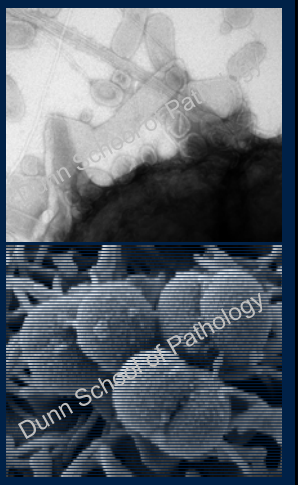
SIR WILLIAM DUNN
SCHOOL OF PATHOLOGY

UNIVERSITY OF OXFORD

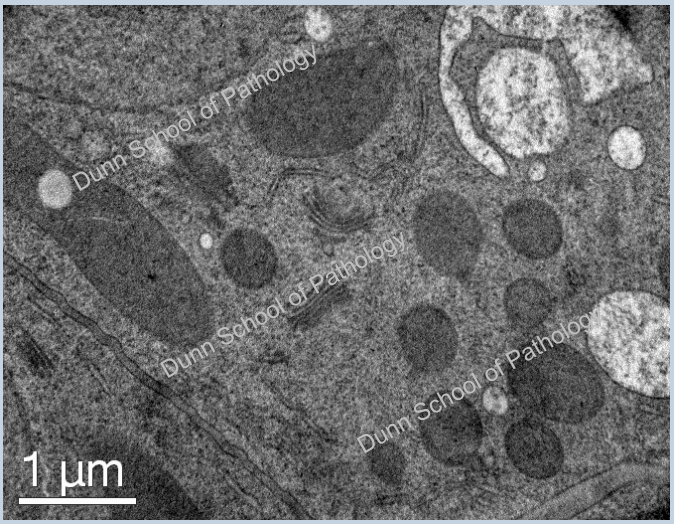
BIOLOGICAL SAMPLE PREPARATION FOR ELECTRON MICROSCOPY

Anna Pielach

Dunn School Bioimaging Facility



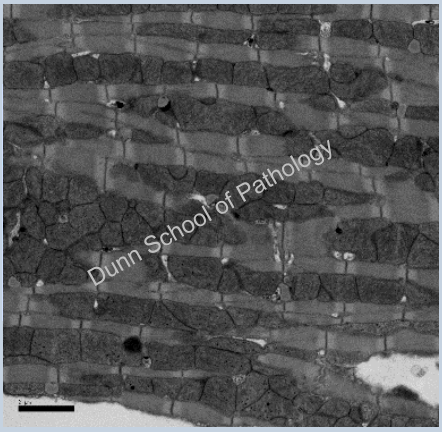
Transmission Electron Microscopy (TEM)



Arabidopsis root tip cell , JEOL 1400 TEM, (E Johnson)

Electron microscopy
Specimen requirements

TEM
Stable in the vacuum
Well preserved internal structure
Electron dense staining
Very thin (eg: 70 nm)
Particulate samples can be stained and viewed quickly
Cells and tissue require extensive specimen preparation

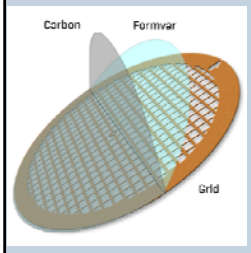


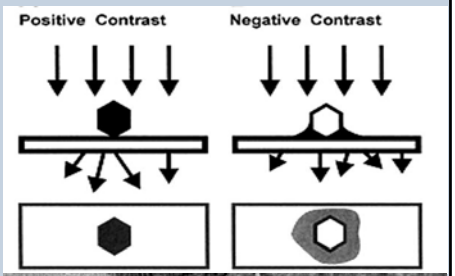
TEM of resin-embedded mouse cardiac tissue (scale bar = 2 µm), Tecnai12 TEM, E Johnson

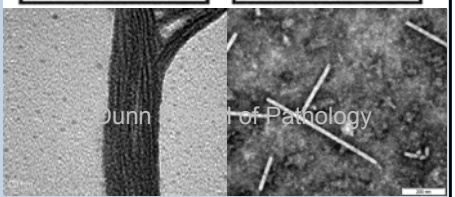
Specimen Preparation for TEM
Particulate samples

Negative Staining:

- Coat grids with plastic film and carbon
- Apply the particulate specimen eg: proteins, viruses, DNA)
- Stain with heavy metal solution, eg: uranium salts
- Blot dry and view in the TEM



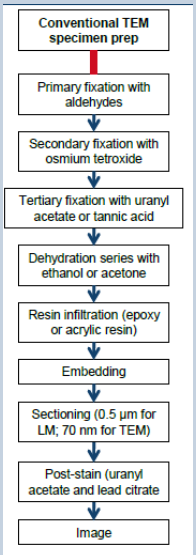


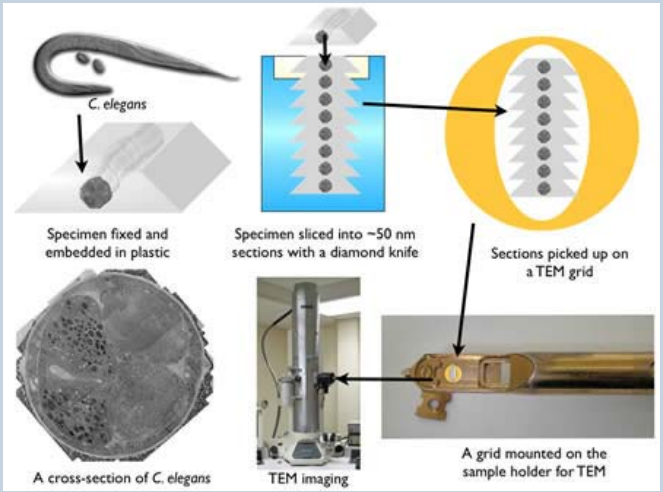


Bacterial protein stained with uranyl acetate; Tobacco mosaic virus negatively stained with sodium silicotungstate (E. Johnson)

Specimen Preparation for TEM
Cells & Tissue

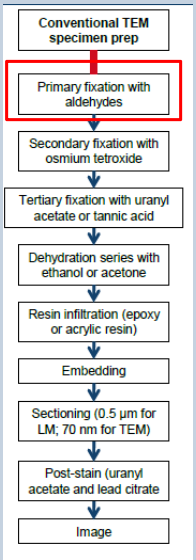
Specimen Preparation for TEM
Cells & Tissue – Overview





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

Specimen Preparation for TEM
Cells & Tissue – Primary Fixation



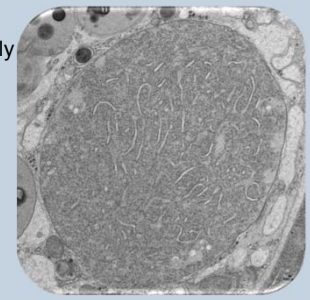
Fixation stops cellular processes and aims to preserve the specimen as close as possible to its natural state.

Characteristics of a good fixative:

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

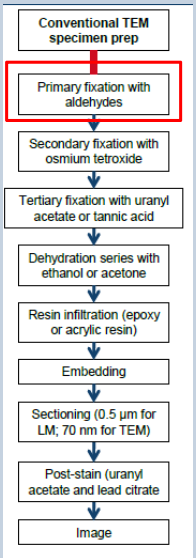
Methods of fixation include:

- Chemical fixation with aldehydes
- Cryo-fixation with liquid nitrogen



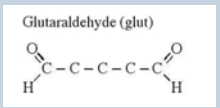
C elegans, A Moloney/E Johnson

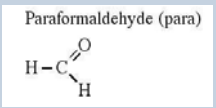
Specimen Preparation for TEM
Cells & Tissue – Chemical Fixation



Glutaraldehyde
irreversible cross-linking of proteins via amino groups

Paraformaldehyde:
reversible cross-linking, small molecule, penetrates quicker

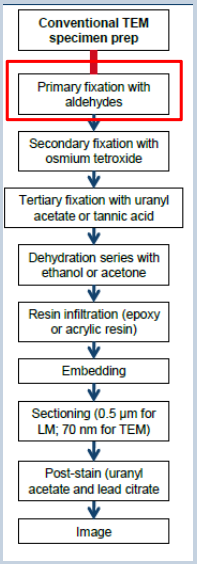




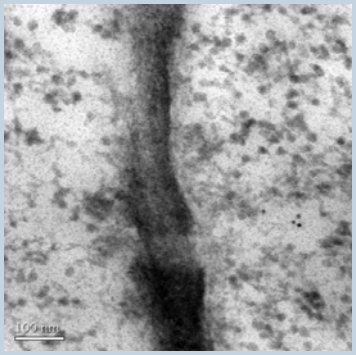
Standard TEM fix: 2.5% glutaraldehyde + 2-4% PFA for 30 mins to overnight.

Specimen Preparation for TEM

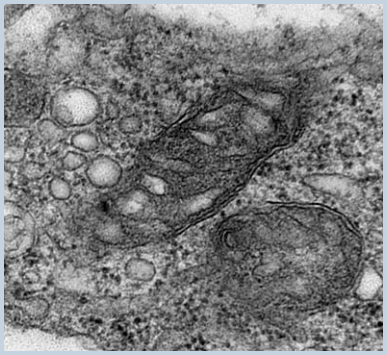
Cells & Tissue – Chemical Fixation artifacts



Loss of membranes

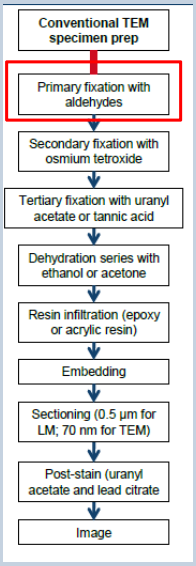


Deformed mitochondria

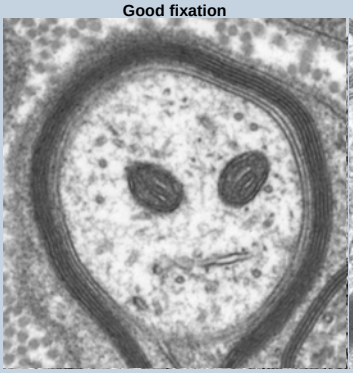


Specimen Preparation for TEM

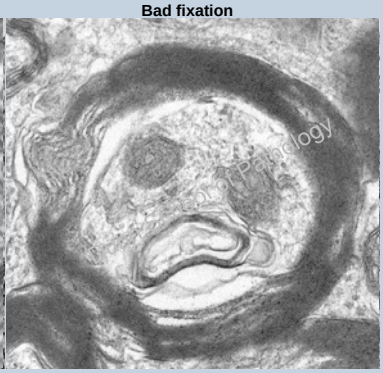
Cells & Tissue



Good fixation



Bad fixation



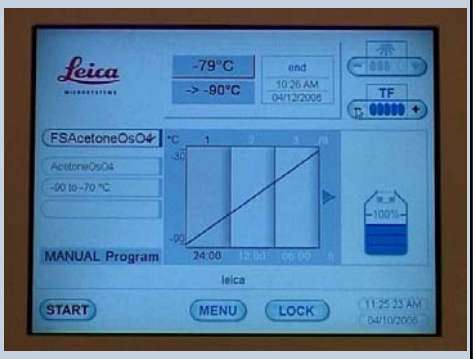
Specimen Preparation for TEM
Cells & Tissue – Cryo-fixation

- Tissue can be cryo-fixed using LN_2 in the High Pressure Freezer and then further processed for TEM (adds 1 week)
- 3-6 mm specimen carriers
- cryo-fixedation with LN_2 under high pressure (~2000 bar) to prevent damaging ice crystal formation up to 200 µm into the tissue



Specimen Preparation for TEM
Cells & Tissue – Cryo-fixation

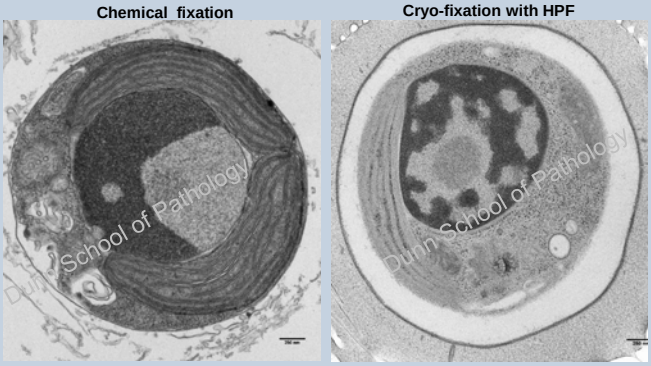
Samples are then carefully transferred to the AFS and freeze-substituted with solvent (+ osmium and/or glutaraldehyde or uranyl acetate) at sub-zero temperatures.

Specimen Preparation for TEM
Cells & Tissue – Cryo-fixation

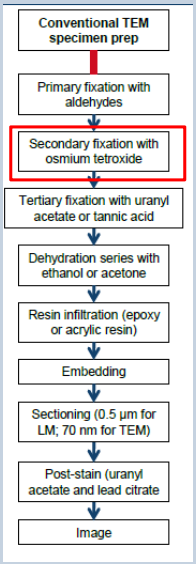
- **Cons** of cryofixation: time consuming, finicky and restrictions on sample size, possible ice crystal issues
- **Pros** of cryo-fixation: best possible ultrastructural preservation, maintains fluorescence and antigenicity

Chemical fixation | **Cryo-fixation with HPF**

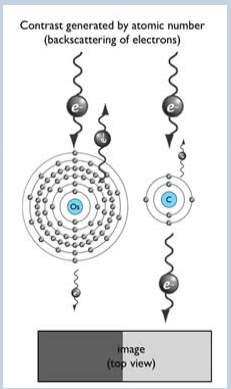


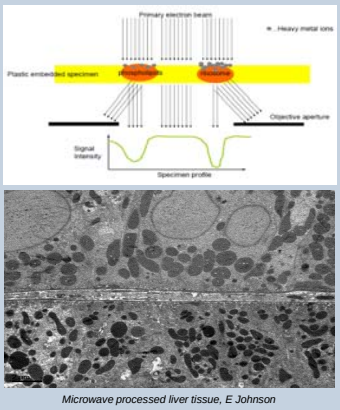
Emiliania huxleyt algae fixed using chemical (left) and cryo methods (rught; M Eason-Hubbard/E Johnson

Specimen Preparation for TEM
Cells & Tissue – Secondary Fixation



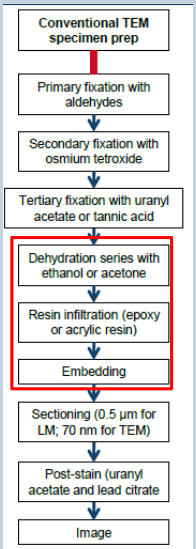
- **Osmium tetroxide** is a heavy metal that fixes unsaturated lipids.
- Used as both a **secondary fixative and an electron stain**, it significantly improves specimen preservation (especially of membranes) and contrast.





Microwave processed liver tissue, E Johnson

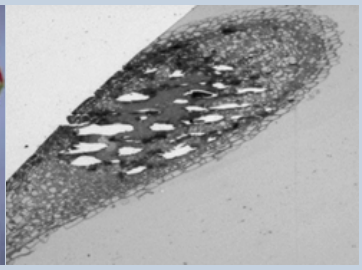
Specimen Preparation for TEM
Cells & Tissue – Dehydration & resin infiltration



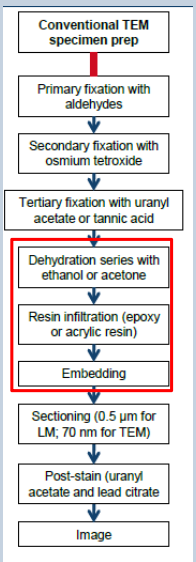
- Dehydration is the process of gradually replacing water in the sample with a solvent (usually acetone or ethanol).
- The solvent is then gradually replaced with resin. This process can be lengthy and depends on both the sample and type of resin used.

Resin blocks

Poor resin infiltration



Specimen Preparation for TEM
Microwave-assisted sample processing

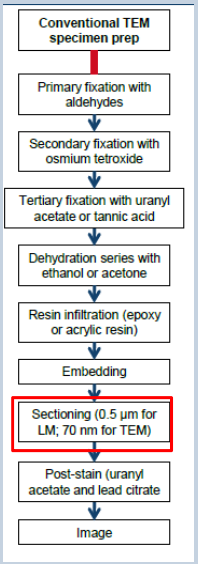


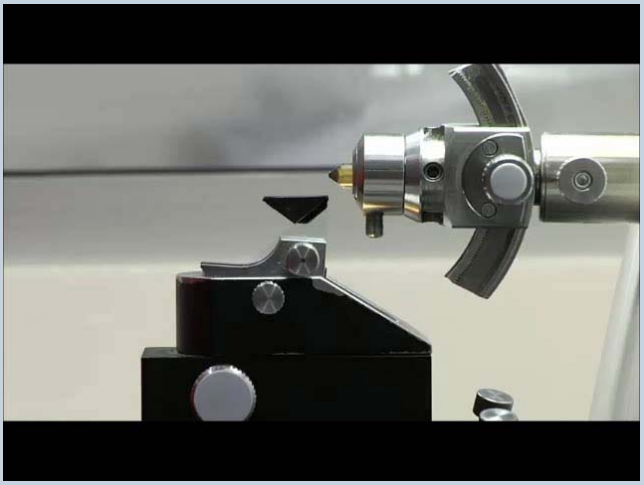
Leica EM AMW

From 5 days to 5 hours

Specimen Preparation for TEM

Cells & Tissue - Ultramicrotomy

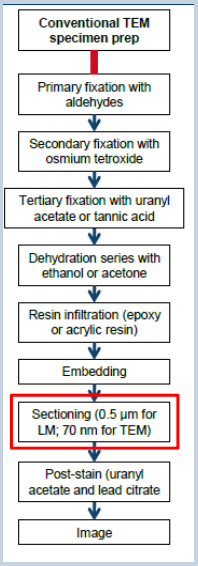


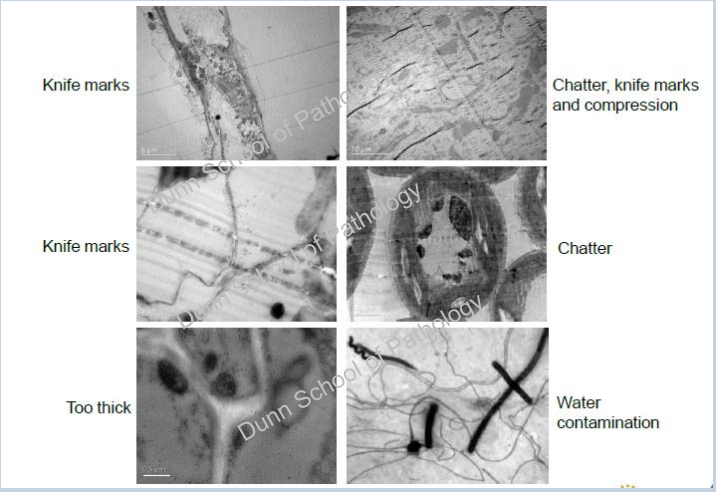


Introduction to ultramicrotomy video, University of Sydney

Specimen Preparation for TEM

Cells & Tissue – Ultramicrotomy artifacts

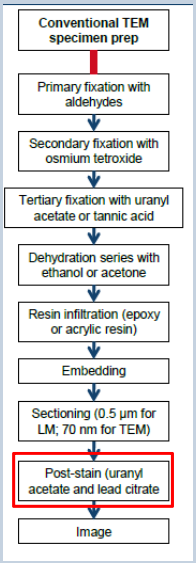




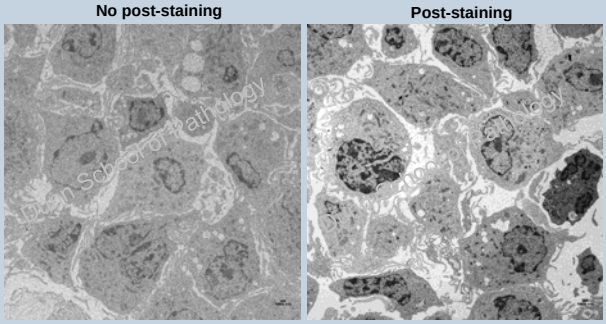
Images by E Johnson (unfortunately!)

Specimen Preparation for TEM

Cells & Tissue – Post-staining



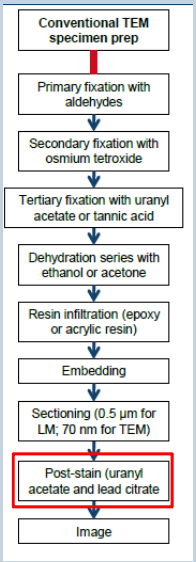
Contrast can be increased by post-staining sections with salts of heavy metals, specifically **uranyl acetate** and **lead citrate** solutions. Uranyl acetate stains protein and DNA and also acts as a mordant for lead citrate, which is a more general stain.

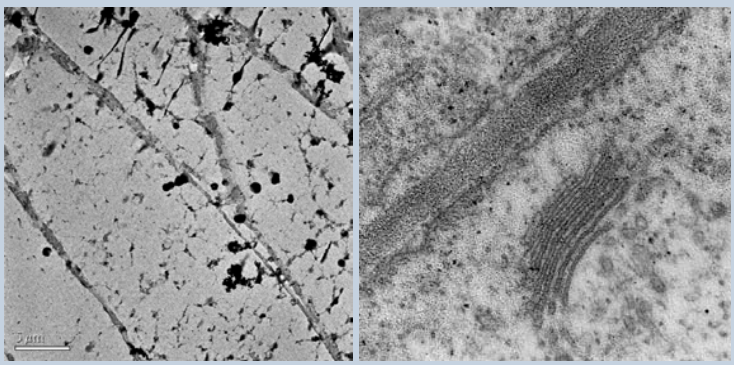


Dendritic cells (S Hackett/E Johnson)

Specimen Preparation for TEM

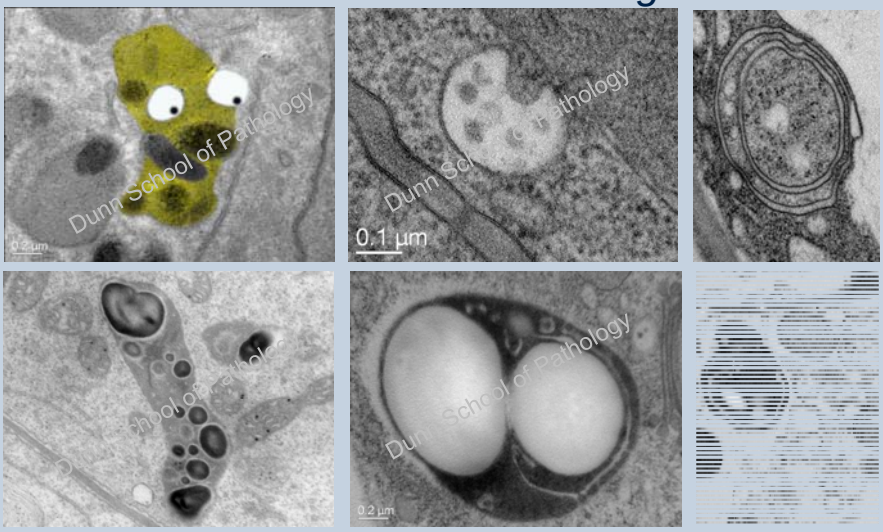
Cells & Tissue – Post-staining artifacts





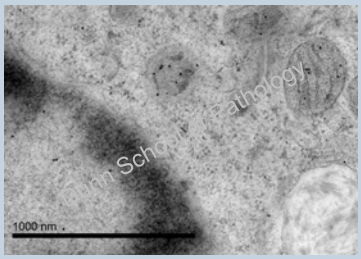
Images by E Johnson (unfortunately!)

TEM Specimen Preparation
Critical evaluation of images

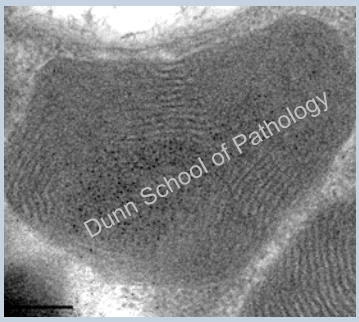


Specimen Preparation for TEM
Protein localisation – Immunogold labelling

- Secondary antibody is conjugated to a colloidal gold particle.
- For cells and tissue, post-embedding labelling is usually the best option
- A lighter chemical fixation or cryo-fixation
- The osmium tetroxide step is omitted
- Acrylic resins are used instead of Epoxy resins.



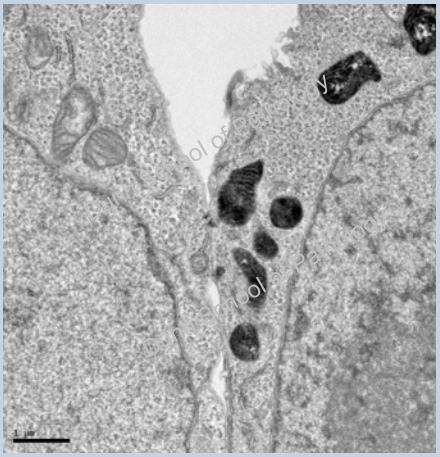
Immunogold labelled mitochondria in HeLa cells (A Pielach)



Immunolabelled mitochondrion in mouse cardiac muscle (P Ostrowski/EJohnson)

Specimen Preparation for TEM
Protein localisation – EM genetic tags

- Two new **genetically encoded tags** for identifying proteins of interest at the EM level whilst using a **standard TEM prep**
- **APEX** (Martell et al, Nature Biotech 30, 2012)
  - 28kDa **peroxidase that catalyses with DAB** (with H_2O_2) to produce a localised osmophilic precipitate
- **miniSOG** (Shu et al PLOS Biology 9, 2011)
  - Small **fluoresecent flavoprotein that can be photo-oxidised to react with DAB** to produce a localised osmophilic precipitate - CLEM



Chemically fixed HEK cells transfected with APEX tagged to a mitochondrial matrix protein (J Long/E Johnson)

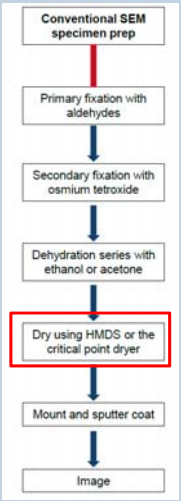
Scanning Electron Microscopy (SEM)

Penicillium (E Johnson)

Sample Preparation for SEM
Overview

- SEM specimens must be:
  - Well preserved with no surface contamination or damage
  - Stable in the vacuum
  - Conductive
  - Composed of high atomic number elements
- The conventional preparation for SEM samples is similar to that for TEM, although the resin and sectioning steps are omitted.
- There are less size restrictions on SEM samples compared to TEM.

Sample Preparation for SEM
Drying the sample

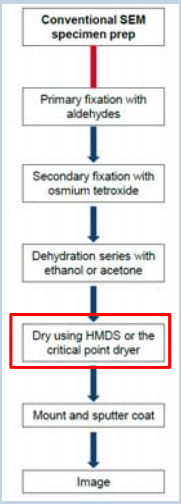


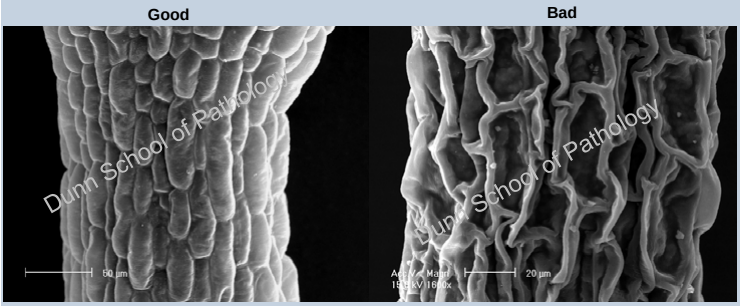
Air drying is not recommended, as ethanol evaporation generally causes severe surface tension artifacts.

Ethanol is instead flushed out using transitional fluid of very low surface tension:

- manually, using hexamethyldisilazane (HMDS) – cell monolayers
- in a Critical Point Dryer (CPD) using liquid CO_2 – tissues and whole organisms

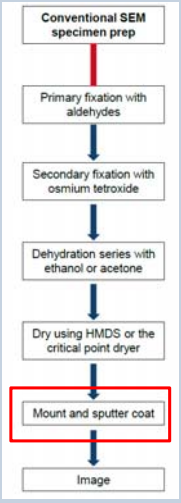
Sample Preparation for SEM
Drying the sample





Arabidopsis stem, Phillips XL30 SEM, E Johnson

Specimen Preparation for SEM
Sputter coating

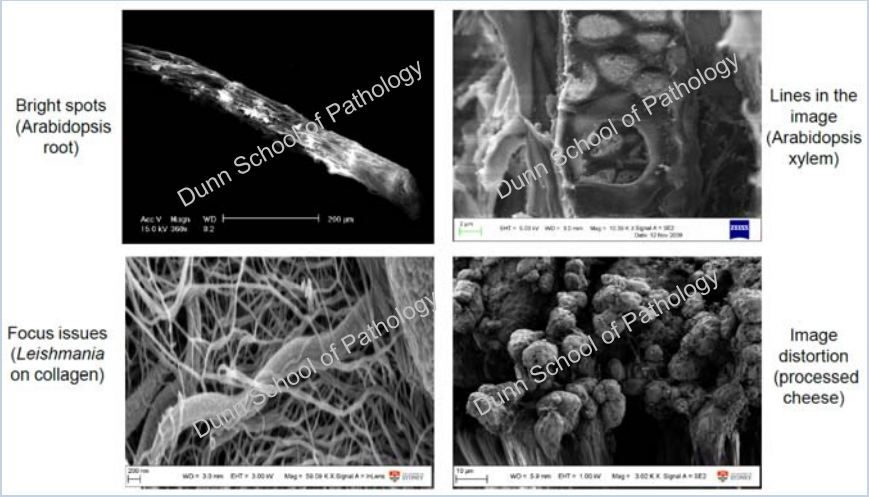


- Mounting – immobilization and grounding on a conductive backing
- Sputter coating with metal ions

  →continuous conductive layer over the sample

  → increased SE signal (and therefore contrast)

Specimen Preparation for SEM
Charging artifacts

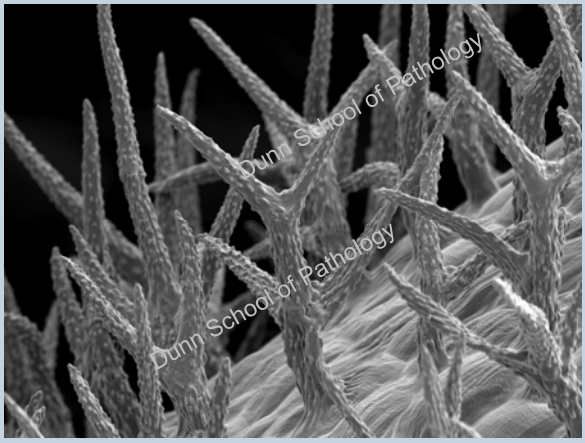


Images, E. Johnson

Specimen Preparation for SEM
Surface contamination and deformation

Images, E. Johnson

Specimen Preparation for SEM
No problems!



Questions?