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Immunomicroscopy: Resin Techniques and On-Section Labelling with Immunocolloidal Gold or Immunoperoxidase - Planning a Protocol

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IMMUNOMICROSCOPY: RESIN TECHNIQUES AND ON-SECTION LABELLING WITH IMMUNOCOLLOIDAL GOLD OR IMMUNOPEROXIDASE - PLANNING A PROTOCOL

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Abstract

On-section immunocytochemistry is divided into two parts: (i) processing of biological tissue for section microscopy and (ii) immunolabelling of sections. Many of the more successful microscopical methods employ delicate aldehyde fixation of biological tissue followed by "sympathetic" processing into an acrylic resin. Processing regimens do not have to be complicated. Simple and cost effective room temperature protocols utilising partial dehydration have been devised and they can be as effective as the more complex low temperature techniques in preserving both ultrastructure and antigenic reactivity. The embedded material can be investigated by either light or electron microscopy. Frozen sections can be cut and immunolabelled but only if the tissue is chemically fixed first, as in resin embedding. Fixation with low concentrations of aldehyde will normally better preserve tissue immunoreactivity but this may be at the expense of good ultrastructure with these protocols. If so, low temperature resin embedding methods or rapid freezing and cryosubstitution can be tried. The choice of processing protocol will determine which acrylic resin to use, as will the preference for subsequent immunolabelling with either colloidal gold or peroxidase/diaminobenzidine (DAB). Both types of labelling system offer advantages to localisation studies and can be used in combination for double or even triple labelling. Silver enhancement of the colloidal gold or DAB allows for improved observation by light microscopy.

Key Words: Full dehydration, partial dehydration, progressive lowering of temperature, cryo-techniques, London Resin (LR) White, Lowicryl, immunocolloidal gold, immunoperoxidase, immuno-light microscopy, immuno-electron microscopy.

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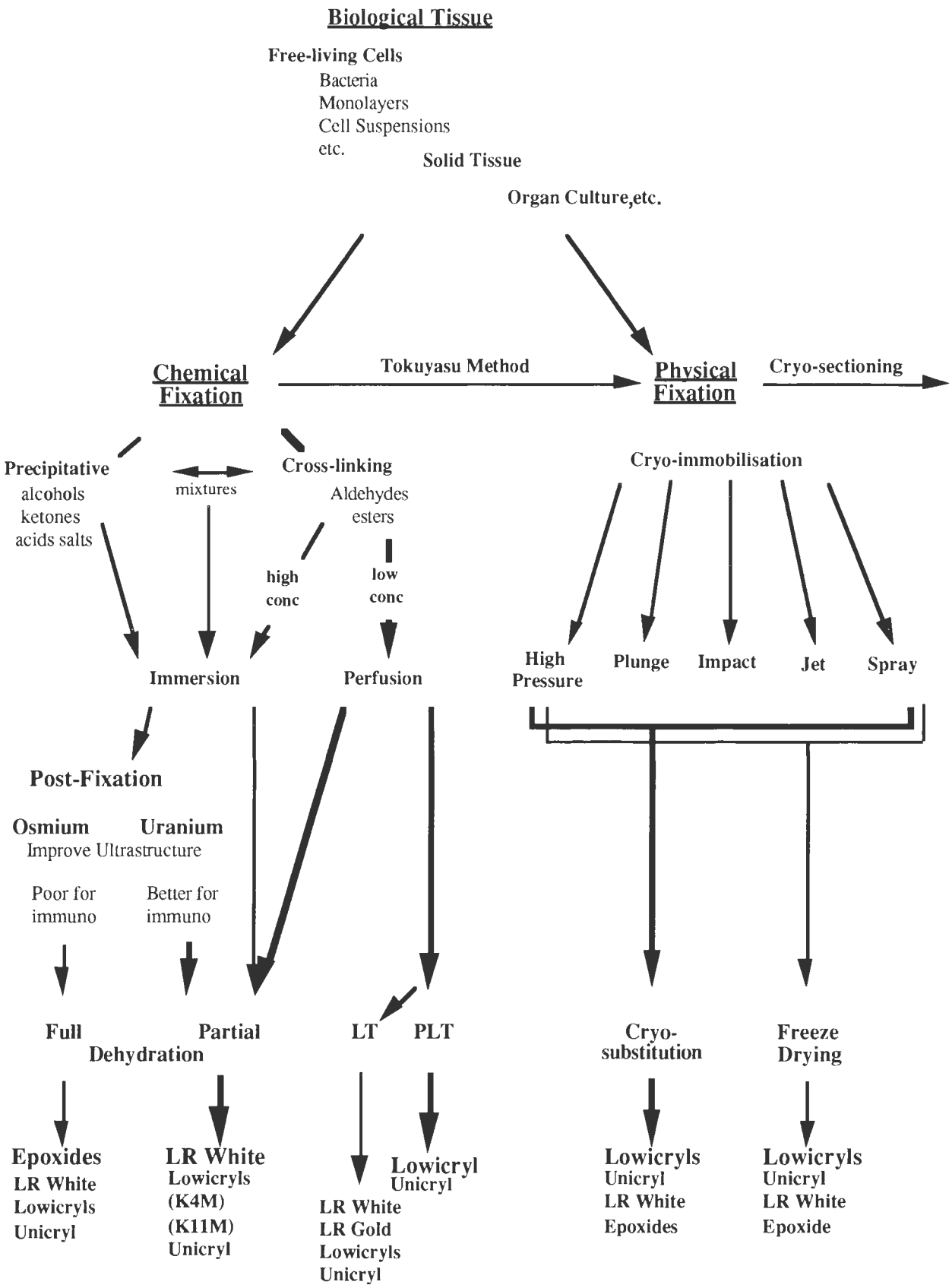
Introduction

Successful scientific enquiry depends on the quality and direction of the practical work carried out in the laboratory. Investigations requiring an immunomicroscopical approach are not exempt from this premise. To solve their biological problems, both the uninitiated and the expert face the need to choose an appropriate protocol from what appears to be a bewilderingly complex technology. Hoping for a straightforward answer, newcomers can be faced with conflicting evidence in published articles which often fail to take into account the resources and expertise immediately available to them in their laboratories. It is not surprising, therefore, that in some cases beginners avoid the issue by simply following the path already taken by others in previous investigations. If the novice is to make a start on planning a protocol or the expert is to develop something new, it is essential that they have an understanding of the underlying logic by which all the different approaches are linked. Only then can an informed choice be made.

The main methodologies and their relationships to one another can be summarised in diagrammatic form (Fig. 1; Newman and Hobot, 1993). These methods will form the basis of discussion in this paper, and it will be seen that the questions required to formulate a working protocol will take the microscopist along certain pathways as illustrated in the diagram. Full details of actual protocols can be found in Newman and Hobot (1993).

The methods listed in Figure 1 only cover one part of the work required by the immunomicroscopist. It deals with preparing biological tissues for resin embedding and observation in the microscope. It does not proffer guidance on how to actually label the resin sections of the prepared tissue with selected antibodies and their detection by marker systems, e.g., colloidal gold or peroxidase/diaminobenzidine (DAB). This important aspect will also be considered in this paper, as the preparative protocol for microscopy can influence the choice of marker system which can be effectively employed (Hobot and Newman, 1991; Newman and Hobot, 1989, 1993).


Biological Tissue
Free-living Cells
Bacteria
Monolayers
Cell Suspensions
etc.
Solid Tissue
Organ Culture,etc.
Chemical Fixation
Tokuyasu Method
Physical Fixation
Cryo-sectioning
Precipitative
alcohols
ketones
acids salts
mixtures
Cross-linking
Aldehydes
esters
high conc
low conc
Cryo-immobilisation
High Pressure
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Jet
Spray
Immersion
Perfusion
Post-Fixation
Osmium
Uranium
Improve Ultrastructure
Poor for immuno
Better for immuno
Full Dehydration
Partial
LT
PLT
Cryo-substitution
Freeze Drying
Epoxides
LR White
Lowicryls
Unicryl
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(K11M)
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LR Gold
Lowicryls
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Epoxides
Lowicryls
Unicryl
LR White
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Figure 1 (on the facing page). Resin embedding strategies for immunomicroscopy. The relationships between the main technologies available for resin embeddings are shown increasing in complexity and decreasing in tissue block size from left to right. Preferred routes are indicated by thicker arrows. LT: low temperature, 0°C to -20°C; PLT: progressive lowering of temperature technique, -35°C to -50°C. (Newman and Hobot, 1993; reproduced by kind permission of Springer-Verlag).

Microscopical Preparative Techniques

The huge variety of biological tissues and research demands makes it impossible to simply quote a protocol for use on each specific type of material. General principles can, however, be applied to limit the developmental research and fine down to a basis of understanding, ready-to-introduce changes to the protocol if necessary. The aim is to optimally preserve structure and antigenic reactivity within the embedded tissue. In fact, this is a contradiction in terms because the optimisation of tissue structure involves chemical and physical alteration, and this inevitably leads to reduced immunoreactivity (Hayat, 1986). Alternatively, a reduction in chemical alteration may improve tissue immunoreactivity but has to be accompanied by milder forms of embedding procedure, or structure will be sacrificed. However, an appreciation of the major contributory factors enables the best compromise to be struck.

The main steps involved in the preparation of the biological specimen for resin section immunolabelling are fixation, dehydration, resin infiltration and resin polymerisation. For simplicity, these steps will be briefly discussed individually, but they are very much interrelated. For example, the optimal methods for dehydration, resin infiltration and resin polymerisation very much depend on the kind of fixation chosen. The choices taken at one level influence all the others. The following methods and protocols are suitable for either light or electron microscope immunocytochemistry.

Chemical-fixation

The structure of biological tissue has to be stabilised to withstand the subsequent adverse effects of processing into resin. This is achieved by chemical fixation (Hayat, 1981). For conventional electron microscopy (EM), heavy fixation with neutrally buffered glutaraldehyde followed by post-fixation with neutrally buffered osmium tetroxide (Sabatini *et al.*, 1963) is necessary to offset the rigours of full dehydration with organic solvents (i.e., up to 100%) and embedding in an epoxy resin. Osmium post-fixation irreversibly affects the structure of some tissue components (Baschong *et al.*, 1984; Emerman and Behrman, 1982), rendering them unrecognisable by antibodies (Bendayan, 1984; Hayat, 1986; Roth *et al.*, 1981). It often masks other specific antigenic sites within tissue, and so for on-section immunolabelling to occur, it is necessary to remove the osmium with sodium metaperiodate (Bendayan, 1984; Bendayan and Zollinger, 1983). Frequently, this pretreatment of resin sections does not optimally restore antigenic sensitivity. "Etching" with hydrogen peroxide (Baskin *et al.*, 1979) will also remove osmium although its main purpose appears to be to reduce resin hydrophobia which generates an unspecific attraction for immunocytochemical markers, in particular, colloidal gold. The etching of the surface of resin sections can adversely affect tissue structure (Coggi *et al.*, 1984). Osmium post-fixation, therefore, should be avoided, but in conventional processing into epoxy resin, this will generally lead to poor structural preservation and still leave the problem of unspecific immunolabelling (Roth *et al.*, 1981). In order to facilitate the greatly reduced levels of chemical fixation that are compatible with improved tissue immunoreactivity, less destructive processing and embedding protocols than are used for epoxy resin embedding become necessary. To achieve this, epoxy resin regimens are replaced by new processing procedures employing modern, electron beam stable acrylic resins (Carlemalm *et al.*, 1982; Newman and Hobot, 1987; Newman *et al.*, 1982; Roth *et al.*, 1981). These resins have the added advantage of being hydrophilic and therefore having virtually no non-specific attraction for immunolabels.

Aldehydes, then, remain the most commonly used and effective fixatives for the preparation of tissue for on-section immunocytochemistry and are used without osmium post-fixation. Formaldehyde on its own may be adequate for light microscopy and gives good preservation of antigenicity, but if electron microscopy is also required, the ultrastructural preservation is often poor. Glutaraldehyde rapidly and irreversibly cross-links tissue protein, resulting in good ultrastructure but antigenicity can be adversely effected (Hayat, 1986). Mixtures of formaldehyde with glutaraldehyde have been used with considerable success, and for example, neutral buffered 2% formaldehyde/0.2% glutaraldehyde has been advocated. Such mixtures can only be tested empirically, and there is evidence that immunocytochemical results with some formaldehyde/glutaraldehyde mixtures can be the same as those obtained with 1% glutaraldehyde alone (Bowdler, 1991; Hobot and Newman, 1991; Newman and Hobot, 1993). Whatever is preferred, solutions of formaldehyde in the laboratory and commercial preparations of glutaraldehyde can greatly vary in composition (Gillett and Gull, 1972) even on a day to day basis. For improved experimental reproducibility and comparability, stable, high purity, depolymerised reagents should

Figure 2 (on the facing page). Rat pancreas sections incubated in rabbit anti-rat anionic trypsin antibody (diluted 1/1000) and labelled with a 1/5 dilution of goat anti-rabbit IgG conjugated to 10 nm colloidal gold (see Hobot and Newman, 1991). Counterstained with uranyl and lead acetate. (a) Rat pancreas, immersion fixation for 60 minutes in 1% glutaraldehyde and processed by room temperature full dehydration into Lowicryl K4M, polymerised by UV-light at 4°C (Newman and Hobot, 1993). Two adjoining cells show very different aspects of cellular organisation of their rough endoplasmic reticulum (RER), exemplifying the uneven structural preservation that can be obtained by immersion fixation. (b) Rat pancreas prepared as for Figure 2a. In well-preserved areas of the immersion fixed tissue, the zymogen granules (Z) and Golgi apparatus (G) are immunolabelled. There is no label in the RER. (c) Rat pancreas, perfusion fixation with 1% glutaraldehyde for 15 minutes, immersed in 1% glutaraldehyde for a further 45 minutes, and processed by PLT into Lowicryl K4M and polymerised by the chemical catalytic method at -35°C (Newman and Hobot, 1993). As in the preparation at room temperature (Fig. 2b), the zymogen granules (Z) and Golgi apparatus (G) are immunolabelled. There is no label in the RER. (d) Rat pancreas, perfusion fixation with 0.1% glutaraldehyde for 15 minutes, and processed by PLT into Lowicryl K4M and polymerised by UV-light (Newman and Hobot, 1993). The zymogen granules and Golgi apparatus (not shown) were immunolabelled as for Figures 2b and 2c (Hobot and Newman, 1991; Newman and Hobot, 1989), but having reduced the concentration of glutaraldehyde fixation, more sensitive secondary or low concentration antigenic sites in the RER are now immunolabelled. The same result is obtained with tissue processed by room temperature partial dehydration protocols using low glutaraldehyde concentrations (Hobot and Newman, 1991; Newman and Hobot, 1989, 1993). Bars = 0.5 μm.

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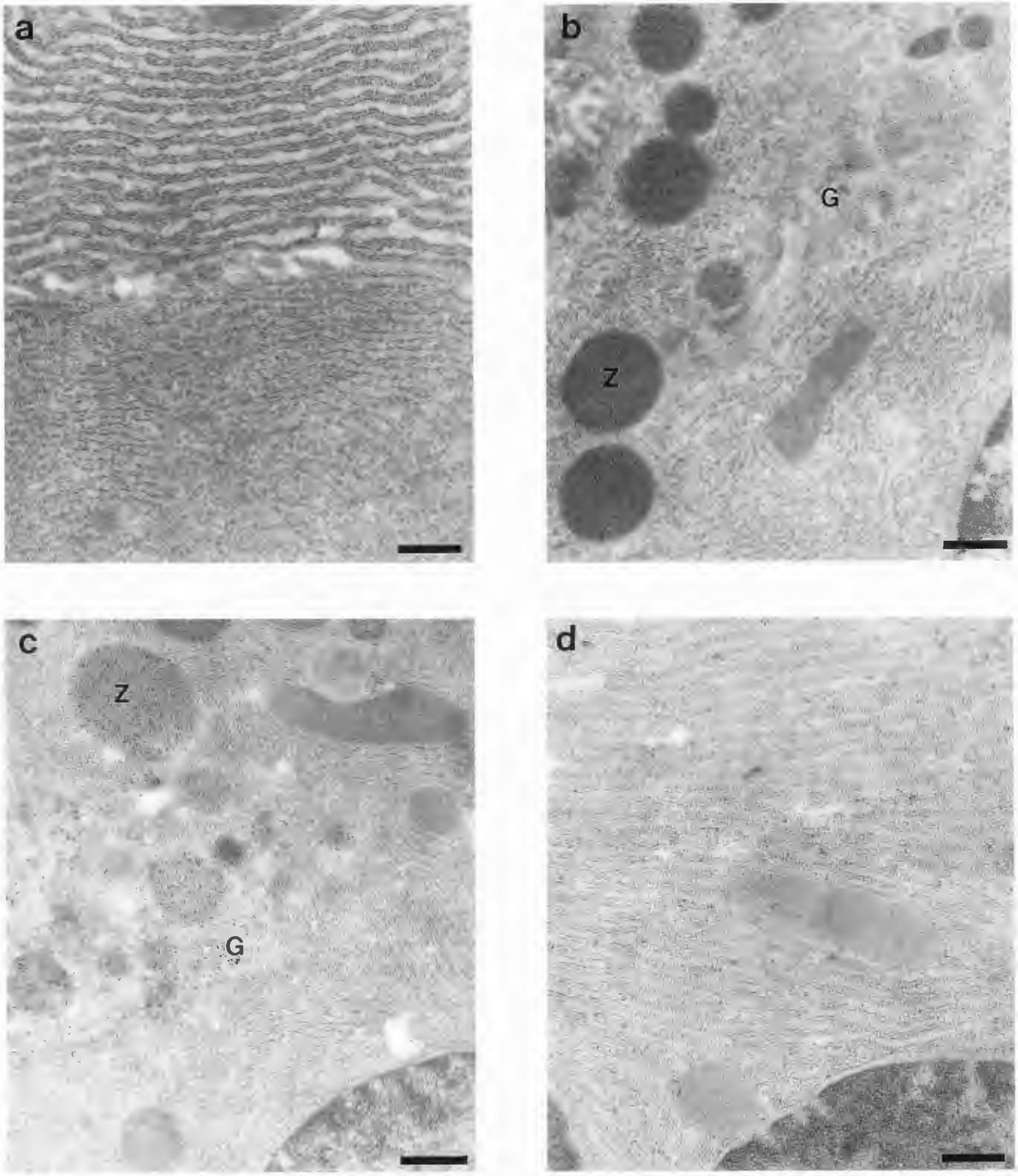
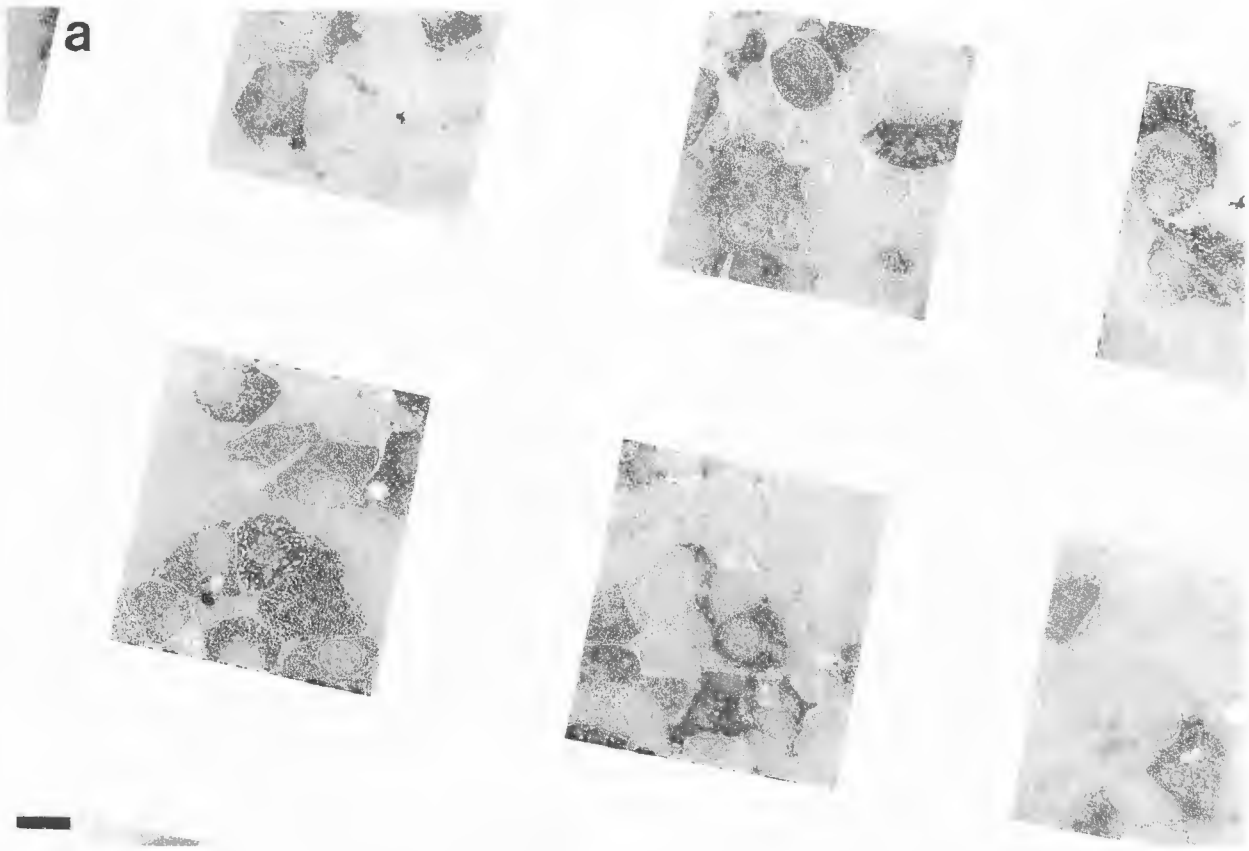
always be used (Hayat, 1986; Newman and Hobot, 1993; Prentø, 1995). For the optimal preservation of structure, the aldehyde should be in a neutral buffer that is at a similar osmolarity to the tissue (Glauert, 1975; Hayat, 1989). We have generally buffered our solutions with a 0.1 M Sorensen's phosphate buffer of pH 7.4, freshly made just before use to avoid any adverse reactions or loss of buffering. However, the use of low concentrations of aldehyde (see below) give some freedom in the choice of buffer and less strong buffering systems such as HEPES (N-[2-Hydroxy]piperazine-N'-[2-ethane sulphonic acid]) and PIPES (piperazine-NN'-bis-2-ethane sulphonic acid) have been successfully used. To balance the colloid osmotic pressure of the fixative/buffer solutions to that of the tissue, it may be necessary to add sucrose {2% weight/volume (w/v)} or dextran (1.5% w/v; 25,000 molecular weight) to these solutions. Cacodylate is a powerful buffer but toxic and therefore not recommended.

The retention of tissue antigenic reactivity following aldehyde fixation is inversely proportional to the concentration of the aldehyde, particularly glutaraldehyde, and the duration of its use (Hayat, 1986). Low concentrations (0.2%) of glutaraldehyde are less harmful to the antigenic reactivity of proteins *in vitro* than high concentrations (1%) (Kraehenbuhl *et al.*, 1977) and in conjunction with short fixation times (10 minutes), preserve enzyme reactivity within isolated mitochondria (Wakabayashi *et al.*, 1975). Fixation in low concentrations (0.1-0.2%) of glutaraldehyde also preserves antigenic sites within tissue that are not detected by immunolabelling after fixation in high concentrations (1%), even when using short fixation times of 15 minutes (Hobot, 1989; Hobot and Newman, 1991; Newman, 1989; Newman and Hobot, 1989). Long times (> 60 minutes) for fixation are discouraged, as the antigenic reactivity of tissue falls with increasing times of fixation. Tissue structure can still be well preserved after only 15 minutes of fixation with very low (0.1-0.2%) glutaraldehyde concentrations (Hobot and Newman, 1991; Newman and Hobot, 1989).

The method by which biological tissue is chemically (or physically) fixed is also an important consideration. Chemical fixative should be introduced to the tissue as quickly and directly as possible. Where only minimal diffusion of the fixative through the tissue is required, e.g., in bacterial or fungal cultures and cell monolayers or suspensions, low concentrations of glutaraldehyde or formaldehyde/glutaraldehyde mixtures can be added to the growth medium or suspension buffer. Removal of the fixative can be achieved quickly so that different times of fixation can also be tested. However, there are two problems concerning the treatment of solid tissue for immersion-fixation, the first of which applies equally to methods that depend on cryo-immobilisation (rapid freezing) and is the removal of tissue from the body. This separation of tissue from its blood supply can result in artefact because of anoxia and autolysis. The second occurs when the tissues are immersed in aldehyde fixative because then diffusion becomes an important factor governing the speed, the extent and the completeness of tissue cross-linking. Even with very small tissue pieces (< 1 mm^3), a low concentration of glutaraldehyde will not penetrate quickly or evenly enough to give uniform fixation and this will result in poor ultrastructure and uneven immunolabelling (Fig. 2a; Hayat, 1986; Newman and Hobot, 1993). Therefore, to increase penetration rates, high concentrations of aldehyde are used



which may compromise antigenicity, but the priority has to be given to structure. Formaldehyde has the advantage of penetrating tissue more quickly than glutaraldehyde but does not preserve ultrastructure as well. For this reason, formaldehyde with a small inclusion of glutaraldehyde has become very popular for immersion fixation. In spite of the limitations of immersion-fixation methods, it is impossible to prepare some tissues, e.g., surgical biopsies, in any other way, and a great deal of excellent work has been published using them.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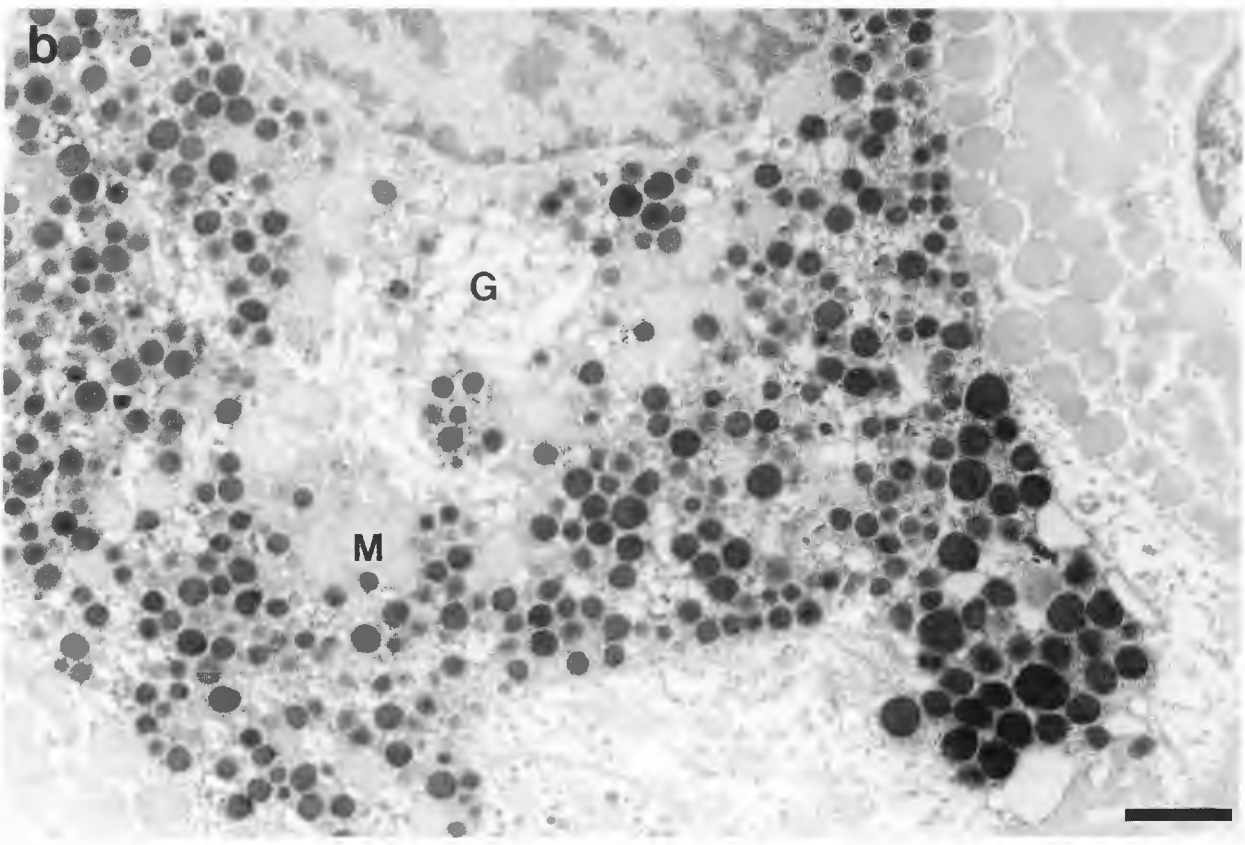
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Figure 3 (on the facing page). Surgically removed human pituitary immersion-fixed in 1% neutral buffered glutaraldehyde/0.2% picric acid and embedded in LR White by the cold catalytic method following room temperature partial dehydration (Newman and Hobot, 1987). Unsupported sections on nickel grids were immersed for 20 minutes in a 1:500 dilution of rabbit anti-synthetic adrenocorticotrophic hormone antibody, anti-ACTH (adrenocorticotrope hormone) (see Newman *et al.*, 1989). Immunoperoxidase/diaminobenzidine (DAB)/hydrogen peroxide stained. The DAB was made very electron dense with 0.05% sodium tetrachloroaurate (yellow sodium gold chloride). No counterstaining. **(a)** Even at very low magnification the electron dense endocrine storage granules in the corticotrophs make the cell population stand out clearly against an unstained background. The grid-bars have been masked out in order to improve photography. Bar = 10 μm. **(b)** Detail from (Fig. 3a) at higher magnification showing the accuracy of the DAB localisation. In addition to the endocrine storage granules, the Golgi membranes (G) and endoplasmic reticulum have stained. Other types of endocrine storage granules and mitochondria (M) are clear of DAB. Bar = 1 μm.

Chemical fixation is usually carried out at room temperature, but it is also possible to use microwave techniques which can considerably shorten immersion-fixation times or even avoid chemical fixation entirely. This opens up another avenue for exploration in improving tissue processing for microscopy (Login and Dvorak, 1994).

The most practical way to fix solid or organ tissue with low concentrations of glutaraldehyde is by perfusion. With this technique, there is only minimal disturbance of the organ before fixation and the fixative is distributed around the body or the selected organ via its own blood-vascular system before anoxia and autolysis become significant. All parts of it very rapidly and intimately receive a good supply of fixative so that no concentration gradients are built up across the tissue as occurs with immersion fixation. Following perfusion fixation, therefore, tissue usually has a more organised and less shrunken appearance than is seen after immersion methods (compare Figs. 2a and 2c). These differences between perfusion and immersion fixation are seen irrespective of the processing protocol used, be it at room temperature or low temperature. In addition, both immersion fixation and rapid freezing, where as mentioned above, the method of handling the specimen usually necessitates removal (dissection/excision) of the cells/organ from their natural location, therefore probably upsetting their cellular physiological equilibrium (Hongpaisan and Roomans, 1995), generally give rise to the same overall organisational appearance of cells, especially the rough endoplasmic reticulum {cf. figures of rat pancreas in: (a) immersion fixation, Bendayan *et al.*, 1980; Roth *et al.*, 1981; (b) perfusion fixation, Newman and Hobot, 1993; (c) cryo-immobilisation, high pressure freezing, Studer *et al.*, 1989}.

In the absence of post-fixation in osmium, lipid losses from tissue during processing can cause a lack of membrane contrast or even reduce overall levels of ultrastructure. In lipid-rich tissues, e.g., the central nervous system, free lipid will cause problems for acrylic resin embedding, often leading to uneven polymerisation and poor beam stability. In these situations, post-fixation in aqueous uranyl acetate (2%) will often help. It has been found to improve the preservation of lipids (Weibull *et al.*, 1983; Humbel *et al.*, 1983; Voorhout *et al.*, 1991), and as a consequence, it will increase the contrast of cellular membranes in aldehyde-fixed, acrylic-embedded tissue. Some authors have suggested that it does not generally affect the level of immunolabelling of most tissue structures (Berryman and Rodewald, 1990; Erickson *et al.*, 1987), but its use in studies involving nucleic acids, which it can gel (Kellenberger and Ryter, 1964), should be avoided. In our experience, there can be a small but significant reduction in tissue immunoreactivity so that to secure the fullest advantages of fixation with low glutaraldehyde concentrations, this post-fixation step should be avoided. Uranyl salts are easily precipitated by ionic buffers which should therefore be washed out of the tissue with distilled water before post-fixation. In addition, after post-fixation (1-2 hours), unbound uranyl acetate should be thoroughly removed from the tissue with distilled water washes. Washes that are too prolonged can lead to extraction of soluble tissue components (Newman and Hobot, 1993) but free uranium salts left in the tissue can act as accelerators in the polymerisation of acrylic resins. In any event, there are implications for immunolabelling following post-fixation in uranium salts (see **On-Section Immunolabelling Techniques**, below). Solutions of uranyl acetate can also be used buffered with, e.g., veronal acetate (Ryter *et al*, 1958) or Tris-maleate (Berryman and Rodewald, 1990). Phosphate buffers cannot be used as they cause the deposition of unwanted precipitates.

Dehydration

Water is gradually removed from a chemically fixed biological specimen with a gradient of increasing concentration of organic solvent in order to make it compatible with resin embedding. Depending on the extent to which tissue is stabilised by chemical fixation, the use of organic solvents can lead to a variety of structural artefacts and reduce antigenic reactivity through extraction.

Figure 4 (on the facing page, top). Surgically removed neonatal human pancreas fixed and embedded as for Figure 3. The section, unsupported on a nickel grid, was immersed for only 15 minutes in a 1:1500 dilution of rabbit anti-porcine glucagon antibody (see Newman *et al.*, 1986). Immunoperoxidase as in Figure 3. No counterstaining. The distribution of endocrine storage granules in unusual situations (arrows) can be analysed without the need for high magnification searches (cf. colloidal gold). Bar = 1 μm.

Figure 5 (on the facing page, bottom). Rat pituitary perfused with 0.5% glutaraldehyde for 30 minutes and fixed in an excess of fixative for a further 30 minutes. Embedded in LR White as in Figures 3 and 4. Unsupported sections were immersed for 20 minutes in a 1:500 dilution of rabbit anti-human thyroid-stimulating hormone beta chain antibody (anti-TSH, see Newman *et al.*, 1989). Immunoperoxidase as in Figures 3 and 4. No counterstaining. The tiny TSH storage granules are precisely stained and easily detectable at low magnification. Bar = 1 μm.

There are various manouevres to off-set these problems.

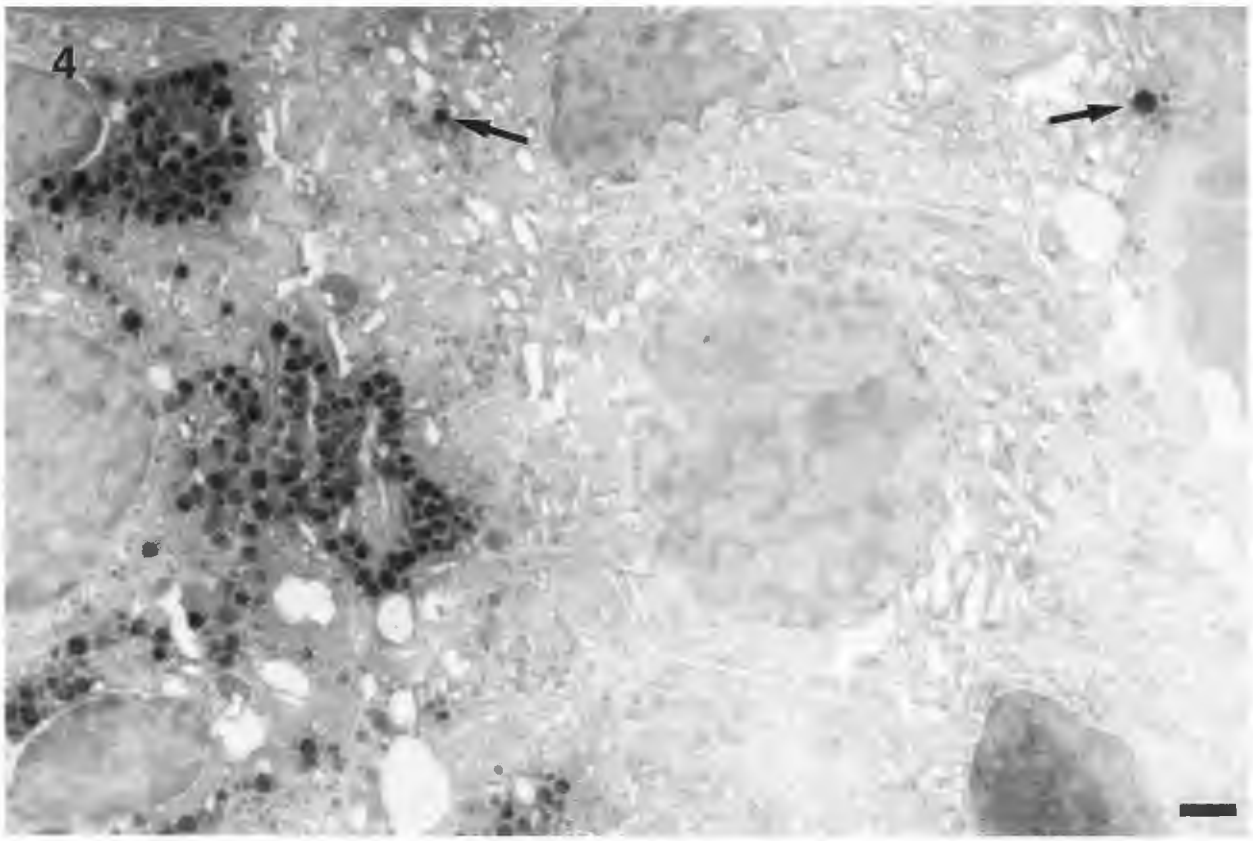
Full dehydration Fixation in high glutaraldehyde concentrations (0.5-1%) strongly stabilises the tissue so that the preservation of its ultrastructure is less dependant upon the processing steps to follow. Here, full dehydration (up to 100% organic solvent concentrations) can be simply employed at room temperature.

The use of low concentrations of glutaraldehyde (< 0.2%) will improve the antigenic reactivity that can be retained within tissue. However, all subsequent processing steps will have to take into account the fact that the tissue is only delicately stabilised for the preservation of ultrastructure. Full dehydration can be carried out, but this has to be at low temperatures by following the complex progressive lowering of temperature (PLT) technique. Briefly, the tissue is progressed through a dehydration gradient, usually of ethanol, of increasing concentration (30%, 50%, 70% and 100%), whilst the temperature is reduced from 0°C and -20°C for the initial stages through to -35°C for the final stages (Carlemalm *et al.*, 1982; Newman and Hobot, 1993, for full details of PLT requirements). The preservation of ultrastructure is much improved over room temperature full dehydration methods, even to the extent that new aspects of ultrastructure have been observed in bacterial cell envelope (Hobot *et al.*, 1984) and in Golgi apparatus (Roth *et al.*, 1985).

However, if fully stabilised tissue, fixed in 0.5-1% glutaraldehyde is embedded by PLT, retention of antigenic reactivity within the tissue is the same as that provided by full dehydration and processing at room temperature (Figs. 2b and 2c; Hobot and Newman, 1991; Newman and Hobot, 1989, 1993). Full dehydration at room temperature requires no special equipment and is by far the easiest course to follow, unless a specific structure that can only be preserved by PLT is to be immunolabelled.

Partial dehydration It may not be necessary to expose the tissue to the extractive process of full dehydration in concentrated organic solvent. The versatile acrylic resins are compatible with small amounts of water, making it possible to follow a protocol employing partial dehydration at room temperature. Here the dehydration steps stop at concentrations of 70% organic solvent, providing a rapid system of preparing tissue for immunocytochemistry (Newman *et al.*, 1982, 1983a). The next step can be directly into pure resin (100%) or an intermediate step of 2:1 of pure resin:70% organic solvent to prevent distortion caused by osmotic shock. The choice of organic solvent is important, as not all are miscible with resin at concentrations of 70% (Newman and Hobot, 1993). Ethanol is probably the best. Of greater importance is the choice of resin for infiltration and embedding. Epoxy resins can be miscible with 70% organic solvents, but early attempts at partial dehydration, although successful (Idelman, 1964, 1965), provided no advantages. Acrylic resins are more versatile and can be used for comparative studies with the light and electron microscopes (Newman, 1987; Newman and Hobot, 1993; Newman *et al.*, 1983a; Steiner *et al.*, 1994, Wynford-Thomas *et al.*, 1986). The commercially available acrylic resins are hydrophilic, some being more so than others: Lowicryl K4M > Lowicryl K11M > LR (London Resin) White = Biocryl/Unicryl > Lowicryl HM20 > Lowicryl HM23 (Hobot and Newman, 1991). Of these, the more hydrophilic resins LR White (Newman *et al.*, 1982, 1983a), Lowicryls K4M/K11M (Hobot, 1990; Hobot and Newman, 1991) and Biocryl/Unicryl (Scala *et al.*, 1993) are compatible with 70% organic solvents, and therefore can be used for partial dehydration protocols.

After strong fixation with 1% glutaraldehyde and partial dehydration, preservation of novel ultrastructure is the same as that observed following PLT in bacterial cell envelope and Golgi apparatus (Newman and Hobot, 1987). However, the level of immunoreactivity retained within such tissue by either partial dehydration or PLT is not better than that seen following full dehydration at room temperature (Hobot and Newman, 1991). The full capacity of partial dehydration protocols at room temperature or PLT to greatly increase the preservation of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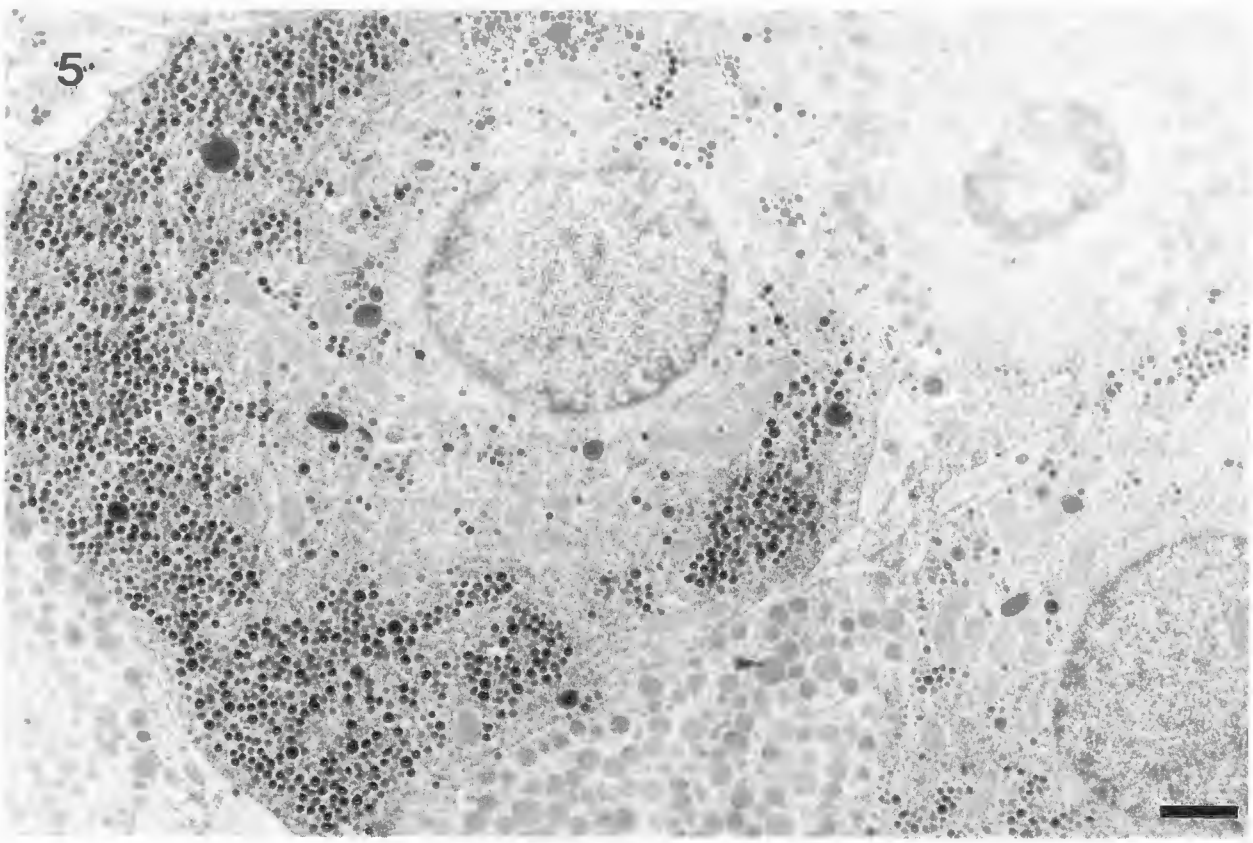
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antigenic reactivity for immunocytochemistry is only realised if tissue is fixed in low glutaraldehyde concentrations of less than 0.2% for relatively short periods of time (< 1 hour) (Fig. 2d; Hobot and Newman, 1991; Newman, 1989; Newman and Hobot, 1989). Partial dehydration is almost certainly less deleterious and extractive to tissue fixed with low concentrations of glutaraldehyde than full dehydration at room temperature. This is borne out by the similar improvements in ultrastructural preservation obtained after either partial dehydration at room temperature (Hobot, 1990; Newman and Hobot, 1987) or PLT (Hobot *et al.*, 1984; Roth *et al.*, 1985), where PLT also minimises the deleterious effects of dehydration (Carlemalm *et al.*, 1982; Kellenberger *et al.*, 1980).

The preservation of lipids after partial dehydration (Mazzotti *et al.*, 1995; Zini *et al.*, 1989) is similar to that obtained after cryosubstitution (Voorhout *et al.*, 1991; see **Cryosubstitution or freeze-drying** below). It has been suggested that the preservation of hydration shells, essential for protein structure and function, are equally well preserved by both these dehydration methodologies (Hobot and Newman, 1991). From a purely practical point of view, it is easier to work initially at room temperature, but the PLT method has an edge over partial dehydration in preserving ultrastructure in some situations (Newman and Hobot, 1993). These can only be found empirically.

Resin embedding

Acrylic resins have been in and out of favour for use in EM since 1949 (Kushida, 1961; Newman *et al.*, 1949; Rosenberg *et al.*, 1960; Wichterle *et al.*, 1960), but it was following their reintroduction by Kellenberger *et al.* (1980) that their use in electron immunomicroscopy really came to the fore (Roth *et al.*, 1981). They are very adaptable and can be used at various temperatures and polymerised by several methods. Based upon this versatility, Kellenberger *et al.* (1980) suggested a program to study the effects of the different microscopical preparative steps upon ultrastructural preservation, which was put into practice and included a study of the effects of these steps on the immunocytochemical properties of the tissue (Hobot and Newman, 1991; Newman and Hobot, 1987, 1989, 1993). The acrylic resins share a common chemistry, and although their usage is very similar (Newman and Hobot, 1993), different chemical blends can be used to cover special requirements such as compatibility with water or use at low temperature. The most popular and successful proprietary mixtures are the Lowicryls and the LR resins, and these will be the centre of attention in this article. The recently introduced resin Biocryl/Unicryl (Scala *et al.*, 1992) has a formulation similar to the Lowicryls (Acetarin *et al.*, 1986; Carlemalm *et al.*, 1982) and, like the Lowicryls, can be used down to temperatures of -35°C with the PLT protocol. Indeed, it is possible for anyone to come up with an entirely original acrylic resin formulation of his very own, though whether this would exhibit any desirable fresh properties is another matter.

For partial dehydration protocols at 70% ethanol or less, only LR White and the Lowicryls K4M and K11M are sufficiently compatible with water. For PLT methods, any of the Lowicryls may be used. LR Gold has been advocated for low temperature embedding at -20°C with polymerisation by blue light. In fact, LR White can also be used down to temperatures of -20°C, with polymerisation by either chemical catalysts or ultraviolet (UV) light. These methods allow a much more accurate control of the temperature of polymerisation than does blue light which is a strong heat source (Newman and Hobot, 1993). Results with LR Gold show the same levels of immunolabelling as that obtained with LR White (Berryman and Rodewald, 1990, Takada *et al.*, 1992).

The method of polymerisation is important because acrylic resins are exotherms and the production of heat during polymerisation can seriously damage structure and lower the antigenic reactivity retained within the tissue. The most acceptable methods control the heat rise that occurs during polymerisation, where necessary employing a heat sink to remove excess heat production (Newman and Hobot, 1993; Weibull, 1986). In our experience, the three most acceptable methods of polymerisation are: heat, chemical catalysis and UV-light. Of these, heat is the most deleterious. Indirect UV-light has been advocated for the Lowicryls (Carlemalm *et al.*, 1982), although chemical polymerisation is possible (Acetarin and Carlemalm, 1982). Initially polymerisation by heat at 50°C for 24 hours was advocated for LR White protocols (Newman *et al.*, 1982, 1983a), but later it was established that a rapid method reducing the tissue's exposure to resin monomer and allowing for hardening of the resin to occur within 30 minutes by polymerisation with chemical catalysts at room temperature, preserved higher levels of tissue antigenicity in 1% glutaraldehyde fixed tissue (Yoshimura *et al.*, 1986). Yoshimura *et al.* (1986) found that the temperature rose during polymerisation to over 50°C, although only briefly. However, tissue distortion can result if tissue, which has only been lightly fixed in low aldehyde concentrations, is too rapidly embedded in this way. In order to slow down the rate of polymerisation, chemical catalysis is carried out in the cold at 0°C (Newman and Hobot, 1987). Indirect UV-light can also be used for the cold polymerisation of LR White. Indirect UV-light reduces the heat rise that would occur if direct sources of UV-light were used (Carlemalm and Villiger, 1989).

The adaptability of the acrylic resins means that they can be polymerised at any given temperature mainly by either chemical catalysts or UV-light. By restricting the amount of chemical catalyst added to the resin monomer, the degree of resin cross-linking can be readily controlled (Newman and Hobot, 1993). Reducing the cross-link density of the resin can increase the final level of on-section immunolabelling (Hobot and Newman, 1991) but may destabilise the section in the electron beam. With LR White, when following either the room temperature, full or partial dehydration protocols, the cold chemical catalytic method at 0°C is used to reach the optimum in structural preservation (Newman and Hobot, 1987) and immunocytochemical reactivity (Bowdler *et al.*, 1989; Hobot and Newman, 1991; Newman, 1989; Newman and Hobot, 1989, 1993; see also legends to Figs. 3, 4 and 5 where very short times are required for incubating sections with low antibody dilutions). Further polymerisation of blocks is possible in an accurately set oven at 50°C for 1-2 hours and will often be necessary to increase electron beam stability. Unfortunately, in the literature many immunocytochemical studies involving LR White still use crude heat polymerisation methods and not the more efficient cold chemical catalytic method (Newman, 1987). Further, inappropriate comparisons are then often made between tissue embedded by poor heat polymerisation methods (even after high glutaraldehyde fixation and full dehydration) and other very sophisticated preparative methods (e.g., cryo-procedures). A true comparison would compare the optimum protocol from each method (cf. Newman and Hobot, 1993). The Lowicryls can also be polymerised by chemical catalytic methods in exactly the same way as LR White (Hobot, 1990; Newman and Hobot, 1993) following either full dehydration or partial dehydration protocols (the latter only with Lowicryls K4M/K11M). For PLT, it is better to use indirect UV-light, as although chemical polymerisation is possible at -35°C (Fig. 2c; Acetarin and Carlemalm, 1982), it requires large amounts of the appropriate chemical catalysts. In all cases involving chemical catalytic methods, and also where partial dehydration protocols are employed, it is very important to use fresh LR White resin, no more than three months old from the date of production (Newman and Hobot, 1987, 1989, 1993). This is because LR White already contains benzoyl peroxide which slowly causes the resin to polymerise, reducing its property of being miscible with 70% organic solvents. Using the commercially available "Uncatalysed" LR White, to which the catalyst still has to be added, can reduce this problem (Newman and Hobot, 1993).

The degree of cross-linking of a resin can be reproducibly controlled with chemical catalytic polymerisation (Newman and Hobot, 1993). Less cross-linked resins give a better immunocytochemical response (Newman, 1987) and the final degree of cross-linking can be controlled to some extent by the amounts of catalyst(s) added (Newman and Hobot, 1993). Both LR White and the Lowicryls can be polymerised chemically, whilst published protocols for Biocryl/Unicryl only refer to either heat or UV-light polymerisation (Manara *et al.*, 1993; Scala *et al.*, 1992, 1993). However, by following the procedures detailed in Newman and Hobot (1993), any acrylic resin can be polymerised chemically. The amount of catalyst given for LR White (hard grade) chemical polymerisation yields blocks that are not fully cross-linked but if sections are insufficiently beam stable, further polymerisation is possible (Newman and Hobot, 1987).

In general, acrylic resins give similar results in terms of structural preservation and immunolabelling when used in appropriate protocols (Bendayan *et al.*, 1987; Dürrenberger *et al.*, 1988; Hobot, 1989; Hobot and Newman, 1991; Hobot and Rogers, 1991; Hobot *et al.*, 1984; Newman and Hobot, 1989; Schwarz and Humbel, 1989). Some thought, however, needs to be given in choosing which resin to employ depending on whether colloidal gold or peroxidase/DAB is to be the detection system for immunocytochemistry. All acrylic resins are compatible with colloidal gold and any subsequent silver enhancement techniques. Not all are compatible with peroxidase/DAB or even for the basic dyes used in semithin section light microscopical studies. Those that are compatible are LR White and Lowicryls HM20/HM23 (Hobot and Newman, 1991). This immediately shows that the simplest system to use at its optimum sensitivity for both light and electron microscopical studies is low glutaraldehyde fixation (< 0.2%), room temperature partial dehydration, and LR White with cold chemical catalytic polymerisation. If structural preservation presents some problems, then this protocol can be replaced by low glutaraldehyde fixation (< 0.2%), PLT and Lowicryl HM20 polymerised by indirect UV-light. For comparisons to investigate the effects of the different processing steps upon structure and subsequent colloidal gold immunolabelling, Lowicryl K4M as an initial choice will suffice.

Cryoultramicrotomy

Cryoultramicrotomy is an alternative sectioning method that avoids resin embedding. However, once again, tissue must be chemically fixed first. It is then infiltrated with sucrose as a cryoprotectant and rapidly frozen in liquid nitrogen. Thin frozen sections are cut, thawed and immunolabelled (Tokuyasu, 1973, 1984). In some cases, cryoultramicrotomy can yield tissue with improved retention of antigenic reactivity (Tokuyasu, 1986) probably because organic solvent dehydration and

resin infiltration and polymerisation have been avoided, but the problems of whether to use high versus low fixative concentrations remain. Low fixative concentrations give poor structure and unstable thin sections of frozen tissue that upon thawing can easily loose small peptides and the more soluble proteins, leading to reduced levels of immunolabelling (Hobot and Newman, 1991; Newman and Hobot, 1993). Cryoultramicrotomy is a fairly rapid method when compared to most other protocols, though it is possible to process and embed practically just as rapidly into LR White (see sec. 3.3.2 in Newman and Hobot, 1993). Resin blocks are more easily stored, serially sectioned and reused than frozen tissue pieces.

Cryosubstitution or freeze-drying

The discussion so far has centred around using chemical fixatives to stabilise tissue structure. However, in rare cases, antigenic reactivity within tissue may be adversely affected by chemical fixation (Young *et al.*, 1995). If even low concentrations of fixative result in negative immunocytochemical results, then, in these cases, chemical fixation may be the cause and can be replaced by physical fixation in the form of rapid freezing of the biological tissue to cryo-immobilise structures in ice (Kellenberger, 1991). There are several methods for cryo-immobilising or rapidly freezing tissue (Echlin, 1992; Robards and Sleytr, 1985; Steinbrecht and Zierold, 1987). The ice can then be removed by substituting it with an organic solvent (acetone or methanol) at very low temperatures of -80°C (cryosubstitution) or by subliming it away (freeze-drying). Equipment is commercially available for these procedures or can be constructed in the laboratory (Linner *et al.*, 1986; Plattner and Knoll, 1984; Ryan, 1992; Sitte *et al.*, 1994; Studer *et al.*, 1989). It is in this area that it is probably best to use the Lowicryl resins, which were specially formulated to be used at low temperatures, in the range -80°C to -30°C (Acetarin *et al.*, 1986; Carlemalm *et al.*, 1982, 1985b; Appendix 1). The evidence from the literature points to the importance of maintaining the processing steps below -30°C (Carlemalm *et al.*, 1982; Edelmann 1989; Humbel *et al.*, 1983; MacKenzie, 1972) and avoiding the use of fixatives in the processing steps following freezing (Edelmann, 1986, 1989; Hobot, 1989, 1990; Hobot *et al.*, 1987; Humbel and Muller, 1984; Humbel *et al.*, 1983; Monaghan and Robertson, 1990; Schwarz and Humbel, 1989). Cryosubstitution has yielded some results showing new aspects of ultrastructure and an improved immunocytochemical response in tissue (Björnsti *et al.*, 1986; Hobot, 1989, 1990; Hobot *et al.*, 1985, 1987; Howard, 1981; Humbel *et al.*, 1983; Hunziker and Herrmann, 1987; Kandasamy *et al.*, 1991; Schwarz and Humbel, 1989) more so than freeze-drying (Chiovetti *et al.*, 1987; Jesaitis *et al.*, 1990; Jorgensen and McGuffee, 1987; Livesey *et al.*, 1989; Van Winkle, 1991).

The limitation in using cryo-procedures is the very small sample size that can be frozen: less than 0.5 mm^3. The amount of well-frozen material then available for detailed analysis is generally in the region of 5-40 μm, or up to 200 μm with high-pressure freezing apparatus (Studer *et al.*, 1989). Dedicated equipment can be very expensive and needs considerable expertise, so it is advisable to investigate thoroughly the possibility of using, e.g., partial dehydration or PLT before proceeding to cryosubstitution.

On-Section Immunolabelling Techniques

The tissue has been embedded, sectioned and is now ready for immunolabelling using the method chosen, which is likely to be immunocolloidal gold or possibly immunoperoxidase/DAB. Colloidal gold is an electron dense marker which can be manufactured in a variety of sizes from approximately 1 nm to over 40 nm (Handley, 1989). It can be conjugated to a variety of substances including various antibodies (De Mey, 1983), protein A (Romano and Romano, 1977), protein G (Björck and Kronvall, 1984) and protein AG (Eliasson *et al.*, 1988), designed to recognise a primary antibody raised against the tissue component under study. Colloidal gold can also be linked to enzymes which can localise their substrates (Londoño *et al.*, 1989) and lectins (Roth, 1983a) which are used to recognise a specific carbohydrate moiety.

Colloidal gold particles are discrete, very electron dense markers and precise in their localisation (Roth, 1983b). They are visible with the electron microscope but not at the light microscopical level. Here silver enhancement of the colloidal gold is necessary to visualise areas of antigen localisation (Bowdler *et al.*, 1989; Christensen *et al.*, 1992; Danscher and Rytter Nørgaard, 1983; Danscher and Rytter Nørgaard, 1985; Danscher *et al.*, 1987; Hacker, 1989; Holgate *et al.*, 1983; Schiønning *et al.*, 1993).

The sensitivity of immunocolloidal gold methods is improved by using smaller gold particles, and for this reason, very small (1-5 nm) colloidal gold particles have become popular. Even at the electron microscopical level, it is often necessary to employ silver enhancement techniques to enlarge these very small particles before they can be seen (reviewed in Hayat, 1995). However, an extreme enlargement is required if such particles are to be seen at low magnification (1-5,000x) which can result in obtrusive background and unspecific deposits and may undermine the precise and discrete localisation usually available with colloidal gold particles. In addition, variability in the size of small colloidal gold parti-

cles prior to enlargement and the unreliability of some of the silver techniques leads to silver-enhanced particles of varying size. This uneven enhancement gives problems in double immunolabelling with colloidal gold of different sizes. These problems do not exist with DAB methods. Therefore, to obtain a more panoramic view of antigen localisation with the electron microscope, especially at very low magnifications where colloidal gold particles are not readily visible, immunoperoxidase/DAB techniques still have a lot to offer (Fig. 3a).

Poor localisation and the obscuring of ultrastructure have been criticisms of immunoperoxidase/DAB methods. They have probably arisen from the early practice of immunolabelling epoxy resin sections with massive complexes such as occur in the peroxidase antiperoxidase (PAP) and avidin/biotin peroxidase (ABC) methods, and the subsequent use of osmium tetroxide to enhance the electron density of such complexes. However, on permeable acrylic resin sections, simple indirect immunoperoxidase techniques produce much more precise localisation and the electron density of the peroxidase/DAB complex is enhanced using a 0.01% solution of sodium tetrachloroaurate (yellow sodium gold chloride; Newman *et al.*, 1983c), which is specific for DAB and gives a significantly more electron dense marker than that provided by osmium (Fig. 3; Newman and Hobot, 1993; Newman and Jasani, 1984; Newman *et al.*, 1983b). Unlike osmium, sodium tetrachloroaurate does not react with the embedded tissue, thereby keeping background or unspecific staining to a minimum. Observation of DAB labelled tissue is also possible at higher magnifications, demonstrating the specificity and discrete localisation available with sodium tetrachloroaurate enhancement (Figs. 3, 4 and 5). Silver enhancement of the peroxidase/DAB complex is available for light microscopical studies (Amersham DAB Silver Amplification Kit, Amersham International, Amersham, Bucks., U.K.; Newman *et al.*, 1983c; Wynford-Thomas *et al.*, 1986), as with colloidal gold. Actual procedures are given in detail in Newman and Hobot (1993). Further, double or even triple immunolabelling is possible, utilising both immunocolloidal gold and immunoperoxidase methodologies (Newman and Hobot, 1993; Newman *et al.*, 1986, 1989). Whilst colloidal gold particles cannot penetrate acrylic resin sections, thus limiting observation of labelled antigen to the section surface, there is evidence that immunoperoxidase reagents do, which improves the sensitivity of immunocytochemical detection (Newman and Hobot, 1987).

Establishing an immunolabelling protocol

Whatever detection system is chosen to observe immunolocalisation in the light or electron microscope, a protocol for immunolabelling is formed of three parts: pretreatment of the resin sections, incubation with the appropriate antibody and marker and visualisation of the tissue and/or marker. The first and third parts of the protocol are generally common to all methods, whilst the second part can vary depending on the antibody and marker system chosen. Direct methods use the primary antibody (or other primary reagent, see below) linked or conjugated to the marker. These methods generally have low sensitivity. In indirect methods, the unlabelled primary reagent is followed by a secondary detection system such as protein A or an antibody conjugated to peroxidase or colloidal gold. The primary reagent may also be labelled with a hapten such as biotin and localised with an anti-hapten conjugate or, in the case of biotin, with an avidin conjugate. These much more sensitive methods will form the basis of the discussion below.

Resin section pretreatment Pretreatment prepares the resin section for immunolabelling. Semithin epoxy and acrylic resin sections are adhered to glass slides; thin epoxy resin sections are mounted unsupported on the dull or matt side of nickel grids, thin acrylic resin sections on the shiny side. Semithin epoxy resin sections will need to be etched with sodium methoxide and thin sections with hydrogen peroxide. Etching is unnecessary and may be deleterious with acrylic resin sections. If the tissue was post-fixed in osmium, it can be removed from either epoxy or acrylic resin sections with sodium metaperiodate.

For immunoperoxidase/DAB protocols only, hydrogen peroxide solution can also be used to remove endogenous peroxidase present in some tissues (Hittmair and Schmid, 1989). However, this can be damaging to the retention of antigenic activity within acrylic resin sections and less deleterious methods are now available (Andrew and Jasani, 1987; Jasani *et al.*, 1986; Schmid *et al.*, 1989).

With or without these steps, all resin sections should be equilibrated prior to immunolabelling for 5-10 minutes in a buffered saline (preferably the same as that used for primary antibody/antiserum dilution, see below) containing a non-specific blocking protein. The protein can be albumin (e.g., bovine serum albumin or ovalbumin), gelatin or even powdered milk and will help to block any non-specific affinity that may occur between tissue or resin and the primary reagent or secondary detection system, thus lowering non-specific background labelling. Equilibration also hydrates the resin sections, causing acrylic resin sections to swell due to their hydrophilia, which is a factor in improving the "noise to signal" ratio of labelling on them.

Following equilibration, immunolabelling can begin. It is only after careful examination of the final immunolabelling results and those of controls that further refinements such as the need for aldehyde abolition or specific

blocking reagents to reduce non-specific labelling can be assessed. Aldehyde groups can be removed by pre-embedding treatment of aldehyde fixed tissue with ammonium chloride solution, but ultrastructure may be damaged. In fact, the resin pretreatment steps outlined above will usually neutralise aldehyde groups without compromising structural integrity. Where they persist, however, treatment with 0.028% freshly made sodium borohydride solution for 3 minutes will chemically destroy them but will almost certainly reduce antigenicity. The same is true of indiscriminate blocking, for example, with non-immune sera or immunoglobulin fractions which can actually lower specific immunolabelling. For thawed cryosections following the methods of Tokuyasu (1984, 1986), more extensive blocking steps are usually required, but experimentation should reduce these to a minimum, depending on the tissue and type of fixation used.

Incubation Sections adhered to glass slides are simply covered by the immunoreagents and incubated in a humidity chamber. They are rinsed free of reagents between changes, in Coplin jars or the equivalent.

The immunolabelling of thin sections on grids requires 30-50 μl droplets of the immunoreagents placed on a hydrophobic surface, such as dental wax or Nescofilm/parafilm, and held in a humidity chamber. Floating the grids on the surface of the droplets is an option that is normally reserved for epoxy resin sections or acrylic sections on a support film. It is also necessary for the very tricky two-faced double immunolabelling method of Bendayan (1982). Floatation methods of immunolabelling are usually accompanied by jet-washing, used to rinse off the immunoreagents and reduce non-specific immunolabelling. It can give rise to variable results when jets of different strengths with large volumes of reagent (up to 5 ml) are used; very probably jet-washing also detaches specific immunolabel from the section. The immunolabelling response is usually greater and the background less when the grids (without a support film) are fully immersed in the immunoreagents (Newman and Hobot, 1993). Only small amounts of sometimes costly reagents are necessary, and the washing of grids between incubation steps simply involves passing the grids through 2-3 droplets of washing solution. (All the immunolabelling results presented in this article were obtained by using the immersion method).

The antibody of choice is diluted in an appropriate buffer {usually 0.01 M phosphate buffered saline (PBS)} containing the same concentration of non-specific protein (approximately 0.6% w/v) as used in the equilibration buffer for the pretreatment step. If uranyl acetate has been used as an en bloc stain or fixative, then to avoid any adverse precipitation, 20 mM Tris buffered saline (TBS) must be used in place of PBS. Grids can be passed directly from the equilibration buffer into the primary antibody solution. When testing a new antibody, a dilution profile is absolutely essential. Generally a series of solutions doubling in antibody dilution can be constructed. Typically this would take the form of, for example, 5 solutions from 1/100 to 1/1600 for the high affinity polyclonal antibodies, 1/10 to 1/60 for the lower affinity monoclonal antibodies. The grids are incubated at a constant temperature, e.g., 22°C, for a set time, e.g., 60 minutes. These ranges are only rough guides and there are many exceptions where higher or lower ranges would be more appropriate, but in the absence of any indication to the contrary, they are a good place at which to start and usually they will provide solid data to indicate in which direction the next experiment should be taken. The dilution profile should theoretically take the immunolabelling out to extinction and will then characterise the kind of sites localised. Higher concentrations of antibody will show up low and high affinity sites, but as the dilution increases so the low affinity sites will drop out, leaving only the higher affinity, specific sites. It can be very informative to see for how long the higher affinity sites persist with antibody dilution. Overnight incubations at room temperature or at 4°C are rarely necessary with acrylic resin sections but high antibody dilution for long periods of time can improve background levels of labelling with epoxy resin sections.

Following primary antibody incubation and washing, the grids are ready for secondary detection by colloidal gold or peroxidase/DAB. The secondary marker system should be specific for detecting only the primary reagent. The level of its dilution is not so critical, maybe 1/10 for immunocolloidal gold and 1/100 for immunoperoxidase, but the dilution can be increased if in the control sections, where the primary antibody has been omitted (see **Controls** below), labelling gives high background levels caused perhaps by unspecific attraction of tissue or resin for the marker.

Visualisation Visualisation incorporates both the marker and the tissue structure.

For light microscopy, all resin semithin sections following immunolabelling will need the marker to be amplified before it can be easily seen. Immunocolloidal gold is visualised by direct silver staining and following treatment with sodium tetrachloroaurate (see below), DAB can also be silver intensified. Epoxy resin sections can be counterstained with any of the usual stains or dyes. Most acrylic resins will also stain with toluidine blue, methyl green or haematoxylin and/or eosin (Bowdler *et al.*, 1989; Newman and Hobot, 1993). Lowicryls K4M and K11M are notable exceptions and should instead be viewed by phase-contrast microscopy.

Colloidal gold appears as spherical, electron dense particles, and usually can be easily visualised in the electron microscope without further ado. However, with very small particles of colloidal gold, silver enhancement may be necessary to enable it to be seen at low magnifications in the EM. DAB can be made much more electron dense by applying aqueous 0.01% sodium tetrachloroaurate to the sections for 1 minute.

For electron microscopy, tissue structure in epoxy resin sections can be contrasted as usual by double staining with uranium and lead salts; however, care should be taken when counter-staining acrylic resin sections because they can become over-contrasted very quickly. Stain for a few minutes only with 2% uranyl acetate alone at first, and if this proves insufficient, follow up with dilute lead acetate or citrate. In the case of DAB, even this may not be necessary, as the acrylic resin section will, because of its low electron cross-scattering density (Carlemalm *et al.*, 1985a), provide enough contrast to visualise the tissue and locate precisely the peroxidase/DAB complex (Figs. 3, 4 and 5). Heavy counterstaining may interfere with the observation of both colloidal gold and peroxidase/DAB labelling (Newman and Jasani, 1984).

Controls Controls are vital to establish the specificity of labelling (Newman and Hobot, 1993). The most obvious is the omission control in which the primary reagent is left out of the immunolabelling protocol. This control shows how much of the labelling is due to the primary reagent and validates the secondary detection system. It allows dilution of the secondary detection system reagents to their optimum concentrations. If non-specific labelling persists, it is tempting to introduce blocking with say pre-immune serum or an isologous serum such as whole swine serum, but over use of these can inhibit specific immunolabelling; in any event, there can be other causes requiring other measures (see **Resin section pretreatment**). This is such a simple control to perform it must be included routinely with all immunocytochemical experiments.

Even if the omission control is clear, immunolabelling from the primary reagent could still be non-specific. An inappropriate primary reagent control can help to validate the specificity of immunolabelling. The primary reagent is substituted at the same concentration with another very similar substance that is known to have no specific reaction with the tissue. Immunolabelling of the control will cast doubts on the specificity of the labelling of the experimental sections. The cause could be too high a concentration of primary reagent, but if a dilution profile has been completed (see above), this should already have been eliminated. In fact, the dilution profile is made more meaningful if a similar profile with an inappropriate primary reagent at the same dilutions is run alongside it.

Positive and negative tissue controls may also help to support a view formed of any given result. Negative immunolabelling can be difficult to analyse, but if a positive control such as a section of tissue or even an immunoblot that is known to be strongly reactive with the primary reagent also fails to immunolabel it places great suspicion on the secondary detection reagents. Negative tissue controls are very infrequently needed because there will usually be negative areas of tissue in the section which will act as internal controls.

Where the primary reagent is an antibody, preabsorption of it with high concentrations of its isolated antigen is often cited as the most convincing way to validate its specificity. It can be a valuable control but may also be very expensive because of the high concentrations of reagents involved. Often 100% antibody-antigen binding does not occur so that preabsorption controls frequently show a residual level of immunolabelling. If the antigen is unknown, as is the case with many monoclonal antibodies, preabsorption may be totally impracticable.

Conclusions

A logical spectrum of resin immunomicroscopy techniques exists progressing from the simple, easily accomplished methods to those that are highly complex and for which specialist knowledge will be necessary. The simplest methods can produce unexpectedly satisfying results and cost little to try. **Appendix I and II** attempt to distill some of the main points by prompting the sort of questions that the investigator should be asking, and if thought about early enough, can save time. They also provide examples of protocols and a work schedule showing what can be achieved with a simple approach.

Appendix I: Towards a Full Protocol

Designing a protocol means asking some relevant questions aimed at organising a complete experimental approach:

(1) What type of tissue?

(2) What type of antigen is to be localised?

(3) What type of investigation?
- (A) cytochemistry?
  - (i) lectin? (ii) enzyme histochemistry?
- (B) immunocytochemistry?
  - (i) monoclonal or polyclonal antibodies?
  - (ii) colloidal gold or peroxidase/DAB?
  - (iii) *in situ* hybridisation?

(4) What type of fixative? What concentration? What time?

Table 1. Room-temperature protocols for resin embedding.

Full Dehydration		Partial Dehydration	
50% ethanol	10 minutes	50% ethanol	10 minutes
70% ethanol	10 minutes	70% ethanol	3 x 10 minutes
90% ethanol	10 minutes	2:1 Neat LR White:70% ethanol	30 minutes
100% ethanol	2 x 30 minutes	Neat LR White	4 x 20 minutes
1:1 Neat LR White: 100% ethanol	15 minutes	Cold chemical catalytic polymerisation 0°C/24 hours	
Neat LR White (hard grade)*	4 x 30 minutes	Transfer to 50°C/2 hours	
(*or then overnight and 2 x 20 minutes)		N.B. 70% ethanol made from 100% (anhydrous) ethanol	
Cold chemical catalytic polymerisation 0°C/24 hours		Use recently purchased LR White (hard grade)	
Transfer to 50°C/2 hours			

Table 2. Progressive lowering of temperature (PLT) protocol.

30% ethanol	0°C	30 minutes
50% ethanol	-20°C	60 minutes
70% ethanol	-35°C	60 minutes
100% ethanol	-35°C	120 minutes
1:1 Lowicryl: 100% ethanol	-35°C	60 minutes
2:1 Lowicryl: 100% ethanol	-35°C	60 minutes
1:0 Lowicryl*	-35°C	60 minutes
1:0 Lowicryl	-35°C	overnight
1:0 Lowicryl	-35°C	2 x 60 minutes
Polymerisation:		
Indirect ultra-violet light	-35°C	24 hours
Direct ultra-violet light	room temp.	72 hours

*Either Lowicryl K4M or HM20

(A) formalin?
(B) glutaraldehyde?
(C) mixtures?

(5) What method of fixation?
(A) chemical?
(i) immersion?
(ii) perfusion?
(B) cryo-immobilisation?

(6) What type of resin? For colloidal gold? For peroxidase/DAB? Both?

(7) What type of dehydration and processing?
(A) full?
(B) partial?
(C) PLT?

Table 3. Cryosubstitution protocol.

Preparation of specimen

Rapid freezing of specimen

Substitution: 100% acetone or 100% methanol at -80°C to -90°C for 3-4 days

Raise temperature slowly to temperature of resin embedding* (over a period of not less than 1 hour)

***Maximum lowest temperature for using a particular Lowicryl resin:**

(1) Lowicryl HM23: -80°C;
(2) Lowicryl K11M: -60°C;
(3) Lowicryl HM20: -50°C;
(4) Lowicryl K4M: -35°C.

Infiltration schedule/times for Lowicryls K4M and HM20 as for PLT in Table 2, as also for polymerisation.

Infiltration schedule/times for Lowicryls K11M and HM23 are prolonged (up to 8 hours and/or overnight) to allow for complete infiltration of specimens at the very low temperatures of use. Polymerisation by indirect ultra-violet light for 72-96 hours at low temperature of use; then direct ultra-violet light at room temperature for 72 hours.

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(8) What type of polymerisation?
(A) chemical catalysts?
(B) UV-light?

The answers to these questions are covered in the discussion in the preceding sections, but any study will also need to include labelling to localise a (new) tissue substance (e.g., protein/enzyme, carbohydrate fraction, nucleic acid) that has to be optimally preserved. In typical protocols, Tables 1, 2 and 3, we give examples of

basic regimes that have proved routinely successful in our laboratory. By understanding the science behind the points discussed, these protocols can be customised to suit the particular problem to be studied (cf. Hobot and Newman, 1993 and references cited in text above; it would be impossible to discuss in this tutorial paper the initial variations possible or the ways of handling the different reagents used). And, we do stress, that if success is not obtainable straight away, a sound empirical approach will yield results to the patient researcher. Science is not an instant product, where one just adds water as in the kitchen!

Appendix II: A Simple Start

By way of an example, a case study is presented. Bacterial cells are usually optimally embedded for structural preservation by using low temperature techniques (Hobot, 1991). However, some of the advantages of low temperature can be found using partial dehydration protocols at room temperature (Newman and Hobot, 1987). Even embedding by the room temperature full dehydration method has led not only to satisfactory structural preservation (Hobot and Newman, 1990) but also has yielded new data in respect of cell wall synthesis (Clarke-Sturman *et al.*, 1989; Merad *et al.*, 1989).

The very complexity of some embedding schemes can limit their experimental value. Where numerous blocks of tissue are needed for analysis, the weight of work dictates the use of a relatively simple, quick and easily reproducible protocol. This was the case with the germinating spores of *Bacillus (B.) subtilis* which were prepared as described in the legend for Figure 6 by the room temperature, full dehydration protocol followed by embedding into LR White. Resin sections were immunolabelled by primary antibody (polyclonal rabbit IgG) directed against N-acetylmuramyl-L-alanine amidase, a cell wall lytic enzyme involved in cell wall synthesis, and shown previously to be localised to the cell wall and septa of normally growing, vegetative cells of *B. subtilis* (Hobot and Rogers, 1991). In the example shown here, the localisation of the cell wall amidase is followed during bacterial spore germination (Fig. 6). The amidase is found mainly within the spore cytoplasm even 20 minutes into germination (Figs. 6a and 6b). By 40 minutes, most of the amidase is located not within the spore cytoplasm but in the newly synthesised and developing vegetative cell wall (Fig. 6c). After 60 minutes, this is still the case, although some amidase is now again present in the cytoplasm (Fig. 6d). By 105 minutes, the germination process is over and vegetative cells now have the pattern of amidase localisation found before by Hobot and Rogers (1991) within the cell walls, cytoplasm and septa of all cells (Fig. 6e).

This very simple time course experiment demonstrates that inactive amidase is present in the dormant spore cytoplasm and is then transferred to the newly developing vegetative cell wall to be involved in its synthesis. Only after this event does the germinating spore cytoplasm (or probably now, vegetative cell cytoplasm) switch on production of amidase. EM shows very clearly the timed sequence of events between "spore amidase" and "vegetative amidase" being utilised by the cell and the necessity for amidase to be present in the cell wall during its synthesis and development. The micrographs also give a visual guide as to when amidase can be isolated from the spore cytoplasm (inactive amidase) or cell walls (active amidase) and examined biochemically for differences of structure relating to its cellular location and function.

This study underlines the fact that initially with a very straight-forward technique much can be accomplished, and that although more sensitive approaches are available, these may be over complex and first priority should be given to room temperature (partial dehydration) methods. Only if these prove insufficient should it be necessary to proceed to more costly low temperature procedures.

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Figure 6 (on the facing page). *Bacillus subtilis* germinating spores, fixed directly in growing culture with 1% glutaraldehyde for 45 minutes, and processed by room temperature full dehydration into LR White and polymerised by the cold chemical catalytic method at 0°C (Hobot and Newman, 1990). Sections incubated in rabbit anti-cell wall lytic enzyme (N-acetylmuramyl-L-alanine amidase) antibody and labelled with 10 nm protein-A colloidal gold (see Hobot and Rogers, 1991). Counterstained with uranyl and lead acetate. **(a)** 0 minute: The labelled amidase is located mainly within the dormant spore cytoplasm. **(b)** 20 minutes: The amidase is still localised mainly within the germinating spore cytoplasm. **(c)** 40 minutes: Most of the amidase is now localised to the new cell wall being synthesised by the germinating spore, which will eventually become the vegetative cell wall. Little label, or amidase, remains in the spore cytoplasm. **(d)** 60 minutes: The amidase is still localised to the cell wall, but more label is now reappearing in the cytoplasm. **(e)** 105 minutes: In vegetative cells, the distribution of amidase in both cell walls and cytoplasm is as expected (cf. Hobot and Rogers, 1991). Bars = 0.25 μm.

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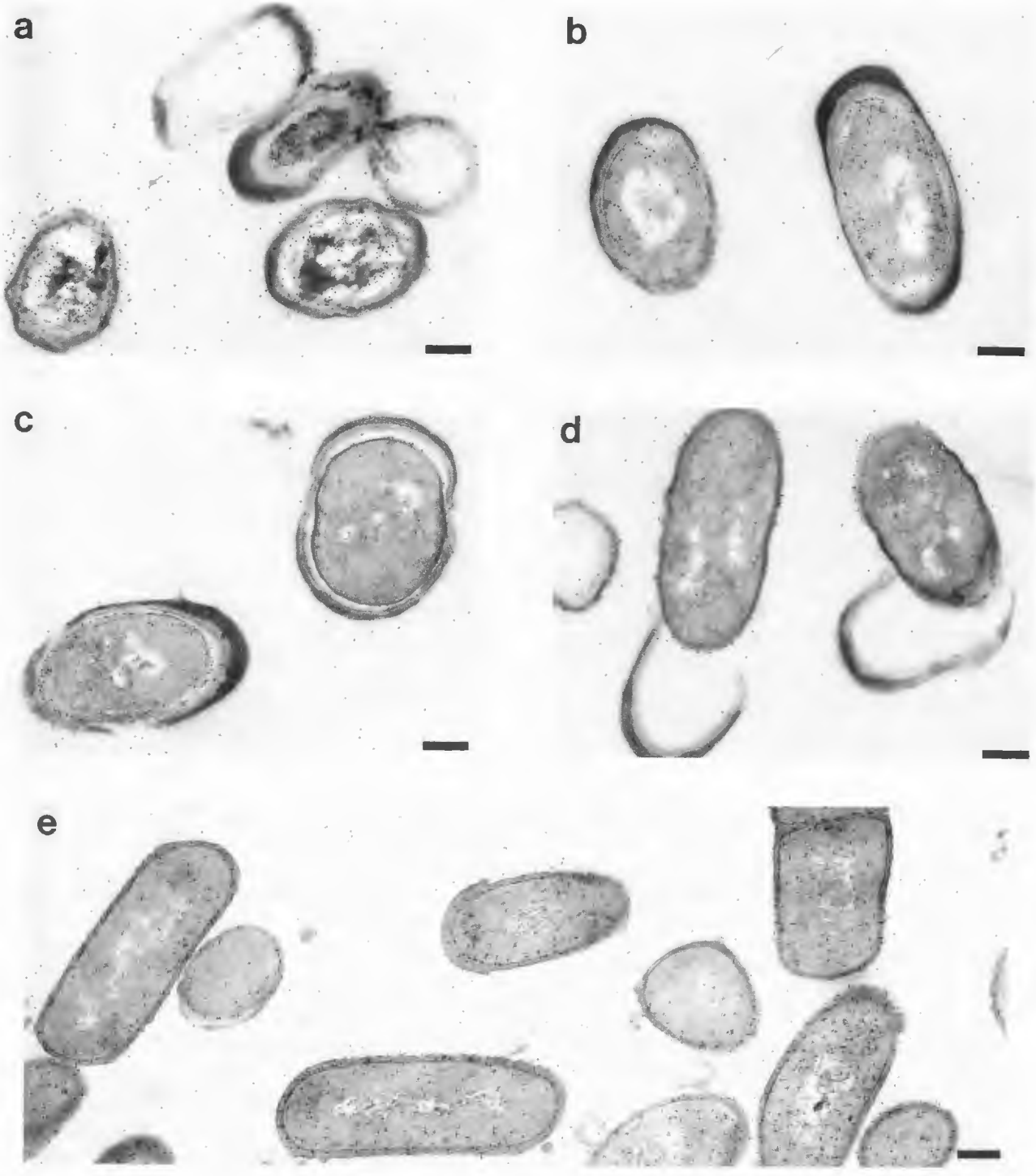

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Discussion with Reviewers

Reviewer I: In Figure 2a, differences in RER (rough endoplasmic reticulum) organisation between two adjacent cells is reported as the result of uneven fixation. This is not true. Differences in RER are not due to fixation but to differences in physiological state of the cells. Pancreatic tissue fixes in various different ways, even by perfusion, will show these differences in RER organisation. Not everything is due to the fixation protocol!

Authors: Perhaps the choice of the phrase "result of uneven fixation" in the legend of Figure 2a was unfortunate. There is a limit to what can be included in a legend and this can lead to ambiguity. We agree with the reviewer that "not everything is due to the fixation protocol," but then neither is everything due to natural variation. We are aware of the reviewer's opinion that the physiological differences between cells of the pancreas result in structural differences, particularly in the appearance of ER, but so do artifacts introduced during tissue handling and immersion-fixation. It is universally accepted that, when done properly, the perfusion-fixation of organs results in a higher level of consistency and well-organised structure than does immersion-fixation. Handling of tissue before fixation (e.g., dissection/excision) and the relatively slow process of diffusion of the fixative through the tissue during immersion-fixation can lead to anoxia and autolysis and causes both uneven fixation and the exaggeration of any differences in appearance between cells because artifact is superimposed on the natural variation. The RER highlighted in Figure 2a is a feature of both immersion-fixed and rapidly frozen pancreatic tissue and, therefore, probably results largely from the method of handling of the specimen, i.e., its removal (dissection/excision) prior to fixation or freezing. Differences in RER structure are also seen in perfusion-fixed pancreas but not as grossly and as frequently as those seen following immersion-fixation. Incidentally, perfusion-fixation is essential for the rapid, even distribution of low concentrations of fixative around organs (discussed in text). Further, these differences between perfusion- and immersion-fixation are seen **irrespective of the embedding protocol used**, be it at room temperature or low temperature.

Reviewer I: In spite of what is being reported, immunogold can also be examined at low magnification with silver-enhancement. The advantage of this over DAB would be that gold labeled sections can also be examined at high magnification with excellent resolution, while DAB will not give you excellent resolution at high magnification.

Authors: Silver-enhancement of immunogold can be used at low magnifications in the light microscope. However, we are unhappy with some of the recommendations for the silver-enhancement of small gold particles for transmission electron microscopy (TEM) and deliberately underplayed this technology. In the first place, when we talk of low magnification we mean 1,000 times or less, at which magnification DAB/sodium tetrachloroaurate will often give easily visible, clean, accurate

localisation. Assuming that a gold particle would need to be minimally 1-2 mm in diameter for it to be visible, at this magnification 5 nm gold would need to be amplified 200-400 times and 1 nm gold 1,000-2,000 times. Even assuming heavy immunolabelling with particles closer together than 1-2 mm at 1,000 times so that less amplification would be needed to aggregate them, it is clear that very powerful silver amplification is still needed. In our experience, problems such as serious levels of background and unspecific silver deposit become conspicuous in the TEM when such heroic silver methods are used, and in any event, amplification obscures ultrastructure and prevents high magnification viewing. It should also be remembered that DAB/sodium tetrachloroaurate, as well as being intrinsically very electron dense, can also be cleanly amplified with silver adding yet another dimension to its low and high magnification examination.

Secondly, uneven particle size is often observed in the electron microscope following silver amplification of immunocolloidal gold, leading to problems not only at low magnifications, but also at higher magnifications. There are two reasons for this. Some immunocolloidal gold preparations include a variation of sizes to start with and some silver methods amplify very unevenly. This phenomenon limits its use in double immunolabelling with colloidal gold of different sizes. More flexibility is available when DAB methods, which are very good for low and high magnification work in the electron microscope, are combined with immunocolloidal gold techniques.

C. Scala and G. Pasquinelli: You state that by controlling chemical catalytic polymerisation, the degree of acrylic resin cross-linking can be readily varied. This means that one can obtain less cross-linked acrylic resins giving, experimentally, a better immunocytochemical response. However, it is generally accepted that due to the large size of the immunoreagents, i.e., colloidal gold and immunoglobulins, on-grid immunostaining reasonably involves antigens which are exposed on the surface of the section. If this premise remains true, can you explain the improved immunocytochemical response you found on the less cross-linked acrylics?
Authors: We do explain the improved cytochemical response by the detection of immunoglobulins that have penetrated hydrated acrylic resin sections (Hobot and Newman, 1991; Newman, 1987; Newman and Hobot, 1993). Colloidal gold particles, however, are inflexible and prevent penetration into anything other than the surface of such resin sections. Therefore, an alternative detection system for immunoglobulins has to be used. This alternative is the peroxidase/DAB method. Observations of cross-sections of re-embedded, immunoperoxidase/DAB/sodium tetrachloroauarate labelled LR White sections showed labelling of antigenic sites within the depth of the sections (Newman, 1985; Newman and Hobot, 1987). These results are probably not due to diffusion artefacts as the very specific labelling of R1 antibody reactive sites on membrane surfaces of human carcinoma A431 cell monolayers demonstrates (Wynford-Thomas *et al.*, 1986). Indeed, the longer a semi-thin LR White section is exposed to the primary antibody the further into the section the DAB/sodium tetrachloroauarate label is found, even though in all experiments, the hydrogen peroxide activated DAB is used at the same dilution, 0.005%, for the same time of only 3 minutes. Experiments that are carried out carefully, using appropriate antibody dilutions for short times, show no build up of DAB deposits, which could erroneously give the impression of diffusion artefacts being present (Newman and Hobot, 1987). Perhaps it should be added that the use of sodium tetrachloroaurate (Newman *et al.*, 1983b, 1983c), instead of osmium tetroxide, to visualise DAB in the electron microscope, also helps to eliminate background staining, which could lead to the supposed presence of diffusion artefact.

J.E. Beesley: Why is it necessary to mount acrylic sections on the shiny side of the grid, whereas epoxy resin sections can be mounted on either side?
Authors: Practise has shown that acrylic resin sections stick more firmly to the shiny side of "naked" (i.e., no support film present) metal grids, than to the matt/dull side. The reverse is true for epoxy resins, i.e., they stick better to the matt/dull surfaces of grids. This is important in view of the long incubation times for which the grids (e.g., use nickel, gold, but not copper) plus sections are immersed in the various reagents during immunolabelling.

J.E. Beesley: The authors suggest that indiscriminate use of non-immune sera or immunoglobulin fractions may inhibit specific immunolabelling. Will the authors please expand this concept, describing under what conditions and at what concentrations of sera it may occur and give details of recommended concentrations and conditions for the use of blocking sera?
Authors: Specific blocking with non-immune sera or purified immunoglobulin fractions should only be used if high background still persists after all other efforts have failed. By definition, blocking reagents are used in high concentration so that there is a real danger that there may be fractions present in the sera which can have a non-specific attraction for the embedded tissue or resin and contribute to an increased background label (Dürrenberger, 1989), where in fact the initial aim was to reduce background. Such fractions can also compete

with, and therefore reduce, specific labelling.

If it becomes necessary to use blocking, then it can be carried out in two ways. By inhibitive blocking (pre-blocking), where the blocking solution is added before incubation with a primary or secondary reagent; or by competitive blocking, where the primary or secondary reagents are prepared by dilution in the blocking solution. The blocking solution can contain a 1/5 dilution of heterologous, normal swine serum.

Sometimes the secondary detection system can give a high background. This can usually be overcome by diluting the secondary reagent, (generally used in high concentrations), but not at the expense of reducing the quality of detection. If dilution is ineffective, then non-immune sera (inhibitive blocking) can be used. The sera (diluted 1/5) are from the same species of animal that has contributed the antibodies for conjugation to the colloidal gold or peroxidase. In practice, normal goat serum is used for goat anti-rabbit or goat anti-mouse conjugated colloidal gold, or normal sheep or normal rabbit sera for peroxidase conjugates.

It is important to note that extra checks must be run when using these extensive blocking reagents/steps, where the effects on labelling are studied by comparing combinations of blocked and unblocked sections (cf. Newman and Hobot, 1993).

Reviewer V: What is meant by "sympathetic" processing?
Authors: Less deleterious in terms of harming the biological tissue's ultrastructure or antigenicity.

Reviewer V: Can the authors provide data on the relative shrinkage for each of the common resins, and how the magnitude of shrinkage is affected by each of the curing methods?
Authors: We have no hard data on tissue shrinkage, as we have not undertaken any such studies. But from observations by Audrey Glauert (personal communication) on pure resin, it would seem that epoxy resins shrink by as little as less than 2% during curing and acrylics can be as high as 10-15%. Introducing cross-linkers and tissue to the resin will reduce these values. Gerrits *et al.* (1990) suggested that the extent to which resin infiltrates the different parts of a tissue block varies depending on the composition of the tissue locally and can affect on-section staining following resin curing or polymerisation.

Reviewer V: In Figure 3b, why do the cells appear to have lost their cytoplasmic contents?
Authors: As mentioned in the figure legend, this is a surgically removed human pituitary. Clinical biopsies are frequently delayed for hours before immersion-fixation and EM-processing into acrylic resin for immunocytochemistry (compare with Fig. 5, a perfusion-fixed pituitary of rat). Nonetheless, much structure remains, including the main organelles.

Additional References

Dürrenberger M (1989) Removal of background label in immunocytochemistry with the apolar Lowicryls by using washed protein A-gold precoupled antibodies in a one-step procedure. J Electron Microsc Techn **11**: 109-116.

Gerrits PO, Horobin RW, Wright DJ (1991) Staining sections of water-miscible resins. 1. Effects of the molecular size of the stain, and of resin cross-linking, on the staining of glycol methacrylate embedded tissues. J Microsc **160**: 279-290.

Newman GR (1985) Water, wax or plastic? Proc Royal Microsc Soc **20**: 41M (abstract).