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

TEM
SEM

Electron microscopy
Resolution

1 m 1 dm 1 cm 1 mm 100 µm 10 µm 1 µm 100 nm 10 nm 1 nm 0.1 nm
1 m 10^{-1} m 10^{-2} m 10^{-3} m 10^{-4} m 10^{-5} m 10^{-6} m 10^{-7} m 10^{-8} m 10^{-9} m 10^{-10} m

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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

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. Eldridge

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

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
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.

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

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).
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
Electron Microscopy at the Dunn School

Biological Specimen Preparation Laboratory
Equipment & reagents (214.00.21)

Transmission Electron Microscope (TEM)
FEI Tecnai 12 TEM (214.00.21)

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

eg: ultramicrotomy

Internal ultrastructure
Blood monocyte, B. van Wilgenburg, E. Johnson

Surface morphology
Blood monocyte, B. van Wilgenburg, E. Johnson

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

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.
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 (e.g., protein and viruses) can be stained and viewed quickly
  - Cells and tissue samples require extensive preparation for TEM

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**Cells & Tissue – Overview**

- Specimen fixed and embedded in plastic
- Specimen sliced into ~50 nm sections with a diamond knife
- Sections picked up on a TEM grid
- A cross-section of C. elegans
- A grid mounted on the sample holder for TEM

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**Specimen Preparation for TEM**

- 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)
- Epoxy cementing
- Sections, 0.5 cm for LM, 70 nm for TEM
- Post-stain (e.g., osmium tetroxide and lead citrate)
- Image
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

Specimen Preparation for TEM  
**Cells & Tissue – Methods of Fixation**

- 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)
Specimen Preparation for TEM
Cells & Tissue – Secondary Fixation

- Osmium tetroxide is a heavy metal that fixes unsaturated 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.

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!
Specimen Preparation for TEM

Critical evaluation of images

Particulate samples

There are a number of ways to prepare particulate samples (e.g.: 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, (e.g.: uranyl acetate, phosphotungstic acid, sodium silicotungstate) for ~1 min
   - Blot dry and view in the TEM
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


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

Ultrastructural analysis

3D tomography

Protein localisation

Correlative microscopy

TEM Applications

Ultrastructural imaging – Particulate samples

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

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

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

Dunn School Bioimaging Facility

Sir William Dunn School of Pathology

Micron Advanced Microscopy Course

May 23, 2014
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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

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
TEM Applications
Ultrastructural imaging – Particulate samples

Resin embedded trypanosome flagellum isolation prep (T. Benecke/E. Johnson)
Resin embedded mitochondrial isolation prep (J. Long)

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)
TEM Applications

Ultrastructural imaging – Tissue

- Bacterium in gut of cryo-fixed *C. elegans* (A Moloney/E Johnson)
- Centrioles in *Drosophila* spermatocytes (M Pratt)
- Mouse blood/brain barrier (A Douglas)

TEM Applications

Protein localisation – Immunogold labelling

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

- Immunolabelled influenza (Ed Hutchinson/E Johnson)
- Immunolabelled amylolibins (J Alegre/E Johnson)
- Whole mount immunolabelled Trypanosome cytoskeleton (S Dean)
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.

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 H₂O₂) to produce a localised osmophilic precipitate

  - miniSOG (Shu et al PLOS Biology 9, 2011)
    - Small fluorescent flavoprotein that can be photo-oxidised to react with DAB to produce a localised osmophilic precipitate - CLEM
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

   ![Correlative microscopy example]


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)
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 combined backprojection, in which each tilted view is used to reconstruct a three-dimensional image of the original structure. [McLennan et al. (2010) Trends Cell Biol. 20:43-49]
Cryo-electron tomography and modelling of trimetric SIV Env virions (White et al. 2010, PloS Pathog, 6(12): e1001246)

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.

Unstained mouse pancreas with elemental contrast using EELS (NIH)

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. Ou et al. Sun Yat-sen University, PR China)
Scanning Electron Microscopy (SEM)

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.

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**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 (e.g. pollen, insects) can be imaged without much sample prep at all.
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 CO₂. Air drying is not recommended, as ethanol evaporation generally causes severe surface tension artifacts.

- Liquid CO₂ 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

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.

- 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.
Protein localisation
Correlative microscopy
Chemical composition

3D tomography

Microtubules (D Barton, University of Sydney)

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

Leishmania on collagen (R Jain/E Johnson)

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

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

SEM Applications
Topography – Particulate samples
Monocytes and macrophages (B van Wilgenburg/E Johnson)

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

Drosophila rough eye phenotype (M Elschami, NDCN)

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

Exotic arthropod (E Johnson)
One method for generating a 3D high resolution image stack is to use serial block face sectioning with the Gatan 3View system.

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.
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 (e.g. sample preparation, practical considerations), please contact me.
Sem Applications – 3D
Focussed ion beam

Arabidopsis leaf, Zeiss Auriga FIB (S Moody & Johnson)

Serial Block Face Sectioning – Focussed ion beam

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 (i.e., not a vacuum!).

Electron microscopy: Get into it!

Darth Vader