# Cellular Microrheology


**Author:** Antoni Garcia de Heerreros Dunyac

**Supervisor:** Xavier Escaler Puigoriol

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**Abstract**

A multitude of cellular and subcellular processes with pathological relevance depend critically on the mechanical deformability of the cytoplasm. Particle-tracking microrheology measures the viscoelastic properties of the cytoplasm locally and with high spatiotemporal resolution. In this project I worked on different methods to study microrheology on living cells (mouse embryo fibroblasts) and select the one that gives better results to analyze the changes in the cytoplasm under flow conditions.

# Summary

# 1. Glossary

**MEF**: Mouse Embryo Fibroblasts

**Rheology**: Is the study of the flow of matter under conditions. When applied to very small magnification it is called microrheology.

**MSD**: Mean Squared Displacements. Is a measure of the deviation over time between the position of a particle and some reference position.

**PDS-1000**: It is a helium injection system commonly used to microinject DNA to transfect cells.

**D-MEM**: Is a cell culture medium that can be used to maintain cells in tissue culture. It contains amino acids, salts and glucose. Often FBS (Fetal Bovine Serum) is added.

**Lysosome**: Is a spherical intracellular organelle where the degradation of a wide variety of macromolecules happens.

**Mitochondria**: Is a double membrane-bound organelle found in all eukaryotic organisms that takes part in cellular respiration.

**Pinocytosis or endocytosis**: It is a process in which small particles are brought into the cell, forming an invagination, and then suspended within small vesicles

# *2.* Preface

## 2.1. Project background

This project was made in collaboration with the Mechanical and Aerospace Engineering Department (MAE) in UCSD (University of California San Diego, US). First of all, before starting this project I spoke with one of the professors there, Dr. Juan Carlos de Alamo, to focus my work. He explained me that there were some students who just finished doing their PhD on cellular microrheology and he suggested it would be an interesting field for me to study.

## 2.2. Motivation

Last year I took the Fluids Mechanical Course and I like it very much; so I began to think which kind of project I would like to do. The motivation to start this project came from the idea of applying this physics knowledge in some other fields. After thinking of many other possibilities I finally chose the biological field because I believe it has a lot of potential and there is still much to be explained and discovered. The fact that the MAE Department was working in some related topics also gave me an incentive, as I really wanted to see how the investigation in engineering topics works.

## 2.3. Previous requirements

Learning cell culture

This work can be catalogued as a multidisciplinary project, with some parts related to Biology and others completely focus in the mechanical and mathematical field.

Therefore, before starting, I spent some time in a biomedical laboratory in the PRBB (Parc de Recerca Biomèdica de Barcelona) where I was introduced in the biological environment and laboratory workstyle.

The first problem I had with my project was that I did not know anything related with cell culture and maintenance. So, before doing all the experiments I planned, I did a short stay in a laboratory in Barcelona called IMIM (Institut d'Investigació Mèdica) during one month.

There they taught me the most important things of cell culturing. I learned how to use the equipment in the lab, like the hood or the centrifuge, how to look at the cells in the microscope, and to use the main tools such as dishes and pipettes. The most important thing I learned during the one month stay was how to properly culture my cells and avoid any kind of contamination that could damage the cells. They also taught me how to use an optical microscope.

In order to continually have cells ready to use in my experiments I had to learn how to grow them. The full process has a few steps:

The first thing to do is to defreeze cells usually stored in a liquid nitrogen tank at a temperature of -180ºC. Using special gloves and a coat I had to take out the cells from the tank and slowly warm them up until 37 ºC; that is our body temperature and when cells work at their finest.

Once the cells are warmed, you can put them inside a flask with pre-warmed media. I filled the flask with 10 ml of media to prevent cell dryness and death. The media I use to grow my cells is (Dubecco's-modified Eagle's medium a (DMEM) + 10% FBS(Fetal Bovine Serum); it also has 4,5 g/L glucose, 2 mM L-Glutamine, 56 U/ml penicillin, 56 µg/L streptomycin. This media has all the components cells need to properly develop and also has some antibiotics (Penn and Step) to kill the bacteria to prevent contamination. Once the flask is ready it can be stored in the incubator at a temperature of 37ºC and with an atmosphere with 5% of CO2, the best conditions for cells to grow and develop.

After a couple of days in the incubator cells are completely grown and they spread through all the flask surface; so it is time to pass them to another flask. The first thing to do is remove the media as the cells are attached to the flask surface. Then using PBS I washed the cells during a few seconds. PBS (Phosphate Suffered Saline, 20 mM phosphate buffer pH 7,6; 135 mM NaCl, 5 mM KCl ) is a water-based salt solution that with an osmolarity matching that of the human body. After a few seconds I removed the PBS and I added Trypsin, a protease that digests the extracellular proteins.

I used around 3 ml of Trypsin (0.25% in DMEM) to detach cells from the flask. Trypsin is an enzyme commonly used to pass cells; it breaks the molecular junctions they made with its surrounding. It has an optimal operational temperature of about 37ºC and after waiting 5 minutes using the microscope you can clearly see that all the cells are no longer attached and now are floating in the medium.

Once all the cells are detached I pour them in a small vial and put it in the table-top centrifuge. Using a speed of 2000 rpm during 4 minutes, cells are separated from the medium; all cells are now pelleted down at the bottom of the tube.

The next step and final one is pouring out the Trypsin and re-suspend all the cells using few milliliters of media. Then you only take a small part of that volume and add it to a flask full of media; so you will have fewer cells than before and they can keep growing in the new flask. This process is called to pass the cells. Depending on how many days you seed to the plates they will take longer or shorter to grow and cover all the surface and stop growing.

I chose to work with MEF (Mouse Embryo Fibroblast) cells not just because of their cancer relevance but because they are quite big cells (10-15 υm), grow fast and can be easily manipulated. The main function of fibroblasts is to maintain the structural integrity of connective tissues by continuously secreting fibers, such as collagen, to form the extracellular matrix. MEFs are usually branched cells and the nucleus has an elliptical shape and is located in the center of the cell. As said before, fibroblasts are responsible of secreting many different fibers to the extracellular matrix like collagens, glycosaminoglycan, reticular and elastic fibers, glycoproteins and cytokine TSLP. Fibroblasts have the ability to migrate slowly over substratum: a very important property in order to study how the cytoplasm flows.

# 3. Introduction

## 3.1. Objectives

My project is focused on the study of the rheological properties of some specific kind of cells. I decided to work with a very common variety of cells called MEF (Mouse Embryo Fibroblasts). I chose these cells because they have physiological relevance in our body and they also play an important role in cancer cell migration.

In this project I will be using these cells to track some particles inside them and then, using advanced software, I will be able to calculate the main physical properties of the intracellular fluid such as its anisotropy and viscosity. The main objective of this project is to compare different microrheology methods and use the best one to do and extra experiment.

## 3.2. Project scope

*As microrheology is a highly specific topic and it is hard to understand and study, I focused on understanding the main concepts in order to decide which method is better. I had six months to figure out how microrheology works, run my experiments and analyze my results. Of course there is further work that can be done in this area that has a lot of potential and is very recent. Many scientists are still trying to figure out how the mechanics of the cell works.*

# 4. Project theory

## 4.1. Viscosity

The viscosity of a fluid is a property that measures the resistance to gradual deformation when a stress is applied.

A fluid that has no resistance to shear stress is known as an ideal or inviscid fluid. Otherwise, all fluids have positive viscosity, although it is very frequent to compare a liquid viscosity with water in order to say if it is viscous or not.

The dynamic viscosity of a fluid expresses its resistance to shearing flows, where adjacent layers move parallel to each other with different speeds. It can be defined through the idealized situation known as a Couette flow, where a layer of fluid is trapped between two horizontal plates, one fixed and one moving horizontally at constant speed, *u*. This fluid has to be homogeneous in the layer and at different shear stresses.

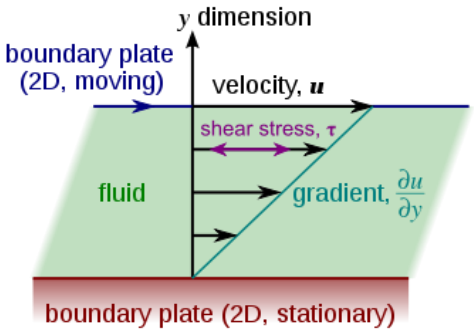



Figure 1: Laminar shear of fluid between two plates

If the speed of the top plate is small enough, the fluid particles will move parallel to it, and their speed will vary linearly from zero at the bottom to *u* at the top. Each layer of fluid will move faster than the one just below it, and friction between them will give rise to a force resisting their relative motion. In particular, the fluid will apply on the top plate a force in the direction opposite to its motion, and an equal but opposite one to the bottom plate. Obviously in order to keep the top plate moving at a constant speed it has to be an external force with the same modulus.

The modulus of the shear stress can be found with this equation: $\tau = \mu \frac{\Delta u}{\Delta y}$ and $\tau = \frac{F}{A}$

where *u* is the speed of the top plate, A the area and μ the dynamic viscosity which is measured in Poises (1P= 0.1Pa·s).

Depending on how the ratio $\frac{\Delta u}{\Delta y}$ in the shear stress equation behaves, the fluids are classified as Newtonian or non-Newtonian. Many common fluids such as water, oil and many gases are said to be Newtonian, their viscosity $\mu$ is stress independent. However, there are some other fluids that do not follow this law:

- Shear thickening liquids, whose viscosity increases with the rate of shear strain.
- Shear thinning liquids, whose viscosity decreases with the rate of shear strain.
- Thixotropic liquids, that become less viscous over time when shaken, agitated, or otherwise stressed.
- Rheopectic liquids, that become more viscous over time when shaken, agitated, or otherwise stressed.
- Bingham plastics that behave as a solid at low stresses but flow as a viscous fluid at high stresses.

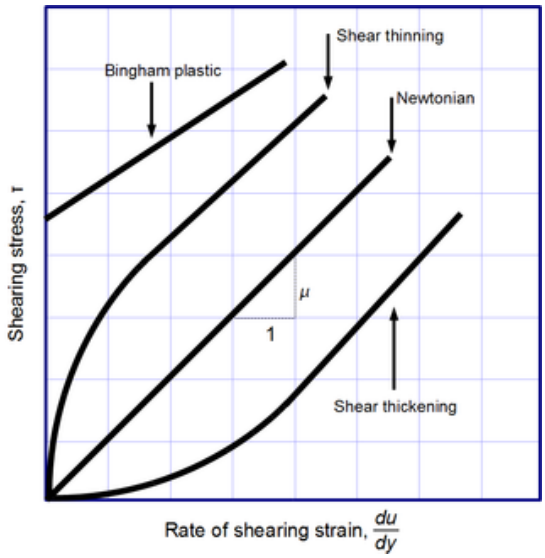



Figure 2: Types of fluids

Using the dynamic viscosity we can calculate the kinematic viscosity (υ) just dividing by the fluids density. This kinematic viscosity is used to calculate the Reynolds number that expresses the ratio between inertial forces and viscous forces. $\upsilon = \frac{\mu}{\rho}$

$$Re = \frac{Inertial\ Forces}{Viscous\ Forces} = \frac{uL}{\upsilon}$$

In order to measure the viscosity of a fluid is very common to use a rheometer or a viscometer.

A rheometer is a laboratory device used to measure how a liquid reacts when applying

forces. Newtonian fluids can be characterized by a single coefficient of viscosity for each temperature. Although this viscosity will change with temperature, it does not change with the strain rate. Only a small group of fluids exhibit such constant viscosity, the Newtonians, the other fluids (non-Newtonian) as told before experiment a change of their viscosity when modifications in their stress rate. The rheometer is used for those fluids which cannot be defined by a single value of viscosity and therefore require more parameters to be set and measured.

There are two different types of rheometer, the ones that control the applied shear stress or shear strain which are called rotational or shear rheometers and rheometers that apply extensional stress or extensional strain, called extensional rheometers. The most common rotational rheometers are: the Dynamic shear rheometer, the Pipe or Capillary, the Rotational cylinder and the Cone and plate rheometer. On the other side the extensional rheometers are less used due to the difficulty to generate homogeneous extensional flow, also the strain history of the material elements has to be known . The most common extensional rheometer is the Rheoten.

A viscometer or viscosimeter is a device used to measure the viscosity of a fluid only measure under one flow condition.

In general, either the fluid remains stationary and an object moves through it, or the object is stationary and the fluid moves past it. The drag caused by relative motion of the fluid and a surface is a measure of the viscosity. The flow conditions must have a sufficiently small value of Reynolds number for there to be laminar flow. These devices are usually calibrated with the water viscosity at 20ºC. The U-tube, the Falling sphere and the rotational viscometers are the most commonly used to measure fluid's viscosity.

All these devices are used to measure viscosity in fluids that are easy to handle and we have enough quantity of them. In this project I will not be using any of those devices because they do not work in microscopic scales. So in order to measure the viscosity of the intra cell's medium I have to use another method.

## 4.2. Microrheology

Rheology is the study of how complex materials flow and deform under stress. The rheological properties of a soft material determine its flow and processing behavior, and provide a window into its microstructural makeup. As told before, traditional rheometers typically measure the frequency-dependent linear viscoelastic relationship between strain and stress on milliliter-scale material samples. Microrheology measures these rheological

quantities using colloidal probes directly embedded in a soft material. So microrheology is the study of flow and deformation of very small volumes of materials, typically involving thermal or forced excitations. Microrheology advantages over traditional rheology are that it requires very small (e.g., microliter) sample volumes, which enables studies of materials that are either expensive or impossible to acquire in larger quantities, including biomaterials and living cells. The first rudimentary examples of micro-rheological measurements date back many decades, when Crick and Hughes (1950) employed magnetic fields to force iron filings inside small biomaterial samples. Since then colloids have been improved in order to obtain more information of the samples. Colloids are small structure, such as a particle, droplet, micelle, organelle, or macromolecule, having a size between a nanometer and several micrometers. Although the first microrheology studies were in 1950 it wasn't until 1995 that Mason and Weitz used a diffusing wave spectroscopy to measure the mean square displacement (MSD) of fluctuating spherical colloids in concentrated suspensions in order to quantitatively relate the measured MSD of tracer beads with the rheological properties of these fluids, they developed and applied a generalized Stokes-Einstein equation.

Few years later, in 1997, they introduced the laser-microrheology techniques to measure trajectories of individual particles, using the generalized Stokes-Einstein relation, to extract rheological moduli.

The modern microrheology improvements have been related with the ability to obtain high frequency images using advanced laser microscopes. Modern microrheology is typically concerned with flows around microscale and nanoscale probe particles that are thermally excited or forced using external fields. The goal of microrheology is to extract the rheological properties of a material from the motion of colloidal probes embedded within it. Unlike traditional rheology, in which each frequency is generally measured separately, modern microrheology employs probe statistics to effectively measure a wide range of frequencies simultaneously. We can distinguish two different branches in microrheology, passive microrheology: the use of thermal fluctuations of embedded colloidal probes to measure material rheology (also called thermal diffusion microrheology) and active microrheology: the use of externally forced colloidal probes to measure the linear or nonlinear rheological response of a material.

### 4.2.2. One point particle tracking microrheology

In my project I have applied microrheology to study the cytoplasm mechanics of a specific kind of cells. Many cellular and subcellular processes depend critically on the mechanical deformability of the cytoplasm. For instance, the translocation of organelles (e.g., nucleus, mitochondria, and endoplasmic reticulum) within the cytoplasm is partly controlled by their

frictional drag and therefore by the local viscoelastic properties of the cytoplasm. Migrating cells at the edge of a wound significantly increase the stiffness of their cytoplasm to enable dendritic filamentous actin (F-actin) assemblies to produce net protruding forces against the plasma membrane. Cells need to adapt their intracellular physical properties to the physical properties of their extracellular medium to grow, differentiate, and migrate. Moreover, changes in the mechanical properties of cells often correlate with disease state. For instance, cells derived from mouse models of progeria (premature aging) or muscular dystrophy display significantly softer (i.e., less elastic) cytoplasm than wild-type controls. This affects the ability of these cells to resist shear forces and to migrate to the edge of a wound.

Recently, the method of particle-tracking microrheology was introduced to measure the viscoelastic properties of the cytoplasm locally and with high spatiotemporal resolution. Usually fluorescent beads of less than 1 μm in diameter are injected directly into the cytoplasm of live cells. These beads rapidly disperse throughout the cytoplasm and are subsequently tracked by fluorescence microscopy. The recorded movements of the beads are analyzed in terms of viscosity and elasticity of the cytoplasm. Particle-tracking microrheology allows probing the viscoelastic properties of many types of cells in a wide range of conditions. Recent experiments have revealed important new mechanistic insights into how the physical properties of the cytoplasm adapt to various chemical and physical stimuli, how they can control basic cell functions, and how these properties can be significantly altered in diseased cells.

Particle-tracking microrheology shows that the cytoplasm of adherent cells at rest, such as endothelial cells and fibroblasts on planar substrates, is typically more elastic than viscous for short timescales (high frequencies) between 0.1 and 10 s. However, at timescales greater than 10 to 20 s, these cells show a predominantly viscous response to shear forces; the cytoplasm behaves like a liquid. In general, the viscoelastic properties of adherent cells are dominated by the actin filament cytoskeleton. Furthermore, there have been some experiments that disassembly the actin filaments and eliminates the deformability of the cytoplasm and makes the cytoplasm mostly viscous at all timescales. So, the elasticity level in the cell is highly related with the local concentration of actin present in the cytoplasm, high actin concentration zones are much stiffer than other parts of the cell containing less actin.

In my project I have used a powerful microscope that allowed me to get many images per second; so I could track particles with high precision. With this microscope I could get more than 100 frames per second and with a very high magnification using oil-immersion lenses. I have tracked different particles and checked how they move inside the cell. These particles move because they are affected by different processes, but one of the most important is the Wiener process or Brownian motion.

## 4.3. Brownian motion or Wiener process

Brownian motion is the random motion of particles suspended in a fluid resulting from their collision with the other atoms or molecules in the gas or liquid.

This transport phenomenon is named after the botanist Robert Brown. In 1827, he was looking through a microscope at small pollen grains in water, he noted that the particles moved through the water; but he was not able to determine the mechanisms that caused this motion. Long after that, Albert Einstein published a paper in 1905 that explained in precise detail how the motion that Brown had observed was a result of the pollen being moved by individual water molecules when colliding. This explanation of Brownian motion served as convincing evidence that atoms and molecules exist, and was further verified experimentally by Jean Perrin in 1908 who was awarded with the Nobel Prize in Physics in 1926. The direction of the force of atomic bombardment is constantly changing, and at different times the particle is hit more on one side than another, leading to the seemingly random nature of the motion.

This motion can be imagined as a big balloon in a big crowd of people if seen from above. Many people will try to hit the balloon at the same time, some to the right and some to the left but if there are more hitting left, the balloon will move that direction and then it will find another group of people and move to a different direction. Same happens with the pollen particle and the water molecules but in a different scale. In a gas there will be more than $10^{16}$ collisions in a second and even greater in a liquid, where there will be $10^{20}$ collision per second.

In 1906 Smoluchowski studied this process and realized that the velocity of the Brownian particle depends on the collisions that tend to accelerate and decelerate it. The faster the particle moves, the greater will be the collisions that will retard it, so the velocity of a Brownian has a limit. As the Brownian particle gets the kinetic energy from the collisions, the maximum velocity will be the corresponding to the kinetic energy of the other fluid particles.

According to the theory previously suggested by Maxwell, Boltzmann and Clausius, the temperature of a substance is proportional to the average kinetic energy with which the molecules of the substance are moving or vibrating.

Einstein wrote in 1905 about Brownian motion in an article and he discussed on the basis of statistical mechanics. He showed that for such a microscopic particle the random difference between the pressures of molecular bombardment on two opposite sides would cause it to constantly wobble back and forth. A smaller particle, a less viscous fluid, and a higher temperature would each increase the amount of motion one could expect to observe. Over a period of time, the particle would tend to drift from its starting point, and, on the basis of the

kinetic theory, it is possible to compute the probability (P) of a particle moving a certain distance (x) in any given direction during a certain time interval (t) in a medium whose coefficient of diffusion (D) is known, D being equal to one-half the average of the square of the displacement in the x-direction.

$$P = \frac{e^{\frac{-x^2}{4Dt}}}{2\sqrt{\pi Dt}}$$

In statistical mechanics, the mean squared displacement (MSD) is a measure of the deviation over time between the position of a particle and some reference position. It is the most common measure of the spatial extent of random motion, and can be thought of as measuring the portion of the system "explored" by the random walker. The MSD is defined as:

$$MSD = \frac{1}{T}\sum_{t=1}^{T}(x(t) - x_0)^2$$

Using the MSD we can obtain the coefficient of diffusion, D, with $\overline{X^2} = 2Dt$. George Stokes studied the dynamical equilibrium of the particles under the action of gravity and diffusion. He proved the following equation of diffusivity.

$$D = \frac{K_B T}{6\pi\eta r}$$

Where $K_B$ is the Boltzmann constant which is the universal gas constant divided by the Avogadro number, T is the absolute temperature, $\eta$ is the viscosity and r the particle radius. I will use this formula to calculate the viscosity of the cells once I have the MSD of the particles inside the cell.

## 4.4. Microrheology of living cells

The rheological response of the cytoplasm can be either predominantly viscous or elastic, depending on the time of application and the magnitude of the force. This can be seen the same way as a silly putty ball; when quickly thrown into a surface the ball bounces due to the fast deformation and the regain of its original shape, but if we leave the ball in a surface for a long time it will partially flatten due to its own weight. So the ball behaves elastic for short time (high frequency) excitations and viscous for longer times: it is viscoelastic.

The induced stress in the cell cytoplasm can be decomposed in two phases, a sine (in-phase) component and a cosine (out-of-phase) component: $\tau(t) = \tau' \sin \omega t + \tau'' \cos \omega t = \gamma_0( G'\sin \omega t + G''\cos \omega t)$. If the material is an elastic solid (no or little viscosity), such as rubber, then the induced stress is exactly in phase with the input deformation and $\tau(t) = \tau'\sin \omega t$. In this case, $G''= 0$. If the material is a viscous liquid (no elasticity), such as water or glycerol, then the induced stress is out of phase with the input deformation, and $\tau(t) = \tau'' \cos \omega t$. In this case, $G'=0$.

Typical cell's cytoplasm has rheological properties that depend on the rate of deformation, $\omega$, both G' and G'' depend on $\omega$. At low frequencies, the cytoplasm has the time to reorganize its cytoskeleton and can flow, behaving as a viscous liquid. At high frequencies, the cytoskeleton does not have the time to reorganize and the cytoplasm behaves as an elastic solid, which resists the deformation. This underlies the importance of measuring the full frequency-dependent response when doing the different experiments.

# 5. Experimental methods

## 5.1. Methodology introduction

The experimental part of this work was carried out in the laboratory of Professor Juan Carlos del Alamo at the Mechanical and Aerospace Engineering Department in the University of California San Diego (UCSD). Before starting there, I also did a short stay in the laboratory of Epithelial Tumor Invasion at the Institut Hospital del Mar d'Investigacions Mèdiques (IMIM) in Barcelona, to learn how to culture immortalized cells. As soon as I did this, I sent the cells from Barcelona to San Diego in order to get them ready and start my experiments as soon as I arrived there.

When I arrived to the lab, I had to thaw the cells and split them into different flasks to let them grow and develop again. I cultured my cells in two big flasks one for each type of cell (Murine embryo Fibroblasts Snail1 knock out and Snail1 wild-type or control). The mutant cells deficient in Snail1 gene prevent their activation and were used as a control in my experiments. Occasionally I also used small dishes to visualize them at the microscope. The first problem I got was that when I looked to the cells using a high magnification in the microscope, 40x or 63x, the dish plate was too thick and I was not able to correctly focus my image; so I had to improvise something. I solve the problem by making a hole in the bottom of the dish and sticking a coverslip that is much thinner. With this solution I was able to focus the bottom of the plate but, as I used a glass coverslip instead of the plastic treated plate dish, the cells had more difficulty to attach to the substrate. To improve the attachment, before culturing the cells on the coverslip, I added Collagen, an extracellular matrix protein that helps the cells to attach better.

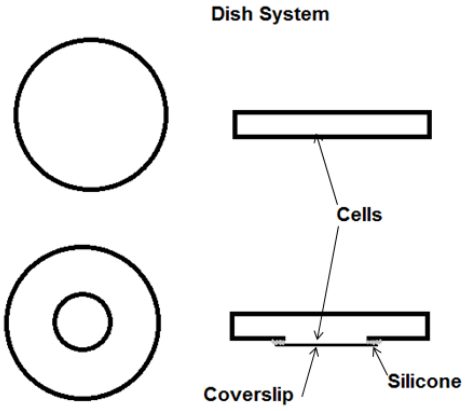



Figure 3: Dish system design

## 5.2. First method: Bright field

Once I had the cells grown in the coverslip I looked at them using a high magnification lens to check if there were organelles which I could use for as tracking elements in my experiments. These cells were grown for a couple of days using the media described in section 2.3 that contains L-glutamine and 10% FBS. After checking the cells in the bright field of the microscope I saw that I could track some particles that look like small spherical vacuoles. These endogenous particles are lysosomes or small vacuoles of about 1 μm in diameter that float around the cytoplasm. In order to do so I needed to change some lines in the informatics program that I used to track the particles.

Before analyzing the results with the program I had to take the best possible focused images with the microscope. I usually took more than 500 images in 5 seconds; so, the frame rate is about 100 fps that allowed me to see the particle moving continuously and not jumping from frame to frame. Once I had the stack of images and a file that contains important information I could use the program.

This program was created two years ago by a former PhD student in the lab, but it presents some problems which I corrected to adapt it better to my experiments. It is a very complex and long program that has two different parts; the first one is written in Python and the second one in Cython, both are similar programing languages. The first part of the program deals with the reading of the images and tracking the particles; the second one uses the trajectories to calculate the physical properties.

The program starts by showing a window to inquire the images stored and the dimensions of the rectangles used to track the images. It also requests some information that it is going to be used in the second part of the program, such as the temperature and the particle radius. Finally it asks if the user wants a video to be displayed at the end of the program. Here you can see the steps you go through when running the program.

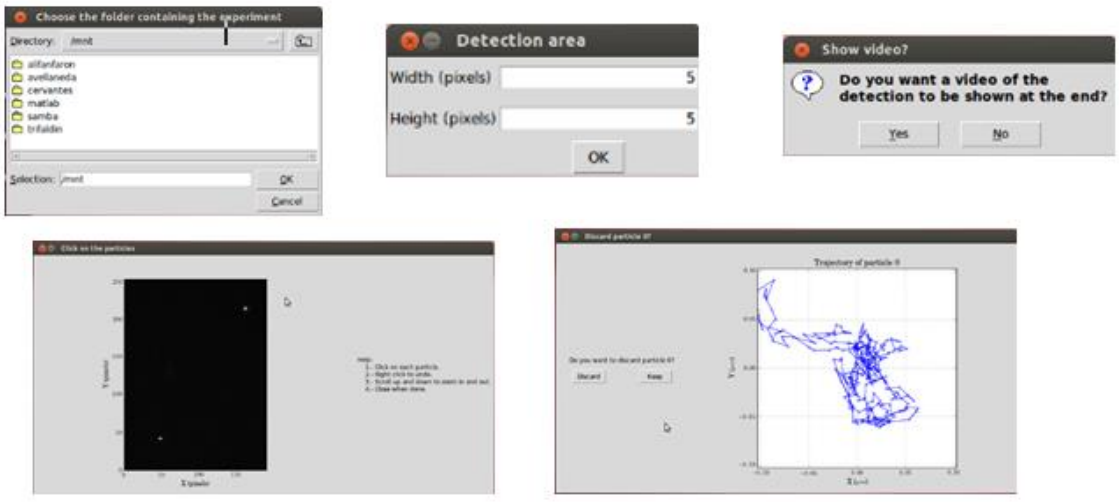



Figure 4: Parts of the program

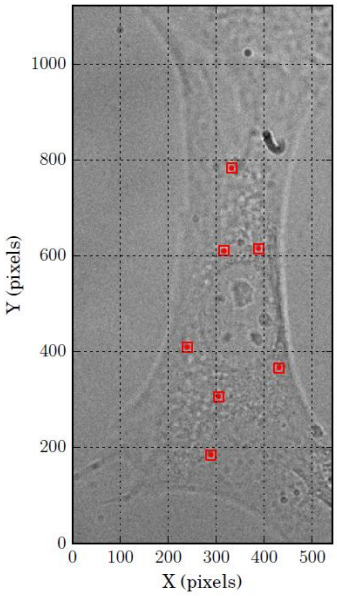



Figure 5: cell with selected particles (red rectangles)

The program shows a window where you select the particles that are going to be tracked. As shown in the image in Figure 5, around every selected particle there is a rectangle with the dimensions specified before, that will be used to calculate the particle's trajectory.

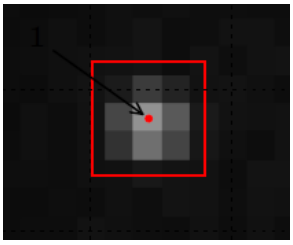
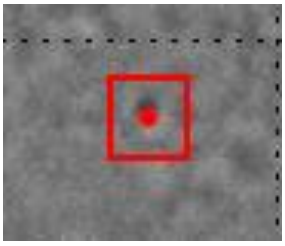


Fig 6: Rectangle to crop the particle

To track the particles the program calculates the centroid of each rectangle and finds the pixel with the maximum luminosity. Then it goes to the next frame and checks where the position of this pixel is; repeating this for all the images gives the trajectory of the particle.



The problem I have had is that when tracking particles in the bright field (see right image), the particles are darker than the rest of the cell; so the part of the program that detects the lightest pixel does not work here. In order to solve this difficulty, I changed that part for a few lines that let the program detect the darkest pixel in the rectangle.

Once the program has obtained the trajectory of all the particles you have selected, they are

shown and it asks if you want to keep them all or discard some of them. The program stores the x coordinates for all the time points in a different file and does the same with the y coordinates. After this point, the first part of the program is completed.

In the second part, the program uses the trajectories measured before to extract the MSD (Mean Squared Displacements) and calculate the viscosity and the physical properties of the medium. To measure the MSD the program calculates all the possible time- steps. The time between one frame and the next one is the minimum time step and the time of the whole image acquisition is the biggest time-step. Once it has got all the time-steps the program calculates the square of the displacement the particle moves in that time-step for every single time point if possible. When it has all the displacements, it calculates the mean of them for every time-step; that is why is called Mean Squared Displacements. The program repeats this process for all the particles and plots them in a graph MSD--time-step. Sometimes it is commonly used the frequency concept to concern time-step; as frequency is the inverse of time, short time-steps are equivalent to high frequencies and the other way around.

When the MSDs of all particles have been measured, the program calculates the mean and the standard deviation between them and plots the result in a different graph.

This bright field tracking will be considered as the first method of particle tracking and its results will be shown later. The second and third experiments consist in finding different ways to introduce fluorescent particles inside the cells in order to watch them later using a laser-assisted microscope.

## 5.3. Second method: Microinjecting

In this case my first approach consisted in using a special machine called biolistic injector. The PDS-1000 helium injection system is commonly used to microinject DNA to transfect cells. Biolistic particle delivery is a method of transformation that uses helium pressure to introduce DNA-coated microcarriers into cells. Micro-projectile bombardment can transform such diverse targets as bacterial, fungal, insect, plant, and animal cells and intracellular organelles. The Biolistic PDS-1000/He instrument uses pressurized helium to accelerate subcellular sized micro-projectiles coated with DNA (or other biological material) over a range of velocities necessary to optimally transform many different cell types. The system consists of the bombardment chamber (main unit), connective tubing for attachment to vacuum source, and all components necessary for attachment and delivery of high pressure helium to the main unit (helium regulator, solenoid valve, and connective tubing).

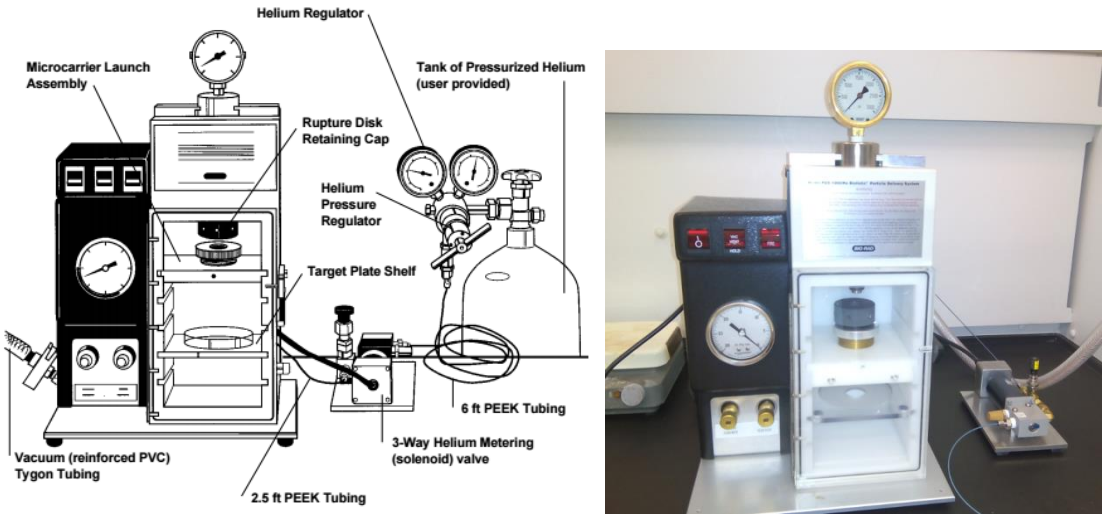



Figure 7: Scheme of the Biolistic PDS-1000/He system

The Biolistic PDS-1000/He system uses high pressure helium, released by a rupture disk, to shoot a carrier sheet loaded with millions of microscopic microcarriers toward target cells at high velocity. The microcarriers are coated tungsten or gold particles with DNA or other fluorescent biological material that can be easily seen under a laser field. The carrier is stopped after a short distance by a metallic screen allowing the DNA-coated microcarriers continue traveling toward the cells. The chances of success for each bombardment relay upon the helium pressure (rupture disk selection), the amount of vacuum in the bombardment chamber, the distance from the rupture disk to the carrier (A), the macrocarrier travel distance to the stopping screen (B), and the distance between the stopping screen and target cells (C).

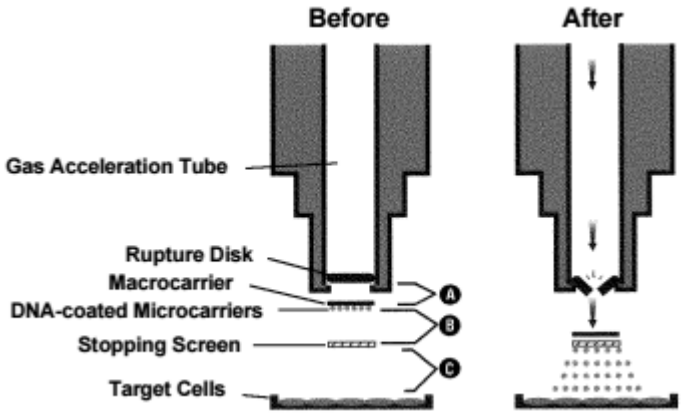



Figure 8: PDS-1000/He operation

The problem I had initially with the microinjector was that nobody in the laboratory had used it before, so I had to calculate all the parameters described above to obtain the best results. In

order to get those parameters I used the trial and error method until I found the exact parameters that worked better with my cells. The problem with this injector is that it is very easy to damage the cells; if I use too much vacuum the cells detach from the dish layer; if you use to much helium pressure, the beads kill the cells; if the beads are not dried well enough, you will have many clusters in your sample.

The first thing to do before injecting the beads is to make sure that the cells are confluent so I have a higher probability that a bead reaches a cell. The next step is to wash the cells with PBS to remove the debris that may collide with the beads and after that I inject the particles using the PDS-1000. To inject the particles first I had to prepare the macrocarrier with the beads on top what requires to prepare the beads solution. The beads come from the fabricant in a 2% water solution, but as the macrocarrier is hydrophobic you have to dilute them in 10% ethanol. Once you have the 10% solution the next step is to pour a few drops in the macrocarrier and let them dry during 2 hours in a dark place. Once the beads are dried I have to prepare the rupture disk and decide which pressure I am going to work with, and wash it with propanol to sterilize it. After that the PDS-1000 is ready to start working; I just need to open the helium tank and turn on the machine.

First I set the vacuum to the chamber by using the vacuum button, if I worked with 900 psi rupture disks, the vacuum indicator should reach 11 in. Hg¸ if I work with the 450 psi disks, the chamber should be at 6.5 in. Hg. When the exact pressure was reached I pressed the hold button and then fired. This raises the pressure in the gas acceleration tube until it reaches the rupture disk limit pressure. When this happen the disk brakes and a string of high velocity helium hits the macrocarrier making the beads spread all over the target cells.

Once the cells are injected, the following step consists in washing them again with PBS to remove the beads that had not been able to get inside a cell and are floating on the media; so I eliminate the noise they may produce when looking through the microscope.

The main problem I had when using this method is the preparation of all the components and the operation of the machine. As told before If the vacuum pressure is too high the cells will detach and die; same happens when the velocity of the beads is too high. If those parameters are too low the beads will not be introduced inside the cells. Another problem is the preparation of the macrocarrier. As explained before the macrocarrier is highly hydrophobic and repels the water solution of the beads. When I tried to put the beads directly over the macrocarrier, the drop will not spread all over the surface due to the high superficial tension of water. Diluting this water solution in ethanol helps the drop to spread but at the same time as the ethanol evaporates faster than water you can clearly see that the beads are not completely spread around the macrocarrier and there are areas with a higher concentration. When I watched the cells in the microscope after injecting them, I could clearly visualize big cluster, many beads stuck together, that could not be tracked because of their

size. Sometimes I saw that these clusters killed the cells of a small area due to the impact when injected.

In order to solve the problem described above, we designed a method to exchange the water solution of the beads by ethanol using a process known as osmosis. To do this process we designed a system that had two compartments, one with water and the other one with the beads solution dissolved in ethanol. The two compartments were separated by a membrane that only allowed water molecules to go through. With this method we obtained a bead solution only with ethanol that spreads easier on the macrocarrier and causes less clusters when dried.

## 5.4. Third method: Endocytosis

The third approach I used consisted in introducing the beads inside the cells without injecting them, just letting the cells internalize the beads from the media. This process is called endocytosis or pinocytosis.

To do so, I cultured the cells in normal growth media and conditions and waited for one or two days until they had grown and attached the dish layer. Once the cells were ready I changed the normal media for a media without L-glutamine but with the beads dissolved. Glutamine is an α-amino acid that is used in the biosynthesis of proteins and is required for the cells in culture; to respond to Glutamine deprivation cells increase the process of micropinocytosis to capture external proteins and use Glutamine derived from those proteins for their needs.

I cultured cells with this special media (DMEM plus FBS, plus antibiotics but without Glutamine) to make cells starve and stimulate pinocytosis. I treated the cells with the non L-glutamine media supplemented with the beads for one night and then change the media to the regular one to prevent cell from dying. Before changing the media I washed the cells with PBS to remove all the beads that may have attached to the external matrix and those which are floating on the media.

This method worked fine, because the cells endocytosed many beads and gives us a lot of information of every cell. With the other methods I was able to track just a few particles per cell, less than 10, but with this method I can track as many as I want. An example of cell showing beads internalization is shown below.

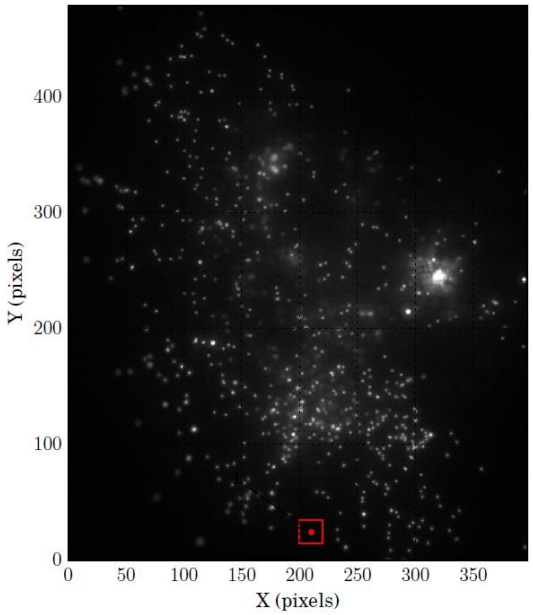



Figure 9: Beads internalized by pinocytosis

As each method, it has its pros and cons. The cons of the pinocytosis are that the beads in the cell are not free in the cytosol but inside small vesicles called lysosomes. Lysosomes are spherical intracellular organelles where the degradation of a wide variety of macromolecules happens, including proteins, complex carbohydrates, glycolipids and nucleic acids. The role of lysosomes in supporting cellular activities is especially important under conditions of nutritional deprivation, in which the lysosome can be viewed as a major recycling center for providing the basic building blocks for synthesis of macromolecules necessary for cell survival. Many of the metabolites generated as a result of lysosomal macromolecule breakdown are transported back into the cytosol, where they can be reutilized for the synthesis of new macromolecules. The lysosomes of MEF and many more cells have acid media at a pH of 4.5-5 inside them to help them in the enzyme degradation. Those enzymes work fine just in acid conditions, so if the enzymes leak out, they will not damage the cell because the cytosol is slightly basic.

To check if the beads were incorporated into lysosomes I did the following experiment. First I labelled the lysosomes with the specific colorant LysoTracker® Red DND-99 in order to mark and be able to watch them using the laser scope of the microscope. Once I marked the lysosomes with the red dye I put the green beads inside the cells. Then using the laser scope I watched the two different particles in different phases. I did that regulating the laser wavelength and analyzing the specter of the emitted light by the particles. As these two colors have entirely different wavelength in the light spectra there is not much noise between them. The left image shows the green beads and the right image the LysoTracker-labelled

lysosomes.

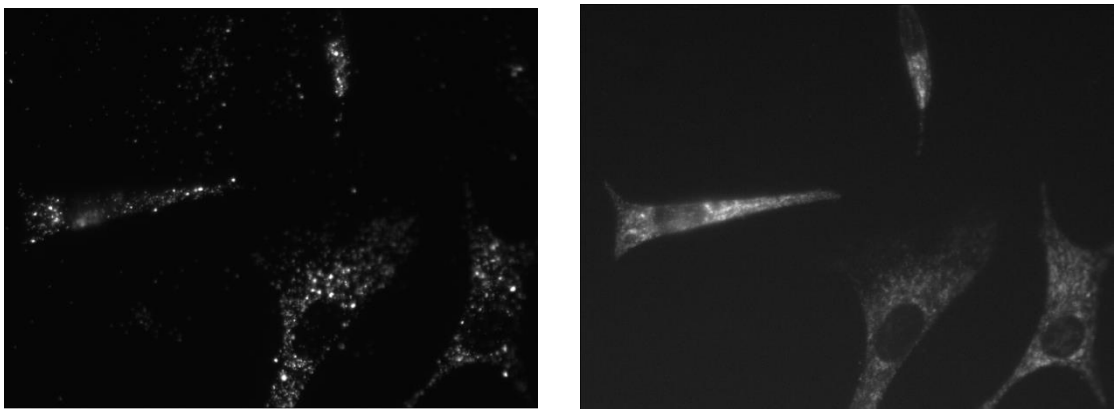


Figure 10: On the left, green beads internalized by pinocytosis; on the right, lysosomes marked with LysoTracker

The right image shows the big amount of lysosomes a cell has. Although I had some noise when merging the images, every red dot is in the same position as a green dot.

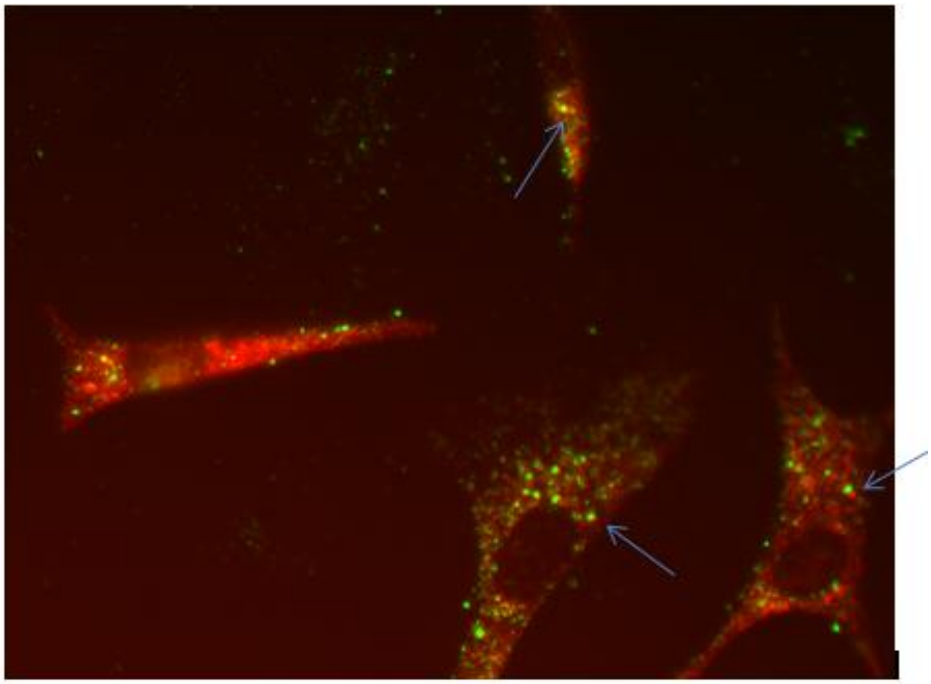


Figure 11: Merging both images in figure 9. Red dots correspond to lysosomes, green dots, to beads.

In this image you can see the merging of the red (lysosomes) and green (beads) channels. As it can be seen, the red and green dots do not match in the same position but if we take a closer look, (see the particle marked with the arrow) all the green dots are located in the

upper left side of a red dot. This can be explained because there has been a time gap between the moments of acquisition of two images. The microscope I was using has a chamber that can be heated to 37ºC to simulate as closely as possible the body conditions. This microscope has this heat system that moves air through all the chamber and it may have moved the cell dish a few microns in the direction we see. So, it can be said that the beads introduced inside the particles are included inside lysosomes.

This introduces a limitation to the study since, when analyzing the mobility of these particles you cannot extrapolate these results for particles that are free in cytoplasm, just to the movement of lysosomes.

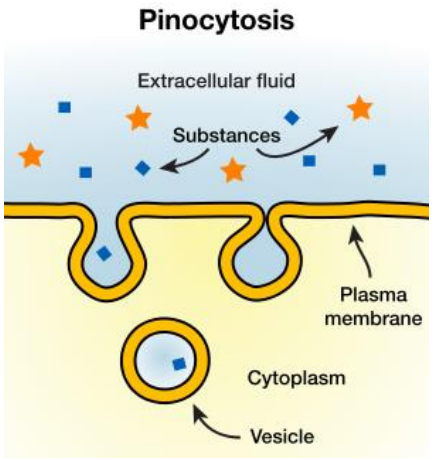



Figure 12: Pinocytosis scheme

As the lysosomes experiments did work in the way I wanted, I tried to mark the mitochondria to see if they work the same way. To track them I use the same procedure I did with the lysosome. I cultured the cells in different dishes and once they were all grown I added different concentrations of MitoTracker® Red CMXRos in every dish. This substance is a red-fluorescent dye that attaches to the mitochondria membrane in living cells. I wait one day for the cells to retain this dye and after that I washed them with PBS and explored the cells using the laser microscope. Mitochondria are double membrane-bound organelles found in all eukaryotic organisms. They have an ellipsoid shape between 1 and 3 um of diameter. The number of mitochondria inside the cell may vary depending on the cell type, but MEF cells may have more than 3000 which is much more than the lysosomes number. One of the main advantages of using mitochondria is the possibility to see the directionality of the cytoplasm flow. This is caused by the ellipsoid shape of the mitochondria that aligns with the flow of the cytoplasm.

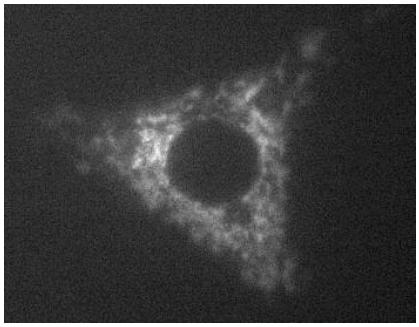


Figure 13: Cell marked with MitoTracker

But as it can be seen in the image above, the concentration of mitochondria is too big to identify single particles. Cells have so many mitochondria because they generate most of the cellular chemical energy through the Krebs' cycle. In addition to supplying cellular energy, mitochondria are involved in other tasks, such as signaling, cellular differentiation, and cell death, as well as maintaining control of the cell cycle and cell growth. Due to the impossibility to run the program with mitochondria I did not follow these organelles.

# 6. Results

## 6.1. Bright field

As soon as I finished doing the first experiments I analyzed the results using the program. To start I ran the program on the Wild-Type (WT) cells and the Snail1 Knock-Out (KO) to have a general idea of the way they behave. As told before these cells were culture on glass coverslips and initially I took all the images using the bright field of the microscope. I changed the part of the program that locates the brightest pixel on the cropped rectangle to the darkest one, so I could run the program in the bright field. The particles I tracked were small vacuoles moving around the cytoplasm. I made sure the vacuoles were moving and not attached to the Golgi complex; I also tried to select the particles that were not surrounded by any others so there won't be any interaction between them. In Fig 14 I show the cells I use to track the particles.

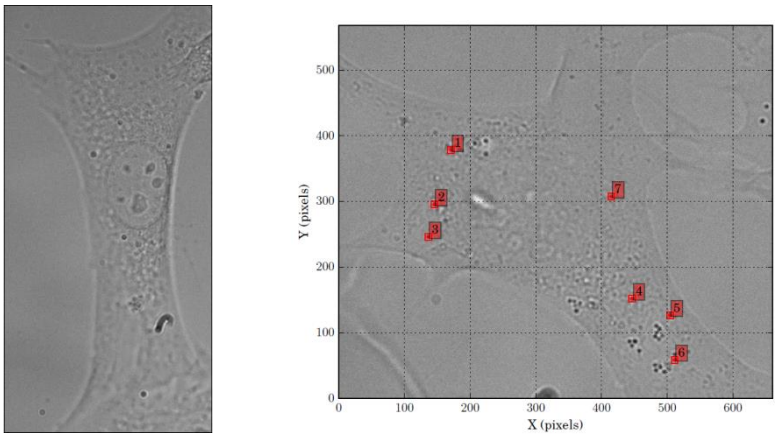



Fig 14: Bright field microscopic analysis of WT MEF cells.

One of the main problems I detected in the bright field images is that the contrast between the vacuoles (black particles) and the cytoplasm is too low and might cause errors when detecting the darkest pixel. To check if this was an important problem I analyzed the trajectory plot of each particle to see if there were some unexpected jumps.

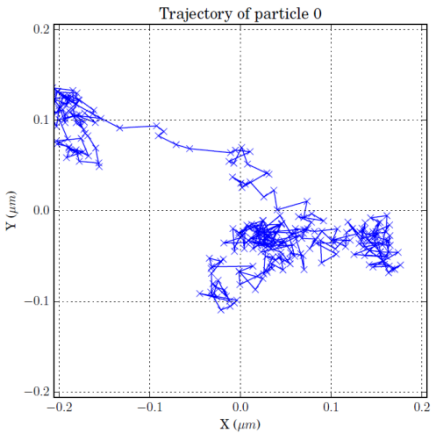



Figure 15: Trajectory of a particle of cell in figure 14, with jumps in X=-0.05 μm and Y=0.05 μm

The image above show the problem explained before, since the program cannot detect the darkest pixel and it jumps to another pixel located in the wrong coordinates. The only solution I thought about was using another microscope with a higher magnification. The images from Fig 15 were taken using a 40x objective and the factor of calibration is 0.16 microns per pixel. I consider that if I use the 63x objective the calibration factor drops to 0.1029 microns per pixel and maybe I would be able to follow the vacuoles more clearly.

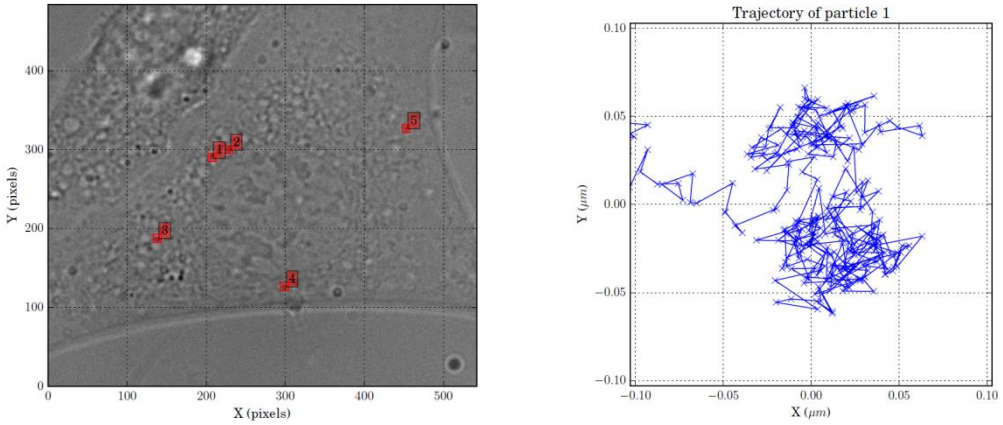



Figure 16: (Left) Particles tracked using a 63x objective. Trajectories of one particle (right)

As seen in the trajectory plot (Fig. 16), the particles keep having the same behavior. At this point there is not such a solution for this problem, so I decided to use the laser field for future experiments.

Even with this limitation, I collected all the data and try to analyze some particles that do not show this behavior. First of all I discarded all the “jumping particles” and then ran the second part of the program.

The results of both experiments, using the 40x and 63x objectives, are pretty similar. The first graphic corresponds to the histogram of the steps (Fig 17). This graph gives an idea of how many steps I have analyzed and how long they have been. I used this kind of graph to detect possible jumps. If the histogram is centered on 0 μm but there is a column on one side with a few larger steps, it indicates that there may have been a problem in the particle tracking. This plot also gives you the idea if the particles tend to go in the vertical or the horizontal direction just by checking the x-step and y-step. On the graph below (Fig 17) the average of the 7 particles tracked they tend to move more in the y direction; most of the x-steps are close to 0.0 μm and the y-steps are more separated; so there will be a lot of steps where the particle travels a longer distance in the y direction than in the x.

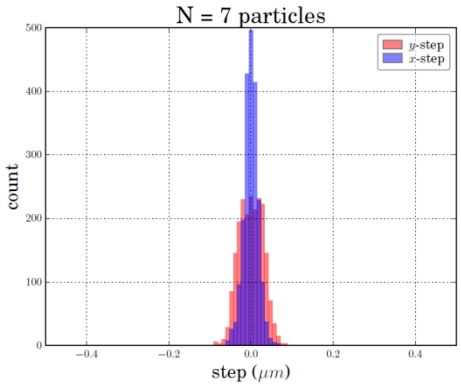



Figure 17: Histogram of steps for the seven particles

Once the histogram is checked and I did not detect errors to discard I started studying the MSD (Mean Squared Displacements) graphs. The MSD calculates the average distance traveled in a time lapse. For example, if I have an experiment with 300 images taken every second I would start by calculating the shortest time lapse, which is one second and doing the average of the distance traveled in that second for all the 300 seconds. This would be the highest frequency of the MSD, to calculate a shorter frequency we could do the same by taking a time lapse equal to 150 seconds for example. Then I should check the distance traveled by the particle between the second 0 and 150; then 1 and 151 and so on, until I reach the last time point. Finally I should do the average of all those distances to have the mean of the 150 seconds time lapse. If there is more than one particle tracked I should do and average of the value of the MSD for all of them.

The program does all this procedure but considered the distance traveled in two directions and calculating a 2x2 matrix for every time lapse (or frequency).

$$\begin{bmatrix} \Delta x \Delta x & \Delta x \Delta y \\ \Delta y \Delta x & \Delta y \Delta y \end{bmatrix}$$

This matrix is symmetric as ΔxΔy is the same as ΔyΔx. Once the program has the matrix it calculates its eigenvalues and the eigenvectors to see the main directions of the MSD for that frequency and particle. One graph we can look at now is the eigenvalues plot and the eva-ratio plot to see the directionality of the MSD depending on the frequency.

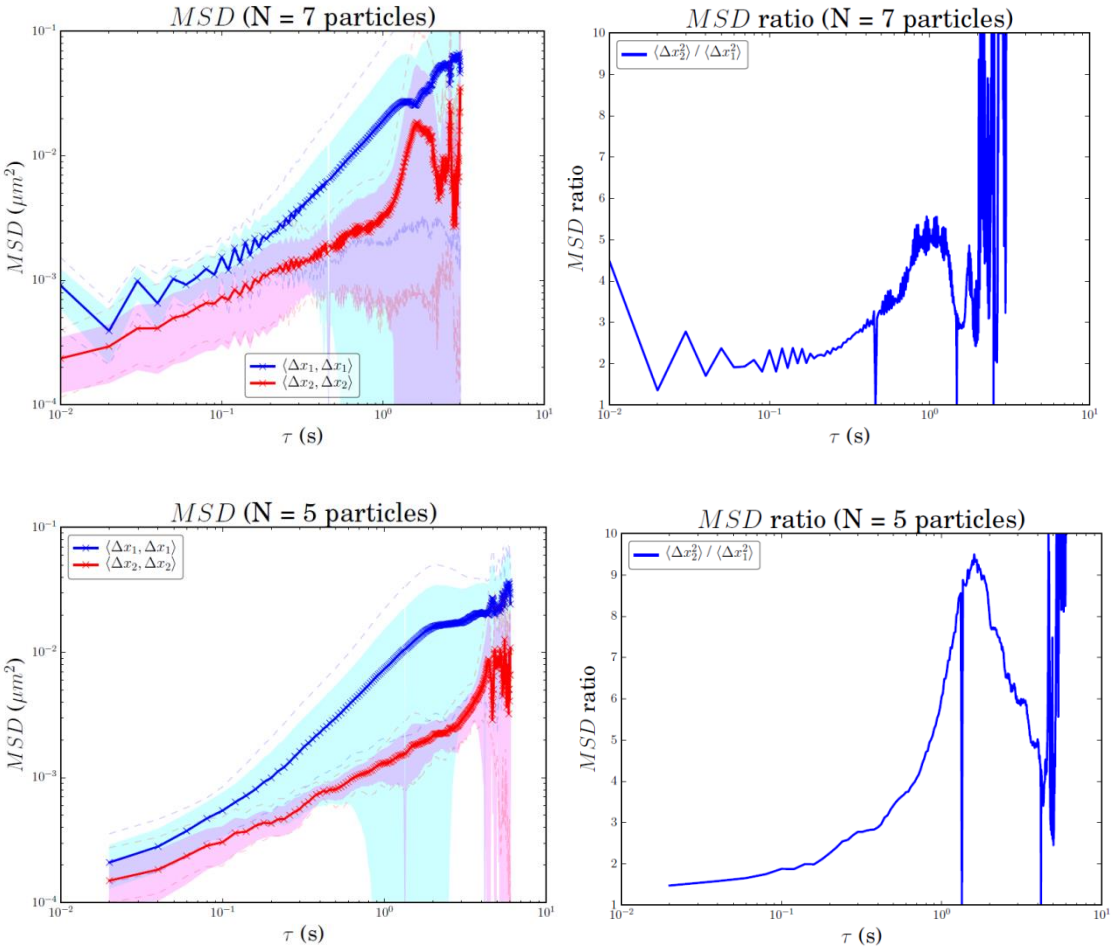



Figure 18: (Left, top and bottom) MSD of the principal directions; (right) eigenvalues ratio

The top two graphs in Fig.18 correspond to the 40x experiment and the bottom two are from the 63x objective. Since they were generated using the same type of cells (WT) the results are very similar.

The two graphs in the left (Fig. 18) are the result of plotting all the eigenvalues for the entire time lapses. The blue and red lines correspond to the average of the N particles and the colored area surrounding the lines shows the variation for each frequency. In the right graphs it is shown the ratio between the eigenvalues of the matrix. This can be also seen in the left graphs but it is clearer in eva-ratio plots.

In all of these plots, for low-frequencies (high time lapse, τ) it appears a higher variation and

the average of the eigenvalues shakes. This happens because the way the MSD is calculated; for longer time lapse the displacements will be lower, so the average will have a higher variation. So for all the experiments in the future I will not take any conclusions by looking into this part of the graph.

Using these two types of graph it can be seen the directionality of the movement. If one of the eigenvalues is much bigger than the other, it means that the movement of the particles is mainly anisotropic; thus, they move in one direction. This fact will appear as very high values for the eva-ratio plot, and the MSD of the biggest eigenvalue will have bigger values.

Now that the first MSD plots have been analyzed and there are not any errors in them it is time to look at one of the most important graphs, the MSD-time lapse graph with the eigenvalues and the slopes in it.

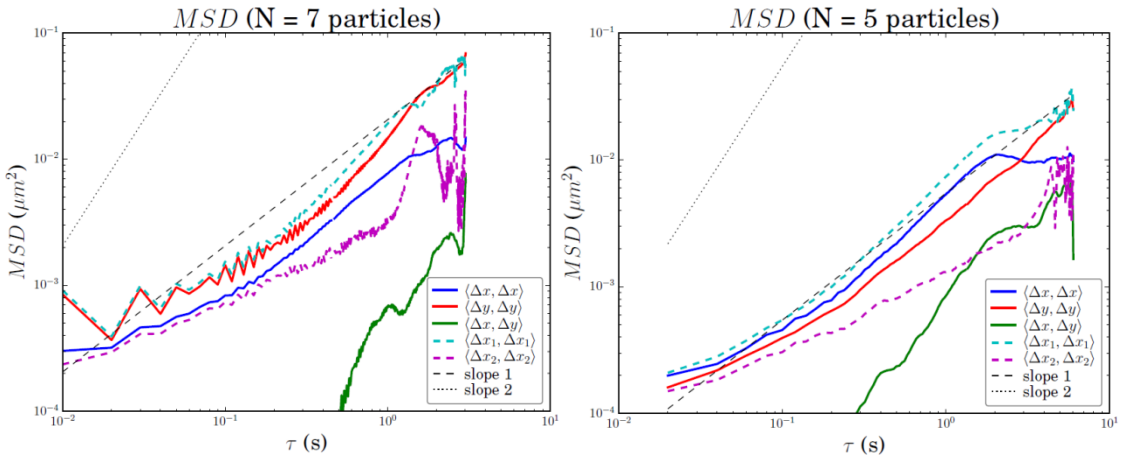



Figure 19: MSD with slopes plotted, left 40x, right 63x

In this plot we can see three different types of lines; the cyan and purple dashed lines correspond to the same eigenvalues plot already seen before. The program calculates for each frequency the average of the eigenvalues obtained when linearizing the MSD matrix for all the N particles. The blue red and green continuous lines represent the mean of the MSD matrix components for each time lapse which are slightly different from the eigenvalues. Finally the black dashed and dot lines are just a plot for a slope equal to 1 or 2. Although this line looks useless, it gives an idea of the behavior of the particles.

Both experiments look very alike in this plot but we can take some conclusions and observations. First it can be seen that the left plot (Fig. 19) starts with a time lag equivalent to 0.02 s and the one in the right (Fig. 19) with 0.01 s. This can be explained because the frame per second rate was modified between the acquisitions of the different experiments. The next thing to be seen is the shaking of the red lines in Fig .19. This happens because the plot uses logarithm scales; so the distance between two short consecutive time points is bigger

than for two big consecutive times. During the entire plot there is a variation between frequencies but for the shortest time lapses the distance between them is bigger causing the variation to amplify.

Finally the most important thing is that both graphs tend to adopt a slope equal to one. This can be explained because they come from a random particle walk or Brownian diffusion; there is not any factor forcing the particles to move in a particular direction. The 1 slope means that the distance covered for every particle in a time lapse is proportional to the square root of the time. A particle submitted to Brownian motion moves randomly to any direction but if it moves during a long period of time the area covered will look like a circle in 2D and a sphere in 3D. This proportion equation gives us an idea on how fast the area explored by the particle grows, how the fast the radius of the circle increases.

Another important graph is that obtained plotting the main directions the particles move. Using this plot it can be seen if some particles move in the same way; for example, in this case of the particles 4, 5 and 6 (Fig. 20) they move approximately in the same direction. The red plotted arrow gives an idea of how much the particle moves in that direction; the longer the arrow is the larger the distance traveled in that direction. As seen, particles 3, 4 and 5 are much more mobile than 1 or 7 just by looking at their arrows. The other black arrows are just to show the particle number, they do not have any important value.

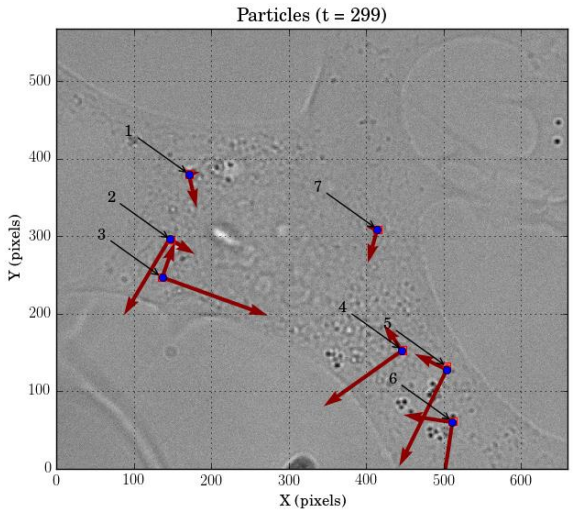



Figure 20: Red arrows mark the principal direction for each particle

Once the MSD graphs have been checked, the final plots can be prepared to get more conclusions. But first it is important to explain how the cytoplasm works under different conditions. The intracellular mechanics of a living cell is best characterized by multiple rheological parameters, including viscosity, elasticity, and creep compliance. The shear viscosity of a liquid measures its propensity to flow under random or applied forces. Viscosity

generates the drag forces that slow down the motion of organelles and protein complexes in the cytoplasm and nucleus. The elasticity (also called the elastic modulus) of a material measures its stretchiness. Elasticity measures the ability of cytoplasmic structures to resist forces and store energy caused by deformation. A material that is only elastic (and not viscous) can deform under stress but cannot flow. As no viscous dissipation occurs during its deformation, the elastic material snaps back to its original shape upon cessation of the stress.

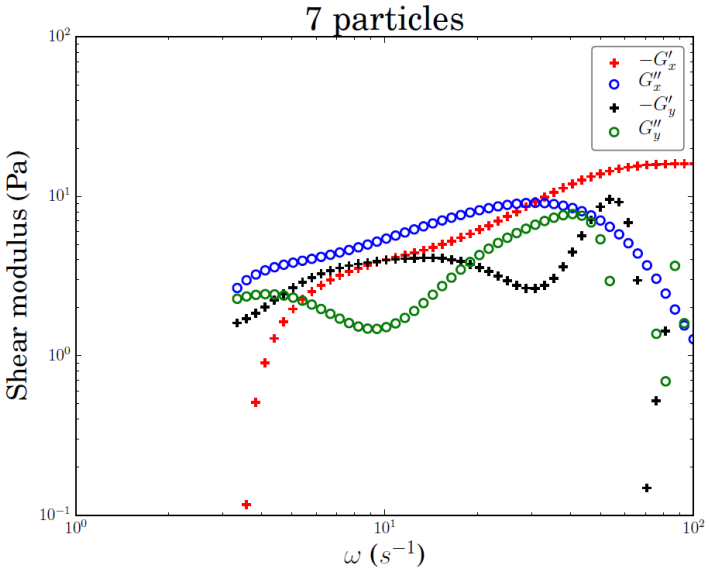



Figure 21: Shear modulus-frequency plot

As you can see in the Fig. 21, for low frequencies, the cytoplasm has a viscous behavior in the x direction (see red and blue dots) because the viscous modulus, G''(blue dots) is much bigger than the elastic, G' (red dots). For the y direction we can clearly see that it has a viscoelastic behavior due to the similarity of both elastic and viscous modulus (see black and green dots). However, at intermediate frequencies the cytoplasm behaves a little more elastic than viscous or the other way probably due to the cytoskeleton reorganization. Finally for high frequencies it starts to behave more elastic, clearly seen for the x direction as the elastic modulus grows while the viscous decreases. These results show that the cells are as expected, more viscous for short frequencies and elastic for the high frequencies. Finally to double check these results it is possible to analyze the viscosity plot (Fig. 22). Again it can be seen that for short frequencies the viscosity is higher than for high frequencies. With this graph it is easy to compare the directionality of the cytoplasm properties. The cell it is mainly isotropic except for one range of frequencies where the viscosity for the y direction is slightly less than for the x direction.

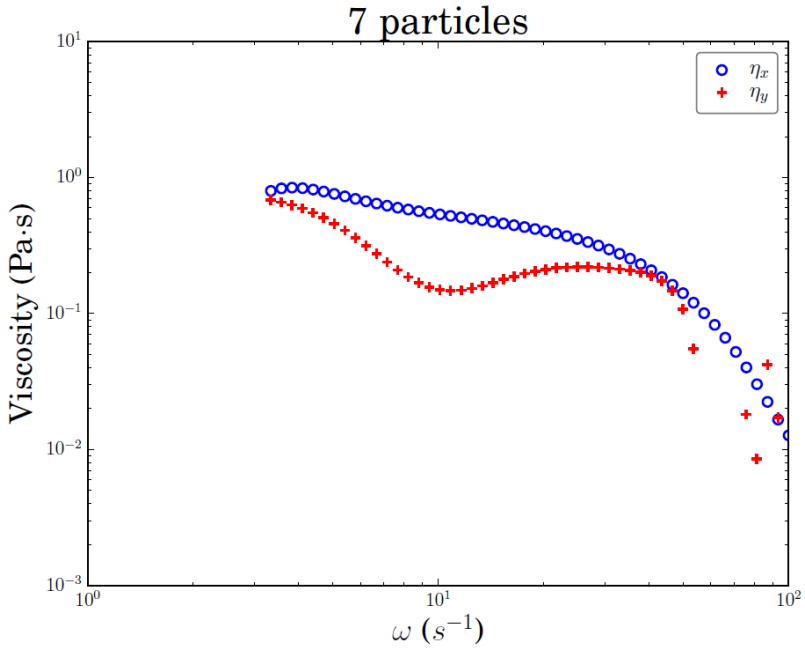



Figure 22: Viscosity-frequency plot

Now that the first results have been analyzed it can be said that this tracking method worked more or less as expected but it can be improved. To do so I decided to start using the laser field of the microscope and use fluorescent particles to improve my imaging.

## 6.2. Microinjection

As explained before, the method I used to get the following results is the biolistic injector. To introduce the beads inside the cells I used the PDS-1000, a rupture disk of 450 psi and a bead size of 0.2 um in diameter. After a first approach using the microscope laser field I realized that not so many beads had reached the cells and that there were many clusters that could not be tracked. I chose one of the cells that had more beads inside, but as seen there is a small cluster (group of beads attached) in the left side. On the other hand there are some beads which cannot be tracked because they get out of focus during the acquisition of data, top-right of the image.

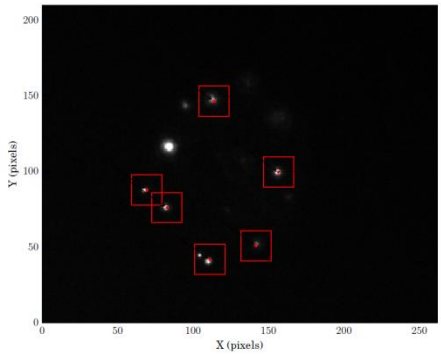



Figure 23: Particles selected to track

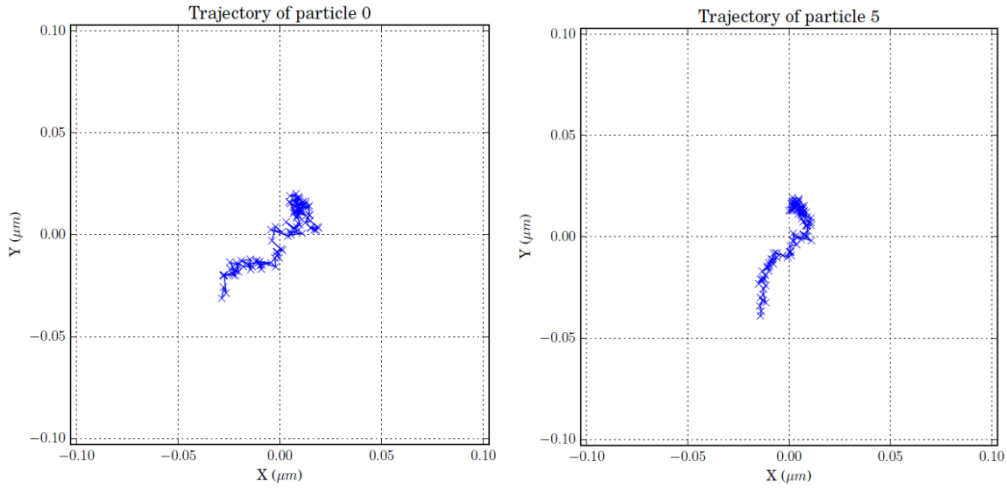



Figure 24: Trajectories of the particles tracked

To start the analysis the first thing to do is to check that the particles trajectories do not have errors, as done in the previous experiment. In this experiment it can be seen that the particles have less mobility, they do not even reach a 0.05 μm distance in any direction. This problem might be caused because the beads are not inside the cytoplasm and remain attached to the cell membrane which has less mobility.

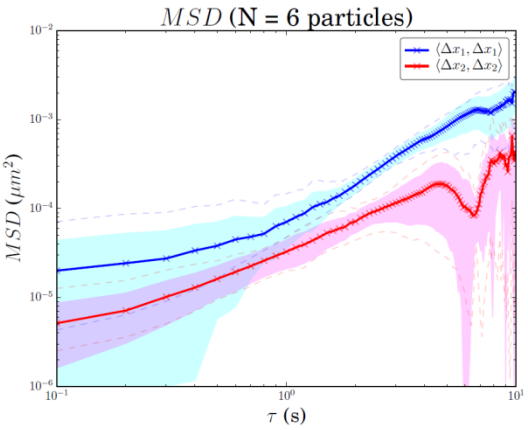



Fig 25: Eigenvalues plot

In the eigenvalues plot (Fig. 25) it can be seen that they have a similar behavior than the last experiment but with a lower MSD magnitude. They reach a minimum value around $10^{-5}$ due to the short movement of the beads. The variation for short time lapse is very high also related with the movement problem. All this is caused because the microscope works with a maximum resolution of 0.01 pixels (equivalent to $10^{-5}$ $\mu m^2$ in the MSD) and it cannot detect the short movements for these particles. Even with this drawback, the results for lower frequencies can be trusted and I will keep analyzing the results.

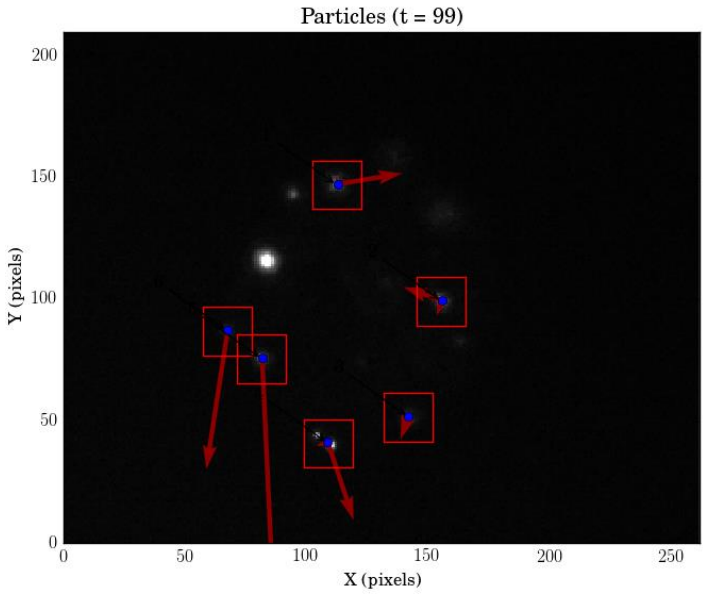



Figure 26: Main direction plot

The directionality of the particles trajectory is pretty obvious; in the bottom-left side the particles tend to move more than on the top-right side of the cell. This may be due

to the cell movement that causes cytoskeleton to stretch, making the cytoplasm to flow more in some parts of the cell.

When looking at the MSD it can be seen that the particles have random motion because the slope of the MSD is close to one. Due to the mobility problems the MSD values for high frequencies cannot be trusted and neither the modulus nor viscosity plot. As it can be seen the value of the average viscosity for all the frequencies it is almost 100 Pa·s which is equivalent to 1000 Poise, a value too high. In some articles of previous experiments they show that the cytoplasm viscosity is about 10 Poises which means that my results are 100 times more than what they should be.

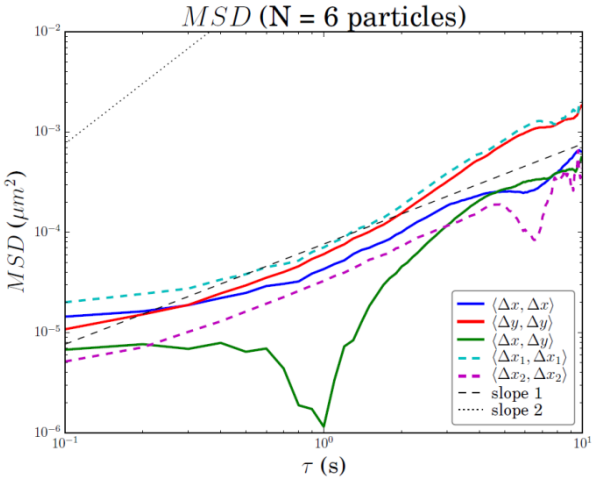



Figure 27: MSD with slopes plot

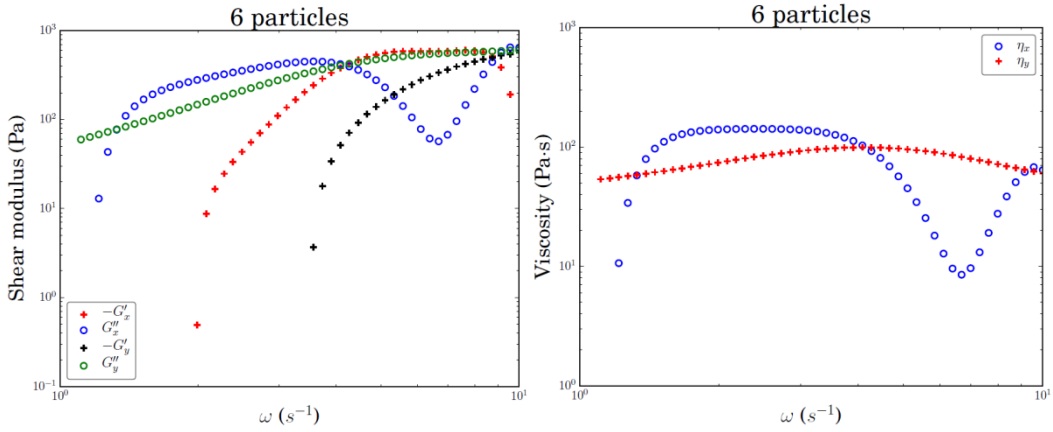



Figure 28: Left, shear modulus-frequency plot. Right, viscosity-frequency plot

On the other side the cells behaves a little bit as expected, although the magnitude of the viscosity the cell is slightly more viscous for short frequencies and more elastic for high frequencies, clearly seen for the results in the y direction. This can also be seen in the modulus plot just by looking to the difference between the elastic modulus G'

and the viscous modulus G''. For short frequencies the elastic modulus is lower giving the cell a viscous behavior.

I decided to repeat the experiment using my two different kinds of cells, WT and KO. I did the whole process again and after shooting the beads with the biolistic injector and looked with the microscope I realize I did not have enough single beads to be tracked. There were too many clusters that could not be tracked because they were too big. In both cell culture dishes I got the same problem and this caused that there were not cells with more than one bead inside. In the two images below, the beads are located inside different cells.

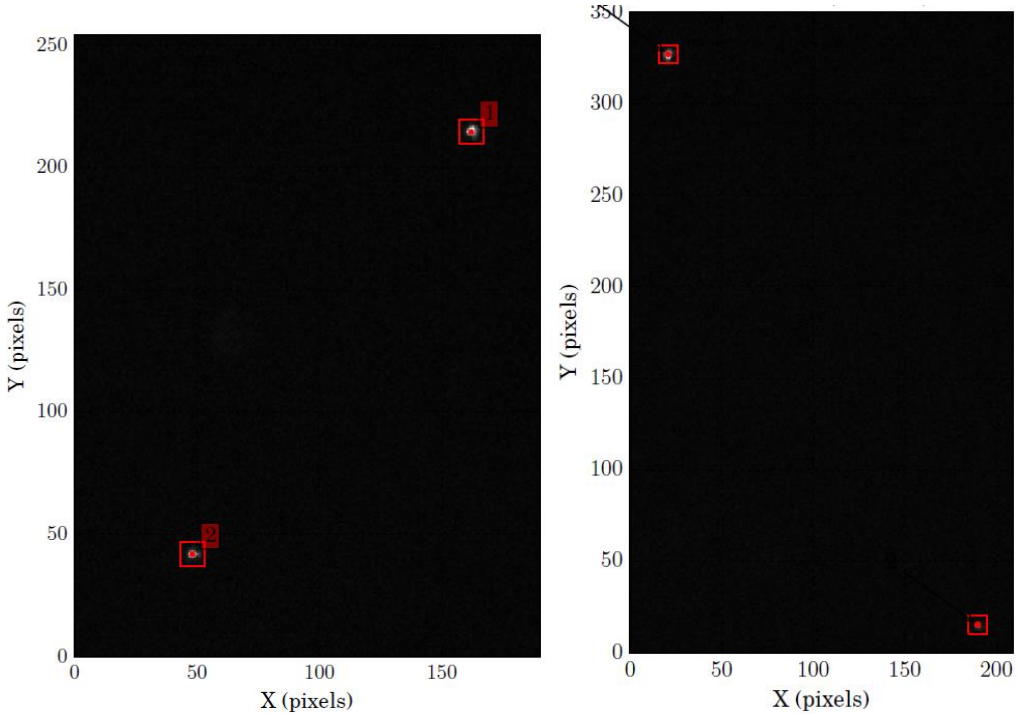



Fig 29: Particles tracked in different cells

For both lines I use the same bead diameter size, 0.2 μm. The images were taken both with the 40x objective but using the 3x magnification lens. With this extra lens have a total magnification of 64x due to the 1.6 multiplier given by the extra lens. With this magnification the calibration factor of the microscope drops to 0.1 μm per pixel. Once all the previous steps had been checked and seen that there are no errors it's time to look at the MSD plot and look how these cells behave. It can be seen that the particles are subjected to random motion because the slope of the MSD plots have a slope similar to 1. It can be seen that in the last part of both graphs the MSD have a strange behavior, which I did not analyze because at very low frequencies the MSD presents some intrinsic drawbacks.

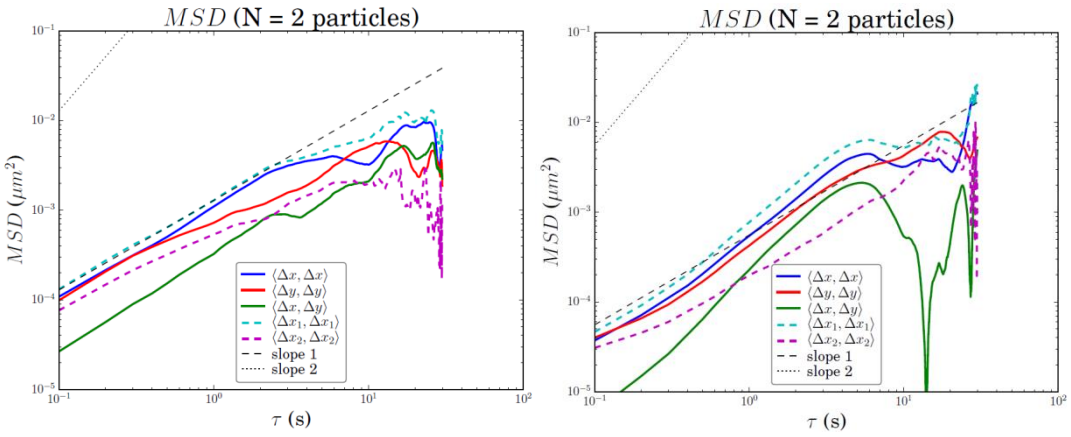



Fig 30: MSD with slopes for both experiments in Fig. 29

The problem I got with these experiments is that due to the lack of beads I cannot calculate the viscosity and the modulus plot. This happens because the program needs at least three particles to calculate those values.

Since the results I got after analyzing this method were not very conclusive, I tried to improve the beads injection technique. As told before one of the ways to develop this technique is to do the dialysis of the beads solution to exchange water for ethanol in order to decrease the surface tension, allowing a better bead dispersion over the micro carrier.

## 6.3. Endocytosis

I also analyzed cells that were labelled with the beads by a third method. I cultured WT cells in normal media, and after a couple of days I changed the media by a special culture media lacking L-glutamine and supplemented with the beads. Glutamine deficiency exacerbates the process of pinocytosis, allowing the entrance of extracellular nutrients and the beads. After an overnight incubation I replaced the media by the complete one without beads and analyzed the cells at the microscope. To get these images I use the same lens system as the experiments before getting a calibration factor of 0.1 um per pixel.

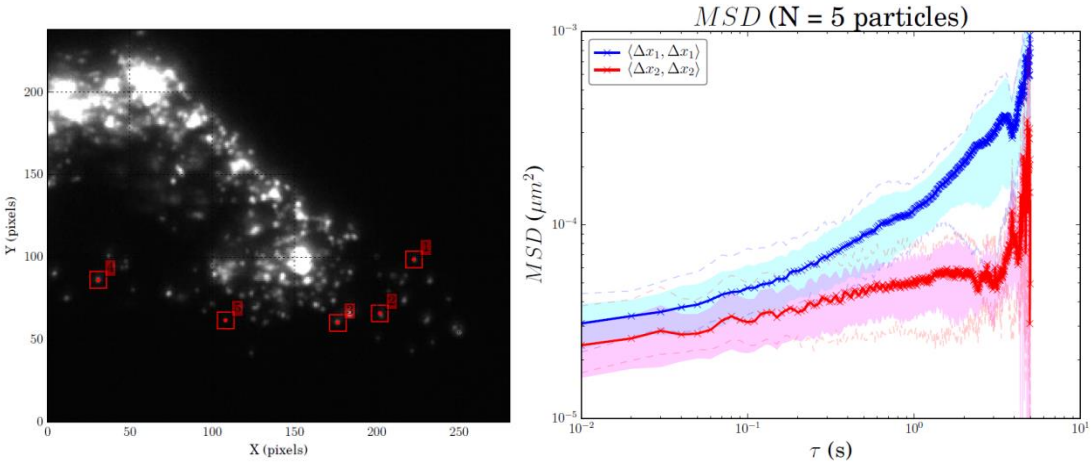



Figure 31: Left, particles tracked. Right, eigenvalues plot

I tracked the particles that at the video have more mobility in the focus plane and can be easily identify. When looking to the eigenvalues plot (Fig 31 right) the first thing to see is that they do not show a lot of variation in any direction which is a good symptom that the results are precise.

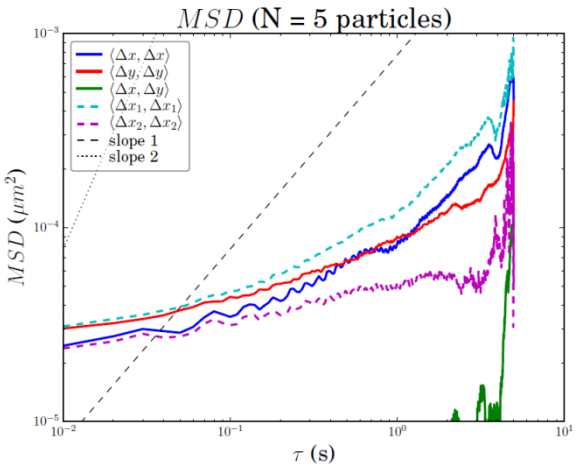



Figure 32: MSD with slopes

As always the graph that gives the most information is the MSD plot (Fig. 32). For high frequencies these cells exhibit an elastic behavior but as the time lapse gets bigger the cells start to show a more viscous behavior due, demonstrated by a slope close to one. This is the standard behavior for normal cells, elastic for high frequencies and viscous for low frequencies. This happens due to the stretchiness of the cytoskeleton, since for short time lapses the cytoskeleton does not have time to reorganize. When this happens the cytoskeleton contracts and when the stress is over it expands making the cell elastic. For larger time lapses, the cytoskeleton moves and aligns with the direction of the stress allowing

the cytoplasm to flow and deforming the cell. If the deformation of the cell is very notorious and all the cytoplasm particles flows under one direction it will be clearly seen in the MSD with a slope similar to 2. Under flow conditions the MSD has a slope equal to 2 because the MSD is proportional to the square of the time. It means that the distance covered by a cell is proportional to the time lapse and not to the square root of time as it happens in random motion.

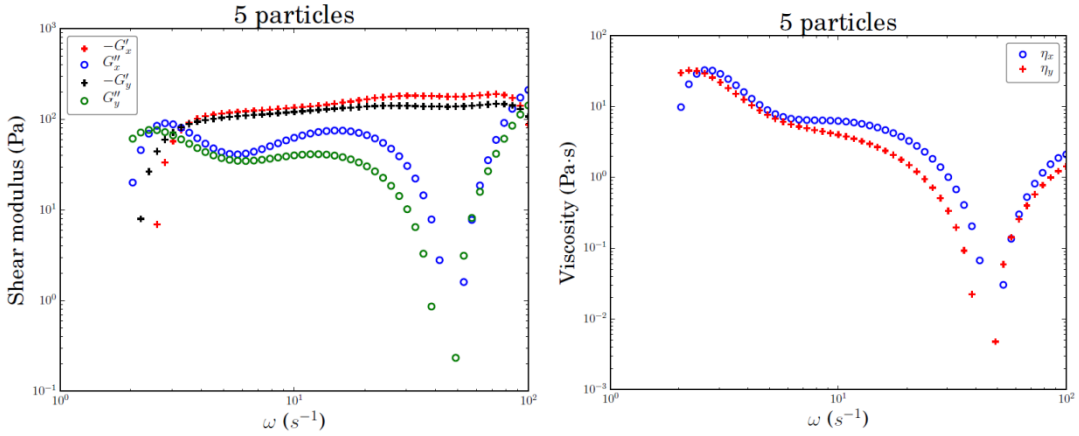



Figure 33: Left, shear modulus-frequency plot. Right, viscosity-frequency plot

When looking to the shear modulus plot, it can be seen that the elastic modulus is always a little bit higher than the viscous modulus for both directions. This is unexpected because ideally the cells tend to be elastic for high frequencies and more viscous for the shorter ones. However, if we take a closer look to the low frequencies area (Fig 33) it can be seen that the elastic modulus start to drop while the viscous modulus remain the same level. This could have been clearer if I would have taken images during a longer period of time in order to have longer time lapse expanding the plot in the left side.

Although the experiment was not long enough, the viscosity plot is informative. The cell is more viscous for low frequencies with a viscosity around 10 Pa·s. There is a drop in the viscosity probably caused by a jump in the particles tracking or any other external factor. It can also be seen that the cell is very isotropic due to the similarity of all the properties for both directions clearly seen in all the plots above.

The main problem of this method as told before is that the beads are not free in the cytoplasm but included into lysosomes. The problem with that is that the conclusions taken above are not fully applicable to the cytoplasm. For high frequencies the beads move around the lysosomes randomly bouncing into the membrane probably causing the elastic behavior seen above. On the other hand for lower frequencies the beads have to move the same direction the lysosomes flowing allowing us to see the viscosity of the cytoplasm. To conclude this method works fine for low frequencies but lacks of cytoplasm information for

short time lapse.

After analyzing these results I tried extract some conclusions and focused on the more efficient methods. On the next experiments I added both green and red beads to the cells and tried to track and see the difference between them. To do so I introduced one type of beads using the PDS-1000 biolistic injector and the other type of beads using the pinocytosis method. With the following experiments I tried to determine the difference between the mobility of the beads inside lysosomes and the beads free on the cytoplasm and take some conclusions. Also I incubate the cells with a growth factor called TGFβ that modify their shape. I wondered if this treatment makes the cytoplasm anisotropic due to the movement of the cytoplasm just in one direction.

To do the final experiment I cultured both cells types in normal plastic dishes and with regular medium. Then I switched them to the special media without glutamine supplemented with the green beads as I did in the third method. After an overnight labelling in this condition, and trypsinized the cells, attached to new plates and let them grow for one day. I had four dishes, two for each type (WT and KO cells) in order to control all the variables in the experiment. I injected the red beads using the PDS-1000 in all the dishes and check if they had reached the cells. To do so I use the dialyzed solution of the beads explained before. Once everything looked fine, I passed the four l samples to special dishes with the coverslip system in order to be able to focus with a 40x or higher magnification. I waited until all the cells were completely attached and used the laser field of the microscope to look if there are some differences. As I did before I use a special wavelength interval to detect the green beads and another one to detect the red ones to prevent noise between them. The laser uses light of a special wavelength that allows us the microscope to detect the particles. When the light emitted by the laser hits a particle, it is absorbed and re-emitted with a little bit less energy. That means that the light retuned by each particle has a bigger wavelength than the light emitted by the laser. The microscope is able to detect both particles analyzing the wavelength of the light emitted: between 525 and 575 nm it detects the green beads and between 650 and 700nm, the red beads.

In order to start analyzing the results first I checked if the microscope detection system worked as expected. The two images showed below (Fig. 34) were taken for the same area, and as expected, the position of the particles is not the same at one wavelength than in the other. This means that the microscope works fine and there is not cross-reactivity. Although in these images is not very clear, it is also remarkable that the number of beads introduced using the pinocytosis method is much higher than with the injection method.

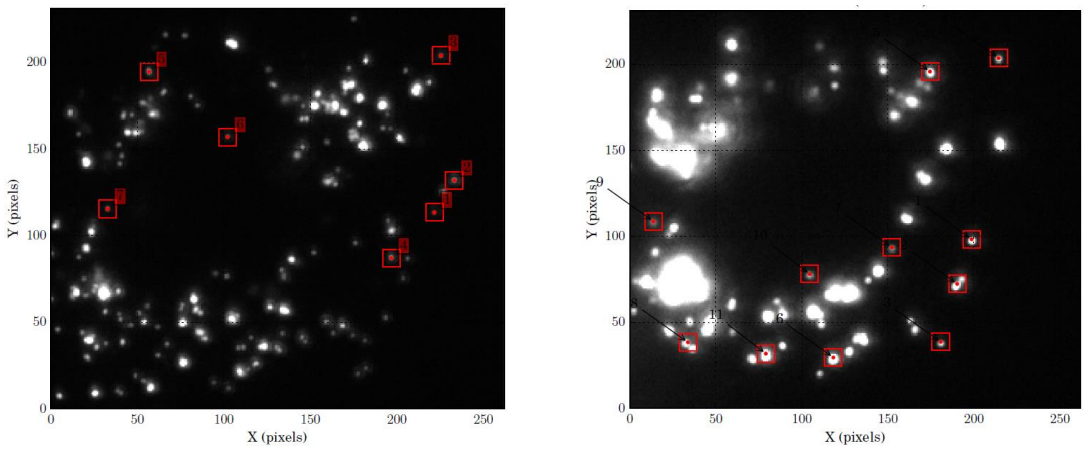



Figure 34: Left, particles introduced by pinocytosis. Right, particles injected

When looking to the MSD of both particles of WT cells cell it is very clear that both particles are subjected to random motion as their slope is closer to 1. They also have more or less the same MSD values, reaching 1 $\mu m^2$ for the biggest time lags and $10^{-4}$ $\mu m^2$ for the shortest ones. This similar behavior might be due to the fact that red beads (the ones I injected) are also inside lysosomes, like the green beads.

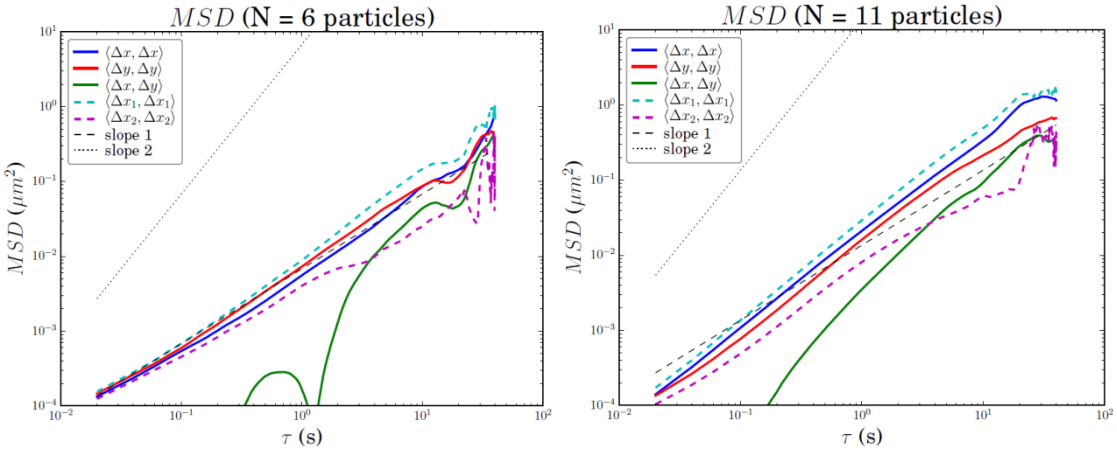



Figure 35: MSD with slopes for both experiments in Fig. 34

When looking to the viscosity (Fig. 36) the green beads have slightly higher values than the red beads. In both cases the cell is almost isotropic although when analyzing the red beads for low frequencies the cell behaves a little bit different for the x and y direction.

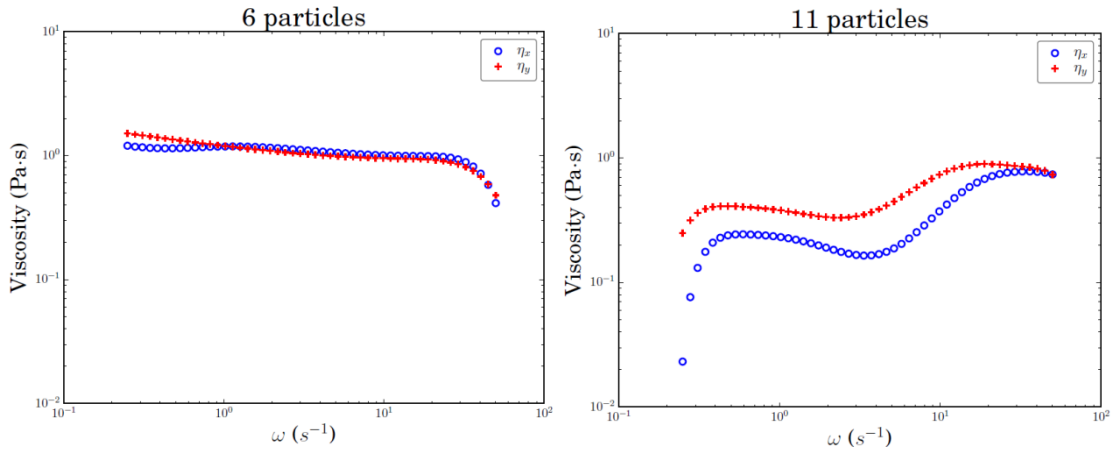



Figure 36: Viscosity- frequency plot for both experiments in Fig. 34

For the KO cells the results are pretty similar to those obtained with the WT cells. Although the areas analyzed (Fig. 37) are not the same size, I made sure all the particles were inside the same cell.

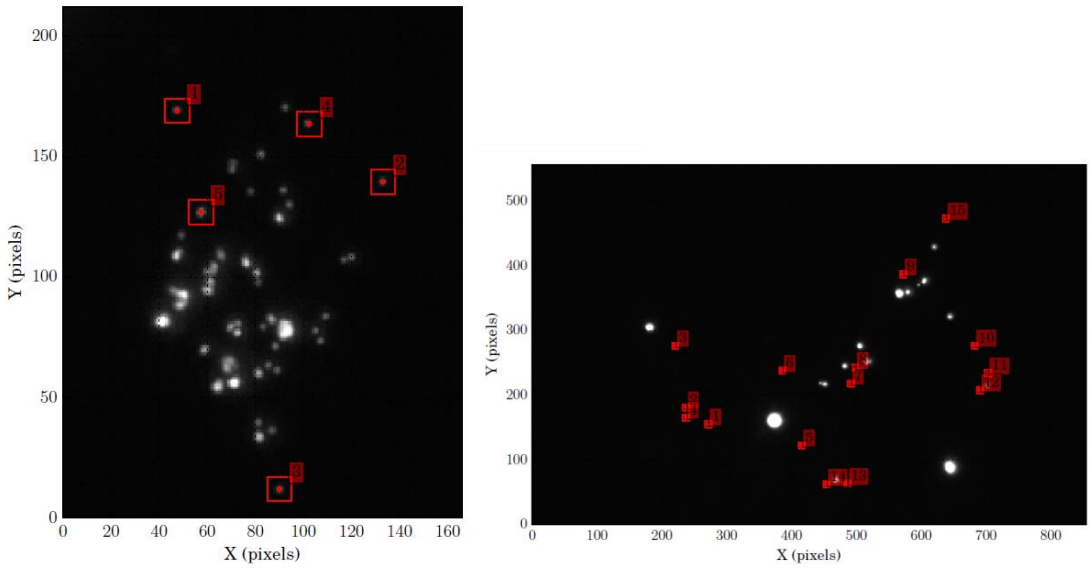



Figure 37: Particles analyzed in the same cell. Left pinocitosis, right injection.

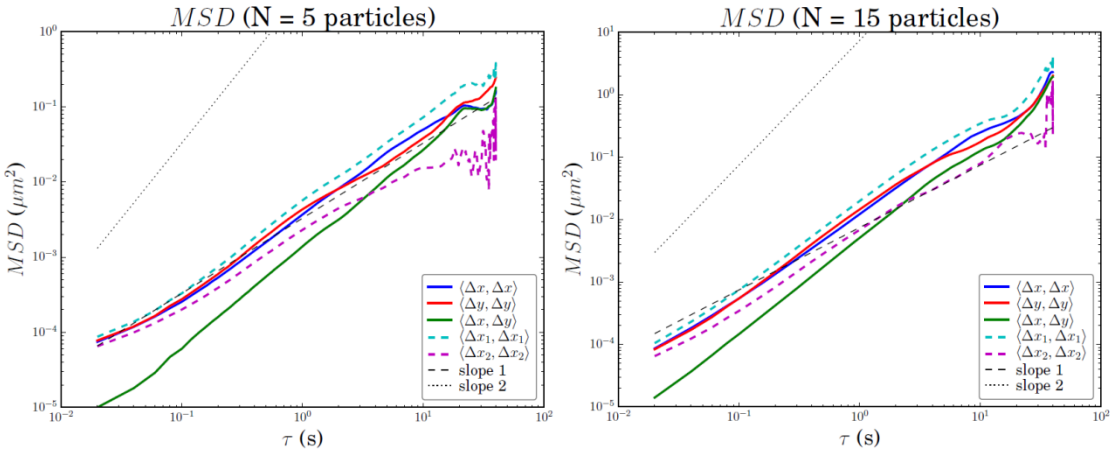



Figure 38: MSD with slopes

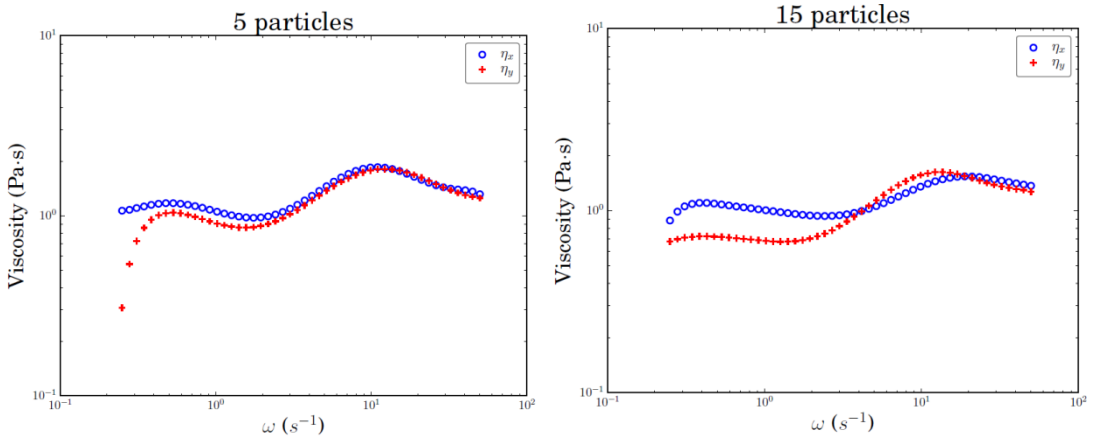



Figure 39: Viscosity-frequency plots

Again it can be seen that the bahaviour for the KO cells is pretty much isotropic for both directions, as showed by the MSD and the viscosity plots. For this cell the MSD has a slope equal to 1 because the particles are subjected to Brownian motion again. For long time lapse the cell behaves a little more viscous showed by a slope more than 1 in the MSD. This may be due to a movement of the cell as they don't feel confortable under the laser light and they tend to contract. When looking at the viscosity graph it can be seen that for both particles types the viscosity has a fluctuation in a specific frequency, arround 2 Hz probably due to that cell contraction.

# 7. TFG-β experiment

Once that those experiments had been analyzed it's time to go a step forward and make the cells change their shape to try to determine anisotropy in the cytoplasm and see how this affects the MSD and the viscosity. To do so I used a growth factor called TGF-β that makes them to grow faster and changes their shape making a group of cells align in one direction. For this final experiment I used the two cell lines and studied how this growth factor acts on both of them. Since, as checked before the particles behave more or less the same way when using the pinocytosis method and when injecting them using the PDS-1000, I decided to use only the pinocytosis method for this experiment as I can get more beads inside each cell and more easily. I proceeded the same way as always and once the cells were loaded and attached to the coverslip I added TGF-β to in a concentration of 5 ng/ml and then I let them grow for a day.

Once the cells were ready I checked them using again both green and red wavelength of the laser field. After analyzing KO cells I got these results:

For the control group of the KO cells I got the same results as always.

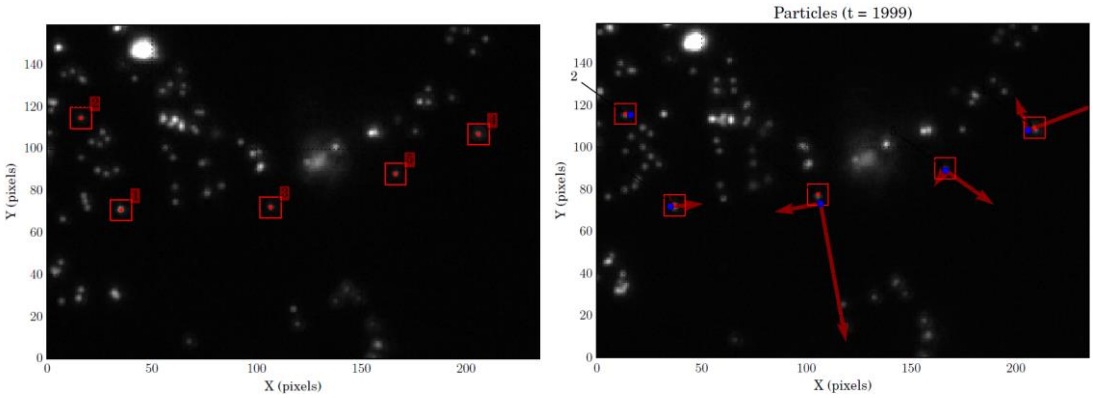



Figure 40: Left, particles selected. Right, main directions

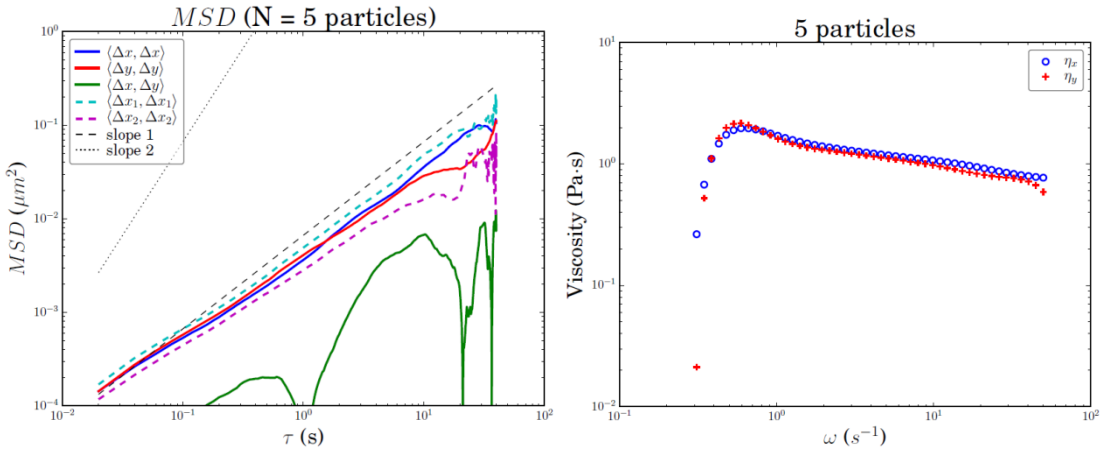



Figure 41: Left, MSD with slopes plot, right viscosity-frequency plot

As expected the KO cell behaves the same way than before. This cell is completely isotropic; although is not evident in the trajectories plot, it is clearly seen in the similarity between the viscosities of both main directions. Also the particles are submitted again to random motion as the slope of the MSD is equal to one for all time lapses.

I also analyzed these cells after Once the KO control group is analyzed it's time to see how these cells react when adding TGF-β.

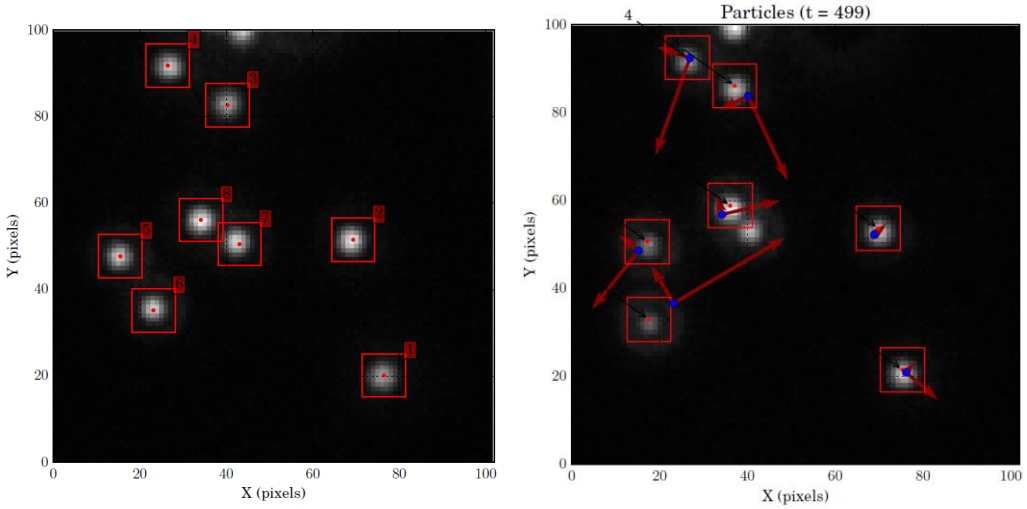



Figure 42: Left, particles selected. Right, main directions plot

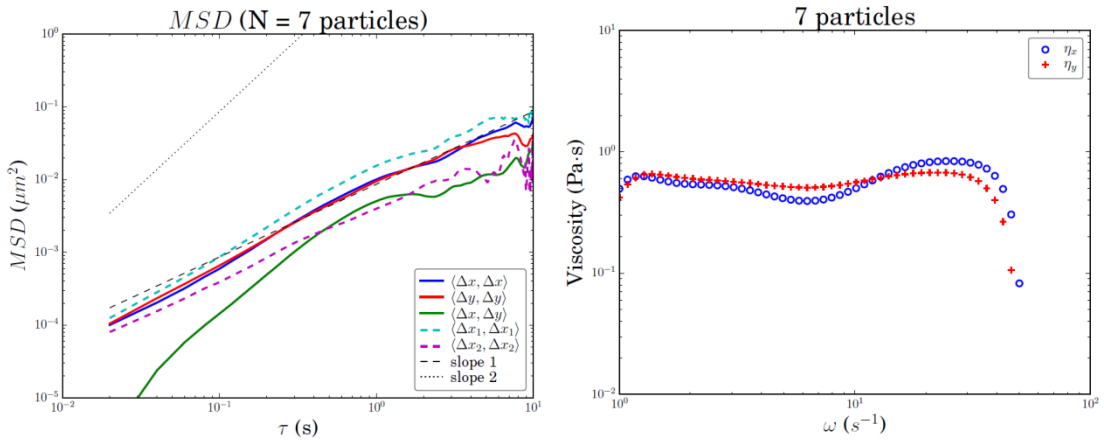



Figure 43: (Left panel) MSD with slopes plot. (Right panel) viscosity-frequency plot

After looking at the results above it can be said that TGF-β does not alter the behavior in KO cells. The MSD has again a slope equal to one, so the particles are also submitted to Brownian motion for all frequencies. TGF-b did not modify the directionality of the cell as the viscosity remain isotropic for both x and y directions. As conclusion, the first part of this experiment it can be approved that TGF-β does not modify the cell structure in KO cells, making them remain with the same properties as always.

I also analyzed WT cells in the same way I did for the KO cells, first checking the control group to see if they behave as always.

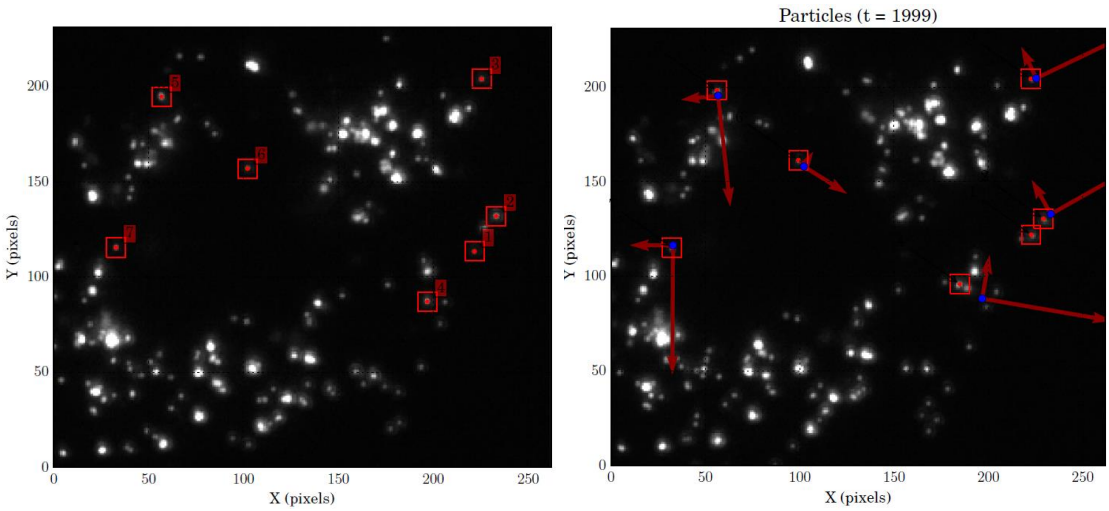



Figure 44: (Left panel) particles selected. (Right panel) main directions plot

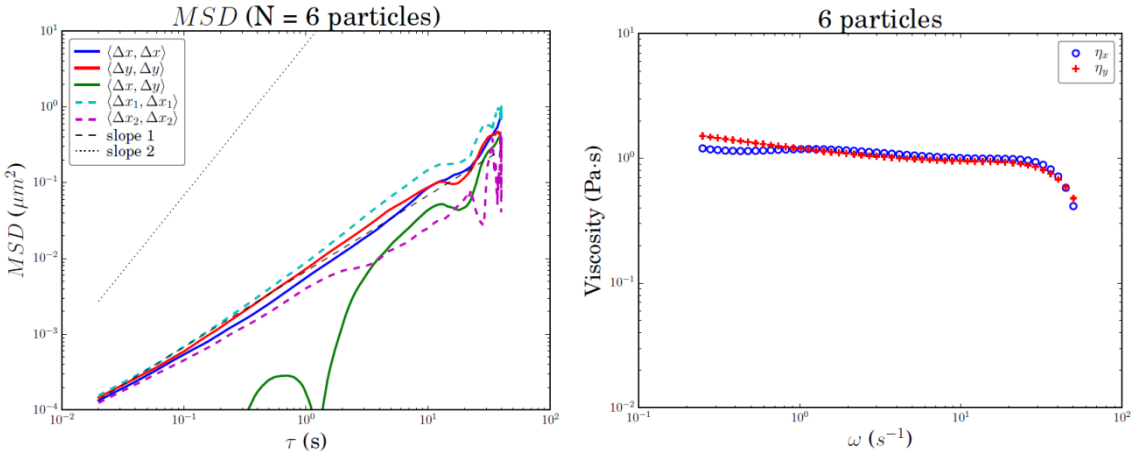



Figure 45: (Left panel) MSD with slopes plot. (Right panel) viscosity-frequency plot

It can be seen that this cell has the same properties all the previous had. It has a isotropic viscosity with an average value of 1 Pa·s for all frequencies. The MSD shows the random motion of the beads inside the citoplasm.

After adding TFG-β to Wil type cells and letting them grow for one day I got the following results. I tracked four particles that moved more in the video so I can have an MSD with a higher magnitude. I used again the 40x lens  with the 1.6 multiplier magnification that allowed a total of 64x.

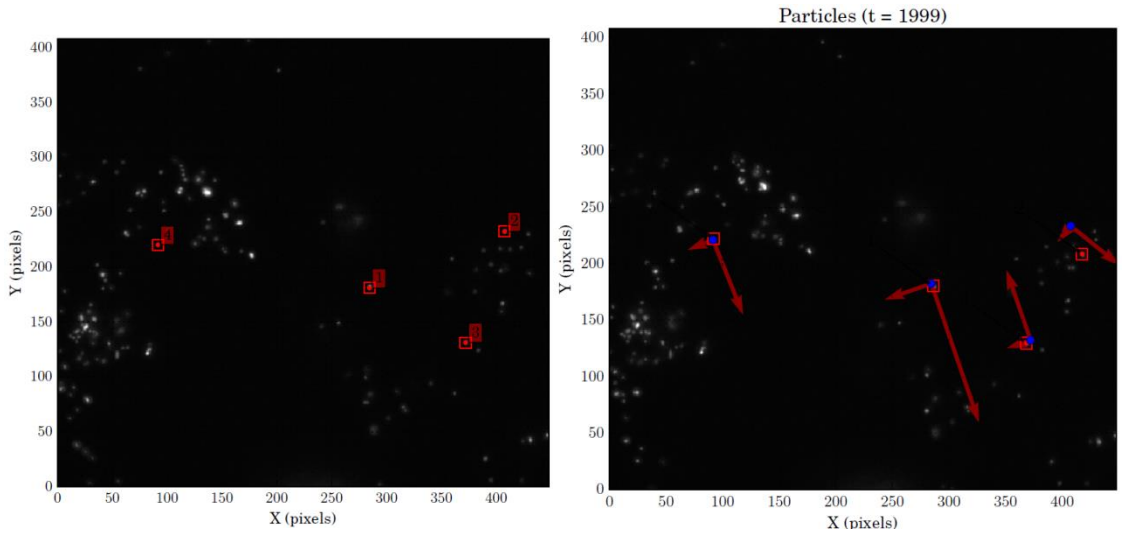



Figure 46: (Left panel) particles selected. (Right panel) main directions plot

After checking the trajectories of the particles I realize they all are moving in the same direction probably due to the new shape of the cell which is more elongated.

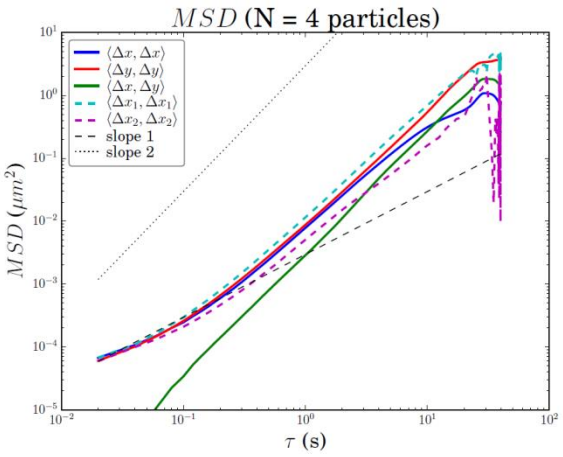



Figure 47: Viscosity-frequency plot

As you can see the MSD now is a little bit different than the one of the control group. For high frequencies the particles are submitted to random motion as it can be seen when looking to the slope equal to 1. As time lags began to grow the particles tend to have now a conducted motion as the slope is similar to 2. This is caused due to the movement of the cytoplasm making the particles flow just in one direction. It can be deduced that when the particles are submitted to random motion the distance explored is proportional to the time, double time double distance. Due to the reshape of the cell this conducted motion was more or less expected but it was also expected some anisotropy in cell cytoplasm which is not reflected in the graph above. But this can be explained when looking to the image below. Cells are oriented with an angle of 45º from the x and y axes, and so do the trajectories of the particles tracked, making the values of the MSD equal for both directions.

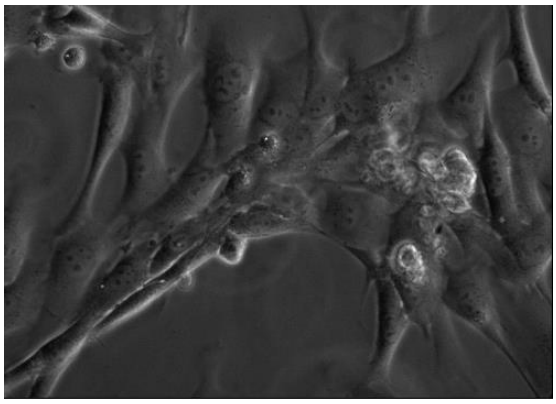


Figure 48: TFG-β modifies cells shape

To solve this problem I just have to track and other group of particles and check how it's their response.

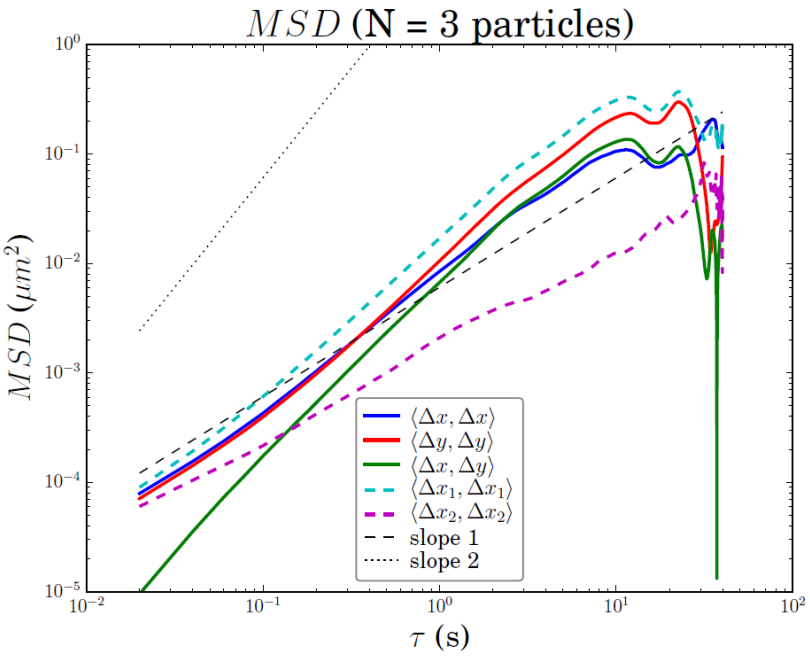



Figure 49: MSD with slopes plot

In this new group of particles the conducted motion it is not as obvious as before because the particles trajectory is not the bisector anymore. Now it can be seen the anisotropy of the cytoplasm when looking to the main directions, see cyan and purple dashed lines. When looking at low frequencies one direction, purple, is mainly submitted to random motion and the other, cyan, is almost pure conducted motion.

# 8. Additional experiments

## 8.1. Cardiomyocyte

In this lab I had the chance to work with some other PhD students who were working in microrheology. The following results were obtained by Ernesto Criado who was working with cardiomyocytes in order to learn who the microrheology of these special cells behaves. Cardiomyocytes are the muscle cells that make the cardiac muscle so they are the responsible for the beating of the heart. The movement of the cardiomyocytes consists of two cycles, a rest phase and an active phase. These two phases are commonly understood as systole and diastole. To prepare this experiment and introduce the beads inside the cells Ernesto used 0.2 um diameter particles and he introduced them using the PDS-1000 ballistic injector. When looking through the microscope he used a total augment of 64x, 40x for one len and the extra len of 3x which gave a multiplier to the first one of 1.6. After checking for his results he realized he actually did have one bead inside one cell and started studying it.

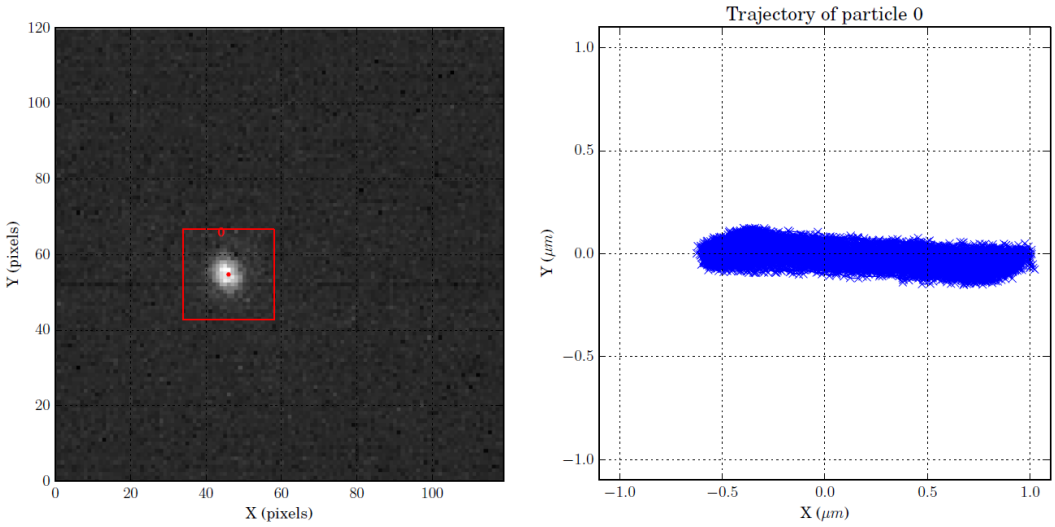



Figure 50: (Left panel) particle selected. (Right panel) trajectory of the particle

The movement of this particle is completely different to the ones analyzed in previous experiments due to the beat of the cell. The particle move back and forward in a horizontal direction resulting the trajectory above.

After he analyzed the MSD of the particle he obtained the following results.

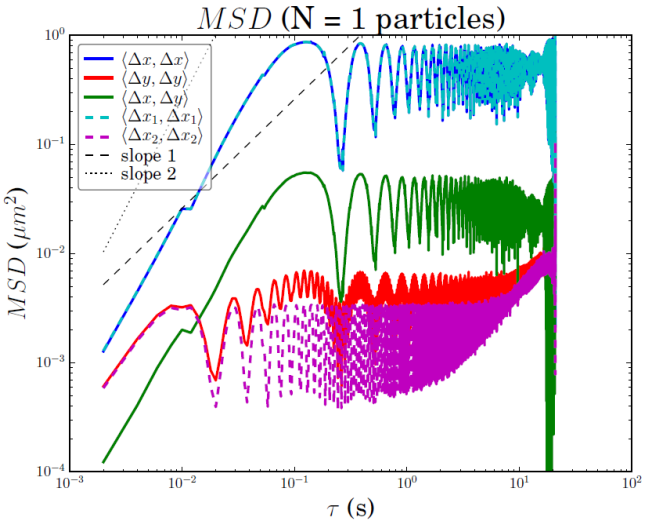



Figure 51: MSD with slopes of the particle

The MSD of this particle shows that the particle is completely anisotropic as it was expected when looking to its trajectory. For the x direction, which is the approximately one of the main directions the cells behaves under a conducted motion for high frequencies. For short time lags the particle moves in the horizontal direction following the flow, this is represented in the MSD with a slope equal to two. For the vertical direction the particle is not affected by the beat so it has more of a viscous behavior as the slope of its MSD is closer to one.

For larger time lags there's a point, around 0.3 s, where the MSD drops and starts a sinusoidal behavior. When looking to the video it can be seen that this time lag correspond to the period of the cardiomyocyte beating. After this point the cardiomyocyte keeps with this sinusoidal behavior of about three beads per second but it looks as if it was getting faster due to the logarithm scale. For low frequencies it can be seen that the movement of the beads has an elastic behavior as the slope of the MSD gets closer to zero. This can be explained with the cardiomyocytes fibers that stretch the cell making the cytoplasm flow back and forward with the beat frequency.

With this experiment I showed how different cells can behave when having different functions. As it was shown MEF cells tend to be elastic for high frequencies and more viscous for larger ones opposite to the elastic behavior of cardiomyocytes. Although it cannot be done in our lab because our microscope does not have enough power resolution it would be very interesting to study this cells for higher frequencies and see how the conducted motion evolves.

# 9. Economic analysis

To calculate the total cost of this project the student has worked a total of 6 months equal to 24 weeks.

- The student worked for 6h every day, five days a week valued at 8€ per hour (if the student would have done an internship in a Spanish Company, the legal agreement rules that the maximum compensation would be 8 €/h).

- The supervisor work involved 1h per week due to project guidance and orientation. Every hour is worth 60€.

- The informatics program used for this project was mainly made by a PhD student and took him about 6 months to finish it. So the lease of this program is estimated in 2500€.

-The biological material used in the experiments has a very large range of prices but the total value rises up to 2000€.

- Office furniture was purchased at 200€ and will last for 10 years.

- Office material was bought for 20€ and will be consumed for 2 years.

- The average energy consumption is estimated by 500W. It includes all the office and laboratory equipment that need electricity: PC, cell culturing hood, spinner… It is assumed that 1 kWh costs 0.10907€ after taxes.

| Concept | Lifespan (years) | Acquisition cost (€) | Usage (hours) | Variable cost (€/h) | Total (€) |
|---|---|---|---|---|---|
| Student work | | | 720 | 8 | 5760 |
| Surpervisor work | | | 24 | 60 | 1440 |
| Office lease | | 3000 | | | 3000 |
| Office furniture | 10 | 200 | | | 200 |
| Office material | 2 | 20 | | | 20 |

| | | | | | |
|---|---|---|---|---|---|
| Energy consumption | | | 675 | 0.035 | 23.625 |
| Biological material | | 2000 | | | 2000 |
| Informatic software | | 2500 | | | 2500 |
| Laboratory lease (microscope, Hood, …) | | | 150 | 100 | 15000 |
| TOTAL | | | | | 29043,63 |
| **TOTAL IVA(21%)** | | | | | **35166,23** |

Chart 1: Costs of the project

As can be observed, the total budget of this project is very high since it is a research project and I have also considered the lease of all the laboratory material which is very expensive.

In the following Gantt diagram it can be seen how the student assign the hours shown above

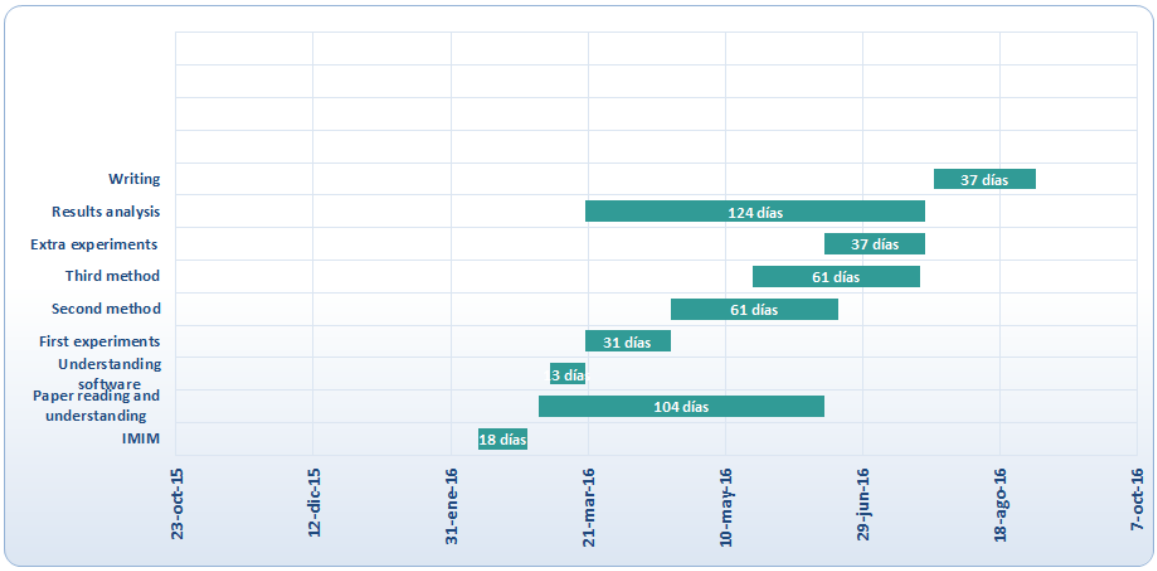



Diagram 1: Gantt diagram for the whole project

# 10. Environmental impact

Considering that this project has been mainly developed using computer systems and laboratory experiments, its environmental impact is very low.

In order to start my experiments in the laboratory, the University of California San Diego made me take some exams to guarantee that I had the knowledge of all the laboratory hazards and environmental impacts.



For the laboratory equipment I will consider the fact that many tools used for the realization of my project were just used once and then safely thrown them away. During the first laboratory stay I realized in IMIM, the glass pipettes were deposited in a special box and then at the end of the day they were sterilized and ready to use them again. This recycling program was not established in UCSD as they throw all the tools they use in order to prevent eventual contaminations.

For the office work I only have to consider the $CO_2$ emissions produced while I was working (400W for 6h per day, 5 days per week and 24 weeks in total).

# 11. Social impact

Although this project does not affect directly to any sector of population it is a small progress for science with its battle to discover and explain cells behavior. This project may be useful for those who want to get introduced in microrheology and the study of cells motion as it's not very precise or hard to understand.

# 12. Conclusions

Now that this project is finished it is time to see if the objectives proposed have been accomplished. As it was explained before the goal of this project was to work and be familiarized with microrheology and study this property in cells motion under different conditions. In this project I explained the most basic concepts in order to understand cell motion but as I worked with some PhD students I realize how complicated and precise this topic can be.

After finishing this project and once all the experiments are performed I can finally extract some conclusions on which method of particle tracking works better. As it was shown in the results both pinocytosis and particle injection methods offer similar results, which are much better than those obtained using the tracking in the bright field. The problem with the particle tracking using the bright field is that the color contrast between the particles and the rest of the image is very low so the program has some difficulties when detecting the particles. For future experiments I would select the biolistic injection method using the PDS-1000 but with a dialyzed solution. Using this method I injected fewer particles than with the pinocytosis method which initially may look as a disadvantage. However, as mentioned before, with the second method, pinocytosis, particles are inside lysosomes so I cannot extrapolate the results for the whole cytoplasm. When the particles are injected and tracked right after, the cell has no time to wrap them into vesicles, therefore we see the pure motion of the cytoplasm. Of course this is highly related to cell type, since some cells may encapsulate the particles really fast, while some others do not do it at all.

After working six month at the Mechanical Aerospace Engineering Department in UCSD I got the experience of how a group of investigators works. In this department they work in some different topics such as blood flow in the heart and cell forces. They collaborate with many other departments and universities. They also work with some undergraduate students to teach them and get them involved in their program and laboratory techniques.

# 13. Extensions and future work

In this project, I studied one point particle tracking microrheology to acquire information of a specific cell type. There are more methods we can use to get information about cell mechanics; for instance two point particle tracking microrheology. Until now we have considered only the movement of *n* single particles inside the cell but this change with the two point technique is used where we study the movement of pairs of particles. The key behind two-point microrheology is that the hydrodynamic interactions between two beads are mediated through the material between the particles, so that the complex viscosity $\tilde{\eta}$(s) that they encode is more sensitive to the bulk material rheology than (one-point) self-mobility measurements. With this method theoretically we have much more precision when obtaining microrheology results because we have in mind how the movement of one particle affects the other. To use this method particles have to be close so the interaction of both particles can be compared to the one point particle tracking result. This interaction is inversely proportional to the distance between the particles so if they are very the results are quite similar to one point particle.

In one of my PDS-1000 experiments I actually got two particles very close so I decided to run the two point particle tracking program and look how the results look.

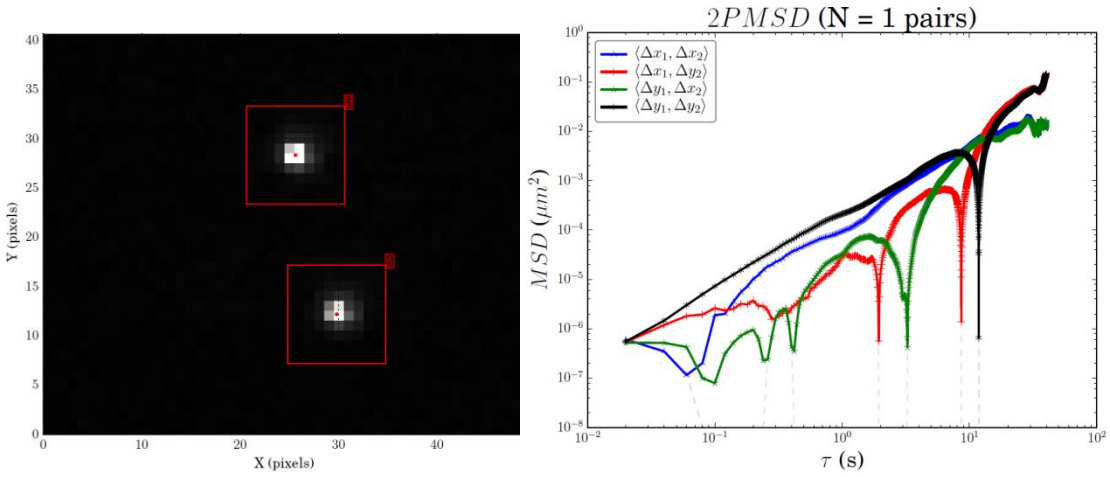



Figure 52: Two point particle tracking example

The MSD shown above plots the four components of the correlation two point tracking matrix in this case only for one pair of particles.

After reading some papers about one and two points particle tracking microrheology I realized it does not have as much precision as thought before. This method works perfetly fine when applied to samples of pure viscous liquids that do not have any forces aplied.

However, when this method is used for microrheology on living cells there are a lot of factors to consider that can modify particle movement such as attachment to ther cytoskeleton and many others.

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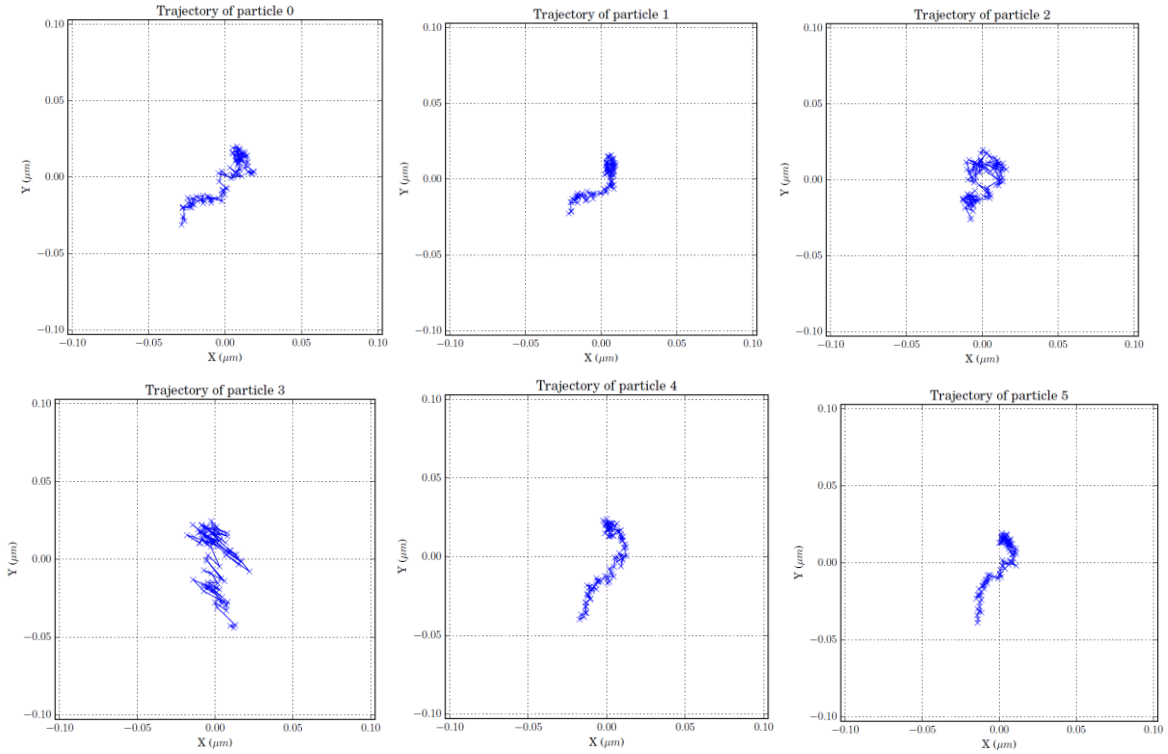
# 15. Gratitude

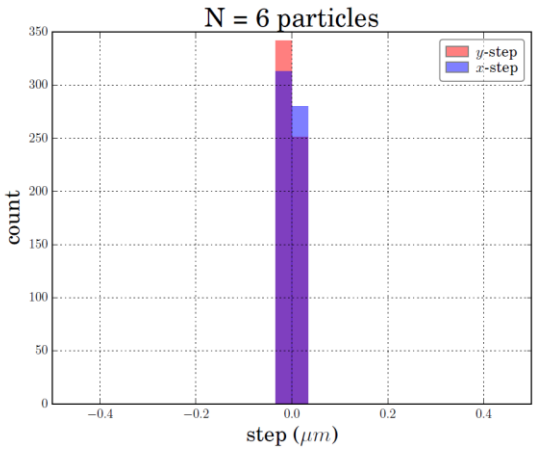
I really appreciate the support of the whole MAE Department in the University of California San Diego for allowing me to work with them during my six month stay. I specially thank my supervisor Professor Juan Carlos del Alamo and all his PhD students for helping me while I was there. I also thank Raul Peña for teaching me how to culture cells when I was doing my previous short stay in the IMIM-PRBB in Barcelona.

# 16. Appendix

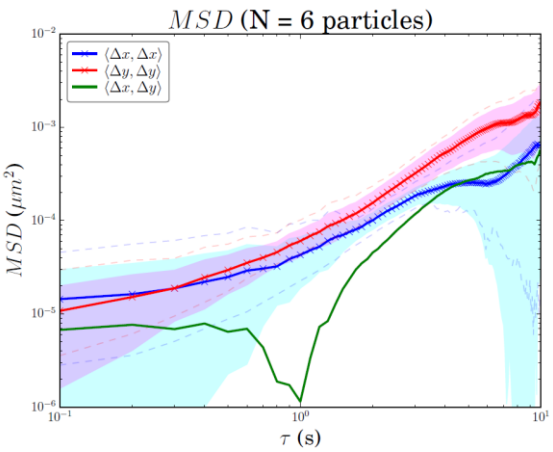
## I. Microinjection results



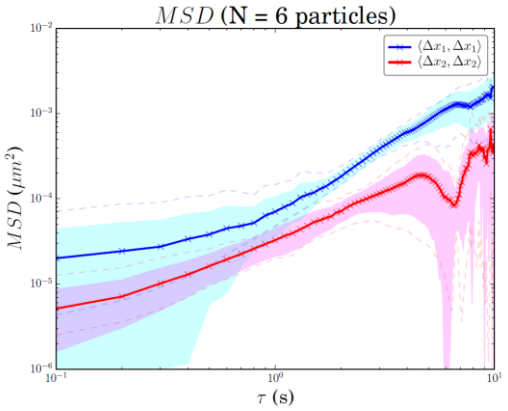

Trajectories



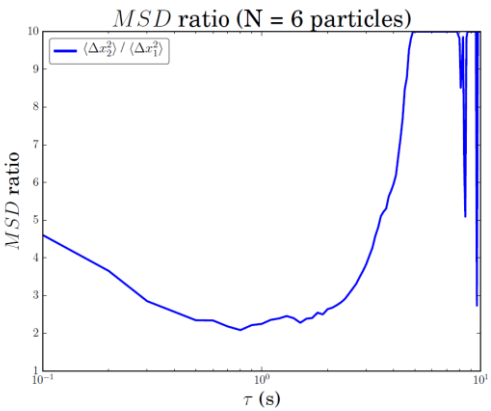

Histogram of steps




MSD 1 particle

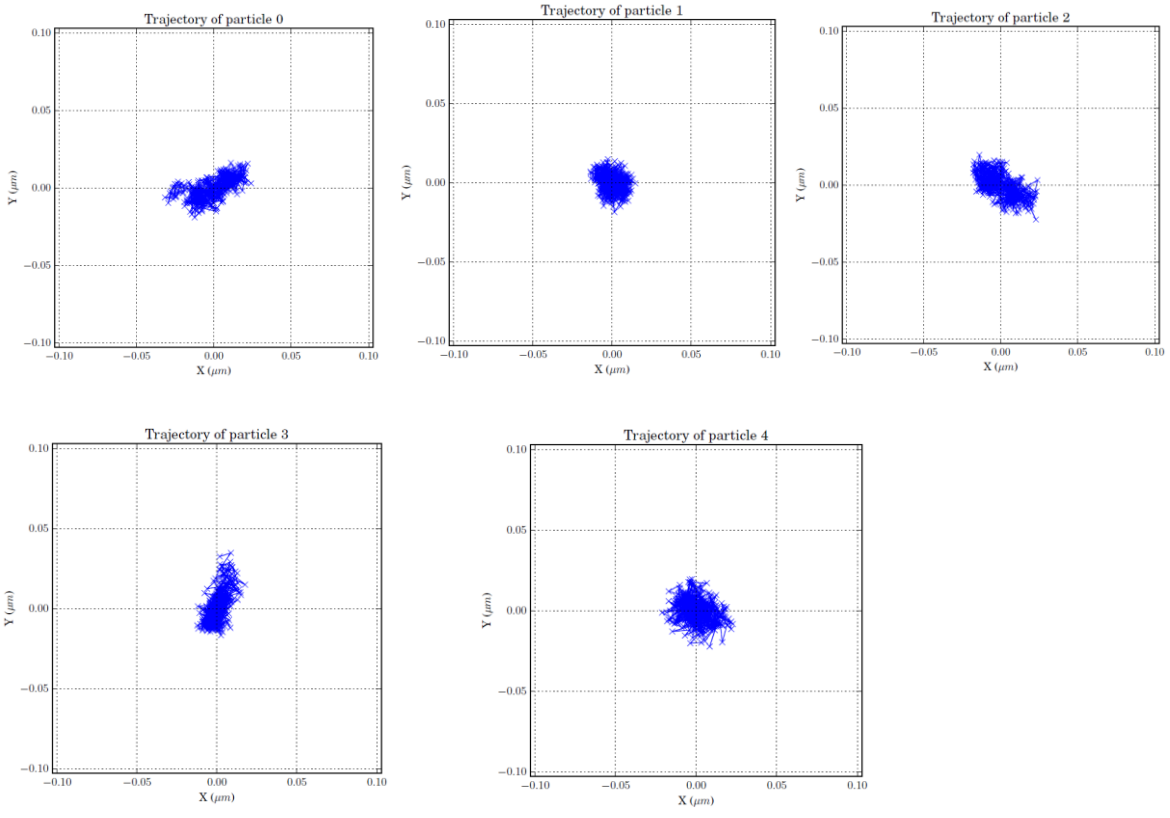



Eigenvalues

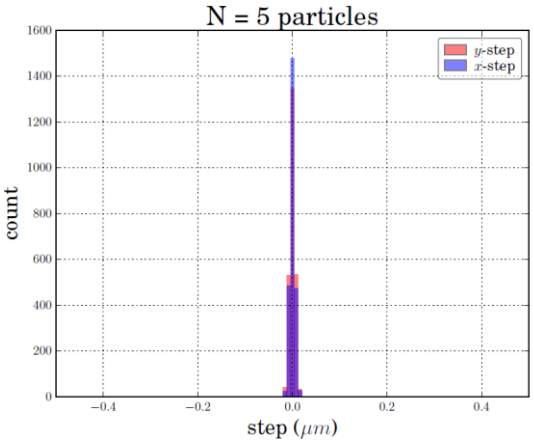



Eigenvalues ratio

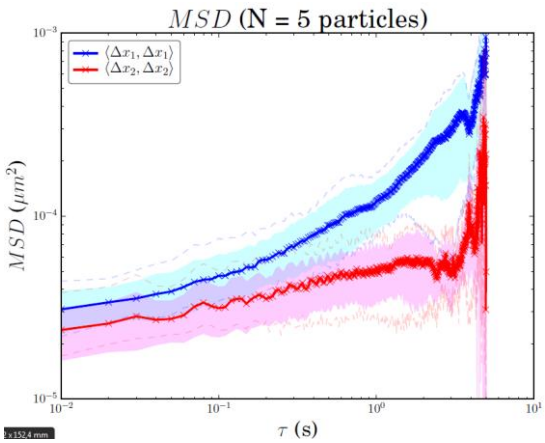
## II. Pinocytosis results




Trajectories of each tracked particle




Histogram of steps

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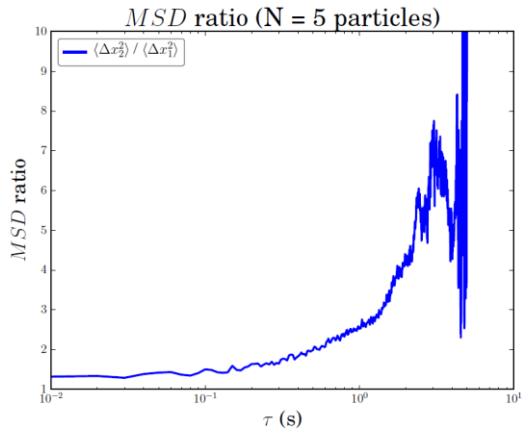
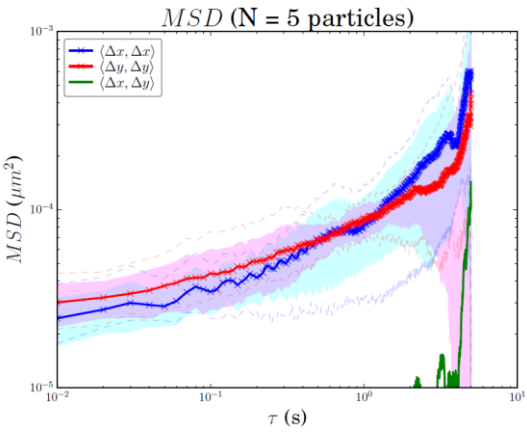


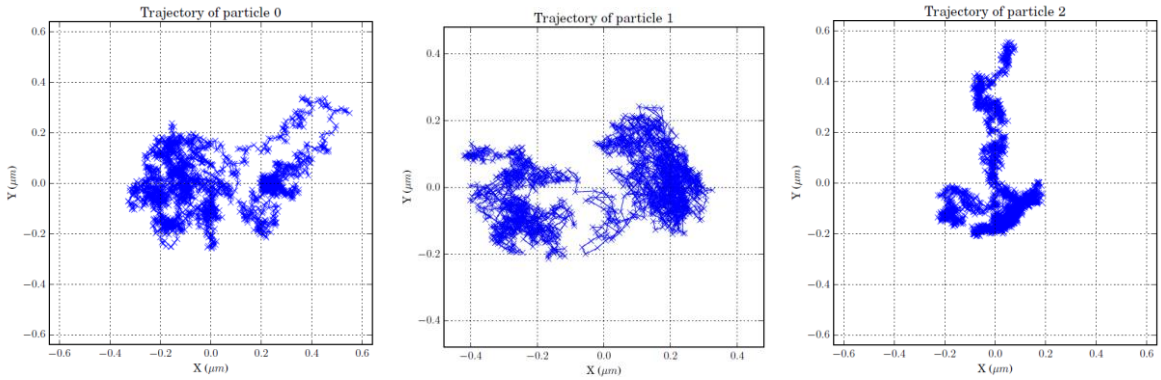

Eigenvalues ratio

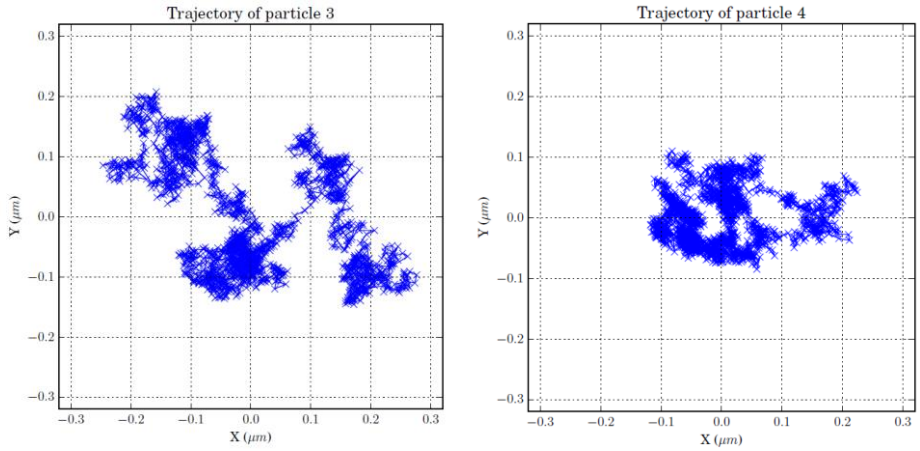



MSD 1 particle

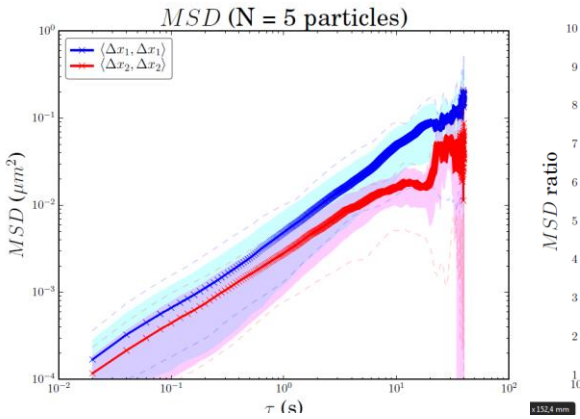
## III. TFG-β

### III.a KO






Particle trajectories

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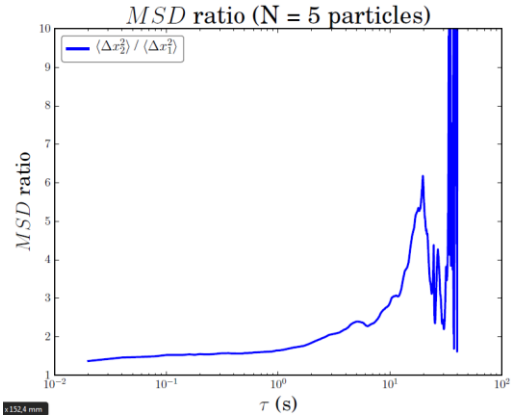
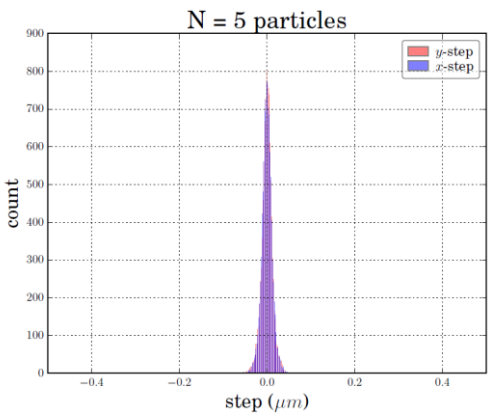
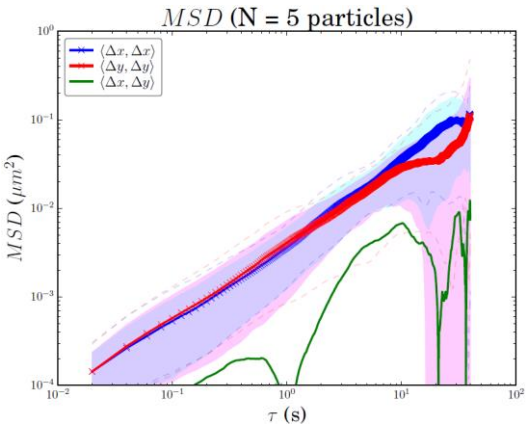



Eigenvalues ratio

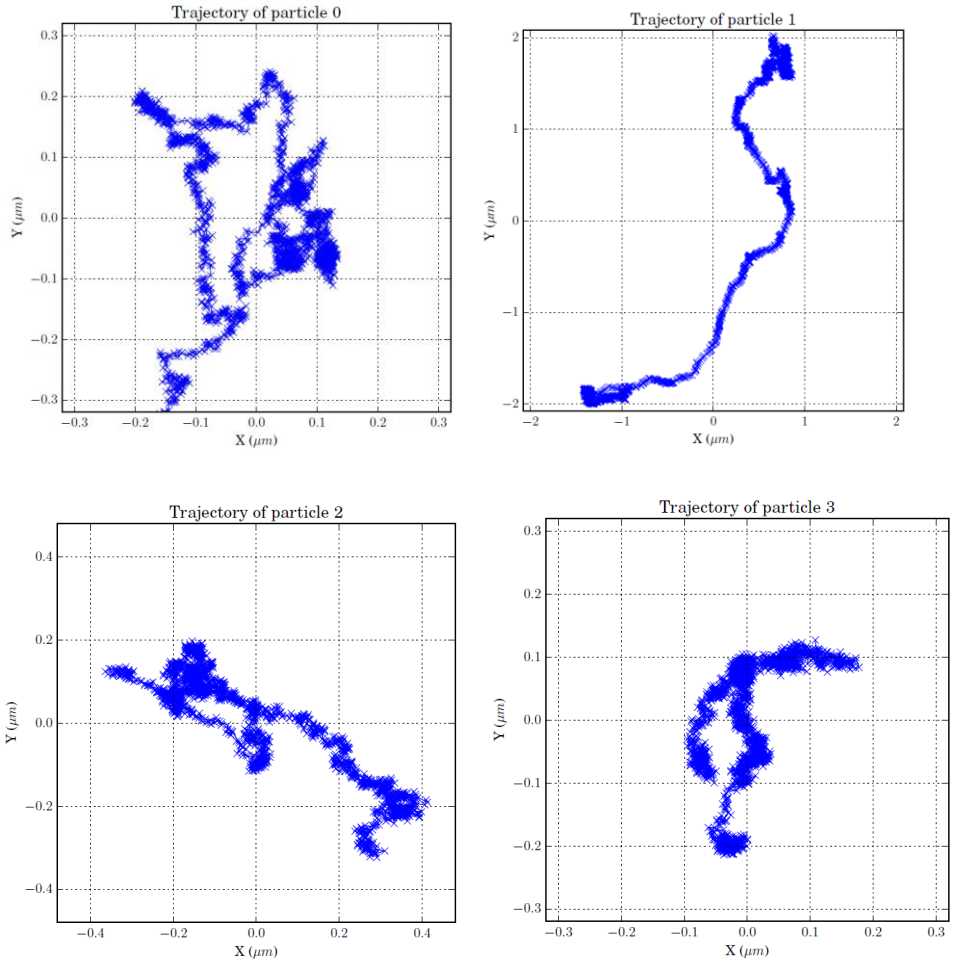



Histogram of steps

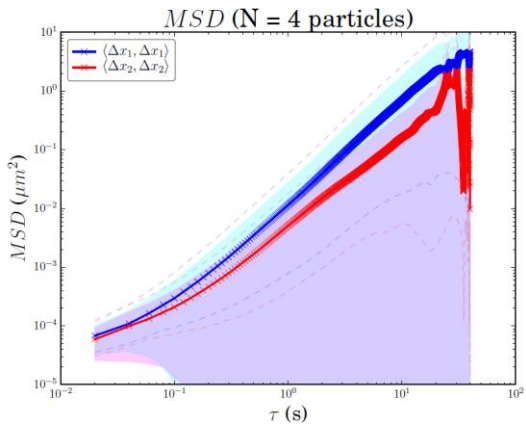



MSD 1 particle

## III.b WT




Particles Trajectories

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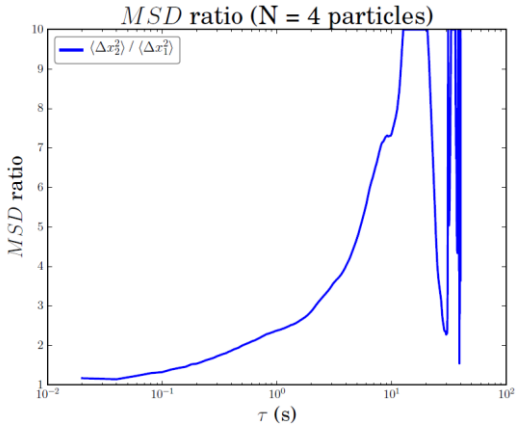
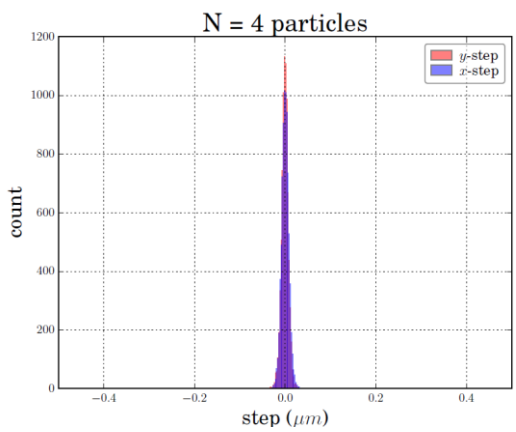
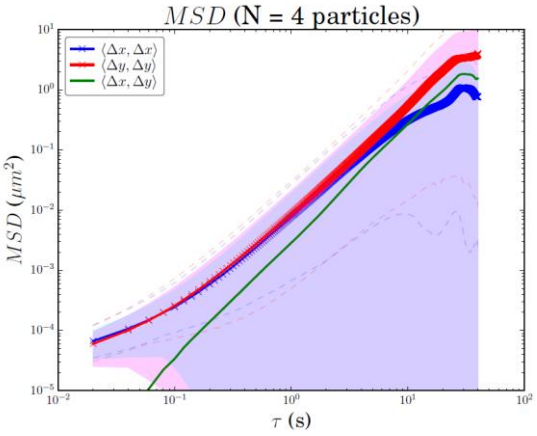



Eigenvalues ratio




Histogram of steps




MSD 1 Particle