

Introduction

Fixation is an attempt at stabilizing biological systems for viewing in a vacuum with minimal distortion to their cytomorphology and cytochemistry. Biological electron microscopy generally employs two methods for obtaining these results, chemical and/or physical fixation. While chemical fixation remains the most common method of specimen preservation being less expensive, physical techniques (mainly cryo) are gaining in popularity. Certain procedures combining chemical and physical fixation are both useful and advisable.

Chemical fixation

Chemical fixation prepares the cell for a whole series of rather traumatic events imposed by the physical characteristics of the electron microscope, (i.e., improved resolution, extreme vacuums and the intense heat of the electron beam). Ideally, the reagent selected as a fixative should transform the viscous colloidal protein solution (protoplasm) into a stabilized material. Simultaneously, the spatial relationship of all organelles should be maintained; the phospholipids, which form the framework of the cell, should be stabilized and the remaining chemical constituents rendered insoluble. This rather large undertaking probably contributes to the popularly held notion that “the perfect fixative” has yet to be developed. There are, however, a number of fixatives, which are capable of performing at least some of the desired effects, provided appropriate steps, are taken to adjust certain physiological parameters to approach the cell’s natural milieu.

Rate of penetration

Determines the speed at which the reagent effects the cell’s structure and function. The rapidity with which this is accomplished is paramount to good fixation. High pressure freezing stabilizes the cellular structure in milliseconds. Formaldehyde penetrates the cell faster than glutaraldehyde. For further reading see “Principles and Techniques of Electron Microscopy Biological Applications” M. A. Hayat.

Buffers

Are used to maintain the desired pH regardless of the chemical nature of the fixative selected. Most animal cells are fixed at a pH of 7.3 - 7.4. Certain highly hydrated tissues prefer a more alkaline pH (i.e. 8.0 - 8.4), while plant cells, nuclear material and the fibrils of mitotic spindles appear to favor the more acid pH (i.e. 6.8 - 7.2).

Tonicity

A solution is said to be isotonic with a cell if the cell neither swells nor shrinks when immersed in it. Hypertonic solutions cause shrinkage while hypotonic solutions induce swelling. The tonicity of a solution can be adjusted by adding electrolytes (e.g., sodium chloride) or a non-electrolyte (e.g. sucrose). Electrolytes are favored over non-electrolytes because the latter tend to decrease the rate of penetration and increase cellular extraction. Most fixatives are preferentially prepared slightly hypertonic.

Temperature

Fixation in this lab is at room temperature or for cells in culture at the temperature of the incubator. In some labs, fixation is initiated at a low temperature (e.g. 4°C) and allowed to slowly rise to room temperature. It is thought that reduced temperatures diminish “leaching” of cellular components and aid in preserving some enzymatic activity. At lowered temperatures, enzymatic activity is reduced, thus preventing catabolism. At room temperature, fixation occurs more rapidly and the cytoskeleton does not depolarize.

Size of sample

Often determines the quality of fixation. In general the size of the sample should be not exceed 1mm to assure good penetration and fixation.

Glutaraldehyde

Glutaraldehyde is a 5-carbon molecule with an aldehyde group at each end. It is a good general-purpose fixative. Since it is a strong fixative, it generally reduces antigenicity for immunolabelling. For immunolabelling formaldehyde is used with as much glutaraldehyde as can improve the ultrastructure and not reduce the antigenicity too far. A typical general fixative for good ultrastructure would be 2.5 – 3% glutaraldehyde in 0.1 M sodium cacodylate buffer. A typical immunolabeling fixative would be 4% paraformaldehyde + 0.1 -0.5% glutaraldehyde in phosphate buffer.

When made up, the shelf life for good ultrastructural studies is one week when kept in the fridge.

Using sealed vials of 25% glutaraldehyde

1. Take 50 mls 0.2M sodium cacodylate buffer
2. Add 10 mls 25% glutaraldehyde
3. Make up to 100 ml with ddw
4. Adjust pH

A formaldehyde - glutaraldehyde fixative of high osmolarity

A combination of formaldehyde - glutaraldehyde fixative has yielded excellent fixation of a variety of tissues. It is interesting that the osmolarity of this fixative is about 2010 milliosmols per kilogram. Despite the high osmolarity, shrinkage is minor except when perfused or applied to free-floating cells and minelayers. Tissue fluids in slabs of tissue probably dilute the fixative as it penetrates, reducing the osmolarity somewhat. Myelin figures are less common than with formaldehyde or glutaraldehyde alone, and all cell components are well preserved except lipid droplets, which are extracted. Microtubules are particularly well preserved. It is surmised that the formaldehyde penetrates faster than the glutaraldehyde and temporarily stabilizes structures, which are subsequently more permanently stabilized by glutaraldehyde.

1. Dissolve 2 g. of paraformaldehyde powder in 25 ml.-distilled water, heat to 60 – 70°C and stir.
2. Add 1 - 3 drops of 1N NaOH while stirring until solution clears.
3. Cool solution and add 5 ml. Of 50% glutaraldehyde.
4. Bring total volume to 50 ml. with 0.2M cacodylate buffer.
5. Add 25 mg. of CaCl₂ (anhydrous).
6. Final pH about 7.2.
7. Post fix
8. Dehydrate and embed

Osmium tetroxide

Stock solution is 2% osmium tetroxide in ddw

1. 1 part 2% OsO₄
2. 1 part 0.2M sodium cacodylate buffer
3. Makes 1% OsO₄ in 0.1M cacodylate buffer

Uranyl acetate

Used as enbloc stain for animal tissue and cells in culture. Adds considerable contrast if used enbloc.

- 2% aqueous UA
- Also used as a grid stain with lead citrate

Physical fixation

Physical fixation utilizes extremely low (-196° C.) temperatures, applied to very small samples, to provide “a frozen slice of life.” Freeze drying and/or freeze etching are two physical techniques commonly used to circumvent some of the adverse effects inherent in chemical fixation. The major attributes of these techniques are the rapidity with which they fix the sample and often their failure to “kill” the cells. Freeze-etch replicas have, in particular, been especially interesting for the three-dimensional images they produce and the new information gained on membrane fine structure.

Chemical - physical fixation

It is sometimes advisable to combine chemical and physical fixation for specific samples. Certain preparations are simply too awkward to handle in the brief time required to remove and physically fix the sample. A “one of a kind” sample may be lost forever if the non-chemically treated cell is inadvertently lost or damaged during physical processing. Others may require additional support to withstand collapse in the high vacuum of the instruments employed. Finally, a word of caution: as was previously stated, physical fixation does not always “kill” a cell. This phenomena assumes major importance in laboratories using live pathogens. It is highly recommended that all necessary precautions be employed to determine the viability of such

preparations immediately upon completion of physical fixation. For example, certain unfixed fungi are capable of withstanding the rigors of scanning electron microscopy with no apparent effect on their viability.