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THESIS TO OBTAIN THE MASTER OF SCIENCE DEGREE IN

BIOMEDICAL TECHNOLOGIES

Protocol optimization for the ultrastructural preservation of cilia in *Drosophila melanogaster's* antenna

Candidate

Susana Patrícia Oliveira Mendonça

Supervisors

Dr. Raúl Daniel Lavado Carneiro Martins

(Professor of the Bioengineering Department at Instituto Superior Técnico)

Dra. Erin Marie Tranfield

(Head of the Electron Microscopy Facility in Instituto Gulbenkian de Ciência)

Examination Committee

Chairperson: Prof. Maria Margarida Fonseca Rodrigues Diogo

Supervisor: Dra. Erin Marie Tranfield

Members of the Committee: Dra. Maria João Lopes Gonçalves de Brito Amorim

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Glossary

% – Percentage

ºC – Degrees *Celsius*

ChF – Chemical fixation

CrF – Cryo fixation

Dm – Drosophila melanogaster

EM – Electron Microscopy

FM – Formaldehyde

FS – Freeze substitution

Glut – Glutaraldehyde

HPF – High pressure freezing

MT – Microtubules

IPE – Individual Protection Equipment

LC – Lead citrate

OsO_4 – Osmium tetroxide

PBS – Phosphate Buffered Saline

PHEM – Pipes – HEPES- EGTA – $MgCl_2$

RT – Room Temperature

TEM – Transmission Electron Microscope

UA – Uranyl Acetate

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Abstract

Cilia are microtubule-based organelles involved in a variety of processes, such as sensing, motility and cellular architecture-organizing functions. Moreover, they are altered in several human conditions called ciliopathies and are involved in cancer.

To understand these pathologies, a detailed knowledge of the biology of cilia is required. These organelles are remarkably well conserved throughout eukaryotic evolution and have been well studied in *Drosophila melanogaster (Dm)*.

Dm is an advantageous model organism to study several biological and physiological properties since they are conserved between the fly and mammals, and nearly 75% of human disease-causing genes are believed to have a functional homolog in the fly. Other advantages are the availability of powerful genetics tools, highly conserved disease pathways, very low comparative costs, rapid life cycle and no ethical problems.

Despite these advantages, there is still a gap in the application of transmission electron microscopy (TEM) to study the ultrastructure of *Dm*. One of the reasons is the difficult-to-process chitin exoskeleton that surrounds the fly body, in particular the antenna, an organ that contains ciliated neurons. Also, basic sample preparation procedures for resin embedding of biological specimens have not evolved much since the 1960's and newer methods such as cryo techniques have not been used to study this organ.

Therefore, the goal of this project is to develop an optimized TEM protocol, both for chemical and cryo processing in the antenna of *Dm*, which can help to provide the necessary leap to expand the utilization of this model in research.

KEY-WORDS: Ultrastructure, protocol optimization, cilia, *Drosophila melanogaster*, chemical fixation, cryo fixation.

Resumo

Cílios são organelos constituídos por microtúbulos e são necessários para várias funções da célula como mover-se, detectar estímulos e organizar a arquitectura celular. Alterações nestes organelos podem levar ao surgimento de um conjunto de doenças humanas chamadas ciliopatias e ao desenvolvimento de cancro.

Para compreender estas patologias é necessário um conhecimento detalhado da biologia dos cílios que se encontram conservados ao longo da evolução em organismos eucariontes, como a *Drosophila melanogaster (Dm)*.

A Dm é utilizada no estudo destes organelos pois é um modelo organismo vantajoso para o estudo de várias propriedades biológicas e fisiológicas. Estas encontram-se conservadas entre a mosca e os mamíferos e cerca de 75% dos genes humanos causadores de doenças têm homologia na mosca. Este modelo também dispõe de ferramentas de engenharia genética, baixo custo comparativo, rápido ciclo de vida e ausência de problemas éticos.

Contudo, existe ainda uma falha na aplicação de microscopia electrónica de transmissão (MET) no estudo da ultra-estrutura da *Dm*. Uma das razões é exosqueleto rígido de quitina que envolve a mosca, em particular a antena, um órgão que contém neurónios ciliados. Ao mesmo tempo, os protocolos de preparação de amostras biológicas não evoluíram muito desde a década de 60 e ainda não foram utilizadas novas técnicas de crio-processamento para o estudo deste órgão.

Consequentemente, este projecto tem como objectivo obter um protocolo optimizado para MET tanto para o processamento químico como para o crio-processamento da antena da *Dm*, o que poderá ampliar a utilização deste modelo em investigação.

KEY-WORDS: Ultra-estrutura, optimização de protocolo, cílios, *Drosophila melanogaster*, fixação química, criofixação.

1. Introduction

1.1 Motivation

Most methods for processing tissue and analyzing its ultrastructure have not progressed much since their first development in the 1960s. However, since 1960 there has been an increase in tools that can be used to study different model organisms, such as high pressure freezing. One very important model organism currently used in research is *Drosophila melanogaster (Dm)*. There is therefore a need for the electron microscopy protocols to be developed so these tools can be used as a complementary or standalone tool in research.

Currently working in a laboratory that focus its research on cilia and centrioles, where *D*m is used as a model organism and working as an electron microscopy (EM) technician, I have seen firsthand the need and importance to combine these two fields using a standard and optimized protocol for the ultrastructural preservation of *Dm*'s antenna.

To do so, this study will focus on a more classical approach of EM, chemical fixation (ChF) and on a more recent technology for optimized ultrastructural preservation, cryo fixation (CrF).

1.2 Questions to start with

As a first approach to this study it is necessary to think about the factors of specimen preparation that influence the ultrastructural preservation of a tissue. There are some questions that need to be taken into consideration for this study that should be evaluated and answered:

- Does the buffer in which the fixative is suspended affect the quality of sample preservation?
- If there is a difference in quality between buffers; what are the differences?
- How can the ultrastructural preservation of a tissue be quantitatively evaluated?
- Can different durations of the freeze substitution (FS) procedure used on high pressure frozen samples influence ultrastructural preservation?
- Are there any differences between the best chemically fixed samples and the best cryo processed samples?
- Can two different techniques (chemical processing and cryo processing) be compared in a quantitative way?

1.3 Goals

Taking into consideration the questions listed above, the general goal of this project was to create an optimized protocol for chemical and cryo processing that preserves the ultrastructure of the antenna of *Dm*.

The specific goals designed for this works are 1) to create a standardized protocol to fix the antenna of *Dm* as close as possible to its native state by minimizing fixation artifacts both by chemical and cryo techniques; 2) to compare the preservation quality of the antenna attained by different

processing strategies; 3) to create a quantitative tool to assess the quality of ultrastructural sample preservation that also allows quantitative comparisons between samples and protocols.

1.4 Strategies to answer the questions

The strategies were adapted for the different protocols, accordingly to the properties of each type of fixation.

For chemical processing:

- Compare ultrastructural preservation quality of samples processed using a standard fixative for electron microscopy – a modified Karnovsky's fixative - in different buffers (Phosphate, Cacodylate, PHEM, PBS and Water).

For cryo processing:

- Compare ultrastructural preservation quality of cryo-immobilized samples processed using freeze substitution protocols of different durations (short – 8 hours; medium – 24 hours; and long – 52 hours).

For quantitative analysis:

- Review the literature to assess the major criteria to define ultrastructural quality for electron microscopy samples and create a table that uses those criteria to quantitatively grade the ultrastructural preservation of the sample.

1.5 Hypothesis

Different buffers have been used in electron microscopy for several decades but little has been published regarding their contribution to tissue preservation and comparative studies between them are scarce. Although some uses are known for Phosphate, PBS and Cacodylate buffers, the action of PHEM buffer remains a topic to be studied.

In terms of general preservation, based on the convention in the field Phosphate is described to be the best buffer for electron microscopy followed by Cacodylate buffer. Also, Phosphate is less expensive and less toxic than Cacodylate (Bozzola & Russel, 1999). Hence, Phosphate buffer should offer a good preservation of the ultrastructure of *Dm* at a lower cost.

The PHEM buffer was first developed for preserving microtubules, therefore it is expected to give the best preservation of the antenna cells that contains cilia, a microtubule based structure that is my structure of interest (Schilwa & Blerkom, 1981). This buffer also seems to be an ideal candidate for regular usage because it is an organic buffer. Organic buffers show fewer detrimental effects on fine cell structure and are nontoxic (Kuo, 2014).

Fast cryo techniques (rapid freeze substitution protocols) have been shown to give good results for the processing of *Dm* samples and since faster freeze substitutions are less costly (less amount of reagents, less instrument usage and less technician time) this should be the best choice for cryo processing the antenna samples (Shanbhag, 1999; Shanbhag, 2000; McDonald, 2014). It has also been shown that cryo techniques improve the tissue quality when compared to chemical techniques (Shanbhag, 1999; Shanbhag, 2000; McDonald, 2012).

1.6 Thesis outline

This thesis is comprised of 10 chapters. Chapter 1 introduces the reader to the motivation behind this work, the thesis goals and the general hypothesis driving the work. Chapter 2 reviews the basic concepts and history of electron microscopy. Chapter 3 is dedicated to the traditional techniques for specimen preparation while Chapter 4 focuses on cryo techniques for specimen preparation. In Chapter 5 there is a brief explanation about cilia and *Dm.* Chapter 6 details the methodology used to develop this work. Chapter 7 will present all the results, followed by its discussion and conclusion in Chapter 8. Chapter 9 contains the references and Chapter 10 contains the appendices.

2. Transmission Electron Microscopy

Transmission electron microscopy (TEM) has proven to be crucial in research. However like all microscopy techniques TEM is not perfect. Accelerated electrons propagate only under a high vacuum environment, where water evaporates, which is a hitch in the TEM technique since biological samples contain liquid water. As a consequence, for a sample to be imaged under vacuum it must have all the water removed from it (Studer, 2008).

Furthermore, the electron beam can only penetrate thin structures (Hayat, 2000). In the standard procedure, biological samples are fixed with a cocktail of aldehydes and osmium tetroxide (OsO_4), dehydrated to remove the water, embedded into a hard resin that works as a support to the tissue, and ultra-thin sections are cut and stained with heavy metal ions (Luft, 1961). The contrast seen in classical EM micrographs is based on differential adsorption of heavy metal cations to various sample components rather than to the biological structures themselves. Heavy metals are added during sample processing with osmium tetroxide fixation and uranyl acetate (UA) *en block* staining and if needed during an additional step of post-staining (Hayat, 2000).

However, all preparation steps can introduce artifacts. Fixation with glutaraldehyde and dehydration with organic solvents leads, for example, to aggregation of proteins, collapse of highly hydrated glycans, and loss of lipids (Cope, 1968; Cope 1969 and Kellenberger, 1992).

Although some artifacts might be introduced with this technique, it was thanks to the much higher resolution than the one achieved with a light microscope, that many cellular organelles and substructures were first discovered by TEM (Palade, 1954). This resolution is achieved thanks to the small wavelength of an electron and it can be expressed by the Rayleigh criterion:

$$\rho = 0.6\lambda/(n.\sin\mu) = 0.6\lambda/NA \qquad (2.1)$$

ρ - resolution, λ - wavelength, n - refractive index, μ - semi-angle at the specimen and NA - numerical aperture (Egerton, 2005).

Thus, although in theory atomic resolution is possible with the electron microscope (and in practice with inorganic samples), in practice the preparation and imaging artifacts limit the effective resolution for biological specimens for TEM to about 2 nm (Hayat, 2000; Batson, 2002; Studer, 2008).

2.1 Historical Context

Max Knoll and Ernst Ruska invented the electron microscope in 1931 at the Berlin Technische Hochschule. This breakthrough surpassed the limitations of visible light yielding higher resolution microscopy. This new technique of electron microscopy allowed, for the first time, the visualization of viruses, DNA and many smaller organelles (Egerton, 2005).

There are two basic types of electron microscopes: transmission and scanning. The transmission electron microscope (TEM) projects electrons that pass through a thin section of tissue (sample) and interact with it. A two-dimensional picture is produced where the brightness of an area is proportional to the number of electrons that are transmitted through the sample. The scanning electron microscope (SEM) uses electrons to scan the surface of the sample that give rise to secondary electrons. These are captured by a detector and the image is produced over time, as the sample is

scanned. A picture of the sample's surface is produced with a three-dimensional look (Bozzola & Russel, 1999).

The development of the electron microscope drove the evolution of the techniques used in microscopy. However, since the first electron microscopists where physicists and engineers, the first applications for this type of microscope where focused on material sciences. Only afterwards, in the 1950's, the tissue preparation techniques evolve, allowing the biological sciences to profit with the usage of this equipment. At first, chemical fixation (ChF) was conventionally used in EM and with the development of the techniques and the machines used, cryo fixation (CrF) techniques were employed to try to decrease some of the artifacts caused by ChF and to help achieve a more close to native state of the final preserved EM sample (Bozzola & Russel, 1999).

2.1.1 Conventional fixation

Most of the first micrographs acquired by TEM were not much better than a light microscopy picture. Despite the fact that the first focus of TEM analysis was applied to material sciences, it led researchers to consider the possibility of applying it to biological sciences. This new application would allow acquiring information regarding some small size cell components that could not be achieved with light microscopy, such as viruses, small organelles as endoplasmic reticulum and cytoskeleton elements.

One of the limitations to TEM application in biological samples was that specimens being observed (like tissues and whole mounts) where too thick. It was necessary to develop a way to section these samples into thinner slices. Although the problem was straightforward, it took some time to develop a technique for sample sectioning. Initially in the 1950's Hartmann employed glass knives to acquire thin sections. This was followed by the usage of diamond knives introduced by Fernandez-Moran (Latta & Hartmann, 1950; Fernandez-Moran, 1953).

Furthermore, to achieve the desired thin sections thickness an embedding medium to serve as a support was needed, as well as a suitable microtome for the newly developed knives. Moreover, to preserve the samples a good fixative was needed. A landmark in fixation was the development of buffered osmium tetroxide fixative known as "Palade's pickle" that was used until the mid 1960's when double fixation with glutaraldehyde and osmium tetroxide became the new standard fixation (Palade, 1952; Sabatini, 1963).

Until today, this fixation method continues to be used by many laboratories. Usually, the first fixation is with formaldehyde (sometimes in a cocktail with glutaraldehyde, known as Karnovsky's fixative) and lasts about one hour after which a rinse with buffer is done. It is followed by a post-fixation with osmium tetroxide for about another hour after which a rinse with buffer and/or water prior to dehydration in an alcohol or acetone series. After these steps, the sample is infiltrated in resin and embedded. *En bloc* fixation/staining with uranyl acetate has also become common practice in conventional electron microscopy, which is usually done after the double fixation (McDonald, 2014).

This is the essence of a double fixation protocol but many variations to this can be made regarding concentrations, buffer composition, timing, pH and so forth, accordingly to the sample in question and laboratory preferences (Hayat, 2000).

2.1.2 Cryofixation

To cryo-immobilize a sample, the temperature of a sample needs to be decreased very rapidly (in ms) which causes the water present in the sample to create ice crystals. If the ice created is smaller than the resolution of the microscope then although ice is present in the sample it is not visible under the TEM, meaning that no damage was done to the quality of the sample for data collection. However, if the resolution is improved, then the ice crystal size has to become smaller, otherwise it will become visible.

After the evolution of sectioning techniques for material embedded in resin, rapidly frozen samples were tested where freeze-dried tissues were infiltrated with wax. This showed that plunging tissue into cooling media (like propane or isopentane) resulted in tissue damage by ice crystal formation (McDonald, 2014). These samples could still be used for histochemical studies but their quality for morphological studies was low. Trying to overcome this situation, Moor and Muhlethaler developed a cryo-ultramicrotome, aiming to produce thin sections of frozen material under vacuum. However, they were not successful in achieving thin-sectioning but instead, with the introduction of Balzers freeze-fracture machine, they found that they could apply Steere's replica technique to the fractured surfaces, developing like this the freeze etching technique (Steere, 1957; Muhlethaler, 1973).

With the help of this new technique it became possible to look inside the cells without the usual artifacts created by the chemical processes of fixation, dehydration and resin embedding. In spite of this achievement, artifacts (ice crystals) were still created with this technique. To overcome this, it was necessary to use a cryoprotectant that would protect the sample and minimize ultrastructural distortions. Furthermore, with the development of high pressure freezing (HPF) it was now possible to freeze larger samples reducing ice damage (McDonald, 2000).

2.1.3 High pressure freezing

The importance of using the HPF technique in resin-based EM is comparable to the importance of the introduction of glutaraldehyde as a primary fixative, in the increase of cell preservation (Sabatini, 1963). Although HPF was initially used for freeze-fracture work, with freeze substitution methods it started being used for resin-embedded samples, evolving into the technique that is used nowadays (McDonald, 2000).

The main reason HPF is still broadly used for EM sample preparation is its ability to overcome some of the drawbacks from chemical fixation, like processing artifacts and section thickness. These artifacts impair the resolution of the images obtained with chemically fixed samples to a few nanometers in contrast to the theoretical resolution power on the atomic scale of some electron microscopes. On the other hand, cryofixing the samples allows better resolution to be achieved and the sample to retain a closer to native state.

It was the evolution of cryofixation that pushed the resolution of the electron microscope towards the imaging of macromolecular assemblies, and kept advancing the technique well into the 21st century (McDonald, 2000).

3. Traditional techniques for specimen preparation

3.1 Chemical fixation

The electrons emitted in the TEM plus its high vacuum make a severe internal environment that can easily damage the biological tissue. In order to prepare the samples to withstand these conditions they must be processed in a series of steps. The initial step is called fixation and its purpose is to stop all biological activity and to prevent necrosis processes that would alter the cell ultrastructure. This can be accomplished with the usage of fixatives. Ideally, these fixatives should maintain cell size and shape, preserve the chemical nature of constituents as much as possible (such as antigenic proteins and enzymes), not cause distortions in the spatial relationship between the cell components and give the cells sufficient stability to endure the following harsh processing steps (Kuo, 2007; Dikstra, 2003).

In EM the most commonly used type of fixatives are aldehydes, such as formaldehyde and glutaraldehyde. Aldehydes have a fast penetration action, quickly stopping biological activity by crosslinking proteins. These fixatives mainly preserve proteins and its associated macromolecules and glycogen, although during the following processing steps carbohydrates can be extracted (Kuo, 2007).

After this fist fixation step, the sample is postfixed with osmium tetroxide, a strong oxidizer, to enhance the fixation. Osmium tetroxide has a slower penetration action than aldehydes but it is helpful since it reacts with the double bonds of unsaturated lipids. Osmium tetroxide is a heavy metal, and after oxidation it is reduced onto macromolecules providing some contrast to the sample, when afterwards viewed in the TEM (Kuo, 2007).

During fixation the sample loses most of its immunological and enzymatic activity and the cells become hard and brittle and therefore can be easily damaged. To protect the cells from pH changes during this process a buffer should be used in combination with fixatives. There are several buffers that can be used and the most common ones are Cacodylate and Phosphate. However, buffers may also cause some ultrastructural alterations to the sample, which should be taken into consideration when choosing one. For instance, mitochondria can be altered by high concentrations of Phosphate. Since buffers are not innocuous, buffer concentration should be maintained low, just allowing the pH of the final solution to stay within the right pH range (Kuo, 2007).

Although Phosphate and Cacodylate are the most used buffers in EM, organic buffers should be considered as a good substitute as they are non-toxic and produce fewer artifacts in the fine structure of the sample. With organic buffers the sample, when imaged with the TEM, appears to be denser, suggesting less cellular extraction. Furthermore, microtubules and other cytoskeleton components are better preserved in samples processed with organic buffers when compared to samples processed using Phosphate and Cacodylate buffers (Kuo, 2007).

After fixation and post-fixation, the sample is dehydrated (usually with ethanol) to gradually replace the water by the solvent. Doing this step gradually, in a graded series of ethanol helps to minimize cytoplasmatic extraction and tissue shrinkage. Once the sample is in 100% solvent, the samples are infiltrated with an epoxy resin (a plastic monomer). If wanted, a transitional solvent (for

example propylene oxide) that is highly miscible with resin can also be used to improve infiltration (Kuo, 2007).

Subsequently, the resin is polymerized providing the chemically fixed sample a hard support that allows it to be ultrathin sectioned in the ultramicrotome. Finally the sections are stained with heavy metals, usually in a two-step process, with uranyl acetate and lead citrate (LC) creating further sample contrast and allowing improved visualization under the electron beam. Uranyl acetate binds to proteins, lipids and phosphate groups of DNA and RNA. Due to uranium's atomic weight (238), it produces high electronic density and contrast, which gives the image a fine grain. On the other hand, lead citrate binds to several structures in the cytoplasm such as cytoskeleton, ribosomes, lipid membranes and others, enhancing their contrast. The type of fixation, especially fixation with osmium tetroxide, also influences lead citrate. Its reduction allows the lead ions to connect to polar groups of molecules. Also, lead citrate reacts with uranyl acetate (albeit less than it does with osmium tetroxide) thus it should be used after Uranyl acetate staining to maximize contrast improvement (Pandithage, 2013).

General Tissue Preparation Scheme for Electron Microscopy		
Activity	**Chemical used**	**Time involved**
Primary fixation	Tissue is fixed with 2-4% Glutaraldehyde and 2-4% Formaldehyde in buffer	1 - 2hr
Washing	Buffer (three changes)	1hr
Secondary Fixation	Osmium tetroxide (1-2%: can be buffered)	1 - 2hr
Washing	Distilled water (three changes)	1 - 12hr
En bloc staining	1% aqueous Uranyl acetate	20min – overnight
Dehydration	50% Ethanol 70% Ethanol 95% Ethanol (2 changes) absolute Ethanol (2 changes)	5 - 15min 5 - 15min 5 - 15min 20min each
Transitional solvent	Propylene oxide (3 changes)	10min each
Infiltration of resin	Propylene oxide and resin mixtures (gradually increasing the concentration of resin)	Overnight to 3 days
Embedding	Pure resin mixture	2 - 4hr
Polymerization (at 60ºC)		1 – 3 days

Table 1: A standard tissue preparation scheme to process biological samples for electron microscopy. Originally dapted from (Bozzola & Russel, 1999).

3.1.1 Aldehydes

Aldehydes (with the exception of formaldehyde) have been introduced latter as a fixative for EM. Their use started after Sabatini *et al.* (1963, 1964) demonstrated that they are very useful, especially glutaraldehyde ($C_5H_80_2$). These studies showed that a good structural and enzymatic preservation can be achieved using glutaraldehyde as a primary fixative, followed by a secondary fixation with osmium tetroxide. In the primary fixation aldehydes stabilize proteins by creating inter and intra-chain cross-links. The secondary fixation is necessary to prevent lipid extraction from the tissue since aldehydes do not react with these cellular components (Hayat, 1981). Due to the good results demonstrated by this two-step fixation, it is still used nowadays as standard for many sample types in many laboratories.

3.1.1.1 Formaldehyde

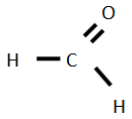


Fig. 1: Formaldehyde molecule. Source: http://www.ammrf.org.au/mys

Formaldehyde, a monoaldehyde, is the simplest of the fixatives from the aldehyde family. It is a colorless gas, easy soluble in water and it is usually sold commercially as formalin solution (37-40%). EM grade formalin should be used since the commonly available ones contain methanol and formic acid, which makes them unsuitable for EM (Hayat, 1981).

Formaldehyde alone causes swelling and distortion of cytoplasmic organelles making it not advisable to use for ultrastructural preservation. For this reason it should be used in combination with other fixatives such as glutaraldehyde, in a smaller concentration than the last (Robinson *et al*., 1987). Despite being less efficient at preserving the ultrastructure there are some cases where formaldehyde usage is recommended, such as immune-detection due to its ability on preserving antigenicity. Since it has a rapid penetration rate in the tissues it has also proven useful for fixing very dense tissues such as seeds that are not as easily penetrated with other fixatives.

Another advantage of formaldehyde is that it is mostly removable by washing with water and the cross-links it creates are reversible (Hayat, 1981).

3.1.1.2 Glutaraldehyde (Glutaric Acid Dialdehyde)

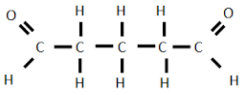


Fig. 2: Glutaraldehyde molecule. Source: http://www.ammrf.org.au/myscope/tem/practice/prep/fixation/

Glutaraldehyde is a simple five-carbon dialdehyde with a straight hydrocarbon chain with two aldehyde moieties (Hayat, 1981).

It is normally sold commercially as a 25 or 50% solution and it is osmotically active (a 3%, v/v, solution has an osmolarity of 300 mOsm) which can cause cell shrinkage. Even so, it allows some enzymes to remain active, biomembranes to retain their permeability and it is recommended to preserve cytoplasmatic microtubules, rough and smooth endoplasmic reticulum, platelets and pinocytic vesicles (Robinson *et al*., 1987).

In Sabatini' studies (1963, 1964) from all the aldehyde tested, glutaraldehyde proved to be the one wielding better ultrastructural preservation on both prokaryotes and eukaryotes and it is still one of the fixatives of choice for the preservation of biological specimens for routine electron microscopy (Hayat, 1981).

3.1.1.3 Glutaraldehyde – Formaldehyde

As stated before, better ultrastructural preservation can be achieved with a mixture of glutaraldehyde and formaldehyde for a wide variety of specimens. This superiority results mainly from two factors. First, due to its small size formaldehyde has a fast penetration rate, faster than glutaraldehyde. This results in the fast but temporarily stabilization of cellular structures. Secondly, although glutaraldehyde has a slower-penetration rate, it permanently fixes the structures previously temporarily fixed with the formaldehyde. As a final outcome, this mixture generally results in good fine structure preservation.

The usage of this mixture was suggested in 1965 by Karnovsky referring to a fixative containing 5% glutaraldehyde and 4% formaldehyde in 0.08 mol/L Cacodylate buffer (pH 7.2) containing 0.05% (5 mM) $CaCl_2$. However, this formula is extremely hypertonic, with an osmolality of 2010 mOsmols and more recent protocols use lowers concentrations of glutaraldehyde (1-3%) and formaldehyde (0.5-2%)(Hayat, 1981).

Method I. Glutaraldehyde (2.5%) - paraformaldehyde (2%)

Cacodylate buffer…… (0.2 mol/L) 25mL
Paraformaldehyde (10%) ……10mL
Glutaraldehyde (25%) ……5mL
Distilled water …… to make 50mL

This formula, or its modification, is the most commonly used fixative for animal and plant species (Hayat, 1981).

3.1.2 Osmium tetroxide (OsO_4)

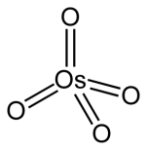


Fig. 3: Osmium tetroxide molecule. Source: http://commons.wikimedia.org/wiki/File:Osmium-tetroxide-2D-structural.png

Osmium tetroxide is a tetrahedral and symmetrical molecule, and consequently nonpolar. This characteristic eases the penetration of this fixative into the charged surfaces of the specimen, making osmium tetroxide an effective fixative. Nonetheless it is almost never used alone but instead it is combined with aldehyde fixation or used after it. This happens since osmium tetroxide has a slow rate of penetration into the majority of the tissues and it cannot cross-link most proteins. This can produce some artifacts in the ultrastructural preservation if osmium tetroxide is used alone as a primary fixative. However, if the sample has been already primarily stabilized with aldehydes, the slow penetration rate of osmium tetroxide is not detrimental.

Osmium tetroxide does not work only as a fixative. When reduced, osmium tetroxide acts as an electrondense stain that reacts mainly with lipids and with other osmiophilic structures in the sample. This molecule also acts as a mordant, as it enhances

lead staining (Hayat, 1981).

Osmium tetroxide is sold commercially as crystals in sealed ampoules or as a solution. The crystals have a greater storage life, so this form should be preferred. Also, osmium tetroxide solutions are relatively stable at 4ºC due to its poor solubility (maximum solubility is *7%, w/v,* at 25°C). If osmium

tetroxide is reduced to metallic osmium the solution develops a brown coloration and therefore cannot be used.

The fixatives containing osmium tetroxide have very low osmolarity when compared with aldehyde fixatives. The membranes of the cells fixed with osmium tetroxide lose their differential permeability properties and therefore the cells cease their osmotic activity (Robinson *et al*., 1987).

Fixative	Speed of Infiltration	Tissue stabilization	Preservation	Main Target	Fixation Reversibility	Precautions
Glutaraldehyde	Slow	Fast	Very good	Proteins	Not possible	Dangerous to handle because of its toxicity and vapor pressure – use fumehood
Formaldehyde	Fast	Slow	Reasonable	Antigens and Proteins	Possible	
Glutaraldehyde and Formaldehyde	Intermediate	Intermediate	Good	Proteins	Not possible	
Osmium Tetroxide	Slow	Slow	Good	Lipids	Not possible	

Table 2: Summary of some fixative characteristics.

3.1.3 Fixative vehicle

3.1.3.1 Buffers in fixation

Wood and Luft (1965) did the first systematic study on the specific effect of a buffer in the fixative solution. This investigation led to the conclusion that for a chosen pH the nature of a buffer medium is important and there was no universally better buffer.

Good *et al* (1966) reported the inadequacy of many buffers used at that time for biological research that may have lead to erroneous results. Their criticisms are based on the physicochemical action of buffers during fixation of tissue since the main role of buffers is to establish the microenvironment in which the fixative works more efficiently.

Pentilla *et al* (1974) reinforced the idea that not all cellular constituents are equally properly fixed by any of the common fixatives. The grade of extraction or rearrangement caused in these cellular components is expected to vary accordingly to the medium that buffers the fixative. Besides that, the cellular molecules can react with reduced and unreduced buffer components, by constantly changing physicochemical states with the advancing front of the fixative solution. During diffusion into the tissue it is thought that the buffer front presumably precedes the fixative front since fixative molecules react with biochemical moieties and are removed from the solution, whereas the buffer molecules may or may not react. If physiologically active cations and anions, such as Ca^{2+}, K^{+}, PO_4^{3-}, Na^{+}, are present in the buffered fixative the cells may suffer some biochemical alterations before they are immobilized by the fixative (Schiff & Gennaro, 1979).

The differential ability of structural components to form complexes, also know as Werner salts, induces electron scattering, allowing the tissue to be differentially stained for TEM (Beer 1965). If ionic species from buffers are present they can mask or expose sites of organic molecules than in another

environment would interact differently with the fixative or the stain. For example, calcium that can be added to fixative solutions to stabilize membranes may form insoluble phosphate salts that can precipitate within the tissue and cannot be removed through rinsing, post-fixing or dehydration (Schiff & Gennaro, 1979).

3.1.3.2 pH, dissociation constant and pKa

To know how acidic or alkaline an aqueous solution is a numeric scale is required, which we define as the **pH** scale. It was defined that "p" represents a value of any quantity as the negative logarithm of the hydrogen ion concentration. Therefore, pH can be described by the following equation (Mohan, 2003):

$$pH = -\log [H^+] \quad (3.1)$$

In biological systems, solutions are usually composed of weak acids and bases. Weak acids and bases do not completely dissociate in solution, contrary to strong acids and bases that are completely ionized in aqueous solutions. Buffer solutions are composed of a weak acid (the proton donor) and its conjugate base (the proton acceptor). Therefore, in buffer solutions, the weak acid and base are not completely dissociated, but instead they exist as an equilibrium mixture of non-dissociated and dissociated species. The relationship of this equilibrium interaction can be written as an equation (Mohan, 2003):

$$HA \leftrightharpoons A^- + H^+ \quad (3.2)$$

At equilibrium, the rate of dissociation of HA is equal to the rate of association of $[A^-]$ and $[H^+]$ So, at equilibrium, $K_2 [HA] = K_1 [A^-][H^+]$, where $[A^-]$ and $[H^+]$ are the concentration of product and [HA] is the concentration of the reactant. This **dissociation constant** can be written as:

$$K_a = \frac{[A^-][H^+]}{[HA]} \quad (3.3)$$

Similarly to pH, **pKa** can be defined as –log Ka. If the equilibrium expression is converted to –log then we get the Henderson-Hasselbalch (HH) equation (Mohan, 2003):

$$pH = pK_a + \log \frac{[A^-]}{[HA]} \quad (3.4)$$

With this equation, the pH of a buffer solution can be estimated and also the equilibrium pH in acid-base reactions can be found.

Usually, pKa of weak acids or bases values are determined by titration and the pKa value indicates the middle of the buffering range. Many times the terms pK and pKa are used interchangeably in the literature.

3.1.3.3 Buffers, Buffer Capacity and Range

A **buffer** prevents pH changes in a solution when a small amount of acid or base is added to it. The buffering works when the concentration of proton donor and its conjugated proton acceptor are equal in the solution and equilibrium of these two reversible reactions is achieved. This is known as the isoelectric point and at this moment small amounts of acid or base can be added to the solution without any detectable pH variation. Since at the isoelectric point $[HA] = [A^-]$ then if we look at the HH equation we can quickly conclude that at the isoelectric point pH is equal to pKa (log 1 =0):

$$pH = pK_a + \log 1 \qquad (3.5)$$

This ability of the buffer to resist the changes in pH with the addition of acid or based is called **buffering capacity**. A buffering capacity of 1 is when 1 mol of acid or alkali is added to 1 liter of buffer and the pH changes by 1 unit. When the individual pKa values are in close proximity in a mixed acid-base buffer then the buffer capacity is much greater.

The buffering capacity usually depends on the buffer concentration in the solution, in the sense that higher concentrations offer higher buffering capacity. However, pH is not dependent on the concentrations of buffer but on their ratio.

Buffering capacity exists in the **range** from $\frac{[A^-]}{[HA]}$ = 0.1 to $\frac{[A^-]}{[HA]}$ = 10.0. Beyond this range, the buffering capacity might get significantly reduced (Mohan,C. 2003).

3.1.3.4 Biological Buffers – What makes a "Good Buffer"

Originally several inorganic substances were used as buffers (such as Phosphate and cacodylate) for biological research and later on organic acids were also employed. However, many of these buffers present some disadvantages such as being hazardous, making them hard to work with and expensive to dispose and they are not inert (Mohan,C. 2003). Currently, many other buffers are used, especially biological buffers that were developed in 1966 by Good and his research colleagues. They defined several rules that a buffer should follow to be considered a good buffer. Although it is very hard to fulfill concomitantly all the criteria, Good *et al* were able to design several buffers, such as PIPES and HEPES, that follow some of these criteria and have shown better results than the commonly used buffers. The criteria outlined by those researchers are:

1) **pK_a**. Buffers should have pKa values near neutral pH (between 6 and 8) since most biological reactions take place at this pH range;
2) **Solubility**. Buffers should be soluble in water since the majority of biological systems are aqueous. Also, low solubility in non polar solvents is considered beneficial since it prevents the buffer components to accumulate in non polar compartments of the cell, such as cell membranes;
3) **Membrane impermeability.** Buffers should not easily pass through cell membranes, preventing its components from accumulating inside the cell;
4) **Minimal salt effects.** Buffers on their own should have low ionic strength since some ions can interact with some biological components;
5) **Influences on dissociation.** The dissociation of the buffer should not be influenced by factors such as buffer concentration, temperature and ionic compositions of the medium.
6) **Well-behaved cation interactions.** Ideally, buffering compounds should not form complexes. However, if they do form complexes with cationic ligands they should remain soluble;
7) **Stability.** Buffers should be chemically stable, enduring several types of degradation;
8) **Biochemical inertness.** Buffers should not participate in or influence any biochemical reactions;

9) **Optical absorbance.** Buffers should not absorb light in wavelengths that might interfere with commonly used spectrophotometric assays;
10) **Ease of preparation.** Buffers should be easy to prepare and inexpensive.

3.1.3.5 Choosing a Buffer

When designing an experiment several aspects should be taken into consideration when choosing the buffer:

1) Select a buffer with a pKa value near the middle of the needed range. If the pH is expected to decrease during the experiment then a buffer with a pKa slightly lower than the working pH should be chosen. If on the contrary, the pH is expected to increase then a pKa slightly higher than the working pH is required. This will increase the buffer capacity;
2) Adjust pH of the solution at desired temperature since pH may vary with temperature;
3) Prepare buffers at working conditions. Prepare the buffer at the same temperature and concentration that you planned to use during the experiment. If using a stock solution then only dilute prior to use;
4) Pay attention to purity and cost. High purity and moderate cost compounds should be preferred. Also, the highest possible quality water should be used to dilute the buffer in;
5) Buffer compounds should have no significant absorbance between 240 to 700 nm range;
6) Be careful with special cases. For example, highly calcium-dependent systems cannot have either citrate (citric acid and its salts are calcium-chelators) or Phosphate as buffers (calcium phosphates are insoluble and therefore will precipitate). Tris (hydroxymethyl) aminomethane also chelates calcium and other essential metals;
7) Many buffer reagents are supplied both as a free acid (or base) and its corresponding salt. When making a series of buffers with different pH this might be very helpful.
8) Use stock solutions of monobasic and dibasic sodium phosphates to prepare Phosphate buffers. Mix the appropriate amounts of monobasic and dibasic sodium Phosphate solutions buffers to achieve the desired pH;
9) Use buffers without mineral cations when appropriate;
10) If necessary, use a graph like the one shown in Fig. 4 to calculate the relative amounts of buffer components required for a particular pH. The buffers more commonly used show very small deviations from theoretical value in the pH range (Mohan,C. 2003).

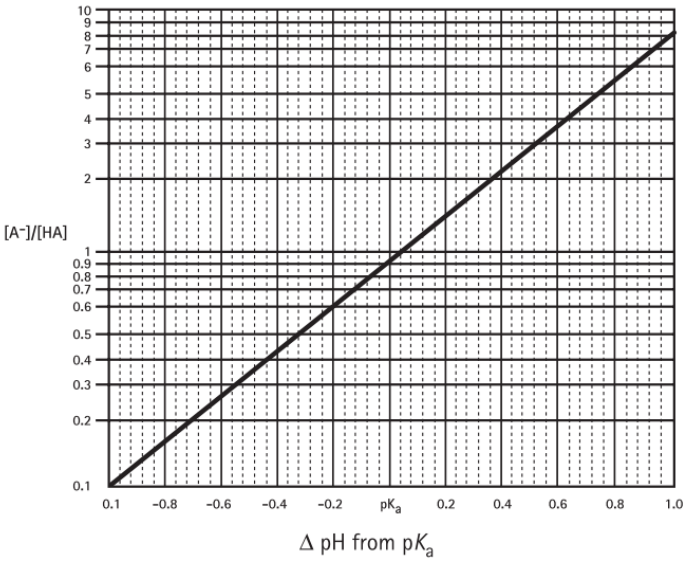


Fig. 4: Theoretical plot of Δ pH versus [A-]/[HA] on two-cycle semilog paper (Mohan, 2003).

3.1.3.6 Buffers used in this study

Today, the most commonly used buffers are Phosphate based buffers, Cacodylate and organic acid buffers (such as the ones described by Good *et al*.). Five were chosen for this comparative study: bi-Phosphate, PBS, cacodylate, PHEM, and water as a control for the buffer action. Below is a brief description of the main characteristics of each of the selected buffers.

- **Phosphate buffers**

They are commonly used with aldehydes and osmium fixatives. Since they mimic certain components found in living systems they are called “physiological buffers”. They are non-toxic and their pH is maintained more effectively than the other most commonly used buffer, Cacodylate. The buffering capacity of Phosphate buffer is best at physiological pH or in a slightly alkaline formulation (pH 7.2-7.4). If the solution pH is above or below this range, the buffering capacity of Phosphate buffer decreases drastically (Dykstra, 2012).

Phosphate buffer precipitates easily, may become slowly contaminated with microorganisms and should not be used in fixatives containing divalent cations such as Ca^{2+} (Weakly, 1981; Glauert, 1975).

There is a big variety of Phosphate buffers but there is not much evidence that one is superior to the other, as long as the osmolarity is the same. The majority of the Phosphate buffers used are based on Sörensen’s buffer, a mixture of monobasic and dibasic sodium phosphates (Glauert, 1975).

- **PBS**

Amongst biological buffers, Phosphate-buffered saline (abbreviated PBS) is one of the most commonly used in biochemistry. This buffer contains several salts in a water-based solution. It contains sodium chloride and sodium phosphate. In some cases potassium Phosphate and potassium chloride can also be added to the mixture. This is a non-toxic buffer and it is isotonic, matching the osmolarity and ion concentration of the human cells (“SmartBuffers”, 2014)

The constitution of the PBS used in this study is mainly sodium chloride, Phosphate buffer and potassium chloride and its buffering range goes from pH 7.2 to 7.6 (Morris, 2001)

- **Cacodylate buffer**

This buffer has been proposed as the buffer of choice for glutaraldehyde fixatives by Sabatini *et al* (1963). When Cacodylate buffer is used during primary fixation with aldehydes, the quality of the preservation is usually similar to the one achieved with Phosphate buffers, however Cacodylate is more expensive than them (Dykstra, 2012). Cacodylate is also effective with osmium fixatives (Weakly, 1981).

Cacodylate is much less reactive than Phosphate buffer and thus can be employed in cytochemical reaction mixtures and also with media containing various ions without the danger of co-precipitation with other solution components. Also, Cacodylate buffer capacity ranges from pH 6.4-7.4, and like Phosphate buffer it is used primarily at physiological pH or at slightly alkaline conditions. Although it prevents bacterial contamination, its arsenic content makes it highly toxic (Weakly, 1981).

- **PHEM**

PHEM buffer is composed of PIPES, HEPES, EGTA and $MgCl_2$. Both PIPES and HEPES are Zwitterionic compounds (latter inserted in Good's buffers list). They are little used in electron microscopy. However, their usage for several other research techniques can help them start to be seen as a possible good vehicle for EM fixatives (Dykstra, 2012). Hayat (1981) reported that these two compounds increase the retention of proteins and phospholipids which might preserve a relatively high cellular density. Also, he claimed that they do not contain ions that would compromise elemental analysis.

Although there is not much described in the literature regarding PHEM applications, it is known that it is a very good microtubule stabilizing buffer. Below is a brief description of the individual components of PHEM:

PIPES (piperazine-N,N′-bis(2-ethanesulfonic acid)) is a commonly used buffering agent in biochemical research. When buffering glutaraldehyde solutions it is described to reduce lipid extraction in plant and animal tissues. It has an effective buffering range from 6.1 to 7.5 at 25ºC (Good, 1966; Schiff, 1979).

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) is an organic chemical buffering agent. As the temperature decreases, HEPES dissociation decreases as well, making it a more efficient buffering agent for preserving enzyme function and structure at low temperatures. It has an effective buffering range from 6.8 to 8.2 at 25ºC. When exposed to light it produces hydrogen peroxide making HEPES phototoxic. It is therefore suggested that solutions containing this reagent are kept protected from light (Lepe-Zuniga *et al., 1987)*

EGTA (ethylene glycol tetraacetic acid) is a common buffer ingredient due to its chelating activity. It is related to EDTA but it has a lower affinity for magnesium, making EGTA more selective for calcium ions. It is commonly used in buffer solutions that simulate the intracellular environment in living cells where calcium ions are usually at least a thousand fold less concentrated than magnesium (Bett, G. Ramusson, R. 2002). Also, Schliwa (1981) showed that as long as the pH is kept close to neutral, a high EDTA concentration (around 10mM or more) helps to preserve the structural integrity of all fibrous components of the cytoskeleton.

$MgCl_2$ helps to dissolve the EGTA (Scott, 2014).

- **Water**

About 70% of the mass of most living organisms is water making them aqueous chemical systems. All biological reactions take place in an aqueous medium and therefore all aspects of cellular function and structure are tailored to the physical and chemical properties of water.

Most tissues are fixed near the physiological pH values (from 7.2 to 7.5 for animals). Therefore, in unbuffered fixative solutions the pH of the solution may not be maintained, producing inferior results than buffered fixatives. Also, as the fixative penetrates the cell, the buffer solution prevents a possible acidic wave of injury (Dawes, 1971; Crang, R., Klomparens, K., 1988).

Buffer	Buffer Range at 25ºC
Phosphate	7.2 - 7.4
PBS	7.2 - 7.6
Cacodylate	6.4 - 7.4
PHEM	Not known for buffer solution PIPES - 6.1 - 7.5 HEPES - 6.8 - 8.2 EGTA - Neutral
Water	Neutral

Table 3: Summary of buffer range at 25ºC of the different used buffers.

3.1.4 Effects of chemical fixation

Samples fixed by different methods usually show some variation in ultrastructural details but the overall structural relationship is maintained and subcellular organelles exhibit, in general, great similarity. This means that whether samples were fixed with different chemicals as aldehydes (cross-linkers), osmium tetroxide (oxidizer) or organic solvents (coagulators) or whether the sample was vitrified, it shows the same characteristics, allowing it to be identified by an experienced observer. These observations are evidence that the commonly seen relationships and structures are not an artifact caused by a particular preparation method, but are a true representation of the *in vivo* specimen (Crang, R., Klomparens, K., 1988).

However, it is helpful to know what are the main artifacts caused by each individual technique since it is not always possible to do comparative studies for the same sample. A perfect fixation should be artifact free (Crang, R., Klomparens, K., 1988). Therefore, knowing the artifacts produced by each technique and how they arise can help us to optimize the fixation procedure so that artifacts do not impair us to answer our biological question.

Discussed below are some of the most common artifacts caused by chemical fixation.

3.1.4.1 Changes in volume

During the processing of samples for EM analysis the specimens go through several preparatory steps where changes of volume can occur (Fig. 5). Also, specimen weight can fluctuate during these processes. It is important to know how and when these changes occur during processing since they are an indicator for the quality of preservation of the fine structure.

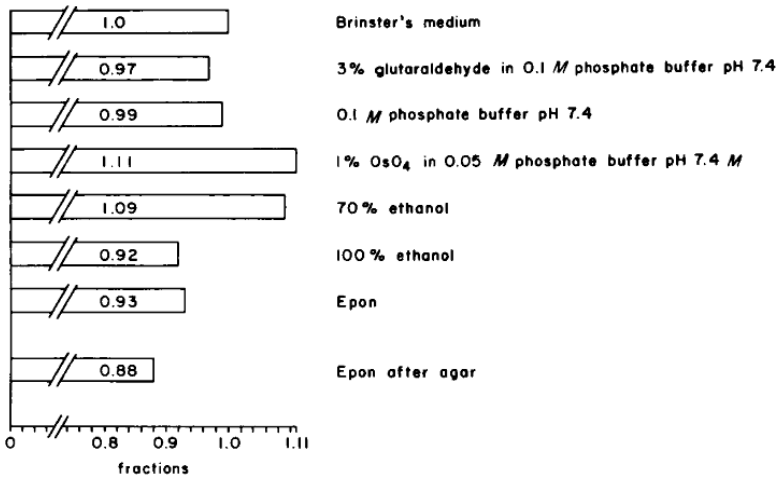


Fig. 5: Mean diameter of mouse ova during successive steps of preparation regarded as optimal (expressed as fraction of their initial mean diameter in Brinster's medium – egg culture medium that contains mainly bovine serum albumin). The specimen showed the lowest volume in 100% ethanol and remained unchanged after embedding in Epon (Konwinski *et al*., 1974).

In addition, inhibition of cellular respiration will cause tissue

swelling if the osmolarity is not maintained. When the tissue is fixed, cellular respiration stops immediately and this can cause the tissue to swell. This swelling happens with an increase of water flow towards the inside of the cell. This causes an uptake of ions that are dragged along with the water. This is one of the reasons why during fixation slightly hypertonic solutions should be used.

Therefore, amount of shrinkage or swelling of the cell is a consequence of the osmolarity of the fixative vehicle and the ion species that it contains, the concentration of the fixative and the inherent characteristics of the tissue.

The fixation step is not the only step responsible for defining the final size of the specimen. The conditions of dehydration and embedding can also influence the final size of the sample, as shown in Fig. 5. This change in cell size is mainly a consequence of changes in the amount of water inside the cell with changes in the size of subcellular components playing a minor role. However, it is not clear that subcellular components undergo the same transitory changes in volume as the entire cell. It is likely that the plasma membrane and the membranes of subcellular components have different permeability values, and therefore it is expected that they respond differently to the same osmotic gradient. Moreover, subcellular components encounter different osmotic gradients than the plasma membrane (Eisenberg and Mobley, 1975). Therefore, the accurate volume of subcellular components cannot be deduced from only measuring the cell. As an example, the cytoplasm and nucleus can have different degrees of shrinkage. For instance, for the nucleus, fixation with glutaraldehyde causes more shrinkage that fixation with osmium tetroxide or formaldehyde but the contrary is true for the cytoplasm. Therefore, in the same cell, different size changes can be happening at the same time for different subcellular components (Hayat, 1981).

As previously stated, aldehyde fixatives should be slightly hypertonic to help reducing cellular artifacts. Hypotonic fixative solutions cause swelling of the tissue, while too hypertonic solutions cause shrinkage. To avoid these artifacts, the solution would be to use an isotonic solution to fix the sample. However, in practice isotonic fixative solutions have proven to be non satisfactory in preserving the normal volume of intact tissues. Indeed, the total osmotic pressure that solute particles in a fixative solution apply to a cell is not always related to its volume changes. The main reason for the failure of isotonic fixative solutions in maintaining the cell volume has to do with the impossibility of measuring the extracellular and intracellular osmolarity. Not having this information hinders the preparation of isotonic fixative solutions. Also, different cell types have different intracellular osmotic pressure. In addition, after removing a tissue from the body and depriving it from its *in situ* conditions may change its cells osmolarity. And lastly, while fixing the tissue new ionized groups are formed.

Although fixation rapidly changes membrane permeability, there is maintenance of colloid-osmotic equilibrium. Therefore, it is to this equilibrium that fixative osmolarity should be adjusted to and not to the extracellular osmolarity, since they might differ.

Furthermore, when the samples are fixed with an isotonic fixative solution, the fixative penetrates the membrane rather slowly. This is not desirable since the speed of membrane penetration by the fixative influences the quality and rate of fixation. A slower penetration of fixative may cause anoxia, since it takes longer to cease the cell metabolism, which in turn may cause swelling (Hayat, 1981). Morphometric studies showed superior results in slightly hypertonic fixatives

since a constant surface-to-volume ratio is preserved by cell components under hypertonic conditions of fixation (Hayat, 1981).

However, adverse effects may occur if a tissue is fixed with excessive hypertonic solution. These include artifactual widening of extracellular spaces and separation of cytoplasm from the nuclear membrane. Also, cell organelles that don't have direct contact with the interstitial fluid, such as mitochondria, shrink in volume proportionally to the decrease in cell volume in hypertonic solutions, while organelles in direct contact with the interstitial fluid (*e.g.*, transverse tubules) either swell or remain unchanged.

It is clear that the changes in tissue volume that occur in the cell are caused by the artifacts introduced during the many sample preparatory steps. However, in part, some of the final shrinkage or swelling of the cell is directly connected to the ability of the primary fixative solution to maintain or not its original volume. The reliability of the preparatory procedure and its capacity of producing quality of specimen preservation can be assessed by the ability of maintaining the sample's original *in vivo* size (Hayat, 1981).

3.1.4.2 Reorganization in the plasma membrane

In vivo, there is equilibrium between the osmolarity in the extracellular and intracellular space (isosmolarity). The membrane is permeable to small hydrophobic molecules and to small uncharged polar molecules such as water. This means that water can enter or exit the cell simply through diffusion or it can go through a special channel, called aquaporin. The latter is a passive process that follows the direction of osmotic pressure across the membrane although many aquaporins function as always-open channels. Conversely, large uncharged polar molecules and ions have to be actively transported inside or outside the cell through the help of specialized channels or transporters (Cooper, 2000).

In the cytosol there are some macromolecules that, although few in number, are high in size and highly charged, attracting many inorganic ions of opposite charge (counterions). Also, as a result of active transport and metabolic processes there is a high concentration of small organic molecules, such as amino-acids, which also attract counterions. There is also an abundance of inorganic ions since they can only slowly leak across the plasma membrane to the cytosol. If they were not pumped-out of the cell and if there were no interaction with other molecules inside the cell they would eventually achieve an equilibrium state, where the extracellular concentration of ions would match the intracellular concentration of ions. However, in the cytosol the macromolecules and small organic molecules interact with the small inorganic ions and transporters pump them out of the cell. This causes the environment in the interior of the cell to be electrically negative relative to its exterior, a phenomena known as the Donnan effect. At the same time this difference in electric potential favors the entry of positively charged ions into the cell such as Na^+ (and the amino-acids "dragged" with it) and opposes the entry of negatively charged ions, such as Cl^-.

The most important transporter that helps the cell pump out the inorganic ions is the sodium-potassium pump (Na^+/K^+). It actively transports three Na^+ ions to the extracellular space while importing two K^+ ions concomitantly, by ATP hydrolysis (Lodish *et al*., 2000).

Since the Na^+/K^+ pump drives different amounts of charged species in and out of the cell, it is electrogenic. It drives a net current across the membrane, contributing to the maintenance of the electrical potential of the cell, preserving the inside negative charge relative to the outside. Therefore, energy is required to make these specific ion pumping systems work, maintaining a low concentration of Na^+ in the interior of the cell.

However, one of the major changes that happens after fixing a tissue is cellular membrane reorganization which causes changes in membrane permeability and osmotic behavior. Different types of cells may suffer different changes in their membranes after fixation but changes in osmotic equilibrium happen to all plasma membranes (Cooper, 2000).

When the tissue is separated from the body and fixed, cellular respiration is inhibited and the energy available for maintaining specific ion concentrations is diminished. With this, Na^+ diffuses into the cells without being actively extruded, and at the same time, K^+ leaks out of the cells. This results in Na^+ accumulation inside the cells and loss of K^+, diminishing the negative potential of the plasma membrane, allowing Cl^- to enter the cells.

The net gain of solutes (especially osmotically active ones) by the cell causes a water flow towards the inside of the cell and generates swelling of the tissue. This increased intracellular osmotic pressure, if too extensive, may lead to autolysis of the cell (Hayat, 1981).

Although cellular membrane reorganization happens after fixation, it can happen in different degrees. The most severe alteration happens with osmium tetroxide fixation. This fixative destroys the differential permeability of the cellular membrane, allowing low-molecular-weight substances to pass through it, including vital dyes. At the same time, this fixative decreases the fluidity of the membranes, by perturbing lipid chains (Jost *et al*., 1973). Any damage caused to the cellular membrane will usually lower its electrical resistance, and fixation with osmium tetroxide is not an exception.

On the other hand, a milder alteration happens to the cellular membrane when fixed with glutaraldehyde, since it does not impair the relative impermeability of cellular membranes to the majority of the ions. It is believed that after fixation with glutaraldehyde some reactive groups persist in the membrane, and there is some evidence that suggest that glutaraldehyde-fixed cells remain osmotically active (Fahimi and Drochmans, 1965).

According to Jard *et al*. (1966) and others, not only glutaraldehyde but also formaldehyde fixed cells remain partly impermeable to ions (such as Na^+). This is the reason that the osmolarity of solutions used after fixation with glutaraldehyde may affect the cell structure.

The above stated superiority of glutaraldehyde over osmium tetroxide in terms of preserving membrane osmotic selectivity does not seem to arise from the difference in penetration rates between the two fixatives. It is likely that osmium tetroxide destabilizes the cell membrane by denaturing its surface proteins. Another possibility suggested by Robertson (1959) is that this fixative is only able to stabilize the inner most layer of the cell membrane while the most external layer breaks down into chains of vesicles. Another factor that may contribute to the alteration of membranes is the action of osmotic forces that can expand or contract the cytoplasm in a way that can cause the disruption and recombination of the membranes (Doggenweiler and Heuser, 1967).

Additionally, it is assumed that reactive groups of adjacent cellular membranes interact, which can lead to membrane reorganization if catalyzed by a substance used in subsequent steps of the sample processing (*e.g*. organic solvents or unpolymerized embedding media) or by an increase in temperature.

Finally, fixation with different reagents results in different alterations in cell membrane but also fixation with a specific reagent can result in different alterations in different membrane (Hayat, 1981, Daughty, 2004). It is obvious that the phenomenon of structural reorganization of membranes is still not fully understood nor are all the factors that influence it.

3.1.4.3 Microtubules preservation

In vivo, microtubules are unstable structures and their preservation can be affected by several factors. Low temperatures (around 4ºC), trauma during tissue preparation, autolysis, certain types of fixatives and buffers, non-optimal osmolarity in fixative solutions, and excessive divalent cations (especially Ca^{2+}) are some of the factors that can induce microtubule disassembly (Freeman, 2000).

There are various classes of microtubules and each one of them reacts differently to temperature and fixation processes. Axonemal microtubules are usually considered to be more stable, whereas cytoplasmatic microtubules are more unstable. Even microtubules of the same type can present different stability. When microtubules are fixed with osmium tetroxide without a prefixation with glutaraldehyde it is believed that they are no longer recognizable in most tissues. This happens since the commonly used 1-2% aqueous solutions of osmium tetroxide at low temperature penetrate the tissue too slowly to preserve microtubules. Therefore, it can be concluded that the fixative penetration rate into the tissue and the interaction between fixative and sample are both important factor for microtubule preservation. Taking this into consideration, for routine preservation of microtubules a prefixation with glutaraldehyde at room temperature seems to be the most advisable choice. Furthermore, if a secondary fixation with osmium tetroxide is used, then between the primary and secondary fixation the temperature should be gradually decreased to 4ºC, to allow the specimen to gradually acclimatize (Hayat, 1981).

4. Cryo techniques for specimen preparation

Since the initial development of electron microscopy, structural biologists have aimed to preserve biological samples as close as possible to their native state. The ultrastructural preservation techniques have been improving concomitantly to the technology available.

Some limitations have been hindering the achievement of native-state-like preservation but at the same time they are the boost needed to improve technological development. One limitation is the use of conventional chemical fixation methods. These methods have a relatively slow penetration rate when compared to the rates of cellular events (Dahl and Staehelin, 1989). This produces a time delay in fixation that is proportional to the depth of the tissue which in turn results in the opportunity for cellular events to change throughout the fixation process.

The advance of microscopy technology, such as improvements in the correction of aberrations, new types of microscopes and increased computer performance and data storage has improved the resolution of electron microscopy and required the improvement of fixation protocols. This has lead to an increased interest in technologies that allow rapid immobilization and preservation of biological fine structure, such as ultra rapid freezing (cryofixation). Cryofixation has two main advantages over chemical fixation: a faster rate of fixation (full sample fixation in milliseconds instead of minutes to hours) and stabilization of all cellular components.

After rapidly freezing, the samples undergo freeze-substitution (FS). The underlying principle of FS is that fixatives and dehydrating agents diffuse into the sample at very low temperatures where the fixative components are still not active. Then, when the temperature is raised fixation occurs in all parts of the sample at the same time. Therefore, the fixation gradient that is usually seen in conventionally fixed samples is prevented and the removal of water with damaging effects, like the ones caused by dehydration at room temperature, is avoided in this technique (Morphew, 2002).

Nonetheless, there are some technical difficulties in the application of cryo techniques. It is still challenging to work with very small sample size, it is hard to obtain optimally frozen samples and it remains a time consuming technique. This limits the application of cryo techniques for ultrastructural studies. Still, in the cases where cryo preservation showed successful results, they have consistently displayed superior quality on preserving cellular ultrastructure over chemical fixation methods (Dahl and Staehelin, 1989).

4.1 High Pressure Freezing

The ideal situation for imaging biological specimens should be preservation as close to the *in vivo* condition as possible, *i.e* fully hydrated. Taking into consideration the vacuum conditions of the electron microscope, the visualization of fully hydrated cells can only be achieved if all the water present in the sample is frozen. However, if a living tissue is frozen at low cooling rates this has harmful effects on the sample preservation. There can be cytoplasmatic formation of ice, and since this ice can be ten times larger than water it will destroy the cells. Also, there can be induction of phase segregation between water and solutes (organic matter and salts). This can lead to precipitation

of cellular components between ice crystals which can be visible in electron micrographs (Dubochet 2007).

Ice formation in the cell can be deleterious since ice crystals can grow and create holes in the cellular membranes leading to the destruction of organelles. When these badly frozen samples are thawed again they are already completely destroyed. This harmful ice crystal formation can be avoided if the biological samples are protected with anti-freeze agents that reduce the concentration of free water (Dubochet 2007).

When freezing a biological sample, the freezing rate should be fast enough so that the ice crystals formed are smaller than the resolution of the electron microscope (smaller than 10-15nm) or that vitreous ice is formed. The method to obtain these conditions is called ultrarapid freezing. The most commonly used methods of cryo immobilization are: 1) plunge freezing, 2) propane jet freezing, 3) cold metal block freezing and 4) high-pressure freezing. However, for the first three methods successful preservation is only achieved on very thin samples, such as a monolayer of cells. Only the fourth one, high pressure freezing, allows successful preservation of samples up to theoretically 600µm of thickness.

To achieve acceptable freeze-fixation, freezing rates of more than - 10,000ºC/sec are required for most biological specimens. However, for samples thicker than 20 µm this rate cannot be achieved in the middle of the sample due to the low heat conductivity of water. Because of this, some types of samples cannot be preserved by ultrarapid freezing methods at atmospheric pressure.

For successful preservation on thicker samples, the physical properties of the water need to be changed by reducing the critical rate of freezing in order to create a small size ice crystal (Moor, 1987). One way of achieving this is with the application of cryoprotectants. Although the use of cryoprotectants helps to reduce water's freezing rate to between -100 and -500ºC/sec it is not always innocuous. The addition of natural cryoprotectants in most cases is not possible and therefore the use of artificial cryoprotectants is adopted. However, high concentration (20-30%) of these artificial cryoprotectants and their dehydrating activity can lead to changes in cell morphology (such as severe shrinking and specific responses to osmotic stress) which essentially removes the advantages of cryo fixation. To overcome this problem, freezing under high pressure should be adopted for preserving samples thicker than 20µm. As discussed in several papers (Meryman, 2007; Dahl and Staehelin, 1989) the reduction of the freezing rate by high pressure freezing is achieved by some changes in the freezing properties of water, namely:

1) Decreasing the freezing point of water;

2) Reducing the nucleation rate of ice crystals;

3) Diminishing the growth of ice crystals.

4.2 Physics of ice formation in biological specimens

There are three forms of pure water in the solid state. Two of them are crystalline forms, hexagonal crystal and cubic crystal. The third, also called vitreous form, happens when water is frozen so rapidly that crystals don't have enough time to be formed and water remains amorphous. Since crystal formation causes water expansion as it freezes and vitreous ice is the same size as liquid

water this makes this makes it the only advantageous form of ice for biological samples (Jongebloed *et al*, 1999).

For ice crystals to grow they require a “nucleus” of at least a critical size (minimal size of a cluster to inevitably initiate ice crystal growth, for a certain temperature) to which ice molecules are added. In biological samples, nucleation is a homogeneous process, meaning that the nucleation site is a small cluster of water molecules. If the nucleation site has any type of contaminant or if it is not composed entirely by water then the process is called heterogeneous nucleation (Rasmussen *et al*., 1975).

With the decrease of water temperature: (1) there is a decrease in magnitude of the surface free energy. Therefore, there is also a **decrease** in the **critical size** of a homogenous nucleus; (2) there is an **increase** in the average **cluster size** which means that the probability of the formation of a cluster as big or bigger than the critical size increases and consequently nucleation occurs sooner at lower temperatures. Close to -40ºC the average cluster dimension is equal to the critical size and homogeneous nucleation happens. This is the temperature of homogeneous nucleation (TH), for water. If water is frozen at or below TH then ice crystal growth will be minimal, as long as the heat produced by crystallization can be withdraw quickly enough to keep the temperature at or below TH.

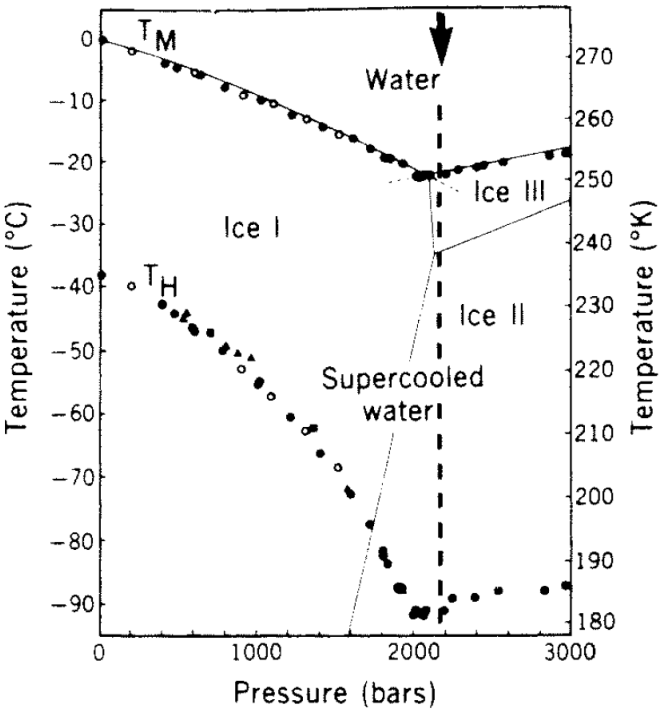


Fig. 6: Homogeneous nucleation (TH) and equilibrium melting temperatures (TM) for water in emulsion form as a function of pressure. The dashed, vertical line indicates the conditions under which ice II and ice III may be produced in high pressure frozen samples (adapted from Kanno *et al*, 1975).

This means that all samples ultrarapidly frozen contain ice crystals but as long these conditions are maintained the ice crystals formed will be too small to be seen in the electron microscope.

On Fig. 6, there is a water/ice phase diagram. It is know that at ~2050 bar the melting point of pure water is reduced to a minimum of -22°C**.** When analyzing the diagram it is possible to determine that for the most beneficial pressure zone that affects freezing behavior of water (~2,050 bar) and to maintain water below its temperature of homogeneous nucleation (TH), high pressure freezing should be performed at a temperature of ~ -90ºC or below.

In theory, the application of 2,100 bar pressure results in a cryoprotective effect equivalent to about 20% glycerol and allows satisfactory freezing of up to 0.6 mm thick planar samples (although in practice this value is closer to 200µm) when cooling is applied from two sides (Muller and Moor, 1984).

4.2.1 Technology of high pressure freezing

To freeze a sample in the HPF machine, first the sample has to be introduced into a special sample holder (carrier). There are different types of carriers depending on what type of sample is used and also for different applications (Studer, 2008) and different machines.

First, the carrier should be selected to fit the sample size without damaging it and at the same time preventing extra space (unnecessary volume creates heat that must be extracted). Also, there should not be any space filled with air inside the carrier. Air bubbles within the carrier act as insulators and collapse under pressure which can lead to sample damage (Dahl, Staehelin 1989). Therefore, all enclosed air spaces should be filled with sample or fluid.

There are a wide variety of carriers. The ones used in this study were aluminum. They have two hats. Generally one type is "welled" and the other smooth so they can be combined. If two welled hats are used, a larger cavity is created while a combination of a welled and a flat hat decreases the size of the cavity by half. This type of carriers can suffer damage during freeze substitution and therefore are usually discarded after one usage (Studer, 2008).

To achieve the best freezing rate the type of holder and the medium that surrounds the sample must be optimized. As previously said, the volume inside the carrier that is filled with air should be replaced with a medium. The success of the freezing process relies upon the type of sample being frozen since different samples possess different water content and even the same cell has different water content between cytoplasm and organelles. Also, the freezing success can also vary due to naturally cryoprotective action within a sample. For the majority of the samples, the best choice of "filler" is one that possesses some cryoprotection capacity. Cryoprotectants suppress ice crystal formation and expansion, which leads to reduced heat release in the crystallization process. This improves the cooling rate of the sample, increasing the quality of freezing (Dahl, Staehelin, 1989).

There are a wide variety of cryoprotectants that can preserve the samples without having any interaction with them, and without influencing any cellular process (Morphew, 2002). They can be **non-penetrative and hydrophobic** substances that have low osmotic activity such as 1) serum albumin (10-20%); 2) low melting agarose (0.5% - 2.0%); 3) dextran (15-20%); 4) cold water fish gelatin (50-100%); 5) polyvinylpyrolidone (15%); 6) Ficol (5-15%); 7) 1-hexadecene; or they can be **penetrative** on some tissues such as sucrose (150mM) or methanol (10%) in yeast paste for filler.

Since the goal of vitrifying a sample is to allow its ultrastructural observation in a close to native state, it is important to minimize as much as possible the time between sample collection and freezing.

The Wohlwend HPF machine, developed according to Moor *et al*. (1980), pressurizes liquid nitrogen to ~2050 bar and then “shoots” it onto the sample holder to freeze the specimen, making the cryogen to act as a pressurizing agent. Pressurization and cooling of the sample are synchronized to occur within 20 ms in both systems (Fig. 7).

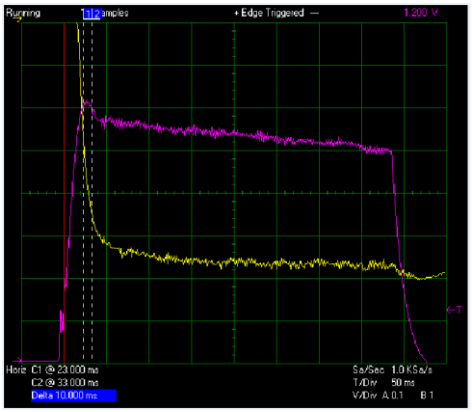


Fig. 7: Record of pressure (purple line) and temperature (yellow line) changes during freezing (total time = ~380 ms) in the Wohlwend Compact 02 high pressure freezing machine. Temperature drops approximately -120ºC.

4.3 Freeze substitution

With the pioneering work of Müller and Steinbrecht an old EM method called freeze substitution regained importance since it has proven to the best routine method for processing high pressure frozen samples. It allows relatively high quality structural preservation and also allows the use of immunogold and fluorescent techniques by combining cryofixation with resin embedding.

In freeze substitution, fixatives are added to the sample at very low temperatures (around -90ºC). They will diffuse into the cells at this temperature but not react (cross-linking) with cellular components. The fixatives only start to act at higher temperatures (~-50ºC for glutaraldehyde and ~-30ºC for osmium tetroxide) (Humbel and Müller 1986). Since chemical fixatives are already diffused throughout the sample when the cross-linking temperature is achieve, there is no diffusion-related fixation artifacts like the ones caused by conventional room-temperature methods and therefore no osmotic effects occur (Studer *et al*. 1992).

For freeze substitution the fixatives (glutaraldehyde or osmium tetroxide) are diluted in an organic solvent such as methanol or acetone. In the same cocktail uranyl acetate can be added to help improve membrane contrast. Under these conditions formation of cubic ice crystals may occur but typically their size is below the TEM resolution therefore the small crystals do not noticeably damage cellular ultrastructure (Kent, 2013).

Since the fixatives are diluted in organic solvent this means that dehydration of the samples starts at the same times as fixation. At these low temperatures, large molecules (such as proteins) are immobilized but smaller molecules (such as water) can be dissolved and replaced with the organic solvent.

After the freeze substitution is complete the samples can be brought up to room temperature without the risk of recrystallization since water is now absent from the sample.

The main advantages of this freeze substitution are:

1) Fixatives are already diffused through the sample prior to warming, which is when their cross-linking activity begins.
2) Dehydration takes place at very low temperatures, which helps to prevent ultrastructural artifacts as caused by room temperature dehydrations.
3) Samples can be processed for immunochemistry techniques. Since the sample is infiltrated and resin polymerized at low temperature, epitopes are more protected from damaging effects (Morphew, 2002).

5. Cilia

Cilia are 9-fold symmetric microtubule-based structures that protrude from the cell membrane. They can be motile or immotile and they have several functions in the cells such as promoting motility (in the sperm flagellum), stirring particles around (in the respiratory epithelium) and responding to various external signals (intercellular communication). As shown in Fig. 8, the cilium has two compartments: (1) the transition zone which is linked to a basal body docked to the cell membrane and (2) the axoneme (Jana *et al*, 2014).

At the transition zone the doublet microtubules are heavily cross-linked to the surrounding ciliary membrane by structures called y-linkers. The transition zone may act as a gatekeeper for material that goes into the cilia. The doublet MTs of the transition zone are thought to be a template for the MT skeleton of the cilium, also called axoneme, which consequently exhibits a 9-fold symmetry (Carvalho-Santos, 2012).

Since cilia are a component of most eukaryotic cells, when these organelles are altered they can lead to a wide variety of human diseases, which are referred to as ciliopathies. These diseases present a large spectrum of conditions including various syndromes, sterility, microcephaly, *situs inversus*, polycystic kidney disease, retinal degeneration, and dwarfism (Waters, A. Beales, P., 2011). Also, alterations of these organelles have been linked to cancer. All of these implications caused by cilia alterations stress the need of having a better understanding of cilia biology and structure.

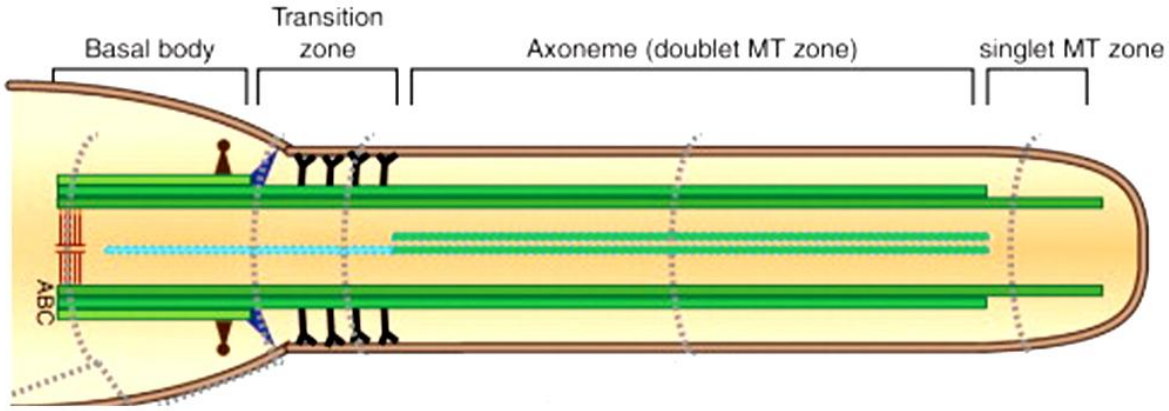


Fig. 8: Schematic representation of a longitudinal view of the cilium and its compartments. (Jana *et al*, 2014).

The structures described above were initially studied using conventional TEM, revealing remarkable features and structural complexity (Jana *et al*, 2014). Much more can be studied and learned using both the conventional electron microscopy approach and cryo techniques for tissue fixation. Also employing other modern techniques such as super-resolution light microscopy, x-ray crystallography, and cryoelectron tomography can gives us new insights (Carvalho-Santos, 2012).

To carry out these techniques, model organisms need to be used. Since cilia are conserved eukaryotic organelles several different model can be used such as *Drosophila melanogaster* (*Dm)* (Jana *et al*, 2014).

5.1 *Drosophila melanogaster*

Dm is a model organism widely used to research the fundamental mechanism of biological processes. For many reasons it is an excellent model to study the biology of centrosomes and cilia by fluorescent microscopy and also electron microscopy (Jana *et al*, 2014). Some of these reasons include the following: Many diverse cilia found in several uni and multi-cellular organisms are present within this single organism, the fruit fly (Briggs *et al*, 2014). Secondly, several genetic tools are already available and fly mutants of ciliary proteins are not embryonic lethal (Basto el al, 2006). Moreover, fertility, sensory responses and behavioral assays can serve as an output for fly ciliary functions (Enjolras, 2012). Also, nearly 75% of human disease-causing genes are believed to have a functional homolog in the fly. Lastly, short generation time, very low comparative costs and minimal ethical problems make *Dm* a unique organism to study cilia (Pandey, U. Nichols, C., 2011)

A pair of antennae is located on the front of the head of *Dm*, between the eyes. Each antenna serves as a sound and mechanical stimuli transducer, creating an electrochemical answer in the peripheral nervous system (Todi, 2004). Olfactory and auditory stimuli are detected by ciliated sensory neurons, on the third and second segment of the antenna respectively. At the distal tip of those neurons there is a cilium, serving as a bridge between the received stimuli and the cell body of the neuron (Vincensini, 2011).

The antenna is caped with an exoskeleton, the cuticle. This structure contains lipids and polysaccharide chitin, serving as a protective barrier and as an interface with the environment (Boseman *et al*, 2013). However, since chitin is a tough material it is hard to process for electron microscopy.

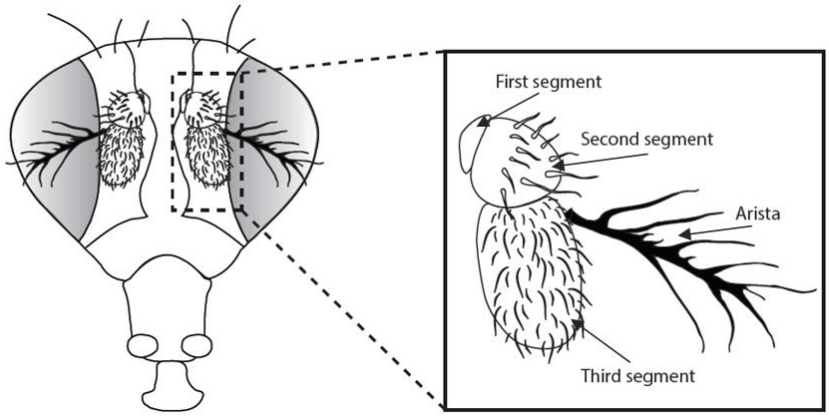


Fig. 9: Schematic representation of the head of *Drosophila melanogaster*. The inset shows one of the two antennae that are positioned between the eyes of the fly. In the inset the several segments of the antenna are represented as well as the arista, another sensory organ.

Each antenna consists of three segments (first segment – scape, second segment – pedicel and third segment – funiculus) and a thin arista (Fig. 9). The arista is the main sensory organ that receives sound. When there is a stimulus, the arista vibrates and activates the third segment of the antenna that twists relatively to the second segment. A thin stalk connects both segments and encloses the antennal nerve that descends until the third segment. The signal goes until the second segment, to the Johnston's organ where the chordotonal sensory neurons are. The axons of the

chordotonal neurons project to the antennal mechanosensory region of the brain. This way, the Johnston's organ can detect the sound signal and serve as a transducer (Todi, 2004).

The third segment of the antenna contains several sensillum, a sensory organ that protrudes from the cuticle and that encapsulates olfactory neurons. These sensilla can be divided into several types and there are approximately 200 basiconic (BS), 150 trichoid (TS) and 60 coeloconic sensilla (CS) surrounding this segment. Male flies have about 30% more TS but 20% fewer BS than female flies (Stocker, 1994).

Each sensilla are innervated with two to four olfactory receptor neuron (ORNs). Each of these olfactory neurons contains a cilium. The cilium of these neurons is constituted by a basal body, transition zone, axoneme and at the distal end there is a branched outer segment. As show in Fig. 10, in the transition zone of these neurons, as in many other types of ciliated cells, there are some structures that connect the microtubules to the membrane called y-linkers (Jana *et al*, unpublished).

All these morphologically different cilia are an attractive model for comparative studies regarding basic cilia assembly and differentiation mechanisms.

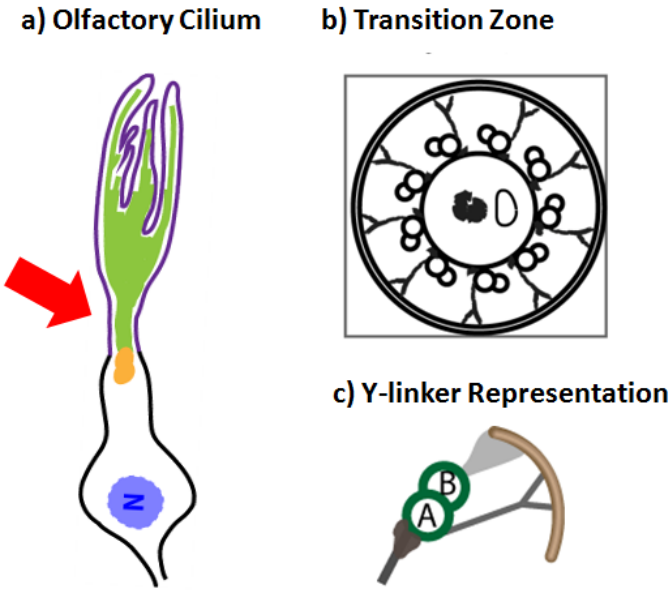


Fig. 10: Schematic representation of an olfactory cilium. a) Longitudinal scheme of olfactory cilium. The arrow represents the transition zone area. b) Cross section representation of the transition zone. c) Schematic representation of the Y-linker structures (dark grey) connecting the microtubules (green circles with A and B) to the membrane (brown). (Jana, S. unpublished)

5.2 Electron Microscopy in the antenna of *Drosophila melanogaster*

Some specimens can be difficult to fix if they have a barrier that does not allow diffusion of fixative and other chemicals in and out of the sample (McDonald, 2013). One example is the *Dm* that possesses an impermeable chitin coat that some say is impossible to preserve by direct aldehyde fixation. Conversely, after high pressure freezing and freeze substitution, their ultrastructure is maintained (Shanbhag, 1999; Shanbhag, 2000; Studer, 2008).

However, some studies on the antenna of *Dm* have been done using chemical fixation proving that it is possible to preserve such structure if an optimized protocol is used. In Fig. 11 there is an example of chemically fixed antenna, showing the cilia organization on the third segment of the antenna.

Nonetheless, chemical fixation very commonly introduces artifacts in the samples. If the goal of a researcher is to do ultrastructural studies of a specific organelle or cellular structure this can decrease the strength of the results obtain with this technique.

To overcome this problem, and to achieve a greater level of precision, cryo fixation techniques can be used. This method usually produces improved structural as preservation when compared to chemical fixation methods (Fig. 12) (McDonald, 2013).

Several cryo-fixation techniques were adapted for *Dm* tissues. However, so far there are no reports in the literature of a cryo-fixation study that focuses on the antenna of the *Dm*, in particular the third segment.

One of the challenges with this tissue is that it has a wide amount of empty space to allow neurons to vibrate. Air, however affects the success of high pressure freezing since while applying high pressures to a tissue, if it contains air it can collapse (McDonald, 2007).

One way to overcome this problem can be trying to fill these empty spaces before high pressure freezing the sample. It is described that the quality of samples fixed with aldehydes prior to being high pressure frozen is very similar to the ones high pressure frozen without the fixation step and superior to conventional glutaraldehyde fixation methods (Sosinsky *et al.,* 2008). Therefore, one solution could be to put the sample in fixative prior to high pressure freezing and allow the fixative to penetrate the sample, fixing it and filling the empty spaces.

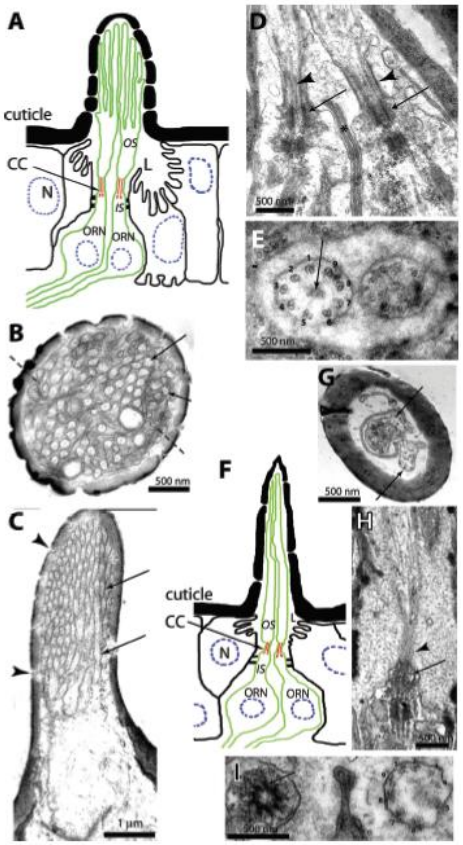


Fig. 11: Anatomy of olfactory cilium (Jana, 2011).

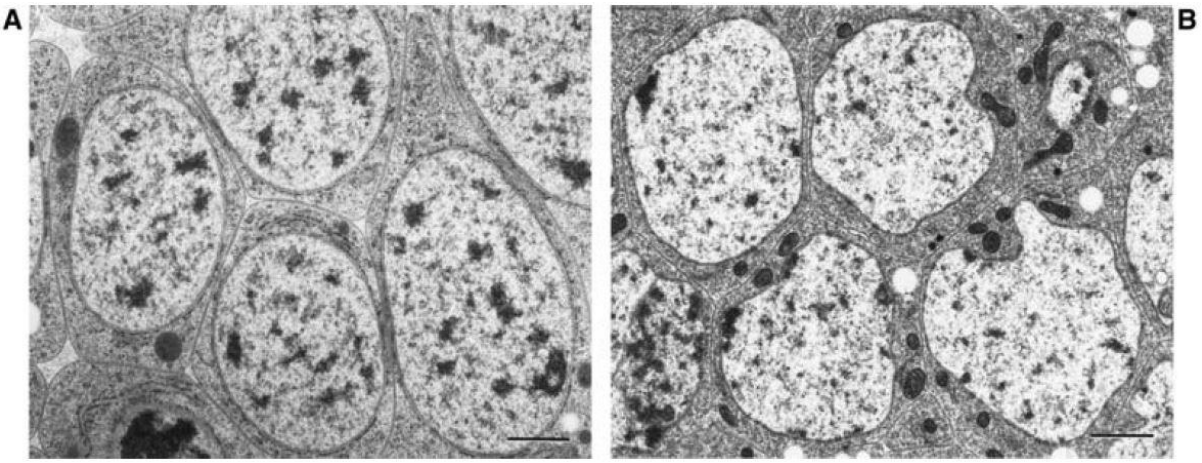


Fig. 12: Low-magnification views of epidermal cells processed by HPF-FS (A) or conventional fixation (B). Scale bars, 1 µm (McDonald, 2014).

6. Methodology

In this study, relationships between phenomena are going to be analyzed. Therefore, accordingly to Fortin this study can be classified as a correlational study (Fortin, 2000). As for the manipulation of variables, this is an experimental study, because the independent variables are going to be manipulated and an alteration of the dependent variables is expected. This is also a quantitative study, because the methodology is going to be based on a systematic process of data analysis quantifying objective outputs that occur independently of the researchers (Fortin, 2000).

6.2 Sampling and sample

Sampling was non probabilistic because there is a deliberate choice of the sample elements, and it is not representative of the entire population. It is accidental, since the organ was chosen by convenience, not being possible to generalize the results to other organs.

For chemical fixation the sample consists of 40 antennas. The samples were divided as outlined in Table 4 (times 2 rounds of processing):

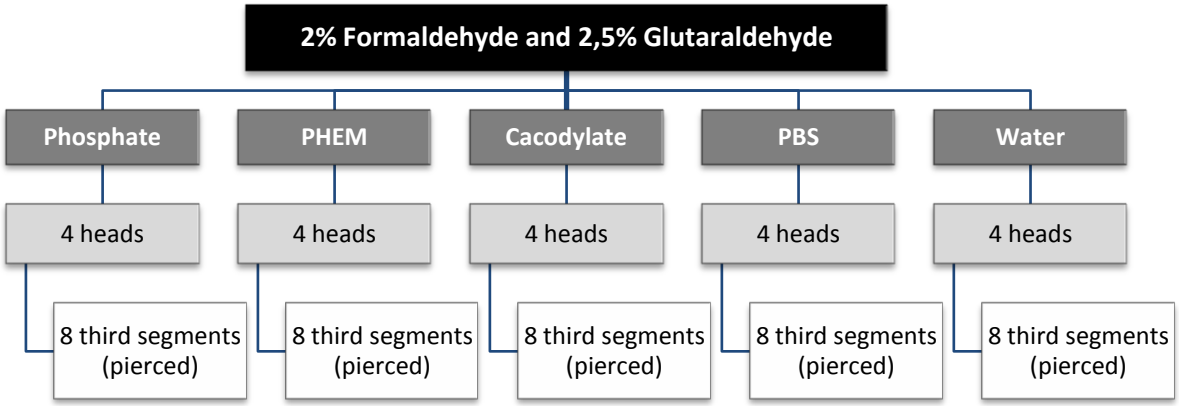


Table 4: Sample division for chemical processing according to each analyzed buffer.

For the freeze substitution protocol the samples were divided as shown in Table 5. There are 48 antennas (4 antennas x 4 carriers per condition x 2 rounds of processing per condition):

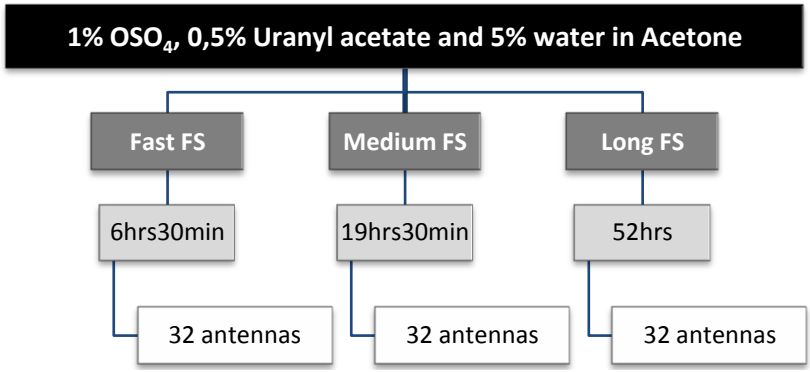


Table 5: Sample division for cryo processing according to each parameter to analyze.

6.3 Lab work

All the lab work was done at the Instituto Gulbenkian de Ciência in the Cell Cycle Regulation Laboratory and in the Electron Microscopy Facility.

6.4 Methods for chemical fixation

6.4.1 Sample processing solutions

6.4.1.1 Sodium bi-Phosphate buffer

- Stock solution A and B was first made:
    - Stock solution A: 0.2M monobasic sodium phosphate, monohydrate (27.6 g NaH_2PO_4 was dissolved in 1 L Milli-Q water).
    - Stock solution B: 0.2M dibasic sodium Phosphate (28.4 g Na_2HPO4 was dissolved in 1 L Milli-Q water).
- 28mL of stock solution A was mixed with 72mL of stock solution B to obtain 100mL of 0.2M of Phosphate buffer pH 7.2.

6.4.1.2 PBS buffer

- Phosphate buffered saline tablets were used.
    - 1 tablet of PBS was diluted in 150mL of WFI quality water to obtain 150mL of 0.2M PBS buffer.

6. 4.1.3 Cacodylate buffer

- 375mL of WFI quality water were measured.
- 21.4g of sodium Cacodylate (Na(CH3)2 AsO2 • 3H2O) were added to the water.
- The solution was mixed and the pH adjusted to 7.4
- When the pH was stabilized more water was added to the solution until made up to obtain 500mL of 0.2M Cacodylate buffer.

6. 4.1.4 PHEM buffer

- 4 NaOH pellets (approximately 1g) were diluted in 75mL of WFI quality water.
- 3.63g of PIPES (120mM) were added to the solution.
- The pH was set to 7.0. After the solution turned clear the following steps were performed:
- To the solution the following was added: 1.3g of HEPES (for a final concentration of 50mM), 0.08g of MgCl2.6H20 (final concentration 4mM), 0.76g of EDTA (final concentration 20mM).
- The pH was adjusted to 7.4.
- The volume was made up to 100mL with WFI quality water to obtain a final solution of 100mL of 0.2M PHEM buffer.

6. 4.1.5 Fixative for chemical Fixation

To make 100mL of fixative solution (2% Formaldehyde, 2,5% Glutaraldehyde in 0.1M buffer), 12.5mL of 16% formaldehyde (EM grade), 10mL of 25% glutaraldehyde (EM grade), 27.5mL WFI quality water and 50mL of 0.2M buffer (one of each of the previously mentioned) were mixed.

6.4.1.6 Uranyl Acetate solution

2% UA in d-water (20 g/L) and 2% UA in 70% methanol (20 g/L) were used for sample fixation/processing and for the staining of ultra-thin sections, respectively.

6.4.1.7 Reynold's Lead Citrate

30mL de-carbonated water was made by boiling the d-water in a microwave and by cooling the water in a freshly washed sealed container. 30mL of de-carbonated water was added to 1.33g of lead nitrate and 1.76g of sodium citrate into a volumetric flask and shaken vigorously for 1min followed by sonication for 30min. The container was agitated at five minutes intervals. 8.0mL of 1N sodium hydroxide (NaOH) was added in the opaque mixture and the mixture would then turn clear. The volume was brought up to 50mL with the de-carbonated water. The solution was stored in a tightly sealed volumetric flask. NOTE: The solution should be stored in a sealed container, since lead citrate reacts with carbon dioxide (CO_2) to form lead carbonate precipitate.

6.4.1.8 EPON^{TM} Epoxy resin

- Stock solutions were first made:
    - Stock EPON I: 62mL of EMbed 812 and 100mL of dodecenyl succinic anhydride (DDSA) were mixed.
    - Stock EPON II: 100mL of EMbed 812 and 90mL of nadic methyl anhydride (NMA) EPON working solution were mixed according to Luft (1961).
- NOTE: These two stock solutions should be stored at 4ºC separately. Since both EPON I and EPON II are strongly hydroscopic, the stock solutions should be mixed just before the mixture is used and warmed up before the containers are opened.
- To make the working EPON solution, 30mL of EPON I was then mixed with 70mL of EPON II and 1.5mL of Tris-(dimethylaminomethyl) phenol (DMP-30) (accelerator) was added.

6.4.2 Chemical Fixation Protocol

1) Flies were put to sleep with CO_2 and the heads were removed from the fly.
2) The heads were immersed in fixative (2% formaldehyde, 2.5% glutaraldehyde in 0.1M buffer (either Phosphate, PHEM, cacodylate, PBS or simply WFI quality water) pH 7.4) for 30min.
3) The third antennal segments were pierced using a thin tungsten needle.
4) Heads were transferred to the same fixative and were fixed overnight at 4ºC with rotation.
5) The samples were washed for 5 x 5min in the buffer used for the fixative solution.
6) The samples were post-fixed in 1% osmium tetroxide (OsO_4), for 1hr30min at 4ºC.
7) The samples were washed with Milli-Q water for 5 x 5min

8) The samples were incubated in 2% uranyl acetate for 20min in RT with rotation.
9) The samples were washed in Milli-Q water 3 × 10min.
10) The samples were dehydrated in a graded series of alcohol (50%, 70%, 90% and 100%) for 10min in each solution. Dehydration in 100% alcohol was repeated twice more to obtain complete dehydration.
11) The samples were treated in propylene oxide for 2 x 15min.
12) The samples were incubated with rotation in 1:1 propylene oxide: EPON resin for 3hr.
13) The samples were incubated in EPON resin overnight at 4ºC with rotation.
14) The next day, the samples were incubated in fresh resin (same batch as used in step 13) for 2hr at RT with rotation.
15) The samples were aligned in the molds with resin and the samples were labeled and polymerized overnight in 60ºC oven.

6.4.3 Randomization of samples

To ensure the buffer used in the sample fixation could not be identified throughout the rest of the sample processing, imaging and scoring the samples were randomized. A colleague of the electron microscopy facility was asked to give a random number to the samples during labeling and to do the labeling herself. During the entire processing and analysis only this person knew which number corresponded to each sample and the analyzer was completely blind. Only after the scoring of all the samples was the identity of the samples revealed to the analyzer.

6.4.4 Sample selection and sectioning

For every sectioning session five samples (one per condition) were selected and sectioned. To maintain the sample blinding, the person who labeled the samples selected the blocks to section. Each block was trimmed until the target area and then serial sectioning was made for a total of twelve grids per conditions. For the first block analyzed per condition (samples 1a, 1b and 1c of each buffer) the sectioning was done as follows: with an ultramicrotome, serial ultrathin sections (~70 nm) were cut using a diamond knife. For samples 1a, sixteen serial sections were obtained and divided into four groups of four sections. Each group of four sections was collected on a Formvar-coated copper grid. Between sample 1a and sample 1b a distance of 2µm was trimmed to have different cells to analyze in the following sample. Then sixteen serial sections were taken using the same criteria as used of sample 1a. Again, between sample 1b and sample 1c distance of 2µm was trimmed and another sixteen serial sections were taken.

16 serial sections	Trimmed area	16 serial sections	Trimmed area	16 serial sections
(4 sections per grid x 4 grids)	Discarded	(4 sections per grid x 4 grids)	Discarded	(4 sections per grid x 4 grids)
1.120 µm	2µm	1.120 µm	2µm	1.120 µm

Table 6: Schematic of how the sectioning was done for samples 1a, 1b and 1c.

For samples 2 and samples 3 of each buffer, the sectioning was done in the same way but only the first round of sixteen samples was collected, giving a total of four grids with four sections each per condition.

6.4.5 Sample post-staining

The grids (with sections) were post-stained with uranyl acetate 70% methanol and lead citrate solutions. The post-staining was done as follows:

Uranyl acetate staining: The grids (with sections) were post-stained by putting them (with the section side down) on a drop of 2% uranyl acetate in 70% methanol for 3min at RT. NOTE: The grids should be protected from the light to avoid precipitate formation.

The sections were rinsed by passing the grid through two drops of 70% methanol.

The grids were then washed in four drops of d-water and the grids were air-dried.

Lead citrate staining: In a closed chamber with NaOH pellets (used to absorb humidity), the grids were put with the section side down) on a drop of lead citrate for 1min. NOTE: A closed chamber with NaOH should be used to protect the sections from CO_2, avoiding precipitate formation.

The grids were washed in five drops of d-water and the grids were air-dried. The sections were ready to be imaged in a Transmission Electron Microscope (TEM).

6.4.6 Sample imaging

The samples were imaged using a Hitachi H-7650 120kV TEM. Each sample was imaged with the observer still blinded. For each sample the first grid of the group was analyzed. Six representative pictures of the areas of interest were taken to make a panel of picture per condition. Each panel was collected following the criteria defined in the following Table 7.

Structure	Area	Answers which criteria?	Magnification
Overall	Part of total sample	General Preservation	1 K
Cell body	Between two cells	Cellular membrane/ Cytoplasm/ artifacts	3K
	Nucleus and between cells	Nucleus and intercellular space	8k
Cilia	Longitudinal cilia	Helps to understand general preservation of the cilia	8k
Mitochondria	Mitochondria in the cytoplasm	Mitochondria preservation	12k
Transition Zone	One transition zone	Cilia evaluation table	12K

Table 7: Summary of the pictures needed to use the table for evaluation.

If not all six pictures were obtained in the observation of the first grid then grid two was analyzed, then grid three and four until all pictures were obtained. If not all pictures could be obtained after the evaluation of grid four then the sample was discarded. However, for this study there was no need to discard any samples.

6.4.7 Sample evaluation

6.4.7.1 Variables

In accordance with the objectives outlined, the following are considered dependent and independent variables for each type of processing (chemical and cryo processing):

Independent variables: In this study, the independent variable for the chemical processing protocol is the type of buffer (Phosphate, PHEM, cacodylate, PBS or water. For the cryo processing protocol the independent variable is the duration of FS protocol (rapid- 6hr30min, medium – 19hr30min or slow – 52hr).

Dependent variables: The dependent variables, conditioned by manipulating the previously mentioned independent variables, are considered to be the final quality of samples observed by TEM, which is based on ten parameters:

- General preservation – any electron microscopy technique should maintain the cell structures in a close to native state of preservation, to allow proper observation of areas of interest and to ensure a reliable characterization of the entire sample;
- Cellular membrane preservation – the membrane should be continuous, have a smooth contour along the entire cell and there should not be any vesiculation (blebbing) present;
- Cytoplasm preservation – the cytoplasmic content should be evenly distributed throughout and not show areas of clumping or empty spaces;
- Intercellular space preservation – the space between cells should be narrow and empty space should not be visible. The intercellular space was measured in several HPF pictures (Sanbhag, S. *et al*. 1999, 2000) to determine a mean value that resembles a close to native state;
- Nuclear preservation – Nucleus should maintain the expected shape for the type of cell and cell cycle; the double membrane should be visible and evenly spaced through the entire nuclear contour; the contour should be smooth around the entire nucleus;
- Mitochondria preservation - Mitochondria should have the expected shape and the membrane should be visible and not ruffled;
- Artifacts – there should be no artifacts resulting from the fixation method - neither extraction in the chemical fixation protocol nor ice crystal in the cryo fixation protocol;
- Ciliary membrane preservation – the ciliary membrane should be continuous and round shaped;
- Microtubule preservation – On a clear cross-section of the transition zone, the doublet microtubules should be visible;
- Preservation of other structures - On a clear cross-section of the transition zone, the y-linker structures should be visible and the electron density surrounding the microtubules should not impair their visualization.

To analyze these dependent variables and be able to understand the influence of the independent variables in the ultrastructural preservation of the sample a table with a set of criteria were defined. The criteria defined are based on the dependent variables that were to be evaluated.

The table was constructed specifically to allow the comparison between each protocol (either the chemical fixation protocol or the cryo fixation protocol). The table is divided into two main topics: general preservation of the sample and the cilia preservation in the tissue. Together, these two topics make the Criteria table for judging ultrastructural preservation. The example of the table created to evaluate each protocol is presented below (Tables 8 and 9).

To ensure an unambiguous interpretation, each parameter was defined by assigning it a value for both Table 8 and Table 9 (an evaluation of “no” corresponds to a 0 and an evaluation of “yes” corresponds to a 1). For each value a specific description was created that clarifies the criteria to be used by the observer, with the aim of standardizing the evaluation process across different samples and different observers. The maximum total that can be obtained with this quantitative analysis for the final evaluation score (general score + cilia score) is a total score of 20 (all parameters with positive evaluation) and the minimum total that can be achieved is a total of 1 (all parameters with negative evaluation except parameter G15). The minimum value that can be achieved for the final evaluation cannot be 0 since giving a negative evaluation to parameter G15 in the general evaluation table automatically invalidates the evaluation of the sample.

The observer was only allowed to know the identity of each sample after all the evaluation tables were filled, so that the final results could be analyzed.

After the observer analyzed all the samples, four of them were selected. These samples are representative of different difficulties to analyze. It was chosen one easy, two medium and one hard sample to analyze, corresponding to samples A- Water (sample 3), B- Phosphate (sample 1b), C- Water (sample 1a) and D- PBS (sample 3). These four samples were coded with the letter just mentioned (A, B, C and D) and given to three other observers, with different backgrounds and EM experience, to judge if the table can be used by different EM experienced users. The observers selected were one EM technician, one biologist and one air traffic controller. The identity of all the samples remained unknown to all the observers until the end of the evaluation.

General Evaluation Table

Structure	Criteria	Yes	No
General Preservation	G1 - Entire tissue shows overall preservation throughout the entire picture when observed at lower magnification		
Cellular Membrane	G2 - Is visibly continuous around the entire cell border		
	G3 - Shows a smooth contour along the entire cell		
	G4 – Absence of membrane associated artifacts (blebbing - vesiculation of membranes – and/or detachment between inner and outer membrane layer)		
Cytoplasm	G5 - Content is homogenously distributed through the entire cytoplasm, not confined to a specific area and with no empty spaces		
Intercellular space	G6 - Is narrow (below 32.7 ± 10.4 nm of space between cells → mean and standard deviation of 4 measurements – 3 random locations in the intercellular space and another 1 that represents the maximum distance between membranes)		
Nucleus	G7 - Nucleus maintains the expected shape for the type of cell or cell cycle (select a shape, like oval, and use it as a reference to analyze the nucleus shape)		
	G8 - Nuclear membrane is visibly continuous around the entire nucleus border		
	G9 – In the areas where there is nuclear membrane, the double membrane looks evenly spaced		
	G10 - Nuclear membrane shows a smooth contour along the entire nucleus		
Mitochondria	G11 - Mitochondria have the expected shape		
	G12 – Outer membrane shows a smooth contour		
	G13 – Cristae have a smooth contour (are not ruffled)		
Artifacts	G14 - Absence of specific artifacts for the fixation technique: • Chemical fixation – extraction • Cryo fixation – Ice damage (either extracellular or intracellular)		
	G15 - * Absence of artifacts that invalidate the evaluation of the sample. *E.g.*: • Total or partial absence of sample due to poor infiltration • Sectioning artifacts that unable the visualization of the sample, like knife marks or incomplete sample • Staining artifacts that unable the visualization of the sample		

Table 8: Classification table for the evaluation of the general features of the samples.

Cilia Evaluation Table

Structure	Criteria	Yes	No
Ciliary membrane	C1 - Ciliary membrane is continuous		
	C2 – Cilia membrane is round-shaped (doesn't show a flower-shape pattern)		
Microtubules	C3 - On a clear cross-section is visible that all doublets are present		
Other structures	C4 - On a clear cross-section y-linkers are visible in the transition zone		
	C5 - The electron density surrounding the microtubules is clear enough to allow their visualization		

Table 9: Classification table for the evaluation of the ciliary features of the samples.

6.4.8 Statistical analysis

To analyze the data, the following statistical procedures were used:

- Evaluation tables created using the worksheet in Microsoft Office Excel 2010;
- All the statistical calculations done in GraphPad Prism 6 ® statistical analysis software;
- Statistical inference done using the non parametric Kruskall-Wallis test and Dunn's multiple comparisons test with statistical analysis software;
- Descriptive statistics (calculation of maximum, minimum, median and quartile range) also done using statistical analysis software.

6.5 Methods for cryo fixation

6.5.1 Sample processing solutions

6.5.1.1 Freeze substitution cocktail

- 9.25mL of low water acetone were first cooled down for a few minutes inside the automatic freeze substitution machine.
- 0.5mL of Milli-Q water was added to the solution followed by 0.25mL of 20% uranyl acetate in methanol.
- 0.1g of osmium tetroxide was added to the solution to obtain 10mL of 1% OsO_4, 0.5% UA and 5% water in acetone.
- The solution was mixed with a Pasteur pipette (by bringing the solution in and out of the pipette).
- The solution was transferred to the automatic freeze substitution machine and stayed there cooling down until the temperature of the solution reached close to the temperature necessary for freeze substitution (approximately -90ºC)

6.5.2 Cryo Fixation Protocol

1. HPF machine was turned on (takes about 30min until it is ready)
2. Flies were put to sleep with CO_2 and the heads were removed from the fly.
3. The heads were immersed in fixative (2% formaldehyde, 2.5% glutaraldehyde in 0.1M Phosphate buffer pH 7.4) for 30min.
4. The third antennal segments were pierced using a thin tungsten needle.
5. Heads were transferred to the same fixative and were fixed for another 15min.
6. The samples were transferred to a 0.150µm thick aluminum carrier previously dipped in 1-hexadecene (to fill the empty spaces of the carrier) and containing 10% BSA in Phosphate buffer.
7. A flat carrier was added to close the carrier sandwich and it was transferred to a carrier holder.
8. The carrier holder was loaded in the HPF machine and the sample was frozen.
9. The carrier holder was quickly transferred to a small box filled with liquid nitrogen.
10. At this point the sample can be either freeze substituted or can be stored in liquid nitrogen until freeze substitution can be done. To continue with freeze substitution go to step 13. For cryo storage continue to step 11.
11. The carrier holder was opened and the carrier was transferred into a cryo-storage holder containing several 1mL labeled eppendorfs.
12. The cryo storage holder was stored in a cryo storage tank until the sample was required (ideally should be less than four weeks from sample freezing)
13. The carrier was put inside a container submerged with liquid nitrogen.
14. When the automatic freeze substitution machine was ready and at the right temperature (-90ºC) the carriers were transferred into a custom made metallic support.
15. The carrier were opened and transferred into a plastic support that has specific places to keep the carrier through the whole processing.
16. The freeze substitution cocktail (1% OsO_4, 0.5% UA and 5% water in acetone) was added to the samples
17. The samples were infiltrated for 3hr (fast freeze substitution), 10hr (medium freeze substitution) or 36hr (slow freeze substitution).
18. After the infiltration the temperature was allowed to rise to 0ºC over 3hr (fast freeze substitution), 9hr (medium freeze substitution) or 15hr30min (slow freeze substitution) in slopes of 30min.
19. The samples were washed in fresh acetone at 0ºC for 1hr30min.
20. The samples were embedded in a graded series of acetone and Epon resin: 50% Epon in resin, 100% Epon for 30min each.
21. The 100% Epon resin was changed to a new one and the carrier with the samples was put in the oven overnight at 60ºC for the resin to polymerize.

6.5.3 Randomization of samples

Done similarly to described in 6.4.3

6.5.4 Sample selection and sectioning

Done similarly to described in 6.4.4

6.5.5 Sample post-staining

Done similarly to described in 6.4.5

6.5.6 Sample imaging

Done similarly to described in 6.4.6

6.5.7 Sample evaluation

Done similarly to described in 6.4.7

6.5.8 Statistical analysis

Done similarly to described in 6.4.8

6.6 Materials, Reagents, Equipment and IPE

For this chapter consult appendix, subchapter 10.6.

7. Results

7.1 Chemical fixation results

7.1.1Evaluation chart with the buffer scores

The results of the sample quality scoring that was done using the evaluation tables developed (see tables 7 and 8) is shown as the score per criteria per sample for all the five different buffers, as described in the methods section and can be consulted in appendix I. There is only one criteria that achieves the maximum value (total score of 25 out of 25 samples) for all the samples which is the G15 (artifacts that invalidate evaluation). On the other hand there are two criteria, G3 (cell membrane contour) and G10 (nuclear membrane contour) that get the minimum possible value (total score of zero out of 25 samples). Also, the criteria G5 (cytoplasm homogeneity), G6 (intercellular space), G12 (mitochondria membrane contour), G13 (mitochondria cristae contour), G14 (artifacts of the technique) and C2 (cilia membrane shape) achieve only a very low total value (total score equal or lower than five out of 25 samples). The criteria G2 (cell membrane continuity) achieved a very high total value (total score of 20 out of 25 samples).

7.1.2 Sample scoring by different evaluators

Four different samples were given to three independent evaluators, each with different scientific backgrounds and levels of electron microscopy knowledge. These evaluators scored the same four samples using the “Criteria for judging ultrastructure” table. Fig. 13 presents the final score median and range for each sample, for the four different evaluators. There are no significant differences between evaluators. The minimum (ranging from 0.20 - 0.30) and maximum (ranging from 0.60 – 0.65) score of the samples is very similar between evaluators. The median score from the evaluation made by the electron microscopy technicians is lower than the non electron microscopy technicians.

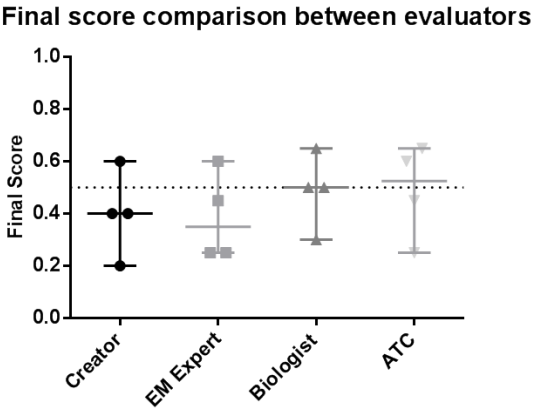


Fig. 13: Graphic representing the final score of the samples analyzed by four independent evaluator (creator of the table, an electron microscopy expert, a biologist and an air traffic controller) to assess evaluation table utility.

7.1.3 Buffer final evaluation scores

The “Criteria for Judging Ultrastructure Quality” table was used to score the five different buffers. Each criteria was analyzed blindly by me, using the panel of pictures created for each buffer, for each sample (see tables 7 and 8). The values given to each criteria (0 or 1) were registered in a Microsoft Excel sheet (see appendix I) representing the quantitative analysis of each buffer.

The final scores obtained with this method (which are a ratio - the sum of the score for all the 20 criteria divided by 20) were then used to create the "Buffer Evaluation Scores" table. Fig. 14 presents the median and range for each buffer, for the five different samples analyzed per buffer. Looking at Fig. 14, from all the 25 samples analyzed (divided by five buffers), only four have score values above half of the maximum score (0.5). One corresponds to Cacodylate buffer (score 0.55) and the other three to PBS buffer (scores 0.5, 0.60 and 0.65). Also, only one buffer median hits the 0.5 score corresponding to PBS. The difference in final score between PBS and water is statistical significant.

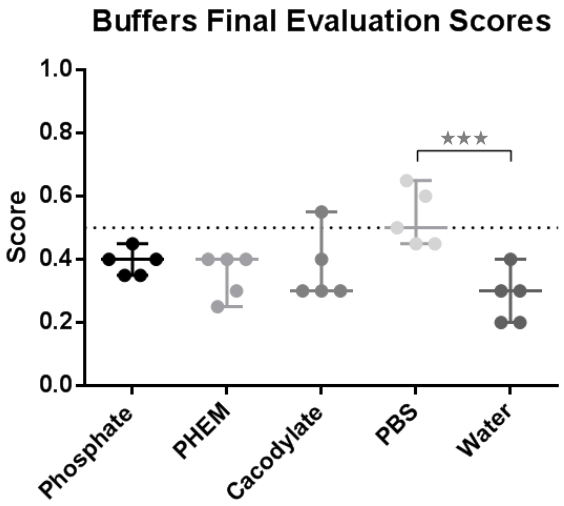


Fig. 14: Graphic representing the final evaluation score of the samples analyzed for the five different buffers used in the chemical fixation protocol.

As for descriptive statistics, we can see that the buffer with the lowest minimum is Water (0.2) and the buffer with the highest maximum is PBS (0.65). The buffers with lowest median are Cacodylate and Water (both with 0.3) and the buffer with the highest median is PBS (0.5).

7.1.4 General evaluation scores

As mentioned above, the final scores obtained are a sum of the general evaluation scores and the cilia scores. Fig. 15 shows the general evaluation scores for each buffer and presents median and range for each buffer, for the five different samples analyzed per buffer.

As shown in Fig. 15, from all the 25 samples analyzed (divided by five buffers), only four have score values above half of the maximum score (0.5). One corresponds to PHEM buffer (score 0.53) and the other three to PBS buffer (scores 0.53, 0.6 and 0.67). Also, only one buffer median is above the 0.5 score corresponding to PBS (0.53). However, there are no statistically significant differences between buffers.

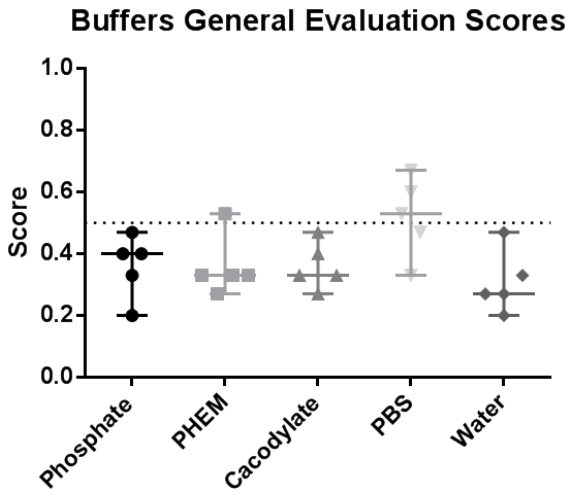


Fig. 15: Graphic representing the general evaluation score of the samples analyzed for the five different buffers used in the chemical fixation protocol.

As for descriptive statistics, we can see that the buffers with the lowest minimum are both Phosphate and water (0.2) and the buffer with the highest maximum is PBS (0.67). The buffer with lowest median is Water (0.27) and the buffer with the highest median is PBS (0.53).

7.1.5 Cilia evaluation scores

Fig. 16 shows the cilia evaluation scores for each buffer. This graphic presents median and range for each buffer, for the five different samples analyzed per buffer.

Looking at Fig. 16, from all the 25 samples analyzed (divided by five buffers), nine have score values above half of the maximum score (0.5). Three correspond to Phosphate buffer (scores 0.6, 0.6 and 0.8), two to PHEM buffer (both scores 0.6), one to Cacodylate (score 0.8) and finally three correspond to PBS buffer (scores 0.6, 0.8 and 0.8). Two buffer medians are above the 0.5 score corresponding to Phosphate (0.6) and to PBS (also 0.6). However, there are no statistically significant differences between buffers.

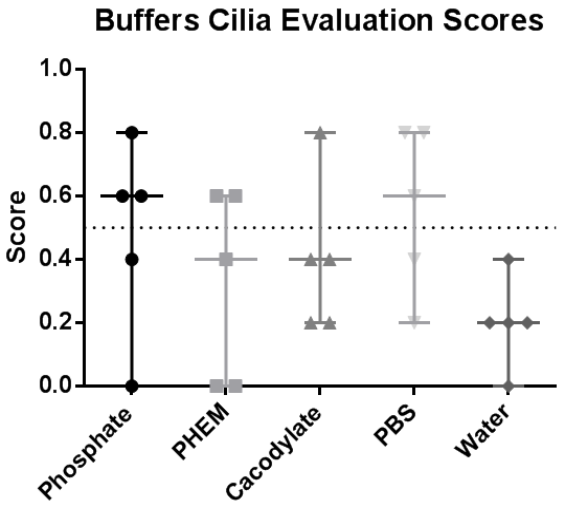


Fig. 16: Graphic representing the cilia evaluation score of the samples analyzed for the five different buffers used in the chemical fixation protocol.

As for descriptive statistics, we can see that the buffers with the lowest minimum are Phosphate, PHEM and water (0.0) and the buffers with the highest maximum are Phosphate, Cacodylate and PBS (0.8). The buffer with lowest median is Water (0.2) and the buffer with the highest median is Phosphate and PBS (0.6).

7.1.6 Panel Analysis

Fig. 17 shows the representative pictures of the best cilia score (top row) and the worse cilia score (bottom row) for each of the five different buffers analyzed. The best and worst cilia score pictures depict the best and worst cilia scores obtained using the evaluation tables (see appendix 10.1).

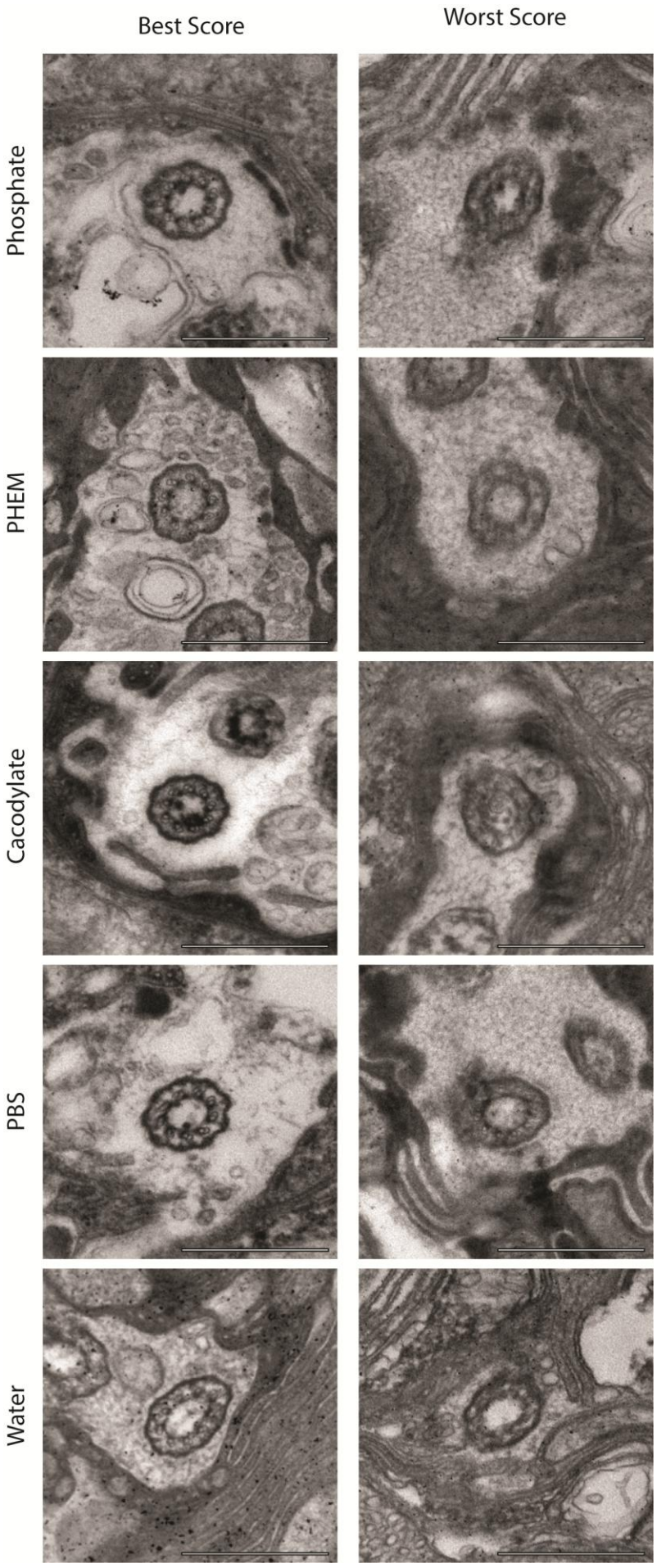


Fig. 17: Pictures representing the best and worst cilia scores for all the different buffers analyzed in the chemical fixation protocol. Scale bars represent 500nm.

7.2 Cryo fixation results

7.2.1 Evaluation chart with the buffer scores

The results of the sample quality scoring that was done using the evaluation tables I developed (see tables 7 and 8) are shown as the score per criteria per sample for all the three different freeze substitution times (see appendix II). There are four criteria that achieve the maximum value per criteria for all the samples, which are the G11 (mitochondria shape), G15 (artifacts that invalidate evaluation), C1 (cilia membrane continuity) and C2 (cilia membrane shape) in a total score of six out of six samples. On the other hand there are two criteria, G10 (nuclear membrane contour) and C4 (y-linkers visibility) that get the minimum possible value (total score of zero out of six samples). Also, the criteria G2 (cell membrane continuity) and G4 (cell membrane artifacts) achieve a very high total value (total score of five of six samples).

7.2.2 Freeze substitution final evaluation scores

The "Criteria for Judging Ultrastructure Quality" table was used to analyze the three different freeze substitution times. Each criteria was analyzed blindly by me, using the panel of pictures created for each freeze substitution time, for each sample (see appendix II). The evaluation per sample was done similarly to the chemically fixed samples (see 7.1.3 Buffer final evaluation scores). However, since only two samples were analyzed per freeze substitution time statistical analysis was not done for the cryo fixation. The points corresponding to each sample score per freeze substitution time are shown in Fig. 18.

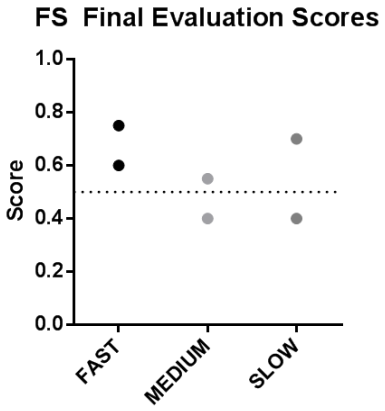


Fig. 18: Graphic representing the final evaluation score of the samples analyzed for the three different freeze substitution times used in the cryo fixation protocol.

From the six samples analyzed (divided by three freeze substitution protocols), four have score values above half of the maximum score (0.5). Two correspond to the fast freeze substitution (score 0.6 and 0.75), one to the medium freeze substitution (score 0.55) and the other one to slow freeze substitution (score 0.7). The fast freeze substitution protocol has the highest score from the three protocols (Fig. 19).

7.2.3 General evaluation scores

The final scores obtained are a sum of the general evaluation scores and the cilia scores. Fig. 19 shows the general evaluation scores for each freeze substitution.

Looking at Fig. 19, from all the six samples analyzed (divided by three freeze substitution times), four have score values above half of the maximum score (0.5). Two correspond to the fast freeze substitution (score 0.67 and 0.73), one to medium freeze substitution (score 0.6) and the other one to slow freeze

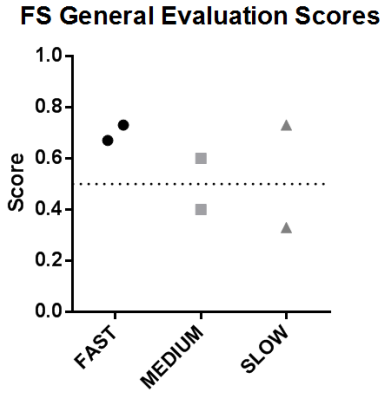


Fig. 19: Graphic representing the general evaluation score of the samples analyzed for the three different freeze substitution times used in the cryo fixation protocol.

substitution (score 0.73). The fast freeze substitution protocol has the highest score from the three protocols.

7.2.4 Cilia evaluation scores

Fig. 20 shows the cilia evaluation scores for each freeze substitution.

Looking at Fig. 20, from all the six samples analyzed (divided by three freeze substitution times), three have score values above half of the maximum score (0.5). One corresponded to the fast freeze substitution (score 0.8) and the other two to slow freeze substitution (both scores 0.6). The fast freeze substitution protocol has the highest score from the three protocols although with the range shown all three conditions are scoring about the same.

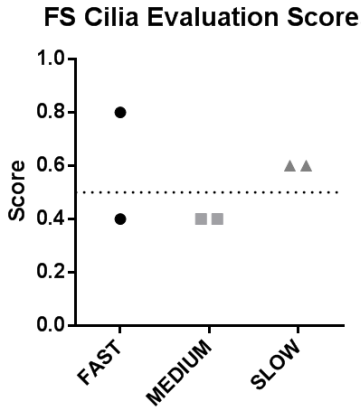


Fig. 20: Graphic representing the cilia evaluation score of the samples analyzed for the three different freeze substitution times used in the cryo fixation protocol.

7.2.5 Panel Analysis

Fig. 21 shows the representative pictures of the best cilia score (top row) and the worst cilia score (bottom row) for each of the three freeze substitution times analyzed. The best and worst cilia scores pictures depict the best and worst cilia scores obtained using the evaluation tables (see appendix II). However, it is necessary to take into consideration that besides the fast and medium freeze substitution protocol showed for best score, all other protocols show ice crystal damage so the best and worst score pictures may not reflect the true preservation of the tissue.

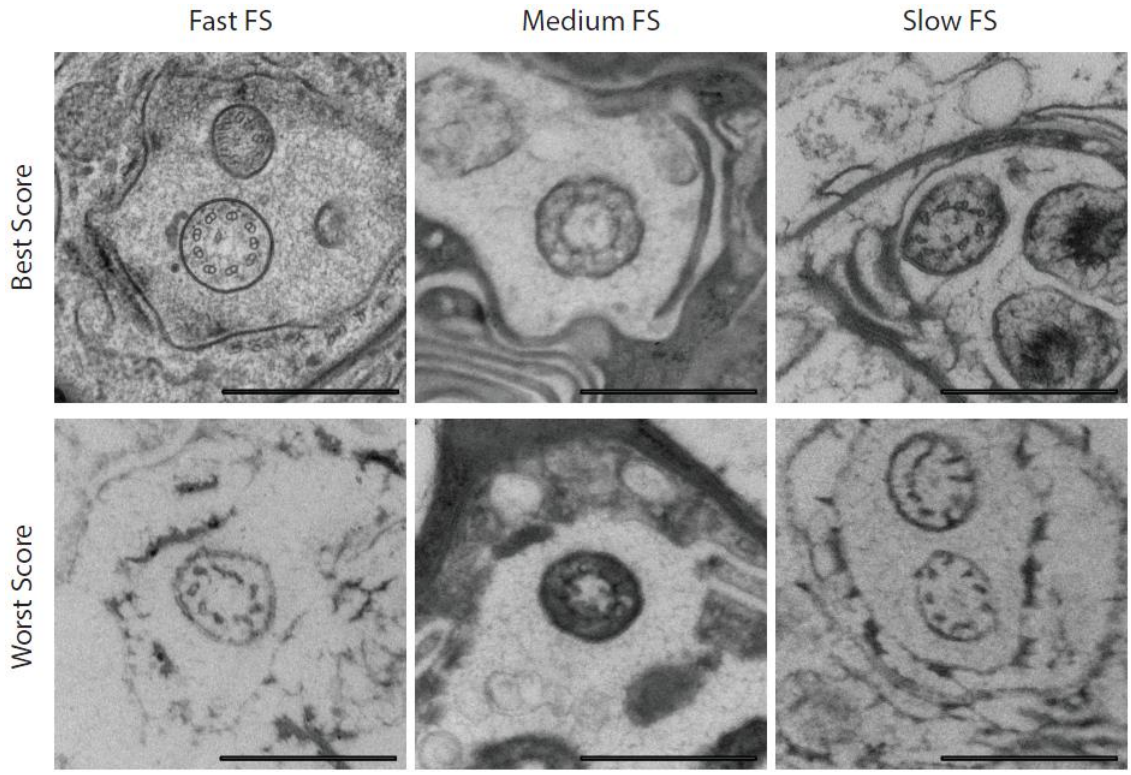
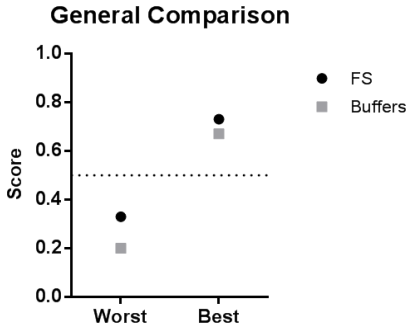


Fig. 21: Pictures representing of the best and worst scores for all the freeze substitution times analyzed in the cryo fixation protocol. Scale bars represent 500nm.

7.3 Chemical vs Cryo fixation

7.3.1 Comparison of the general score between the best and worst buffer and the best and worst freeze substitution protocol

Fig. 22 shows the representative pictures of the best general score (top row) of all the buffers and freeze substitution times analyzed and the worse final score (bottom row) of all buffers and freeze substitution times analyzed. The graphic represents the values of the general score from the samples represented in the figure.



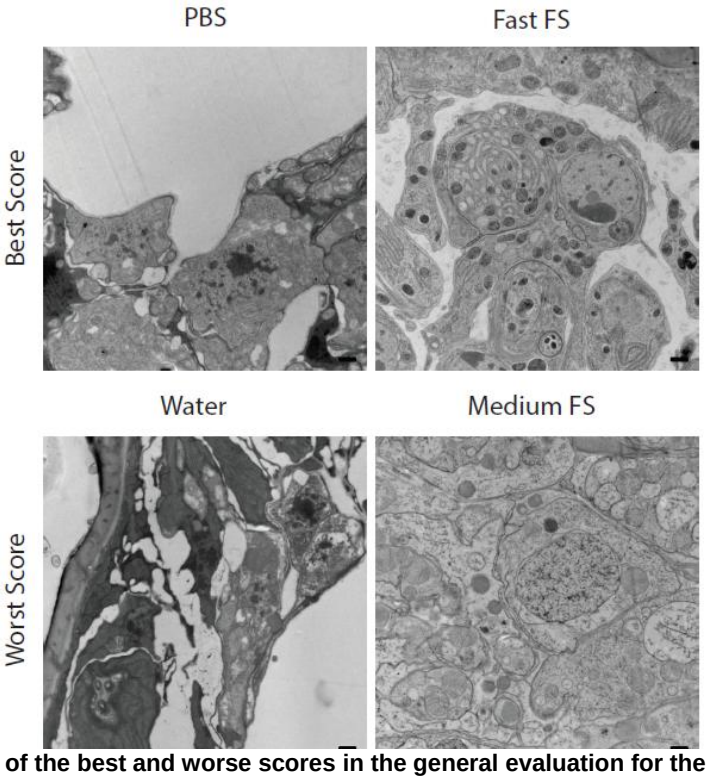
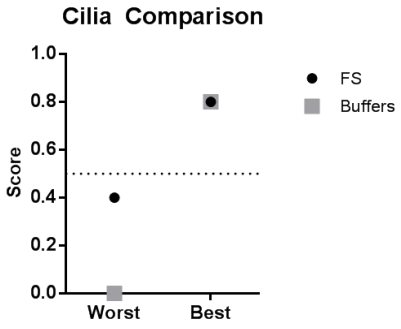


Fig. 22: On the left: Representative graphic of the best and worse scores in the general evaluation for the samples analyzed for chemical and cryo fixation. On the right: Pictures representative of the values of the general score from the samples represented in the panel. Scale bars represent 500nm.

7.3.2 Comparison of the cilia score between the best and worst buffer and the best and worst freeze substitution protocol

Fig. 23 shows the representative pictures of the best cilia score (top row) of all the buffers and freeze substitution times analyzed and the worse cilia score (bottom row) of all buffers and freeze substitution times analyzed. The graphic below represents the values of the cilia score from the samples represented in the figure.



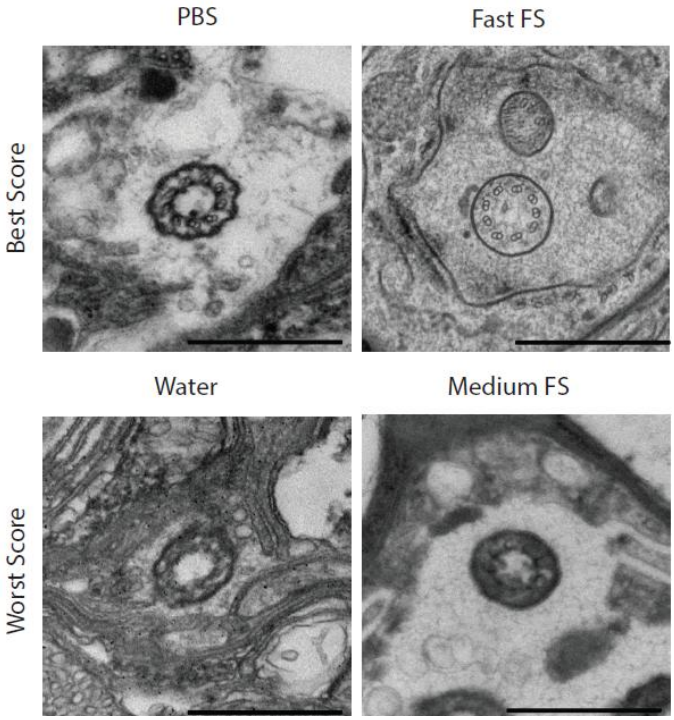


Fig. 23: On the left: Representative graphic of the best and worse scores in the cilia evaluation for the samples analyzed for chemical and cryo fixation. On the right: Pictures representative of the values of the cilia score from the samples represented in the panel. Scale bars represent 500nm.

8. Discussion and Conclusion

Chemical Fixation achieves lower scores that Cryo Fixation

When analyzing the evaluation chart results for the buffer scores (appendix 10.1) it becomes apparent that for general chemical fixation the criteria scores are low for all buffers. On the other hand, for cryo fixation (appendix 10.2) the majority of the criteria have very high scores along all the different freeze substitution protocols. If we compare the results obtained with both techniques it is visible that the criteria G10 (nuclear membrane shows a smooth contour along the entire nucleus) has a total score of zero for all the samples analyzed, in other words, that the nuclear membrane of the cells analyzed in all the samples had an irregular contour. This might mean that the nuclear membrane is one of the most difficult structures to preserve regardless of the fixation technique used or that the protocols used are not adapted well to preserve this structure. This is useful information for studies that focus on nuclear membrane components since this distortion of the membrane might not be only physical but could represent loss of some of its structural components.

Although the samples can be evaluated, both Chemical Fixation and Cryo Fixation show specific artifacts of the technique

The criteria G15 (absence of artifacts that invalidate the evaluation of the sample) obtained a score of one for all the samples analyzed. This means that both chemical and cryo fixation techniques were done properly, allowing the imaging and evaluation of all the samples. However, in both techniques specific artifacts of the fixation technique were visible, as showed by the low score of criteria G14 (artifacts of the technique). For the chemical fixation, all the samples but one from PHEM buffer showed overall extraction and for the cryo fixation four samples out of the six in total showed ice damage.

For chemical fixation, these results were not expected. A study comparing the amount of extraction of different cell components by fixation with glutaraldehyde in different buffers in plant cells for the most commonly used buffers for electron microscopy showed the following order of increasing extraction: phosphate, HEPES, no buffer (water), PIPES, Cacodylate (Coetzee and van der Merwe. 1984). Although plant cells and animal cells behave differently during fixation (since the latter doesn't have a cell wall) the buffer effect on extraction level between both should be similar. Therefore, it was expected that Phosphate (and PBS) buffer showed the best cytoplasmatic matrix preservation, followed by PHEM and that water and Cacodylate caused some extraction. This fact makes me believe that the extraction seen in the chemical fixed samples might be caused by the secondary fixation in osmium tetroxide, since "a substantial amount of cellular protein in glutaraldehyde-fixed tissue is extracted following postfixation with osmium tetroxide" (Hayat, 1981). To reduce the number of variables in this study, instead of secondary fixing the samples in osmium tetroxide in the corresponding buffer, aqueous osmium tetroxide was used. Although several references state that osmium tetroxide can be diluted in water (Claude, 1961; Barer and Cosslet, 1968) others say that it should be diluted in the same buffer as the primary fixative (Glauert, 1971; Dawes 1975; Hayat 1986).

In the future, to improve the current chemical fixation protocol the secondary fixation should be done diluting the osmium tetroxide in the same buffer used for the primary fixation.

Also, according to Hayat (1981) the degree of extraction can also be dependent on the duration of osmium tetroxide fixation. The longer the fixation with osmium tetroxide the more cellular proteins are destroyed and the more extraction during dehydration. This might mean that another adjustment that can be made to the current chemical fixation protocol is decreasing the secondary fixation time with osmium tetroxide, for example to only one hour.

Cryo-fixation improves the ultrastructural preservation of the cell components, however some lack contrast

Continuing with the criteria evaluation, the criteria G8 (nuclear membrane is visibly continuous around the nucleus border) is better for chemical fixation than for cryo fixation. This was expected since samples cryo fixed usually have membranes with a lack of contrast, making it more difficult to visualize (Gidding, 2003). However, not being able to visualize a structure does not necessarily mean that the structure is not preserved. It can mean that the data need to be post-processed to improve the visualization.

Some criteria had a big improvement when cryofixed. This is visible for criteria G6 (the intercellular space is narrow) and especially for the mitochondria criteria G12 (outer membrane shows a smooth contour) and criteria G13 (cristae have a smooth contour – are not ruffled). These improvements were expected. Larger intercellular spaces are most probably caused by shrinkage during the chemical fixation protocol (Rostgaard and Tranum-Jensen, 1980), so it is expected that cryo fixation and freeze substitution reduce the intercellular space, keeping it closer to its original value. Also, it is already reported that membranes of high pressure frozen and freeze substituted samples are morphologically better preserved than the ones chemically fixed (Giddings, 2003; McDonald, 2012).

Following the idea of achieving better membrane morphology preservation, as expected, the cilia criteria C2 (cilia membrane is round shaped – does not show a flower-shape pattern) achieves a very high total score in the cryo fixed samples, in contrast to what happens in the chemical fixed samples. On the other hand, the criteria C4 (on a clear cross-section y-linkers are visible in the transition zone) achieves the lowest score in the cryo fixed samples. These two observations lead me to speculate they might be related. The flower shape pattern that is visible in the chemically fixed samples is present in the area analyzed, called the transition zone. So far, it has never been reported anywhere else in the cilia. At the same time, the transition zone is the only area of the cilia where the y-linker structure was reported and they are not artifacts caused by the chemical fixation technique since they have already been observed with a cryo technique, freeze etching (Gilula, N., Satir P., 1972). The y-linkers connect the microtubules to the membrane. It might be that they are applying a force to the membrane that confers it that flower like pattern or the y-linkers stabilize the membrane and prevent it from widening following fixation. On the other hand, the y-linker structures are not visible by cryo-fixation. It may be that the proteins that make up the y-linkers are not visible due to reduced extraction with this technique or they were not stained with the fixation protocol used.

Although there are no significant differences between buffers, PBS shows better general ultrastructural preservation of the antenna

When comparing buffers based on the final evaluation scores (Fig. 14) it is visible that there is no significant statistical difference between their score. However, the highest score is PBS and the one with the lowest score is water, with their score being statistically different. According to Weakley (1981), formaldehyde and glutaraldehyde are small molecules that can easily penetrate the tissues, giving the buffer a reduced importance in tissue preservation than for example with osmium tetroxide fixative. It is therefore normal that the difference in the ultrastructural preservation obtained with different buffers is not big. This is also supported with the observations of Maunsbach (1966) and Busson-Mabillot (1971) where no differences were found with differently buffered glutaraldehyde fixatives, as long as the buffers have approximately the same osmolarity. Osmolarity is a key element to reduce cell shrinkage or swelling. The gradient should be kept as small as possible between intra and extracellular space otherwise water will flow in one direction or the other. Another reason for the small differences observed between buffers can be related to the fact that the proteins in the tissue help to keep pH in a narrow range, and therefore the tissue functions as a buffer itself (Claude, 1962; Glauert, 1975; Crang, R., Klomparens, K., 1988). This could mean that the main importance of a buffer in a tissue is not its buffering capacity but actually the capacity of improving the fixative action. However, since the buffers used for this study all have a very close buffering range (close to neutrality) it is not possible to confirm this theory. It would be useful to do a study using a buffer with basic buffering range, one with neutral buffering range and another with acidic buffering range to test this hypothesis and to assess if maintaining the pH of the fixative solution and the tissue being fixed close to neutrality is really critical for good ultrastructural preservation. Moreover, the buffer can help improve the fixative action by giving specific ions to the fixative solution, which can help the penetration of the fixative into the tissue or can help to maintain slightly higher osmolarity in the fixative solution. It might be that the ions that PBS buffer contains do this function and help to slightly improve the general ultrastructural preservation in these samples.

Also, the similarities between buffers might have to do with the nature of the tissue. Different tissues have different osmolarities and can be permeable to different ions (Hayat, 1981). Therefore, if this study had been made in a different organ or model organism the results could change and differences between buffers may possibly arise. Another factor influencing the similarity between buffers could be the piercing of the antenna. This is a technically challenging step yet a very crucial step. It is through the hole made with the needle that the fixative will get inside the organ and be able to penetrate the tissue. Therefore, if there was a difference in penetration rate caused by the buffer solution, and therefore a difference in fixation velocity, with the piercing this difference is decreased. However, piercing can also decrease the quality of preservation of the tissue. If the piercing is not done properly (too far from the target area) or if the hole made is too small this can impair the fixative penetration after piercing. Also, if the piercing is not done properly the tungsten needle can crush structures of interests, damaging the tissue.

The criteria for judging the ultrastructure are too stringent

It is also observed that the final evaluation scores are low. This could mean that the sample preservation is in general not very good, but when analyzing the pictures in the panel from the different samples it is visible that is not the case. Although some samples indeed have bad preservation others have better preservation but it is not reflected on the results. This can be due to a strict evaluation or from not having broad enough criteria. In the comparison of the evaluation between EM experts and non-experts, the experts give more strict evaluations and give slightly lower results than the non-experts. Also, expanding the amount of criteria to evaluate and perhaps giving more emphasis on the specific area of interest could help increase the difference in final quality between buffers. Another change that could be done to the evaluation table would be to increase the number of categories for scoring. Instead of giving a score of zero or one to a sample, if for example another category in between (as 0.5) was created the sample score would be more spread. This way, the samples that were good, but not perfect or that were bad but not terrible could move to this new category, decreasing or improving respectively the values of the final score for those samples. The challenge becomes is the definition of good the same for all users.

There is variability between samples

Still analyzing Fig. 14, it is evident that the buffer with the least sample variability is phosphate. Nonetheless, all samples present variability between them, across all buffers. This might have to do with the technical challenges of processing these samples and variability created in the preparatory steps, such as when piercing the antenna with the tungsten needle as described above.

Using unbuffered fixatives reduces the quality of structural preservation

When comparing the data from the general evaluation scores (Fig. 15) PBS again shows a slightly better score. Yet, as expected, there is not much difference in the general evaluation scores between buffers for the same reasons as discussed above. However, an odd result comes through the observation of the data. There is no major difference between water and the buffers. It is believed that unbuffered fixative solutions produce inferior results than buffered fixatives (Dawes, 1971; Crang, R., Klomparens, K., 1988). However, the results obtained don't depict that inferiority. It can be that the evaluation table is not sensitive enough for the score obtained to reveal the inferiority exhibited in the panel pictures of this condition. If the alterations to the table (as described above) were implemented then the water scores would probably decrease. Also, it might be that also the magnification of the pictures of certain areas is not high enough for the artifacts to come across, since the areas evaluated at higher magnification (12k), the mitochondria and cilia, are the areas with very low scores for water.

Cilia preservation in the transition zone of the olfactory neurons in the antenna is similar between buffers but lower for unbuffered fixatives

If we analyze the data from the cilia evaluation scores (Fig. 16) regarding the comparison between buffers here we can observe that the water evaluation score is much lower than the others, consistent with the idea that some organelles are more sensitive to buffering (Hayat, 1970). Cilia are highly specialized and have different membrane components than other organelles. This might lead to

different membrane diffusion of the fixative to the interior of the cilia, requiring more buffering function than other cellular components to protect the structure from the harmful fixative action. Another interesting finding is that phosphate, Cacodylate and PBS are able to achieve the same high score (0.8), very close to the maximum possible and PHEM achieves a little less than them.

Extraction of cytoplasmatic components can help to improve the visualization of certain cellular components

If we analyze the pictures from Fig. 17 we can easily correlate the better scores achieved by these buffers with the level of extraction they cause in the sample. It seems that the more extracted the cilia matrix is, the easier it is to visualize the microtubules and the y-linkers. The latter structures were never visible with PHEM, since this buffer has a much lower extractive action, preserving the cilia cytoplasm which makes it denser and conferring less contrast with the other structures. This is also a very interesting finding since it goes against the belief that extraction is always harmful. Obviously that with extraction comes information loss (Crang, R., Klomparens, K., 1988) but if the structure that we want to analyze is actually not altered then the greater relative contrast gained with the extraction of cytoplasmatic matrix can be very helpful, as portrayed in this situation.

Fast Freeze substitution yields good ultrastructural preservation of the antenna

Although this study was more focused on chemical fixation since is the routine technique in the majority of the electron microscopy laboratories in Portugal, a brief study of cryo fixation was also one of the goals, since this technique has gained importance over the last years.

Before discussing the results, it is important to emphasize that these conclusions are based on a very low number of samples, so it is very hard to extrapolate the results. Despite the low number of samples for the comparison between different freeze substitution times, some interesting results were observed.

The freeze substitution time that showed the best results in final, general and cilia evaluation (Figures 19, 20 and 21 respectively) was the fast freeze substitution protocol. This result was expected since there are protocols for fast freeze substitution in *Dm* (although for different organs than the antenna) published showing good results (McDonald, 2012, 2014). This is a very encouraging result since less time doing freeze substitution implies having results much faster, having less equipment time being used and spending less technician time therefore decreasing the overall cost of the technique.

Cryo-fixation methods have a very low sample survival rate

Besides being a very time consuming and expensive protocol, cryo fixing samples achieves a very low sample survival rate. This means that medium and low freeze substitution time are not necessarily bad for the overall quality of the samples but only that the samples analyzed were not preserved. If we take a look at the Fig. 21 we can immediately see that the samples analyzed had ice damage. In a normal evaluation scenario these samples that are completely damaged by ice formation should not be used for analysis. However, I wanted to convey the difficulty of this technique and show how low the survival rate can be. The truth for this technique seems to be that the majority of the

samples are badly preserved but the ones that are correctly cryo fixed show a very good ultrastructural preservation (Fig. 22), as has been already described by many authors.

This bad ultrastructural preservation can be a result of manipulation artifacts prior to high pressure freezing the sample. The most important step in HPF is sample loading into the specimen holder (McDonald, 2012). In this protocol this is a step very difficult to perform. First the sample is slightly fixed (approximately 30min) to allow the piercing of the antenna. After piercing the sample is transferred to fixative for about 15min. The goal of such a short fixation time is to avoid chemical fixation artifacts. However, the antenna is hollow inside and therefore 15min might not be enough for the fixative to penetrate the tissue and fill the empty spaces inside the third segment of the antenna. If there is still air in the sample during HPF the tissue can collapse and be damaged. One solution for this problem would be to increase the fixation time after piercing. However, increasing the time of fixation could lead to chemical fixation artifacts which hinder the goal of cryo fixing the sample: keeping the sample as close as possible to its native state. One proof that this could happen is visible in sample one from the protocol of medium freeze substitution. If comparing this sample with the images of chemically fixed samples or cryo fixed samples it is visible that the sample of medium FS look more similar to samples chemical fixed. It is likely that this particular sample was kept in fixative longer after piercing than the other samples. All the samples are pierced sequentially, which means that the samples that are pierced first will stay in fixative more time that the one pierced last. This happens since time of fixation after piercing was only started to count after all samples were pierced. Therefore, one alternative to prevent chemical fixation artifacts would be to leave the sample in buffer after piercing instead of fixative. This way, the buffer has the opportunity to fill the empty spaces inside the antenna and chemical fixation artifacts will be avoided.

To identify the real morphology of a cell component it is necessary to combine chemical and cryo-fixation techniques

Analyzing the results from the cilia evaluation (Fig. 23) for both techniques we can see that both achieve the same maximum score (0.8). This means that both techniques have their own advantages. In the case of chemical fixation, although we have some artifacts like the membrane contour having a flower shape pattern, we can see the y-linker structures that are not visible by cryo fixation. However, cryo fixation preserves much better the cilia membrane maintaining it round. Therefore, what we can conclude from this result is that to fully be able to analyze the ultrastructure of a cell or a cell component, the best is to combine several techniques and compare specimen morphologies from different fixation techniques. This is the best way of obtaining reliable morphological, biochemical and physiological information, for EM studies but can be used as well as a general rule for all biomedical research techniques (Crang, R., Klomparens, K., 1988).

Otherwise, there is a risk of taking erroneous conclusions from the sample observation like the case of the bacterial “mesosome”, a membranous structure found in electron micrographs of chemically processed gram positive bacteria. This structure is actually an artifact of chemical fixation that was never found in cryo fixation studies (Silva *et al*, 1976). This example illustrates why it is important to know what is the aim of each study and to understand how each technique can contribute to achieve that goal and which artifacts each technique can introduce to the sample.

Conclusion

This study gives some insights on the contribution of each method of fixation (chemical and cryo) to preserve the ciliary structure on the third segment of the *Drosophila* antenna and what kind of artifacts arise from each preservation technique. Also, having an evaluation table that summarizes the desirable characteristics of a sample processed for electron microscopy observation helps to understand what to look for in the sample when looking for artifacts specific for each technique. It is also helpful to have a tool that helps to compare between samples and even between techniques not only qualitatively but also in a quantitative way. As shown here, a huge amount of information can be extracted when the samples are analyzed this way and this evaluation table has shown to be a valuable tool not only for experts in electron microscopy but also for people without any background in the area that might want to start doing some work in this field.

As a final remark I consider this to be a very useful study. It raises the question of what is the true action of a buffer in a fixative solution, since until now not much is known and discussed regarding this subject. It would be of great importance if in the future someone would address this question in a detailed chemical analysis. Also it allowed me to assess the chemical fixation protocol that preserves better the structures my research group is interested in studying and gave me the opportunity to work with a new technique (cryo fixation) from which I was able to apply a protocol that provides good ultrastructural preservation. Also, this might be useful to investigate further the ultrastructure of cilia in *Drosophila melanogaster*'s antenna and maybe incorporate it in other studies such as immune detection of ciliary proteins in the transition zone.

Last but not the least, from this study arises a new tool, an evaluation table for judging ultrastructure. This table might be very useful since it can be adapted to whatever sample or criteria being analyzed and it can be specified accordingly to a structure of interest. This can be applied not only for my routine in the laboratory to assess the quality of the samples I will work with, but also for everyone that is interested in standardizing new protocols, comparing techniques or even for routine quality control in electron microscopy facilities.

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10. Appendix

10.1 Quantitative evaluation of the samples chemically fixed using the criteria for judging ultrastructure quality

		1st Processing - 1st Round														
		bi-Phosphate			PHEM			Cacodylate			PBS			Water		
	Criteria	**S 1a**	**S 1b**	**S 1c**	**S 1a**	**S 1b**	**S 1c**	**S 1a**	**S 1b**	**S 1c**	**S 1a**	**S 1b**	**S 1c**	**S 1a**	**S 1b**	**S 1c**
	G1	0	0	0	1	1	1	0	0	0	1	0	1	1	1	1
	G2	0	1	1	0	0	1	1	1	1	1	1	1	1	0	1
	G3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	G4	1	1	1	1	0	0	1	1	1	1	1	1	1	0	0
	G5	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
G6 measurements	M1	63.52	106.19	53.91	53.43	263.12	81.14	21.75	100.66	19.53	18.57	259.55	68.44	117.17	57.18	191.07
	M2	45.8	132.98	192.33	58.7	288.52	116.28	18.49	120.71	24.19	21.04	93.31	53.77	237.59	281.43	272.18
	M3	98.08	93.64	60.51	83.2	118.93	77.78	16.41	64.01	20.03	43.37	366.25	91.57	166.85	450.15	80.31
	Max	102.27	252.79	258.33	87.71	337.64	216.55	51.28	130.15	38.46	84.22	543.49	136.3	350.19	497.12	579.12
	Mean	77.42	146.40	141.27	70.76	252.05	122.94	26.98	103.88	25.55	41.80	315.65	87.52	217.95	321.47	280.67
	std	27.31	72.80	100.77	17.20	93.98	64.79	16.35	29.29	8.85	30.40	188.90	36.05	101.06	199.05	213.94
	Score	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0
	G6	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0
	G7	0	0	1	1	1	0	1	0	1	1	0	0	1	1	0
	G8	0	1	1	0	0	0	0	1	0	1	0	1	1	0	1
	G9	1	1	1	1	1	1	0	1	1	0	0	1	1	0	0
	G10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	G11	0	1	1	1	1	0	0	1	1	1	1	0	0	0	0
	G12	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
	G13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	G14	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	G15	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	C1	1	0	0	0	1	1	1	1	1	0	1	1	1	0	1
	C2	0	1	0	0	0	0	0	1	0	1	0	0	0	1	0
	C3	1	0	0	0	1	1	0	0	1	0	1	0	0	0	1
	C4	1	0	0	0	0	0	0	0	1	0	1	1	0	0	0
	C5	1	1	0	0	1	0	0	0	1	0	1	0	0	0	0
	Total G	3	6	7	8	5	4	5	6	7	9	5	7	7	3	4
	Score G	0.20	0.40	0.47	0.53	0.33	0.27	0.33	0.40	0.47	0.60	0.33	0.47	0.47	0.20	0.27
	Total C	4	2	0	0	3	2	1	2	4	1	4	2	1	1	2
	Score C	0.80	0.40	0.00	0.00	0.60	0.40	0.20	0.40	0.80	0.20	0.80	0.40	0.20	0.20	0.40
	G + C	**7**	**8**	**7**	**8**	**8**	**6**	**6**	**8**	**11**	**10**	**9**	**9**	**8**	**4**	**6**
	F Score	**0.35**	**0.4**	**0.35**	**0.4**	**0.4**	**0.3**	**0.3**	**0.4**	**0.55**	**0.5**	**0.45**	**0.45**	**0.4**	**0.2**	**0.3**

		1st Processing - 2nd Round					2nd Processing						
		bi-Phos	PHEM	Caco	PBS	H20	bi-Phos	PHEM	Caco	PBS	H20		
	Criteria	**S 2**	**S 2**	**S 2**	**S 2**	**S 2**	**S 3**	**S 3**	**S 3**	**S 3**	**S 3**	**Total**	
	G1	0	0	0	1	0	1	0	0	0	0	10	out of 25
	G2	1	1	0	1	1	1	1	1	1	1	20	out of 25
	G3	0	0	0	0	0	0	0	0	0	0	0	out of 25
	G4	0	1	0	1	1	1	0	1	1	0	17	out of 25
	G5	0	0	0	1	0	1	0	0	0	0	4	out of 25
G6 measurements	M1	37.88	103.2	66.76	129.65	300.89	208.01	106.38	80.82	40.16	93.58		
	M2	57.82	48.19	66.76	86.54	203.89	183.35	42	58.61	15.02	46.51		
	M3	19.61	38.46	60.01	163.18	100.76	107.78	272.32	57.6	16.54	126.52		
	Max	82.84	213.73	160.54	265.95	505.91	363.87	333.47	120.31	40.43	130.99		
	Mean	49.54	100.90	88.52	161.33	277.86	215.75	188.54	79.34	28.04	99.40		
	std	27.14	80.44	48.12	76.48	172.60	107.56	136.93	29.34	14.17	39.01		
	Score	0	0	0	0	0	0	0	0	1	0		
	G6	0	0	0	0	0	0	0	0	1	0	4	out of 25
	G7	1	0	1	1	1	1	1	0	1	1	16	out of 25
	G8	0	1	1	1	0	0	0	1	1	1	13	out of 25
	G9	1	0	0	0	0	0	1	0	1	0	13	out of 25
	G10	0	0	0	0	0	0	0	0	0	0	0	out of 25
	G11	1	1	1	1	1	0	1	1	1	0	16	out of 25
	G12	0	0	0	1	0	0	0	0	0	0	3	out of 25
	G13	0	0	0	1	0	0	0	0	0	0	1	out of 25
	G14	0	0	0	0	0	0	0	0	0	0	1	out of 25
	G15	1	1	1	1	1	1	1	1	1	1	25	out of 25
	C1	1	0	1	1	1	1	1	1	1	0	18	out of 25
	C2	0	0	1	0	0	0	0	0	0	0	5	out of 25
	C3	1	0	0	1	0	1	1	0	1	0	11	out of 25
	C4	0	0	0	0	0	0	0	0	1	0	5	out of 25
	C5	1	0	0	1	0	1	1	0	1	0	10	out of 25
	Total G	5	5	4	10	5	6	5	5	8	4		
	Score G	0.33	0.33	0.27	0.67	0.33	0.40	0.33	0.33	0.53	0.27		
	Total C	3	0	2	3	1	3	3	1	4	0		
	Score C	0.60	0.00	0.40	0.60	0.20	0.60	0.60	0.20	0.80	0.00		
	G + C	**8**	**5**	**6**	**13**	**6**	**9**	**8**	**6**	**12**	**4**		
	F Score	**0.4**	**0.25**	**0.3**	**0.65**	**0.3**	**0.45**	**0.4**	**0.3**	**0.6**	**0.2**		

10.2 Quantitative evaluation of the samples cryo fixed using the criteria for judging ultrastructure quality

		Fast	Fast	Medium	Medium	Slow	Slow		
	Criteria	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2	Total	
	G1	1	1	0	1	0	1	4	out of 6
	G2	1	0	1	1	1	1	5	out of 6
	G3	1	1	0	0	0	1	3	out of 6
	G4	1	1	1	1	0	1	5	out of 6
	G5	0	1	0	1	0	1	3	out of 6
G6 measurements	M1	34.22	30.04	48.23	13.71	21.66	4.25		
	M2	15.68	20.03	13.65	9.69	21.81	12.75		
	M3	17	18.13	12.93	11.56	45.13	15.68		
	Max	34.22	59.02	124.83	13.7	71.43	20.291		
	Mean	25.28	31.81	49.91	12.17	40.01	5080.92		
	std	10.337079	18.880569	52.593209	1.9351916	23.67413	10140.054		
	Score	1	1	0	1	1	0		
	G6	1	1	0	1	1	0	4	out of 6
	G7	1	1	0	0	1	1	4	out of 6
	G8	1	0	1	0	0	0	2	out of 6
	G9	0	1	1	1	0	1	4	out of 6
	G10	0	0	0	0	0	0	0	out of 6
	G11	1	1	1	1	1	1	6	out of 6
	G12	1	1	0	1	0	1	4	out of 6
	G13	1	1	0	0	1	1	4	out of 6
	G14	0	1	0	1	0	0	2	out of 6
	G15	1	1	1	1	1	1	6	out of 6
	C1	1	1	1	1	1	1	6	out of 6
	C2	1	1	1	1	1	1	6	out of 6
	C3	0	1	0	0	0	1	2	out of 6
	C4	0	0	0	0	0	0	0	out of 6
	C5	0	1	0	0	1	0	2	out of 6

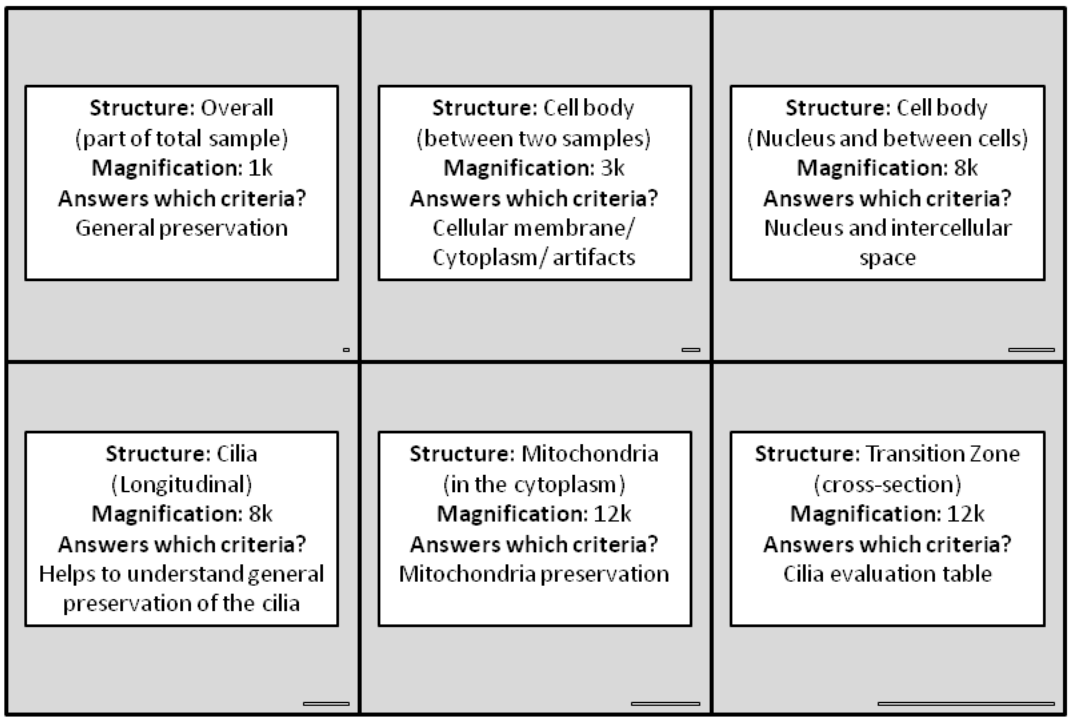
Total G	10	11	6	9	5	11
Score G	0.67	0.73	0.40	0.60	0.33	0.73
Total C	2	4	2	2	3	3
Score C	0.40	0.80	0.40	0.40	0.60	0.60
G + C	**12**	**15**	**8**	**11**	**8**	**14**
F Score	**0.6**	**0.75**	**0.4**	**0.55**	**0.4**	**0.7**

10.3 Quantitative evaluation of the samples by different evaluators using the criteria for judging ultrastructure quality

		Creator				Expert in EM				Biologist				Air Traffic Controller			
	Criteria	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
	G1	0	0	1	0	0	0	0	1	0	1	0	1	0	1	0	0
	G2	1	1	1	1	0	0	0	0	1	1	0	0	1	1	1	0
	G3	0	0	0	0	1	0	0	0	1	1	1	1	1	1	0	1
	G4	0	1	1	1	0	1	1	1	1	1	1	1	0	0	0	1
	G5	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
G6 measurements	M1	93.58	106.19	117.17	40.16	63.43	39.22	17.31	20.80	61.54	127.96	244.77	18.13	57.85	152.69	322.07	406.24
	M2	46.51	132.98	237.59	15.02	106.38	48.19	23.08	28.85	69.23	75.93	18.24	12.82	83.03	211.64	169.41	194.15
	M3	126.52	93.64	166.85	16.54	133.51	36.49	114.52	23.79	103.85	25.77	23.08	15.39	57.18	209.25	427.74	511.47
	Max	130.99	252.79	350.19	40.43	134.84	138.68	363.10	33.64	126.92	271.96	375.31	20.67	177.80	408.27	598.42	736.26
	Mean	99.40	146.40	217.95	28.04	109.54	65.64	129.50	26.77	90.38	125.41	165.35	16.75	93.96	245.46	379.41	462.03
	std	39.01	72.80	101.06	14.17	33.42	48.94	161.97	5.66	30.53	106.24	175.38	3.40	57.17	111.91	180.45	225.48
	Score	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0
	G6	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0
	G7	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1
	G8	1	1	1	1	0	0	0	1	1	1	1	1	0	1	0	1
	G9	0	1	1	1	0	0	1	1	0	1	1	1	0	1	0	1
	G10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	G11	0	1	0	1	0	0	0	1	0	1	1	1	0	1	1	0
	G12	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0
	G13	0	0	0	0	0	0	1	1	0	0	1	1	0	0	1	1
	G14	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
	G15	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	1
	C1	0	0	1	1	0	0	1	1	0	0	1	1	1	0	1	1
	C2	0	1	0	0	1	1	0	0	1	1	0	0	0	1	0	0
	C3	0	0	0	1	0	0	1	1	0	0	0	1	0	0	0	1
	C4	0	0	0	1	0	0	1	1	0	0	1	1	0	1	1	1
	C5	0	1	0	1	1	1	1	1	0	0	0	1	0	0	1	1
	Total G	4	6	7	8	3	3	5	8	5	9	8	9	4	10	6	9
	Score G	0.27	0.40	0.47	0.53	0.20	0.20	0.33	0.53	0.33	0.60	0.53	0.60	0.27	0.67	0.40	0.60
	Total C	0	2	1	4	2	2	4	4	1	1	2	4	1	2	3	4
	Score C	0.00	0.40	0.20	0.80	0.40	0.40	0.80	0.80	0.20	0.20	0.40	0.80	0.20	0.40	0.60	0.80
	G + C	**4**	**8**	**8**	**12**	**5**	**5**	**9**	**12**	**6**	**10**	**10**	**13**	**5**	**12**	**9**	**13**
	F Score	**0.2**	**0.4**	**0.4**	**0.6**	**0.25**	**0.25**	**0.45**	**0.6**	**0.3**	**0.5**	**0.5**	**0.65**	**0.25**	**0.6**	**0.45**	**0.65**

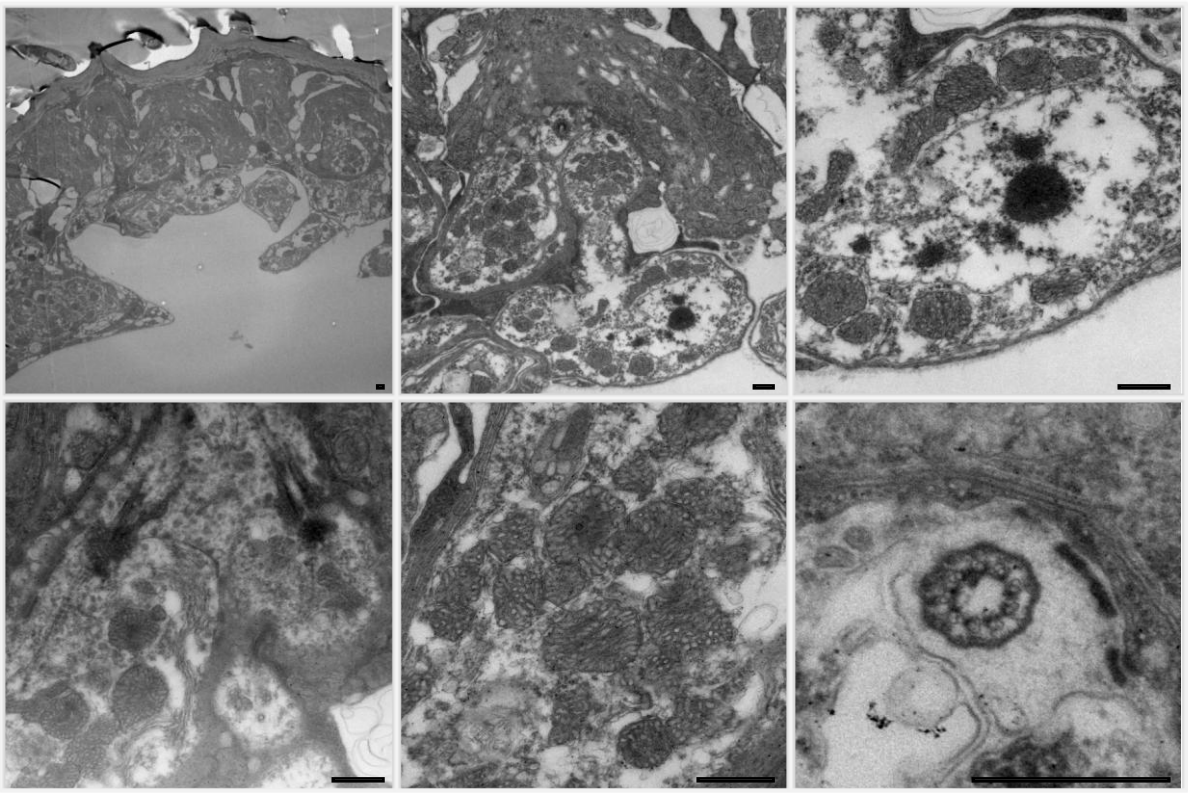
10.4 Chemical Fixation Panels

10.4.1 Schematic Representation on how the panels are organized

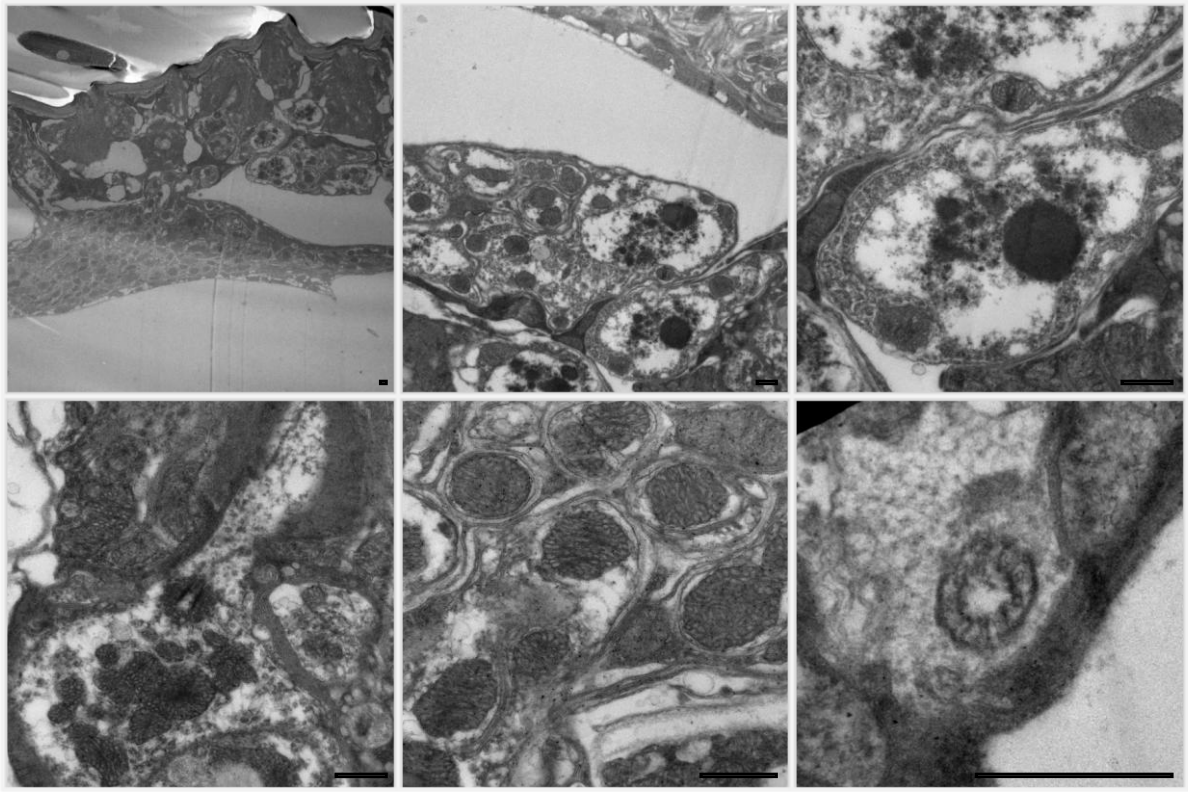


Scale bar represents 500 nm

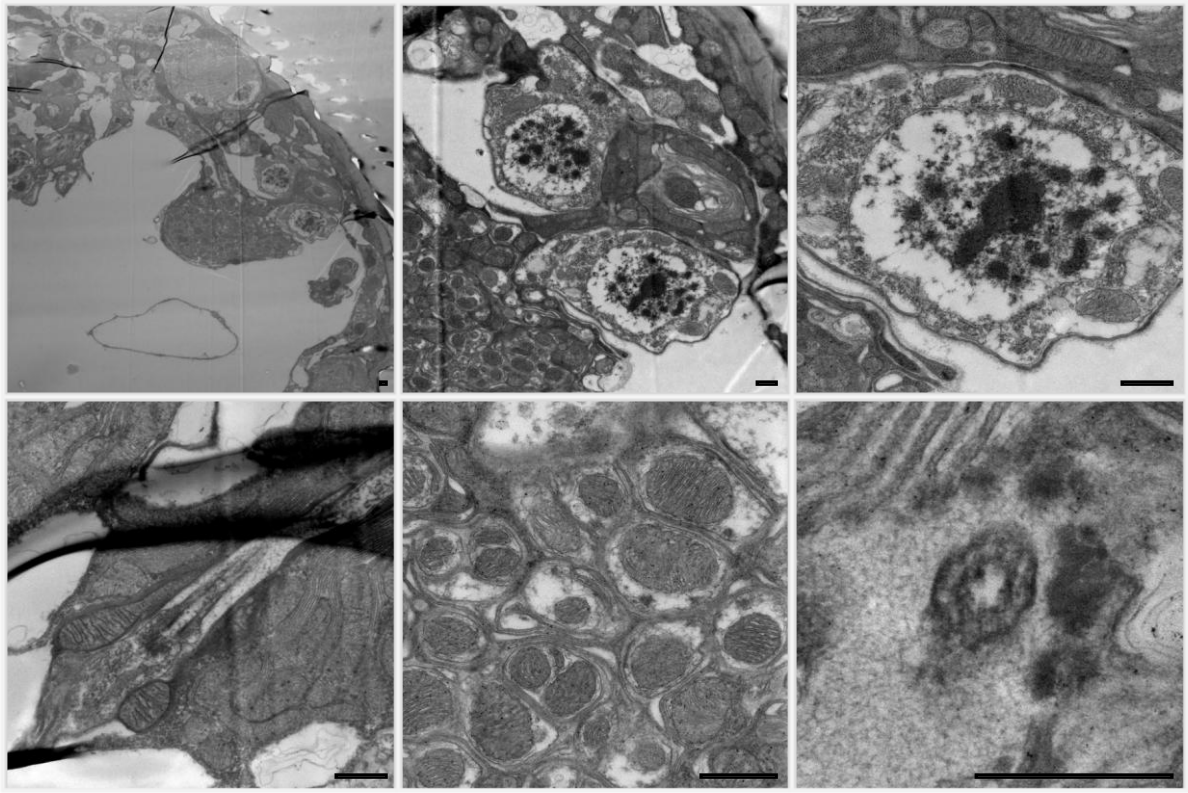
10.4.2 Phosphate Sample 1a



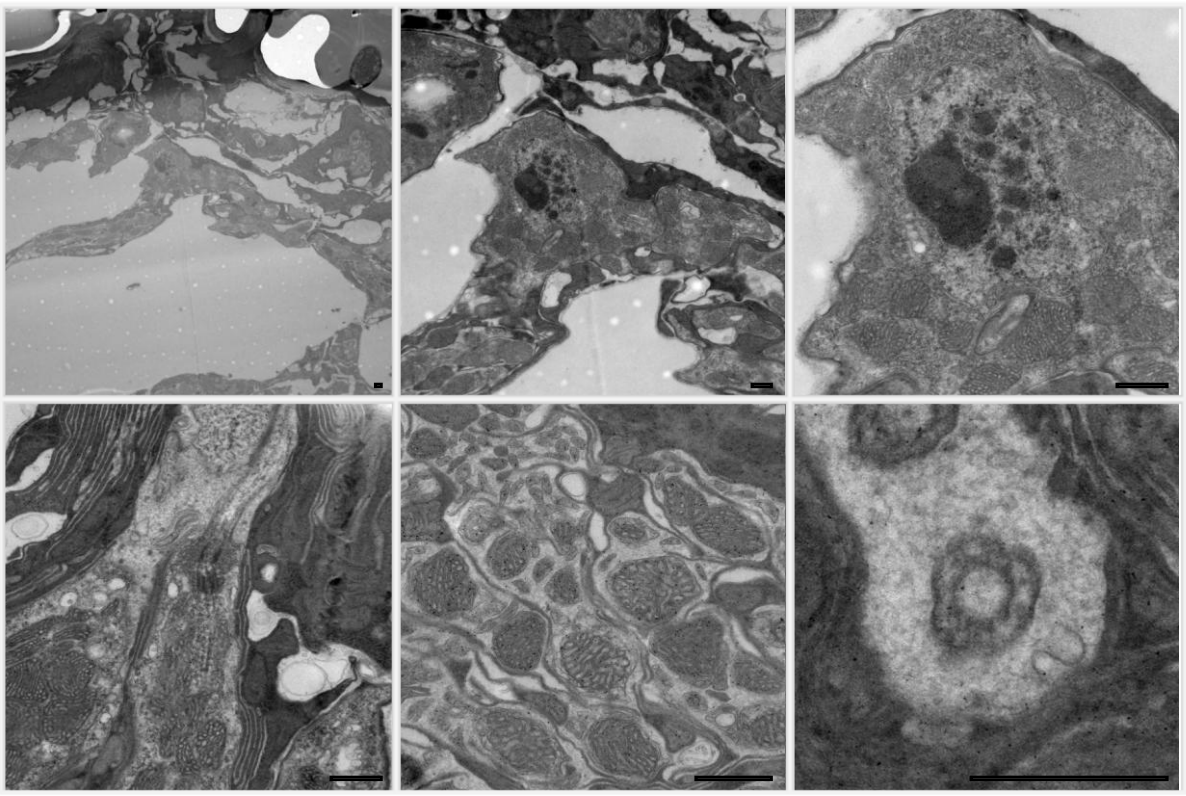
10.4.3 Phosphate Sample 1b



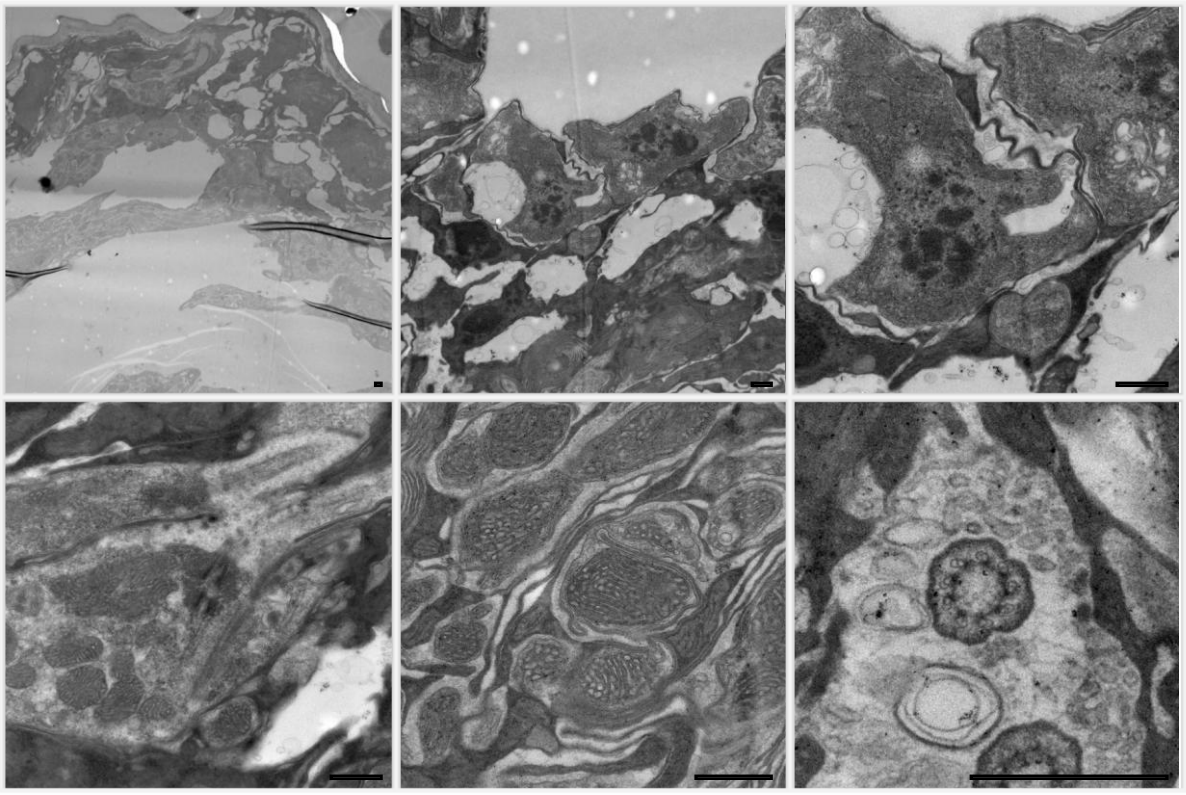
10.4.4 Phosphate Sample 1c



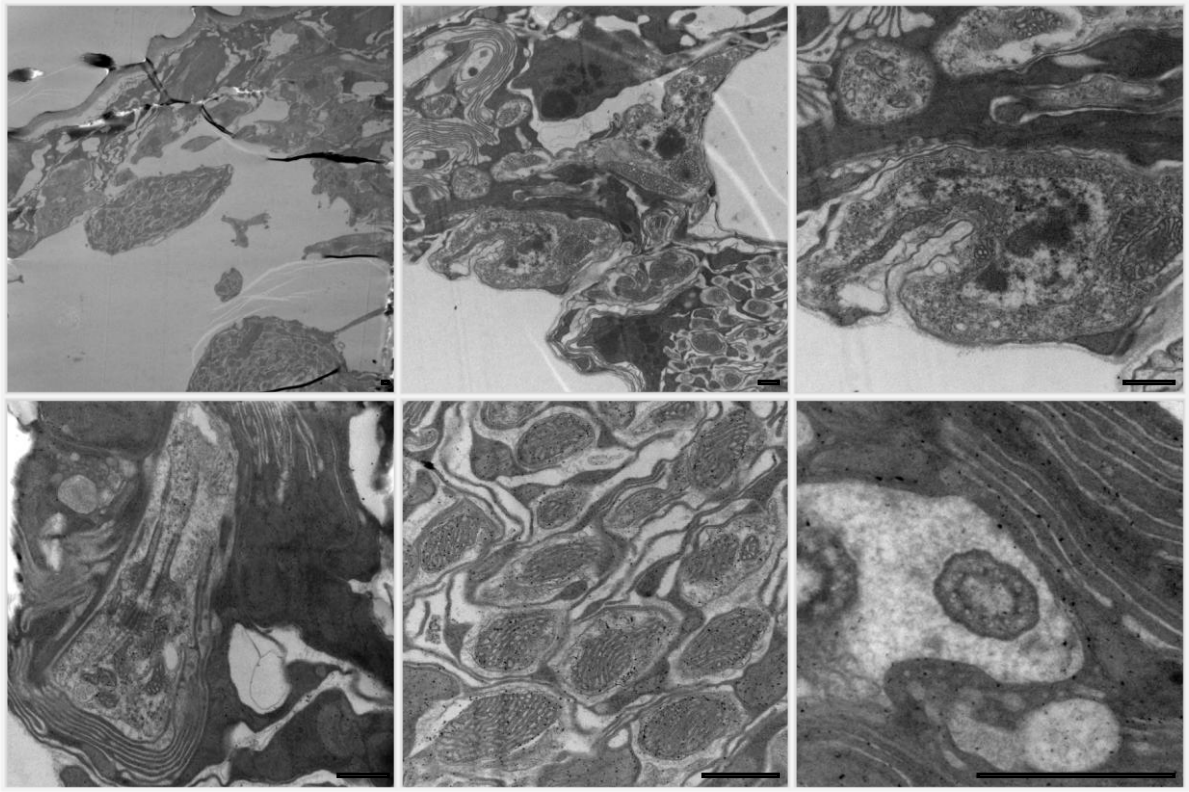
10.4.5 PHEM Sample 1a



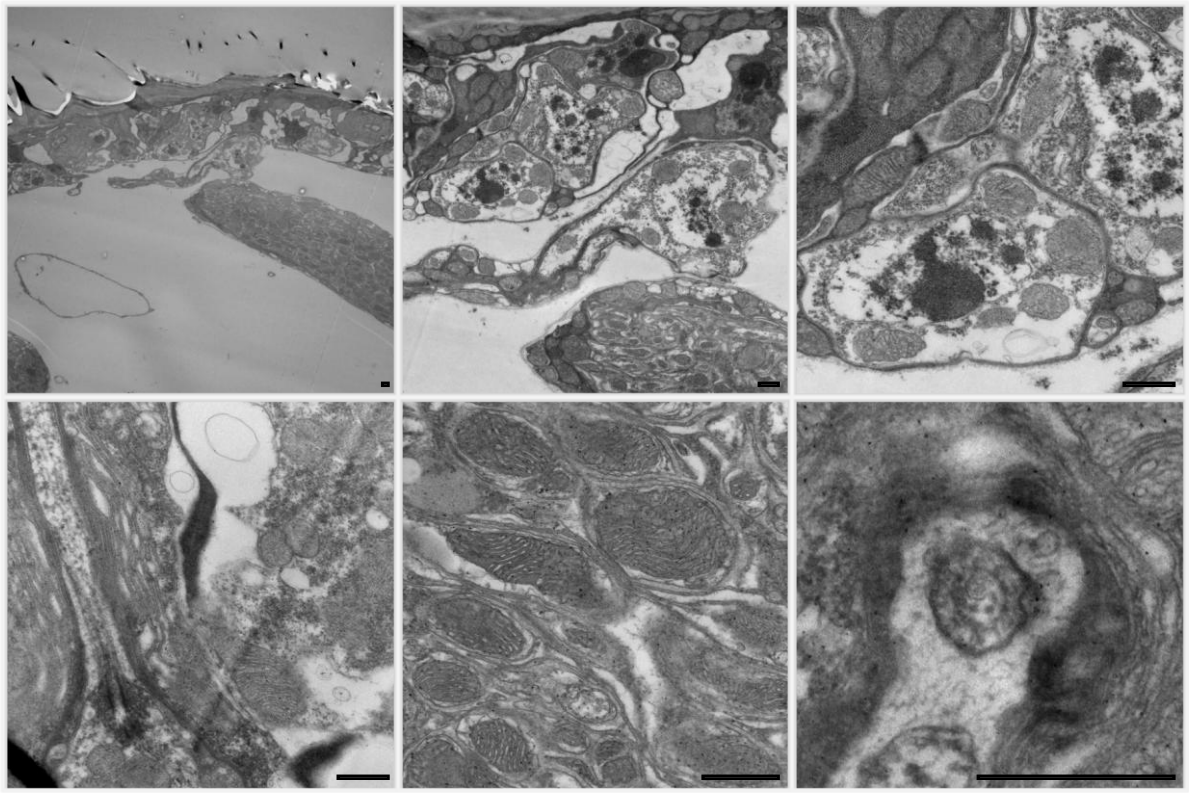
10.4.6 PHEM Sample 1b



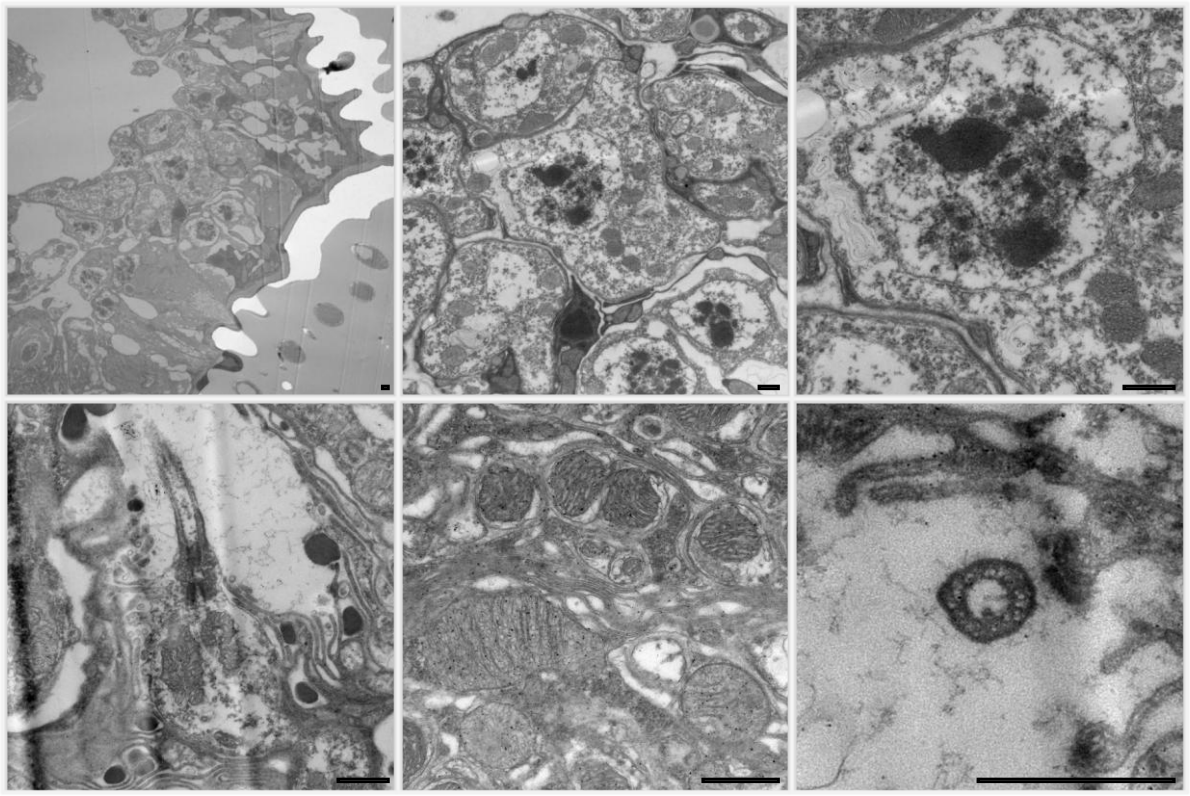
10.4.7 PHEM Sample 1c



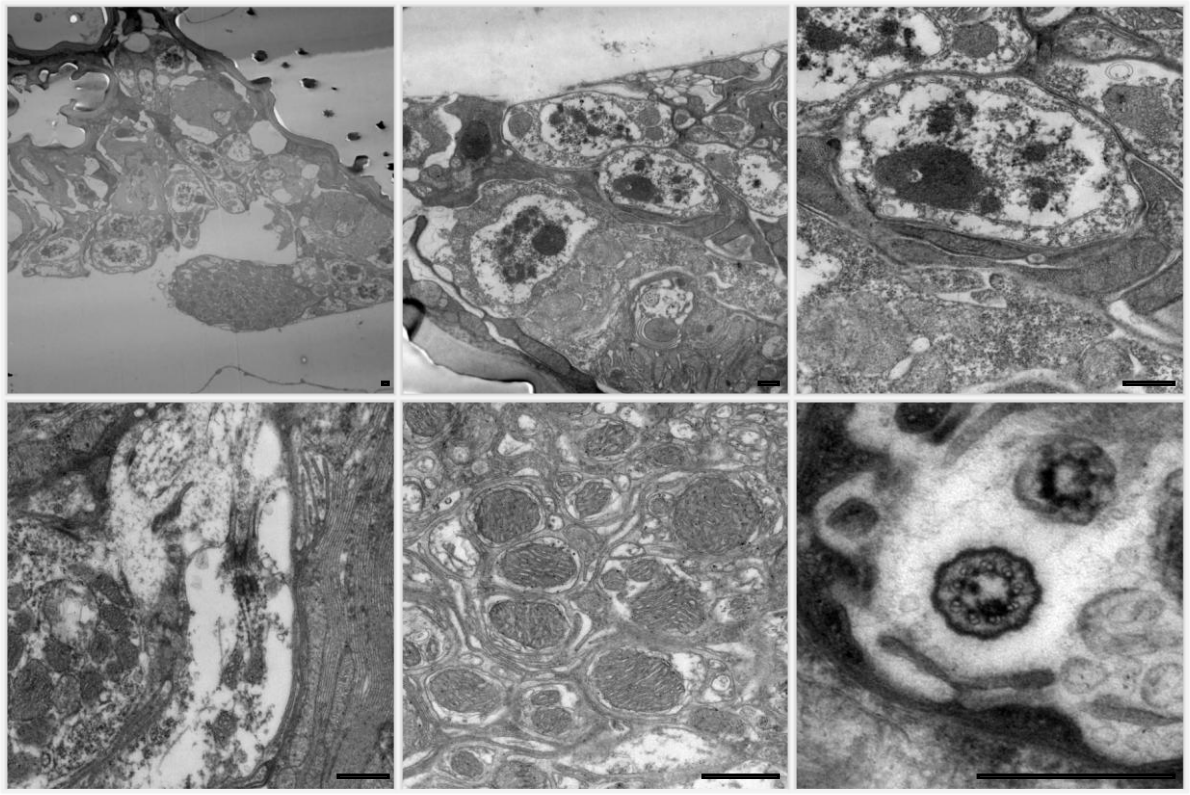
10.4.8 Cacodylate Sample 1a



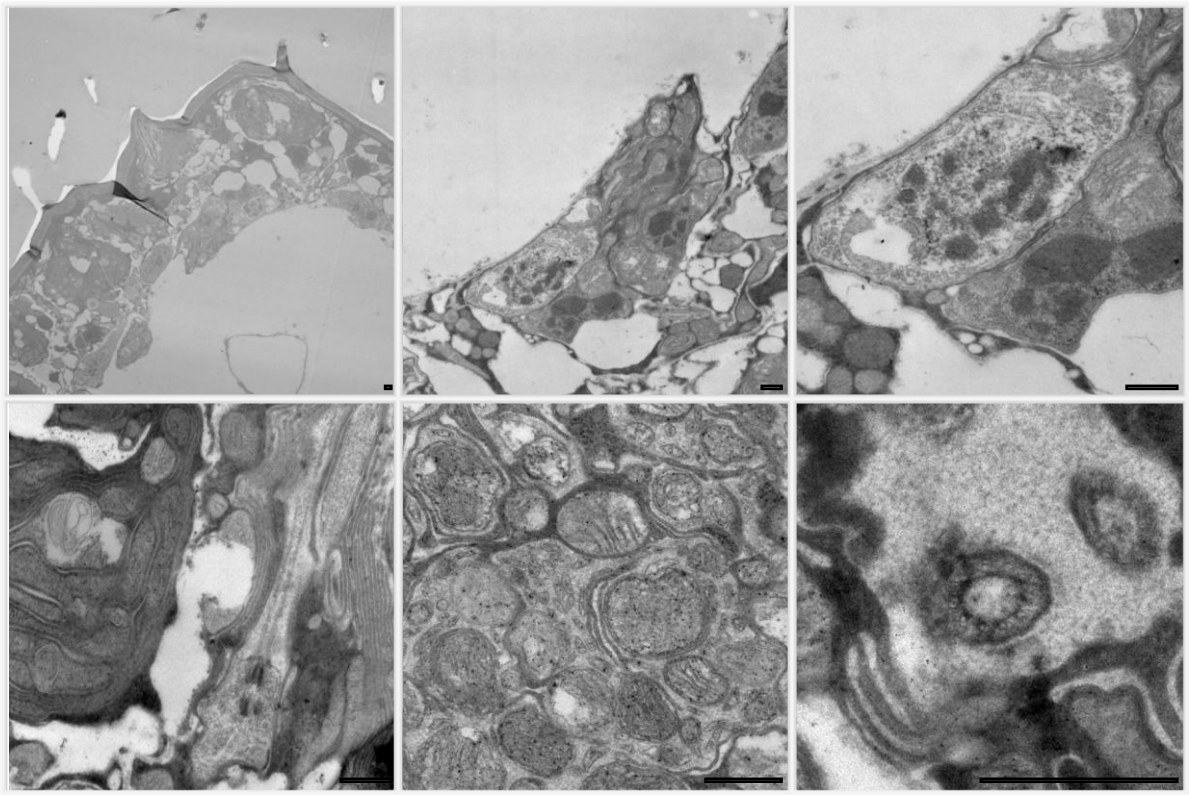
10.4.9 Cacodylate Sample 1b



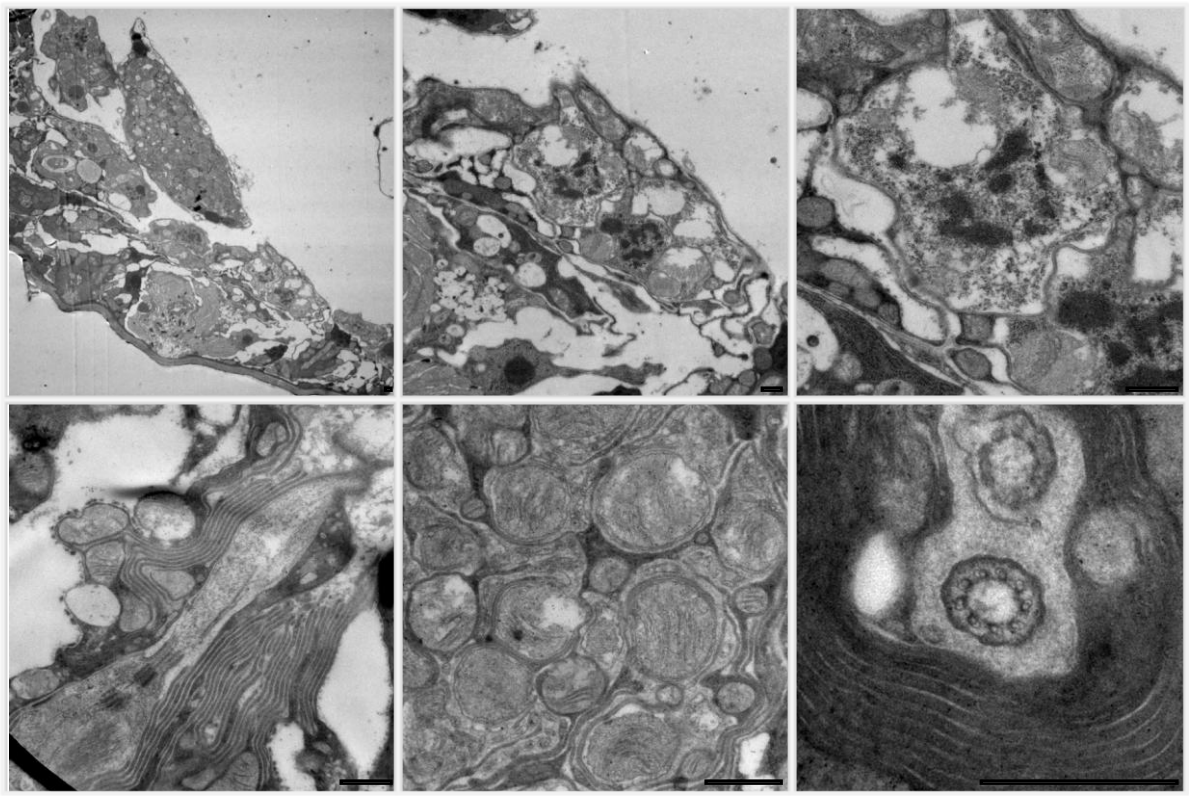
10.4.10 Cacodylate Sample 1c



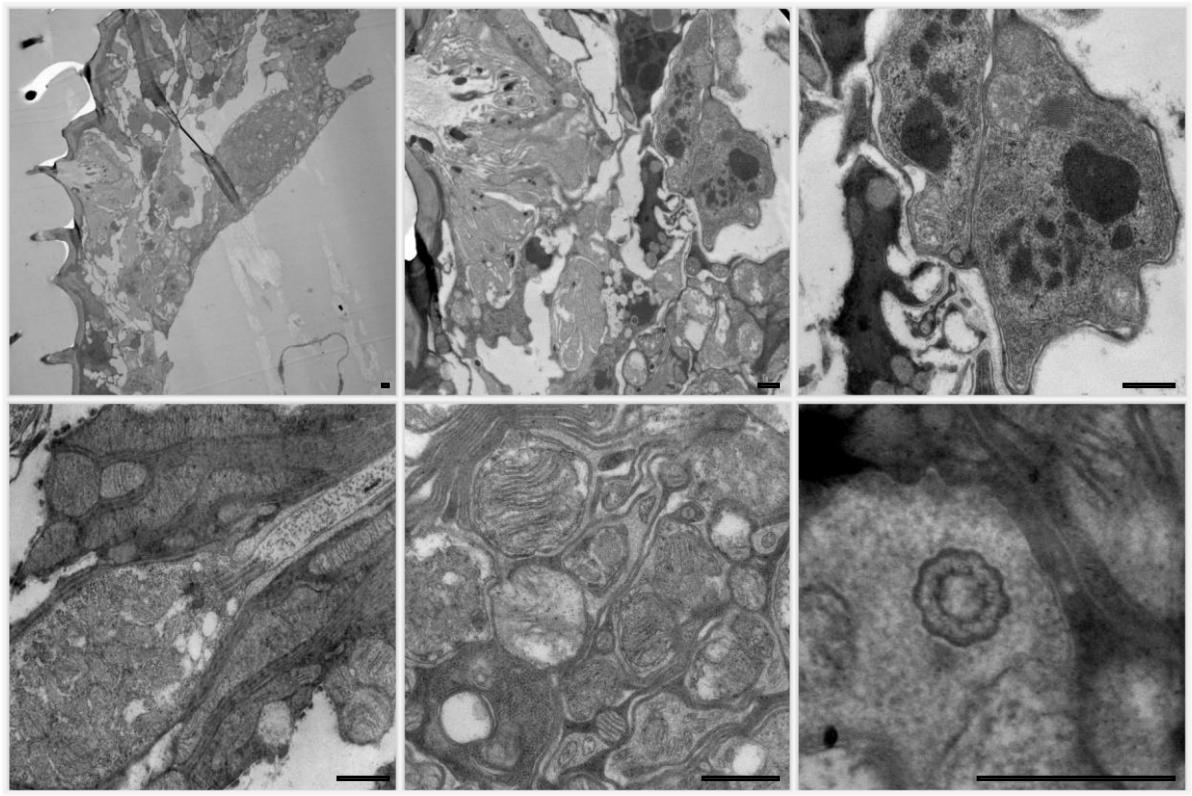
10.4.11 PBS Sample 1a



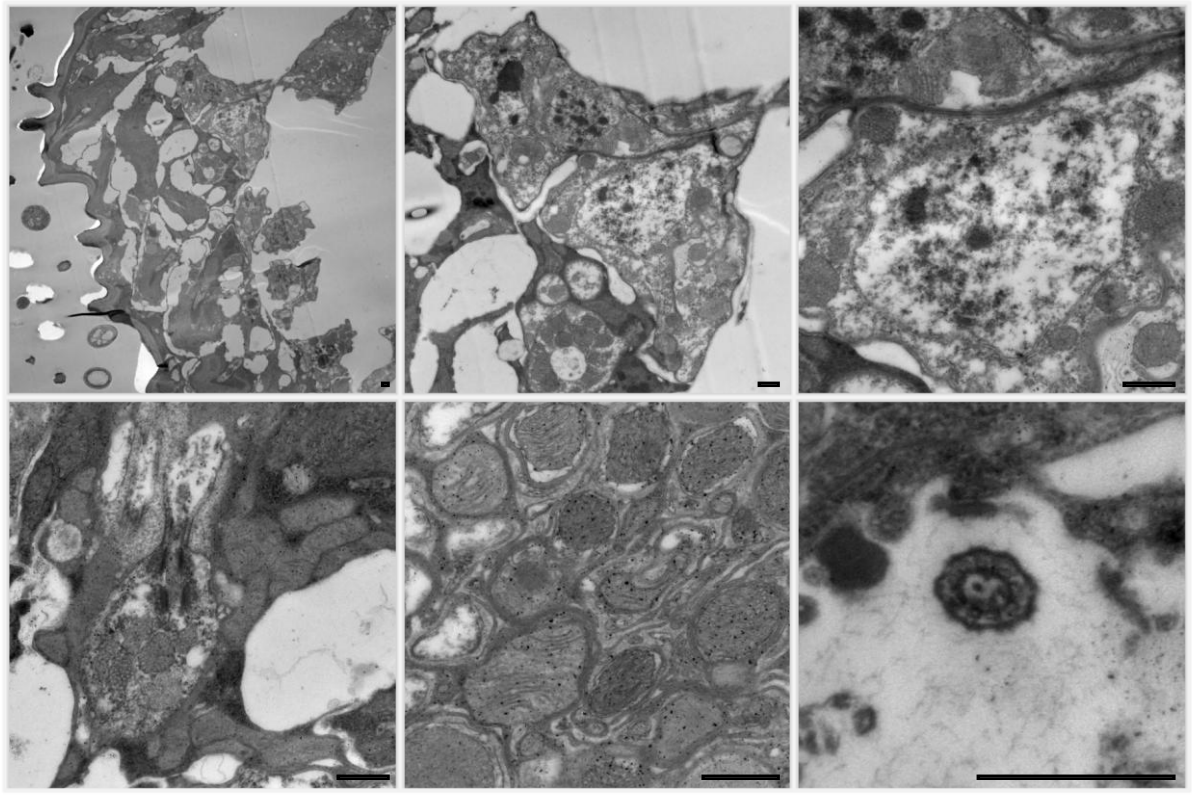
10.4.12 PBS Sample 1b



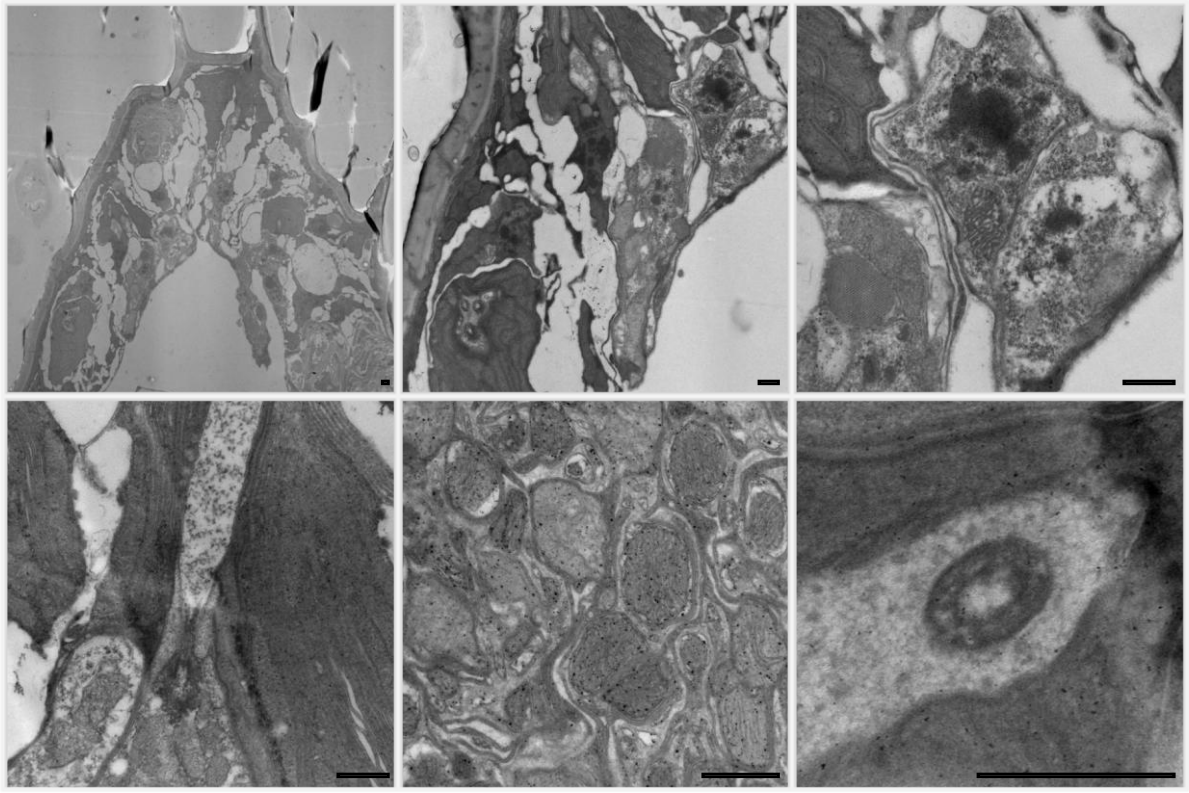
10.4.13 PBS Sample 1c



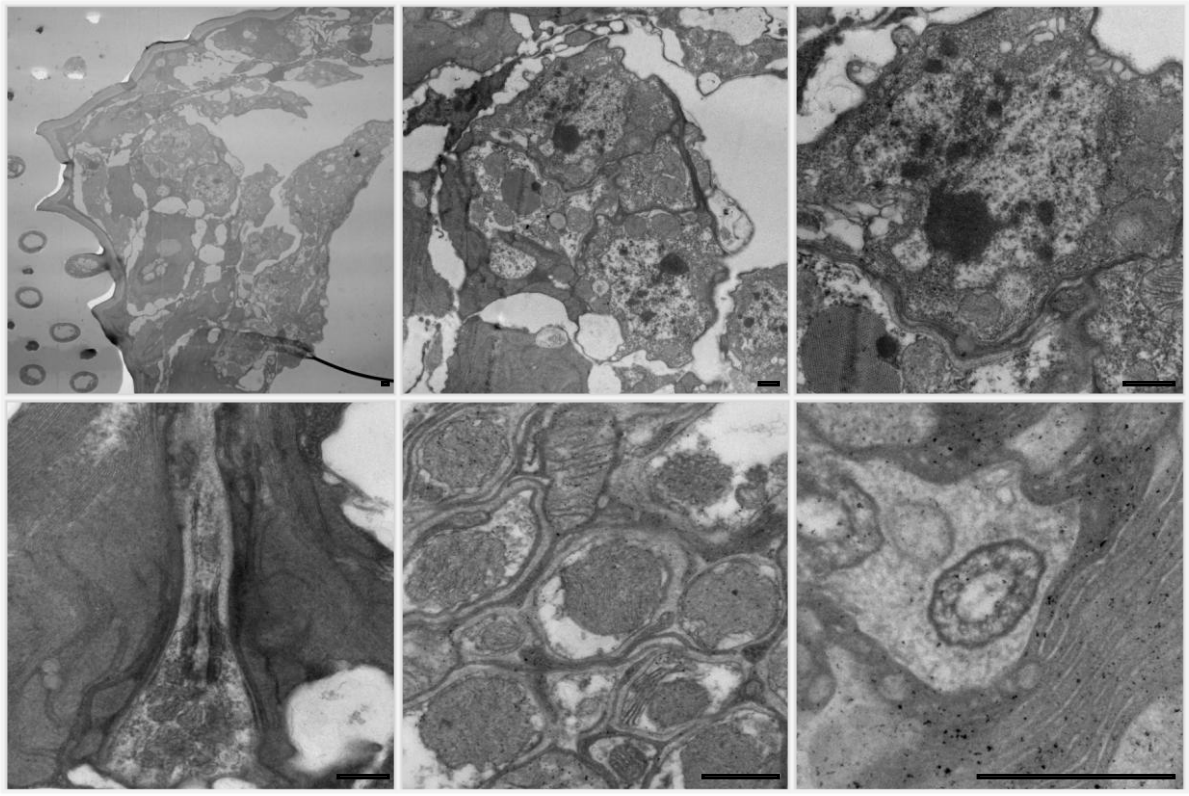
10.4.14 Water Sample 1a



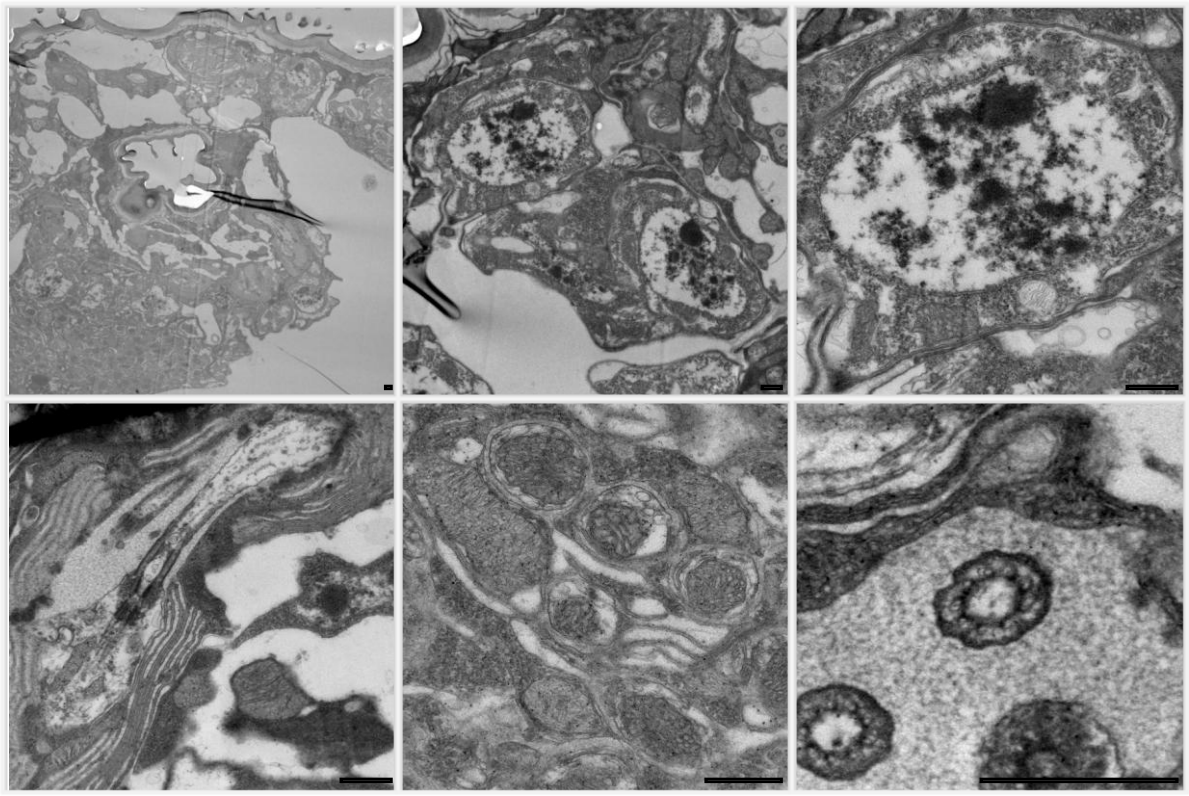
10.4.15 Water Sample 1b



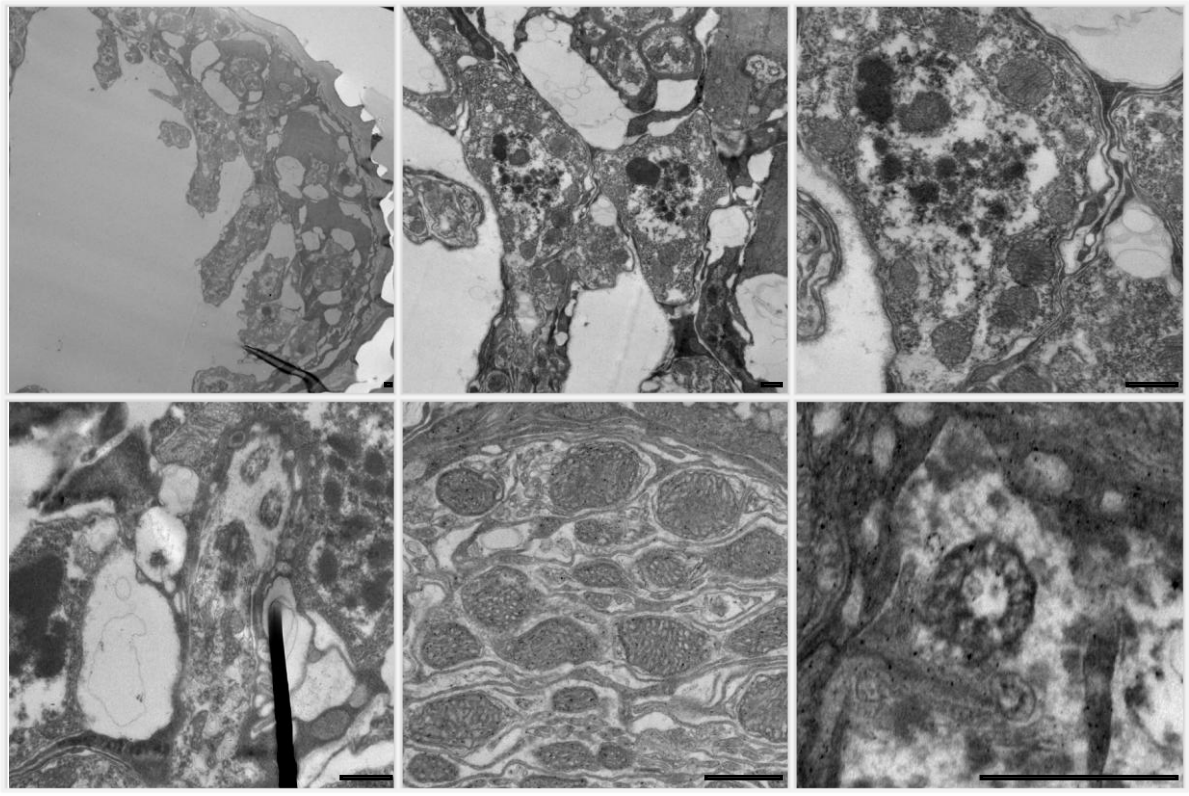
10.4.16 Water Sample 1c



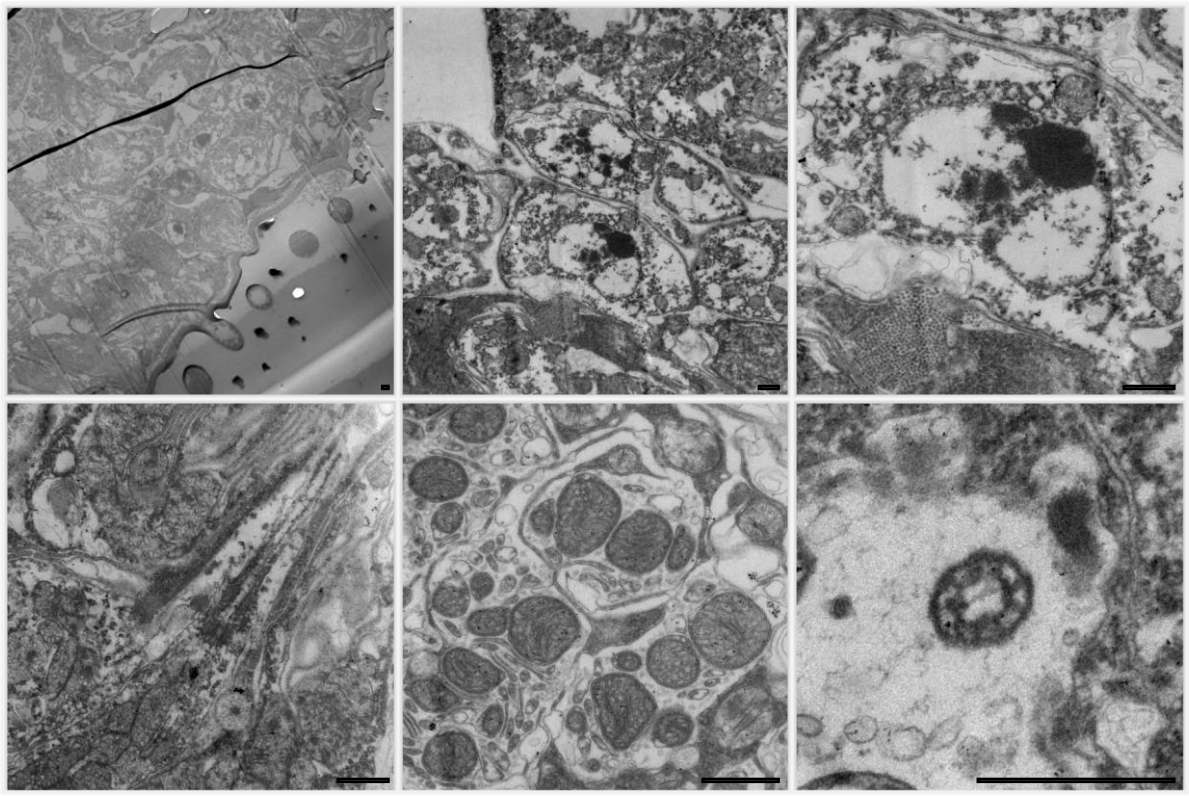
10.4.17 Phosphate Sample 2



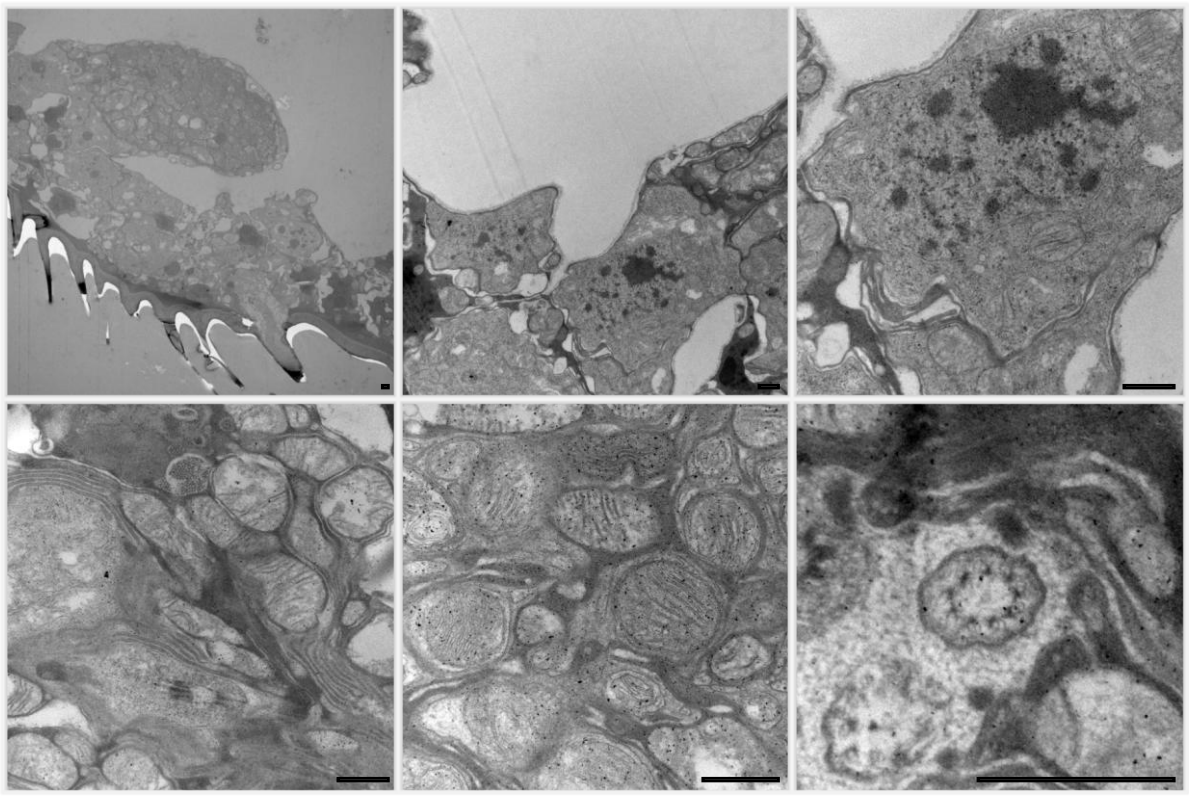
10.4.18 PHEM Sample 2



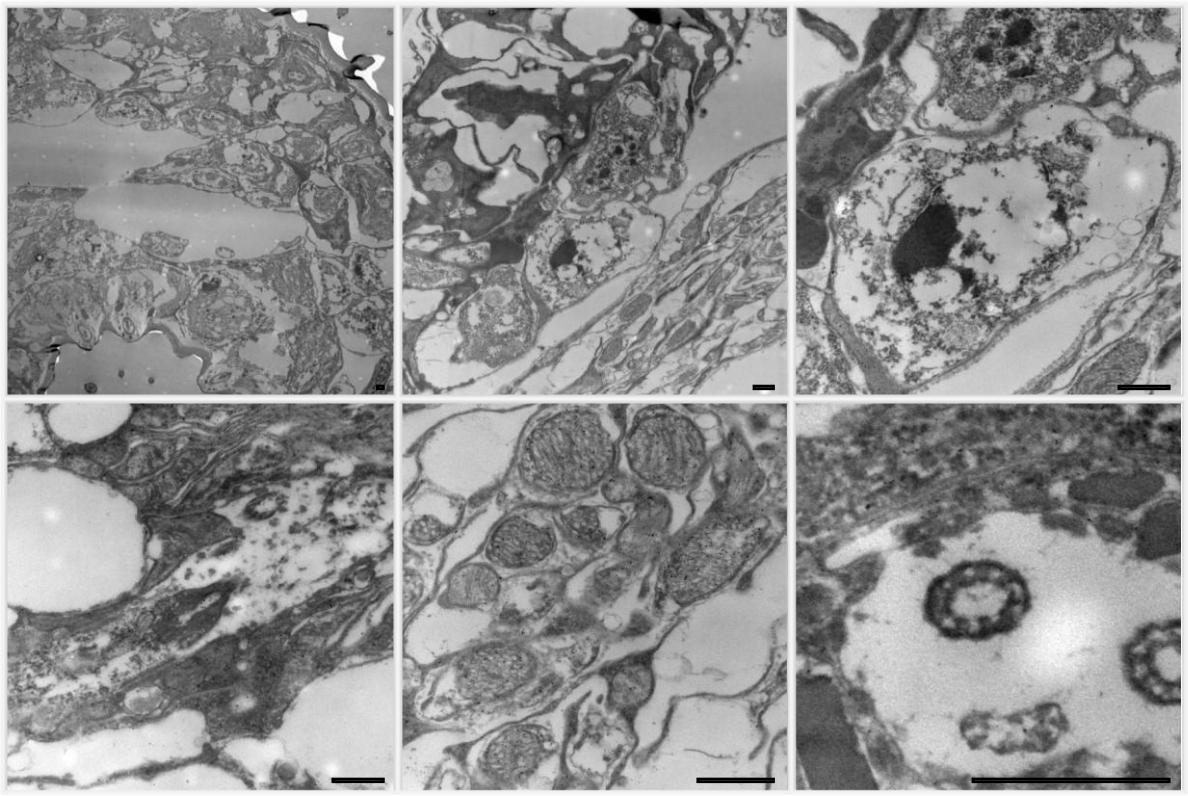
10.4.19 Cacodylate Sample 2



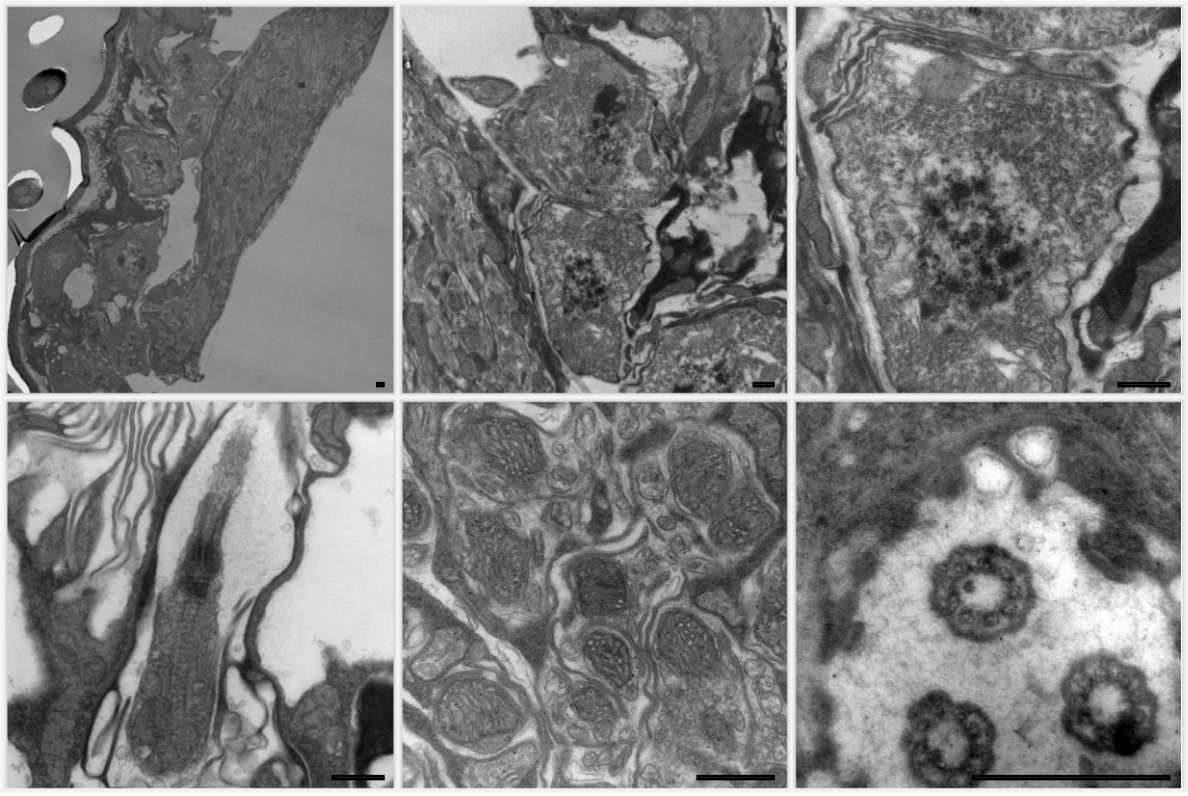
10.4.20 PBS Sample 2



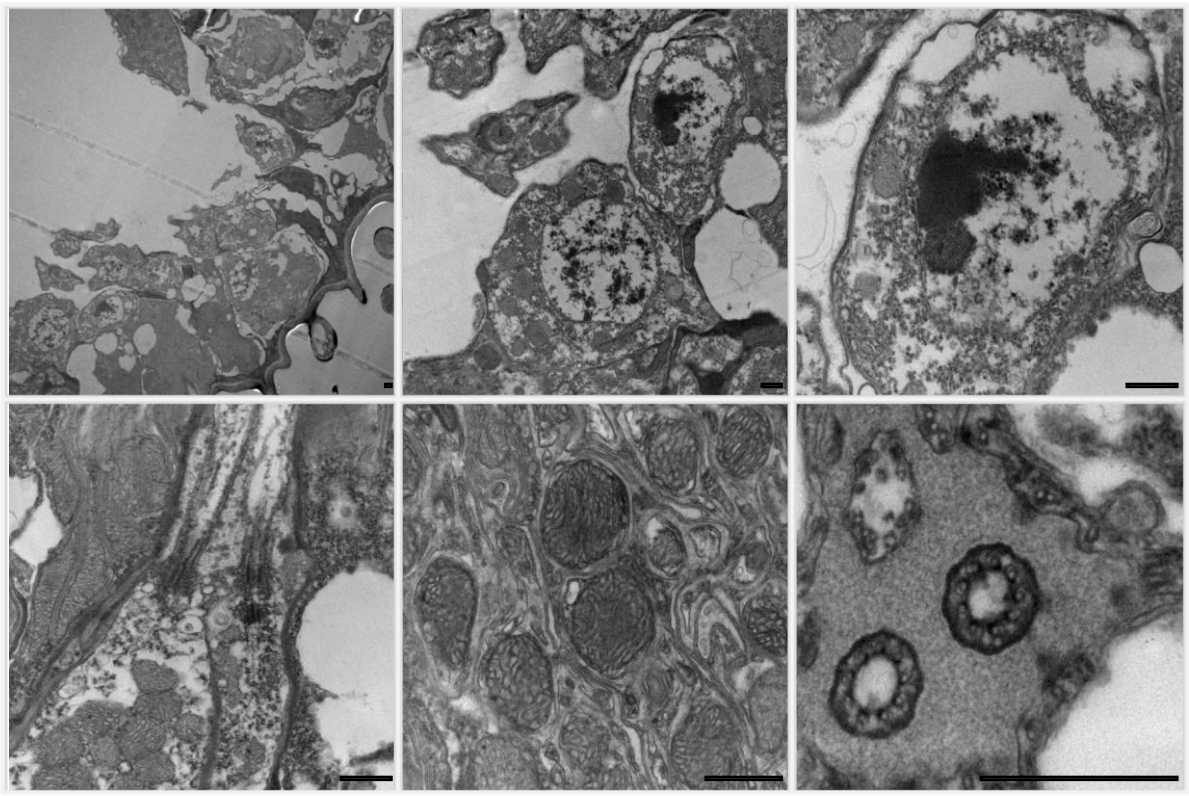
10.4.21 Water Sample 2



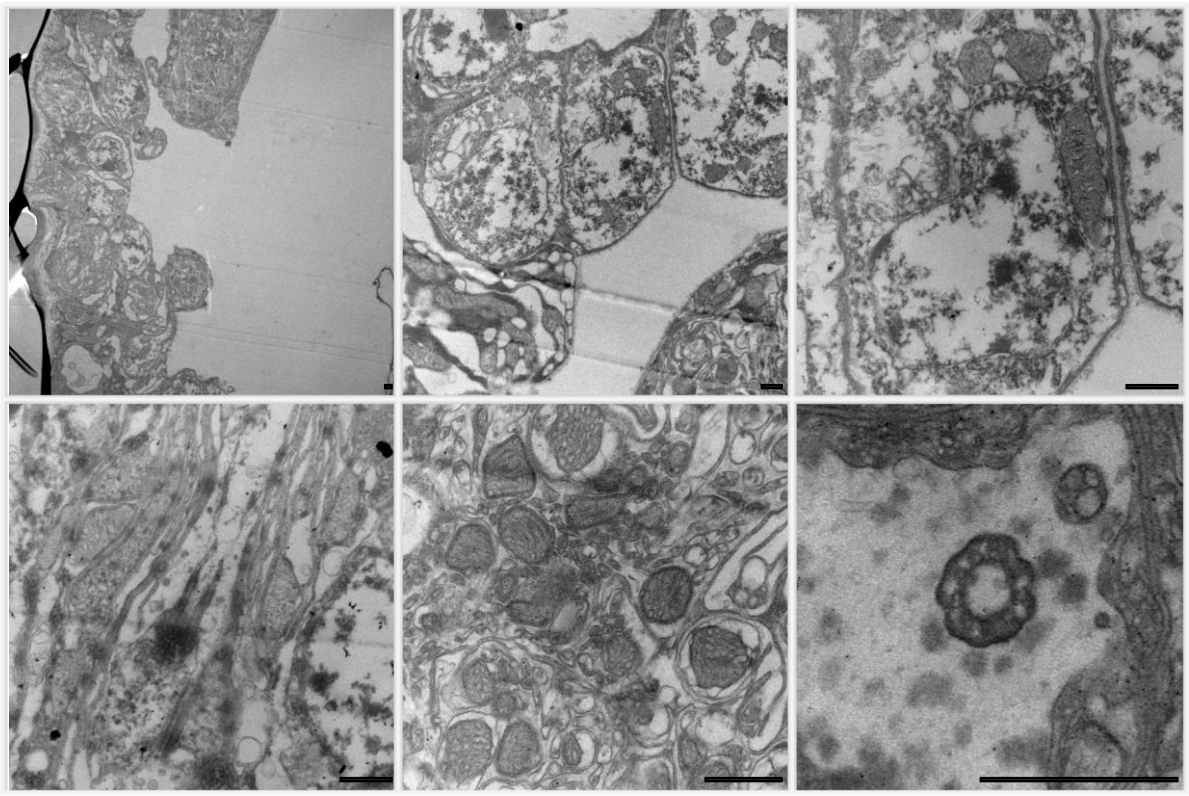
10.4.22 Phosphate Sample 3



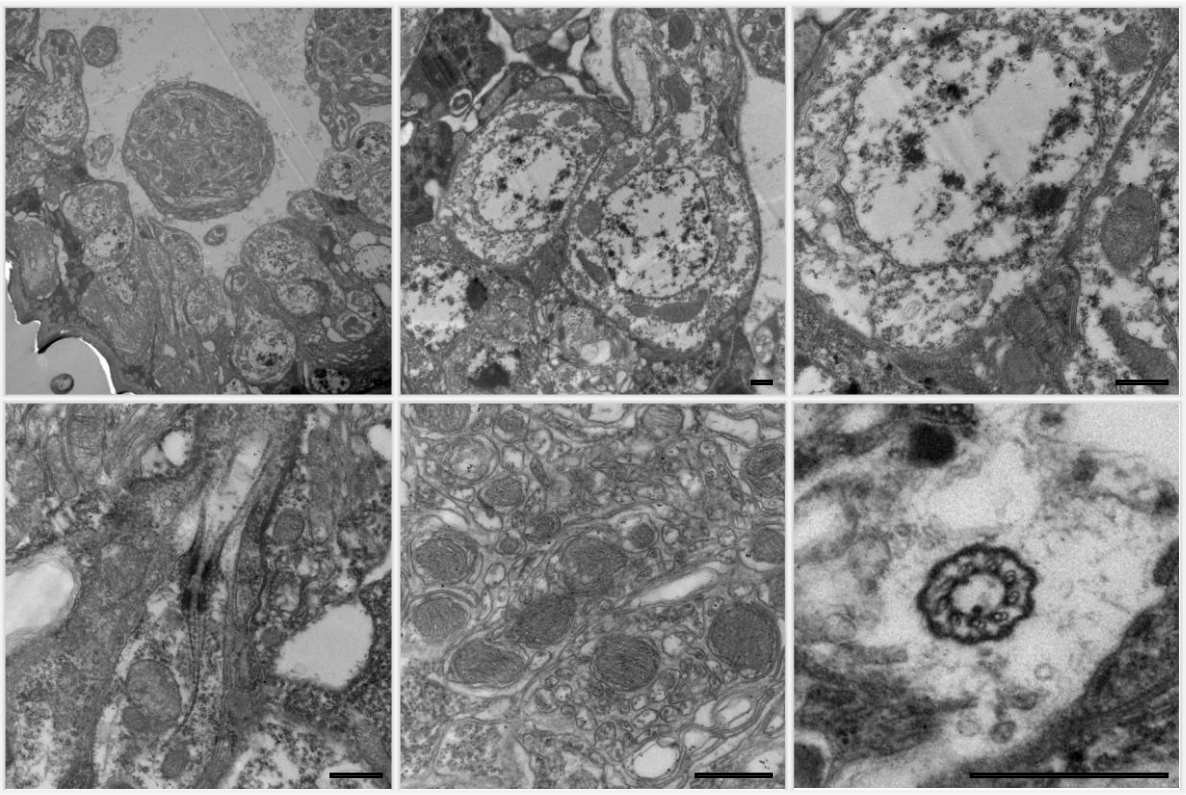
10.4.23 PHEM Sample 3



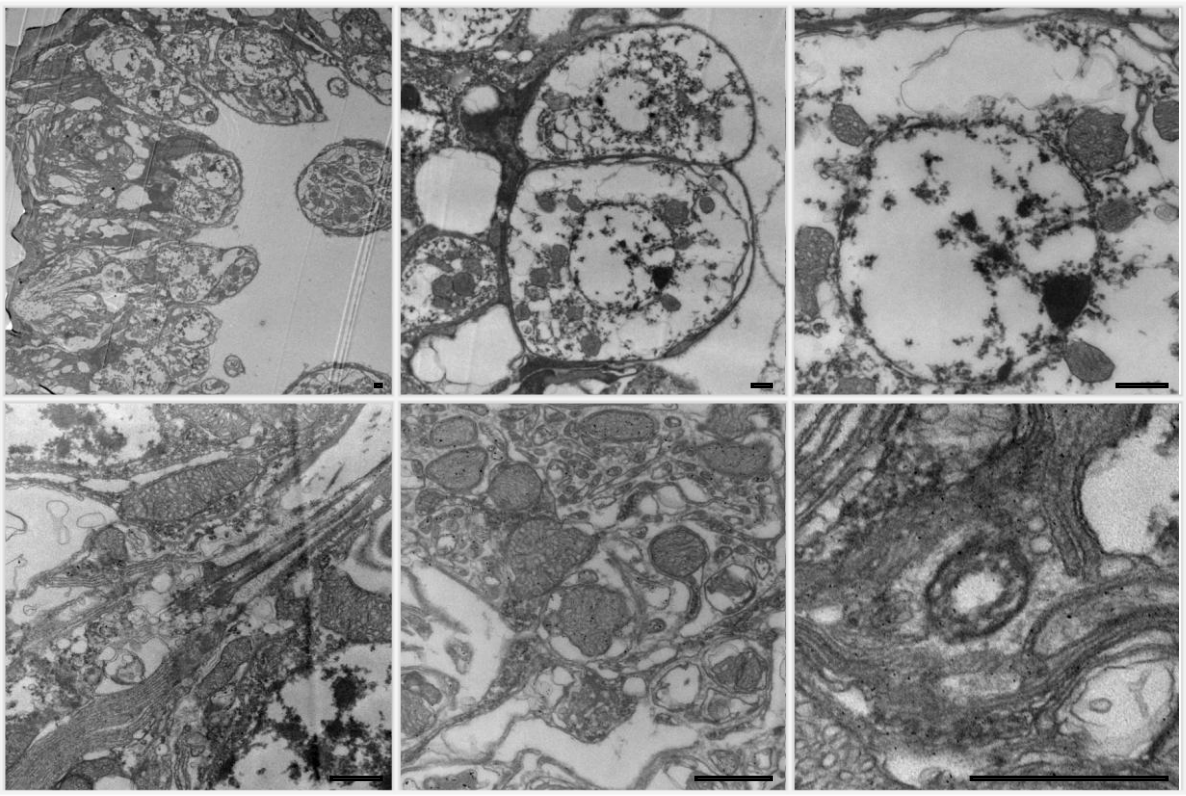
10.4.24 Cacodylate Sample 3



10.4.25 PBS Sample 3

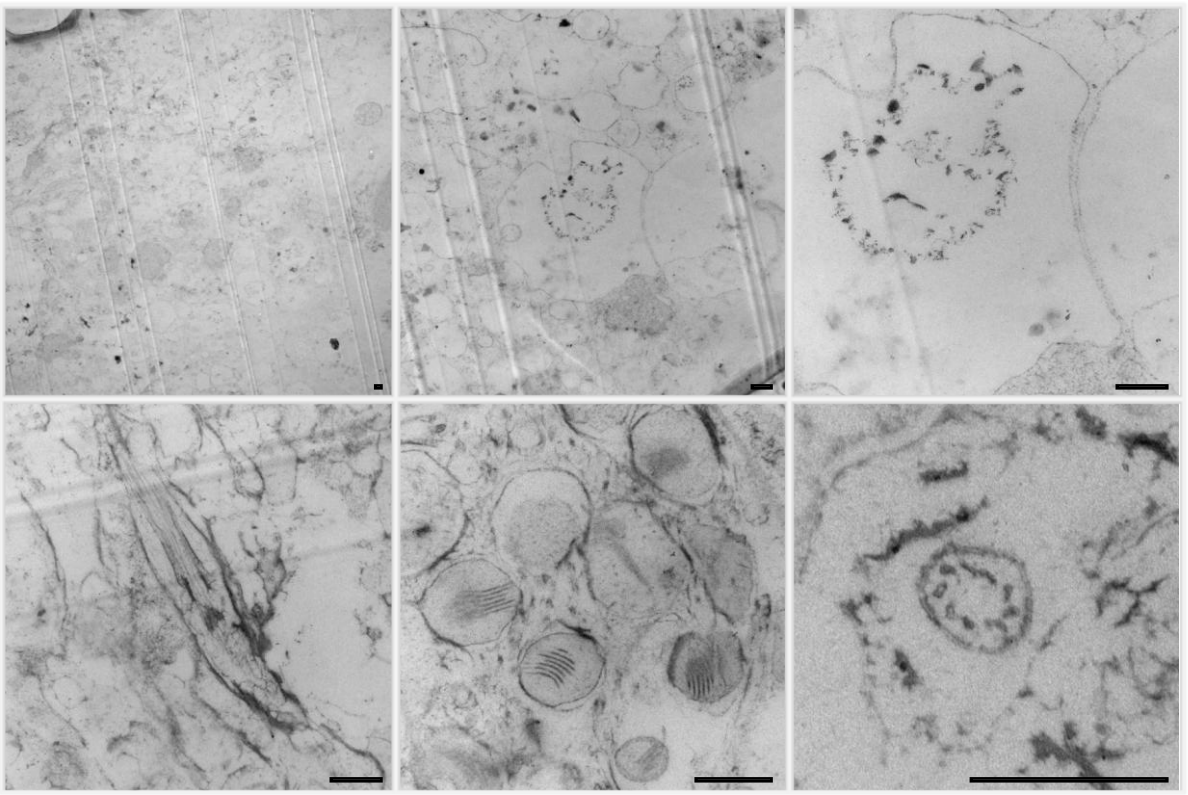


10.4.26 Water Sample 3

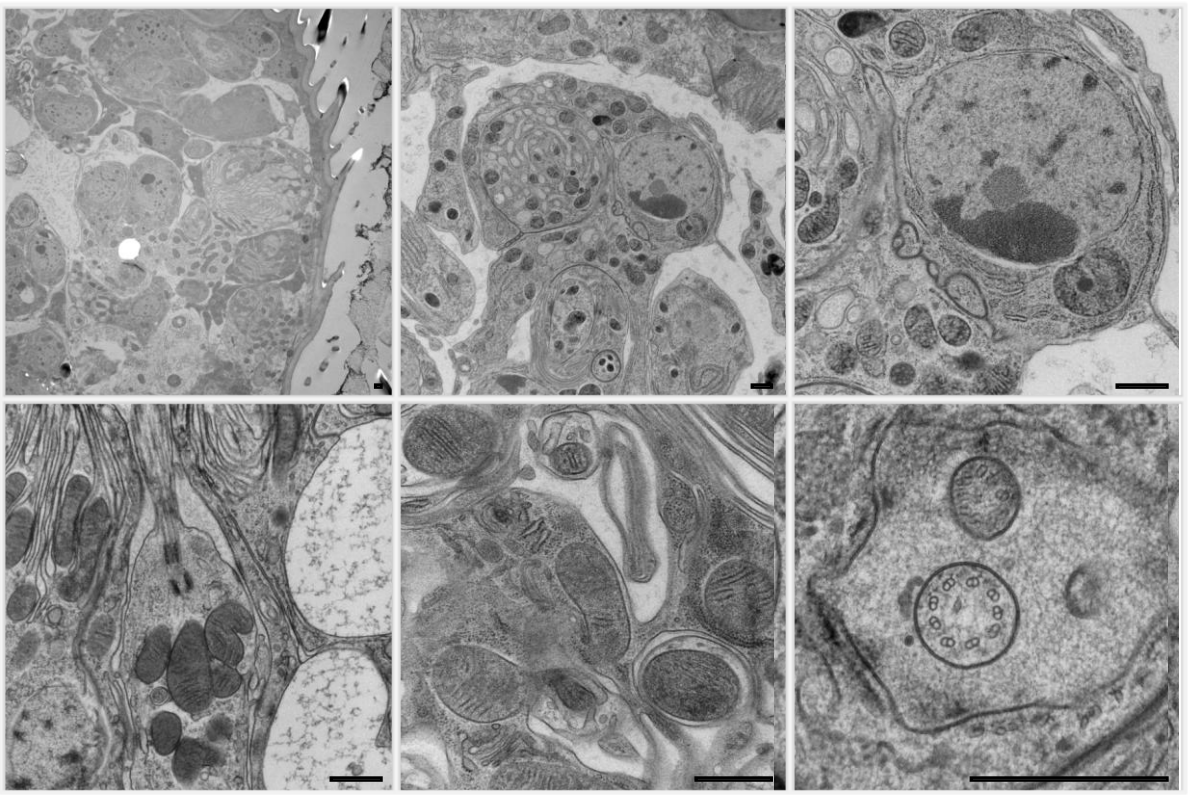


10.5 Cryo Fixation Panels

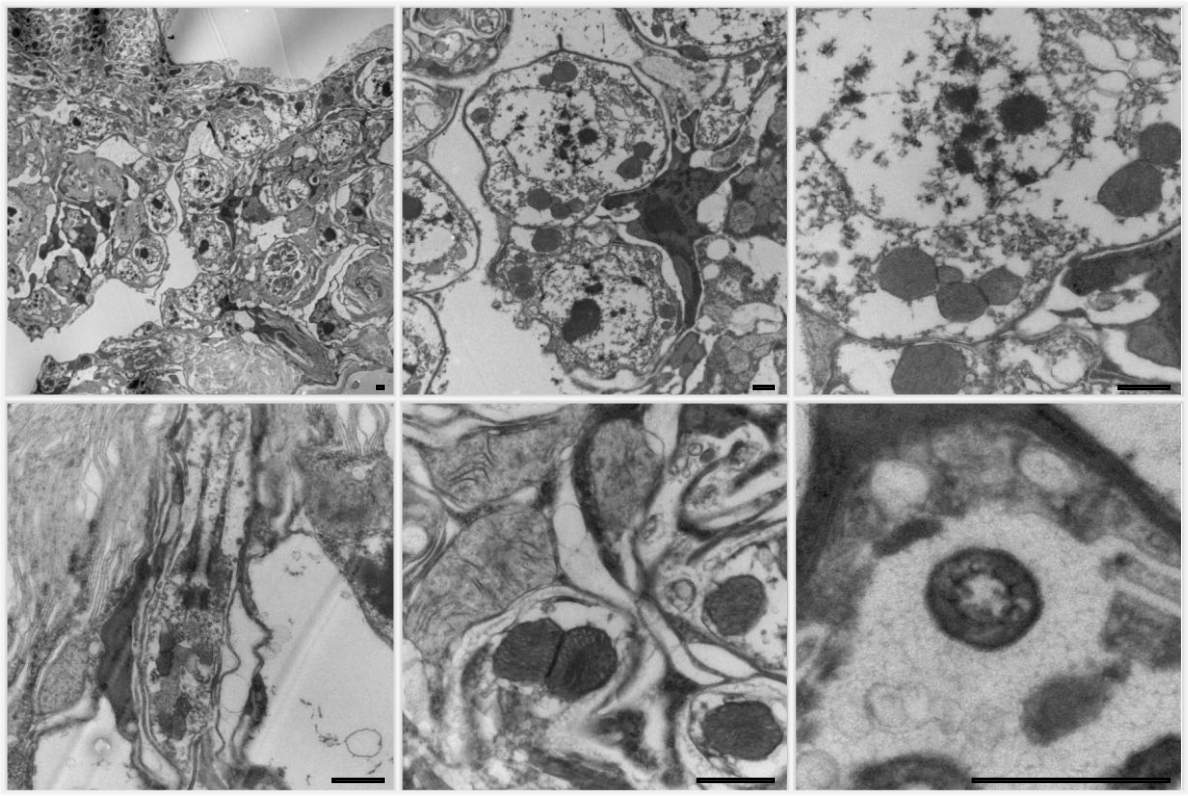
10.5.1 Fast Freeze Substitution Sample 1



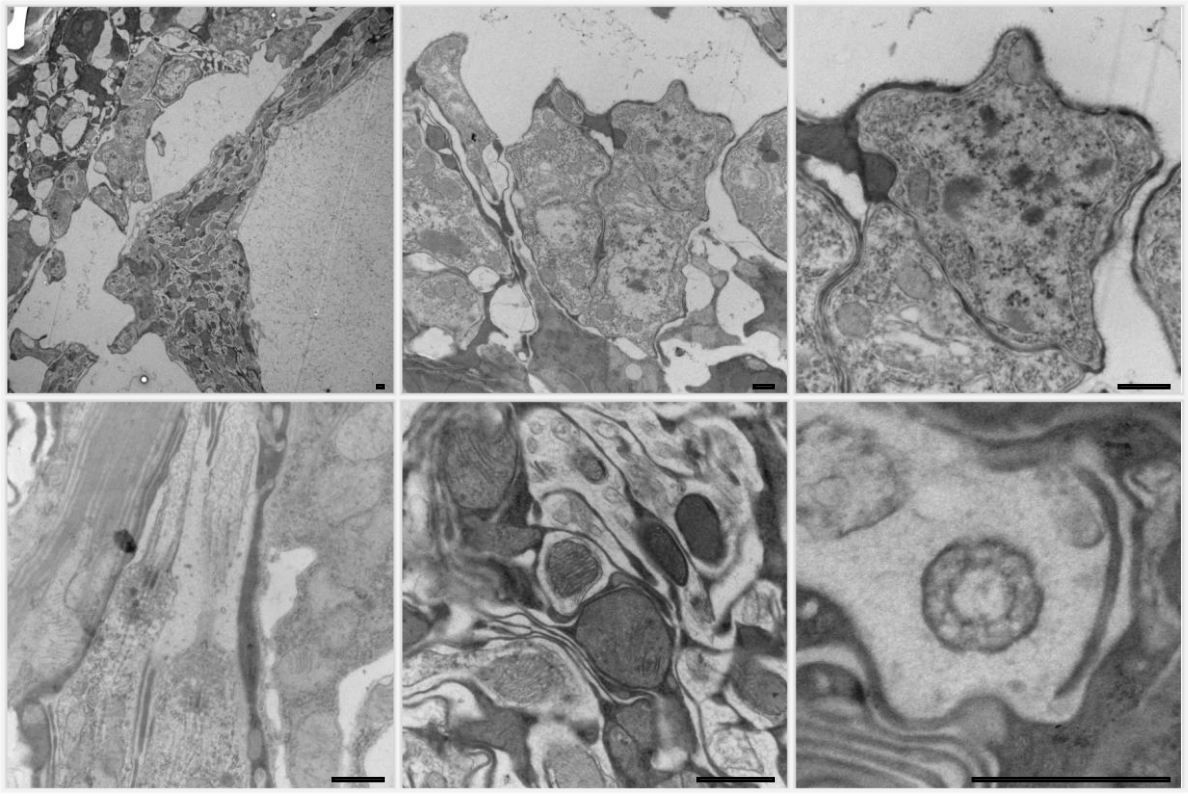
10.5.2 Fast Freeze Substitution Sample 2



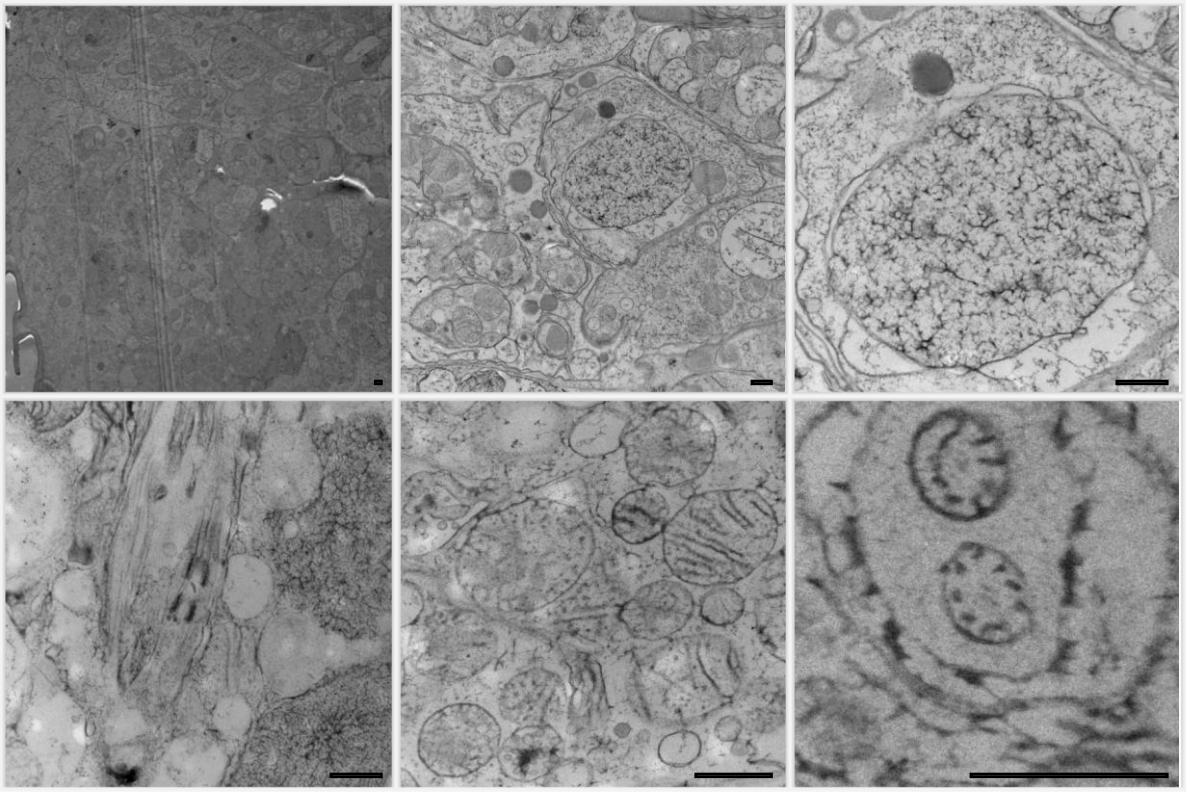
10.5.3 Medium Freeze Substitution Sample 1



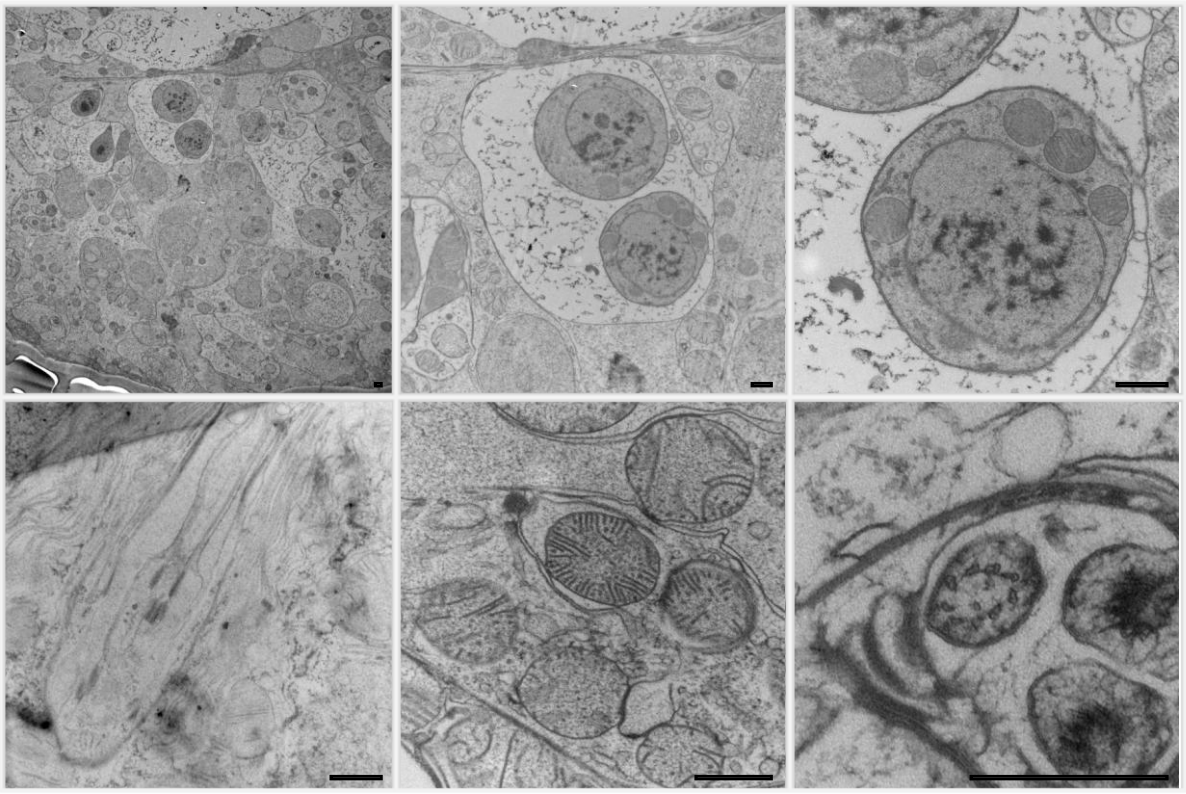
10.5.4 Medium Freeze Substitution Sample 2



10.5.5 Slow Freeze Substitution Sample 1



10.5.6 Slow Freeze Substitution Sample 2



10.6 Materials, Reagents and Individual Protection Equipment

- 1-hexadecene, Merck, CAS# 629-73-2, Lot# S6852764
- Acetone EM grade, Polysciences, CAS# 67-64-1, Lot# 648214
- Bovine Serum Albumin, Sigma Aldrich, CAS# 9048-46-8, Lot# SLBH2718V
- Centrifuge 5415D,ARS Plus, Eppendorf
- Chloroform, Merck, CAS# 67-66-3, Lot# K45872845433
- Cotton swabs
- Dalmatian hair for section tips
- DDSA, Electron Microscopy Sciences, CAS# 25377-73-5
- Diamond Knife 45º, Diatome
- Dibasic sodium phosphate, Calbiochem, CAS# 858-450-5558, Lot# D00140223
- DMP-30, Electron Microscopy Sciences, CAS# 90-72-2
- *Drosophila melanogaster* wild type, Bloomington
- EGTA, Sigma Aldrich, CAS# 67-42-5, Lot# SLBB6797V
- EMBed-812 Resin, Electron Microscopy Sciences, CAS# 25068-38-6
- Eppendorfs, 1.5mL, Fisher Scientific
- Ethanol Absolute, VWR Chemical, CAS# 64-17-5, Lot# 14G290521
- Formaldehyde 16% ULTRA PURE Polysciences, Inc., CAS# 50-00-0, Lot# 647935
- Formvar Powder, Agar scientific, CAS# 63148-64-1, Lot#R1201
- Freeze substitution machine S6E, Leica
- Gloves, Nitrile, Powder Free, Semper guard
- Glutaraldehyde, EM grade 25% Polysciences, Inc., CAS 111-30-8, Lot# 667935
- HEPES, Calbiochem, CAS# 858-450-5558, Lot# D00137935
- High Pressure Freezing machine Compact 02, Engineering Office M. Wohlwend GmBH
- Labcoat
- Laboratory Glasswear
- Lead Nitrate, Sigma Aldrich, CAS# 10099-74-8, Lot# BCBJ9164V
- Liquid nitrogen recipients for transportation (custom made, designed by E. Tranfield)
- Liquid nitrogen tank
- Magnesium chloride hexahydrate ($MgCl_2.6H_2O$), Sigma Aldrich, CAS# 7791-18-6, Lot# BCBF2018V
- Methanol, VWR, CAS# 67-56-1, Lot# 14B210516
- Molds for resin, Electron Microscopy Sciencies
- Monobasic sodium Phosphate monohydrate, Sigma Aldrich, CAS# 7558-80-7, Lot# 021M00134V
- Multiuse Labels 70x36mm, Staples
- NMA, Electron Microscopy Sciences, CAS# 25134-21-8
- Osmium tetroxide 0.1g, Electron Microscopy Sciences, CAS# 20816-12-0, Lot# 140708

- Osmium tetroxide 1g, Electron Microscopy Sciences, CAS# 20816-12-0, Lot# 121011
- Oven, Gentlab
- Parafilm, roll 10cm wide, Reagente 5
- PBS tablets, Sigma Aldrich, Product# P4417, Lot# 051M8213
- pH meter Five Easy Plus, Mettler Toledo
- PIPES, Sigma Aldrich, CAS# 5625-37-6, Lot# 110M54022V
- Pipettes, Gilson
- Propylene oxide, Sigma Aldrich, CAS# 75-56-9, Lot# 110205-1L
- Razor blades, single edge extra keen, Electron Microscopy Sciences, Cat#71962
- Rotator, Glas-Col
- Scale Scout Pro 600g, DHAUS
- Scissors
- Slides Thermo Scientific Menzel-Glässer
- Sodium Cacodylate Trihydrate, Sigma Aldrich, CAS# 6163-99-3, Lot# SLBF8273V
- Sodium Citrate, Electron Microscopy Sciences, CAS# 6132-04-3, Lot# 080828
- Sodium hydroxide pellets, Sigma Aldrich, CAS# 1310-73-2, Lot# BCBH5016V
- Sodium tetraborate decahydrate, Fluka ANalytical, CAS# 1303-96-4, Lot# 1311713
- Stereomicroscope SteREO Discovery. V8, ZEISS
- Support for coating grids with Formvar (Custom made by UMC Utrecht, NL)
- Syringe filters, Acrodisc 25mm w/ 0.2µm supor membrane, PALL, Lot#12463417
- Syringe without needle, Terumo, Ref. SS+10ES1, Lot#140530W
- Tags
- Tape
- Tips for pipettes
- Toluidine Blue, Electron Microscopy Sciences, CAS# 92-31-9, Lot# 30113
- Toothpicks
- Transmission Electron Microscope model H-7650
- Tungsten needle
- Tweezers
- Ultramicrotome Leica Reichert Ultracuts
- Uranyl acetate, BDH chemicals Ltd, Product# 10288, Lot# 6437050
- WFI (water for injection) quality water, Omnipur, Calbiochem, CAS# 7732-18-5, Lot# 98072062