Implementation and Application of a Method for Measuring Traction Forces to Characterize Deficient CNP Signaling in Mouse Fibroblasts

by

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A thesis submitted in conformity with the requirements for the degree of Master of Applied Science

Department of Mechanical & Industrial Engineering
Institute of Biomaterials and Biomedical Engineering
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Abstract

Adherent cells respond to changes in their microenvironment by generating internal forces that are transferred to the substrate to which they are attached. Manipulation of cellular signaling pathways also influences the response to these changes and can direct cells to pathological outcomes. C-type natriuretic peptide (CNP), through the NPR-B receptor, has been shown to protect quiescent fibroblasts in heart valves from differentiating into myofibroblasts that contribute to development of calcific aortic valve disease (CAVD). Here, I implemented and validated a method for quantifying cellular traction forces in vitro, and used this method to characterize fibroblasts’ response to haploinsufficiency of Npr2, the gene that encodes NPR-B. I showed that CNP significantly lowered traction forces in Npr2+/+ fibroblasts, but not Npr2+/− fibroblasts. These studies demonstrate a novel effect of CNP on fibroblast biomechanical function, adding to our knowledge of the mechanisms by which CNP may protect against CAVD.
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Chapter 1

1 Thesis Overview

1.1 Motivation

17.3 million people die every year as a direct result of cardiovascular disease, making it the leading cause of death around the world [1]. Calcific aortic valve disease (CAVD) is one of the most prevalent cardiovascular diseases with 25% of the population over the age of 65 years having some form of the disease [2]. CAVD is characterized by the stiffening and thickening of the leaflets that comprise the aortic valve. Leaflet stiffening can prevent the valve from fully opening and thus the heart must work harder to pump the required volume of blood through the valve. CAVD can lead to further complications that result in increased morbidity and mortality [3]. Currently the only treatment for CAVD is replacement of the valve; there are no therapeutic treatments, indicating that the underlying mechanisms of the disease remain incompletely understood.

1.2 Current Research Problem

The majority of cells in the interior of healthy leaflets (valve interstitial cells, or VICs) are quiescent fibroblasts. Histological stains of diseased leaflets show increased expression of α-SMA, a marker of myofibroblasts [4]. In the presence of transforming growth factor β1 (TGF-β1) and increased local extracellular matrix (ECM) stiffness, quiescent fibroblasts can change their phenotype and differentiate into myofibroblasts [5]. Myofibroblasts are characterized by increased synthesis of ECM proteins and increased expression of α-smooth muscle actin (α-SMA) stress fibers within the cell. These mechanisms synergistically promote stiffening and thickening (fibrosis) of the valve in a positive feedback loop that advances the progression of CAVD.

Stress fibers within fibroblasts and myofibroblasts are anchored to focal adhesions, which also serve as attachment points for the cell on its surrounding ECM. When actin-myosin cross-bridges are activated, the cell contracts and transfers force to the ECM substrate via the focal adhesions. These forces are known as traction forces, and are quantified using a technique called traction
force microscopy (TFM). The process typically involves observing the displacement of the substrate caused by the contraction of the cell and using this displacement in combination with the known mechanical properties of the substrate to calculate the forces. Cells with more stress fibers and particularly stress fibers containing α-SMA, such as myofibroblasts, have a greater capability for contraction and thus can be characterized by the increased amount of traction forces that they generate.

C-type natriuretic peptide (CNP) has been shown to protect VICs from myofibroblastic differentiation via the second natriuretic peptide receptor (NPR-B), indicating that CNP and the signaling pathway it activates are important homeostatic elements that prevent the initiation and development of CAVD [6]. Ongoing studies in our lab have shown that impairing the function of NPR-B in mice by genetic manipulation results in accelerated CAVD development (M. Blaser, unpublished data). The mechanisms by which CNP protects against accelerated disease are being actively investigated in our lab. CNP may protect against maladaptive valve ECM remodelling by reducing VIC traction forces, which are associated with fibrosis. An aim of this thesis is to test this novel hypothesis by quantifying traction forces exerted by (myo)fibroblastic cells from NPR-B-deficient mice by TFM.

1.3 Thesis Organization

This thesis is organized into six chapters. The second chapter summarizes existing knowledge of traction forces and their role in the progression of CAVD, and reviews current technologies used to quantify traction forces generated by adherent cells. The third chapter outlines the objectives and specific aims of the thesis. Chapter 4 describes the implementation of traction force microscopy with a detailed description of the algorithms used to calculate traction forces, followed by a description of validation experiments for the platform. Chapter 5 makes use of the platform for traction force microscopy described in Chapter 4 to characterize deficiency in CNP signaling in mouse lung fibroblasts by quantifying their traction force generating capabilities. Chapter 6 recapitulates the conclusions of the thesis and outlines recommendations for future work. Lastly, a list of references and detailed experimental protocols are appended to the end of the document.
1.4 Overview of Contributions

In this section, I list the specific contributions from all those who directly aided in experimental design or execution in each chapter. Every component of the thesis was guided by continual input and thorough discussion with my supervisor, Dr. Craig Simmons.

The ImageJ plugins used to align slices in a stack, track two-dimensional displacement of markers, and calculate traction forces were developed by Dr. Qingzong Tseng when he was a PhD student at Université de Grenoble. These plugins were made freely available online by him. Also, source code for the traction force microscopy plugin was provided by him upon request, which assisted in my understanding of how Tikhonov regularization was integrated into the force calculation algorithm. Alan Lam isolated the VICs from the heart valves of freshly-slaughtered pigs, and Haijiao Liu measured the stiffness of the polyacrylamide gels with and without beads using AFM in Dr. Yu Sun’s laboratory at the University of Toronto.

Rachel Adams and Mark Blaser contributed to the design of experiments to establish a time point for image analysis of mouse lung fibroblasts. Rachel Adams also isolated lung fibroblasts from freshly-sacrificed mouse pups and assisted with α-SMA staining, while Laura-lee Caruso genotyped the mice with respect to the Npr2 gene using gel electrophoresis.
Chapter 2

2 Literature Review

2.1 Introduction

This chapter is divided into two main sections, with the first part describing the anatomy of the aortic valve, progression of calcific aortic valve disease (CAVD), a review of factors being investigated as potential initiators of CAVD, and a discussion about C-type natriuretic peptide as a possible protective agent against CAVD. The second section focuses on the different methods used to quantify traction forces generated by single cells and the mathematical algorithms used to calculate the forces.

2.2 The Aortic Valve

The aortic valve is a 1-mm thick tissue formed by three leaflets that passively prevents backflow of blood from the aorta to the left ventricle of the heart during diastole. Each leaflet is covered on both sides by a single confluent layer of valve endothelial cells (VECs), which selectively permits transport of certain soluble factors from the blood to the valve interstitium and protects the interstitium and embedded cells from hemodynamic forces.

The interior of the tissue consists of three distinct layers, each characterized by a unique composition of extra-cellular matrix (ECM) proteins (Figure 2-1). The ventricularis on the ventricular side of the leaflet features a dense, radially-oriented network of collagen and elastin fibers [8]. The fibrosa on the aortic side of the leaflet contains mostly circumferentially-oriented Type I and III collagens, which serve as the primary load-bearing fibers for the whole leaflet [9]. The spongiosa is the middle layer of the leaflet that links the fibrosa and ventricularis together, and permits these layers to shear past each other when the leaflet is in motion. The spongiosa consists primarily of proteoglycans and glycosaminoglycans with some collagen fibers scattered throughout the layer [10],[11]. The varying compositions of each layer of the leaflet give rise to the anisotropic nature of the leaflet as determined by biaxial tensile testing [12]. In particular, the elasticities for each layer are reflective of their ECM composition. Tissue stiffness varies over a relatively large range within a layer as well as between layers, and there is significant overlap between the range of stiffnesses for both the fibrosa and ventricularis. However, stiffness testing
with micropipette aspiration showed a statistically significant difference between the average stiffnesses of the fibrosa and ventricularis, with the fibrosa being the stiffer layer. In addition, some regions of the fibrosa had local stiffnesses greater than 22 kPa, while the upper bound of local stiffnesses for the ventricularis never exceeded 22 kPa [14].

![Figure 2-1: Normal porcine aortic valve leaflet stained with Movat’s pentachrome showing the trilayer structure: (F) fibrosa, (S) spongiosai, (V) ventricularis. Both sides of the valve are lined with the single-cell layer of valve endothelial cells (VlvECs). VICs (which include quiescent fibroblasts and some activated myofibroblasts) reside in the interior of the valve. Reprinted with permission from Wolters Kluwer Health: Circulation Research [7], © 2013.](image)

The leaflets also contain valve interstitial cells (VICs), which are sparsely interspersed throughout all three layers, and are a heterogeneous population of different types of cells. While most VICs are quiescent fibroblasts, activated myofibroblasts, smooth muscle cells, and progenitor mesenchymal stem cells are also present in the interstitium of the valve [15].

### 2.3 Calcific Aortic Valve Disease

CAVD is a progressive disease characterized by stiffening of the valve leaflet (sclerosis) or narrowing of the valve’s cross-sectional area (stenosis) due to calcium and lipoprotein deposition in the interstitium of the leaflets (Figure 2-2). CAVD is prevalent among the elderly; it is present in about 25% of people 65 to 74 years of age and 48% of those older than 84 years of age [2],[16]. CAVD in its early stages is typically asymptomatic, but it is associated with increased
morbidity and mortality; individuals with CAVD are 40% more at risk of myocardial infarction and 50% more at risk of cardiovascular death [3]. There are currently no treatment options for CAVD except for replacement of the valve, which is an indication that the molecular mechanisms for the development of CAVD continue to be incompletely understood.

Figure 2-2: Photographs of normal aortic valve (left) and stenotic aortic valve (right). Reprinted with permission from Wolters Kluwer Health: Circulation Research [17], © 2005.

Lesions preferentially begin to form on the disease-prone, aortic side of the leaflets in the early stages of CAVD. The formation of lesions may be caused by dysfunction of the endothelium of the valve, initiating chronic inflammation, or perhaps by an increase in mechanical or hemodynamically-induced shear stress disrupting the endothelium of the valve [17]. Immunohistochemical staining showed that early-stage lesions are primarily composed of proteoglycan deposits between the endothelial layer and distorted elastic lamina [18],[19]. In addition, cells in the vicinity of these lesions on porcine aortic valves were found to express Sox9, an osteochondral marker [20]. As CAVD progresses, myofibroblasts begin to appear at the base of the lesion [19],[20].

2.3.1 Myofibroblastic Differentiation

The quiescent fibroblasts that account for the majority of VICs normally synthesize components of the ECM that surround them, namely collagen, elastin, proteoglycans, and glycoproteins. In areas of tissue injury, these fibroblasts undergo differentiation to become myofibroblasts, which are instrumental in wound healing and tissue repair. The differentiation process from fibroblasts
to myofibroblasts is a two-step process (Figure 2-3): under external mechanical stress, fibroblasts begin to form additional intracellular actin stress fibers that are anchored to focal adhesion sites. These cells acquire the proto-myofibroblast phenotype, which is characterized by cells with increased contractility but without expression of alpha smooth muscle actin (α-SMA). In the presence of TGF-β1, ED-A fibronectin, and continued external mechanical tension, these proto-myofibroblasts become fully differentiated myofibroblasts that express α-SMA, have larger focal adhesion sites, and are even more contractile than proto-myofibroblasts [5].

![Figure 2-3: Pathway for fibroblast-to-myofibroblast differentiation. In the presence of external mechanical tension, fibroblasts respond by expressing more cytoplasmic actin filaments anchored at focal adhesions, assuming a proto-myofibroblast phenotype. When further mechanical tension is added in the presence of ED-A fibronectin and TGF-β1, proto-myofibroblasts differentiate into activated myofibroblasts with significant expression of α-SMA stress fibers. When the differentiating stimuli are removed, it is possible for myofibroblasts to revert back to a quiescent phenotype but most disappear by apoptosis. Reprinted with permission from Macmillan Publishers Ltd: Nature Review Molecular Cell Biology [5], © 2002.](image)
disappear after the tissue has healed or appear in above-normal levels in healthy tissue, the tissue enters a fibrotic state where the myofibroblasts continually produce excess amounts of ECM protein, actively remodel the ECM, and recruit more inflammatory cells via chemoattraction [21].

2.3.2 The Role of the RhoA Signaling Pathway in Myofibroblast Differentiation and Traction Force Generation

Differentiation of quiescent fibroblasts to myofibroblasts has been shown to be mediated by the RhoA signaling pathway. RhoA belongs to a family of GTPases that regulate intracellular actin dynamics and are involved with mechanosensing and mechanotransduction [22],[23]. When RhoA is activated by guanine exchange factors (GEFs), it activates Rho-associated kinase (ROCK), which affects other proteins involved with regulation of the cytoskeletal structure. Some of these proteins include focal adhesion kinase (FAK), which helps order and align the stress fibers that comprise the actin cytoskeleton [24], LIM kinase-1 (LIMK-1), which phosphorylates cofilin and prevents actin depolymerisation [25], and myosin light chain (MLC), which induces interaction between actin and myosin [26]. The combined effect of FAK, cofilin, and MLC results in an overall increase in cell contractility and the traction forces exerted by the cell on its surrounding extracellular matrix. Cellular traction forces are generated by myosin cross-bridges along the length of the cytoskeletal actin filaments that contract when ATP. The actin filaments then transfer the force to the substrate to which the cell is attached via focal adhesions, which bind the cell to the ECM and provide an anchor point for actin filaments within the cell (Figure 2-4) [27]. Myofibroblasts are highly contractile and thus increased traction forces are a functional phenotypic marker of myofibroblasts. The ability of cells to contract and exert forces on their matrix is essential to many of the processes by which myofibroblasts contribute to CAVD, including matrix synthesis, remodeling, and contraction.
RhoA and traction force generation can be regulated by a variety of stimuli in the cellular microenvironment. One example of such a stimulus is stiffening of the substrate to which the cell is attached, resulting in increased expression of F-actin and stress fibers within the cell, leading to increased adhesion strength and contractility, thus increasing its capability for generating traction forces [28]. This process of increasing the activation of stress fibers and reorganization of focal adhesions is mediated by the RhoA signaling pathway in vascular smooth muscle and fibroblastic-type cells [29],[30]. In addition to activation by changing mechanical conditions, RhoA can be activated or inhibited biochemically. For example, bioactive sphingolipids such as sphingosine-1-phosphate are capable of activating RhoA [31]. In mechanistic studies, RhoA activity can be manipulated biochemically or genetically: for example, C3 exoenzyme inhibits its activity [32] or cells can also be infected with adenoviruses that genetically modify target cells to constitutively express active or inactive RhoA to trigger or suppress the signaling pathway, respectively [33].

Cell tractions mediated via RhoA play important roles in a number of basic cellular functions, including the maintenance of cell shape, generation of mechanical signals [27], migration [34],[35], remodeling of the ECM [36], and making decisions about cell fate [33],[37]. Of particular relevance to CAVD and other fibrotic disease, RhoA regulates myofibroblast differentiation. Inhibition of RhoA and ROCK suppressed α-SMA expression at the mRNA and
protein level in lung fibroblasts, whereas activation resulted in increased phosphorylation of MLC [39]. The in vitro RhoA activity (amount of activated RhoA relative to total amount of RhoA) and differentiation of VICs into myofibroblasts has been shown to be sensitive to the stiffness of the substrate on which the VICs are cultured. Chen [40] showed that RhoA activity in VICs increased as the substrate stiffness increased, with statistical significant changes occurring between 11 and 22 kPa, and 50 and 144 kPa. Pho et al [43] demonstrated that a significantly higher proportion of VICs cultured on stiff substrates expressed SMA-containing stress fibers (and thus a higher occurrence of myofibroblasts) compared to VICs grown on softer substrates. This result was confirmed by Benton et al [44], who showed using qRT-PCR analysis that α-SMA mRNA expression was significantly higher in VICs cultured on stiff substrates compared to soft ones. In a study by Kloxin et al [45], VICs were cultured on a photodegradable hydrogel and the stiffness was locally varied between 7 and 32 kPa by adjusting the length of time areas were exposed to irradiation. They found that the percentage of cells expressing α-SMA stress fibers increased as the substrate stiffness increased. In addition, myofibroblastic differentiation was not observed in areas where the substrate stiffness was lower than 15 kPa. This threshold stiffness is higher than the average elasticity of the ventricularis, which gives a partial explanation for why CAVD initiates preferentially on the fibrosa side of the leaflet.

Increased traction forces generated by VICs can also be a factor potentiating the development of CAVD. After treatment with potassium chloride to stimulate cellular contraction, the overall flexural stiffness of the valve against its natural curvature was shown to be significantly higher [51]. On a local level, increased traction forces in isolated areas of the valve can lead to a concentration of ECM proteins, with the resulting effect being a local increase in stiffness [7]. This increase could lead to further activation of the RhoA signaling network, completing the positive feedback loop leading to myofibroblastic differentiation of quiescent VICs and eventually CAVD.

2.3.3 C-Type Natriuretic Peptide

C-type natriuretic peptide (CNP), one of three natriuretic peptides, is expressed primarily in the brain, cardiovascular system, and skeletal system. The NPs are initially produced as pro-forms and later cleaved into their active forms. In particular for CNP, pro-CNP is proteolytically cleaved by furin to form a 53-amino acid peptide, the active form of CNP [52]. In normal human
valve leaflets, CNP is constitutively expressed [53], but its expression can be stimulated or suppressed by cytokines and shear stress [54],[55],[56]. The NPs can bind natriuretic peptide receptors (NPRs), designated NPR-A, NPR-B, and NPR-C. Of the three known types of NPRs, CNP only binds NPR-B and NPR-C. NPR-C mediates clearance of all NPs from the circulation by facilitating their internalization and degradation [57]. NPR-B is a transmembrane guanylyl cyclase that catalyzes the intracellular synthesis of cGMP [52].

When CNP binds to NPR-B, the guanylyl cyclase element of the receptor synthesizes intracellular cGMP, which through cGMP-dependent protein kinases (typically cGK1 in cardiovascular cells) leads to the phosphorylation of myosin light chain phosphatase. This results in dissociation of MLC from actin, which causes the relaxation of smooth muscle [58]. Also, cGK1 binds pSmad3 to prevent its interaction with the SMA promoter, thus reducing the transcription of SMA (Kuiru Wei and Craig Simmons, unpublished data).

CNP also acts as an anti-fibrotic agent throughout the body: continuous infusion of CNP in mouse lungs inhibited bleomycin-induced fibrosis [59], and local expression of CNP in rat kidneys inhibited proliferation of mesangial cells and ECM accumulation following renal injury [60]. In cases where fibrosis has taken hold, expression of CNP is reduced: Sangralingham et al [61] showed an inverse correlation between the degree of fibrosis and the plasma levels of CNP in the circulation. In addition, they showed that CNP is excreted to a greater degree in cases of renal fibrosis, leaving less available CNP in the circulation [62].

Previous studies by Simmons et al [63] showed by microarray analysis of VECs that CNP expression on the disease-protected ventricular side of the leaflet was significantly higher than on the disease-prone aortic side. Furthermore, CNP expression was shown to be downregulated in human stenotic valves [53]. Histological staining also showed a decrease in CNP expression with a corresponding increase in α-SMA and Runx2 in sclerotic valves [6]. In vitro experiments of VICs in osteogenic media showed that CNP prevents the formation of aggregates and calcific deposits as well as α-SMA expression [6]. Knockdown of the gene for the NPR-B receptor by siRNA showed that CNP is protective against the formation of calcific deposits and α-SMA expression only when NPR-B is actively expressed [6]. These results imply that the presence of CNP has a protective effect against the onset of CAVD and that the effects of CNP are mediated at least in part through the NPR-B receptor.
Mice with inactivating mutations in both copies of the gene encoding NPR-B (Npr2\(^{-/-}\), or KO) experience dwarfism and do not survive longer than seven days after birth. Mice with an inactivating mutation in only one copy of the Npr2 gene (Npr2\(^{+/-}\), or Het) do survive to adulthood, but the aortic valves exhibit an accelerated rate of CAVD development compared to mice without the inactivating mutations (Npr2\(^{+/+}\), or WT). In a recent study, the maximum transvalvular velocity and reduced cross-sectional area of the valve opening was higher in Het mice than in WT mice. Histological staining using Movat’s pentachrome also showed cusp thickening, fibrosis, and accumulation of proteoglycans consistent with the development of CAVD [64]. These results suggest that impaired function of the Npr2 gene is a potential marker for vulnerability to CAVD.

### 2.4 Traction Force Measurement Methods

In order to use traction forces generated by cells as a phenotypic marker for cellular responses to external stimuli, methods for quantifying the traction forces must be developed and utilized. Here I review three methods for measuring traction forces on a two-dimensional surface: observing the wrinkle pattern in a deformable substrate, directly measuring the force using cantilever microposts, and inferring force generation from the movement of markers embedded in a continuous thin substrate. Next, the calculation methods for traction forces on a thin substrate are examined. Lastly, advances into measuring traction forces in three dimensions are discussed.

#### 2.4.1 Substrate Wrinkles

The field of traction force microscopy as a means of quantifying cell tractions was pioneered by Harris et al. who showed that fibroblasts were capable of deforming silicone substrates to the point that wrinkles were formed and could be observed in the substrate (Figure 2-5) [65]. Although quantifying wrinkling is a complex non-linear problem, this represented the first effort to quantify the traction forces generated by a single cell. Recently, Yu et al [66] described a method for quantifying traction forces from imaging wrinkles using a combination of digital holography microscopy and quantitative phase microscopy (DH-QPM). Three-dimensional holograms that track the width and depth of the wrinkles were captured using a Mach-Zehnder interferometer, and the force was estimated by multiplying the stiffness of the silicone film by the measured displacement of the wrinkle pattern. One drawback of this method is that
intracellular organelles or other particulate matter can interfere with the imaging of wrinkle patterns, leading to an incorrect force measurement in the vicinity of the interfering matter. Furthermore, the method for estimating the forces oversimplifies the nonlinear interaction between forces generated by the cell and the geometry of the substrate wrinkles. To date, there is no easy method of extracting the forces from a wrinkle pattern, with most of the literature making use of wrinkles solely for qualitative observations.

Figure 2-5: Images of silicone rubber substrate being deformed and wrinkled by fibroblasts. a: A single 3T3 fibroblast compressing the substrate beneath the cell, forming “compression wrinkles” (scale bar = 10 μm). b: Group of chick heart fibroblasts creating compression wrinkles as well as pulling on the substrate to form “tension wrinkles” (scale bar = 100 μm). Reprinted with permission from Macmillan Publishers Ltd: Nature [65], © 1981.
2.4.2 Cantilever Beams and Microposts

Later studies employed a platform where cylindrical cantilever beams or microposts of polydimethylsiloxane (PDMS) were fabricated on a silicon substrate. In [67], the diameter, height, and spacing of the microposts were 5 μm, 25 μm, and 7 μm, respectively. The top surface of these PDMS beams was printed with ECM proteins to facilitate cell adhesion, and cells were seeded on top of these beams. The deformed beams were imaged from above (Figure 2-6) and the deflection measured by subtracting the position of the beam’s attachment point to the substrate from the beam’s attachment point to the cell. The force was then calculated for each beam using Euler-Bernoulli beam theory [67],[68],[69]. The primary advantage of this method is that each beam is independent of every other beam, so coupling effects do not need to be considered when calculating the traction forces. In addition, cells cultured on microposts do not need to be detached from the microposts in order to calculate the displacement. The cells can also be fixed and stained for expression of proteins after imaging of the microposts has taken place. However, the array of microposts limits the attachment points for the cell and they force cells to assume morphologies differing from their in vivo morphology, potentially altering their behaviour and internal processes. Also, if the elasticity and separation distance of the microposts are decreased to enhance the sensitivity of the array to weak traction forces, the posts become prone to sticking to each other when the array is being fabricated, functionalized, or even transported.

Figure 2-6: Optical image of a single vascular smooth muscle cell cultured on an array of microposts, captured from above (a) and below (b). Reprinted from [67] with permission from Elsevier.
2.4.3 Measuring Traction Forces on Thin Substrates

As the surface area and separation distance between microposts decreases, the system’s geometry begins to approach optically-clear substrates that are elastic, have embedded markers, and are functionalized with ECM proteins that allow for cell attachment anywhere on the substrate. These markers can either be patterned on the surface of the substrate [70] or infused throughout the substrate [71]. While this method imposes no restriction on where cells can attach, measuring the displacement and calculating the traction forces becomes more difficult on the continuous substrate because of the coupling between measurement points in close proximity. For this method, two images are captured: one with the cell attached to the substrate and one with the cell detached, and the displacement field is obtained by observing the movement of markers from the second image to the first [71],[72]. The displacement field is then used to calculate the traction field across the substrate, in combination with the substrate’s mechanical properties.

Figure 2-7: Schematic drawing of traction force microscopy on thin substrates. Displacement of fiducial markers embedded in substrate (in blue) are measured and used to calculate traction forces (red vectors) generated by the cell (green). Reprinted from [72] with permission from Elsevier.

Cells adhering to the surface of flat substrates spread out across the xy-plane and are fairly flat. In this case, force generation in the vertical direction is typically neglected, yielding a two-
dimensional problem. However, recent studies have shown that these simplifying assumptions may not be valid for adherent cells; the cell pulls up on the substrate at the edge of the cell and pushes down near the nucleus, and these forces are significant [73]. Despite the presence of these vertical forces, little error is introduced in only calculating the surface forces. For substrates with a Poisson’s ratio near 0.5 and situations in which the displacement is much smaller than the height of the substrate, displacements in the vertical direction are decoupled from surface displacements, so the calculation of surface forces is unaffected by the lack of information about normal displacements [74]. Because the Poisson’s ratio of polyacrylamide is \(0.480 \pm 0.012\) [75], surface force calculations fall in the low-error region. Therefore, while neglecting the contribution of normal forces omits potentially useful information about traction force generation, it does not affect the accuracy of calculations for forces along the surface of the substrate.

The displacement field \(u(x)\) (where \(x = (x_1, x_2)\)) is related to the traction field \(f(x)\) by the following integral equation:

\[
   u_i(x) = \int \sum_j G_{ij}(x - x') f_j(x') \, dx'
\]

(Eq. 1)

For a sufficiently thick substrate, the substrate can be approximated by an elastic half-space which is described by the Boussinesq Green function:

\[
   G_{ij}(x) = \frac{1 + \nu}{\pi E \mu^2} \left[ (1 - \nu) r^2 + \nu x_1^2 \right] \left[ (1 - \nu) r^2 + \nu x_2^2 \right]
\]

(Eq. 2)

where \(\nu\) and \(E\) are the Poisson’s ratio and Young’s modulus of the substrate, respectively, and \(r\) is the vector norm of \(x\) [76]. Many studies use the reasoning that if the surface displacements are much smaller than the substrate thickness, then the elastic half-space assumption is valid for their experimental design. Polyacrylamide gels used as a substrate for TFM in [71] had a thickness of about 70 \(\mu\)m, whereas silicone elastomer gels used in [70] had a film thickness of 40 \(\mu\)m. In both cases, surface displacements were on the order of a few microns.
2.4.3.1 Boundary Element Method

There are three commonly-used methods of calculating the traction force field in the literature. First, the boundary element method (BEM) used by Dembo and Wang [71] takes a finite-element approach that discretizes the area bounded by the cell membrane into mesh elements sufficiently small enough that the Eq. 1 can be approximated using an interpolation scheme. A boundary condition of zero force is applied to the region outside of the cell boundary because cells can only generate forces at focal adhesions, which can only exist within the cell boundary.

In theory, the boundary conditions make sense at it is not possible for a cell to generate force outside of the reach of its cell membrane. However, the applicability of this theory is limited by the ability to exactly determine where the cell boundary is as well as the spacing between vectors of the displacement field. If the boundary is drawn too restrictively, forces that appear to be outside of the boundary will be concentrated at the boundary, which does not normally occur within adherent cells.

One benefit of BEM is that in areas of interest such as near the cell boundary and in proximity to cell extensions and processes, the mesh can be adaptively refined to include more mesh elements in those areas, enabling greater resolution in those areas without significantly increasing computational time across the entire cell [72].

2.4.3.2 Fourier Transform Traction Cytometry

The second method commonly used is Fourier Transform Traction Cytometry (FTTC), which transforms the integral equation (Eq. 1) into the Fourier domain [77]. Because Eq. 1 is a convolution integral, applying the Fourier transform on it causes the right side of the equation to become a multiplication operation. This has the effect of reducing the system of equations that need to be solved, significantly improving computational time. The resulting field is then transformed back to real space using the inverse Fourier transform.

The FTTC method can be modified to solve the same mixed boundary value problem as BEM – displacements within the cell boundary are specified by measurement and traction forces outside the cell boundary are specified to be zero [77]. In order to obtain a solution, the forces must be calculated iteratively – first, the traction field is calculated from the displacement field as described above. The traction field is modified to set traction vectors outside the boundary to
zero. The displacement field induced by the modified traction field is then calculated using Eq. 1 in the Fourier domain. This field is modified again to replace displacement vectors within the cell boundary with the measured displacements. These steps are repeated until the modified displacement field across the entire field of view sufficiently converges to the measured displacement field.

When comparing the performance of FTTC to BEM, the results obtained by both methods are substantially similar [72]. The primary difference comes in computational time, because the system of equations that need to be solved in Fourier space is much smaller than with BEM, so the traction field can be calculated much faster with FTTC.

### 2.4.3.3 Traction Reconstruction with Point Forces

The third method is Traction Reconstruction with Point Forces (TRPF), which improves upon FTTC by calculating forces only at points where the cell is adhered to the substrate, that is, the cell’s focal adhesions [70],[78]. The primary assumption of this method is that traction forces are only acting through the focal adhesion. If this is true, then the traction field should be zero everywhere except for the locations of the focal adhesions. This method requires information about the position of focal adhesions, and this can be achieved by fluorescently tagging focal adhesions with GFP-vinculin.

### 2.4.3.4 Regularization and Smoothing

The weakly singular and long-ranged nature of the Green’s function (Eq. 2) [72] implies that the inverse problem of determining the force given the displacement field could be sensitive to small displacements. While this is desirable for tracking small traction forces, the introduction of noise in the experimental data from any source would be reflected in the traction field. Thus, it is necessary to smooth signals that may be derived from noise while preserving signals derived from actual cell tractions. Some noise suppression schemes include the application of a Wiener [79] or Gaussian [80] filter on the displacement fields prior to force calculation. A common filtering method employed by some studies as a means to suppress noise in the displacement and traction force fields is zero-order Tikhonov regularization. This scheme minimizes the discrepancy between the calculated force field and measured displacement field, while restricting
the magnitude of the force vectors [81]. Mathematically, zero-order Tikhonov regularization can be expressed as follows:

\[
\min_F \left\{ \left( \frac{\mathbf{u}}{\mathbf{u}} \right)^2 + \lambda^2 |\mathbf{F}|^2 \right\}
\]  
(Eq. 3)

Minimizing this equation is a trade-off between the accuracy of the solution (the first term) and the smoothing of noise from high-frequency sources in the force field (the second term). Thus, the choice of the regularization factor is important to balance these competing interests. While this choice is generally a subjective procedure [82], plotting $|F|$ against $|GF - u|$ to form an L-curve and selecting the regularization factor corresponding to the corner of the curve is an approach that multiple studies have used in their analyses [72],[83],[84].

2.4.4 Traction Force Microscopy in Three Dimensions

More recently, investigators have applied displacement tracking and force calculation algorithms used to quantify two-dimensional traction forces to quantify traction forces in three dimensions. Most adherent cells are embedded in the tissue to which they belong and thus exist in a three-dimensional environment. Traction forces measured in a three-dimensional environment are thus more likely to be a more accurate reflection of the forces generated by cells in vivo. In a three-dimensional volume, displacement tracking of embedded markers is handled by subdividing the volume of the null-force substrate into small chunks or voxels that contain at least a few markers. The position of the voxel is then found in the volume of the stressed substrate using correlation and the displacement vector is formed by subtracting these positions [85]. The method used by Legant et al. [86] improved the resolution of the displacement field by calculation a displacement vector for each marker. This was achieved by drawing vectors from a target bead in the stressed volume to its closest neighbouring beads, and these vectors served as a unique signature for the target bead. Their algorithm then searched the relaxed volume for the same vector signature and the displacement vector was found by subtracting the position of the target bead in the stressed volume from its position in the relaxed volume (Figure 2-8).
Figure 2-8: A: Volume rendering of an EGFP-expressing NIH 3T3 fibroblast spreading into a 3D hydrogel containing fluorescent beads. B: Color-coded displacement vectors of fluorescent beads. Scale bar = 50 μm. Reprinted with permission from Macmillan Publishers Ltd: Nature Methods [86], © 2010.
Chapter 3

3 Thesis Objectives

Aortic valve homeostasis is maintained by the cellular microenvironment, including appropriately “soft” tissue mechanical properties and constitutive expression of protective molecules like CNP. An imbalance of homeostatic versus pathological factors can lead to valve dysfunction, due in part to myofibroblast differentiation of VICs. Myofibroblasts cause fibrosis via contraction and dysregulated ECM protein synthesis. Unless arrested by homeostatic mechanisms, including myofibroblast apoptosis or the action of anti-fibrotic agents like CNP, this process proceeds unchecked, as cell tractions can cause a local increase in substrate stiffness by densification of ECM proteins. When cells detect this increase in substrate stiffness, RhoA is activated and the cells generate even greater traction forces, further driving pathological differentiation of quiescent fibroblasts into myofibroblasts. Disruption of this positive feedback loop via inhibition of traction generation therefore may be a treatment strategy. In this context, understanding the impact of CNP and deficiency of its receptor on traction force generation is important towards assessing the therapeutic potential of CNP and the impact of CNP signaling deficiency on valve disease development.

With this rationale, the overall goal of this thesis is to implement a custom system capable of quantifying traction forces generated by adherent cells in order to characterize their response to an external stimulus. The specific objectives of this study are as follows:

1. Implement a method for quantifying traction forces generated by single cells adhered to an elastic substrate
   a. Validate this method by quantifying and comparing traction forces generated by RhoA-activated and RhoA-inhibited porcine aortic VICs

2. Use traction force as a phenotypic marker for the function of the NPR-B receptor in mouse lung fibroblasts
   a. Determine suitable culture conditions and a time point for traction force microscopy
b. Quantify and compare the traction forces generated by $Npr2^{+/+}$ (WT) and $Npr2^{+/-}$ (Het) mouse lung fibroblasts treated with CNP using the method developed in objective 1
Chapter 4

4 Implementation and Validation of a Method for Measuring Traction Forces

This chapter describes the implementation and validation of a method for measuring traction forces generated by single cells. The chapter is divided into three main sections. First, the implementation of the method, including a detailed description of the algorithms used to calculate force from a measured displacement field is discussed. Second, I describe the method for determining the regularization parameter for the purpose of force calculation. Lastly, I describe validation experiments in which the RhoA signaling pathway was manipulated in porcine VICs and the traction forces they generated were measured.

4.1 Implementation of the Traction Force Microscopy Method

In this section, I describe my implementation of a method for quantifying traction forces generated by single cells. First, I describe the process by which a continuous elastic substrate with embedded fluorescent markers is fabricated in glass-bottom Petri dishes. Next, the process for functionalizing the substrate with collagen to permit cell adhesion is described. Then the imaging protocol for capturing images of the substrate under stress from the cell and in a relaxed state without the cell is described. This is followed by a description of the process for analyzing these images to calculate the displacement of the fluorescent markers embedded within the elastic substrate.

4.1.1 Polyacrylamide Gel Fabrication

Polyacrylamide (PA) substrates were fabricated using a protocol adapted from Pelham and Wang [87]. Glass bottom Petri dishes with a microwell diameter of 20 mm, overall diameter of 35 mm, and #0 cover glass bottom (D35-20-0-N, In Vitro Scientific, Sunnyvale, CA) were used as the base for gel fabrication. The cover glass was treated to facilitate chemical bonding of the PA

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1 Work presented in this chapter was published in:

solution to the glass by coating with 0.1 M sodium hydroxide for 10 minutes, and then with (100% 3-aminopropyl)trimethoxysilane (3-APTMS; 281778, Sigma-Aldrich, Oakville, ON), which was removed after 3 minutes. The glass was thoroughly rinsed with deionized water to remove all trace of 3-APTMS. The cover glass was treated with 1% solution of glutaraldehyde (340855, Sigma-Aldrich, Oakville, ON) and left to incubate for 30 minutes at room temperature, after which the solution was removed and glass thoroughly rinsed with deionized water again. The Petri dishes were left to sit at room temperature to allow residual water to evaporate. PA solution was sandwiched between the cover glass at the bottom of the dishes’ microwells and cleaned glass cover slips. Top glass cover slips with a diameter of 12 mm were cleaned by immersion in a 3 M solution of hydrochloric acid for 24 hours, 100% ethanol for 24 hours, and then dried under a nitrogen stream.

PA substrates with 11 kPa elastic modulus as determined by compression testing [88] were obtained by mixing 8% acrylamide (161-0140, Bio-Rad, Mississauga, ON), 0.2% bis-acrylamide (161-0142, Bio-Rad, Mississauga, ON), and 10 mM HEPES buffer (H0887, Sigma-Aldrich, Oakville, ON). Polymerization was initiated by adding 1/200 (v/v) 10% ammonium persulfate (215589, Sigma-Aldrich, Oakville, ON) and 1/2000 (v/v) n,n',n'-tetramethylethylenediamine (161-0800, Bio-Rad, Mississauga, ON) and mixed by pipetting up and down. Fluorescent beads (0.18-μm diameter; excitation maximum of 441 nm and emission maximum of 485 nm; 9003-53-8, Polysciences Inc., Warrington, PA) were added to the gel solution (1/150 v/v) immediately after addition of the polymerizing agents and mixed thoroughly to ensure uniform distribution of the beads. 4 μL of the gel solution was pipetted on the treated cover glass in the microwell of the Petri dish and then the treated cover glass was quickly placed on top. After polymerization, the cover glass was removed.

4.1.2 Gel Functionalization

Polymerized gels were activated for protein conjugation by placing them in 0.5 M N-sulfosuccinimidyl-6-(4’-azido-2’-nitrophenylamino) hexanoate (sulfo-SANPAH, CovaChem, Loves Park, IL) solution in 0.25% dimethyl sulfoxide (D2650, Sigma-Aldrich, Oakville, ON), 50 mM HEPES and deionized water, and exposed to UV light for 10 minutes. Photoactivation was repeated with fresh sulfo-SANPAH solution. The gels were thoroughly rinsed with 50 mM HEPES, and then sterilized under UV for 30 minutes. The gels were incubated with 100 μg/mL
of rat tail type I collagen (354236, Becton Dickinson, Mississauga, ON) in PBS at 4 °C overnight. The collagen-functionalized gels were rinsed three times with PBS prior to cell seeding.

4.1.3 Imaging

The plated VICs were imaged on a Nikon Ti-Eclipse inverted confocal microscope. The glass bottom Petri dishes with VICs on PA gel substrates were placed on a stage heated to 37 °C and cells visualized using an oil immersion objective with 60x magnification (Plan Apo 60x λS, NA 1.40). Once the cell was centered and focused in the field of view, an image was captured under differential interference contrast without moving the stage. A Z-stack of the cell and embedded fluorescent beads was then captured using confocal fluorescence microscopy to serve as the images of the deformed substrate. To remove the cells from the substrate and restore the substrate to its initial, undeformed state, 10 μL of 10% SDS was added to the media to lyse the cells. After 90 seconds, another Z-stack of images was captured. The confocal images were captured at a resolution of 0.414 μm/pixel, and each image had width and height of 512 pixels.

4.1.4 Image Analysis

An image from the Z-stack captured before SDS application where the focal plane was at the top surface of the gel (the point at which the fluorescent beads were most in focus and the fluorescence signal from the CellTracker Red was bright) was extracted for use in force and displacement calculations. An image from the Z-stack captured after SDS application in which the fluorescent beads were visually at the same focal plane as the pre-SDS image was also extracted. The images extracted from the two Z-stacks were placed in a new image stack with the “after” image appearing first.

Three custom ImageJ plugins published online by Dr. Qingzong Tseng were used to calculate the displacement and traction fields generated by the VICs [89]. First, the images were aligned in the xy-plane using the Template Matching and Slice Alignment plugin, using the normalized correlation coefficient algorithm with the subpixel registration option checked. Second, the displacement of the beads was tracked using the Particle Image Velocimetry (PIV) plugin. Last, the traction field was calculated using the Traction Force Microscopy plugin, which uses the
Fourier transform traction cytometry (FTTC) method with zero-order Tikhonov regularization, as described in [72].

The traction vector plot was overlaid on the DIC image of the cell such that the centers of both images were coincident, and the cell boundary was manually drawn over the vector plot. Because calculating tractions with FTTC generates vectors originating in a square grid over the entire image, it is possible that forces originating close to, but within the boundary are represented as being outside. To account for this, the cell boundary was adjusted to vectors pointing towards the cell body that were adjacent to the edge of the cell and had magnitudes larger than the background.

To compare the traction force generation between treatment conditions and replicates, the magnitudes of traction vectors originating within the cell boundary were summed and divided by the cell area. In addition, the maximum traction force generated within the cell was recorded. The summing of traction forces was done in a custom-written MATLAB script (Appendix A on p. 78), and the cell area was found using the “Analyze Particles” function in ImageJ.

4.1.4.1 Displacement Measurement: Iterative PIV

The displacement field is measured from the motion of the fluorescent beads using an iterative PIV algorithm, which is an adaptation of the standard PIV algorithm. Like standard PIV, the null-force image is subdivided into interrogation windows. For each interrogation window, its position in the stressed image is located using the normalized cross-correlation function within a search window of defined size. Subtracting the position of the interrogation window in the null-force image from its corresponding position in the stressed image yields the displacement vector with its origin at the center of the interrogation window at its original position (Figure 4-1A).

The iterative PIV algorithm repeats this process multiple times, beginning with interrogation and search windows that are very large. With each progressive iteration, smaller interrogation and search windows, as well as smaller separation distances between interrogation windows are used to refine the measured displacement vector (Table 1). The interrogation and search windows are centered on the final position determined from the previous iteration, and PIV is performed again. The origin of the interrogation window’s final position in the most recent iteration becomes the final position used to calculate the displacement vector (Figure 4-1B). The
resolution of the displacement field is controlled by the image resolution (0.414 μm/pixel) and the separation distance between interrogation windows in the final iteration. The Iterative PIV plugin sets the separation distance between adjacent displacement vectors to be 6.62 μm.

<table>
<thead>
<tr>
<th></th>
<th>Iteration 1</th>
<th>Iteration 2</th>
<th>Iteration 3</th>
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<td>Side Length of Interrogation Window</td>
<td>128 pixels</td>
<td>64 pixels</td>
<td>32 pixels</td>
</tr>
<tr>
<td>Separation Distance</td>
<td>64 pixels</td>
<td>32 pixels</td>
<td>16 pixels</td>
</tr>
<tr>
<td>Side Length of Search Window</td>
<td>256 pixels</td>
<td>128 pixels</td>
<td>64 pixels</td>
</tr>
</tbody>
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Table 1: Dimensions of interrogation and search windows and separation distance between interrogation windows for iterative PIV algorithm, in pixels
Null Force

Interrogation Window

Search Window

New location of Interrogation Window

Stressed

Overlay

Displacement vector
Figure 4-1: Schematic of iterative PIV algorithm. A: First iteration of displacement tracking. Null-force image of green fluorescent beads embedded in polyacrylamide gel is divided into square interrogation windows (square with dashed red outline). For each interrogation window, correlation is used to find its position in the stressed image (square with solid red outline) within a search window (square with solid blue outline) that is four times the size of the interrogation window and centered on the original position of the interrogation window. The displacement vector (orange arrow) is obtained by subtracting the interrogation window's centroid in the null-force image from its centroid in the stressed image. The process is repeated for all other interrogation windows. B: Subsequent iterations of displacement tracking. Null-force image is subdivided into interrogation windows that are smaller in dimension and closer together (squares with dotted red outline). As with the first iteration, correlation is used to find the position of the interrogation window in the stressed image (squares with solid red outline); however, the centroid of the search window is set to be the final position of the displacement vector obtained from the previous iteration.
### 4.1.4.2 Force Calculation: FTTC with Regularization

The traction field is calculated from the displacement field using the Fourier Transform Traction Cytometry method, which transforms the integral equation (Eq. 4) and Green’s function for tangential forces in a two-dimensional surface (Eq. 5) into the Fourier domain. The derivation of (Eq. 2) is described in [77].

\[ u_i(x) = \int \sum_j G_{ij}(x - x') f_j(x') \, dx \quad \text{(Eq. 4)} \]

\[ \tilde{G}_{ijk} = \frac{4(1+\nu)}{\mu k}\left[ \begin{array}{cc} (1 - \nu)k^2 + \nu k_y^2 & -\nu k_x k_y \\ -\nu k_x k_y & (1 - \nu)k^2 + \nu k_x^2 \end{array} \right] \quad \text{(Eq. 5)} \]

The Tikhonov method for regularization uses a scalar factor \( \lambda \) to balance the competing objectives of obtaining an accurate solution while ensuring that the magnitude of the forces do not decrease too much. The target function to be minimized in this method is as follows:

\[ \| \tilde{G}f - \tilde{u} \|^2 + \lambda^2 \| Lf \|^2 \quad \text{(Eq. 6)} \]

This is a variational expression where \( \lambda \), the regularization parameter, is to be varied. \( \| Gf - u \|^2 \) is a measure of the solution’s accuracy, and is known as the residual norm. When the solution is 100% accurate the matrix product of the Green’s function and the calculated traction field exactly matches the measured displacement field and so the solution norm becomes zero. \( \| Lf \|^2 \) is a measure of the magnitude of all force vectors in the field, and is known as the solution norm. Plotting the solution norm versus the residual norm using a log-log scale yields an L-shaped curve that varies with \( \lambda \). Lowering \( \lambda \) increases the solution accuracy at the cost of greater noise in the traction field, whereas increasing \( \lambda \) has the opposite effect. Once \( \lambda \) has been selected, the minimum of the variational expression is the solution of the following equation [90]:

\[ (\tilde{G}^T \cdot \tilde{G} + \lambda^2 L \cdot L)f = \tilde{G} \tilde{u} \quad \text{(Eq. 7)} \]
Solving for the traction field then gives the following:

\[ f = (\tilde{G}^T \cdot \tilde{G} + \lambda^2 L \cdot L)^{-1} \tilde{G}^T \tilde{u} \]  

(Eq. 8)

Performing an inverse Fourier transform on the traction field converts the field vectors back into spatial coordinates.

4.2 Determination of the Regularization Parameter

As previously described, the regularization parameter in the Tikhonov regularization scheme is the balancing term between solution accuracy and noise in the traction field. Here, I analyze the sensitivity of the force calculation algorithm to the regularization parameter and choose one that meets a selection criterion used in the literature. The selected parameter is used throughout all force calculations in this thesis.

4.2.1 Methods

4.2.1.1 Cell Culture

VICs were harvested from freshly-slaughtered pigs (Quality Meat Packers, Toronto, ON). At the time of slaughter, the pigs weighed about 270 lbs and were 5-6 months old. The pigs were bred from the progeny of Yorkshire-Landrace sows mated to boars ranging through cross-breds of Duroc, Hampshire, Large White, and Pietrain. Leaflets were excised from the heart within an hour of slaughter. VICs were extracted from the leaflets by collagenase digestion [6], and plated at 10,000 cells/cm² on tissue culture-treated culture flasks. The VICs were cultured with DMEM containing 10% fetal bovine serum (FBS), 10000 U/mL penicillin and 10 mg/mL streptomycin at 37 °C in a humidified incubator with 5% CO₂. The cells were allowed to grow to confluence, at which point the cells were subcultured into new flasks. This process was repeated once.

To establish a consistent cell population for all experiments, all second-passage (P2) cells were trypsinized, aliquoted into separate vials, and cryopreserved for later use. After centrifugation of the suspended cells, the supernatant containing trypsin was aspirated and the cells were resuspended in DMEM with 45% FBS and 10% DMSO, and aliquoted in cryovials. They were then placed in a freezing container, which was put in a -80 °C freezer for 24 hours, after which the cryovials were transferred to a liquid nitrogen tank.
4.2.1.2 Cell Seeding

Frozen P2 cells were thawed and cultured to confluence in culture flasks. At this point, cells were labelled with a 5 μM solution of CellTracker Red (C34552, Invitrogen, Eugene, OR) for 30 minutes at 37 °C in an incubator with 5% CO2. The staining solution was removed, cells washed twice with PBS with Ca2+ and Mg2+, and incubated for 30 more minutes with supplemented media. The cells were then trypsinized and plated on gels fabricated as described in Section 4.1.1 and 4.1.2 at a density of about 400 cells/cm², and incubated for 24 hours to allow for cell attachment.

4.2.1.3 Sensitivity Analysis

The VICs were imaged and the images were analyzed to calculate the displacement field as described in Section 4.1.4. For force field calculation, a sensitivity analysis of the regularization factor on the magnitude of traction force vectors was conducted in order to select an appropriate λ. The regularization factor was varied by powers of magnitude from 10^{-12} to 10^{-6} and the normalized traction force was calculated each time.

4.2.2 Results and Discussion

4.2.2.1 Traction magnitudes and background noise are sensitive to choice of regularization parameter

The regularization parameter λ controls how much weight is given to obtaining an accurate solution that is vulnerable to noise versus smoothing out noise at the cost of completely eliminating the magnitude of traction force. In this sensitivity analysis, the regularization factor was increased from 10^{-12} to 10^{-6} by orders of magnitude. To compare traction force generation between cells, the magnitude of all force vectors originating within the cell boundary were summed and divided by the cell area. This quantity, the normalized traction force, was re-calculated as the regularization parameter was varied. In addition, the background noise was quantified by calculating the standard deviation of force vectors’ magnitudes outside the cell boundary.

Without regularization, cell traction forces were localized to the extremities of the cell (Figure 4-2A). As the regularization parameter was increased, the area of the force footprint increased and the peak magnitude decreased (Figure 4-2B). If too much regularization was applied, the
smoothing operation reduced the magnitudes of the force vectors to near zero (Figure 4-2C). As a result of the sensitivity analysis, I selected $4.7 \times 10^{-10}$ as the regularization parameter to be used for all experiments using this platform because the 25% reduction in force magnitude along with the 25% reduction in noise was deemed to be an acceptable balance between the competing interests of obtaining an accurate solution and reducing noise in the force field (Figure 4-2D). This point on the curve also coincided with the region of the curve before the steep descent in traction magnitude, a selection criterion characterized as yielding the “truest measure” of quantifying traction stresses [91].

![Figure 4-2: Increasing regularization parameter increases the area of effect of traction forces at the cost of reduction in force magnitude. Vector plots of the force field generated by the same cell treated with RhoA activator, using (A) $\lambda = 0$ (no regularization), (B) $\lambda = 10^{-9}$, and (C) $\lambda = 10^{-7}$. All force magnitudes are in Pascals. Scale bar = 25 µm applies to all vector plots. D, Increasing regularization parameter from 0 decreases background noise, but is associated with a reduction of traction force magnitudes as well. Red square marks $\lambda = 4.7 \times 10^{-10}$.](image)
While this selection criterion can also be characterized as being fuzzy or arbitrary, the process of choosing from one of many possible smoothing methods and then assessing the amount of smoothing to apply is a subjective procedure by its nature [82]. One example of a smoothing method used in the literature is the application of an optimal Wiener filter to the displacement field [79]. The Wiener filter is effective at eliminating white noise from an image or vector field; however, this requires advance knowledge of the power spectrum of the white noise, which may not be available. It is also spatially invariant, meaning that the edges of the image will be smoothed. This would introduce edge artifacts in the displacement field and the resulting traction field. Regardless of the choice of method used, the important part is that some method is used to suppress noise in the force field.

Of the studies that utilize the Tikhonov regularization scheme in traction force microscopy, the value of the regularization parameter is typically much higher that used in this thesis (on the order of $10^{-5}$). The significant discrepancy is most likely due to the higher sampling frequency of displacement field vectors in the literature. Here, the separation distance between adjacent displacement vectors is 6.62 μm, whereas other studies use grid sizes such as 1.71 μm [91]. The lower sampling rate acts as a cut-off to high-frequency noise, so less regularization is required to reduce noise in the force field for this particular configuration.

### 4.3 Validation Experiments

The stiffness of the polyacrylamide gel fabricated with monomeric proportions as described in Section 4.1.1 has been shown to be 11 kPa by compressive testing [88]. However, the addition of polystyrene fluorescent beads could significantly increase the stiffness of the substrate. It is necessary to show that the increase in substrate stiffness, if any, is not beyond the threshold stiffness for activation of the RhoA signaling pathway in order to decouple the effect of the stimulus being tested from the mechanical activation of RhoA.

To validate the ability of my implementation of the traction force microscopy protocol to support cell culture and quantify traction forces generated by those cells, a signalling pathway known to affect cell contractility (namely the RhoA signalling pathway) was manipulated using biochemical reagents. I hypothesized that activating RhoA in VICs would increase their contractility, while inhibiting RhoA would decrease their contractility. Traction forces generated
by the VICs after treatment with these reagents were measured using the platform described in Section 4.1.

4.3.1 Methods

4.3.1.1 Polyacrylamide Gel Stiffness Verification

Gels with and without fluorescent beads were tested using a commercial AFM (Bioscope Catalyst, Bruker, Santa Barbara, CA) mounted on an inverted optical microscope (Nikon Eclipse-Ti). Force mapping was accomplished using a spherical tip with diameter 4.99 μm according to the procedure in [92]. All AFM measurements were done in a fluid environment at room temperature. The gels’ elastic moduli were determined using the standard Hertz model.

4.3.1.2 Cell Culture and Seeding

VICs were cultured and seeded on polyacrylamide gels as described in Sections 4.2.1.1 and 4.2.1.2.

4.3.1.3 Chemical Treatment

Reagents based on the catalytic domain of bacterial CFN toxins (CN03, Cytoskeleton, Denver, CO) and C3 transferase covalently linked to a cell-penetrating moiety (CT04, Cytoskeleton, Denver, CO) were used to activate and inhibit RhoA in VICs, respectively. Because the recommended range of reagent concentrations in culture was large, it was necessary to determine an appropriate concentration to achieve a marked change in actin filament expression (putatively reflective of a change in RhoA activity) without compromising cell attachment or viability. Culture media for VICs was replaced with unsupplemented media, i.e. only DMEM, and incubated for 24 hours to serum starve the cells. The reagents were reconstituted in sterile distilled water, and then added to the unsupplemented media to achieve concentrations ranging from 0.25 to 1.00 μg/mL for the activator, and 0.5 to 2.0 μg/mL for the inhibitor. Some VICs were treated only with sterile distilled water as a control to the RhoA treatments. The volume of water added was the same as that of the reconstituted activator (10 μL in 500 mL media).

The VICs were then stained with phalloidin four hours after treatment, which is the time at which imaging would begin in a typical traction force mapping experiment. The cells were first fixed with 10% neutral-buffered formalin (Sigma-Aldrich HT 501128, Oakville, ON) for 15
minutes, then permeabilized with 0.1% Triton X-100 for 10 minutes. After thorough washing with PBS +/-, 1% phalloidin was added to the cells and incubated for one hour at room temperature. The cells were then rinsed with PBS +/- four times and twice with deionized water. Lastly, the water was removed and one drop of Permafluor mounting media was added to each gel before imaging with a fluorescent microscope.

The concentrations of RhoA activator and inhibitor identified through this experiment (1.00 μg/mL and 2.0 μg/mL for activator and inhibitor, respectively) were used for the RhoA traction force validation study.

4.3.1.4 Statistical Methods

Data are reported as mean ± standard deviation and analyzed by one-way ANOVA with the Fisher LSD pairwise comparison for post-hoc analysis. For each comparison, the significance threshold was set to an alpha of 0.05 or lower.

4.3.2 Results and Discussion

4.3.2.1 Embedded fluorescent beads do not significantly alter substrate stiffness

Previous work showed a significant increase in RhoA activation in VICs cultured on substrates with an elastic modulus of 22 kPa compared to VICs cultured on 11-kPa substrates [40]. Embedding polystyrene fluorescent beads could increase the elastic modulus of the substrate. Therefore, it was necessary to verify that this increase in elastic modulus was not high enough so as to trigger RhoA activation alone. AFM testing of 11-kPa gels with and without the fluorescent beads showed that there was no statistical difference in elastic modulus (p = 0.498). When compared to 22-kPa gels without fluorescent beads, there was a significant difference in elastic modulus (p < 0.001; Figure 4-3).
4.3.2.2 Exposure to RhoA activating and inhibiting reagents affects F-actin expression in VICs

Treatment of VICs with RhoA activating reagent increased F-actin filament expression and treatment with the RhoA inhibiting reagent reduced actin filament expression compared to distilled water control (Figure 4-4). Increasing the concentration of activator and inhibitor appeared to induce a dose-dependent response, i.e. actin filaments were more numerous and defined with higher concentrations of the activator, whereas they were less numerous as the concentration of inhibitor increased. Neither cell attachment nor viability appeared to be compromised within the range of reagent concentrations tested. As a result, the highest concentration tested for both activator (1.00 µg/mL) and inhibitor (2.0 µg/mL) were selected for use in the validation study in order to maximize the difference in measured traction forces between cells treated with activator, inhibitor, and vehicle control.
Figure 4-4: Fluorescent images of VICs after staining for F-actin using phalloidin. Cells were treated with RhoA activator (0.25 μg/mL: A, 0.50 μg/mL: B, 1.00 μg/mL: C), sterile distilled water (D), RhoA inhibitor (0.5 μg/mL: E, 1.0 μg/mL: F, 2.0 μg/mL: G). Scale bar represents 50-μm length.
Exposure to RhoA activating and inhibiting reagents affects traction force generation in VICs. Traction forces generated by VICs treated with RhoA activating or inhibiting reagents at the concentrations determined from the F-actin staining experiment were measured using the platform described previously in this chapter. To compare contractility between cells, the magnitudes of all force vectors originating within the cell boundary was summed, and then normalized to the area of the cell. As expected, VICs treated with the activator generated significantly more force per unit area than the control, and treatment with the inhibitor significantly reduced the cells’ capability for traction force generation (p<0.05, Figure 4-5).

This result confirms the initial hypothesis that direct activation of RhoA leads to increased contractility and traction force generation, while inhibition does the opposite, validating this implementation of traction force microscopy as a tool for quantifying cellular responses to input stimuli. However, there are some limitations to its applicability as a platform to study other biological questions. First, normal forces are neglected in this implementation as a simplifying assumption. However, as previously discussed in Section 2.4.3.1, while neglecting the contribution of normal forces omits potentially useful information about traction force generation, this implementation of traction force microscopy can still be used to make accurate conclusions about traction force generation on the surface of the substrate.

The lowered spatial resolution also creates a limitation for usage of this implementation. Because the displacements are measured and forces calculated in a sparse grid, detailed information such as gradients within focal adhesions during cell migration [93] cannot be recovered. Therefore, this implementation can only be used to quantify traction force generation on a whole-cell scale.
Figure 4-5: Increased active RhoA increases contractility and traction force generation. Representative vector plots of the traction field generated by VICs are shown treated with RhoA activator (A), control (B), and RhoA inhibitor (C). Cell boundaries are marked in white. Scale bar applies to all plots; all force magnitudes are in Pascals. D, Sum of all traction magnitudes within cell boundary normalized to cell area (mean ± SD; *p<0.05 by ANOVA/Fisher LSD pairwise comparison test; N = 5-6). Regularization parameter $\lambda = 4.7 \times 10^{-10}$ used for all replicates.

4.4 Conclusions

In this chapter, an implementation of single-cell traction force microscopy was described, and a validation study was conducted to determine its suitability as a platform for measuring traction forces of cells subjected to different stimuli. The results of the validation study confirmed the hypothesis that activation of RhoA increases VICs’ capability for traction force generation, whereas inhibition decreases their contractility. Now, this implementation can be applied to answer other biological questions where stimuli are applied to a cell and traction forces are a measurable output to those stimuli.
Chapter 5

5 Characterization of Deficiency in CNP Signaling in Mouse Lung Fibroblasts by Quantification of Traction Forces

5.1 Introduction

The protective effect of CNP against myofibroblastic differentiation and development of CAVD has been shown to be mediated by the NPR-B receptor.\(\textit{Npr2}^{+/-}\) (Het) mice with only one functional copy of the \(\textit{Npr2}\) gene show increased development of CAVD compared to mice with two functional copies of the gene (\(\textit{Npr2}^{+/+}\) or wild-type, WT), suggesting that deficient CNP signaling caused by haploinsufficiency for NPR-B can also lead to myofibroblastic differentiation and valve dysfunction. In this chapter, I describe the methods and results of experiments designed to use traction force generation as a phenotypic marker of NPR-B function in mouse lung fibroblasts.

This chapter is split into two main sections. The first describes experiments designed to establish culture conditions and a time point at which the fibroblasts should be imaged for the purpose of measuring their traction forces. The second section describes traction force measurements of the fibroblasts at the time point established in the first section.

5.2 Culture Length Experiment and \(\alpha\)-SMA Staining of Lung Fibroblasts

The process of fibroblasts differentiating into proto-myofibroblasts and myofibroblasts is gradual and can vary based on culture conditions. Culturing on stiffer substrates and the addition of exogenous TGF-\(\beta\)1 have been shown to trigger and accelerate the differentiation process, whereas culturing on softer substrates and withholding TGF-\(\beta\)1 slows it down [96],[97]. To confirm the hypothesis of the study that \(\textit{Npr2}\) haploinsufficiency leads to increased traction force generation despite the presence of CNP, I needed to establish a CNP concentration and time point at which WT fibroblasts treated with CNP generated less traction forces (thus remaining quiescent fibroblasts) than WT and Het fibroblasts without CNP treatment (indicating differentiation into myofibroblasts). To achieve this, a combination of \(\alpha\)-SMA staining and
traction force measurements were used to evaluate the suitability of various conditions for testing of the study hypothesis.

In this chapter, I switched cell type from porcine aortic VICs to mouse lung fibroblasts for efficiency reasons. Mice are much easier to genetically manipulate and more cost-effective to breed than pigs. However, the small size of mice leads to very small valves that are difficult to excise. This means that every mouse has a limited number of VICs, especially at an early stage in development. Expansion of VICs in vitro is not an option for studies aimed at investigating myofibroblast differentiation of native cells, as expansion on stiff polystyrene induces myofibroblast differentiation. It is also impractical to isolate VICs from large numbers of mice at the same time, which can increase the risk of contamination. Therefore, it was necessary to use another source of fibroblasts, and the lungs can provide an ample supply of fibroblasts and lung fibroblasts exhibit many similarities to VICs.

5.2.1 Methods

5.2.1.1 Lung Fibroblast Isolation

Mouse lung fibroblasts were harvested from a colony of Npr2 mice (JAX ID 7658). The colony produced Mendelian proportions of WT, Het, and Npr2-/- (knockout, or KO) pups, although KO pups typically died within 6 days of birth. Each pup was genotyped with respect to the Npr2 gene five days after their birth by having their toe(s) clipped for identification purposes and tail clipped to obtain DNA material. The DNA was isolated from the clipped tail by treating with NaOH/EDTA for 1 hour at 98 °C followed by addition of TrisHCl buffer. The section of the genome coding for the NPR-B receptor was amplified using PCR and then subjected to gel electrophoresis. Samples with only one band at 490 base-pairs on the ladder are WT mice, samples with only one band at 234 base-pairs are KO mice, and samples with two bands (one at 490 bp and one at 234 bp) are Het mice.

All pups were sacrificed by decapitation on day 6. Fibroblasts were extracted from the lungs by collagenase digestion [6] then resuspended in fully-supplemented media (DMEM, 10% FBS (lot #1117138, Life Technologies, Burlington, ON), 1% P/S). The resuspension was pipetted either into 12-well plates or onto collagen-functionalized polyacrylamide gels in glass-bottom Petri dishes fabricated as described in Sections 4.1.1 and 4.1.2. Because the resuspension contained
both fibroblasts and red blood cells, counting of the cells using a hemocytometer was not possible. To control for density, it was assumed that each lung contained a similar number of fibroblasts. All cells from one lung were spread over an area of 37.5 cm$^2$. The wells and/or gels were rinsed twice with PBS $+/+$ 24 hours after initial seeding to remove the red blood cells, leaving only adherent fibroblasts.

5.2.1.2 Culture Conditions

The fibroblasts were stored in a humidified incubator with temperature set to 37 °C and ambient CO$_2$ concentration set to 5%. To remove red blood cells existing in culture, the wells and/or gels were washed twice with PBS $+/+$ 24 hours after initial seeding. For cells designated for CNP treatment, CNP (Bachem H1296, Torrance, CA) was added to the resuspension media in amounts depending on the experiment. Freshly-unfrozen CNP was added to these cells every time media was exchanged (every 24 hours).

5.2.1.3 α-SMA Staining

Cells were fixed with 10% neutral-buffered formalin (Sigma-Aldrich HT 501128, Oakville, ON) for 15 minutes, then permeabilized with 0.1% Triton X-100 for 10 minutes. After thorough washing with PBS $+/+$, 3% bovine serum albumin (Sigma-Aldrich A9647, Oakville, ON) in PBS $+/+$ was added to the cells and incubated for 30 minutes at room temperature to block non-specific binding. The cells were then washed again thoroughly with PBS $+/+$, after which 1% Cy3-conjugated monoclonal anti-actin α-smooth muscle antibody (Sigma-Aldrich C6198, Oakville, ON) dissolved in 3% BSA was added to the cells and incubated for one hour at 37 °C/5% CO$_2$. The cells were then rinsed with PBS $-/-$ four times, and then stained with 0.1% Hoechst 33342 (Life Technologies, H3570, Burlington, ON) for five minutes at room temperature. The cells were then rinsed four times with PBS $-/-$ and twice with deionized water. Lastly, the water was removed and one drop of Permafluor mounting media was added to each well or gel before imaging with a fluorescent microscope.

5.2.1.4 Traction Force Measurement

Cells seeded on collagen-functionalized polyacrylamide gels fabricated in glass-bottom Petri dishes were imaged and analyzed as described in Sections 4.1.3 and 4.1.4.
5.2.1.5 Statistical Methods

Data are reported as mean ± standard deviation and analyzed by one-way ANOVA with the Fisher LSD pairwise comparison for post-hoc analysis. For each comparison, the significance threshold was set to an alpha of 0.05 or lower.

Sample size estimation based on pilot study results was performed using SigmaStat v3.5 (Systat Software, Inc., San Jose, CA). The desired statistical power was set to 0.8.

5.2.2 Results

5.2.2.1 WT fibroblasts cultured on plastic differentially express α-SMA with increasing CNP concentration

Fibroblasts from all genotypes with and without CNP treatment were fixed using neutral-buffered formalin 24, 72, and 120 hours after initial seeding on tissue culture-treated 12-well plates. The CNP concentration was 100 nM, which matched the concentration used in previous studies [6].

All cells were stained for α-SMA expression following the 120-hour time point. There was no α-SMA expression for any of the genotypes at the 24-hour time point, but there was significant expression for all genotypes at 72 and 120 hours post-seeding (Figure 5-1). The lack of differential expression of α-SMA between genotypes at any of the time points implied that the concentration of CNP was insufficient to protect WT fibroblasts from myofibroblastic differentiation.

The above experiment was repeated with 10 times the original concentration of CNP (1 μM). α-SMA expression by WT cells remained limited at the 24-hour time point regardless of CNP concentration. As the culture time increased, α-SMA expression increased significantly for the 72- and 120-hour time points for WT fibroblasts without CNP treatment and with 100 nM CNP; however, there was no α-SMA expression by WT fibroblasts treated with 1 μM CNP at both the 72- and 120-hour time points (Figure 5-2). This shows that the increased CNP concentration was able to prevent myofibroblastic differentiation in WT fibroblasts. The increased CNP concentration was used in all experiments that followed.
5.2.2.2 WT fibroblasts transferred from plastic after 48 hours to PA gel for 24 hours do not generate significantly lower traction forces with CNP treatment

Because treatment with 1 μM CNP causes differential expression of α-SMA in WT fibroblasts at 72 hours, I tried seeding the fibroblasts on tissue culture plastic for 48 hours, transferring them to collagen-coated polyacrylamide gels, and then imaging 24 hours after passaging. As in Section 5.1.1.2, the cells were washed at the 24-hour mark to remove red blood cells, and media with freshly-unfrozen CNP was added every 24 hours. The images were analyzed to measure traction forces generated by the cells as described in Section 4.1.4.

WT fibroblasts did not generate significantly different traction forces with or without CNP treatment (Figure 5-3). It was expected that WT fibroblasts with CNP treatment would generate less traction forces corresponding to their lack of α-SMA expression. However, passaging adherent cells can cause them to become more contractile [94]. This means that trypsinizing the cells from tissue culture plastic and transferring them to gels could introduce a selection bias for contractile and motile cells as they are able to create new focal complexes quickly due to already-activated focal adhesion kinases. Therefore, the experimental protocol for measuring traction forces of mouse lung fibroblasts must avoid passaging to maximize the difference between WT fibroblasts treated with and without CNP. To satisfy this requirement, the fibroblasts must be seeded directly on the gels immediately following the isolation procedure.

Because there was no α-SMA expression at the 24-hour time point on tissue culture plastic, it is unlikely that there would be α-SMA expression at the same time point on a softer substrate such as an 11-kPa polyacrylamide gel. Based on the results presented in this section, the mouse lung fibroblasts must be cultured for at least 48 hours on the polyacrylamide gels in order to see differential contractility between the genotype and treatment conditions.
Figure 5-1: Mouse lung fibroblasts cultured on tissue culture plastic do not express clear α-SMA fibers until after 24 hours, and do not respond to 0.1 μM CNP treatment after 72 hours in culture. Fluorescent images of α-SMA in WT, Het, and KO mouse lung fibroblasts cultured on plastic for 24, 72, and 120 hours (1st, 2nd, and 3rd columns respectively) treated with 0 μM (-CNP) and 0.1 μM (+CNP). White scale bar in first image represents distance of 50 μm, and applies to all images.
Figure 5-2: Increasing CNP concentration suppresses α-SMA expression in WT mouse lung fibroblasts. Fluorescent images of α-SMA in WT mouse lung fibroblasts cultured on plastic for 24, 72, and 120 hours (1st, 2nd, and 3rd rows, respectively) treated with 0 μM, 0.1 μM, and 1.0 μM CNP (1st, 2nd, and 3rd columns, respectively). White scale bar in first image represents distance of 50 μm, and applies to all images.
Figure 5-3: WT fibroblasts do not generate significantly different traction forces with CNP treatment after 48 hours on plastic and 24 hours on PA gel. Sum of all traction magnitudes within cell boundary normalized to cell area (mean ± SD, N = 3-4 except for Het+CNP, where N = 1). Regularization parameter $\lambda = 4.7 \times 10^{-10}$ used for all replicates.

5.3 Traction Force Microscopy of Lung Fibroblasts

5.3.1 Methods

Isolated lung fibroblasts were seeded directly on 11-kPa collagen-coated polyacrylamide gels fabricated in glass-bottom Petri dishes as described in section 4.1.1 and 4.1.2. The fibroblasts were rinsed twice with PBS +/- 24 hours after seeding to remove red blood cells. At about 44 hours after isolation, the fibroblasts were stained with 5 μM CellTracker Red in unsupplemented DMEM for 30 minutes at 37 °C/5% CO$_2$, after which they were rinsed with PBS +/- and supplemented media was re-added. The cells were imaged as described in Section 4.1.3 and analyzed as described in Section 4.1.4.
5.3.2 Results and Discussion

5.3.2.1 CNP treatment did not significantly affect traction force generation by Het lung fibroblasts

Traction forces generated by cells in each of the treatment groups were compared using the same process as described in Section 4.3.2.2: the magnitudes of all force vectors originating within the cell boundary were summed and divided by the area of the cell. Traction forces generated by Het fibroblasts treated with CNP were not significantly different from Het fibroblasts not treated with CNP, while WT fibroblasts treated with CNP were significantly lower than those that did not receive treatment (Figure 5-4). These results indicate that CNP is protective against traction generation associated with myofibroblast differentiation in WT mice, but not in Hets, which supports the hypothesis that haploinsufficiency for \textit{Npr2} in mouse lung fibroblasts limits the protective effect of CNP against increased traction force generation, possibly explaining in part why CAVD development is accelerated in \textit{Npr2}^{+/-} mice.

While the differences were not statistically significant, there was a trend for Het fibroblasts treated with CNP to generate more traction force than WT fibroblasts treated with CNP but less than both WT and Het fibroblasts without CNP treatment, suggesting that the CNP treatment had some protective effect in Hets but not as much as with two functional copies of \textit{Npr2}. This is consistent with my hypothesis, but the differences between genotypes lacked statistical significance as the standard deviation for each genotype/treatment group was large relative to its mean value. Further, the relatively high variability limited the statistical power to distinguish differences in the Hets with and without CNP treatment. Therefore, while we have high confidence that CNP reduces traction forces in WT fibroblasts, we cannot be certain that the lack of effect of CNP in Hets is real or indistinguishable due to insufficient statistical power. To improve statistical power, the standard deviation should be decreased, the number of replicates should be increased, or both.
Figure 5-4: CNP does not significantly lower traction force generation in Het fibroblasts. Representative vector plots of the traction field generated by WT cells with (A) and without (B) CNP treatment, and Het cells with (C) and without (D) CNP treatment are shown. Color bar applies to vectors in all plots, white scale bar represents distance of 25 μm, all force magnitudes are in Pascals. E, Sum of all traction magnitudes within cell boundary normalized to cell area (mean ± SD, **p < 0.1 by one-tailed unequal variance t-test; N = 9-12). Regularization parameter $\lambda = 4.7 \times 10^{-10}$ used for all replicates.
The large standard deviation for each treatment group could be caused by the heterogeneity of cell types that comprise VICs. A possible method of to reduce the variance would be to use a live stain for F-actin such as the LifeAct Plasmids described in [95] and then select for cells that express less F-actin for treatment conditions where less traction force generation is expected, and vice versa for treatment conditions where more traction force generation is expected. There is a concern though about introducing a selection bias for different levels of actin-expressing cells, which can leave any conclusions open to questions about their accuracy. To safeguard against this, the cell populations’ actin-expressing capability must be characterized for each condition to ensure that actin-expressing cells are indeed representative of treatment conditions where more traction force generation is expected, and vice versa. In addition, the plasmids themselves are costly to purchase, which would limit the use of the TFM platform for future experiments. Lastly, plasmid transfection is never 100% effective, so cells that could fit the desired phenotype of F-actin expression but do not accept the plasmid would be excluded from the study.

Another way to achieve statistical significance would be to obtain more biological replicates by imaging multiple cells on per gel. If the choice of cells is truly random, the average traction force of cells on a single gel should be representative of the average phenotype of the cells on that gel. Since SDS application to the gel instantly destroys all cells, images of all cells must be taken before SDS application, and then the stage must be moved back to the exact same location after SDS application to capture the corresponding null-force images. The software that controls the confocal microscope is capable of automating the movement of the stage between pre-programmed positions in multi-point acquisition mode, but the precision of the motors can create problems for displacement tracking. If the motors move the stage to a position even a micron away from its original position, the marker displacement caused by translocation could dominate the marker displacement caused by the cell itself. While translocation can be corrected to a degree by slice alignment using correlation, it comes at the cost of limiting the field of view with a potential loss of information, depending on the size of the cell.

The number of replicates could also be increased by fabricating more gels. However, it would be prohibitive to fabricate the number of gels required from a time and cost perspective. If the data presented in Figure 5-4 were to be considered as a pilot study for the purposes of a power analysis to determine the required sample size to achieve statistical significance in a one-way ANOVA test, 108 samples for each treatment condition would be necessary to achieve a
statistical power of 0.8 with the alpha criterion equal to 0.05. Therefore, it would be more advantageous and efficient to image multiple cells on the same gel rather than fabricating more gels, as long as the precision of the stage motors on the confocal microscope can be characterized.

Ideally it would have been preferable to also characterize the traction force generation capability of KO fibroblasts as well. However, the occurrence of KO pups in litters was much lower than predicted by Mendelian proportions, raising the possibility that these mice either did not survive during gestation or they died shortly after birth and prior to genotype identification.

5.4 Conclusions

The platform for traction force microscopy described in the previous chapter was used to quantify traction forces generated by mouse lung fibroblasts. I identified 48 hours after seeding the fibroblasts on 11-kPa PA gels as a suitable time point for image analysis because α-SMA expression in WT fibroblasts treated with CNP was lower and less clear than WT fibroblasts that did not receive CNP treatment. I then showed that haploinsufficiency for the Npr2 gene in mouse fibroblasts led to increased traction force generation on average, indicating that deficient CNP signaling can lead to myofibroblastic differentiation and accelerated CAVD progression. However, the certainty of this conclusion is limited by the lack of statistical significance. I then discussed the possible reasons for the high variance within the treatment conditions, and suggested methods for decreasing this variance and/or increasing the number of replicates.
Chapter 6

6 Conclusions and Recommendations

6.1 Conclusions

The first objective of this thesis was to implement a protocol for quantifying traction forces generated by single cells. Cells are cultured on 11-kPa polyacrylamide substrates fabricated in the microwells of glass-bottom Petri dishes. The gels are imaged on a confocal microscope with and without the cell. These images are input into ImageJ plugins that measure the displacement of the fluorescent beads embedded in the substrate and calculate the forces that caused this displacement. I then validated the system by treating VICs with biochemical RhoA activating and inhibiting reagents and measured their traction forces. Through this validation experiment, I was showed that the system was able to differentiate between each of the treatment conditions.

The second objective of this thesis was to characterize deficiency in CNP signaling in the lung fibroblasts of mice by quantifying the traction forces that they generated using the protocol implemented in Chapter 4. First, I determined that the CNP concentration needed to be increased to 1 μM in order for CNP to protect WT fibroblasts from myofibroblastic differentiation, and that culturing fibroblasts on gels for at least 48 hours would achieve differential expression of α-SMA between WT fibroblasts treated with CNP and WT and Het fibroblasts not treated with CNP. Using the protocol described in Section 4.1, I showed that WT fibroblasts were responsive to CNP treatment, as WT fibroblasts treated with CNP generated significantly lower traction forces than WT fibroblasts not treated with CNP. I also showed that CNP did not have a statistically significant effect on traction forces generated by Het fibroblasts, suggesting that Npr2 haploinsufficiency impaired the fibroblasts’ response to CNP.

6.2 Future Work

6.2.1 Improvements to imaging workflow

Although the protocol for measuring traction forces generated by single cells described in this thesis was functional, using only one cell on the entire gel surface could be less wasteful by making use of the multi-point acquisition feature available through the confocal microscope control program. This would allow multiple cells on the same gel to be imaged, thus increasing
the number of replicates without a corresponding increase in material cost or preparation time. However, there remain concerns about the precision of the stage motors and whether the z-stacks will be reasonably aligned after SDS application and movement of the stage. The precision of the stage motors should be characterized to determine whether it is feasible to move the stage and still be able to discern between image translocation and cell-induced bead displacement.

6.2.2 Investigate contribution of normal forces

The method for measuring traction forces described in this thesis assumed that all traction forces were in the plane of the substrate and not normal to its surface because the cell is typically thin when spread on a substrate. However, recent studies have shown that this assumption is not always valid, with cells exerting upwards forces near the edges and downwards forces near the nucleus [73]. Quantifying these normal forces could add additional information about the cellular response to mechanical and/or biochemical stimuli. Because the surface forces are decoupled from normal forces for substrates with a Poisson’s ratio near 0.5, the normal forces could be calculated separately. This would require applying the correlation algorithm on interrogation windows across slices of a Z-stack sequence.

6.2.3 Further optimization of culture conditions

In experiments designed to establish a suitable time point for image analysis, I showed that a culture time of at least 48 hours was necessary to show differential α-SMA expression between WT fibroblasts with and without 1 μM CNP. However, it is possible that further increasing the concentration of CNP and/or increasing the culture time could increase the difference in traction force generation between each of the treatment groups. An optimization study could be performed to vary the CNP concentration over a greater range and culture the fibroblasts for multiple lengths of time exceeding 48 or even 72 hours. The fibroblasts would be stained for both F-actin and α-SMA and an optimal time point could be selected on the basis of differential expression of either form of stress fiber.
References


Appendix A – Protocols

Valve Interstitial Cell Isolation

Reagents:

- Penicillin/streptomycin (P/S) mixture (Sigma-Aldrich, P4333)
- Sterile PBS with Ca\(^{2+}\) and Mg\(^{2+}\) (PBS +/-)
- Amphotericin B
- 0.125% trypsin with EDTA diluted in PBS without Ca\(^{2+}\) and Mg\(^{2+}\) (PBS -/-)
- DMEM + 10% FBS + 1% P/S
- TES buffer

Equipment:

- Large and small dissection scissors
- Scalpel
- Biohazard waste bags
- Dissection tray
- Large and small tweezers
- Cell strainers
- Cell scrapers
- Sterile Petri dishes

Procedure:

*Steps 1-3 can be done outside of the biosafety cabinet*

1. Remove heart from non-sterile PBS and cut in half
2. Use large dissection scissors to cut open aorta to the aortic valve
3. Use small scissors and tweezers to remove individual leaflets and immerse the leaflets in PBS +/-, 1% P/S, and 0.5% amphotericin B

*Steps 4 and later must be done in biosafety cabinet*

4. Rinse leaflets (2-3 times) with clean, sterile PBS +/-, 1% P/S, and 0.5% amphotericin B; hold leaflets in the last wash
5. To remove endothelial cells (ECs), place all leaflets (6 leaflets/15 mL tube) in 5 mL of 150 U/mL collagenase dissolved in TES buffer. Then incubate for 20 minutes at 37 °C/5% CO\(_2\)*
6. Transfer leaflets to new tube containing 0.125% trypsin with EDTA, incubate for 7 minutes at 37 °C/5% CO₂

7. Vortex at maximum speed for 1 minute

8. In a Petri dish, rinse away loose ECs with sterile PBS +/- twice

9. In another Petri dish, soak leaflets in 0.125% trypsin with EDTA for 1-2 minutes

10. Transfer 3 leaflets at a time into a new Petri dish, and mince them into small pieces with scissors

11. Transfer leaflet pieces to 150 U/mL collagenase solution dissolved in PBS +/-

12. Incubate for 2 hours in collagenase/PBS solution at 37 °C/5% CO₂

13. Following incubation, vortex at maximum speed for 1 minute. Check that the pieces are actually being agitated

14. Strain tissue using cell strainers (1 strainer per 3 leaflets)

15. Rinse filter once with an equal volume of DMEM

16. Centrifuge cells to a pellet (large centrifuge: speed 1150 rpm for 7 minutes; small centrifuge: speed 0.9 x g for 5 minutes)

17. Resuspend pellet in supplemented DMEM (DMEM + 10% FBS + 1% P/S)

18. Strain cell mixture using cell strainers to break up cell clumps

19. Count viable and dead cells with hemocytometer or with Vi-Cell cell viability analyzer

20. Plate at 10,000 cells/cm² in tissue culture-treated flasks

21. Two hours after plating: check cells to see if they begin to adhere (should be rounded, but not floating)

22. Three hours after plating: cells should be adherent and beginning to spread; note relative number of adherent to floating cells

23. Remove media and dead cells; replace with new supplemented media the following day
**Npr2 Genotyping of Mice**

Reagents:

- Solution A: 25 mM NaOH + 0.2 mM EDTA
- Solution B: 40 mM Tris-HCl (pH 5.5)

Primers (5’ – 3’):

- 9950 (common): CGG CTA TCA GGC TCA GTT TT
- 9951 (wild-type reverse): CAG CAT TCT GGA GGC TAA GG
- olMR7415 (mutant reverse): GCC AGA GGC CAC TTG TGT AG

Procedure:

1. Add 75 μL of solution A to PCR tube containing tail or ear clip. Place tube in thermocycler at 98 °C for 1 hour, reduce temperature to 15 °C for 3 min, then hold at 4 °C.

2. Add 75 μL of solution B. Pipet up and down to dissolve the tissue.

3. Add all three primers to the PCR tubes. Set the thermocycler to the following temperature settings:
   - 94 °C for 30 seconds
   - 64 °C for 30 seconds
   - 72 °C for 30 seconds
   - Repeat for 35 cycles

4. Transfer the contents of the PCR tube(s) to gel for gel electrophoresis. WT shows single band at 490 base pairs (bp), KO shows single band at 234 bp, and Het shows two bands – one at 490 bp, and one at 234 bp.
Npr2 Neo-natal Mouse Lung Fibroblast Isolation

Solutions:
- PBS -/- solution (PBS without Ca$^{2+}$ or Mg$^{2+}$ + 1% P/S, 0.5% amphotericin B (AmpB))
- Growth media (DMEM + 10% FBS, 1% P/S)
- Enzyme solution (PBS -/- + 0.2% trypsin, 300 U/mL collagenase IV) – 3.5 mL enzyme solution/4 lungs

Materials:
- 2 Petri dishes per pup
- 1 15-mL tube per 2 pups
- 70% ethanol in 500 mL bottle
- PBS -/- solution
- Dissection tools (scissors, tweezers, scalpel)
- Ice box

Procedure:

At lab, prior to leaving for Toronto Centre for Phenogenomics (TCP):
1. UV tools for about 30 minutes, then place in Ziploc bag
2. Make PBS -/- and growth media solutions
3. Aliquot about 5 mL PBS -/- solution into 15-mL Falcon tubes (1 tube per 2 mice)

At TCP, before dissection:
4. Clean hood (clidox, blower 5 minutes, hood closed), keep hood half-closed
5. Set up contents of hood (keep sterile)
6. Place tools in 70% ethanol immediately and leave for about 10 minutes
7. Collect pups, place in new cage

Dissection at TCP:
8. Sacrifice pup, invert on napkin immediately for about 10 seconds

---

2 Protocol developed by Kathak Vachhani and Rachel Adams
9. Clip skin, clip tissue, and open chest

10. Remove heart and lungs, place them in a new Petri dish. Leave carcass on napkin

11. Remove heart from lungs


13. Transfer lungs to tube containing PBS -/- solution, label tube, and place on ice

14. Dispose of carcass and any remaining tissue in plastic bag

Fibroblast isolation in lab:

Immediately:

- place tools (tweezers and scissors) in 70% EtOH beaker ~20 mins
- weigh out collagenase aliquots
- measure PBS-/- *only* for enzyme solution
- thaw trypsin

15. Rinse lungs in PBS-/- solution (petri dish)

16. Make collagenase solution: add trypsin and collagenase to PBS-/- tube, invert to mix well, aliquot into 15 mL falcon tubes (1.75 mL / 2 lungs, 3.5 mL / 4 lungs, 5 mL / 6 lungs)

17. Transfer lungs to new petri dish, mince, add tissue to collagenase solution

18. Incubate 80 min in 37°C incubator, inclined/horizontal

- incubate 20 min, vortex 20 sec (*to release cells*), incubate 20 min
- add newly made collagenase solution (50% addition), vortex 20 sec
- incubate 20 min, vortex 20 sec, incubate 20 min

19. Add equal volume of growth media to tubes (*to neutralize enzymes*)

20. Vortex 1 min

21. Pipet through 70 µm strainer; rinse tube and strainer with growth media

22. Centrifuge @ 1150 rpm, 15 min

23. Resuspend pellet in 5 mL growth media
24. Pipet through 40 µm strainer; rise strainer with growth media
25. Centrifuge @ 1150 rpm, 8 min
   - thaw CNP; prepare media with and without 100 mM CNP
26. Remove supernatant but leave ~0.5 mL to resuspend cells in (mix well)
27. Pipette half cells into media with and without CNP
28. Plate in flask(s)
**Gel Fabrication**

Materials:

- 12-mm diameter #1.5 cover glass (Fisherbrand)
- 6 M hydrochloric acid
- 100% ethanol
- 35-mm glass-bottom Petri dish with 20-mm microwell #0 cover glass (In Vitro Sci, D35-20-0-N)
- 0.1 M NaOH
- 100% (3-aminopropyl)trimethoxysilane (3-APTMS) (Sigma-Aldrich, 281778)
- Deionized water
- 1% gluteraldehyde (Sigma-Aldrich, 340855)
- 40% acrylamide (Bio-Rad, 161-0140)
- 2% bis-acrylamide (Bio-Rad, 161-0142)
- 1 M HEPES buffer (Sigma-Aldrich, H0887)
- Ammonium persulfate (APS) (Sigma-Aldrich, 215889)
- TEMED (Bio-Rad, 161-0800)
- Green 0.18-μm diameter fluorescent beads (Polysciences, Inc., 9003-53-8)
- PBS +/-

Procedure:

Clean top cover glass

1. Create 80 mL of 3 M HCl (40 mL of 6 M HCl + 40 mL deionized water) and put in sealable bottle. Note: Add acid to water, not the other way around
2. Immerse 12-mm cover glass in bottle and seal
3. Tape bottle sideways to surface of orbital rotator and turn on rotator, maximum setting
4. After at least 24h, dispose of acid and replace with 100% ethanol
5. After at least 24h, extract cover glass one by one and dry under nitrogen stream. Place in clean test tube when dry

Silanize cover glass in glass-bottom Petri dish

1. Clean bench surface with 70% EtOH and place glass-bottom Petri dishes on bench. Remove lids and set aside
2. Put enough 0.1 M NaOH on the glass bottom to cover majority of glass surface but do not let any liquid touch the plastic

3. Remove NaOH and use glass pipette to add 3-APTMS to glass bottom. Only put enough to cover same surface area as NaOH; do not let any liquid touch the plastic. Wait 3 minutes. Note: 3-APTMS dissolves plastic

4. Remove 3-APTMS and wash 3x with deionized water

5. Add 1% gluteraldehyde, wait 30 minutes

6. Remove gluteraldehyde, wash 3x with deionized water

7. Let dry at room temperature – do not add gel solution until all moisture has evaporated

Gel Fabrication

1. Prepare 11-kPa gel solution in test tube:
   - 1430 μL water
   - 300 μL 2% bis-acrylamide
   - 250 μL 40% acrylamide
   - 20 μL 1 M HEPES buffer

2. When ready to polymerize, add polymerizing reagents:
   - 10 μL 10% APS
   - 1 μL TEMED

3. Mix thoroughly by pipetting up and down

4. Withdraw 300 μL of gel solution and put in small test tube

5. Add 2 μL of fluorescent beads to 300 μL of gel solution. Mix thoroughly by pipetting up and down

6. Add 4 μL of gel + bead solution to each microwell. Place cleaned cover glass on top of gel solution to sandwich it. Try to eliminate air bubbles

7. Wait 15-20 minutes for gel to polymerize – should see faint ring when polymerization is complete. Can also look at remainder of gel solution to see if that has polymerized

8. Pipette PBS +/- onto gel and put Petri dishes in 4 C refrigerator for at least 30 minutes
Gel Functionalization

Materials:
- Sulfo-SANPAH (CovaChem)
- 0.25% DMSO (Sigma-Aldrich, D2650)
- Deionized water
- 50 mM HEPES buffer
- UV lamp
- Rat tail Type I collagen (Becton Dickinson, 354236)
- 0.02 N acetic acid

Procedure:

Cross-linking
1. Take Sulfo-SANPAH vial out of 4 °C refrigerator at least 1 hour before using. Note: Sulfo-SANPAH is light-sensitive
2. Use needle and tweezers to peel top cover glass off gel. Take care not to damage the gel
3. Prepare Sulfo-SANPAH solution:
   - Measure 3 mg of Sulfo-SANPAH powder using balance (use small test tube as container)
   - Add 30 μL DMSO to dissolve (make sure all powder is dissolved)
   - Add 30 μL of Sulfo-SANPAH dissolved in DMSO to 11.34 mL deionized water and 600 μL 50 mM HEPES buffer
4. Aspirate PBS +/+ from Petri dish and place 500 μL of Sulfo-SANPAH solution in microwell of each dish. Note: When pipetting to microwell in presence of gel, take care not to pipette too quickly
5. Place dishes under UV lamp for photoactivation. Move stage to maximum height. Wait 10 minutes
6. Remove dishes and aspirate photoactivated Sulfo-SANPAH. Wash 1x with HEPES buffer and put dishes on shaker (setting 4) for at least 10 minutes
7. Repeat steps 4-6 once
8. Wash dishes 3x10min with 50 mM HEPES buffer

Functionalization with Collagen
1. Place dishes with lids in biosafety cabinet and sterilize with UV for 20-30 minutes. Note: All steps after this must be done in biosafety cabinet
2. Prepare 100 µg/mL rat tail type I collagen solution in 0.02 N acetic acid
3. Aspirate HEPES buffer and replace with 500 µL collagen solution
4. Place lids on all dishes and move them to the 4 C refrigerator. Wait overnight

Cell Seeding
1. Wash dishes 3x with PBS +/-
2. Prepare cell suspension with desired density
3. Add 500 µL of cell suspension to each dish
4. Place dishes in incubator for at least 24 hours to allow for cell attachment
Confocal Imaging

1. Turn on temperature control for live cell imaging stage and place in on the main stage of the confocal microscope. Use tape to secure its position. Do not proceed to step 5 until temperature reaches 37 °C

2. Turn on confocal stack, motorized stage control, transmitted light supply, microscope, QImaging camera, and lasers

3. Open Nikon software (Confocal grabber)

4. Ensure height of objective nose is at minimum, then switch objective to PA 60x and add a drop of immersion oil to the lens

5. Move the stage in the xy-direction such that the objective is centered in one of the circular holes of the live cell imaging stage

6. Place a dish in that hole, ensuring that the fit is secure

7. Click “Find” to begin fast image acquisition mode. Move stage in z-direction until oil is in contact with the dish, then adjust sensitivity to medium and continue moving stage up until green fluorescent beads can be seen

8. Adjust z-height to highest level such that beads remain in focus

9. Move stage in xy-direction to find a cell; re-focus if necessary

10. Close Nikon software and reopen using QImaging grabber

11. Capture DIC image of cell

12. Close Nikon software and reopen using confocal grabber

13. Capture Z-stack with top surface of gel as center of stack. Settings for Z-stack: +/- 4 µm, step 0.5 µm

14. Add 10 µL SDS to microwell. Use “Find” to verify that cells are dying (red signal from CellTracker red should disperse quickly). Add more SDS if necessary.

15. Wait 1.5-2 minutes, and then capture another Z-stack with the exact same settings.

16. Lower objective nose to minimum and dispose of dish

17. Repeat steps 6-16 for each dish. Reapply immersion oil as necessary (usually every 3-4 dishes)
18. Reopen all .nd2 files and export to TIF (each channel should have its own grayscale image, convert to 8-bit)

19. Save files to USB or upload to lab server
**Image Analysis**

Software Required:

- ImageJ v1.48u or later
- ImageJ Plugins:
  - Template Matching/Slice Alignment: [https://sites.google.com/site/qingzongtseng/template-matching-ij-plugin](https://sites.google.com/site/qingzongtseng/template-matching-ij-plugin)
  - Particle Image Velocimetry: [https://sites.google.com/site/qingzongtseng/piv](https://sites.google.com/site/qingzongtseng/piv)
  - Traction Force Microscopy: [https://sites.google.com/site/qingzongtseng/tfm](https://sites.google.com/site/qingzongtseng/tfm)

Procedure (Displacement Tracking/Force Calculation):

1. In ImageJ, use the ND2 viewer to open the “before” z-stack and find the highest slice where beads are uniformly focused across the entire image and cell body is clearly visible
2. Open the image file linked to that slice
3. Close the ND2 viewer and reopen it to view the “after” z-stack
4. Visually compare the slice opened from the “before” z-stack to the slices of the “after” z-stack. Select the slice that has a similar focus and intensity level, and open it in ImageJ. Close the NS2 viewer
5. Create a stack of the two images: Image > Stacks > Images to Stack
6. Reverse order of the stack so slice from the “after” z-stack is on top: Image > Stacks > Tools > Reverse
7. Press “play” on the stack to check for whole-image displacement – if not present, skip to step 11
8. Align slices: Plugins > Template Matching > Align slices in stack…
9. Check the “Sub-pixel registration?” checkbox, then click OK
10. Draw a rectangle on a region of the image away from the cell, then press OK
11. Measure displacement field: Plugins > PIV > iterative PIV(Basic)…, then press OK
12. If there are any unusually large vectors, apply either dynamic or normalized mean test from drop-down menu
13. Save PIV displacement file to folder of your choice

14. Calculate force: Plugins > FTTC > FTTC

15. Input information:
   - Pixel size: 0.414
   - Poisson ratio: 0.48
   - Young’s modulus: 11000
   - RF: 4.7e-10
   - Plot width: 512
   - Plot height: 512

16. Open desired PIV displacement file

17. Save scale graph and vector plot images

18. Repeat steps 1-17 for each set of images
Integrate Traction Forces

Required Software:

- ImageJ
- GIMP v2.8 or later: [http://www.gimp.org/downloads/](http://www.gimp.org/downloads/) (Photoshop can also be used)
- MATLAB (R2010b or later)
- calc_int_traction.m MATLAB script

Procedure:

1. In GIMP, open the vector plot image and DIC image of cell
2. Scale DIC image of cell to same scale as vector plot image (set width to 475 px)
3. In vector plot image, create alpha channel: Layer > Transparency > Add Alpha Channel
4. Delete black background: Select > By Color, then click on anything black and hit delete
5. Create two new layers: one called “Background” that is entirely black; the other called “Cell Outline” which is completely transparent
6. Reorder layers so Background is on the bottom and Cell Outline is on the top
7. Cut and paste scaled DIC image to vector plot, and move pasted content to new layer
8. Move layer to be between background and vector plot
9. Select “Cell Outline” layer
10. Set foreground colour to white and use pen tool to trace outline of cell. If force vectors likely caused by the cell are outside it, adjust drawn outline to include vectors
11. Hide all layers except for Cell Outline. Select all (Control-A) and copy everything (Control-C)
12. Create new image with height and width = 512px with white background
13. Paste contents into new image. Send pasted contents to new layer and invert colour (Colors > Invert)
14. Change foreground colour to black and use fill tool to fill in the cell outline
15. Save image file with filename ending with “_cellfill” and export to .png (or preferred) image format
16. In vector plot image, un-hide the Background and Vector plot layers
17. Save image file and export to .png (or preferred) image format

18. Optional: Open vector plot with cell outline image in ImageJ and add scale bar
   a. Analyze > Set Scale…
      Distance in pixels: 1
      Known distance: 0.414
      Pixel aspect ratio: 1.0
      Unit of length: um
   b. Analyze > Tools > Scale Bar

19. Open _cellfill image in ImageJ

20. Convert file type to 8-bit grayscale: Image > Type > 8-bit

21. Image > Adjust > Threshold, then click “Apply”

22. Calculate area of cell: Analyze > Analyze Particles…
    Show: Nothing
    Make sure “Display results” checkbox is checked

23. Record cell area in spreadsheet/notebook

24. Open calc_inttraction.m file in MATLAB and run

25. Open the text file containing the calculated traction force field and the _cellfill image

26. Record all output information in spreadsheet/notebook

27. Repeat steps 1-26 for each imaged cell
Source code for calc_int_traction.m

clear all

%% Obtain data and import to workspace

ForceDialogTitle = 'Select the file containing the traction data';
CellDialogTitle = 'Select the image of the cell shadow';

force_data = uigetfile('Traction*.txt',ForceDialogTitle);
image_data = uigetfile('*cellfill.png',CellDialogTitle);
traction_map = importdata(force_data);
cell_image = imread(image_data);

%% Initialize variables
integrated_traction = 0;
array_length = size(traction_map,1);

%% Calculate integrated traction
% Using magnitude
j = 1;
k = 1;
for i = 1 : array_length
    if cell_image(traction_map(i,2),traction_map(i,1)) == 0
        reduced_array(j,:) = traction_map(i,:);
        j = j + 1;
    else
        background_tractions(k,:) = traction_map(k,:);
        k = k + 1;
    end
end

%% Plot traction vectors inside cell boundary
% figure(1)
% quiver(reduced_array(:,1),reduced_array(:,2),reduced_array(:,3),reduced_array(:,4))
% set(gca,'YDir','reverse');
% xlim([0 512]);
% ylim([0 512]);
% axis square
% figure(2)
% quiver(traction_map(:,1),traction_map(:,2),traction_map(:,3),traction_map(:,4))
% set(gca,'YDir','reverse');
% axis square
% figure(3)
% imshow(cell_image);
% saveas(1,'tractions_inside','png');
% saveas(2,'full_map','png');
% saveas(3,'cell','png');
%% Output data to command window

output.filename = force_data;
output.integrated_traction = sum(reduced_array(:,5));
output.max_traction = max(reduced_array(:,5));
output.mean_traction = mean(reduced_array(:,5));
output.stdev_background = std(background_tractions(:,5));

output
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