

Tipps and Tricks for using LR White

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Technical Tips

Distinguishing LR White from Tissue

If you are having difficulty locating your tissue in LR White after it polymerizes due to the fact that the color of your tissue is the same as LR White once polymerized, there is a solution. Dye the LR White using any oil soluble dye. Remember, however, that the intensity of the dye needs to be very high if contrast is to be seen at high magnification. Dyes with phenol or tertiary amine groups should be avoided because they effect the rate of cure of the resin. It is our experience that Sudan Black (cat. # 21610) is the best dye to use due to its color intensity. To make up a stock solution, add the dry stain to the resin until a saturated solution is obtained. Then proceed as usual.

Adjusting pH

If you require the pH of LR White to be neutral (the standard is 5) you can adjust it by adding small amounts of caustic soda or a primary amine. The best primary amine to use is Ethanolamine (absolute) for it will mix into the resin very easily. Small amounts are to be titrated into the resin and the pH should be constantly checked until 7 is reached. Do not add more than 50ml.

Adherence of LR White Sections to Glass Slides for Silver Enhancement Immunogold Labeling

A continuing problem in immunogold labeling of 1 micron thick sections from blocks embedded in the hydrophilic acrylic resin LR White for Light Microscopy is the lack of adherence of the sections to the glass slide during the silver enhancement.

- This problem can be solved by using a 2% formvar solution to coat the glass slide.
- Cleaned glass slides are dipped in a solution of 2% formvar in chloroform and left to dry horizontally.
- Mark the coated surface with a diamond tip scribe and clean the under side of the slide with acetone.
- Transfer the section to the coated slide, which will then be placed in an oven to dry at 50°C.
- Use the Pap-Pen (Cat. #71310) to draw a water repellent circle around the section, which will facilitate the labeling procedures.

Reference:

Slater, M. (1989) Adherence of LR White Sections to GlassSlides for Silver Enhancement. Immunogold Labeling. Vol. 65, No. 6 Stain Technology. pp. 297-299

Using LR White for Light Microscopy

Catalog #14380-14382

Resin embedding for light microscopy provides greatly improved cellular definition compared to paraffin embedding, and for this reason is now widely used in diagnoses particularly of Renal disease, Lymphomas and bone marrow trephines as well as research.

The acrylic resin currently used however are not suitable for EM and the epoxy resin used for EM are not easily stained for light microscopy. LR White however can be used for both purposes and a lymph node for example (12x10x1mm) can be processed, cut and stained for light microscopy then the same block trimmed down cut and stained for electron microscopy.

LR White can also be used for the Histochemical demonstration of some of the more resistant enzymes, and for the immunocytochemical demonstration of intracellular immunoglobulins.

For those laboratories already using an acrylic resin e.g. Hema or Glycol Methacrylate no alteration need to be made to the current processing schedule, but we have laid out here a typical schedule for LR White as guidance for its use.

Fixation

No change from normal fixation need be made if LM only is required from the final blocks (Neutral Buffered Formalin recommended). If however EM is required subsequent to LM then we have found the use of freshly depolymerised Paraformaldehyde (3-4%) in a phosphate buffer pH 7.2 with 2 1/2% w/v sucrose is the best compromise. Glutaraldehyde-Formaldehyde mixtures may lead to very pale staining with Hematoxylin and patchy eosin, whereas normal Formalin fixation gives unacceptable EM structure. For the dual LM/EM role osmium Tetroxide should be avoided due to its effect on many LM stains but 1% Phosphotungstic acid (w/v) in the first absolute ethanol step of dehydration improves electron contrast without adversely affecting most LM stains. If this does not provide adequate electron density then "staining" of ultrathin sections can be carried out with osmium (a brief exposure to 1% aqueous osmium Tetroxide or osmium Tetroxide vapor on a copper grid) or lead citrate.

Dehydration

A graded ethanol series is the method of choice when using LR white. Acetone acts as a radical scavenger in the resin system and traces of acetone left in the tissue at curing can interfere with polymerization.

Infiltration

The extreme low viscosity of LR White allows the use of short infiltration times, but these will obviously depend on the size of the tissue. Infiltrated tissue will become translucent and sink to the bottom of the container.

A typical dehydration and infiltration schedule for a block (12x10x3mm) on a mixer would be:

1. Two changes 70% alcohol 30 minutes each
2. Two changes Absolute alcohol 30 minutes each
3. Infiltrate with L.R. White at RT 2-3 changes 60 minutes each or leave overnight.

Polymerization

Either heat or cold curing can be used for LM, cold curing gives slightly better cutting and staining qualities. When cold curing it is important to cool the molds in a bath of cold water, during polymerization, to disperse the heat produced by the exothermic reaction, but it is not necessary to exclude oxygen from the surface of the curing block.

Some polymerization problems have been experienced when embedding very flat pieces of tissue, which stick to the base of the embedding mold. The way to avoid this is to smear the base of the mold with accelerator before adding mixed resin and allow the tissue to sink to the base of the mold rather than applying pressure.

When thermal curing it is important to limit the contact of oxygen with the resin while polymerization occurs. The most convenient way of achieving this is to use gelatin capsules for small pieces of tissue. Fill up to the brim and slide the other half of the capsule on. For larger specimens the surface of the resin must be covered and one convenient method is to utilize the JB-4 type molds, one being used as a lid for another, or to polymerize in a nitrogen environment.

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Polymerization time and temperature are fundamental to the physical character of the final block, to a much greater extent than with undercured epoxy systems.

We strongly recommend a temperature of 60°C ± 2°C for a period of 20-24 hours. Some ovens are not capable of controlling temperature so closely and if faced with over brittle blocks this is the first parameter to check.

Resin may be used straight from the refrigerator and has a very low toxicity in both monomeric and polymerized states unlike epoxies (see Proc. Roy. Mic. Soc.(1981), 16, Pt.4, p. 265-271). The cold cure accelerator does have some toxic risk and contact with skin and eyes should be avoided.

For cold curing the accelerator should be used at one drop per 10ml of resin and this should cause polymerization in 10-20 minutes. If polymerization occurs faster than this we recommend either more careful metering of the one-drop of accelerator or a higher volume of resin per drop of accelerator.

Cutting and Mounting

Although it is possible to cut LR White on a standard microtome with a steel knife the method of choice would be to use a heavy duty motorized microtome, and glass (Ralph type) knife.

LR White can cut as thin as 0.25 microns on some microtomes, but is very difficult to obtain a satisfactory stain intensity with anything other than Toluidine blue at this thickness, simply because there is so little tissue present in the sections.

For Hematoxylin and eosin staining as well as most other routine stains we recommend sections of 2-3 microns. It is of course possible to cut thicker (up to 15 or 20 microns) if required.

Blocks can be cut dry, the sections picked up and floated out on a 30-40% acetone on a hot plate @ 60-70°C, and then allowed to dry at this temperature. For hard tissues, and blocks which contain a combination of hard and soft tissues, such as marrow trephines, the following floating out fluid is recommended, again on a hot plate @60-70°C.

To 20ml acetone add 0.5ml benzyl alcohol mix then make up to 50ml with distilled water. A section adhesive such as egg albumin can be added to this if required.

Section Staining

Most routine stains give good results on tissue embedded in LR White resin using standard times and temperatures although it may occasionally be necessary to extend some staining times e.g. methyl green pyronin. Stains made up in ethanol or methanol should be avoided as these solvents soften the resin and may remove sections from the slide. Dehydration of sections through graded alcohols after staining should also be avoided. Sections should be blotted air dried and then mounted in resinous mounting medium.

Using LR White for Hard Tissue

Catalog #14380-14382

LR White can be used for the microtomy of decalcified bone and teeth and also for microtomy or "sawing or grinding" of undecalcified tissues.

Decalcified Tissue

Decalcified tissue may be processed, cut and stained similarly to soft tissue (see using LR White for Light Microscopy), except that dehydration and infiltration times may need to be extended depending on the size of tissue. It is also recommended that bone be "de-fatted" to improve the penetration of resin into marrow cavities. This can be achieved by using chloroform after dehydration, returning to absolute alcohol to remove the chloroform before infiltrating with resin and polymerizing.

Undecalcified Tissue

Dehydration and infiltration times will vary depending on size and density of tissue. Those laboratories using Methyl or Butyl Methacrylate at present can use similar dehydration times, but infiltration will probably be shortened due to the low viscosity of the resin.

Dehydration

A graded series of alcohols should be used for dehydration of tissue, and when processing bone "de-fatting" is recommended to improve the penetration of resin into marrow cavities. This can be done using chloroform, for the same length of time that would be necessary to clear the tissue. The bone should then be taken back to absolute alcohol and given sufficient changes to remove the chloroform before infiltration with LR White (Hard grade).

Infiltration

Several changes of resin will be necessary and impregnation under vacuum is recommended.

Polymerization

The tissue can be heat or accelerator cured after embedding in strong plastic molds, such as JB4 or Peel-A-Way type, or aluminum foil dishes. (#70176-10 to 70176-30).

When heat curing the molds should first be filled with resin then the tissue added and orientated. Polymerization will occur in 18-24 hours at $60^{\circ}\text{C} \pm 2^{\circ}\text{C}$. The surface of the block exposed to oxygen may remain slightly sticky, but this will not affect the cutting quality of the face of the block. Some ovens are not capable of controlling temperature so closely and if faced with over brittle blocks this is the first parameter to check.

When accelerator or cold curing the molds should be placed in a bath of ice cold water to disperse the heat produced during the exothermic polymerization. The base of the molds should first be smeared with accelerator using a cotton wool bud or swab, the accelerator is then added to the resin, 1 drop per 10ml resin, and thoroughly mixed before pouring into the mold, the tissue is then placed into the mold and orientated. Polymerization should occur in 10-20 minutes, if it occurs faster than this we recommend either more careful metering of the one-drop of accelerator or a higher volume of resin per drop of accelerator. NB: the accelerator does have some toxic risk and contact with skin and eyes should be advised.

Cutting and Mounting

Bone marrow trephines and small pieces of cancellous bone may be cut using a motorized heavy duty microtome, but larger pieces of cancellous bone, cortical bone and teeth offer too much resistance to the microtome knife and preparations of this material must be prepared by sawing and grinding.

Microtomy

Sections can be cut, using Ralph type glass knives for trephines or a tungsten carbide knife for larger pieces of cancellous bone, from 2-10 micron. Blocks can be cut dry, the sections picked up and floated out on a hot plate at $60^{\circ} - 70^{\circ}\text{C}$ using the following solutions: to 20ml acetone add 0.5ml benzyl alcohol mix then make up to 50ml with distilled water. A section adhesive such as egg albumin can be added to this if required. Sections should be allowed to dry on the hot plate for at least 30 minutes before staining.

Sawing and Grinding

Thick slices 150-200 micron can be cut using a milling machine and then ground to the required thickness, usually 20micron for staining of 70micron for microradiography; the section is inclined to fragment if grinding is continued much below 20 micron.

Using the newer types of saw microtome, such as the Leitz 1600, which has a diamond coated internal hole saw, sections can actually be cut at 20micron and no further grinding is necessary.

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Section Staining

Sections of material embedding in LR White are stained "free floating", times of staining are usually longer than those for paraffin, sections and dehydration through alcohols should be avoided. A recommended schedule for Hematoxylin and Eosin staining is as follows:

1. Remove Ca deposits, which would otherwise interfere with the staining, from the section by treating it with Kristensen's Decalcifying Solution for about 15 minutes.
2. Wash in running tap water for a few minutes to remove the formic acid from the tissue.
3. Transfer the section to several changes of distilled water, a few minutes each and then into a 0.5% w/v solution of periodic acid in distilled water where it is left for 5 minutes.
4. Wash the section with several changes of distilled water and stain with Harris Hematoxylin for about 1 hour.
5. Transfer the section, after a short time in distilled water, into running tap water to "blue" the Hematoxylin stained tissue.
6. Check the Hematoxylin stain; if the tissue should be overstained or the surface of the resin has become stained with Hematoxylin, this may be corrected by a short differentiation in acid alcohol (0.5% HCl in 70% ethanol) and "reblueing" of the stained tissue.
7. Rinse the section in distilled water and Counterstain it, using a 5% solution of Eosin Y in distilled water; leave the section in the Eosin stain for 30 minutes to 1 hour, wash briefly in running tap water and check the staining of the tissue. Nuclei and Hematoxophilic elements should be bright blue, cytoplasmic structures in various shades of red-pink.
8. Rinse the section in distilled water, blot dry with filter paper and either clear briefly in Xylene and mount in DPX, or mount directly in LR White resin by adding a drop of accelerator to 1ml of resin.

Using LR White for Electron Microscopy

Catalog LR White Medium grade #14380

When using LR White embedding resin for dedicated electron microscopy, very few changes need to be made to the regime used for epoxy resin embedding. Every laboratory has its own individual embedding schedule but we have laid out here a typical schedule for LR White as guidance for its use.

Fixation

No change from normal fixation should be made, if EM only is required from the final blocks.

If, however, good ultrastructure and a wide range of LM, staining is required then we have found that the use of freshly depolymerised Paraformaldehyde (3-4%) in a phosphate buffer pH 7.2 with 2 1/2% w/v sucrose is the best compromise. Glutaraldehyde alone and Karnovsky's Glutaraldehyde de-formaldehyde mixtures may lead to patchy LM staining and some stains not working or giving "false positives" (e.g. PAS) whereas normal Formalin fixation yields unacceptable EM ultrastructure.

For the Dual LM/EM role Osmium Tetroxide should be avoided due to its effect on many LM stains, but 1% Phosphotungstic acid (w/v) in the first absolute ethanol step of dehydration improves electron contrast without adversely affecting most LM stains. Osmium Tetroxide may be used if the blocks are required for dedicated electron microscopy only.

Dehydration

A graded ethanol series is the method of choice when embedding in LR White. Acetone acts as a radical scavenger in the resin system and therefore traces of acetone left in the tissue at curing can interfere with this polymerization. For this reason the use of graded acetone series and 2,2-dimethoxypropane (which generates acetone) are best avoided. If the use of 2,2-dimethoxypropane is considered vital we recommend either a protracted resin infiltration or washing the tissue with dry ethanol prior to infiltration in order to minimize the chance of acetone contamination of the final resin.

Infiltration

The extreme low viscosity of LR White may be exploited by allowing the use of short infiltration times or large specimens BUT NOT BOTH! A 1mm cube of animal tissue will be adequately infiltrated in a bout 3 hours if 46 changes of LR White at 60°C are employed during this period. An overnight infiltration at room temperature, followed by two short changes of resin will often be more convenient, however. The long shelf

life and low extraction rate of LR White allows specimens (perhaps reserve tissue) to be stored safely in resin for many weeks at 40°C if required. Larger blocks do require significantly longer infiltration times than small ones.

Polymerization

Osmium Tetroxide reacted tissues should not be “cold-cured” with the accelerator. This process is strongly exothermic and the dark color of the tissue leads to a focal heat accumulation, which can cause local problems in and around the tissue.

If the tissue is not Osmium Tetroxide post fixed then curing with LR White accelerator may be employed. As with cutting blocks for light microscopy we recommend cooling the molds during polymerizing, but there is no need to exclude oxygen from the surface of the curing block.

Thermal curing should be used for osmicated specimens and may be used for all specimens. Here it is important to limit the contact of oxygen with the resin while polymerization occurs. The most convenient way of achieving this with capsule type embedding is to use gelatin capsules, fill up to the brim and slide the other half of the capsule on.

If flat embedding is required for cutting orientation then the surface of the resin must be covered to prevent contact with oxygen. One convenient method is to utilize the JB-4 type molds and chucks, useful for light microscopy, and after polymerization the block may be sawn off the stub and mold re-used.

Polymerization time and temperature are fundamental to the physical characteristics of the final block, to a much higher degree than with under cured epoxy systems.

We strongly recommend a temperature of 60° ± 2 for a period of 20-24 hours. Some ovens are not capable of controlling polymerization temperature so closely, and if faced with over brittle blocks, this is the first parameter to check.

LR White has extremely good powers of penetration and can penetrate and soften some low-density polyethylene capsules. This causes them to distort and collapse. Also polyethylene is not impermeable to oxygen and may allow enough contact with atmospheric oxygen to give the blocks an inhibited “tacky” surface. Both these problems may be overcome by the use of gelatin capsules (size 00 is similar to the popular polyethylene capsule size, Cat #70100) and these are much cheaper and easier to seal during polymerization.

Resin may be used straight from the refrigerator and has a very low toxicity both in monomeric and polymerized states, unlike epoxies (see Proc. Roy. Mic. Soc. 16, Pt 4, p. 265-271). The cold cure accelerator does have some toxic risk and contact with the skin and eyes should be avoided.

For cold curing the accelerator should be used at one drop per 10ml of resin and this should cause polymerization in between 10 and 20 minutes. If polymerization occurs faster than this we recommend either more careful metering of the one-drop of accelerator or a higher volume of resin per drop of accelerator.

Trimming and Cutting

Trimming the block may be done with jewelers saw, razor blade or with a glass knife on the ultramicrotome as with epoxy resin blocks.

Cutting, too, may be performed in the same way as for epoxy resin with glass or diamond knives. A typical cutting speed of about 1mm per second is suitable.

Section Staining

All the common section stains give good results on tissue embedding in LR White resin. Stains made up in ethanol or methanol should be avoided as these solvents soften the resin and may remove sections from grids. As an alternative to uranyl acetate, 1% Phosphotungstic acid has proved a good general-purpose stain, both as a block stain, as mentioned earlier and as a section stain.

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In the Electron Microscope

An initial reduction in electron density may accompany the initial exposure of the resin to the beam. This is thought to represent a loss of water, imbibed from knife boat or staining solutions. Thinning as such does not occur and specimens have been kept stationary under a 120 Kv electron beam for 3 hours with no obvious signs of damage.

Using LR White for Immunohistochemistry

Catalog #14380-14382

Sections from LR White embedding tissue have been used successfully for immunocytochemistry at both the light microscope and electron microscope levels. This demonstrates quite clearly that the visualization steps of the immunocytochemical procedure will penetrate the resin and react with tissue antigens if they have been preserved in tissue.

As with all immuno-localizations the key factor is whether or not the tissue antigen has survived fixation, processing and embedding in such a form as to be recognizable to the specific antibody. This is difficult to predict with certainty, but some antigens have been shown to be highly resistant whilst others are fickle even in unfixed frozen sections.

Much interest has centered on using immunocytochemistry to detect protein hormones and the various classes of immunoglobulin and generally these classes of antigen have proved resistant to alteration both in processing to paraffin wax and to LR White resin.

It is the special hydrophilic nature of LR white which allows immunochemicals to permeate the supporting resin and reach its sites of binding and no resin pretreatment is necessary, or indeed possible, to facilitate this penetration.

We have been successful with LR White blocks only when they have been thermally cured, probably because when accelerator-curing the resin the exotherm produced is sufficient to damage the integrity of the tissue antigen. Some workers have also reported that a slight under-cure of LR white say at 55°C for 20-24 hours aids subsequent penetration of antisera, but we have obtained good results without deviating from the standard polymerization schedule.

If the particular antigen under consideration has already been localized in paraffin wax sections then a trusted fixation regime will be established and should be adhered to. For those approaching the problem for the first time there is an extensive bibliography available regarding fixation for immunocytochemistry, much of it contradictory, and a reference list is provided as some guidance. Rules of thumb seem to be to avoid Glutaraldehyde and perhaps use an acid rather than a neutral fixative, but there are many conflicting and strongly held views on the topic.

Similarly, the need to enzyme digest sections prior to reaction is fraught with controversy and may indeed be linked to the fixation regime chosen. We have used both protease type VII and trypsin type II to good effect on our neutral buffered Formalin fixed materials.

If frozen, dewaxed or etched epoxy resin sections are used for immuno-staining, the tissue is not surrounded by a supporting matrix when they are being reacted. When using LR White sections the resin is still intact and therefore diffusion to the sites of reaction must occur prior to reaction of antisera with antigen. For this reason we have found it necessary to use antisera at approximately ten times the concentration that would work on dewaxed sections. The exact titre of each antibody will depend upon its source and how well it has been stored, but we have used many commercial anti-immunoglobulins at about a 1 in 10 dilution.

For the same reason the antibody stages of the reaction often benefit from a longer incubation time. Up to two hours at room temperature or overnight at 4°C in a moist chamber may be used.

Various immunoperoxidase techniques have given results on LR White tissue sections including the peroxidase-antiperoxidase complex method (PAP) (Sternberger, 1970), the hapten sandwich technique

(Jasani et al, 1981), and the indirect peroxidase method. The Avidin-biotin-peroxidase complex method if Hsu has not been successful in our hands with LR White embedding material, probably due to the molecular size of Avidin. As fairly strong antibody concentrations are required, a highly sensitive method of detection is to be preferred and for this reason the PAP or hapten sandwich techniques are more suitable than a two-layer indirect peroxidase reaction.

Visualization for the bound peroxidase is achieved with the diaminobenzidine-peroxide reaction as described by Graham & Karnovsky (1966).

Any technique where the sections are subjected to hydrogen peroxide solutions twice during staining is likely to tend to lift sections from the slides. We have found that poly L lysine (MW 350,000) is an excellent adhesive for immunocytochemical work, and also care should be taken to dry sections onto slides very thoroughly in an oven rather than a hot plate at 60°C for two hours. This step should not have any effect on the antigenicity of the tissue, as it will already have spent 20-24 hours at 60° during polymerization.

It is clear that not "standard" immunohistochemical staining regime can be cited as there are some any variables, but a "typical" regime is described below for general guidance.

Pap Procedure for L.R. White Sections (3 micron)

1. Block Endogenous Peroxidase with 1% Phenylhydrazine Hydrochloride in PBS (optional) .. 30 minutes
2. Wash in PBS 1x5 minutes, and 2x5 minutes at 37°C
3. 0.1% Trypsin in 0.1% CaCl₂ (aqueous) ... 20 minutes
4. Wash in ice cold distilled water ... 10 minutes
5. 2% Goat serum in PBS... 20 minutes
6. 1° antibody (approx. 1:10 dilution) 2 hours at 37°C or overnight at 4°C (usually Dako or Nordic)
7. Wash in P.B.S...10 minutes
8. Goat anti rabbit antibody (approx. 1:20 dilution) 2 hours at 37°C or overnight at 4°C.
9. Wash in P.B.S. ... 10 minutes
10. PAP at 1:10 dilution (Dako) 2 hours at 37°C or overnight at 4°C
11. Wash in P.B.S. ... 10 minutes
12. Wash in Tris HCl pH 7.6 ... 10 minutes
13. DAB/H₂O₂ (Graham and Karnovsky) ... 15 minutes
14. Wash in distilled water.
15. Counterstain as required.

Note: FOR IMMUNOCYTOCHEMISTRY LR WHITE MUST BE THERMALLY CURED AND NOT ACCELERATOR CURED.

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Using LR White for Electron Microscopic Immunocytochemistry

Catalog #14380

LR White resin has five advantages, which can be exploited for the localization of antigens in sections of fixed and embedded tissue under the electron microscope.

1. It is a hydrophilic embedding agent, which means that ultrathin sections allow the passage of aqueous solutions even at neutral pH as opposed to epoxides, and polyesters, ultra thin sections of which are much less permeable.
2. Its lipid solvency is apparently low for a plastic embedding agent and therefore membrane and cytosol structures can be observed under the electron microscope even when osmium has not been used to stabilize lipids. No low temperature methods are required although tissue structure is much improved by perfusion fixation methods.
3. It does not, where it has so far been tested, prevent the demonstration of antigens by immunochemical techniques. No "etching" or protease digestion has so far been necessary.
4. It is beam-stable, standing up well to even quite low KV's thus representing a considerable advance over the more commonly used methacrylates.
5. It tolerates rapid, partial dehydration, accepting tissue from 70% ethanol. Such tissue has an improved antigenicity over tissue, which has been fully dehydrated.

Fixation

To preserve antigenicity post-fixation in osmium Tetroxide is best avoided but then a variety of possibilities is available depending on the requirements of the investigator. If tissue blocks are kept small (1-3mm³) four to six hours fixation in freshly depolymerised and purified 4% Paraformaldehyde in 0.1M phosphate (Sorensen's) buffer pH 7.3, is recommended for the preservation of maximum tissue antigenicity. Tissue is then washed overnight in buffer solution. Picric Acid, included with formaldehyde with its greater power to cross-link proteins, undoubtedly stabilizes tissue structure to a greater degree than does formaldehyde, but in turn, tissue thus fixed demonstrates a reduced antigenicity. It is strongly recommended that vacuum-distilled, purified forms of monomeric or trimeric Glutaraldehyde are used, to increase method reproducibility and avoid deleterious fixative impurities. Three to four hours in 1-2% Glutaraldehyde in 0.1M phosphate (Sorensen's) buffer pH 7.3, is perfectly adequate, remembering that the lower the concentration of Glutaraldehyde the higher the antigenic yield. Tissue is then washed overnight in buffer.

The effects of the avoidance of osmium Tetroxide can be partially compensated, without detriment to tissue antigenicity, by the inclusion of picric acid in the Glutaraldehyde solution. The suggested solution is as follows: 50% purified Glutaraldehyde – 2ml, picric acid (sat. aq) – 15ml and 0.1M phosphate (Sorensen's) buffer pH 7.3 – 83ml (BGKPA) giving a 1% Glutaraldehyde, 0.2% picric acid solution. The percentage of Glutaraldehyde and picric acid can be increased to give more stable ultrastructure but this may be at the expense of antigenicity. Two to four hours for the fixation of 1-3mm³ blocks is recommended. More membrane structure is seen with this fixative than when Glutaraldehyde is used alone, especially if it is perfused into animal tissue, when the molarity of the buffer should be decreased (0.05M-0.08M).

Further steps, such as post-osmication or "block-staining" should not be carried out because they may reduce immunocytochemical sensitivity – either through reduced antigenicity or enhanced background.

Dehydration

Tissue fixed in aldehyde alone, after buffer rinses, is dehydrated in a graded ethanol series. Tissue fixed in aldehyde/picric acid is placed directly into 70% ethanol where some of the picric acid can be washed out. Reducing the time in, and concentration of, ethanol used in dehydration will often increase antigenic yields. LR White will accept tissue from 70% ethanol so that after two washes of 30-60 minutes each blocks may be transferred into LR White. When osmium is avoided tissue shrinkage can be a problem and tissue taken from fixative to 70% ethanol and then straight into LR White may show shrinkage as fact. This can be lessened by introducing an intermediate step of diluted LR White which is 2:1 LR White to 70% ethanol. Be careful not to carry over large amounts of 70% ethanol when transferring tissue.

Infiltration

Even though the tissue may be taken from 70% ethanol into LR White no special procedures are necessary. One change for an hour, followed by an overnight change (preferably on a rotamix) and then a final change the following morning before embedding, using gelatin capsules, is usually sufficient unless the blocks of tissue are particularly large (i.e. in excess of 3mm³). As in the application sheet on LR White in routine electron microscopy, blocks may be stored in unpolymerized resin at 40°C for weeks if necessary.

Polymerisation

Some special care must be taken to make sure that tissues undergoing embedding are not exposed to temperatures in excess of 55°C if antigenicity is not to be impaired. The "cold-cure" procedure (see "Using LR White for EM"), therefore, even though the tissue is not osmicated, should not be used (the exothermic reaction may exceed 60°C). Instead an accurate oven or incubator set at 50°C is preferred and a 24 hour polymerization time is given. Although anaerobic polymerization is advised for Beem capsules, in fact LR White, when contained in gelatin capsules, fully filled and tightly capped, polymerizes well in a 50°C oven without further precautions.

Trimming, cutting and use in the electron microscope are as for the applications sheet for electron microscopy.

Staining

The choice of immunolocalization technique is entirely up to the user and PAP, hapten-anti hapten, Avidin-biotin or gold-colloidal methods may all be adaptable. Some useful publications to help with this choice are given in the reference at the end of this application sheet.

Of course, from time to time, ultra-thin section so tissues fixed, processed and embedded as described above should still be stained with uranyl acetate (15 mins) followed by lead citrate (3 mins) on the grids as for routine electron microscopy. The appearance of such tissues is different from that of post-osmicated tissue and it is important that the observer understands the material with which he's working.

Immunostained sections can be counterstained with lead citrate but if peroxidase techniques are in use this may confuse the picture.

References

Fixation

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Fixation and Embedding in L.R. White

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