American Journal of
Biomedical Science & Research

www.biomedgrid.com
ISSN: 2642-1747

Review Article

Methodological Approaches to Preparing Tissues for Electron Microscopic Examination

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To Cite This Article: *Bon E I*, Maksimovich N Ye, Zimatkin S M, Ostrovskaya O B, Rybchinskaya V and Pauliuchenkava D. Methodological Approaches to Preparing Tissues for Electron Microscopic Examination. Am J Biomed Sci & Res. 2023 20(3) AJBSR.MS.ID.002715,* DOI: 10.34297/AJBSR.2023.20.002715

Received: October 17, 2023; **Published:** October 27, 2023

Abstract

Assessment of cell ultrastructure is an important and integral part of complex scientific research. To correctly interpret the results, an adequate approach to the methodology is required. This article is devoted to the methodology of preparing tissue samples of living organisms for electron microscopic examination.

Keywords: Methodological Approaches, Tissues, Electron Microscopic Examination

Preparing Replicas

Replicas are especially widely used in the study of the sub-microscopic structure of metals, but in some cases, they are also used in biological research. For example, replicas are used to study the structure of the surface of teeth [1]; very important studies have been conducted with prints of the surface of crystals of biologically important compounds. When studying hard or cured surfaces, collodium and formalin are most often used. These substances are deposited from the solution onto the test surface and form a sufficiently strong film on it, which can be separated, sprayed, and examined for lumen. Of course, the replica obtained in this way will be negative, but, if necessary, a positive replica can be obtained; for this, a film of beryllium, silicon monoxide or plastics soluble in various solvents is applied to the resulting negative replica in the same way. Usually, a negative remark is washed off with a suitable solvent and thus separated from the positive one. If they want to get a positive replica, then the first (negative) cast should be thick enough, but not too thick, as this may affect its quality. When making a positive replica of metal or silicon monoxide, spraying should be carried out in a special apparatus, and the sample should be positioned perpendicular to the beam. Sometimes a slightly different method is used to study fine details: the sample is sprayed and at a small angle, the resulting layer is covered with a synthetic film, and then both films are separated together and examined as a replica. When working with soft tissues, the surface layer of the sample adheres to the replica and separates with it; such a replica, together with the layer adhering to it, is called a pseudo-replica [2]. It is often possible to sequentially separate layer after layer from the sample and examine each layer separately in an electron microscope; because of such a layered study of the tissue, valuable information about its structure can be obtained. Two methods are successfully used to obtain high resolution when working with replicas.

If one of them gave good results in the study of protein crystals. In this case, the preparation is first sprayed with a heavy metal, and a thin layer of plastic is deposited on top of it. The first is to separate the plastic together with the metal, or to do otherwise -to remove the sample itself by dissolution or enzymatic cleavage, after which only a sprayed layer of metal remains, for which the plastic serves as a supporting film [3]. Another method, also used in the study of

protein crystals, is that the drug is sprayed first with a heavy metal, and then (at right angles) with silicon monoxide, which acts as a substrate film. Silicon monoxide scatters electrons worse than plastic, because of which this method allows you to achieve very high resolution. However, both methods involve evaporation in a high vacuum, and under these conditions crystals very rarely retain their original structure. To reduce the drying rate, attempts were made to make replicas of silicon monoxide in vacuum using samples frozen in a wet state, but at the same time they had to face the problem of ice crystals formation. Nevertheless, in this way it is still possible to get an idea of the true size and shape of the particles. Recently, the same principle has received a slightly different embodiment: a freshly cut surface (frozen biological tissue) is subjected to short-term heating to remove the surface layer of water or ice, and then a replica is prepared by spraying a layer of coal. All these processes are carried out in a vacuum chamber and in this case the fabric is subjected to minimal preparative effects, and ultra-thin details are visible on the microphotographs of replicas, which are in good agreement with the results of studying thin sections, the manufacture of which is associated with stronger effects on the fabric [4].

Obtaining Ultra-Thin Slices

Biological tissues are in most cases too massive to be directly studied in an electron microscope. There are numerous physical and chemical methods that make it possible to obtain fragments of such tissues that are suitable in size for electron microscopic examination. At the same time, the question arises to what extent the results of the study of individual fragments allow us to get an idea of the structure of intact tissue. Thus, individual thin fibrils that can be split, the studied fibrillar structures, often turn out to be the same and therefore can be considered as the main structural units. However, there may be several different structural units in other sources, and then, when using fragmentation methods, it is difficult to imagine their relationship in an intact tissue. The study of thin slices gives the most complete idea of these relationships. This method should be considered as a kind of extension of the field of optical microscopy since the same problems arise in both cases [5]. However, the use of electrons instead of visible radiation imposes new requirements for the manufacture of drugs and creates additional difficulties in interpreting the results.

Cutting on a Microtome

In the manufacture of preparations for a conventional microscope, microtomes are needed, with the help of which it is possible to obtain slices with a thickness of about 1 mk; methods of filling the sample and concluding the slice were calculated for such a thickness of the slice. In electron microscopy, the maximum allowable slice thickness is about 0.5 microns (for fresh tissues), and even thinner slices are needed to obtain high resolution. Nevertheless, in the early period of the development of electron microscopy, it turned out to be possible to adapt existing microtomes to make sections that corresponded to the resolution achievable at that time. To do this, it was enough to change the gear ratio and thereby slow down the sample feed rate; otherwise, it was only necessary to monitor the condition of the knife and apply special filling methods; if these conditions were met, it was possible to prepare slices with a thickness of 0.1 mk or even less. Attempts to apply a high cutting speed did not give the desired results. Thin slices turned out to be possible to prepare from frozen tissue. To do this, the sample is mounted on a massive block, strongly frozen, and then the block is slightly heated (but so that the sample does not thaw); in this case, the sample feed rate is determined by the thermal expansion of the block. With due attention to detail, it is possible to obtain very thin slices, so this method serves as a useful alternative to filling the sample [6]. However, it is believed that to obtain a high resolution, the fill gives the best results; therefore, special microtomes were created to obtain sections of tissues poured into polymers [7].

The devices for mechanical feeding of the sample have been improved so much that it has become possible to obtain serial slices with a thickness of less than 200 A; when obtaining particularly thin slices, the use of thermal feeding gives equally reliable results. The linear expansion of the metal rod is easy to adjust using electric current for heating. The rod is mounted on the sample holder or on the microtome knife holder. In this way, it is possible to obtain ultra-thin slices. The microtome is driven by an electric motor; the thickness of the slice is determined by the ratio between the sample feed rate and the rotation speed of the microtome drive. Currently, there are heat-fed ultratoms on sale, equipped with a device for a single step feed, which ensures that a cut is obtained as soon as the sample is at the edge of the knife (these devices can be of different types). Particular attention was paid to the elimination of vibration, as well as to the fact that the area of contact between the block and the knife was the same all the time, if possible.

A lot of effort was spent on the selection of the knife. They tried to use standard steel knives, but at the same time it was necessary to constantly make sure that the cutting edge was not blunted; therefore, many preferred to use a specially sharpened razor blade, paying special attention to the shape of the edge and the angle between the tip and the line of movement of the block with the sample. However, the selection of this angle is important in any case. Each blade serves for a very short time, after which it is replaced by another. Comparable results are obtained by using glass knives, which can be obtained by carefully breaking into pieces sheet (preferably mirror) glass; each such piece is used only for one series of slices. Usually they take a sheet of glass with a thickness of a quarter of an inch (6.3 mm), cut into strips 2.5-3.7 cm wide and then break them into pieces in the form of triangles or parallelograms; for this purpose, short notches are made on it at an angle of about 45° to the long edge of the glass, and then a heated glass stick is brought or simply struck on the glass. In skillful hands, special glass tongs turned out to be very effective. They are made so that they touch the glass at once at several points located in a certain way; sometimes, for the same purpose, the edges of the forceps are given a curved shape. On sale there are devices for breaking glass, allowing

you to standardize this process and quickly get pieces of glass with smooth edges. Usually, the cutting edge is an edge perpendicular to the glass surface. But even with such perfect methods, the cutting edge, completely free from the slightest irregularities, still turns out to be an exception; therefore, the selection of a suitable glass knife requires attention and some experience. It is equally important to choose the right angle between the cutting edge of the knife and the cutting plane; its value depends on the angle of the knife and on the hardness of the block. From here it can be seen that the process of cutting fabric with glass knives is not easy to standardize [8].

The possibility of complete standardization of this process appeared in connection with the use of diamond knives. In practice, a good diamond knife is installed in a microtome, after which it can be used for quite a long time for rare hard enough tissue blocks. Unfortunately, the quality of diamond knives on sale often leaves much to be desired; this circumstance, along with the specific difficulties encountered when working with some media for pouring, and the high cost of diamond knives limit their use. The slices obtained with the help of ultratoms are extremely small and thin; in order to make it easier to work with them, a bath with water is attached to the cutting edge of the knife, to which a little organic solvent is sometimes added; the slices fall directly into the water and are straightened out, and then they are transferred to a mesh. If the block is carefully trimmed from the end, so that a square or trapezoid turns out in the section, a whole tape of serial slices is built on the grid. When working with glass knives, a metal or plastic bath is soldered to the glass; if a diamond knife [9] is used, which is fixed in a metal frame even before sharpening, then it is inserted into a special holder equipped with a recess for water.

Fixing, Filling and Staining of the Sample

Even though microtomes and electron microscopes have already entered the daily practice of biological laboratories, it is not always possible to fully use the capabilities of these advanced devices, since the problem of preparing biological samples, themselves has not yet been solved. This problem can be formulated as follows: how should the fixation, dehydration and filling of the sample, preparation and staining of the cut be performed to preserve all the details of several angstroms in size that characterize the structure of the original tissue? Fixation is necessary to minimize structural changes that may occur at all stages of preparation of the drug. Fixation methods, commonly used in histology, allow you to preserve the main structural details, distinguishable with a light microscope, and give some of them additional contrast. At the same time, the task is to preserve the general features of the distribution of the material in the fabric in such a way that they do not change during drying and can be identified through selective interaction with dyes. In other words, the cell membrane and the entire contents of the cell can undergo chemical changes, but if it is possible to preserve the membranes and prevent the diffusion of the contents, fixation can be considered successful. The situation is different in electron microscopy, where the resolution reaches the molecular level; therefore, the details of the membrane structure and the exact distribution of material inside the cell fall within the resolution limits. It is no longer enough to preserve the details discernible in a light microscope (note, by the way, that electron micrographs are better compared with the pictures observed in an interference or phase contrast microscope, since the contrast of the image is also determined by the distribution of mass, and not by selective absorption of the sample). In electron microscopic studies, the ideal fixative should preserve the molecular structure of the drug.

Thus, the search for ideal fixators turns into a chemical problem of replacing weak molecular bonds with stronger bonds capable of withstanding all further effects on the drug during its processing. In fact, this search has just begun. Hydrogen bonds and salt bridges that break down when water is removed from the preparation should be replaced with more stable bonds, and substances soluble in reagents used at the pouring stage should be turned into insoluble, without disturbing their distribution in the tissue. Obviously, some structures are more difficult to maintain than others; structures that contain a lot of water inevitably shrink or coagulate when dried. If measures are not taken before drying to fix weak bonds, then, likely one of two general effects will be observed. In some cases, molecules can shift towards each other, resulting in a more compact system; for example, when fibrillary proteins are compressed, polypeptide chains approach each other and form a more compact fiber. In such systems, preliminary fixation from the point of view of the preservation of the ultrastructure is not so necessary since the structure is compacted during the drying itself. However, in other cases, components that are loosely bound in the presence of water may turn out to be incompatible when, because of water removal, they come closer to each other; then, during drying, there is a tendency to rearrange molecules and divide the system into different phases consisting of similar molecules. For example, it is well known that molecules of various lipids in water sometimes associate, forming a single lipid system; when water is removed, such a system usually breaks down into separate lipid phases. When studying such systems, it is very important to introduce stronger bonds into the structure before drying to prevent the separation of components. Fixation serves this purpose. However, the search for chemical agents that stabilize specific molecular systems has not yet been systematic.

Currently, those fixators that have proven themselves well in histology are used in electron microscopy; only the most general information is available about their chemical action. The most widely known are formalin and osmium tetroxide, which are successfully used in histology to fix a wide variety of tissues. Osmium tetroxide reacts especially actively with unsaturated lipids, but to some extent interacts with saturated lipids as well as with proteins; since it is also an effective dye, the tissue acquires a well-defined contrast. Formalin is not a dye, but it preserves most tissues well [10]. In electron microscopy, osmium tetrachloride proved to be the best general-purpose fixative for working with all tissues. At first, as in histology, just its aqueous solution was used, now for more effective preservation of small structures, a buffer is added to the solution

and made isotonic. Recently, osmium tetroxide has been used together with other fixatives and a number of additional dyes. The nature of the chemical interaction of tetroxide with tissues is still not fully understood. It has long been known that tetroxide interacts with double bonds in hydrocarbon chains of lipids, but for the interpretation of electron micrographs it would be more important to know the final localization of the interaction products and the degree of interaction with ionic groups of lipids, proteins and other tissue components. Gradually accumulating data indicate that after treatment with osmium tetroxide, the densest regions of lipid-containing systems, in particular lipoprotein membranes, correspond not to hydrocarbon chains, but to ionic sites. According to X-ray diffraction analysis, in the myelin sheath of the nerve, the increase in density due to osmium reaches 100%, and osmium is localized mainly at the border between lipid and protein.

Osmium tetrachloride slowly penetrates into the fabric, so when working with this retainer, you have to use relatively small blocks of fabric (less than 1 mm thick). This can also explain why additional structural details are often found in tissues previously fixed with saturated formaldehyde or glutaraldehyde and then treated with osmium tetroxide [11]. In most cases, preparations fixed with osmium tetroxide can be made more stable by injecting heavy metal salts into the tissue at the stage of dehydration and pouring or after the preparation of the cut. The contrast of protein structures is often enhanced if, at the end of dehydration and before pouring, the drug is dipped in an alcoholic solution of phosphoric-tungstic acid. By washing the sections already mounted on the grid with aqueous or organic solutions of uranyl acetate or lead hydroxide, it is possible to increase the contrast of membrane structures and reveal additional details. According to the existing opinion, such "staining" is nonspecific. It gives contrast to membrane structures, but nothing is known about the identification of any particular molecular component [12].

In electron microscopy, specific "staining" is currently used mainly to detect the localization of enzymes. After fixing the tissue with osmium tetroxide, the enzymatic activity almost completely disappears, however, if the tissue is fixed with formalin or (even better) glutaraldehyde before staining, then many methods of this kind are quite effective. If these fixators are used in the form of cooled and buffered solutions, then after staining, the fabric can be treated with osmium tetroxide; as a result, the fine structure of the fabric is well preserved, and the localization of the dye is almost not affected [13]. Many of the most effective methods for detecting enzymes used in electron microscopy are based on the release of a phosphate group from a substrate molecule under the action of an enzyme; then the phosphate group interacts with lead ions and is deposited on the tissue in the form of lead phosphate [14]. Thus, when acid phosphatase is detected, the enzyme releases a phosphate ion from sodium glycerophosphate; when a suitable solution containing lead nitrate is added, lead phosphate is immediately precipitated. Localization of salt in the tissue is then easy to establish on electronic microphotographs of sections. The localization of adenosine triphosphatases and some esterase's is detected in the same way; naturally, other phosphate-containing substrates are used. There are a number of other methods for detecting enzymes that were originally intended for light microscopy and have found application in electron microscopic studies; these methods are also based on the introduction of heavy metal ions. Some progress has been made in the use of aromatic dyes, which can produce effects visible on electronic microphotographs. Buffered solutions of potassium permanganate and bichromate can also be named among the fixators successfully used in electron microscopy. Perhaps it is important that these fixatives, like osmium tetroxide, are oxidizing agents, but the exact mechanism of their action on the tissue has not yet been clarified.

When making thin sections with the use of pouring, the fabric must be dewatered. Usually, for this purpose, the fabric is sequentially "passed" through solutions of alcohol or acetone of increasing concentration (up to absolute) before pouring. Alcohol easily destroys molecular complexes containing protein (apparently, the protein is simply denatured in this case); therefore, such complexes must be stabilized beforehand. Usually, a retainer is used for the total; in some cases, for electron microscopic studies, a section of frozen tissue was first prepared and dried in a frozen state. Frozen drying (freeze drying) to some extent prevents the shrinking of structures during dehydration, but, apparently, does not prevent changes at the molecular level; thus, during lipophilic drying of tissues fixed with osmium tetroxide, lipids in lipoprotein structures will still be separated from protein [15]. Despite all precautions, many tissue components (especially lipids) are still partially extracted by both the dehydrating liquid and the filling medium. It is currently almost impossible to counteract the effects of drying by specific stabilization of certain complexes, however, we can at least limit major structural changes and, when interpreting electron micrographs, try to consider the most likely molecular changes that occur during drying [16].

The ability to obtain increasingly thin slices has created the need for harder media for pouring [17], which at the same time would best match the type of fabric, the thickness of the cut, the cutting speed and the type of knife. Previously, collodion with paraffin was most often used for filling, and the collodion later remained on the slice, acting as a substrate [18]. Later, a polymerization method was developed; in this case, the fabric is impregnated with a monomer, for example, butyl methacrylate [19], which is then polymerized [20]. By selecting all kinds of mixtures of monomers and changing the polymerization conditions, it is possible to obtain blocks of various hardness that are most suitable for a particular cutting method. Removing the polymer from the cut increases contrast but requires the use of strong solvents. In the past, this undoubtedly led to significant distortions of the sample structure, however, with modern technology of manufacturing thin slices, the medium for filling is left as a substrate, and the contrast is increased by the introduction of "dyes" [21-23]. Some components of the filling medium are sublimated by the action of an electron beam, which again

leads to distortion and loss of fine details of the structure. Even at low beam currents, the thickness of the slices can be reduced by almost half. This effect can be reduced to some extent by increasing the number of crosslinking in the polymer, but to obtain a high resolution, it is better to use other plastics that are less sensitive to the action of the electron beam [24]: for example, you can take epoxy resins (araldite and epon) or polyesters (vestopal) [25]. Such an approach to the production of thin tissue sections could be considered ideal: first, all important bonds that are stable only in the presence of water are replaced with stronger bonds that can preserve the structure of the tissue during dehydration; then the tissue is treated with substances that are not permeable to electrons, so that the components of the structure of interest to the researcher acquire a clear contrast; dehydration and filling of the sample they are performed in such a way that they do not cause any distortion of the structure; finally, thin uniform slices are prepared, which are necessary to obtain high resolution in cases where the filling medium is left on the slice as a substrate. When interpreting electronic microphotographs, it should always be remembered that none of these operations has been perfected.

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