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Transmission Electron Microscopy of Biological Samples

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Additional information is available at the end of the chapter

Abstract

During the last 70 years, transmission electron microscopy (TEM) has developed our knowledge about ultrastructure of the cells and tissues. Another aim is the determination of molecular structure, interactions and processes including structure-function relationships at cellular level using a variety of TEM techniques with resolution in atomic to nanometre range. Even with the best transmission electron microscope, it is impossible to obtain real results without optimal sample preparation, respecting both the structure and the antigenicity preservation. Preparation techniques for high-resolution study of both macromolecular complex and organelles within cellular complex are based on fast cryoimmobilisation process, where the sample is in the most native, hydrated state. Next, thin samples are directly visualised under cryo-transmission electron microscopy (cryo-TEM), while thicker samples require a thinning step via cryo-electron microscopy of vitreous sections (CEMOVIS) or cryo-focused ion beam (cryo-FIB) before visualisation. Alternatively, vitrified samples are freeze substituted and embedded in chosen resin for room temperature ultramicrotomy. This preparation technique is suitable for morphological study, 3D analysis of cellular interior and immunoelectron microscopy. A different route for immunolocalisation study is cryosectioning according to the Tokuyasu technique that is a choice for rare or methacrylate-sensitive antigens. Most recently, new hybrid techniques have been developed for difficult-to-fix organisms and antigens or labile and anoxia-sensitive tissues. Another preparation technique is, the oldest but still important, conventional chemical fixation dedicated in a wide range of research interest, involving morphological and immunolocalisation study. In this chapter, we present different sample preparation approaches for transmission electron microscopy of biological samples, including its methodological basis and applications.

1. Introduction

The first transmission electron microscope was constructed in the early 1930s by Ernst Ruska and Max Knoll [1]. Roughly a decade later, the first electron microscope picture of eukaryotic cells was taken by Keith Porter [2]. Since then TEM made it possible to study cells and tissue structure and function at nanoscale. This technique is placed between high-resolution methods like X-ray crystallography or nuclear magnetic resonance and lower-resolution light/fluorescence microscopy techniques. Although fluorescence techniques allow for imaging dynamic process in living cells and modern fluorescence microscopes overcoming the diffraction limits that makes it possible to zoom in on cellular structure with resolution under 100 nm [3], TEM remains the main technique which makes it possible to study biological systems owing to its near-atomic-level resolution [4]. Moreover, TEM gives opportunities to visualise an interesting target with surrounding structure, when unlabelled surroundings still remain hidden at fluorescence sample [5]. Additionally, TEM comprises different branches: electron crystallography and single-particle analysis are dedicated to study proteins and macromolecular complexes, (cryo-)electron tomography and CEMOVIS for cellular organelles and molecular architectures and conventional TEM for gross morphology. Such a wide range of electron microscope techniques gives opportunity to find the relation between different macromolecules, their supramolecular complexes and organelles assembled into an intricate network of cellular compartments. Knowledge of the cellular ultrastructure can contribute to an understanding of how cells and tissues function in both normal physiological and pathological state.

Since the invention of the first TEM, the aim has been to image liquid samples at higher resolution but as easily as with light microscopy. At the beginning, it was impossible to accomplish due to low technological knowledge and lack of appropriate tools. Thus, scientists have introduced sample preparation techniques for observing soft and frail living matter in the inhospitable environment of an electron microscope. The TEM column is under ultrahigh vacuum, where electrons as a coherent beam are directed on the sample. From a biological point of view, living matter consists of up to 80% water; therefore, without appropriate sample preparation, the high vacuum literally sucks out every trace of liquid. Moreover, biological matter compose mainly of light elements (e.g. carbon, hydrogen, oxygen, nitrogen, sulphur, etc.) and for that reason, electrons which travel at a fraction of the speed of light, do not interact strongly with these atoms. Consequently, native biological materials are of extremely low contrast. On the other hand, the two aforementioned factors oriented sample preparation strategies. Due to the electron's scattering phenomena within the sample, only small objects can be observed directly. Larger samples need to be sectioned for analysis, but cells and tissues are too soft to be sectioned thinly enough without earlier sample preparation. Many laboratories have been ingenious in designing and implementing different preparation techniques over the years, and as a result, scientists have found at least a partial remedy to these problems.

Therefore, a biological sample can be prepared either by removing or by freezing water. The oldest method is conventional sample preparation which uses chemical fixation, sample dehydration at room temperature and embedding with chosen resin. In the 1970s, Tokuyasu introduced an alternative to conventional sample preparation dedicated for immunocyto-chemistry. An alternative method to chemical fixation is cryofixation via vitrification process. Taking into consideration the size of the sample, electron microscopists have a wide range of freezing techniques at their disposal. Small or thin sample after plunge freezing can be directly observed at low temperature under cryo-transmission electron microscope (cryo-TEM). Thicker samples are first vitrified by using high-pressure freezing (HPF) or self-pressurised rapid freezing (SPRF) technique and then proceed to thinning process at liquid nitrogen temperature by CEMOVIS procedure or cryo-FIB milling. Finally, thin frozen-hydrated samples are directly observed under cryo-TEM. Another option is freeze substitution (FS) which bridges the gap between vitreous states and room temperature ultramicrotomy. Lastly, different combinations of mentioned techniques offer a new research possibility, especially for difficult-to-fix organisms or antigens. In a particular situation, chemical pre-fixation step is a prerequisite for successful sample vitrification, although it seems to be contradicted.

Biological systems are very complex; thus, it is impossible to understand structure-function relationship outside the surrounding context. These days, dynamic developing of correlative light and electron microscopy (CLEM) approach can be observed. This approach relies on two steps. Firstly, the object of interest is located and imaged with fluorescence microscopy (FM), and then the sample is imaged in TEM. This technique is highly demanding according to cell biologists because high-resolution data can be fitted in the cellular context. However, new possibilities introduce new challenges in the preparative stage, and protocols for TEM and FM often are incompatible. Therefore, it is worth to mention that technological progress stimulates new sample preparation design, but often existing preparation schemes initiate new ideas.

In this chapter, we present different specialised preparation techniques dedicated to cells and tissues; but at the beginning, we would like to impress the importance of water in life on readers, because for a long time, its role in living organisms was neglected. Another point is that for a long time, water was treated as a foe by electron microscopists. Nevertheless, readers should bear in mind that selection of an appropriate technique strongly depends on the material and aims of the study. Thus, a general rule of thumb is that the higher the resolution is important, the closer to the native state sample preparation is desired. Moreover, the higher the resolution, the thinner sample should be, but at the same time, less information is achievable. During morphological study, more important is the sample size; hence, the preparation technique based on resin-embedded sample is an adequate choice. However, for immunolab-elling research, compromise between antigens and ultrastructure preservation is the major challenge. Although the main aim of this chapter is to present different preparation techniques of biological specimens for TEM, we would like to also point out that the preparation step is important for correlative approach.

We strongly encourage further reading of proposed positions where the reader can find practical insights of the presented subject, e.g. chosen volumes from *Methods in Molecular Biology* [6–8] and *Methods in Enzymology* [9–11], and with practical application in different model systems, positions from the Methods in Cell Biology series [12–14]. Many important hints in sample preparation for TEM are also connected with CLEM field [15,16] and immu-

noelectron microscopy [17]. In our opinion, a complete library should also include the *Handbook of Cryo-Preparation Methods for Electron Microscopy* because this position is strongly oriented to the practical side of sample preparation art [18]. It is also important to know what was done so far and thus where we should go. Among many old books, but with still-current knowledge, *Cryotechniques in Biological Electron Microscopy* [19] captured our attention. The last but not the least position is the *Principles and Techniques of Electron Microscopy: Biological Applications* [20].

2. Water and its vital role in life

Organisms consist in major part of liquid water which is the medium in which life takes place. Hence, life on our planet and its probability elsewhere in the universe cannot have evolved or continue without water. In view of the abundant presence of water in living organisms, this substance cannot be perceived as an inert diluent. Water performs many functions: it transports, reacts, lubricates and structures and is used in signalling. Water is also a metabolite and a temperature buffer. The physical properties of water, which result from its structure, play a key role in the orchestration of the cell machinery. Biological molecules and water should be thought as equal partners where one is required and structured by the other.

From a chemical point of view, a water molecule contains one oxygen atom covalently bounded with two hydrogen atoms. Due to positively charged hydrogen atoms and a negatively charged oxygen atom, where negative charge comes from two lone electron pairs, water is a dipole. Water as a dipole has the most important property: water molecules are able to form multiple hydrogen bonds between each other. A hydrogen bond occurs when a partially positively charged hydrogen atom lies between partially negatively charged oxygen of H_2O molecules. A hydrogen bond is naturally formed from a complex combination of different interactions: an electrostatic, a polarisation and a covalent attraction, and a dispersive attractive interactions, an electron repulsion and a nuclear quantum effects. In theory, one water molecule can interact with four other water molecules, thereby forming a tetrahedron configuration. In practice, hydrogen bonds are very dynamic and heterogeneous structures, both on energetic and structural levels, and a single water molecule can form two or four hydrogen bonds. As a result, in liquid water, hydrogen bonds behave in cooperative and anticooperative manner [21]. At the higher level of organisation, water molecules in liquid state tend to create tetrahedral pentameric clusters, which linked to other water molecules and clusters to form a complex network or liquid phases [22]. Such a network of hydrogen bonds is dynamic and ordered in a nanometre range structure.

From a biological point of view, a cell's interior consists of membranes and cytoskeleton together with proteins, carbohydrates and nucleic acids which are strongly and inseparable dependent on water molecules. The dipolar nature of water enables to arrange molecules of water into an ordered, very constrained manner on the surface of biological molecules. Depending on the chemical nature of surface domains, hydrophobic or hydrophilic, water order is different. Water molecules are strongly attracted by ionised and hydrophilic domains than by apolar domains, where H_2O molecules arrange themselves into clathrate-like struc-

tures [23]. They form a hydration shell, called also interfacial water, built from several water layers. Hydration shells are critical for solubility of molecules and prevent them from aggregating. When two particles meet, they do not stick together, but separate [24]. Moreover, protein folding is mediated and guided by aqueous solvation, and protein structure is stabilised by water clusters and their hydrogen bonding capabilities. Water also gives proteins flexibility during conformational changes, and its molecules mediated protein-ligand interaction. Another interesting example of water role in the cellular world is nucleic acid-water interactions. Firstly, water molecules stabilise structure of double helix. Secondly, water hydrates both the major and the minor grooves of DNA. The hydration shells have a characteristic pattern reliant on bases and their sequences and thus create a 'hydration fingerprint' for a given DNA sequence. The specific arrangement of interfacial water governs protein binding to the DNA. The enumerated examples are further discussed in detail in [25]. Water inside the cell, which is not bound in hydration shell, is unaffected by the biomolecules. Additionally, cellular unbounded water behaves differently from water outside, e.g. intracellular water has higher viscosity. The cytoplasm has a sol-gel nature. The local parts of the cytoplasm may manifest itself as a more highly viscous and stiff environment, likened to a gel-state, or as a low-viscosity sol-state solution [21]. In the former case, water molecules form more strongly hydrogen-bounded water clusters. This reduces local fluctuations in the nearby macromolecules and slows down metabolite and ion migration [23]. An additional function of strong hydrogen-bonded network existence is transmission of information about solutes and surfaces at distances of several nanometres. In this way, biomolecules 'sense' each other, thereby changing their solubility and activity. In the latter case, proteins can release a significant amount of water, changing the fluidity and activity of the intracellular 'sol'-state environment. The state of the water is thus essential for the biological activity of the cell, and the state of metabolites controls water structure. Thus, water is defined as an engine of life [26].

From the physical point of view, water is usually perceived as an ordinary substance because people interact with it all the time in their everyday lives. Very often, people think that other liquids have similar properties to this liquid; however, nothing could be further from the truth. Water is the only liquid that exists in all three states on Earth: liquid, solid and gas. Although some researchers believe that intracellular water is the fourth phase of water [27]. Some properties of water, such as large heat capacity and high thermal conductivity, allow the control of body temperature. The high latent heat of evaporation is a protection from dehydration and considerable evaporative cooling [21]. To sum up, water is essential for life existence at different levels: from molecular to cellular and organisms to whole-planet level. All properties of this substance are not yet known; therefore, new discoveries in this field will have an interesting impact in understanding how life works.

3. Conventional TEM

As the vital role of water was outlined in the last paragraph, it must be borne in mind that the knowledge about water and its role in living organisms has evolved over decades. Recently, however, the role of water in cell and molecular biology has become clearer and much more

important than in the past. At the beginning of the sample preparation, biologists carefully eliminate every trace of water. Conventional specimen preparation is the most commonly used method for TEM. The major advances in conventional specimen preparation technique are summarised at the end of the section.

The first step in this procedure is chemical fixation to preserve the biological sample with minimal alteration of volume and morphology from the native state. Chemical fixation can be carried out in various fixatives which are used separately or in combination, i.e. glutaraldehyde (GA), paraformaldehyde, osmium tetroxide, uranyl acetate (UA) and tannic acid solution are usually used. Glutaraldehyde, as a dialdehyde, preserves ultrastructure well but penetrates slower than the monoaldehyde, i.e. paraformaldehyde. Glutaraldehyde is used alone for small pieces of material, but a mixture of the two aldehydes may be used for fixation of larger items. The most popular fixation strategy in conventional preparation is double fixation with GA and osmium tetroxide. The aldehydes preserve mainly proteins, but reaction with lipids is limited. Therefore, to stabilise the lipid part of the sample, the post-fixation with osmium tetroxide is required [28]. Furthermore, osmication enhances contrast which is important during the analysis in the TEM, but it is important to remember that prolonged process can destroy proteins; as a consequence, the biological material becomes brittle. It should also be noted that the common practice in conventional electron microscopy is en bloc fixation/staining with UA and tannic acid. The first fixative decreases protein and phospholipid extraction; the second one reveals ultrastructure of microtubules.

The chemical fixation depending on its sample type and size can be performed in four various ways: by in situ fixation (applied to cell cultures), by immersion (small pieces of tissues are carefully excised and immersed in fixative as soon as possible), by vascular perfusion (the fixative is pumped through the vascular system of deeply sedated animals) or by vapours (the small delicate specimens, such as membranes, are suspended over a solution of osmium tetroxide). Usually, in standard conditions, fixation is carried out by immersion method. After fixation with buffered fixatives, the sample is dehydrated in increasing concentration of a solvent (a combination of either alcohol or acetone with propylene oxide) to enable infiltration with a liquid resin. An epoxy resin is most commonly used in conventional TEM. Finally, supersaturated and surrounded by resin, the sample is polymerised by applying gentle heat. Cured resin block containing biological material is thinly sectioned (40–150 nm), and subsequently post-stained with heavy metal salts, such as uranyl, and lead in order to introduce contrast inside the sample.

Previous reports revealed that chemical fixation, dehydration, heavy metal staining and plastic embedding can introduce various artefacts. Fixation with GA 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 [24,29,30]. To sum up, artefacts introduced during conventional sample preparation limit the resolution of biological samples to about 2 nm [20]. Further, 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 [31]. Therefore, the improvement of biological sample preparation technique has become the challenge for many scientists, and nowadays, some interesting alternatives are available.

On the other side, it should be stressed that TEM with conventional preparation is still an essential tool in many fields of tissue and cell biology, as well as in medicine. Where the approach is quality control method or gold standard to complement, support or confirm the results of specific histopathological diagnosis (neoplastic, renal, neuromuscular, infectious, hereditary and metabolic diseases) [32,33]. Therefore, the advantages of the conventional sample preparation should be emphasised, starting from simplicity of this fixation technique. There is no requirement for any specialised equipment; indeed, simple vessels with fixatives are sufficient. At the same time, numerous 1-mm^3-in-volume blocks of tissue can be fixed; thus, large areas of sample are accessible to analysis. When the tissue autolyses quickly, organs are too large or dissecting is difficult, chemical fixation is carried out by perfusion. Appropriate optimisation of fixation parameters, i.e. fixative concentration, buffer pH, temperature of fixative and time fixation, results in optimal ultrastructure preservation. Therefore, the unique advantage of conventional fixation is its ability to fix human tissue biopsies and study different animal organs without biopsy need.
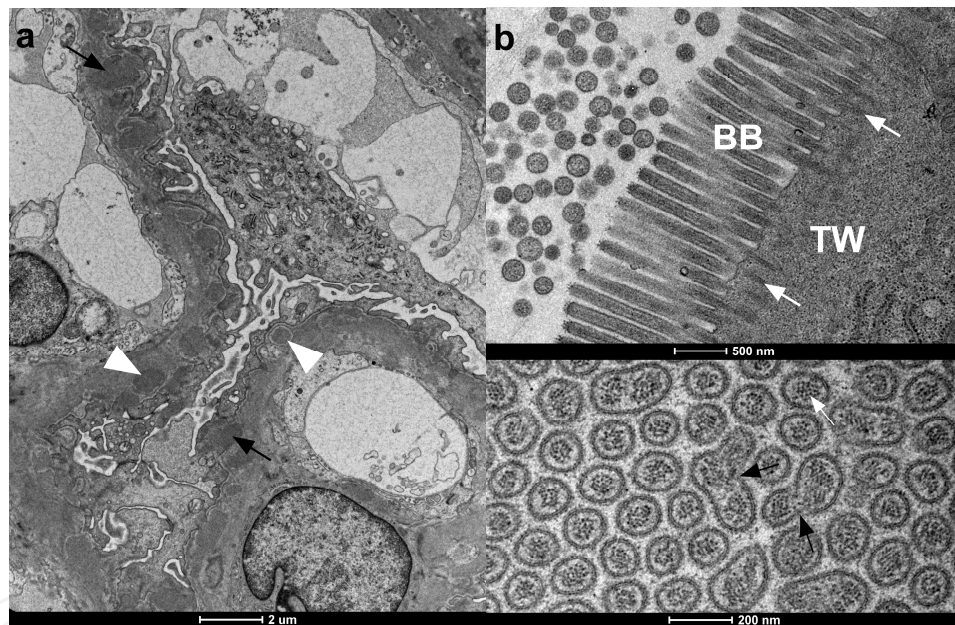

Figure 1. (a) Example of membranous glomerulopathy. Electron photomicrograph shows large amounts of electron-dense deposits in the glomerular basement membrane (black arrows); the sub-epithelial deposits are covered by a bridge of newly formed glomerular base membrane (white arrowheads). The foot processes of the epithelial cells are obliterated. In the cytoplasm of the epithelial and endothelial cells are numerous organelles and vacuoles. **(b)** Electron micrograph chemically fixed and an Epon-embedded rat's small intestine. Ultrastructure of the brush border (BB) and terminal web (TW) region of an enterocyte. Notice the actin filaments (white arrows) that descend from each microvillus deep into the underlying terminal web; below, organelles are visible. Lower part: transverse section through brush border showing numerous microvilli containing actin filaments (white arrow) and fusion of microvilli (black arrows).

In particular cases, pre-fixation with aldehydes is a prerequisite for further sample processing (see Sect. 8). Another advantage of chemical fixation is the possibility to perform sample preparation in different locations, e.g. in an operating room, in a laboratory other than EM lab and in the natural environment.

Adding to this knowledge about artefacts that are introduced during sample preparation, these advantages constitute strong position of conventional TEM in modern science. To prove this statement, some examples are presented. The conventional sample preparation in ultrastructural pathology is often irreplaceable (Figure 1a), as evidenced by different books [32,34]. A human biopsy from a patient with mitochondrial cardiomyopathy may serve as another example, where pathogenic giant mitochondria probably are formed to compensate effects caused by mutation of mitochondrial DNA [35]. Also, some interesting discoveries at scientific ground are contributed by conventional TEM. Among examples, electron microscopic analysis of a spherical structure of mitochondria is quite interesting [36]. Conventional sample preparation combined with ET revealed that mitochondria under oxidative stress were able to undergo a structural transformation in spheroid form. This novel mitochondrial dynamic process is probably involved in some pathological conditions; however, further study is needed.

Key events in the history of conventional TEM in the life science	
1932	Ruska and Knoll built the first transmission electron microscope
1932–1934	The first images of unfixed biological material were obtained (the wings and legs of a housefly)
1939	Ruska obtained the first bacterium and virus TEM pictures
1943	Sjöstrand built the first ultramicrotome and developed a method of producing ultrathin tissue samples which were used to study the skeletal muscle. Rapid development of ultramicrotomy since 1948
1945	Porter, Claude and Fullam imaged whole eukaryotic cells that were fixed in osmium vapour and then dried
1949	Newman et al. introduced methacrylates which became the first embedding media of quality for TEM samples
1950	Latta and Hartmann used glass knife in ultramicrotomy
1952	Palade recommended buffered osmium fixative for the preservation of cell ultrastructure
1956	Fernandez-Moran used for ultrathin sectioning a diamond knife
	Potassium permanganate fixation was introduced by Luft
1956–1982	Introduction of new resins for electron microscopy: Araldite (1958), epoxy resin (1961) and Lowicryl media (1982)
1958	Kellenberger used UA to stabilise viral and bacterial DNA
1959	Singer employed ferritin coupled with immunoglobulins to recognise the location of the antigen of interest
1963	David D. Sabatini introduced the aldehyde fixation; previously used fixatives were replaced by double fixation with glutaraldehyde and osmium tetroxide. This fixation strategy revolutionised the field of biological electron microscopy, and it is still the method of choice for many laboratories nowadays
1963	Reynolds used lead citrate as electron-dens stain in TEM
1966	Graham and Karnovsky developed a cytochemical method for horseradish peroxidise localisation
1971	Faulk and Taylor introduced colloidal gold labelling technique for ultrathin sections

Telocytes are a new type of interstitial cells characterised by the presence of telopodes, visible only by TEM [37]. These cells can be found in most organs, including the heart. The recent study showed the new type of extracellular vesicles released by heart telocytes that probably are an essential component in the paracrine secretion and may be involved in the heart physiology and regeneration process [38]. The combination of classical TEM and cell culture is an unbiased approach to identify unrecognised pathogen, provide the first clue about investigated pathogen and guide further laboratory study and epidemiology [39]. Another example is ultrastructure of the glomerular filtration barrier in the kidney [40]. ET in combination with scanning electron microscopy (SEM) confirmed suggestions that the glomerular filtration barrier comprises five layers instead of three. This discovery should allow for deeper understanding of kidney physiology and diseases.

Although conventional TEM is also appropriate for immunogold labelling, we leave this approach out. For more information about immunogold labelling on resin section, refer to Chapter **X**.

4. The Tokuyasu technique

Cryosectioning according to Tokuyasu is one of the most reliable and sensitive immunolocalisation techniques for different types of sample. It was introduced in the 1970s by Tokuyasu, using a cryo-ultramicrotome developed by A. Christensen [41], although the first attempts at cryosectioning were pioneered by Fernandez-Moran in 1952 [42] and further developed by Bernhard and Leduc [43]. Similarly to the conventional specimen preparation techniques, the biological material is first chemically fixed with aldehydes at low concentration. Instead of dehydration with organic solvent and resin embedding, the fixed material is infiltrated with sucrose, subsequently frozen in liquid nitrogen and sectioned with a dry knife at low temperature. What is interesting is in the past, it was suggested that wet sectioning based on water mixtures with sucrose, dimethyl sulfoxide or glycerol may resemble conventional resin ultramicrotomy. This idea dies a natural death because of limited temperature range for optimisation of the cryosectioning and chemical properties of proposed mixtures [44]. Obtained cryosections are retrieved from the knife surface, thawed and placed either onto microscope slides for light microscopy applications or electron microscopy formvar-carbon-coated grids. Such prepared cryosections are ready to perform immunolocalisation step on both fluorescence microscopy [45] and EM level [46]. For the latter, immunolabelled cryosections are stained with UA, embedded in methylcellulose to prevent drying and cell organelle irregular shrinkage artefacts and examined in the TEM [41,47]. The final step for fluorescence microscopy is mounting the coverslip with immunolabelled material on a slide with a drop of mounting medium before examination.

At a recent time, a new and improved contrasting procedure called 2+Staining was introduced for Tokuyasu cryosections for correlative approach [48]. 2+Staining consists of contrasting immunolabelled sections with 1% osmium tetroxide, 2% UA and lead citrate and followed by embedding in 1.8% methylcellulose. In comparison with the Griffith method, where membrane

contrast is excellent but fluorescence signal on the section is strongly reduced or lost altogether [49], the introduced procedure yielded a positive contrast of the cellular organelles and membranes, as in the contrast obtained from stained resin-embedded sections. The 2+Staining procedure shows little to no effect on the signal of the fluorescence labelling on the sections.

In comparison with conventional technique, the sucrose infiltration step dehydrates chemically fixed specimen in lesser degree. Thus, the advantage of thawed cryosection labelling over resin sections stems from the preservation of a natural hydrophilic environment. Moreover, resin sections are created with a polymeric cross-linked matrix; thus, antigen accessibility is lower and mainly restricted to the resin surface. In consequence, thawed cryosections enable better access to the antigens, particularly rare ones, for the probes and high detection sensitivity [50]. Over the years, the technique has been further improved by Tokuyasu and his colleagues [51–54]. These improvements become basis for the future research on various biological organisms and hybrid technique developments [48,55–59] including correlative approach [4,48].

5. Vitrification as an alternative to conventional fixation

An alternative way to deal with water is keep it inside the biological specimen by cryofixation. Cryofixation is based on vitrification that is not fixation per se, because vitrification is a physical process of the solidification of a liquid into noncrystalline or amorphous solid known as glass, usually induced by rapid cooling [60]. Water molecules do not have time to start to crystallise because heat is extracted from the system with high efficiency. Accordingly, term fixation cannot be understood in a chemical sense, where covalent bond is created. In the case of water, its vitreous state can be seen as liquid water with extremely high viscosity. Hence, cryofixation of biological matter allows instantaneous immobilisation of all cell constituents in their current positions which translates to obtain a true snapshot of the cell at the moment of ultrarapid freezing [61]. To précis, diffusible ions and molecular components are not shifted or extracted from the cell [62–64]; ultrastructural morphology is preserved close to the nature state, and rapid physiological processes can be characterised in a precise point in time.

When cooling rate is not sufficient, the formation of crystalline water destroys cellular ultrastructure. Another anomalous property of water is its increasing volume during crystallisation. Water freezing is not the reverse process of ice melting [65]. The freezing process is complex and influenced by nucleation centre, crystal growth and cooling rate. Slow cooling rate at ambient pressure results in hexagonal ice, whereas cubic ice forms under faster cooling rate, especially in the presence of nucleation centre. However, liquid water below its melting point is supercooled water. Liquid water is easily supercooled down to about -25 ºC and in 9-µm-diameter tiny droplet, down to about -46 ºC [66] and -92 ºC at 204,8 MPa [60]. Below these temperatures, crystal formation rapidly takes place as supercooled water has to go through the nucleation process by itself, generating nucleation centre distributed uniformly over the phase. In the end, ice crystals are formed. In the context of biological matter, cellular interior is a complex solution of different soluble materials, such as ions, proteins and sugars that

interact with the water molecules, thus changing water freezing behaviour. The presence of salts and hydrophilic solutes increase the extent of supercooling by lowering the freezing point of the solution. Furthermore, increasing solution concentration increases the temperature at which water becomes vitreous. In general, there is lack of heterogeneous nucleation centres inside the cell, and cellular solution concentration is usually higher compared to the surrounding medium. For these reasons, freezing starts somewhere in the external medium or on the surface where cells or tissues are placed. Growing ice crystals outside the cell are avid for any water molecules, including intracellular water, resulting in osmotic pressure changes and eventually bursting of the membrane [67]. Finally, biological matter after freezing consists of ice crystals and dehydrated solution between ice ramifications. Therefore, the water in the cell and around it should always be vitrified to avoid deleterious effects of ice formation. The mentioned issue underlies the success of the Tokuyasu method, where cryoprotected chemically fixed sample is vitrified by dropping it into liquid nitrogen. Nevertheless, the time when vitrification becomes more understandable and easier to perform had yet to come.

The preparation of biological samples by vitrification process is relatively a new technique. Until 1980, it was thought that water cannot be vitrified because of its thermodynamic properties. However, the big breakthrough came in 1980 when Mayer and Bruggeler had used X-ray diffraction to prove that small water droplets can be vitrified [68]. One year later, Dubochet and McDowall published an article where they demonstrated that small droplets of water or aqueous solution can be vitrified on electron microscopic grids [69]. These discoveries marked a turning point in biology, especially structural biology, and changed the point of view on water in electron microscopy from being the foe to being the best friend of electron microscopists [70]. The vitrification process depends on biological and physical factors such as the thermal diffusivity, the thickness and solute concentration of the sample as well as the cooling rate and the pressure applied. Nonetheless, the cooling rate seems to be the most important factor and decides which technique should be used [60]. Different preparation techniques are available for different sample sizes and the microscopy approach.

5.1. Plunge freezing

The simplest way to obtain a vitrified sample is through the so-called bare grid method [71] or plunge freezing (PF). Typically, vitrification is done by an immersion of small biological objects within a thin water film into properly chosen cryogen. After vitrification, the sample is directly imaged in a frozen state under a cryo-electron microscope. Under atmospheric pressure, cooling rate as high as 10^8 K/s is possible, at least in theory [72]. The successful vitrification obtained by PF is strongly dependent on properties of the grid, the temperature and nature of cryogen and the environment of the sample. The cryogen of choice is ethane, cooled to their freezing points by thermal contact with liquid nitrogen [71]. However, the usage of the mixture composed of propane and ethane has two advantages over pure ethane. Firstly, pure alkane is solid at 77 K, whereas the above mixture remains as a liquid at this temperature. For that reason, ethane-propane mixture does not require repeated cooling and warming cycles in order to ensure proper vitrification conditions. Secondly, the mixture produces less damage to carbon layer on the grids and to specimens mounted on C-flat holey carbon grids [73]. In

addition, the surrounding environment has an influence on the success of the final vitrification. In the case of thin films, usually 100 nm thick, prepared before plunging, sample is subjected to evaporation and heat transfer processes. In consequence, sample is exposed to risk of concentration, pH, ionic strength and temperature changes. Therefore, a new method was devised by Bellare et al. [74] and further improved by Frederik [75], who has constructed a new robot for sample vitrification by this technique. Plunge freezing can be applied to viruses [76], bacteria [77,78], isolated protein complexes [79,80] and whole cells [81,82], however, only 10 µm thick. In the last case, the flat thin periphery of cells [81] or whole thin cells, e.g. *Ostreococcus* [83] and *Plasmodium* sporozoites [84], is directly observed.

Although high cooling rate values are obtained during vitrification by PF, this technique is suitable for samples as thick as few micrometres. The reason is poor heat conduction properties of water because even an infinitely high cooling rate applied at the sample surface rapidly decays within the sample. Thicker-sample vitrification is only possible when cooling rate is reduced under particular conditions [60,85].

5.2. High-pressure freezing

The high-pressure freezing (HPF) technique was developed in the 1960s by Moor and Riehle [86], but as a routine laboratory technique, it became available from the mid-1980s. This freezing technique is based on Le Chatelier's principle and on the fact that physical properties of water can be manipulated in some range. Water during crystallisation expands its volume and forms crystal. According to Le Chatelier's principle, the increase in ice volume can be hindered by external high pressure which suppresses ice nucleation and ice crystal growth. Hence, high pressure is physical cryoprotectant which additionally changes the physical behaviour of water. High pressure lowers both the melting point and the freezing point, with the effect that at 2,048 bar, the melting point of water reaches its minimum of -22 ºC. At the same time, the possibility for supercooling is expanded, and the freezing point is shifted to -92 ºC [87]. Consequently, less heat is produced by crystallisation and less heat has to be extracted from the sample during freezing. In practice, it means that only several 1,000 K/s are required to vitrify biological matter at 2,048 bar [85]; thus, the sample thickness that can be vitrified increases tenfold, up to 200 µm [88]. Vitrification process during high-pressure freezing is obtained by pressurising the sample at 2,048 bar and subsequent rapid cooling at -196 ºC using liquid nitrogen jets within tens of milliseconds [72]. However, in very special cases, if the concentration of intrinsic cryoprotectants is high, samples even as thick as 600 µm can be vitrified [89,90]. Usually, the space around samples during vitrification has to be filled with appropriate filler. The role of the filler is to effectively transfer heat across the sample as well as cryoprotection during cooling process. Therefore, an important issue is physiological compatibility with the cells or tissues [67].

Except that HPF technique enables vitrification of thick samples, this technique has evident limitations. Firstly, gaseous compartments collapse at a high pressure. To counteract this phenomenon, intracellular air-filled spaces (e.g. lung tissue, plant leaves) need to be filled with a chemically inert solvent not mixable with water [91,92]. Secondly, high pressure solidifies the lipid bilayers and leads to a change in biological membrane structure [93]. Similarly, cholesteric liquid crystal of DNA cannot be retained by using HPF [94]. The

variety of samples prepared by HPF is enormous, and in the literature, there can be found numerous articles about the advantage of cryoimmobilisation over chemical fixation. Naturally, some exceptions to this rule exist. The HPF technique is less successful for labile tissue, such as brain or nerve tissue, where excision of tissue can last for quite some time, which results in anoxia and osmotic effects [95]. The inner ear tissue is another example of difficult to preserve sample by HPF [96]. In the case of stereocilia, which are mechanosensing organelles of hair cells, the actin core had a distorted appearance after freezing, described as 'tangled'. Fortunately, a hybrid technique resolves this problem and amplify the strength of HPF as cryopreparation technique (see Sect. 8).

The handling time during sample preparation is crucial because short time enables to obtain sample quality close to the native state. This requirement has brought several interesting solutions for sample preparation step prior HPF. While whole organisms (e.g. *Caenorhabditis elegans*, *Drosophila* [67], zebrafish embryos [97]) or human and animal cell culture is quite easy to load into the carrier, tissue sampling is more demanding. In the former case, cells are either directly loaded into the carriers or first cultured on chosen substrates (e.g. sapphire coverslips [98], Aclar disc [99] or gold grids [100]) and subsequently transferred to the live carriers and vitrified. For different tissues, short handling times can be reduced by using a rapid microbiopsy system. The microbiopsy system allows to accomplish the excision of a small sample and freeze it in less than 30 s [101]. Another solution for improving both the fast sample transfer and freezing reproducibility process is the rapid transfer system [102]. The principal advantage of this tool is the ability to correlate light microscopy and HPF with about 5 s time resolution [103]; thus, a catching of dynamic processes at known point and rare event localisation is possible. For faster cellular events, such as ultrafast endocytosis, flash-and-freeze approach was designed [104]. Flash-and-freeze electron microscopy combines optogenetics with HPF; viz. a brief single light stimulus is applied to the sample with subsequent freezing step. This tool can capture cellular dynamics with millisecond temporal resolution and nanometre spatial resolution.

5.3. Self-pressurised rapid freezing

Self-pressurised rapid freezing (SPRF) has been introduced in 2007 as a novel and low-cost cryofixation method. Sample is loaded inside the copper tube which is then clamp sealed at both ends and plunged directly into the cryogen [105]. The essence of vitrification by SPRF is isochoric freezing process. This technique is based on the fact that water expands upon freezing, and formed crystal ice builds pressure inside the confined volume of the tube. Thus, formation of internal pressure by hexagonal ice and/or supercooled water expansion supports the vitrification of the sample, at least in parts of the tube. Additionally, growing ice crystals close to the tube wall might concentrate the cryoprotectant in the centre and support vitrification [106]. Another factor that influence cryofixation is higher heat diffusion coefficient of crystalline ice, and this means a faster cooling system [107]. Although this explanation is still only hypothetical, the fact is that for vitrification by SPRF, lower concentration of dextran is required in comparison with HPF, thereby causing less osmotic changes [108]. The ultrastructural quality of vitrified specimens by SPRF and HPF is comparable, although on average, only 50% of the

structures will be vitrified and 50% will be in crystalline form. To prove the concept of self-pressurised vitrification, different organisms were used including bacterial strains, yeast cells, eukaryotic cell culture and *C. elegans* nematodes. In the case of eukaryotic cell culture, it was noted that the copper tubes are poisonous, even in a typical time range (30–60 s) during sample preparation prior vitrification. Thus, the physiological or 'close-to-native' state is definitely lost [106]. An aluminium container was proposed as a substitute for poisonous copper tubes, although authors suggested developments of new biocompatible coatings of the copper tube's internal surface. The reason was a slightly higher degree of crystalline ice volume in aluminium tubes. The necessity to prepare cell culture as a suspension is a different obstacle; therefore, although SPRF technique is cheaper and less laborious in comparison with HPF, this technique cannot be used in morphological research where cell adhesion is of critical point. More recently, SPRF was used to vitrify *Arabidopsis* inflorescence stems with subsequent cryosectioning in order to obtain architecture information about plant cell walls. This knowledge is another step to improve understating of biofuel plant material and to rationalise reengineering of second-generation lignocellulosic biofuel crops [109].

6. Post-freezing processing

After vitrification, water within the samples is amorphous as long as samples are stored below devitrification temperature (<-136 ºC). Further processing of the sample depends on its size and aims of the study. The electron beam penetration limit in TEM is about 1 μm. Therefore, many different small-enough samples, such as macromolecular complexes, viruses or bacteria as well as subcellular structures at a periphery of the cell, can be imaged directly under cryo-TEM. Water inside a column of TEM evaporates, unless a special cold stage holder is used at temperature close to that of liquid nitrogen. At this temperature, water evaporation rate is almost negligible and vitrified material can be observed without loss of image quality [110]. However, thicker samples require distinct follow-up procedures – specimen sectioning is the basic need. Vitrified material can be sliced at a temperature below devitrification point into ultrathin sections and subsequently analysed under cryo-microscope (CEMOVIS). As another option, the focused ion beam technology is adequate for the purpose of thinning vitreously frozen biological material. Besides application of pure cryotechniques, specimens can be prepared by cryo-to-room temperature techniques.

6.1. CEMOVIS

In principle, cryo-electron microscopy of vitreous sections (CEMOVIS) technique is as simple as possible. The first step is vitrification of biological material; then vitrified material is sectioned in a cryo-ultramicrotome. Frozen sections are transferred to a grid, and subsequently the grid is imaged in a cryo-TEM under low-dose condition [111]. Critical issue leading to final success is hidden in performing all these manipulations below devitrification temperature. In spite of the fact that CEMOVIS seems to be a simple technique, the modern state of this approach was strongly connected with indispensable laboratory work and technological development of electron microscopy field.

In the first cryosectioning-based studies [42,43], samples were freeze-dried before analysis under electron microscope. Another important development was sectioning of unfixed biological material at -150 ºC [112], together with transfer and observation of frozen-hydrated specimens in the transmission electron microscope [113]. Similar to the previous studies, also in these works, obtained sections were not analysed as vitreous specimens.

However, the first successful trails in cryosectioning of vitreous material were built on precursors' work and existing cryo-microtomes and took place in the eighties of the last century [111,114]. Obtained good results were not reproduced each time, and they were difficult to extend to other specimens [70]. It took another 20 years, where new vitrification techniques were established, and scientists obtained better knowledge of the vitreous state of water and cryosectioning method was optimised. Although promising results have been obtained at the beginning of this century, 2004 was a pivotal year because CEMOVIS has reached maturity. Since then, CEMOVIS is regarded as a routine laboratory technique, at least by some researchers, and reveals the native state of cells and tissues with remarkable quality and resolution [115].

Frozen-hydrated sections are prepared without any additional purification, fixation, dehydration and staining steps. As a result, all cellular components are immobilised inside vitrified water, with preservation to the atomic level [116]. During vitrification process, using HPF or SPRF, usually a 20% solution of a high-molecular-mass dextran is used. Addition of dextran to the sample mimics the vitrification properties of typical cells. Consequently, surrounding environment of the cells is vitrified and thus eliminates possibility of crystalline ice formation. Hexagonal or cubic ices make the sample brittle for sectioning. From another point of view, addition of dextran polymer ensures better sectioning of the sample [117]. In spite of the fact that observed specimen remains fully hydrated, unstained and close to the native state, it is not absolutely free of artefacts.

Vitreous water is a liquid with very high viscosity. This viscous nature of water entails difficulties during cryosectioning process and results in cutting-induced deformation. Resin-embedded and vitrified materials differ in cutting properties. During the cutting process of resin-embedded material, obtained thin sections from diamond knife are subsequently straightened during floating on the water surface through a high surface tension [118]. The required liquid for CEMOVIS techniques probably does not exist [117]. Therefore, a dry diamond knife is dedicated for cryosectioning of vitreous samples. The absence of liquid to float vitreous sections results in increasing interaction between the forming section and the knife [118]. These factors results in the formation of cutting artefacts such as chatter, compression, knife marks and crevasses [119]. In fact, sectioning of vitreous biological samples is technically difficult, but what is more important is some of the artefacts can be eliminated to some extent [119,120]. For example, knife marks are reduced through the use of undamaged diamond knife and through elimination of frost and debris at the edge of the knife [121]. The humidity inside the chamber of the cryo-ultramicrotome can be reduced to below 1% by using a protective glove box surrounding the cryo-ultramicrotome. It ensures that cryo-tools remain clean from frost contamination during vitreous sectioning [122]. The forming section flows during cutting process, thus applied force yields in different deformations. Crevasses are

fractures on the surface of the section and they are more severe in thick sections and high cutting speed [115,119]. To minimise this artefact, sections should be thick but less than 70 nm. Chatter is defined as a periodic variation in section thickness along the cutting direction and depends on the gliding properties of the knife surface. It can be minimised by increasing cutting speed [117,123]. Chatter is associated with irregular compression which is considered as the most prominent cutting artefact. Compression is formed due to irregular friction of the knife surface, and it makes the section shorter along cutting direction compared to the length of sample's block face. The main problem associated with compression is its heterogeneous nature which is discerned at cellular and molecular level [119,124–127]. Microtubule may serve as an example [124,125]: very often microtubules were observed as noncircular-shaped structures in case when long axes of these structures were not perpendicular to the cutting direction. What is more interesting, microtubules that were close to each other did not always display the same degree of deformation. A more detailed explanation can be found in [125]. Compression is reduced either through the use of low-angle knives [128] or through increasing the cutting thickness. Each sample is different, and different combinations of artefacts are possible. For that reason, it is important to find appropriate sectioning conditions to obtain optimal results [126]. Another solution is application of an oscillating knife to reduce cutting-induced artefacts of vitreous sections [120].

Probably the most difficult step during cryo-ultramicrotomy is transfer of vitreous ribbon of sections onto the carbon grid. To accomplish this step successfully, different approaches have been developed. One of the solutions is micromanipulator to hold and control the vitreous cryosections by eyelash when they come off from the knife edge. During the entire process, the ribbon is under constant tension as it grows longer, thereby keeping the ribbon as straight as possible. When the appropriate length is obtained, the ribbon is attached to the grid surface by lowering the micromanipulator and holding it in optimal position while a second eyelash is used to affix the other end of the ribbon to the grid surface from the knife edge. It is possible to affix a few ribbons to a single grid. However, the discussed solution is time consuming and prone to ice contamination [129]. The ribbons on the grid can be flattened by pressing with tools. The aim of this step is to reduce the probability of losing the sections during storage and transfer the grid, as well as improve the stability of section under electron beam [117]. Another solution is electrostatic charging for attaching the sectioned ribbon to the grid [122]. In comparison with micromanipulator solution, this method increases the successful attachment of frozen-hydrated sections to the carbon film, albeit both methods cannot guarantee uniform attachment of cryosections to the carbon film. This results in higher sensitivity of the section to the beam exposure and section movement during image acquisition, especially during electron tomography [123]. Recently, a new tool based on an aforementioned solution was presented [130]. One of the micromanipulator is used to manipulate the section ribbon by electrically conductive fibre; the second one positions the grid beneath the newly formed ribbon, and with the help of an ioniser, the ribbon is attached to the grid. This tool greatly facilitates manipulations, but sectioning artefacts remain. In summary, although much is known about CEMOVIS procedure and its artefacts, there is no remedy so far for discussed limitations. For this reason, CEMOVIS is still far from a routine application and general use.

6.2. Focused ion beam milling of vitreous samples

Focused ion beam (FIB) is an alternative method for sample thinning, free of artefact characteristic for CEMOVIS. This technique is widely used in material science; however, Marko et al. [131] proved that FIB milling can be applied in preparing biological material. FIB milling of vitreous samples is conducted using a dual-beam microscope. The dual-beam microscope is a combination of FIB system and scanning electron microscope. During FIB milling of vitreous sample, a finely focused beam of ions, usually gallium, is used to ablate the surface of the specimen through sputtering process. The whole procedure is under visual control of the SEM to ensure optimal procedure of sample preparation [132]. However, direct interaction between the ion beam and vitreous material must be taken into consideration due to possible sample damage. The application of a gallium ion beam with current of 10 pA and 30 kV acceleration does not cause sample devitrification [131]. Moreover, interaction of FIB with vitreous sample results in implantation of an ion layer, as thick as 5–20 nm into the FIB milling surface [133]. Indeed, the thickness of implanted gallium layer is almost negligible in a vitreous specimen with a thickness of 100–300 nm. Furthermore, the ion layer is much thinner in comparison with crevasses found in vitreous sections.

Cryo-FIB micromachining is a relatively new technique and remains in its early stages. Nevertheless, few sample preparation strategies have been introduced in the last 10 years. To date, bacteria and small eukaryotic cells, like *Mycobacterium smegmatis*, *Saccharomyces cerevisiae* and *Dictyostelium discoideum* [134,135], *Escherichia coli*, HeLa cells [136,137], BHK-21 cells [138] and *Aspergillus niger* [139], are deposited for culturing on the TEM grids and vitrified by plunge freezing technique. Next, vitrified material is transferred into the dual-beam microscope for a thinning process with a precision in the 10–100 nm range. At this stage, different FIB milling strategies for vitrified cellular samples are possible. The optimal geometry for small prokaryotic cells is wedge shaped, where frozen material is sputtered away at an oblique angle (~10°) with respect to the plane of the grid. Consequently, wedge-shaped vitreous material can be imaged with up to 3 µm transparency length and a thickness gradient less than 400 nm [134]. Eukaryotic and other cells similar in shape and size are milled in a thin self-supported membrane. During this process, a specific region of interest is localised and then rectangular sector below and above the selected volume is sputtered away, leaving behind a thin membrane, commonly referred to as a lamella, supported by the surrounding unmilled cells and ice [135]. Another option to obtain lamellas is a traditional FIB lift-out method, although it was deemed impossible because of the difficulty in obtaining platinum deposition at cryogenic temperatures [139,140]. Shortly, after vitrification, the feature of interest is defined through SEM; next the sample is cryo-coated with platinum (Pt) and two trenches are milled on each side of the lamella to be extracted. In the next steps, the sides and bottom of the lamella are sputtered away, and by using cold nanomanipulator, lamella is then lifted out from the sample and finally attached to the TEM grid by cryo-Pt deposition. During the last step, the attached lamella is thinned enough to be transparent to electron beam. The lamella-based sample preparation has an advantage over the wedge-shaped strategy, because the simple ablation geometry would not permit to easily find and target structures of interests embedded deeply

in cellular volume [135,136]. Thick samples and suspension of cells, e.g. *S. cerrevisiae* [140,141] and muscle tissue [142,143], are vitrified in copper tube or dedicated carriers via high-pressure freezing. After that, material hidden inside both the tube and the carrier is exposed during pretrimming step inside the cryo-ultramicrotome. Subsequently, vitreous sample is transferred to the dual-beam microscope and milled according to H-bar strategy, ultimately resulting in lamellas with the required thickness and surface area. Finally, prepared sample is transferred to the cryo-TEM in order to perform visualisation under low-dose mode. Critical point is when each of the described steps must be carried under devitrification temperature and minimising frost or warming during transfer steps implicates customised transfer device introduction. For samples vitrified by plunge freezing, different cryo-FIB transfer stations and cryo-FIB shuttles were introduced [135–137], while high-pressure frozen samples require sophisticated and complex transfer systems like cryo-nano-bench system [140] or an intermediate specimen holder [142,143].

The cryo-FIB technique provides controlled access to specific supramolecular structures buried inside the cell. Moreover, many macromolecular complexes that are present in low copy numbers may be studied in their native cellular environment because homogenously thick lamellas with more than 100 μm^2 areas can be prepared in a controlled and targeted manner. However, this preparation technique is low throughput due to several factors. As it was mentioned, an incidence of the ion beam should be as low as possible because a smaller milling angle produces a larger viewing area for analysis and minimises the deposition effect of milled material. The latter factor is not interrelated with milling currents or other parameters during sample thinning as such [135,137]. Inhomogeneous and varied composition of the vitreous samples is the main cause for curtaining effect which finally results in strong inhomogeneous lamella thickness [134,142]. These effect is reduced by deposition of organometallic platinum with a gas injection system without electron or ion beam radiation, prior to lamella preparation [144]. Another issue is the amount of information obtained from prepared samples during cryo-electron tomography. During cryo-FIB milling process, part of the vitreous specimen is physically destroyed along all axes [134]. In contrary, after vitreous sectioning, a series of cryosections is obtained, and information along these axes is partly remained, especially along *z*-axis. A further problem is the time needed to prepare sample prior to visualisation. Due to the large size of eukaryotic cells, longer milling time is required in comparison with the prokaryotic samples [135–137]. Other challenges related to the increased size of eukaryotic cells are identifying and targeting specific sites for processing. Small organisms, such as *E. coli*, are readily distinguished from the vitreous ice. Inversely, eukaryotic cells are surrounded by thick ice, thus identifying the area of interest is not simple. A method to overcome this limitation is either milling of many adjacent places to find features of interest or application of correlative light and electron microscopy techniques. Cells are cultured on EM finder grids and optical images are recorded before vitrification. Appropriate regions are selected for subsequent cryo-FIB milling based on light microscopy photos [136,145]. Alternatively, the frozen-hydrated sample is imaged under cryo-fluorescence microscopy before sample preparation by cryo-FIB for further analysis. The second approach allows direct correlation of the prepared vitreous sample between the two imaging modalities [134,138,146]. Moreover, localisation of smaller molecules or structures that exist in low copy number is simplified because targeting is based

on clonable labels, such as green fluorescent protein [134]. The new idea presented recently is to localise structure of interest by fluorescent labels using cryo-light microscopy and then use it for coordinate transformation-based approach in the FIB-SEM system for milling [138].

At present, cryo-FIB milling of vitreous samples remains cumbersome and far from routine [143]. Much effort is required to improve the efficiency and repeatability of cryo-FIB milling process, such as reproducibility of lamella quality, i.e. overall thickness and thickness homogeneity, localisation feature of interests or better avoidance of frost during cryo-transfer steps. Therefore, the main issue in the presented articles is improving sample preparation protocols. Nevertheless, some interesting results have been achieved. For example, ten nuclear pore complexes in *D. discoideum* cells were identified in situ in one tomogram. Subtomogram averaging process yields the structure of nuclear pore with resolution of 7.9 nm. To achieve 6 nm resolution, 4,182 protomers are required from isolated nuclei using the same type of analysis [135]. Another result comes from bacterial cells, where membrane invaginations into both the cytoplasmic and periplasmic spaces of *E. coli* were found [136]. This technique will greatly facilitate high-resolution imaging of dynamic process, such as HIV particles travelling into the deep side of the host cell at different stages of infection, especially when viral capsid interacts with nuclear pore complex components [136]. By using FIB milling process, ryanodine receptors in toadfish swimbladder muscle were determined. Obtained results agreed remarkably well with those described previously, albeit further study will be required to understand structural features of ryanodine receptor connected to the T-tubule [143]. Cryo-FIB is also a promising thinning tool for describing new bacterial cytoplasmic structures termed as a stack. Stacks were defined as piles of oval disc subunits which are surrounded by a membrane-like structure. These structures are localised in the cell cytoplasm and are presented separately or grouped together in variable number within each cell. One can only speculate about stacks' function, but it is suggested that they could be related to the bacterial cell replication process. Due to compression created during CEMOVIS and visualisation limitations arising from plunged frozen samples, cryo-FIB technique could provide new insight into macromolecular assembly of membrane-enclosed discs [147].

6.3. Nature of vitreous material

The sample in native state is very different from what has been seen before with conventional microscopy. Vitrified material is as close as possible to the native state because during sample preparation, neither chemical fixation, staining nor dehydration is used. Therefore, the final images represent the real distribution of the immobilised biological material within vitrified water. With frozen-hydrated samples, the contrast is proportional to the density and distribution of molecular inhabitants within the thickness of the sections. Moreover, structures in vitreous material are equally visible over the entire thickness, thus the native-state inherent low contrast due to low signal-to-noise ratio. In contrary to stained material, imaging of native biological material relies on phase contrast, which strongly depends on focus [71]. An additional issue is plethora of overlapping information for the reason that the typical fine details are much smaller than the section thickness. The solution overcoming this limitation is both

preparation of thin sections or lamellas and electron tomography to obtain a three-dimensional model of the material distribution in the vitreous material. Frozen-hydrated specimens behave differently under electron beam in comparison with plastic sections. A characteristic phenomenon that may be developed is bubbling. Bubbling is a result of gas accumulation produced by electron beam decomposition of biological matter. What is the most interesting is that different substances have different electron radioresistance. Another problem is beam-induced deformations which are seen twofold. Vitreous material can be considered as high-viscosity liquid and can be rearranged by the electrons. On the one hand, sharp irregularities, such as crevasses and knife marks, under electron beam are removed from the vitreous section due to increasing the flow of the section. On the other hand, some biological structures, i.e. chromatin, aggregate under the effect of beam producing locally apparent higher-density area [148].

The main disadvantage of frozen-hydrated specimens is its uselessness to perform post-immunolocalisation of studied target. Antibodies require proper conditions for working, that is, ambient conditions and water solution. Accordingly, some researchers have developed a specific label for the identification of molecules for cryo-electron microscopy. These labelling techniques are base either on a clonable ferritin FtnA protein [149] or a biotin-streptavidin complex [150], called STAMP approach [151] and SNAP-tag technology [152]. However, ligand labelling for cryo-EM is still an emerging field; hence, another preparation technique is dedicated for immunogold labelling and structural studies, namely, freeze substitution. Indeed, vitreous and freeze-substituted materials are very complementary [123]. The latter preparation solution should be considered as the method of choice when high-resolution study is not the major aim. Resin-embedded material is easier to obtain and is less sensitive to electron beam in comparison with vitreous material, and for this reason, analysis of larger sample area is possible. Additionally, plastic sections can be thicker than vitreous ones, and thus, the former enable studying a larger volume. The other advantage of resin sections is the possibility of immunogold labelling.

6.4. Freeze substitution

Freeze substitution (FS) is a hybrid method that bridges the gap between vitrified material and room temperature ultramicrotomy of resin-embedded material. Biological material after vitrification process is gradually dehydrated in the presence of chemical fixatives at low temperature. Later, the whole process is gradually warmed, and finally, the sample is embedded in resin. This technique was first introduced in 1941, as a preparative technique for light microscopy samples [153]. The potential of FS at electron microscopy filed was explored by Fernandez-Moran [154] and was further developed by others [155–158].

Freeze substitution process consists of dehydration and chemical fixation step followed by either low- or room-temperature embedding in chosen resin. Dehydration process usually starts at -90 ºC in an organic substitution medium containing chemical fixatives. The key point is that sample must be kept below devitrification temperature. In case of biological material, this temperature remains well below -100 ºC. What happens during water substitution at low temperatures is not fully understood. Nevertheless, vitrified water turns into cubic ice, and then transition takes place into hexagonal ice. Cubic ice is a metastable state, thermodynami-

cally more stable form of water. The second transition process, from cubic to hexagonal form, occurs at around -80 ºC. The most important event during these transitions is that water molecules probably rearrange by rotation with only small transitional displacement which leads to embedding the biological structure by ice without any segregation. Then the result is that the structural preservation is excellent down to molecular dimension. In reality, FS process deals with cubic and sometimes with hexagonal ice but never with vitreous water [60]. This would imply that cubic ice has no significant influence on the observed morphology at the level of resolution of biological samples during FS process under controlled conditions. Besides these theoretical bases, other aspects must be considered.

From the biophysical side, low temperature influences on ultrastructure preservation through hydration shell preservation and infiltration of chemical fixatives. FS process preserves the hydration shell at least partially, although less hydrophilic organic solvents are used, e.g. acetone or methanol. It is well known that organic solvents cause protein aggregation, and chemical fixatives react relatively slowly; therefore, it cannot preserve all the cellular components simultaneously. The consequences of chemical fixation are seen as osmotic changes and redistribution or extraction different molecules, i.e. lipids and ions [85,157,159,160]. The reason for the superior structural preservation is infiltration of stabilising or fixative compounds together with the dehydrating agents. After raising the temperature, fixatives react in situ, between -90 ºC and -30 ºC, avoiding penetration and diffusion artefacts [157]. Many different substitution media compositions were developed. The main fixatives are GA, osmium tetroxide and UA used in different combinations and at different percentages in acetone, methanol or ethanol [67,161–164]. Acetone is the most commonly used dehydrating agent because it substitutes at a slower rate than methanol, thus resulting in better structural preservation [165, 166]. The most-used fixatives are OsO_4 with or without UA in acetone for morphological study and low concentrated GA in acetone for immunolabelling detection [167–169]. The interesting observation concerning reactivity of used fixatives was made. Osmium tetroxide at low temperature does not react as protease, but begins cross-linking, via cis-diol covalent bonding to unsaturated lipid chains at -70 ºC [170]. Uranyl acetate binds to proteins and phospholipids at an even lower temperature. Glutaraldehyde starts cross-linking at -50 ºC, but it acts readily only at or above -30 ºC [161]. For some immunolabelling study, pure solvent preserves well both the antigenicity and the ultrastructure of the cells [159,171]. A distinct feature of well-vitrified samples, without visible ice segregation artefact, is a 'reverse contrast' seen as pale membrane against a more electron dense background [172,173]. In order to improve the membrane contrast and preservation of cultured cells, a few different substitution media were introduced. The most interesting substitution medium contains 5% water in acetone [173]. It has been proved that substitution cocktails can include up to 20% of water without deleterious ice damage or extending dehydration time [174]. These protocols totally contradict the long-standing theory that a low amount of water as 1% in acetone extends freeze-substitution process four times [161]. A disadvantage of a medium containing water is antigen loss by extraction. Similar results were observed for pure solvents; thus, low concentration of glutaraldehyde can be generally used [175], but the exact substitution protocol requires an individual approach. Alternatively, membrane contrast can be enhanced by using tannic acid-mediated osmium impregnation method [176], tannic acid in acetone during FS [95,177] or different combina-

tions of glutaraldehyde, UA and OsO_4 [178,179]. An attractive protocol omitting osmium tetroxide is based on 20% Araldite/Epon in acetone as fixative in the first step and subsequent embedding in pure epoxide resin. Epoxide compounds react with proteins and lipids and provide interesting results which may become an important tool in getting information about influence of different reagents and protocols on ultrastructure preservation [180]. Current FS procedures are measured in wide time range from less than 24 h for cell culture [162,172,174,181] to longer period of time such as four days for plant material [182,183]. However, other tissues are usually substituted during 2–4 days, e.g. rat liver [184], mouse skin [185] or *C. elegans* [186]. On the one hand, such a wide range of different fixative cocktails gives opportunity to study different structures of interests in both structural and immunolocalisation research. On the other hand, the variety of possibilities become a challenging task for optimisation of FS process because each sample is unique [67].

The last step during sample preparation for the room-temperature ultramicrotomy is sample embedding. Epoxide-based polymers are dedicated for morphological analysis including electron tomography by virtue of a larger stability in the electron beam and ease of sectioning. In contrast to epoxide resins, methacrylates do not bind covalently to cellular structures; hence, the antigens of interests remain unaltered and section surface has higher roughness, thereby higher access to antigens. Another advantage of methacrylic resins is low-temperature embedding and polymerisation by UV light (Figures 2 and 3); thus, harmful heat effects on epitopes is avoided [157,165]. However, this division is not a rule, because Epon sections were used to identify the subcellular localisation of proteins [187], lipids [188] or carbohydrates [189]. Special attention should be directed to McDonald's FS protocols [168,190]. Super-Quick FS takes only about 6 h from freezing process to resin blocks preparation ready to section. Substitution process from -90 ºC to 0 ºC is performed during 2,5 h; then rapid infiltration in LR White or Epon resin takes another two hours followed by polymerisation at 100 ºC for 1,5–2 h. For this protocol, organisms considered as difficult to fix were chosen. As a result, presented ultrastructure preservation was comparable to standard FS protocols, and high-temperature polymerisation does not affect antigen preservation.

7. Sample examples prepared by cryotechniques

High-resolution study requires superior sample preparation via vitrification process. Sample such as protein, protein complexes, viruses, bacteria or organelles in vitro and in situ within whole organisms or single cell are prepared by plunge freezing. Moreover, in modern structural biology, the main goal is in situ structure determination within unperturbed cells because purified objects are disintegrated during sample purification. Thus, for structural biology at cellular and tissue level, thicker samples are prepared by CEMOVIS (30–200 nm) or cryo-FIB (100–300 nm) techniques. The visualisation of frozen-hydrated biological samples is performed by single-particle analysis or cryo-electron tomography. The former technique enables to achieve near-atomic resolution and is applied to purified viruses, macromolecular complexes and single proteins [80,191,192]. On the other hand, cryo-ET bridges the gap between cellular ultrastructure and the structural analysis of macromolecular complexes

within the cell with resolution in the sub-nanometre [193] to 10 nm range [194–196]. Electron tomography of plastic sections is another technique dedicated for cellular structural biology, where the more important aim is to reveal functional-morphological relationships than macromolecular details. Sample is prepared by cryoimmobilisation followed by freeze-substitution process. Next, polymerised specimen is sectioned in 100–400 nm range and analysed [194]. Combination of subsequent section tomograms extended the depth of analysed volume to several micrometres. These advantages led to large-scale imaging where both detail and overview are necessary. Another worth-noting point for ET study is a scanning transmission electron microscopy (STEM) mode. Due to lack of inelastic scattering of electrons and chromatic aberration in a STEM mode, resin sections as thick as 1 μm can be analysed [197–200]. Besides three-dimensional analysis, both frozen-hydrated and plastic sections are also analysed at lower resolution at 2D morphological level.

CEMOVIS has already provided unusual views of different structures with a molecular resolution in native cellular context including microtubules, mitochondria, Golgi apparatus [194] or desmosomes [116]. Combination of CEMOVIS with electron tomography, called TOVIS [201], becomes a powerful tool in virology [76], microbiology, cellular biology and tissues. The reader is referred to review articles that cover mentioned topics with further references [79,202–205] due to limited space. However, two examples of CEMOVIS application will be pointed out. Cryo-EM of vitreous sections gives opportunity to study different tissues, including skin biopsies. The unravelling of molecular organisation of the skin lipids will significantly improve molecular understanding of the tissue. Until recently, six theoretical models for the molecular arrangement of the extracellular lipid matrix have been proposed. Nevertheless, combination of TOVIS, molecular modelling and EM simulations has revealed a new model of lipid organisation, which rationalises the skin nature and functions [206]. These results will influence dermatology field and thereby further translates in technological developments of new, transdermal drug delivery systems, the development of noninvasive diagnostic sensors and dealing with toxicity from topical exposure to chemicals. Without CEMOVIS, it would be impossible to perform the simultaneous quantification of water and elements in the native state [63]. In combination with fluorescence microscopy, it was showed that the induction of nucleolar stress in cancer cells resulted in both an increase in water content and a decrease in the element content in all cell compartments. The presented study opens new way to understand cell functions, and future research could extend our knowledge about cell activities, depending on actual concentration of ions and the hydration status.

The classical examples of superiority of the cryopreparation techniques, based on HPF followed by FS process, over conventional TEM were showed on the method-dependent bacterial mesosomes [55] and articular cartilage [85], although in the former, further investigation led to revisiting the mesosome as a site of hydrogen accumulation [208] using quick-freezing preparation of TEM. Among the advantages of plastic sections, one is the section thickness; thus, comparatively large cellular volume can be analysed that was exemplified by microtubule cytoskeleton architecture in yeast [209], organellar relationship in the Golgi region of the pancreatic cells [210] or architecture of the caveolar system [211]. Besides, the reader can find many articles where either different techniques were compared [55,91,96,212–216] or examples were collected in reviews [194,217].
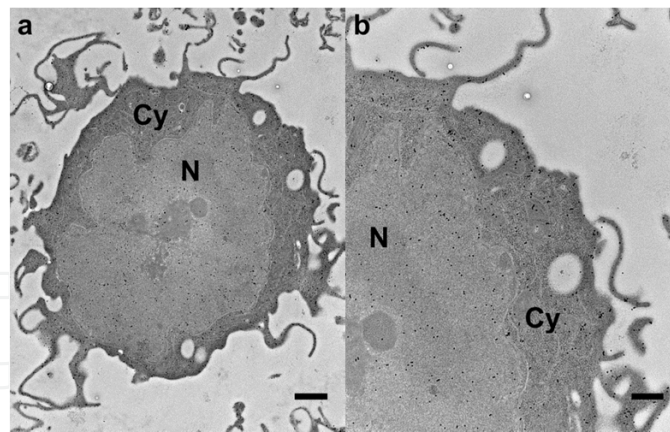
Figure 2. Ultrastructural demonstration of immunogold labelling of visfatin particles or small clusters consisting of number gold particles were demonstrated in the subcellular compartments of human colorectal HCT-116 mono-nucleated cells which were cultured in log phase of growth (Cy cytoplasm, N nucleus, Scale bars: a) 1 μm, b) 500 nm). Cells were chemically fixed, dehydrated and embedded in LR White resin [207]. Visfatin is an enzyme which overexpression is correlated with poor prognosis in cancer patients. In this study, we tried to explore the association between visfatin distribution in subcellular compartments and increased apoptosis in cells treated with cytochalasin B. For further study, our aim is to optimise of HPF-FS protocols for cytoskeleton ultrastructure. Figure and legend adapted and changed from [207] under terms of the CC BY 3.0 license.
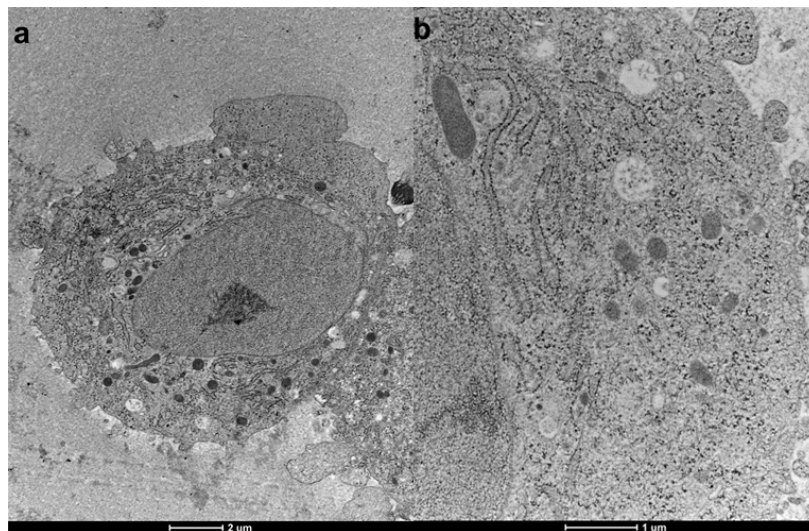

Figure 3. Human colorectal HCT-116 were vitrified by HPF followed by FS and embedding in LR White resin. During FS process, cells were fixed with only 0,25 % GA. In comparison with cells prepared by conventional method (Figure 2), vitrified and freeze-substituted material show outstanding ultrastructure preservation. Even without OsO_4 fixation, membranes are clearly visible, mainly rough endoplasmic reticulum and nuclear membranes. Nevertheless, further protocol optimisation is required.

8. Unity is strength: The hybrid techniques

Immunoelectron microscopy bridges the information gap between molecular biology and ultrastructural studies providing information regarding the function of the internal structures

of the cell. The main requirements to obtain valuable results are suitable fixation protocol and functional antibody conjugated with an appropriate nanoparticle to be detected. For many years different protocols were developed for structure localisation study [167] based mainly on Tokuyasu technique, HPF followed by FS and low-temperature embedding [169] or conventional chemical fixation [207,218] together with progressive-lowering temperature technique. Nevertheless, this field is still amenable to new solutions, especially for difficult-to-fix samples and difficult-to-fix antigens. The hybrid techniques combine advantages of different cryopreparation techniques in order to eliminate the particular step limitations of each one, at least in part.

Cryosectioning according to Tokuyasu is one of the most reliable and efficient immunolocalisation techniques for different types of sample. An inherent limitation of Tokuyasu cryosectioning is mild chemical fixation at the beginning of the sample preparation. Thus, small molecules, including molecules of interests, may be dislocated or extracted during chemical fixation. Besides, it should be mentioned that process of chemical fixation is selective and results in pH-related and osmotic changes in the different organelles [58]. Another restriction is intractability of samples, which contain a hydrophobic cuticle or a rigid wall, such as *C. elegans*, *Drosophila* or plants. Cryoimmobilisation should be used to overcome difficulties arising from chemical fixation during sample fixation. In spite of the all above-mentioned issues, one more should be considered – the nature of the antigen. Some antigens are sensitive on chemical fixation at room temperature or resin components and solvents and thus cannot be immunolabelled either in thawed cryosections or after cryoimmobilisation, freeze substitution and resin embedding. Therefore, the main aim was to introduce hybrid methods that combine the high-efficiency Tokuyasu cryosectioning labelling technique with an initial cryoimmobilisation step. Different approaches have been introduced with different results. The first attempts were taken by the group of Slot and Geuze [53,56] by combination of a frozen-hydrated cryosections with subsequent material fixation during thawing and after transfer to a grid. This method turned out to be unsuitable to routine use because, besides technical requirements and lack of reproducibility, only a small area of obtained sections has got desired morphological quality [56].

Another strategy to improve the antigenicity and ultrastructure preservation is rehydration method (RHM) based on cryofixation, freeze substitution and rehydration process before entering Tokuyasu cryosectioning and immunolabelling [56]. Sample after vitrification is dehydrated at low temperature with a substitution medium containing UA, glutaraldehyde and/or OsO_4 and water to improve membrane contrast. After freeze substitution step, a rehydration process is carried out on ice. Rehydration step is necessary to enter the Tokuyasu procedure, i.e. sample embedding in gelatine, infiltration with high concentrated sucrose and freezing in liquid nitrogen. During dehydration step, additional chemical fixation is performed because fixation during FS step turns out to be insufficient. This approach resulted in excellent preservation of HepG2 cells, primary chondrocytes, cartilage and exocrine pancreases and immunolabelling efficiency comparable to Tokuyasu method. In the case of tested samples, authors suggested using the standard Tokuyasu technique because it is much easier, faster and allows the preparation of larger samples. The real power of the RHM methods was showed on *Arabidopsis* tissues, anthers containing pollen grains, *D. melanogaster* embryos and young

adult nematodes. For these organisms, the RHM method was slightly modified [58,59] on dehydration step which was started at subzero temperatures. Obtained results were similar to the Van Donselaar et al. study: 1) water addition to FS cocktail appeared to be necessary for improved visibility of the membrane's bilayer structure and 2) sample fixation with osmium tetroxide, UA and glutaraldehyde during FS and post-fixation with glutaraldehyde at 0 ºC did not influence immunolabelling. Moreover, fixation-sensitive antigens were not inactivated, despite of using high concentration of fixatives. An additional benefit is usefulness of the hybrid techniques for fluorescence microscopy and CLEM due to the optimised ultrastructure preservation. Green fluorescent protein signal could be observed even after OsO_4 treatment [58]. High lateral and axial fluorescence resolution can be obtained using thin cryosections; thereby, blurred signals are eliminated. Fluorescence-tagged antibody is much more sensitive than gold markers; thus, if fluorescence signal is not detected, then immunogold labelling is not worth to perform [59].

Hybrid techniques provide an alternative for worthlessness of vitrified cryosections in immunogold labelling. Recently, a novel hybrid technique called VIS2FIX was presented. First, vitrified material is cut into vitreous sections (VIS) and adhered to the EM grid. Next, the sections on the grid are fixed and brought to room temperature by means of FS and immuno-labelling. Vitreous sections are fixed (FIX) either by VIS2FIX$_{FS}$ method using a high-speed FS procedure with subsequent rehydration procedure or by VIS2FIX$_H$ approach based on water-based frozen fixation, hence 'H' for hydrated [219]. Different combinations of fixatives were tested, including osmium tetroxide, glutaraldehyde, formaldehyde, acrolein and UA. The variety of mentioned fixatives and short time needed for sample preparation give huge possibilities in protocol optimisation for different antibodies and samples. The unique feature of VIS2FIX$_H$ method is fixation of lipids droplets, offering an interesting application in the lipidomics field. Although vitreous section is open structure and thus fixatives can penetrate it in high extent, the lack of embedding medium does not cause the material extraction. The high accessibility for the fixatives results in outstanding preservation of vesicles, particularly in the Golgi area and organelles. Further superiority over other techniques was proved through immunolabelling of both resin and aldehyde-sensitive antigens. As another option for making the impossible possible is vitrification of Tokuyasu-style immunolabelled sections, in brief VOS (vitrification of sections) technique. This approach was first time presented nearly a quarter of century ago by Sabanay et al. [220], and further afresh used in reconstruction of adhesion structures in tissues by cryo-ET [221]. In contrary to the described hybrid approach, in VOS technique, the first step is based on Tokuyasu sample preparation. The common steps are mild chemical fixation and cryoprotection in sucrose followed by immunogold labelling. After that, sample is re-vitrified in liquid ethane instead of treatment with methylcellulose and air-drying steps, as in the Tokuyasu technique [220]. The interesting thing is that the refrozen cryosections are free from cutting artefacts related with CEMOVIS procedure. Therefore, VOS technique provides meaningful 3D information on 300-400 nm thick sections. This method was used for 3D reconstruction of desmosomal adhesions in stratified epithelium and membrane-dense plaques and flanking caveolae in smooth muscle tissue [221]. The VOS technique is a valuable tool in fluorescence-based CLEM study, but together with Cryo-ET, its possibility to gain localisation of the targeted object in three-dimensional context is unique [222]. The

advantage of VOS technique over standard Tokuyasu cryosectioning is that re-frozen cryo-sections are visualised by phase contrast results in more native sample preservation and overall better resolution. Moreover, structures invisible in Tokuyasu cryosections, i.e. cytoskeleton and ribosomes, should be possible to reveal with VOS.

In some conditions, chemical pre-fixation is an essential step prior vitrification process. This is true when safety consideration must be fulfilled, especially when pathogen organisms are the aims of the study. An aldehyde fixation step was introduced before vitrification of *Bacillus anthracis* spores [223]. Chemical pre-fixation step (CAF) followed by CEMOVIS made it possible to describe two new structures present in the spore. In some cases, chemical fixation is the only alternative for sample preservation. Biopsy specimen is particular situation, considering the place of sample collection where immediate processing of samples by HPF is rarely possible, i.e. a hospital or external laboratory. Thus, even sample is prolonged and stored in fixatives, subsequent processing by HPF followed by FS and polymer embedding results in better ultrastructure preservation compared to the conventional methods [224]. Some tissues are challenging specimen for ultrastructural preservation due to varied morphologies across its entity. Brain tissue integrity is degraded due to anoxia, unless chemical pretreatment is applied after the excision. The utility of this hybrid technique was demonstrated for variety of nervous system tissues. Aldehyde fixation prior to cryoimmobilisation and tailored FS protocol provides ultrastructural preservation superior to that obtained by conventional preparation and close to that obtained by HPF-FS protocol for tested samples [95]. Another challenging structure to preserve by HPF are stereocilia on the apical part of epithelial cells inside the inner ear. Preservation of the inner ear tissue using HPF is a challenging task, because the overall preservation of the sample was generally very good except for stereocilia. The explanation for this observation is unusual freezing properties of stereocilia actin bundles [96]. Authors considered different factors that could account for these properties, such as structure of the stereociliary actin bundles, high pressure during vitrification process and the treatment of the sample after freezing. Based on their research, authors were not able to unambiguously explain this phenomenon due to further study is required. Most importantly, chemical pre-fixation before cryoimmobilisation step resulted in preservation of cellular structure close to that prepared by HPF alone, and stereocilia actin bundle was preserved in a consistent manner.

9. Conclusions and outlook

The resolving power of TEM made it possible to visualise different objects at various resolution level ranging from angstroms for macromolecular complexes to nanometre scale for subcellular complexes and cells to micrometres for tissue gross morphology. Thus, electron micrographs have contributed significant understating of cellular structure and functions in physiological state as well as disease process. The sample preparation methods, however, still pose the main issue in biological TEM. It is important to realise that there is no single preparation technique that could be applied universally. Appropriate choice of the preparation technique is determined by different factors:

1. A model organism. Vitrification should be method of choice for formidable to fix organisms such as plants or *C. elegans* where a hydrophobic cuticle, thick wall or starch granules slow down chemical fixatives and thereby induce artefactual morphological changes.

2. When the time matters. Significant advantage of cryoimmobilisation over chemical fixation is the possibility to catch a dynamic cellular process at a known point and localisation of a rare event and structures, e.g. endomembrane transport in tissue culture cells, syncytial mitoses in early *Drosophila* embryos, nuclear division in *C. elegans* [103,225] or viral dynamics in cell [203].

3. The aim of the study and the object size.

The first aim is structural analysis. Sample architecture should be changed as less as possible for high-resolution analysis; therefore, cryoimmobilisation-based techniques are the best choice. Sample in near to native state can be prepared as follows:

• By plunge freezing, dedicated to proteins, macromolecular complexes and viruses suspension, for atomic or near-to-atomic resolution study

• By high-pressure freezing or self-pressurised rapid freezing for CEMOVIS or cryo-FIB, dedicated to samples thicker than 10 μm and by plunge freezing dedicated to small organisms, for analysis in sub-nanometre to nanometres range of macromolecular complexes and organelles within the cells

For morphological and cellular structural biology studies, sample is prepared as follows:

• By conventional sample preparation, dedicated for different samples, especially human tissue biopsies and whole organs of different animals. In some instances, this technique is more preferred, especially when the final contrast after cryoimmobilisation followed by FS is insufficient for further analysis. Good examples of this are synaptic vesicles in nerve cells and thylakoid chloroplasts [190]. If the extractions and/or distortions of the cytoplasm do not influence on data analysis then conventional TEM is a good choice.

• By HPF/SPRF followed by FS combination; thereby, structural artefacts are limited. Resin sections give opportunities to obtain large-scale imaging at nanometre resolution.

The second aim of TEM is the immunocytochemical localisation of biochemically defined antigens within the cell landscape. Pure frozen-hydrated samples are useless for this task; thus, vitrified material is resin embedded after FS. Also specimen chemically fixed can be embedded in methacrylic resins for immunolocalisation study. Cryosectioning according to Tokuyasu is an alternative for both rare and sensitive to ethanol or methacrylates antigens. Tokuyasu technique excels in membrane contrast; hence, it is best suited for locating antigens in correlation to cellular compartments. On the other hand, resin sections have a larger area to analysis and better contrast that can be optimised for specific task. As it was mentioned in previous sections, hybrid techniques can offer a solution to antigens and samples difficult to fix for various reasons.

Another problem where preparation techniques pose the main issue is element distribution within analysed organism. Indeed, it is critical point to avoid and prevent diffusion of water

and ions between cell compartments and outside the cell. To study element distribution within the cells and tissue, the best choice is CEMOVIS where sample remains at native state [62,63], at least in theory. Distribution of elements in resin sections obtained after vitrification and freeze substitution process are closer to natural state [64,226] in comparison with conventionally prepared sample. Nevertheless, a conventional TEM is also used for analysis of ions in biological study [227,228].

Last but not the least is technical level of difficulty and equipment requirements. Cryotechniques are generally more technically demanding, including skills (e.g. cryosectioning of vitreous samples) and devices (e.g. cryo-ultramicrotome, cryo-EM, vitrification machines) in comparison with conventional TEM.

On the whole, the wide variety of available preparation techniques for biological samples enables to answer specific questions dependent on the study aims, model organisms and required resolution.

Considering the technological advances at EM field during the last decade, it is nearly impossible to predict future developments. Currently, some interesting solutions in both imaging and sample preparation at different level are under development, and presented ideas may significantly improve the ability to investigate and understand the world around us. Continuous software development for images acquisition, 3D reconstruction and further image processing and interpretation during electron tomography pushes the resolution limit down [229]. Another factor for improving resolution is new direct detection system with better quantum detection efficiency and high speed that allows corrections of beam-induced movements [230]. Further reduction of image distortions can be achieved by applying a holey carbon grid modified with graphene sheets [231]. An alternative way to improve final image of vitrified specimen is introduction of Zernike phase plate. This modification permits to record higher contrasted images with better resolution [232]. On the other side of scale of resolution, correlative light and electron microscopy has its own place. This modern approach is strongly explored in cell biology at various levels of resolution, and further progress is driven by scientific needs. Most recently, an integrated system for live microscopy and vitrification (MAVIS) was presented [233]. MAVIS combines a light microscope with a plunger to vitrify the specimen, and in this time lapse, imaging in a few second time resolution could be performed without the need for transfer step. An elegant solution called integrated light and electron microscope (ILEM) joins light and electron microscopes within one set-up and in this way greatly simplifies sample handling and navigation between the two modalities, therefore increasing final success of image correlation of both plastic sections and vitrified material [48,234]. Fluorescence signal during sample observation inside the column of ILEM is detected at dry environment; thereby, fluorescence marker should be picked carefully. Additionally, to avoid quenching and loss of signal, the en bloc staining, for example, via FS process, is a prerequisite for resin sections post-labelled with fluorescent dyes [48]. The ultimate goal of every microscopy technique which contribute to biology is visualisation of working life in as close to the native state as possible. Incompatibility of a liquid sample with the vacuum needed for electron microscopy seems to be apparent since electron microscopy of specimens in liquid is possible [235]. Another dream of electron microscopists has come true, but even so, appropriate sample preparation is still a basic need.

The last decades have seen exponential technological progress that improves electron microscopes. Nevertheless, even if modern TEM will reach the electrons resolution limits, the sample preparation step remains a critical issue, limiting final achievements. Arguably, the optimisation of sample preparation is key issue for the integration different microscopy techniques and joining data acquired at different-length scale into one view.

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