UNIVERSITY OF SOUTH BOHEMIA

FACULTY OF SCIENCE

Preparation of Specimen for Transmission Electron Microscopy using Freeze Substitution and Agitation

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Bachelor's Thesis

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Annotation:

The quality of biological samples for Transmission Electron Microscopy prepared using High Pressure Freezing and Freeze Substitution with a novel agitation module invented by Helmuth Goldhammer and Siegfried Reipert was compared to Cells prepared without agitation and the Freeze Substitution protocol by Obornik et al.

The amount of extraction was quantified via immunolabelling of RuBisCO inside the plastids of the Alveolate *Chromera Velia*

Mechanical Damages were quantified by counting of obviously damaged and intact cells at low magnification

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Abstract:

The aim of this Thesis is to test the novel Agitation module developed by Goldhammer and Reipert (2016) [13] for the high pressure freezing (HPF) /freeze substitution (FS) specimen preparation technique with the Alveolate *Chromera Velia* as the model organism.

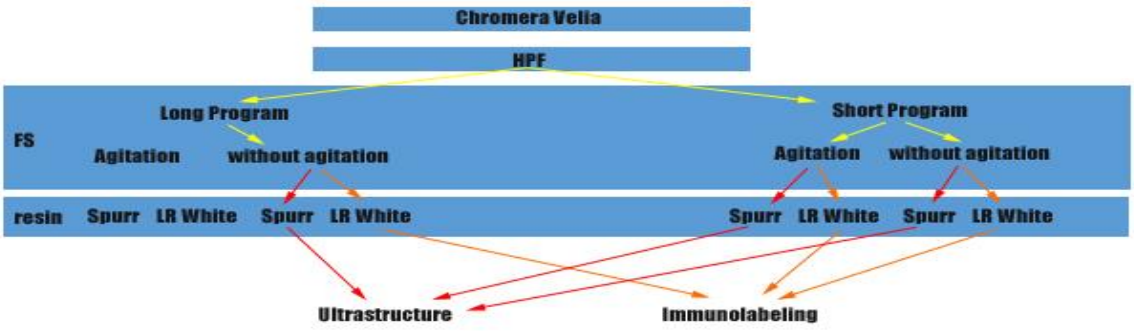
In the present experiment transmission electron microscopy (TEM) shall be used to compare preservation of ultrastructure and influence on the density of the immunogold labelling of the samples prepared by FS protocols (short, long, agitation) and verify that the agitation system is not a source of artefacts caused by mechanical damages or the extraction of biological material. In order to compare the Morphology, damaged and well preserved cells are counted and compared using an ANOVA as well as a Tukey-HSD test.

In a second sample TEM is used to compare the influence of uranylacetate (UA) and $HfCl_4$ on the preservation of the cell structures, the labeling density and contrast of biological samples.

Material Safety Datasheets can be retrieved on the emsdiasum website:

00 (Safety Data Sheet acc. to OSHA HCS, retrieved from https://www.emsdiasum.com/microscopy/technical/msds/22400.pdf on 12.03.2018 , 14:05)

Experimental design:



Introduction

High-pressure-Freezing :

High-pressure-Freezing (HPF), firstly proposed by Moor and Riehle (1968) [33], is used for the immobilization of biological activity with the aim to preserve the structures under their physiological conditions., During the HPF process the pellet of cells is loaded into specimen carriers and immediately frozen using the HPF device under high pressure with liquid nitrogen as a cooling agent . The main advantage of HPF is proper vitrification of samples up to 200 µm in thickness. The increase in pressure opposes the expansion of water that occurs during freezing and at 210 MPa the melting point of water is at its minimum at -22°C. While the volume requirement of ice crystals is greater than for liquid water up to a pressure of 210 MPa, due to the difference in density, amorphous ice is about as dense as water and thus takes up a similar volume (Huebinger et al. 2016 [20])

Besides the pressure the cooling rate also plays an important role (Fig 1.1). The red area depicts the temperature range where crystals can form. Below the so called vitrification temperature of about -130°C no more ice crystals will occur. If the cooling rate is high enough to cool the sample below the temperature threshold rapidly, no crystals can form [20]

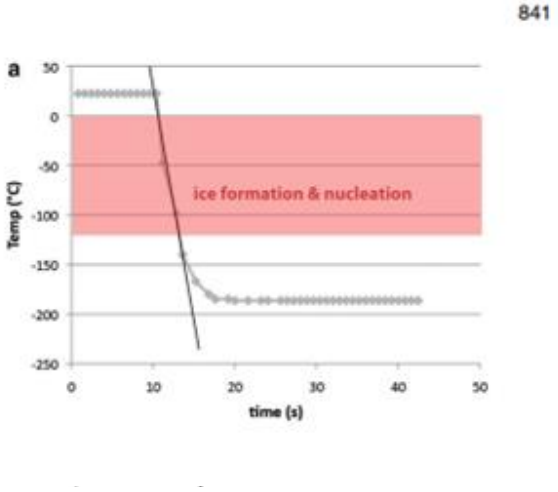


Fig 1.1: A faster cooling rate reduces the amount of crystals as the vitrification temperature is reached faster. Retrieved from Huebinger et al. [20]

HPF does not induce any major structural changes to the cell, as well as enabling fixation and concurrent immobilization of all macromolecular components, preventing labile protein systems from falling apart due to changes in osmotic pressure or temperature that could occur during chemical fixation [2].

Furthermore the sample frozen in amorphous ice can be stored in liquid nitrogen since the thermal energy at -196°C is not high enough to induce chemical reactions [42]

Freeze Substitution:

Freeze Substitution (FS) with initial cryoimmobilization provides improvements in structural preservation and avoids the creation of aggregation artifacts compared to conventional resin embedding (Humbel and Schwarz 1989 [14]; Van Harreveld and Crowell 1964 [40] Dubochet and Sartori-Blanc 2001[19]).

Additionally the sample is fixed with osmium tetroxide, uranyl acetate, or aldehydes during the warming-up period.

During the fixation the Osmium tetroxide most likely reacts with membrane phosphatides and unsaturated carbons to form cyclic monoesters, while the positive anions move between the two layers of the membrane. [18]
However osmium tetroxide may also react proteolytically [7] making it not suitable for preparation of the specimen used for immunolabelling experiments.

Osmium tetroxide begins to fix the specimen by crosslinking carbon double bonds at -70°C (White et al. 1976 [41]) and glutaraldehyde begins crosslinking at -40°C (Humbel et al. 1983 [15]) As there are no distinct procedures for FS up until today, researchers tend to use fixatives such as OsO_4 or Uranyl-Acetate in Acetone for morphological work and low concentrations of glutaraldehyde to perform immunolabeling (Kent L. McDonald 2014 [23]). Once the sample is dehydrated it is embedded in resin either epoxy resin (at room temperature) or methacrylates (e.g. LR White, at range between -50°C to -10°C based on the used resin). [4]

There are multiple FS procedures described in the “Leica EM AFS Recipe Book” (available at http://otolic.stanford.edu/documents/Recipe_book.pdf).. The wide range of methods mentioned in different literature suggests that FS is very forgiving and can even be completed within three hours or less (McDonald and Webb 2011 [30]).

Regarding the temperature programs used for FS, many adjustments can be made. Usually the temperature is kept constant for about three days after starting the process, although this step can be significantly shorter or longer (Kent L. McDonald 2014 [23]). Especially speeding up the procedure can be achieved by continuous agitation, as it provides a constant contact with the substitution medium (Kent L. McDonald 2014 [23])

Contrasting agents

- **Uranyl acetate (UA)**

Uranyl acetate stains have a pH of 3.5–4 and reacts strongly with phosphate and amino groups to stain nucleic acids [16] and proteins [26]
When using UA is important to exclude light from the staining process since uranyl acetate is sensitive to light [25] and use alcohols as a solvent, since the solubility of UA in water is lower.

A molecule of uranyl acetate in water is present as an uranyl cation ($UO2^{2+}$). At a pH of approximately 4.5, which is below the isoelectric point of most proteins, proteins are positively charged and the charged protein residues repel positively charged $UO2^{2+,}$ resulting in negative staining (Hayat and Miller, 1990 [11]).

Additionally UA can act as a fixating agent for proteins, lipids and nucleic acids by interaction with negatively charged groups (Hayat 2000) [10]. The fixing effect becomes evident when comparing stained specimen with unstained specimen via cryo-EM or x-ray diffraction (Lehmann et al. 1995)[24]

- **Hafnium Chloride (**$HfCl_4$)

Since UA is radioactive a less problematic alternate Staining agent would be desirable. When taking into account the staining mechanism of UA (Hayat 2000 [12]) among other lanthanoids , $HfCl_4$ was chosen as a suitable substitute that achieved contrasts as good as UA while causing no damages to the cell. Additionally it has been observed to aid in the staining of carbohydrates [22]

$HfCl_4$ previously has been used to increase the contrast of cytoskeletal fibres by increasing their electron density, however the mechanism of the contrasting is not known. (Hatae et al. 1984 [38], Kakimoto and Shibaoka 1987 [39]).

Materials and Methods

- *C. velia* (kindly provided by Dr. Tomčala, Laboratory Evolutionary Protistology, BC CAS) were cultured in f/2 media*

HPF:

- Cells were centrifuged for 10 min at 560 × g at room temperature

A mixture of 20% bovine serum albumin (BSA), sea water and f/2 culture medium is used as a substitution medium with BSA acting as a cryoprotectant to prevent formation of crystals [31]

Copper carriers are treated at first with a release-agent, such as 1% lecithin in chloroform to prevent the sample from sticking to the carrier during the further preparation. [31]

A drop of the lecithin solution is put on the carrier, which will evaporate quickly. After the drop is fully evaporated the sample is transferred to a copper carrier using a pipette and a small amount of medium containing BSA is added. The carrier should not be overfilled. The sample is then put into the machine using the rapid loader. Once the sample is put into the machine the sample will be cryofixated at about 2050 bar and stored in a small reservoir of

liquid nitrogen. It can then be transferred into another vial filled with liquid nitrogen and put into a polystyrene box for further storage.

FS and embedding resins:

The cryo-immobilized samples were placed in cryovials filled with the solvent precooled in the FS unit at -90°C.

The instrument used for FS: AFS from Leica.

FS protocols for ultrastructural studies

For Comparison of the ultrastructure across the different methods different temperature programs for FS were used (Fig. 2).

- Agitation and Without agitation:

  -90°C for eight hours | -90°C to -15°C for nine hours | -15°C for 52 hours

- Long Procedure according to Obornik et al. [35]:

  -90°C for 96 hours | -90 to -60°C for 7,5 hours | -60°C for 24 hours | -60 to -15°C for 9 hours | -15°C for 52 hours

Spurr's resin consists of vinylcyclohexene dioxide (ERL 4221), the diglycidyl ether of polypropylene glycol (DER 736,) and nonenyl succinic anhydride (NSA) [6]
The aliphatic DER functions as a flexibilizer, while ERL acts as a hardener. Depending on the amount of ERL used the hardness of the sample can be varied. Due to its low viscocity ERL is especially potent at penetrating the thick cell walls of i.e. plant tissues. Dimethylaminoethanol is added to increase the viscocity and quicken the polymerization, however it should not be mixed with NSA alone, as the reaction is exothermic. [6]

The Resin used was obtained by mixing:

- EMS, catalog #15004 | ERL 4221 (10g)
- EMS, catalog #13000 | DER 736 (8g)
- EMS, catalog #19050 | NSA (25g)
- EMS, catalog #13300 | DMAE - dimethylamino ethanol (0.3g)

The exact amounts should be weighed in, and one should add the catalyst, DMAE, last after mixing the other components. The mixture can either be used immediately or stored in a tightly capped syringe in the freezer for a few weeks. Before use the resin should be allowed to warm to room temperature.[6]

The substitution media for embedding in Spurr low-viscosity embedding resin had the following composition:

- 2% Osmium-Tetroxide (EMS) diluted in 100% Acetone

FS protocols for immunolabeling:

For Comparison of immunolabeling across the different methods different temperature programs for FS were used (Fig. 2).

- Agitation and Without agitation – short procedure:

  -90°C for eight hours | -90°C to -15°C for nine hours | -15°C for 52 hours

- Long Procedure according to Obornik et al. [35]:

  -90°C for 96 hours | -90 to -60°C for 7,5 hours | -60°C for 24 hours | -60 to -15°C for 9 hours | -15°C for 52 hours

The substitution media and LR-White resin (medium, EMS Catalog #14381) had the following composition:

- 0.25% Uranyl-Acetate (EMS Catalog #22400) and 0,01% glutaraldehyde (EMS) diluted in 100% Acetone

- 0.25% $HfCl_4$ (Sigma, cat no. 590592) and 0,01% GA diluted in 100% Acetone

- LR-White Resin (EMS Catalog #14381) was mixed with the UV catalyst Benzoin Methyl Ether (EMS Catalog #11290) 5mg per 1gram of medium or 0.5 % (w/v) shortly before the Polymerization step

FS and Embedding of the samples:

When the last step of the protocol is reached the samples are washed three times in pure Acetone for 20 minutes each. In the following step three resin/acetone mixtures are added to each sample and left to infiltrate for one hour each.

Depending on the preparation methods the following mixtures are used (table 1):

Table 1:

	Step 1	Step 2	Step 3	Step 4	Agitation Module
Agitation	Resin/Aceton (1:2)	Resin/Aceton (1:1)	Resin/Aceton (2:1)	Pure Resin	yes
No agitation					no
Long					no

For the infiltration the Spurr resin is mixed with a dehydration fluid, in this case Acetone and the resin in a 1:2 ratio. It is advised to use a specimen rotator. The mixture is swirled and left for two to three hours. The same procedure is repeated with a 1:1 ratio of Acetone and Resin. The sample is then left overnight followed by a repetition with a 1:3 Acetone/resin mixture for two to three hours. Finally the sample is left for infiltration in pure Resin for five to six hours and cured at 60°C for 16 hours to a day.[6].

For the infiltration in LR-white resin each vial was filled with about 0,5 ml of mixture. After the samples were treated with all three mixtures for one hour the samples are embedded in pure resin overnight at -15°C. The next day vials for polymerization are precooled in the FS unit and the samples are taken out and kept cool on ice. The samples are put into a Petri dish filled with a bit of resin one at a time and removed from the sample holder with a needle. The sample is then transferred to one of the tubes and left in the FS unit. After all samples are transferred the UV lamp is mounted on the FS unit and the specimens are left to polymerize for 48 hours at -15°C. After the polymerization is finished the tubes containing the samples can be cut off with a scalpel and the blocks can be prepared for further processing under the microscope.

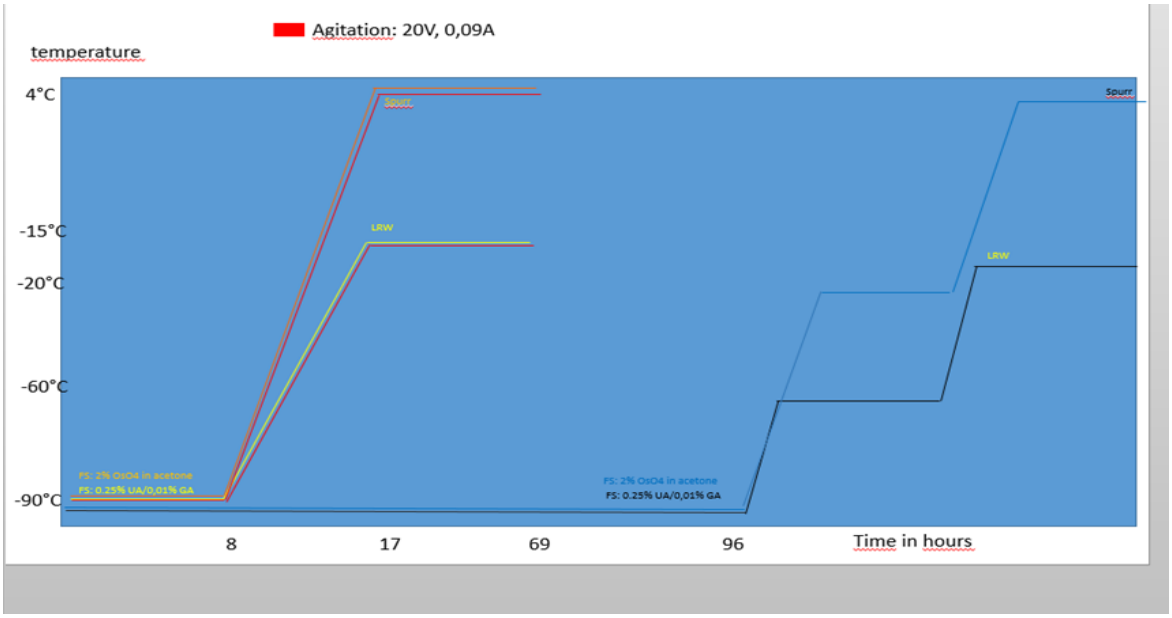


Fig 2: Visualization of the temperature protocols

Using Hafnium as a contrasting agent:

A solution of 0.25% $HfCl_4$ (Sigma, cat no. 590592) and 0,01% Glutaraldehyde diluted in 100% Acetone was used instead of the UA solution.

The Agitation Module:

In the present experiments the Agitation module (Fig. 3) was operating at about 20 V and 0,09 A. The samples prepared by HPF are transferred into small vials inside the freeze substitution unit. One should be careful to not take the vials out of the unit, as water may condense on the surface or the samples might thaw, which will reduce the quality of the sample considerably in terms of stability. The agitation device is a ring with radially aligned sample holders (Fig. 3 A). Each tube is put into one of the special sample holders of the agitation module (Fig 3 B). To each tube a bit of the solution is added, so the samples are covered, the protocol of the FS unit is started and the agitation module (fig 3. C marked by 1) mounted on a plexi glass plate (Fig 3 C, marked by 2) is placed on top of the cryochamber) so that the rotor blade (Fig 3 C, marked by 3) can rotate freely. One should make sure that the sample holders can move while the rotor blade is turned on [13].

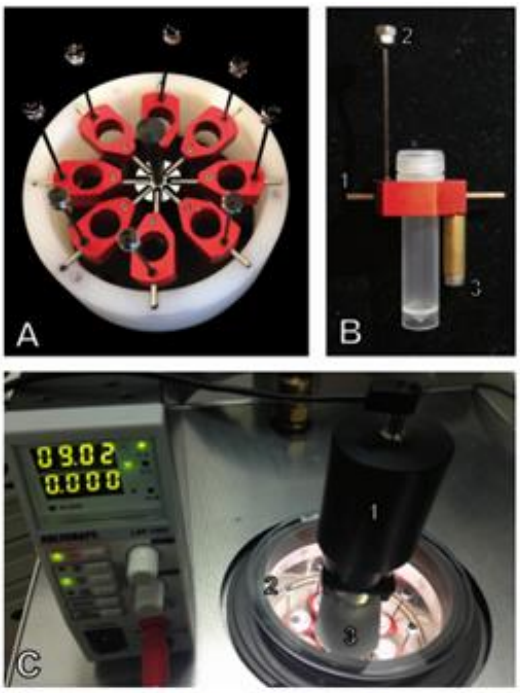


Fig. 3: The Agitation Module [13]

Sectioning:

Preparation of glass-knives:

In order to prepare semi thin sections glass knives can be used using the knife maker. There are three pins visible in the knife maker. On the bottom side resides one balance pin and on the top side there are two breaking pins. A bar of glass is put inside the apparatus and broken into squares by applying pressure via the breaking pins. The square is then placed on the balance pin, so that the one corner points towards oneself, resulting in a diagonal break (Fig. 4). The pressure should be applied in such a way, that it takes about three minutes for the glass to break into two pieces. As the breaking line will not be perfectly diagonal there will

be a small rectangular area on one side. This is called the “counter piece” (Fig. 5) which should be smaller than 0.1 mm and parallel [36]. When putting the knife down one should take care that the side with the counter piece faces the surface the knife stands on, as the knife might be damaged otherwise. The other side is the sharp edge used for cutting. Under the microscope a curved stress line will be visible (Fig. 6). For cutting the side where the stress line coincides with the knife edge should be used [36]. Before the knife can be used for cutting a knife reservoir has to be prepared. A small strip of metal tape band is used to form an arch that starts on one side of the knife and ends on the other. The reservoir has to line up with the knife edge. In order for the reservoir to become waterproof nail enamel is used to cover open spaces between the knife and the reservoir. After the enamel has dried the knife is ready for cutting [36]

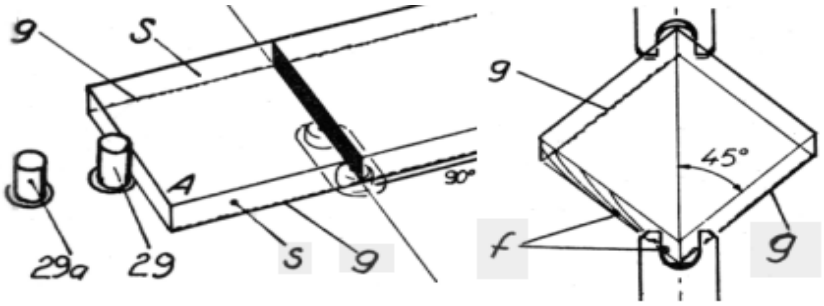


Fig. 4 Preparation of squares and triangles:
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https://depts.washington.edu/if/lkb7801b_inst.shtml [44]

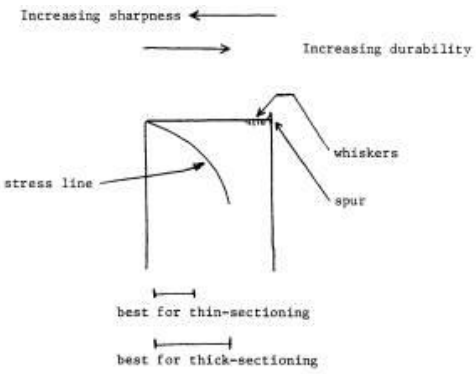


Fig. 6 Stressline:
http://bomi.ou.edu/bmz5364/making-knives.html
[28]

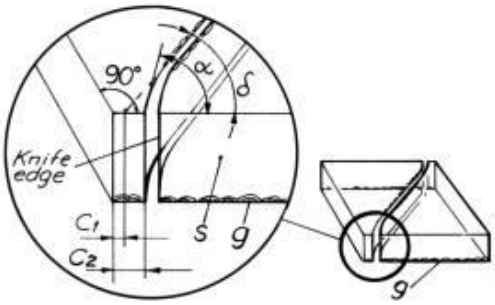


Fig. 5 Counterpiece
http://bomi.ou.edu/bmz5364/knife-maker.html
[28]

Preparation of pyramids and cutting of resin blocks:

Before cutting a pyramid has to be cut out of the block. This can be done by using a razor blade and is performed under the microscope of the ultramicrotome (Leica UCT). The sides of the pyramid should be cut in an approximately 45° angle and the specimen itself should be in the tip of the pyramid. This tip does not have to be in the center of the pyramid [36]. Now an ultramicrotome is used to cut semi-thin sections with a thickness of about 0,5 µm from the block. The block is placed in the specimen arm of the microtome and a glass knife is put into the knife holder. The reservoir of the knife is now filled with 10% Acetone until the liquid lines up with the edge of the knife. Now the flat tip of the pyramid is placed parallel to the knife. This can be achieved by constantly moving the pyramid up and down. The reflection of the glass knife on the pyramid will be visible as a rectangular shape. If the height of this shape stays the same while moving the block it is parallel to the knife. Should the block not be parallel to the knife it can be adjusted using the specimen arc [36]. The first cut should be done at a higher speed, however the subsequent cuts should be performed at a slower speed. A drop of 10% acetone is placed on a glass slide. The sections are then transferred from the knife reservoir to the glass slide using a glass rod. The Acetone is then evaporated using a heating plate (60-80°C). Once the Acetone has evaporated several drops of 1% toluidine blue were placed

on the sections using a syringe. After one minute the sections were washed off with distilled water. The specimen can then be observed under the light microscope.

Before ultrathin sections are cut the size of the pyramid is reduced if necessary. The ultrathin section's thickness should be 70nm. The ultrathin sections are cut using the diamond and the knife reservoir is filled with distilled water instead of 10% Acetone. After cutting a cotton swab is dipped into chloroform and slowly moved over the sections floating on the surface of the water. This will remove wrinkles and reduce the compression[34].
To pick up the sections one can simply lower the grid onto the swimming sections using tweezers [36].

Antibody labeling:

- Primary antibodies: polyclonal RuBisCO antibody (large subunit, form II, Agrisera, Vännäs, Sweden)
- Secondary antibodies: Protein A conjugated to 10 nm gold particles, as gold particles have excellent electron scattering properties and are thus clearly visible under the microscope [32].
    - Both antibody solutions are diluted 1:50 with blocking buffer
- Blocking Buffer: 10% goat serum (Sigma-Aldrich), 0.05% Tween (Sigma-Aldrich), 0,02 M Glycine (sigma-Aldrich) in 0,1 M HEPES (sigma-Aldrich)
- Washing Buffer: 1:10 diluted Blocking buffer
- 0,1 M HEPES
- 2,5% Glutaraldehyde (EMS) in 0.1 M HEPES
- Distilled water
- Parafilm

The labeling procedure was performed in a humidity chamber to prevent evaporation of drops during incubation of grids on drops of smaller volumes. Pieces of wet paper were placed on the top part of the parafilm. Furthermore the parafilm must be covered during all waiting periods to prevent contamination by dust particles in the air and the grids may only be transferred using clean tweezers. In the beginning every grid was placed on a 50 µl drop of blocking solution for one hour. In the next step the sample is transferred to 10 µl of primary antibody solution and left for 1 hour followed by washing six times in 50 µl of washing buffer for 3 Minutes per drop. After washing the specimen were transferred to drops of secondary antibody solution (10 µl) and again left for one hour followed by another set of washing steps. Next up is transferring the grids to Glutaraldehyde (10 µl) for 1-3 minutes. In this step a fume hood should be used if possible, as Glutaraldehyde is an irritant and toxic. If no fume hood is available the drops should be removed again as soon as this step is over. Each sample is washed in three drops of HEPES for 2 minutes followed by washing in three drops of distilled water. In the end each grid is transferred to ethanoic UA (10 µl) for 5 -10 minutes for staining which is applied using a filter. It is important to note, that UA is very sensitive to light, thus the drops must be protected from light [25] during the staining. After

completing this last step, the grids are washed in 30% ethanol using three different Petri dishes. Before observation the grids can be coated with carbon.
(see below)

Statistical Evaluation

In order to evaluate the efficiency of the labeling across the different methods the average amount of nanoparticles per µm2 was counted. This was done using ImageJ which can calculate the areas using a scale bar from the image taken. For every picture the amount of gold nanoparticles inside a plastid and outside (calculated as area of the whole image minus area of the plastid) was recorded. As the antibodies are specific to the RuBisCO inside the pyrenoids of the plastid the antibodies outside the plastid are the amount of antibody background which should be taken into account.

Contrasting agents:

In order to improve the contrast of the sample, the specimen is stained with UA and lead citrate

- Ethanolic UA (saturated solution from 50-70% Ethanol)
    - Prepared by weighing in 2.6 g of UA powder in a dark bottle followed by adding 20 ml of 50% Ethanol. The mixture is stirred until all of the powder is dissolved (about 2 hours) and filtered three times through the same filter. The solution must be stored at 4°C and can be used for about 3 weeks. When applying the solution one should use a syringe with a 0.45 µm filtertip to avoid contamination by undissolved solid particles
    - (EMS Catalog #22400)

- Lead Citrate solution
    - 0.02 to 0.04 g of lead citrate are added to 20 ml of boiled and cooled distilled water. To the mixture 0.2 ml of 10N NaOH are added and the solution is stirred until the lead citrate dissolves. The solution must be stored in a dark bottle and tightly covered. The solution should be stored at 4°C and can be used for a month, however it has to be centrifuged before use.

- 30% Ethanol
- NaOH pearls

The staining was conducted in the dark as UA is susceptible to light [25]. Parafilm was spread on the bottom of, for example, a metal box with ethanolic atmosphere to minimize evaporation of UA ethanolic solution. Two 50-100 µl drops of UA were applied using a syringe with a 0.45 µm filter tip. The grids were then left to incubate for 30 minutes [25].

In the next step the grids were washed in 30% Ethanol (Same as in immunolabeling) and left to dry.

After drying the grids were stained using lead citrate in an NaOH environment for 20 minutes. NaOH pearls are therefore put next to the parafilm and a few drops of water are added. This step was carried out in a covered dish to prevent atmospheric CO2 from touching the sample. After staining the grids were rinsed again with double distilled water, as a too high content of CO2 might lead to the precipitation of toxic Lead Carbonate [25].

Carbon coating:

In order to protect the specimen form beam damage the grids were coated by carbon using the carbon coating device (JEOL JEE 4C). This was achieved by mounting a carbon thread or rod between two high-current sockets in a vacuum space. The current then will heat the carbon to evaporation and a thin film of carbon is applied to the grid. Note that it is important that the space is fully evacuated, as carbon will burn in the presence of oxygen [8]. One of the factors that make this method so widely usable are the low background signal and good electrical conductance of the carbon [17].

TEM

The grids were observed under the Transmission Electron Microscope Jeol 1010 at accelerating voltage 80 kV equipped with CCD SIS MegaView III.

Results

Preservation of ultrastructure using different FS protocols

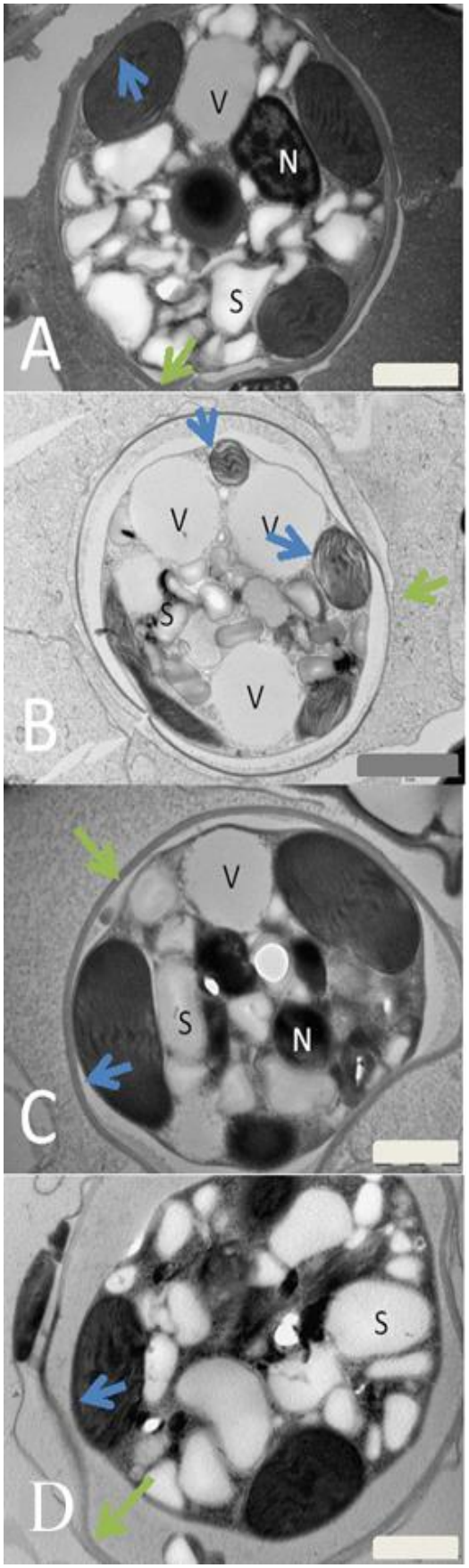


Fig.7:
A comparison of morphology of C. velia cells prepared by freeze substitution techniques short procedure without agitation (A), short procedure with agitation (B) and the long procedure (C, D).

V = vacuole; S = starch granule; N = nucleus
Green arrows indicate cell walls
Blue arrows indicate plastid membranes
The bars are 1 µm.

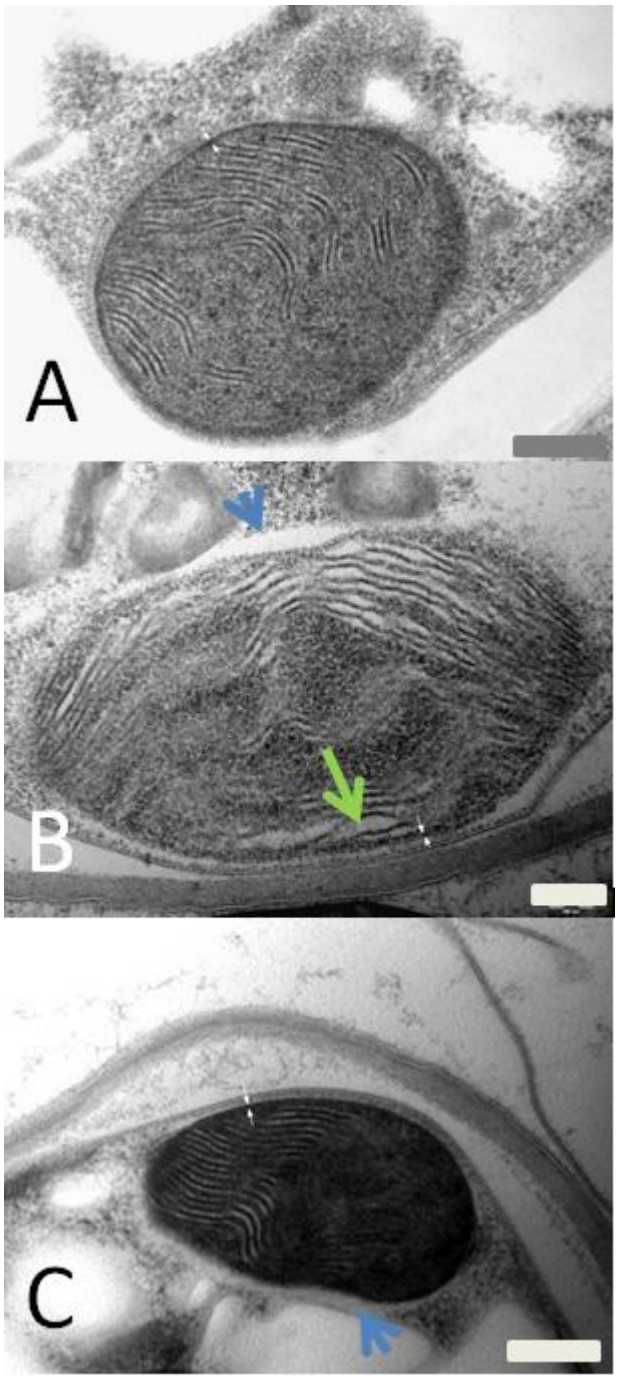
The amylopectin granules (Fig. 7, A-D, marked by S) were still intact and seem to be similar in shape across all tested methods. However they have taken on greyish color in the sample prepared with agitation. (Fig 7, B) which can be related to the different thickness of sections.

The other organelles of cells, for example large electron-lucent vacuoles (Fig. 7, A-D, marked by V) seem to be intact also.

The cell wall, indicated by the green arrows in Fig. 7 A-D was detached from the cells and probably fractured during the FS procedure, however the cell membrane remained intact as no obvious leakage of cytoplasm was observed.

When comparing the membrane of the plastids, indicated by the blue arrows in Fig 7 A-D, it appears that they are better visible in the picture taken from the sample prepared with agitation (Fig 7.B). The specimen prepared without agitation (Fig 7. A) and with the standard long procedure (Fig 7. C,D) they seem to be much fainter, which can be caused by orientation of structures to the beam of the primary electrodes [43].

Extraction of cell material



If the plastids are observed at a higher magnification one can indeed confirm that the lipid bilayer surrounding the plastids is still present regardless of the method used for preparing the sample.

Missing (white) areas of stroma and "better visible" or wavy appearance of thylakoid membranes in plastids in Fig. 8 B and C could be artificially created by higher extraction of Biological Material

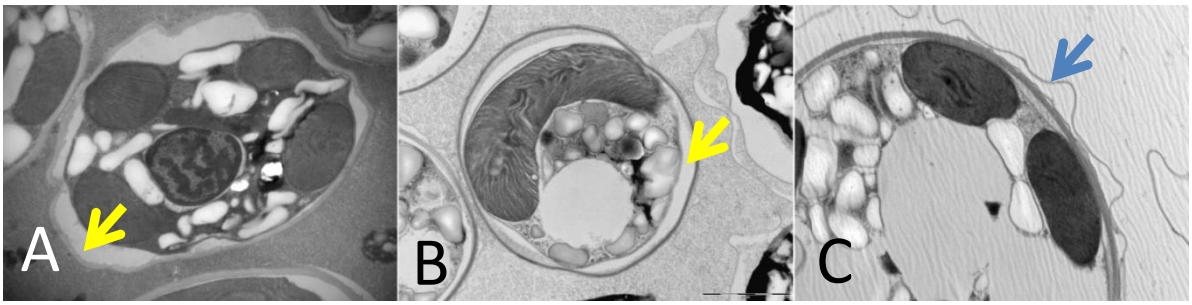
In Fig 8. B and C the blue arrow points at slight 'halos' visible around the plastids that can occur if tension acts on the cytoplasm [13]

One can also observer blurry edges at some parts of the plastid, mainly visible on thylakoid membranes which can be caused by orientation of the structures to the electron beam [43] .

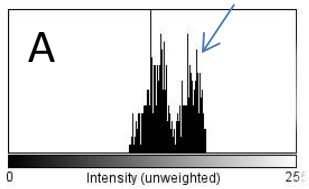
The actual amount of extraction of organic material (RuBisCO) from the plastids will be showed further in the section "Evaluation of immunolabeling".

Fig .8:
Detailed images of plastids prepared by freeze substitution techniques short procedure without agitation (A), short procedure with agitation (B) and the long procedure (C)
The lipid bilayer is indicated by the white small arrows
The blue arrow shows artificial areas in the cytoplasm created after the detachment/shrinkage of the plastid. Other Artificial areas were found in the stroma of plastids (green arrows). The bars are 200 nm.

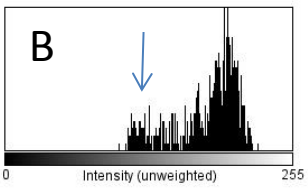
Evaluation of Staining Intensities



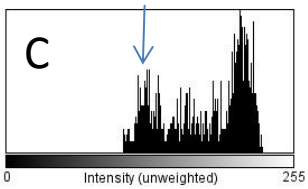
Cell wall:



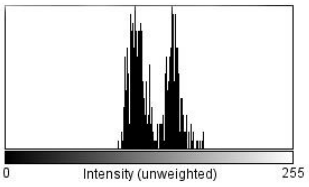
Count: 464 Min: 107
Mean: 143.345 Max: 175
StdDev: 18.125 Mode: 126 (18)



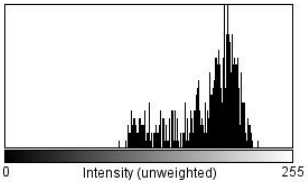
Count: 580 Min: 101
Mean: 178.414 Max: 225
StdDev: 29.344 Mode: 195 (19)



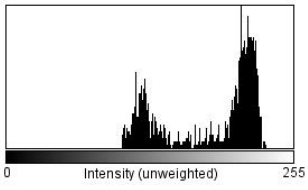
Count: 975 Min: 104
Mean: 176.077 Max: 228
StdDev: 36.452 Mode: 208 (24)



Count: 468 Min: 100
Mean: 131.152 Max: 176
StdDev: 18.185 Mode: 115 (17)

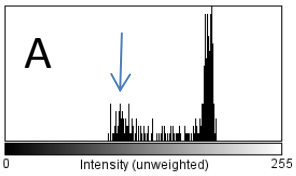


Count: 580 Min: 101
Mean: 178.414 Max: 225
StdDev: 29.344 Mode: 195 (19)

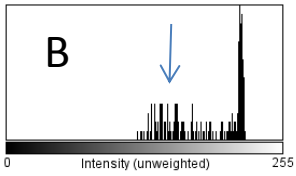


Count: 1197 Min: 103
Mean: 181.159 Max: 231
StdDev: 41.948 Mode: 209 (41)

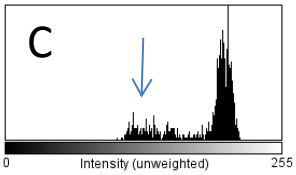
Cell membrane:



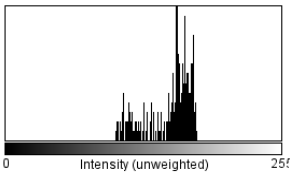
Count: 266 Min: 95
Mean: 162.846 Max: 195
StdDev: 33.733 Mode: 191 (18)



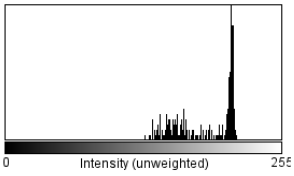
Count: 191 Min: 121
Mean: 184.764 Max: 221
StdDev: 32.597 Mode: 216 (15)



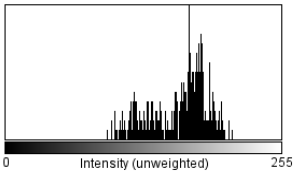
Count: 738 Min: 103
Mean: 183.976 Max: 217
StdDev: 30.885 Mode: 206 (43)



Count: 254 Min: 102
Mean: 151.453 Max: 177
StdDev: 21.011 Mode: 158 (14)



Count: 245 Min: 129
Mean: 185.086 Max: 214
StdDev: 26.679 Mode: 209 (26)



Count: 350 Min: 94
Mean: 160.337 Max: 210
StdDev: 26.788 Mode: 170 (14)

Fig 9: Ultrathin section (70 nm thick) of C. velia cells prepared by FS protocols without agitation (A), with agitation (B) and the standard long FS procedure (C). The sections were stained with ethanolic UA and lead citrate. The Source Images shown at the top row were used for measuring the pixel intensity values for the cell wall and resin with the aim to the differences in staining between cell membrane and cell wall of obtained images. The sown histograms were recorded from different areas and shall be representative of the intensity of the staining. The arrows represent the peak originating from the cell wall or membrane

The pixel intensity values obtained from the histograms (Fig. 9) proved different intensities of cell wall and cell membranes in samples prepared by different FS protocols. Less intense cell walls were found in the samples prepared by short FS protocols without agitation (Fig. 9 A), but short FS protocol with agitation and long FS procedure (Fig 9, B and C) reports similar pixel intensity values. The cell wall of the specimen in picture Fig. 9 C, indicated by the arrow seems to be slightly wrinkled

Mechanical Damage

For a comparison of the mechanical damage of cells across the three different preparation FS methods the amount of damaged cells per square of the grid were counted and an ANOVA test combined with a tukeyHSD test was applied to the results of the counting. For this evaluation the statistics program "R" was used. The Null-hypothesis for the test is "There is no significant difference between the different methods". The samples were embedded in Spurr, as epoxy resins will give a better ultrastructure over all (see embedding). Ultrathin sections with a thickness 70nm were cut and collected with copper grids. Coating with formvar and Carbon was performed to reduce the amount of beam damage.

The cells were evaluated according their integrity. Cells with heavily damaged cell walls, distorted or heavily wrinkled shape or completely black color were labeled as damaged in the evaluation. For the statistical evaluation only the amount of good cells was compared. On average the amount of good cells was 62,05% for the short FS procedure with agitation, 61,82% for the long FS procedure without agitation and 65,86% for the short FS without agitation. (table 2)

Table 2. Ultrastructural preservation of Chromera velia cells prepared by three different freeze substitution protocols (short with and without agitation, long). The results were statistically evaluated using an ANOVA and Tukey HSD test in R

Short FS - ag			Short FS - No ag			Long FS - no ag		
	damaged	good		damaged	good		Damaged	good
Mean	0.3795	0.6205	Mean	0.3414	0.6586	Mean	0.3818	0.6182
St. Dev	0.0498	0.0498	St. Dev	0.0414	0.0414	St. Dev	0.0718	0.0718

Summary					
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Procedure	2	0.0453	0.022625	7.256	0.00103**
Residuals	129	0.4023	0.003118		
TukeyHSD					
	diff	lwr	upr	p adj	
Long - ag	-0.0022	-0.0304	0.026	0.981	
No ag - ag	0.0381	0.0099	0.0663	0.0049	
No ag - Long	0.0403	0.0121	0.0686	0.0027	

In the test a p-Value of less 0.05 will indicate a significant difference with 95% certainty for the given Null-Hypothesis. As the results of the ANOVA test show, there is no significant difference in the ratio of well-preserved and damaged ultrastructure between the samples with agitation and the long procedure, as indicated by the p-value of about 0.98. However, there is a significant difference between the sample prepared without agitation (short FS) compared to both the long FS protocol and short FS with agitation, as the as shown by the p-values of 0.0049 and 0.0027 respectively. This means that our Null-hypothesis is rejected when comparing the preparation method without agitation to the other two.

Evaluation of the immunolabeling:

For the preparation of samples for immunolabeling LR-White was used, as the acrylic LR-White is better suited for this task than, epoxy resins due to its “looser” matrix and possible better accessibility of the antigens on the surface of the section [21].

On the other hand, the ultrastructure of the samples embedded in LR White was only moderately preserved (Fig. 6). Inside the Plastids of the cells pyrenoids containing RuBisCO can be found. In order to determine the difference in extraction of organic material among the different FS protocols, labeling with primary antibodies targeting RuBisCO and secondary antibodies conjugated to 10 nm gold nanoparticles (NPs) were used. These gold particles, visible as electron dense spots in the TEM image, are counted for statistical evaluation. The results of the immune-gold-labelling were evaluated by counting the NPs inside the plastid, where specific binding occurs, and outside the plastid. The Labeling density was obtained by dividing the amount of nanoparticles by the area. A Chi-square analysis with the null hypothesis “There is no significant difference from random distribution” was conducted and the relative labelling index (RLI) was calculated according to Mayhew et al (2002) [29]. The labelling density data can be found in Table 3.

It is evident from the RLI value and the labelling densities that the agitation module-short FS protocols greatly decreased the amount of extraction from the cells compared to the long FS procedure. The short FS procedure without the agitation module also shows lower extraction, which is likely due to the lower exposure time to the substitution medium

One problem is that the antibodies sometimes formed aggregates. In this case, clusters of antibodies consisting of four or more antibodies that are ten or less nm apart were considered as an aggregate and counted as one antibody. Centrifugation to remove the aggregates was not successful. In order to evaluate the results, a chi-square test was performed as it is used to evaluate the deviations of observed and expected frequencies. (table 3.)

Table 3: Statistical evaluation of RuBisCO immunolocalization in plastids of *C. velia* prepared by three different freeze substitution (FS) protocols, the long FS protocol without agitation (L), the short protocol without agitation (S) and with agitation (M)

	LD PI	LD Os	∑ Au PI	∑ Au Os	∑ A PI	∑ A Os
	(particles/µm^2)	(particles/µm^2)	(particles)	(particles)	(µm^2)	(µm^2)
S Control	3.85	0.7	38	40	9.88	57.43
L Control	6.75	1.72	43	76	6.37	44.18
M Control	6.46	0.6	53	21	8.2	35
S RuBisCO	19.52	2.13	257	108	13.16	50.6
L RuBisCO	15.1	2.78	199	165	13.18	59.39
M RuBisCO	45.72	4.77	536	184	11.72	38.54
Sum			1682		347.66	
LD (particles/µm2)		4.84				

	EXPECTED gold		**χ2 PI**	**RLI PI**	**χ2 Os**	**RLI Os**
	gold Pl	gold Os	/E	RLI=O/E	/E	RLI=O/E
S Control	47.78	277.85	2	0.8	203.61	0.14
L Control	30.84	213.75	4.79	1.39	88.77	0.36
M Control	39.69	169.34	4.46	1.34	129.95	0.12
S RuBisCO	63.69	244.79	586.69	4.04	76.44	0.44
L RuBisCO	63.78	287.33	286.72	3.12	52.08	0.57
M RuBisCO	56.72	186.44	4049.99	9.45	0.03	0.99

Abbreviations used:

O: observed E: expected

Au: gold particles A: area

LD: Labeling density (gold particles per µm^2)

Pl: Plastid Os: Outside (the plastid)

Sum of all chi values: 5485.54 | degrees of freedom: 11 | $p < 0{,}005$

The degrees of freedom are calculated as follows:

(r-1)*(k-1)= (12-1)*(2-1)=11

r=S Control Pl, S Control BG, L Control Pl, L Control BG, …, M RuBisCO PL, M RuBisCO BG

k= Pl, Os

RLI values less than one mean a less than random distribution, while a value bigger than one indicates a more than random, or specific, distribution [29]. In this case a value bigger than one indicates a specific labeling and thus the presence of RuBisCO. The samples that were used as a control group show RLI values close to 1 which indicates a more or less random distribution of the antibodies, while the values for the labeled samples, displayed as red numbers above, are much bigger than one.

This means a significantly difference from a random distribution, with the sample prepared using the agitation module being the highest at a value of 9,45. If one compares this value to the value of 3.12 of the conventional long protocol it is safe to say that the extraction during sample preparation is considerably smaller when the agitation module is used instead of the long protocol. The RLI values of the background staining are always lower than one and thus less than random, which is the result of the unbound antibodies being washed away during the washing steps, while the specifically bound antibodies in the plastids will not be washed away.

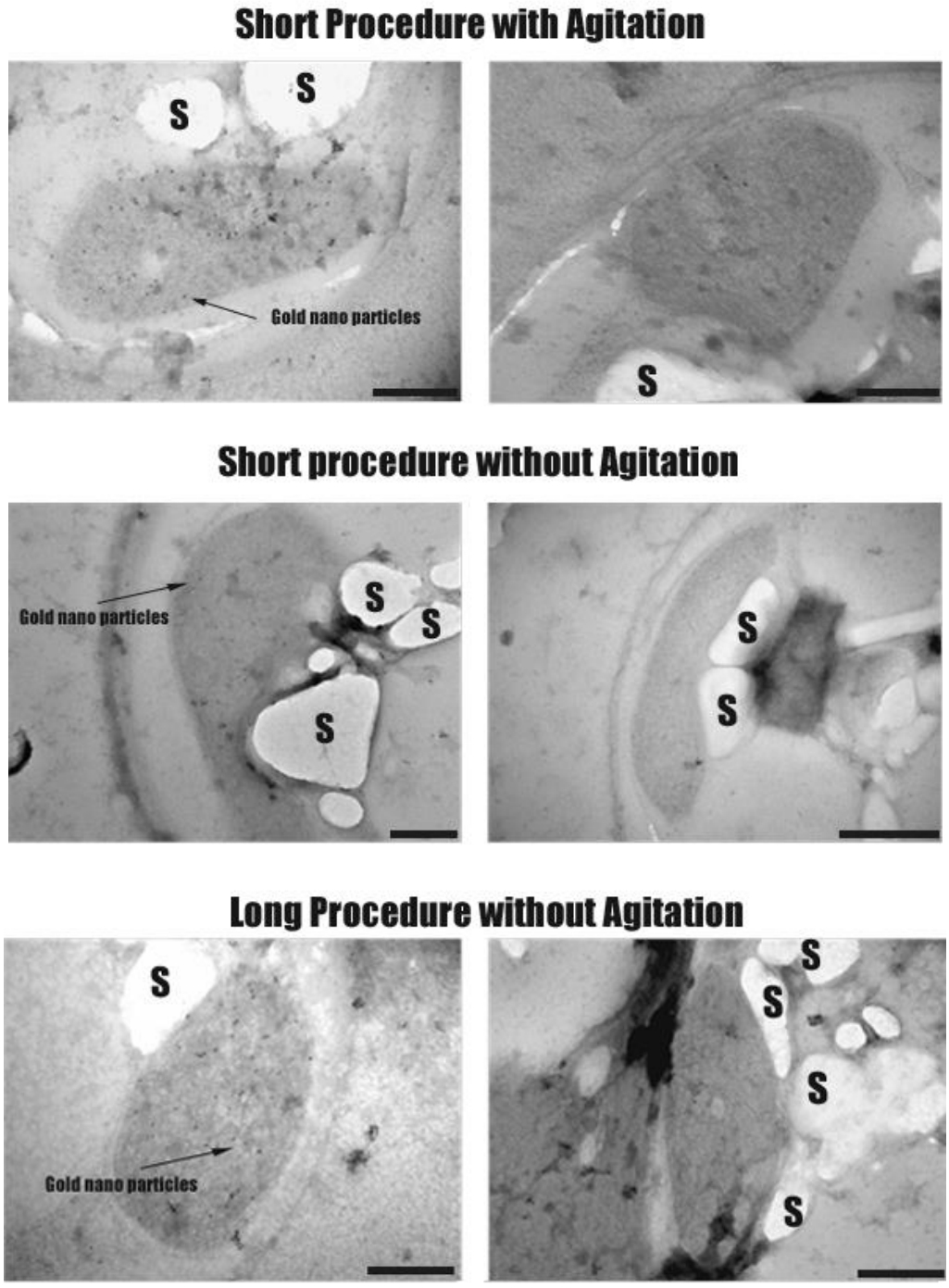


Fig. 12:

Immunogold localization of the enzyme RuBisCO in the plastids of *C. velia* cells prepared by different FS protocols. Gold particles are marked by black arrows.

The left side shows the specifically labeled LR-White section and the right side shows the controls, sections labeled only with the secondary gold conjugated antibodies. Starch granules (S). The bars are 500 nm.

En-Bloc staining with hafnium tetrachloride

The $HfCl_4$ was proposed to replace the UA usage during *en block* staining of specimens. I evaluate the influence on the staining and preservation of cell structures. The ultrastructure of cells was heavily damaged (Fig 13.) as shown by the dissolved plastids indicated by the arrows, which might be the result of the lower pH-value of the $HfCl_4$ solution. Furthermore, $HfCl_4$ had the deleterious effects on preservation of antigen binding sites (immune labeling did not work in the samples stained with $HfCl_4$).

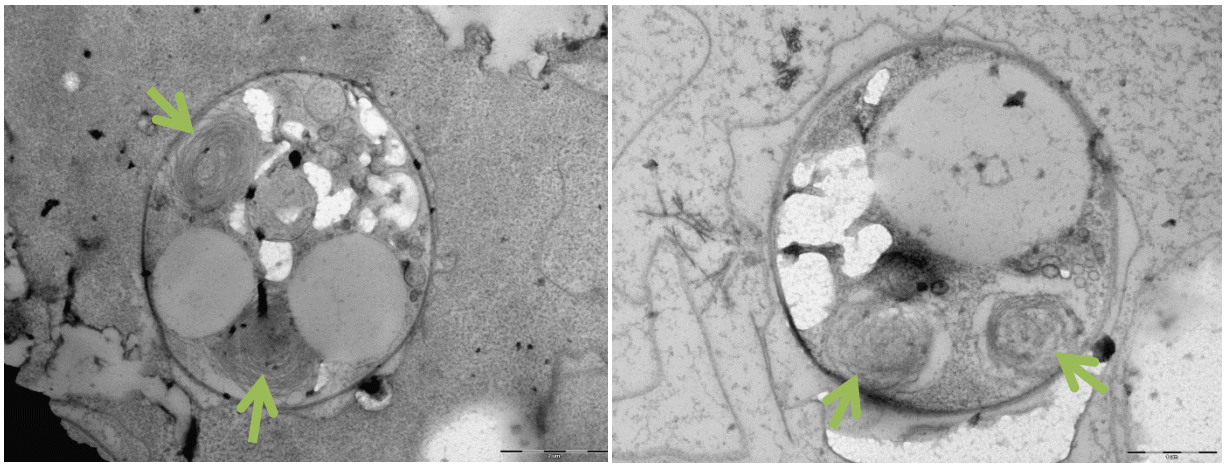


Fig. 13: Cross-section of *C. velia* cells prepared with the HPF-FS protocol in the presence of 0.25% $HfCl_4$ and 0,01% glutaraldehyde in 100% Acetone. The bars are 1 μm.

Discussion

FS was chosen over chemical fixation since cell walls and vacuoles can greatly reduce the penetration of fixatives into the cells (personal communication). Intercellular air and cuticles may then further slow down the fixation, which increases the time required to stop subcellular dynamics (Hoch 1991 [9]; Mersey and McCully 1978 [1]). Further the resulting sudden stop of flow of the cytoplasm or vesicular transport may cause distort ions (McCully and Canny ; 1985 [27]; Orlovich and Ashford, 1993 [3]; Wilson et al., 1990 [37]), Additionally leaking of the the acidic content of the vacuoles into the cytoplasm may add to this effect (Hayat, 2000 [10]; Kellenberger ,1991 [5])

The experiments of Goldhammer and Reipert (2016) [13] have shown that FS with agitation significantly reduced the degree of extraction compared to the long-term FS processing (Oborník et al. 2011 [35]), which could be confirmed by the present experiment. Observation of ultrastructural details of C. velia, mainly sensitive starch granules and the inner matrix of plastids, as well as a higher labelling density that were achieved by using agitation. The lower extraction might make FS with agitation a powerful method for the observation of biological processes.

Next the contrast of the cell wall and the cell membrane in C. velia across the different FS protocols was compared. Histograms indicate that the cell wall appears brighter when using FS without agitation, which might be caused by the lower exposure to the substitution medium.

The Histograms also indicate that the cell membrane is less electron dense when using the agitation device compared to the other procedures.

In terms mechanical damage, such as disruption of the cell membranes and starch granules there is no major difference between the long- and short FS protocol with agitation, however the Tukey-HSD test suggests a significantly lower amount of mechanical damage when using FS without agitation, which may be important to consider if one wants to observe systems that are very susceptible to mechanical damage.

Conclusions

The combination of lower extraction and reduction of the time required for the preparation by half may lead to better results for samples prepared using FS, which may make the method especially suitable when observing symbiont/host interaction or bacterial as well as viral infections. Additionally it would also make Freeze substitution a more viable preparation method because of the increased amount of samples that can be prepared.

	preservation of structure	contrast	labelling/extraction	mechanical damages
long FS protocol without Agitation	Comparable among all methods	Less dark Cell wall. Membrane less bright than when using Agitation.	LD lower by a third	Comparable to agitation
short FS protocol without Agitation		Membrane less bright than when using Agitation.	higher LD	Less Mechanical Damage
short FS protocol with Agitation		Cell Wall more electron dense compared to membrane.	Highest LD	

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