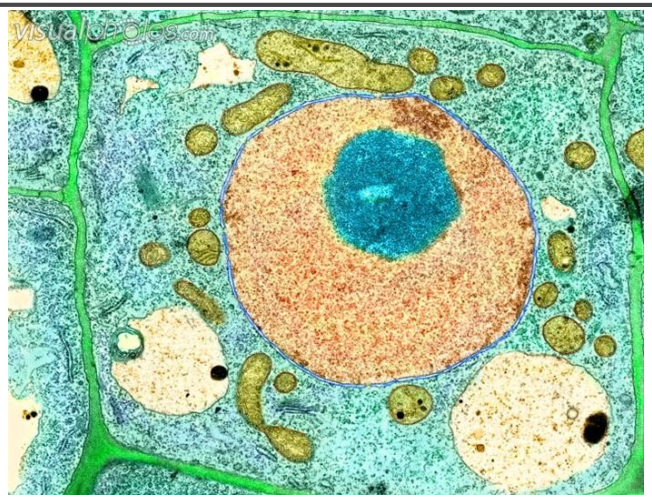
BIOLOGICAL SAMPLE PREPARATION FOR TEM OBSERVATION



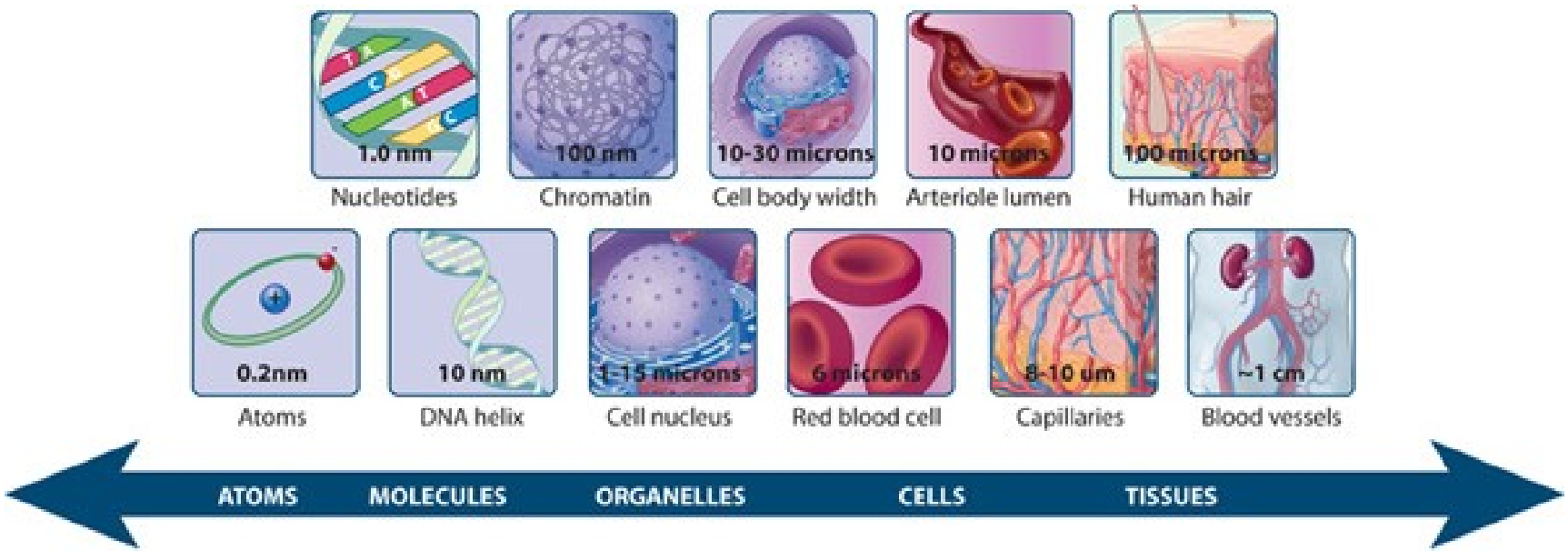
TEM Seminar
Nov 16, 2017
Astari Dwiranti, Ph.D

UNIVERSITAS INDONESIA

DIREKTORAT
RISET &
PENGABDIAN
MASYARAKAT

Laboratorium
[TEM] TRANSMISSION ELECTRON MICROSCOPY
UNIVERSITAS INDONESIA

Why do we need EM for biological samples?



(O'Connor and Adams, 2010)

Why do we need EM for biological samples?

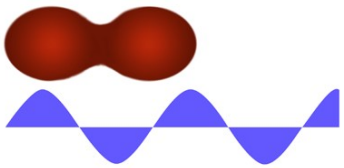
High Resolution

High magnification

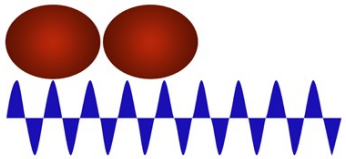
High Depth-of-field

Inner structure investigation

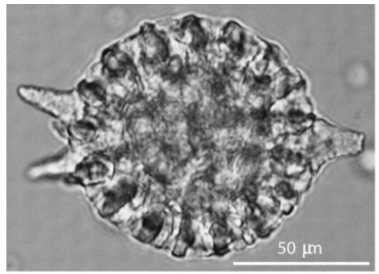
3D construction



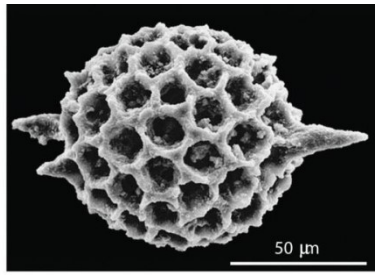
light



electron

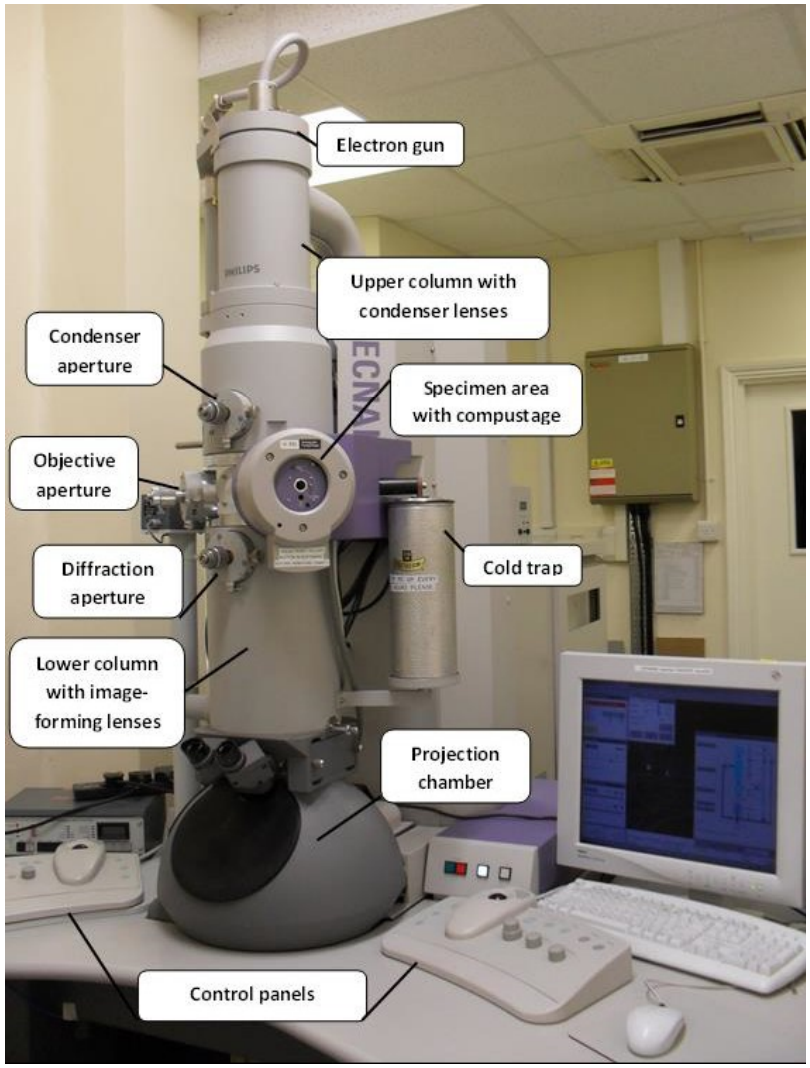


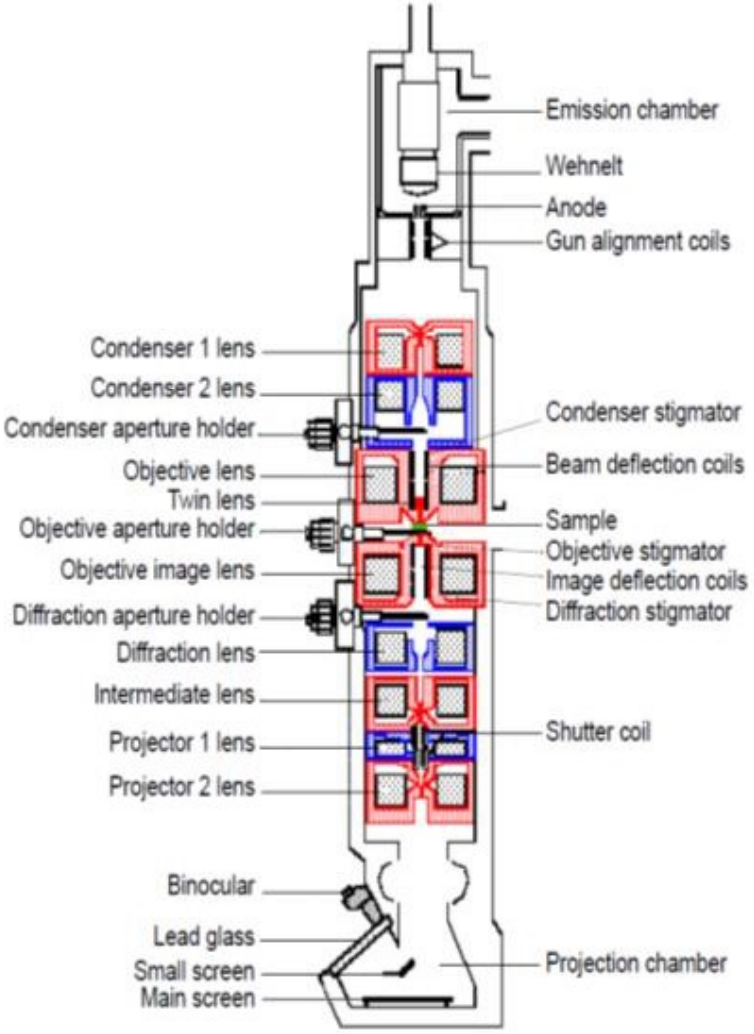
(a) Radiolarian under light microscope



(b) Radiolarian under electron microscope

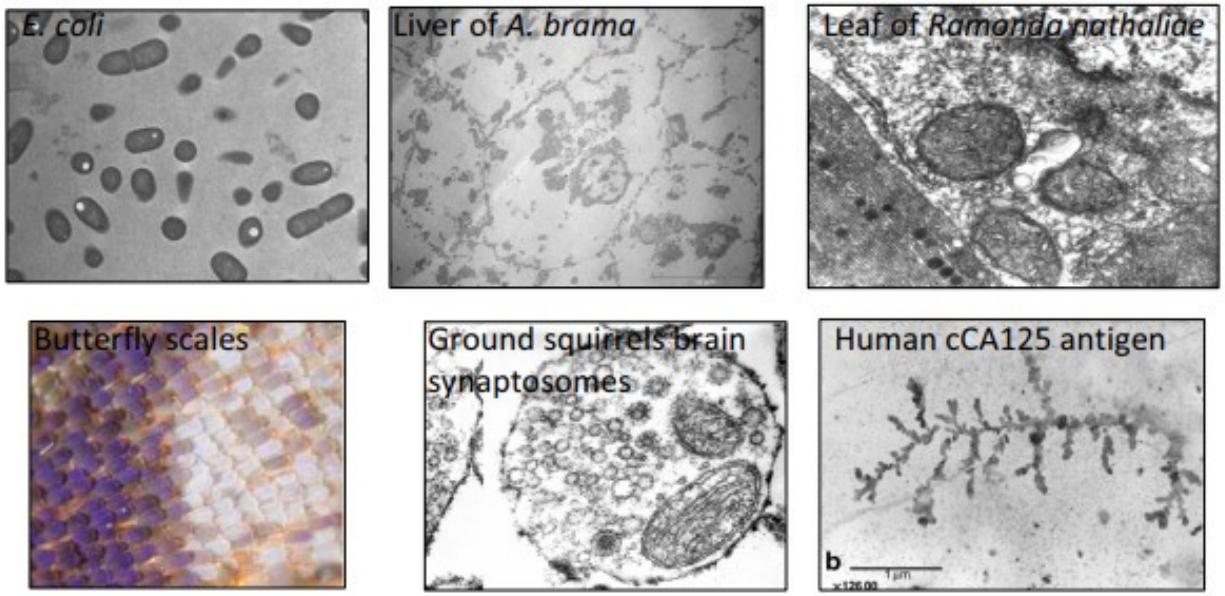
Seeing with electrons!



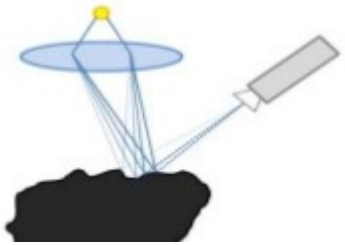


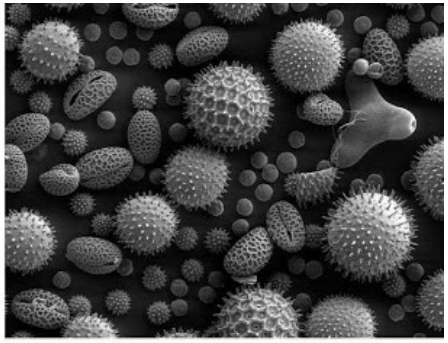
Biological samples types

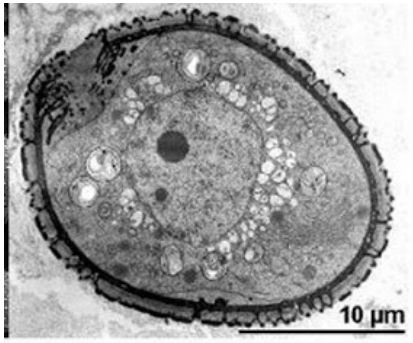
- Animal tissues (ex. Liver, spleen, bone, etc)
- Cell culture
- Bacteria, biofilms
- Cellular organelles
- Plant tissues (ex. Seeds, pollen, leaves, etc).



SEM vs TEM





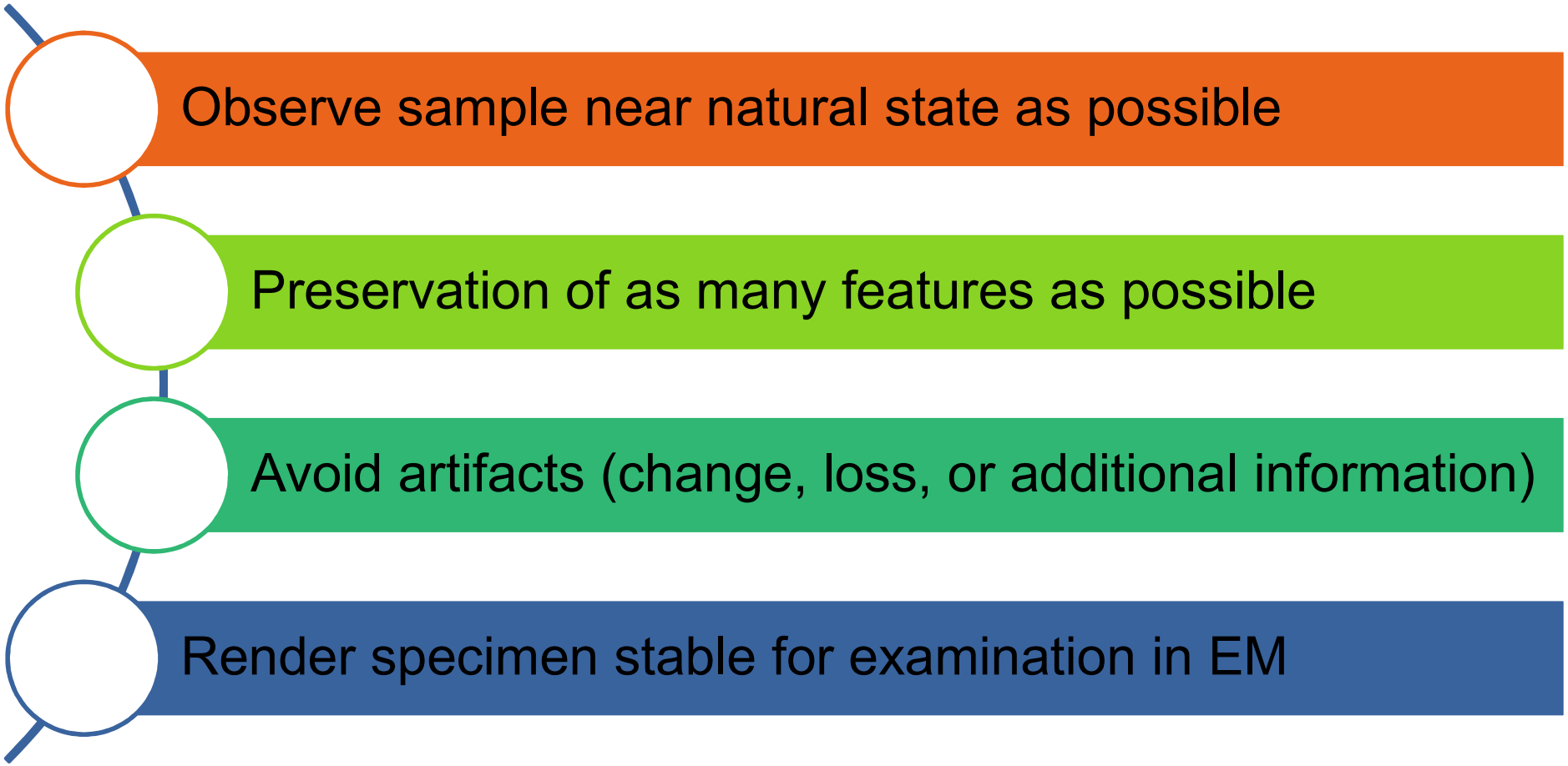


	SEM	TEM
Imaging	Beam formation	Direct imaging
Accelerating voltage	50-30,000 volts	~120,000 volts
Specimen	Need not be thin	thin
Image	3D	2D
Magnification	~2 million	~50 million
Resolution	~ 0.4 nm	~ 0.5 Å

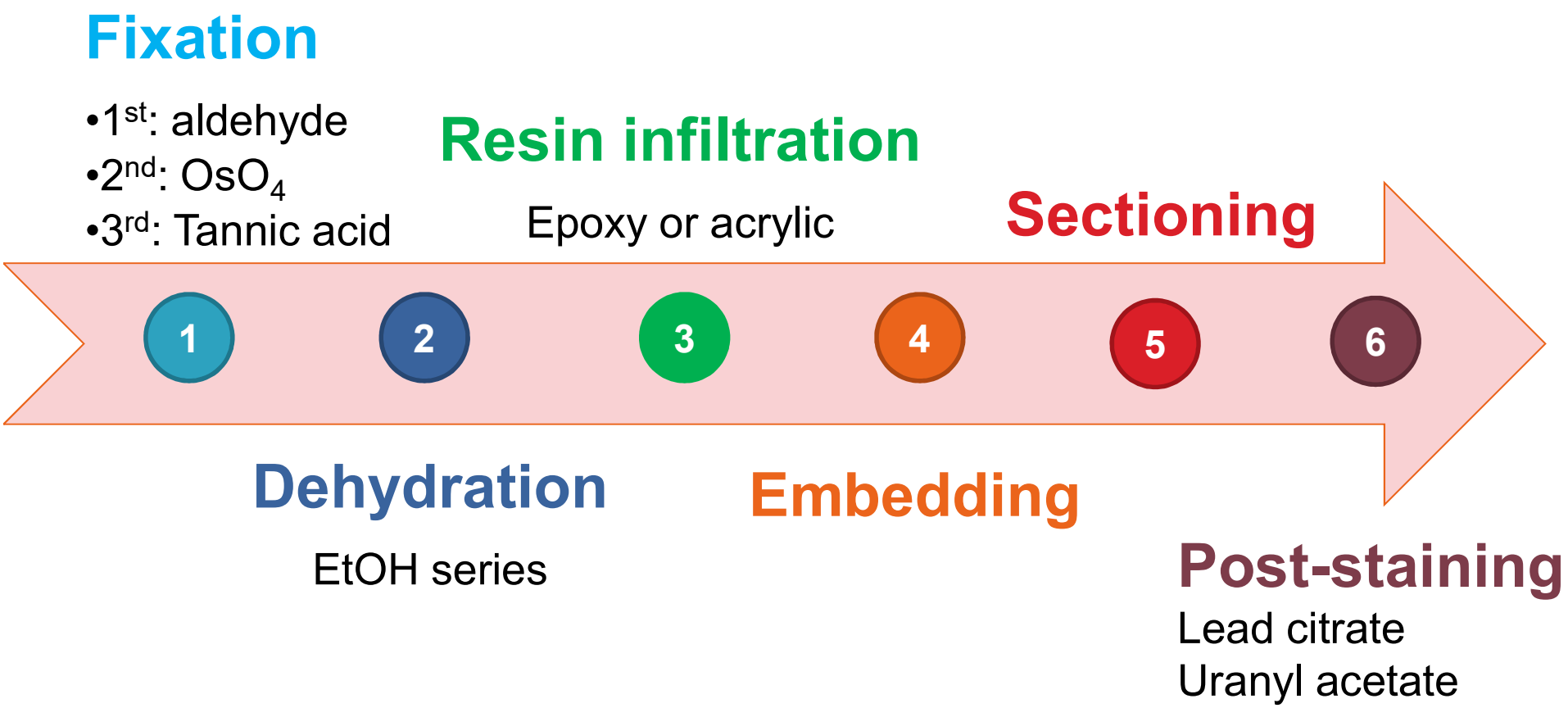
Biological samples

- Non-electronically conductive
- Contains water
- Soft and can easily be destroyed
- Various size of specimen.

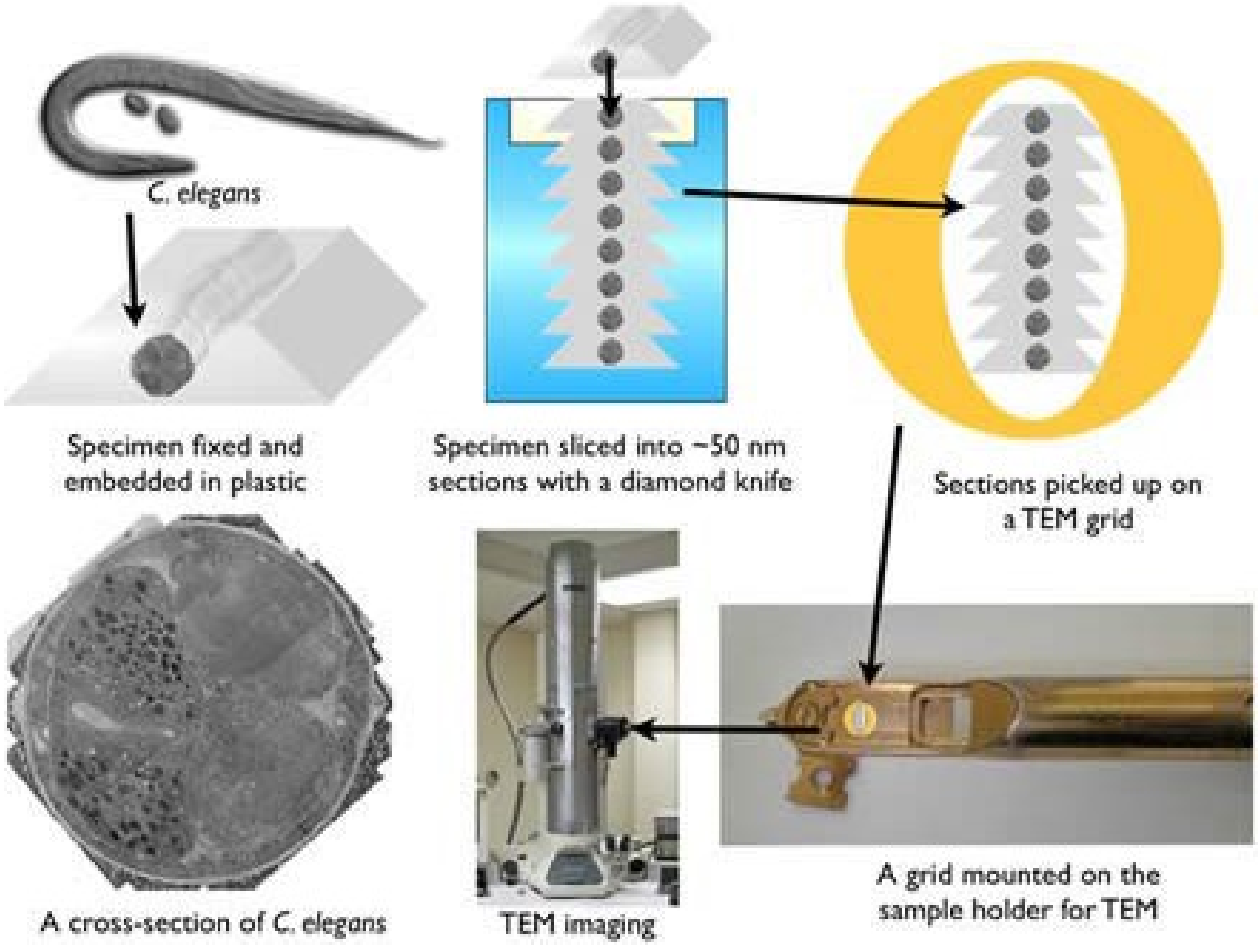
Goals of sample preparation



Conventional TEM sample preparation



Sample preparation for TEM



http://www.research.utah.edu/advanced-microscopy/education/electron-micro/index.html

Fixation

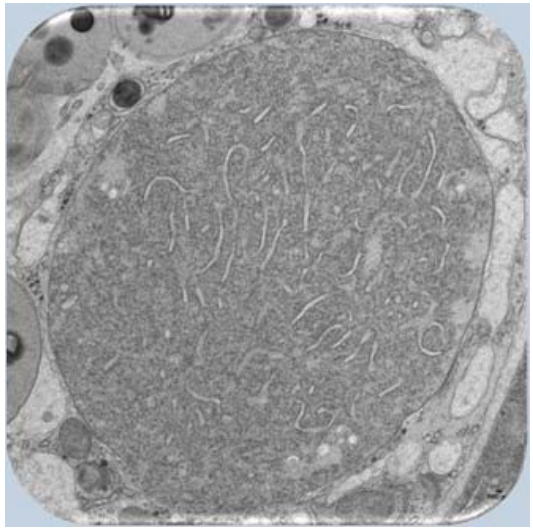
- preserve the sample structure in a state that most closely resembles the structure of the original living state (minimum alteration)
- protect and stabilize sample structure from changes during subsequent treatments and irradiation.

Characteristics of a good fixative:

- Permeates cells readily and acts quickly
- Is irreversible
- Does not cause artifacts

Methods of fixation:

- Chemical fixation
- Freeze-fixation



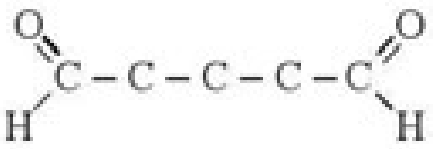
Fixation

Standard TEM fix: 2.5% GA + (2~4) % PFA, 30 min ~ overnight

Glutaral dehyde

Irreversible cross-linking of proteins

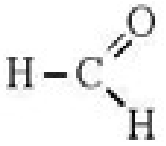
Glutaraldehyde (glut)



PFA

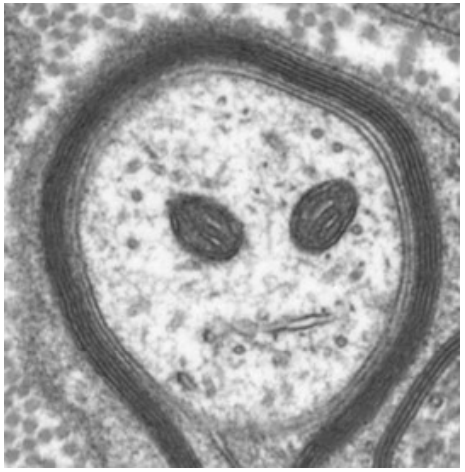
Reversible cross-linking, small molecule, penetrates quicker

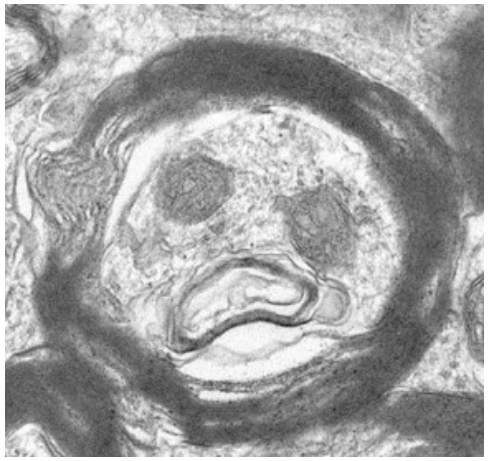
Paraformaldehyde (para)



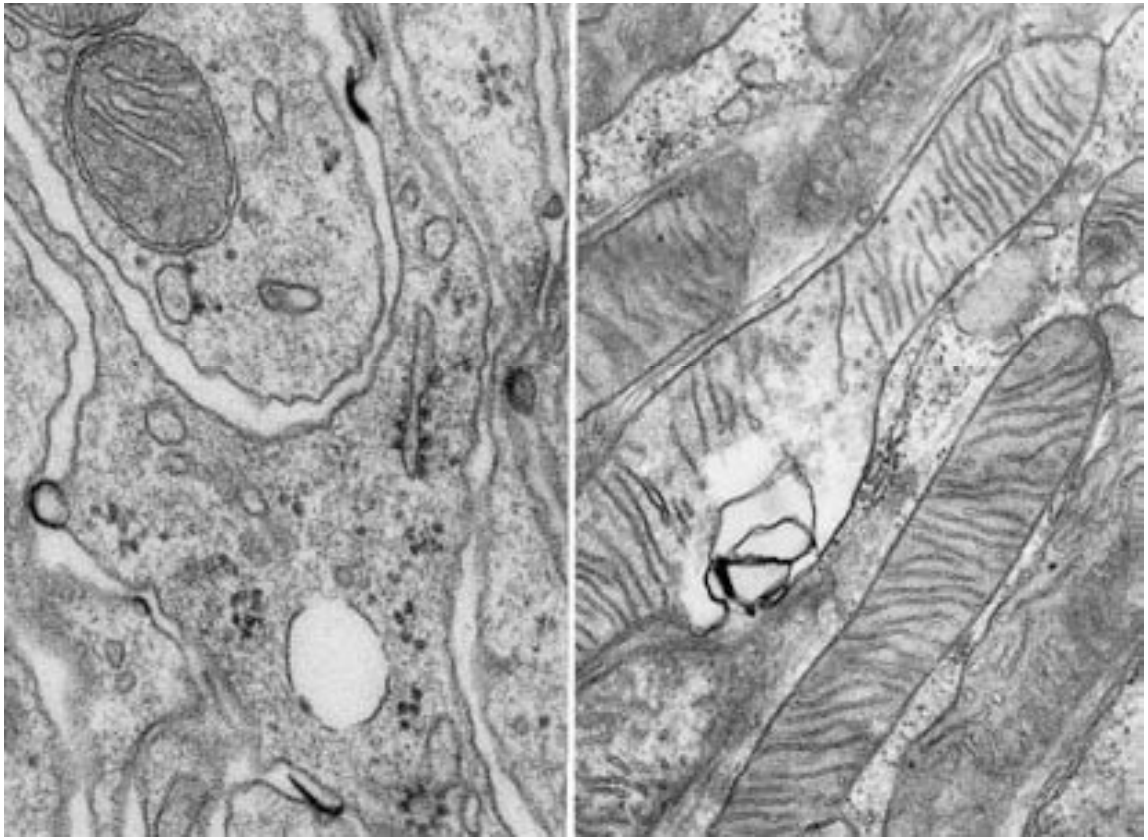
Consideration of chemical fixation

- pH (isoelectric point)
- Total ionic strength of reagents
- Osmolarity
- Temperature
- Length of fixation
- Method of application of fixative.





Artefacts



Over-fixing

Muscle tissues fixed for 3 days at RT in 2.5% glutaraldehyde

(Knott, 2009)

Freeze fixation

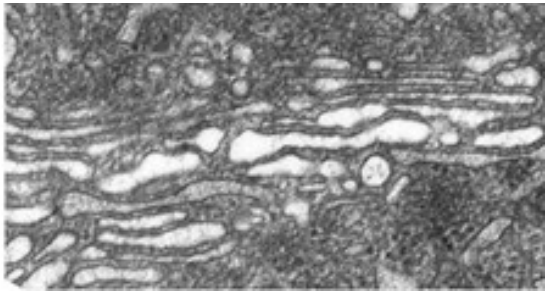
Reasons:

- Halt rapid events
- Structures are fixative sensitive
- Removal of water changes morphology.

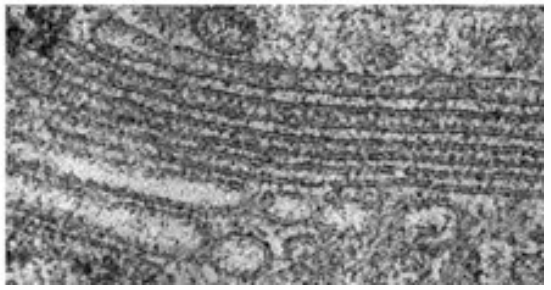
Disadvantages:

- Specialized equipment required
- Freeze damage
- Limited view of specimen
- Hazards.

Chemical Fixation



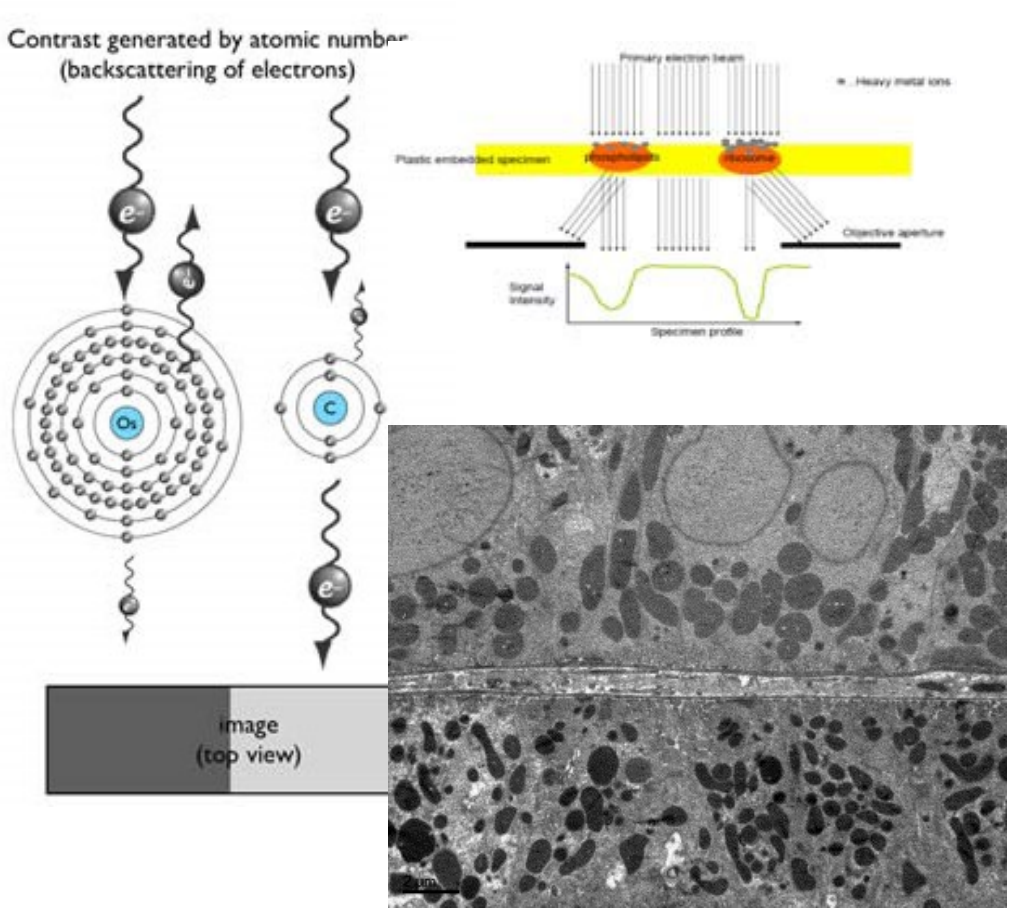
Plunge-Freeze/ Freeze-Sub.

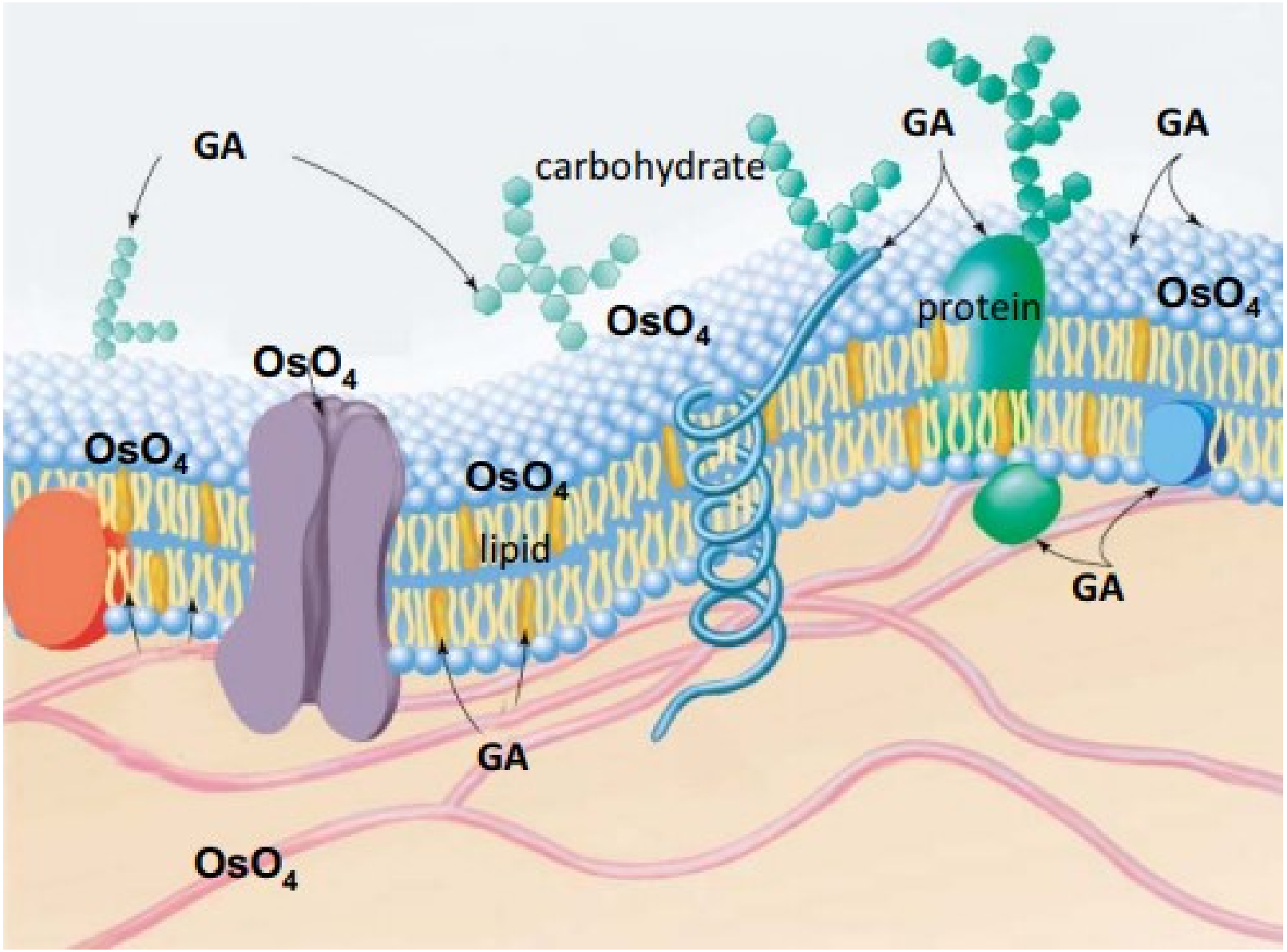


(Knott, 2009)

Secondary fixation

- Osmium tetroxide is a heavy metal that fixed unsaturated lipids.
- Used as both a secondary fixative and an electron stain
- Significantly improves specimen preservation and contrast.




GA
carbohydrate
GA
GA
protein
OsO_4
OsO_4
OsO_4
OsO_4
OsO_4
lipid
GA
GA
OsO_4

Dehydration

- The process of gradually replacing water in the sample with a solvent (acetone or ethanol).
- The solvent is then gradually replaced with resin.

35 % EtOH

50 % EtOH

70 % EtOH

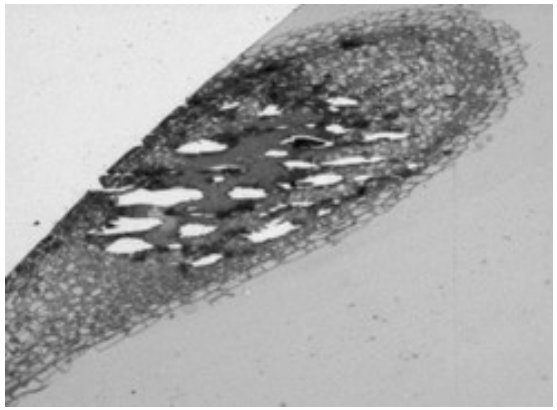
95 % EtOH

100 % EtOH

Propylene Oxide

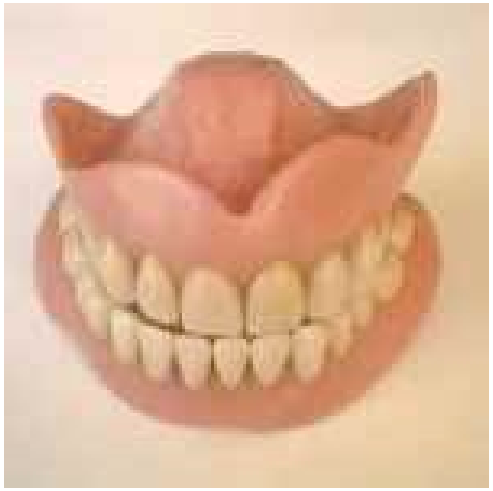
1:1 Propylene Oxide : Epon resin

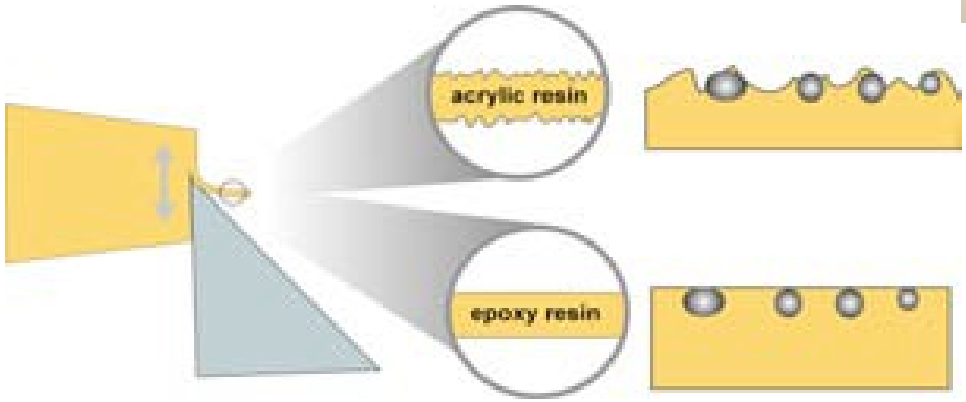
The dehydrating times should be adjusted to size and kind of tissue.



Embedding

- Epoxy resin – araldite
- Acrylic resin – methyl methycrylate.

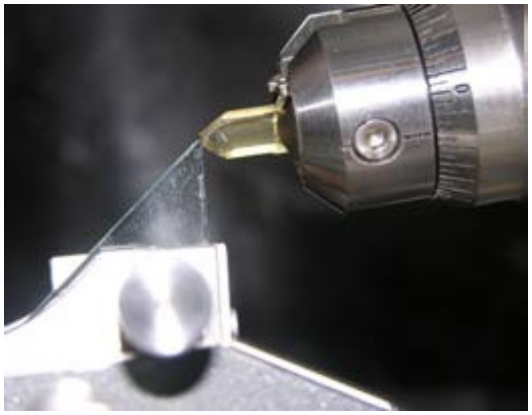


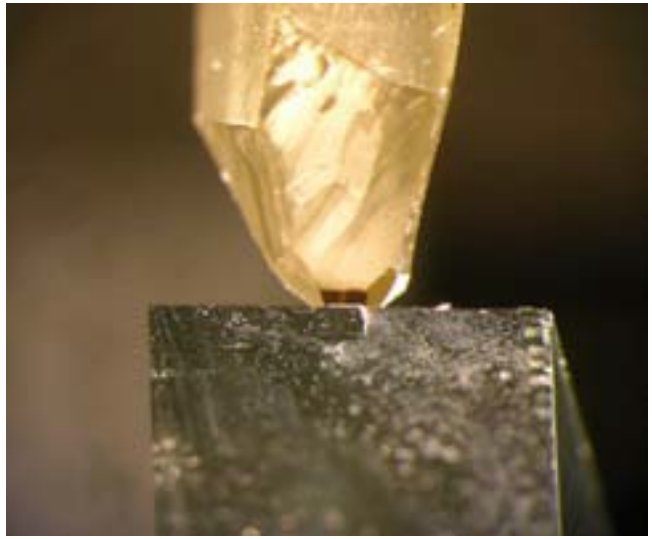


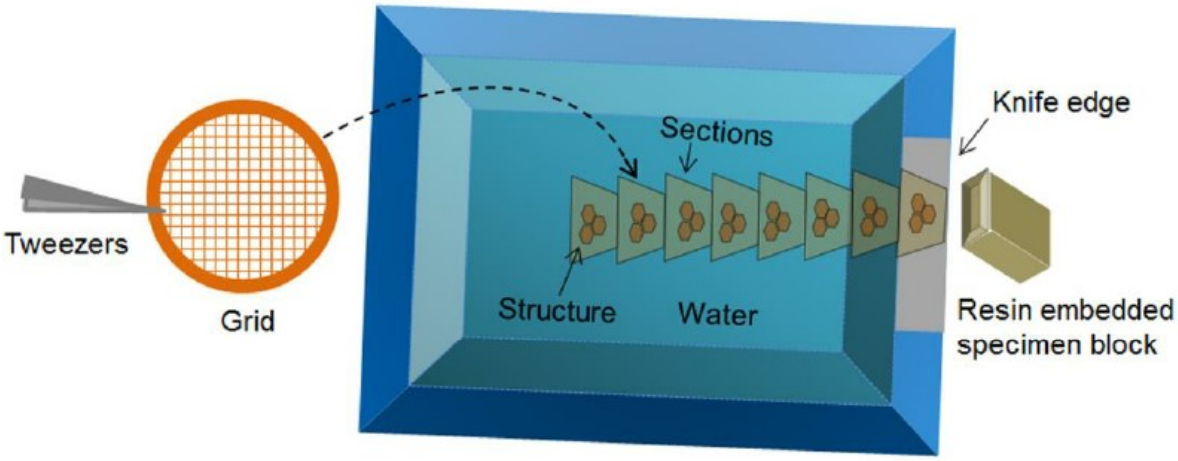
Embedding with Epon (1-3 hours)
The ‘block’ are cured for 48 hours in a 60 degree oven.

Sectioning

Block trimming with a glass knife



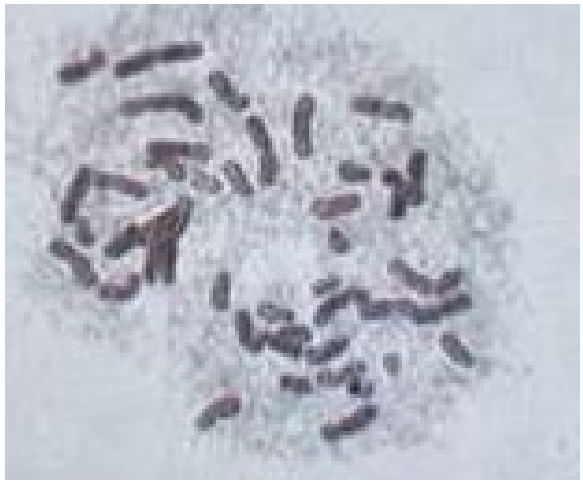




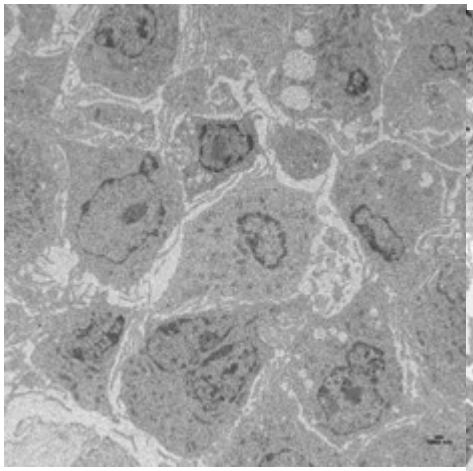
- Sample is cut with ultramicrotome
- Thin sections (70nm)
- Place on grids.

Post staining

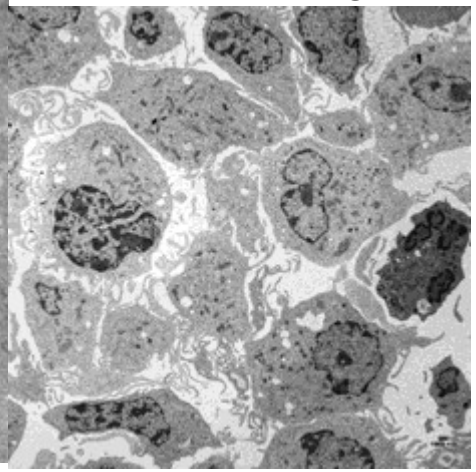
- Increases the image contrast
- Lead citrate (stains polysaccharides, eg. glycogen)
- Uranyl acetate (basic stain eg DNA).

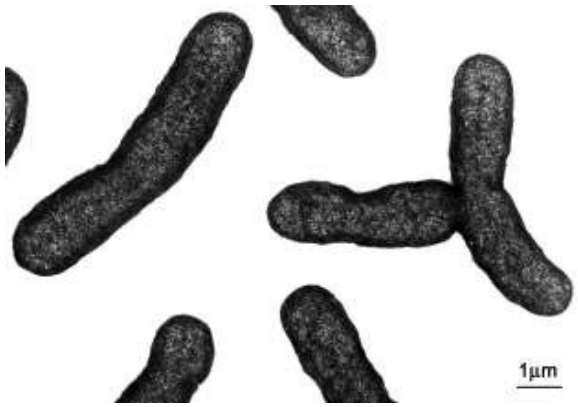


No post-staining



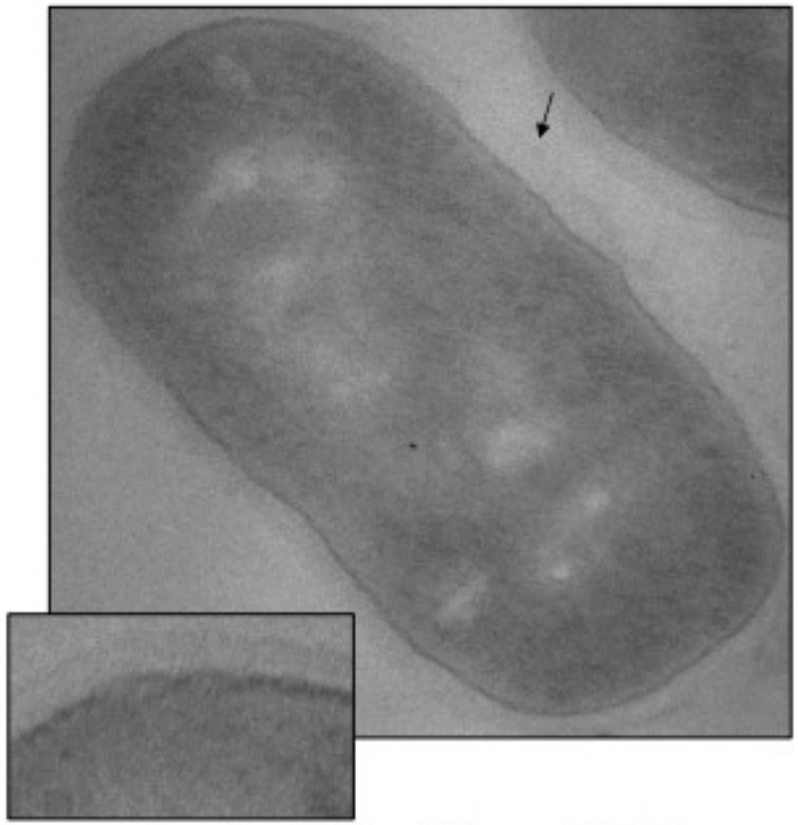
Post-staining



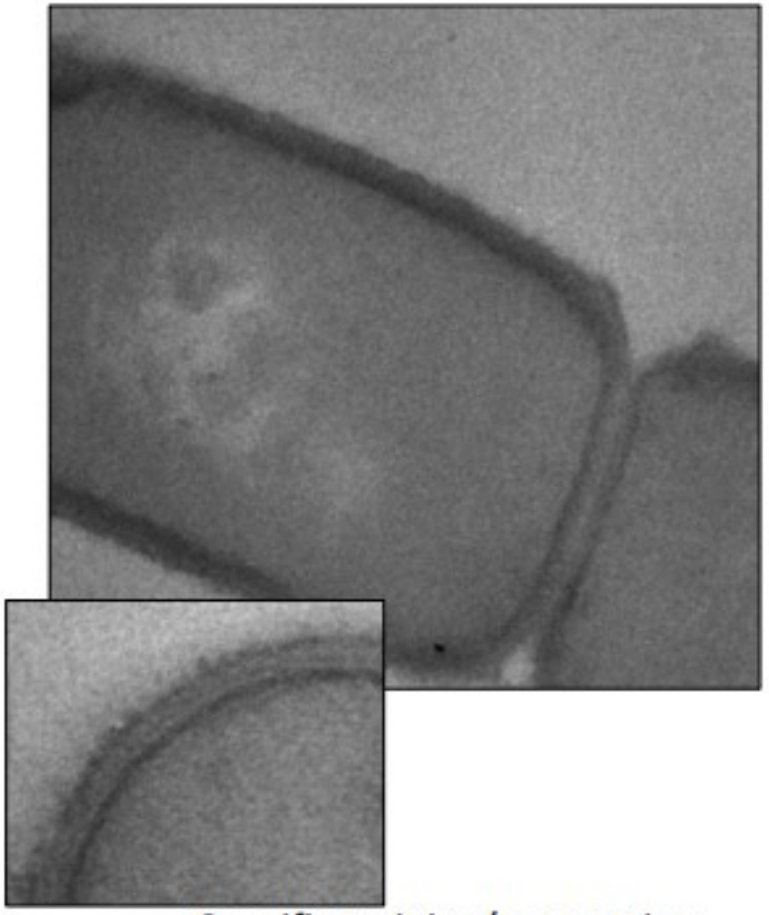


(Oguro and Ghazizadeh, 2015)

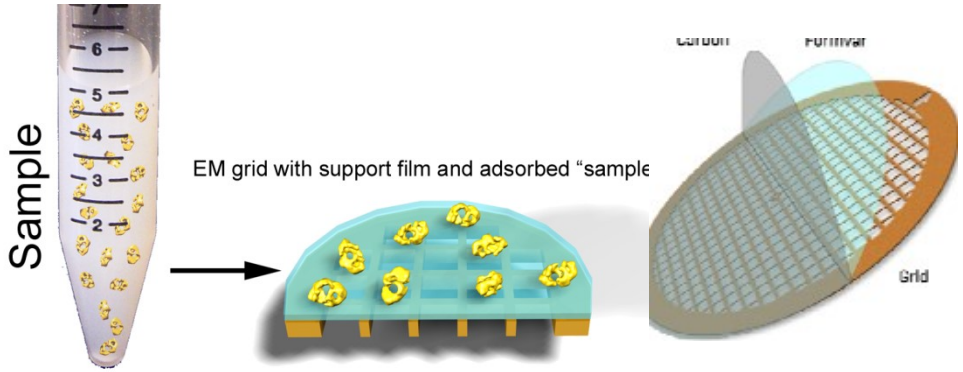
Staining enhances the image contrast



Routine TEM staining x88.000

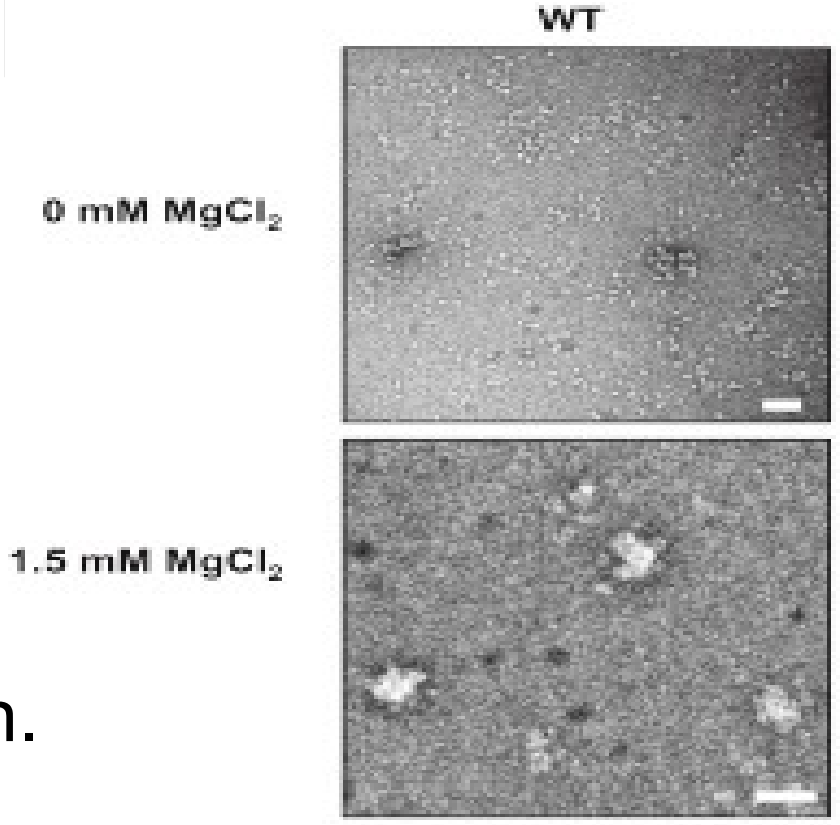


Specific staining/contrasting



Very small samples do not need embedding/sectioning

- Coat grids with plastic film and carbon.
- Apply the particulate specimens (eg. Protein, viruses, DNA).
- Stain with heavy metal soln. (eg. Uranyl acetate).



(Equilibrina, et al. 2015)

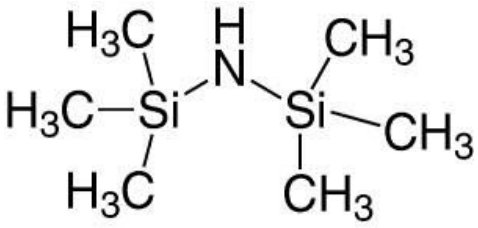
The procedure for every biological sample may be different

(need to be adjusted

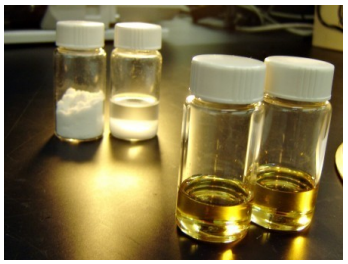
to obtain the best results).

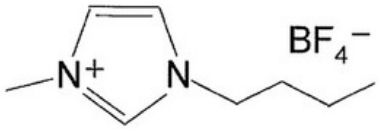
- Other methods:

- Hexamethyldisilazane (HMDS)



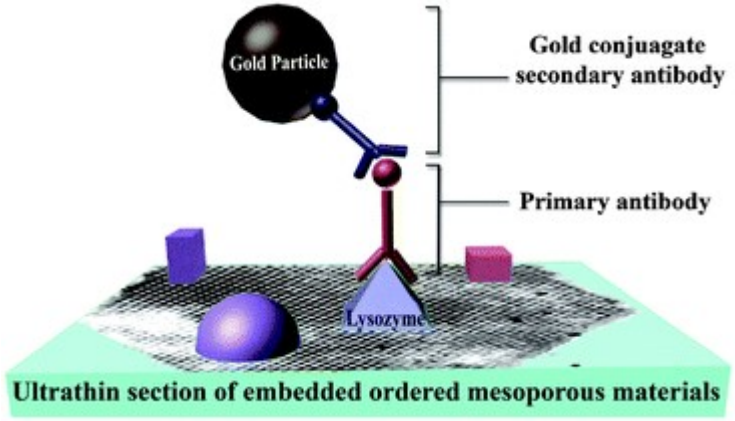
Ionic Liquids

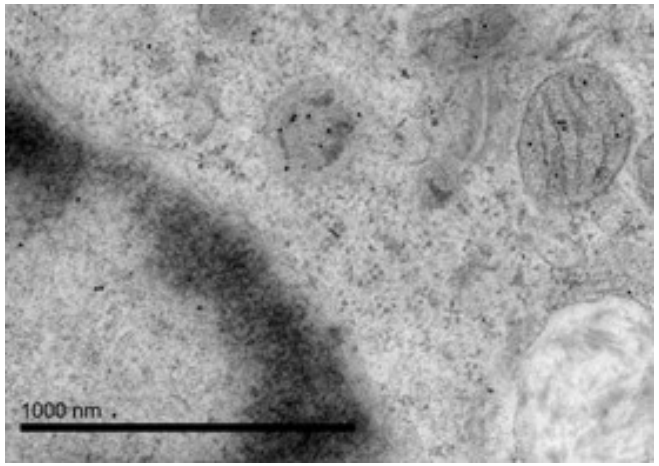




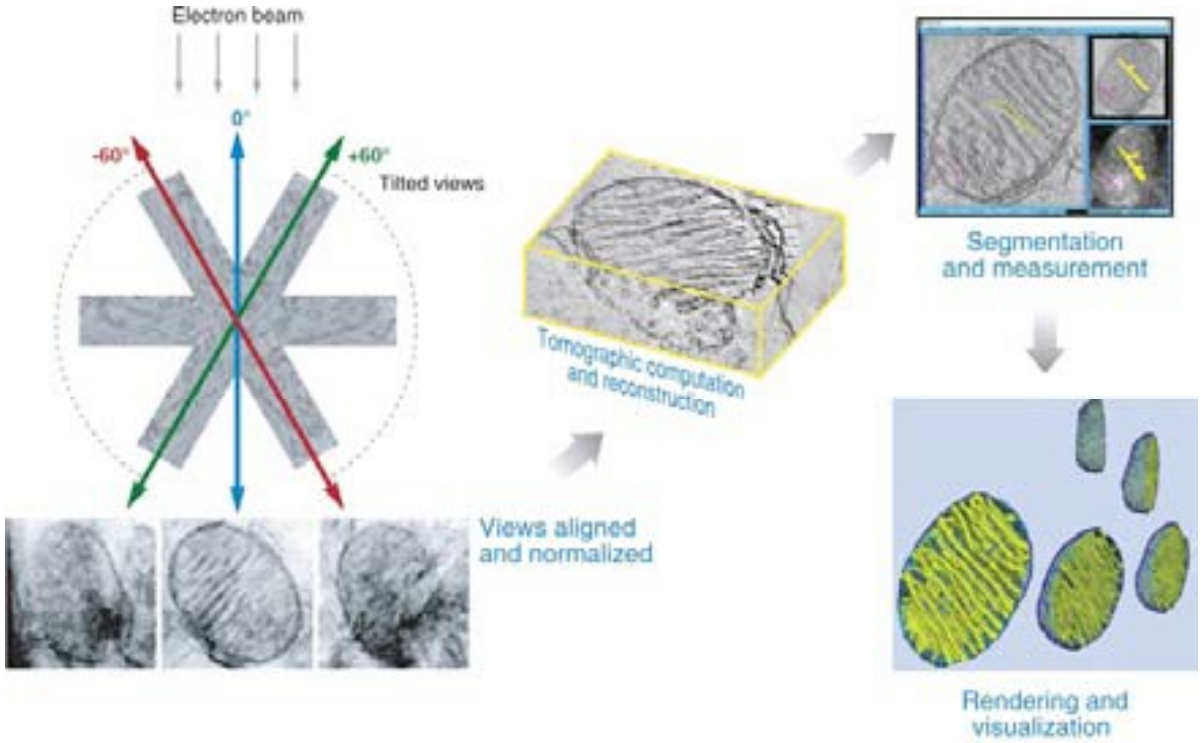
Protein localization (Immunogold labelling)

- Secondary antibody is conjugated to a colloidal gold particle.
- For cells and tissue, post-embedding labelling is usually the best option.
- The osmium tetroxide step is omitted.
- Acrylic resins are used instead of epoxy resin.

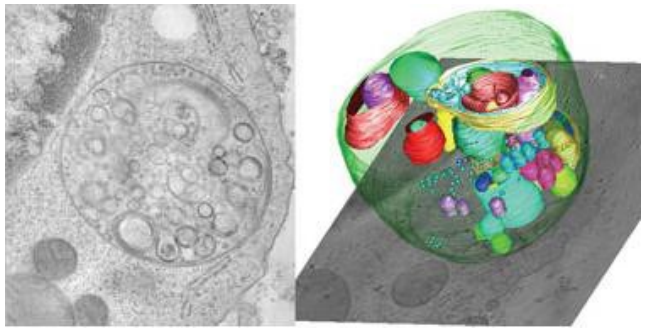




3D Tomography



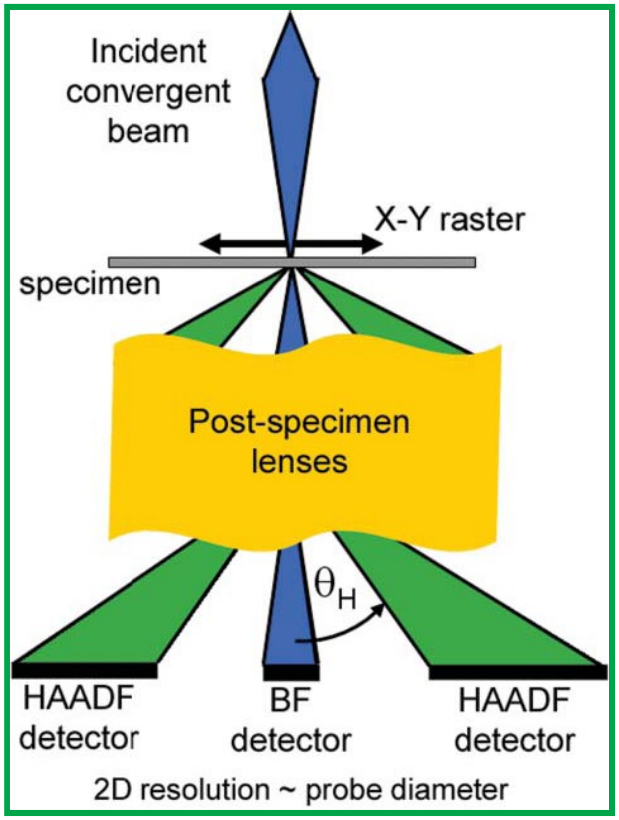
Frey TG, et al. 2006.
Annu. Rev. Biophys. Biomol. Struct. 35:199–224

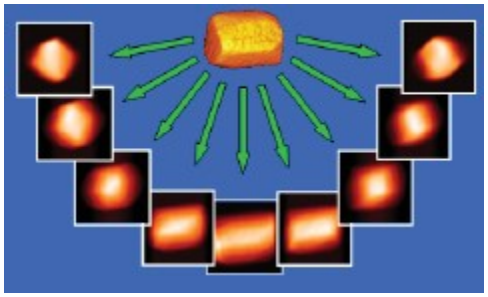


Multi-lysosomal body
A.J. Koster and W.J.C. Geerts, Utrecht University

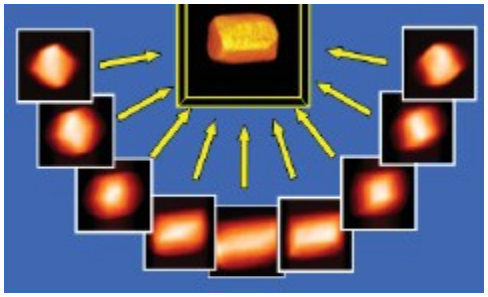
STEM tomography

Schematic diagram of STEM





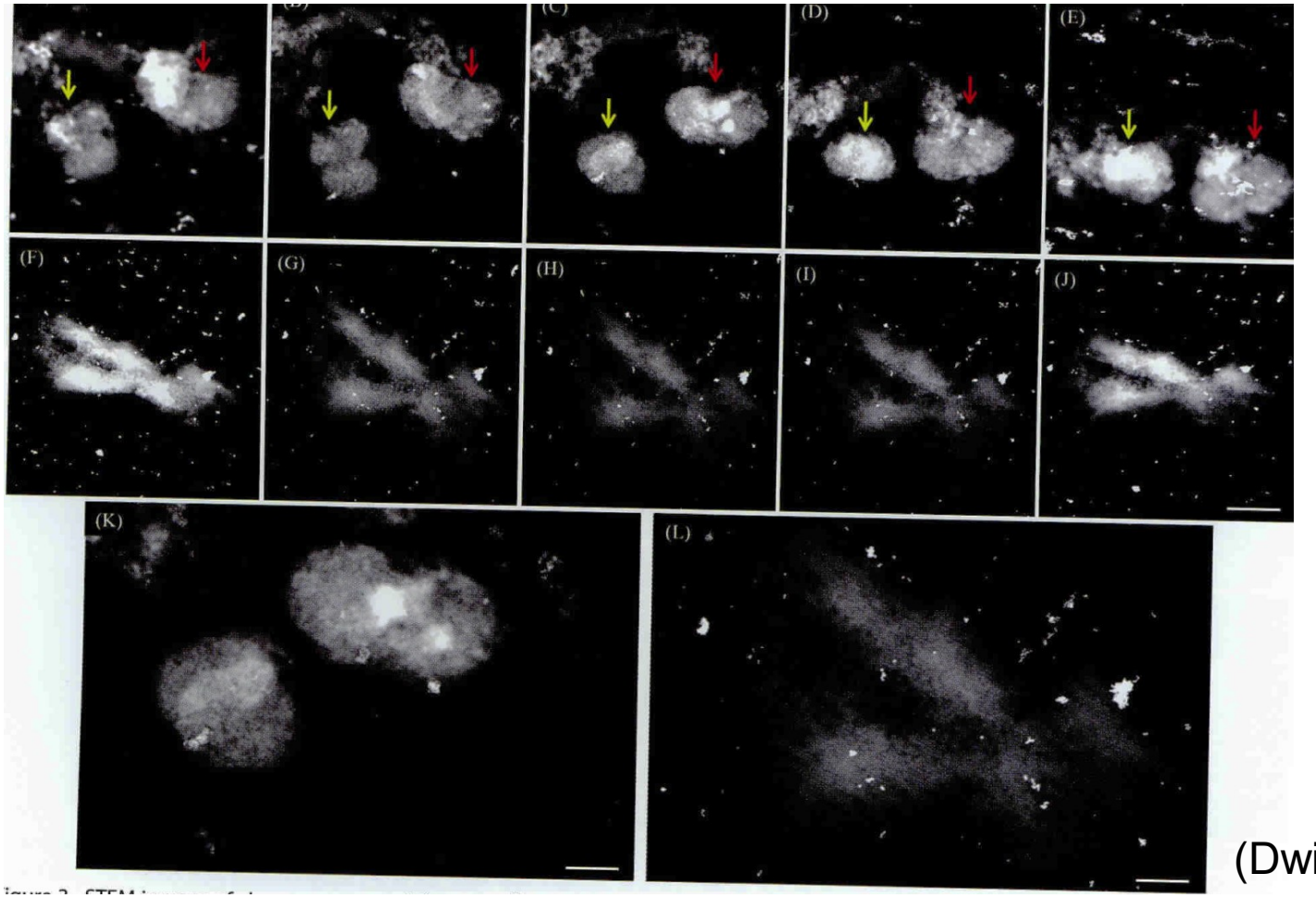
Single axis tilt series



Back projection

STEM tomography would provide a 3D data of the samples in a rapid and practical manner.

Tomography images of chromosomes



(Dwiranti, 2016)

Conclusions

- The general procedure for biological sample preparation are fixation, staining, dehydration, and sectioning.
- Different sample may require different preparation method.
- Good preparation considering the character of the samples would give the best TEM images.

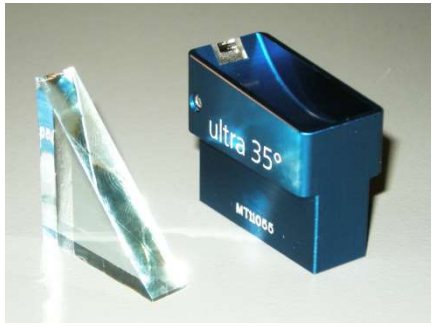
Life Science Facilities

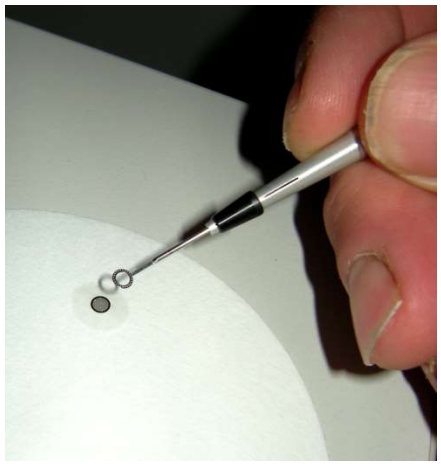
-Ultramicrotome

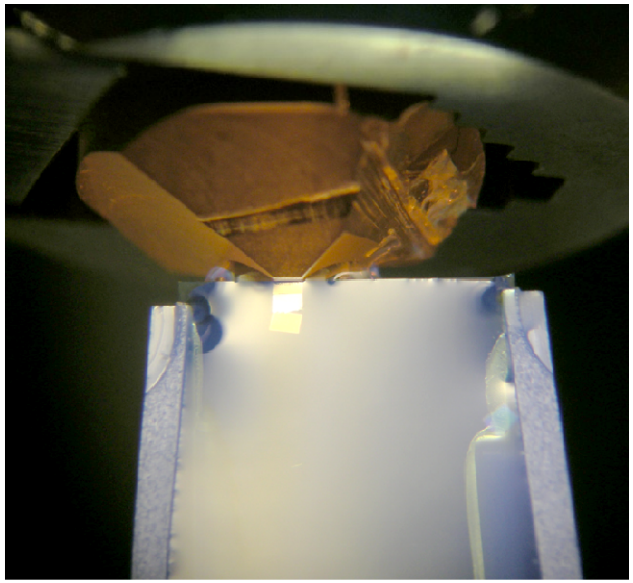
-Chemical Kit

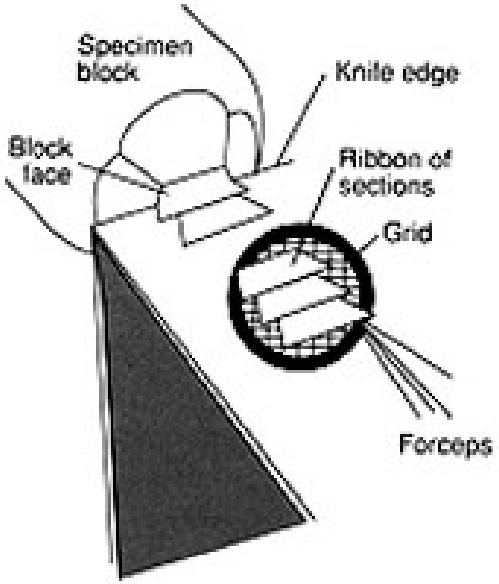
--> Epoxy Resin

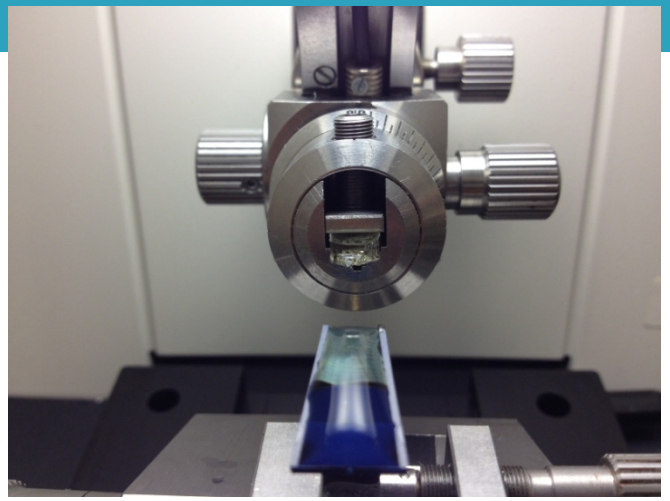

face
Pyramid

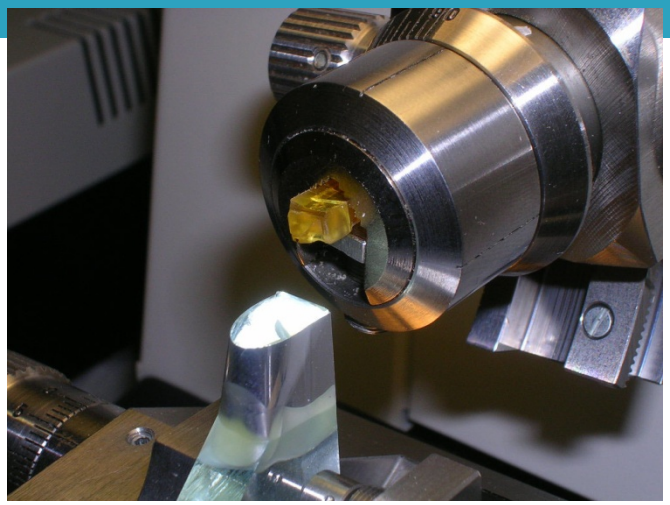

ultra 35°

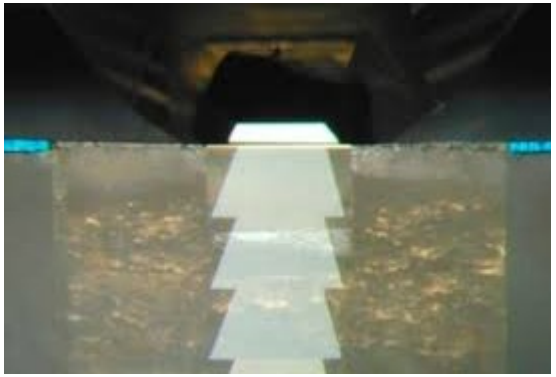


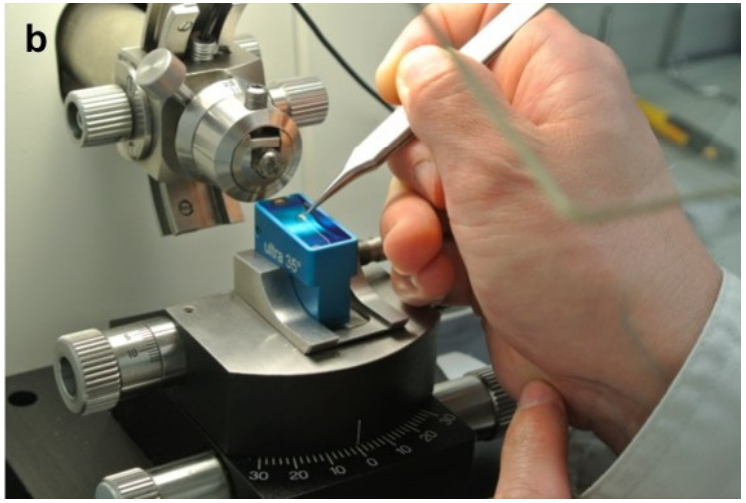


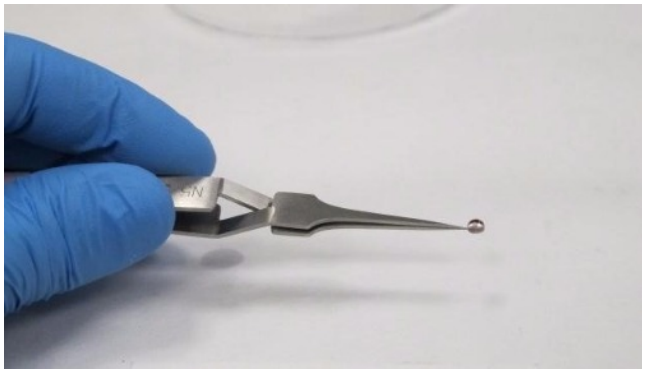

Specimen block
Knife edge
Block face
Ribbon of sections
Grid
Forceps








b



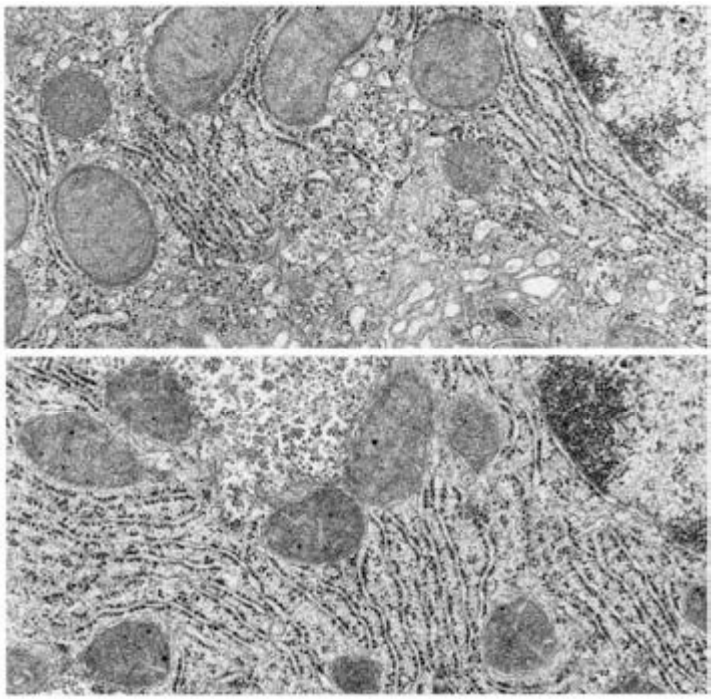

TECNAI

Thank you

Effects of aldehydes fixatives

Glutaraldehyde

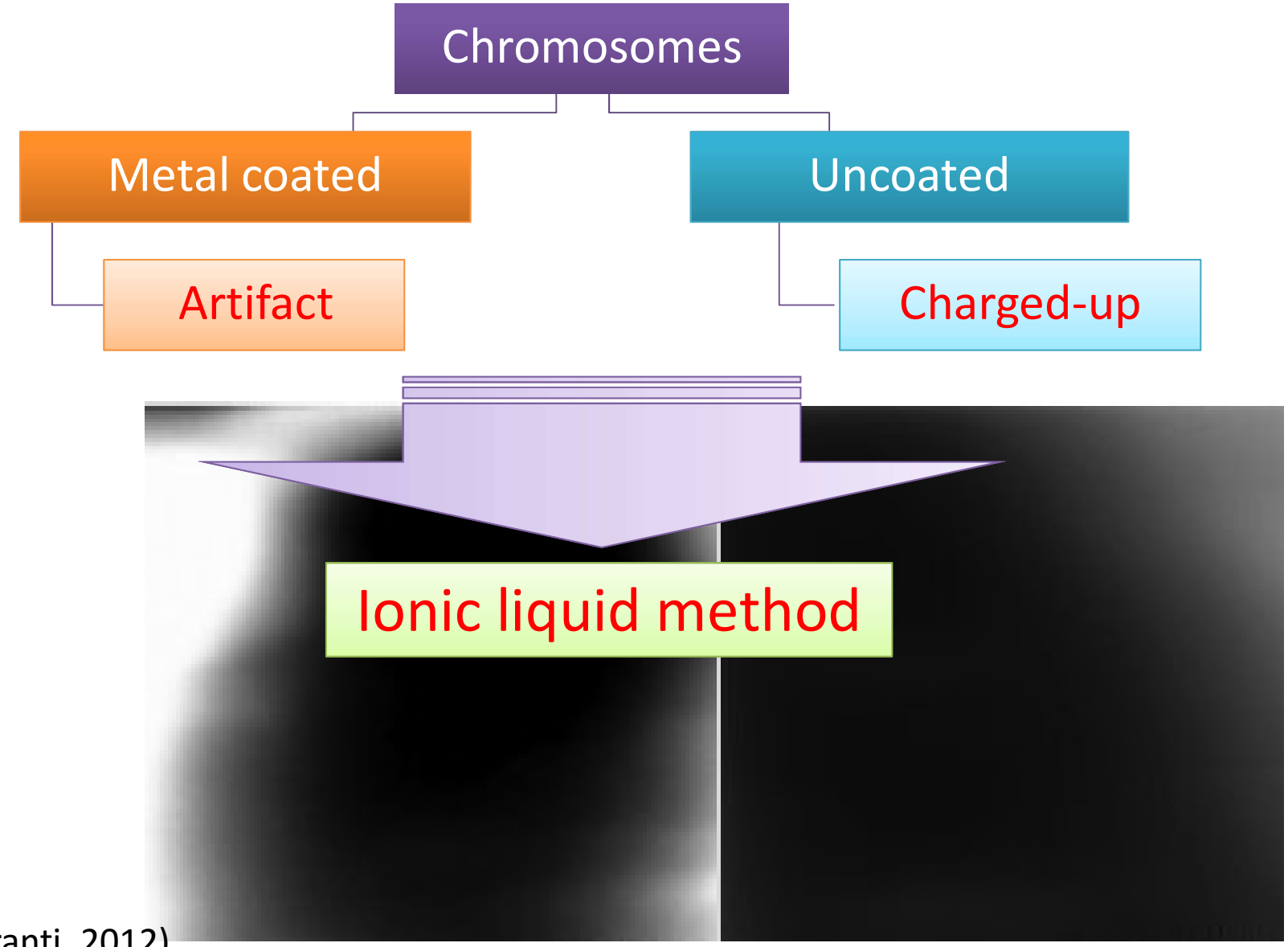
Formaldehyde



TEM images of rat liver

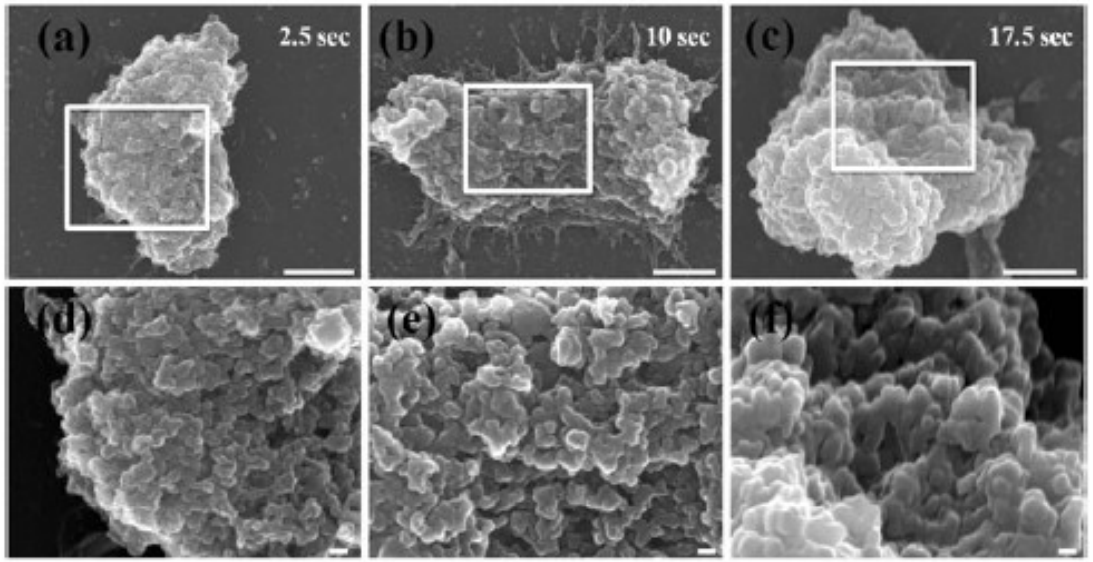
(Knott, 2009)

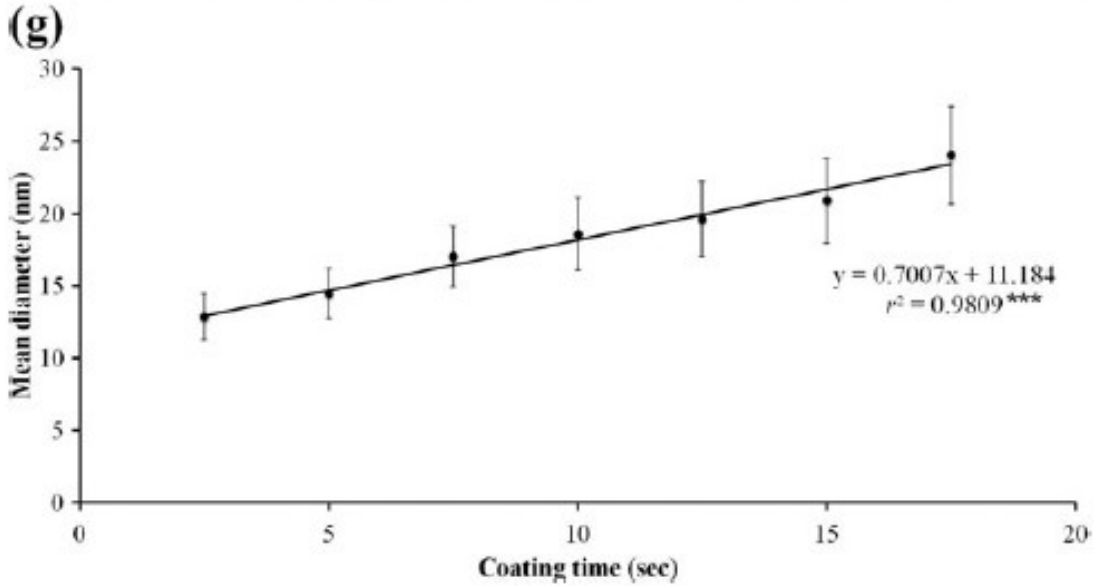
IL method



(Dwiranti, 2012)

The effect of OsO_4 coating



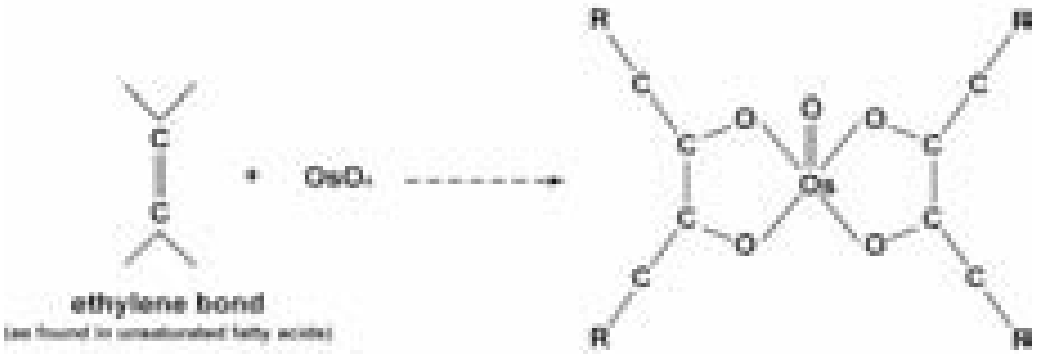


Chromatin diameter dynamics for different OsO_4 coating times observed bySEM. Graph shows the effect of OsO_4 coating time on mean chromatin diameter.

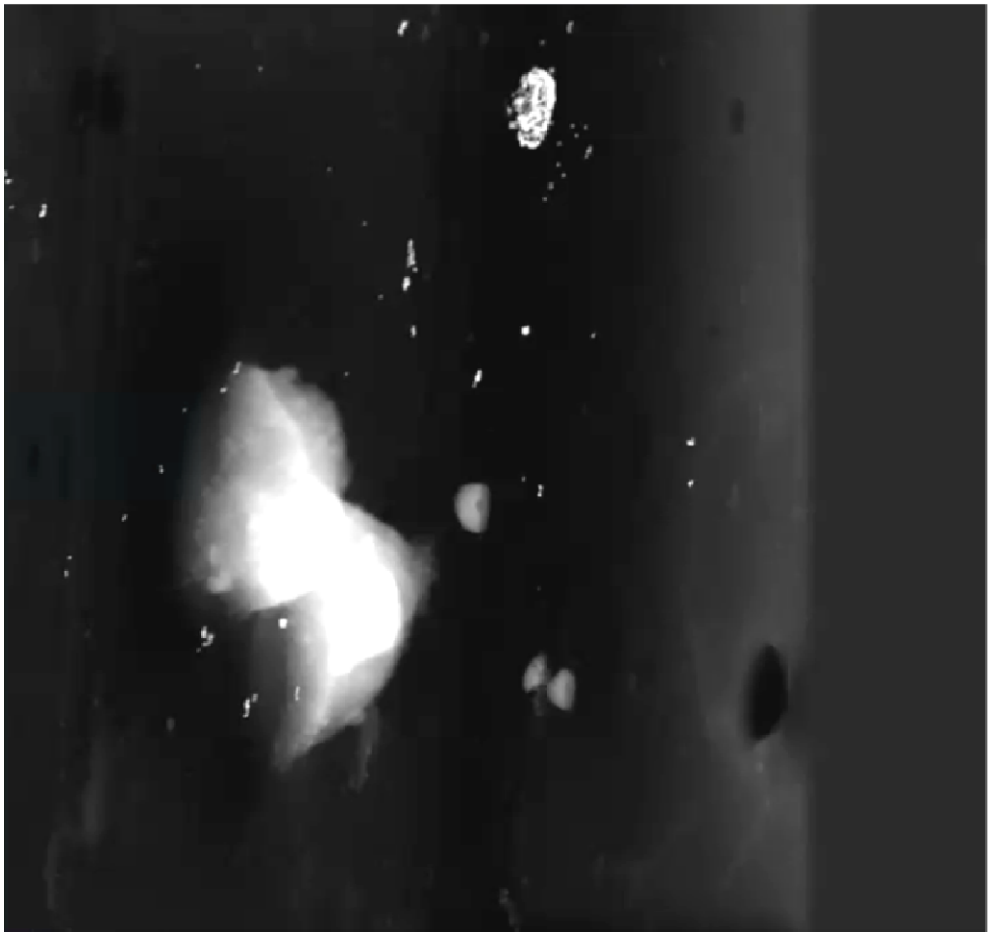
(Dwiranti, 2013)

Osmium tetroxide

- Non-polar tetrahedral molecule MW 254, solubility in water and a variety of organic compounds.
- Ability to stabilize and stain lipids
- Mode of action: reacts primarily with double bonds and sulfihydryl groups of proteins, causing major conformational changes in the structure of proteins.



Chromosome 3D visualization by STEM tomography



IL method

Fixation → IL treatment → SEM

Time requirement: 50 min

IL method is applicable for chromosome 3D observation by STEM tomography.

Bar: 1 µm

Artifacts induced during TEM preparation and observation

Table 6.1 Table summarizing the various artifacts formed by the preparation techniques or during TEM observation

Mechanical preparation-induced artifacts	Ionic preparation-induced artifacts	Chemical preparation-induced artifacts	Physical preparation-induced artifacts	Artifacts induced during observation
Deformation	Redeposition	Material displacement	Deformation	Dehydration
Material displacement	Implantation	Selective dissolution	Microstructure change	Charge effects
Material tearing	Vacancies	Composition change	Segregation of liquid phases	Destruction
Cracks	Dislocation loops	Structural change		Contamination
Fractures	Cavities	Microstructure change		
Inclusion of abrasive grains	Fractures	Change of molecular bonds		
Dislocations	Roughness	Change of natural contrast		
Glide planes	Selective abrasion	Protein reticulation		
Twinning	Structural change	Residues		
Strain hardening	Microstructure change			
Selective abrasion				
Particle aggregation				
Roughness				
Structural change				
Microstructure change				
Crystal-network change				
Composition change				
Residues				
Secondary thermal damage				
Fusion	Fusion	Microstructure change	Deformation	Demixing
Phase transformation	Phase transformation	Changes in distribution in the phases	Particle aggregation	Amorphization
Loss of chemical elements	Loss of chemical elements		Fusion	Phase transformation
Amorphization	Amorphization		Phase transformation	Loss of chemical elements
	Demixing		Frost	Migration
				Fusion