Fixation and Fixatives – Factors influencing chemical fixation, formaldehyde and glutaraldehyde

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This Fixation and Fixatives series covers the factors that influence the rate and effectiveness of tissue fixation as well as looking at two common fixatives: formaldehyde (histology) and glutaraldehyde (ultrastructural electron microscopy studies).

Factors influencing chemical fixation

There are a number of factors that will influence the rate and effectiveness of tissue fixation.

Temperature: Increasing the temperature of fixation will increase the rate of diffusion of the fixative into the tissue and speed up the rate of chemical reaction between the fixative and tissue elements. It can also potentially increase the rate of tissue degeneration in unfixed areas of the specimen. For light microscopy initial fixation is usually carried out at room temperature and this may be followed by further fixation at temperatures up to 45°C during tissue processing. This is really a compromise that appears to be widely accepted to produce good quality morphological preservation. Microwave fixation may involve the use of higher temperatures, up to 65°C, but for relatively short periods. See Part 5 for further discussion.

Time: The optimal time for fixation will vary between fixatives. For fixation to occur the fixative has to penetrate, by diffusion, to the centre of the specimen and then sufficient time has to be allowed for the reactions of fixation to occur. Both diffusion time and reaction time depend on the particular reagent used and the optimum time will vary from fixative to fixative. In busy diagnostic laboratories there is considerable pressure to reduce turnaround time and this can result in incompletely-fixed tissues being processed. This can lead to poor quality sections showing tissue distortion and poor quality staining because poorly fixed tissue does not process well. Remember that if incompletely-fixed tissue is taken from formalin and placed in ethanol during processing, ethanol will continue to fix the tissue and the morphological picture at the centre of the specimen will be that of ethanol fixation.

Penetration rate: The penetration rate of a fixing agent depends on its diffusion characteristics and varies from agent to agent. As devised by Medawar it can be expressed as $d = K\sqrt{t}$, where d is the depth of penetration, K is the coefficient of diffusion (specific for each fixative), and t is the time. 1 In practical terms this means that the coefficient of diffusion (K) is the distance in millimeters that the fixative has diffused into the tissue in one hour. For 10% formalin K = 0.78. This means that your formalin fixative should not be

expected to penetrate more than say 1 mm in an hour and it will take approximately 25 hours to penetrate to the centre of a 10 mm thick specimen , i.e. 5 mm (= 5^2 hours).

Volume ratio: It is important to have an excess volume of fixative in relation to the total volume of tissue because with additive fixatives the effective concentration of reagent is depleted as fixation proceeds and in a small total volume this could have an effect on fixation quality. A fixative to tissue ratio of 20:1 is considered the lowest acceptable ratio but I would advocate a target ratio of 50:1.

pH and buffers: At the light microscope level the pH of a fixative does not appear to affect the quality of preservation greatly as a number of formulations have quite a low pH, such as those containing acetic or picric acids. However pH can be important for other reasons as in the case of formaldehyde solutions, where breakdown of formaldehyde to form formic acid produces an acidic solution which in turn reacts with hemoglobin to produces an artefact pigment (acid formaldehyde hematin). The most popular formaldehyde solution in use today is therefore buffered to pH 6.8 – 7.2 for this reason. For electron microscopy pH is more important and should match physiological pH. 2

Osmolality: The osmotic effects exerted by the fixative are again more important at the ultrastructural level than at the level of the light microscope because it is the phospholipid membranes that are easily damaged by excessively hypotonic or hypertonic solutions, but osmolality does have some relevance in routine histopathology. Generally it is the osmolality of the vehicle (buffer) that is most important and in some formulations this is adjusted to resemble that of tissue fluid (eg. formalin in isotonic saline). Before fixation occurs cells can certainly be damaged by non-isotonic fluids such as water and if specimens cannot be immediately fixed they can be kept moist with gauze soaked in isotonic saline for a short time. It is not a good idea to hold tissue immersed in saline for extended periods.

Fixing agents

There are a number of reagents that can be used to fix tissues. Formaldehyde, by far the most popular agent used for histopathology and glutaraldehyde, widely used for ultrastructural studies requiring electron microscopy, are described here. Other reagents are discussed in Part 3.

Formaldehyde: Formaldehyde (CH_2O) is the only gaseous aldehyde and is dissolved in water to saturation at 37% – 40% w/v. This solution is generally referred to as “formalin” or “concentrated formaldehyde solution”. For fixation, one part formalin is usually diluted with nine parts of water or buffer. This produces a 10% formalin solution which contains about 4% formaldehyde w/v, an optimal concentration for fixation. In concentrated solutions formaldehyde exists as its monohydrate methylene glycol and as low molecular weight polymeric hydrates. In its diluted form the monohydrate predominates. Paraformaldehyde, a highly polymerised form of formaldehyde may be deposited as a white precipitate in concentrated formaldehyde

solutions. To prevent this, small quantities of methanol (up to 15%) are commonly added to proprietary solutions. Paraformaldehyde can be purchased as a dry powder and used to make up highly pure solutions of formaldehyde such as those required for electron microscopy. 2, 3

Unbuffered formalin will slowly oxidize to formic acid resulting in a fall in pH. Under these conditions the formic acid will react with hemoglobin forming acid formaldehyde hematin, a brown-black granular artefact pigment which is deposited in blood-rich tissues. This pigment is a nuisance as it can be confused with micro organisms or other pathological pigments. 4 Although the pigment can be removed from sections with saturated aqueous picric acid before staining, it is preferable to avoid its formation in the first place. For this reason and because formaldehyde reacts most effectively at about a neutral pH, 10% formalin solutions are usually buffered to pH 6.8 – 7.2.

.Formaldehyde can react with some groups in unsaturated lipids particularly if calcium ions are present, but tends to be unreactive with carbohydrates. 5 Formaldehyde can react with groups on lysine, arginine, cysteine, tyrosine, threonine, serine and glutamine forming reactive complexes which may combine with each other forming methylene bridges (cross-links) or with hydrogen groups. 5 It is widely accepted that washing tissues after formalin fixation can reverse some of these reactions but important cross-links remain. 6 It is the ability of formaldehyde to preserve the peptides of cellular proteins which have made it so useful as a general purpose fixative.

Glutaraldehyde: Glutaraldehyde or glutaric dialdehyde (CHO(CH2)3CHO) Is regarded as a bi-functional aldehyde, possessing aldehyde groups at either end of the molecule which have the potential to react with the same chemical groups as formaldehyde. They will form addition compounds and methylene bridges but also a single glutaraldehyde molecule may form direct cross links if the steric arrangement of adjacent peptides allow it. The amino groups of lysine are particularly important in this respect. Tissue fixed in glutaraldehyde will be more extensively cross-linked than tissue fixed in formalin and will also possess some unreacted aldehyde groups that, unless chemically blocked, can cause background staining in methods such as PAS. The extensive cross-linking adversely affects immunohistochemically staining but does provide excellent ultrastructural preservation which explains its extensive use as a primary fixative for electron microscopy. Cross-linking reactions of glutaraldehyde are largely irreversible. Glutaraldehyde penetrates very slowly and it is recommended that tissue be less than 1mm in thickness in at least one dimension. 5, 11

Glutaraldehyde will slowly decompose to form glutamic acid and will also polymerize to form cyclic and oligomeric compounds. Glutaraldehyde is therefore best obtained in sealed ampoules in a convenient form “stabilized for electron microscopy” and this can be added to a suitable buffer at pH 7.2 – 7.4 (usually cacodylate, phosphate or maleate) to produce a 3% glutaraldehyde concentration for use. For electron

microscopy glutaraldehyde primary fixation is commonly followed by secondary fixation in osmium tetroxide. Glutaraldehyde is not normally used for routine histopathology. 11

Effect of heat during fixation

When the temperature of a fixative is raised or lowered (as is sometimes recommended for particular histochemical procedures), the rate of diffusion into the specimen is affected, as is the rate of the chemical fixation reactions occurring with the various tissue components. Increasing temperature accelerates the process of fixation. Excessive heat however, particularly if it is prolonged, can damage cells and cause substantial shrinkage and hardening of the specimen.

In the days before the widespread use of the cryostat it was standard practice to rapidly fix small specimens in boiling formalin prior to preparing frozen sections using the freezing microtome. This process produced specimens which could be sectioned but showed indifferent and very variable microscopic results, apart from often exposing the microtomist to unacceptable levels of formaldehyde vapor.

Today most laboratories carry out primary fixation of specimens at ambient temperature and only after specimens are loaded onto the processor, where staff have some protection from the vapors produced, would fixative temperatures be increased. Temperatures of between 37°C and 45°C are commonly employed.

Another of the problems of using hot fixative solutions to initially fix larger specimens (greater than 3mm thick), is that the outside of the specimen fixes rapidly whilst it may take quite some time for the fixative to penetrate to the center of the block and this area may be poorly fixed or not fixed at all. Blocks then show an exaggerated “zonal” fixation effect with different morphological and staining characteristics on the outside as compared to the inside of the specimen. It is for these reasons that microwave fixation is used in some laboratories

Practical procedures to optimize quality

By following simple common-sense principles high quality, consistent fixation outcomes can be achieved. Here are **three essentials** to good fixation and **twenty rules** to follow to ensure they are achieved.

Essential 1: Fresh tissue

1. Fix as soon as possible.
   Remember that degeneration commences as soon as cells are deprived of a blood supply.

2. If fixation is not immediately possible refrigerate, do not freeze.
   Slow freezing of tissue will produce considerable damage due to the formation of ice crystals.

3. Fresh tissue may be infectious.
   Consider any fresh or incompletely-fixed tissue as potentially infectious to you and other workers in your lab.

4. Do not allow specimens to dry out.
   Dessication of specimen surfaces will cause permanent damage and may mask pathological change. Small endoscopic specimens are particularly susceptible to this type of damage.

5. Do not distort tissue.
   Do not be rough with fresh tissue. Distortion or other mechanical damage will cause permanent morphological changes which can make interpretation difficult.

6. Label fully and accurately.
   An absolute essential for diagnostic and research material.

Essential 2: Proper penetration of fixative

7. Fixative should penetrate from all sides.
   Always place specimens into containers that already contain fixative. This will prevent adhesion of the specimen to the container.

8. Cavities should be opened.
   Where possible hollow organs or specimens with natural cavities should be opened to allow immediate access of fixative.

9. Perfusion of some specimens is advantageous.
   Fixation by perfusion of the vascular system of whole organs or small experimental animals may produce excellent results.

10. Thickness is important (4mm maximum).
    The thickness of any specimen or tissue slice should not exceed 4 mm if optimal fixation is to be achieved.

11. Some agitation is useful.
    Some occasional, gentle agitation (swirling) of the specimen during its first few minutes in fixative will aid penetration.

12. An adequate volume is vital (20:1 at least).
    An excess of fixative is required as its effective components may be depleted as part of the reactions of fixation.

13. Allow sufficient time.
    The fixative has to penetrate to the centre of the densest part of the specimen and then the chemical reactions of fixation have to take place.

14. Room temperature is best.
    Initial fixation is best carried out at room temperature (20°C).

Essential 3: Right choice of a correctly formulated fixative

15. Fixatives should be carefully made up from reagents of suitable quality, fresh if so specified.
    Poor quality reagents can produce poor quality fixation. Some formulated fixatives should be made up from stock solutions immediately before use because they are unstable (eg. Helly's fluid).

16. Specimens received in fixative should be checked and fixative replaced if necessary.
    If a specimen is received in fixative of dubious quality replace it with fresh fixative – it can only improve the results.

17. Fixatives should be used once only.
    Specimens shed cells and tissue fragments into the fixative solution which could contaminate any subsequent specimen. In addition components are used up during the reactions of fixation.

18. Avoid metal lids.
    Some fixatives are highly corrosive and will attack metals (eg. mercury salts).

19. A suitable treatment following fixation should be applied.
    Some fixatives require that specimens be washed in water prior to processing (eg. Zenker or Helly) or some other requirement may exist (phosphate may precipate from buffer in concentrations of alcohol of greater than 70%).

20. All fixatives are toxic and irritant.
    In order for a fixative to fix it must be toxic and is likely to be irritant although they do vary in extent. Anyone using fixatives should be aware of the potential hazards.

TEM Fixation protocol for pathology samples

Fix tissues in a mixture of 2.5% glutaraldehyde, 2% (para)formaldehyde in 100 mM cacodylate buffer (pH 7.0) with 2 mM $CaCl_2$
.
After an initial ~30 min fixation,
cut the specimens into small (~1 mm_3) pieces and continue
fixation in fresh fixative for 16-24 h at 4°C.
Wash briefly with 200 mM
cacodylate buffer (pH 7.0).

Post-fix with 1% osmium tetroxide in 100 mM cacodylate buffer (pH 7.0); 2 h at 4°C.
Wash with excess distilled water [to remove any free cacodylate and/or phosphate ions]. En bloc stain with 2.0% aqueous uranyl acetate, ~2 h at 4°C (in dark).
Dehydrate with acetone (or ethanol), propylene oxide and embed in resin as outlined above

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