

# Fixation of biological specimens

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Fixation is usually the first step to prepare biological specimens for microscopy. Any treatment which will preserve cell structure and its biochemical composition can be deemed to be fixation. Of course, the quality of fixation is the key for all following steps which are necessary in histological research. Hence, preservation of cells with minimal alteration of morphology and virtually no loss of molecules is essential in tissue preservation. In this sense, tissue fixation has to preserve the cells in as life-like a state as possible. Moreover, fixation should protect biological specimens from the denaturing effects of dehydration and all further processings.

Historically, the majority of fixative solutions and fixation procedures were developed for light microscopic studies. The choice of a fixation protocol will largely depend on the analyses to be performed. All processes of tissue preparation should be as reproducible as possible. Obviously, there exists no ideal fixative for all study types. It can be even necessary to apply different fixation protocols for different structural elements within a given tissue. Organic and inorganic reagents have been used successfully for tissue stabilisation and a number of histological stainings. One of the most commonly used fixatives in histology is still formalin, i.e. formaldehyde dissolved in aqueous solutions (FOX CH et al., 1985; KIERNAN JA, 2008).

With the invention of the electron microscope it was soon realized that the usual fixation schedules developed for light microscopy are not adequate for ultrastructural studies. With regard to high resolution morphology, electron microscopy is going to be more exacting than light microscopy. Common histopathological fixatives and tissue preparations allow not to reveal ultrastructural details satisfactorily because of inherent artefacts such as shrinkage, extraction and precipitation phenomena.

In the search for adequate fixatives for electron microscopy, major advances were made with the introduction of glutaraldehyde as a primary fixative which is followed by osmium tetroxide as a secondary fixative (SABATINI DD et al., 1963; SABATINI DD et al., 1964). The convenience of this double fixation and the excellent preservation of fine structure has made this procedure to be the standard in biological electron microscopy. The success of glutaraldehyde, however, led to the reexamination of other aldehydes as primary fixatives. It became obvious that f.e. formaldehyde, freshly prepared from paraformaldehyde (ROBERTSON JD et al., 1963), compares favourably with glutaraldehyde. Furthermore, mixtures of both aldehydes and were found to be superior to either of the aldehydes used alone (KARNOVSKY MJ, 1965).

Histological fixatives contain one or more reactive components. It can be expected that no matter what reagent is used the molecular structures may become altered: either chemically by reaction with molecules (e.g. cross-linking of proteins) or physically by precipitation. Both actions can result in denaturation. Likewise, structures and biomolecules will be disrupted or being lost during fixation (as well as afterwards during subsequent tissue processing). We know by experience that fixation methods which offer good morphological preservation are

prone to pitfalls in cytochemical methods such as enzyme histochemistry or antigen-antibody reactions in immunohistology. For this reason, cell and tissue fixation must be adapted to the special needs. This is usually done by a number of defined trials to monitor morphological integrity and molecular reactivity likewise.

## Fixatives and some impacts of fixation

Many fixation types exist for various purposes. Fixatives which are chosen for *anatomical* studies (whole tissue structure) are often different from those for *cytological* purposes (cell smears, cell suspensions from body fluids or prepared from tissues). Apart from such aspects, fixation has to reconcile with *histochemical* features of the studied material (enzymes, antigens etc.). This discrimination is not exclusive because fixatives can be satisfactory for different purposes.

Fixatives which combine with proteins are called *additive fixatives*, and those which precipitate proteins are *coagulant* or *precipitant fixatives*. On the basis of this categorization, fixatives may be roughly classified into the following groups.

- Additive fixatives, e.g. aldehydes, potassium dichromate, osmium tetroxide.
- Coagulant fixatives, e.g. acetone, ethanol, methanol, trichloroacetic acid.
- Additive coagulant fixatives, e.g. chromic acid, mercuric chloride, picric acid.
- Non-additive non-coagulant fixatives, e.g. acetic acid.

A great number of factors will influence the fixation process. Many variables must be considered and controlled.

- Time interval from removal of tissue to fixation: fixation should be started as fast as possible. Also, avoid drying of tissue between the steps of tissue removal and fixation.
- pH, buffering capacity and osmolality: fixation of animal cells is best carried out near neutral pH. Hypertonic fixatives will lead to shrinkage, and hypotonic solutions result in cell swelling.
- Penetrability of fixative: this depends on the diffusibility of the individual components and the size of the tissue specimens.
- Volume: a high ratio of fixative to tissue will ensure good fixation process. In this sense, it is best to change the fixative solution several times during the fixation process.
- Temperature: fixation is mainly a chemical process, thus, increasing the temperature will increase the fixation speed.
- Concentration: too low and too high a concentration will have adverse effects, and artefacts can be produced.
- Vehicles and additives: some salts can have denaturing effects while others such as ammonium sulfate can stabilise proteins. Tannic acid can be useful because it penetrates tissues easily and precipitates polypeptides and proteins. Phenol has an accelerating effect on formaldehyde fixation. Transitional metal salts such as zinc sulfate helps in the formation of insoluble protein and polypeptide complexes, thus enhancing antigen preservation. It is possible to supplement fixatives with detergents with the aim to enhance subsequent microtechniques. It must be noted, however, that this type of primary fixation is limited to special applications because detergents in general have deleterious effects on cellular details.
- Duration of fixation: the optimal fixation time depends on multiple factors, f.e. on the thickness of the tissue specimen and, of course, most of the above mentioned features of

the fixation process (temperature, buffering capacity, penetrability of the fixative substances, volume ratios). Apart from loss of antigenic reactivity, prolonged fixation will cause shrinkage and hardening of specimens. In certain cases, degradations can be also expected.

The influence of different fixation methods such as osmolality, ionic strength etc. on the fine structure and molecules of cells was shown impressively in the electron microscope (MAUNSBACH AB, 1966a, 1966b; DOGGENWEILER CF and HEUSER JE, 1967).

## Fixation procedures

Fixation of biological specimens can be realized by two typical ways: (a) *perfusion fixation* via bloodflow; and (b) *immersion fixation* by which tissue samples are immersed into the fixative solution. It depends on the type of tissue which procedure is to favour. Alternatively, phase partition fixation may be preferred under certain conditions (LEIST DP et al., 1986).

Fixation is in most cases done by immersion of specimens into the fixative solution. Tissue specimens should be placed in fixative as rapidly as possible after they have been removed from the body. The fixative solution should be at least 20 times the volume of the tissue. Furthermore, the fixative should be changed several times; the time of fixation is dependent on the thickness of the tissue.

Perfusion fixation is generally preferred for large organs or when the fine structure of an organ is critically dependent on a continuous blood supply. A number of perfusion methods have been published and, most probably, many more unpublished variants will exist. A helpful description of perfusion fixation, perfusion fluids and the route of injection is given by AM GLAUERT (1975).

In the case of cell smears or single cells which are collected on glass slides by cytocentrifugation, merely air-drying may also act as a form of preservation. The requirements of further fixation vary according to the techniques needed for the visualization of morphological details and the molecules (cell function) to be studied. Cells in culture growing as monolayer are first rinsed with balanced salt solution, then treated with fixative (by immersion fixation). The choice of the right fixative and the right buffer solution is a matter of careful selection since monolayer cells are unprotected against osmotic changes as compared with cells in tissues.

The handling of isolated cells or cell fractions is sometimes difficult. Therefore, numerous methods were developed to collect the cells and to perform a primary fixation. Usually, centrifugation steps are included (RYTER A and KELLENBERGER E, 1958; MALAMED S, 1963; ANDERSON DR, 1965; GLAUERT AM and THORNLEY MJ, 1966; CHARRET R and FAURÉ-FREMIET E, 1967; MARIKOVSKY Y and DANON D, 1967; HIRSCH JG and FEDORKO M, 1968; MCCOMBS RM et al., 1968; SHANDS JW, 1968; FURTADO JS, 1970; SAWICKI W and LIPETZ J, 1971; KUHLMANN WD and VIRON A, 1972).

- Cells are fixed in suspension by adding adequate volumes of fixation solution, followed by centrifugation in order to obtain a pellet. Pellets are washed and further treated depending on the selected study protocol.
- Cells are first pelleted by centrifugation. After removal of the supernatant, fixative is carefully added to the pellet so as to keep the pellet intact; alternatively, the pelleted cells can be resuspended within the added fixative. Washed pellets (or centrifugation steps for cell washings) are submitted to selective study protocols.

- Cell suspensions or cell pellets which tend to disintegrate upon further handling can be encapsulated by several means such as fibrin clotting, suspension in agar gel, cross-linking with bovine serum albumin and others.

In some cases, primary fixation will not be sufficient for cellular preservation so that the specimens (tissue blocks, cells in suspension or as monolayers) have to be submitted to postfixation. Postfixation in osmium is very common preparative step in electron microscopy. In this case, fixed cell preparations are transferred from the washing buffer into a postfixation solution of buffered osmium tetroxide which is followed by dehydration and embedding.

For a number of reasons, chemical fixation should be avoided at all so as not to disturb cellular fine structures and their molecular compositions. Thus, possible alternatives were searched for, and with the advances made in freezing techniques, vitreous ice was found as a useful tool in ultrastructural research by which both chemical fixation and chemical embedding matrix can be avoided (PLATTNER H and BACHMANN L, 1982).

## Cellular components and the process of fixation

Unfortunately, fixation is often associated with variable loss of molecular properties (f.e. antigenicity), or fixation will render molecular sites (tissue antigen) inaccessible. In order to optimize tissue fixation, one has to implement a strict strategy of tissue sampling which allows to control the nature and duration of fixation. This will at least help to compromise on morphology and preservation of function (f.e. antigenicity). Since results of tissue are difficult to predict, attempts of antigen retrieval are straightforward to render the desired antigen accessible for immuno-staining.

Tissues are composed of both more and less soluble substances. Secretory products are part of the soluble ones whereas membranes and other high molecular complexes including components of the cytoskeleton are organized as relatively insoluble structures. Hence, it is readily understood that tissue molecules not being bound to solid structures can easily diffuse from their sites unless sufficient stabilization is performed by appropriate tissue sampling. Fixation may break membrane structures. Consequently, molecules are allowed to penetrate hitherto “forbidden” compartments or to escape from their original sites.

The stabilization step must be in consent with all subsequent stages of tissue preparation: dehydration, embedment, microtomy and staining schedules. An ideal fixative is characterized by rapid penetration into the specimens and compatibility with conventional and molecular specific stains. Unfortunately, all these properties are difficult to find in a single fixative. Different fixatives will give different degrees of fixation outcomes. Some of them can be of advantage for routine stainings but at the same time of great disadvantage for the more selective ones.

Fixation is a compromise and will vary according to the techniques applied. The requirements of fixation for *cytology* are different from those for *histopathology* or for *electron microscopy*. In special cases, fixation solutions must be designed for the selected application.

For most purposes, tissue fixation is done by chemical reactions. Two main types of fixatives are regularly used in histology: cross-linking fixatives and coagulating fixatives. Especially aldehydes and organic solvents are common. “Fixation procedures” based on water replacement by substitution with inert molecules (e.g. ethylene glycol) and physical means (freeze-drying, freeze-substitution etc.) can be very useful under certain conditions. Fixation procedures based on water-soluble carbodiimides, periodate-lysine-paraformaldehyde

mixtures and diethylpyrocarbonate vapors have also been suggested. Such fixations, however, have not yet found widespread application.

We have to keep in mind: in performing their role in tissue stabilization, fixatives will change the chemical and physical nature of cells, and this leads to denaturation of macromolecules by additive condensation, intra- and intermolecular cross-linkages or coagulation. Sol-gel transformations, protein networks, conformational changes are some of the results which can make the tissue components inaccessible for molecular probes in histochemical studies.

## **Denaturation by fixation**

There is a great variability in protein masking or denaturation among the different tissues by use of the various fixatives. At least various effects of cross-linkage are obtained with proteins possessing varying amounts of reactive lysine groups. It can be expected that different polypeptide chains are linked randomly to give a blend of soluble and insoluble polymers or copolymers. The final outcome in cells will be further modulated by factors such as pH, ionic strength and the ratio of fixative to protein.

Differences in reactivity are intrinsic to the different antigenic epitopes under study. It must be kept in mind that fixation is usually accompanied by alterations of the specific biological nature, and that the extent of denaturation is always difficult to predict. Whereas the antigenicity of small peptides like hormones appears to withstand fixation quite well, proteinous antigens behave capriciously due to unpredictable influence on foldings of the polypeptide chains and its antigenic structure. Upon the action of aldehydes on antigenic sites, one can expect changes of immunochemical reactivity due to modification of amino groups in some antigenic sites and due to conformational changes outside those sites. All changes may occur at random, thus, fixation is a process which is difficult to control.

Changes of cellular characteristics are regular features in tissue fixation and a compromise between structural conservation and retention of biological activity must be made. On the other hand, attempts to “reconstitute” antigenicity can be tried. For instance, some epitope retrieval may be achieved by tissue treatments with proteolytic enzymes (f.e. pronase), heat (microwave), certain buffer solutions or by other formulas (which are usually individual developments for the tissue under study) which enhance the sensitivity of histological antigen staining and other molecular probe reactions.

## **Fixation solutions**

Fixatives may be organic or inorganic in nature and irrespective of the classification as additive or coagulant fixative (see above), the routine laboratory designates fixatives as

- Aldehydes.
- Organic solvents.
- Mercurials.
- Oxidizing agents.
- Picrates.

Aldehydes fix mainly by cross-linkages and are most widely used for fixation and also well known from the tanning industry (GUSTAVSON KH, 1956). Then, mixtures of aldehydes (e.g. paraformaldehyde) with periodate and lysine have been proposed (MCLEAN IW and NAKANE PK, 1974; HIXSON DC et al., 1981). Organic solvents such as alcohols are protein denaturants which are mainly employed for cytologic smears but rarely in classical histopathology. Mercurials fix by a virtually unknown mechanism. Their best application is for fixation of hematopoietic and reticuloendothelial tissues. Also, the way of fixation of oxidizing reagents (e.g. permanganate, dichromate and osmium tetroxide fixatives) is not well known. They may cross-link proteins and cause denaturation of proteins much more extensively than aldehyde fixatives. Chromic salts form complexes with water and have a cross-linking effects which are similar to that of aldehydes. Picrate fixatives are based on picric acid with an additive coagulant mechanism of action. For detailed descriptions of fixative formulations and for relevant references see also the chapter *Fixatives*.

## Formaldehyde

Since the introduction of formaldehyde by F BLUM and O LOEW as potential fixative of biological specimens (LOEW O, 1886; BLUM F, 1893; BLUM F, 1894; BLUM F, 1896), this chemical has become one of the most commonly used fixative for routine histology and immunohistology. Formaldehyde can be used either alone or in combination with other chemicals. Though rapidly penetrating into tissue blocks, formaldehyde is not ideally cross-linking as compared with glutaraldehyde. The latter, however, penetrates tissues more slowly and interferes with macromolecules more intensely than formaldehyde. To achieve advantages and minimize the disadvantages of both fixatives for an optimal outcome, one can prepare combinations and vary their concentrations in buffered solutions by trial and success.

Methanol free formaldehyde (preferentially prepared freshly from paraformaldehyde and used either alone or in combination with other chemical substances) is a widely employed fixative for immunohistological work. In principle, formaldehyde (HCHO) is a gas and this term should be in fact restricted to that gas itself. Solutions of formaldehyde gas dissolved in water are usually called formalin. Yet, the correct use of this terminology is not strictly applied (see chapter *Fixatives*).

The molecular mechanisms of tissue fixation with formaldehyde are not well understood. A number of studies indicate that formaldehyde reacts readily with biological macromolecules such as proteins, nucleic acids and polysaccharides by the formation of cross-links, methylene bridges according to BLUM (BLUM F, 1896). The most reactive sites are primary amino groups (f.e. lysine) and thiols (cystein). Denaturation, intra- and intermolecular cross-links and polymerization will considerably alter the characteristics of organic structures (FRAENKEL-CONRAT H et al., 1945; FRAENKEL-CONRAT H et al., 1947; FRAENKEL-CONRAT H and OLCOTT HS, 1948a, 1948b; FRAENKEL-CONRAT H and MECHAM DK, 1949; HOPWOOD D, 1967; HOPWOOD D, 1969a, 1969b; 1969d; 1969e; HOPWOOD D, 1970; MAYS ET et al., 1984; MASON JT and O'LEARY TJ, 1991; HELANDER KG, 1994).

Aqueous formaldehyde solutions preserve cellular structures by its reaction with peptides and nucleic acids; in the presence of calcium, formalin is also useful as fixative for lipids. Basically, fixation with formaldehyde molecules leads to addition products between aldehyde and reactive amino groups (primarily with the residues of the basic amino acid *lysine*) by the formation of reactive hydroxymethyl groups. Subsequent condensation occurs with other neighboring amino groups to form methylene bridges between polypeptide chains (methylene bridge cross-links); some of these reactions are partially reversible. Formalin fixation is

temperature dependent and progressive with time with profound change of macromolecular conformation by changes of tertiary and quaternary structures of proteins. The final result can lead to failure of subsequent antibody reactions and other molecular probe reactions.

In the selection of formalin as fixative and in consideration of special staining methods, one has to reconcile that most of the formaldehyde induced cross-links occur at neutral pH. With buffered formalin fixatives at neutrality, more cross-links are generated because hydrogen ions from charged amino groups of protein side chains are dissociated resulting in uncharged amino groups which contain reactive hydrogen to form the above described addition products. In contrast, less cross-links occur with acid formalin solutions.

## Glutaraldehyde

Fixation of tissue is more efficient with glutaraldehyde than with formaldehyde and relies on its cross-linking properties. For reproducible results, highly purified glutaraldehyde is needed. Besides the monomer, glutaraldehyde frequently may contain large amounts polymers,  $\alpha$ ,  $\beta$ -unsaturated aldehydes after aldol condensation, glutaric acid and inorganic substances (see BEILSTEIN's Handbuch der Organischen Chemie E III 1.3111) which all together can initiate unexpected reactions. In these cases and when highly purified preparations are not available, the degree of purification of glutaraldehyde can be improved by treatment with activated charcoal and chromatography (e.g. Sephadex G-10), or better, by vacuum distillation over a Vigreux column; the degree of purity  $P.I < 0.2$  ( $P.I = E_{235\text{ nm}} : E_{280\text{ nm}}$ ) being strived for. Methods of purification and standardization for fixation purposes have been published (FAHIMI HD and DROCHMANS P, 1965; ANDERSON PJ, 1967).

The stabilizing effect is attributed to rapid and persistent intra- und intermolecular cross-linkages of tissue components. Glutaraldehyde is believed to react by a similar mechanism to formaldehyde. Glutaraldehyde, however, will give a more tightly linked product: its greater length as compared with formaldehyde and its two aldehyde groups allow glutaraldehyde to link more distant pairs of protein molecules as formaldehyde. This type reaction is a factor which makes glutaraldehyde an efficient fixative. The degree of cross-linking is progressive with time, and depends on the accessibility of  $\epsilon$ -amino groups by aldehyde groups which leads to the formation of Schiff bases. Important aspects of the reaction of glutaraldehyde on proteins, the cross-linking and the fixation process have been published (QUIOCHO FA and RICHARDS FM, 1964; HOPWOOD D, 1967; HABEEB AJ and HIRAMOTO R, 1968; RICHARDS FM and KNOWLES JR, 1968; HOPWOOD D, 1969a, 1969b, 1969d, 1969e; HOPWOOD D, 1970; HOPWOOD D et al., 1970; KORN AH et al., 1972; PAYNE JW, 1973; MONSAN P et al., 1975).

Cross-linking fixatives tend to preserve the secondary structure of proteins and may also protect tertiary structures as well. Yet, it must be kept in mind that this type of fixation can be accompanied by an alteration of specific biological activity. The extent, however, is difficult to predict and varies from tissue to tissue and from cell to cell. In the case of antigenic epitopes, the action of aldehydes will modify one or several amino groups and may change the reactive regions of the molecule. Thereby, the charge pattern of proteins can be changed inducing conformational changes. Furthermore, conformational changes outside the antigenic epitopes must be considered which will also influence protein denaturation. For more details see chapter *Artefactual staining in immunohistology*.

## Miscellaneous aldehydes

A number of other aldehydes are known and may be used for fixation procedures. For example acrolein (acrylic aldehyde) which is mainly used in tanning industries. Solutions of 4% acrolein may be employed for histochemistry. This aldehyde produces more cross-links than formaldehyde, it is, however, unstable at alkaline pH and tends to polymerize into disacryl when exposed to light.

Glyoxal, malondialdehyde, 2,3-butanedione and other di- or polyaldehydes are possible alternatives to the above mentioned aldehydes for the purpose of histological fixation. They are, however, rarely employed.

## Other cross-reacting fixatives

There exist a number of other reagents as protein cross-linking fixatives. They are, however, not widely employed. From the bulk of possible reagents, just a few are mentioned, e.g.

- Water-soluble carbodiimides: compounds that react with and cross-link carboxyl groups. They form cross-links between soluble proteins by joining C-termini and side chains of glutamic and aspartic acid units. Carbodiimides are mainly used to prepare conjugates of peptides and larger proteins.
- Diisocyanates and diazonium compounds: reagents often used to conjugate antibodies with fluorescent labels; in histology used to reduce diffusion artefacts of soluble enzymes in tissue preparations.
- Diimidoesters: these reagents react rapidly with protein molecules by the formation of cross-links (amidines). They have proved useful as fixatives in light and electron microscopy.
- Diethylpyrocarbonate: compound consisting of diethyl oxyformate and ethoxyformic anhydride and mainly used for cold sterilization of beverages; introduced in histology as a vapour phase fixative (freeze-dried tissue) for subsequent immunostaining. With improved water-solubility, this compound can be used as a liquid phase fixative.
- Maleimides: reagents with mild cross-linking properties, useful for the preparation of protein conjugates such as antibodies labeled with enzymes as markers.
- Benzoquinones: cross-linking reagents often used in immunochemistry for the conjugation of enzymes and various protein molecules. Comparable to diimidoesters, benzoquinones can be applied as fixatives in histology.

Over the years, these reagents have never caught great importance. If the antigenicity of proteins is critically dependent on free amino groups of an epitope, then carbodiimides can be an alternative to aldehydes. In the case of light microscopic immunohistology, we prefer chemically unreactive fixatives like ethanol-acetic acid mixtures (see chapter *Fixatives*).

## Non-aldehyde fixatives

In order to avoid cross-linkage and other aldehyde-induced loss of reactivity of cellular molecules, other fixatives were looked for as substitutes for aldehydes. Many of them are *coagulating fixatives* which precipitate proteins, and ethanol is a typical representative. By water removal with ethanol, hydrophobic interactions, which give many proteins their tertiary



structure, are (often) disrupted. These events will reduce the solubility of protein molecules and result in denaturation.

The most common precipitating fixatives are ethanol, methanol and acetone. Acetic acid is also a denaturant that is used in combination with other precipitating fixatives. Alcohols are known to cause shrinkage of tissue while acetic acid alone leads to tissue swelling. The combination of both reagents results in better preservation of morphology. In a number of histological and immunohistological studies with a variety of organs, the great value of ethanol-acetic acid fixatives could be clearly shown. Apart from immunohistology, this type of fixation competed well with conventional formalin fixation with respect to routine stainings and histochemical reactions (KUHLMANN WD, 1975; WURSTER K et al., 1978; KUHLMANN WD and PESCHKE P, 2006).

*Oxidising fixatives* react with various side chains of biomolecules (including proteins) leading to the formation of cross-links which stabilize the tissue structure. Potassium permanganate, potassium dichromate, chromic acid and osmium tetroxide are useful in certain specific histological studies (BAKER JR, 1965a, 1965b; LUFT JH, 1956). Then, osmium tetroxide has been often used as fixing reagent in light and electron microscopy (SCHULTZE M and RUDNEFF M, 1865; FLEMMING W, 1895; PORTER KR, 1950; PALADE GE, 1952; PORTER KR and KALLMAN F, 1953; RYTER A and KELLENBERGER E, 1958; BENNETT HS and LUFT JH, 1959; MILLONIG G, 1961). The effect of some of the above fixatives on proteins has been described in model experiments (LUFT JH and WOOD RL, 1963; HOPWOOD D, 1969c).

The value of *osmium tetroxide* for the preservation of cell structure has been already reported in 1927 (STRANGWAYS TSP and CANTI RG, 1927). With the development of electron microscopy in the early 1950s, it became apparent that osmium tetroxide was by far the best choice for the study of fine structure as compared with the then employed fixatives for light microscopy (PALADE GE, 1952). The main disadvantage of osmium tetroxide is its slow penetrability into tissue blocks. Furthermore, the ionic constitution of the buffer influences the cellular fine structure (TRUMP BF and ERICSSON JLE, 1965) as well as the rate of osmium penetration. Today, osmium tetroxide has still its important role as secondary fixative in electron microscopy.

Other fixatives include *picric acid* and *mercuric chloride*. Both types of fixatives have proved useful for special histological studies. The type of fixation, however, is poorly understood.

Recently, new fixative formulations have been proposed as alternatives to aldehydes, organic solvents etc. with respect to histopathological diagnosis and immunohistology. One of these promising fixatives is *HOPE* (HEPES-glutamic acid buffer-mediated organic solvent protection effect) fixation. This fixative can be employed for cytopins and tissue blocks. Fixation starts by overnight immersion of fresh tissue specimens into the aqueous protection solution at low temperature followed by acetone dehydration, paraffin embedment and microtomy (OLERT J et al., 2001; UMLAND O et al., 2003). Then, special kinds of ionic liquids containing a heterocyclic cation and based on a substituted imidazole such as 1-methyl-3-octyloxymethylimidazolium tetrafluoroborate were shown to be good fixatives with similar effects as formalin (PERNAK A et al., 2005). All these new fixative formulations are of some interest as formalin substitutes if they really function as fixatives rather than as tissue preservatives that prevent autolysis. Further studies will show if those formalin substitutes have the advantage of little or no need for antigen retrieval since in many instances the use of appropriate epitope retrieval allows immunostaining on formaldehyde fixed specimens.

## **Microwave irradiation for tissue fixation**

Fast tissue fixation methods are of interest in the study of precise localization of highly dynamic cell processes. Fast tissue fixation techniques are also of advantage in diagnostic histopathology at least for reasons of rapidness. Quick-freezing and freeze-drying preparations are often successful solutions, but the versatility of these approaches is limited with respect to simultaneous cytochemical, immunological and conventional stainings in histology. Hence, the development of other methods are looked for. One promising alternative with special regard to primary chemical fixatives is fixation of tissues by microwave energy.

Microwaves for tissue fixation were introduced by CP MAYERS (1970). Later on, the basics of microwave heating patterns in tissue models were described (HAND JW, 1977). The first approaches focussed on its use for light microscopy, but soon this techniques was also successfully applied for electron microscopic studies (BERNARD GR, 1974; GORDON HW and DANIEL EJ, 1974; HOPWOOD D et al., 1984; LOGIN GR and DVORAK AM, 1985; LOGIN GR et al., 1986; OHTANI H, 1991; LEONG AS, 1993; GIBERSON RT and DEMAREE RS, 1995; GIBERSON RT et al., 1997). In the meantime, numerous reports on microwave fixation support the usefulness of this type of fixation for a variety of tissues and purposes (LEONG AS et al., 1985; LOGIN GR et al., 1991; LOGIN GR et al., 1992; LOGIN GR and DVORAK AM, 1994a, 1994b; TINLING SP et al., 2004).

Microwaves are a form of non-ionizing radiation which may be generated by domestic microwave ovens at a frequency of 2.5 GHz and 600 Watt output. The exposure of dipole molecules such as water or the polar side chains of proteins to the electromagnetic field results in oscillation corresponding to the frequency of the generator. Immediately, the kinetics result in heat which is proportional to the energy flux until radiation ceases; the heat being controlled by the energy level and the duration of exposure. The optimal temperature for fixation is in the range of 60°C and 85°C. Temperature and time are usually established for each tissue.

The heat in microwave fixation is probably the primary factor in tissue fixation. Yet, the rapid movement of molecules (induced by the oscillating electromagnetic field) will also have a direct influence on the outcome of fixation. In the case of combined fixation by microwaves and aldehydes as cross-linking reagents, the diffusion rate of the chemical fixative is enhanced and will accelerate the process of cross-linkage (LOGIN GR et al., 1986). Furthermore, other still unknown mechanisms are expected to occur with irradiation. Influences on non-covalent secondary bonding, hydrogen bonds, van der Waals' interactions etc or disruption of bound water are possible which all together can alter biological systems.

## **Selected publications for further readings**

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