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Analyzing the Effects of E-hook Peptides on Kinesin-1

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ANALYZING THE EFFECTS OF E-HOOK PEPTIDES ON KINESIN-1

by

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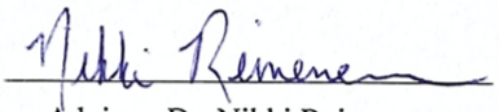

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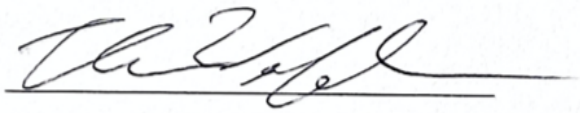

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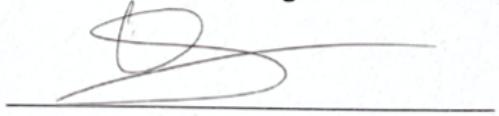

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ABSTRACT

Cancer is the second leading cause of death in the United States. Cancerous growth is a result of oncogenes, or mutated genes that increase the rate of cell division in an uncontrolled manner. Cell division, which consists of mitosis and cytokinesis phases, is dependent upon the active movement of kinesin motor proteins along microtubules to rearrange the cytoskeleton for equitable distribution of genetic material to daughter cells. As kinesins are vital to this process, if we could prevent kinesin from binding to the microtubules, cell division would cease.

The goal of this study is to develop a method to prevent cell division by targeting and disrupting kinesin's microtubule binding sites to prevent them from generating the necessary forces to foster cell growth. The "walking" of the kinesin on microtubules is facilitated by a series of negatively charged residues in the carboxy terminal tail of tubulin known as E-hooks. The processivity of kinesin motor proteins is strongly reduced if E-hooks have been removed from the microtubule surface. It is thought that this is because the E-hooks' negative charges interact with positively charged domains of the kinesin motor domain. Therefore, we hypothesize that similarly structured, negatively charged peptides could be used to saturate kinesin's binding sites.

We found that a short E-hook like peptide inhibited kinesin-1 resulting in a decrease in force generated; however, there was minimal effect on velocity. Kinesin-5 is less processive and generates weaker forces than kinesin-1; thus we propose that this effect will be amplified in kinesin-5 and other mitotic kinesin. We hypothesize that inhibiting these motors that play a vital role in mitosis will stop cell division and halt cancerous growth.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
CDK	cyclin dependent kinase
CNB	cover-neck bundle
CS	cover strand
CTT	carboxy-terminal tail
ddH_2O	double distilled water
DMSO	dimethyl sulfoxide
GTP	guanosine triphosphate
KBD	kinesin bead dilution
KOH	potassium hydroxide
MAP	microtubule-associated protein
MT	microtubule
OT	optical trap

CHAPTER 1

INTRODUCTION

According to the American Cancer Society, cancer is the second leading cause of death in the United States[1]. In January 2019, it was predicted that cancer would take the life of 606,880 people[2]. Scientists across the world are working relentlessly to find a cure for this neoplastic disease. While there are many types of cancer cells, they all share the ability to proliferate irregularly. The mitogenic signaling of most of these cells is mutated, producing their own growth factors or elevating the levels of receptor proteins[3]. We must find a way to decrease this rapid proliferation rate.

Cell division is dependent on cellular filaments and machinery. Once the cell has replicated the chromosomes and other important organelles, the cell begins to divide. Tubulin polymerizes within the cell forming microtubules that establish a mitotic spindle to organize and separate the chromosomes[4]. Many inhibitory drugs that target tubulin polymerization are currently used in cancer chemotherapy[5,6]. By quenching microtubule dynamics, cell division is not possible[7]. However, it has been shown that microtubules can acquire resistance to this therapy[6]. To combat this problem, this study alternatively focuses on the role of the motor protein, kinesin. Mitosis is dependent on kinesin binding to the microtubules. Studies suggests that the tubulin's carboxy-terminal tail, also known as an E-hook, stabilizes the kinesin microtubule bound statebon[8]. Kinesin-5, a motor

protein, binds two microtubules and slides them to balance the forces during segregation [8]. This helps to align the chromosomes for subsequent segregation to complete mitosis. Research of antimitotic drugs used to inhibit kinesin is a topic of intense research focus[9]. In our study, by targeting mitotic kinesins using an E-hook peptide, our goal is to inhibit kinesin binding to the microtubule and prevent their motility. Thus, the force generation necessary to accomplish cell proliferation is inhibited. Using these biologically inspired peptides to exploit blocking microtubule interactions with kinesin is a novel approach. Studies using subtilisin to cleave E-hook peptides from the tubulin have analyzed the effects on kinesin motors[7]. However, studies have not been performed that introduce an E-hook like peptide to the kinesin motors to prevent the tubulin's carboxy-terminal tails from stabilizing the motor.

In our study, conventional kinesin is analyzed which, by nature, is more processive than mitotic kinesin. This leads to the assumption that the inhibitory effects by E-hook peptides on conventional kinesin will be amplified in mitotic kinesin[10–13]. Even with success on the molecular level, the process of drug development and testing will require further research in cells and clinical trials in animals and humans. The ultimate goal of this research is to inhibit mitotic kinesin associated with aggressive cancer cells in order to slow or stop the tumor growth.

CHAPTER 2

BACKGROUND

2.1 Cell Cycle

To understand where cell division goes wrong, we need to understand how cells normally divide. The cell cycle is fundamental to life. Cells constantly spread and multiply to promote reproduction, growth, and repair. The cell cycle represents one cell division cycle, and it is characterized by four events[9]. For cell division to occur, there must be a reproductive signal, replication of DNA, segregation, and cytokinesis. In the eukaryotic cell cycle (**Figure 2.1**), these four events occur through two phases: interphase and M phase.

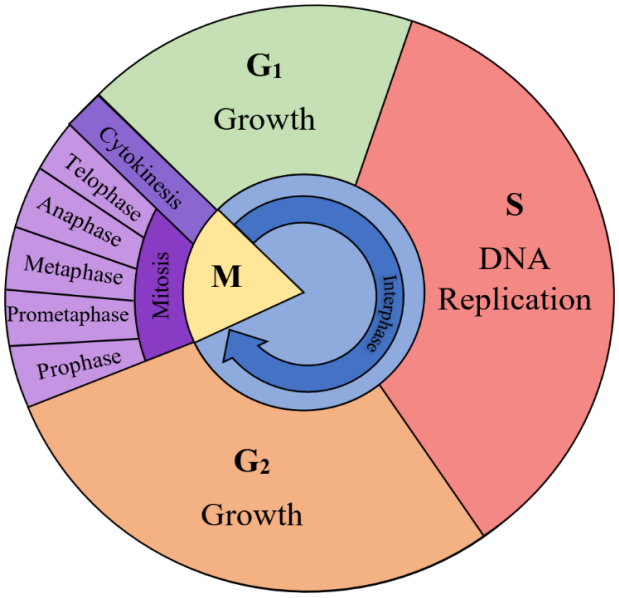

Figure 2.1: **Eukaryotic Cell Cycle.** There are two phases of the Eukaryotic cell cycle. Interphase, the first phase, is composed of three subphases: G1, S, and G2. M phase, the second phase, is composed of mitosis and cytokinesis. Cell division marks the period of one cycle to the next.

DNA is replicated during interphase, which encompasses the subphases Gap 1 (G1), Synthesis (S), and Gap 2 (G2). During interphase, all the required components of cells are sufficiently produced so that proper segregation can occur to form two self-sustaining daughter cells[9]. Interphase is where cells spend the majority of their time. The amount of time spent in each phase depends on the type of cell[9]. Each cell begins its life in G1 phase, also known as gap phase in which normal cellular activity occurs. Cells that do not divide regularly can spend years confined to G1 phase while others may only spend minutes in G1 phase[9]. The cell cycle is regulated by checkpoints that rely on cyclin-dependent kinases (CDKs)[14]. CDKs are able to catalyze certain target proteins that allow the cell to pass from checkpoint to checkpoint[14]. The G1-S checkpoint is responsible for ensuring the cell is prepared for replication. Healthy cells pass through this checkpoint and continue to S phase; however, cells that do not meet these qualifications are stuck in G0 phase until the cell environment improves[15]. Replication is an important step in the process. During replication, successful copies of the genetic material contained in chromosomes must be established, so that each daughter cell will receive one copy of each chromosome[15]. Once the replication occurs, the cell enters G2 where it prepares to enter mitosis and complete cell segregation. The cell must pass through another checkpoint in order to enter into mitosis[15]. This checkpoint only detects damaged DNA or chromosomes that have not been replicated completely[9].

Passing through the G2 checkpoint allows the cell to enter M phase, which consists of the subphases mitosis and cytokinesis[8]. When cell segregation occurs in mitosis, a parent nucleus gives rise to two genetically identical nuclei and forms two daughter cells[4]. Mitosis is completed through five phases (**Table 2.1**). The initial phase, prophase, is

characterized by the condensation of the chromosomes, which each consists of a set of sister chromatids, and the beginning formation of the mitotic spindle. Next, the cell transitions into prometaphase where the nuclear envelope breaks down and the two duplicated centrosomes begin to separate, finalizing the formation of the mitotic spindle[9]. Once the spindle forms, it attaches to the chromosomes through a search and capture method employed by the kinetochores to connect to microtubules[7]. As the cell enters metaphase, the spindle pulls the chromosomes into the equatorial position at the midline of the cell[9]. Metaphase organizes the chromosomes into a symmetric structure that allows the chromosomes to be separated through anaphase. During anaphase, the miotic spindle pulls on each chromosome, separating each chromatid by dragging them towards opposite poles. Once the chromatids have fully separated, the transition to telophase is exhibited by the establishment of two full functional nuclei[7]. The formation of the components of two separate cells marks the end of mitosis.

Mitosis

Prophase	The first phase of mitosis. The chromosomes are condensed, and the mitotic spindle forms.
Prometaphase	The nuclear envelope breaks down. The compacted chromosomes attach to the mitotic spindle.
Metaphase	The chromosomes, each consisting of two chromatids, align in the equatorial position.
Anaphase	The chromatids are separated as they are each pulled toward opposing poles.
Telophase	The nuclear envelope redevelops around each set of chromosomes, that are now decondensed. The nucleoli appear and the mitotic spindle disappears.

Table 2.1: **Phases of Mitosis.** Mitosis occurs in the M phase of cell division. During mitosis, the replicated chromosomes are aligned and segregated, forming all of the necessary components of two complete daughter cells.

The cell separates to form two daughter cells through cytokinesis. The two sets of cellular components are pulled apart, allowing the cell's cytoplasm to separate by the cinching of a contractile ring[4]. Two daughter cells are formed, and the cell membrane forms to surround each respective cell. Each daughter cell will now have one complete set of chromosomes that are identical not only to each other but the parent cell as well. Each newly formed cell contains all the necessary components to grow and divide again[16]. At the end of cytokinesis, the cell once again enters interphase or more specifically the G1 phase and the process can repeat.

Cell division is a crucial aspect to all living organisms as it allows cells to spread and multiply. One of the driving components of cellular division is the mitotic spindle, a self-assembling macromolecular machine[9]. The mitotic spindle is the machine behind chromosome segregation. Accurate segregation during mitosis is vital to all life on Earth[17]. In order to develop a method to inhibit cellular division as an implication of cancer, we must understand the structure and mechanics of the mitotic spindle.

2.2 The Mitotic Spindle

Eukaryotic cell division is accomplished through the formation of the mitotic spindle. The mitotic spindle has two main functions: 1.) it serves as a framework allowing the two poles to separate and 2.) it attaches to the chromosomes in order to segregate the two chromatids[18]. These functions ultimately allow the mitotic spindle to ensure the distribution of chromosomes to each daughter cell during mitosis which is a vital element of cell division[7]. Improper chromosome segregation can have detrimental effects that stem

from a cell with an abnormal number of chromosomes[3]. Also known as the spindle apparatus or simply the spindle, the mitotic spindle is a bipolar machine made from microtubules (MTs)[4]. The formation and the function of the mitotic spindle relies on the direction of MTs with the assistance of multiple kinesin and dynein motors. These biological motors walk along the MT to move structures within the cell.

MTs are long, hollow polymers composed of α/β-tubulin dimers[9,19] (**Figure 2.2**). MTs differ from other cytoskeletal filaments in their ability to grow and shrink by the addition and loss of α/β-tubulin termed polymerization and depolymerization respectively[7]. These unbranched cylinders are measured to be approximately 25 nanometers in diameter and as long as several micrometers[7]. Before MTs can form the mitotic spindle, the interphase MTs must disappear to allow new MTs to form[7]. These new MTs are more labile than the interphase MTs, giving them the ability to move as needed to form the mitotic spindle[7]. MTs are shown to prod the nuclear envelope, promoting the dispersal of the envelope[9]. Once the envelope dissipates, the MTs are free to form between the chromosomes and poles[17]. This allows the construction of the mitotic spindle during prophase and prometaphase[20]. MTs have opposing ends with one designated the plus end and the other the minus end. The MTs are arranged so that their minus ends are concentrated at each pole and their plus ends are directed towards the chromosomes[20]. This structure, shown in **Figure 2.3**, helps develop the bipolar structure of the spindle and connection to chromosomes.

The mitotic spindle is composed of three types of MTs: astral microtubules, polar microtubules and kinetochore microtubules.

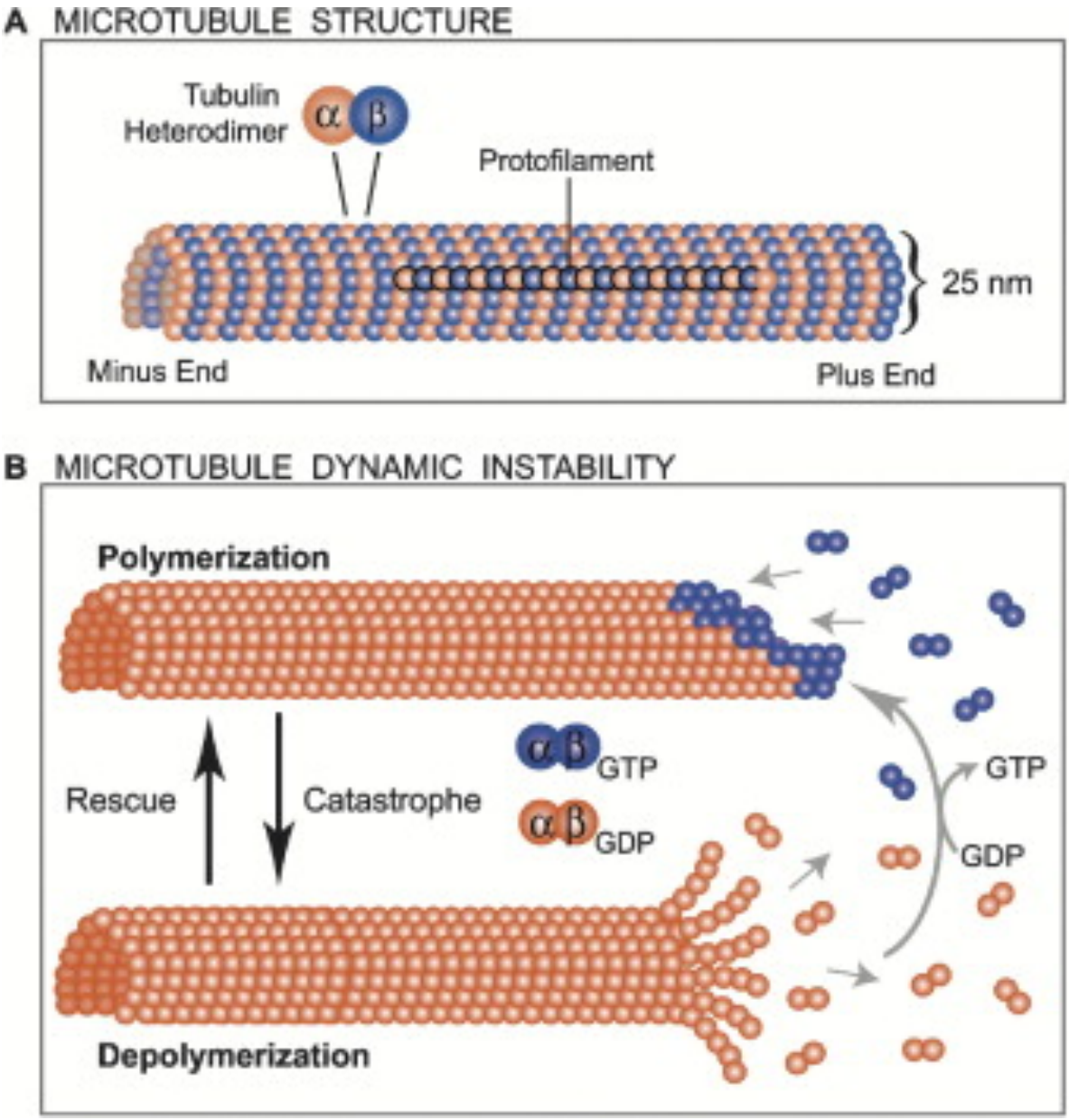

Figure 2.2: **Microtubule. a.** Microtubules are composed of α/β-tubulin dimers. Each microtubule has a designated plus and minus end. **b.** Microtubules are able to grow by the addition of α/β-tubulin through polymerization and they are able to shrink by the loss of α/β-tubulin in depolymerization. Figure adapted from Kline-Smith el at. (2004). Molecular Cell, 15, 317-327.[19]

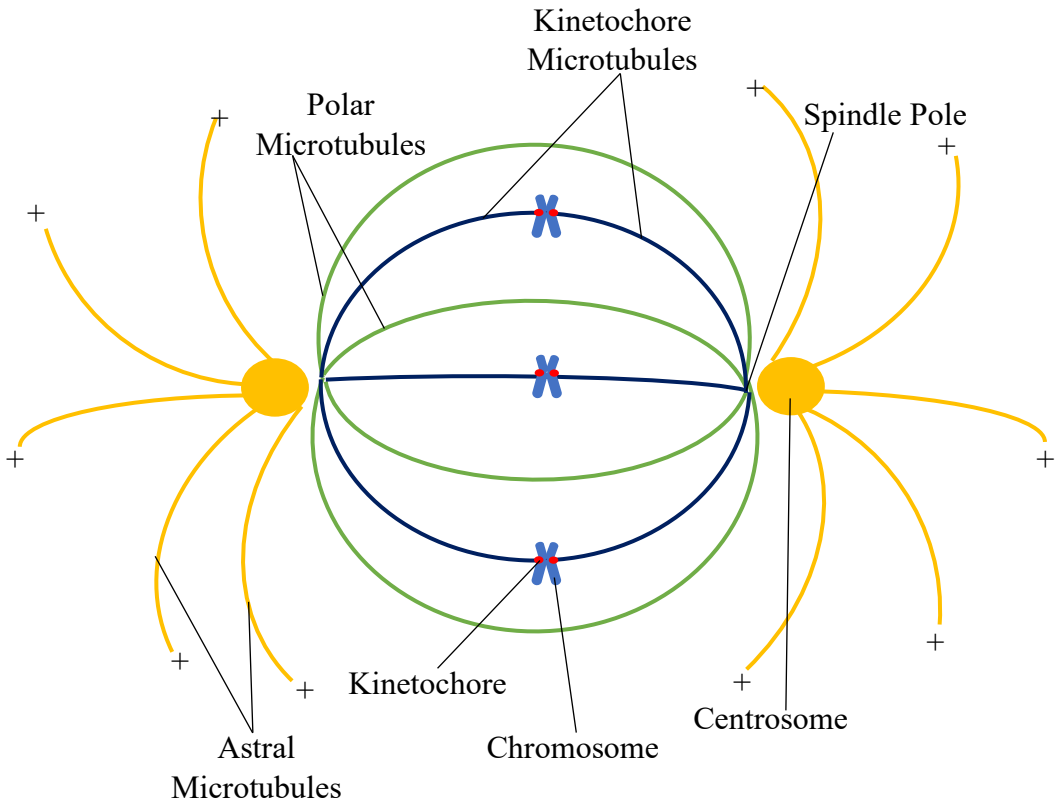

Figure 2.3: **The Mitotic Spindle.** The mitotic spindle is composed of microtubules, long thin tubes of protein that extend from each pole. Each microtubule is connected to the spindle pole at its minus end with the plus end protruding from the pole. There are three types of microtubules in the mitotic spindle. Astral microtubules connect each pole to the cell membrane, anchoring the pole in place. Polar microtubules extend from pole to pole and push the poles apart. Kinetochore MTs bind to the kinetochore on each chromatid allowing for chromosome segregation.

Astral MTs are responsible for anchoring the opposing poles of the mitotic spindle to the cell membrane[9]. These MTs are essential for maintaining spindle location and pole separation[9]. While astral MTs anchor the spindle in place, polar MTs are responsible for separation of the poles[17]. Polar MTs serve as the frame of the mitotic spindle and run from one pole to the other [7]. These polar MTs are the basis of the bipolar structure of the mitotic spindle by pushing each pole to opposite sides of the cell. This bipolar organization properly aligns the chromosomes down the center of the spindle so that proper chromosome

segregation can occur[9]. When the cell is ready to separate into two daughter cells, it is critical that each duplicated chromosome is separated evenly. The spindle arranges the already duplicated chromatids so that one copy is attached to each end of the spindle[7]. Before arrangement of chromosomes can occur, the mitotic spindle must connect to the chromosome through kinetochore MTs.

The ability of the mitotic spindle to move and segregate the chromosomes stems from the kinetochore MTs' ability to bind to the kinetochores[4]. Kinetochores are constructed of several fibrous proteins and are present on each chromatid[4]. MTs probe their surrounding area in a system termed "search and capture" in effort to encounter a kinetochore[17]. This allows the kinetochores to connect chromosomes to the bipolar spindle[4]. The microtubule plus ends attach to the chromatids from opposing poles[17]. The MTs' repeated exploration of the region that contains chromosomes ensures compete attachment to every chromatid[9]. Kinetochores prevent further procession through the cell cycle until complete connection and proper alignment is achieved[3]. Once proper alignment is established, the cell is able to move into the next phase of the cell cycle.

2.3 Biological Motors

During cell division, proper alignment of MTs to form the mitotic spindle would not be possible without the aid of microtubule-associated proteins (MAPs). Many types of MAPs are present in the cell; together, they are responsible for the organization, movement, and crosslinking of MTs[8]. A particular family of MAP motors that play an essential role in the formation of the mitotic spindle is the Kinesin family[21]. MTs serve as tracks for

these motor proteins[9]. Kinesins are a family of motor proteins that have more the 650 identified members with different functions, roles, and structures[22]. As seen in **Figure 2.4**, all kinesins are made up of four main regions: head, neck, stalk, and tail[22]. The head and neck are often termed the motor region and used in the power stroke to provide motility for the kinesin to walk along a microtubule. The neck-linker attaches the neck to the catalytic core of the head domains and facilitates communication between the head groups[23]. The tail has various functions, in most families, it is used to bind cargo[16]. The stalk is a parallel, α/β coiled-coil central rod.

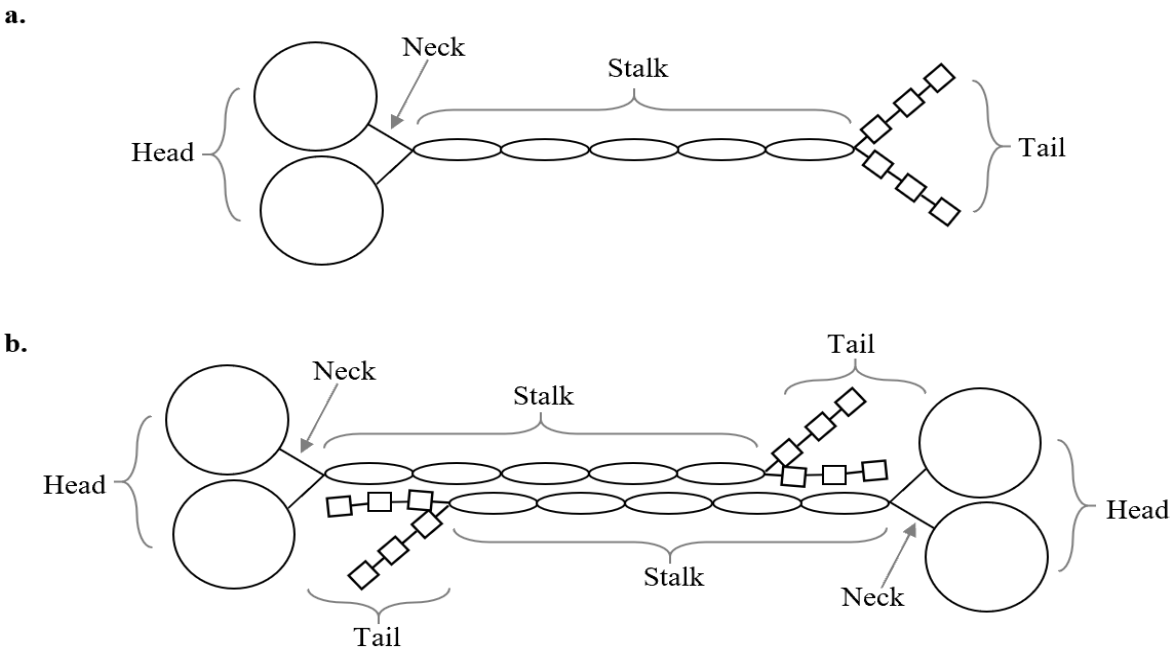

Figure 2.4: **Kinesin Comparison. a.** Kinesin-1 exhibits a homodimeric organization. The protein is made up of four significant regions: head, neck, stalk, and tail. The head binds the microtubule and the tail binds cargo. **b.** Kinesin-5 exhibits a bipolar homotetrameric organization with two dimers on each side of a central rod. This structure gives the motor protein its characteristic 'dumb-bell' shape. The parallel, α/β coiled-coil central rod of kinesin-5 is structurally essential for successful operation of the protein.

Kinesin motor proteins are classified into three groups based on the location of the head or motor group. The motor for C-kinesin and N-kinesin is located at the carboxyl and amino terminal region respectively. C-kinesin and N-kinesin have MT minus end and plus end-directed motilities respectively[22]. The motor for M-kinesin is in the middle of the protein; these are responsible for depolymerization of microtubules. Each of these are further divided into families and subfamilies based on the structure of the motor region.

Motor proteins function to actively move within the cell's cytoskeleton. Specifically, regarding kinesin, the rod domain allows the motor domains on either side of the rod to walk in a hand-over-hand fashion to foster motility[24]. This is hand-over-hand walking shown in **Figure 2.5** is described by a series of docked and disordered positions that facilitate the power stroke.

During each power stroke, adenosine triphosphate (ATP) is hydrolyzed, and the neck-linker region alternates from a docked to disordered conformation as the kinesin steps down the microtubule[22]. Prior to attaching to the microtubule, the kinesin dimer is randomly moving until the first head encounters and binds to a microtubule. Binding to the microtubule initiates the release of adenosine diphosphate (ADP) leaving a nucleotide binding site for ATP[24]. When ATP binds this head, it triggers the neck-linker region to "zipper" or "dock" to the microtubule[25,26]. This zippering movement positions the second head to bind to the microtubule in front of the first[26,27]. The ATP is hydrolyzed releasing a phosphate which allows the neck-linker region to return to a disordered conformation. Simultaneously, the second head now binds to the microtubule, releasing ADP and binding ATP. The cycle continues, allowing the kinesin heads to "walk" along the microtubule. As the kinesin "walks" down the MT, it develops a force that is transmitted from end to

end as the protein is propelled forward. Typically, the kinesin is transporting cargo such as a vesicle, organelle, or chromosome[27]. This is the most common function of kinesin motor proteins; however, there are many families with various functions such as depolymerizing MTs and sliding MTs apart[28].

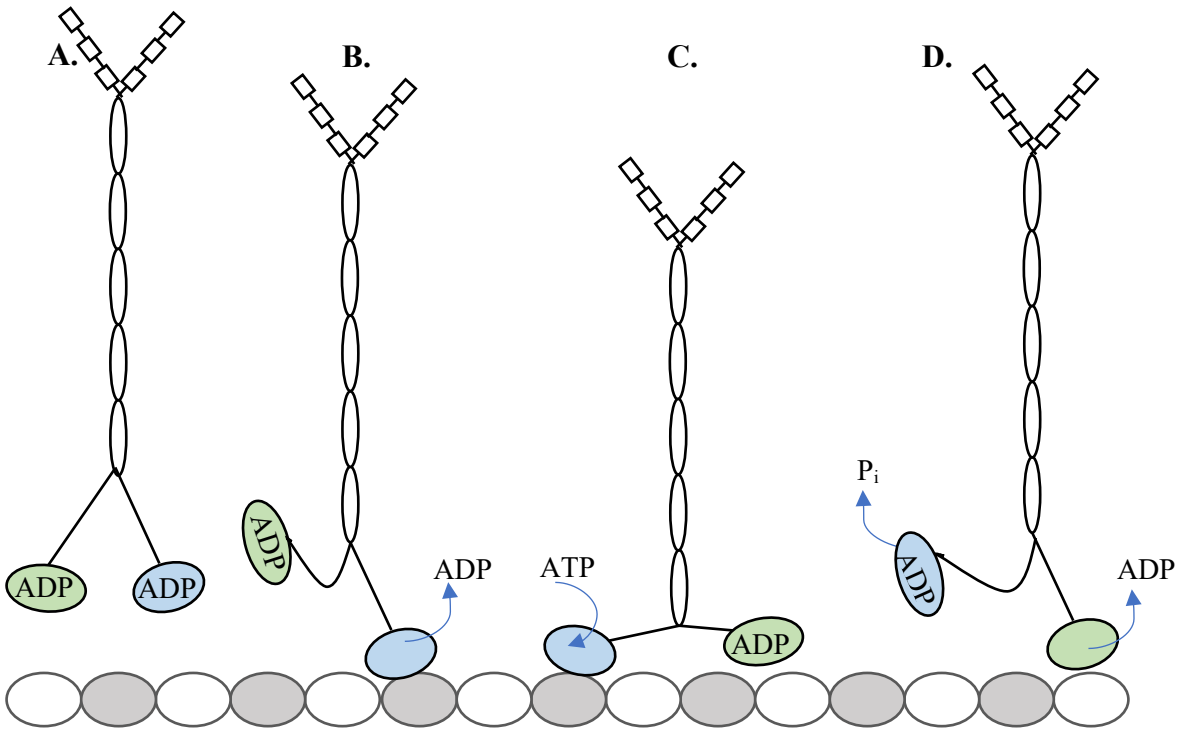

Figure 2.5: **Conventional Kinesin Mechanochemical Cycle. A.** Random movement until the kinesin motor head is near the microtubule. **B.** The first kinesin head binds to the microtubule by releasing ADP. **C.** ATP binds to this open nucleotide site causing the neck-linker to "zipper" into a docked position near the microtubule. This positions the second head to bind the microtubule. **D.** The second head binds the microtubule, releasing ADP. Simultaneously, the first head hydrolyzes the ATP releasing a free phosphate. The neck-linker moves to a disordered position.

Many kinesin proteins are required to accomplish the incredible feat of mitosis[28]. Kinesin-4 family has the ability to bind to DNA; therefore, it can regulate the completion of anaphase and cytokinesis[28]. Kinesin-10 is a family of N-kinesin proteins with a tail that can bind DNA[27]. Specifically, kinesin-10 family member 22 is responsible for spindle microtubule stabilization and pushing the chromosomes to the equator[29]. The kinesin-13

family is responsible for shortening the microtubules during telophase[29]. Kinesin-5 motor proteins establish the force necessary to form and maintain spindle bipolarity as well as assisting with spindle elongation[30].

Kinesin-5 is a motor protein that has a bipolar homotetrameric organization[29]. It has two motor dimers on each side of the central rod giving it a 'dumb-bell' configuration[3]. Kinesin-5 molecules are found on polar and kinetochore MTs but are not evident on astral MTs[29]. Their presence on polar MTs explains their function in mitosis, to develop the mitotic spindle bipolarity. A key feature of kinesin-5 that produces this bipolarity is the motor's ability to walk on two MTs at once; in this instance the MT becomes both the track and cargo[29]. This is possible due to the availability of a motor unit on each side of the central rod. With kinesin-5 attached to both MTs, *in vitro* studies have proven the motors' ability to slide antiparallel microtubules apart through crosslinking[3]. Its movement down the MTs allows kinesin to aid in cell division by providing a sliding element that works to generate an outward force pushing the opposing poles apart[3,30].

During prophase, kinesin-5 motors have shown the ability to move polar MTs that extend from each centrosome relative to each other, driving the establishment of the bipolar mitotic spindle[3,30]. MTs take on an antiparallel position meaning their positive ends meet in the middle while the negative ends are concentrated at the spindle poles. Therefore, the MTs extending from one pole lie in a different direction than the MTs of the other pole. Kinesin-5 is a plus-end-directed motor protein meaning it moves from the spindle poles towards the outer limbs of the MTs[3]. Kinesin-5's alignment along with its plus-end-directed movement allows it to push two antiparallel MTs apart[2]. This attachment to MTs

allows kinesin-5 not only to facilitate MT sliding, but it is also able to constrain unwelcomed movement[3].

For successful cell division to occur, a mitotic spindle must form to enable each chromatid to be separated from the chromosome. Therefore, when the cell divides, each cell contains exactly one copy of the genetic material of the cell. When kinesin-5 is inhibited from functioning properly, the spindle fails to form, creating a monopolar spindle[2]. This prohibits the cell division process to continue and often leads to cellular malfunction and eventually death[3]. Kinesin-5 has become a target for cancer research due to its significant link to the cell division process. Understanding the many structures and functions of kinesins within the cell is significant; however, to fully understand their role, it is crucial to analyze kinesin's physical capabilities on a molecular level.

2.4 Properties of Kinesin-1 and Kinesin-5

Studying the molecular movement of a single molecule of kinesin, scientists have been able to analyze the run length, processivity, stall force, mechanochemical coupling, and velocity of these motor proteins[31,32]. Run length is the distance the motor travels on the microtubule. Processivity is a function of the run length; it is a value based on the distance the protein is able to travel along the microtubule before detaching[31]. Stall force is proportional to the displacement. Generally, continuous the hand over hand mechanical walking cycle enables kinesin to move processively along the microtubule; however, this processive movement is also dependent on "gating" or the ability of the head domains to communicate their nucleotide binding state to prevent detachment[32].

The properties of kinesin are dependent on the nucleotide binding states and the hand-over- hand walking mechanism[31,32]. Guo and colleagues performed a thorough experiment to study the processivity of kinesin-1 as it relates to the neck-linker region and nucleotide binding states[31]. This study revealed that when the head is in the ADP state, it has weak affinity for the microtubule; however, all other binding states (ATP, free, and ADP + P_i) have strong interactions with the microtubule[31]. This proves that most often the kinesin has a strong affinity for the microtubule which increases its ability to remain in association with the microtubule. The binding state also affects the neck-linker docking formation. The neck-linker is in the fully docked position once ATP is hydrolyzed to ADP and P_i and before P_i is released[31]. As mentioned in the previous section, the neck-linker must completely dock in order to position the other head to bind the microtubule. Kinesin's mechanical walking patterns encourage processive movement as one head is attached to the microtubule at all times[32]. Toprak and colleagues associate this phenomenon with the process they refer to as "gating"[32]. Gating is described as the ability of the head domains to communicate their binding state to one another to prevent dissociation from the microtubule[32]. Kinesin proteins are categorized as processive motors; however, some families are able to take many more consecutive steps than others.

Kinesin-1, for example, can take over hundreds of steps before detaching from the microtubule; whereas kinesin-5 is only able to take approximately 10 steps[13,33]. The neck-linker, present in all kinesin proteins, is extended in kinesin-5 which results in a decrease in processivity[34]. The functions of the neck-linker region are not fully understood. It is assumed that the neck-linker affects the affinity for the microtubule, may facilitate communication between the heads, and plays a major role in generating force[34]. The length

of the neck-linker region may affect the strength of the interaction with the microtubule[34]. Shastry and Hancock performed an experiment to determine the effect of kinesin-5's 18 residue neck linker[35]. By shortening kinesin-5's neck-linker to 14 residues, the length of kinesin-1's neck linker, the run length increased from near 0.25 to 2.0 micrometers[35]. This proves that the extended neck linker is partially responsible for the low processivity of kinesin-5. The neck-linker also plays a role in force generation.

As kinesin travels along the microtubule, it must convert chemical energy to mechanical energy in order to move. Both kinesin-1 and kinesin-5 hydrolyze ATP as the main source of chemical energy[12]. This hydrolyzation creates the energy for conformational changes to occur. As the neck-linker switches between the docked to disordered position a mechanical force to pull the motor is generated[12]. To take one step, the kinesin-5 motor uses approximately two-thirds of the energy produced by hydrolyzing a single molecule of ATP[33]. This energy per step was calculated based on an 8-nanometer step length and a maximum force of 7 piconewton[33]. Although kinesin-5 has low processivity, it is able to generate the same amount of force as kinesin-1. Studies reveal that the stall force for each is approximately 5-6 piconewton of force[33,36,37]. Other research has identified another structural component, the cover strand, that works with the neck-linker to generate force[26]. The cover strand (CS) is located at the N-terminal of the kinesin motor head. When the neck-linker is in the docked position, it combines with the CS to form a 2- stranded beta sheet known as the cover-neck bundle (CNB). When the neck-linker switches to the disordered position, the CNB dissociates. Khalil and colleagues revealed that by mutating the CS, the force generated is significantly reduced[26]. These mechanisms that enable kinesin to generate force and move processively are theoretical

and not fully understood. In order to better comprehend kinesin's abilities, the structure of the microtubule must also be analyzed.

2.5 E-Hooks

The polymerization of alpha (α) and beta (β) tubulin creates a microtubule. The core structure of these proteins is made up of about 400 amino acid residues; this structure is highly conserved among various tubulin genes[11]. However, the structure and sequence of the carboxy-terminal tail (CTT), or E-hook, of tubulin is subject to post-translational modifications and varies significantly[11]. The E-hooks on α and β tubulin are typically composed of 10 and 18 residues, respectively. A majority of these negatively charged amino acids are glutamate and aspartate. The E-hook does not contain a secondary structure of helices or sheets causing the tail to stick out from the tubulin core structure as seen in **Figure 2.6**[38]. This allows the E-hook to have easy access to interact with MAPs near the microtubule. The neck region of the kinesin is positively charged facilitating and electrostatic interaction with the E-hook.

E-hook and kinesin have a complex interaction that is not completely understood. Using a protease, subtilisin, the c-terminal tails along a microtubule can be removed resulting in digested microtubules[2]. This process can be utilized to understanding the role of E-hooks in kinesin motility. Although kinesin-1 can walk along the digested microtubules, the processivity, or how far the kinesin walks along the MT without diffusing away, is less than normal[39]. This suggests that E-hooks stabilize the kinesin's attachment to the microtubules. In a similar experiment, Reinemann and colleagues

revealed that kinesin-14 was unable to bind the microtubule when the E-hook was removed[40]. Conventional kinesin is influenced by the E-hook; however, the E-hook is critical to the function of mitotic kinesin such as kinesin-5 and kinesin-14. In a study on fungal kinesin, the E-hook played a role in the ADP kinetic turnover but did not interact with the neck region[1]. The E-hook's electrostatic interactions with kinesin are favorable.

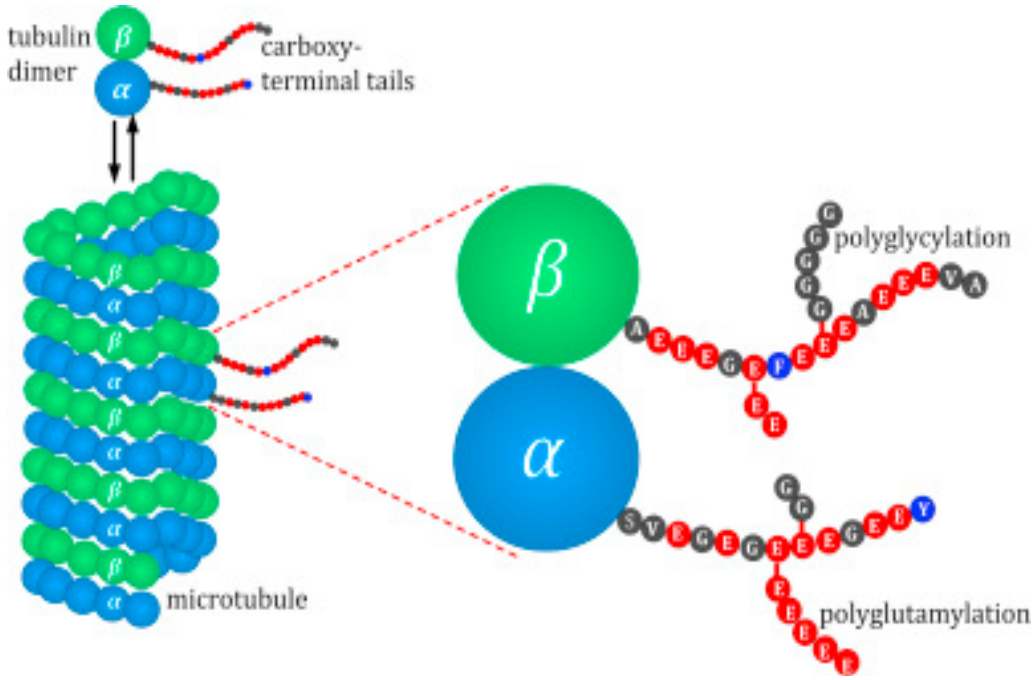

Figure 2.6: **E-hook.** Alpha (blue) and Beta (green) tubulin dimers have a negatively charged carboxy-terminal tail known as an E-hook. This tail lacks secondary structure and extends outward from the polymerized tubulin allowing easy access to any MAPs. Figure adapted from Sataric el at. (2017). Journal of Theoretical Biology, 420, 152-157.[51]

In hopes of stopping cell division, our study focuses on exploiting the mitotic kinesin's vulnerability to E-hook digestion. Sirajuddin and colleagues revealed the sequence of the carboxy-terminal tail on human alpha and beta tubulin[11]. The last 6 residues of beta tubulin are EGEDEA as seen in the **Figure 2.7**. This study also proved that the kinesin-1 is dependent on the beta E-hook to have normal velocity and processivity[11]. Without the beta E-hook the velocity and processivity decreased by 50%[11]. If this affect is seen in kinesin-1, it can be assumed that the effect on mitotic kinesin would

be greater. We will use this EGEDEA peptide to saturate the kinesin binding sites; our goal is to interfere with the kinesin's binding to MTs and ultimately disrupt mitosis in cancer cells.

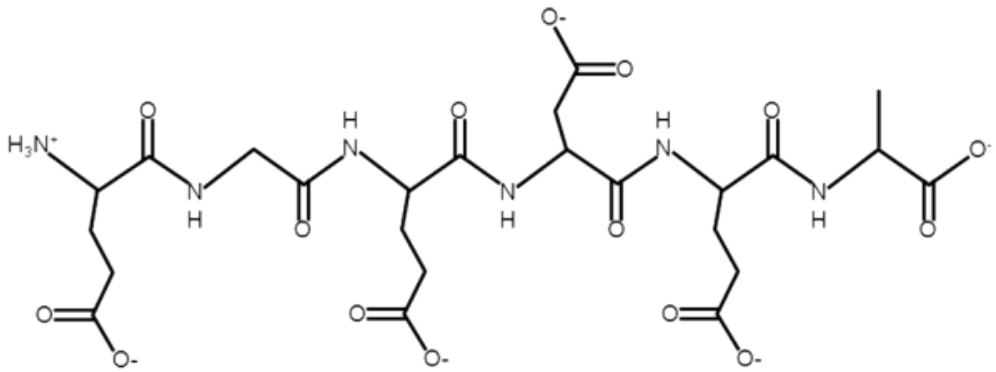

Figure 2.7: **E-hook Terminal Amino Acid Sequence.**
Glu-Gly-Glu-Asp-Glu-Ala [EGEDEA]. Glutamate and Aspartate residues are responsible for providing the net negative charge of the peptide.

2.6 Cancer Implications

Cancer is an invasive disease that has no contact inhibition (**Figure 2.8**)[9,41]. Cancer cells do not stop dividing upon contact with other cells, instead they invade surrounding tissue ultimately forming tumors[9]. According to Dumon and colleagues, there is not a four-step linear process in which there is an initial cause, an oncogenic event, and a signal transduction pathway that results in a phenotypic tumor[1]. When a cell becomes corrupted, a programmed series of events known as apoptosis occurs that results in cell death[42]. When cellular division occurs at a rate in excess of the rate of apoptosis, excess cell growth occurs, a hallmark of cancer. It was once thought that cell death was a random loss of normal cell function, but it is now perceived as a vital regulatory aspect of the cell division

process[43]. While all cancer cells exhibit some form of contact inhibition, there are many alterations in the deformities of the cell. These alterations make it difficult to develop targeted therapy and have led researchers to focus their attention on common cell cycle

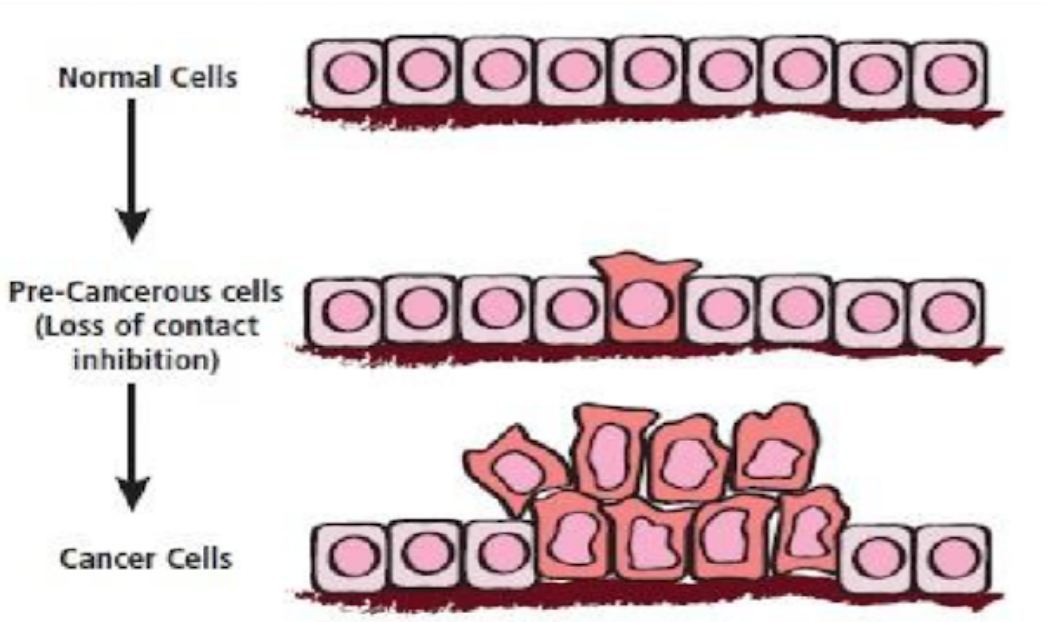

Figure 2.8: **Loss of Contact Inhibition.** Normal cells exhibit contact inhibition which prevents them from dividing once they come into contact with other cells. A hallmark of cancer is the loss of contact inhibition, causing cancerous cells to grow on top of each other in disarray, invading surrounding tissue. Figure adapted from EDVOTEK® protocol "Morphology of Cancer Cells".[41]

machinery[6].

A positive feedback loop of growth factors and increasing production of growth-factor receptors allows cancer cells to replicate indefinitely[6]. The checkpoints throughout interphase should arrest the cell if there is DNA damage. These checkpoints rely on cyclin-dependent kinases and proteins. A common occurrence in mammalian cells is DNA damage that results in increased p53 production. Excess p53 induces transcription of specific genes causing arrest during G1-phase or apoptosis[8]. However, in cancer cells, this G1-S checkpoint is often lost due to p53 mutations. To induce cell-cycle arrest, cancer cells

are exposed to ionizing radiation, also known as chemotherapy. This treatment is not ideal. According to Hartwell and Karstan, all cancer cells could be killed by irradiating the whole body with 12,000 centi-Gray of ionizing radiation; however, this is toxic to healthy cells[8]. Chemotherapy is not the only cancer treatment; there are many other drugs that focus on arresting the cell cycle in various manners.

The mitotic spindle is essential to the cell cycle by allowing proper segregation of chromosomes and is therefore, a target for cancer research. The spindle itself is hard to target due to acquired resistance when spindle specific poisons, such as Taxol, are used[8]. Therefore, it is important to determine how to inhibit spindle formation without attacking the spindle directly. This thought process has led to the study of the motor protein kinesin which is a crucial element of mitosis.

By targeting kinesins, the antimitotic effects of spindle positions can be avoided, but kinesins provide their own set of challenges. One of the major setbacks in kinesin research is the many functions of each kinesin. Kinesin-5 is a popular target for cancer research due to its well-defined function within the cell cycle[44]. It only has one function in the cell cycle; therefore, its inhibition will not affect other aspects of the cell the way manipulation of other kinesin subgroups may[45]. By preventing proper crosslinking of the microtubules and therefore spindle formation, the cell cycle can be corrupted, inhibiting cell division and continued cancerous cell growth.

2.7 Optical Trap Technology

By inhibiting cell division associated with aggressive cancer cells, this study strives to slow or stop tumor growth. We hypothesize that using an E-hook peptide to target mitotic kinesins, will prevent kinesin motility and thus inhibit cell division. We will first test this hypothesis *in vitro* using a biophysical technique called optical trapping (OT). OT measures forces in the piconewton range, suitable for the forces generated by kinesins. In our research, we use the JPK NanoTracker optical trapping system to measure the force generated by kinesin-1 in the presence of E-hook peptides to test our hypothesis. While we analyze the effects on kinesin-1, we conjecture mitotic kinesins such as kinesin-5 are more vulnerable to E-hook binding than conventional kinesins such as kinesin-1. Our work with kinesin-1 will call for future research with kinesin-5 to measure how force differs between kinesins in the presence of E-hook peptides. This will allow better understanding of the level and mechanism of inhibition.

OT allows us to analyze the properties of kinesin such as force generation, velocity, and step size. It also allows us to monitor kinesin motility while walking along microtubules and calculate the force kinesin generates. OT, also referred to as optical tweezers, allows manipulation and measurement of micron-sized particles. OT is able to manipulate micron-sized, dielectric, polystyrene beads by applying piconewton-level forces[46]. For most experiments, these beads are attached to the object of study. For the force to be generated, the index of refraction of the dielectric object must be larger than the refractive index of the surrounding medium (water in the case of biological studies) shown in **Figure 2.9**[47]. Since the dielectric object has a higher refractive index, the light will pass through it and then bend inward[48]. The generated force is established by the change of momentum caused by the change in direction of light. Based on Newton's third law, this

change will result in an equal and opposite force[48]. This refraction will result in a refractive force that moves the bead toward the center of the beam[48].

Once an object is trapped by the OT, nanometer-level displacements and piconewton forces can be measured. The trap acts like a Hookean spring thus the forces of the optical tweezers can be characterized by Hooke's Law:

$$F = -k\,x$$

Hooke's Law states that the force (f) is equal to the negative of the spring constant (k) multiplied by the displacement of an object (x)[49] . In our analysis, the spring constant is equivalent to the trap stiffness and the displacement of the bead is measured by x[48].

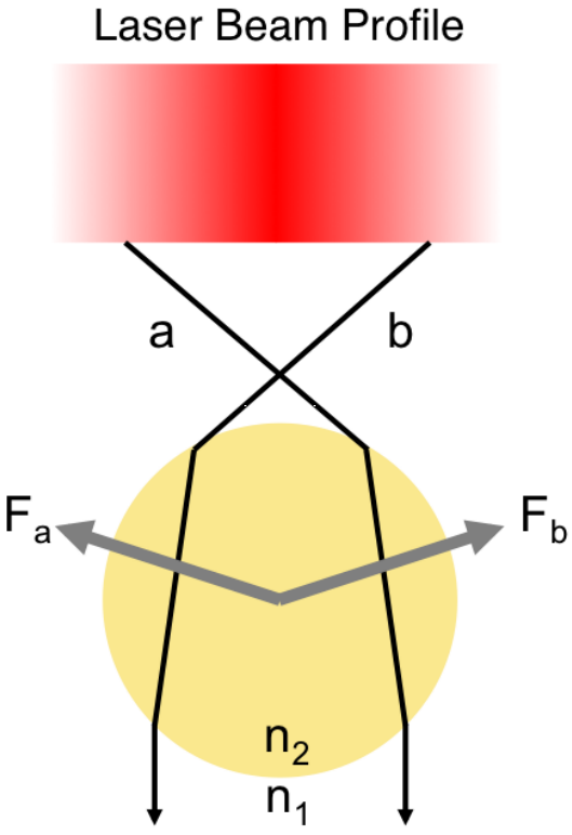

Figure 2.9: **Laser Beam Profile of Stable Trap.** When the trap is stable, a three-dimensional intensity is established. A radial intensity gradient is shown. The n_1 and n_2 notations represent the refractive index differences where $n_2 > n_1$. The refractive force is depicted by the gray arrows. These forces are balanced, trapping the bead in the equilibrium position. Figure adapted from Reinemann, 2018.[47]

The trap stiffness and the force of the laser beam must be taken into account when calculating the force generated by kinesin[48]. By using a bead smaller than the wavelength of light, the force calculations of the optical trap are dependent on an electromagnetic Lorentz force. The position detector must be calibrated before each trial. The x, y, and z voltage signals from the photodetector are monitored as the bead moves[45].

The Nano Tracker is able to calibrate the system using a power spectrum analysis for optimal calculations[46]. The Lorentz function that describes the theoretical spectrum is:

$$S(f) = \frac{k_b T}{\gamma \pi^2 (f_c^2 + f^2)}$$

The corner frequency (f_c) is proportional to the trap stiffness (K) and the viscous drag(γ).

$$f_c = \frac{K}{2\gamma\pi}$$

$$\lambda = 2\pi * \eta * d$$

The only values that the user must input are the viscosity (η) of the medium and the diameter (d) of the bead. The trap stiffness can be adjusted by the user based on the laser power controls. Extra noise within the trap's position signal can cause the measured stiffness to be underestimated[45].

The Nano Tracker can also generate a force spectroscopy oscilloscope that displays x-signal versus time[46]. The x-signal is a voltage measurement that correlates to the position displacement of the bead. The displacement of the bead can be used to calculate the force the kinesin generates as it walks along the microtubule.

CHAPTER 3

METHODS

3.1 Etching Slides

Since the optical trap detects micrometer sized particles, the coverslip must be etched to remove all particles from the surface. Although the process of etching takes a couple of hours, it is simple and requires minimal reagents. Using a magnetic stirrer and stir bar, begin dissolving 100 grams of potassium hydroxide (KOH) in 300 milliliters (mL) of ethanol. While this is dissolving, place 10 slides in a Teflon rack, prepare two beakers of 300 mL of double-distilled water (ddH_2O), and pour 300 mL of ethanol into a beaker. All four of these beakers should be degassed in a sonicator for five minutes. Vigorously dip the Teflon rack in and out of the KOH solution. Continue until the ethanol flows from the slide smoothly. Repeat this dipping process in a beaker of ddH_2O. Place the Teflon rack into the second beaker of ddH_2O and sonicate for five minutes. Spritz the slides with water until the water flows off smoothly; repeat the spritzing process with ethanol. Finally, dry the rack in the oven for 15 minutes. To prevent contamination, store in airtight container until usage.

3.2 Taxol Stabilized Tubulin Polymerization

This protocol describes the production of Taxol-stabilized microtubules that will be used in the kinesin assay. The initial step is to make the polymerization mixture. All the reagents and the new solution must remain in ice throughout the work period. Before beginning, ensure that the bead bath is warming to 37ºC. Mix all these reagents into an epp tube and allow to sit on ice for five minutes allowing the tubulin to bind to the guanosine triphosphate (GTP). To induce polymerization, place the tube into the 37 ºC bead bath for one hour.

While waiting, prepare Taxol dilutions. Using dimethyl sulfoxide (DMSO), prepare 3 Taxol dilutions of 5, 50, and 500 micromoles per liter (μM). It is critical that the Taxol is added stepwise, or it will precipitate out of the solution. Since Taxol is highly insoluble in aqueous solutions, the DMSO is used to help prevent precipitation. The volume of the current reagents in the polymerization mixture is 5.

0 μL. For a 1:10 volume ratio, add 5 μL of 5 μM Taxol. Incubate in the bead bath for 10 minutes. Repeat this step using 50 μM Taxol and 500 μM Taxol; to ensure 1:10 volume ratio, add 5.5 and 6.05 μL respectively. After the final step, incubate for 15 minutes. The Taxol-stabilized microtubules should be stored at room temperature.

3.3 Constructing a Flow Cell

Every assay requires the construction of a flow cell. The materials required include a microscope slide, etched coverslip, double-sided tape, and a microcentrifuge, or epp, tube. Initially, place two pieces of tape a couple of millimeters apart across the width of

the slide (**Figure 3.1a**). In order to view the flow cell using the optical trap, it is crucial for the channel to be in the center of the slide. The edge of the microscope slide will easy cut any excess tape. Place the coverslip perpendicularly on the microscope slide (**Figure 3.1b**). Use the epp tube and carefully push the coverslip onto the tape to ensure it is attached well. When preparing to flow the slide, be sure that the cover slip is below the slide. This flow channel will hold about 10 – 20 microliters.

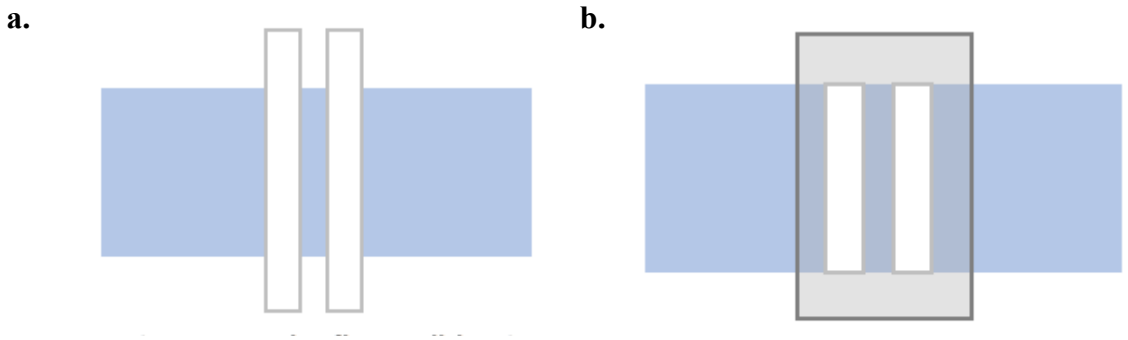

Figure 3.1: **Flow Cell Construction. a.** The flow cell begins with just the microscope slide and two pieces of tape. **b.** Flow cell after removal of excess tape and addition of coverslip.

3.4 Kinesin Optical Trap Motility Assay

The Kinesin Optical Trap Motility Assay measures the movement of the kinesin on the microtubules (see Appendix A.3 for protocol). This procedure calls for a Poly-l-lysine surface, Taxol stabilized microtubules, and kinesin linked anti-His beads. Microtubules stick to the poly-l-lysine slide and kinesin saturated anti-His beads attach to the microtubules. An optical trap is used to analyze and track the kinesin/bead movement along the microtubule.

Appropriate buffers must be prepared to begin; this includes: PemTax, Assay Buffer, and C-Tax (protocols found in Appendix A.1). The kinesin is subsequently diluted

to form a 10,000x dilution that is combined with the beads and incubated at 4°C for an hour in a rotator. While the kinesin-bead dilution (KBD) incubates, the poly-l-lysine coverslips are prepared by diluting 400 μL poly-l-lysine in 30 mL of ethanol. An etched coverslip is placed in the diluted poly-l-lysine solution and allowed to soak for 15 minutes. The coverslip is dried using the air line and is now ready to prepare the flow channel.

A flow cell is constructed by flowing in 15 μL of diluted microtubules. When the microtubules are first prepared, a 20x dilution in PemTax is adequate. After 10 days, the dilution must be increased to 10x in order to have a sufficient amount of polymerized microtubules. The flow cell is allowed to incubate for 10 minutes, giving the microtubules time to bind. After the 10 minutes, 20 μL of PemTax is washed through the channel. Next, the flow cell is washed with 15 μL of C-Tax, 15 μL of PemTax, and 15 μL of Assay Buffer with 5 minutes of incubation between each new addition.

Once the hour is up, the KBD is removed from the rotator and the following is added: 1 μL of 100x glucose oxidase4, 1 μL of beta-D-glucose, and 1 μL of 100x catalase5. 20 μL of the KBD is washed in the flow cell. The flow cell is allowed to incubate for five minutes before the slide is loaded into the optical trap.

3.5 Focusing the Optical Trap

The JPK NanoTracker is a valuable asset that enables us to observe and record data. We continually learn new functions and methods for using this complex instrument. For the purpose of our research, our protocols are relatively simple.

Once the microscope slide is prepared for observation, begin turning on the control box, laser, and computer monitor. Begin the JPK software, open the camera and objective

controller, and set the mode to DIC. Remove the stage and place the microscope slide on top with coverslip facing upward. Pipette 30 μL of ddH_2O on the lower objective lens before reloading the stage. Be sure the stage is fully inserted before raising the lower objective. Using the controller raise the objective until the water reaches the slide; it will appear as if the water bubble pops. Pipette 170 μL of ddH_2O on top of the coverslip. Lower the upper objective until it is inserted into the water; similarly, it will appear to "pop" the water. Each step is shown in **Figure 3.2**. With the objectives are immersed in water, prepare to focus by moving the stage so that the edge of the tape is near the center of the objectives.

Begin by raising the lower objective until large bubbles and the edge of the tape are distinct (**Figure 3.3a**). To focus the upper objective, change the mode settings by turning the gain boost and the auto gain off. Shut the iris completely, and move the stage off of the tape towards the flow channel. Depending on the initial position of the upper objective, move the objective until an octagon appears. Slightly open the iris and adjust the position of the upper objective until the edges of the octagon are sharp (**Figure 3.3b**). Lock the objectives together, open the iris, and return to DIC mode.

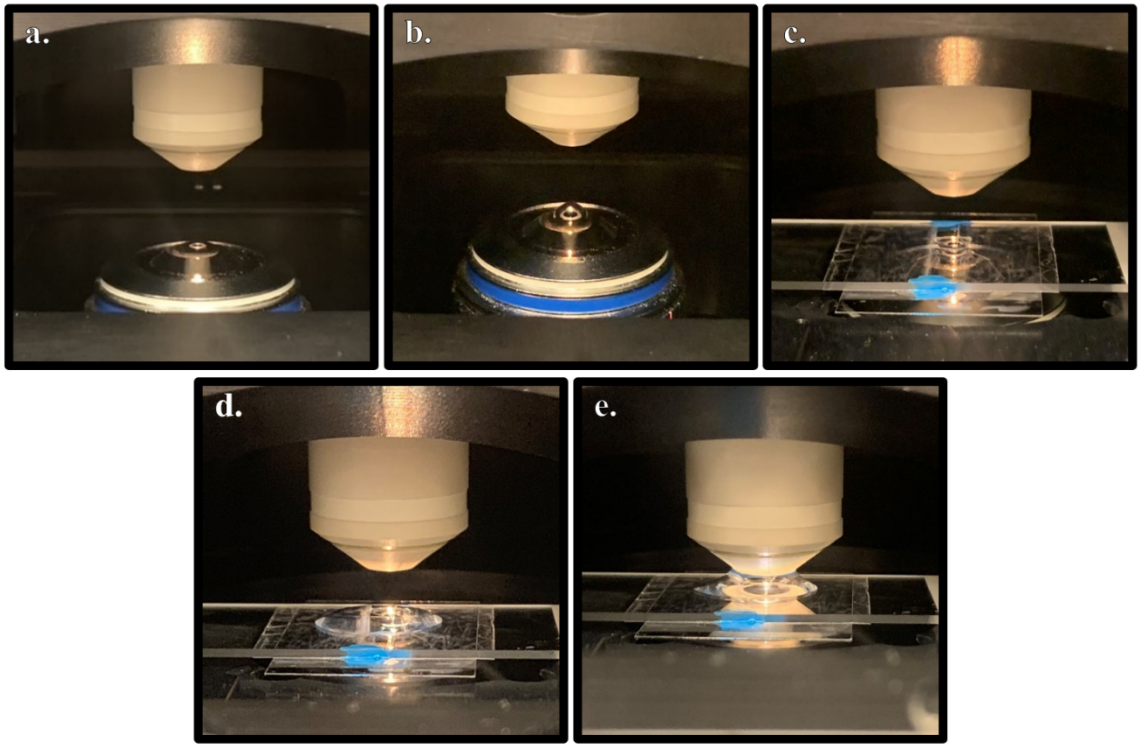

Figure 3.2: **Optical Trap Setup. a.** The initial objective placement with the stage removed is shown. **b.** 30 µL of ddH_2O is added on the bottom objective. **c.** The bottom objective is raised until the water "pops" against the slide. **d.** 70 µL of ddH_2O is placed on top of the slide. **e.** The top objective is lowered until it makes contact with the water.

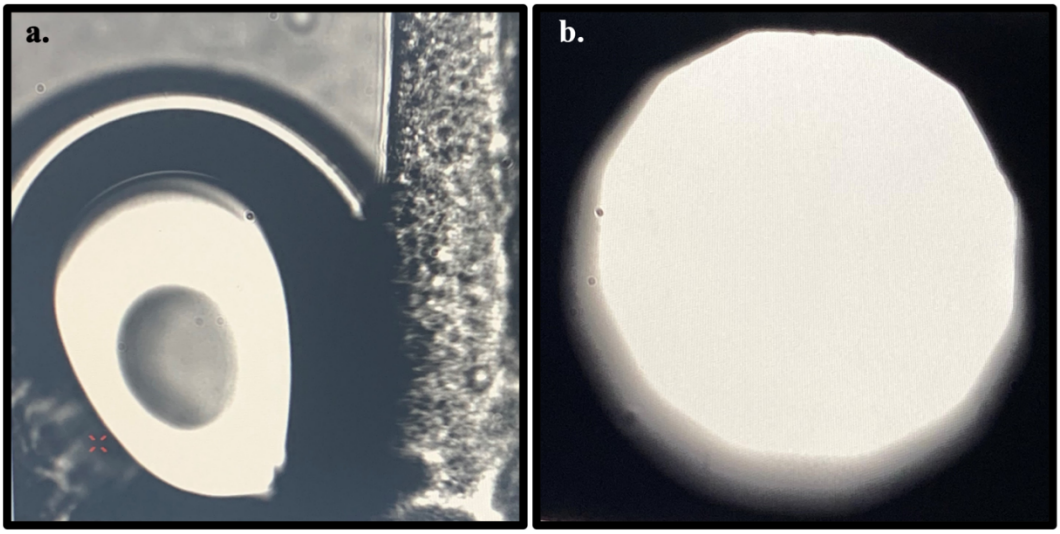

Figure 3.3: **Focusing the Optical Trap. a.** The height of the bottom objective is adjusted until the bubble and the edge of the tape become distinct. **b.** With the iris shut the upper objective height is adjusted until a hexagonal shape becomes visible.

3.6 Operating the Optical Trap with Kinesin Motility Assay

When analyzing the Kinesin motility assay, the first step is to find the microtubules on the surface of the slide. Move the objectives down in 10 (μm) steps until the particles are no longer moving but appear stuck to the surface. Take 1 μm steps up and/or down to get the microtubules in focus as seen in **Figure 3.4**. After ensuring the microtubules are abundant and straight, find a bead.

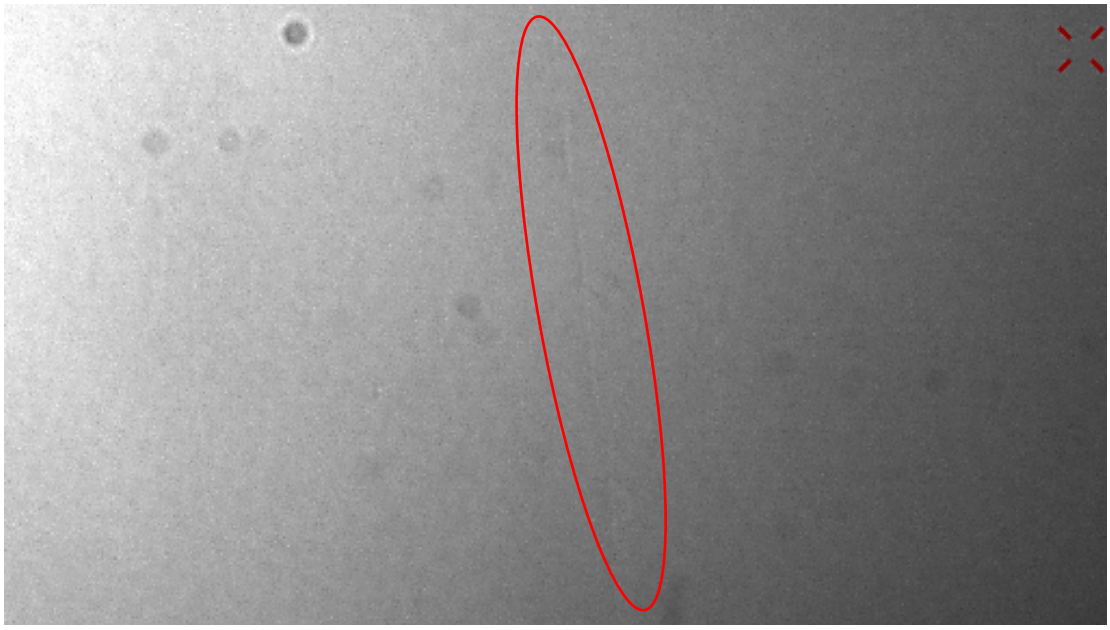

Figure 3.4: **Microtubule in Focus.** Shown above is a microtubule in focus using the optical trap. Once the MTs come into focus, we know that we are in focus with the surface of the slide.

There are two places to find a bead: 1) sticking on the surface and 2) floating in the solution. If a bead is found on the surface, it must be pulled off by the laser. Open the laser shutter, increase power to 500 mM and pass the laser across the bead swiftly to pluck it from the surface. If this does not work, find a bead above the surface. Move the objectives up in 10 μm steps; count the displacement to return to the MTs focal plane easily.

Once a bead is located, open the laser shutter and decrease the power. Do not turn the laser on directly on top of the bead or it will often be pushed away. With the laser on a low power, slowly approach the bead to trap it. To return to the surface without losing the bead, lower the objectives using a maximum of 5 μm step size.

A few nanometers above the surface, calibrate the bead to achieve precise data and force calculations. Curve fit each X, Y, and Z position graph until the R value approaches 1.0.

Taking 1 μm steps, return to the surface and locate a vertical, straight microtubule. Lower the objectives 2 μm to push the bead on the MT. Graze the bead up and down along the MT to encourage the kinesin to attach. Open the force vs time graph and switch from X to Y displacement values. Click start, record, and save data. Observe the graph for a few minutes and save the data for further analysis. To obtain more data using the same assay, use the same bead on another MT or trap a new bead and recalibrate it.

3.7 Diluting the Peptide

The stock GenScript EGEDEA Peptide is 100 mg. Dilute this solid into 1 mL of reverse osmosis water to have a stock solution of 100 mg per mL. The peptide's molecular weight is 648.229 moles per gram. Simple calculations reveal the molarity to be 0.154 molar (M). Adding 1 μL of stock solution to a microtube with 99 μL of reverse osmosis water, the peptide is diluted to a concentration of 1 mg/mL [1.54 mM]. Divide this solution into 20 aliquots of 5 μL each and store in –80°C.

CHAPTER 4

RESULTS

4.1 Kinesin Optical Trap Motility Assay Control

For our analysis of the force generation of kinesin-1, we performed a single molecule bead motility assays using optical tweezers. The optical trap allowed us to generate force graphs, exemplified in **Figure 4.1**, by monitoring the movement of a bead once it has been introduced to a MT.

Once the kinesin attached to the MT, it would begin to walk, pulling the bead away from the trap. This movement represents a walking event. When the kinesin is no longer able to generate enough force to overcome the force of the laser, the motor releases from the MT, snapping the bead back into the equilibrium position of the laser trap.

The control data was collected by performing the Kinesin Optical Trap Motility Assay (**Appendix A.5**). For each plot, we were able to determine the number of walking events the kinesin was able to take and the force each step generated in piconewtons. From this data, we were able to determine the average force, run frequency, and standard error of the mean individually for each of our control data sets. This data is shown in **Table 4.1**. We also performed each of these statistical measurements for the combination of these trials shown in **Table 4.2**.

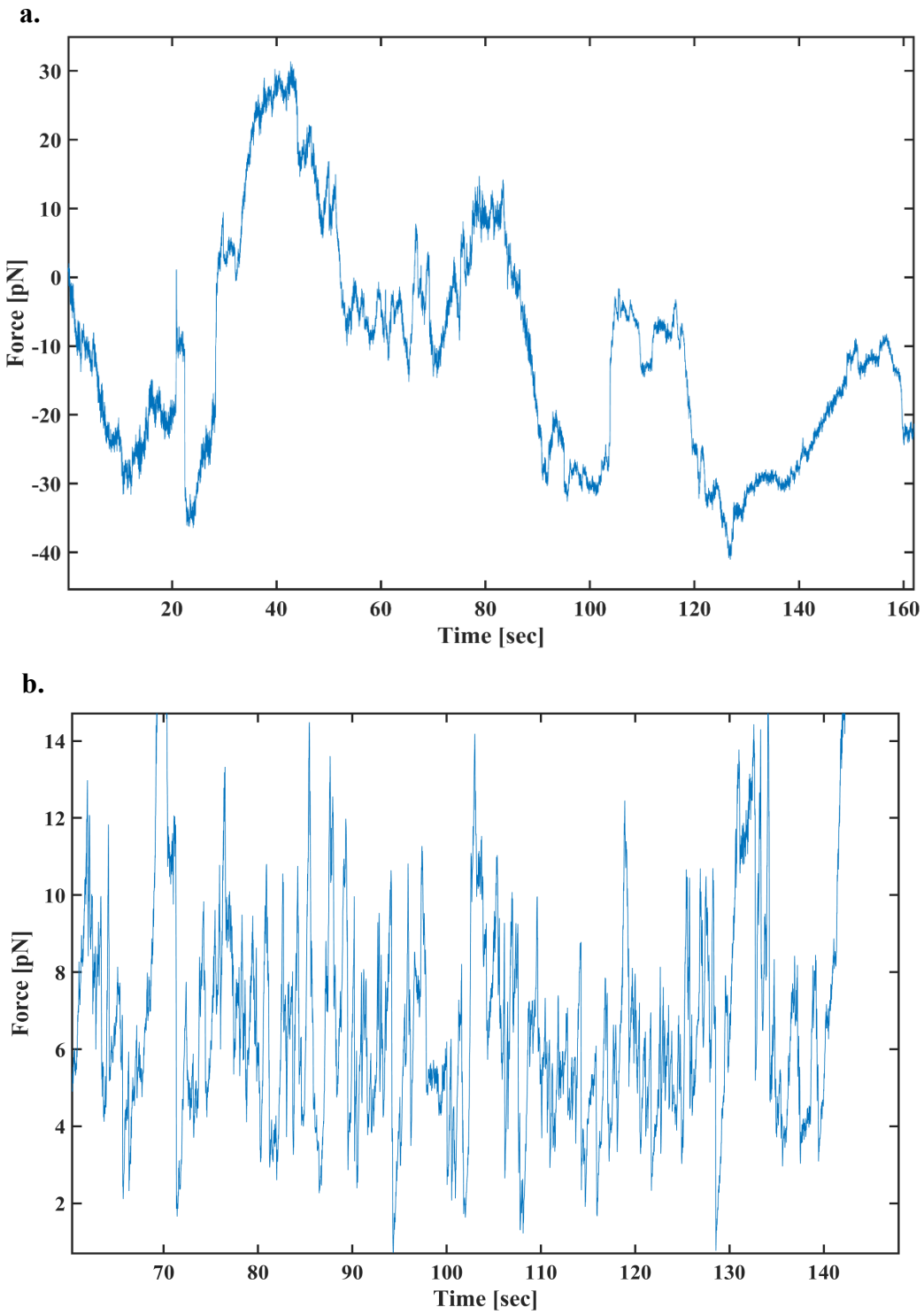

Figure 4.1: **Control Data Force vs. Time. a.** Control Data Set 1 shows a wide range in force generation over about 60 seconds. **b.** Control Data Set 2 show quick steps occurring over 140 seconds.

	Data Set 1	*Data Set 2*
Count	36	62
Run Frequency (steps/min)	13	26
Average Force (pN)	8.8	6.5
Standard Error of the Mean	1.1	0.5

Table 4.1: **Control Measurement Statistics.** Statistical measurements are shown as calculated for each control data set. We assume that the relatively large standard error of the mean stems from the presence of multiple motors.

	Total
Count	98
Average Force (pN)	7.3
Run Frequency (step/min)	19
Standard Error of the Mean	0.5

Table 4.2: **Total Control Measurement Statistics.** These values were determined from combining data from 206 steps from each of the four data sets in Table 4.1 to give a true average.

Our statistical analysis of our control measurements showed relatively surprising results with an average force generation of 7.3 pN. Previous studies have shown that kinesin-1's average force generation abilities to be ~5 to ~6 piconewton[13,33]. Due to the presence of slightly higher average force generation in both of the control data sets, we acknowledge the presence of multiple kinesin motors. We believe the difference in force generation stems from the effects of varying amounts of kinesin motors present on the measured beads. This assumption is also supported by the Force vs. Time graph for both data sets.

In the Force vs. Time graph for Control Data Set 1, there is a large range in the force generated by the steps. Due to this measurement and the shape of the graph we conclude that at times there was only one motor present where at other times multiple motors would pull together establishing forces up to ~30 piconewtons. Alternatively, the Force vs. Time graph for Control Data Set 2 shows quick steps with the bead frequently snapping in and out of the equilibrium position. We propose the presence of multiple motors in this graph as well to create the relatively high run frequency exhibited by this graph. While the motors were attaching sequentially, they were not doing so fast enough to build off of each other. Therefore, the laser was able to snap the bead back each time resulting in a graph differing from the one Data Set 1 produced. Both data sets provide the control measurements that allowed us to begin collecting experimental data by introducing the peptide to the assay.

4.2 Kinesin Optical Trap Motility Assay With E-Hook Peptide

The goal of the use of the E-hook peptide is to mimic the decrease in processivity found in kinesins in which the E-hooks have been removed using subtilisin. To analyze the effects of the E-hook peptide on kinesin-1, one μL of one mg/mL EGEDEA peptide was mixed with 80 μL of kinesin bead dilution. After the KBD rotated for one hour, we removed 20 μL to perform the normal kinesin assay before adding the peptide. This solution was placed back on the rotator for at least 15 minutes to ensure that the protein had adequate time to saturate the kinesin motors.

While in the laboratory, we made notes of visual observational differences. The main observation being that the kinesin motors were never spontaneously walking on the

MT. In the normal assay, we witnessed on multiple occasions, a bead that appeared to be processively moving along the microtubule. For the assay with the peptide, we were able to trap beads with the laser to encourage the kinesin to associate with the MT in order to record a force vs. time graph.

Analysis of the data reveals that the run frequency ranges from ~8 up to ~25 runs per minute (**Table 4.3**). Our normal kinesin assay has an average run frequency that ranges from ~13 to ~26 steps per minute, which is in the same range as the run frequency recorded for protein + KBD. Our data exhibits minimal change in the velocity of the kinesin. Well-defined steps of a single kinesin motor appear to have slower movement; however, all steps were not this precise (**Figure 4.2**).

	Data Set 3	*Data Set 4*	*Data Set 5*	*Data Set 6*
Count	38	70	79	19
Run Frequency (runs/min)	11	11	26	8.3
Average Force (pN)	1.1	3.0	1.8	3.2
Standard Error of the Mean	0.1	0.2	0.1	0.4

Table 4.3: **Kinesin + Protein Measurement Statistics.** All values in each data set were recorded from the same kinesin + protein assay.

It is likely that the peptide decreased the kinesin's processivity. Calculations of average run length are often correlated to processivity. This can be measured directly in similar unloaded video tracking assays that should be performed by future students. However, as noted previously, studies have shown that kinesin-1 is capable of taking hundreds of steps before releasing the MT[13,33]. In data set 5 (**Table 4.3**), 79 walking events

were recorded, but 38 occurred in the first 100 seconds, then a gap of 40 seconds occurred before the kinesin began to move again. The inability of the kinesin to rapidly rebind the MT and begin walking again during these time gaps also suggests a level of lowered affinity for the MT.

Another property of kinesin that was significant in determining if the peptide affected kinesin, is the average force generation. As mentioned previously, our studies show that kinesin-1's average force generation is ~7 pN while other studies report forces around 5 to 6 pN[13,33]. The average force generated by kinesin-1 when combined with peptide is ~2.2 piconewtons (**Table 4.4**). A single kinesin motor generating approximately 3 piconewtons of force is shown in **Figure 4.2**. This indicates that the E-hook peptide,

Combining the runs from all four data sets, a histogram of the 209 runs was

	Total
Run Frequency (runs/minute)	13
Average Force (pN)	2.2
Standard Error of the Mean	0.1

Table 4.4: **Total Kinesin + Protein Statistics.** These values were determined from combining data from 206 steps from each of the four data sets in Table 4.3 to give a true average. These numbers reveal that kinesin-1 was inhibited by the E-hook peptide.

generated (**Figure 4.3**). This reveals that the numbers are skewed to the right. The majority of the values are low, between one and two piconewtons, but a few high values are responsible for raising the average.

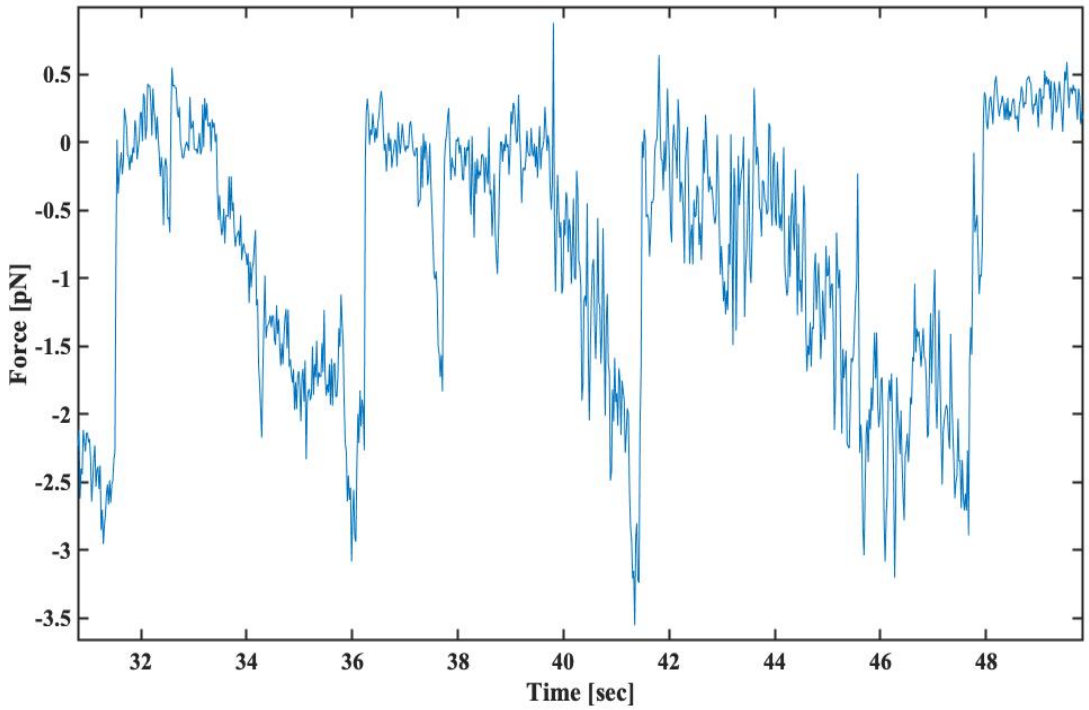

Figure 4.2: **Single Molecule Steps Inhibited by E-hook Peptide.** There are three steps in this image with a force ~3 pN. The slow downward movement is generated by the kinesin; the sharp upward movement is due to the laser pulling the motor back to its center.

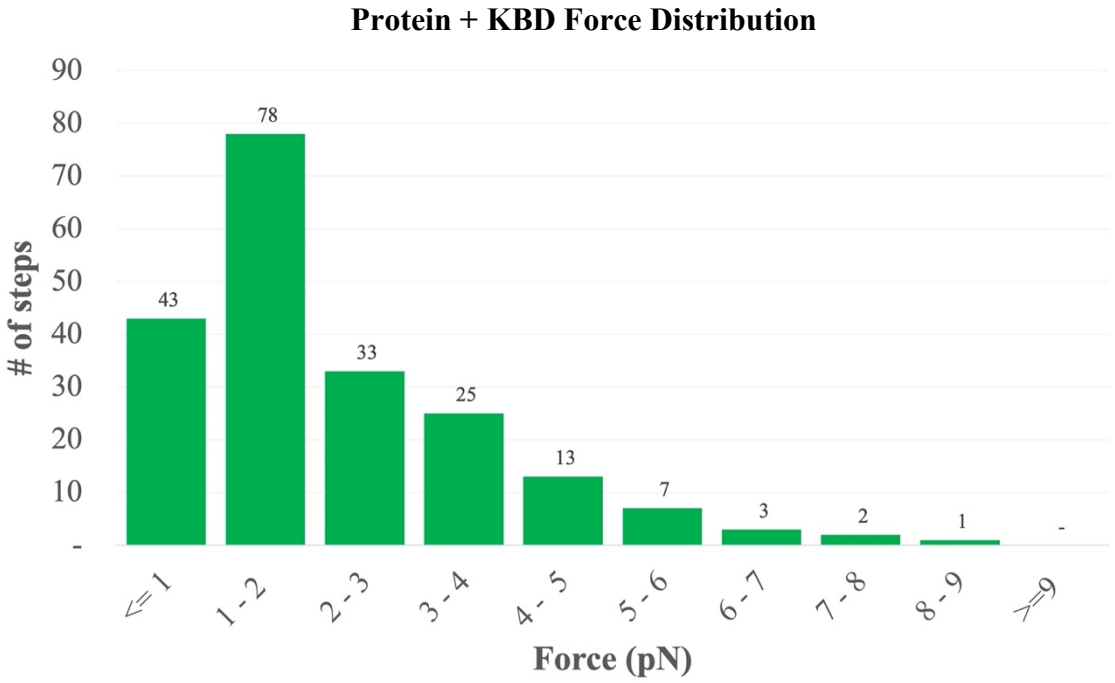

Figure 4.3: **Protein + KBD Force Distribution.** This histogram depicts the distribution of force generation of 206 steps. It is apparent that the curve is skewed right. Majority of the values are less than 3 pN and few greater than 5 pN.

4.3 Implications of Data

Based on our experimental results, the E-hook peptide had minimal effect on run frequency, but it decreased the force generated 70%. This supports our hypothesis by proving that the peptide inhibited kinesin-1's normal function. Understanding that mitotic kinesin, such as kinesin-5, is more vulnerable to E-hook binding, we presume that this affect would be amplified in kinesin-5[10–13]. Our work with kinesin-1 will call for future research with kinesin-5 to measure how force differs between kinesins in the presence of E-hook peptides. Previous studies on the kinesin-5 dimer reveal that the average force generated is 3.0 pN[10]. Beginning with a lower stall force, we assume decreasing this force will inhibit its motility. Kinesin-5 is also considered a weakly processive motor and dependent on the E-hook stabilization[10,33]. Our results convey positive trajectory towards the ultimate goal of creating an anti-tumor drug to inhibit mitotic kinesin to prevent cell division.

CHAPTER 6

DISCUSSION

Dr. Reinemann's Molecular and Biophysics Lab was just getting underway when we joined the team in the Fall of 2019. We took safety training courses and learned to use the laboratory equipment. The JPK NanoTracker was installed during this semester and we began learning the basic procedures of loading the slide, focusing the objectives, and calibrating beads. Over the next two years, we worked on improving current protocols, building assays, recording data, and analyzing this data. Throughout this time, we overcame many obstacles, from dealing with the technical malfunctions of the optical trap to learning to work remotely during the COVID-19 pandemic.

Establishing a new lab requires all of the protocols to be re-evaluated for our products and equipment. Analyzing and adjusting the protocols has required significant trial and error to determine the optimum procedure. One of the first protocols we faced was polymerizing tubulin (shown in **Appendix A.3**). The MTs would not polymerize, and pieces of tubulin were floating on the slide. Determining that the pH of the PEM80 was awry, we decided to remake a new buffer solution. When this did not fix the issue, Dr. Reinemann recommended that we try the Tubulin PEM buffer purchased from PurSolutions. Indeed, this fixed the issue, and we were able to see long but wiggly MTs on the slide. After examination we noticed the poly-lysine, employed to hold the MTs to

the slide had expired that month; thankfully, we were able to purchase and receive more quickly. At that point, we had been using 200 microliters of poly-lysine. Even with new poly-lysine, we had to increase to 400 microliters to see straight, motionless MTs.

Our next obstacle came as we began working with the Kinesin Optical Trap Motility Assay (**Appendix A.5**). However, we used the MTs on the slide the following week and they had depolymerized and formed large bundles. We quickly learned that the microtubules would not last longer than 3 weeks. With fresh microtubules, we were able to use the optical trap to view the assay and see MTs and beads. We began to calibrate a bead in the trap and record it walking on a MT. At first, we recorded minimal force over time, so we tested various concentrations of kinesin, from 1:10,000 to 1:100. By increasing the concentration, there was a more likely chance that the bead we selected would have at least one kinesin motor tethered to it. At one point, it was so difficult to find a bead on the slide, we accidentally trapped tubulin pieces that resembled the spherical, shiny beads. It took many weeks to improve this issue. Eventually, we resolved the issue by changing the sonication method and concentration of the beads. To prevent the beads from sticking to the tube and forming clumps, we aggressively flick the microtube of beads then sonicate the tube for 1 minute. We stopped diluting the beads before combining with assay buffer and added a second sonication. Sonicating 1 μL of beads in 90 μL of assay buffer, the beads have more space to separate and prevent clumping. The diluted kinesin and oxygen scavenging reagents are added after sonication. With these changes, by simply observing the assay, it appeared ideal to begin recording data with the optical trap.

After a prolonged break due to the COVID-19 pandemic, we were determined to finally record data when we returned to the laboratory. To our surprise, the tubulin, again

would not polymerize. This time, we determined the issue was with the Taxol dilutions. Using DMSO, we diluted the Taxol to 5, 50, and 500 mM. Instead of wasting the solutions, we froze the three dilutions in the -80°C freezer. Taxol is necessary to stabilize the polymerization of MTs; however, freezing, the Taxol caused a loss of function[50]. We learned that making fresh Taxol dilutions each time is required for the MTs to polymerize adequately. After addressing all the issues that we have witnessed thus far, the protocols are set and performing them is simply like following a recipe. Using the optical trap throughout this time to visualize the issues required patience and persistence.

We have dealt with the stage and objectives moving randomly, the laser trap door not opening, and even settings being lost due to power outages. For these complications with the optical trap, we assess a series of possibilities of underlying causes that could be creating the issue and work to resolve it. Other obstacles we were challenged with include "trash" falling into the trap while recording runs, the slide drying out, and beads sticking to the surface. We would re-evaluate our protocols to add wash steps to decrease the amount of unpolymerized tubulin and other unwanted particles. We also made sure to seal the coverslip with polish to prevent desiccation and work efficiently to decrease the amount of time the beads have to settle to the surface.

Problem solving, being patient and persistent, and asking for help are key life lessons we have learned throughout our research project. As citizen scholars, we are prone to believing that we are expected to have all the answers. However, working on this project taught us the importance of having mentors to learn and grow from. We learned the significance of never giving up. There were many months that we would spend at least 12 hours a week in the lab and collect no results. This was discouraging but taught us another

lesson: the importance of being an optimistic and encouraging team member. Working as a team has proved to have significant benefits; we have learned to be better team members and team leaders that do not shy away from a challenge. Our hope is that we have paved the way for future student researchers to dive into further studies of the molecular mechanics of kinesin and E-hook interactions. The goal we set for future research is to develop a drug that will effectively target kinesin motors to halt cancerous growth.

CHAPTER 7

DIRECTION FOR FUTURE RESEARCH

By researching kinesin, microtubules, and the roles of E-hooks and analyzing the effect of the EGEDEA peptide on kinesin-1, we have uncovered questions and identified a need for further research. Concluding here, we present a series of questions that could be developed for future studies.

1) Does the E-hook peptide decrease the processivity as a function of run length? Using an optical trap to record kinesin walking on the microtubule; what does the run length analysis reveal?
2) Does the EGEDEA peptide used in our study present amplified effects on the processivity and stall force of kinesin-5 or other mitotic kinesins?
3) What is the effect of using a 12-residue E-hook peptide (GEFEEEEGEDEA) to inhibit kinesin-1? Kinesin-5? Does this length change the effect observed from the shorter peptide? Why?
4) Moving from molecular to cellular studies, what are the effects of the E-hook like peptide on replication kinetics?
5) In order to produce a medication, drug delivery must be studied. What are the possible avenues for drug delivery to target specific cancerous tumors?

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APPENDIX A

PROTOCOLS

A.1 Buffers

1. PEM80, pH 6.9

   Materials:
   PIPES
   EGTA
   $MgCl_2$
   KOH
   Deionized water

   Procedure:
   1. Prepare 250 mL of PEM80 by mixing
      a. 6.048 g PIPES
      b. 95.1 mg EGTA
      c. 204.1 mL of 4.9 M $MgCl_2$ into 250 mL final volume
   2. Add small volumes of concentrated KOH solution to adjust the pH to 6.9

2. PemTax

   Materials:
   PEM80
   10 mM Taxol

   Procedure:
   1. Prepare 501 μL of PemTax by mixing
      a. 500 μL PEM80
      b. 1 μL 10 mM Taxol

3. Assay Buffer

Materials:
PEM80
DTT
Taxol
ATP
10 mg/mL Casein in PEM80

Procedure:

1. Prepare 1,500 µL of Assay Buffer by mixing
    a. 1329 µL PEM80
    b. 3 µL DTT
    c. 3 µL Taxol
    d. 15 µL ATP
    e. 150 µL 10 mg/mL Casein in PBT
2. Store on ice

4. <u>C-Tax</u>

Materials:
PemTax
10 mg/mL casein

Procedure:

1. Prepare 80 µL of C-Tax by mixing
    a. 72 µL PemTax
    b. 8 µL 10 mg/mL Casein
2. Store on ice

A.2 Dilutions

1. <u>100mM ATP stock in PEM80</u>

Materials:
Solid ATP
PEM80
KOH

Procedure:

1. Prepare solution by mixing
    a. 0.061 g solid ATP
    b. 1 mL of PEM80
2. Allow solute to dissolve completely
3. Add small volumes of concentrated KOH solution to adjust the pH to 7
4. Make 15 μL aliquots
5. Store at -80°C

2. <u>1 M DTT Stock</u>

Materials:
DTT
Deionized water

Procedure:
1. Prepare solution by mixing
    a. 0.154 g DTT
    b. 1 mL dH_2O
2. Allow solute to dissolve completely
3. Make 10 μL aliquots
4. Store at -80°C

A.2 Etching Slides

Materials:
100g KOH
Ethanol
Coverslips

Procedure:
1. Dissolve 100g of KOH in 300mL of ethanol in a beaker. Stir with a stir bar until the KOH is completely dissolved.
2. Put coverslips in Teflon racks.
3. Fill one beaker with 300 mL of ethanol and two more beakers with 300 mL of ddH_2O. Degas all four beakers (KOH in ethanol, ethanol, two beakers with water) for 5 minutes in the bath sonicator on the degas setting.
4. Submerge the rack of coverslips in the beaker with the KOH and sonicate for 5 minutes.
5. Dip the rack of coverslips up and down in the beaker with ethanol until the ethanol runs off the coverslips smoothly (no beading).

6. Dip the rack of coverslips up and down in a beaker of water until the water runs off the coverslips smoothly (no beading).
7. Submerge the rack of coverslips in the other beaker of water and sonicate for 5 minutes.
8. Spritz with water until the water flows off the coverslips smoothly. Don't be afraid to use a little force when it comes to the spritzing steps.
9. Spritz with ethanol until the ethanol flows off the coverslips smoothly.
10. Dry the rack in the oven for at least 15 minutes.

A.3 Taxol Stabilized Tubulin Polymerization

Materials:
H_2O
PEM buffer
100 mM DTT
100 mM GTP
20 mg/mLTubulin
5 μM Taxol
50 μM Taxol
500 μM Taxol
Bead Bath

Procedure:
1. Assemble polymerization mixture by mixing
    a. 26.5 μL H_2O
    b. 10 μL PEM buffer
    c. 0.5 μL 100 mM DTT
    d. 0.5 μL 100 mM GTP
    e. 12.5 μL 20 mg/mL Tubulin
2. Incubate polymerization mixture on ice for 5 minutes to allow tubulin to bind to GTP.
3. Incubate polymerization mix in 37°C bath for 1 hour.
4. Add 5 μL of 5 μM Taxol.
5. Incubate in 37°C bath for 10 minutes.
6. Add 5.5 μL of 50 μM Taxol.
7. Incubate in 37°C bath for 10 minutes.
8. Add 6 μL of 500 μM Taxol.
9. Incubate in 37°C bath for 15 minutes.
10. Store at room temperature.

A.4 Constructing a Flow Cell

Materials:
Coverslip
Microscope slide
Double-sided tape
Small epp tube

Procedure:
1. Take a microscope slide and apply two strips of double-sided tape a few millimeters apart.
2. Use a razor blade to cut off excess tape.
3. Carefully place coverslip on top perpendicular to the microscope slide.
4. Use epp tube to gently press against the coverslip to ensure the flow cell is properly stuck together.
5. Flip flow cell over so that the coverslip is on the bottom. Flow cell channel should hold 10-20 μL of liquid.
6. Use pipets to add solutions slowly. Use Kimwipes or vacuum to slowly wick the solution through without causing the flow cell to dry out.

A.5 Kinesin Optical Trap Motility Assay

Materials:
Biotinylated penta-His antibody
Streptavidin coated polystyrene bead
PEM80
Casein (10mg/mL)
DTT (0.5 M in 10 mM K-acetate)
Taxol (10 mM in DMSO)
Poly-l-lysine
ATP (100 mM in PEM80)
Centrifuge
Sonicator

Procedure:
1. Make PemTax
2. Make Assay Buffer
3. Make C-Tax
4. Make kinesin dilutions

a. K/100: 1 μL K into 99 μL AB
b. K/1,000: 10 μL K into 90 μL AB
c. K/10,000: 10 μL K into 90 μL AB

5. Make kinesin/bead dilution
    a. Incubate for at least 1 hour on rotator at 4 degrees Celsius
6. Prepare poly-l-lysine coated coverslips
    a. Dilute 400 μL poly-l-lysine into 30 mL EtOH
    b. Place two KOH cleaned coverslips into the solution
    c. Let sit for 15 minutes
    d. Dry with airline
7. Prepare kinesin bead dilution solution (KBD)
    a. Add 1 μL 100X glucose oxidase4
    b. Add 1 μL beta-D-glucose
    c. Add 1 μL 100 X catalase5
8. Prepare flow channels from KOH etched slides
    a. Flow in 15 μL MT diluted 20 times in PTX
        i. Let bind for 10 minutes
    b. Wash in 20 μL PTX
    c. Wash in 15 μL CTX
        ii. Let sit for 5 minutes
    d. Wash in 15 μL PTX
        iii. Let sit for 5 minutes
    e. Wash in 15 μL AB
        iv. Let sit for 5 minutes
    f. Wash in 20 μL KBD
9. Seal channel

A.6 Focusing the Optical Trap

Materials:
Optical Trap
Flow Cell
200 μL of H_2O

Procedure:
1. Turn on the control box, the trapping laser, and the computer.
2. Open the JPK optical trap computer software.
    a. Open camera to view image of stage.
    b. Open the objective controller.
3. Add 30 μL of H_2O to the bottom objective.
4. Place slide with the coverslip up on the stage.
5. Insert stage between the two objectives.

6. Raise the bottom objective until the water on the objective "pops" and attaches to slide.
7. Add 170 μL of H_2O to the top of the coverslip.
8. Lower the top objective until it makes contact with the water on the coverslip.
9. Focus the bottom objective.
    a. Begin to get either the tape or a bubble in the tape in focus. This should be achieved by bringing the bottom objective up.
10. Close the iris.
11. Unenable the Auto gain and the Gain boost.
12. Focus the top objective by finding a white octagon shape.
13. Once the top objective is focused, reset the trap by reenabling the Auto gain and the gain boost, and opening the iris.
14. Lock the objectives

A.7 Operating the Optical Trap with the Kinesin Motility Assay

Materials:
Kinesin Optical Trap Motility Assay
Focused Optical Trap

Procedure:
1. Turn on the control box, the trapping laser, and the computer.
2. Open the JPK optical trap computer software.
    a. Open camera to view image of stage.
    b. Open the objective controller.
3. Add 30 μL of H_2O to the bottom objective.
4. Place slide with the coverslip up on the stage.
5. Insert stage between the two objectives.
6. Raise the bottom objective until the water on the objective "pops" and attaches to slide.
7. Add 170 μL of H_2O to the top of the coverslip.
8. Lower the top objective until it makes contact with the water on the coverslip.
9. Focus the bottom objective.
    a. Begin to get either the tape or a bubble in the tape in focus. This should be achieved by bringing the bottom objective up.
10. Close the iris.
11. Unenable the Auto gain and the Gain boost.
12. Focus the top objective by finding a white octagon shape.
13. Once the top objective is focused, reset the trap by reenabling the Auto gain and the gain boost, and opening the iris.
14. Lock the objectives

A.8 Analyzing .out Files from JPK Optical Trap

Materials:
Excel

Procedure:

1. Open the .out file in Excel. Your computer may ask you to choose a program with which to open the file. Choose Excel.
2. To separate the data into separate columns, save this file as a .txt file. Go to File, Save As, from the drop-down menu, choose Tab delimited Text and save. You may get an error message that says "some features in your workbook might be lost if you save as Tab delimited Text (.txt)." Just ignore for now and click OK.
3. Now open a new, blank Excel file. Then go to file, Import, and import the .txt file you just saved. A series of windows will show up.
4. Choose "Text File", click Import.
5. Choose "Delimited", click Next.
6. In the next window, since our data is separated by spaces, we should choose "Spaces" and click Next,
7. Click Finish.
8. Click OK.
9. Open a new Excel file, and copy and paste the time data into the first column. To highlight the data quickly, highlight the first cell of the time data. Then press command+shift+down at the same time. Then just command+c and command+v into the new Excel sheet.
10. The y data is in N, and we need it in pN. To make this change in units, multiply he y direction force cell by :1E12". Click enter, and then double click the cell to make the value propagate down the column.
11. Copy and paste the pN force data into the new Excel sheet. Copy the pN column you just made, right click the third column in the new Excel sheet, click "paste special", and then click "Values" and OK. If you just straight copy and paste, it pastes the formula, and we need the numbers that are already calculated.
12. Now you need to get position in nanometers. You can get this from the y stiffness value in the data file. The stiffness value is given in μN/m, but we need it in pN/mnm so that we can convert our force values in the new Excel sheet from pN to nm. So we need to divide the stiffness in the data file by 1000.
13. In the second column of the new Excel file, divide the force in pN by the stiffness in pN/nm to get position in nm. Double click the cell to make it propagate all the way down. Save the file as a .txt file.
    a. Now the .txt file has the time in seconds in the first column, position in nm in the second column, and force in pN in the third column.

14. Open Matlab, and make sure the Matlab code NR_dataVisualization.m and your .txt file are all “added to the path”.
15. Go to the command window and type in NR_dataVisualization and then press Enter.
16. You will now be prompted with a pop up window to import your data file. Find the data file with three columns you just made, and click Open.
17. The code will run now. It will plot the raw position vs. time and force vs. time as Figure 1 and Figure 2, respectively. Then it will filter the data and replot position vs. time and force vs. time as Figures 3 and 4, respectively.
18. If you have data that is shorter than 209 seconds or 1048576 lines, you may stop here. If you have data that is longer do the following steps.
19. Make a duplicate of your original .out file. It will be called *title*COPY.out
20. Open the file. Click Format – Make Rich Text.
21. Excel can only handle 1048576 lines. Delete the first portion of data from the .rtf file.
    a. Scroll down to 209.5 seconds. Highlight this row. Hold Command+Shift while pressing left arrow and then up arrow. Delete this highlighted portion.
22. Change the format back to a plain text file. Click Format – Make Plain Text.
23. Using this file, begin with step 3 from above to import to excel.
    a. If there is more than 418 seconds in the original .out file, you will have to repeat this process for every 209 seconds you recorded.