

Closer to the native state. Critical evaluation of cryo-techniques for Transmission Electron Microscopy: preparation of biological samples

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Abstract: Over the years Transmission Electron Microscopy (TEM) has evolved into a powerful technique for the structural analysis of cells and tissues at various levels of resolution. However, optimal sample preservation is required to achieve results consistent with reality. During the last few decades, conventional preparation methods have provided most of the knowledge about the ultrastructure of organelles, cells and tissues. Nevertheless, some artefacts can be introduced at all stages of standard electron microscopy preparation technique. Instead, rapid freezing techniques preserve biological specimens as close as possible to the native state. Our review focuses on different cryo-preparation approaches, starting from vitrification methods dependent on sample size. Afterwards, we discuss Cryo-Electron Microscopy Of Vitreous Sections (CEMOVIS) and the main difficulties associated with this technique. Cryo-Focused Ion Beam (cryo-FIB) is described as a potential alternative for CEMOVIS. Another post-processing route for vitrified samples is freeze substitution and embedding in resin for structural analysis or immunolocalization analysis. Cryo-sectioning according to Tokuyasu is a technique dedicated to high efficiency immunogold labelling. Finally, we introduce hybrid techniques, which combine advantages of primary techniques originally dedicated to different approaches. Hybrid approaches permit to perform the study of difficult-to-fix samples and antigens or help optimize the sample preparation protocol for the integrated Laser and Electron Microscopy (iLEM) technique. (*Folia Histochemica et Cytobiologica* 2014, Vol. 52, No. 1, 1–17)

Key words: cryo-TEM; cryo-fixation; cryo-FIB; CEMOVIS; freeze substitution; iLEM; Tokuyasu method; hybrid technique

Abbreviations:

CEMOVIS — Cryo-Electron Microscopy Of Vitreous Sections
CLEM — Correlative Light and Electron Microscopy
Cryo-EM — Cryo-Electron Microscopy
Cryo-ET — Cryo-Electron Tomography
Cryo-FIB — Cryo-Focused Ion Beam
ET — Electron Tomography

FIB — Focused Ion Beam
FS — Freeze Substitution
HPF — High Pressure Freezing
IEM — Immuno-Electron Microscopy
iLEM — integrated Laser and Electron Microscopy
PF — Plunge Freezing
RHM — ReHydration Method
ROI — Region Of Interest
SEM — Scanning Electron Microscopy
SFM — Section Fixation Method
SPRF — Self-Pressurized Rapid Freezing
TEM — Transmission Electron Microscopy
TRITC — TetramethylRhodamineIsoThioCyanate
TSEM — Transmission Scanning Electron Microscopy
UA — Uranyl Acetate
VFS — Vitrified Frozen Sections

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Introduction

The introduction of Transmission Electron Microscopy (TEM) nearly 70 years ago in the life science have made it possible to study the structure and function of cells and tissues at new level of resolution. Different TEM techniques permit the study of the architecture of cells and tissues on the scale of micrometers (conventional TEM), cellular organelles and molecular architecture on the scale of nanometers (CEMOVIS, ET, cryo-ET) and single molecular complexes at near atomic resolution (Single Particle Analysis, Electron Crystallography). The resolution range of TEM locates this technique next to X-ray crystallography and Nuclear Magnetic Resonance on the one side and light microscopy on the other. Therefore, TEM gives unique possibilities to fit biochemical and atomic scale models into structure-function relationships of macromolecules and organelles in the cellular context. Ultrastructural resolution is obtained by using a coherent beam of electrons in the TEM column under high vacuum. Therefore electron microscopists have to deal with a few aspects during sample preparation. First of all, water as the most abundant cellular constituent must be removed or kept immobilized since water under vacuum at room temperature evaporates. Secondly, it is not possible to observe an entire cell or tissue because the specimen has to be thin enough to allow the electrons to penetrate it and create an image. Additionally, biological samples are composed of light elements, so the sample should be stained with heavy metals or some computational method can be used to create specimen contrast. Over the years different preparation techniques have been developed in order to obtain results as close as possible to the native state.

The scope of this review covers different specialised preparation techniques dedicated to cells and tissues. Readers may ask, why another review which covers cryo-preparation topics in TEM sample preparation if there are a lot articles about this subject. The aim of our review is to refresh introduced cryo-preparation techniques and describe some new solutions in this field. We would like to particularly focus on the vitreous cryo-sectioning technique, the limitations of this technique and an emerging solution in the form of cryo-Focused Ion Beam thinning. Other important and remarkable techniques from our point of view are hybrid techniques that are mainly dedicated to high resolution immunolocalization of selected antigens and a new Correlative Light and Electron Microscopy (CLEM) solution — the integrated Laser and Electron Microscopy (iLEM).

The review starts with a commonly used specimen preparation technique, vitrification of water and

a short description of the state of the art preservation techniques. We focused our attention on cryo-fixation techniques, such as Plunge Freezing (PF), High Pressure Freezing (HPF) and recently introduced Self-Pressurized Rapid Freezing (SPRF). We did not introduce Slam Freezing and Jet Freezing, because HPF has almost completely replaced these methods [1]. Subsequently, we present different basic processing routes after vitrification in case of pure structural research (CEMOVIS or cryo-FIB) or localization of gene products with immunolabelling. Finally, we describe hybrid techniques, which combine different basic approaches into one optimized protocol dedicated to solving specific problems, such as difficult-to-fix material or antigens. Furthermore, a hybrid approach enables to fulfil specific requirements of novel advanced tools for application in biological studies.

Conventional sample preparation

For a few decades the ‘conventional electron microscopy technique’ has been one of the methods for sample preparation in TEM. Conventional specimen preparation uses chemical fixation at room temperature. During this process, the biological sample is chemically fixed, usually using aldehydes and osmium tetroxide (OsO_4), and dehydrated with organic solvents prior to infiltration and embedding in resin. Embedded biological materials are ultra-thinly sectioned and post-stained with salts of heavy metals, such as uranyl, and lead to the introduction of contrast inside the sample. Previous reports revealed that chemical fixation, dehydration, heavy metal staining and plastic embedding can introduce various artefacts. Fixation with glutaraldehyde prior to dehydration results in cross-linking causing aggregation of proteins, collapse of highly hydrated glycans and loss of lipids. Heavy metals can cause additional artefacts in the form of precipitation [2–5]. To sum up, artefacts introduced during conventional sample preparation limit the resolution of biological samples to about 2 nm [6]. Modern transmission electron microscopes with a field emission gun can obtain sub-angstrom resolution, thus the resolution is mainly limited by the properties of the sample [7]. Therefore the improvement of biological sample fixation has become the challenge for many scientists.

Alternatives to the conventional sample preparation

An alternative to chemical fixation is cryo-fixation, *i.e.* physical preservation. The aim of this method is to vitrify biological samples *via* rapid freezing. Vitrifica-

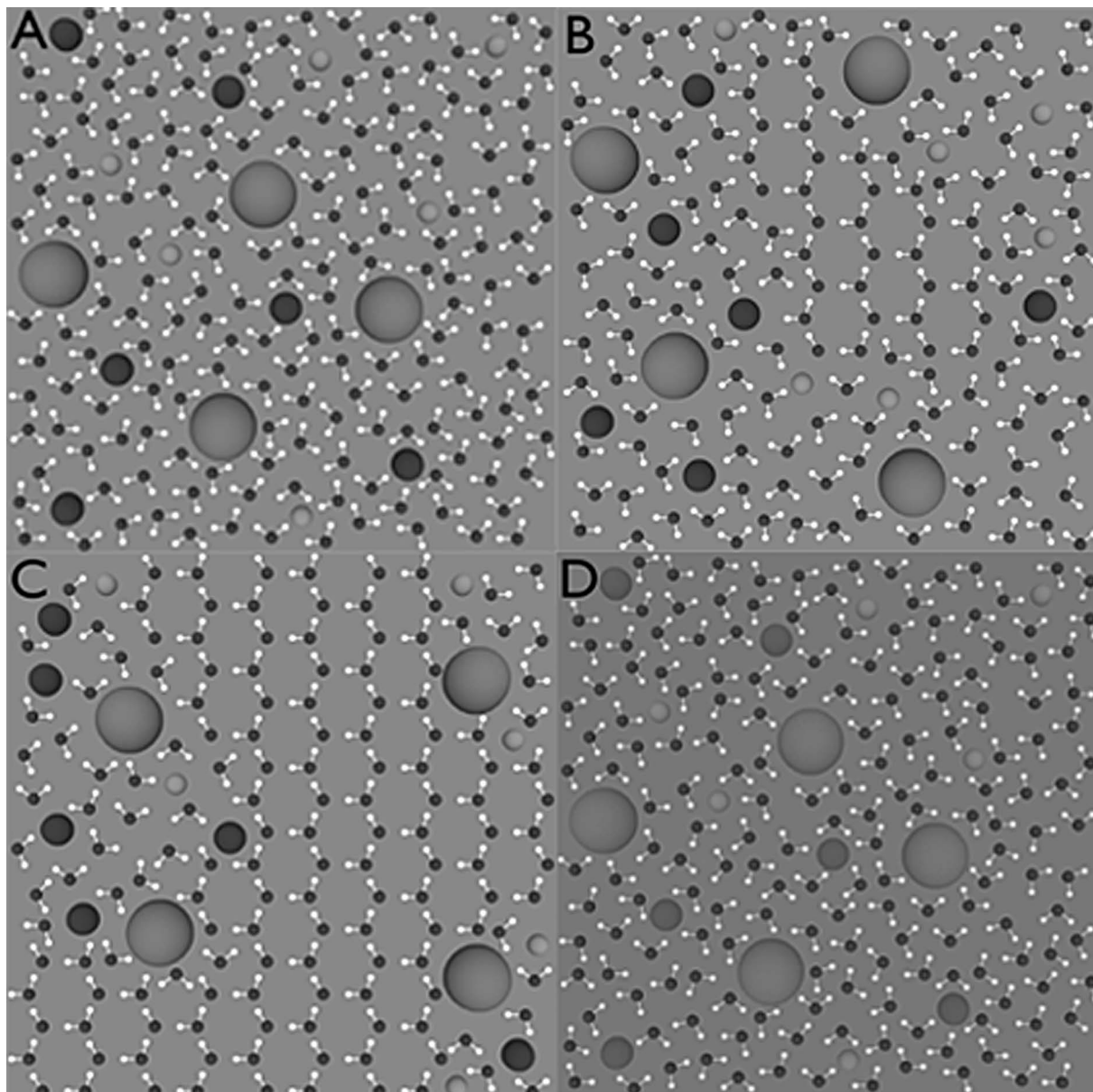


Figure 1. Water state under different cooling rate. **A–D.** Ice crystal formation in water solutions, known as pattern segregation occurs under lower cooling rate. Water in liquid state with ions (the smallest and medium size) and sugar molecules (the biggest) (**A**) start to create ice crystals under freezing temperature (**B**). Created ice crystals devours water molecules which results in separation of solutes from growing ice crystals (**C**). Water solution under high cooling rate and high pressure transform into amorphous state (**D**)

tion is a transformation process from the liquid to the amorphous state with the exclusion of the formation of ice crystals within microseconds. Amorphous water can be regarded as a liquid with an extremely high viscosity, thus at the cellular level bioparticles are immobilised in their native state without a change in the cell morphology (Figure 1D). In consequence, cryo-fixation suspends biological processes at a point in time, namely preserving a particular physiological state. Improper vitrification leads to crystalline ice

that destroys cellular ultrastructure through a phase segregation process (Figure 1A–C) [8, 9].

For a long time it was thought that vitrification of liquid water is impossible for thermodynamical reasons, in spite of the fact that Taylor and Glaeser [10] showed the presence at near atomic resolution of vitrified, fully hydrated catalase crystals. The real breakthrough in the electron microscopy field took place at the beginning of the 1980s. At this time, Bruggeller and Mayer [11] had demonstrated by jet

freezing and X-ray diffraction that small amounts of water can be vitrified by rapid cooling. A year later Dubochet and McDowell [12] observed that sprayed water drops cooled by immersion in liquid ethane looked structureless. Their surprising finding was confirmed by electron diffraction [13]. Therefore “water is beautiful and its traditional status of foe must be revisited; when treated with suitable care water is electron microscopist’s best friend” [14]. This discovery became the basis for the development of cryo-Electron Microscopy (cryo-EM).

Vitrification strongly depends on the cooling rate, cryo-protection and pressure. Thermal properties of water in the biological material are the physical reasons for improper vitrification of thick samples. Under atmospheric conditions only a very thin film (up to 1 μm) of pure water can be vitrified using an extremely high cooling rate [12]. The probability of proper vitrification is higher in the presence of cryoprotectants because water molecules are immobilised. This fact is used in the preparation of biological samples for cryo-sectioning by the Tokuyasu method. Vitrification is obtained due to sample infiltration by sucrose followed by freezing in liquid nitrogen (see Tokuyasu cryo-sectioning method). Although biological samples consist of about 80% water, cells can be directly vitrified because intracellular components of cells (protein, glycoprotein, lipids, etc.) act as natural cryoprotectants. Thereby the required cooling rate is reduced.

Plunge Freezing

Plunge Freezing (PF) is the method of choice for a wide range of thickness: from cellular macromolecules and complexes to whole cells up to 10 μm thick. This simple method works under atmospheric conditions and enables to obtain high enough cooling rates (equal to at least 104 K/s). Biological samples as small objects are suspended in a thin water film (100 nm) over holes in the perforated ‘support layer’ coating EM grids. Larger samples, for example eukaryotic cells, can be grown on EM grids. The prepared sample is plunged into cryogen such as liquid ethane, propane or ethane-propane mixture [15, 16]. Both liquid ethane and propane in contact with liquid nitrogen solidify, whereas the mixture of these liquid alkanes has a lower freezing point. Thus an ethane-propane mixture remains liquid at 77 K and therefore this mixture is more convenient to use. Another advantage of the discussed mixture is the fact that it produces less damage to the thin, fragile, holey carbon film on C-grids [17]. After vitrification the sample is either transferred and directly observed at -180°C under

the cryo-electron microscope or cryo-thinned. Evaporation is a very important factor during sample preparation because if not controlled it can change the osmotic pressure or surface tension, which can lead to flattening of the sample [13].

High Pressure Freezing

Samples thicker than 10 μm are too thick to ensure proper vitrification without ice crystallisation by PF and cannot be directly imaged in cryo-EM. The nucleation of ice crystals is temperature and pressure dependent, and this influence is exploited in High Pressure Freezing (HPF) [6]. This method was first introduced by Moor and Riehle in the 1960’s [18] but the HPF machine only became commercially available in the 1980’s. The volume of water expands during the crystallisation process. According to Le Chatelier and Braun principle, the increase in ice volume can be hindered by application of high pressure which works against expansion and acts as a physical cryo-protectant. High pressure strongly reduces crystal growth and the nucleation rate of ice. As a result, specimens up to $\sim 250 \mu\text{m}$ can be vitreously frozen by application of high pressure (*i.e.* 2048 bar) and subsequent rapid cooling using liquid nitrogen jets for $\sim 50 \text{ ms}$ [19].

In order to avoid extracellular ice crystallisation, the space around the sample has to be filled with appropriate filler [20]. Usually, the filler is transmission fluid or an extra- or intracellular cryo-protectant solution which does not affect the sample. Another function of filler is to increase the overall cooling rate of the process.

Self-Pressurized Rapid Freezing

Self-Pressurised Rapid Freezing (SPRF) is a relatively new method, introduced by Leunissen and Yi in 2007 and further developed by Leunissen and Yi [21]. In this method the material is loaded into standard copper HPF capillaries which are sealed at both ends and plunged into liquid nitrogen, liquid propane or liquid ethane. The essence of this constant volume process is the increase in pressure when the volume of water increases during the freezing process in a sealed capillary [21, 22]. The transformation of water into different crystalline forms of ice at constant volume results in a strong pressure build-up and promotes amorphisation of the sample, which was determined by cryo-EM and electron diffraction [23]. Amorphisation of the sample in a sealed capillary is promoted by the addition of cryo-protective compounds albeit it has been shown that in certain cases the addition of cryo-protectants is unnecessary.

In comparison with HPF, it is possible to achieve similar pressure and higher rates of heat extraction. In the SPRF method, the concentration of cryo-protectants is lower due to a higher rate of heat transfer through crystalline ice compared to amorphous ice or water. As a result, during the crystallisation process the cooling rate increases, which may minimise cell dehydration [1]. Lower concentrations of cryo-protectants may help to avoid difficulties in sample preparation, such as unfavourable osmotic effects or contrast matching in the images. The depth and quality of vitrified samples through the SPRF method is comparable to samples prepared by HPF, however, during vitrification by SPRF ca. 50% of the original water mass is changed into crystalline ice, therefore part of the sample is damaged by the formed ice.

Post-processing after vitrification

Only thin vitrified samples such as purified isolated organelles, macromolecular complexes, viruses, small prokaryotic cells and thinner parts of eukaryotic cells (up to 0,5 μm) can be directly imaged under the cryo-electron microscope [24, 25]. Thicker vitrified samples require a thinning step under cryo-conditions in order to obtain slices thin enough to visualize prior to imaging. Vitreous material can be either cut with a cryo-ultramicrotome by the CEMOVIS procedure or, more recently, thinned with Focused-Ion Beam milling. Alternatively, vitrified samples can be visualized in a standard TEM microscope after a Freeze Substitution process followed by resin embedding or the Tokuyasu technique.

Cryo-Electron Microscopy Of Vitreous Sections

The first attempts at cryo-sectioning were pioneered by Fernandez-Moran [26] and Bernhard and Leduc [27]. Vitreous sectioning was introduced by Christensen [28] and later developed by McDowall et al. [29]. It was not until 25 years later that the work of Hsieh et al. [30] and Al-Amoudi et al. [31, 32] showed that Cryo-Electron Microscopy Of Vitreous Sections (CEMOVIS) is a reliable method for observing cells and tissues in the native state with molecular resolution. Almost a quarter of century time span was necessary to achieve a technological progress and improvements of specimen preparation protocol [14].

In CEMOVIS all the artefacts of the standard EM preparation technique due to chemical fixation, dehydration, staining and embedding are eliminated [31, 32]. Biological samples are vitrified by HPF or SPRF usually with 20% dextran (MW 40 kDa) to ensure homogeneous vitrification. In the next step

of the technique the vitrified sample is transferred in liquid nitrogen to a cryo-chamber in the ultramicrotome where it is mounted with cryo-glue [33]. It is subsequently trimmed and sectioned on the dry knife under adequate temperature (below -140°C). An increase in temperature leads to devitrification, therefore all the steps with the vitreous material must be performed below this temperature. The obtained sections are placed on carbon coated EM grids, and then transferred to the cryo-EM using a cryo-holder where they are observed at -180°C under low-dose of electron beam conditions or may be stored in liquid nitrogen for long periods of time [31].

As already mentioned, in CEMOVIS chemical treatment is avoided, and the specimen remains close to the native state, fully hydrated and unstained. Cryo-EM generates contrast from the protein densities themselves and allows observation at the atomic level [34]. Therefore, on the one hand CEMOVIS is regarded as the 'gold standard' of electron microscopy by some scientists [14, 35] but on the other hand the method is not without artefacts.

First, cryo-sections are more prone to suffer cutting artefacts than resin sections because conventional plastic sections are cut with a wet knife and the water's high surface tension allows the compressed sections to float and unfold. A liquid working the same way with the vitreous section has not been found yet, and as such cryo-sections are obtained with a dry knife. Pierson et al. [36] implemented a protective glove box in order to reduce the humidity around and within the cryo-ultramicrotome.

Secondly, knife marks, compression, chatter and crevasses become worse with increasing thickness of the sections; nonetheless some of them can be eliminated to some extent. These artefacts have been described during CEMOVIS [37] and cryo-ET studies. Knife marks are the mildest of the cutting artefacts and have no negative impact on the resolution of the observable image or quality of the 3D reconstruction [38]. A periodic variation in section thickness along the cutting direction, so called chatter, can be reduced by increasing the cutting speed [37]. Crevasses depend on the cutting parameters such as feed, cutting speed and the knife angle [32, 37] and can be minimized by reducing the thickness of sections below 70 nm [38]. According to Al-Amoudi, compression (30–60%) is the most prominent cutting artefact in cryo-sectioning and is dependent on the sample [9, 38, 39]. It involves the shortening and thickening of sections and leads to a loss of resolution. To minimise this problem, it is recommended to decrease the knife angle [40] and keep the diamond knife scrupulously clean [41]. The application of an oscillating cryo-knife is an alterna-

tive way to reduce compression [40]. Unfortunately, none of the aforementioned adjustments can completely eliminate compression.

Thirdly, after cryo-sectioning vitreous sections are attached to the EM grid support film. Using a polished metal stamping tool, the integrity of the vitreous ribbon and individual sections is altered. Moreover, this method is not adequate for proper attachment of cryo-sections to the support film. Pierson et al. [36] introduced electrostatic charging for attachment, which is devoid of stamping-related artefacts, but it cannot guarantee uniform attachment of cryo-sections to the carbon film. Consequently, the sections can be affected by the beam exposure and may move during image acquisition [38]. However, the recent developments in vitreous cryo-sectioning permit the application of this technique to study different samples. The comparison between the images obtained using a conventional preparation and CEMOVIS showed differences in the organization of bacterial DNA and cell wall of *Mycobacterium smegmatis* [35] and *Shigella flexneri* [42] and presented new insights into the morphology of bacteria [32, 43–45]. This technique enabled to show details of the design of melanosomes [46], the human skin barrier [47] and the molecular architecture of cadherins in native epidermal desmosomes [34].

Cryo-Focused Ion Beam for thinning vitreous sample

A fundamentally different method for thinning vitreous samples is Focused Ion Beam (FIB) micromachining. FIB instruments were primarily developed for material science applications such as semiconductor devices modifications, to fabricate optoelectronic components or sample preparation for high resolution TEM imaging [48, 49]. During cryo-FIB milling, the vitreous sample is maintained in a vacuum at low temperatures and is irradiated by the Focused Ion Beam, usually gallium (Ga^+) ions. The controlled bombardment of the sample with a Focused Ion Beam enables to remove material with very high precision from the specimen surface through the process of sputtering [50]. However, the interaction of gallium ions with vitreous material must be taken into consideration. In the preliminary study, Marko et al. [51] proved that heat transfer to vitreously frozen water during FIB milling, with current of 10 pA and acceleration of 30 kV, did not cause devitrification. Another result of the interaction of the Focused Ion Beam with vitrified biological material is an implantation layer of gallium ions. Simulations indicate that the ion implantation layer in a vitreous material is in the range of 10–20 nm [52]. Indeed, in comparison with the crevasses found in 100–300 nm thick vitreous section, the thickness of

the implantation layer is much thinner. Furthermore, the angle of incidence, along the surface of the support, should be low to minimize the deposition effect of milled material during the milling process.

All experimental steps, ranging from vitrification, cryo-FIB to visualization in cryo-EM, have to be kept below the devitrification point of -135°C . A critical issue is to avoid additional ice contamination and prevention of other potential damage during the sample transfer and handling steps. Thus, a preparation system to mount the grids (*i.e.* appropriate holders, transfer devices for insertion and extraction of the samples) are generally excogitated mainly for specific applications. The process of sample thinning is performed in the Dual Beam microscopes, *i.e.* a combination of FIB with Scanning Electron Microscopy (SEM), enables to select area of interest and guide the milling process. During the thinning process high current is used to remove bulk material, while low current permits further thinning and obtaining final geometry [53]. The Cryo-FIB technique was used to study the structure of *E. coli*, *M. smegmatis*, *S. cerevisiae*, *D. discoideum*, HeLa and neuronal cells vitrified by Plunge Freezing [54–60] and *Aspergillus niger* spores and yeast vitrified *via* High Pressure Freezing [57, 61, 62].

Depending on the sample size and feature of interest, different preparation strategies are required. Possible FIB-milling strategies include parallel milling, wedge-shaped milling and more sophisticated cryo-lamellas. During parallel milling, the Focused Ion Beam is parallel to the EM grid surface. Alternatively, the incident ion beam mills the sample at oblique angles, resulting in a wedge-shaped area. In order to obtain sufficiently large areas for imaging, the shallow angles should be small ($< 10^\circ$). This type of geometry is dedicated to small bacterial cells or relatively small eukaryotic cells. During foregoing cryo-FIB approaches, material along the z -axis is lost that makes it impossible to analyse structures of interest located deep in cellular volumes. In consequence, thinning of large mammalian cells and tissues remains a challenge. A solution for this issue is milling vitrified cells into a lamella, which enables to open a window into the interior of the cell. This can be done by micro-machining areas below and above the region of interest, leaving a thin enough area for imaging by TEM. The obtained electron transparent membrane is supported by the surrounding unmilled cell and ice. The width of the lamella should not exceed more than two thirds of the cell's visible width [56]. This approach gives access to anywhere in the sample through exclusionary selection of the milling areas.

An alternative method to obtain lamellas is a traditional FIB lift-out method used in material science. This method had been deemed impossible because of technical aspects [57]. However, deployment of cryogenic platinum deposition [63] and the adaptation of nanomanipulators to cryogenic conditions enable to implement the lift-out method in the vitreous sample preparation field [57]. After identification of a feature of interest through SEM imaging, the sample is cryo-coated with platinum. The latter step protects the chosen area from undesirable ion beam erosion, and preserves topographical features against re-deposition of milled material and curtaining effects [63]. Next, two trenches are milled on each side of the lamella and the bottom part of the obtained structure is once again milled, leaving only two small bridges connecting the lamella to the bulk. The cooled nanomanipulator is attached to the lamella by cryo-platinum deposition followed by sputtering away the bridges holding the lamella to the bulk. The lamella is lifted out from the sputtered trenches, moved to the TEM grid and welded to a support with cryo-platinum deposition. The nanomanipulator is cut out and the lamella is finally thinned to the desired thickness.

A slightly different strategy is applied for samples vitrified by HPF. Samples can be vitrified both in copper tubes [61] and in membrane carriers [62]. Initially, either the tip of the copper tube or membrane carrier has to be trimmed with the cryo-ultramicrotome to a wedge shape in order to expose a frozen-hydrated material, which is covered with a protection layer of platinum. A lamella with the required dimensions and thickness is obtained during an iterative milling process under different currents.

In general, identifying and targeting specific sites for milling in case of smaller organisms is relatively easy and efficient. On the contrary, large vitrified eukaryotic cells, for example HeLa cells, are embedded in amorphous ice with significantly varying thickness that results from heterogeneity in cell morphology and size. Therefore, vitrified material is difficult to find and distinguish from the ice background by SEM. In order to expose large material embedded in the ice, Rigort et al. [55] adopted a cryo-planning method to generate large, homogeneously thin samples. Furthermore, a combination of light and electron microscopy can be used to expedite and widen the search for features of interest. The combination of specific fluorescent labelling of structure permits to localize features of interest of vitrified specimens under cryo-fluorescence microscope and subsequent imaging with backscattered electrons within the SEM. This approach significantly enhances finding

and targeting molecules with a low number of copies or smaller structures for FIB milling [55]. Another solution is the culturing of cells on EM finder grids and taking optical images of the cells of interest before vitrification and cryo-FIB milling [59].

On the one hand, thinning samples by cryo-FIB gives unique possibilities to study structures buried deep inside cellular volumes and for selective thinning of the sample. Moreover, mammalian cells can grow directly onto the substrate in native conditions, without the need of additional steps like cell trypsinization or scrapping. Depending on the need of different geometries, thickness (< 100–500 nm) and size of the vitreous material, cryo-EM or cryo-ET studies can be chosen. On the other hand, the FIB milling technique is time consuming and requires correlative solutions in order to localize cells on the grid in case of larger cells. Otherwise, the process of sampling takes more time and involves milling of many adjacent places in order to find features of interest. Another important point is the need of very complex transfer systems and handling steps between devices, thus the risk of ice contamination. On the surface of the milled vitreous samples milling artefacts occur, resulting from deposition of milled material. The artefacts are characteristic of milled regions and they are not present in all samples. Interestingly, the occurrence of the artefacts is not inter-related with milling currents or other parameters of the process [58, 59]. Uneven vertical striations, the so called curtaining effect, is another artefact produced from different sputtering rates as a consequence of compositional changes within the vitreous sample [55, 59]. The advantage of CEMOVIS over the cryo-FIB technique is the capability to prepare tissue samples in a more controllable manner. During the cryo-FIB thinning process, part of the material is physically destroyed. CEMOVIS permits to obtain serial cryo-sections, and therefore information along the z-axis is not entirely lost.

The cryo-FIB technique is still under development; however, some interesting results have been achieved. In *E. coli* cells Wang and co-workers [59] found a new structural feature as invagination of the cytoplasmic membrane curves into both periplasmic and cytoplasmic spaces. Rigort et al. [56] present the potential of using cryo-FIB during structural studies of nuclear pore complexes *in situ*. Obtained results allow pushing resolution to the limits and opening new possibilities for microscopic studies of eukaryotic cells.

A characteristic feature of vitreously frozen material is poor contrast, due to the absence of staining with heavy metal salts. As a result of the nature of the vitreous sample, the main obstacle is low signal-to-no-

ise ratio. This fact stems from the low phase contrast, which is based on small differences in the density and uniform distribution of the native cellular components. Another issue is the plethora of overlapping information, which results from equal distribution of the structures through the entire thickness of the sample. Additionally, vitreous material is beam sensitive, therefore a too high dose of electrons leads to the 'bubbling' (the specimen seems to be boiling and turning into small bubbles which disappear under the beam) and moving of the sections [13]. Unquestionably, computer control of the microscope is a crucial step to obtain full information from the vitreous sections. Huge progress in the computing systems and electron microscopy technologies permits to introduce low-dose mode, to minimize the total electron dose, and electron tomography technique which give insight into the three dimensional structure of the cell. For the description of the electron tomography technique in detail and its computational aspects, such as image alignment and 3D reconstruction, post processing, analysis and interpretation, the reader is referred to exemplary works [25, 64–72].

The main disadvantage of vitreously frozen specimens is the uselessness of on-section immunogold labelling, because it requires ambient conditions to work. Therefore, the Tokuyasu technique and freeze substitution followed by resin embedding are complementary techniques to the vitrified sample.

Freeze Substitution

Freeze Substitution (FS) is a cryo-fixation method which bridges the gap between the vitreous material and conventional plastic embedding on one hand and room temperature ultramicrotomy on the other. Freeze substituted and vitreous samples are very complementary [38]. Resin sections permit to obtain an excellent overview of the cell ultrastructure with enhanced contrast of the object of interest. Information obtained from cryo-EM images can complement the images obtained from resin sections. Moreover, resin in comparison with vitreous sections enables the analysis of larger sample areas, is more stable in the electron beam and is easier to obtain. Last but not least, resin sections permit post-labelling with gold nanoparticles. This method was introduced for light microscopy by Simpson [73] and in the following years further developed in the field of electron microscopy [74–76].

The process of Freeze Substitution [5, 77] is performed at low temperature, yet above the devitrification temperature. Presumably, during the FS process sequential transformation from the vitreous state to

cubic ice and from cubic to hexagonal ice preserve the biological structures without any segregation [9]. Low temperatures result in a reduction of the extraction of lipids and aggregation of protein [78], avoiding osmotic effects and chemical artefacts which result from the penetration and diffusion of fixative [79–81]. Freeze Substitution consists of dehydration and chemical fixation followed by embedding in resins. In the first step, a sample after cryo-immobilisation is dehydrated by organic solvents, such as acetone, methanol or ethanol. This step typically takes place in the temperature range from -78°C to -90°C . In the second step, the biological material can be slowly infiltrated with nonreactive fixatives in chosen organic solvents in the same temperature as in the first step. As soon as the temperature is raised, fixatives start to react in situ, between -90°C and -30°C . The main constituents of fixative solutions are aldehydes, OsO_4 and uranyl acetate (UA) in mixed configurations in acetone or alcohols. It is worth mentioning that OsO_4 at low temperature does not react as protease, but fixes unsaturated chains of plasma membrane fatty acids. Alternatively, Muller and Matsko showed that instead of the above mentioned stabilizing agent, reactive epoxy resins are an interesting alternative in morphological studies [82]. A characteristic feature after cryo-fixation is a weaker contrast of the sample, especially of membranes against more electron dense background — *i.e.* 'reverse contrast' [83]. In order to enhance membrane contrast to the substitution medium, small amounts of water may be added [84]. However it is possible to wash out the antigens, which should be taken into account at the immunocytochemistry level [85]. Other media may contain tannic acid in acetone [86, 87] in combination with OsO_4 [88], different combinations of UA, glutaraldehyde and OsO_4 [83, 89, 90] or potassium permanganate [89]. The duration of substitution depends on the chosen medium and the sample but the range of time fluctuates between a few hours and a few days.

It is worth mentioning the latest McDonald and Webb's studies, with subsequent McDonald's [91] modifications, where FS can be obtained in 3 hours or less [92]. After the FS process, the sample is embedded in resins. Epoxy resins are dedicated to the morphological approach by virtue of good preservation of ultrastructure, a larger stability in the electron beam and ease of sectioning. Methacrylic resins are used for immunocytochemical research because of their properties such as impregnation and UV polymerisation at low temperatures, lack of sample cross-linking and higher roughness of the section surface [2, 76, 93]. Interestingly, total processing time from vitrification followed by freeze substitution and resin embedding

to section can be completed in 6 hours [91, 94]. Results obtained by above rapid specimen preparation procedures was comparable to standard FS and resin embedding methods that take days to complete.

A combination of HPF and FS followed by resin embedding offers tremendous research capabilities in cell and tissue biology. *E.g.*, studies on the aorta prepared by HPF provided a new basis for understanding of cell/cell and cell/matrix cross-talk in aortic media [95] and new data about structure and function of Weibel-Palade body in endothelial cells were obtained [96, 97]. Cryo-fixation with immunogold labelling was used for subcellular localisation of H^+/K^+ -ATPase in gastric parietal cells [98], immunolocalisation of collagen in human articular cartilage [99] and of Connexin43 proteins in rat myocardium [100]. It is worth mentioning that cryo-fixation in combination with ET [16, 66, 101] and CLEM [102–104] gives a chance to understand not only cell ultrastructure but also dynamics of the cellular processes.

Tokuyasu cryo-sectioning method

The Tokuyasu technique was introduced in the 1970's by Tokuyasu, using a cryo-ultramicrotome developed by A. Christensen [105, 106]. According to his protocol the biological material is chemically fixed with low concentrations of aldehydes, cryo-protected in sucrose, frozen in liquid nitrogen and sectioned at low temperature (ca. -100°C) in a cryo-ultramicrotome with a dry knife. Cryo-sections are retrieved from the knife edge, thawed and transferred to microscope slides or formvar carbon-coated grids. Semi-thin cryo-sections can be used for immunofluorescence microscopy [107]. Thin sections may be directly observed in EM, labelled (mostly by antibodies) [108] (Figure 2A), or used for mRNA localisation by in situ hybridization [109]. After labelling, unstable and delicate sections are embedded in a thin film of plastic containing a contrasting agent [106, 110]. Over the years, the technique has been improved and perfected by its inventor and other researchers [111–115]. What makes this method so preferred? The Tokuyasu method eliminated harmful processes *i.e.* dehydration by organic solvents and embedding in resin. Dehydration was replaced by cryo-protection which reduces the water content in cells resulting in partial dehydration. However, antigens still remain in their natural aqueous environment, which is important for antigens sensitive to ethanol. Additionally thawed cryo-sections enable better access to the antigens (particularly rare ones) for the probes because they are not embedded in resin, that is to say a high density matrix. These advantages determined that cryo-sectioning of chemically fixed

and cryo-protected samples became a widely used method for immunolabelling [77, 116].

Unfortunately, the technique has drawbacks. A limitation inherent to the Tokuyasu cryo-sectioning method is chemical fixation at ambient temperature. On the one hand, it is a prerequisite to stabilize the cell structure prior to cryo-protection and cryo-sectioning in order to reduce extraction of cytoplasmic proteins. On the other hand, aldehyde fixation is a slow and selective process which results in pH-related and osmotic changes in the different organelles [117]. Besides, it should be mentioned that not all specimens are suited for this method. The penetration of the fixatives is hampered in samples containing intercellular air spaces or large vacuoles (leaves), cell walls (plants, fungi) or cuticles, hydrophobic surfaces (insects, nematodes) [114]. In consequence, improperly cross-linked molecules can be dislocated or get lost which could lead to misconception about their internal organization [117, 118]. The main advantage of the Tokuyasu technique, however, can also be a disadvantage because the absence of resin matrix may result in the partial extraction of the material.

Despite the drawbacks for several types of biological specimens, the Tokuyasu cryo-sectioning technique is a valuable and preferred immunolabelling method in combination with the highly efficient immunogold labelling [77]. Therefore many researchers have applied this technique to study various biological objects [35, 110, 117–122].

At the edge of possibilities — hybrid techniques

It has been well documented that cryo-fixation by HPF combined with FS and resin embedding can significantly improve the preservation of ultrastructure and antigenicity [123, 124]. Nevertheless, not all antigens can be immunolabelled in such a way due to a limited number of accessible antigens at the resin section surface (especially important in locating rare antigens) [125]. As it is known the Tokuyasu cryo-sectioning is the most efficient, localization technique for immunolabelling. Therefore different groups of scientists have tried to establish a new solution using combinations of different cryo-techniques. Hybrid techniques have been developed in response to difficulties which have occurred during the sample preparation process. They include the advantages of two or more techniques creating new methods with prospective research potential.

Hybrid techniques include the method which introduces the initial chemical fixation prior to HPF and FS (Hybrid Fixation Technique). The aldehyde fixation is necessary in order to minimize structural

damage during long dissection times. This procedure is intended for the labile tissue (especially the brain) exposed to damage due to lack of oxygen at the time of collection. Sosinsky et al. [87] showed that chemical pre-fixation before HPF and FS followed by resin embedding provided a better preservation of ultrastructure than a single chemical fixation. However, exposure of fresh, microdissected tissue to highly concentrated cryo-protectants can lead to osmotic stress and physical damage [126]. Using aldehyde fixation before HPF-FS and resin embedding, Ewald et al. [127] achieved high-quality ultrastructural preservation of cell membranes in multilayered epithelium. If we have to deal with biopsies, immediate processing of samples by HPF may not always be possible and chemical fixation is the only alternative, thus, the hybrid fixation method seems to be a good solution for this problem. Work of Venter et al. [128] proved that ultrastructure after prolonged storage in fixatives and subsequent HPF-FS followed by resin embedding was better retained in comparison with conventional sample processing.

Liou et al. [112] and van Donselaar et al. [120] developed a technique that combines high-efficiency of Tokuyasu cryo-section labelling with initial cryo-fixation by HPF. This can be achieved in two ways: the first is the Section Fixation Method (SFM) (Figure 2B), in which native ultrathin cryo-sections are chemically fixed (in a mixture of sucrose/methyl cellulose/glutaraldehyde/formaldehyde/uranyl acetate) and subsequently immunolabelled. It has turned out that this method is impractical in the Immuno-Electron Microscopy (IEM) standard research (well-preserved material has been obtained only on a small area of the section's surface). According to the authors, it was probably due to the critical interweaving of thawing and chemical fixation [112, 120].

Another hybrid technique is the ReHydration Method (RHM) in which cryo-fixed material is subjected to Freeze Substitution, subsequent rehydration, cryo-sectioning following the Tokuyasu method and finally immunogold labelling (Figure 2A). This method has been applied for tissues and mammalian cells [120] and difficult to chemically fix material such as plant, nematodes and insects tissues [117, 118].

It has been shown that the hybrid technique is superior to the conventional method for the following reasons. Cryo-immobilization is a fast and non-selective process and therefore reduces the changes linked to a slowed down diffusion of fixatives during conventional fixation in ambient temperature. Freeze Substitution enables the use of glutaraldehyde, uranyl acetate or OsO₄ as primary fixatives and these approaches resulted in the good preservation of the

cellular ultrastructure as well as the antigenicity. It has been proved that antigens which do not react with aldehydes and are sensitive to chemical processes maintain their activity despite the use of high concentrations of fixatives. The hybrid method is suitable for antigens that are sparse and sensitive to embedding in resin. The visibility of the cellular membranes was enhanced when the Freeze Substitution medium was supplemented with low amount of water [117, 120]. According to Stierhof and Kasmi the water's effect on membrane preservation was not observed in all plant tissues. Therefore, they preferred OsO₄ fixation alone during FS, because it resulted in sufficient visibility of the ultrastructural details of different plant tissues [118]. The improved preparation methods combined with sensitive marker systems, such as 1 nm gold markers, allow detection of sparse antigens.

It should be mentioned that immunofluorescence microscopy may benefit from the hybrid technique: the good quality and adequate section thickness guarantee high resolution data. The simultaneous detection of several fluorescence markers together with labelled antigens is possible. The fluorochromes do not affect the binding properties of the antibodies which results in high selectivity and sensitivity in this method. If the sample does not emit any fluorescent signal in immunolabelling experiments, it means that immunogold labelling for TEM does not make sense due to the lower sensitivity of TEM markers (gold nanoparticles) in contrast to the fluorescent markers [118]. However, a serious disadvantage of the method is its long duration.

Sabanay et al. [129] presented a new approach to obtain Vitrified Frozen Sections (VFS) for high resolution ultrastructural analysis of cells and tissues (Figure 2A). This technique combines the classic Tokuyasu method with re-vitrification in liquid ethane of the sections collected on the EM grid. The immunolabelling step was performed following the rinsing of the sections and prior to their embedding in ice. Twenty years later Bokstad et al. [130] applied this technique to 3D imaging. This experiment used comparatively thick sections (300–400 nm) with acceptable structural preservation of the tested sample which enabled the reconstruction of the 3D structure by cryo-ET. The VFS technique avoids artefacts associated with the CEMOVIS approach due to the thickness and also by thawing the sections on the grid prior to the vitrification process.

Two new methods for the fixation of sections from cryo-immobilized samples for Immuno-TEM were developed by Karreman et al. [131]. These high-speed fixation techniques known as VIS2FIX are an alternative to the more time-consuming approaches mentioned above. In the first step of the VIS2FIX

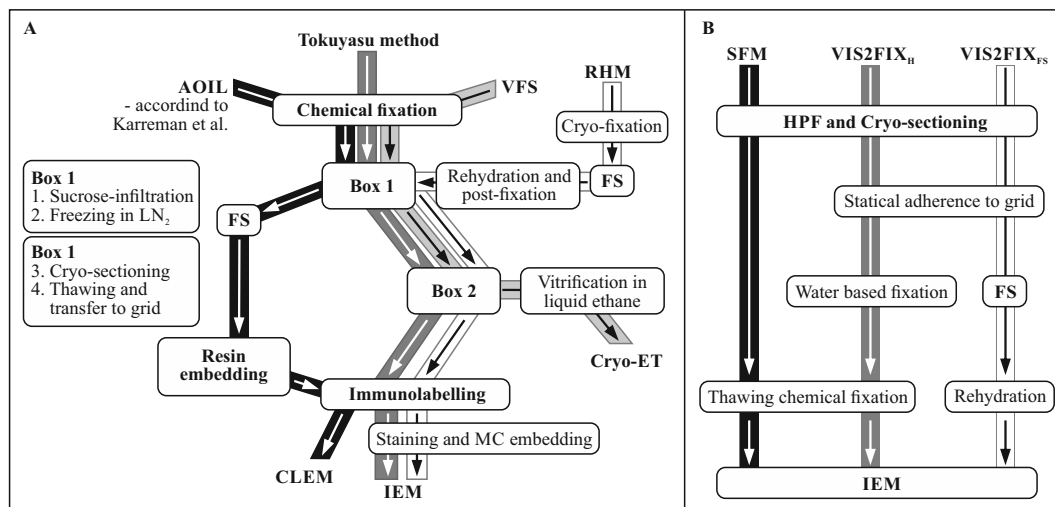


Figure 2. The flow chart shows the steps involved in Hybrid Technique. **A.** Chemical fixation is a prerequisite to the Tokuyasu, VFS and AOIL methods. The Tokuyasu method contains the steps shown in the Box1 and Box2 followed by the final labelling step. The VFS method includes the Box1 and Box2, the final step of this method is vitrification in liquid ethane, subsequently, the sample can be analysed by cryo-ET. The AOIL method is characterized by Box1, (2) Freeze Substitution (FS) with resin embedding, (3) labelling, finally the sample can be analysed in IEM or iLEM. The RHM method starts from cryofixation, there follows (1) FS; (2) rehydration and postfixation; (3) Box1; (4) Box2; (5) immunolabelling; (6) staining; (7) analysing in IEM. Box 1 includes the following steps: (1) sucrose infiltration; and (2) freezing in liquid nitrogen (LN₂). Box 2 involves: (3) cryo-sectioning; (3) cryo-sectioning; (4) thawing and transfer to grid. **B.** The SFM and the VIS2FIX methods are based on cryo-immobilization and cryo-sectioning. Subsequently, in the SFM sections are simultaneously thawed and fixed, then labelled and finally analysed in IEM. In the VIS2FIX sections attached to the grids are preserved under different conditions, more details are describes in the text. In the last step the labelled sample may be viewed in IEM and CLEM

process, vitreous sections (VIS) of the cryo-immobilized sample are cut and statically adhered to the grid. Next, the sections on the grid are fixed in two ways (FIX); VIS2FIXFS, and VIS2FIXH (Figure 2B). VIS2FIXFS is based on FS and subsequent rehydration of the sections. In VIS2FIXH („H”-hydrated) the grids with sections are placed on a water based frozen fixative which is then thawed and fixed in liquid fixative. The VIS2FIX technique reduces the time needed for preparing samples and allows the use of a wide variety of fixatives which enlarge the research potential, *e.g.* the VIS2FIXH preserves the neutral lipids. The fixation and retention of neutral lipids is a unique feature of the VIS2FIXH method. Both techniques provide good membrane contrast and excellent preservation of vesicles especially in the Golgi area. The lack of embedding medium does not cause the loss of epitopes. The VIS2FIX offers new possibilities for IEM (the cross-linked antigens are more accessible because only a thin layer of section is fixed using this method) and for the integrated Laser and Electron Microscopy (iLEM — a novel tool for correlative microscopy combining fluorescence microscopy and TEM in a single setup). Both methods provided high fluorescence signals and abundant immunogold labelling.

The iLEM allows for quick correlation between fluorescence microscopy and transmission electron microscopy in the imaging of a sample. The optical-imaging mode is used to identify fluorescent markers and then the region of interest (ROI) is analysed at high resolution in the TEM mode. In this way, the time-consuming relocation of the ROI across microscopy platforms is avoided (with non-integrated solutions for correlative microscopy the sample needs to be transferred from one imaging set-up to another and specialised procedures are required to correlate the results) [132, 133].

In preparing specimens for analysis in the iLEM, two distinct and conflicting demands need to be met: the preservation of cellular ultrastructure and bright fluorescent labelling. These requirements, which a sample must meet to be imaged in iLEM, have become an impulse to develop new sample preparation techniques or to optimize the ones that exist. It is commonly known that if heavy metal and fluorescent label particles are localized at the distance of ~100 Å from each other, quenching of the fluorescence signal may occur [132]. In order to prevent this, the material must be prepared in such a way that increasing the distance between markers' particles is possible. Karreman and co-workers [134] used a procedure in which chemically

Table 1. Hybrid techniques and its advantages

Hybrid technique	Characteristic features
Hybrid Fixation Technique Purpose: stabilization of dedicated labile and fragile tissues	Minimizes structural damage due to anoxia and excision in labile tissues ¹ Convenience in preservation of biopsies
ReHydration Method Purpose: suitable for difficult-to-fix specimens and antigens	Eliminates the limitations related to diffusion of the fixatives into problematic samples ² Allows the application of different fixatives and their combinations ³ The reagents act gently at low temperatures, OsO ₄ is less proteolytical to antigens ³ Antigens remain in an aqueous environment prior to immunolabelling, <i>i.e.</i> improves the accessibility of epitopes ⁴ The visibility of membranes can be improved by adding water to FS medium ³ Can be used for <i>in situ</i> hybridization for CLEM
VFS Method Purpose: structural research by ET	Enables direct examination of unstained, hydrated sections in conjunction with immunogold labelling ^{2,4} The sample remain hydrated during preparation and examination ^{2,4} Thickness of vitrified ice layer does not change under vacuum column Offers a simple approach for 3D reconstructing thick volumes of hydrated frozen sections ^{2,4} Allows obtaining appropriate thickness sections and circumvent artefacts associated with CEMOVIS ^{2,4}
VIS2FIX Methods for IEM Purpose: new potential for conventional immuno-TEM, CLEM and ET	Fast, reproducible and many fixatives can be tested on single sample ^{3,5} Unique feature of VIS2FIX _H — preservation of the lipid droplets Good membrane contrast of the ER, the nuclear envelope, the Golgi, lysosomes and cristae of the mitochondria Gives new possibilities for immuno-TEM The labelling of covered by cross-linking epitopes is possible ^{3,4,5} The loss of material is not found (Forssman glycolipid was excellently labelled ^{3,5}) Suited for use in CLEM (iLEM)
AOIL — An Optimized Immuno Labelling for iLEM	Offers <i>en bloc</i> staining of the biological material before embedding in resin to prevent quenching of the fluorescence ^{3,4}

Numbers 1–5 indicate which cryo-technique is used in hybrid approach: ¹chemical fixation, ²cryo-fixation, ³FS, ⁴Tokuyasu cryo-sectioning, ⁵vitreous cryo-sectioning. AOIL according to Karreman et al., 2012.

fixed cells were embedded in 12% gelatin and cryo-protected in sucrose. The sample blocks were plunge frozen in liquid nitrogen and subsequently freeze substituted and embedded in the Lowicryl HM20 resin (Figure 2A). The material was stained during a FS process, before resin embedment. Simultaneously, an appropriate TEM fluorescent marker had been chosen (such as marker which in the vacuum conditions emits a strong fluorescence signal). Fluorophores' labelling was made on sections obtained from *en bloc* stained samples. As a result of this experiment, the authors established, that *en bloc* staining did not quench the fluorescence signal that was emitted by a given marker (TRITC) and, ultimately, guaranteed a good contrast of the analysed sample [134].

The different approaches have their own sets of advantages and might well be the methods of choice in experiments under various experimental conditions (Table 1). The advantages of the application of hybrid techniques can be summarised in a following way:

1. they enable to deal with different materials and also with those that are difficult to fix,

2. can be used when conventional fixation at ambient temperature is not satisfactory or not useful due to the inactivation of antigens and the presence of artefacts caused by slow fixative diffusion,
3. can be applied when cryo-fixation and accessibility of antigens (two in one) is required.

Conclusions and perspectives

Even if due to technological advances, transmission electron microscopes will reach the resolution of Ångstrom limit, the sample preparation process will always remain a crucial issue. Technological progress and sample preparation methods are linked with each other and create a kind of feedback. Since the mystery of water vitrification was unveiled by Dubochet and co-workers [13], various cryo-preparation methods have been developed. This discovery has also stimulated development of different instruments as the solution of specific problems which evolved during material preparation. Nowadays, the results of these developments enable vitrification of various types of

samples. Direct observation of vitrified specimens, especially in combination with electron tomography, permits visualisation of 'near to native state' cellular architecture. The CEMOVIS technique first enabled the direct visualization of the cell ultrastructure in the vitreous state. The inherent feature of the CEMOVIS is section compression due to the different flexibility of cellular structures [135], which is difficult to correct by the specialized software. A solution for this problem was the adaptation of FIB and SEM microscopes to cryogenic conditions in order to obtain perfectly flat cryo-lamellas with no evidence of mechanical distortions. On the other hand, the research potential of cryo-techniques has been well established, which makes it possible to simultaneously exploit various preparation routes to reach the goal in mind. One excellent instance is the iLEM microscope, because this correlative technique requires the optimization of sample preparation protocols for optimal results. It was shown that a hybrid approach, a combination of the Tokuyasu technique with FS, enables to prevent the quenching effect of the contrasting agent on the immunofluorescent labelling on the section. This approach enables to localize fluorescence signals at a high resolution level. Other hybrid approaches were introduced for either difficult to fix samples or antigens (Rehydration Method and VIS2FIX method). However, depending on the sample, the Tokuyasu technique and FS followed by resin embedding rather became complementary to immuno-TEM.

Modern EM focuses on a correlative approach at different microscopic levels. Cryo-preparation techniques play a very important role during imaging of cell ultrastructure by new, powerful tomography solutions such as Serial Sectioning SEM and Correlative Array Tomography or FIB-SEM correlated with Confocal Laser Scanning Microscopy [136]. Current trends focus also on all-in-one solutions. The combination of FIB with TSEM (Transmission Scanning Electron Microscopy) microscopes enables to obtain cryo-lamellas with navigation in the SEM mode and higher magnified images in transmission mode. TSEM microscopes permit to check the quality of the frozen material by orientation contrast or by Electron BackScatter Diffraction detectors [62]. Finally, Cryo-iLEM, as a new approach proposed by Faas et al. [137], increases the successful correlation rate and permits to avoid contamination and damage of vitrified samples during transfer between different microscopes.

The combination of TSEM with fluorescence microscopy into a single device could potentially provide, at the in situ level, assignment of location, preparation and integrity check of the cryo-lamella,

and subsequent observation and tomography of the sample.

However, it must be taken into account, that conventional TEM has a well established position in biological and medical sciences because of the possibility to study samples with a large size. Conventional TEM helps to make diagnosis in different kinds of illness, for example kidney glomerular disease and primary ciliary dyskinesia. A very interesting example which proves the research capabilities of conventional TEM was the discovery of telocytes (or re-discovery of Cajal cells) found in different tissues [138, 139]. Therefore, the choice of the sample preparation option depends on the size of the specimens being studied, how the sample is obtained (for example in a hospital) and what type of study will be carried out.

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