The Use of Micropipette Aspiration to Measure Cortex Tension in HeLa Cells and Cardiac Myocytes

By

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Thesis

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

in

Biomedical Engineering

January 31, 2021

Nashville, Tennessee

Approved: Date:

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CHAPTER 1

INTRODUCTION

Heart failure is the leading cause of mortality in the United States[1]. Despite current interventions to treat heart failure, life expectancy remains low, and half of patients with heart failure die within 5 years of diagnosis[1]. A principal defect in heart failure is a loss in cardiac contractility, and current treatments for heart failure include various interventions to increase this contractility[2]. Under normal conditions, heart muscle cells generate contractile forces that pumps blood throughout the body, but abnormalities in these contractile forces result in disease states in the body[3]. Therefore, it is essential to investigate the mechanisms underlying the heart's contractile system, because in doing so, we will gain a greater understanding of how this contractile machinery can be reassembled when diseased or otherwise damaged. A greater understanding of heart function in healthy and disease states will contribute to the treatment and prevention of this leading cause of death and disability.

The well-studied biochemical changes occurring during the progression of heart disease are accompanied by changes in cellular mechanics. Cardiomyocytes are the essential muscle cells which drive the beating of the heart, and cardiac function is dependent on their contraction. Cells, including cardiomyocytes, modify their shape and surroundings to drive processes vital for eukaryotic life, including cell division, cell migration, and muscle contraction[8]. Shape changes are controlled by changes in tension of the cellular cortex, which is a thin actin network beneath the plasma membrane. It is well known that

dysregulation of cortical mechanics often causes disease[4], and while our understanding of this topic is rapidly progressing, many open questions still remain. Investigating cortical mechanics by way of cortex tension will provide insight into the many biological functions – and disfunctions – it is involved in.

Non-muscle myosin II (NMII) is the motor protein that generates the forces that drive cortical tension and is therefore is the major driver of key cellular processes[5-6]. Understanding how NMII contributes to force generation inside of cells is central to our understanding of force-dependent progression of diseases such as heart disease, which makes NMII a fundamental target for experimental investigation[3]. Generation of cortex tension is important to understand, because when it goes awry, it can result in devastating consequences in the body. In the heart, changes in cardiomyocyte tension are associated various forms of heart failure. Thus, the goal of this thesis is to investigate the biophysical property of cortex tension at the level of a single cell. Micropipette aspiration was the method used throughout the experiments described in this thesis in order to measure cortical tension levels under different conditions. In this method, a micropipette is brought into contact with a cell and a known suction pressure is applied through the pipette. As pressure is increased, a portion of the cortex is drawn into the micropipette, forming a protrusion whose length can be measured. By tracking the length of the aspirated region as it moves into the pipette, the imposed pressure is related to the resulting deformation, and can be used to calculate cortex tension based on established mathematical relationships.

This thesis is divided into five chapters: Chapter 2 consists of a comprehensive guide I wrote on the method of micropipette aspiration, going into detail about its history and

implementation. Chapter 3 outlines the experimental work carried out in HeLa cells, in which this method was applied to study the role of the motor protein non-muscle myosin II (NMII) in modulating cortex tension. Chapter 4 describes how micropipette aspiration was used for the first time to directly measure the cortical tension of cardiomyocytes. Chapter 5 proposes future directions for the research going forward, based upon the findings presented in this thesis, as we anticipate the experiments described in chapter 4 will provide the foundation for further studies in defining new potential targets for investigating the development of heart failure.

CHAPTER 2

MICROPIPETTE ASPIRATION

2.1 Project Motivation

Micropipette aspiration is a technique that allows us to precisely measure the mechanical properties of single cells. The greater part of my work in the Burnette lab has involved setting up our lab's micropipette aspiration system and using it to perform experiments. In my experience, the process of getting started in micropipette aspiration without prior experience can be challenging. Methods sections in published literature involving micropipette aspiration tend to give a concise description of the technique; these explanations are sufficient to allow readers to understand the study, but they omit important details which make them difficult to reproduce. This project presents a comprehensive guide to micropipette aspiration, which includes what I have learned from my personal experience with this technique, as well as a literature review of its history and applications. The objective of this project was to develop a resource that facilitates the use of this technique, with the goal of increasing its accessibility for future research. This guide aims to explains micropipette aspiration in a detailed, yet straightforward way, such that it can understood by someone with no prior experience. The intended use of this project is for current and future members of my lab to understand our system, as well anyone else interested in implementing this technique in their own work.

The methodology and instrumentation have been refined to streamline the process as much as possible. A method commonly used to control pressure in micropipette aspiration systems is adjusting the height of a water reservoir. However, this method suffers from problems of evaporation and the need to calibrate prior to each use. Our system uses a microfluidic pump system to provide the pressure for the experiment with an associated computer program that allows the pressure applied to the cell to be varied. Our protocol further simplifies the aspiration process through the use of automated pressure control; by creating a protocol to steadily increase pressure, we avoid the variability of manual pressure control of this system and are able to incorporate a smoother pressure increase. This automated pressure control program has parameters that can easily be modified to the fit the requirements of different experiments. After the micropipette is positioned on the cell of interest and the program is started, the rest of the experiment proceeds automatically. The rest of the experiment simply involves stopping the imaging program and pressure protocol when the cell has been sufficiently aspirated. This releases the cell from the pressure and can then be used find another cell and repeat the process as desired.

This guide begins with an overview of the micropipette aspiration method, how it works, and why it is useful. This is followed by the explanation of a semi-automated method for micropipette aspiration, detailing the necessary materials and equipment, set-up of the system, and an experimental protocol. It further describes how data is collected and used to determine cortex tension, including the mathematical model that underlies these calculations. It concludes with a discussion of micropipette aspiration in my own research, describing its use in studying the roles of non-muscle myosin II paralogs in cortex tension.

2.2 Background

Studying mechanical changes at the cellular scale enables us to further our understanding of the relationship between a cell's biophysical properties and its biological function. A cell's stiffness is a major biophysical property that drives its ability to change its shape and can be quantified by measuring the tension of the cell's cortex. This *cortex tension* is defined as force per unit length exerted on a piece of the cortex by the cortical network around it[6,9]. The cell cortex is a thin network of actin filaments located directly underneath the cell's plasma membrane. Through the modulation of its mechanical properties, the cortex allows a cell to maintain and change shape in response to internal and external signals, and thus generate the forces that determine cortical tension[9-12].

> "Investigating the mechanical properties of the surface of so small an object as a cell presents considerable difficulties. It would hardly be desirable, even if it were possible, to isolate a piece of cell membrane. It is necessary, therefore, to deform the intact cell in some way, such that the deformation and the deforming force can both be measured."
>
> - *Mitchison and Swann, 1954*

Thus began micropipette aspiration.

J. M. Mitchison and M. M. Swann published *The Mechanical Properties of The Cell Surface I. The Cell Elastimeter* in 1954, laying the groundwork for what would come to be known as micropipette aspiration[13]. Their paper introduces the cell elastimeter, a device that was used to determine the extent to which a cell surface is drawn up the pipette by a given negative hydrostatic pressure. Although the scientific field has advanced significantly in the past 70 years, this technique of micropipette aspiration has stood the test of time and remains a widely adopted technique to study cell mechanics.

With the cell elastimeter, cell deformation is measured by eye and a water reservoir is used to control the applied pressure, but the working principle is the same as modern micropipette aspiration systems. In order to measure the mechanical properties of a cell, the cell must be deformed in some way by a known force and its deformation must be measured. This principle is the basis for micropipette aspiration: known negative pressure (suction) is applied to a cell through a micropipette, and as pressure is increased, this causes a deformation of the cell surface into the micropipette. By tracking the length of this aspirated region as it moves into the pipette, the imposed force is related to the resulting deformation, and this relationship can then be used to determine the cell 's mechanical properties. Micropipette aspiration systems analogous to the original elastimeter have since been used on various cell types to study a wide range of topics such blood cell mechanics, nuclear dynamics, and bleb growth, to name a few[14-18].

2.3 Set-Up of the Micropipette Aspiration System

Materials and Equipment

- Glass capillaries – 0.75 mm Inner Diameter, 1 mm Outer Diameter capillaries
- Needle puller – Narishige PC-100
- Microforge – Narishige MF-900
- Pipette storage container – 10-cm dish and non-hardening clay
- Microscope capable of DIC imaging
- Micromanipulator – Eppendorf TransferMan
- Pipette holder – Eppendorf capillary holder with associated grip head
- Microfluidic pump – Fluigent Flow EZ Module, 25 mbar
    - Tubing to connect to vacuum supply
    - LineUp Power Supply Kit
    - LineUp Link PC Communication Module
    - PCAP Reservoir, 2mL, high pressure
    - Connection Kit for PCAP Reservoir

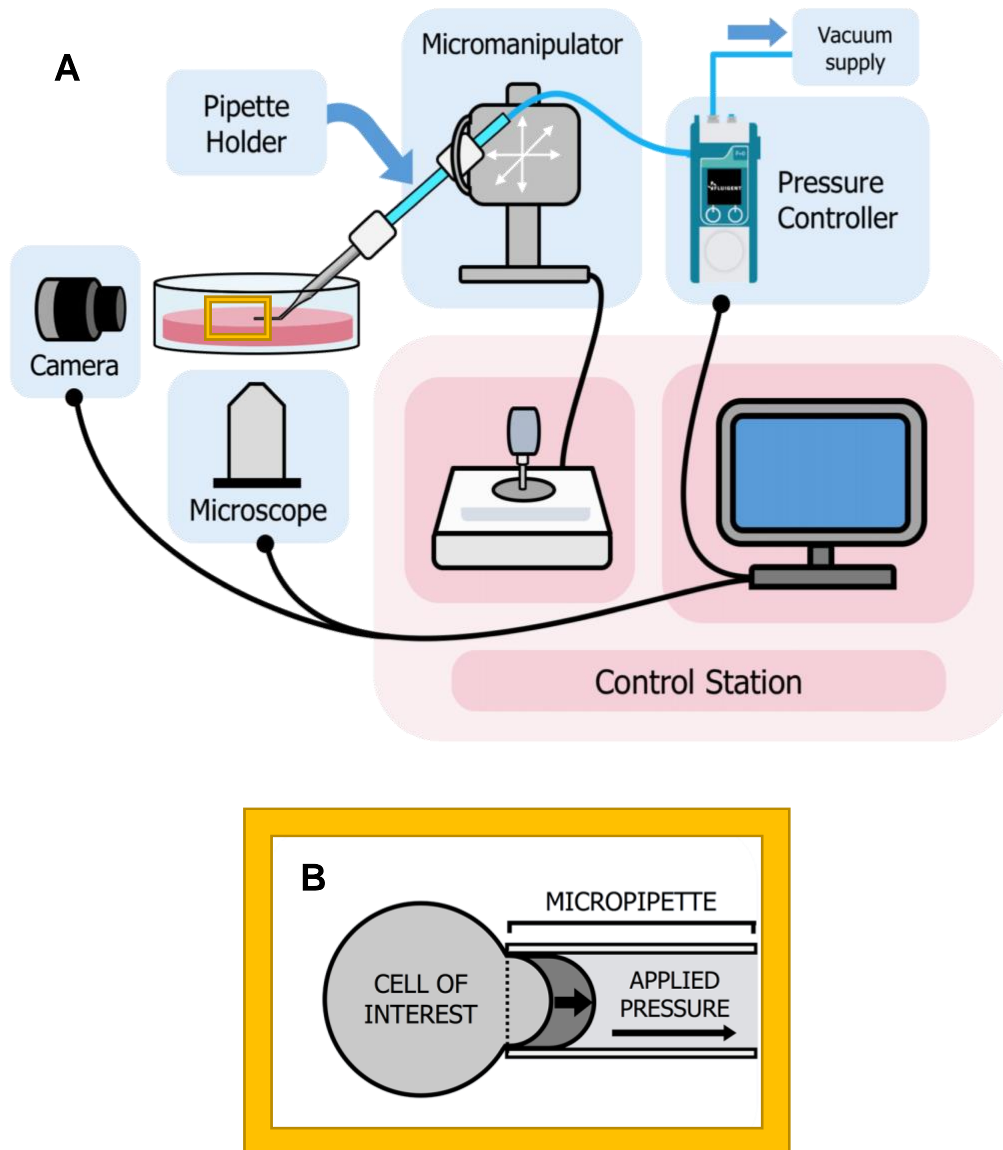


Figure 1: (**A**) Components and set-up of the micropipette aspiration system described in this protocol. The pipette holder secures the micropipette needle at one end and attaches to the micromanipulator at the other. The micromanipulator allows for positioning of the micropipette; a joystick is used to control movement in three dimensions. The pressure controller controls the applied pressure via a computer interface. An optical microscope is used to observe the cell deformation and to capture video of the process. (**B**) Magnified schematic of micropipette aspiration at the cellular level.

2.4 Preparing Micropipette Needles

Micropipettes

A micropipette is a glass tube that tapers to a diameter of several microns at the tip, through which negative pressure is applied to a cell. Producing reliable micropipette needles is critical to the success of the experiment. These needles can either be acquired commercially or made in the lab. Given how fragile these needles are, many are broken throughout the experimental process; the advantage to making them yourself is that they can be made rapidly, in a large volume for a negligible cost. Micropipette needles are prepared in two steps: (1) Micropipette needles are pulled from glass capillaries using a needle puller, (2) micropipettes are cut to the desired diameter, fire polished, and bent to the desired angle using a microforge.

Needle Puller

A micropipette needle-puller is used to pull micropipette needles from glass capillaries. A filament at the center of the capillary is heated by a current, making the glass more pliable, while weights attached to the lower half provide the necessary force to pull this softened region apart, resulting in two micropipette needles per capillary. The input current and added weight can be adjusted in order to obtain an optimal tip shape for a given experiment. For our purposes, this was done using 55% maximum current and one light weight (mass = 23.5g) which results in a centimeter-long taper.

Microforge

Using a microforge, the taper of the micropipette needle is cut to the desired pipette diameter, approximately half of the cell diameter. A 0.5 mm glass bead sitting on the

microforge filament is positioned next to the needle at the required breaking point. The filament and bead are heated up for less than a second so that the glass bead is slightly displaced due to thermal expansion and briefly contacts the needle. After contact and extinction of the heat source, the retraction of the bead causes a blunt break at the contact point on the needle. The end of the needle is then fire polished by bringing it close to the heated bead to obtain a smooth edge that will attach to the aspirated cell without disrupting the membrane.

The microforge is used to bend the tip of the micropipette needle to or an angle at which its tip will be parallel to the bottom of the microscope stage upon setup, typically 30-50 degrees. This is done by positioning the needle vertically at a 0.1 mm distance from the glass bead. The filament and bead are heated up so that the needle bends toward the bead until it reaches the desired angle.

Pipette Storage

After pipettes are prepared, they can be stored until use. It is good to have extra on hand in case one is broken, to avoid having to make more mid-experiment. The pipettes can be kept in ten- centimeter dishes and secured, tip up, in a piece of clay at the bottom of the dish. It is best to use clay that does not harden, to prevent the pipettes from getting stuck in the clay.

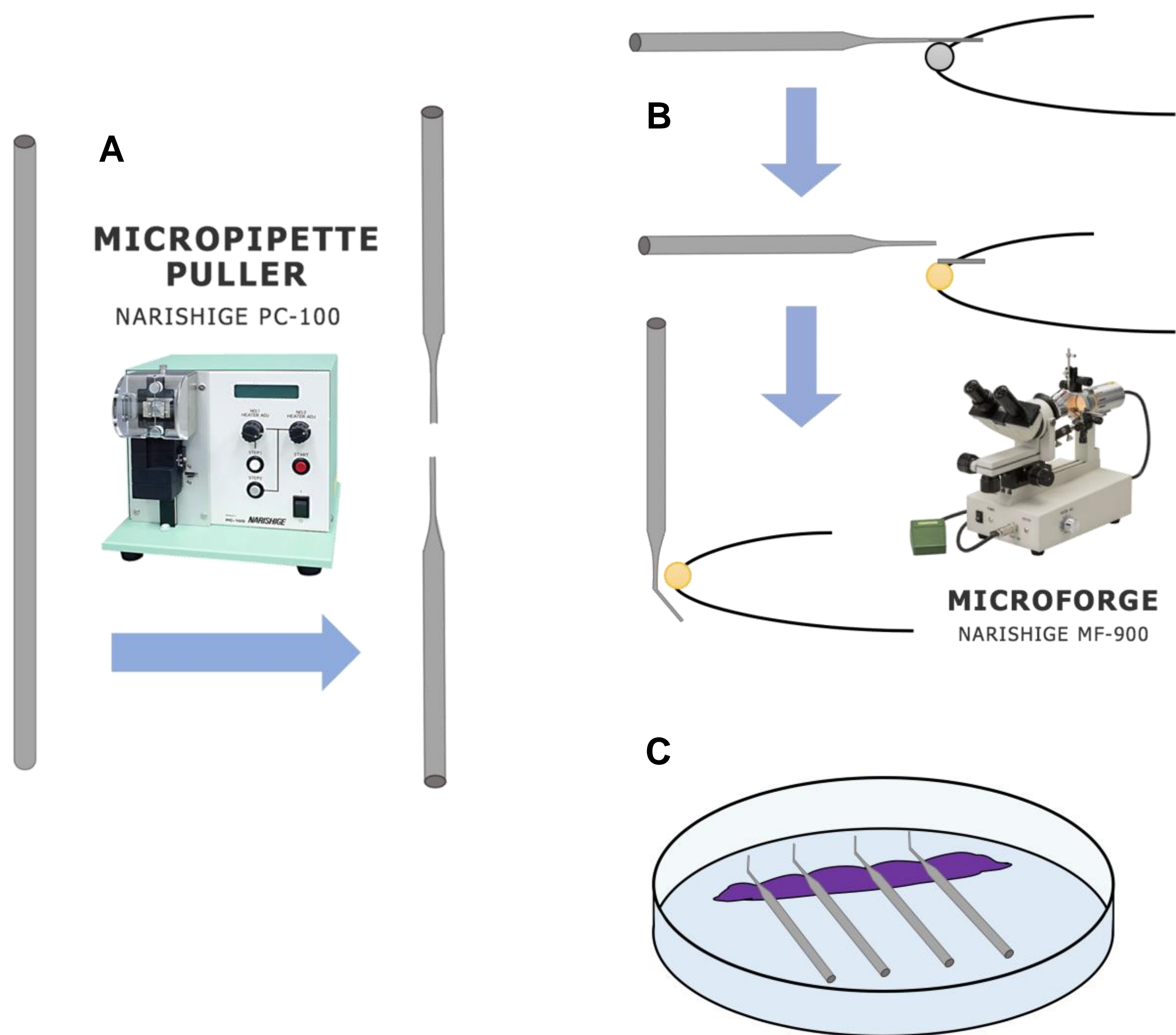


Figure 2: Preparing the micropipette needles. (**A**) A Micropipette puller is used to pull the needles to a taper. (**B**) A Microforge is used to cut and bend the tip of the micropipette needles. (**C**) Prepared micropipette needles are stored in clay to prevent breakage of their fragile tips.

2.5 Experimental Protocol

Overview

A micropipette is brought into contact with a cell and a known negative pressure is then applied within the pipette, causing a seal to form and the cell to be drawn into the micropipette. The applied pressure is the independent variable manipulated in this experiment. The response of the cell to this pressure, the extension of its surface into the micropipette, is the dependent variable we measure.

Finding the Cell

In order to begin the experiment, the micromanipulation system is manually operated with a joystick to move the micropipette needle in the x- y- and z-directions and position its tip on a cell of interest.

1. Secure the micropipette in the needle holder.
    - **i.** Adjust the angle of the holder to ensure the tip is positioned parallel to the bottom of the plate.
2. Locate the micropipette tip in the microscope field.
    - i. By eye, approximately center the tip over the objective lens.
    - ii. Locate the cells and set the focal plane of the microscope to a height of at least 1000 microns above this level, to minimize the potential for breaking the pipette tip on the bottom of the plate.
    - iii. While viewing the field through a 20x objective, lower the pipette until it enters the cell media, beginning at a course setting and switching to finer

movement as the tip approaches the media. Adjust the x- and y- positions of the pipette until the tip is located in the field of view – using a lower magnification objective makes locating the tip much easier. At this point, the pipette will appear as a shadowy outline above the focal plane of the microscope.

iv. Upon location of the tip, lower the pipette until it is focused in the field of view, center it in the frame and switch to a 40x objective, which will be used for the experiment.

3. Locate a cell of interest

    i. Return the microscope focus to the level of the cells, keeping the micropipette at its current position.

    ii. Move the microscope stage until a desirable cell is found, this can be done on cells in suspension or sufficiently round substrate-attached cells.

    iii. Confirm that the pressure control and imaging parameters are set-up and ready to operate.

4. Position the pipette tip on the surface of the cell

    i. Lower the pipette tip to the focal plane of the cell.

    ii. Move pipette in the x- and y- directions until its tip contacts the cell surface.

    iii. Upon attachment, immediately start pressure protocol and begin imaging.

    iv. For our experiments, an image was captured every 2 seconds in order to obtain sufficient temporal resolution for our calculations.

Aspirating the Cell

A method commonly used to control pressure in micropipette aspiration systems is through adjusting the height of a water reservoir[14,18]. However, this method suffers from problems of evaporation and the need to re-calibrate the system prior to each experiment. Instead, our system uses a microfluidic pump system to provide the pressure for the experiment with an associated computer program that allows the pressure applied to the cell to be varied.

Our protocol further simplifies the aspiration process through the use of automated pressure control; by creating a protocol to steadily increase pressure, we avoid the variability of manual pressure control of this system and are able to incorporate a smoother pressure increase. Fluigent's Microfluidic Automation Tool software enabled us to create this automated pressure control program. For our experiments, we started at a pressure of zero and increased the pressure at a rate of 0.01 mbar per second – these parameters can easily be modified to the fit the requirements of a particular experiment.

After the micropipette is positioned on the cell of interest and the program is started, the rest of the experiment proceeds automatically. To complete the experiment and release the cell from the micropipette needle, the imaging program and pressure protocol are stopped when the cell has been sufficiently aspirated.

2.6 Calculating Cortex Tension

Liquid Drop Model

The liquid-drop model is a simple cell model that can be used to analyze the cell's behavior in a micropipette aspiration experiment[11,19]. Using this model, we consider the inside of the cell to be a homogenous viscous liquid and the cell cortex to be a thin layer under constant surface tension. Since the outer membrane is very thin compared to the radius of the cell, we consider it to be infinitely thin for the sake of this model. Despite the complex and heterogenous composition of the cytoplasm within an actual cell, the liquid drop model provides reliable predictions of a cell's mechanical response during micropipette aspiration.

Law of Laplace

By modeling the cell as a liquid drop, we can use the Law of Laplace to relate the difference in internal and external pressure of this spherical liquid drop with the surface tension in its thin outer layer[11,19]. The Law of Laplace can be derived from a free body diagram of the cell at the point of aspiration when the protrusion length is equal to the pipette radius.

Back half of the cell (Fig. 4A):

$\mathbf{\Sigma}FX = PC\ (\pi * RC^2) - TC\ (2\pi * RC) = 0$

Simplifying our force-balance equation brings us to the Law of Laplace. However, we still have two unknown variables.

$$P_C = 2 * \frac{T_C}{R_C}$$

Aspirated portion of the cell (Fig. 4B):

$$\Sigma FX = 0 = PC\ (\pi * RP^2) + PP\ (\pi * RP^2) - TC\ (2\pi * RP)$$

Substituting the Law of Laplace in for Pc, we obtain the equation:

$$P_P = 2T_C * \left(\frac{1}{R_P} - \frac{1}{R_C}\right)$$

We can calculate cortical tension by taking measurements from our data of the relevant parameters needed for the Law of Laplace: cell radius (R_C), pipette radius (R_P), and length of the cell tail aspirated into the pipette (Lp). The aspiration length of the cell tail into the pipette can be determined with respect to time and applied pressure (Pp) by manual tracking of an edge with a cursor on the video screen, producing the graph shown in Figure 4A.

Cell Measurements

The Law of Laplace requires a spherical shape, so the protrusion length is often scaled with respect to the radius of the pipette. At the point when these lengths are equal, Lp/Rp=1, the aspirated region is a hemisphere, and this law can therefore be applied (Fig. 4B). The suction pressure at which this occurs is called the critical pressure. The values of Pp, Rp, and Rc are now known and can be used in the law of Laplace to calculate the cell's cortical tension.

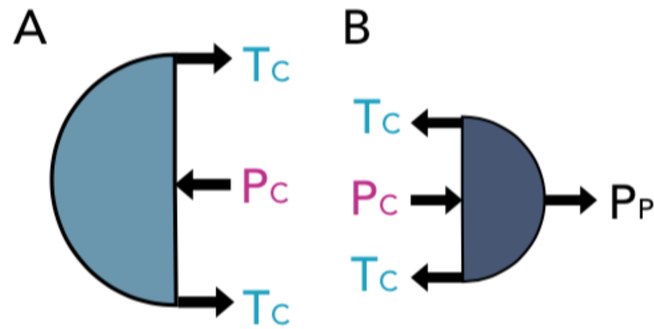


Figure 3: Free body diagrams of the back (**A**) and aspirated portion (**B**) of a cell undergoing micropipette aspiration. The relevant forces on the cell are shown; internal pressure (Pc, units of force/area) and surface (Tc, units of force/length).

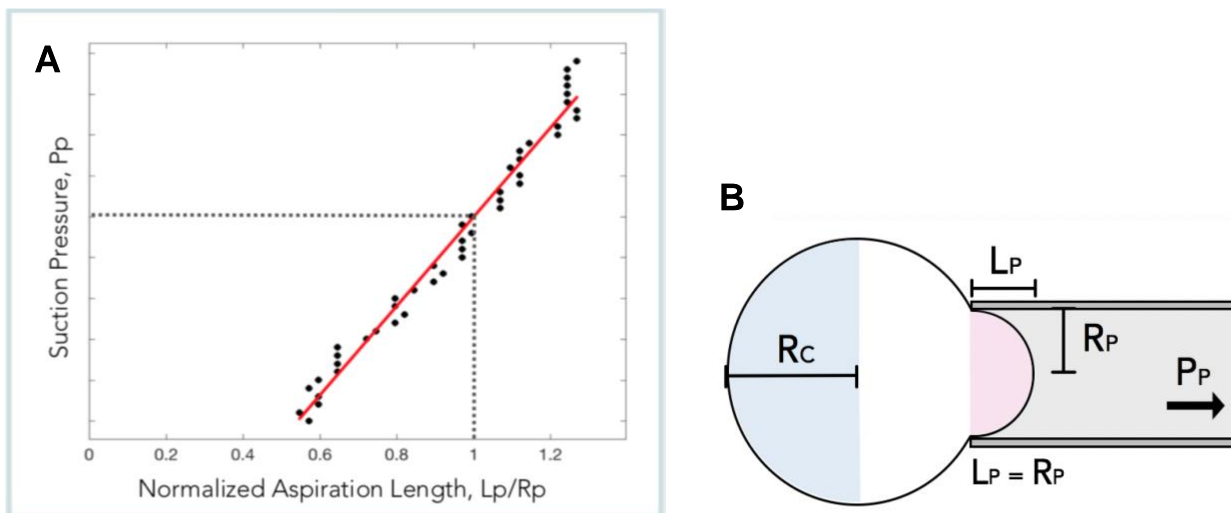


Figure 4: (**A**) Representative plot of normalized aspiration length versus suction pressure, which is used to determine the critical pressure at which Lp/Rp = 1. (**B**) A cell's cortex tension (TC) is calculated from the Law of Laplace by using the applied pressure (PP) and taking measurements of relevant parameters; cell radius (RC), pipette radius (RP), and length of the cell tail aspirated into the pipette (LP).

CHAPTER 3

Micropipette Aspiration of HeLa Cells

3.1 Overview

In the study described in this chapter, Precise Tuning of Cortical Contractility Regulates Cell Shape during Cytokinesis[7], we define the roles of two non-muscle myosin II (NMII) paralogs, NMIIA and NMIIB, in fine-tuning of polar cortex contractility to allow cells to maintain shape during cytokinesis. Our results reveal that NMIIA generates cortex tension, while NMIIB acts as a stabilizing motor, and its inclusion in NMII hetero-filaments reduces cortex tension. Tension generation by NMIIA drives faster cleavage furrow ingression and bleb formation. We show that modulation of myosin II filament composition allows tuning of surface tension at the cortex to maintain cell shape during cytokinesis. These findings also uncover a general mechanism that may allow cells to attain a broad range of contractile states in order to perform various cellular functions requiring cortex contractility. These experiments provided specific biophysical measurements at a cellular level, which allowed us to deepen our understanding of the role of NMII in cellular function.

3.2 Introduction

The mechanical properties of the actin cortex, specifically cortical tension, regulate many vital biological processes. The actin cortex is a thin network of actin filaments that allows cells to change shape and generate tension. On the molecular scale, cortical tension can be modulated by changes in the composition, spatial organization, and dynamics of the cortical actin network. The main molecular motor that is responsible for controlling changes in cortex tension is non-muscle myosin II (NMII), which generates stress by pulling on actin filaments in the cortex[5,18]. Cellular contractile forces generated by NMII drive processes such as cell division, cell migration and heart muscle contraction. For instance, during the cytokinetic phase of cell division, accumulation of NMII at the cleavage furrow results in an increase in cortex tension, that drives the deformation of the membrane and an increase in intracellular pressure. This creates a gradient of cortex tension, with maximal tension at the equator and lower tension at the polar region of the cell. Deregulation of this gradient results in shape instabilities and cytokinetic failure.

Non-muscle myosin II functions in virtually all mammalian cells in various cell- and tissue-specific combinations of three paralogs: NMIIA, NMIIB and NMIIBC, which have both unique and shared roles in force generation within cellular contexts[8,20-22]. Localization of different NMII paralogs in the cortex is nonhomogeneous, which results in the local increases or decreases in cortical tension that drive biological processes[22-23]. Consequently, experimentally modifying NMII localization in the cortex enables us to predict changes in the cell's tension[7].

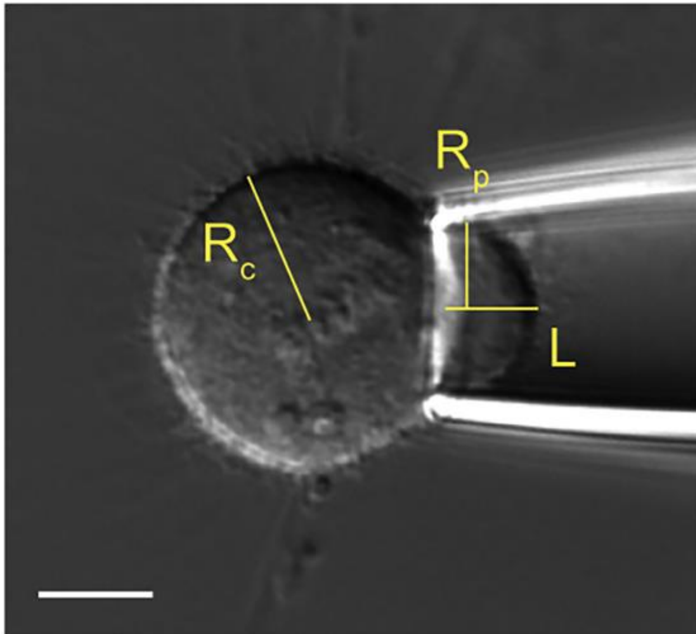
One of the focuses of the Burnette lab is investigating the role of non-muscle myosin II (NMII) in cellular function, including how myosin II-based contractile systems assemble within cells. It has been long established that contraction of non-muscle myosin II (NMII) filaments generates the contractile forces driving cortex tension[6], however, it was previously not known which of the three NMII paralogs were responsible for generating these forces. To investigate this question, I depleted different NMII paralogs in HeLa cells and used micropipette aspiration to measure the cortex tension of these cells. These micropipette aspiration experiments were completed as part of a larger study in which the roles of NMIIA and NMIIB were defined in fine-tuning the surface tension of the polar cortex to allow cells to maintain shape during cytokinesis.

3.3 Experimental

We investigated how changes in NMII filament composition altered cortex tension and stiffness. Specifically, we knocked down NMIIA and NMIIB to determine their individual effects on cortical tension as measured by micropipette aspiration. We hypothesized that, based on its biophysical properties, NMIIA knockdown in metaphase cells should result in lower cortical tension as compared to control and NMIIB knockdown cells. We chose to perform these measurements during metaphase, since we found compensation in localization during metaphase; the metaphase cortex is uniform and devoid of the fluctuations in NMII localization that occur at the polar cortex, and polar cortex blebbing would interfere in cortex tension measurements.

Micropipette aspiration was performed according to the protocol described in chapter 2. For each cell, the pressure was initially set to 0 mbar and was increased by -0.05 mbar per second up to -25 mbar, or until the cell was sufficiently aspirated. Live microscopy videos were captured of each cell as its membrane was aspirated into the pipette, and a MATLAB program was used to determine cortical tension based on measurements from these videos. The parameters used to calculate tension values were the cell radius (R_c), pipette radius (R_p), and length of the cell tail aspirated into the pipette (L). The cortical tension of different cells could then be compared.

Representative DIC



$$T = \frac{\Delta P}{2\left(\frac{1}{R_p} - \frac{1}{R_c}\right)}$$

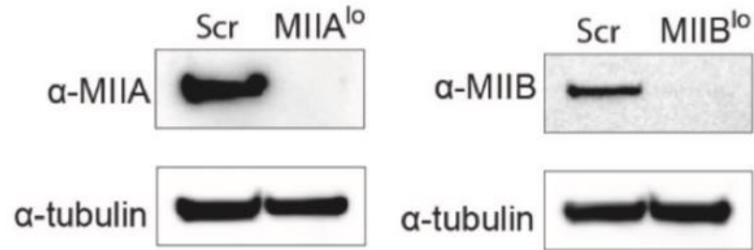
T= tension
ΔP= pressure change
R_p= radius of pipette
R_c= radius of cell
L= aspiration length

Figure 5: Representative micropipette aspiration experiment on a HeLa cell, showing the relevant parameters used to calculate cortical tension.

3.4 Results and Discussion

Depletion of myosin-II paralogs

Knockdown of NMIIA and NMIIB was done using single siRNA. Validation of knockdown of myosin-II paralogs was done to confirm that the proteins of interest, NMIIA and NMIIB, were depleted in knock-down cells by western blot and immunofluorescence. NMIIA and NMIIB expression was be determined by western blot upon depletion of the other paralog, depicted in Fig. 6A[7]. Knockdown efficiency should be 70% or greater when quantified from western blot; the western blot validated knockout, showing decreased expression of the depleted paralog. NMIIA- and NMIIB-knockdown cells were stained for F-actin, DAPI and the depleted paralog, NMIIA or NMIIB, and immunofluorescence imaging was used to confirm knockdown of the desired protein (Fig. 6B). Immunofluorescence with anti-MIIA and anti-MIIB antibodies confirmed depletion of the corresponding protein following siRNA knockout. Successful knockdown was visible upon imaging as a significant reduction in fluorescence of the knocked-down paralog.



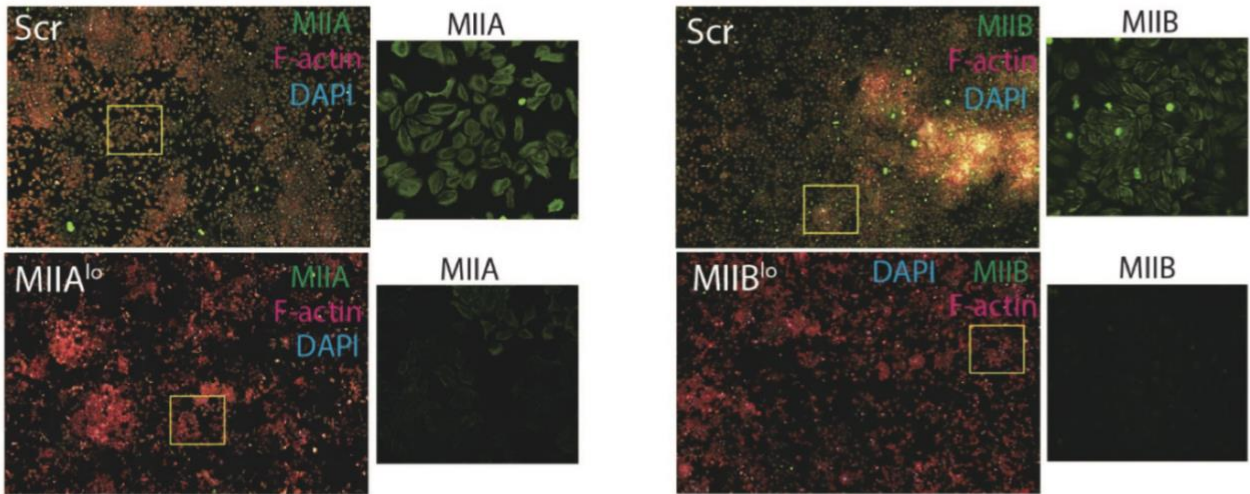


Figure 6*:* (**A**) Verification of NMIIA and NMIIB knockdown using single siRNA by Western Blot[7]. (**B**) Validation of knockdown using immunofluorescence[7].

Inhibition of Myosin II decreases tension

Cortical tension is generated by actomyosin contractility[9], and disrupting myosin activity using blebbistatin (a myosin-II inhibitor) is known to reduce the cortical tension of the cells, and thus was used to validate the suitability of this approach. HeLa cells treated with 50 uM blebbistatin (BB) were seen to have significantly lower cortex tension than control cells, when measured using micropipette aspiration (Fig. 7). This provided confirmation that the experimental approach was valid and showed that inhibition of myosin-II results in a decrease in cortical tension.

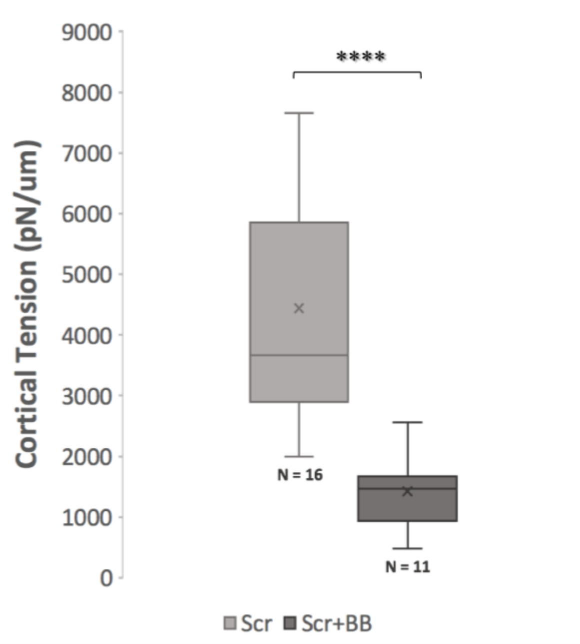


Figure 7: Comparison of cortical tension between control HeLa cells and those treated with blebbistatin.

MIIA knockdown results in decreased cortex tension

We depleted either NMIIA or NMIIB in HeLa cells, which express only these two paralogs. We measured cortex tension in HeLa cells using micropipette aspiration and found a significant reduction in cortex tension in NMIIA-knockdown as compared to control (Scr) cells (Fig. 8, $p < 0.0001$). NMIIB-knockdown cells had higher cortex tension compared to control (Scr) cells (Fig. 8, $p = 0.0082$). The phenotypes that occurred upon depletion of one the NMII paralogs resulted from increases in localization of the other paralog, resulting in a change in the relative concentrations of the two paralogs at the cortex. Depletion of NMIIA resulted in reduced tension generation and furrowing. Depletion of NMIIB resulted in destabilization of cell shape.

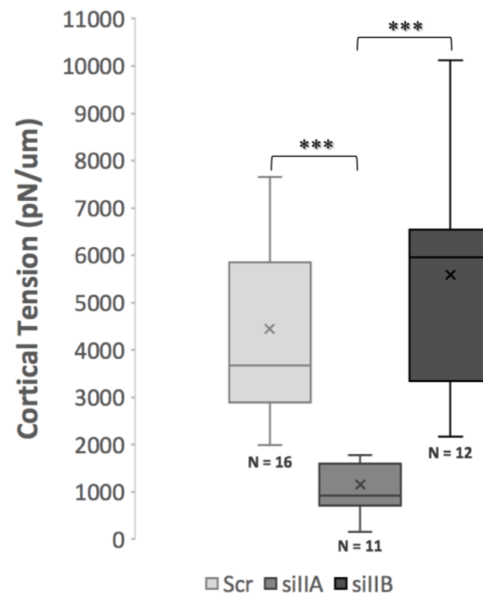


Figure 8: Comparison of cortical tension between control HeLa cells, NMIIA knockdown cells, and NMIIB knockdown cells.

MIIA is sufficient to generate cortex tension

To confirm that NMIIA is the specific paralog of NMII that is necessary and sufficient to generate cortex tension, we rescued NMIIA knock-down cells with NMIIA and measured their cortical tension. When NMIIA knockdown cells were rescued with NMIIA, cortical tension was restored to the level of the control.

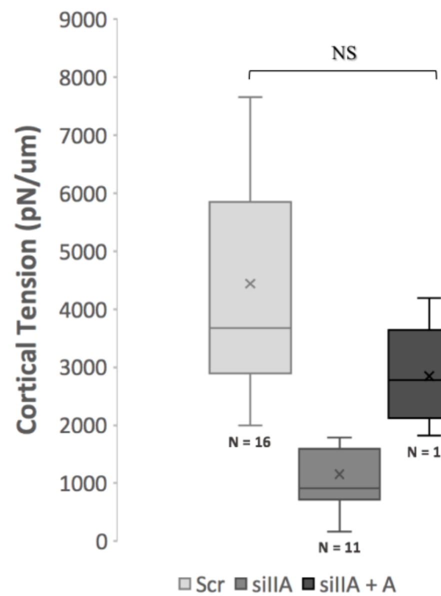


Figure 9: Comparison of cortical tension between control HeLa cells, NMIIA knockdown cells, and knockdown cells rescued with NMIIA.

3.5 Conclusion

As a result of this series of experiments, we were able to identify NMIIA as the sole generator of cortex tension[7,24]. Our data further suggested that cortical contractility is determined by the local concentrations of NMII, with the differences in NMIIA and NMIIB allowing for a fine-tuning mechanism of cortex tension. We found that knockdown of NMIIA resulted in significantly reduced tension as compared to both control and NMIIB-knockdown cells, which was consistent with our hypothesis. Taken together, our results show that NMIIA depletion results in lower cortex tension, while NMIIB depletion results in higher cortex tension. We found the increase in cortex tension and blebbing upon NMIIB depletion was driven by an increase in NMIIA localization at the cortex. A similar compensatory localization by NMIIB was observed upon NMIIA depletion. This data reveals a mechanism to tune cortex tension by altering the relative levels of NMIIA and NMIIB at the cortex. Furthermore, particular phenotypes were observed upon depletion of each of these the NMII paralogs; depletion of NMIIA resulted in reduced tension generation and furrowing and depletion of NMIIB resulted in destabilization of cell shape. As a result of this series of experiments, we showed that NMIIA is the specific paralog of non-muscle myosin II that is necessary and sufficient to generate cortex tension.

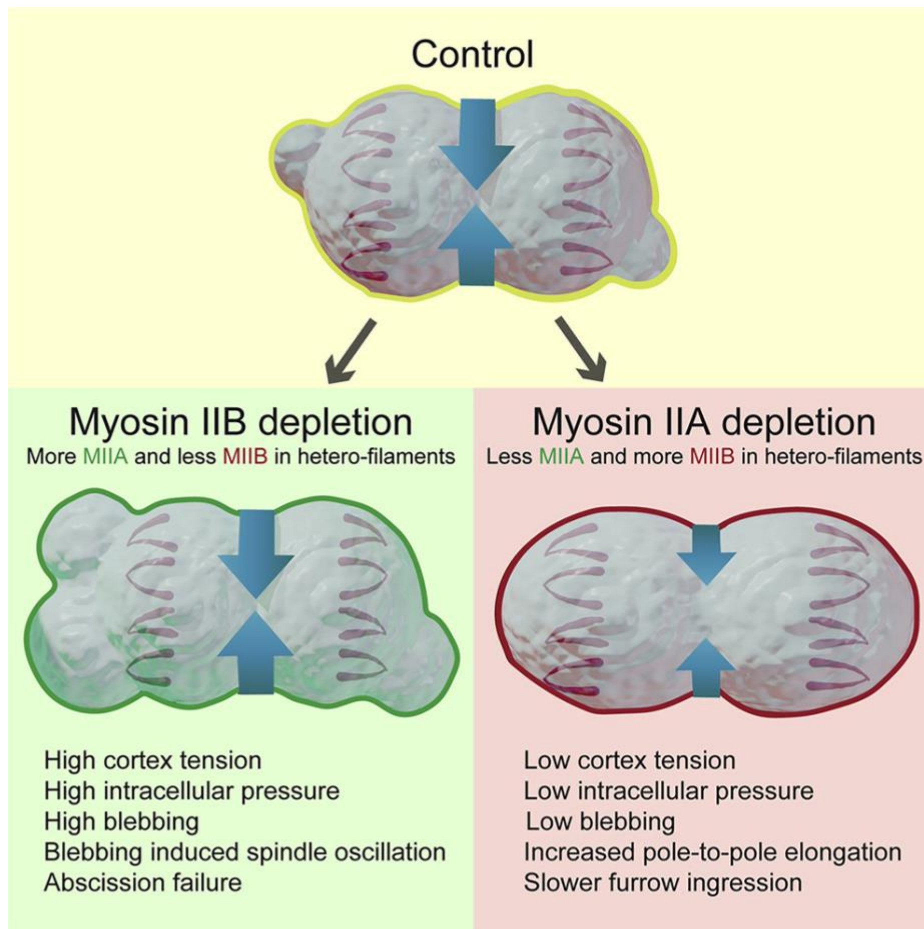


Figure 10: Overview of the results of the experiments described in this chapter.

CHAPTER 4

MICROPIPETTE ASPIRATION OF CARDIOMYOCYTES

4.1 Introduction

Cardiac function is dependent on contraction, and our long-term goal in the Burnette lab is to understand the mechanisms underlying contractile systems. In doing so, we are working toward a greater understanding of how contractile systems, such as the heart, can be reassembled when diseased or otherwise damaged. Micropipette aspiration allows for the measurement of cortex tension on a single cell level, and mechanical effects at the cellular level have a significant impact on the integrated function of tissues and organs such as the heart[6,9]. Therefore, we used the micropipette aspiration method to measure the cortical tension in human induced pluripotent stem cell-derived cardiomyocytes, in order to explore cardiovascular disease on a single cell level.

Micropipette aspiration systems have been used on various cell types to study a wide range of topics such blood cell mechanics, nuclear dynamics, and bleb growth[14-18]. This project established, for the first time, the use of micropipette aspiration to study the physical properties of cardiomyocytes. The micropipette aspiration method allows the experimenter to *directly* measure values of cortex tension, thereby furthering scientific understanding of the signals that regulate these values in cardiomyocytes.

These experiments were done in order to further scientific knowledge on cardiomyocyte mechanodynamics, and thus open the door to further experiments to investigate the

relationship between a cardiomyocyte's mechanical properties and its function. This is important because an improved understanding of the effects of drugs and genetics on cardiomyocyte function will allow for identification of potential targets for therapeutic intervention. Such further experiments would provide insight into potential treatment methods to reassemble damaged contractile systems such as the heart under pathophysiological conditions.

Objective

The overall goal of this project was to better understand fundamental mechanisms of cardiomyocyte cells. The described experiments represent a novel method to characterize cardiomyocyte mechanical properties during the contraction and relaxation of the beating cycle. Achieving this aim will provide knowledge on cardiomyocyte contraction that is currently an unknown and will open the doors to future experiments using this method to investigate cortical tension in the beating cycle under various conditions.

4.2 Experimental

Overview

The objective of these experiments was to measure cortex tension in the beating cycle of cardiomyocytes. This was done by first developing a protocol for rounding cardiomyocytes, to ensure that these cells could physically be aspirated by the micropipette aspiration system. This rounding protocol was then implemented in order to perform micropipette aspiration experiments on beating myocytes. These experiments allowed us to investigate changes in cortex tension in the contraction and relaxation phases of the beating cycle. We hypothesized that cortical tension would increase during contraction and decrease during relaxation of the beating cycle.

Rounding Protocol

The rounding protocol developed in this study addresses the primary barrier to micropipette aspiration of cardiomyocytes, which is that under normal conditions, these cells are extremely flat once they have been plated, which means that they are unable to be aspirated because a micropipette needle cannot physically be attached to the side of the cell (Fig. 11). This problem can be overcome by obtaining a shape for cardiomyocytes in which they can be successfully aspirated. This can be done through partial trypsinization of the cells, causing them to round up, but still remain attached to the bottom of the plate. In order to create an effective partial-trypsinization rounding protocol, trypsinization and post-trypsinization times needed to be optimized to ensure that the cardiomyocytes could physically be aspirated and that they also continued to beat. A successful protocol would meet the criteria that (1) the cells were no longer flattened to

the bottom of the plate and had instead assumed a ‘rounded-up’ state that would allow them to be aspirated, and (2) the cells were visibly beating.

Development of an optimized partial-trypsinization rounding protocol involved applying the same amount of three different concentrations of trypsin (0.025%, 0.05%, and 0.1%) to cells for varied amounts of time to determine the optimal outcome, which is the highest percent of round, beating cells. For each concentration of trypsin, DIC images were acquired as the cells rounded up to observe how long it took the cells to exhibit a rounded phenotype, in order to determine at what point media should be added. This was repeated three times for each concentration of trypsin. After the rounding time was determined, media was added, and cell flattening was observed using DIC image acquisition. This was also repeated three times for each concentration of trypsin. The protocol with the most successful outcome, in terms of percent of rounded cells that are beating and able to be aspirated at the end of the protocol was selected for use in experiments. This was found to be 0.05% trypsin applied to cells for 7 minutes, followed by DMEM addition and 45 minutes of incubation before performing micropipette aspiration.

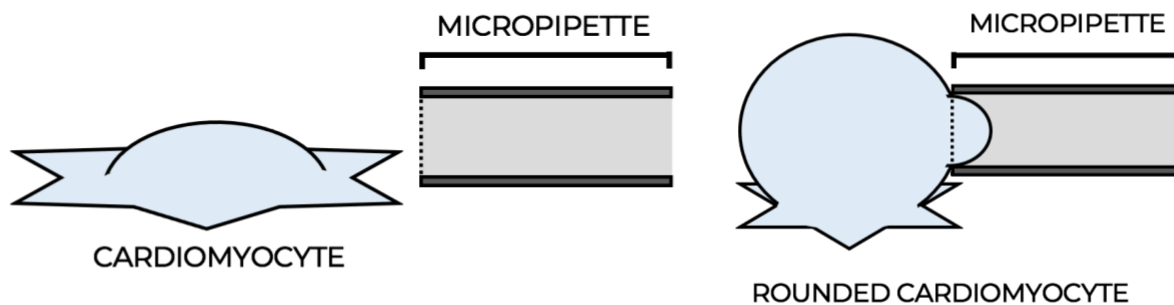


Figure 11: Representation of cardiomyocyte shape before (left) and after (right) rounding protocol is applied to the cells.

Alternative Design

The primary barrier to applying the micropipette aspiration method to cardiomyocytes was that the cells were too flat on the bottom of the plate, making it impossible to attach a pipette to the side. This problem can be overcome by obtaining a shape for cardiomyocytes in which they can be successfully aspirated. This was done through partial trypsinization of the cells, causing them to round up, but still remain attached to the bottom of the plate. The partial trypsinization protocol has been optimized to ensure that cells are still round from trypsinization but have stopped blebbing and begun beating again. An alternative approach to solve this problem that could be used as a backup plan is to plate the cardiomyocytes on a soft Matrigel substrate. Matrigel is a collagen and laminin matrix that mimics extracellular matrix in body. While this offers the benefit of providing a more physiologically accurate environment for the cardiomyocytes, it is more difficult to obtain data because the gel provides a barrier to pipette access during micropipette aspiration.

Micropipette Aspiration

Micropipette aspiration (MPA) is a method that is used to directly measure a cell's cortex tension. Therefore, this method enabled us to measure cortex tension during the contraction and relaxation phases of the beating cycle, and thus understand how a cardiomyocyte's cortex tension changes throughout this cycle. Preliminary work in HeLa cells has shown this method to be appropriate in evaluating cortex tension. Transitioning to cardiomyocytes comes as a logical next step to expand upon existing scientific knowledge by applying the micropipette aspiration method to another cell line.

The rounding protocol was applied to cardiomyocytes to prepare them for aspiration. Micropipette aspiration was then performed on the cells and the resulting measurements were used to determine the relationship between cortex tension and the contraction and relaxation of the beating cycle of cardiomyocytes. Indicators of success of this experiment were that (1) the aspirating pipette remains attached to the cell throughout the beating cycle, (2) there is visible movement of myocyte beating observed throughout the aspiration, and (3) that acquired images can be analyzed with a MATLAB program.

Micropipette aspiration works according to the basic idea that in order to measure the mechanical properties of a cell, the cell must be deformed in some way by a known force and its deformation must be measured. In the specific context of micropipette aspiration, a known suction pressure is applied to the cell, causing a deformation of the cell surface, which can be measured by the length of the extension of the surface of the cell into the pipette. The use of this technique on cardiomyocytes allowed us to determine and compare cortex tension during contraction and relaxation.

4.3 Results and discussion

Cortex tension is greater during contraction

The beating cycle of a cardiomyocyte consists of a relaxed and a contracted phase, which are visibly distinguishable as the cell is aspirated into the pipette. As cells were aspirated, high-temporal imaging captured the movement of the cell tail into the pipette. Frames in the contraction and relaxation phases of the beating cycle were isolated by selecting for local maxima and minima of the cell tail aspiration length. These frames were separated and run through a MATLAB program for the calculation of cortex tension in each phase, to produce unbiased interpretation of the results. A paired t-test was used to compare the cortical tension levels of cardiomyocytes in the contraction and relaxation phases of their beating cycles and revealed cortical tension to be greater during the contraction phase of the beating cycle (Fig. 12).

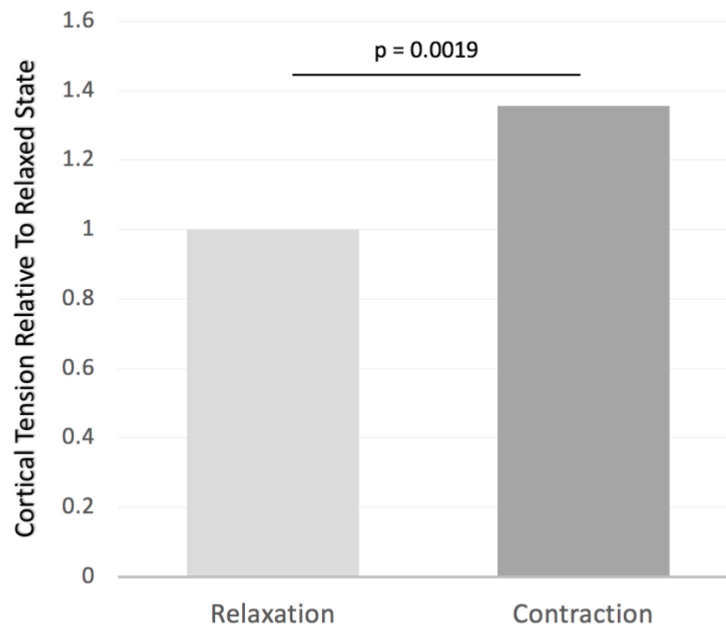


Figure 12: Relative cortical tension during contraction and relaxation phases of the cardiomyocyte beating cycle, shown relative to the cortical tension in the relaxed state.

Inhibition of Myosin II decreases tension

Blebbistatin treatment inhibits myosin-II activity and is known to reduce cortical tension, as discussed for HeLa cells in chapter 3. Cardiomyocytes were treated with 50 uM blebbistatin to validate the suitability of this approach, and blebbistatin-treated cells were revealed to have significantly lower cortex tension than control cells, when measured using micropipette aspiration (Fig. 13). This provided confirmation that the experimental approach was valid and showed that inhibition of myosin-II in results in a decrease in cortical tension in cardiomyocytes.

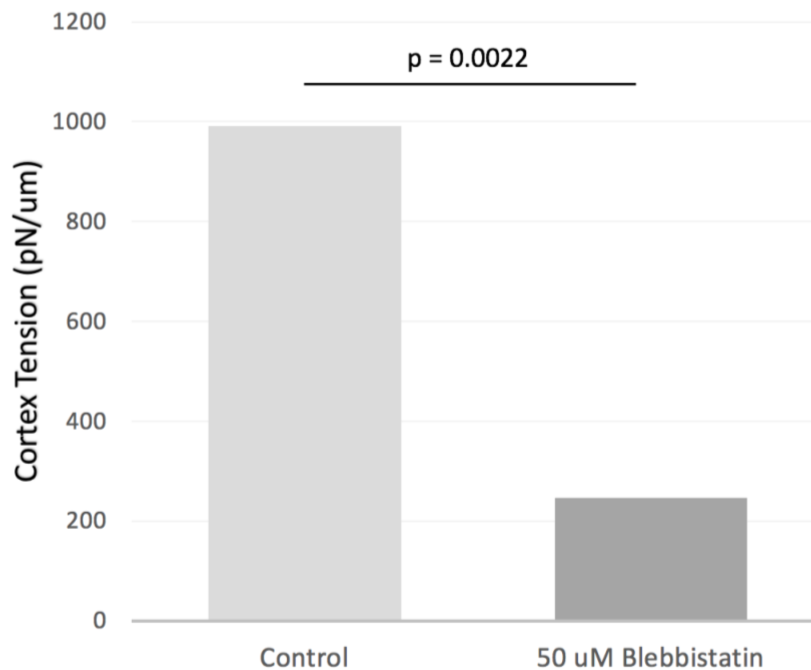


Figure 13: Cortical tension in control and blebbistatin-treated cardiomyocytes.

4.4 Conclusion

In these studies, micropipette aspiration was used to measure cortex tension, a direct contributor to force generation within cells. Thus, these experiments are working towards an increased understanding of heart function and the dysregulation of force generation in heart disease. Success of this project opens the doors to further studies of the biomechanical properties of cardiomyocytes in response to different perturbations. These could include drug treatments, knock-down of particular proteins, or other genetic changes to observe their effects on cardiomyocyte contractility. The experiments described in this chapter have allowed us to learn more about the physiological changes that occur in the membranes of cardiomyocytes as they beat. Going forward, studies using the experimental setup we describe here will not only continue to clarify previous models but also reveal new insights into cardiac cell biology. Furthermore, this will have a positive impact on human health by providing a greater mechanistic understanding of pathologic processes in the heart and subsequently helping to identify targets for intervention.

CHAPTER 5

FUTURE DIRECTIONS

5.1 Introduction

Non-muscle myosin II (MII) is the motor protein that generates the forces that drive cortical tension and is therefore the major driver of key cellular processes[5-6]. Understanding how NMII contributes to force generation inside cells is central to our understanding of force-dependent progression of diseases such as heart disease, which makes NMII a fundamental target for experimental investigation[3]. Although mutations in NMII isoforms have been implicated in human cardiomyopathy, few studies have specifically investigated cortical changes that accompany such mutations. Specifically, myosin II isoforms, NMIIA and NMIIB are required for proper heart development in mice, and mutation of NMII has been shown to underlie human cardiomyopathy[25-28]. This therefore represents a gap in scientific knowledge that we seek to fill by investigating the link between cortical regulation and heart disease.

The role of myosin II in modulating the mechanical property of cortex tension has previously been investigated in HeLa cells using micropipette aspiration[7]. These experiments in HeLa cells, described in chapter 3, have shown this method to be appropriate in evaluating the role of NMII paralogs in modulating cortex tension. Transitioning to cardiomyocytes comes as a logical next step to expand upon our previous experiments.

Research by the Burnette lab demonstrated distinct roles for myosin-IIA and myosin-IIB during cell division[7]. These studies revealed NMIIA as the major driver of cortex tension. Given NMIIA's role in driving cortex tension and its implication in cardiomyopathy, investigating cortex tension in cardiomyocytes would bridge an evident gap in scientific knowledge and provide a foundation for further studies. Our hypothesis is that NMIIA plays a role in driving cortex tension in cardiomyocytes. The objective of these experiments is to learn more about the physical changes that occur in the membranes of cardiac myocytes as they beat.

5.2 Objective

The objective of this future experiment would be to determine the role of Myosin-II paralogs NMIIA and NMIIB in modulating cortex tension of cardiomyocytes. We propose to use the methods described in chapter 4 on cardiomyocytes that have undergone genetic perturbation. We will deplete NMIIA and NMIIB from cardiomyocytes using siRNA and validate this knockdown by western blot and immunofluorescence, as previously described for HeLa cells in chapter 3. We will then treat cardiomyocytes with blebbistatin, a myosin-II inhibitor, as an additional control to validate the experimental approach. We will then use micropipette aspiration on NMIIA- and NMIIB-knockdown cells to determine cortex tension under each condition. Based on its biophysical properties, we hypothesize that NMIIA knockdown in cardiomyocytes will result in lower cortical tension as compared to control and NMIIB knockdown cells. Achieving this aim will provide insight into how the NMII proteins implicated in cardiomyopathy affects cardiomyocyte cortical mechanics.

5.3 Anticipated Results

Anticipated results of these experiments are that cortical tension is decreased upon NMIIA-knockdown, and increased upon NMIIB-knockdown, which would correspond with the results observed in HeLa cells. Given this novel method for single cell measurements of cardiomyocytes, there are many possible alternative experiments that can be done with the micropipette aspiration set-up. These include other genetic perturbations by knockdown or overexpression, or pharmacological perturbations by treatment with drugs or small molecules.

5.4 Broader Significance

These experiments will develop fundamental understanding on how NMII contributes to force generation inside of cardiomyocytes, which will advance our understanding of the relationship between a cardiomyocyte's physical properties and how it functions in the heart. Successful completion of the proposed studies investigating the myosin II-based contractile system of the heart at the single cell level has significant promise to gain a mechanistic understanding of pathologic processes and subsequently identify targets for intervention, providing a health benefit to the public at large. Additionally, these experiments will further scientific knowledge on cardiomyocyte mechanodynamics, and thus open the door to further experiments to investigate the relationship between a cardiomyocyte's mechanical properties and its function. A better understanding of force generation in cardiomyocytes will provide insight into what happens to heart tissue following heart attacks or other diseases in this tissue, which would provide insight into potential treatment methods to reassemble damaged contractile systems such as the heart under such conditions.

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