $j$  is an integer counter denoting the last time point the interrogation window size was changed.

#### 2.2.4 3D Displacement Gradient Calculations

Correlation techniques serve as a powerful tool to measure strains in many research fields; e.g. investigations of cell traction force microscopy [175, 194, 114], biological tissues characterization [158], material fracture [221, 39] and full field soft matter characterization [230]. All of these examples rely on accurate measurements of the 3D internal material strains. Care must be exercised when differentiating the displacement field in the presence of noise and large displacement gradients. Traditionally, differentiation kernels in conjunction with low-pass filters used to produce optimal noise mitigation and sample dealiasing strategies. Based on the work of Farid et al. [72], the optimal differentiation and interpolation kernel is based on 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 compute 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  $n$  reduce errors due to noise, it may cause 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.

## 2.3 Validation of the FIDVC Algorithm

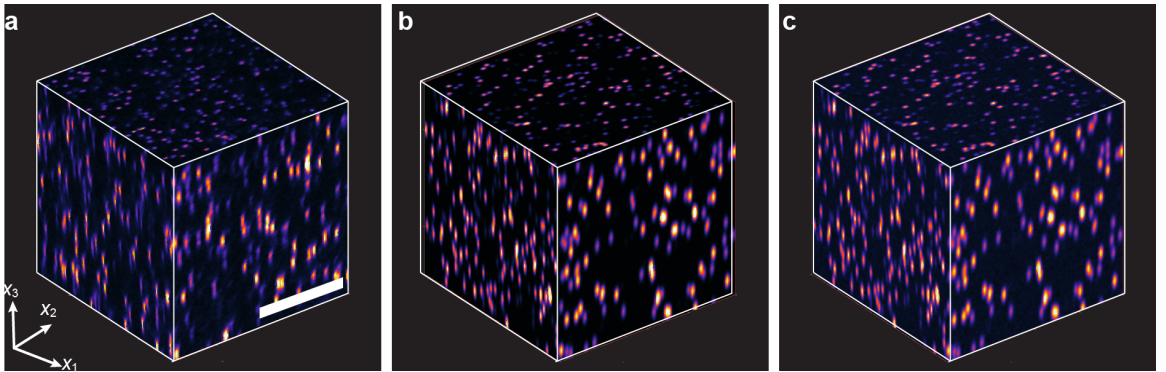
This section presents the quantitative validation of the FIDVC detailing the spatial resolution limits and average processing times.

### 2.3.1 Generation of Simulated Volumetric Images and Displacement Fields

The validation examples presented here are primarily based on simulated deformation fields. To mimic experimental conditions, all simulated images were corrupted with experimental-like noise determined from actual laser scanning confocal microscopy (LSCM) images (**Fig. 2.3**). The choice in numerical rather than experimental validation generates analytically exact, nonlinear localized material deformations to assess the FIDVC spatial resolution limits quantitatively. All validation results were compared to the previous DVC algorithm from LSCM developed by Franck et al. [78].

The optical system's point spread function (*PSF*) was determined by least-squares fitting of a 3D Gaussian profile to the intensity values of 1,000 randomly sampled micron-sized fluorescent particles within the image [308]. The gray-scale intensity of a PSF with amplitude  $A$  and standard deviation,  $\sigma = (\sigma_1, \sigma_2, \sigma_3)$  is expressed as

$$I_{PSF}(\mathbf{x}) = A \exp \left( - \sum_{i=1}^3 \frac{x_i^2}{2\sigma_i^2} \right), \quad (2.3.1)$$



**Figure 2.3:** Slices of three volumetric images used for FIDVC validation. (a) Actual experimental image of a transparent polyacrylamide gel embedded with 0.5 micron-sized fluorescent particles recorded via LSCM. Scale bar, 128 voxels. (b) Numerically simulated image based on the image properties of (a) without noise. (c) Numerically simulated image in (b) with added Gaussian noise.

where  $\sigma = (5, 5, 10)$  from the least-squares fitting procedure. A 12-bit grayscale undeformed image  $I(\mathbf{x})$  was digitally generated by superimposing the intensities of multiple PSFs at random locations throughout the image (**Fig. 2.3b**). The superposition of PSF intensities is given as

$$I(\mathbf{x}) = \sum_{n=1}^N I_{PSF}(\mathbf{x} - \mathbf{x}^n) \quad (2.3.2)$$

where  $\mathbf{x}_n = (x_1^n, x_2^n, x_3^n)$  is the center location of the  $n$ th micron-sized fluorescent particle. The total number of beads  $N$  was calculated by multiplying the marker seeding density  $\rho$  with the number of voxels of the image  $V$ , i.e.  $N = \rho V$ . From the experimental data,  $\rho = 4 \times 10^{-13}$  markers per voxel was used to generate all subsequent synthetic images. Image intensities greater than the maximum 12-bit gray scale level, were saturated to simulate true LSCM images. The same procedure was utilized to digitally generate the deformed image  $\hat{I}(\mathbf{x})$  except each marker location  $\mathbf{x}^n$  was translated to a new location,  $\mathbf{X}^n = (X_1^n, X_2^n, X_3^n)$  by the prescribed displacement field  $\mathbf{u}(\mathbf{x}^n)$ , i.e.

$$\mathbf{X}^n = \mathbf{x}^n + \mathbf{u}(\mathbf{x}^n). \quad (2.3.3)$$

Following a typical experimental approach for DVC, all simulated volumetric images were deconvolved using the Lucy-Richardson deconvolution prior to calculating the displacement field using FIDVC [224].

To characterize the spatial resolution of the FIDVC, a superposition of Gaussian displacement functions was applied to a set of volumetric images, i.e.

$$g(\mathbf{x}) = \sum_j A^j \exp \left( - \sum_{i=1}^3 \frac{(x_i - c^j)^2}{2(\sigma_i^j)^2} \right) \quad (2.3.4)$$

where

1. The number of Gaussian functions  $j$  was chosen by the user.
2. The Gaussian width was 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 was equal in all directions.

3. The amplitude  $A$  was chosen such that the applied material deformation field falls into the finite deformation regime. The analytical Lagrangian strain was computed from the deformation field given in equation (2.3.4), and  $A$  was adjusted such that the maximum value of any strain component equals 15%.
4. The center position of the peak  $c$  for each Gaussian displacement field was chosen not to overlap with any neighboring functions.

This approach allows simultaneous application of several well defined spatial deformation wavelengths to assess FIDVC resolution limits. It also provides an analytical benchmark for quantitative assessment of various finite difference schemes in estimating the Lagrangian strains. Physical examples of such displacement fields can found in many cell-induced material deformations [58, 159, 236, 79].

The FIDVC was 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, equation (2.2.7) was implemented on a GPU using the commercially available AccelerEyes Jacket library (AccelerEyes, Atlanta, GA).

### 2.3.2 Validation Parameters

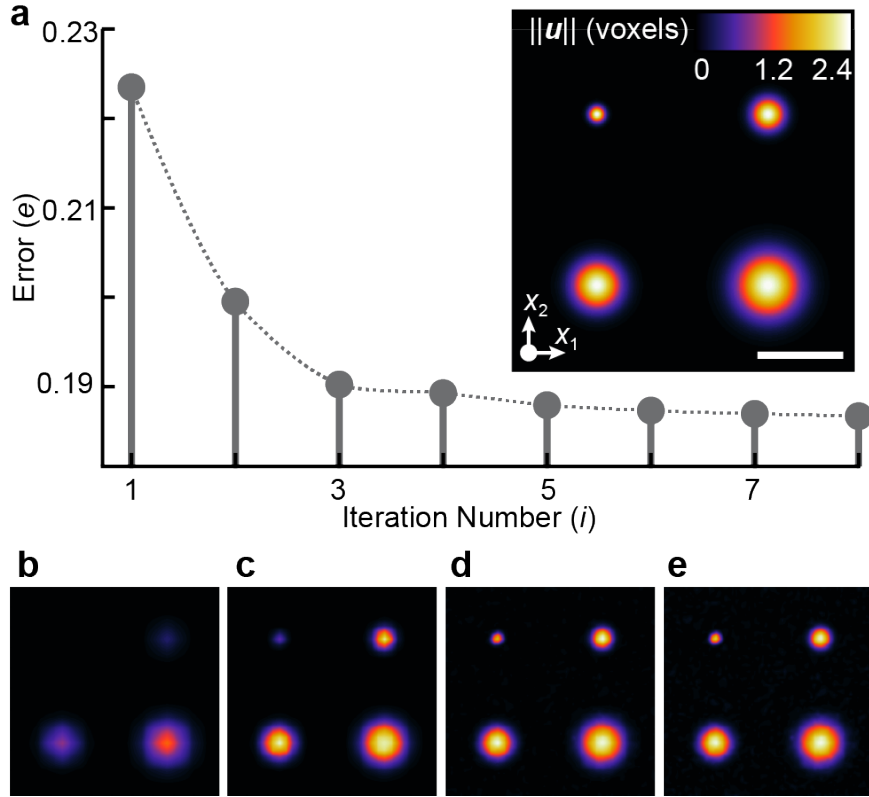
The undeformed and deformed images were processed using the FIDVC with an initial interrogation window side length of 64 voxels with 50% overlap. The universal outlier removal filter was performed with a threshold of 2 and a fluctuation level of 0.1 based on previously published displacement standard deviation values [4,35,40]. The polynomial degree of the predictor filter was set to  $z = 7.5 \times 10^{-3}$ , the convergence threshold for the interrogation window refinement was set to  $\epsilon_1 = 0.25$  and the stopping threshold was set to  $\epsilon_0 = 0.0625$  based on empirical evidence of various validation cases.

For all numerical experiments performed using DVC, the interrogation window side length and window overlap was fixed at its optimal value of 64 voxels with a 75% overlap. The final spatial resolution for both FIDVC and DVC results in an equal grid spacing of 8 voxels,

producing  $49 \times 49 \times 33$  correlation points.

### 2.3.3 FIDVC Convergence

Since FIDVC is an iterative technique, the convergence behavior of the algorithm needs to be characterized. A simulated image of size  $512 \times 512 \times 256$  voxels was deformed according to a displacement field given by a superposition of four Gaussian functions of widths 32, 64, 96, and 128 voxels (**Fig. 2.4a**). The normalized residual error  $e$  between the undeformed and deformed images given in equation (2.2.14) was used as the convergence metric (**Fig. 2.4a**). The corresponding contour maps of the computed displacement field magnitude  $\|\mathbf{u}(\mathbf{x})\|$  show the iterative recovery of the displacement field (**Fig. 2.4b-e**). The displacement contours fields at the first iteration shows the typically recovered displacement field using the previous DVC method. Qualitative comparison with the prescribed solution clearly shows that most



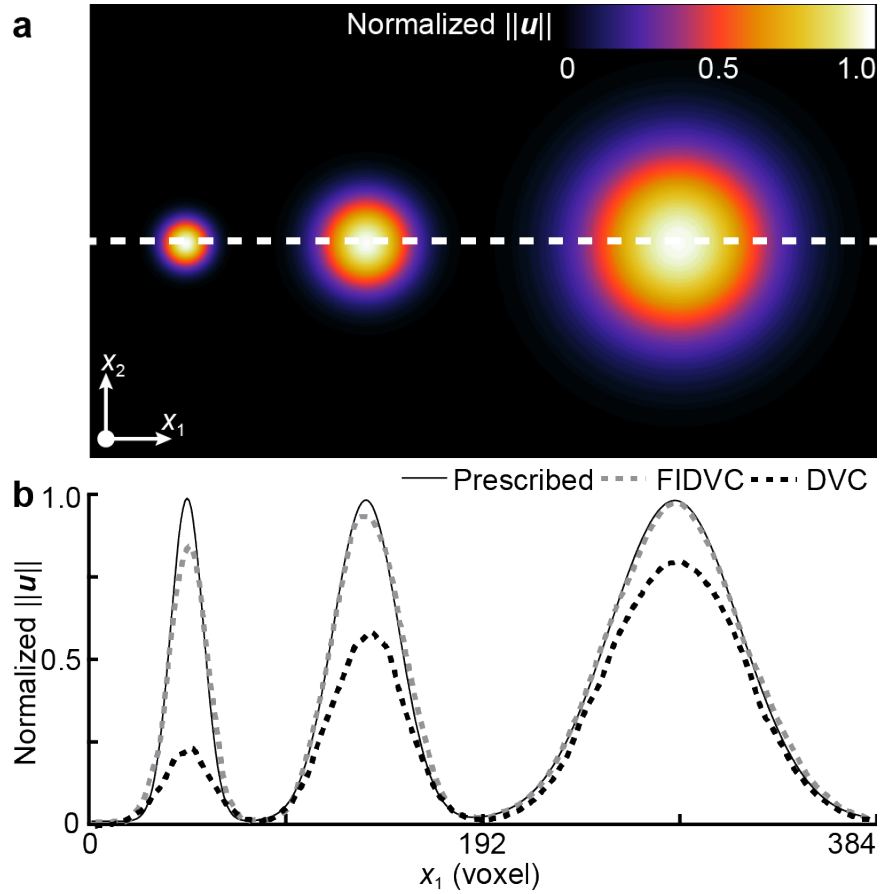
**Figure 2.4:** FIDVC converges within seven iterations for a multiple frequency deformation field. (a) Normalized residual error  $e$  between the prescribed displacement field and FIDVC results at every iteration. (Inset) Contour map of a central  $x_1 - x_2$  cross-section of the prescribed  $\|\mathbf{u}(\mathbf{x})\|$ , and FIDVC results for iterations (b) 1, (c) 3, (d) 5, and (e) 7. Scale bar, 128 voxels.



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.

#### 2.3.4 Spatial Resolution

To quantify the spatial improvements of FIDVC over the previous DVC technique and to investigate the spatial limitations of both algorithms, an image of size of  $384 \times 256 \times 256$  voxels was deformed by a superposition of three Gaussian displacement functions of widths 32, 64, and 128 voxels (**Fig. 2.5**). The displacement field was recovered using the FIDVC and DVC and compared to the analytically prescribed displacement field.

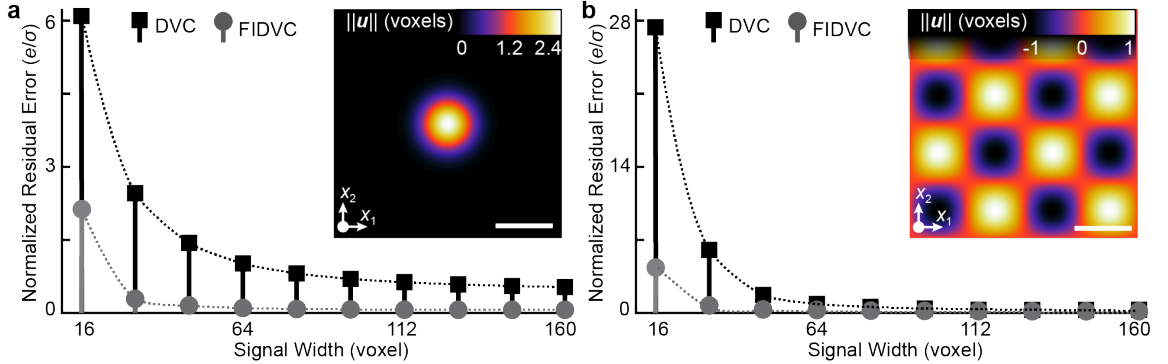


**Figure 2.5:** FIDVC successfully captures high spatial frequencies. (a) Contour map of the central  $x_1 - x_2$  plane of the prescribed  $\|\mathbf{u}(\mathbf{x})\|$  used to compare the spatial resolution of FIDVC and DVC. The widths of the Gaussian functions are, from left-to-right, 32, 64, and 128 voxels. (b) A line plot across the dotted line in (a) compares the results of DVC (dashed black) and FIDVC (dashed gray) to the prescribed  $\|\mathbf{u}(\mathbf{x})\|$  (solid black) .

For low spatial frequencies both algorithms capture the extent of the applied deformations accurately. However, for high spatial frequencies DVC underpredicts the amplitude of the imposed displacements. For high spatial frequencies the original DVC algorithm is unable to accurately predict the correct peak amplitude, and shows an approximately 80% difference compared to the prescribed solution at a 32 voxels width. This effect stems from the intrinsic low-pass smoothing behavior of the cross-correlation, in which case, DVC can be regarded as a moving-average operation [191, 242, 19]. In contrast, FIDVC successfully captures most of the applied deformations even at 32 voxels widths.

To quantify the resolution limits and errors associated with each algorithm over multiple frequencies, a single Gaussian and sinusoidal function was prescribed to deform images of size  $256 \times 256 \times 256$  voxels. The normalized residual error between the numerical and analytical displacements was computed with equation (2.2.14) for signal widths of 16 to 160 voxels (**Fig. 2.6**).

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



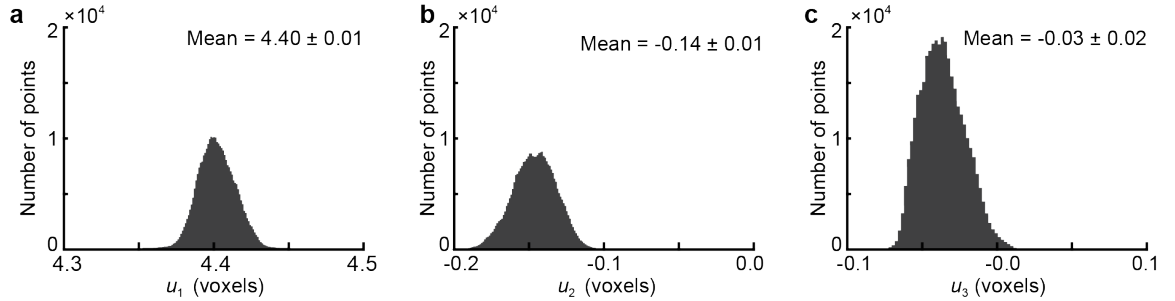
**Figure 2.6:** FIDVC produces better spatial resolution for multiple wavelengths. Plot of the normalized residual error associated with (a) prescribed Gaussian and (b) sinusoidal displacement field of widths 16 to 160. (Inset) Contour map of the  $x_1 - x_2$  mid-plane of  $\|\mathbf{u}(\mathbf{x})\|$  for each function. Scale bars, 128 voxels.

field. FIDVC was 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 in equation (2.2.5), which plays an important role in detecting higher spatial frequencies.

### 2.3.5 Measurement Precision and Accuracy

To determine the measurement precision of FIDVC, a zero-strain deformation was experimentally acquired using LSCM. A polyacrylamide hydrogel embedded with 0.5 micron-sized fluorescent particles was rigidly translated by 4.4 voxels along the  $x_1$  direction. Images before and after translation were processed with FIDVC to calculate the displacement field. The histogram shows that each displacement component is normally distributed (**Fig. 2.7**). The mean and standard deviation of the corresponding Lagrangian strain field were computed using an Optimal-11 tap gradient kernel and are summarized in Table 2.1.

The standard deviation of the recovered displacements are an order of magnitude smaller



**Figure 2.7:** Histograms of the displacement field corresponding to a experimental rigid translation of 4.4 voxels along the  $x_1$  direction. (a)  $u_1(\mathbf{x})$ , (b)  $u_2(\mathbf{x})$ , (c)  $u_3(\mathbf{x})$ .

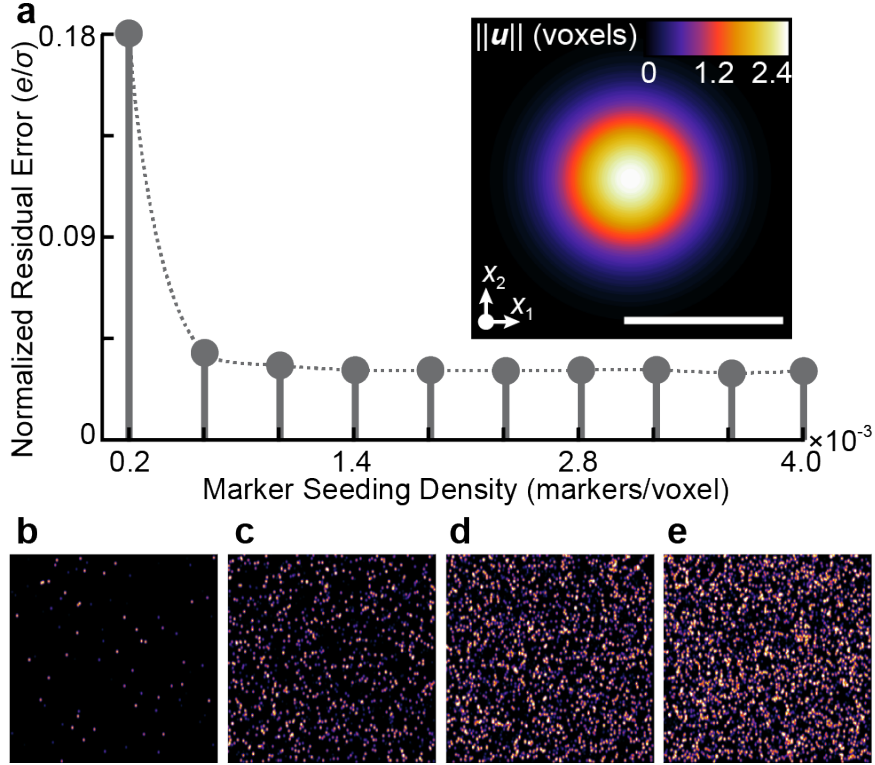
**Table 2.1:** Mean and standard deviation values corresponding to the experimental zero-strain deformation

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

than those previously reported [78, 230]. This large increase in precision was likely due to the universal outlier removal process that was performed during each iteration. The axial precision of the displacements and strains in the  $x_3$  direction was approximately 3 – 4 times lower than the transverse precision in the  $x_1, x_2$  directions, caused by an axially elongated PSF (**Fig. 2.3.1**). The values of all strain components were close to zero and smaller than their corresponding standard deviation values confirming a zero-strain deformation.

### 2.3.6 Displacement Recovery at Multiple Bead Densities

To quantify FIDVC performance with volumes containing lower marker seeding densities, a single Gaussian displacement function of width 128 voxels was applied to deform images of size  $256 \times 256 \times 256$  voxels with bead densities ranging from  $0.2 \times 10^{-3}$  to  $4 \times 10^{-3}$  markers per voxel. The error computed by equation (2.2.14) shows that FIDVC is still able to recover

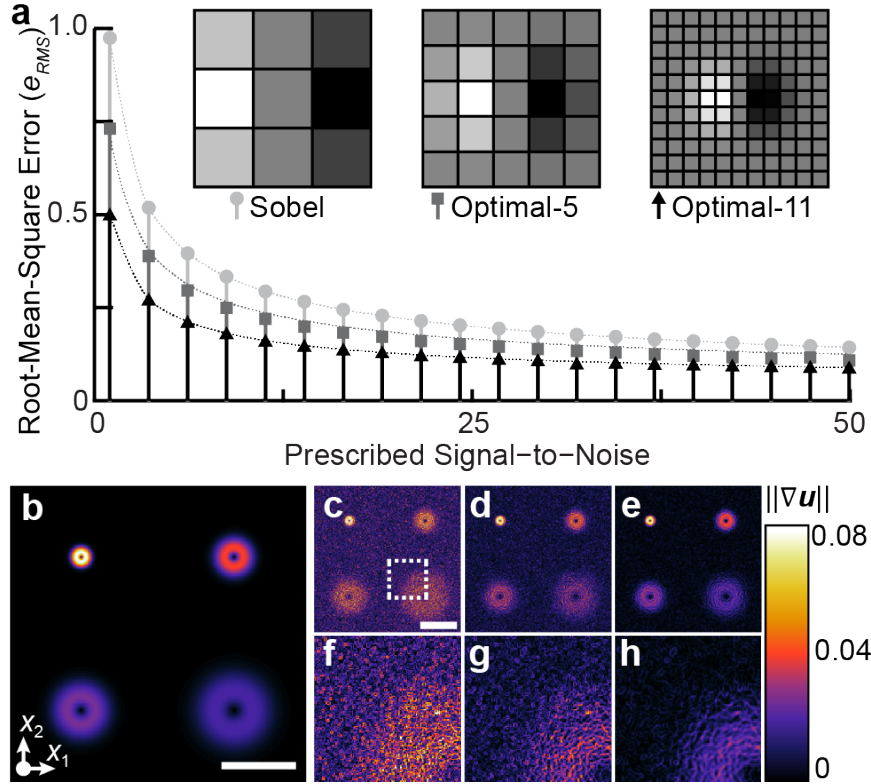


**Figure 2.8:** FIDVC recovers displacement fields at low marker seeding densities. (a) Plot of the normalized residual error associated with the prescribed Gaussian displacement field generated in the volumes with seeding densities ranging from  $0.2 \times 10^{-3} - 4 \times 10^{-3}$  markers per voxel. (Inset) Contour map of the  $x_1 - x_2$  mid-plane of the prescribed  $\|u(x)\|$  of width 128 voxels. Scale bars, 128 voxels.  $x_1 - x_2$  LSCM image of the markers at seeding density of (b)  $0.2 \times 10^{-3}$ , (c)  $1.4 \times 10^{-3}$ , (d)  $2.8 \times 10^{-3}$ , and (e)  $4.0 \times 10^{-3}$  markers per voxel.

the deformation at lower seeding densities (**Fig. 2.8**). However, with an increase in bead seeding density, it is possible to decrease the smallest interrogation window size to 16 voxels thereby further improving the spatial detection capability of FIDVC.

### 2.3.7 Computing Displacement Gradients in the Presence of Noise

The performance of different differentiation schemes was used to compute the displacement field gradient  $\nabla \mathbf{u}(\mathbf{x})$  in the presence of noise. Motivated by the normal distributions in the displacement noise (**Fig. 2.7**), a four-pole Gaussian displacement field with widths 32, 64, 96, and 128 voxels was corrupted with Gaussian white noise with varying signal-to-noise ratios (SNRs). The root-mean-square error  $e_{RMS}$  was computed between the prescribed



**Figure 2.9:** Optimal-11 tap finite difference kernels shows the best gradient recovery across SNRs. (a) Plot of the root-mean-square error  $e_{RMS}$  computed between the prescribed  $\|\nabla \mathbf{u}(\mathbf{x})\|$  and the one calculated with the Sobel (inset, left), Optimal-5 tap (inset, center), and Optimal-11 tap (inset, right) kernels for SNR of 1 to 50. Inset shows the grayscale value of each differentiation kernel at the central slice. (b) Contour map of the  $x_1 - x_2$  mid-plane of the prescribed  $\|\nabla \mathbf{u}(\mathbf{x})\|$  with no additive noise. Scale bar, 128 voxels. Recovered  $\|\nabla \mathbf{u}(\mathbf{x})\|$  using a (c) Sobel, (d) Optimal-5 tap, and (e) Optimal 11-tap for SNR of 5. Scale bar, 128 voxels. (f – g) zoomed contour maps of (c – (e))

$\|bv\nabla u(\mathbf{x})\|$  and the one calculated with the Sobel [96], Optimal-5 tap, and Optimal-11 differentiation kernels for SNRs of 1 to 50 (**Fig. 2.9**). For  $SNR = 5$ , the contour map of  $\|\nabla \mathbf{u}(\mathbf{x})\|$  along the central  $x_1 - x_2$  slice was shown for the three differentiation kernels. The Optimal-11 tap performs the best in handling noise and recovering the correct displacement gradient. The Sobel kernel show the most fluctuations in the recovered displacement gradient and is an unreliable predictor of the correct strain field at low SNRs.

### 2.3.8 Spherical Inclusion Problem

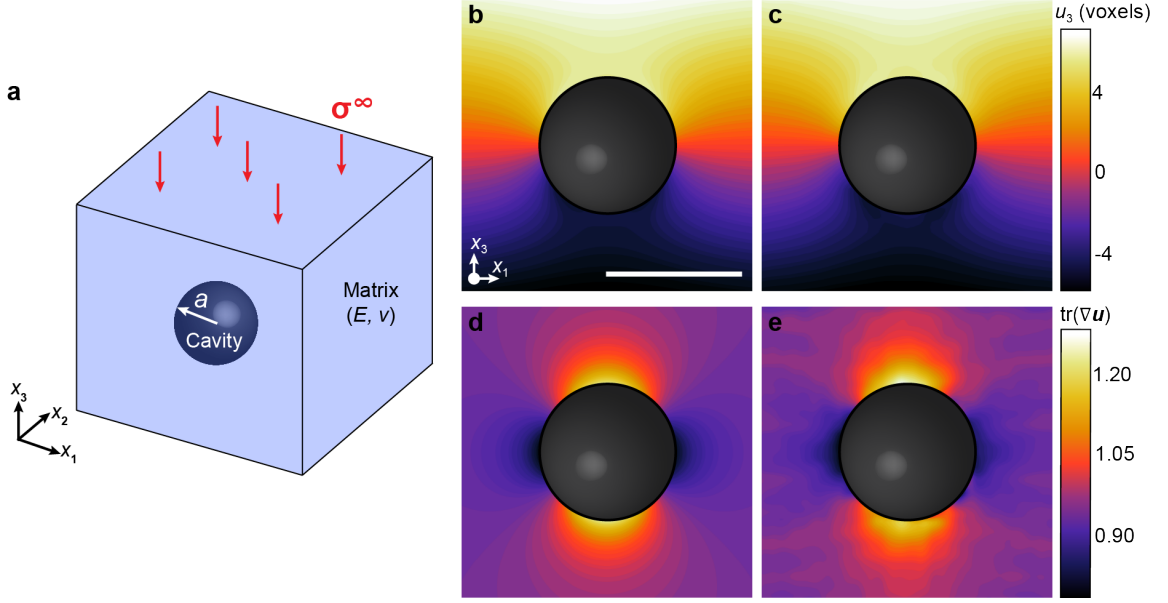
FIDVC was used to measure a ubiquitous non-uniform 3D deformation, i.e. the classical solution of a spherical cavity in an infinite, isotropic, linear-elastic solid with Young's modulus  $E$  and Poisson's ratio  $\nu$  subjected to a remote compressive stress  $\sigma_\infty$  along the  $x_3$  direction. (**Fig. 2.10a**) [68, 69]. The displacement field  $u_i$  is given in index notation as

$$u_i = \frac{(1 + \nu)\sigma_\infty}{2E} \left( \left[ 2 + \frac{5(5 - 4\nu)}{(7 - 5\nu)} \frac{a^3}{R^3} + \frac{6}{(7 - 5\nu)} \frac{a^5}{R^5} \right] x_3 \delta_{i3} + \left[ \frac{-2\nu}{(1 + \nu)} + \frac{(5\nu - 6)}{(7 - 5\nu)} \frac{a^3}{R^3} + \frac{3}{(7 - 5\nu)} \frac{a^5}{R^5} \left( 1 - \frac{5x_3^2}{R^2} \right) \right] x_i \right) \quad (2.3.5)$$

where  $a$  is the cavity radius and  $R$  is the radial coordinate [26], i.e.  $R = \sqrt{x_i x_i}$  [26]. Under stress, the material both deforms non-uniformly around the cavity perimeter and homogeneously due to compression. To perform the numerical experiment, the displacement field given in equation (2.3.5) of an inclusion of radius  $a = 48$  voxels under a remote stress of  $\hat{\sigma}_\infty = \sigma_\infty/E = 0.04$  was applied to a  $256 \times 256 \times 256$  voxel image. The images were processed with FIDVC, and the calculated  $\|\mathbf{u}(\mathbf{x})\|$  and the first invariant of the displacement gradient  $\text{tr}(\nabla \mathbf{u}(\mathbf{x}))$  were compared to the analytical solution (**Fig. 2.10b-e**). For visualization purposes, the spherical cavity is shown in black and the region around the cavity was cropped to  $128 \times 128 \times 128$  voxels for better visualization of the high gradients in proximity to the cavity.

Qualitative comparison of the contour maps of the displacement fields between the FIDVC computed results and the prescribed solution show excellent agreement in both the spatial distribution and magnitude of the color contour levels. When examining the first invariant, there was some deviation between the analytical solution and the FIDVC results due to

slight numerical error associated with FIDVC.



**Figure 2.10:** (a) Schematic of a spherical cavity with radius  $a$  in an infinite, isotropic, linear-elastic solid matrix with Poisson's ratio  $\nu$  and Young's modulus  $E$  subject to a uniaxial compressive stress at infinity  $\sigma^\infty$  [68, 69].  $x_1 - x_3$  slice comparing the (b) prescribed displacement along the loading direction  $x_3$  with the (c) FIDVC results. The corresponding (d) analytical and (e) numerical calculation of the displacement gradient's first invariant,  $\text{tr}(\nabla \mathbf{u}(\mathbf{x}))$ .

### 2.3.9 Computational Efficiency

**Note:** Ronnie Bar-Kochba assisted the GPU implementation of the code.

The computational cost of DVC computations was 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  $m \times m$  points with a square interrogation window side length of  $L$  pixels, and one with a DVC displacement grid of  $m \times m \times m$  points with a cubic interrogation window side length of  $L$  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  $L = 64$  voxels. For DVC applications to be competitive and widely applicable, fast computation speeds without compromising high-accuracy measurements were imperative.

The computational efficiency of FIDVC was investigated and compared against DVC by

**Table 2.2:** Processing times and speed up factors for an image of size  $512 \times 512 \times 256$  with  $65 \times 65 \times 33$  computation points deformed with a four-pole Gaussian displacement field. The images were processed with DVC and FIDVC executed on CPU and GPU. The speed up factor was computed with reference to DVC processed on the CPU.

| Algorithm | Processor | Total<br>Time (min) | Time per<br>Iteration (min) | Speed Up<br>Factor |
|-----------|-----------|---------------------|-----------------------------|--------------------|
| DVC       | CPU       | 35:30               | —                           | —                  |
| DVC       | GPU       | 01:36               | —                           | 22.2×              |
| FIDVC     | CPU       | 12:12               | 1.75                        | 2.90×              |
| FIDVC     | GPU       | 02:08               | 0.31                        | 16.6×              |

processing a series of images using the CPU and GPU. Each image of size  $512 \times 512 \times M$  voxels, where  $M$  was varied from 32 to 256 voxels in increments of 32 voxels, was deformed with a four-pole Gaussian displacement field (**Fig. 2.4**). Due to the refinement of the interrogation window size in FIDVC, the final grid spacing was set to 8 voxels for a window side length of 32 voxels, producing a 75% overlap. To accomplish an equal grid size or number of computation points for the DVC execution, we set an 8 voxel grid spacing, or 87.5% overlap for  $L = 64$  voxels. It is possible to use smaller window sizes that would yield lower computation times; however there is a trade-off between spatial resolution and capturing large displacements [149]

Table 2.2 summarizes the average computation speed up factors for the different combinations of processors and algorithms. As a baseline comparison without GPU acceleration, the FIDVC is  $\approx 3$  times faster than DVC because of the optimized interrogation window and overlap sizes. When executing each algorithm separately on the GPU, substantial speed improvements of over 16 times are found, resulting in 1–2 min processing times. These computational benefits were especially important since the purchasing cost of GPUs is far less than both supercomputing time and high end CPUs. The same GPU processing times can be maintained within a low to medium range personal computer, allowing many end-users to take advantage of FIDVC for their studies.



## 2.4 Conclusion

A new fast iterative digital volume correlation (FIDVC) algorithm capable of capturing large nonlinear deformation fields in 3D is presented. FIDVC utilizes the well-known iterative deformation method in conjunction with a window weighting function allowing it to significantly increase the displacement field spatial resolution while maintaining low computational cost. A finite difference scheme that addresses the typical sampling issues of discrete signal differentiation is presented and validated for computed 3D deformation gradients, useful in strain and stress measurements. This approach has shown to drastically improve strain measurement accuracy by up to 50% in low signal-to-noise displacement fields. Validation of FIDVC was performed using prescribed analytical displacement fields applied to experimentally simulated volumetric images and compared against a previous DVC algorithm [78].

FIDVC was shown to successfully capture displacement information with a subset size resolution as small as 16 voxels, which is an approximate four-fold increase in resolution compared to previous DVC techniques [230, 78]. Implementation of the FIDVC on a GPU offers computational speed up times of up to  $17\times$  when compared to traditional DVC methods run on a CPU. Replacing expensive parallel implementations on high performance computers with GPU implementation on average personal computers will open new doors in allowing DVC to be run by a wide-spread user base similar to DIC.

Having the capability to compute nonlinear deformation fields accurately and at reasonable times will greatly benefit any application that requires non-invasive 3D measurement of material deformations. These measurements are length scale and time independent, as long as volumetric images with sufficient motion tracking patterns are supplied. In the context of cellular biology, these measurements can be applied to understand the physical interaction between cells and their micro-environment. This physical interaction does not necessarily rely on the material properties of the matrix (Chapter 3). However if known, FIDVC deformations can be easily converted to measure cell-induced forces using traction force microscopy (Appendix A,B).

### 3. Quantifying Nonlinear Deformations for Neutrophil Migration in the Absence of Material Properties

**Note:** A version of this chapter is currently being prepared as a manuscript with Jon B. Estrada and David S. Stout as first co-authors. Data has been included in the section with co-authors' consent.

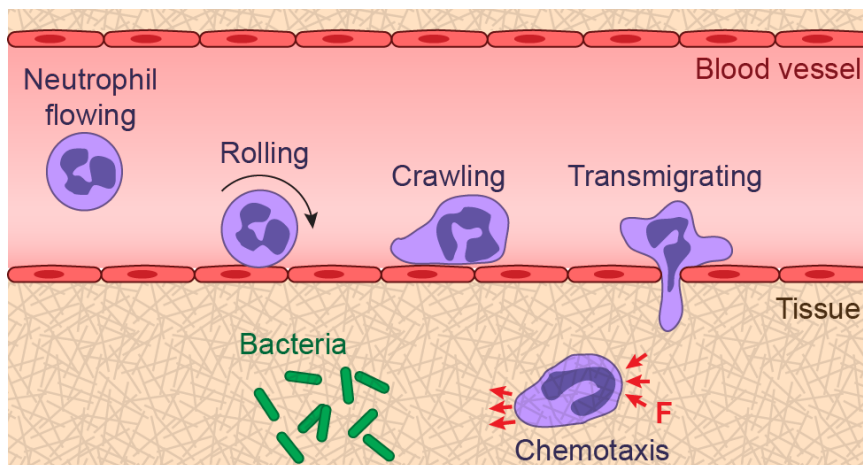
#### 3.1 Introduction

The ability to perform analysis of cellular forces and tractions generated by cells migrating through fibrillar matrices is limited due to the lack of well-characterized material properties at the cellular scale. Additionally, current traction force microscopy (TFM) measurements are limited to purely linear materials [152], which cannot be applied to describe fibrillar matrices that display significant nonlinear deformation behavior [285].

In this chapter, fast iterative digital volume correlation is applied to measure the surface features of cell-induced matrix deformations of migrating human neutrophils in a fibrillar three-dimensional (3D) collagen matrix (acquired from previous experiments). New visualization techniques are presented that assist in interpreting the complex spatial variations of the deformation field. To circumvent the TFM limitations in materials that are not well characterized, a mathematical framework is presented that extracts meaningful kinematic quantities from the surface deformation without the need for the material properties of the matrix.

Polymorphonuclear leukocytes or neutrophils are key players in the innate immune response against invading pathogens [141]. Compared with the acquired immune response, neutrophils can immediately attack microorganisms such as bacteria without past exposure. During inflammation or infection, Neutrophils, which are normally found in the blood stream, become activated by signaling molecules such as chemokines and transmigrate through the endothelium to enter the affected tissue (**Fig. 3.1**) [134]. Interstitial migration through tissue

relies on the exchange of physical forces between the cell and the 3D extracellular matrix [257, 195, 121]. These cell induced forces are essential for proper defense as dysregulation can impede migration and ultimately lead to tissue or organ failure.



**Figure 3.1:** Illustration showing mechanisms of neutrophil migration towards tissue infected by bacteria. Migration through complex tissue depends on the transmission of force  $F$  (red) between the cell and the three dimensional extracellular matrix

## 3.2 Methods

**Note 1:** Experimental procedures were performed by David S. Stout and detailed in Stout, D. S. Analysis of Naïve vs Lipopolysaccharide (LPS) Activated Neutrophil Chemotaxis in 3D Collagen Matrices Using Traction Force Microscopy. PhD dissertation, Brown University, 2014

**Note 2:** Jon B. Estrada assisted in developing and implementing the analysis methods described.

### 3.2.1 Measurement of Cell Induced 3D Displacements

Cell-induced full-field displacements were measured using the fast iterative digital volume correlation algorithm was detailed in Chapter 2 [15]. In this analysis, the final interrogation window spacing, which sets the displacement spatial grid resolution was eight voxels or  $1.7 \mu\text{m}$  for measurements in  $x_1 - x_2$  and  $2.4 \mu\text{m}$  for measurements in  $x_3$ .

### 3.2.2 Surface Displacements Features

#### 3.2.2.1 Estimation of Discretized Cell Surface

The 3D cellular surface boundary was determined from fluorescently labeled cell membrane volumetric images. A median filter with a  $5 \times 5 \times 5$  voxel window was applied to the raw volumetric image to remove noise. Following a contrast adjustment where 1% of intensity values get saturated, a binary image was generated by setting all intensity values less than 50% of the maximum intensity of the image to 0 (black) and all others to 1 (white). The final binary mask of the cell was generated by removing all but the largest connected component. The triangular surface mesh of the binary mask was computed using the marching cubes algorithm [162] and then smoothed using the algorithm in [273, 272].

#### 3.2.2.2 Interpolation of Cell Induced 3D Displacements onto the Cell Surface

Matrix displacements are mapped onto the triangulation of the cell surface  $\partial V$  via a trilinear surface element interpolation scheme (**Fig. 3.2**). Each triangle has an associated surface normal  $\mathbf{n}$ , which is used to decouple the surface displacements into a surface normal  $\mathbf{u}_\perp$  and surface tangential  $\mathbf{u}_\parallel$  components using

$$\mathbf{u}_\perp = (\mathbf{u} \cdot \mathbf{n})\mathbf{n} \quad (3.2.1)$$

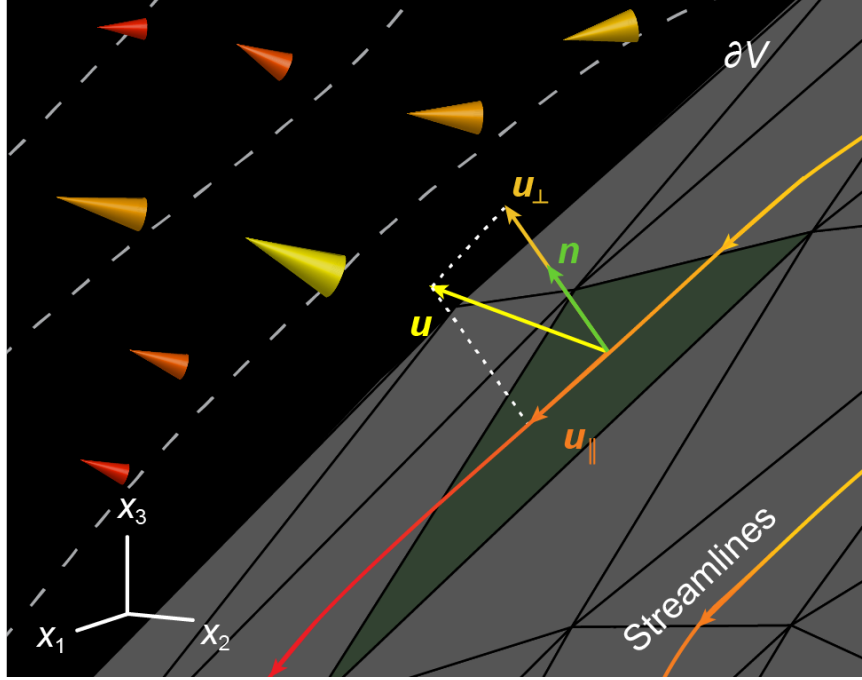
$$\mathbf{u}_\parallel = \mathbf{u} - \mathbf{u}_\perp \quad (3.2.2)$$

Streamlines are computed and plotted using Tecplot 360 2013R1 (Bellevue, WA). Streamlines show the manner in which  $\mathbf{u}_\parallel$  varies over  $\partial V$  and are generally computed using numerical path integration along parametric paths  $\mathbf{x}_S(s)$ , originating from a set of seed point positions at time  $t$ . The parametric equation for a streamline is

$$\frac{d\mathbf{x}_S(s)}{ds} = \mathbf{u}_\parallel(\mathbf{x}_S(s), t) \quad (3.2.3)$$

where  $s$  is the arc-length parameter of the line. In this study, the streamlines are mathematically equivalent to streamlines computed for fluid flow velocities constrained to a surface.

The spatial gradient of the local surface displacement  $\nabla \mathbf{u}$  is calculated numerically via



**Figure 3.2:** Schematic outlining the interpolation of the 3D displacement field onto  $\partial V$  to produce the surface displacements  $\mathbf{u}$ . The (green) surface normal  $\mathbf{n}$  is used to calculate the corresponding normal  $\mathbf{u}_\perp$  and tangential  $\mathbf{u}_\parallel$  component of  $\mathbf{u}$  with respect to  $\partial V$ . Streamlines are calculated via path integration of  $\mathbf{u}_\parallel$  along  $\partial V$ .

convolution with a Optimal-9 differentiation kernel [72, 15].

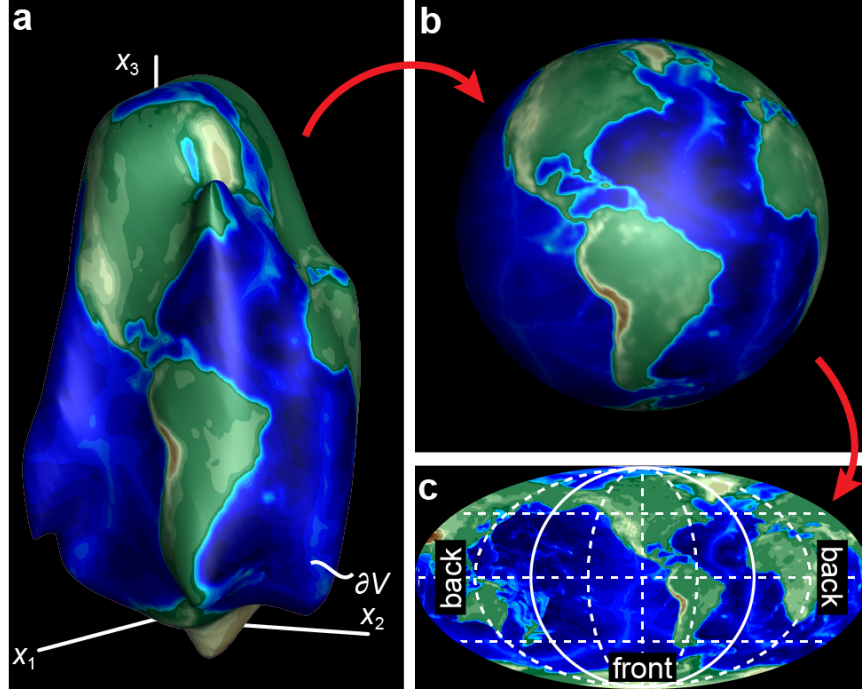
### 3.2.2.3 Mapping Surface Displacements onto a 2D Plane

To visualize and interpret data constrained to a closed surface more effectively, the surface displacement  $\mathbf{u}(\mathbf{x})$  is decoupled into normal  $\mathbf{u}_\perp$  and tangential  $\mathbf{u}_\parallel$  and mapped to a 2D ellipse using the Mollweide projection method (**Fig. 3.3**). The Mollweide projection, used commonly in cartography, is a homolographic (iso-area) technique for mapping a spherical surface to an ellipse. Mapping was performed by first converting the position vectors from the centroid of each cell to spherical coordinates  $(r, \theta, \phi)$  and then transforming the  $(\theta, \phi)$  values to normalized map coordinates  $x^M$  and  $y^M$ ) using

$$x^M = \sqrt{2} \sin \beta, \quad (3.2.4)$$

$$y^M = \frac{2\sqrt{2}}{\pi} \theta \cos \beta, \quad (3.2.5)$$

$$2\beta + \sin(2\beta) = \pi \sin \phi. \quad (3.2.6)$$

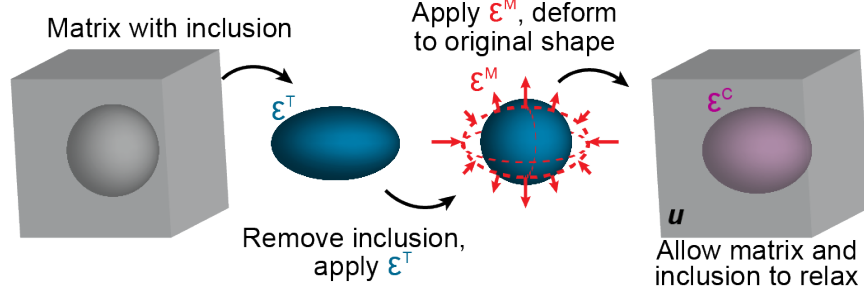


**Figure 3.3:** Illustration of the Mollweide transform for Earth’s elevation data. (a) Elevation data transformed onto 3D neutrophil surface and converted back to (b) spherical coordinates. (c) Mollweide mapping of elevation data in b to allow for user-friendly visualization of all 3D data onto a plane. Dashed grid lines represent spacing of  $45^\circ$  along latitude and longitude lines. Data interior to the solid circle (white) is located on the front hemisphere ( $x_1 >$  cell centroid on  $\partial V$ ) while data outside lies on the back hemisphere ( $x_1 <$  cell centroid on  $\partial V$ ) of the cell.

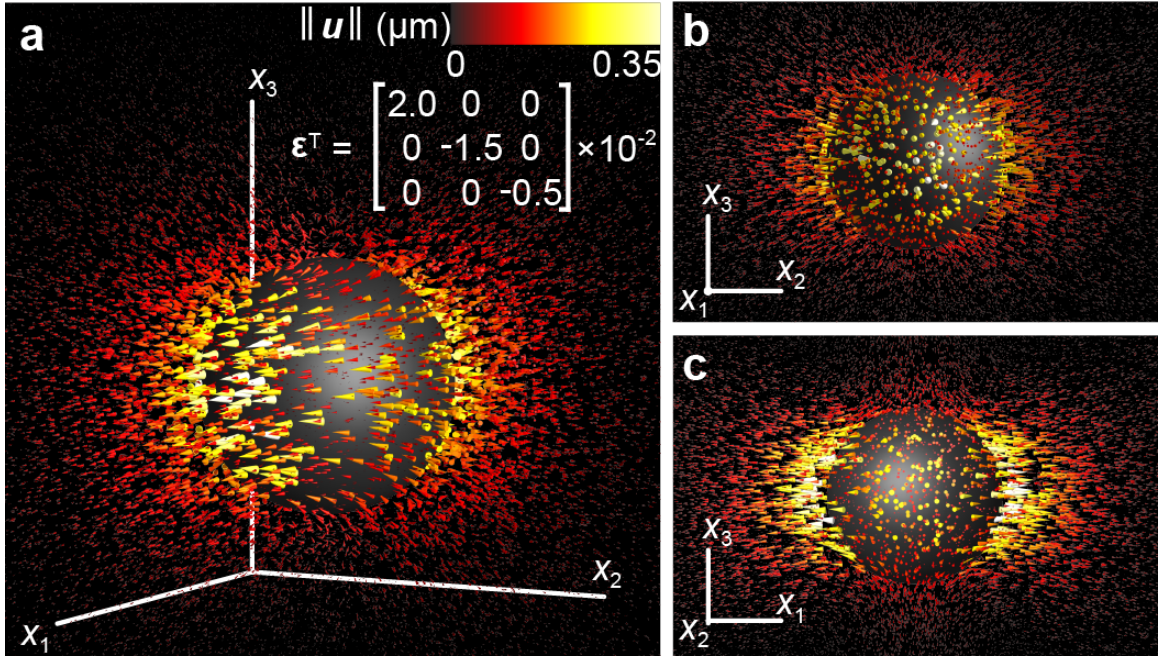
Corresponding surface and mapping locations are computed using the ‘mfwdtran’ function in the MATLAB Mapping Toolbox and interpolated onto a rectangular grid using the ‘scatteredInterpolant’ function. Surface streamlines are mapped similarly from 3D to 2D using the Mollweide projection.

#### 3.2.2.4 Application to the Eshelby Inclusion Solution

An analytical comparison to cells embedded in a 3D matrix exists in the form of the Eshelby inclusion problem [68, 69]. The Eshelby problem considers an infinite, isotropic, linear-elastic solid matrix with Poisson’s ratio  $\nu$  and Young’s Modulus  $E$  (**Fig. 3.4**). The solid is initially stress-free, with all displacement and strain initially zero. A spherical inclusion in the matrix undergoes a transformation strain (or eigenstrain)  $\epsilon^T$ , which is resisted by the matrix. Due to the inclusion embedded in the solid, strain  $\epsilon^M$  and displacement  $\mathbf{u}$  fields are induced in the matrix. The final strain field in the inclusion is given by the sum of the



**Figure 3.4:** Schematic illustrating the Eshelby inclusion problem [68, 69, 26], where an initially-spherical inclusion (gray) in an infinite matrix undergoes a uniform transformation eigenstrain  $\epsilon_T$ , and produces a known analytical displacement field  $\mathbf{u}$  and confinement strain ( $\epsilon_C$ ) external to its boundary.

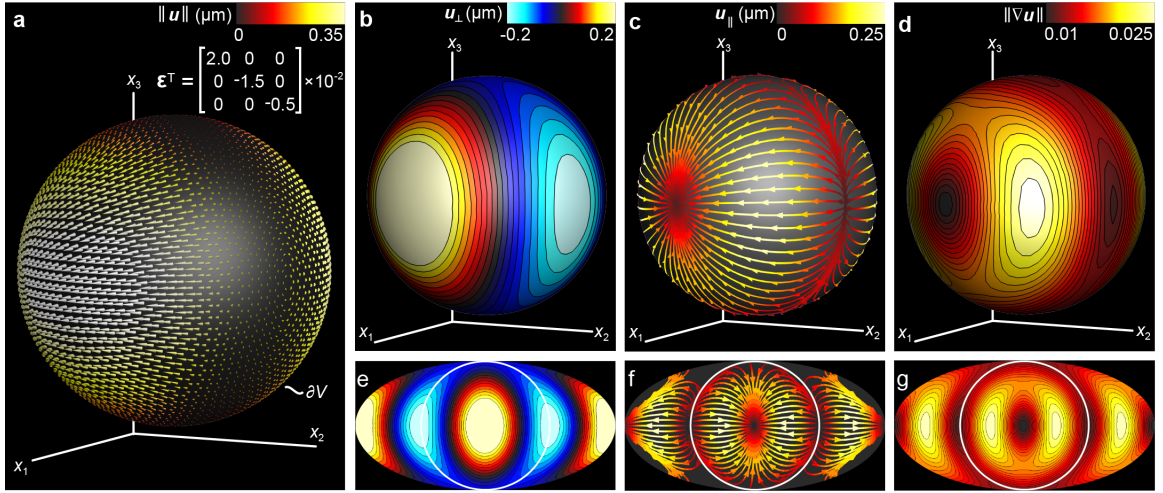


**Figure 3.5:** Calculation of full-field matrix displacements using the closed-form solution for an inclusion undergoing an eigenstrain. (a) Cone plot of the solution of the matrix displacements calculated analytically from the inclusion eigenstrain  $\epsilon_T$ . For visualization purposes, the displacement field is interpolated onto random locations as cones color coded by magnitude  $\|\mathbf{u}\|$ . (b)  $x_2 - x_3$  and (c)  $x_1 - x_3$  plane views of the solution in (a).

transformation and matrix strain  $\epsilon^C$ . The strain field  $\epsilon^C$  inside and on the surface of the inclusion is constant, while the displacement is found by taking the dot product of  $\epsilon^C$  with the position vector  $\mathbf{x}$ . The displacement field outside the inclusion  $\mathbf{u}(\mathbf{x})$  has an analytical solution given by Eshelby [68, 69, 26] and is a function only of inclusion radius (32 voxels),



transformation strain  $\boldsymbol{\varepsilon}^T$ , spatial position  $\mathbf{x}$ , and Poisson's ratio ( $\nu = 0.2$ ) (**Fig. 3.5**). Matrix displacements are interpolated onto the surface of the spherical inclusion and decoupled into surface normal  $\mathbf{u}_\perp$  and surface parallel  $\mathbf{u}_\parallel$  components using equations (3.2.2) and (3.2.2) (**Fig. 3.6**). Displacement gradient magnitude  $\|\mathbf{u}\|$  is used to check validity of small-strain, linear-elastic theory, which should be  $\ll 1$ .



**Figure 3.6:** Calculation of surface displacements using the analytical solution of Eshelby outside an inclusion undergoing an eigenstrain ( $\boldsymbol{\varepsilon}_T$ ). (a) Cone plot of the displacement field interpolated onto the surface ( $\partial V$ , gray) of the inclusion, color coded by magnitude ( $\|\mathbf{u}\|$ ). (b) Contour plot of the normal component of  $\mathbf{u}$ ,  $\mathbf{u}_\perp$ , with respect to  $\partial V$ , color coded by direction and magnitude (red is outward and blue is inward normal surface displacement). (d) Streamline plot of the tangential component of  $\mathbf{u}$  ( $\mathbf{u}_\parallel$ ) along  $\partial V$ , color coded by magnitude and arrow heads are pointed along the vector field. (d) Contour plot of the displacement gradient norm  $\|\nabla\mathbf{u}\|$  on  $\partial V$ , color coded by magnitude. (e – g) Mollweide projection of corresponding surface data in b – d onto a 2D plane. Data interior to the solid circle (white) is located on the front hemisphere ( $x_1 >$  inclusion centroid on  $\partial V$ ) while data outside lies on the back hemisphere ( $x_1 <$  inclusion centroid on  $\partial V$ ) of the inclusion.

### 3.2.3 Mean Deformation Metrics

#### 3.2.3.1 Calculating Mean Deformation Metrics

In continuum mechanics, shape changes in solids (and in fluids) are described by a displacement function,

$$\mathbf{X} = \mathbf{x} + \mathbf{u}(\mathbf{x}, t) \quad (3.2.7)$$

that maps a point at position  $\mathbf{x}$  in the reference configuration to a new position  $\mathbf{X}$  in the deformed configuration at time  $t$ . The deformation gradient  $\mathbf{F}$ , the quantity that describes



the shape change of infinitesimal line elements, is given by the spatial vector gradient or Jacobian of  $\mathbf{y}$ , i.e.

$$\mathbf{F} = \nabla \mathbf{X} = \mathbf{I} + \nabla \mathbf{u}(\mathbf{x}), \quad (3.2.8)$$

where  $\mathbf{I}$  is the identity matrix and  $\nabla$  is the spatial vector gradient operator of  $\mathbf{x}$ .

The overall or average shape change of the solid is given by the mean displacement gradient tensor  $\langle \nabla \mathbf{u} \rangle$  [104] in the reference volume  $V_0$  as

$$\langle \nabla \mathbf{u} \rangle = \frac{1}{V_0} \int_V \nabla \mathbf{u} dV \quad (3.2.9)$$

where the volume  $V_0 = \int_V dV$ . Since the full-field displacements are not known within  $V$ , equation (3.2.9) must be derived using information only known at the cell boundary,  $\partial V$ .

Let  $\mathbf{a} \in \mathbb{R}^3$  be an arbitrary constant vector. The tensor  $\langle \nabla \mathbf{u} \rangle$  maps  $\mathbf{a}$  to a vector by

$$\begin{aligned} \langle \nabla \mathbf{u} \rangle \cdot \mathbf{a} &= \left( \frac{1}{V_0} \int_V \nabla \mathbf{u} dV \right) \cdot \mathbf{a} \\ &= \frac{1}{V_0} \int_V \nabla \mathbf{u} \cdot \mathbf{a} dV. \end{aligned} \quad (3.2.10)$$

In the second step,  $\mathbf{a}$  is factored into the integral since it is constant. Using the identity,

$$\nabla \mathbf{u} \cdot \mathbf{a} = \nabla \cdot (\mathbf{u} \otimes \mathbf{a}) - \mathbf{u} \otimes (\nabla \cdot \mathbf{a}), \quad (3.2.11)$$

equation (3.2.11) becomes

$$\langle \nabla \mathbf{u} \rangle \cdot \mathbf{a} = \frac{1}{V_0} \int_V \nabla \cdot (\mathbf{u} \otimes \mathbf{a}) dV. \quad (3.2.12)$$

Upon applying the divergence theorem for tensors, equation (3.2.12) is given by

$$\begin{aligned} \langle \nabla \mathbf{u} \rangle \cdot \mathbf{a} &= \frac{1}{V_0} \int_{\partial V} \mathbf{u} (\mathbf{a} \cdot \mathbf{n}) dA \\ &= \left( \frac{1}{V_0} \int_{\partial V} (\mathbf{u} \otimes \mathbf{n}) dA \right) \cdot \mathbf{a} \end{aligned} \quad (3.2.13)$$

where  $\mathbf{n}$  are the unit vectors normal to  $\partial V$ . In the second step,  $\mathbf{a}$  is factored out of the

integral since it is constant. Since equation (3.2.13) holds for any arbitrary vector  $\mathbf{a}$ ,

$$\langle \nabla \mathbf{u} \rangle = \frac{1}{V_0} \int_{\partial V} (\mathbf{u} \otimes \mathbf{n}) dA. \quad (3.2.14)$$

The symmetric and skew-symmetric parts of  $\langle \nabla \mathbf{u} \rangle$  physically represent the mean expansion or contraction, and rotation of  $V$  respectively, which are defined by

$$\langle \nabla \mathbf{u} \rangle_{\leftrightarrow} = \frac{\|\text{sym } \langle \nabla \mathbf{u} \rangle\|}{\|\langle \nabla \mathbf{u} \rangle\|} = \frac{\|\langle \nabla \mathbf{u} \rangle + \langle \nabla \mathbf{u} \rangle^T\|}{2\|\langle \nabla \mathbf{u} \rangle\|} \quad (3.2.15)$$

and

$$\langle \nabla \mathbf{u} \rangle_{\circ} = \frac{\|\text{skew } \langle \nabla \mathbf{u} \rangle\|}{\|\langle \nabla \mathbf{u} \rangle\|} = \frac{\|\langle \nabla \mathbf{u} \rangle - \langle \nabla \mathbf{u} \rangle^T\|}{2\|\langle \nabla \mathbf{u} \rangle\|}. \quad (3.2.16)$$

Equation (3.2.14) utilizes the available displacement data directly without invoking a spatial derivative or inverse formulation, and does not require any displacement information within the cell.

The results in equation (3.2.14) can be used to compute the mean deformation gradient tensor,  $\langle \nabla \mathbf{u} \rangle$ , expressed mathematically as,

$$\begin{aligned} \langle \mathbf{F} \rangle &= \frac{1}{V_0} \int_V (\mathbf{I} + \nabla \mathbf{u}) dV \\ &= \mathbf{I} \left( \frac{1}{V_0} \int_V dV \right) + \frac{1}{V_0} \int_V \nabla \mathbf{u} dV \\ \langle \mathbf{F} \rangle &= \mathbf{I} + \langle \nabla \mathbf{u} \rangle. \end{aligned} \quad (3.2.17)$$

Other mean deformation metrics can be computed from the results in equation (3.2.17), e.g. the mean volume ratio is given by

$$\langle \mathbf{J} \rangle = \det \langle \mathbf{F} \rangle \quad (3.2.18)$$

The mean Lagrangian strain tensor associated with  $\langle \nabla \mathbf{u} \rangle$ , is given by

$$\langle \mathbf{E} \rangle = \frac{1}{2} \left( \langle \mathbf{F} \rangle^T \langle \mathbf{F} \rangle - \mathbf{I} \right) \quad (3.2.19)$$

with eigenvalues (principal strains)  $\langle E_i \rangle$ . The mean deformation gradient,  $\langle \mathbf{F} \rangle$ , can be uniquely decomposed into its mean right stretch  $\langle \mathbf{U} \rangle$  tensor

$$\langle \mathbf{U} \rangle = \left( \langle \mathbf{F} \rangle^T \langle \mathbf{F} \rangle \right)^{1/2} \quad (3.2.20)$$

and rotation  $\langle \mathbf{R} \rangle$  tensor

$$\langle \mathbf{R} \rangle = \langle \mathbf{F} \rangle \langle \mathbf{U} \rangle^{-1}. \quad (3.2.21)$$

The mean rotation angle between time steps  $\langle \Delta\theta \rangle$  is given by

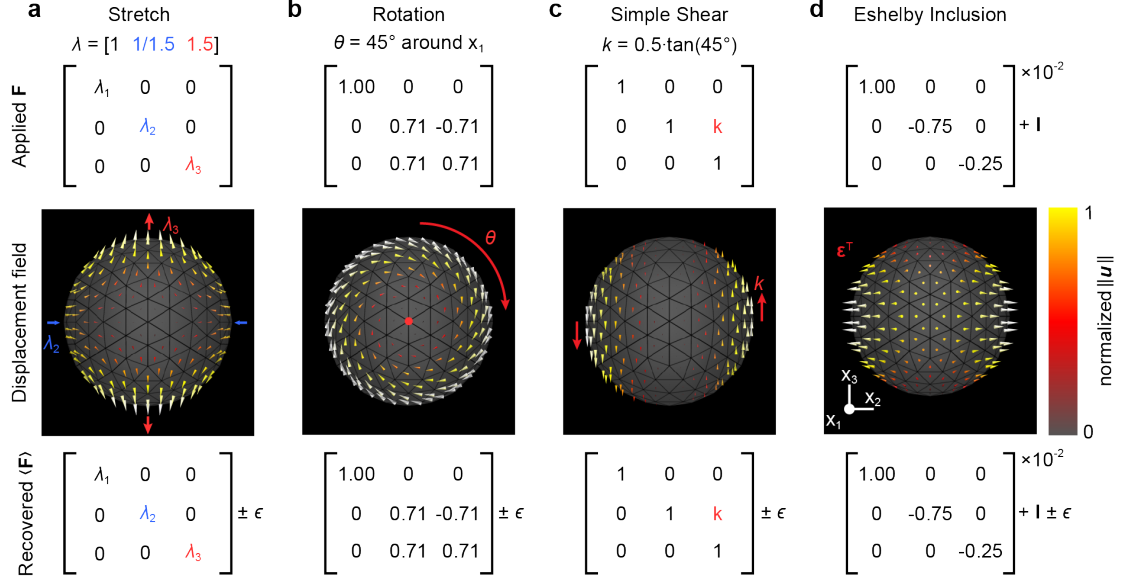
$$\langle \Delta\theta \rangle = \arccos \left( \frac{1}{2} (\text{trace } \langle \mathbf{R} \rangle - 1) \right) \quad (3.2.22)$$

with cumulative rotation  $\langle \Theta \rangle$  over total time  $t$  as

$$\langle \Theta \rangle = \int_t |\langle \Delta\theta \rangle|(\tau) d\tau. \quad (3.2.23)$$

### 3.2.3.2 Validation of Mean Deformation Metrics

Validation of the mean deformation metrics was performed using four classical mechanical examples with analytical forms; **(a)** simple stretch, **(b)** axial rotation, **(c)** simple shear, and **(d)** the Eshelby inclusion solution (**Fig. 3.7**) [68, 69]. For each case, a deformation gradient tensor  $\mathbf{F}$  was specified to apply a known 3D displacement field. The mean deformation gradient tensor was calculated from the displacement field and surface normals using equations (3.2.14) and (3.2.17). The test surface for the deformation was a sphere, meshed using recursive subdivision starting from an icosahedron. For cases **a** – **c**, the number of subdivisions was 2 resulting in 320 discrete triangles, and for the last case, **d**, the number of subdivisions was 4, resulting in 5120 discrete surface triangles. Error from the analytical solution was on the order of  $10^{-12}$  for cases **a** – **c** due to numerical error, and on the order of  $10^{-5}$  for the Eshelby inclusion solution, predominantly due to the assumption of small-strain conditions.



**Figure 3.7:** Validation of the mean deformation metrics on a sphere (gray) undergoing (a) stretch with three stretch ratios ( $\lambda_1, \lambda_2, \lambda_3$ ), (b) rotation with angle  $\theta$  around axis  $x_1$ , (c) simple shear with magnitude  $k$ , and (d) a transformation strain  $\epsilon_T$ . (Top row) Applied deformation gradient  $\langle \mathbf{F} \rangle$ . (Middle row) Cone plot of the displacement fields interpolated onto the surface of the sphere for a-d, color coded by normalized magnitude  $\|\mathbf{u}\|$ . (Bottom row) Results for the recovered mean deformation gradient  $\langle \mathbf{F} \rangle$  for a-d with error  $\epsilon$  less than  $10^{-12}$  for a – c due to numerical error, and less than  $10^{-5}$  due to small strain assumption for d.

### 3.2.4 Statistical Analysis

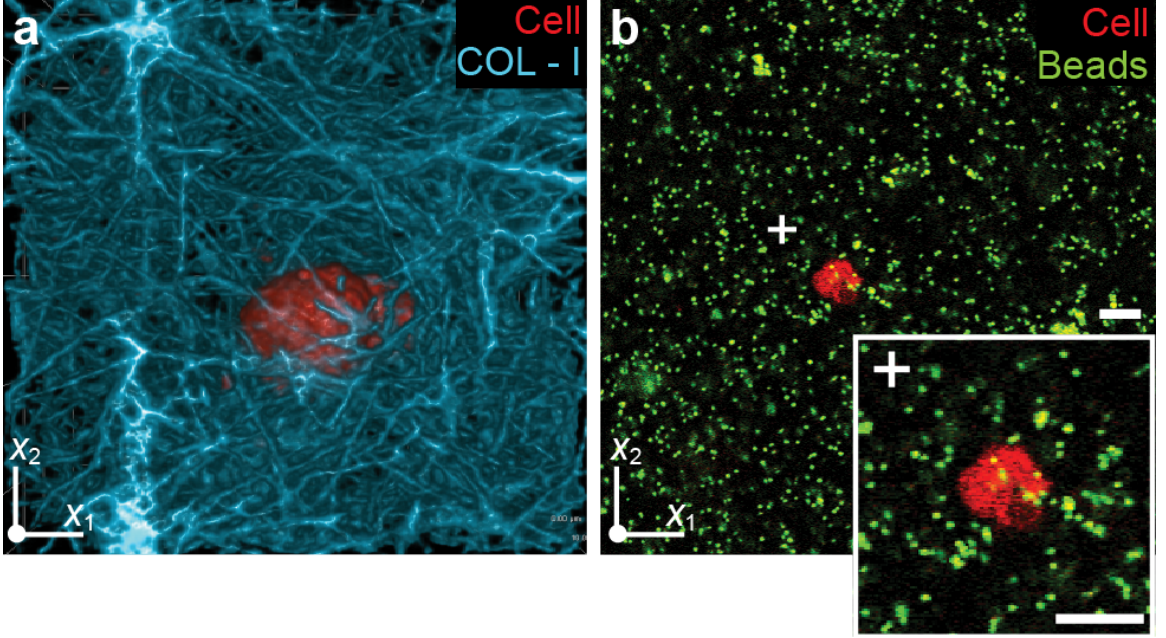
Data was subjected to the Shapiro-Wilk test ( $p > 0.05$ ), to test whether the data is normally distributed. In all cases data was non-normal, and groups were subjected to a nonparametric test, Mann-Whitney U, to compare the rank. A  $p < 0.05$  was considered to be significant.

## 3.3 Results and Discussion

The fast iterative digital volume correlation algorithm was applied to resolving the spatiotemporal surface features of cell-induced matrix deformations of a small, migrating human neutrophil ( $< 10 \mu\text{m}$ ) in a fibrillar 3D collagen matrix. Specifically, primary human neutrophils were encapsulated in a reconstituted type I collagen gel of final concentration of 2.2 mg/mL resulting in the fibrillar microstructure (**Fig. 3.8a**).

The collagen fibers were visualized in blue via confocal reflectance microscopy and the neutrophil was visualized via the membrane stain DiI in red. Simultaneous injection of

0.5  $\mu\text{m}$  green fluorescent spherical particles (**Fig. 3.8b**, right panel) during the collagen polymerization process serves as means for tracking the 3D matrix deformations imparted by the locomoting cell.

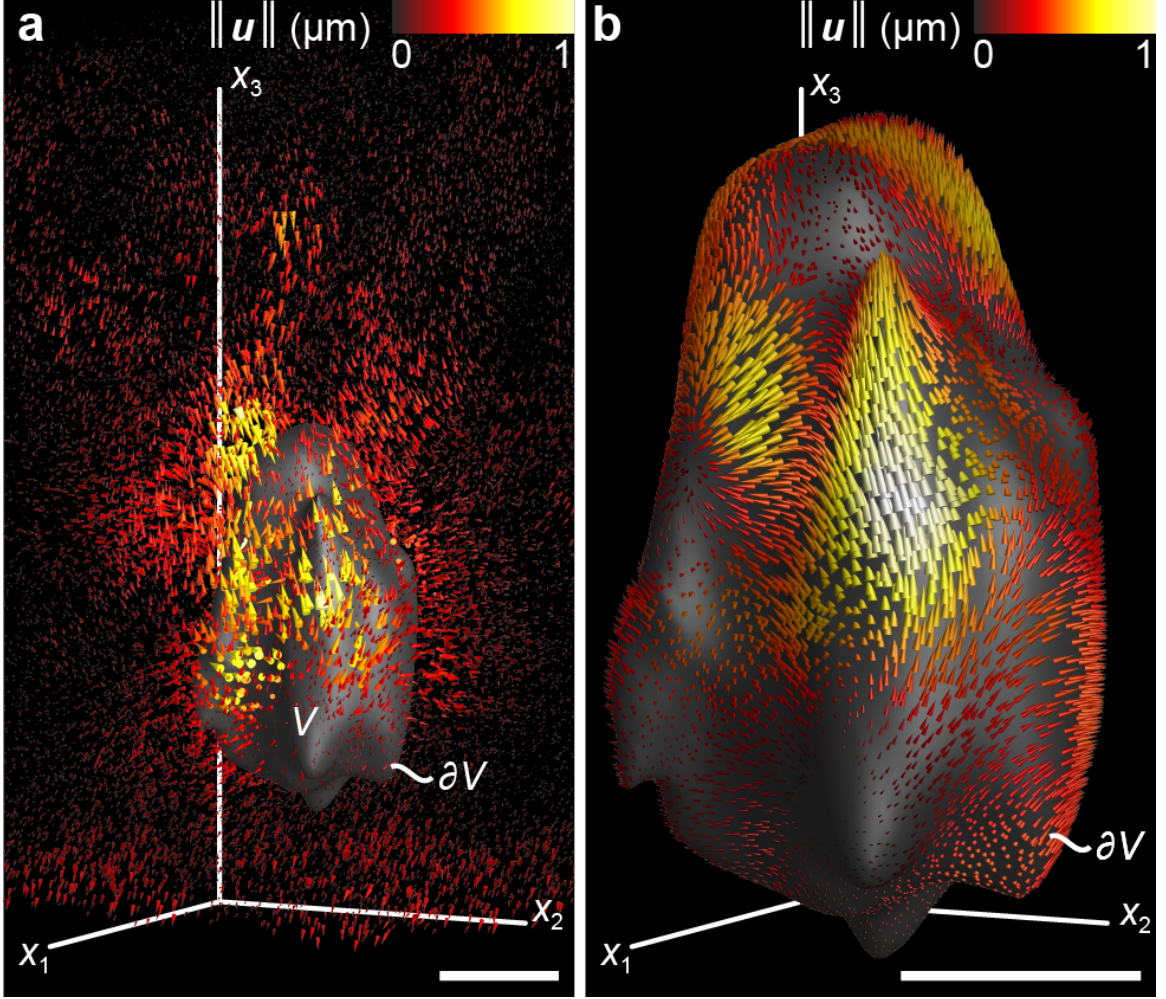


**Figure 3.8:** (a) Volume rendering of a DiI-stained neutrophil (red) embedded in a fibrillar type I collagen matrix (cyan) imaged with reflectance confocal microscopy. (b) Confocal microscope image of a neutrophil (red) migrating in a corresponding 3D collagen matrix containing randomly distributed 0.5  $\mu\text{m}$  fluorescent microspheres (green) used to measure cell-induced displacements. Scale bars, 10  $\mu\text{m}$ . Experiments were performed by Stout D. S.

### 3.3.1 Surface Deformation Features

Full-field vector maps of the displacement fields during neutrophil migration through collagen show complex distributions (**Fig. 3.9a**). Areas of large displacement (yellow) occur in close proximity to the cell surface  $\partial V$  (gray) and quickly decay in the far-field. At the interface between the cell and the matrix, the displacement features show significant non-linearities that may be difficult to interpret (**Fig. 3.9b**) without further processing. Additionally, many of the non-linearities are in the finite deformation regime, i.e.  $\nabla \mathbf{u} \gg 0$  (**Fig. 3.10a-c**), therefore validating the use of FIDVC.

To assist in interpreting the surface localization of the displacement field,  $\mathbf{u}$  was decoupled into the surface normal  $\mathbf{u}_\perp$  and tangential  $\mathbf{u}_\parallel$  components (**Fig. 3.11a,b**). Tangential

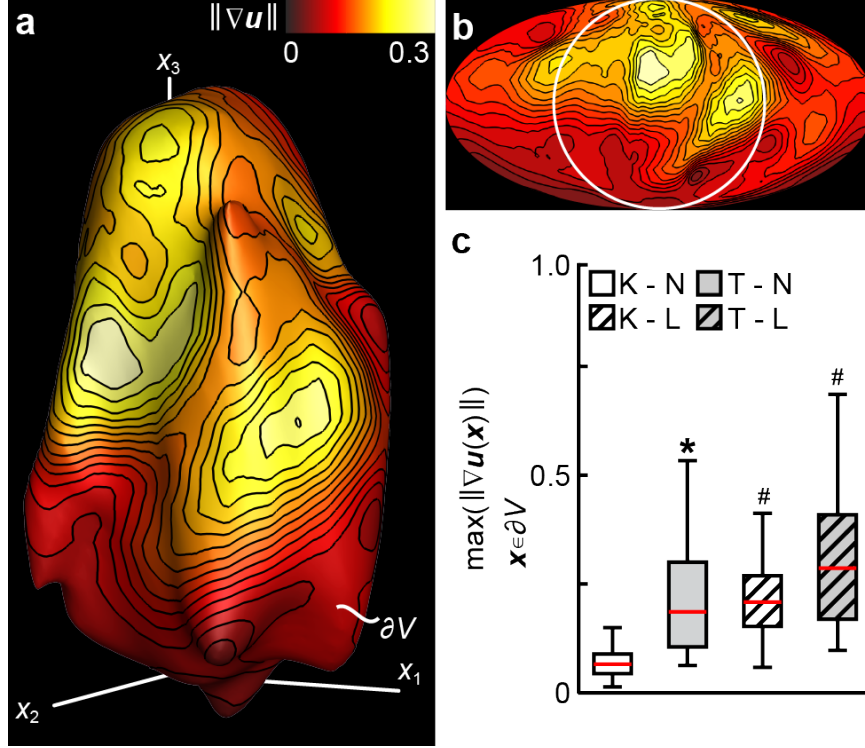


**Figure 3.9:** Cell-induced hydrogel deformations show significant nonlinearities. (a) Cone plot of cell-generated 3D displacement field measured by fast iterative digital volume correlation (FIDVC) surrounding a migrating cell (gray) with volume  $V$  and boundary  $\partial V$ . Displacement grid resolution is set to 8 voxels ( $1.7 \mu\text{m}$  for  $x_1$ ,  $x_2$  and  $2.4 \mu\text{m}$  for  $x_3$ ). Scale bar,  $5 \mu\text{m}$ . (b) Vector cone plot of the 3D displacement field interpolated onto the surface  $\partial V$  (gray) of the neutrophil, color coded by  $\|u\|$ .

displacements are represented with streamlines to assist in visualizing deformation patterns. The spherical inclusion problem by Eshelby [68, 69] was used as quantitative validation of the full-field displacement interpolation procedure, matching the analytical solution to within  $10^{-3}\%$  error (Figs. 3.5, 3.6).

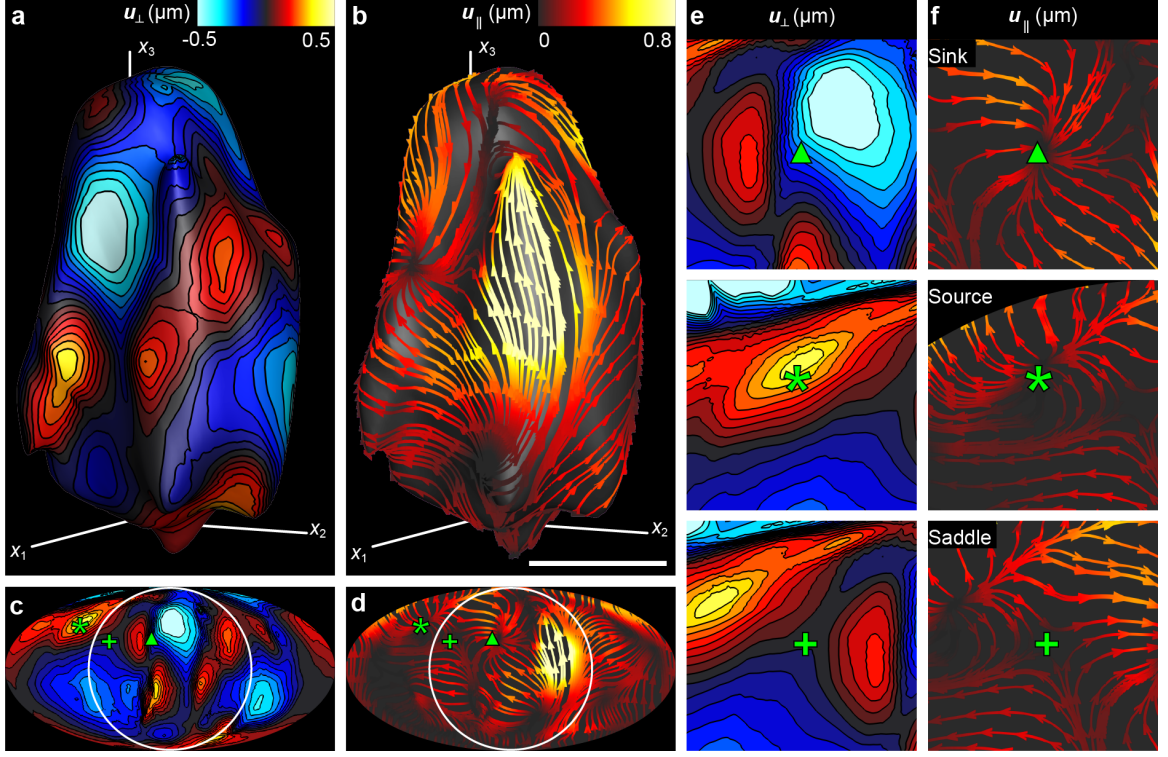
The surface displacement field (Fig. 3.11a) varies spatially across the  $\partial V$  and is viewpoint-dependent, complicating feature analysis for the viewer. To address this issue, the homographic Mollweide mapping technique (Fig. 3.3) is used to cast 3D surface data onto





**Figure 3.10:** Measurement of large surface displacement gradients exerted by a chemotactic neutrophil on the collagen matrix. (a) Contour plot of the displacement gradient norm  $\|\nabla \mathbf{u}\|$  on the cell surface  $\partial V$  (gray), color coded by magnitude. (b) Mollweide projection of corresponding surface data in a onto a 2D plane. Data interior to the solid circle (white) is located on the front hemisphere ( $x_1 >$  cell centroid on  $\partial V$ ) while data outside lies on the back hemisphere ( $x_1 <$  cell centroid on  $\partial V$ ) of the cell. (c) Box plot of the maximum value of  $\|\nabla \mathbf{u}\|$  on  $\partial V$  for all time points and tested condition, K - N (solid white,  $n = 30$ ), T - N (solid gray,  $n = 48$ ), K - L (hatched white,  $n = 44$ ), and T - L (gray hatched gray,  $n = 57$ ). Red lines: medians, top and bottom boxes: upper and lower quartiles, whiskers: maximum and minimum values.

a viewpoint-independent 2D contour plot. The Mollweide mapping is used on  $\mathbf{u}_\perp$  and  $\mathbf{u}_\parallel$  (Fig. 3.11c,d) on  $\partial V$  to visualize the complex local out-of-plane and in-plane deformations in convenient 2D contour maps (Fig. 3.11f,g). Specific regions along the cell surface (Fig. 3.11f,g) reveal previously undocumented deformation patterns reminiscent of sink, source, and saddle point flow structures (Fig. 3.11h,i) commonly found in moving fluids. These deformation structures can be used by modelers to intracellular dynamics.



**Figure 3.11:** Measurement of 3D surface displacements exerted by a chemotactic neutrophil and its associated homographic 2D projections. (a) Contour plot of the normal component of  $\mathbf{u}$ ,  $u_{\perp}$ , with respect to  $\partial V$ , color-coded by direction and magnitude (red is outward and blue is inward normal surface displacement). (b) Streamline plot of the tangential component of  $\mathbf{u}$ ,  $u_{\parallel}$ , along  $\partial V$ , color-coded by magnitude with arrowheads pointing along the vector field. Scale bar,  $5 \mu\text{m}$ . (c - d) Mapped contour plot of  $u_{\perp}$  in a and streamline plot of  $u_{\parallel}$  in b using the Mollweide projection. (e - f) Magnified view of c and d highlighting sink-like (top, green triangle), source-like (middle, green star), and saddle-like (bottom, green plus) features of  $u_{\parallel}$  and  $u_{\perp}$ .

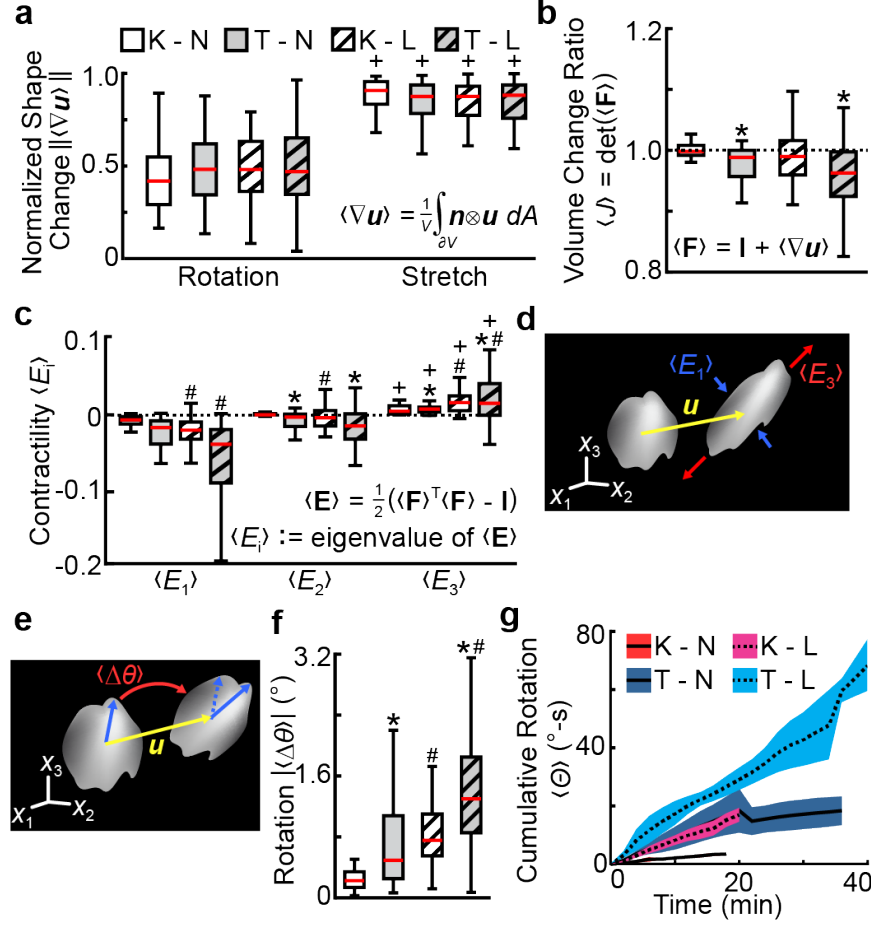
### 3.3.2 Mean Deformation Metrics

While applying FIDVC can provide a detailed map of matrix deformation along the cell surface, for many practical applications a simpler metric for calculating overall shape change of the cell. Traditional TFM studies include root-mean-squared tractions, total forces, strain energies, and contractility measures such as the trace of the dipole tensor [152, 37, 161, 271]. To provide similarly useful kinematic metrics of deformation in the absence of material properties, the mean displacement gradient tensor [104]  $\langle \nabla \mathbf{u} \rangle$  is presented (3.2.14). Calculating  $\langle \nabla \mathbf{u} \rangle$  directly from the measured displacement field additionally circumvents the need to solve inverse formulations, which assume a linear-elastic framework, or spatial



derivatives, which introduce numerical error. Quantitative validation of  $\langle \nabla \mathbf{u} \rangle$  shows that the error between the analytically prescribed and numerically recovered deformation fields is  $10^{-10}$  for homogeneous deformations, i.e. stretch, shear, and rotation, and  $10^{-3}$  for the non-linear Eshelby inclusion solution (**Fig. 3.7**).

$\langle \nabla \mathbf{u} \rangle$  is decoupled into its symmetric and skew-symmetric parts to physically describe the mean expansion or contraction of the cell, and mean rotation respectively. These quantities are given in equation (3.2.15) and (3.2.16) and are plotted for several naïve (N) and lipopolysaccharide (LPS)-treated (L) neutrophils undergoing either chemokinesis (K) or chemotaxis (T). Most of the applied matrix deformations are along the principal cell axes resulting in either a direct push or pull but to a lesser extent in a shearing or rotational motion (**Fig. 3.12a**). The mean principal strains, which give further insight into the cellular expansion/contraction behavior reveal that neutrophils contract isochorically (**Fig. 3.12b**) along two of their principal axes  $\langle E_1 \rangle$ ,  $\langle E_2 \rangle$  while extending along the third axis  $\langle E_3 \rangle$  (**Fig. 3.12c**). Such behavior is indicative of a contractile, integrin-dependent mode of motility as opposed to a pushing, or integrin-independent mechanism [223]. This is consistent with recent findings showing that integrins continue to play a significant regulatory role for traction generation and motility under confinement in the presence of ligands [277]. Using this mean strain metric, there are statistically significant differences between naïve and LPS-activated human neutrophils. LPS-activated neutrophils demonstrate a significant increase in overall contractility (**Fig. 3.12c**) and a greater capacity to undergo changes in volume (**Fig. 3.12c**). Additionally, in the presence of LPS, neutrophils undergo significant rotational motion as quantified by an increase in the average rotation angle (**Fig. 3.12f**) and cumulative rotation (**Fig. 3.12g**). These measurements are currently not possible to deduce from fluorescent imaging alone but might provide significant insight into the dysregulation of neutrophil chemotaxis during states of disease.



**Figure 3.12:** Mean deformation metrics for neutrophils calculated from the mean displacement gradient tensor  $\langle \nabla \mathbf{u} \rangle$ . **(a)** Box plot of the normalized norm of the skew (rotational) and symmetric (contraction/expansion) parts of  $\langle \nabla \mathbf{u} \rangle$  for all time points of the tested conditions; K - N, chemokinesis and naïve (solid white,  $n = 30$ ); T - N, chemotaxis and naïve (solid gray,  $n = 48$ ); K - L, chemokinesis and LPS - activated (hatched white,  $n = 44$ ); and T - L, chemotaxis and LPS - activated (gray hatched gray,  $n = 57$ ). Red lines: medians, top and bottom boxes: upper and lower quartiles, whiskers: maximum and minimum values. **(b)** Box plot of the mean volume change ratio  $\langle J \rangle$  of the cell (dotted line, constant volume), defined as the determinant of the mean deformation gradient  $\langle \mathbf{F} \rangle$ . **(c)** Box plot of the three mean principal strain values  $\langle E_i \rangle$  calculated from the mean Lagrangian strain tensor  $\langle \mathbf{E} \rangle$ . Negative values of  $\langle E_i \rangle$  signify mean contraction while positive values of  $\langle E_i \rangle$  are mean expansion of the cell along the associated principal axis. **(d)** Schematic illustrating maximum  $\langle E_3 \rangle$  and minimum  $\langle E_1 \rangle$  principal strains exerted by the cell. **(e)** Schematic illustrating mean cell rotation angle  $\langle \Theta \rangle$  and **(f)** corresponding box plot for all time points and conditions. **(g)** Integrated  $\langle \Theta \rangle$  for each cell over its migration time,  $t$  for all tested conditions; K - N (solid red,  $n = 4$  cells), T - N (solid blue,  $n = 3$  cells), K - L (dashed magenta,  $n = 5$  cells), T - L (dashed cyan,  $n = 3$  cells). Middle line: mean, shaded area: standard deviation of integrals evaluated at the specified time point.  $p < 0.05$  by Mann-Whitney U test across corresponding (\*) naïve and LPS activated groups, (#) chemokinesis and chemotaxis groups, and (+) metric groups (skew and symmetric in **a**, and across  $E_1$ ,  $E_2$ ,  $E_3$  in **b**).

### 3.4 Conclusion

Having the capability to extract meaningful kinematic measurements without knowledge of material properties provides new experimental tools for analyzing fibrous networks, multilayer scaffolds, and living tissue. In this study, FIDVC was applied to show previously unknown displacement patterns exerted by neutrophils in a complex 3D collagen matrix. A new set of mean kinematic metrics were defined and validated against well-known analytical solutions. These mean kinematic measurements do not rely on inverse solutions or explicit spatial derivatives, thus producing a lower measurement noise floor compared to other techniques that are applied to study cell-matrix interaction [37, 54, 152]. As an example, this mathematical framework was applied to show significant differences in the overall cell induced deformation between naïve and LPS-activated human neutrophils.

## 4. Characterizing Injury Evolution of Neurons in Nonlinear Materials

### 4.1 Introduction

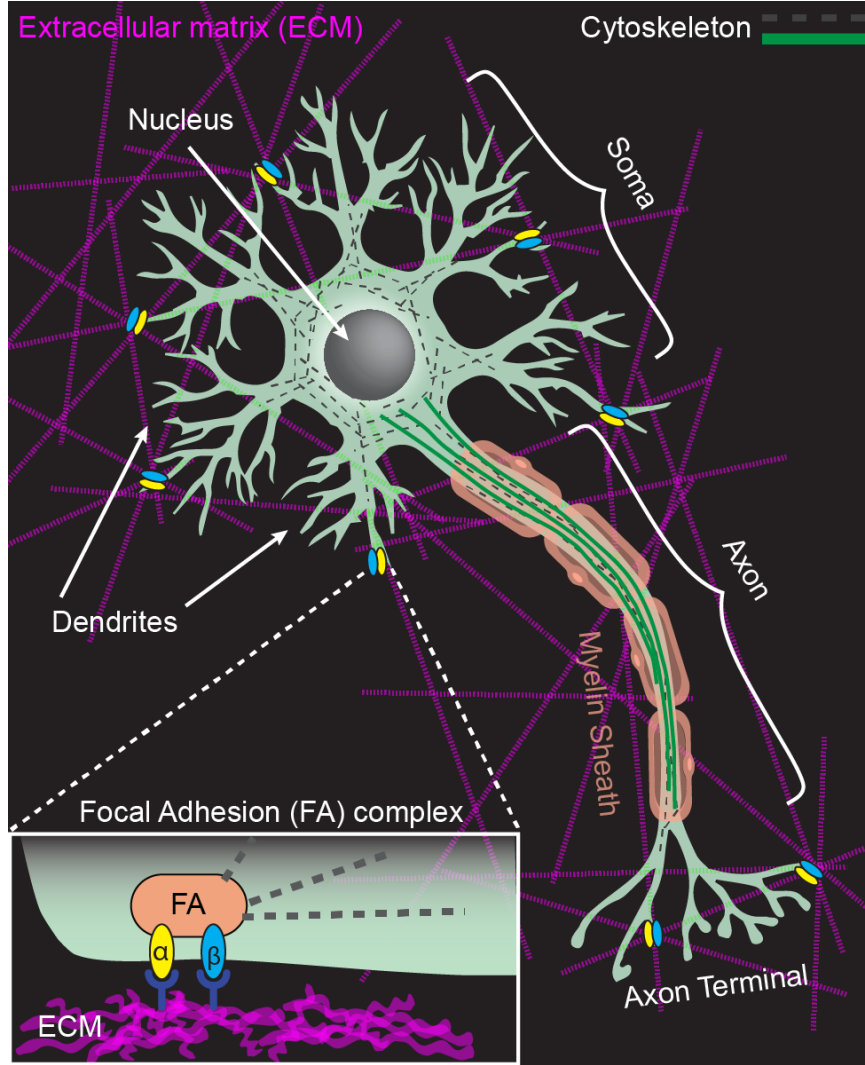
Traumatic brain injury (TBI) is a worldwide problem that causes devastating health and economic challenges. In the United States alone, over 1.7 million people suffer TBI every year [75] at an annual healthcare cost of over \$60 billion [76]. The incidence of TBI continues to grow with the adoption of motorized vehicles in developing countries [196], the faster pace of sports [237], and the increased destructive power of modern weaponry [157, 252]. Despite the wealth of literature available, there is no comprehensive solution to TBI; not only are there no proven treatments and interventions [289], but also no dependable methods of detection given current technology and clinical knowledge [225, 202].

TBI results from direct or indirect loading to the head that disrupts normal brain function [91, 170, 180]. The loading can be mechanistically classified into one or a combination of: (1) impact, where the head makes direct contact with an object; (2) inertial, caused by rapid accelerations within the brain; and (3) blast, induced by a high energy pressure wave generated by an explosion. The severity of neurological disability depends on an array of complex factors that may be unrelated to the traumatic event, for example, genetic predisposition [60, 296], and medical history [197, 179]. However, in most TBIs, the severity is directly related to the parameters of primary insult and ensuing secondary events [216, 202, 235, 165], which may be complex and persistent. The most common secondary event across all severities is diffuse axonal injury (DAI) [1, 216, 126], a micro-scale pathology that is linked to acute symptoms such as loss of consciousness, confusion, and headache [6, 117, 126], as well as persistent deficits in attention, memory, and motor skills [6, 244, 126]. Recent evidence has suggested that DAI plays a role in the development of long-term neurodegenerative diseases such as Alzheimer's [254, 43, 179, 125, 126]. Although the risk associated with DAI is recognized, a comprehensive understanding of the multiscale

mechanics and biology that initiate this injury remains a topic of current research.

During traumatic loading, external kinetic energy that transfers into the brain produces complex mechanical deformations of the neural tissue. The spatiotemporal signature of these deformations is a function of material properties, and biological structure, both of which are spatially heterogeneous and vary at multiple length and time scales [184, 183, 218, 62, 63]. In DAI, macroscopic deformations transfer to the local microstructures, initiating a devastating cellular cascade that ultimately leads to neuron failure [126]. The extent of deformation transfer is highly dependent on the local orientation of neurons [47, 136], e.g., highly aligned neuronal fibers in the white matter may be sensitive to strain directionality [133, 5, 136].

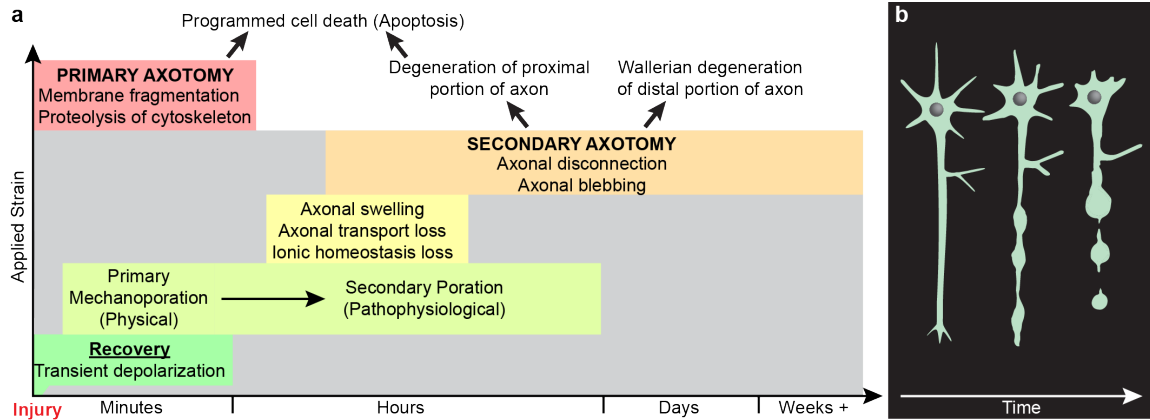
To study how mechanical deformations are transmitted to neurons it is necessary to gain a basic understanding of the cellular structure. Although there are phenotypical differences between neurons, generally there are four distinct anatomical regions: the soma or cell body, dendrites, axon, and the axon terminals (**Fig. 4.1**) [189]. The cell body contains the nucleus and many other essential organelles that synthesize virtually all neuronal proteins and regulate homeostasis. Dendrites are extensions that branch away from the cell body and receive electrochemical signals from other neurons. These signals are synthesized by the cell body and transmitted through the axon in the form of action potentials. Generally, longer axons in the central nervous system are covered by oligodendrocytes that create a myelin sheath, which provides electrical insulation. When an action potential reaches the axon terminal, it triggers the release of signaling molecules called neurotransmitters to communicate with a target neuron. All of these structures are mechanically supported by filamentous polymers including actin, microtubules, and intermediate neurofilaments that comprise the cytoskeleton. Each load bearing element is specialized for a certain function, i.e. actin (5 – 9 nm in diameter) and neurofilaments (10 nm in diameter) bear tension [35, 137], and microtubules (25 nm in diameter) bear compression [131]. External mechanical deformations are transmitted from the extracellular matrix (ECM) to dynamic protein assemblies called focal adhesions (FAs) located in the cell membrane. These FAs are tied to the actomyosin machinery and cytoskeleton which can actively generate internal forces [36, 14]. The dynamic network interaction of these load bearing elements and FAs



**Figure 4.1:** Structure of typical mammalian neurons.

provide the architectural basis of cellular mechanotransduction, or how cells sense and respond to external deformations [115, 116, 90].

Depending on the degree of mechanical strain at the time of injury, DAI exhibits two distinct pathways that lead to axonal degeneration or axotomy (**Fig. 4.2a**) [176, 22, 153]. In the minority of DAI cases, high axonal strains produce primary axotomy within an hour and causes severe membrane fragmentation and proteolysis of the axonal cytoskeleton leading to eventual cell death [92]. At much more innocuous strains, the generation and propagation of action potentials is temporary disabled due to ion influx into the cell causing depolarization [92]. In this case, ionic homeostasis is restored within minutes and damaged neurons recover

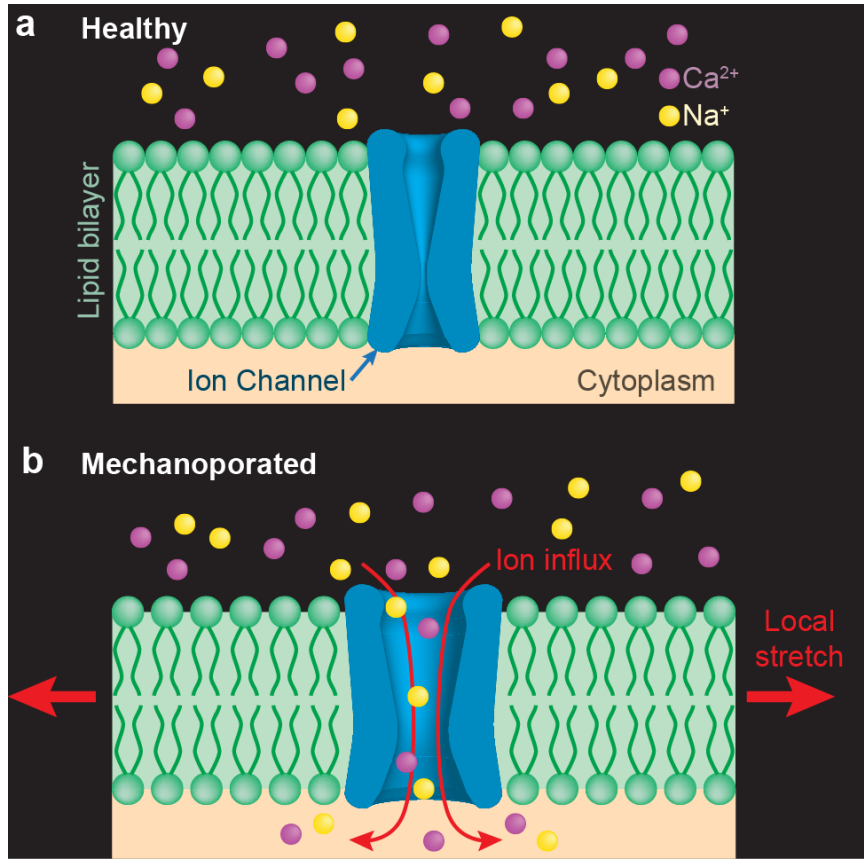


**Figure 4.2:** (a) Two pathways lead to axonal degeneration or axotomy in DAI. (b) Secondary axonal pathology in DAI shows healthy neuron (left) undergoing axonal swelling leading to bleb formation (center) and disconnection (right).

function completely. If axonal strain is increased, physical stretch of the cell membrane causes primary mechanoporation within minutes, allowing the influx of extracellular molecules and ions into the cell. The influx of molecules internally activates destructive enzymes such as calpains and caspases that digest the cell membrane, leading to pathophysiological secondary poration that is irreversible [51]. These digestive enzymes also degrade the cytoskeleton [32, 128, 118, 112, 178], which, when combined with direct mechanical failure of the cytoskeleton due to the primary insult, impairs axonal transport. Over the time course of a few hours, vesicles and organelles accumulate at cytoskeletal failure locations to produce axonal swelling and eventual bulbing or blebbing [198, 73, 130, 103]. Because of this obvious morphological change of neurons, axonal blebbing is currently the hallmark symptom of DAI (**Fig. 4.2b**) [83, 216]. Following blebbing, axonal disconnection and fragmentation occurs [255] due to continued proteolysis and compaction of neurofilaments, ultimately leading to Wallerian degeneration [1, 215]. Despite the widespread study of DAI, the molecular and cellular mechanisms that underlie and trigger the sequence of morphopathological changes have remained elusive to researchers.

*In vitro* cellular injury models have shown direct evidence of axonal pathologies associated with DAI without large systemic variabilities found *in vivo* (see [187] for review). Cellular models have produced injury to a population of neurons cultured on planar, two-dimensional substrates through uniaxial [12, 210, 163, 164, 270, 102, 269] or biaxial [64, 38, 309, 275,

94, 293, 211, 86, 294, 87, 148, 95, 129, 42, 11, 88] membrane tension and fluid shear [145, 146, 217, 250, 130, 144]. However, models in three-dimensions (3D) better mimic the complex heterogeneous deformations that occur within the brain. By applying high rate simple shear to 3D hydrogel cultures [143, 50, 51], Laplaca et al. found that neuron viability decreased with higher strains and strain rates. The rate dependence of viability suggests viscoelastic effects underlying cytoskeletal failure [2]. The same study showed that neurites pointing in the direction of maximum shear strain were less numerous 24 hours after mechanical insult. These measurements were assuming no cytoskeletal reorganization and solely made for large neurites that pointed directly away from the soma. Even with these estimations, neuronal orientation in 3D was shown to be an important factor in elucidating neuronal injury thresholds for the first time. For many materials, failure is highly dependent on the underlying anisotropy [279].

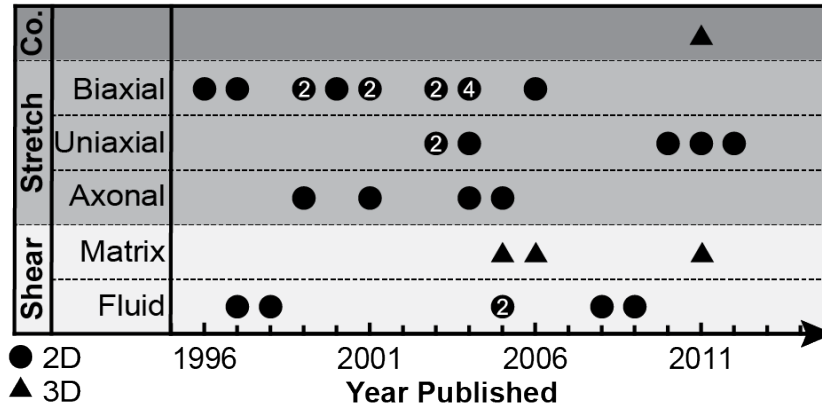


**Figure 4.3:** (a) Healthy ion channel in plasma membrane properly regulates ionic homeostasis of neuron. (b) Transient Local stretch induces opening of mechanosensitive ion channels which allows the influx of ions during a traumatic event.



As previously discussed, the cascade of secondary events in DAI has been suggested to be a result of increased membrane permeability, causing intracellular ionic dysregulation [92]. *In vitro* and *in vivo* models have shown that permeability increase is due to the local transient stretch of mechanosensitive ion channels [32, 303], which repair within minutes of injury (**Fig. 4.3**) [87]. However, during this acute time, a sharp rise of intracellular calcium ( $\text{Ca}^{2+}$ ) and sodium ( $\text{Na}^{+}$ ) ionic concentration following injury [146, 86, 303] can lead to the activation of proteases such as calpains and caspases [32, 128, 118, 112, 178]. These enzymes may facilitate the formation of non-resealable secondary pores [73] that ultimately lead to cytoskeletal breakdown, and necrotic or apoptotic cell death.

Our micropathological understanding of DAI is primary derived from *in vitro* models applying shear and stretch to neurons (**Fig. 4.4**). However, *in vivo*, compressive deformation may be a major contributor to the cell death caused by DAI. In this chapter, it is hypothesized that compression will minimize the stretch of neurons locally, and therefore limit the secondary effects of mechanoporation. As a result, traditional injury indicators such as axonal blebbing, may have an alternate manifestation. To test this hypothesis, a high speed cell compression device was developed to deliver repeatable and homogeneous deformations to 3D collagen cultures of neurons at two different strain rates. The long-term cell membrane permeability



**Figure 4.4:** Literature history of *in vitro* TBI models applying shear (white, bottom) [145, 146, 217, 143, 250, 50, 130, 144, 51], stretch (light gray, middle [309, 275, 94, 256, 293, 211, 86, 293, 297, 87, 12, 148, 163, 164, 118, 95, 1, 129, 42, 11, 46, 88, 270, 102, 269], and compression (dark gray, top) [51] to 2D (circle) or 3D (triangle) neuronal cell cultures plotted by year published. The number within the symbol denotes the number of publications at the given year for two or more publications.

of individual neurons was assessed after mechanical loading to determine the severity of mechanoporation in compression. The micropathological role of mechanoporation were assessed by investigating the morphological injury evolution of neurons as a function of strain rate. The local orientation of each neuron was computed to establish local injury metrics which were then correlated to axonal swelling or blebbing. Analysis of the neurite orientations also allows for the computation of global injury metrics, which are hypothesized to disrupt the mechanical homeostasis of the cell, leading to eventual death.

## **4.2 Methods**

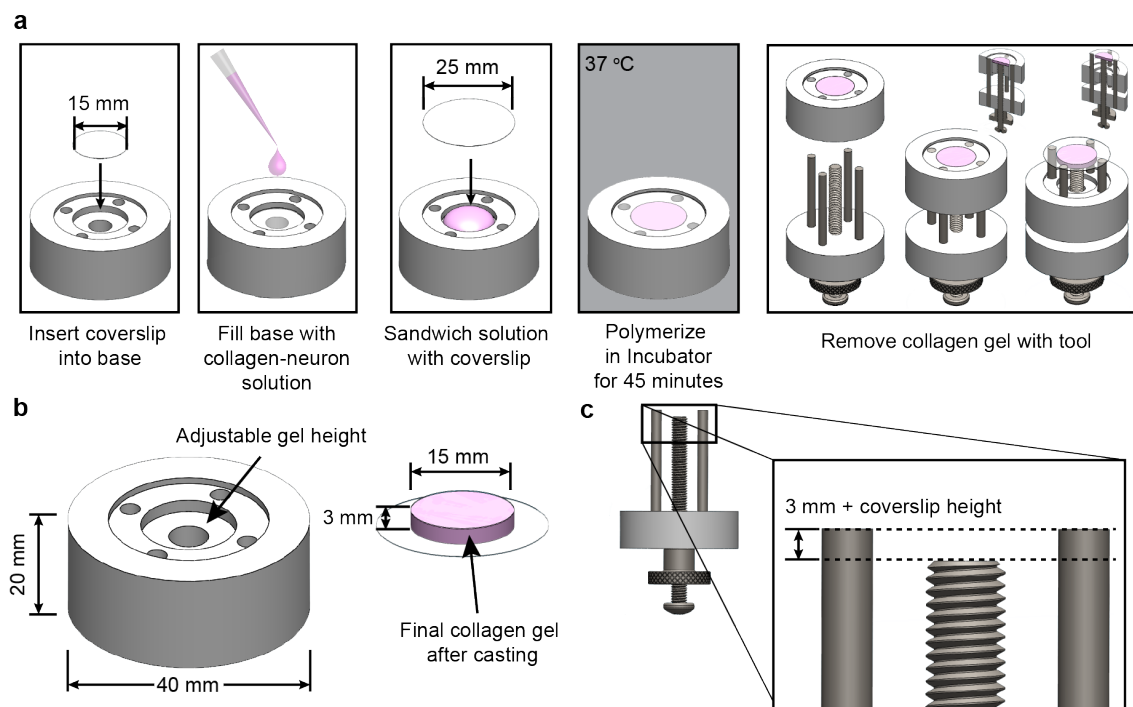
### **4.2.1 Isolation of Primary Cortical Neurons**

All procedures involving animals were approved by the IACUC of Brown University. All reagents were from Life Technologies (Grand Island, NY) unless otherwise noted. Primary rat cortical neurons were isolated from postnatal day 0 Sprague-Dawley rats (Charles River, Wilmington, MA). The pups were rapidly decapitated, and the cerebral hemispheres were placed in a cold solution of Hibernate A (HA; Brain Bits LLC, Springfield, IL) + 2% B-27 (50 $\times$ ) + 0.25% GlutaMAX. Cerebral cortices were minced and digested in pre-warmed Hibernate A Minus Calcium (Brain Bits LLC) with Papain (2 mg/mL; Worthington Biochemical Corporation, Lakewood, NJ) for 30 minutes at 37°C with gentle agitation every 5 minutes. Half of the papain was replaced with pre-warmed HA/B-27 and centrifuged at 150 $\times$  g for 5 minutes. Dissociated tissue was resuspended in culture media (Neurobasal medium + 2% B-27 + 1% mixture of Penicillin Streptomycin, L-Glutamine, and Fetal Bovine Serum), and gently passed through sterile cell strainer (40  $\mu$ m mesh size, Thermo Fisher Scientific Inc., Waltham, MA) to filter large debris. Following another centrifuge step (150 $\times$  g for 5 minutes), the cells were gently resuspended by mechanical trituration in culture media, and counted using a hemocytometer (Hausser Scientific, Horsham, PA). Cell solution was diluted with culture media to 10<sup>7</sup> cells/mL prior to preparation of 3D collagen gel system to yield a cell density of approximately 3,750 cells/mL [147]

### **4.2.2 Preparation of 3D Collagen Gel**

3d collagen (type I, rat tail; Dow Corning, Tewksbury, MA) matrices were prepared to a final concentration of 2.2 mg/mL according to manufacturer’s standard protocol. 10 $\times$  phosphate

buffer saline (PBS; Life Technologies) was mixed with 1 N NaOH (Sigma-Aldrich, St. Louis, MO) (Table 4.1). Collagen I stock solution with a concentration of 3.3 – 3.7 mg/mL in 0.02N acetic acid was added to 10× PBS/1 N NaOH solution, thoroughly vortexed, and held in ice. The pH of the solution was tested with a pH spear (Oakton Instruments, Vernon Hills, IL) and incrementally adjusted to 9.2 – 9.5 by addition of 1 - 10  $\mu$ L 1 N NaOH. Cell solution was slowly added to the collagen to yield a final gel concentration of 2.2 mg/mL and pH of 8.5 – 8.8, and gently mixed prior to polymerization.



**Figure 4.5:** Collagen gel casting system produces accurate and reproducible 3D neuron cultures for compression. (a) Schematic of the casting procedure to create the collagen gel system. (b) Key dimensions of the (left) custom mold used to produce a disk shaped collagen gel (right). (c) Custom tool to remove collagen gel out of the mold after polymerization. The center screw height is offset from the four posts to allow gel removal without damage.

Custom made Delrin molds were used to cast cylindrical gels of 3 mm height and 15 mm diameter (**Fig. 4.5**). The center base of the mold was sterilized with 99% isopropyl alcohol,

| Collagen Gel Concentration | PBS | NaOH | Collagen I, Stock | Cell Solution |
|----------------------------|-----|------|-------------------|---------------|
| 2.2 mg/mL                  | 10% | 1.4% | 56.2%             | 32.4%         |

**Table 4.1:** v/v% of each component to produce a collagen gel concentration of 2.2 mg/mL.

coated with a thin layer of vacuum grease, and covered with a 15 mm cover slip. Collagen-cell solution was inserted into the base cavity, sandwiched with a chemically activated 25 mm top cover slip, and polymerized in a humidified cell incubator at 37°C and 5% CO<sub>2</sub> for 45 minutes. Polymerized gels were removed with a custom made tool (**Fig. 4.5c**) designed to not damage the gel during removal, and transferred to six-well plates filled with 3 mL of culture media. To maximize nutrient diffusion, the 15 mm cover slip was carefully removed. Collagen gel cultures were maintained in humidified cell incubators at 37°C and 5% CO<sub>2</sub>, and culture medium was exchanged at 24 h and every 2 days thereafter. Experiments were initiated at day 7 *in vitro* (DIV).

### 4.2.3 Fluorescence Staining

For all compression experiments, neuron viability and geometry were assessed using calcein AM (Life Technologies), a membrane-permeant dye that converts nonfluorescent calcein AM to green-fluorescent calcein upon acetoxymethyl ester hydrolysis by intracellular esterases. Calcein AM has shown to be well retained within live cells, and has a high quantum yield [53, 201]. Acute alterations in plasma membrane permeability following compressive loading were assessed by evaluating uptake of Alexa Fluor 568 hydrazide (; Life Technologies), a relatively low molecular weight (730 Da) and cell impermeant fluorophore used for intracellular labeling experiments [262]. Used extracellularly, AFH cannot infiltrate the cell membrane until pores, approximately the size of AFH, develop due to mechanical insult. Previous studies have shown that this mechanoporation occurs even for significantly larger fluorescent dyes (MW 150 kDa) after mechanical stretch [87].

Prior to mechanical loading, cell cultures were placed into a Chambridge chamber (Live Cell Instruments Inc., Seoul, Korea), and incubated with calcein AM (2  $\mu$ M) and AFH (10  $\mu$ M diluted with dH<sub>2</sub>O) in prewarmed HA + 2% B-27 for 45 minutes. After rinsing with 1 $\times$  PBS, a 15 mm cover slip was gently placed on top of the gel, and a solution of AFH (10  $\mu$ M) in HA/B-27 was left in the chamber during mechanical insult and imaging. Studies have shown maintenance of neuronal cell viability and function at ambient CO<sub>2</sub> for up to 48 hours. [28, 132, 27] in HA/B-27.

#### 4.2.4 Confocal Microscopy and Live Cell Imaging

For cell compression experiments, three-dimensional image volumes of neurons immediately after mechanical insult 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). To ensure physiological conditions, temperature within the microscope chamber was controlled at 37°C using a closed-loop heater (Air-Therm heater, World Precision Instruments Inc., Sarasota, FL), and the high-speed compression device was allowed to thermally equilibrate for 2 – 3 hours prior to imaging. A CFI Plan APO VC 60× water immersion objective (NA = 1.2; Nikon) was used for all mechanical compression experiments. Calcein AM stained neurons were excited with an Argon ion laser (488 nm), and extracellular AFH was excited with a HeNe diode laser (561 nm). Confocal image stacks of  $1024 \times 1024 \times 100$  voxels ( $177 \times 177 \times 100 \mu\text{m}^3$ ) were recorded with a z-step of  $1 \mu\text{m}$ , and within a depth of 100 – 200  $\mu\text{m}$  into the collagen gel. For each experiment 6 – 12 cells were imaged directly after mechanical insult, at four hours, and every hour thereafter until cell death or fading of calcein AM dye.

For reflectance microscopy, cells stained with calcein AM were imaged with an APO 40× water immersion objective (NA = 1.15, Nikon) and Z-step of  $0.63 \mu\text{m}$ , to yield an image volume of  $1024 \times 1024 \times 176$  voxels ( $212 \times 212 \times 110 \mu\text{m}^3$ ).

#### 4.2.5 Control experiments

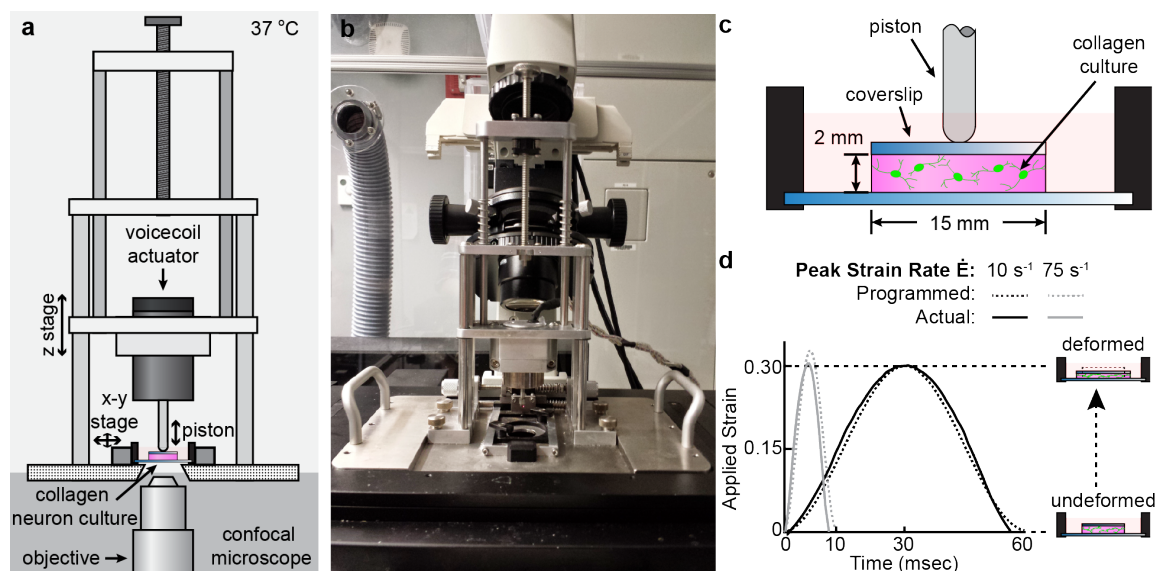
**Note:** Mark T. Scimone performed the population studies of cell viability.

Control experiments to assess the cell damage associated with the cell stains, and confocal microscopy imaging were performed under same conditions as the impact experiments without applying mechanical insult.

Control experiments to induce cell membrane poration were performed by introducing 0.1% Triton X-100 in HA/B-27 after staining with Calcein AM and AFH. Confocal images were acquired every ten minutes with the same imaging parameters as the mechanical insult experiments.

### 4.2.6 3D Cell Compression Device

The 3D cell compression device applies mechanical deformation to cultured neurons in a 3D collagen gel. The primary goals of the cell compression device are (1) uniaxial compression across the 3D collagen cell culture with high strain rates and magnitudes that are characteristic of inertial TBI [169, 171, 170, 180]; (2) displacement (as opposed to force) controlled deformation with well characterized boundary conditions; (3) user defined temporal characteristics of the strain field; and (3) cell accessibility to confocal microscopy before, during, after mechanical insult so that the immediate and long term biochemical events are measurable. The cell compression device consists of a voice coil actuator (VCA) that is mounted on a custom frame attached to the confocal microscope stage (**Fig. 4.6a**). Uniaxial compressive strain is generated by linear motion of the VCA piston that is in contact with a cover slip resting on top of the collagen gel (**Fig. 4.6c**). To ensure normal contact with the cover slip during deformation, the piston is fitted with a spherical tip. To overcome the limited range of motion of the piston and to increase sample accessibility, the VCA is mounted on a manual z-stage with a full range of motion of 100 mm (**Fig. 4.6a**).



**Figure 4.6:** 3D Cell Compression Device that produces cell injury. (a) Schematic representation of the cell compression device fitted into a confocal microscope for cell injury tracking. (b) Photo of the cell compression device. (c) Schematic representation of the 3D collagen culture system. Linear motion of the voice coil actuator piston drives the displacement of the cover slip, inducing compressive deformation in the 3D collagen gel culture. (d) Strain profiles generated by the cell compression device used in this study.

The sample chamber is situated in an independent x-y translation stage that is used to precisely center the sample with the piston. Prior to mechanical deformation, a custom cylindrical tool (with same diameter as the sample chamber) and a centered spherical cutout (with the same radius as piston tip) is inserted into the x-y stage. The VCA is lowered and the x-y stage is adjusted so that the piston tip fits into the centered cutout. Motion of the piston is produced via a linear VCA (LAS 13-18; BEI Kimco, San Marcos, CA) and a closed loop control system consisting of a servo controller (SilverSterling S2-IG; QuickSilver Controls, Inc., Covina, California) and integrated hall-based displacement transducer. A computer is used to program and communicate with the controller for any desired piston motion. In the study, the VCA was programmed to deliver strain magnitudes of 0.30 and peak strain rates of  $10 \text{ s}^{-1}$  and  $75 \text{ s}^{-1}$  (**Fig. 4.6c**). Due to the inertia of the system, the actual measured strain differed slightly from the programmed.

## 4.2.7 Strain Field Characterization

### 4.2.7.1 Experimental Parameters

The uniaxial compressive strain field was validated under quasistatic loading on the 3D collagen neuron cultures ( $n = 7$ ). Fluorescent microspheres ( $0.5 \mu\text{m}$  in diameter, carboxylate-modified, Lift Technologies) were thoroughly mixed at 6 v/v% into the collagen gel during cell culture and maintained in the same conditions as the mechanical insult experiments. At day 7 *in vitro*, collagen cell cultures stained with calcein AM were mounted on the cell compression device and compressed by a 2% strain increment with a 2 minute loading time every 6 minutes to a total strain of 38%. During the hold time, confocal image volumes of size  $512 \times 512 \times 350$  voxels ( $212 \times 212 \times 210 \mu\text{m}^3$ ) were acquired within a depth of 100 – 200  $\mu\text{m}$  using an APO 40 $\times$  water immersion objective (NA = 1.15, Nikon).

### 4.2.7.2 Full-field displacement computation

Cell-induced full-field displacements were measured using the fast iterative digital volume correlation algorithm as detailed in Chapter 2 [15]. The final interrogation window spacing, which sets the displacement spatial grid resolution was 8 voxels or  $3.3 \mu\text{m}$  for measurements in  $x_1 - x_2$  and  $4.8 \mu\text{m}$  for measurements in  $x_3$ . The displacement gradient tensor  $\nabla \mathbf{u}$ , computed with an optimal-9 tap finite difference kernel, was used to calculate the Lagrangian strain

field  $\mathbf{E}$ , given as

$$\mathbf{E} = \frac{1}{2} \left( \nabla \mathbf{u} + \nabla \mathbf{u}^T + \nabla \mathbf{u} \cdot (\nabla \mathbf{u})^T \right). \quad (4.2.1)$$

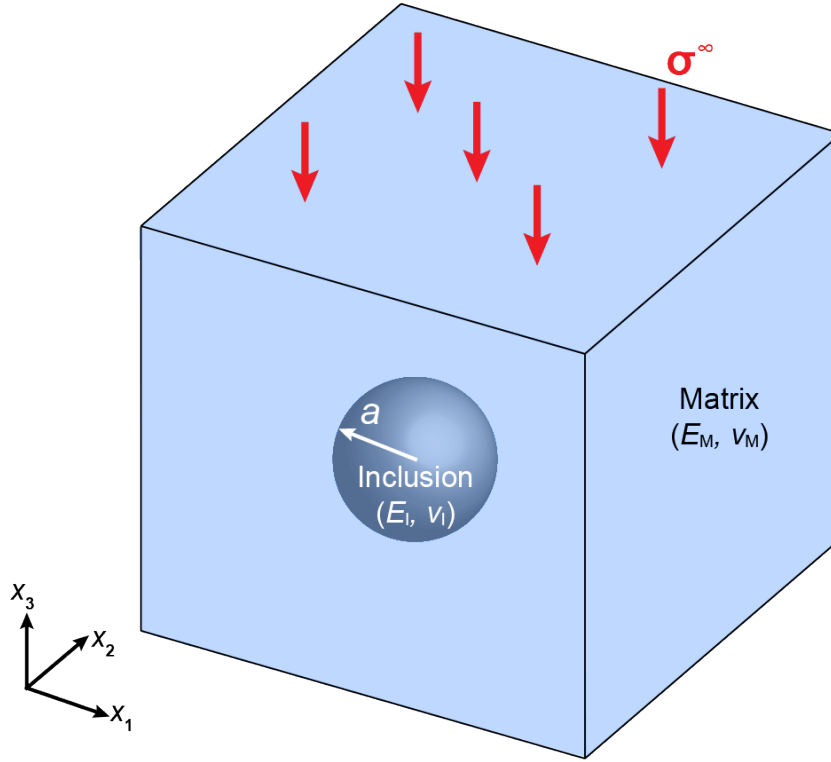
The quasistatic Poisson's ratio  $\nu$  was computed as the negative ratio of mean transverse  $E_{11}$  and  $E_{22}$  to axial  $E_{33}$  averaged over all increments, i.e.

$$\nu = -\frac{1}{N} \sum_n^N \left( \frac{2E_{33}}{E_{11} + E_{22}} \right), \quad (4.2.2)$$

where  $N$  is the total number of loading increments.

#### 4.2.7.3 Spherical Inclusion Problem

Cells being compressed in a 3D matrix can be analytically compared to an Eshelby inclusion solution given by Goodier [97]. Consider a spherical inclusion with radius  $a$ , Poisson's ratio  $\nu_I$ , and Young's modulus  $E_I$  in an infinite, isotropic, linear-elastic solid matrix with Poisson's ratio  $\nu_M$  and Young's modulus  $E_M$  subject to a uniaxial stress at infinity  $\sigma^\infty$  (**Fig. 4.7**).



**Figure 4.7:** Schematic of a spherical inclusion with radius  $a$ , Poisson's ratio  $\nu_I$ , and Young's modulus  $E_I$  in an infinite, isotropic, linear-elastic solid matrix with Poisson's ratio  $\nu_M$  and Young's modulus  $E_M$  subject to a uniaxial far field stress  $\sigma^\infty$



The elastic mismatch between the matrix and inclusion gives rise to nonlinear displacements around the interface. The closed-form solution of the radial displacements  $u_r$  and tangential displacements  $u_\theta$  solely due to the elastic mismatch is given by

$$\begin{aligned} u_r &= -\frac{A}{r^2} - \frac{3B}{r^4} + \left( \frac{5-4\nu_M}{1-2\nu_M} \frac{C}{r^2} - \frac{9B}{r^4} \right) \cos 2\theta \\ u_\theta &= -\left( \frac{2C}{r^2} + \frac{6B}{r^4} \right) \sin 2\theta \end{aligned} \quad (4.2.3)$$

where  $A$ ,  $B$ , and  $C$  are constants determined by equating the displacements and tractions at the interface, i.e.  $r = a$ . The values of the constants are

$$\begin{aligned} A &= -a^3 \frac{\sigma^\infty}{8\mu_M} \frac{\mu_M - \mu_I}{(7-5\nu_M)\mu_M + (8-10\nu_M)\mu_I} \\ &\quad \times \frac{(1-2\nu_I)(6-5\nu_M)2\mu_M + (3+19\nu_I-20\nu_M\nu_I)\mu_I}{(1-2\nu_I)2\mu_M + (1+\nu_I)\mu_I} \\ &\quad + \frac{\sigma^\infty}{4\mu_M} \frac{\left( (1-\nu_M) \frac{1+\nu_I}{1+\nu_M} - \nu_I \right) \mu_M}{(1-2\mu_I)2\mu_M + (1+\nu_I)\mu_I} \\ B &= a^5 \frac{\sigma^\infty}{8\mu_M} \frac{\mu_M - \mu_I}{(7-5\nu_M)\mu_M + (8-10\nu_M)\mu_I} \\ C &= a^3 \frac{\sigma^\infty}{8\mu_M} \frac{5(1-2\nu_M)(\mu_M - \mu_I)}{(7-5\nu_M)\mu_M + (8-10\nu_M)\mu_I} \end{aligned} \quad (4.2.4)$$

where the matrix shear modulus  $\mu_M$  and inclusion shear modulus  $\mu_I$  is related to the Young's modulus and Poisson's ratio by

$$\begin{aligned} \mu_M &= \frac{E_M}{2(1+\nu_M)} \\ \mu_I &= \frac{E_I}{2(1+\nu_I)}. \end{aligned} \quad (4.2.5)$$

The full-field displacements in the matrix are obtained by superposing the displacements in

equation (4.2.3) with the solution for uniaxial compression/tension along  $x_3$ ,

$$\begin{aligned} u_r &= \frac{\sigma^\infty a}{2E_M} ((1 - \nu_M) + (1 + \nu_M) \cos 2\theta) \\ u_\theta &= -\frac{\sigma^\infty a}{2E_M} (1 + \nu_M) \sin 2\theta. \end{aligned} \tag{4.2.6}$$

To compare between the experimentally determined displacement field around the cell and the Eshelby solution, an inclusion ( $a = 5$  voxels,  $\nu_I = 0.49$ ) in a matrix ( $\nu_M = 0.06$ ) is subjected to a remote stress given by

$$\sigma^\infty = \frac{E_M}{1 + \nu_M} \left( E_{33}^\infty + \frac{\nu_M}{1 - 2\nu_M} (E_{11}^\infty + E_{22}^\infty + E_{33}^\infty) \right). \tag{4.2.7}$$

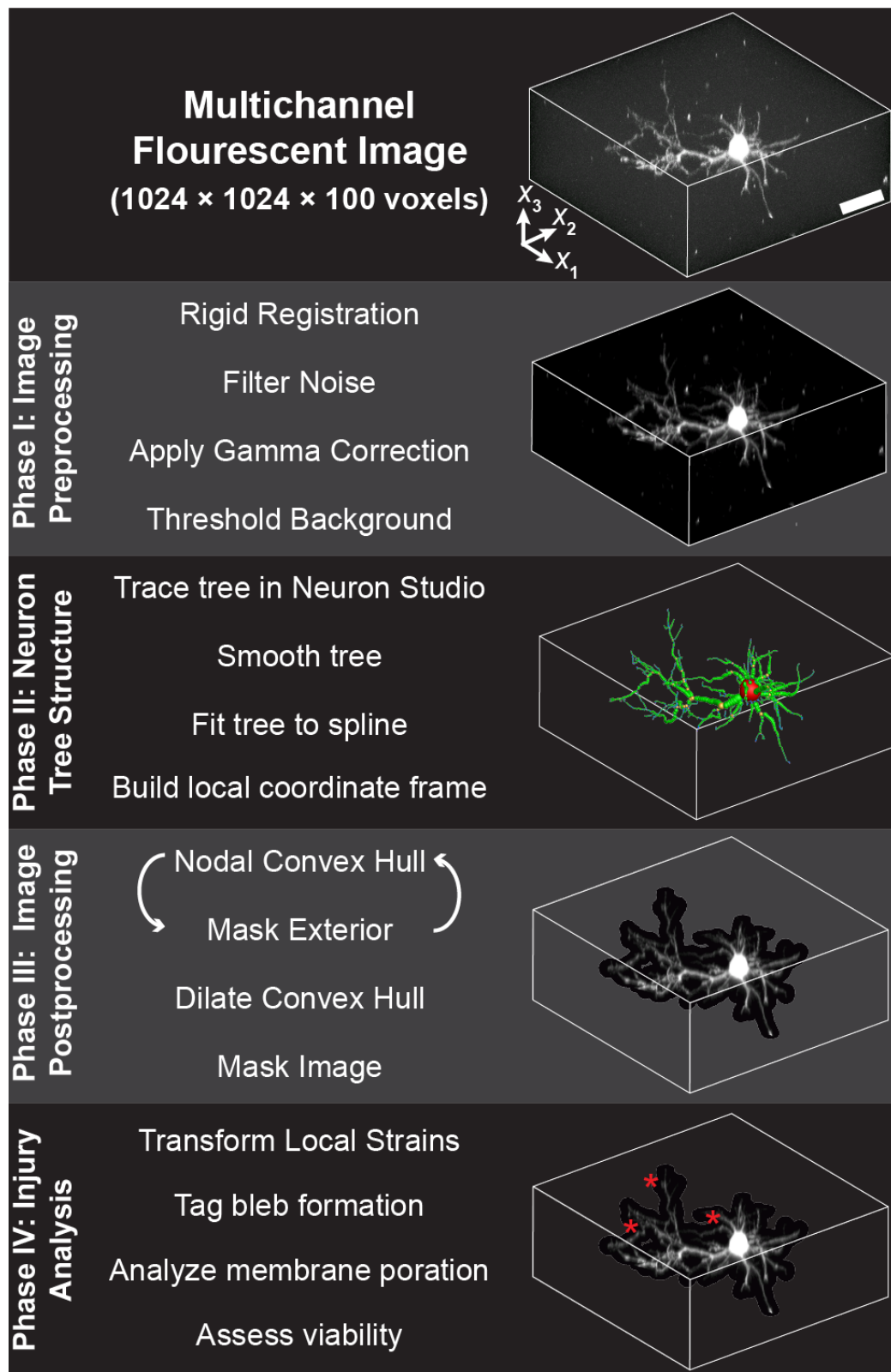
$E_{33}^\infty$  is the far field strain applied by the cell compression device, and  $E_{11}^\infty$  and  $E_{22}^\infty$  are the strain components that arise due to the Poisson effect. The displacement along the loading axis  $u_3$  is computed after transforming equations (4.2.3) – (4.2.6) to Cartesian coordinates for varying ratios of  $E_I/E_M$ . It is important to note that there are values of  $E_I/E_M$  that produces well known deformations;  $E_I/E_M = 0$  is the Eshelby solution of a spherical cavity subject to a remote stress;  $E_I/E_M = 1$  is the solution for uniform strain; and  $E_I/E_M = \infty$  is the Eshelby solution for a rigid inclusion subject to a remote stress.

## 4.2.8 Image Processing

Converting raw confocal images of neurons to quantitative metrics that could be used for injury analysis requires a multiphase image processing routine

### 4.2.8.1 Image Pre-Processing

Volumetric confocal images of the calcein AM and Alexa Fluor hydrazide channel were converted to .tif files and loaded into MATLAB (Natick, MA) for preprocessing. Images were rigidly translated to correct for experimental drift by maximizing the cross-correlation between an image and the image at the next time point using the calcein AM channel (Algorithm 3). A median filter with a  $3 \times 3 \times 3$  voxel window size was then applied twice for noise reduction, followed by a gamma correction with a coefficient of 0.7 ( $I(\mathbf{x}) = I(\mathbf{x})^{0.7}$ ) for contrast enhancement. To remove background noise, all intensity values below the mode



**Figure 4.8:** Main image processing steps for analysis.

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**Algorithm 3** Correct for spatial drift.

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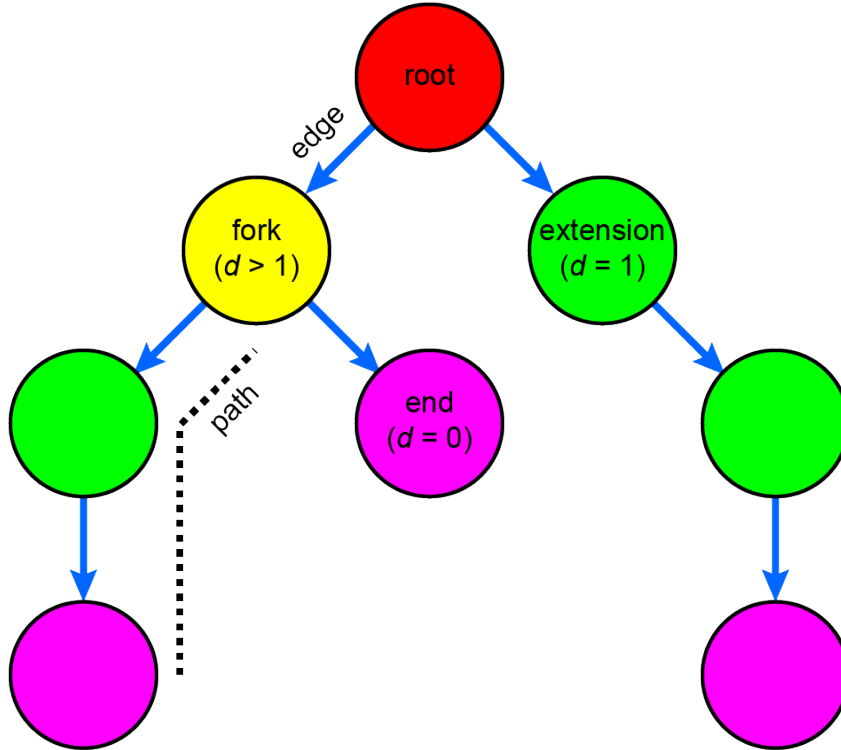
```
1: for ( $t = 1; t < T - 1; t = t + 1$ ) do                                 $\triangleright$  for each time of  $T$  times
2:    $\chi(\mathbf{x}) = I_t(\mathbf{x}) \otimes I_{t+1}(\mathbf{x})$                          $\triangleright$  compute 3D cross-correlation of images
3:    $\mathbf{u}(\mathbf{x}) = \underset{\mathbf{u}(\mathbf{x})}{\operatorname{argmax}} \chi(\mathbf{x})$            $\triangleright$  compute drift from cross-correlation maximum
4:    $I_{t+1} = I_{t+1}(\mathbf{x} + \mathbf{u})$                                  $\triangleright$  shift image with drift
5:    $t = t + 1$ 
6: end for
```

---

of the image were set to zero. (**Fig. 4.8**).

#### 4.2.8.2 Building Neuron Tree Structure

The tree data structure, commonly used in graph theory is a powerful way to describe the morphology of a neuron. In this study, the neuron tree structure,  $T = \{V, E\}$ , is composed a set of  $n$  nodes or vertices  $V$  connected by a set of  $n - 1$  edges  $E$  that are directed away

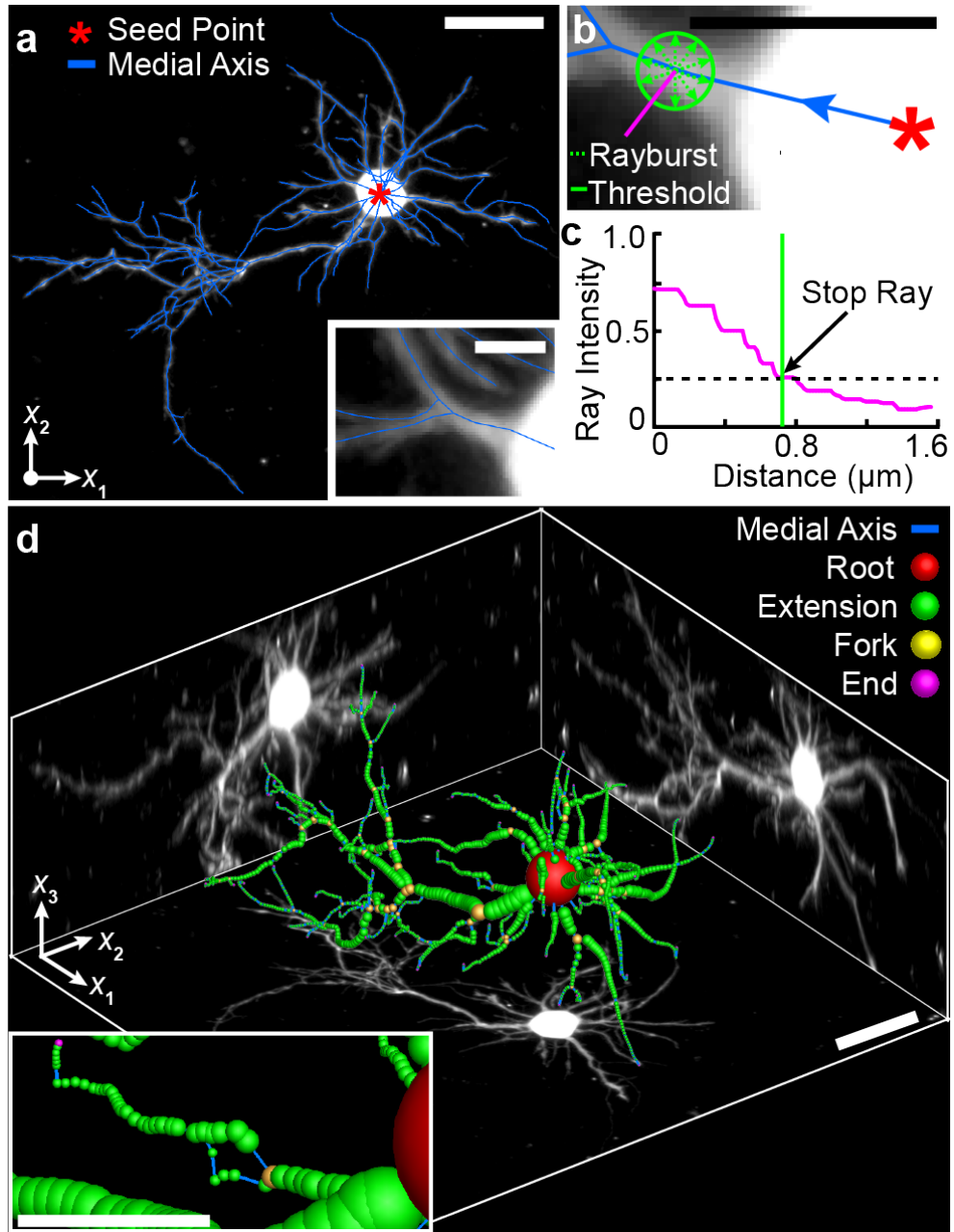


**Figure 4.9:** Tree data structure with a set of nodes connected by edges directed away from a root (red). Nodes are categorized by the number of directly connected children or degree ( $d$ );  $d = 1$  (green), extension;  $d > 1$ , fork (yellow); and  $d = 0$ , end (magenta). Tree traversal is performed along paths, defined by a sequence of connected nodes without repetition

from a root that has no parent node (**Fig. 4.9**). Nodes are classified by the degree ( $d$ ) or number of children that directly follow, i.e.  $d = 1$ , extension;  $d > 1$ , fork, and  $d = 0$ , end or leaf. For quantitative geometric and injury analysis, nodes and edges can store important information, e.g. position, radius, and strain. Implementation of this structure also allows easy traversal along any paths defined by a sequence of nodes connected with edges without repetition.

To build a tree structure, each calcein AM image was exported as tiff format and loaded into Neuron Studio, a software that performs semi-automatic tracing and reconstruction of neuron structures from confocal images [228]. From a manually selected seed point at the soma center, Neuron Studio automatically thresholds and skeletonizes the image with a topology and geometry-preserving algorithm that computes the medial axis [292, 229]. (**Fig. 4.10a**). Branch diameters are estimated at each node along the skeleton or medial axis using the Rayburst sampling algorithm [292, 229]. In this algorithm, the image is trilinearly interpolated along ray vectors that are casted from a node, and are allowed to grow until a specified intensity is reached (**Fig. 4.10b,c**). This intensity value is automatically adjusted by Neuron Studio according to the local intensity distribution of the image. Final node diameters of the Rayburst spheres are estimated by computing the median ray vector distances for each node, which are less affected by local surface irregularities.

The Neuron Studio tracing and re-construction procedure as described produced a tree structure of the neuron, which was carefully edited for validity, i.e. extensions automatically built from other neurons in the image were carefully removed using the built in 3D viewer. To prevent undersampling, the "Discretization Ratio" parameter, which sets the sampling density of nodes was set to the minimum value of zero, producing the most dense nodal connectivity. Additionally, spurious extensions that were produced from image noise were removed by setting the "Minimum Length" parameter to 5  $\mu\text{m}$ . The image channel of the cell impermeant Alexa Fluor hydrazide dye was manually traced since the neuronal extensions were not as well defined. In most cases, the tree consisted of a soma and one to two short neurites.



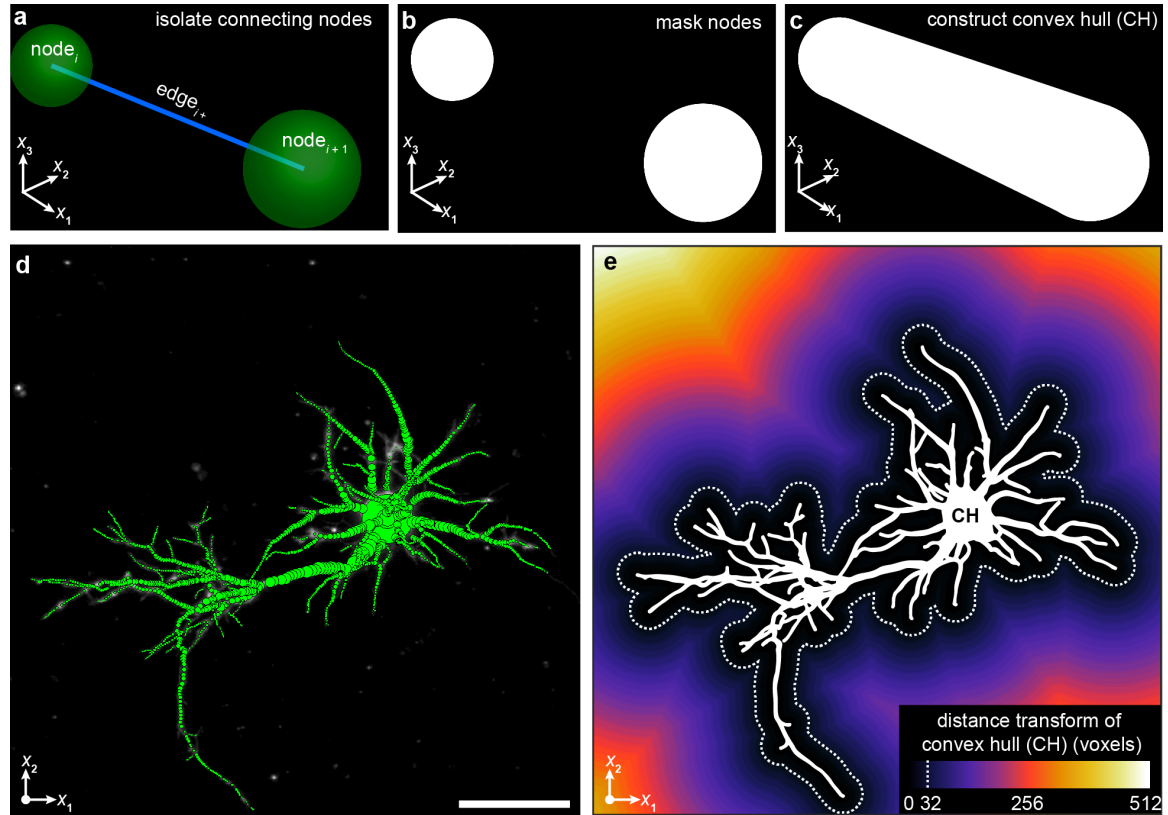
**Figure 4.10:** Building a tree structure from a volumetric image of a neuron. (a) Maximum intensity projection (MIP) of a neuron overlaid with its medial axis (blue) starting at a manually selected seed point (red, star). Scale bar, 20  $\mu\text{m}$ . Inset scale bar, 5  $\mu\text{m}$ . (b) Schematic of the Rayburst sampling algorithm showing ray vectors (green) being cast from a node. Scale bar, 5  $\mu\text{m}$ . (c) Image intensity along line (pink) shown in b. Ray vector growth is stopped at a prescribed intensity threshold (green, solid). (d) Neuron tree projected along the three coordinate planes. Node spheres are color coded by degree; root (red); extension (green); fork (yellow); and end (pink). Scale bar, 20  $\mu\text{m}$ . Inset scale bar, 5  $\mu\text{m}$ .

After editing, the neuron trees were exported as ‘swc’ format, read into MATLAB, and converted to a tree structure for quantitative injury analysis. Functions that were not custom written for a specific application came from the TREES toolbox [52], a graph theory library specifically for neuron tree structures in MATLAB.

#### 4.2.8.3 Image Post-Processing

**Note:** Jon B. Estrada assisted in developing the post-processing algorithm.

To accurately tag the formation of blebs or to determine when cell death occurs, all image information outside of the tree was removed (**Fig. 4.11a**). To perform this masking procedure, connecting nodes in the tree were isolated and converted to a nodal mask by setting interior voxels to 0 and exterior voxels to 1 (**Fig. 4.11b,c**). For each nodal mask,



**Figure 4.11:** Image post-processing to remove image information outside the neuron. (a) Neighboring nodes connected by an edge are isolated from (d) the neuron tree and converted into a (b) binary mask. (c) The convex hull (CH) of the binary mask in b was computed and iteratively superimposed to construct (e) the CH of the whole neuron (white). The final image mask was computed by setting all voxels within 32 voxels (dotted) away from CH to 1. Scale bar, 40  $\mu\text{m}$ .

a convex hull (CH) was computed and superimposed with the previous nodal mask using an OR logical operation to construct the CH of the whole cell (**Fig. 4.11d**). The final image mask was computed by dilating the CH of the whole cell using a Euclidean distance transform, ‘bwdist’ function, where all values within a distance of 32 voxels were set to 0 and outside were set to 1 (**Fig. 4.11e**).

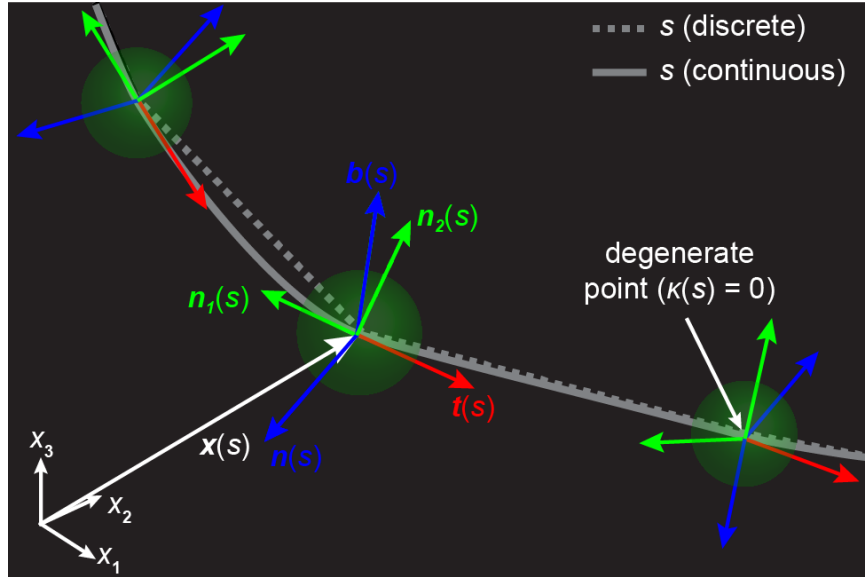
## 4.2.9 Quantitative Injury Analysis

### 4.2.9.1 Preliminary definitions of local coordinate frame for trees

Defining quantitative injury metrics requires the definition of a local coordinate frame that moves along the extensions of the neuron. Each extension, if treated like a smooth space curve,  $\mathbf{x}(s)$  parametrized as a function of  $s$ , can be described by a orthonormal Frenet frame,  $\mathbf{f}(s)$  [138]. At each point on the curve, the Frenet frame is defined as

$$\mathbf{f}(s) = \{\mathbf{t}(s), \mathbf{n}(s), \mathbf{b}(s)\}, \quad (4.2.8)$$

where  $\mathbf{t}(s)$ ,  $\mathbf{n}(s)$ , and  $\mathbf{b}(s)$  are the tangent, normal, and binormal unit vectors (**Fig. 4.12**(green)).



**Figure 4.12:** Schematic representation of local Frenet frame  $\mathbf{f}(s) = \{\mathbf{t}(s), \mathbf{n}(s), \mathbf{b}(s)\}$ , and Bishop frame ( $\mathbf{b}(s) = \{\mathbf{t}(s), \mathbf{n}_1(s), \mathbf{n}_2(s)\}$ ) on nodes (green) at positions  $\mathbf{x}(s)$ . Discrete node-to-node edges (solid black) are fitted with piecewise cubic splines (dotted black) to produce a  $C^2$  differentiable smooth curve that allow computation of tangent vectors  $\mathbf{t}(s)$  (red). Frenet frame normals,  $\mathbf{n}(s)$  and  $\mathbf{b}(s)$  (blue) can erratically change direction at degenerate points where  $\kappa(s) = 0$ , whereas Bishop frame normals,  $\mathbf{n}_1(s)$  and  $\mathbf{n}_2(s)$  (green) preserve direction.



They are mathematically expressed as

$$\mathbf{t}(s) = \frac{\mathbf{x}'(s)}{\|\mathbf{x}'(s)\|}, \quad (4.2.9)$$

$$\mathbf{n}(s) = \frac{\mathbf{t}'(s)}{\|\mathbf{t}'(s)\|} = \frac{\mathbf{x}'(s) \times (\mathbf{x}''(s) \times \mathbf{x}'(s))}{\|\mathbf{x}'(s)\| \|\mathbf{x}''(s) \times \mathbf{x}'(s)\|}, \quad (4.2.10)$$

and

$$\mathbf{b}(s) = \mathbf{t}(s) \times \mathbf{n}(s) = \frac{\mathbf{x}'(s) \times \mathbf{x}''(s)}{\|\mathbf{x}'(s) \times \mathbf{x}''(s)\|}. \quad (4.2.11)$$

The curvature  $\kappa(s)$ , and torsion  $\tau(s)$ , of  $\mathbf{x}(s)$  is related to  $\mathbf{t}(s)$ ,  $\mathbf{n}(s)$ , and  $\mathbf{b}(s)$ , and their derivatives by the Frenet-Serret system of differential equations [251]. Given in matrix form they are expressed as

$$\begin{bmatrix} \mathbf{t}'(s) \\ \mathbf{n}'(s) \\ \mathbf{b}'(s) \end{bmatrix} = \|\mathbf{x}'(s)\| \begin{bmatrix} 0 & \kappa(s) & 0 \\ -\kappa(s) & 0 & \tau(s) \\ 0 & -\tau(s) & 0 \end{bmatrix} \cdot \begin{bmatrix} \mathbf{t}(s) \\ \mathbf{n}(s) \\ \mathbf{b}(s) \end{bmatrix} \quad (4.2.12)$$

where

$$\kappa = \|\mathbf{t}'(s)\| = \frac{\|\mathbf{x}'(s) \times \mathbf{x}''(s)\|}{\|\mathbf{x}'(s)\|^3} \quad (4.2.13)$$

and

$$\tau = -\mathbf{n}(s) \cdot \mathbf{b}'(s) = \frac{(\mathbf{x}'(s) \times \mathbf{x}''(s)) \cdot \mathbf{x}'''(s)}{\|\mathbf{x}'(s) \times \mathbf{x}''(s)\|^2}. \quad (4.2.14)$$

Although the Frenet frame,  $\mathbf{f}(s)$ , can be computed along the extensions of the cell, its rotation about  $\mathbf{t}(s)$  can behave erratically at degenerate points where the curvature vanishes, e.g. inflection points or straight segments (**Fig. 4.12**). This undesirable rotation of  $\mathbf{n}(s)$  and  $\mathbf{b}(s)$  causes  $\tau(s)$  to have unphysical origin. In 1975, Bishop introduced an alternative frame based on parallel transport that forces the normal vectors to vary smoothly throughout the path regardless of  $\kappa(s)$  [23]. At each point along the curve, the orthonormal Bishop frame  $\beta(s)$  is defined as

$$\beta(s) = \{\mathbf{t}(s), \mathbf{n}_1(s), \mathbf{n}_2(s)\}, \quad (4.2.15)$$

where  $\mathbf{t}(s)$  is the tangent vector given in equation (4.2.9), and  $\mathbf{n}_1(s)$ , and  $\mathbf{n}_2(s)$  are the two

normal vectors (**Fig. 4.12**(blue)). This frame has been successfully applied to simulate the physics of elastic rods [20], and ropes and strings [16]; model trees [24]; and design stable motion of moving cameras [74]. In this study, the well defined geometric properties of the curve,  $\mathbf{t}(s)$  and  $\kappa(s)$ , will be computed and used to define a discrete Frenet frame and Bishop frame.

#### 4.2.9.2 Implementation of a discrete local coordinate frame for trees

In practice, using equations (4.2.8) – (4.2.15) to define a local coordinate frame is numerically challenging for many reasons. Extensions are not made of smooth curves, but piecewise linear functions that can sharply change direction at certain nodes due to image noise. Additionally, node sampling is highly dependent on the tracing parameters in Neuron Studio. To circumvent these issues, a series of processing steps was performed before computing the local coordinate frame.

##### *Tree resampling*

To begin the processing procedure, the neuron tree was resampled using the ‘resample\_tree’ function in the TREES toolbox, which redistributes the nodes at a user defined equal distance away from each other. The resampling distance was chosen as the 25th percentile of all edge lengths, such that most geometric features are not lost. Using the Euclidean distance metric, the length of the  $i$ th of  $n - 1$  edges,  $l_i$  is given by

$$l_i = \sqrt{\sum (\mathbf{x}_i - \mathbf{x}_{i+1})^2}, \quad (4.2.16)$$

where  $\mathbf{x}_i$  and  $\mathbf{x}_{i+1}$  are locations of neighboring nodes.

##### *Tree smoothing*

After resampling, trees were smoothened to eliminate sharp corners using Taubin smoothing, an algorithm commonly used to remove noise on polygonal meshes while preserving salient shape features [273, 272, 274]. To adapt this algorithm for trees, first the discrete Laplacian of  $\mathbf{x}$  is computed for each extension node ( $d = 1$ ) with the expression

$$\Delta \mathbf{x}_i(s) = \frac{1}{2}(\mathbf{x}_{i-1}(s) - \mathbf{x}_i(s)) + \frac{1}{2}(\mathbf{x}_{i+1}(s) - \mathbf{x}_i(s)). \quad (4.2.17)$$

Geometrically,  $\Delta \mathbf{x}_i$  is the average positions of neighboring nodes to the  $i$ th node. In general there are more advanced weighting schemes for the discrete Laplacian operator. However, since the tree is resampled at equidistant points, the effects would be minimal. A Laplacian filter is then applied by updating the position  $\mathbf{x}$  with  $\Delta \mathbf{x}$ , given by the equation

$$\mathbf{x}(s) = \mathbf{x}(s) + \lambda \Delta \mathbf{x}(s) \quad (4.2.18)$$

where  $\lambda$  is a scale factor that controls the speed of smoothing, and is  $0 < \lambda < 1$ . The Laplacian filter must be applied iteratively a large number of times to produce significant smoothing. However, by doing so would result in large tree contraction or shrinking (**Fig. 4.13c**). To overcome this issue, Taubin incorporated an expansion step following each contraction step (**Fig. 4.13d**). Mathematically, the expansion step is similar to equation (4.2.18), but  $\lambda$  is replaced with a negative scale factor  $\mu$  such that  $\mu < -\lambda$ . The resulting transfer function of the expansion and shrinking operation has a pass-band frequency given by

$$k_{PB} = \lambda^{-1} + \mu^{-1} > 0. \quad (4.2.19)$$

The practical application of Taubin smoothing is shown in algorithm 4.

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**Algorithm 4** Taubin smoothing of tree structure.

---

```

1: for ( $n = 0; n < N; n = n + 1$ ) do                                 $\triangleright$  N is number of iterations
2:    $\Delta \mathbf{x}(s) = \text{Laplacian}(\mathbf{x}(s))$                          $\triangleright$  compute discrete Laplacian, equation (4.2.17)
3:   if  $n$  is even then
4:      $\mathbf{x}(s) = \mathbf{x}(s) + \lambda \Delta \mathbf{x}(s)$                          $\triangleright$  update position with shrinking step
5:   else
6:      $\mathbf{x}(s) = \mathbf{x}(s) + \mu \Delta \mathbf{x}$                              $\triangleright$  update position wiht expansion step
7:   end if
8: end for

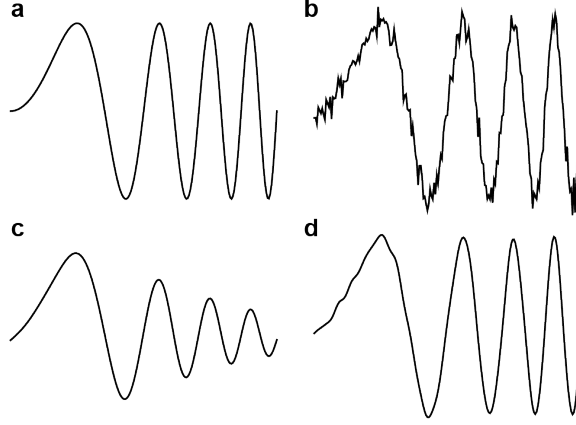
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As in **Fig. 4.13d**, all trees in this study were smoothed 100 times with  $\lambda = 0.63$  and  $k_{PB} = 0.1$ , chosen from [274].

#### *Computing discrete tangent and curvature of a tree*

Now that the trees are sufficiently smooth, the well defined properties of the curve,  $\mathbf{t}(s)$  and  $\kappa(s)$ , can be computed. There are many methods to compute these quantities numerically



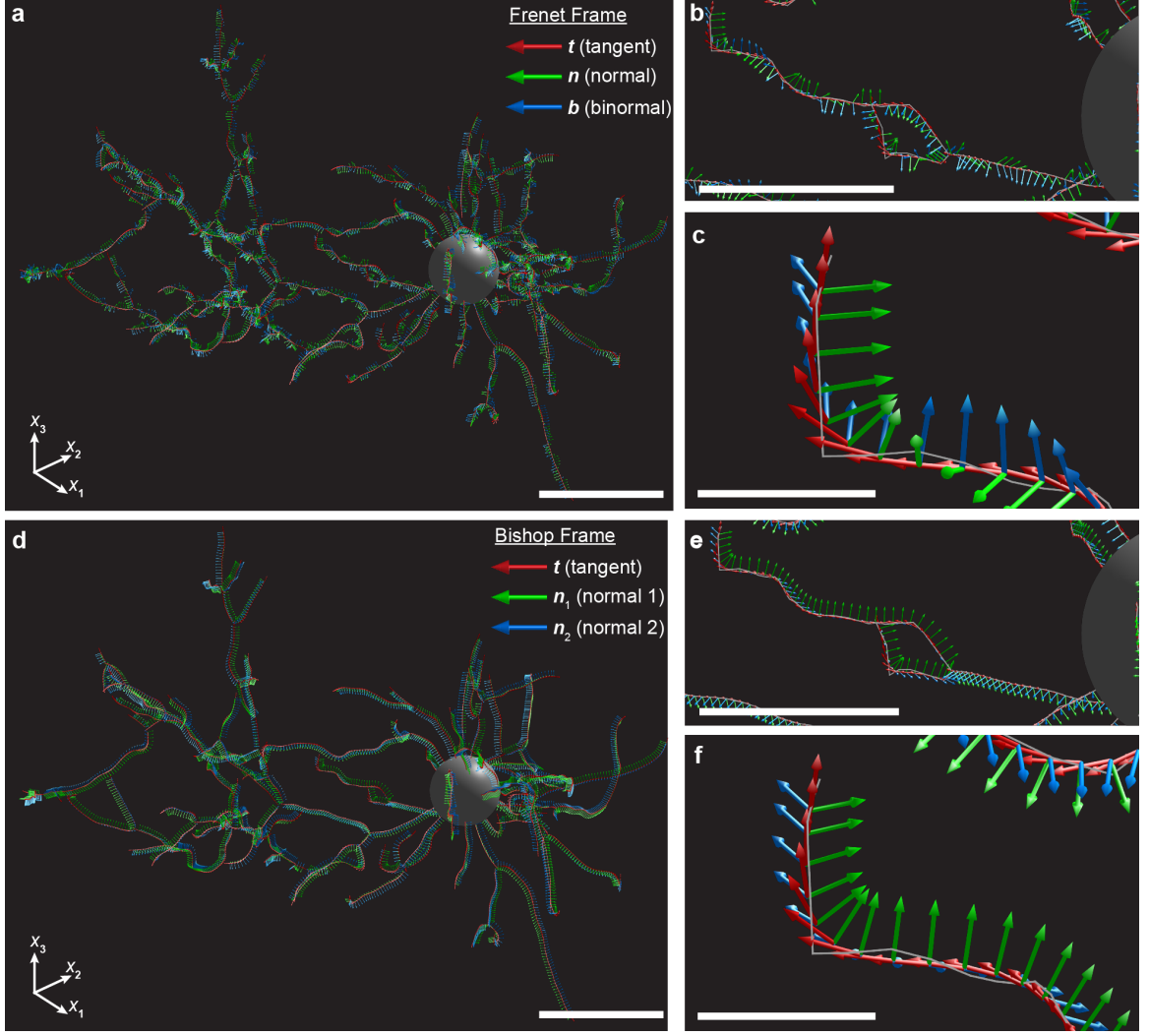
**Figure 4.13:** Comparison of smoothing algorithms. (a) Input test function defined as  $x(s) = \sin(2\pi s^2)$ . (b)  $x(s)$  corrupted with normally distributed noise (standard deviation = 1). (c) Significant shrinking of  $x(s)$  after 100 iterations of Laplacian smoothing with  $\lambda = 0.63$ , and clamped end points (d)  $x(s)$  restoration after 100 iterations of Taubin smoothing with  $\lambda = 0.63$ ,  $k_{PB} = 0.1$ , and clamped end points.

[108, 264]. In this study, a piecewise cubic spline ( $C^2$  differentiable) was fit to a set of tree paths using the “spline” function in MATLAB. Each path was defined as set of extension type nodes that connect a root and fork, root and end, fork and fork, or fork and end. As oppose to other methods, cubic splines return a set of third order polynomial coefficients that can be analytically evaluated to compute the geometric features at any point. The piecewise polynomial function that describes  $\mathbf{x}(s)$  and its first, second, and third derivatives is given by

$$\begin{aligned}
 \mathbf{x}(s) &= \mathbf{C}_0 + \mathbf{C}_1 s + \mathbf{C}_2 s^2 + \mathbf{C}_3 s^3 \\
 \mathbf{x}'(s) &= \mathbf{C}_1 + \mathbf{C}_2 s + \mathbf{C}_3 s^2 \\
 \mathbf{x}''(s) &= \mathbf{C}_2 + \mathbf{C}_3 s \\
 \mathbf{x}'''(s) &= \mathbf{C}_3
 \end{aligned} \tag{4.2.20}$$

where  $\mathbf{C}_0$ ,  $\mathbf{C}_1$ ,  $\mathbf{C}_2$ , and  $\mathbf{C}_3$  are an array of fit coefficients for each dimension of  $\mathbf{x}(s)$ .

Using equations (4.2.20) and (4.2.9) – (A.5.10), the Frenet frame can be computed at any point (**Fig. 4.14a,c,d**). It should be noted that since  $\mathbf{x}'''(s)$  is disconnected at nodes, the torsion and thus the twist of  $\mathbf{n}(s)$  and  $\mathbf{b}(s)$  will exhibit erratic behavior. This undesired behavior is not seen in  $\mathbf{t}(s)$  and  $\kappa(s)$  since  $\mathbf{x}'(s)$  and  $\mathbf{x}''(s)$  are smoothly connected at nodes.



**Figure 4.14:** Comparison of local (a-c) Frenet frame  $\mathbf{f}(s) = \{\mathbf{t}(s), \mathbf{n}(s), \mathbf{b}(s)\}$ , and (d-f) Bishop frame  $\mathbf{b}(s) = \{\mathbf{t}(s), \mathbf{n}_1(s), \mathbf{n}_2(s)\}$  computed for a neuron tree. (a,d) Scale Bar, 20  $\mu\text{m}$  (b,e) Scale Bar, 10  $\mu\text{m}$  (c,f) Scale Bar, 2  $\mu\text{m}$ .

Higher order splines can be implemented to resolve this issue but that is left for future research. For comparison, the discrete Bishop frame was computed along the tree using a simple algorithm detailed in [100] (Fig. 4.14b,e,f).

#### 4.2.9.3 Local Strain Transformation

The strain that neurons experience is highly dependent on the local orientations of their neurites. For example, a neurite that is aligned with the principal loading axis will experience more strain than one that is transversely aligned with the loading axis. Analysis for this orientation dependent deformation draws from classic laminate theory [127] and strain

rosette calculations [18], where the global strain or stress is transformed to coordinates of the material fibers or strain gauges. For both of these cases, the analysis is simplified using plane stress assumptions, which is not valid for neurons. To extend the theory into 3D, the far field strain  $\mathbf{E}^\infty$  must be rotated to the direction of each edge in the neuron tree. The transformation is expressed as

$$\mathbf{E}^{(f)} = \mathbf{Q} \cdot \mathbf{E}^\infty \cdot \mathbf{Q}^T. \quad (4.2.21)$$

where  $\mathbf{E}^{(f)}$  is strain matrix in the local basis,  $\mathbf{Q}$  is the rotation matrix.  $\mathbf{E}^\infty$  is given from the strain applied by the cell compression device and the quasistatic Poisson's ratio  $\nu$  as

$$\mathbf{E}^\infty = 0.3 \cdot \begin{bmatrix} \nu & 0 & 0 \\ 0 & \nu & 0 \\ 0 & 0 & -1 \end{bmatrix}. \quad (4.2.22)$$

Assuming a discrete Frenet frame ( $\mathbf{f}$ ) along  $\mathbf{x}(s)$ ,  $\mathbf{Q}$  is given by

$$\mathbf{Q} = \begin{bmatrix} \mathbf{b} \cdot \mathbf{e}_1 & \mathbf{b} \cdot \mathbf{e}_2 & \mathbf{b} \cdot \mathbf{e}_3 \\ \mathbf{n} \cdot \mathbf{e}_1 & \mathbf{n} \cdot \mathbf{e}_2 & \mathbf{n} \cdot \mathbf{e}_3 \\ \mathbf{t} \cdot \mathbf{e}_1 & \mathbf{t} \cdot \mathbf{e}_2 & \mathbf{t} \cdot \mathbf{e}_3 \end{bmatrix} \quad (4.2.23)$$

where  $\mathbf{e}$  is the global Cartesian coordinate frame and the tangent vector,  $\mathbf{t}$  is aligned with loading axis,  $\mathbf{e}_3$ . Applying the rotation in equation (4.2.23) to  $\mathbf{E}^{(f)}$ , equation (4.2.21) becomes

$$\mathbf{E}^{(f)} = \begin{bmatrix} \mathbf{b} \cdot \mathbf{E}^\infty \cdot \mathbf{b} & \mathbf{b} \cdot \mathbf{E}^\infty \cdot \mathbf{n} & \mathbf{b} \cdot \mathbf{E}^\infty \cdot \mathbf{t} \\ & \mathbf{n} \cdot \mathbf{E}^\infty \cdot \mathbf{n} & \mathbf{n} \cdot \mathbf{E}^\infty \cdot \mathbf{t} \\ & & \mathbf{t} \cdot \mathbf{E}^\infty \cdot \mathbf{t} \end{bmatrix} \quad (4.2.24)$$

Since  $\mathbf{t}$  is the most uniquely defined geometric quantity in the system, the axial components of  $\mathbf{E}^{(f)}$  will only be used to quantify the injury of a neuron. The axial strain is defined as

$$E_c = \mathbf{t} \cdot \mathbf{E}^\infty \cdot \mathbf{t}, \quad (4.2.25)$$

and the axial shear strain is defined as

$$E_s = \sqrt{(\mathbf{n} \cdot \mathbf{E}^\infty \cdot \mathbf{t})^2 + (\mathbf{b} \cdot \mathbf{E}^\infty \cdot \mathbf{t})^2}. \quad (4.2.26)$$

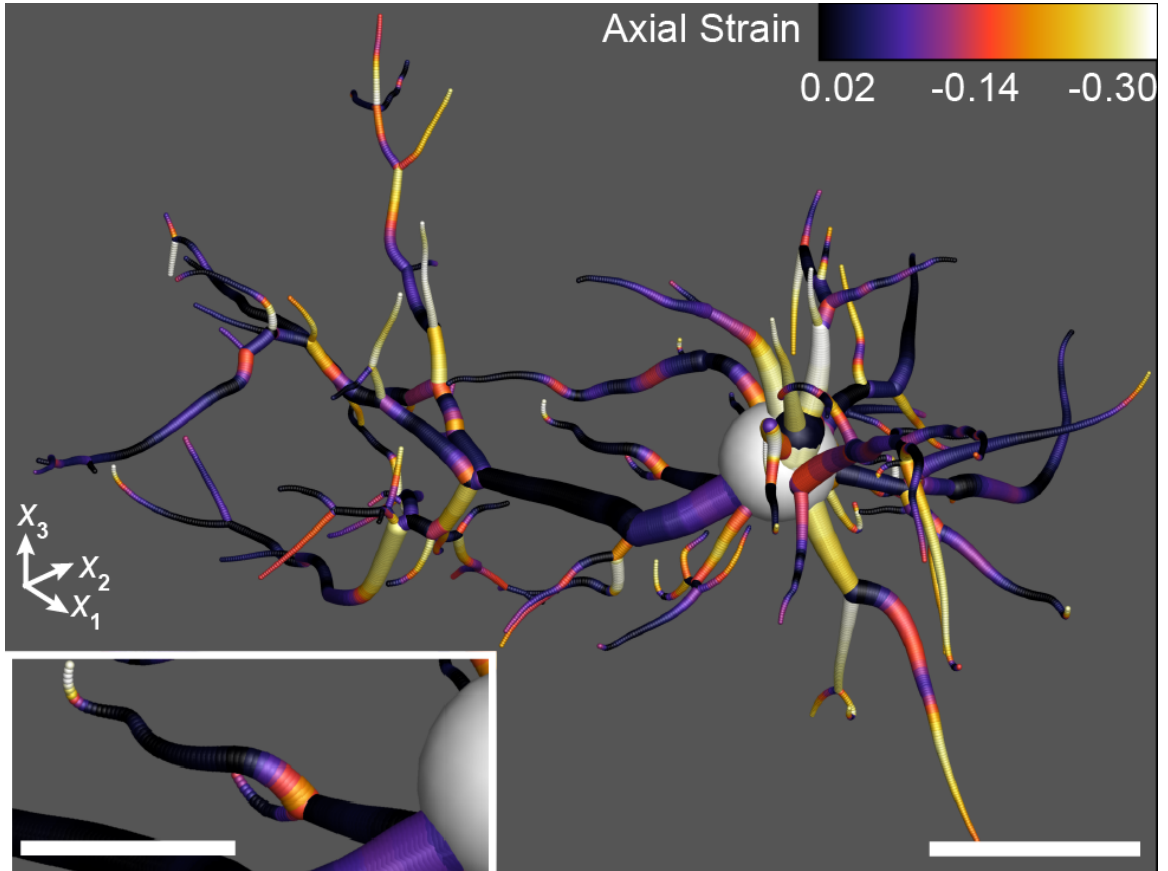
After equations (4.2.25) and (4.2.26) are computed for every point along a neuron, the mean axial strain is defined as

$$\langle E_c \rangle = \frac{1}{L} \int_{\mathbf{x}(s)} E_c(s) ds \quad (4.2.27)$$

and the mean axial shear strain is defined as

$$\langle E_s \rangle = \frac{1}{L} \int_{\mathbf{x}(s)} E_s(s) ds \quad (4.2.28)$$

where  $L$  is the total length of the neuron, i.e.  $L = \int_{\mathbf{x}(s)} ds$ .



**Figure 4.15:** (a) Axial strain computed with equation 4.2.25 along a neuron tree. Scale bar, 20  $\mu\text{m}$ . Inset scale bar, 10  $\mu\text{m}$ .

#### 4.2.9.4 Injury Assessment

##### *Bleb Formation*

For each neuron that exhibited clear blebbing, a time series of maximum intensity projection images was created. The image at the time point at which severe blebbing occurred was loaded into flNeuronTool [185], a software for 3D visualization and proofreading of neuronal morphologies. All blebs were tagged using the ‘probing’ mode and imported into MATLAB for analysis. The maximum compressive axial strain and axial shear strain from the soma of all nodes within a specified radial distance of the bleb was computed. This distance was equal  $3.35 \pm 0.73 \text{ } \mu\text{m}$ , computed as the mean bleb radius of 26 blebs as manually recorded from three neurons

##### *Membrane Poration*

AFH trees were loaded into MATLAB and each node sphere was converted into a binary mask. Neurons were considered porated if the mean intensity within the mask increased to the mean background intensity. The same analysis was performed for the membrane poration controls.

##### *Cell Death Assessment*

Time of cell death was defined at the time at which fading of viability dye calcein AM, somatic lysis, or severe morphological deformities occurred.

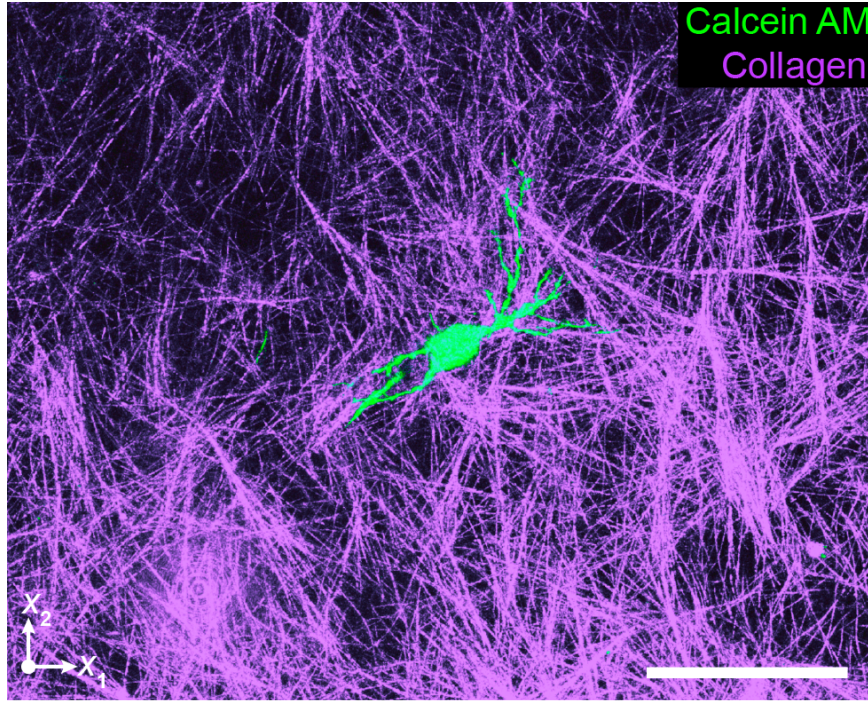
##### *Bivariate compressive axial Strain Fitting and Probability Density Estimation*

The scatter data of the mean compressive axial strain  $\langle E_c \rangle$  as a function of time at cell death  $t_d$  was robustly fit to a bivariate normal distribution using the minimum covariance determinant (MCD) estimator [232, 233] implemented in the LIBRA MATLAB library [280]. MCD is commonly used in bivariate fitting since outliers in the data can greatly influence the fitting. The major and minor axis of the 95 % hyperellipse were computed from the eigenvalues of the covariance matrix of the fit. To qualitatively confirm the validity of the bivariate normal distribution, the 2D probability density was computed using the commonly used non-parametric adaptive algorithm for kernel density estimation [25].



### 4.3 Results and Discussion

A new 3D *in vitro* model was developed to investigate the secondary effects of DAI in compression. Cortical neurons were embedded in fibrillar type I collagen gels of final concentration of 2.2 mg/mL, resulting in a heterogeneous fibrillar microstructure as imaged with confocal reflectance microscopy (**Fig. 4.16**). After maturation for seven days *in vitro*, the collagen cultures demonstrate healthy network formation and viability (**Fig. 4.24a,b**). To reproduce DAI, the collagen cultures were subjected to compressive far field loading applied by a cell compression device at strain magnitudes of 0.30 and peak strain rates of  $10 \text{ s}^{-1}$  and  $75 \text{ s}^{-1}$  (**Fig. 4.6**).

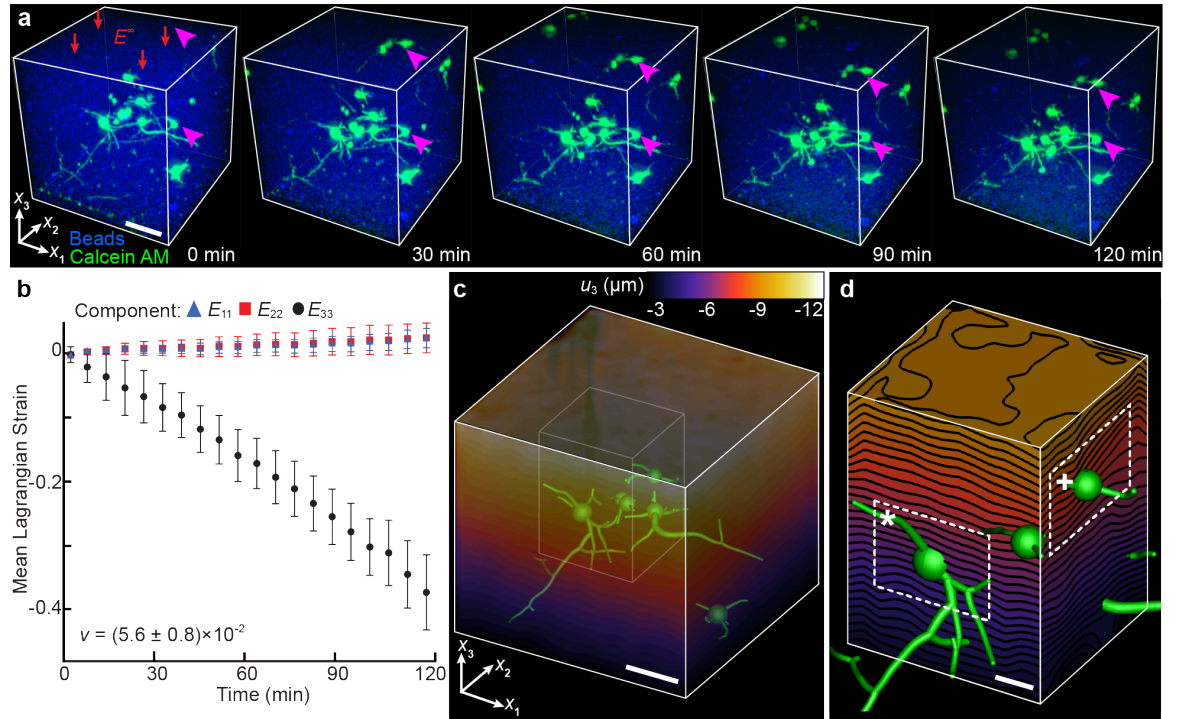


**Figure 4.16:** Maximum intensity projection of a neuron (green) stained with calcein AM that is encapsulated in a dense fibrillar collagen matrix (light purple) imaged with confocal reflectance microscopy. Scale bar,  $50 \mu\text{m}$ .

#### 4.3.1 Local Deformation Field Characterization

The far field strain  $\mathbf{E}^\infty$  given in equation (4.2.22) applied by the cell compression device to the collagen needs to be validated in order to apply the local strain transformation to the cells given in equations (4.2.25) and (4.2.26). The deformation transfer was analyzed by measuring the spatial distributions of the local displacement field around the neurons with fast iterative

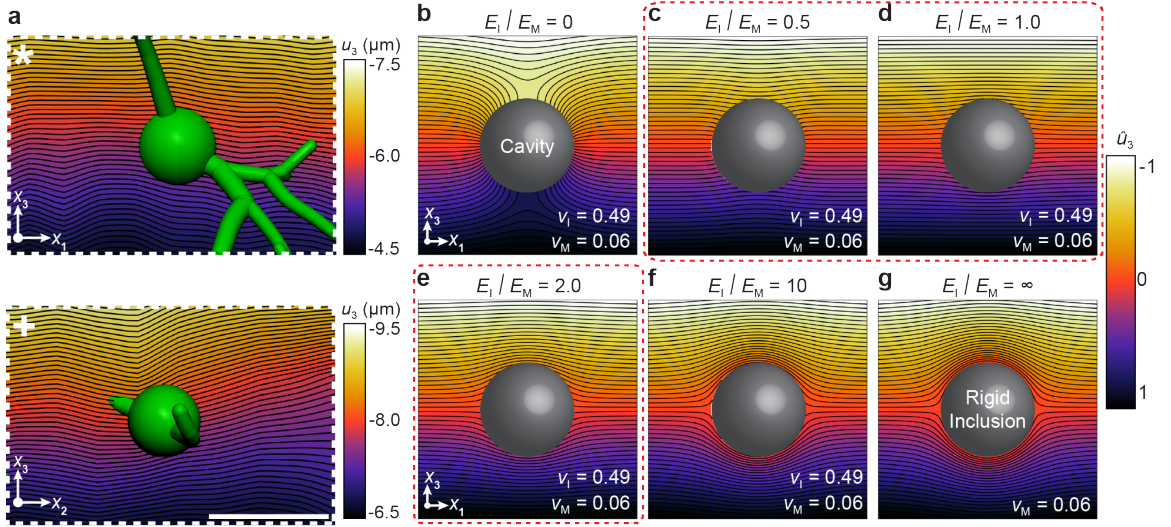
digital volume correlation (FIDVC) [15]. In this experiment, collagen cultures embedded with tracker beads were compressed at increments of 2% far-field strain  $E^\infty$  and then imaged with confocal microscopy (**Fig. 4.17a**). The corresponding mean Lagrangian strain computed from the displacements (equation (4.2.1)) shows that collagen is very compressible with a Poisson's ratio of  $(5.6 \pm 0.8) \times 10^{-2}$  (**Fig. 4.17b**). Qualitative assessment of the spatial distribution of the displacement along the loading axis  $u_3$  shows that the collagen surrounding the neurons is being uniformly compressed due to  $E^\infty$  (**Fig. 4.17c,d**). These



**Figure 4.17:** Characterization of the uniform applied far field strain  $E^\infty$  under quasistatic loading. (a) Maximum intensity confocal micrographs of collagen culture with fluorescent beads (blue) and neurons (green) used to characterize the deformation field. Two neurons are tagged (magenta) for every time point to show the incremental compression of the collagen. Scale bar,  $50 \mu\text{m}$ . (b) Plot of the mean and standard deviation of the transverse  $E_{11}$  (blue triangle) and  $E_{22}$  (red square), and axial  $E_{33}$  components of the mean Lagrangian strain tensor per loading increment as computed from volumetric images shown in a using fast iterative digital volume correlation (FIDVC) [15]. The collagen is highly compressible with a Poisson's ratio  $\nu = (5.6 \pm 0.8) \times 10^{-2}$ . (c) Contour plot of the displacement along the loading axis  $u_3$  in the collagen matrix surrounding the neurons (green). The neurons renderings were built from the tree data structure. Scale bar,  $20 \mu\text{m}$ . (d) Magnified results of box (white) embedded in c. Box dimensions are chosen to cut the midplane of neuron somas along the  $x_1 - x_3$  (\*) and  $x_2 - x_3$  (+) planes. Magnified views of the rectangle (dotted) surrounding \* and + are provided in **Fig. 4.18a**. Scale bar,  $20 \mu\text{m}$ .

results show that the far field uniaxial compression  $\mathbf{E}^\infty$  applied by the cell compression device is transferred uniformly to the collagen matrix surrounding the cells with minimal transverse stretch due to the Poisson effect.

To obtain an order of magnitude estimate of the displacement continuity across the cell-matrix interface, the experimentally determined  $u_3$  (**Fig. 4.17**) was compared to the analytical solution of a spherical inclusion (with radius  $a = 5$  voxels, Young's Modulus  $E_I$ , Poisson's Ratio  $\nu_I = 0.49$ ) in an infinite matrix (with Young's Modulus  $E_M$ , Poisson's Ratio  $\nu_M = 5.6 \times 10^{-2}$ ) subject to a uniaxial far-field stress [97] (**Fig. 4.18**). The solution for the displacement  $u_3$  normalized by its maximum value was computed for different mismatch ratios of Young's modulus; e.g.  $E_I/E_M = 0$  is the spherical cavity solution,  $E_I/E_M = 1$  is the uniform strain solution, and  $E_I/E_M = \infty$  is the rigid inclusion solution. Qualitative assessment of  $u_3$  near the soma shows good agreement with  $\hat{u}_3$  for  $E_I/E_M = 0.5 - 2.0$ , suggesting that there is displacement continuity at the cell-matrix interface.



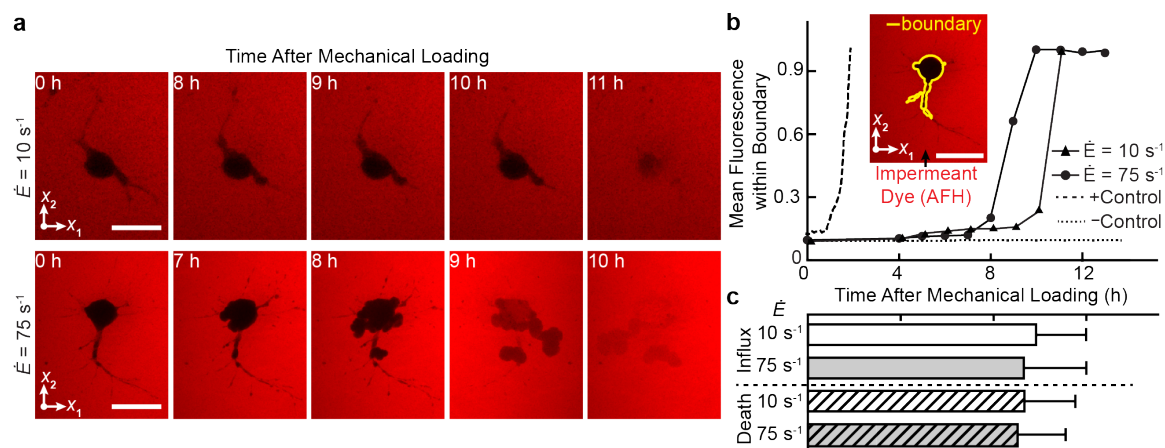
**Figure 4.18:** Comparison of local displacement field around neurons to the spherical inclusion solution. (a) Magnified contour plots of the displacement field along the loading axis  $u_3$  **Fig. 4.18d**. Scale bar,  $20 \mu\text{m}$ . (b-g) Analytical solution of a spherical inclusion (gray) (with radius  $a = 5$  voxels, Young's Modulus  $E_I$ , Poisson's Ratio  $\nu_I = 0.49$ ) in an infinite matrix (with Young's Modulus  $E_M$ , Poisson's Ratio  $\nu_M = 5.6 \times 10^{-2}$ ) subject to a uniaxial far-field stress [97]. The solution for the displacement normalized by its maximum value  $\hat{u}_3$  along the loading axis  $x_3$  is presented for Young's modulus mismatch ratios  $E_I/E_M$  of (b) 0 (spherical cavity solution), (c) 0.5, (d) 1.0 (uniform strain solution), (e) 2.0, (b) 10, and (b)  $\infty$  (rigid inclusion solution). c-e (dotted red) are in good agreement with  $u_3$  in a.



The quasistatic assumption may not hold for dynamic loading since many fibrillar materials are viscoelastic [80]. However, given the current limitations in 3D imaging, these measurements serve as an estimation of the true strain field, used to determine the contribution of cell orientation to injury. Other studies have characterized the strain field by dynamically loading and holding the cell culture at maximum strain [146, 143] but were unable to capture the local spatial variations in the displacement field since the motion of three particles were traced.

### 4.3.2 Cell Membrane Mechanoporation in Compression

The normally cell-impermeant dye Alexa Fluor 568 hydrazide (AFH) was utilized to evaluate the pathophysiological cell membrane disruptions following mechanical loading. A cell with an intact membrane will not allow influx of AFH, and therefore will not internally fluoresce, until membrane integrity is compromised (**Fig. 4.19a**). To quantitatively assess



**Figure 4.19:** Mechanoporation does not occur in compression since there is no influx of normally cell-impermeant dye Alexa Fluor 568 hydrazide (AFH) until cell lysis and death. (a) Time series of minimum intensity confocal micrographs of cell in the presence of AFH after mechanical loading at strain rates  $\dot{E}$  of  $10 \text{ s}^{-1}$  (top) and  $75 \text{ s}^{-1}$  (bottom). Scale bar,  $20 \mu\text{m}$ . (b) AFH influx was quantitatively assessed by computing the mean fluorescence intensity within the cell boundary (inset) for  $\dot{E} = 10 \text{ s}^{-1}$  (solid triangle),  $\dot{E} = 75 \text{ s}^{-1}$  (solid circle), a positive control (long dash) by introduction of Triton X-100, and a negative control (short dash) without mechanical loading. Scale bar,  $20 \mu\text{m}$ . (c) The mean and standard deviation of the influx time for  $\dot{E} = 10 \text{ s}^{-1}$  (solid white;  $n = 16$  cells,  $N = 5$  experiments) and  $\dot{E} = 75 \text{ s}^{-1}$  (solid gray;  $n = 23$  cells,  $N = 8$  experiments), and cellular death time for  $\dot{E} = 10 \text{ s}^{-1}$  (hatched white;  $n = 19$  cells,  $N = 8$  experiments) and  $\dot{E} = 75 \text{ s}^{-1}$  (hatched gray;  $n = 22$  cells,  $N = 8$  experiments). No significant difference was found using t-test. The influx time was computed as the peak rise of internal fluorescence of each cell as seen at time 9 h in b.

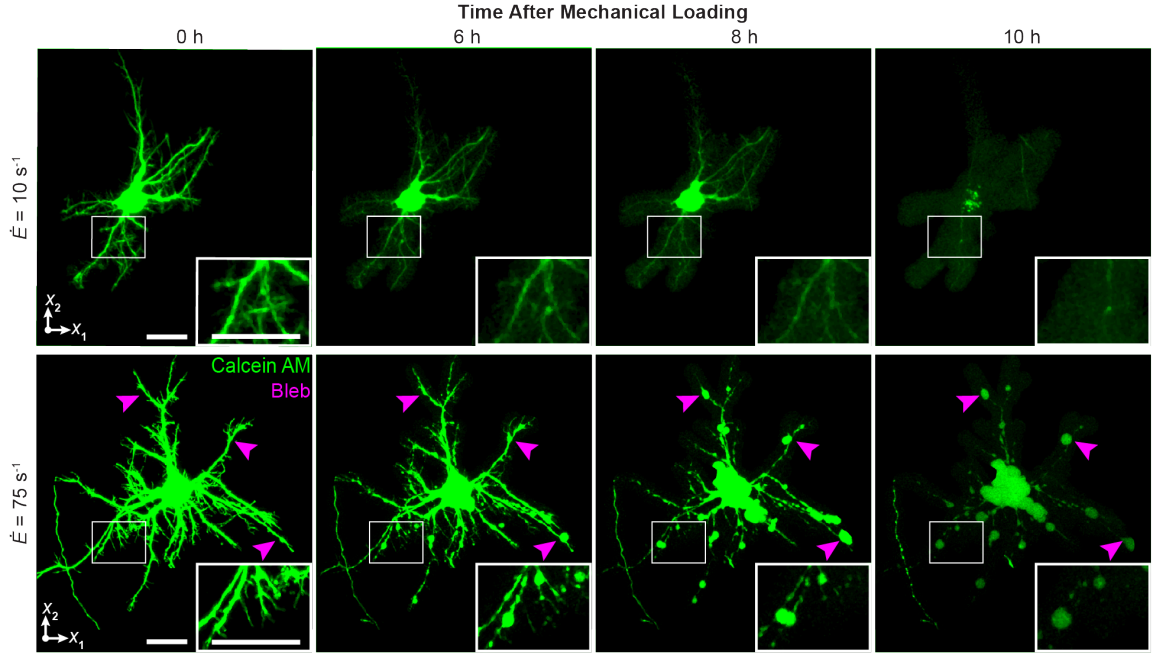
the AFH influx after mechanical loading, the mean intensity within the cell boundary was computed for both strain rates, and compared to a positive control by introducing Triton X-100 and a negative control without mechanical loading (**Fig. 4.19b**). The average influx time, computed as the peak rise of internal fluorescence for each cell, was compared to the average cellular death time for both strain rates (**Fig. 4.19c**).

Neurons undergoing both strain rates did not intake AFH until the cell membrane was severely fragmented (**Fig. 4.19a**). Importantly, these membrane disruptions are associated with cell death and not increased membrane permeability since the average influx time ( $9.8 \pm 2.2$  h for  $\dot{E} = 10 \text{ s}^{-1}$ , and  $9.3 \pm 2.7$  h for  $\dot{E} = 75 \text{ s}^{-1}$  h), as indicated by a sharp increase internal fluorescence (**Fig. 4.19b**), was not significantly different then the mean death time of the cell ( $9.3 \pm 2.2$  h for  $\dot{E} = 10 \text{ s}^{-1}$  and  $9.0 \pm 2.1$  h for  $\dot{E} = 75 \text{ s}^{-1}$  h) (**Fig. 4.19c**).

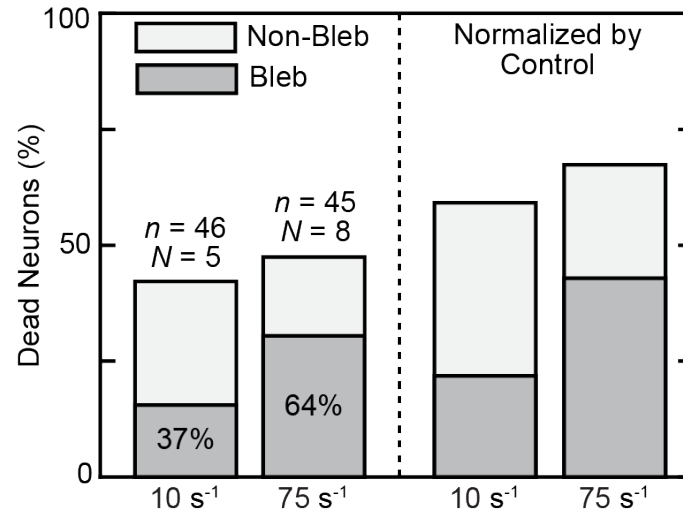
Previous *in vitro* models applying tension and shear have shown that membrane permeability increases after loading [64, 38, 86, 143, 50], even for significantly larger fluorescent dextran molecules (MW 150 kDa) [87]. In this study, the sharp rather than gradual increase of internal fluorescence (**Fig. 4.19b**) suggests that compression does not initiate the enzymatic formation of non-resealable secondary pores due to transient stretching (or mechanoporation) of the cell membrane. This finding is important since mechanoporation is suggested to lead to a host of secondary events associated with DAI such as axonal blebbing [92, 73, 32, 128, 118, 112, 178], which may not be present in areas of the brain that are undergoing compression.

### 4.3.3 Strain Rate Dependence on Bleb Formation

Local morphological injury indicators such as blebbing, retraction, and thinning of neurites were assessed for each strain rate after mechanical loading using the live cell dye calcein AM. Representative confocal micrograph time series of a neuron for each strain rate show that there is a strong morphopathological dependence on strain rate (**Fig. 4.20**). Neurons deforming at lower rates ( $\dot{E} = 10 \text{ s}^{-1}$ ) exhibited gradual retraction, thinning, and fading of their extensions, ultimately leading to somatic lysis, indicating cell death (**Fig. 4.20(top)**). Neurons at high strain rates ( $\dot{E} = 75 \text{ s}^{-1}$ ) also exhibited this retraction, thinning, and



**Figure 4.20:** Time series of maximum intensity confocal micrographs shows the strain rate ( $\dot{E}$ ) dependence of the morphological injury evolution of neurons after mechanical loading. Neurons undergoing  $\dot{E} = 10 \text{ s}^{-1}$  (top) exhibit gradual retraction, thinning, and fading of their extensions, ultimately leading to somatic lysis. Neurons undergoing  $\dot{E} = 75 \text{ s}^{-1}$  (bottom) exhibited retraction, thinning, and fading, and additionally formed large blebs at distinct points along the neurites (magenta) until somatic lysis. Scale bars,  $20 \mu\text{m}$ .



**Figure 4.21:** Stacked bar plots of the percentage cell death for  $\dot{E} = 10 \text{ s}^{-1}$  ( $n = 46$  cells,  $N = 5$  experiments) and  $\dot{E} = 75 \text{ s}^{-1}$  ( $n = 45$  cells,  $N = 8$  experiments) for cells exhibiting non-bleb (light gray) and bleb (dark gray) formation. The same data was normalized by the 7 days *in vitro* viability control (see supplementary **Fig. 4.24b**).

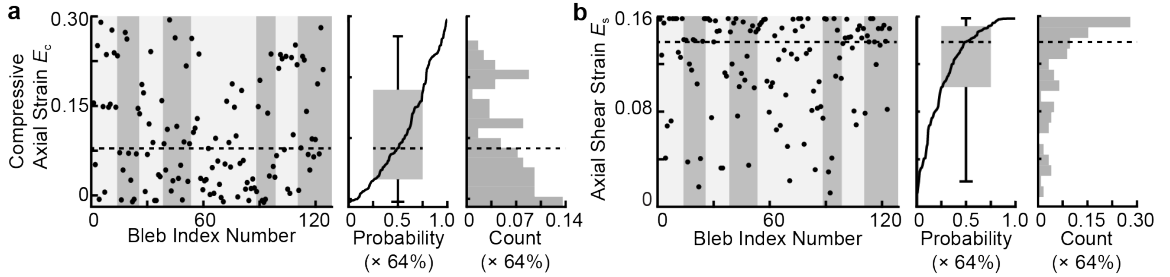
fading pathology, and additionally formed large focal swellings and blebs at distinct points along the neurites (**Fig. 4.20**(bottom)). Larger blebs for each cell tended to initiate at approximately the same time and grow in size until somatic lysis, whereas smaller blebs gradually increased in number and size producing local cell fragmentation. To quantify the symptomatic differences in blebbing, the population of dead cells for each strain rate was classified according to large bleb and non-bleb morphologies (**Fig. 4.21**). There was an approximately  $1.7\times$  increase in occurrence of bleb formation vs. strain rate, i.e. 37% of dead cells formed blebs at  $\dot{E} = 10 \text{ s}^{-1}$  and 64% formed blebs at  $\dot{E} = 75 \text{ s}^{-1}$ . This large difference in bleb vs. non-bleb occurrences is significant considering the small differences in percentage of total cell death, i.e. 42% (or 59% normalized by the 7 days *in vitro* viability control in supplementary **Fig. 4.24b**) for  $\dot{E} = 10 \text{ s}^{-1}$  ( $n = 46$  cells,  $N = 5$  experiments), and 48% (or 67% normalized by the 7 DIV viability control in supplementary **Fig. 4.24b**) for  $\dot{E} = 75 \text{ s}^{-1}$  ( $n = 45$  cells,  $N = 8$  experiments).

The bleb dependence on strain rate observed in this study may be due to the unique viscoelastic properties of the cytoskeleton [238, 219], i.e. along axons, elastic microtubule bundles are cross-linked by viscoelastic tau proteins [34, 2]. Ahmadzadeh et al. numerically modeled the failure of these viscoelastic structures and reported a critical strain rate of  $22 - 44 \text{ s}^{-1}$  [2], which is in agreement with previous experimental studies [256, 270]. At subcritical strain rates (e.g.  $\dot{E} = 10 \text{ s}^{-1}$  in this study), viscoelastic tau proteins allow the reversible sliding of microtubules [2]. However at supercritical rates, (e.g.  $\dot{E} = 75 \text{ s}^{-1}$  in this study), the strain stiffening of tau proteins facilitates the load transfer onto microtubules, causing local failure [2]. This microtubule failure at high strain rates may disrupt neurite transport causing the accumulation of vesicles and organelles, manifesting morphologically as bleb formation [130]. In regions of the brain that undergo compression, bleb formation associated with DAI may represent just one pathological subset of damaged neurons. Injured tissue containing neurons not exhibiting bleb formation may have been previously undetected using magnetic resonance imaging, computed axial tomography scans, or histological staining of postmortem brain tissue.

#### 4.3.4 Correlating Local Axial Strains with Bleb Formation

To assess if bleb formation is spatially correlated with strain magnitude, the local compressive axial strain  $E_c$  and axial shear strain  $E_s$  were computed via equations (4.2.25) and (4.2.26) for neurons exhibiting clear bleb formation. Specifically, the maximum value of  $E_c$  and  $E_s$  around the neighborhood of each manually tagged bleb was computed for eight neurons at  $\dot{E} = 75 \text{ s}^{-1}$  (**Fig. 4.22**). The inverse cumulative distribution function shows that 50% of blebs form at above or below  $E_c = 0.08$  and  $E_s = 0.14$ , corresponding to 32% of total dead cells exhibiting bleb formation at  $\dot{E} = 75 \text{ s}^{-1}$ . The histogram of  $E_c$  shows a multimodal distribution with the highest peak at  $E_c \approx 0.22$ , suggesting that bleb formation is not correlated with  $E_c$ . The histogram of  $E_s$  shows a skewed distribution with the highest peak at  $E_s \approx 0.16$ , suggesting that shear may be driving mechanical deformation behind bleb formation.

Bleb formation at locations not corresponding to high strain may be explained by the integrin mediated mechanical coupling between the extracellular matrix and the cytoskeleton [290]. By focally pulling on neurons with magnetic tweezers, Hemphill et al. found that depending on integrin (i.e. fibronectin and polylysine), blebs either form locally, i.e. at the injury site, or distally, i.e. away from the injury site [102]. This injury transmission may be caused by



**Figure 4.22:** Quantification of the local axial strains computed at locations with bleb formation on neurons. **(a)** Compressive axial strain  $E_c$  at each bleb location for eight neurons at  $\dot{E} = 75 \text{ s}^{-1}$  (circle). Alternate shading pattern denotes data points for every cell at  $\dot{E} = 75 \text{ s}^{-1}$ . (center) Corresponding inverse probability function and box and whiskers plot. Dotted band: medians, top and bottom boxes: upper and lower quartiles, whiskers: 5th and 95th percentiles. Probability of finding a bleb given a certain value of  $E_c$  is multiplied by 64%, the percentage of bleb formation at  $\dot{E} = 10 \text{ s}^{-1}$  (**Fig. 4.21**). (right) Corresponding histogram normalized by total count. **(b)** The same plot formats in **a** computed for the axial shear strain  $E_s$ .

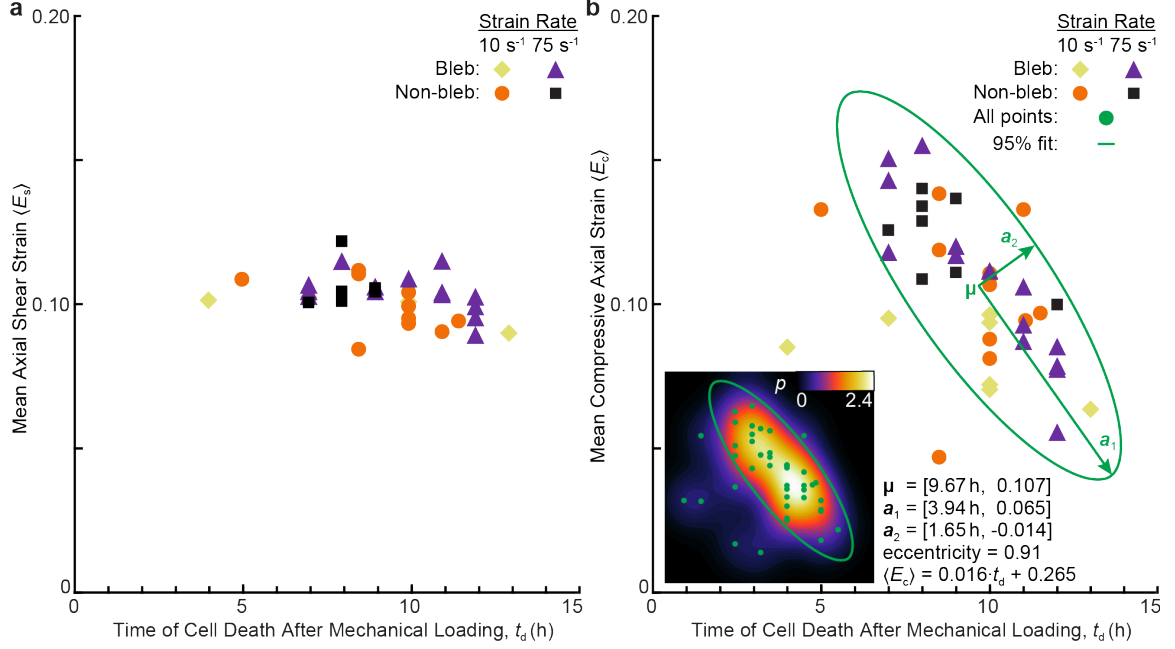


mechanical force propagation through the cytoskeleton, activating biochemical cascades that lead to bleb formation [182, 290, 40, 119]. Even with these results, blebs formed only in a subset of the total dead cells for both strain rates (**Fig. 4.21**), suggesting that cell death is driven by a different mechanism.

#### 4.3.5 Establishing Global Metrics for Cell Injury

Recent studies have shown that axonal tension is required for the essential functions of neurotransmission [253] and active vesicle transport [3]. This tensional homeostasis is carefully regulated by neurons [219] via the dynamic rebuilding of their cytoskeleton, which is tied with the focal adhesion complex [115, 116, 90]. It is hypothesized that global mechanical disruptions in this tensional homeostasis may lead to cell death.

To test this hypothesis, global injury metrics defined by the mean shear strain  $\langle E_s \rangle$  and the mean compressive axial strain  $\langle E_c \rangle$  were computed using equation (4.2.27) for all cells exhibiting bleb and non-bleb formation at both strain rates, and plotted as a function of time at which cell death was observed after mechanical loading  $t_d$  (**Fig. 4.23**). Regardless of strain rate and blebbing, data points were condensed to a narrow region in both plots of  $\langle E_s \rangle$  and  $\langle E_c \rangle$ . The shape of this region indicates the functional correlation between  $t_d$  and the respective global strain metric, e.g. the time of cell death  $t_d$  was the same regardless of  $\langle E_s \rangle$ , suggesting that cell death is not driven by  $\langle E_s \rangle$ . Qualitative examination of the plot of  $\langle E_c \rangle$  shows that there is a correlation between  $t_d$  and  $\langle E_c \rangle$ . This correlation was quantified by computing the minimum covariance determinant (MCD) estimator, which fit the data of  $\langle E_c \rangle$  excluding outliers to a bivariate normal distribution [232, 233]. To qualitatively confirm the fitting, the contour map of the bivariate probability density shows a normal-like distribution within the ellipse (**Fig. 4.23(inset)**) [25]. The hyper-ellipse shown represents an isocontour at 95% confidence of the normal distribution. The average time of death  $t_d$  and corresponding  $\langle E_c \rangle$ , given by the spatial location of the coordinate  $\mu$ , was 9.67 hours and 0.107 respectively. The stretch or elongation of an ellipse is quantified by its eccentricity  $e$ . For reference, as  $e \rightarrow 0$  an ellipse becomes a circle, and as  $e \rightarrow 1$  an ellipse collapses to a line. The eccentricity of the fitted ellipse was 0.91, signifying a highly elongated ellipse which can be primarily described by its major axis, i.e.  $\mathbf{a}_1 = [3.94 \text{ h}, 0.064]$ . The linear



**Figure 4.23:** Global injury drives cell death. (a) Mean axial shear strain  $\langle E_s \rangle$  computed for all cells exhibiting bleb formation at  $\dot{E} = 10 \text{ s}^{-1}$  (yellow diamond,  $n = 7$ ) and  $\dot{E} = 75 \text{ s}^{-1}$  (purple triangle,  $n = 12$ ), non-bleb formation at  $\dot{E} = 10 \text{ s}^{-1}$  (orange circle,  $n = 14$ ) and  $\dot{E} = 75 \text{ s}^{-1}$  (black square,  $n = 8$ ) as a function of time at which cell death is observed after mechanical loading  $t_d$ . (b) Mean compressive axial strain  $\langle E_c \rangle$  computed for all cells exhibiting bleb formation at  $\dot{E} = 10 \text{ s}^{-1}$  (yellow diamond,  $n = 7$ ) and  $\dot{E} = 75 \text{ s}^{-1}$  (purple triangle,  $n = 12$ ), non-bleb formation at  $\dot{E} = 10 \text{ s}^{-1}$  (orange circle,  $n = 14$ ) and  $\dot{E} = 75 \text{ s}^{-1}$  (black square,  $n = 8$ ) as a function of time at which cell death is observed after mechanical loading  $t_d$ . The hyper-ellipse (green) represents an isocontour at 95% confidence of the bivariate normal distribution fitting of all data points excluding outliers.  $\mu$  is the mean value of  $t_d$  and  $\langle E_c \rangle$ ,  $a_1$  is the major axis of the ellipse, and  $a_2$  is the minor axis of the ellipse. (inset) Contour map of the bivariate probability density  $p$  of all data points (green circle).

function computed from the major axis is given as  $\langle E_c \rangle(t_d) = 0.016t_d + 0.265$ , but it is only valid within the ellipse since external non-linear behavior might be expected.

These results suggest that cell death is primarily driven by global injury and in compression, the mean compressive axial strain. Specifically, the highly elongated shape shows that the time at which cells die is a linear function of the mean compressive axial strain. The quantitative envelope computed from the bivariate fitting of  $\langle E_c \rangle$  provides a more accurate description of the cellular death response to loading, i.e. cells within this envelope are most likely to undergo necrosis or apoptosis. This quantitative analysis also establishes guidelines of the time frame for therapeutic intervention.

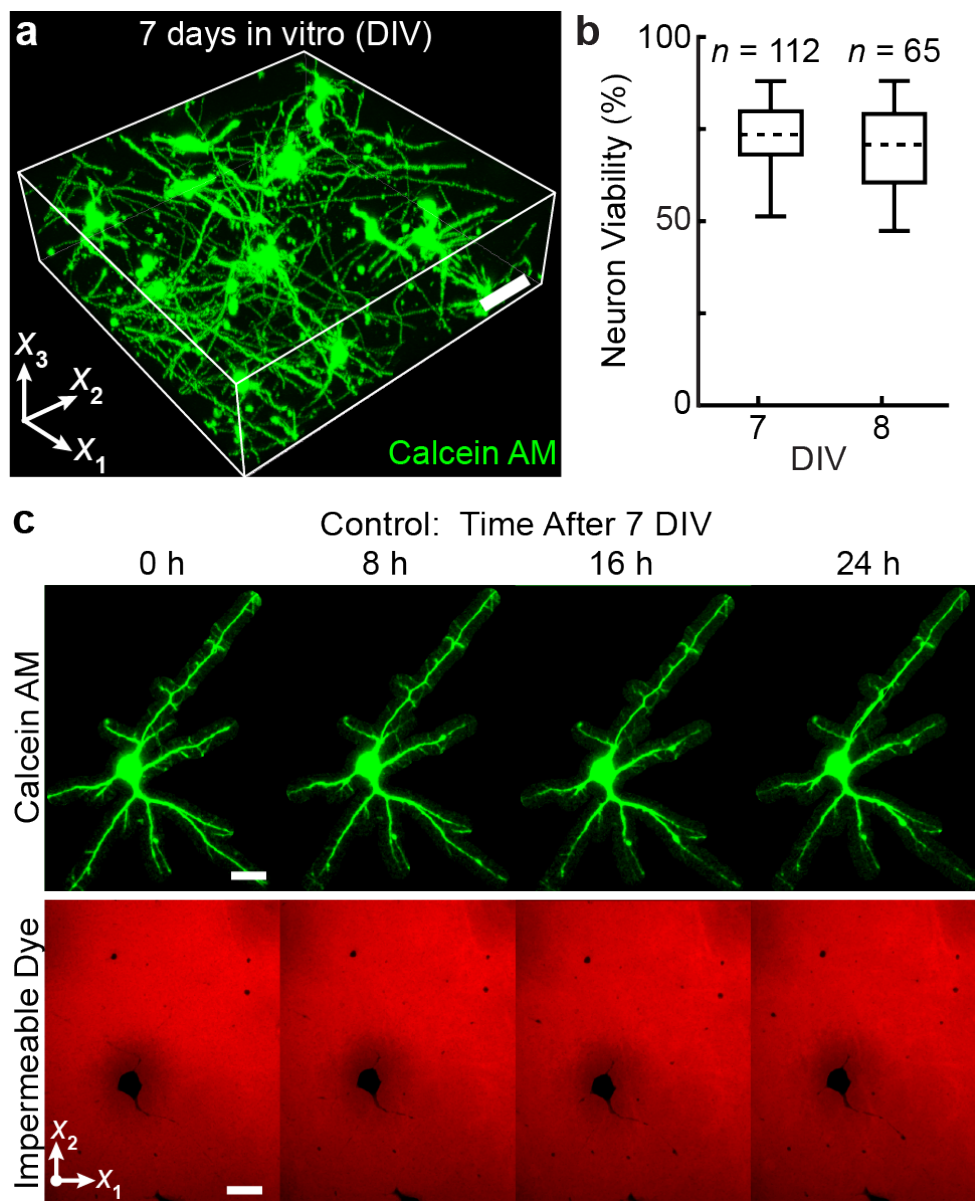
## 4.4 Conclusion

A new *in vitro* model of DAI was developed to investigate the injury evolution of neurons following compressive deformation in 3D. A high speed cell compression device was developed to mechanically load 3D collagen cultures at two strain rates. Characterization of the spatial distributions in the local deformation field shows homogeneous strain transfer to the neurons from the far-field. By assessing cell membrane permeability, there was no initiation of secondary pore formation due to local mechanoporation of the cell membrane. These findings are significant since mechanoporation has been suggested to initiate DAI in tension and shear [92, 73, 32, 128, 118, 112, 178], leading to important physiological secondary effects such as axonal blebbing [198, 73, 130, 103]. Morphological injury analysis following mechanical loading shows that the neuronal micropathologies in compression were found to be highly dependent on strain rate. In particular, bleb formation was more frequent at high strain rates, suggesting that viscoelastic effects may underpin local mechanical failure of the cytoskeleton [2]. Additional studies need to be performed to elucidate possible biochemical cascades that initiate cellular death at lower strain rates. The results of these studies may guide future TBI detection protocols for mild cases of traumatic loading that may have been previously overlooked. The local orientation of each neuron was computed to determine if bleb formation is correlated to the local strain magnitudes. The results of this strain localization analysis showed that bleb formation is not linked to the compressive axial strains, but linked to the axial shear strains. Even with these findings, blebs were present in a subset of the total dead cells, suggesting that cell death is driven by a different mechanism. Motivated by the findings that cellular viability is minimally dependent on bleb formation, a global injury metric that quantifies the overall deformation of the cell was established. Using this global injury assumption, time of cell death was correlated with the mean compressive axial strain regardless of bleb formation and strain rate. A quantitative injury envelope was developed to correlate the mean axial compressive strain and time at which cell death was observed.

The cellular injury analysis performed in this study may serve as important input for better predictive TBI models. For example, the local injury metrics can be incorporated into

cytoskeletal mechanics models of neurons that predict local failure, specifically bleb formation. The global injury metrics that were found to be correlated with cell death may be integrated into finite element models that require cellular injury thresholds. Additionally, these global injury metrics may establish quantitative time frames for therapeutic intervention.

## 4.5 Supporting Information



**Figure 4.24:** (a) Maximum intensity confocal micrograph of collagen culture at 7 days in vitro (DIV) with neurons stained with live dye calcein AM. Scale bar, 50  $\mu\text{m}$ . (b) Box and whiskers plot of the viability control for collagen culture populations at 7 DIV ( $n = 112$ ) and 8 DIV ( $n = 65$ ). Dotted bands: medians, top and bottom boxes: upper and lower quartiles, whiskers: 5th and 95th percentiles. No significant difference found with two-way ANOVA. Experiments and analysis performed by Mark T. Scimone. (c) Control for the mechanical loading experiments with the same imaging intervals and settings. (top) Maximum and (bottom) minimum intensity confocal micrographs of neuron stained with calcein AM and cell-impermeant dye Alexa Fluor Hydrazide showing clear viability over 24 hours. Scale bar, 20  $\mu\text{m}$

## 5. Conclusion

### 5.1 Summary

The aim of this thesis is twofold: first, to develop new experimental tools that are capable of analyzing the kinematics of nonlinear deformations in three-dimensions; and secondly, to demonstrate the capability of these tools by studying problems of cell-matrix interactions. In chapter 2, a new experimental analysis technique has been developed to noninvasively quantify the internal motion of materials undergoing nonlinear deformations within pragmatic computation times. This technique has applications to a broad array of imaging modalities regardless of dimensionality, and is independent of length and time scales. The capability of this technique has been demonstrated in chapter 3 to measure the nonlinear surface deformations generated by neutrophils migrating through a heterogeneous collagen matrix that is not mechanically characterized. A new mathematical framework has been developed to convert these complex surface measurements to simple yet powerful metrics that may be used by researchers to investigate the cellular migration mechanisms in more physiologically relevant materials. Instead of applying kinematic analysis to characterize cell-induced deformations, chapter 4 reports the cellular response to externally applied kinematic deformations. An electromechanical device has been built to apply uniaxial compression to neurons embedded in a heterogeneous microenvironment under dynamic loading conditions. New kinematic analysis techniques have been developed to show that neuron death is correlated with the orientation-dependent mean axial strain experienced by the cell during deformation. The techniques presented in this thesis have shown that kinematic analyses can be successfully applied to nonlinear cell-matrix interactions in 3D, the study of which would be not possible with traditional methods.

### 5.2 Recommendation for Future Works

Large computational gains can be achieved by performing DVC locally in applications that do not contain global deformation. For example, in studying cell-induced deformations,

displacements rapidly decay in the farfield and performing DVC everywhere in the image is computationally wasteful. Instead, an algorithm can be implemented that coarsely estimates the displacement field everywhere in the image and then iterates on areas that contain non-negligible values of displacement, e.g. areas around the cell. This can be easily implemented by assigning local convergence to the interrogation windows. Another potential area for improvement of DVC is in the numerical computation of the material strains. Formulations that consider the underlying mechanics of the problem could guide more accurate computation in the presence of noise. Although computationally expensive, compatibility conditions for finite deformations can be used as a global minimization condition during the deformation gradient calculation. On the experimental side, deformations measurements on the cell surface (as observed in chapter 3) can be coupled with single particle tracking of intracellular structures. These combined techniques may provide further insight into the force generation mechanisms and mechanotransduction pathways in the cell.

For future researchers applying DVC, it is important to be mindful of the reconstruction of the surface topology in the image. For example, in traction measurements and in the techniques presented in chapter 3, the estimation of the surface topology has large implications in the reported measurements. This is especially significant since the cell induced deformations decay rapidly, and the assumption of a flat substrate may not always be valid in 2D analyses. To assist in this surface reconstruction in 2D, nontoxic dyes can be applied to make the culture media fluoresce. The substrate surface can then be estimated by inverting the intensity of the image. However, in 3D this problem becomes much more complicated due to the axial spreading of the image and diffusion limitations. Companies need to address this issue by developing new dyes that are suitable for 3D cultures.

Even with the wealth of literature available, our understanding of TBI is still in its infancy. Future studies on multiple length scales should investigate the electrical disruptions associated with TBI. These electrical disruptions may correlate with diffuse micropathologies since neurons are the fundamental electrical components in the body. This research can be drive the development of new noninvasive electrical recording techniques that may also be applied to other injuries or diseases. Looking at TBI prevention, hyperbaric oxygen

conditions have shown a promising future in mitigating concussive injury [226]. Although animal studies have begun to evaluate hyperbaric conditions after TBI [282], in vitro models may provide further insight into the possible healing mechanisms of neurons. For future applications of deformation kinematics, the mechanical state of neurons can be analyzed after mechanical loading using the methods described in chapter 2 and 3. Distressed neurons may exhibit a state of hypertension which has implications in neurotransmission. As a final note to conclude this thesis, the future of studying cell-matrix interactions is very promising. The long-term results will provide health care professionals better options when caring for individuals suffering from a wide array of ailments.



## A. High Resolution, Large Deformation 3D Traction Force Microscopy

**Note:** A version of this appendix is published in *PLoS ONE* with Jennet Toyjanova as a first co-author. Published data has been included in the section with co-author’s consent.

Bar-Kochba, E., Toyjanova, J., Fagundo-López, C., Reichner, J., Hoffman-Kim, D., & Franck, C. (2014). High Resolution, Large Deformation 3D Traction Force Microscopy. *PLoS ONE*, 9(4):e90976. doi: 10.1371/journal.pone.0090976.

### A.1 Abstract

Traction force microscopy (TFM) is a powerful approach of quantifying cell-material interactions, which over the last two decades has contributed significantly to our understanding of cellular mechanosensing and mechanotransduction. In addition, recent advances in three-dimensional (3D) imaging and traction force analysis (3D TFM) have highlighted the significance of the third dimension in influencing various cellular processes. Yet irrespective of dimensionality almost all TFM approaches have relied on a linear elastic theory framework to calculate cell surface tractions.

Here we present a new high resolution 3D TFM algorithm which utilizes a large deformation formulation to quantify cellular displacement fields with unprecedented resolution. The results feature some of the first experimental evidence that cells are indeed capable of exerting large material deformations, which require the formulation of a new theoretical TFM framework to accurately calculate traction forces. Based on our previous 3D TFM technique we reformulate our approach to accurately account for large material deformation and quantitatively contrast and compare both linear and large deformation frameworks as a function of the applied cell deformation. Particular attention is paid in estimating the accuracy penalty associated with utilizing a traditional linear elastic approach in the presence of large deformation gradients.

## A.2 Introduction

Traction Force Microscopy (TFM) is a powerful methodology of quantifying cellular forces during cell-material interactions. From the early work developed by Harris et al. in 1980 to the pioneering work by Oliver et al. and Dembo et al. in the mid 1990s, TFM studies have demonstrated the important role that mechanical cues play in cell biology by providing rigorous means of quantifying cellular traction forces [159, 58, 203, 236, 37, 174, 120, 199, 121]. Starting in 2009 Maskarinec et al. and Hur et al. showed the importance of recording all three traction components in gaining a deeper and fuller understanding of how cells interact with their substrate materials during locomotion and spreading [114, 175, 79]. Recent studies have underscored the importance of measuring the full three-dimensional (3D) traction profiles during cell migration, locomotion and cell-cell interactions [152, 135, 56, 113].

TFM computes cell generated surface tractions from a set of measured cell-induced displacement fields that are typically recorded by using either a single particle tracking or image correlation approach such as digital image (2D) or digital volume (3D) correlation [58, 174, 121, 114, 175, 79, 152]. Image correlation techniques are generally advantageous over single particle tracking algorithms if a high enough fiduciary tracker particle density can be achieved. They are generally less prone to error in the presence of noise and they can be readily computationally implemented. Once the cell-generated displacement fields are computed the surface tractions can be calculated through either an inverse Boussinesq formulation or in cases where 3D displacement data is available, in a forward formulation as presented by Franck et al. [175, 79]. Alternatively, one can utilize a finite element framework to compute surface tractions from the measured displacement values [305, 114].

In almost all of the currently reported TFM methods the underlying assumption is that the cell-generated material strains are small enough to be analyzed within a linear elastic continuum framework [55, 288]. This assumption seems justified for most published works showing mostly small cellular strains.

Utilizing a recently developed advanced high resolution digital volume correlation (DVC) technique [15] we show that cells actually exert large, or finite, deformations with strain

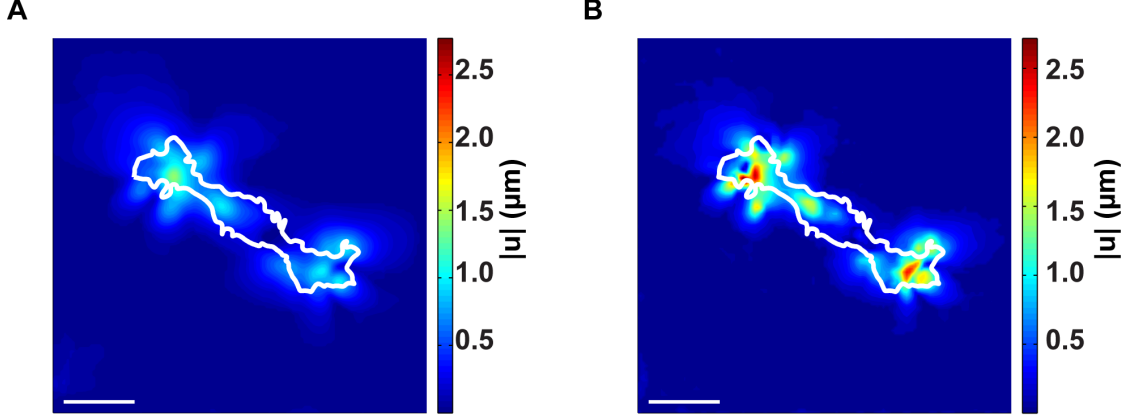
magnitudes of up to 40%. Motivated by these observations, we present a new reformulated approach to calculating cellular traction fields in 3D using a large deformation formulation. This new methodology is capable of providing unprecedented spatial detail of 3D cellular traction forces with a four to five fold signal-to-noise improvement over our previous small deformations 3D TFM approach. At the same time we reduced the overall computation times by one order of magnitude by implementing our algorithm on a personal computer’s graphics processing units (GPU) [15]. Finally, we quantitatively compare and contrast the differences in calculated cell tractions between the traditional small deformation (SD) (linear) TFM framework to our new large deformation (LD) (non-linear) methodology. We will show that for small strains the LD approach automatically simplifies to the commonly used linear framework thus providing the most general and robust overall approach. Our entire 3D TFM large deformation algorithm will be freely available from our website (franck.engin.brown.edu).

### A.3 Results

The large deformation 3D TFM (LD 3D TFM) methodology presented in this paper consists of two basic components, analogous to our previous small deformation 3D TFM (SD 3D TFM) technique. First, a novel fast iterative digital volume correlation algorithm (FIDVC) [15] is used to compute the 3D cell-generated displacement fields from a series of laser scanning confocal microscopy (LSCM) 3D images. Second, the measured 3D displacement fields are converted to cellular tractions using a LD continuum mechanics formulation.

#### A.3.1 High Resolution Improvements in Measuring 3D Cellular Displacement Fields

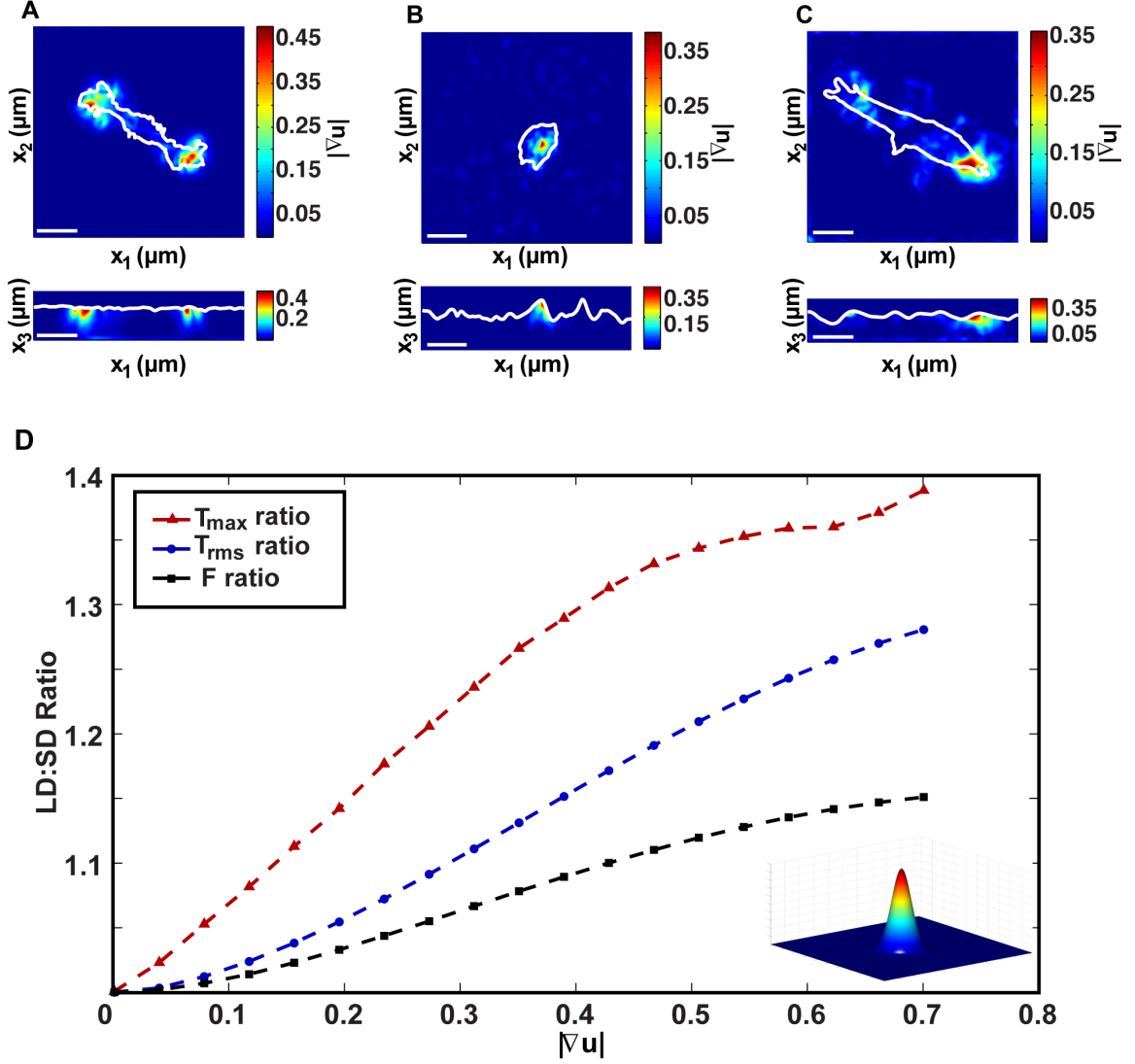
Our newly developed FIDVC algorithm is capable of producing spatial resolutions and accuracies far beyond most current 3D methods, and is a significant advancement over our previously reported DVC displacement algorithm. **Fig. A.1** depicts the cell-induced 3D displacement fields of a Schwann cell during locomotion. For comparison purposes **Fig. A.1a** presents the cell displacement analyzed with our previous DVC algorithm [78], whereas **Fig. A.1b** shows the results analyzed with our high resolution FIDVC technique [15]. As can be seen from **Fig. A.1** the recovered displacement magnitudes as well as their



**Figure A.1:** Top-view displacement contours of a migrating Schwann cell measured by DVC and FIDVC. Side by side comparison of the 3D cell displacements measured with (a) our previous DVC [78] and (b) our new FIDVC algorithm [15]. Cell outlines are shown in white. Scale bars = 40  $\mu\text{m}$ .

spatial gradients are significantly better resolved using the FIDVC providing higher overall signal-to-noise ratio and finer spatial detail of the cell-induced material displacement fields than our previous methodology.

Due to the improvement in spatial resolution, we found cells are indeed capable of producing large deformations. **Fig. A.2** presents color contour plots of three different cells: (a) a Schwann cell, (b) a polymorphonuclear neutrophil, and (c) a NIH 3T3 fibroblast seeded on polyacrylamide gels all generating large displacement gradients, or material strains. The choice of plotting the magnitude of displacement gradient rather than a particular strain component is to provide the reader with specific numbers to show that the non-linear component can no longer be neglected, and that the finite deformation behavior of the material and its associated Lagrangian strains (**E**) need to be accounted for in equation (A.5.4). This is further illustrated in **Fig. A.2d** showing significant deviations in the traction estimates between the small strain, linear and finite deformation theory as the displacement gradient term increases. **Fig. A.2d** quantifies the deviation in surface tractions and total force between the LD and SD analysis 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. We believe these observations are primarily the

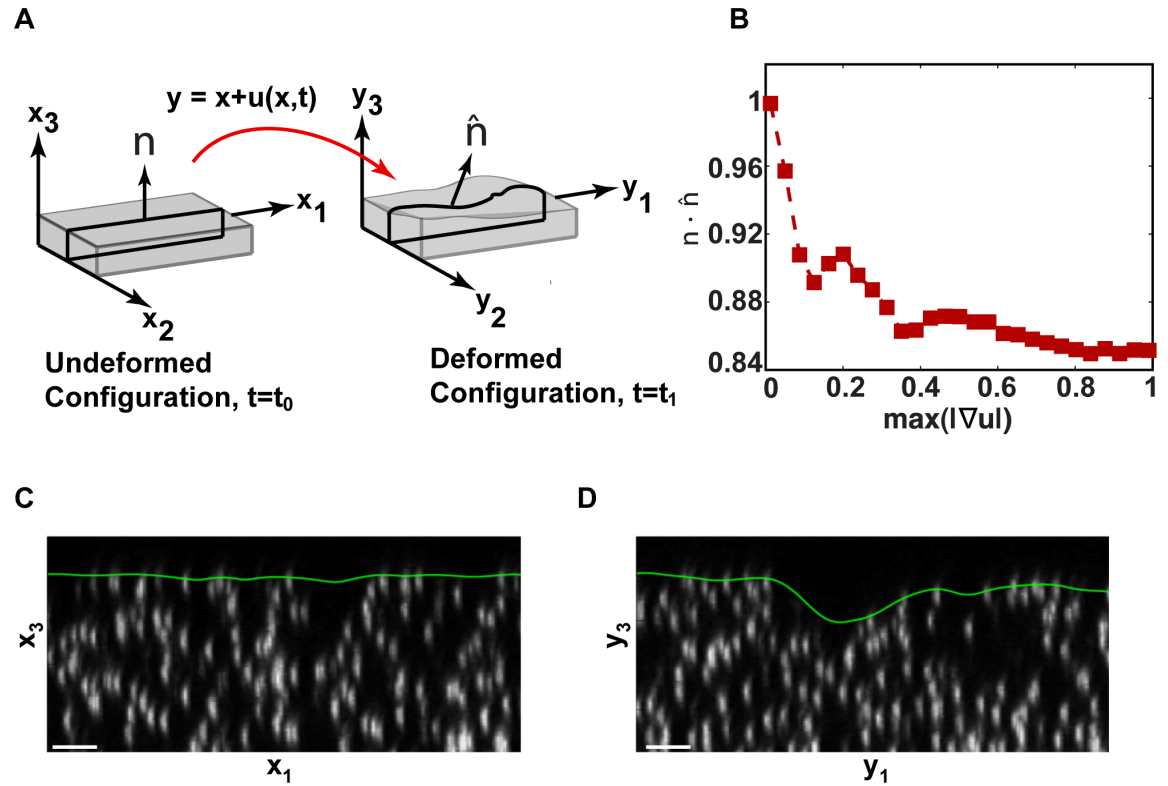


**Figure A.2:** Displacement gradient comparison for large deformation.  $x_1 - x_2$  and  $x_1 - x_3$  cross-sections of the calculated 3D displacement gradient for (a) a Schwann cell (scale bar =  $40 \mu\text{m}$ ), (b) a polymorphonuclear leukocyte (scale bar =  $20 \mu\text{m}$ ) and (c) a NIH 3T3 fibroblast (scale bar =  $20 \mu\text{m}$ ). (d) 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.

result of our improved spatial accuracy in better resolving the actual cell displacement fields. Many of the currently available 3D displacement motion estimation algorithms intrinsically neglect to account for LD in their formulations, which as a result underpredict both the magnitude and gradients of the cell-induced displacement fields [15].

This deviation is illustrated in more detail in **Fig. A.3**. 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. A.2a–c**. **Fig. A.3a** 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. There are two important conclusions 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),



**Figure A.3:** Undeformed and deformed surfaces due to a large deformation. (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  $t_1$ . (b) Angle change between the undeformed and deformed surface normals in (a)  $\mathbf{n}$  and  $\hat{\mathbf{n}}$ , under the application of a cell-simulated Gaussian displacement field profile. 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. Actual LSCM  $x_1 - x_3$  cross-sectional images with (c) in the absence of a cell, and (d) directly underneath a locomoting Schwann cell. Scale bars = 5  $\mu\text{m}$ .

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. This is illustrated in **Fig. A.3b**. 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. However, as shown in **Fig. A.3b** 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. A.3b** is caused by significant material rotations at the free surface. These large surface rotations are seen in **Fig. A.3c,d** which presents an experimental  $x_1 - x_3$  cross-sectional image obtained via confocal microscopy before and after applied cell deformation. The material shown is a polyacrylamide substrate with embedded  $0.5 \mu\text{m}$  fluorescent particles and the green line represents the free surface of the sample. As a result even 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 fiduciary 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.

To provide the reader with a more comprehensive analysis of using a small (SD) versus large (LD) deformation framework to calculate tractions, we present two specific examples. In the first example, an analytical, simulated displacement dipole is generated with a displacement gradient magnitude,  $|\nabla \mathbf{u}| = 0.5$ , whereas in the second example a displacement field is experimentally measured from a locomoting Schwann cell. In both cases, the surface tractions, forces, and corresponding strain energies are compared using a SD and LD framework.

### A.3.2 Comparison between Large and Small Deformation Traction Results (Analytical Example)

To validate our LD 3D TFM methodology and to quantify the differences between a SD (linear) and LD TFM approach, we generate two analytical displacement dipoles applied

along a free surface of the  $x_1 - x_2$  plane in the form of

$$\mathbf{u}(\mathbf{x}) = A \exp \left( -\frac{(x_1 - b_1)^2 + (x_2 - b_2)^2 + (x_3 - b_3)^2}{2\sigma^2} \right) \quad (\text{A.3.1})$$

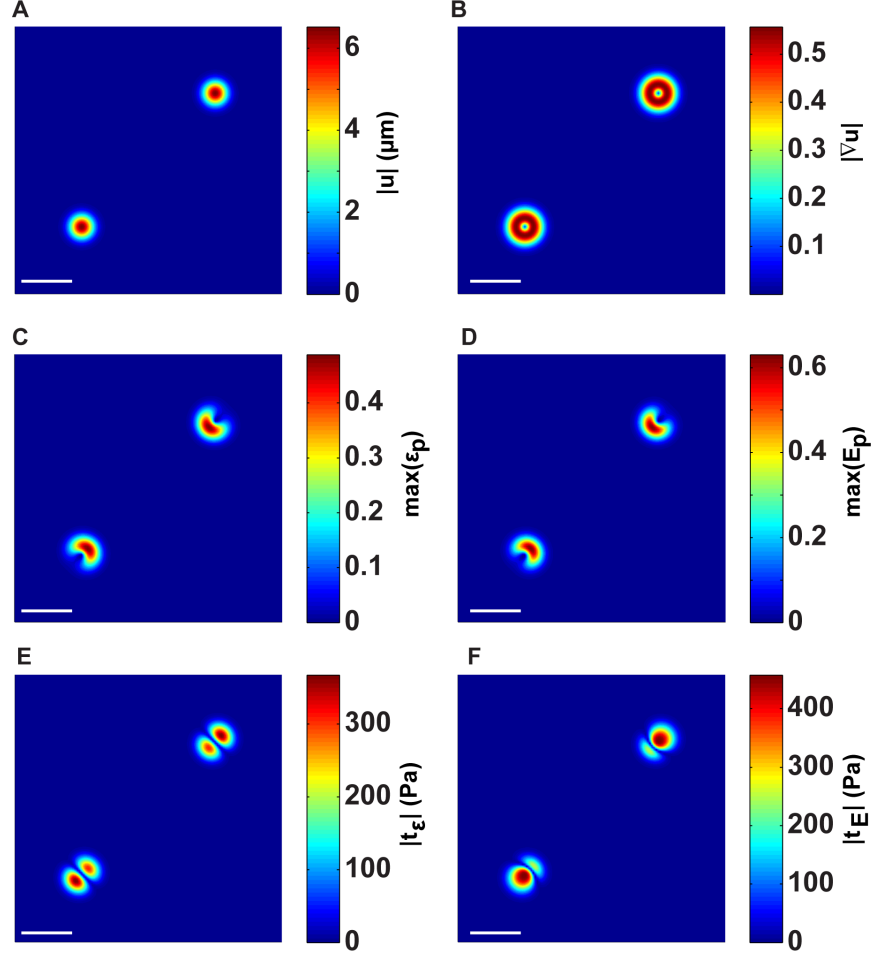
with  $A$  being the amplitude,  $\sigma$  the spread, and  $\mathbf{b}$  the position of the center peak of the Gaussian function (Fig 2d insert). Here,  $A$  and  $\sigma$  were chosen such that the displacement gradient magnitude,  $|\nabla \mathbf{u}|$ , yields a value of  $\sim 0.5$ , which is similar to the ones experimentally observed (**Fig. A.2**).

**Fig. A.4a** plots the magnitude of the 3D displacement vector,  $|\mathbf{u}|$ , along the  $x_1 - x_2$  free surface plane, whereas **Fig. A.4b** depicts the magnitude of the displacement gradient,  $|\nabla \mathbf{u}|$  of the analytically applied displacement fields along the same plane. **Fig. A.4a,b** serve as the input parameters to calculate the surface tractions shown in **Fig. A.4c-f**. For comparison, **Fig. A.4c** displays the maximum principal infinitesimal strain (SD),  $\epsilon_p$ , whereas **Fig. A.4d** displays the maximum principal Lagrangian strains (LD),  $\mathbf{E}_p$ . The difference between the two arises from the non-linear displacement gradient term in equation (A.5.4). The final row in **Fig. A.4e,f** compares the resulting surface tractions between the SD and LD approaches. **Fig. A.4e** presents the surface tractions as calculated by our previous SD 3D TFM methodology which assumes the material to be linearly elastic, whereas **Fig. A.4f** presents the tractions calculated using a finite LD Neo-Hookean material behavior, which has been shown to adequately describe the material behavior of PA at large strains [160].

Comparison between **Fig. A.4e,f** shows that both the magnitude as well as the spatial distribution of calculated surface tractions are different. The  $\sim 25\%$  difference in the recovered traction magnitude is a strong function of local displacement gradient as illustrated in **Fig. A.2d**. The larger the displacement gradient the stronger the deviation between the two traction fields. The spatial differences in the traction fields are primarily attributed to the underlying topographical change of the substrate surface, which as shown in **Fig. A.3** changes the spatial distribution of the in-plane and out-of-plane tractions.

To conclude our validation we compare the total applied surface forces for both the SD and LD 3D TFM approaches to the analytically computed force. In **Fig. A.5** we show a comparison

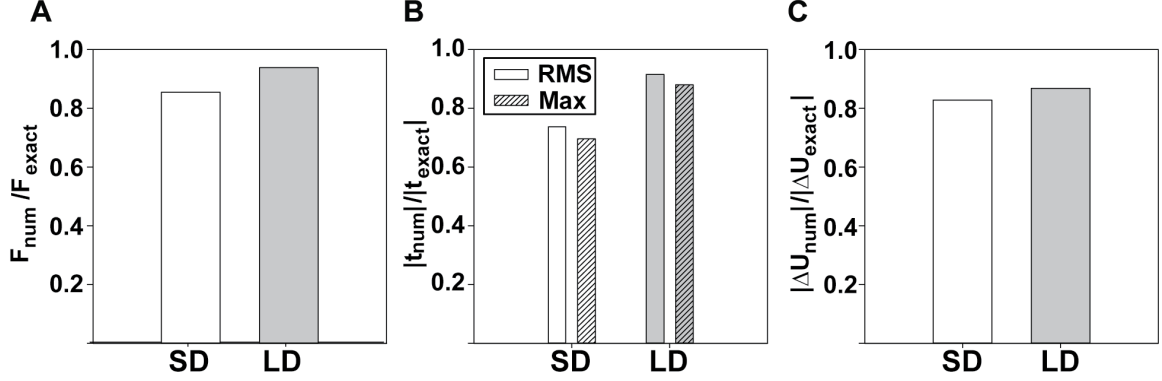




**Figure A.4:** Analytically example of prescribed Gaussian displacement dipoles on the surface of a 3D LSCM imaging volume. The (a) 3D surface displacement magnitude,  $|u|$ , and (b) displacement gradient magnitude,  $|\nabla u|$ . Profiles of calculated maximum 3D principal strains calculated from the infinitesimal ( $\epsilon_p$ ) (c) and Lagrangian ( $E_p$ ) strains d. The corresponding traction magnitudes calculated on the (e) undeformed surface,  $\mathbf{x}$ , using a linear elastic constitutive model,  $|t_\epsilon|$ , and on (f) the actual deformed surface,  $\mathbf{y}$  using a large deformation (LD) constitutive model  $|t_E|$ . Scale bars = 40  $\mu\text{m}$ .

of different metrics that are commonly reported in TFM studies [121, 278, 37, 54, 55]. This result is shown in **Fig. A.5a**, which plots the ratios between the total force magnitudes of the SD and LD 3D TFM approaches to the exact analytical value.

As expected in the presence of high displacement gradients, the linear, small strain (SD) approach underestimates the total force by  $\sim 15\%$ , whereas our large deformation formulation (LD) predicts it more accurately. The corresponding root mean squared and maximum traction values are compared with the exact solution and presented in **Fig. A.5b**. **Fig. A.5c**

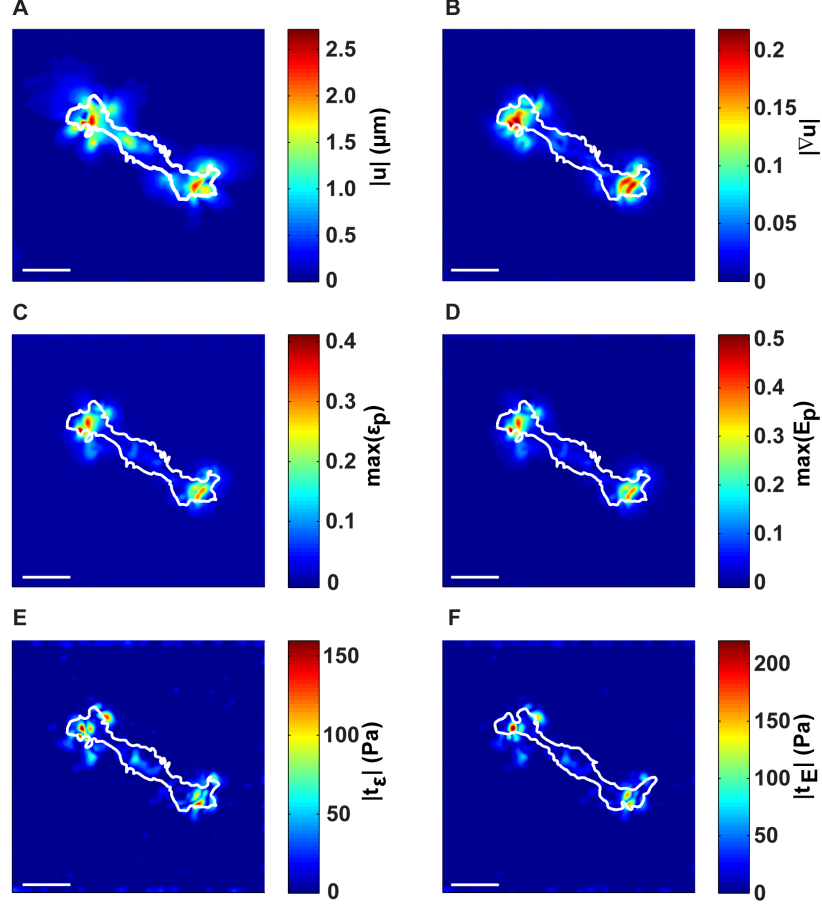


**Figure A.5:** Comparison of commonly reported metrics in TFM for the analytical example. Side by side comparison of the (a) total force, (b) root mean squared (RMS) tractions and maximum tractions, and (c) strain energy for both the linear elastic, small deformation (SD) and non-linear, large deformation (LD) models. All of the values are normalized by the exact analytical solution.

depicts the ratio in the elastic strain energy ratios between the calculated values and the exact solution.

### A.3.3 Comparison between Large and Small Deformation Traction Results (Experimental Cell Example)

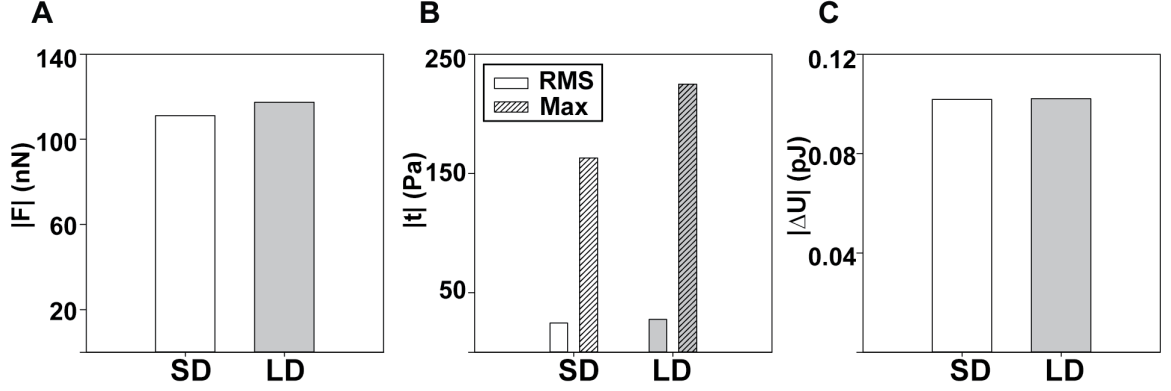
To highlight the differences in calculated cellular strain and traction fields, we applied the same comparative approach from the previous section to analyzing actual experimental cell displacement data. **Fig. A.6a** is a plot of the 3D displacement vector field magnitude,  $|\mathbf{u}|$  directly underneath a migrating Schwann cell. **Fig. A.6b** plots the corresponding displacement gradient magnitude,  $|\nabla \mathbf{u}|$  of the measured displacements in **Fig. A.6a**. Analogous to **Fig. A.4**, **Fig. A.6a,b** serve as the input data to calculate the cell surface strains and tractions shown in **Fig. A.6c-f**. **Fig. A.6c** depicts the maximum principal infinitesimal strain,  $\epsilon_p$ , whereas **Fig. A.6d** displays the maximum principal Lagrangian strain,  $\mathbf{E}_p$ . As before, the difference in the strains arises from neglecting the squared gradient term in equation (A.5.4). **Fig. A.6e,f** plots the respective surface traction magnitudes for the linear elastic method (SD 3D TFM),  $|\mathbf{t}_\epsilon|$ , and the finite deformation method (LD 3D TFM),  $|\mathbf{t}_E|$ . As in **Fig. A.6e** the surface tractions are calculated based on our previous SD 3D TFM technique, whereas in **Fig. A.6f** the tractions are calculated using our new LD 3D TFM methodology. Similar to our analytical example the traction magnitude is underpredicted



**Figure A.6:** Experimental example of a migrating Schwann cell on the surface of a 3D LSCM imaging volume. (a) Magnitude of the 3D Schwann cell surface displacement field,  $|\mathbf{u}|$ , and its (b) resulting displacement gradient magnitude ( $|\nabla \mathbf{u}|$ ). Calculated maximum principal strains from the infinitesimal ( $\epsilon_p$ ) c, and Lagrangian ( $E_p$ ) strains d. The corresponding traction magnitudes calculated on the (e) undeformed surface,  $\mathbf{x}$ , using a linear elastic constitutive model,  $|\mathbf{t}_\epsilon|$ , and on the (f) actual deformed surface,  $\mathbf{y}$  using a large deformation (LD) constitutive model  $|\mathbf{t}_E|$ . Cell outlines are shown in white. Scale bars = 40  $\mu\text{m}$ .

in the linear case. More importantly the spatial distribution and features of the traction patterns are significantly different between the SD and LD formulation due to changes in the underlying surface topography as illustrated in **Fig. A.3**.

While total force and strain energy are convenient scalar quantities to classify cellular contractility, root-mean-squared and maximum traction measures present another useful metric that are not dependent on the actual projected cell surface area. **Fig. A.7a-c** highlights the differences in total force, surface tractions, and strain energy between the linear elastic and finite deformation methodologies. When comparing **Fig. A.7a-c**, the



**Figure A.7:** Comparison of commonly reported metrics in TFM for the experimental example. Side by side comparison of the (a) total force, (b) root mean squared (RMS) and maximum tractions, and (c) strain energy for both the linear elastic, small deformation (SD) and non-linear, large deformation (LD) models.

deviations between the tractions, in particular between the maximum tractions are much higher than for the forces or strain energies. This is not surprising since the average cell-applied surface tractions generally feature small displacement gradients as to not produce significant differences between the two models. The maximum tractions on the other hand will almost always feature very steep underlying displacement gradients,  $\nabla \mathbf{u}$ , making the calculation extremely sensitive to the deviation in the non-linearity of the particular model used.

## A.4 Discussion

This paper presents an advanced high resolution 3D TFM technique capable of calculating fully non-linear cell-induced material stresses and traction fields. By utilizing a recently developed FIDVC technique, cellular displacement fields can be measured with unprecedented resolution and at computation times an order of magnitude faster than our previous 3D DVC algorithm. The increase in computational speed and accuracy is achieved through the extension of the well-known iterative deformation method (IDM) [243, 192] into three dimensions, and the implementation of the algorithm on the GPU. GPU implementation on a personal computer's graphics card allows a widespread user base to utilize our new LD 3D TFM code without the need to resort to powerful computational cores. The motivation of applying finite or LD continuum mechanics theory in analyzing cellular traction fields

was warranted through the experimental observation of existing significant displacement gradient profiles.

To illustrate the significance of the displacement gradient and its role in determining the most appropriate theoretical TFM framework, we provide a quantitative side-by-side comparison of the traditionally SD 3D TFM framework to our LD 3D TFM approach. As shown in **Figs. A.2-A.7** the deviation in the recovered surface traction is significant at values of  $|\nabla \mathbf{u}| \geq 0.5$ , exceeding 30% error in the determined maximum traction values. Therefore we recommend to always compute the displacement gradient at every time point to assess the appropriateness of a SD 3D TFM versus a LD 3D TFM mathematical framework. We also show that for quantities such as the total cell force and the strain energy, the deviation errors between the two approaches is small, and a linear framework might provide adequate results. Lastly, we present a simple forward formulation of calculating LD 3D cell surface tractions. Our entire LD 3D TFM algorithm will be freely accessible via download from our website (<http://franck.engin.brown.edu>).

In conclusion, our new LD 3D TFM algorithm presented here provides unprecedented spatial detail of 3D cellular traction fields at computation times of a few minutes on an average personal computer equipped with a GPU graphics card. We show that depending on the cell-induced displacement gradient magnitude, the traditional linear TFM frameworks such as the Boussinesq theory can introduce significant error (**Figs. A.2-A.7**) in estimating cellular tractions and a large deformation formulation should be used instead. To this end we provide a straightforward method to account for finite deformations to accurately calculate cell tractions in any material for which the constitutive material behavior is known.

## A.5 Materials and Methods

### A.5.1 Large versus Small Material Deformations

In continuum mechanics, a material point undergoing a deformation from location  $\mathbf{x}$  to location  $\mathbf{X}$  can be represented by its material displacement,  $\mathbf{u}(\mathbf{x})$  by

$$\mathbf{X} = \mathbf{x} + \mathbf{u}(\mathbf{x}). \quad (\text{A.5.1})$$

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

$$\nabla \mathbf{X} = \nabla(\mathbf{x} + \mathbf{u}(\mathbf{x})) = \mathbf{I} + \nabla \mathbf{u}(\mathbf{x}) = \mathbf{F}, \quad (\text{A.5.2})$$

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  $\mathbf{X}$ . To calculate the material strains associated with that deformation, we compute the Lagrangian strain tensor as

$$\mathbf{E} = \frac{1}{2} \left( \mathbf{F}^T \cdot \mathbf{F} - \mathbf{I} \right). \quad (\text{A.5.3})$$

Equation (A.5.3) can be rewritten in terms of the material displacement as

$$\mathbf{E} = \frac{1}{2} \left( \nabla \mathbf{u} + (\nabla \mathbf{u})^T + \nabla \mathbf{u} \cdot (\nabla \mathbf{u})^T \right), \quad (\text{A.5.4})$$

where the term  $\nabla \mathbf{u}$  is known as the displacement gradient. Equations (A.5.1) - (A.5.4) are valid for any non-linear finite (large) homogeneous deformation. In small strain and linear elasticity, which is the fundamental framework of almost all TFM methodologies, the displacement gradient term is assumed to be small, i.e.,  $\nabla \mathbf{u} \ll 1$ , which simplifies equation (A.5.3) to

$$\boldsymbol{\epsilon} = \frac{1}{2} \left( \nabla \mathbf{u} + (\nabla \mathbf{u})^T \right). \quad (\text{A.5.5})$$

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 [65, 61, 54]. While several numerical papers have suggested accuracy improvements in calculating cell tractions by using a finite deformation formulation, no prior experimental observation had been made to corroborate the necessity of such approaches.

The observation presented in this paper that cells can indeed generate non-linear material strains should not only have implication for traditional TFM methodologies but also for cell traction assays that utilize very compliant micropillars. 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 strains are indeed small, however in the presence of large strains may warrant additional large deformation (LD) analysis.

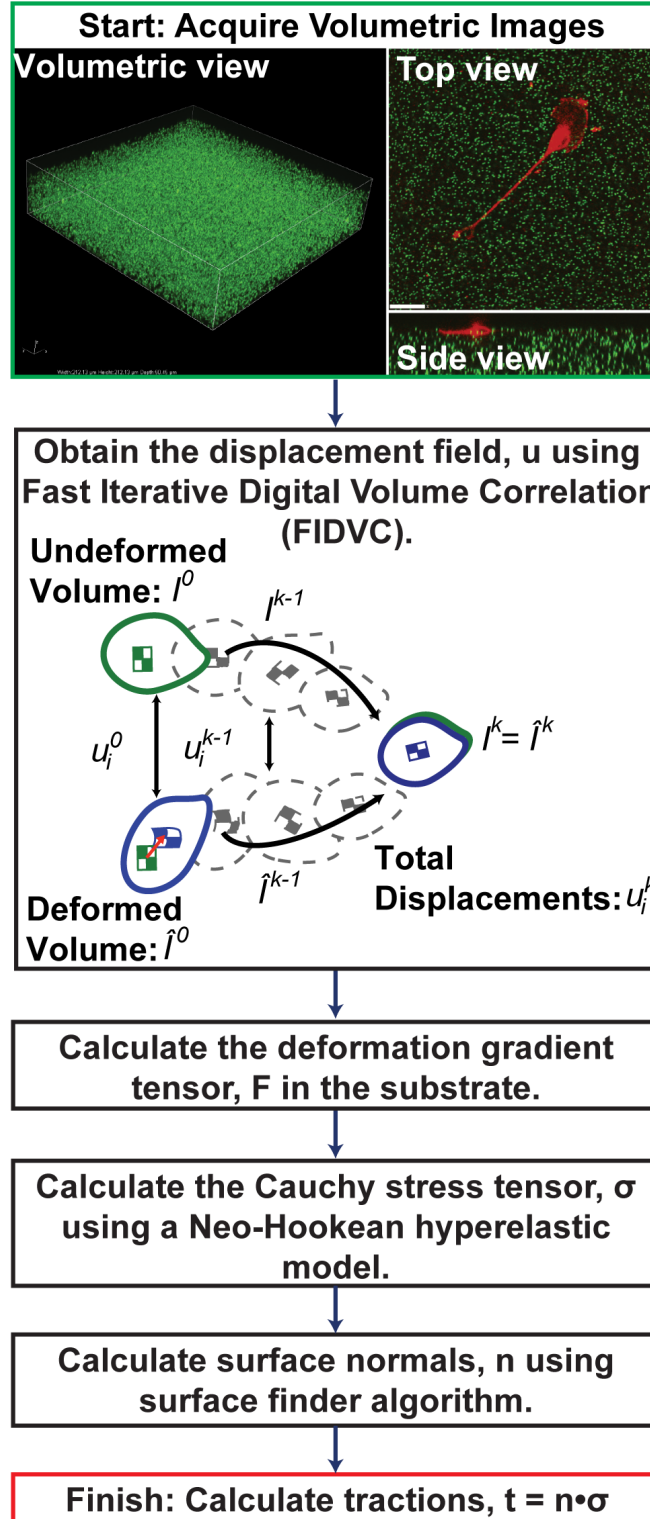
### A.5.2 LD 3D TFM

The two significant advances in improving our traction resolution capability are: **(a)** a completely new approach to measuring the cell-imposed displacement fields via our recently developed Fast Iterative Digital Volume Correlation (FIDVC) algorithm [15], and **(b)** a LD continuum mechanics formulation to determine more accurate cell surface tractions. **Fig. A.8** illustrates the flow of our new LD 3D TFM technique. We will address each part of **Fig. A.8** in detail below.

Analogous to our previous 3D TFM technique, 3D volumetric image stacks are acquired using laser scanning confocal microscopy (LSCM). 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. To estimate the cell-generated displacements, the motions of embedded submicron-diameter fluorescent particles are estimated using our FIDVC algorithm [15]. The top image in **Fig. A.8** shows an example of a three-dimensional volumetric image recorded using LSCM denoting the starting point for our calculations. The submicron particles are shown in green and the cell is superimposed in red.

#### A.5.2.1 Estimating 3D LD Cellular Displacements

Motivated by the Gaussian-like material displacement field signature that cells generate [174, 114, 152, 151, 79], we developed a new volumetric displacement finding scheme particularly suited to handle cellular deformation fields. While our previous DVC approach provided a robust approach in estimating cellular displacement fields it suffered from both slow computation times and lack of high spatial resolution close to the origin of traction application. Capturing highly localized displacement gradients such as those found near the peak traction locust is challenging with any cross-correlation metric due to the intrinsic low-pass filtering



**Figure A.8:** Flowchart of the large deformation high-resolution 3D TFM technique illustrating how cell surface tractions are being calculated.



characteristics of such a technique. However, their numerical robustness, high precision and easy implementability makes them the preferred choice over single particle tracking algorithms. To address the challenges of achieving high spatial resolution at low computational costs we recently developed a new technique called the FIDVC approach [15].

While the mathematical and implementation details of this new FIDVC algorithm can be found elsewhere [15], we provide a brief summary of its key technical points. LD motion estimates are generally captured by a 12 degree of freedom minimization formulation that accounts for rigid body as well as affine transformation between small regions, or subsets, within an image pair [30]. Such approaches have been well-documented in the DIC communities but they generally perform poorly in DVC measurements due to the large computational cost associated with the third dimension [85]. To solve the issue of remaining computationally efficient while computing large deformations in 3D volumetric data sets, we extended the well-known image deformation method (IDM) into three dimensions and applied it to calculating volumetric displacement fields. The working principle of the IDM is schematically shown in the second step in **Fig. A.8**, where the deformation field between two volumetric images is linearized into  $k$ -increments by means of incrementally warping both image pairs by estimates of the cumulative displacement field,  $\mathbf{u}^k$ . For each linearized increment the DVC cross-correlation algorithm from our previous 3D TFM method is used to calculate the material displacement fields with high accuracy.

This process is repeated until both the reference and deformed image have converged to the same intensity pattern producing the cumulative displacement field measure,  $\mathbf{u}^k$ . One of the key benefit of the IDM method is the spatial refinement of the local interrogation window size. In our previous SD 3D TFM method we used a fixed interrogation, or subset, size of 64 voxels, similar to many correlation-based algorithms. This particular choice in subset size is typically governed by the maximum intensity information that can be obtained within the subset, such as the fiduciary marker size, density and distribution. Higher particle densities allow for smaller interrogation windows to be used. To provide maximum spatial resolution, the smallest possible subset size is sought while still maintaining high cross-correlation robustness, which is often found at a window size of 64 voxels. However, by employing an

interrogation window refinement during the iterative IDM process, our FIDVC technique is capable of reducing its subset size to 32 voxels and in some instances even lower without introducing significant correlation error. The result provides overall higher spatial resolution than previously possible, which is exemplified in **Fig. A.1**.

### Estimating 3D LD Cellular Traction

After the cellular displacements,  $\mathbf{u}$ , are determined the deformation gradient tensor,  $\mathbf{F}$ , is calculated via finite differences. As shown in equation (A.5.2) the displacement gradient,  $\nabla \mathbf{u}$ , and the deformation gradient tensor,  $\mathbf{F}$ , are directly related to the gradients of the measured displacement field. In order to provide the most robust measure of the displacement gradient we evaluated several differentiation kernels specifically designed to mitigate sampling dealiasing errors and measurement noise. 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. [72].

Similar to our SD 3D TFM approach, calculating cellular surface tractions first requires determination of the material's true stress tensor, or Cauchy stress  $\boldsymbol{\sigma}$ . Given that for the LD the reference and deformed configuration are no longer identical, computation of the Cauchy stress tensor involves the deformation gradient tensor. While our technique is general enough to allow numerical implementation of any constitutive material law, here we present the formulation of the stresses in polyacrylamide (PA), which can be well approximated by a Neo-Hookean material model [160]. Thus its Cauchy stress,  $\boldsymbol{\sigma}$ , can be expressed [26] as

$$\boldsymbol{\sigma} = \frac{\mu}{J^{5/3}} \left( \mathbf{B} - \frac{1}{3} \text{trace}(\mathbf{B}) \cdot \mathbf{I} \right) + K(J - 1)\mathbf{I} \quad (\text{A.5.6})$$

where the parameters  $\mu$ , 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

$$K = \frac{E}{3(1 - 2\nu)}, \quad \mu = \frac{E}{2(1 + \nu)}. \quad (\text{A.5.7})$$

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

$$J = \det(\mathbf{F}) \quad (\text{A.5.8})$$

and

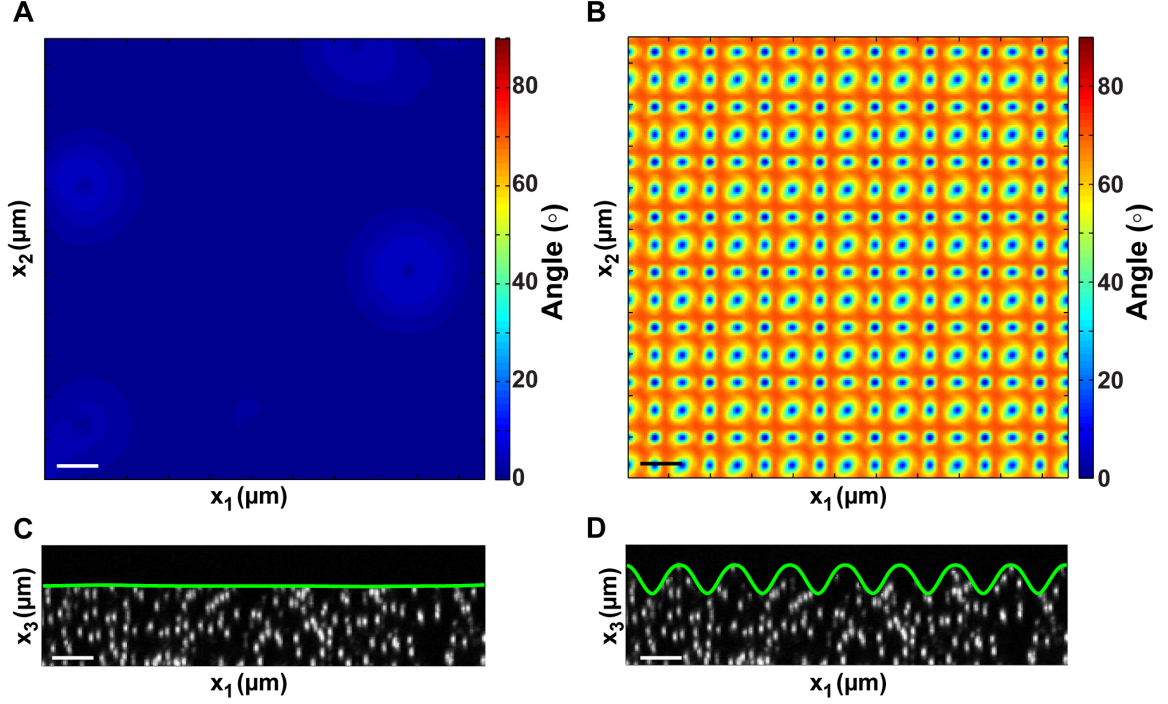
$$\mathbf{B} = \mathbf{F} \cdot \mathbf{F}^T. \quad (\text{A.5.9})$$

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

$$\mathbf{t} = \mathbf{n} \cdot \boldsymbol{\sigma}. \quad (\text{A.5.10})$$

Before equation (A.5.10) can be evaluated the surface normals,  $\mathbf{n}$ , need to be determined. Although it is theoretically possible to estimate the surface normals in the deformed state via the deformation gradient tensor and the reference surface normals, this procedure is practically challenging since the reference normals are often not known. To address this issue we developed a surface finding algorithm that uses the LSCM images to determine the true surface plane, i.e.,  $\mathbf{n}(\mathbf{x})$ . Here, 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 maximum 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 [59]. The surface normals,  $\mathbf{n}(\mathbf{x})$ , are calculated using the Delauney triangulation representation of the surface. **Fig. A.9** 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. A.9a** 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. A.9c** with the superimposed outline (green) that is found by the surface finding algorithm. Similar validation was performed on a surface that is defined by

$$f(x_1, x_2) = A \left( \sin \left( \frac{2\pi}{\lambda} x_1 \right) + \cos \left( \frac{2\pi}{\lambda} x_2 \right) \right), \quad (\text{A.5.11})$$



**Figure A.9:** 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 sinusoidal surface undulations. Scale bars =  $20\ \mu\text{m}$ .

where  $A$  is the amplitude and  $\lambda$  is the wavelength of the surface profile which was 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 equation (A.5.10).

#### A.5.2.2 Resolution and Measurement Sensitivity

Since the determination of the surface tractions involves calculations of strains and experimental determination of material constants, the sensitivity of our LD 3D TFM technique in terms of traction forces needed to be assessed. This was accomplished by converting the measured displacement and strain fields of control samples (no cells) into surface tractions. Using standard statistical error analysis, we determined the noise floor to be similar to our SD 3D TFM technique. Specifically, we can accurately resolve any displacement and strains greater than  $0.5\ \mu\text{m}$  and 1.0%, respectively, and hence by means of Eqs. (A.5.6)–(A.5.10), stresses and traction forces that are greater than  $50\ \text{Pa}$  or  $50\ \text{pN}/\mu\text{m}^2$  for all samples with a

Young’s modulus of 1.70 kPa. A similar analysis of the maximum resolution sensitivity for 2D and 3D TFM methodologies can be found elsewhere [248, 79, 151].

#### **A.5.2.3 Global Force and Moment Balance**

Analogous to our SD 3D TFM methodology, we compute the sum of all forces and moments acting on any given control volume inside each PA gel to verify that static force and moment equilibrium are satisfied. The overall procedure is identical to our previous methodology [79]. Under all experimental conditions and imaging time points we found static equilibrium satisfied with force and moment magnitudes on the order of  $10^{-10} - 10^{-9}$  N, and  $10^{-15} - 10^{-14}$  N·m respectively, which is consistent with our determined experimental noise floor, and similar to our previously reported values [79].

#### **A.5.3 Materials Preparation**

The remainder of the Material and Methods section is dedicated to briefly describing the experimental conditions under which cell-displacement data was acquired. Most of the described materials procedures are based on our previously developed methods and protocols [175, 195, 79, 186].

#### **A.5.4 Glass Cover Slips and Microscope Slides Surface Modification**

Circular cover slips (25mm, Thermo Fisher Scientific, MA) are chemically modified to allow covalent attachment of polyacrylamide substrates using previously developed protocols [205, 58, 175]. Briefly, glass cover slips are rinsed with ethanol and placed in a petri dish containing a solution of 0.5% (v/v) 3-aminopropyltrimethoxysilane (Sigma-Aldrich, MO) in ethanol for 5 minutes. Next, cover slips are washed with ethanol and submersed in a solution of 0.5% glutaraldehyde (Polysciences, Inc., PA) in deionized (DI) water for 30 minutes. Activated cover slips are washed with DI water and left to dry. Rectangular cover slides (75×25 mm, Fisher Scientific) are chemically modified to create hydrophobic surface to facilitate gel detachment. Cover slides are placed in a petri dish containing 97% (v/v) hexane (Thermo Fisher Scientific, MA), 2.5% (v/v) (tridecafluoro-1,1,2,2-tetrahydrooctyl)-triethoxysilane (SIT) (Gelest Inc., PA), and 0.5% (v/v) acetic acid (Sigma-Aldrich, MO) for approximately 1 minute. Cover slides are removed and left to dry.

### A.5.5 Preparation and Mechanical Characterization of PA Substrates

Thin films of different PA gels are prepared from 40% w/v acrylamide (Bio-Rad, CA) and 2% w/v N,N-methylene-bis-acrylamide (BIS) (Bio-Rad, CA) stock solutions as described previously [205, 58, 175]. The final acrylamide-BIS concentration ratios yield Young's moduli of  $\sim 238$  Pa for Schwann cells,  $\sim 1500$  Pa for neutrophils, and  $\sim 820$  Pa for 3T3 fibroblasts. All substrates contain 14% (w/v) of fluorescent microspheres (0.5  $\mu\text{m}$  in diameter, carboxylate-modified, Life Technologies, NY). Crosslinking is initiated through the addition of ammonium persulfate (Sigma-Aldrich, MO) and N,N,N,N-tetramethylethylenediamine (Invitrogen, CA). The PA gel solution is vortexed for about 30 seconds and 35  $\mu\text{l}$  of PA solution is pipetted on the surface of microscope slide and sandwiched with an activated glass coverslip (25 mm in diameter), yielding a final thickness of around 60  $\mu\text{m}$ .

### A.5.6 Mechanical Characterization of PA Substrates

Mechanical characterization of all PA substrates is based on previously established testing protocols on a custom-built uniaxial compression apparatus. Briefly, gel samples are cast in circular nylon molds (16 mm in diameter and 10 mm in height). Following polymerization, the molds are removed and the samples are submersed in DI water. The custom-built compression device consists of a centrally positioned linear actuator (Series A, Ultramotion, NY) and 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., CT) is attached to the end of the linear actuator. To ensure uniaxial compression conditions, a ball point tip-platen top is placed on each gel sample, and all samples are carefully aligned along the central compression axis of the linear actuator [78, 175]. Nominal stress ( $\sigma$ ) is computed by dividing the measured applied force by the circular contact area. Nominal strain ( $\epsilon$ ) is calculated by measuring the height change of each sample divided by the sample's original height. Each sample is compressed at three different strain rates ( $10^{-2}$ ,  $10^{-1}$ , and  $10^0$ ) to capture any time-dependence in the material behavior. Young's modulus is calculated from the slope of each linear stress-strain curve. As shown previously, PA can be described as NH solid that has the same Young's modulus as the linear stress-strain relationship in the limit of

small strains [160]. The Poisson's ratio is taken to be 0.45, which is within the range of the typical values chosen for TFM studies [65, 55].

### **A.5.7 Functionalization of PA Substrates**

To promote cell attachment to polyacrylamide films, substrates are functionalized with laminin (Schwann cells) and fibronectin (neutrophils and fibroblasts) using the bifunctional crosslinker, sulfo-SANPAH (Pierce Chemicals, TX) [205, 58, 175, 195, 186]. Excess water is removed prior to deposition of 100  $\mu$ l of sulfo-SANPAH (1mg/ml) onto the surface of each film, following a 15-minute exposure to UV light. The darkened sulfo-SANPAH solution is aspirated and the procedure is repeated. The samples are thoroughly washed with DI water and are covered with a solution of 0.2 mg/ml laminin (Thermo Fisher Scientific, MA) diluted in 50 mM HEPES (pH 8, Sigma Aldrich, MO). Samples are then left undisturbed at 4°C overnight. Following overnight incubation, the substrates are rinsed three times with  $1\times$  phosphate buffered saline and sterilized with UV irradiation before depositing cells.

### **A.5.8 Cell Culture**

Schwann cells were cultured as previously described by Mitchel et al. [186]. Fibroblasts and neutrophils were cultured according to protocols published by Franck et al. and Oakes et al. [195, 175, 79]. Schwann cells, fibroblasts and neutrophils were seeded at a density of 1,000, 80,000, and 50,000 cells/cm<sup>2</sup> on PA gels. Cells were allowed to attach for 4 hours before initiation of the time-lapse.

### **A.5.9 Live Cell Imaging**

After an initial seeding period, three-dimensional image stacks of individual cells on PA gels are acquired using a Nikon A-1 confocal system mounted on a TI Eclipse inverted optical microscope controlled by NI-Elements Nikon Software. Red fluorescent microspheres are used as fiduciary markers and excited with a Diode (561 nm) laser.  $512\times 512\times P$  voxels ( $209\times 209\times P$   $\mu$ m) confocal volume stacks are recorded at user specific time intervals (e.g., 10 seconds to 30 minutes) with  $P$  ranging from 64 - 256 voxels ( $\sim 20$  -  $77$   $\mu$ m). To ensure physiological imaging conditions within the imaging chamber, temperature and pH are controlled at 37°C and 7.4 during time-lapse recording as previously described [175, 79]. Cellular surface outlines are determined either from phase images or from fluorescent

membrane labels.

## **A.6 Acknowledgments**

The authors wish to thank Cindy Oh for her help with the Schwann cell experiments, and Professor Allen F. Bower for his helpful discussions.



## B. 3D Traction Forces of Schwann Cells on Compliant Substrates

**Note:** A version of this appendix is published in *Journal of the Royal Society Interface* with Cristina Fagundo-López as a first co-author. Published data is included in the section with co-author’s consent.

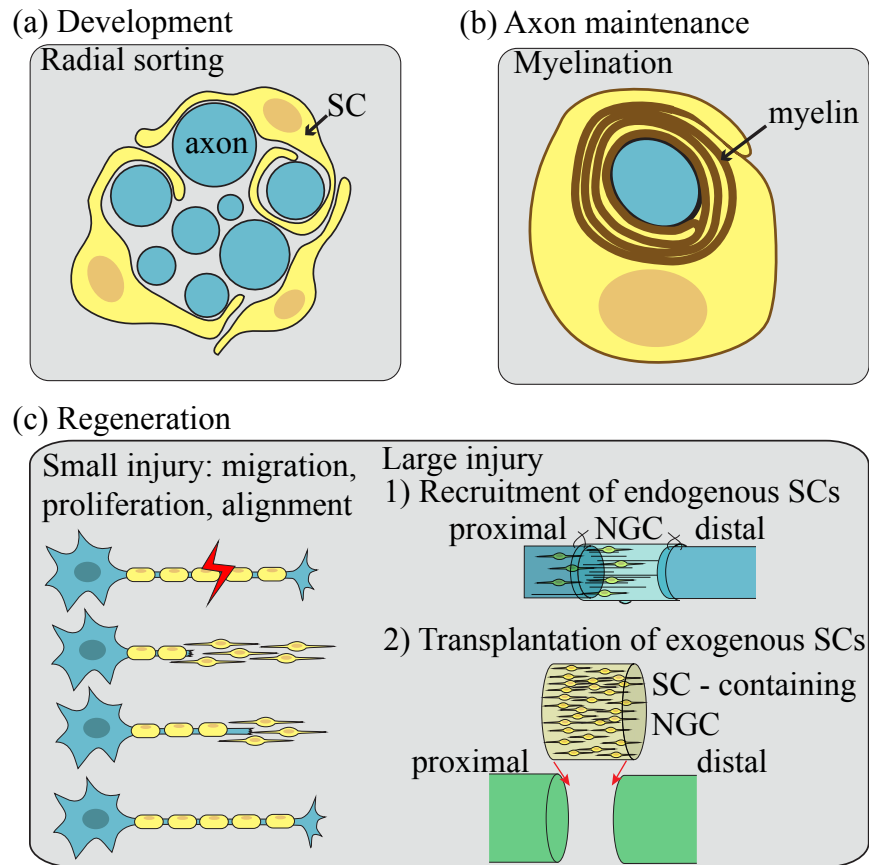
Bar-Kochba, E., Fagundo-López, C., Hoffman-Kim, D., & Franck, C. (2014). Three-dimensional traction forces of Schwann cells on compliant substrates. *J. R. Soc. Interface*, 11(97):20140247. doi: 10.1098/rsif.2014.0247

### B.1 Abstract

The mechanical interaction between Schwann cells (SCs) and their microenvironment is crucial for the development, maintenance, and repair of the peripheral nervous system. In this paper we present a detailed investigation on the mechanosensitivity of SCs across a physiologically relevant substrate stiffness range. Contrary to many other cell types we find that the SC spreading area and cytoskeletal actin architecture was relatively insensitive to substrate stiffness with pronounced stress fiber formation across all moduli tested (0.24 kPa to 4.80 kPa). Consistent with the presence of stress fibers we found that SCs generated large surface tractions on stiff substrates and large, finite material deformations on soft substrates. When quantifying the 3D characteristics of the SC traction profiles we observed a significant contribution from the out-of-plane traction component, locally giving rise to rotational moments similar to those observed in mesenchymal embryonic fibroblasts. Taken together these measurements provide the first set of quantitative biophysical metrics of how SCs interact with their physical microenvironment, which are anticipated to aid in the development of tissue engineering scaffolds designed to promote functional integration of SCs into post-injury *in vivo* environments.

## B.2 Introduction

The biophysical capabilities of Schwann cells (SCs) in the context of their vivo-like mechanical environments have remained understudied until now. SCs in the body require the maintenance and versatility of their bipolar phenotype across a range of biomechanical environments to perform their functions. They play a pivotal role in the development, maintenance and regeneration of axons in the peripheral nervous system (PNS) [122, 107, 123]. SCs are derived from neural crest cells and can either be myelinating or nonmyelinating [123]. In a process called ‘radial sorting’ during development of peripheral nerves, SCs associate with, and segregate axons based on their thickness (**Fig. B.1a**). After sorting



**Figure B.1:** SCs perform a range of functions in the body. **(a)** During development SCs sort axons based on their diameter and **(b)** deposit myelin on larger axons to increase conduction velocity of action potentials. **(c)** SCs aid axon regeneration by migrating into an injury site after a small injury, or into a transplanted nerve guidance channel (NGC) after a large injury. Once there, SCs proliferate and align. NGCs can also be pre-seeded with exogenous SCs to enhance regeneration.

is complete, myelin sheaths are deposited by SCs around axons that are larger than 1  $\mu\text{m}$  in diameter. This provides axons with the required insulation for fast moving, saltatory conduction of electrical signals [21] (**Fig. B.1b**). After injury in the PNS (**Fig. B.1c**), SCs present at the e stump of the nerve become activated, and migrate to the injury site. There they enter mitosis, mitigate Wallerian degeneration and provide axons with a path to grow along by forming an aligned trail of bipolar cells, commonly referred to as bands of Büngner [227, 106]. In injuries, even at the scale of millimeters, endogenous SCs respond and are capable of guiding nerve fibers toward the distal stump (**Fig. B.1c**). Moreover, although naturally not found in the central nervous system (CNS), transplantation of exogenous SCs into the brain [139] or the spinal cord [302, 33] has promoted axonal regeneration. The means by which SCs accomplish successful migration and morphological organization across these scenarios remain unknown. Understanding the mechanosensitive behavior of SCs in tailorable mechanical environments could play an important role in guiding scaffold designs to promote efficient functional integration of SCs *vivo*.

It is known that not only do cells interact with their environment biochemically but also physically; e.g. substrate stiffness modulates cell functions such as cell adhesion, motility and spreading [205, 287, 65, 67, 195]. While the role of substrate stiffness on SCs has begun to be acknowledged [98], here we present an experimental system of significance to the nervous system environment, in its investigation of stiffnesses relevant to the nervous system, mediated by laminin, a molecule present in developmental peripheral axon tracts and upregulated following injury. To gain insight into this physical interaction, we investigated and quantified the three-dimensional (3-D) tractions, cell morphologies, and cell spreading behavior of SCs on polyacrylamide (PA) gels ranging in stiffness from 0.24 to 4.80 kPa. This range is consistent with reported stiffnesses in the nervous system [105, 184]. We also characterized the correlation of morphology with substrate stiffness since SCs are reported to exhibit different types of morphologies *in vitro* [186, 291]. Interestingly, we found that the bipolar morphology, the most common morphology *vivo*, is the most prevalent across all stiffnesses in our study.

Perhaps our most striking finding is the observation of similar cytoskeletal architecture

with the existence of pronounced actin stress fibers across all elastic moduli tested, even on moduli as soft as 0.24 kPa. Since the formation and existence of stress fibers usually indicates a highly contractile stress state, we quantified the 3-D displacement and traction signature of SCs as a function of substrate stiffness. Using a recently reformulated 3-D traction force microscopy (3-D TFM) approach capable of accounting for finite material deformations, we found that on the most compliant PA gels, SCs generate strains large enough to cause significant substrate surface deformations, highlighting the contractile capability of these cells. By analyzing the spatial distribution of the 3-D SC displacement and traction fields, we found that SCs generate highly localized out-of-plane moments and that their size, distribution and characteristics are similar to recent findings for mouse embryonic fibroblasts [151]. Taken together these results provide important insights into the mechano-characteristics of SCs that should be considered in the design of regenerative tissue engineering scaffolds and nerve conduits.

## B.3 Methods

### B.3.1 3-D Traction Force Microscopy

Cell-generated full field displacements and tractions were computed using our recently developed large deformation 3-D traction force microscopy (3-D LDTFM) method [276]. Briefly, three-dimensional time-lapsed volumetric images of fluorescent beads embedded in polyacrylamide substrates were recorded using laser scanning confocal microscopy (LSCM). A new fast iterative digital volume correlation (FIDVC) algorithm was used to track the motion of the embedded fluorescent beads in all three dimensions between each time increment [15]. Due to the spatial refinement of the subvolume correlation windows, the FIDVC is capable of capturing large non-linear displacement fields with high spatial resolution while maintaining low computation times ( $\approx 1.5$  min per time increment for a typical image size of  $512 \times 512 \times 160$  voxels). The final subvolume spacing, the parameter that controls the displacement spatial grid resolution, was set to 8 voxels ( $\approx 3.3 \mu\text{m}$  for in-plane measurements and  $2.4 \mu\text{m}$  for out-of-plane measurements).

After the full-field displacements,  $\mathbf{u}$ , were determined, the deformation gradient tensor,  $\mathbf{F}$ ,

of the substrate is calculated via finite differentiation. Extra precaution is taken to choose a differentiation kernel that mitigates sampling dealiasing errors and measurement noise. The differentiation kernel we used in this study is an optimal-9 tap filter described in detail by Farid et al. [72]. The computation of  $\mathbf{F}$  is necessary to accurately calculate the Cauchy stress tensor,  $\boldsymbol{\sigma}$ , since for large deformations, as those seen in this study, the reference and deformed configurations are no longer identical [276]. For this reason, Hooke's law of linear small-strain elasticity used in most previous studies [37, 305, 175, 114, 135] is replaced by a neo-Hookean finite deformation material model [26]. The relation between the Cauchy stress ( $\boldsymbol{\sigma}$ ) and the deformation gradient ( $\mathbf{F}$ ) is mathematically expressed [26] as

$$\boldsymbol{\sigma} = \frac{\mu}{J^{5/3}} \left( \mathbf{B} - \frac{1}{3} \text{trace}(\mathbf{B}) \cdot \mathbf{I} \right) + K(J - 1)\mathbf{I} \quad (\text{B.3.1})$$

where the parameters  $\mu$  and  $K$  are the shear and bulk modulus for PA. By using our mechanical characterization results (see Supplemental Material) we can relate these quantities to the elastic modulus and Poisson's ratio of the material by

$$\mu = \frac{E}{2(1 + \nu)}, \quad K = \frac{E}{3(1 - 2\nu)}. \quad (\text{B.3.2})$$

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

$$J = \det(\mathbf{F}) \quad (\text{B.3.3})$$

and

$$\mathbf{B} = \mathbf{F} \cdot \mathbf{F}^T. \quad (\text{B.3.4})$$

The tractions acting on the deformed surface,  $\mathbf{T}$ , with surface normals  $\mathbf{n}$  are calculated via the Cauchy relationship,

$$\mathbf{T} = \mathbf{n} \cdot \boldsymbol{\sigma}. \quad (\text{B.3.5})$$

The root mean squared tractions,  $T_{RMS}$  are defined as

$$T_{RMS} = \sqrt{\frac{1}{N} \sum_{i=1}^N \mathbf{T}_i^2}, \quad (\text{B.3.6})$$

where  $\mathbf{T}_i$ , is the traction vector located at every  $i$ th point along the surface within the boundary, enclosing  $N$  points (see **Fig. S2** in the Supplemental Material). More details about the computation of the root-mean-squared integration boundary can be found in the Supplemental Material.

Before proceeding to calculate the tractions ( $\mathbf{T}$ ) we must first determine the true surface normals ( $\mathbf{n}$ ). In brief, we take advantage of the spatial locations of the embedded fluorescent beads in the raw LSCM images. A scattered data representation of the surface is built by finding the local maximum height of the bead locations using a sliding window. After least-squared fitting and surface gradient smoothing [59], the normals were calculated from a Delaunay triangulation of the surface. The subsequent surfaces in the time-lapse were computed by translating the surface points in the reference configuration via the displacement field calculated from the FIDVC algorithm.

Finally, to calculate the work done by the cell to elastically deform the substrate, we compute the strain energy between time increments as the volume integral of the strain energy density  $\bar{U}$ , i.e.

$$U = \int_V \bar{U} dV. \quad (\text{B.3.7})$$

For a Neo-Hookean solid the strain energy density is given by [26]

$$\bar{U} = \frac{\mu}{2} \left( \frac{\text{tr}(\mathbf{B})}{J^{2/3}} - 3 \right) + \frac{K}{2} (J - 1)^2 \quad (\text{B.3.8})$$

### B.3.2 Fabrication of Polyacrylamide Gels

Thin films of different polyacrylamide (PA) gels were prepared from 40% (w/v) acrylamide (Bio-Rad Laboratories, Hercules, CA) and 2.5% (w/v) N,N'-methylene-bis-acrylamide (BIS,

Bio-Rad Laboratories) stock solutions as described previously [205, 58, 175] with 20% (w/v) red fluorescent microspheres (0.5  $\mu\text{m}$  in diameter, carboxylate-modified, Life Technologies, Grand Island, NY). The final acrylamide-BIS concentration ratios, 3.0%–0.08%, 3.0%–0.2%, and 5.0%–0.1% produced PA gels with elastic moduli of 0.24 kPa, 1.70 kPa, and 4.80 kPa respectively (see **Fig. S1** in the Supporting Material). Crosslinking was initiated by the addition of 0.125% ammonium persulfate (APS, Sigma-Aldrich, St. Louis, MO) and N,N,N',N'-Tetramethylethylenediamine (TEMED, Life Technologies). The PA gel solution was vortexed for 30 seconds and 20  $\mu\text{l}$  of PA solution was pipetted onto the surface of a microscope slide and sandwiched with an activated glass coverslip (25 mm in diameter, Thermo Fisher Scientific, Waltham, MA), yielding a final gel thickness of approximately 40 – 60  $\mu\text{m}$ .

PA gels were chemically functionalized with laminin (LN, Life Technologies) using the heterobifunctional crosslinker sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate (sulfo-SANPAH, Thermo Fisher Scientific) to promote cell adhesion [58, 175]. After removal of the excess water, 100  $\mu\text{L}$  of sulfo-SANPAH (1 mg/mL) was deposited onto the surface of each sample, and irradiated with ultraviolet light for 15 minutes to initiate activation. The darkened sulfo-SANPAH solution was aspirated and the procedure was repeated. The samples were rinsed three times with 1 $\times$  phosphate buffered saline (PBS, Life Technologies) and their surfaces covered with 0.2 mg/mL LN in Hank's balanced salt solution (Life Technologies) overnight at 4  $^{\circ}\text{C}$ . Excess LN was removed and gels were rinsed three times with PBS for 10 minutes before cell deposition.

### B.3.3 Cell Culture

All cell culture reagents were from Life Technologies unless otherwise stated. SCs from adult rat sciatic nerve (generous gift from Dr. Mary Bunge, University of Miami, Coral Gables, FL, USA) were cultured on tissue culture plastic flasks pre-coated with 100  $\mu\text{g}/\text{mL}$  poly(L-lysine) (PLL, Sigma-Aldrich) in Dulbecco's modified eagle's medium with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100  $\mu\text{g}/\text{mL}$  penicillin and 100  $\mu\text{g}/\text{mL}$  streptomycin supplemented with 2  $\mu\text{M}$  forskolin (Sigma-Aldrich), 10  $\mu\text{g}/\text{mL}$  bovine pituitary extract (Sigma-Aldrich) and 2  $\mu\text{M}$  heregulin (Genentech, San Francisco, CA) (SC media). Cells used in experiments

were between passages 5 and 9. Cultured cells were maintained in a humidified chamber at 37 °C with 5% CO<sub>2</sub>. Cells were trypsinized using 0.25% trypsin and seeded in SC media on functionalized gels at a density of 950 SCs/cm<sup>2</sup> for 3 hours to allow for cellular attachment. Cell-labeling solution, Vybrant DiD (far red, 30 μL/mL of SC media) was applied to each sample for 30 minutes at 37 °C and 5% CO<sub>2</sub> followed by 3 rinses with media for 10 minutes at 37 °C and 5% CO<sub>2</sub>.

#### **B.3.4 Confocal Microscopy**

After an initial seeding period, three-dimensional image stacks of individual SCs on PA gels 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). The temperature in the microscope chamber was controlled and regulated to 37 °C using a feedback-controlled heater (Air-Therm heater, World Precision Instruments Inc., Sarasota, FL). PA gel samples containing stained SCs were mounted into a Chamlide magnetic chamber (Live Cell Instrument, Seoul, Korea), filled with SC media and placed in the microscope chamber where 5% CO<sub>2</sub> was injected. All samples had an equilibration time of one hour before the start of the time-lapse experiments to avoid thermal drift. A 40× CFI APO Lambda S water objective with a numerical aperture of 1.25 was used in all experiments. The green fluorescent microspheres used as fiduciary markers were excited with an Argon ion laser (488 nm) and the fluorescent cell membrane label was excited with a Diode (640 nm) laser. Confocal image stacks of size 512 × 512 × 160 voxels (212 × 212 × 48 μm<sup>3</sup>) were acquired every 10 minutes for 1 hour (see Movie S1 in the Supporting Material). For all cases, the area surrounding a cell was scanned to ensure the selection of an isolated cell. Experiments for each stiffness were performed in triplicate and at least 15 cells were recorded per experiment. Cellular surface outlines were determined either from phase images or from fluorescent membrane labels.

#### **B.3.5 Fluorescence Staining**

All reagents were from Life Technologies unless otherwise stated. Unlabeled SCs were plated on gels without beads and cultured under the same experimental conditions as previously described in the Confocal Microscopy section. After 5 hours in culture, cells were fixed with

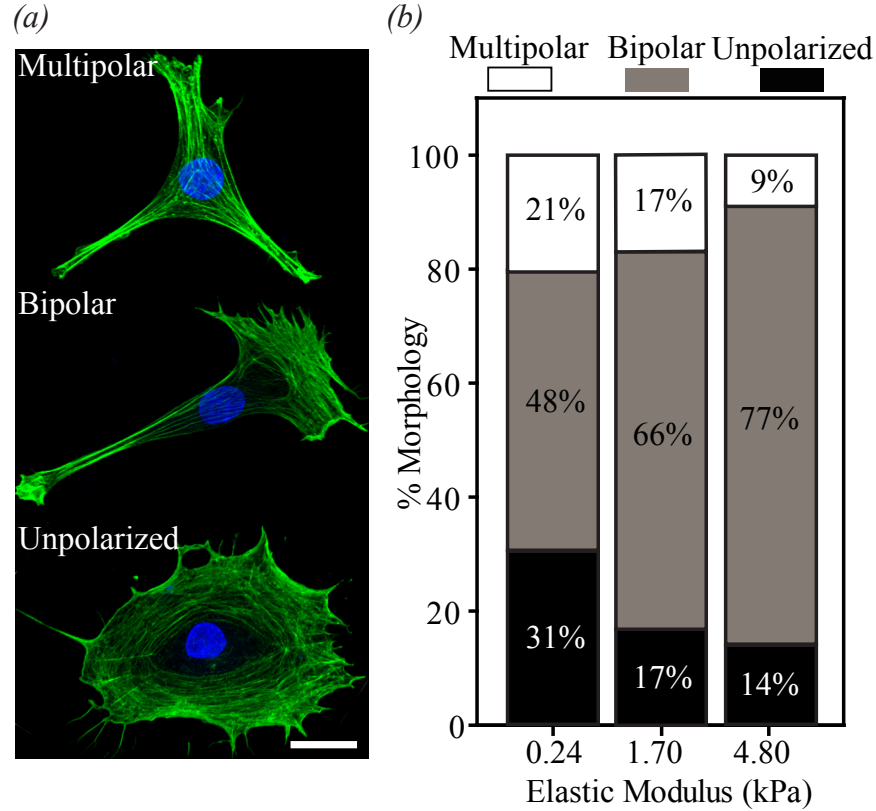


2% paraformaldehyde in 0.1 M PBS (pH 7.4) for 30 minutes. After rinsing 3 times with PBS, samples were incubated with 0.1% Triton X-100 (VWR, Radnor, PA) in 0.1 M PBS for 5 minutes, rinsed 2 $\times$  with 0.1 M PBS, and incubated with 1% bovine serum albumin (BSA, Sigma-Aldrich) in 0.1 M PBS for 30 minutes. Alexa 488-phalloidin methanolic stock stain solution was diluted 1 in PBS and added to samples for 1 hour followed by 2 rinses with PBS. Cell nuclei were stained with TO-PRO-3 iodide (Sigma-Aldrich) diluted to 1  $\mu\text{g}/\text{ml}$  in PBS. Samples were rinsed two times with PBS before they were imaged. To image the cells, two different lasers were used. A diode laser (640 nm) was used to image the nuclei of the cells in the far red spectrum and an Argon ion laser (488 nm) was used to image phalloidin-488.

## B.4 Results and Discussion

### B.4.1 Schwann cells exhibited varying morphologies depending on substrate stiffness

When SCs were cultured on PA gels with elastic moduli of  $0.24 \pm 0.05$  kPa,  $1.70 \pm 0.09$  kPa, and  $4.80 \pm 0.29$  kPa, three distinct types of morphologies were observed; unpolarized, bipolar, and multipolar (**Fig. B.2a**). The unpolarized morphology was characterized by a general circular shape with the cell nucleus located symmetrically in the center of the cell. The bipolar morphology was characterized by two processes extending away from the nucleus. In general, the bipolar extensions were thin and elongated, sometimes displaying wide lamellar-like features at the tip of each extension. The multipolar morphology was characterized by the presence of three or more processes. **Fig. B.2b** shows the percent occurrence of each morphology across elastic moduli to assess the influence of matrix stiffness on SC phenotype. These results suggest that the substrate's elastic modulus can be used to predetermine the morphology of SCs. This result takes particular significance considering studies showing that cellular morphology can influence growth and function [41]. Additionally, we found that the bipolar morphology was dominant across elastic moduli with increasing occurrence on stiffer substrates within this physiological range. Incorporating these stiffnesses in nerve regeneration strategies for targeting SCs to the injury site could be of great benefit since recent studies have shown that the bipolar phenotype is characteristic

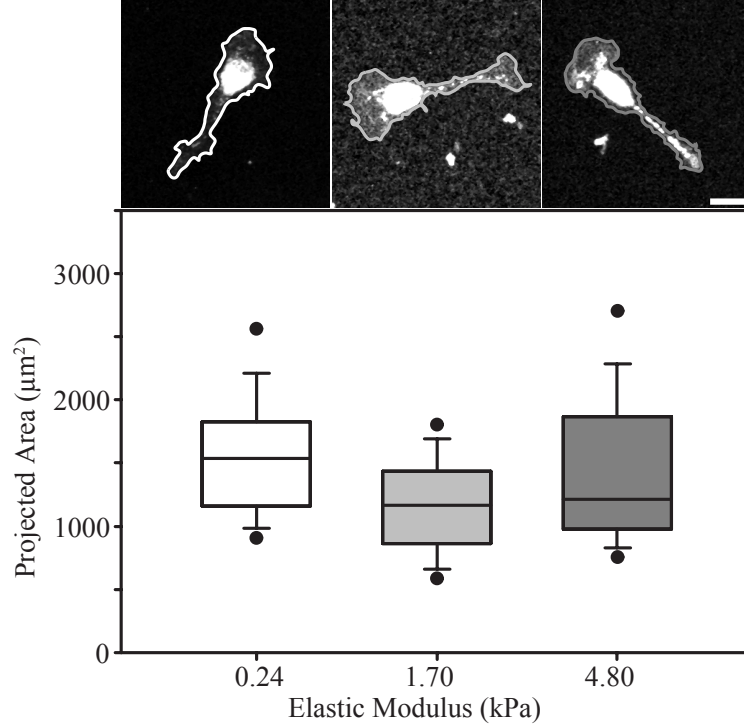


**Figure B.2:** Substrate stiffness affected morphological distribution of SCs *in vitro*. (a) Maximum projection confocal micrographs of representative SCs exhibiting unpolarized, bipolar, and multipolar morphologies (bottom to top) stained with Phalloidin for actin, and TO-PRO-3 iodide for nuclei (green and blue respectively). Scale bar represents 20  $\mu\text{m}$ . (b) Bar plot of the percent occurrence of unpolarized (black), bipolar (gray), and multipolar (white) morphology occurrence per stiffness shows that the bipolar phenotype is most prevalent across all elastic moduli; 0.24 kPa ( $n = 298$ ), 1.70 kPa ( $n = 382$ ), and 4.80 kPa ( $n = 156$ ).

of motile and migratory SCs [186].

#### B.4.2 Schwann cells' spreading area was unaffected by substrate stiffness

Focusing on bipolar SCs, the most common phenotype and the most relevant to vivo function, we examined SC spreading. It is well known that the mechanical rigidity of the substrate alters the cellular shape and spreading area [61, 260]. Furthermore, cell shape has been reported to affect cell fate [41, 306, 61] and cytoskeletal tension has been shown to be important for SC cycle progression [291]. Kidney epithelial cells [205], fibroblasts [205, 306], vascular smooth muscle cells [29], aortic endothelial cells [306] and neutrophils [195] cultured on substrates of varying stiffness all showed reduced spreading area on the softest substrate.

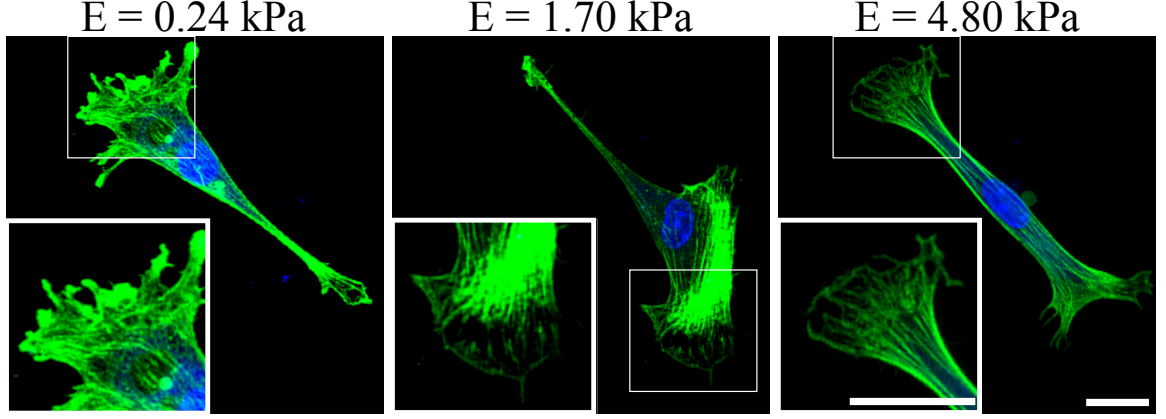


**Figure B.3:** SC spreading area was similar across elastic moduli ( $E$ ). Box and whiskers plot of SC spreading area for the three tested elastic moduli; 0.24 kPa ( $n = 143$ ), 1.70 kPa ( $n = 252$ ), and 4.80 kPa ( $n = 120$ ). Solid bands: medians, top and bottom boxes: upper and lower quartiles, whiskers: standard deviations, and dots: 5th and 95th percentiles. Representative confocal micrographs of SCs for each elastic modulus are shown. Scale bar represents 20  $\mu\text{m}$ .

Unlike many other cells, SCs were able to adhere and extend protrusions regardless of substrate stiffness. Additionally, **Fig. B.3** shows that SC spreading area was comparable across elastic moduli; median values of 1540  $\mu\text{m}^2$  for  $E = 0.24$  kPa, 1160  $\mu\text{m}^2$  for  $E = 1.70$  kPa, and 1210  $\mu\text{m}^2$  for  $E = 4.80$  kPa respectively. This suggests that SC spreading behavior is relatively insensitive to the underlying mechanical substrate stiffness, contrary to almost all other mesenchymal or ameboid cell types.

#### B.4.3 Schwann cells exhibited stress fibers on soft materials

To investigate how SCs maintained a consistent spreading area across the different elastic moduli tested, we examined the structural organization of the SC cytoskeleton with the primary focus on filamentous actin. SC were grown on the different elastic moduli as described before and their actin filaments were stained with Phalloidin. As can be seen from **Fig. B.4**, SCs exhibited a mature actin cytoskeleton with the presence of pronounced stress fibers

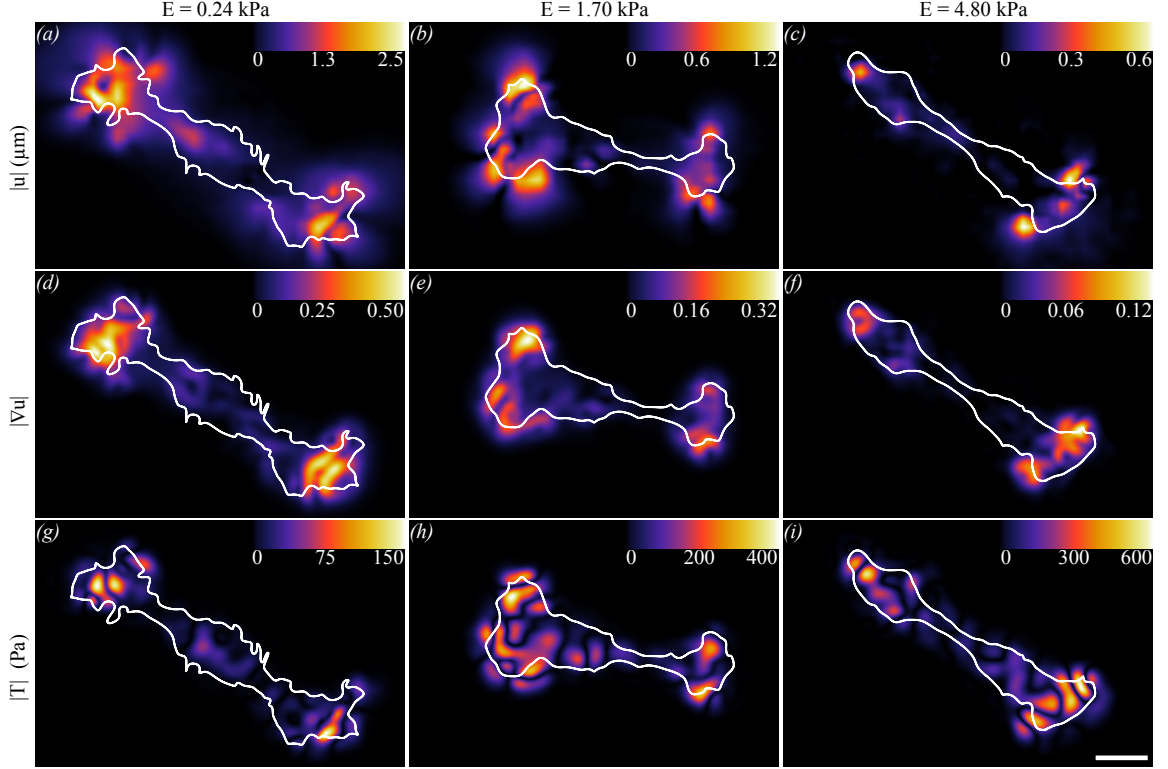


**Figure B.4:** SCs exhibited a mature actin cytoskeleton including stress fibers across all moduli. Maximum projection confocal micrographs of representative SCs cultured on elastic moduli of 0.24 kPa, 1.70 kPa, and 4.80 kPa that were stained with phalloidin for actin, and TO-PRO-3 iodide for nuclei (green and blue respectively). White box shows a  $2\times$  zoomed in image to highlight the stress fibers. Scale bars represents  $20 \mu\text{m}$ .

across all moduli. In contrast to most other cells, SCs produced clearly visible stress fibers on gels of 0.24 kPa, which is indicative of a contractile phenotype with significant cell traction generation [247, 249, 84]. Intrigued by these findings, we quantified SC tractions across the different moduli to investigate what parameters supported the observed cytoskeletal arrangement and cell spreading areas presented in **Figs. B.3 – B.4**.

#### B.4.4 Schwann cells induced large 3-D tractions and material deformations

Motivated by our results, that both SC spread area and F-actin arrangement appeared to be unaffected by substrate stiffness, we quantified the material deformations caused by SCs across our substrate stiffnesses. Using our recently improved 3-D large deformation traction force microscopy (3-D LDTFM) technique [276] we quantified the 3-D SC displacement and traction field as a function of substrate stiffness. One of the key improvements in our technique is the incorporation of finite deformation constitutive models that allow proper determination of cell tractions in the presence of large deformations. This is a significant departure from most traditional TFM approaches that intrinsically assume infinitesimal material deformations. **Fig. B.5** shows color contour maps of the magnitudes of the 3-D displacement vector (**a-c**), the 3-D displacement gradient (**d-f**), and the 3-D surface tractions



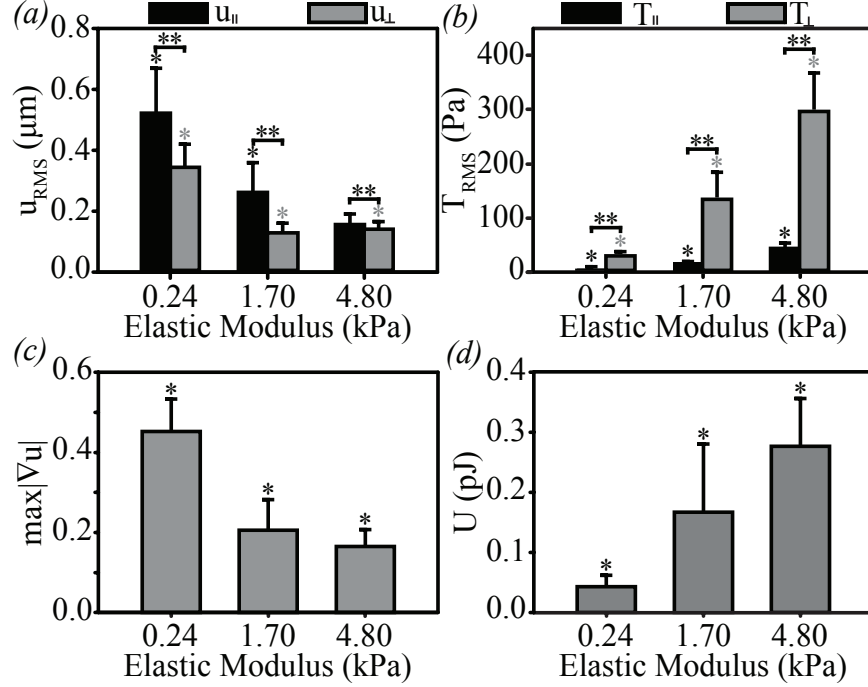
**Figure B.5:** SC displacements and tractions varied across substrate stiffness. Surface contour maps of the magnitude of (a–c) the 3-D displacement field ( $|u|$ ), (d–f) the displacement gradient ( $|\nabla u|$ ), and (g–i) the traction field ( $|T|$ ) vector for each elastic modulus; 0.24 kPa, 1.70 kPa, and 4.80 kPa. The cell outline (white) is superimposed onto the contour maps to show its position with respect to each field. Scale bar represents 20  $\mu\text{m}$ .

(g–i) across our three substrate moduli. To provide a robust comparison between cellular traction generation and cytoskeletal arrangement, we analyzed both immunohistological images and SC tractions of a large population of cells and time points under identical experimental conditions. Consistent with the F-actin arrangement shown in **Fig. B.4**, the spatial distribution of SC displacement and surface tractions was concentrated at both distal ends of the cells, indicating a highly polarized cell. The spatial distribution of displacement and traction profiles looked similar across the three substrate moduli, which is qualitatively consistent with the cytoskeletal arrangement shown in **Fig. B.4**. While the distributions of SC displacements and tractions appeared minimally affected by the underlying substrate stiffness, the magnitude of the displacement values decreased as the substrate became stiffer whereas the tractions increased. These results are consistent with general trends observed in other cell types; however SCs’ displacement attenuation in our study is distinct and contrasts

with other cells’ across stiffnesses ranging from 1 to 10 kPa or greater [175]. Interestingly, when we performed additional TFM experiments of SC on PA gels of moduli  $> 5$  kPa, we found no noticeable cell-generated material displacements during spreading and locomotion.

Since SCs were capable of forming visible stress fibers on the soft PA gels, we investigated the material deformations that SCs might generate to maintain their stress fibers. **Fig. B.5d-f** shows the magnitude of the displacement gradient across stiffnesses. In linear elastic theory, the basis of most traditional TFM formulations that employ the Boussinesq solution approach, the gradient term is deemed small, i.e.,  $\ll 1$ . However, as shown in **Fig. B.5d** the deformation gradient on the 240 Pa gels was significant with peak levels above 0.5, indicating the existence of large deformations. Given the compliant nature of these gels, and the existence of stress fibers, it was not surprising that the cell would need to apply large enough deformations to maintain stable adhesion contacts conducive to the formation of stress fibers. Examining **Figs. B.5a,d,g** and **Fig. B.4**, we see that stress fiber formation was supported both by large material tractions and also through large material deformations. Given that the material deformations originate from intracellular force generation and regulation, we note that the magnitude of the displacement gradient decreased as the substrate stiffness increased, which supports the idea that less compliant substrates require less material deformation to achieve the necessary adhesion to produce stress fibers [84].

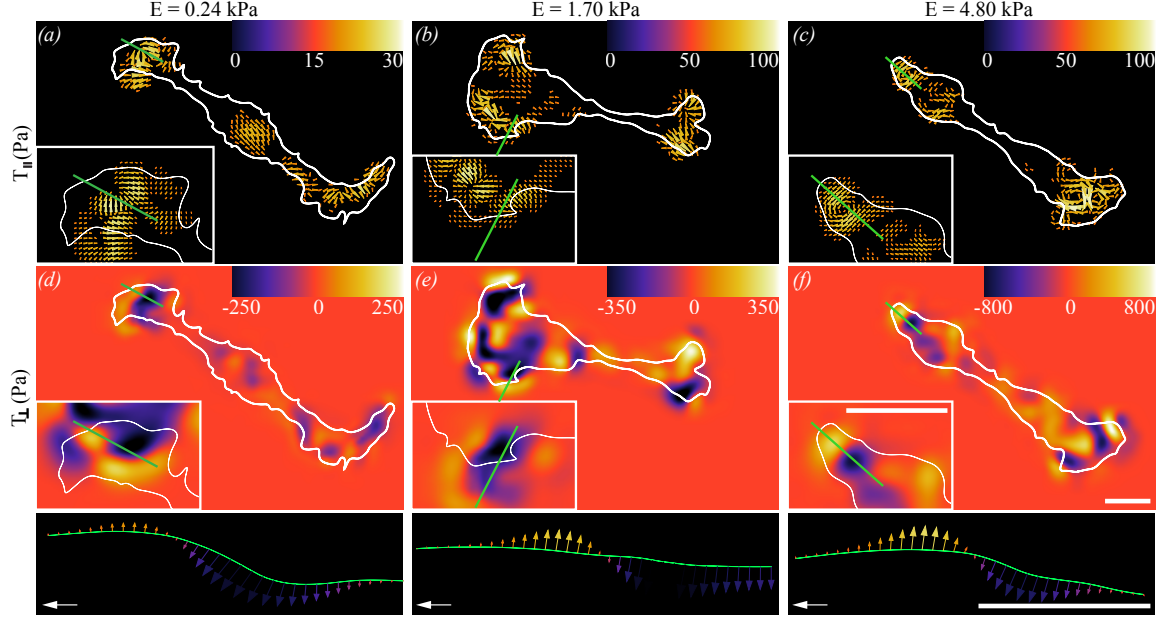
**Fig. B.6a-c** summarizes our displacement and traction results in a statistical bar plot format across all moduli to present a cumulative distribution of our findings for the bipolar morphology (see **Figs. S3 – S4** in the Supplemental Material for more information about the unpolarized and multipolar phenotypes). We included calculations of the applied strain energy as an indication of the work done by the cells on the material (**Fig. B.6d**). We believe this metric is useful as it is an integral measure of both the applied displacements and tractions. The strain energy of the SCs followed the same trend as the tractions across moduli showing that cells worked harder on the stiffer substrates to spread and locomote. Furthermore, as shown in **Fig. B.6a,b**, we found significant differences between the in-plane (shear) and out-of-plane (normal) displacement and traction components, which are strongly attenuated by the underlying substrate stiffness. In particular, the normal traction



**Figure B.6:** SC displacements, tractions, and strain energies varied with elastic moduli. Bar plots of the (a) root mean squared displacements magnitude ( $u_{RMS}$ ), (b) root mean squared tractions ( $T_{RMS}$ ), (c) maximum displacement gradient magnitude ( $\max|\nabla u|$ ), and (d) strain energy ( $U$ ) as a function of elastic modulus; 0.24 kPa ( $n = 143$ ), 1.70 kPa ( $n = 252$ ), and 4.80 kPa ( $n = 120$ ). The  $u_{RMS}$  and  $T_{RMS}$  values are split into their respective shear components ( $u_{||}$ ,  $T_{||}$ ) and normal components ( $u_{\perp}$ ,  $T_{\perp}$ ) (black and gray respectively). Geometric mean ( $\mu_g$ ) and geometric mean absolute deviation ( $MAD$ ) are shown. \* indicates  $p < 0.05$  by one-way ANOVA on ranks across elastic moduli, and \*\* indicates  $p < 0.001$  by t-test between normal and shear components respectively.

component plays a significant role in the SC traction signature as the material stiffness increases.

To investigate the observed shear and normal traction differences further, we plotted the shear and normal traction distribution of representative SCs across the three different moduli in **Fig. B.7**. Consistent with the stiffness scaling observed in **Fig. B.6a-c**, the shear tractions increase only slightly with increasing substrate stiffness whereas the normal tractions show a significant increase in their magnitude (**Fig. B.7a-c**). The spatial distribution of both the shear and normal tractions show a highly polarized cell with most of the tractions applied at the distal ends.



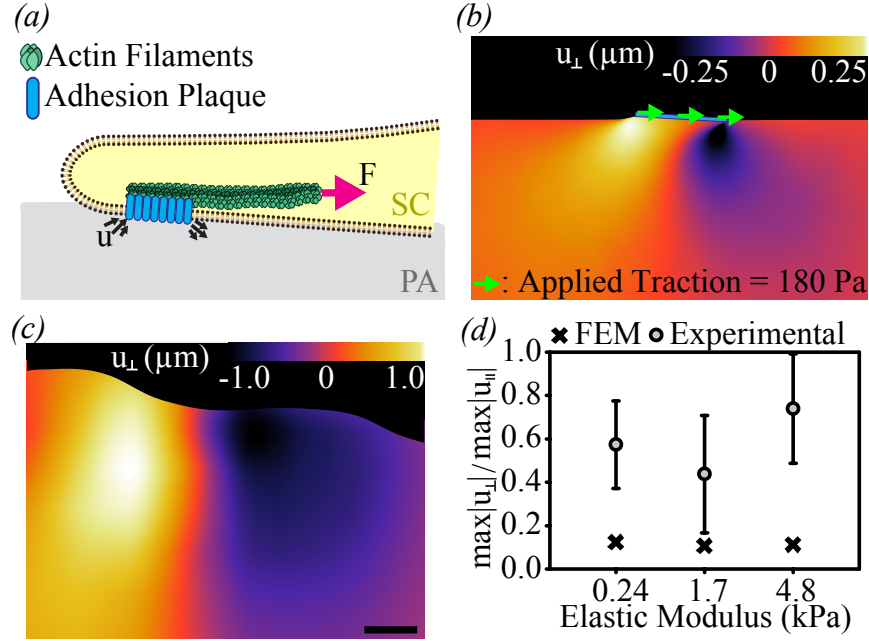
**Figure B.7:** SC induced normal tractions played a significant role in the SC traction profile and exhibited an alternating dipole pattern across substrate stiffness. (a–c) Vector field of the SC applied shear tractions ( $T_{\parallel}$ ) and (e–f) contour map of the SC applied normal tractions ( $T_{\perp}$ ) for the three tested substrate stiffnesses. The color bar represents the magnitude of the respective component and the reference vector shows a shear traction magnitude of 250 Pa, 350 Pa, and 800 Pa for  $E = 0.24$  kPa,  $E = 1.70$  kPa,  $E = 4.80$  kPa respectively. The cell outline (white) is superimposed to show its position with respect to the tractions. Scale bar represents  $20 \mu\text{m}$ .

#### B.4.5 Schwann cells generated significant normal tractions and out-of-plane moments

Perhaps even more interesting is the alternating dipole pattern in the normal SC traction profile prevalent across all stiffnesses (**Fig. B.7**). These normal traction dipole patterns give rise to local rotational moments, which can be seen clearly in the bottom row of **Fig. B.7**. Here, the local variation in the cell traction vector is plotted along the arbitrarily drawn line in **Fig. B.7a-f**. Motivated by recent findings [151] showing that embryonic mesenchymal fibroblasts are capable of generating local rotational out-of-plane moments stemming from an intracellular force transmission of actomyosin onto an adhesion patch of discrete size, we investigated whether our observed traction patterns shared a similar structural origin. Analogous to Legant et al. we constructed a simple finite element model (FEM) of a discrete cellular adhesion patch with similar dimensions, diameter  $\approx 6 \mu\text{m}$ , height = 150 nm. **Fig. B.8a** shows a schematic representation of how a SC might apply force onto the discrete



adhesion patch. We assumed firm bonding of the adhesion patch to the underlying substrate and applied uniform shear traction across the adhesion patch simulating the intracellular actomyosin contractile force. The shear traction magnitude loading was determined by matching the maximum displacement magnitudes of the simulated to the experimentally observed ones. This procedure allows us to obtain an order of magnitude estimate of the intracellular force generation that is transmitted via the SC stress fibers. All simulations were performed using 39123 four-node, linear, tetrahedron continuum elements (C3D4) in Abaqus/Standard 6.12-2 (Dassault Systèmes SIMULIA, Providence, RI USA). Based on the geometry of our simulations, the applied shear tractions we determined are 180 Pa, 700 Pa, and 1,000 Pa for each increasing stiffness, which fall within the range of previously reported



**Figure B.8:** Finite element simulation approximates the experimentally observed dipole and out-of-plane SC displacement behavior. (a) Schematic cartoon showing how SC might transmit force at an adhesion plaque of discrete size. Intracellular actin filaments contract with a force  $F$ , inducing dipole like displacements ( $u$ ) and rotational moments about the adhesion plaque. (b) Contour map of the FEM results along the symmetry plane showing the normal displacements ( $u_{\perp}$ ) induced in the deformed configuration of the PA gel ( $E = 0.24$  kPa). The adhesion plaque is modeled as a rigid plate that is firmly bonded to the PA gel with a uniform shear traction of magnitude 180 Pa applied to the top surface of the FA plaque. (c) Experimentally measured out-of-plane SC displacement field. Scale bar represents 5  $\mu\text{m}$ . (d) Plot of the mean normal and shear maximum displacement ratios from our experimental measurements and our finite element analysis across elastic moduli.

stress values sustained at cellular adhesion sites [8, 9, 10, 267]. Our finite element simulation shows that it qualitatively reproduces the experimentally observed dipole behavior in the measured out-of-plane displacements (**Fig. B.8b,c**) (see **Fig. S5** in the Supplementary Material for the FEM results for each displacement component). **Fig. B.8d** depicts the average normal and shear maximum displacement ratios from our experimental measurements and our finite element analysis across the three different elastic moduli. As can be seen in **Fig. B.8d** the qualitative behavior of the displacement ratios across the different stiffnesses is similar in the simulations when compared to our experimental measurements, and on the same order of magnitude, suggesting that our model is capable of qualitatively describing the appropriate cellular deformation behavior. The quantitative differences between the experimental and modeling ratios are not surprising given the simple nature of our model and the large possible parameter space within a real adhesion plaque. Furthermore, these qualitative observations are consistent with the cellular deformation signature previously reported for embryonic mesenchymal fibroblasts [151].

## B.5 Conclusion

In this study we have presented a detailed biophysical characterization of SCs across a range of elastic moduli typically encountered in the PNS. Given the importance of SCs during nerve development, axon maintenance and neuronal regeneration following an injury [122], our results provide significant insight into the mechanosensitivity of these cells. In particular, we find that SCs have a strong preference to acquire the prevalent vivo bipolar morphology even in the absence of environmental cues. This preference seems to be further stabilized by increasing substrate stiffness. We found that approximately 48% of all SCs exhibited a bipolar phenotype on our soft (0.24 kPa) substrates, a number that increases to 77% on 4.80 kPa, our stiffest substrate examined. Of note and contrary to most anchorage-dependent cells, we observed that SC spread area was unaffected by the modulus of the underlying substrate and that cells on all stiffnesses showed pronounced stress fibers as part of their actin cytoskeleton. This was true even for SCs attached and locomoting on 0.24 kPa polyacrylamide gels. Motivated by previous results showing that the existence of actin stress fibers requires substantial force transmission onto the cell substrate, we quantified

the 3D SC deformation fields. We found that on our softest (0.24 kPa) polyacrylamide gels, SCs generate large deformations, albeit small tractions, that required careful analysis within a finite deformation, rather than a traditional linear elastic framework. We believe that these large deformations are necessary to stabilize the formation of actin stress fibers on such highly compliant surfaces. Consistent with previous observations, SC tractions increased with increasing substrate stiffness; however, cell-generated displacement magnitudes decreased to the point of negligible cell displacements during locomotion on substrates with stiffnesses  $> 5$  kPa. When comparing SC in-plane to out-of-plane tractions we find that SCs exert significant out-of-plane tractions on all moduli investigated. Furthermore we show that these out-of-plane tractions give rise to local rotational moments, which we qualitatively attribute to the deformation signature of actomyosin force transmission at discrete adhesion plaques similar to that shown by Legant et al. for mesenchymal embryonic fibroblasts. Taken together, these findings provide the first set of quantitative biophysical measurements on the mechanosensitivity of SCs across a range of tissue stiffnesses typically encountered in the PNS vivo. We believe these results will provide critical information for the development of improved nerve guidance channels and regenerative scaffolds by considering the mechanosensitive characteristics of SCs.

## B.6 Acknowledgments

The authors thank Jennet Toyjanova for her helpful discussions and Yu-Ting Liu Dingle for her assistance in preparing **Fig. B.1**

This work is supported by NIH IMSD R25GM083270-01 and NSF Graduate Research Fellowship to C.L.F; NSF CBET 1134166 to D.H.K; NSF Graduate Research Fellowship to E.B.K..

## B.7 Supplementary Material

### B.7.1 Statistical analysis

Data were subjected to the Shapiro-Wilk normality test, to verify that the distribution of the data was normal,  $p > 0.05$ . For groups with normal distributions, parametric tests (one-way

analysis of variance (ANOVA) for three or more groups or Students t-test for two groups) were used to compare the means. Post Hoc analyses (Tukey) for equal variance in the population were also employed for multiple comparison of the means and their interactions. For groups with non-normal distributions, non-parametric tests (Kruskal-Wallis one-way ANOVA on ranks for three or more groups or Welch's t-test for two groups) were used to compare the means. Post Hoc analyses (Dunn's) for unequal variance in the population were also employed for multiple comparison of the means and their interactions. A  $p < 0.05$  was considered to be significant. The geometric mean,  $\mu_g$ , and geometric mean absolute deviation,  $MAD$ , (error bars) that are reported in this study are defined as

$$\mu_g = \left( \prod_{i=1}^n x_i \right)^{1/n} \quad (\text{B.7.1})$$

and

$$MAD = \left( \prod_{i=1}^n |x_i - \mu_g| \right)^{1/n}. \quad (\text{B.7.2})$$

All statistical tests, including power analysis were performed using SigmaPlot 12.3 (Systat Software Inc).

### **B.7.2 Mechanical characterization of polyacrylamide gels**

The elastic moduli of polyacrylamide (PA) gels were measured using an unconfined uniaxial compression test and a custom built compression testing apparatus. PA gels were cast using circular molds of 16 mm in diameter and 10 mm in height (McMaster Carr) and allowed to polymerize at room temperature for 30 – 40 minutes. The resulting cylindrical specimens were submerged in deionized water for 24 hours prior to testing to match the experimental cell time-lapse conditions. Samples were immersed in deionized water in a 35 mm culture dish (Corning Inc.) and covered with a 25 mm diameter, 1 mm thick polystyrene disc. This step was performed while the culture dish was located within the sample holder of the testing apparatus, consisting of a centrally positioned linear actuator (Series A, Ultramotion) equipped with a built-in encoder that provided displacement (strain) feedback. A tension/compression force transducer (LCFA-50G, Omega Engineering Inc.)

attached to the moving end of the linear actuator provided force (stress) feedback during the compression of the sample. Prior to testing, the center of the PA gel sample was aligned along the central axis of the linear actuator and force transducer to ensure purely uniaxial stress conditions. Each sample was compressed at a strain rate of  $0.02 \text{ s}^{-1}$  while being monitored to ensure that the circumferential walls were free to expand. The engineering stress ( $\sigma$ ) is calculated as the measured force divided by the circular contact area, and the engineering strain is calculated as the sample's change in height divided by its original height. **Fig. S1**(inset) shows the loading stress-strain curve for a typical PA sample. The elastic modulus is calculated for each sample from the stress-strain curve using the relationship

$$E = \frac{\sigma}{\epsilon} = \frac{F/A}{\Delta h/h}. \quad (\text{B.7.3})$$

The mechanical compression results are summarized in Fig. S1. The Poisson's ratio is taken to be 0.45, a value that falls within the typical range of values chosen for TFM studies [55, 65].

### B.7.3 Global force and moment balance

Net forces and moments acting on different control volumes within the PA gels satisfied static equilibrium. Under all conditions and time points the force and moments magnitudes were on the order of  $10^{-9} - 10^{-6} \text{ N}$ , and  $10^{-14} - 10^{-11} \text{ N}\cdot\text{m}$ , which is consistent with our zero traction, zero displacement noise floor measurements. These numbers are similar to our previously reported values [175].

### B.7.4 Automated calculation of the Schwann cell surface boundary

The cellular surface boundary was determined from the fluorescently labeled cell membrane. The sum intensity projection along the  $x_3$  axis of the volume stack was computed. Following the application of a median filter to remove noise, contrast adjustment, and intensity normalization, the image was thresholded such that any values of less than 90% of the maximum intensity were set to 0. A binary mask of the cell was generated by computing the largest connected component of the thresholded image. A coarse boundary of the binary mask was computed using the Moore-Neighbor tracing algorithm [96] and then smoothed

using the algorithm developed by Taubin [273, 272].

### B.7.5 Signal to noise measurements

The root mean squared traction values shown in equation (B.3.6) in the main text are sensitive to the integration boundary used in the summation. To circumvent this issue, the integration boundary was chosen such that it maximized the signal to noise ratio ( $SNR$ ) of the root mean squared tractions magnitude,  $|\mathbf{T}|_{RMS}$ . **Fig. S2** shows a contour map of the tractions magnitude  $|\mathbf{T}|$ , the cell boundary (**white**), and all values interior and exterior of the integration boundary (**pink**),  $\partial B$  are defined as  $B_S$  and  $B_N$ . The signal-to-noise ratio,  $SNR$ , value is

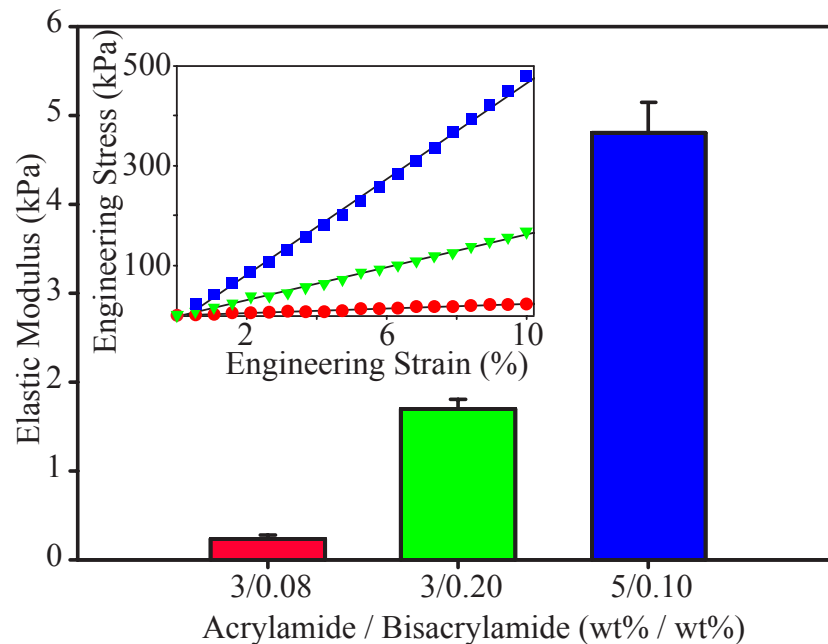
$$SNR = \frac{\text{signal}}{\text{noise}} = \frac{(|\mathbf{T}|_{RMS})_{\subset B_S}}{(|\mathbf{T}|_{RMS})_{\subset B_N}}. \quad (\text{B.7.4})$$

where  $(|\mathbf{T}|_{RMS})_{\subset B_S}$  and  $(|\mathbf{T}|_{RMS})_{\subset B_N}$  are the  $|\mathbf{T}|_{RMS}$  values computed interior and exterior of  $\partial B$  respectively. To begin the optimization procedure, the initial  $SNR$  value, using  $\partial B$  of the cell boundary is computed. Each iteration thereafter, the  $\partial B$  is dilated and the  $SNR$  value is recomputed until convergence is reached. All traction values that are less than the noise level are not considered in the calculation for the  $(\mathbf{T}_{RMS})_{\subset B_S}$ . As an extra precautionary measure, any time point with a  $SNR$  value of less than two is excluded from the analysis. Additionally, the minimum resolvable traction is conservatively computed as a value that is less than  $\mu_g + 2 \cdot MAD$  of all of the samples binned by each elastic modulus. Table S1 summarizes the noise floor results for the tractions and displacements as computed by the above method.

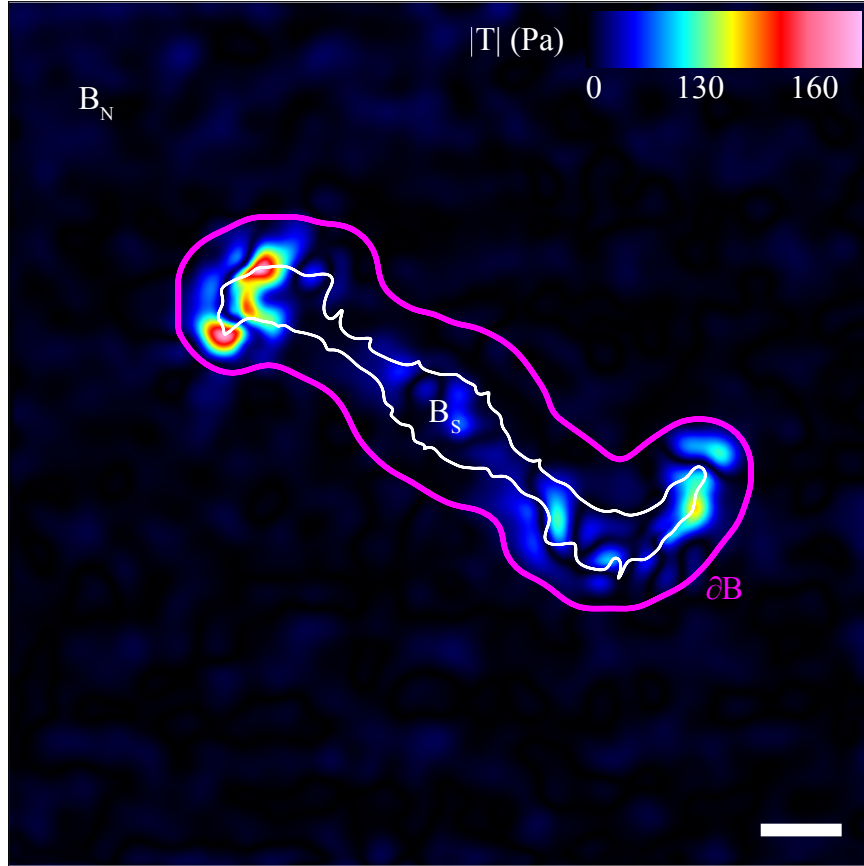
## B.8 Supplementary Figures & Tables

**Table S1:** Noise floor results used to determine the signal-to-noise ratio or the image.

| E (Pa) | Displacements ( $\mu\text{m}$ ) |                    | Tractions (Pa)        |                    |
|--------|---------------------------------|--------------------|-----------------------|--------------------|
|        | Shear ( $\parallel$ )           | Normal ( $\perp$ ) | Shear ( $\parallel$ ) | Normal ( $\perp$ ) |
| 240    | 0.09                            | 0.20               | 2.0                   | 9.9                |
| 1700   | 0.06                            | 0.14               | 15.9                  | 81.1               |
| 4800   | 0.06                            | 0.06               | 43.4                  | 200                |

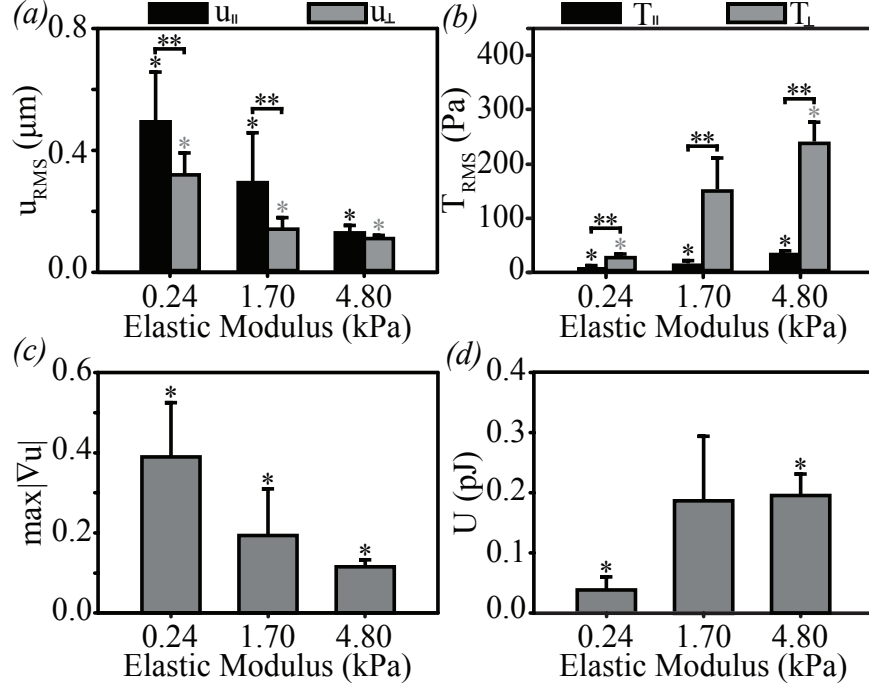


**Figure S1:** Polyacrylamide (PA) substrates were fabricated with three different physiologically relevant stiffnesses. Young's modulus for PA gels with different degree of crosslinking were calculated: (**red**)  $0.24 \pm 0.05$  kPa, (**green**)  $1.70 \pm 0.09$  kPa, and (**blue**)  $4.80 \pm 0.29$  kPa. Stress-strain curve for polyacrylamide gels with different degrees of crosslinking was linear across stiffnesses (inset): (**●**) 3/0.08, (**▼**) 3/0.20, and (**■**) 5/0.10.

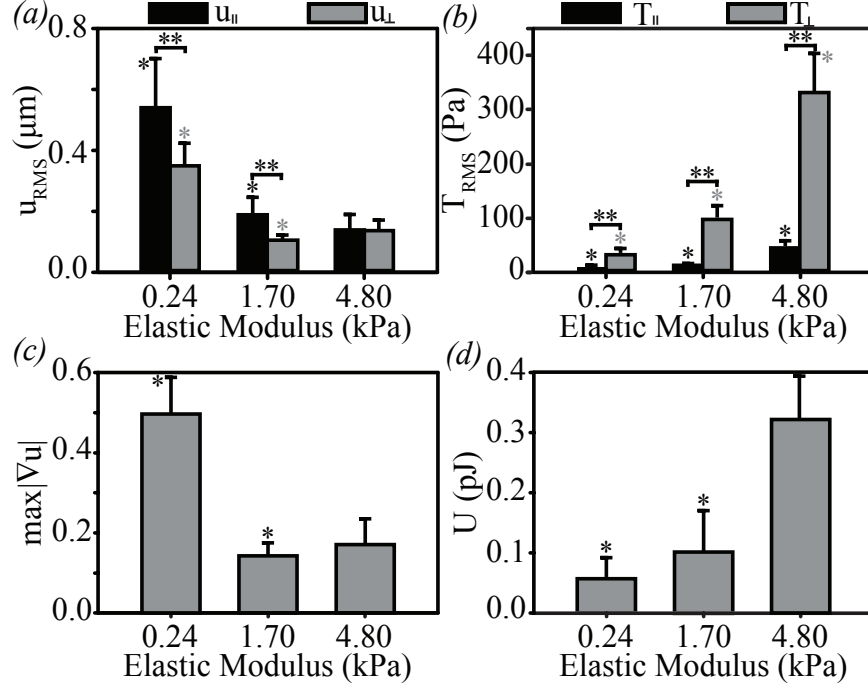


**Figure S2:** Surface contour map of a representative SC used to illustrate the integration boundary computation (pink,  $\partial B$ ). The integration boundary was computed such that it maximized the signal to noise ratio of the root mean squared tractions magnitude. All values interior and exterior of the integration boundary are defined as  $B_N$  and  $B_S$  respectively. The cell surface boundary (white) was the initial guess for the optimization procedure. Scale bar represents 20  $\mu\text{m}$ .

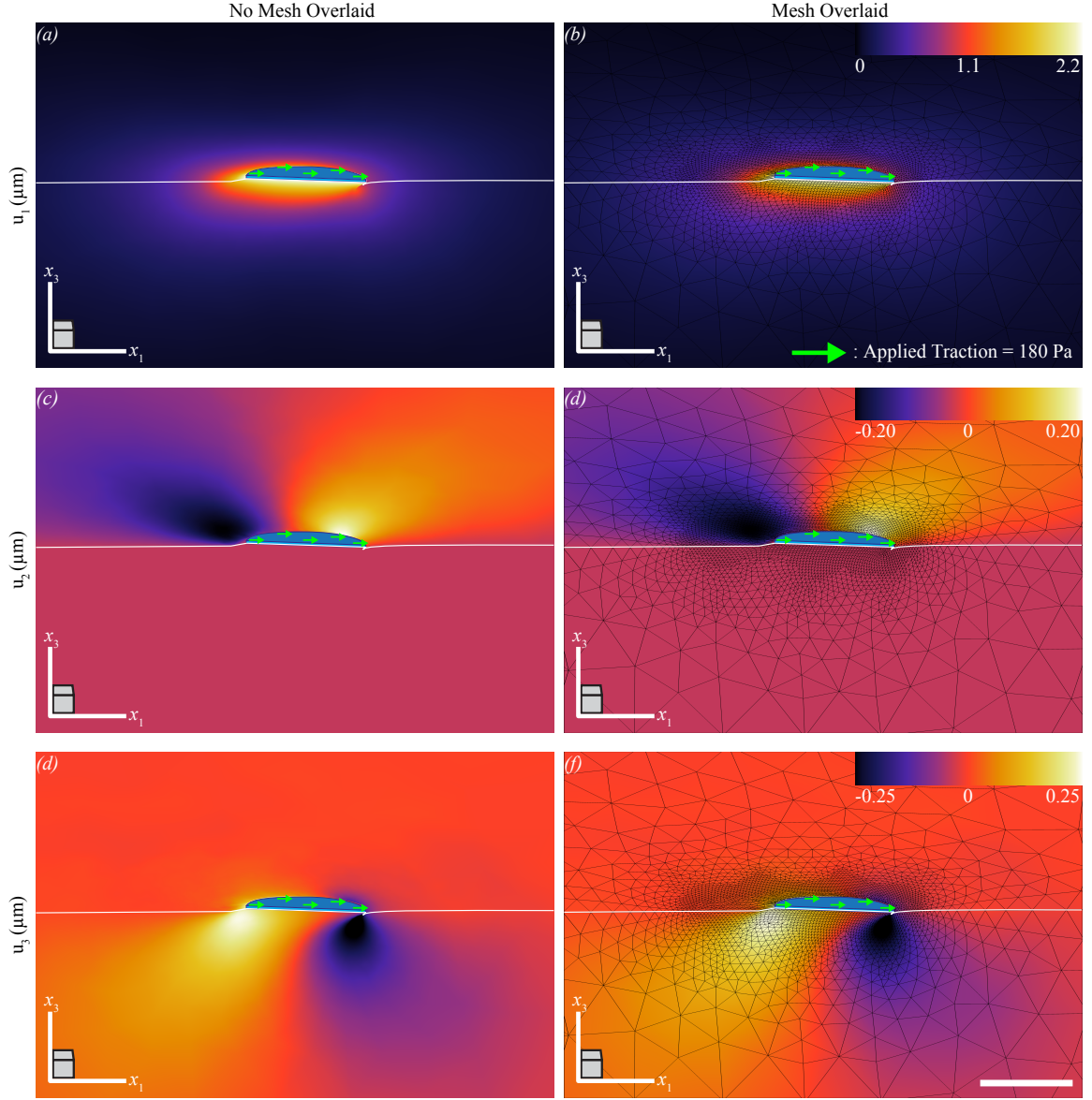




**Figure S3:** SC displacements, tractions, and strain energies varied with elastic moduli for the unpolarized morphology. Bar plots of the (a) root mean squared displacements magnitude ( $u_{RMS}$ ), (b) root mean squared tractions ( $T_{RMS}$ ), (c) maximum displacement gradient magnitude ( $\max|\nabla \mathbf{u}|$ ), and (d) strain energy ( $U$ ) as a function of elastic modulus; 0.24 kPa ( $n = 92$ ), 1.70 kPa ( $n = 65$ ), and 4.80 kPa ( $n = 22$ ). The  $u_{RMS}$  and  $T_{RMS}$  values are split into their respective shear components ( $u_{||}, T_{||}$ ) and normal components ( $u_{\perp}, T_{\perp}$ ) (black and gray respectively). Geometric mean ( $\mu_g$ ) and geometric mean absolute deviation ( $MAD$ ) are shown. \* indicates  $p < 0.05$  by one-way ANOVA on ranks across elastic moduli, and \*\* indicates  $p < 0.001$  by t-test between normal and shear components respectively.



**Figure S4:** SC displacements, tractions, and strain energies varied with elastic moduli for the multipolar morphology. Bar plots of the (a) root mean squared displacements magnitude ( $u_{RMS}$ ), (b) root mean squared tractions ( $T_{RMS}$ ), (c) maximum displacement gradient magnitude ( $\max|\nabla u|$ ), and (d) strain energy ( $U$ ) as a function of elastic modulus; 0.24 kPa ( $n = 63$ ), 1.70 kPa ( $n = 65$ ), and 4.80 kPa ( $n = 14$ ). The  $u_{RMS}$  and  $T_{RMS}$  values are split into their respective shear components ( $u_{\parallel}$ ,  $T_{\parallel}$ ) and normal components ( $u_{\perp}$ ,  $T_{\perp}$ ) (black and gray respectively). Geometric mean ( $\mu_g$ ) and geometric mean absolute deviation ( $MAD$ ) are shown. \* indicates  $p < 0.05$  by one-way ANOVA on ranks across elastic moduli, and \*\* indicates  $p < 0.001$  by t-test between normal and shear components respectively.



**Figure S5:** Finite element simulation results along the symmetry plane showing the displacement field induced in the deformed configuration of the  $E = 0.24$  kPa PA gel. The displacement field is shown for the  $u_1$ ,  $u_2$ , and  $u_3$  components with no mesh overlaid (a, c, and e) and with mesh overlaid (b, d, and f). The adhesion plaque was modeled as a rigid plate that is firmly bonded (surface-to-surface \*TIE constraint) to the PA gel with a uniform shear traction (green) of magnitude 180 Pa applied to the top surface (using \*DSLOAD) of the FA plaque (blue). The shear traction magnitude loading was determined by matching the maximum displacement magnitudes of the simulated to the experimentally observed ones. Surface Scale bar represents 5  $\mu\text{m}$ .

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