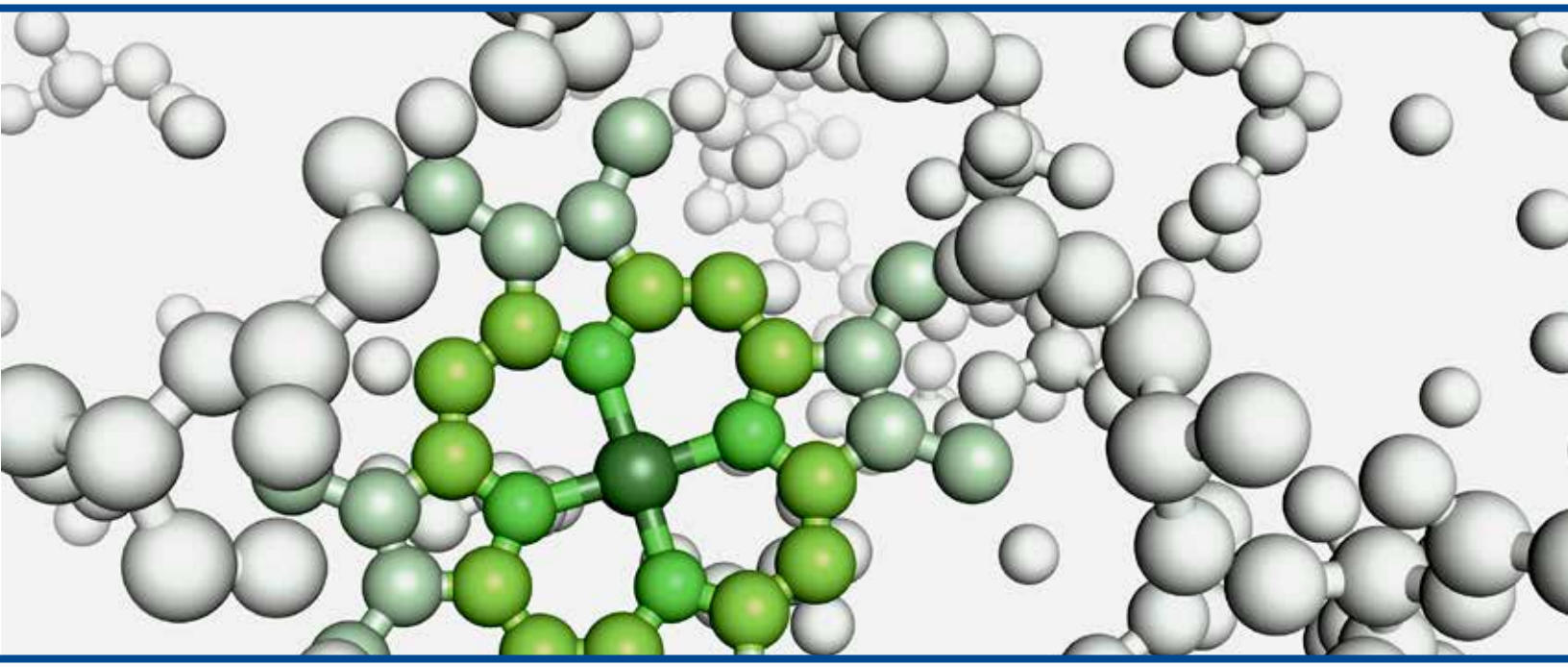
MonoStep® Application Notes



We recommend bubbling nitrogen gas for 5 minutes into the MonoStep to mix it well and to displace dissolved gases which can impede polymerization.

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Introduction

History of Lowicryl and Development of Monostep

Initially, low-temperature embedding with LOWICRYL resins was developed to improve the preservation of the cellular ultrastructure [Kellenberger et al., 1980; Carlemalm et al., 1982], and to image unstained sections of biological material in the scanning transmission electron microscope (STEM) [Carlemalm and Kellenberger, 1982]. Depending on the actual composition of the LOWICRYL resins, the two basic types K4M (polar) and HM20 (nonpolar) may be used at temperatures down to -35ºC (K4M) or -50ºC (HM20). Exploring cryofixation and freeze-substitution led to the development of two additional LOWICRYL resins, the polar K11M, and the nonpolar HM23 [Carlemalm et al., 1985; Acetarin et al., 1986]. The latter two resins may be used at temperatures down to -60ºC (K11M) or -80ºC (HM23). It was demonstrated quite early that low-temperature embedding with the polar LOWICRYL resin K4M also led to significantly better preservation of organelle structure and antigenicity [Roth et al., 1981] compared to that obtained after classic fixation and embedding protocols. Both polar and nonpolar LOWICRYL resins are highly suitable for immunolabeling and lectin-labeling [see e.g. Düerrenberger et al., 1991; Roth, 1989], thus explaining their frequent use in immunocytochemistry both at the light and electron microscopic level.

The Lowicryl K and HM series of resins are made by mixing three components by the user.

The new **MonoStep** resin (polar and nonpolar) were the successors of these resins and were developed by Polysciences Inc. (USA). All the well known properties of Lowicryls were kept and new ones were added:

- **immediately applicable without mixing**
- **improved mechanical properties**

Since the Lowicryl resins were introduced in 1980, numerous papers have reported results obtained by embedding human, animal and plant tissues, or microorganisms such as bacteria or yeast. The protocols used were often optimized to meet specific demands and thus expanded from that originally proposed by Carlemalm et al. [1982].

To this end, the following protocols may serve as a basis for embedding animal tissues or microorganisms in **MonoStep**.

Fixation

Good preservation of cellular and subcellular structures generally requires fixation of the constituting components. Conventionally, fine-structural preservation is achieved by chemical fixation. Often, results obtained with cryofixed material are much better, and at the same time the antigenicity of the embedded specimens is significantly increased. For any biological object the precise conditions for fixation (i.e., the method and, if chemical fixation is employed, the fixative(s) used, its/their concentration and time of fixation) and the sample size have to be optimized and may vary widely among different types of specimens. The effects of different fixatives and different times for fixation on the preservation of antigenicity can be estimated as described by Hortsch et al. [1985] and Roth [1989].

Chemical Fixation

Conventional chemical fixation with aldehydes and osmium tetroxide is performed at temperatures above 0ºC [Hayat et al., 1981]. For immunocytochemical purposes, improved fixation was achieved using buffered aldehyde solutions. While glutaraldehyde primarily preserves cellular fine structure, formaldehyde seems to improve the retention of the antigenicity. In addition, for subsequent dehydration by the progressive lowering of temperature (PLT) method, chemical fixation with either formaldehyde or glutaraldehyde or—most frequently—a mixture of both is required. After such chemical fixation and subsequent low-temperature processing (dehydration, resin infiltration, embedding and polymerization), a wide variety of specimens exhibit good structural preservation combined with high immunological reactivity.

With the following standard fixation for cells, we observed good preservation of fine structural details and antigenicity. Fixation is performed for 15 to 60 min with 0.1-1% (v/v) glutaraldehyde in phosphate buffered saline (PBS), or with 2% (v/v) formaldehyde/0.1% (v/v) glutaraldehyde in PBS. For the fixation of mammalian tissue, 10-min perfusion with 3% (v/v) formaldehyde/0.1% (v/v) glutaraldehyde in oxygenated Hank's balanced salt solution is recommended [for details, see e.g. Roth, 1989].
As already mentioned, osmium tetroxide fixation should only be employed if definitely required (e.g., to fix lipid-rich samples), and, if so, only at moderate concentrations such as e.g. 0.1 % (w/v). For subsequent PLT-dehydration and MonoStep embedding, osmium tetroxide fixation has to be stopped as soon as the color of the sample turns into a pale yellow since strong UV-absorption of a sample prevents the UV-induced polymerization.

For a general review on fixation, see Bullock [1984].

For immunocytochemical applications, see Leenen et al. [1985], Roth [1982, 1986, 1989], Hobot [1989] (animal tissues), Schwarz and Humbel [1989] (microorganisms), or Herman [1989] (plant tissues).

Cryofixation

The purely physical stabilization of biological structures by rapid-freezing was called cryofixation. It is now well established that cryofixation results in excellent preservation of both, the ultrastructure of the cellular components as well as the antigenicity of biological material. Moreover, cryofixation allows a temporal resolution in the milliseconds range and thus enables to trap and visualize even highly dynamic processes in cells and organelles [Knoll. et al., 1987; Heuser et al., 1978]. In contrast, depending on the sample and the fixation protocol used, conventional chemical fixation takes several seconds to minutes to complete.

For many years, most biological samples (particularly tissues) could only be cryofixed in the presence of cryoprotectants such as glycerol or sucrose to decrease the rate of ice crystal formation. However, the current state-of-the-art cryofixation even allows the fixation of completely untreated samples (i.e., no cryoprotectants and/or chemical fixatives are required) at ambient pressure [see Sitte et al., 1987]. The actual quality of cryofixed material [for criteria, see Sitte et al., 1987] is limited by the sample properties (e.g., its size, the heat-transfer rate from the sample to the coolant), the method employed (e.g., slamming or plunging), and the system used. With rapid freezing systems (i.e. plunge, slam, or jet freezers), well-preserved layers of 10-15 μm thickness may be obtained while high-pressure freezing [Moor, 1987; Studer, 1989] may yield up to 600 μm thick layers.

Good and reproducible results can be obtained with a slam freezer system "Cryoblock" [Escaig, 1982] operated at liquid helium temperature. For cryofixation samples are applied or attached to small pieces of filter or cigarette paper that are then mounted onto slamming supports [Escaig, 1982]. Cryofixed samples can be stored for several months under liquid nitrogen. Prior to low-temperature embedding, they are dehydrated by either freeze-substitution or freeze-drying [for review, see Plattner and Bachmann, 1982; Robards and Sleytr, 1985; Menco, 1986; Zierold and Steinbrecht, 1987; Müller, 1988]

Dehydration

The Progressive Lowering of Temperature (PLT) Method

Biological samples that have been chemically fixed (preferentially using aldehydes) are transferred into a dehydrating agent, generally an organic solvent. To minimize extraction, aggregation, precipitation, or dislocation of cellular components during dehydration, the temperature is decreased stepwise while simultaneously increasing the concentration of the dehydrating agent. It is obviously of crucial importance to prevent the sample from freezing. Therefore, care has to be taken to keep the temperature of the dehydration mixture above its freezing point (see Table I). In addition, the dehydrating agent has to be well miscible with the resin used for subsequent embedding. Dehydrating agents that have successfully been used in our laboratory are listed in Table II.

Table I: Concentration Dependence of Solvent Freezing-Points:

Resin	Concentration of solvent	Time (min)	T (º C)
MonoStep K4M polar	30%	30 min	0
	50%	60 min	-20
	70%	60 min	-35
	100%	2x60 min	-35
MonoStep K4M polar	30%	30 min	0
	50%	60 min	-20
	70%	60 min	-35
	100%	2x60 min	-50

Acetone, ethanol and methanol are the most commonly used solvents for PLT-dehydration. At each step of the dehydration process, care has to be taken to keep the temperature above the freezing-point of the actual aqueous solvent mixture.

Table II: The polarity and some suitable dehydrating agents for MonoStep resins:

Resin	Polarity	Dehydrating agent
MonoStep K4M polar	polar	ethanol, methanol, acetone, ethyleneglycol, glycerol, dimethyl formamide
MonoStep K4M polar	nonpolar	ethanol, methanol, acetone

PLT Dehydration Protocol

An aldehyde-fixed sample (volume_0.5 mm^3) in a 10-ml vial is either directly, or after a brief wash in ice cold PBS, transferred into 5 ml of the dehydrating agent and processed according to the protocol for PLT dehydration of biological specimens with ethanol or methanol for subsequent MonoStep embedding. During dehydration, the sample should slowly and carefully be agitated from time to time either by hand (gentle swiveling) or using a mechanical stirrer (which should not get in contact with the sample).

Important: The samples should be kept in the same vial during the whole procedure. Any exposure of the sample to air (condensation of ice; drying) has to be avoided (i.e., while changing the dehydrating agent, the sample is kept submerged). The dehydrating agent for the next step of the dehydration protocol has to be added pre-chilled in e.g. 5-ml aliquots. During the dehydration steps, the vials are kept closed by polyethylene lids. After the 100% dehydration step the sample is ready for resin infiltration (see Infiltration).

Freeze-Substitution

Dehydration of cryofixed samples in the frozen state by organic solvents at temperatures between -80ºC to -90ºC is known as freeze-substitution. The frozen water will slowly exchange with the surrounding solvent (e.g., acetone or methanol) that may contain classic chemical fixative(s) (e.g., glutaraldehyde, osmium tetroxide, uranyl acetate, or mixtures thereof) [Humbel and Müller, 1986].

The kinetics of the chemical reactions between the fixative(s) and the biological macromolecules are very slow at these conditions [Hum bel et al., 1983]. For instance, no significant differences in the preservation of fine structural details were revealed when comparing sections of bacterial cells after freeze-substitution for 85 hours with either pure acetone or 3% glutaraldehyde in acetone. Freeze-substitution with pure solvents is certainly not a standard procedure and absolutely requires subsequent very

low-temperature embedding in LOWICRYL K11M or HM23 resins at temperatures below -60ºC [see e.g. Edelmann, 1989; Villiger and Bremer, 1990].

Samples that have been cryofixed as outlined (see Cryofixation) are transferred rapidly from liquid nitrogen to the substitution medium. For routine applications, 0.56 ml glutaraldehyde of a 70% stock solution (Polysciences, Inc. Warrington, PA) in 13 ml of acetone per sample (final glutaraldehyde concentration: 3% v/v) can be used. The dehydrating agents should be pre-dried using molecular sieve (0.4 nm). In addition, molecular sieve may also be added to the vial containing the sample during freeze-substitution. The time required for freeze-substitution depends on the physical-chemical properties (e.g., polarity, viscosity) of the organic solvent used (e.g., methanol, ethanol, acetone) and has to be determined experimentally. With microorganisms such as bacteria, 80 to 90 hours at -85ºC were sufficient for acetone substitution. After freeze-substitution, the temperature is slowly raised at the rate of 5-10ºC per hour to the temperature required for resin infiltration (see Infiltration). For reviews on freeze-substitution, see Robards and Sleytr [1985), Sitte et al. [1986), Humbel and Müller [1986), Steinbrecht and Müller [1987).

Freeze-Drying

In contrast to freeze-substitution, freeze-drying of biological samples followed by subsequent low -temperature embedding has only rarely been used in the past [Wroblewski and Wroblewski, 1986; Chiovetti et al., 1986, 1987; Edelmann, 1986; Steinbrecht and Müller, 1987). For subsequent X-ray microanalysis and cytochemical analysis, freeze-dried samples may be low-temperature embedded *in vacuo* in the MonoStep resins polar or nonpolar [Wroblewski and Wroblewski, 1990].

The MonoStep Resins

General Remarks
The highly crosslinked **polar** and **nonpolar** acrylate-methacrylate-based MonoStep resins are distributed by Polysciences Inc., Warrington, P A. **MonoStep kits are shipped ready to use**.

Important:

- Oxygen strongly inhibits the polymerization of methacrylates. Therefore, mixing of MonoStep with any other components should be done in a stream of bubbling nitrogen. Alternatively, if dry nitrogen is not available, mixtures may also be gently shaken by hand until the components are completely mixed.

- **MonoStep can be stored at 4ºC or at temperatures up to 25ºC.**

- **MonoStep can act as a sensitizer. Direct contact with the skin should be avoided and a well-ventilated fume-hood should be used whenever possible.**

  Since latex or vinyl gloves are rapidly penetrated, they should only be used for a few minutes before replacement. The acrylate and methacrylate esters in Lowicryl kits are potential skin irritants and sensitizers. Prudent care should be used in handling the material. A pair of neoprene gloves are being included, but care should be used in handling the material and any spills on the gloves need to be cleaned up. The ultimate choice and care of gloves and other protective materials rests with the user and his exact use. Gloves should not be used if contaminated and replaced with a suitable fresh pair of good chemical retardant gloves chosen by the operator based on his operating conditions. For longer exposure, multi-laminated gloves, e.g. 4 H gloves, are recommended. In addition, a protective silicone hand cream may be used.

- **MonoStep resins are designed for UV polymerization at temperatures below 0ºC.**
  Exceptionally MonoStep resins can also be UV-polymerized at higher temperatures (i.e., 0ºC to +30ºC). To achieve this, 0.5% (w/w) benzoinethylether (Polysciences, Warrington, PA) must be added to the MonoStep resins.

Infiltration with MonoStep

After dehydration by the PLT method (see section The Progressive Lowering of the Temperature (PLT) Method, 3.1. above), biological samples (cultured cells. microorganisms. or small pieces of tissue) are infiltrated by the LOWICRYL resins as outlined below.

Protocol for the Infiltration of the MonoStep Polar and Nonpolar After PLT-Dehydration

Concentration of the Resin	Temperature		Time (min)
	Polar	Nonpolar	
50%	-35°C	-50°C	60 min
66%	-35°C	-50°C	60 min
100%	-35°C	-50°C	60 min
100%	-35°C	-50°C	overnight

During resin infiltration, the samples are kept in closed 10-ml vials as used for dehydration. Slight agitation from time to time with a toothpick or using a slow rotator is recommended.

Infiltration after Freeze-Substitution

Cryofixed samples were infiltrated after freeze-substitution and slow warming (i.e., 5 to 10ºC per hour) to the desired temperature according to the following protocol:

Preparation Step	Temperature		Time
	Polar	Nonpolar	
Freeze Substitution	-85°C	-85°C	3-4 days
Temperature Increase 5 to 10ºC per Hour			
Acetone Wash	-40°C	-50°C	1 h
50% (v/v) resin	-35°C	-50°C	2 h
66% (v/v) resin	-35°C	-50°C	2 h
Pure resin	-35°C	-50°C	2 h
Pure resin	-35°C	-50°C	overnight
Pure resin	-35°C	-50°C	4 hr

This protocol requires the presence of chemical fixatives during freeze substitution.

Embedding And Polymerization

General Remarks

Conventional embedding and polymerization is performed at temperatures ranging between 25ºC and 80ºC. This requires no special devices. Better preservation of the hydration shell of biological macromolecules [Kellenberger, 1987] may be achieved with low-temperature processing. It is conceivable that this hydration shell plays an important role in determining the antigenicity of biological matter. For low-temperature embedding and polymerization at temperatures ranging from approximately 0ºC to -50ºC, the use of commercially available freezers is necessary. The sample is rapidly transferred into 0.5-ml gelatin capsules (No.1, Lilly Co., Indianapolis, IN; only one sample per capsule!) that are pre-filled with the chilled resin to be used. To minimize contaminations

due to condensing moisture, only one capsule should be prepared at a time. To prevent a warming of the resin, both the sample transfer and the filling of the capsule are performed with pre-chilled pipettes (glass or polypropylene). After complete filling and closing of the capsules, they are kept for about one hour in the cold before UV-polymerization is started. Optimal polymerization should be induced by indirect (i.e., reflected) UV-irradiation.

UV sources from various manufacturers may be used provided their emission maximum is close to 360 nm wavelength. To optimize the degree and rate of polymerization of the resin at a fixed temperature, the tube to sample distance may be varied. The polymerization velocity is correct if the resin block after hardening does not show any deformation or bubbles. It has to be kept in mind, that UV-induced polymerization is efficiently inhibited by strongly light absorbing samples (intense color from osmium tetroxide fixation or dark pigments).

Minimal Temperatures and UV-Irradiation Time Required for the Polymerization of the MonoStep Resins Resin:

Resin	Minimal temperature for polymerization	Time of UV-irradiation
Polar	-35°C	1 day
Nonpolar	-50°C	1 day

After low-temperature polymerization the resin blocks were warmed to room temperature and irradiated for another three days in the direct UV-light, to improve the cutting properties of the blocks.

Sectioning

General Remarks

For ultrathin sectioning of MonoStep-embedded samples, glass or diamond knives with cutting-edge angles of ~35-45° may be used. Ultrathin sectioning of properly polymerized blocks of nonpolar MonoStep resins may be performed as with epoxy-embedded (e.g., EPON 812) samples. However, due to the physical-chemical properties of the acrylates/methacrylates, the contact between the resin and the biological material is much weaker than that with epoxies. Therefore, to avoid pressure-induced deformation in the sample area, the blocks should not be trimmed by hand, but by either a microtome equipped with a glass knife or a special trimming device such as the Ultratrim (Leica Instruments/ Reichert-Jung). The pyramid angle should be about 30 and the pyramid surfaces should be clean and shiny. The optimal cutting-speed depends on various parameters (e.g., the size and shape of the pyramid, the hardness of both the resin and the embedded biological material, the knife quality) and usually ranges between 2-5 mm/s. The resulting sections are deposited on collodion/carbon or Formvar-coated EM grids.

Recommendations for Polar MonoStep Sectioning

The following precautions are recommended for sectioning specimens that are embedded in the polar MonoStep resins;

- Since wetting of the pyramid may cause a swelling-induced deformation, the water level in the knife-through should be reduced such as to just keep the cutting-edge wetted (dark grey reflex).
- To reduce capillary suction with not perfectly trimmed block faces, a high initial cutting-speed of 5-10 mm/s has to be employed. As soon as the full pyramid surface is cutting, the cutting-speed can be reduced to 2 mm/s.
- To avoid swelling-induced deformation of the sample, polar MonoStep sections have to be picked up immediately after cutting

- To provide enough stability to reduce the swelling effects, the thickness of the sections should be at least 50 to 70 nm.

Usually, deformed or soft pyramids result from water uptake during sectioning and should therefore immediately be removed from the microtome and dried either in a desiccator or in the presence of a desiccant for at least one day. After that time, the block should be newly trimmed. After sectioning, the polar resin blocks should be stored moisture-protected.

Staining

General Remarks

The generally low inherent contrast of biological matter upon imaging in the CTEM calls for enhancement by e.g. staining with heavy metal compounds such as uranyl acetate, lead acetate or lead citrate. The procedure for staining conventionally embedded samples [Lewis and Knight, 1974] does also apply for MonoStep sections.

However, the physical-chemical properties of the MonoStep resins require different staining times. Using the data given below, good results should be obtained with MonoStep embedded specimens.

Basic Protocol for Staining MonoStep-Embedded Specimens

Monostep Resin	Staining Time	
	Uranyl Acetate	Lead Acetate or Citrate
Polar	25-35 min (4-5 min)	1-3 min (45 sec)
Nonpolar	5-15 min	1-3 min

EM grids with deposited thin sections are first floated on a drop of uranyl acetate solution for the time indicated, followed by a brief wash, and floated on a drop of lead acetate prepared according to Millonig [1961] or lead citrate. For polar MonoStep sections, the reduced times listed above in parenthesis have been reported to be sufficient [Roth, 1989; Roth et al., 1990].

Although lead citrate as well as lead acetate can be used as the second staining step, the use of lead acetate [according to Millonig, 1961] gave better results compared to lead citrate staining [according to Reynolds, 1963; Venable and Coggeshall, 1965].

Protocol for Lead Staining

- Deposit the thin sections on a collodion/carbon- or Formvar-coated EM grid
- As indicated in the Table above, with the sections facing the droplet, place the grid on a 25-30 µl drop of 2% to 6% (w/v) aqueous or alcoholic uranyl acetate solution
- Remove the grid, rinse it for a few seconds with distilled water, then blot the water off
- For the time indicated in the Table above, float the grid (section-face down) on a 25-30 µl drop of lead acetate or lead citrate.
  Note: To prevent the formation of lead carbonate precipitates, staining should be performed under nitrogen or another protective gas atmosphere
- Remove the grid, rinse it for a few seconds with distilled water, then blot the water off

For specimens embedded with the polar MonoStep resin, uranyl acetate/methyl cellulose staining protocol was developed [Roth, 1989; Roth et al., 1990] and reported to improve the staining of fine-structural details in the cytoplasm while being performed much quicker (i.e., 5 min).

Trouble-Shooting

General Remarks

Low-temperature processing always harbors the danger of unintended temperature fluctuations and contamination by the condensation of moisture during the various steps involved. Therefore, all instruments should be pre-chilled, and all operations should be performed as quickly as possible!

Instruments for sample transfer such as insulated tweezers are commercially available.

Provided good resin infiltration and polymerization are achieved, the different MonoStep resins warrant good cutting properties over the whole low-temperature range.

The Most Frequently Encountered Difficulties

Diagnosis	Problem	Remedy
Specimen “milky”	Incomplete dehydration and/or infiltration	• use smaller samples (0.5 mm^3) • prolong infiltration time • increase agitation
Specimen too soft	Incomplete infiltration	• use smaller samples (0.5 mm^3) • prolong infiltration time • increase agitation
	Sample rich in lipids	• change conditions for fixation (include osmium tetroxide)
	Incomplete polymerization	• use smaller samples (0.5 mm^3)
Resin block too soft	Incomplete polymerization (inhibition by oxygen or water)	• mix resin components in a stream of nitrogen • dry the resin for several hours at room temperature with molecular sieve • keep vials or capsules closed • close capsules immediately • check UV irradiation conditions (source, absorption of sample) • repeat polymerization step at room temperature
Deformed block or bubbles within the block	Polymerization too rapid	• increase UV source-sample distance • reduce light intensity and/or temperature

Immunolabeling on Ultrathin Sections

General Remarks

Immunolabeling is a technique that is performed on thin sections of acrylate-based embedding to localize proteins (in common elements) with respect to the specimen. This can be in common any specific reaction, but in the immunolabeling it is always an antigen to antibody reaction. A second step involves the visualization of the specific reaction in the electron microscope that involves usually the specific binding of protein A-gold or protein G-gold grains of various sizes to the Fc part of the specific antibody. The dynamic reactivity at pH 7.4 of protein A and protein G with certain species of Immunoglobulins is listed below:

Immunoglobulin	Protein A	Protein G
Human IgG1	+	+
Human IgG2	+	+
Human IgG3	-	+
Human IgG4	+	+
Mouse IgG1	+ pH 9 / -Ph 7.4	-
Mouse IgG2a	+	+
Mouse IgG2b	+	+
Mouse IgG3	+	+
Rat IgG1	Weak	Weak
Rat IgG2a	-	-
Rat IgG2b	-	Weak
Rat IgG2c	Weak	+
Pig IgG	Weak	+
Rabbit IgG	+	+
Bovine IgG1	-	+
Bovine IgG2	+	+
Sheep IgG1	-	+
Sheep IgG2	-	+
Goat IgG1	Weak	+
Goat IgG2	+	+
Horse IgG(ab)	Weak	+
Horse IgG(c)	Weak	+
Horse IgG(t)	+	Weak
Dog IgG+	+	Weak

In the literature there is a tremendous variety of labeling procedures of which only a selection of standard procedures is listed below.

During a labeling procedure grids with sections are usually incubated for hours on salt solutions that induce oxidation of the copper of standard grids. There are ways to avoid oxidation. The simplest is to protect the copper grids by immersing them in diluted Formvar or collodion before the actual foil is prepared on them. Copper can also be replaced by gold grids. Gold is a soft metal and skill of the operator is needed to handle gold grids. Very widespread is the use of nickel grids. These are ferromagnetic and stick to normal steel forceps. This magnetism also influences the image forming electrons in a microscope in a way that low magnification images are impossible to perform. Also astigmatism correction for high resolution images on nickel grids is impossible to perform.

Fixation is another topic heavily discussed in the literature. Too strong (even aldehyde) fixation leads to a reduced reactivity of antibody to antigen. Some fixatives like OsO_4 or uranyl acetate cover the antigen preventing a reaction with the antibody (except during freeze-substitution and cryosectioning). These effects can be partially reversed, if needed, by etching (OsO_4 or uranyl acetate) or incubation of the sections (aldehydes) on diluted HCl (pH2) prior to labeling.

Standard pAG Two Step Procedure

- Incubate grids with the section face to a drop of 20mM PBS (pH 7.4) containing 1% pure Ovalbumine for 5 min to cover unspecific binding sites.
- Transfer grids directly and incubate with the section face to a drop of diluted primary antibody in 20mM PBS (pH 7.4) containing 1% pure Ovalbumine for one hour.
- Wash well with 20mM PBS (pH 7.4) containing 1% pure Ovalbumine.
- Incubate grids with the section face to a drop of diluted protein A-gold in 20mM PBS (pH 7.4) containing 1% pure Ovalbumine for one hour.
- Wash well with 20mM PBS (pH 7.4).
- Incubate grids with the section face to a drop of 1% Glutaraldehyde in 20mM PBS (pH 7.4) for 3 min.
- Wash well with distilled water.

Staining:

- Incubate grids with the section face to a drop of 6% uranyl acetate in H_2O for 5 to 45 min (see staining section).
- Wash well with distilled water.
- Incubate grids with the section face to a drop of lead acetate or lead citrate for 45 sec to 3 min (see staining section).
- Wash well with distilled water.

Standard Secondary Antibody Two Step Procedure

- Incubate grids with the section face to a drop of 20mM PBS (pH 7.4) containing I % pure Ovalbumine for 5 min to cover unspecific binding sites.
- Transfer grids directly and incubate with the section face to a drop of diluted primary antibody in 20mM PBS (pH 7.4) containing 1% pure Ovalbumine for one hour.
- Wash well with 20mM PBS (pH 7.4) containing 1% pure Ovalbumine.
- Incubate grids with the section face to a drop ofdiluted secondary antibody-gold in 20mM PBS (pH 7.4) containing 1% pure Ovalbumine for one hour.
- Wash well with 20mM PBS (pH 7.4).
- Incubate grids with the section face to a drop of 1% Glutaraldehyde in 20mM PBS (pH 7.4) for 3 min.
- Wash well with distilled water.

Staining:

- Incubate grids with the section face to a drop of 6% uranyle acetate in H_2O for 5 to 45 min (see staining section).
- Wash well with distilled water.
- Incubate grids with the section face to a drop of lead acetate or lead citrate for 45 sec to 3 min (see staining section).
- Wash well with distilled water.

Precoupled Single Step Procedure (Dürrenberger, 1989)

Precoupling:

- Prepare in an Eppendorf tube a total volume of 200 µl diluted primary antibody in 20mM PBS (pH 7.4) containing 1% pure Ovalbumine, containing 25 µl concentrated 15 nm protein A-gold (15 µl 10 nm protein A-gold; 10 µl 5 nm protein A-gold) and incubate for 2 hours at room temperature.
- Centrifuge full speed in an Eppendorf table top centrifuge. A pellet should be formed.
- Wash pellet 3 times with 20 mM PBS (PH 7.4) containing 1% pure Ovalbumine.
- Add 400 µl 20 mM PBS (pH 7.4) containing 1% pure Ovalbumine and put it on ice.
- Sonicate with 40kHz (max. 10W) until slight red color (colloidal distribution) reappears (within seconds). Prevent cooking or foaming.

Labeling:

- Incubate grids with the section face to a drop of 20 mM PBS (PH 7.4) containing 1% pure Ovalbumine for 5 min to cover unspecific binding sites.

- Transfer grids directly and incubate with the section face to a drop of **freshly sonicated** precoupled antibody in 20 mM PBS (pH 7.4) containing 1% pure Ovalbumine for maximum one hour.
- Wash well with 20 mM PBS (pH 7.4).
- Incubate grids with the section face to a drop of 1% Glutaraldehyde in 20 mM PBS (pH 7.4) for 3 min.
- Wash well with distilled water.

Staining:

- Incubate grids with the section face to a drop of 6% uranyl acetate in $H_2O$for 5 to 45 min (see staining section).
- Wash well with distilled water.
- Incubate grids with the section face to a drop of lead acetate or lead citrate for 45 sec to 3 min (see staining section).
- Wash well with distilled water.

Direct Gold Single Step Procedure

- Incubate grids with the section face to a drop of 20 mM PBS (pH 7.4) containing 1% pure Ovalbumine for 5 min to cover unspecific binding sites.
- Transfer grids directly and incubate with the section face to a drop of diluted direct antibody-gold in 20 mM PBS (pH 7.4) containing 1% pure Ovalbumine for one hour.
- Wash well with 20 mM PBS (pH 7.4).
- Incubate grids with the section face to a drop of 1% Glutaraldehyde in 20 mM PBS (pH 7.4) for 3 min.
- Wash well with distilled water.

Staining:

- Incubate grids with the section face to a drop of 6% uranyl acetate in H_2O for 5 to 45 min (see staining section).
- Wash well with distilled water.
- Incubate grids with the section face to a drop of lead acetate or lead citrate for 45 sec to 3 min (see staining section).
- Wash well with distilled water.

Immunofluorescence on Ultrathin Sections

General Remarks

Ultrathin methacrylate (e.g. MonoStep polar and nonpolar) or epoxy (Epon/Araldite, Spurr) sections are transferred with a loop on polylysine coated round cover slips. Residual water is drained with filter paper along the outside of the loop. Note: air-dried resin sections can be stored for months prior to labeling.

Procedure

- Mark sections with a water repellent silicon pen {e.g. PAP-Pen from Polysciences, Inc. Warrington, PA)
- Incubate sections with blocking buffer, e.g. PBG (0.2 % gelatin, 0.5 % BSA in PBS or TRIS) or 1% milk powder in PBS for 10 min.
- Remove blocking buffer, add 25 μl of the primary antibody solution per cover slip (with a final concentration in the range of 1-5 μg specific IgG/ml) and incubate for 30-60 min
- Wash 5 times with buffer and incubate with fluorochrome-labelled second antibodies, analogue to the primary antibody staining conditions.
- Wash 5 times with buffer and counterstain nuclei with DAPI, Hoechst or propidium iodide (0.4-0.1, ug/ml in H_2O) for 5 min.
- After a final wash with buffer, mount covers lips on glass slides using a small drop of mounting medium (Aqua PolyMount or MowioI 4-88) for semi-permanent embedding. The addition of anti-fading agents like DABCO (25-10 mg/ml), p-phenylenediamine (1 mg/ml) or n-propyl gallate (10 mg/ml) is strongly recommended (see references). Use oil immersion objectives.

ATTENTION: All solutions should be very clean or centrifuged before use for 2 min at 10'000 rpm in an Eppendorf centrifuge.

Safety

The LOWICRYL types are (meth-)acrylic acid ester preparations. (Meth-)Acrylic acids can cause allergies. The following points should always be taken into consideration when working with LOWICRYL products:

- Keep the area in which you are working well ventilated.
- Use protective clothing and protective glasses with side protection.
- Use protective skin creams and appropriate gloves (Latex gloves are not fully protective-the protection can be limited to only a few minutes.) 4H gloves are recommended, although these tend to go brittle at very low temperatures and are awkward to work with.
- Avoid direct skin contact of the liquid and vapors by using the appropriate utensils such as tweezers or other recommended mechanical instruments for such work.

Precautions and Storage

The chemical, physical and toxicological properties of these products are not fully known. Avoid contact with skin and eyes. Avoid inhalation of resin vapor. Use well-ventilated fume hood for mixing resins. It has been shown that methacrylate resins can cause irritation to skin and eyes and may cause sensitization to some individuals. The use of disposable utensils and tools is

recommended. Note: Kits should not be stored in the refrigerator.

In case of contact, promptly wash affected area of skin with plenty of soap and water. Flush eyes with plenty of water. Get medical attention immediately.

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Semipermanent mounting medium containing polyvinylalcohols (Elvanol / Mowiol):

Rodriguez J, Deinhardt F. (1960). Virology 12:316

Antifading agents:

1,4-diazabicyclo[2.2.2]octane (DABCO):

G.D. Johnson et al., 1982. J. Immuno!. Meth. 55, 231-242; G. Langanger et al., 1983. Mikroskopie, 40, 237-241

p-phenylendiamine:
Johnson GD, Araujo GMDCN. (1981). *J. Immunol. Meth.* 43:349-350
n-propyl gallate:

Giloh H, Sedat JW. (1982). *Science* 217:1252-1255

Appendix

Enhancement Protocols
From: Stierhof, YD, Hermann R, Humbel BM, Schwarz H. (1995). Use of TEM, SEM, and STEM in imaging 1-nm colloidal gold particles. In: Hayat MA (Ed.), Immunogold-silver Staining. Principles, Methods, and Applications. CRC Press, Boca Raton, pp. 97–118.

Sections have to be thoroughly washed with bidistilled water to remove ions (especially chloride ions) which could interfere with the silver-enhancement process. Enhancement is carried out by incubating the grids on a drop of 100 μl of the enhancer solution. Although most of the enhancers can even be used in daylight, we recommend covering the grids or working under red safe light conditions, as this delays self-nucleation of silver ions. The reaction is stopped by transferring the grids to bidistilled water or to a photographic fixative (Larsson, 1989). Before staining with uranyl acetate and lead citrate, components like gum arabic have to be completely removed by thoroughly washing the grids. It has to be noted that higher temperatures and moving of the grids during incubation on the enhancer solution speed up the deposition of silver on the gold surface (Humbel and Biegelmann, 1992).

Acidic Silver Lactate (Danscher, 1981)
Combine 0.6 ml gum arabic (33% in bidistilled water), 0.1 ml citrate buffer (2.55 g citric acid plus 2.35 g trisodium citrate dihydrate, to bidistilled water to make 10ml, pH 3.8). Then add 0.15 ml hydroquinone (0.85 g in 15 ml bidistilled water) and 0.15 ml silver lactate (0.11 g in 15 ml bidistilled water). Gum arabic, citrate buffer, and hydroquinone can be premixed and stored in a freezer. Silver lactate has to be stored separately in a freezer. Incubation temperature 20 to 22°C; incubation time is about 20 min; for Nanogold about 25 min.

Neutral Silver Lastate (Lah et al., 1990)
See above, replace citrate buffer by 0.2 M Hepes buffer, pH 6.8. Incubation temperature 20º to 22°C, incubation time approximately3 min for 1nm gold; for Nanogold 4 to 5 min.

Silver Acetate (Hacker et al., 1988)
Mix equal amounts of 0.5% hydroquinone in 0.5 M citrate buffer (see above), and 0.2% silver acetate. Final concentration of 16.5% gum arabic was prepared from a stock solution of 33% gum arabic. Incubation temperature is 20 to 22°C; incubation time is approximately90 min for 1nm gold (but approximately20 min without gum arabic).

HQ SILVER™ (Nanoprobes)
Equal amounts of the three components initiator, moderator, and activator were mixed before use (see instructions of Nanoprobes). Incubation temperature is 20 to 22°C, incubation time about3 min for 1nm gold and approximately 5.5 min for Nanogold.

IntenSE M (Amersham), R-Gent (Aurion), SEK:L15 (British BioCell)
Equal amounts of developer and enhancer were mixed before use (see instructions of Amersham, Aurion, and British BioCell).

Final concentrations of 20 or 33% gum arabic in IntenSE M and R-Gent were prepared from stock solutions of 50% gum arabic. Incubation temperature 42°C, incubation time 20 min (20% gum arabic) or 30 min (33% gum arabic) (but only about 6 min without gum arabic at 20 to 22°C).

Grid Material (Stierhof et al., 1992)

Nickel grids are useful for all enhancers mentioned above. If sections have already been mounted on copper grids, the neutral silver lactate enhancer is recommended. Nonprotected copper grids are not suitable for IntenSE M, R-Gent, and for the acidic silver lactate and the silver acetate solution. Gold grids are useful for all enhancers with the exception of R-Gent. The commercial HQ SILVER™ enhancer and the SEKL15 kit were only tested with nickel grids.