EXAMINING SINGLE MOLECULES IN AN EFFORT TO ANALYZE SUBDIFFUSION IN LIVING CELLS

By

David P. Richards

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requirements for the degree of

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Signature of Author……..……..
Department of Physics
September 18, 2014

……..
Dr. Mark Yuly
Professor of Physics
Research Supervisor

……..
Dr. Brandon Hoffman
Associate Professor of Physics

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Abstract

Brownian motion is named after Scottish botanist Robert Brown, who observed the motion of particles in a fluid in the 1820s. In the first years of the 20th century, Einstein developed a random-walk theory about the thermal energy induced motion of a liquid's particles causing a relatively small number of particles suspended in the liquid to be moved in the process of diffusion, and it was soon accepted that this motion was what Brown had observed. Based on the then-current view of the cell as a membrane containing particles in a fluid, it was expected that molecules within a living cell would move according to Einstein's theory. However, recent experiments have revealed that the diffusion of molecules within living cells occurs slower than Brownian motion would predict. This is not surprising, given the modern understanding of the cell as a diverse environment filled with many particles and structures. Scientists are currently conducting experiments to see if other mathematical models can describe the motion of such particles accurately.

Thesis Supervisor: Dr. Mark Yuly
Title: Professor of Physics

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INTRODUCTION

1.1 Brownian Motion

Brownian motion is the irregular motion of particles suspended in a liquid. It is named after Scottish botanist Robert Brown, who was one of the first to observe the phenomenon. However, it was not until many decades after Brown's observations that scientists realized the cause of Brownian motion: "the random thermal motion of the liquid's molecules" [1]. Traditionally, Brownian motion has been viewed as the mechanism for diffusion in living prokaryotic cells.

1.1.1 Robert Brown's Observations

In 1827, Robert Brown was using a microscope to examine the pollen of plants in an effort to study the process of fertilization. While doing so, he noticed particles contained within the grains of pollen. When placed in water, Brown observed that the particles were in motion, and he noted that "[t]hese motions were such as to satisfy me, after frequent repeated observation, that they arose neither from currents in the field, nor from its gradual evaporation, but belonged to the particle itself" [2]. He observed these particles, along with much smaller objects, which he termed "molecules," in many different types of plants, both living and dead.

Brown went on to examine "various animal and vegetable tissues, whether living or dead" [2] and he always found "molecules." He again found them when he examined minerals (inorganic materials), noting "in every mineral which I could reduce to a powder, sufficiently fine to be temporarily suspended in water, I found these molecules more or less copiously…" [2]. In fact, he eventually observed "molecules" in any material he could suspend in water. As to the cause of this motion, Brown was uncertain.

1.1.2 Development of Theory

In 1905, Albert Einstein published a paper [3] that showed that, according to the molecular-kinetic theory of heat, particles suspended in a liquid should exhibit motions that can be described with a random walk model, provided the ratio of suspended particles to liquid molecules was small. This is the mechanism by which standard diffusion, the motion of particles from an area of higher concentration to an area of lower concentration, takes place. By the following year, Einstein had been informed that other scientists had concluded that his theory was, in fact, talking about Brownian motion [1], a possibility that Einstein himself had considered [3].

1.1.3 Early Experiments

In 1908, Jean Perrin was trying to determine Avogadro's number [4]. To do so, he examined the Brownian motion of small grains of putty in an aqueous solution by looking at the trajectories of single particles. This method, known as single-particle tracking, is advantageous because the different particles may not be identical, causing them to behave differently. Since the trajectories of the individual particles were too short to provide meaningful statistics by themselves, Perrin used ensemble averages of those trajectories. This provided him with the meaningful statistics he needed, but at the cost of making the results less accurate, because, again, the trajectories were from non-identical particles [5].

In 1914, Ivar Nordlund [6] was recording the Brownian motion of a single drop of mercury that was moving through an aqueous solution by projecting it on a moving film. He was able to obtain meaningful statistics from a single trajectory by performing a time average, thus avoiding the need to average the trajectories of non-identical drops, as Perrin had done [5].

1.1.4 Connection to Living Cells

The traditional view of a prokaryotic cell (e.g. a bacterium) was that it essentially consists of particles suspended in a liquid. Because of that view, scientists assumed that the conditions were dilute enough for diffusion to occur within the cell with Brownian motion as the mechanism. Since diffusion is a

result of thermodynamics [3], this would allow the cell to have molecules (e.g. nutrients) transported to areas of lower concentration without expending additional energy in the process [7].

1.2 Examining Single Molecules with Spectroscopy and Statistics

Often, when studying a molecule, scientists will treat it as a system within an ensemble of identical systems. This approach yields the averaged values for properties of the entire ensemble (i.e. an ensemble-averaged property). However, in a living cell the molecules are not identical, prompting an alternative approach: examining the molecules individually. This is what Perrin and Nordlund set out to do in their experiments. It is possible to use single-molecule spectroscopy to obtain the information necessary to perform single-particle tracking. In recent years, these processes have revealed that Brownian motion does not correctly describe the motion of molecules inside a living cell.

1.2.1 Single-Molecule Spectroscopy and Single-Particle Tracking

In single-molecule spectroscopy, a molecule is bombarded with photons. This causes the molecule to transition to an excited state, which it then leaves by emitting more photons. These emitted photons will have different energies, as they are emitted by molecules in a heterogeneous system, as the resonance frequency of the molecule is affected by the surrounding environment. Single-particle tracking can then be used to analyze the photons. Researchers examine aspects such as their location, time dependence, polarization, and spectral content. This reveals details about the region surrounding the molecule [8].

1.2.2 Subdiffusion

These single-molecule techniques have revealed that the diffusion inside living cells cannot be easily explained with standard Brownian motion as the mechanism [7]. A bacterial cell, for example, is more complex than scientists originally thought, containing a cytoskeleton and many particles of various sizes. A eukaryotic cell (e.g. a human stomach cell) is more complex still. This weakens the approximation that a bacterial cell is merely a dilute liquid containing suspended particles. In fact, it is found that diffusion occurs slower than Brownian motion would predict. Thus, this process is known as subdiffusion.

1.3 Significance in Living Cells

It might be assumed that subdiffusion would be detrimental to a living cell, considering that it would result in chemical reactions happening less rapidly. However, there are potential benefits to a slower diffusion process. For example, fractional Brownian motion [9], a mathematical model of subdiffusion, describes a three-dimensional random walk with a fractal dimension [10]. In contrast to the region explored by the traditional random walker [3], this model allows the walker (physically, a reactant in a living cell) to explore a larger region. This process does take more time, but it increases the chance that the reactant will find its target and the reaction will take place [10]. As for cellular processes that require speed, it has been speculated that subdiffusion can be overcome with the use of motor proteins to move molecules [11].

1.4 Thesis Objective

This thesis will describe the theoretical basis behind Brownian motion and its connection to ergodic theory. In addition, it will give brief descriptions of the Continuous-Time Random Walk model (CTRW) and of the Fractional Brownian Motion (FBM) model, mathematical models that could describe subdiffusion. It will also examine recent experiments done to test the validity of Brownian motion as a model of diffusion in living cells. These experiments have revealed that Brownian motion cannot be used as a model of diffusion in living cells.

Chapter 2

THEORY

2.1 Brownian Motion

The first section of this chapter will focus on Brownian motion as described by Albert Einstein. It will also connect Brownian motion to basic ergodic theory.

2.1.1 Einstein's Theory

In his 1905 work "On the Movement of Small Particles Suspended in a Stationary Liquid Required by the Molecular-Kinetic Theory of Heat" [3], Albert Einstein began by using two conceptions of thermal energy to examine particles of a non-electrolyte in a liquid. According to traditional thermodynamics, if z moles (Einstein used the term "gram-molecule," which is so similar to the modern concept of a mole that one can use either concept in explaining this theory [12], [13]) of a substance are dissolved in a liquid of volume V^* that is a part of a larger volume V of the same liquid, such that V^* is separated from the rest of V by a wall that is permeable to the solvent, but not to the solute, then that wall will experience an osmotic pressure p from the dissolved particles according to Equation 1:

$$pV^* = iRTz, \tag{1}$$

where T is the liquid's temperature, R is the universal gas constant, and i is the van't Hoff factor, which is one for non-electrolytes [14], [15] (in his paper, Einstein omitted the van't Hoff factor from this equation, but since he was considering non-electrolytes, the result is the same). On the other hand, if small particles are suspended in the partial volume V^* and cannot pass through the wall then those suspended particles will not exert an osmotic pressure on the wall.

However, using the molecular-kinetic theory of heat, the only real difference between a dissolved particle and a suspended particle is its size. Thus, if n suspended particles are in that partial volume V^*, they will exert an osmotic pressure p on the wall according to Equation 2:

$$p = \frac{RT}{V^*}\frac{n}{N^*}, \tag{2}$$

where N^* is the number of actual molecules in a gram-molecule or mole. Einstein then used thermodynamics to show that the molecular-kinetic theory of heat gives this result, provided that the liquid containing the suspended particles remains dilute.

Einstein then moved on to discuss the theory of diffusion of suspended particles. He used the concept of thermodynamic equilibrium to show that the coefficient of diffusion D, a proportionality factor [11] with units of length squared per time [5], of a dissolved substance consisting of spherical particles of radius P is given by Equation 3:

$$D = \frac{RT}{N^*}\frac{1}{6\pi kP}, \tag{3}$$

where k is the liquid's coefficient of friction. This is significant because it showed that "apart from universal constants and the absolute temperature, the coefficient of diffusion of the suspended substance depends only on the coefficient of friction of the liquid and the size of the suspended particles" [3].

Finally, Einstein came to a description of what he would eventually realize to be Brownian motion itself. He noted that each suspended particle's motion is independent of the motion of all the other particles. He also noted that a particle's motion during one small time interval is independent of its motion during a second small time interval, provided that the time intervals are not too small [3].

Einstein basically treated each suspended particle as a one-dimensional random walker. His approach is essentially as follows [16]. The random walker starts at a position $x = 0$. From there, it can move

along the x-axis in either direction in N total steps, each of length l. Let n_r and n_L represent the number of steps to the right and left, respectively, such that

$$N = n_r + n_L. \tag{4}$$

Let m represent the net number of steps to the right, such that

$$m = n_r - n_L. \tag{5}$$

Thus, after N steps, the walker will be a distance

$$x = ml \tag{6}$$

from its starting point. From Equations (4) and (5), we see that

$$n_r = \frac{1}{2}(N + m). \tag{7}$$

Now, since the steps are independent of each other, if we let p represent the probability of the walker making a step to the right and q the probability of the walker making a step to the left, then the probability of the walker moving in a sequence of n_r steps to the right and n_L steps to the left is

$$P = p^{n_r} q^{n_L}. \tag{8}$$

There are

$$\binom{N}{n_r} = \frac{N!}{n_r!\,(N - n_r)!} \tag{9}$$

ways for the walker to move n_r steps to the right and n_L steps to the left. Thus, the probability of the walker being n_r steps to the right is given by

$$P(n_r) = \binom{N}{n_r} p^{n_r} q^{N - n_r}. \tag{10}$$

The probability of a step to the right is $\frac{1}{2}$, and the probability of a step to the left is also $\frac{1}{2}$. Thus, the probability of a random walker being a distance $x = ml$ from a point of origin is given by

$$P(m) = \frac{1}{2^N} \frac{N!}{\left(\frac{N+m}{2}\right)! \left(\frac{N-m}{2}\right)!}. \tag{11}$$

If the walker is in motion for a total time t and the average time between steps is τ, then we have

$$N = \frac{t}{\tau}. \tag{12}$$

Then, using Sterling's approximation

$$M! = \sqrt{2\pi M} M^M e^{-M} \tag{13}$$

we obtain from Equation (11)

$$P(m) \approx \frac{\sqrt{2\pi N} N^N e^{-N}}{2^N 2\pi \sqrt{\left(\frac{N+m}{2}\right)} \left(\frac{N+m}{2}\right)^{\left(\frac{N+m}{2}\right)} e^{-\left(\frac{N+m}{2}\right)} \sqrt{\left(\frac{N-m}{2}\right)} \left(\frac{N-m}{2}\right)^{\left(\frac{N-m}{2}\right)} e^{-\left(\frac{N-m}{2}\right)}}. \tag{14}$$

Define

$$U(m) = \ln P(m) \tag{15}$$

to get, after some simplification

$$U(m) = \frac{1}{2}\ln(2\pi) + \left(N + \frac{1}{2}\right)\ln N - \frac{1}{2}(N+m+1)\ln\left(\frac{N+m}{2}\right) \tag{16}$$

$$-\frac{1}{2}(N-m+1)\ln\frac{(N-m)}{2} - N\ln 2.$$

Now, using a Maclaurin Series, we can expand this about $\frac{m}{N} = 0$. Upon simplifying, we obtain

$$U(m) \approx -\frac{1}{2}\ln(2\pi) + \ln 2 - \frac{m^2}{2N} - \frac{1}{2}\ln N + \frac{m^2}{2N^2}. \tag{17}$$

Let's consider the behavior when $\frac{m}{N} \ll 1$, which allows the final term in this approximation to be neglected

$$U(m) \approx -\frac{1}{2}\ln(2\pi) + \ln 2 - \frac{m^2}{2N} - \frac{1}{2}\ln N. \tag{18}$$

Solving for $P(m)$ now gives

$$P(m) = e^{U(m)} \approx \frac{2}{\sqrt{2\pi N}} e^{-\frac{m^2}{2N}}. \tag{19}$$

Since the diffusion coefficient (or coefficient of diffusion) is defined

$$D = \frac{l^2}{2\tau} = \frac{l^2 N}{2t}, \tag{20}$$

we see that

$$Nl^2 = 2tD. \tag{21}$$

Since $x = ml$ and

$$P(m)dm = P(x)dx, \tag{22}$$

it follows that

$$P(x)dx = \frac{dx}{\sqrt{4\pi Dt}} e^{\frac{-x^2}{4Dt}}. \tag{23}$$

This is the Brownian probability function, the probability of finding a particle undergoing Brownian motion at position x and time t.

2.1.2 Connection to Basic Ergodic Theory

The ergodic hypothesis can be explained as follows [5]. A large amount N^{μ} of identical particles are distributed randomly into various boxes. The probability $< p >_j$ of N_i particles being in box j is given by

$$< p >_j = \frac{N_j}{N^{\mu}}. \tag{24}$$

An individual particle hopping randomly among the boxes will be inside box j for a time t_j. For an overall experimental time t, that particle has a probability $\bar{p_j}$ of being in box j, such that

$$\bar{p_j} = \frac{t_j}{t}. \tag{25}$$

Now, the ergodic hypothesis states that if the number of particles and the averaging time are large enough, $< p >_j$ (the ensemble mean) and $\bar{p_j}$ (the time average) will be the same. Symbolically, we have

$$\lim_{N^{\mu}\to\infty} < p >_j = \lim_{t\to\infty} \bar{p_j}. \tag{26}$$

Brownian motion is ergodic, as can be shown as follows. For a particle whose probability function is given by $f(x,t)$, the ensemble mean $< x^2(t) >$ is defined as [17]

$$< x^2(t) > \equiv \int_{-\infty}^{\infty} x^2 f(x,t) dx. \tag{27}$$

Note that t is only a parameter in this equation.

The probability function for a particle undergoing Brownian motion is given by Equation 23. Using that expression for $f(x,t)$ and integrating, we obtain [5]

$$< x^2(t) >= 2Dt. \tag{28}$$

For a particle with a trajectory $x(t)$, the time average $\overline{\delta^2(\Delta,T^*)}$ is defined as [17]

$$\overline{\delta^2(\Delta,T^*)} = \frac{1}{T^*-\Delta}\int_0^{T^*-\Delta}[x(t+\Delta)-x(t)]^2 dt. \tag{29}$$

Where T^* is the overall measurement time and Δ is the lag time, a "time window swept along the time series" [17]. Averaging $\overline{\delta^2(\Delta,T^*)}$ over many trajectories yields "a unique, smooth result also at finite measurement times" [5]:

$$< \overline{\delta^2(\Delta,T^*)} >= \frac{1}{N^\mu}\sum_{\kappa=1}^{N}\overline{{\delta_\kappa}^2(\Delta,T^*)} = \frac{1}{T^*-\Delta}\int_0^{T^*-\Delta} < [x(t+\Delta)-x(t)]^2 > dt. \tag{30}$$

We are able to write the average $< [x(t+\Delta)-x(t)]^2 >$ over the square particle position as the number of steps performed during the time interval $(t, t+\Delta)$ times the length of an individual step squared. On average, the former is given by $\frac{\Delta}{\tau}$, where τ is again the average time between steps.

For a Brownian particle, we can used the definition of the diffusion coefficient (Equation 20) to find the time average

$$\overline{\delta^2(\Delta,T^*)} = 2D\Delta. \tag{31}$$

This is the same result we would have obtained had we used Δ instead of t in Equation 28. Therefore, for long measurements of the Brownian motion of a particle, the number of steps self-averages, meaning Brownian motion is ergodic.

2.2 New Theories

Single-molecule spectroscopy and single-particle tracking have revealed that, inside living cells, the ensemble mean of a molecule's position is given by [5]

$$< x^2(t) > \propto Dt^{\alpha}, 0 < \alpha < 1. \tag{32}$$

This is in contrast to the ensemble mean of the position of a particle undergoing Brownian motion, which is proportional to t, as shown in Equation 28. This means that, inside a living cell, the motion of molecules has a weaker time dependence (this is why this process is known as subdiffusion [10]). Since Brownian motion does not correctly describe the motion of these molecules, scientists have been examining other mathematical models to explain diffusion in living cells. Two of these models are the Continuous-Time Random Walk model (CTRW) and the Fractional Brownian Motion (FBM) model.

2.2.1 Continuous-Time Random Walk Model

The CTRW model was introduced by Elliott Montroll and George Weiss in their 1965 paper [18]. Their model differs from the conventional random walk model [3] in that their random walker remains immobile for a random waiting time τ_γ after each step, and $\varphi(t)$, the random distribution of waiting times, is given by [10]

$$\varphi(t) \sim \tau_\gamma{}^{-1-\beta}, 0 < \beta < 1. \tag{33}$$

Of note, the average waiting time $< \tau_\gamma >$ of the CTRW walker diverges: $< \overline{\delta^2(\Delta, T^*)} >$ never converges to $< x^2(t) >$. Thus, the CTRW model is not ergodic.

2.2.2 Fractional Brownian Motion Model

In their 1968 paper, Benoît Mandelbrot and John van Ness introduced the Fractional Brownian motion model [9]. In the FBM model, $< \boldsymbol{r}(t) >$ (the ensemble mean in three dimensions) has a component $x(t)$ that is described by "a stochastic differential equation with random noise"[10]:

$$\frac{d(x(t))}{dt} = \varepsilon(t), \tag{31}$$

where $\varepsilon(t)$ is the random noise. In this model, the dynamics are stationary, meaning the noise correlation function $< \varepsilon(t_2)\varepsilon(t_1) >$ depends on nothing but the time difference $|t_2 - t_1|$. This results in the time average and ensemble mean eventually correlating. Thus, the FBM model is ergodic.

Chapter 3

EXPERIMENTS

3.1 mRNA Molecules in *E. coli*

In 2006, Ido Golding and Edward C. Cox of Princeton University published a paper [7] detailing their observation of mRNA molecules in living *E. coli* cells. They made an mRNA detection system that consisted of a green fluorescent protein (GFP) fused to the bacteriophage MS2 coat protein, and a reporter RNA that contained 96 tandemly repeated sites where the MS2 coat protein can bind. A large number of the tagging proteins bind to each RNA molecule when the protein fusion is coexpressed with the target RNA. This forms bright fluorescent particles that can be observed with a microscope. It was found that the tagged RNA molecules moved randomly in the cell, traversing the complete length of the cell multiple times within a 30 minute observation period, as shown in Figure 1.

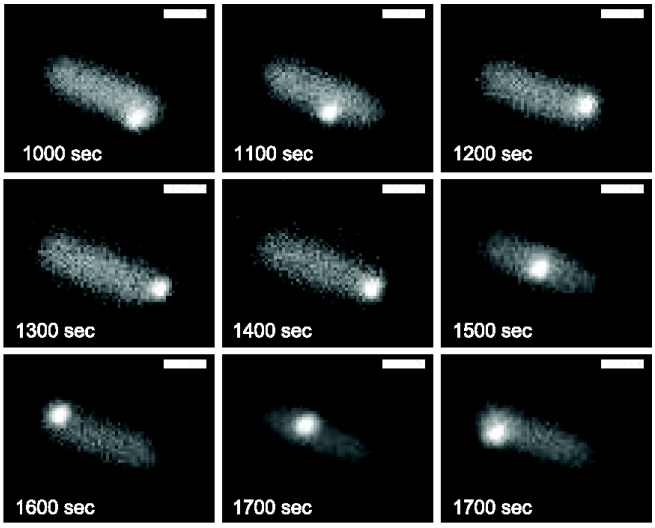

Figure 1. Tagged RNA molecule in an *E. coli* cell. These epifluorescent images were taken 100 seconds apart. Notice how the RNA molecule (the white dot) moves through the entire cell (figure from Ref. [7]).

Golding and Cox then categorized the motion of the RNA molecules by measuring the time average $< \delta^2(\tau) >$ during a time interval $\tau = |t_1 - t_2|$. They found that, on the time scale of seconds to minutes, the time average is given by

$$< \delta^2(\tau) > \sim \tau^{\alpha}, \alpha = 0.70 \pm 0.07. \quad (32)$$

If the RNA molecules were moving according to Brownian motion, we would have $\alpha = 1$. This is illustrated in Figure 2, which compares the motion of an RNA molecule in the cell to its motion in a solution of 70% glycerol, the latter being an environment that fits the conditions necessary for the Brownian motion model to apply.

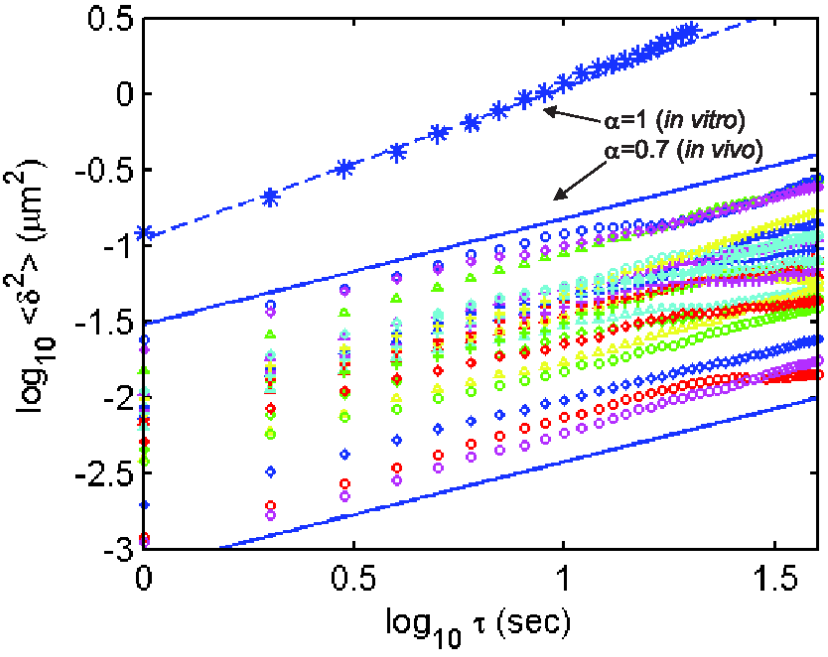

Figure 2. A plot of $< \delta^2(\tau) >$ vs. τ. Here, $< \delta^2(\tau) >$ is the time average and τ the time between measurements for the movement of a tagged RNA molecule in an *E. coli* cell. Different trajectories are denoted by different colors and markers. The slope of the lines that are fit to the data represents the value of α in Equation 32. Notice how the value of α is larger *in vitro* (in a glycerol-based solution) than *in vivo* (inside an *E. coli* cell) (figure from Ref. [7]).

3.2 Telomeres in Human Cells

In 2009, Eli Barkai and six other scientists submitted a paper [19] detailing an experiment similar to that of Golding and Cox. However, this group was examining telomeres (sequences of DNA on the end of eukaryotic chromosomes) in living human cells. The telomeres were labeled with a GFP fused to the shelterin subunit TRF2, which recognizes the telomeres by attaching to the telomeric sequences in human DNA. The fused protein was expressed transiently in the cells, and the scientists typically observed about 60 telomeres in each cell.

They ended up finding that at short time scales of 0.02 to 100 seconds, the two-dimensional ensemble average $< r^2(t) >$ ($< x^2(t) >$ is the ensemble average in one dimension) follows the pattern of subdiffusion, as shown in Figure 3. Symbolically, we have

$$< r^2(t) > \sim t^{\alpha}, \alpha < 1. \tag{33}$$

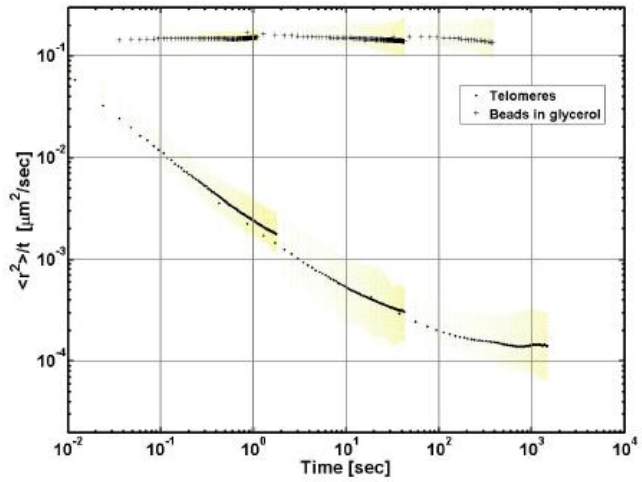

Figure 3. A log-log plot of $\frac{<r^2(t)>}{t}$ vs. t. Notice how the ensemble average indicates subdiffusion until about 100 seconds. The diffusion of fluorescent beads in glycerol is also included; notice how it follows standard Brownian motion (figure from Ref. [19]).

3.3 Lipid Granules in *S. Pombe* Cells

In 2011, Jae-Hyung Jeon and seven other scientists published a paper [20] detailing an experiment in which they studied the motion of single lipid granules inside *S. pombe* fission yeast cells. One of the ways the scientists studied the single-granule trajectories was with an optical tweezers setup. The setup initially centers a trap on the granule so that no force is exerted. A restoring Hookean force acts on the granule when it starts to move away from the trap center.

The researchers examined time averages from granules inside cells in two different stages of the cell cycle (early mitotic and early telophase). They observed that the time average $< \delta^2(\Delta) >$ (Δ is again the lag time) was initially proportional to Δ (standard Brownian motion). However, the time average eventually experienced a turnover, resulting in it being proportional to Δ^β, with $\beta \cong 0.10 \ldots 0.20$, as shown in Figure 4. The time average had turned over to indicate subdiffusion.

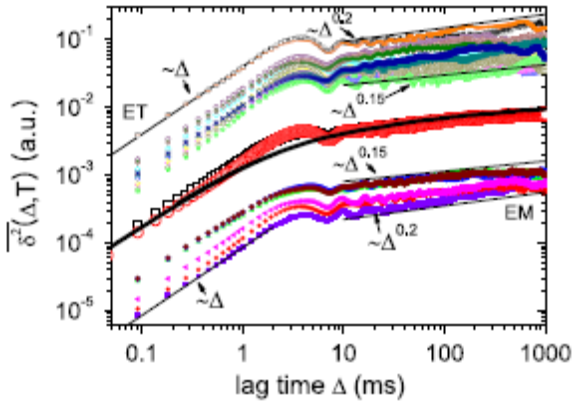

Figure 4. $< \delta^2(\Delta) >$ from individual trajectories of lipid granules in *S. pombe* cells. The upper curves (labeled "ET") correspond to cells in early telophase, and the lower curves (labeled "EM") correspond early mitotic cells. Notice that the time average is not consistently what would be expected based on Einstein's theory of Brownian motion. Instead, standard Brownian motion turns over to subdiffusion (figure from Ref. [20]).

Chapter 4

Conclusion

This thesis examined the motion of particles inside living cells. Previously, it was thought that such particles would behave as particles in a fluid, with Brownian motion. However, recent experiments have shown that Brownian motion does not explain the motion of these particles. These particles diffuse slower than Brownian motion would predict, which makes sense, given that the environment inside a living cell is not similar to that inside a container with a dilute solution in it, and the latter is the only environment where Einstein's theory of Brownian movement is applicable.

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