LR White Sections as Slot Grid Support Films for Transmission Electron Microscopy

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Abstract

The utility of LR White sections as slot grid support films for the examination of thin resin-embedded tissue sections by TEM was investigated and compared with traditional formvar-carbon films. Throughout a variety of staining procedures, which involved the use of organic solvent, oxidising agents, strong acid and prolonged incubation, LR White support films remained intact and the attached tissue sections remained adherent. In contrast, complete loss of formvar-carbon support films occurred in 25% of preparations during routine staining with aqueous reagents. This loss increased to 62% following staining with either alcoholic or oxidising and acidic stains, and to 66% following prolonged (immunohistochemical) staining. Tissue contrast, ultrastructural detail and immunohistochemical staining intensity were comparable between sections on the two types of support film. The use of LR White sections as support films for slot grids represents a quick, cheap, simple and robust alternative to traditional support films and, furthermore, requires no carbon coating.

Introduction

The examination of attenuated structures, such as transverse sections of cultured cell monolayers, by TEM can often be frustrated by obscuring grid bars. Coated slot grids for the uninterrupted examination of such specimens can be prepared or purchased, but these must be handled with the utmost care less they become damaged and therefore a simple and robust alternative seems desirable.

Support films for EM grids have traditionally been prepared from collodion, formvar or pioloform (Anderson & Stanley, 1941; Drummond, 1950; Hawn & Porter, 1947; Stockem, 1970) and these are often further stabilised by coating with carbon. Beam-stable embedding resins have been available for many years yet their utility as support films has not, to our knowledge, been investigated.

Ideally, a support film should be mechanically strong, electron beam-stable and of low cross-scatter, a property met, in particular, by the acrylic resins (Carlemalm et al., 1985).

Here we describe a novel technique for producing thin, electron beam-stable support films using acrylic resin sections that require no carbon coating, which are sufficiently strong to be used on slot grids and which can withstand prolonged immersion through a variety of staining solutions without obvious damage.

Materials and Methods

Preparation of LR White Support Films

Hard grade pre-catalysed LR White acrylic resin (London Resin Company, Reading, U.K.) was dispensed into size '0' gelatine capsules and polymerised for 24 hours at 50^{o}C. Following removal of the gelatine capsule, the block face was trimmed to a trapezium and thin (60nm) sections cut with either a glass or diamond knife on an Ultracut E ultramicrotome. Optimal sectioning speed was found to be 1.5 mm/second. Copper or nickel

slot grids (2 x 1 mm) were cleaned (5 seconds agitation in acetone, ethanol and then in 0.2µm filtered double distilled water) and used immediately, while the grid surface was still wet. Sections were collected onto either the dull or shiny side of the grids, either by immersing the grid in the knife boat and approaching the sections from underneath or by lowering the grid onto the section from above, and then allowed to air dry.

Tissue Sections

In preliminary experiments, to evaluate the stability of acrylic resin support films during routine staining procedures, thin (80nm) sections of archival 2.5% glutaraldehyde-fixed, 2% osmium tetroxide-postfixed araldite-embedded cultured cells grown on cell culture inserts were collected onto LR White or formvar-carbon coated copper slot grids (Agar Scientific Ltd., Essex, U.K.) by either lowering the coated grids onto the sections from above, or immersing the grids and approaching the sections from underneath.

To further evaluate the robustness of the acrylic resin support films, additional staining procedures were performed which involved the use of organic solvent (methanolic uranyl acetate), oxidising conditions and strong acid (which are used in the initial stages of the osmium-ammines technique (Cogliati and Gautier, 1973) and prolonged staining (immunohistochemistry). In case the formvar-carbon coated slot grids did not survive the staining procedures, additional sections were collected onto 100 mesh formvar-carbon coated gold grids (Agar Scientific Ltd., Essex, U.K.).

For immunohistochemical staining, thin (80nm) sections of archival 1% glutaraldehyde perfusion-fixed, 2% osmium tetroxide-postfixed epon-embedded rat pancreas (Newman & Hobot, 2001) were collected onto LR White or formvar-carbon coated nickel slot grids (Agar Scientific Ltd., Essex, U.K.). Determination of an appropriate antibody titre was performed on tissue sections collected onto naked 300 mesh nickel grids.

Staining

Staining and washing of sections was performed by passing the grids through droplets of solution. In all staining procedures, unsupported sections were stained by immersion whilst those on LR White or formvar-carbon films were stained by flotation.

In the routine staining experiment, above, sections were stained for 15 minutes in 4% aqueous uranyl acetate and 5 minutes in Reynolds' lead citrate (Reynolds, 1963). For methanolic uranyl acetate staining, sections were stained in saturated uranyl acetate in methanol for 10 minutes, washed for 2 x 1 minute in methanol and 1 minute in H_2O and air dried. For the initial stages of the osmium-ammines technique, sections were treated with 2% H_2O_2 for 20 minutes, washed for 3 x 1 minute in H_2O and then treated with 5M HCl for 15 minutes, prior to washing in H_2O and air drying.

Immunohistochemical Staining

Sections were pre-treated for 1 hour in saturated aqueous sodium periodate (Bendayan and Zollinger 1983), washed for 3 x 1 minute in double distilled water followed by equilibration for 10 minutes in 0.6% bovine serum albumin (BSA) in 100mM phosphate buffered saline pH 7.4 (PBS/BSA). Sections were immunohistochemically stained for 1 hour with mouse anti-amylase monoclonal antibody (Autogen Bioclear, Calne, U.K.) diluted in PBS/BSA (initially at doubling dilutions from 1/50 to 1/3200, including a primary antibody omission control on unsupported sections, to determine an appropriate titre, and applied thereafter on experimental sections at 1/500). Following a 1 minute wash in PBS/BSA and 2 x 1 minute washes in 0.6% BSA in 20mM Tris HCl buffer pH 8.5 (TB/BSA), sections were incubated for 1 hour in goat anti-mouse IgG 10nm colloidal gold conjugate (prepared in-

house) in TB/BSA. Sections were washed for 1 minute in TB/BSA, 2 x 1 minute in double distilled water and counterstained with uranyl acetate and Reynolds' lead citrate as above.

Light and Electron Microscopy

Light micrographs of LR White films on slot grids were acquired by attaching the grids to a coverslip, tilting it a approximately 45^{o} to the horizontal and collecting reflected light from an angle poise lamp through a x2 (0.05 NA) objective of an Olympus BX51 microscope (Olympus Optical Co. (U.K.) Ltd, London, U.K.). A focal series of images were acquired and combined using a Zeiss Axiocam and Axiovision software (Carl Zeiss Vision GmbH, Hallbergmoos, Germany).

For transmission electron microscopy, samples were examined in a Philips CM12 TEM at 80kV and digital photomicrographs captured with a Megaview III digital TEM camera and iTEM software (Soft Imaging System GmbH, Münster, Germany) with and without automatic gain correction, the latter being comparable to traditional photographic plates and thus facilitating comparison of staining contrast under otherwise identical imaging conditions.

Results

Perhaps not surprisingly, the diamond knife gave superior results to glass knives in that it provided a constant ribbon of more uniform sections. Sections produced using glass knives were perfectly acceptable although quality began to deteriorate after about 10 sections had been cut. Attempts to cut sections that were thinner that 60nm did not usually meet with success and a thickness of 60nm seemed, in our hands, to be the lower practical limit for cutting such large sections. Sections collected onto immersed grids tended to be much less wrinkled (Fig. 1a) than those collected by placing the grid onto them from above (Fig. 1b),

even though the latter technique was slightly easier to perform. When picking up sections from underneath, the dull side of the grid wetted much better than the shiny side and facilitated complete coverage of the slot with the section. If the grid failed to wet properly, it was best to repeat the washing procedure. Where coverage was incomplete, sections could often be gently dragged across the wetted grid surface with an eyebrow hair attached to a cocktail stick to completely cover the slot.

Tissue sections, that were collected by immersing the coated grids in the knife trough and approaching the sections from underneath, were less wrinkled after air drying (Fig. 2a) than those that had been collected by lowering the support film onto them from above (Fig. 2b). When picking up tissue sections from underneath, it was sometimes found that the uncoated part of the grid did not wet properly, causing the meniscus to rapidly retract from the grid and thus making controlled collection of tissue sections difficult. This problem was overcome by tilting the grid at approximately 45^{o} as it was lifted.

Throughout all the staining procedures, resin support films remained attached to the slot grids, as did the tissue sections attached to them. The forceps holding the grids could even be gently tapped against the finger during the staining procedure to remove surplus reagents with no apparent damage to the film. In the case of methanolic uranyl acetate staining, wrinkling of the LR White support film was observed which, while restricted mainly to the periphery, occasionally traversed the whole support film. The wrinkles were thin and only restricted observation of structures directly overlying them. Even when the resin support film did not completely cover the slot, they remained stable throughout the staining procedure and during subsequent examination by TEM. In contrast, there was a 25% (3/12) loss of formvar-carbon films following routine staining using aqueous reagents. For both methanolic uranyl acetate staining and treatment with H_2O_2/HCl, this loss increased to 62% (5/8) and to 66%

(8/12) following immunohistochemical staining, even though the utmost care was taken and no tapping was performed.

LR White-supported sections benefitted from preliminary equilibration of the support film in the electron beam at low magnification prior to examination at higher magnifications. Once the LR White support film had stabilised and cleared in the beam, staining contrast (as judged by comparison of images without automatic gain correction) (Fig. 3a) and ultrastructural detail (with automatic gain correction) (Fig. 3c) in supported tissue sections was comparable with those on formvar-carbon film (Figs. 3b and 3d respectively) as was immunohistochemical staining intensity between the LR White-supported (Fig. 4a) and formvar-carbon film-supported (Fig. 4b) sections.

Discussion

Coated slot grids are 5 to 10 times more expensive than their uncoated counterparts. Although they can be prepared in-house, the process is time consuming, requires considerable skill, and carbon coating is essential. By contrast, the technique of preparing support films from LR White sections for the examination of thin biological tissue sections is both quick, cheap and requires no carbon coating equipment. In addition, LR White support films are more stable under a variety of staining conditions than their formvar-carbon counterparts. LR White was chosen for its ease of use, but similar results might well be expected from other commercially available cross-linked acrylic resins and the reader is invited to experiment.

The greater stability of the LR White support films during the staining procedures may have been due, in part, to their greater thickness, 60nm, compared to 40-50nm for the formvar-carbon film (Agar Scientific, personal communication) but also to the 3-dimensional cross-linked nature of the acrylic resin. Heat polymerisation of the LR White resin is

recommended, as this leads to a more thorough cross-linking of the resin than is achieved by the chemical catalytic method (Newman & Hobot, 2001).

The LR White support film appears initially translucent when examined in the electron microscope, but rapidly clears in the electron beam to become as transparent as the formvar-carbon film and allows an unimpeded view of fine ultrastructural detail.

Ordinarily, immunohistochemical staining is performed on acrylic resin-embedded tissue sections. In this study epoxy resin-embedded tissue was chosen as part of the evaluation of the robustness of the LR White support film, since epoxy resins require the additional step of prolonged treatment with sodium periodate to reveal antigenic sites.

The use of slot grids is of particular advantage where uninterrupted examination of specimens is required such as at high tilt angles in electron tomography, serial sections and attenuated specimens such as transverse sections of cultured cells. In addition, support films in general are of particular value for specimens that embed poorly, such as some cell culture inserts and dermatological samples. LR White support films on slot grids are now routinely used by the Electron Microscopy Unit, University Hospital of Wales for examining thin sections of epoxy resin-embedded dermatology specimens for diagnostic purposes (Pitman, R. personal communication).

Here we have described the use of LR White support films for slot grids, but they can, of course, find equal utility when used on ordinary mesh grids. In this case, much thinner sections can be cut if the block face is made smaller. If desired, these can then be manoeuvred to form a raft of sections with a view to covering the greater part of an ordinary grid.

In conclusion, LR White sections provide a quick, simple and robust alternative to traditional support films for slot grids.

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Figures

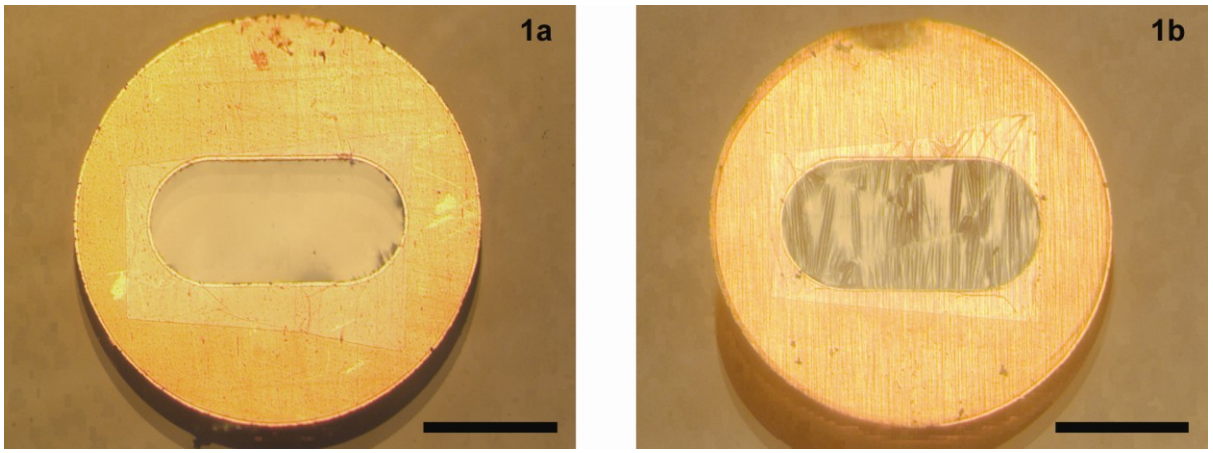

Fig. 1. Light micrographs of LR White support films on 2 x 1 mm slot grids showing their appearance when collected by approaching them (a) from underneath or (b) from above. Images have been stretched in the y axis, for purely aesthetic reasons, to correct for the foreshortening that resulted from tilting the slot grids at 45^{o}. Scale bar 1mm.

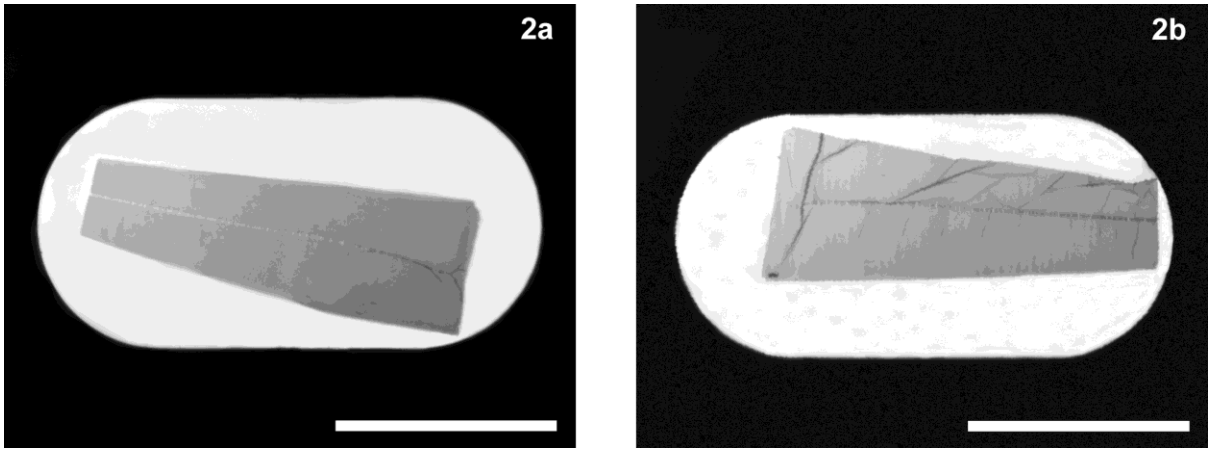

Fig. 2. Electron micrographs of thin section of resin-embedded cultured cells showing the effects collecting the sections by immersing the slot grid in the knife boat and approaching them (a) from underneath or (b) by lowering the slot grid onto the section from above. Scale bar 1mm.

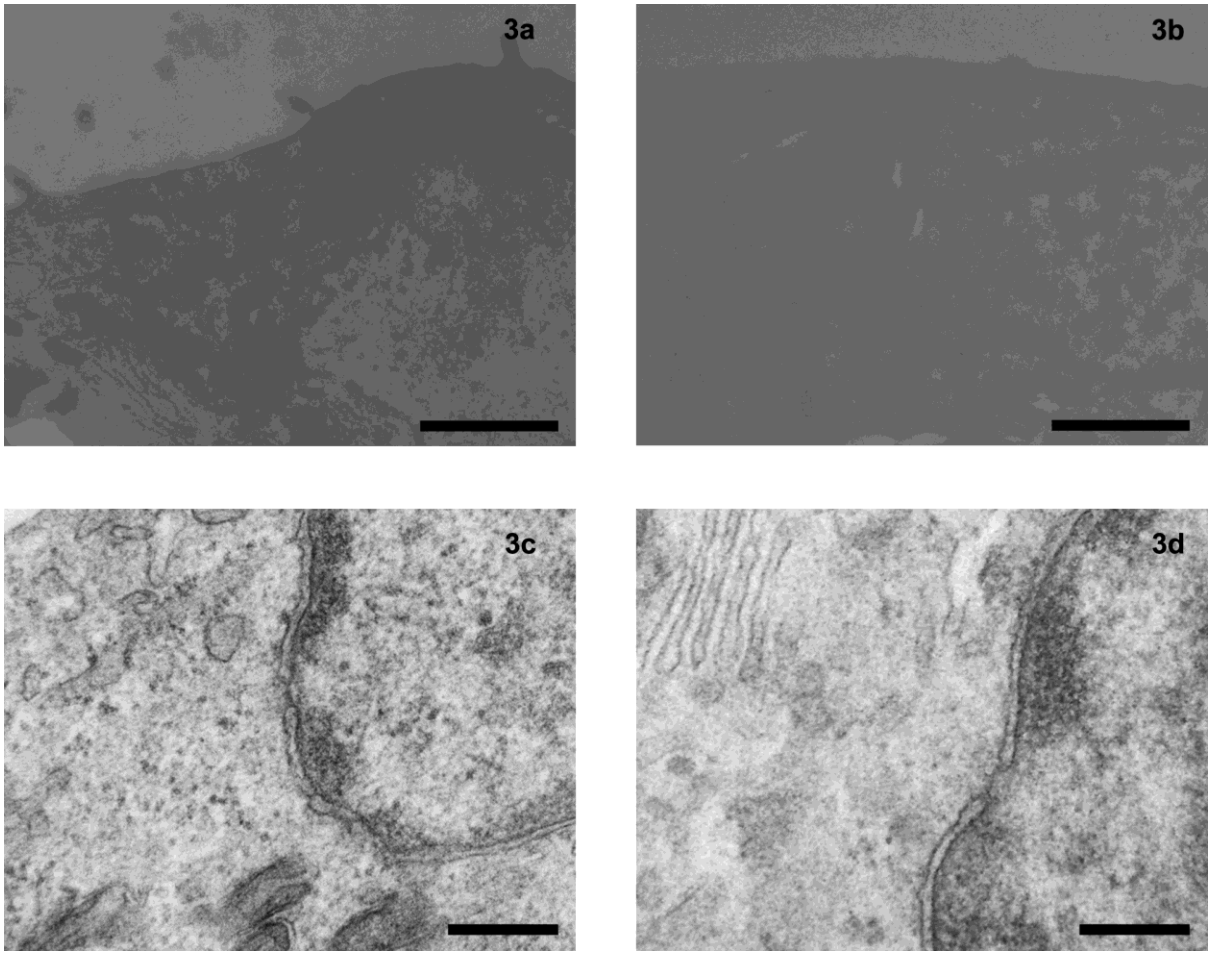

Fig. 3. Electron micrographs of sections of araldite-embedded cultured cells, on LR White (a and c) and carbon-formvar support films (b and d), without automatic gain correction to show staining contrast (a and b) and with automatic gain correction showing ultrastructural detail (c and d). Scale bar 1μm (a and b) and 500nm (c and d).

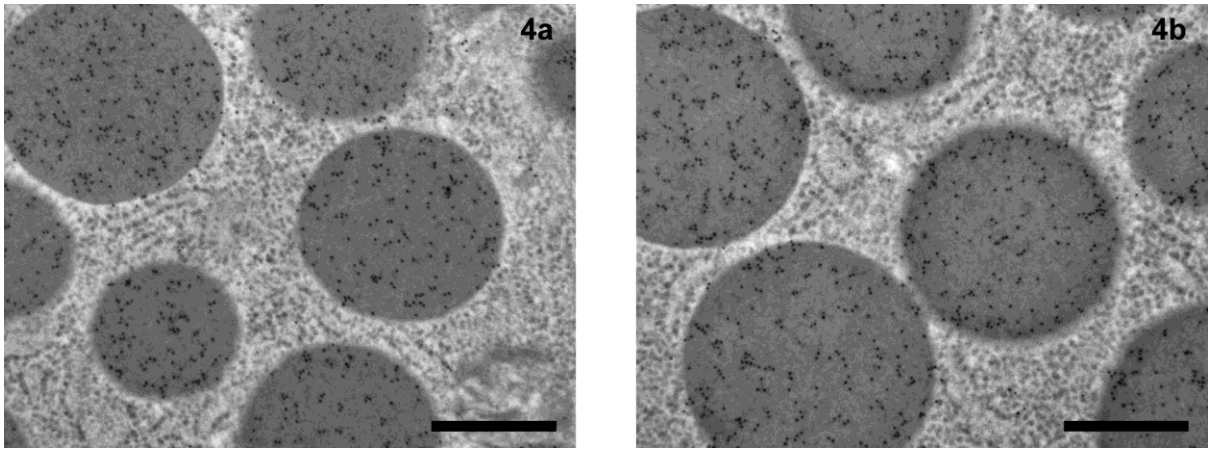

Fig. 4. Electron micrographs of sections of epon-embedded rat pancreas on (a) LR White and (b) carbon-formvar support films immunohistochemically stained for amylase. Scale bar 500nm.