Technical Data Sheet

Lowicryl Letters

#14330

Trends and Developments in Low Temperature Embedding

K4M has found rather wide application in immunolabelling. HM20, as we mention below, has opened new avenues in the observation of unstained sections and thus - finally - the observation of proteins under presumed conditions of minimal denaturation. We found it reasonable to create a means of communication within the "Lowicryl-family", for the dissemination of new successes and new uses as well as difficulties. The Lowicryl Letters should promote also joint projects. Indeed many of the potential possibilities need still laborious efforts, which could be handled much more efficiently if the involved laboratories would become known to each other and thus be able to communicate.

We very much hope that small advances of a technical nature would be made accessible, particularly in all those cases where the concerned laboratory might not have the possibilities to continue systematic studies acceptable for publication. Our own laboratory is for example also not able to develop all the techniques as outlined in parts C and G of this issue. As part of a Microbiology Department, it has to concentrate on biologically relevant applications.

In TIBS 6, XI (1981) we made a proposal for certain new forms of publications which consist of communicating short abstracts while simultaneously depositing the corresponding complete manuscript at the editorial office from which copies can be ordered to minimal costs. If this proposal is not taken up by others, the Lowicryl Letters might later become such an abstract journal in the field of electron microscopy applied to biology.

Since the present Lowicryl Letters are not yet a publication accessible to everybody, its contents should be considered a "personal communications".

We hope very much to receive contributions from everywhere, both in the form as exemplified in the present issue and in the form of questions, which according to each case, will be answered individually or, when we feel it to be of general concern, published in the letters.

Eric Carlemalm, Werner Villiger & Eduard Kellenberger, Editorial Board of the Lowicryl Letters

Recent Publications:

B.L. Armbruster et al., Specimen preparation for electron microscopy using low temperature embedding resins., J. Microscopy, Vol. 126, pp. 77-85, 1982

E. Carlemalm et al., Resin development for electron microscopy and an analysis of embedding at low temperature., J. Microscopy, Vol. 126, pp. 123-143, 1982

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TheMain Goal for HM20 is Achieved

E. Carlemalm & E. Kellenberger

In the EMBO-Journal we published the first observations, demonstrating that we have achieved the main goal of our resin development (1), namely the reproducible imaging with high contrast and sufficient resolution of completely unstained sections!

Through numerous observations, most of us electron microscopists have come to believe that the usual staining of tissue blocks and sections with Os, U, Pb, is in reality a sort of impregnation. Staining is initiated by a stoichiometric reaction with a suitable side chain or a double bond, according to organic chemistry, but this is then followed by a non-specific addition reaction which increases the heavy metal deposit many fold. This is also confirmed by recent experiments which will be published soon. We knew therefore that any decrease of fine structure due to denaturation is likely not visible as long as staining still has to be involved.

Dark field imaging of unstained sections was shown to be possible (CTEM 2,3; STEM 3,4) but the results were disappointing due to the lack of sharpness and of reproducibility. During more than a year of attempts we succeeded only once to repeat - even with less quality - the beautiful picture of unstained phage with their 40 Å tail fibers (Carlemalm and Villiger in 5).

By considering theoretically and numerically the different modes of contrast formation (Carlemalm, Wurtz, Engel and Kellenberger, paper in preparation) we reached the conclusion that Z-contrast, formed by the ratio of elasticity to inelastically scattered electrons, should be able to provide enough contrast also with unstained sections.

Unfortunately this mode is possible only with a STEM, equipped with an adequate energy analyzer (electron spectrometer). Dr. Ch. Colliex (Universite de Paris-Sud, Laboratoire de Physique des Solides, Orsay-Cedex-France) has recently acquired a spectrometer (Gatan) and through his kindness we were able to check our theoretical predictions. Within a few working days we were able to get as good a yield of usable micrographs as with CTEM. On septate junctions of Drosophila testis we were able to image an integral transmembrane protein (6,7). This was not possible by staining sections from the same embedding and observing them in CTEM.

The way is now open to investigate biological membranes which are rich in proteins. Lipids are not fixed by aldehydes, as shown in work by Prof. C. Weibull's group in Lund, Sweden (8). The are apparently replaced by the resin in a similar manner as cellular water. Lipid-rich membranes are therefore not yet accessible to our new method of observation and we are actively investigating the possibility to fix lipids without stain.

December 1981 - Eric Carlemalm and Eduard Kellenberger

References

1. Carlemalm, E., Garavito, R.M. and Villiger, W.; Resin development for electron microscopy and an analysis of embedding at low temperature. , J. Microsocpy, Vol. 126, pp. 123-143, (1982).

2. Weibull, C., Electron Microscopy Studies on Aldehyde-Fixed Unstained Microbial Cells., J. Ultrastruct. Res., 43, 150-159 (178).

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4. Jones, A.V., and Leonard, K.R., Scanning Transmission Electron Microscopy of Unstained Biological Sections., Nature, Vol 271, 659-660, (1978).

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6. R.M. Garavito, E. Carlemalm, C. Colliex and W. Villiger, Septate junction ultrastrucutre as visualized in unstained and stained preparations. JUR in press.

7. E. Carlemalm and E. Kellenberger, The reproducible observation of unstained embedded cellular material in thin sections: visualization of an integral membrane protein by a new mode of imaging for STEM., The EMBO Journal, Vol. 1, 63-67, (1982).

8. C. Weibull and A. Christiansson, E. Carlemalm, Extraction of membrane lipids during fixation, dehydration and Embedding of Acholeplasma Laidlawii cells for electron microscopy., Submitted.

The Setup for UV-Polymerization

E. Carlemalm, W. Villiger, M. Garvito, and J.D Acetarin

The 15W lamps, emitting mainly at 360nm are currently used in biochemical laboratories for thin layer chromatography. They are fluorescent coated; to the eye they are bluish white. Philips sold them formerly as TLAD 15W/05 and now TLD 15W/05. Similar types of lamps are available from other makers.

It is obvious that UV should have access to the capsules from all sides. Polyethylene and polypropylene are relatively transparent to 360nm UV light and thus BEEM®-capsules are very suitable. The medical gelatin capsules (unstained) can also be used. It is recommended to check the set-up by polymerizing the resin without sample.

The normal low-temperature procedure is at least 24 hours at -35 to -50 degrees C with the initiator contained in the kit. The blocks should then be hard (fingernail check) but sectioning can be difficult. Complete hardening is obtained after 2-3 days of UV-irradiation at room temperature by the same type of lamps as before.

Cold room temperature (4 degrees C) polymerization is easily possible, but the standard initiator should be replaced by the same amount of benzoin ethyl ether. This initiator is suitable up to +20 degrees C.

IMPORTANT NOTE: If too much oxygen becomes dissolved into the resin, for example through vigorous mixing of the components, the polymerization might be incomplete. We recommend mixing the resin with a Pasteur-Pipette through which one slowly streams some nitrogen.

The Chemical Polymerization of Lowicryls

J.D. Acetarin and E. Carlemalm *

UV-polymerization as recommended for the Lowicryls works well with unstained blocks which also do not contain pigments absorbing at 360nm. Such a pigment is for example contained in the slime mold Physarum. Carotenoids, Hemes and Flavins apparently do not affect UV-polymerization if present in the usual amounts in cells. Os-fixed blocks are necessarily also opaque to UV-light and thus UV-polymerization is not possible.

It would be useful for any cytological E.M.-Laboratory, if the same resin could be used for all purposes. Every resin has somewhat different cutting properties and the microtomist is in general not very happy, when he or she has to change resins all the time. It is also of general experience that optimal sections are only achieved when concentrating on a single resin. Realizing this, we have also investigated the chemical polymerization of Lowicryls. The successful and also partial results are described in an appendix to our chemical papers on the Lowicryls *) and in what follows.

An essential limit in chemical polymerization is the requirement for a temperature jump. Since polymerization should not start during infiltration, the temperature has to be raised by some 20 degrees C for curing.

(1) Polymerization at 50 to 60 degrees C is reproducible by using dibenzoylperoxide as initiator, 0,2% (by weight) for K4M and 0,5% (by weight) for HM20. Polymerize overnight.

*) See also: J.D. Acetarin and E. Carlemalm, Appendix, J. Microscopy 126, 140-141, (1982).

The polymerization reaction is exothermic and therefore generates a temperature rise which is unfavorable. It is therefore recommended to put the capsules into holes drilled in a 15-22mm thick aluminum plate which acts as a heat sink. Gelatin capsules are in this case better than BEEM® capsules.

(2) Preliminary results of room-and low-temperature chemical polymerization. For achieving a suitable polymerization rate, this requires an activator in addition to the initiator. Tertiary alkylarylamines such as N,N-dimethyl para-toluidine has been used as activator in combination with dibenzoyl peroxide.

Chemical initiation of the polymerization is critically dependent on the ratio between activator and initiator. It is also very easily inhibited by oxygen. For the time being our results are not yet reproducible. For those who cannot use UV-polymerization and prepared to take some risks, (see note at the end of this section) we give in the following the preliminary data for polymerization at room temperature and -35 degrees C. (For 0 degrees C the statistics of successes is too meager to even mention the conditions):

	Room Temperature		**- 35 degrees C**	
	HM20	K4M	HM20	K4M
Dibenzoyl peroxide	0.08%	0.05%	1%	0.1%
N.N.-dimethyl para-toludine	0.05%	0.03%	0.6%	0.25%

Points to be emphasized:

1. Mix the resin in two parts, one containing the peroxide, the other the activator.
2. Cool the two parts separately.
3. Mix them immediately before use.
4. Do not forget that the polymerization reaction starts as soon as the activator and initiator and are combined in the resin, unlike in the case of UV-polymerization that starts only with irradiation. One has thus to infiltrate the resin below the planned polymerization.
5. Since oxygen is a very efficient inhibitor, it is essential to perform the polymerization in an inert atmosphere (nitrogen or carbon dioxide).

IMPORTANT NOTE: We discovered recently that the stabilizer, used to ensure long shelf-life of the resin components, does not influence UV-polymerization; it has however an influence in chemical polymerization. With the above described recipes only little reproducibility is achieved. If you have problems, contact the editors or the firm.

Problems of Flat Embedding of Tissue Cultures

E. Carlemalm and W. Villiger

Epon is currently used for embedding cell monolayers in the culture dish. We have investigated the two Lowicryl resins for the same purpose. Two main problems arose:

A) The polymerization is quite inhibited by oxygen due to its increased uptake through the large resin surface in contact with air. This is avoided by polymerization in a protective atmosphere (N_2 or CO_2). A simple set-up, that can easily be improvised in any laboratory, is shown below.

B) Petri-dishes and other supports used for growing cells are frequently made of polystyrene which is dissolved by the Lowicryl resins. Heraseus supplies petri-dishes with an oxygen-permeable membrane at the bottom. These are not dissolved in Lowicryls and alcohol. Two types are offered either with a hydrophilic or hydrophobic surface available for the cell growth under the name of Petriperm. The following drawbacks are associated with the use of these vessels: the hydrophilic surface, which normally allows cell growth, binds very strongly to Lowicryl K4M. The hydrophobic surface does so to HM20. In these combinations the membrane cannot be removed and the embeddings have thus to be sectioned with the membrane present. This can cause some problems. The following procedure has worked well in the hands of some people in our laboratory and less well for others:

1. Fix, dehydrate and infiltrate according to the desired procedure.
2. Polymerize by UV-light in a protective atmosphere (N_2 or CO_2) for at least 24 hours at -45 to -35 degrees C. If the resin is insufficiently polymerized by indirect light, remove the reflector below the lamps and use direct irradiation (see figure from chapter B)
3. Continue the polymerization at room temperature for 3-5 days with direct irradiation at a distance of 200-300mm.

C) Petri-dishes made of glass can be used. The detachment of the polymerized layer of cells and resin from the glass is difficult. Although somebody will certainly invent a procedure for doing this, the people of our laboratory have not continued to investigate this possibility.

Is Very Low Temperature-Processing Needed for Immunolabelling on K4M?

It is known to immunologist that denaturation of proteins need not lead to substantial losses of the affinity towards antibodies. This might turn out differently for some particular monoclonal antibodies. Everything suggests that low-temperature embedding might not be essential for successful immunolabelling with protein-A-gold. Cold room processing, possibly even room temperature, might be fully sufficient (see chapter 3)

Dr. Jurgen Roth, Dept. of Histology, University of Geneva (Director Prof. L. Orci), and Dr. Barbara Armbruster in our laboratory are presently investigating these possibilities and will report on the results in a later issue of these letters.

A Few Important References for Immunolabelling with K4M

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The Use of HM20 for Freeze-Substitution

Freeze-substitution is a very interesting alternative to our procedure of progressive lowering of the temperature during dehydration.

Martin Muller (1) has described a successful procedure. He now uses currently HM20 as resin, because of its low viscosity at low temperatures.

J. Escaig (2) has designed a rapid freezing device obtainable from Reichert. He is presently investigating the use of HM20.

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(2) Escaig, J., New instruments which facilitate rapid freezing at 83 K and 6 K. J.Microscopy (1982), 126, 221-229.