SIR WILLIAM DUNN
SCHOOL OF PATHOLOGY

UNIVERSITY OF OXFORD

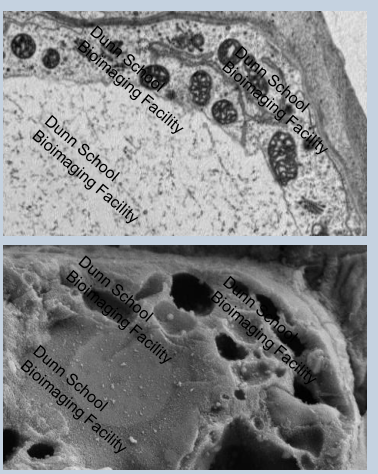
BIOLOGICAL ELECTRON MICROSCOPY

Dr Errin Johnson
EM Facility Manager

May 23, 2014

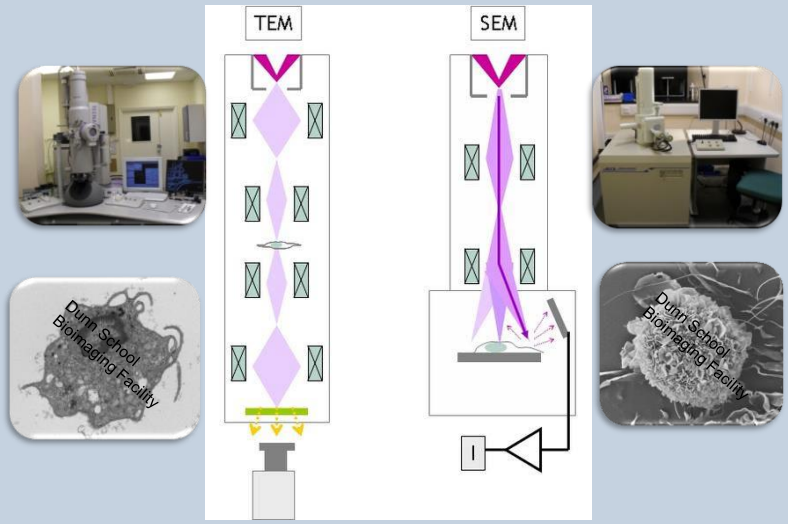
Lecture Overview

- Introduction to Electron Microscopy (EM)
  - Features of Electron Microscopes
  - EM facilities at The University of Oxford
- 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

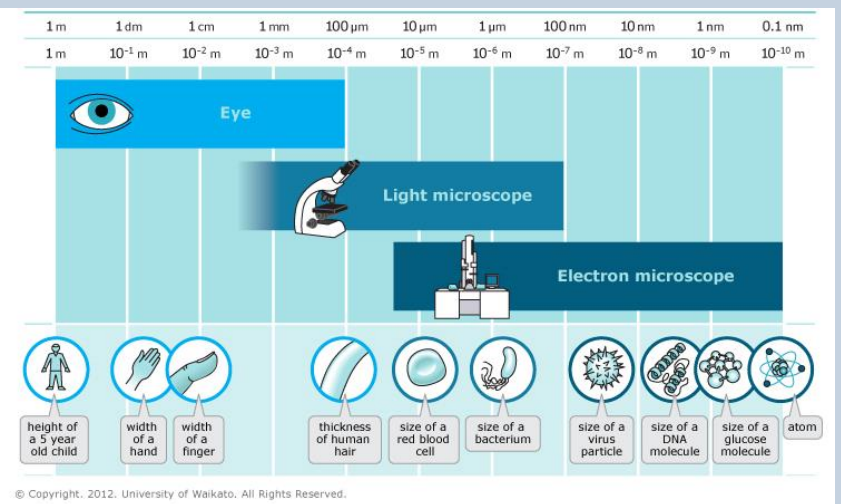


Leaf epidermal cells imaged by TEM (top) and SEM (bottom)
E Johnson, Dunn School

Electron microscopy
Overview

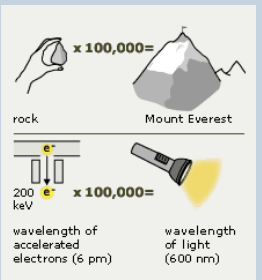


Electron microscopy
Resolution

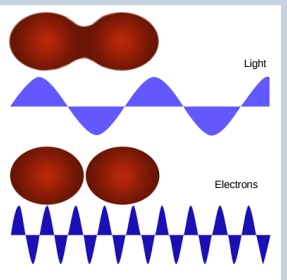


Electron microscopy
Resolution

- Resolution is the smallest distance at which two neighbouring points can be distinguished and is dependent on wavelength
- The wavelength of electrons is MUCH shorter than that of light
  - Confocal microscope resolution = 200 nm
  - Electron microscope resolution < 1 nm

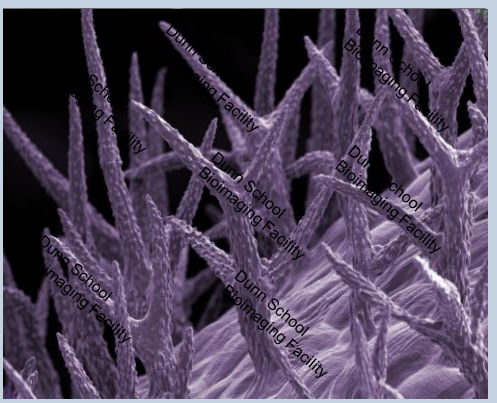


www.nobelprize.org



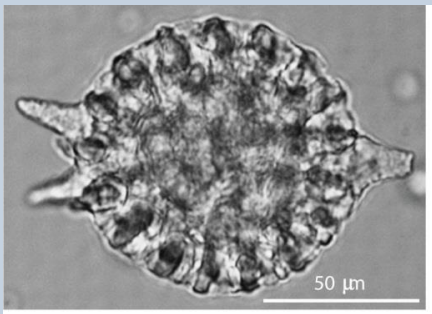
www.ammrf.org

Electron microscopy
Resolution

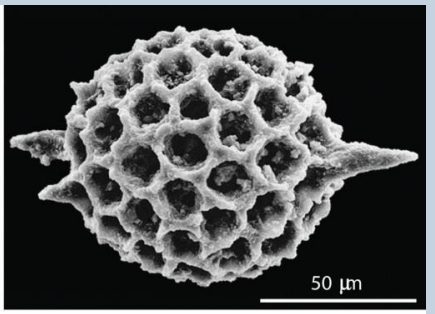


Lavender flower imaged using SEM (right; E Johnson, Dunn School)

Electron microscopy
Resolution



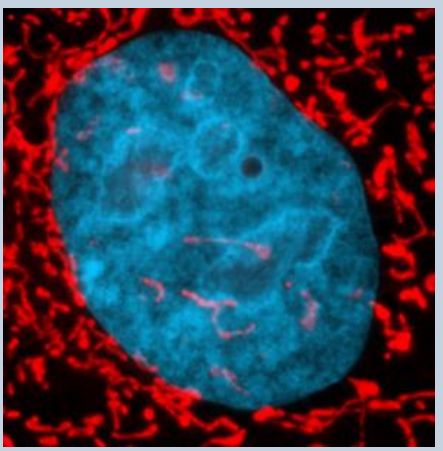
(a) Radiolarian under light microscope



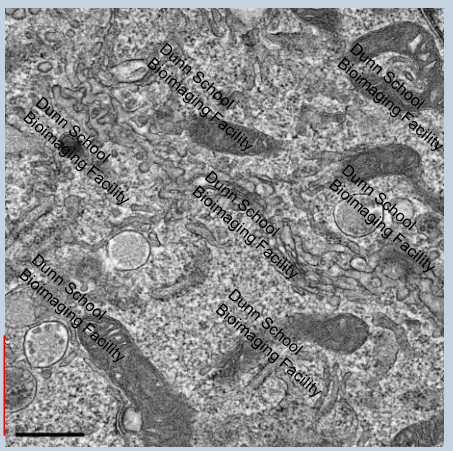
(b) Radiolarian under electron microscope

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

Electron microscopy
Resolution



Confocal image of a kidney cell stained with DAPI and MitoTracker (Hammamatsu.magnet.fsu.edu)

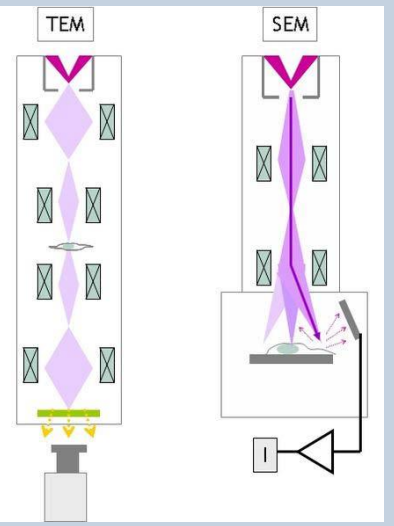


TEM image of fibroblast cell stained non-specifically (E Johnson, Dunn School)

Electron microscopy
Overview

The main components of an electron microscope are:

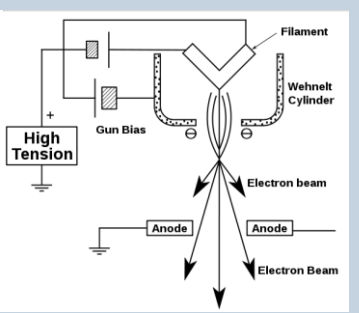
- An electron gun
- Electromagnetic lens system
- Vacuum system
- Camera/detector
- Computer



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

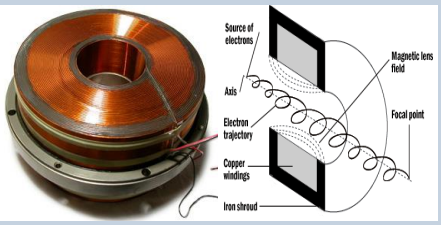
www.ammrf.org



www.wikipedia.org

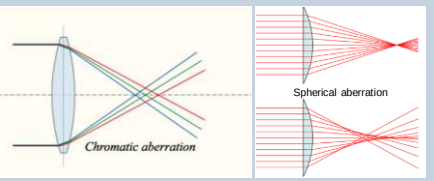
Electron microscopes
Electromagnetic Lenses

- 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

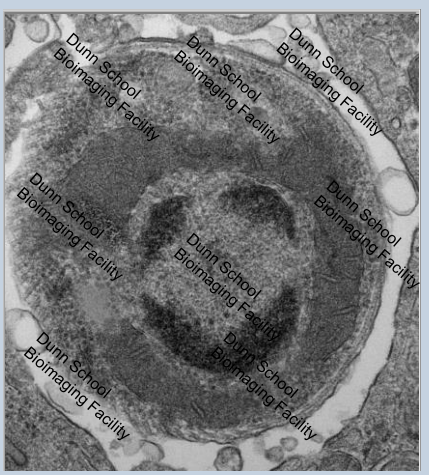
- Similarly to optical lenses, electromagnetic lenses are also susceptible to aberrations
  - Chromatic aberration
  - Spherical aberration
  - Astigmatism



A. Kach, University of Zurich

Electron microscopes
Vacuum systems

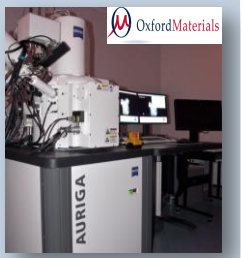
- EMs have elaborate pumping systems to ensure that the microscope is operated under a high vacuum (10^{-4} Pa)
  - Maintains the integrity of the electron beam, as any interaction with gas atoms will cause the beam to scatter
  - Avoids arcing between the cathode and ground (and damage to the filament)



TEM of [illegible] (Dunn School Bioimaging Facility)

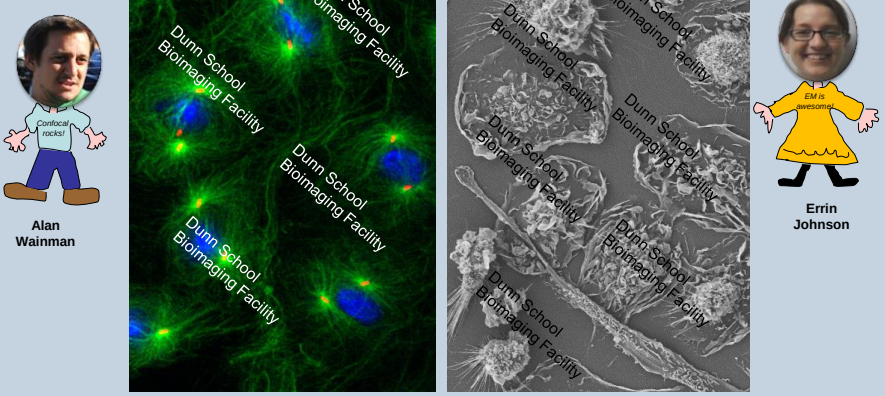
EM Facilities at The University of Oxford

- 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



The Dunn School Bioimaging Facility
Associated with Micron Oxford

Light Microscopy | **Electron Microscopy**



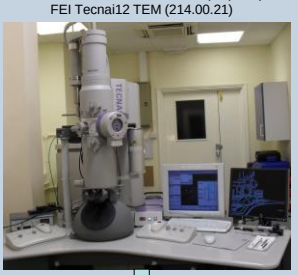
A Franz, Dunn School | *E Johnson, Dunn School*

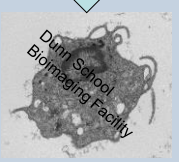
Electron Microscopy at the Dunn School

Biological Specimen Preparation Laboratory
Equipment & reagents (214.00.21)

eg: ultramicrotomy

Transmission Electron Microscope (TEM)
FEI Tecnai12 TEM (214.00.21)





Internal ultrastructure

Blood monocyte, B. van Wilgenburg/ E. Johnson

Scanning Electron Microscope (SEM)
JEOL JSM-6390 SEM (214.00.33)

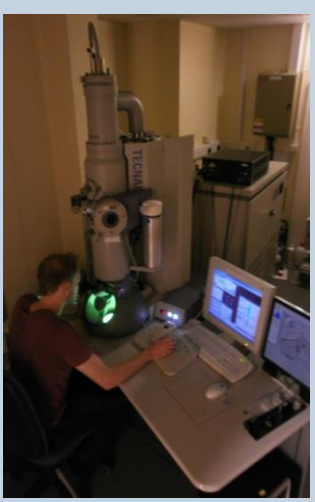
Surface morphology

Blood monocyte, B. van Wilgenburg/ E. Johnson

Electron Microscopy at the Dunn School
Access

Multi-user facility with three modes of usage:

- Independent
  - Medium to long-term projects
  - 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
  - Specimen preparation and/or microscopy performed by Errin
  - Cost: technician time, consumables & instrument time
- Collaborative
  - Technique development, performed by Errin
  - Cost: consumables and instrument time

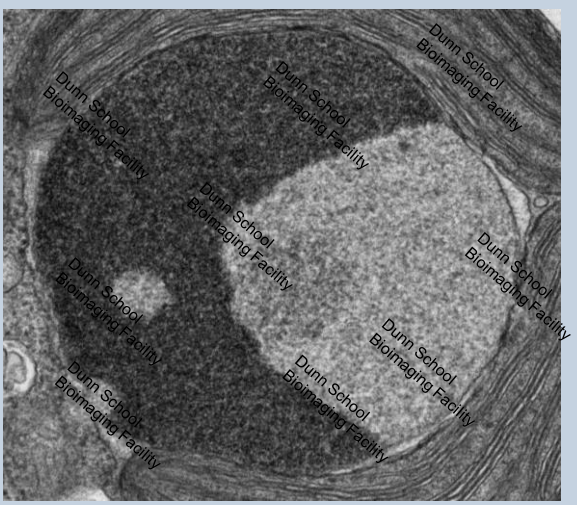


Dunn School PhD student Joshua Long (Fodor group) using the TEM to study the mitochondrial localisation of an influenza protein

Electron Microscopy at the Dunn School
Access

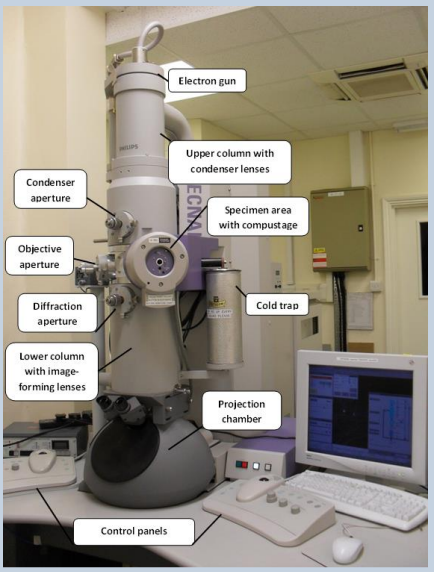
- Keep up to date with EM papers, talks and news via our Twitter feed (@DunnSchoolBIF)
- Courses
  - In-house courses throughout the year
  - Med Sciences Skills Training Programme
  - Variety of external courses
- Search the literature!
- Contact me to discuss options for your research and to setup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

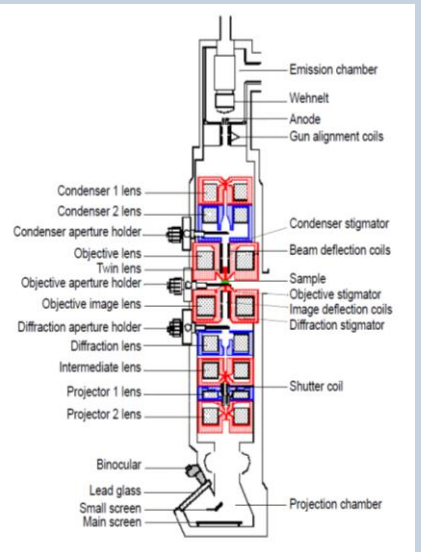
Transmission Electron Microscopy (TEM)



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

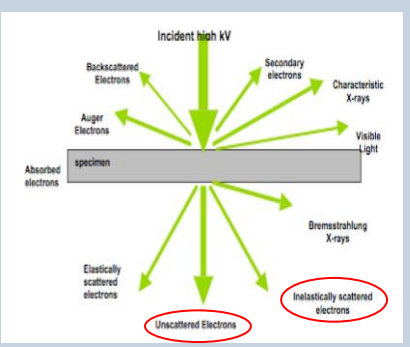
The TEM

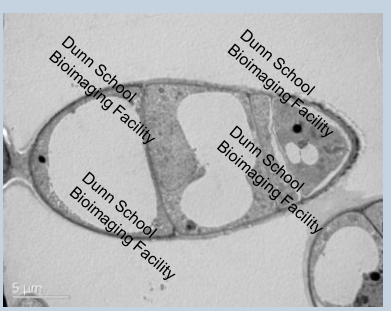




The TEM
Contrast

- 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.

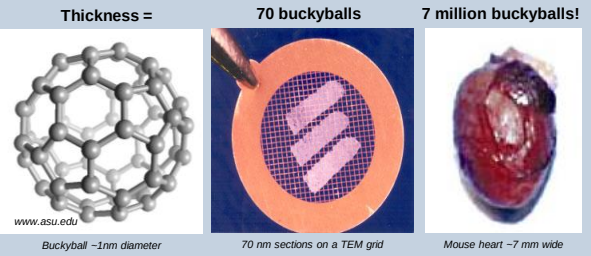




Lavender trichome, E Johnson

Specimen Preparation for TEM
Overview

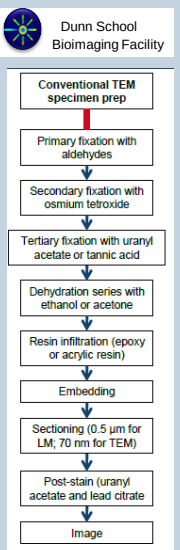
- TEM specimens must be:
  - Very thin (typically 70 nm)
  - Well preserved
  - Electron dense
  - Stable in the vacuum

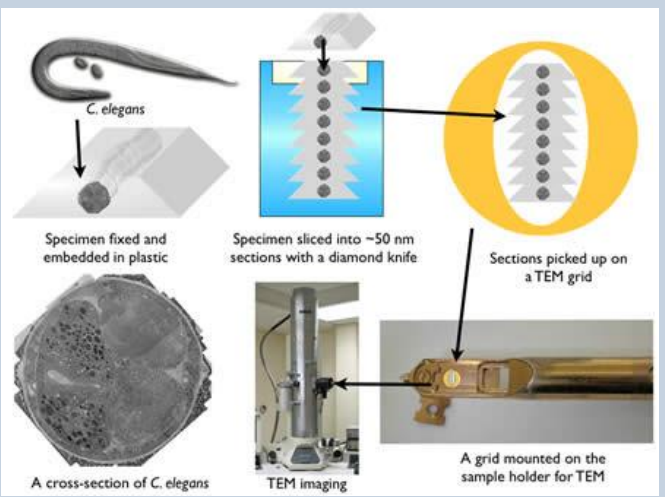


- The degree of specimen preparation for biological TEM depends on the specimen
  - Particulate samples (eg: protein and viruses) can be stained and viewed quickly
  - Cells and tissue samples require extensive preparation for TEM

Specimen Preparation for TEM
Cells & Tissue – Overview

Dunn School Bioimaging Facility



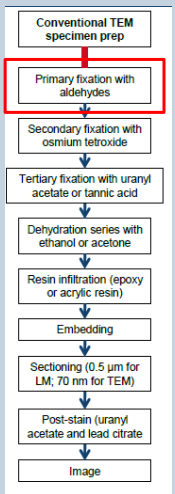


http://www.research.utah.edu/advanced-microscopy/education/electron-micro/index.html

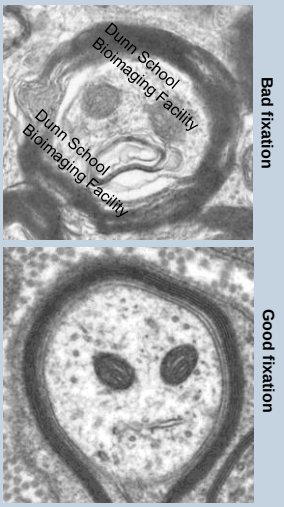
Specimen Preparation for TEM

Cells & Tissue – Primary Fixation

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

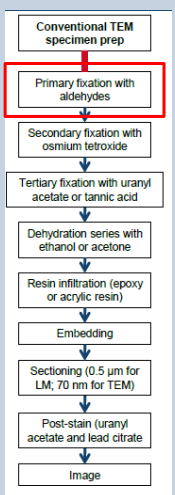


Top: E Johnson (modified) Bottom: remf.dartmouth.edu

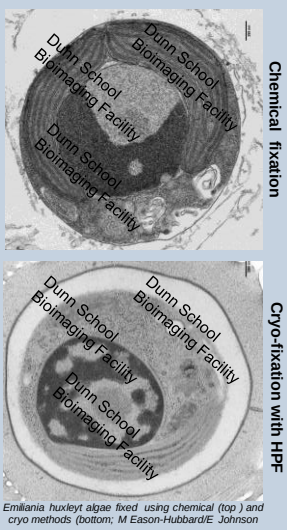
Specimen Preparation for TEM

Cells & Tissue – Methods of Fixation

Dunn School Bioimaging Facility



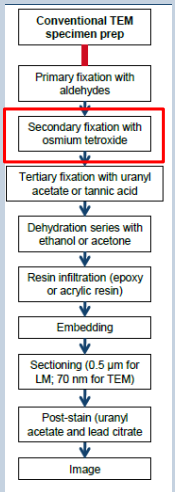
- Chemical fixation with aldehydes, most commonly 2.5% glutaraldehyde, which quickly and irreversibly cross-links proteins via their amino groups
- Microwave fixation can aid reagent penetration and reduce fixation time
- Cryo-fixation using High Pressure Freezing (HPF), which preserves specimens as close as possible to the native state (bit trickier than chemical fixation though)



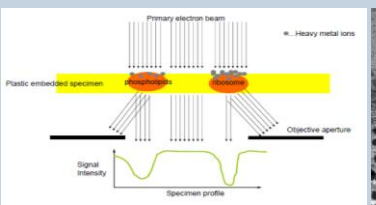
Emiliania huxleyi algae fixed using chemical (top) and cryo methods (bottom; M Eason-Hubbard/E Johnson

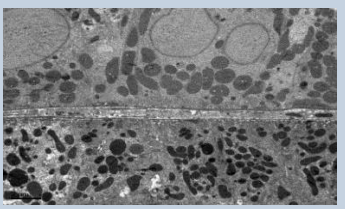
Specimen Preparation for TEM
Cells & Tissue – Secondary Fixation

Dunn School Bioimaging Facility



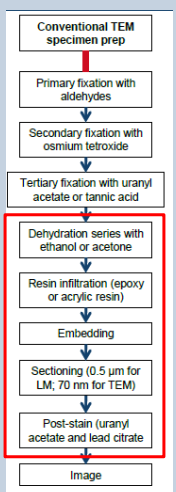
- Osmium tetroxide is a heavy metal that fixes unstaturated lipids and is also electron dense.
- Used as both a secondary fixative and an electron stain, it significantly improves specimen preservation (especially membranes) and contrast.





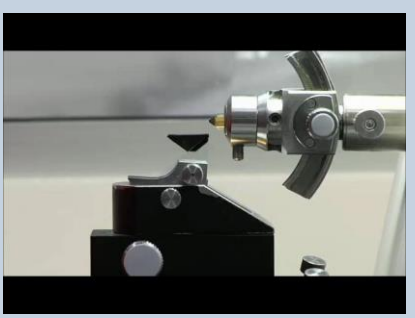
Microwave processed liver tissue, E Johnson

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



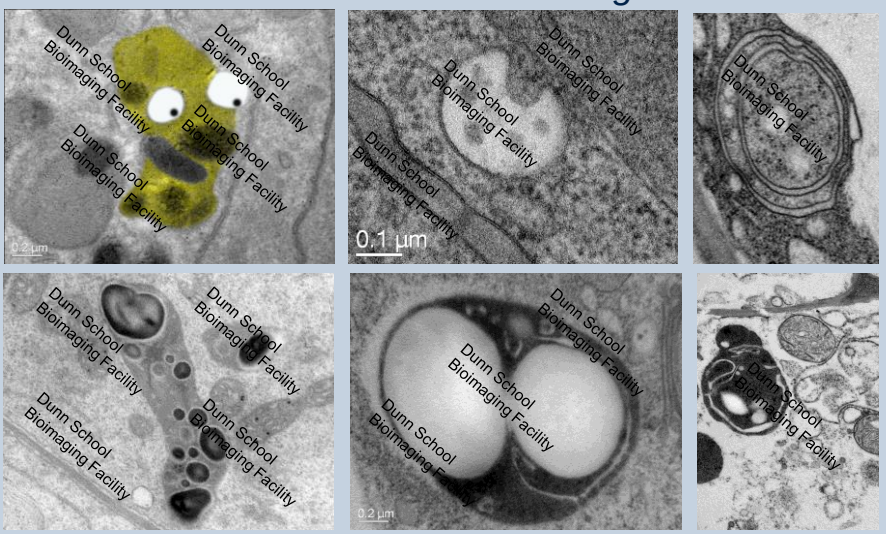
- Dehydration gradually replaces water in the sample with a solvent.
- The solvent is then gradually replaced with resin. The sample is embedded in resin and polymerised. The block is sectioned on an ultramicrotome and post-stained with even more heavy metals!

Leica Ultracut 7 ultramicrotome, Dunn School



Introduction to ultramicrotomy video, University of Sydney

Specimen Preparation for TEM
Critical evaluation of images



Specimen Preparation for TEM
Particulate samples

There are a number of ways to prepare particulate samples (eg: proteins, liposomes, DNA and viruses) for TEM:

1. 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

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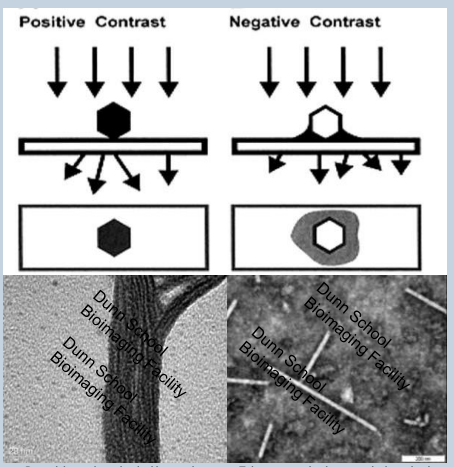


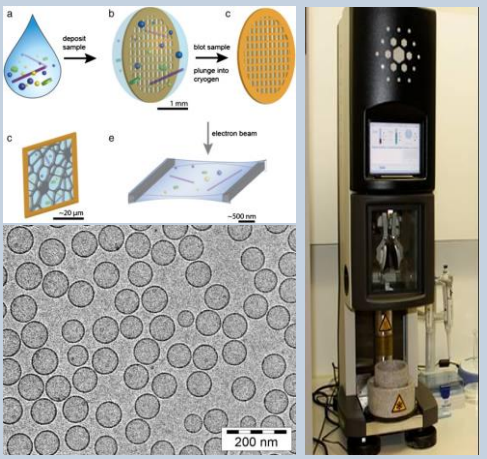
Bacterial protein stained with uranyl acetate; Tobacco mosaic virus negatively stained with sodium silicotungstate (E. Johnson)

Specimen Preparation for TEM
Particulate samples

There are a number of ways to prepare particulate samples (eg: proteins, liposomes, DNA and viruses) for TEM:

2. Plunge freezing

- Coat grids with plastic film and carbon
- Apply the particulate specimen
- Vitrify using a cryogen (eg: ethane)
- Transfer to cryo-TEM under liquid nitrogen and image frozen



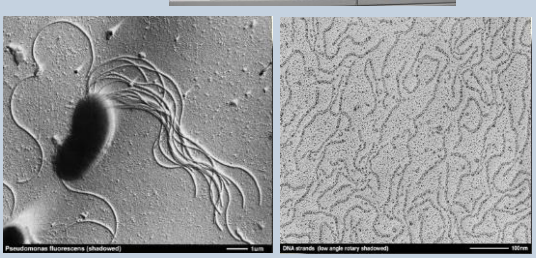
Top: Newcombe et al (2012) Current Opinion in Colloid & Interface Science, 17(6): 350-359.
Bottom: Cryo-TEM liposomes, www.pharmtech.uni-freiburg.de

Specimen Preparation for TEM
Particulate samples

There are a number of ways to prepare particulate samples (eg: proteins, liposomes, DNA and viruses) for TEM:

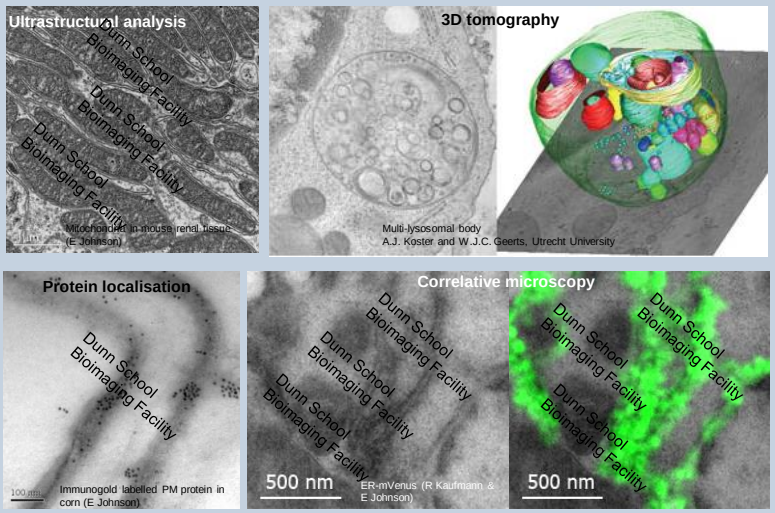
3. Rotary shadowing

- Apply particulate specimen to mica sheet
- Shadow with platinum
- Coat with carbon
- Remove carbon replica from mica and attach to grid, then image



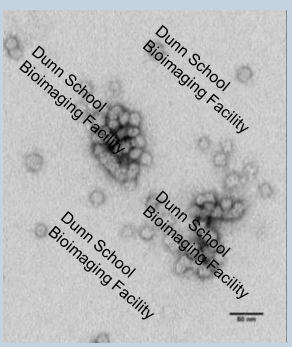
www.quorumtech.com

Transmission Electron Microscopy
Biological Applications

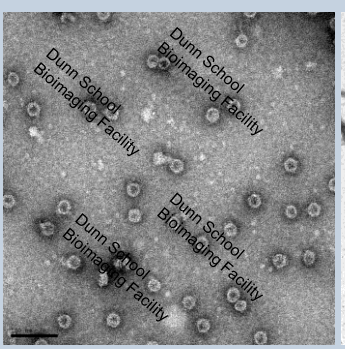


Dunn School Bioimaging Facility

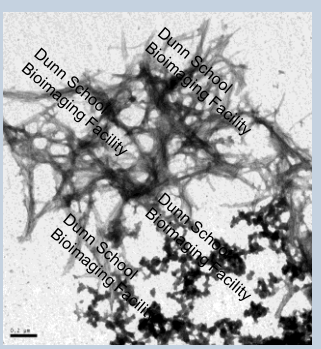
TEM Applications
Ultrastructural imaging – Particulate samples



Positively stained Hepatitis B vaccine (A Crook/E Johnson)



Negatively stained virus-like particles (A Lull/E Johnson)



Positively stained amyloid fibrils (E Johnson/C Lee, Dunn School)

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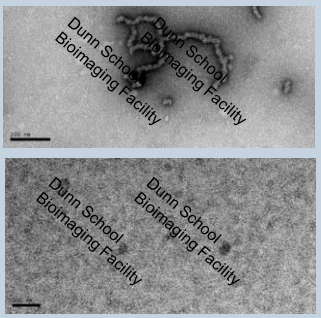
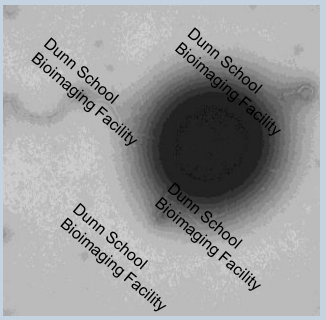
TEM Applications

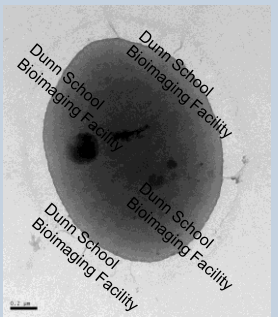
Ultrastructural imaging – Particulate samples



Negatively stained SAS-6 protein
WT aggregates, top; Mutant dimers, bottom
(M Cottee/E Johnson)



Negatively stained Neisseria meningitidis
(M Woermann/E Johnson)

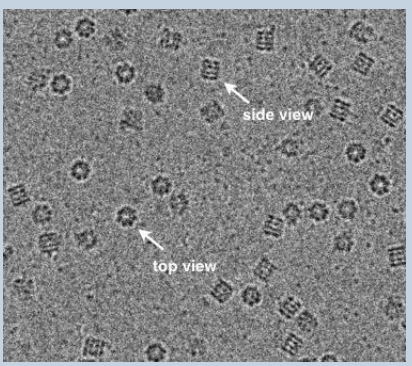


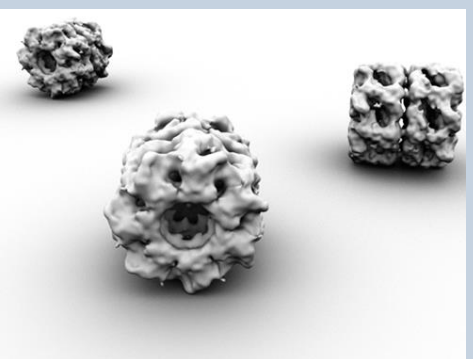
Rhodobacter sp.
(E Johnson/I Stuart, Biochemistry)

TEM Applications

Single particle imaging

PIC





Cryo-TEM of the GroEL chaperonin (image: N Vossman), in a range of different orientations which can be averaged to reconstruct and render the complex in 3D to 1 nm resolution (image: D Nowakowski), both images from http://bsp.med.harvard.edu

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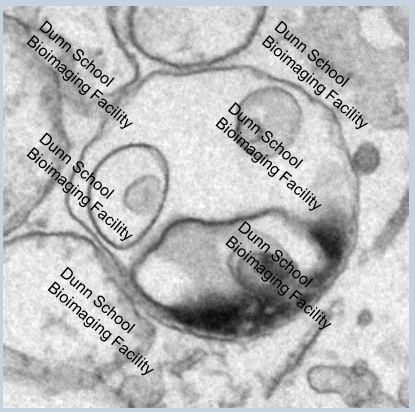
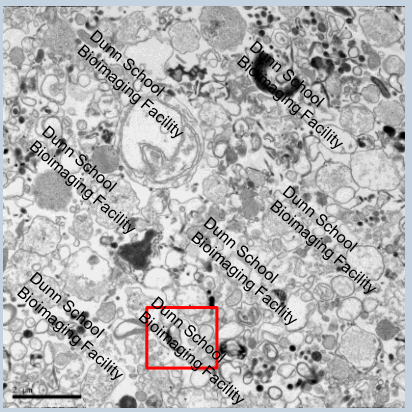
TEM Applications

Ultrastructural imaging – Particulate samples



Resin embedded trypanosome flagellum isolation prep (T Benecke/E Johnson)



Resin embedded mitochondrial isolation prep (J Long)

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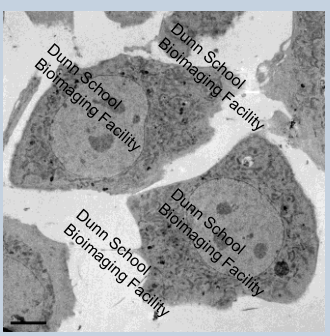
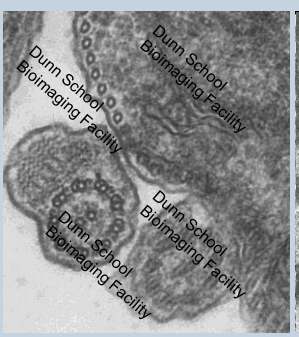
TEM Applications

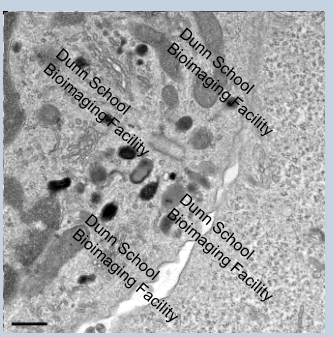
Ultrastructural imaging – Cells



Mouse fibroblast cells (E Johnson)



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



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

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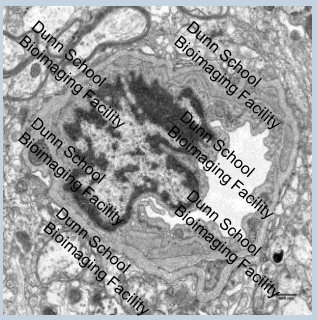
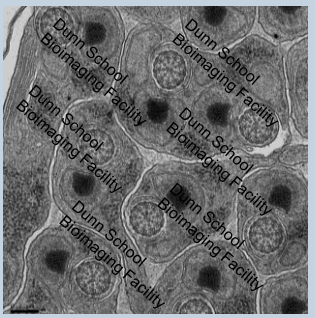
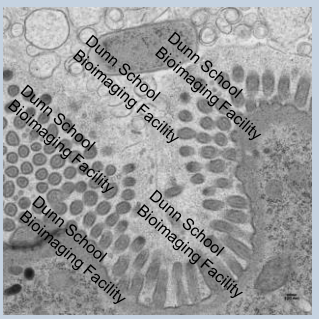
TEM Applications
Ultrastructural imaging – Tissue



Mouse blood/brain barrier (A Douglas)



Centrioles in Drosophila spermatocytes (M Pratt)



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

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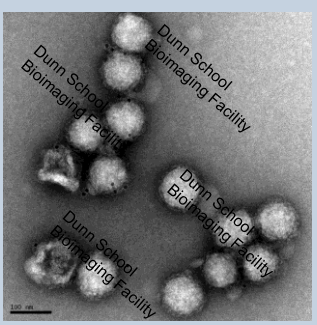
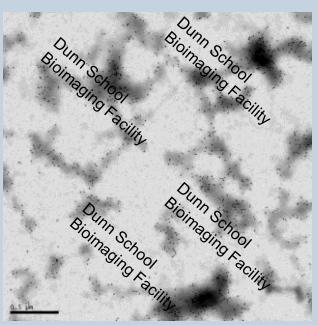
TEM Applications
Protein localisation – Immunogold labelling

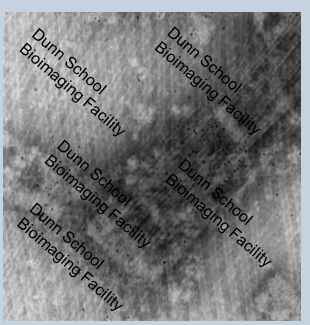
- As for immunofluorescence labelling, but the secondary antibody is conjugated to a small (1-4 nm) collodial gold particle instead of a fluorophore



Immunolabelled influenza (Ed Hutchinson/E Johnson)



Immunolabelled amylofibrils (J Alegre/E Johnson)



Whole mount immunolabelled Trypanosome cytoskeleton (S Dean)

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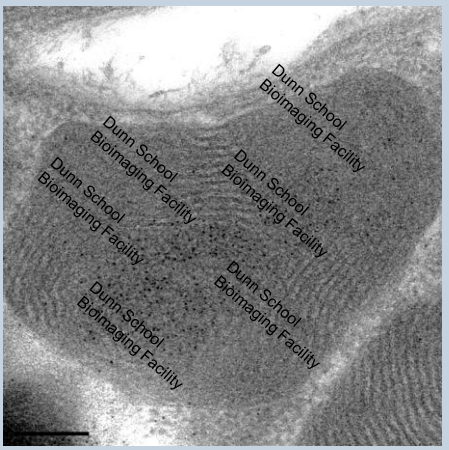
TEM Applications

Protein localisation – Immunogold labelling

- For cells and tissue, post-embedding labelling is usually the best option.
- A lighter chemical fixation is required, as glutaraldehyde affects antigenicity. Cryo-fixation is highly recommended.
- The osmium tetroxide step is omitted (as it also reduces antigenicity). Acrylic resins are used instead of Epoxy resins.
- The Tokuyasu cryo-sectioning technique is another option.



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

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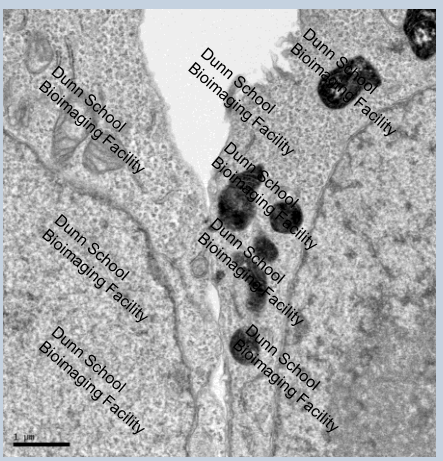
TEM Applications

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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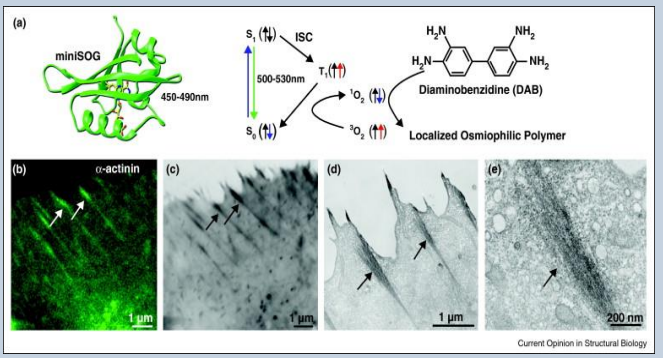
TEM Applications

Protein localisation – Correlative microscopy

- Correlative microscopy allows you to place your fluorescent protein in ultrastructural context. There are two main ways to achieve this:

1. Confocal of GFP -> Standard TEM prep -> TEM of same cell



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

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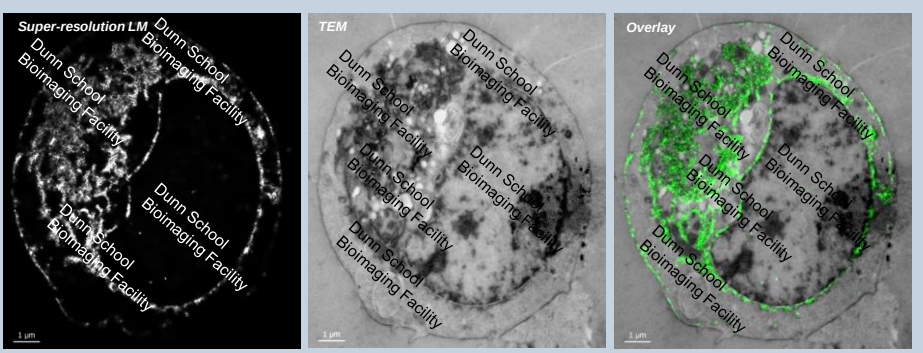
TEM Applications

Micron OXFORD

Protein localisation – Correlative microscopy

- Correlative microscopy allows you to place your fluorescent protein in ultrastructural context. There are two main ways to achieve this:

2. Specialised TEM prep -> Confocal on TEM section -> TEM of same cell



Correlative light & electron microscopy of HEK cells expressing mVenus
(E Johnson & R Kaufmann, Micron)

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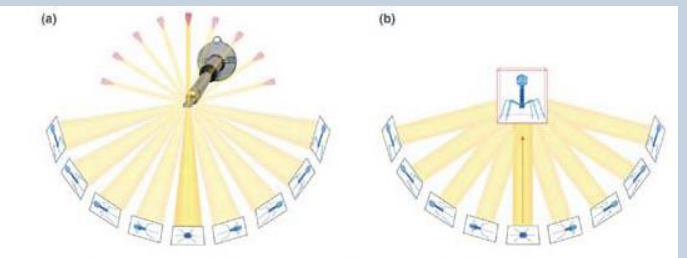
TEM Applications
Electron tomography

- 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].

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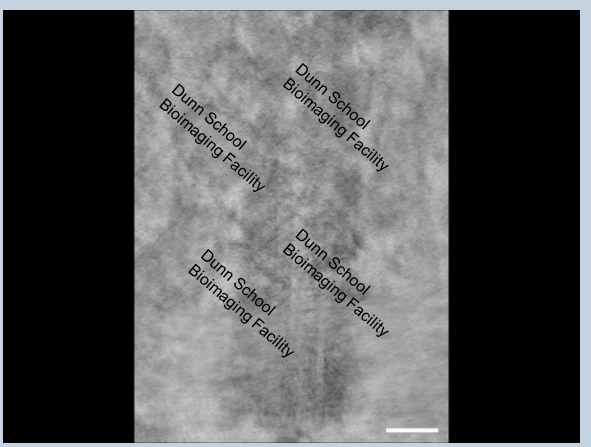
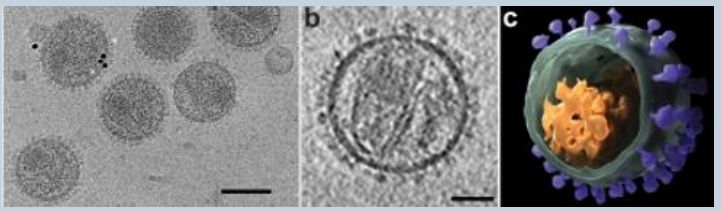
Advanced TEM techniques
Electron tomography

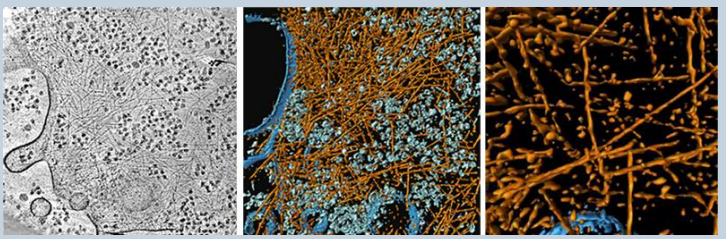


Drosophila primary spermatocyte centrioles, H Roque (Dunn School)

TEM Applications
Cryo-TEM tomography



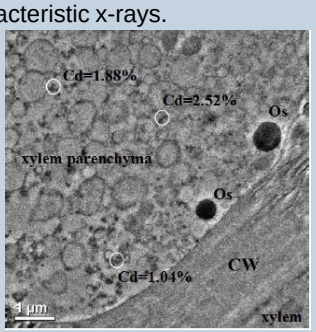
Cryo-electron tomography and modelling of trimeric SIV Env virions (White et al 2010, PLoS Pathog, 6(12): e1001249)



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

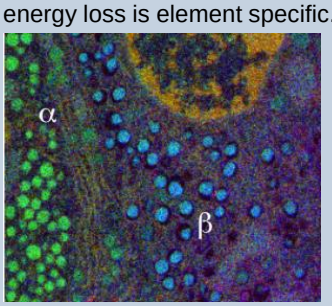
TEM applications
Chemical characterisation

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



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

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.

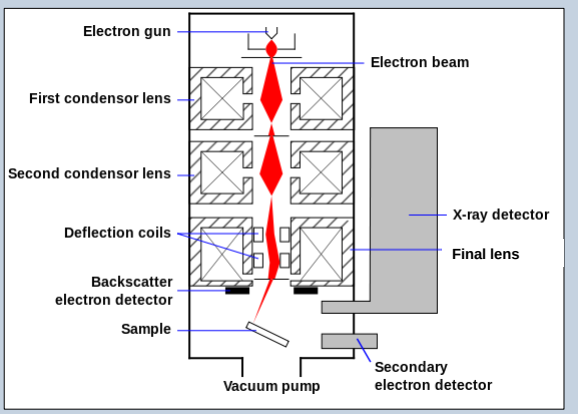


Unstained mouse pancreas with elemental contrast using EELS, NIH

Scanning Electron Microscopy (SEM)

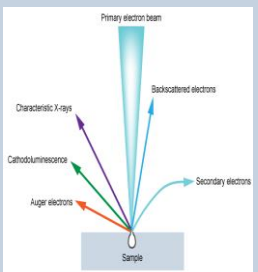
Penicillium (E Johnson)

The SEM

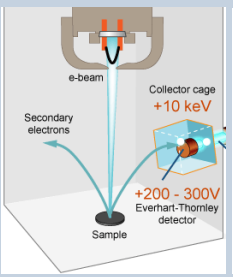


How the SEM works
Signal detection

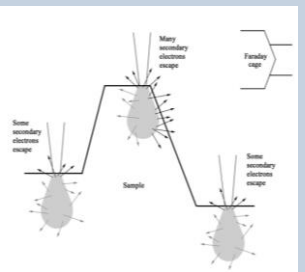
- Secondary electrons (SEs) provides surface morphology and topology information.
- SEs are captured by the Everhart-Thornley detector



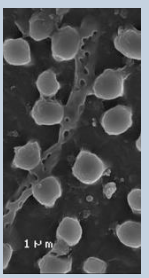
www.ammrf.org



www.ammrf.org



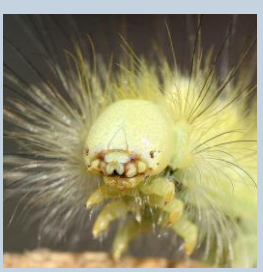
Dept Biological Sciences, Smith College Northampton USA

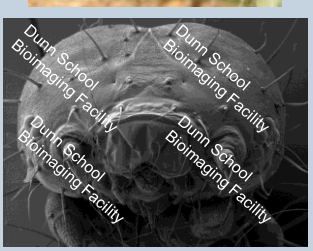


www.ammrf.org

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. Some samples (eg: pollen, insects) can be imaged without much sample prep at all.





Bottom: SEM of a caterpillar (E Johnson)

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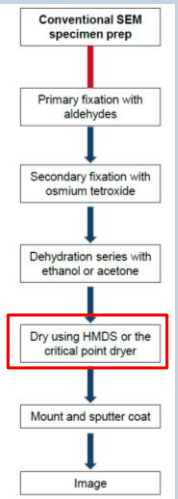
Sample Preparation for SEM

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).

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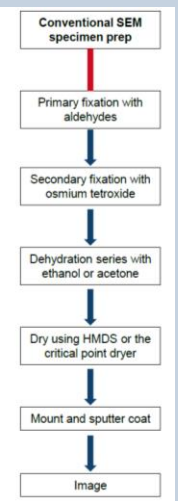
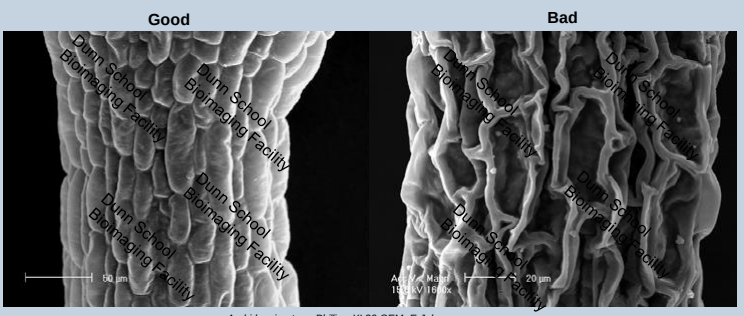
Sample Preparation for SEM

Drying the sample





Arabidopsis stem, Phillips XL30 SEM, E Johnson

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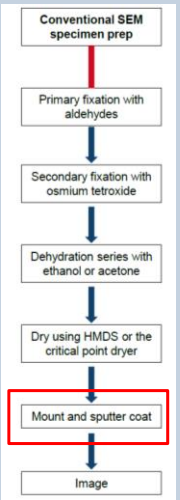
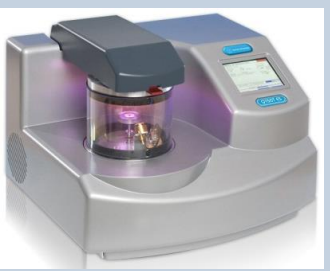
Sample Preparation for SEM
Mounting and sputter coating



- Mounting immobilizes the sample on a conductive backing, grounding it. Sputter coating with metal ions deposits a thin continuous conductive layer over the sample, so that charge does not build up on the sample.



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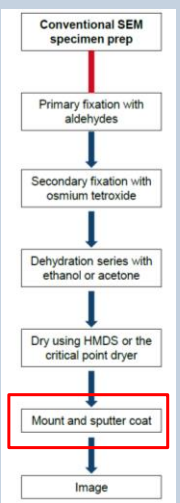
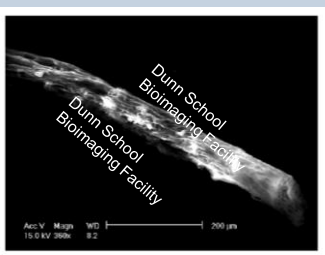
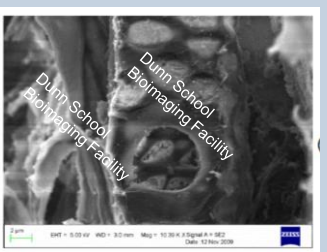
Sample Preparation for SEM
Mounting and sputter coating



- If a 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.



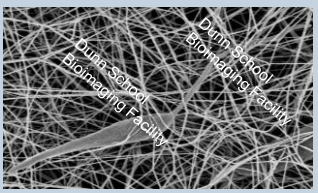
Arabidopsis root (E Johnson)



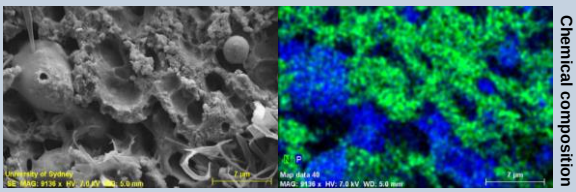
Arabidopsis xylem (E Johnson)

Scanning Electron Microscopy
Biological Applications

Topography



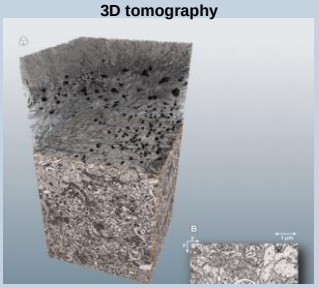
Leishmania on collagen (R Jain/E Johnson)



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

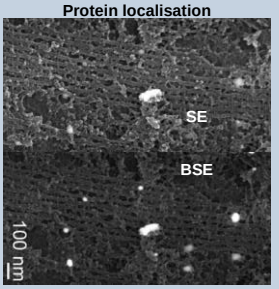
Chemical composition

3D tomography



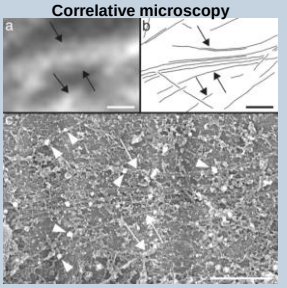
Moritz Helmstaedter, Max-Planck Institute for Medical Research

Protein localisation



Microtubules (D Barton, University of Sydney)

Correlative microscopy



Microtubules (D Barton, University of Sydney)

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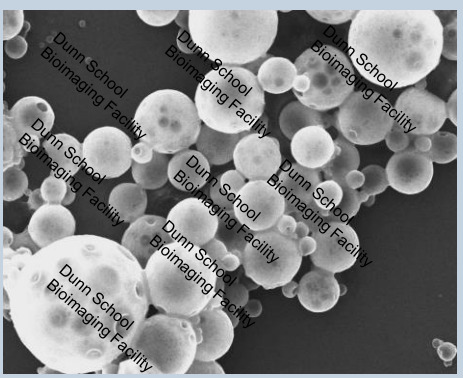
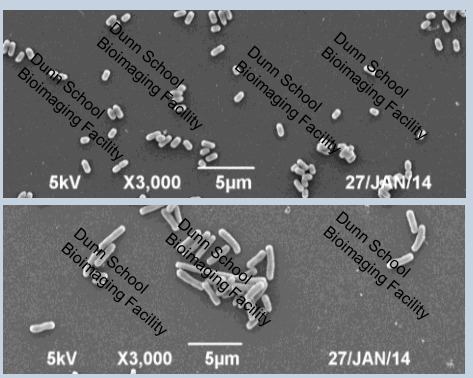
SEM Applications
Topography – Particulate samples



Plastic beads conjugated to vaccine particles (A Walters/E Johnson)



E coli (WT at top, +vector at bottom) (R Harding/E Johnson)

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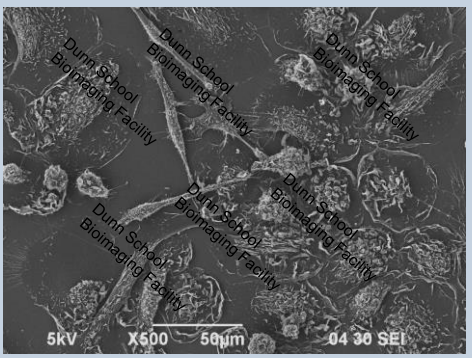
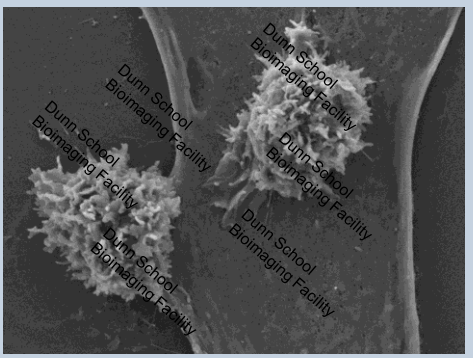
SEM Applications
Topography – Cells



Monocytes and macrophages (B van Wilgenburg/E Johnson)



T-cells interacting with a cancer cell (E Johnson, Dunn School)

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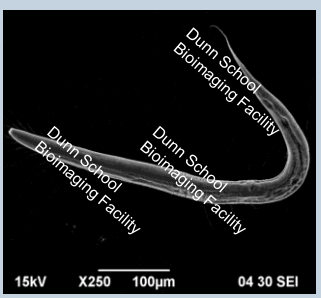
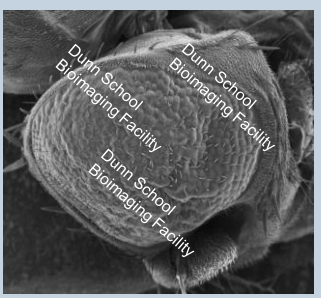
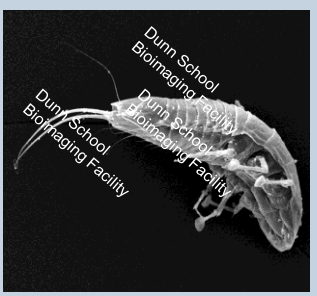
SEM Applications
Topography – Whole organisms



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



Drosophila rough eye phenotype
(M Elschami, NDCN)



Exotic arthropod (E Johnson)

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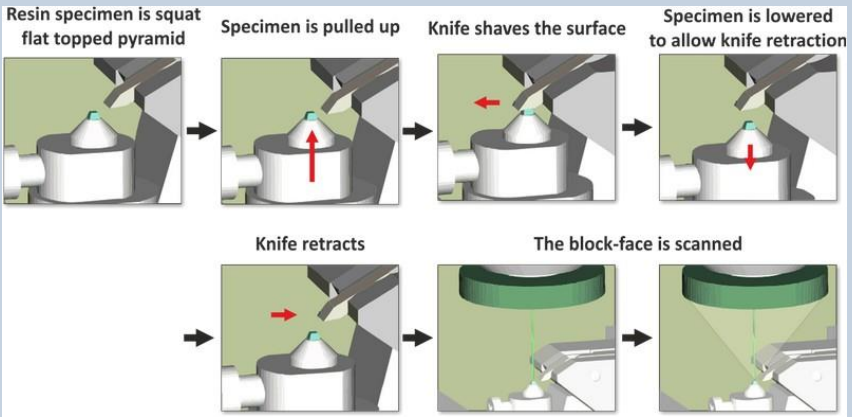
SEM Applications – 3D

Serial Block Face Sectioning – 3View

OXFORD BROOKES UNIVERSITY

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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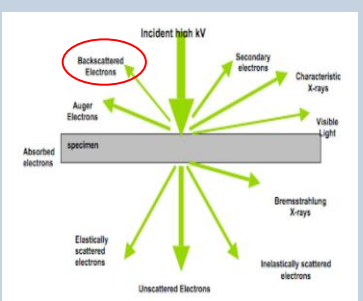
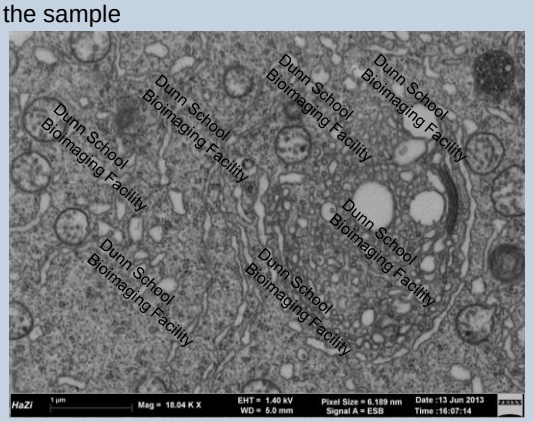
SEM Applications – 3D

Serial Block Face Sectioning – 3View

OXFORD BROOKES UNIVERSITY

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





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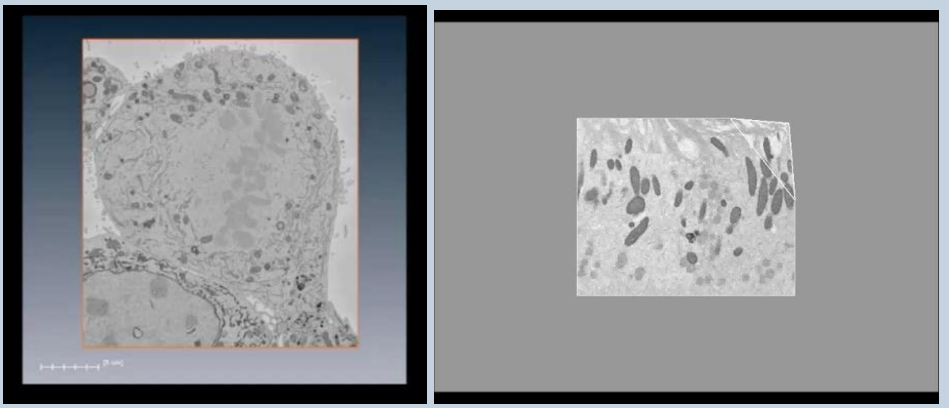
SEM Applications – 3D

Serial Block Face Sectioning – 3View

OXFORD BROOKES UNIVERSITY



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

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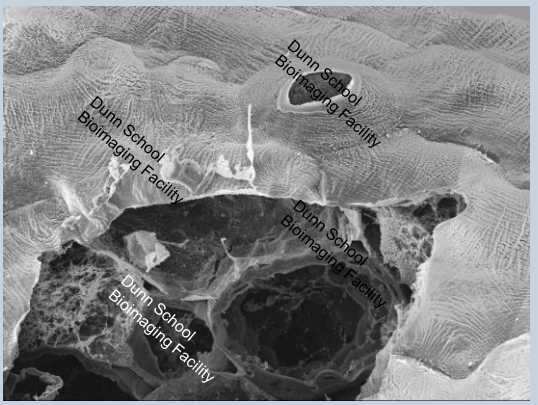
SEM Applications – 3D

Serial Block Face Sectioning – 3View

OXFORD BROOKES UNIVERSITY

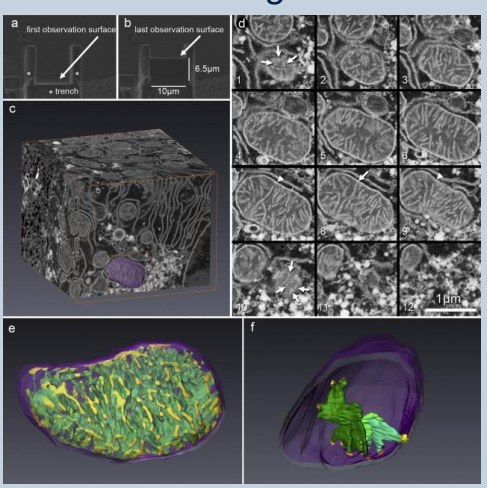
- Zeiss Merlin compact VP FEG-SEM with 3View2 XP system is being installed right now at Oxford Brookes in collaboration with the Dunn School. The system will soon be fully operational!
- Oxford University researchers can access the 3View through the Dunn School Bioimaging Facility and will have equal access rights to it
- There will be two dedicated computer stations in the Dunn School for modelling 3View datasets using IMOD/Imaris
- For more detailed information on the 3View (eg: sample preparation, practical considerations), please contact me

SEM Applications – 3D
Focussed ion beam



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

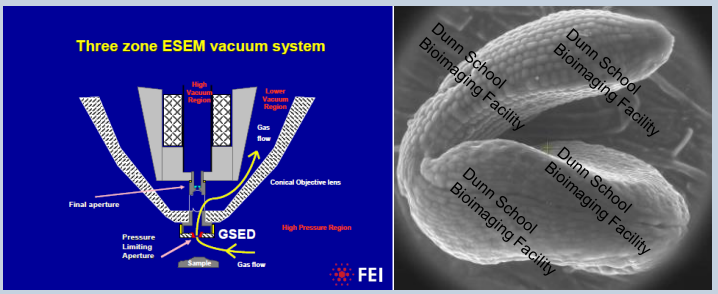
SEM Applications – 3D
Serial Block Face Sectioning – Focussed Ion beam



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

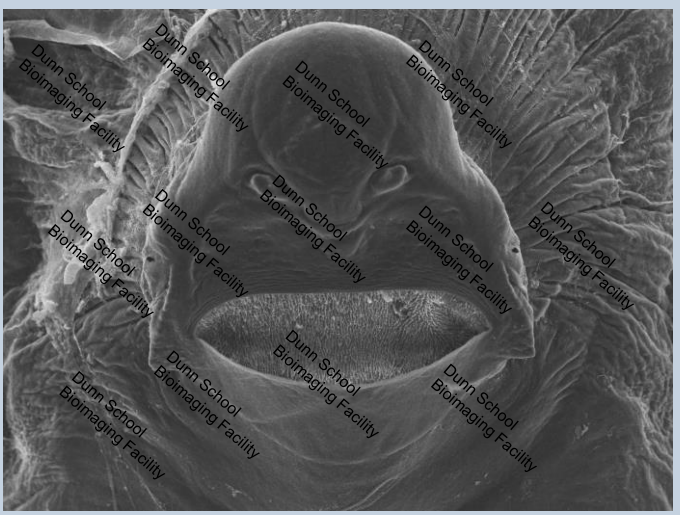
SEM Applications – 3D
Environmental SEM

- 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!).



ESEM of an Arabidopsis embryo, E Johnson

Electron microscopy: Get into it!



Darth Vader