NEURONS UNDER TENSION: A STUDY OF VESICLE DYNAMICS

BY
WYLIE WEERA AHMED

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Mechanical Engineering
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2013

Urbana, Illinois

Doctoral Committee:

Professor Taher A. Saif, Chair and Director of Research
Assistant Professor Randy Ewoldt
Professor Rhanor Gillette
Associate Professor Sascha Hilgenfeldt
Abstract

Neurons are the basic communication element in the nervous system of nearly all animals. They communicate with other neurons and other types of cells via chemical and/or electrical signaling. For instance: Our thoughts are processed by a complex network of neurons in our brains; Our vision is mediated by light sensitive optical neurons in our eyes; We move our bodies by actuating muscles signaled by motor neurons; And we are able to sense using sensory neurons wired throughout our bodies.

Growing experimental evidence suggests that mechanical tension plays a significant role in determining the growth, guidance, and function of neurons. Recent developments in experimental techniques have made quantitative studies at the level of individual cells possible. The purpose of this dissertation is to introduce the growing field of cellular neuromechanics and present my Ph.D. research on the dynamics of vesicles in neurons under tension.

To understand the role of mechanics in neuron function, we must take a closer look at the underlying subcellular dynamics. The basic goal of this research is to explore the currently unknown relationship between applied mechanical strain and vesicle transport in neurons. Vesicle transport in neurons is a critical process since it is necessary for neurotransmission, growth, synaptic plasticity, and several other functions. Thus understanding how mechanical strain affects vesicle transport will shed light on its role in neuronal function (and dysfunction in neurodegenerative diseases). In this dissertation I will discuss: A method for applying mechanical strain to cells; A technique for analyzing nonequilibrium vesicle dynamics; And our main scientific findings that mechanical tension in neurons modulates synaptic vesicle clustering at the *Drosophila* neuromuscular junction and active transport of vesicles in *Aplysia* neurons.
To my family
Acknowledgments

First, I would like to express my sincere gratitude to Prof. Taher Saif for his continued support throughout this process. His scientific curiosity, infinite energy, and challenging questions were an unending source of inspiration. Also, his encouragement for further study of physics and the freedom to explore new research ideas was instrumental in my development. Prof. Saif has provided me with endless opportunities to develop myself scientifically and professionally, and for that I am deeply indebted to him. Without him, I would certainly not be in this position today.

Furthermore, I would like to thank my committee members, Prof. Randy Ewoldt, Prof. Rhanor Gillette, and Prof. Sascha Hilgenfeldt, for their guidance and advice on my research. Their insightful questions were always thought provoking, and often led to a new line of thought. I would like to thank all of the current and former members of the Saif Lab, Chiba Lab, and Sweedler Lab, for their support in completing experiments and discussing research ideas.

From abroad, I would like to thank Prof. Dr. Ralf Kemkemer and Prof. Dr. Erich Sackmann. Ralf’s mentorship during my visit at the Max Planck Institute for Intelligent Systems helped open my eyes to a wide-spectrum of science. His insight, patience, and generosity made the transition to interdisciplinary research fun and made Germany feel like a second home. My interactions with Erich have been few but tremendously fruitful. His patience and genuine curiosity are inspiring. Spending a few hours with him on shores of the Mediterranean Sea, in deep discussion of molecular biophysics, had a permanent effect on my personal interests and outlook on science.

My sincere thanks goes to Cameron Talisch. Our deeply intellectual and sometimes profoundly stupid conversations have shaped who I am. Pondering the meaning of life, and realizing that an overconstrained system may not have a solution... But mostly, I thank you for always giving good advice. My deepest thanks goes to Sofie Leon for her love, support, and encouragement. For teaching me how to be a human being, enjoy life, and making me a better person. But most of all, I thank you for patiently teaching me how to "write a program" with all those "ones and zeros".

My deepest gratitude are for my father, Pui, my mother, Veena, and my sister, Marie. Their unconditional love and encouragement have motivated me to constantly seek new challenges, improve myself, care for others, and contribute to society. To my parents, thank you for instilling in me the confidence to do anything, and indulging my ridiculous hobbies as a child. Both factors have shaped my character as well as my love for engineering and science. To my sister, thank you for teaching me to be adventurous, and to see the world as my oyster. You certainly lead by example.
"The predominant factors are no longer those of our scale; we have come to the edge of a world of which we have no experience, and where all our preconceptions must be recast." - D’Arcy Thompson, "On Growth and Form" (1917).
"We must therefore not be discouraged by the difficulty of interpreting life by the ordinary laws of physics. For that is just what is to be expected from the knowledge we have gained of the structure of living matter. We must be prepared to find a new type of physical law prevailing in it." - Erwin Schrödinger, "What is Life?" (1944).
"A poet once said, 'The whole universe is in a glass of wine.' We will probably never
know in what sense he meant it, for poets do not write to be understood. But it
is true that if we look at a glass of wine closely enough we see the entire universe.
There are the things of physics: the twisting liquid which evaporates depending on the
wind and weather, the reflection in the glass; and our imagination adds atoms. The
glass is a distillation of the earth’s rocks, and in its composition we see the secrets of
the universe’s age, and the evolution of stars. What strange array of chemicals are
in the wine? How did they come to be? There are the ferments, the enzymes, the
substrates, and the products. There in wine is found the great generalization; all life is
fermentation. Nobody can discover the chemistry of wine without discovering, as did
Louis Pasteur, the cause of much disease. How vivid is the claret, pressing its existence
into the consciousness that watches it! If our small minds, for some convenience, divide
this glass of wine, this universe, into parts – physics, biology, geology, astronomy,
psychology, and so on – remember that nature does not know it! So let us put it all
back together, not forgetting ultimately what it is for. Let it give us one more final
pleasure; drink it and forget it all!" - Richard Feynman, "Surely you’re joking, Mr.
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4.6 Velocity decreases upon unstretching. Anterograde motion is labeled (+) and retrograde motion is labeled (−). When stretch is applied, there is no significant difference in active or passive vesicle velocity from the control. However, upon unstretching (relaxation of tension) both active and passive vesicle velocity decreases significantly. * indicates statistical difference by Student’s t-test ($p < 0.05$) ($n > 16$ cells, > 100 vesicles per video, > 20 videos per cell) (error bars = SEM).

5.1 Active transport of vesicles in neurons is mediated by molecular motor activity. (a) Simplified schematic of vesicle transport in a neurite. Vesicles alternate between active transport along microtubules and passive brownian-like motion. Mechanical strain is used to modulate tension along neurite length while tracking vesicle dynamics. (b) Plot of a representative trajectory, where the tMSD algorithm is used to determine active ($\alpha \geq 1.4$) and passive motion ($\alpha < 1.4$). This example clearly shows the vesicle switching between active and passive behavior. (c) Probability of active motion of vesicles in control (red) neurons exhibit stable behavior with $P_{a,\text{control}} \approx 0.09$. Under stretch (blue), vesicles exhibit more active motion, which peaks at $P_{a,\text{stretch}} \approx 0.18$ after 25 min. The activity of small (×) vesicles remains high whereas large (□) vesicle activity decreases. Vesicles in compressed (green) neurons exhibit slightly decreased activity relative to the control (red). [control (red), stretch (blue), compress (red), all vesicles (○), large vesicles (□), small vesicles (×)].
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Chapter 1

Introduction

Summary1. Growing experimental evidence in recent decades suggests that the mechanical micro-environment plays a role in growth and guidance of neurons. Recent studies have shown that mechanical tension plays a role in neuronal function, including neurotransmission and synaptic vesicle clustering. In this chapter, we discuss the role of mechanical tension in determining the structure and function of neurons and its implication in learning and memory. To interpret the mechanical behavior of neurons a simple mechanics model is proposed based on force generation by molecular motors on cytoskeletal elements. The review concludes by highlighting some unanswered questions in cellular neuromechanics.

1.1 The neuron

In recent years it has become increasingly evident that mechanical stimuli play an important role in the differentiation, growth, development, and motility of cells [11, 12, 13, 14, 15, 16]. Cells sense and respond to cues from their mechanical microenvironment as well as externally applied mechanical stimuli. Most studies of cell mechanics have focused on cell types that are obviously subjected to mechanical forces during everyday function, such as: smooth muscle and endothelial cells, which experience strain as blood vessels expand and contract [17]; cardiac and skeletal muscle cells, which undergo contraction to pump blood and generate movement [18]; and epithelial cells throughout the body, such as in the lungs during breathing [19]. However, studies of mechanical forces during development and embryogenesis have highlighted their importance in all types of cells [20, 21, 22, 23, 24, 25].

Growing experimental evidence suggests that mechanical tension plays a significant role in determining the growth, guidance, and function of neurons [26, 27, 28, 29]. Recent developments in experimental techniques have made quantitative studies at the

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1Parts of this chapter have been previously published as, W. W. Ahmed, J. Rajagopalan, A. Tofangchi, T. A. Saif, "Neuromechanics: The role of tension in neuronal growth and memory", Nano and Cell Mechanics, 2012 (invited book chapter)
level of individual cells possible [30, 31, 32]. Here, we discuss cellular neuromechanics, which focuses on the mechanics of neurons at the cellular level and their function. The purpose of this introductory chapter is to present a brief review of some significant studies supporting the role of mechanical tension in neuronal growth and its implications in memory. We begin with an introduction to the structure and function of the neuron (Section 1.1). Then we discuss experimental measurements (in vitro and in vivo) of neuronal force and its role in structural development (Section 1.2). Next, we explore the role of tension in neuronal function, including neurotransmission and vesicle dynamics (Section 1.3). In Section 1.4 we discuss models of the mechanical behavior of axons to interpret their behavior. Finally, we conclude the chapter by highlighting some unanswered questions in cellular neuromechanics and defining the focus of the remaining parts of the dissertation (Section 1.5).

What is a neuron? Neurons are the basic communication element in the nervous system of nearly all animals. They communicate with other neurons and other types of cells via chemical and/or electrical signaling. For instance:

- Our thoughts are processed by a complex network of neurons in our brains;
- Our vision is mediated by light-sensitive optical neurons in our eyes;
- We move our bodies by actuating muscles signaled by motor neurons; and
- We are able to sense using sensory neurons wired throughout our bodies.

Many specialized types of neurons exist; however, most neurons share common biological structures [33]. The neuron is composed of four main parts: the soma (cell body), the dendrite, the axon, and the synapse, as shown in Figure 1.1. The soma of a neuron is very similar to that of other cells. The majority of the cell body is the nucleus, which holds the genetic information in the form of DNA. The cell body also contains the usual organelles, such as mitochondria, endoplasmic reticulum, ribosomes, the Golgi complex, and so on [34]. The dendrites of a neuron are a highly branched network of processes originating from the cell body. The branches contain many small protrusions, called dendritic spines, which function as locations for synaptic input from other cells. Through the dendritic network, a single neuron can receive signals from thousands of cells simultaneously. The axon is a long, thin, tube-like structure that originates from the soma and can extend for meters to terminate at a synapse. They usually do not branch along their length (except very near their terminals) and maintain an approximately constant diameter. Axons are able to rapidly transmit electrical
signals (action potentials) from soma to synapse and this process is crucial to neuronal signaling. Like most cells, neurons have an underlying support structure called the cytoskeleton consisting of actin, neurofilaments, and microtubules, as shown in Figure 1.2. Actin is an important structure known to regulate a variety of processes including the growing tips of axons, membrane shape, and dendritic spines [14, 35, 36, 37]. Neurofilaments are the least understood cytoskeletal element, but it is known that their organization is related to disease pathogenesis [38]. Microtubules function as support structures as well as tracks for molecular motor based transport of organelles and vesicles [39, 40]. Microtubules have a polar structure and their organization in neurons is specialized. Axons of vertebrate neurons exhibit “plus-end out” microtubules, meaning that the plus-end of the microtubule is pointing towards the distal end of the axon (synapse), and vertebrate dendrites usually have mixed polarity [41, 42].

The synapse is a highly specialized structure, which serves as a communication element between cells (Figure 1.2). It is one of the most important elements of a neuron and its structure and function have been the focus of cellular neurobiology. There are two kinds of synapses: chemical and electrical. Here we will focus on chemical synapses because they are the most well-studied. Chemical synapses are composed of distinct presynaptic and postsynaptic sides that are separated by a 20–30 nm gap called the synaptic cleft. The presynaptic terminal is the swollen axon terminal that contains neurotransmitter vesicles to be released to elicit a change in the postsynaptic cell.
Figure 1.2: The subcellular structure of a neuron is composed of actin, microtubules, and neurofilaments (not shown). Microtubules serve as structural support and as the main tracks for molecular-motor-based transport of organelles and vesicles. Actin serves as structural support in advancing growth cones, as well as being involved in signaling processes, including tethering synaptic vesicles (SV) at the presynaptic terminal for neurotransmission. Synaptic vesicles are released from the presynaptic terminal and activate receptors on the postsynaptic cell for signal transmission.
Understanding synaptic function is a key goal of modern neuroscience. Malfunctioning synapses are associated with a large number of debilitating diseases, such as Parkinson’s disease, depression, and schizophrenia [43]. It is widely believed that changes in synaptic properties are a key factor in determining the plasticity of the nervous system and the processes of learning and memory [33, 44, 45, 46].

**How does a neuron function?** The primary function of a neuron is to receive, integrate, and transmit signals. Supporting this function are the processes of neuronal transport, electrical signaling, and synaptic plasticity.

Neuronal transport involves the directed movement of organelles and vesicles throughout the intracellular space of the neuron. Since an axon can be over a meter in length, a protein or molecule produced in the cell body would require several days to diffuse to the synapse. To distribute components along axons and dendrites, neurons use a system of directed transport, which utilizes molecular motors to transport cargo along microtubules. Molecular motors are a large family of motor proteins that use adenosine tri-phosphate (ATP) as an energy source to drive movement. The molecular motors that function as transporters on cytoskeletal elements are myosin, kinesin, and dynein. Myosin interacts with actin filaments to allow short-range transport and contractile force generation [47, 48, 49, 50]. Kinesin and dynein are the primary molecular motors involved in neuronal transport and function by moving along microtubule structures in a polarity-dependent fashion [39, 51, 52, 53]. Kinesin moves towards the plus-end of the microtubule and dynein moves towards the minus-end. Since the plus-ends of the microtubules point towards the distal end of the axon, kinesin transports molecules towards the synapse and dynein towards the cell body. Therefore, different types of cargoes, such as organelles, proteins, neurotransmitter vesicles, and so on, can be transported throughout the cell by associating with the appropriate motor.

One function that is crucial to the process of neurotransmission is the transport of vesicles containing neurotransmitters to the synapse, where they cluster at regions called active zones. These vesicles are called synaptic vesicles since they cluster at, and are released from, the presynaptic terminal. Synaptic vesicles are transported back and forth along the axon to maintain a steady pool of neurotransmitters ready to be released to communicate with the postsynaptic cell [54, 55].

Neurons rapidly send messages down their axons. Like all other cells, the neuron is enclosed by a plasma membrane, which is an electrically insulating barrier that separates the inside of the neuron from the extracellular space. This plasma membrane prevents passive diffusion of charged ions in and out of the cell and maintains a potential
difference across the membrane, which is important for signaling. Ion channels in the membrane have selective permeability to different ions, and this results in an unequal distribution of electrical charge (carried by ions) across the membrane. The primary ions that determine the membrane potential are K\(^+\), Na\(^+\), Cl\(^-\), and organic ions (A\(^-\)). Of these ions, Na\(^+\) and Cl\(^-\) are more concentrated outside the cell and K\(^+\) and A\(^-\) are more concentrated inside. The membrane potential of a neuron at rest is dominated by K\(^+\) channels, and thus the resting potential can be calculated to be \(-70\) mV [46]. When a nerve cell is at rest, the steady influx of Na\(^+\) is balanced by the steady efflux of K\(^+\), so that the membrane potential is constant.

During signal transmission, a depolarizing stimulus above a certain threshold causes large and rapid change in membrane potential, resulting in the propagation of an action potential down the axon from the cell body to the axon terminal. The disturbance in membrane potential rapidly opens voltage-gated Na\(^+\) channels, which increases membrane permeability to Na\(^+\) and leads to a net influx of positive charge. As the positive charge increases, more Na\(^+\) channels open and this positive feedback system quickly drives the system towards the Na\(^+\) equilibrium potential of +55 mV (the peak of the action potential). The action potential is terminated by inactivation of Na\(^+\) channels and the opening of voltage-gated K\(^+\), leading to efflux of K\(^+\). Thus, since voltage-gated channels of Na\(^+\) open faster than K\(^+\) channels do, a depolarization of the cell results in rapid depolarization and repolarization process known as the action potential [46]. The action potential is considered an “all-or-none” event, which means that the depolarizing stimulus must be above a certain threshold to elicit a response. However, the amplitude of the action potential is fixed and does not reflect the amplitude of the depolarizing stimuli.

The transmission of an action potential along an axon results in many processes, including the release of neurotransmitters at the synapse. When the neuronal membrane becomes depolarized, cytosolic Ca\(^{2+}\) levels increase due to influx through voltage-gated ion channels. The increased level of intracellular Ca\(^{2+}\) activates exocytosis of vesicles, which results in neurotransmitter release from the presynaptic terminal to the postsynaptic cell [56]. Additionally, action potentials can induce release of neuropeptides that diffuse into the extracellular environment for long-range communication with distant cells [57].

Synaptic plasticity is defined loosely as synaptic change as a function of activity. This synaptic change results in modulation of synaptic efficiency in terms of neurotransmission and is believed to be the basis of learning and memory [44]. When an organism develops, its genetic map ensures that there are fixed networks of neurons with (rela-
tively) invariant connections. Although the physical connections are predetermined, the precise strength of the synapses is not specified genetically. Rather, experiences tune the strength and effectiveness of the synapses [58]. One instance of synaptic plasticity is observed when the amount of neurotransmitter release in response to electrical stimulation changes due to activity. An example is when a neuron is stimulated, an action potential is elicited that leads to exocytic vesicle fusion and neurotransmitter release. A repetitive sequence of stimulation may result in increased sensitivity (potentiation) of the synapse; that is, the synapse “remembers” its usage in the past. As a result, the synapse can release more neurotransmitters in response to the same stimulus. And vice versa: if the neuron becomes less sensitive (depression), then the number of neurotransmitters released will decrease. This seemingly simple change in sensitivity or the strength, known as synaptic plasticity, is mediated by changes in structural, biochemical, and electrical changes of the synapse [59, 60, 61, 62, 63]. The concept of synaptic plasticity, originally proposed by Ramon y Cajal during the early twentieth century [46], forms the basis of memory and learning, and hence is a hallmark of neuroscience. It involves the clustering of neurotransmitter vesicles at the presynaptic terminal, without which neurotransmission or change of synaptic strength would not be possible.

How does a neuron grow? Neurons are unique, since, unlike most cells, terminally differentiated neurons do not divide. Instead, many neurons will increase their size dramatically to accommodate development and growth of an organism. Axonal growth can be simplified into two stages: pathfinding and towed growth (Figure 1.3). During development, a neuron must extend an axon to reach its synaptic target, and this process is known as pathfinding. The axon terminal exhibits an enlarged and spread-out morphology, called the growth cone, which explores the extracellular environment in search for guidance cues to reach its postsynaptic partner [27]. As neuronal growth cones navigate, they apply traction forces to the underlying substrate to generate motion [64]. These traction forces applied by the growth cone generate a tension along the axon, which results in growth and elongation. By this method, the growth cone continuously elongates the axon by pulling until it reaches its final destination. Once the growth cone has reached its target, it structurally and biochemically differentiates into a synapse. After a synapse has formed (synaptogenesis), the axon is anchored between the cell body and the presynaptic terminal. As the embryo develops further, and increases its size, the synapse is stretched or towed outward, and the axon undergoes towed growth to accommodate expansion of the organism.
Figure 1.3: Neuronal growth can be divided into two stages. (1) Pathfinding occurs when the growth cone of a growing axon explores the extracellular environment in search for its postsynaptic partner. During this stage the growth cone applies traction forces to the underlying substrate to generate tension along the axon and stimulate elongation and growth. (2) Towed growth occurs after a synapse has formed (post-synaptogenesis), and the axon is stretched to accommodate expansion of the embryo during growth.

This dependence of extension on tension provides a plausible mechanism to regulate neuronal growth during development [8, 65]. For example, if tension does not induce growth, then, as the organism grows, tension in the axon will continue to increase or the axon would thin significantly due to viscoelastic extension. Thus, it is reasonable to hypothesize that axons grow in response to tension; and in order to avoid uncontrolled growth, there must be a threshold above which growth is initiated. Similarly, axons may have a lower force threshold, below which axonal tension is generated. This lower force threshold would give rise to a rest tension in axons. These upper and lower force thresholds may regulate axonal elongation during growth of an organism and axonal retraction during specification of neural networks, respectively. In between the lower and upper thresholds there may exist a regime of passive axonal behavior. This regime could provide a buffer between stimulating force generation and relaxation in the axon induced by normal movement. Later, it will be highlighted that such a tension might also be essential for clustering of neurotransmitter vesicles at the synapse, and hence for their core functionality.

1.2 Tension in neuronal growth

The concept of mechanical tension in neuronal growth is not new. Qualitative observations were reported in 1945, when Weiss observed that nerve tissue advancing along
grooves generated tension and distorted the nerve explants [66]. Decades later, Bray showed that growth cones of cultured neurons exert mechanical tension and that there was a correlation between the tension in a neurite and its diameter [67]. More importantly, these experiments showed that the direction of growth cone advance (and hence neurite growth) was dictated mainly by the tension existing in the neurite. This suggested that mechanical tension, and not the growth cone itself, was the primary determinant of neurite growth [67]. A subsequent study by Bray [68] showed that externally applied tension can indeed both initiate new neurites (from rounded cell bodies) and elongate existing neurites. Furthermore, the ultrastructure of the neurites initiated by external tension was identical to spontaneously generated (growth-cone-mediated) neurites, with abundant longitudinally aligned microtubules and neurofilaments. This observation was also confirmed in a later study by Zheng et al., who showed that tension-initiated neurites exhibit rapid and normal microtubule assembly [69].

Based on these observations it was postulated that tension was a primary signal for neuronal growth during normal development, with the tension being supplied by the locomotory activity of the growth cone before synaptogenesis and later by the movement of the innervated tissues in a growing organism. While it was fairly obvious that the moving tissues would exert tension on the neurites, the question as to whether growth cones applied a similar pulling force on neurites was unclear. This question was clarified by Lamoureux et al. [64], who showed that an increase in neurite tension was directly correlated to growth cone advance and apparent neurite growth in vitro. In contrast, no increase in tension was seen in neurites with stationary growth cones. These studies established that mechanical tension is essential for neurite initiation and growth in vitro and, quite likely, in vivo as well.

**In-vitro measurements of tension in neurons.** The first quantitative measurements of tension in cultured neurites were performed by Dennerll et al. [1], who examined the mechanical properties of PC-12 neurites. They applied force on the neurites with calibrated glass needles and measured their force-displacement characteristics. Their measurements showed a linear force-displacement relationship for small, rapid distensions and the presence of a positive rest tension (300–400 pN) in the neurites (Figure 1.4). In addition, their experiments revealed a complementary force interaction, with microtubules under compression resisting the tension exerted by the actin network. This interaction appeared to dictate neurite shape and size: an increase in the compressive load on microtubules led to their depolymerization and consequent retraction of the neurite. Along similar lines, it was suggested that a reduction in compressive
load on microtubules (by shifting of tension to the substrate by an advancing growth cone) could lead to their assembly and, hence, neurite elongation.

In a subsequent study using similar techniques, Dennerll et al. uncovered three distinct phases of response to tension in PC-12 neurites and chick sensory neurons [8]. When the tension in the neurites was reduced below a lower threshold they actively contracted to restore tension, sometimes to a value above their resting tension. It has been suggested that the ability to actively develop tension and contract could provide a potential mechanism for in vivo axonal retraction from neuromuscular junctions [70, 71, 72], which results in a pattern of innervation where only one motor neuron synapses with each skeletal muscle fiber. On the other hand, when the applied tension exceeded an upper threshold (1 nN), the neurites grew in response, with the growth rate being proportional to the applied tension. For intermediate values of applied tension the
neurites behaved as passive viscoelastic solids; that is, an initial elastic response followed by force relaxation to a steady-state value.

Following the work of Dennerll et al., Zheng et al. examined the relationship between applied tension and neuronal growth rates in greater detail [2]. They applied force on chick sensory neurons in increasing steps, holding the force fixed for 30–60 min in each step. They found a linear relationship between applied force (in excess of a tension threshold) and growth rate (Figure 1.4) and a surprisingly high sensitivity (150 μm h⁻¹ nN⁻¹) of growth rate to tension. They reasoned that the linear relationship between growth rate and tension provides a simple control mechanism for axons to accommodate tissue expansion in growing animals that consistently maintain a moderate rest tension on axons. Furthermore, the force required to initiate new neurites was found to be similar to the tension threshold for elongating neurites, suggesting that the basic mechanism underlying both initiation and elongation of neurites was similar.

**In-vivo measurements of tension in neurons.** As described previously, numerous studies have revealed the important role of mechanical tension in the initiation, development, elongation, and retraction of neurites in vitro. A similar role has been long suggested for mechanical forces in vivo. For example, Van Essen [73] hypothesized that tension in axons may underlie many aspects of morphogenesis of the brain, especially of the cortical regions of the brain. As a case in point, it was suggested that the folding of the cerebral cortex is due to the tension exerted by axons that connect relatively distant regions of the brain and that the folding minimizes the communication time between interconnected brain regions. While experiments examining the in vivo mechanical behavior of neurons have been scarce, two recent studies have provided evidence of the role of forces in neuronal development and function in vivo.

Experiments by Siechen et al. [28] (discussed in greater detail in Section 1.3) have shown that vesicle clustering in the presynaptic terminal of the neuromuscular junction in *Drosophila* embryos is dependent on mechanical tension in the axons. That study also provided preliminary evidence for the presence of a rest tension and viscoelastic behavior in *Drosophila* axons.

Following this work, Rajagopalan et al. examined the in vivo mechanical response of *Drosophila* neurons using high-resolution micromechanical force sensors (Figure 1.5) [3]. They found that:

1. *Drosophila* axons have a rest tension in the range 1–13 nN.

2. In response to fast deformation, axons behave like elastic springs, showing a linear
Figure 1.5: In vivo measurements of force in neurons. (a) Force–deformation response of an axon during fast stretch showing a linear response. Note the presence of a rest tension (∼ 2 nN) at zero elongation. (b) Force relaxation over time for the axon shown in (a). The force in the axon decays exponentially to a steady state over approximately 20 min. (c) Force buildup after unloading for axon shown in (a). The force increases exponentially to a value close to the initial rest tension over approximately 20 min. (d) Elongation of the axon during force decay shown in (b). Adapted from [3].
Figure 1.6: Stiffening of in vivo axons. (a) Ratio of the stiffness of the axons during the first and second loadings plotted as a function of their force relaxation after the first loading. Axons that show a large force relaxation show diminished stiffness during the second loading, and vice versa. (b) Force–deformation response for the first and second loadings of an axon that underwent a large force relaxation after the first loading. Adapted from [3]

force–deformation response, which is followed by force relaxation to a steady-state value after 15–30 min.

3. When the applied deformation is sufficiently large, the axons adopt a slack appearance upon force removal. However, the axons tauten and build up the tension, often to a level close to their rest tension, in a period of 15–60 min. In other words, the axons actively generate force and contract to restore tension.

These observations of in vivo neuronal mechanical behavior were remarkably similar to those from in vitro studies, suggesting that mechanical forces could prominently influence neuronal growth and function in vivo. Furthermore, Rajagopalan et al. [3] uncovered two other interesting facets of axonal response to applied tension. The axons that underwent large force relaxation (80–90%) became considerably longer and thinner during the process. More remarkably, axons that showed low relaxation (30% or less) became stronger (with their stiffness doubling in some cases) even though their length increased during force relaxation (Figure 1.6).

Both these observations, however, are consistent with the observations of Lamoureux et al., who showed that in vitro axonal growth proceeds through a combination of steps: lengthening by viscoelastic stretching and intercalated addition of material [74]. In their study, during viscoelastic stretching, a noticeable thinning of the axons was observed, but the axons eventually regained their thickness through material addition. On the other hand, material addition to the axon (and an increase in axon diameter) was also found to precede lengthening in both spontaneously growing (growth-cone-mediated)
and towed axons. If this increase in axonal diameter is sufficiently large then the axon can become stiffer in spite of an increase in length as seen in the in vivo study of Rajagopalan et al. [3]. In other words, not only are the mechanical responses of axons in vivo and in vitro remarkably similar, but the mode through which tension induces axonal growth also appears identical. Thus, the force response and sensitivity seem to be highly conserved properties of neurons.

In this context, it is worth noting the observations of Pfister et al., who found that integrated axon tracts could be stretched up to 8 mm per day to lengths over 10 cm without disruption [75]. During this elongation process the axons increased in diameter by 35% and maintained normal density of cytoskeletal structures and organelles, and also generated normal action potentials [76]. For a more comprehensive review of the role of force in axonal elongation, we suggest a recent article by Suter and Miller [77].

The role of tension in structural development. As discussed in the previous sections, neurons actively regulate their tension both in vivo and in vitro and tension stimulates both the initiation and elongation of neurites. But in addition to regulating neurite initiation, mechanical tension has been shown to specify axonal fate. While a neuron can have many neurites emanating from the cell body, usually only one of them becomes an axon. Previous work on axonal specification had suggested that neurites need to attain a critical length before showing axonal character [78, 79], implying that neurite length was the major determinant of axonal fate. However, Lamoureux et al. showed that by applying mechanical tension one can induce axonal characteristics even in minor neurites [4]. In other words, neurites under external tension would express axonal characteristics even when their length was lower than sibling neurites. Figure 1.7 shows a minor neurite being initiated by external tension from a neuron with many existing neurites. After external tension is removed, the neurite continues to elongate and expresses axonal characteristics, as shown by the fluorescent staining. This suggests that tension, rather than length, specifies axonal specification.

Apart from its role in the initiation and growth of neurites, tension appears to be capable of influencing the development of the neural system in two other important ways. The first concerns growth-cone guidance, the process through which developing axons find their synaptic targets. As mentioned earlier, axonal elongation in early development (before synaptogenesis) is mediated by growth-cone-generated tension. But tension also appears to influence growth-cone pathfinding. A simple and elegant study by Suter et al. showed that the direction of growth-cone pathfinding can be altered by applied tension [5]. They attached beads to cell surface receptors and showed that, by
Figure 1.7: Neurite initiation and axonal specification. (a) A neuron with several long processes before manipulation. (b) A neurite is initiated by mechanical pulling with a glass needle (arrowhead). (c) The glass needle is removed. (d, e) The neurite initiated by mechanical tension continues to elongate. (f) The neurite was fixed and stained to show that only the neurite initiated by mechanical tension expresses axonal proteins. Scale bar = 20 µm. Adapted from [4]
restraining bead motion, growth cones would generate tension in the direction of the bead, followed by cytoskeletal polymerization and growth-cone advance. Thus, tension could determine the direction of cytoskeletal polymerization and subsequent growth-cone pathfinding. Figure 1.8 shows time-lapse images of actin and microtubules responding to tension generated by bead manipulation. The second way in which tension could influence neural development is through the elimination of unnecessary axonal branches. As growth cones extend during the pathfinding phase, branches are often extended and retracted to explore the extracellular environment. Anava et al. discovered that tension along a growing axon promotes stabilization of one set of axon branches while causing retraction or elimination of collaterals [80]. Thus, by the elimination of unnecessary axon collaterals, tension could influence the wiring of the neural network.

As an aside, it is worth noting that several other mechanical cues also influence neuronal development. For example, neurons show significantly higher neurite branching activity on softer polyacrylamide substrates that mimic the stiffness of brain tissue rather than stiff cell culture surfaces [81]. Growing neurons also use substrate stiffness as a guidance cue to avoid stiff substrates [82], a possible mechanism to avoid stiff scar tissue that develops as a result of injuries.

1.3 Tension in neuronal function

It is clear that mechanical tension exists and is actively regulated in neurons both in vivo and in vitro. Now, the question that arises is: What is the purpose of mechanical tension in neurons? The following discussion highlights the role of tension in neurotransmission and vesicle dynamics.

**Tension increases neurotransmission.** Uncovering the role of mechanical tension in neuronal signaling is a topic in its infancy, despite the fact that the first observations were reported 60 years ago. In 1951, Fatt and Katz reported that stretching a muscle (and presumably its motor neurons) by 10–15% beyond its resting length resulted in a reversible increase of 2.5–3 times in the rate of spontaneous electrical potentials at the muscle endplate [83].

A later study conducted by Chen and Grinnell showed that stretching a whole frog muscle could more than double the release of neurotransmitters from motor nerve terminals [26]. They also showed that the stretch enhancement of neurotransmitter release was mediated by integrin, a well-known mechanosensor. Interestingly, they found that the stretch enhancement of neurotransmission bypassed the usual Ca\(^{2+}\) triggering step.
Figure 1.8: Tension can change the direction of growth-cone advance and cytoskeletal polymerization. (a, b) A glass pipette was used to restrain a bead attached to the cell surface and to generate a force on the growth cone. (c–e) Fluorescent staining shows actin accumulation around the bead as well as microtubule polymerization in the direction of the force. (f) Sequence of images showing the microtubule extension in the direction of force (line indicates the initial position of the needle). (g) Velocity of the extending central domain of microtubules towards the glass needle plotted as a function of time. Adapted from [5]
in vesicle fusion [84, 6]. Vesicle fusion is usually triggered by an influx of extracellular Ca\(^{2+}\) ions through stretch-activated ion channels. Their experiments showed that stretch enhancement of neurotransmission is reduced, but still occurred in the absence of extracellular Ca\(^{2+}\) influx and even when internal stores of Ca\(^{2+}\) were buffered. These results suggest that Ca\(^{2+}\) plays a role in determining the amount of stretch enhancement but that it is not necessary for it to occur. Based on these findings, Chen and Grinnell concluded that second messenger pathways or chemical modification are not plausible mechanisms for stretch-enhanced neurotransmission [84]. Figure 1.9 shows the increased electrical firing frequency as a function of muscle stretch. In experiments with Drosophila, Grinnell et al. [6] showed that hypertonic swelling also induced enhancement of neurotransmission, which was strongly dependent on the amount of cAMP/PKA activity. They hypothesized that the cAMP/PKA cascade regulates the size of the vesicle pool available for release, and thus determines the amount of enhanced neurotransmission [6]. These studies suggest that mechanical tension can modulate neurotransmission on time scales of seconds.
**Tension affects vesicle dynamics.** Since tension is involved in modulating neurotransmission, it must also affect vesicle dynamics. A recent study showed that synaptic vesicle clustering is dependent on mechanical tension within the axon [28]. Siechen et al. found that vesicles failed to cluster when tension was removed by axotomy. However, if tension is reapplied to the severed axon, vesicle clustering was restored. In addition, they showed that axons have a rest tension and that if this tension is perturbed then axons respond to maintain tension. They showed that if axonal tension is increased by external stretching, then the amount of synaptic vesicle accumulation at the presynaptic terminal increases by more than two times [28]. And as hypothesized by Grinnell et al. [6], this could lead to increased amount of neurotransmission. The study by Siechen et al. [28] suggests that mechanical tension is a necessary regulatory signal of presynaptic vesicle clustering, and thus is involved in the tuning of synaptic efficiency (i.e., synaptic plasticity).

To investigate the time evolution of tension-induced vesicle accumulation in vivo, Ahmed et al. stretched axons while observing by live imaging [7]. They found that vesicle accumulation increased by approximately 30% after approximately 5 min of mechanical stretch, as shown in Figure 1.10. This stretch enhancement of vesicle accumulation remained for at least 30 min after stretch was released (maximum time of experiment), indicating a persistent change. Experiments by Ahmed et al. [7] were conducted in Ca$^{2+}$-free medium, suggesting that influx of external Ca$^{2+}$ is not necessary for stretch enhancement of synaptic vesicle accumulation. In comparison with Siechen et al. [28], the stretch-enhanced vesicle accumulation was attenuated in both time and amount, which may be explained by the absence of extracellular Ca$^{2+}$ influx, as observed by Grinnell et al. [6]. Ahmed et al. [7] also found that vesicle accumulation is not affected when tension is removed from an intact axon. Since it is known that relaxed axons will rebuild their tension quickly [3], Ahmed et al. hypothesized that this rebuilding of tension does not allow vesicle accumulation to decrease [7]. This may be a protective mechanism to maintain vesicle clustering at synapses. These studies show that mechanical tension can modulate synaptic vesicle accumulation on time scales of minutes to hours.

If mechanical tension modulates vesicle clustering, then it must also affect local vesicle dynamics. Ahmed et al. found that reducing the mechanical tension in *Aplysia* neurites resulted in a significant decrease of vesicle motion [7]. Figure 1.11 shows that the range of vesicle motion and processivity decreases dramatically when neurite tension is decreased. This suggests that mechanical tension may also be necessary for normal vesicle motion. The results in Ahmed et al. [7] are discussed in detail in Chapter 3.
Figure 1.10: Stretch-induced accumulation of synaptic vesicles. (a) A control axon is shown on the PDMS surface. The inset on the right shows a magnified image of the presynaptic terminal where a 2.5 μm square region was used to quantify synaptic vesicle accumulation. (b) An axon is shown stretched by substrate deformation, notice the straightness of the axon (increased tension). (c) An axon is shown compressed by substrate deformation; notice the axon is squiggly (decreased tension) (scale bar: 5 μm). The plot shows the fluorescence intensity of GFP-tagged synaptic vesicles at the presynaptic terminal of the Drosophila neuromuscular junction as a function of time. Control samples show no significant change in synaptic vesicle accumulation. When axons are stretched, increased accumulation is observed after approximately 50 min and the effect persists for at least 30 min after stretch is removed. In compressed axons, no significant change occurs in accumulation during compression or after it is removed [7].
Figure 1.11: Decreased neurite tension disrupts vesicle transport in *Aplysia* neurons. (a) A representative plot showing the range and largest processive motion of a single vesicle. (b) Vesicle range of motion was approximately 550 nm in the control samples. Mechanical compression caused an immediate decrease to 200 nm and the range continued to decrease to 140 nm. (c) In control samples the largest processive motion of vesicles was approximately 380 nm. This decreased to 140 nm immediately after compression and continued to decrease to 80 nm. In both cases, the effect persists for over 15 min after compression was removed [7].
Figure 1.12: Tension is known to increase actin polymerization. Increased actin polymerization may lead to increased synaptic vesicle accumulation due to a larger number of actin–synapsin binding sites.
When cells are stretched, it is known that actin polymerization increases [85, 86]. This increase in actin polymerization due to stretching may also increase the amount of actin at the synapse, which could increase the number of actin–synapsin binding sites [61, 60]. Therefore, assuming that axonal transport properties do not change, then, based on a higher number of binding sites for synaptic vesicles, the vesicle accumulation at the synapse could increase (Figure 1.12). This conceptual framework suggests that mechanical tension could play a role in synaptic plasticity and ultimately in learning and memory.

The emergent view is that axons actively maintain a rest tension, and such tension is essential to maintain vesicle transport and clustering at the synaptic terminal [28, 7]. Synaptic vesicle accumulation is necessary for neurotransmitter release at a functional synapse [46]. Therefore, it seems natural to conclude that axonal tension is critical in maintaining neuronal function.

1.4 Mechanical behavior of axons

It is clear from the above that in vitro neurons in culture show the following mechanical characteristics:

1. Axons actively maintain mechanical tension.

2. Axons behave as linear springs under fast stretch. Under a fixed stretch, the increased tension decays with time to a steady value.

3. When the tension is relaxed mechanically, axons shorten to rebuild the tension.

4. Axons can grow in response to mechanical stretch, which may serve as a mechanism of force relaxation under fixed stretch.

Dennerll et al. [8] postulated that there are two force thresholds for axons. When the upper threshold is exceeded due to stretching (mechanically or during development), the axon grows in length by synthesizing cytoskeletal structure. If the stretch is held fixed, this growth relaxes the tension. On the other hand, if the tension falls below the lower threshold, axons shorten by undoing the growth of the current cytoskeleton. In between the two thresholds, axons behave as a viscoelastic material. Thus, a mechanics model of the axon is represented as a combination of a “growth dashpot” (Figure 1.13) and a classical viscoelastic element. The growth dashpot is linear, in the sense that the rate of growth is linearly dependent on the applied tension above the upper threshold, as observed in experiments with chick sensory neurons [2]. The spring constants of the
linear springs and the time constants of the dashpots of the model can be estimated from experiments.

It is hypothesized that molecular motors serve as force actuators in axons. A mechanical model that represents the motors and viscoelasticity of neurites was proposed by Bernal et al. [9] (Figure 1.14). Here, molecular motors are attached to parallel fibers (such as F-actin). The motors slide the fibers with respect to each other. If the fibers are anchored at both ends, then tensile force develops within the fibers, which results in a net tensile force in the neurite. The model does not require any remodeling of the cytoskeleton, either under stretch or during shortening.

Force evolution in a network of neurites of a single neuron was presented in a seminal paper by Bray [67]. Here, each neurite, mostly on a straight line, is considered as a unit vector pointing towards the tip of the neurite. The neurites orient themselves such that their vector sum is close to zero. This implies that the neurites possibly generate similar forces, and their evolution (growth, morphology, substrate attachment) is determined by force equilibrium. If this equilibrium is perturbed (e.g., by mechanical cutting of a neurite), then the network responds by reorienting and generating new neurites that also develop tension and maintain equilibrium. When a neurite branches into two new neurites, then the forces $T$ in the parent neurite and those in the branches ($t_1$ and $t_2$) are related by a power-law relation: $T^n = t_1^n + t_2^n$, where $n = 1.6 \pm 0.09$. 

![Figure 1.13: Mechanical model of neurite behavior with growth dashpot. The axon is represented as a combination of a “growth dashpot” and a classical viscoelastic element. The “growth dashpot” allows the axon to elongate when the force is above an upper threshold and shorten when the force is under a lower threshold. Adapted from [8]](image-url)
Studies on the mechanical behavior of in vivo neurons are limited. The only report, to the best of our knowledge (described above [3]) shows that motor neurons that have formed neuromuscular junctions (i.e., post synaptogenesis) in embryonic fruit flies behave very similarly to those of in-vitro-cultured neurites. The significant features are: (a) force response of axons is linear with fast stretches; (b) axons maintain a rest tension actively. When stretched rapidly and the stretch is held fixed, axons relax tension to a steady value with time. When axon tension is released mechanically by bringing the neuromuscular junction closer to the central nervous system, the slack axons shorten with time linearly and regain tension in minutes. (c) Stiffness or the spring constant of the axons along the longitudinal direction changes after an applied stretch and after the tension relaxes to a steady value. When considering many experiments, for small relaxation (less than 30% of the force immediately after stretch), axon stiffness increases compared with the initial (unstretched) value. Stiffness progressively decreases with increasing force relaxation. When the relaxation is large (>80%), the stiffness is significantly lower than the initial value (Figure 1.6).

In order to interpret the above observations, we propose a simple mechanics model based on the hypothesis that axons have cytoskeletal fibers (actin, microtubule, and potentially other filaments), pairs of which are bridged by motor proteins. Various other proteins, such as tau, also bridge the fibers. The motors slide the fibers with respect to one another. When the fibers are restrained from motion (e.g., due to attachment with a substrate such as the neuromuscular junction), tensile force develops. As a result, tension generates in the axons macroscopically.

Consider such a pair of fibers that are pointing towards opposite directions. A motor is bridging the two. The heads of the motor can “walk” along either directions of the fibers by consuming ATP, but the activation barrier for taking a step along one direction
is higher than in the other direction. Hence, it walks more frequently along the easier
direction. Let $E_a \pm F_0 d_0$ be the activation barriers for walking along the harder and
easier directions. Here, $d_0$ is the activation distance, $F_0$ is a characteristic force, often
called the stall force [87]. $\pm F_0 d_0$ results in asymmetry in the energy landscape. When
the fibers are free from any restraints, the motor keeps walking by consuming ATP, and
the constant average velocity of the fibers with respect to each other is,

$$
\dot{\gamma}_{sl} = \gamma_0 \left\{- \exp \left[ \frac{-(E_a - F_0 d_0)}{k_B T} \right] + \left[ \frac{-(E_a + F_0 d_0)}{k_B T} \right] \right\}
$$

(1.1)

where $\dot{\gamma} = \frac{d\gamma}{dt}$, $\gamma_0$ is a constant, and $k_B T$ is the thermal energy in the system. Note that
$\dot{\gamma}_{sl}$ is positive towards the right in Figure 1.15. If a force $F$ is applied at the ends of
the fibers as shown in Figure 1.15, then the energy landscape will change. For $F < F_0$,
sliding continues to be in the same direction as when $F = 0$. When $F > F_0$, the motion
is reversed. In the presence of $F$, the rate of change of displacement of the fibers with
respect to each other is given by

$$
\dot{\gamma}_{sl} = \gamma_0 \left\{- \exp \left[ \frac{-(E_a - F_0 d_0 + F d_0)}{k_B T} \right] + \left[ \frac{-(E_a + F_0 d_0 - F d_0)}{k_B T} \right] \right\}
$$

(1.2)

The displacement of the fibers with respect to each other has two components: an
elastic component $\gamma_1 = F/G$, which accounts for the elastic deformation of the fibers
and the motor due to $F$ and the spring constant $G$, and a sliding component $\gamma_{sl}$. Thus,
the net rate of displacement at the fiber ends $\dot{\gamma}$ is

$$
\dot{\gamma} = \frac{\dot{F}}{G} + \dot{\gamma}_{sl}
$$

(1.3)

Now consider a special case where the ends of the freely sliding fibers are suddenly
brought to a stop by holding them at time $t = 0$. Then, $\dot{\gamma}$ is zero for all times $t > 0$; that is:

$$
\dot{\gamma} = \frac{\dot{F}}{G} + \dot{\gamma}_{sl} = 0
$$

(1.4)

Owing to the anchorage at the ends (the ends are held fixed), the constraining force $F$
increases and the energy landscape changes until the activation barriers for the motor
for its forward and backward steps become equal at $F = F_0$. From this point onwards,
$F = F_0$ remains steady.

In the second case, let the fibers be pulled suddenly by a force $F = F_* > F_0$ at
time $t = 0$. The ends of the fibers are held fixed thereafter. Corresponding elastic
Figure 1.15: Mechanical model for a molecular motor bridging two fibers. (a) The molecular motor is biased to walk in one direction more often than the other. The activation barriers for walking along either direction is $E_a \pm F_0d_0$, where $F_0$ is the characteristic stall force and $d_0$ is the activation distance. Thus, $\pm F_0d_0$ results in asymmetry in the energy landscape. (b) When a force is applied, the energy landscape changes accordingly and may change the direction of sliding. For $F < F_0$ the sliding continues to be in the direction opposite of $F$ and when $F > F_0$ the motion is reversed.
displacement of the ends is \( F_s/G \). Owing to the high force, the energy landscape for the motor becomes skewed, such that walking backwards becomes easier than walking forward. Thus, the fibers slide in the reverse direction compared with the case of \( F = 0 \). Thus, the force on the fibers relaxes with time until the force reaches a steady value \( F = F_0 \).

For \( \frac{|F_0 - F|d_0}{k_B T} \ll 1 \), equation 1.4 can be linearized, retaining only the first-order terms:

\[
\dot{F} + \frac{2G\gamma_0 \exp(-E_a/k_B T)}{k_B T} (F - F_0) = 0
\]  
(1.5)

Consider two initial conditions: first, when ends of the fibers are held fixed from \( t = 0 \) (i.e., \( F(t = 0) = 0 \)); second, when the ends of the fibers are pulled by a force \( F_s \) at \( t = 0 \) and the stretch is held fixed (i.e., \( F(0) = F_s \)). Then the solutions for \( F \) given these two initial conditions are

\[
F = F_0 \left[ 1 - \exp \left( \frac{-t}{\tau} \right) \right]
\]  
(1.6)

\[
F = F_s \exp \left( \frac{-t}{\tau} \right) + F_0 \left[ 1 - \exp \left( \frac{-t}{\tau} \right) \right]
\]  
(1.7)

where

\[
\frac{1}{\tau} = \frac{2G\gamma_0 \exp(-E_a/k_B T)}{k_B T}
\]  
(1.8)

which predicts: (a) if the fiber ends are held fixed, tensile force will develop in the fibers reaching a steady value of \( F_0 \) with a time constant \( \tau \); (b) if the ends are pulled by a force \( F_s > F_0 \) and the ends are held fixed, the force will decay to a steady force \( F_0 \) with the same time constant \( \tau \). If no force or constraint is applied at the ends, the fibers will slide with respect to each other at a constant velocity. These three qualitative features were observed in in vivo experiments by Rajagopalan et al [3]. If the above model is applied macroscopically (i.e., \( F \) is the force on the axon, \( F_0 \) is the rest tension, and \( F_s \) is the force when a sudden stretch is applied on the axon), then \( \tau \) is the time constant for force generation or relaxation. Figure 1.16 shows the force relaxation and force generation with time for the same axon. The solid line is an exponential fit to the data. Both the fits have the same time constant, \( \tau = 12.4 \) min. The rest tension of the axon was about 2 nN. After a stretch and force relaxation, the axon was slackened when the tension in the axon was reduced to zero. The axon then began to shorten its length and generate force until the force reached a steady value of about 1.8 nN. In a separate experiment to study axon shortening, the neuromuscular junction of an
Figure 1.16: Force relaxation and generation in the in vivo axon. Force relaxation and generation have the same time constant of $\tau = 12.4$ min, as shown in the two upper plots. The lower plot shows that the axon shortens at a constant speed when the force is removed. The axon exhibits two distinct speeds during shortening.

The axon was brought close to the central nervous system to slacken the axon (Figure 1.16). The axon (free of force) shortened at a constant rate. However, the axon exhibits two distinct rates of shortening. Initially the rate is high, and then it abruptly decreases. The reason for two distinct rates of shortening is currently unknown [3].

The model above does not account for any change in stiffness of the axon after an applied stretch. Stiffness may increase due to alignment of fibers in a polymer. However, in an embryonic Drosophila axon with diameter less than a micrometer and length of about 100 \( \mu \)m, cytoskeletal structures, particularly microtubules and neurofilaments, are typically aligned along the longitudinal direction. Thus, there is not much room for further alignment. But how could stiffness be increased after stretch? Prior experiments show that in vitro axons can add cytoskeletal materials under stretch [74, 75]. However, the mechanism by which axons initiate polymerization in response to tension or stretch remains elusive. A mechanism-based mechanics model can be developed when further biological insight is acquired on cytoskeletal growth and remodeling in axons due to forces.
1.5 Outlook

In this chapter, we have discussed some important studies highlighting the role of mechanical tension in neuronal function. Neurons actively regulate tension along their axons, and this regulates growth, guidance, and signaling. Mechanical tension may serve as a regulatory signal to modulate both neurotransmission and vesicle clustering in functional synapses. There remain many unresolved issues in the field of cellular neuromechanics. A few include:

- What is the origin of force generation in axons?
- How does neuronal tension lead to vesicle clustering?
- What role does tension play in the plasticity of synapses (i.e., learning and the formation of memory)?

The remainder of this dissertation mainly focuses on one of the above questions: “How does neuronal tension lead to vesicle clustering?” The connection between mechanical tension and vesicle clustering is striking, but the mechanism is completely unclear. If the spatial organization of vesicles is altered due to tension, it is reasonable that the underlying transport properties may change. To the best of our knowledge, the effect of mechanical strain on vesicle transport in neurons has not been studied previously. In the subcellular environment, vesicles are jostled around and constantly bombarded by molecules undergoing thermal agitation as well as molecular motors [88, 89, 39]. Vesicle transport is a highly dynamic process that consists of many different types of motion, which may be separated into passive and active regimes [90, 91]. Passive motion is characterized by a particle (or vesicle) undergoing a random walk driven by thermal energy [89]. Active motion is characterized by super-diffusive motion which could be driven by a variety of sources including the cytoskeleton, cytoplasmic streaming, and molecular motors [88, 90, 92]. Considering that vesicle transport is dependent on many different processes occurring inside the cell, and that mechanical strain also affects many such processes, it is easy to imagine that vesicle dynamics may change in response to mechanical stretch/compression. For instance mechanical strain may affect the availability of Ca$^{2+}$ [19] or ATP [93], which could affect both vesicle transport and fusion. In addition, the local subcellular stiffness may change [94], which could also affect the viscous drag on the vesicle. Vesicle transport is a critical process, and by investigating the mechanoresponse in detail we may uncover the role of mechanical tension in neuronal function. This additional layer of understanding will aid in understanding early stage
neuronal development [95, 96, 97, 98, 99] as well as treatments for neurodegenerative diseases and injury [43, 100].

The importance of the mechanical micro-environment has created a new looking-glass through which to view biological systems. These topics stand to benefit from interdisciplinary work among biologists, neuroscientists, engineers, and physicists. Two main ways engineering and physics can contribute are: (1) Engineering of experimental systems to probe biological systems with high precision; and (2) Investigating the biological system from the physical perspective, offering new insight on old problems. This thesis outlines my contributions to the emerging field of cellular neuromechanics through these two channels. More specifically, this dissertation describes our understanding of the effect of mechanical strain on the dynamics of vesicles in neurons. The remainder of the dissertation includes: the development of a stretching system (Chapter 2); experiments on local and global vesicle dynamics (Chapter 3); measurement of nonequilibrium vesicle dynamics (Chapter 4); analysis of the neuron as an active matter system (Chapter 5); and conclusion and future work (Chapter 6); Some useful theoretical derivations are included in Appendix A.
Summary. The mechanical micro-environment influences cellular responses such as migration, proliferation, differentiation and apoptosis. Cells are subjected to mechanical stretching in vivo, e.g., epithelial cells during embryogenesis. Current methodologies do not allow high-resolution in situ observation of cells and tissues under applied strain, which may reveal intracellular dynamics and the origin of cell mechanosensitivity. A novel polydimethylsiloxane substrate was developed, capable of applying tensile and compressive strain (up to 45%) to cells and tissues while allowing in situ observation with high-resolution optics. The strain field of the substrate was characterized experimentally using digital image correlation, and the deformation was modeled by the finite element method, using a Mooney–Rivlin hyperelastic constitutive relation. The substrate strain was found to be uniform for > 95% of the substrate area. As a demonstration of the system, mechanical strain was applied to single fibroblasts transfected with GFP-actin and whole transgenic Drosophila embryos expressing GFP in all neurons during live-imaging. Three observations of biological responses due to applied strain are reported: (1) dynamic rotation of intact actin stress fibers in fibroblasts; (2) lamellipodia activity and actin polymerization in fibroblasts; (3) active axonal contraction in Drosophila embryo motor neurons. The novel platform may serve as an important tool in studying the mechanoresponse of cells and tissues, including whole embryos.

2.1 Motivation

The mechanical micro-environment influences cellular responses such as migration, proliferation, differentiation and apoptosis [11, 101, 13, 102]. When the natural state of the micro-environment is altered, cells often transit to a malfunctioning or diseased state [103, 104, 105]. Vascular cells such as smooth muscle cells or endothelial cells experi-

\footnote{Parts of this chapter have been previously published as, W. W. Ahmed, M. H. Kural, T. A. Saif. "A novel platform for in-situ investigation of cells and tissues under mechanical strain", Acta Biomaterialia, 6: 2979-90. 2010}
ence strain along their long axis as blood vessels expand and contract [17]. Striated muscle such as cardiac and skeletal muscle cells undergo strain upon every heartbeat and movement [18]. Epithelial cells are subjected to various types of strain throughout different regions of the body, such as the lungs during breathing [19]. Recent literature highlights the influence of mechanical forces in cell and tissue development [20, 106, 107] and embryogenesis [21, 22, 23, 24].

To understand how cells and tissues respond to mechanical stretch, it is important to investigate their morphological organization and subcellular structure. The combination of new imaging techniques and modern biosensors provides visualization tools for investigating subcellular dynamics previously unavailable for living cells [108, 109, 110], motivating the development of compatible experimental techniques. Cell stretching systems, which allow simultaneous application of strain and high-resolution imaging, are needed to understand the role of stretch in cell functionality [111, 112, 113, 114]. Ideally, such a system should be easily reproducible for use in many different laboratories, and be highly compatible with a wide variety of imaging systems and biological specimens. In addition, the applied strain field must be precise, well characterized and highly uniform, since it is known that strain gradients have varying affects on gene regulation, intracellular signaling and alignment [115, 116, 117, 118]. Such a system would allow investigations probing different scales, including subcellular dynamics of single cells and large-scale organization of cells in whole tissues in response to applied strain.

Earlier cell stretching systems, such as that offered by Flexcell International, use vacuum pressure to deform an elastomeric membrane which is stretched over a cell culture well [119], but these systems are primarily used with fixing and staining for biochemical studies. Furthermore, the strain field in the pressure-driven system may not be uniform [120], which may lead to ambiguous experimental results. Most current systems grip and stretch the substrates in an attempt to achieve a more uniform strain field. However, they do not allow live imaging with high numerical aperture optics because of incompatibility with the short working distance (100–200 μm) objectives. This is typically due to either limitations of the stretching device itself [121, 122, 123, 124, 125] or the thickness of the stretchable substrate [126, 127, 128, 129]. Stretching systems which are compatible with high-resolution optics tend to be complicated and require integration with a specific microscopy system [130, 131]. In addition, most studies measure the strain field experimentally at discrete locations and assume uniformity over the substrate surface. This may not be adequate to characterize the strain field precisely, since complicated boundary conditions due to clamping may lead to locally non-uniform strains, especially under large deformations.
2.2 Design and working principle

A novel platform was designed and characterized for precise application of mechanical strain to single cells and tissues, while allowing in situ live-imaging with high-resolution optics. The system specifications are as follows: (a) portable and easily replicated; (b) compatible with various types of microscopes and imaging chambers; and (c) uniform strain over a large area.

The novel platform is a polydimethylsiloxane (PDMS) substrate with two thicknesses. A thin region (diameter 15 mm, thickness 170 µm) serves as the culture surface and is surrounded by a thicker region (thickness 1.2 mm) as shown in Fig. 2.1. The thick substrate around the thin region provides the structural support necessary for handling. The ends of the thick substrate are clamped and stretched, and the strain is transferred to the thin culture surface (Fig. 2.2). The PDMS substrate is supported by a linear stage and actuator (Newport Inc.) mounted on an aluminum base with adjustable clamps. Aluminum was chosen for its corrosion resistance in cell culture/incubator environments. Uniaxial stretch of the flexible substrate, which is unconstrained in the lateral direction, results in a Poisson contraction. Thus as \( \varepsilon_x \) is applied, there is a lateral contraction of \( \varepsilon_x = -\nu\varepsilon_y \) for the ideally linear elastic case (Poisson’s ratio, \( \nu \approx 0.5 \) for PDMS). To describe the material behavior under large deformations adequately, a non-linear constitutive relation must be used, which is discussed later.
Polymer Casting. PDMS substrates were prepared by thoroughly mixing a 10:1 ratio of Dow Corning Sylgard 184 silicone elastomer and curing agent resulting in a Young’s modulus of $E \approx 1 \text{ MPa}$ [132]. The mixture was cast in an aluminum mold and cured at $100 \, ^\circ\text{C}$ for 12 h (Fig. 2.1). The surface of the aluminum mold was coated with a thin layer of polytetrafluoroethylene (PTFE) prior to polymer casting to facilitate mold release.

Surface Roughness. The surface of the culture reservoir is the mirror image of the surface of the polished aluminum mold. Thus, to ensure a smooth surface finish, the mold was polished with alumina nanoparticles and metal polish. The surface roughness of the resulting PDMS substrate was found to have an rms value of $\sim 30 \, \text{nm}$ by atomic force microscopy (AFM). To achieve a smoother finish, mica discs were adhered to the surface of the aluminum mold, and the PDMS was cured in contact with this surface, as shown in Fig. 2.1. The resulting surface roughness of the PDMS was found to have an rms value of $< 2 \, \text{nm}$. The surface of the culture well was probed by AFM after plasma treatment, and no irregular surface patterns were observed, as shown in Fig. 2.3.
Surface Functionalization. PDMS can be functionalized in a variety of ways [133, 134, 135]. Here, two different types of surface functionalization were employed, using (1) silane for *Drosophila* embryos and (2) fibronectin for cell adhesion. Silane-based functionalization [136] involves pretreatment with O$_2$ plasma (300 W, 500 mtorr, 3 min) followed by immediate incubation in 10% (3-aminopropyl)triethoxysilane (APTES) for 2 h. After incubation, the PDMS surface was washed with phosphate buffered saline (PBS) and allowed to air dry to make the surface hydrophobic [137].

Fibronectin functionalization [138] involves sterilization of the PDMS substrates with ethanol and rinsing with PBS. The substrates were then exposed to low-intensity ultraviolet (UV) light for 20 min to ensure sterilization and modify the surface [139]. Subsequently, the culture surface of the substrate was incubated in 0.5 mL of 50 µg /mL human-derived fibronectin for at least 2 h to allow uniform adsorption.

2.3 Characterization of substrate deformation

To study the effect of mechanical strain on cells and tissues quantitatively, it is important to know the magnitude and the type of strain being applied. If the strain is applied by culturing the cell or the tissue on a stretchable substrate, the substrate strain must be known at any point on the surface. In the case where the substrate strain is
non-uniform, in order to determine the strain on the cell, the location of the cell with respect to a reference must also be known. The latter is non-trivial when a cell that is orders of magnitude smaller than the substrate size is being observed. A non-uniform strain field also induces a strain gradient on the cell or the tissue, which may result in additional unwanted functionalities. As an example, the strain field for a circular film induced by a pressure differential is non-uniform: it varies radially [120]. A uniform strain field of the substrate is thus most desirable, which ensures that any cell or a tissue is subjected to a prescribed strain that is independent of their location. Such uniformity is particularly important when the applied strains are large and material behavior is non-linear. In that case, small geometric non-uniformities of the substrate (due to fabrication) can result in large unaccounted variation in the strain field [140].

The substrate strain was investigated both experimentally and numerically using finite element analysis. The in-plane displacements and strains measured experimentally match well with the numerical simulation. The simulation reveals the uniformity of the strain field and the out-of-plane deformation of the substrate [141, 142].

**Experimental observation of strain field.** To measure the strain field experimentally, markers were added to the substrate surface, and stretch was applied incrementally using the stretching system. The surface was imaged using a Canon 5D Mark II Digital SLR camera with a 65 mm 1x to 5x macro lens mounted perpendicular to the substrate surface. Strains were calculated using the built-in digital image correlation (DIC) function in Matlab. The DIC algorithm located multiple trackable points within each marker and tracked their displacement through sequential images. An example of the images used for the DIC analysis is shown in Fig. 2.4. Strains were measured both at room temperature and at 37 °C in a cell culture incubator. In the latter case, a fixed stretch was applied, and strain was measured over 24 h later to check for any time-dependent change in the strain field in the thin substrate.

The deformation of the PDMS substrate was found to be completely reversible and time-independent, even for large strains (\(\varepsilon_x > 45\%\)). The strain field remained steady even after 24 h of applied stretch at an elevated temperature (37 °C). Thus it can be concluded that there is no slippage in the clamps and no strain redistribution in the substrate due to relaxation of higher stresses at the stress concentration regions (near the edges of the circular thin substrate). The lack of relaxation is an important observation for two reasons: (1) this is highly desirable for controlled experiments; and (2) a time-independent material model can be used for the finite element analysis. The stretching system has a resolution of \(\sim 0.1\%\) substrate strain.
Figure 2.4: Images of substrate strain for Digital Image Correlation (DIC) analysis. The DIC algorithm locates multiple trackable points within each marker and calculates their displacement through sequential images. $\varepsilon_x$ denotes tensile strain in the horizontal direction and $\varepsilon_y$ denotes the compressive strain in the vertical direction in the image plane (scale bar = 5 mm).

The DIC analysis yielded the experimentally observed substrate strain in Fig. 2.5. When the substrate is stretched by 8 mm, the resulting tensile strain in the thin membrane is 30% with a lateral contraction of 10%. When the substrate is stretched by 14.5 mm, the resulting tensile strain is 45% with a lateral contraction of 15%.

Numerical simulation of strain field

Constitutive behavior of PDMS. Rubber-like materials such as PDMS are able to sustain large deformations with elastic recovery, and they exhibit a non-linear stress–strain behavior (Fig. 2.6). Here, the Mooney–Rivlin constitutive relation is used to describe the hyperelastic material behavior [143, 144, 145]. In this model, PDMS is assumed to be ideally elastic, isotropic and incompressible. The strain energy function $W$ is a polynomial function of the strain invariants,

$$W = \sum_{i=0}^{\infty} \sum_{j=0}^{\infty} c_{ij}(I_1 - 3)^i(I_2 - 3)^j$$  \hspace{1cm} (2.1)

where $c_{ij}$ are materials constants, and $I_1 = \text{tr}\mathbf{C}$, $I_2 = \frac{1}{2}((\text{tr}\mathbf{C})^2 - \text{tr}\mathbf{C}^2)$, where $\mathbf{C}$ is the right Cauchy-Green deformation tensor, and the Green strain tensor is defined as $\mathbf{E} = \frac{1}{2}(\mathbf{C} - \mathbf{I})$ [146]. For strains of $<100\%$, the first-order term of the strain energy is often used which is a linear function of the strain invariants [147],

$$W = c_{10}(I_1 - 3) + c_{01}(I_2 - 3)$$  \hspace{1cm} (2.2)
where \( c_{10} \) and \( c_{01} \) are the two material constants required to completely specify the material behavior, the strain invariants are \( I_1 = \lambda_1^2 + \lambda_2^2 + \lambda_3^2 \), \( I_2 = \lambda_1^2\lambda_2^2 + \lambda_2^2\lambda_3^2 + \lambda_3^2\lambda_1^2 \), and the principle stretch ratios are \( \lambda_i = 1 + \varepsilon_i \), where \( \varepsilon_i \) is the principle value of the engineering strain in the \( i \)th direction. The first-order Mooney–Rivlin model for a hyperelastic material was used with \( c_{10} = 90.35 \) kPa and \( c_{01} = 12.82 \) kPa, which were previously reported to be valid for strains up to 50% [10].

**Boundary conditions for numerical simulation.** The PDMS substrate geometry was created in ANSYS, and displacement boundary conditions were applied to the clamping area of the substrate to mimic application of strain by the stretching system. One-quarter of the platform was modeled with symmetric boundary conditions: (1) all the nodes on the \( yz \) surface were restrained against motion along the \( x \) direction; (2) all the nodes on the \( xz \) surface were restrained against motion along the \( y \) direction; and (3) the clamping surface was fixed in the \( z \) direction, and an \( x \) displacement was applied to stretch the platform (refer to Fig. 2.7 for axis orientation).

**Simulation results**

**Uniformity of strain field.** Most importantly, the FEM simulation shows that the strain in the thin substrate is uniform for the majority of the surface area, as shown in Fig. 2.7. In over 95% of the surface area of the thin substrate, the surface strain varies by \( \sim 1\% \). This verifies that the strain field is uniform, and any biological specimen will be under approximately the same strain regardless of its location on the substrate. This allows a highly controlled experiment where the applied strain in known regardless of the location of the specimen on the substrate.

**Out-of-plane deformation.** The simulation shows that, at 30% tensile strain, the center of the thin membrane displaces upward by 400 \( \mu m \) above the surrounding edge. Relative to the diameter of the membrane (15 mm), this deformation is small, and the resulting membrane curvature is low and thus does not affect imaging or the uniformity of the surface strain in the thin membrane.

**Comparison with experiment.** The FEM results (Fig. 2.7) show close agreement with the measured experimental strains (Fig. 2.5). When an equivalent substrate stretch of 8 mm was applied in the simulation, the resulting tensile strain in the circular well along the loading direction was 30%, with a lateral contraction of 14%. Thus, the
Figure 2.5: Average substrate strain as calculated by the DIC analysis: (a) the tensile strain in the $x$ direction; (b) the compressive strain in the $y$ direction.
experimentally measured tensile strain (30\% in response to 8 mm grip displacement) is in direct agreement with the results predicted by the simulation, but the predicted value of the lateral contraction is 4\% higher than observed experimentally. This deviation from experiment in the lateral contraction is expected to be a result of idealized clamping boundary conditions in the simulation in contrast to the experiment where clamping is achieved by a pair of aluminum plates held by bolts at the ends. In the experiment, after tightening the bolts, the aluminum clamps bend, and the gap between the plates increases towards the center. This results in a complex state of stress in the substrate with a boundary condition that is pinching the substrate near the edges.

**Biological investigation.** The novel platform is capable of applying precise amounts of strain to cells and tissues, including whole embryos, while allowing in situ live imaging. To illustrate this advantage, the mechanoresponse of single fibroblasts and whole *Drosophila* embryos was investigated.

### 2.4 Stretching fibroblasts

Fibroblasts are a robust model system for studying the basic response of the cytoskeleton to mechanical stimulus. They are the primary cells present in connective tissue and play a critical role in wound healing. In addition, they are easily cultured in vitro and have been extensively studied. The present system allows observation of real time subcellular dynamics in single cells to observe their mechanoresponse to applied strain.
Figure 2.7: Finite-element method simulation of substrate deformation using the two-parameter Mooney–Rivlin hyperelastic constitutive relation: (a) and (b) are the contour plots of $\varepsilon_x$ and $\varepsilon_y$, respectively. The average predicted tensile strain and lateral contraction are $\varepsilon_x = 30\%$ and $\varepsilon_y = 14\%$, respectively.
Cell culture and transfection. Monkey kidney fibroblasts from ATCC were cultured in an incubator at 37 °C and 5% CO$_2$ in growth medium consisting of Dulbecco’s modified Eagle medium (BioWhittaker Cat. No. 12-604F) with 10% fetal bovine serum and 1% penicillin/streptomycin (Pen/Strep) (Gibco). PDMS substrates were sterilized with 70% ethanol and washed with PBS. Cells were transfected with Cellular Lights Actin-GFP (Cat. No. C10126) using standard protocols from Invitrogen. After transfection, cells were trypsinized and sparsely plated on fibronectin functionalized PDMS substrates and allowed to attach for 24 h prior to the stretching experiment. Fibroblasts remained attached to the PDMS substrates for over 5 days, indicating potential for long-term stretching studies.

Microscopy and image analysis. To illustrate compatibility with high-resolution imaging systems, the cytoskeletal dynamics in fibroblasts as a response to mechanical strain were observed using a state of the art Zeiss LSM 710 NLO microscope capable of single-photon and two-photon laser excitation. Fluorescence and differential interference contrast images were collected simultaneously at 2-min intervals for $\sim$ 1 h. The images were processed using 3-D blind deconvolution in Autoquant, and maximum intensity projection and data analysis were carried out in ImageJ [148] to measure actin fiber strain and angle change.

Dynamic rotation of intact stress fibers begins within minutes. This novel platform is capable of applying both tensile and compressive strain to the cells. To apply tensile strain, the fibroblasts were seeded on unstretched substrates and allowed to attach overnight. Then the substrates were loaded into the stretching device and stretched. The strain rate is limited by the actuator speed and, in the present case, the maximum strain was applied over $\sim$ 30 s. The resulting substrate strain is transferred to the cell, as shown in the GFP-actin transfected fibroblasts in Fig. 2.8. To apply compressive strain, the fibroblasts were seeded on prestretched substrates and allowed to attach overnight. The substrates were then unloaded, transferring a compressive strain to the cells, as shown in Fig. 2.9.

Realtime high-resolution imaging allowed observation of interesting cytoskeletal dynamics, including change in actin fiber angle in response to applied strain. It is known that fibroblasts reorient themselves in response to stretching, and it has been reported that cell bodies begin to show alignment within 2–3 h [149]. It was observed that subcellular reorganization begins much earlier where actin stress fiber reorientation occurs within minutes of the applied strain. An earlier study [150], found that actin fibers in
the direction of applied tensile strain depolymerized, and new actin fibers formed at an oblique angle. Also, a recent mathematical model predicts reorientation of actin stress fibers as a function of turnover (i.e., polymerization and depolymerization processes) [151]. It seems to be widely believed that actin fiber reorganization occurs mainly due to new stress fiber formation.

In contrast, dynamic rotation of intact stable stress fibers was observed (see Supplementary Video S1). In response to an applied axial strain of 11%, the actin stress fibers reoriented by rotating counter clockwise, away from the direction of applied strain (Figs. 2.10 and 2.11). Multiple stress fibers were measured, and their rotation is plotted in Fig. 2.10c. Thus, actin fiber reorientation is not only due to fiber turnover, but intact and stable stress fibers also undergo dynamic rotation oblique to the direction of applied strain. Actin stress fibers are connected between focal adhesion complexes, which are anchored to the underlying substrate. Thus, for an intact actin stress fiber to undergo dynamic rotation, its anchor points must move relative to each other. This observation suggests mobilization of focal adhesion complexes in response to applied strain. Interestingly, a recent study [152] found that focal adhesion sliding and actin fiber reorientation are key players in force-induced cellular reorganization independent of microtubules. This is in direct contrast to cellular reorganization during migration, where microtubules are known to regulate focal adhesion dynamics [153]. These results [152] highlight the importance of in situ observation in uncovering the mechanisms of cellular mechanotransduction. It should be noted that the actin stress fibers which underwent active dynamic rotation extended through the center region of the cell, whereas the stress fibers located at the cell periphery remained stable. This indicates that actin fiber reorganization varies spatially throughout the cell, and it may be interesting to visualize microtubules simultaneously to investigate filament interaction.

It is also interesting to note that multiple stress fibers originally oriented around 45° rotated by approximately the same amount (7°) away from the applied strain to 52°, whereas a stress fiber originally at 52° rotated by nearly 15° to 67° over a similar period of time. This suggests that the amount and/or rate of rotation may be a function of the initial stress fiber configuration. The mechanism for this oblique orientation of actin stress fibers is unclear.

If the driving force is minimization of axial strain [154, 155], one would expect the fibers to align to ~60°, which is the direction of minimal strain in the present substrate, as calculated from the transformation equation for plane strain:

\[
\varepsilon'_x = \frac{\varepsilon_x + \varepsilon_y}{2} + \frac{\varepsilon_x - \varepsilon_y}{2} \cos 2\theta + \frac{\gamma_{xy}}{2} \sin 2\theta
\]  
(2.3)
Figure 2.8: Images of fibroblasts under applied tensile strain. Cells were seeded on unstretched substrate and allowed to adhere overnight and then stretched. Due to the applied strain, the actin fibers are stretched axially by (b) 16% and (c) 28% (scale bar = 30 µm).
where $\varepsilon_x$, $\varepsilon_y$ are the normal strain in the $x$ and $y$ directions, $\gamma_{xy}$ is the shear strain, and $\theta$ is the angle relative to the applied stretch. By setting Eq. (2.3) equal to zero and noting that $\gamma_{xy} = 0$, one can solve for $\theta$ which is the direction of zero axial strain. This may explain the rotation of stress fibers with an initial angle of $\sim 45^\circ$, as shown in Fig. 2.10c. However, the stress fiber with an initial angle of $52^\circ$ continues to rotate well beyond $60^\circ$, and this cannot be explained by minimization of axial strain. A recent model predicts a more perpendicular orientation of the stress fiber if the cell senses stress rather than strain [156].

**Lamellipodia activity and actin polymerization.** Fibroblasts were stretched, inducing 10% axial strain in the actin stress fibers. After 8 min of applied tensile strain, one observed an abrupt onset of lamellipodia activity throughout the cell, as shown in Fig. 2.12. The lamellipodia exhibited wave-like motion emanating outward from the actin fibers. This continuing lamellipodia motion resulted in the formation of new actin stress fibers (see Supplementary Video S2). The propagation of actin waves may be a mechanism of cytoskeletal reorganization in response to mechanical stretching [157]. It has been shown that lamellipodial actin mechanically links myosin activity with adhesion-site formation [158]. Thus, the observations of lamellipodial activity and actin polymerization in response to applied strain may suggest the formation of new adhesion sites, which is crucial to cellular motility. Assembly and disassembly of actin filaments drives cellular motility and has been shown to involve proteins such as Arp2/3, profilin and ADF/cofilin [159, 160]. Arp2/3 complexes nucleate actin filaments, which grow from pre-existing filaments, and it has been shown to be distributed throughout actin waves [157]. Thus it is reasonable to hypothesize that Arp2/3 may be mechanically activated and is mobilized to these regions of lamellipodial activity resulting in nucleation of new actin fibers emanating from pre-existing filaments. Possible mechanisms for this mobilization are unclear and are a topic currently under investigation.

### 2.5 Stretching *Drosophila* embryos

*Drosophila* embryos are a convenient model system for studying neurons in vivo. They have long been used to study synaptic development and neurotransmission, and they are known to have structurally plastic neuromuscular junctions [161, 162]. It is believed that mechanical forces may play a role in neuronal sensing [163], and a recent in vivo study showed that mechanical tension leads to presynaptic clustering of neurotransmitters [28], although the underlying mechanisms are not well understood. Studying
Figure 2.9: Images of fibroblasts under applied compressive strain. Cells were seeded on a prestretched substrate overnight, and the substrate was unloaded. Owing to applied strain, the actin fibers are compressed axially by 7% (scale bar = 30 µm).
Figure 2.10: Actin stress fiber rotation in response to applied static strain (horizontal arrows indicate stretch direction): (a) and (b) are the original and stretched (11%) configuration, respectively, where the red dotted region indicates the stress fibers tracked; (c) actin stress fiber rotation as a function of time (shown for five stress fibers). Here, it is clear that the actin stress fiber reorientation occurs within minutes of applied strain. The small inset in the lower right schematically illustrates the stress fiber rotation, where $\theta_1$ is the initial angle, and $\theta_2$ is the final angle relative to the strain direction (scale bar = 30 $\mu$m).
Figure 2.11: Images of actin stress fiber rotation in response to applied strain (horizontal direction). An actin stress fiber is shown with its angle and corresponding time after applied strain. The stress fiber rotates by 15° within 1 h (scale bar = 10 µm).
Figure 2.12: Lamellipodia activity and actin polymerization induced by applied tensile strain (10% horizontal direction) was observed throughout the cell. The left column shows images of the entire fibroblast. The region within the red box is magnified and shown in the right column. The red triangle indicates an example of lamellipodia activity and actin polymerization. Lamellipodia activity begins after 8 min and results in new actin fiber formation by 45 min (left scale bar = 30 µm; right scale bar = 10 µm).
the role of mechanical forces in functional neurons is difficult, owing to experimental limitations. In vitro studies of neuronal mechanics [130, 164, 165, 8, 166, 167] use both primary and cultured cells, which lack the supporting glial cells present in in vivo systems. The present system allows mechanical stretch of the entire embryo to study the mechnaresponse of the functional neurons in their in vivo environment.

**Drosophila culture and embryonic dissection.** *Drosophila* (elav-gal4/UAS-gapGFP) embryos expressing green fluorescent protein (GFP) in all neurons were used for this investigation. *Drosophila* were cultured on standard grape agar plates under ambient light at 25 °C. Embryonic dissection was carried out on the silane functionalized PDMS substrates using techniques previously described by Budnik et al. [168]. Briefly, the embryos were dechorionated using a 50/50 bleach and water solution for 1 min. Embryos of the correct age (20 h after egg laying) were placed on double-sided tape, and then the PDMS reservoir was flooded with saline solution, and the embryos were de-vitellinized. The embryos were oriented such that the ventral nerve cord was closest to the PDMS surface, and the tape was removed. Upon contact with the functionalized APTES surface, the embryos stick strongly and cannot be removed without damage. Glass micro-needles were created for dissection with a Sutter Instruments laser-based micropipette/fiber puller. The micro-needle was used to create a dorsal incision in the embryo, remove the guts and lay the body walls down flat on the PDMS surface, resulting in a specimen that is ~ 25 µm thick (see Ref. [168] for detailed procedure). The motor neuron axons are attached to the cell body at one end and to the neuromuscular junction at the other; thus, along its length, the axons are not anchored.

**Microscopy and Image Analysis.** The mechnaresponse of *Drosophila* motor axons was observed using a Zeiss SteREO Luminar.V12 microscope. Fluorescence images were acquired for 30 min of applied strain and 30 min after unloading. ImageJ [148] was used to measure the strain in individual axons by two methods: (1) the change in straight line distance between the ends of the axon (P–P strain); and (2) the change in length of the axon itself (L strain). To measure the axon lengths consistently, the image sequences were thresholded and skeletonized, and NeuronJ [169] was used to trace the axon.

**Drosophila** motor neuron axons actively contract in response to applied strain. An image of a *Drosophila* embryo and a plot of active axonal contraction in response to the applied strain are shown in Fig. 2.13. The embryo was oriented such
that the central nervous system is along the direction of tensile strain, and the motor neuron axons of the peripheral nervous system are along the direction of the lateral Poisson contraction. A tensile strain of 30% was applied to the substrate, resulting in a lateral compressive strain of 10%. This effectively moves the anchor points of the motor neuron axons closer together, owing to Poisson’s effect in the substrate. This leaves the axons free of tension, and additional shortening of axonal length over time implies an active process. Note that compressive strains are denoted by negative numbers, and tensile strains are positive numbers.

Immediately after the applied strain, the axon became more “zig-zagged” and exhibited compressive strain (decrease in length) of $-5.9\%$ relative to its original in vivo length. In this state, it is assumed that the axon is in a stress-free state, because it is slack. Owing to a slight delay between applied stretch and the first image, this value was calculated by extrapolating the time–strain relation (1 min before the first image).

Over the next 10 min, the axon exhibited active contraction to a strain of $-4.6\%$ relative to its stress-free state at an average rate of 7.2 nm/s, as seen in Fig. 2.13b. After this period of active contraction, the axon’s length reached a steady state of $-4.9\%$ strain. During this time, the end-to-end axon strain (P–P strain) exhibited similar behavior. After a total duration of 30 min, the substrate was unloaded (returned to zero strain) and the axons were effectively put under tension, because they had contracted. As labeled by “unloading” in Fig. 2.13b, the end points of the axon follow the substrate strain almost exactly. After unloading, the axon length was observed to have a tensile strain of 1.9%, relative to its stress-free state, and it actively contracted to a compressive strain of $-1.0\%$ over the next 30 min at an average rate of 1.7 nm/s. It is worth noting that the average rate of axonal contraction is higher when measured along the length of the axon (L strain) compared with measuring the distance between the end points of the axon (P–P strain). This suggests that, during active contraction, the axons may be building tension (see Supplementary Videos S3 and S4).

The present authors believe the active axonal contraction observed in the motor neurons of the *Drosophila* embryos is a response to the applied mechanical strain. Axonal contraction is observed twice: (1) after applied strain; and (2) after the substrate is unloaded as shown in Fig. 2.13b. One possible mechanism for the observed contraction could be explained by the maintenance of an internal tension within the axon, which may be involved in axonal development [170] or synaptic plasticity [28].
Figure 2.13: Active axonal contraction was observed in response to applied strain. (a) A representative fluorescent image of the *Drosophila* embryo expressing GFP in all neurons; the arrow indicates a motor neuron axon. (b) Axon strain as a function of time. Here, axonal contraction is observed twice: (1) after initial applied strain; and (2) after substrate unloading. L strain is the change in length of the axon, and P–P strain is the change in distance between the axon end points.
2.6 Conclusion

A novel platform was designed and characterized to investigate the response of cells and tissues to applied strain. This system is capable of applying prescribed uniform strain over a large substrate area for cell or tissue culture, while allowing in situ observation by high-resolution live imaging techniques. As a demonstration of the system, the mechanoresponse of single fibroblasts and whole *Drosophila* embryos under applied strain was examined. In situ live-imaging allowed three interesting observations.

1. Intact actin stress fibers in fibroblasts underwent dynamic rotation away from the direction of applied strain without depolymerizing. This may suggest mobilization of focal adhesion complexes in response to applied strain.

2. Lamellipodia activity and actin polymerization were induced by applied strain and resulted in new stress fiber formation in fibroblasts, suggesting mechanical mobilization of actin filament assembly proteins such as Arp2/3.

3. In *Drosophila* embryos, in vivo motor neuron axons exhibited active contraction in response to applied strain, possibly in an attempt to maintain a steady-state internal tension necessary for neurotransmission.

These observations show the utility of the system and the importance of in situ observation during applied strain to investigate the mechanoresponse in biological systems.
Chapter 3

Tension Modulates Vesicle Dynamics

Summary\textsuperscript{1}. Growing experimental evidence suggests that mechanical tension plays a significant role in determining the growth, guidance, and function of neurons. Mechanical tension in axons contributes to neurotransmitter clustering at the \textit{Drosophila} neuromuscular junction (NMJ) and is actively regulated by neurons both in vitro and in vivo. In this work, we applied mechanical strain on in vivo \textit{Drosophila} neurons and in vitro \textit{Aplysia} neurons and studied their vesicle dynamics by live-imaging. Our experiments show that mechanical stretch modulates the dynamics of vesicles in two different model systems: (1) The global accumulation of synaptic vesicles (SV) at the \textit{Drosophila} NMJ and (2) the local motion of individual large dense core vesicles (LDCV) in \textit{Aplysia} neurites. Specifically, a sustained stretch results in enhanced SV accumulation in the \textit{Drosophila} NMJ. This increased SV accumulation occurs in the absence of extracellular Ca\textsuperscript{2+}, plateaus after approximately 50 min, and persists for at least 30 min after stretch is reduced. On the other hand, mechanical compression in \textit{Aplysia} neurites immediately disrupts LDCV motion, leading to decreased range and processivity. This impairment of LDCV motion persists for at least 15 min after tension is restored. These results show that mechanical stretch modulates both local and global vesicle dynamics and strengthens the notion that tension serves a role in regulating neuronal function.

3.1 Motivation

Neurons are the key rapid signal generating and processing components of the nervous system. They generate and transport vesicles containing neurotransmitters and neuromodulators used in communication with other cells by exocytic release of vesicle contents. Neurotransmitter filled synaptic vesicles (SV) cluster at the synapse prior to release for signaling a postsynaptic cell. Previous studies of vesicle clustering have focused primarily on biochemical signaling \cite{97}. But recent investigations show that

\textsuperscript{1}Parts of this chapter have been previously published as, W. W. Ahmed, T. Li, S. Rubakhin, A. Chiba, J. Sweedler, T. A. Saif. "Mechanical tension modulates local and global vesicle dynamics in neurons", Cellular and Molecular Bioengineering, 2012 (DOI: 10.1007/s12219-012-0223-1) (invited paper)
mechanical tension contributes to clustering of neurotransmitter vesicles at the presynaptic terminal [28]. Furthermore, the modulation of presynaptic terminal machinery is critical in synaptic plasticity [44]. This suggests a functional role for mechanical tension in neuronal signaling.

While in vivo studies of mechanical tension in neurons are fairly recent, the presence of tension in cultured neurons has been known for several decades. Mechanical tension was observed to exist in neurons in cell culture many decades ago. One of the first observations of in vitro axonal tension was made in 1945 by Weiss, who suggested that axons straighten along their length, possibly due to an “elastic tension” [66]. Decades later, Bray [67] showed that growth cones exert mechanical tension and that the direction of tension determines growth direction. Subsequently, it was discovered that growth cones pull on their underlying substrate to induce neurite growth [64].

Growing evidence of the mechanical sensitivity of neurons has led to many recent in vitro studies that highlight how mechanical forces affect neuronal behavior. For instance, axons undergo continuous elongation under mechanical tension and exhibit normal subcellular structures and electrophysiology [76, 75, 167]. Coupling of the cytoskeleton to the underlying substrate mediates cytoskeletal polymerization and growth cone steering [5, 171], and tension generated along the axon may serve as a signal for branch pruning [172, 82]. Mechanical tension has been shown to exist in in vivo Drosophila motor neurons and this tension is actively regulated [3]. In an effort to explain the mechanical sensitivity of neurons, mathematical models have been developed to explain force generation, elongation, and axonal transport [9, 8, 173, 174, 175]. For in depth reviews of the role of forces in neuronal growth see Franze et al. [176], Franze and Guck [29], and Suter and Miller [77].

While many recent studies have focused on neuron growth, guidance, and structure, studies of the effect of mechanical tension on neuronal signaling are limited. Previous studies have shown an immediate and reversible increase of neurotransmitter release due to muscle stretch in frogs [26, 83, 6]. Chen et al. [26] hypothesized that stretch enhancement of neurotransmitter release was derived from either elevated intracellular Ca$^{2+}$ concentrations or by increased sensitivity to Ca$^{2+}$ in the nerve terminal. They later showed that Ca$^{2+}$ influx was not necessary for stretch enhanced neurotransmitter release but the magnitude of the effect was attenuated in the absence of extracellular Ca$^{2+}$. As a result they postulated that tension on integrins mechanically transduces changes in molecular conformations causing increased sensitivity to Ca$^{2+}$ [84]. Stretch enhanced neurotransmitter release occurred on a millisecond time scale and was reversible after stimulation was removed. However, recent experiments have suggested
that tension induced SV accumulation occurs over a much larger time scale [28]. Additionally, the effect of tension on the global behavior of vesicle accumulation naturally leads to the question: does tension affect the local behavior of individual vesicles?

We investigated three main questions: (1) Does stretch enhanced SV accumulation occur in the absence of extracellular Ca$^{2+}$, and what are the time dynamics? (2) Does decreased tension in intact neurons affect SV accumulation? (3) Does mechanical tension affect local vesicle dynamics?

Our experiments show: (1) Increased mechanical tension enhanced SV accumulation at in vivo Drosophila NMJ’s in the absence of extracellular Ca$^{2+}$ and accumulation saturated after approximately 50 min of elevated tension. This effect persisted for at least 30 min after axonal tension was reduced. (2) Compressive strain along Drosophila motor neuron axons did not affect SV accumulation. (3) Decreased tension in neurites of cultured Aplysia neurons disrupted motion of large dense core vesicles (LDCV), including the local range and processivity, and this effect persisted for at least 15 min after tension was restored. A significant difference between the in vivo Drosophila and the in vitro Aplysia neurons studied here was that the former had a neuromuscular synapse, while the latter had not formed any synapse and had a growth cone as their neurite terminal.

3.2 Materials and methods

Stretching experiments. In this study we used a stretching system for high-resolution live-imaging of cells and tissues under applied mechanical strain [86]. The system applied a static deformation to a stretchable polydimethylsiloxane (PDMS) cell culture substrate. The substrate consisted of two different thicknesses, an outer thick region (1.2 mm) to provide mechanical stability and an inner thin region (170 µm), which served as a cell culture well. The thin region allowed imaging through the PDMS substrate with high-resolution optics. When the substrate was stretched in one direction, it experienced a Poisson contraction in the orthogonal direction. Thus, a cell aligned along the stretch direction would be under tensile strain, and a cell aligned perpendicular to the stretch direction would be under compressive strain. In this study, cells experienced up to 20% tensile strain and up to 8% compressive strain. The substrate deformation was characterized experimentally by digital image correlation and computationally by the finite element method and it was found that the mechanical strain of the cell culture surface was uniform over greater than 95% of the surface area [86].

We investigated the change in vesicle dynamics in neurons in response to mechanical
tension by using a three step experiment:

1. Control: Cells were attached to the cell culture substrate in their natural undeformed state.

2. Applied deformation: Mechanical stretch or compression was applied along the axis of the neuron.

3. Deformation removed: The deformation applied in step 2 was completely reversed by unstretching the substrate back to its original state.

Vesicle dynamics were observed by live-imaging as described below for all stages of the experiment.

*Drosophila* embryo motor neurons. *Drosophila* embryonic in vivo motor neurons were used in this study to investigate SV dynamics. They are amenable to live-imaging and are known to have structurally plastic neuromuscular junctions (NMJ) [162]. To study SV dynamics, transgenically modified *Drosophila* (eve-GAL4;UAS-sytEGFP) were used to express synaptotagmin–GFP [177]. Synaptotagmin is an integral vesicle membrane protein which can be tagged with GFP to serve as a SV marker in living synapses [178]. *Drosophila* were cultured on standard grape agar plates under ambient light at 25 °C. Embryonic dissection was carried out on silane functionalized PDMS substrates, which was described in detail in previous studies [86, 179]. Briefly, embryos were dechorionated in 50/50 bleach and water solution for 2 min. Embryos of the correct age (~16 h after egg laying) were placed on the substrate and the PDMS reservoir was flooded with Ca$^{2+}$ free PBS. Ca$^{2+}$ free solution minimized muscle twitching in the embryos. Such twitching caused imaging artifacts. Glass micro-needles were used to devitellinize and dissect the embryo such that the motor neuron axons were parallel to the PDMS surface. The motor neuron axons were attached to the cell body at one end, and to the NMJ at the other, and thus the axon was not anchored along its length. Since the embryo was adhered to the PDMS surface, substrate deformation induced mechanical strain along the length of the axon. Sample sizes for experiments were: control (n = 10), stretch (n = 8), compress (n = 8).

*Aplysia* neurons. *Aplysia californica* pedal ganglion neurons were used for tracking local vesicle dynamics because this in vitro system had large neurites that allow high-resolution imaging of LDCVs [180]. *Aplysia* neurons were isolated and cultured as in previous studies [181]. Briefly, A. californica were obtained from the National Resource
for Aplysia (University of Miami/RSMAS, Miami, FL) and maintained in a 14 °C tank of circulating artificial sea water until use. Neurons were mechanically dissociated from the Aplysia CNS after a 30–60 min incubation in 1% protease. Cells were plated on polylysine coated PDMS substrates overnight at room temperature and then at 14 °C for 24–48 h in artificial sea water antibiotic solution. Experiments were conducted with cells from multiple animals (n > 10) and multiple vesicles (n > 11) were tracked for each cell.

**Light microscopy and image processing.** All images were collected on a Zeiss LSM 710 laser scanning confocal microscope using a 40x (1.2 NA) water immersion objective lens (Carl Zeiss, Germany). All imaging parameters (e.g., laser power, pixel dwell time, pinhole size, gain, etc.) were kept constant for a given set of experiments. Image analysis was carried out in MATLAB (The MathWorks, Natick, MA) or ImageJ [148].

For Drosophila, fluorescent images of SVs were captured as Z-stacks at approximately 10 min intervals. It was assumed that the amount of SVs present is proportional to the fluorescence intensity of the synaptotagmin–GFP signal. Thus, the amount of SVs was quantified using the fluorescent intensity calculated from the collapsed Z-stack. The average intensity in a 2.5 µm by 2.5 µm region at the presynaptic terminal was used to quantify the SVs at the NMJ.

For Aplysia, differential interference contrast (DIC) image sequences of LDCVs were captured at a rate of 2.5 frames per second. Videos were captured approximately every 5 min. Dynamics of LDCVs were tracked using an algorithm for precise particle tracking by polynomial fitting with Gaussian weight in MATLAB [182]. Note that this method of particle tracking allows particle displacements to be tracked with nanometer precision.

### 3.3 Results

**Increased axonal tension induces synaptic vesicle accumulation at the in vivo Drosophila NMJ.** Global accumulation of SVs was investigated in motor neurons of Drosophila embryos [161, 162]. The tension in Drosophila axons was modulated by conducting stretch and compression experiments. Under stretch, axonal tension was increased and leads to increased SV accumulation at the presynaptic terminal of the NMJ. Under compression, axonal tension was decreased and no significant change in SV accumulation was observed.
Figure 3.1: Axons of a *Drosophila* motor neuron under mechanical strain where the color map represents the fluorescent intensity, blue indicates low synaptotagmin and red indicates high. (a) A control axon is shown on the PDMS surface. The inset on the right shows a 10 µm by 10 µm region near the presynaptic terminal where a 2.5 µm by 2.5 µm region was used to quantify synaptic vesicle accumulation. (b) An axon is shown stretched by substrate deformation, notice the straightness of the axon (increased tension). (c) An axon is shown compressed by substrate deformation, notice the axon is squiggly (decreased tension). Note that images in (a) and (b) show the same axon, while the image in (c) is a different axon (scale bar = 5 µm).

Figure 3.2: Synaptic vesicle accumulation (in terms of fluorescent intensity) at the *Drosophila* NMJ as a function of time. Control samples (n = 10) show no significant change in synaptic vesicle accumulation. When axons are stretched (n = 8), increased accumulation by 30±15% is observed after approximately 50 min and the effect persists for at least 30 min after stretch is removed. In compressed axons (n = 8), no significant change occurs in accumulation during compression or after it is removed. Statistical significance evaluated by Student’s t-test (p <0.01) and denoted in figure by * (error bars = SEM).
Figure 3.1 shows representative images of axons under mechanical strain where the color map represents the fluorescent intensity, blue indicates low synaptotagmin and red indicates high. A control axon is shown in Fig. 3.1a from a dissected embryo on the PDMS surface in the absence of applied deformation. The zoomed-in inset at the right shows a 10 µm by 10 µm region near the presynaptic terminal of the NMJ. A 2.5 µm by 2.5 µm region in the synapse was used to quantify synaptic vesicle accumulation. Figures 3.1b and 3.1c show a stretched and compressed axon, respectively. Note that the stretched axon is straight (increased tension) and the compressed axon is squiggly (decreased tension). In the stretch experiments on axons, a static tensile strain was applied and held for 90 min to increase tension, after which the stretch was removed and the substrate strain was reduced to zero. In the compression experiments, a static compressive strain was applied and held for 60 min to decrease axonal tension, after which the deformation was reversed and the substrate strain was reduced to zero. All experiments with Drosophila motor neurons were carried out in the absence of extracellular Ca$^{2+}$. The overall duration of the experiments was maintained at approximately 2 h to avoid complications with embryo aging. In stretch experiments, the tensile strain was held for 90 min to investigate if accumulation continued to increase with time. Then the substrate was unloaded and the axon was observed for 30 min to investigate if stretch enhanced accumulation persisted after axonal strain was decreased. For compression experiments, the compressive strain was held for 60 min because it was known the axon would shorten and rebuild its rest tension well within 60 min [3]. Then the substrate was unloaded, stretching out the axon to restore its original length, and the axon was imaged for an additional 60 min to observe the effect of stretch from the shortened state.

The time dynamics of vesicle accumulation are shown in Fig. 3.2. When the axons were subjected to sustained stretch, the tension increased and we observed a significant ($p < 0.01$) increase in SV accumulation of 30 ± 15% after approximately 50 min as shown in Fig. 3.2. The increased SV accumulation saturated by 50 min. Then after 90 min of static stretch, the substrate was undeformed and the axon was unstretched. In this state of decreased axonal tension, SV accumulation persisted for at least 30 min (end of experiment). Therefore, we observed that SVs accumulated during the stage of increased axonal stretch in the absence of extracellular Ca$^{2+}$, the increase saturated after approximately 50 min, and this effect persisted after axonal strain was decreased by unloading the substrate. When static substrate compression was applied, the tension in the axon was decreased. During this stage of decreased axonal tension the accumulation of SVs seemed to fluctuate but did not significantly differ from the
control as shown in Fig. 3.2. When the substrate was undeformed, the axonal tension increased due to stretching it back out to its original length. In this stage the SV accumulation also did not significantly differ from the control case.

Representative images of the synaptic regions of the *Drosophila* NMJ throughout the experiment are shown in Fig. 3.3. Here, variations in SV accumulation can be observed qualitatively by fluorescence intensity of the SVs in the presynaptic terminal. In the control case, the amount and distribution of SVs remains approximately the same throughout the experiment. Under stretch, the SV accumulation increased significantly by 60 min and remained at the elevated level until 120 min (end of experiment), even though the stretch was released at 90 min. Under compression, fluctuations in SV accumulation are observed, however, they are not statistically significant.

**Decreased neurite tension disrupts vesicle motion in in vitro Aplysia neurons.** To investigate the effect of tension on local dynamics of individual LDCVs we used in vitro *Aplysia* neurons because they have large growth cones, which are easily visualized [183], are known to generate high levels of tension [5], and have large neuropeptide containing LDCVs [184, 185, 186]. The tension in *Aplysia* neurites was modulated by conducting stretch and compression experiments. Under stretch, neurite tension was increased and no discernable difference in the LDCV velocity distribution could be detected throughout the course of the experiment. Under compression, neurite tension was decreased and LDCV motion was disrupted.

Figure 3.4 shows the velocity distributions of LDCV motion from the stretch and compression experiments where the vertical axis is the relative frequency and the horizontal axis is the vesicle velocity. When the neurite was stretched, the velocity distribution of LDCVs did not change significantly (Figs. 3.4a and 3.4c). Similarly, upon unloading the substrate, there was no observable difference in the velocity distribution of LDCV motion (Fig. 43.4). This experiment shows that an increase in neurite stretch did not significantly affect the velocity distribution of LDCVs in *Aplysia* neurites. On the other hand, when neurite tension was decreased (by compression), then the velocity distribution narrows significantly ($p < 0.01$) and the peak at zero velocity increased as shown in Figs. 3.4e–f. The magnitude of this effect increased as compression is held static (Fig. 3.4g). This indicated that the motion of the LDCVs decreased and they spend more time not moving (zero velocity). Upon stretching from the compressed state, the neurite length was restored. In this case the velocity distribution remained narrow for at least 15 min (end of experiment) as shown in Fig. 3.4h. Videos of vesicle motion are shown for a control (see Movie S1 in the Supporting Material), imme-
Figure 3.3: Snapshots of representative *Drosophila* presynaptic terminals during the experiment. Blue indicates low fluorescence intensity of SV and white indicates high intensity. (a) Control samples show no significant change in vesicle clustering throughout the entire experiment. (b) Increased tension by stretch showed increased accumulation in the 60 min frame and this effect persists until the end of the experiment at 120 min (stretch was removed at 90 min). (c) Decreased tension by compression showed fluctuations in vesicle accumulation, but the amount of vesicles was similar to that of the control throughout the experiment. (Each image is 10 \(\mu\)m by 10 \(\mu\)m).
Figure 3.4: Histograms showing the velocity distribution of LDCVs in *Aplysia* neurons. (a–d) When *Aplysia* neurons are stretched, the velocity distribution of the LDCVs remains the same throughout the time-course of the experiment. Mean ± standard deviation are indicated in the charts and were not found to be statistically different. This shows that increased tension in *Aplysia* neurites does not affect the velocity distribution of LDCVs. (e–h) When *Aplysia* neurons are compressed, the velocity distribution of the LDCVs narrows in width and increases in magnitude throughout the experiment. Mean ± standard deviation are indicated in the charts and velocity distributions were found to be statistically different (Student’s t-test, \( p < 0.01 \)). This shows that decreased tension in *Aplysia* neurites leads to disrupted motion of LDCVs.
ately after compression (Movie S2), 10 min thereafter (Movie S3), and 15 min after re-stretching to original length (Movie S4).

To illustrate this effect on compressed neurites a representative plot of position vs. time for a LDCV (Fig. 3.5) shows that in the control case a vesicle moved back and forth over a large range (black). When the neurite was compressed, LDCV motion immediately decreased (blue). After 10 min of static compression the vesicle motion decreased even further (pink). LDCV motion was not restored even 15 min (end of experiment) after the neurite’s original length was restored (see supplementary videos). Figure 3.6 shows the effect of mechanical compression on LDCV range and the largest processive movement. Here, range refers to the range of vesicle motion and the largest processive movement is the longest distance of continuous vesicle motion as shown in Fig. 3.6a. The range of LDCV motion started out around 550 nm, decreased to 200 nm immediately after compression, and decreased further to 140 nm after an additional 10 min of compression. LDCV range did not recover and remained around 140 nm after removal of compression for 15 min (Fig. 3.6b). Similarly, before stimulation LDCVs exhibited an average largest processive movement of approximately 380 nm, which decreased to 140 nm immediately after compression and further decreased to 80 nm after 10 min of static compression. Vesicle processivity does not recover and remains around 80 nm even 15 min after the neurite is restored to its initial length (Fig. 3.6c).

3.4 Discussion

Our results show that mechanical tension affects both global and local vesicle dynamics. Increased axonal tension (by mechanical stretch) led to increased SV accumulation at the in vivo NMJ of Drosophila. Decreased neurite tension (by mechanical compression) disrupted LDCV motion in in vitro neurites of cultured Aplysia neurons.

SV Accumulation. Increased axonal stretch resulted in enhanced SV accumulation at the in vivo Drosophila NMJ. This accumulation occurred in the absence of extracellular Ca\(^{2+}\), saturated after approximately 50 min of sustained stretch, and persisted for at least 30 min after the stretch was removed.

When Drosophila neurons are stretched the axonal tension increases linearly with applied deformation, and when the deformation is held fixed the tension relaxes to a steady state value in about 25 min. This steady state tension is usually larger than the rest tension [3]. Thus, when the axons are subjected to sustained stretching, they experience an increased level of tension for an extended period of time. It has been
Figure 3.5: Motion of an individual *Aplysia* vesicle as a function of time. Vesicles in control samples show a large motion back and forth about a central region. Immediately after compression is applied the vesicle motion decreases dramatically (blue) and continues to decrease while compression is held (pink) for over 10 min. Even after removal of compression (neurite tension is restored), vesicle motion does not recover (red) for over 15 min. In this plot the motion of one representative vesicle is shown.
Figure 3.6: Range and processivity of vesicles in *Aplysia* neurons. (a) A representative plot showing the range and largest processive motion of a single vesicle. (b) Vesicle range of motion was approximately 550 nm in the control samples. Mechanical compression caused an immediate decrease to 200 nm and the range continued to decrease to 140 nm. (c) In control samples the largest processive motion of vesicles was approximately 380 nm. This decreased to 140 nm immediately after compression and continued to decrease to 80 nm. In both cases, the effect persists for over 15 min after compression was removed. Statistical significance evaluated by Student’s t-test (*p* < 0.05) and denoted in figure by * (error bar = SEM).
hypothesized that actin acts as a structural scaffold to cluster vesicles at the synapse and transport them to the active zone [61, 60]. It has also been shown that tensile strain promotes polymerization of both actin and microtubules [86, 85, 187]. Thus, it is possible that increased axonal tension promotes polymerization of actin scaffolding in the cytomatrix of the synapse, providing more sites for actin-synapsin based tethering of vesicles [188, 61]. As mentioned earlier, the time scale of this vesicle accumulation is orders of magnitude greater than that of stretch induced neurotransmitter release [26, 84, 83, 6]. The amount of time required for polymerization of actin scaffolding structures could explain the slow time scale observed for tension induced SV accumulation. Equivalently, scaffolds would not immediately depolymerize upon decreased tension, which may explain the persistent SV accumulation after stretch is removed. Also due to active regulation of axonal tension, the neuron will rebuild tension shortly after it is reduced [3]. This suggests a possible mechanism for neurons to utilize axonal tension to modulate synaptic structure.

In contrast to stretching, compression of Drosophila neurons has much less effect since the axons actively generate force in response to a loss of tension. This force generation restores axonal tension to a value close to its rest tension in less than 15 min [3]. Thus, when axons are compressed they are subjected to reduced tension only for a short period of time because they are actively shortening to build axonal force. This short perturbation in axonal tension may not be adequate to alter SV accumulation, and thus no effect is observed in the compression experiments. It is, however, worth noting that while a temporary reduction in axonal tension does not alter SV accumulation, a permanent removal of tension through axotomy leads to the dispersion of SVs from the NMJ [28]. These experiments further suggest if neurons do use axonal tension as regulatory signal, then their ability to rapidly rebuild tension may be a mechanism for maintaining synaptic structure.

When the compression was removed, axons were stretched back to their original length causing an increase in tension above the rest tension. In this case we still do not see a significant change in synaptic vesicle accumulation. One possible reason is that there exists a threshold that must be crossed to induce a neural response. Dennerll et al.[8] proposed a three stage model for neural response to axonal tension. They suggest there exists an intermediate regime of axonal tension where neurons respond to deformations with passive viscoelasticity. When tension is decreased below this regime, neurons regulate tension by actively shortening to build force. When tension is increased above this regime, neurons will grow in length to decrease axonal force. In our experiments, when the compression was removed the axons were stretched back out to

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their original length. This deformation back to its original length may be insufficient to elicit a stretch enhancement of SV accumulation. Unfortunately, the current experimental setup does not allow us to stretch the compressed axons beyond their original length.

Our experiments with *Drosophila* showed stretch enhancement of SV accumulation occurred in the absence of extracellular Ca\(^{2+}\). Previous experiments by Siechen et al. [28] showing tension induced (stretch< 10%) SV accumulation were conducted in the presence of extracellular Ca\(^{2+}\) [28]. We observed a similar qualitative response in the absence of extracellular Ca\(^{2+}\). However in comparison to the study by Siechen et al. [28], where the increase in accumulation was close to 300%, the magnitude of increase in our experiments is much smaller (30%). This suggests the attenuated stretch enhancement observed in our experiments could be due to reduced levels of extracellular Ca\(^{2+}\). Previous experiments demonstrated that the stretch enhancement of neurotransmitter release is attenuated in the absence of extracellular Ca\(^{2+}\) in frogs and still occurs when internal stores of Ca\(^{2+}\) are buffered [84]. Chen et al. suggests the stretch enhancement does not occur via secondary messenger pathways or chemical modification but rather could be mediated by mechanical coupling via integrin mechanosensors [84]. Thus, it seems that extracellular Ca\(^{2+}\) may contribute to the magnitude of stretch enhanced SV accumulation, but is not necessary for it to occur. This suggests that the mechanism of stretch enhanced SV accumulation is not completely dependent on extracellular Ca\(^{2+}\).

**Disruption of local LDCV dynamics.** Local dynamics of LDCVs in cultured *Aplysia* neurites was disrupted immediately after neurite tension was reduced (by compression). Vesicle range and processivity continued to decrease as compression was held fixed, and remained low for at least 15 min after compression was removed. Interestingly, increased neurite tension does not seem to affect the LDCV velocity distribution. This may suggest that neurons are able to maintain normal LDCV dynamics under mechanical stretch, such as occurs during motion or natural growth of an organism [65]. On the other hand, this may also suggest LDCV dynamics could be disrupted when neurite tension is lost as occurs in axonal branch pruning and retraction [71, 70, 72].

When *Aplysia* neurites are compressed, any preexisting tension decreased immediately. Reduced neurite tension could affect vesicle dynamics in many ways including altering: microtubule depolymerization, microtubule binding proteins, or molecular motor function. It has been shown that microtubule depolymerization leads to disrupted vesicle transport in *Aplysia* [189]. Depolymerization of microtubules could occur due to the mechanical compression along the neurite [187]. Additionally, if microtubules ex-
hibit buckling [190], this may promote mechanical severing of the microtubule binding protein Tau which leads to disrupted vesicle transport [191] due to polar mismatching or discontinuities.

When neurite tension was restored by reversing substrate compression, vesicle motion did not recover. This suggests that a structural change has occurred that persists after reversal of substrate deformation. Shemesh et al. [192], found that microtubule polar mismatching or discontinuities that lead to disrupted vesicle transport are structurally stable. Therefore, in our experiments, any microtubule reconfiguration due to compression along the neurite may remain after the deformation is reversed. Thus, vesicle motion would remain disrupted even after neurite tension is restored.

**Vesicle transport and accumulation.** Transport and accumulation of vesicles appear related given the collective changes in the spatiotemporal organization of vesicles with stretch. Here we discuss a hypothesis linking the transport of vesicles along the axon to their accumulation at the synapse as shown schematically in Fig. 3.7. In its natural resting state (Fig. 3.7a), an axon has microtubules (green) running along its length, which are crosslinked (black) together to form a network. Vesicles (blue) are attached to microtubules via molecular motors (brown) that transport them along the axon. Some of these vesicles may attach to actin scaffolding (red) and accumulate at the synapse. When the axon is compressed along its length (Fig. 3.7b), tension in the axon is reduced as shown by its wavy morphology. Vesicle transport is disrupted, perhaps due to microtubule depolymerization. Thus, vesicles previously located at the synapse would remain trapped in the actin scaffolding. When the axon is stretched (Fig. 3.7c), tension in the axon is increased and vesicle transport is not disrupted. Increased tension may induce actin polymerization at the synapse allowing more vesicles to attach to the scaffold at the synapse leading to increased accumulation. This putative mechanism between vesicle transport and accumulation may explain an increase in vesicle accumulation under stretch and no effect under compression, although further studies are certainly needed to validate the proposed relationships between mechanical perturbation, actin polymerization, and vesicle movement/accumulation.

### 3.5 Conclusion

Our results show that mechanical stretch affects vesicle dynamics at the local scale of individual LDCV motion as well as the global scale of SV accumulation at the synapse. Increased axonal stretch induced enhanced SV accumulation at the NMJ of *Drosophila*.
Figure 3.7: A connection between vesicle transport and accumulation. (a) A schematic diagram of an axon in its normal resting state. Microtubules (green) extend along the axon and are crosslinked (black) together to form a network. Vesicles (blue) are attached to molecular motors (brown) that transport them along the microtubule network and some accumulate in the actin scaffolding (red) at the synapse. (b) Microtubules depolymerize under compression leading to disrupted vesicle transport while maintaining normal vesicle accumulation at the synapse. (c) A stretched axon exhibits increased vesicle accumulation at the synapse due to tension induced actin polymerization creating more vesicle binding sites.
motor neurons, which saturated after approximately 50 min. This increase in vesicle accumulation occurred in the absence of extracellular Ca\(^{2+}\) and persisted for at least 30 min after axonal stretch was decreased. Mechanical compression disrupted LDCV dynamics in *Aplysia* neurites, including range and processivity. This decreased LDCV motion occurred immediately after compression was applied, and persisted for at least 15 min after compression was removed. These results strengthen the hypothesis that mechanical tension could serve as a mechanism to regulate neuronal function including vesicle transport and synaptic plasticity. The underlying molecular mechanisms that link mechanical tension and vesicle dynamics warrant further investigations.
Chapter 4

Nonequilibrium Vesicle Dynamics

Summary. Vesicle transport in neurons is a highly complex nonequilibrium process. Their subcellular environment is undergoing constant fluctuations from thermal energy and molecular motors. Vesicle transport is an interplay between random motion (passive) and directed motion (active) driven by molecular motors along cytoskeletal filaments. It has been shown that growth, guidance, and vesicle dynamics of neurons is affected by mechanical tension. Here we present a method to analyze vesicle transport via a temporal Mean Square Displacement (tMSD) analysis while applying mechanical strain to neurons. The tMSD analysis allows characterization of active and passive vesicle motion as well as many other parameters including: power law scaling, velocity, direction, and flux. Our results suggest: (1) The tMSD analysis is able to capture vesicle motion alternating between passive and active states, and indicates that vesicle motion in *Aplysia* neurons is primarily passive (exhibiting active motion for \( \sim 8\% \) of the time). (2) Under mechanical stretch (increased neurite tension), active transport of vesicles increases to \( \sim 13\% \), while vesicle velocity remains unchanged. (3) Upon unstretching (decreased tension), the level of active transport returns to normal but vesicle velocity decreases. These results suggest that vesicle transport in neurons is highly sensitive to mechanical stimulation. Our method allows precise characterization of vesicle dynamics in response to applied mechanical strain.

4.1 Motivation

Active transport in the narrow confines of the axon is necessary to moderate growth, maintenance, and function of the neuron [193, 95, 194, 96]. Axons extend up to 1 meter in length in the human body and 30 meters in the blue whale, while remaining just a few microns in diameter [195]. As such, passive diffusion driven by thermal excitation in the subcellular environment is inadequate for efficient vesicle transport. Active transport,

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1Parts of this chapter have been previously published as, W. W. Ahmed, B. Williams, A. Silver, T. A. Saif. "Measuring the non-equilibrium vesicle dynamics in neurons under tension " Lab on a Chip, 2012 (DOI:10.1039/C2LC41109A)
most commonly driven by molecular motors, supplements passive diffusion to increase the efficacy of intracellular transport [88, 89, 39].

The mean square displacement (MSD) is a common tool in quantifying the motion of an object with stochastic behavior. A vesicle embedded in an elastic medium is stationary within the matrix and does not exhibit any time-dependence (MSD\(\propto t^0\)); the motion of a vesicle that is freely moving in a viscous medium will follow the diffusion equation (MSD\(\propto t\)); and the motion of a vesicle moving ballistically will move with a constant velocity (MSD\(\propto t^2\)). These examples depend on ideal environments, unlike the cytoplasm. In practice, when a vesicle in a neuron undergoes passive motion due mainly to thermal energy, its MSD exhibits sub-diffusive behavior between that of an elastic solid and a viscous liquid (viscoelastic) [89, 196]. When vesicles undergo active motion due to a directed force input (e.g. molecular motors, cytoskeletal fluctuations, and cytoplasmic streaming), its characteristic MSD behavior is super-diffusive indicating behavior between a viscous liquid and ballistic motion [88, 90, 92]. On the timescale of observation of vesicle motion the interplay of random interactions and directed motor input develops a continuum of transport type, which can be separated into active and passive regimes [90, 197, 198].

Mechanical tension plays a significant role in determining the growth, guidance, and function of neurons [29, 199, 77]. During development, neurons apply force to their underlying substrate to generate a tension along their axons [64] which directs the growth of the neurite [67]. Further studies show that externally applied tension could initiate axons [2], and that neurons actively regulate their tension [1, 8, 164]. In addition to growth and guidance, mechanical tension also increases the release of neurotransmitters from motor nerve terminals [26]. Recent experiments have shown that neuronal tension is also regulated in in vivo neurons [3] and it modulates in vitro vesicle transport and in vivo accumulation at the synapse [28, 7].

Mechanical tension has also been shown to affect the velocity distribution of vesicles in neurons [7], however the effect on active and passive transport is not clear. Experiments in Aplysia neurons by Ahmed et al. [7] suggest that when neurons are stretched the velocity distribution of vesicle motion remains unchanged. When neurons are compressed the vesicle velocity decreases significantly, suggesting neurite tension is necessary to maintain normal transport. However, the velocity distribution does not contain information on the cumulative motion of vesicles and thus the relationship to overall vesicle transport is not clear. In addition, the previous study could not discriminate by transport type, further limiting interpretation. To understand the role of neuronal tension in vesicle transport, it is necessary to dissect vesicle motion into active
and passive regimes to measure the change in each transport type. Specifically, active transport is an indication of molecular activity, because it requires energy input to drive directed motion, and may provide insight on the molecular mechanisms of mechanosensitivity in vesicle dynamics in neurons. To address this, we use an extension of the MSD calculation to temporally resolve the local transport type and observe switching between active and passive motion during the vesicle trajectory.

In this study, we present a methodology to analyze the active and passive transport of vesicles in neurons while applying mechanical strain and observing via live-imaging. A deformable polydimethylsiloxane cell culture substrate was used to apply mechanical strain to cells while recording vesicle motion using high-speed imaging and high-resolution optics. Vesicle trajectories were analyzed using a temporal Mean Squared Displacement (tMSD) to determine active and passive motion. We found that vesicles spend more time in the active state in response to mechanical stretch, even though their velocities do not change. These results highlight the ability of the tMSD analysis to detect changes in the nonequilibrium dynamics of vesicles in neurons.

4.2 Materials and methods

**Neuron stretching.** We used a stretching system developed for high-resolution live-imaging of cells under applied mechanical strain [86]. Briefly, the system applied a static deformation to a stretchable polydimethylsiloxane (PDMS) cell culture substrate (Fig. 4.1a). The substrate consisted of two different thicknesses, an outer thick region (1.2 mm) to provide mechanical stability and an inner thin region (170 mm), which served as a cell culture well (diameter = 15 mm). The thin region allowed imaging through the PDMS substrate with high-resolution optics (Fig. 4.1b), which is critical for studies of vesicles in neurons. In this study, cells were stretched to 20% tensile strain for 40 min and then the deformation was removed. The substrate deformation was characterized experimentally by digital image correlation and computationally by the finite element method. It was found that the mechanical strain of the cell culture surface was uniform over greater than 95% of the surface area. The details of the stretching system have been previously published [86].

**Cell culture and imaging.** *Aplysia* neurons were isolated and cultured as in previous studies [181]. Briefly, *A. californica* were obtained from the National Resource for *Aplysia* (University of Miami/RSMAS, Miami, FL, USA) and maintained in a 14 °C tank of circulating artificial sea water until use. Neurons were mechanically dissociated
from the Aplysia CNS after a 30–60 min incubation in 1% protease. Cells were plated in artificial sea water antibiotic solution on fibronectin coated PDMS substrates at room temperature for 12 h and then at 14 °C for 24–48 h. Aplysia cells exhibited highly polarized outgrowth exhibiting long straight neurites. Cells with neurites growing parallel with the axis of stretch were used to ensure uniaxial stretching. Experiments were conducted with cells from multiple animals (n > 16) and multiple vesicles (n > 100) were tracked for each cell.

All images were collected on an Olympus IX81 using a (1.15 NA) water immersion objective lens (Olympus America, Center Valley, PA, USA). An Andor Neo sCMOS camera cooled to -30 °C was used to record images (Andor Technology, Belfast, UK). All imaging parameters (e.g., light intensity, exposure time, gain, etc.) were kept constant during all experiments. Differential interference contrast (DIC) image sequences of neurons were captured at a rate of 200 frames per second. Videos were captured approximately every 5 min (n > 20 videos per cell). Image stack alignment to remove drift was completed with a subpixel registration algorithm [200]. Vesicle motion was tracked using an algorithm for precise particle tracking by polynomial fitting with Gaussian weight in MATLAB (The MathWorks, Natick, MA, USA) [182]. A representative image of an Aplysia neurite with particle tracking overlaid is shown in Fig. 4.1c.

Temporal mean square displacement analysis. The Mean Square Displacement (MSD) of a particle is often used to characterize its random motion in space [201, 202, 203]. The MSD is computed over a range of timescales, τ, ranging from zero to the total observation period, t_{max}:

\[
\text{MSD}(\tau) = \langle |r(t + \tau) - r(t)|^2 \rangle_{0 < t < t_{\text{max}}}
\]  

(4.1)

Here, \langle \cdots \rangle indicates an average over the available timesteps \( \tau \) in \([0, t_{\text{max}}]\), and \( r(t) \) is the position vector. The MSD can be fitted to a power law of the form \( \text{MSD}(\tau) = C\tau^\alpha \) where the \( \alpha \)-parameter provides information on the transport behavior. In an ideal system, passive transport by pure diffusion and ballistic motion with constant velocity would be associated with \( \alpha \) values of 1 and 2, respectively. Intermediate values are expected in complex environments.

We extract \( \alpha \) as a function of time to study transport in the intracellular environment. To accomplish this, we use a temporal MSD (tMSD) analysis [197], where a rolling window of width \( \tau_{\text{max}} \) centered on time \( t \) is used to generate the function \( \alpha(t) \). To observe rapid shifts in transport behavior, the width of the window should be
Figure 4.1: (a) An image of the stretchable PDMS substrate with the self-contained cell culture surface (diameter = 15 mm). (b) A schematic side view of the PDMS substrate showing the thick region for ease of handling and the thin region for high-resolution imaging. (c) A representative image of an Aplysia neurite cultured on a stretchable substrate. Neurons were stretched 20% along the x-axis of the image frame. Vesicle trajectories are overlaid to show that many vesicles could be tracked. Trajectories that did not show significant motion or were less than 100 frames were not analyzed.
minimized while maintaining capacity to estimate $\alpha(t)$. For vesicle motion in *Aplysia* neurons, we used $\tau_{\text{max}}$ of 500 ms. The $t$MSD function associated with a time $t$ is:

$$t\text{MSD}(\tau) = \langle |r(t' + \tau) - r(t')|^2 \rangle_{(t-\tau_{\text{max}}/2)<t'<(t+\tau_{\text{max}}/2)}$$

(4.2)

where $t'$ indicates time within the rolling window. To compute the $t$MSD obtained from vesicle tracking experiments we rewrite the equation using displacements of non-overlapping time-spans $\tau$:

$$t\text{MSD}_n = \frac{1}{E[(N-1)/n]} \sum_{i=1}^{E[(N-1)/n]} |r_{m+1} - r_{(i-1)n+1}|^2$$

(4.3)

for $n = 1, \ldots, N - 1$, where $E[(N-1)/n]$ denotes the integer part of $(N - 1)/n$. $r_j = (x_j, y_j)$ is the position in frame $j$ of the rolling window, $\tau_{\text{max}} = (N - 1)\Delta t$ is the local time window, where $N$ is the total number of frames in the rolling window, and $\Delta t$ is the time step between frames. Accordingly, the timescale is $\tau = n\Delta t$ where $n$ is an integer value ranging from 1 to $N - 1$, varying the timescale from the time between frames to the duration of the rolling window. By utilizing non-overlapping displacements the correlations between measurements are avoided [203, 204]. Thus a $t$MSD curve is associated with each time point. The $t$MSD curves are fitted with a power law on the interval 100–160 ms, and the $\alpha$-parameter is extracted as a function of time, $\alpha(t)$. To eliminate spurious values in $\alpha(t)$ attributed to noise, a smoothing filter was applied. The $t$MSD analysis was verified and calibrated experimentally and computationally via particle tracking of fluorescent nanoparticles in water and simulations of Brownian and ballistic motion.

In the complex intracellular environment of the living cell, a continuous spectrum of $\alpha(t)$ is observed. In this viscoelastic system [196, 205], clear examples of active transport exhibit $\alpha$ values of approximately 1.6. We partition transport states as passive for $\alpha < 1.4$ and active for $\alpha > 1.4$. These values of $\alpha$ were chosen in an attempt to ensure that all diffusive and sub-diffusive processes were considered passive due to the difficulty of interpreting the transition from diffusive and sub-diffusive regimes. The transition from diffusion to sub-diffusion is often associated with transitioning from a purely viscous solution to viscoelastic [206], but interpretation in a living cell is not so clear [90]. We use the $t$MSD analysis to analyze endogenous vesicle motion in neurons as a function of time.
4.3 Results

Extraction of physical parameters. The time evolution of the $\alpha$-parameter characterizes the nonequilibrium behavior and allows identification of transitions between active and passive transport. The entire trajectory for an individual particle is then dissected into segments based on transport state. Fig. 4.2a plots a sample trajectory of vesicle motion consisting of 3 segments. The green and blue segments exhibit active transport, while the red segment is passive. For each of these 3 segments, Fig. 4.2c plots the representative $t$MSD curves, bounded by ideal curves for ballistic ($\alpha = 2$) and Brownian motion ($\alpha = 1$). The two active segments (green and blue) have distinctly steeper slopes than the passive segment. Fig. 4.2d plots the $\alpha$-parameter for the entire trajectory as a function of time, illustrating the drop in $\alpha$ associated with a transition to passive transport.

In the neuron, vesicle transport away from the cell body (anterograde) and back towards the cell body (retrograde) often serve different functions. This interplay between anterograde and retrograde motion controls the spatial organization of vesicles and thus their function in the neuron [43]. To distinguish between anterograde and retrograde motion we simplify the vesicle trajectory with the Douglas–Peucker algorithm, which eliminates high frequency motion associated with thermal excitation [207]. The resultant trajectory, illustrated in Fig. 4.2b, is used to determine the transport direction.

Other parameters of interest for each segment of the trajectory are calculated with respect to classification of segment type (active or passive). The vesicle velocity is calculated from the time-derivative of the smoothed particle trajectory. The duration of each segment is determined by the number of time steps comprising the segment. The distance traveled for each active segment is computed by the length of the Douglas–Peucker fitted trajectory associated with the segment. The “%-Active” is calculated by finding the duration of all active states and normalizing by the total time of particle motion. The “% Forward” is calculated as the ratio of forward motion (anterograde) to the total vesicle motion. Interesting transport properties are readily observed with multivariate analysis using these parameters. Calculating parameters such as these allow characterization of vesicle transport in neurons and detection of changes with time.

Characterising vesicle dynamics. To provide an experimental control, vesicle dynamics were analyzed in unperturbed Aplysia neurites. Fig. 4.1 shows a representative image of an Aplysia neurite with particle trajectories overlaid. Here it is evident that many vesicles can be tracked per neurite. Particles that are tracked for less than 100
frames or do not exhibit significant motion are not analyzed. Fig. 4.2a shows a single vesicle switching between active to passive motion as indicated by the a-parameters on the tMSD and the $\alpha(t)$ plot (Fig. 4.2c–d). This behavior is reflective of the stochastic nature of intracellular transport where vesicles alternate between directed motion along filaments and random motion when not driven by molecular motors. Thus it may be expected that vesicles moving passively have no apparent direction and actively transported vesicles should be directional.

Fig. 4.3 plots angular histograms of transport direction where $0^\circ$ represents anterograde motion away from the cell body and towards the neurite terminal, and $180^\circ$ is motion back towards the cell body. Histograms show relative frequency as normalized by each transport type. Due to the high alignment of microtubules in the neurite, the angular histogram for active transport is highly polarized (Fig. 4.3a). On average, no overall preference for anterograde or retrograde motion is observed. In the passive state the vesicles move in all directions with the same frequency (Fig. 4.3b).

Fig. 4.4 plots histograms of the active and passive vesicle velocities. Similarly, relative frequency is normalized by transport type. Median values of anterograde (+) and retrograde (2) motion were nearly identical, thus only one value for both directions is reported. Velocities of active vesicles peaked at a median of 259 nm/s, approximately twice the value measured for velocities of passive vesicles (133 nm/s). Thus in the active state, the $\alpha$-parameter is greater (indicating greater directional persistence) as well as the effective velocity of the vesicles.

These three parameters: $\alpha$, $\theta$, and $v$, allow for characterization of the vesicle motion in Aplysia neurons. Vesicles exhibit behavior ranging from sub-diffusive to super-diffusive with most values of $\alpha$ between 0 and 1.8, showing that their motion ranges from being almost pinned in an elastic medium ($\alpha = 0$) to moving nearly ballistically ($\alpha = 2$).

**Active behavior increases due to stretch.** Aplysia neurons were stretched 20% along the direction of their neurite, resulting in increased tension in the neurite. Tensile strain of the substrate was held static for 40 min, then the stretch was removed and the substrate returned to its original unstrained configuration for an additional 40 min. When tensile strain is applied it is expected that the neurite tension increases instantaneously followed by a viscoelastic relaxation. Similarly when strain is removed, tension instantaneously drops followed by a force build up [8, 164, 3].

The following results are split into three stages. (1) Control: the resting state of the cell before mechanical perturbation. (2) Stretch: the average behavior during 40
Figure 4.2: Sample plots showing temporal Mean Square Displacement (tMSD) analysis of a single vesicle. (a) Trajectory of a single vesicle that is alternating between active (green and blue) and passive (red) motion. (b) The Douglas–Peucker algorithm was used to simplify trajectories to determine direction of motion. (c) Characteristic tMSD plots are shown for the states of vesicle motion above as well as ideal curves for ballistic ($\alpha = 2$) and random motion ($\alpha = 1$). Sample tMSD data correspond to black dots in matching color regions of (d). Active motion shown by green and blue curves exhibit clearly steeper slopes than the passive curve shown in red. (d) Plot of $\alpha(t)$ color coded to correspond with trajectory plots. It is clear that vesicles exhibit a large range of $\alpha$-values and that the green and blue segments exhibit significantly higher $\alpha$ than the red.
Figure 4.3: Angular histograms showing the direction of vesicle motion in terms of relative frequency. A direction of $0^\circ$ indicates anterograde motion from the cell body towards the neurite terminal. A direction of $180^\circ$ indicates retrograde motion back towards the cell body. (a) Vesicles undergoing active transport exhibit highly polarized motion along the axis of the neurite. (b) Vesicles in the passive state show random motion in all directions with no specific preference.
Figure 4.4: Velocity distributions of active and passive motion. Anterograde (+) and retrograde (2) motion showed similar velocities so only one value is reported for both. (a) Active velocities show a wide spread with a median value of 259 nm/s. The distribution tails show velocities over 500 nm/s in both directions as well as a secondary peak in anterograde transport around 550 nm/s. (b) Passive velocities show a narrower spread as expected with strong peaks. The median value of passive velocity was 133 nm/s, which is close to the value expected of diffusion.
Figure 4.5: Active transport increases due to stretch. (a) The average $\alpha$-parameter increases from $\alpha = 0.8$ (red) to $\alpha = 0.9$ (green) when stretched (increased tension). Upon unstretching, the activity returns to its previous state $\alpha \approx 0.8$ (blue). (b) Under stretch the percentage of active transport increases from 7.5% (red) to 12.7% (green). Upon unstretching it decreases to 9.5% (blue), which is not significantly different from the control. (c) Throughout the experiment the vesicles show approximately 50/50 motion forward (anterograde) and backward (retrograde). Thus the net flux does not change. * indicates statistical difference by Student’s t-test ($p < 0.05$) ($n > 16$ cells, $> 100$ vesicles per video, $> 20$ videos per cell) (error bars = SEM).

min of static stretch. (3) Unstretch: the average behavior during the 40 min following unloading of the substrate back to 0% strain.

Fig. 4.5a shows that in the control state vesicles exhibit $\alpha = 0.8$, on average, suggesting most vesicle motion is considered sub-diffusive and passive. In the stretched state this value increases significantly to $\alpha = 0.9$ (Student’s t-test, $p < 0.05$). This suggests that the overall behavior of vesicles is shifting towards a more active regime. This result is supported by the average percentage of time that vesicles are active. Fig. 4.5b shows the vesicles in the control state spend 7.5% of the time moving actively. When the neuron is stretched, the vesicles spend significantly more time in the active state (12.7%) (Student’s t-test, $p < 0.05$). In both cases, when the neuron is unstretched, the $\alpha$-parameter and the percentage of time active decrease to values not significantly different than before they were stretched. Interestingly, while the activity of the vesicles seems to change in response to stretch, the directionality does not show any change (Fig. 4.5c). Thus even though vesicles become more active, their time averaged flux is not expected to change (assuming all vesicles are identical).
Figure 4.6: Velocity decreases upon unstretching. Anterograde motion is labeled (+) and retrograde motion is labeled (−). When stretch is applied, there is no significant difference in active or passive vesicle velocity from the control. However, upon unstretching (relaxation of tension) both active and passive vesicle velocity decreases significantly. * indicates statistical difference by Student’s t-test ($p < 0.05$) ($n > 16$ cells, > 100 vesicles per video, > 20 videos per cell) (error bars = SEM).

**Vesicle velocity decreases upon unloading.** Fig. 4.6 shows the response of vesicle velocity to mechanical strain. The average active and passive velocities for each stage of the experiment are shown for both anterograde (+) and retrograde (−) motion. Notice that the speed of anterograde and retrograde motion is nearly identical for both active and passive motion.

When mechanical stretch is applied, there is consistently a small decrease in velocity for all cases, but they are not found to be statistically significant. However, upon unstretching the substrate back to its 0% strain configuration, both active and passive velocities decreased significantly ($p < 0.05$) (anterograde and retrograde). Thus upon relaxation of neurite tension, vesicle velocity decreases.

### 4.4 Discussion

Neuronal behavior is sensitive to mechanical forces as shown in studies of morphology, development, and signaling [29, 199, 77, 163]. Vesicle transport is a critical process supporting all of these functions, but its response to neuronal force is not well understood. Studying the mechanical sensitivity of vesicle transport in neurons may lead to a deeper understanding of the role of force in neuronal function.
Our results show that active and passive vesicle motion are clearly observed in vesicle transport in *Aplysia* neurons. For control neurons, a mean value of $\alpha = 0.8$ characterized the vesicle motion as primarily passive (Fig. 4.5), consistent with behavior reported for transport of quantum dots in PC12 neurites [208]. This suggests that vesicles are predominantly in a state of passive motion with sparse periods of active transport. Interestingly, in stretched neurites we observe an increase to $\alpha = 0.9$ indicating a shift towards more active behavior associated with an increase in the %-Active from 7.5% (control) to 12.7% when stretched. Thus the increase in active motion skews the overall distribution of the $\alpha$-parameter upwards. Our results are the first evidence of increased active transport of vesicles in neurons due to mechanical stretch. Upon unstretching, the mean $\alpha$-parameter returned to a level similar to the control state. The mechanism of this stretch-induced increase in activity is not understood and is a current topic of research. One possible mechanism is cytoskeletal fluidization [209] or realignment [86] could decrease drag on vesicles, allowing them to undergo active transport for longer durations before encountering an obstacle. In this case the % Active would increase, but the vesicle velocity could stay the same since it is mainly dependent on available ATP concentration [87].

While the amount of active transport in neurons increased due to stretch, there was no effect on the velocity during the active states. The median velocity of active transport was approximately 259 nm/s, which is in agreement with previous studies of microtubule based transport via kinesin and dynein [210, 211, 212, 213, 214]. It is interesting that anterograde and retrograde transport exhibit the same velocity since their motion is driven by different motors. This lack of change in velocity may suggest that ATP availability and drag on the vesicle do not change due to stretch. However, upon unstretching and relaxing tension in the neurite, both passive and active vesicle velocities decreased significantly below the velocities in the control neurons. This may be due to cytoskeletal crowding leading to higher density of obstacles for vesicle motion [215, 90]. These results agree with those previously reported by Ahmed et al. [7], where the overall velocity distributions did not change due to stretch but narrowed under compression.

This study highlights the power of the tMSD analysis to characterize transport dynamics. Previous studies of vesicle motion often use velocity as a measure of activity [86, 211, 213]. However, changes in the amount of active transport are not necessarily manifested in changes in vesicle velocity. The tMSD analysis is able to uncouple these two parameters and show their independent behavior.

This methodology will help develop an understanding of vesicle transport during
development and growth as well as impaired transport in neurodegenerative diseases. It can also be directly extended to analyze motion of any subcellular constituents in a cell that can be imaged and tracked. Furthermore, a multivariate analysis would allow investigation of active transport as a function of space, time, direction etc. This could uncover spatial and temporal inhomogeneities in subcellular dynamics that could be correlated with cell structure and externally applied mechanical strain.

4.5 Conclusion

While the complexity of biological systems is daunting, recent advancements in microscopy, image processing, and theoretical frameworks provide promising tools for analysis. Here we presented a method that combines a polymer stretching system, high spatio-temporal vesicle tracking, and recently developed tools in nonequilibrium statistical physics to investigate the mechanosensitivity of neurons. Our experiments are the first evidence that mechanical stretch increases the active transport of vesicles in Aplysia neurons. We found: (1) Stretching (increased tension) results in more active transport of vesicles with no change in their velocity. (2) Subsequent unstretching (decreased tension) returns active transport back to its original level but leads to decreased vesicle velocity.
Chapter 5

Active transport is modulated by tension

Summary. Effective intracellular transport of proteins and organelles is critical in most cells, but especially for proper neuron functionality. In neurons, most proteins are synthesized in the cell body and must be transported through long and thin structures over long distances where normal diffusion is insufficient. Neurons transport subcellular cargo along axons and neurites through a stochastic interplay of active and passive transport. Mechanical tension is critical in maintaining proper function in neurons, but its role in transport is not well understood. We observe the active and passive transport of vesicles in in vitro Aplysia neurons while modulating neurite tension via applied strain, and analyze the resulting dynamics as an active matter system. We found that tension in neurons modulates active transport of vesicles by increasing molecular motor activity, probability of active motion, effective diffusivity, and induces a retrograde bias. Our results suggest that mechanical tension modulates active transport processes in neurons and that external forces can couple to internal (subcellular) forces and change the overall transport dynamics.

5.1 Introduction

A living cell is a natural example of an active matter system that is by definition out-of-equilibrium. Energy input occurs at the molecular scale and gives rise to nonthermal fluctuations of the cytoskeleton and directed transport through the medium. These systems maintain complex nonequilibrium dynamics in the absence of external force fields, which is a hallmark of living or active matter [216, 217]. In addition to exhibiting interesting bulk mechanical properties [218, 219, 220], biological active matter systems often exhibit transport properties due to the collective behavior of molecular motors [91, 221, 222, 223, 90, 224].

Active transport is critical in maintaining biological function in living cells. This is

Parts of this chapter are currently under review for publication as, W. W. Ahmed, T. A. Saif. "Active transport of vesicles in neurons is modulated by mechanical tension"
especially true in neurons where axons and dendrites have long aspect ratio geometry, which limits the effectiveness of passive diffusion. Cargo transport in cells is mediated by a stochastic interplay of passive diffusion and active transport along cytoskeletal elements driven by molecular motors [88, 39]. This process allows the neuron to control the spatial organization of vital proteins and molecules throughout its complex structures. For instance, if a synaptic protein is synthesized in the cell body, it may need to be transported the entire length of the axon (which could be over 1 meter in a human) to reach its functional target. Proper transport of vesicles and cargo to specific locations in the cell is critical in building and maintaining synaptic machinery as well as modulating synaptic plasticity [97, 44, 225]. Additionally, a deficit in neuronal transport is an early pathogenic event and possibly the cause of several neurodegenerative diseases [43, 100]. Hence, investigating the mechanisms of neuronal transport is critical in understanding neuronal function.

Mechanical tension exists in neurons and recently has been implicated in maintaining normal vesicle dynamics [28, 3, 7, 226], but the underlying mechanism is not well understood. To this end, we analyzed active transport of endogenous large dense core vesicles of in vitro Aplysia neurons under external mechanical strain, and interpreted their dynamics under the framework of active matter physics. A schematic diagram of vesicle transport and a representative trajectory are shown in Fig 5.1a,b. Our results suggest that external forces applied to the neuron can couple to the internal (subcellular) forces to change overall transport dynamics of the system. Mechanical tension increases active transport of vesicles in neurons via modulating collective and single motor dynamics. When neurons are stretched we observe: Increased activity of molecular motors; Increased probability of active motion of vesicles; Increased mean squared displacement (MSD) of vesicles; Increased effective diffusivity; and a retrograde bias of vesicle motion. When neurons are compressed, we observe decreased active transport. These results highlight the role of mechanics in neuronal function.

5.2 Materials and methods

Cell Culture. Aplysia neurons were isolated and cultured as in previous studies [181]. A. californica were obtained from the National Resource for Aplysia (University of Miami/RSMAS, Miami, FL, USA). Neurons were mechanically dissociated from the Aplysia CNS after a 30-60 min incubation in 1% protease. Cells were plated in artificial sea water antibiotic solution on fibronectin coated PDMS substrates at room temperature for 12 h and then at 14 °C for 24-48 h. Aplysia cells exhibited highly
polarized outgrowth exhibiting long straight neurites. Experiments were conducted with cells from multiple animals ($n > 16$) and multiple vesicles ($n > 200$) were tracked for each cell.

**Cell Stretching.** Neurons were stretched using a system developed for high-resolution live-imaging of cells under applied mechanical strain [86]. The system applied a static deformation to a stretchable polydimethylsiloxane (PDMS) cell culture substrate. In this study, cells were stretched (20%) or compressed (8%) for 40 min and then the deformation was removed. All time points prior to $t = 0$ min are internal controls. The substrate deformation was characterized experimentally by digital image correlation and computationally by the finite element method. It was found that the mechanical strain of the cell culture surface was uniform over greater than 95% of the surface area. The details of the stretching system have been previously published [86].

**Microscopy and Image Analysis.** All images were collected on an Olympus IX81 using a (1.15 NA) water immersion objective lens (Olympus America, Center Valley, PA, USA). An Andor Neo sCMOS camera cooled to -30 °C was used to record images (Andor Technology, Belfast, UK). All imaging parameters (e.g., light intensity, exposure time, gain, etc.) were kept constant during all experiments. Differential interference contrast (DIC) image sequences of neurons were captured at a rate of 200 frames per second. Videos were captured approximately every 5 min ($n > 20$ videos per cell). Image stack alignment to remove drift was completed with a subpixel registration algorithm [200]. Large dense core vesicle motion was tracked using an algorithm for precise particle tracking by polynomial fitting with Gaussian weight [227] in MATLAB (The MathWorks, Natick, MA, USA). Active and passive vesicle motion was determined using the $t$MSD algorithm [226]. All computations, statistical analysis, and plots were generated in MATLAB.

### 5.3 Results and discussion

**Active transport of vesicles.** In this study, the primary interest is in active transport of vesicles. To discriminate between active and passive motion, vesicle dynamics were analyzed using a temporal Mean Squared Displacement ($t$MSD) algorithm to extract the local behavior of the vesicle as a function of time [226]. The algorithm estimates the local behavior for time, $t$, by calculating the $t$MSD of a rolling window
centered about the time of interest,

\[ t \text{MSD}(\tau) = \langle |r(t' + \tau) - r(t')|^2 \rangle_{(t-\tau_{\text{max}}/2)<t'<(t+\tau_{\text{max}}/2)} \]  \hspace{1cm} (5.1)

where \( \langle \cdots \rangle \) indicates an average over timescale \( \tau \), \( r(t) \) is the position vector as a function of time, \( t' \) is the time in the rolling window, \( \tau \) is the timescale, and \( \tau_{\text{max}} \) is the width of the rolling window (\( \tau_{\text{max}} = 500 \text{ ms} \)). The \( t \text{MSD} \) is then fitted to a power law of the form, \( t \text{MSD}(\tau) = C\tau^{\alpha} \) on the interval 100 – 160 ms, and the power law scaling, \( \alpha(t) \), can be extracted as a function of time to indicate the type of diffusion the vesicle is undergoing (\( \alpha < 1 \), subdiffusive; \( \alpha = 1 \), Brownian; \( \alpha > 1 \), superdiffusive). It should be noted that MSD analyses can be highly sensitive to noise and should be interpreted carefully [228, 229]. Previously it has been shown in \textit{Aplysia} neurons that \( \alpha > 1.4 \) is indicative of active motion driven via molecular motors, and \( \alpha < 1.4 \) is considered passive motion [226]. By convention, positive values indicate direction forward towards the neurite terminal (anterograde) and negative values are rearward (retrograde) towards the cell body. This method was used to determine if vesicles were undergoing active or passive motion as used throughout the study. A sample trajectory is shown in Fig 5.1b where a vesicle alternates between active and passive motion.

**Probability of active motion.** As vesicles move around inside the neuron they stochastically switch between molecular motor driven active transport and passive diffusion. To quantify the amount of active transport the probability of active motion, \( P_a \), is estimated from each image sequence by dividing the time of active vesicle motion by the total tracked time,

\[ P_a = \langle \frac{t_{\text{active}}}{t_{\text{total}}} \rangle \]  \hspace{1cm} (5.2)

where \( \langle \cdots \rangle \) indicates an ensemble average. In control neurons (Fig 5.1c, red), the probability of active motion is steady and remains \( \sim 0.09 \). This value is similar to the probability of active motion observed for tracer beads in a remodeling actin-myosin gel [230]. This similarity is surprising because the neuron is highly structured and significant reorganization is not expected, yet it maintains dynamics similar to a far from equilibrium remodeling actin-myosin gel. In stretched neurons (Fig 5.1c, blue), \( P_{a,\text{stretch}} \) increases sharply and peaks at \( \sim 0.18 \) after 25 min of stretch. Interestingly, after 25 min, \( P_{a,\text{stretch}} \) begins to decay for large vesicles (\( r_{\text{large}} > 350 \text{ nm} \)) but remains elevated for small vesicles (\( r_{\text{small}} < 350 \text{ nm} \)). In compressed neurons, \( P_{a,\text{compress}} \) decreases slightly to \( \sim 0.07 \) and remains low for the duration of the experiment (Fig 5.1c, green). It is interesting that small vesicles consistently have a higher probability of active motion.
Figure 5.1: Active transport of vesicles in neurons is mediated by molecular motor activity. (a) Simplified schematic of vesicle transport in a neurite. Vesicles alternate between active transport along microtubules and passive brownian-like motion. Mechanical strain is used to modulate tension along neurite length while tracking vesicle dynamics. (b) Plot of a representative trajectory, where the $tMSD$ algorithm is used to determine active ($\alpha \geq 1.4$) and passive motion ($\alpha < 1.4$). This example clearly shows the vesicle switching between active and passive behavior. (c) Probability of active motion of vesicles in control (red) neurons exhibit stable behavior with $P_a,\text{control} \approx 0.09$. Under stretch (blue), vesicles exhibit more active motion, which peaks at $P_a,\text{stretch} \approx 0.18$ after 25 min. The activity of small (×) vesicles remains high whereas large (□) vesicle activity decreases. Vesicles in compressed (green) neurons exhibit slightly decreased activity relative to the control (red). [control (red), stretch (blue), compress (red), all vesicles (○), large vesicles (□), small vesicles (×)].

This may be due to less resistance to motion when compared to larger vesicles.

Mean Squared Displacement. The MSD($\tau$) of vesicles provides a measure of overall vesicle motion. When neurons are stretched, the MSD of vesicles increases significantly. This effect is greatest at $t \sim 25$ min after stretch has been applied, and is most noticeable at large timescales, $\tau > 10$ sec (Fig 5.2b). Conversely, when neurons are compressed (Fig 5.2c), the MSD decreases, exhibiting significantly decreased motion for all timescales, $\tau$.

If the particle motion is a combination of normal diffusion and drift then,

$$\text{MSD}(\tau) \approx 4D_e\tau + V_d^2\tau^2$$

(5.3)

can be used to estimate the effective diffusion coefficient, $D_e$, and the effective drift velocity, $V_d$ [203]. It is worth noting that the effective diffusion coefficient, $D_e$, will differ from the equilibrium diffusion coefficient due to the existence of active processes. Using a least squares fit of the MSD, we estimate the effective diffusion coefficient to be $D_e \approx 4 \times 10^{-3} \mu m^2/s$, which is similar to values found previously [91]. By the same method we find the effective drift velocity of the ensemble of particles to
be $V_d \approx 30$ nm/s. The drift velocity, $V_d$, is the ensemble averaged drift of particles at large time scales. This is significantly lower than the instantaneous active vesicle velocity calculated from the tMSD analysis, $V_i \approx 260$ nm/s, which is indicative of short timescale velocity [226]. The instantaneous vesicle velocity is the run velocity as driven by molecular motors and is thus related to the drag force on the vesicles.

While it is unclear if the fluctuation-dissipation theorem (FDT) can be applied in the complex subcellular environment of living cells, it is illustrative to consider its consequences. Given the Einstein-Smoluchowski relation, $D = \mu k_B T$ where, $\mu = V/F$ is the particle mobility, we can estimate the force on the vesicles, $F = V k_B T / D_e$ (in a system obeying FDT). If we estimate the force on vesicles at short timescales ($V = V_i \approx 260$ nm/s), we find that $F \approx 250$ pN. This estimated force value is very high, and it is unlikely that an ensemble of motors could generate this force on one vesicle, suggesting violation of the FDT. Breakdown of FDT has been observed in several other out-of-equilibrium biological systems [205, 231, 232, 90], but to our knowledge this is the first instance in vesicle transport in neurons.

**Velocity Autocorrelation.** The velocity autocorrelation function is a time-dependent correlation function that reveals information on the underlying dynamical processes in a system [233]. The normalized velocity autocorrelation function,

$$\psi(\tau) = \frac{\langle v(t_0) \cdot v(t_0 + \tau) \rangle}{\langle [v(t_0)]^2 \rangle}$$

(5.4)
provides insight on the strength of interactions in the system. For a completely non-interacting system, $\psi(\tau) = 1$, because the particle maintains its initial velocity independent of $\tau$. Thus the rate of decay of $\psi(\tau)$ is a measure of how quickly the particle velocity decorrelates. Accordingly, the self diffusion coefficient can be evaluated from the integral of the velocity autocorrelation function, $D_s = v_0^2 \int_0^{\infty} \psi(\tau) d\tau$, which is a special case of the Green-Kubo relations for obtaining transport coefficients [233].

In control neurons, $\psi(\tau)$ shows a characteristic decay and a self diffusion coefficient, $D_{s,\text{control}} \approx 4 \times 10^{-3} \mu m^2/s$ (Fig 5.3a), in agreement with the previously calculated value from fitting the MSD($\tau$). When the neuron is stretched, $\psi(\tau)$ shows a slower decay, indicating weaker interactions and a higher self diffusion coefficient, $D_{s,\text{stretch}} \approx 1 \times 10^{-2} \mu m^2/s$ (Fig 5.3b). Under compression, the behavior of $\psi(\tau)$ decays slightly more rapidly than the control, $D_{s,\text{compress}} \approx 2 \times 10^{-3} \mu m^2/s$. Notice that $\psi(\tau)$ at large time exhibits small oscillations around zero, which is characteristic of a strongly interacting material. These results suggest that the self diffusivity of the vesicle increases when the neuron is stretched, and decreases due to compression.

**Van Hove Correlation.** The Van Hove Correlation function (VHC) is the probability distribution of the vesicle displacements, as a function of timescale, $P(\Delta x, \tau)$, where $\Delta x(\tau) = x(t + \tau) - x(t)$ (5.5)

The VHC for a passive actin gel is expected to be mainly Gaussian [234], and deviations from this behavior can be attributed to active (nonequilibrium) behavior. It has been shown that in active gels, the VHC of embedded probe particles exhibits a broader Gaussian regime and marked non-Gaussian tails [230]. The broader Gaussian region has been attributed to the collective action of many motors [230], and the non-Gaussian tails suggest the action of a single motor [234].

The results of the calculated VHC’s and Gaussian fits are shown in Fig 5.4, where active and passive behavior were separated using the tMSD analysis ($\alpha_{\text{active}} \geq 1.4$ and $\alpha_{\text{passive}} < 1.4$)[226]. The statistics of the Gaussian fit are shown as $\mu = \bar{x} \pm \sigma$. A non-zero mean, $\bar{x}$, of the distribution indicates a bias in vesicle motion. The standard deviation, $\sigma$, is a measure of the Gaussian width and is indicative of collective molecular motor activity [230]. A non-Gaussian parameter is defined as,

$$\xi = \frac{\langle \Delta x(\tau)^4 \rangle}{3\langle \Delta x(\tau)^2 \rangle^2} - 1$$ (5.6)
Figure 5.3: Normalized velocity autocorrelation functions. (a) Vesicles in control neurons exhibit a characteristic decay of $\psi(\tau)$ and $D_{s,\text{control}} \approx 4 \times 10^{-3} \mu m^2/s$. (b) When neurons are stretched, vesicles exhibit a slower decay of $\psi(\tau)$ and thus have a higher estimated self diffusion coefficient of $D_{s,\text{stretch}} \approx 1 \times 10^{-2} \mu m^2/s$. (c) Under compression, vesicles in neurons exhibit behavior similar to the control case. The estimated self diffusion coefficient is slightly lower, $D_{s,\text{compress}} \approx 2 \times 10^{-3} \mu m^2/s$. 
which is a dimensionless parameter that is zero for a Gaussian distribution but takes on non-zero values to characterize deviation from Gaussianity, and provides a measure of single motor activity [234]. The VHC functions are plotted where the subscript “a” indicates active vesicles and the subscript “p” indicates passive vesicles (Fig 5.4).

Overall, in all experimental conditions and at all timescales observed, \( \sigma_a > \sigma_p \) and \( \xi_a < \xi_p \). The larger Gaussian width, \( \sigma_a \), observed in active motion suggests that it is due to a collective ensemble of several molecular motors [230]. The larger non-Gaussian parameter, \( \xi_p \), observed in passive motion suggests many events are caused by single motor activity [234] that lead to jumps in vesicle motion but are not directed enough to generate active transport.

When neurons are stretched, \( \xi_{\text{stretch}} > \xi_{\text{control}} \) for shorter timescales (\( \tau = 0.15 \) and 1 sec), indicating increased activity of single molecular motors. In addition, \( \sigma_{\text{stretch}} > \sigma_{\text{control}} \) for larger timescales (\( \tau = 1 \) and 5 sec), suggesting higher activity of molecular motors giving rise to greater vesicle motion. It is worth noticing, that for stretched neurons the VHC at \( \tau = 1 \) sec (Fig 5.4b) exhibits a local minima at \( \Delta x \approx 0 \), indicating that active vesicles are more likely to move than stay stationary. When neurons are compressed, they exhibit the opposite behavior: decreased motor behavior at all timescales.

Interestingly, control neurons show no significant bias in vesicle motion (\( \bar{x}_{\text{control}} \approx 0 \)), but when neurons are stretched/compressed they show a retrograde bias of vesicle motion (\( \bar{x}_{\text{stretch}} = -26 \) nm and \( \bar{x}_{\text{compress}} = -10 \) nm) at larger timescales. This is most clear in the stretched neuron at \( \tau = 5 \) sec (Fig 5.4b), where the VHC is clearly not symmetric, but biased towards retrograde movements. To quantify the bias we estimate the probability of retrograde motion as,

\[
p_r(\tau) = \int_{-\infty}^{0} P(\Delta x, \tau) \, d\Delta x
\]

where \( p_r(\tau) \) is the probability that a particle is moving in the retrograde (\( \Delta x < 0 \)) direction for a given timescale, and \( P(\Delta x, \tau) \) is the VHC. In all other experimental conditions we see no significant bias, \( p_r \approx 0.5 \). However, for stretched neurons we see \( p_{r,\text{stretch}}(\tau = 5 \text{ s}) = 0.59 \) for active vesicles, indicating there is a significantly larger probability for vesicles to move in a retrograde direction. The retrograde bias emerges at long timescales, suggesting it is a result of coordinated activity of many molecular motor events.

The dependence of \( \mu \) and \( \xi \) on timescale is expected because of the type of phenomenon being measured. The bias and collective activity (measured by \( \bar{x} \) and \( \sigma \))
respectively) of vesicle motion are processes that require a large number of molecular interactions to occur. Thus their behavior occurs over longer periods of time and becomes visible at longer timescale. The activity of single molecular motors (measured by $\xi$) are single (short) events which are evident at short timescales, but are averaged out at over longer timescales. Hence, changes in $\mu$ are more noticeable at long timescales, and $\xi$ at short timescales.

In summary, these results suggest that neuronal stretch leads to increased molecular motor activity at all timescales at the level of single motors as well as collective ensembles. Conversely decreased tension leads to decreased activity. These results highlight the possible role of neuronal tension in both neurodegenerative diseases and synaptic plasticity.

**Neurodegenerative Disease.** Deficits in axonal transport are early pathogenic symptoms and possibly causes of several neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, and amyotrophic lateral sclerosis (ALS) [43]. Extreme stretch growth of axons for nerve repair and regeneration have also showed decreased axonal transport at large strains ($>24\%$), possibly due to subcellular damage [235]. However, our results indicate that an intermediate level of strain ($\sim 20\%$) leads to an increase in active transport. This suggests a possible “sweet spot” for neuronal tension that may be utilized in therapies and treatments of neurodegenerative diseases. For instance, in ALS mice, one of the earliest observed pathologies is a decrease in retrograde transport [100] that eventually leads to axon degeneration. If an intermediate level of tension could be applied to these ALS axons, it may be possible to both increase active transport as well as induce a retrograde bias to restore normal axonal transport and prevent axon degeneration. Overall, our results indicate that manipulating neuronal tension may be a possible method to restore axonal transport and combat neurodegeneration.

**Synaptic Plasticity.** The basis of synaptic plasticity is a change in synaptic strength in response to stimuli, and it is believed to be a critical process in mediating the storage of memory in animals [45]. Synaptic plasticity is partially regulated by the amount of neurotransmitter released at the presynaptic terminal [236]. Neurotransmitter release is a cyclic process involving transport of vesicles to active zones, clustering of vesicles at the presynaptic terminal, exocytosis of neurotransmitters via vesicle fusion, and retrieval and recycling of vesicle membrane after release [44, 97]. Mechanical tension plays a role in many of these processes. Neurons regulate their membrane tension [237, 96], and increased tension increases the probability of exocytosis and release [238, 239, 240].
Figure 5.4: Van Hove correlation functions. Here $\mu = \bar{x} \pm \sigma$, where $\bar{x}$ is the mean and $\sigma$ the standard deviation of the Gaussian fit, and $\xi$ is the non-Gaussian parameter. The subscript “a” indicates active vesicles and “p” are passive vesicles. (a) Vesicles in control neurons exhibit no significant bias ($\bar{x} \approx 0$ nm) and small values of the $\xi$ for both active and passive vesicles at all timescales. (b) When neurons are stretched the activity of vesicles increases. At short timescales ($\tau = 0.15$ s), $\xi$ increases for active and passive vesicles, indicating an increase in activity of single molecular motors. At intermediate timescales ($\tau = 1$ s), $\sigma_{\text{stretch}} > \sigma_{\text{control}}$ indicating an increase in the activity of collective molecular motor ensembles, and $\bar{x}_{\text{stretch}} = -26$ nm suggesting a retrograde bias of vesicle motion. At large timescales ($\tau = 5$ s), $\xi$ values are low suggesting little influence from single motor activity and $\mu$ values remain large suggesting increased activity of collective motors and a retrograde bias. The probability of retrograde motion of active vesicles was estimated as $p_{\text{stretch}}(\tau = 5$ s$) = 0.59$ whereas for all other cases no bias was observed ($p_{\text{stretch}} \approx 0.5$). (c) When neurons were compressed, vesicle activity decreased overall for all timescales as indicated by decreased values of $\sigma_{\text{compress}}$ and $\xi_{\text{compress}}$ in comparison to the control.
Axonal tension is critical in maintaining clustering of vesicles at the presynaptic terminal, and axonal stretching leads to a further increase in vesicle clustering [28, 7]. It is hypothesized that increased tension leads to actin polymerization in the synapse, which results in greater actin-synapsin binding sites for vesicles to remain localized at the presynaptic terminal [61, 241, 225]. However, with increased neurotransmitter release under tension [6], the available supply of vesicles may become depleted. Our results indicate that neuronal tension modulates the active transport of vesicles in neurons by increasing activity of molecular motors and increasing the effective self-diffusivity of the cargo. This is a plausible mechanism to increase delivery of vesicles (containing neurotransmitters and other synaptic machinery) to the presynaptic zone to provide the building blocks for structural plasticity, increase the available pool of neurotransmitters, and maintain a sufficient supply during elevated release. The emerging scenario is that mechanical tension in neurons increases vesicle transport, presynaptic clustering, and neurotransmission; all of which are critical in the process of synaptic plasticity.

5.4 Conclusion

Our results suggest that global tension in neurons modulates the internal activity at the level of molecular motors. This is a plausible reason that mechanical tension is involved in regulating the amount of clustering of neurotransmitter vesicles at the synaptic terminal [28, 7]. It’s involvement in neuronal transport also suggests a possible role in treatment or prevention of neurodegenerative disease. Furthermore, all adherent cells apply tractions to their underlying substrate resulting in cytoskeletal tension. This cell level tension could be a general regulatory mechanism for tuning active transport of subcellular components. More generally, our results indicate that global forces can modulate internal activity at the molecular level and alter the overall dynamics of a living matter system.
This dissertation presents an investigation of the effect of mechanical tension on vesicle dynamics in neurons. It includes technology development and a report of our scientific findings.

Two technologies were developed to investigate cellular neuromechanics. The first was a simple system for applying high-precision mechanical strain to living cells while allowing high-resolution live-imaging (2). The simplicity of this tool makes it amenable to a broad variety of studies in studying the mechanics and physics of cellular systems. The second technology was an algorithm for studying the stochastic motion of vesicles using tools from statistical mechanics (Chapter 4). This algorithm is based on simple theories in near-equilibrium statistical mechanics but allows interpretation of the highly nonequilibrium vesicle dynamics in neurons. This tool is directly applicable to studying the stochastic dynamics of particles that can be tracked in any system and used to extract diffusion exponents as a function of timescale.

The main scientific finding in this dissertation is that increased tension in neurons leads to an increased vesicle clustering in Drosophila (Chapter 3) and increased active transport of vesicles in Aplysia mediated by molecular motors (Chapter 5). These findings suggest that neuronal tension could be a regulatory signal to maintain normal function. Furthermore, this raises many questions regarding the biophysical mechanisms at play. All parts of the neuron work together to form a complex interconnected system that will require careful experiments and theory to understand their relationship. Thus, I would like to conclude this dissertation with a brief discussion of possible future directions.

**Slow axonal transport.** The mechanism of slow axonal transport is still unclear, but it has been hypothesized that it is a combination of active transport, passive diffusion, and cytoplasmic flow during growth [242]. It is critical to the long range transport of necessary molecules for neuronal function. Thus by using a temporal MSD algorithm and a correlation analysis over a large range of timescales, it should be possible to
dissect out the different contributions to slow axonal transport. In this dissertation, we discussed active and passive transport which occur at relatively short time scales (on the order of seconds). However cytoplasmic flow or flux of the axonal framework occur over much longer time scales (minutes and hours), and thus an extension of timescale may uncover new behavior. This analysis could lead to a better understanding of the molecular processes occurring in slow axonal transport, which could be critical in understanding neurodegenerative diseases and developing improved treatment.

**Plasma membrane tension.** Neurons have an enormous amount of plasma membrane that they must regulate due to their highly elongated structures [96]. This membrane is a complex jungle of transmembrane proteins, ion channels, lipid rafts, etc., all swimming in a sea of thermal energy. In addition, the membrane is most likely being driven by nonthermal fluctuations originating from ion pumps and other dynamic processes occurring inside the cell [243]. These fluctuations, in addition to forces along the axon/neurite, must be giving rise to some effective tension in the plasma membrane. It is known that membrane tension affects many processes, including exocytosis of vesicles [244]. It would be interesting to directly measure the tension in the plasma membrane, while modulating global neurite tension, and quantify the exocytosis of vesicles. Newly developed experimental techniques may make this possible [86, 245, 246].

**The actin-spectrin cortex.** The cortical actin network exists adjacent to the neuron membrane in many structures. A very recent paper, highlighting the power of super-resolution microscopy, has shown that the actin in axons is organized in periodic ring-like structures wrapped around the axonal circumference with inter-ring spacing of approximately 180-190 nm [247]. Spectrin also exhibited periodic structures that were alternating with actin. Strikingly, actin structures were not periodic in dendrites, but instead formed long continuous structures. These findings suggest that axons in particular have a continuous periodic actin-spectrin network. This actin-spectrin network could be another force/tension bearing structural element in the axon that is different from the usual suspects (microtubules, neurofilaments, etc.). Investigating the role of this actin-spectrin network in axons may be critical in understanding how an axon is fundamentally different from a dendrite. It should be noted that the existence of this actin-spectrin network will make measuring the membrane tension in the previous paragraph much more difficult.
**Forced self-assembly.** It is possible to initiate an axon based only on application of mechanical tension [4]. These mechanically initiated axons show biochemical markers consistent with naturally occurring axonal development. In addition, neurons usually have only one axon, but by applying mechanical tension a second axon can be initiated. This raises the question of why applied force is sufficient to generate an axon. It would be interesting to investigate from a physics perspective, to see if force could give rise to self-assembly of structures resembling an axon. With the development of reconstituted biological systems [248], these types of experiments may soon be possible. For instance, a reaction vessel made of a plasma membrane could be created that contains the molecular components of an axon (including an energy source, ATP). Then the reaction vessel could be deformed or stretched and the resulting dynamic organization of structures could be investigated. This would provide a minimal system for investigating the biophysics of axonal organization.

**Extraction of subcellular structure.** Rheology has been used to extract information about the molecular architecture of polymer gels [249]. At its most basic, this involves applying an input force/deformation and measuring the output response of the system. In neurons, we have vesicles (which can act as rheological probes) that are subjected to forces via molecular motors as they are dragged through the complex subcellular environment. In an ideal environment (e.g. spherical particles driven by deterministic motors through water), we would expect the measured particles to alternate between Brownian and ballistic motion. In the actual environment (e.g. complex vesicles driven by stochastic motors through a non-Newtonian cytoplasm), we observe vesicles undergoing anomalous diffusion. By using the molecular motor force as the input and the vesicle motion as the measured response, it may be possible to use the natural activity occurring in the neuron to extract information about the subcellular structure. This would be interesting for two reasons: It is non-invasive, except for the incident photons required for imaging; And most importantly it would provide information about the *dynamic* structure of *living* neurons (which is something electron microscopy cannot provide).

**Simulation at the correct scale.** Stochastic models of molecular motor dynamics have been extensively studied in the past decade [250, 251, 252, 253]. These studies usually investigate one or more molecular motors in an asymmetric energy potential and focus on understanding the process of converting chemical energy (ATP) into mechanical energy (motion). With rapid advancements in computational power and techniques,
it is imaginable to actually simulate vesicle motion in a more realistic environment. Recent studies have begun to extend molecular motor models to simulate transport of vesicles interacting with their environments on one-dimensional tracks [254, 255, 256]. However, to truly understand the complexities of vesicle transport I believe that further extension is needed. Models should be developed to simultaneously simulate the dynamics of the several coupled systems in the neuron. To extract meaningful information from these simulations, an appropriate level of description must be adopted. This will require some models to be “coarse-grained”, and others to be “fine-grained”, to couple several processes together and achieve a useful physical description for simulations. This includes models of molecular motors, cytoskeletal fluctuations, cytoskeletal polymerization, vesicle interactions with the subcellular network, etc. While incremental progress in simulating each component in detail will surely contribute to our understanding, it is hard to predict how each new element will affect the overall system. Such is the beauty of complexity [257].
Appendix A

Useful theoretical derivations

Summary. The purpose of this section is to outline a few useful theoretical derivations encountered during my Ph.D. coursework that I found particularly helpful or inspired new ways of thinking. The notes presented here are derived from lectures notes from courses in math, physics, and theoretical and applied mechanics. In addition to my traditional coursework in Mechanical Engineering, I found these courses to be particularly interesting:

- Physics 427 - Thermal and Statistical Physics (Prof. Yoshi Oono)
- Physics 504 - Statistical Mechanics and Kinetic Theory (Prof. Nigel Goldenfeld)
- Physics 554 - Nonequilibrium Statistical Mechanics (Prof. Klaus Shchulten)
- Math 558 - Methods of Applied Mathematics (Prof. Robert Lee Deville)
- Math 595 - Random Walks and Queueing Theory (Prof. Robert Bauer)
- TAM 598 - Soft Solids (Prof. Sascha Hilgenfeldt)

To any students who are reading this, I strongly encourage you to explore any coursework that you find interesting even if it does not seem directly related to your current work. And don't be afraid if it seems outside of your discipline! It will give you a broader perspective on science and engineering and allow you to develop important analytical skills. Also, a keen observer might notice, there is significant overlap between topics in the courses listed above. For me to understand some of these unfamiliar concepts, I had to see them a bajillion different times in a quadrillion different ways. And I believe this is true for many different people and topics, so do not let this deter you. Many of the topics discussed in the courses listed above are surprisingly general, with applications to just about anything. Many are also deceptively complex in their theoretical foundations. I do not claim to understand all of these topics in their entirety, but I certainly enjoyed learning about them. The following sections include some theoretical derivations from these courses that helped shape my view of the world.
A.1 The Boltzmann Distribution

The Boltzmann distribution is ubiquitous in physical processes and shows up in many different problems. In this section we will discuss a derivation of the Boltzmann distribution outlined in lecture notes from a course in physics\(^1\).

First, we adopt the hypothesis that a closed system in thermal equilibrium is equally likely to be in any of the quantum states accessible to it. This is the principle of equal and *a priori* probabilities. If all quantum states have equal probability, then statistical mechanics reduces essentially to the task of counting states. Now we can define some basic thermodynamic relations. Let us suppose that for a system of \(N\) particles with energy \(E\) and volume \(V = L^3\), the number of possible states is \(\Gamma\). Then we define the entropy to be,

\[
S \equiv k_B \log \Gamma \tag{A.1}
\]

where \(k_B = 1.38 \times 10^{-23}\) J \(\cdot\) K\(^{-1}\). We will see that \(S\) has the correct properties of the thermodynamic entropy. Since \(\Gamma = \Gamma (E, N, V)\) we have that,

\[
S = S (E, N, V) \tag{A.2}
\]
or

\[
E = E (S, N, V) \tag{A.3}
\]

The differential is,

\[
dE = \left( \frac{\partial E}{\partial S} \right)_{N,V} dS + \left( \frac{\partial E}{\partial N} \right)_{S,V} dN + \left( \frac{\partial E}{\partial V} \right)_{S,N} dV \tag{A.4}
\]

and the coefficients are defined as

\[
\begin{align*}
T &= \frac{\partial E}{\partial S} \quad \text{temperature} \\
-p &= \frac{\partial E}{\partial V} \quad \text{pressure} \\
\mu &= \frac{\partial E}{\partial N} \quad \text{chemical potential}
\end{align*} \tag{A.5}
\]

We can also define the heat capacity as the amount of energy that must be transferred to raise the temperature of a system by an amount \(dT\). If this amount is \(dE\), then we

\(^1\)Nigel Goldenfeld. “Lectures notes for Physics 504 - Statistical Mechanics and Kinetic Theory”
can define the heat capacity as,

\[
C_x = \left( \frac{\partial E}{\partial T} \right)_x
\]  

(A.6)

where \( x \) denotes \( p, V, \ldots \) or any other constraint that is applied during the energy transfer. Since energy is extensive, then \( E \propto N \). A measure of the heat capacity which is independent of the size of the system is the specific heat which is conventionally defined as, \( c_x = C_x/M \) where \( M \) is the mass of the system.

Now given these thermodynamic relations, we can continue with our derivation of the Boltzmann distribution. Here we address the general question: what is the probability of a system being in a particular quantum state, given that it is held at some fixed temperature \( T \). Such a system is by definition, not an isolated system, and so the principle of equal and \( a \) priori probabilities does not apply. In particular, the system is not in any well-defined eigenstate. This argument is quite subtle and a mathematically rigorous derivation is printed in section 9.1 of the book by K. Huang\(^2\). Here the procedure is outlined in three steps.

1. First, we must figure out how to maintain the system of interest, \( S \), at a temperature \( T \). This can be simply achieved by placing in thermal contact with another system, called the reservoir \( R \), which is already at thermal equilibrium at a temperature \( T \). That is, the parts of the large system \( R \) are in thermal equilibrium with themselves. System \( S \) need not necessarily be large. When \( S \) and \( R \) are placed in contact, they will come into equilibrium at a new temperature \( T' \). To evaluate the change in temperature of the reservoir, we note that by definition of the specific heat we have,

\[
T' - T = \frac{\Delta E}{C \cdot M}
\]  

(A.7)

where \( \Delta E \) is the energy transferred to the reservoir from \( S \) during the process of equilibration, and \( M \) is the mass of the reservoir. By making \( M \to \infty \), we have \( T' - T \to 0 \). Thus in a “large” system, the temperature does not change when a finite amount of energy is transferred to it. We shall assume that \( R \) is sufficiently big that the system \( S \) and \( R \) do come into equilibrium at temperature \( T \).

2. The joint system is isolated, so the total energy is

\[
E = E_R + E_S = \text{constant}
\]  

(A.8)

Suppose that when the system $S$ is in quantum state $i$, it has energy $E_S = E_i$. Let $p_i$ be the probability that it is in quantum state $i$. Then from the principle of equal a priori probabilities applied to the joint system we have,

$$p_i \propto \left( \begin{array}{c} \text{# of quantum states of the} \\
\text{joint system when $S$ is in state $i$} \end{array} \right)$$

\[ \propto \left( \begin{array}{c} \text{# of q. states of the} \\
\text{reservoir when $S$ is in state $i$} \end{array} \right) \times \left( \begin{array}{c} \text{# of q. states of the} \\
\text{reservoir when $S$ is in state $i$} \end{array} \right) \times 1 \]

and thus we can write

$$p_i \propto \Gamma_R (E_R) = \Gamma_R (E - E_i) \quad (A.10)$$

The probability that $S$ is in state $i$ is proportional to the number of states of $R$ consistent with $S$ being in state $i$.

3. Now we use the basic property of the reservoir discussed in step 1. Being large, its temperature stays constant when we add or substract the energy $E_i$. Thus,

$$\text{constant} = \frac{1}{T} = k_B \frac{\partial}{\partial E_R} \log \Gamma_R (E_R) \quad (A.11)$$

and integrating we obtain

$$\Gamma_R (E_R) = (\text{constant}) \times \exp \left( \frac{E_R}{k_B T} \right) \quad (A.12)$$

But

$$p_i \propto \Gamma_R (E - E_i) \quad (A.13)$$

which means that

$$p_i = (\text{constant}) \exp \left( \frac{E}{k_B T} \right) \cdot \exp \left( \frac{-E_i}{k_B T} \right) \quad (A.14)$$

or

$$p_i = \frac{1}{Z} \exp \left( \frac{-E_i}{k_B T} \right) \quad (A.15)$$

which is known as the Boltzmann distribution where the normalization, $Z$, is the
partition function, determined by the fact that $\Sigma_i p_i = 1$,

$$Z = \sum_i \exp \left(-\frac{E_i}{k_B T}\right)$$  \hspace{1cm} (A.16)

Note that the above formula is a sum over the quantum states $i$, NOT the energy levels. Equation A.15 gives the probability of finding the system in a given quantum state, not the probability it has energy $E_i$. This is NOT the same as the probability of finding a system with energy $E_i$, because there are many quantum states with energy $E_i$. The probability, $f(E)$, of finding the system with energy $E_i$ is given by,

$$f(E_i) \propto p_i \Gamma \langle E_i \rangle$$  \hspace{1cm} (A.17)

where $p_i$ is a decreasing function of $E_i$, whereas $\Gamma$ is a rapidly increasing function. The net result is an approximately Gaussian distribution, peaked at the average energy.

When making experimental measurements, the value that is obtained is always a statistical average of the quantity of interest. Given a physical quantity, $Q$, which is a function of the state $i$ of the system, in thermal equilibrium, the thermal average is,

$$\langle Q \rangle = \sum_i Q_i p_i = \frac{1}{Z} \sum_i Q_i \exp \left(-\frac{E_i}{k_B T}\right)$$  \hspace{1cm} (A.18)

The average energy, which is usually written as $U$ is,

$$U \equiv \langle E \rangle = \sum_i E_i p_i = \frac{1}{Z} \sum_i E_i \exp \left(-\frac{E_i}{k_B T}\right)$$  \hspace{1cm} (A.19)

The partition function, $Z$, is a generating function, and thermal averages may be computed from it by differentiation. First define, $\beta = 1/k_B T$. Then,

$$E_i \exp \left(-\beta E_i\right) = -\frac{d}{d\beta} \left(\exp \left(-\beta E_i\right)\right)$$  \hspace{1cm} (A.20)

and

$$\langle E \rangle = -\frac{1}{Z} \cdot \left(\frac{\partial Z}{\partial \beta}\right)_{N,V} = -\frac{\partial}{\partial \beta} \langle \log Z \rangle_{N,V}$$  \hspace{1cm} (A.21)

which we can rewrite in terms of $T$ as

$$\langle E \rangle = k_B T^2 \frac{\partial}{\partial T} \langle \log Z \rangle_{N,V}$$  \hspace{1cm} (A.22)
We can restate the calculation of averages in the framework of classical statistical mechanics by describing a system in terms of coordinates, \( q = (q_1, q_2, \ldots, q_n) \) and momenta \( p = (p_1, p_2, \ldots, p_n) \), where the number of each is equal to the number of degrees of freedom of the system\(^3\). Then the Boltzmann distribution can be written as,

\[
\exp(\beta E(p_1, \ldots, p_n, q_1, \ldots, q_n)) \, dq_1 \, dp_1 \ldots dq_N \, dp_N = 1
\]

where \( E \) is the Hamiltonian or total energy of the system and \( d\Omega = dq_1 \, dp_1 \ldots dq_N \, dp_N \).

Similarly as before we introduce the classical partition function,

\[
Z_c = \exp(-\beta E) \, dq_1 \, dp_1 \ldots dq_N \, dp_N = \int \exp(-\beta E) \, d\Omega
\]

and then we can calculate the average value of any function of \( p \)'s and \( q \)'s (denoted \( A(p, q) \)) as,

\[
\langle A(p, q) \rangle = \frac{1}{Z_c} \int A \exp(-\beta E) \, d\Omega
\]

Now let's use this property to calculate an interesting result (of mean kinetic energy for a particle) of statistical mechanics. In equilibrium statistical mechanics, it is assumed that for a stationary process the time-averaged behavior of a system is equal to the ensemble-average behavior (ergodic hypothesis). Or in particular (similarly as above), the average total energy is given by,

\[
\langle E \rangle = Z_c^{-1} \int E \exp(-\beta E) \, d\Omega = \frac{\partial}{\partial \beta} \ln Z_c
\]

The average energy of each coordinate is,

\[
\langle q_i \rangle = Z_c^{-1} \int q_i \exp(-\beta E) \, d\Omega
\]

Suppose that the coordinates are chosen so that the kinetic energy is expressed as a sum of squares of the momenta with constant coefficients as, \( \sum_i p_i^2/(2m_i) \), and thus the Hamiltonian is,

\[
E = \sum_i \frac{p_i^2}{2m_i} + V(q)
\]

Then in calculating \( Z_c \), or the average of any function of the coordinates only, the integrals that occur are the products of integrals over the position and momentum

coordinates respectively. Then when evaluating the average energy, \( \langle E \rangle \),

\[
\frac{1}{2m_i} \langle p_i \rangle = \frac{1}{2m_i} \int p_i^2 \exp \left( -\beta p_i^2 / 2m_i \right) dp_i
\]  

(A.29)

Now,

\[
\int_{-\infty}^{\infty} \exp \left( -ap^2 \right) dp = \sqrt{\pi/a}
\]  

(A.30)

and,

\[
\int_{-\infty}^{\infty} p^2 \exp \left( -ap^2 \right) dp = \frac{1}{2a} \sqrt{\pi/a}
\]  

(A.31)

and the mean value of \( p_i^2 / 2m_i \) is given by

\[
\frac{1}{2m_i} \langle p_i^2 \rangle = \frac{1}{2} k_B T
\]  

(A.32)

and thus we have the well known result of the equipartition theorem which states the mean value of the kinetic energy in any coordinate (one degree of freedom) is \( k_B T / 2 \).
A.2 Entropic Elasticity

In this section we will derive an example of entropic elasticity. It is based on lecture
notes from courses in physics\textsuperscript{4} and theoretical and applied mechanics\textsuperscript{5}.

In general, the Helmholtz free energy, $F$, is defined as

$$ F = U - TS $$

(A.33)

where $U$ is the internal energy, $T$ is the absolute temperature, and $S$ is the entropy,
for a closed system at a constant temperature and volume. Similarly, the Gibbs free
energy, $G$, or free enthalpy is defined as,

$$ G = U - TS + pV $$

(A.34)

where $p$ is the pressure, and $V$ is the volume, for a closed system at constant temperature
and pressure. Now for a closed system with no change in energy we have,

$$ dG = dU - TdS + pdV = 0 $$

(A.35)

which we can rearrange to find,

$$ -pdV = dU - TdS $$

(A.36)

Equation A.36 tells us that the pressure (or stress), $p$, is dependent on the change of
internal energy, $U$, and the change of entropy, $S$. Now imagine an engineering material
composed of atoms arranged together to form a solid with a specific internal energy and
entropy. The internal energy of this engineering solid arises from the atoms arranged in
a lattice linked together by atomic bonds that can be modeled as springs. The entropy
arises from the possible number of configurations in which that atoms could be ordered
to create this atomic lattice. If we apply a small elastic deformation to this engineering
solid, the atomic spacing will change, and as a result the internal energy will also change,
$dU \neq 0$. However, the configuration of the atoms relative to each other changes very
little, and as a result the entropy also changes very little, $dS \approx 0$. Therefore for
a traditional engineering solid, the stress in the system is mainly dependent on the
change of internal energy, and entropic effects can be neglected. However, in many
other cases, the configuration of the atoms in the material does change and thus the
entropic term plays a significant role. Thus let us explore an example of elasticity based

\textsuperscript{4}Yoshi Oono. “Lectures notes for Physics 427 - Thermal and Statistical Physics”
\textsuperscript{5}Sascha Hilgenfeldt. “Lecture notes for TAM 598 - Soft Solids”
only on configurational change.

Imagine a one-dimensional polymer composed of rigid rods connected to each other, known as the freely-jointed-chain (FJC). Let’s suppose that this FJC is composed of \( N \) number of links, each with length \( b \), such that the contour length of the entire polymer is \( L_c = Nb \). From equation A.36 we have may write down the force displacement relation as,

\[
F = \frac{\partial U}{\partial x} - T \frac{\partial S}{\partial x}
\]  \hspace{1cm} (A.37)

and since each rigid rod is inextensible, there can be no change in the internal energy and \( \frac{\partial U}{\partial x} = 0 \) so we are left with

\[
F = -T \frac{\partial S}{\partial x}
\]  \hspace{1cm} (A.38)

Recall that the entropy arises from the configuration as well as the kinetics,

\[
S = S_{\text{config}} + S_{\text{kinetic}}
\]  \hspace{1cm} (A.39)

and in our case we assume that the kinetics do no change and thus \( S_{\text{kinetic}} = \text{constant} \) and we leave it out of our calculation. Now imagine the possible configurations the polymer could have. For example, each link of the polymer can be pointing in either the positive or negative direction. If we picture one configuration of the polymer it may look something like, \(+---+++--++--++--\cdots\). Thus the overall end-to-end length of the polymer, \( x \), would be,

\[
x = (N_+ - N_-)b
\]  \hspace{1cm} (A.40)

where \( N_+ \) is the number of links pointing in the positive direction, \( N_- \) is the number pointing in the negative direction, and the total number of links is \( N = N_+ + N_- \). Now the configurational entropy of the FJC is

\[
S = S_{\text{config}} = k_B \ln \Omega(x)
\]  \hspace{1cm} (A.41)

where \( k_B \) is the Boltzmann constant and \( \Omega(x) \) is the number of possible configurations. We may rearrange equation A.40 to get,

\[
\frac{x}{b} = N_+ - (N - N_+) = 2N_+ - N
\]  \hspace{1cm} (A.42)

which gives us,

\[
N_\pm = \frac{1}{2} \left( N \pm \frac{x}{b} \right)
\]  \hspace{1cm} (A.43)
and accordingly we can calculate the number of possible configurations as

$$\Omega(N_+) = \binom{N}{N_+} = \frac{N!}{N_+!N_-!} \quad (A.44)$$

which we can simplify using Stirling’s approximation ($N \gg 1$),

$$N! \approx N^N \sqrt{2\pi} \exp(-N)\sqrt{N} \quad (A.45)$$

or

$$\ln(N!) \approx \left(N + \frac{1}{2}\right) \ln N - N + \frac{1}{2} \ln 2\pi \quad (A.46)$$

which gives us

$$\ln \Omega(x) = \left(N + \frac{1}{2}\right) \ln N - \frac{1}{2} \left(N + \frac{x}{b} + 1\right) \ln \left[\frac{1}{2} \left(N + \frac{x}{b}\right)\right] \quad (A.47)$$

and recalling from equation A.38, we have

$$F = -k_B T \frac{\partial}{\partial x} (\ln \Omega(x)) \quad (A.48)$$

which gives us

$$F = \frac{k_B T}{2b} \left[ \ln \left(\frac{1 + \frac{x}{N_b}}{1 - \frac{x}{N_b}}\right) + \left(1 + \frac{x}{N_b} + \frac{1}{N}\right) - \left(1 - \frac{x}{N_b} + \frac{1}{N}\right) \right] \quad (A.49)$$

and for large $N$ we have,

$$F = \frac{k_B T}{2b} \ln \left(\frac{1 + \frac{x}{N_b}}{1 - \frac{x}{N_b}}\right) \quad (A.50)$$

This is the result for the force-displacement relation for the FJC one-dimensional polymer. Equation A.50 shows us that based only on configurational entropy (inextensible rods), we can derive the nonlinear force behavior of a simplified polymer. If we restrict our model to small displacements we get,

$$F = \frac{k_B T}{Nb^2} x \text{ for } \frac{x}{Nb} \ll 1 \quad (A.51)$$

where the behavior is now that of a linear spring with spring constant,

$$k_{sp} = \frac{k_B T}{Nb^2} \quad (A.52)$$
A.3 A Random Walk

The purpose of this section is to illustrate an asymptotic approach for derivation of the random walk. It is based on lecture notes from courses in applied mathematics\(^6\).\(^7\). This will include the microscopic model of a biased random walk, which if scaled appropriately leads to the macroscopic description of probability flow, known as the Fokker-Planck equation (FPE). The constant coefficient FPE can be solved exactly, however the situation is not so simple with nonconstant coefficients. In this case, it is possible to use the corresponding stochastic differential equation (SDE) to simulate the individual particle trajectories. In principle, the simulation of the correct SDE will approach the solution of the FPE as \(1/\sqrt{n}\), where \(n\) is the number of particle paths.

Imagine a one-dimensional lattice where the particle is moving as a random walk. Begin with the definition of the one-dimensional biased random walk where the particle can make steps to the left and right of \(\Delta x\) over a time step of \(\Delta t\),

\[
X_{t+\Delta t} = \begin{cases} 
X_t + \Delta x, & \text{prob, } \alpha \\
X_t - \Delta x, & \text{prob, } \beta \\
X_t + 0, & \text{prob, } 1 - \alpha - \beta
\end{cases}
\]

(A.53)

where \(\alpha + \beta \leq 1\). This means that there are three ways for a particle to arrive at position \(x\). Namely it could have moved from the left (with probability \(\alpha\)), it could have moved from the right (with probability \(\beta\)), or it could have stayed stationary, meaning it was there in the previous timestep (with probability \(1 - \alpha - \beta\)). The probability that a particle is at \(x\) at a certain time \(t + \Delta t\) depends only on the location at the previous time and is called the master equation as follows,

\[
p(x, t + \Delta t) = \alpha p(x - \Delta x, t) + \beta p(x + \Delta x, t) + (1 - \alpha - \beta) p(x, t)
\]

(A.54)

Now if we let \(\Delta x, \Delta t\) be small and assume \(p(x, t)\) is smooth we can use a Taylor expansion to get,

\[
p(x, t + \Delta t) = p(x, t) + \Delta t \frac{\partial p(x, t)}{\partial t} + O(\Delta t^2)
\]

(A.55)

\[
p(x + \Delta x, t) = p(x, t) + \Delta x \frac{\partial p(x, t)}{\partial x} + \frac{(\Delta x)^2}{2} \frac{\partial^2 p(x, t)}{\partial x^2} + O(\Delta x^3)
\]

(A.56)

\[
p(x - \Delta x, t) = p(x, t) - \Delta x \frac{\partial p(x, t)}{\partial x} + \frac{(\Delta x)^2}{2} \frac{\partial^2 p(x, t)}{\partial x^2} + O(\Delta x^3)
\]

(A.57)

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\(^6\)Robert Lee Deville. “Lectures notes for Math 558 - Methods of Applied Mathematics”

\(^7\)Robert Bauer. “Lecture notes for Math 595 - Random walks and Queueing Theory”
and now combining the above Taylor expansions into the master equation while ignoring higher order terms we get,

\[
p(x, t) + \Delta t \frac{\partial p(x, t)}{\partial t} = \alpha \left( p(x, t) - \Delta x \frac{\partial p(x, t)}{\partial x} + \frac{(\Delta x)^2}{2} \frac{\partial^2 p(x, t)}{\partial x^2} \right) + \beta \left( p(x, t) + \Delta x \frac{\partial p(x, t)}{\partial x} + \frac{(\Delta x)^2}{2} \frac{\partial^2 p(x, t)}{\partial x^2} \right) + (1 - \alpha - \beta)p(x, t)
\]

which we can simplify to get \((p = p(x, t))\),

\[
p + \Delta t \frac{\partial p}{\partial t} = p + (\beta - \alpha)\Delta x \frac{\partial p}{\partial x} + (\beta + \alpha) \frac{(\Delta x)^2}{2} \frac{\partial^2 p}{\partial x^2}
\]

where we can divide through by \(\Delta t\) to get,

\[
\frac{\partial p}{\partial t} = (\beta - \alpha) \Delta x \frac{\partial p}{\partial x} + (\beta + \alpha) \frac{(\Delta x)^2}{2\Delta t} \frac{\partial^2 p}{\partial x^2}
\]

Now here we have an equation describing the probability flow as a function of microscopic parameters. To scale this problem and see its relation to the macroscopic probability flow, allow

\[
\frac{(\Delta x)^2}{2\Delta t} \to D
\]

where \(D\) is a constant. Now in this scaling we also get \(\frac{\Delta x}{\Delta t} \to \infty\) which we dont want. We would like the two terms \((\frac{\Delta x}{\Delta t}\) and \(\frac{(\Delta x)^2}{2\Delta t}\) to scale the same way, but if \(\alpha, \beta\) are constant then this is not possible. So then we allow \(\alpha, \beta\) to change in the limit, where \((\beta - \alpha)\) is \(O(\Delta x)\). So now lets be more precise about our definitions of \(\alpha, \beta\):

\[
\alpha = \frac{\gamma}{2} - a\Delta x \quad (A.62)
\]

\[
\beta = \frac{\gamma}{2} + a\Delta x \quad (A.63)
\]

where both \(\gamma\) and \(a\) are constants. Notice that the units are, \([\gamma] = 1\) and \([a] = 1/L\). Now notice that,

\[
\beta + \alpha = \gamma \quad (A.64)
\]

\[
\beta - \alpha = 2a\Delta x \quad (A.65)
\]

and plugging these terms in we have,

\[
\frac{\partial p}{\partial t} = 2a \frac{(\Delta x)^2}{\Delta t} \frac{\partial p}{\partial x} + \gamma \frac{(\Delta x)^2}{2\Delta t} \frac{\partial^2 p}{\partial x^2}
\]

\(115\)
where plugging in for \((\Delta x)^2 \rightarrow D\) we get,

\[
\frac{\partial p}{\partial t} = 4aD \frac{\partial p}{\partial x} + \gamma D \frac{\partial^2 p}{\partial x^2}
\]

which is the constant coefficient FPE. Now notice that the units are \([4aD] = L/T\), which is the same as velocity, so we can think of this as representing the drift velocity, \(v\). Notice that the diffusion coefficient is directly linked with the drift velocity. This point will be discussed again later. This relatively simple version of the FPE can be solved analytically and can be thought of as a spreading gaussian which is translating with a drift velocity. It is also known as the Smoluchowski equation for diffusion with drift.

However in the general case where the coefficients of the FPE are not constant we have the form,

\[
\frac{\partial p(x, t)}{\partial t} = -\frac{\partial}{\partial x} \left[ D_1(x, t)p(x, t) \right] + \frac{\partial}{\partial x} \left[ D_2(x, t) \frac{\partial}{\partial x} p(x, t) \right]
\]

where \(D_1(x, t)\) corresponds to the drift coefficient (velocity) and \(D_2(x, t)\) is the diffusion coefficient. In general, solving this equation can be very difficult for dimensions higher than 1. To this FPE there is a corresponding SDE (shown without derivation),

\[
dX_t = D_1(X_t, t)dt + \sqrt{2D_2(X_t, t)}dW_t
\]

where \(W_t\) represents a Wiener process. The Wiener process is a continuous time stochastic process that represents standard Brownian motion. The above equation relies on only local information. Thus we can define the iterative process to calculate the trajectory of a single particle,

\[
X_{t+\delta t} = X_t + D_1(X_t, t)\delta t + \sqrt{2D_2(X_t, t)}\sqrt{\delta t}N(0, 1)
\]

where \(dW_t \rightarrow N(0, 1)\) by the central limit theorem, where \(N(\mu, \sigma^2)\) is the normal distribution function with mean \(\mu\) and variance \(\sigma^2\). Now the above stochastic equation can be iterated to find the path of a single particle.
A.4 Brownian Motion

Now let us look at a model for the Brownian motion of a particle in a fluid, which is also based on a random walk. This section is based on lectures notes from courses in physics. First imagine a Brownian particle immersed in a fluid. The particle is experiencing molecular impacts from the surrounding fluid where the mean time between impacts is \( \tau \), and the mean path a particle travels between impacts is \( \ell \). At each step, this particle moves in a random direction for a distance \( \ell \) and a time \( \tau \), corresponding to a random walk. The particle has an equal probability of moving in any direction in the absence of any bias.

Now let us calculate \( p(\mathbf{r}, t) \), which is the probability that the particle is at \( \mathbf{r} \) after a time \( t \) has elapsed since it left the origin. If a particle is at a location \( \mathbf{r} \) at a time \( t \), that means that on the previous time step, \( t - \tau \), the particle must have been on a sphere of radius \( \ell \) centered about the point \( \mathbf{r} \) (assuming we are in three-dimensional space). And since there is an equal probability that the particle was anywhere on the sphere at time \( t - \tau \) we have,

\[
p(\mathbf{r}, t) = \int \frac{d^2 \hat{n}}{4\pi} p(\mathbf{r} - \ell \hat{n}, \tau), \quad \text{where } |\hat{n}| = 1
\]  

(A.71)

This equation states that for a particle on the sphere of radius \( \ell \), centered around \( \mathbf{r} \), that it has a \( \frac{1}{4\pi} \) chance of moving in the correct direction to reach the desired point \( \mathbf{r} \). Thus we integrate the probabilities of getting to all points on the sphere and multiply by the probability \( \left( \frac{1}{4\pi} \right) \) of going in the right direction to reach point \( \mathbf{r} \). Doing a Taylor expansion about the point \( (\mathbf{r}, t) \) we have,

\[
p(\mathbf{r}, t) = \int \frac{d^2 \hat{n}}{4\pi} \left[ p(\mathbf{r}, t) - \ell \hat{n} \cdot \nabla p + \frac{1}{2} \ell^2 \hat{n}_i \hat{n}_j \frac{\partial^2 p}{\partial x_i \partial x_j} + O(\ell^3) - \tau \frac{\partial p}{\partial t} + O(\tau^2) \right] \]  

(A.72)

where the \( O(\ell) \) term vanishes because \( \int \frac{d^2 \hat{n}}{4\pi} \hat{n} = 0 \) and the second order term is written with index notation with summation over repeated indices as,

\[
\hat{n}_i \hat{n}_j \frac{\partial^2 p}{\partial x_i \partial x_j} = n_x n_x \frac{\partial^2 p}{\partial x \partial x} + n_y n_x \frac{\partial^2 p}{\partial x \partial y} + n_z n_x \frac{\partial^2 p}{\partial x \partial z} + n_x n_y \frac{\partial^2 p}{\partial x \partial y} + \cdots
\]  

(A.73)

and the term,

\[
\int \frac{d^2 \hat{n}}{4\pi} \hat{n}_i \hat{n}_j = \frac{1}{3} \delta_{ij}
\]  

(A.74)

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as we can see when \( i = j \) we have \( \int n_x^2 = \int n_y^2 = \int n_z^2 \), which can be rewritten as \( \int n_x^2 = \frac{1}{3} \int n_x^2 + n_y^2 + n_z^2 \). Where we can recall that \( n_x^2 + n_y^2 + n_z^2 = 1 \). Now simplifying equation A.72 using the above relations we get,

\[
\frac{\partial p}{\partial t} = \frac{\ell^2}{6\tau} \nabla^2 p
\] (A.75)

which is an equation known as the diffusion equation. The diffusion coefficient in this case (three-dimensional) is

\[
D = \frac{\ell^2}{6\tau}
\] (A.76)

The initial condition of this partial differential equation is \( p(\mathbf{r}, 0) = \delta(\mathbf{r}) \), meaning that particle is originally at the origin. The boundary condition on \( p(\mathbf{r}, 0) \) comes from the normalization condition, \( \int p(\mathbf{r}, t) d^3\mathbf{r} = 1 \), where the probability of the particle existing somewhere at a time \( t \) is finite.

The solution to equation A.75 can be derived using the initial and boundary conditions using Green function techniques which are not shown here. The solution is,

\[
p(\mathbf{r}, t) = \begin{cases} 
\frac{1}{(4\pi Dt)^{3/2}} \exp \left( \frac{-|\mathbf{r}|^2}{4Dt} \right) & \text{for } t > 0 \\
0 & \text{for } t < 0 
\end{cases}
\] (A.77)

which is an example of a Markov Process because the future evolution of the system \((t > 0)\) is only dependent on the present time, and not the history \((t < 0)\).

Now that the solution to the diffusion equation is known, it is possible to calculate many properties of the motion of the Brownian particle. One interesting parameter used frequently to describe a particle motion is the mean square displacement. The mean square displacement for a particle from the origin after a time \( t \) is,

\[
\langle r^2 \rangle = \int d^3\mathbf{r} \ r^2 p(\mathbf{r}, t) \\
= -\frac{\partial}{\partial a} \int d^3\mathbf{r} \exp(-ar^2) \\
\] (A.79)

where \( a = (4Dt)^{-1} \). We may simplify this by noticing that,

\[
\int d^3\mathbf{r} \exp(-ar^2) = \left( \frac{\pi}{a} \right)^{3/2}
\] (A.80)
and thus we have,

$$\langle r^2 \rangle = \frac{3}{2a} \left( \frac{\pi}{a} \right)^{3/2} = \frac{3}{2} \cdot 4Dt$$  \hspace{1cm} (A.81)

which is,

$$\boxed{\langle r^2 \rangle = 6Dt}$$  \hspace{1cm} (A.82)

This is the famous result that a random walker goes a distance $\sqrt{N}$ for $N$ steps. The above relation for the mean squared can also be extended for other dimensions as $\langle r^2 \rangle = d2Dt$ where $d = 1, 2, 3$ is the spatial dimension. This derivation for Brownian motion via a random walk is a fundamental basis of the analysis of vesicle dynamics discussed in chapter 4.
A.5 Brownian Motion in a Force Field

The following discussion is an extension of Brownian motion discussed in the previous section to particles in a force field. This section is based on lectures notes from courses in physics\textsuperscript{9,10}. Now consider a large number of particles that are interacting with their surrounding environment via Brownian interactions, but sufficiently dilute that they are not interacting with each other. Let us denote the number density of these particles as $n(r,t)$. Now we may write down two equations governing the dynamics of these particles. The first is a simple statement of particle conservation, in that no particles are gained or lost in our system,

$$\frac{\partial n(r,t)}{\partial t} + \nabla \cdot J = 0 \quad (A.83)$$

where $J$ is the total flux of the particles. The second equation is due to our previous observation that each of these individual particles obeys diffusion. Thus we have Fick’s Law,

$$J_B = -D \nabla n \quad (A.84)$$

where $J_B$ is the flux due to Brownian motion, and $D$ is a phenomenological parameter. Substitution of Fick’s Law in the conservation equation gives us,

$$\frac{\partial n}{\partial t} = D \nabla^2 n \quad (A.85)$$

which is equivalent to equation A.75 derived in the previous section. Now assume the particles are subjected to an external force, $F$, which causes the particles to experience a viscous drag from the medium. Then for each particle the equation of motion is,

$$m \frac{dv}{dt} = F - \gamma v \quad (A.86)$$

where $\gamma$ is the coefficient of friction arising from the fluid viscosity. Now if the particles have a small mass $m$, or the friction coefficient is very high $\gamma \gg 1$, then the inertial forces can be neglected and the velocity is determined directly by the external force,

$$v = \frac{1}{\gamma} F \equiv \mu F \quad (A.87)$$

where $\mu$ is defined as the particle mobility. This condition of neglecting inertial effects is often referred to as an overdamped system, which is a criteria often (but not always)
met in biological systems. The flux of the particles experiencing due to the external force is

\[ J_F = n \mathbf{v} = n \mu \mathbf{F} = \frac{n}{\gamma} \mathbf{F} \]  (A.88)

Now the total particle flux due to Brownian effects and the external force can be written as,

\[ J = J_B + J_F = -D \nabla n + \frac{n}{\gamma} \mathbf{F} \]  (A.89)

Substituting the total flux into the conservation condition (equation A.83) we get,

\[ \frac{\partial n}{\partial t} = \nabla \cdot \left( D \nabla n - \frac{n}{\gamma} \mathbf{F} \right) \]  (A.90)

which is an equation known as the Smoluchowski equation and is very similar to equation A.67 derived via a biased random walk previously. In fact these relations are identical in that the number density \( n(r, t) \) of particles can also be thought of as the probability density, \( p(r, t) \).

Now suppose that the external force, \( \mathbf{F} \), can be written as the gradient of a potential,

\[ \mathbf{F} = -\nabla U(r) \]  (A.91)

As \( t \to \infty \) we should expect the system to reach thermal equilibrium with its environment which gives us a steady-state system,

\[ \frac{\partial n}{\partial t} = 0 \]  (A.92)

where

\[ n(r, t) = n_{\text{eqm}}(r) \propto \exp \left( \frac{-U(r)}{k_B T} \right) \]  (A.93)

and the total flux of the system is zero, \( J = 0 \). Now if we substitute \( n_{\text{eqm}} \) into equation A.89 and set it to zero (as in equilibrium) then we get,

\[ J = -D \nabla n_{\text{eqm}} + n_{\text{eqm}} \mu \mathbf{F} = 0 \]  (A.94)

which simplifies to

\[ \frac{D}{k_B T} \nabla U(r) - \mu \nabla U(r) = 0 \]  (A.95)

and leads to the famous result,

\[ D = \mu k_B T \]  (A.96)
which is known as Einstein’s relation, and is a form of the Fluctuation Dissipation Theorem of the first kind (FDT-I). It should be interesting that the diffusion and particle mobility are directly related, even though they may seem to be independent quantities. Also it should be interesting that temperature is involved. Recalling that the mobility is \( \mu = v / F \) then we can see that \( D = \frac{V k_B T}{F} \), which allows us to directly relate the diffusion of a particle and the force. As an aside, many interesting experiments have been done in the past by varying the temperature as these quantities were measured to establish deviations or a breakdown in the FDT-I.

As an example consider a one-dimensional system where a particle experiences a linear potential,

\[ U(x) = Cx \quad \text{(A.97)} \]

where \( C \) is an arbitrary scaling constant of the potential. Now the force experienced by the particle is

\[ F = -\nabla U(x) = -C \quad \text{(A.98)} \]

Now rewriting the Smoluchowski relation (equation A.90) for the probability density of the particle location, \( p(x, t) \), and utilizing the FDT-I relation,

\[ D = \mu k_B T = \frac{k_B T}{\gamma} \quad \text{(A.99)} \]

we have,

\[ \frac{\partial p}{\partial t} = \nabla \cdot (D \nabla p - D \beta F p) \quad \text{(A.100)} \]

where \( \beta = \frac{1}{k_B T} \). Substituting in for the force, \( F \), and simplifying we have,

\[ \frac{\partial p}{\partial t} = D \frac{\partial^2 p}{\partial x^2} + D \beta C \frac{\partial p}{\partial x} \quad \text{(A.101)} \]

where we can see that the drift velocity is \( v = -D \beta C \). To solve the above equation we have the initial condition,

\[ p(x, t_0|x_0, t_0) = \delta(x - x_0) \quad \text{(A.102)} \]

similarly as before. And we have the boundary condition on an infinite space,

\[ \lim_{x \to \pm \infty} p(x, t|x_0, t_0) = 0 \quad \text{(A.103)} \]

because at time zero the probability that a particle could exist far away from where it was started is vanishingly small. Now using a change of variable and Green Function
techniques, equation A.101 can be solved to get the probability density of a particle undergoing diffusion and drift,

\[
p(x, t|x_0, t_0) = \frac{1}{\sqrt{4\pi D(t - t_0)}} \exp \left[ -\frac{(x - x_0 + D\beta C(t - t_0)^2)}{4D(t - t_0)} \right]
\]

(A.104)

where again the drift velocity is \( v = -D\beta C \). Thus by examining the terms in the solution above it can be seen that the solution to the diffusion-drift problem is a Gaussian that is spreading with drifting mean over time.
A.6 Langevin Equation

The Langevin equation is a stochastic differential equation (SDE) that describes the equation of motion of a brownian particle. It is stochastic because it explicitly incorporates the random force that represents molecular collisions. This use of a stochastic or random force to represent the “noise” in the system is entirely non-trivial and its origin is deeply rooted in mathematics. Here, we will use it superficially to understand its application in Brownian motion. This section is based on lectures notes from courses in physics\textsuperscript{11} and math\textsuperscript{12}.

We begin with a statement of the Central Limit Theorem (CLT). Consider \( n \) independent random variables \( \Delta x_1, \Delta x_2, \Delta x_3, \ldots, \Delta x_n \) with zero mean,

\[
\langle \Delta x_n \rangle = 0
\]  

(A.105)

and variance \( \sigma^2 \),

\[
\langle \Delta x_n^2 \rangle = \sigma_n^2
\]  

(A.106)

Then consider the quantity,

\[
\chi_n = \Delta x_1 + \Delta x_2 + \Delta x_3 + \cdots + \Delta x_n
\]  

(A.107)

and define,

\[
S_n^2 \equiv \sigma_1^2 + \sigma_2^2 + \cdots + \sigma_n^2
\]  

(A.108)

and the CLT states that the probability distribution of \( \chi_n \) approaches the form

\[
p(\chi_n) = \frac{1}{\sqrt{2\pi S_n^2}} \exp \left( -\frac{\chi_n^2}{2S_n^2} \right)
\]  

(A.109)

as \( n \to \infty \). Which we can rewrite as,

\[
p(\chi_n) = \frac{1}{\sqrt{2\pi \sigma^2}} \exp \left( -\frac{(x - \langle x \rangle)^2}{2\sigma^2} \right)
\]  

(A.110)

where \( \sigma^2 = \langle (x - \langle x \rangle)^2 \rangle \). A variable with this probability distribution is considered a Gaussian random variable.

For this discussion it is assumed that the observed molecular collisions are completely independent of each other with a correlation time, \( \tau \). Thus if \( \xi_i(t) \) is the force exerted on the Brownian particle by molecular impacts in the \( i \)th direction \( (i = x, y, z) \), then

\textsuperscript{11}\textsuperscript{11}Nigel Goldenfeld. “Lectures notes for Physics 504 - Statistical Mechanics and Kinetic Theory”
\textsuperscript{12}\textsuperscript{12}Robert Lee Deville. “Lecture notes for Math 558 - Methods of Applied Mathematics”

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on average the force is zero,

$$\langle \xi_i(t) \rangle = 0$$  \hspace{1cm} (A.111)

and the different components are uncorrelated,

$$\langle \xi_i(t) \xi_j(t) \rangle = 0 \text{ for } i \neq j$$  \hspace{1cm} (A.112)

The variance, $\langle \xi_i(t)^2 \rangle$, is a measure of the strength of the force, but it is more useful to consider a more general function called the noise correlation function,

$$\langle \xi_i(t) \xi_i(t') \rangle = \frac{A}{2\tau} \exp \left( -\frac{|t-t'|}{\tau} \right)$$  \hspace{1cm} (A.113)

In equation A.113, we can see that when $t$ and $t'$ are close, the $i$th component of the force at $t$ is nearly the same as $t'$. But as $|t-t'| \to \infty$ the value of the forces at the two different times is completely unrelated. To understand the meaning of the noise correlation function note that,

$$\langle (\xi_i(t) - \xi_i(t'))^2 \rangle = \langle \xi_i(t)^2 \rangle + \langle \xi_i(t')^2 \rangle - 2\langle \xi_i(t) \xi_i(t') \rangle$$  \hspace{1cm} (A.114)

measures how different on average the random force is at two instants in time. Thus

$$\langle \xi_i(t) \xi_i(t') \rangle = \langle \xi_i(t)^2 \rangle - \frac{1}{2} \langle (\xi_i(t) - \xi_i(t'))^2 \rangle$$  \hspace{1cm} (A.115)

measures how much the RMS noise strength is reduced for its value at $t$. Now that the random force $\xi_i(t)$ is defined, we may write down the Langevin equation.

The Langevin equation is the equation of motion for a Brownian particle. In its general form we have

$$m\frac{d^2x(t)}{dt^2} = -\gamma \frac{dx(t)}{dt} + F + \xi(t)$$  \hspace{1cm} (A.116)

where $m$ is the particle mass, $x$ is the particle position, $t$ is time, $\gamma$ is the friction coefficient, $F$ is the deterministic force, and $\xi(t)$ is the random force. Neglecting inertia, the left hand side of the equation goes to zero, and we have the overdamped form of the equation,

$$\gamma \frac{dx(t)}{dt} = -\frac{\partial U}{\partial x} + \xi(t)$$  \hspace{1cm} (A.117)

Starting with $x(t) = x_0$ at $t = 0$, we solve equation A.117, with $\xi(t)$ taking on some value at each time $t$. Thus over the evolution with time, the value of $\xi(t)$ is the same set of numbers chosen from a probability distribution. So we solve the Langevin equation
with a particular $\xi(t)$, which is chosen at random from a set of all functions with the same probability distribution with moments,

$$
\langle \xi(t) \rangle = 0 \\
\langle \xi_i(t)\xi_j(t) \rangle = \delta_{ij} \frac{A}{2\tau} \exp\left(-\frac{|t-t'|}{\tau}\right) \tag{A.118}
$$

That means that if we solved the Langevin equation several times, starting from the same $x_0$, we would get a different realization of $\xi(t)$ each time. This is because the values of $\xi(t)$ are chosen from the same set of numbers, but each realization is different. So the solution to equation A.117 will depend on the actual noise (realization of $\xi(t)$) that is used,

$$
x = x(t, \{\xi(t)\}) \tag{A.119}
$$

If we do this enough times, we can average $x(t)$ over the different repetitions (i.e. over the probability distribution of $\xi$) to find an average behavior of the system.

As an example lets solve the Langevin equation for for a free particle. Starting with equation A.117, lets solve for $U = 0$, which corresponds to pure Brownian motion. The Langevin equation is,

$$
\gamma \frac{dx}{dt} = \xi(t) \tag{A.120}
$$

For simplicity lets work in one dimension, then,

$$
x(t) = \frac{1}{\gamma} \int_0^t \xi(t') dt' + x_0 \tag{A.121}
$$

and from this equation we can see explicitly that $x(t)$ depends on the entire function $\xi(t)$ and whatever it happens to be for a given realization. If we average over realizations we see that,

$$
\langle x(t) \rangle = \frac{1}{\gamma} \int_0^t \langle \xi(t') \rangle dt' + x_0 = x_0 \tag{A.122}
$$

because $\langle \xi(t) \rangle = 0$. Thus on average, in pure Brownian motion, a particle’s displacement from its starting point is zero (as expected). Now lets suppose that our timescale of observation is much larger than the correlation time, $\tau$, of the molecular impacts. Thus taking $\tau \to 0$ in equation A.118 we obtain,

$$
\langle \xi(t)\xi(t') \rangle = A\delta(t-t') \tag{A.123}
$$
Then by squaring equation A.121 we have
\[ x(t)^2 = \frac{1}{\gamma^2} \int_0^t \int_0^t \xi(t_1)\xi(t_2)dt_1dt_2 + x_0^2 + \frac{2}{\gamma} x_0 \int_0^t \xi(t')dt' \] (A.124)
and now averaging and using \( \langle \xi(t) \rangle = 0 \) we have
\[ \langle x(t)^2 \rangle - x_0^2 = \frac{1}{\gamma^2} \int_0^t \int_0^t \xi(t_1)\xi(t_2)dt_1dt_2 - \frac{1}{\gamma^2} \int_0^t \int_0^t A_1\delta(t_1 - t_2)dt_1dt_2 \] (A.125)
which gives us
\[ \langle x(t)^2 \rangle - x_0^2 = \frac{1}{\gamma^2} \int_0^t A_1dt_1 = \frac{A}{\gamma^2} t \] (A.126)
which is a similar result as we found previously in equation A.82, that the mean squared displacement is proportional to \( t \).

This result is most interesting because we have derived the mean squared displacement in two different ways with two similar, but different results. The one-dimensional version of equation A.82 is,
\[ \langle x(t)^2 \rangle = 2Dt \] (A.127)
where \( D \) is the diffusion coefficient. Also recall from equation A.99 that the FDT-I gives us, \( D = \frac{k_BT}{\gamma} \). Combining the results of the mean squared displacement analysis from diffusion (equation A.82) and the Langevin analysis (equation A.126) we have
\[ \frac{A}{\gamma^2} = 2D = \frac{2k_BT}{\gamma} \] (A.128)
and we can rearrange to get,
\[ A = 2\gamma k_BT \] (A.129)
which is known as the Fluctuation Dissipation Theorem of the second kind (FDT-II). The conclusion here is that the noise strength is proportional to the temperature and the friction coefficient!

Another insightful way to derive the mean squared displacement from the Langevin equation for a free particle is as follows\(^\text{13}\). Starting with the Langevin equation for a free particle we have,
\[ m\frac{d^2x}{dt^2} = -\gamma \frac{dx}{dt} + \xi(t) \] (A.130)

where we can multiply both sides by $x$ to get,

$$m x \ddot{x} = m \left[ \frac{d(x \dot{x})}{dt} - \dot{x}^2 \right] = -\gamma x \dot{x} + x \xi(t) \quad (A.131)$$

where $\dot{x}$ and $\ddot{x}$ denote the time derivatives. Then taking ensemble averages we find $\langle x \xi(t) \rangle = 0$ because the random force has a zero first moment, and we have,

$$m \frac{d\langle x \dot{x} \rangle}{dt} = m \langle \dot{x}^2 \rangle - \gamma \langle x \dot{x} \rangle \quad (A.132)$$

Since particles are in equilibrium, we know from the Equipartition theorem that the kinetic energy of the particle is,

$$\langle KE \rangle = \langle \frac{1}{2} m \dot{x}^2 \rangle = \frac{1}{2} m \langle \dot{x}^2 \rangle = \frac{1}{2} d k_B T \quad (A.133)$$

where $d = 1$ for one-dimensional space. So we can rewrite the Langevin equation as,

$$\left( \frac{d}{dt} + \eta \right) \langle x \dot{x} \rangle = \frac{k_B T}{m} \quad (A.134)$$

where $\eta = \gamma/m$, which has the solution,

$$\langle x \dot{x} \rangle = \frac{1}{2} \frac{d \langle x^2 \rangle}{dt} = C \exp(-\eta t) + \frac{k_B T}{\gamma} \quad (A.135)$$

and we know at $t = 0$ the mean squared displacement is zero, so that $0 = C + k_B T/\gamma$, and we have

$$\frac{1}{2} \frac{d \langle x^2 \rangle}{dt} = \frac{k_B T}{\gamma} (1 - \exp(-\eta t)) \quad (A.136)$$

which can be integrated to find the solution,

$$\langle x^2 \rangle = \frac{2 k_B T}{\gamma} \left[ t - \frac{1}{\eta} (1 - \exp(-\eta t)) \right] \quad (A.137)$$

Now in the limit of very small times where $t \ll 1/\eta$ (much shorter than the collision time), the solution to the mean squared displacement is of the form,

$$\langle x^2 \rangle \propto t^2 \quad (A.138)$$

(expanding the exponential up to second order), which is the case of ballistic motion. This is expected between collisions. In the other limit, $t \gg 1/\eta$, the solution has the
form,

\[ \langle x^2 \rangle \propto \frac{2k_B T}{\gamma} t \] (A.139)

which is expected for normal diffusion and is the same as calculated earlier in equation A.126.
A.7 Kubo Formula

In this section we will derive an equation relating the diffusion coefficient to the velocity autocorrelation function. This is a special case of a set of formulas known as the Green-Kubo relations that relate transport coefficients with time-correlation functions. This section is based on lectures notes from a course in physics. We begin by using our previous results from equation A.126 and A.128,

\[ \langle x(t)^2 \rangle - x_0 = \frac{A}{\gamma^2} t = \langle (x(t) - x_0)^2 \rangle \quad (A.140) \]

and

\[ D = \frac{A}{2\gamma^2} \quad (A.141) \]

which can be combined to get

\[ D = \lim_{t \to \infty} \frac{\langle x(t)^2 \rangle - x_0^2}{2t} \quad (A.142) \]

in one-dimension. Now notice that we can calculate the position by integrating the velocity, \( v(t) \), as

\[ x(t) - x_0 = \int_0^t v(t') dt' \quad (A.143) \]

Therefore we can rewrite the expression in equation A.142 to be,

\[ D = \lim_{t \to \infty} \frac{1}{2t} \left[ \int_0^t v(t') dt' \cdot \int_0^t v(t'') dt'' \right] \quad (A.144) \]

Now lets rewrite the quantity in the square brackets above as,

\[ I(t) = \int_0^t \int_0^t dt_1 dt_2 \langle v(t_1) \cdot v(t_2) \rangle \quad (A.145) \]

In equilibrium the correlation function, \( \langle v(t_1) \cdot v(t_2) \rangle \), can only depend on \( |t_1 - t_2| \). We split the integral into two parts where \( t_1 > t_2 \) and \( t_1 < t_2 \) and we use,

\[ \langle v(t_1) \cdot v(t_2) \rangle = \langle v(t_1 - t_2) \cdot v(0) \rangle = \langle v(0) \cdot v(t_2 - t_1) \rangle \quad (A.146) \]

to get

\[ I(t) = \int_0^t dt_1 \int_0^t dt_2 \langle v(t_1 - t_2) \cdot v(0) \rangle + \int_0^t dt_1 \int_0^t dt_2 \langle v(0) \cdot v(t_2 - t_1) \rangle \quad (A.147) \]

\[ ^{14}\text{Nigel Goldenfeld. “Lectures notes for Physics 504 - Statistical Mechanics and Kinetic Theory”} \]
To simplify this integral we must make a change of variable. In the first integral let \( \tau = t_1 - t_2 \) and \( s = t_2 \). In the second integral let \( \tau = t_2 - t_1 \) and \( s = t_2 \). Thus we have,

\[
I(t) = 2 \int_0^t d\tau \int_0^{t-\tau} ds \langle v(\tau) \cdot v(0) \rangle + \int_0^t d\tau \int_\tau^t ds \langle v(0) \cdot v(\tau) \rangle \tag{A.148}
\]

which is equal to

\[
I(t) = 2 \int_0^t (t - \tau) \langle v(\tau) \cdot v(0) \rangle d\tau \tag{A.149}
\]

Now assuming these integrals are bounded,

\[
\int_0^\infty t \langle v(t) \cdot v(0) \rangle d\tau < \infty \quad \int_0^\infty \langle v(t) \cdot v(0) \rangle d\tau < \infty \tag{A.150}
\]

Then from equation A.144 we have,

\[
D = \lim_{t \to \infty} \frac{1}{2t} I(t) = \lim_{t \to \infty} \left[ \int_0^t \langle v(\tau) \cdot v(0) \rangle d\tau + \frac{1}{t} \int_0^t \tau \langle v(\tau) \cdot v(0) \rangle d\tau \right] \tag{A.151}
\]

where the second integral vanishes and we have our final result,

\[
D = \int_0^\infty \langle v(\tau) \cdot v(0) \rangle d\tau \tag{A.152}
\]

which is the Kubo-Formula relating the diffusion coefficient and the velocity autocorrelation function. The velocity autocorrelation function, \( \langle v(t) \cdot v(t + \tau) \rangle \), is a measure of how closely the velocity at time \( t + \tau \) resembles the velocity at time \( t \). Thus it is a measure of strength of interactions (or correlations) in the system.
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