

A microscopic investigation of cytoplasmic streaming in the inner epidermis of *Allium cepa*

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Preamble

This paper describes simple but powerful experiments of important life events at a microscopic scale observed with a light microscope. - In Spring of 2012, I presented the following work in the botany section during the Synopsys Silicon Valley Science and Technology Championship [1]. I received very positive feedback and decided to share my findings with a larger audience. I hope my study stimulates others to repeat similar experiments for pleasure or educational purposes. - I want to thank Walter Dioni for his interest and his valuable feedback. I also want to thank my father, Gregor Overney, for supporting me during this project. Last but not least, I owe my thanks to my brother, Normand Overney, for helping me with recording the images and videos through the microscope.

Introduction

The study of cytoplasmic streaming in the inner and outer epidermis of an onion bulb scale (*Allium cepa*) is a fascinating activity of life events. These easily obtainable specimens are most suitable for microscopic investigation.

I studied the dynamic process of cytoplasmic streaming in the inner epidermis as a function of the molarity of a sucrose solution by measuring the speed of microbodies along acting filaments (also referred to as microfilaments). Microbodies are cytoplasmic organelles that comprise degradative enzymes and are specialized as containers for metabolic activity. For all my experiments, I anticipate a direct link between cytoplasmic streaming and the speed of microbodies.

I also investigated whether cytoplasmic streaming is controllable. For my experiments, I used several different sucrose solutions to study the speed of cytoplasmic streaming and the impact sugar has on this dynamic process. I determined the optimal molar concentration at which the speed of cytoplasmic streaming has its maximum.

I prepared five different molar solutions of sucrose, **0.1**, **0.2**, **0.4**, **0.6** and **0.8**. The procedure on preparing an exact molar solution is explained later in this paper.

The speed of mitochondria and other small movable features is measured using a 40x Nikon CFI₆₀ objective and a stopwatch. In the following, I will call all these small movable features (including mitochondria) microbodies. The 40x objective offers a resolution of 0.4 μm , which is sufficient to resolve these tiny structures, which are between 0.5 to 1.0 μm in size. The illumination technique used for microscopic investigation is known as darkfield (or dark-ground) illumination [2].

Background information

Cytoplasmic streaming by motion of microbodies, which is especially common in large plant cells, is a circular flow of cytoplasm within cells that speeds the distribution of materials. This movement aids in the delivery of nutrients and genetic information. In addition, these moving microbodies are responsible for metabolic processes within the cell.

To regulate cytoplasmic streaming, I decided to use various molar solutions of sucrose. Sucrose is an integral part in activating motor proteins, which move the microbodies along the actin filaments. These filaments composed of actin are tracks for the microbodies. I decided to find a direct link between sucrose concentration and the locomotion of microbodies within cells. To better illustrate this process, please see **Figure 1**.

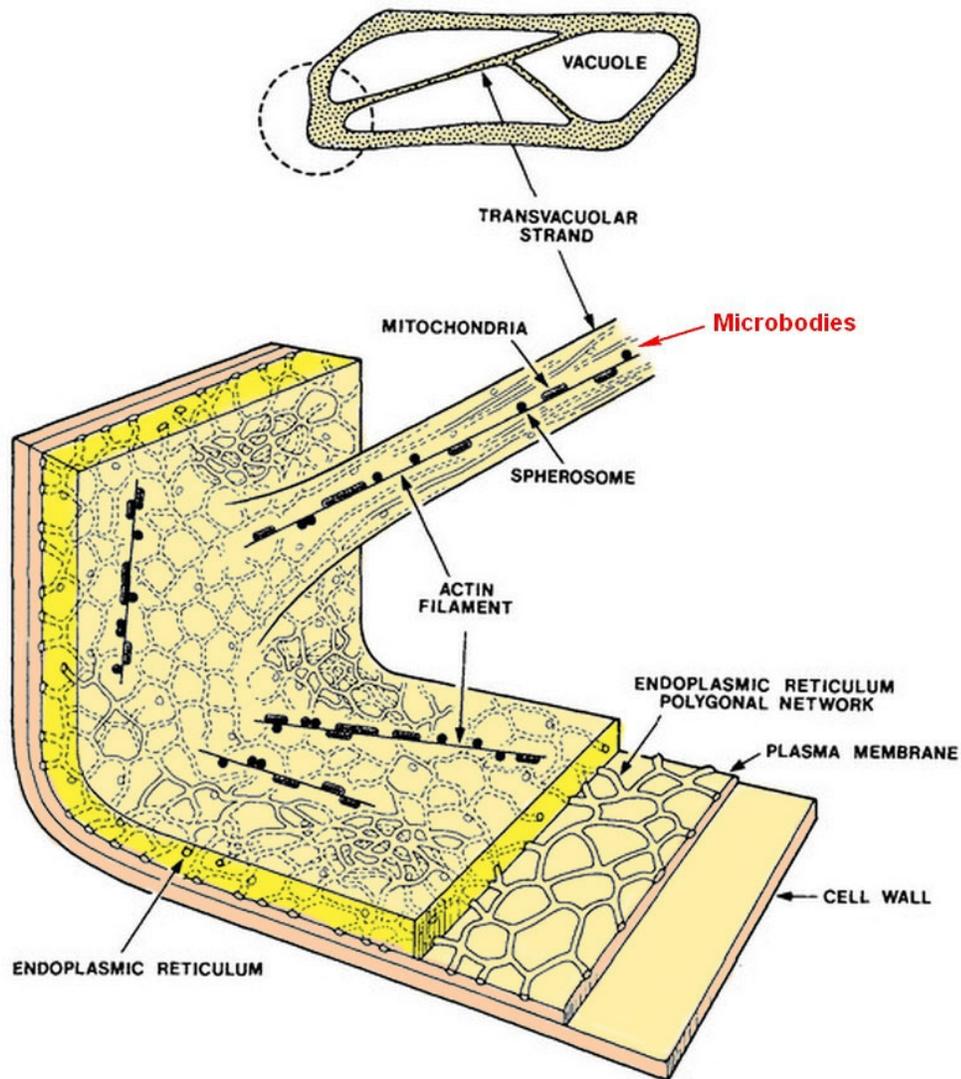


Figure 1: The process of cytoplasmic streaming due to motion of microbodies along actin filaments. Image is a slightly modified version of the one from N. S. Allen and D. T. Brown, *Cell Motility and the Cytoskeleton* 10:153-163 – Wiley-Liss Inc., 1988.

How does a plant cell utilize sugar for its locomotion of microbodies? - To illustrate this rather complex process, please refer to **Figure 2**.

From Sugar Molecules to Cytoplasmic Streaming

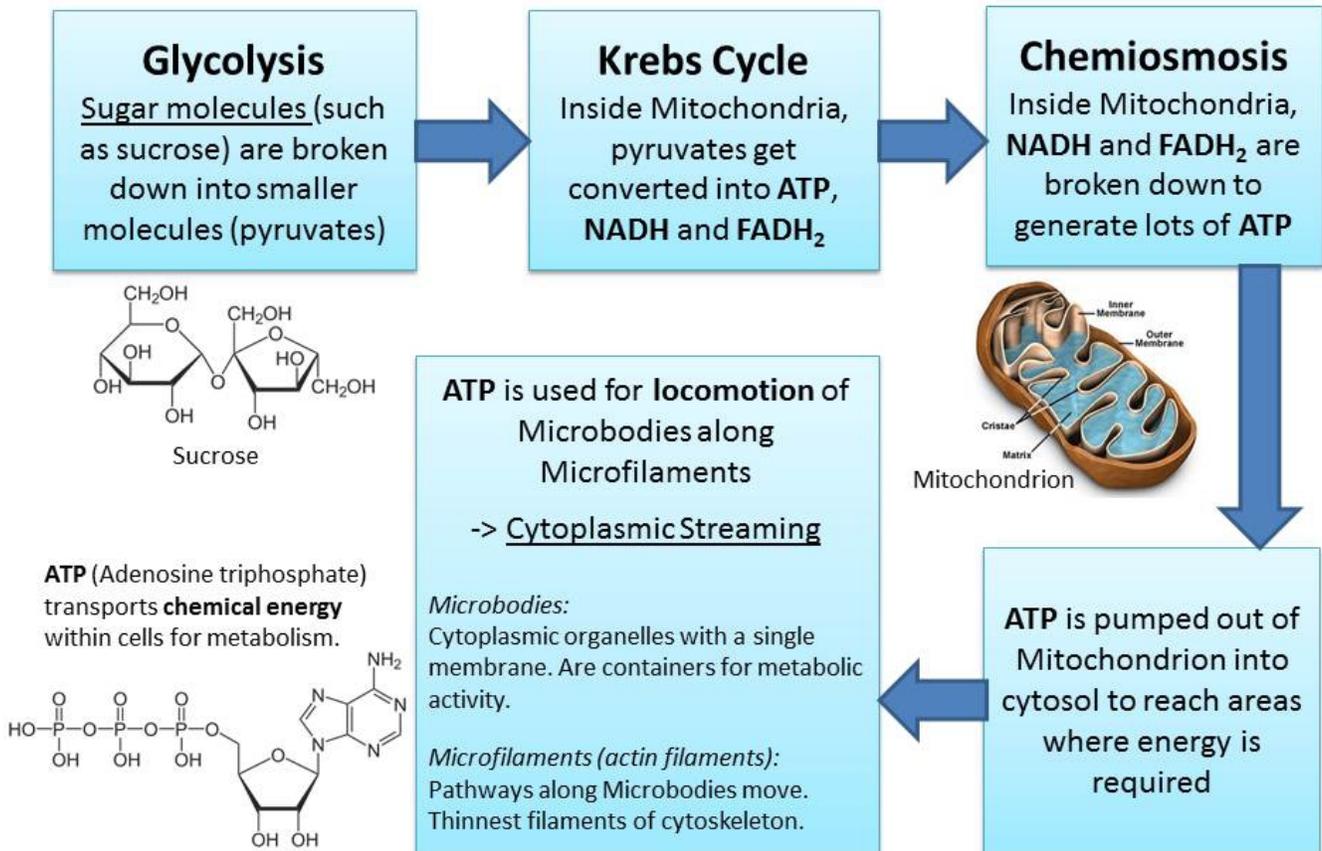


Figure 2: Process of ATP production inside plant cells.

The entire process starts out with breaking down sugar molecules inside the plant cell. This process is known as Glycolysis. Glycolysis does not create a lot of energy. Only when the pyruvates, which are an ionized form of a three-carbon acid called pyruvic acid, move inside a mitochondria to invoke the so called Krebs cycle and a process known as Chemiosmosis, a significant amount of ATP is produced. Pyruvates are taken in to the Krebs cycle to produce 2 ATPs and additional compounds. Chemiosmosis breaks down the NADH and FADH₂ created by the Krebs cycle and finally pumps ATP out of the mitochondria. During Chemiosmosis, around 32 ATPs get produced. ATP serves as a source of energy for many metabolic processes. It releases energy when it is broken down into ADP via a process known as hydrolysis. ATP is vital for many functions inside the plant cells such as locomotion of microbodies along actin filaments.

What got me interested in this project?

During the school year of 2011, I learned about the animal and plant cells including the shape and function of their individual organelles, which are structures inside cells that are responsible to perform particular functions. To further deepen my understanding of microscopic life inside eukaryotic cells, I began to read my brother's high school biology textbook [4]. While I was reading about mitochondria, I was fascinated by the complex process of producing ATP with sucrose. I started to explore the direct link between cytoplasmic streaming and table sugar. These early experiments gradually matured into my scientific explorations on cytoplasmic streaming. In 2012, I presented my work during our local science fair [1].

Hypothesis

Every good science project must start with a hypothesis, which is then scrutinized by conducting suitable experiments. Results are obtained from these experiments to draw certain conclusions. This scientific method (or process) can then restart with a modified and hopefully improved hypothesis (see **Figure 3**).

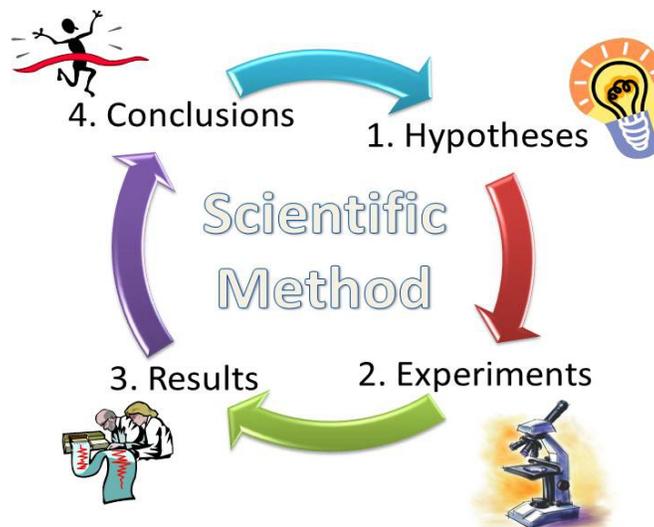


Figure 3: The cycle of the scientific method.

As we learned, nutrients (food) are necessary to provide energy for moving microbodies along microfilaments. I hypothesize that this process known as **cytoplasmic streaming** is influenced by the **sucrose concentration** inside plant cells. – I expect the average speed of microbodies along microfilaments to increase as the sugar concentration rises until plasmolysis starts to occur. Plasmolysis, a process where the cytoplasm pulls away from the cell wall, reduces cytoplasmic streaming activity (see **Figure 4**). Hence, I predict that the activity of cytoplasmic streaming peaks at a certain measurable molarity of sucrose solution.

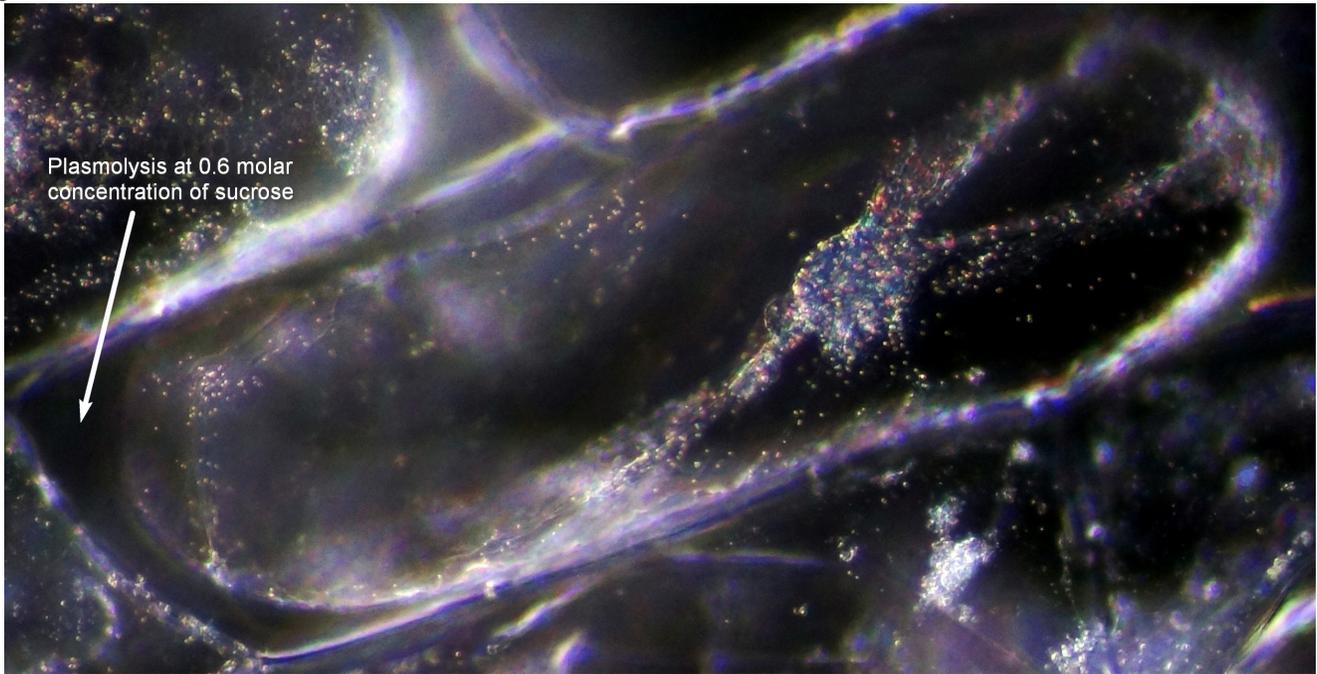


Figure 4: Plasmolysis inside cell of *Allium cepa* observed in darkfield illumination (400x).

Equipment and materials used for these science experiments

1. Red onions (*Allium cepa*) were freshly obtained from local grocery stores (see **Figure 5(a)**).
2. The microscopic setup consisted of a Nikon Eclipse E200 with darkfield slider condenser (see **Figure 5(b)**). Most observations were conducted at 400x total magnification using darkfield illumination.
3. To determine the speed of microbodies, a calibrated ocular reticle and stopwatch were used. Exact calibration of the ocular reticle was done with a stage micrometer.
4. A digital camera was connected to the microscope and used to record images and small videos. For this task, the binocular viewing head was replaced with a trinocular viewing head. Primarily, a Sony NEX-5N digital camera was used.
5. A laptop computer with Windows Excel 2010 and Word 2010 was used.
6. Other equipment required: bottles, flasks, microscope slides, cover glasses, plotting paper, tweezers, pipettes, precision weight scale, and various volumetric flasks (see **Figure 5(c)**).

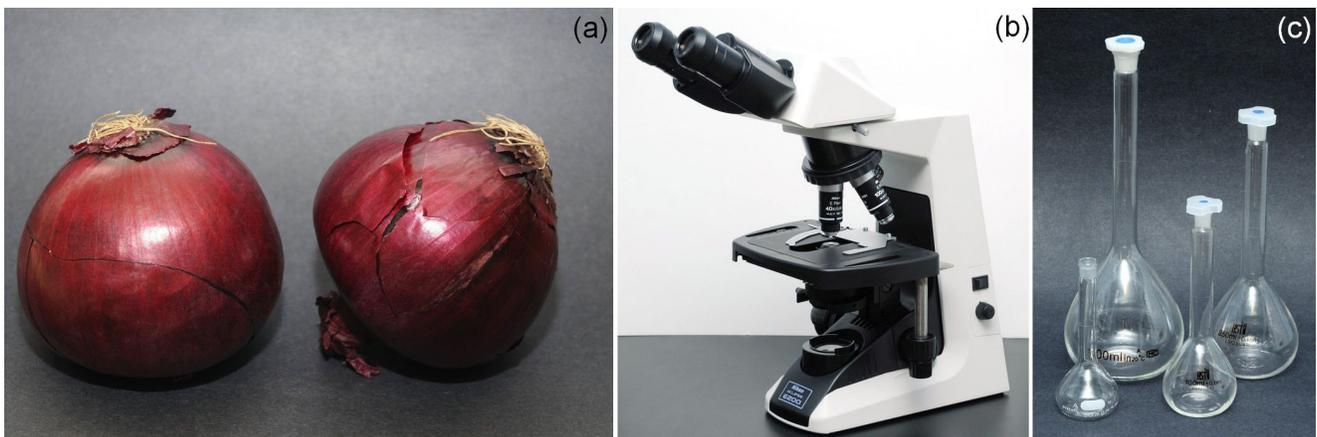


Figure 5: (a) Red onions (*Allium cepa*); (b) Nikon Eclipse E200 microscope; (c) volumetric flasks.

More details about equipment for darkfield illumination

I used the following components to establish darkfield illumination with my Nikon E200 microscope:

1. Sufficient illumination using a 20-Watt halogen bulb.
2. An achromat 40x objective with a numerical aperture (NA) of 0.65.
3. An Abbe condenser with an NA of at least 0.7 (when used without immersion oil). I measured that my Nikon Abbe condenser offers at least an NA of 0.75 when used dry. It's an Abbe 1.25 oil condenser that allows a slider to be moved into the condenser (see **Figure 6**).
4. A darkfield stop (or central opaque 'patch stop') for the condenser mentioned under point "3".



Figure 6 - The Nikon Abbe slider condenser NA 1.25 oil. The darkfield slider is also depicted.

From literature [2], we know that the smallest, resolvable separation d is given by (λ is wavelength of light)

$$d[\mu\text{m}] = 0.61 \lambda[\mu\text{m}] / NA_{\text{Objective}}$$

Using a wavelength of 0.546 μm (green light) and a NA of 0.65, I obtain for the smallest, resolvable distance d , also called resolving power of a microscope, a value of 0.512 μm . With blue light (440 μm), d is just around 0.4 μm . Since the diameter of most microbodies is between 0.5 to 1.0 μm , I could easily observe mitochondria and microbodies with this setup. More information about this powerful and practical illumination can be found at various Websites [2].

The procedure to measure the speed of microbodies along microfilaments

1. Extraction of onion sections for microscopic investigation:
 - From freshly purchased red onions, extract **18** layers of onion skins (three onion samples for each molar solution tested) from the inner epidermis of an onion bulb scale using a pair of tweezers.
 - Take onion samples from the concave side of the onion, because it is easier to peel off [3].
2. Create five different molar solutions using sucrose, distilled water, volumetric flask, and lab scale. The following molar solutions are needed **0.1, 0.2, 0.4, 0.6, and 0.8**. (Procedure explained in more detail below.)
3. Put specimen into small tray containing certain molar solution of sucrose. **Three** thin layers of onion samples are necessary for each of the following molar solutions: **0.0** (distilled water), **0.1, 0.2, 0.4, 0.6** and **0.8**. Distilled water only is used as reference (also known as “blank”).
4. Leave specimens inside the molar solution for at least **20 minutes** to stimulate enough cytoplasmic streaming. (During a cold season, onions tend to be “lazy”.)
5. Creating the microscope slide:
 - Move specimen from tray onto microscope slide. Ensure that upper layer of the inner epidermis is facing up to avoid that specimen rolls up.
 - Using micropipette, add a small drop of sucrose solution of correct molarity to specimen and cover it carefully with very clean cover glass.
6. Put freshly created microscope slide under the microscope. Use darkfield illumination and a 40x objective. If available, other illumination techniques, such as phase contrast or annular illumination, can be used, too.
7. Measuring the speed of microbodies:
 - Measure the speed of cytoplasmic streaming from most active cells in each preparation. Speed is calculated by measuring the time of travel for a given microbody along a known distance. A known distance is determined with calibrated ocular reticle, and time is measured with a stopwatch.
 - Measurement is repeated at least **ten times** for each preparation. This ensures sufficient statistical relevance of data.
 - Hereby, it is important that outliers are not considered (i.e. microbodies that reverse direction should not be measured.)
8. Consequently, at least **30** total measurements are done for every molar concentration of sucrose solution (including distilled water).
9. Average speeds are calculated for each molar concentration in $\mu\text{m}/\text{sec}$ and plotted as a function of molarity of sucrose solution.

Creating a molar solution

During the experiment, molar solutions are required to stimulate cytoplasmic streaming. I used **0.1, 0.2, 0.4, 0.6** and **0.8** molar concentration of sucrose. Below is a short instruction on how to prepare these molar solutions.

To create a molar solution, you multiply the desired amount of liters by the desired molarity and the formula weight.

The best thing is to look at an example. Let us assume that a chemical has a formula weight (FW) of 180 grams/mole. (One mole corresponds to a value of $6.02214179(30) \times 10^{23}$ elementary entities.). Let us further assume that we need 25 ml of 0.15 M (M = moles/liter) solution. How many grams of the chemical must be dissolved in 25 ml water to make this solution? The equation is

$$\text{number of grams} = \text{desired volume (in liter)} * \text{desired molarity (in mole/liter)} * \text{FW (grams/mole)}$$

Therefore, we get for the amount in grams = 0.025 liter * 0.15 mole/liter * 180 grams/mole = 0.675 grams. This is the amount, we need to dissolve in 25 ml water.

First, we decide the total volume of solution we want to prepare and select the appropriate volumetric flask (e.g. 100 ml). For sucrose, the formula weight is 342.3 grams/mol. After we calculated the amount of grams necessary for the molar solution, we put the correct amount of sucrose into a Petri dish (cell culture dish) and place it on a scale. We need to cancel out the weight of the Petri dish (calibrate scale) in order to exactly determine the weight of the sugar. After measuring the correct amount of sucrose, we need to dissolve it in water of the desired volume (e.g. 100 ml). This can be accomplished by filling a beaker with sufficiently less water than the total volume of the final solution in order to compensate for the volume of the added sugar. A heating stage is recommended to ensure that all the sugar dissolves. Now we pour the solution into a volumetric flask and then fill the rest of the volumetric flask with distilled water all the way to the fill level (e.g. up to the 100 ml mark). After shaking the freshly created solution, we pour the molar solution into a sterilized bottle. (Of course, we have to make sure that the Petri dish, the beaker and the volumetric flask were steam sterilized before mixing the solution.)

Using this procedure, I created **0.1, 0.2, 0.4, 0.6** and **0.8** molar solution of sucrose.

Calibration of the ocular reticle

In order to calibrate the ocular reticle, I used a calibrated stage micrometer. With my microscope, I focus on the stage micrometer and measure the distance between ten ticks of the ocular reticle. This process is repeated several times. I obtained the following data:

Number of ticks	Measured distance in μm using stage micrometer at 400x total magnification
10	25 μm
10	26 μm
10	24 μm
10	25 μm

From the above data, I estimate my error in distance measurement to be around $\pm 1 \mu\text{m}$. For all following experiments, I convert 10 ticks of the ocular reticle into a distance of 25 μm . This is important for calculating the speed (velocity) in $\mu\text{m}/\text{sec}$. Of course, this is only valid for a 40x objective and this particular ocular reticle.

Observations during experimentation

The following are the observations made during my measurements of the speed of microbodies in cells of *Allium cepa*.

1. In distilled water (my so-called “blank”), I was not able to record any significant, directional motion of microbodies along active filaments. The only detected motion was purely random and is known as Brownian motion. I conducted these experiments with onions that may have been exposed to lower temperatures before I purchased them from the local grocery store. However, the absence of cytoplasmic streaming at a **0.0** molar solution of sucrose provided me with a good baseline to study the impact of sucrose on cellular activity.
2. At **0.1**, **0.2**, **0.4**, **0.6**, and **0.8** molar solutions of sucrose, motion of microbodies was consistently detected. Plasmolysis was systematically observed at a **0.8** molar solution. (Plasmolysis is the contraction of the protoplast as a result of loss of water from the cell). Plasmolysis starts to occur at around **0.6** molar solution (see **Figure 10** and **11**). When plasmolysis occurs, the time it takes the microbodies to travel 25 μm increases substantially. Based on these observations, I conclude that the sweet spot for cytoplasmic streaming is between **0.1** and **0.8** molar solution.
3. At a **0.4** molar solution of sucrose, I measured noticeably the fastest average speeds from all five molar solutions (see **Figure 7**). All experiments were repeated three times with different red onions.

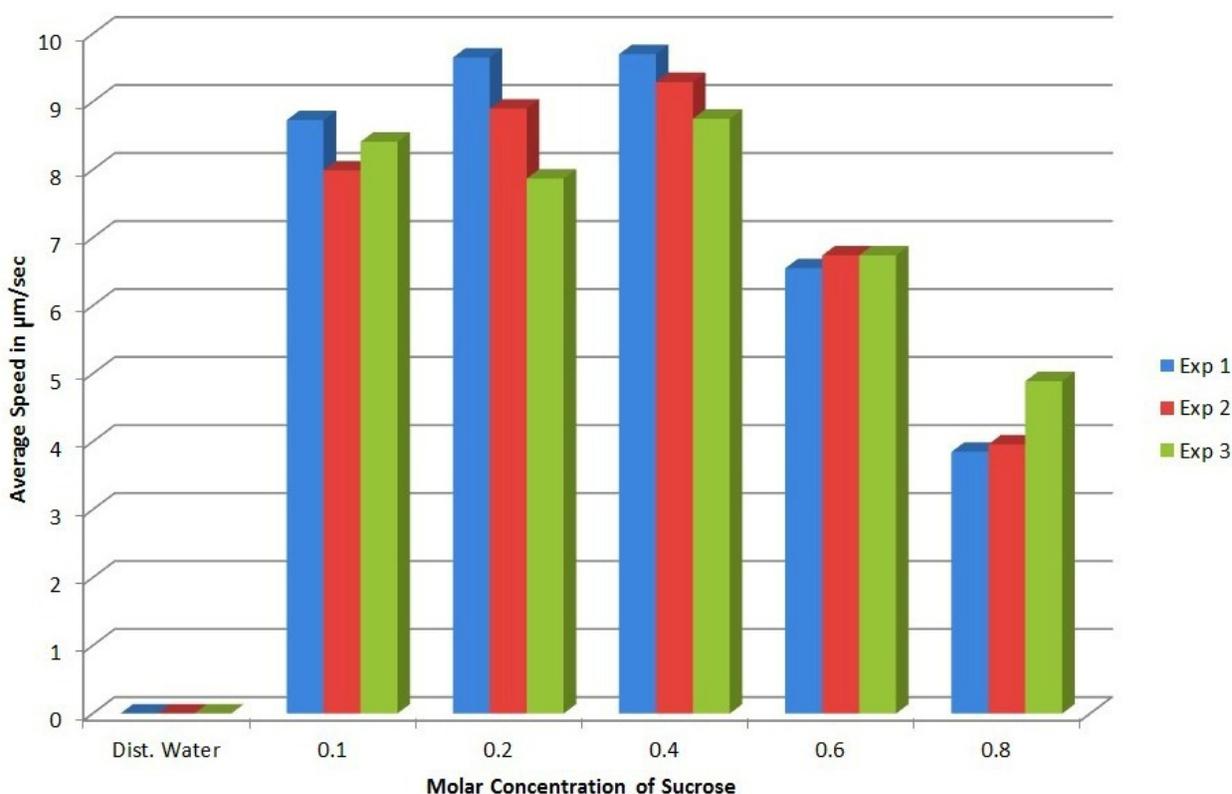


Figure 7: Average speed in $\mu\text{m}/\text{sec}$ for different molar concentration of sucrose. The experiment was repeated three times to reduce the impact of systematic errors.

Histogram analysis of experimental data

For my a comprehensive data analysis, I used Microsoft Excel 2010 to create graphs and histograms. The histogram analysis is necessary to ensure that my data has statistical relevance.

In order to plot histograms, I normalized all the measured speeds by introducing a **speed-index**, which is defined as the ratio between measured speed divided by the average-speed for a given sequence of measurements ($\text{speed-index} = \text{speed} / \text{average-speed}$). Using speed-indexes allows to combine multiple data sets for a given molarity that were obtained with different onions. This takes care of so-called “onion-to-onion” variations. Then I determined the smallest and the biggest speed-indexes. The number of bins (meaning the number of columns for the histogram) is preselected to match the data set. Now, I only had to count how many microbodies have a speed-index within the boundaries of a given bin. – All the results are plotted in **Figure 8(a) to 8(e)**. **Figure 8(f)** is used to analyze the speed profile of each histogram, and its purpose will be explained later.

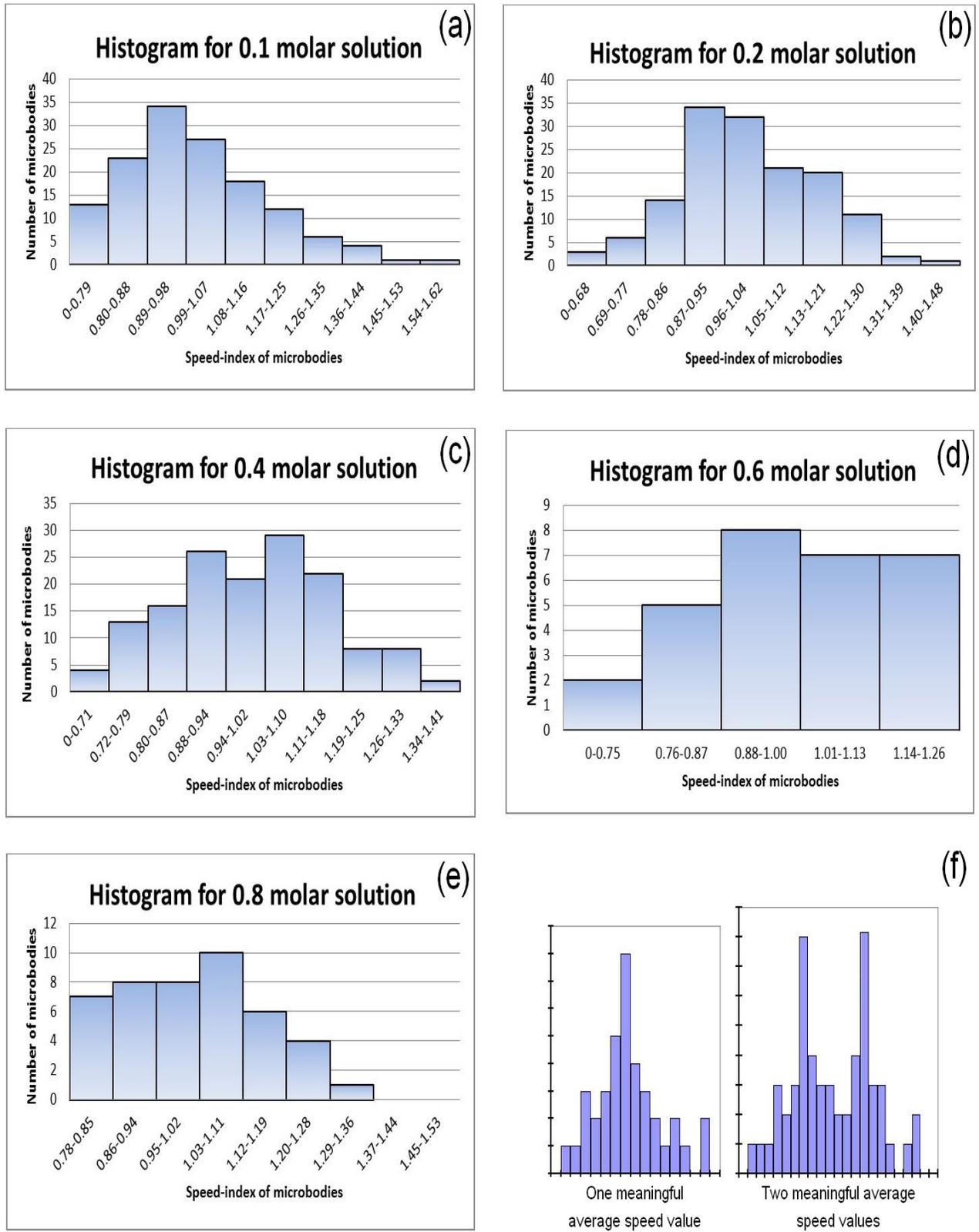


Figure 8: (a) to (e) shows experimentally measured histograms at different molar concentration of sucrose. Number of microbodies is plotted as a function of their average speed-index (see (a) to (e)). (f) shows two theoretical cases where either one or two meaningful average speed values are observed.

All the histograms have a climax around a speed-index of **1.0** (x-axis). Within tolerances, each histogram has only one distinct maximum. This is evident of a speed distribution caused by microbodies traveling at around one average speed. Consequently, I determined that the measured average time is indeed a statistically relevant quantity to determine the speed of cytoplasmic streaming.

The purpose of creating these histograms was to verify whether my data is free from effects that could invalidate the relevance of determining just one single speed average per data set. To clarify this fact, let us model the process of moving microbodies along actin filaments (microfilaments) as motor vehicles driving on roads. In this model, the actin filaments (the path microbodies travel during cytoplasmic streaming) are the roads, and the microbodies are the motor vehicles. Let us now imagine that we have two types of motor vehicles, passenger cars and trucks. With two enforced speed limits (e.g. 55 and 65 mph), one for cars and one for trucks, most drivers will now operate their vehicle at a speed close to their respective speed limit. In this case, we will measure two distinct average speeds (see **Figure 8(f)** as illustration). If only one speed limit is enforced (e.g. 60 mph), we will immediately measure only one distinct average speed. – My experiments show that the rate of cytoplasmic streaming is independent on the type of microbody moving along the microfilaments.

Results

With **0.4** molar solution of sucrose, cytoplasmic streaming shows peak activity (highest average speed). Cytoplasmic streaming is clearly evident from **0.1** to **0.8** molar solution. Statistical relevance of taking one single average speed to quantify activity of cytoplasmic streaming has been proven by histogram analysis over many data sets (see section “Histogram analysis of experimental data”). Plasmolysis starts to appear at around **0.6** to **0.8** molar solutions. Plasmolysis strongly impacts activity of cytoplasmic streaming. The effect of plasmolysis is reversible by adding distilled water to the onion specimen. Measurable activity of cytoplasmic streaming has not been observed without adding sucrose. With distilled water, only Brownian motion was seen. The onions obtained from supermarkets in winter were rather inactive and adding sucrose was necessary to trigger cytoplasmic streaming. For **0.0** (distilled water), **0.1**, **0.2**, **0.4**, **0.6**, and **0.8** molar solution of sucrose, I plotted average speeds for three consecutive measurements to show peak average velocity at around **0.4** molar solution (see **Figure 7**).

Conclusions

My hypothesis that cytoplasmic activity peaks at a certain molarity of sucrose solution has been confirmed. Plasmolysis has a detrimental effect on dynamic activity of cytoplasmic streaming suggesting a reduction of ATP production. (ATP is used to power the motion of microbodies along microfilaments.) More experiments need to be conducted in order to better understand the connection between various mechanisms controlling cytoplasmic streaming (speed of microbodies) and the availability of nutrients to the cell (molarity of sucrose solution). My experiments clearly show that there is a relationship between sucrose concentration and cellular activity that promotes cytoplasmic streaming. Last but not least, I conclude that cytoplasmic streaming is independent on the type of microbody moving along actin filaments.

Personal comments

I was amazed when observing these life events inside a living cell using a light microscope. With rather simple means of darkfield illumination, I was able to conduct an interesting experiment that allowed me to find a relationship between provided nutrients and individual dynamic processes inside a living cell. I hope that my work will stimulate others to conduct similar experiments in order to enjoy these fantastic processes in real-time, which happen in every living plant and animal cell all the time. Although many available photomicrographs and videos very nicely demonstrate these life events, they are by no means a replacement of seeing them with our own eyes through the microscope.

For a short video of cytoplasmic streaming, please visit my YouTube section at

<http://www.youtube.com/watch?v=6Inf7VUcyiA&feature=youtu.be>.

References

- [1] More information about the Synopsys Silicon Valley Science and Technology Championship can be found at <http://www.science-fair.org/>.
- [2] Darkfield illumination is explained at <http://www.olympusmicro.com/primer/techniques/darkfield.html> and in the article "Why I Like Darkfield Illumination" Micscape Magazine **101** (2004) at <http://www.microscopy-uk.org.uk/mag/artmar04/godarkfield.html>.
- [3] Walter Dioni, "How many onion skins are there?" Micscape Magazine, England, February 2011 at www.microscopy-uk.org.uk/mag/artfeb11/wd-onion1.html.
- [4] N.A Campbell, "Biology", pg.112-118, 153-165, 5th edition, Pearson Benjamin Cummings, San Francisco, 2008. - This book contains lots of information on glycolysis, Krebs cycle, and Chemiosmosis. It is a good textbook about Biology. More information is available at <http://www.pearsonhighered.com/campbell/>.

Appendix A: Photomicrographs of microbodies inside *Allium cepa*

The following photomicrographs were recorded with a Sony NEX-5N digital camera attached to a trinocular viewing head of a Nikon E200 microscope. The objective magnification is 40x and the total magnification is around 400x (see **Figure 9, 10 and 11**).

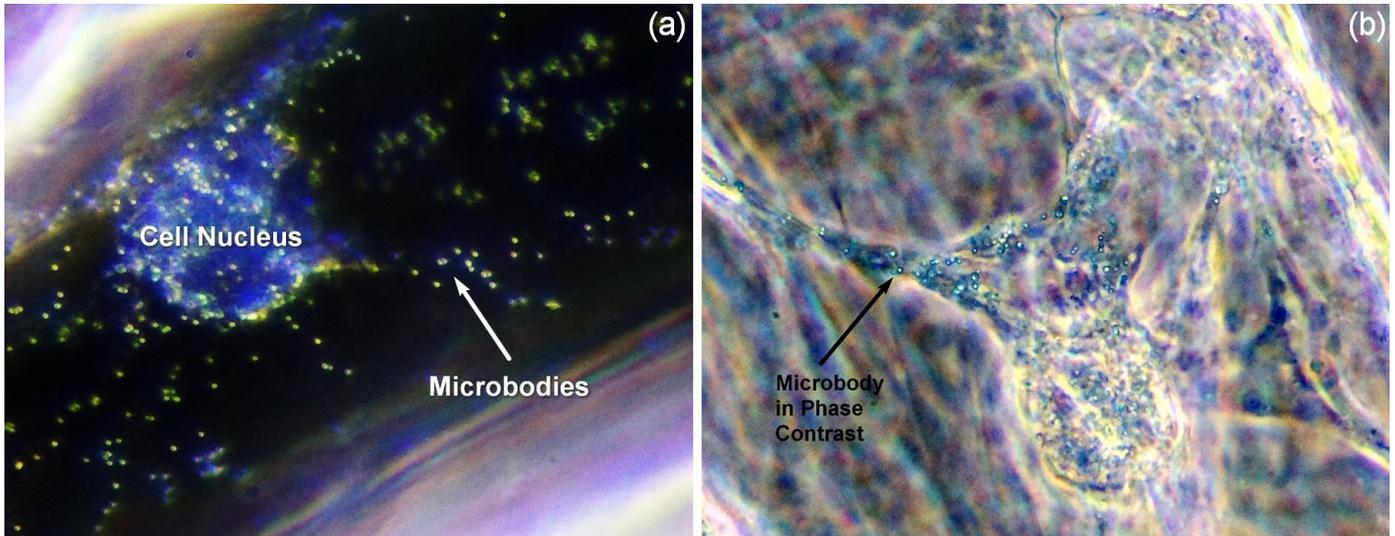


Figure 9: (a) showing microbodies in darkfield illumination. (b) shows microbodies in phase contrast. The total magnification is 400x. In both cases, the molar concentration of sucrose is 0.6.

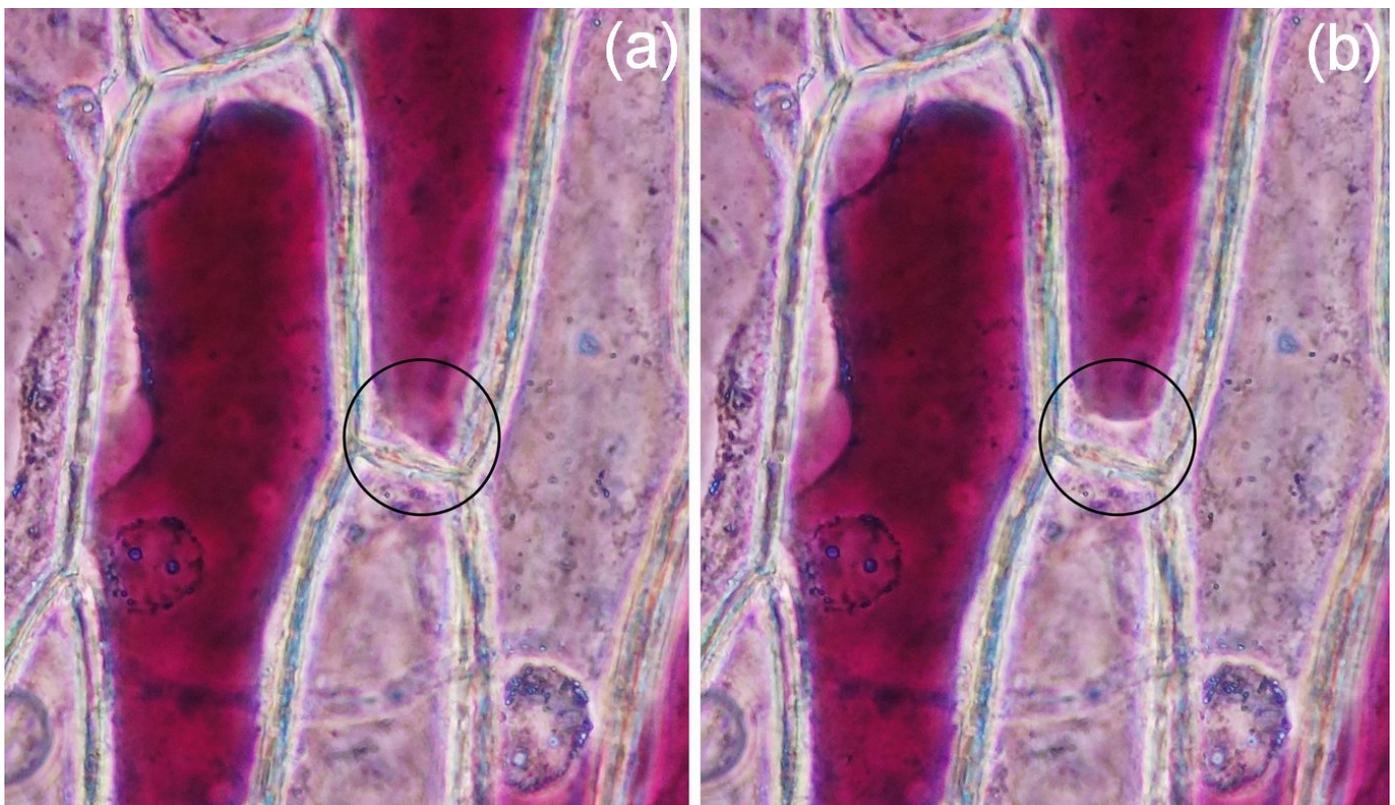


Figure 10: The dynamic process of plasmolysis shown inside onion cells (*Allium cepa*). (a) shows onion cells after 5 minutes exposure to 0.6 molar concentration of sucrose. (b) shows the same location 35 seconds later (see location indicated by circle).

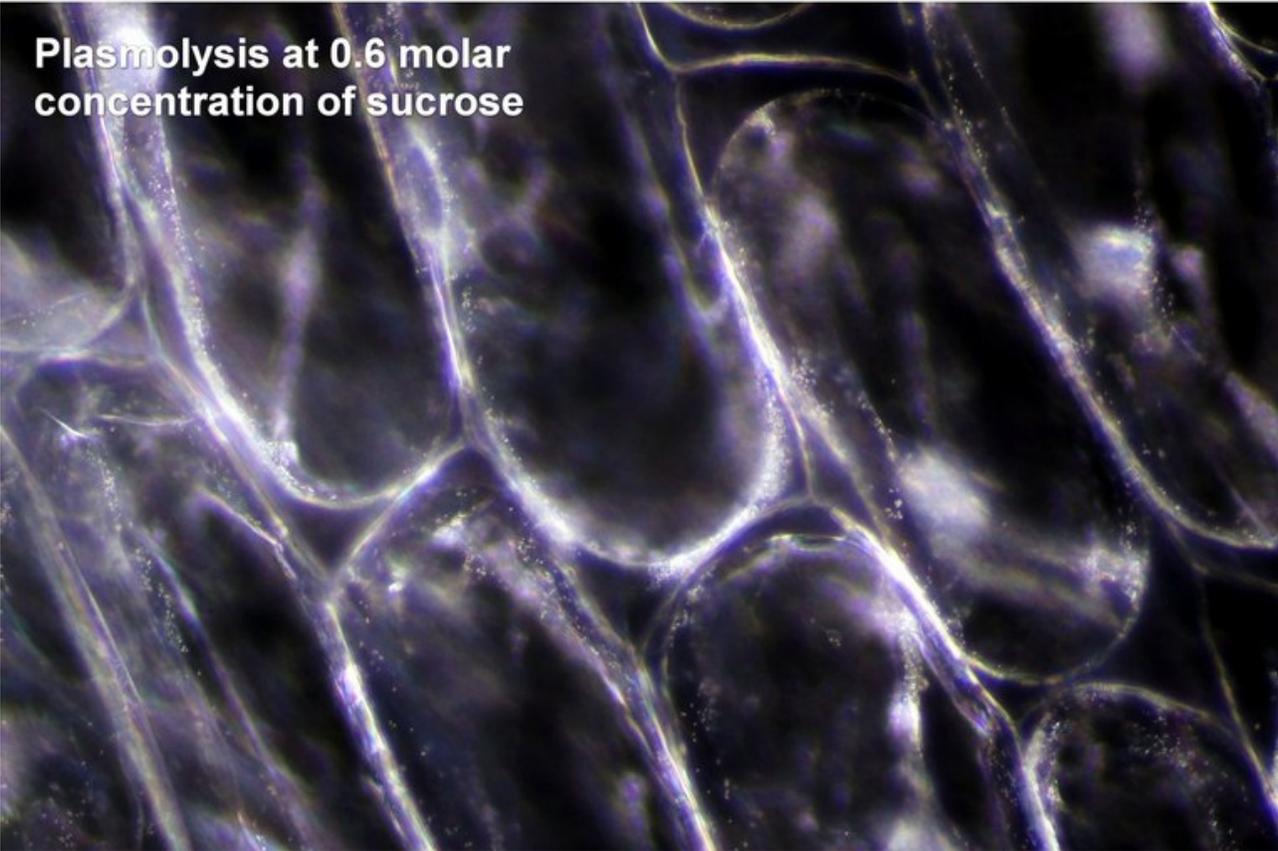
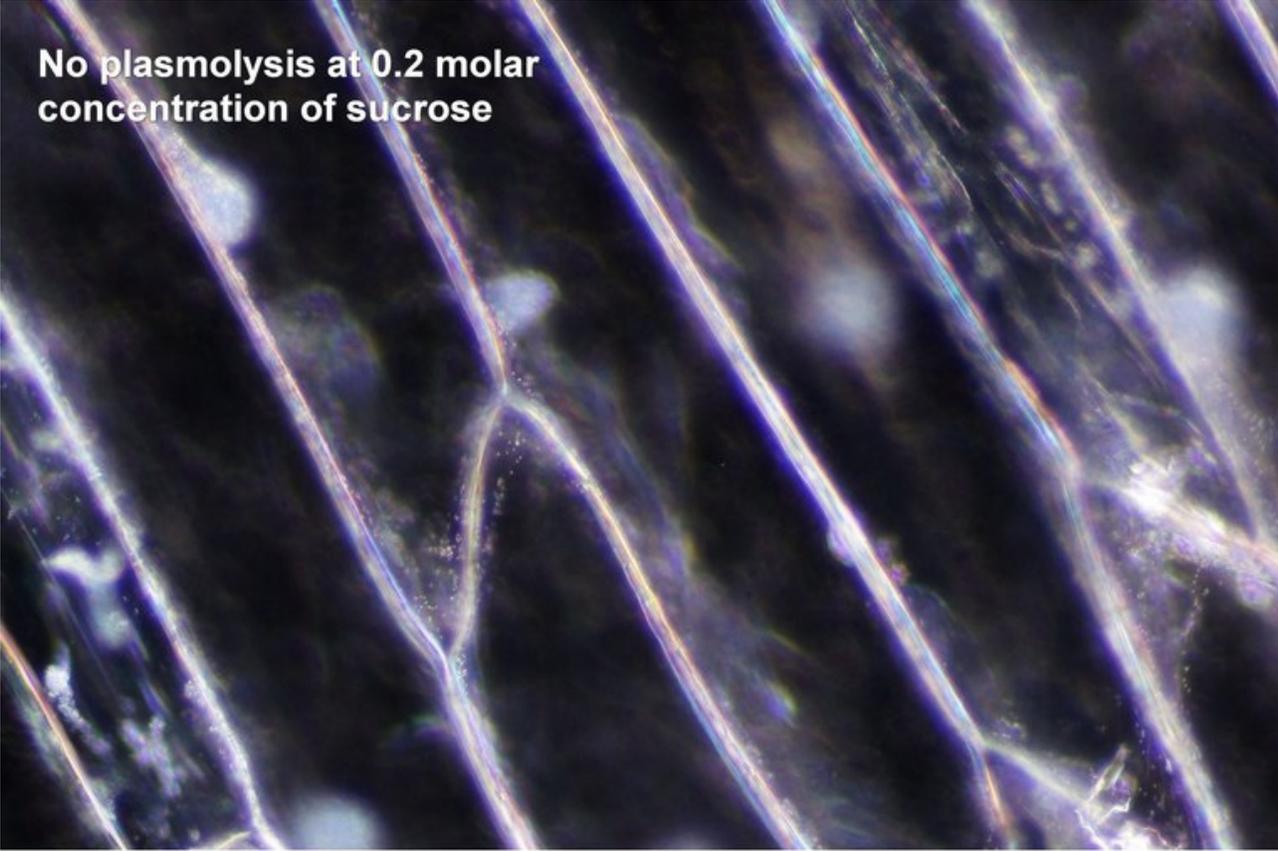


Figure 11: The effect of sucrose concentration on the occurrence of plasmolysis inside onion cell (*Allium cepa*).