Tissue dehydration and embedment

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Biological specimens contain up to 80% or more water which must be substituted in fixed cells (with the exception of special cryo-techniques) by some kind of embedding media to allow mechanical support for the sectioning process. Usually, embedding media cannot directly replace water, and fixation is followed by a sequence of dehydrating solutions from which the last must be miscible with the embedding matrix. The most employed dehydrating agents are ethanol and acetone.

It depends on the employed embedding medium which dehydration schedules should be chosen. For example, paraffin or polyester resins are not soluble in ethanol, and specimens may be either dehydrated in acetone are passed after dehydration in ethanol through acetone as intermedium. Even if epoxy resins are soluble in ethanol or acetone, they mix more readily with propylene oxide. Furthermore, propylene oxide is a reactive diluent so that small amounts can remain in specimens which will not disturb epoxy resin polymerization.

Physico-chemical changes of biological molecules including denaturation of antigenic structures must be expected during such tissue processing. The combinatory effects of fixation, dehydrating solvents and heat in the course of paraffin embedding or resin curing is difficult to predict. The major effects of dehydration are the extraction of lipids and proteins. Both effects are accompanied by shrinkage of cells and subcellualr components.

Generally, the effects on the reactivity of tissue antigens produced during dehydration are less than compared with the changes during fixation, and the dehydration schedule seems not to be the critical point. Dehydration of specimens may be performed in the cold or at room temperature. When in the cold and the dehydrating solution has reached about 95%, the temperature can be raised to room temperature (see chapter *Laboratory methods*).

Tissue dehydration

Dehydration is usually done by low polarity organic solvents, e.g. ethanol. Ethanol has a great tradition and is probably the earliest experienced chemical for dehydration and hardening of tissues in histology, f.e. in form of the so-called "spirits of wine". The drawbacks of dehydration with ethanol and other solvents have become apparent with the evolution of modern microtechniques. Extraction artefacts by ethanol-water mixtures as well as changes of the hydration shells of protein antigens have been observed. Consequently, loss of molecules, native structure and antigenicity must be realized (LUFT JH and WOOD RL, 1963; MOLLENHAUER HH, 1993). When these chemical effects have to be avoided in the experimental material, then the possibility of alternative agents including water-soluble embedding media must be examined.

In contrast to the standard solvent effects (ethanol, acetone), the native conformation of proteins will be less affected when substitution of water is done for example by the more

polar ethylene glycol. Due to its two hydroxyl groups, ethylene glycol fits well into water shells of proteins so that their native conformation is less affected and better preserved from denaturation (TANFORD C et al., 1962). Ethylene glycol was found suitable for the electron microscopic preparation of biological specimens (PEASE DC, 1966; SJÖSTRAND FS and BARAJAS L, 1968; SJÖSTRAND FS and KRETZER F, 1975). When sufficient stabilization of tissue structure by inert dehydration is achieved, the final steps of embedding can be completetd in different ways. This physical method of preservation can be compared with freeze-substitution techniques.

Other proposed dehydration techniques include polyethylene glycol (Carbowax 200), hexylene glycol, propylene oxide, acrolein or potassium dichromate. Such methods are not widely employed, but they may be of interest for some special research work (KUSHIDA H, 1961; KUSHIDA H, 1963; ROBISON WG and LIPTON BH, 1969; SPURR AR, 1969; KUSHIDA H and FUJITA K, 1970).

The above considerations are relevant for both morphological and immunohistological studies. Despite its denaturation effects, low polar solvents are still in use under routine conditions. Moreover, numerous immunohistological studies in clinical histopathology have shown that a wide range of antigens will resist to these harsh dehydration procedures. It is adviced, however, that the usefulness of dehydrating solvents is proven under experimental conditions.

Other possibilities of dehydration which are of great promise include procedures of freeze-drying and freeze-substitution. Such cryo-techniques date back to the pioneering works of R ALTMANN (1890) and I GERSH (1932) to avoid alterations noted in the use of chemical fixatives and dehydration. Since then, techniques of freeze-drying and freeze-substitution became adopted to a number of experimental models for ligth and electron microscopy (SIMPSON WL, 1941; ANFINSEN CB et al., 1942; LOWRY OH, 1953; GRUNBAUM BW et al., 1956; SJÖSTRAND FS and BAKER RF, 1958; BULLIVANT S, 1960; FERNÁNDEZ-MORÁN H, 1960; STUMPF WE and ROTH LJ, 1964; STUMPF WE and ROTH LJ, 1965; MERYMAN HT, 1966a, 1966b; PEASE DC, 1973; KELLENBERGER E, 1991; PLATTNER H et al., 1997). Even if several modifications improved handling of those techniques (for references cf. TERRACIO L and SCHWABE KG, 1981; DUDEK RW et al., 1982 and references cited these papers) they have not found expansion in histopathological units and are mainly employed for distinct studies in research laboratories.

Tissue embedment (infiltration)

The final stage in tissue preparation is to prepare specimens which allow the cutting of sections thin enough for microscopy. Thus, tissue dehydration is followed by infiltration with a suitable matrix. The choice of embedding substance depends mainly on the type of histological study to be performed. Paraffin wax is the usual embedding medium for histopathology and many other light microscopical purposes. However, other embedding media such as polyethylene glycol (PEG), diethylene glycol distearate (DGD) and other waxes may be also used (ORTON ST and POST J, 1932; CUTLER OI, 1935; STEEDMAN HF, 1947; CHESTERMAN W and LEACH EH, 1956; GRAHAM ET, 1982; SALAZAR H, 1964; TALEPOROS P, 1974; TALEPOROS P, 1976; WOLOSEWICK J, 1980). These, however, are mainly of interest in studies where paraffin is not the first choice.

Many dehydrating agents are immiscible with paraffin, and a transition solvent (so-called clearing substance) miscible with both dehydration and embedding medium are needed to

facilitate paraffin infiltration. Benzene and xylene are classical clearing agents which, however, are quite toxic. Both can be replaced by chloroform (originally proposed by O BÜTSCHLI [1881] and W GIESBRECHT [1881]), acetone or other commercially available and less toxic substances. They are often called "xylene substitutes". Once dehydrated, tissue blocks are infiltrated by classical paraffin.

Tissue infiltration is always done with liquid media. In the case of paraffin, tissue blocks are treated with hot liquified which becomes solid when cooled down to room temperature. Paraffins with different hardness and melting points are available; the higher the melting point, the harder the wax. Some products contain plasticizers to make tissue blocks easier to cut. In general practice, paraffins (e.g. Paraplast® and Paraplast® Plus) with a melting point of 56°C are used.

Paraffin is still the usual embedding medium for light microscopy. For electron microscopic studies, however, as well as under certain conditions when semithin sections are to be preferred, tissue specimens are embedded in one of the available resins. The advantage of resin embedded tissue is that one can obtain better morphological details than with paraffin sections. But most importantly, one can cut ultrathin sections for electron microscopy.

Modern embedding resins for plastic embedment of tissues are liquid solutions which must be cured by heat or by UV irradiation to produce solid tissue blocks with sufficient hardness for microtomy. Three main types of resins can be used: methacrylates, polyester resins, and epoxy resins (NEWMAN SB et al., 1949; GLAUERT AM et al., 1956; KELLENBERGER E et al., 1956; MAALØE O and BIRCH-ANDERSEN A, 1956; RYTER A and KELLENBERGER E, 1958; GLAUERT AM and GLAUERT RH, 1958; GIBBONS IR, 1959; KUSHIDA H, 1959; FINCK H, 1960; KUSHIDA H, 1960; ROSENBERG M et al., 1960; LUFT LH, 1961; LOW FN and CLEVENGER MR, 1962; KUSHIDA H, 1974; KUSHIDA H and KUSHIDA T, 1982). The epoxy resins are the most widely employed resins because they possess most of the desired properties of an embedding medium for ultrastructural studies.

When the tissue has to be "lightly" fixed (f.e. omitting osmication which leaves the tissue more vulnerable to extraction during dehydration) as proposed for the preservation of tissue antigens in subsequent section staining by cytochemical and immunohistological means, one suggestion is to choose cold tissue dehydration and and embedding in plastic at a low temperature using UV polymerization. To this aim, electron beam-stable methacrylates and other acrylic plastics can be employed. The hydrophilic properties of these plastics make them especially useful as embedding media for post-embedding immunostaining. Examples are Lowicryl, LR White and other acrylic resins (CARLEMALM E et al., 1980; NEWMAN GR and HOBOT JA, 1987; NEWMAN GR, 1999). Apart from acrylic resins, water-soluble embedding media such as cross-linked polyampholytes appear to be useful alternative (MCLEAN JD and SINGER SJ, 1964).

The introduction of water-miscible resins of low solvent power was a significant progress in cell research because the undesirable effects of dehydration extraction by organic solvents could be reduced. 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 are in use since the 1980s which were designed 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).

From the newly developed spectrum of acrylate-methacrylate resins one has the choice of either polar (hydrophilic) K4M and K11M resins or non-polar (hydrophobic) HM20 and HM23 resins, just depending on the type of reseach. The freezing points allow applications down to -35°C (K4M), -60°C (K11M), -70°C (HM20) and even to -80°C (HM23), respectively. The resins can also be used in freeze-drying and freeze-substitution experiments. The Lowicryl resins are usually photopolymerized by ultraviolet light (360 nm). Because this type of polymerization is largely independent from temperature, resin infiltration and polymerization of the specimens can be done at the same temperature. Moreover, the hydrophilic properties of K4M and K11M 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; KELLENBERGER E et al., 1987).

In order to overcome problems in tissue embedment for subsequent cytochemistry, reversible embedding media have been proposed (GORBSKY G and BORISY GG, 1986). Reversible embedding media are those in which tissue specimens are embedded and which are removed after sectioning. Apart from those media and water-miscible waxes being employed in cryo-techniques, water-miscible hard waxes may be also used in non-cryo-techniques. To this aim, JJ WOLOSEWICK (1980) proposed polyethylene glycol (PEG) and DG CAPCO et al. (1984) described diethylene glycol distearate as reversible media.

Protective measures prior to tissue embedment

In principle, immuno-staining of resin sections is possible. However, pitfalls in antigen staining of resin sections must be expected. These are due to of alteration of biomolecules in the course of tissue preparation such as (1) fixation; (2) dehydration; and (3) resin curing. The latter will introduce further modifications of tissue structure by the formation of cross-links including cross-linkages between resin and tissue molecules.

In view of denaturation effects by fixation, nonpolar dehydration and embedment, attempts can be made to protect amino groups of proteins by procedures of reversible modification. Imidoester ethyl acetimidate is a candidate for this type of modification (imidoester). The conversion of free amino groups into unreactive amidines by monofunctional imidates follows aldehyde fixation and prior to dehydration and resin embedment. It can be expected that by this measure the blocked amino groups will no longer react with resin in the curing process. Thus, antigenic sites will be more readily exposed in the tissue sections upon partial removal of resin and removal of the artificially introduced acetimidyl groups by use of ammonia-acetic acid at pH >11. The effectiveness of these measures could be demonstrated in some models (KUHLMANN WD and KRISCHAN R, 1981).

Furthermore, tissue conditioning with inert compounds can be tried. Experiments with the linear polymer polyvinylpyrrolidones (PVP, originally developped by W REPPE as plasma expander, see review by W REPPE, 1954) as filler material during tissue dehydration were promising. PVP molecules can form micelles by adsorption to achieve tissue stabilization. The addition of PVP to wash buffers, ethanol and embedding media has two objectives: (1) antigen protection by micellar distribution of PVP; (2) incorporation of PVP in tissue as ready-for-use wash-off filler material. Micellar distribution of PVP may help tissue molecules to remain nearer to their native states (see chapter *General aspects in tissue preparation*).

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