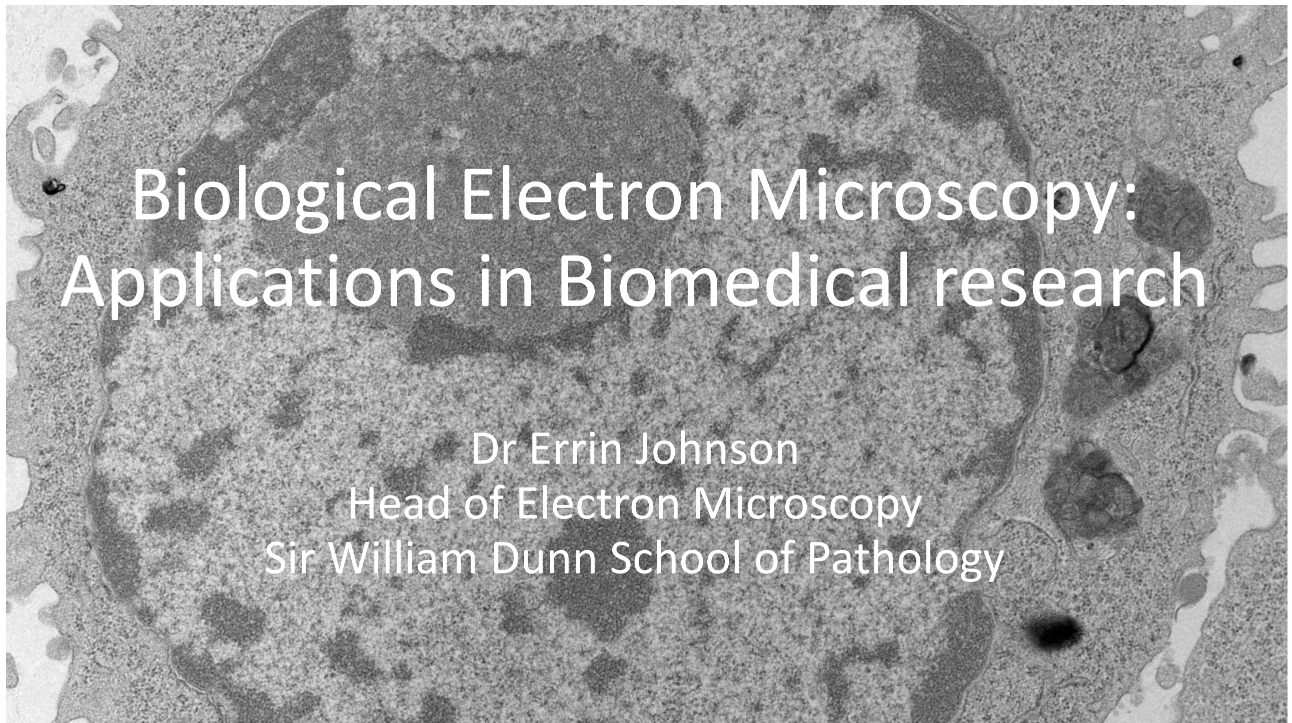
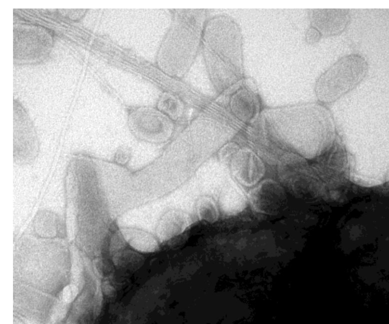


Biological Electron Microscopy: Applications in Biomedical research

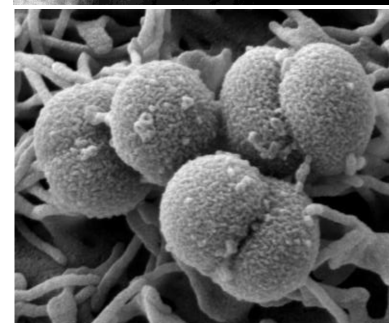
Dr Errin Johnson
Head of Electron Microscopy
Sir William Dunn School of Pathology

Lecture Overview

- Recap of imaging techniques covered so far
  - Applications and limitations of cryo-EM and light microscopy
- Introduction to Biological EM
  - What is it and how does it fit with other imaging techniques?
  - Applications and drawbacks/limitations
  - How electron microscopes work
- The importance of sample preparation for EM
  - Standard protocol and critical steps
  - How to spot artifacts
- Protein localization with nanometer resolution
  - Tools: Immunogold labelling and genetic EM tags
- Correlative microscopy to pinpoint rare events
  - Tools: Correlative light and electron microscopy (CLEM)
- 3D cellular ultrastructure with volume EM
  - Tools: Serial block face imaging, FIB-SEM & EM tomography



Transmission Electron Microscopy (TEM)

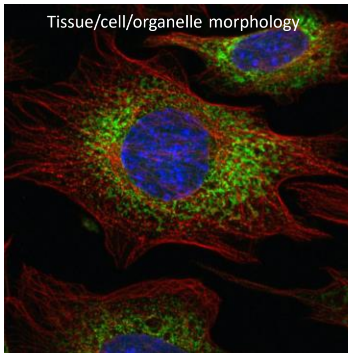


Scanning Electron Microscopy (SEM)

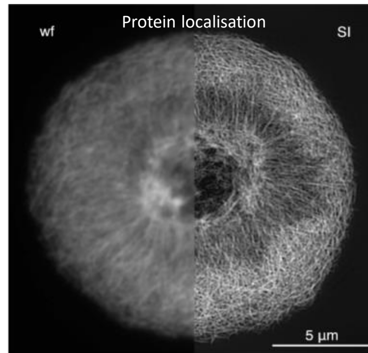
Neisseria cinerea

Recap: Light microscopy as a tool for cell biology

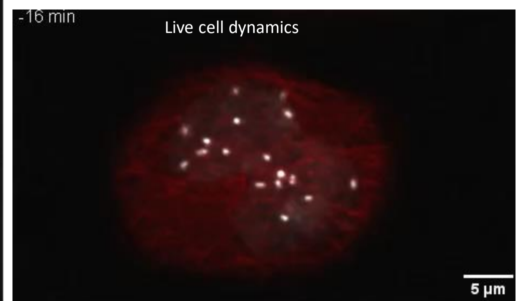
There are a huge number of ways that light microscopy can be applied to visualise proteins and events in cells and tissue. Eg: proteins can be genetically tagged with fluorescent proteins or labelled with fluorescent antibodies, while organelles and ions can be tracked with specific dyes. These can be imaged over a range of different scales and in fixed or live cells. Super-resolution microscopy techniques enable imaging down to ~20 nm resolution.



Nucleus, actin & endoplasmic reticulum in mouse fibroblast cells, V Leibe (Dunn School)



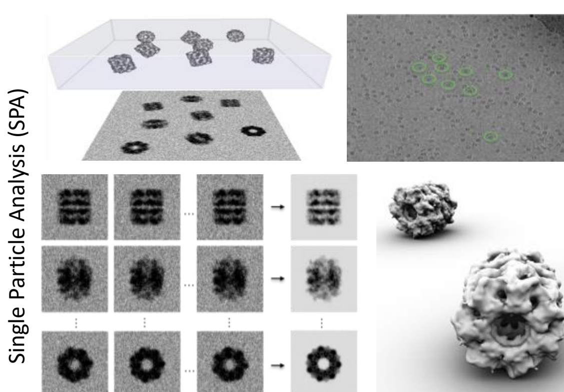
Actin in a Drosophila macrophage imaged with FM (left) & SRM (right), E Wegel (Micron)



Tracking kinetochores during cell division, Dan Hayward (Dunn School)

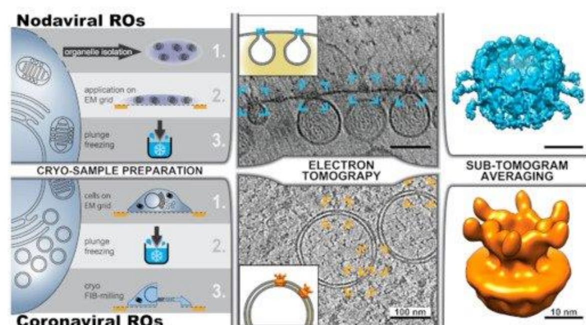
Recap: Cryo-EM as a tool for structural biology

Proteins, macromolecular complexes, viruses and whole cells are vitrified and imaged in a near native, frozen hydrated state. Hundreds/thousands of images are then processed to generate a 3D structure at sub-nanometer resolution. Recent technological advances in cryo-EM have revolutionized the field of structural biology.



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 10 Å resolution. From: http://people.csail.mit.edu/gdp/cryoem.html

Cryo-electron tomography and sub-tomogram averaging



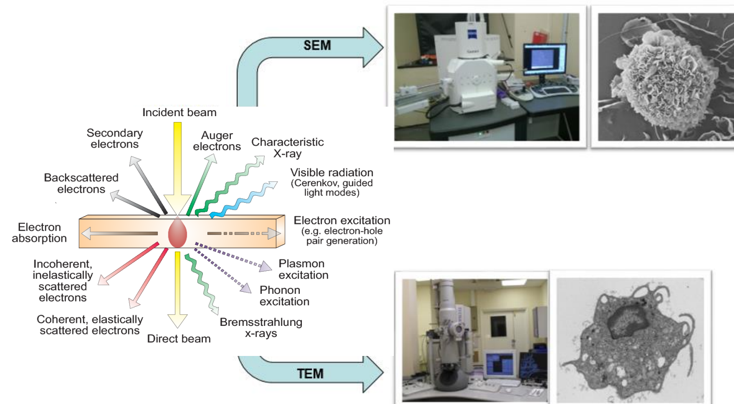
Cryo-electron tomography of viral replication organelles
(Wolff & Barcena 2021, *Viruses* **2021**, *13*(2), 197; **https://doi.org/10.3390/v13020197**)

Limitations and challenges of LM & cryo-EM

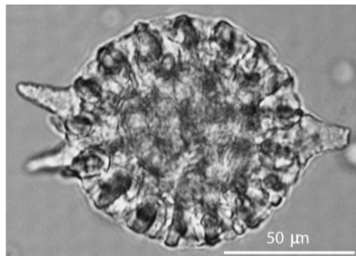
- Whilst excellent for solving protein structures, cryo-EM is not as well suited to routine imaging of proteins, cells & tissues since:
  - Cryo-EM images have a very low signal to noise ratio due to low dose imaging conditions (to help protect the frozen sample from electron beam damage) and no additional contrast agents (except in special cases)
  - Data processing is computationally expensive and time consuming
  - Sample preparation is technically demanding (particularly for cells/tissue which often need to be thinned first under cryo-conditions) and extensive optimisation of protein preps is often required
  - Microscope time on high resolution cryo-EMs can be very expensive (££££)
- By comparison, light microscopy is a relatively high-throughput, accessible suite of techniques for routine imaging of cells and tissues. However:
  - Even super-resolution techniques are still an order of magnitude below the resolution of EM!
  - Organelles and sub-compartments need to be specifically labelled, which can be problematic if you want to image multiple proteins/organelles due to fluorophore constraints
  - Super-resolution microscopy can also be technically demanding

Biological EM as a complementary tool for cell biology

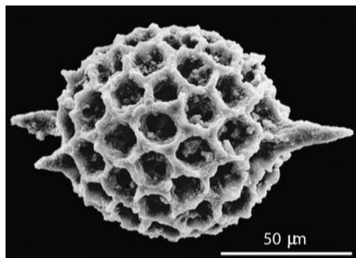
- Biological EM encompasses a suite of techniques where fixed, stained and dehydrated specimens are imaged at ultrastructural resolution at room temperature
- Biological EM overcomes some key limitations of cryo-EM and LM



Comparison of microscopy techniques - Resolution

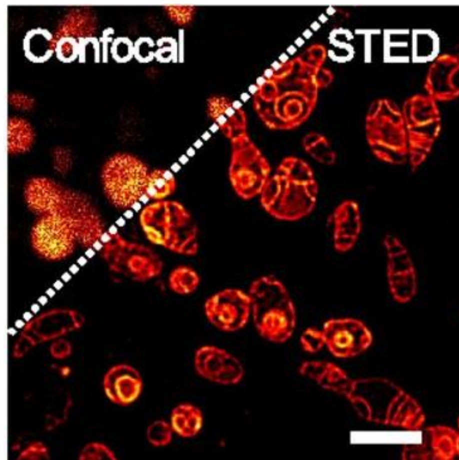


(a) Radiolarian under light microscope

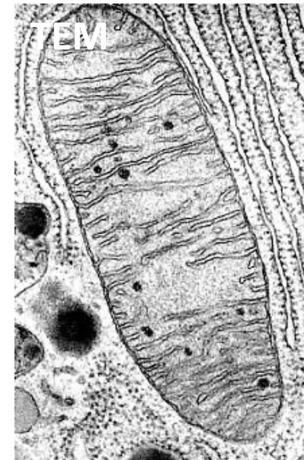


(b) Radiolarian under electron microscope

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



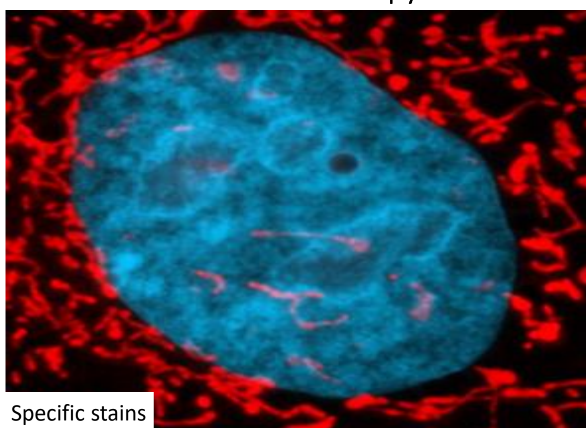
Wang et al (2019) PNAS August 6, 2019 116 (32) 15817 - 15822; https://doi.org/10.1073/pnas.1905924116



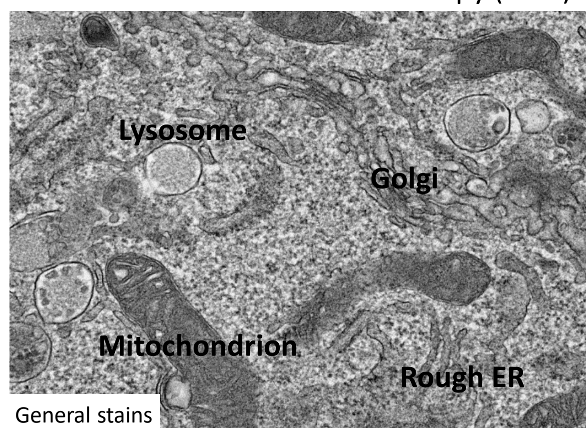
Keith R Porter

Comparison of microscopy techniques - Contrast

Confocal microscopy



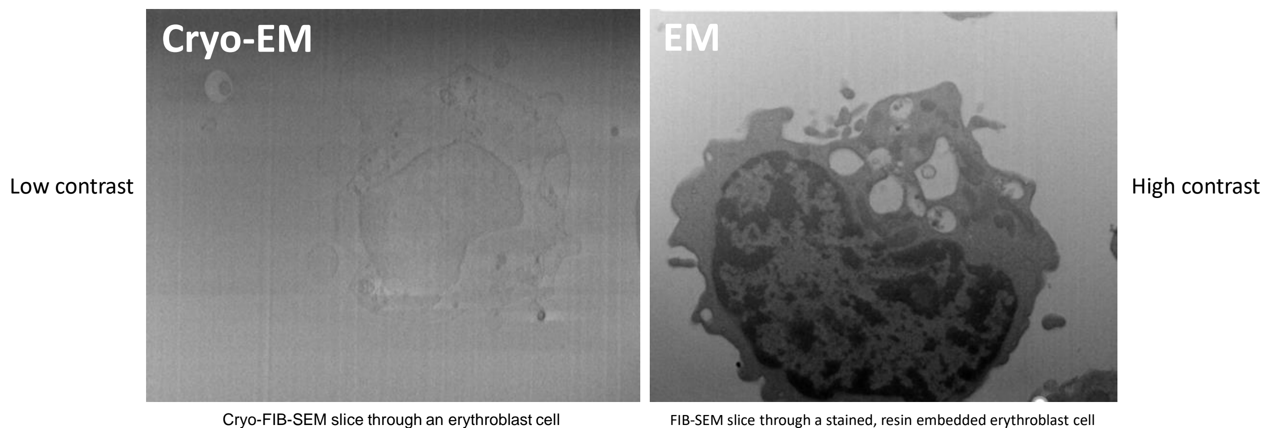
Transmission Electron Microscopy (TEM)



Left : Confocal image of a kidney cell stained with DAPI (blue) and MitoTracker (red) (Hammamatsu.magnet.fsu.edu). Right: TEM image of fibroblast cell (E Johnson).

Comparison of microscopy techniques - Contrast

- In EM, 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 (eg: heavy metal stains).

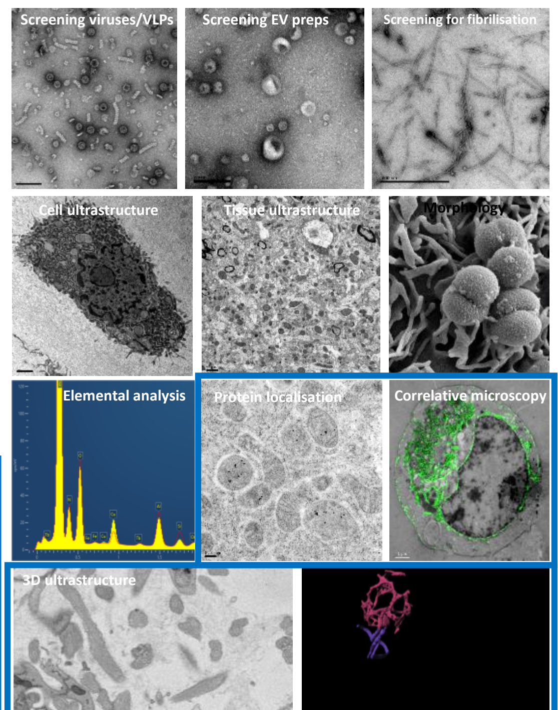


Cryo-FIB-SEM slice through an erythroblast cell

FIB-SEM slice through a stained, resin embedded erythroblast cell

Biological EM applications

- **Particles:**
  - Screening (eg: checking purification, aggregation) -> TEM
  - Protein localisation -> Immuno TEM
- **Cells and tissue:**
  - 2D cellular ultrastructure (drug treatment, knockouts etc) -> TEM
  - Cell morphology (drug treatment, knockouts etc) -> SEM
  - Elemental composition & mapping -> EDS
  - 3D cellular ultrastructure -> Volume EM: serial sectioning TEM, 3View, FIB-SEM, EM tomography, array tomography
  - Localising proteins of interest -> immuno TEM, EM genetic tags, correlative microscopy
  - Identifying specific cells/rare events -> immuno TEM, correlative, EM genetic tags

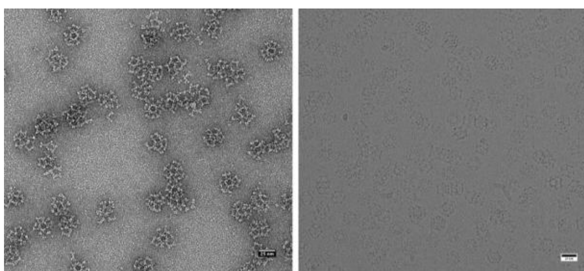


Limitations and challenges of biological EM

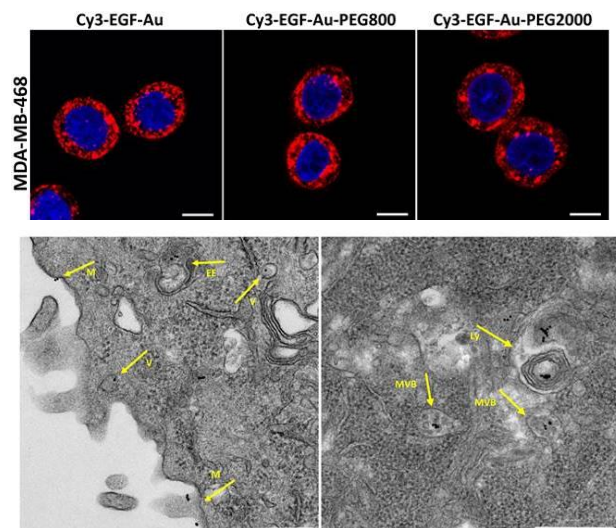
- Samples must be fixed, which means no live cell imaging
- Samples are not imaged in their native state. They are fixed, dehydrated and stained, which induces changes at the macromolecular level and also sometimes at the ultrastructural level
- EM sample preparation involves the use of toxic chemicals (eg: uranium based salts)
- Sample preparation of cells/tissue for TEM & 3D EM can be technically demanding
- 3D EM involves time consuming data segmentation and reconstruction
- Immunolabelling/correlative microscopy can be difficult and may require extensive optimisation/troubleshooting

Choosing the right microscopy technique

- Which microscopy technique you use depends on:
  - Your research question, the resolution and contrast you need to answer it
  - Your sample requirements, plus the tools and expertise available
- LM and EM techniques, including cryo-EM, are often employed in parallel on the same project to provide complementary information
- These techniques can also be correlated on the same sample to provide more information about the sample



Protein cage imaged with negative staining (left) and cryo-EM (right). From: Gonen (2020) Methods in Molecular Biology, 2215.

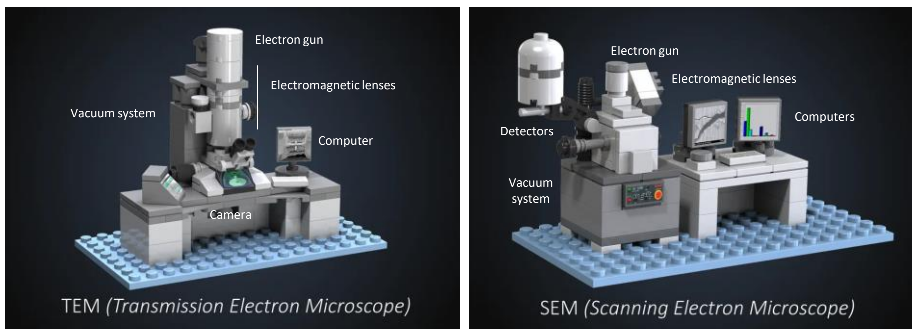


Using light and electron microscopy to characterise the uptake of gold nanoparticles conjugated to EGF and stabilised with PEG. From: Song et al (2017) Nanotheranostics, 1(3).

Electron microscope components

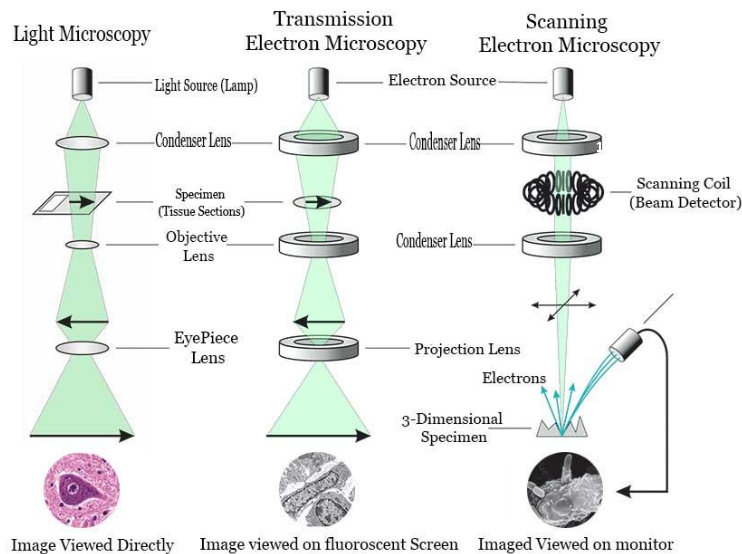
Both SEMs and TEMs share the following main components:

- An electron gun – source of electrons, operated at high voltages under vacuum to accelerate the electrons
- Electromagnetic lens system – creates precise circular electric fields to manipulate the electron beam
- Vacuum system – protects beam integrity and prevents electrical discharging at the gun
- Camera/detector – fast and sensitive signal detection
- Computer – microscope control

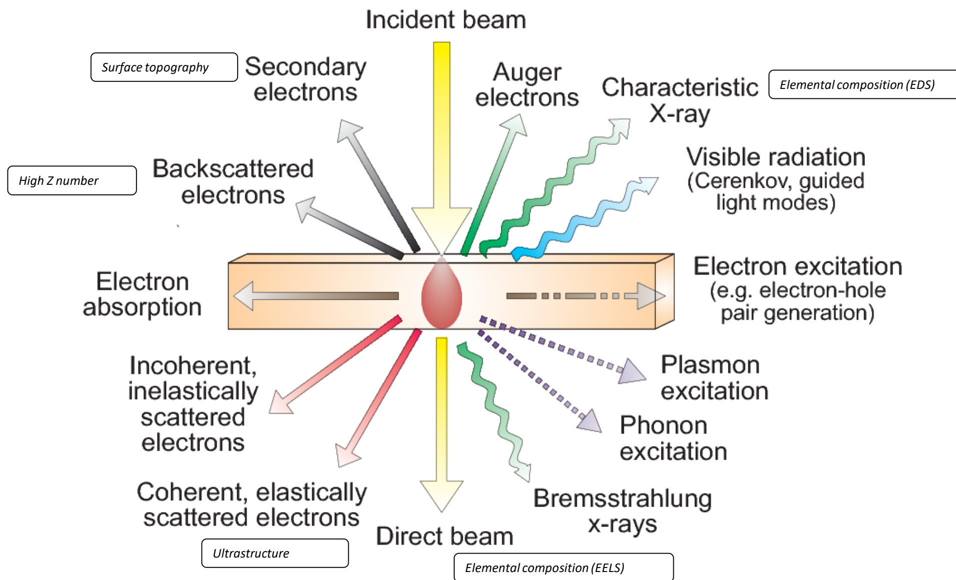


https://ideas.lego.com/projects/102281

Electron microscope components & image formation



Signal generation and detection



Specimen requirements for SEM

- SEM specimens must be:
  - Stable in the vacuum
  - Well preserved surface structure
  - Conductive

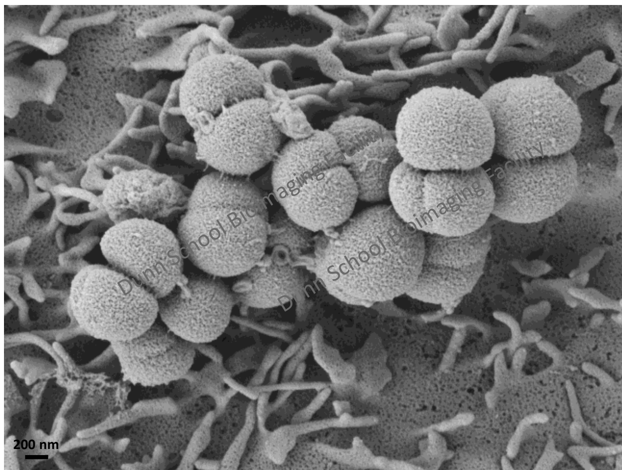
Live caterpillar (left) and fixed caterpillar imaged with SEM (right)

- The degree of specimen preparation required for biological SEM depends on the specimen
  - Particulate samples can be prepared and viewed quickly
  - Cells and tissue samples require some sample prep, although this is much less extensive than for TEM

Example SEM project:

Colonisation modes of different Neisseria strains

***N. cinerea* strain 346T**

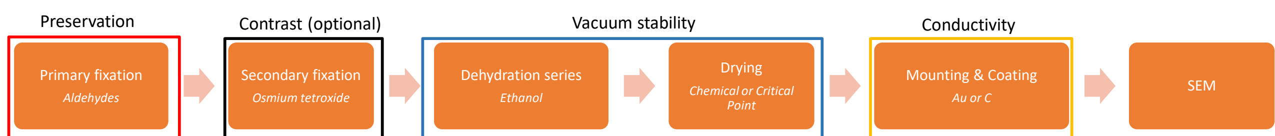


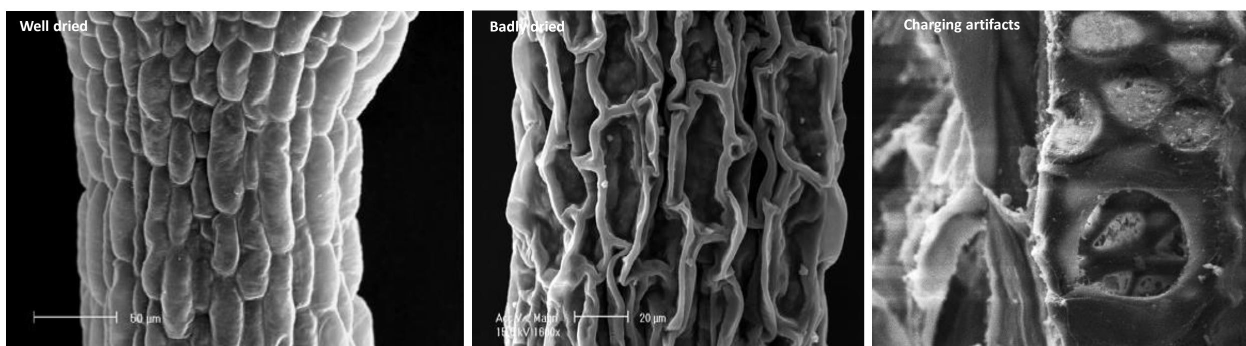
***N. meningitidis* strain S4**

E Johnson & Tang group

Preparation of cells & tissues for SEM

A standard SEM sample preparation protocol involves less steps than a TEM prep and can be done within a day.



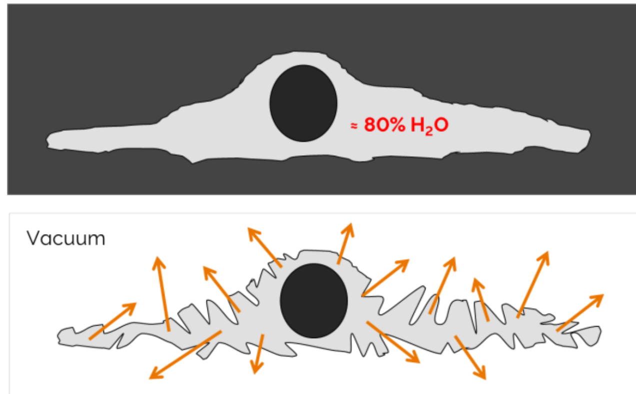


SEM images of Arabidopsis stem (left, centre) and xylem (right; E Johnson)

Specimen requirements for biological TEM & vEM

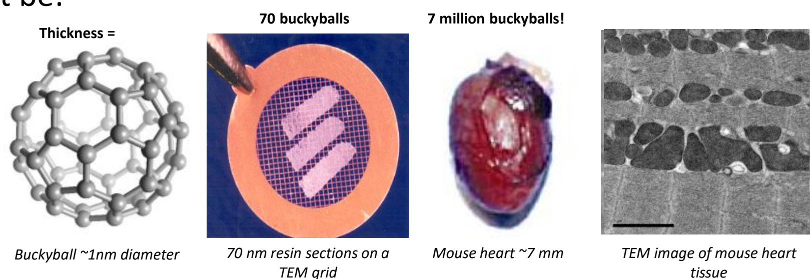
- TEM & vEM specimens must be:
  - Stable in the vacuum

Many biological samples are unstable in a vacuum due to their high water content



Specimen requirements for biological TEM & vEM

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

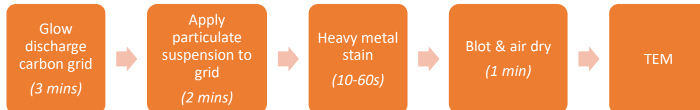


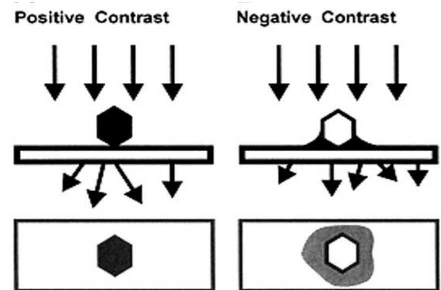
Buckyball ~1nm diameter | *70 nm resin sections on a TEM grid* | *Mouse heart ~7 mm* | *TEM image of mouse heart tissue*

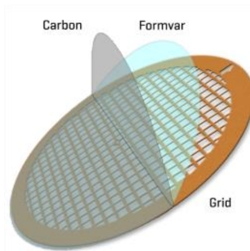
- The degree of specimen preparation required depends on the specimen
  - Particulate samples (eg: protein and viruses) can be stained and viewed quickly
  - Cells and tissue samples require extensive preparation for TEM

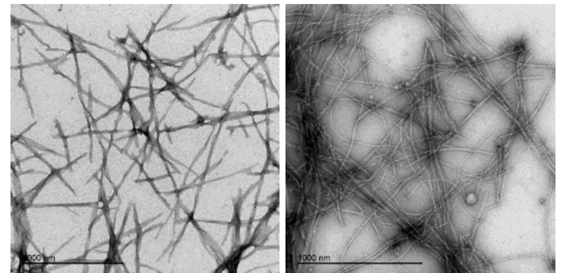
Preparation of particulate samples for TEM

Particulate samples (eg; proteins, fibrils, nanoparticles, vesicles, DNA & viruses) are prepared using a quick staining technique





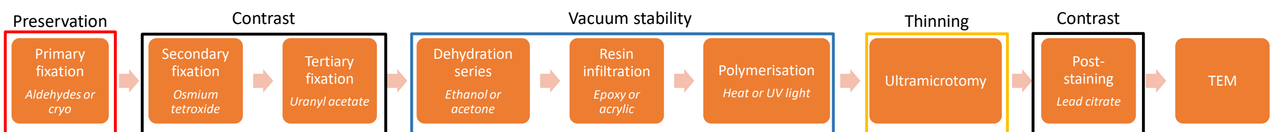




Positively (left) and negatively (right) stained amyloid fibrils (E. Johnson)

Preparation of cells & tissues for TEM & vEM

A standard TEM sample preparation protocol involves many steps (with washes in between each step) and typically takes 1-2 weeks. The exact protocol used depends on the sample itself and on the goal of the EM experiment.



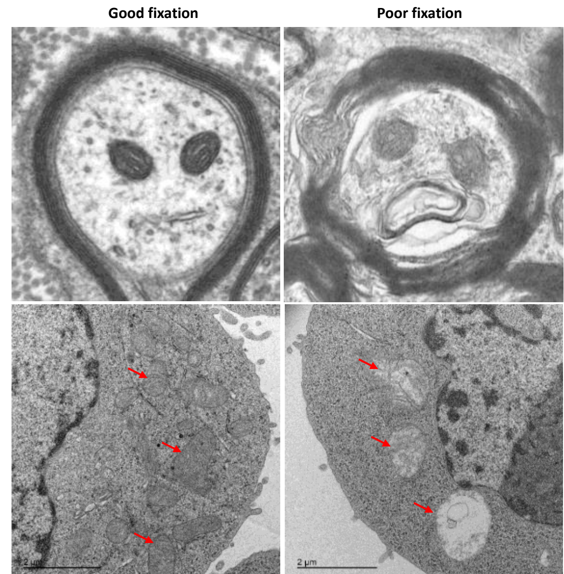
Factors affecting prep quality include:

- Sample constraints, size and density
- Type of fixative (incl buffer type)
- Mode of fixation
- Type & concentration of heavy metal stains used
- Duration & temperature of dehydration series
- Which resin you use and how its polymerised

Preparation of cells & tissues for TEM & vEM

Preservation - Primary Fixation

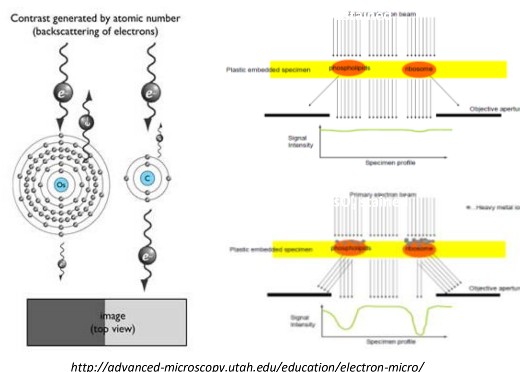
- Fixation stops cellular processes and aims to preserve the specimen as close as possible to its natural state.
- Chemical fixation with buffered aldehydes is the most commonly used primary fixation method for TEM:
  - Glutaraldehyde (irreversibly cross-links proteins via their amino groups, essential for good ultrastructural preservation)
  - Formaldehyde (light cross-linker, faster penetration into cells)
  - Standard fixative = 2.5% glut + 2-4% FA in buffer
- There are several different modes of chemical fixation:
  - Immersion (eg: monolayer & suspension cells, tissue pieces)
  - Perfusion (eg: mouse, rat)
  - Microwave-assisted (eg: plants)
- Alternatively, cryo-fixation with liquid nitrogen will give almost native state preservation, but it is more technically demanding, more time consuming and requires special equipment



Biological Specimen Preparation for TEM

Preservation & Contrast – Secondary Fixation

- Osmium tetroxide (OsO_4) is a (very toxic) heavy metal that fixes unstaturated lipids and is also electron dense.
- It acts as both a secondary fixative and an electron stain, to significantly improve specimen preservation (especially of membranes) and contrast.

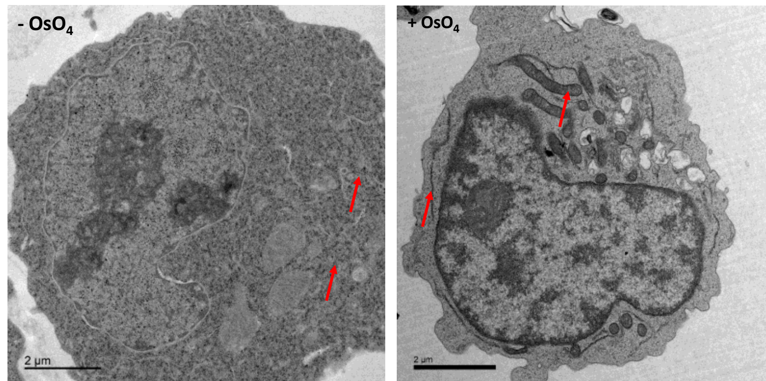


http://advanced-microscopy.utah.edu/education/electron-micro/

Biological Specimen Preparation for TEM

Preservation & Contrast – Secondary Fixation

- Osmium tetroxide (OsO_4) is a (very toxic) heavy metal that fixes unstaturated lipids and is also electron dense.
- It acts as both a secondary fixative and an electron stain, to significantly improve specimen preservation (especially of membranes) and contrast.



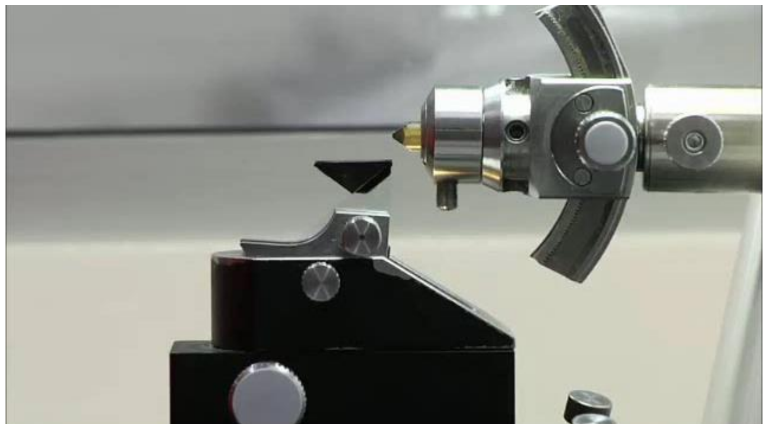
Erythroblast cells, E Johnson & Buckle Group (WIMM)

Preparation of cells & tissues for TEM

Ultramicrotomy

An ultramicrotome is an instrument which enables ultra-thin (70 nm!) sectioning of resin embedded material using a glass or diamond knife. The sections are then transferred to a TEM support grid (3mm in diameter and typically copper or nickel) and post-stained.

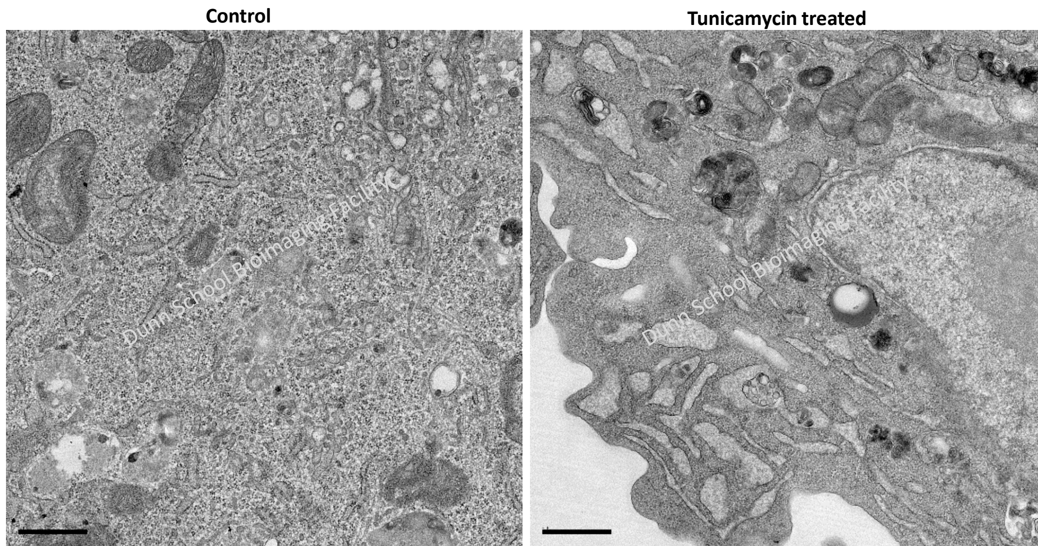
Leica UC7 @ Dunn School EM Facility



Introduction to ultramicrotomy video, University of Sydney

Example TEM project:

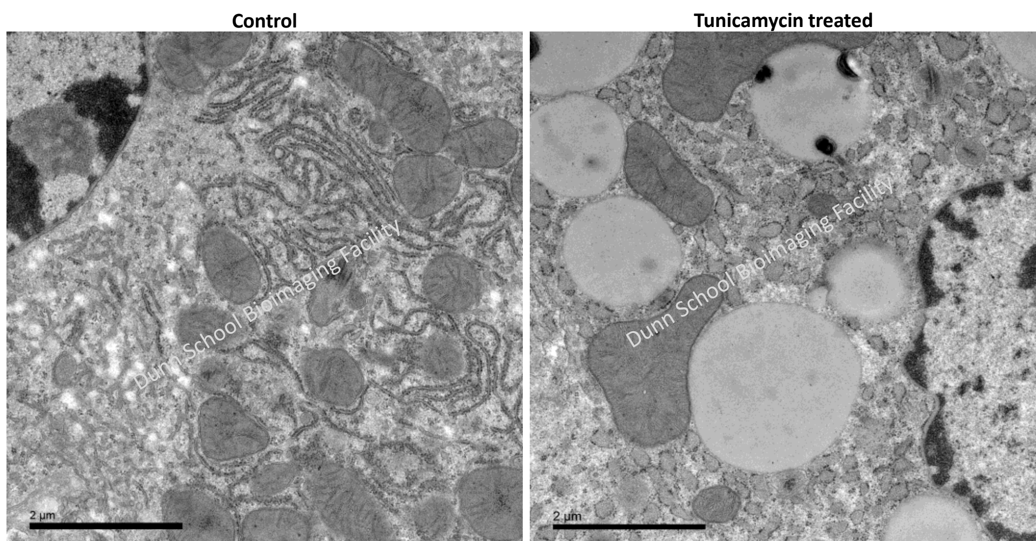
Visualising the effects of ER stress in mouse fibroblast cells



Mouse fibroblasts controls (left) and treated with tunicamycin (right), E Johnson/V Liebe

Example TEM project:

Visualising the effects of ER stress in mouse liver tissue

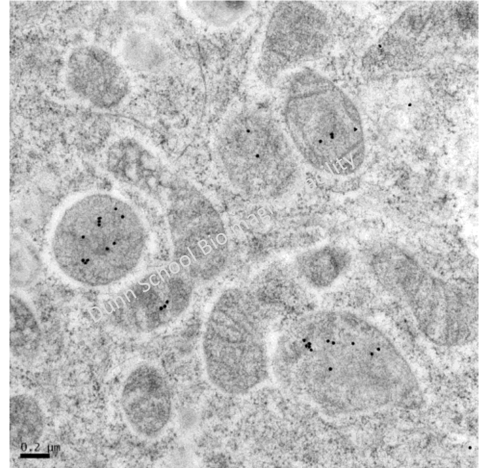


Mouse liver tissue, untreated (left) and under ER stress (right) (V Liebe & E Johnson)

Protein localisation in cells and tissues

Immunogold

- Similar to immunofluorescence labelling, but the secondary antibody is conjugated to a small (1-40 nm) collodial gold particle instead of a fluorophore, so that it can be visualised in the EM
- For LM, cells/tissues are usually permeabilised with detergents to allow antibodies access into the cell. However, this damages ultrastructure and results in poor preservation at the EM level
- Therefore, for EM, post-embedding labelling is usually the best option, as it avoids the use of permeabilisation agents needed for pre-embedding labelling
- For post-embedding labelling, a lighter chemical fixation is required, as glutaraldehyde affects antigenicity. However, PFA alone results in poorer quality fixation. Therefore, cryo-fixation and freeze subsitution is highly recommended.
- The osmium tetroxide step is omitted (as it also reduces antigenicity). but may be replaced with uranyl acetate instead.
- Epoxy resin does not allow reagent penetration, so acrylic resins are used instead.

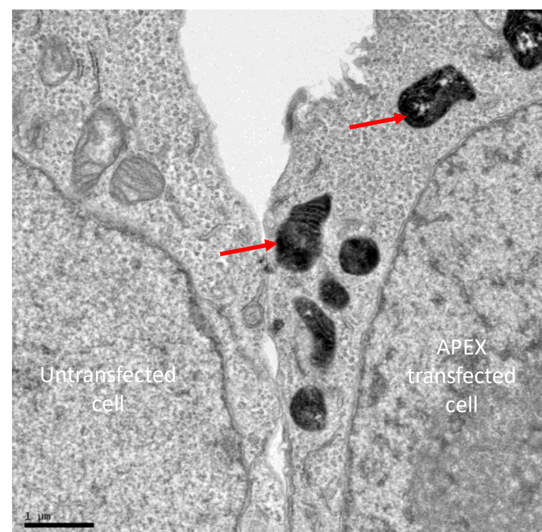


Immunogold labelled mitochondria in a mammalian cell (A Dhir/Ejohnson, Dunn School)

Protein localisation in cells and tissues

EM genetic tags

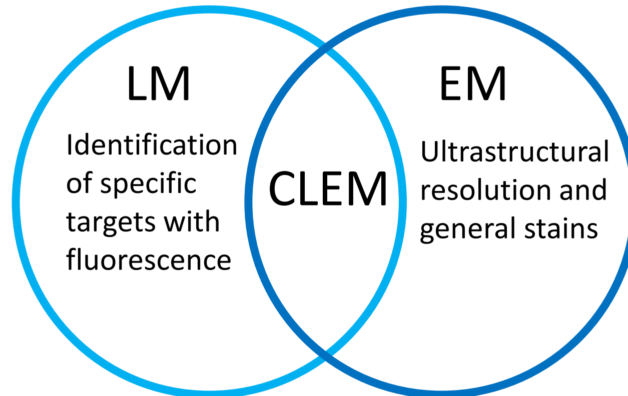
- Several new genetically encoded tags are now available as alternatives to using immunogold labelling for identifying proteins of interest at the EM level whilst using a standard TEM prep
- miniSOG (Shu et al PLOS Biology 9, 2011)
    - Small fluoresecent flavoprotein that can be photo-oxidised to react with DAB to produce a localised osmophilic precipitate - CLEM
- APEX (Martell et al (2012) Nature Biotech 30 & Martell et al (2017) Nature Protocols, 12(9):1792-1816)
    - 28kDa peroxidase that catalyses with DAB (with H2O2) to produce a localised osmophilic precipitate
    - See also Ariotti *et al.* (2018) *PLOS Biology* 16(4): e2005473 and Rae *et al* (2021) *eLife* for new APEX applications



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

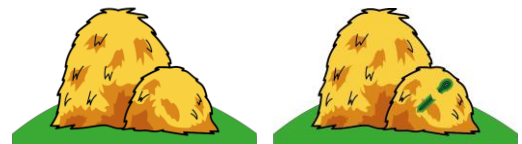
Correlative microscopy

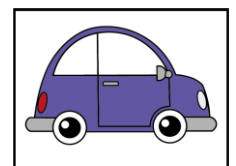
- Correlative microscopy is where the same cell/area of tissue is imaged using two or more microscopy techniques, such that the resulting images are correlated/combined to provide more information about the sample than could be obtained by using one technique on its own.
- The most commonly used correlative method is correlative light and electron microscopy (CLEM). This combines the strengths of both techniques:

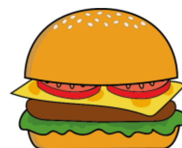


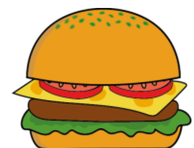
Correlative Light & Electron Microscopy (CLEM)

- The three main applications of CLEM are:
  - **Targeting** specific cells or rare cellular events at the EM level by first using fluorescence imaging to identify them
  - Placing fluorescently labelled proteins/organelles within **ultrastructural context**
  - **Localisation** of proteins at the ultrastructural level without the need for antibodies





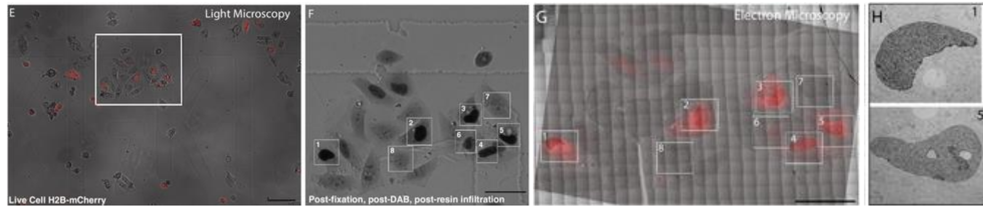




Thank you to Charlotte Melia for these custom graphics

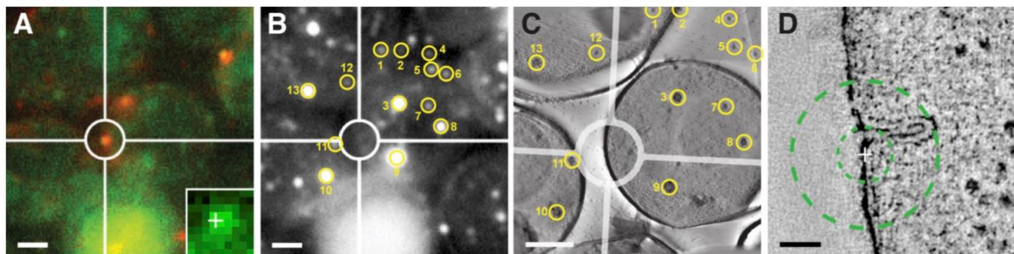
CLEM applications - Targeting

Locating specific cells in a mixed population



CLEM of mCherry expressing cells co-transfected with APEX-ChBP (Ariotti *et al.* 2018, *PLOS Biology* 16(4))

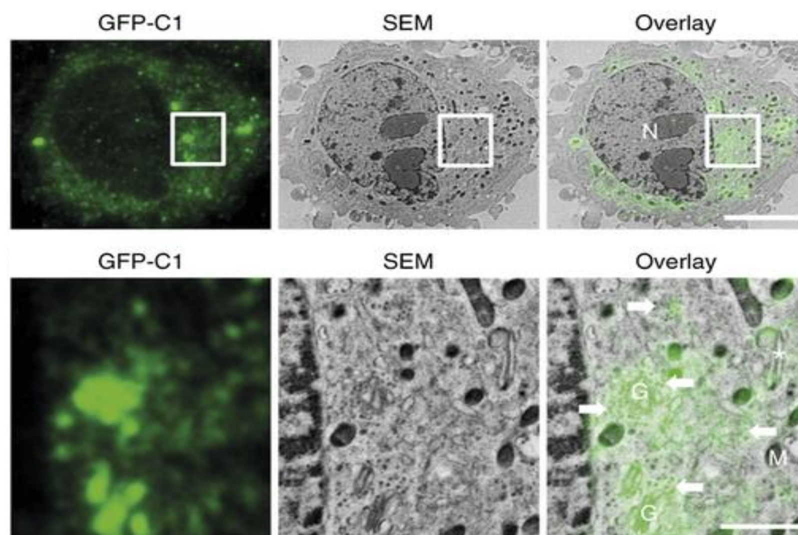
Pinpointing rare cellular events



CLEM of in-resin fluorescing endocytic patches in yeast cells (Kukulski et al. 2011, JCB, 192)

CLEM applications - Context

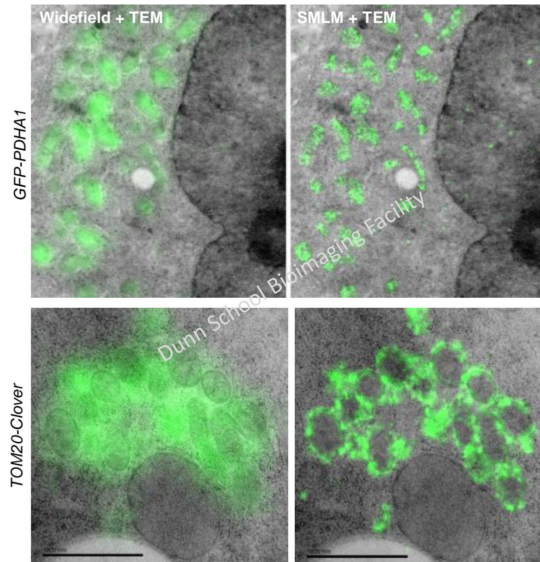
Providing ultrastructural context to fluorescent signals



CLEM of GFP-C1 in resin sections of HeLa cells (Peddie *et al.* 2014, *Ultramicroscopy*, 143)

CLEM applications – Protein Localisation

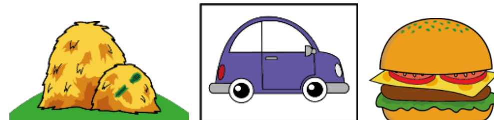
Protein localisation using super-resolution microscopy



Super-resolution CLEM of GFP/TOM20 resin sections of HeLa cells (E Johnson & R Kaufmann)

CLEM workflows

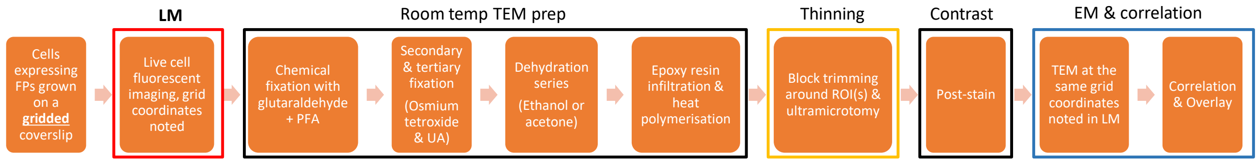
- There are a **HUGE** number of ways to do CLEM
- Which approach you take depends on multiple factors, including:
  - Your overall aim, including desired resolution
  - Choice/availability of fluorophore(s)
  - Sample type (eg cells vs tissue)
  - Availability of instrumentation and expertise
  - Special considerations (eg: 3D, cryo, containment restrictions, additional microscopy techniques, eg x-ray)

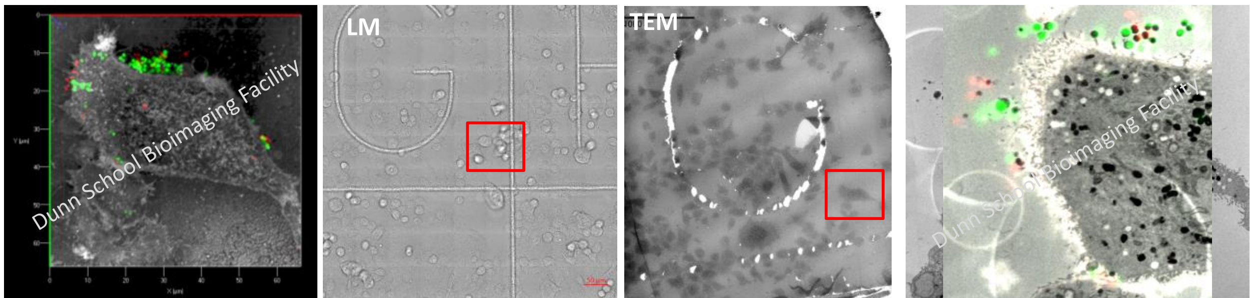


- CLEM can broadly be divided into two categories defined by the timing of the light microscopy relative to the EM prep:
  - Pre-embedding LM
  - Post-embedding LM

Pre-resin embedding CLEM workflows

Aim: Track the sample position

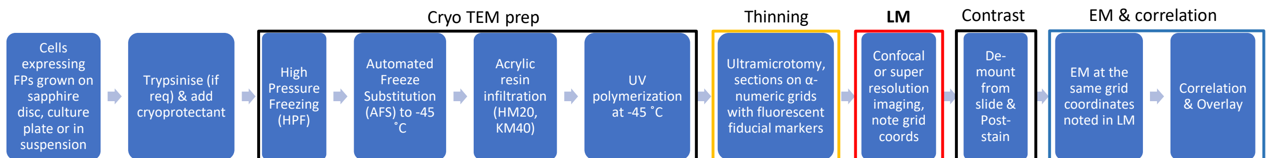


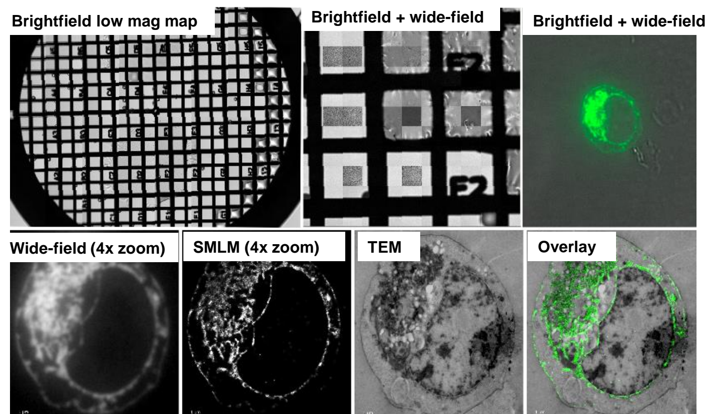


Correlative imaging of epithelial cells co-infected with Niesseria cinera (green) and Neisseria meningitidis (red) (E Johnson & Tang Lab, Dunn School)

Post-resin embedding CLEM workflow

Aim: reserve fluorescence/antigenicity

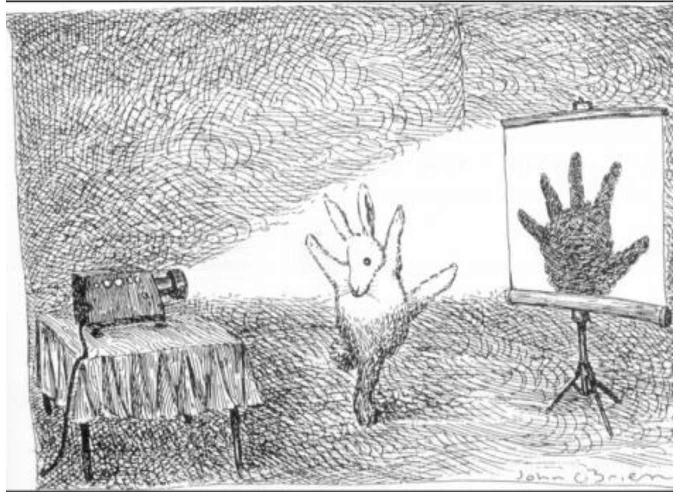




CLEM of HEK cells expressing EphA2-mVenus (From: Johnson & Kaufmann (2017) Correlative Light & Electron Microscopy III)

3D Cellular ultrastructure with volume EM

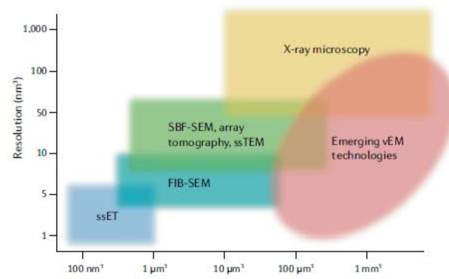
- TEM images are 2D projections of complex 3D objects and this can sometimes be misleading
- For instance, mitochondria look very different when sectioned transversely compared to longitudinally, or could be mistaken for being damaged if cut obliquely. A single mitochondrion can also be highly branched, which in thin section would appear as multiple mitochondria when it is in fact just one mitochondrion through different planes of section -> use volume EM!



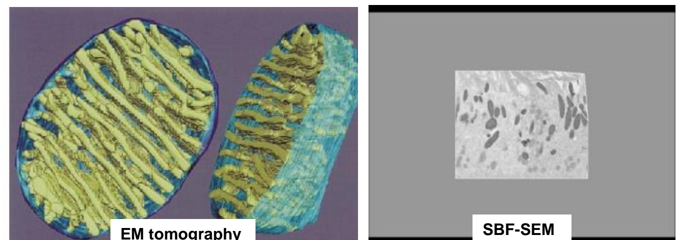
Drawing by John O'Brien, The New Yorker Magazine (1991)

3D Cellular ultrastructure with volume EM

- There are several 3D or volume EM techniques available, including EM tomography, serial block face SEM (SBF-SEM) and Focused ion beam SEM (FIB-SEM).
- Which technique choose depends on lateral resolution, z resolution, field of view, and total depth you need to answer your research question
- For instance, to investigate changes in mitochondrial structure, you would use EM tomography. However, to visualize how mitochondrial morphology is affected (eg: by a mutation/drug treatment) in a cell then FIB-SEM would be best or if through a larger volume in tissue, then SBF-SEM would be best



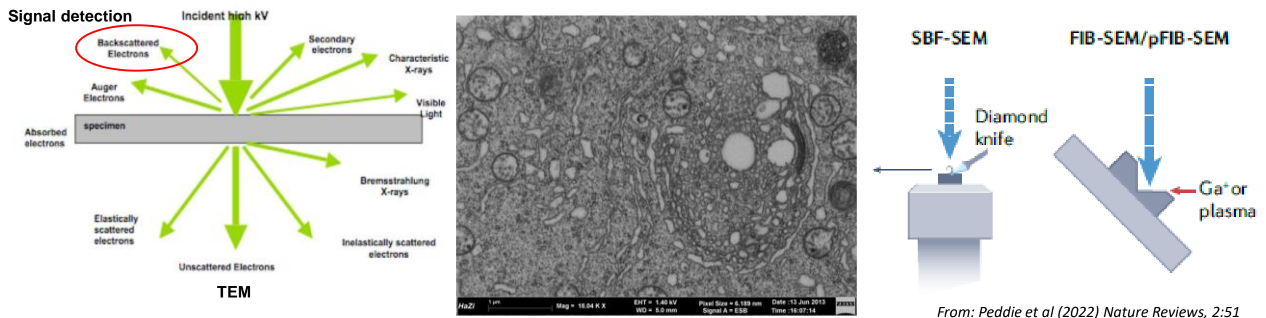
Scale and resolution ranges of various volume EM techniques.
From: Peddie et al (2022) Nature Reviews, 2:51



Perkins & Frey (2000) Micron

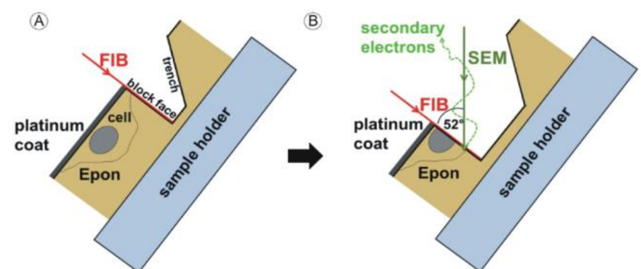
SEM-based volume EM Techniques

- SEM can also be used to generate 'TEM' like images of resin embedded samples by detecting backscattered electrons, which are beam electrons that have been elastically scattered/deflected by high atomic number elements (heavy metals) in the sample
- In SEM-based vEM, a resin block is mounted in the SEM chamber and the resin is then removed in a controlled and automated manner using either a diamond knife (SBF-SEM) or focused beam of ions (FIB-SEM), with the fresh block face imaged at each pass, to produce a high resolution z-stack



From: Peddie et al (2022) Nature Reviews, 2:51

Volume EM with FIB-SEM



Best for: 3D imaging of a whole cell or small area of tissue

Z resolution: ~5-100nm

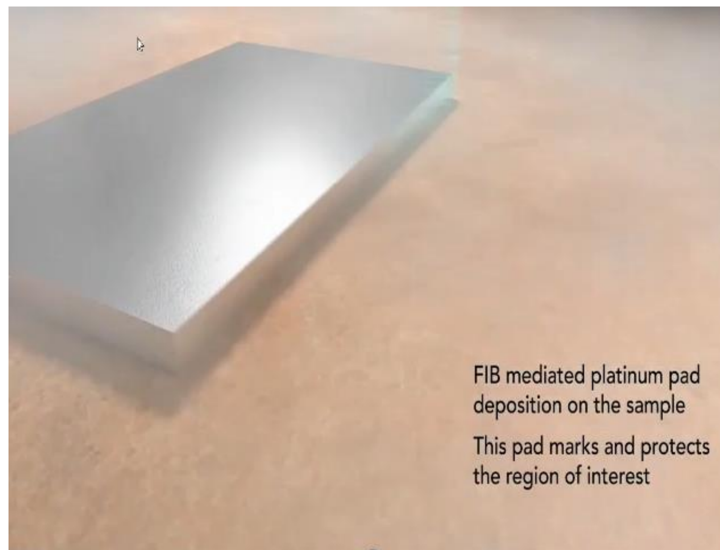
Sample prep: similar to standard TEM, with extra OsO_4

Destructive technique? Yes

Acquisition time: ~24 hrs for 1 cell or months for tissue (depending on the settings)

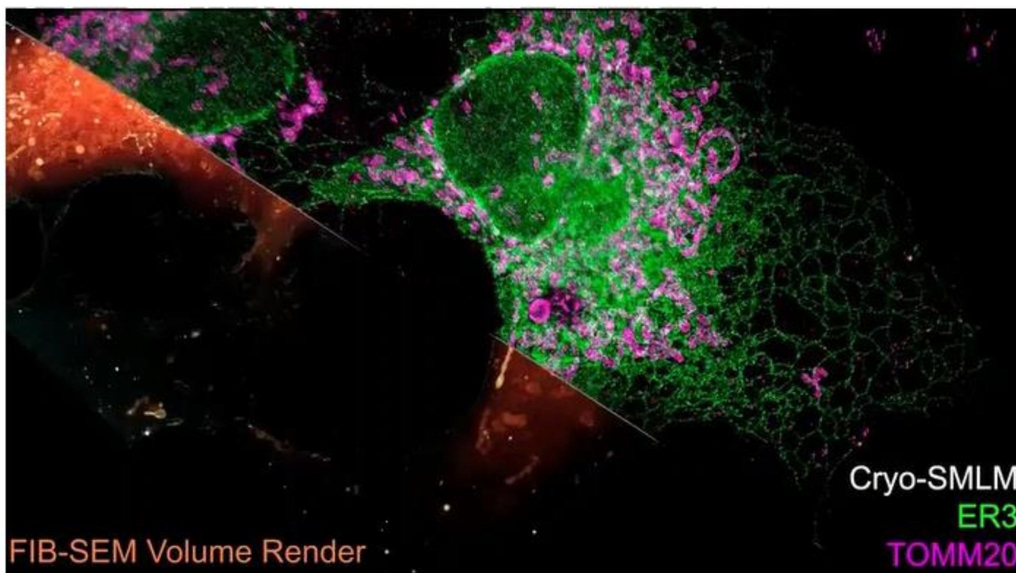
Data processing: Stack alignment, segmentation, 3D modelling

Volume EM with FIB-SEM - video



Video excerpt from FIB-SEM video by Kadar Narayan, National Cancer Institute, USA
https://www.youtube.com/watch?v=zqW6pXaU4Go

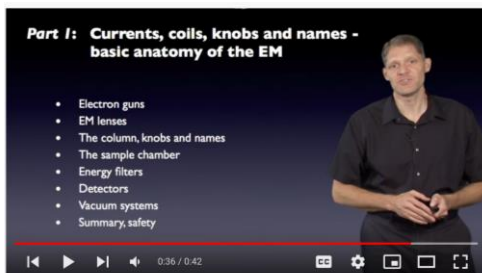
Volume CLEM with FIB-SEM - video



Correlative volume EM of ER and mitochondrial markers in cos-7 cells. From: Hoffman et al (2020) Science, 367 (6475)

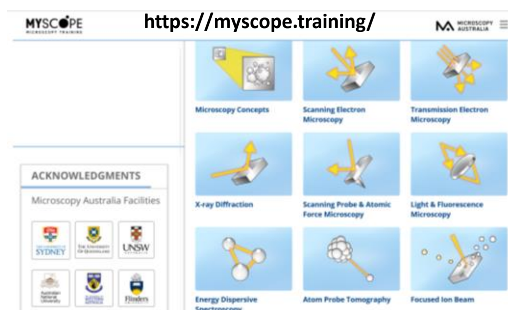
Microscopy Resources

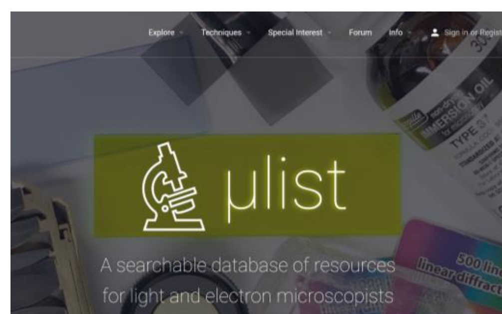
- To learn more about the components of TEMs and how they work, Grant Jensen's 'Getting started in cryo-EM' video series is an excellent introduction to TEM and cryo-EM.
- Microscopy Australia's MyScope is a fabulous resource to learn about many different imaging techniques, including EM and LM.
- The Microlist database is an excellent resource for light microscopy as well as for volume EM, and has links to many great resources to discover more about these techniques.



Search 'Getting started in Cryo-EM' on Youtube

https://myscope.training/

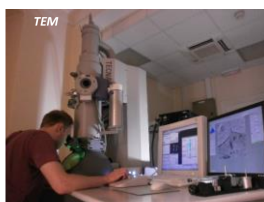




www.microlist.org

The Dunn School EM Facility

- **Staff:**
  - Errin Johnson (EM Facility Manager)
  - Charlotte Melia (EM support scientist)
- **Instruments:**
  - JEOL 1400Plus120 kV TEM
  - JEOL 2100Plus TEM
  - JEOL 4700F FIB-SEM
  - Zeiss Sigma 300 FEG-SEM
  - Zeiss Merlin compact FEG-SEM + 3View (sited at & shared with Oxford Brookes)
  - Biological EM specimen preparation lab
- **Access options:**
  - Full training for independent usage
  - Full service for sample prep & imaging
  - Collaboration for EM methods development



JEOL 1400Plus TEM

JEOL 2100Plus TEM

JEOL 4700F FIB-SEM