Dehydration and resin embedment of tissues

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The purpose of embedding biological specimens is to replace water by a matrix which is sufficiently stable to maintain cell structures. Furthermore, the tissue block must be penetrable by a cutting device (microtome) to deliver high quality sections (thin and thick sections, equally) which are resistant to the following chemical and staining treatments and to the electron beam in the microscope. For this purpose, several resins can be used, and usually low polarity solvents such as ethanol are employed for dehydration. *

Schedules of dehydration

Apart from a possible extraction of tissue proteins by ethanol-water mixtures, a change of hydration shells of proteins occurs and is accompanied by denaturation. In contrast, the native conformation of proteins is less affected by the more polar ethylene glycol. In several experimental approaches, ethylene glycol has shown its usefulness as dehydrating substance for biological specimens (TANFORD C et al.1962; PEASE DC 1966; SJÖSTRAND FS and BARAJAS L 1968; SJÖSTRAND FS and KRETZER F 1968) Due to its two hydroxyl groups, ethylene glycol fits well into water shells of proteins so that these are not seriously disturbed and denatured. In practice, a variety of dehydration schedules should be compared, and examples for subsequent epoxy resin embedment are given in this chapter.

Table: Possible schedules of tissue dehydration for epoxy resin embedment

Ethanol method (1) [a]	**Ethanol method (2)** [a]	**Ethylene glycol method**
30 % v/v ethanol/water for 30 min	70 % v/v ethanol/water for 30 min	10 % ethyl. glycol/water for 30 min
50 % v/v ethanol/water for 30 min	95 % v/v ethanol/water for 20 min	gradually to 65 % ethyl. glycol by addition of pure ethyl. glycol within 60 min
70 % v/v ethanol/water for 30 min	95 % v/v ethanol/water for 20 min	65 % ethyl. glycol/water for 60 min
95 % v/v ethanol/water for 30 min	95 % v/v ethanol/water for 20 min	gradually to 100 % ethyl. glycol by addition of pure ethyl. glycol within 60 min
100 % v/v ethanol/water for 20 min	100 % v/v ethanol/water for 20 min	100 % ethyl. glycol for 30 min
100 % v/v ethanol/water for 20 min	100 % v/v ethanol/water for 20 min	100 % ethyl. glycol for 30 min
100 % v/v ethanol/water for 20 min	100 % v/v ethanol/water for 20 min	100 % ethyl. glycol for 30 min
100 % v/v ethanol/water for 20 min	100 % v/v ethanol/water for 20 min	100 % ethyl. glycol for 30 min

* Dehydration and resin embedding chemicals can be toxic. They must be handled with care

100 % v/v ethanol/water for 20 min	100 % v/v ethanol/water for 20 min	100 % ethyl. glycol for 30 min
100 % v/v ethanol/water for 20 min	100 % v/v ethanol/water for 20 min	100 % ethyl. glycol for 30 min
propylene oxide for 15 min	propylene oxide for 15 min	propylene oxide for 15 min
propylene oxide for 15 min	propylene oxide for 15 min	propylene oxide for 15 min
propylene oxide/resin mixture for 30 min	propylene oxide/resin mixture for 30 min	propylene oxide/resin mixture for 30 min
fresh epoxy resin mixture	fresh epoxy resin mixture	fresh epoxy resin mixture

[a] All steps at 0°C until 100 % ethanol is reached

Resins for embedding of biological specimens

For ultrastructural studies, special embedding resins have been developed. With progress in microtomy and ease of sectioning of resin embedded tissues, some defined advantages of this preparation technique became also evident for light microcopy, even if mainly for research purposes (GLAUERT AM 1975; NEWMAN GR and HOBOT JA 1999).

Three types of resins are used in histology: epoxy, polyester and methacrylate resins. The most employed resins are those with epoxy groups. Commercial exploitation of epoxy resins was initiated in the mid 1930s by I.G. Farben in Germany. Usually, epoxy resins are thermosetting liquids of which the most widely used are derived from reaction between bisphenol A and epichlorohydrin in the presence of NaOH; further reaction of the chlorohydrin intermediate with NaOH then gives diglycidyl ether of bisphenol A.

This manufacturing principle still holds true today. From the available epoxy resins, Epon® 812 (registered trade mark by Shell) is the most common embedding material in electron microscopy. Epon 812 is a glycerol based aliphatic epoxy resin and its major components are di- and monoglycidyl but also some triglycidyl ethers of glycerol. When epoxy monomers are cured (e.g. with phthalic anhydride), three-dimensional structures are formed by cross-links. These are principally based on di-ester bridges and ether bridges (FISCH W et al. 1956; LEE H and NEVILLE K 1967).

In the course of Epon curing, not all monomers and oligomers will react. Incompletely reacted compounds occur especially when Epon is cured stepwise with increasing temperatures. Exotherm profiles from curing Epon 812 mixtures have shown that considerable heat is liberated over the period of polymerization. Yet, exotherm reaction in small castings of few grams (which is usually done in histological tissue embedding) is only slightly higher than the oven temperature, and temperature increase is practically immeasurable when curing is performed in three steps: at 35°C for 24 h, then at 45°C for 24 h and finally at 60°C for 24 h (LUFT JH 1961; KUHLMANN WD 1984).

Since resin monomers can react with amino groups, loss of immunoreactivity will be added to that due to fixation and dehydration. The extent of such a loss is almost difficult to appreciate. The usefulness of a resin for immunohistological work has to be established by trial. All such experiments should accompanied by studies with antigen retrieval methods.

Polar media and low-temperature embedding procedures offer the advantage to reduce denaturation and conformational changes which are generally associated with nonpolar dehydration and with conventional resin curing. Several Lowicryl® embedding media have

been developed since the 1980s for a wide range of embedding conditions. These resins consist of highly cross-linked acrylate-methacrylate media with the outstanding feature of low viscosity at low temperature (CARLEMALM E et al. 1980; KELLENBERGER E et al. 1980); ARMBRUSTER BL et al. 1982).

Standard method of Epon® 812 embedment

After aldehyde fixation (and eventually preembedding immuno-staining) of tissue blocks or after double fixation by aldehydes and OsO_4, specimens may be dehydrated and embedded following one of two alternative schedules.

Chemicals *p.a*. are used according to the recommendations of the manufacturer:

Fixatives (aldehydes, osmium tetroxide for fixation of specimens)

Ethanol, 100 %

Epon® 812

Hardener and accelerator: dodecenyl succinic anhydride, nonenyl succinic anhydride, methyl nadic anhydride, 2,4,6-tri (dimethylaminomethyl) phenol

Propylene oxide

Standard method after double fixation (aldehyde, osmium tetroxide)

Procedure	Reagent	Time
1	70 % v/v ethanol in distilled water	20 min at room temperature
2	95 % v/v ethanol in distilled water	20 min at room temperature
3	100 % ethanol	20 min at room temperature
4	100 % ethanol	20 min at room temperature
5	100 % ethanol	20 min at room temperature
6	propylene oxide	20 min at room temperature
7	propylene oxide	20 min at room temperature
8	propylene oxide/epoxy resin mixture (ratio 1:1), epoxy resin mixture	30-60 min at room temperature
9	epoxy resin mixture	2 h at 37°C or overnight at room temperature
10	embedment in capsules or flat molds with fresh epoxy resin mixture and polymerization	24 h at 60°C

Modified procedure for postembedment immunocytochemistry

Procedure	Reagent	Time
1	30 % v/v ethanol in distilled water	30 min at 0-4°C
2	50 % v/v ethanol in distilled water	30 min at 0-4°C
3	70 % v/v ethanol in distilled water	30 min at 0-4°C
4	90 % v/v ethanol in distilled water	30 min at 0-4°C
5	95 % v/v ethanol in distilled water	30 min at 0-4°C
6	100 % ethanol	20 min at 0-4°C
7	100 % ethanol	20 min at 0-4°C
8	100 % ethanol	20 min at 0-4°C
9	100 % ethanol	20 min at room temperature
10	100 % ethanol	20 min at room temperature
11	100 % ethanol	20 min at room temperature

12	propylene oxide	20 min at room temperature
13	propylene oxide	20 min at room temperature
14	propylene oxide/epoxy resin mixture (ratio 1:1), epoxy resin mixture see Table 1	30-60 min at room temperature
15	epoxy resin mixture	2 h at 37°C or overnight at room temperature
16	embedment in capsules or flat molds with fresh epoxy resin mixture and polymerization	24 h at 35°C 24 h at 45°C 24 h at 60°C

Epoxy resin mixtures for embeddingy

Resin	**Hardener, accelerator**	**Intermediate**	**Polymerization**
Epon 812 (A:E = 0.70) 87 mL	Dodecenyl succinic anhydride (DDSA) 80.0 mL *plus* Methyl nadic anhydride (MNA) 23.0 mL *plus* 2,4,6-Tri (dimethylamino-methyl) phenol (DMP-30) 3.0 mL	Propylene oxide *followed by* Propylene oxide/Epon mixture (ratio 1:1)	Schedule (a) 35°C for 24 h 45°C for 24 h 60°C for 24 h Schedule (b) 60°C for 24 h
Diepoxide octane DEO (A:E = 0.60) 72 mL	Nonenyl succinic anhydride (NSA) 128.0 g *plus* 2,4,6-Tri (dimethylamino-methyl) phenol DMP-30 2.6 mL	Propylene oxide *followed by* Propylene oxide/DEO mixture (ratio 1:1)	Schedule (a) 35°C for 24 h 45°C for 24 h 60°C for 24 h Schedule (b) 60°C for 24 h

Resin embedment with Quetol 651

Apart from Epon 812 as embedding medium, other epoxy resins will work as well. For example Quetol 651®, a water miscible epoxy resin (polyethylene glycol diglycidyl ether) of low viscosity (viscosity of 15 cps at 25°C), has been shown to be suitable for light and elecron microscopy (KUSHIDA H and KUSHIDA T 1982). The resin is miscible with water, alcohol, acetone, n-butyl glycidyl ether etc.

Chemicals *p.a*. are used according to the recommendations of the manufacturer:

Fixatives (aldehydes, osmium tetroxide for fixation of specimens)

Ethanol, 100 %

Quetol 651®

Hardener and accelerator: Nonenyl succinic anhydride, nadic methyl anhydride, 2,4,6-tri (dimethylaminomethyl) phenol

n-Butyl glycidyl ether (n-BGE)

Procedure	**Reagent**	**Time**
1	70 % v/v ethanol in distilled water	20 min
2	95 % v/v ethanol in distilled water	20 min
3	100 % ethanol	20 min
4	100 % ethanol	20 min

5	100 % ethanol	20 min
6	ethanol/n-BGE mixture (ratio 1:1)	30 min
7	n-BGE	30 min
8	n-BGE/Quetol 651 resin mixture (ratio 1:1)	1-2 h
9	Quetol 651 resin mixture	2-3 h
10	embedment in capsules (gelatin or polyethylene) with fresh Quetol 651 resin mixture and polymerization	24 h at 60°C

Quetol 651 resin mixture for embeddingy

Resin	**Hardener, accelerator**	**Intermediate**	**Polymerization**
Quetol 651 35 mL	Nonenyl succinic anhydride (NSA) 54.0 mL *plus* Nadic methyl anhydride (NMA) 11.0 mL *plus* 2,4,6-Tri (dimethylamino-methyl) phenol (DMP-30) 1.5-2.0 mL	n-Butyl glycidyl ether (n-BGE) *followed by* n-BGE/Quetol 651 mixture (ratio 1:1)	60°C for 24 h

LR White resin embedment

LR White is a polyhydroxy-aromatic acrylic resin (polyhydroxy substituted bisphenol A dimethacrylate resin) of very low viscosity (viscosity of 8 cps at 25°C). The resin has a limited miscibility with water; polymerized resin is hydrophilic. LR White can be cured by heat, microwave or UV light (365 nm) with a chemical accelerator (aromatic tertiary amine). After addition of the catalyst the resin should be kept at 4ºC or at -80ºC; shelf life is a minimum of 1 year when stored at 4ºC.

Chemicals *p.a*. are used according to the recommendations of the manufacturer:

Fixatives (formaldehyde freshly prepared from paraformaldehyde in phosphate buffer and supplemented with sucrose for fixation of specimens)

Ethanol, 100 %

LR White™ resin system

Benzoyl peroxide (prior to use, mix catalyst with resin at room temperature until completely dissolved; let the resin sit for about 24 hours in the refrigerator)

Procedure	**Reagent**	**Time**
1	70 % v/v ethanol in distilled water	20 min
2	70 % v/v ethanol in distilled water	20 min
3	95 % v/v ethanol in distilled water	20 min
4	100 % ethanol	20 min
5	100 % ethanol	20 min
6	ethanol/LR White resin mixture (ratio 1:1) on a rotator	overnight
7	LR White resin mixture on a rotator	1 h
6	LR White resin mixture on a rotator	1 h
8	embedment in capsules (gelatin) with fresh LR White resin mixture and polymerization	24 h at 50-60°C

LR White resin mixture for embeddingy

Resin	Hardener, accelerator	Intermediate	Polymerization
LR White resin 100 g	Benzoyl peroxide 1.98 g	Ethanol/LR White resin mixture (ratio 1:1)	50-60°C for 24 h

The resin can be cured equally well by UV with 2 x 15 W UV bulbs at temperatures between 0ºC and -20ºC with a curing time of 12-24 hours; distance of specimens from the light source is about 15 cm. For further informations on the use of LR White as embedding medium and for applications see GR NEWMAN (1989, 1999), GR NEWMAN and JA HOBOT (1987, 1999).

Lowicryl K4M resin embedment

Lowicryl K4M is based on cross-linked acrylate-methacrylate and is proposed as a polar low-temperature embedding medium for special ultrastructural work. K4M may serve as an alternative to the so-called thermosetting resins. Polar media and low-temperature embedding procedures can reduce denaturation and conformational changes usually associated with nonpolar dehydration and resin curing. Lowicryl K4M resin has shown its potential for several immuno-stainings of ultrathin and semithin tissue sections.

Lowicryl resins are usually photopolymerized by ultraviolet light (360 nm). Because this type of polymerization is largely independent from temperature, both resin infiltration and polymerization can be done at the same temperature. Moreover, the hydrophilic properties of K4M allow dehydration and infiltration of biological specimens in partially hydrated state (about 5% water). The hydrophilic Lowicryl resins are of particular interest for immunohistological labelings (ROTH J et al. 1981; BENDAYAN MJ and SHORE GC 1982; LEMANSKI LF et al. 1985).

Chemicals *p.a*. are used according to the recommendations of the manufacturer:

Fixatives (formaldehyde freshly prepared from paraformaldehyde and glutaraldehyde may be used for fixation of specimens)

Ethanol, 100 %

Lowicryl®K4M system: Monomer B, Cross-linker A and Initiator C

Procedure	Reagent	Time
1	30 % v/v ethanol in distilled water at 0ºC	30 min
2	50% v/v ethanol in distilled water at -20ºC	60min
3	70% v/v ethanol in distilled water at -35ºC *	60min
4	95% v/v ethanol in distilled water at -35ºC *	60 min
5	100 % ethanol at -35ºC *	60min
6	100 % ethanol at -35ºC *	60min
7	ethanol K4M resin mixture (ratio 1:1) at -35ºC *	60 min
8	ethanol K4M resin mixture (ratio 1:2) at -35ºC *	60 min
9	K4M resin mixture at -35ºC *	60 min
10	K4M resin mixture at -35ºC *	overnight
11	embedment in capsules (gelatin) with fresh K4M resin mixture and polymerization at -35ºC	24 h at -35°C

*	Suggested temperature; working temperature can be as low as -50ºC Schedules for other temperature and other polar dehydrating agents can be developed as long as solubility allows it including freeze substitution

Lowicryl K4M resin mixture for embeddingy

Resin	**Hardener, accelerator**	**Intermediate**	**Polymerization**
Lowicryl K4M monomer B 86.5 g	Cross-linker A 13.5 g *plus* Initiator C 0.5 g	K4M resin/ethanol mixture (ratio 1:1) *followed by* K4M resin/ethanol mixture (ratio 2:1)	Indirect UV (360 nm) 0°C for 12 h

By varying the ratio of monomer to cross-linker, one can influence the resin hardness just to the needs of the biological specimens. Resins are polymerized by indirect long-wave UV (360 nm) with 2 x 15 W bulbs at -35ºC with a curing time of 24 hours; distance of specimens from the light source is about 30-40 cm. Slow polymerization may be preferred (less shrinkage effects). Improved sectioning quality is obtained when tissue blocks are further hardened under UV light at room temperature for 1-2 dys. For further informations on the use of Lowicryl K4M as embedding medium see (CARLEMALM E et al. 1980; KELLENBERGER E et al. 1980); ARMBRUSTER BL et al. 1982).

References for further readings

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Full citation of publications is given in chapter *References*
link: https://www.kuhlmann-biomed.de/wp-content/uploads/2020/12/References.pdf