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Technical Overview

Cross-linking fixatives:
What they are, what they do, and why we use them

Focus on: Formaldehyde, Glutaraldehyde, and Osmium tetroxide

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EM processing and imaging demands better tissue fixation

Light Microscopy

- Visible light
- No vacuum (ambient pressure)

- Live cells/tissue can be imaged.

- Samples can contain water.
- Biological tissue has sufficient contrast.

- Formaldehyde fixation is often sufficient to preserve the tissue/cell.

Electron Microscopy

- Electron beam (high energy)
- High vacuum

- Samples must be embedded into plastic.
- Samples must be dehydrated.
- Biological tissue is not electron-opaque enough. → need heavy metal stains
- Tissue must be protected against subsequent EM processing and imaging.

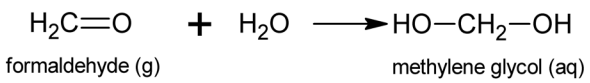
Commonly used chemical fixatives for microscopy

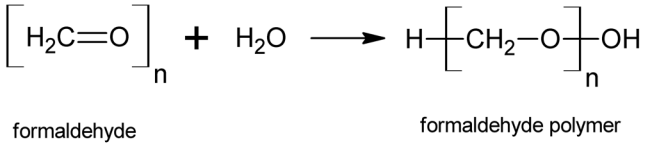
- Cross-linking (additive) fixatives: e.g., formaldehyde, glutaraldehyde, acrolein, osmium tetroxide
  - Fixative molecules form cross-linkage with their targets.
  - Formaldehyde-glutaraldehyde mixture is the most commonly used primary fixative for EM (introduced by Karnovsky in 1965).

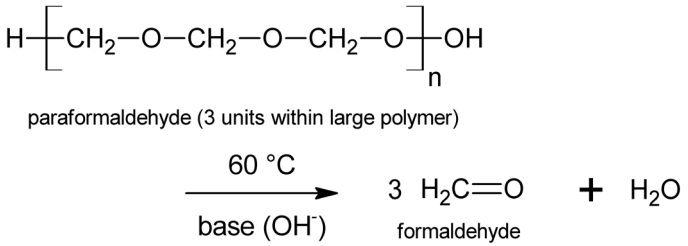
- Coagulants: e.g., EtOH, MeOH
  - Coagulate and/or precipitate proteins
  - Do not fix carbohydrates and lipids
  - LM only

- Acids: e.g., acetic acid, picric acid
  - precipitate proteins
  - Do not fix carbohydrates and lipids
  - LM only

Formaldehyde
(or is it *para*formaldehyde? What is formalin, anyway?)



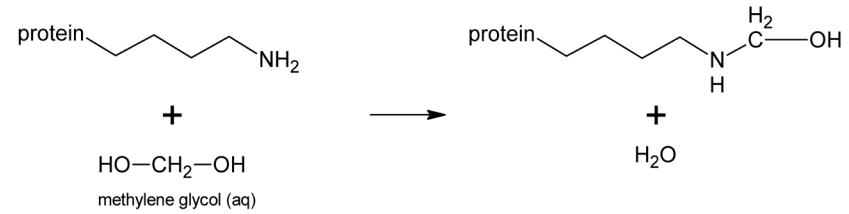




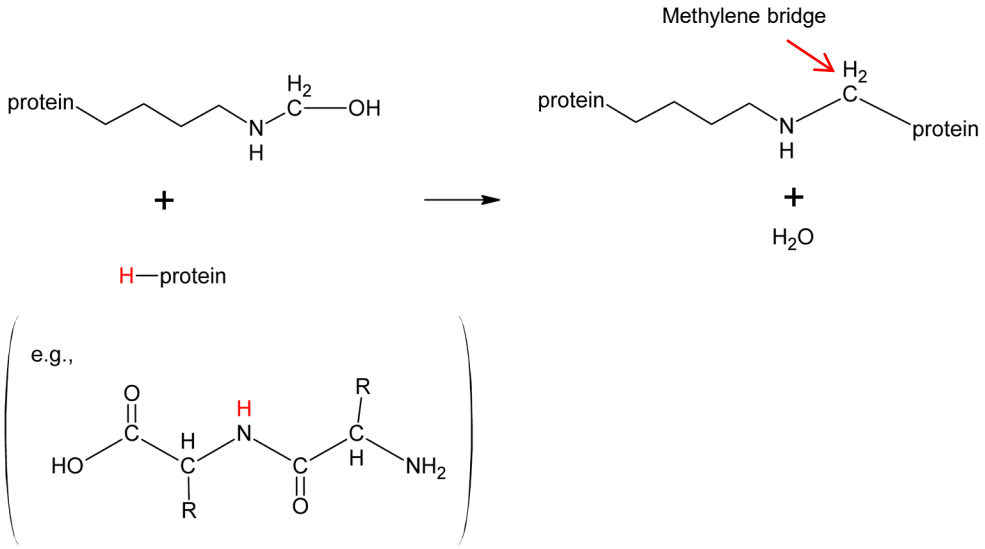
- **Formaldehyde** = water soluble gas
  - When dissolved, it forms methylene hydrate
  - Methylene hydrate molecules can react with one another to form polymers
  - Small molecule = rapid penetration into tissue
- **Formalin** = 37-40% formaldehyde (aq) with methanol (up to 15%), which prevents polymerization
- **Paraformaldehyde** = higher polymers (n = up to 100) of formaldehyde
  - insoluble in water
  - requires heat and high pH to de-polymerize in water.

Formaldehyde reacts primarily with proteins

Addition of formaldehyde (methylene glycol) to a lysine side-chain (fast)

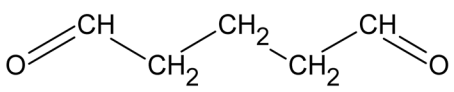


Formation of methylene bridge with a neighboring nitrogen atom (slow)

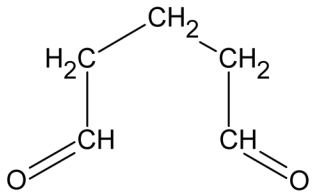


- The aldehyde group can react with nitrogen and some other atoms of proteins.
- Methylene bridge (-CH_2-) is formed between two reactive atoms in proteins that are very close together.
- Other molecules (carbohydrates, lipids, nucleic acids) are thought to be trapped in a matrix of cross-linked proteins.

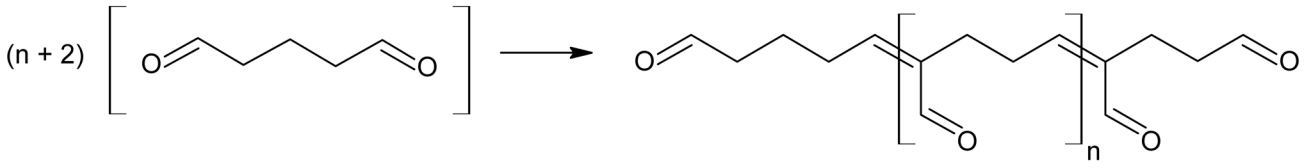
Glutaraldehyde



glutaraldehyde

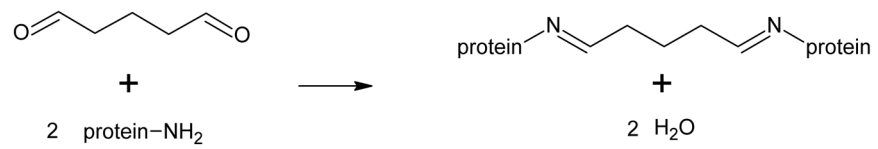


- First introduced by Sabatini et al. in 1963 as a primary fixative for EM
- Two aldehyde groups per molecule, with a longer, flexible hyrocarbon chain
  - More efficient cross-linking
  - Small enough to penetrate tissue (slower than formaldehyde)
- Present in aqueous solutions as monomers and polymers of variable size

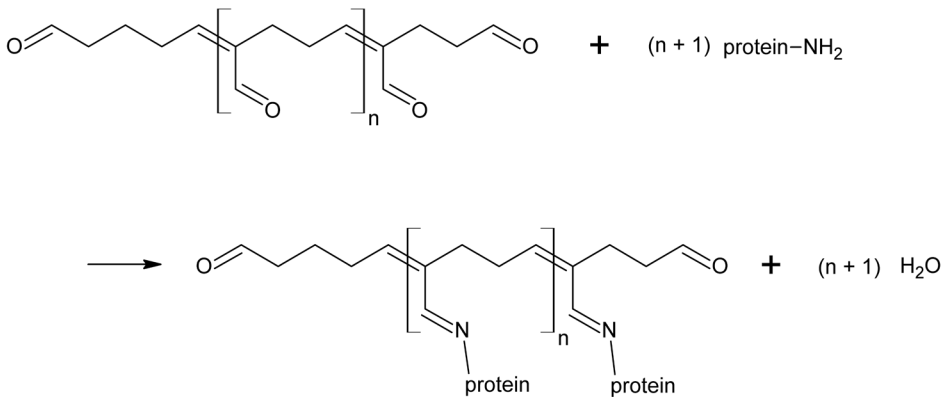


Glutaraldehyde readily cross-links proteins

Cross-linking of proteins with glutaraldehyde monomer



Cross-linking of proteins with glutaraldehyde polymer

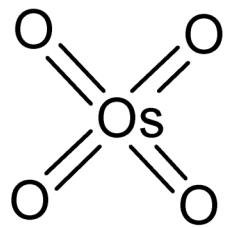


- Both aldehyde groups of a single glutaraldehyde molecule react with proteins to form cross-linkage.
- Glutaraldehyde can also react with phospholipids containing free amino groups (e.g., phosphatidylserine, phosphatidylethanolamine).
- Glutaraldehyde introduces free aldehyde groups to the fixed tissue
  - Can pose problems for immunolabeling

Osmium tetroxide
(the multi-tasker)

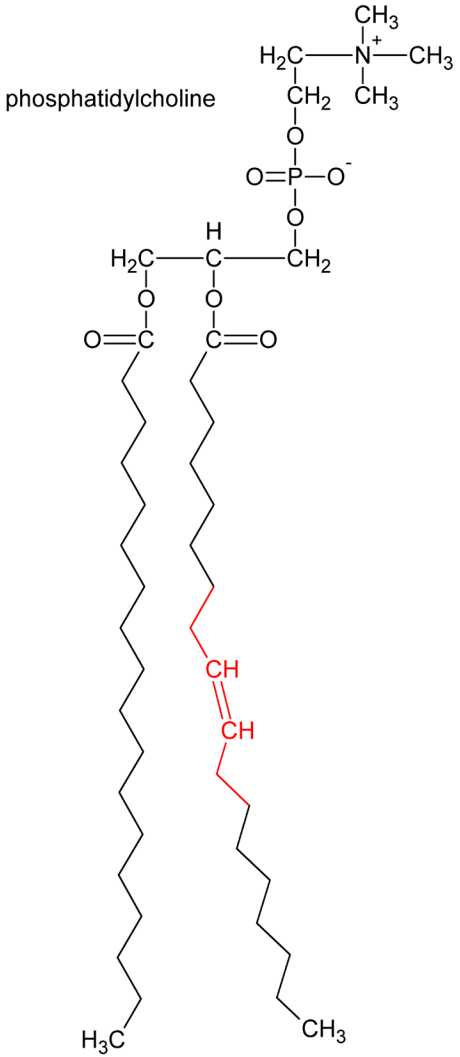
- Introduced for EM in 1940s (Claude & Fullam, 1945; Porter et al., 1945)

- Os can exist in nine oxidative states, five of which are reasonably stable.
  - Many potential chemical reaction pathways with many substrates
- OsO_4 is soluble in both polar (aqueous) and non-polar media.
  - Penetrate into, and react with, hydrophobic regions of tissue/cell (e.g., membrane phospholipids)
  - Water solubility: ~ 7% at RT
- Os is electron opaque.
  - Works as a stain, as well as a fixative
- OsO_4 also acts as a mordant.
  - Enhancement of staining with other heavy metals (e.g., Pb)



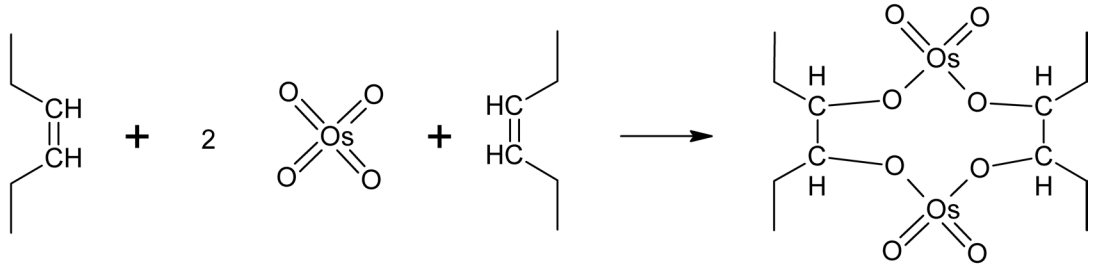
- Slow penetration into tissue
  - Limit tissue section thickness to < 100μm
- Highly toxic

OsO_4 reacts primarily with unsaturated lipids



- OsO_4 reacts with C=C in unsaturated fatty acid chains of phospholipids
  - Reduction of Os during cross-linking reaction produces dark brown color in the processed tissue.
- OsO_4 can also react with some proteins and lipoprotein complexes
  - But does not cross-link proteins

Cross-linking of unsaturated fatty acid chains with OsO_4



Ideal vs. practice of chemical fixation

- Ideally, a good fixation method should preserve the cell/tissue as a whole.
- In practice, a chemical fixative is usually selective (e.g., aldehydes → proteins).
  - Choose a fixative for molecule/structure of your interest, OR,
  - Use a combination of fixatives, OR,
  - Use a physical fixation method (i.e., rapid freezing)

- Ideally, a good fixation method should preserve the cell structure with minimum change from the living state (volume, morphology, localization of macromolecules and organelles, etc.).
- In practice, fixation and tissue processing usually induce artifacts...
  - Minimize avoidable ones (e.g., swollen mitochondria)
  - Interpret the tissue structure in the context of the fixation and processing

References and Notes

- Claude A & Fullam EF (1945) An electron microscope study of isolated mitochondria: method and preliminary results. J Exp Med 81:51-62. https://doi.org/10.1084/jem.81.1.51
- Porter KR et al. (1945) A study of tissue culture cells by electron microscopy: methods and preliminary observations. J Exp Med 83:233-246. https://doi.org/10.1084/jem.81.3.233
- Karnovsky MJ (1965) A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J Cell Biol 27:137A-138A. https://www.jstor.org/stable/1604673
- Sabatini DD et al. (1963) Cytochemistry and electron microscopy. J Cell Biol 17:19-58. https://doi.org/10.1083/jcb.17.1.19

- Much of the information presented here can be found in the following books:
  - Hayat MA (1981) Fixation for Electron Microscopy. Academic Press.
  - Bozzola JJ & Russell LD (1992) Electron Microscopy: Principles and techniques for biologists. Jones and Bartlett Publishers.
- This technical overview was originally created by Masaaki Kuwajima in 2011 as a short summary presentation for the Harris lab at The University of Texas at Austin.
- This technical overview is available online at SynapseWeb (http://synapses.clm.utexas.edu/) and Harris Lab wiki (https://tinyurl.com/harrislabwiki), maintained by Kristen M. Harris (Principal Investigator)
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