A NOVEL MICROPIPETTE ASPIRATION TECHNIQUE TO STUDY NUCLEAR CYTOSKELETAL RUPTURE FORCES

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2012

To my loving family

ACKNOWLEDGMENTS

I wish to acknowledge the teaching and support of my advisors Dr. Tanmay Lele and Dr. Richard Dickinson who helped me start this project. Their insights at various steps helped me finish this thesis and I thank them for their guidance. I also want to thank all the members of the Lele lab for their contributions. In particular, TJ Chancellor taught me the basics of cell culturing and micromanipulation and collaborated with me on this project. I have also learnt a lot from Jun Wu and Nandini Shekhar who helped me with Matlab codes and discussed various developments. My other colleagues David Lovett, Steve Winter, Brittany Hicks and Aniruddha Mungale have also helped me during my time in lab.

I extent my heartfelt gratitude to my grandparents, parents and aunt for motivating me to learn more. Despite the geographical distance they have been very supportive and have ensured that I reached this academic milestone in my life. I also wish to thank my friends both here and abroad, particularly Sherin Thomas, Henna Tangri, Meher Anand, Jayesh Joy and Nitasha Gupta. Lastly, special thanks to Jennifer Mendonca, Suchismita Mohapatra and Derek Starkey for their sense of humor and encouragement.

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AFM	Atomic Force Microscopy
DMEM	Dulbecco’s Modified Eagle’s Medium
EDMD	Emery Dreifuss Muscular Dystrophy
hPa	Hectopascal = 100 Pa
INM	Inner Nuclear membrane
KASH	Klarsicht, Anc-1, Syne-1 homology
ONM	Outer Nuclear Membrane
PNS	Perinuclear space
SUN	Sad1p, Unc-84

Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

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May 2012

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Major: Chemical Engineering

The nucleus is the largest organelle in a eukaryotic cell and is centrally positioned by cytoskeletal forces that are transferred to the nucleus through connections mediated by nuclear envelope proteins. The extent to which individual cytoskeletal elements contribute to nuclear forces is unclear. In this thesis we developed a novel micropipette aspiration technique for applying controlled forces to the nucleus. The technique involves inserting a microneedle into the cell, creating suction at the nuclear surface and then withdrawing the microneedle, resulting in nuclear motion. Knowledge of the capillary pressure in the needle allowed the estimation of the force at which the nucleus was detached from the filament network while still within the cell. Additionally, experiments were carried out to remove the nucleus entirely from the cell, past the cortical network and stress fibers. On decoupling the nucleus from the cytoskeleton or inhibiting actomyosin contractility, a decrease was observed in the critical detachment force. We conclude that this method can be used for estimating the cytoskeletal forces acting on the nuclear surface. We expect this technique to be valuable to basic studies of nuclear and cell mechanics.

CHAPTER 1
NUCLEAR CYTOSKELETAL CONNECTIONS

Introduction

Cells constantly interact with their environment and perform a multitude of functions that involve mechanical connections with their surroundings[1]. Mechanical interactions of the cells with the substrate in turn dictate a large number of processes such as cell migration[2] and gene regulation[3]. This process is termed as mechanotransduction or the process of converting physical forces acting on the cell to biochemical signals followed by integration of these signals into cellular responses[4]. Thus, forces acting on the cell during various processes are an important topic of study since they act as mechanistic signals that direct downstream biochemical pathways.

It is known that external mechanical forces can propagate through the intracellular cytoskeleton to the nuclear surface and cause nuclear distortion[5]. This thesis focuses on cytoskeletal forces generated on the nuclear surface.

Nuclear Positioning and Dynamics

The nucleus is centrally located in cells and has been shown to be 5-10 times stiffer than the surrounding cytoplasmic matter[6]. Fig 1-1 of this thesis shows an image of 3T3 cells crawling on a glass substrate where the nucleus occupies a central position in most cells and is positioned rearward in migrating cells. Despite its large size, the nucleus undergoes significant repositioning during cellular processes such as differentiation, cell migration etc[7]. An example of the importance of nuclear positioning in the cell, is the active migration of male and female pronuclei towards each other during the early phases of animal cell development[8]. In other studies, nuclear migration from apical to basal surfaces was shown in pseudostratified basal epithelium[8]. The

movement of the nucleus in a migrating cell is discussed in a review by Freidel et al where coupled nuclear cytoskeletal dynamics at the leading and trailing edge cause changes in cell polarity and migration[2]. Summarily, the nucleus actively moves towards the leading edge or rearwards of a migrating cell depending on the cell type and is extensively affected by the cytoskeletal network[2]. Essentially, while the nucleus is centrally positioned in most cells, it does undergo repositioning during many major cellular processes like cell differentiation, migration, mitosis and meiosis[9]. The centrality of the nucleus and its role in important cellular processes suggests that irregular nuclear positioning and function can be detrimental to an organism.

The elimination of nuclear cytoskeletal connections such as Nesprin-1 and KASH domain knockout studies in mice have shown increased growth retardation, perinatal death[10] and incidences of muscle diseases[11]. A family of diseases known as “laminopathies” which include Emery-Dreifuss Muscular Dystrophy (EDMD), Dilated cardiomyopathy, Dunnigan-type familial partial lipodystrophy, axonal neuropathy and Hutchinson-Gilford progeria syndrome have been linked to deficiencies of the lamin network of the nucleus[12]. Apart from inadequate nuclear connections to the cytoskeleton, irregular positioning has also been associated with carcinogenesis[13].

Since nuclear positioning is important role in normal cellular functioning, it is important to explore how the cytoskeletal elements hold it in place. This can be accomplished by studying the forces that the cytoskeletal elements exert on the nucleus within the cell and the factors that affect these forces. This chapter focuses on the structure of the nucleus and its connections with the surrounding cytoskeletal framework.

Structure of the Nucleus

The nucleus is divided into two distinct regions: the nuclear envelope which is a bilipid layer and the nuclear interior or matrix.

Nuclear interior

Briefly, the nuclear interior consists of almost two meters of DNA wound around histones to form the chromatin fibers. Cajal bodies, chromosomes (formed by arrangement of chromatin fibers)[14] and nuclear actin and myosin filaments form structural components of the nuclear interior[15]. Chromatin has various structural areas consisting of nucleosomes, loops of chromatin fibers and specific spots where it forms connections to the nucleolus and the lamina. Nucleoli which are the largest structural components of the nucleus produce ribosomes and have been shown to be stiffer than the surrounding nuclear matrix in studies on the human ganglion[16]. Cajal bodies are coiled bodies that associate with nucleolus and small nuclear riboproteins and respond to cellular stresses[14]. Nuclear actin and myosin have been observed in the nucleus and possibly contribute to the mechanical and structural balance within the nuclear interior[15]

Nuclear lamina is a protein meshwork on the inner surface of the inner nuclear membrane. The main components of the lamina are the nuclear lamin proteins which regulate DNA activity[17], nuclear positioning and gene expression through protein complexes formed by binding with emerin and lamin associated polypeptides[18].Lamins are divided into two types: A type lamins and B type lamins which are coded by separate genes and are type V intermediate filaments proteins[14].The A type lamins consist of lamin A and C. A study of the role of lamin A in physiological ageing by Scaffidi et al showed that accelerated ageing in patients with Hutchinsons Gilford Progeria Syndrome could result from an accelerated lamin A dependant mechanism[19].

Other studies by Lammerding et al comparing the contributions of lamin B1 and lamin A/C[20] and Dahl et al[6] have linked the lamins A/C to maintaining nuclear structure, shape and stability.

Lamin binding proteins form a linker between the inner nuclear membrane and the lamin network. These proteins comprising of emerin, lamin-B1 receptor, etc have a transmembrane domain and a lamin binding domain[14]. Mechanically inducing changes in nuclear shape has shown that the nuclear interior is very porous and aqueous with a 60-70 % decrease in size occurring upon micropipette aspiration of isolated nuclei[21].

Nuclear envelope

The nuclear envelope is divided into three major parts, the Outer Nuclear Membrane (ONM) which is contiguous and a part of the Endoplasmic Reticulum(ER), the ribosome free Inner Nuclear Membrane(INM) and the nuclear pore complex(NPC). A space between the two membranes called the Perinuclear space (PNS) separates them by a distance of 30-50nm[8,22]. The ONM and INM are connected at annular junctions known as nuclear pore complexes which assist in the facilitated diffusion of macromolecules between the cytoplasm and the nucleus[14]. Within the nuclear envelope, a bridging structure is required to direct the flow of membrane proteins from the ER which interact with the lamina in INM or to the ONM for interaction with the cytoskeleton [23]. The connections of the nucleus with the rest of the cytoplasm, which determine its positioning and movements during cellular processes, are directed through this nuclear envelope. The bridging of the ONM and INM over the luminal space is essential for connecting the nucleus to the cell exterior.

LINC Complex

Connection between the nucleus and the cytoplasm are established by the Linker of Nucleoskeleton and the Cytoskeleton (LINC)[24]. The LINC complex comprises of the SUN domain containing proteins of the INM and the KASH domain containing proteins of the ONM which form a linking bridge. This has been illustrated in Fig 1-2 where the elements of the LINC complex have been outlined.

Nuclear migration and positioning studied in *Caenorhabditis elegans* have shown that worms deficient in the membrane proteins UNC 83 and UNC 84 display defective phenotypes of slow nuclear motions and defective migration[25]. These genetic mutations studies of off-centered nuclei in *c elegans* led to the discovery of the role of the unc 84 gene which codes for UNC 84[25]. The C terminus of UNC 84 was found to be conserved in Sad1 and two human proteins having a 175 amino acid domain called the SUN domain[23]. The proteins UNC, Sad1, etc have a SUN domain and some of their common features include a transmembrane domain that allows them to localize in the nuclear envelope[26]. They are proteins of the INM with the SUN domain present in the PNS and the nucleoplasmic domain bound to lamins[27].

Studies in *Drosophila* later led to the discovery of the KASH family of proteins which include Drosophila Karlsicht, C elegans ANC -1 and mammalian paralogs, Syne/Nesprin-1 and -2[8]. These proteins have a conserved C terminal KASH domain (Karlsicht, ANC-1, and Syne Homolgy) which has a transmembrane span followed by 6-30 residues at the C terminus[25]. In mammalian cells, the LINC complex consists of nesprins which have multiple spectrin repeats in the cytoplasmic N terminus and a transmembrane KASH segment. Of these the Nesprin-1 and Nesprin-2 are very large proteins (800kDa) and have actin binding domains[28]. Nesprin-3 and Nesprin-4 bind to

plectin and kinesin-1. Zhang et al (2005) have shown that the KASH domain is essential for the localization of nesprins to the nuclear envelope[29]. KASH proteins have a conserved transmembrane C terminal domain and a large divergent N terminus in the cytoplasm while being localized to the ONM[30]. This localization and anchoring to the ONM is a result of interactions with the SUN domain essentially forming a bridging structure along the PNS[14,31]. These interactions are dynamic and the KASH-SUN interaction pairs change during various instances of cell migration. Over-expression of the KASH domain has been shown to have a significant anchoring impact which is due to the competition with endogenous ANC-1 sites for limited UNC-84 sites[30].

The cytoskeletal elements that anchor the nucleus in place are the actin microfilaments, the intermediate filaments and the microtubules. The nature of forces exerted on the nucleus are the pushing and pulling forces exerted by actomyosin, tensile forces by the microtubules and compressive or tensile forces by the intermediate filaments[32]. The physical connections between these elements and the nucleus are established by the LINC complex. The nucleoplasmic face of the SUN proteins is linked to the lamins and the SUN domain is connected to the KASH domain of KASH proteins which extend into the cytoplasm.

The LINC complex connects the nucleus to the elements of the cytoskeleton, namely actin, microtubules and intermediate filaments. These connections are formed by a number of different proteins. Nesprin-1 and -2G are KASH proteins that contain an actin binding domain on the cytoplasmic face, thus providing a binding site for actin filaments[33]. There is a hypothesis of transmembrane actin-associated nuclear (TAN) lines which are comprised of actin cables, nesprin-2G and SUN2. These are analogous

to focal adhesions and transmit forces generated in the actomyosin cytoskeleton to the nucleus[34]. Mutations in Nesprin-1 and -2 have been linked to Emery-Dreifuss muscular dystrophy [29]. Other members of this family, such as Nesprin-3 have been shown to have plectin binding domains (plectin connects to intermediate filaments), shown by Postel et al in studies on Zebrafish[35]. Nesprin-3 is expressed in nuclear envelopes of skeletal and epidermal cells and loss of Nesprin-3 was shown to sever connections between the nucleus and intermediate filaments[35]. Other lamin-associated proteins such as Emerin are linked to chromatin, lamins and are responsible for maintaining stable nuclear structure and shape[36]. The SUN-KASH bridge is also essential for proper coupling of the centrosomes to the nucleus which plays a role in pronucleus migration in *C elegans*[23]. In centrosomes independent processes, the nucleus is associated with the minus end directed microtubule motor dynein. The plus end directed motor, kinesin interacts actively with the nucleus and kinesin mutant animals have been shown to have severe migration defects[37]. Nesprin-4 interacts with kinesin light chain and induces the nucleus to move away from the centrosomes and toward the plus end of microtubules suggesting its role in nuclear positioning[38]. The exact functionality of Nesprin-4 is unknown but due to its capacity to bind with kinesin-1which in turn interacts with lateral microtubules, it could be responsible for positioning the nucleus close to the basal membrane[38]. In the light of all these connection forms by proteins containing the KASH an SUN domains, the LINC complex may function in mechanotransduction of physical signals from the extracellular matrix directly to chromatin[39].

Measuring Forces Acting on the Nucleus

From the above discussion it is clear the cytoskeletal elements position the nucleus in an active manner and that mechanotransduction is a complex interlinked

event between the nucleus and the cytoplasm. The mechanical adaptation of the nucleus to shear forces shows that the cell reorganizes nuclear elements in response to the force. The ability of the nucleus to withstand these deformation forces is expected to depend on the values of bulk modulus, shear modulus, viscosity, etc[40]

The mechanism by which these forces are transmitted, as well as the value of the forces, are as yet unclear. Since the cytoskeletal elements are dynamic and the number of attachments and number of motors acting at any time are unknown, it is difficult to measure the forces acting on the nucleus by direct methods. An indirect approach would measure the forces resisting the nuclear displacement away from the center of the cell. The cytoskeletal connections and LINC complex proteins could then be sequentially eliminated in order to determine the contributions of each. This would determine the relative contribution of an individual element with respect to others in positioning and anchoring the nucleus. A number of techniques exist to apply small-magnitude forces in order to test the response of the nucleus and the cell. This thesis describes a quantitative experimental technique to measure nuclear cytoskeletal rupture by employing micropipette aspiration on live cells. The benefits and drawbacks of this method with respect to other methods have been determined as part of understanding the feasibility of carrying out the procedure. Elimination of various cytoskeletal connections by over-expressing the KASH domain and using the drug blebbistatin, have been carried out to determine the relative force balance on the nucleus.

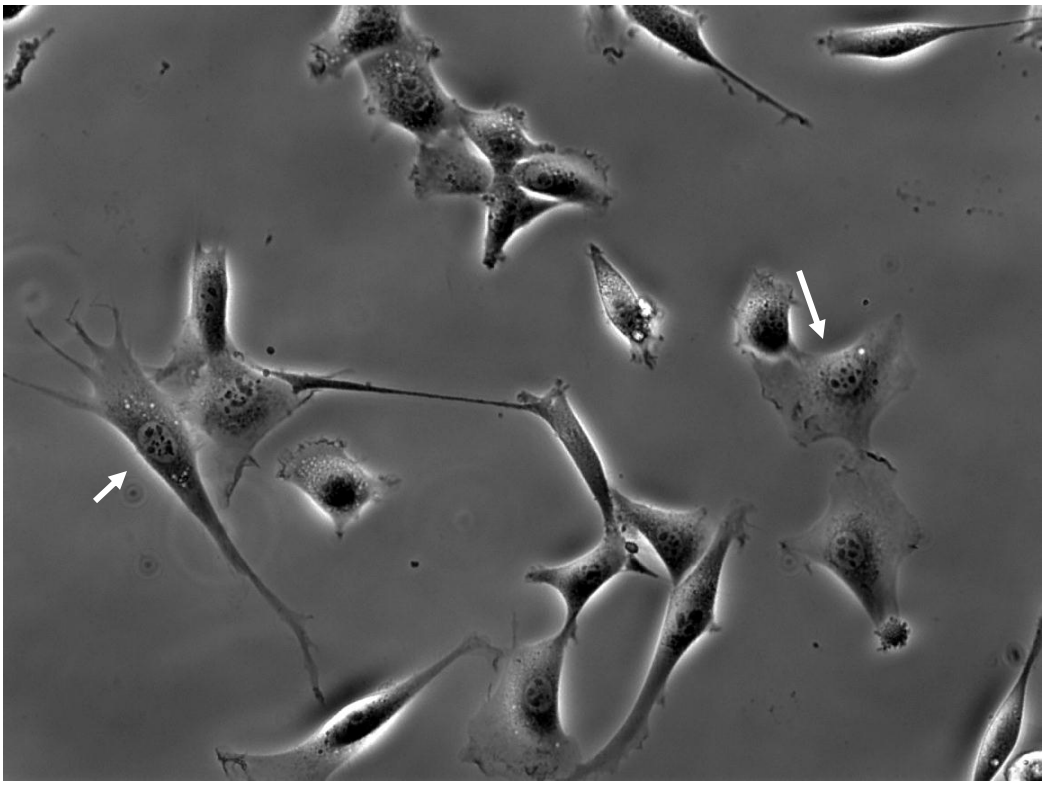

Figure 1-1. 3T3 cells crawling on a glass substrate. Note the centrality of the nucleus in most cells and its rearward positioning in migrating cells.

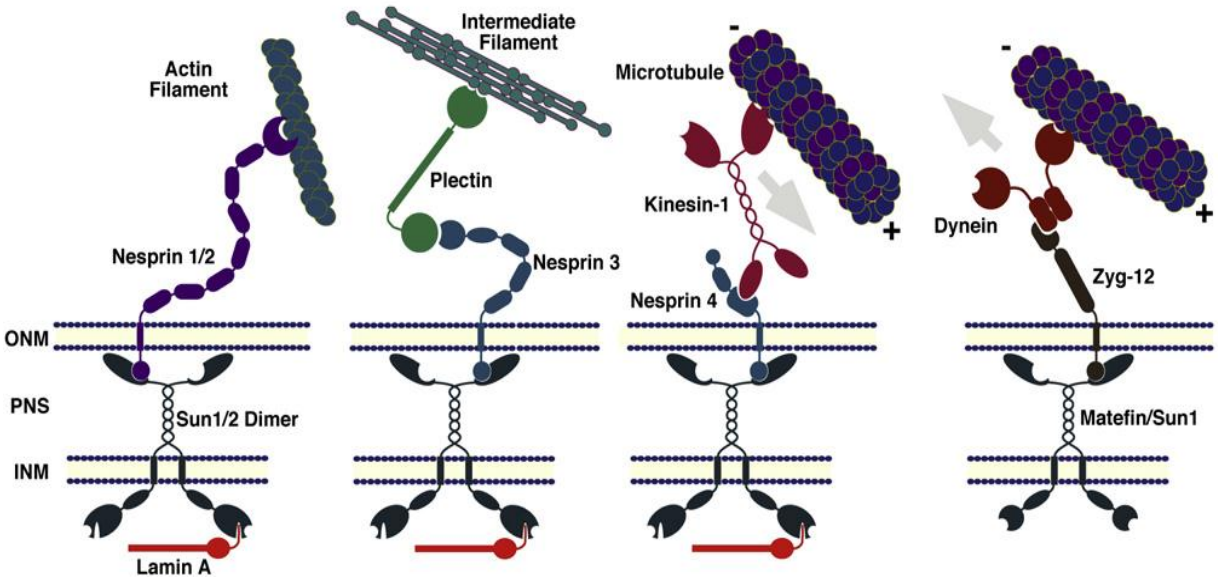

Figure 1-2. Schematic of the nuclear envelope proteins or the LINC complex showing connections between the nucleus and the cytoskeleton[9]. The SUN and KASH proteins form a bridge which connects to the cytoskeletal elements .

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CHAPTER 2
METHODS OF PROBING THE NUCLEUS

Introduction

Alterations in the nature and linkages of the nuclear envelope proteins are associated with a host of diseases. These changes are linked to alterations in nuclear shape, structure and stability. In order to study these mechanical properties of the nucleus, there has to be a method to apply a force or stress on the nuclear body and measure the response. Nuclei can be studied both within the cell and as isolated bodies. These two approaches have their own advantages: in situ nuclei are subjected to all membrane connections thus providing information about nuclear properties in relation to other cellular elements while isolated nuclei can be studied to quantify nuclear properties exclusively [41]. Since the nucleus is 5-10 times more rigid than the surrounding cytoskeleton, isolation of stable nuclei can be achieved by both single cell and bulk methods[41]. Usual methods of isolation include using a microneedle to tear though the cell membrane or mechanically rupture the cell. Bulk methods include the use of hypotonic solutions to cause swelling of the cell body and release of the nucleus by ultracentrifugation[42]. Once isolated, the integrity of the nuclei have to be maintained using buffer solutions which mimic cellular conditions. It is difficult to predict if the isolated nuclei have identical properties as the nuclei within cells [43].

Depending on the difference in methods employed and the cell type, nuclear stiffness has been estimated to be around 0.1-10kPa[14]. Specific nuclear components such as the chromatin, lamina and envelope proteins can be labeled with fluorescent probes to test the response of each contributing element. The techniques employed so far can apply forces in a range of 10^{-7} to 10^{-9} N, and forces acting on the nucleus are

estimated to be of a magnitude of 10^{-9} N[1,42]. Some common techniques employed in force application are atomic force microscopy, micropipette aspiration of isolated nuclei, substrate strain, microneedle manipulation, cell compression and magnetic manipulation of the nucleus. A brief introduction to these methods with their results, benefits and shortcomings is provided below

Techniques

Atomic Force Microscopy

AFM uses a flexible tip to indent a cell surface by applying a range of forces. The graph between the forces and extent of indentation provides insight into the elastic properties of the material by the Hertz model which generates the Young's modulus[44]. Its use in probing the properties of the nucleus and cytoskeleton are however complicated by the inhomogeneity of the cell and the non-mutually exclusive response of the nucleus and the cytoskeleton[45]. This method was successfully applied by Schaoe et al in quantifying the contribution of Lamin A protein to the stiffness of amphibian oocyte nuclei by generating force curves and stiffness values. Assuming a nuclear radius of 0.25 µm and a laminar thickness of 200nm, an elastic modulus of 200MPa was obtained for the average stiffness of 7 mN/m[46]. An advantage of using AFM is that it can be applied to single cells giving excellent spatial resolution on the scale of nm. The models used to interpret the data must include the contact area and spatial variance of the substrate[2,44]. The cantilever tips used are flexible with a diameter in the range of nm to µm and are used to perturb single cells. The deflection of cantilever tips is measured and the force calculated by knowing the stiffness. This method has been applied in testing the interactions between biomolecules as well as in intra molecular interactions[45].

Micropipette Aspiration of Isolated Nuclei

Micropipettes have been successfully used to perform aspirations on isolated nuclei and cells. By aspirating cell material, membrane tension and adhesion properties of the cell to the substrate can be tested[47,5]. Micropipettes are usually prepared by using a pipet puller and have varying tip diameters of 1-4µm and aspiration is performed into the tip. In aspirations performed on living cells, forces of around 10pN to 10000nN could be applied[48].Application of this technique to nuclei requires their isolation from the cell body. In a pioneering work by Dahl et al, micropipette aspiration of isolated nuclei in TC7 cells helped in exploring the role of chromatin and lamin B in nuclear viscoelasticity[43]. The nucleus was aspirated into a micropipette to study the creep properties. Swelling of cells was used to separate the effects of cytoskeletal elements, and an effective area increase of almost 70% was measured[43].

In the work by Guilak et al, a micropipette with a tip diameter of 4-6 µm was used to repeatedly aspirate articular condrocyte cells, rupturing the cell membrane and isolating the nucleus. The pressure gradient on the cell, initial cell diameter and length of aspirated cell were used to calculate the viscoelastic properties of intact and isolated nuclei giving a nuclear Young's modulus of 1 kPa[40].

This technique is effective for studying the properties of isolated nuclei and obtaining viscoelastic parameters. A limitation of this method is that once the nucleus has been isolated, buffer conditions have to be maintained accurately[1].

Cell Compression

Cell compression can be achieved by holding the cell between two glass microplates as the micromanipulation system for applying force. The work of Thoumine et al[49] describes the protocol for compression of chick fibroblasts between two glass

microplates. The plates are connected to two micromanipulators with the bottom plate being rigid and adhesive and the top flexible and non adhesive. Traction force in the range 10^{-7}- 10^{-8} was applied. Compression and traction were achieved by the top plate; cell orientation in parallel and perpendicular direction and the deflection of the plate was noted[49]. Elastic moduli in the range of 600-1000N/$m^2$and a viscosity of 10^4 were obtained.

An extension of this technique in studying the mechanical behavior of the nucleus was performed by Caille et al[50]. The uniaxial force was measured in round and spread cells to eliminate the effect of cell shape on nuclear mechanics. Isolated nuclei held between glass microplates exhibited non linear force deformation curves which indicated round cells deformed at lower forces than spread cells. An advantage of this method is that it allows for global strain application the whole cell, while still being a single cell technique.

Substrate Strain

Nuclear aspiration on isolated nuclei might distort the properties of the nucleus or alter structures due to the interference of the removal process. An alternate method of applying strain to the nucleus is the use of silicon membranes which transmit stresses to the cytoskeleton through focal adhesions. This work, extensively described by Lammerding et al[51] examines lamin A/C mutations which are implicated in Emery-Dreifuss muscular dystrophy. A transparent silicon membrane was employed to generate strain on cells by deformation. The strain was transmitted via the focal adhesion complexes to the cytoskeleton in proportion to the applied mechanical strain[52]. A limitation of this technique is that strain application tests only the relative stiffness of the nucleus and the cytoplasm without elucidating the absolute nuclear stiffness value.

It does have the advantage of simultaneous strain application on a number of cells increasing the experimental throughput[53]. Variations can be introduced by using micropatterned substrates and this has been employed in examining the stiffness in *Drosophila melanogaster* and *Caernohabditis elegans* nuclei.

Micro-Needle Manipulation

Microneedle manipulation uses fine needles to test the mechanical response of the cell and the nucleus. In an early study by Kishino et al, glass needles were used to test the elasticity of single actin filament, giving a value of around 108 pN[54]. In literature so far, many variations of this technology have been employed. Forces required to bend the fibroblast ruffles in BC3H1 cells were tested using a thin needle to bend the ruffles[55] where the ruffles resisted a force value of 15-20µdyn/µm. Maniotis et al[5] used fibronectin coated microbeads on bovine capillary endothelial cells as substrate. The beads were then pulled away laterally by using an uncoated microneedle, demonstrating a pull on the nucleus even when it was situated away from the site of force application[5]. With this method it is difficult to quantify the forces acting on the cellular structure and to interpret the molecular details while an advantage is that the cell remains intact.

Magnetic Manipulation of Intra Nuclear Particles

Magnetic manipulation is a method that utilizes magnetic beads, which can be manipulated under an external magnetic field. De Vries et al describe an experiment with magnetic tweezers where a magnetic bead was injected into the nucleus of HeLa cells using a micromanipulator. The viscosity within HeLa cells was measured to obtain a Young's modulus of 250Pa [56].This method was eventually used to study the elastic modulus of chromatin within the cell. In a subsequent work by Celdon et al, nanorods

were inserted into the nucleus and controlled by a magnetic field to cause micron-sized displacement within the nucleus in LMNA-/- cells. The viscosity data calculated indicated that the viscosity of these cells is almost seven times lower than the LMNA+/+ and shear modulus that is three times lower[57]. This is an important technique for precisely controlling nuclear deformation, but the variation in contact area between cell surface and bead can be difficult to interpret.

Development of a Novel Aspiration Technique

While the techniques discussed so far give an insight into the viscoelastic properties of the cell and isolated nuclei, it is difficult to infer the forces acting on the nucleus in living cells. Probing isolated nuclei can distort their properties due to the methods used in separation. Since a direct method of determining the values of these forces is difficult to develop due to the indeterminate number of connections and motors, an indirect approach was used in the technique described in this thesis. The cytoskeleton maintains a dynamic force balance on the nucleus where any motion off-center must result in a corresponding restoring force from the cytoskeleton. Micropipette aspiration was employed with several modifications to existing methods to measure the forces contributing to nuclear positioning. This method involves pulling the nucleus away from the cell in order to overcome the restoring forces that hold it on place estimating the resistive contribution of the elements. Further, selectively disrupting nuclear connections to specific elements of the cytoskeleton by severing LINC complex connections gives an insight into relative force contributions.

Utility of Making these Measurements

The result of aspirating the nucleus out of the cell would help in extending understanding of nuclear positioning during many important events in the cell cycle. It

would provide a quantitative value of the dynamic force balance on the nucleus by assessing the relative contribution of each element. Motile cells reposition the nucleus towards the rear of the migrating cell, and these experiments would help identify the extent to which cytoskeletal elements remodel the nuclear forces in order to position it. The micropipette aspiration technique described in this thesis advances previously employed methods to test nuclear cytoskeletal rupture forces. Usually micropipette aspiration is used to measure the affinity of cells for various substrates and the adhesion strength by using suction pressure and a fine needle. These techniques have been limited to isolated nuclei aspirated by larger diameter needles while the method employed in this document uses a very fine diameter needle to establish initial contact with a nucleus present in a live cell. This needle is an Eppendorf Femtotip II® (Eppendorf, Hauppaugh, NY) which has a tip diameter of 0.2-0.5 μm at the tip. The needle geometry is such that it tapers down to a point exhibiting varying diameter along the length of the needle. On touching the nucleus, the capillary pressure in the nucleus is removed and the nucleus gets sucked by the tip of the needle. Upon withdrawing the needle, the nucleus may also be removed with it depending upon the value of the capillary pressure. The extent of nuclear removal is contingent upon the forces opposing the motion of the nucleus away from the cytoskeleton. These are the forces anchoring the nucleus in place due to cytoskeleton and the forces due to the cortical and stress fiber network. Forces due to the cell membrane which are almost negligible compared to other forces were not included in this study.

In most cases at lower pressures, the needle is able to detach the nucleus while still maintaining it within the cell body. However the force acting on the nucleus is not

sufficient to pull it past the cortical network. At higher pressures, the force was sufficient to cause a complete rupture where the nucleus was removed entirely from the cell.

Capillary Action of the Needle

As shown in Fig 2-1, the needle is immersed in the sample medium of h ~ 3 cm depth and aspirates a liquid column of length l, diameter d and angle α. The hydrostatic force balance requires that air pressure inside the capillary P_1 plus the fluid head in the column $l\rho g cos\alpha$ balance the fluid head ρgh in the column plus the capillary pressure $p_c = 4\gamma cos\theta/d$. Here θ is the wetting angle of water in the capillary and γ is the surface tension. That is

$$P_1 + l\rho g cos\alpha = \rho gh + 4\gamma cos\theta/d \quad (2\text{-}1)$$

The fluid heads can be assumed to be negligible relative to the capillary pressure, in which case pipette air pressure approximately balances the capillary pressure.

$$P_1 \approx 4\,\gamma cos\theta/d \quad (2\text{-}2)$$

In a pulling experiment, the capillary is pressed against the nuclear surface, and the air pressure is reduced in order to create a suction pressure on the nucleus by the capillary tip. This suction pressure is approximately equal to the original capillary pressure $4\gamma cos\theta/d$. Because the tip is tapered (d is variable), the suction pressure on the nucleus can be controlled.

Table 2-1. Pressure balance acting on the liquid column within the needle. The height of media in the dish is estimated to be 3 cm and the length of liquid column is around 1mm. The diameter of the tip is 0.5 μm and surface tension of water is 72 mN/m. The angle of inclination of the needle is 45^0 while the angle of contact of water and glass is 30^0

Pressure	Formula	Value
P1	Capillary pressure	1600 hPa
P2	Pgh	294 Pa
Pressure due to liquid column	$lg\rho\cos\alpha$	6.9 Pa
Surface tension	$4\pi\gamma\cos\theta/d$	~1800 hPa

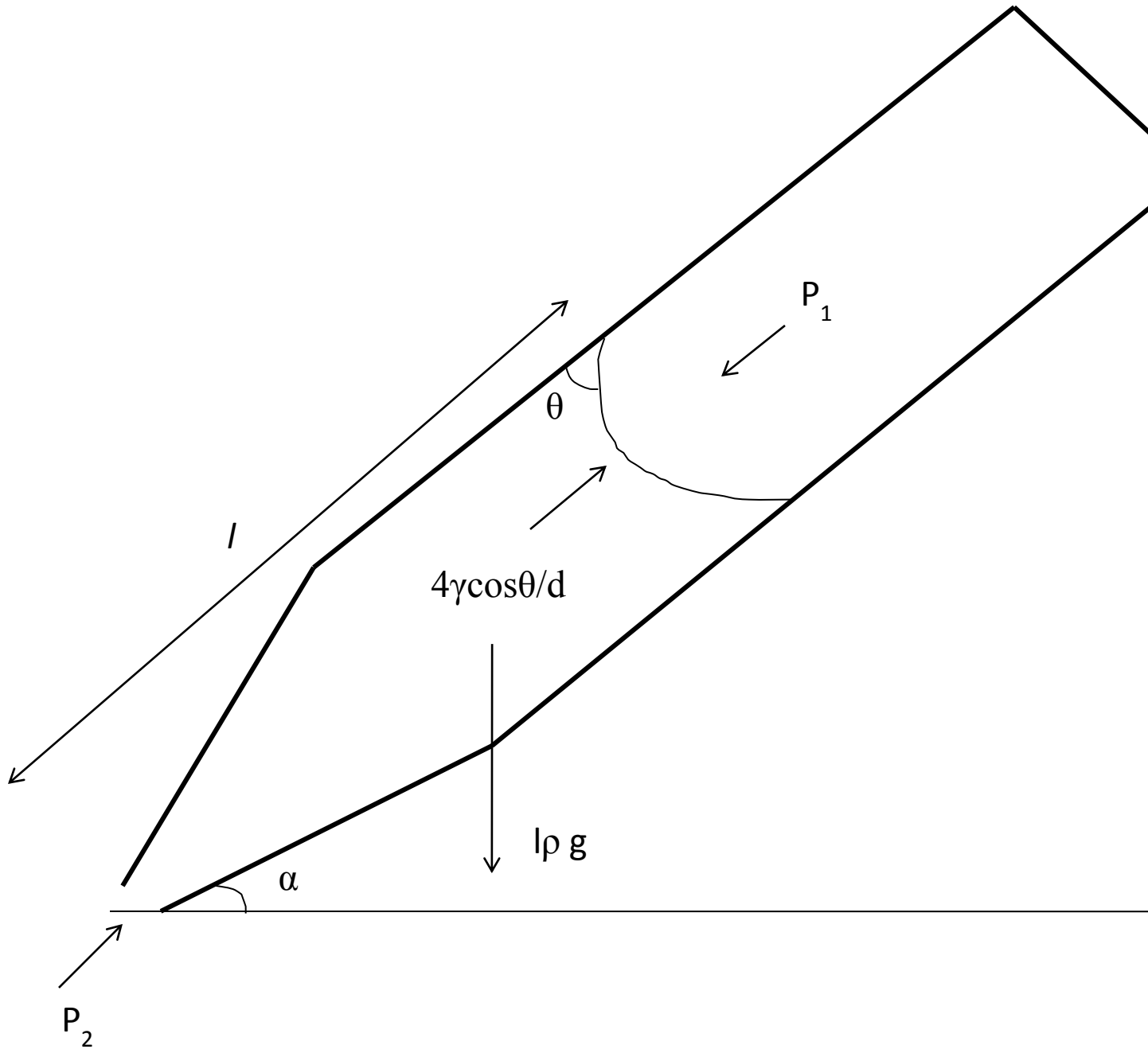


Figure 2-1. Schematic of the capillary and the pressure balance showing the surface tension forces and the capillary pressure. The length of liquid in the column is l, the angle that the needle makes with the horizontal is α and the wetting angle of water and glass is θ. The needle used here is an Eppendorf Femtotip® which has a tapering diameter as shown in figure.

CHAPTER 3
MICROPIPETTE ASPIRATION OF THE NUCLEUS

Micropipette aspiration is a method commonly employed to perturb single cells and isolated nuclei in order to measure their viscoelastic properties. It employs a micromanipulator, a pressure system and a fine needle or capillary for contact and aspiration. In studies on human neutrophils by Shao et al[47], this technique was successfully employed to determine the adhesion forces between the cell and its substrate. A system of two microneedles, one containing a coated bead and the other, an aspirated neutrophil were brought in contact and the adhesion force determined by applying a suction pressure to pull the bead away. A force value of greater than 45 pN caused the adherent cells to move downstream with a constant velocity profile.

The force dynamics of living cells was studied by micropipette aspiration in liposomes and HeLa cells by employing an L shaped pipette[58]. The deflection of the micropipette was used as a force transducer and the rupture force of a lipid vesicle estimated to be 20 nN. Aspiration experiments performed on isolated nuclei involve aspirating the body of the nucleus into the needle. Also, the isolation process is usually achieved by repeated aspirations of the cell body or by chemical means which cast some doubt on the process since nuclear properties may be irreversibly altered.

The experimental method described in this thesis involves using a micromanipulator and a microneedle with an associated pressure system. This method differs from those used so far in two ways: firstly, the nucleus is not being isolated from the cell prior to aspiration, thus maintaining the force balance on it and causing no alteration in its properties. Secondly, the needle tip used is smaller than those generally employed in the literature quoted above. Diameter of the tip in the experiments is 0.5

μm while in the past a diameter of 1-6 μm was employed. By this method, the nuclear body is not sucked into the needle and remains at the tip of the aspirator.[58,40]

The main aim of these experiments was determining the force at which the nucleus can be pulled away from a living cell. There a combination of forces that acts on the nucleus due to the cytoskeleton which are difficult to resolve experimentally. The relative force balance can be determined by selectively eliminating each connection and this was incorporated into the experimental procedure. Three major contributors to the force balance were identified, the first being the cytoskeletal forces that hold the nucleus in place, the second being the force contribution of the cortical network and the stress fibers and the third being the cell membrane. The force contribution of the latter is negligible as demonstrated by the work of Hochmuth[48] which demonstrated that the shear modulus for the red cell membrane is one fourth the value of the cortical tension. In the light of these forces acting on the nucleus, three cases of nuclear aspiration were differentiated, the first being detachment from the cytoskeletal elements where the nucleus still remained within the cell as a free body. The second case was a complete detachment from the cell body where even the cortical network forces were surpassed. The third was a case of insufficient pressure acting on the nucleus where no detachment was seen.

Experimental Procedure

Materials and Methods

Cell culture and transfection

Experiments were conducted on NIH 3T3 fibroblast cells which were used at passages of 10-11 and maintained at low confluence. Cells were grown in a medium of Dulbecco's Modified Eagle's Medium (DMEM, Mediatech, Manassas, VA)

supplemented with 10% Donor Bovine Serum (DBS, Gibco, Grand Island, NY) and 1% antibiotics.

Cells once sufficiently confluent were passed on sparsely at a 1:10 dilution on a 3.5 cm glass bottom dish. The dishes were then filled with 3 ml media to avoid image defects due to reflection caused by entry of the needle. Transfection was performed using plasmids and lipofectamine reagent in Optimem as a solvent. A concentration of 1.5-2µg of plasmid DNA and 4 µg of lipofectamine was found to be effective. Cells were transfected in a 12 well cell culture dish overnight and were then plated at dilutions of 1:20 on a glass bottom dish (MatTek, Ashland, Texas).

Treatment with the drug blebbistatin was done at controlled concentrations where 1µl of 50 µM blebbistatin was used for every 1 ml of media in the dish. Using cells coated sparsely on a glass bottom dish, 1 ml of media was removed from the dish. 3 µl of blebbistatin was mixed with this volume until completely dispersed and the mixture was added to the cells. The dish was incubated for half an hour before experiments were performed on them.

Imaging and micromanipulation

Cells were imaged on a Nikon TE 2000-E microscope on a 20X objective in Phase mode. The microscope was equipped with a covered glass chamber used to maintain the temperature at 37.0C. A CO_2 tank was used to supply CO_2 to a small chamber covering the cells that maintained CO_2 levels at 5%. Results were recorded and analyzed using the NIS Elements software (NIKON)

An Eppendorf InjectMan® NI 2 Micromanipulator system was used for detaching the nucleus from the cell. The micromanipulator arm was attached to an inbuilt module on the microscope. The flexible arm was maneuvered to reach the dish through the CO_2

chamber thus maintaining the CO_2 levels for the duration of the experiment. The arm of the needle was set to a constant of 45^o indicating the angle at which it approached the adherent cells. This system is equipped with a joystick that controls the position of the needle in the x-y plane by moving back and forth. It can also be twisted up and down to lower or raise the needle in the z direction. The InjectMan® system was coupled with a Femtojet® system for varying the aspiration pressure on the nucleus. The needles used for aspiration procedure were the Femtotip II® microinjection needles with a tip diameter of 0.5 µm

Description of microneedle setup

The cells were imaged in 3.5 cm glass bottom dish that screwed on to a holder within the CO_2 chamber. A 20 X objective was employed to focus on the cells and note their confluence. The Femtotip® needle screws onto the micromanipulator arm and has a capillary connecting it to the Femtojet® system. The capillary pressure within the needle can be adjusted by varying the capillary pressure knob on the Femtojet®. The needle was then maneuvered through the CO_2 chamber on *Coarse* control and lowered till it was just above the level of media and roughly positioned over the objective.

The plane of focus was raised to focus above the cells and on the needle. The needle settings were then changed to *Fine* and the joystick used to move the needle back and forth. This was done until a faint shadow of the needle was spotted moving over the objective. The joystick was then twisted down to lower the needle below the level of the media. By successively lowering the objective and the needle, it was bought down to the level of the cells, making sure that the length of the needle did not impact the sides of the dish.

It must be noted here that the needle is very fragile since the diameter of the tip is very small. Under these conditions lowering the needle and scraping it on the dish surface in an incorrect manner can lead to breakage. This step had to be performed carefully after some training and familiarization with the setup.

Experimental Procedure

Once the needle was lowered into the dish and positioned at the level of the cells, the stage was moved around in order to find suitable cells. Since the angle of approach of the needle is fixed, cells had to be chosen accordingly. The preferred orientation of the cells was vertical which resulted in a straight pull that corresponded to the angle of raising the needle from the nucleus. The needle was lowered till it made contact with the nucleus without piercing the cell body and the nucleus, both of which lead to instantaneous tearing and cell death. On establishing contact, the capillary was detached from the pressure system, creating an instant vacuum within the needle as the pressure returned to atmospheric pressure. The needle was then removed from the contact point at an even speed as soon as aspiration began. This speed was held constant for all experimental conditions and the needle was removed away from the cell body and out of the dish. Three distinct cases were observed: In the first, there was no apparent movement of the nucleus and it failed to detach from the forces holding it in place. An illustration of this is provided in Fig 3-1 where the nucleus has not been moved along with the needle. This was treated as a null pull and a case of no detachment from the cell. In the next case observed, the needle pulled the nucleus with sufficient suction force to dislodge it from the cell body, as in Fig 3-2. However the nucleus could not be removed past the cortical membrane and emerge out of the cell completely. The needle thus did not bring the nucleus out of the cell with it but left it

behind as a dislodged body within the cell. In the last case, shown in Fig 3-3, the aspiration pressure was large enough to pull the nucleus out of the cell completely past the cortical and stress fiber network. The experiment was repeated for the same pressure value on a different cell. On an average 12 pulls were conducted at each pressure and the behavior of the nucleus was noted. Then the pressure was increased by 100 hPa and the procedure was repeated on different cells. Aspirations were conducted at successively increasing pressures until an 80% or higher success rate was attained.

For GFP-KASH transfected cells, a similar procedure was followed for cells that were successfully transfected. Blebbistatin treated cells were incubated with the drug half an hour prior to imaging in order to see the effects of the drug. The dish was used for an hour before being switched to maintain uniformity in concentration for various findings.

Observations

Since this is a new technique, a number of observations were made regarding the reproducibility and technical problems that can occur during the process. The cell body was seen to detach rapidly at the site of aspiration as illustrated in Fig 3-4.

Contact of the Needle with the Cell

Improper contact resulted in failure to pull the nucleus out of the cell where media was aspirated out and no effect was seen on the cell. Deep contact of the cell with the nucleus, where the needle pierced the nucleus caused the whole cell to be pulled off the surface. This is a case of the needle dragging the cell along the surface as opposed to aspiration which acts as a suction force on the nucleus. When the needle tip broke very slightly, the nucleus was pulled out of the cell and into the needle. To test if the needle

tip was broken, the *Clean* function of the Femtojet®, which pumps air to unclog the needle, was used periodically within the dish. As long as the needle tip emitted bubbles, it was considered unclogged.

Cell Orientation

If the cell and the nucleus were not roughly perpendicular to the needle's approach, aspiration could be difficult to achieve because of geometry. In a confluent dish, detachment is difficult since surrounding cells interfere with the needle's path and sever connections between the needle and nucleus.

Cell Shape

Cell shape was noted to affect the aspiration process. Elongated cells with oval shaped nuclei showed a better success rate with aspiration than spread cells with more rounded nuclei. This is a hypothesis which needs to be verified by carrying out aspiration on micropatterned cells. This observation could be because the force balance on the nucleus differs in these two cases and is difficult to quantify without having uniform cell shape. However the nucleus can be removed from many cell types by varying the angle of pulling

Results

Two types of observations were made based on the extent of removal of the nucleus from the cell.

Detachment within the Cell

Here the nucleus was removed from the cytoskeletal elements holding it in place without completely leaving the cell body. Cases of success and failure were scored as 1 and 0 which was then used to calculate the probability of detachment at each pressure value. The net probability for pulling at each successive pressure increment was noted

and plotted against the pressure value as shown in Fig 3-5. These experiments were done for control cells and cells in which the KASH domain was over-expressed. It was expected that in the latter case, the nucleus is not firmly connected to the cytoskeletal elements and can thus be removed with a higher probability than control nuclei. Indeed it was seen that control nuclei were aspirated with a probability approaching 92% at a pressure if 1600 hPa while transfected cells were aspirated with a probability of 1 at a pressure of 900 hPa. There was a significant difference observed in probability of pulling at every pressure.

The results of pulling in control and KASH cells showed a sigmoidal trend represented by the following equation within the probability limits (0<P<1):

$$y = \frac{1}{(1+EXP(\frac{-(P-P_0)}{W})} \quad (3\text{-}1)$$

Here P is the applied pressure. The value of the characteristic pressure of detachment P_0 was fit to 837 hPa representing the pressure at which 50% success in aspiration was seen. The value of P_0 in the case of KASH transfected cells was much lower at 472 hPa showing a significant departure from the value obtained for control cells. The graph demonstrating the model fit is shown in Fig 3-7. The width of the interval in each case was 326 and 160 hPa respectively, demonstrating the range over which aspiration was observed.

Complete Removal from the Cell

In the case where the nucleus was removed completely from the cell, a larger pressure was required for aspiration. A 70% probability of aspiration was noted at a pressure of 1600hPa in control cells and a 60% probability in transfected cells at a pressure of 900hPa.The contribution of cortical forces is expected to make aspiration of

the nucleus out of the cell slightly more difficult since larger forces are now opposing the motion of the nucleus. A sigmoidal curve fit these cases as well.

BlebbistatinTreated Cells

In cells treated with the drug Blebbistatin, an inhibitor of non-muscle myosin II, a probability of 80% was observed at a pressure of 900 hPa. This is lower than that of KASH cell and higher than that of control cell. The results for the blebbistatin treatment on nuclear aspiration has been shown in Fig 3-6 where the three cases have been compared simultaneously.

Statistical Significance

A z-value test was undertaken to determine the statistical significance of these results. This test is applied to samples with different population sizes and probabilities. In this case, the different populations are the control and KASH cells with different aspiration probabilities. The data from aspiration experiments in different pressure ranges was compared to test the statistical significance. The critical value of z is 1.96 and test values have to be greater than this to be statistically different. Applying the test to experimental data of aspiration at pressures above 600 hPa, a z-value of 2.00 was obtained. Combining the entire data range from 100-1700 hPa was not significantly different for control and KASH cells, but combined data from 600-1700 hPa showed a z-value above the critical. This implies that at very low pressures, the aspiration trend is not very different for control and KASH cells, but shows greater variance at pressures above 600 hPa.

Calculation of Force Values

The force values anchoring the nucleus in place due to the cytoskeletal elements were estimated assuming a tip diameter of 0.5 μm and the equation $F = PA$. Control

cells were aspirated with a success rate of 90% at a pressure of 1600 hPa. This gives a calculated force value of around 31 nN which is much larger than the force value of 17nN obtained for KASH cells which detach with a probability of 100% at a pressure of 900 hPa. Blebbistatin treated cells with inhibited myosin showed an intermediate trend with a force of detachment of 17nN at a pressure of 900 hPa with a 70% success of aspiration.

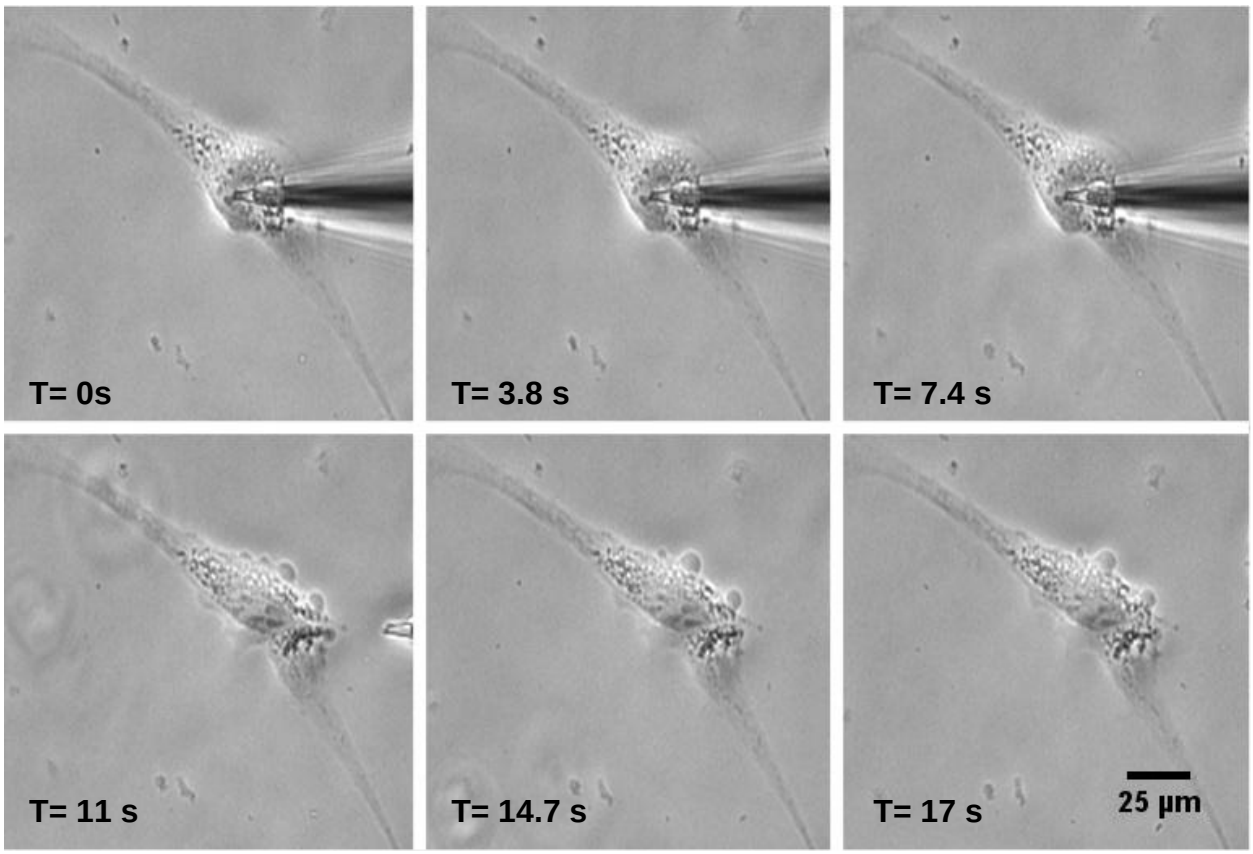


Figure 3-1. No detachment observed at a pressure of 800hPa in control cells. The nucleus does not have sufficient pressure acting on it to be aspirated out by the needle. Cases like these were considered to have a success of 0.

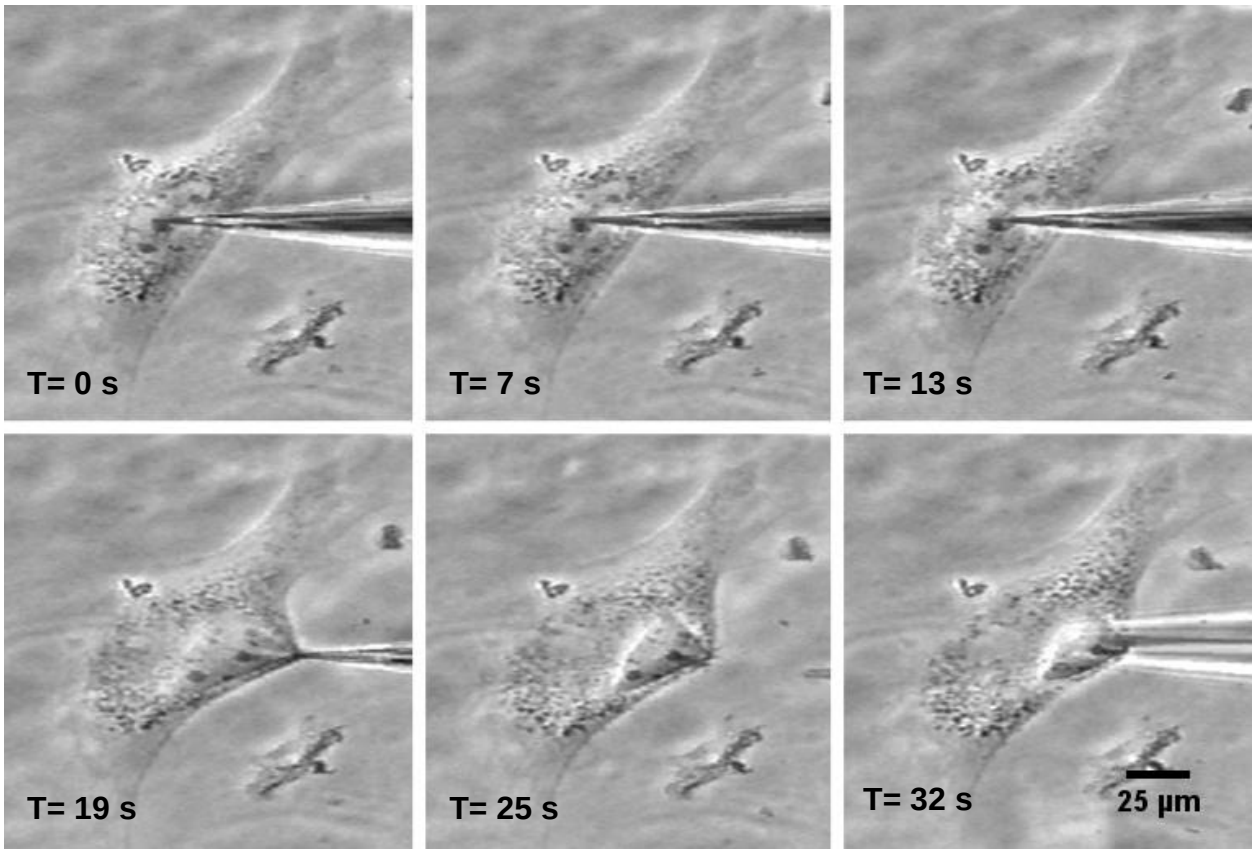


Figure 3-2. Detachment of the nucleus within the cell at a pressure of 1400 hPa. Here it is no longer connected to cytoskeletal elements but cannot move out of the cell due to the presence of the cortical network. Experimentally, this had a success value of 1.

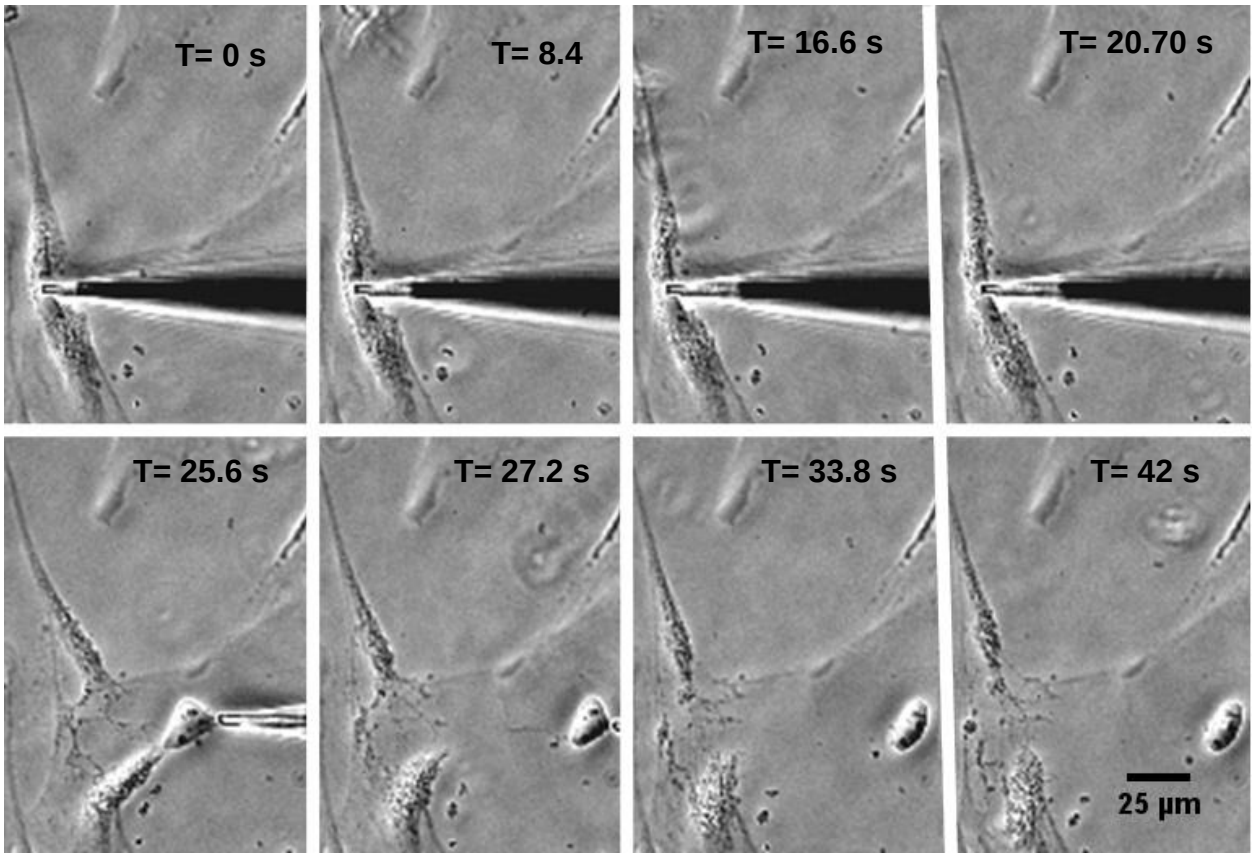


Figure 3-3. Complete removal of the nucleus past the cortical network and out of the cell at 1000hPa.

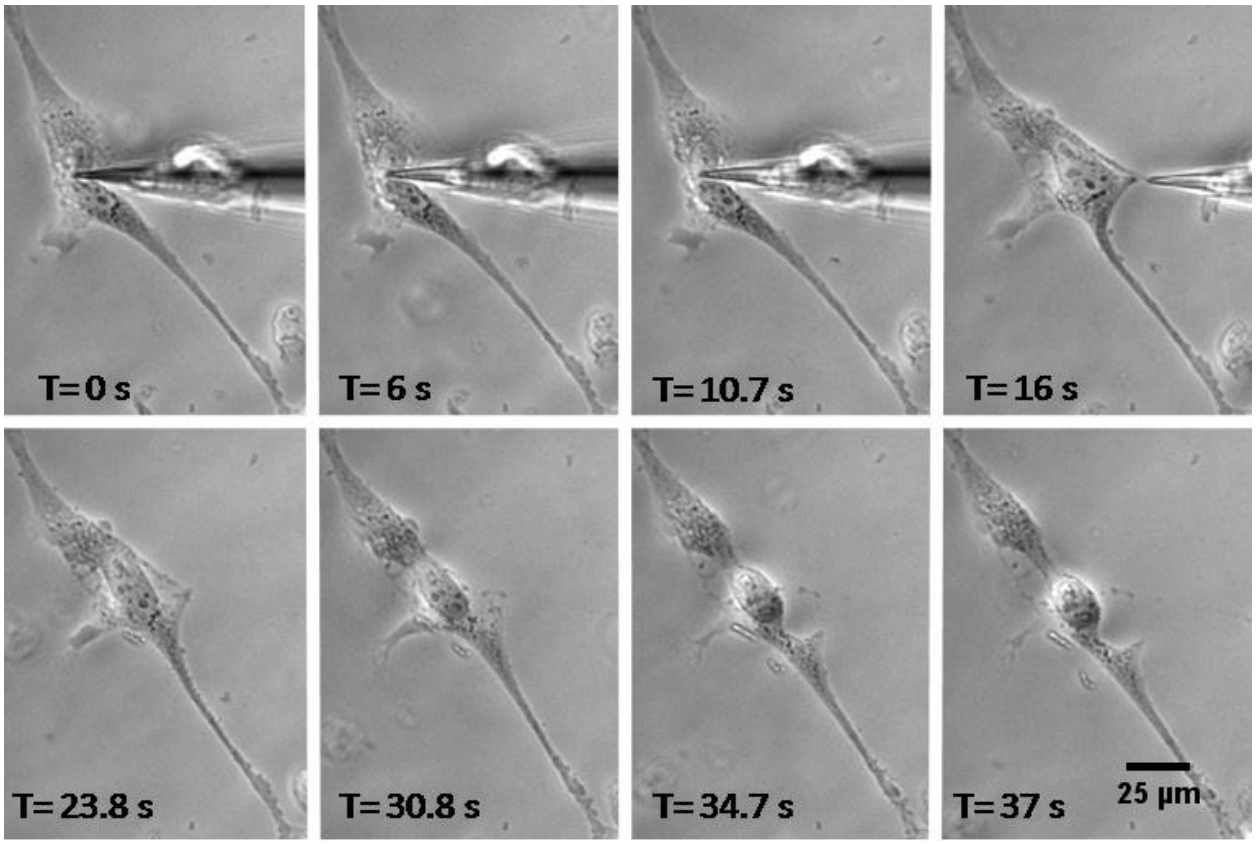


Figure 3-4. Rapid tearing of the cell after removal of the needle. This was observed in some cases, where the cell body retreated away from the nucleus and the aspiration site.

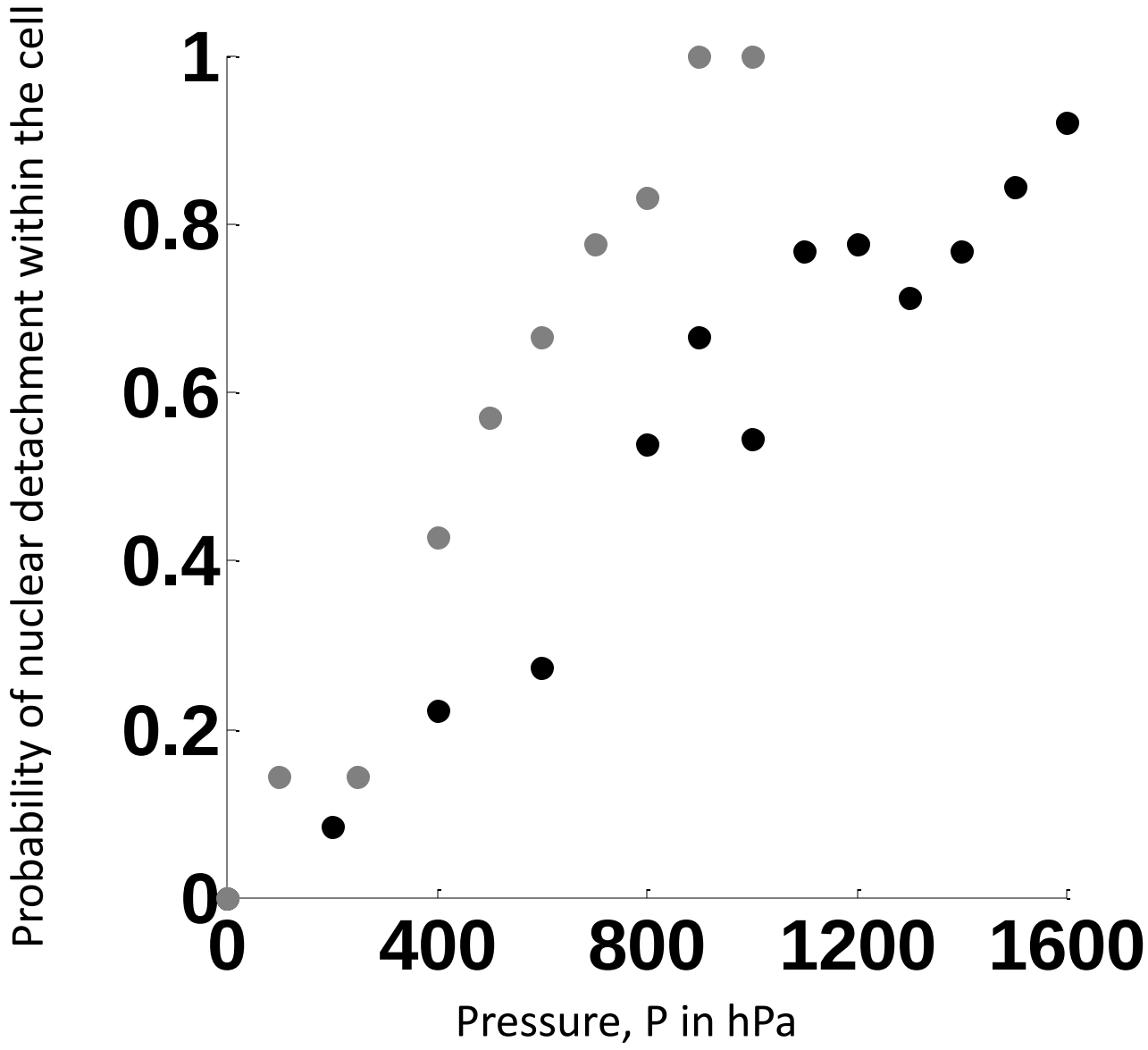


Figure 3-5. Graph showing the pressure versus probability of detachment in control and KASH cells. Control cells have stronger nuclear cytoskeletal forces due to the higher pressure needed to cause aspiration. The black dots represent control cells and the gray dots represent KASH cells

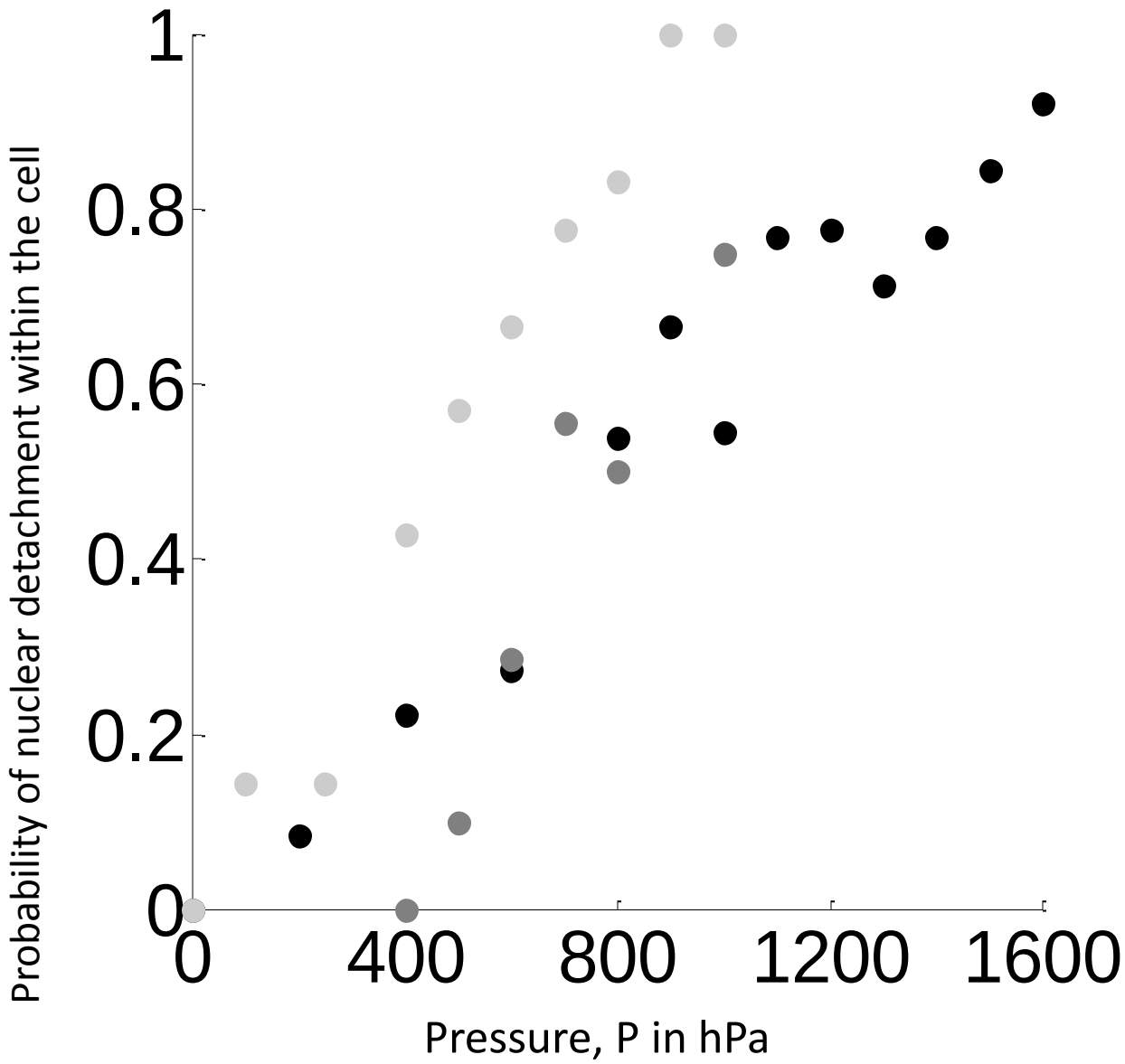
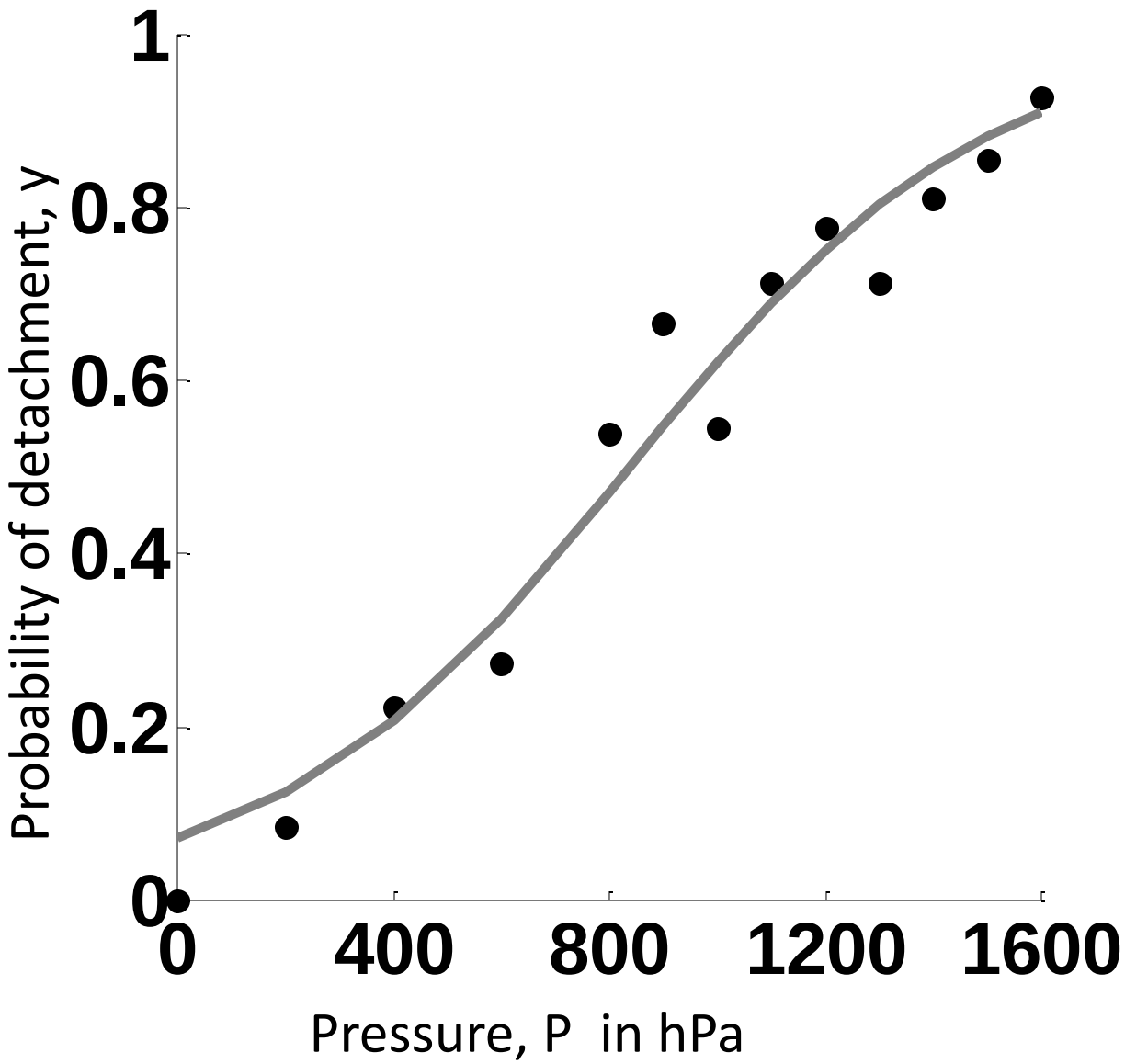


Figure 3-6. Graph comparing probability of detachment for blebbistatin, control and KASH cells. Blebbistatin treated cells show an intermediate aspiration success rate between control and KASH cells and are indicated by the gray dots. Control cells are in black and KASH cells are in light gray.


Probability of detachment, y
1
0.8
0.6
0.4
0.2
0
0
400
800
1200
1600
Pressure, P in hPa

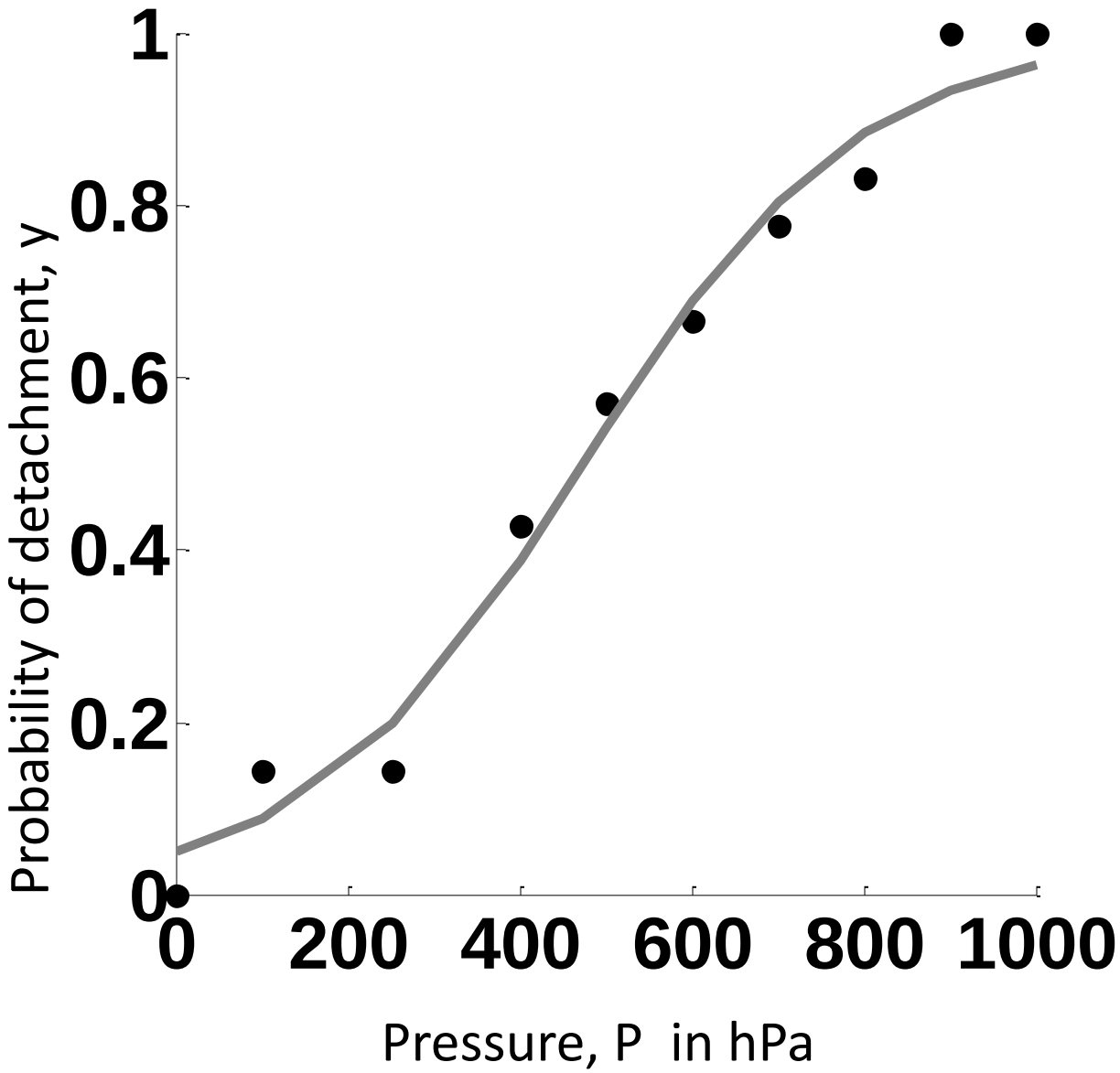


Figure 3-7. Graph showing the sigmoidal fit of the control nuclear detachment within the cell in the first graph and KASH nuclear detachment within the cell in the second graph. The black dots represent experimental values and the solid gray line indicates the sigmoidal fit according to equation 3-1.

CHAPTER 4
CONCLUSIONS AND FUTURE WORK

Conclusions

Micropipette aspiration to remove the nuclei from living cells is a technique that promises a new insight into nuclear positioning in living cells. By probing the nucleus in this manner, results could be drawn upon the relative force balance that anchors the nucleus in place due to the effect of cytoskeletal forces. As anticipated, cells in which the KASH domain was over expressed showed very little resistance to nuclear detachment, validating the importance of the LINC complex in securing the nucleus in place and providing a quantitative value to the force of anchorage. The highest probability of detachment in Control, KASH and Blebbistatin treated cells was seen at pressures of 1200, 700 and 900 respectively. Force values for removal were calculated to be 31nN, 17nN and 17nN respectively for different cases and probabilities.

A sigmoidal curve describing the aspiration trend was proposed assuming a zero aspiration at low probabilities which eventually evens out to 100% at higher pressures with the intermediate zone exhibiting an S-shape. The two parameters of the curve agree with experimentally obtained results and the mean and width of the distribution can be basis factors for further work.

This method can be easily reproduced and applied to other experiments where treatment with drugs such as Nocodazole for microtubule inhibition and Latrunculin B for actin inhibition can be done to follow the effects of these connections on nuclear attachment to the cytoskeleton. Another advantage of the technique is the versatile approach it provides to studying the effect of nuclear removal on stress fibers, focal adhesions etc. A drawback of the method is that it is a single cell technique and hence

identifying the statistical significance is difficult. Like any single cell technique, these experiments are slightly intensive but account well for the variance in cellular geometry. The use of the micromanipulator setup requires skill but adequate training can produce consistent results. It takes an average of 4 hours to learn the basics of locating the needle and using it to probe the cell. These experiments are reproducible and uniform results were obtained on probing cells with similar geometries. Force values obtained from the pressure measurements and the graphs are in agreement with biology of severing connections between the nucleus and the cytoskeleton.

Future Work

Transfecting cells with GFP-Actin and pulling on the nucleus would provide an interesting approach to visualize the behavior of various cytoskeletal components during nuclear removal. A trial run of this experiment showed that the actin fibers moved with the nucleus during aspiration as illustrated in Fig 4-1. Another avenue of interest is studying the effect of various inhibitors like Latrunculin B, nocodazole and blebbistatin. This would provide a picture of the contributions of various elements to the overall force balance. Studying the nuclear connections to focal adhesion through the actin cytoskeleton can also be undertaken by staining for paxilin and vinculin. Nuclear-cytoskeletal forces on different substrates like soft gels can also be tested in order to see how stiffness of the substrates influences connections. The most important scope of study lies in aspiration of micropatterned cells in order to eliminate the effect of cell shape variance. This would give a clear picture of the force required for aspiration by having uniformity in the experimental parameter of shape.

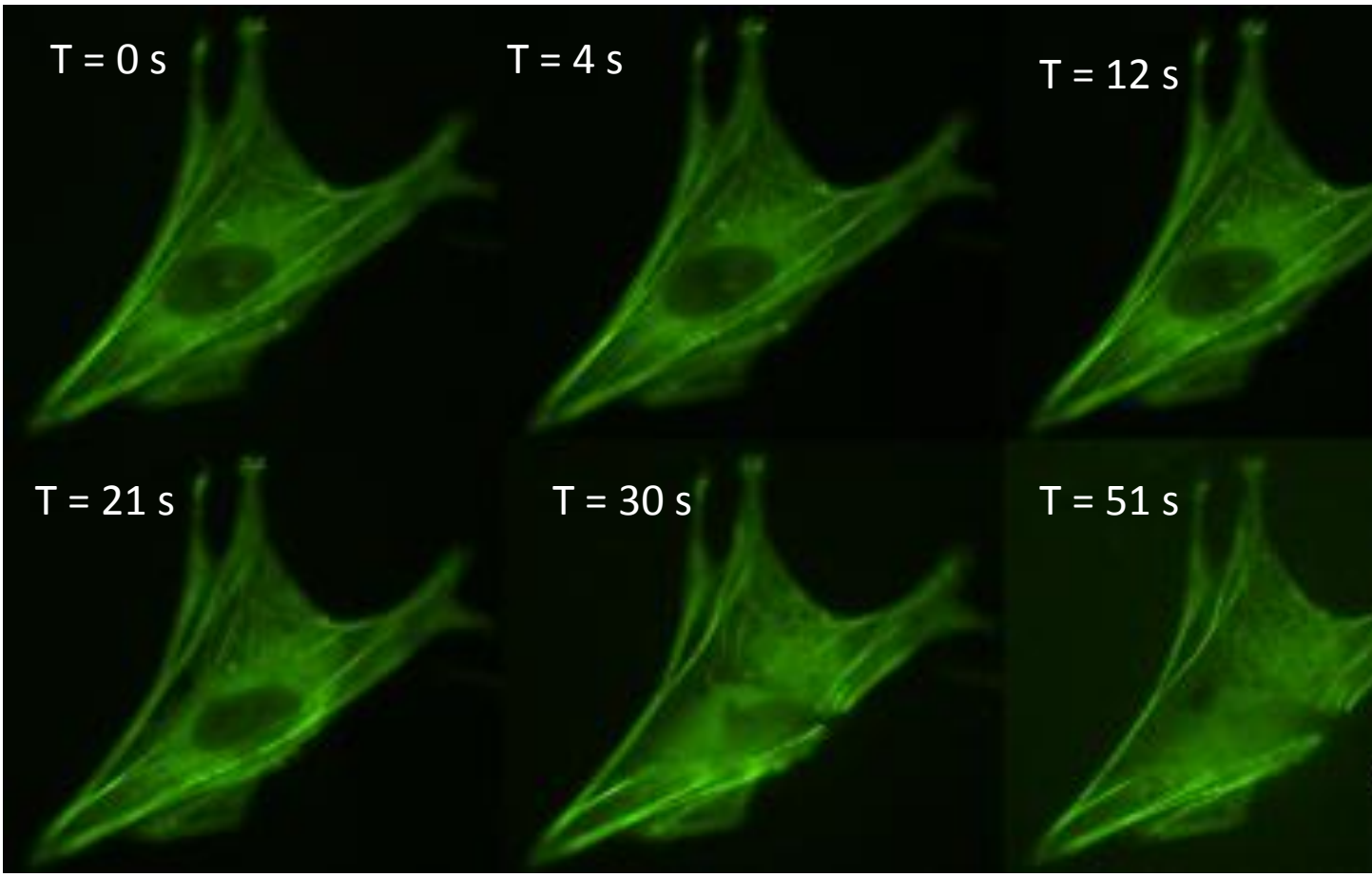


Figure 4-1. Aspiration of the nucleus in a cell transfected with GFP-Actin. This causes the actin cytoskeleton to move with the nucleus showing an interesting application of this technique in studying the relationship between the nucleus and the cytoskeleton.

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BIOGRAPHICAL SKETCH

Agnes Mendonca was born in Mumbai, India to John Mendonca and Fabiola Mendonca. She graduated from Apeejay School in 2006 and enrolled in the National Institute of Technology, in Nagpur, India. Here, she majored in chemical engineering and graduated with a bachelor of technology degree. While at NIT, she interned with Herdillia Petrochemicals and won a Summer Fellowship award at the Indian Institute of Technology, Chennai. Upon graduation from NIT in May 2010, she entered the Department of Chemical Engineering at the University of Florida as a graduate student in August 2010. She started work under Professor Richard Dickinson in May 2011 and worked with Professor Tanmay Lele from September 2011. During her time with Professors Lele and Dickinson she has studied microneedle aspiration of nuclei.