19.5.89

SEMT

Society of Electron Microscope Technology

Affiliated to the Royal Microscopical Society

You are cordially invited to a meeting of the SEMT on Friday, 19th. May on

FIXATION AND SPECIMEN PREPARATION

at the Imperial Cancer Research Fund, Lincolns Inn Fields, London at 2.00 p.m.

Programme

2.00 Chairman's Welcome and Introduction

2.10 "Fixation for Morphology"
Dr. Gillian Bullock (President, Royal Microscopical Society)

2.50 "Pre-embedding and post-embedding techniques for E.M. Immunocytochemistry"
Dr. F.J.Alvarez (St. Thomas's Hospital Medical College)

3.20 "Freeze substitution : possibilities for immunocytochemistry"
Dr, Paul Monaghan (Institute of Cancer Research, Sutton)

3.50 Tea

4.10 "Ultrastructure as revealed by freeze-fracture and cytoplasmic maceration".
Ms. Sue Barnes (British Museum of Natural History)

4.40 Chairman's Summing-up and General Discussion.

There will be a small Trade Exhibition.

If you expect to be able to come, please send the slip below to :-
Dr. Jill Lewis, EMU, St. Bartholomews' Medical College, Charterhouse Square, EC1M 6BQ

ABSTRACT.

FIXATION FOR MORPHOLOGY.

Dr Gillian Bullock, Ciba-Geigy Ltd, Basel.

It has become very apparent over the years that when we say that we are 'fixing' a cell, we are in fact inducing a situation full of artifacts.The cell is killed and every thing in it loses the constraints which would normally keep the cell contents in place. As the good preservation of cellular ultrastructure is dependent on minimising such artifacts, what can we do to ensure this minimum and also to recognise the artifacts when they occur.

In order to reduce extraction from the cell or tissue, we have,for example,a number of chemical fixatives which act by cross-linking protein thus providing an anchor within the cellular framework. Proteins are relatively easy to stabilise whilst lipids and carbohydrates are more difficult. However, cross-linking of a protein introduces two recognizable artifacts, firstly that of shrinkage and secondly protein structural distortation.Whilst the first introduces problems for the would-be morphologist, the second can create difficulties for the immunocytochemist who finds that his antibody cannot recognize the cellular antigen. In addition, in the case of enzymes, activity may be severely reduced or even lost. This is just one of the dilemmas that we have to face.

In this talk, examples of the more and less commonly recognizable artifacts will be given and their relative importance will be discussed. Finally, an overview of the standards we can hope to achieve will be included.

PRE-EMBEDDING AND POST-EMBEDDING TECHNIQUES FOR EM IMMUNOCYTOCHEMISTRY

Dr FJ Alvarez
Departments of Physiology and Biochemistry
UMDS St Thomas's Hospital Medical School Campus, London

Electron microscopic (EM) immunocytochemistry can be carried out in two basically different ways. In pre-embedding techniques immunostaining is carried out on thick sections before embedding the tissue for ultrathin sectioning while in post-embedding techniques the immunostaining is carried out directly on the ultrathin sections. These techniques can be further divided according to the marker used and the way in which the marker is attached to the antigenic sites (eg peroxidase anti-peroxidase (PAP), indirect colloidal gold or avidin/biotin techniques).

The main difficulty with pre-embedding staining is to obtain an adequate immunoreaction within well preserved thick sections. This will depend on many different factors but two of the most important are the type of tissue fixation and the size and properties of the immuno-reagents. The commonly used fixatives are based on a mixture of paraformaldehyde (eg 4%) and glutaraldehyde. However since glutaraldehyde may diminish staining by excessive cross-linking of tissue components, its concentration is normally kept low (0.05% - 0.5%) and alternative fixatives have also been developed (eg picric acid, acrolein, carbodiimide). With a given fixation and set of reagents, penetration into sections can be enhanced by freeze/thawing of tissue blocks, by treatment with detergents or by increasing the antigen or antibody concentration.

Freeze Substitution: Possibilities for Immunocytochemistry

Freeze substitution as an electron microscope preparation technique consists of rapid sample freezing followed by replacement of the frozen water at low temperature by solvent, usually containing fixatives. Embedding takes place either after warming to room temperature, or at low temperature in Lowicryl resins. The initial rapid freezing of the sample halts rapid cellular processes and can provide improved resolution of cellular detail when compared with conventional fixation.

We have been interested in the possibilities that this method provides when processing cells for immunocytochemistry where particular antigens are sensitive to even minimal fixation protocols. Our approach has been to freeze samples by impact onto a liquid nitrogen cooled copper block using a Reichert MM80, and transfer them to the chambers of a Reichert CSAuto. Substitution of ice takes place with methanol, and the samples are warmed to -50°C to -35°C for infiltration and embedding in Lowicryl resin. Polymerisation is by UV light at low temperature for 1 to 3 days, depending upon temperature.

For such an approach to be valuable, two features need to be demonstrated. Firstly, the retention of good cellular and organelle morphology, and secondly the ability to demonstrate labile antigens in freeze substituted material.

Despite the lack of conventional fixation, light microscope sections show excellent morphology extending a considerable distance from the impact face. At the electron microscope level, however, only the 10-20 μm nearest the impact face show good morphology. Further into the block, freezing rates are inadequate to prevent ice crystal formation and subsequent cellular distortion.

Lowicryl resins have a good reputation for allowing post-embedding immunocytochemistry, and our results indicate that this remains true for freeze substituted material.

Many parameters need to be optimised in this system, but for particularly fixation-labile antigens at least, freeze substitution has enormous potential.

P43/PM/sjt/2.5.89.

Ultrastructure as revealed by freeze-fracture and cytoplasmic maceration

Susan H. Barnes

British Museum (Natural History), Cromwell Road, London SW7 5BD

With modern scanning electron microscopes and new preparation techniques interesting details of cell interiors can be observed. In this presentation the freeze-fracture and cytoplasmic maceration technique, and its applications to specific research problems in plant biology, will be described.

The method used to reveal the internal structures of plant cells is a modification of the original technique devised by Tanaka and Naguro (1981). Fresh material is fixed in osmium tetroxide, cryoprotected, frozen on a liquid nitrogen cooled block and fractured with a razor blade and hammer. Thawed fragments are treated for extended periods with dilute osmium tetroxide, fixed, dehydrated and critical point dried.

The technique has been applied to several aspects of botanical ultrastructure including a study of mesophyll chloroplast membrane systems in Aucuba japonica and a comparative study of pollen development in the Lactuceae.

Barnes, S.H. & Blackmore, S. 1984. Freeze fracture and cytoplasmic maceration in botanical scanning electron microscopy. Journal of Microscopy **136**:RP3-4.

Barnes, S.H. & Blackmore, S. 1984. Scanning electron microscopy of chloroplast ultrastructure. Micron and Microscopica Acta **15**:187-194.

Barnes, S.H. & Blackmore, S. 1986. Plant ultrastructure in the scanning electron microscope. Scanning Electron Microscopy /1986/I:281-289.

Blackmore, S. & Barnes, S.H. 1987. Pollen development in Tragopogon porrifolius (Compositae: Lactuceae) and its taxonomic significance. Review of Palaeobotany and Palynology **138**:233-246.

Tanaka, K. & Naguro, T. 1981. High resolution scanning electron microscopy of cell organelles by a new specimen preparation method. Biomedical Research **2**:63-70.

Fixation for Morphology

Dr. Gillian Bullock

Chemical fixation is a compromise between retention and the introduction of structural changes. We can and must live with the artefacts and recognise them. A white empty space emans that something has been lost.

Gross changes: shrinkage, swelling, change of shape, change of surface e.g. by retraction of processes. Shape changes especially with isolated cells.

Medium changes: organelles, vacuoles distorted, granules extracted or discharged, membrane structure changed.

Fine changes: extraction of e.g. lipid, relocation, loss of enzyme activity, loss of ions.

Kellenberger maintains that many antigens survive fixation, but it is the embedding treatment which damages them.

Gross changes can make morphometry impossible. We must balance the osmolarity of the buffer with that of the cell contents. For surface structure, check the temperature and pH, mix with albumin etc; if necessary change the fixation. Cross-linking proteins alone produces shrinkage.

Redistribution is due to the buffer being at the wrong osmotic pressure, producing e.g. wide tubules, expanded endoplasmic reticulum, larger vacuoles. PIPES buffer especially does not enter the cell.

N.B. the polymorph nuclei membranes are not normally closely apposed. Mitochondria are liable to swell and shrink. Use a shallow slab of tissue. Nick coat of a spore (!!!). Reduce the time of transfer fo tissue from body to fixative; for morphology, preferably perfuse. Thrombocytes are especially labile. Lipid-containing bodies e.g. liposomes are very difficult.

Fine changes are moee difficult to detect. To minimise extraction of lipid, use short glutaraldehyde fixation and low concentration of osmium, and fast processing. Carbohydrates can be lost with the wrong buffer. Nucleic acids cannot be fixed. Use a test block to check for loss of soluble proteins. Nucleic acid loss is particularly important with in situ hybridisation. The remedies include change in fixation procedure; precipitating ions; use of frozen sections; additives e.g. malachite green, tannic acid, ferrous compounds. Formalin fixation is not reversed by hydrolysis if the tissue is thoroughly fixed.

NMR can show swelling of mitochondria. Every tissue is different! Piperazine preserves microtubules; cacodylate does not. The buffer can also affect the junction regions between cells a lot.

The dark line around lipid droplets with glutaraldehyde/osmium fixation is a precipitate formed later in processing, when osmium is left after lipid extraction.

The important stage is the beginning of fixation; after this, there is NO possible control of volume change during dehydration. Different things are extracted at different pH; best is to use pH 7.
The osmolarity of the BUFFER is VITAL. Karnovsky fixative and glutaraldehyde give the same picture with the same buffer, although their osmolarities are very different.
Procaine anaesthesia may relax the tissue, to improve the picture.
If thd tissue is left in absolute alcohol for a long time, the membrane can be extracted to give a negative picture.
Liver needs 0.1 mM, heart 0.08 mM fixative.
Detergents such as Brij or Tween shouldn't damage the tissue.

Pre-embedding and post-embedding techniques for EM immuno-cytochemistry

Dr. F.J. Alvarez

Resolution for cytochemistry is 1 nm. Most is done pre-embedding, with osmium after the immuno stain and before embedding. The best labels are gold or peroxidase.

Pre-embedding: high non-specific staining
limited choice of immuno reagents
poor antibody penetration

post-embedding: poor preservation of antigenicity

Penetration of the antibody depends on the fixation and the size of the immuno reagents, the affinity of the antibodies, and the sensitivity of the technique. Glutaraldehyde affects the antigenicity most, depending on the time and concentration:

4% formalin + up to 0.4% gluaraldehyde, in 0.1 M phosphate pH 7.4
2% formaldehyde in 0.1 M periodate + 0.075 M lysine in 0.04 M phosphate pH 6.2
(McLean & Nakane 1974)
Zamboni & de Martino 1967
Willingham & Yamada 1979
King et al 1983 - acrolein

human IgG	molecular weight	c. 150,000
Peroxidase conjugated with IgG		c. 200,000
PAP complex	more than	500,000
monoclonal PAP		c. 340,000
hybrid hybridomas		c. 90,000
avidin		c. 60,000
biotin		c. 244,000
IgM		c. 900,000

Saponin is better than Triton-X

Thick sections cna be put on a drop of Durcupan on a slide, covered with a plastic coverslip, and weighted down lightly.

The use of Lowicryl affects the final ultrastructure.

Freeze substitution: possibilities for immuno-cytochemistry

Dr. Paul Monoghan

Low temperature techniques are very useful for antigens which are sensitive to processing, or are present only in small amounts.

With Progressive Lowering of Temperature, you are liable to get a sorbet !

With cryo-sectioning, no embedding is employed, but care is needed in the fixation, cutting, storage, interpretation.

Pre-treatment for cryo methods can be death to antigens.

It is difficult to cut good cryosections of cell pellets.

Freeze-substitution is easy to interpret and no fixation is necessary; but the specimen is then resin- embedded, so only the antigens on the surface of the section can be demonstrated; and only a small depth, 10-20 um, is well frozen.

Stages:

1. Cryofix - fast onto a cold copper block, to stabilise;
2. Substitution - of the ice (water) with acetone (over days) or methanol (over hours) at -80'C.
3. Lowicryls at low temperature
4. POlymerise at low temperature by UV light

Lowicryls	K4M	at -40'C	hydrophylic
	K11M	-60'C	
	HM20	-50'C	hydrophobic
	HM23	-80'C	

Hydrophilic resins should be better, but he gets good results with HM20.

Keep the tissue damp.

Lowicryl heats up a bit as it polymerises, but not significantly. At the light microscopy level, large areas of good morphology are seen; for EM only smaller areas are really good, then ice crystals are seen. Pellets of cells can give good results. There is excellent staining even though there is NO fixation.

It si possible to see coated vesicles in process of fusing with other vesicles.

Resin sections, of course, only stain at the surface.

Microtubules are seen in freeze-substituted material, but not in cryosections.

The substitution method may be determined by the antigen; some are sensitive to acetone, some to methanol.

Silver enhancement can be used on 1 nm gold label.

-25'C is the warmest that one can go without fixation, before the tissue falls to pieces. Cryoprotectants are not used.

Lowicryl K11M tends to chatter when cut.

Ultrastructure as revealed by freeze-fracture and cytoplasmic maceration

Ms. Sue Barnes

(Bullock - piperazine is VERY GOOD for microtubules)

DMSO is used as cryoprotectant for freeze-fracturing. Osmium fixation is used to extract the cytoplasmic matrix.

1. Fix in 1% osmium in M/15 phosphate - must be HYPERtonic
2. 15%, 30%, 50% DMSO
3. Freeze-fracture with a razor blade
4. Thaw in DMSO
5. Wash wash wash
6. Macerate in 0.1% osmium in M/15 phosphate for 2 weeks in the fridge, replenishing frequently
7. Fix in 1% osmium, treat with 2% tannic acid, refix in 1% osmium
8. dehydrate, mount, coat.

USE EYE PROTECTION

A thin coat of Au/Pd can be sued, because there is so much osmium in the specimen. The best cells are at the edge of the specimen, because of the freeze-fracturing. The nuclei are liable to swell.

Callose is produced in the vesicles; it is electron-lucent, and si very important in the formation of the pollen wall.

The specimen can be supported by inlcusion in chitosan.