TECHNICAL NOTE ON FIXATIVES

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WARNING: fixatives are dangerous chemicals that should always be handled in a chemical hood. Formaldehyde, paraformaldehyde and glutaraldehyde are especially dangerous. Carefully observe all chemical safety rules…!

I Introduction

Tissue preparation includes multiple steps of cell/tissue handling procedures. One of the critical steps is tissue fixation, which is to preserve tissue with fixatives, as such to maintain cell/tissue morphology and retain the integrity of biomolecules of interest.

Fixatives prevent postmortem decay or cellular degradation by deactivating proteolytic enzymes and destroying microorganisms in tissue, as well as preserving the sample as close to the natural state as possible. In addition, most fixatives increase mechanical strength and stability of the specimen by cross-linking proteins.

Three types of fixatives are commonly used in tissue preparation: aldehyde fixatives, organic solvent fixatives and other specific fixatives. Each fixative has its own advantages and disadvantages. The selection of a fixative depends on the type of tissue and its characteristics as well as the goal of the projects.

II Aldehyde fixatives

Formaldehyde and glutaraldehyde are the most common aldehyde fixatives. They fix the tissue by forming cross-links within and between proteins.

Formaldehyde

Formaldehyde reacts with functional groups in biomolecules, particularly with side-chain amino group of lysine residues in proteins, to form methylene bridges that cross-link and eventually fix tissues (Fig 1).

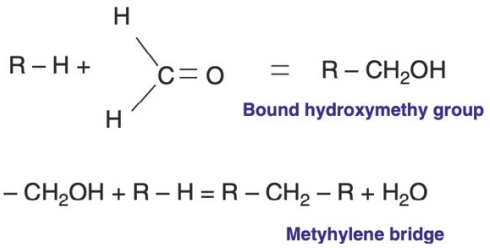

Fig 1 (from Basic and Advanced Laboratory Technique in Histopathology and Cytology)

Pure formaldehyde is a gas that can be dissolved in water to form a 37-40 % formaldehyde solution known as formalin.10% neutral buffered formalin (NBF) is commonly used to fix tissue in biological laboratory, the actual formaldehyde concentration of 10% NBF is 3.7-4% (0.37-0.40 × 0.10 = 3.7-4%). Commercial formalin may contain 10-12% methanol as a stabilizer to prevent the formation of paraformaldehyde precipitates. Alternatively, 4% formaldehyde solution can be prepared from paraformaldehyde. Paraformaldehyde is formaldehyde polymer; it degrades into formaldehyde monomer when heated with sodium hydroxide (Fig 2).

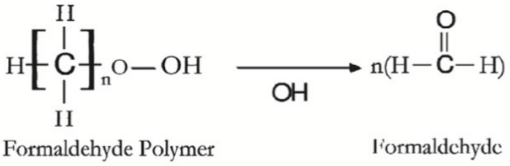

Fig 2 (from Thavarajah R et al. Journal of Oral and Maxillofacial Pathology 2012)

Paraformaldehyde is commercially available as powder or high concentration solutions. Paraformaldehyde powder need to be dissolved in water at 60°C with NaOH to make formaldehyde solution. For example: to make 100 ml 4% formaldehyde solution, add 4 g paraformaldehyde powder into 85 ml water; heat at 60°C with addition of 2 N NaOH until dissolved, add 10 ml 10X PBS and bring the volume to 100 ml final volume, cool down completely and adjust the pH.

Commercial 16%, 20%, 32% pre-made paraformaldehyde solutions are sold in ampules to prevent oxidation and precipitation. These solutions can be easily diluted to make 4% working solution. For example: mix 10 ml of 16 % pre-made paraformaldehyde solution (one ampule) with 4 ml 10X PBS and 26ml of water, and adjust the pH.

4% formaldehyde solution should always be FRESHLY PREPARED to avoid acidification and polymerization. Formaldehyde undergoes acidification in the presence of atmospheric oxygen with conversion to formic acid which lowers the pH and decreases the fixation efficiency. Formaldehyde also forms polymers and precipitates slowly over the time.

4% formaldehyde solution penetrates the tissues slowly, at a rate of 1mm per hour. As such, perfusion fixation is recommended for whole animal fixation. In this technique, a 4% formaldehyde solution is quickly brought into the whole animal with trancardial infusion through the whole circulatory system. Following perfusion, tissues should be post-fixed by immersion in 4% formaldehyde for 16 hrs to a few days at 4 degree.

Cross-links induced by formaldehyde fixation may conceal protein epitopes targeted by antibodies. In this case, an antigen retrieval step is required for optimum immuno-reactivity.

Formaldehyde may also cause background autofluorescence, especially in the green channel for tissues. A lower concentration of formaldehyde (2%), a shorter fixation time, or an extended period of photobleaching with a strong LED white light at 4 degree may potentially reduce the autofluorescence.

Glutaraldehyde

Glutaraldehyde monomer is a five-carbon chain with two aldehyde groups locating on each end. Like formaldehyde, the aldehyde groups react with the amino group of proteins, particularly lysine side chains (Fig 3). Glutaraldehyde also forms oligomers or polymers that cross-link proteins more extensively, providing better ultrastructure preservation than formaldehyde (Fig 4). Tissue preparation for electronic microscopy (EM) requires glutaraldehyde fixation.

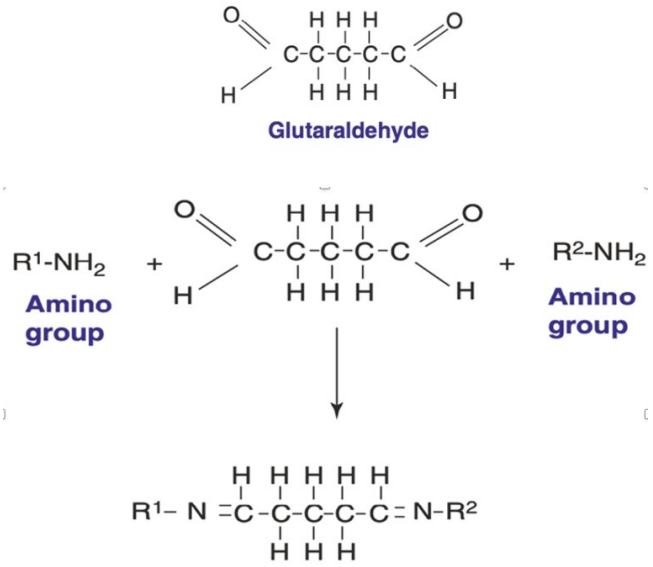

Fig 3 (from Basic and Advanced Laboratory Technique in Histopathology and Cytology)

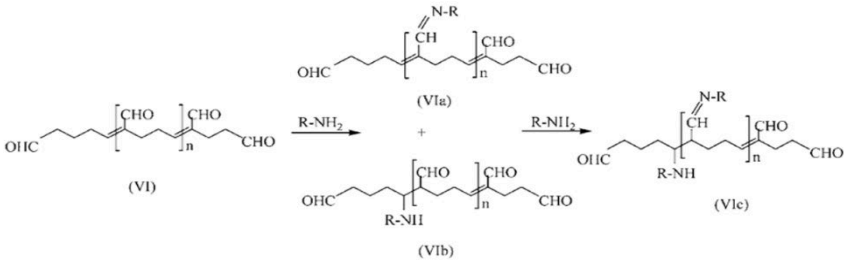

Fig 4 (from Migneault I. et al. BioTechniques 2004)

Due to its larger size, glutaraldehyde penetrates tissue more slowly than formaldehyde. Transcardial perfusion and extended post-perfusion fixation is recommended when using glutaraldehyde.

One major disadvantage of glutaraldehyde is the high fluorescence background, a combined effect of aldehyde autofluorescence and remaining aldehyde groups binding antibodies. Because

of this, enzyme-based immune-histochemistry methods are more suitable for glutaraldehyde-fixed samples than immunofluorescence staining.

III Organic fixatives: Ethanol, Methanol, Acetone

Certain organic solvents, such as **ethanol**, **methanol**, and **acetone**, can be used as fixatives. These solvents break hydrogen bonds, remove and replace water, and precipitate proteins within the cellular architecture, as such, fix and preserve tissue integrity. Organic solvents act very fast and are normally prechilled and used ice-cold or at -20 ° C with short incubation times (1 to 10 mins depending on the sample).

Methanol and acetone also permeabilize tissues. Thin (< 20 μm) tissue sections and cells fixed by these two solvents may not require further permeabilization.

On the other hand, the destruction of hydrogen bonds by organic solvents may disturb the tertiary structure of proteins and lower immuno-reactivity. Organic solvents also deplete lipids in cell membrane and are not suitable for membrane-bound epitopes.

IV Other alternative fixatives

Other types of fixatives can be used when neither aldehyde fixation nor organic solvents are suitable. Among others:

Carnoy`s Solution, made of 60% ethanol, 30% chloroform and 10% glacial acetic acid, is commonly used for fixation of DNA and RNA for in- situ hybridization.

Helly`s Solution, is composed of mercury chloride, potassium dichromate, and formaldehyde. It is used for fixation of blood-forming organs such as bone marrow, liver and spleen.

Zinc Fixative, made with calcium acetate, zinc acetate, and zinc chloride in tris buffer. This solution is useful for fixation and staining of immune cell-related protein markers.

V Fixation of endogenous markers

Fluorescent proteins (FPs) are widely used as expression reporters in cells or tissues, commonly as fusion proteins. However, their fluorescence intensity is adversely affected by fixation. Most FPs lose 40% to 80% of their brightness upon fixation, regardless of the fixative types. There is no effective method that could completely recover or retain original fluorescence intensity of FPs, but several options can help alleviate the problem:

Test different fixatives: FPs may retain more fluorescence when fixed by certain fixatives. In general, 4% FPA preserves 30% to 60% of original fluorescence of GFP, but organic solvents (methanol, ethanol and acetone) may lead to a complete loss of fluorescence.

Adjust fixative concentration and/or fixation time: for example, decrease the concentration of PFA to 2% and/or shorten the fixation time to 5 mins (cells / thin tissue sections).

Test or exchange fixative buffers: PBS and PB are commonly used as fixative buffers. In certain circumstances, one buffer may retain more FP fluorescence than the other.

Use alternative FPs: new chemically stable FPs were created to overcome or rescue the unfavorable fixation problem, for example, “hyperfolder YFP” (hfYFP) was reported to retain 75% of its fluorescence after fixation by 4% PFA + 5% Glu (Campbell BC et al, Nature Methods 2022: 1612-1621).

Counter-staining: if possible with fixed specimen, counter-stain FPs with a primary and secondary antibody. Counterstaining improves the brightness 10 to 50 times. Primary anti-FP antibodies are generally not very specific and may bind to different types of FPs at once, so, carefully check their specificity.