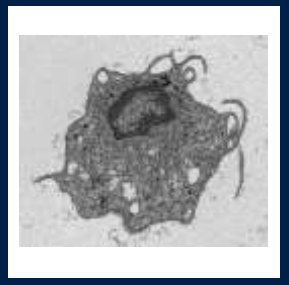
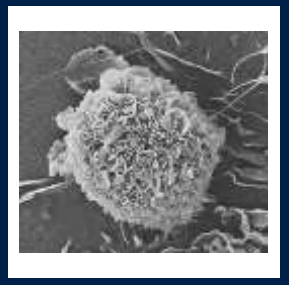
SIR WILLIAM DUNN
SCHOOL OF PATHOLOGY

UNIVERSITY OF OXFORD

# ELECTRON MICROSCOPY OF BIOLOGICAL SPECIMENS



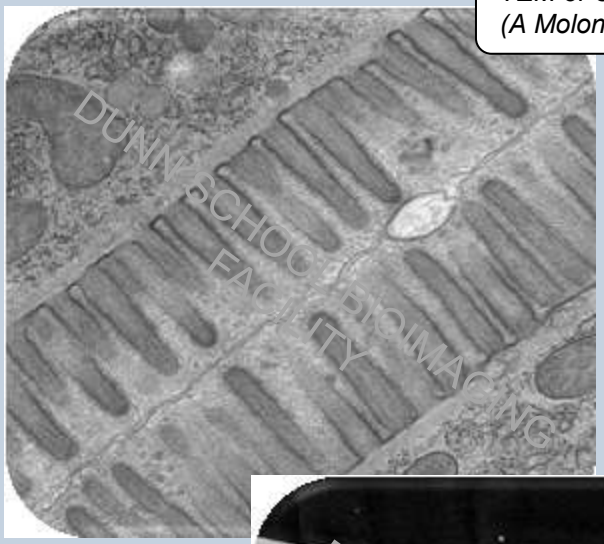


Errin Johnson
EM Experimental Officer

March 20, 2013

# Lecture Overview

- Introduction to Electron Microscopy (EM)
  - Basic principles
  - Applications to biological research
  - EM facilities at The University of Oxford
- Transmission Electron Microscopy (TEM)
  - How the TEM works
  - Biological specimen preparation for TEM
  - Advanced TEM techniques
- Scanning Electron Microscopy (SEM)
  - How the SEM works
  - Biological specimen preparation for SEM
  - Advanced SEM techniques




*TEM of C. elegans gut*
*(A Moloney & E Johnson)*


*SEM of C. elegans*
*(A Moloney & E Johnson)*

# Introduction to Electron Microscopy

# Introduction to Electron Microscopy

# Basic Principles
## *Brief history of EM*

**1873** – Hermann von Helmholtz & Ernst Abbe show that the wavelength of light affects optical resolution

**1924** – Louis de Broglie theorised the wave/particle duality of electrons

**1926** – Hans Busch demonstrated that magnetic lenses can manipulate the path of electrons in the same way as optical lenses do with light

**1932** – Ernst Ruska & Max Knoll invent the TEM

**1934** – Ladisalus Marton publishes the first biological EM micrograph

**1937** – Manfred von Ardenne builds the first SEM

**1951** – Erwin Muller develops the field emission microscope for atomic resolution

**1951** – Albert Claude *et al* publish the first TEM image of an intact cell



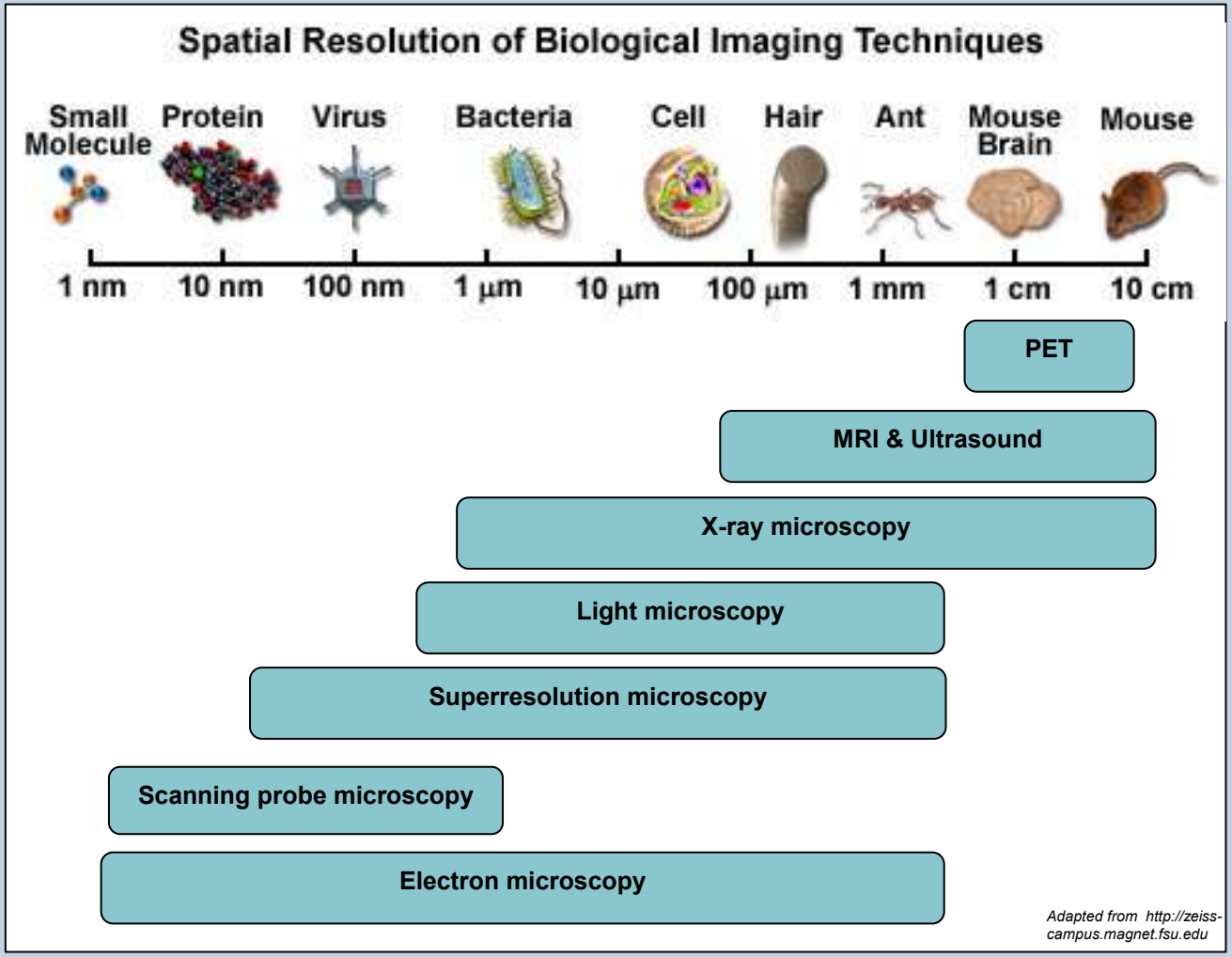
For a detailed history of EM, see:

1. Haguenau et al. (2003) Key events in the history of electron microscopy. *Microscopy & Microanalysis*, 9(2): 96-138.

2. Masters, B (2009) History of the electron microscope in cell biology. *eLS*, Wiley.

# Basic Principles

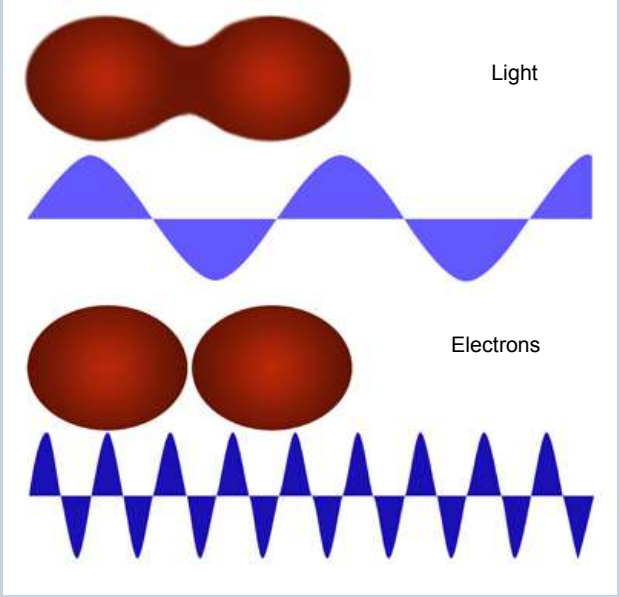
## *Advantages of EM - Resolution*



# Basic Principles
## *Advantages of EM - Resolution*

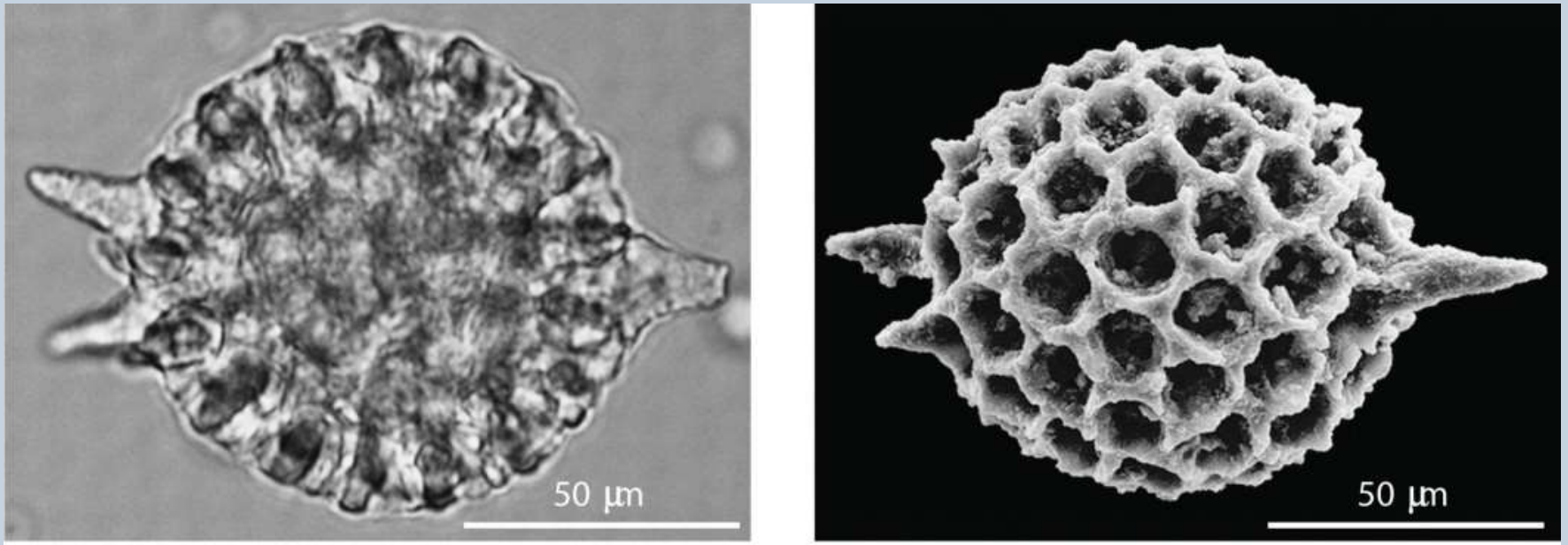
- The wavelength of electrons is MUCH smaller than that of light
- Confocal microscope resolution = 200 nm
- Electron microscope resolution < 1 nm


*www.nobelprize.org*




*www.ammrf.org*

# Basic Principles
## *Advantages of EM - Resolution*




(a) Radiolarian under light microscope

(b) Radiolarian under electron microscope

*General Chemistry: Principles, Patterns, and Applications, B. Averill & P. Elderege*

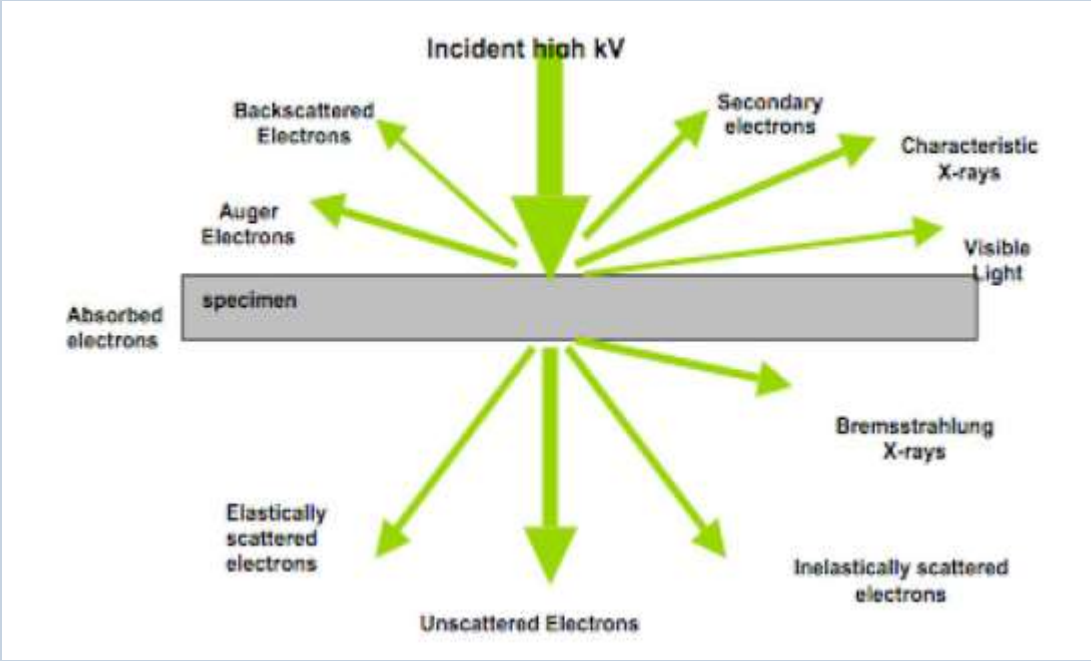
# Basic Principles
## *Advantages of EM - Depth of field*

- Depth of field is ~300x greater in EM than in LM, providing topographical information of your specimen


*www.ammrf.org*

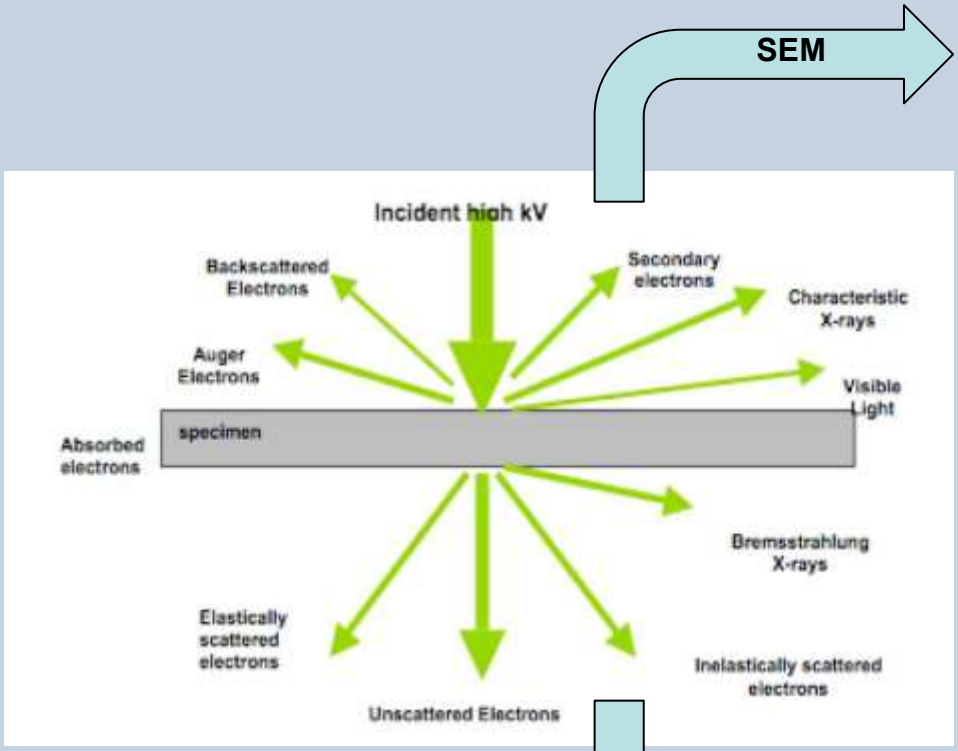
# Basic Principles
## *Advantages of EM – Microanalysis*

- Electron microscopy also allows the chemical composition (as well as crystollographic, electrical and magnetic properties) of a specimen to be characterised.



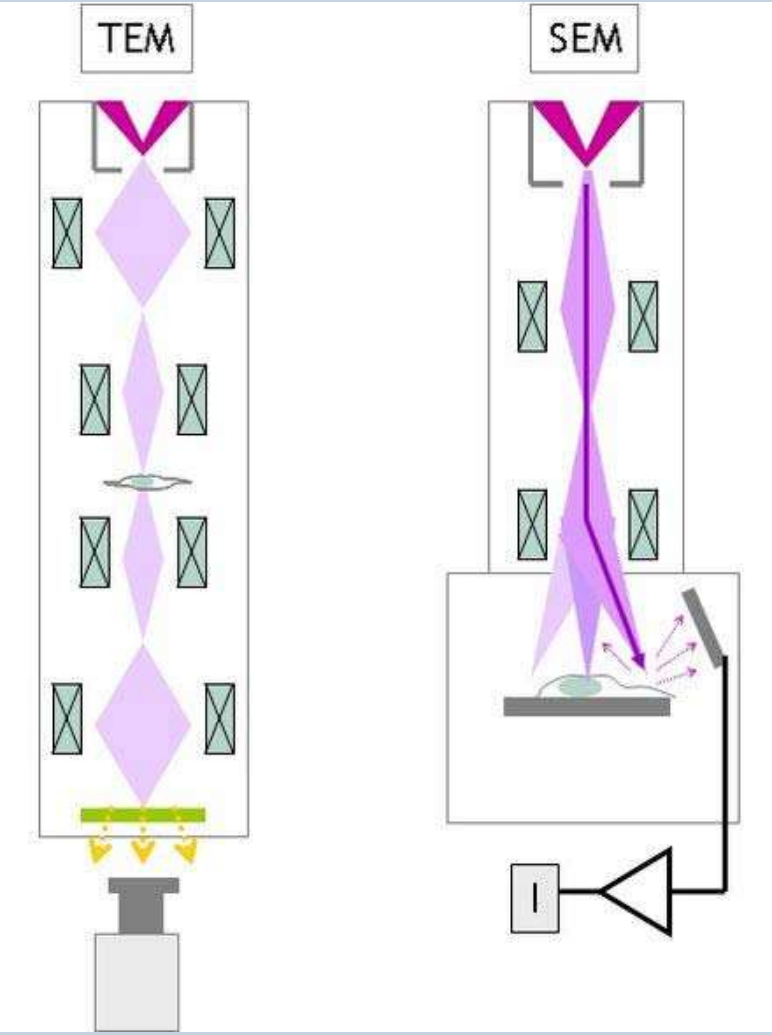
# Basic Principles

## *Electron Microscopes - Overview*




# Basic Principles

## *Electron Microscopes - Overview*



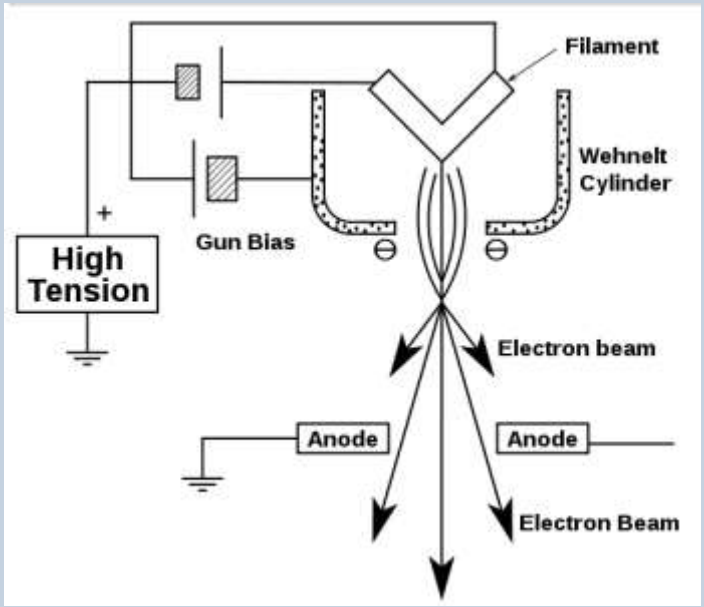

The main components of an electron microscope are:

- An electron gun
- Electromagnetic lens system
- Vacuum system
- Camera/detector
- Computer

# Basic Principles
## *Electron microscopes – Electron gun*

- The gun consists of an electron source, electrode, Wenhelt assembly and anode
- A current is run through the filament/crystal to heat it, resulting in the emission of electrons from the tip. The high voltage difference between the cap and the anode causes the electrons to accelerate and form a beam

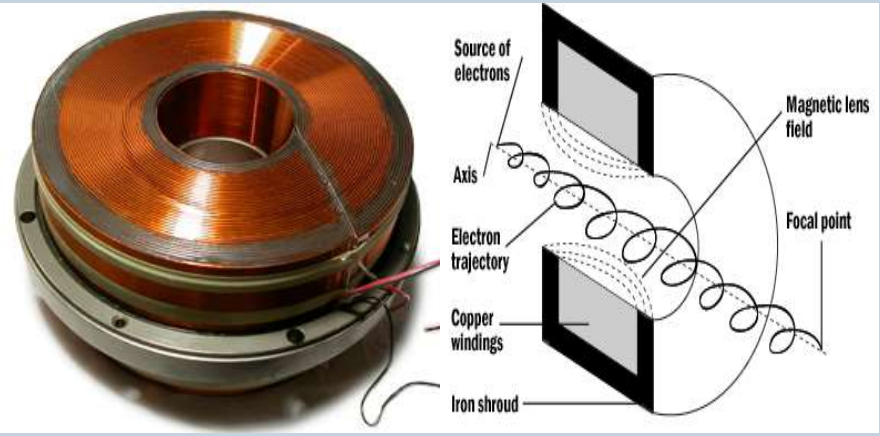
*www.ammrf.org*




*www.wikipedia.org*

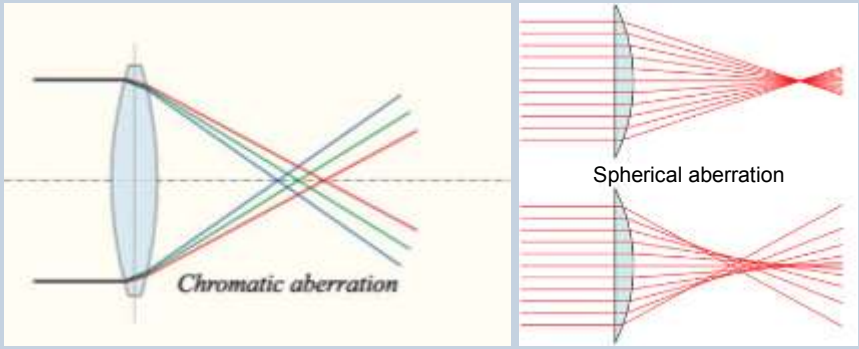
# Basic Principles
## *Electron microscopes – Lenses*

- TEM lenses are electromagnetic, creating precise, circular magnetic fields that manipulate the electron beam, much the same way that optical lenses focus and direct light




*www.ammrf.org* *www.uiowa.edu*

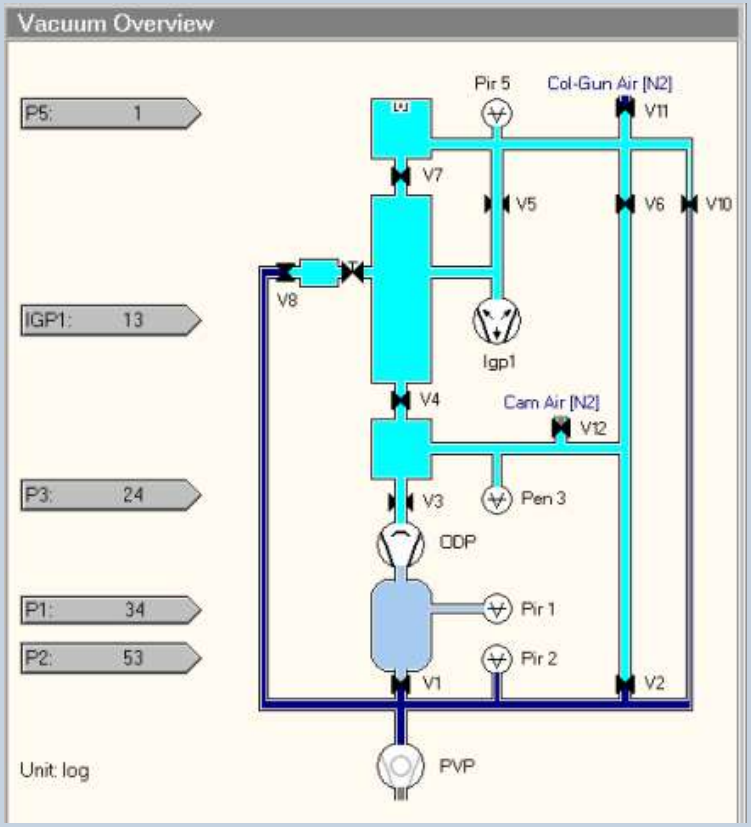
- Similarly to optical lenses, electromagnetic lenses are also susceptible to aberrations
  - Chromatic aberration
  - Spherical aberration
  - Astigmatism




*A. Kach, University of Zurich*

# Basic Principles
## *Electron microscopes – Vacuum*

- EMs have elaborate pumping systems to ensure that the microscope is operated under a high vacuum ($10^{-4}$ Pa)
  - Maintains the integrity of the electron beam, as any interaction with gas atoms will cause the beam to scatter
  - Avoids arcing between the cathode and ground




*Overview of vacuum system on the Tecnai12 TEM*

# Basic Principles
## *Electron microscopes – Signal detection*

- EM images are monochromatic and are essentially intensity maps of the number of electrons that are detected from a given point. False colour may be added during post-processing of the image, if desired.

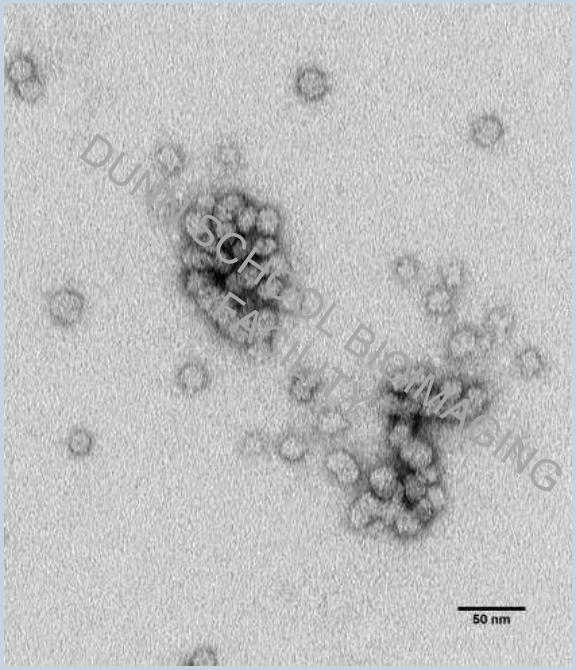
*Original SEM image*

*False-coloured SEM image*

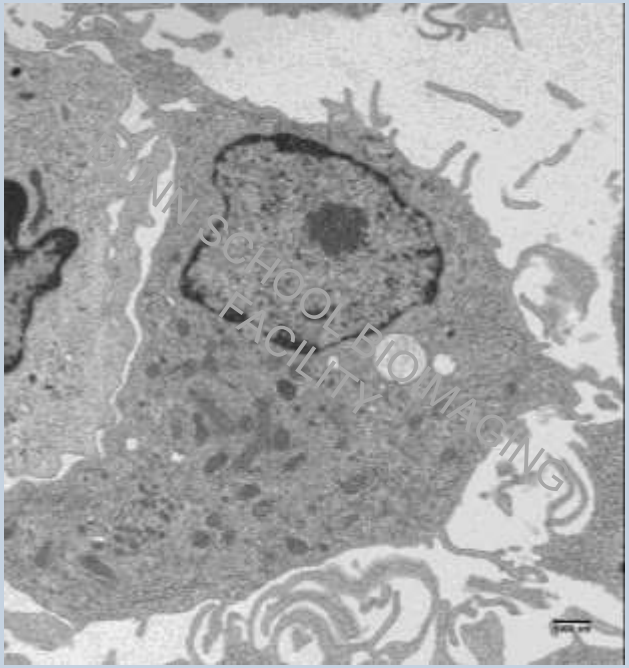
*Hydrothermal worm (x525) by Philippe Crassous, FEI.com*

# Biological Applications

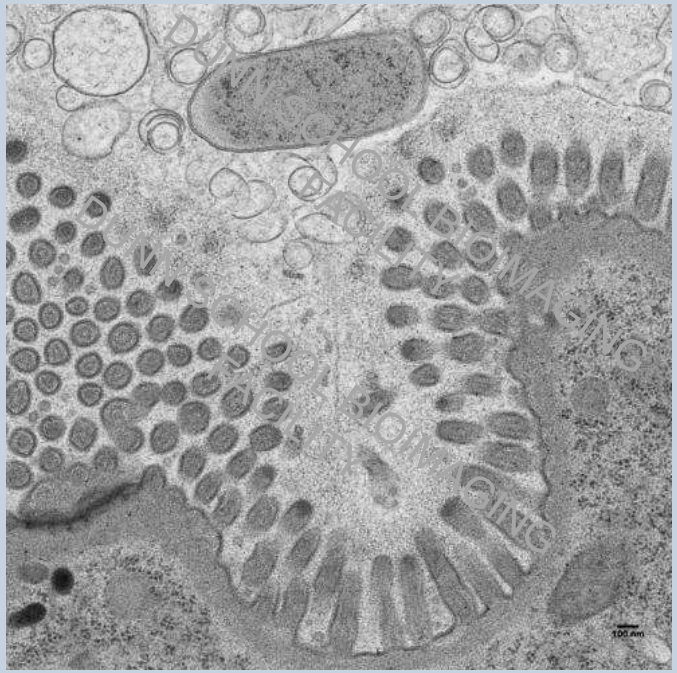
## *TEM - Ultrastructure*




*Negatively stained Hepatitis B vaccine*
*(A Crook/E Johnson)*

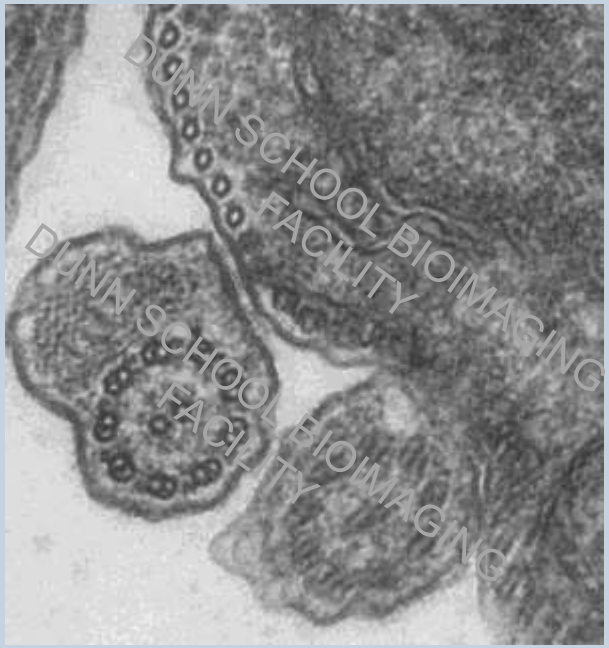


*Macrophage*
*(C Duncan/E Johnson)*

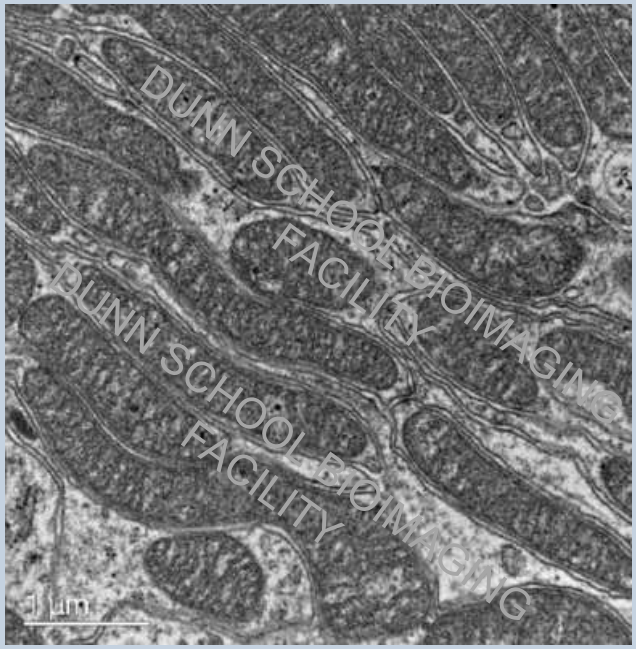


*Bacterium in gut of cryo-fixed C. elegans*
*(A Moloney/E Johnson)*

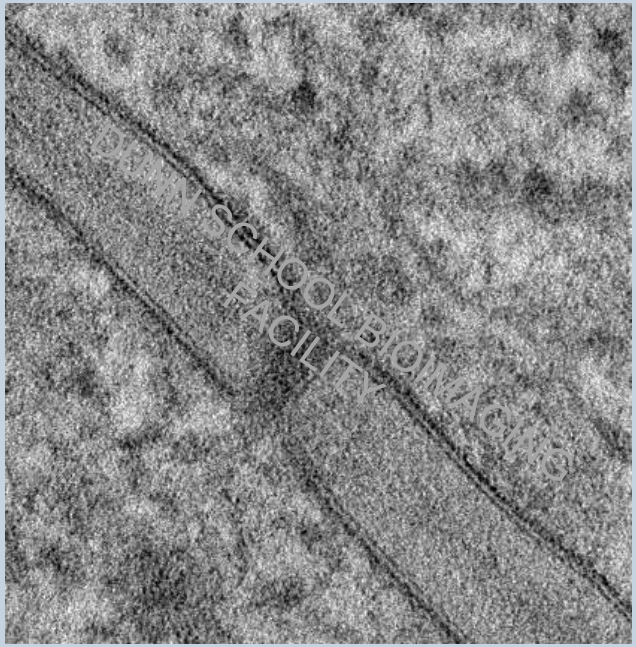
# Biological Applications
## *TEM - Ultrastructure*




*Cross-section of flagella in Trypanosoma brucei*
*(J Sunter)*




*Mitochondria in mouse renal tissue*
*(E Johnson)*

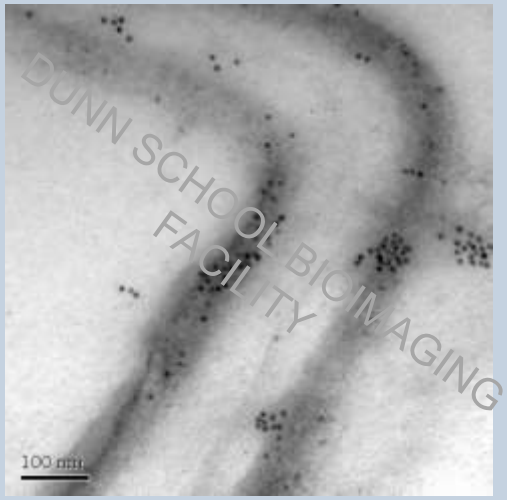



*Plasmodesmata in Arabidopsis root meristem*
*(E Johnson)*

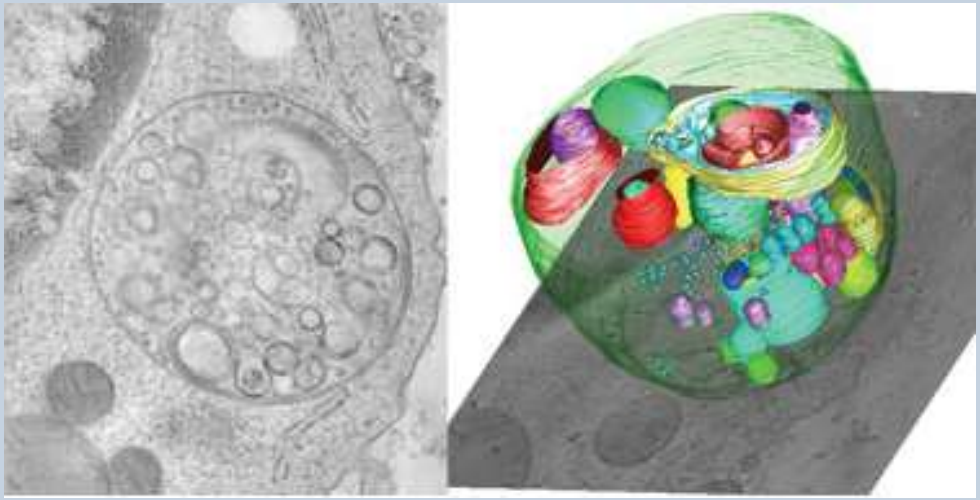
# Biological Applications

## *TEM - Advanced*

**Protein localisation**



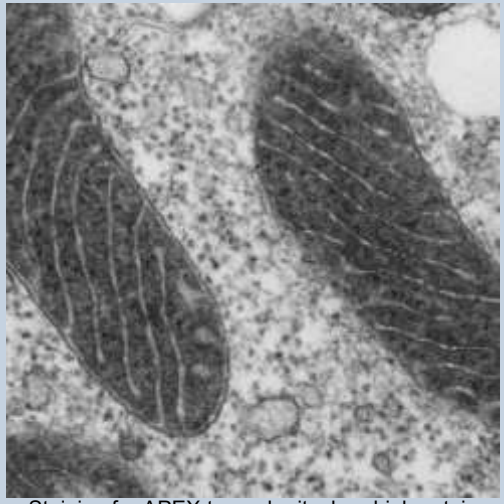

Immunogold labelled PM protein in corn (E Johnson)



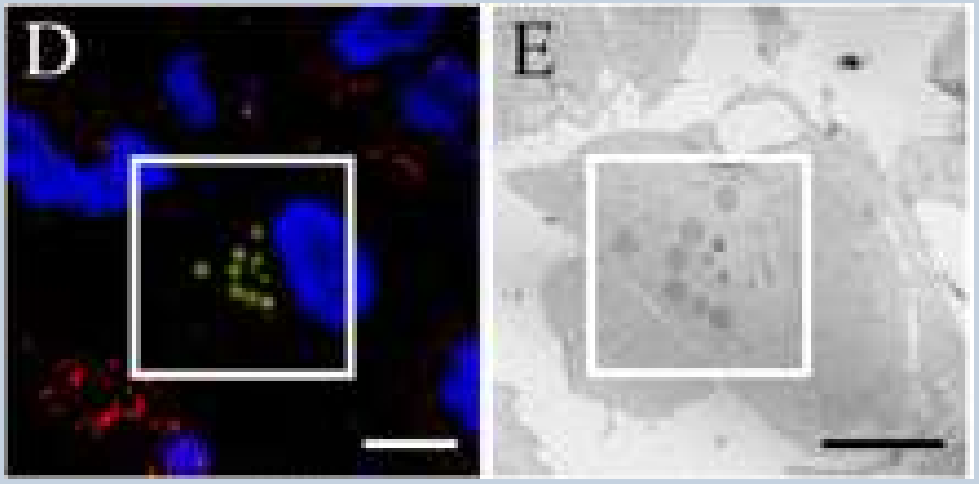
Multi-lysosomal body (A.J. Koster and W.J.C. Geerts, Utrecht University)

**3D tomography**

**Protein localisation**



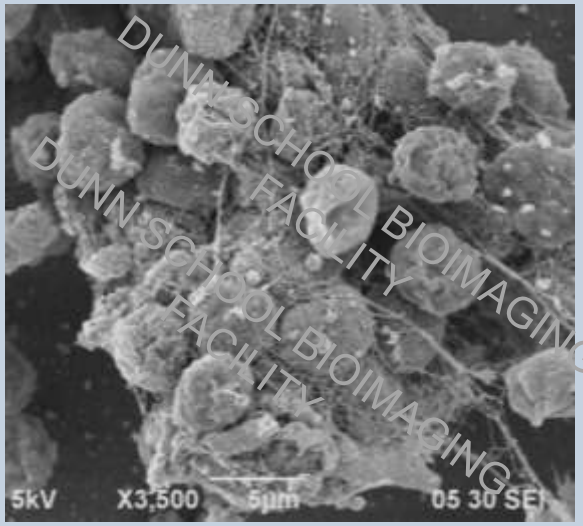
Staining for APEX-tagged mitochondrial protein
(T Derrinck & K Martell)



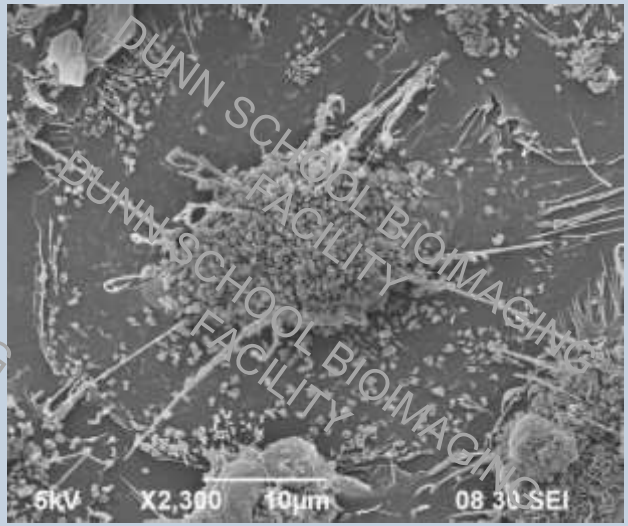

Correlative light and electron microscopy of cryo-sections (Vicidomini et al. Traffic 9:1828–1838, 2008)

**Correlative microscopy**

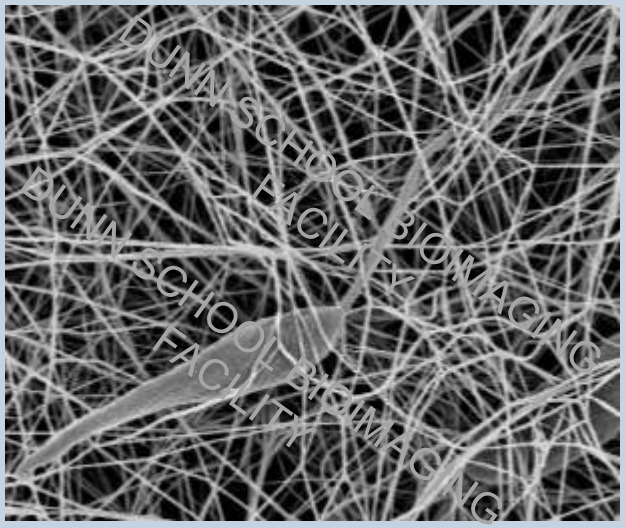
# Biological Applications
## *SEM – Morphology*




*Maleria-infected red blood cells*
*(D Llewellyn/E Johnson)*

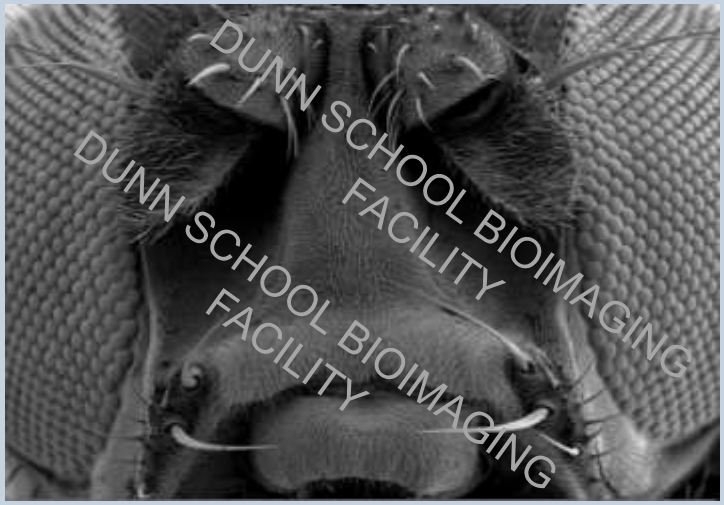



*HIV-infected macrophage & T-cell interaction*
*(C Duncan/E Johnson)*

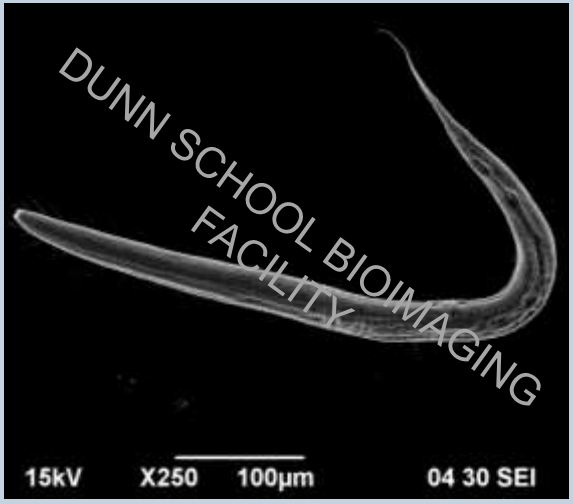



*Leishmania on collagen*
*(R Jain/E Johnson)*

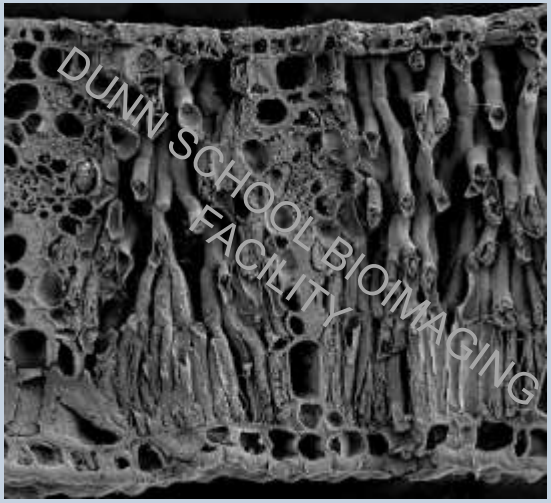
# Biological Applications
## *SEM – Morphology*




*Drosophila (E Johnson)*



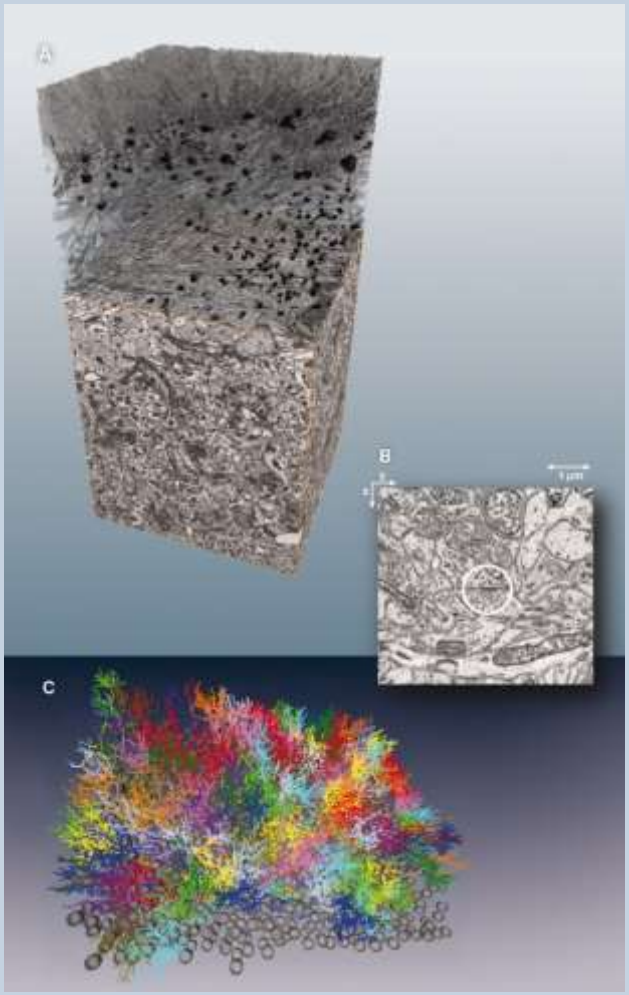

*C elegans (A Moloney/E Johnson)*



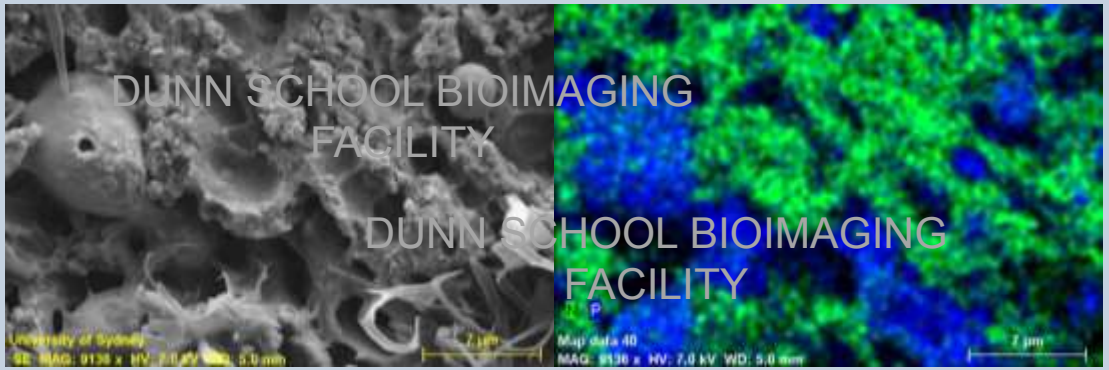

*Cross section of a spinach leaf (E Johnson)*

# Biological Applications
## *SEM - Advanced*

**3D tomography**



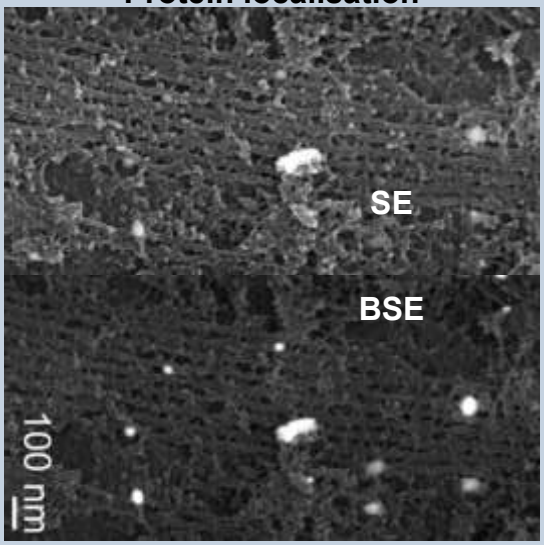
Moritz Helmstaedter, Max-Planck Institute for Medical Research




**Chemical composition**

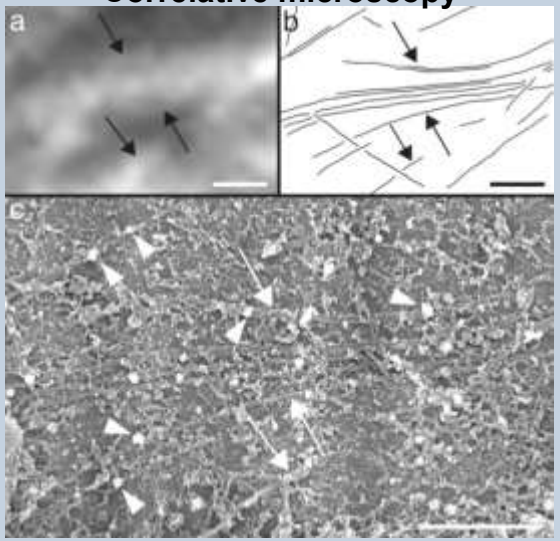
EDS analysis of cheese (M Foley, University of Sydney)

**Protein localisation**




Microtubules (D Barton, University of Sydney)

**Correlative microscopy**




Microtubules (D Barton, University of Sydney)

# EM Facilities @ The University of Oxford
## *Overview*

- Oxford Particle Imaging Centre (OPIC)
  - Henry Wellcome Building for Particle Imaging
  - Website: http://www.opic.ox.ac.uk
  - Biosafety containment (ACDP3/DEFRA4)
  - Electron cryo-microscopy and tomography

*Tecnai Polara Cryo-FEG-TEM, OPIC*

- Materials Department
  - Parks Rd & Begbroke Science Park
  - Website: http://www-em.materials.ox.ac.uk/
  - Large EM unit


*Zeiss Auriga FIB-SEM, Materials Dept*

- Dunn School of Pathology

# EM Facilities @ The University of Oxford

## *Dunn School Bioimaging Facility*


*http://web.path.ox.ac.uk/~bioimaging//bioimaginghome.html*

**Light Microscopy**
alan.wainman@path.ox.ac.uk
(Raff Group) Room 214.10.25
Ph: 01865 275531

**Electron Microscopy**
errin.johnson@path.ox.ac.uk
Room 214.00.21
Ph: 01865 285742

# EM facilities @ Oxford Uni

## *Dunn School Bioimaging Facility - EM*

**Biological Specimen Preparation Laboratory**

- **TEM**
  - Edwards Auto306 Coating unit
  - Glass knifemakers (2x LKB & KMR3)
  - Leica UC7 & Reichert UCE ultramicrotomes
  - Leica EMPACT2 HPF
  - Leica AFS1 & AFS2 units
  - Leica UCS cryo-ultramicrotome
- **SEM**
  - Touismis AutoSamdri CPD
  - Quorum Tech Dual ES Coater

**FEI Tecnai12 TEM**

- Accelerating voltage: up to 120 kV
- Lanthanum hexaboride (LaB6) crystal
- Resolution: ~1 nm (120 kV)
- Magnification: 440x to 300,000x
- Single tilt specimen holder
- Gatan Dual Orientation specimen holder
- 4 Megapixel Gatan Ultrascan™ 1000 CCD camera, plus plate camera with film

**JEOL JSM-6390 SEM**

- Accelerating voltage: 0.5 kV to 30 kV
- Tungsten filament
- Resolution: ~10 nm (5 kV)
- Magnification: 63x to 300,000x
- Secondary electron detector only
- Maximum specimen diameter: 150 mm
- Frame size: up to 2560 x 1920 pixels

# EM facilities @ Oxford Uni

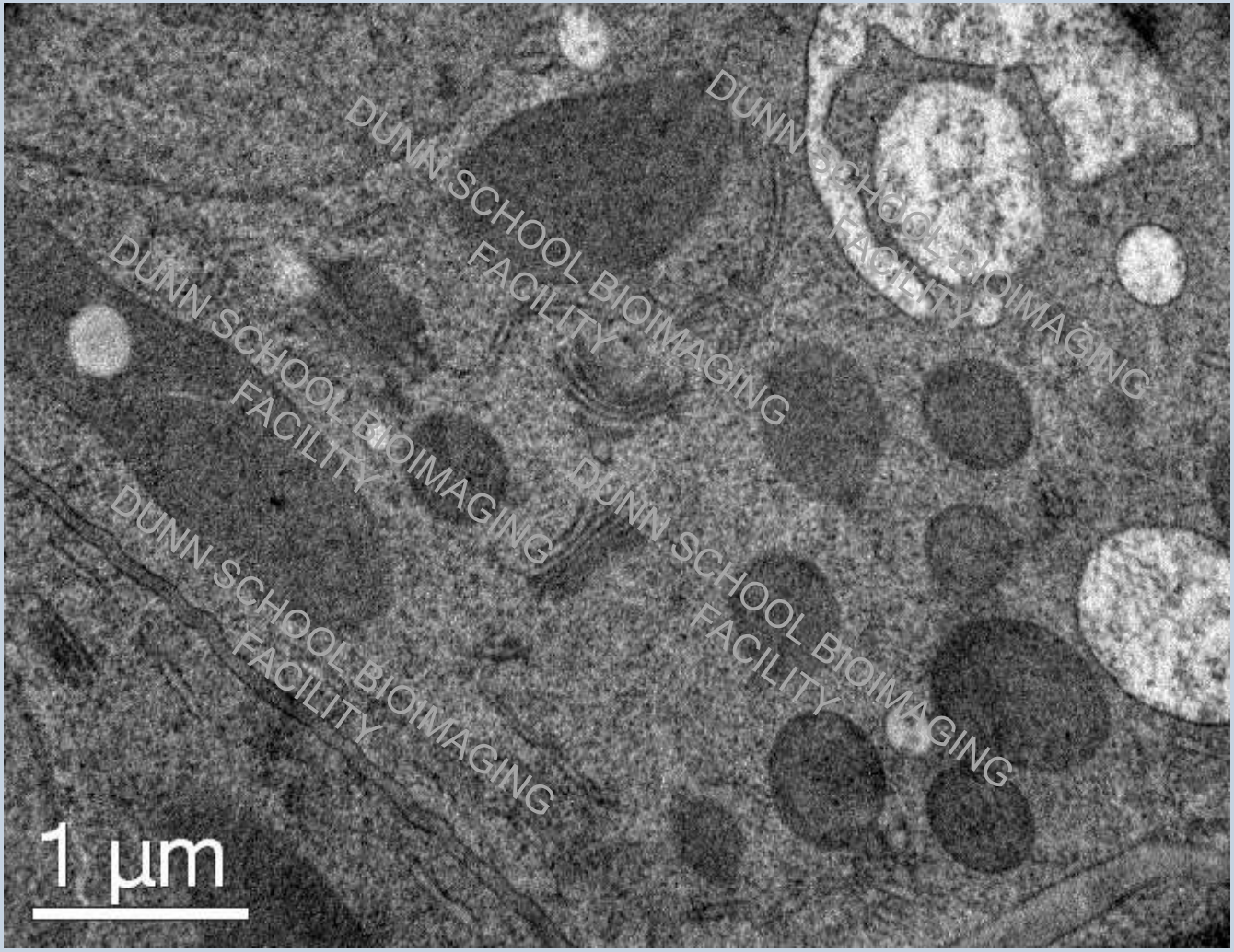
## *Dunn School Bioimaging Facility - EM*

- Multi-user facility with three modes of usage:
  - Independent
    - Medium to long-term projects from research institutions only
    - User is fully trained to use relevant microscopes & equipment
    - Errin available to help with troubleshooting and image analysis
    - Cost: consumables & instrument time
  - Service
    - One-off/short-term projects from research institutions only
    - Specimen preparation and/or microscopy performed by Errin
    - Cost: technician time (£25/hr), consumables & instrument time
  - Collaborative
    - Technique development, performed by Errin
    - Cost: consumables and instrument time

| Type of sample prep | Price |
|---|---|
| Negative staining | £1/sample |
| Standard TEM prep | £5/sample |
| Cryo-TEM prep | £6/sample |
| SEM prep | £4/sample |

| Instrument | Price |
|---|---|
| Tecnai12 TEM | £35/hr |
| JEOL 6390 SEM | £25/hr |
| High Pressure Freezer (HPF) | £30/hr |
| Automated Freeze-substitution Units (AFS) | £132/run |
| Dual Carbon/Sputter Coater | £10/run |
| Critical Point Dryer (CPD) | £20/run |
| Ultramicrotomes | £20/hr |

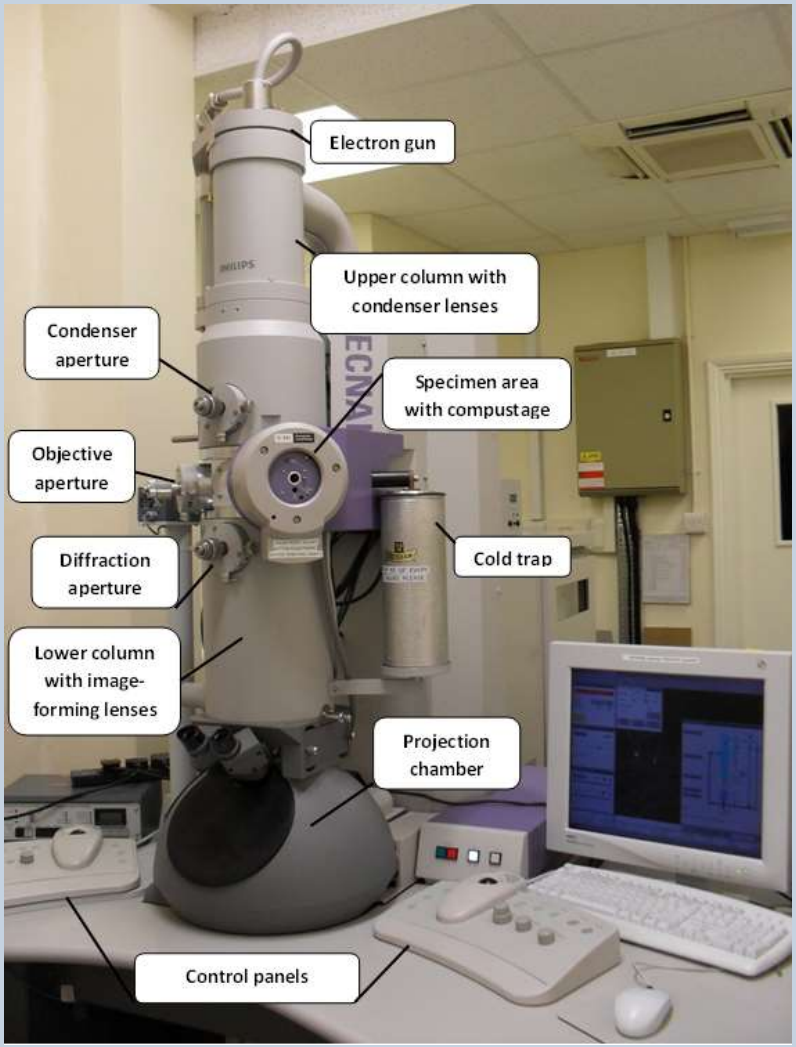
# Transmission Electron Microscopy (TEM)

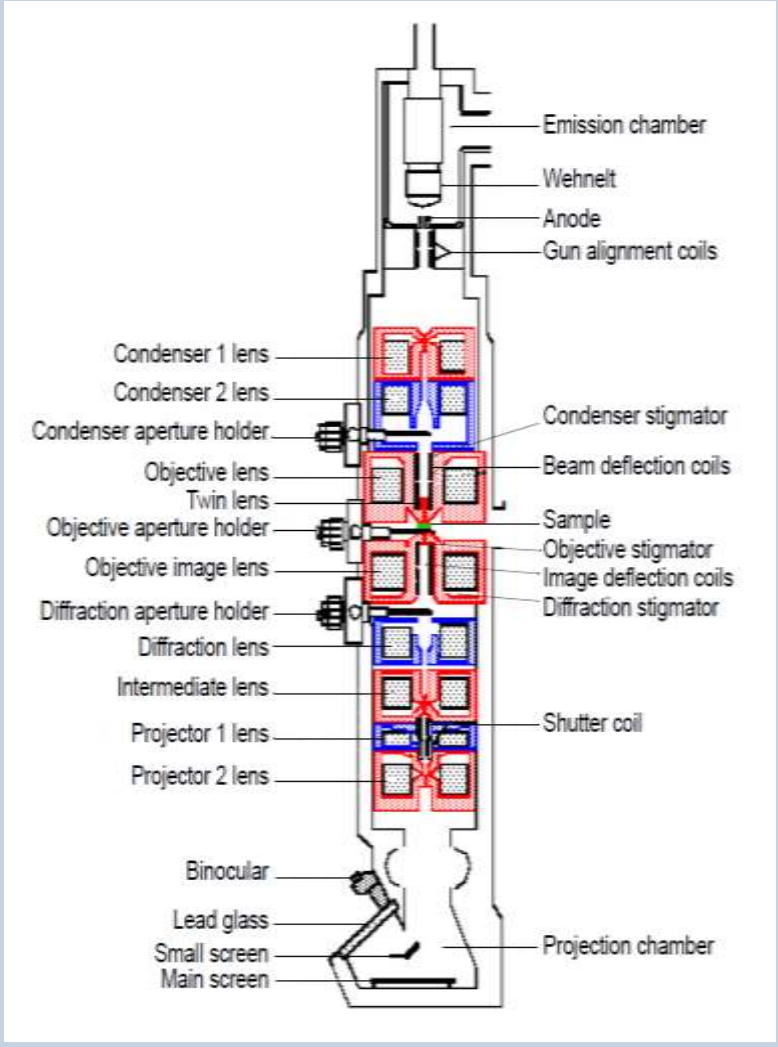



*Arabidopsis root tip cell , JEOL 1400 TEM, (E Johnson)*

# How the TEM Works

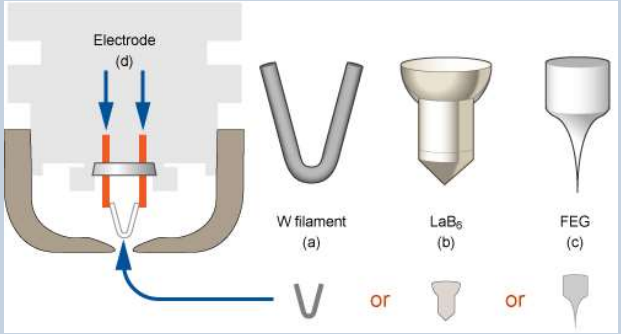
## *Overview*



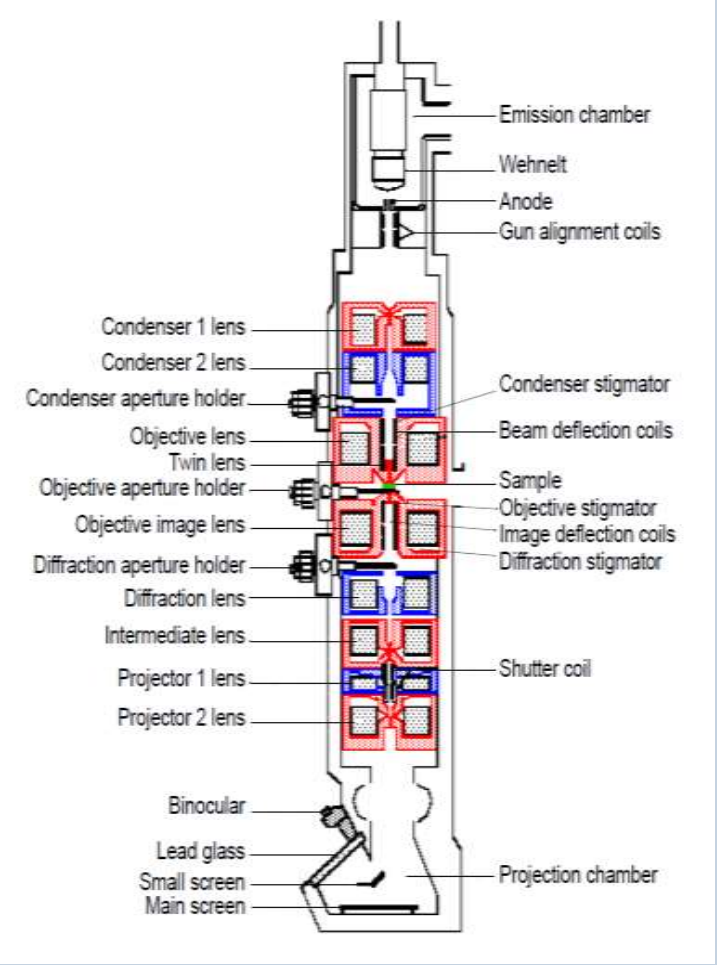



# How the TEM Works
## *Overview – Electron gun*

- Resolution depends on a number of factors, including the accelerating voltage and the type of electron source used
- Electron sources are typically Tungsten or LaB6 and can be thermionic or field emission (FEG)
- Accelerating voltage (kV) is typically 80-120 kV for biological specimens

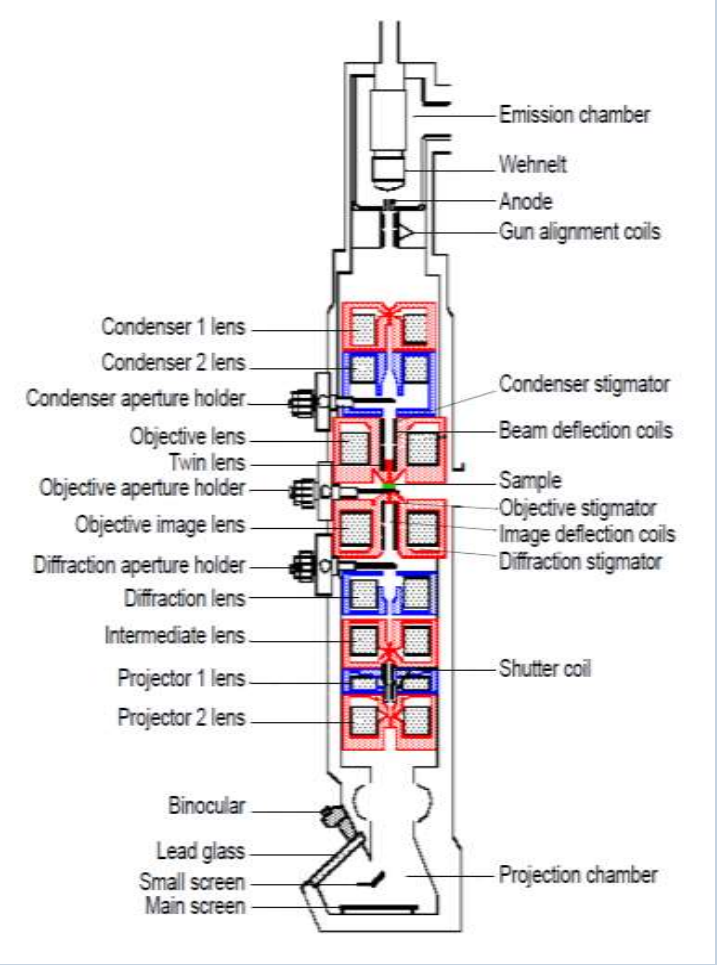



*www.ammrf.org*



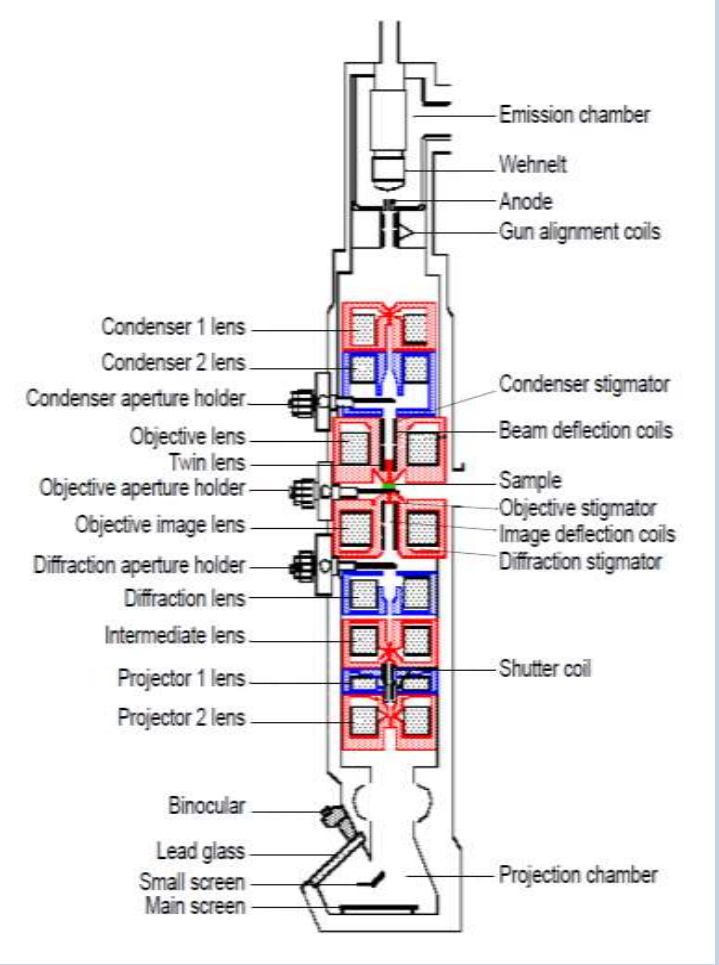
# How the TEM Works
## *Overview – Condenser lens*

- The condenser lens system focuses the emitted electrons into a coherent beam.
  - The first condenser controls the spot size of the beam. This is controlled by the spot size setting in the TEM software.
  - The second condenser focuses the beam onto the sample (this is controlled by the 'brightness' knob on the microscope).
- The condenser aperture restricts the beam by excluding high angle electrons. Usually a middle sized condenser aperture is suitable.



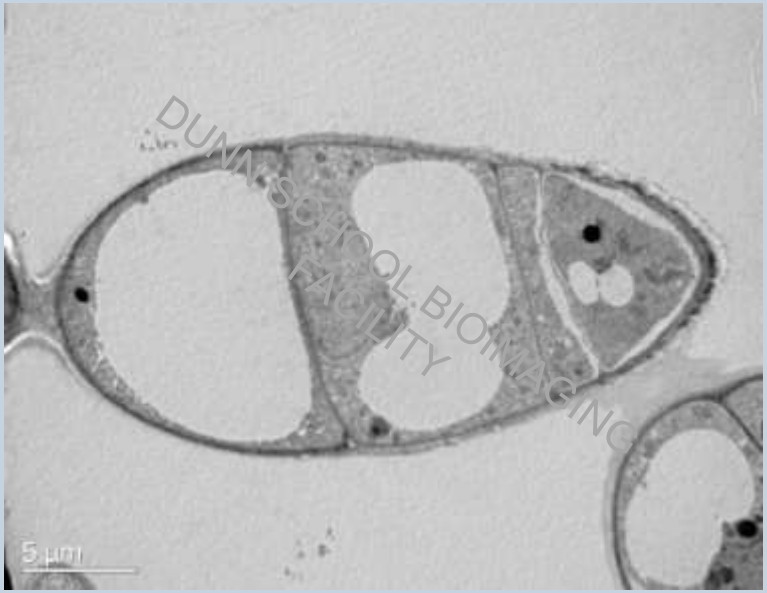
# How the TEM Works
## *Overview – Imaging lenses*

- The objective lens focuses the electrons transmitted through the sample into a magnified image.
  - The objective aperture can be used to increase contrast by excluding high angle transmitted electrons.
- The intermediate and projection lenses enlarge the image. When the electrons hit the phosphorescent screen, it generates light which allows the human eye to view it.
- Images can be acquired using a high resolution CCD camera or with film

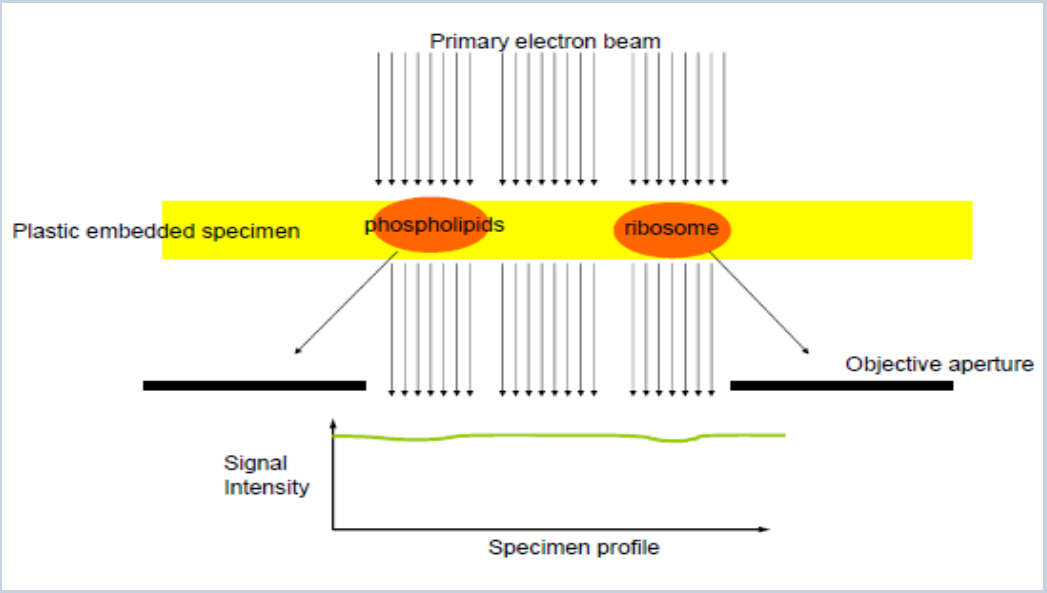


# How the TEM Works
## *Overview – Contrast*

- Contrast is generated by density differences within the sample, just as in LM.
- Darker areas in the image are where few electrons have been transmitted through the sample, due to thickness or high atomic number.




*Lavender trichome, E Johnson*



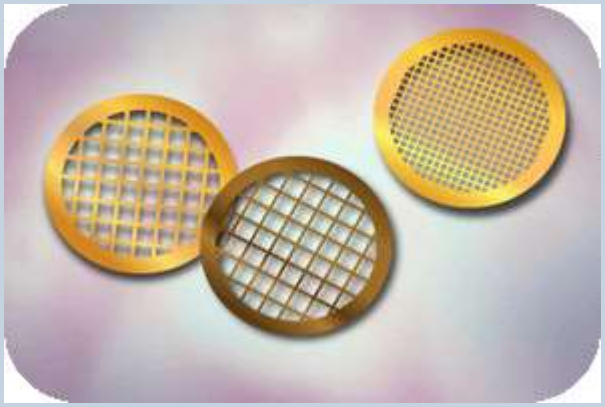
# Specimen Preparation for TEM
## *Overview*

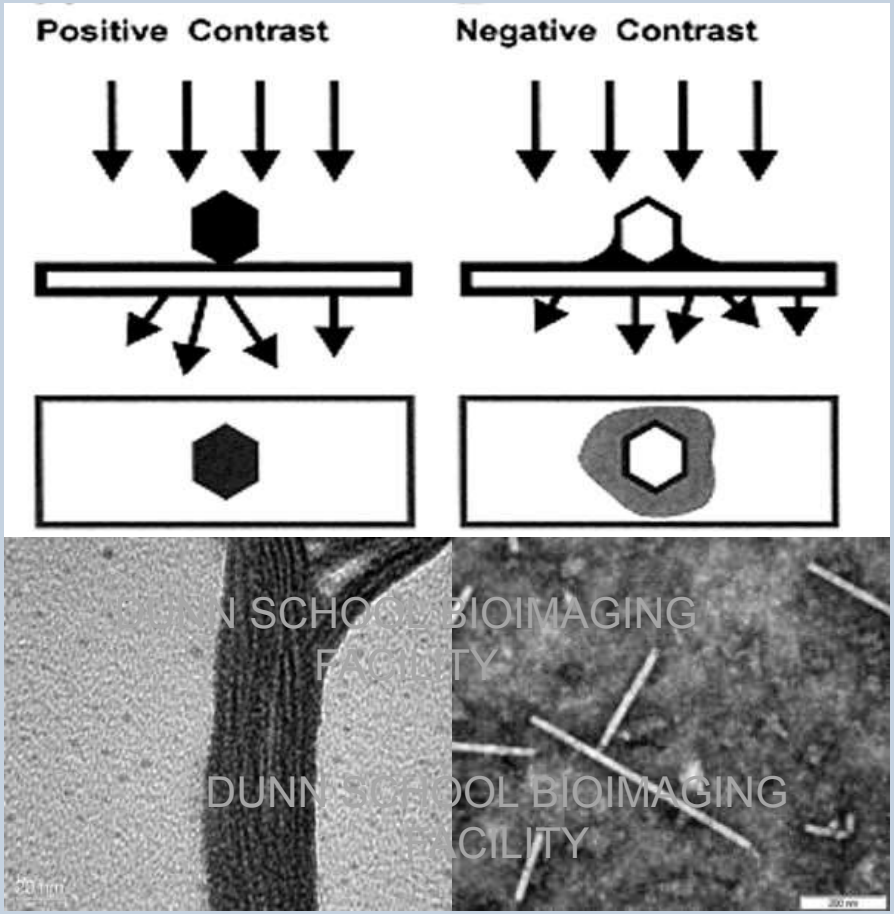
- TEM specimens must be:
  - Very thin
  - Well preserved
  - Electron dense
  - Stable in the vacuum
  - ie; everything that most biological samples are not!


- The degree of specimen preparation for biological TEM depends on the specimen
  - Particulate samples (eg: protein and viruses) can be stained and viewed quickly
  - Cells and tissue samples require extensive preparation for TEM

# Specimen Preparation for TEM
## *Particulate samples*

- **Negative Staining:**
  - Coat grids with plastic film and carbon
  - Apply the particulate specimen
  - Stain with heavy metal solution, eg: uranyl acetate, phosphotungstic acid, sodium silicatungstate
  - Blot dry and view in the TEM





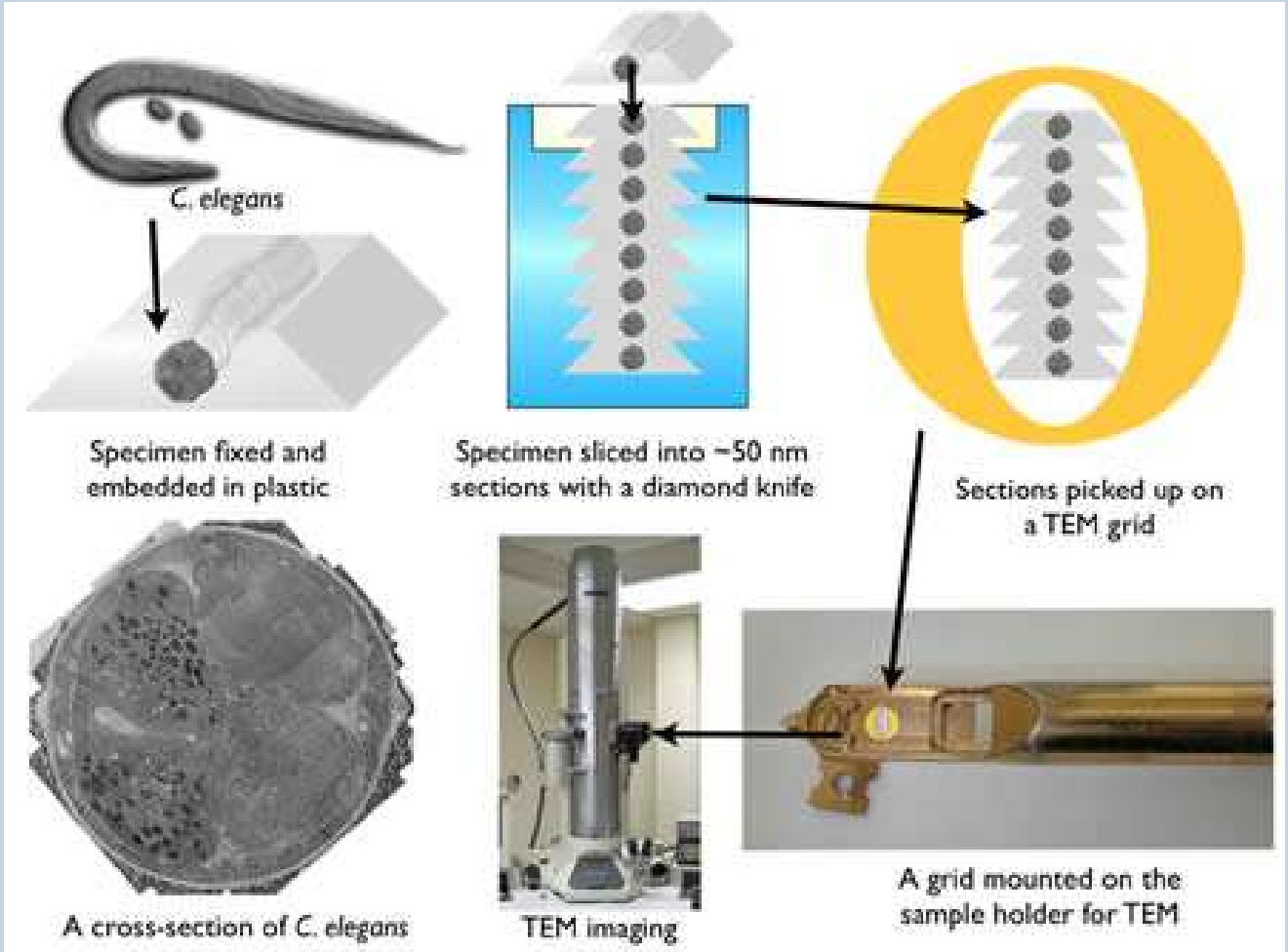

*Bacterial protein stained with uranyl acetate; Tobacco mosaic virus negatively stained with sodium silicotungstate (E. Johnson)*

# Specimen Preparation for TEM

## *Cells & Tissue – Overview*

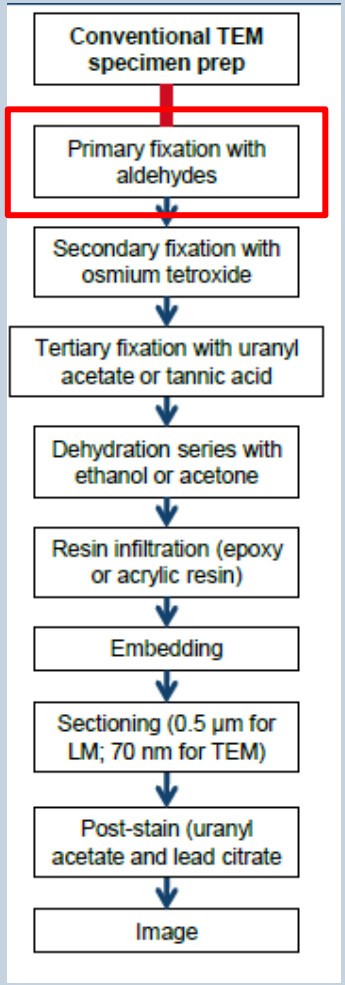
**Conventional TEM specimen prep**

- Primary fixation with aldehydes
- Secondary fixation with osmium tetroxide
- Tertiary fixation with uranyl acetate or tannic acid
- Dehydration series with ethanol or acetone
- Resin infiltration (epoxy or acrylic resin)
- Embedding
- Sectioning (0.5 µm for LM; 70 nm for TEM)
- Post-stain (uranyl acetate and lead citrate
- Image

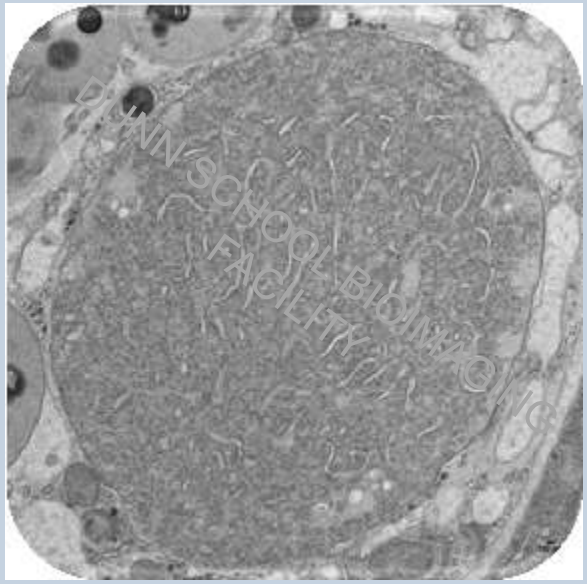



*http://www.research.utah.edu/advanced-microscopy/education/electron-micro/index.html*

# Specimen Preparation for TEM
## *Cells & Tissue – Primary Fixation*

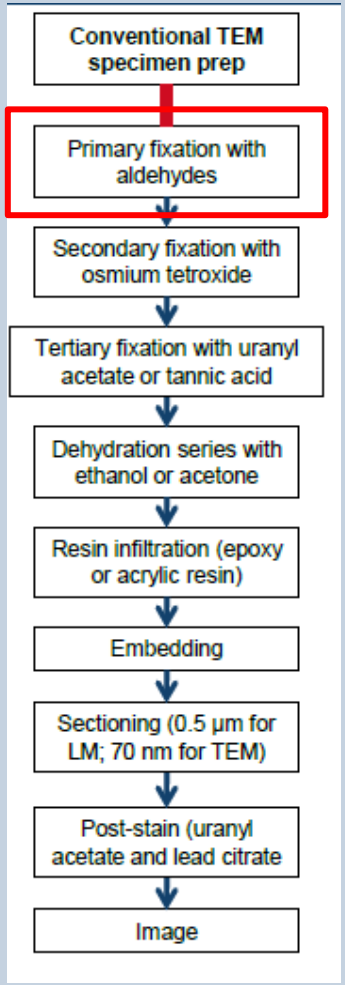



- Fixation stops cellular processes and aims to preserve the specimen as close as possible to its natural state.
- Characteristics of a good fixative:
  - Permeates cells readily and acts quickly
  - Is irreversible
  - Does not cause fixation artifacts
- Methods of fixation include:
  - Chemical fixation with aldehydes
  - Cryo-fixation with liquid nitrogen
  - Microwave fixation

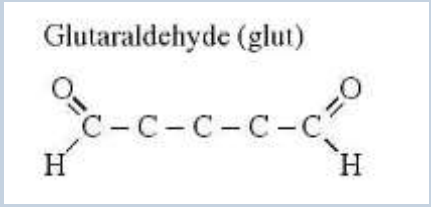


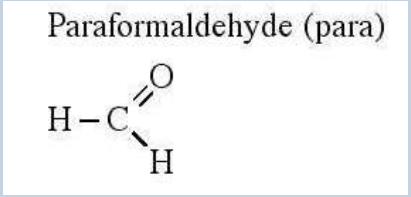

*C elegans, A Moloney/E Johnson*

# Specimen Preparation for TEM
## *Cells & Tissue – Chemical Fixation*




- Glutaraldehyde quickly and irreversibly cross-links proteins via their amino groups. However, it does penetrate tissue quite slowly and is therefore often used in combination with paraformaldehyde.

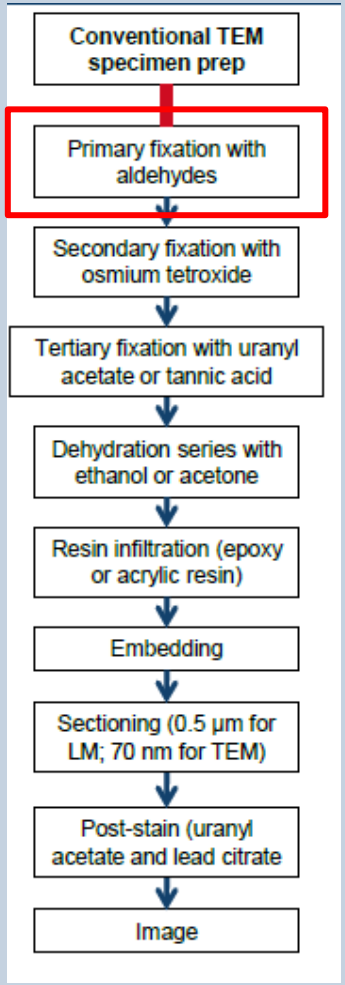


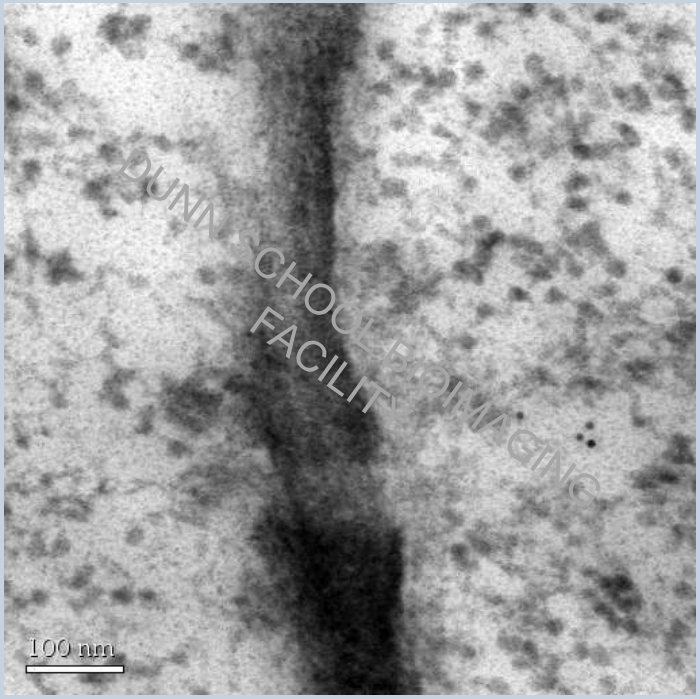

- Paraformaldehyde reversibly cross-links proteins, but is a small molecule and penetrates tissue quite quickly.
- Standard TEM fix: 2.5% glutaraldehyde + 2-4% PFA for 30 mins to overnight.

# Specimen Preparation for TEM

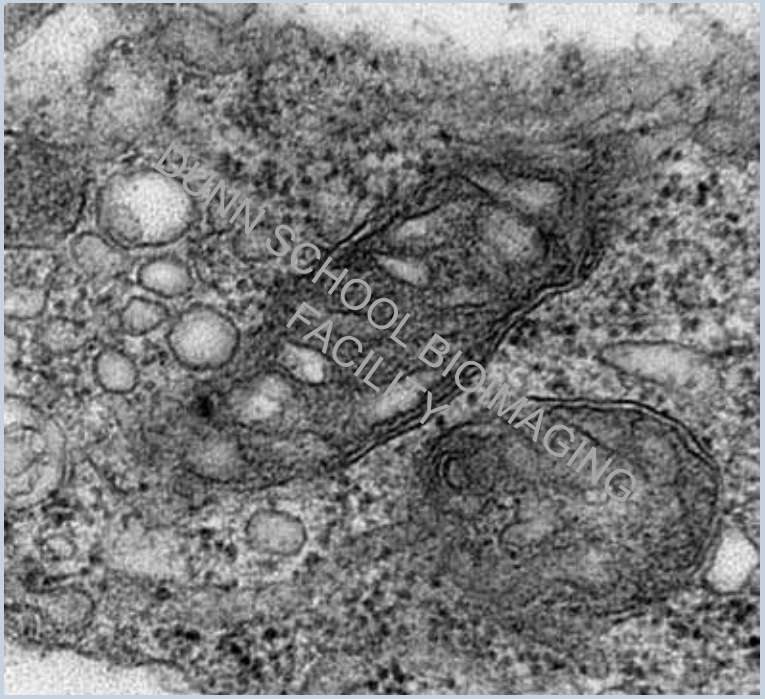
## *Cells & Tissue – Chemical Fixation artifacts*




**Loss of membranes**




**Deformed mitochondria**

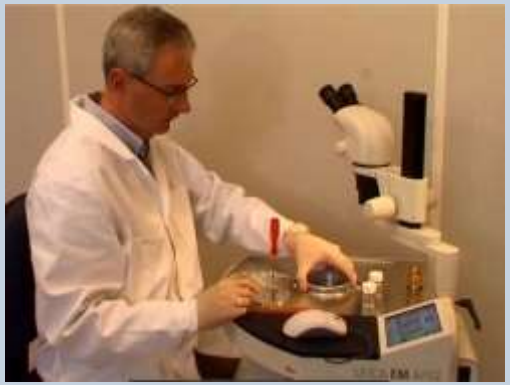


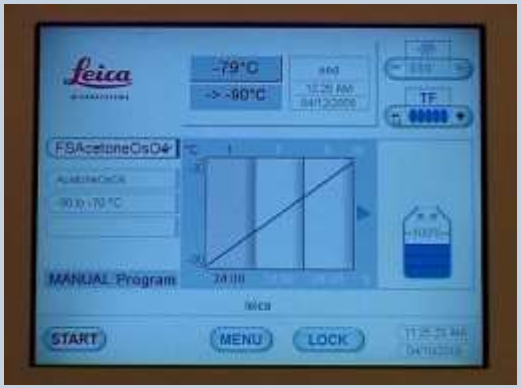
# Specimen Preparation for TEM
## *Cells & Tissue – Cryo-fixation*

- Tissue can be cry-fixed using $LN_2$ in the High Pressure Freezer and then further processed for TEM (adds 1 week)
- Specimens are mounted into specimen carriers and cryo-fixed 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.
- **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

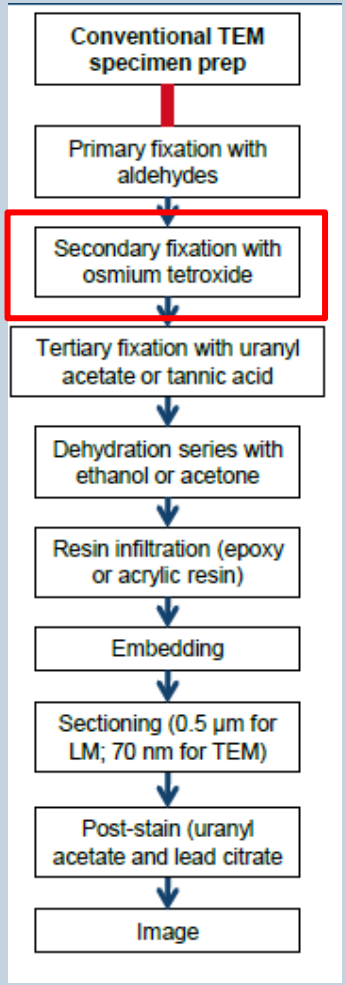




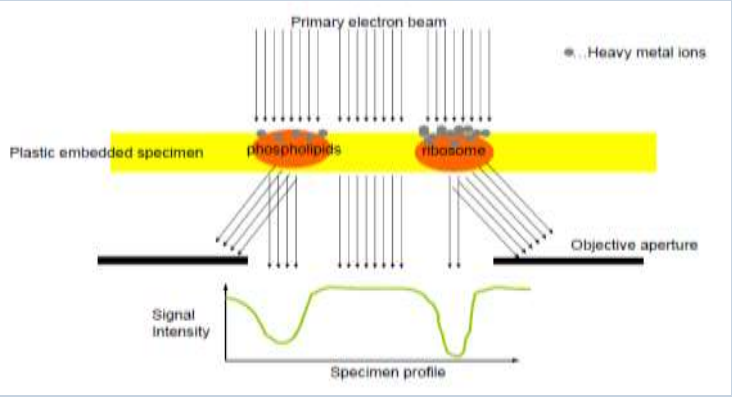


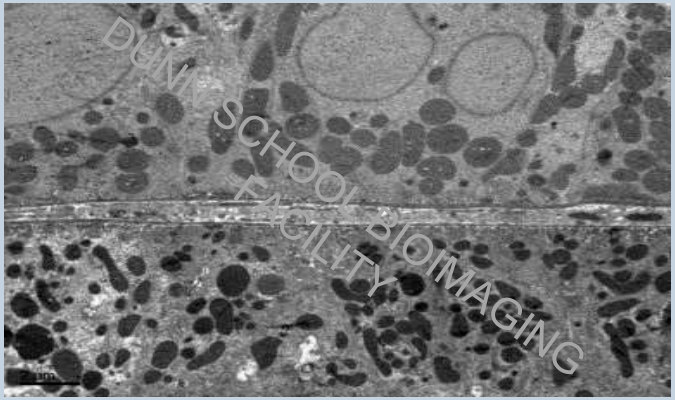
*Cryo-fixed root cell, E Johnson*

# Specimen Preparation for TEM
## *Cells & Tissue – Secondary Fixation*




- Osmium tetroxide (very toxic!) is a heavy metal that fixes unstaturated lipids and is also electron dense.
- Used as both a secondary fixative and an electron stain and significantly improves specimen preservation (especially membranes) and contrast.

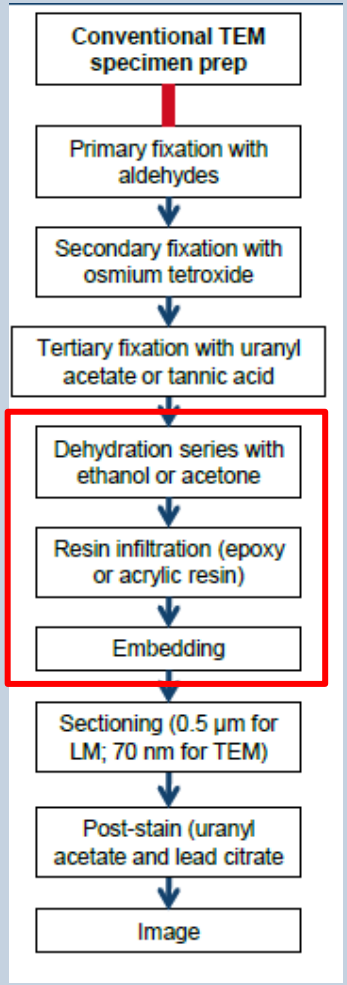



*Microwave processed liver tissue, E Johnson*

# 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**

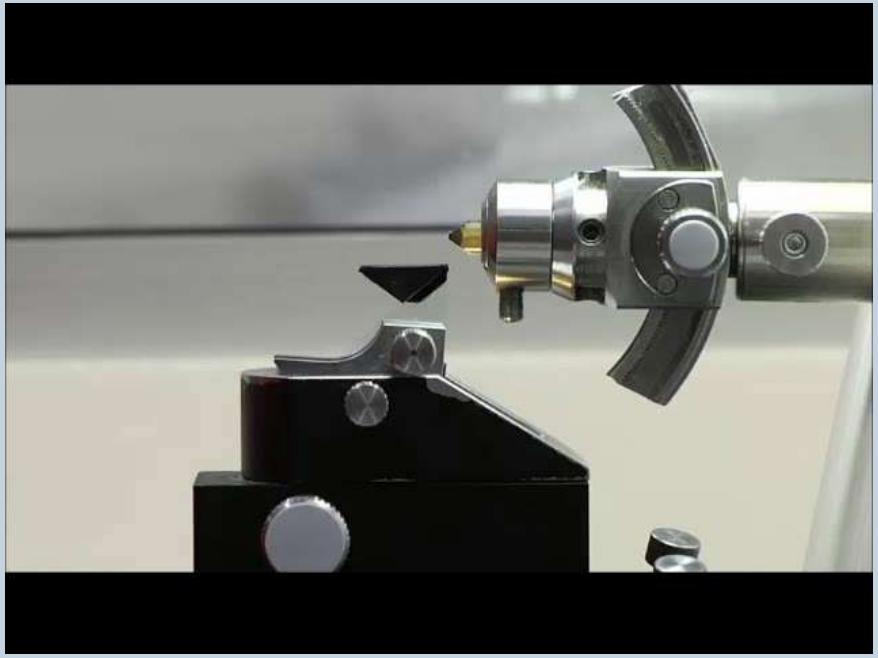
# Specimen Preparation for TEM

## *Cells & Tissue - Ultramicrotomy*

**Conventional TEM specimen prep**

- Primary fixation with aldehydes
- Secondary fixation with osmium tetroxide
- Tertiary fixation with uranyl acetate or tannic acid
- Dehydration series with ethanol or acetone
- Resin infiltration (epoxy or acrylic resin)
- Embedding
- Sectioning (0.5 µm for LM; 70 nm for TEM)
- Post-stain (uranyl acetate and lead citrate
- Image

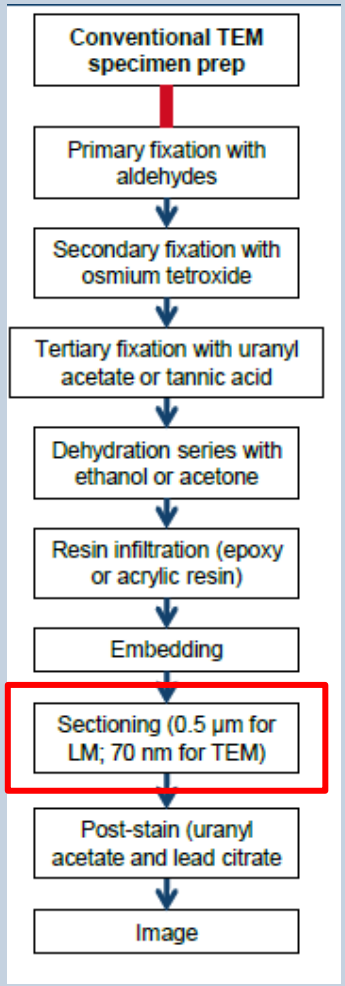
*Leica Ultracut 7 ultramicrotome, Dunn School*

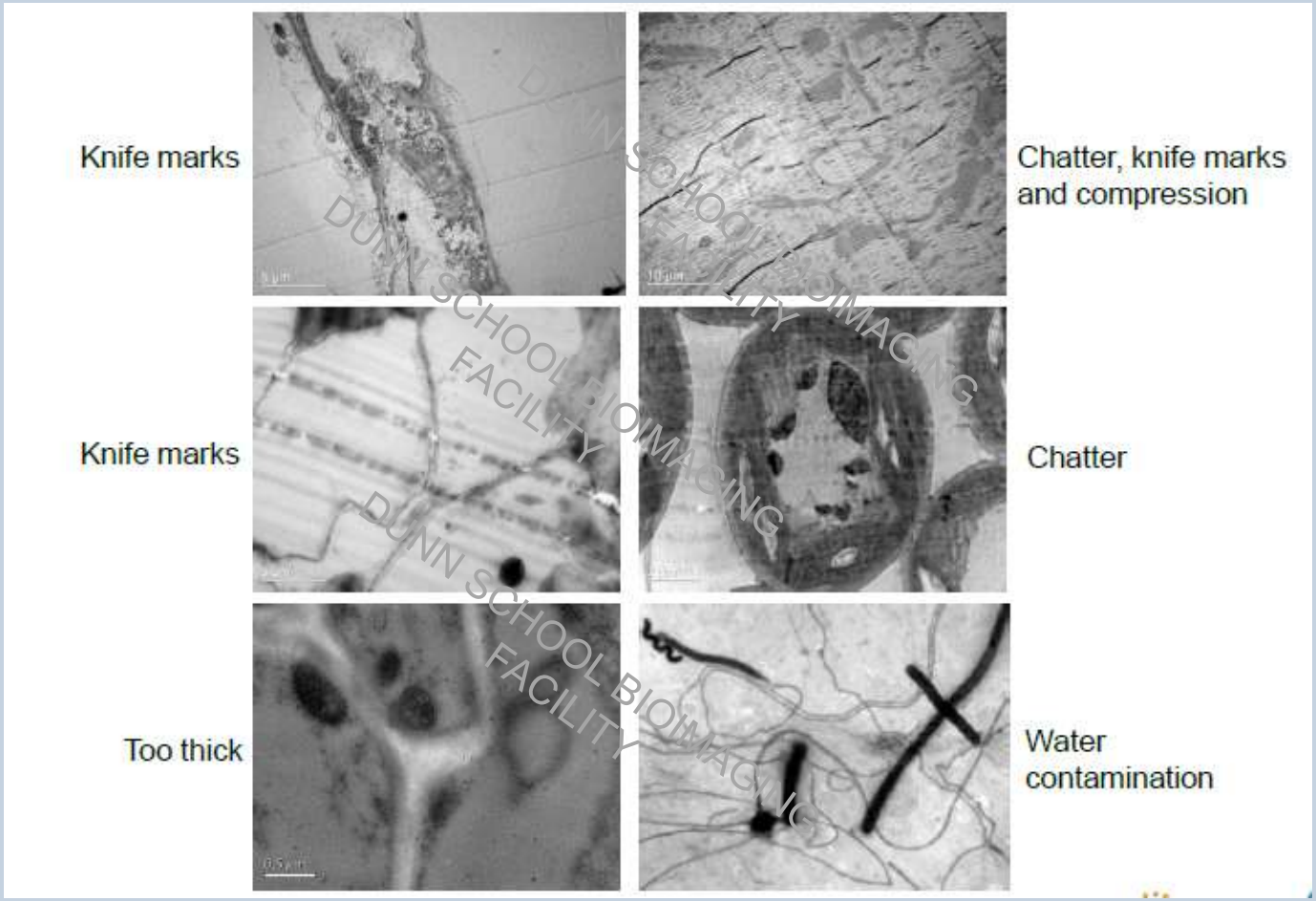


*Introduction to ultramicrotomy video, University of Sydney*

# Specimen Preparation for TEM

## *Cells & Tissue – Ultramicrotomy artifacts*

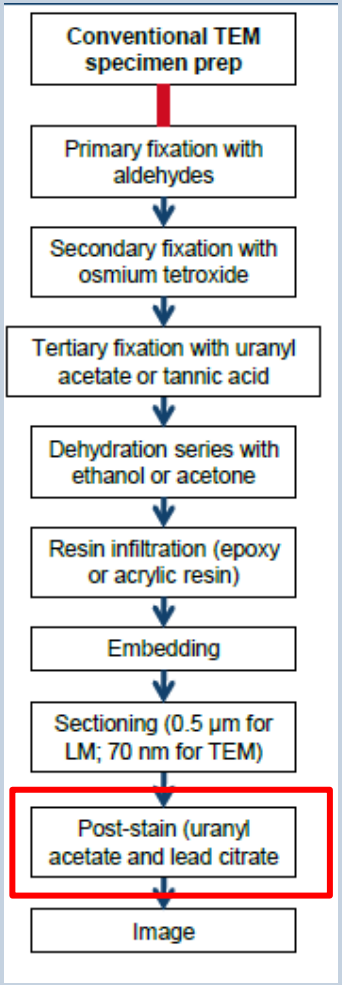



*Images by E Johnson (unfortunately!)*

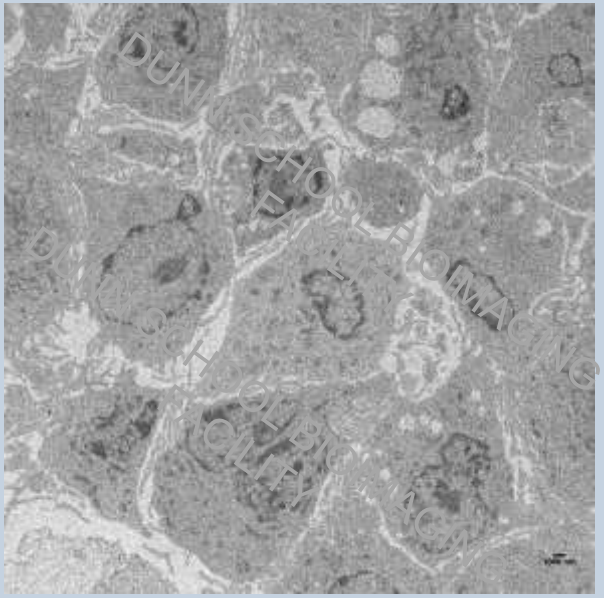
# Specimen Preparation for TEM

## *Cells & Tissue – Post-staining*

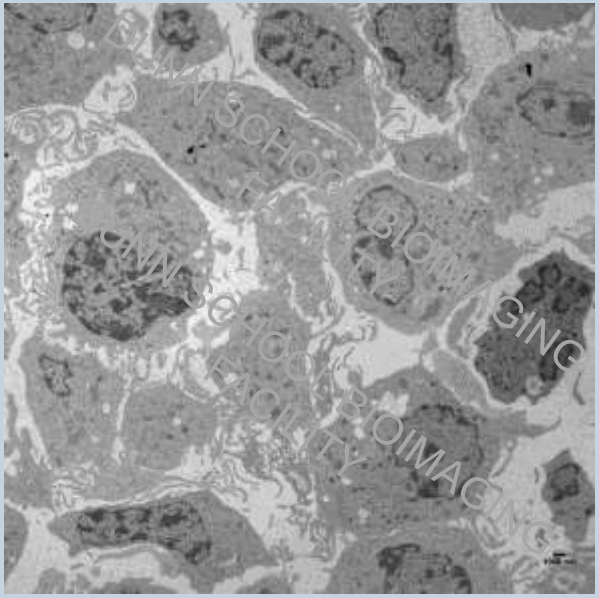



Contrast can be increased by post-staining sections with salts of heavy metals, specifically uranyl acetate and lead citrate solutions. Uranyl acetate stains protein and DNA and also acts as a mordant for lead citrate, which is a more general stain.

**No post-staining**



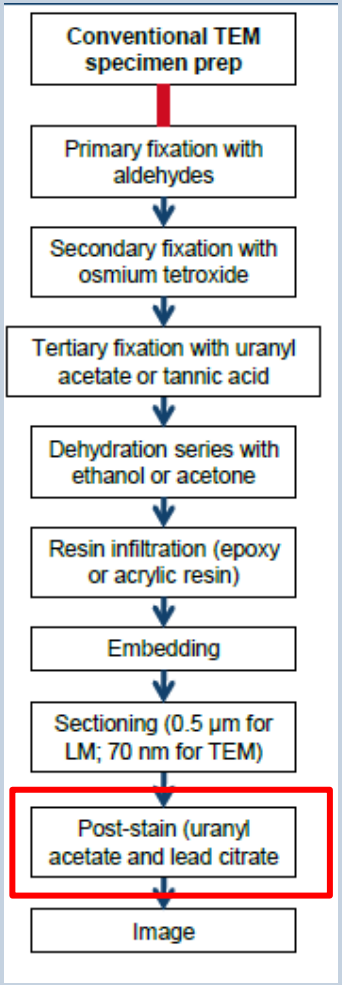
**Post-staining**

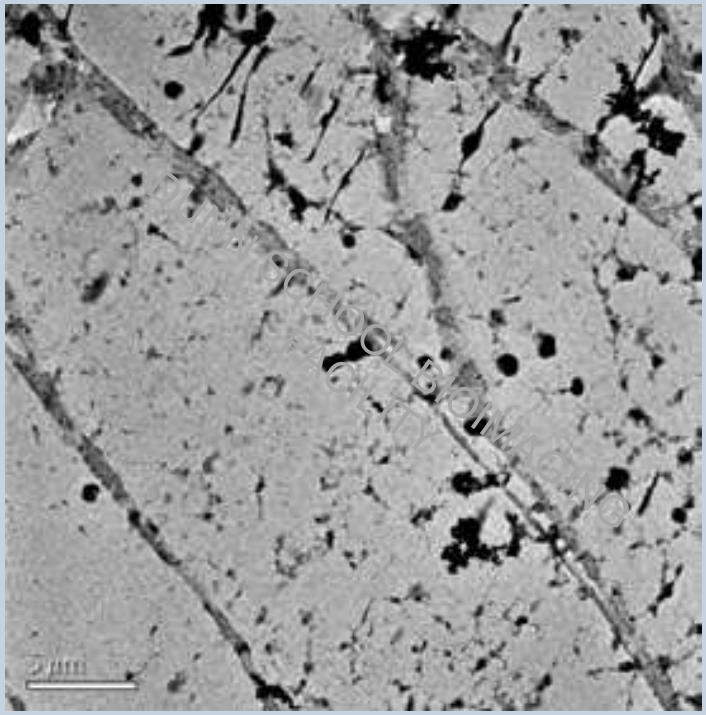


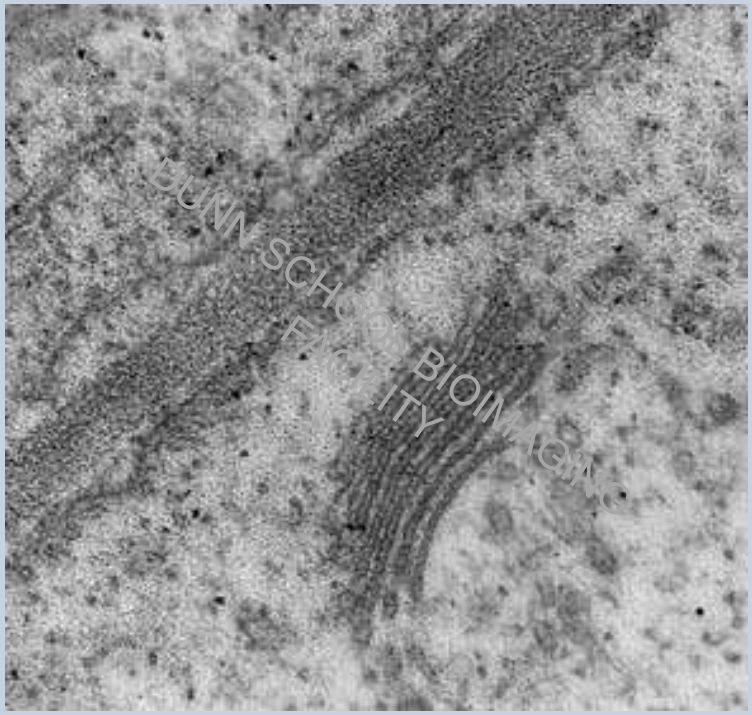
*Dendritic cells (S Hackett/E Johnson)*

# Specimen Preparation for TEM

## *Cells & Tissue – Post-staining artifacts*

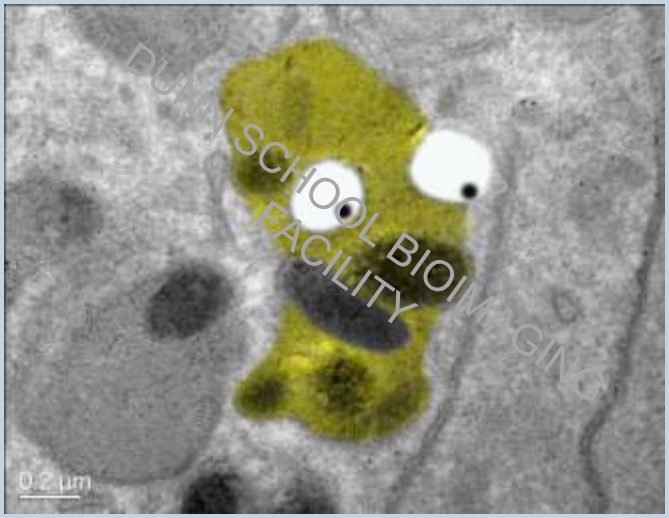


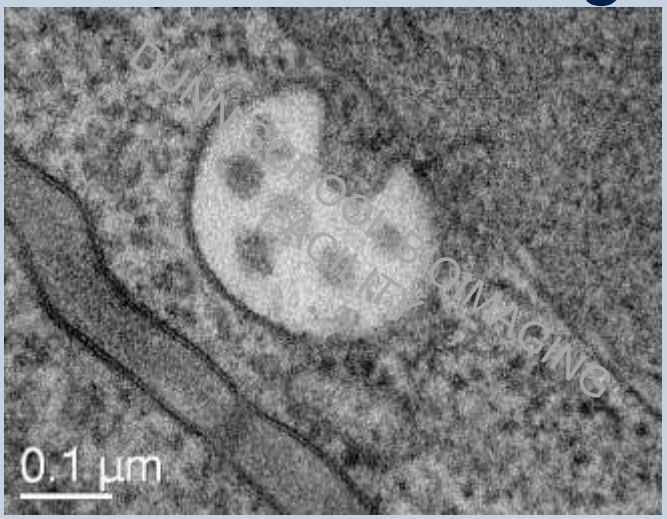



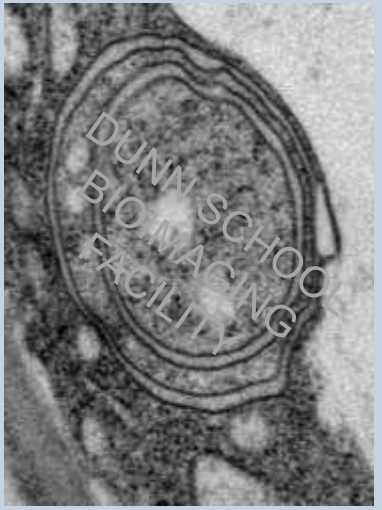


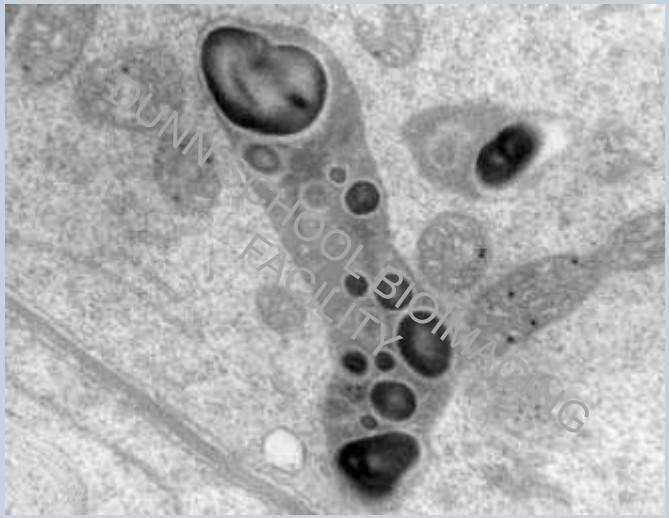
*Images by E Johnson (unfortunately!)*

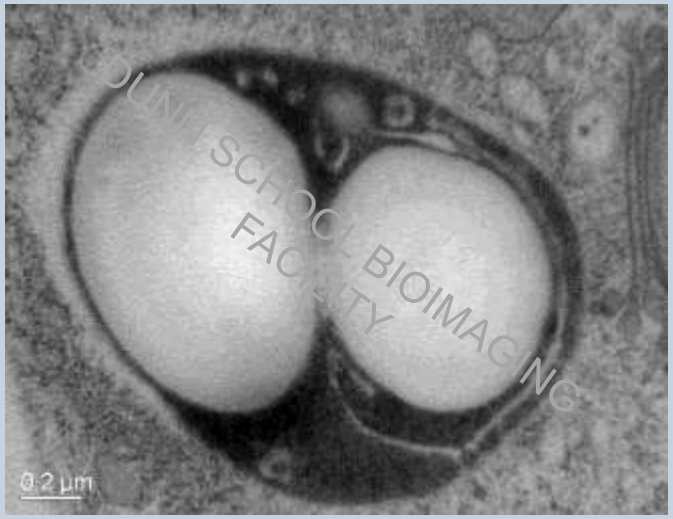
# TEM Specimen Preparation
## *Critical evaluation of images*

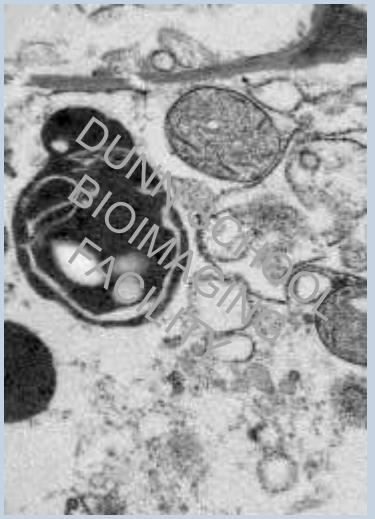


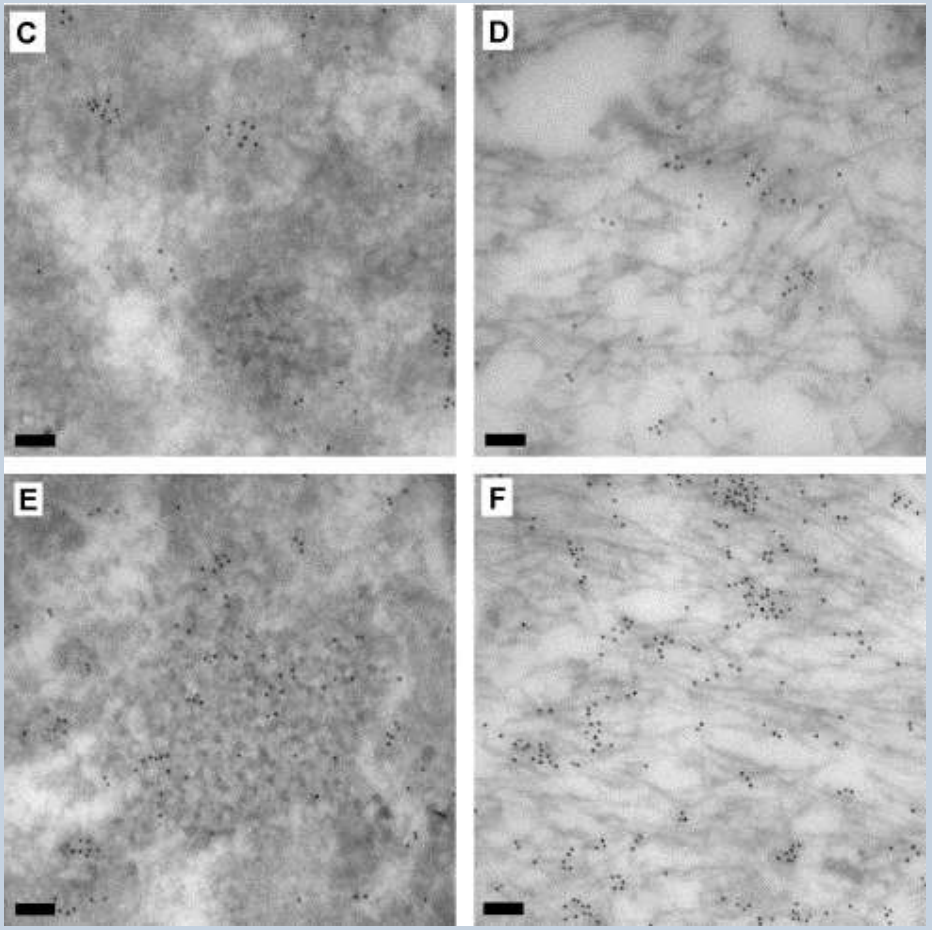




# Specimen Preparation for TEM
## *Advanced techniques - Immunolabelling*

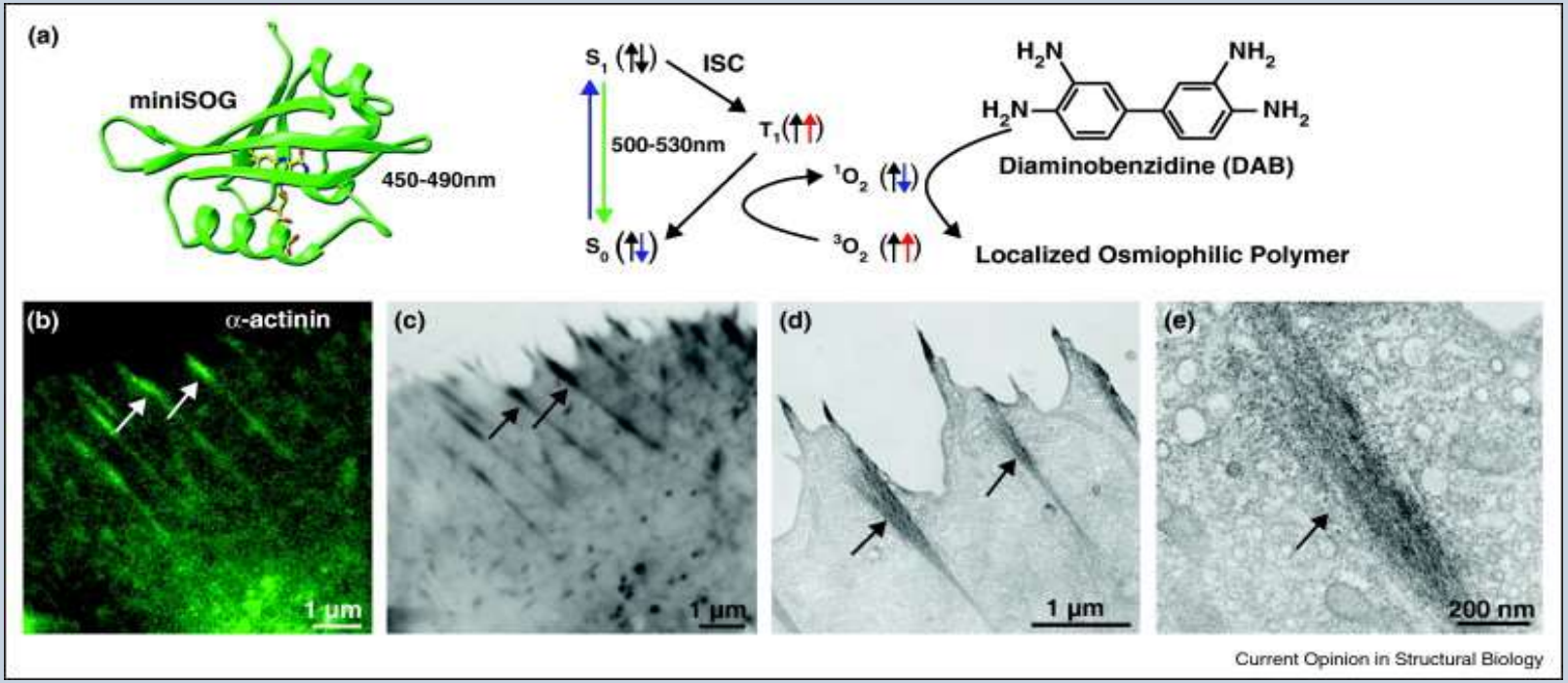
- A lighter chemical fixation is required, as glutaraldehyde affects antigenicity. Cryo-fixation is highly recommended for immunocytochemistry.
- The osmium tetroxide step is omitted (as it also reduces antigenicity), but may be replaced with uranyl acetate instead.
- Epoxy resin does not allow reagent penetration, so acrylic resins are used instead.




Immunolabelling of Pin1 protein in human neurons
(Thorpe et al., 2004 *Neurobiology of Disease,* 17(2): 237-249.

# Specimen Preparation for TEM
## *Advanced techniques – Correlative microscopy*

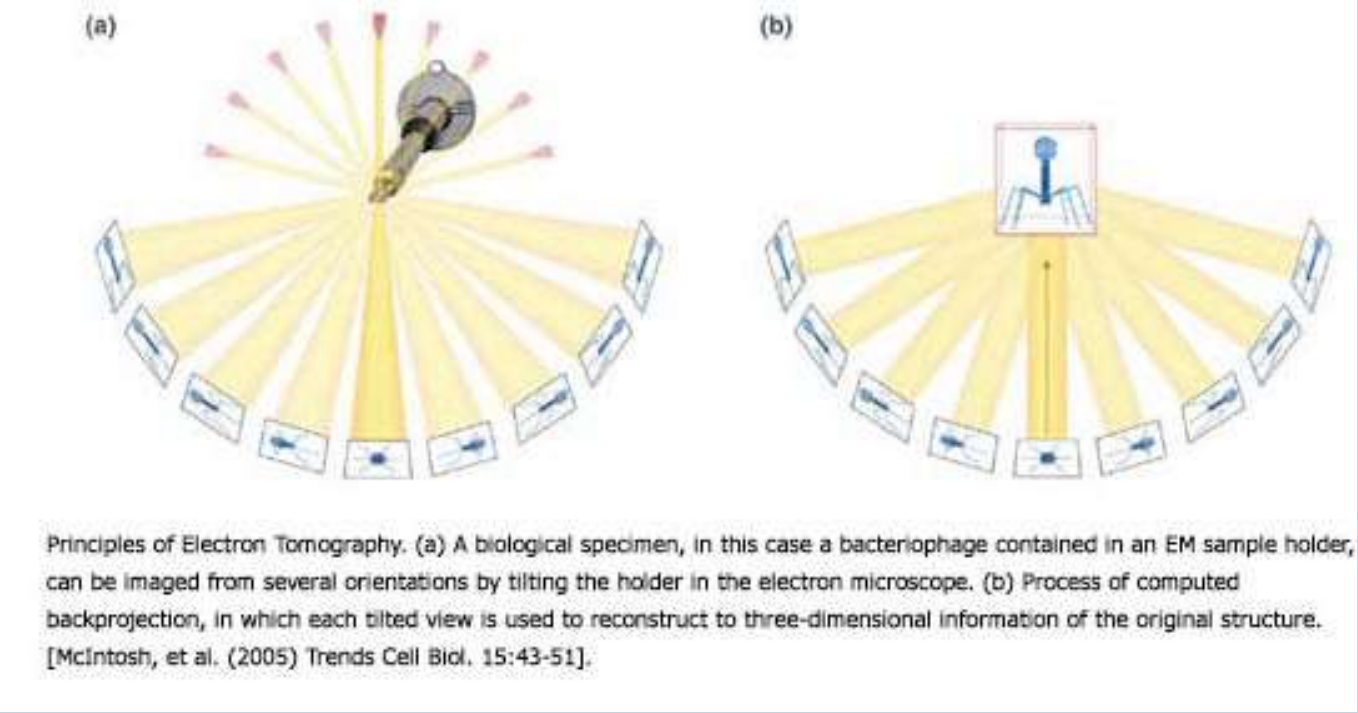
- Tricky, but an emerging field
- Must maintain fluorescence (or use dual antibody or expressed tag), but not at the risk of ultrastructural preservation, while at the same time keeping track of the same cell




*Shu et al (2011) PLoS Biol, 9 (2011), p. e1001041*

# Advanced TEM techniques
## *Electron tomography*

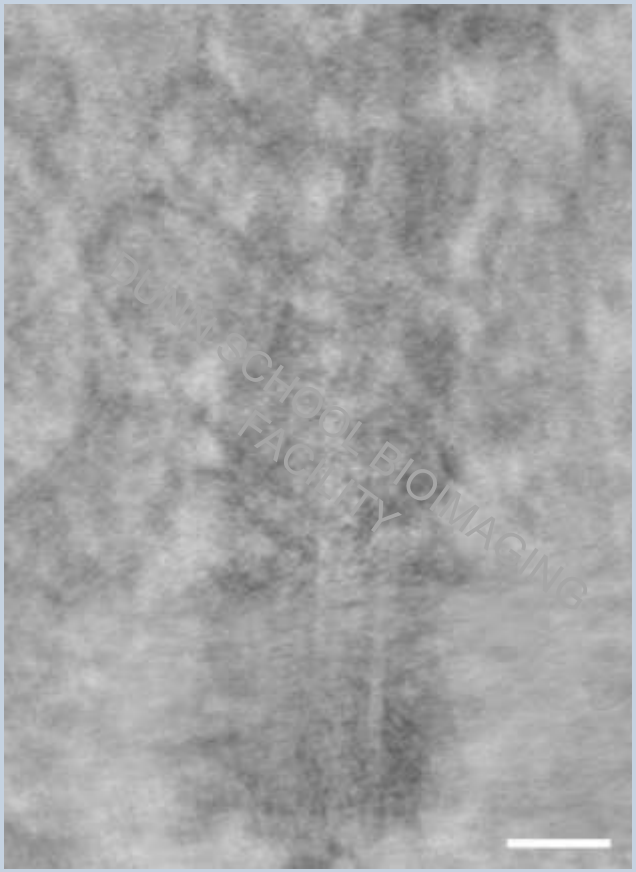
- Thicker sections (150-300 nm) on filmed slot grids with gold fiducial markers
- Use special tomography holder for dual axis tilting of the specimen
- Reconstruct using computer modelling (eg: IMOD)




Principles of Electron Tomography. (a) A biological specimen, in this case a bacteriophage contained in an EM sample holder, can be imaged from several orientations by tilting the holder in the electron microscope. (b) Process of computed backprojection, in which each tilted view is used to reconstruct to three-dimensional information of the original structure. [McIntosh, et al. (2005) Trends Cell Biol. 15:43-51].

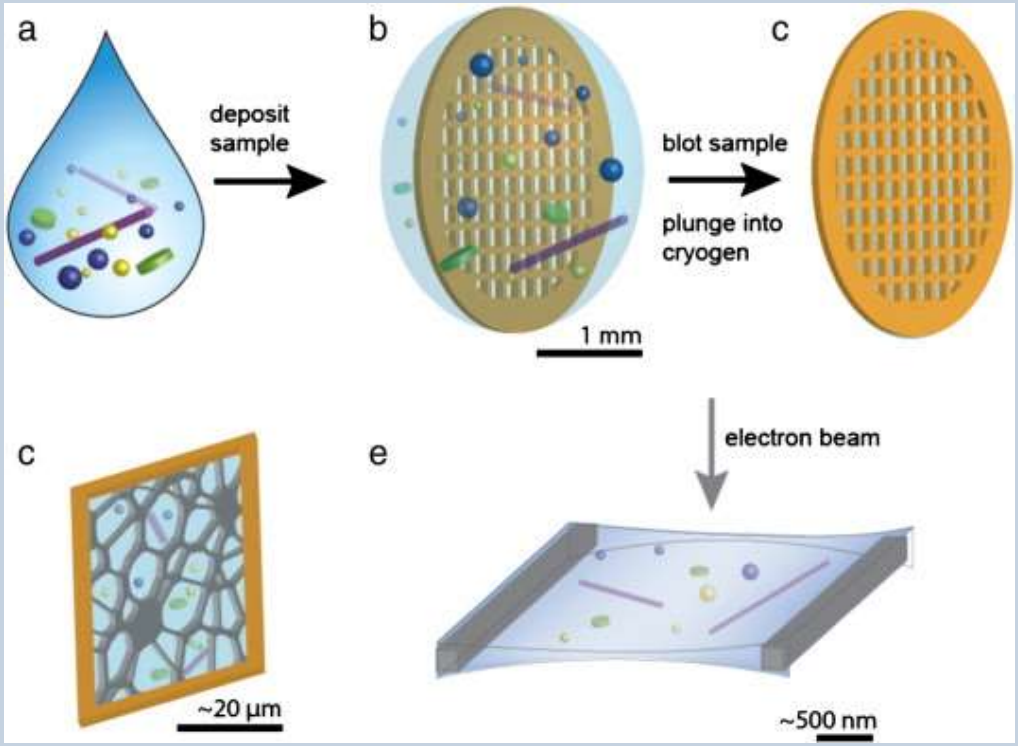
# Advanced TEM techniques

## *Electron tomography*

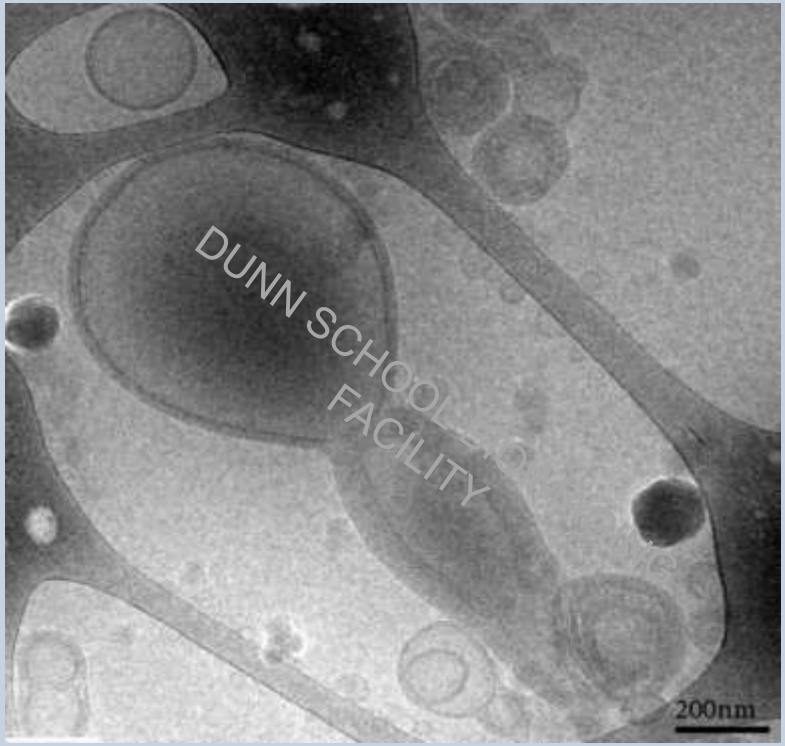


*Drosophila primary spermatocyte centrioles,*
*H Roque (Dunn School)*

# Advanced TEM techniques
## *Cryo-TEM*



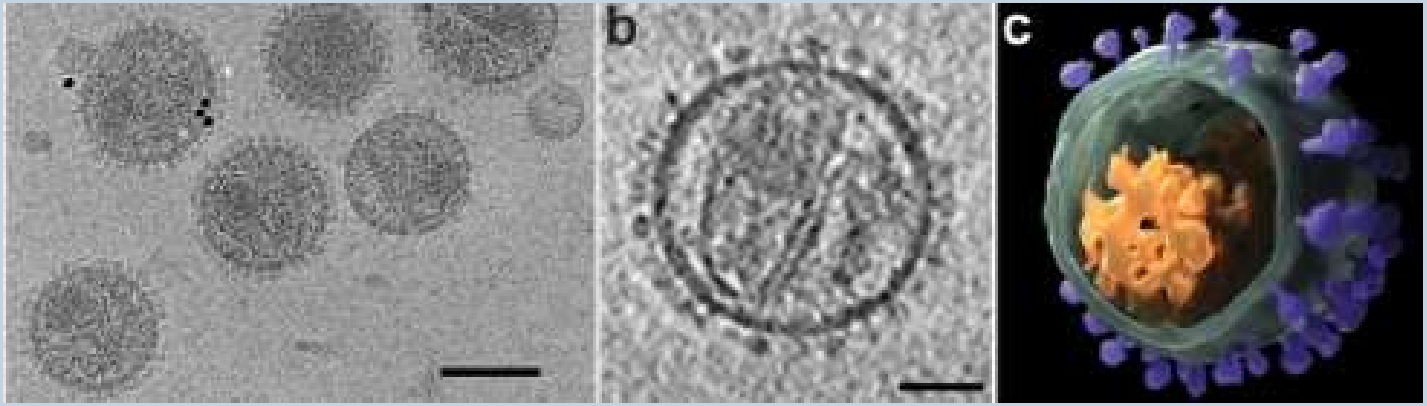

*Cryo-fixation of particulate samples*
*Newcombe et al (2012) Current Opintion in Colloid & Interface Science, 17(6): 350-359.*



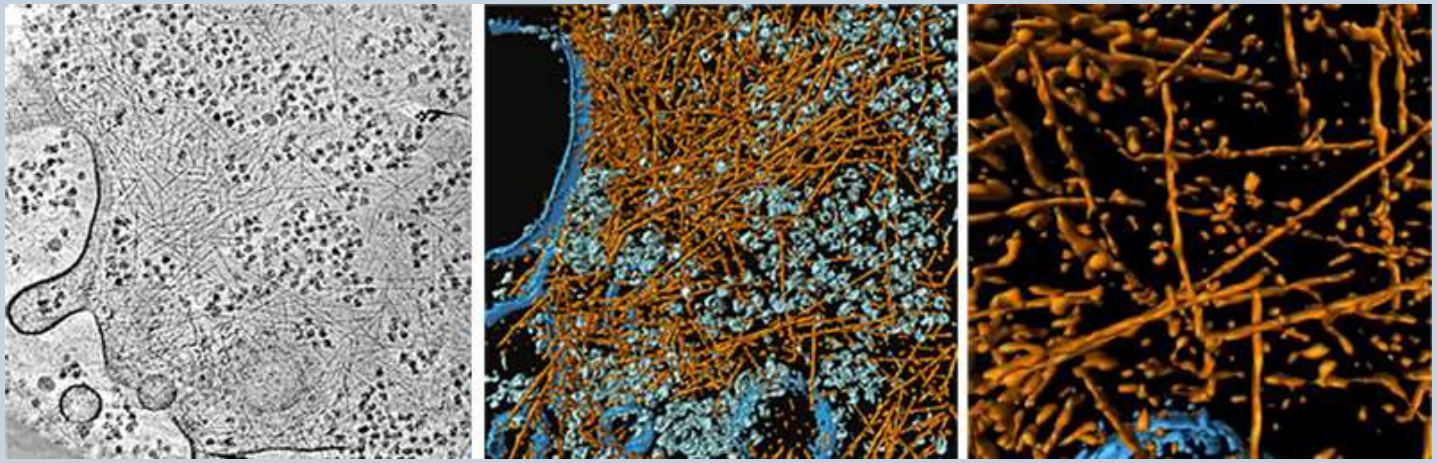

*Cryo-TEM of vitrified liposomes (D Cheng, University of Sydney)*

# Advanced TEM techniques

## *Cryo-Electron tomography*




Cryo-electron tomography and modelling of trimeric SIV Env virions (White et al 2010, PLoS Pathog, 6(12): e1001249)

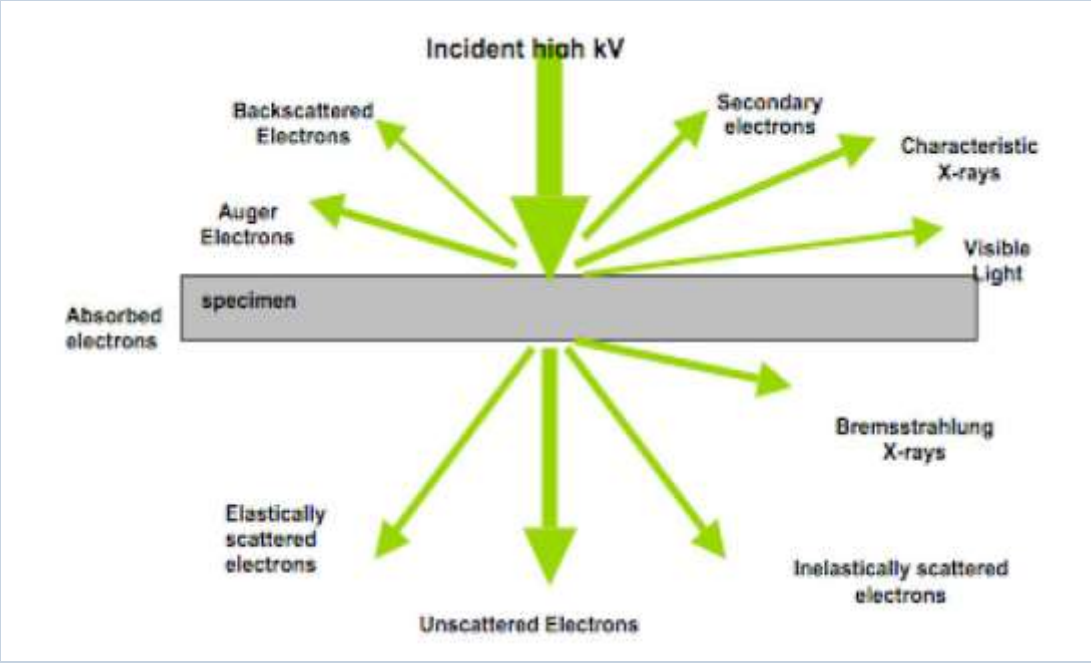


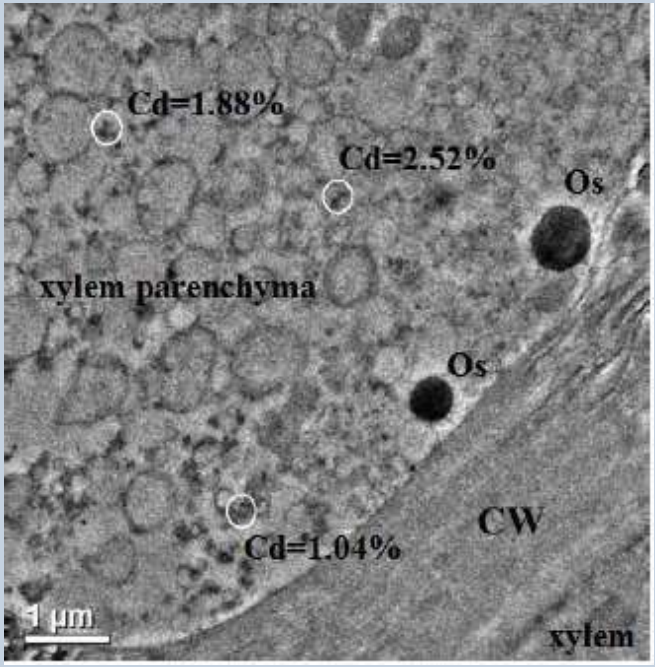
Cryo-electron tomography of the actin network in a slime mold (Wolfgang Baumeister lab, Max Planck Institute)

# Advanced TEM techniques

## *Chemical characterisation*

Energy-dispersive x-ray spectroscopy (EDS) allows chemical characterisation of specimens, based on the emission of x-rays that are characteristic for each element.

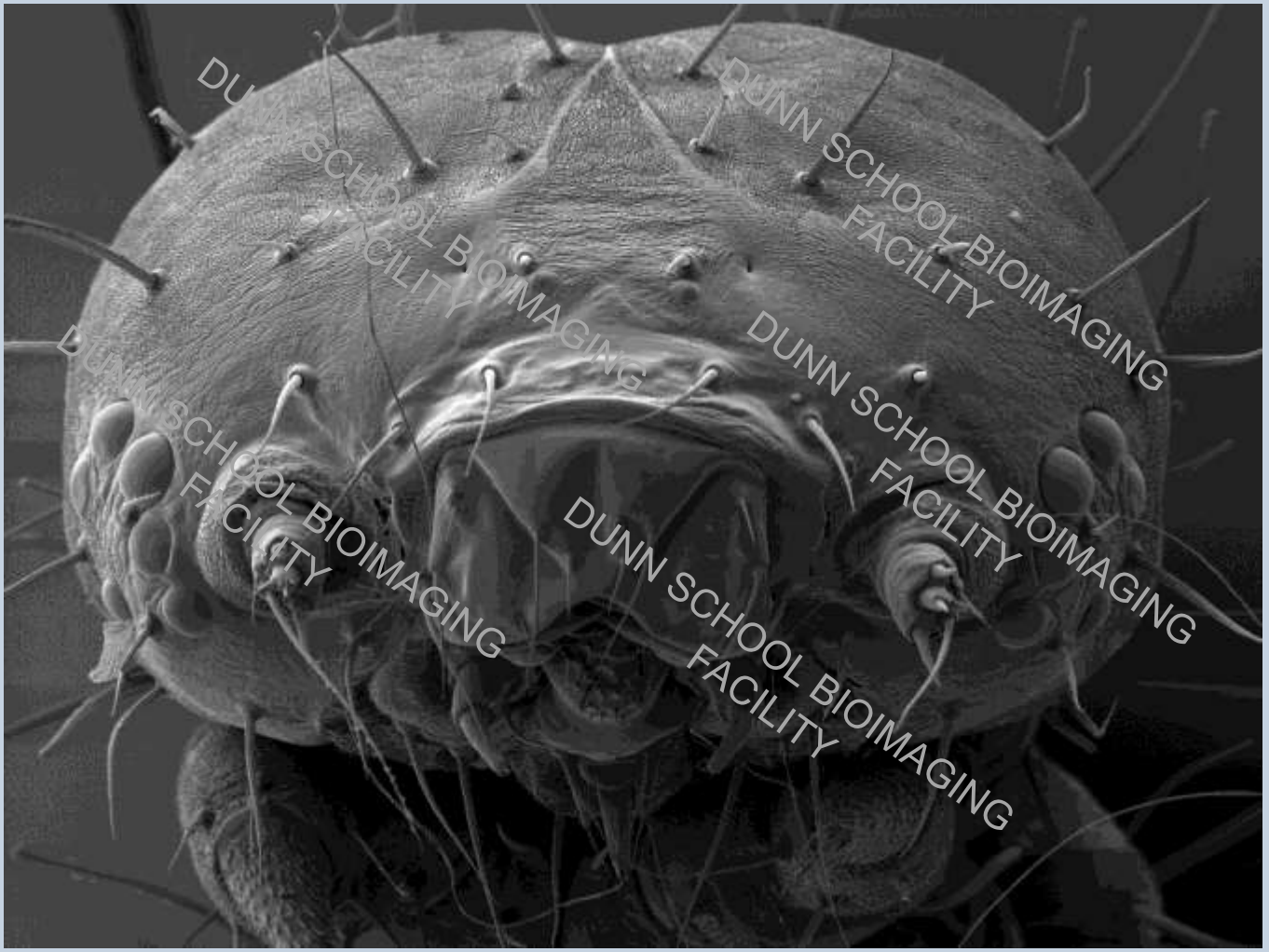



Cd Distribution in roots of Arabis paniculata
*(Y. Tang, R. Qiu et al. Sun Yat-sen University. PR China)*

*Images courtesy of Y. Tang, R. Qiu et al. Sun Yat-sen University. PR China.*

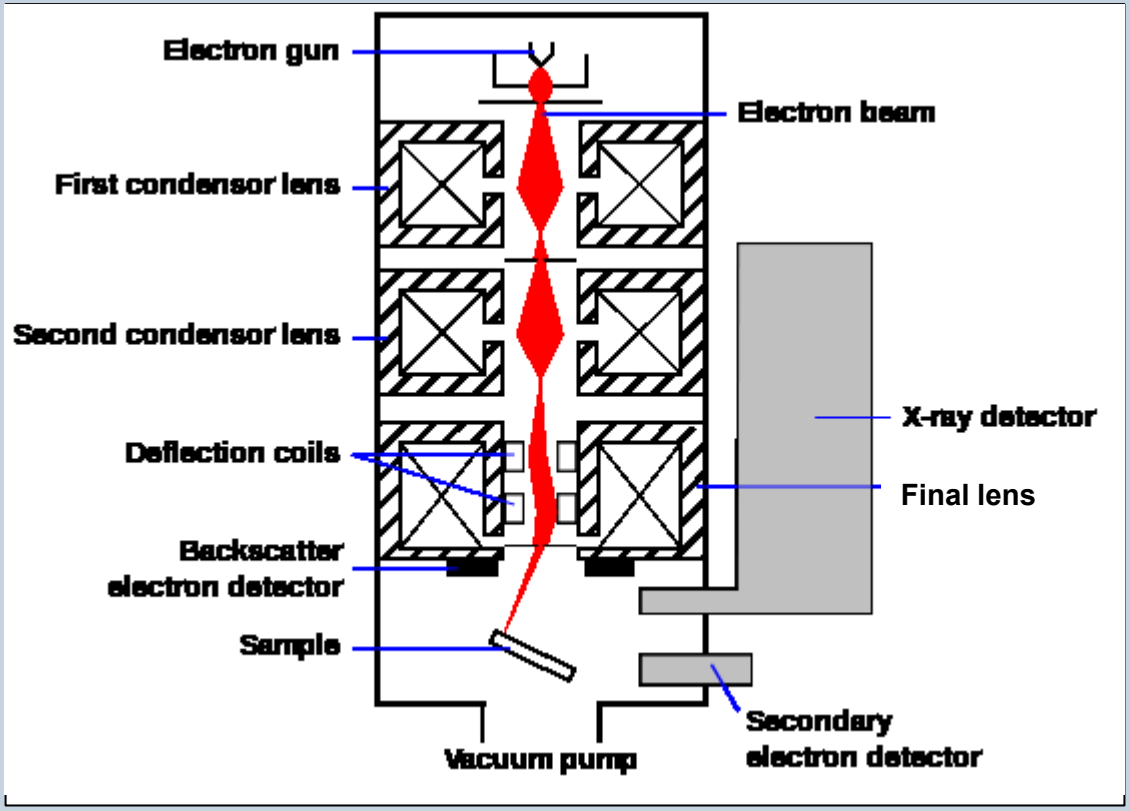
# Scanning Electron Microscopy (SEM)




*Caterpillar, Zeiss Ultra Plus, ACMM (E Johnson)*

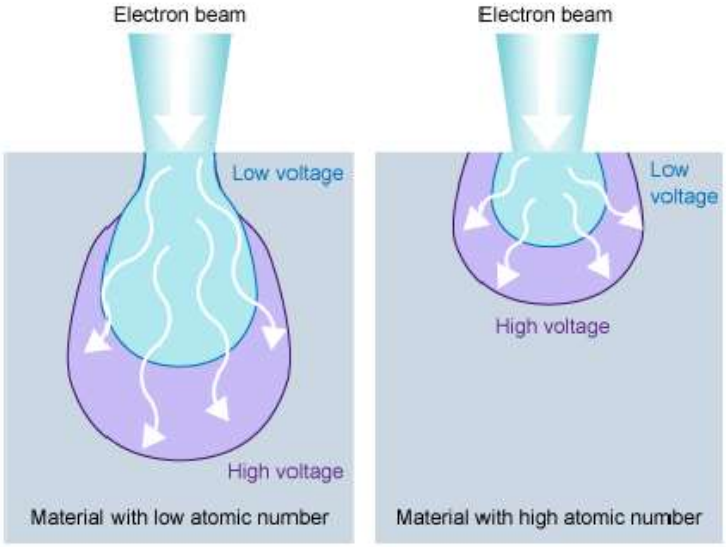
# How the SEM works

## *Overview*



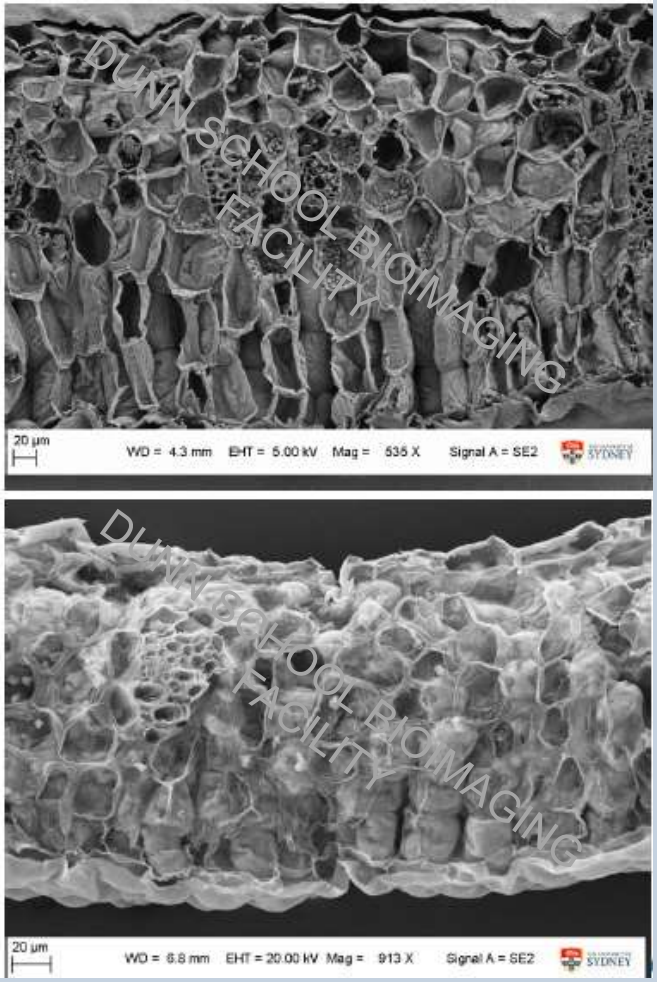
# How the SEM works

## *Overview - Accelerating voltage*



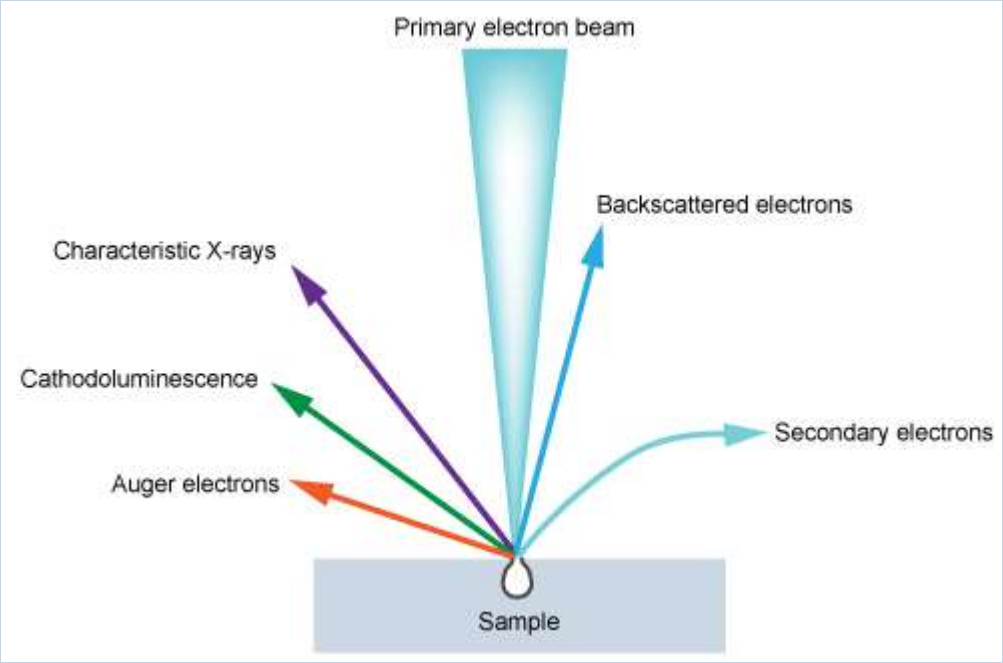

*From: ammrf.org.au.myscope*

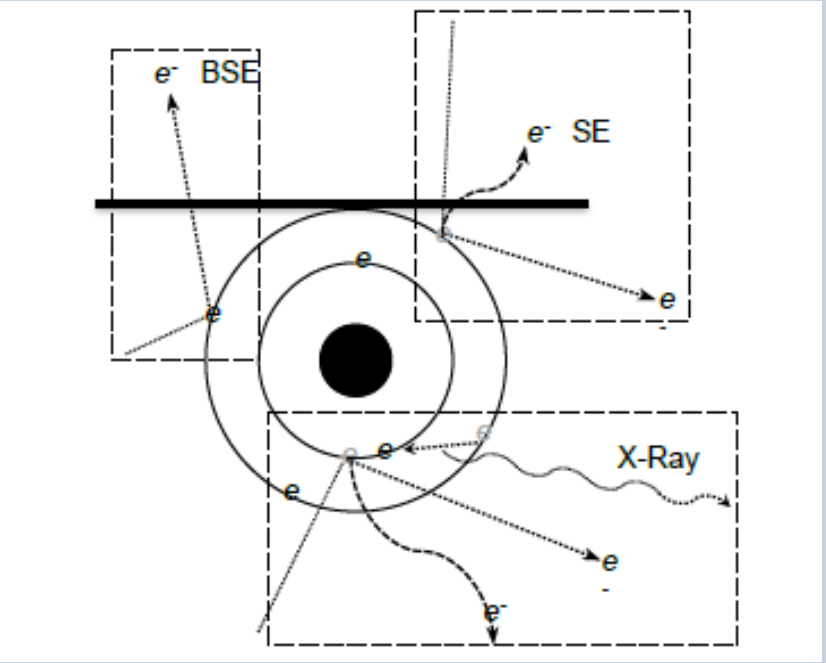
30 kV for material samples
~5 kV for biological samples




*Spinach leaf section, Zeiss Ultra Plus, ACMM (E Johnson)*

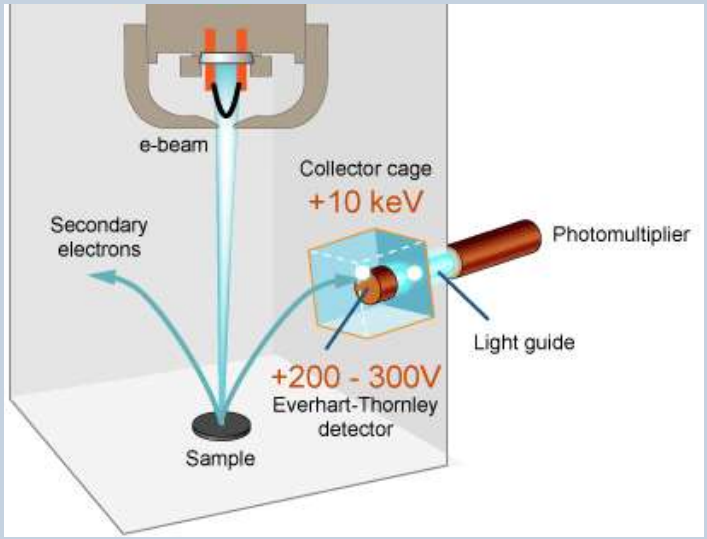
# How the SEM works
## *Overview - SEM signals*



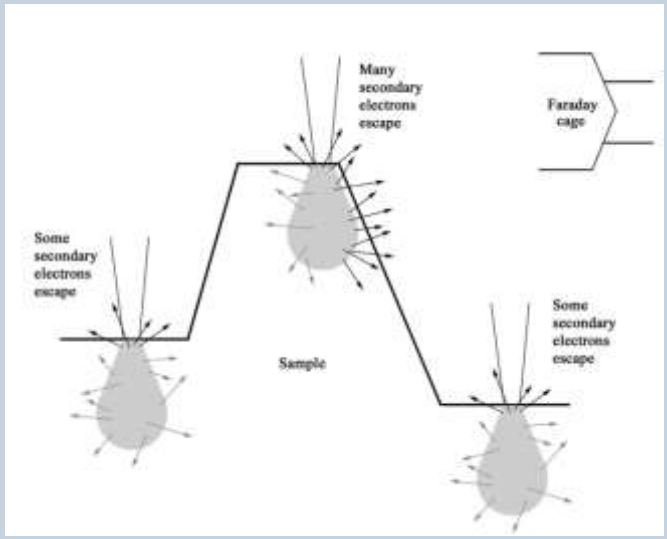



# How the SEM works
## *Overview - Signal detection*

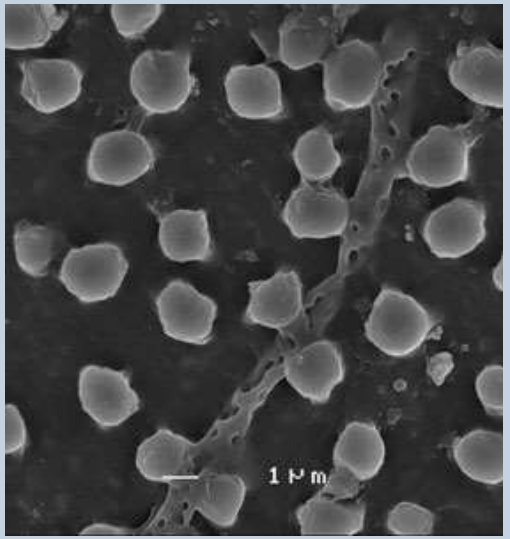
- Secondary electrons (SEs) provides surface morphology and topology information.
- SEs are captured by the Everhart-Thornley detector




*www.ammrf.org*



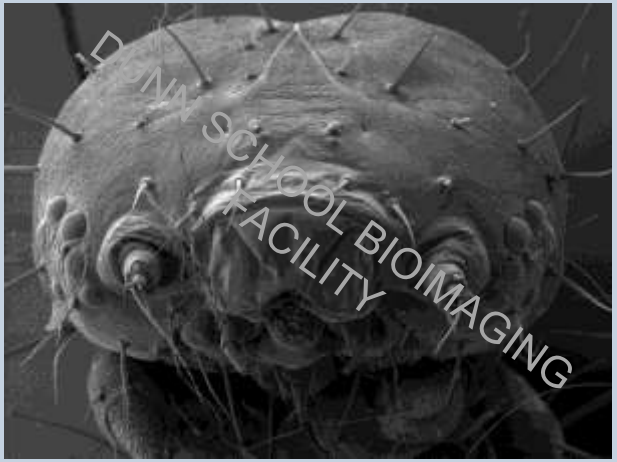

*Dept Biological Sciences, Smith College Northampton USA*



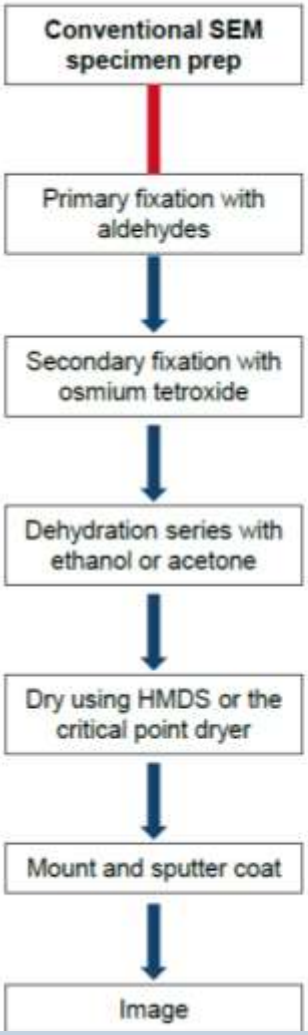

*www.ammrf.org*

# 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.



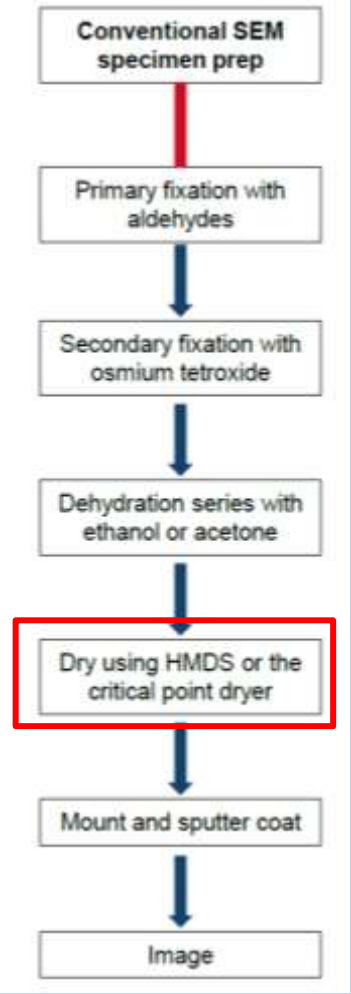
# Sample Preparation for SEM
## *Overview*




| Specimen | Examples | General comments on specimen preparation |
|---|---|---|
| Particulate | Pollen, freeze-dried starch granules, dried soil, seeds | Dry, particulate samples can be deposited directly on conductive carbon tape and coated with a thin layer of Au, Au/Pd, Pt or C. |
| Cells | HeLa cells, Tobacco BY-2 suspension, fungal cultures | Cells must first be immobilised: adherent cells may be grown directly on a coverslip or on a scaffold, while non-adherent cells can be run through a filter (where they are retained on the filter membrane) or can be allowed to settle on 'sticky' coverslips[#]. Centrifuging suspension cells is not recommended as this can result in cell deformation and/or rupture of fine structures. Once immobilised, samples must be fixed, dehydrated and dried, then mounted and coated as above. |
| Micro-organisms | Dinoflagellates, Chromera, bacteria | Can be freeze-dried, then treated as for particulate samples. Can also be prepared as for cells. |
| Plant tissue | Wood, flowers, roots, leaves, apical buds | Woody samples may be air dried, then mounted and coated as above. Soft tissue samples should be cut to size[*] and fixed by immersion. Vacuum infiltration may aid this process. Tissue can then be sectioned using a vibratome or freeze-fractured if required, before proceeding as for cells. Leaf and wood samples are well suited to ESEM (pg ). |
| Food | Cheese, potato | Treat as for soft plant tissue. Also well suited to ESEM. |
| Whole organisms | Plant seedlings, insects, small fish. | If the specimen has a hard outer coat (*eg:* many insects), allow to dry naturally, then coat and image directly. 'Soft' organisms should be treated as for soft plant tissue. Increase incubation times in proportion to sample size. |

# Sample Preparation for SEM

## *Drying the sample*

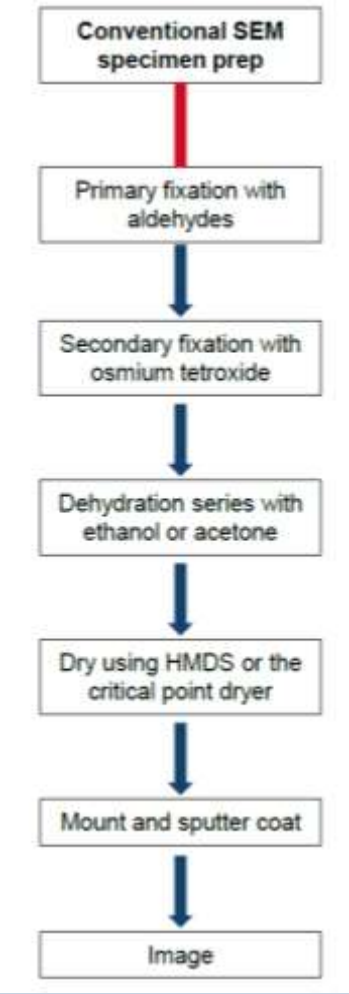



- Once the dehydration series is complete, the solvent itself must be removed from the tissue without introducing surface tension/drying artifacts into your sample. This is achieved through the use of a transitional fluid, most commonly hexamethyldisilazane (HMDS) or liquid $CO_2$. Air drying is not recommended, as ethanol evaporation generally causes severe surface tension artifacts.

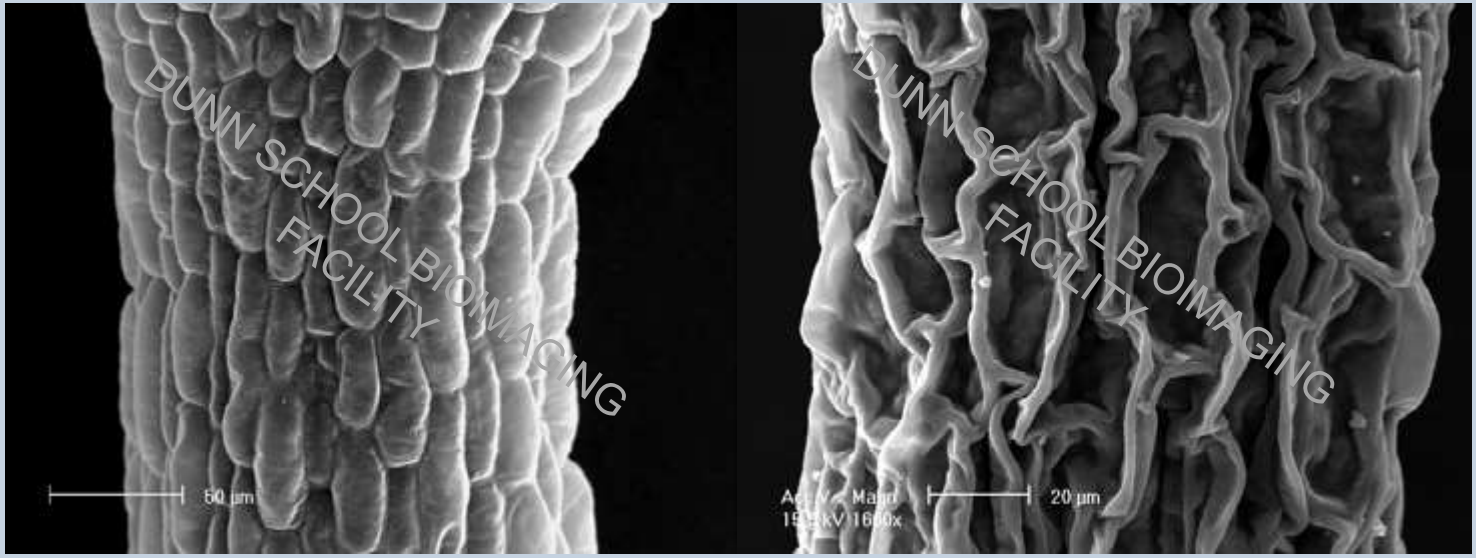
- Liquid $CO_2$ can be used to flush the solvent from tissue using a technique called Critical Point Drying (CPD).

# Sample Preparation for SEM

## *Drying the sample*



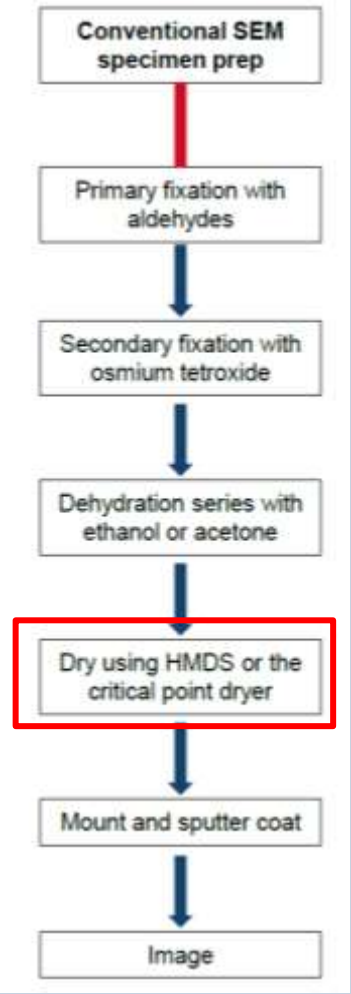

**Good** **Bad**




*Arabidopsis stem, Phillips XL30 SEM, E Johnson*

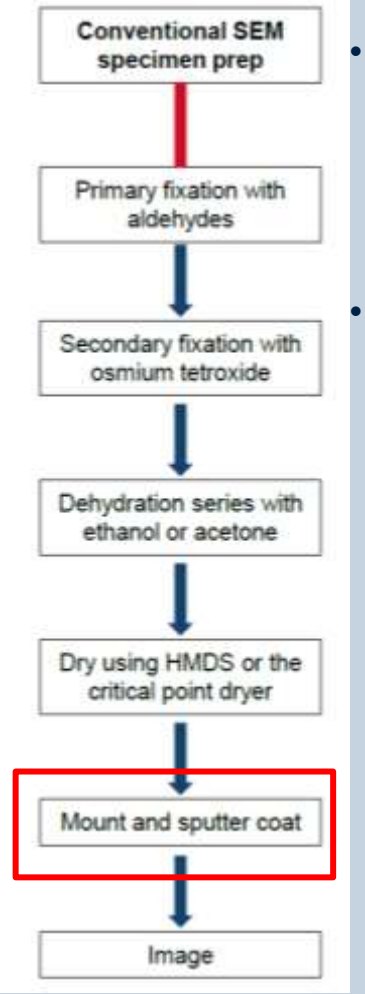
# Sample Preparation for SEM

## *Mounting and sputter coating*




- If a biological specimen is not mounted and coated correctly, it will react to the electron beam (an effect called charging), resulting in sample damage and/or image distortion.
- Mounting immobilizes the sample on a conductive backing, grounding it. Ensure that your sample is in full contact with the conductive backing; if not, use conductive glue (eg: carbon and silver) to ensure conductive continuity.

# Specimen Preparation for SEM
## *Sputter coating*

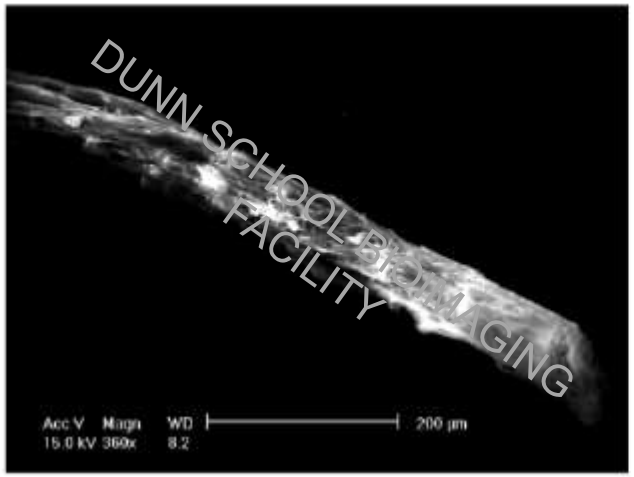



- Sputter coating with metal ions deposits a thin continuous conductive layer over the sample, such that charge from the electron beam flows to the ground and does not build up on the sample.
- Sputter coating also increases the SE signal (and therefore contrast), high Z elements have a higher yield of SEs than low Z elements (biological material!).

# Specimen Preparation for SEM

## *Charging artifacts*

Bright spots (Arabidopsis root)





Lines in the image (Arabidopsis xylem)

Focus issues (*Leishmania* on collagen)

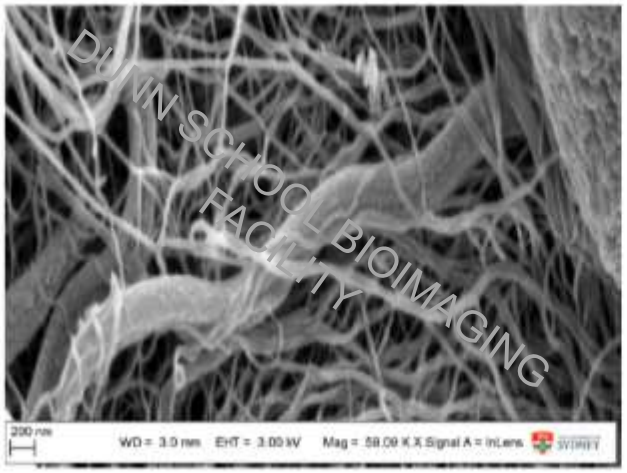




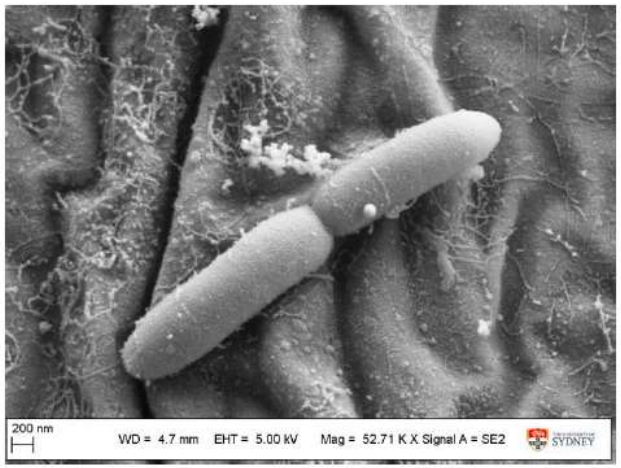
Image distortion (processed cheese)

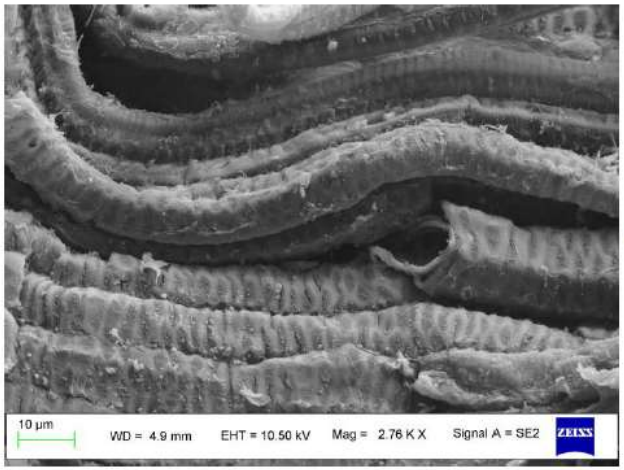
Images, E. Johnson

# Specimen Preparation for SEM

## *Surface contamination and deformation*

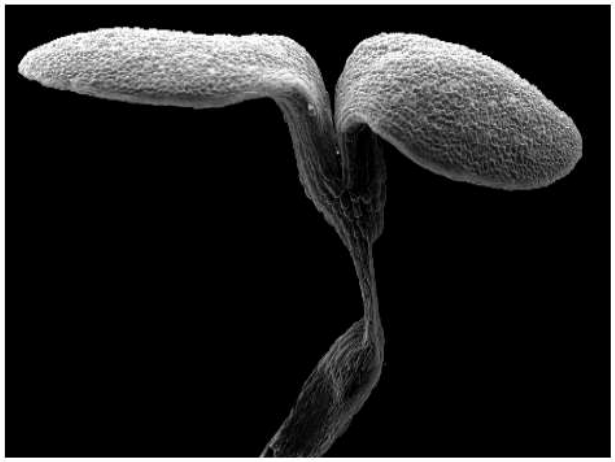
Bacteria

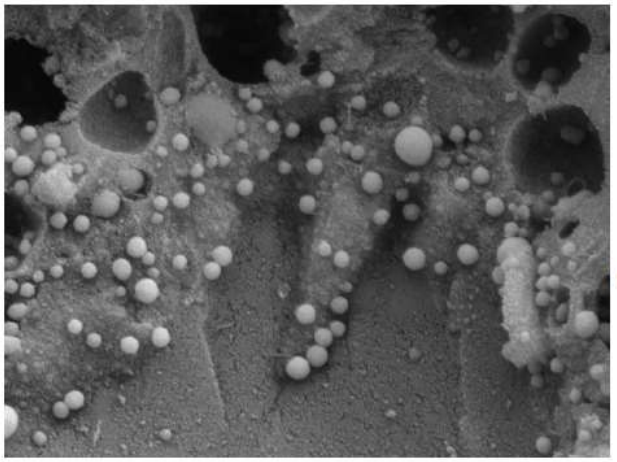



Cellular debris

Tweezer marks

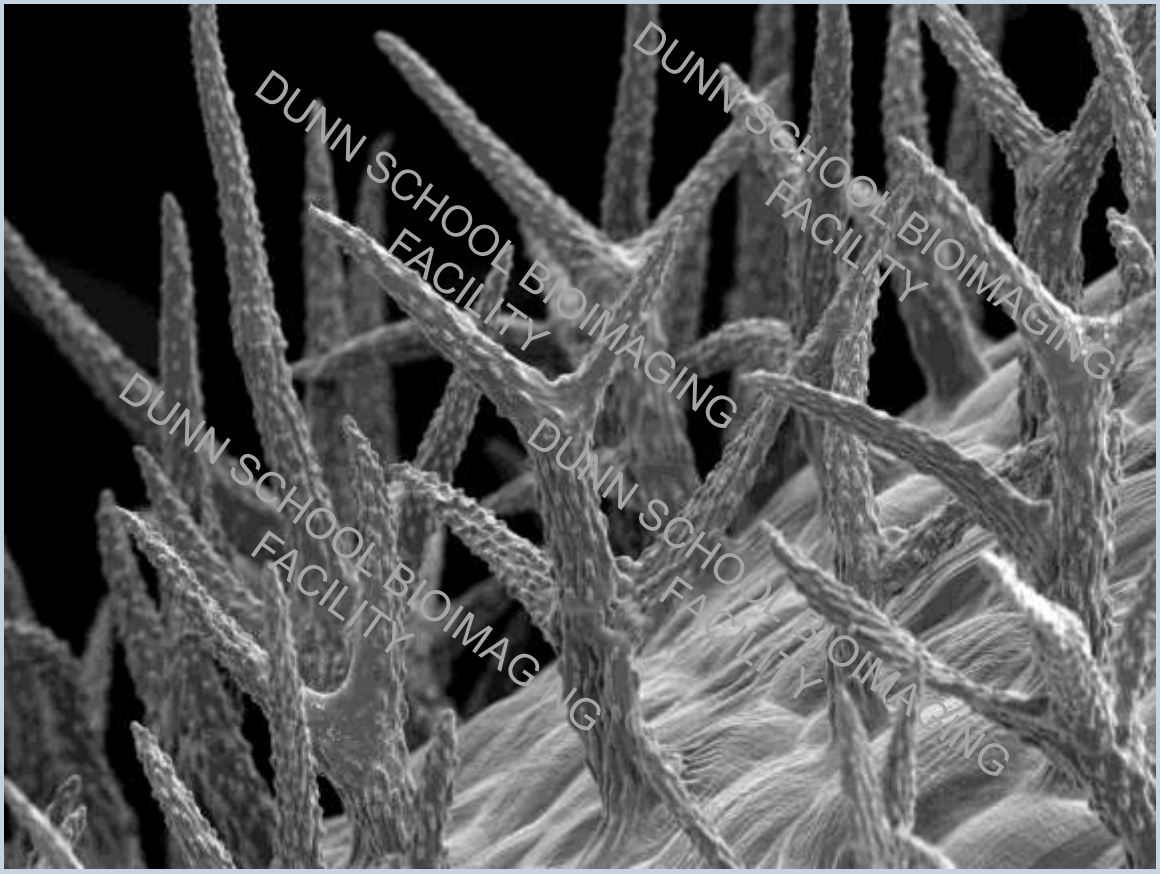




Blebs or other oddities

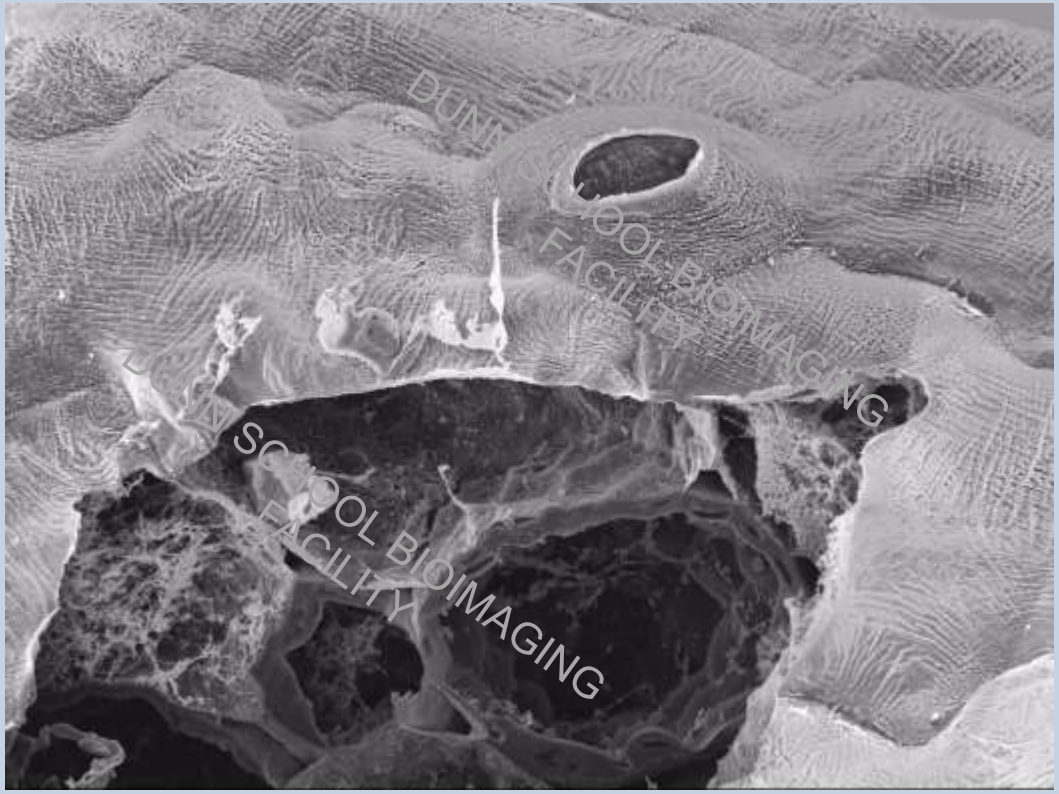
Images, E. Johnson

# Specimen Preparation for SEM
## *No problems!*



# Advanced SEM techniques

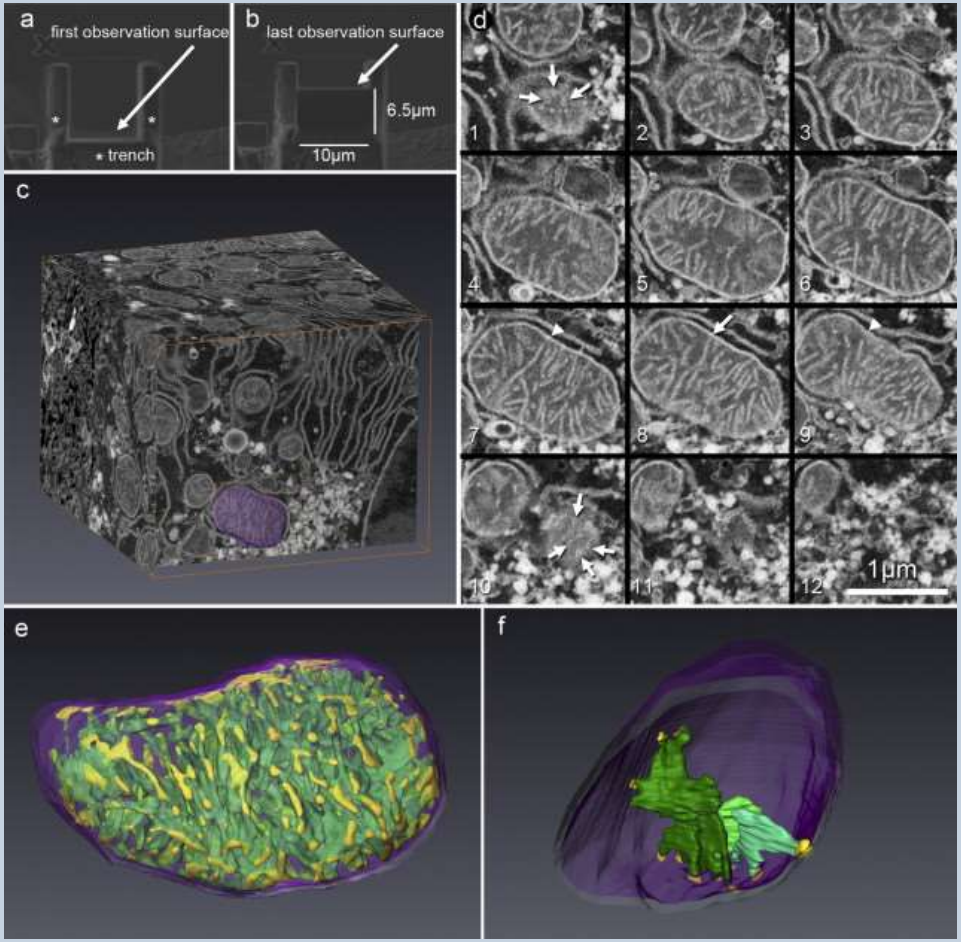
## *3D imaging – Focussed Ion Beam (FIB)*




*Arabidopsis leaf, Zeiss Auriga FIB (S Moody/E Johnson)*

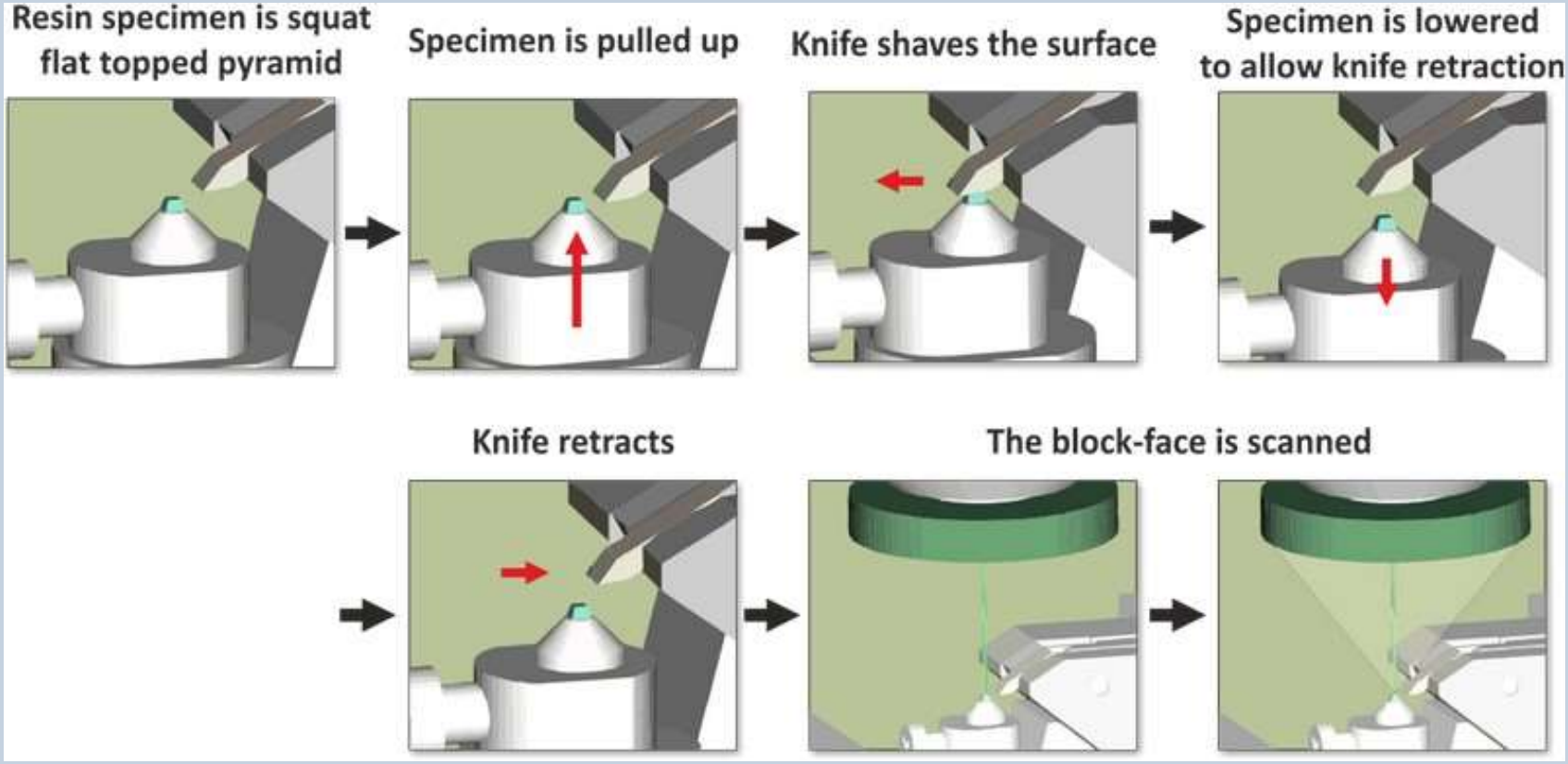
# Advanced SEM techniques

## *3D imaging – Focussed Ion Beam (FIB)*




*FIB serial-sectioning of resin-embedded hepatocyte (Ohta et al (2012) Micron, 43(5): 612-620)*

# Advanced SEM techniques
## *3D imaging – in situ microtome*

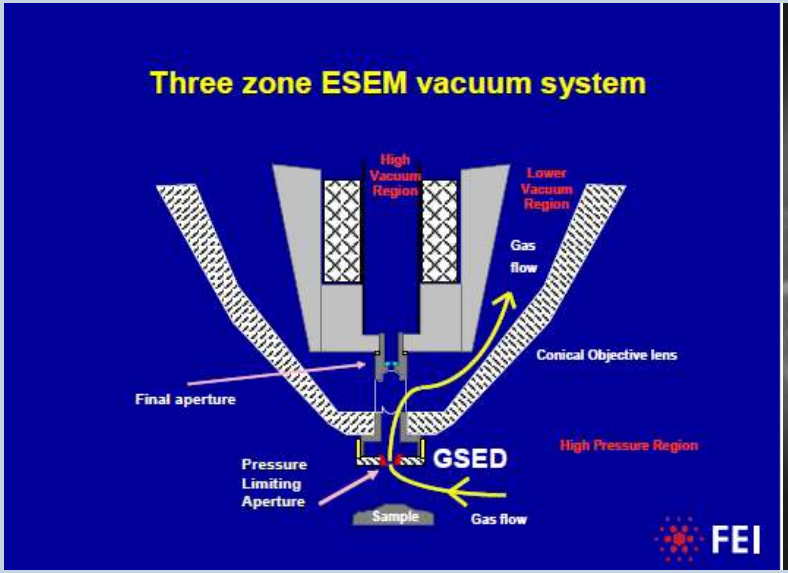



*http://www.biocenter.helsinki.fi/bi/em/emu_methods_3view.html*

# Advanced SEM techniques

## *Environmental SEM*

- Variable pressure and environmental SEM (ESEM) allows untreated, hydrated specimens to be imaged at high resolution.
- Utilises a specialised detector and vacuum system that enables imaging under low pressure conditions (ie; not a vacuum!).




# Questions?