General aspects in tissue preparation

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Immunohistology was invented as a specific tool for the identification of *biomarkers* in the microscope and to understand structure-function-relationships. The term biomarkers may be defined or used for biomolecules possessing particular features that make them useful in the context of histological tracing or measuring or monitoring under certain conditions which include a variety of aspects in phylogenetic, ontogenetic and disease development. Today, immunohistological techniques are essential tools for proteom and transcriptom analysis as well as for histopathologic diagnostics. In order to get familiar with histological techniques in general it is recommended to begin the studies with one or more of the classical histology handbooks (GRAUMANN W and NEUMANN K, 1958; ROMEIS B, 1968; GLAUERT AM, 1975; LILLIE RD and FULLMER HM, 1976; BÖCK P, 1989; BANCROFT JD and GAMBLE M, 2007; KIERNAN JA, 2008).

An important requirement for valid immunohistological assays is to have adequate test material. This is because biochemical and morphological alterations (autolysis) will occur immediately after removal of cells and tissues from the body. Even under best conditions, minor changes seem to be inevitable. Adequate test material means that the tissue sample must be representative of the organ (and the lesion to be studied) and must be prepared for preservation of both morphological detail and antigenicity. Otherwise invalid results will result. Compensation of inadequate tissue preparation cannot be obtained by refinements of the immunohistological technique. It appears quite dull, but one has always to realize that fixations, dehydrations, embedments etc. which usually proceed histological staining will cause various changes of the natural specimen. With respect to reliable interpretation, one needs knowledge about the effect of autolysis and changes of biomolecules caused by tissue preparation.

Since the introduction of histological immuno-stainings, a variety of tissue sampling methods has been developed for light and electron microscopic studies. Single cells in suspension, solid organ fragments and *in vitro* grown cell cultures are the most employed biological specimens. No serious difficulties are encountered when cell suspensions or monolayer cultures are submitted to immuno-staining of cell surface and intracellular molecules. Cell suspensions (stabilized or not by fixation) may be directly stained in the test tube by sequential application of all the necessary reagents. Alternatively, cells can be smeared or centrifuged onto microscope slides and are then submitted to stainings.

Very complex situations are encountered with solid organs for which a series of preparative steps are needed before one can observe the cells in the microscope. Many experiments with different organ fixations and preparations are usually necessary. This holds true for both, light and electron microscopy. The experimental design may include paraffin embedded tissue samples or samples obtained from non-embedded tissues (usually stabilized by fixation) such as small tissue blocks (hand-cut tissue fragments), sections obtained with a tissue sectioner (e.g. Sorvall TC2) and thick frozen cut sections (LEDUC EH et al., 1968; KUHLMANN WD and MILLER HRP, 1971; KUHLMANN WD, 1977; LALIBERTE F et al., 1981).

Finally, all experimental beginning has to consider the possibly deleterious effects of tissue fixation, dehydration and embedment on the desired stainings. Thus, tissue sampling needs critical considerations in studies on structure-function relationships.

Fixatives and fixation

The main goal of tissue preparation is conservation of the living state, i.e. stabilization of morphological detail and stabilization of biomolecules in their native form. Reasons for disappointing staining results are loss of immunoreactivity of antigens by irreversible denaturation. This must be expected in the course of tissue sampling; fixation, dehydration and embedment (paraffin, resin etc.) are the most important factors. The extent of qualitative and quantitative denaturation is most difficult to predict due to the lack of direct controls. Denaturation studies with isolated molecules in test tubes cannot be compared with the physiological situation of molecules in tissue compartments (f.e. complex sol-gel situation in the cytoplasm of cells). Phenomena of interphases, collapse, thermal movements etc. are difficult to predict in both conditions; their influence on intramolecular forces is difficult to measure.

From the above it becomes clear that the quality of tissue preparations depends on skillful handling. All persons involved in the collection and processing of specimens must be properly trained. Apart from tissue fixation and embedment, the freshness of specimens whether obtained from surgery or from cell culture is proportional to the ultimate product.

From quite early times, the natural product ethanol (in form of *spirits of wine*) was a popular means for the preservation of organic material because it could harden the tissue enabling it to be cut into sections. The earliest documented example of a compound fixative was CLARKE's alcohol-acetic acid solution of 1851. Subsequent popular compound fixatives included ZENKER's solution of 1894 and BOUIN's fixative of 1897. When AW HOFMANN in 1868 was able to synthesize formaldehyde from methanol, O LOEW (1886) and F BLUM (1893) examined the properties of this substance extensively and tested it as a potential histological fixative. Since then, fomaldehyde fixation has become and remained the most commonly used fixative in histology.

With the development of chemistry, several other substances were found to harden tissues, and their particular effects were exploited as single reagents or in various mixtures. Some examples are (a) mercuric chloride; (b) osmium tetroxide; (c) chromium trioxide; (d) potassium dichromate; many of them continue to be in use today. Another classical reagent derived from organic chemistry is picric acid (2,4,6-trinitrophenol).

Much later, and with the need of suitable fixatives for electron microscopy, glutaraldehyde was introduced as a fixative for biological specimens (SABATINI DD et al., 1963). Yet, basic knowledge of the chemistry of glutaraldehyde came first from the tanning industry who were able to demonstrate the potential use of glutaraldehyde as stabilizer for proteins due to the rapid formation of cross-links. Furthermore, it was quite early found that glutaraldehyde is a useful agent to denature proteins and that it was more efficient than formaldehyde or phenolic derivatives as a disinfectant (SNYDER RW and CHEATLE EL, 1965). In the meantime, glutaraldehyde has become one of the most important fixatives in electron microscopy, and much work is published on the effect of glutaraldehyde on proteins and enzymes in connection with histology (HOPWOOD D, 1967; HOPWOOD D, 1969a, 1969b; HOPWOOD D et al., 1970).

Principles of tissue embedment

In the beginning of histology, tissue preparation and specimen sectioning were difficult goals to reach. In a way to obtain sections sufficiently thin to be examined in the microscope, the use of alcohol (spirits of wine), chromic acid (and its salts) or picric acid was very popular for preserving and hardening of tissues. With further developments of embedding techniques, the production of sections improved steadily (for review of historical data see F BLOCHMANN, 1884). Advances were reached with collodion (DUVAL M, 1879), glycerol-gelatine (KAISER E, 1880), wax-oil mixture (STRICKER S, 1871) or celloidin (SCHIEFFERDECKER P, 1882) mainly as embedding media but also for adhesion of sections to microscopic slides. Yet, the introduction of paraffin by E KLEBS (1869) proved to be a real breakthrough in histology. In order to cut paraffin blocks, an important progress was the the invention of microtomes which replaced the usual freehand method of sectioning. JL VINCENT and CL CHEVALIER coined already in 1830 the term microtome; further details, see chapter *Microtomy of tissue specimens, collection of sections*.

Many dehydrating agents are immiscible with embedding media, and a transition solvent (clearing substance) miscible with both dehydrating and embedding medium is needed (see chapter *Tissue dehydration and embedment*). Dehydrated specimens are either infiltrated by classical paraffin or by one of the modern resins. While paraffin is still the most employed embedding medium, semithin sections prepared from resin embedded tissue may be preferred under certain conditions. The advantage of resin embedment is that one can obtain better morphological details than with paraffin, and, furthermore, ultrathin sections can be cut for electron microscopy.

Three main types of resins can be used to prepare plastic embedded specimens for histology: methacrylate esters, Vestopal polyester resin, and Araldite epoxy resin (NEWMAN SB et al., 1949; RYTER A and KELLENBERGER E, 1958; GLAUERT AM and GLAUERT RH, 1958). Yet, it must be borne in mind that molecules such as proteins can be denatured by water-miscible, organic solvents in the course of classical tissue embedding. The introduction of water-miscible resins of low solvent power was a progress because the undesirable effects of dehydration extraction by organic solvents could be reduced. For research work, acrylamide gels and special polar low-temperature embedding media have been proposed.

Polar media and low-temperature embedding procedures offer the advantage to reduce denaturation and conformational changes which are usually associated with nonpolar dehydration and with conventional resin curing. Several Lowicryl® embedding media are in use since the 1980s which have been designed for a wide range of embedding conditions. These resins are highly cross-linked acrylate-methacrylate embedding media with the special characteristic of low viscosity at low temperature (CARLEMALM E et al., 1980; KELLENBERGER E et al., 1980); ARMBRUSTER BL et al., 1982).

One has the choice of either polar (hydrophilic) K4M and K11M resins or non-polar (hydrophobic) HM20 and HM23 resins. The freezing points allow applications down to -35°C (K4M), -60°C (K11M), -70°C (HM20) and even to -80°C (HM23). The resins may be also used in freeze-drying and freeze-substitution experiments. Lowicryl resins are usually photopolymerized by ultraviolet light (360 nm). Because this type of polymerization is largely independent from temperature, infiltration by resins 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).

Cryo-microtomy

A specific measure of tissue sectioning is the preparation of frozen sections as already used in 1842 by B STILLING. He prepared serially cut frozen sections for his studies on the spinal cord. Later on, frozen sections were introduced for diagnostic purpose. In order to obtain sections with solid quality, tissue were fixed with formaldehyde. TS CULLEN (1895), H PLENGE (1896) and L PICK (1896) independently proposed formaldehyde fixed specimens for the preparation of frozen sections in surgical pathology. Subsequently, LB WILSON (1905) described a rapid and reliable technique for the preparation frozen sections from fresh tissues taken intraoperatively, and, thus allowed for a diagnosis within minutes while the patient was still on the operating table. The significance of these dicoveries is clear and still today frozen cut sections are important for rapid diagnosis in surgery.

Frozen sections received a boost when K LINDERSTROM-LANG and KR MORGENSON (1938) presented the first cryostat-microtome for quantitative and qualitative cytochemical work with the major advance of an anti-roll plate which allowed the cutting of flat sections to be attached directly onto the microscope slide.

Cryostat techniques are an alternative to paraffin histology. Since the early times of immunohistology, sections from rapidly frozen tissue have been employed for all types of immuno-staining. With cryostat sections, antigen preservation is usually much better than with paraffin sections. Cryostat sections, however, suffer from the drawback of inferior morphology. Yet, in the beginning of experimentation the antigen staining pattern in frozen sections will serve as reference for all other tissue preparation schedules. There exist almost as many cryostat procedures as laboratories, so the individual laboratory has to determine what sequential steps (prefixation or not, freeze protection and sectioning followed by fixation and drying etc.) will give the best results with the molecules to be detected.

The preparation of ultrathin frozen sections for electron microscopic studies was a great challenge for several decades. Major contributions were published by H FERNÁNDEZ-MORÁN (1952), W BERNHARD and MT NANCY (1964), AK CHRISTENSEN (1971) and KT TOKUYASU (1973). In the meantime, this technique has significantly advanced, and many applications including immunolabeling studies could prove the usefulness of this approach (LIOU W et al., 1996).

Cryoprotection, freeze-drying and freeze-substitution

For most histological studies, tissues being removed from its natural site are chemically fixed, dehydrated by organic solvents and embedded in supports such as paraffin or resin for the preparation sections. These procedures are inherent with morphological and molecular artefacts which often influence selective studies. In the search for alternate methods, the principles of freeze-drying and freeze-substitution have received attention.

Freeze-drying and other 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 light 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). Later on, many modifications followed including thermoelectric freeze dryers which improved those techniques (for references cf. TERRACIO L and SCHWABE KG 1981; DUDEK RW et al. 1982 and references cited these papers).

Cryo-techniques enable improved preservation of native tissues without loosing molecules. The reliability of cell preservation which is possible with ultrarapid freezing can be well coupled with freeze-substitution and low temperature embedding enabling convenient imaging of thin sections. Those cryo-techniques are beneficial to selective cell labeling in the sense of immunohistology (HUNZIKER EB and HERRMANN W, 1987; SAWAGUCHI A et al., 2004).

The potential of cryo-electron microscopy became apparent with GLAESER'S work (GLAESER RM, 1971; TAYLOR KA and GLAESER RM, 1976) and opened the way to obtain high resolution images of unstained frozen-hydrated specimens. Since the 1980s, low-temperature techniques combined with cryo-electron microscopy have gained some popularity, and, together with electron tomography, these methods have become standard for the ultrastructural characterization of multisubunit structures such as membrane-associated proteins, ribosomes etc. (DUBOCHET J et al., 1988; BAUMEISTER W et al., 2000; BAUMEISTER W, 2002; AL-AMOUDI A et al., 2004; DUBOCHET J, 2007; WAGENKNECHT T et al., 2002). Future studies will show the contribution of those techniques to the detection of molecules in cells by immunostaining.

Antigen protective measures, antigen retrievals

In view of denaturation effects by fixation, nonpolar dehydration and embedment, attempts can be made to protect protein amino groups by procedures of reversible modification. Several antigen protective measures (for example carbobenzoxychloride, originally introduced in peptide synthesis in 1937 by M BERGMANN and L ZERVAS) and restoration techniques of masked antigens by either trypsin, protease or other enzymes were proposed. However, antigen protection (prior to dehydration and embedment) as well as unmasking experiments (with enzyme treatment of tissue sections prior to immuno-staining) proved not to be reliable under all circumstances (more details in chapter *Retrieval of antigenic determinants*).

In another approach, imidoester ethyl acetimidate was used for reversal chemical modification of proteins (imidoester) in order to protect antigenic epitopes in the course of tissue embedment. The monofunctional imidate was applied after aldehyde fixation but prior to dehydration and resin embedment for the conversion of free amino groups into unreactive amidines. We expected that the blocked amino groups will no longer react with epoxy groups in resin curing. Thus, antigenic sites were thought to be more readily exposed in the tissue sections upon partial removal of resin and further removal of acetimidyl groups by ammonia-acetic acid pH > 11. The effectiveness of these measures could be demonstrated in some models (KUHLMANN WD and KRISCHAN R, 1985).

There is no question that dehydration and embedment with low-polarity substances will alter tissue molecules. Inert dehydration schedules e.g. with ethylene glycol may solve some of the inherent problems. In another approach, we have studied some tissue conditioning with inert

compounds as filler material and introduced polyvinylpyrrolidones (PVP) during tissue dehydration. PVP is a synthetic and linear polymer originally developped by W Reppe (review at W Reppe, 1954) as plasma expander. Today, PVP serves for multiple purposes as an inert product of pharmaceutical and cosmetic relevance such as a binder, emulsion stabilizer, film former and suspending agent-nonsurfactant.

PVP molecules are able to form micelles by adsorption, so that tissue stabilization can be achieved. The addition of PVP to wash buffers, ethanol and embedding media is proposed for 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. On the one hand, dehydration has less deleterious effects on the conformation. On the other hand, fragile structures are protected from reactive resin and copolymerization being prevented to a large extent. Finally, inert filler material might be readily washed off from tissue sections after removal of embedding media in order to enhance the penetration of histochemical staining reagents.

Apart from antigen protective measures in the course of tissue processing, one can try to retrieve antigens in histological sections from embedded tissue. In many cases of classically processed tissue blocks (e.g. formaldehyde fixation and paraffin embedment as usually done in routine histopathology) several special techniques have been described to unmask antigenic epitopes to a certain extent in order to enable immuno-stainings; see chapter *Retrieval of antigenic determinants*.

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Full citation of publications is given in chapter *References*
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