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Review

Beginners guide to sample preparation techniques for transmission electron microscopy

ENA DUMANČIĆ
LEA VOJTA
HRVOJE FULGOSI*

Laboratory for Molecular Plant Biology and Biotechnology, Division of Molecular Biology, Ruđer Bošković Institute, Zagreb, Croatia

***Correspondence:**
Hrvoje Fulgosi
E-mail address: hrvoje.fulgosi@irb.hr

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Abstract

Background purpose: *The revolution in microscopy came in 1930 with the invention of electron microscope. Since then, we can study specimens on ultrastructural and even atomic level. Besides transmission electron microscopy (TEM), for which specimen preparation techniques will be described in this article, there are also other types of electron microscopes that are not discussed in this review.*

Materials and methods: *Here, we have described basic procedures for TEM sample preparation, which include tissue sample preparation, chemical fixation of tissue with fixatives, cryo-fixation performed by quick freezing, dehydration with ethanol, infiltration with transitional solvents, resin embedding and polymerization, processing of embedded specimens, sectioning of samples with ultramicrotome, positive and negative contrasting of samples, immunolabeling, and imaging.*

Conclusion: *Such collection of methods can be useful for novices in transmission electron microscopy.*

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INTRODUCTION

Many biological structures that we would like to study are so small that naked eye is unable to see them. For this purpose, numerous variations of microscopes have been developed. The revolution in microscopy came in 1930s with the invention of electron microscope, which made it possible to study specimens at first on the ultrastructural level, and later even atomic level *(1)*. There are two types of electron microscopes: transmission electron microscope (TEM) and scanning electron microscope (SEM). Here, we are going to describe basic protocols for TEM specimen preparation. TEM has resolution of up to 0.1 nm, which is thousand times greater than light microscope. This is possible because of the short wavelength of electron beam *(2)*. With TEM, significant advancements in analyses of cellular compartments, such as cytoskeleton, membranes, organelles, cilia, etc. have been made *(3)*. TEM is using a beam of electrons, produced by the electron gun operating at high voltages, usually from 25 kV to more than 200 kV, to illuminate the specimen. The passage of electron beam is controlled by two pairs of electromagnetic lenses, condenser and objective lens, and this layout is similar to the one in the light microscope. Electron beam produced by the electron gun is focused on the specimen by condenser lens and after that projector lens is creating enlarged picture of the specimen on the fluorescent screen. All these components of TEM are under a high vacuum in order to avoid collisions between electrons and air molecules. In these conditions specimens must be dehydrated, otherwise the presence of water could cause their collapse under vacuum. The electron beam must be

able to penetrate the specimen. For this reason, biological specimen must be very thin, not thicker than 100 nm *(2)*. Although TEM is more than appropriate device to study the internal structure of biological cells, conditions inside TEM and preparation of specimens may damage the cells. Steps needed to prepare specimens for TEM include fixation, dehydration, infiltration with transitional solvents, embedding with resins, resin polymerization, sectioning, and contrasting *(1)*. Since the current literature lacks basic, simple protocols about TEM samples preparation techniques for novices, the aim of this review is to explain the basics of TEM, introduce those techniques to young researchers without much electron microscopy experience, and help them to learn fundamental sample preparation techniques.

PREPARATION OF BIOLOGICAL SPECIMENS

Firstly, methods of electron microscopy did not include sample fixation which we practice today. Prior to microscopy samples were just dried out onto a grid, but because of that, quality of images was not much better than when light microscopes were used. Shortly after these first images were obtained, it became clear that specimens need to be fixed, embedded, sectioned, and stained. Till this day, basic specimen preparation procedures from 1960s are still in use in many laboratories, with only slight modifications *(4)*. As already mentioned, procedure of sample preparation with thin sectioning consists of fixation, dehydration, infiltration with gradually increasing concentration of transitional solvents, resin embedding, and its polymerization, sectioning, and contrasting. The goal of fixation is to preserve biological specimens from damage, to maintain its natural conditions, and to stabilize molecules against disruption by subsequent procedures such as dehydration and resin infiltration *(4)*. There are two variations of this procedure: chemical fixation and cryo-fixation *(2)*. For maximum tissue preservation it is important to choose proper method of fixation corresponding to the type of samples and structures of interest that need to be visualized. Here, the selection of fixatives and buffers plays the major role. In all these procedures, tissue needs to be handled carefully, in order to preserve cellular ultrastructures as close to the natural state as possible *(5)*. Damage of tissue structures must be minimized, and the sample should be able to withstand subsequent vigorous handling, such as dehydration *(6)*. Quality of specimens depends on each step of the procedure, and because of that, it is important to follow defined protocols *(1)*.

CHEMICAL FIXATION

The most common procedure of chemical fixation is primary fixation using glutaraldehyde, mostly because of its capability of protein cross-linking and preservation of lipid structures, carbohydrates, and nucleic acids *(2)*. The amount of proteins to be cross-linked with the fixative depends on the temperature and the pH of the fixation medium *(5)*. Primary fixation is followed by secondary fixation or post fixation. Post fixation is done with osmium tetroxide that mostly reacts with unsaturated lipids, but also with proteins, nucleic acids, and carbohydrates. Tissue needs to be cut with razor blade into small pieces (not larger than 1 mm^3) in a drop of primary fixative, in order for fixative to penetrate the sample evenly. After that, tissue is infiltrated in primary fixative for 1 hour. Glutaraldehyde is replaced with cacodylate buffer and rinsing lasts for additional 2 x 10 minutes. Afterwards, cacodylate buffer is replaced with osmium tetroxide for further 1h of infiltration. The last step of fixation includes replacement of osmium tetroxide with distilled water, 4 x for 10 minutes *(2)*. All steps of fixation, besides the last one, need to be performed on ice and with freshly made reagents, to reduce the interaction between the components, tissue autolysis, structural changes, and protein extraction caused by osmium tetroxide *(5)*.

DEHYDRATION

After these two fixations, specimen needs to be dehydrated to prevent the collapse of its structural elements under the vacuum. Although necessary, this process may cause alterations in cell ultrastructures *(1)*. In this procedure, water is replaced with less polar solvent, ethanol. Ethanol is used in a decreasing dilution series. In the beginning, distilled water is replaced with 50% ethanol for 10 minutes. Subsequently, after every 10 minutes, tissue is placed in the decreasing dilutions of ethanol, 60%, 70%, 80%, 90%, and finally 96%. In the 96% ethanol tissue can be stored for several weeks *(7)*. Dehydration also can be performed by freeze substitution, the process which is used for cryoimmobilized samples. This is a physiochemical process of specimen immobilization and stabilization in which vitrified water within cells is replaced by organic solvents at subzero temperatures, followed by specimen fixation with fixatives and embedding with the resin. Two procedures of freeze-substitution are commonly in use: dehydration of frozen tissue in ethanol, methanol, organic or aqueous solution that contains a metallic salts or acids as fixatives (acetic acid, piric acid, potassium dichromate, mercuric chloride, zinc salts, diimidoesters, acrolein), and dehydration without fixation. The solvents are well mixed with embedding resin. The next step of the procedure is specimen embedding with resins that are nonvolatile in the vacuum *(8,9)*.

EMBEDDING

Resin embedding process was developed as a result of microscopists' observation that most cells are too thick to

be viewed directly in the electron microscope and that some kind of ultrathin sectioning is needed *(4)*. Sections must be thin enough so that electron beam can penetrate through the material. To cut such thin sections from soft biological samples they need to be infiltrated with a liquid resin that is then polymerized. Resin serves as the matrix that supports the tissue while sample is being sectioned by the special device made for that purpose, the ultramicrotome. Various resins are nowadays in use, mostly consisting of epoxy monomers. Most frequently used embedding epoxy resin is the Spurr's resin, which is compatible with ethanol or acetone as dehydrants *(2)*. Tissue is gradually incubated in the mixed resin and dehydrant solutions of different ratios (1:2, 1:1, 2:1), and after this series of dilutions, tissue is placed in pure Spurr's resin overnight. On the next day, the sample is ready for polymerization. For this purpose, several samples are placed in a silicone mold, filled with pure Spurr's resin, with the specimen's name label also included *(7)*. Besides silicon mold, there is possibility of using gelatin capsules as embedding container. Tissue is placed into the resin to gradually sunk to the pyramidal bottom of the capsule *(2)*. Polymerization of specimens in the silicone mold is done at 63 °C for 2 days. Alternatively, it can also be done under UV light (acrylic resins). After polymerization, specimens are ready for further processing. Specimen is located on the top of the resin block and needs to be trimmed out by hand with a razor blade or, with the trimming device in 0.5 mm by 1 mm long trapezoidal shape pyramid. Such pyramidal shaped specimen block is placed in ultramicrotome's specimen arm which moves up and down over a diamond knife. Knife with a water reservoir on top of it is fixed on the immobilization stand. Hand-made glass knives can also be used. On such knives water reservoir can be made with silver coated polyester tape which is sealed to the knife with dental wax. Sections made with the knife are floating on the water surface and are picked up with a specimen grid *(3)*. Grids are round and made of metal that cannot be magnetized, such as copper, gold, platinum, nickel, rhodium. Grids also have various patterns of holes, so the sections can be imaged through. Non-magnetic metals are used so that they can't interfere with the microscope's image formation system, since image is created by electron passage thought the specimen *(2)*. Sections need to be grouped in 2 to 4 with an eyelash tool and collected with bended grid by submerging grid into the water, under the sections and lifting them up. Grids frequently need to be coated with a thin plastic layer, usually Formvar, to support sections over the holes. The film can additionally be strengthened with carbon coating *(3)*.

CRYO-FIXATION

The other term for cryo-fixation technique is cryo-immobilization since usual chemical fixation of molecules is absent and is replaced with immobilization by freezing. It is very important that, whichever technique is used, arrest of molecules in the sample occurs quickly, in as much as possible natural state. Fixation speed rate in chemical fixation proceeds at the minute to hours scale, while cryo-fixation proceeds at the millisecond rate *(4)*. Cryo-fixation also minimizes osmotic changes in samples, that can develop during chemical fixation. Variations of sample preparing techniques for cryo-TEM are plunge freezing for small samples (1-2 µm vitrification depth, *10*), high pressure freezing (HPF) for larger samples (up to 500 µm vitrification depth, *11*), propane jet freezing (up to 40 µm vitrification depth, *12*), slam freezing (10-15 µm vitrification depth, *13*), and freeze substitution (up to 200 µm vitrification depth, *9*). Plunge freezing is performed by plunging grid with the sample in cryogen, mostly liquid ethane, or propane, then the sample is transferred into liquid nitrogen in a grid storage box. Sample is observed on cryo-specimen holder (*10*). Due to fast freezing water molecules do not form crystals, which preserves cells in their near native state *(1)*. High pressure freezing applies 2000 bar pressure in a liquid nitrogen surrounding, which prevents ice crystal formation. Specimens prepared in this way can be immediately sectioned with a diamond knife, as long as they are not thawing, or can be further processed by chemical fixation at below freezing temperature *(1)*. However, high pressure freezing has some disadvantages too. High cost of equipment, only small amount of sample that can be used and, in many cases, it is still necessary to use some reagents to prevent the formation of ice crystals *(3)*. The third possibility is plastic resin embedding prior to warming to the room temperature and subsequent sectioning. This procedure is called low temperature embedding. The method avoids ultrastructural changes due to dehydration, resin infiltration, and staining, but it can lead to sample crumbling when it is picked up on the grid. Crumbling can be prevented by gently pressing blunt glass rod against the sample to flatten it out. Still, the sample can be damaged in the form of compression effects, or knife marks and crevasses. Many of these limitations can be overcome by using focused ion beam (FIB) milling for preparations of specimens. FIB technique uses focused ion beam to ablate specimen and forms thin slices *(1)*. FIB can, however, produce its own set of artefacts.

CONTRASTING OF BIOLOGICAL SPECIMENS

For the formation of image and to achieve contrast in TEM it is very important how electron beam passes through the specimen and how electrons scatter on the specimen atoms. Usually, thicker and denser parts of specimens have greater potential of electron scattering. For the purpose of contrast enhancement, biological specimens naturally consisting mostly of light elements (carbon atoms) are stained with atoms of heavy metals.

Specimens are already partly contrasted by using osmium tetroxide as the secondary fixative, but in most cases, this is not enough for high quality images. Two types of contrasting techniques have been developed, positive and negative contrasting. Most widely used heavy metals are uranium, lead, and tungsten *(2)*. An additional option for increasing of contrast is shadow casting technique. The specimen is placed on a rotating platform in a chamber that contains a heavy metal filament and that is under vacuum. By passing through the metal, electric current heats it up and causes evaporation of the metal that rains down on the specimen as the plate rotates. This produces a metal coating on one side of the specimen, while creating a shadow effect behind *(1)*.

Positive contrasting

Heavy metal salts used in positive contrasting bind to the macromolecules in the sample and increase their ability to scatter electrons, thereby increasing contrast across the specimen *(2)*. Commonly used chemicals for positive contrasting are uranyl acetate and lead citrate. Those are nonspecific contrasting media. Uranyl acetate reacts with phosphorus and amino groups of nucleic acids and proteins. Lead citrate binds to the negatively charged molecules, such as hydroxyl groups and molecules which have already been stained with osmium tetroxide. When contrasted with uranyl acetate in a Petri dish, as many drops of uranyl acetate as there are grids with specimens are dropped onto a piece of dental wax, or Parafilm. Bottom of the Petri dish can be covered with moist filter paper to avoid evaporation of uranyl acetate. Grids are placed onto drops with specimens facing downwards and are incubated in this way for 5-10 minutes. After that, the grids are washed with a gentle stream of distilled water and dried with a filter paper. For optimal results, it is best to proceed with contrasting process with lead citrate right after drying. This step is also done in a Petri dish on a dental wax with several granules of sodium hydroxide to remove CO_2. Petri dish needs to be open as little as possible, to reduce CO_2 influx. Rinsing of grids is performed in the same way as with the uranyl acetate. Grids are again washed with a gentle stream of lukewarm distilled water. After drying, specimens are ready for microscopy *(2)*. Uranyl acetate contrasting can be done either before or after resin embedding. Additionally, pre-embedding contrasting technique has a strong fixative effect on specimen ultrastructures *(2)*.

Negative contrasting

Negative contrasting is a simple and fast procedure, and it is mostly used when fast diagnostics is needed. Contrasting medium accumulates around biological structures and it enhances the form and the outline of the samples *(2)*. It is used for objects that are smaller than 100 nm and can be imaged directly without sectioning *(1)*. In negative contrasting, specimen stays non contrasted and is brighter than the background. Salts of heavy metals such as uranium, tungsten, and molybdenum are used as the contrasting media. There are two methods for negative staining: drop and flotation method. Since negative contrasting is usually used for whole biological structures such as viruses, bacteria, cellular organelles, etc. and not for the sectioned samples, for both methods sample of interest must be in suspension. In the drop method procedure, the drop with the sample is touched with a Formvar-coated grid (additional carbon coating can also be performed) and after 30 seconds the drop of stain is added. After 15 to 30 minutes the sample is ready for TEM imaging. It is also possible to pre-mix equal amounts of the sample and the stain, and the mixture drop is touched with a grid. In the flotation method, coated grid is floated on a drop of the sample for 1 minute, and then transferred onto a drop of the stain for 30 seconds. Again, it is also possible to use a pre-mixed drop of the sample and a stain and float grid on it. Any excess liquid is dried with a filter paper (*2*). In the end, the grid is dried for 30 minutes.

Immunolabeling

Principle of immunolabeling is rather straight forward, location of the antigen within the samples is detected with the specific antibody. Antibodies can be conjugated with marker molecules, such as diverse metals and enzymes, or indirectly recognized by binding to the marker-conjugated secondary antibodies. In both cases, the antigen-antibody complexes are forming electron-dense contrast points which are detected by TEM (*2*). Electron microscopic immunocytochemical techniques can be conducted by applying the immunocontrasting prior to resin embedding (pre-embedding technique) or after resin embedding (post-embedding technique). The decision when to perform the immunocontrasting for the detection of an antigen at a certain location will depend to a large extent upon the distribution of the antigen and the characteristics of the primary antibody. Before starting immuno-EM labeling, a test for the characteristics and dilution of the primary antibody should be performed at light microcopy level. One common technique for immunolabeling TEM specimens is immunogold labeling. For the visualization of immunolabeling, a method using colloidal gold particles has been developed by Faulk and Taylor in 1971 (*14*). Colloidal gold particles are generally attached to secondary antibodies which interact with the primary antibodies designed to bind a specific antigen or other cell component. Gold particles are easily visible under the electron microscope due to their high electron density and can be made in different sizes from 5 nm to 30 nm, therefore enabling multiple contrasting at the electron microscopic level, most easily by direct labeling of several first layer antibodies with different sized particles. The indirect techniques can also be used in a double or triple labeling by parallel approach, if the primary an-

tibodies are from different species, and by sequential approach, if the primary antibodies are from the same species. Immunogold labeling allows localization of proteins at the electron microscopy level of resolution and quantification of signals *(14)*. The immobilization of the biological material is the prerequisite for successful application of this method to preserve the location of the molecules of interest and to preserve as many related structures and molecules as possible. The immobilization must withstand subsequent processing steps such as dehydration, sectioning, and immunolabeling. Molecules that are not immobilized may be displaced from their normal location, resulting in either false localization or a negative result, such as when molecules are washed away *(15)*. For immobilizing of specimens, the vitrification of biological material and chemical fixation could be applied. Protein molecules in the biological material prepared for immunocytochemical experiments are usually cross-linked in lower concentrations of aldehyde to preserve antigenicity. The ability of the specific antibodies to access and bind the target antigen after fixation while still obtaining adequate specimen preservation must be considered. The more effective the cross-linking the less likely is the antigen in the section to be accessible to the antibodies. Aldehyde solutions that have been successfully used for immunolabeling include 0.1% glutaraldehyde in 100 mM phosphate buffer (pH 7.2), or 2% formaldehyde in 100 mM PIPES buffer (pH 7.2) *(15)*. Generally, the more glutaraldehyde used for cross-linking, the better the morphology will be, but at the expense of antigenicity *(17)*. Probably the most preferred method for preparing biological material for immunolabeling is the Tokuyasu cryosectioning technique *(18,19)*, since it is the only post-embedding technique immunolabeling approach that does not require dehydration of the samples by polar solvents before the application of affinity markers. Thawed cryosections have been proven to give the highest accessibility of antigens to the antibodies *(17)*. Biological material is chemically fixed using buffered aldehyde solution, cryoprotected in sucrose, and vitrified by immersion in liquid nitrogen *(20,21)*. The vitrified, cryoprotected specimen blocks are subsequently sectioned at low temperature (between 60 °C and 120 °C) in a cryo-ultramicrotome and thawn onto metal specimen grids. The thawn sections are labeled with specific affinity markers and colloidal gold probes and finally embedded in a thin film of plastic *(21)*. Pre-embedding technique includes the procedure with the entire tissue block, while for post-embedding technique, specimen can be sectioned several times and can be studied with different antibodies. Pre-embedding technique is more sensitive and results in denser labeling. Embedding media can lead to either loss, or preservation of antigen sensitivity, depending on many circumstances, such as the type of sample preparation procedure, chemicals used, nature of embedding resin, and ultrastructure resistance *(22)*. Although pre-embedding technique generally provides better results than the post-embedding technique, there are also some disadvantages of pre-embedding technique. Due to the variations in permeability of different cell membranes antibodies can't reach all cell compartments equally; antibodies conjugated with labels are often too large, and results are harder to quantify *(23)*. On the other hand, post-embedding technique results are more easily reproducible, subcellular antigen localization is more specific and there is no reduction in labeling from diffusion since antibodies are applied directly onto sections. However, post-embedding technique suffers from inevitable antigen reduction, so the sensitivity is reduced. Also, antigen of interest may not be present in every section *(24)*. Besides immunogold labeling there is immuno-peroxidase labeling, and these two techniques can be used in combination for even better results *(23)*. Optimal immunolabeling is accomplished when maximum preservation of the antigen is achieved, when high specificity binding of antigens and antibodies is present, and when final conjugate is clearly visible in TEM *(24)*.

IMAGING

After preparation procedures, samples are ready for imaging in TEM. TEMs have a mechanism in which a grid is inserted by using the holder that places it in the electron beam path. Modern TEMs have detailed and easy to handle computer interfaces *(3)*. In recent decades, the processing of TEM images of biological specimens became more advanced. This was largely accomplished due to the development of optical diffraction, computer image processing methods *(6)*, software, and digital cameras that are builtin the microscope and are recording and displaying images. Sample observation begins at low magnification, 100–1000x, in order to locate sections on the grid and choose the area of interest to be viewed at higher magnifications *(3)*.

PREPARATORY ADVICES

Since specimen preparation is a multistep procedure, each step can affect the outcome. A precise sample preparation method is essential. The mistake in any step can have an impact on the final result. Good micrographs can reveal abnormal ultrastructural features. If the tissue appears to be inadequately fixed, one should check the fixative condition and recheck the procedure. Almost all chemicals that are used for processing of samples for electron microscopy are toxic, allergenic, carcinogenic, and flammable, so protective wear and equipment placed in fume hoods need to be used *(1)*. Tissues used for specimen preparation must be intact, in optimal physiological condition, and razor blades must be sharp to minimize unwanted tissue damage. The procedures should be done as quickly as possible, but also carefully. The samples must not dry out or be excessively warmed up. Samples must

be treated gently to avoid mechanical damage. Practice is the key to good performance in TEM specimen preparation, so getting help from experienced colleges in the beginning may be useful *(4)*.

CONCLUSIONS

The invention of the electron microscope made a huge breakthrough in studying different kinds of specimens on ultrastructural and atomic level. Today we have TEMs and SEMs with a wide range of different performances. Although the first protocols of specimen preparation for electron microscopy are still in use today in many laboratories with only slight changes, over the years huge advancements in electron microscopy quality were made. These were achieved by advancements in technology, such as cameras, software, computer image processing, development of the new, different, and more suitable techniques for sample preparation, application of most suitable technique for the specific sample, etc. Since every step of the specimen preparation needs to be correctly executed in order to obtain high-quality results, it is very important to have sufficient practice and to properly teach young researchers, starting with the basis of specimen preparation and electron microscope operation, so they can acquire quality skills, and develop deeper interest in electron microscopy. Finally, advancements in this field of science could prosper even more in the future. This guide was written with that in mind.

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APPENDIX

1. STANDARD PREPARATION OF SPECIMENS

1. Fixation

Chemicals: cacodylate buffer, ($Na(CH_3)_2AsO_2$)
deH_2O
2%-4% glutaraldehyde (GA, $(CH_2)_3(CHO)_2$) in cacodylate buffer
1:1 osmium tetroxide, OsO_4 in cacodylate buffer

Procedure: all steps are done on ice
Primary fixation: slice tissue with razor blade in a drop of GA (< 3 mm^3)
place tissue in vials filled with GA for 1 h on ice
Rinsing: replace GA with cacodylate buffer on ice for 10 min, repeat 2x
Secondary fixation: replace buffer with OsO_4 on ice, incubate for 1h
Rinsing: replace OsO_4 with deH_2O, 10 min on RT (room temperature), repeat 4x

2. Dehydration

Chemicals: ethanol, C_2H_6O dilution series, 50%-96% deH_2O

Procedure: replace deH_2O with 50% ethanol, 10 minutes, RT
repeat with 60%, 70%, 80%, 90%, 96% ethanol, RT

3. Infiltration with transitional solvents

Chemicals: acetone, $(CH_3)_2CO$
96% ethanol

Procedure: replace 96% ethanol with mixture of 96% ethanol and acetone in 1:1 ratio, 30 minutes
Replace mixture with pure acetone, 30 minutes

4. Embedding and polymerization

Chemicals: acetone
ERL 4221-D, vinylcyclohexene dioxide, $C_8H_{12}O_2$, (less toxic substitute for the traditional component in Spurr's embedding medium ERL 4206)
DER 736, diglycidyl ether of polypropylene glycol, $C_{12}H_{22}O_5$
NSA, nonenyl succinic anhydride, $C_{13}H_{20}O_3$
DMAE, 2-dimethylaminoethanol, $C_4H_{11}NO$

Procedure: replace acetone with mixture of acetone and Spurr's resin in 2:1 ratio, 1h
replace mixture with same mixture in 1:1 ratio, 2h
replace mixture with same mixture in 1:2 ratio, 3h
replace mixture with pure Spurr's resin, overnight on a shaker and RT
put labels into the silicon mold and cover with a small amount of pure
Spurr's resin following with putting the samples in the molds and covering with pure Spurr's resin
the resin covering samples must be aligned with the silicon mold surface
polymerization is carried out for 2 days on 63 °C

Equipment: pipette
silicon mold
glass vials with cap
spatula

ice bath
heat shaker
razor blade
digestor
laboratory oven (incubator)

Chemical preparation:

Sodium cacodylate ($Na[CH_3]_2AsO_2 \times 3H_2O$) 0,2 M:
Dissolve 4.28 g sodium cacodylate in 100 ml reH_2O and keep in refrigerator

Cacodylate buffer:
For preparation of 100 ml buffer (0.05 M) pH 7.2 (5.5 – 7.5, depending on the tissue), measure out 25 ml of 0.2 M sodium cacodylate and add reH_2O to 100 ml. Adjust pH to 7.2 with 0.2 M HCl.

Primary fixative (glutaraldehyde 2%-4 %):
Dissolve 200 µL 50% stock solution of glutaraldehyde (2%) in 10 ml cacodylate or phosphate buffer.

Secondary fixative (osmium tetroxide 2%):
Stock solution is made day in advance because of slow solubilization. Wash capsule with osmium tetroxide in 96% ethanol and rinse with reH_2O. Break the capsule and put it in 50 ml reH_2O.

Spurr's resin:
Mix 40 g ERL 4221-D with 32 g DER 736 in a glass and stir with a glass rod. Add 104 g NSA and stir again. Drop 1.6 g DMAE and stir. Keep in refrigerator.

5. Ultrathin sections contrasting

Chemicals: uranyl acetate, $UO_2(CH_3CO_2)_2(H_2O)xH_2O$
lead nitrate, $Pb(NO_3)_2$
sodium citrate, $C_6H_5Na_3O_7$
sodium hydroxide, NaOH
reH_2O

Procedure:

Sectioning:
Adjust thickness of sectioning on ultramicrotome to 70 nm to 90 nm slices, usually 70 nm. Move sections in the cluster of 2 to 4 with an eyelash tool. Grasp a grids rim with forceps and bend grid against filter paper. Submerge grid under the sections and collect them. Dry any excess of water by touching filter paper with underside of the grid.

Uranyl acetate staining:
Place dental wax tile in a Petri dish and drop as many drops of uranyl acetate as there are grids with sections. Immerse grids in drops with sections facing down. Close Petri dish and let it stand for 5-10 minutes. After that, rinse grids with fine stream of room temperature reH_2O. Dry out grids with filter paper.

Lead citrate staining:
Place dental wax tile in a Petri dish together with a couple granules of sodium hydroxide to remove CO_2. Drop as many drops of lead citrate as there are grids with sections. Immerse grids into drops with sections facing down. Close the Petri dish and let it stand for 5-10 minutes. Petri dish needs to be opened as little as possible to reduce CO_2 influx. Rinse grids with fine stream of lukewarm reH_2O. Dry out grids with filter paper.

Chemical preparation:

Uranyl acetate (aqueous solution) 1%-4%:
Make solution a day in advance because of slow dissolution. Dissolve stock solution in reH_2O and keep in dark bottle wrapped up in foil. Keep in a refrigerator.

Lead citrate:
Keep it in a syringe because it forms lead carbonate in contact with CO_2. Dissolve 1.33 g lead nitrate and 1.76 g sodium citrate in 30 ml reH_2O. Shake it for 1 minute and then 5-6 times in the next 30 minutes until lead nitrate becomes lead citrate (milky white color). Prepare fresh 1M sodium hydroxide (40 g in 1L reH_2O) and add 8 ml. Add reH_2O to 50 ml. Keep it in a refrigerator.

Equipment: dental wax
Petri dish
pipette
grids
forceps
filter paper
wash bottle

Grid rinsing with ultrasound

Fill ultrasound bath with small amount of water. Put used grids in a glass, add 100% chloroform and place the glass in ultrasound bath. Expose it to ultrasound for 5 minutes. Replace chloroform and repeat 3 times. After the last chloroform change, overflow grids with acetone and expose to the ultrasound one more time. In the end, put grids in a Petri dish, cover it in half and let it dry out.

2. METHODOLOGICAL EXAMPLE OF IMMUNOGOLD LABELING

Preparation of plant material for transmission electron microscopy and immunogold labeling of glutathione molecules

1. Take small samples of the youngest, fully developed leaves (about 1.5 mm^2).
2. Fix them in 2.5% paraformaldehyde/0.5% glutardialdehyde in 0.06 M phosphate buffer (pH 7.2) for 90 min at room temperature (RT).
3. Microwave fixation is performed in the same fixation solution for two times 25 seconds at 300 W microwave irradiation. In between these steps, cool off samples gently to about 20 °C. The maximum temperature of the solution, which must be constantly aerated to reduce the risk of an uneven heating, during fixation in the microwave oven has to be 30 °C *(25,26)*. Microwave irradiation reduces the time required for fixation and staining for 1/3. Macromolecules morphology and antigen reactivity are preserved, retention of lipids and soluble proteins is better and there is no nonspecific binding of dyes or antibodies. It also yields low-background, high-contrast images. Power of microwave oven and sample temperature needs to be very precise because of specimen sensitivity *(2)*. Embedding media for immunocytochemistry must not physically or chemically affect antigenicity and enzyme activity of specimen. Glutaraldehyde must be used in concentrations less than 1%, and formaldehyde under 4%. Osmium is also rarely used prior to labeling reaction *(2)*.
4. Rinse samples fixed conventionally and with the help of microwave irradiation in a buffer and dehydrate in increasing concentrations of acetone (50%, 70%, and 90%) at RT, 2 times for 10 min for each step.
5. Gradually infiltrate specimens with increasing concentrations of LR White resin (30%, 60%, and 100%) mixed with acetone (90%) and finally embed in LR White resin.
6. Polymerize at 50 °C for 48 h in small plastic containers.
7. Cut ultrathin sections (80 nm) of the samples using an ultramicrotome.
8. Immunogold labeling of glutathione in ultrathin sections can be performed on coated nickel grids with the automated immunogold labeling system Leica EM IGL (Leica Microsystems, Vienna, Austria) according to *(26)* and *(27)*.
9. For cytohistochemical analysis, block the samples with 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS, pH 7.2) and then treat with the primary antibody (e.g., anti-glutathione rabbit polyclonal IgG) diluted 1:50 in PBS containing 1% goat serum for 2 h at RT.
10. Rinse the samples three times for 5 min in PBS.
11. Incubate samples with a 10 nm gold-conjugated secondary antibody (e.g., goat anti-rabbit IgG) diluted 1:50 in PBS for 90 min at RT.
12. Wash samples three times for 5 min in PBS and two times for 5 min in distilled water.
13. Labeled grids are either immediately observed in a transmission electron microscope or post-stained with 2% uranyl-acetate for 15 s, which facilitates the distinction of different cell structures.

– Please note that the antibody does not discriminate between free reduced and oxidized glutathione. The specificity of the immunogold-labeling procedure is tested using negative controls: 1) pre-immune serum instead of the primary antibody, 2) gold-conjugated secondary antibody (goat anti rabbit IgG) without the primary antibody, 3) non-specific secondary antibody (goat anti mouse IgG), and 4) primary antibodies pre-adsorbed with an excess of glutathione for 2 h at RT prior to labeling of the sections. For the latter, incubate solution containing 10 mM of glutathione (GSH or GSSG) with 0.5% glutardialdehyde for 1 h. Saturate the excess of glutardialdehyde by incubation in 1% (w/v) BSA for 30 min. The resulting solution is used to saturate the glutathione-antibody for 2 h prior to its use in the immunogold labeling procedure described earlier.

Quantitative analysis of immunogold labeling: After taking a micrograph, count gold particles in the cell structures (organelles) of interest either manually or automatically, using proper software package. Take care to analyze enough sections and enough number of structures of interest to gain a reliable result, after statistical analysis. Unspecific background labeling has to be determined on different sections outside the specimen and subtracted from the values obtained in the sample.

REFERENCES

1. TIZRO P, CHOI C, KHANLOU N 2019 Sample preparation for transmission electron microscopy. In: Yong W (ed) Biobanking. Methods mol biol. Humana Press, New York, p 417–424. https://doi.org/10.1007/978-1-4939-8935-5_33
2. BOZZOLA J J, RUSSELL L D 1999 Electron microscopy: principles and techniques for biologists. Jones & Bartlett Learning, Boston, p 7–282. https://doi.org/10.1017/S1431927602029975
3. WINEY M, MEEHL J B, O'TOOLE E T, GIDDINGS JR T H 2014 Conventional transmission electron microscopy. Mol biol cell 25: 319–426. https://doi.org/10.1091/mbc.e12-12-0863
4. McDONALD K L 2014 Out with the old and in with the new: rapid specimen preparation procedures for electron microscopy of sectioned biological material. Protoplasma 251: 429–448. https://doi.org/10.1007/s00709-013-0575-y
5. BULLOCK G R 1984 The current status of fixation for electron microscopy: A review. J microsc 133: 1–15. https://doi.org/10.1111/j.1365-2818.1984.tb00458.x
6. HORNE R W 1978 Special specimen preparation methods for image processing in transmission electron microscopy: A review. J microsc 113: 241–256. https://doi.org/10.1111/j.1365-2818.1978.tb00103.x

7. GLAUERT A, LEWIS P 1999 Biological Specimen Preparation for Transmission Electron Microscopy. Princeton univ press 80: 108–338. https://doi.org/10.1515/9781400865024
8. SHIURBA R 2001 Freeze-substitution: Origins and applications. Int rev cytol 206: 45–96. https://doi.org/10.1016/S0074-7696(01)06019-3
9. HURBAIN I, SACHSE M 2011 The future is cold: Cryo-preparation methods for transmission electron microscopy of cells. Biol cell 103: 405–420. https://doi.org/10.1042/BC20110015
10. DE CARLO S 2009 Plunge-Freezing (Holey Carbon Method). In: Cavalier A, Spehner D, Humbel B M (ed) Handbook of cryo-preparation methods for electron microscopy. CRC Press, Boca Raton, p 49–69. https://doi.org/10.1201/9781420006735
11. MCDONALD K L, MORPHEW M, VERKADE P, MÜLLER-REICHERT T 2007 Recent advances in high-pressure freezing. In: Kuo J (ed) Electron Microscopy. Methods mol biol. Humana Press, New York, p 143–173. https://doi.org/10.1007/978-1-59745-294-6_8
12. DING B, TURGEON R, PARTHASARATHY M V 1991 Routine cryofixation of plant tissue by propane jet freezing for freeze substitution. J electron microsc 19: 107–117. https://doi.org/10.1002/jemt.1060190111
13. HOCH H C 1991 Preservation of cell ultrastructure by freeze-Substitution. In: Mendgen K, Lesemann D E (ed) Electron Microscopy of Plant Pathogens. Springer, Berlin, Heidelberg, p 1–16. https://doi.org/10.1007/978-3-642-75818-8_1
14. FAULK WP, TAYLOR G M 1971 An immunocolloid method for the electron microscope. Immunoch 8: 1081–1083. https://doi.org/10.1016/0019-2791(71)90496-4
15. TAO-CHENG J H, CROCKER V, MOREIRA S L, AZZAM R 2021 Optimization of protocols for pre-embedding immunogold electron microscopy of neurons in cell cultures and brains. Mol brain 14: 86. https://doi.org/10.1186/s13041-021-00799-2
16. VAN GENDEREN I L, VAN MEER G, SLOT J W, GEUZE H J, VOORHOUT W F 1991 Subcellular localization of Forssman glycolipid in epithelial MDCK cells by immuno-electronmicroscopy after freeze-substitution. J cell biol 115: 1009–1019. https://doi.org/10.1083/jcb.115.4.1009
17. WEBSTER P, SCHWARZ H, GRIFFITHS G 2008 Preparation of Cells and Tissues for Immuno EM. Methods cell biol 88: 45–58. https://doi.org/10.1016/S0091-679X(08)00403-2
18. GRIFFITHS G, SIMONS K, WARRE G, TOKUYASU K T 1983 Immunoelectron microscopy using thin, frozen sections: Application to studies of the intracellular transport of Semliki Forest virus spike glycoproteins. Methods enzymo 96: 466–485. https://doi.org/10.1016/s0076-6879(83)96041-x
19. TOKUYASU K T 1980 Immunochemistry on ultrathin frozen sections. Histochem j12: 381–403. https://doi.org/10.1007/BF01011956
20. GRIFFITHS G, MCDOWALL A, BACK R, DUBOCHET J 1984 On the preparation of cryosections for immunocytochemistry. J ultrastruct res 89: 65–78. https://doi.org/10.1016/s0022-5320(84)80024-6
21. WEBSTER P, WEBSTER A 2007 Cryosectioning fixed and cryoprotected biological material for immunocytochemistry. In: Kuo J (ed) Electron Microscopy. Methods mol biol. Humana Press, New York, p 257–289. https://doi.org/10.1007/978-1-59745-294-6_13
22. BENDAYAN M, NANCI A, KAN F W 1987 Effect of tissue processing on colloidal gold cytochemistry. J histoche cytochem 35: 983–996. https://doi.org/10.1177/35.9.3302022
23. PETRALIA R S, WANG Y X 2021 Review of post-embedding immunogold methods for the study of neuronal structures. Front neuroanat 15: 763427. https://doi.org/10.3389/fnana.2021.763427
24. SARRAF C E 2000 Immunolabeling for electron microscopy. In: George A J T, Urch C E (ed) Diagnostic and Therapeutic Antibodies. Methods mol med, Humana, Totowa, p 439–452. https://doi.org/10.1385/1-59259-076-4:439
25. ZECHMANN B, MÜLLER M, ZELLNIG G 2006 Intracellular adaptations of glutathione content in *Cucurbita pepo* (L.) induced by reduced glutathione and buthionine sulfoximine treatment. Protoplasma 227: 197–209. https://doi.org/10.1007/s00709-005-0129-z
26. ZECHMANN B, MÜLLER M 2008 Effects of ZYMV-infection on the subcellular distribution of glutathione and its precursors in a highly tolerant *Cucurbita pepo* cultivar. Botany 86: 1092–1100. https://doi.org/10.1139/B08-048
27. ZECHMANN B, MÜLLER M 2010 Subcellular compartmentation of glutathione in dicotyledonous plants. Protoplasma 246: 15–24. https://doi.org/10.1007/s00709-010-0111-2