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BROWNIAN MOTION AND SINGLE PARTICLE TRACKING

ABSTRACT

This lab begins with the ground-breaking physics of a century ago demonstrating the atomic nature of matter and ends with today's biophysics state-of-the-art of intracellular transport and molecular motors. The pairing of advanced light microscopy with automated image analysis and particle tracking software provides a powerful tool for investigating the motion of molecules, organelles, and cells. In the first part of this lab, Perrin's work will be replicated with such modern equipments: the motion of synthetic beads suspended in liquids of various viscosities will be tracked and studied. In the second part, the motion of particles inside living cells will be observed. Thereby this practical will introduce the bases of bead suspension sample preparation, microscopy aspects, particle detection and tracking as well as data analysis using matlab.

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Please find the exercise on Brownian motion simulation on the web site, and please do it at home before the practical session.

1. THEORY

1.1 What is Brownian motion?

Although it was Jan Ingenhousz who made the first known documented observations of fluctuating movements of carbon dust particles in alcohol in 1765, the discovery of Brownian motion is credited to Robert Brown due to his observations of pollen in water in 1827. Also because the previous description by Ingenhousz was not well known, the chaotic movement was for a long time considered to be a property of living or at least organic matter. Brownian motion is stochastic movements of small particles suspended in a solution. The molecules (for example water molecules) constituting the fluid constantly hit the immersed objects which results in chaotic and non-directed movements. These movements can be measured by the mean square displacement $\left\langle (\Delta r)^2 \right\rangle$ and the lag time Δt and is characterized by the diffusion coefficient D which is a measure of the speed of diffusion. For three-dimensional brownian motions these terms can be put into an equation as follows

$$\left\langle (\Delta r)^2 \right\rangle = 6 \cdot D \cdot \Delta t \tag{0.1}$$

This is only true for isotropic and unrestricted translational diffusion. Brownian motion is actually observed for many different dynamical phenomena. Here we concentrate on isotropic translational displacements (random walk) but brownian motion can be also of rotational, undulating etc. nature. Translational diffusion or random walk in three dimensions can mathematically be described by a differential equation:

$$\frac{\partial \rho(\vec{r})}{\partial t} = D \cdot \Delta \rho(\vec{r}) \tag{0.2}$$

Where $\rho(\vec{r})$ is the particle location distribution and Δ is the Laplace-Operator which is a second order differential operator.

In 1905, Einstein published a paper that predicted a relationship between the mean squared magnitude of Brownian excursions and the size of molecules [1-2]. Now all that remained was to do the experiment. Jean Perrin [3-5] won the Nobel Prize in 1926 for his work confirming Einstein's hypothesis. Perrin's experimental confirmation of Einstein's equation was an important piece of evidence to help settle a debate about the nature of mater that had begun nearly 2000 years earlier in the time of Democritus and Anaxagoras. Since then, a thorough understanding of Brownian motion has become essential for diverse fields are ranging from polymer physics to biophysics, aerodynamics to statistical mechanics, and even stock option pricing.

Albert Einstein has calculated the diffusion coefficient to for a spherical particle

$$D = \frac{k_B T}{3 \cdot \pi \cdot \eta \cdot d} \tag{0.3}$$

where k_B is the Boltzmann constant, T the temperature, η the viscosity of the medium and d the diameter of the diffusing particle. The dimension of the diffusion coefficient is m^2/s. The given relation between diffusion coefficient, temperature, viscosity and particle size is only true for isotropic, non-hindered diffusion of a spherical particle. The diffusion coefficient therefore gives us information about the temperature and viscosity of the system and size and shape of the diffusing particle.

For two and one dimensions the time dependence of mean square displacements for isotropic diffusion differs only in the numerical factor:

$$\begin{aligned} two-\dim: &\quad \left\langle (\Delta r)^2 \right\rangle = 4D\cdot\Delta t \\ one-\dim: &\quad \left\langle (\Delta x)^2 \right\rangle = 2D\cdot\Delta t \end{aligned} \tag{0.4}$$

The diffusion coefficient does not depend on the dimensions in which the diffusion takes place. Hindered or restricted diffusion is, for example, the case where the particle has to diffuse in a porous or structured environment as in cells. Anisotropic diffusion takes place in cases when the particle itself has an asymmetric shape. Then the diffusion coefficient is no simple scalar like in eq.0.3 anymore but becomes a complex tensor.

1.2 Calculation of the Mean Square Displacement

In this introduction we want to limit ourselves to a movement in two dimensions. Consider a trace of arbitrary movements (random walk) of a particle as depicted in Figure 1a). The mean square displacement can be then calculated as follows. For each time point separated by a fixed lag time Δt one obtains a position x_i and y_i. A displacement is then calculated as:

$$\begin{aligned} &x_1 = x(t_1) \quad x_2 = x(t_1+\Delta t) \quad \Delta x_1(\Delta t) = x_2 - x_1 \\ &y_1 = y(t_1) \quad y_2 = y(t_1+\Delta t) \quad \Delta y_1(\Delta t) = y_2 - y_1 \\ &\\ &x_i = x(t_i) \quad x_{i+1} = x(t_i+\Delta t) \quad \Delta x_i(\Delta t) = x_{i+1} - x_i \\ &y_i = y(t_i) \quad x_{i+1} = y(t_i+\Delta t) \quad \Delta y_i(\Delta t) = y_{i+1} - y_i \end{aligned} \tag{0.5}$$

The Δt in brackets shall indicate that this step takes place during a time period of a single lag time Δt. The square displacement $(\Delta r)^2$ is then the sum of the displacements in respect of the different dimensions:

$$\left(\Delta r_1(\Delta t)\right)^2 = \left(\Delta x_1(\Delta t)\right)^2 + \left(\Delta y_1(\Delta t)\right)^2 \tag{0.6}$$

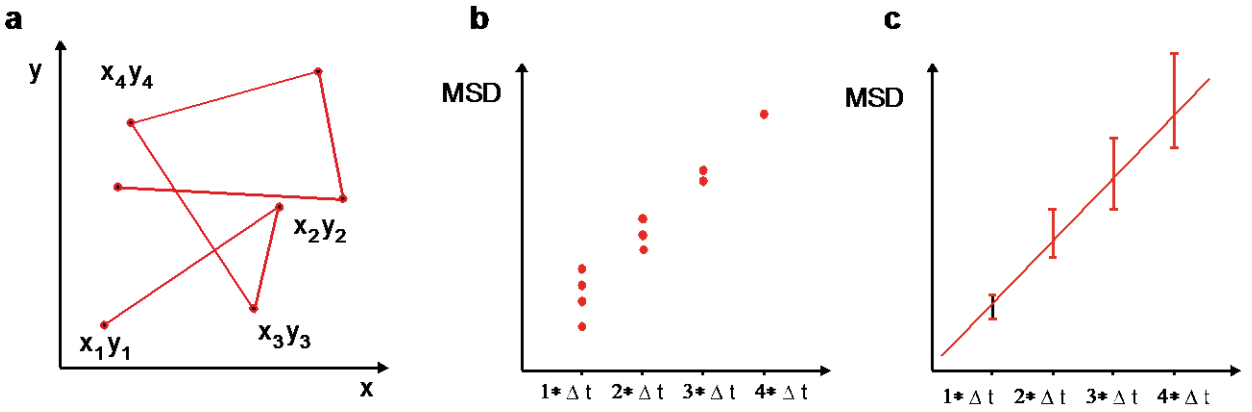


Figure 1. a) Random walk in 2D: intermediate positions and traces of a diffusing particle. Continuous lines indicate the displacement corresponding to single steps, dotted lines to double step during two time intervals. **b)** Squared displacements can be plotted according to the time intervals. Note that for longer steps the number of data points becomes less **c)** Data points corresponding to one time interval merge into on average value. Fitting should give a straight line for unrestricted and isotropic diffusion.

Q1. Note that the error bar becomes larger for larger steps. Why?

The displacement and the square displacement can be calculated for every step of the same trace corresponding to the same step size of stepping time (step during the time length Δt .).

$$\begin{aligned}\left(\Delta r_2(\Delta t)\right)^2 &= \left(\Delta x2(\Delta t)\right)^2 + \left(\Delta y_2(\Delta t)\right)^2 \\ \left(\Delta r_3(\Delta t)\right)^2 &= \left(\Delta x_3(\Delta t)\right)^2 + \left(\Delta y_3(\Delta t)\right)^2 \\ &\vdots \\ \left(\Delta r_i(\Delta t)\right)^2 &= \left(\Delta x_i(\Delta t)\right)^2 + \left(\Delta y_i(\Delta t)\right)^2\end{aligned} \tag{0.7}$$

The mean square displacement is obtained as an average of all steps corresponding to a single lag time Δt :

$$\left\langle \left(\Delta r(\Delta t)\right)^2 \right\rangle = \frac{1}{n}\left(\left(\Delta r_1(\Delta t)\right)^2 + \left(\Delta r_2(\Delta t)\right)^2 + \left(\Delta r_1(\Delta t)\right)^2 + \ldots\right) = \frac{1}{n}\sum_{i=1}^{n} r_i^2(\Delta t) \tag{0.8}$$

The same procedure applies to double step during a time length of $2\Delta t$.

Q2. Please provide step by step calculation for $2\Delta t$ and $3\Delta t$.

Now the mean square displacement (MSD) can be plotted to its corresponding step time interval which gives characteristic curves. If the analyzed diffusion is of isotropic nature then one would expect a linear correlation. In this case the slope of the line corresponds to the diffusion coefficient multiplied with its factor (normally 2, 4 or 6). Diffusion or random walk can be hindered or restricted which changes the characteristic form of the MSD plots. In the case of diffusion restricted to a confined space the MSD

naturally does not exceed the diameter of this space as shown in Figure 2. In biological matter one often finds a combination of both.

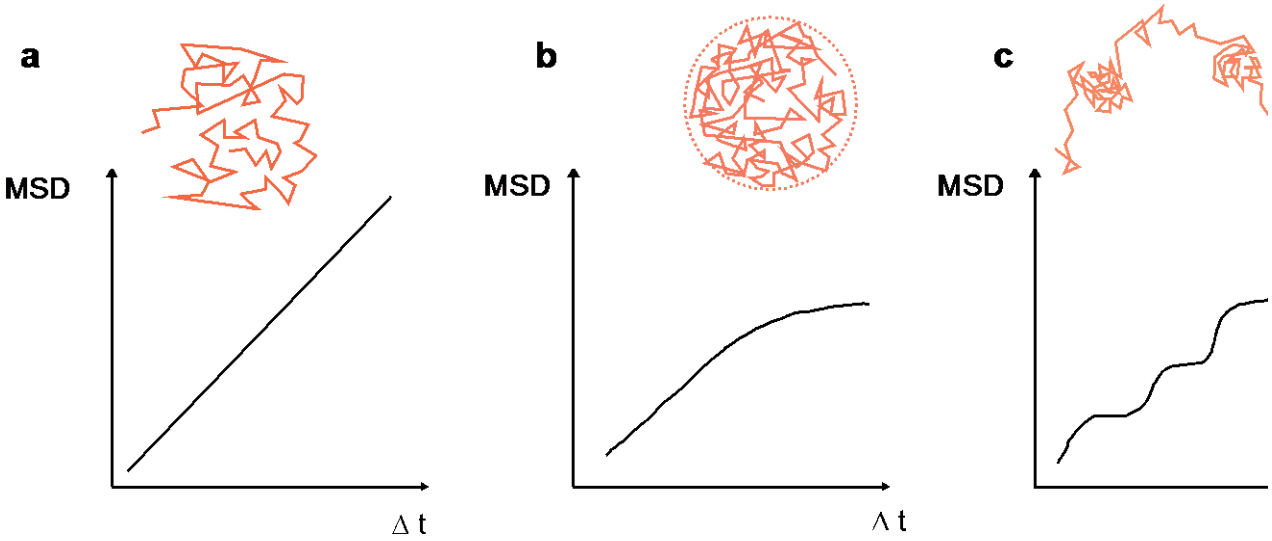


Figure 2. Different types of random walk and their corresponding MSD plot. From left to right: Isotropic random walk, confined random walk and partially confined random walk (hopping).

In the first part of this lab, you will replicate Perrin's work with modern equipment. You will track the motion of synthetic beads suspended in liquids of various viscosities on a research-grade inverted microscope. A CCD camera will transfer images of the beads to a computer for automated particle tracking and analysis. You will explore use of algorithms to improve the identification and tracking of particles and analyze the effects of particle size, viscosity of the solution, and molecular weight of the dissolved solute on the motion of the beads. One of the aims of this lab is to directly reproduce the experiments of J. Perrin that led to his Nobel Prize. He used latex spheres, and we will use polystyrene spheres; otherwise the experiments will be identical. In addition to reproducing Perrin's results, you will probe further by looking at the effect of varying solvent molecule size

Techniques developed in this lab include bright-field/dark-field microscopy, pipetting, image data acquisition, theory and software design for image filtering and particle tracking, and data analysis in ImageJ, Matlab or Excel. Previous programming experience is not required.

1.3 Intracellular Transport

The second part of this experiment consists of observing the motion of particles inside a living cell. Cells transport nutrient, waste, information, etc. in membrane-bound vesicles, which are visible under a light microscope. An old-fashioned view of a cell was that it is a "bag of water" containing various enzymes in which matter is transported passively by diffusion. Though diffusion is an important mechanism, it is too slow and random for long distance transport and directing materials where they are most needed, especially in larger cells. It is now understood that cells have highly developed and intricate mechanisms for directed transport of materials.

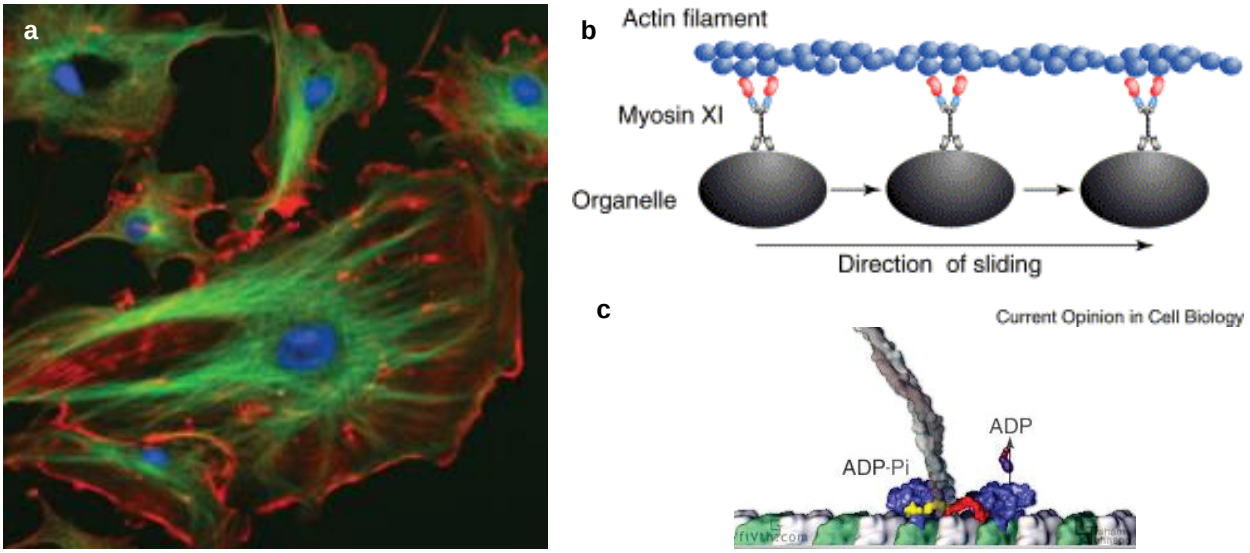


Figure 3. a) The eukaryotic cytoskeleton. Actin filaments are shown in red, microtubules in green, and the nuclei are in blue. **b)** Cartoon of myosin motors pulling organelles along an actin filament .**c)** Binding of kinesin motor to microtubule.

Most motions within and of cells involve two components, a cytoskeletal fiber that serves as a track, and a motor protein that does the work (Figure3. a and b). The motor molecule uses energy from the hydrolysis of one ATP molecule to bind to the fiber, bend to pull itself along the fiber, and release, all of which constitutes one "step". For an animation of this stepping process, see this movie animation from the Vale lab web site at UC San Francisco. One can divide cellular motility mechanisms into two classes based on the cytoskeletal fibers involved. Microtubule-based mechanisms involve dynein or kinesin motors pulling on microtubules made of the protein tubulin. Actin-based mechanisms involve myosin motors pulling on actin fibers, also called microfibers.

Virtually all cell types exhibit directed intracellular transport, but some cell types are particularly suitable for transport studies. Fish-scale pigment cells work well, since a large fraction of the cargoes that are transported are pigmented and thus easy to observe – the disadvantage is that you would need to bring a living fish into lab as a source of these cells. For convenience, we will use epidermal cells from onion bulbs that you can easily acquire in a grocery store (Coop or Migros). With some care, a single layer of cells can be peeled off from an inner section of the onion bulb and mounted flat on a slide.

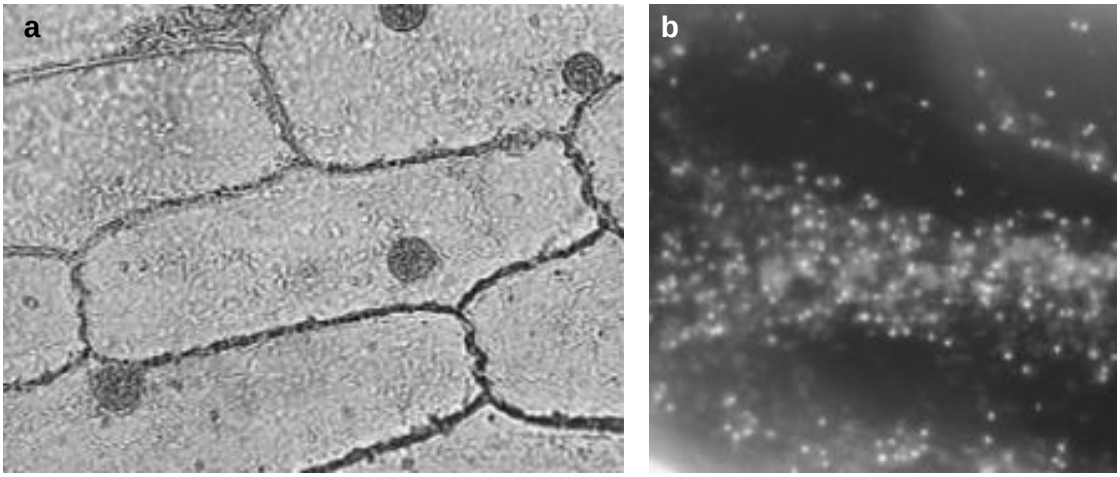


Figure 4. a) Onion cells in bright-field illumination. Round object in each cell is the nucleus. **b)** Vesicles in the cytoplasm of a plant cell, as seen in dark-field.

In this experiment, we will be viewing the movement of vesicles within the cytoplasm of onion epidermal cells, shown above as they appear in bright-field and dark-field microscopy Figure 4. a and b. The layers you see in an onion bulb develop into leaves when it sprouts. Both sides of the leaf are covered with an epidermis consisting of brick-shaped cells, each with a cell wall and cell membrane on the outside. Most of the interior of the cell is filled with a clear fluid vacuole that functions in storage and in maintenance of hydrostatic pressure essential to the stiffness of the plant (the difference between crisp lettuce and wilted lettuce). The cytoplasm, containing all of the other cell contents, occurs in a thin layer between the cell membrane and the vacuole, and in thin extensions through the vacuole called transvacuolar strands. It is within the cytoplasm that you will be observing directed transport of vesicles by an actin-based mechanism. These vesicles are spherical or rod-shaped organelles such as mitochondria, spherosomes, and peroxisomes ranging in size from 0.5 to 3 microns..

The diagram of an onion cell (Figure 5) shows the location of the cell wall, cytoplasm and vesicles in a typical cell; you will not be able to see much of the endoplasmic reticulum or the vacuole depicted because of their transparency. Under the microscope, you will notice the vesicles are located just along the edges of the cell, or near the top and bottom surface if you focus up and down. When you see a narrow band of moving vesicles in the center of the cell, it is located in a transvacuolar strand, which may be a handy place to study motion. In plant cells, vesicles are transported along actin fibers by myosin motor molecules. An actin filament is composed of two intertwined actin chains, about 7 nm in diameter. An actin fiber is considered structurally polar, having a (+) end and a (-) end, and most myosin motors move only towards the (+) end of the actin fiber. In order to reverse the direction of a vesicle's motion, the vesicle must grab on to another actin fiber oriented in the opposite direction. There are at least eighteen described classes of myosin, of which three, myosin VIII, XI, and XII are found in plant cells. Some myosin motors are processive, meaning that they remain bound to an actin fiber as they move step-by-step along it (analagous to this movie animation of kinesin. Other myosins are non-processive, releasing from the actin fiber after each step. Myosin II found in muscle cells is non-processive, as illustrated in this video animation. In the muscle functional unit, there are many myosin motors acting together, so there are always some attached to the actin fiber. The myosin XI responsible for transport of plant cell vesicles is the fastest myosin known and is processive. It is not certain how many myosin molecules are attached to the surface of a vesicle or how many of those are active at one time in pulling the vesicle along an actin fiber.

In some plant cells and algal cells, a large-scale streaming motion of the cytoplasm is observed, logically called ***cytoplasmic streaming***. This bulk flow is believed to be caused by myosin motors pulling the extensive endoplasmic reticulum along actin fibers lining the cell membrane. Many other vesicles are then dragged along with the endoplasmic reticulum. Lodish and Berk, et al. provide a detailed explanation of this process and a video of cytoplasmic streaming in the pond weed Elodea can be viewed here.

In your observations of vesicles in onion epidermal cells, you should distinguish between the random Brownian motion of vesicles that are unattached (or at least not actively moving along) actin filaments, the directed transport of vesicles by attached myosin motors, and possibly (though we are not sure this really happens in onions) bulk flow of vesicles in cytoplasmic streaming.

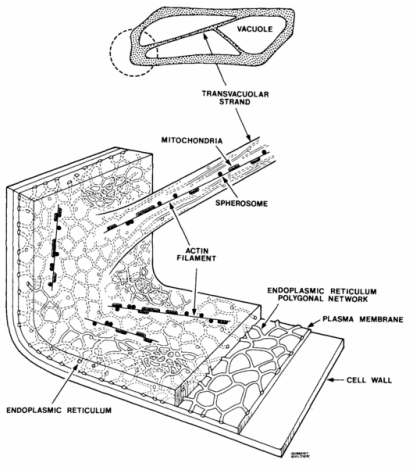


Figure 5. A 3D cross-section model of an onion epidermal cell, showing actin filaments and vesicles in the narrow bands of cytoplasm within the cell.

2. PRACTICAL WORK

2.1 Material requirements

- *Handling.* Safety glasses, gloves, tweezers, pipettes, spoons, razor blades, scalpels,
- *Machines* .IX 71 microscope, dark field bright field, Pipettor: Finpette 10-100µL
- *Products.* Synthetic beads from Bangs Laboratories, http://www.bangslabs.com (10,4,1µm)

* Solvents: Glycerol, PBS, water

Please read the microscopy part, as well as the Koehler/dark field illumination part of the master handout BEFORE using the microscopy.

2.2 Calibration and Testing (done by TA beforehand)

Before taking data in your first investigation, you must calibrate the microscope and learn the experimental techniques involving pipetting, microscopy, and data-taking. Your first slide preparation of 10µm beads will be used to determine the conversion from pixels in the image to µm on the actual specimen. This calibration must be done separately for the microscope's 20x objective lens and 40x objective lens. For testing purposes, you will make a slide of 1µm beads in water, set up dark-field illumination on the microscope, and experiment with settings of the lighting, focus, and particle tracker software to successfully track beads and save data on particle motion. Setting up bright-field Kohler illumination and dark-field illumination requires careful alignment and some practice, but this will pay off later in the quality of your images and data.

2.3 Viscous suspensions preparation

A solution will consist of three parts, PBS buffer, which makes up most of the solution, a solute glycerol to provide the viscosity, and the beads which we will then observe. PBS buffer together with glycerol and beads solution is located in the 4 °C in the fridge and are also clearly marked. Note that the glycerol is extremely sticky (i.e. viscous) and will not be measured precisely using the pipette as it will stick to both the inside and outside of the filter tip. To measure glycerol it is best to use the scale located at the lab station. Note (1:1 ratio water glycerol (labeled 50% glycerol by weight) 1:2 (labeled 75% glycerol by weight) are also located in the 4 °C fridge.

Use the table below to compute the required dilution of the solvent. The stock Glycerol is a thick liquid with >99% purity. It will require some care to measure the pure glycerol accurately, since it tends to stick to the sides of the pipette tip. For this reason, weighing the glycerol before adding the water is probably a better technique than adding the glycerol to the water. You may either use these values or interpolate between them.

PLEASE do ONLY 3 different viscosities pure water and the other two marked in red!

Glycerol Viscosity versus Concentration	
Viscosity (cP)	**Glycerol to Water Ratio (by weight)**
1.66	18%:82%
2.5	32.2%:67.8%
4.65	44.2%:55.8%
13.2	64.5%:35.5%

Note : 1 cP = 1 mPa·s = 0.001 Pa·s

1) Take out a new plastic vial to contain the viscous solution
2) Turn on the balance
3) Place vial on balance and ensure all of the doors on the enclosure are shut.
4) Zero/Tare the balance by pressing the 0/T button to cancel out the weight of the vial
5) Place a moderate amount of solute into vial
6) Weigh the amount of glycerol using the balance
7) Calculate how much water is needed to obtain the desired viscosity using the table above
8) Add the appropriate amount of water to the vial by pippeting

2.4 Polystyrene suspensions preparation

Beads are located (and should be kept) in the refrigerator. Each of the vials is clearly marked with the size of bead that it contains. (Note: The sizes reported on the vials are mean particle diameter, not radius.) These vials are often extremely concentrated and you may wish to create your own diluted solution to work with. We have created 1:25 dilutions already.

Note that the densities vary to a small extent between the different size particles. For this reason, the following procedure may need to be adjusted slightly for each of the different particle sizes.

1) Remove a bead vial of the desired size from refrigerator shake it vigorously to ensure it is mixed uniformly.
2) Using a NEW filter tip extract 20µL from the vial containing (1:25 bead dilution) and deposit into a new plastic vial.
3) Use the large volume micropipette to add 80µL of desired viscous solution to the same vial.
4) Cap vial using a plastic vial top and shake it vigorously to ensure it is mixed uniformly.
5) Repeat this process with each experimental condition selected.

2.5 Floating bead slide preparation

Now that we have a proper vial of viscous bead solution made up we need to transfer a sample of it onto a slide so that we can observe the beads' behavior.

1) Take out a slide from its box and carefully rest in a position to minimize dust contamination.
2) Place a self-adhesive reinforcement ring onto the center of a new slide. This will create a well for the solution and keep it from drying out. See Figure 6.
3) Make sure that this label is well pressed down onto the slide to ensure that liquid isn't sucked out towards the open air. Rubbing the edge of another slide over the coverslip provides a good method of pushing down the well without contaminating the slide with oils from your hands.
4) Remove outer adhesive liner

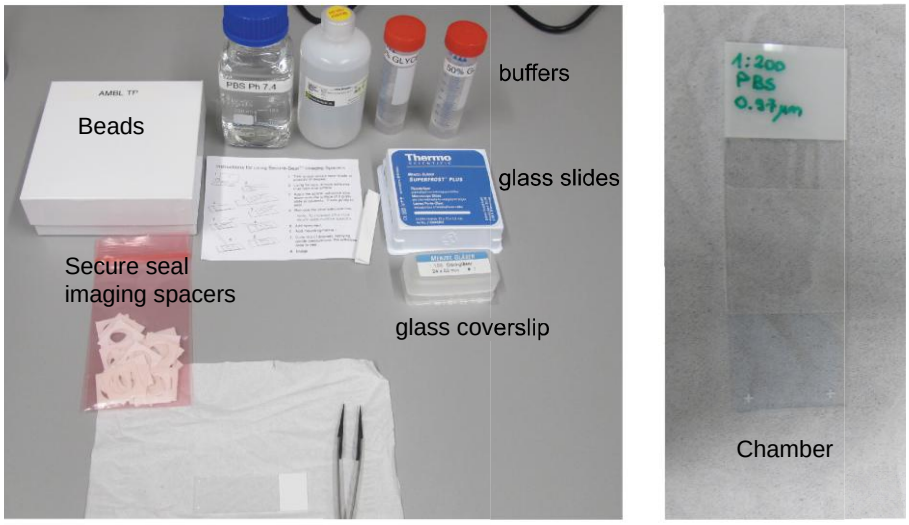


Figure 6. Make a beads slides

5) Use the pipette to transfer roughly 30-35µL of your bead solution into the center of the well
6) Cover the slide with one of the small 24 x 60mm coverslips. It is important to ensure that air bubbles do not form beneath the coverslip. To prevent this, rest one edge of the coverslip on the slide and then let the other side drop onto the slide. (Capillary action will adhere the coverslip to the slide.)

2.6 Slide loading

1) Move the objective lens away from the microscope stage first before placing the slide onto the stage.
2) The entire carrier arm that holds the HAL 100 illuminator and the condenser tilts backward on a hinge to facilitate access to the microscope stage. Push back gently on the angled metal nosepiece until the arm comes to a rest.
3) Our microscope is an inverted microscope and as such the sample (i.e. side with the coverslip) should be positioned such that the coverslip is on the bottom side of the slide. This will ensure that the sample is closest to the objective. You will not be able to focus on the sample using the 40x objective if this is not the case!

2.7 View the slide in transmitted light

In this step, you will view your sample under Köhler illumination to achieve uniform illumination with little reflection or glare and minimal sample heating. Please find the Koehler illumination protocol in the master handout.

The samples in this lab are difficult to focus on because they have very little contrast. If you have trouble focusing, try starting with the 10x objective. At higher magnification, it is sometimes helpful to focus on the edge of the slide first to get the setting close.

SAFETY (MICROSCOPE MANIPULATION)

Stay away from slower particles for they are close to the edges. The eyepieces are designed to be used while wearing eyeglasses. If you do not wear glasses, **DO NOT** get too close to them.

2.8 Set-up darkfield illumination

Now, you can set up the dark field illumination. Please find the Dark field illumination protocol in the master handout.

1) Select the 20x objective and establish the Koehler illumination.
2) Set the dark field illumination.
3) Open the field iris all the way.
4) Increase the light intensity using the Toggle Switch for Illumination Intensity. You will need to turn the light level up significantly in order to see the small amount of light scattered by the smaller nanoparticles (even though the PS spheres will be easily visible).

2.9 Viewing / Tracking Particles in Viscous Suspensions

Now that suspensions and sample chambers have been made for each experimental condition, and the microscope has been fully configured, we are now ready to take data.

1) Use 10μm beads sample to establish the pixel size of your camera. (already done by your TA)
2) Turn on the Andor camera.
3) Open Andor camera software called Solis.
4) Turn the switch on the microscope to send an image to CCD camera.
5) Click on the movie camera icon to get a live image from your sample.
6) Set up the exposure time to 0.05s *by pressing exposure button (Figure 7).
7) To take pixel calibration image open in the main menu acquisition, under setup CCD, select **Single** and enter the following value: exposure time 0.01-0.075s. Then under Setup acquisition, open binning to 512-512 pixels, you can move binning box to the region around your 10μm bead press Ok and close Acquisition menu.
8) Press Record and save image as sif file.

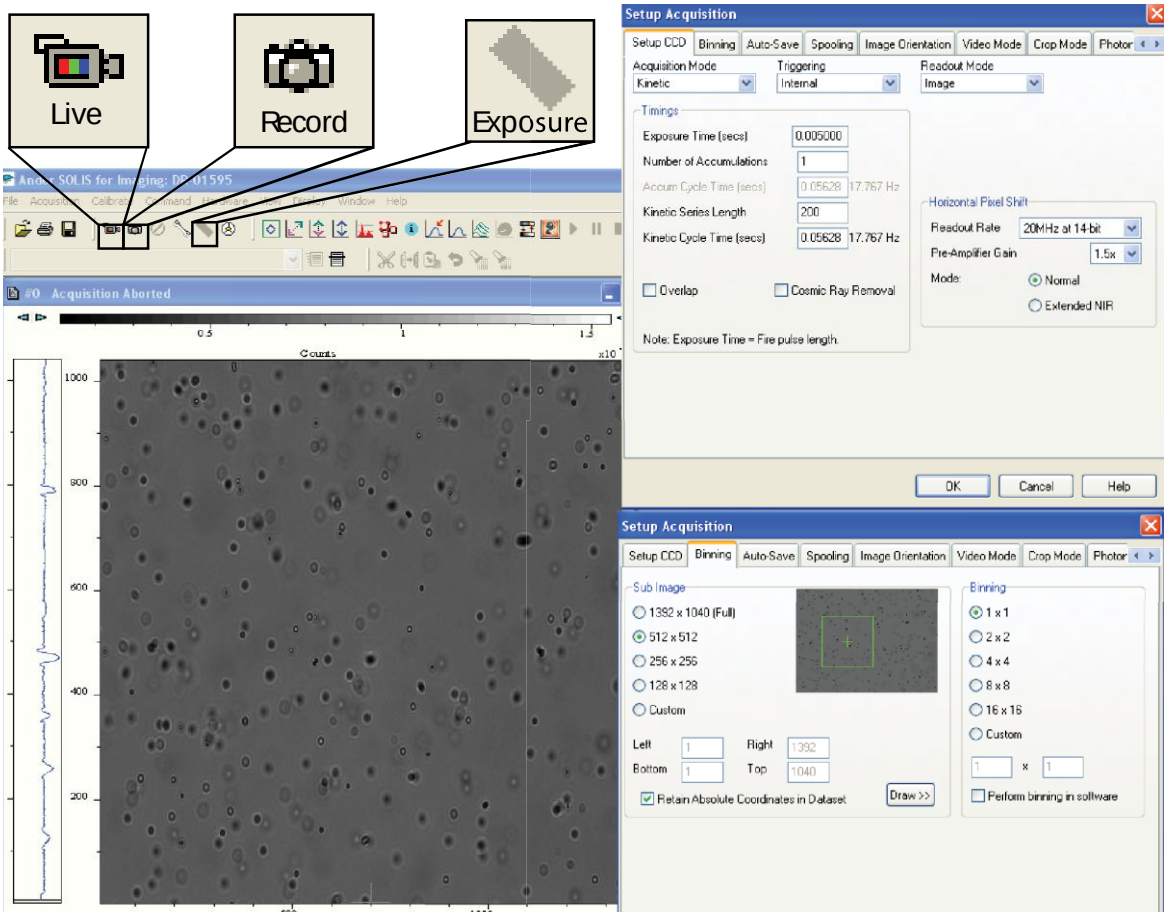


Figure 7. Andor Solis program for data acquisition. Bright field image of 0.97 μm beads.

9) Watch out for bulk flow. If you see a number of particles moving in one direction, they are likely undergoing bulk flow. This could be due to evaporation of liquid from beneath the coverslip or an air bubble popping, or various other conditions. If you see bulk flow occuring, your data will be skewed. **MAKE SURE** there is no bulk flow when collecting data.

10) Now we are ready to collect movies for your analysis session.

11) To setup your movies, exposure time Δt , kinetic series length (number of frames in your movies) open in the main menu acquisition, under setup CCD, select Kinetic series enter following values, exposure time 0.01-0.1s, kinetic series length 200, next under Setup acquisition open binning to 512-512 pixels , you can move binning box to the region containing the most beads. Mark in your notebook the values you entered.

12) Press record.

13) Save files as sif . Collect all necessary data and save them in your folder.

14) Repeat this for each experimental condition selected, select three viscosities for 0.97 μm beads.

15) Once you have finished data collection, you will need to convert all sif. files in raw files. You can do it file by file or using a batch conversion option in File menu (Main Menu). Make sure

that you convert it in 16 bit unsigned integer (with range 0-65322). This is format required for the analysis session.

16) *For those of you interested in a challenge, you can attempt to create a program in C++ or Matlab to adjust for bulk flow in the slide.*

17) *If you have enough time, repeat this experiment for other bead size or add one more viscosity*

2.10 Making an onion slide

We will now try to track particles in a living cell (onion!). Before coming to lab obtain an onion from your favorite produce store. If you have forgotten one you can try to find some in 4 °C fridge.

1) Use a knife, box-cutter, razorblade or whatever other cutting tool is provided to cut out an one inch cube from the onion.
2) Take one of the lower layers (activity depends somewhat on depth) and remove the lower membrane using the forceps, this is similar to pulling off a sticker. The membrane is a single layer of cells which makes it particularly clean when viewing through a microscope. It should appear translucent and should be relatively strong. Make a slide using this membrane.
3) Place a drop of PBS onto a clean slide (don't use water).
4) Place the membrane onto the slide.
5) Drop a couple more drops onto the onion and cover with a large 24x60mm cover glass slide.
6) Blot excess liquid using a paper towel and mount it onto microscope.

⚠ Keep in mind that the lifetime of an onion slide is about 30 minutes before it dries out.

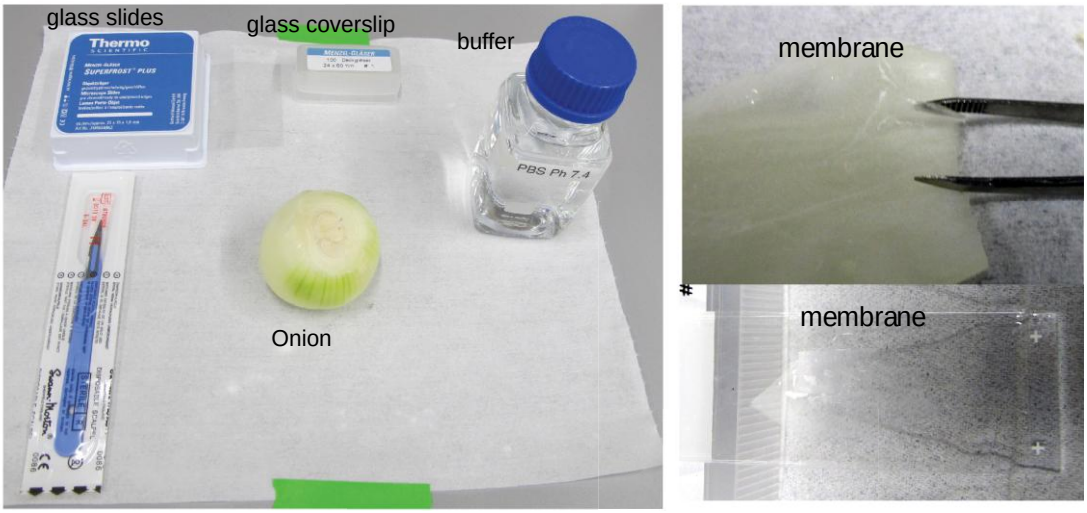


Figure 8. Make an onion slide

2.11Making Observations

First you should spend a little while looking around, trying to find some regions of interest. Note the different types of movement and where they tend to occur. In particular, be sure to investigate regions around the cell walls, around the nucleus, and also see if you can find anything happening within the otherwise empty center of the cell. Most of the activity happens on the lower and upper layers of the cell as the center is occupied with the vacuole which should be devoid of anything except water. If you scan through the depths of a few cells carefully (using the focus knob to move in depth), you should be able to find isolated actin fibers which make for very clean data-taking. Your data analysis will be much easier if you can isolate the forms of movement within the cell and only take data on one type at a time.

If you don't find much activity you could try a different section of onion or another onion altogether.

1) Locate a particle that does not appear to be moving around very much (i.e. look for a particle undergoing Brownian motion rather than active transport).
2) Take several movies of a number of particles undergoing Brownian motion. See Figure 9.
3) Take then some movies of particles undergoing active transport within the actin filaments.
4) Repeat this for a number of different cells.
5) Change the microscope to obtain a transmitted light image of the onion cells.
6) Determine the size of the particles by counting the number of pixels each one take up on the screen as you did with the 10μ polystyrene spheres to obtain the pixel to meter conversion.
7) Repeat this step as necessary to obtain a statistically acceptable number.

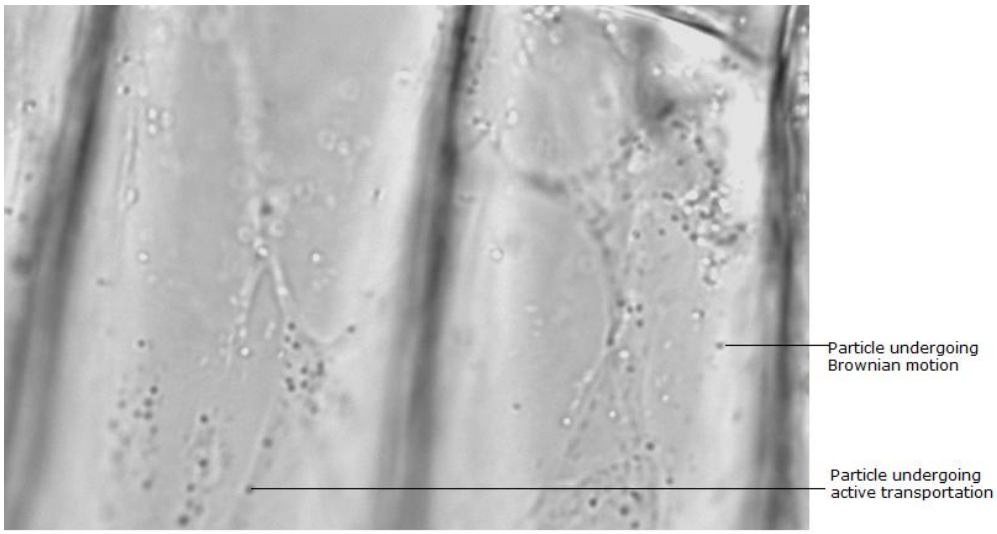
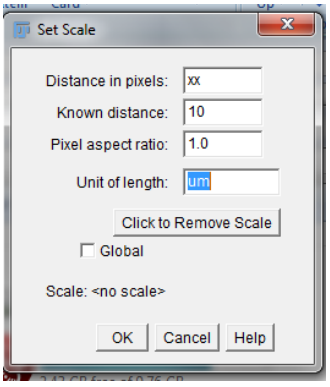


Figure 9. Visualized vesicles in onion cells (x20)

3. DATA ANALYSIS

3.1 Calibration (already done by your TA)



1) Open in ImageJ your dark filed image of 10μm beads taken for bin size of 512*512 pixels.
2) Use a line tool in ImageJ toolbox to draw a line across the selected bead. Below the Image J toolbox you will notice x,y coordinates of your line together with the angle and the length of the drawn line. Make sure to draw the line straight across the bead diameter.
3) Next open **Analyze\Set Scale** from File menu where you enter the length of 10μm bead as known distance. It will calculate the pixel aspect ratio. Use these parameters to set a scale on all movies that you will be processing.

Make sure you have used same objective! For 40x objective 1μm size bead corresponds to 7.7 pixels

3.2 Particle Tracking

To obtain single particle trajectories from recorded movies you will need to use Particle Detector and Tracker which is an ImageJ Plugin for particles detection and tracking from digital videos.

The plugin implements the feature point detection and tracking algorithm as described in recent publication by *Sbalzarini et al.*[6] This plugin presents an easy-to-use, computationally efficient, two-dimensional, feature point-tracking tool for the automated detection and analysis of particle trajectories as recorded by video imaging in cell biology. The tracking process requires no apriori mathematical modelling of the motion, it is self-initializing, it discriminates spurious detections, and it can handle temporary occlusion as well as particle appearance and disappearance from the image region. The plugin is well suited for video imaging in cell biology relying on low-intensity fluorescence microscopy. It allows the user to visualize and analyze the detected particles and found trajectories in various ways: i) Preview and save detected particles for separate analysis; ii) Global non progressive view on all trajectories; iii) Focused progressive view on individually selected trajectory and iv) Focused progressive view on trajectories in an area of interest.

It also allows the user to find trajectories from uploaded particles position and information text files and then to plot particles parameters vs. time - along a trajectory.

1) Before the plugin can be started you must open an image sequence or a movie in ImageJ. For opening your saved movie use the **File\Import\ Raw.** You should input following parameters as indicated in Figure 10. ***(Check your lab notes for number of frames and binning size).*** Upon file import you should obtain video sequence of your moving beads.

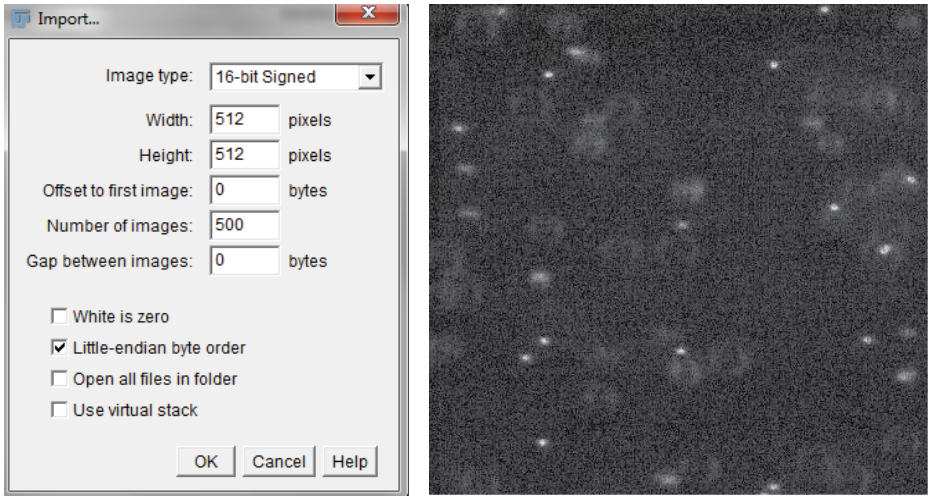


Figure 10. Import parameters.

2) Next you need to improve contrast and adapt your movie so that it can be treated with ParticleTracker plugin. To do so use the **Image\Type\ 8 bit** option from File menu. Next you need to increase contrast you will do it by using **Process\Enhance Contrast** option from File menu. It is safe to select 0.1%saturated pixels under Use Stack Histogram see Figure 11. To filter out noise use P**rocess\Filter\Gaussian blur option** from File menu. Again safe sigma value to use is 1.2.Befor aoplying this filtering you can preview your movie.

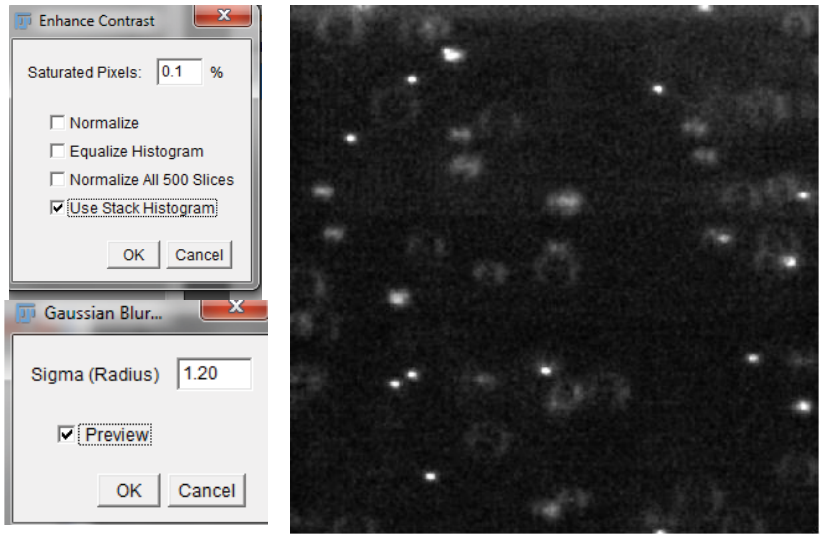


Figure 11. Parameters for better movie quality.

3) Now, that the movie is open and compatible with the plugging, you can start the plugin by selecting ParticleTracker from the Plugins -\ Particle Detector & Tracker menu. After starting the plugin, a dialog screen is displayed. The dialog has two parts "Particle Detection" and "Particle Linking".

- **Particle Detection:** This part of the dialog allows you to adjust parameters relevant to the particle detection (feature point detection) part of the algorithm

- **Preview the detected particles:** in each frame according to the parameters. This options offers assistance in choosing good values for the parameters. Save the detected particles according to the parameters for all frames. The parameters relevant for detection are:

- **Radius:** Approximate radius of the particles in the images in units of pixels. The value should be slightly larger than the visible particle radius, but smaller than the smallest inter-particle separation.

- **Cutoff**: The score cut-off for the non-particle discrimination

- **Percentile:** The percentile (r) that determines which bright pixels are accepted as Particles. All local maxima in the upper rth percentile of the image intensity distribution are considered candidate Particles. Unit: percent (%).

4) Clicking on the Preview Detected button will circle the detected particles in the current frame according to the parameters currently set. To view the detected particles in other frames use the slider placed under the Preview Detected button. You can adjust the parameters and check how it affects the detection by clicking again on Preview Detected. Depending on the size of your particles and movie quality you will need to play with parameters.

Note that very rarely you detect all particles in the field of view mostly due to the fact that they quickly go out of focus

5) To start on 0.97μm beads Enter these parameters: radius = 5, cutoff = 0, percentile = 0.4 and click on preview detected. Check the detected particles at the next frames by using the slider in the dialog menu. With radius of 5 they are rightly detected as 2 separate particles. If you have any doubt they are 2 separate particles you can look at the 3rd frame. Change the radius to 10 and click the preview button. With this parameter; the algorithm wrongfully detects them as one particle since they are both within the radius of 10 pixels.

6) Try other values for the radius parameter. Go back to these parameters: radius = 5, cutoff = 0, percentile = 0.4 and click on preview detected. It is obvious that there are more 'real' particles in the image that were not detected. Notice that the detected particles are much brighter then the ones not detected. Since the score cut-off is set to zero, we can rightfully assume that increasing the percentile of particle intensity taken will make the algorithm detect more particles (with lower intensity). The higher the number in the percentile field - the more particles will be detected. Try setting the percentile value to 2. After clicking the preview button, you will see that much more particles are detected, in fact too many particles - you will need to find the right balance (for our dark filed movies between 0.3-0.7)

Remember! There is no right and wrong here - it is possible that the original percentile = 0.1 will be more suitable even with this film, if for example only very high intensity particles are of interest.

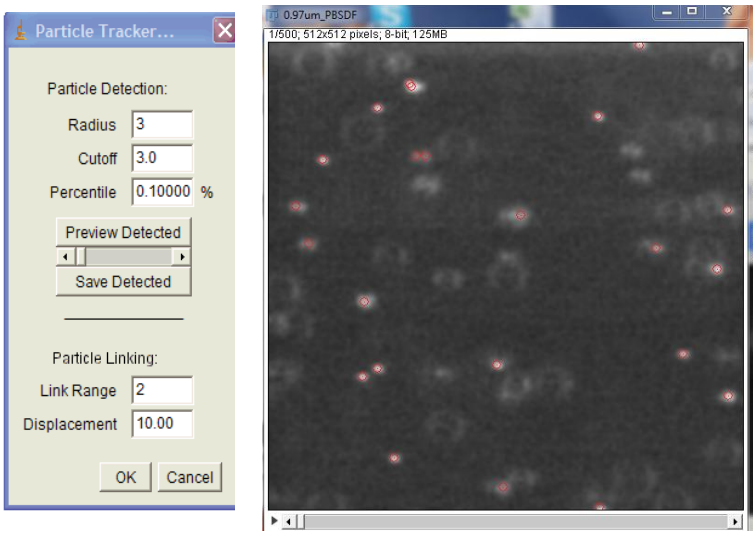


Figure 12. Parameters for particle detection. On the left panel with default values. In the right movie with particles identified using following parameters. radius = 5, cutoff = 0, percentile = 0.4

7) After setting the parameters for the detection (we will go with radius = 5, cutoff = 0, percentile = 0.6) you should set the particle linking parameters. The parameters relevant for linking are:

- **Displacemen**t: The maximum number of pixels a particle is allowed to move between two succeeding frames

- **Link Range:** The number of subsequent frames that is taken into account to determine the optimal correspondence matching.

8) These parameters can also be very different from one movie to the other and can also be modified after viewing the initial results. Put following initial guess for the displacement=5 and link range=3.You can now go ahead with the linking by clicking OK.

9) After completing the particle tracking, the result window will be displayed Click the Visualize all Trajectories button to view all the found trajectories.

10) Window displays an overview of all trajectories found (see Figure 13). It cannot be saved! It is usually hard to make sense of so much information. One way to reduce the displayed trajectories is to filter short trajectories. Click on the Filter Options button to filter out trajectories under a given length. Enter 75 and click OK. (Be careful, if you select to long length you might end up with very few trajectories and lose information!).

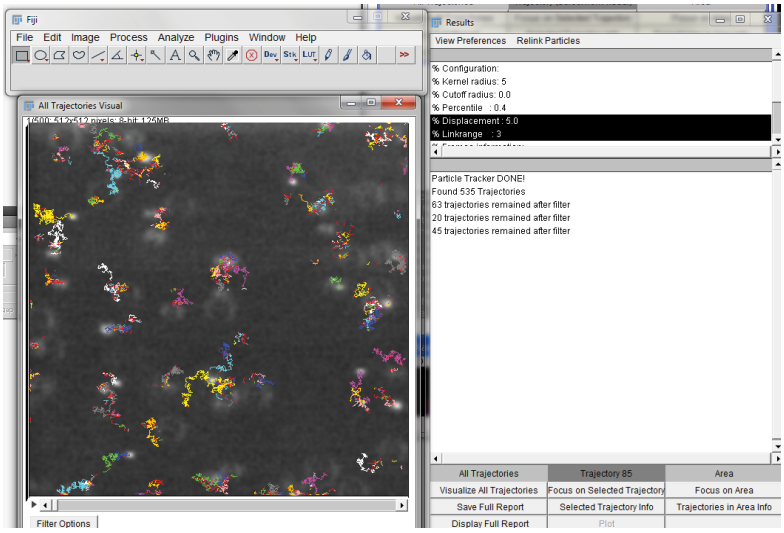


Figure 13. Selected 45 trajectories plotted.

11) Select a trajectory by clicking it once with the mouse left button. A rectangle surrounding the selected trajectory appears and the number of this trajectory will be displayed on the trajectory column of the results window.

12) Now that a specific trajectory is selected, you focus on it to get its information. Click on Selected Trajectory Info button. The information about this trajectory will be displayed in the results window.

13) Click on the Focus on Selected Trajectory button - a new window with a focused view of this trajectory is displayed. This view can be saved with the trajectory animation through the File menu of ImageJ. Look at the focused view and compare it to the overview window - in the focused view only the selected trajectory is displayed. See Figure 14.

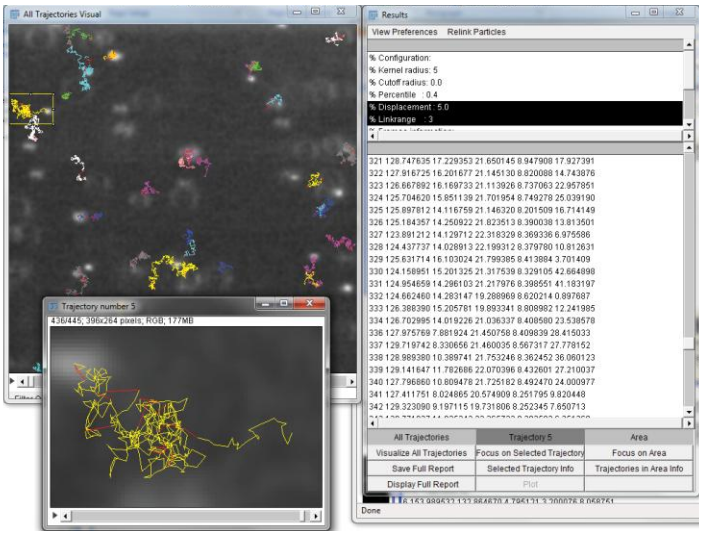


Figure 14. Focus on selected 5 trajectories.

14) Finally you can save the data by pressing Save Full report. Repeat particle tracking for all 3 experimental conditions measured in the first part of the practical work (3 different glycerol viscosity and water). Track particles as well in your onion movie.

3.3 Matlab analysis

Once single particle tracks for your bead and onion sample obtained, you can use those tracks to:

1) Use functions loadTraj.m PlotAllTrajectories.m or just run loadTraj2.m. You can find these mfiles on the web server.
2) To visualize trajectories, use this code

```
figure
hold on;
c = ['b' 'g' 'r' 'c' 'm' 'y' 'k'];
for i=1:size(data,2)
plot(data{i}(:,2), data{i}(:,3),c(mod(i,length(c))+1));
end
hold off;
```

3) Plot trajectories of certain length (not shorter than 50 frames)
4) Calculate diffusion coefficients

Q3. Does the experimentally estimated value match with the theoretical one? What relationship do you observe between viscosity, particle size, and diffusion coefficient? Is it consistent with Newton's theory? Compute according to the Error Propagation Handout the standard deviation of the diffusion coefficient.

5) Find and plot MSD.

Q4. What is their average velocity? How do the velocities due to active transport compare to the velocities you observed in your experiments involving Brownian motion? (You may wish to plot a histogram of particle velocities you observe). Compute according to the Error Propagation Handout the standard deviation of the average velocity.

15) Plot the cross correlation functions

Q5. Are your particle trajectories auto-correlated or cross-correlated? Over what timescalse? What might lead to correlations?

3.4 Questions

Q6. What is a Newtonian fluid?

Q7. Are we really in the "inertia-less" regime? Why or why not?

Q8. What would happen if your particles interacted through some potential?

Q9. What is meant by viscous coupling? Is this something you had to take into account?

Q10. What is the amount of work needed to transport a vesicle from the perimeter of the cell to the center? (You can calculate this quantity based on Stokes Law using the particle size and viscosity of the cytosol that you have already determined).

Q11. Knowing that hydrolysis of an ATP to ADP release 30.5kJ/mol, compare this quantity to the amount of work needed to transport a vesicle from the perimeter of the cell to the center.

Q12. Calculate the minimum number of myosin motors required to transport a vesicle from the perimeter of the cell to its center. (Each power stroke consumes the energy involved in converting a single molecule of ATP to ADP, remember to correct for the efficiency of the myosin motors (about 0.18-0.30).

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