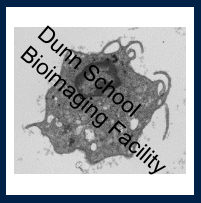
SIR WILLIAM DUNN
SCHOOL OF PATHOLOGY

UNIVERSITY OF OXFORD

BIOLOGICAL ELECTRON MICROSCOPY

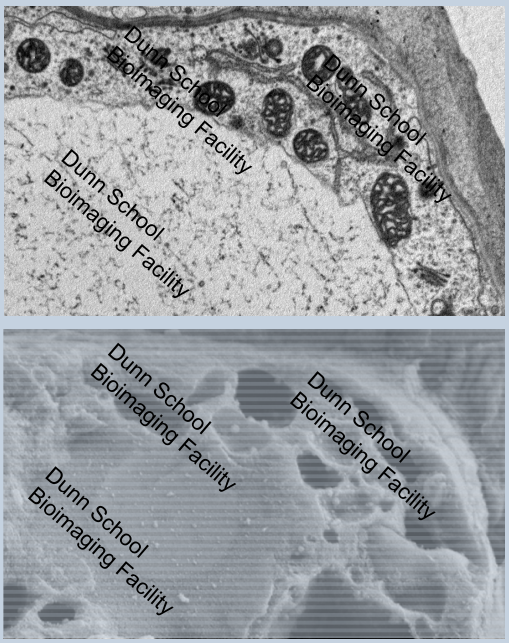


Dr Errin Johnson
EM Facility Manager

March 19, 2015

Lecture Overview

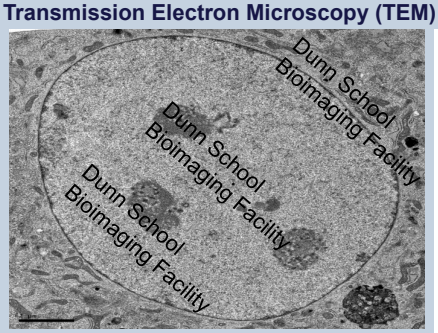
- Introduction to Electron Microscopy (EM)
  - Features of Electron Microscopes
- Transmission Electron Microscopy (TEM)
  - Overview of the microscope
  - Biological specimen preparation for TEM
  - TEM applications
- Scanning Electron Microscopy (SEM)
  - Overview of the microscope
  - Biological specimen preparation for SEM
  - SEM applications
- 3D EM techniques
- EM Facilities at Oxford

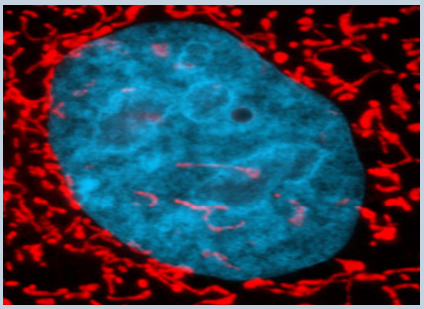


Leaf epidermal cells imaged by TEM (top) and SEM (bottom)
E Johnson, Dunn School

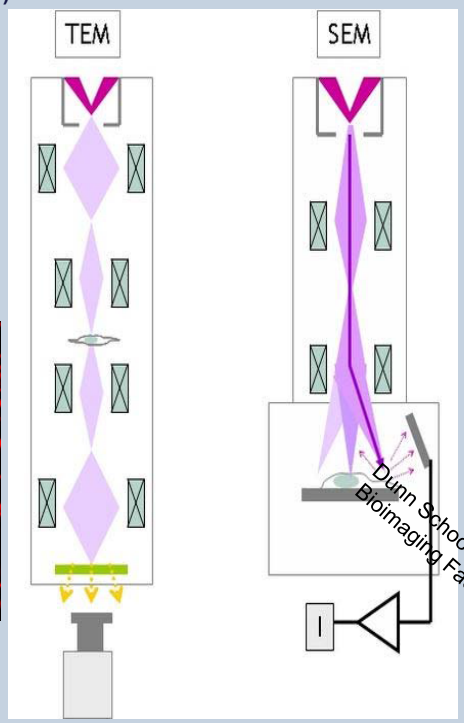
Electron microscopy
Overview

Transmission Electron Microscopy (TEM)

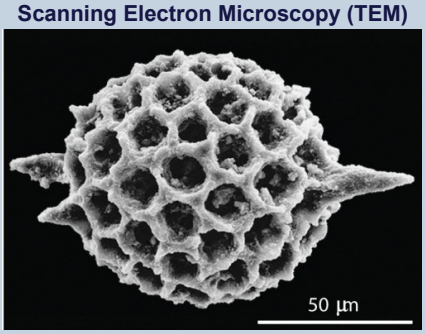


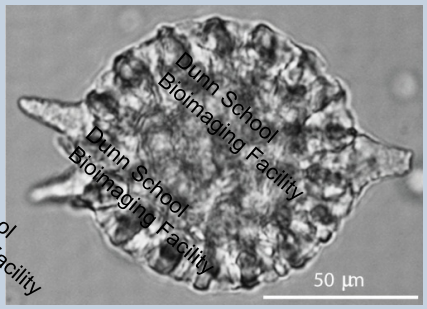


Top: TEM image of fibroblast cell stained with no specific stain (E Johnson, Dunn School). Bottom: Confocal image of a kidney cell stained with DAPI (blue) and MitoTracker (red) (From: Hammamatsu.magnet.fsu.edu)



Scanning Electron Microscopy (TEM)



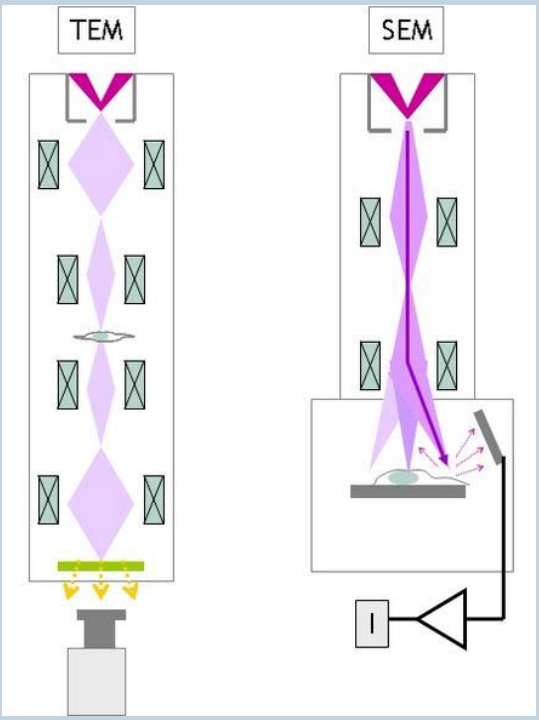


Radiolarian imaged with both SEM (top) and light microscopy (bottom). From: General Chemistry: Principles, Patterns, and Applications, B. Averill & P. Elderege

Features of Electron Microscopes
Overview

The main components of an electron microscope are:

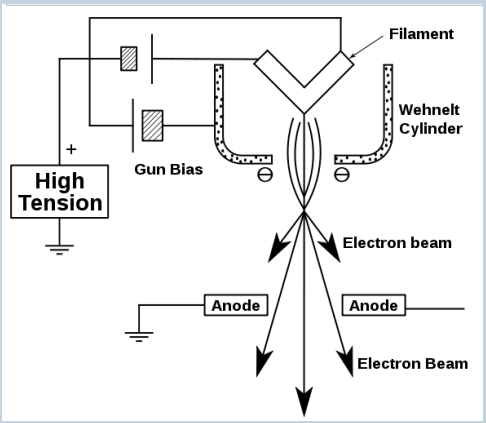
- An electron gun
- Electromagnetic lens system
- Vacuum system
- Camera/detector
- Computer



Features of Electron Microscopes
The 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

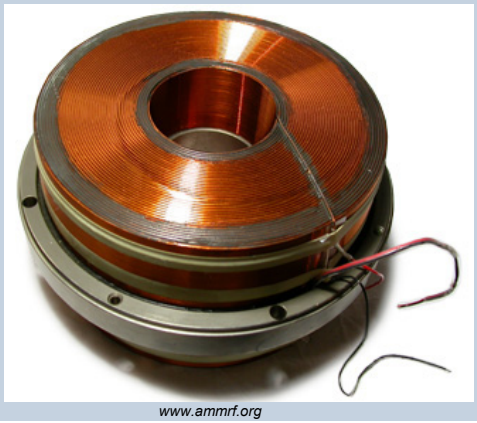
www.ammrf.org



www.wikipedia.org

Features of Electron Microscopes
Electromagnetic Lenses

- EM lenses are electromagnetic, creating precise, circular magnetic fields that manipulate the electron beam, much the same way that optical lenses focus and direct light
- Similarly to optical lenses, electromagnetic lenses are also susceptible to chromatic and spherical aberrations, as well as astigmatism



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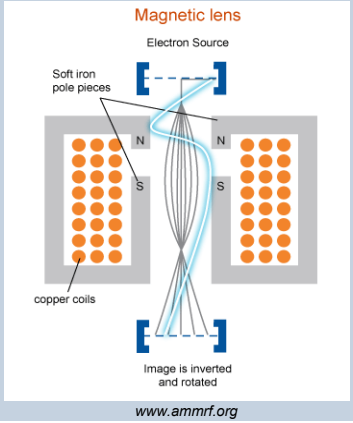

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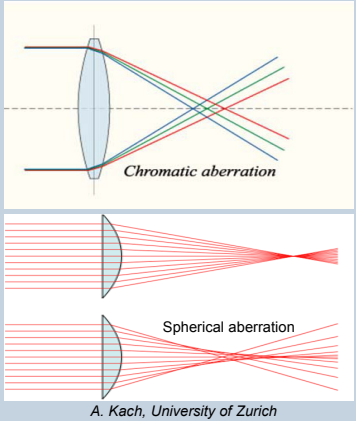
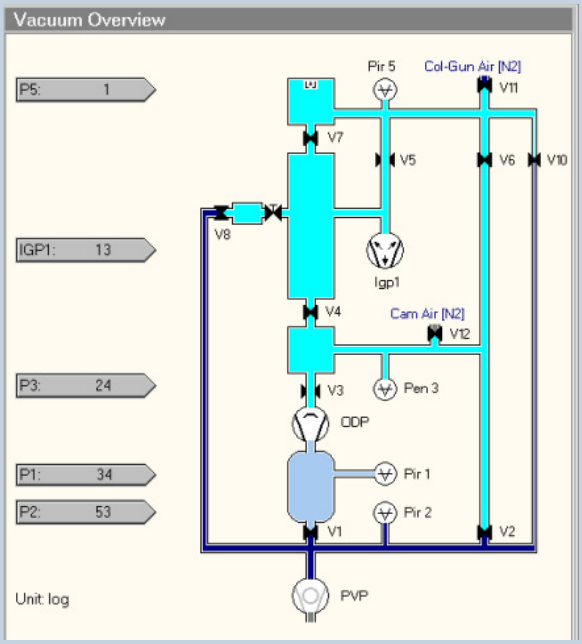


A. Kach, University of Zurich

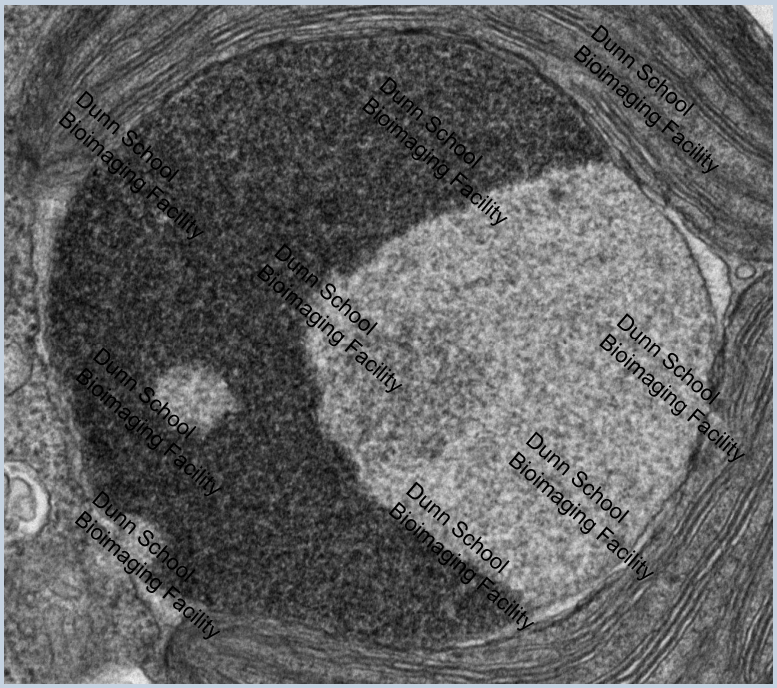
Features of Electron Microscopes
Vacuum systems

- 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 (and damage to the filament)



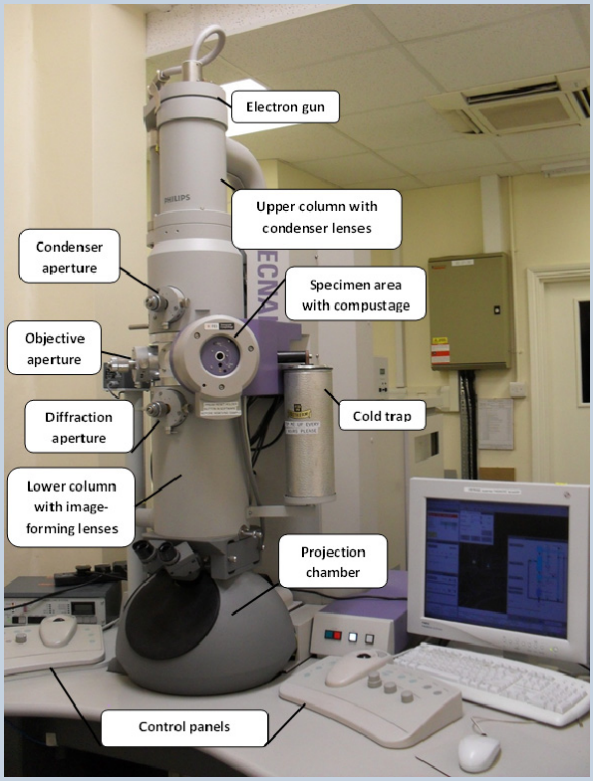
Overview of vacuum system on the Tecnai12 TEM

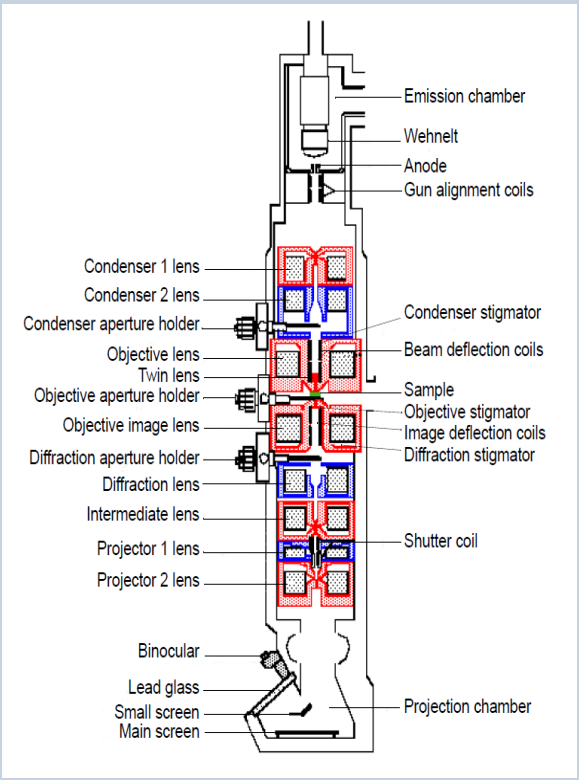
Transmission Electron Microscopy (TEM)



Nucleus of a single celled algae (M Eason-Hubbard & E Johnson)

The TEM

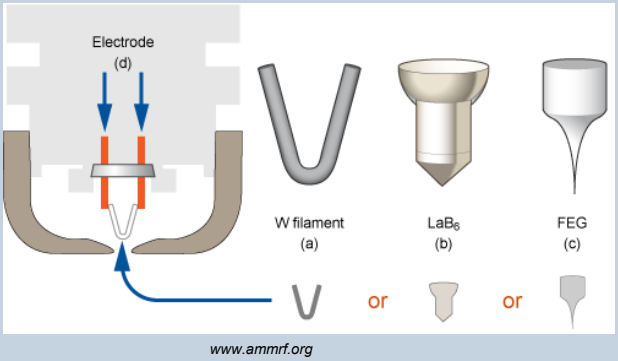




How the TEM Works

Electron gun

- Resolution depends on a number of factors, including the accelerating voltage, the type of electron source used and how you setup the microscope
- Electron sources are typically Tungsten or Lanthanum hexaboride (LaB6) and can be thermionic or field emission (FEG)
- Accelerating voltage (kV) is typically 80-300 kV for biological specimens

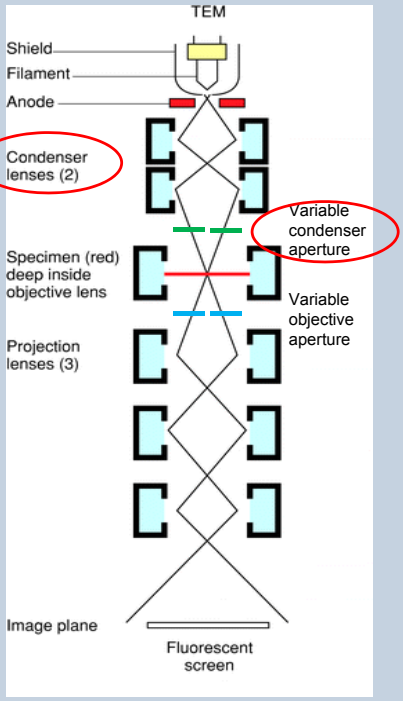


www.ammrf.org

Accelerating Voltage (kV)	Wavelength (nm)
80	0.0043
120	0.0035
200	0.0027
300	0.0022

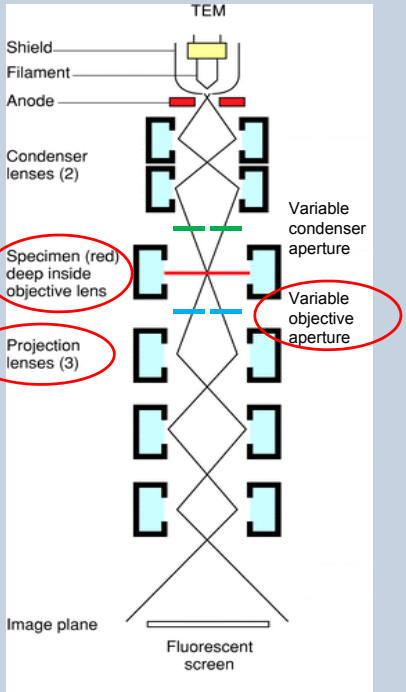
How the TEM Works
Condenser lenses

- The condenser lens system focuses the emitted electrons into a coherent beam.
  - The first condenser controls the spot size of the beam.
  - The second condenser focuses the beam onto the sample.
- The condenser aperture restricts the beam by excluding high angle electrons.



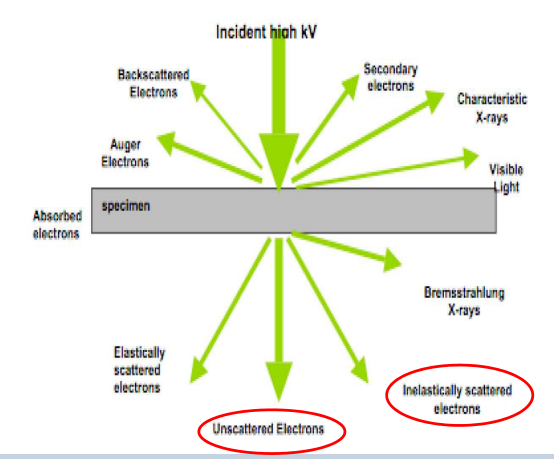
How the TEM Works
Overview – Imaging lenses

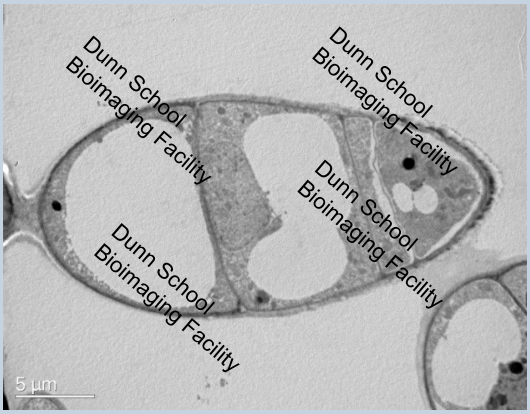
- The objective lens focuses the electrons transmitted through the sample into a magnified image.
  - The objective aperture can be used to increase contrast by excluding high angle transmitted electrons.
- The intermediate and projection lenses enlarge the image.
- When the electrons hit the phosphorescent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



The TEM
Contrast

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



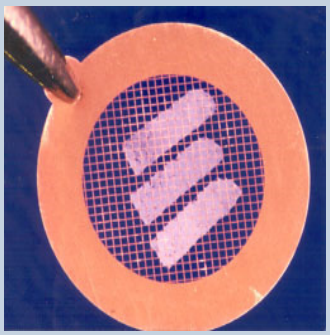


Lavender trichome, E Johnson

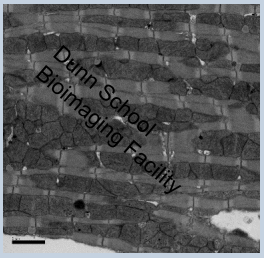
Electron microscopy
Specimen requirements

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

Mouse heart ~7 mm wide



70 nm thick resin-embedded tissue sections on a TEM grid



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

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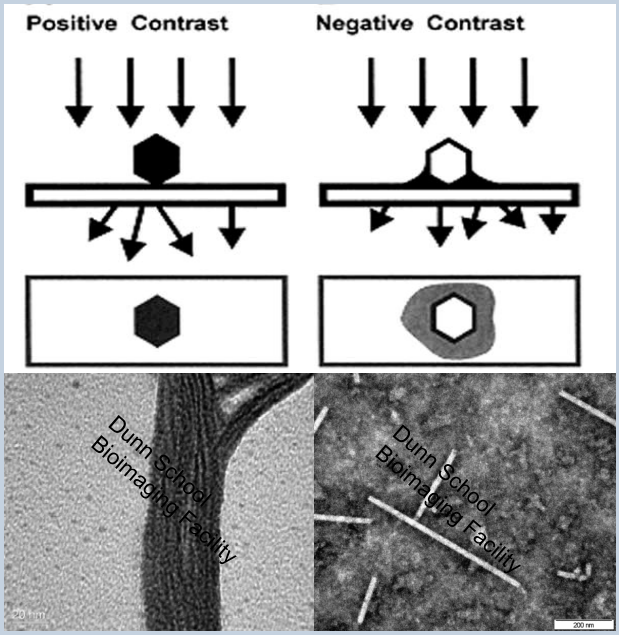
TEM techniques
Negative staining

- There are a number of different ways to prepare particulate samples (eg: proteins, liposomes, DNA and viruses) for TEM
- The easiest and quickest method is 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) for ~1 min
  - Blot dry and view in the TEM



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

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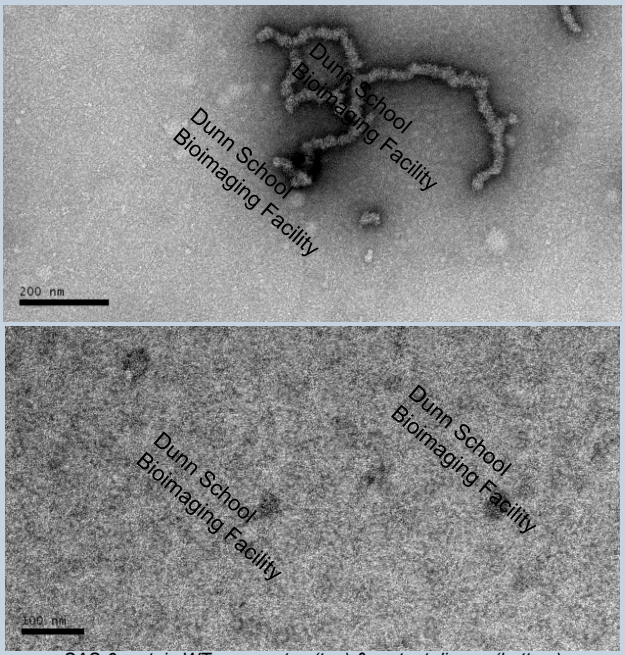
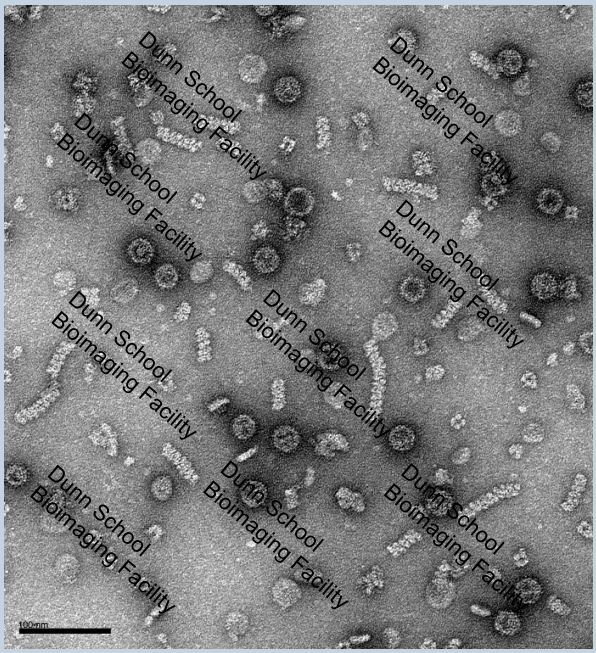
TEM techniques
Negative staining – Proteins and viruses



SAS-6 protein WT aggregates (top) & mutant dimers (bottom)
Tecnai12 TEM, M Cottee/E Johnson



Virus-like particles
Tecnai12 TEM, Darren Lenegham/E Johnson

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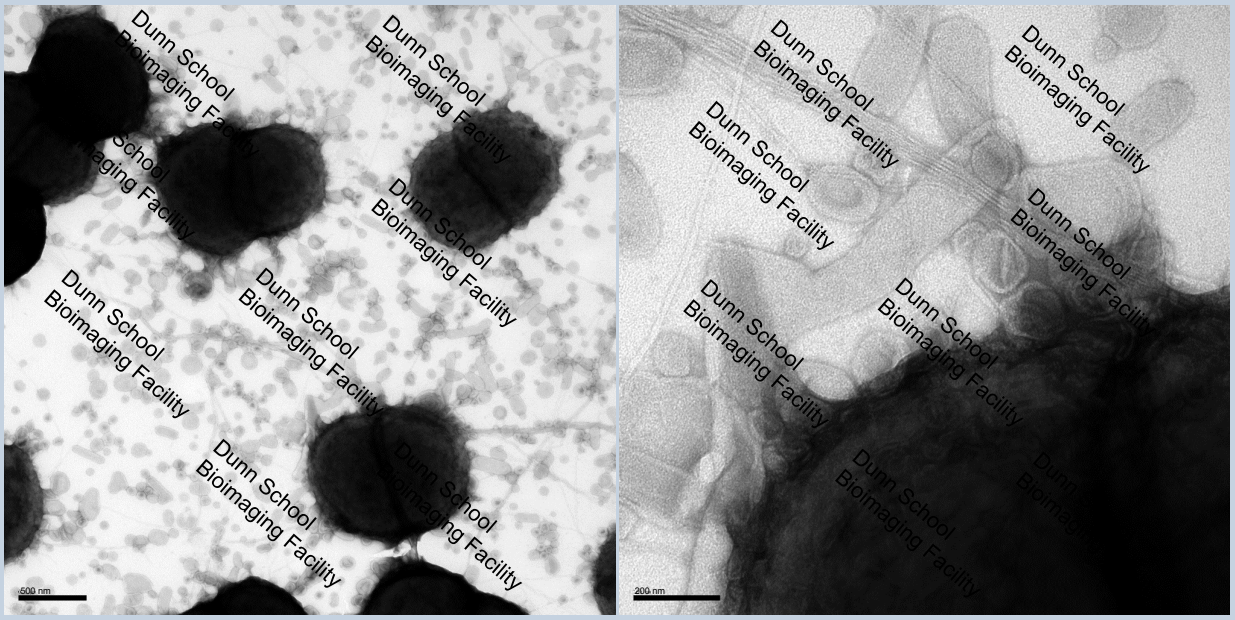
TEM techniques
Negative staining – Bacteria

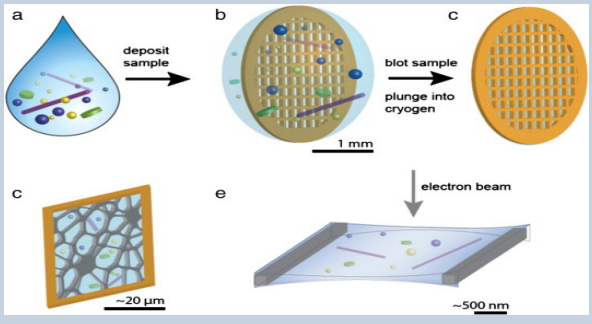


Negatively stained Neisseria sp. (R Exley/EJohnson)

PIC

TEM techniques
Cryo-TEM

- Alternatively, you can freezing particulate samples and image under cryo conditions, which allows you to view them as close as possible to their native state.
  - Coat grids with plastic film and carbon
  - Apply the particulate specimen
  - Vitrify by plunge freezing into a cryogen (eg: ethane or propane)
  - Transfer to cryo-TEM under liquid nitrogen and image frozen

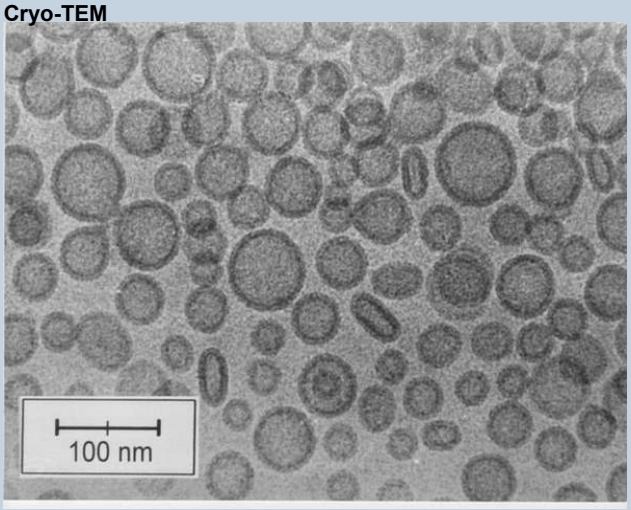


From: Newcombe et al (2012) Current Opinion in Colloid & Interface Science, 17(6): 350-359.

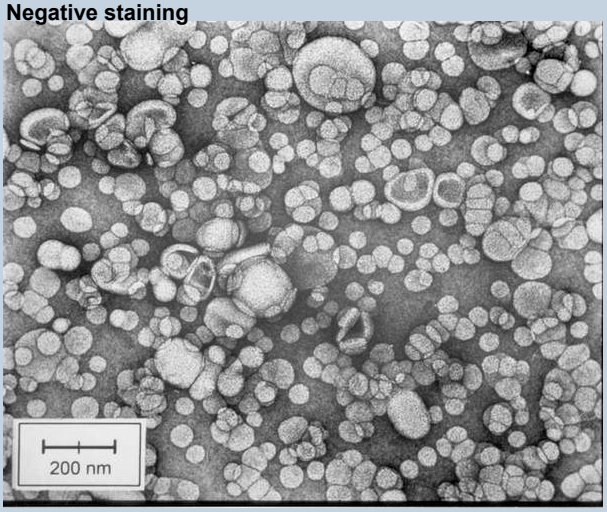
FEI Vtrobot for automated plunge freezing of grids

TEM techniques
Cryo-TEM vs Negative staining

Cryo-TEM



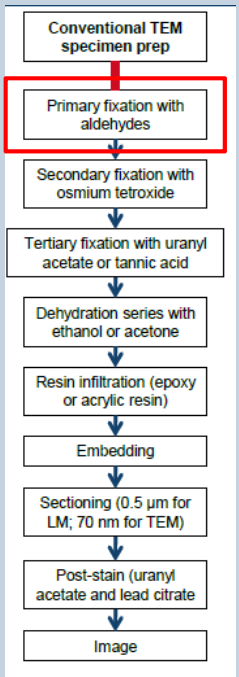
Negative staining



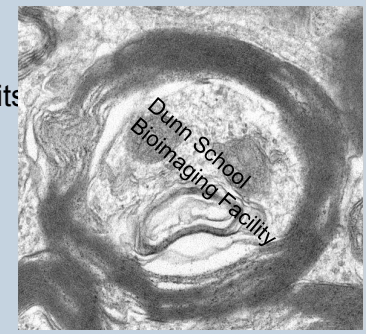
Dispersion of Egg-PC liposomes imaged frozen with Cryo-TEM (left) or negatively stained with uranyl acetate (right)
From: Laboratory for Soft Matter Electron Microscopy, University of Bayreuth

Specimen Preparation for TEM
Cells & Tissue

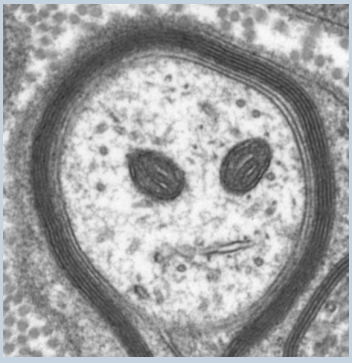
Dunn School Bioimaging Facility



- 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
- Chemical fixation with aldehydes, most commonly 2.5% glutaraldehyde, which quickly and irreversibly cross-links proteins via their amino groups



Bad fixation

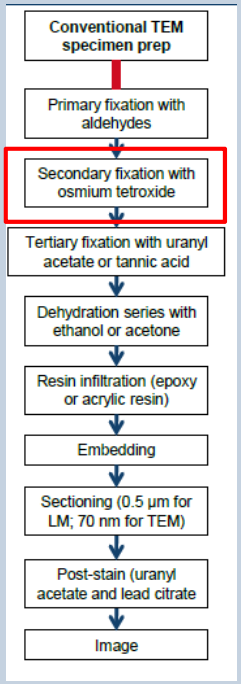


Good fixation

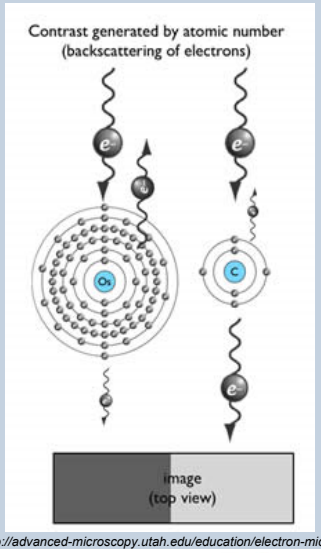
Top: E Johnson, Dunn School Bioimaging Facility
Bottom: remf.dartmouth.edu

Specimen Preparation for TEM
Cells & Tissue

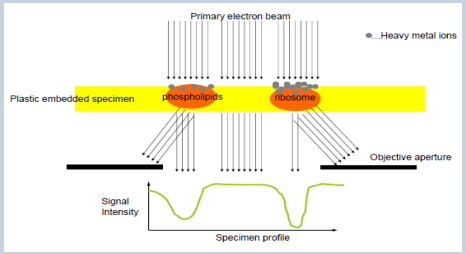
Dunn School Bioimaging Facility

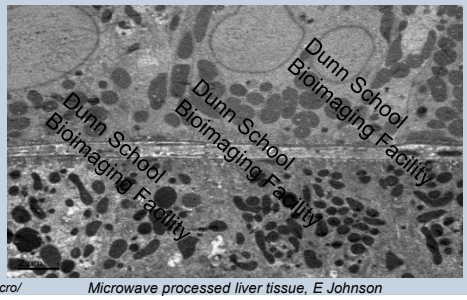


- Osmium tetroxide is a heavy metal that fixes unsaturated lipids.
- Used as both a secondary fixative and an electron stain, it significantly improves specimen preservation (especially of membranes) and contrast.



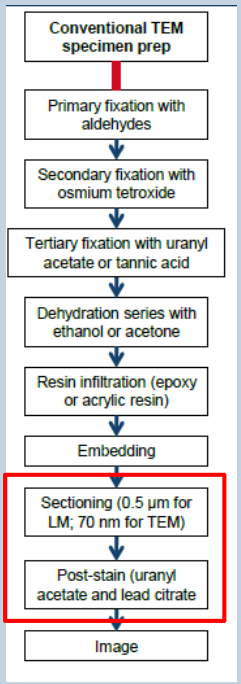
http://advanced-microscopy.utah.edu/education/electron-micro/





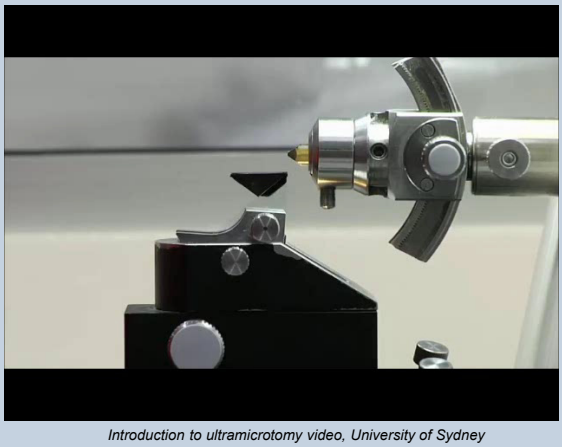
Microwave processed liver tissue, E Johnson

Specimen Preparation for TEM
Cells & Tissue



- Dehydration gradually replaces water in the sample with a solvent.
- The solvent is then gradually replaced with resin. The sample is embedded in resin and polymerised. The block is sectioned on an ultramicrotome and post-stained with even more heavy metals!

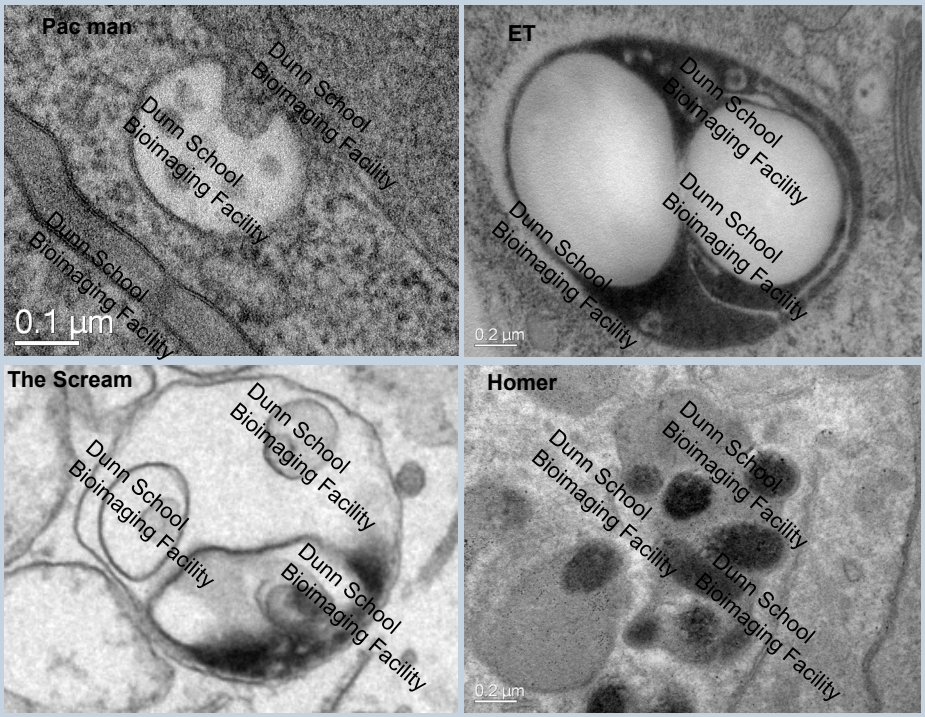
Leica Ultracut 7 ultramicrotome, Dunn School



Introduction to ultramicrotomy video, University of Sydney

Specimen Preparation for TEM

Critical evaluation of images



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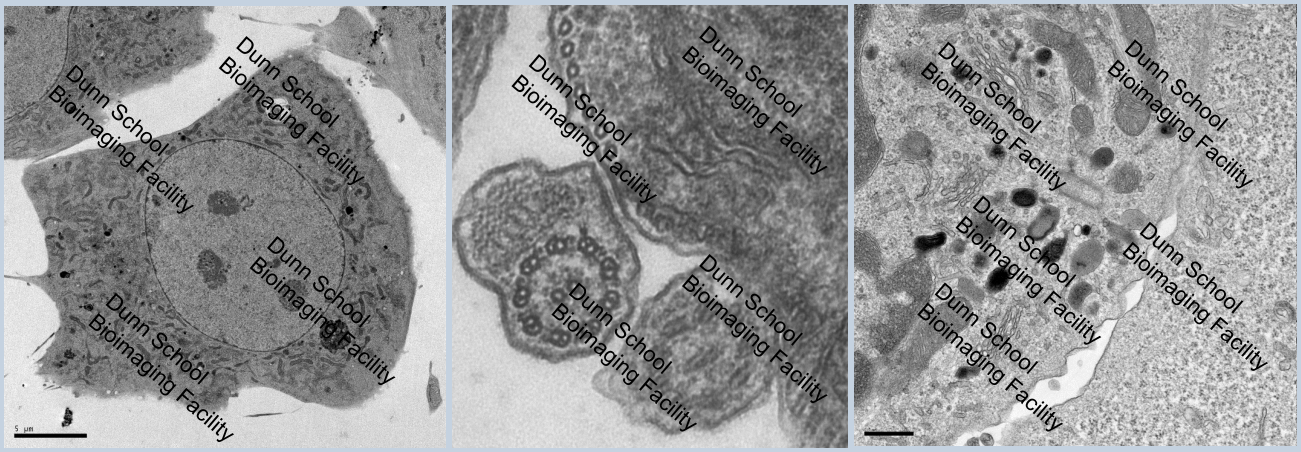
TEM Techniques

Ultrastructural imaging – Culture cells



Mouse fibroblast (E Johnson/A Moncada Pazos)

Cross-section of flagella in T. brucei (J Sunter)

HRP labelled T-cell interacting with a melanoma cell (E Johnson/G Bossi)

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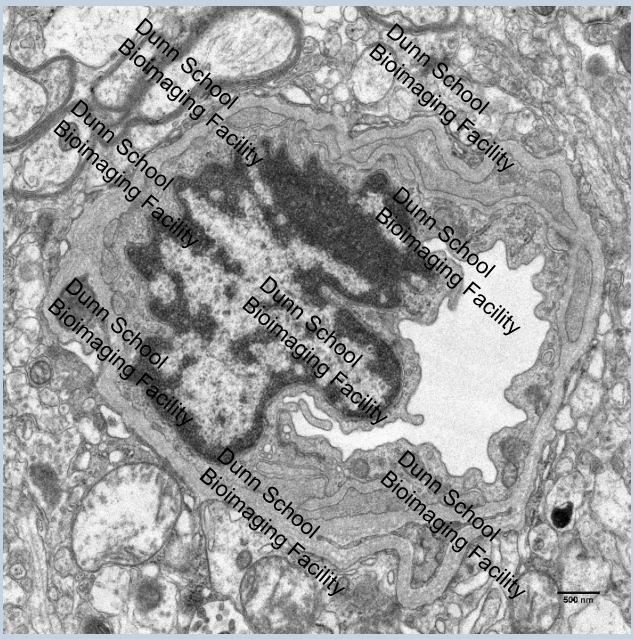
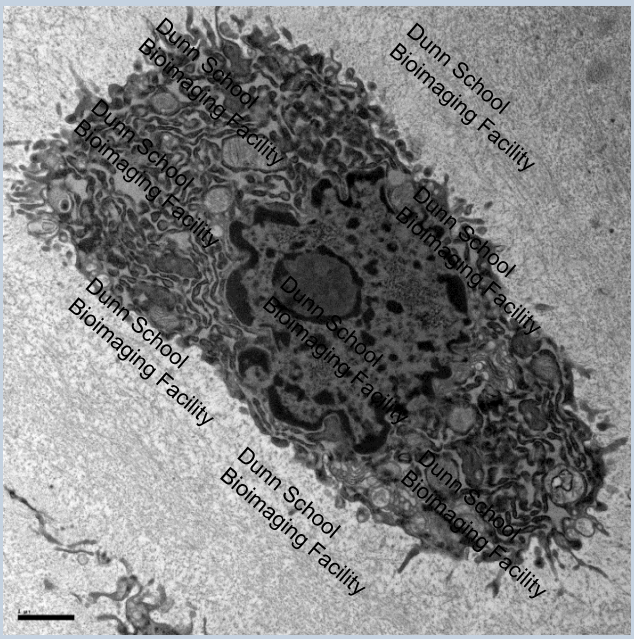
TEM Applications

Ultrastructural imaging – Tissue



Mouse blood/brain barrier
Tecnai12 TEM, A Douglas



Chondrocyte in mouse cartilage tissue
Tecnai12 TEM, P Sacitharan/A Pielach

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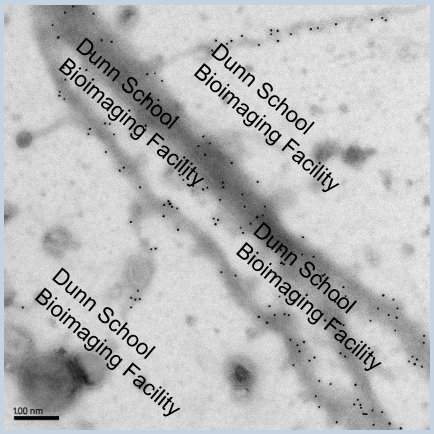
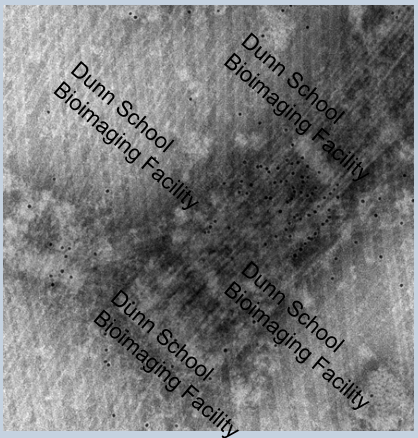
TEM Techniques

Protein localisation – Immunogold labelling

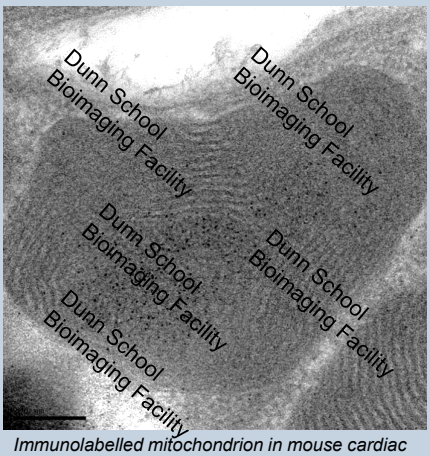
- Similar to immunofluorescence labelling, but the secondary antibody is conjugated to a small (1-40 nm) collodial gold particle instead of a fluorophore



Immunogold labelled Type IV pili from Neisseria meningitides (M Woermann/E Johnson)



Whole mount immunogold labelled Trypanosome cytoskeleton (S Dean)



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

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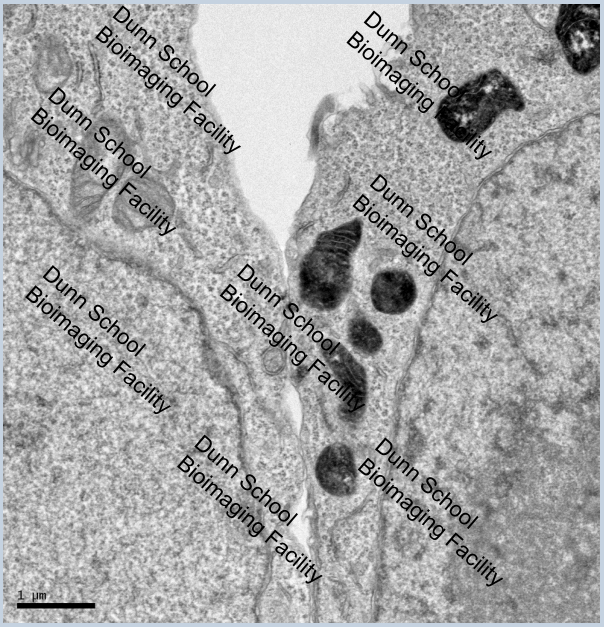
TEM Techniques

Protein localisation – EM genetic tags

- Two new genetically encoded tags are now available as alternatives to using immunogold labelling 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)

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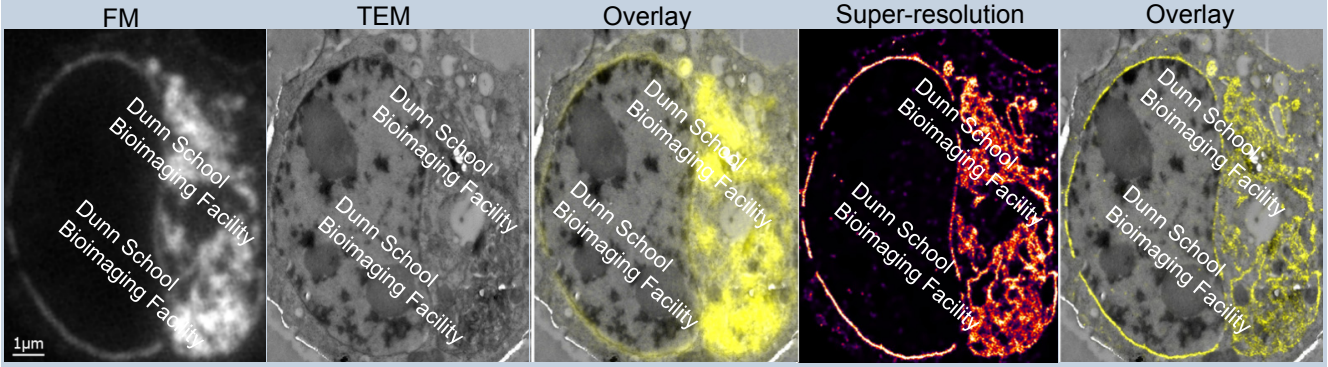
TEM Techniques

Micron OXFORD

Protein localisation – Correlative microscopy

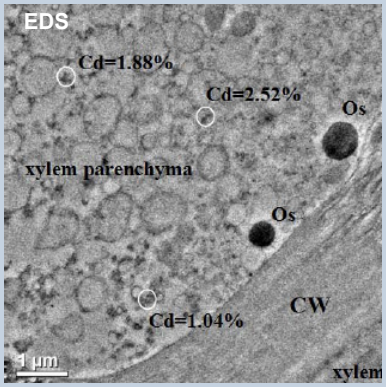
- Correlative microscopy allows you to place your fluorescently labelled protein in ultrastructural context.
- It is technically challenging and there are many different ways to perform it.
- Example: Cells expressing GFP -> Specialised TEM prep -> Widefield or localisation microsocpy on TEM section -> TEM of same cell ->Overlay LM image



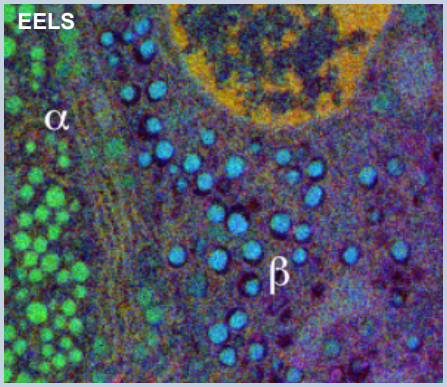
Correlative light & electron microscopy of HEK cells expressing an ER receptor tyrosine kinase tagged with mVenus (E Johnson & R Kaufmann, Micron)

TEM techniques
Chemical characterisation

- Energy-dispersive x-ray spectroscopy (EDS) allows chemical characterisation of specimens, based on the emission of characteristic x-rays.
- Electron energy loss spectroscopy (EELS) measures the amount of energy lost by inelastically scattered electrons as they pass through the sample. The energy loss is element specific.

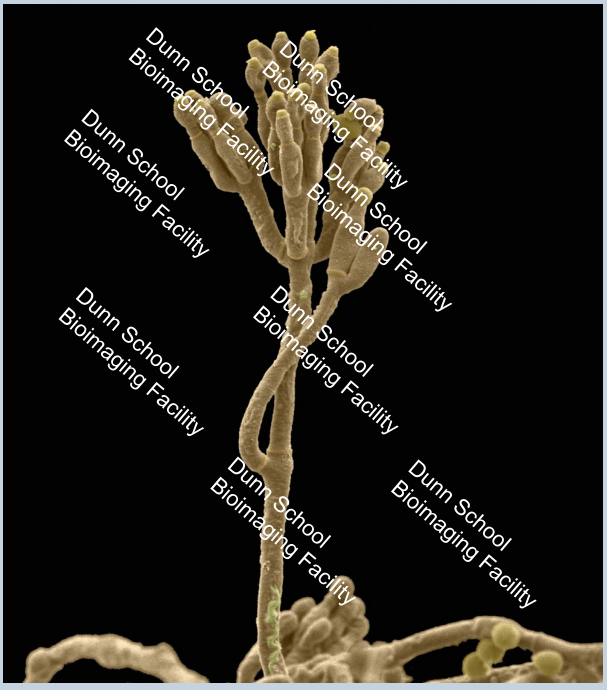


Cd Distribution in roots of Arabis paniculata
(Y. Tang, R. Qiu et al. Sun Yat-sen University. PR China)



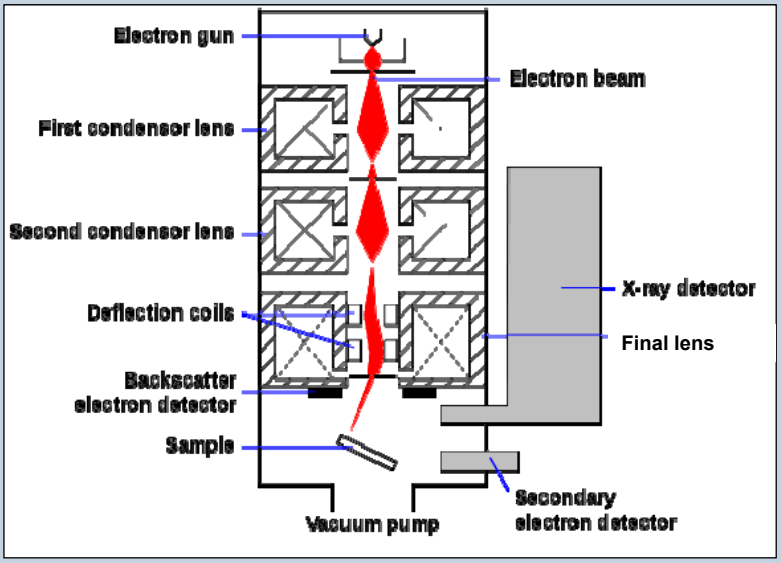
Unstained mouse pancreas with elemental contrast using EELS, NIH

Scanning Electron Microscopy (SEM)

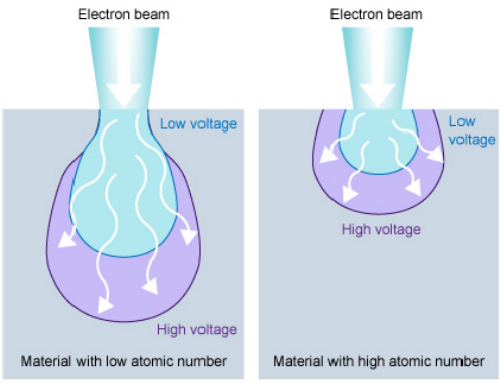


Penicillium (E Johnson)

The SEM

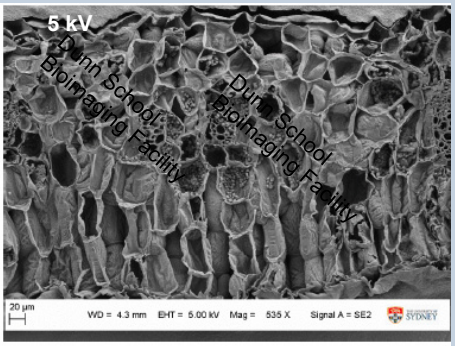


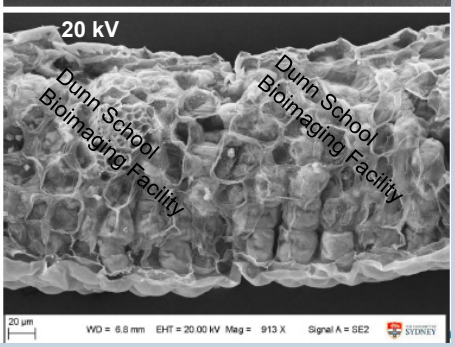
How the SEM works
Accelerating voltage



From: ammrf.org.au.myscope

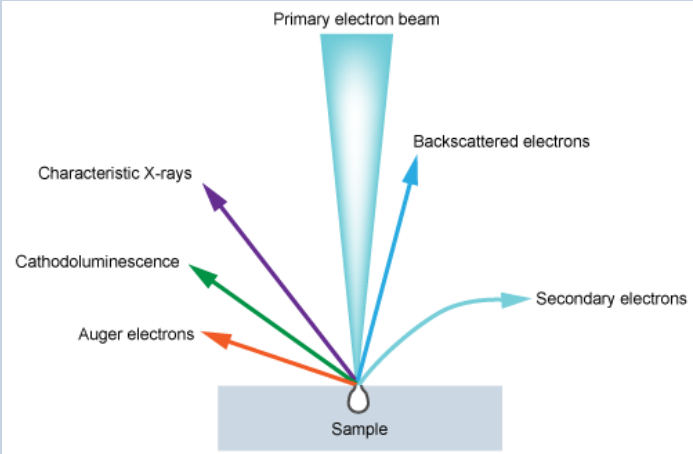
30 kV for material samples
~5 kV for biological samples

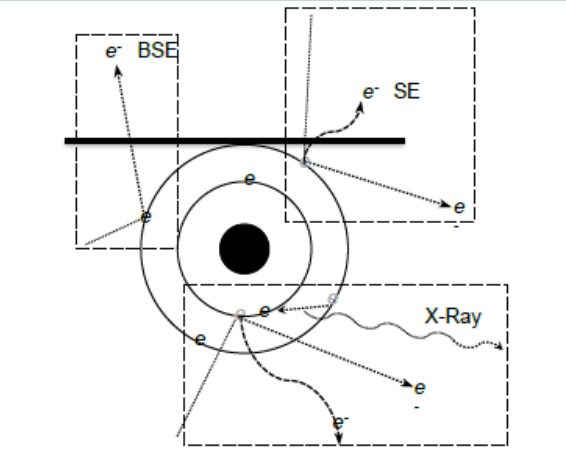




Spinach leaf section, Zeiss Ultra Plus, ACMM (E Johnson)

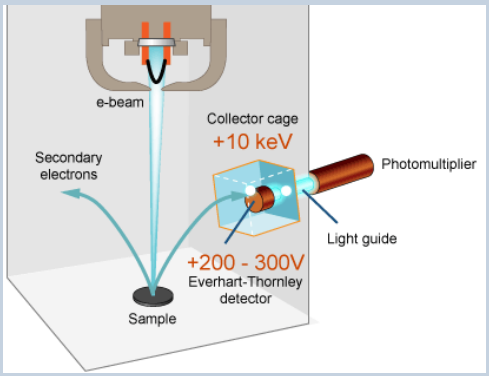
How the SEM works
SEM signals



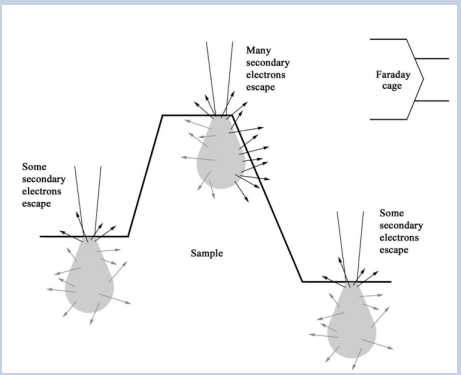


How the SEM works
Signal detection

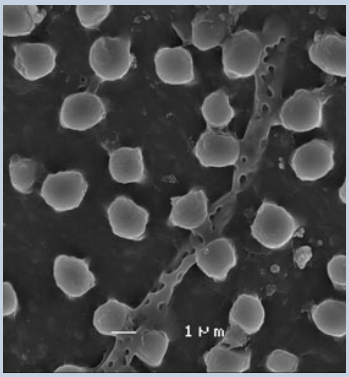
- Secondary electrons (SEs) provides surface morphology and topology information.
- SEs are captured by the Everhart-Thornley detector



www.ammrf.org



Dept Biological Sciences, Smith College Northampton USA

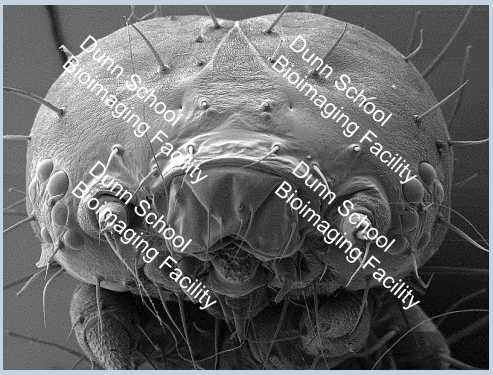


www.ammrf.org

Scanning Electron microscopy
Specimen requirements

SEM
Stable in the vacuum
Well preserved surface structure
Conductive surface
Whole mount
Particulate samples can be coated and viewed quickly
Cells and whole organisms require some specimen preparation

Little dude going about his business



SEM of a fixed and Au coated caterpillar (E Johnson)

SEM techniques
Specimen preparation

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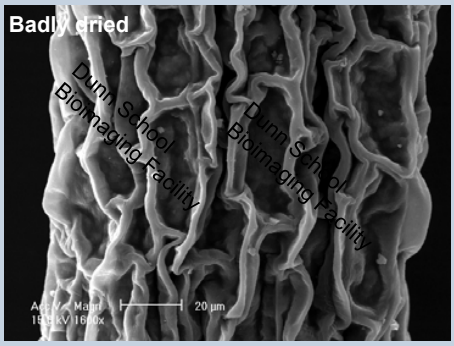
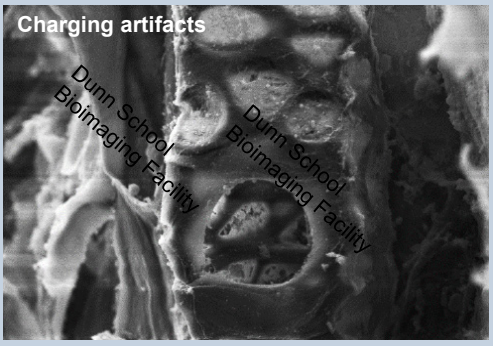
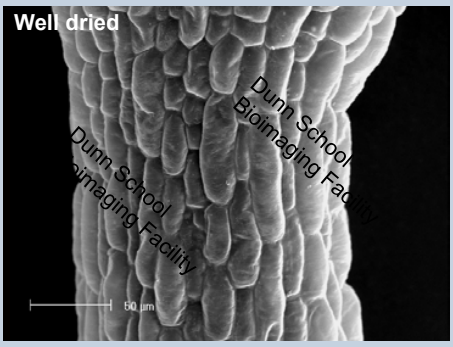
Conventional SEM specimen prep

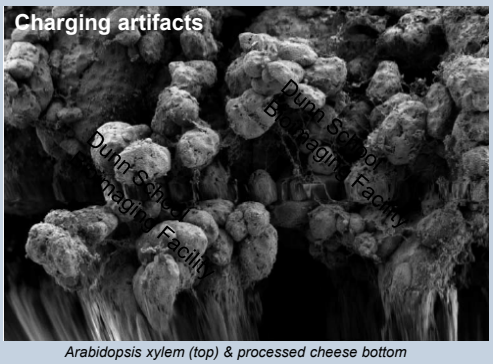
- Primary fixation with aldehydes
- Secondary fixation with osmium tetroxide
- Dehydration series with ethanol or acetone
- Dry using HMDS or the critical point dryer
- Mount and sputter coat
- Image







Arabidopsis stem, Phillips XL30 SEM, E Johnson



Arabidopsis xylem (top) & processed cheese bottom Zeiss UltraSEM, E Johnson

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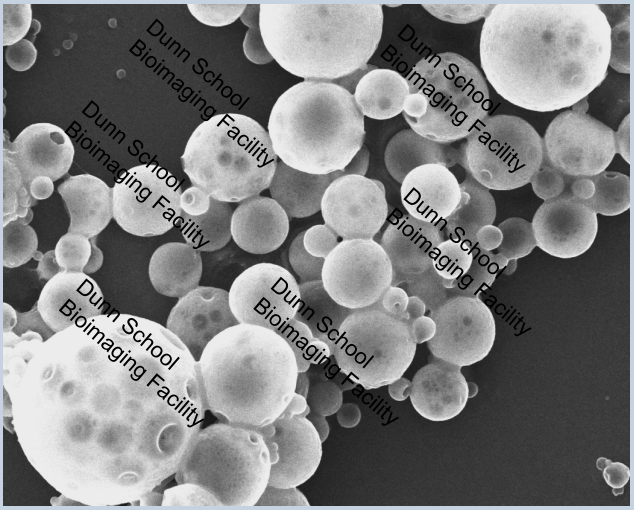
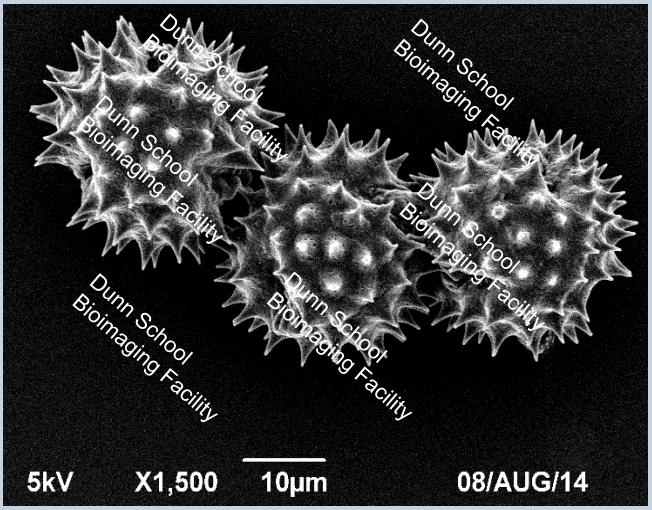
SEM Applications
Topography – Particulate samples



Plastic beads conjugated to vaccine particles (JEOL 6390 SEM, A Walters/E Johnson)



Pollen grains (JEOL 6390 SEM, E Johnson)

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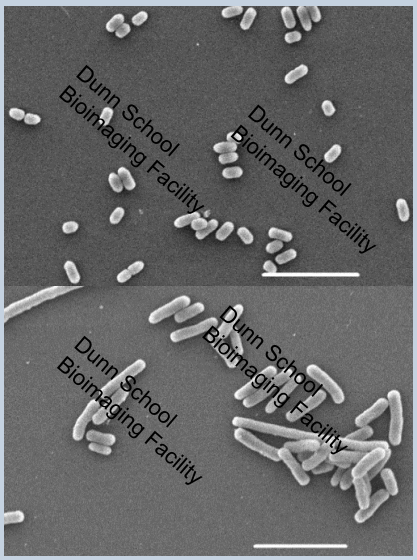
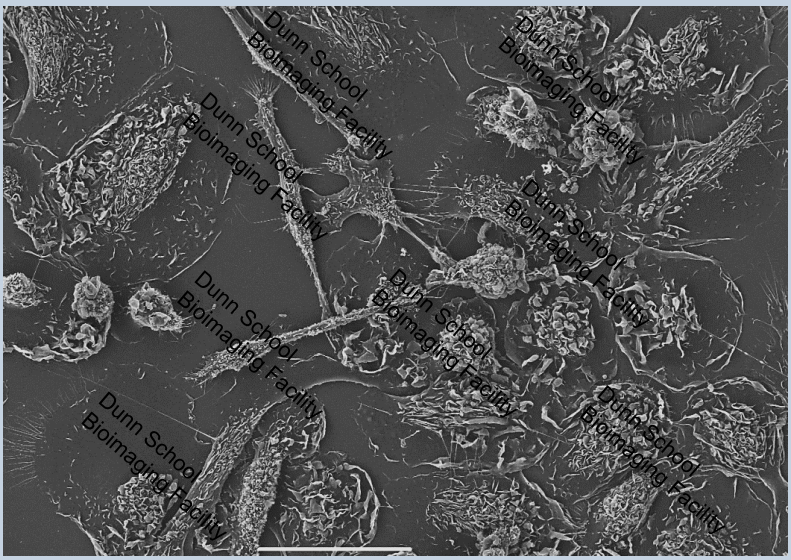
SEM techniques
Topography – Cells



E coli (WT at top, +vector at bottom) Scale bar 5 µm (R Harding/E Johnson)



Monocytes and macrophages, scale bar 50 µm (B van Wilgenburg/E Johnson)

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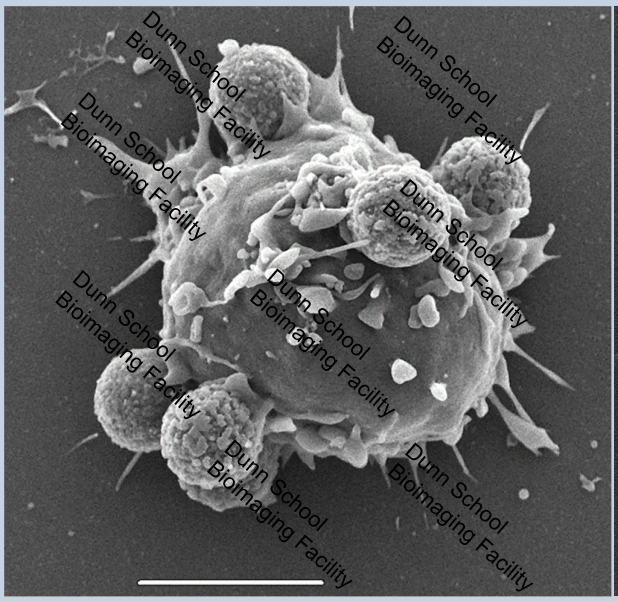
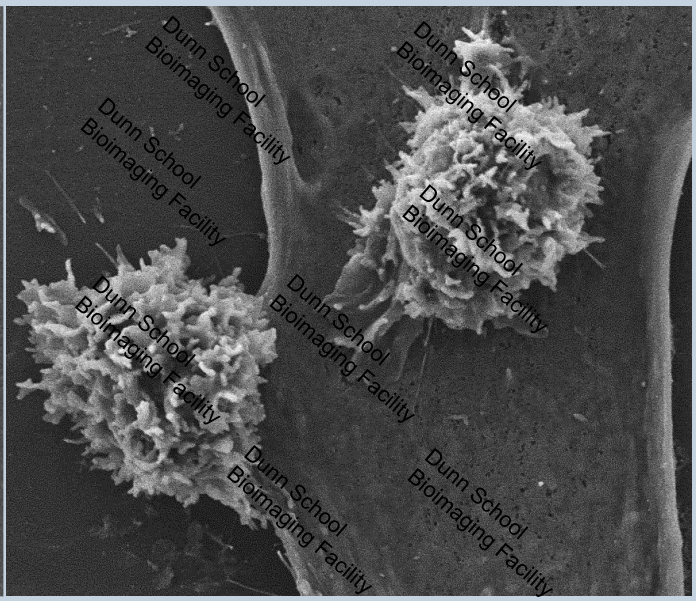
SEM techniques
Topography – Cells



HEK 293 cell interacting with streptavidin coated dynabeads plus biotinylated anti-EPCAM, Scale bar 5 µm (JEOL 390 SEM, M Brenner/E Johnson)



T-cells interacting with a melanoma cell (JEOL 390 SEM, E Johnson, Dunn School)

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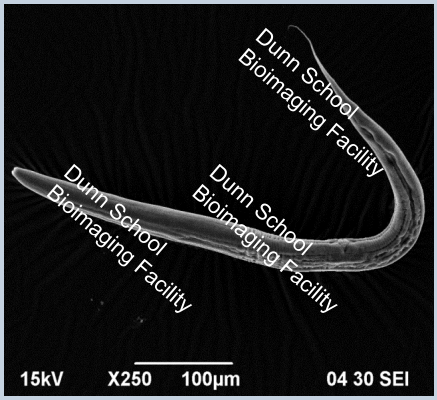
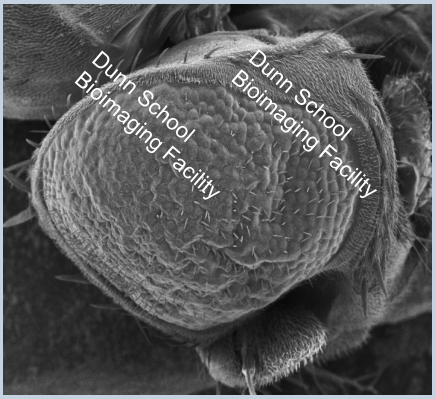
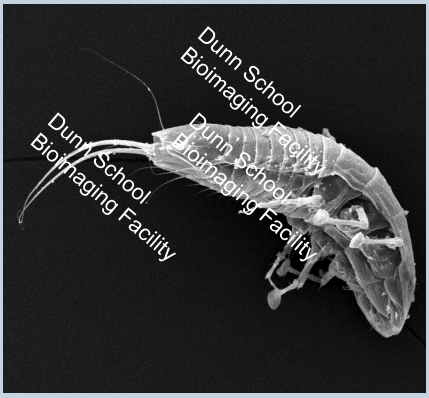
SEM Applications
Topography – Whole organisms



C. elegans
(E Johnson/A Moloney, Dunn School)



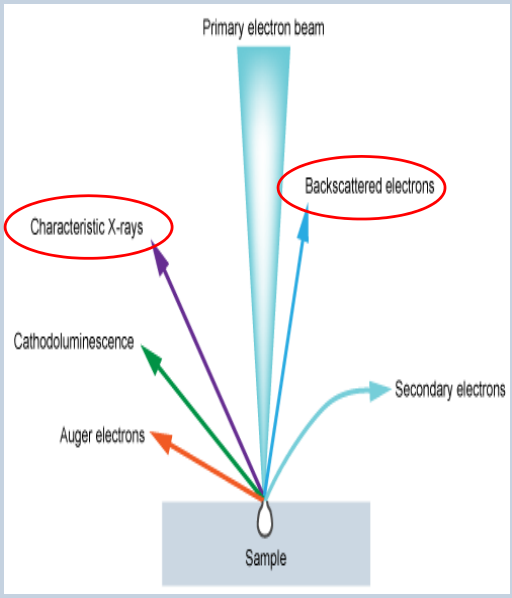
Drosophila rough eye phenotype
(M Elschami, NDCN)



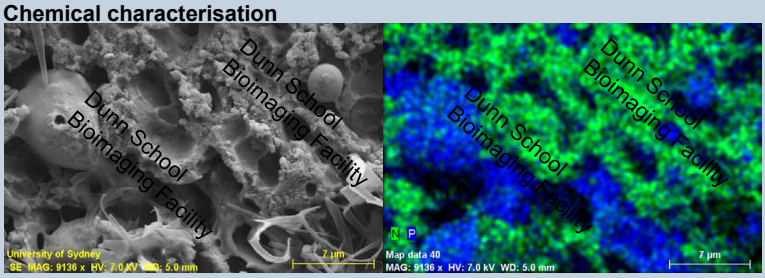
Exotic arthropod (E Johnson)

SEM Techniques

Other options

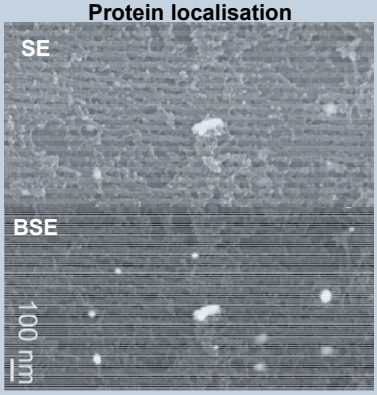


Chemical characterisation



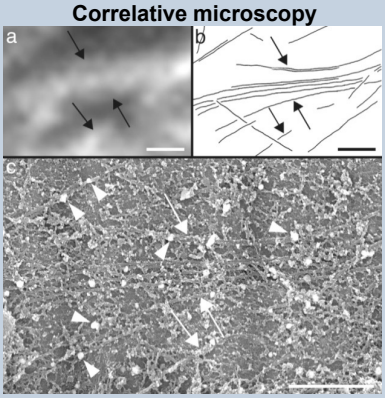
EDS analysis of cheese (M Foley, University of Sydney)

Protein localisation



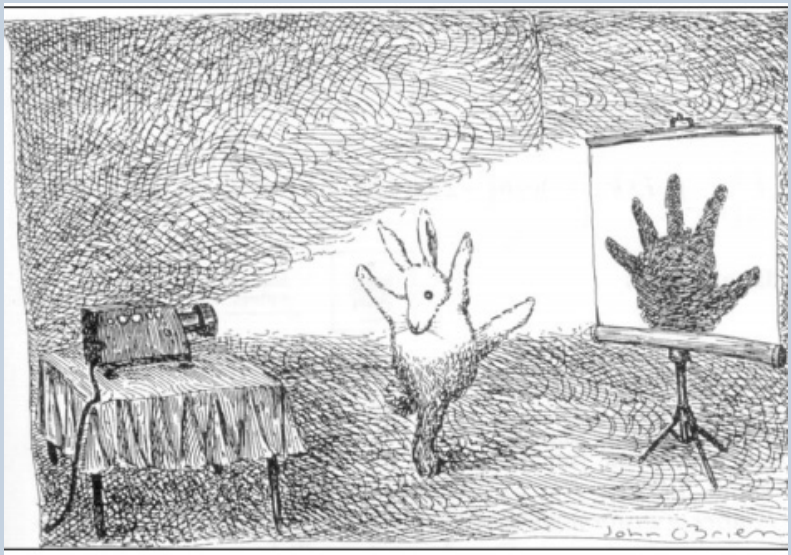
Microtubules (D Barton, University of Sydney)

Correlative microscopy



Microtubules (D Barton, University of Sydney)

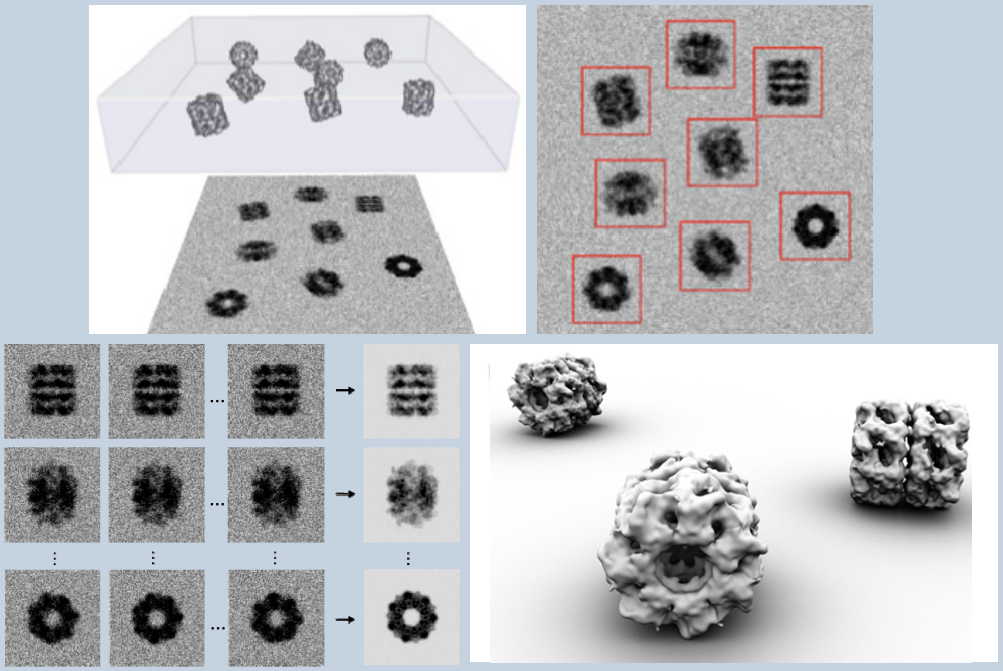
3D EM Techniques



Drawing by John O'Brien, The New Yorker Magazine (1991)

3D EM Techniques

TEM - Single particle imaging & reconstruction



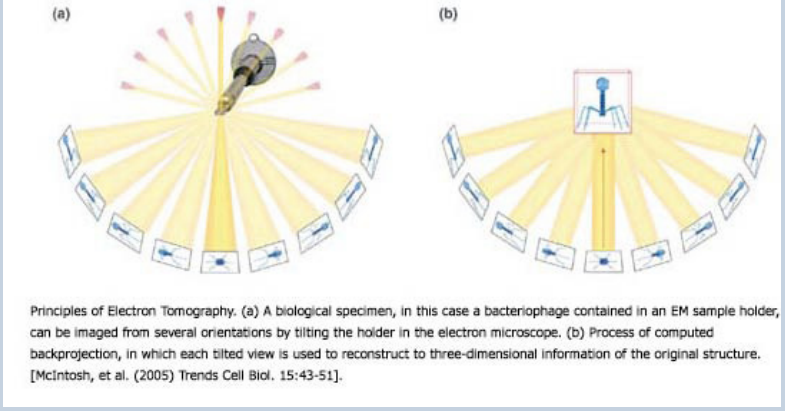
Single particle imaging and reconstruction of the GroEL chaperonin: purified complexes were applied to a grid and vitrified, then imaged with cryo-TEM. Thousands of images are collected and the same orientations are clustered together, averaged and back projected to render the complexes in 3D to 1 nm resolution. From: http://people.csail.mit.edu/gdp/cryoem.html

3D EM Techniques

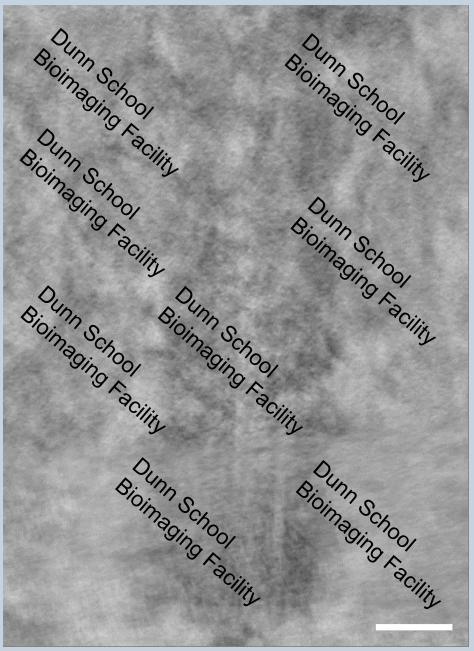
TEM - Electron tomography

Dunn School Bioimaging Facility

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



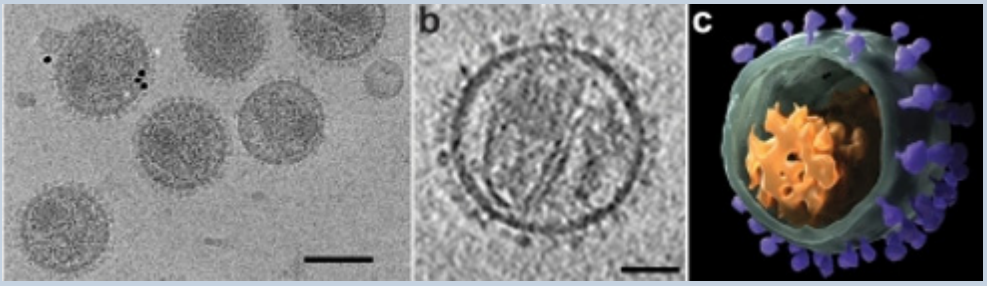
Principles of Electron Tomography. (a) A biological specimen, in this case a bacteriophage contained in an EM sample holder, can be imaged from several orientations by tilting the holder in the electron microscope. (b) Process of computed backprojection, in which each tilted view is used to reconstruct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



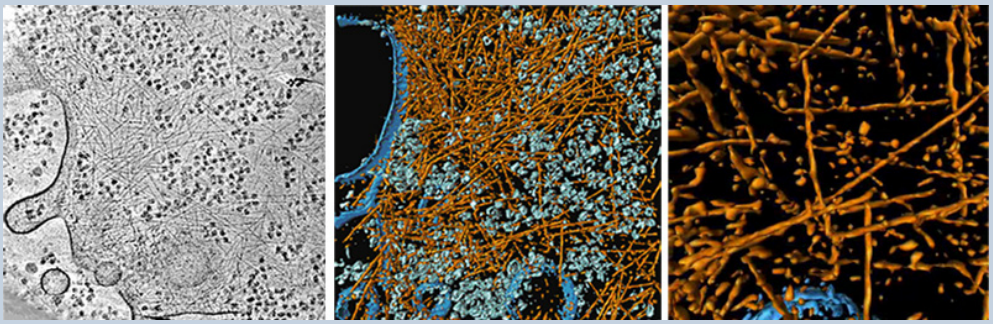
Drosophila primary spermatocyte centrioles, H Roque (Dunn School)

3D EM Techniques

TEM - Cryo-TEM 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)

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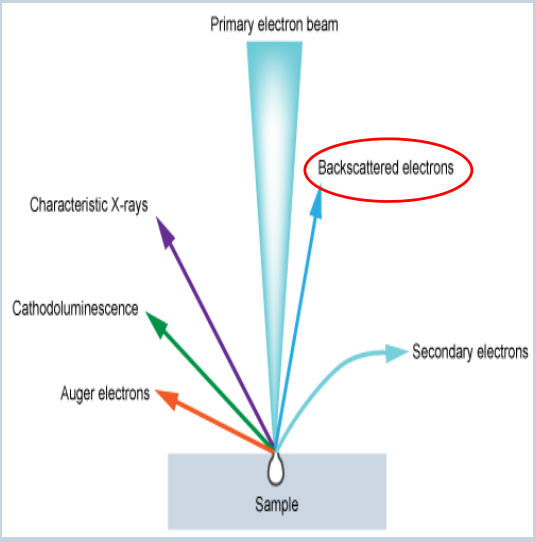
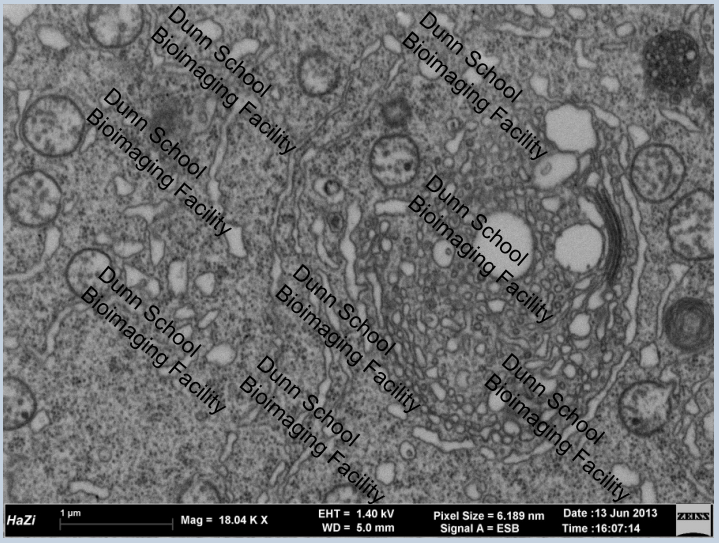
3D EM Techniques

OXFORD BROOKES UNIVERSITY

SEM - Serial Block Face Sectioning with Gatan 3View

SEM can be used to generate 'TEM' images by detecting backscattered electrons, beam electrons that have been elastically scattered/deflected by high atomic number elements (heavy metals) in the sample

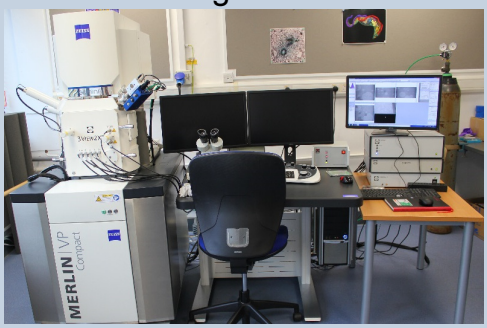


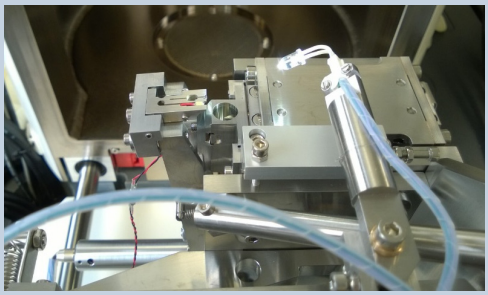


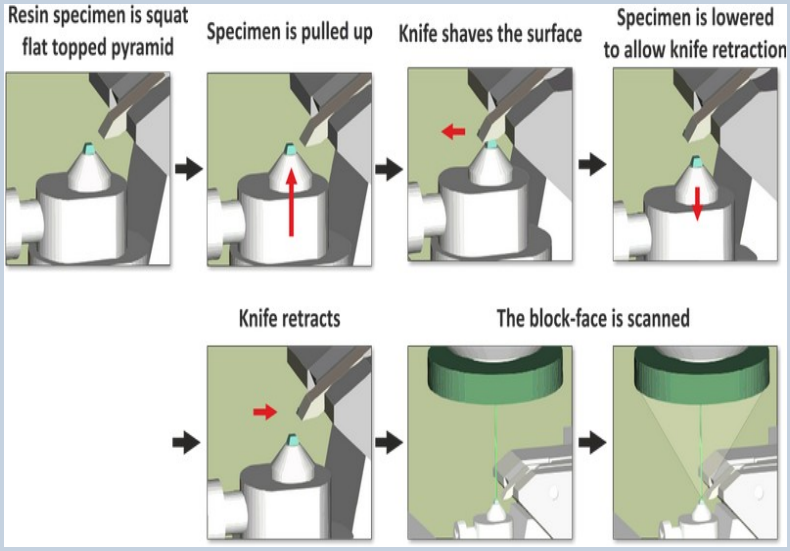
3D EM Techniques

SEM - Serial Block Face Sectioning with Gatan 3View

One method for generating a 3D high resolution image stack is to use serial block face sectioning with the Gatan 3View system







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

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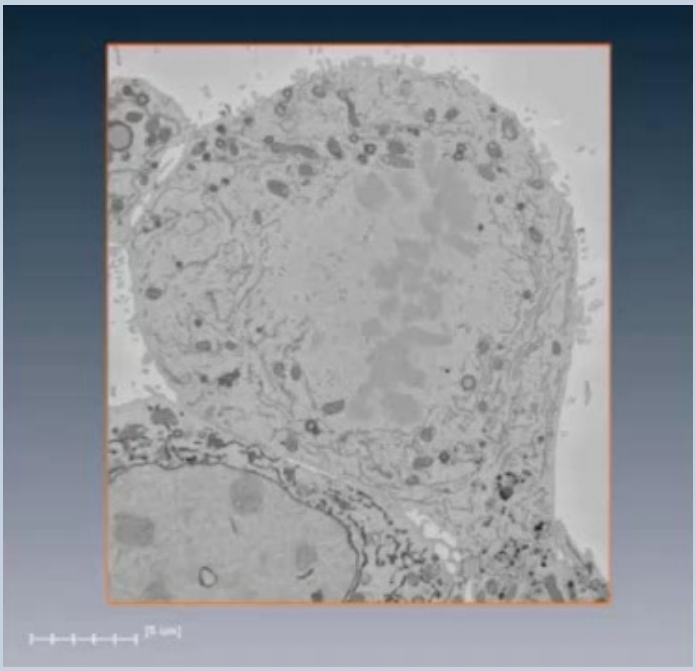
3D EM Techniques

OXFORD BROOKES UNIVERSITY

SEM - Serial Block Face Sectioning with Gatan 3View

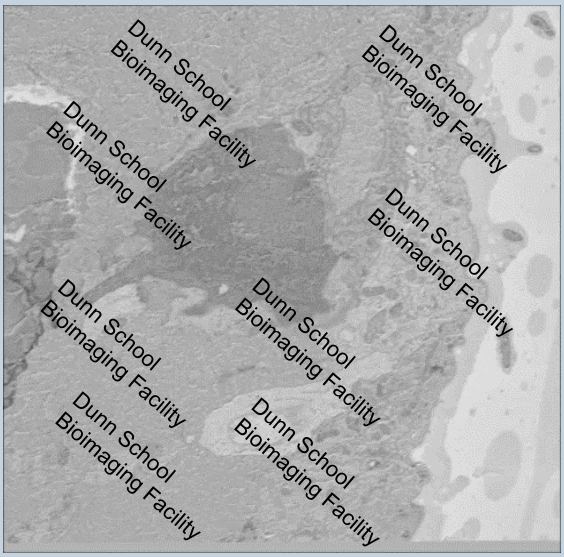


Huh-7 cell in early-metaphse with chromosomes and ER segmented and modelled
Puhka et al (2012) Mol Biol Cell 23(13)

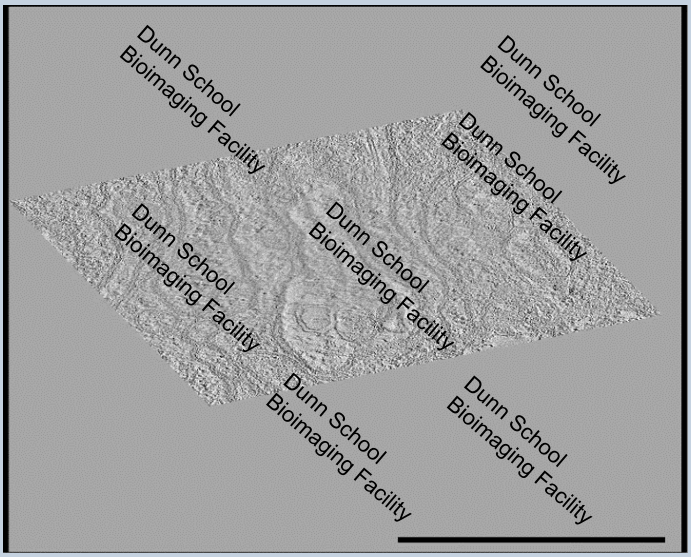
3D EM techniques

Comparing 3View and TEM tomography

3View

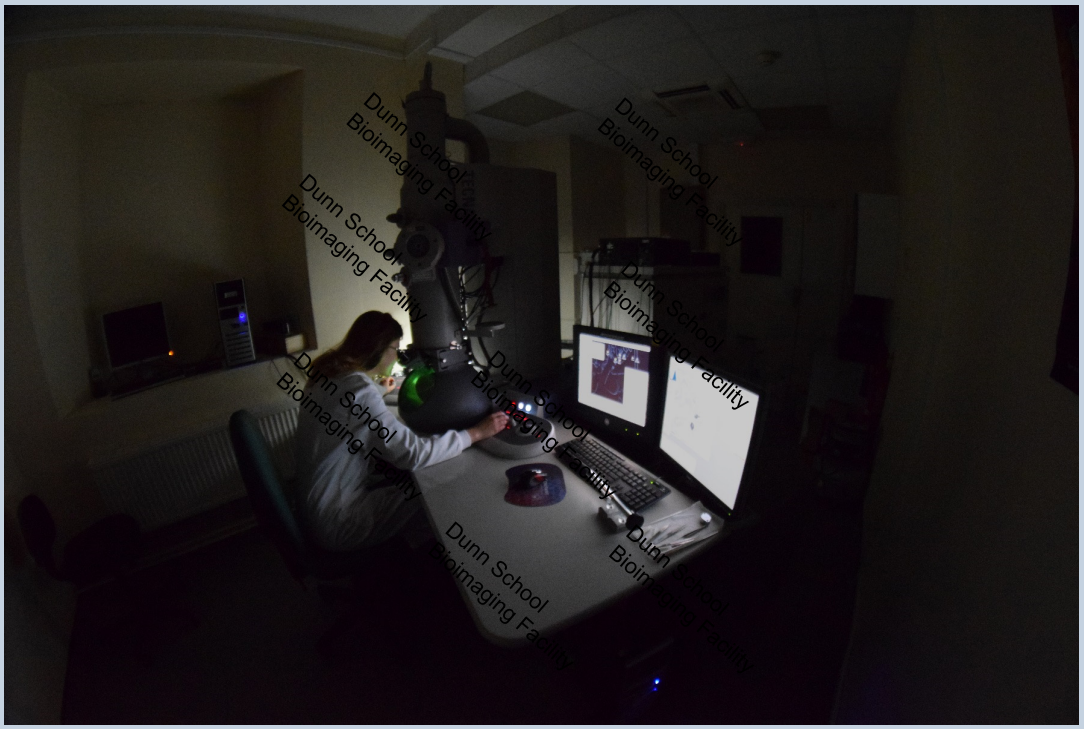


TEM tomography



Sensory cilia in Drosophila notum tissue imaged on the Zeiss Merlin Compact 3View SEM (left) and with serial section EM tomography on the Tecnai12 TEM (H Roque/E Johnson)

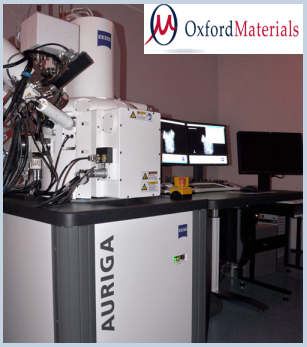
Electron microscopy at Oxford



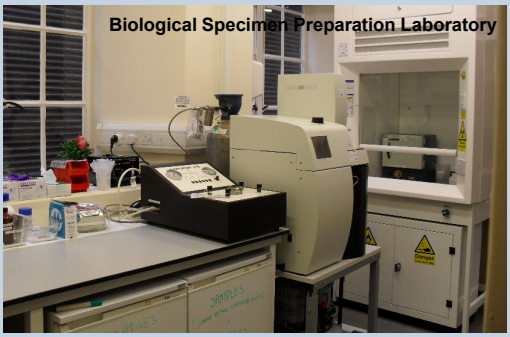
EM Facility staff member Dr Anna Pielach using the Tecnai12 TEM

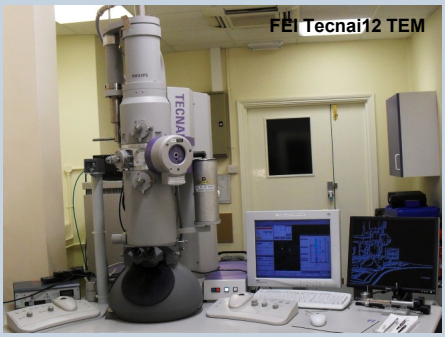
EM Facilities

- A full list of EMs at Oxford is available at https://www.research-facilities.ox.ac.uk/
- The main EM facilities are:
  - Materials Department: http://www-em.materials.ox.ac.uk/
    - Parks Rd & Begbroke Science Park
    - Wide range of EMs, though little biological experience!
  - Physics Department: https://www2.physics.ox.ac.uk
    - Nanofabrication and SEM facility
  - Oxford Particle Imaging Centre (OPIC): http://www.opic.ox.ac.uk
    - Henry Wellcome Building for Particle Imaging
    - Biosafety containment (ACDP3/DEFRA4)
    - Cryo-TEM and Cryo-electron tomography
  - The Dunn School Bioimaging Facility: http://web.path.ox.ac.uk/~bioimaging//bioimaginghome.html



Dunn School EM Facility
Overview

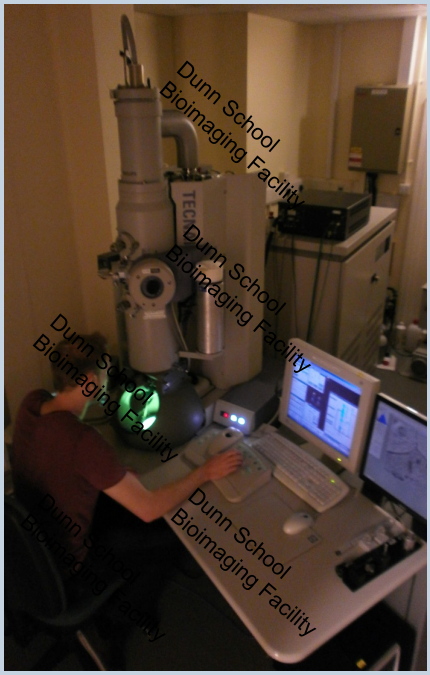




Dunn School EM Facility
Access

Multi-user facility with three modes of usage:

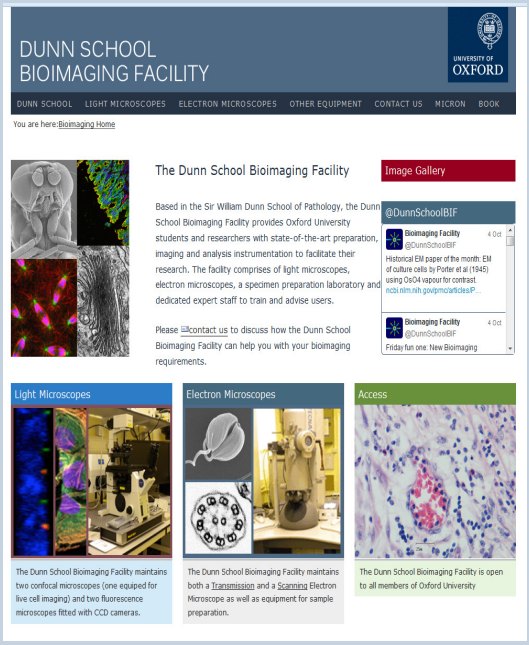
- Independent
  - Medium to long-term projects
  - User is fully trained to use relevant microscopes & equipment
  - Staff available to help with troubleshooting and image analysis
  - Cost: consumables & instrument time
- Service
  - One-off/short-term projects
  - Specimen preparation and/or microscopy performed by staff
  - Cost: technician time, consumables & instrument time
- Collaborative
  - Technique development, performed by staff
  - Cost: consumables and instrument time



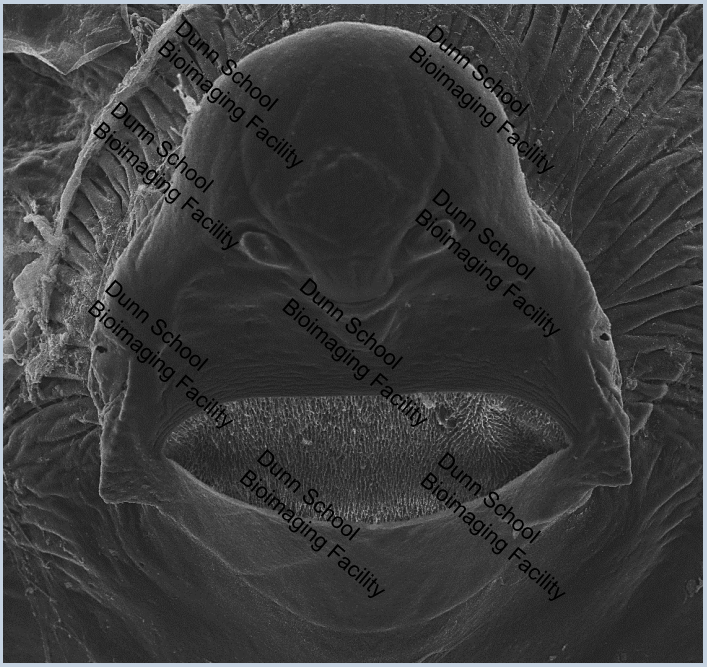
Dunn School PhD student Joshua Long (Fodor group) using the TEM to study the mitochondrial localisation of an influenza protein

Dunn School EM Facility
Access

- Keep up to date with EM papers, talks and news via our Twitter feed (@DunnSchoolBIF)
- Courses
  - In-house courses throughout the year
  - Med Sciences Skills Training Programme
  - Variety of external courses
- Search the literature!
- Contact me to discuss options for your research and to setup an EM project.



Electron microscopy: Feel the Force!



Darth Vader