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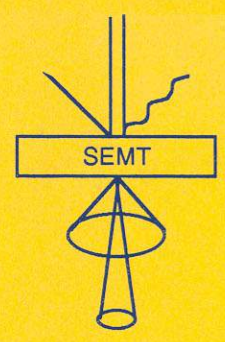
Society of
Electron
Microscope
Technology



Tea and Tuition

with

❄ Pat Echlin - Cryo-techniques ❄

✂ Jan Hobot - Preparation for immunocytochemistry ✂

❀ Alice Warley - X-ray micro-analysis ❀

Paul Monaghan - Immuno-gold labelling

on Wednesday 18th May

For details contact Dr G Lewis tel : 081 428 426

S.E.M.T. 18th May 1994 TEA AND TUITION

Pat Echlin - Cryotechniques

Cryofixation is a physical process, adding mechanical strength to a soft sample. It stops physiological processes and immobilises liquids. It reduces contamination and radiation damage; avoids using chemicals; retains the natural structure and the soluble constituents. BUT the big limitation is the formation of ice crystals. Radiation damage does still occur to some extent because of the many free radicals. It requires complex protocols and expensive instrumentation.

The cooling procedures used depend on the properties and structure of water and ice. Echlin routinely fixes at room tmeperature, then dehydrates through alcohols at progressively lower temperatures, and infiltrates at low temperature.

Vitrified water may form the perfect embedding matrix for bio-organisms. Frozen water is an important milieu for dissolved ions in the sub-cellular components; it stops dynamic processes, and permits the resolution of time-dependent inter-actions. A smaple up to 25 um across can contain virtually no ice crystals.

Use liquid nitrogen to cool ethane, pentane etc, for immersion freezing; immersing in liquid nitrogen direct is not good. Or slam the specimen onto cold copper, for a large surface; or jet-spray onto copper, etc. Slamming is done at high speed, so there is some mechanical damage; the first 5-6 sections should be OK. Hyperbaric freezing (Monaghan has a machine) will give satisfactory freezing to ½ um depth. Echlin prefers not to use any anti-freeze agents.

Jan Hobot - Preparation for Immunocytochemistry

The effects of fixation, dehydration and embedding are all inter-linked. If cross-linking with aldehyde, a high concentration for immersion fixation is greater than 0.5% glutaraldehyde; low concentration for perfusion is 0.1% Post-fixation with osmium or uranyl - uranyl is good for membranes; osmium may obliterate visualisation of the immunocytochemistry. Low concentration fixation allows demonstration of some labelling. If "high concentration" glutarladehyde is used, any differences in dehydration schedules will not make a ny difference to the immuno result.

"Low concentration" glut fixation leaves the tissue very sensitive to the dehydration technique; it is better not to go right to 100% alcohol, but from 70% alcohol to acrylic resin - LRWhhite or Lowicryls - at room temperature.

With perfusion fixation, it is possible to go at low temeprature into LRWhite or Lowicryl; or with Progressive Lowering of Temeprature, into Lowicryl. If you need to correlate with the light microscopy appearance, remember that Lowicryls K4M and K11M Lowicryls react with basic stains.

In the pancreas, 1% glut will allow reaction in the zymogen granules; 0.1% glut will allow reaction in the rough endoplasmic reticulum as well.

With peroxidase, use a short low concentration of DAB, or you will get diffusion of the reaction product.

Resin Microscopy and On-Section Immunocytochemistry
G.R.Newman & J.A.Hobot
Springer-Verlag 1993 £27

Alice Warley - X-ray Microanalysis

Electron probe microanalysis gives quantitative results; the high-energy beam knocks out electrons depending on the energy of their shells; the peaks also give a measure of teh specimen mass.

EPXRMA is best when there is only a small amount of specimen. It is a visual technique, and also a multi-element technique if the elements are at sufficiently high concentration. It is relatively non-destructive, and you can (hopefully) go back to the same specimen again. It is especially useful for pathology, both for disease and for the legal implications.
In cystic fibrosis, nail clippings show raised sodium and chloride,
It is also possible to de-wax sections and use SEM (Levinson at Guy's)

Specimen pre-aration is critical; with damage, the ion gradients alter. E.g. if using cell cultures of fibroblasts, the stimulated cells do have different diffusible elements.

It is possible to quantify the technique, but ti is sensitive to mass loss. The grid and fupporting film give a constant contribution to the background. With higher background, there is less chance of seeing the smaller peaks.

Paul Monaghan - Immuno-gold Labelling

Are you sure that the antigen is present in the sample?
Is it recognisable?
With a new antibody, do your homework! - frozen sections by light microscopy, positive control, background, fixation.

Some antigens are very sensitive to fixation. Monaghan recommends a mixture of 2% paraformaldehyde + 0.5% glutaraldehyde.
Blocking procedures for non-specific labelling: BSA, foetal calf serum, fish gelatine (from Amersham with certain of their antibodies - very good).
With Lowicryl HM20, better labelling is obtained if the incubation with the first antibody is overnight; with K4M, 1½ hours is sufficient.

Colloidal gold from commercial sources is Ok and will last for years. Smaller gold particles, less than 10 nm, give more label but are more difficult to see. On resin sections there is little difference in sensitivity between 5 nm and 1 nm particles, as it appears that not all the gold particles are enlarged by the silver enhancement.
On thawed cryosections, 1 nm particles are definitely better than 5 nm, if silver enhancement is used.
In SEM, you can use smaller particles for a back-scattered electron detector than for secondary electrons.
Silver enhancement is very good for LM and EM, but not perfect. For LM, use epipolarised and transmitted light together; this system (i.e. the epipolarisation) will show all the background gold stain.
The silver enhancement process is temperature-dependent; get the solutions out of the 'frig in good time.
With a good specimen, the primary antibody can be used at a diltuion of 1:2000.

Tea with Hobot

The key to partial dehydration is the freshness of the LRWhite; if it is good, it is miscible with 70% alcohol; less fresh resin needs higher alcohol concentration. If the mixture with alcohol is only slightly milky, it may be OK; but it is supplied mixed with a small amount of benzoyl peroxide, and at room temperature it will begin to polymerise and reduce the amount of free monomer. So for partial dehydration, keep the resin only 3 months from the date of manufacture. Prepare the 70% alcohol from 100%; then mix 2 parts of LRWhite with 1 of 70% alcohol. Store the LRWhite at 4'c, and allow to warm to room temperature before using; store the 100% alcohol over molecular sieve.
The older resin will still be OK for complete dehydration schedules.

You can stipulate uncatalysed resin (this was prepared originally for hot countries), and the catalyst will then be supplied separately; the catalyst is only added as required, and the components separately will last a year or more. Benzoyl peroxide is supplied as a 25% paste with water; it is explosive when dry. Biocryl does contain catalyst, is the same as Unicryl, much more expensive than LRWhite, and has no advantage over LRWhite.

Progressive Lowering of Temperature goes down to 100% alcohol at -35'C.
Partial Dehydration goes from 70% alcohol at room temperature.

For perfusion studies for research, 0.1% glut is good for the immunogenicity but less good for the ultrastructure. If 1% glut is used, there is no point in bothering with PLT embedding. After several hours of high-concentration fixation, reactivity does decrese slowly. At low concentrations of glut, 1 hour fixation is fine, but longer fixation will allow some extraction.

Amines in the tissue act as accelerators in polymerisation.
BSA blocking will deal with free aldehdyes, so there is no need to quench for free amines.

Lowicryls K4M and K11M cannot be used with DAB.

The hardness of the resin can be varied by adding chemical catalyst and accelerator.
Cross-linking improves beam stability, but may interfere with immunogenicity.
10 ml resin requires 15 ul activator.
Flat embedding can be done on Thermanox coverslips etc., in an anaerobic atmosphere, e.g. dry nitrogen.
Cryoprotectants do affect the ultrastructure of memberanes.
LRWhite is the best acrylic generally - cheapest, for LM and EM, at room temperature for full or partial dehydration; partial dehydration is easier technically than PLT, but the window of success is greater with PLT. Since PLT must be done below -30'C, only Lowicryls can be used for this.

There is no difference between LRWhite and LRGold, except possibly for tissues with high lipid content. Some fixation is required with LRGold too.

For partial dehydration, 70% alcohol is on the limit of miscibility; from this, put into pure resin and mix on rotamixer; or into 2 resin:1 alcohol.
Lowicryl HM20 at low temperature, is good for lipids.
For lipid, you can post-fix with uranyl acetate; but then you cannot subsequently use phosphate buffer - use Tris instead.

For visualisation, gold chloride reacts only with DAB; osmium reacts with the tissues as well.

Do the immuno staining on naked nickel grids, by immersion, to increase the sensitivity.
0.5% collodion in amyl acetate can be used to coat the copper grid bars, to protect the copper against the stain.
Wash the sections by immersion several times for 1 minute each time, rather than dislodge the antibody by using wash-bottle. This gives good clean repreducible labelling.

Hobot recommends Nesca film rather than Parafilm, for doing the staining on.

Polysciences glutaraldehyde is very pure, and the 50% soltion should be kept frozen in the vial. It does polymerise sbowly. Taab glut is supplied at 25%