Chemical and Physical Fixation of Cells and Tissues

Fixation

- In order to keep the cellular components as "**lifelike**" as possible, it is essential that all biochemical and proteolytic processes are inactivated and structures are immobilized and locked in space by a "**fixation**" step.

Two approaches are normally used to “fix” biological samples:

- Chemical fixation methods
- Physical fixation methods.

Chemical fixation

- The most common approach used in specimen preservation.
- The tissues are immersed in a fixative that kills and stabilizes the cell contents.

Chemical Fixation for General Histological Studies

- Chemical fixation is the most common approach used in the fixation of biological specimens for light microscopy (LM) and electron microscopy (EM).
- Relatively large specimens can be fixed when compared to the physical methods.

Fixing agents

- Coagulants - Non-coagulants
- Additive – Nonadditive
- Acidic - Basic

Coagulant fixing agents

- Such as ethanol, methanol, and acetone
- They can cause protein denaturation.
- These fixing agents precipitate proteins by replacing water, resulting in conformation change of protein molecules and their solubility.

Non-coagulant fixing agents

- Such as aldehydes and osmium tetroxide (OsO_4)
- They react with proteins and other components, forming intermolecular and intramolecular cross-links resulting in a better retention of the cellular organization.

Solution is Solution???

- A buffer solution with additional compounds such as inorganic salts added to optimize fixation of targeted organelles.

Buffer

- Selection of a buffer is based on its ability to maintain a constant pH in the desired pH range during fixation.
- The buffer chosen has to be compatible with other components of the fixative and stains
- The fixative also needs to have a suitable osmotic concentration compatible with cells and tissues

The most commonly used fixatives for botanical specimens are:

- Ethanol
- Methanol
- Acetone
- Formaldehyde
- Glutaraldehyde
- Osmium tetroxide (OsO_4)

Ethanol and methanol

- Coagulant fixing agents.
- Denature proteins by replacing water in the tissue usually combined with other fixing agents as a fixative for LM.
- The alcohols, especially ethanol, are also used as dehydrating solvents for both LM and EM.

Ethanol and methanol

- It is important to note that lipids are not preserved by methanol and ethanol, and some lipids may be extracted during the course of fixation and later processing.

Acetone

- It denatures proteins and causes rapid precipitation of proteins and dehydration of tissues.
- It is also an effective lipid solvent but makes the tissue more brittle.
- Acetone is a highly volatile and flammable liquid and should be handled with care.

Acetone

- Acetone can also be used as a dehydrating solvent for the epoxy embedding method.
- Since it is miscible with epoxy resins, the use of a transitional fluid, that is, propylene oxide is not needed.

Acetic acid

- Non-coagulated fixing agent; however, it causes coagulation of nuclear proteins and indirectly stabilizes and helps to prevent the loss of nucleic acids.
- Acetic acid does not react with proteins.
- It is commonly used in conjunction with ethanol as a cytological fixative and aids in the preservation of nucleic acid.

Farmer’s fixative

- (ethanol:acetic acid; 3:1, v:v)
- Used for the preservation of the nucleus and cytological studies.

Formaldehyde

- The chemical properties and its reactions towards proteins and other macromolecules are numerous and complex.
- Its molecular weight is 30.
- The small size allows it to penetrate into the tissue quickly.

Formaldehyde

- The aldehyde group can react with protein nitrogen, primarily the basic amino acid lysine, to form methylene bridges.
- The initial binding to protein is relatively fast, but the formation of methylene bridges occurs slowly.
- Therefore, if formaldehyde is used alone, a longer fixation time is needed for proper fixation.

Glutaraldehyde

- Glutaraldehyde is a much more efficient cross-linker for proteins than formaldehyde.
- Its reactions with macromolecules are believed to be irreversible
- Inhibits enzyme activity more than formaldehyde.
- However, the rate of penetration (0.34 mm/h) is slower than formaldehyde.

Glutaraldehyde

- Similar to formaldehyde, self-polymerization occurs in solution.
- In order to ensure the quality of the stock solution, that is, having mainly the monomeric form, an “electron microscope grade” glutaraldehyde solution should be used.

Osmium tetroxide

- A fixing agent for light and electron microscope studies.
- It reacts with unsaturated lipids and results in the reduction of osmium during the cross-linking process with the formation of dark brown to black compounds.
- When used alone, not all proteins can be fixed and results in the loss of protein during fixation.

Osmium tetroxide

- The key drawback of this compound is that it penetrates tissue very slowly (0.25 mm/h)
- Therefore only small pieces of tissues can be fixed.
- As a result, this compound is seldom used as a fixing agent at the LM level
- Now mainly used as a secondary fixing agent for postfixation after aldehyde in an ultrastructural study.

Osmium tetroxide

- Since OsO_4 is electron dense, it also serves as a “stain”, imparting contrast when viewed with an electron microscope.
- Osmium tetroxide is usually sold as a crystalline solid in a sealed glass ampule.

Osmium tetroxide

- It is well known that OsO_4 crystals sublimate into vapor from a solid state.
- Exceptional caution is recommended when working with this fixative because unexpected exposure to OsO_4 vapor can lead to blindness and other ailments.

Formalin–acetic acid–alcohol (FAA)

- The most common botanical fixative for the paraffin-embedded method.
- The fixative penetrates tissue quickly, and the shrinkage effect of ethanol is counterbalanced by the swelling effect of acetic acid.

Common Buffers and Their Properties

- Phosphate
- Cacodylate
- PIPES
- HEPES

Phosphate buffer

- The most common buffer used for LM and EM, as it is nontoxic and has a stable buffer capacity at the physiological pH range.
- It is relatively inexpensive to make, and once prepared, it is stable for several weeks at 4°C.
- The disadvantage of a phosphate buffer is that it can form precipitates in the presence of calcium and other metallic ions, and it can be contaminated with microorganisms during storage.

Sodium cacodylate

- This buffer is easy to prepare and can be stored for a long time without contamination, as arsenic can serve as a preservative.
- No precipitation occurs in the presence of low concentrations of calcium and magnesium salts.
- It is mainly used for EM when phosphate buffer cannot be used and for cytochemical staining protocols.
- The main disadvantage of this buffer is that it may cause a redistribution of cellular material along osmotic gradients, and arsenic is toxic.

Good's buffers: PIPES and HEPES

- excellent for physiological pHs.
- nontoxic to cells and are compatible with divalent cations.
- They appear to resist chemical extraction of cell components.
- PIPES buffer is often used as the buffer for the fixation of cytoskeletal elements.
- Good's buffers are relatively more expensive to prepare.

Laboratory Safety

- Almost all fixing agents, certain buffers and histochemical-related compounds are toxic and require proper handling and storage.

Laboratory Safety

- As a general rule, fixing agents, fixatives, and tissue samples should be stored in their own refrigerator
- It is advisable to conduct a majority of fixation and processing operations inside a **fume hood**. The handling of liquid gases and other cryogenic chemicals for Chemical Fixation requires extreme care.