

# Transmission Electron Microscopy in Cell Biology: sample preparation techniques and image information

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**Abstract.** Transmission electron microscopy is a proven technique in the field of cell biology and a very useful tool in biomedical research. Innovation and improvements in equipment together with the introduction of new technology have allowed us to improve our knowledge of biological tissues, to visualize structures better and both to identify and to locate molecules. Of all the types of microscopy exploited to date, electron microscopy is the one with the most advantageous resolution limit and therefore it is a very efficient technique for deciphering the cell architecture and relating it to function. This chapter aims to provide an overview of the most important techniques that we can apply to a biological sample, tissue or cells, to observe it with an electron microscope, from the most conventional to the latest generation. Processes and concepts are defined, and the advantages and disadvantages of each technique are assessed along with the image and information that we can obtain by using each one of them.

## 1. Introduction

The preparation of a biological sample, cells or tissue, for transmission electron microscopy (TEM) requires several stages, some of which are quite complicated and some are critical. There is a wide range of possible techniques and processes and the use of one particular process rather than another will depend on the sample (type of tissue, cells, biofilm, etc.), on the size of the specimen being studied, on how the sample is obtained (in a laboratory, a hospital, or in the field, etc.), on the equipment that is available to us and finally, and very importantly, on what type of study we wish to carry out (ultrastructural, molecular localization, immunolocalization, electron tomography, correlative techniques, etc.). There are different possibilities for each type of study and before starting, it is necessary to decide which is the best option or options that we can use based on the information that we want to obtain from the sample and on the equipment that is available to us to carry out the study.

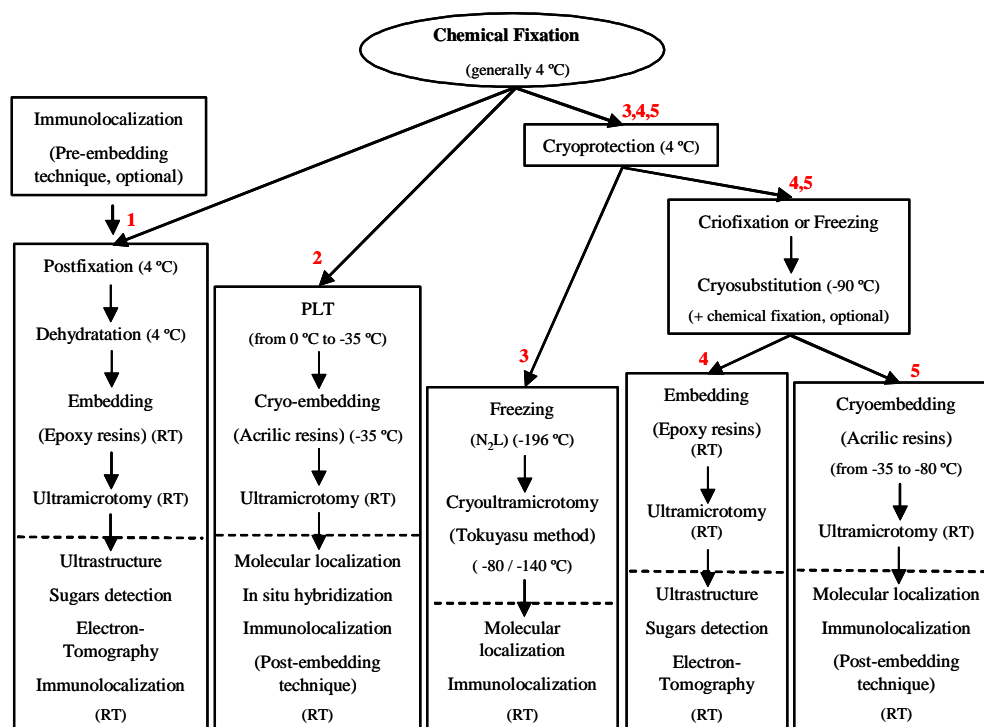
The first stage in preparing a biological sample is the fixation, one of the most important and most critical stages. The objective of this is to halt cellular activity without altering the cellular characteristics, the components that make up the sample or their distribution: to preserve the internal three-dimensional organization, the size and the shape of the sample.

The fixation process can be carried out in two different ways, by chemical fixation or by cryofixation. Chemical fixation [1,2] has been used since the 1950s and consists of the use of a whole range of chemical components (glutaraldehyde, paraformaldehyde, acrolein, osmium tetroxide, uranyl acetate, etc.) which, when used separately or in combination, in different proportions and dissolved in a buffer solution that acts as a vehicle, will serve as the fixative, by combining with the components of the cell and halting its processes. Within what is known as chemical fixation there are different systems, perfusion, immersion, by vapour, etc., and the use of one or another will depend on different factors: the type of sample, its size, etc. However, we need to bear in mind that chemical fixation produces structural artifacts and can result in the redistribution of ions and small soluble proteins in the sample [15].

The best way to immobilize and preserve the cell architecture is probably cryofixation, which consists of freezing the sample very quickly without crystals formation. There are different systems and equipment for carrying out the process: immersion in a liquid agent, impact on a pre-cooled metallic block, high pressure, etc. The use of one or another will depend on the type of sample and also on the equipment that can be used. However, the only system that permits maximum depth of cryofixation, of around 200  $\mu\text{m}$ , is high pressure freezing (HPF). This consists of stopping cellular activity by freezing a sample at a rate of more than 10,000°C/s and at a pressure of around 2100 bars [5]. At this rate of freezing and this pressure, the water molecules lose their usual mobility and crystals do not form (this is the phenomenon of nucleation) resulting in what is called vitreous ice [14]. However, in many cases the sample has to be treated with cryoprotectants prior to cryofixation [3,4]. Cryofixation is the most modern method for preserving the ultrastructure, but we must bear in mind that the instability and the anoxic sensitivity of tissues prior to cryofixation mean that in some cases and for certain types of samples a combination of chemical fixation with cryofixation by HPF can be a very useful compromise [4,9].

## 2. Methodology

After the fixation stage, the sample can be processed by different routes before it is studied in the electron microscope. The most common routes are indicated in the following schematic diagrams: Chemical fixation (Fig.1) and Cryofixation (Fig.7).



**Figure 1.** Chemical fixation. The most important processing routes for a biological sample based on chemical fixation.

### 2.1. Chemical fixation – dehydration – embedding (see Fig. 1, technique 1) [1,2,18].

If the sample has been chemically fixed (see Fig. 1), it can then undergo some postfixation, which is usually performed using osmium tetroxide, to improve the quality of the fixation and especially to preserve the lipid part of the sample. Afterwards, it is dehydrated in an organic solvent (alcohol, acetone, propylene oxide, etc.) in order to eliminate the water from the sample and to embed it in an epoxy-type resin that is not miscible with water (Epon 812, Spurr, Araldite, etc.). The sample will then be sectioned, contrasted with heavy metals and observed in a TEM.

#### Advantages:

- Most biological samples can be fixed and processed using this method.
- The process of chemical fixation requires no special equipment and it can be carried out in any setting (laboratory, operating theatre, in the field, onboard ship, etc.).
- The block containing the sample can be stored for many years.
- The sample can be redirected, after dehydration, to the critical point process so that it can later be observed in a scanning electron microscope (SEM).

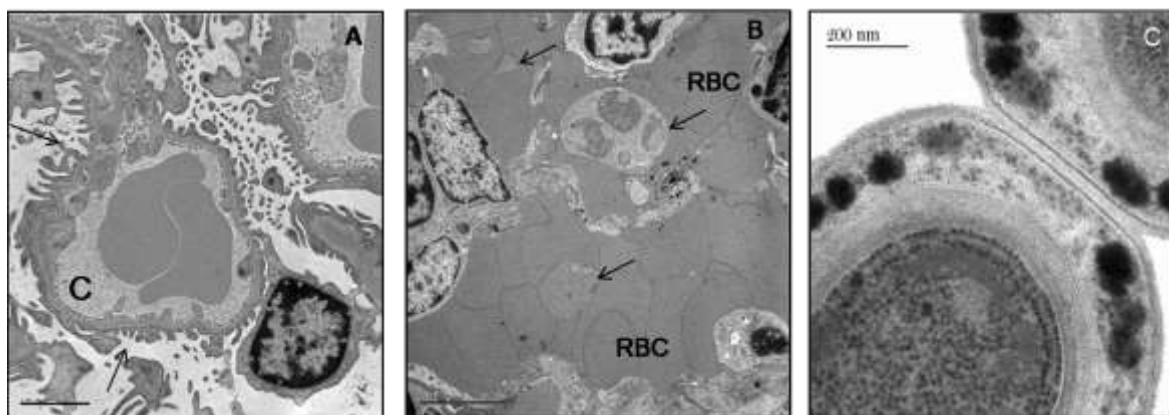
#### Disadvantages:

- It can create structural artefacts.
- Modifications may be produced in the cellular volume due to osmotic changes.
- It can lead to the redistribution of ions and of small soluble proteins.

#### Image and information:

- The image that is obtained is a contrasted image, with well-defined membranes, and it is easy to bring into focus in the TEM.
- We can work with a large area of the specimen and the fixation is homogeneous.
- This processing route allows us to carry out optical studies, ultrastructural studies, detection and localization of sugar residues using lectins and colloidal gold, cytochemical techniques, enzymatic digestions, electron tomography and molecular localization techniques with nanogold or quantum-dots (pre-embedding technique) (see Fig. 1).
- It allows correlatives techniques to be used: optical microscopy–electron microscopy.

- It also allows the elements of the sample to be analysed by energy filtered transmission electron microscopy (EFTEM) or by secondary ion mass spectrometry (SIMS).
- Semithin sections can be observed by SEM.



**Figure 2.** Chemical fixation – dehydration - embedding (A) Kidney capillary and filtration barrier. Bar 2 µm. (Courtesy F..Pardo and R. Gomis, Idibaps), (B) Spleen malaria infection , *Plasmodium yoelii* (→). Bar 5 µm. (Courtesy H. del Portillo, L. Martin, M. Ferrer – Cresib), (C) Bacteria detail of *Clostridium bifermentans*. Bar 200 nm. (Courtesy of R. Guerrero and L.Villanueva - UB).

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## 2.2. Chemical fixation–PLT–cryoembedding method. (see Fig. 1, technique 2) [5,6,7].

After chemical fixation we can also perform what is called progressive lowering of the temperature (PLT), which is specific for immunolocalization techniques and molecular localizations such as “in situ” hybridization. The technique uses a gentle aldehyde fixation followed by dehydration of the sample which is achieved by progressively lowering the temperature from 4°C to –35°C [5,6]. This method, working at temperatures below 0°C, reduces the loss of components from the sample and minimizes the denaturation of the proteins. Afterwards, the sample is embedded at –35°C in an acrylic resin (Lowicryls, LRWhite, LRGold, etc.) that has low viscosity and is miscible with water. Most of the resins are hydrophilic and they have good electron beam stability when they are polymerized [7]. Furthermore, they polymerize with ultraviolet light, which means that the antigenicity of the tissue is preserved.

### Advantages:

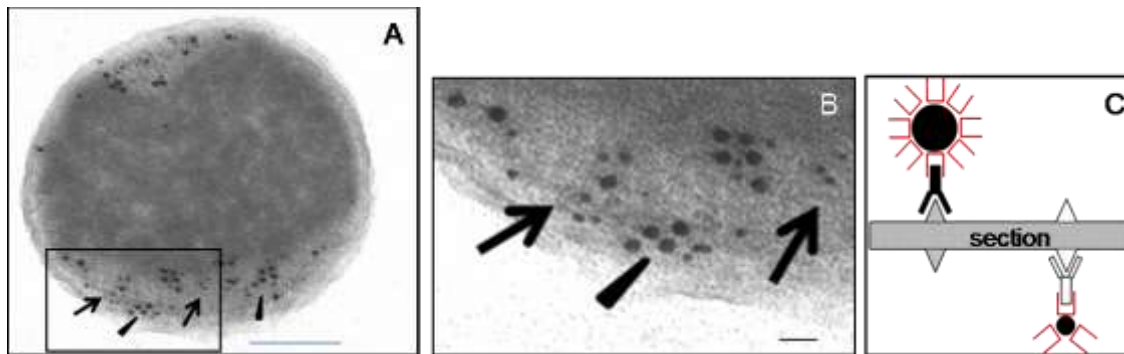
- The method is fast and an easy way to localize proteins or a genetic sequence by “in situ” hybridization.
- Labelling or co-localization can be performed using different sizes of colloidal gold.
- This method allows co-localization with antibodies from the same species to be performed, incubating both sides of the ultrathin slice.
- The use of acrylic resins allows 5% of the content of the sample to be water.
- The sectioning is not easy but, with experience in ultramicrotomy, very good quality slices can be obtained.
- The block containing the sample can be stored for years.

### Disadvantages:

- If the quantity of protein to be localized or the genetic sequence is small, it is difficult to obtain a signal using this method and so it will be better to use the Tokuyasu method which is more sensitive.
- The technique requires commercial equipment or a homemade system.

Image and information:

- The image allows the structures to be clearly identified, but even so it is useful to know the tissue well at the ultrastructural level by other methods.
- Since osmium tetroxide is not used, the membranes are not very electron dense and in general the sample has a gentle contrast, which means that the gold labelling stands out clearly over the structure that we are labelling.
- It is a good technique for immunolocalization and “in situ” hybridization techniques. This can be performed using an optical microscope, on semithin slices, using colloidal gold and intensification in silver or directly on ultrathin sections with colloidal gold.
- It is suitable for correlative techniques (optical–electron microscopy).



**Figure 3.** Technique Chemical fixation–PLT–cryoembedding method. Immunolocalization (A) Double detection of chaperones DnaK (▶) (15 nm gold) and GroEl (→) (5 nm gold) in *E. coli* inclusion bodies using antibodies from the same species. (Bar 200 nm). (B) Detail inset in A. (Bar 50 nm). (C) Technique detail. (Courtesy of M. Carrió and A. Villaverde – UAB).

### 2.3. Chemical fixation–Tokuyasu method (see Fig. 1, technique 3) [7,8]

The Tokuyasu method is based on cryosectioning of the sample and is used for immunolocalization techniques. In this case, the chemical fixation that is carried out is at low concentrations of aldehydes in order to preserve the antigenicity of the sample and so that molecular detection can be performed afterwards. The chemically fixed sample is enveloped in gelatine and is then impregnated with sucrose or with a sucrose/polyvinylpyrrolidone mixture. After that, it is mounted on a special sample holder and frozen immediately in liquid nitrogen. Finally, it is cryosectioned in a cryoultramicrotome at a temperature between  $-80^{\circ}\text{C}$  and  $-140^{\circ}\text{C}$ . The immunolocalization is carried out on ultrathin sections (80-100 nm) and finally these slices have to be contrasted and embedded in methyl cellulose (a thin layer which embeds the slices and allows them to be embedded and dried without the sample shrinking).

Advantages:

- The sample is not dehydrated or embedded in resin: the proteins remain in their aqueous state.
- The immunolocalization is highly efficient; this is extremely useful for antigens that are present in small quantities.
- Very high definition of the intracellular membrane systems.
- The method is very quick and results can be obtained in a day, if necessary.

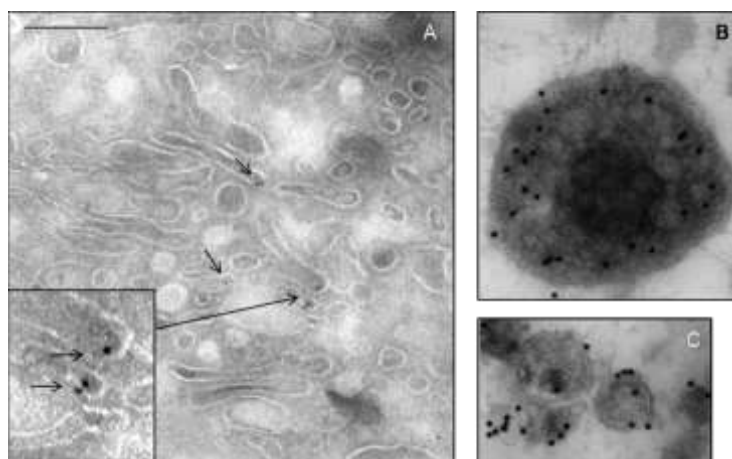
Disadvantages:

- Cryoprotection in sucrose can cause problems of a reduction in cellular volume and soluble proteins can be lost, since the fixation that is usually used is gentle.
- The level of contrast is generally low and depends on the fixation; strong fixation leads to less loss of proteins.

- The sectioning and handling of the slices can lead to relocalization of antigens and the loss of material.
- The difficulty involved in cryosectioning certain samples.
- The sample has to be kept frozen in liquid nitrogen.

Image and information:

- In general, the sample exhibits only a gentle contrast, but the membranes and cellular compartments can be very clearly observed.
- It is specifically aimed at molecular localization techniques and is very useful for these.
- The technique can be used to work on semithin sections with gold and silver intensification or on ultrathin sections with colloidal gold.
- The sectioned side of the block can be observed by cryo-SEM.



**Figure 4.** (A) Caveolin (→) localization in CHO cells. Bar 0.1  $\mu\text{m}$ . (B and C) Multivesicular bodies isolated from rat liver and labelled with anti-annexin A-6 (Courtesy of C.Enrich – UB).

2.4. Chemical fixation – cryofixation - (hybrid method) – embedding (see Fig. 1, technique 4). There is evidence that the chemical fixation itself produces fewer alterations or artefacts than the later stages after fixation, such as osmium tetroxide postfixation and the process of dehydration. The hybrid method consists of chemical fixation of the sample and then cryoprotection (usually in sucrose) and cryofixation (by HPF, for example) or freezing in liquid nitrogen (if the sample is large in size). The sample then undergoes the process of freeze substitution (FS), which consists of eliminating the water from the sample at a temperature around  $-90^{\circ}\text{C}$  through the use of organic solvents, and in this case we use methanol as the organic solvent, since it is miscible with sucrose. This freeze-substitution medium will also contain different types of chemical fixatives (glutaraldehyde, osmium tetroxide, uranyl acetate, etc.) which will depend on the sample and the type of study that we wish to carry out and will provide better contrast and ultrastructural preservation [4,9].

Advantages:

- If a long dissection time is required to localize the area before cryofixation, then anoxia can be avoided if they are first fixed chemically (for example, nervous system)
- We replace the dehydration process by FS which is less aggressive since it is carried out at very low temperatures ( $-90^{\circ}\text{C}$ ) and it reduces the loss of components from the sample.
- It is possible to work with large samples and to obtain homogeneous fixation.
- The block can be stored for years.

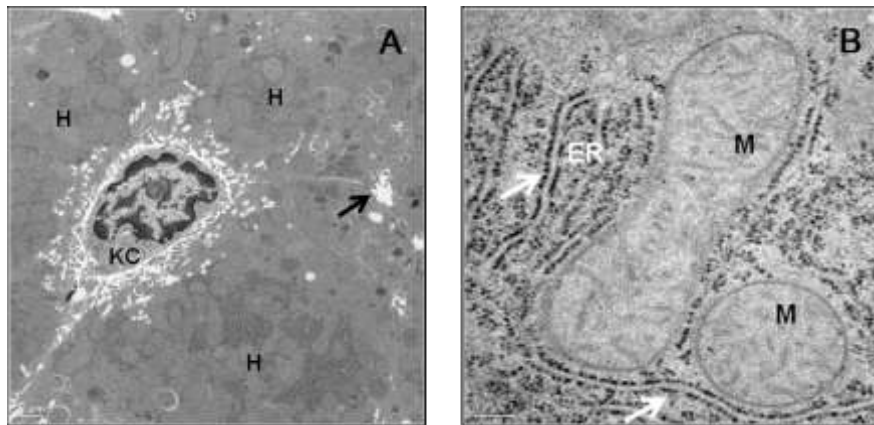
Disadvantages:

- The process starts with chemical fixation that can cause structural changes in the sample.

- Commercial equipment or a homemade system is required (in the latter case there are certain difficulties in controlling the stability of the temperature and the rates of temperature increase).

Image and information:

- It preserves the ultrastructure well and produces an image similar to cryofixation.
- It is useful for ultrastructural studies, detection of sugar residues, cytochemical techniques, enzymatic digestion, correlative techniques (optical–electronic), electron tomography by TEM, and electron tomography by SEM.



**Figure 5. Chemical fixation – cryofixation - Hybrid method,** (A) Liver general view. Hepatocytes (H), bile canaliculus (→), Kupffer cell (KC) in a sinusoid. Bar 1  $\mu\text{m}$ . (B) Liver cytoplasm detail. Mitochondria (M) endoplasmic reticulum (ER) and ribosome (→). Bar 0.2  $\mu\text{m}$ .

2.5. Chemical fixation– cryofixation (hybrid method) – cryoembedding (see Fig. 1, technique 5) [4,9].

The hybrid method can also be used to carry out immunolocalization, in which case the aldehyde-type chemical fixation will have to be gentle in order to preserve the antigenicity of the sample. The sample is then cryoprotected in sucrose and afterwards we perform FS at  $-90^{\circ}\text{C}$  using methanol as the organic solvent. Different types of chemical fixatives, or a mixture of different proportions of several fixatives, can also be added to the FS medium. Once FS has been carried out, the temperature is raised in order to embed the sample in acrylic resin. How the temperature is raised will depend on the type of resin in which we wish to embed the sample; so, for example, we raise it to  $-35^{\circ}\text{C}$  for Lowicryl K4M or to  $-50^{\circ}\text{C}$  for Lowicryl K11M. Finally we polymerize it with ultraviolet light at the chosen temperature.

Advantages:

- FS allows a better preservation of cellular structures.
- This method allows us to embed the sample in different types of acrylic resin at different temperatures.

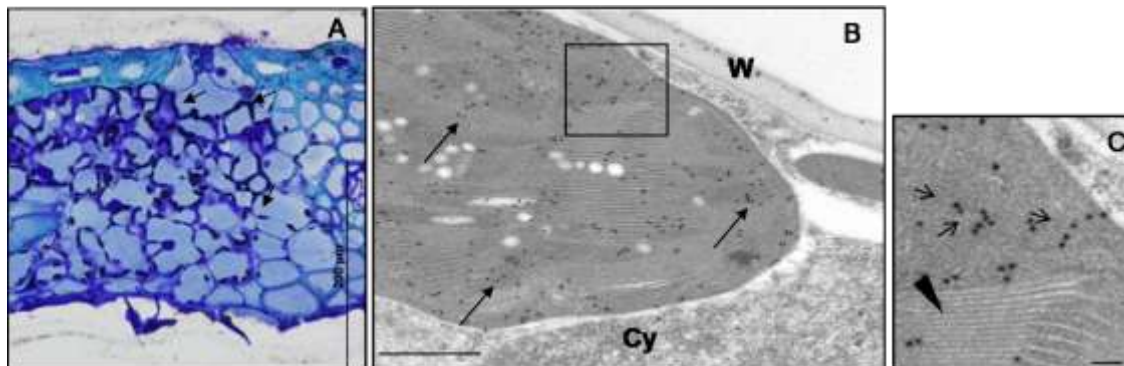
Disadvantages:

- Commercial equipment or a homemade system is required (see the explanation of the previous technique).
- The difficulty involved in visualizing and orienting the sample.
- The difficulty involved in sectioning, depending on the type of acrylic resin used.

Image and information:

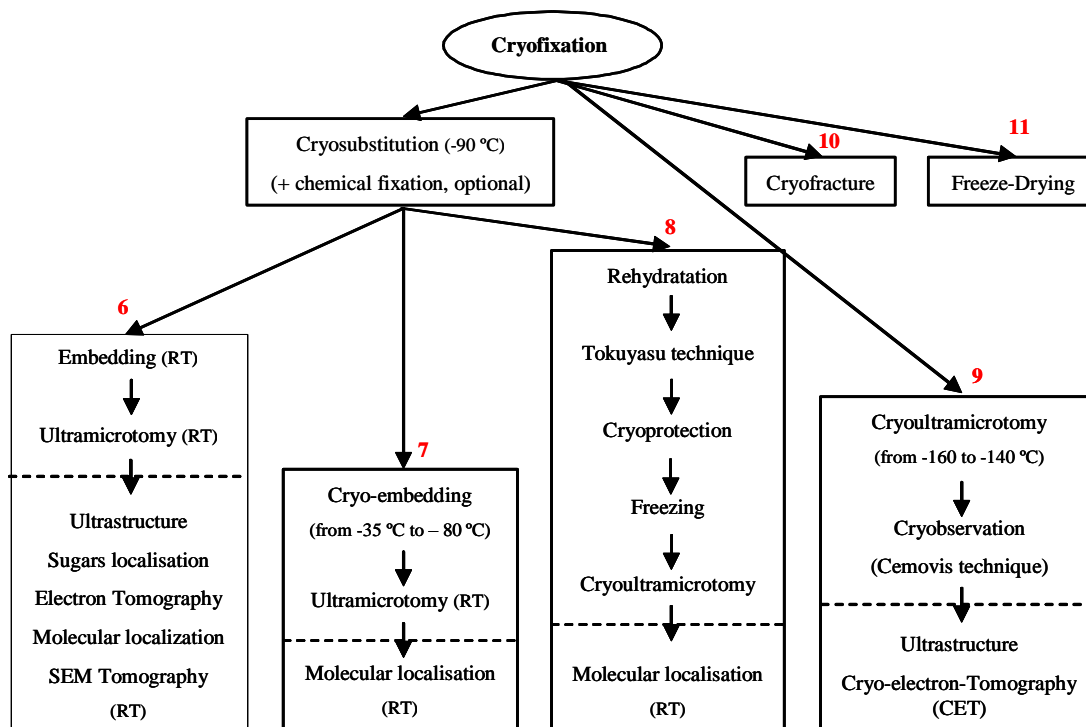
- The contrast is gentle, which means that labelling with gold can easily stand out over the structure that we are labelling.

- It is a good technique for immunolocalization and “in situ” hybridization. It can be performed both by optical microscopy (semithin section) with colloidal gold labelling and silver intensification or by electron microscopy (ultrathin section) with colloidal gold labelling, or by correlative techniques.



**Figure 6.** Glutamine synthetase immunolocalization in wheat flag leaf mesophyll spongy parenchyma cell. (A) Light microscopy image – gold silver enhancement (→). Bar 200 µm. (B) Electron micrograph spongy cell chloroplast. Bar 0.5µm (C) Inset detail. Bar 50 nm. Arrows indicate immunogold label, Cytosol (Cy), cell wall (W) and thylakoid (arrowhead). (Courtesy of M. Lopes and JLL. Araus – UB).

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**Figure 7.** Cryofixation. The most important processing routes for a biological sample, based on cryofixation.

2.6. Cryofixation–FS–embedding (see Fig. 2, technique 6). [14,15,16,17, 18].

A sample that has undergone cryofixation by HPF can be processed by different routes (see Fig. 2). One route is to carry out FS using different organic solvents (acetone, alcohol, methanol, etc.) in order to replace the aqueous content of the sample by an organic solvent that allows us to embed the sample afterwards. We can add different types of chemical fixatives (glutaraldehyde, osmium tetroxide, uranyl acetate, etc.) to the FS medium in different proportions (depending on the sample



and the type of study we wish to perform) in order to preserve the structures better and to intensify the contrast. During the heating process the chemical fixatives begin to act: uranyl acetate as soon as the negative charges of the nucleic acids and phosphate groups become accessible; osmium tetroxide at  $-70^{\circ}\text{C}$  and glutaraldehyde between  $-40^{\circ}\text{C}$  and  $-30^{\circ}\text{C}$ . Since FS is usually carried out at around  $-90^{\circ}\text{C}$ , afterwards the temperature of the sample is gradually increased to room temperature and then the sample is embedded in epoxy resin, followed by sectioning and observation of the sample.

#### Advantages:

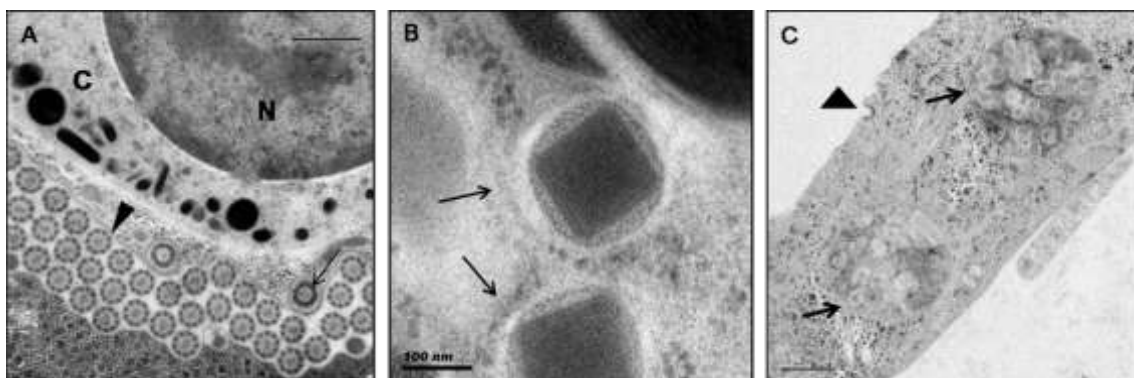
- It is the best way to preserve the cell architecture.
- The processing time of cryoimmobilized samples is no greater than it is for chemical methods.
- The quality of the fixation depends on the size of the specimen and on its composition, e.g., whether the sample naturally contains antifreezing agents or whether it has a low water content.

#### Disadvantages:

- Although there are routine protocols, on many occasions it is necessary to experimentally work out the necessary conditions for each sample in order to avoid the formation of crystals (the type of cryoprotectant, type of sample holder, etc.).
- The sample must be of the order of 2 mm in diameter and 200  $\mu\text{m}$  thick.

#### Image and information:

- Very good ultrastructural preservation, the images give a feeling of turgidity, the microtubules are preserved, but it does not have the membrane definition that we achieve with chemical fixation.
- This processing route allows us to perform optical studies, ultrastructural studies, detection and localization of sugar residues using lectins and colloidal gold, cytochemical techniques, enzymatic digestions, electron tomography, elemental analysis by energy-filtered transmission electron microscopy (EFTEM) or by secondary ion mass spectrometry (SIMS).



**Figure 8.** Technique 6. Cryofixation – FS- embedding (A) Embryonic structure (miracidium) from *Mediogonimus jourdanei* (Tremathode), centriole ( $\rightarrow$ ), cilium ( $\blacktriangleright$ ), nucleus (N) and cytoplasm (C) (Bar 0.5 $\mu\text{m}$ ). (Courtesy of J. Miquel – UB). (B) Trichocyst ( $\rightarrow$ ) of a dinoflagellate, an organelle that releases long filamentous proteins (Courtesy of E. Garces, ICM-CSIC). (C) Caco-2 cells directly cryofixed without cryoprotection, coated-pit (arrowhead) and autophagosomes ( $\rightarrow$ ).

#### 2.7. Cryofixation–FS–cryoembedding (see schme 2, technique 7) [14,15,16,17,20].

Starting from cryofixation we can also perform “in situ” hybridization and immunolocalization techniques. The process is similar to that above described, although generally acetone is used as the

organic solvent and we always have to be careful with the type of chemical fixatives used and in what proportions (we have the option of not using any chemical fixative) as the antigenicity of the sample has to be preserved. After FS and similarly as in Technique 5 above, we can embed the sample in different types of acrylic resin at different temperatures and finally polymerize it with ultraviolet light.

Advantages:

- FS together with cryofixation preserves the cellular structures very well while, at the same time, maintaining the antigenicity of the sample.
- The method allows us to embed the sample in different types of acrylic resins and at different temperatures.
- The block can be stored for years

Disadvantages:

- The same as for the previous process of cryofixation-FS-cryoembedding
- Visualization of the sample during the process, making the blocks and the orientation of the sample all involve a certain degree of difficulty

Image and information:

- Good preservation for molecular localization (immunolocalization, “in situ” hybridization, etc.)
- This processing route allows us to carry out optical studies with colloidal gold and silver enhancement in semithin sections, and immunolocalization using colloidal gold on ultrathin sections.

2.8. Cryofixation–FS–rehydration–Tokuyasu method. (see Fig. 2, technique 8) [19].

A sample that has been cryofixed by HPF and freeze substituted can be rehydrated, and then we can apply the Tokuyasu method to cryosection it.

Advantages:

- This technique combines the ultrastructural preservation of cryofixation with a very efficient immunolocalization system.
- Chemical fixation during FS is useful for antigens that are sensitive to fixation. It may be used for samples for which chemical fixation is difficult, such as yeasts, bacteria, plants, insects, etc

Disadvantages:

- The size of the sample is small given that we start with cryofixation.
- Artefacts may be caused during rehydration

Image and information:

- The image is very similar to that obtained by the Tokuyasu method, a gentle contrast, but the membranes and the cellular compartments can be seen very well.
- It is a specific technique which is useful for molecular localization techniques of samples that may be difficult to fix chemically.

However, starting from a cryofixed sample, we can use other techniques (all of them require special equipment) such as:

- **Freeze-fracturing**, which consists of freezing the sample and fracturing it in such a way that we obtain a double-sided fracture, a replica of the sides is made using platinum-carbon at different angles and they are observed in the microscope. There is also the “freeze-

etching” variation in which the fracture face is gently freeze dried. This is a technique for studying membranes and for localization and distribution of membrane proteins [3].

- **Freeze-Drying**, this technique consists of cryofixation of the sample and extraction of the water from the sample in order to sublimate it at low temperatures in a vacuum; once it is dry, it can be embedded in any type of resin and sectioned [12].
- **CEMOVIS**: it consists in freezing the sample at high pressure, cryosectioning it and cryoobserving it directly on the electron microscope; this technique allows us to see the tissue in its hydrated state, without the necessity to remove the water from the sample and therefore in its most natural state [13].

### 3. Examples of applications

All these techniques can be applied to:

- Animal tissues (ex. liver, spleen, bone...) (see Fig. 2A/B, 4A, 5A/B, 5A)
- Cell culture (in liquid medium, on substrate...) (see Fig. 8C)
- Bacteria, cellular organelles, biofilms, ... (see Fig. 2C)
- Plant tissues (ex. seeds, pollen, leaves...) (see Fig.6)

### 4. Conclusions

Electron microscopy is essential to understanding how cells and tissues work; it allows us to visualize structures with high resolution and to relate their functions of the components as well as being able to detect and localize molecules “in situ”. It is very important to consider the different techniques, be able to work with several of them, and assess and compare them in order to arrive at a good final conclusion.

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