Bidirectional Interactions Between Mitochondrial Function and Cell Mechanics

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Abstract
Energetic and structural maintenance are both critical to cellular homeostasis, and clinical disease is often characterized by alterations in both of these realms. While the manifestation of pathology in each of these fields has been extensively studied, little research has been done to characterize basic, direct interactions between mitochondrial function and cell mechanics. The experiments described in this dissertation endeavored to address that gap, first by investigating the cytoskeletal and mechanical effects of mitochondrial dysfunction and then by considering the mitochondrial consequences of cytoarchitectural breakdown.

Mechanical integrity of the cell following mitochondrial dysfunction was investigated through multiple experimental platforms, including the quartz crystal microbalance with dissipation (QCM-D). Early work thus focused on improving the suitability of QCM-D for cell experimentation by developing a method of covalently conjugating fibronectin to QCM-D sensors. We then subjected cells to mitochondrial toxins in order to address whether and how mitochondrial dysfunction affects cell mechanics and the cytoskeleton. Cells showed characteristic rounding after long-term exposure to rotenone, an inhibitor of complex I of the mitochondrial respiratory chain. Since mitochondrial dysfunction can also be caused by genetic defects in the mitochondrial DNA (mtDNA), we also studied the cytoskeletal and mechanical variations in cells heteroplasmic for the m.3243A>G mutation. We found a conserved, nonmonotonic relationship between m.3243A>G heteroplasmy and cell mechanics, originating in expression of actin-related genes and persisting at the levels of protein production, cytoskeletal structure, and single cell stiffness.

The second half of the dissertation considered how cytoarchitectural breakdown influences mitochondria. We first developed a novel tool for tracking individual mitochondria throughout entire cells, and then used this method to demonstrate that microtubule and microfilament depolymerization affect mitochondrial motility in opposing ways. Another set of experiments found that cytoskeletal breakdown significantly decreased mitochondrial respiration, which sometimes only occurred when mitochondria were pre-stressed by increased demands of calcium maintenance.

Together, these studies highlight direct, bidirectional interactions between mitochondrial function and cell mechanics. These findings will inform future mechanistic studies focused on a comprehensive understanding of human disease at the cellular level, which will hopefully contributing to advancing development of therapeutics.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Bioengineering

First Advisor
David M. Eckmann

Keywords
actin, atomic force microscopy, cell mechanics, mitochondria, mitochondrial motility, mitochondrial
respiration

Subject Categories
Biomedical | Cell Biology
BIDIRECTIONAL INTERACTIONS BETWEEN MITOCHONDRIAL FUNCTION AND CELL MECHANICS

Judith Kandel

A DISSERTATION

in

Bioengineering

Presented to the Faculties of the University of Pennsylvania

In Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2016

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ACKNOWLEDGMENTS

This work would not have been possible without the support of many people. First and foremost, thank you to my advisor, Dr. David M. Eckmann, for your research mentorship over the last 6 years. You have invested a tremendous amount of time, money, and energy in my development as a scientist, and I will forever be indebted to you for that. I would also like to thank my dissertation committee members, Drs. Paul Janmey, Marni Falk and Rob Mauck for their support and mentorship.

There have been many other lab members, both from the Eckmann lab and beyond, who were instrumental in the success of this research. Drs. Peter Sobolewski and Alexandra Klinger were so giving of their time and knowledge in training me when I first came to the lab. Peter in particular gave me my very first exposures to live imaging of mitochondria and trained me in cell culture techniques. Dr. Hyun-Su Lee has also been dedicated to my success and has been particularly helpful with QCM-D research and any other projects involving chemical synthesis. Dr. M. Carme Coll Ferrer was also a wonderful friend and research mentor during her time in the lab. I would like to thank our lab technicians, Nancy Tomczyk, Jessica Campo, Abhay Ranganathan, and Bruce Braender for keeping the lab running smoothly. Nancy was heavily involved in the chemical conjugation of fibronectin onto QCM-D crystals (Chapter 3) and is co-authored on the paper relating that work. Bruce Braender was an integral part of the QCM-D experiments presented in Chapter 4. Dr. Meg Grady, a post-doc in our lab, has been a valuable source of scientific feedback, particularly with AFM experiments. I would also like to thank Eric Abhold, who joined us for a summer research project, for teaching me Western blot fundamentals.

I was lucky enough to enjoy the core facilities belonging to the Anesthesia and Critical Care research department on the third floor of the John Morgan Building. Thank you to Dr. Roderic Eckenhoff for his service as the Vice Chair for research in the Anesthesia department. In addition to Eric, Brian Weiser, Kellie Woll and Tim DeYoung were amazingly helpful with Western blotting. Dr. Bo Han has been a wonderful source of general scientific advice and
immunofluorescence in particular. Drs. Qing Cheng Meng and Weiming Bu were great guides to both general and specific procedural questions. Thank you also to Drs. Max Kelz and Maryellen Eckenhoff for providing a listening ear and professional guidance when I needed it.

I have also relied on our support staff on JM3 throughout my training: Cindy Bliven, Ann Marie Tenuto, and Randall Hardie. Thank you for doing everything from organizing potlucks to placing orders for reagents. My work certainly would not have gone as smoothly as it did without you.

Like many interdisciplinary projects, my work has benefited from the collaboration of many other scientists in other laboratories. Thanks to Dr. Russell Composto, a longtime collaborator of the Eckmann lab, for his general involvement and encouragement, and his insights particularly on the chemFN functionalization of QCM-D crystals. The AFM experiments, as well, could not have been done without many other people. Dr. Prathima Nalam conjugated colloids onto my AFM tips and was helpful with general AFM questions. Dr. Matt Caporizzo’s expertise in the TIRF-AFM instrumentation saved my experiments on many different days, and Dr. Matt Brukman of the Nano-Bio Interface Center was also always available for AFM troubleshooting. Emmabeth Parrish Vaughn from Russ’s lab has been a great friend and collaborator in our push to publish the kurtosis method of measuring actin stress fiber alignment (Appendix A).

Collaboration was also critical to some of our mitochondrial work. Thank you to Dr. Douglas C. Wallace and his lab members, Drs. Martin Picard and Alessia Angelin, for their help in bringing these projects to fruition. Martin was the one who shared the initial data showing altered actin gene expression in the m.3243A>G cybrids with me, which developed into the great story of mitochondrial genetics affecting single-cell mechanics told in Chapter 5. In addition to helping with Seahorse XF Analyzer experiments (Chapter 7), Alessia initially showed me some images of actin-stained cells in the hopes of quantifying them somehow. This work developed into the single-cell kurtosis analysis related in Appendix A. I would also like to thank Drs. Xilma Ortiz-Gonzalez and Tal Yardeni from the Wallace lab for their friendship and scientific collaboration.

Perhaps more important than the network of scientific collaborators contributing to this work were the people standing behind me over the course of this degree. I would like to thank my
parents and grandparents (those with us and those who have passed on) for their love and support. Now that I am a parent myself, I appreciate even more how much parents invest in their children and recognize that I would not be who I am today without my parents and ancestors. I would also like to acknowledge my siblings, in-laws, and siblings-in-law for their love and support over the last few years. Additional thanks are due to my friends for being a wonderful network of support over the last few years. I feel fortunate that there are too many of you to list here, but I am thinking of all of you. I hope that I have given you in return a fraction of the friendship that you have extended to me and my family.

Having children while completing a Ph.D. in experimental science is quite uncommon, and I can definitely understand why after having done it. At the same time, my sons Ezra and Elan are without question the best things I have ever produced, and I am so grateful to have had them join me in this experience. There were countless times when I arrived home after a particularly frustrating day in the lab, but was reminded by my husband and children of how blessed and fulfilled my family life is. Becoming a parent also imbued with me with a new sense of mission, and I truly believe that this drive has pushed me to produce more and better scientific work. Ezra and Elan, thank you for bearing with me in this delicate balancing act between being your mom and a scientist. I am hoping that as you grow older, you can increasingly relate to the scientist side of me in addition to the mom side.

Finally, to my husband Ben: words cannot suffice to thank you for everything you have done over the last 6 years while also pursuing your own Ph.D. in the process. It’s hard to believe that we have been working towards these degrees for virtually our entire marriage. I would be remiss if I did not acknowledge your scientific contributions to my work, including valuable statistical input and general scientific feedback. Much more than that, though, you have been my anchor throughout this journey, and what a journey it has been. Thank you for enriching my life in so many immeasurable ways, and for being there to celebrate with me in my triumphs and comfort me in my failures and frustrations. Most of all, thank you for believing in me when I didn’t believe in myself. I truly would not be here without you.
ABSTRACT

BIDIRECTIONAL INTERACTIONS BETWEEN MITOCHONDRIAL FUNCTION AND CELL MECHANICS

Judith Kandel
David M. Eckmann

Energetic and structural maintenance are both critical to cellular homeostasis, and clinical disease is often characterized by alterations in both of these realms. While the manifestation of pathology in each of these fields has been extensively studied, little research has been done to characterize basic, direct interactions between mitochondrial function and cell mechanics. The experiments described in this dissertation endeavored to address that gap, first by investigating the cytoskeletal and mechanical effects of mitochondrial dysfunction and then by considering the mitochondrial consequences of cytoarchitectural breakdown.

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CHAPTER 1: INTRODUCTION

1.1 OVERVIEW

Mitochondrial function and cellular mechanical integrity are both fundamental regulators of mammalian cell function. All cells need energy to survive, and in most cases mitochondrial metabolism provides that energy. Mitochondria are also involved in other critical aspects of cellular function, such as reactive oxygen species generation and calcium homeostasis. Cell mechanics, which involves maintaining structure as well as responding to and generating force, is also crucial to many basic cellular processes such as tissue morphogenesis and cell migration. Some cell types are also particularly involved in the push and pull of mechanical forces, including endothelial and bone cells. When studied at the cellular level, human disease often manifests as changes in both mitochondrial and mechanical homeostasis. The experiments described in this dissertation probe causational interactions between alterations in these two critical aspects of cellular maintenance.

The following introduction is intended to familiarize the reader with basic aspects of both mitochondrial function and cell mechanics in cellular pathology and pathophysiology. Many of the topics touched upon in the following pages are themselves the products of research spanning lifetimes, and it would be impossible to present a comprehensive overview of each one of them. Therefore, the references provided are frequently review articles which distill decades of findings on a given subject into a few pages for the readers’ understanding.
1.2 MITOCHONDRIA IN CELLULAR HEALTH AND DISEASE

1.2.1 The roles of mitochondria in cellular function

Mitochondria are fundamentally known as the “powerhouses” of the cell for their role in producing adenosine triphosphate (ATP) to provide energy for cellular processes. Glycolysis, which takes place in the cytosol, involves the breakdown of glucose to pyruvate. Pyruvate enters the mitochondrial matrix, where its catabolism leads to the release of carbon dioxide and the reduction of nicotinamide adenine dinucleotide (NADH, in reduced form). Beginning a process known as oxidative phosphorylation (OXPHOS), NADH then donates two electrons to complex I of the respiratory chain, which resides in the mitochondrial inner membrane. Protons are pumped from the mitochondrial matrix to the intermembrane space as these electrons are passed through various components of the respiratory chain, building up an electrochemical gradient. Oxygen serves as the final electron acceptor which combines with hydrogen ions to form water, and the flow of protons back into the mitochondrial matrix powers ATP synthase to produce ATP.

Beyond energy production, mitochondria are involved in many other signaling pathways critical to cellular maintenance. The process of apoptosis, or “cellular suicide” originates in the mitochondria. Mitochondria contain both pro- (e.g., Bax, Bak) and anti-apoptotic proteins (e.g., Bcl-2, Bcl-w) belonging to the Bcl-2 family. When activated, the pro-apoptotic proteins cause permeabilization of the outer mitochondrial membrane, which releases cytochrome c and triggers the apoptotic cascade (reviewed by Brunelle and Letai, 2009). Like many other cellular processes, apoptosis requires a delicate balance between too much and too little: uncontrolled or inappropriate apoptosis involves the death of healthy cells, while failure to perform apoptosis when warranted allows the proliferation of unhealthy cells (Czabotar et al., 2013).
Mitochondria are also key players in regulating calcium within the cell (reviewed by Rizzuto et al., 2012) The same inner membrane potential which draws hydrogen ions into the mitochondrial matrix in order to produce ATP also allows calcium to enter the mitochondria. Thus, mitochondria serve as important buffers of calcium within the cell, entering via the mitochondrial calcium uniporter and leaving via Na⁺/Ca²⁺ or H⁺/Ca²⁺ exchangers. Increases in cytosolic calcium are generally followed by increases in mitochondrial calcium (e.g., Sobolewski et al., 2012, reviewed by Finkel et al., 2015), which can stimulate dehydrogenases of the tricarboxylic acid cycle and increase ATP production at low levels (Finkel et al., 2015). At sustained high concentrations, however, continued calcium accumulation into the mitochondrial matrix causes protons to exit into the intermembrane space, effectively uncoupling respiration and inhibiting ATP production (Lemasters et al., 1997; Petronilli et al., 2001; Reed and Lardy, 1972; Wong et al., 1973). Related to this, calcium overload can trigger opening of the mitochondrial permeability transition pore (mPTP), which spans the inner and outer mitochondrial membranes, initiating cell death (Finkel et al., 2015).

Finally, reactive oxygen species (ROS) are produced chiefly in the mitochondria (reviewed by Valko et al., 2007) during both physiological and pathological situations. As many as 1-2% of electrons prematurely leak out of the respiratory chain, primarily at complexes I and III (Circu and Aw, 2010), to reduce oxygen. The resulting free radical, superoxide (O₂⁻), is the most common ROS molecule produced by mitochondria and a relatively stable one. It can be catalyzed by the manganese-dependent mitochondrial superoxide dismutase enzyme (MnSOD) to form hydrogen peroxide (H₂O₂) or oxygen (O₂) (Murphy, 2009). At physiological levels, ROS can serve as “redox signaling” molecules in critical processes such as cell growth and proliferation, activation of the innate immune system, and stem cell differentiation (Schieber and Chandel, 2014). ROS
levels are kept in check by a delicate balance between mitochondrial ROS and antioxidants, including MnSOD. However, elevated ROS can increase mitochondrial outer membrane permeability (Circu and Aw, 2010; Turrens, 2003), leading to the release of cytochrome c into the cytoplasm and triggering the apoptotic cascade. Additionally, overabundance of superoxide can cause H$_2$O$_2$ to form hydroxyl radical (OH$^-$), or react with nitric oxide (NO$^-$) to form peroxynitrite (ONOO$^-$) (Turrens, 2003). These more powerful ROS species can oxidize a wide variety of biological molecules, including proteins and DNA, altering their chemical makeup and potentially their functions (Turrens, 2003). Ultimately, elevated levels of ROS have been implicated in major diseases such as Alzheimer’s disease (Markesbery, 1997), cancer (Benhar et al., 2002), ischemia/reperfusion injury (Dhalla et al., 2000), diabetes (Newsholme et al., 2007), and rheumatoid arthritis (Filippin et al., 2008).

Other characteristics of mitochondria beyond their function have also become focal points of study in recent years. These include mitochondrial fusion/fission, morphology and motility. Mitochondria are continually fusing and breaking apart, and defects in either fusion or fission are associated with cellular pathology (Chen et al., 2005; Ikeda et al., 2015; Ishihara et al., 2009). Relatedly, mitochondrial fragmentation has been observed in neurodegeneration (Knott et al., 2008) and diabetes (Makino et al., 2010) among other disorders, and occurs during apoptosis (Arnoult et al., 2005). Other work suggests that mitochondrial fission may actually be protective in that it potentiates removal of dysfunctional mitochondria (Twig et al., 2008), and at least one study demonstrates that impairing fusion partially protects mitochondria from ROS-mediated mPTP opening (Papanicolaou et al., 2012). Mitochondrial motility is also critical to cellular health, as mitochondria tend to migrate to subcellular regions with elevated energetic requirements (e.g., growth cones of neuronal axons (Morris and
Mitochondrial toxins impair mitochondrial motility (Giedt et al., 2012), and cellular models of neurodegenerative diseases show decreased mitochondrial motility (Calkins and Reddy, 2011; Wang et al., 2011).

Mitochondria thus represent diverse organelles which are involved in regulating many essential aspects of cellular function including energy production, calcium homeostasis, ROS production, and apoptosis. Furthermore, these processes are heavily interrelated, and injury in one realm may likely manifest in other areas of mitochondrial function as well.

1.2.2 Mitochondrial dysfunction in disease

Mitochondrial dysfunction often becomes apparent when studying disease at the cellular level. This can manifest as basic up- or down-regulation of OXPHOS or as changes in other aspects of mitochondrial function.

Perhaps the most well-known example of mitochondrial dysfunction in human disease is cancer. Cancer cells famously upregulate glycolysis even in the presence of oxygen in order to produce ATP, a phenomenon known as the Warburg effect (Warburg, 1925). However, cancer cells by no means rely universally and exclusively on such “aerobic glycolysis” for survival and function, and many cancer cell types rely largely on OXPHOS to meet their metabolic needs (Moreno-Sánchez et al., 2007). Recent studies have also demonstrated increased reliance on OXPHOS in metastatic cancer cells (LeBleu et al., 2014) and decreased viability of cancer cells under conditions of impaired OXPHOS (Zhou et al., 2014). In addition to altered energetic pathways, cancer cells produce more ROS than other cells types (Sztrowski and Nathan, 1991), a feature which some hope to exploit for specific therapeutic targeting (Trachootham et al., 2009).
Another facet of mitochondrial dysfunction in cancer is highlighted by the discovery of increased mitochondrial DNA (mtDNA) mutations in tumor cells as compared to normal cells (Brandon et al., 2006), particularly in protein-encoding regions of the mtDNA (He et al., 2010). All of these aspects of mitochondrial dysfunction in cancer cells are likely intertwined, and many studies have demonstrated connections between them.

Cardiovascular disease and the metabolic syndrome are part of another class of diseases in which mitochondrial function is profoundly altered. Mitochondria-mediated changes in these disorders include decreased OXPHOS and increased ROS production (Nisoli et al., 2007; Ren et al., 2010). Ischemia has also been shown to cause mitochondrial fragmentation, and interfering with mitochondrial fission significantly attenuated cell death resulting from ischemia/reperfusion injury (Ong et al., 2010). Additionally, mtDNA mutations are more prevalent in hearts from patients with coronary artery disease in comparison to hearts from healthy patients (Corral-Debrinski et al., 1992).

Many other widespread clinical pathologies feature mitochondrial dysfunction, among them neurodegenerative diseases such as Parkinson’s (Hauser and Hastings, 2013) and Alzheimer’s (Wang et al., 2014) diseases. The evidence of pervasive mitochondrial involvement in general human disease is too detailed to fully present in this context; the interested reader is encouraged to further research this topic.

A specific class of diseases exists which originate in mtDNA mutations. These mutations often cause defects in the electron transport chain, since mtDNA principally encodes respiratory chain subunits. Patients suffering from mitochondrial diseases exhibit a wide variety of clinical symptoms, ranging from optic atrophy to cardiomyopathy (Taylor and Turnbull, 2005). This is further complicated by the fact that mtDNA mutations may be present in different percentages since many copies of mtDNA exist in each cell,
a state termed heteroplasmia. A given mutation may cause different clinical presentation depending on heteroplasmatic level. For example, the mtDNA 3243A>G (m.3243A>G) mutation commonly presents as diabetes and deafness at low heteroplasmatic levels (van den Ouweland et al., 1992). However, at higher (50-90%) mutation loads, patients exhibit mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) (Goto et al., 1990), and at least one report in the literature showed very high heteroplasmatic level associated with Leigh syndrome (Koga et al., 2000), which involves degeneration of the central nervous system and is fatal in early life. Chapter 5 of this work includes an in-depth study of the mechanical features of cells harboring different m.3243A>G heteroplasmatic levels.

1.3 CELL MECHANICS IN HEALTH AND DISEASE

1.3.1 Overview of cell mechanics

One of the first monumental papers describing the impact of mechanical forces on cell function was written by Pelham and Wang in 1997. They showed that cells on stiffer substrates exhibited greater adhesion, increased spreading and more stable focal adhesions (Pelham and Wang, 1997). Cells plated on more flexible substrates, on the other hand, were more prone to migration than adhesion and displayed small, irregular focal adhesions. Substrate stiffness has also been shown to be an important determinant of stem cell differentiation among other major cellular processes (Engler et al., 2006). Other types of forces, such as shear stress, also influence cellular homeostasis. Cells even modify their own mechanical properties in response to external forces. For instance, Solon et al. (Solon et al., 2007) demonstrated that substrate rigidity and cell stiffness are often coupled. Cellular stiffness is also specific to cell type
reviewed by Kuznetsova et al., 2007), and alterations in cell mechanics can be symptomatic of disease, as discussed below.

Microfilaments, which are composed of actin, are the cytoarchitectural components whose role in cell mechanics is most established (Janmey and McCulloch, 2007). Breakdown of the actin cytoskeleton leads to a massive decrease in cellular elastic modulus (Grady et al., 2016; Rotsch and Radmacher, 2000). Unlike actin, the role of microtubules in cell mechanics is a matter of debate, with some studies showing no direct relationship between the two (Rotsch and Radmacher, 2000; Trickey et al., 2004) and others finding that microtubule depolymerization contributes to cellular mechanical behavior (Barreto et al., 2013; Pelling et al., 2009). Recent work from our laboratory suggests that the role of microtubules in cell mechanics may actually be cell type dependent, with microtubule depolymerization exhibiting some effect on mechanics of cancer cells but not normal cells (Grady et al., 2016). A third category of cytoskeletal components is vimentin, whose integrity is important for preserving cytoplasmic cell stiffness in several cell types (Wang and Stamenović, 2000; Haudenschild et al., 2011). However, recent work distinguishes between stiffness of the cytoplasm, and stiffness of the cell cortex, which is not affected by vimentin (Guo et al., 2013).

Focal adhesions are large complexes linking the cytoskeleton with the extracellular matrix, and are also important players in cellular mechanosensing and force generation (Ross et al., 2013). They are chiefly composed of integrins, heterodimeric proteins which span the plasma membrane and contact the extracellular matrix, and molecules such as talin or vinculin, which link integrins with the actin cytoskeleton (Geiger et al., 2009). The roles of focal adhesion complexes include cell spreading which in turn influences cell stiffness, as well as both generating and responding to mechanical forces.
Motor proteins are also important in cytoskeletal organization, with myosin sometimes playing a critical role in cross-linking and maintaining the actin cytoarchitecture (Fletcher and Mullins, 2010). Myosin is also heavily involved in cell contractility, which is the basis by which cells achieve migration.

Cell mechanics is a critical aspect of many physiological and pathological processes. The molecular processes involved in cell migration, for example, involve close coordination between actin-mediated membrane protrusion at the leading edge of the cell and detachment from the substrate at the rear (Lauffenburger and Horwitz, 1996; Ridley et al., 2003). The migratory capabilities of cells are crucial to physiological processes such as wound healing and neutrophil activity in the immune response (Lauffenburger and Horwitz, 1996). Epithelial-mesenchymal transition (EMT) describes a process by cells switch from a more stationary epithelial-like phenotype to a more motile mesenchymal-like phenotype. This transition involves major cell mechanical remodeling, including dissolution of cell-cell junctions and reorganization of the actin cytoskeleton (Morita et al., 2007). EMT is critical in several stages of embryonic morphogenesis, including gastrulation and differentiation in specific cell types (Thiery et al., 2009).

Cellular mechanosensing is also involved in a wide range of physiological signaling processes. Shear stress, for example, is well known to regulate endothelial cell signaling via activation of ion channels, growth pathways, and changes in gene and protein expression (reviewed by Davies, 1995). Downstream effects include blood vessel dilation, inflammation, growth factor expression and the balance between thrombosis and fibrinolysis (Chien et al., 1998). Furthermore, disturbances in flow patterns predispose cells to an atherogenic phenotype (Davies, 2009). Platelet endothelial cell adhesion molecule 1 (PECAM-1), an intercellular adhesion molecule in endothelial cells, is known to mediate shear stress mechanosensing (Tzima et al., 2005).
Osteocytes are another cell type which responds to mechanical forces. In a process called adaptive remodeling, the presence of a load triggers bone maintenance or growth, whereas the absence of load promotes bone resorption (Bonewald and Johnson, 2008). The mechanisms by which this mechanotransduction occurs are still under debate (Bonewald, 2011). Osteocytes also produce biochemical responses to shear stress, as might occur in bone fluid (Bonewald, 2011).

A final example of the importance of mechanotransduction in physiological processes is evidenced by hearing. Hair cells of the inner ear are exquisitely sensitive to motion caused by head movements or sound waves. Resulting displacement causes opening of mechanosensitive channels which then allows for neurotransmitters to send auditory signals to the brain (Vollrath et al., 2007).

1.3.2 Pathological changes in cell mechanics

With the increasing development of new tools for studying mechanical properties of cells and their surroundings, more research began to focus on how changes in cell mechanics occur in diseased states. Most famously, it was discovered that cancer cells are softer (Cross et al., 2007; Paszek et al., 2005; Xu et al., 2012) and produce weaker traction forces than normal cells (Munevar et al., 2001). This observation even extends to the comparison between highly invasive and less invasive cancer cells, with highly invasive cancer cells showing decreased elastic moduli and more disordered actin cytoskeletons than their less invasive counterparts (Xu et al., 2012; Swaminathan et al., 2011). Interestingly, extracellular matrix (ECM) in tumors is much stiffer than in normal tissue (Levental et al., 2009). This increased stiffness is induced by increased collagen crosslinking, and ultimately promotes cancer cell invasion.
Cardiovascular diseases are another pervasive group of diseases which demonstrate altered mechanics on the cellular level. For example, carotid arteries from patients with cardiovascular disease tend to be less flexible than those from healthy patients (Simons et al., 1999). Aortic stiffness is characteristic of hypertension and is correlated with resulting mortality (Laurent et al., 2001). One underlying cause of aortic stiffness is increased vascular smooth muscle cell stiffness (Sehgel et al., 2013), which also often occurs as a consequence of aging (Qiu et al., 2010). One relatively recent cell study also highlighted the importance of the ECM in cardiac function, showing that the percentage of beating cardiac cells greatly decreased when cells were plated on stiffer substrates reminiscent of fibrotic tissue (Engler et al., 2008).

Other examples of pathological changes in cell mechanics span the entire gamut of human disease. Studies of blood disorders show increased stiffness in red blood cells infected with malaria (Suresh et al., 2005) and abnormal red blood cells from patients with sickle cell trait (Maciaszek and Lykotrafitis, 2011). Alterations in tissue stiffness have also been associated with renal (Wyss et al., 2011) and liver (Georges et al., 2007) diseases.

1.4 LINKAGES BETWEEN MITOCHONDRIAL AND MECHANICAL FUNCTION

Connections between mitochondria and the cellular cytoarchitecture have long been explored, with particular emphasis placed on how microtubules and mitochondria are interrelated. Structurally, mitochondrial are known to be associated with microtubules in particular (Ball and Singer, 1982; Heggeness et al., 1978). Inhibiting microtubule polymerization clearly impedes mitochondrial motion (Morris and Hollenbeck, 1995; Müller et al., 2005), and much is known about the motor proteins which aid directed
mitochondrial motion along microtubules (see, e.g., Brickley and Stephenson, 2011 and Nangaku et al., 1994).

As discussed in section 1.3.1, the contribution of microtubules to cell mechanics is debated, so linkages between mitochondria and microtubules do not necessarily indicate a mitochondria-mechanics connection. Most work exploring structural associations between mitochondria and microfilaments was done in yeast models (reviewed by Boldogh and Pon, 2006), but recent research suggests that actin may be important for mitochondrial fission in particular (Korobova et al., 2013). Furthermore, studies as far back as 1995 showed that impairing microfilament formation markedly reduced directional transport of mitochondria towards the growth cone in neurons (Morris and Hollenbeck, 1995).

Functional connections between mitochondria and cell mechanics are largely inferred from observations of heterogeneity in mitochondrial localization or potential. While mitochondrial performance varies among a given cell population (Huang et al., 2004) and even within a single cell (Collins et al., 2002), several influential papers have shown that highly energized mitochondria actually migrate to cellular regions where energy needs are particular high. This is demonstrated, for example, by intense mitochondrial movement along neurons to regions such as synapses and active growth cones (Hollenbeck and Saxton, 2005), or mitochondrial clustering around viral assembly sites in infected cells (Rojo et al., 1998). The principle of energetic requirement dictating mitochondrial localization may explain several related observations showing increased localization or elevated inner membrane potential of mitochondria within cellular regions associated with mechanosensing. For instance, it has been shown that lymphocyte migration is dependent on mitochondrial ATP and is associated with mitochondrial concentration in the uropod (Campello et al., 2006). Traction forces are highest in the
uropod (Jannat et al., 2010), and mitochondrial clustering in this region suggests that energetic needs are particularly high there. Greater energetic need due to increased mechanical activity may also explain the reason that mitochondria with higher potentials are often more concentrated at the cell periphery, which we and others have observed, since traction forces tend to be concentrated there due to directional cell migration (Collins et al., 2002). The proposition that mitochondrial potential correlates with increased mechanical energy demanded by cell migration supports work done as early as 1981, when Johnson et al. (Johnson et al., 1981) showed that mitochondrial potential was highest at the migrating edge of an epithelial sheet in a wound-healing model.

Directional signaling between mitochondria and cell mechanics has been explored to some extent as it relates to specific cellular phenomena. Anoikis is perhaps the most widely studied example in this regard, and describes a unique type of apoptosis resulting from cell detachment from the extracellular matrix. As reviewed by Reddig and Juliano (Reddig and Juliano, 2005), the molecular pathways involved in anoikis are in direct opposition to those involving cell growth in some studied cell lines. Several different pro-apoptotic mitochondrial proteins, including Bim and Bax, have been shown to become activated upon loss of integrin engagement with the substrate. Zhang et al. also showed that integrin activation increases the expression levels of Bcl-2, an antiapoptotic mitochondrial protein (Zhang et al., 1995). ROS are also involved in the deleterious cell signaling which follows cell detachment from the substrate. Li et al. note that this process is accompanied by a strong burst in ROS (Li et al., 1999), a finding later supported by the observation that α5β1 integrin inhibition resulted in an ROS increase (Werner and Werb, 2002).

Other studies investigating direct relationships between mitochondrial function and cell mechanics present intriguing findings. Observations include increased ROS
production by mitochondria in response to high shear stress (Kudo et al., 2000), as well as differential caspase activity depending on substrate stiffness (Zhang et al., 2011). Several recent studies have also specifically investigated mitochondrial requirements for tumor invasiveness (Caino et al., 2015; Desai et al., 2013). These studies suggest a complex interplay between mitochondrial and mechanical function in cells. However, a foundational characterization of this relationship is lacking. The experiments described in this thesis therefore attempt to characterize fundamental directional signaling between mitochondria and cell mechanics.

1.5 SUMMARY

Both mitochondrial function and cell mechanics are critically involved in cellular physiology and pathology. Mitochondria are centrally involved in energy production, calcium homeostasis, ROS production, and apoptosis. A vast number of human diseases are characterized by changes in one or more of these fundamental mitochondrial roles. The mechanical integrity of the cell is mediated primarily by the actin cytoskeleton and proteins linking actin with the extracellular matrix. Cellular mechanical properties as well as mechano sensing and force generation are important in many physiological processes, and disturbances in cell mechanics are hallmarks of cell and tissue dysfunction.

Despite vast research on both mitochondrial and mechanical function in cells and implied connections between them, fundamental questions about this interplay have yet to be answered. How does cytoskeletal breakdown affect mitochondrial motility, structure, and function? Conversely, how does impaired mitochondrial function influence the cytoskeleton and cell mechanical properties? These basic questions inspired the
research presented herein, and is it my hope that this work provokes further investigation into these issues.
CHAPTER 2: RESEARCH OVERVIEW

2.1 OBJECTIVE

As Chapter 1 presented, both mitochondrial and mechanical function are critical regulators of cellular homeostasis. Clinical pathologies persistently show coinciding alterations in both of these realms, but a foundational characterization of their interaction is lacking in the literature. The objective of this dissertation was to elucidate basic components of the causational relationships between mitochondrial and mechanical function, with the broader goal of shedding light on how the dynamics of these relationships may be exploited for therapeutic purposes.

2.2 SPECIFIC AIMS, HYPOTHESES AND OUTCOMES

The global hypothesis of this dissertation is that mitochondrial function and the cellular mecanostructure exhibit direct interactions with one another. As such, I endeavored to investigate the dynamics of this relationship in both directions, comprising Specific Aims 1 and 2. Each of these aims is further divided into sub-aims which address particular questions borne out of the more general Specific Aims.

2.2.1 Specific Aim 1: Assess whether mitochondrial dysfunction influences cytoarchitectural integrity and cell mechanics

*Specific Aim 1a: Characterize the effects of pharamacologically-induced mitochondrial dysfunction on the cytoskeleton and cell mechanics*

*Motivation:* Based on the often concurrent dysfunction in mitochondria and cell mechanics, we wanted to investigate directional effects of mitochondrial toxicity on cell mechanics. Our experimental setup included subjecting mitochondria to relatively low
doses of the complex I inhibitor rotenone and complex III inhibitor antimycin A for
periods of 24 hours. We then examined whether these cells showed alterations in
cytoskeletal structure and mechanics.

**Hypothesis:** Because mitochondria are critical regulators of many basic aspects of cell
function, pharmacological inhibition of mitochondria will cause cytoskeletal and
mechanical alterations in cells.

**Outcomes:** We found a slight but significant decrease in viability of cells subjected to 2
µM rotenone. Fluorescence microscopy revealed that while the microtubule structures
remained largely intact in treated cells, rotenone-treated cells frequently showed a
rounded morphology which was evident in actin cytoskeletal restructuring. However, this
reorganization was not reflected by any detectable changes in cell mechanical
properties. Future work should examine whether cell rounding induced by rotenone
changes the distribution and magnitude of force generation in these cells.

**Specific Aim 1b: Describe the cytoskeletal and mechanical features of cells with
genetically dysfunctional mitochondria**

**Motivation:** Mitochondria contain their own DNA which largely encodes components of
the respiratory chain. The percentage of mutant mtDNA copies per cell can vary, a state
termed heteroplasmy, and can influence clinical presentation of a given mitochondrial
disease. A famous example of this is the m.3243A>G mutation (the most common
genetic cause of mitochondrial encephalomyopathy, lactic acidosis and stroke-like
episodes, MELAS syndrome). Previous work using m.3243A>G cybrid cells showed
heteroplasmy-induced changes of expression of many nuclear genes. This study
endeavored to assess whether heteroplasmy level of the m.3243A>G mutation affected
expression of cytoskeletal genes, and resulting cytoskeletal structure and cell mechanics.

**Hypothesis:** Cytoskeletal gene expression, protein production, and cell mechanical properties will vary among cell lines harboring different heteroplasmy levels of the m.3243A>G mutation.

**Outcomes:** We showed that expression of various cytoskeletal genes varied among cell lines harboring different heteroplasmy levels in a manner strikingly uncorrelated with heteroplasmy level. This particular pattern persisted in measures of protein production, quantification of fluorescent cytoskeletal images, and measurements of cell stiffness. We believe this to be the first demonstration of mechanical effects of mtDNA mutation. The nonmonotonic pattern of effect suggests a complex regulatory circuit between m.3243A>G mutation and expression of genes encoding production of cytoskeletal proteins.

### 2.2.2 Specific Aim 2: Characterize the effects of cytoskeletal breakdown on mitochondrial function

**Specific Aim 2a: Demonstrate how cytoskeletal toxins alter motility of individual mitochondria on the whole-cell level**

**Motivation:** Mitochondrial motility is subject to increasing study, and has been shown in many cases to correlate with mitochondrial function. We wished to characterize the effects of cytoskeletal breakdown on mitochondrial motility. However, tools to characterize mitochondrial motility tend to be limited to a small number of well-resolved mitochondria or to a whole-cell motility “index” which does not relate to motion of individual mitochondria. The goal of this work was to develop a method to measure
motility of individual mitochondria across the entire cell and then use it to characterize changes in mitochondrial motility resulting from cytoskeletal disintegration.  

**Hypothesis:** Motility of individual mitochondria can be tracked over a two-dimensional time-lapse recording of an entire cell. The distribution of mitochondrial tracks will change to reflect impeded or accelerated mitochondrial motion resulting from cytoskeletal breakdown.

**Outcomes:** Our method of mitochondrial tracking first involved image-preprocessing in order to best resolve mitochondria. Then we created a custom Matlab algorithm which tracked individual mitochondria on a frame-by-frame basis over time. We found that in a given cell or group of cells, net distances traveled by mitochondria followed a lognormal distribution. The lognormal distribution provided a metric by which different groups of cells could be compared to one another. Cells treated with nocodazole to inhibit microtubule polymerization showed a leftward shift of the lognormal distribution, indicating shorter net distances traveled. Microfilament disintegration via cytochalasin D, on the other hand, resulted in greater net distances traveled by mitochondria. This study thus demonstrated that cytoskeletal integrity is critical to maintenance of normal mitochondrial motility.

**Specific Aim 2b:** Assess changes in mitochondrial inner membrane potential, morphology, and oxygen consumption following cytoskeletal depolymerization

**Motivation:** Our findings on the effects of cytoskeletal toxins on mitochondrial motility led us to question whether cytoskeletal breakdown also affected other mitochondrial parameters including oxygen consumption. Oxygen consumption is a basic indicator of mitochondrial function, but to our knowledge had not been measured followed cytoskeletal disintegration in intact cells. Since cytoskeletal changes occur in a variety of
human pathologies, characterizing the direct effects of cytoskeletal breakdown on mitochondrial function may suggest signaling pathways between mitochondrial and mechanical dysfunction in cells.

**Hypothesis:** Cytoskeletal breakdown will alter mitochondrial oxygen consumption in intact, adherent cells.

**Outcomes:** We found that although they did not reduce mitochondrial inner membrane potential, cytoskeletal toxins induced significant decreases in basal mitochondrial respiration. In some cases, basal respiration was only affected after cells were pretreated with the calcium ionophore A23187 in order to stress mitochondrial function. In most cases, mitochondrial morphology remained unaffected, but extreme microfilament depolymerization or combined intermediate doses of microtubule and microfilament toxins resulted in decreased mitochondrial lengths. Interestingly, these two particular exposures did not affect mitochondrial respiration in cells not sensitized with A23187, indicating an interplay between mitochondrial morphology and respiration. In all cases, inducing maximal respiration diminished differences between control and experimental groups, suggesting that reduced basal respiration is a largely adaptive rather than pathological symptom of cytoskeletal impairment.

### 2.3 CHAPTER OUTLINE

The thesis is organized into eight chapters and three appendices. Chapter 1, which preceded this chapter, gave a general introduction to familiarize the reader with mitochondrial and mechanical function in cellular physiology and pathophysiology. Chapter 3 is a prelude to Chapter 4, presenting a method for chemically coating QCM-D sensors with fibronectin in order to optimize the QCM-D setup for cell mechanical measurements following mitochondrial toxicity. In Chapter 4, I consider direct effects of
pharmacologically induced mitochondrial dysfunction on cell mechanics and the cytoskeleton (Specific Aim 1a). Chapter 5 considers Specific Aim 1b by examining cytoskeletal and mechanical effects of genetically-induced mitochondrial dysfunction.

Directional signaling from mitochondria to the cytoskeleton and cell mechanics are discussed beginning in Chapter 6. Chapter 6, which relates to Specific Aim 2a, presents a novel approach to characterizing mitochondrial motility and employs it to measure changes in mitochondrial motility following cytoskeletal disintegration. Specific Aim 2b is then addressed in Chapter 7, which considers other mitochondrial effects of cytoskeletal breakdown with particular emphasis on mitochondrial oxygen consumption. Conclusions, limitations and future directions are presented in Chapter 8.

Appendices A and B present shorter, related projects which did not entirely fit into the general dissertation structure. Appendix A considers mitochondrial and mechanical effects of cell swelling. Appendix B presents a novel approach to measuring actin stress fiber alignment in cells, and uses a specific example to demonstrate that alignment correlates with other measures of cellular health. Appendix C contains important MATLAB codes constructed for analysis of mitochondrial and cytoskeletal structure and motility.
CHAPTER 3: CHEMICALLY GRAFTED FIBRONECTIN FOR USE IN QCM-D CELL STUDIES

3.1 CONTEXT AND ACKNOWLEDGEMENTS

The intention of the work presented in this chapter was to enable use of the QCM-D for later studies in cell mechanics, with a particular goal to investigate changes in cell mechanics resulting from mitochondrial dysfunction. In traditional cell culture methods, surfaces for cell seeding are often coated with a physisorbed layer of fibronectin. Coating a QCM-D sensor in this way with fibronectin led to a layer which was not stable under flow and potentially interfered with the ability of QCM-D to detect mechanical changes in the cells seeded on the surface. To address these problems, we developed a method of covalently conjugating fibronectin to surfaces, including QCM-D sensors, which resulted in a thin and stable fibronectin layer. We demonstrate that cells adhere to this layer and behave similarly to cells on traditionally physisorbed fibronectin layers. Furthermore, QCM-D experimentation is more predictable and sensitive when sensors are coated with a chemically rather than physically attached layer of fibronectin.

This work was published in 2014 in *Biosensors and Bioelectronics* under the title “Chemically grafted fibronectin for use in QCM-D cell studies” by Judith Kandel, Hyun-Su Lee, Peter Sobolewski, Nancy Tomczyk, Russell J. Composto and David M. Eckmann. The first two authors equally contributed to the work. Hyun-Su was primarily responsible for developing the coating method while I designed and executed cell-related experiments. Nancy Tomczyk served as a technician in our laboratory who assisted with experiments, and Dr. Peter Sobolewski was instrumental in cell culture training and
experimental design. Dr. Russ Composto advised Hyun-Su and was involved in experimental design and manuscript writing.

In addition to the co-authors of the work, the following are acknowledged for help with this work: Dr. Matt Caporizzo and Boris Rasin for AFM imaging; Jessica Campo for help with flow chamber experiments; Dr. M. Carme Coll Ferrer for FTIR; Dr. Prathima Nalam for helpful discussions regarding QCM-D; Dr. GeLiang for help with imaging phallloidin-stained cells. Drs. Roderic and Maryellen Eckenhoff are also acknowledged for enabling use of the spectrofluorimeter with specific thanks to Dr. Weiming Bu for training on the instrument. This work was supported by the ONR (N00014-08-1–0436), NSF/NSEC (DMR08-32802), and NIH (R01-HL-060230, T-32-HL-007954).

3.2 INTRODUCTION

Fibronectin is a critical component of the extracellular matrix (ECM) which has binding sites to cellular integrins, heparin, collagen and fibrin (Pankov and Yamada, 2002). It usually exists as a dimer of two monomers, each containing three types of repeating subunits. The third subunit contains the RGD peptide, a tripeptide arginine-glycine-aspartic acid sequence. This is the primary binding site for α5 integrins (Pytela et al., 1985; Takada et al., 1987), transmembrane receptors which mediate cell adhesion to substrates, such as neighboring cells and the ECM. In addition to preventing a particular type of apoptosis deemed anoikis (Frisch, 1996), integrins are heavily involved in various cell signaling mechanisms, such as enhancing cell proliferation, governing platelet activation, and directing cell migration (Miranti and Brugge, 2002).

Because of fibronectin’s important role in cell adhesion, it has been used extensively as a thin surface coating in cell culture experiments by our laboratories and many others (Toworfe et al., 2009; Ostuni et al., 2000; Uttayarat et al., 2010). These
coatings are physically absorbed to existing surfaces (physFN), with excess solution aspirated before cells are plated. While suitable for most research, the physical method of fibronectin deposition results in coatings that are too thick, nonuniform, and unstable for studies where these qualities are important. Such research includes the use of certain microfluidic devices and flow chambers (Kent et al., 2010), as well as quartz crystal microbalance (QCM) studies where cellular properties are of interest. QCM detects changes in resonance frequencies and dissipation (for quartz crystal microbalance with dissipation, QCM-D) of a quartz crystal oscillated by a shear wave resonator in order to model changes in mass and viscoelastic properties of the surface. Since decay length of the shear wave can be less than 250 nm (Fredriksson et al., 1998), the surface layer deposited on the crystal must be as thin as possible to maximize detection of mass or mechanical changes of cells resting on the substrate. In addition, substrate uniformity helps ensure that an observed effect is occurring to a similar degree across the crystal’s surface area, a common assumption in QCM modeling (Vig and Ballato, 1998). Covalent attachment, rather than physical adsorption, of fibronectin would be more appropriate for such studies because it can provide an exceptionally thin, uniform and stable surface.

Previous QCM research involving cells has been limited, probably due in part to the challenges presented by physically coating QCM crystals for biofunctionalization. Thus far, most QCM studies involving cells have used QCM in order to sense and characterize cell adhesion to the crystal surface, and some have correlated changes in frequency with the known value of cell density (Fredriksson et al., 1998; Zhou et al., 2000; Modin et al., 2006). A significant subset of this research investigates changes in cell adhesion in response to substrate modification (Saitakis and Gizeli, 2011). Several studies have gone one step further by investigating real-time mechanical changes in
cells on QCM crystals in response to cytoskeleton-disrupting drugs (Saitakis et al., 2010; Marx et al., 2007). Only a few QCM research studies have used QCM for sensing both short- and long-term changes in cellular viscoelastic properties in more biologically relevant situations, and they tend to be limited. Elsom et al. (Elsom et al., 2008) used QCM to examine epithelial cell uptake of microspheres, and Chen et al. (Chen et al., 2012b) employed QCM to study the effects of epidermal growth factor on cell mechanics. These are reviewed along with other studies by Saitakis and Gizeli (Saitakis and Gizeli, 2011) and Xi et al (Xi et al., 2013). In none of these studies is the QCM crystal functionalized as is traditionally done in cell culture experiments.

Changes in cellular mechanical properties are a critical feature of many cellular processes, such as stem cell differentiation (Darling et al., 2008), apoptosis (Pelling et al., 2009), and cancer (Cross et al., 2007), and currently there is considerable biomedical and bioengineering research focused on methods of measuring such changes. As such, the adaptation of QCM for this purpose is paramount.

Perhaps because of the drawbacks of physically coating surfaces with fibronectin, Völcker et al. demonstrated a way to functionalize silicon rubber in order to covalently attach fibronectin (Völcker et al., 2001). Their technique involves grafting acrylic acid (AAc), methacrylic acid (MAac), or glycidyl methacrylate (GMA) onto silicon substrates. GMA, immobilized using radical polymerization, provides the substrate with epoxy groups which easily bind fibronectin by reacting with primary amine groups on fibronectin’s lysine residues. The radical polymerization method presents a significant drawback, however, as it creates an epoxide-functionalized layer with an indeterminate number of monomers and therefore varying thickness, which is unsuitable for applications requiring thin, uniform surfaces.
Here, we report a new method of chemically grafting fibronectin (chemFN) to silicon oxide surfaces. We improve upon Völcker et al.’s method by using 3-glycidoxypropyltrimethoxysilane (GPTMS) instead of GMA for epoxy functionalization, closely following our recently published work on grafting chitosan to silicon oxide surfaces (Lee et al., 2012). This allows for the epoxide-containing molecules to covalently attach to glass, quartz or silicon surfaces in a characteristic single-molecule layer. Our laboratory has previously reported studies of physical adsorption of fibronectin onto various silane self-assembled monolayers (Toworfe et al., 2009), as well as the resulting effects on cell adhesion (Lee et al., 2006). This study is the first in which is described both the chemical grafting of fibronectin onto a GPTMS monolayer via a well-known epoxide-amine reaction (Hermanson, 1996) and the resultant grafted layer’s particular suitability for cell-based research using QCM-D. We characterize the chemFN surface using surface ellipsometry (SE), atomic force microscopy (AFM) and QCM-D. In addition, we use human umbilical vein endothelial cells (HUVEC) to assess biocompatibility by measuring cell adhesion, viability, cytoskeletal structure and metabolic properties on both chemFN and physFN substrates. We then compare QCM-D sensitivity to the presence of cells on both surfaces, and study the effect of cell density on the average thickness, viscosity, and shear modulus of the adherent cell layer on the chemFN-coated crystal surface. Finally, we demonstrate that QCM-D can detect viscoelastic changes in fibroblasts subjected to cytochalasin D, an actin depolymerizing agent, when plated on chemFN coated crystals.
3.3 MATERIALS AND METHODS

3.3.1 Surface Preparation

N-Type, (100) oriented silicon wafers (CZ silicon, dopant; Ph, 20–30 Ω resistivity) were purchased from Silicon Quest International. QCM sensor crystals, AT-cut piezoelectric quartz crystals (14 mm in diameter and 0.3 mm thickness) coated with a 50 nm thick layer of silicon dioxide, were purchased from Biolin Scientific, Inc. Microscope coverslips (24-40-1) were purchased from Fisher Scientific. Silicon wafers (20 mm × 20 mm for SE measurements), microscope coverslips (24 mm × 40 mm for cell culture), and SiO₂-coated QCM sensor crystals were cleaned by immersion in piranha solution (3:1 (v:v) H₂SO₄/30% H₂O₂ (Fisher Scientific)), rinsed with ultrapure water (Millipore Direct-Q, 18 MΩ cm resistivity), dried with N₂, and exposed to UV–ozone to produce a homogeneous hydroxylated surface and to remove impurities. GPTMS (≥98%, Aldrich Chemical Co.) deposition on silicon oxide surfaces was performed by immersion of the wafers, coverslips, and crystals into 10% (v/v) GPTMS in anhydrous toluene (99.8%, Aldrich Chemical Co.) at 80°C for 12 hours under N₂. The deposited samples were sonicated in toluene to remove physically absorbed GPTMS and impurities on the surface. The GPTMS surface was then covered in a 10 µg/mL fibronectin (BD Biosciences) solution, water was evaporated slowly, and the fibronectin film was formed by direct contact with the GPTMS surface at 60°C, overnight (∼12 h). The surface was immersed in deionized (DI) water with shaking at 200 rpm for 1 day to remove physically adsorbed fibronectin and other surface impurities.

To prepare physFN layers, cleaned silicon oxide surfaces were immersed in a 50 µg/mL fibronectin solution for either 30 minutes or 12 hours in a 37°C incubator receiving 5% CO₂. The surfaces were gently rinsed (1x) with ultrapure water to remove loosely absorbed fibronectin and other surface impurities.
3.3.2 Surface Characterization

The thicknesses of dry substrate on the surface were measured using an alpha-SE ellipsometer (J.A. Woollam Co., Inc., Lincoln, NE) equipped with a wavelength range from 380 to 900 nm (70° angle of incidence). Contact angles were measured using a 1 μL sessile drop method. Additionally, a Rhodamine Red™-X, Succinimidyl Ester dye (Invitrogen, Abs/Em = 560/581 nm), which reacts with residual amine functional groups of the lysines in fibronectin, was used as a red fluorescent label to characterize the chemFN layers conjugated to SiO$_2$-coated QCM crystals. A standard TRITC filter (see Fluorescence Microscopy section) was used to illuminate the surfaces and measure the resulting fluorescence. The GPTMS-coated underside of the same crystal served as a negative control.

3.3.3 FTIR

Fourier transform infrared spectroscopy (FTIR) spectra of the drop casted solutions on cleaned silicon wafers were recorded using an attenuated total reflection accessory as a sampling system on a Perkin Elmer infrared spectrophotometer (Spectrum RX I FTIR system) at a resolution of 8 cm$^{-1}$ averaging 256 scans. Data were analyzed using Omnic E.S.P v3.2 software.

3.3.4 AFM measurements

Atomic force microscopy (Digital Instruments, Santa Barbara, CA: Dimension 3000 AFM) was used to measure surface topography and roughness of GPTMS, chemFN and physFN coated QCM crystals. Tapping mode was performed using a single crystal Si tip with a spring constant of 48 N/m, a radius of curvature of about 10 nm, and a resonance frequency of approximately 190 kHz. AFM images were taken over scan sizes of 5
μm × 5 μm and 1 μm × 1 μm. The root mean square roughness ($R_{\text{rms}}$) values were determined from 25 separate 1 μm$^2$ images for each substrate type. All images were analyzed using Picoview 1.6 software (Agilent Technologies).

The as-received silicon oxide coated QCM sensor was characterized as a control. This gave an $R_{\text{rms}}$ of 1.30 ± 0.19 nm, in agreement with the manufacturer’s description of an $R_{\text{rms}}$ less than 3 nm.

### 3.3.5 Cell culture and viability assays

All cells and cell culture media for in vitro cell culture models were obtained from Lifeline Cell Technolog (Walkersville, MD). HUVEC were cultured in VascuLife VEGF cell culture media as previously described (Sobolewski et al., 2011). Cytochalasin D experiments used human dermal fibroblasts cultured in FibroLife cell culture media. Briefly, cells between passage 2 and 5 were plated onto chemFN and physFN surfaces approximately 48 hours before planned experiments. Surfaces were rinsed with ethanol and sterile water before cell plating. For adhesion assays, cells were plated 3 hours before rinsing and counting. Most cell experiments included control physFN surfaces which were coated at a fibronectin concentration of 50 µg/mL for 30-40 minutes before aspiration. All dye loading and incubation was performed in the dark.

For comparison of cell metabolic activity, cells were dye-loaded with 500 nM Calcein-AM (Invitrogen) for 20 minutes at 37°C, then rinsed three times and incubated for an additional 15 to allow for de-esterification. Calcein imaging was carried out using 10 ms exposure times at room temperature in Recording HBSS (HBSS pH 7.4 with 1.3 mmol/L CaCl2, 0.9 mmol/L MgCl2, 2 mmol/L glutamine, 0.1 g/L heparin, 5.6 mmol/L glucose, and 1% FBS).
In order to image cellular cytoskeletal structure, cells were fixed at 4°C using 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 20 min. The cells were then rinsed three times in Recording HBSS and subsequently permeabilized for three minutes in 0.1% Triton (Sigma Aldrich, St. Louis, MO). Then, the cells were incubated in Alexa Fluor 594 phalloidin (Invitrogen) diluted 1:100 for 20 minutes at room temperature, and rinsed three times before imaging.

Cell adhesion onto chemFN and physFN surfaces was compared using a cell adhesion assay. Cells were trypsinized from tissue culture flasks, resuspended, counted using a hemacytometer (Hausser Scientific, Horsham, PA), and then seeded at equal, known densities onto both surfaces. After 3 hr, surfaces were rinsed gently with Recording HBSS. Phase contrast images were acquired of multiple regions on each surface, which were then averaged to calculate the cell density on each surface.

To assess proliferative capacity, cells were seeded at equal densities on chemFN and physFN surfaces. After 24 hr, when the cells were presumed to be in their exponential growth phases, the media was replaced with a 1:10 alamarBlue (Invitrogen): media mixture. Viable cells convert the blue, non-fluorescent resazurin in alamarBlue to bright pink, fluorescent resorufin. Thus, the fluorescence intensity of alamarBlue cell media is a measure of cell proliferation and metabolism. 18 hours after alamarBlue addition, 1-2 mL of media was removed from each of the cell culture dishes and placed into cuvettes. Fluorescence of these samples were measured at a 565 nm excitation wavelength and a 585 nm emission wavelength using a Shimadzu RF 5301 PC spectrofluorophotometer (Columbia, MD). When the fluorescence detector was saturated by the samples, both samples were diluted to the same concentrations with Recording HBSS to obtain accurate measurements. The fluorescence of the
alamarBlue-media solution was measured in all cases as a negative control and found to be negligible.

To determine whether cells respond to extracellular ATP by releasing calcium, as healthy endothelial cells should (Sobolewski et al., 2011; Hallam and Pearson, 1986), cells were dye-loaded with the calcium sensitive dye 1 µM Fluo-4 AM (Invitrogen) plus 0.005% Pluronic F-127 (Invitrogen) for 15 minutes at room temperature, then washed three times and incubated for an additional 15 minutes to allow for de-esterification. Live fluorescence microscopy was used to monitor the cellular response to 20 µM ATP (Sigma Aldrich). These experiments used 50% Recording HBSS mixed with 50% serum-free HBSS to minimize the ATP binding effects of serum, which may attenuate the calcium signal.

### 3.3.6 Fluorescence Microscopy

Cells were imaged using a SensiCam QE camera (The Cooke Corp., Romulus, MI) (2x2 binning, 688x520) attached to Olympus IX70 microscope (Olympus, Melville, NY) with Olympus LUCPlanFL N 4x, 10x and 20x objectives (Olympus) and Photofluor light source (89 North, Burlington, VT). Computer control of the microscope was facilitated by LUDL programmable filter wheels, shutters, and focus control (LUDL Electronic Products, Hawthorne, NY) and images were collected using IPL 3.7 software (BD, Rockville, MD). Coverslip-coated surfaces were visualized using 4-section 100 mm diameter dishes (Fisher Scientific) or 60 mm diameter dishes (BD, Franklin Lakes, NJ) while silicon and QCM-D crystals were visualized by placing them, inverted, onto 35 mm diameter MatTek glass bottomed Petri dishes (MatTek, Ashland, MA). Slides of phalloidin-stained cells were viewed using an Olympus IX70 microscope equipped with a SensiCam SVGA high-speed cooled digital camera (The Cooke Corp.) and a 60x oil
immersion lens. Images were collected using IPLab 4.0 Imaging software (Biovision Technologies, Exton, PA).

The ATP experiments were performed similarly to what we have previously described (Sobolewski et al., 2011). Images were collected every 2 seconds for a period of 5 minutes each, beginning before and lasting for several minutes following ATP addition. ImageJ software (NIH, Bethesda, MD) was used to analyze the resulting image stacks. Regions of interest (ROI) for background fluorescence (cell-free region) and around the nucleus were defined. Next, the Time Series Analyzer plugin was used to calculate the mean fluorescence intensity of the ROIs at each time point. The fluorescence ratio ($F_R$) was then calculated for each time point as:

$$F_R = \frac{F - F_{bg}}{F_0 - F_{bg}}$$

(3.1)

where $F$ is intensity of the nuclear ROI, $F_{bg}$ is the intensity of a background ROI, and $F_0$ is the intensity of the nuclear ROI before ATP stimulation.

### 3.3.7 Flow chamber experiments

HUVEC plated on chemFN or physFN coverslips were dyed with calcein-AM, then mounted in a parallel-plate flow chamber (RC-30HV; Warner Instruments Inc., Hamden, CT, USA) as in (Calderon et al., 2009). Recording HBSS (described above) was used to perfuse the chamber from a re-circulating reservoir using a peristaltic pump. Shear stress was increased stepwise at a rate of $\sim$1.1 dyne/cm² per minute while cells were imaged every 30s to assess whether they remained adhered to the coverslips.

### 3.3.8 QCM-D experiments

The QCM-D measurement is based on the resonance frequency change of a vibrating quartz crystal, a piezoelectric material, in response to mass deposition. The
deposited mass, \( \Delta m \), is related to the frequency change, \( \Delta f_n \), according to the Sauerbrey equation (Sauerbrey, 1959; Jhon et al., 2006):

\[
\Delta m = -C(\Delta f_n/n)
\]  

(3.2)

where \( C \) is the mass sensitivity constant (\( C = 17.7 \text{ ng} \cdot \text{cm}^{-2} \cdot \text{Hz}^{-1} \) for an AT-cut, 5 MHz crystal) and \( n \) is the vibrational mode number (\( n = 1, 3, 5, \ldots \)). In addition, the dissipation change, \( \Delta D_n \), the loss of energy stored in a vibration cycle, indicates the mechanical characteristics of the deposited layer such as viscosity, elasticity, and so on. An elastic film has \( \Delta D_n \) less than \( 2.0 \times 10^{-6} \) and superimposable plots of \( \Delta f_n/n \) under several modes; the Sauerbrey equation (Sauerbrey, 1959; Vogt et al., 2004) can be used to calculate the layer’s mass and thickness. On the contrary, a viscoelastic layer has a \( \Delta D_n \) of more than \( 2.0 \times 10^{-6} \) and plots of \( \Delta f_n/n \) which cannot be superimposed. The physical properties (thickness, shear modulus, and viscosity) of the layer can be estimated by fitting the QCM-D experimental data (\( \Delta f_n/n \) and \( \Delta D_n \)) to a Voigt-based viscoelastic model incorporated in Q-Sense software Q-Tools (Lee et al., 2011; Lee and Penn, 2008; Höök et al., 2001). An E4 QCM instrument (Q-Sense Inc., Gothenburg, Sweden) was used for all QCM-D experiments.

For stability assessments, chemFN and physFN coated sensors were monitored in the QCM-D instrument when subjected to DI water flowing at 40 \( \mu \text{L/min} \) for 24 hours at 21°C. For evaluation of the physFN and chemFN modified sensors containing cells, both sensors were first monitored for frequency and dissipation at 21°C in PBS containing calcium and magnesium at a flow rate of 100 \( \mu \text{L/min} \) (stage I in Figure 3.9(a)). Data was collected when frequency and dissipation reached constant values. Then, crystals were removed from the QCM-D and sterilized with ethanol. Cells were plated on both crystals and placed in the incubator for approximately 48 hours in cell culture media. Both QCM sensors were then reloaded into flow modules, and frequency and
dissipation were measured in PBS using the same method as stage I (stage II in Figure 3.9(a)). Cells were then stained with calcein-AM and imaged to demonstrate their viability and measure cell density on the crystal. Finally, a published oxygen plasma method (Lee et al., 2012a; Lee et al., 2012b) was used to remove the organic layer (in this case, the underlying fibronectin layer and the overlying adherent cell layer) of both sensors without damaging the underlying silicon oxide surface. The cleaned crystals were then reloaded (stage III in Figure 3.9(a)) and frequency and dissipation data were collected using the same method as stage I. This allowed us to estimate the physical properties of the chemFN and physFN fibronectin layers (stage I).

Real-time cytochalasin D (cytD) experiments involved growing fibroblasts on chemFN coated crystals for 48 hours and then placing single crystals into the QCM-D in PBS solution. When a baseline was obtained, the perfusate was switched to 0.1% DMSO and a new, stable baseline was obtained. Finally, a 1µM solution of cytD (containing ~0.1% DMSO as a final concentration) in PBS was added. This stepwise progression in solutions enabled isolation of the cytD effects on cells.

3.3.9 Statistics

SigmaPlot (SysStat Inc., San Jose, CA) was used for data plotting and statistical analysis. Where appropriate, data are reported as mean ± standard deviation. A Student’s t-test was used for comparing chemFN and physFN cells’ calcium flashes in response to ATP stimulation. A paired Student’s t-test was used for comparing adhesion and alamarBlue data from the two groups, since results varied across experiments. In all cases, p < .05 was considered significant.
3.4 RESULTS AND DISCUSSION

3.4.1 Immobilization of fibronectin on silicon oxide surfaces

The well-known epoxide-amine reaction was used to immobilize fibronectin onto silicon oxide surfaces, including microscope cover glasses, hydroxylated silicon wafers, and silicon oxide coated QCM sensors. Figure 3.1(a) gives a graphic description of our method of chemical immobilization of fibronectin onto surfaces. First, silicon oxide surfaces were modified with epoxide functional groups by reacting trimethoxy silane groups of GPTMS and hydroxyl groups on silicon oxide surfaces. The thickness value measured by SE and the water contact angle of GPTMS derivatized layers (shown in the table in Figure 3.1(b)) are in reasonable agreement with values obtained in the literature (Lee et al., 2012b; Lee et al., 2011). Upon fibronectin deposition, primary amine functional groups from lysines in fibronectin react with epoxide groups from GPTMS on the surface, resulting in stable covalent bonds.
Figure 3.1: Method of chemical conjugation of fibronectin and resulting characterization parameters. 
(a) Experimental scheme of fibronectin immobilization onto silicon oxide surfaces, including glass microscope coverslips, hydroxylated silicon wafers, and silicon oxide coated QCM sensors, using the well-known epoxide-amine reaction. (b) Table displaying ellipsometric thickness and contact angle of dry layers. After 130 min and 212 hr exposure of FN solution to SiO2 surfaces, respectively, contact angle and thickness were measured between 3 and 5 times for each surface.

<table>
<thead>
<tr>
<th></th>
<th>Thickness (nm)</th>
<th>Contact angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPTMS</td>
<td>0.9</td>
<td>41.1 ± 1.3</td>
</tr>
<tr>
<td>Chem-FN</td>
<td>3.0 ± 0.5</td>
<td>53.3 ± 2.3</td>
</tr>
<tr>
<td>Phys-FN^a</td>
<td>3.5 ± 0.1</td>
<td>90.7 ± 1.6</td>
</tr>
<tr>
<td>Phys-FN^b</td>
<td>19.4 ± 2.0</td>
<td>104.7 ± 1.5</td>
</tr>
</tbody>
</table>

Preliminary data showed that after the initial rinsing of the chemFN surfaces with DI water, further prolonged rinsing on a shaker at had no effect of surface thickness as measured by ellipsometry (data not shown). PhysFN surfaces treated the same way became thinner by 16% in just 24 hours (from 55.5 ± 1.6 nm to 46.6 ± 3.7 nm, p = 0.012), indicating that the fibronectin coating washed away over time. To more accurately capture the setting and chemistry involved in QCM experiments, we also performed rinsing in the QCM-D for 24 hours. As Figure 3.2 shows, the frequency and dissipation readings of the chemFN surface stabilize after several hours, while the physFN surface readings are still under flux after 24 hours. The decreases in physFN
frequencies, indicating mass adsorption, do not necessarily contradict the ellipsometry measurements showing thinning since the QCM-D experiments are done in solution while ellipsometry measures dry thickness. These data demonstrate the superior stability of chemFN surfaces to physFN ones, an imperative quality for QCM-D research, and propelled our further research involving chemFN surfaces in conjunction with QCM-D.

![Figure 3.2: QCM traces of chemFN and physFN coated QCM-D sensors under flow. (a) Frequency and (b) dissipation of chemFN and physFN coated crystals placed under 40 µL/min flow of deionized water for 24 hours.](image)

As shown in the table in Figure 3.1(b), the chemFN grafted layer has a dry thickness of 3.0 nm and water contact angle of 53°. Figure 3.3 shows contact angle measurements for chemFN and two different physFN surfaces. Since the contact angle
value of chemFN is closer to that of GPTMS, and a reduced thickness is characteristic of a chemical rather than physical deposition, this data suggests that the fibronectin in chemFN is chemically grafted to the GPTMS derivatized surface. The chemFN layer is somewhat less hydrophilic than the GPTMS layer, which has a contact angle of 41˚. The physFN^2 layer, which used a 12 hour exposure of fibronectin solution to the SiO₂ surface, has a larger contact angle and dry thickness than the physFN^1 layer, which used a 30 minute exposure (Figure 3.1(b)). This suggests that a thicker and more hydrophobic layer results from greater exposure time of physFN on silicon oxide surfaces. The contact angle of the physFN^2 layer, of approximately 105˚, is in reasonable agreement with literature values reporting it as 97.14˚ ± 4.28˚ (Daoud et al., 2010). In addition, the thickness of the physFN layer increases with a higher concentration of fibronectin used, whereas the thickness of the chemFN layer is relatively consistent regardless of the fibronectin concentration (data not shown).

Figure 3.3: Water contact angle measurements on a chemFN layer and two physFN layers. PhysFN^a and physFN^b were prepared using 30 min and 12 hr exposure of FN solutions to SiO₂ surfaces, respectively.

A well-known Rhodamine Red™-X, Succinimidyl Ester (Abs/Em = 560/581 nm), which reacts with residual amine functional groups of the lysine present in chemFN.
layers, was used in order to additionally confirm the immobilization of the fibronectin on the SiO$_2$ surfaces. Figure 3.4 shows fluorescent images of rhodamine red treated GPTMS and chemFN QCM crystal surfaces. The control GPTMS surface is simply the underside of the chemFN-coated crystal. The Rhodamine Red treated fibronectin has a nearly 5-fold increase in fluorescence intensity as compared to that of GPTMS, 1355 ± 523 and 274 ± 146 (p < 0.0001), respectively (Figure 3.4(b)). This indicates that fibronectin is chemically grafted to the GPTMS derivatized surface, and that the residual amine groups of the fibronectin grafted layer on the silicon oxide surface remain and react with the succinimidyl ester functional groups of Rhodamine Red™-X.

![Fluorescence microscopy illustrating presence of fibronectin on chemFN surfaces.](image)

(a) Fluorescence microscopy images of Rhodamine Red treated GPTMS and chemFN surfaces. (b) Normalized fluorescence intensities from GPTMS and chemFN surfaces. Data are presented as mean ± standard deviation. Statistical significance: ***p << 0.001 versus GPTMS.
FTIR was also performed on GPTMS, physFN and chemFN surfaces (Figure 3.5). Both chemFN and physFN display similar peaks at ~1639 cm$^{-1}$ and 1536 cm$^{-1}$ which are not present in the GPTMS spectrum. These bands most likely correspond to the amide I and amide II groups observed in fibronectin by others at similar wavenumbers (Cheng et al., 1994), and provide further evidence that fibronectin is immobilized on the chemFN surface.

![Fourier transform infrared spectroscopy of GPTMS, physFN and chemFN. Curves are offset to provide data separation for viewing purposes.](image)

**Figure 3.5:** Fourier transform infrared spectroscopy of GPTMS, physFN and chemFN. Curves are offset to provide data separation for viewing purposes.

### 3.4.2 ChemFN and physFN surface characterization using AFM

To examine the surface differences between chemically chemFN and physFN layers on QCM sensors, the surface morphology and roughness of each dry surface was characterized using tapping mode AFM. Images were also taken of the GPTMS modified surface prior to chemFN coating (Figure 3.6), with a resulting $R_{rms}$ of $1.27 \pm 0.31$ nm.
Figures 3.7a and 3.7b show representative topography and phase-contrast images (1 × 1 μm² scan area) of chemFN and physFN layers on QCM sensors, respectively. Images of the chemFN layer show circular domains with a diameter of ~50 nm, and nanocrystalline particle shapes are observed in the phase image. The $R_{\text{rms}}$ of the chemFN surface is 2.24 ± 0.68 nm (Figure 3.7(c)). In contrast, the particle domains are not observed on the physFN surfaces, with an $R_{\text{rms}}$ of 1.72 ± 0.22 nm ($p = 0.007$ vs. chemFN surfaces). This value is characteristic of a surface that is smooth and rather featureless, and is in reasonable agreement with literature values (Daoud et al., 2010). This suggests that fibronectin fills in the valleys between the particle domains, an assumption which is supported by the SE results reporting a greater thickness resulting from physFN deposition. Despite the difference in roughness between the two surfaces, the low $R_{\text{rms}}$ values for both chemFN and physFN indicate that both of these surfaces are extremely smooth.

Figure 3.6: AFM images of a GPTMS coated sensor. Topology (left) and phase (right) AFM images of a GPTMS modified surface prior to chemFN coating. The $R_{\text{rms}}$ is 1.27 ± 0.31 nm.
Figure 3.7: AFM Images of chemFN and physFN coated QCM-D sensors.
(a) Topology and (b) phase AFM images of chemFN and physFN on SiO$_2$ coated QCM sensors, respectively. The scan area of each image shown is $1 \times 1 \mu m^2$. (c) RMS roughness of SiO$_2$, physFN, and chemFN surfaces. RMS roughness is reported as mean ± SD. Roughness values were determined from 25 separate $1 \mu m^2$ subsections taken from $5 \times 5 \mu m^2$ images for each substrate type. ## represents $p < 0.001$ versus physFN, *** represents $p < 0.0001$ versus the SiO$_2$ surface, with $p < 0.05$ considered to be significantly different.

Overall, the surface characterizations of the chemFN and physFN layers show that the chemFN layer is thinner, rougher at the nanoscale, and more hydrophilic than the physFN layer. To evaluate our method of chemically coating fibronectin in cell culture applications, we studied how the chemFN surface affects cell culturing as compared to physFN layers.
3.4.3 Biocompatibility evaluation of chemFN surfaces

3.4.3.1 Cell metabolic activity and cytoskeletal structure

To confirm the viability of cells on both chemFN and physFN surfaces, cells were stained with calcein-AM. Cells from both surfaces displayed similar calcein staining, suggesting that HUVEC viability is similar on both surfaces (Figure 3.8(a)). In addition, cells on both surfaces displayed normal cytoskeletal morphologies with visibly aligned phalloidin-stained actin filaments (Figure 3.8(a)). While the chemFN surfaces shown in the figures were plated on glass coverslips, similar results were found with chemFN surfaces plated on silicon oxide or QCM crystals (data not shown).

![Figure 3.8: Cellular characterization on chemFN and physFN sensors.](image)

(a) Calcein (vital dye) and phalloidin (actin dye) stained cells on chemFN and physFN surfaces. The brightness-contrast of the calcein images was adjusted to show the same range of intensities. All scale bars are 100 µm. Calcein images were taken with a 20x objective lens, and phalloidin images were taken with a 60x oil-immersion objective. (b) ATP stimulation of fluo-4 loaded cells. (i) A group of fluo-4 labeled chemFN cells before and after stimulation with ATP. T = 0 is considered to be when the ATP is added to the dish. Scale bar is 20 µm. (ii) Representative traces of the calcium signal of a cell plated on a chemFN surface (black) and a physFN surface (gray). Inset: average fluorescence ratios for all responding cells on chemFN (44/49) and physFN (34/36) surfaces. A Student’s t-test comparing the two groups gave p = 0.91.
3.4.3.2 Cell adhesion and adhesion strength

To compare the ability of cells to adhere to chemFN and physFN surfaces, cells were seeded at a density of 10,390 cells/cm². On chemFN surfaces cells adhered at a density of 10,270 cells/cm² after 3 hr, while the physFN surface had 9,790 cells/cm². Thus, the number of seeded cells was almost completely recovered on both surfaces. This experiment was repeated three more times with similar results, with cells on average adhering to chemFN 98.25 ± 10.45% (p = 0.615) as much as to physFN. Cell adhesion is thus not statistically different on the two surfaces.

HUVEC were also placed in flow chambers in order to test whether adhesion strength of cells plated on both surfaces was similar. Shear stress was increased stepwise to a maximum of 52 dyne/cm², corresponding to high physiological arterial shear stress levels (Malek et al., 1999), and no cell detachment from either chemFN or physFN was observed. This demonstrates that cell adhesion is quite robust on both types of surfaces.

3.4.3.3 Cell proliferation

AlamarBlue was also used in order to assess cell viability, proliferation and metabolism. ChemFN cells gave alamarBlue fluorescence intensities of 88.4 ± 13.3% the intensity of control physFN cells plated at the same density. This number represents the mean of three separate experiments performed on different days. A resulting p-value of 0.200 indicates that metabolic activity of cells plated on both surfaces is similar.

3.4.3.4 Calcium release in response to addition of extracellular ATP

A final assessment of cellular health on chemFN and physFN surfaces measured the release of intracellular calcium in response to the addition of extracellular ATP. In the
physFN group, 34/36 (94.4%) cells responded to extracellular ATP addition with calcium transients, and in the chemFN group, 44/49 (89.8%) cells responded. Data are pooled from multiple experiments divided over two separate days. Figure 3.8(b,i) shows a group of fluo-4 loaded chemFN cells before and after ATP stimulation, while Figure 3.8(b,ii) shows representative traces of the calcium signal of a sample chemFN cell and a physFN cell. The inset of Figure 3.8(b,ii) shows the mean $F_R$s and standard deviations for all responding cells measured from both groups. The mean $F_R$ for physFN cells was 6.55, while for chemFN cells it was 6.49, with a Student’s t-test giving $p= 0.9097$. This indicates that the cellular responses on both surfaces were virtually identical.

3.4.4 QCM-D

There were three stages to data collection in the QCM-D experiments with cells (see Methods section, Figure 3.9(a)). Stage I involved obtaining a baseline of the coated chemFN or physFN crystal, stage II measured the properties of the same crystal with adherent cells, and stage III involved measuring the crystal after removing the fibronectin and adherent cell layers. To estimate the physical properties of both the fibronectin layer and the adherent cell layer, frequency and dissipation data were stitched together in the order III-I-II, followed by modeling using the QTools software, as shown in Figure 3.9. Both Figures 3.9(a) and 3.9(b) show good fits for all three vibrational modes displayed for both physFN and chemFN surfaces. After modeling, Figure 3.9(b,iii) shows that the thickness of the physFN layer in PBS solution is 76 nm and that the layer thickness after subsequent cell culture increases to 94 nm. Figure 3.9(b,iv) shows that the viscosity and shear modulus of the crystal with cells are lower than those of physFN layer before cell adhesion: the viscosity decreases from $3.93 \times 10^{-3}$ Ns/m$^2$, while the shear modulus decreases from $16.1 \times 10^4$ N/m$^2$ to $12.6 \times 10^4$ N/m$^2$. The increased thickness of
physFN as compared to chemFN coupled with our observation that prolonged rinsing affects the physFN surface makes it impossible to determine whether the changes in thickness, viscosity and shear modulus are solely attributable to the cellular environment rather than changes in the fibronectin layer as well.

The right side of Figure 3.9 displays an example of the QCM data modeling cell adhesion using a chemFN coated QCM sensor. Figure 3.9(c) shows that the thickness of the chemFN layer in PBS solution is 6 nm, increasing to 52 nm after cell culture. This indicates that chemFN swells in PBS, since SE measured the dry thickness of the chemFN layer at 3.0 ± 0.5 nm (Figure 3.1(b)). The thickness increase after cell culture, 46 nm in this particular experiment, can be attributed to the cellular environment because of the stability of the chemically bonded fibronectin layer. In addition, since the chemFN layer is so thin to begin with, there is little worry that cell deposition is responsible for much more than the 46 nm thickness increase even if chemFN removal was to occur. Figure 3.9(d) shows the viscosity and shear modulus of the chemFN layer before cell adhesion to be $1.64 \times 10^{-3}$ Ns/m$^2$ and $0.453 \times 10^4$ N/m$^2$, respectively, which increase to $2.38 \times 10^{-3}$ Ns/m$^2$ and $1.07 \times 10^4$ N/m$^2$, respectively, after cell culture. Figure 3.10(a,i) shows a representative fluorescence image of calcein-stained adherent cells on the chemFN-coated sensor following the collection of the QCM data shown in Figure 3.9, stage II. The staining demonstrates cell viability, and gives the measured cell density for this particular experiment as 753 cells/cm$^2$ (Table 3.1).
Figure 3.9: QCM-D measurements illustrating superior detection of cells by chemFN sensors as compared to physFN sensors.

(a) Experimental scheme for data collection using QCM-D. Stage I: Collecting frequency ($\Delta f$) & dissipation ($\Delta D$) data using chemFN or physFN coated QCM sensors and PBS solution (flow rate = 100 $\mu$L/min, 21°C); stage II: collecting $\Delta f$ & $\Delta D$ using the QCM-sensor from stage I with cells under the same conditions as stage I; stage III: collecting $\Delta f$ & $\Delta D$ using the cleaned SiO$_2$-coated QCM sensor with the same conditions as stage I.

(b) Overall combined traces of (i) $\Delta f/n$ ($n = 3, 5, 7$) and (ii) $\Delta D_n$ of a cleaned SiO$_2$-coated QCM sensor (stage III), the fibronectin layer on a SiO$_2$-coated QCM sensor (stage I), and the cell adherent layer on representative physFN and chemFN QCM-D sensors (stage II) in PBS. The data from stages I, II, and III were stitched together for modeling in the order III-I-II using Q-soft (Q-Sense). Simulated and experimental curves for $\Delta f/n$ ($n = 3, 5, 7$) and $\Delta D_n$ vs. time show a good fit between the viscoelastic model and the experimental data. (iii) Thickness of the fibronectin and cell layers in PBS as determined from the fits shown in (i) and (ii). (iv) Viscosity and shear modulus of the fibronectin and cell layers as determined from the fits shown in (i) and (ii).
To study how cell density affects the modeled thickness, viscosity and shear modulus, we performed another QCM-D experiment using a much higher cell concentration. The cleaning step (stage III) was not performed for this experiment, so only the properties of the cellular environment were measured. Figure 3.10(a,i) and Table 3.1 give the measured cell density as 24,450 cells/cm² and the estimated thickness, viscosity, and shear modulus of the adherent cell layer as 155 nm, $2.84 \times 10^{-3}$ Ns/m², $9.53 \times 10^4$ N.m², respectively. The actual values of viscosity and shear modulus are not physiologically relevant, since the Voigt model used for QCM modeling is far too simplified for complex biological systems and better models do not currently exist (Tymchenko et al., 2012). However, when comparing samples to one another, viscosity and shear modulus indeed increase with increased cell density as expected (Figure 3.10(b)), demonstrating that these values are meaningful in the relative sense. Again, because the chemFN layer is thin and stable, we can be confident that the signal change from stage I to stage II in these experiments is indeed solely attributable to the cell adherent layer and its surrounding environment rather than to changes in the fibronectin layer as well.
Figure 3.10: Viscosity and shear modulus calculated for sensors coated at different cellular densities.
(a) Representative fluorescent images of (i) low cell density (753 cells/cm$^2$) and (ii) high cell density (24,448 cells/cm$^2$) on chemFN QCM sensors, respectively. Both scale bars are 200 $\mu$m. The image shown in (i) was taken at 4x, while the image shown in (ii) was taken at 10x. (b) Viscosity (black) and shear modulus (gray) versus thickness of the cell layer on the chemFN layer. Exp 1 corresponds to (a(i)) and exp 2 corresponds to (a(ii)). The properties of the chemFN layer (derived from exp 1) are as follows: in-situ thickness $= 6$ nm, viscosity $= 1.64 \times 10^3$ Ns/m$^2$, shear modulus $= 0.45 \times 10^4$ N/m$^2$. 
Table 3.1: Parameters of QCM-D studies with low and high density cell seeding.

<table>
<thead>
<tr>
<th>Cell density (cells/cm²)</th>
<th>Thickness (nm)</th>
<th>Viscosity (Ns/m²)</th>
<th>Shear modulus (N/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>753</td>
<td>52</td>
<td>2.38 x 10⁻³</td>
<td>1.07 x 10⁻⁴</td>
</tr>
<tr>
<td>24448</td>
<td>155</td>
<td>2.84 x 10⁻³</td>
<td>9.53 x 10⁻⁴</td>
</tr>
</tbody>
</table>

Endothelial cell density and corresponding *in-situ* thickness, viscosity, and shear modulus of the endothelial cell layer estimated using the chemFN QCM sensor. *The data from stages I and II were stitched in the order I-II using Q-soft (Q-Sense) and show a good fit between the viscoelastic model and the experimental data.*

We also used the chemFN method in order to investigate real-time changes in QCM-D frequency and dissipation in response to drug-induced changes in cellular viscoelasticity. While frequency changes were negligible, a characteristic decrease in dissipation was observed in fibroblasts treated with 1 µM cytD (Figure 3.11(a)) in agreement with others’ observations (Saitakis et al., 2010, Wegener et al., 2000). Figure 3.11(b,i) shows normal actin filament staining of cells on a crystal treated only with 0.1% DMSO, while Figure 3.11(b,ii) shows the disrupted actin filaments of the cells on the crystal subjected to cytD. No such dissipation decrease was observed for the crystal shown in Figure 3.11(b,i) (data not shown), indicating that the mechanical changes were due to cytD alone. Likewise, no changes in dissipation or frequency were observed when cytD was added to a chemFN crystal without cells (data not shown). These data demonstrate that the chemFN method yields biofunctionalized QCM crystals which preserve the ability of QCM-D to sense real-time cellular mechanical changes. The dissipation decrease observed implies cell stiffening. This does not inherently contradict AFM data showing cell softening in response to cytD treatment (Rotsch and Radmacher,
since different portions of the cell are being interrogated by these two different methods. The results may imply that the basal region of the cell, which is accessed by QCM-D, reacts to actin depolymerization in a totally different manner than does the upper cellular region, which is accessed by AFM.

Figure 3.11: QCM-D measurements of cells treated with cytochalasin D. (a) Real-time frequency and dissipation changes of a chemFN coated crystal plated with fibroblasts and then subjected to 1 µM cytD. (b) Fibroblasts stained with phalloidin to highlight the actin cytoskeleton. (i) shows control cells on a crystal treated with 0.1% DMSO only, while (ii) shows cells on the crystal treated with cytD. The scale bar is 20 µm.
3.5 CONCLUSIONS

We have demonstrated a novel method of chemical immobilization of fibronectin onto various surfaces, including glass, quartz, and silicon. A cell culture model system has shown that cells are similarly healthy on these surfaces as those plated on the traditionally used, physisorbed fibronectin coating. Our surface characterization and QCM results indicate that the chemFN surfaces are thinner and more stable than the physFN ones, properties which are both critical in maximizing detection and repeatability in QCM research. Finally, we show that the presence of cells on the chemFN surface leads to an expected increase in measured thickness, viscosity and shear modulus of the crystal, and that this effect is intensified in the presence of an even greater cell density. These experiments involve the extraction of cellular mechanical properties from repeated measures of a single crystal, a capability which in the future will allow for the mechanical comparison of different groups of cells. We also presented characteristic real-time dissipation changes occurring with cells subjected to cytochalasin D on a chemFN coated crystal surface. These data suggest the potential for future real-time QCM-D experiments using chemFN for surface functionalization. All of these results demonstrate the excellent suitability of chemFN surfaces for QCM research, and we anticipate that the use of our method will greatly expand the capability of QCM experimentation within the increasingly broad field of cell mechanics. In addition, we hope that the experimental improvement constituted by the chemFN method will inspire the development of better QCM-D models appropriate for cellular experimentation.
CHAPTER 4: CYTOSKELETAL AND MECHANICAL EFFECTS OF PHARMACOLOGICALLY INDUCED MITOCHONDRIAL DYSFUNCTION

4.1 CONTEXT AND ACKNOWLEDGEMENTS

In the previous chapter, we focused on a method to maximize QCM-D sensitivity to mechanical changes in cells. Here, we begin formally addressing Aim 1 by examining the cytoskeletal consequences of mitochondrial toxicity. We considered cytoarchitectural changes as well as alterations in single cell mechanics under these circumstances. Additionally, we followed the chemFN coating method presented in Chapter 3 in order to assess mechanical changes in cell populations as detected by QCM-D. We encountered a great deal of difficulty performing these experiments which are detailed in section 4.4. Nonetheless, this chapter presents important findings which lay the foundation for future experimentation in this area.

Bruce Braender, a technician in the Eckmann lab, was extremely helpful in carrying out all stages of the QCM-D experiments described in this chapter. Colloid conjugation to AFM tips was done with the help of Dr. Prathima Nalam. I additionally would like to thank Dr. Douglas C. Wallace for use of the confocal microscope used for AFM images, as well as Drs. Xilma Ortiz-Gonzalez and Alessia Angelin for training on the instrument. Drs. Matt Bruckman and Matthew Caporizzo were extremely helpful with AFM training and troubleshooting. We acknowledge the Office of Naval Research for funding this work via grant N00014-14-1-0538.
4.2 INTRODUCTION

Mitochondria are critically involved in many fundamental aspects of cellular function and dysfunction, including ATP production, calcium buffering (Rizzuto et al., 2012), reactive oxygen species generation (ROS) (Valko et al., 2007), and apoptosis (Brunelle and Letai, 2009). Clinical disease is often featured by altered mitochondrial activity in one or more of these realms. For instance, tumor cells frequently show altered metabolic activity (Warburg, 1925) and increased ROS production (Szatrowski and Nathan, 1991) in comparison to healthy cells. Similarly, alterations in cellular structure and mechanics are often hallmarks of clinical pathology, with cancer cells for example being softer (Cross et al., 2007) and producing weaker traction forces (Munevar et al., 2001) than their healthy counterparts. Despite the ubiquitous concurrence of mitochondrial and mechanical dysfunction at the cellular level, few experiments have explored causative connections between the two, a lack which the experiments describes herein begin to address.

Mitochondria are structurally associated with the cytoskeleton, and particularly to microtubules (Ball and Singer, 1982). Additional studies have suggested that mitochondria may also be physically linked to actin filaments (Korobova et al., 2013), which are of prime importance in cell mechanics (Janmey and McCulloch, 2007), although this connection has been primarily demonstrated in yeast (Boldogh and Pon, 2006). Later work in this dissertation establishes the importance of microfilament integrity for mitochondrial function. Chapter 6 shows that microfilament depolymerization alters mitochondrial motility. In Chapter 7, similar experiments demonstrate that actin disintegration reduces mitochondrial oxygen consumption in cells whose mitochondria are already taxed with increased demands of calcium maintenance.
Many other studies imply a functional connection between mitochondrial and cell mechanics, which further suggests that impairing mitochondrial function may affect cell mechanics. These findings primarily relate to subcellular localization of mitochondria, which tend to migrate toward regions of the cell requiring energy such as the growth cones in neurons (Morris and Hollenbeck, 1993). Particular patterns of subcellular mitochondrial positioning have been observed, for example, in migrating lymphocytes (Campello et al., 2006) and cancer cells (Desai et al., 2013a). Since actin turnover is the primary driving force in cell migration, these studies suggest that mitochondria are critical to sustaining these actin dynamics.

The experiments described in this chapter begin to test that hypothesis by characterizing the cytoskeletal and mechanical consequences of pharmacologically induced mitochondrial impairment. Several studies in the literature have presented similar experiments, with one report of microtubule disintegration following treatment with carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), a mitochondrial uncoupler (Maro et al., 1982). The drug concentration used in this work was 60 µM, more than ten times the concentration needed for mitochondrial uncoupling (in our hands). This implies the possibility that microtubule breakdown is mediated by a mechanism other than mitochondrial dysfunction. Other studies described destruction of microfilaments following treatment with metabolic inhibitors (Bershadsky et al., 1980; Gabai and Kabakov, 1993), but this was only the case when ATP production was completely inhibited by omitting glucose from the medium. As such, both of these studies fail to characterize the cytoskeletal consequences of inhibiting mitochondrial function alone rather than interfering with other cellular processes.

Here, we examine the effects of two well-characterized mitochondrial toxins, rotenone and antimycin A, on cytoskeletal structure and cell mechanics. Rotenone
targets complex I of the electron transport change on the inner mitochondrial membrane. It completely inhibits mitochondrial respiration at an approximate concentration in the range of 1 µM, and is known to increase ROS production at that concentration (Li, 2002). Antimycin A, a complex III inhibitor and potent generator of ROS (Boveris and Chance, 1973), has been used in a wide range of concentrations throughout the literature, spanning from the high nM range (Kulisz et al., 2002) to several hundred µM (Formigli et al., 2000). We use both low (500 nM) and high (2 µM) concentrations of each drug to examine resulting effects on microtubules and microfilaments in intact cells. While microtubules remain intact, cells treated with 2 µM rotenone show rounding and consequent reorganization of the actin cytoskeleton. Further experimentation assessing cell mechanical properties showed maintenance of individual cell stiffness despite actin cytoskeletal remodeling. Additional experiments probing other aspects of cell mechanics may demonstrate mechanical changes in rotenone treated cells mediated by the cytoskeletal rearrangement we observe.

4.3 MATERIALS AND METHODS

4.3.1 Cell culture and reagents

Adult human dermal fibroblasts (Lifeline Cell Technology, Walkersville, MD) between passages 1 and 5 were cultured in FibroLife cell culture media (Lifeline Cell Technology) as previously described (Kandel et al., 2014).

AlamarBlue experiments used 24-well plates (company) with cells plated at a density of 10,000 cells/well approximately 48 hours before experiments. Imaging experiments used MatTek 35-mm glass-bottom dishes (MatTek, Ashland, MA) pre-coated for 30-40 minutes with 5 µg/mL fibronectin (BD Biosciences, San Jose, CA) dissolved in PBS. Cells were plated on the MatTek dishes at a density of approximately
~25,000 cells/plate. Atomic force microscopy (AFM) experiments used 50,000 cells cultured on 22x40 mm coverslips (Fisher Scientific, Pittsburgh, PA) coated with 10 µg/ml fibronectin for 30-40 minutes and placed in 60-mm Falcon dishes (Corning).

4.3.2 Cell viability measurements

AlamarBlue was used to assess proliferative capacity and viability of cells. After 24 hours, when the cells were presumed to be in their exponential growth phases, the media in a 24-well plate was replaced with control or drugged media containing 10% alamarBlue reagent. Two independent experiments were carried out, with each experiment involving treating 3 wells per plate with 0.2% DMSO, 0.05% DMSO, 2 µM antimycin A, 500 nM antimycin A, 2 µM rotenone, and 500 nM rotenone. 3 additional wells per plate were left untreated, and 3 wells contained only the alamarBlue:media solution without cells. A Synergy H1 multi-mode plate reader (BioTek, Winooski, VT) was used to measure absorbance at 570 and 600 nm 24 hours after control or drugged alamarBlue:media addition. The absorbance of the alamarBlue:media solution was measured as a negative control, and blank wells were used to measure background absorbance. For each experiment, background absorbance values were first subtracted from readings. Then, percent difference in reduction between treated and control cells was calculated for each well according to the following equation, according to the manufacturer’s instructions:

\[
\frac{(e_{\text{ox}, \lambda_2})A_{\lambda_1} - (e_{\text{ox}, \lambda_1})A_{\lambda_2} (\text{test well})}{(e_{\text{ox}, \lambda_2})A_{\lambda_1} - (e_{\text{ox}, \lambda_1})A_{\lambda_2} (\text{untreated wells})}
\]

where \( \lambda_1 \) is 570 nm, \( \lambda_2 \) is 600 nm, and \( A \) is absorbance at a wavelength specified in the subscript. \( e_{\text{ox}} \) is the molar extinction coefficient of the oxidized form of alamarBlue, given by the manufacturer to be 80,586 at 570 nm and 117,216 at 600 nm. The denominator
was calculated for each untreated well, and then averaged when evaluating equation 4.1 for each test well.

4.3.3 Fluorescence microscopy

Microtubules were stained by incubating live cells with 250 nM Tubulin Tracker Green (Life Technologies) for 30 minutes at 37˚C in the dark. Cells were rinsed with recording HBSS (pH 7.4 with 1.3 mM CaCl$_2$, 0.9 mM MgCl$_2$, 2 mM glutamine, 0.1 g/l heparin, 5.6 mM glucose, and 1% FBS). Nuclei were then stained by placing cells in a 2 µg/mL concentration of Hoechst 33342 (Life Technologies) diluted with recording HBSS, rinsed and imaged in recording HBSS.

For actin visualization, cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA; diluted with HBSS from a 16% stock solution) for 10 minutes, then permeabilized for 3 minutes with 0.1% Triton, followed by 20 minutes of dye-loading with Alexa Fluor 546 phalloidin (Life Technologies). Nuclei were stained by incubating cells with a 2 µg/mL concentration of Hoechst 33342 (Life Technologies) diluted with PBS for 30 minutes before rinsing. Cells were then imaged in PBS.

Tubulin imaging used wide-field fluorescence microscopy. This setup employed a QImaging QIClick camera (QImaging, Surrey, BC, Canada) (1x1 binning, 1392x1040 pixels, pixel size 0.1625 µm) attached to Olympus IX70 microscope (Olympus, Melville, NY) with an Olympus 40x oil immersion objective lens (Olympus) and Photofluor light source (89 North, Burlington, VT). Computer control of the microscope was facilitated by LUDL programmable filter wheels, shutters, and focus control (LUDL Electronic Products, Hawthorne, NY) and images were collected using IPL 3.7 software (BD, Rockville, MD). For each experiment, cells were visualized using standard FITC and DAPI filters.
Actin imaging was performed using a Zeiss LSM 710 inverted laser scanning confocal microscope with a PlanApo 63x/1.40 oil immersion objective, optovar 1.25x and digital zoom of 1, 1024x1024 image size (final pixel size = 0.132 x 0.132 µm). Excitation was applied with 405nm, and 543nm lasers as separate channels, and emission filters specific to each fluorophore were used. Images were acquired and processed with the Zen 2010 software at the following settings: 1024 x 1024 pixels; pixel dwell time = 3.15 µsec.

Imaging QCM-D sensors and corresponding dishes used a different setup than those used for tubulin or actin imaging. wide-field fluorescence microscopy. This setup involved a wide-field Olympus IX51 fluorescence microscope (Olympus) attached to an ORCA-Flash 4.0 LT camera (Hamamatsu, Hamamatsu City, Shizuoka Pref., Japan). Images were taken with an Olympus 20x objective lens (Olympus), and used 1x1 binning to produce 2048X2048 pixel images with a pixel size of 0.1625 µm. A Lumencor LED White Light Fluorescent light source (Beavorton, OR) was attached to the microscope setup. Computer control of the microscope was facilitated by a LUDL Mac 6000 controller (LUDL Electronic Products) and images were collected using Metamorph software version 7.8.9.0 (Molecular Devices, Sunnyvale, CA)).

4.3.4 Atomic force microscopy

Atomic force microscopy (AFM) experiments used tipless cantilevers with nominal resonant frequencies of 10-20 kHz and nominal spring constants of 0.03-0.09 N/m (NanoAndMore, Lady’s Island, SC, #CSC38/tipless/No Al). Silica colloids were attached to the cantilevers using a micromanipulator attached to a long-distance optical microscope (40X, Alessi REL-4100A, NJ). Silicon dioxide microspheres (C-SIO-5.0,
diameter ~4.86 µm, Microspheres-Nanospheres, Corpuscular, NY) were glued to the cantilevers using two-part epoxy glue (JB Weld, Sulphur Springs, TX) with the help of a sharp tungsten wire (TGW0325, World Precision Instruments, Sarasota, FL). The glue was allowed to cure overnight at room temperature. The cantilevers were cleaned in ethanol (Sigma) and were UV-ozone treated (UVO Cleaner model 42, Jelight Co. Inc., Irvine, CA, USA) for at least 15 minutes before use. The colloid radius was measured optically using the 100x oil immersion lens on the Nikon microscope (described below). A sample image of the colloid is shown in the inset in Figure 7.7a.

An Asylum MFP-3D AFM conjugated to a Nikon Total Internal Reflectance Fluorescence (TIRF) microscope was used in force spectroscopy mode to probe cellular mechanical properties. Colloid-attached cantilevers were calibrated in DI water prior to each experimental session using the thermal-noise method (Hutter and Bechhoefer, 1993). The measured spring constants used for force spectroscopy ranged 0.06-0.21 N/m. Deflection sensitivity of the cantilevers was measured from the slope of the force-distance (FD) curves obtained on a clean glass slide in recording HBSS before the start of the experiment. FD curves on cells were acquired at the perinuclear region of the cell with an approach rate of 500 nm/s. The perinuclear region of the cell was chosen so as to avoid both substrate effects, which can be substantial at the cell periphery (Mahaffy et al., 2004), and mechanical contributions from the nucleus, which may differ from the cytoplasm in its mechanical properties (Haga et al., 2000; Kuznetsova et al., 2007). Repeated cell indentation was performed every few seconds, which is likely infrequent enough to allow for relaxation in between indentations (Caporizzo et al., 2015).

Cells were kept in an incubator with 5% CO₂ at 37°C until experiments, which were performed at room temperature in HBSS solution. FD curves were taken from 6-14
cells per dish, and dishes were discarded 30-60 minutes after experimentation began.

Asylum research software AR 12 version was used to fit the Hertz model to the approach curves. The Hertz model relates the force on the cantilever (F) to the depth of indentation in the sample (δ) by

\[ F = \frac{4}{3} E^* \sqrt{R} \delta^{3/2} \]  

where \( R \) is the tip radius and \( E^* \) is the combined elastic modulus of the contact and is given by

\[ \frac{1}{E^*} = \frac{1-\nu_{cell}^2}{E_{cell}} + \frac{1-\nu_{tip}^2}{E_{tip}} \]  

where \( \nu \) is the Poisson ratio, which is 0.17 for the fused silica tip and assumed to be 0.37 for cells (Shin and Athanasiou, 1999). Combining equations 1 and 2 allows for the determination of \( E_{cell} \), the cell elastic modulus. In this work, we extracted \( E_{cell} \) from the data spanning 20% to 80% of the maximum force used. The amount of indentation this corresponded to depended on \( E_{cell} \) and usually was several hundred nm, which is considerable smaller than the thickness of the cell (~2-3 µm) in the perinuclear region.

The depth of indentation was small in comparison to the radius of the colloid (\( \delta/R <0.1-0.2 \)). Force curves were repeatedly obtained for a given cell until 4-6 consecutive curves gave consistent measures of \( E_{cell} \) (with the extreme measures not differing by more than ~15%). The resulting averaged \( E_{cell} \) was used for data analysis. Each of the control and experimental groups had data from 60-67 cells total, measured over two or more separate days.
4.3.5 Quartz crystal microbalance with dissipation

4.3.5.1 Sensor preparation

Sensors for quartz crystal microbalance with dissipation (QCM-D) were coated with chemFN as described in Chapter 3. Prior to cell experiments, sensors were cleaned with 100% ethanol and then dried with nitrogen gas. Sensors were individually placed into 35 mm cell culture treated petri dish (Corning, Corning, NY).

While cells were the same, the media used for the QCM-D cell experiments was different from the media used in other experiments, and was made up of DMEM with 15% FBS, 1% Penn/Strep and 1% L-Glutamine. Cells were cultured in this media before QCM-D experimentation. Prior to seeding sensors with cells, growth media and 0.05% trypsin were warmed to 37°C. Cells were then detached from a confluent 25 cm² flask using 3 mL trypsin, which was neutralized with 3 mL media following cell detachment. Cells were counted using a Countess II automatic cell counter (Life Technologies, Carlsbad, CA) and plated at a density of 400,000 cells per sensor. Each dish contained a total volume of 2 mL. QCM-D experimentation began on the day following initial cell seeding.

4.3.5.2 Experimental setup

The QCM-D instrument was cleaned prior to experimentation by placing sacrificial sensors in all modules. Temperature was set to 21°C, and a 10% bleach solution was flowed through the modules at a rate of 1.41 mL/minute. After 30 minutes of flowing bleach solution through the modules, the media was then switched to deionized water (DIW) for 1.5 hours. Following this, the temperature was raised to 50°C and a 10% Hellmanex III (Sigma, St. Louis, MO) was flowed through the system for 30 minutes.
DIW was again flowed through the system for another 1.5 hours, after which the temperature was lowered back to 21˚C for cell experimentation.

To prepare cell culture media for experiments, 500 mL wide-mouth bottles were autoclaved for 30 minutes and then cooled. Bottles were filled with sterile media solutions. Drugged media contained 2 µM antimycin A or rotenone taken from 1 mM stocks in DMSO. Control media, which was also used to established experimental baselines, therefore contained 0.2% DMSO. Gas lines were placed within the bottle, and the bottle opening was sealed shut using parafilm. Blood gas (5% CO2, 21% O2 in nitrogen gas) was bubbled into the media at a slow rate to ensure adequate perfusion in the media. The bottle was placed within a 37˚C water bath to allow it to reach the targeted temperature. Next the pump connected to the QCM-D was stopped, and the intake lines were poked through the parafilm layers sealing the baseline media-containing bottles and sealed with another layer of parafilm. After baseline media was seen perfusing the entire system, the flow rate was adjusted to 100 µL/minute.

4.3.5.3 Measuring cellular properties in the QCM-D

The pump was then stopped, and sensors containing cells were placed in the flow module to replace the sacrificial sensors. The pump was restarted to deliver 100 µL/minute baseline media to all flow modules, and the experiment was started. Petri dishes containing cells were treated in the incubator under identical conditions to the sensors in the QCM-D.

Cells were monitored overnight to allow for establishment of a proper baseline. Once a baseline was achieved, the pump was temporarily stopped. Media bottles were then switched to those containing drugged media for experimental groups, which were set up in the same way as bottles with baseline media. Intake lines for control sensors
were kept in baseline media. This exposure was maintained for 24 hours. At the same time, seeding dishes from the incubator were exposed to the same control or drugged conditions as the corresponding sensors in the QCM-D apparatus.

4.3.5.4 Staining cells on sensors and dishes

Measurements were stopped after 24 hours of exposure in the QCM-D. The pump was stopped, and sensors were individually placed into clean, labeled dishes. Recording HBSS (HBSS pH 7.4 with 1.3 mmol/L CaCl2, 0.9 mmol/L MgCl2, 2 mmol/L glutamine, 0.1 g/L heparin, 5.6 mmol/L glucose, and 1% FBS) was used for dye loading and washing. All sensors as well as corresponding dishes were stained with 500 nM Calcein dye (Life Technologies) for 30 minutes at 37°C protected from light. After washing three times with recording HBSS, nuclei were then stained using 2 µg/mL Hoechst 33342 (Life Technologies) at room temperature for 30 minutes. Washing was again performed three times. A solution 1.5 mL recording HBSS containing 0.3 µL propidium iodide (Life Technologies) was placed into each emptied dish immediately prior to imaging. Details of the fluorescence microscopy setup are provided above in section 4.3.3.

4.3.5.5 Cleaning the QCM-D

Following each experiment, the QCM-D instrument was cleaned with 10% bleach flowing at a rate of 1.41 mL/minute for 30 minutes. DIW was then allowed to flow through the system for at least 1.5 hours. Intake lines were then removed from DIW, allowing gas to flow through the system. All modules were then completely dried using nitrogen gas.
4.3.6 Statistics

Pairwise comparisons between experimental and control groups were done using Matlab software, and used the rank-sum test. Adjustments for multiple comparisons via the Benjamini-Hochberg method were done in R. Differences are generally accepted as significant when the adjusted p-value is less than 0.05.

4.4 RESULTS

4.4.1 Rotenone mildly reduces cell proliferation

AlamarBlue assays were performed in order to confirm that the mitochondrial toxin exposures we were using did not drastically affect cell viability and proliferation. Percent reduction of alamarBlue reagent was calculated for each well according to the methods described in section 4.3.2. A 24-hour exposure to 2 µM rotenone resulted in an average 7% decrease in percent reduction of the alamarBlue reagent (adjusted p<0.05) in comparison to untreated cells. DMSO controls and cells treated with antimycin A did not show alamarBlue reductions which differed significantly from untreated cells. These observations indicate a slight reduction in viability of cells in the 2 µM group over the 24 hours of treatment.

4.4.2 Rotenone induces rearrangement of the actin cytoskeleton

The decrease in viability in the 2 µM rotenone group was mild, so we proceeded with experiments assessing changes in cell mechanics and the cytoskeleton following exposure to these mitochondrial toxins. Cytoskeletal structure was examined using fluorescence microscopy following 24-hour treatment with cytoskeletal toxins. As Figure 4.2 shows, the cells in the 2 µM rotenone group overall had rounder, rather than elongated, morphologies. Nonetheless, microtubule structure remained intact in this and other treatment groups.
Figure 4.1: Effects of mitochondrial toxins on cell proliferation.
Graph shows the percent reduction of alamarBlue reagent as calculated according to the methods in section 4.3.2. “Anti” refers to antimycin A. Bars represent mean + standard deviation values from a total of six wells measured over two independent experiments. * indicates p<0.05.

Figure 4.2: Microtubules remain intact following treatment with mitochondrial toxins.
Scale bar is 50 µm.
As opposed to microtubules, some (but not all) cells exhibited rearrangement of the actin cytoskeleton following 24-hour exposure to 2 µM rotenone (Figure 4.3). Cells in the control and antimycin A groups exhibited elongated morphologies with parallel stress fibers throughout their cell bodies. By contrast, cells treated with 2 µM rotenone for 24 hours were rounder, consistent with observations from microtubule imaging. The actin stress fibers in these cells were largely relocated to the cell periphery, as opposed to throughout the cell body, and were oriented around the cell perimeter rather than remaining parallel to one another.

**Figure 4.3: Rotenone causes restructuring of the actin cytoskeleton in some cells.** Scale bar is 50 µm.
4.4.3 Single cell stiffness remains unchanged following treatment with mitochondrial toxins

After observing rearrangement of the actin cytoskeleton in many cells following rotenone treatment, we were interested in assessing whether this cytoskeletal change was reflected in mechanical measurements of single cells. We first used AFM in order to measure changes in the mechanical properties of single cells following exposure to mitochondrial toxins. The particular AFM instrument used was conjugated to a high-power optical microscope in order to facilitate precise spatial control of cellular indentation. We specifically indented cells in the perinuclear region of the cell in order to avoid both substrate effects, which can be substantial at the cell periphery (Mahaffy et al., 2004), and mechanical contributions from the nucleus, which may differ from the cytoplasm in its mechanical properties (Haga et al., 2000; Kuznetsova et al., 2007). These experiments only considered cells subjected to high doses of antimycin and rotenone (2 µM) and corresponding amounts of DMSO for control cells.

As Figure 4.4a shows, stiffness measurements were high non-normally distributed within a given group, with the DMSO group shown as an illustrative example. For this reason, we used rank sum tests to compare between control and treatment groups. Figure 4.4b demonstrates that cell stiffness remains similar across all groups, with a median stiffness of 2.25 kPa in the DMSO group, 2.26 kPa in the antimycin group, and 2.66 kPa in the rotenone group. The slightly higher stiffnesses in the rotenone group were dominated by data collected from one of three experimental days. In any case, no statistically significant difference in cell stiffness was identified between groups.
Figure 4.4: Mitochondrial toxins do not affect cell stiffness.
(a) Histogram of stiffness measurements from all individual cells in the DMSO group. (b) Bar chart representing stiffness measurements from all cells. Bar represent mean ± standard deviation in each group. N=60-67 cells per group, collected over 3 separate days.
4.4.4 Challenges in assessing changes in cell mechanics using QCM-D

Despite our results showing no obvious changes in cell stiffness as measured by AFM, we still believe that the cell rounding caused by exposure to 2 µM rotenone probably affects cell mechanics. As such, we were interested in assessing whether changes in cell mechanics induced by mitochondrial toxins could be detected by tools other than AFM. We turned to QCM-D, which complements AFM by its ability to measure changes in mechanics of a cell population rather than in single cells, and in real time as opposed to discrete time points. Additionally, QCM-D assesses mechanical changes occurring within the thin layer above the QCM-D sensor, in this case the base of the cells, while we used AFM to probe perinuclear areas near the cell apex. We had previously observed that changes in cell mechanics as measured by QCM-D could differ from those measured by AFM following the same drug exposure, and were therefore confident that a negative result from AFM did not necessarily foretell a negative result from QCM-D.

Unfortunately, we encountered many difficulties when attempting to perform these experiments. Our detailed experimental methodology is presented in Section 4.3.5. One persistent issue we had was that cells often did not survive being in the machine for ~36 hours. Figure 4a shows that control cells in the QCM-D were sparser and showed more cell death in comparison to cells originally plated on the same dish and treated identically in the incubator. This issue persisted and was even exacerbated when the QCM-D was set to higher temperature settings (Figure 4.5b) than the 21°C used in Chapter 3. Additionally, QCM-D readings always fluctuated or drifted over the 24-hour measurement period (Figure 4.5c). We could not confidently assert whether these changes reflected cell growth, death, changes in mechanics or simply noise. Furthermore, we previously showed (Chapter 3) that exposure to cytochalasin D, which
presents a massive mechanical upset to cells, results in only a slight change in dissipation as measured by the QCM-D. We therefore concluded that lesser mechanical changes would be extremely difficult to measure over 24 hours given the background changes in frequency and dissipation.

Figure 4.5: Challenges with using QCM-D for long-term cell experimentation. (a) Control cells treated with 0.2% DMSO for 24 hours in (i) the incubator and (ii) at 21°C in the QCM-D. Blue represents DAPI nuclear staining, green is the calcein cell viability dye, and red is propidium iodide, a cell death indicator. (b) Untreated cells on a QCM-D sensor after remaining in the instrument for 24 hours at 30°C. (c) Frequency and dissipation readings from a 24-hour QCM-D experiment with cells.
4.5 DISCUSSION

This work examined the cytoskeletal and mechanical consequences of pharmacologically induced mitochondrial toxicity. Cells were exposed to 500 nM or 2 µM of rotenone or antimycin A, respective inhibitors of complexes I and III of the electron transport chain, for 24 hours. Little cytotoxicity was observed, with only 2 µM rotenone resulting in a slight decrease in cell viability. Fluorescence microscopy revealed that microtubule structure was preserved in all control and experimental groups. The actin cytoskeleton, however, underwent restructuring in some cells exposed to 2 µM rotenone. These cells were rounder than control cells, and showed actin filaments preferentially located at the cell periphery rather than uniformly throughout the cytosol. Despite cytoskeletal rearrangements, stiffness measurements of individual rotenone-treated cells did not differ from control cells. Overall, this research suggests that drug-induced mitochondrial dysfunction does not provoke major mechanical or cytoskeletal changes in cells. However, alternative experimental conditions as well as additional methods for probing cell mechanics may yield different results, and are suggested as part of the discussion below.

In addition to the experiments described above, this work attempted to characterize cell mechanical changes on the population level using QCM-D. These efforts were unsuccessful due to several factors, including poor long-term cell viability in the QCM-D and concerns regarding instrument sensitivity. We hope that improvements to the QCM-D technology will result in better suitability for long-term cell experimentation. As we have had success with experiments involving shorter drug exposures to cells using this instrument (Chapter 3), it may be feasible to detect mechanical changes resulting from short-term, higher doses of mitochondrial toxins using QCM-D.
The concentrations of antimycin A used in this work, 500 nM and 2 µM, did not induce any measurable cytoskeletal or mechanical changes in cells. As discussed in Section 4.2, this drug is used over a very wide range of exposures in cells. It is possible that the concentrations used are too low to significantly impair mitochondrial function and in turn affect the cytoskeleton and cell mechanics. Future work should systematically optimize the antimycin A concentration for mitochondrial toxicity and examine resulting cytoskeletal and mechanical changes in cells.

Rotenone is known to induce apoptosis (Li et al., 2003) mediated by the release of ROS. It is possible that the cell rounding observed following treatment with 2 µM rotenone is indicative of apoptosis. However, only a slight decrease in alamarBlue reduction is observed, suggesting little cell death. Furthermore, cell softening which typically characterizes apoptotic cells (Hu et al., 2009; Kim et al., 2012) does not occur in our experimental system. Thus, rotenone-induced apoptosis causing cell rounding is probably in its early stages if it is happening at all.

Future directions for this work are discussed in greater detail in Chapter 8, but are briefly outlined here. First, we have established a method of measuring actin stress fiber alignment in other cell types (Appendix A) which may lend quantitative support to our observations of rotenone-induced cell rounding. Preliminary experiments subjecting bovine mesenchymal stem cells (bMSCs) to 2 µM rotenone for 24 hours yielded similar results to our findings using human dermal fibroblasts, but stress fibers in control and rotenone treated bMSCs were much more defined than in fibroblasts. The effects of rotenone on actin stress fiber alignment may therefore be quantifiable using this cellular system.

Another interesting direction for this work involves the use of traction force microscopy. In this experimental system, cells are plated on firm gels embedded with
fluorescent beads. The beads move in response to forces exerted by the cell on the substrate. The cell rounding we observe following treatment with rotenone likely affects the distribution and potentially magnitude of traction forces generated by the cell. Subjecting these cells to traction force microscopy will confirm whether any alterations in forces produced by cells indeed occur.

Interfering with mitochondrial function by exposing cells to mitochondrial toxins at the sublethal doses studied did not profoundly affect cell mechanics. Nonetheless, the cell rounding observed following rotenone exposure suggests some interesting follow-up experimentation which will serve to further characterize the mechanical effects of mitochondrial toxicity.
CHAPTER 5: PROGRESSIVE INCREASE IN MTDNA 3243A>G HETEROPLASMY IS ASSOCIATED WITH NONMONOTONIC ALTERATIONS IN CYTOSKELETAL PROTEIN EXPRESSION AND CELL MECHANICS

5.1 CONTEXT AND ACKNOWLEDGEMENTS

Pharmacological toxins are not the only means by which mitochondria can become dysfunctional. Since mitochondria have their own DNA (mtDNA), which primarily encodes subunits of the electron transport chain, defects in the mitochondrial genetic code can also lead to impaired mitochondrial function. Furthermore, since single cells contain many copies of mtDNA, some mtDNA copies can be mutated while others are not, a state termed heteroplasmy. Heteroplasmy level can further influence clinical presentation, as is the case with the m.3243A>G mutation studied in this work.

Here, we utilized a set of cell lines previously developed by our collaborators in the Wallace lab which contained identical nuclear genomes, but varying degrees of m.3243A>G heteroplasmy. These cells were previously shown to vary not only in mtDNA gene expression, but nuclear gene expression as well. Building on this, we set out to determine whether these cell lines differed from one another both structurally and mechanically, and found that this was indeed the case. This work is to our knowledge the first demonstration of mtDNA mutations affecting single cell mechanics.

At the time of submission of this dissertation, this work was in preparation under the title “Progressive increase in m.3243A>G heteroplasmy is associated with nonmonotonic alterations in cytoskeletal protein expression and cell mechanics” by
Judith Kandel, Martin Picard, Douglas C. Wallace and David M. Eckmann. Dr. Martin Picard was involved in collection and analysis of the cytoskeletal gene expression measurements, and was also very involved in crafting of the manuscript. I conducted all experiments and analysis beginning from the protein production experiments. Dr. Douglas C. Wallace served as Martin’s advisor and also oversaw the original creation of the m.3243A>G cybrid lines.

The work presented in this chapter is perhaps the most interdisciplinary study in this dissertation, and benefited from the assistance of many people in addition to the co-authors. Many thanks to the laboratories of Drs. Roderic Eckenhoff and Max Kelz for immunocytochemistry reagents. Dr. Brian Weiser, Dr. Weiming Bu, Dr. Eric Abhold, Tim DeYoung and Kellie Wohl provided invaluable help with Western blotting experiments, and Dr. Bo Han assisted with immunofluorescence protocols. Drs. Xilma Ortiz-Gonzalez and Alessia Angelin were helpful with confocal microscopy. AFM related work was aided by Dr. Prathima Nalam, Dr. Matthew Caporizzo, Dr. Matthew Brukman, and Emmabeth Parrish Vaughn. Benjamin Kandel was extremely helpful with image processing and statistical analyses. Dr. Martha E. Grady is acknowledged for general AFM guidance and overall feedback in manuscript preparation.

This work was funded by the Office of Naval Research grant N000141410538, National Institutes of Health grants T-32-HL-007954, NS21328 and DK73691, the Horatio C. Wood Endowment at the University of Pennsylvania, Simons Foundation grant 205844, and the Canadian Institute of Health Research. The TIRF-AFM instrumentation is additionally funded through the Penn Nano/Bio Interface Center via NSF Major Research Instrumentation Grant DBI-0721913, and NSF Nanoscale Science and Engineering Center grant DMR-0425780.
5.2 INTRODUCTION

Clinical diseases are often studied at the cellular level in order to elicit greater knowledge about their underlying mechanisms. Current trends in cell-based research tend to fall into one of two groups. Biologists tend to consider that metabolic and mitochondrial dysfunction are primary aspects of cellular dysfunction, while bioengineers are largely focusing on how pathology manifests as alterations in cell mechanics.

A wealth of literature now suggests that cellular mitochondrial and mechanical dysfunction are in fact related. Structural studies have mostly focused on the association between mitochondria and microtubules (Ball and Singer, 1982), but recent work show that mitochondria are structurally connected to actin filaments (Morris and Hollenbeck, 1995), and that filament breakdown impairs mitochondrial motility (Kandel et al., 2015). Previous studies further suggest that the physical connection between mitochondria and the cytoskeleton contributes to cell mechanics. For example, mitochondrial subcellular localization is regulated during cell migration (Campello et al., 2006; Desai et al., 2013b), and mitochondria at the cell periphery have a higher membrane potential in some cell types (Collins et al., 2002), where traction forces are usually highes (Munevar et al., 2001). Combined, these observations suggest a functional connection between mitochondria and cellular mechanical activity.

In the pathological realm, both acute and chronic cell dysfunction are often characterized by concurrent alterations in cell mechanics and mitochondrial function. Anoikis, or cell death caused by detachment from the substrate, activates pro-apoptotic mitochondrial proteins (Reddig and Juliano, 2005), whereas engagement of substrate-attaching integrins promotes expression of anti-apoptotic ones (Zhang et al., 1995). Integrin inhibition also triggers the release of mitochondrial reactive oxygen species (Werner and Werb, 2002), which may further contribute to cell death. In addition to acute
cellular toxicity, clinical pathologies often demonstrate parallel mitochondrial and mechanical dysfunctions at the cellular level. Cancer cells exhibit the well-known glycolytic Warburg effect (Warburg, 1925), are softer (Cross et al., 2007) and generate weaker traction forces (Munevar et al., 2001) than normal cells. Metabolic alterations are also pervasive in endothelial and cardiomyocyte cells from patients with cardiovascular disease (Ren et al., 2010), and these cells tend to be less flexible than cells from healthy patients (Simons et al., 1999). Thus, mitochondrial and mechanical alterations frequently occur in parallel in pathological conditions. However, the directionality and causality of this relationship remains unclear.

In this work, we studied a unique cellular model of localized mitochondrial dysfunction, caused by increasing levels of a mtDNA point mutation in the cell cytoplasm. We focused on the m.3243A>G mutation, the most common pathogenic mtDNA defect that ultimately results in electron transport chain deficiency (Sasarman et al., 2008). Because the cytoplasm contains hundreds of mtDNA copies, normal (m.3243A) and mutant (m.3243G) mtDNAs can coexist within a single cell in a state termed heteroplasmy. M.3243A>G heteroplasmy profoundly affects clinical presentation, with low levels (<30%) associated with diabetes and deafness (van den Ouweland et al., 1992), higher levels (50-90%) causing mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS syndrome) (Goto et al., 1990), and extremely high levels causing Leigh syndrome and early death in infancy (Koga et al., 2000). We previously showed that selectively increasing m.3243A>G heteroplasmy perturbed mitochondrial gene expression and resulting bioenergetics, and additionally had a profound, nonmonotonic influence on a variety of nuclear encoded genes (Picard et al., 2014). Since these cell lines are distinguished from one another exclusively on the basis of mtDNA heteroplasmy level, this experimental paradigm is ideal for studying the effects
of an isolated mitochondrial defect on cytoskeletal mechanics. Thus, we conducted further experiments on the cybrid cell lines based on the hypothesis that mtDNA mutations will breed alterations in the cellular cytoskeletal structure and ultimately cell mechanics. Our results demonstrate that increasing mitochondrial dysfunction caused nonmonotonic alterations in gene expression and protein abundance of various mechanically active cytoskeletal components, which translated into abnormal cellular cytoarchitecture and ultimately altered cell stiffness as determined by AFM. Overall, our research suggests that the relationship between mitochondrial mutations and altered cellular mechanics is complex and nonlinear. Rather, our data suggest that mtDNA heteroplasmy causes nonmonotonic alterations in gene expression and cytoskeletal components, which may in turn drive changes in cell mechanics. Further studies of mitochondrial energetics and mechanical alterations in single cells should elucidate the mechanisms linking these layers of regulation.

5.3 MATERIALS AND METHODS

5.3.1 Generation of transmitochondrial cybrids

Cytoplasmic hybrid cell lines (cybrids) with increasing levels of mtDNA mutations (m.3243A>G) were generated as described by Picard et al (Picard et al., 2014). The process is also illustrated in Figure 5.1. Briefly, mtDNA-deficient (rho-0; ρ0) cell line derived from the osteosarcoma 143B thymidine kinase-deficient (TK-) cell line was fused to enucleated lymphoblastoid cells (Trounce et al., 1996) from a patient heteroplasmic for the tRNALeu(UUR) nucleotide m.3243A>G mutation (Heddi et al., 1999). Transmitochondrial cybrids having acquired the lymphoblastoid mtDNAs were then selected, cybrid colonies were isolated, and screened for heteroplasmy for the tRNALeu(UUR) m.3243A>G mtDNA mutation by polymerase chain reaction (Goto et al., 2014).
Clones heteroplasmic for different percentages of the m.3243A>G mutation were generated by partial depletion of the cybrid clones with ethidium bromide (EtBr), followed by removal of the EtBr permitting return to the normal mtDNA content, cloning, and screening for the percentage of m.3243A>G heteroplasmy (Wiseman and Attardi, 1978). Clones were screened for those harboring the desired range of m.3243A>G heteroplasmy ultimately resulting in a series of clones harboring approximately 0, 20, 30, 50, 60, 90, and 100% m.3243G mutant mtDNA, plus the parental ρ0 cell line devoid of mtDNA.

5.3.2 Measurement of mtDNA heteroplasmy

Heteroplasmy levels were monitored by two methods. First, PCR amplification of the 264 nt fragment, digestion with HaeIII (New England Biolabs #R0108), and separation of the fragments initially by agarose gel electrophoresis and densitometric analysis and subsequently by capillary electrophoresis on an Genetic Analyzer 3500 automated sequencer (Applied Biosciences) (Desquiret-Dumas et al., 2012). Second, by sequencing the entire mtDNA using next generation sequencing on an Ion Personal Genome Machine (PGM) using Ion PGM Sequencing 200 v2 kit (Life Technologies). Data was analyzed using the Ion Torrent Suite v4.0.1 and NextGENe software v2.3.3 and A/G heteroplasmy levels at nucleotide position 3243 were verified. Results were in the expected range, are given in greater detail in our earlier work (Picard et al., 2014).
5.3.3 Gene expression measurements

Transcript levels were determined by RNA sequencing (RNA-Seq) as previously described (Picard et al., 2014). Total RNA was extracted with Trizol and depleted from ribosomal units (RiboMinus, Life Technologies #10837-08). RNA was quality checked and quantified on a Bioanalyzer 2100, RNA 6000 Nano kit (Eukaryote Total RNA Nano, Agilent Technolgies) and Qubit 2.0 fluorometer RNA assay kit (Molecular Probes).
Five hundred ng of RNA was used for cDNA library preparation using (Ambion, Total RNA-Seq kit #4445374).

RNA from all eight experimental groups (transmitochondrial cybrid cell lines 0, 20, 30, 50, 60, 90 and 100 % m.3243G heteroplasmy, and ρ₀ parental cell line) were sequenced on the ABI SOLiD 5500 platform. Barcoded triplicates were sequenced on the same slide in different lanes. All sequencing was performed using paired-end chemistry of 50 (forward) x 35 (reverse) base pairs. Sequenced reads were mapped and processed as previously described (McVean et al., 2012).

Data was further analyzed using Partek Genomics Suite™ software version 6.12.0109. Out of a total of 22,449 uniquely annotated genes for which transcripts were detected, 15,652 were differentially expressed (ANOVA model, P < 0.0001). Data was normalized and expressed as reads per kilobase per million reads (RPKM). Normalizing data using R package DESeq yielded similar results. Reads for each gene was extracted, averaged among triplicates, and expressed relative to 0% heteroplasmy. While gene expression was measured in triplicate, error bars were extremely small for all data points, so they are omitted from both graphs in Figure 5.3.

5.3.4 Cell culture

Cells were cultured in T25 and T75 flasks containing DMEM high glucose buffer (Life Technologies, Grand Island, NY) supplemented with 10% FBS (Sigma, St. Louis, MO), 1% non-essential amino acids (Life Technologies), and 50 µg/mL uridine (Sigma). Cells were generally passaged at 80% confluency using 0.25% trypsin (Life Technologies, Grand Island, NY). All experiments were performed within several passages, when the cells had been growing in culture for ~48 hours, except gene
expression which was measured previously on early passage number (Picard et al., 2014).

### 5.3.5 Protein isolation and Western blotting

Cells were grown in T75 flasks and detached using 0.25% trypsin and centrifuged twice in culture media with the supernatant discarded. The cell pellet was lysed in RIPA buffer (Bio-World, Dublin, OH) supplemented with protease inhibitors (Roche, Manheim, Germany), and then centrifuged at 14,000xg to remove the insoluble pellet. The protein concentration of each cell line was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA) as per the manufacturer’s instruction. Standards and samples were appropriately diluted and pipette into a Costar 96-well plate (Corning) in triplicate. Absorbance at 562 nm was measured using a Biotek Synergy H1 microplate reader and Gen5 software (Biotek, Winooski, VT), and sample protein concentrations were extracted from the resulting standard curve.

For Western blotting, 10 µg total protein was separated on 4-15% SDS-PAGE gels (Biorad, Hercules, CA) and transferred to PVDF membranes (Millipore, Billerica, MA). Membranes were blocked with 2.5% BSA (Sigma) in TBS with Tween-20 solution (TBST) for 1 hour at room temperature and incubated with primary antibodies (1:1000) overnight on a rocker at 4°C. After washing 3 times with TBST, membranes were incubated with secondary antibody (1:5000), and washed 3 times with TBST. Protein-antibody complexes were detected using the Amersham ECL Western Blotting Analysis System (GE Healthcare, Piscataway, NJ) according to manufacturer’s instructions, and exposing using a Kodak Image Station 4000MM Pro Digital Imaging System and Carestream MI software (Carestream, Inc, Rochester, NY). When two proteins of similar molecular weights were of interest in the same blot, the membrane was stripped using...
Restore™ Western Blot Stripping Buffer (Life Technologies). Protein isolation followed by Western blotting was performed in duplicate for each protein of interest.

Primary antibodies used for blotting and immunofluorescence included anti-α-actinin 1 isoform (Abcam, Cambridge, MA, #18061), anti-pan-actin (Cytoskeleton, Inc, Denver, CO, #AAN01), and anti-TATA binding protein (TBP, Abcam #51841) as a housekeeping protein. For blotting, secondary antibodies included anti-mouse IgG labeled with horseradish peroxidase (Thermo Scientific) and anti-rabbit IgG labeled with horseradish peroxidase (GE Healthcare).

Quantification of band density was done using ImageJ. Each band was highlighted using an identically sized rectangular selection, and its background-corrected density was normalized to the corresponding TBP-band background-corrected density to yield a final normalized protein level. To determine whether the observed pattern of actin protein abundance was linearly correlated with the gene expression data, we first tested whether the data followed a mixed effects model, with experiment number serving as a random effect. The experiment number was shown to have no effect on the protein expression data, so we pooled data from both experiments when testing for correlation. Each normalized band density was then plotted against the corresponding average normalized value for actin gene expression, shown in Figure 5.3c.

5.3.6 Immunofluorescence and fluorescence microscopy

For imaging, cells were plated at a concentration of 25,000 cells/dish on 30-mm glass-bottom dishes (Mattek, Ashland, MA) coated with 10 µg/mL fibronectin (Corning, Tewksbury, MA) for 30-40 minutes.

For visualization of actinin by immunofluorescence, cells were washed with ice cold PBS 3 times, then fixed on ice for 10 minutes using 4% paraformaldehyde. Cells
were then permeabilized on ice with 0.2% triton for 20 minutes, followed by incubation at room temperature with primary antibody (15 µg/mL diluted in antibody dilution buffer (1:100 goat serum in PBS)) for 1 hour. After washing 3 times with cold PBS, cells were incubated at room temperature with secondary antibody (Alexa Fluor 488 goat-anti-mouse IgG (Life Technologies), 1:200 in antibody dilution buffer) for 30 minutes. Washing was repeated and cells were visualized with a fluorescence microscope.

Microtubules were stained using 250 nM Tubulin Tracker Green (Life Technologies). For actin visualization, cells were fixed and permeabilized as described, and incubated with 100 nM Acti-stain 555 phalloidin (Cytoskeleton, Inc). Nuclei were stained by incubating cells with a 2 µg/mL concentration of Hoechst 33342 (Life Technologies) diluted with PBS for 30 minutes before rinsing. Live cells were imaged in recording HBSS (pH 7.4 with 1.3 mM CaCl₂, 0.9 mM MgCl₂, 2 mM glutamine, 0.1 g/l heparin, 5.6 mM glucose, and 1% FBS), and fixed cells were imaged in PBS.

For preliminary imaging, cells were visualized using wide-field fluorescence microscopy. This setup employed a QImaging QIClick camera (QImaging, Surrey, BC, Canada) (1x1 binning, 1392x1040 pixels) attached to Olympus IX70 microscope (Olympus, Melville, NY) with an Olympus 100x oil immersion objective lens (Olympus) and Photofluor light source (89 North, Burlington, VT). Computer control of the microscope was facilitated by LUDL programmable filter wheels, shutters, and focus control (LUDL Electronic Products, Hawthorne, NY) and images were collected using IPL 3.7 software (BD, Rockville, MD). For each experiment, cells were visualized using standard TRITC, FITC and DAPI filters. Images of the tubulin-stained cells shown in Figure 5.4 were acquired using this setup.

Actin and actinin imaging shown in Figures 5.5 and 5.6 used confocal microscopy. Imaging was performed the same day as staining (~48 hours after plating).
using a Zeiss LSM 710 inverted laser scanning confocal microscope with a PlanApo 63x/1.40 oil immersion objective, optovar 1.25x and digital zoom of 1, 1024x1024 image size (final pixel size = 0.132 x 0.132 µm). Excitation was applied with 405nm, 488nm and 543nm lasers as separate channels, and emission filters specific to each fluorophore used. Images were acquired and processed with the Zen 2010 software at the following settings: 1024 x 1024 pixels; pixel dwell time = 3.15 µsec.

5.3.7 Image processing and analysis

ImageJ was used for initial image processing. Images were separated into appropriate color channels. The actin channel (543 nm) was used to create a mask outlining the shape of each cell. Actin images from each cell were first stacked together. A Gaussian blur with a 2-pixel radius was then applied to the stack, followed by thresholding by eye to include the cells but not noise. Cells other than the cell of interest were cropped out of each frame. Any unfilled holes in the outline of each cell were manually filled in. Figure 5.5a shows resulting masks for several sample cells. Matlab was used for all subsequent analyses, with the custom script provided in the Supplementary Information.

The Matlab built-in object recognition command `regionprops` was used to analyze circularity of cells. Cell masks were read by Matlab and analyzed for area and perimeter. For each image, only the maximum area and perimeters were analyzed in order to exclude small pixels of noise. Circularity $C$ was calculated as

$$C = \frac{4\pi A}{P^2}$$  \hspace{1cm} (5.1)

where $A$ is the cell area and $P$ is the cell perimeter. Circularity is 1 for a perfect circle and increasingly less than 1 for increasingly less circular objects.
To estimate the relative percentages of the cells inhabited by actin filaments, the actin channel images were masked in Matlab using the masks described above so as to only consider the area inside the cell. To avoid any intensity saturation, the 95th percentile of intensity was set as an intensity ceiling for each image, and the number of pixels greater than 55% of this ceiling were counted as actin-containing pixels. This method detected different intensity distributions based on normalizing each cell to its own maximum intensity, so that differences in dye uptake had no effect. 55% was chosen based on visual confirmation that thresholding at this level consistently highlighted actin filaments and minimized noise selection. Figure 5.5a shows some examples of thresholded images. The number of pixels exceeding the threshold set for each cell was normalized to cell area and then expressed as a percentage.

Actinin thresholding was performed similarly to actin thresholding, using 50% rather than 55% of the 95th percentile of intensity based on trial-and-error. Positions of actin and actinin pixels exceeding these thresholds were recorded, and the Matlab function knnsearch was used to find the nearest neighboring actin-containing pixel for each actinin-containing pixel.

5.3.8 Atomic force microscopy

AFM experiments used 100,000 cells cultured on 22x40 mm coverslips (Fisher Scientific, Pittsburgh, PA) coated with 10 µg/ml fibronectin for 30-40 minutes and placed in 60-mm Falcon dishes (Corning). This work used tipless cantilevers with nominal resonant frequencies of 10-20 kHz and nominal spring constants of 0.03-0.09 N/m (NanoAndMore, Lady’s Island, SC, #CSC38/tipless/No Al). Silica colloids were attached to the cantilevers using a micromanipulator attached to a long-distance optical
microscope (40X, Alessi REL-4100A, NJ). Silicon dioxide microspheres (C-SiO-5.0, diameter ~4.86 µm, Microspheres-Nanospheres, Corpuscular, NY) were glued to the cantilevers using two-part epoxy glue (JB Weld, Sulphur Springs, TX) with the help of a sharp tungsten wire (TGW0325, World Precision Instruments, Sarasota, FL). The glue was allowed to cure overnight at room temperature. The cantilevers were cleaned in ethanol (Sigma) and were UV-ozone treated (UVO Cleaner model 42, Jelight Co. Inc., Irvine, CA, USA) for at least 15 minutes before use. The colloid radius was measured optically using the 100x oil immersion lens on the Nikon microscope (described below). A sample image of the colloid is shown in the inset in Figure 5.7a.

An Asylum MFP-3D AFM conjugated to a Nikon Total Internal Reflectance Fluorescence (TIRF) microscope was used in force spectroscopy mode to probe cellular mechanical properties. Colloid-attached cantilevers were calibrated in DI water prior to each experimental session using the thermal-noise method (Hutter and Bechhoefer, 1993). The measured spring constants used for force spectroscopy ranged 0.06-0.21 N/m. Deflection sensitivity of the cantilevers was measured from the slope of the force-distance (FD) curves obtained on a clean glass slide in recording HBSS before the start of the experiment. FD curves on cells were acquired at the perinuclear region of the cell with an approach rate of 500 nm/s. The perinuclear region of the cell was chosen so as to avoid both substrate effects, which can be substantial at the cell periphery (Mahaffy et al., 2004), and mechanical contributions from the nucleus, which may differ from the cytoplasm in its mechanical properties (Haga et al., 2000; Kuznetsova et al., 2007). Repeated cell indentation was performed every few seconds, which is likely infrequent enough to allow for relaxation in between indentations (Caporizzo et al., 2015).
Cells were kept in an incubator with 5% CO$_2$ at 37°C until experiments, which were performed at room temperature in HBSS solution. FD curves were taken from 6-10 cells per dish, and dishes were discarded 30-60 minutes after experimentation began. A representative FD curve measured on a cell is shown in Figure 5.7a. Asylum research software AR 12 version was used to fit the Hertz model to the approach curves. The Hertz model relates the force on the cantilever (F) to the depth of indentation in the sample (δ) by

$$F = \frac{4}{3}E^*\sqrt{R}\delta^{3/2}$$  \hspace{1cm} (5.2)

where $R$ is the tip radius and $E^*$ is the combined elastic modulus of the contact and is given by

$$\frac{1}{E^*} = \frac{1-\nu^2_{cell}}{E_{cell}} + \frac{1-\nu^2_{tip}}{E_{tip}}$$  \hspace{1cm} (5.3)

where $\nu$ is the Poisson ratio, which is 0.17 for the fused silica tip and assumed to be 0.37 for cells (Shin and Athanasiou, 1999). Combining equations 1 and 2 allows for the determination of $E_{cell}$, the cell elastic modulus. In this work, we extracted $E_{cell}$ from the data spanning 25% to 75% of the maximum force used. The amount of indentation this corresponded to depended on $E_{cell}$ and usually was several hundred nm, which is considerable smaller than the thickness of the cell (~2-3 µm) in the perinuclear region. The depth of indentation was small in comparison to the radius of the colloid ($\delta/R < 0.1-0.2$). Force curves were repeatedly obtained for a given cell until 4-6 consecutive curves gave consistent measures of $E_{cell}$ (with the extreme measures not differing by more than ~15%). The resulting averaged $E_{cell}$ was used for data analysis. Each cell line had data pooled from 34-57 cells total, measured over two separate days. For linear regression, the input stiffness value for each cell was the mean elastic modulus, and the input gene expression value was the mean gene expression level for that particular gene in that cell.
line. Regressions were also performed by plotting the geometric means of elastic modulus by cell line against the mean expression level of a given gene for that cell line.

5.3.9 Statistics

Matlab and R were both used for statistical analysis. Linear regressions were performed using the `corrcoef` command in Matlab or the `lm` command in R. R was also used for adjusting p-values for multiple comparisons (p.adjust) using the Benjamini-Hochberg method and linear mixed effects modeling (lm) with ANOVA to test for effects of experiment number. Analysis of variance (ANOVA) was performed using the `anova1` command in Matlab. The p-values are generally accepted as significant when p<0.05.

5.4 RESULTS

5.4.1 Gene Expression

Transcript levels for various cytoskeletal genes in the eight m.3243A>G cybrid cell lines were measured by RNA sequencing. We analyzed genes encoding alpha-actinin (α-actinin), filamin A, vimentin, and expressed isoforms of actin and tubulin. Figure 5.2a illustrates the cumulative gene expression for these genes in each of the cell lines. The graph reveals, for example, that the gamma-actin (Γ-actin) isoform is expressed at higher levels than the beta-actin (β-actin) isoform in all of the cell lines. In comparison, actinin expression levels are substantially lower than other genes examined. Figure 5.2b shows the same data normalized to the 0% cell line (no mtDNA mutation), enabling comparison of relative changes as mitochondrial function increases. This illustrates that actinin and Γ-actin gene expression vary by approximately 3-fold across the cell lines, while β-actin, and vimentin exhibit changes of lower magnitude.
Figure 5.2: Cytoskeletal gene expression varies in the m.3243A>G cybrid cell lines.

(a) RNAseq data showing absolute cumulative gene expression of various cytoskeletal proteins in each of the eight cell lines. (b) Data normalized to the 0% cell line. Gene expression was measured in triplicate, but error bars are omitted in the graphs since they are too small to be easily resolved. \( \rho_0 \) cells lack any mitochondrial DNA, but contain the same nuclear genome as the other cell lines.
Table 5.1: Coefficients of determination and adjusted p-values showing significance of linear relationships between expression of different cytoskeletal genes in m.3243A>G cybrids.

<table>
<thead>
<tr>
<th>Gene</th>
<th>R²</th>
<th>β-actin</th>
<th>α-actinin</th>
<th>Tubulin α1a</th>
<th>Tubulin α1b</th>
<th>Tubulin β</th>
<th>Filamin A</th>
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<tr>
<td>β-actin</td>
<td>0.407</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>X</td>
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<tr>
<td></td>
<td>Adjusted p-value</td>
<td>2.05 x 10⁻³</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
<td>α-actinin</td>
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<td>0.598</td>
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<tr>
<td></td>
<td>Adjusted p-value</td>
<td>1.31 x 10⁻⁴</td>
<td>6.63 x 10⁻⁵</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
<td>Tubulin α1a</td>
<td>0.109</td>
<td>0.127</td>
<td>0.0020</td>
<td>X</td>
<td>X</td>
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<tr>
<td></td>
<td>Adjusted p-value</td>
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<td>0.145</td>
<td>0.833</td>
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<td>0.225</td>
<td>0.0459</td>
<td>0.610</td>
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<tr>
<td></td>
<td>Adjusted p-value</td>
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<td>0.0337</td>
<td>0.383</td>
<td>6.17 x 10⁻³</td>
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<td>Tubulin β</td>
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<td>X</td>
</tr>
<tr>
<td>Filamin A</td>
<td>0.368</td>
<td>0.470</td>
<td>0.906</td>
<td>0.00731</td>
<td>0.0913</td>
<td>0.484</td>
<td>X</td>
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<tr>
<td></td>
<td>Adjusted p-value</td>
<td>3.59 x 10⁻³</td>
<td>6.79 x 10⁻⁴</td>
<td>2.48 x 10⁻¹¹</td>
<td>0.717</td>
<td>0.212</td>
<td>5.55 x 10⁻⁴</td>
</tr>
<tr>
<td>Vimentin</td>
<td>0.315</td>
<td>0.0078</td>
<td>0.0280</td>
<td>0.454</td>
<td>0.243</td>
<td>0.0821</td>
<td>0.0271</td>
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<tr>
<td></td>
<td>Adjusted p-value</td>
<td>8.69 x 10⁻³</td>
<td>0.717</td>
<td>0.496</td>
<td>8.58 x 10⁻⁴</td>
<td>0.0267</td>
<td>0.233</td>
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</table>

The values of gene expression for each gene in each of the cybrid cell lines were tested pairwise for linear regression. N = 3 per data cell line, total 24 total data points per regression. P-values were adjusted using the Benjamini-Hochberg method for the 28 total comparisons. Values with \( P < 0.05 \) are shown in bold.
Overall, as expected from our previous studies (Picard et al., 2014), cytoskeletal gene expression does not monotonically correlate with m.3243A>G heteroplasmy levels in the different cybrid cell lines, but follows a similar pattern across related genes. For example, tubulin α1a and tubulin α1b appear to exhibit a specific pattern, while α-actinin and filamin A appear to follow a different one, suggesting co-regulation of functionally-relates cytoskeletal elements. To test whether associations between different genes are statistically significant we applied linear regression analysis to the dataset and corrected the resulting p-values for multiple comparisons (Table 5.1). The strongest correlation was between α-actinin and filamin A (Figure 5.2b), with an adjusted p-value of 2.48 x 10\(^{-11}\). This is consistent with the data presented in Figure 5.2b, showing that these two genes follow strikingly similar expression patterns. In addition, α-actinin expression was also positively correlated with both isoforms of actin studied (p<0.001 and p<0.0001 for Γ-actin and β-actin, respectively), and filamin A was positively correlated both isoforms of actin (p<0.01 and p<0.001 for Γ-actin and β-actin, respectively). Transcript levels of other cytoskeletal elements, including various tubulin isoforms, were also significantly correlated (Table 5.1).

5.4.2 Protein Abundance

We focused our subsequent analyses on actin due to its well-known role in cell mechanics (Janmey and McCulloch, 2007). To assess whether gene expression variations translated into protein levels, we measured the relative abundance of actin and actinin in these cell lines. To select a housekeeping protein for normalizing actin levels, we avoided traditional glycolytic (i.e., GAPDH) and cytoskeletal elements, and instead selected the nuclear TATA-binding protein. Figure 5.3a shows a representative Western blot of actin across cybrid cell lines, as well as the corresponding TBP bands.
This experiment was repeated, and the quantified band densities from both experiments are given in Figure 5.3b. Data from both experiments were averaged for each cell line, and the percent difference of each data point from the average for that cell line ranged from 1.8-22.8%.

Measured actin protein significantly correlated with y-actin transcripts level (Figure 5.3c). No correlation was found between actin protein and y-actin gene expression. However, combining total mRNA transcripts of both actin isoforms normalized to the 0% cell line yielded a significant correlation with total actin protein abundance.

Similar experiments were performed to quantify actinin protein levels. However, no correlation between protein and gene expression was found (data not shown), possibly because of the relatively low expression of actinin as compared with actin (Figure 5.2a).
Figure 5.3: Actin protein production varies in the 3243A>G cybrid cell lines and correlates with gene expression levels.

(a) Sample actin Western blot showing actin bands and corresponding TBP bands used as a housekeeping protein. (b) Resulting protein expression obtained by normalizing the actin band density to the TBP band density. This experiment was repeated twice, with each experiment represented by a different color. (c) Linear fits between normalized actin protein expression and actin gene expression (from Fig. 1a). Each data point was considered individually in the analysis after a mixed effects model showed that experiment number did not serve as a random effect on the protein level. Linear fits were performed for gene expression of β-actin (blue squares), Γ-actin (purple triangles), and combined β- and Γ-actin (black circles). Significant correlations were found for Γ-actin (adjusted p=0.00573, R²=0.508) and combined β- and Γ-actin (adjusted p=0.01361, R² = 0.395), but not for β-actin (adjusted p=0.407, R² = 0.050).
5.4.3 Actin and Actinin Imaging

We next used wide-field fluorescence microscopy to visualize relative amounts of tubulin, actin and α-actinin in the cybrid lines at the single cell level. For tubulin, we observed no major differences in staining (intensity and sub-cellular distribution) between the different cell lines (Figure 5.4). On the other hand, the actin cytoskeleton filaments appeared differently among the different cell lines, so we used confocal microscopy to obtain higher resolution images of the cells stained for actin and actinin. We focused on the adherent layer of the cell in order maximize visualization of proteins. Figure 5.5a shows representative images from several of the cell lines, while Figure 5.6 displays multiple cells from each of the cell lines. Consistent with the oncogenic nature of cybrid cell lines, actin staining largely presented as disordered aggregates and fewer organized stress fibers than are typically present in normal cells. In general, we observed substantial cell-to-cell heterogeneity in actin staining as might be expected (Figure 5.6). However, when looking at populations of >10 cells, certain trends were apparent. In particular, the 90% heteroplasmic cell line, where mitochondria are highly dysfunctional and energy production is minimal, consistently showed a denser actin network with more pronounced stress fibers than any other cell line (Fig. 3a, Fig. S3). This is consistent with the gene expression data showing that this cell line had among the highest expression levels of Γ- and β-actin. Most other cell lines showed actin structural networks which appeared overall similar to one another, with the 30% and 60% cell lines displaying slightly denser stress fiber networks. Again, this observation correlates with the relative rise in expression of actin and actin-related (actinin and filamin A) genes compared to cells with only normal mitochondria (0% m.3243A>G) (Figure 5.2). We quantified these observations by looking at the relative percentage of actin in each cell line (Figure 5.5b; see Materials and Methods for greater detail on
methodology). Figure 5.5a shows the thresholded actin images used for these measurements. Although the one-way ANOVA comparing the different cell lines did not achieve significance (p=0.0892, the data confirmed that the 90% cell line harbored the highest actin density (median of 27.35%), followed by the 30% (median 25.98%) and 60% (median 26.62%) cell lines. Of these cell lines, the 90% has the smallest spread, indicating a more consistently dense actin cytostructure.

![Figure 5.4: Sample images of different m.3243A>G cells with stained microtubules and nuclei. Scale bar is 20 µm.](image)
Figure 5.5: Sample fluorescent images demonstrate cytoarchitectural heterogeneity among several of the m.3243A>G cell lines. (a) Images are identically contrast-adjusted for better visualization. Scale bar is 20 µm. Also shown are masks corresponding to each sample image, and thresholded actin images, which are both described in detail in the Materials and Methods section. (b) Proportions of cell cytoplasm occupied by actin-containing pixels for each heteroplasmym level, ANOVA p=0.089. The percentages of actin in the individual sample cells shown in (a) measured 21.92% (20%), 32.1% (90%), and 20.3% (ρ0). (c) Boxplot showing circularity measurements by cell line. One outlier is omitted from the plot for better data visualization. The overall ANOVA for the analysis gave p = 0.0407. The circularity measurements of the sample cells shown in (a) measured 0.3108 (20%), 0.0685 (90%), and 0.0927 (ρ0). For both (b) and (c), N=15 cells per group. Box borders show 25th (lowest line), 50th (center line) and 75th (highest line) percentiles for each cell line; whiskers show 10th and 90th percentiles. Outliers are shown as + signs.
Figure 5.6: Sample images of m.3243A>G cells with stained actin, actinin, and nuclei. The columns provide examples of the cell-to-cell heterogeneity observed in each cell line.
We performed linear regressions to model the relationship between the percentages of actin containing pixels in each image with the corresponding expression levels of different actin genes for that cell line (Figure 5.2a). Adjusted p-values for these regressions either achieved or trended toward significance, with Γ-actin p=0.0885, β-actin p=0.0269, and combined Γ- and β-actin p=0.0535. These p-values are potentially elevated because the ρ₀ cells’ cytostructures are morphologically different than those of the 90% cells, as opposed to similar actin gene expression levels among the two cell lines. These findings demonstrate consistent effects of mtDNA heteroplasmy on actin at both the gene expression level and in F-actin structure measured in individual cells.

The ρ₀ cell line, which lacks mtDNA and therefore functional mitochondria, surprisingly lacks dense bundles of stress fibers which might be expected from the gene expression (Figure 5.2) and protein abundance (Figure 5.3) data. However, ρ₀ cells typically contained relatively more actin-ric (Mitchison and Cramer, 1996) filopodia protruding from the cytoplasm than in most other cell lines. We quantified this observation by considering the circularity of a mask of each cell (Figures 3a). The resulting data (Figure 5.5c) showed the lowest circularity measurements for the 90% and ρ₀ cells, with medians of 0.1165 and 0.1299, respectively. An analysis of variance in these cell lines gave an overall p-value of 0.0407. This indicates superior spreading and dynamic actin turnover in the 90% and ρ₀ cell lines. Overall, these observations support the notion that the subcellular organization and localization of actin filaments is influenced, in a dose-response but not monotonic manner, by the degree of mitochondrial dysfunction.

Actinin, which acts as a cross-linker of actin filaments (Sjöblom et al., 2008), tended to colocalize with actin. Higher magnification images (Figure 5.5, insets) illustrate actinin positioning along actin stress fibers and at the cell periphery. Colocalization
analysis (see Materials and Methods for more detail) showed that across all cell lines, 56.39 ± 6.04% of actinin-containing pixels were also occupied by actin, and this measurement rose to 79.96 ± 5.77% when actin pixels within a 2-pixel (264 nm) radius of each actinin pixel were considered to be colocalized with it. Since on average, 25% of a given cell contained actin according to our thresholding analysis, and 20% contained actinin, random colocalization would occur about 5% of the time. Our observation showing over ten times that percentage supports the association between actin and actinin, and is consistent with the significant correlation between actin and actinin gene expression (Table 5.1).

5.4.4 Cell Stiffness Measurements

Finally, we addressed whether the alterations in cytoskeletal gene and protein expression translated to quantitative changes in cell mechanical properties as a function of mitochondrial heteroplasmy. We used AFM in order to measure cell stiffness. Figure 4a shows a typical force-distance curve from a cybrid cell measurement, as well as the fitted Hertz model and resulting elastic modulus. The inset in Figure 5.7a shows an image of an AFM tip with an attached colloid in contact with the perinuclear region of a cybrid cell.

Data were collected from 34-57 cells per line. The cybrid cell lines in general were relatively soft, with elastic moduli on the order of ~500 Pa. This is expected based on the cybrids’ osteosarcoma origin, and is highly consistent with mechanical measurements of other cancer cell lines (Cross et al., 2007). The cell stiffness measurements within each of the cell lines are shown in Figure 5.7b, where each point represents the average modulus from an individual cell (taken from 4-6 measurements). Overlaid boxplots show median and quartiles, and whiskers show 10th and 90th
percentiles. As for the imaging, significant heterogeneity existed in elastic modulus measurements between different cells of each cell line. This is to be expected in a biological system, and such variability has been demonstrated by others (Li et al., 2008a). Still, the overall stiffness pattern appeared to mirror that of gene expression shown in Figure 5.2, with the 90% mutant and $p_0$ cell lines appearing to be the stiffest, and exhibiting the highest expression of $\alpha$-actin and actinin.

Our statistical analysis was focused on the correlation between cellular elastic modulus and gene expression for the corresponding cell type. While this was done on the level of individual cells, plotting average measures of elastic moduli of the cell lines better facilitates visualization of these trends. We plotted geometric means of elastic moduli for each cell line against the corresponding mean gene expression for $\Gamma$-actin, combined $\Gamma$ and $\beta$ actin, $\alpha$-actinin, and filamin A (Figure 5.7c). We used geometric means because the data appear to trend more toward lognormal rather than normal distributions. Linear fits are given for each data set along with the corresponding coefficients of determination.
Figure 5.7: Stiffness measurements highlight mechanical differences among the m.3243A>G cybrids.

(a) Sample force curve resulting from AFM indentation of a cybrid cell. The Hertz model, shown as a solid black line, is fitted to the approach curve, ultimately giving a cell elastic modulus of 535 Pa. Inset: AFM tip indenting a perinuclear region of a cell. Dotted white lines outline the cell periphery and nucleus. (b) Elastic modulus measurements from all 8 cybrid cell lines as measured with AFM. n=34-57 cells per cell line. Box plots showing the 25th, 50th and 75th percentiles are overlaid onto the scattered data for each group. Whiskers show 10th and 90th percentiles. Outliers are outlined in black. Several extreme outliers are not included in this plot. (c) Linear fits between the elastic modulus geometric means and corresponding normalized gene expression for each of the eight cell lines. Data are shown for (i) Γ-actin, (ii) Γ + β actin, (iii) α-actinin, and (iv) filamin A gene expression.
Table 5.2: Coefficients of determination and adjusted $p$-values between stiffness measurements of m.3243A>G cybrids and different cytoskeletal genes.

<table>
<thead>
<tr>
<th></th>
<th>$\Gamma$ actin</th>
<th>$\beta$ actin</th>
<th>$\beta + \Gamma$ actin</th>
<th>$\alpha$-actinin</th>
<th>$\alpha_1$ tubulin</th>
<th>$\alpha_1b$ tubulin</th>
<th>$\beta$ tubulin</th>
<th>Filamin A</th>
<th>vimentin</th>
</tr>
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<tbody>
<tr>
<td>Individual data points</td>
<td>$R^2$ for gene expression vs. E</td>
<td>0.0289</td>
<td>0.0084</td>
<td>0.0041</td>
<td>0.0023</td>
<td>7</td>
<td>0.0033</td>
<td>7</td>
<td>0.0037</td>
</tr>
<tr>
<td></td>
<td>Adjusted $p$-value</td>
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<td>0.174</td>
<td>0.0144</td>
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<td>0.378</td>
<td>0.378</td>
<td>0.0286</td>
<td>0.437</td>
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<tr>
<td>Geometric means of E</td>
<td>$R^2$ for gene expression vs. E</td>
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<td>0.330</td>
<td>0.734</td>
<td>0.600</td>
<td>0.0507</td>
<td>0.111</td>
<td>0.0928</td>
<td>0.441</td>
</tr>
<tr>
<td></td>
<td>Adjusted $p$-value</td>
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<td>0.246</td>
<td>0.0295</td>
<td>0.0722</td>
<td>0.592</td>
<td>0.521</td>
<td>0.521</td>
<td>0.163</td>
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</table>

Both parameters are evaluated by correlating all individual elastic moduli data points with mean gene expression for each gene, and separately by correlating geometric means of elastic moduli for each of cell lines with mean gene expression for each gene. $p$-values are adjusted for multiple comparisons using the Benjamini-Hochberg method. Values with $P < 0.05$ are shown in bold.

Further statistical information is provided in Table 5.2. We used linear regression to test the correlation between cell stiffness and cytoskeletal gene expression both using individual values of cellular elastic moduli and the geometric means of the data. While $R^2$ values using overall data are quite low, this is due to the natural biological variation in cell stiffness. The corresponding $p$-values show that despite this variation, stiffness measurements significantly correlated with $\Gamma$-actin, combined $\beta$- and $\Gamma$-actin, $\alpha$-actinin, and filamin A (adjusted $p<0.05$ for all). Notably, no correlations existed between stiffness data and vimentin or the different tubulin genes measured. Considering geometric means rather than all data points results in higher $R^2$ values, but lower $p$-values in general due to the drastically reduced size of the dataset and resulting reduced power.
Still, adjusted p-values remain significant (p<0.05) when correlating the geometric mean of the elastic modulus with Γ-actin or combined Γ and β actin gene expression by cell line. The correlations for α-actinin and filamin A do not achieve significance when considering the data in this manner due to the small size of the dataset.

5.5 DISCUSSION

The most salient finding in this work is that cells with the same nuclear genome differing only by their degree of mitochondrial dysfunction exhibit nonmonotonic changes in expression levels of various cytoskeletal genes associated with cellular mechanical properties. This parallels our previous findings that increasing mitochondrial dysfunction causes a complex, nonlinear pattern of transcriptional alterations (Picard et al., 2014). Ultimately, the current study shows that nonmonotonic changes in gene expression levels lead to similar changes in cell stiffness. These data suggest the existence of a complex but conserved relationship between mtDNA heteroplasmy and cytoskeletal parameters. Consistent with a model whereby mitochondrial dysfunction directly alters various cellular functions by signaling transcriptional and post-translational processes, our findings suggest that the relationship between mitochondrial dysfunction and cell mechanics is mediated by specific changes in the transcriptional state, protein levels of cytoskeletal elements related to cortical stiffness and cytoplasmic cytoskeletal organization. Our analyses therefore focus on the relationship between cytoskeletal elements (which are regulated by mitochondrial function) and mechanical properties measured by transcriptomics, Western blotting, fluorescence microscopy, and atomic force microscopy. As shown both textually (Figure 5.8a) and graphically (Figure 5.8b), these outcomes are sequentially and biologically related measures, and our analyses demonstrate a conserved relationship among them. This consistent pattern of change,
rather than a direct correlation with mtDNA heteroplasmy, supports our conclusion that mitochondrial dysfunction has a significant but nonmonotonic effect on the cytoskeleton and cell mechanics.

Figure 5.8: M.3243A>G heteroplasmy affects cell mechanics.
(a) The different cytoskeletal effects of mtDNA heteroplasmy are sequentially related to one another. First, nuclear expression of cytoskeletal genes changes in response to mtDNA mutation. This in turn affects expression of cytoskeletal proteins, particularly actin and α-actinin, which then affect cellular cytostructure and ultimately cause relative changes in cell stiffness. (b) Graphic representation of how alterations in mitochondrial DNA affect cell mechanics in a sequential manner.

The nonmonotonic association between the different m.3243A>G heteroplasmic cell lines and certain cytoskeletal genes, such as various actin isoforms, α-actinin and filamin A suggest a complex regulatory circuit. Protein abundance experiments confirmed the linear relationship between gene expression and protein production in the case of actin. The density of the actin stress fiber network overall followed the same pattern shown for gene expression, but structural manifestations of high actin gene
expression varied with mitochondrial genotype. Finally, single cell mechanics followed the same pattern as gene expression of some actin isoforms, α-actinin, and filamin A.

While many types of cellular dysfunction are characterized by both mitochondrial and mechanical dysfunction, we believe this is the first experimental demonstration of a mtDNA mutation directly affecting cellular mechanical properties. A recently published paper showed that accumulated mtDNA mutations caused by a defect in the nuclear gene encoding the mtDNA polymerase-γ led to arterial stiffening in mice (Golob et al., 2015). In this model, the underlying genetic defect is nuclear, and leads to generalized mitochondrial mutations which are not identified by type or quantity. Additionally, cell stiffness is measured on the tissue level. While this work is highly relevant to the study of interactions between mitochondrial defects and cell mechanics, our work differs in that it focuses on mechanical effects on the single cell level which result from graded effects of a particular mtDNA mutation.

One finding from our work is that actin gene expression does not correlate 1:1 with cell stiffness, with large changes in gene expression translating to relatively small alterations in cellular elastic modulus. Changes in actin gene expression translate into variations in protein abundance of roughly the same magnitude. Actin gene and protein levels can dramatically influence the cell phenotype, as illustrated by the denser stress fiber network shown in 90% heteroplasmic cells or the abundance of actin-rich filopodia shown by the \( \rho_0 \) cells. Despite these pronounced alterations in actin gene expression and ultimately cytostructure associated with different heteroplasmy levels, cell stiffness exhibits relatively low variation (~45% maximal change in median elastic modulus between cell lines).

Although actin has mostly been identified for its central role in cell mechanics (Janmey and McCulloch, 2007), several studies have identified roles for α-actinin in cell
stiffness preservation (Sen et al., 2009) and focal adhesion maturation (Roca-Cusachs et al., 2013). Similarly, filamin has been shown to work cooperatively with α-actinin to maintain more solid-like behavior of crosslinked actin gels (Esue et al., 2009), and filamin A in particular has been highlighted for its role in cell mechanosensing in certain environments (Byfield et al., 2009). It is thus reasonable to assume a combined role for actin, α-actinin and filamin A in maintaining cellular elasticity, as actinin and filamin primarily function to crosslink actin filaments and anchor them to the cell membrane (Sjöblom et al., 2008; Feng and Walsh, 2004). This is consistent with the predominant presence of actinin at the cell periphery (see Figure 5.5). The cooperative relationship between the three proteins supports the correlations between actin, actinin and filamin A gene expression observed in the m.3243A>G cybrid cell lines (Table 5.1). Furthermore, the proven role of all three proteins in maintaining mechanical homeostasis supports our observation that cell stiffness is correlated to transcript expression of each of their corresponding genes.

We did not observe any correlation between elastic moduli of the cybrid cells and either tubulin or vimentin gene expression. Unlike actin, the role of microtubules in cell mechanics is a matter of debate, with some studies showing no direct relationship between the two (Rotsch and Radmacher, 2000; Trickey et al., 2004) and others finding that microtubule depolymerization results in lowered elastic moduli in cells (Barreto et al., 2013; Pelling et al., 2009). Our current work supports the former view, demonstrating an absence of correlation between tubulin gene expression and measured m.3243A>G cybrid cell stiffness. The same is true for vimentin gene expression, which is somewhat more surprising since vimentin integrity is important for preserving cytoplasmic cell stiffness in several cell types (Wang and Stamenović, 2000; Haudenschild et al., 2011). However, it was recently shown that vimentin does not contribute to stiffness of the cell
cortex (Guo et al., 2013), which is the sub-cellular region probed by AFM experiments in the current study. The lack of correlation between vimentin gene expression and cell moduli is thus consistent with previous results. Still, because our measurements are limited to gene expression without assessment of protein polymerization status of the corresponding proteins, we cannot rule out the possibility that mitochondrial bioenergetics impact cell stiffness via post-translational mechanisms regulating polymerization of vimentin and/or various tubulin isoforms. Future work should investigate the possible effect of mitochondrial (dys)function on cytoskeletal dynamics, including those of vimentin and microtubules, and cell stiffness.

While we address the roles of multiple cytoskeletal components in determining cell stiffness, other proteins previously related to cytoskeletal structure could not be assessed. One particular such protein, myosin II, has been shown to be critical to maintaining cell stiffness due to its role in actin cross-linking (Martens and Radmacher, 2008). We hope that future work will expand these results in order to provide a more comprehensive picture of the different cytoskeletal effects of m.3243A>G heteroplasmy.

In our Western blotting experiments, we found a linear relationship between actin protein levels and gene expression of Γ-actin, the most highly expressed actin isoform in the m.3243A>G cybrids. These data did not correlate with genetic expression of β-actin, but did with combined β- and Γ-actin. Additional experiments measuring actinin and tubulin protein abundance yielded results which were also not consistent with gene expression. There are several possible reasons for these negative results. First, most traditional housekeeper proteins for Western blot normalization concern either glucose metabolism (e.g., GAPDH) or the cytoskeleton, both of which were not possible in our case. We chose TBP as an alternative, but it is possible that TBP expression is not consistent across the m.3243A>G cell lines, since m.3243A>G heteroplasmy strikingly
affects many nuclear genes (Picard et al., 2014). Another possible reason for the lack of correlation in the case of α-actinin might be low expression overall (Figure 5.2a), which could make it hard to capture small fluctuations in protein production. Quantification of protein abundance by Western blot in general is subject to greater technical variability (Gassmann et al., 2009), so we place more weight on gene expression and cell stiffness measurements.

In a similar vein, while it may be more logical in principle to correlate cell stiffness with measurements of actin density resulting from fluorescent images, we chose instead to compare cell stiffness with gene expression data. While our quantitative measurements of the fluorescent images did support our qualitative impressions, the actin density measurements in particular can be very sensitive to normalization and threshold values. Circularity measurements are less dependent on input parameters, but relating cell stiffness to circularity is not as theoretically straightforward as comparing stiffness with actin density. Additionally, we found that the trend shown by actin density (Figure 5.5b) does not precisely recapitulate differences observed in gene expression or the stiffness measurements. Both gene expression studies and atomic force microscopy show the \( \rho_0 \) line as being similar to the 90% cell line. However, the actin cytostructures of the \( \rho_0 \) cells are far less developed than those in the 90% cell line, and will work against the correlation between imaging data and stiffness data. Since actin quantification and circularity measurements overall indicated a persistence in the original gene expression pattern of actin and other related genes, we ultimately chose to correlate gene expression with cell stiffness based on the objectively quantitative nature of both these experimental systems.

One potential limitation of this work is that while gene and protein expression studies were performed on homogenates of confluent cell monolayers,
immunofluorescence and atomic force microscopy experiments used individual isolated cells. One obvious consequence of this is our inability to directly correlate gene or protein expression in a given cell with the cell’s elastic modulus. Instead, we assumed that each cell expressed a given gene at a level equivalent to the population average. While cell-to-cell structural and mechanical heterogeneity suggested that this assumption is probably incorrect in the strict sense, collecting data from several tens of cells per cell type showed that the population-wide expression patterns of particular genes were indeed quantitatively correlated with mechanical properties within this smaller population. Another possible problem with pooling experimental data from confluent and single cells is that degree of confluency may affect cell mechanical properties (Efremov et al., 2013). We expect, though, that this change would remain similar in the different m.3243A>G lines and that the relative differences in cytoskeletal gene expression, protein production and cell stiffness would persist whether cells were taken from a confluent population or analyzed at the individual level. Although we did not directly confirm this, our experimental results support this hypothesis.

This study firmly establishes the existence of a connection between mitochondrial genotype and single cell mechanics. The particular mechanisms linking mtDNA mutations with nuclear cytoskeletal genes remain unclear, but we hope that future work will address the underlying mechanisms for nonmonotonic effects of mtDNA heteroplasmy level on nuclear gene expression and cell mechanics. We also intend to further study the connections between mitochondrial and mechanical function in cells exposed to different environmental conditions, including situations that promote acute mitochondrial respiratory chain dysfunction (e.g., sepsis) or directly disrupt the cytoskeleton. Overall, we anticipate that this work will lead to greater understanding of
general cellular pathology which so often involves both mitochondrial and mechanical dysfunction.
CHAPTER 6: AUTOMATED DETECTION OF MITOCHONDRIAL MOTILITY AND ITS DEPENDENCE ON CYTOARCHITECTURAL INTEGRITY

6.1 CONTEXT AND ACKNOWLEDGEMENTS

This chapter represents a shift in direction, and is the first to address Aim 2 of the dissertation. Rather than investigate how changes in mitochondrial function affect the cytoarchitecture and cell mechanics, here we begin to study how cytoskeletal integrity is important for mitochondrial function. We begin by first assessing how mitochondrial motility is affected by cytoskeletal breakdown. We first developed a computational tool in order to trace paths taken by individual mitochondria throughout the entire cell, and then used this to evaluate changes in mitochondrial motility in cells subjected to cytoskeletal toxins.

This work was published in July 2015 in the Journal *Biotechnology and Bioengineering*, under the title “Automated detection of mitochondrial motility and its dependence on cytoarchitectural integrity” by Judith Kandel, Philip Chou, and David M. Eckmann. Philip Chou was a talented undergraduate student whose primary role in the work was to make the tracking algorithm easily understood, usable, and available to others.

We thank Drs. Martin Picard, Alessia Angelin, Marni Falk, and Douglas C. Wallace for helpful discussions regarding mitochondrial behavior; Ben Kandel for help with statistical assessments, image processing, and general MATLAB support; and Dr. Eric Abhold for helpful discussions and writing evaluation. The following funding sources are acknowledged for supporting this work: NIH grant T32 HL007954, Office of Naval
Research grant N000141410538, and the Horatio C. Wood Endowment at the University of Pennsylvania.

6.2 INTRODUCTION

Mitochondrial motility has become a subject of increasing study in recent years. This focus was borne out of the observation that mitochondria localize to subcellular areas of high metabolic activity, including the growth cones of neuronal axons (Morris and Hollenbeck, 1993), virus assembly sites in cells infected with African swine fever virus (Rojo et al., 1998), uropods in migrating lymphocytes (Campello et al., 2006) and the leading edge of migrating cancer epithelial cells (Desai et al., 2013a). Such localization implies that mitochondria in healthy cells exhibit characteristic motility which may be impaired in diseased cells. Accordingly, many studies now focus on analysis of mitochondrial motility using live cell imaging, attempting to elucidate the physiological (Morris and Hollenbeck, 1995; Yi et al., 2004) and pathological (Du et al., 2010) factors which affect it.

Different strategies have been employed in order to track mitochondrial movement, with a vast majority of studies investigating mitochondrial transport in neuronal axons. Neuronal mitochondria are individually well-resolved and also migrate towards the growth cone, so this system is by nature uniquely suitable for particle tracking. De Vos and Sheetz (De Vos and Sheetz, 2007) describe two methods to quantify mitochondrial motility in more generalized cell types. In the first, the non-overlapping regions of two successive images of a cell with fluorescently stained mitochondria are measured as a whole-cell index of mitochondrial motility, and is used by the Hajnóczky group (Yi et al., 2004), among others. Rintoul et al. take a similar approach to subcellular analysis of mitochondrial movement by measuring changes in
fluorescent intensity of individual pixels (Rintoul et al., 2003). While this general approach might consider the whole cell, the biological interpretation of the motility index is unclear. Additionally, results can be drastically skewed by movement of the cell substrate or the cell itself, and information about individual mitochondrial tracks is lost.

De Vos and Sheetz also discuss the use of a kymograph, which analyzes space vs. time images for individual mitochondria. Kymographs are used by some (Du et al., 2010), while others have analogously employed the NIH image analysis software ImageJ to analyze individual or groups of mitochondria within cells (e.g., Chen et al., 2012a). Several groups have employed advanced image processing strategies in order to track groups of mitochondria (e.g., Gerencser and Nicholls, 2008) but these strategies remain limited to subcellular areas.

Despite the intense research focus on mitochondrial motility, analysis of individual mitochondrial tracks on the whole-cell level remains elusive. Kymographs are ill-suited for large scale analysis of mitochondrial motility, since they only consider one-dimensional movement and do not represent an automated method for motility detection. A more appropriate approach would be along the lines of Giedt et al. (Giedt et al., 2012) who first pre-processed time-lapse images and then automatically tracked mitochondria using object connectivity in space and time. Their technique is simpler in concept than most single particle tracking (SPT) algorithms, and is well-suited to non-spherical particles which undergo fusion and fission. However, like many others, Giedt et al. only utilized this strategy to analyze groups of mitochondria in subcellular regions instead of at the whole-cell level. We provide a major advancement in mitochondrial motility analysis by characterizing motion throughout the entire cell. In constructing our algorithm, we build on Geidt et al.’s basic approach but implement multiple changes to the overall methodology, technical processing and conditions for data inclusion. The
resulting “whole-cell” analysis we describe refers to a two-dimensional slice of the cell as captured with our fluorescence microscopy setup, which optimizes visualization of most mitochondria in the cell. In addition to eliminating bias potentially introduced by only studying peripheral mitochondria, our technique critically allowed us to establish a whole-cell depiction of mitochondrial motility. This in turn enables the distinction between baseline motility in healthy cells and motility in cells characterized by acute or chronic dysfunction. We additionally made our algorithm publicly available and accessible with a user-friendly graphical interface.

Ultimately, our goal was to use this method to assess the effects of microtubule and microfilament depolymerization on mitochondrial motility in fibroblasts on the whole-cell level. Studies point to the structural association between mitochondria and the cytoskeleton (Boldogh and Pon, 2006; Heggeness et al., 1978). This connection may extend to a functional one, as a higher concentration or greater polarization of mitochondria is often observed in cellular regions associated with mechanosensing (Campello et al., 2006). In the pathological sense, many diseases are characterized by both mechanical and mitochondrial dysfunction. Cancer cells, for instance, abnormally rely on glycolysis for ATP production (Warburg, 1925), and also exhibit decreased elastic moduli and traction force generation as compared to normal cells (Munevar et al., 2001; Paszek et al., 2005). While others have investigated the effects of cytoskeletal impairment on mitochondrial motility in neurons (Morris and Hollenbeck, 1995) and other limited settings, studying this connection on the whole-cell level in generalized cell types will shed light on the general integration between mitochondrial health and cell mechanics.
6.3 MATERIALS AND METHODS

6.3.1 Cell culture and reagents

Adult human dermal fibroblasts (Lifeline Cell Technology, Walkersville, MD) were cultured in FibroLife cell culture media (Lifeline Cell Technology) as previously described (Kandel et al., 2014). MatTek 35-mm glass-bottom dishes (MatTek, Ashland, MA) were coated for 30-40 minutes with 5 µg/mL fibronectin (BD Biosciences, San Jose, CA) dissolved in PBS, and then plated with cells between passages 1 and 5 at a density of approximately ~25,000 cells/plate. The day before experiments, cells were transfected with CellLight Mitochondria-GFP, BacMam 2.0 (Life Technologies, Grand Island, NY) at a concentration of 40 particles per cell, and kept in the dark at 37˚C. Several hours before experiments, some cells were also dye-loaded with 20 nM Tetramethylrhodamine, methyl ester (TMRM, Life Technologies), a mitochondrial potential dye. Cells were placed in Recording HBSS (HBSS pH 7.4 with 1.3 mmol/L CaCl2, 0.9 mmol/L MgCl2, 2 mmol/L glutamine, 0.1 g/L heparin, 5.6 mmol/L glucose, and 1% FBS) for imaging. Changes in mitochondrial potential are not related in this work.

Immediately before imaging, cells were incubated for 30 minutes in either replacement Recording HBSS, cytochalasin D (cytD; Sigma Aldrich, St. Louis, MO; 200 nM, 1 µM, or 5 µM, taken from a 1 mM stock dissolved in DMSO and diluted using Recording HBSS), nocodazole (noco; Sigma; 2 µM, 10 µM, or 50 µM, taken from a 10 mM stock dissolved in DMSO), both 50 µM noco and 5 µM cytD, or carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Sigma; 5 µM, taken from a 10 µM stock dissolved in DMSO). Data shown from each group represent mitochondria pooled from 5 or more cells over multiple days.
The cytoskeletal inhibitors were confirmed to be working in preliminary experiments as follows: cells were incubated in a given concentration of noco (for microtubule depolymerization) or a given concentration of cytD (for microfilament depolymerization) for 30 minutes. Noco-treated cells were then incubated in 250 nM TubulinTracker Green (Life Technologies) for 30 minutes at 37˚C, then rinsed 3 times and placed in recording HBSS for fluorescence imaging with a standard TRITC filter. Fig. S3 in the supplementary data shows that increasing concentrations of noco result in progressive breakdown of microtubules. CytD-treated cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA; diluted with HBSS from a 16% stock solution) for 10 minutes, then permeabilized for 3 minutes with 0.1% Triton, followed by 20 minutes of dye-loading with 100 nM Acti-stain 488 phalloidin (Cytoskeleton, Inc, Denver, CO) taken from a 14 µM stock prepared according to the manufacturer’s instructions. Actin fiber visualization using a standard FITC filter showed that in a similar manner to noco, the concentrations used of cytD affected the actin cytoskeleton in accordance with the level of concentration used (Figure 6.1).

CCCP was confirmed to be working by dyeing CellLight Mitochondria GFP transfected cells with 20 nM tetramethylrhodamine methyl ester perchlorate (TMRM, Sigma), a mitochondrial inner membrane potential dye. Incubation was performed for 1 hour at room temperature in the dark. Cells were illuminated in both the FITC and TRITC channels before treatment with CCCP, and the two dyes showed nearly perfect overlap for a successfully transfected cell. Imaging cells ten minutes after 5 µM CCCP was added showed that the TMRM dye had left the mitochondria and leaked into the cytoplasm (data not shown), indicating inner membrane depolarization.
Figure 6.1: Fluorescent images of control cells with fluorescent labeling of either microtubules or microfilaments. Images are shown of control cells and cells treated with various concentrations of nocodazole (noco) or cytochalasin D (cytD). Scale bar is 50 µm.

6.3.2 Fluorescence microscopy

Fluorescence microscopy was performed similarly to our previous methods (Sobolewski et al., 2011). Cells were imaged using a QImaging QIClick camera (QImaging, Surrey, BC, Canada) (1x1 binning, 1392x1040 pixels) attached to Olympus IX70 microscope (Olympus, Melville, NY) with an Olympus 40x oil immersion objective.
lens (Olympus) and Photofluor light source (89 North, Burlington, VT). Computer control of the microscope was facilitated by LUDL programmable filter wheels, shutters, and focus control (LUDL Electronic Products, Hawthorne, NY) and images were collected using IPL 3.7 software (BD, Rockville, MD). For each experiment, cells were visualized using a standard FITC filter, and isolated cells with well-resolved mitochondria were selected. Cells were imaged for 5-10 minutes every 1.5 or 3 seconds, eventually to be sampled every 3 seconds for a total of 5 minutes.

6.3.3 ImageJ image pre-processing

We preprocessed raw image files in ImageJ using an approach previously presented (Giedt et al., 2012). Briefly, time-lapse images were first convolved using the 5x5 edge-detection filter given in (Giedt et al., 2012). Next, images were converted to the frequency domain using a Fast Fourier Transform (FFT) in ImageJ, and subjected to a bandpass filter ranging from 2 pixels (~0.3 µm with our resolution) to 100 pixels (~16 µm). Finally, the resulting images were thresholded by eye to best eliminate noise, with the results saved as a sequence of individual binary images. Figure 6.2 shows the evolution of a single frame through the ImageJ processing steps. Video 6.1 shows a raw image sequence of a control cell and Video 6.2 shows the same video after pre-processing in ImageJ.
Figure 6.2: Image processing of a sample cell image.
(a) Original image. Scale bar is 50 µm and scale remains the same across all four images, (b) The same image after convolution, (c) the same image after FFT, and (d) the final image after thresholding.

6.3.4 Matlab algorithm

Stacks of images were analyzed by a custom Matlab script designed to track the movement of individual mitochondria. Figure 6.3A displays a flowchart describing the basic structure of the program, and Figure 6.3B shows how the algorithm treats a 4-frame sequence of sample data. The scripts and instructions of our algorithm are included online on Github (www.github.com/kandelj/MitoSPT), a freely available software sharing repository, and are intended for public use with proper citation. The Matlab processing is done via a user-friendly interface with inputs such file names, image speed, image resolution, and desired lifetime minimum, and is usually completed in a
few minutes. Data can be viewed in the interface in the formats shown in Figure 6.5A and Figure 6.7.

Figure 6.3: Matlab based algorithm tracks centroid locations of all objects as they travel throughout a time-lapse recording.
(a) Flowchart of algorithm used to calculate net distances traveled by mitochondria over the course of a 5 minute video. (b) Illustration of the algorithm’s results for artificial sample data. A comparison of frame 1 to frame 2 shows that object 4 has split into objects 5 and 6, necessitating new labels. In addition, all other objects have moved. Object 7 newly appears in frame 3, and object 6 has disappeared in frame 4. The centroid paths from the first frame are traced in the final frame. The path shown below object 5 is from object 6 (sample data lifetime is not limited to 4 frames as true data are).
Here, we relate a brief conceptual outline of the program. First, the algorithm reads the current frame into Matlab, and the built-in function `bwconncomp` is used to find the connected white objects in the image (using 8-point connectivity rather than Giedt’s 4-point connectivity). The Matlab function `regionprops` is used to find object sizes so the image can be recreated to contain only those objects whose area lies within specified limits. `Bwconncomp` is used to automatically number these objects and save their pixel locations, while `regionprops` finds the objects’ centroid locations. Beginning with the second frame of the program, the object pixel locations in the current frame (“frame n+1”) are compared with those of the previous frame (“frame n”), yielding four possibilities for each object:

1. Simple overlap between a single object from frame n+1 and a single object from frame n. We conclude that the two objects are identical, and relabel the object in frame n+1 to match the corresponding object in frame n.

2. Fusion: An object from frame n+1 overlaps with multiple objects from frame n. We assume a fusion event has occurred, and we permanently retire the labels of the corresponding objects in frame n. The fused object in frame n+1 gets a new, unique label. Our approach here differs from that of Giedt et al., which identically labels mitochondrial objects which fuse at some point.

3. Fission: Several objects from frame n+1 overlap with one object in frame n. We infer that a fission event has occurred. As in the case of fusion, we introduce a new label for this object and retire the old label.

4. New object: an object appears which exhibits no overlap with any other object. This may occur e.g., in the case of an object which is hovering around our assigned object area constraints, and suddenly falls into the specified range when it did not previously. We also use new unique labels for such objects.
Once all objects are relabeled so that they are trackable, the new object pixel locations are stored so that the program can then compare the next frame, n+2, to frame n+1. After the program has cycled through all frames, the stored centroid locations are used to calculate distances traveled by each object at each interval, as well as net distances traveled by each object using the simple distance formula:

\[ d_{\text{net}} = \sqrt{(x_{\text{last}} - x_0)^2 + (y_{\text{last}} - y_0)^2} \]  

(6.1)

where \( d_{\text{net}} \) is the net distance traveled by the object, \( x_{\text{last}} \) and \( y_{\text{last}} \) are the x- and y-positions, respectively, of the centroid in the last frame in which the object appears, and \( x_0 \) and \( y_0 \) are the x- and y-positions of the centroid in the first frame in which the object appears.

In order to maximize our signal-to-noise ratio (SNR), we have subjected our algorithm to several restrictions. First, we limit object size to be between 20 and 500 pixels (~0.5 µm\(^2\) to ~13 µm\(^2\) with our microscope’s resolution). We interpret smaller objects as noise. Larger objects represent extremely integrated mitochondrial networks whose movement will be difficult to track, and generally represent <2% of objects from a given cell (see Figure 6.4A). We also established a minimum lifetime of 4 or more consecutive frames (≥ 9 seconds) per object (similar to Giedt et al., 2012 and Jaqaman et al., 2008 in at least one application) in order to increase our SNR, assuming that more temporary objects are often due to imperfect image processing. For example, a fused object which immediately undergoes fission probably never fused in the first place, and including its track in our analysis may result in underestimated values of net distances. A histogram showing object lifetimes for an individual cell (Figure 6.4B) demonstrates that a majority of objects last very temporarily due to fusion, fission, and low SNR resulting from limited resolution. The lifetime cutoff definitely affects the net distance distribution,
as shorter net distances are generally associated with shorter lifetimes (see Figure 6.4C). However, avoiding setting a lifetime threshold likely decreases the SNR. We have chosen our sampling frequency to be every 3 seconds, observing that more frequent sampling increases noise, but less frequent sampling increases our probability of missing true signal.
Figure 6.4: Descriptive data of mitochondrial objects.
(a) Histogram of size distribution of mitochondrial objects in a given cell for a given frame. Each bin corresponds to 10 pixels$^2$. N = 242 mitochondrial objects. When area is restricted to be between 20 and 500 pixels for motility analysis, 176 mitochondrial objects remain. (b) Histogram of lifetimes of all mitochondrial objects for a given control cell. Each bin represents one interval (the time between two frames, which is 3 seconds), with the lowest bin representing zero intervals (an object which only appears in one frame). N = 3207 mitochondrial objects throughout the 5 minute time-lapse video. (c) Net distance in µm vs. lifetimes of all mitochondrial objects for a given control cell. There is a bin for each interval, with the first “bin” representing a lifetime of one interval (3 seconds). N = 1576 mitochondrial objects with a net distance of greater than zero (and lifetime > 0).
6.3.5 Statistics

For computing the coefficient of determination ($R^2$) of the probability plots, a probability plot was first made by applying the Matlab `normplot` function to the log values of the net distances traveled by mitochondria in a given cell or cells. X and y values were extracted from the plot, with each data point represented as $(x_i, y_i)$. The backslash operator was used in Matlab to fit a line to this data, giving expected y values ($f_i$) based on this model. The total sum of squares ($SS_{tot}$) was calculated as:

$$SS_{tot} = \sum (y_i - \bar{y})^2$$  \hspace{1cm} (6.2)

The residual sum of squares ($SS_{resid}$) was calculated as:

$$SS_{resid} = \sum (y_i - f_i)^2$$  \hspace{1cm} (6.3)

$R^2$ was calculated as:

$$R^2 = 1 - \frac{SS_{resid}}{SS_{tot}}$$  \hspace{1cm} (6.4)

The Matlab function `normfit` gave the mean and standard deviation of the normal distribution best fitting the distribution of logs of netdistances. P-values comparing log distributions were computed using two-sample Kolmogorov-Smirnov tests (K-S test; `kstest2` function in Matlab). This statistical method tests the null hypothesis that two sets of data are taken from the same continuous distribution. Since acceptance or rejection of the null hypothesis based on p-value is by definition affected by dataset sizes, we merely report the p-values in supplemental data instead of establishing a threshold for significance when comparing our large datasets.

6.3.6 Videos

All videos are available online through Figshare, and can be accessed via the following link:  https://figshare.com/s/eb5a6f42e99e08146932.
6.4 RESULTS

6.4.1 Algorithm

A description of the image pre-processing performed prior to the particle tracking algorithm is given in the Materials and Methods section and is illustrated in Figure 6.2. In addition, Video 6.1 (sped up 20x, such that 1 second of video equals 1 minute in real time) displays an unprocessed video of a control cell, with Video 6.2 showing the corresponding processed video. Figure 6.3A displays a flowchart representing the algorithm, and Figure 6.3B shows how the algorithm treats a sample 4-frame sequence of data. The algorithm is discussed in greater detail in the Materials and Methods section.

6.4.2 Net distances traveled by mitochondria follow a lognormal distribution

After applying our method to several cells, we performed a log-transformation on the net distances traveled by mitochondria. We found that on a cell-by-cell basis, the data appear to follow a continuous lognormal distribution. Figure 6.5A shows a histogram of log values of the net distances traveled by all objects in a given control cell over the 5 minutes recorded, with the inset graph showing untransformed data. A normal distribution with a mean of \(-2.023\) (= 132.3 nm) and standard deviation of 1.15 is overlaid onto the log-transformed histogram. The mean value corresponds to the geometric mean of the original distribution (Limpert et al., 2001); thus, means of data groups are henceforth reported as geometric means of original data. Since the standard deviations of all groups in this study were similar and exhibited no obvious trends (see Table 6.1), we focus on the geometric means of various groups as their defining parameter. Limpert et al. (Limpert et al., 2001) gives a more thorough description regarding the spatial interpretation of standard deviations in lognormal distributions. In general, we found that
a vast majority of mitochondria traveled very small net distances (< 1 µm), which is consistent with what has been previously reported in the literature (Giedt et al., 2012). Additionally, the net distances traveled by individual mitochondria had no obvious connection to their subcellular spatial location. This is shown in Figure 6.7, which displays an image of a representative control cell at the upper left. Mitochondrial objects are positioned in their final locations, with each mitochondrion being color coded by the log value of its net distances traveled. The lack of dependence of mitochondrial motility on subcellular spatial location persisted when cells were subjected to various mitochondrial and cytoskeletal toxins (presented below). It is possible that this absence of correlation occurs only in resting cells, as it is likely that cells which are metabolically stimulated in some spatially asymmetrical manner will exhibit spatially dependent mitochondrial motility.

We performed a Shapiro-Wilk (S-W) test (using SigmaPlot v11.0) in order to test the goodness of fit of the motility data for a given group to a lognormal distribution. The resulting W-statistics were very high, often above 0.99, but large datasets led the p-values to fall below the standard significance threshold of 0.05. We instead opted to visually assess whether the data followed a normal distribution using normal probability plots. Figure 6.5B shows the probability plot corresponding to the cell shown in Figure 6.5A. The calculated $R^2$ is 0.994, demonstrating an excellent fit. In general, after converting net distances to the log scale, $R^2$ values usually exceeded 0.98 for individual cells and groups of cells. We thus conclude that net distances traveled by mitochondria follow a lognormal distribution and should be quantified as such on the whole-cell level.

While different cells within each group displayed individual geometric means, combining them into a group maintained the lognormal shape of the distribution. Figure 6.6 shows an example of this, with the probability plot of the entire group (10 µM noco)
giving an $R^2$ value of 0.993. Despite the high $R^2$ values, we opted to use the non-parametric K-S test instead of the Student’s t-test (which assumes a normal distribution) when comparing the net distances traveled by mitochondria in different groups, on account of the failed S-W tests. Overall, mitochondria in control cells traveled a geometric mean net distance of 135.5 nm during the 5-minute recording period.

Figure 6.5: Net distances traveled by mitochondria are lognormally distributed. (a) Histogram of log values of net distances traveled by all mitochondrial objects satisfying our size and lifetime inclusion criteria in a given control cell over the video. A normal distribution (red) has been overlaid onto the histogram, with a mean of -2.023 (=135.3 nm) and standard deviation of 1.150. Inset: histogram of net distances traveled by mitochondria without the log transformation. (b) Normal probability plot of the logs of net mitochondrial distances traveled. The data give an R2 of 0.994 when fit to a lognormal distribution.
Figure 6.6: Combining cells in a given group preserves the lognormal distribution of net distances traveled by their mitochondria.

(a-f) Histograms with fit normal distributions for each cell in the 10 µM nocodazole treated group. The center of the fitted distribution, corresponding to its geometric mean, is given in the upper right corner of the diagram. Normal probability plots are also given for each cell, with the $R^2$ value and number of data points listed on each one.

(g) Histogram with a fitted normal distribution for the pooled data set, along with the corresponding normal probability plot.
6.4.3 CCCP decreases mitochondrial motility

For our first experiment comparing mitochondrial motility in normal and diseased cells, we used 5 µM CCCP in order to verify that this uncoupler decreases mitochondrial motility, as shown by others (Giedt et al., 2012; Safiulina et al., 2006). The geometric mean of the mitochondrial net distances traveled by the treated cells was 106.9 nm, compared with 135.5 nm of all mitochondria in control cells. Figure 6.7 compares the logs of the net distances in a representative control cell and CCCP-treated cell. The CCCP-treated cell appears bluer overall, illustrating the shift of its distribution towards decreased motility. Video 6.3 shows the pre-processed movie of this particular cell, and Figure 6.9 plots the parameters of the control and CCCP distributions among other groups. Table 6.1 shows a matrix comparing logs of p-values across different groups using the two-sample K-S test, with cells color coded by the log of their p-value value to illustrate relative significance. Warmer colors indicate smaller, more significant p-values, and cooler colors represent larger, less significant ones. Absolute significance is highly affected by our large sample sizes, resulting in nearly any group comparison falling below the standard threshold of $p = 0.05$. As such, we present logs of p-values in order to highlight their relative, rather than absolute, significance, and colored them according to this scale. A comparison of the control group to the CCCP treated group gives a relatively low p-value, indicating a significant difference.

Others have shown that high concentrations of CCCP can result in microtubule depolymerization (Maro et al., 1982), which itself impairs mitochondrial motility as shown next. However, we verified with fluorescence microscopy that at the concentration and treatment length used, microtubule structure was preserved (Figure 6.8), and appears similar to that of the control cells shown in Figure 6.1. This suggests that the mitochondrial depolarization induced by CCCP primes them for motility dysfunction in a
manner which is independent of microtubule structure, ultimately suggesting an underlying link between mitochondrial function and motility.

Figure 6.7: CCCP and nocodazole lower the distribution of net distances traveled by mitochondria while cytochalasin D raises it.
(a) Images of representative cells from the control, 5 µM CCCP, 5 µM cytD and 50 µM noco groups. Each dot represents a mitochondrial object in its final location after tracking, and is colored by the log of the net distance traveled. Inset into each figure is the first pre-processed binary image from the corresponding video.
(b) Grouped histograms of net distances traveled by mitochondria in control, 50 µM noco and 5 µM cytD groups. Mitochondria are pooled together from 5-17 cells per group, with a total mitochondrial number of 4323 (cytD), 5530 (noco) and 19334 (control). Histograms are normalized to reflect percentages of objects rather than absolute numbers.
Figure 6.8: Cells after 30 minutes of treatment with 5 µM CCCP. Microtubules are fluorescently stained, and appear intact. Scale bar is 50 µm.

6.4.4 Microtubule depolymerization decreases net distance traveled by mitochondria, while microfilament depolymerization increases it

Cells treated with various concentrations of noco in order to depolymerize their microtubules exhibited decreased mitochondrial motility. 2 µM, 10 µM and 50 µM concentrations were used, resulting in geometric means of 108.3 nm, 102.9 nm, and 105.5 nm, respectively. The sample cell from the 50 µM group shown in Figure 6.7 appears overall more blue than the control cell, indicating shorter net distances traveled by the corresponding mitochondria. The pre-processed movie of this cell is given in Video 6.4. The group geometric means did not differ significantly among different noco concentrations, as plotted in Figure 6.9 and shown in the p-value matrix in Table I. Thus, while these concentrations appear to affect the microtubule network in a somewhat
dose-dependent manner (Figure 6.1), the affect on mitochondrial transport does not seem to vary by dose and is exhibited with even partial disruption of the network.

Figure 6.9: Box plots showing motility data from all groups. Whiskers extend to 10th and 90th percentiles of data. The median of the control group is extended throughout the plot as a dashed gray line.

As opposed to noco, cytD increased mitochondrial motility. For the 200 nM and 1 µM concentrations, this effect was hardly pronounced with geometric means of 141.1 and 141.6 nm, respectively. However, at 5 µM the lognormal distribution of net distances exhibited a geometric mean of 167.9 nm as compared to 135.5 nm in the control group. Like in the case of noco treatment, our results show that this effect is not dose-
dependent. However, as opposed to the effects of noco on microtubule-mediated mitochondrial transport, only extreme disruption of the actin cytoskeleton affects mitochondrial motility. A sample cell from the 5 µM cytD group is shown in Figure 6.7, with the object colors tending towards yellow and orange values when compared to control cells. Video 6.5 shows the pre-processed movie of this cell. Figure 6.7B shows overlaid histograms of the 5 µM cytD treated group, the control group, and the 50 µM noco group, with the effect of each drug on the motility distribution appearing quite evident.

### Table 6.1: Motility parameters from all groups and p-values resulting from pairwise K-S tests.

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>5 µM CCCP</th>
<th>200 nM cytD</th>
<th>1 µM cytD</th>
<th>5 µM cytD</th>
<th>2 µM noco</th>
<th>10 µM noco</th>
<th>50 µM noco</th>
<th>cytD + noco</th>
<th>n</th>
<th>geo mean</th>
<th>stdev</th>
<th>R²</th>
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</table>

P-value cells are color coded by log value, with red cells indicating most significant, and blue cells indicating least significant. Additional columns give number of objects per group (n), the geometric mean, the standard deviation (in log scale), and the coefficient of determination to the normal probability plot for each group.
Finally, we were interested in whether the noco or cytD effect on mitochondrial motility would dominate when both drugs were used. We treated cells for 30 minutes with 50 µM noco and 5 µM cytD. Resulting motility was virtually the same as control cells, with a geometric mean of 134.5 nm (see Figure 6.9 and Table I). Thus, the effects of cytD and noco act in opposition to restore mitochondrial motility to its baseline level. Figure 6.9 shows box plots of all groups studied and Table I shows the relative p-values resulting from the K-S tests comparing all groups.

**6.5 DISCUSSION**

We have created a publicly available, easy-to-use method for mitochondrial tracking in whole cells based on fluorescent live imaging. The algorithm is formulated based on the approach described in (Giedt et al., 2012), and uses ImageJ for image preprocessing and Matlab for object recognition and tracking. We used our method in order to easily compare the whole-cell distribution of mitochondrial motility between untreated cells and cells subjected to mitochondrial and cytoskeletal toxins.

One major insight provided by our work is that whole-cell mitochondrial movement follows a lognormal distribution. Until now, it has become standard to categorize mitochondrial movement as either small-scale Brownian-like motion or brief spurts of directed motion (e.g., (Giedt et al., 2012; Nekrasova et al., 2011)), with one group even adding an intermediate category of small-scale directed motion (Saunter et al., 2009). We show, by contrast, that mitochondrial motility follows a continuous distribution, an observation which is also made by Gerencser and Nicholls (Gerencser and Nicholls, 2008). We provide further insight into their observation by showing that the distribution is of a particular type; namely, a lognormal distribution.

Our data slightly deviate from the lognormal distribution at extremely high and
low net distances, indicating fatter tails than expected. This is especially pronounced at the upper extremes (see Figure 6.5B and Figure 6.6), which lends some support to the idea that certain mitochondria undergo bursts of directed motion. However, setting an arbitrary threshold for such bursts within a continuous distribution can make comparison between studies difficult. More problematically, the threshold value heavily affects the resulting analysis. For example, Figure 6.7B shows that control, cytD and nocodazole treated cells all have similar percentages of mitochondria which undergo net distance changes above ~2.1 µm (~0.75 on the x-axis), but a lower cutoff might accurately capture differences between these groups. In contrast to the arbitrary nature of mitochondrial motility categorization, quantification of net distances using a lognormal distribution, as is descriptive of many biological phenomena (Limpert et al., 2001), highlights the substantial shift between different groups even at a glance. Motor-driven events are particularly interesting in their ability to provide insight into mechanisms of mitochondrial translocation, but an accurate understanding of mitochondrial motility at the whole-cell level includes many small-scale movements which reflect mitochondrial drifting or even possibly fluctuations due to microscopy limitations. The lognormal distribution successfully encapsulates all types of mitochondrial movement occurring within a cell, and the pharmacological agents we used (nocodazole, cytD, CCCP) affect the entire range of mitochondrial motility across the cell. Considering the geometric mean of the data rather than the arithmetic mean as is more commonly used enables the determination of a whole-cell index of motility which reflects true biological parameters emanating from individual mitochondria data. This can easily be used to compare different groups of cells subjected to a variety of experimental conditions.

Overall, our findings are consistent with previous research showing that microtubule depolymerization hinders mitochondrial motility in mammalian cells (Morris
and Hollenbeck, 1995; Yi et al., 2004; Ligon and Steward, 2000; Müller et al., 2005). Our ability to demonstrate the same effect across entire microscopy fields encompassing whole cells lends support to our algorithm’s ability to characterize mitochondrial motility, but further expands this finding to fibroblasts on the whole-cell level. Prior results on the role of actin filaments in this regard has been divided, with some studies reporting impaired mitochondrial motility resulting from microfilament depolymerization (Müller et al., 2005; Quintero et al., 2006), and others finding, like us, that it was enhanced (Ligon and Steward, 2000; Morris and Hollenbeck, 1995). One recent study even found that actin depolymerization enhanced mitochondrial motility in neuronal dendrites and axons, but had little effect on mitochondrial motility in the cell body (Narayanareddy et al., 2014). A general perspective on organelle motility presents microtubules and actin as tracks for long-range and short-range movement, respectively (Langford, 1995). With specific regard to mitochondria in eukaryotic cells, some investigators suggest that intermediate filaments impair mitochondrial motility (Nekrasova et al., 2011), while others have proposed that actin filaments are responsible for mitochondrial docking in neurons in particular (Chada and Hollenbeck, 2004). Our data showing that actin depolymerization increases mitochondrial transport supports the extension of this latter theory to fibroblasts. Frederick and Shaw (Frederick and Shaw, 2007) review mitochondrial motility in greater detail, including some discussion of the motor proteins used by mitochondria to travel along cytoskeletal tracks. Our finding that combined effective doses of noco and cytD restore baseline mitochondrial motility are at odds with others’ results in neurons (Morris and Hollenbeck, 1995), and perhaps suggests that neither actin nor tubulin dominates mitochondrial motility in fibroblasts. Much remains to be elucidated regarding the mechanisms of interaction between mitochondria and the actin cytoskeleton in generalized cell types.
We believe that increased optical resolution can further improve our algorithm. Our current limits in microscopy resolution probably lead to increased variability in successive binary images, which may lead to some of the small-scale movements we observe. For example, a one-pixel fluctuation in a large object can lead to a centroid translocation of <10 nm, far less than the ~160 nm length of a pixel in our system. It is impossible to tell whether these fluctuations originate in light detection limitations or truly reflect small-scale mitochondrial movement, and improved resolution might better allow for this differentiation. Greater resolution would also sharpen the appearance of the perinuclear mitochondria. In this work, we were careful to only select cells with well-resolved perinuclear mitochondria following the pre-processing steps. Our resulting analysis shows that these mitochondria exhibit motility on a scale similar to peripheral mitochondrial motility rates. In the Materials and Methods section, we describe other strategies we used to curb other effects of limited resolution, including image pre-processing and imposing a minimum object lifetime.

As a final strategy to maximize SNR, objects resulting from fusion or fission are newly labeled, rather than having their tracks continue as in other studies (Giedt et al., 2012; Jaqaman et al., 2008). Continuing tracks rather than starting new ones in these cases can exaggerate the actual motion traveled by creating jumps in centroid location, especially if the fusion or fission is illusory. Whether this approach is logically justifiable in the case of true fusion and fission is open to debate. One reason to continue tracks of fused or split objects is that poor SNR can cause that illusory fusion, fission, or momentary object disappearance. Since shorter tracks of several such objects may in reality comprise the lengthier track of a single object, prematurely ending the recording of such objects may lead to underestimating their net distance traveled. This
phenomenon was observed by Jaqaman et al. (Jaqaman et al., 2008) using a global optimization technique in order to fuse tracks of various subcellular particles over a time course video and thus reduce such error. While possible application of Jaqaman’s technique may benefit our algorithm in the future, we are reluctant to apply it to mitochondria since fusion and fission can only be detected by connecting tracks. This will lead to jumps in centroid location as discussed above. We would prefer to develop a hybrid approach in which unique identities of mitochondria preceding and following fusion and fission are preserved, but possibly employs track linking to enable more accurate detection of true fusion and fission and disregards momentary disappearance events due to limited resolution. This approach will help us preserve as much data as possible about mitochondrial motility as well as fusion and fission rates.

Another limitation in this work is the possibility of confounding mitochondrial movement with cell movement, which is particularly of concern with the use of cytD. In this work, we were careful to discard any time-lapse images with significant cell movement, as frequently occurred in cytD-treated cells. Video 6.6 (which also exhibits poor resolution) shows an example of one such cell, with a corresponding color-coded plot of the cell shown in Figure 6.10. Despite our meticulousness, it is possible that imperceptible cellular motility can still artificially increase the range of mitochondrial motility in a given cell. Future experiments may be able to account for this by introducing a cellular dye and then correcting for cell movements, in both magnitude and direction, at each time point. This correction may help elucidate whether actin in fact plays a role in mitochondrial docking, as this previously unmentioned confound may relate to conflicting data in this regard.
Figure 6.10: Image of the 1 μM cytochalasin D-treated cell shown in Video 6.6.
Mitochondrial objects are located in their final positions and color coded according to the log value of their net distance traveled. The objects towards the top of the frame tend towards greater values, which is probably a result of the cell movement occurring in that region. The color scale is the same as in Figure 6.7.

This foundational study prompts a variety of additional experiments relating to many different scientific areas of research. In addition to the computational improvements suggested above, some follow-up mechanistic studies may be interesting as well. One obvious question to explore is the mechanism by which microfilaments normally “impair” mitochondrial transport. Since actin filaments are principally involved in the well-studied field of cell mechanics (Janmey and McCulloch, 2007), we additionally intend to investigate general connections between cell mechanics and bioenergetics.
6.6 ANALYSIS OF MITOCHONDRIAL MOTILITY IN PATIENT SAMPLES

6.6.1 Introduction

After publishing the work presented heretofore, we were contacted by a laboratory in Israel studying several patients who presented with severe neurodegenerative symptoms. After extensive analysis, this team had determined that the genetic origin of these symptoms involved a mutation in a protein involved in mitochondrial trafficking. They requested that we use our methodology to demonstrate impaired mitochondrial motility in fibroblast samples from these patients. This work was still in preparation for publication when this dissertation was defended, so details about the study are intentionally left ambiguous.

6.6.2 Materials and methods

Skin biopsies were taken from patients at the Sheba Medical Center in Ramat Gan, Israel after obtaining written informed consent. Families also signed informed consent forms for the specimens to be sent to the NIH for advanced molecular studies.

Mitochondrial motility was analyzed as described above. Briefly, control and patient fibroblasts were plated on MatTek dishes (MatTek, Ashland, MA) at a concentration of 25,000 cells/plate and transfected with CellLight Mitochondria GFP, BacMam 2.0 (Life Technologies, Grand Island, NY) at a concentration of 40 particles per cell. Cells were imaged the following day in Recording HBSS (HBSS pH 7.4 with 1.3 mmol/L CaCl2, 0.9 mmol/L MgCl2, 2 mmol/L glutamine, 0.1 g/L heparin, 5.6 mmol/L glucose, and 1% FBS).

For dual visualization of mitochondria and microtubules, cells were first incubated in 20 nM Tetramethylrhodamine, methyl ester (TMRM; Life Technologies) for 60 min at room temperature. After rinsing, cells were incubated in 250 nM TubulinTracker Green
(Life Technologies) for 30 min at 37°C, then rinsed and placed in recording HBSS for fluorescence imaging with standard TRITC and FITC filters.

Fluorescence microscopy was performed similarly to our previous methods. Cells were imaged using a QIClick camera (QIClick camera, QIImaging, Surrey, BC, Canada) (1 × 1 binning, 1392 × 1040 pixels) attached to Olympus IX70 microscope (Olympus, Melville, NY) with an Olympus 40x oil immersion objective lens (Olympus) and Photofluor light source (89 North, Burlington, VT). Computer control of the microscope was facilitated by LUDL programmable filter wheels, shutters, and focus control (LUDL Electronic Products, Hawthorne, NY), and images were collected using IPL 3.7 software (BD, Rockville, MD). For each experiment, cells were visualized using a standard FITC filter, and isolated cells with well-resolved mitochondria were selected. Cells were imaged for 5 min every 3 seconds.

We preprocessed raw image files in ImageJ and subsequently in Matlab as described above. Video 6.7 shows a raw image sequence of a sample control cell.

We subjected our Matlab analysis to the same restrictions as in our previous work in order to maximize our signal-to-noise ratio (SNR). Only objects measuring between 20 and 500 pixels (~0.5 to ~13 μm² with our microscope’s resolution) and lasting four or more consecutive frames (≥9 s) are considered. Sampling frequency was 3 s since more frequent sampling increases noise, but less frequent sampling increases the probability of missing true signal.

The Matlab function *normfit* gave the mean and standard deviation of the normal distribution best fitting the distribution of logs of netdifferences. Figure 6.12B uses this information and the *normpdf* command to plot the probability density functions for each mitochondrial population. P-values comparing log distributions were computed using the rank-sum test (*ranksum* function in Matlab). Since acceptance or rejection of the null
hypothesis based on P-value is by definition affected by dataset sizes, we simply report the P-values instead of establishing a threshold for significance when comparing our large datasets.

6.6.3 Results

Microtubules appeared intact in control as well as patient cells, confirming that there was no defect in microtubule structure in any of these cells. Mitochondrial distribution, however, differed between control and patient cells. In control cells, mitochondria extended to the cellular periphery, while in patient cells, mitochondria remained clustered around the nucleus. (Figure 6.11)

Figure 6.11: Microtubules and mitochondria in fibroblasts from control and two different patients. Cells are stained with TubulinTracker and TMRM. Scale bar is 50 μm.
Figure 6.12: Mitochondrial tracking in normal and diseased patient fibroblasts.
(a) Image of a sample control cell before and after ImageJ processing. Scale bar is 20 µm. (b) Path lengths of all mitochondrial centroids in this cell. Box indicates inset region. Inset: path length of one particular mitochondrion, with start and finish points indicated. This mitochondrion traveled a total distance of 9.52 µm and a net distance of 2.47 µm. (c) Probability density plots of net distances traveled by control and patient mitochondria.
Consistent with our previous work (Kandel et al., 2015), net distances traveled by mitochondria followed a lognormal distribution. We found that mitochondria from patient cells traveled shorter net distances than control mitochondria, with results summarized in Table 6.2 and Figure 6.12B. The geometric mean net distance traveled by control mitochondria was 192.6 nm (n=3640 mitochondria from 15 cells). Mitochondria in Video 6.7 have a geometric mean net distance of 206.8 nm. For patient misc238, the geometric mean was 160.4 nm (n=2691 mitochondria from 13 cells), giving a p-value of 7.96E-10 vs. control using the rank-sum test. A sample cell from this population is shown in Video 6.8. The mitochondria in this particular cell travel a geometric mean net distance of 164 nm. Patient misc239 showed even more decreased mitochondrial motility than patient misc238, with a geometric mean net distance of 142.9 nm (n=1505 mitochondria from 12 cells). The p-value comparing this population to the control mitochondria was 2.25E-16. Video 6.9 shows a sample cell from this patient. The mitochondria in this cell traveled a geometric mean net distance of 153.8 nm over the course of the 5 minutes recorded.

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<th>Table 6.2: Mitochondrial motility parameters and statistics in patient and control fibroblasts.</th>
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CHAPTER 7: MITOCHONDRIAL RESPIRATION IS SENSITIVE TO CYTOARCHITECTURAL BREAKDOWN

7.1 CONTEXT AND ACKNOWLEDGEMENTS

Having studied the effects of cytoskeletal toxins on mitochondrial motility, we now turned our attention to whether and how cytoskeletal breakdown affects mitochondrial function. This chapter thus characterizes the effects of cytoskeletal breakdown on mitochondrial inner membrane potential, morphology, and most importantly, respiration.

At the time of submission of this dissertation, this work was a manuscript in preparation under the title “Mitochondrial respiration is sensitive to cytoarchitectural breakdown” by Judith Kandel, Alessia A. Angelin, Douglas C. Wallace and David M. Eckmann. Dr. Alessia Angelin was helpful in making up media for the Seahorse experiments as well as training on the instrument. She works in the laboratory of Dr. Douglas C. Wallace, who enabled use of the Seahorse Analyzer as well as provided important intellectual contribution to this work.

In addition to those listed as co-authors of the work, I would like to acknowledge several other people for contributions to these studies. Thank you to Dr. Peter Sobolewski for initial training in mitochondrial imaging and to Benjamin Kandel for help with image processing, statistical analyses and general manuscript feedback. Dr. Martha E. Grady also provided valuable suggestions regarding manuscript preparation. We additionally thank Dr. Marni Falk for general guidance and feedback regarding mitochondrial experimentation.
This work was funded by the Office of Naval Research grant N000141612100 (awarded to DME) and the National Institutes of Health grants NS021328 and CA182384 (awarded to DCW).

7.2 INTRODUCTION

Cellular pathology is often assumed to be reflected by either mitochondrial or structural alterations. In the mitochondrial realm, diseased cells show changes in metabolic pathways (Trushina et al., 2013; Warburg, 1925), respiration rates (Ryan et al., 2014; Wiegman et al., 2015), reactive oxygen species generation (Mackenzie et al., 2013; Wiegman et al., 2015), and even mitochondrial morphology (Knott et al., 2008; Makino et al., 2010) or motility (Giedt et al., 2012; Wang et al., 2011). On the other hand, damaged cellular cytoarchitecture can manifest as changes in cell shape (Kheradmand, 1998), traction force generation (Munevar et al., 2001), or stiffness of the extracellular matrix (Jalil et al., 1989; Levental et al., 2009) or the cell itself (Li et al., 2008b; Sehgel et al., 2013). While the pathways involved in both mitochondrial and mechanical dysfunction are individually complex, there are abundant suggestions in the literature that there are multiple links between them. Here we examine the mitochondrial consequences of cytostructural impairment. This work builds upon our previous research characterizing the effects of cytoskeletal toxins on mitochondrial motility in particular (Kandel et al., 2015).

For decades, mitochondria have been known to be structurally linked to microtubules (Ball and Singer, 1982), and microtubule breakdown impairs mitochondrial motility (Kandel et al., 2015; Morris and Hollenbeck, 1995). Associations between mitochondria and actin filaments, which are largely responsible for cellular mechanics (Janmey and McCulloch, 2007), have mostly been studied in yeast (Lazzarino et al.,
1994), but recent work indicates that actin is involved in mitochondrial motility (Kandel et al., 2015) and fission (Korobova et al., 2013) in mammalian cells. Beyond structure and motility, studies indicating functional links between mitochondria and cytoarchitecture are often predicated on the observation that mitochondria tend to localize to energy-requiring areas of the cell, such as the growth cones of neuronal axons (Morris and Hollenbeck, 1993) and viral assembly sites in infected cells (Rojo et al., 1998). One example is that mitochondria in a freshly plated cell are concentrated at the cell periphery (Bereiter-Hahn, 1990), presumably in order to locally provide energy for cell spreading. Similarly, cells at the edge of a migrating sheet contain mitochondria with elevated inner membrane potentials (Johnson, 1981), and others have even shown increased mitochondrial inner membrane potential at the periphery of more stationary cells (Collins et al., 2002), where traction forces are usually highest (Munevar et al., 2001). Additional studies have demonstrated directed mitochondrial localization during migration of lymphocytes (Campello et al., 2006) and cancer cells (Desai et al., 2013b). While intriguing, these studies are limited to observations regarding subcellular localization of mitochondria, and fall short of directly examining the interplay between cytoarchitectural integrity and mitochondrial function.

Here, we analyzed the direct effects of cytoskeletal inhibitors on mitochondrial inner membrane potential, morphology, and oxygen consumption in intact, adherent human dermal fibroblasts. We believe this is the first demonstration of the effects of cytoskeletal toxins on mitochondrial respiration in particular. Decreased inner membrane potential is perhaps the clearest sign of mitochondrial injury and thus represents an obvious first step to assessing mitochondrial health in response to impending damage. We also considered mitochondrial morphology since it is often, but not always, linked to energetic state (Sauvanet et al., 2010). Finally, we assessed whether cytoskeletal toxins
induced changes in mitochondrial oxygen consumption rates, which may indicate whether mitochondrial energetics are maintained or compromised. Overall, our work shows that while membrane potential remains intact, cytoskeletal breakdown can alter mitochondrial respiration by itself or in concert with known mitochondrial stressors. Our results further suggest that mitochondrial shortening may counteract the effects of cytoskeletal toxins on mitochondrial respiration.

7.3 MATERIALS AND METHODS

7.3.1 Cell culture and reagents

Adult human dermal fibroblasts between passages 1 and 5 (Lifeline Cell Technology, Walkersville, MD) were cultured in FibroLife cell culture media (Lifeline Cell Technology) as previously described (Kandel et al., 2015). Cells were seeded approximately 48 hours prior to experiments.

For experiments involving measuring changes in mitochondrial inner membrane potential and mitochondrial morphology, cells were plated on MatTek 35-mm glass-bottom dishes (MatTek, Ashland, MA) at a density of ~25,000 cells/dish. Dishes were coated for 30-40 minutes with 10 µg/mL fibronectin (BD Biosciences, San Jose, CA) dissolved in PBS prior to cell plating.

For oxygen consumption measurements, cells were plated in 24-well plates (Seahorse Biosciences, North Billerica, MA) at a density of ~10,000 cells/well. This concentration was determined to result in a confluent monolayer at the time of experiments.
7.3.2 Assessing changes in mitochondrial inner membrane potential and morphology

Changes in mitochondrial inner membrane potential and morphology in single cells were assessed using wide-field fluorescence microscopy as previously described (Kandel et al., 2015). This setup employed a QImaging QIClick camera (QImaging, Surrey, BC, Canada) (1x1 binning, 1392x1040 pixels) attached to an Olympus IX70 microscope (Olympus, Melville, NY) with an Olympus 100x oil immersion objective lens (Olympus) and Photofluor light source (89 North, Burlington, VT). Computer control of the microscope was facilitated by LUDL programmable filter wheels, shutters, and focus control (LUDL Electronic Products, Hawthorne, NY) and images were collected using IPL 3.7 software (BD, Rockville, MD). For each experiment, cells were visualized using standard TRITC and FITC filters.

The day before experiments, cells were transfected with CellLight Mitochondria-GFP, BacMam 2.0 (Life Technologies, Grand Island, NY) at a concentration of 40 particles per cell, and kept in the dark at 37°C. Several hours before experiments, cells were also dye-loaded with 20 nM Tetramethylrhodamine, methyl ester (TMRM, Life Technologies), a mitochondrial potential dye, for 60 minutes at room temperature in the dark. After rinsing, cells were placed in Recording HBSS (HBSS pH 7.4 with 1.3 mmol/L CaCl2, 0.9 mmol/L MgCl2, 2 mmol/L glutamine, 0.1 g/L heparin, 5.6 mmol/L glucose, and 1% FBS) for imaging.

In each experiment, a selected cell was first imaged in both the mitoGFP and TMRM channels in recording HBSS. At T=0, recording HBSS was removed from the dish and replaced with either recording HBSS (control), recording HBSS containing 5 µL/mL DMSO, or recording HBSS containing a given cytoskeletal toxin. The DMSO concentration was equivalent to the highest concentration of drug stock solution for
which a morphology effect was observed, and was performed in order to ensure that no observed effects resulted from the DMSO solvent rather than the cytoskeletal drugs. Cytochalasin D (cytD) was taken from a stock solution of 1 mM in DMSO and nocodazole (noco) was taken from a stock solution of 10 mM in DMSO. The cell was then imaged again in both channels at T=30 minutes. Data for each experimental group were collected over two or more days, for a total of 7-22 cells per group. Cytoskeletal toxins were confirmed to be working using Alexa Fluor 546 phalloidin (Life Technologies; for cytD) and TubulinTracker (Life Technologies; for noco) dyes (previously shown in (Kandel et al., 2015)).

Images from the mitoGFP channel were preprocessed using ImageJ as previously described (Kandel et al., 2015). Briefly, images were first convolved using a 5x5 edge detection filter (Process>Filters>Convolve). The resulting image was then bandpass filtered (Process>FFT>Bandpass Filter…) between 2 and 100 pixels. Finally, the image was manually thresholded (Image>Adjust>Threshold) by eye at a level which removed random background pixels in order to maximize signal and minimize noise. Before applying the chosen threshold value, a mitochondrial region of interest (ROI) was first generated by creating a selection (Edit>Selection>Create Selection) and then adding this to the ROI manager (Analyze>Tools>ROI Manager>Add[lt]). The threshold was then applied. Figure 7.1a(iv) shows the resulting image for a sample cell.
Figure 7.1: Measuring changes in mitochondrial morphology and inner membrane potential using fluorescence microscopy.

Image of a sample cell co-stained with CellLight Mitochondria-GFP (mitoGFP) and TMRM. In all rows, (i) shows mitoGFP, (ii) shows TMRM and (iii) shows the two channels merged. 

(a) image of the entire cell before any drug treatment. (iv) shows the image resulting from ImageJ processing of the mitoGFP image in order to measure mitochondrial morphology and create a ROI for measuring mitochondrial inner membrane potential. The red dotted box indicates the area of the cell depicted in panels (b-c). 

(b-c) Magnified section of the cell prior to (b) and following (c) treatment with 5 µM CCCP. (iv) shows how the ROI resulting from the mitoGFP channel is overlaid onto the TMRM channel in order to measure TMRM fluorescence only within the mitochondria.

Mitochondrial inner membrane potential was measured by opening the TMRM channel image in ImageJ and superimposing the corresponding mitoGFP processed image ROI onto it (by selecting the ROI in ImageJ). Figures 1b(iv) and 1c(iv) show examples of mitochondrial ROIs superimposed onto corresponding TMRM images. Since the mitoGFP fluorescence results from transfection, it does not depend on inner membrane potential, and thus allows for mitochondrial identification even when
mitochondria have depolarized. Fluorescence intensity within the mitochondrial ROI was then measured (Analyze>Measure) for a mean TMRM fluorescence within the mitochondrial region of the cell. This process was repeated for the same cell 30 minutes following drug exposure, and the resulting mean fluorescence was normalized to the corresponding value prior to treatment. 5 µM CCCP was used as a positive control (Figure 7.1b-c).

Object recognition functions in Matlab R2010a (Mathworks, Natick, MA) were applied to the preprocessed image of each cell at each time point (Figure 7.1a(iv)) in order to assess mitochondrial morphology. The code constructed for this purpose is publicly available online. Briefly, bwconncomp was used to find the mitochondrial objects in each image (using 8-pixel connectivity) followed by regionprops, which measured the area of each object. The noise floor was set at 5 pixels, and objects with areas less than this were discarded. The image was then reconstructed without these objects. Bwconncomp was used to find mitochondrial objects in the new image, and regionprops measured various attributes of these objects including major axis length. Since lengths were not normally distributed, the metric used to assess overall mitochondrial length at a given time point was the geometric mean. The geometric mean of mitochondrial length was then calculated for each cell following treatment, and normalized to the geometric mean of the same cell prior to treatment.

7.3.3 Oxygen consumption measurements

Prior to beginning respiration measurements, cells were pre-treated for 30 minutes with DMSO, cytD, noco or a combination thereof, as indicated. Some cells were pre-treated prior to the addition of cytoskeletal toxins with 100 nM A23187 (A23; taken from a stock solution of 2 mM) for 20 minutes. This concentration was chosen based on
the lack of effect on respiration at this concentration. After 20 minutes, A23-containing media was removed and replaced with media containing both A23 and appropriate cytoskeletal toxins. Control DMSO groups included a low DMSO concentration and a high DMSO concentration. The low DMSO concentration was 0.2 µL/mL (equivalent to the lowest concentration of noco or cytD stock used) while the high DMSO concentration was 10.05 µL/mL (equivalent to the highest load of DMSO from stock used, in the 5 µM cytD + 50 µM noco + A23 group). These controls were slightly different from those used for morphology and potential measurements, and were chosen to correspond to the lowest and highest DMSO concentrations for which an OCR effect was observed. Control cells (without DMSO) were maintained in their original media.

Mitochondrial oxygen consumption rates (OCR) were evaluated in intact, adherent cells using the Seahorse XF24 Analyzer (Seahorse Bioscience, Billerica, MD). The experiments were performed with unbuffered DMEM XF assay media supplemented with 2mM GlutaMAX, 1mM sodium pyruvate, and 5mM glucose (pH 7.4 At 37°C). The mitochondrial respiration was measured before any pharmacological perturbation (basal respiration). Cells were then subjected to 1 µg/ml oligomycin (oligo) to measure proton leak, followed by 0.4 µM FCCP to induce maximal respiration. Finally, 1 µM rotenone followed by 1.8 µM antimycin A were applied in order to inhibit all mitochondrial respiration. In each experiment, 3 wells were left unseeded to serve as background measurements. Each experimental condition was applied to 4-6 wells per plate, and repeated over 3-4 days for a total of 12-24 wells per condition.

Basal respiration was measured at three consecutive time points, as was respiration following application of each oligo, FCCP, rotenone and antimycin. These three values were averaged to give a single measure of respiration for each state for each well. Outliers were excluded on the basis of one or more visual criteria (1) OCR did
not respond to stimulation with FCCP, visually showing a flat line; (2) OCR (basal, oligo, FCCP, rotenone, or antimycin) was visually well below background well readings; (3) Basal OCR visually lay well below antimycin readings for the same well. The latter two categories rarely occurred. More often, a fourth category was invoked, which considered wells which appeared to have readings significantly different from other wells in the same group from the same day. The OCR readings from these wells were subjected to Grubb’s test using a freely available online calculator (http://graphpad.com/quickcalcs/grubbs2/) to assess whether they were outliers, using either basal OCR or OCR following addition of a mitochondrial inhibitor. Significance was set at p<0.05, and wells meeting this criterion were excluded from analysis. Background readings for each plate were calculated by averaging the OCRs from the background wells for those plates, and subtracted from all subsequent readings. Erratic or extreme background well readings were excluded from this calculation. Prior to data analysis, the OCR from each well after antimycin addition was subtracted from the corresponding basal, oligo and FCCP rates in order to report only mitochondrial OCRs. Each well was considered to be a separate experiment, and data are presented as the mean±standard deviation for all experiments.

7.3.4 Data analysis and statistics

Matlab was used for pairwise t-tests comparing each experimental group to the control group in order to test for significance of a given experimental outcome. For oxygen consumption measurements, all wells from a given experimental group were compared to all wells from the control group (without DMSO). R was then used for adjusting p-values for multiple comparisons (p.adjust) using the Benjamini-Hochberg
method. P-values were adjusted for all comparisons to a given control group. The p-values are generally accepted as significant when p<0.05.

7.3.5 Data availability

All data are available on Figshare via the following links:

https://figshare.com/s/67d71f71c1792498185f (fluorescent images),
https://figshare.com/s/7e4dc6e360753df695be (Seahorse XF Analyzer data), and
https://figshare.com/s/38783bd3357ad8b0c396 (MATLAB codes).

7.4 RESULTS

7.4.1 Mitochondrial potential is unchanged following treatment with cytoskeletal toxins

Figure 7.1 demonstrates how dual staining was used to measure changes in mitochondrial inner membrane potential. MitoGFP staining was used to create a mitochondrial ROI which allowed us to measure TMRM fluorescence only within the mitochondrial boundaries.

Control cells exhibited a ~30% decrease in TMRM fluorescence after 30 minutes (Figure 7.2). This decrease is probably due in part to the photosensitivity of TMRM (Perry et al., 2011), which leads to bleaching under the intense illumination transmitted through the oil-immersion lens. Additionally, replacing the media with either control or drugged media likely leads the dye to redistribute between the cells and the culture dish, exiting the mitochondria and contributing to the observed decrease in TMRM fluorescence. This decrease does not indicate mitochondrial depolarization, as CCCP addition results in a much more drastic decrease in TMRM signal.
Compared to control cells, none of the cytoskeletal toxins used resulted in significant reductions in mitochondrial inner membrane potential. In two cases (nocodazole 50 µM and noco 50 µM + cytD 5 µM), there was surprisingly less of a TMRM fluorescence decrease than in control cells (p<0.05). This observation may suggest less of a potential decrease than in normal cells, but more likely indicates that these cells randomly exhibited fewer effects of photosensitivity and/or dye redistribution than other cells. Overall, these data suggest that the cytoskeletal toxins used have no catastrophic effect on mitochondria that would result in depolarization. However, less extreme mitochondrial effects should not be discounted. We discuss the possibility of changes in morphology and mitochondrial respiration below.

Figure 7.2: Mitochondrial potential remains unchanged following treatment with cytoskeletal toxins.
Before drug treatment, cells were imaged in both the mitoGFP and TMRM channels. The mitoGFP channel was used to create a mitochondrial ROI which was then overlaid onto the TMRM image in order to measure fluorescence only within the mitochondria. The same procedure was repeated at 30 minutes following drug treatment, and resulting TMRM fluorescence for each was normalized to fluorescence for that cell prior to treatment. N=7-22 cells per group. * indicates an adjusted p-value of <0.05, *** indicates adjusted p<0.001 resulting from pairwise t-tests.
7.4.2 Specific cytoskeletal toxins induce shortening of mitochondria

Analysis of mitochondrial morphology was based on mitoGFP staining. MitoGFP images from each time point were processed in order to generate images clearly showing mitochondria within the cell (Figure 7.1a-c(iv)).

In most cases, mitochondrial lengths remained unchanged following treatment with cytoskeletal toxins (Figure 7.3a). However, mitochondrial lengths significantly decreased when cells were exposed to 5 µM cytD, or a combined dose of 10 µM noco and 1 µM cytD. Surprisingly, mitochondrial lengths did not change when cells were subjected to 50 µM noco combined with 5 µM cytD.

Figure 7.3b shows an example of a cell subjected to 5 µM cytD exhibiting mitochondrial shortening. Panel (i) shows the mitoGFP channel of the cell prior to treatment, while panel (ii) shows the same cell after 30 minutes of exposure to 5 µM cytD. In this case, the geometric mean of mitochondrial lengths decreased by approximately 25% following cytD treatment.

We also evaluated changes in numbers of mitochondria in all experimental groups in order to assess whether mitochondrial shortening was indicative of fragmentation. As Figure 7.4 shows, none of these groups showed alterations in mitochondrial number per cell following cytoskeletal inhibition that differed from control cells. This indicates that mitochondria in the 5 µM cytD and 10 µM noco + 1 µM cytD groups probably do not undergo fragmentation as a result of cytoskeletal toxicity.
Figure 7.3: Specific cytoskeletal toxins induce shortening of mitochondria.

(a) ImageJ was used to preprocess the mitoGFP image for each cell at each experimental time point. Matlab object recognition functions then measured physical characteristics of the mitochondria in the resulting image. Geometric means of mitochondrial lengths were calculated for each cell at each time point. Geometric mean length of mitochondria in a given cell at T=30 was then normalized to geometric mean length at T=0 for the same cell. N=8-22 cells per group. * indicates an adjusted p-value of <0.05, *** indicates adjusted p<0.001 resulting from pairwise t-tests. (b) An example cell exhibiting mitochondrial shortening. (i) shows the cell prior to treatment, and (ii) shows the same cell at 30 minutes following treatment with 5 µM cytD.
Figure 7.4: Number of mitochondria does not change following treatment with cytoskeletal toxins.
Pairwise t-tests yielded no significant differences in number of mitochondria following treatment in any experimental group as compared to the control group.

7.4.3 Cytoskeletal toxins caused decreased cellular basal oxygen consumption rates

Next, we measured oxygen consumption rates (OCR) to assess whether mitochondrial function was perturbed in intact cells subjected to various cytoskeletal inhibitors. Figure 7.5a shows experimental results from a typical trial, with outliers removed. Figure 7.6 shows the same experiment before outliers were removed for the purposes of analysis. In this case, it is clear that the noco 50 µM group exhibits lower basal oxygen consumption rates than the A23 and noco 10 µM + cytD 1 µM groups. The noco 50 µM + A23 group shows even further decreased respiration.

Both high and low concentrations of DMSO were confirmed to not affect mitochondrial respiration (Figure 7.5b(i)). Experimental groups were therefore compared to the controls (which were not treated with DMSO) for purposes of uniformity. OCR following antimycin addition was subtracted for each experimental well before analysis in order to only consider mitochondrial respiration. In many cases, exposure to cytoskeletal
toxins decreased basal respiration, either trending towards or achieving significance (Figure 7.5b(ii-iv)). The most significant decrease was shown by the noco 50 µM + cytD 5 µM group, which showed a basal OCR of 34.42 ± 14.22 pmol/min as compared to the control basal OCR of 59.86 ± 23.17 pmol/min (adjusted p<0.01). Significant (adjusted p<0.05) OCR reductions were also shown by the noco 10 µM (42.26 ±14.52 pmol/min) and noco 50 µM (42.00 ± 11.19 pmol/min) groups, with a lower (2 µM) noco exposure leading to OCR reductions which trended toward significance (45.51 ± 11.97 pmol/min). In the case of cytD, 200 nM (51.69 ± 19.05 pmol/min) and 1 µM (49.13 ± 15.36 pmol/min) exposures slightly decreased the basal OCR without achieving significance, but 5 µM left it unchanged (60.00 ± 12.21 pmol/min). Similarly, cells treated with noco 10 µM + cytD 1 µM only slightly decreased the basal OCR (55.78 ± 11.86 pmol/min) without achieving statistical significance.
Figure 7.5: Cytoskeletal inhibitors affect basal mitochondrial oxygen consumption rates in intact cells.

(a) Sample oxygen consumption experiment. Each tracing represents a single well. Wells of the same group are colored identically but differ in their representing symbol. Outliers have been omitted. (b) Oxygen consumption rates for (i) control groups; (ii) groups treated with noco; (iii) groups treated with cytD; (iv) groups treated with both noco and cytD. The OCR from each well following antimycin addition has been subtracted prior to data analysis in order to consider only mitochondrial respiration. Bars represent means with error bars showing standard deviations. N=11-17 wells per group. *indicates p<0.05; ** indicates p<0.01.
Interestingly, any differences in OCR from control cells caused by cytoskeletal toxins are diminished under the effects of oligo, showing that proton leak is the same across control and experimental groups. Similarly, FCCP blurs any OCR distinction between experimental and control groups, suggesting that maximal respiration is preserved under the influence of cytoskeletal toxicity.

### 7.4.4 Cytoskeletal inhibitors sensitize cells to changes in calcium homeostasis

Based on our initial results showing OCR decreases in response to cytoskeletal toxins, we hypothesized that cytoskeletal toxins sensitized cells to further upsets in mitochondrial homeostasis. As such, we challenged the cells with 100 nM A23, a calcium ionophore, prior to addition of cytoskeletal toxins. A23 causes an increase in the intracellular calcium concentration, which must be buffered by mitochondria and thus introduces another level of mitochondrial stress. The chosen concentration did not affect OCR when used alone (Figure 7.7(i)).
Figure 7.7: Cytoskeletal inhibitors sensitize cells to changes in calcium homeostasis, leading to decreased basal mitochondrial oxygen consumption rates.

Oxygen consumption rates for cells treated with (i) A23 alone; (ii) noco and A23; (iii) cytD and A23; (iv) noco, cytD and A23. The OCR from each well following antimycin addition has been subtracted prior to data analysis in order to consider only mitochondrial respiration. Bars represent means with error bars showing standard deviations. N=12-17 wells per group. *indicates p<0.05; ** indicates p<0.01; *** indicates p<0.001.

Cells pretreated with A23 and then subjected to any concentration of noco showed significant decreases in basal OCR (Figure 7.7(iii)). Basal OCRs were 30.87 ± 12.10 pmol/min for the 2 µM group (adjusted p<0.01), 33.31 ± 9.42 pmol/min for the 10 µM group (adjusted p<0.01), and 26.69 ± 14.88 pmol/min for the 50 µM group (adjusted p<0.001). As in the group without A23 exposure, cells treated with A23 followed by noco 50 µM + cytD 5 µM showed significantly decreased OCRs of 29.28 ± 10.90 pmol/min (Figure 7.7(iv); adjusted p<0.01). The A23 groups which differed most from their corresponding groups lacking A23 were cells exposed to cytD and noco 10 µM + cytD 1 µM. In the former groups, basal respiration is decreased in cells pretreated with A23 and
then exposed to 200 nM cytD (37.41 ± 10.18 pmol/min; adjusted p<0.05) or 1 µM cytD (33.24 ± 15.25 pmol/min; adjusted p<0.01). Similarly, cells treated with noco 10 µM + cytD 1 µM showed decreased basal OCR only when pre-treated with A23 (37.36 ± 14.57 pmol/min; adjusted p<0.05). Cells in the 5 µM cytD group showed a slight decrease in basal respiration with A23 pre-treatment (50.27 ± 12.80 pmol/min), but this change did not achieve significance.

OCR decreases in experimental groups somewhat carried over to maximal respiration, and the adjusted p-values achieved significance (p<0.05) for noco 2 µM, noco 50 µM, cytD 200 nM, cytD 1 µM, and noco 50 µM + cytD 5 µM (Figure 7.7). However, the differences between control and experimental groups were overall less extreme for maximal respiration than for basal respiration.

7.5 DISCUSSION

This work establishes that cytoskeletal toxins affect mitochondrial bioenergetics in a manner dependent on the particular cytoskeletal inhibitors used. Table 7.1 summarizes the results of our current work along with our previous findings on the effects of cytoskeletal toxins on mitochondrial motility (Kandel et al., 2015). In all cases, we did not see mitochondrial inner membrane depolarization within 30 minutes of treatment with cytoskeletal toxins, indicating that mitochondrial function is not drastically compromised. However, microtubule depolymerization via noco significantly reduced basal respiration in intact cells, an effect which was exacerbated when cells were pretreated with A23. This correlated with decreased mitochondrial motility in these cells (Kandel et al., 2015). CytD alone (200 nM and 1 µM) did not significantly affect either motility or respiration, but led to decreased basal OCR when cells were preconditioned with A23. The combined effects of actin depolymerization and increased calcium
handling may thus present too great of a metabolic challenge for basal respiration to be maintained. The drastic reduction in basal OCR following severe depolymerization of both microtubules and microfilaments (5 µM cytD + 50 µM noco) indicates an additive energetic consequence of these drugs. Interestingly, these cells show mitochondrial motility similar to controls (Kandel et al., 2015), implying that changes in mitochondrial function are not necessarily reflected in altered mitochondrial motility.

Table 7.1: Summary of mitochondrial effects of cytoskeletal toxins.

<table>
<thead>
<tr>
<th>Group</th>
<th>Motility (Kandel et al., 2015)</th>
<th>Length</th>
<th>OCR</th>
<th>OCR with A23</th>
</tr>
</thead>
<tbody>
<tr>
<td>CytD 200 nM</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>↓*</td>
</tr>
<tr>
<td>CytD 1 µM</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>↓**</td>
</tr>
<tr>
<td>CytD 5 µM</td>
<td>↑</td>
<td>↓*</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Noco 2 µM</td>
<td>↓</td>
<td>--</td>
<td>--</td>
<td>↓**</td>
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<tr>
<td>Noco 10 µM</td>
<td>↓</td>
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<tr>
<td>Noco 50 µM</td>
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<tr>
<td>CytD 1 µM + Noco 10 µM</td>
<td>↓#</td>
<td>↓***</td>
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</tr>
<tr>
<td>CytD 5 µM + Noco 50 µM</td>
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</tr>
</tbody>
</table>

Results detailing our previous work on mitochondrial motility are shown in the “Motility” column. ↑ indicates an increase as compared to control, while ↓ indicates a decrease as compared to control. * indicates adjusted p<0.05, **p<0.01, ***p<0.001. # indicates work which was previously performed but not published together with our earlier work on mitochondrial motility.
Two particular treatment groups showed decreases in mitochondrial lengths: 5 µM cytD and 10 µM noco + 1 µM cytD. The maintenance of basal OCR in these cells suggests a connection between respiration rates and mitochondrial shortening, which is discussed further below. The lack of shortening in the 5 µM cytD + 50 µM noco group, concurrent with decreased basal OCR, further supports this link. The 5 µM cytD and 10 µM noco + 1 µM cytD groups differ in mitochondrial motility, which is increased by 5 µM cytD and decreased by 10 µM noco + 1 µM cytD. Additionally, A23 preconditioning does sensitize cells treated with 10 µM noco + 1 µM cytD, but not 5 µM cytD, to basal OCR reduction.

We did not observe any evidence of A23 itself influencing mitochondrial function. A23 acts as a general calcium ionophore in cells. Increases in cytosolic calcium are generally followed by increases in mitochondrial calcium (e.g., (Sobolewski et al., 2012), reviewed by (Finkel et al., 2015), which can stimulate dehydrogenases of the tricarboxylic acid cycle and increase ATP production at low levels (Finkel et al., 2015). At sustained high concentrations, however, continual calcium accumulation into the mitochondrial matrix can uncouple respiration (Lemasters et al., 1997; Petronilli et al., 2001; Reed and Lardy, 1972; Wong et al., 1973) or initiate cell death via opening of the mitochondrial permeability transition pore (Finkel et al., 2015). We first performed experiments using higher A23 concentrations (2 µM) which resulted in OCR changes without the addition of cytoskeletal toxins, confirming that A23 alone can affect mitochondrial function. We lowered the A23 concentration to 100 nM, which did not alter mitochondrial respiration (Figure 7.7(i)), and seemingly stressed the mitochondria just enough to sensitize them to further energetic upset.

Similarly, we do not expect that the concentration of A23 used affected mitochondrial respiration by exacerbating the effects of the cytoskeletal inhibitors used. 169
Studies showing more global effects of A23 caused by severe disturbances in calcium regulation (Kim et al., 2002; White et al., 1974), including actin disintegration (Kuhne et al., 1993), used A23 concentrations one or more orders of magnitude greater than our 100 nM concentration. More significantly, the additive effects on basal OCR of A23 to cytD and noco concentrations which alone cause extreme depolymerization (Kandel et al., 2015) suggest that A23 does not decrease mitochondrial OCR by affecting cytoskeletal structure. Rather, it induces a distinct and perhaps more direct stress on mitochondrial function which emerges when mitochondria are further stressed by cytoskeletal depolymerization.

In all experimental groups, FCCP application blurs the distinction in OCR between control and treated groups, suggesting that mitochondrial capacity is largely maintained. Thus, lowered basal respiration is likely either a protective mechanism to preserve energy in response to cytoskeletal inhibition, or an indication that energy is no longer needed for cytoarchitectural maintenance. The A23 experiments suggest that the former contributes to the cases of cytD (200 nM-1 µM) or noco 10 µM + cyt 1 µM, since basal respiration is only significantly reduced when calcium regulation is altered by A23 in addition to cytoskeletal breakdown. Reduced maximal respiration in response to 200 nM and 1 µM cytD in A23-pretreated cells indicates that mitochondrial capacity is somewhat decreased by this cocktail of treatments, but this reduction is less extreme than the decrease in basal OCR in these groups. Unlike cytD-treated cells, noco-treated cells show compromised basal OCR without A23 pre-treatment, which is further decreased when cells are pretreated with A23. The aggravated effect following A23 pretreatment suggests some element of change in mitochondrial function which is independent of the energetic requirements of microtubule organization. Moreover, these cells show decreased maximal OCRs which trend toward or achieve significance,
suggesting that mitochondrial respiration is somewhat impaired when both calcium and microtubule maintenance are disturbed. Again, though, the FCCP-induced decreases are less extreme than decreases in basal respiration, indicating a combined effect of impaired mitochondrial function and elective energetic conservation.

The effects of cytoskeletal inhibitors on mitochondrial morphology vary by study. De Vos et al. did not see mitochondrial fragmentation following cytD treatment (De Vos et al., 2005), while another study showed extreme mitochondrial fragmentation following 50 µM cytochalasin B or 10 µM noco in transfected epithelial cells (Kumemura et al., 2008). Our observation that shortening occurs only in the 5 µM cytD and 10 µM noco + 1 µM cytD groups suggests that this response is highly specific.

The decrease in mitochondrial lengths observed in the 5 µM cytD and 10 µM noco + 1 µM cytD groups can either be caused by fragmentation or shortening of mitochondria. While numbers of mitochondria do increase following treatment (Figure 7.4), control cells exhibit a similar change. As such, mitochondria in treated cells do not appear to fragment to a greater degree than those in control cells. We do not expect that defective mitochondria resulting from fragmentation are lysed in the 30 minute duration of the experiment, so the numbers of mitochondria detected at 30 minutes should be a true reflection of the effects of cytoskeletal inhibitors on mitochondrial number. We therefore conclude that the length decrease exhibited by mitochondria in these treatment groups is likely indicative of shortening rather than fragmentation. Most discussion in the literature pertaining to decreases in mitochondrial length relates to mitochondrial fission rather than shortening, with many studies observing mitochondrial fragmentation when mitochondrial energetics are severely impaired (Benard et al., 2007) and as a sign of apoptosis (Olichon, 2003; Jagasia et al., 2005). Several studies relate mitochondrial shortening without concurrent proportional increases in number of
mitochondria. Examples include fibroblasts of patients with complex I deficiency (Koopman et al., 2005) or neurons subjected to complex I inhibitors (Yuan et al., 2007). This phenomenon is overall less studied than mitochondrial fragmentation, and may represent an entirely different effect (Yuan et al., 2007).

In the present study, mitochondrial shortening is observed concurrently with preserved basal OCR and may either cause it, result from it, or neither. As a cause, mitochondrial shortening may represent a protective mechanism that works to prevent cells from reducing respiration rates following severe cytoskeletal impairment. Alternatively, mitochondrial shortening may be an adverse effect of failure to decrease OCR in the face of severe cytoskeletal breakdown. In either case, the reduction of basal OCR in cells preconditioned with A23 and then treated with 10 µM noco + 1 µM cytD suggests that cells exposed to this cocktail of cytoskeletal toxins cannot maintain normal respiration rates when their mitochondria are already stressed by increased demands of calcium maintenance. The combination of A23 and 5 uM cytD, on the other hand, does not result in a reduction of basal OCR, suggesting that calcium-induced stress does not add to the effects of severe disintegration of actin alone. In either case, the interaction between mitochondrial shortening and respiration in these two groups seems to represent an entirely different energetic mechanism than reductions in basal OCR shown in other treatment groups, and merits further mechanistic study.

7.6 CONCLUSIONS

This study overall demonstrates that cytoskeletal impairment directly affects the ability of mitochondria to maintain energetic homeostasis in the cell. Microtubule disintegration leads to decreased basal respiration which is exacerbated when mitochondria are additionally stressed with elevated calcium levels. On the other hand,
actin depolymerization only affects mitochondrial respiration under conditions of disturbed calcium homeostasis. Mitochondrial shortening occurs in specific cases when respiration rates remain unchanged, suggesting an interplay between mitochondrial morphology and respiration. A systematic study of the interplay between cytoskeletal depolymerization and calcium homeostasis as affected by low A23 concentrations may elucidate the mechanisms underlying these findings. Additional experiments using inhibitors of mitochondrial fission may also confirm the potential protective effects of mitochondrial shortening on energetic maintenance in the cell. If the mitochondrial shortening we observe is operating by the same mechanisms as mitochondrial fission, then inhibiting fission may cause reduced basal OCR in cases when shortening appears to be protective.

This is the first report directly investigating the effects of cytoskeletal breakdown on mitochondrial respiration, and our results demonstrate that cell respiration is markedly altered in the face of cytoskeletal toxicity. Beyond indicating a functional connection between mitochondria and the cytoskeleton, this work suggests that cells with distorted cytostructures exhibit changes in metabolism which may contribute to alternate or compromised function. Examples include migrating or dividing cells, which show temporary changes in their cytoarchitecture, as well as cancer cells or cells from patients exhibiting cardiovascular disease, which show permanent mechanical changes.
CHAPTER 8: SUMMARY, LIMITATIONS, AND FUTURE DIRECTIONS

8.1 SUMMARY

Alterations in both mitochondrial function and cell mechanics are ubiquitous at the cellular level of human disease. While countless studies demonstrate that these two areas are interconnected, basic characterizations of the interactions between them are lacking. The work described in this dissertation endeavored to address that lack by studying directional interactions between mitochondrial function and cell mechanics.

Chapters 3-5 relate to the general question of how changes in mitochondrial function affect cell mechanics. Chapter 3 is a prelude to Chapter 4, presenting a new method of functionalizing QCM-D sensors with fibronectin for cell studies. The effects of pharmacologically targeting mitochondrial function on cell mechanics and the cytoskeleton were then examined in Chapter 4, with the major resulting finding being that rotenone application can lead to cell rounding. Future directions for this particular project are discussed below in section 8.3.1. Chapter 5 considered how genetically induced compromises in mitochondrial function affected the cytoarchitecture and cell mechanical properties, particularly focusing on heteroplasmy of the m.3243A>G mutation. This study was the first, to our knowledge, to examine the effects of mtDNA mutation on cell mechanics. The results revealed a conserved but nonmonotonic effect of heteroplasmy level on cell stiffness and actin conformation, suggesting a complex interaction between genetically induced mitochondrial dysfunction and the actin cytoskeleton. These findings provoked many further questions about interactions
between mtDNA mutations and cell mechanics, and Section 8.3.2 therefore suggests more basic experiments to characterize this relationship.

That mitochondrial dysfunction affects cell mechanics is somewhat unsurprising since mitochondria are involved in so many critical cellular processes including ATP generation and calcium handling (reviewed in Chapter 1). However, the reverse hypothesis, that cytostructural alterations will affect mitochondrial parameters, is far less obvious. Experiments addressing this notion were communicated in Chapters 6-7. Chapter 6 presented the development of a computational method to measure tracts of individual mitochondria on the whole cell level, and then demonstrated that cytoskeletal disintegration affected mitochondrial motility as measured by this tool. Since mitochondrial motility is becoming increasingly understood as tied to mitochondrial function, Chapter 7 then considered the functional consequences of cytoarchitectural breakdown on mitochondria. This work showed that basal mitochondrial oxygen consumption was reduced by cytoskeletal inhibitors, sometimes only when mitochondria were pre-stressed with a calcium ionophore. The communicated findings also indicated associations between mitochondrial morphology and respiration, which are suggested for future study in section 8.3.4.

8.2 LIMITATIONS

8.2.1 General need for mechanistic studies

The experiments presented in this work consider relationships between several foundational aspects of cell physiology. As such, this dissertation has focused on extending the breadth, rather than depth, of our knowledge of mitochondrial-mechanical interactions. Studies exploring the mechanisms underlying these interactions are left largely unexplored. It is our hope that future research will delve into these mechanisms,
as greater mechanistic understanding of the various mitochondria-mechanics links explored in this work will be necessary before these results can contribute toward development of new therapeutics.

8.2.2 Limitations of individual studies

8.2.2.1 QCM-D experimentation with cells

Chapter 3 presented a new method of functionalizing QCM-D sensors with fibronectin for cell experimentation. It was demonstrated that this coating was thinner, more uniform and more stable than a traditionally physisorbed layer of fibronectin, and some preliminary short-term experiments using cytoskeletal inhibitors were shown. However, the QCM-D experiments exploring mechanical effects of mitochondrial toxins over a longer time period (Chapter 4) were met with little success. The concerns raised by those studies included cell death at more physiologically relevant temperatures, stability of the QCM-D readings, and general sensitivity of the QCM-D instrument to slight changes in cell mechanics.

QCM-D complements AFM by probing mechanics at the base rather than apical layer of the cell, but other experimental systems may be able to complement AFM in other ways should QCM-D prove unfeasible (see (Janmey and McCulloch, 2007) for a related review). Even though the particular QCM-D experiments probing mitochondrial-mechanical interactions attempted in this work did not generate meaningful results, it is possible that different experimental conditions may yield success with the QCM-D setup. In any case, the chemFN coating method (Chapter 3) has already been used effectively in other experiments probing cell mechanical changes in the QCM-D, and should continue to contribute to such experiments.
8.2.2.2 Clinical applicability of m.3243A>G cybrid studies

The m.3243A>G cybrids studied in Chapter 5 represent a unique system for exploring the effects of mtDNA heteroplasmy on cell mechanics and the cytoskeleton. Because the cells studied were transmитochondrial cybrids, with nuclei and mitochondria derived from distinct cell types, the clinical relevance of this system is somewhat restricted.

An additional limitation to the applicability of this study lies in the osteosarcoma origin of the nuclear donor cells. These particular cells were chosen because they are thymidine kinase deficient, which is a valuable feature in generating the cybrid lines (Moraes et al., 2001). However, as reviewed in Chapter 1, cancerous cells exhibit altered mechanical properties in comparison to normal cells, and are generally softer with disorganized actin cytoskeletons. We indeed observed this when visualizing the cybrids under fluorescence microscopy, seeing disordered actin aggregates rather than organized stress fibers. Additionally, AFM measurements gave an average elastic modulus on the order of ~500 Pa, consistent with what others have observed in other cancer cell lines (Cross et al., 2007). The cancerous origin of the cybrids’ nuclei may affect the interactions between mtDNA mutation and cell mechanics since cell mechanics in this system are altered to begin with. Beyond cell mechanics, an additional concern with the clinical applicability of this experimental system relates to potential mitochondrial changes in the osteosarcoma cells, since cancer cells also show profound and widespread changes in mitochondrial function (Chapter 1).

8.2.2.3 Resolution of mitochondrial motility studies

One important aspect of the findings presented in Chapter 6 was that mitochondrial net distances are lognormally distributed. This is in contrast to many
studies which establish a threshold for motility indicative of directed motion, suggesting instead that mitochondrial motility followed a continuous distribution. However, a large percentage of the net distances traveled by mitochondria are measured to be less than the size of a pixel (162.5 nm in these studies). The ability to calculate net distances of under a pixel can be explained using the simple example in Figure 8.1, which shows two frames of a hypothetical object which differ only by a single pixel. The centroid in this case “travels” approximately one hundredth of a pixel. One could certainly envision more elaborate circumstances of mitochondrial rearrangement allowing for even smaller changes in the centroid location and therefore shorter net distances.

![Figure 8.1](image)

**Figure 8.1: Effects of a single pixel on centroid positioning.**
The only difference between panels (a) and (b) is the addition of an additional pixel to the object, shown in red in panel (b). The centroid location in panel (a) is (15.5208, 13.1667), while in panel b it is (15.5306, 13.1633). The calculated distance is therefore 0.0104, just over one hundredth of a pixel.

While the ability to measure sub-pixel sized net distances is clearly demonstrable, the biological interpretation of these net distances becomes questionable below a certain undefined floor. In the hypothetical case illustrated in Figure 8.1, the addition of one pixel represents the fact that an additional pixel belonging to the object exceeded the set intensity threshold in frame (b) but not in frame (a). The “net distance”
traveled therefore represents a change in resolution rather than true object motility. However, it is difficult, if not impossible, to assess the point at which “net distances” are probably more reflective of resolution changes as opposed to mitochondrial motility.

There are a number of possible ways to address this concern, with the first being to only consider net distances greater than a particular arbitrary threshold as others have done. A completely different approach would be to attempt to more clearly identify the relevant threshold by determining the range of net distances which reflect resolution changes. Experimentally, this could be explored by measuring mitochondrial “motility” in fixed cells. Finally, super-resolution microscopy is becoming increasingly usable for live-cell studies (Jones et al., 2011), and provides superior resolution to the wide-field microscopy methods used in this work. It is certain that employing super-resolution microscopy for studying mitochondrial dynamics will provide valuable insight into this field.

8.3 FUTURE DIRECTIONS

8.3.1 Effects of rotenone on cell mechanics and the cytoskeleton

8.3.1.1 Stress fiber alignment

Chapter 4 explored the cytoskeletal and mechanical effects of different mitochondrial toxins. One interesting observation resulting from that work was the cell rounding induced by 24-hour exposure to 2 µM rotenone. The fibroblasts used in the experiments described in that chapter (and in almost every other chapter of this dissertation) in general show weaker, poorly defined stress fibers. However, many other cell types show thick, parallel stress fibers when plated on glass.

In other work presented in Appendix A, I demonstrate a method for quantifying alignment of strong, defined actin stress fibers in muscle cells. This approach uses
object recognition to measure stress fiber orientation, and then quantifies the orientation distribution by measuring the kurtosis of the resulting histogram. I hypothesize that in cells with stronger, more defined stress fibers, rotenone will alter the stress fiber distribution and orientation throughout the cell. This may be measurable as changes in kurtosis of actin stress fiber orientation.

Preliminary studies have used bovine mesenchymal stem cells (bMSCs) kindly provided by Dr. Rob Mauck with assistance from Claire McLeod. Figure 8.2 shows sample cells from the control (DMSO-treated) and rotenone-treated groups. Confirming our hypothesis, the sample rotenone-treated cell shows more rounding and therefore a lower kurtosis than the control cell.

In using the kurtosis approach to characterize actin stress fiber rearrangement resulting from rotenone treatment, it will be important to assess whether stress fibers are simply broken down, or if they remain intact but become disarrayed. The approach described is relevant only in the case of rearrangement, since measuring orientations of small actin aggregates is less meaningful. Additionally, many cells (both control and treated) show less defined actin stress fibers, and a sound strategy for eliminating these cells from consideration while avoiding researcher bias must be established.
Figure 8.2: Actin stress fiber alignment in control and rotenone-treated bMSCs. Raw images are displayed along with corresponding processed images used for object recognition in Matlab. Also shown are rose plots depicting actin stress fiber orientation. The kurtosis for the sample DMSO-treated cell was 5.67, while for the rotenone-treated cell it was 3.68.

8.3.1.2 Traction force microscopy

Another interesting experimental direction following Chapter 4 is traction force microscopy. Developed in 1999 (Dembo and Wang, 1999), this method involves plating cells on gel substrates embedded with fluorescent beads. Cellular traction forces cause bead displacement, which can then be translated back into a quantity of force.

Since rotenone causes cell rounding, it would be expected that the distribution and possibly magnitude of cellular traction forces is correspondingly altered. Studying how mitochondrial dysfunction affects the forces exerted by cells could have profound implications for critical cellular processes including differentiation and migration (see
Chapter 1). A related setup which may be interesting to explore is the Transwell migration assay (also known as the Boyden chamber assay (Boyden, 1962; Chen, 2004), which may help characterize any changes in cell migration following exposure to cytoskeletal toxins.

8.3.2 Mechanics of cells from patients with mitochondrial diseases

The experimental system studied in Chapter 5, which explores the effects of m.3243A>G heteroplasmy on cell mechanics and the cytoskeleton, does not reflect mitochondrial diseases in the clinical setting. The cell types used are cancer-derived, and engineered to contain different degrees of m.3243A>G heteroplasmy. Perhaps a more relevant approach to examining the mechanical and cytoskeletal effects of mtDNA mutations would be to perform similar experiments on cells from patients harboring different mitochondrial diseases. Collaborating researchers studying mitochondrial diseases have informed us that these cells exhibit altered morphology and substrate attachment in comparison to healthy cells, which implies that they may exhibit cytoskeletal and mechanical disturbances.

The first steps in assessing the mechanical effects of a given mtDNA mutation would be to characterize cytoskeletal structure using fluorescence microscopy and cell stiffness using AFM. Further studies should attempt to link clinical symptoms with mechanical manifestations of disease, with the ultimate goal of developing therapeutic interventions based on the mitochondrial-mechanical axis of interaction for a given mtDNA disease.
8.3.3 Influence of substrate stiffness on mitochondrial motility

In Chapter 6, it was shown that mitochondrial motility is influenced by the integrity of the actin cytoskeleton. While there is more evidence for structural connections between microtubules and mitochondria, our findings are supported by previous work suggesting that microfilaments influence mitochondrial motility (Morris and Hollenbeck, 1995).

Substrates of varying stiffnesses are a well-studied system in the field of cell mechanics. Researchers have observed that substrate stiffness can influence a variety of cellular processes, ranging from cell migration (Lo et al., 2000) to stem cell differentiation (Engler et al., 2006). On an even more basic level, substrate stiffness affects cell shape and the conformation of the actin cytoskeleton. Stiff substrates induce cell spreading and the formation of thick, parallel actin stress fibers, while softer substrates cause cell rounding and poorly defined stress fibers. Since substrate stiffness affects the actin cytoskeleton, and the actin cytoskeleton in turn influences mitochondrial motility, I hypothesize that mitochondrial motility will vary on substrates of different stiffnesses. If confirmed, this finding will strengthen the observation that mechanical environment can influence mitochondrial behavior. This work could potentially be followed with experiments measuring variations in oxygen consumption in cells plated on these substrates.

A common system for studying the effects of substrate stiffness on cellular behavior involves synthesizing polyacrylamide gels of different stiffnesses (e.g., (Pelham and Wang, 1997; Yeung et al., 2005)). The relative concentration of bis-acrylamide cross linker added during the synthesis influences the stiffness of the resulting gel (Tse and Engler, 2010). I have been involved in the fabrication of such gels in collaboration with members of Dr. Russell Composto’s lab (Figure 8.3), who have also functionalized the
gels for cell attachment. Consistent with others’ observations, we have seen that fibroblasts on our softer gels (~1 kPa) appear more rounded, while fibroblasts on stiffer gels (~40 kPa) are more spread out as if on glass (Figure 8.4).

Figure 8.3: Polyacrylamide gels of different stiffnesses.

Figure 8.4: Cells spread different on gels with different stiffnesses. Images were taken using the TIRF-AFM setup describes in Chapters 4 and 5. The colloid-conjugated AFM cantilever is visible in each image. (Images courtesy of Emmabeth Parrish Vaughn)
There are several potential challenges with the proposed study. It will first be necessary to confirm that imaging through the gels will preserve the resolution obtained when imaging on glass. Additionally, it may be challenging to identify cells on softer gels with well-resolved mitochondria. In general, mitochondrial motility studies used cells which were well-spread and therefore had mitochondria which were easily resolved for tracking purposes. Cell rounding on softer gels could potentially interfere with resolution using our current wide-field microscopy setup. Finally, oxygen consumption measurements would probably involve using the Seahorse XF Analyzer, as was used in Chapter 7. This setup requires special 24-well plates, where surface thickness and cell confluence must be tightly regulated. It will be necessary to confirm that the gel system is compatible with this experimental system.

### 8.3.4 Interactions between mitochondrial morphology and respiration

The findings presented in Chapter 7 included an intriguing association between mitochondrial shortening/fragmentation and unexpected maintenance of oxygen consumption rates. Studying this relationship in a more direct and controlled manner might serve to elucidate the structure-function relationship in mitochondria, which is a subject of increasing study.

One of the main proteins involved in mitochondrial fission is Drp1 (Smirnova et al., 2001), which can be inhibited via genetic or pharmacological approaches (Cassidy-Stone et al., 2008). We propose repeating the experiments from Chapter 7 which resulted in mitochondrial shortening (5 µM cytochalasin D and 10 µM nocodazole + 1 µM cytochalasin D), but first pretreating the cells with a Drp1 inhibitor. Fluorescence microscopy will first confirm whether mitochondrial morphology is indeed affected. Following this, oxygen consumption measurements will determine whether inhibiting
Drp1 affects the maintenance of the basal oxygen consumption rate, or whether concurrent mitochondrial shortening and preserved oxygen consumption as shown in Chapter 7 were simply coincidental. It is also possible that the mitochondrial shortening we observed will persist under these conditions, suggesting a lack of involvement of Drp1.

A broader group of experiments would involve monitoring oxygen consumption in cells subjected to Drp1 inhibition to probe general interactions between mitochondrial morphology and respiration. At least one other study showed that mitochondrial fragmentation was crucial to elevated respiration levels in the specific case of hyperglycemic conditions (Yu et al., 2006), and it will be important to determine whether mitochondrial fragmentation relates to oxygen consumption in general or only in specific settings.

8.4 FINAL REMARKS

At my most recent thesis committee meeting, my committee members expressed that they were initially doubtful my work would succeed but were ultimately fascinated by what I had discovered. There were certainly many times when I shared in their early apprehensions, fearing that I was drowning in a never-ending sea of scientific knowledge. So much of what I have studied and presented here suggests new directions which are themselves wholly different fields of study. Entire careers have been devoted to the study of mitochondrial motility, or reactive oxygen species, or single cell mechanics in just a single cell type, to name a few. It would be impossible to comprehensively characterize the relationship between mitochondrial function and cell mechanics in one dissertation, or even in one entire career. It is my hope, though, that the experimental findings presented herein on some level serve to illuminate several
basic aspects of these interactions. The greatest testament to the value of this work
would be its perpetuation and utility in scientific advancement, with the ultimate goal of
improving human healthcare.
APPENDIX A: QUANTIFICATION OF ACTIN ALIGNMENT AS AN INDICATOR OF CELLULAR HEALTH

A.1 BACKGROUND AND RATIONALE

Cell mechanics is increasingly emerging as a critical component of cellular health and disease. Examples include the observations that cancer cells are softer (Cross et al., 2007) and generate weaker traction forces (Munevar et al., 2001) than normal cells, and that cells from patients with cardiovascular disease tend to be less flexible than cells from healthy patients (Simons et al., 1999).

The actin cytoskeleton is known to fundamentally contribute to cellular mechanics (Janmey and McCulloch, 2007). Breakdown of the actin cytoskeleton leads to a massive decrease in cellular elastic modulus (Rotsch and Radmacher, 2000), and the orientation of actin stress fibers often correlates with cellular mechanical properties (Tee et al., 2011). However, variations in actin stress fiber orientation are usually described qualitatively rather than quantitatively (Geiger et al., 2009; Prager-Khoutorsky et al., 2011), making it difficult to assess the degree of a given change. Thus, the ability to quantify changes in the organization of the actin cytoskeleton represents an important tool in our characterization of cell mechanics.

We were approached by members of a collaborating lab who were studying a particular disease using a mouse knockout model. They had observed that the actin stress fibers in cells from knockout mice were far less organized than those in wild-type mice. Furthermore, treating knockout cells with various drugs which protected the mice from the diseased phenotype also seemed to rescue the actin cytoskeleton from disarray. These drugs included a “recovery” drug, which directly addressed the
deficiency present in the knockout mice, as well as cyclosporin A (CsA), which prevents the mitochondrial permeability transition pore (mPTP) from opening. On the basis of this, our collaborators hypothesized that the disease under study could be treated by targeting the mitochondria. They turned to us to develop a method to quantify actin stress fiber alignment in order to support their findings.

What follows is a detailed description of our method of measuring actin stress fiber alignment which was applied to microscopic images taken by our collaborators, and corresponding results from this particular study. We anticipate that our tool will be valuable to a broad array of studies which consider cellular mechanics in many other contexts.

A.2 MATERIALS AND METHODS

A.2.1 Image processing and Matlab object recognition

Once images are collected, they are imported into ImageJ for pre-processing. If images consist of multiple color channels, the channels are separated to retain only the channel displaying actin staining. Additionally, images are manually cropped so as to remove any objects other than the cell of interest. We use a two-step process in order to transform raw images of actin-stained cells to images of white actin filaments resting against a black background. First, we convolve the images (Process>Filters>Convolve in ImageJ) using the following kernel (Giedt et al., 2012; Kandel et al., 2015):

$$K = \begin{pmatrix} -1 & -1 & -1 & -1 & -1 \\ -1 & -1 & -1 & -1 & -1 \\ -1 & -1 & 24 & -1 & -1 \\ -1 & -1 & -1 & -1 & -1 \\ -1 & -1 & -1 & -1 & -1 \end{pmatrix}$$
The second step is to threshold the resulting images. The threshold level is up to the analyst’s discretion, but should remain consistent among all images (assuming exposure times were identical) and should attempt to maximize the signal-to-noise ratio (SNR). An example of the image pre-processing using ImageJ is given in Figure A.1.

Figure A.1: Pre-processing of original images using ImageJ in order to highlight actin filaments.
(a) Original image; (b) the same image showing only the cell of interest in the green (actin-stained) channel; (b) the same image after convolution; (4) and after thresholding to give the final white-on-black image used for quantification. Scale bar is 20 µm.

Next, we use Matlab to extract properties of the actin filaments. The full Matlab script we have constructed is easy to understand, and is given in full in Appendix C.4. Each image requires under one second of total processing time. Here, we relate a brief
conceptual outline of the program. For each image in a set, the built-in Matlab command `regionprops` is used to obtain the length and orientation of each white object identified. We limit analysis to objects above a certain length threshold, which we set at 20 pixels (~5 µm) so as to omit objects representing noise or small fragments of actin rather than actin filaments.

Regionprops measures orientation ranging from -90° to 90°. To create rose plots using the rose command, we first add a mirror image of the vector containing reflected object orientations so that the resulting angles are plotted over an entire circle. The rose plots bin the data into 60 bins, each representing 6°. The rose plot corresponding to the cell shown in Figure A.1 is illustrated in Figure A.2a.

### A.2.2 Measuring kurtosis

Next, we use kurtosis as a quantitative measure of how clustered the actin filament angles are in a given cell. Matlab contains a built-in kurtosis command, which defines the kurtosis of a given dataset as

\[
k = \frac{\mu_4}{\sigma^4}
\]  

(A.1)

where \(\mu_4\) is the fourth moment about the mean and \(\sigma\) is the standard deviation. \(\mu_4\) is given by

\[
\mu_4 = \frac{1}{N} \sum_{i=1}^{N} (Y_i - \bar{Y})^4
\]  

(A.2)

where \(Y\) contains the elements of the dataset (angles in our case), \(N\) is the number of elements in the dataset, and \(\bar{Y}\) is the mean of the dataset elements. The standard deviation \(\sigma\) is defined similarly, as the square root of the variance, which is the second moment about the mean:

\[
\sigma = \sqrt{\frac{1}{N} \sum_{i=1}^{N} (Y_i - \bar{Y})^2}
\]  

(A.3)
Inserting these definitions into eqn 1 gives

\[
k = \frac{\frac{1}{N} \sum_{i=1}^{N}(Y_i - \bar{Y})^4}{\left(\frac{1}{N} \sum_{i=1}^{N}(Y_i - \bar{Y})^2\right)^2} = \frac{\sum_{i=1}^{N}(Y_i - \bar{Y})^4}{\left(\sum_{i=1}^{N}(Y_i - \bar{Y})^2\right)^2}
\]

(A.4)

Using this formula results in a kurtosis value of 3 for a standard normal distribution, with k<3 deemed a platykurtic dataset, meaning the dataset values are more spread out than those in a normal distribution. Similarly, k>3 describes a leptokurtic dataset, indicating more clustered values than a normal distribution.

The distribution must be centered around the peak angle in order to properly calculate kurtosis. Using raw data will probably not successfully result in a particular peak angle, since even essentially parallel actin filaments can differ by an angle or a fraction of an angle. Therefore, we bin the angles into groups of 6°. Since original angles are measured on a scale ranging from -90 to 90 degrees, this results in 30 bins. A mode is chosen within those bins, and the data is shifted so that it centers around that mode in order to calculate kurtosis. Original and shifted histograms for the cell shown in Figure A.1 are given in Figure A.2b. Cells demonstrating high and low kurtosis values are shown in Figures A.2c and A.2d, respectively, along with their corresponding rose plots.

A.2.3 Statistics

Data are given as geometric means due to obvious lack of normal distribution. Matlab and R were both used for statistical analysis. All groups were compared to one another using pairwise rank-sum tests in Matlab, with resulting p-values adjusted for multiple comparisons in R using the Benjamini-Hochberg method. The p-values are generally accepted as significant when p<0.05.
Figure A.2: Quantification of actin filament orientation.
(a) Rose plots are used to pictorially illustrate the distribution of actin filament orientations. This plot corresponds to the cell shown in Figure 1, and gives a kurtosis measurement of 4.96. Each bin contains 6 radial degrees. (b) correct calculation of kurtosis necessitates centering the data distribution around 0°. The original distribution (blue) corresponds to the rose plot shown in panel a. The shifted distribution (red) shifts the data so that the largest bin is at 0°. Each bin contains 6 radial degrees. (c) example of a cell whose actin filaments exhibit high kurtosis, illustrated in the rose plot on the right. The kurtosis is calculated to be 11.92. (d) example of a cell whose actin filaments exhibit low kurtosis, illustrated in the corresponding rose plot. The kurtosis is measured to be 2.26.
A.3 RESULTS AND DISCUSSION

A.3.1 Kurtosis values are associated with cell genotype

Wild-type cells generally exhibited actin stress fibers with similar orientations, resulting in leptokurtic distributions. Cells taken from 6-month-old wild type mice showed kurtosis with a geometric mean of 5.75, while for 9-month-old mice it was 5.27. Cells from knockout mice, on the other hand, exhibited a much greater degree of disorder in stress fiber orientation. The geometric mean kurtosis values were similar for cells from 6- and 9-month-old KO mice, at 3.43 and 3.54, respectively. WT and KO kurtosis values differed significantly from one another in both age groups (adjusted p<0.001 for both age groups). Example WT and KO cells from 9-month old animals, along with corresponding rose plots, are given in Figure A.3, and data is plotted as combined box/scatter plots in Figure A.4.

A.3.2 Therapeutics targeting underlying disease restore actin alignment

Adding back a “recovery” drug which directly addressed the deficiency harbored by the knockout mice restored actin stress fiber alignment (Figure A.4) to 6.21 in 6-month-old animals and 5.00 in 9-month-old ones (adjusted p<0.001 vs. KO in both age groups. The presence of normal actin stress fiber alignment in cells from these mice demonstrates that recovery of actin stress fiber alignment correlated with recovery from disease state.
Figure A.3: Kurtosis values are associated with cell genotype.
Wild-type cells typically show very ordered actin filaments, leading to higher kurtosis, while knockout cells exhibit more disordered actin cytoskeletons, which is evident in their lower kurtosis. Typical 9-month wild type and knockout cells are shown. For each example cell, the original image, the processed image, and the corresponding rose plot are shown. The kurtosis for the wild type cell shown measures 7.05, and is 2.38 for the knockout cell.

Our collaborators also treated cells from knockout mice with various drugs targeting the mitochondria. Treating mice with CsA, a drug which protects cells from mPTP opening, also largely restored actin alignment to wild-type values. Kurtosis showed a geometric mean of 5.35 for 6-month-old animals (adjusted p<0.01 vs. KO) and 6.25 for 9-month-old animals (adjusted p<0.001 vs. KO cells for 9 months). Treating knockout mice with SDZ NIM 511 (NIM) or FK506, two other drugs targeting the opening of the mPTP, resulted in only moderate restorations of kurtosis. In general, these results paralleled other assessments of disease state in the animals the cells were taken from.
Figure A.4: Scatter/boxplots showing all data.
Top is 6 months, bottom is 9 months. Box lines represent median, 25th and 75th percentiles, and whiskers indicate 10th and 90th percentiles. N=29-67 per group. Several high outliers (2 in the 6-month plot, 1 in the 9-month plot) are omitted from the figure for better visualization. P-values are computed using the rank-sum test and then adjusted for multiple comparisons. * indicates p<0.05, ** indicates p<0.01; *** indicates p<0.001 vs. KO values.
The varying effects of different drugs on disease state are explained by different mechanistic effects of these three drugs. CsA binds to cyclophilin A, and the resulting complex binds calcineurin to cause general immunosuppression (Liu et al., 1991). CsA additionally binds cyclophilin D, a mitochondrial analog of cyclophilin A, in order to prevent mPTP opening (Woodfield et al., 1998). FK506 only binds to calcineurin, but does not target cyclophilin D (Liu et al., 1991; Friberg et al., 1998). NIM, on the other hand, does not bind calcineurin and as such does not cause immunosuppression, but binds to cyclophilin (Billich et al., 1995) and confers protection from mPTP opening (Waldmeier et al., 2002). Thus, our results point to a specific therapeutic protection conferred by CsA due to its dual inhibition of calcineurin and mPTP opening.
APPENDIX B: MITOCHONDRIAL AND MECHANICAL EFFECTS OF CELL SWELLING

B.1 BACKGROUND AND RATIONALE

As detailed in the Introduction, a vast array of clinical pathologies present as both mitochondrial and mechanical dysfunction at the cellular level. In line with this observed duality, an early goal of this research was to subject cells to generalized toxins and demonstrate consequent alterations in both mitochondria and cell mechanics. Our focus subsequently shifted to more causational studies as presented in the various chapters of this thesis, but earlier experiments on cell swelling presented herein address this goal by examining swelling-induced changes in various mitochondrial characteristics as well as cell mechanics and the cytoskeleton.

B.2 MATERIALS AND METHODS

B.2.1 Cell culture and reagents

Adult human dermal fibroblasts between passages 1 and 5 (Lifeline Cell Technology, Walkersville, MD) were cultured in FibroLife cell culture media (Lifeline Cell Technology) as described in Chapter 3. Cells were seeded approximately 48 hours prior to experiments.

For experiments involving measuring changes in mitochondrial inner membrane potential, mitochondrial morphology and motility cells were plated on MatTek 35-mm glass-bottom dishes (MatTek, Ashland, MA) at a density of ~25,000 cells/dish. Dishes were coated for 30-40 minutes with 10 µg/mL fibronectin (BD Biosciences, San Jose, CA) dissolved in PBS prior to cell plating.
Each experiment began with cells placed in recording HBSS. Control cells had their HBSS removed and replaced with fresh HBSS. Cells challenged with “50% swelling” had their HBSS removed and replaced with a solution containing 50% water and 50% HBSS. The “67% swelling group” used replacement media containing 67% water, and the 80% group used replacement media containing 80% water. T=0 was considered to occur when media was replaced.

B.2.2 Fluorescence microscopy

Microtubules were visualized by incubating cells in TubulinTracker Green (Life Technologies) in 250 nM for 30 min at 37°C, then rinsed and placed in recording HBSS for fluorescence imaging with a standard FITC filter. Microfilaments were visualized by fixing cells in 4% paraformaldehyde for 10 minutes, followed by 3 minutes of permeabilization with 0.1% Triton and then incubation with Alexa Fluor 546 phalloidin (Life Technologies) used according to the manufacturer’s instructions. Cells were rinsed with HBSS and then visualized using a standard TRITC filter.

For microtubule imaging, cells were imaged using a QIClick camera (QImaging, Surrey, BC, Canada) (1 × 1 binning, 1392 × 1040 pixels) attached to Olympus IX70 microscope (Olympus, Melville, NY) with an Olympus 40x oil immersion objective lens (Olympus) and Photofluor light source (89 North, Burlington, VT). Computer control of the microscope was facilitated by LUDL programmable filter wheels, shutters, and focus control (LUDL Electronic Products, Hawthorne, NY), and images were collected using IPL 3.7 software (BD, Rockville, MD). For each experiment, cells were visualized using a standard FITC filter, and isolated cells with well-resolved mitochondria were selected. For microfilament imaging, slides of phalloidin-stained cells were viewed using an Olympus IX70 microscope equipped with a SensiCam SVGA.
high-speed cooled digital camera (The Cooke Corp.) and a 60x oil immersion lens. Images were collected using IPLab 4.0 Imaging software (Biovision Technologies, Exton, PA).

**B.2.3 Assessing changes in mitochondrial inner membrane potential and morphology**

Changes in mitochondrial inner membrane potential and morphology were assessed using the method described in Chapter 7. The reader is referred to there for a detailed description of the technique.

**B.2.4 Measuring mitochondrial motility**

Mitochondrial motility was assessed using the strategy described in Chapter 6, and the reader is referred there for a detailed explanation of this method. As in Chapter 6, only objects between 20 and 500 pixels were considered, and the minimum object lifetime was set at 9 seconds.

**B.2.5 Atomic force microscopy**

Atomic force microscopy (AFM) was performed similarly to the method described in Chapters 4 and 5, with several key differences related here. This work used triangular probes with nominal resonant frequencies of 12-24 kHz and nominal spring constants of 0.06N/m (Bruker, Camarillo, CA #DNP-S10/cantilever D). Silica colloids attached to the cantilevers were larger than those used for the work detailed in Chapter 5, and measured 7-8 µm in diameter. In this work, we extracted $E_{cell}$ from the data spanning 20% to 80% of the maximum force used.
B.2.6 Statistics

Matlab was used to perform t-tests comparing experimental groups to control (changes in mitochondrial potential, lengths and numbers). The rank-sum test was used to perform pairwise comparisons of cell stiffness, since these data tend to follow a lognormal rather than normal distribution. P-values were adjusted for multiple comparisons in R using the Benjamini-Hochberg method.

B.3 RESULTS

B.3.1 Mitochondria shorten but do not depolarize in response to osmotic swelling

Mitochondrial inner membrane potential remained unchanged following osmotic challenge (Figure B.1A). However, 80% swelling led to a significant reduction in mean mitochondrial lengths as measured by object recognition techniques (p<0.05, Figure B.1B). We also measured changes in numbers of mitochondria in order to assess whether mitochondrial shortening was symptomatic of fragmentation, and found that in this case, mitochondrial lengths were reduced without evidence of fragmentation (Figure B.1C). Thus, mitochondria probably shorten rather than fragment in response to extreme osmotic swelling.
Figure B.1: Mitochondria shorten in response to osmotic swelling.
(a) Changes in mitochondrial inner membrane potential in response to osmotic swelling. 
(b) Changes in the geometric mean length of mitochondria in response to osmotic swelling. (c) Changes in number of mitochondria in response to osmotic swelling. Bars show mean ± standard deviation. N=8-22 cells per group, with data collected over two or more days. * indicates p<0.05 after pairwise t-tests were adjusted for multiple comparisons. 50% indicates that media was replaced with 50% water, 67% indicates that media was replaced with 67% water, and 80% indicates that media was replaced with 80% water. All measurements were taken 30 minutes after osmotic challenge.
B.3.2 Mitochondrial motility is unchanged following osmotic swelling

Mitochondrial motility appeared similar to control cells following osmotic swelling. Figure B.2 shows overlaid histograms of net distances traveled by mitochondria from control cells along with those from cell subjected to different degrees of osmotic swelling. The geometric mean net distance traveled by control mitochondria was 177.8 nm, while in the swelling groups it was 160.9 nm (50% reduction in osmolarity), 174.5 nm (67%), and 168.3 nm (80%). The distributions of net distances do not appear appreciably different from one another, so we conclude that mitochondrial motility remains unchanged following osmotic swelling.

Figure B.2: Mitochondrial net distances are unchanged following osmotic swelling.
Histograms showing net distances traveled by mitochondria following osmotic swelling. Mitochondrial motility was measured 30 minutes following media replacement. N=6653-19334 total mitochondria from 6-17 cells per group.
B.3.3 Osmotic swelling causes structural changes in the cytoarchitecture

Both microtubule (Figure B.3) and microfilament (Figure B.4) structures were examined following osmotic swelling. Compared to control cells, cells subjected to osmotic swelling showed mild restructuring of the microtubule network which did not appear to be dose dependent. Affected cells (indicated by white arrows in the caption of Figure B.3) appeared to have fewer microtubules, and those that were present were more corkscrewed and emanated from a central point.

Figure B.3: Microtubules show slight structural changes following osmotic swelling. Examples of cells showing microtubule restructuring are indicated by white arrows. Scale bar is 50 µm.
The actin cytoskeleton exhibited much more profound dissolution than microtubules. As shown in Figure B.4, increasing reductions in osmolarity caused proportional breakdown of the actin cytoskeleton. While control cells had long, aligned actin stress fibers, cells subjected to the highest degree of osmotic swelling (80%) showed a complete breakdown of the actin cytoskeleton, with f-actin mostly relegated in clumps to the cell periphery. Less extreme degrees of osmotic swelling also caused the f-actin structure to disintegrate in a less severe manner which varied on a cell-to-cell basis.

Figure B.4: Microfilaments disintegrate in proportion to degree of osmotic challenge.
Scale bar is 50 μM.
B.3.4 Cell stiffness is decreased following osmotic swelling

Atomic force microscopy was used to measure cell stiffness. Figure B.5A shows an image of a colloid-conjugated AFM tip in contact with a cell, and Figure B.5B plots the elastic moduli of control cells and cells subjected to osmotic swelling. Osmotic swelling caused cells to soften, with a 50% osmolarity reduction resulting in a geometric mean elastic modulus of 1170 Pa as compared to 1802 Pa from control cells (adjusted p<0.01). 67% and 80% osmolarity reductions resulted in an even greater effect, with geometric mean moduli of 683 Pa and 680 Pa, respectively (adjusted p<0.001 for both groups). The similarity between the 67% and 80% groups suggests that cells may reach a maximum decrease in stiffness following 67% swelling. Since the actin cytoskeleton plays such an important role in determining cell stiffness, it is probable that the breakdown of f-actin leads to this drastic decrease in elastic modulus.

**Figure B.5: Osmotic swelling causes decreases in cell stiffness.**
(a) image of a colloid-conjugated cantilever in contact with a cell. (b) Elastic moduli of control cells and cells subjected to osmotic swelling. N=30-67 cells per group. Horizontal lines of boxes represent 25th, 50th and 75th percentiles (lowest, middle, and highest lines, respectively). Whiskers extend to 10th and 90th percentiles. Open circles represent 5th and 95th percentiles. ** represents adjusted p<0.01, *** represents p<0.001.
B.4 DISCUSSION

That cell swelling would exert effects on cell mechanics is a more straightforward proposition than the suggestion that osmotic swelling would impact mitochondria. Thus, it is not surprising that the cytoskeletal and mechanical effects of osmotic swelling on cells shown here support previous work. Others have observed breakdown of the tubulin and/or actin cytoskeleton in response to cell swelling in other cell types (D’Alessandro et al., 2002; Tilly et al., 1996), and decreases in cell stiffness in response to osmotic swelling have been observed by at least one other group (Steltenkamp et al., 2006).

On the other hand, the effects of osmolarity reductions on mitochondria in intact cells have received little attention, although researchers have long been interested in swelling of isolated mitochondria (e.g., Hunter et al., 1963; Stoner and Sirak, 1969). Here, we demonstrate that despite intense insult to the cellular cytoarchitecture and resulting cell mechanics, mitochondrial retain their membrane potential and show no marked changes in motility. This is consistent with results shown in Chapter 4, which demonstrates that cytoskeletal breakdown does not affect inner membrane potential. Additionally, Chapter 3 shows that microfilament breakdown only affects mitochondrial motility at an extremely high dose, and it is possible that the breakdown shown here as a result of 80% swelling is still insufficient to increase mitochondrial motility. Alternatively, it is possible that the slight degree of microtubule disintegration slows mitochondrial motility enough to counteract any velocity increases caused by breakdown of the actin cytostructure. Unlike other mitochondrial features, we do observe mitochondrial shortening rather than fragmentation which is unaccompanied by decreased inner membrane potential. As discussed more fully in Chapter 4, this process may indicate a self-protective measure. Oxygen consumption measurements would help to provide a more complete characterization of the mitochondrial effects of osmotic swelling.
Overall, we demonstrate that osmotic swelling affects both mitochondria and cell mechanics. While directionality of these effects are not directly addressed in this section, it is likely that primary effects of cell swelling are mechanical, and that these in turn affect mitochondrial size. Experiments in Chapters 3 and 4 showing mitochondrial effects of cytoarchitectural breakdown further support this hypothesis.
C.1 MATLAB CODE FOR MITOCHONDRIAL TRACKING

tic

increments = 1:1:101;

numimages = length(increments);

hz = 1/3;
%how fast the video was taken

ratio = 0.2122;
%how many microns per pixel

for i = 1:length(increments);

    if (increments(i) < 11)
        Pics{i} = sprintf('stack000%d.tif',(increments(i)-1));
        %the name of each image is "stack"
    elseif (increments(i) < 101)
        Pics{i} = sprintf('stack00%d.tif',(increments(i)-1));
    else
        Pics{i} = sprintf('stack0%d.tif',(increments(i)-1));
    end

    fissions(i) = 0;
    fusions(i) = 0;

Image = imread(Pics{i});

CC = bwconncomp(Image,8);
%finds the connected components of the image, 8-connected neighborhood

stats = regionprops(CC, 'Area');
%gets the areas from CC
bigones = intersect(find([stats.Area] >= 20),find([stats.Area] <= 500));
%bigones are the objects greater than a given pixel major axis length
newimage = ismember(labelmatrix(CC),bigones);
%labelmatrix labels each object, if bigones is not in the label matrix, %throw it out
%now we have a new image, called newimage, with our wanted larger objects
newimage = im2uint8(newimage);
%changes to uint8
clear CC areas;

[B,L,N,A] = bwboundaries(newimage,'noholes');
%find the boundaries of the objects in the new image, label the objects
number(i) = N;
%save the number of objects found as the ith index in array number

%HERE IS WHERE WE MATCH UP OBJECTS
%now we need to compare each of the objects to objects of the previous
%image and renumber them

CCnew = bwconncomp(newimage, 8);
%finds objects in newimage
%labels = labelmatrix(CCnew);
%labels them
pixels = [CCnew.PixelIdxList];
%pixels gets the indices of all the objects
allpixels = vertcat(pixels{:});
%gets all of the pixels to one array

if (i >= 2)
    %for each image after the first
    clear repeats;

    overlaps = intersect(allpixels, oldpixels);
    %finds the pixels of overlap between objects

    mapping = unique([oldlabels(overlaps) L(overlaps)],'rows');
    %mapping gives all old object labels in the left column and all new
    %ones in the right column---enables detection of all fusion and
    %fission

    uniquenew = unique(mapping(:,2));
    %gives the unique new object numbers

    uniqueold = unique(mapping(:,1));
    %gives the unique old object numbers

    fusionhist = hist(mapping(:,2),uniquenew);
    %how many times each new object is mapped to the old ones, i.e.
    %fusion

    fissionhist = hist(mapping(:,1),uniqueold);
    %how many times each old object is mapped to the new ones, i.e.
    %fission

    fusionindices = (fusionhist ~= 1);
    %gives the indices of the new objects which appear more than once

    fissionindices = (fissionhist ~= 1);
    %indices of old objects which appear more than once

    fusionobjects = uniquenew(fusionindices);
    %which new object numbers these are
fissionobjects1 = uniqueold(fissionindices);
% gives these "old" object numbers which have undergone fission

numfusion = fusionhist(fusionindices);
% gives how many times fusion has occurred for each object
numfission = fissionhist(fissionindices);
% gives how many times fission has occurred for each object

fusions(i) = fusions(i) + sum(fusionhist - 1);
% the total number of fusions between last and this frame
fissions(i) = fissions(i) + sum(fissionhist - 1);
% the total number of fissions between last and this frame

yesfission = ismember(mapping(:,1),fissionobjects1);
% puts a 1 corresponding to each old object number which has undergone
% fission

newfissionobjects = mapping(yesfission == 1,2);
% gives the corresponding new object numbers

% now we have a list: fusionobjects and newfissionobjects, which are
% all of the "new" object numbers which have fused or resulted from
% fission. we need to treat these objects specially.

specialobjects = unique(vertcat(fusionobjects, newfissionobjects));

% now we need to also relabel the connected pixels
for ii = 1:max(max(L))
    % for each value in L, meaning for each object found
    if (any(L(overlaps) == ii))
        % if this object overlaps at all with an old object
        if (ismember(ii,specialobjects) == 0)
            % if this object is not flagged as the result of
            % fission or
            % fusion--it is just the same object from the previous
            % frame
            % find the corresponding old object number, overwrite
            oldlabel = mapping((mapping(:,2)) == ii,1);
            mapping((mapping(:,2) == ii), 2) = oldlabel + 100000;
            % remaps the new object to have the old object label +
            % 1000
            % (to prevent this overwriting from being the same as a
            % flagged object number
            L(find(L == ii)) = oldlabel + 100000;
        elseif (ismember(ii,specialobjects) == 1)
            % we also do this for all connected pixels--the entire
            % object
            L(find(L == ii)) = oldlabel + 100000;
        else
            % if this object is flagged as a result of
            % fission or
            % fusion
        end
    end
end
%if the object has undergone fusion or fission
if (ismember(ii,fusionobjects) == 1)
    %if the object has undergone fusion
    mapping((mapping(:,2) == ii), 2) = ii + 300000;
    %fused objects get an extra 300000 tacked on to
    %the new number
    L(find(L == ii)) = ii + 300000;
    %same with all connected pixels
end
elseif (ismember(ii,newfissionobjects) == 1)
    %if the object has undergone fission
    mapping((mapping(:,2) == ii), 2) = ii + 400000;
    %fissioned objects get an extra 400000 tacked on to
    %the new number
    L(find(L == ii)) = ii + 400000;
    %same with all connected pixels
end
else
    %if this object has no overlaps--it's not in the mapping
    matrix
    L(find(L == ii)) = ii + 200000;
    %tack on an extra 200000
end
end

%now we need to remove the dummy indices
L(L >= 100000) = L(L >= 100000) - 100000;
%remove the dummy variables for the overlapping objects, the
%fission/fusion
%objects are still >300000/200000, new objects are >100000
allfissionindices = find(L >= 300000);
%fission indices are the indices in L of fissioned objects
allfusionindices = intersect(find(L >= 200000),find(L <= 300000));
%same with fusion
if (any(any(L >= 100000)))
    %if there are any remaining objects, meaning new ones
    newobjects = unique(L(L >= 100000));
    %finds the object numbers of the new objects
    for jj = 1:numel(newobjects)
        %for each new, non-overlapping object
        L(L == newobjects(jj)) = maxobjects(i-1) + jj;
        %renumber the index to be the max of previously numbered
        objects
        %without dummy indices, plus the index of the new object
    end
end

taggedfissionobjects = unique(L(allfissionindices));
taggedfusionobjects = unique(L(allfusionindices));
%these are the objects--with "new" object numbers--which are products of
%fission or fusion
if (i == 1)
    allfissionobjects = taggedfissionobjects;
    allfusionobjects = taggedfusionobjects;
end

if (i >= 2)
    allfissionobjects = vertcat(taggedfissionobjects, allfissionobjects);
    allfusionobjects = vertcat(taggedfusionobjects, allfusionobjects);
    if (max(max(L)) > maxobjects(i-1))
        maxobjects(i) = max(max(L));
        %maxobjects is the total objects ever, and is equal to the highest
        %object index in L
    else
        maxobjects(i) = maxobjects(i-1);
        %keep the maxobjects the same
    end
else
    maxobjects(i) = max(max(L));
    %for the first frame, maxobjects = counted objects in that frame;
end

clear oldpixels oldlabels taggedfissionobjects taggedfusionobjects;

oldpixels = vertcat(pixels{:});
%saves all of the pixel numbers for the object
oldlabels = L;
%gets the object labels for the image and saves them
oldlabels = im2double(oldlabels);
%makes it a double

stats = regionprops(L, 'Area', 'MajorAxisLength', 'MinorAxisLength', 'Centroid');
%gets information about these new objects

areas = [stats.Area];
%gets the areas of the objects
allAreas(1:length(areas),i) = areas(1,:);
%saves all of the areas as the ith column in allAreas
avgarea(i) = mean(areas(areas ~= 0));
%averages the non-zero areas

centroid = [stats.Centroid];
centroidx = centroid(:,1:2:end);
centroidy = centroid(:,2:2:end);
%gets the centroid coordinates for each object, saving the x-coordinates as
%centroidx, and the ycoordinates as centroidy
allcentroidx(1:length(centroidx),i) = centroidx(1,:);
allcentroidy(1:length(centroidy),i) = centroidy(1,:);
%saves centroid coordinates for each object
lengths = [stats.MajorAxisLength];
lengths = round(lengths*10^4)/10^4;
% rounds to 4th decimal place
allLengths(1:length(lengths),i) = lengths(1,:);
% saves the lengths in allLengths
avglength(i) = mean(lengths(lengths ~= 0));
% finds the mean for this frame

widths = [stats.MinorAxisLength];
allWidths(1:length(widths),i) = widths(1,:);
avgwidth(i) = mean(widths(widths ~= 0));
% same for widths

AR(i) = avglength(i) / avgwidth(i);
% finds mean aspect ratio for this frame

normlength(i) = avglength(i) / avglength(1);
normwidth(i) = avgwidth(i) / avgwidth(1);
normarea(i) = avgarea(i) / avgarea(1);
normnumber(i) = number(i) / number(1);
normAR(i) = AR(i) / AR(1);
% normalizes the averages and compares to previous frame

% imshow(newimage)
% plots the newimage
% hold on
% colors=['b' 'g' 'r' 'c' 'm' 'y']
% for k=1:length(B)
%     boundary = B{k};
%     cidx = mod(k,length(colors))+1;
%     plot(boundary(:,2), boundary(:,1),...
%          colors(cidx),'LineWidth',2);
%     randomize text position for better visibility
%     rndRow = ceil(length(boundary)/(mod(rand*k,7)+1));
%     col = boundary(rndRow,2); row = boundary(rndRow,1);
%     h = text(col+1, row-1, num2str(L(row,col)));
%     set(h,'Color',colors(cidx),...
%     'FontSize',8,'FontWeight','bold');
% end

% saveas(gcf, strcat(Pics{i}, '.tif') )
end

allcentroidx(allcentroidx == 0) = NaN;
allcentroidy(allcentroidy == 0) = NaN;
% overwrites any default (0,0) coordinates to NaN--this applies to object A where
% objects A and B have fused to become object B, or an object has disappeared.
% clear B L N A stats areas lengths widths;

normstats = [normlength; normwidth; normarea; normnumber; normAR];
% gives all normalizations in one

for m = 1:max(max(L))
    object = [allcentroidx(m,:); allcentroidy(m,:)];
    % each index in allobjects references a 2xnumframes array which has the
    % xcoordinates of the centroid in the first row and ycoordinates in the
    % second row
    nonans = find(~isnan(allcentroidx(m,:)));
    % finds the index where the object is present
    firstframe(m) = nonans(1);
    % gets the index where the object is first present
    lastframe(m) = nonans(end);
    % gets the last index where the object appears
    allobjects{m} = object;
    % stores all paths in a cell
    for ll = 2:numimages
        distance(ll) = sqrt((allcentroidx(m,ll) - allcentroidx(m,ll-1))^2 + ...
        (allcentroidy(m,ll) - allcentroidy(m,ll-1))^2);
        % for each frame beyond frame one, the distance traveled between
        the
        % last 2 frames is calculated
    end
    distance(1) = NaN;
    % the distance for frame 1 is NaN since the object is not yet moving
    alldistances(:,m) = distance;
    % all of the distances are then stored into an array, with each column
    % representing a new object. this is pixels per frame
    netdistance(m) = sqrt((allcentroidx(m,lastframe(m)) - allcentroidx(m,firstframe(m)))^2 + ...
    (allcentroidy(m,lastframe(m)) - allcentroidy(m,firstframe(m)))^2);
    % calculates the net distance traveled thus far, from the first
    % frame the object is present to the last frame
    netdistance(netdistance == 0) = NaN;
    % those objects that have not traveled anywhere, meaning they appeared
    % and then disappeared, have a NaN net distance
    netangle(m) = atan((allcentroidy(m,lastframe(m)) - allcentroidy(m,firstframe(m))) / ...
    (allcentroidx(m,lastframe(m)) - allcentroidx(m,firstframe(m))));
    % gets the absolute angle of the net path
    clear object;
    clear distance angledistance;
end

allspeeds = alldistances * hz * ratio;
%this converts to microns per second in place of pixels per frame
allspeeds(isnan(allspeeds)) = []; %remove the nans
allspeedslin = allspeeds(:); %make a linear vector
logspeeds = log(allspeedslin);
logspeeds(logspeeds == -Inf) = []; %gets the log distribution
netdistances = netdistance * ratio; %and net distance in microns
netangle = netangle * (180/pi); %translates net movement orientation to degrees rather than radians

allAreas(allAreas == 0) = NaN;
allLengths(allLengths == 0) = NaN;
allWidths(allWidths == 0) = NaN;

for l = 1:length(allobjects)
a = allobjects{l};
cidx = mod(l,length(colors))+1;
plot(a(1,:),a(2,:),colors(cidx), 'LineWidth',2);
hold on end %plots the centroid evolution
toc

C.2 MATLAB CODE FOR ANALYSIS OF MITOCHONDRIAL MORPHOLOGY
tic
Pics = {'before.tif'; '30minafter.tif'};

for i = 1:length(Pics);
    Image = imread(Pics{i});

    CC = bwconncomp(Image,8); %finds the connected components of the image, 8-connected neighborhood
    stats = regionprops(CC, 'Area'); %gets the areas from CC
    bigones = intersect(find(stats.Area >= 5),find(stats.Area <= 10000)); %bigones are the objects greater than a given pixel major axis length
    newimage = ismember(labelmatrix(CC),bigones); %labelmatrix labels each object, if bigones is not in the label matrix, throw it out
    %now we have a new image, called newimage, with our wanted larger objects
newimage = im2uint8(newimage);
%changes to uint8

clear CC areas;

[B,L,N,A] = bwboundaries(newimage, 'noholes');
%find the boundaries of the objects in the new image, label the objects
number(i) = N;
%save the number of objects found as the ith index in array number

CCnew = bwconncomp(newimage, 8);
%finds objects in newimage
%labels = labelmatrix(CCnew);

stats = regionprops(L, 'Area', 'MajorAxisLength', 'MinorAxisLength', 'Centroid');
%gets information about these new objects

areas = [stats.Area];
%gets the areas of the objects
allAreas(1:length(areas),i) = areas(1,:);
%save all of the areas as the ith column in allAreas
avgarea(i) = geomean(areas(areas ~= 0));
%averages the non-zero areas

lengths = [stats.MajorAxisLength];
lengths = round(lengths*10^4)/10^4;
%rounds to 4th decimal place
allLengths(1:length(lengths),i) = lengths(1,:);
%save the lengths in allLengths
avglength(i) = geomean(lengths(lengths ~= 0));
%find the mean for this frame

widths = [stats.MinorAxisLength];
allWidths(1:length(widths),i) = widths(1,:);
avgwidth(i) = geomean(widths(widths ~= 0));
%same for widths

AR(i) = avglength(i) / avgwidth(i);
%finds mean aspect ratio for this frame

normlength(i) = avglength(i) / avglength(1);
normwidth(i) = avgwidth(i) / avgwidth(1);
normarea(i) = avgarea(i) / avgarea(1);
normnumber(i) = number(i) / number(1);
normAR(i) = AR(i) / AR(1);
%normalizes the averages and compares to previous frame

end

normstats = [normlength; normwidth; normarea; normnumber; normAR];
%gives all normalizations in one
allAreas(allAreas == 0) = NaN;
allLengths(allLengths == 0) = NaN;
allWidths(allWidths == 0) = NaN;

toc

C.3 MATLAB CODE FOR ANALYSIS OF CELL CIRCULARITY AND ACTIN CONTENT IN M.3243A>G CYBRIDS

for i = 1:15
  % first read the image
  if (i < 11)
    mask{i} = sprintf('rho0_mask000%d.tif',i-1);
    actin{i} = sprintf('rho0_actin000%d.tif',i-1);
    actinin{i} = sprintf('rho0_actinin000%d.tif',i-1);
  elseif (i > 10)
    mask{i} = sprintf('rho0_mask00%d.tif',i-1);
    actin{i} = sprintf('rho0_actin00%d.tif',i-1);
    actinin{i} = sprintf('rho0_actinin00%d.tif',i-1);
  end

  mask_image = imread(mask{i});
  mask_logical = logical(mask_image);
  mask_double = im2double(mask_image);

  % first we use object recognition to calculate the circularity of the cell shape using the mask
  stats = regionprops(mask_logical, 'Perimeter', 'Area');
  perimeters = cat(1, stats.Perimeter);
  cell_perim(i) = max(perimeters);
  % gets max perimeter, since there may be bits of noise in the image
  areas = cat(1, stats.Area);
  cell_area(i) = max(areas);
  % only look at the max area
  circularity(i) = (4 * pi * cell_area(i)) / ((cell_perim(i))^2);
  % calculates circularity

  % next we calculate percentage of pixels in the cell corresponding to stress fibers
  size = [1024, 1024];
%image size

actin_image = imread(actin{i});
actin_double = im2double(actin_image);
%read the actin image

actin_masked = actin_double.*mask_double;
%mask the actin image to only look at the cell area

cell_only = actin_double;
cell_only(actin_masked == 0) = NaN;
%considers only the cell, everything else is Nans

actin_vect = cell_only(:);
%makes it a vector

actin_95(i) = prctile(actin_vect,95);
%95th percentile of actin intensity within the cell

hot_pixels = actin_masked > 0.55 * actin_95(i);
%the pixels which have actin being greater than 55% of the 95th percentile
num_hot = sum(hot_pixels(:));
%how many pixels are hot
hot_norm(i) = num_hot / cell_area(i);
%normalize to be a percentage of cell area

%%finally we want to look at association between actinin and actin
%%first we need to threshold actinin like we did to actin

actinin_image = imread(actinin{i});
actinin_double = im2double(actinin_image);
%read the actinin image

actinin_masked = actinin_double.*mask_double;
%mask the actinin image to only look at the cell area

cell_only_actinin = actinin_double;
cell_only_actinin(actin_masked == 0) = NaN;
%considers only the cell, everything else is Nans

actinin_vect = cell_only_actinin(:);
%makes it a vector

actinin_95(i) = prctile(actinin_vect,95);
%95th percentile of actinin intensity within the cell

hot_pixels_actinin = actinin_masked > 0.5 * actinin_95(i);
%the pixels which have actin being greater than 50% of the 95th percentile

actinin_locations = find(hot_pixels_actinin > 0);
[actinin_x, actinin_y] = ind2sub(size, actinin_locations);
% gives the actinin locations
actinin_pixels = [actinin_x actinin_y];

actin_locations = find(hot_pixels > 0);
[actin_x, actin_y] = ind2sub(size, actin_locations);
% actin locations
actin_pixels = [actin_x actin_y];

% now find the nearest neighbor in actin to each molecule in actinin
id_actin = knnsearch(actin_pixels, actinin_pixels);
% this gives the row number in actin_pixels. we need the location
id_actin_location = actin_pixels(id_actin,:);
% gives the location

% now calculate the distance
x_dist = actinin_x - id_actin_location(:,1);
y_dist = actinin_y - id_actin_location(:,2);
total_dist = sqrt((x_dist.^2) + (y_dist.^2));

% get some statistics
percent_colocalize(i) = sum(total_dist == 0) / length(actinin_x);
% how many of the actinin pixels colocalize with actin pixels
percent_2pix(i) = sum(total_dist <= 2) / length(actinin_x);
% how many are within 2 pixels

end

C.4 MATLAB CODE FOR ANALYSIS OF ACTIN STRESS FIBER ALIGNMENT

tic
for j = 9:9;
  if (j<11)
    Pics{j} = sprintf('wt9_AA000%d.tif',j-1);
  elseif (j>=11)
    Pics{j} = sprintf('wt9_AA00%d.tif',j-1);
  end
end

Image = imread(Pics{j});

ImageBW = im2bw(Image);

stats = regionprops(ImageBW,
'Area','MajorAxisLength','MinorAxisLength',
'Orientation','PixelIdxList');
% gets the areas and lengths from L

lengths = [stats.MajorAxisLength];
lengths = round(lengths*10^4)/10^4;
% rounds to 4th decimal place

220
lengths(lengths <= 20) = 0;
%gets rid of the < 20 pixel ones

widths = [stats.MinorAxisLength];
widths(lengths <= 20) = 0;
%sets the corresponding length and width parameters to zero

areas = [stats.Area];
areas(lengths <= 20) = 0;
%blocks out all of <20 pixel places;

angle = [stats.Orientation];
angle(lengths <= 20) = NaN;
%same with orientations

allLengths(1:length(lengths),j) = lengths(1,:);
%same for lengths
allWidths(1:length(widths),j) = widths(1,:);
allAreas(1:length(areas),j) = areas(1,:);
% saves all of the areas as the jth column in allAreas
allangles(1:length(angle),j) = angle(1,:);
allangles(allangles == 0) = NaN;

avglength = mean(lengths(lengths ~= 0));
avgwidth = mean(widths(widths ~= 0));
avgarea = mean(areas(areas ~= 0));
%averages the non-zero areas

number = length(areas(areas~=0));
%overwrites the number as the number of nonzero lengths

angle(isnan(angle)) = [];
%deletes the NaN angles from the matrix

for i = 1:length(angle)
    if (angle < 0)
        roseangle(angle < 0) = angle(angle < 0) + 180;
        roseangle = roseangle * (pi/180)
        % since angle is -90 to 90.
    else
        roseangle = angle * (pi/180);
        % converts to radians
    end
    roseangle((1 + length(angle)):(2*length(angle))) =
    roseangle((1:length(angle)) + pi;
    % adds another 180 degrees, with 180-360 mirroring 0-180, just for
    % aesthetics
end

rose(roseangle,60)
%creates a rose plot with 6-degree bins
saveas(gcf, strcat(Pics{j}, '.png') )

divided = angle/6;
% divides angles into bins, each consisting of 6 degrees, so 30 bins
total
% since we start with a 180 range of angles, giving 6 degree rather than
1
% degree bins gives a better chance at getting a mode, so we can center
the
%distribution

divided = round(divided);
% rounding gives the fake integer distribution which approximates the
true
% one

center = mode(divided);
% chooses the center of the distribution

realcenter(j) = center*6;
% gives the center in actual angles, after rounding by bin...

% we want to make realcenter the middle
if (realcenter(j) <= 0)
    centangle = angle - realcenter(j);
    % if the center is negative, add it to all angles
    centangle(centangle >= 90) = centangle(centangle >= 90) - 180;
    % and shift the ones that become >90 to a -90:0 range
elseif (realcenter(j) > 0)
    centangle = angle - realcenter(j);
    % if its positive, subtract
    centangle(centangle < -90) = centangle(centangle < -90) + 180;
    % similarly for those that go below -90--want them in a 0-90 range
end
% centers the distribution around the biggest bin, with the new
distribution
% denoted by centangle

kurt(j) = kurtosis(centangle);

% clear Image B L N A stats areas lengths widths angle divided center
centangle

end

toc


Marx KA, Zhou T, Montrone A, McIntosh D, Braunhut SJ. 2007. A comparative study of the cytoskeleton binding drugs nocodazole and taxol with a mammalian cell


