Systematic Analysis for Ultra-fast Single-Particle Tracking in Live Cells

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UCLA Physics REU, Summer 2007

ABSTRACT
Single-particle tracking at high speeds becomes very convoluted, particularly in biological systems. However, the technique is crucial to understanding processes like the diffusion of proteins in a cell membrane. Therefore, in order to study this motion at very high speeds, a system must be developed with very high sensitivity even as it probes the microsecond time frame. To achieve the needed sensitivity, a thorough noise analysis was undertaken, including an investigation of normal Brownian motion (in the absence of a cell membrane). This paper explores Brownian motion of 200nm gold beads at 40-150kHz and demonstrates the Photron Fastcam SA-1 system's ability to resolve diffusion on the microsecond time scale. This technique will be applied to tracking membrane proteins via 40nm gold beads now that the system has been proven effective.

INTRODUCTION

The Cell Membrane

In the field of cell membrane biology, there is currently a divide between two conflicting models. The traditional theory considers the cell membrane to be analogous to a two-dimensional liquid in which structures, such as proteins and lipids, diffuse freely. Over the last 25 years, however, scientists have come to realize that the diffusion coefficient that describes the speed of a particle's motion is 5-100 times lower in an actual cell membrane than in an artificial bilipid layer. [1] This reduced speed is not easily explained by the traditional model. A new model of the cell membrane proposes that instead of a fluid continuum, the membrane is compartmentalized by immobile proteins anchored to the membrane skeleton. [2] Under this view, membrane proteins do not diffuse freely but rather undergo Brownian motion within a compartment of the membrane before hop-diffusing to another compartment.

This fundamental question of cell membrane structure is hard to address directly. In order to observe the membrane structure, scientists often use 40-50nm gold beads as probes to track membrane proteins. If the trajectories of a gold bead can be determined, and the scientists can say with confidence that each gold bead is attached to exclusively one protein, then some idea of the protein's motion can be extracted. Kusumi and his team of researchers have claimed to see compartmentalized motion of membrane proteins in a variety of cells via this method, but only at high speeds (10-40kHz). [2-3] At lower speeds, the proteins they are tracking appear to undergo regular Brownian motion because the supposed compartment jumps are no longer resolvable. [2-3]

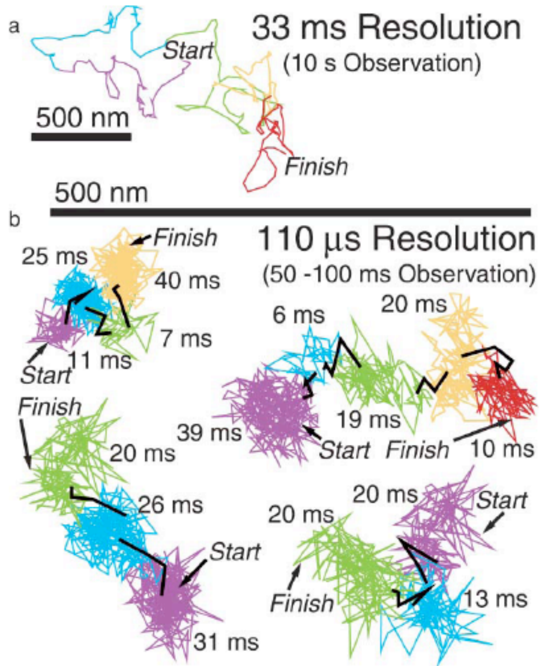

Figure 1. Trajectories of 40nm gold beads attached to membrane proteins published by Kusumi *et al.* showing normal Brownian motion on a slow timescale and potentially compartmentalized Brownian motion on much faster timescales. [2]

High-speed Tracking

As new technologies emerge in the field of microscopy, the potential to push the boundary of current temporal and spatial resolution arises. In the field of Biophysics, faster imaging is especially appealing as it can lead to a better understanding of the rapid processes occurring at a cellular level, such as membrane protein diffusion. Each time imaging is sped up, however, complications materialize that would never have affected a slower system.

This scenario describes the appearance of the Photron SA-1 Fastcam in the arena of biological research. This camera has the ability to probe the microsecond time frame at speeds up to 150kHz, soon to be 500kHz. Its speed can be directly applied to single-particle tracking techniques, such as tracking gold bead labels. Applying this camera to the study of cell membrane dynamics promises a clearer understanding of both the movement of membrane proteins and the structure of the membrane and cytoskeleton themselves, but only once a certain level of reliability and consistency has been established. Ultimately, this camera should be able to achieve 1nm spatial resolution in tracking 40nm gold beads at 150kHz. The rigorous optimization of the system, however, can be a lengthy process. At these high speeds, numerous sources contribute to the systematic noise. Identifying all the noise contributions and calibrating the system for membrane protein tracking require a careful untangling of all the contributions to the protein's motion. The primary contributions include mechanical noise, photostatistical

noise, normal Brownian motion, drift, and possibly confinement. The goal is to be able to distinguish between all of these sources of motion when confronted with a single membrane protein trajectory.

METHODS AND MATERIALS

The Imaging Set-up

The microscope system consists of the Photron SA-1 Fastcam camera coupled to the left port of a Nikon TE2000-U Bio-Inverted Microscope. A 100W Nikon Mercury Arc Lamp illuminates the stage from above and a 100x objective collects light below the stage. Oil-immersion, bright-field microscopy is the default mode of operation, with a magnification factor of 1.5 unless otherwise stated.

Mechanical Noise

Mechanical noise arises in this system as a result of oscillations in the table, vibrations in the stage, vibrations from the fan in the camera, and air movement around the room, among other sources. Minimizing all these contributions required careful analysis of fixed gold beads before and after changes to the environment were made. A certain amount of mechanical noise is inherent in any experimental set-up, but the goal was to approach that minimum value.

The primary method used to accomplish this was preparing a slide where the gold beads had no opportunity to move. These slides were created by placing 20 microliters of the colloidal suspension containing the beads on a glass microscope slide, affixing a 22x22mm no. 1 coverslip on top, and heating the slide until all the liquid had evaporated. The resulting stationary gold beads were imaged at frame rates from 5kHz to 150kHz and analyzed using the program ImageJ.

The ImageJ analysis that was applied to all the gold-bead data sets consisted of the following steps. The data were imported as stacked .raww files. Next the image intensity was inverted using the ImageJ invert function. Then, the background intensity was measured and subsequently subtracted from all the frames leaving only the bead(s) carrying intensity value. A region of interest was specified around the desired bead, leaving a border of only approximately one pixel. Finally, to track the bead, the "Moment Calculator" plug-in was run on the region of interest. This plug-in essentially treats the ADC counts as mass units and finds the center of mass of each frame. Once this point is known for every frame, the center of mass' movement between frames is also known. The results are given as pixel values, but they can easily be converted to nanometers since one pixel corresponds to an area of 133x133 nm (for the magnification previously specified).

Photostatistical Noise

Like mechanical noise, photostatistical noise will always be present, but under the right circumstances, it can be rendered negligible. Poisson noise, for example, always shows a standard deviation equal to the square root of the mean number of intensity units (ADC counts). That means that for one million counts, the standard deviation is only 0.1% of the mean. Dark current is another form of photostatistical noise where even in the absence of photons, some ADC counts will be registered as a result of electrons that

are randomly excited by the thermal energy on the chip. This noise was experimentally evaluated by taking data with the lens of the camera completely shielded from light. These data sets were evaluated simply by identifying the number of registered ADC counts.

Brownian Motion

The third component of the protein's motion that must be isolated is normal Brownian motion. In order to isolate Brownian motion in the trajectory of a membrane protein, it must first be studied in contexts in which the expected result is well-defined. This goal motivated a comprehensive examination of the motion of unattached gold bead probes, usually 200nm for simplicity, in aqueous glycerol solutions of known viscosities.

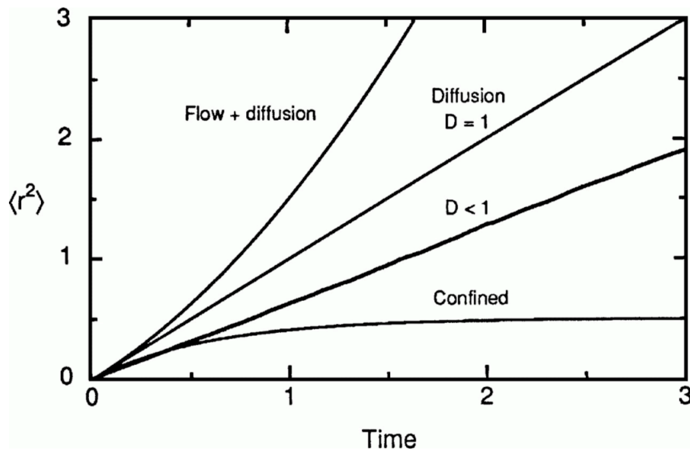

Figure 2. Theoretical Description of Brownian Motion, Confined Motion, and Flow. This graph shows the linear relationship between time and mean squared displacement for a diffusion coefficient of 1, i.e. pure Brownian motion, and how this differs from other possible scenarios. [1]

In order to predict how the data should look, the mathematical model for Brownian motion was carefully applied to this experiment. Pure Brownian motion can be identified based on the mean squared displacements of the particles of interest. This mathematical model predicts average mean squared displacements for spherical particles based on the viscosity of the solution η, the radius of the sphere *r*, and the temperature of the system *T* as seen in Equation 1. [1]

(1) $$\langle x^2 \rangle = \frac{kT\Delta t}{3\pi\eta r}$$

The results of this equation became the theoretical values that were later compared to actual data using the aforementioned imaging system and 200nm gold beads in solutions of water and water to glycerol ratios of 1 to1, 1 to 2, 2 to 1, 1 to 10, and 10 to 1.

The method used to obtain this experimental data started with the sample preparation, which varied slightly based on the desired viscosity. First, the gold bead solution was vortexed and sonicated for approximately one minute each to minimize clumps of beads. Then, typically 20 microliters of the gold bead solution were placed in

a vial. For water measurements, the gold bead solution was placed directly on a 22x22mm no. 1 glass coverslip and contained by the placement of a chamber on top. For all other viscosities, the desired amount of stock glycerol was added to the vial. For one to one ratios of water to glycerol, 20 microliters of glycerol was added to the 20 microliters of gold bead solution. For a ratio of one part water to two parts glycerol, 40 microliters of glycerol was added, and so forth. The vials were then vortexed for at least two minutes to ensure the complete mixing of the water and glycerol. Finally, 10-20 microliters of the final solution was placed onto a 22x22mm no. 1 glass coverslip and held in place with a chamber.

These samples were always made immediately before data was taken. The vials of gold beads in solutions of particular viscosities may have been prepared as much as 5 days in advance, but the slides themselves were always generated right before data-taking. The vials were also vortexed and sonicated for at least one minute each immediately prior to pipetting the solution onto a slide to counteract any settling that may have occurred.

To actually take data, these slides were placed on the stage chamber-side down touching the objective via oil immersion. The lowest possible focal plane above the chamber wall was used to avoid any drift from contact with the objective. Most data sets were taken at 100kHz, and the best 8,000 frames were saved giving slightly less than one-tenth of one second of data. However, data were also taken at frame rates from 5kHz to 150kHz. These data were analyzed as previously described, using ImageJ to isolate a bead of interest and to track its diffraction pattern via "center of mass" calculations. Once a given bead's displacements were known, its mean squared displacement could be determined readily.

RESULTS

Brownian motion results showed better agreement with the theory for lower viscosities and lower frame rates. Some of these data are included below as graphs of the mean-squared displacement versus change in time.

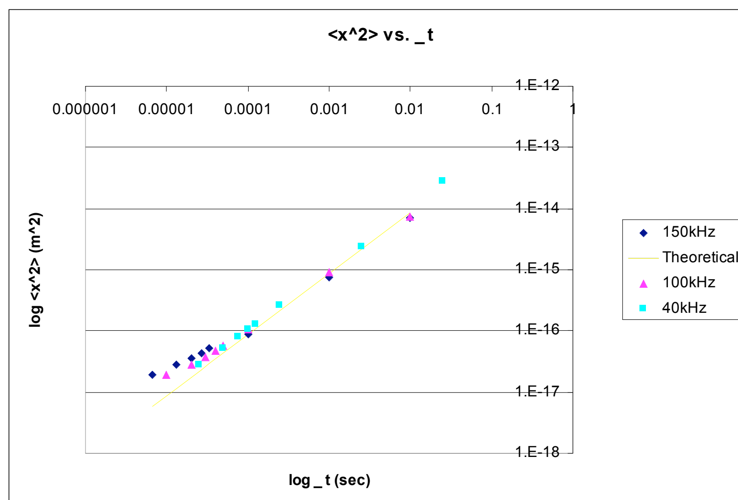

Figure 3. Plot showing relationship between frame rate and agreement with theoretical data for 200nm beads in a 1:1 water:glycerol solution.

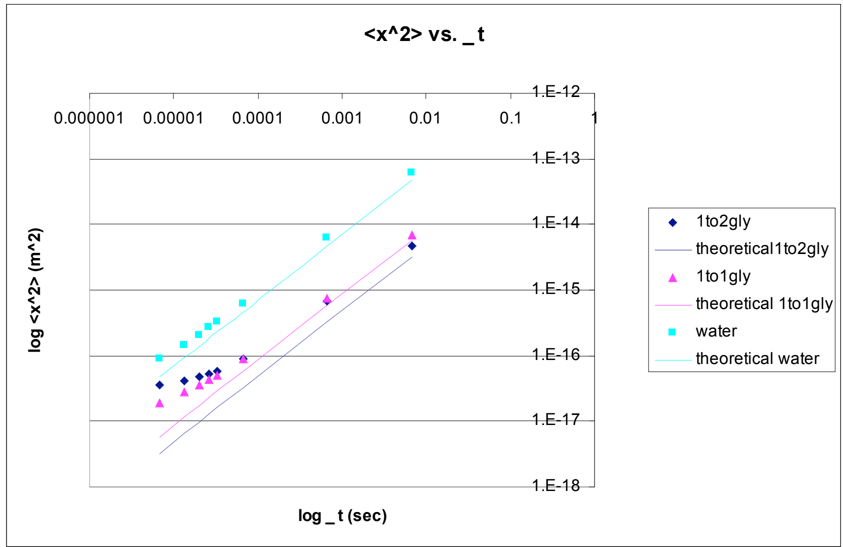

Figure 4. Plot showing relationship between viscosity and agreement with theoretical data for 200nm beads at 150kHz.

These two graphs show that increasing frame rate and increasing viscosity adversely affect theoretical agreement of data. However, they also show that for low viscosities and frame rates as high as 150kHz, Brownian motion can be observed fairly accurately by the current imaging system.
A neutral density filter analysis showed the relationship between accuracy of results and amount of light input.

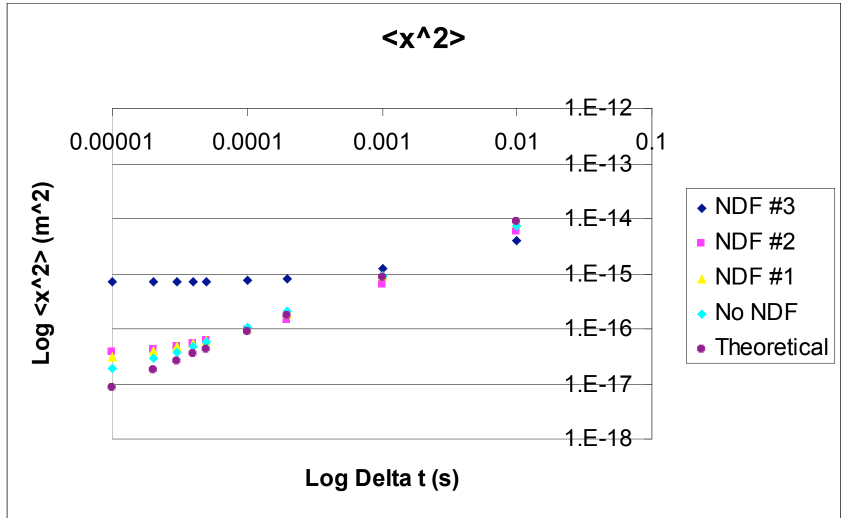

Figure 5. Brownian motion analysis applied to 200nm beads in 1:1 water:glycerol solution. Data taken at 100kHz. Each neutral density filter (NDF) halved the light input of the previous filter (i.e. NDF #1 corresponds to half original intensity, NDF#2 corresponds to one-fourth original intensity, etc.)

DISCUSSION

The Brownian motion curve for 200nm gold beads in a 1 to 1 water to glycerol solution shows that as we probe faster and faster frame rates, our agreement with theoretical predictions decreases. The same decline in agreement with the theoretical curve can be seen for beads in solutions of increasing viscosity. These results are expected because as either frame rate or viscosity is increased, the theoretical step size for the bead's motion decreases. For higher frame rates, the step size between frames is smaller because the motion is being broken down into more and more steps. For higher viscosity, the step size for any given delta *t* is expected to be smaller since high viscosity solutions oppose motion more than low viscosity solutions. The problem with probing smaller and smaller step sizes is that as these steps approach 1nm, they also approach the systematic noise, which is still on the range of 5nm. Therefore, for smaller step sizes, the observed motion reaches a point where it is on the same scale as the noise and is no longer easily resolvable. That is what the flattening out of the Brownian curves corresponds to—the point at which the motion is overwhelmed by noise and smaller steps can no longer be resolved by our system.

These data, therefore, serve to characterize the system in its present state to show how much pure Brownian motion can be resolved and what our limiting displacement is for various conditions (bead size, frame rate, viscosity, etc.). Now that these parameters are substantially known, the cell data can be better interpreted. Reliable and unreliable cell data can now be distinguished based on the probe size and the measured displacements. Of course, the next step is to decrease the noise and develop a more reliable method of tracking to increase the reliability and precision of the system. These improvements are already underway in the form of decreasing mechanical noise, minimizing photostatistical noise, and writing a tracking program that sacrifices less contrast and more accurately reflects the true trajectory of a given gold bead.

Acknowledgments

I would like to thank my advisor, Dr. Katsushi Arisaka, and his talented graduate students, Adrian Cheng and Lea Fredrickson, for their help with every aspect of this project. Additionally, none of it would have been possible without all the work put in by Francoise Queval. Finally, I would like to thank the NSF for funding this research.

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