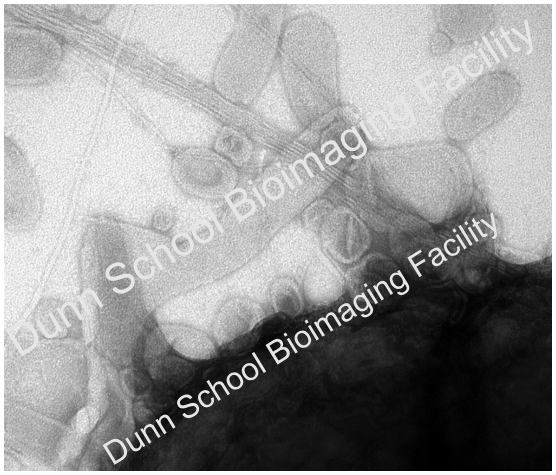
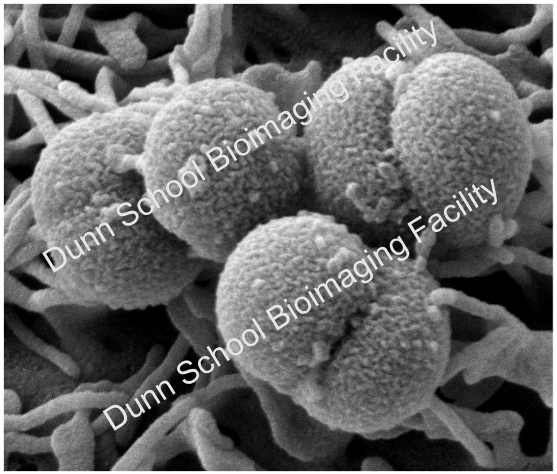
Biological Electron Microscopy: Techniques & Applications

Dr Errin Johnson

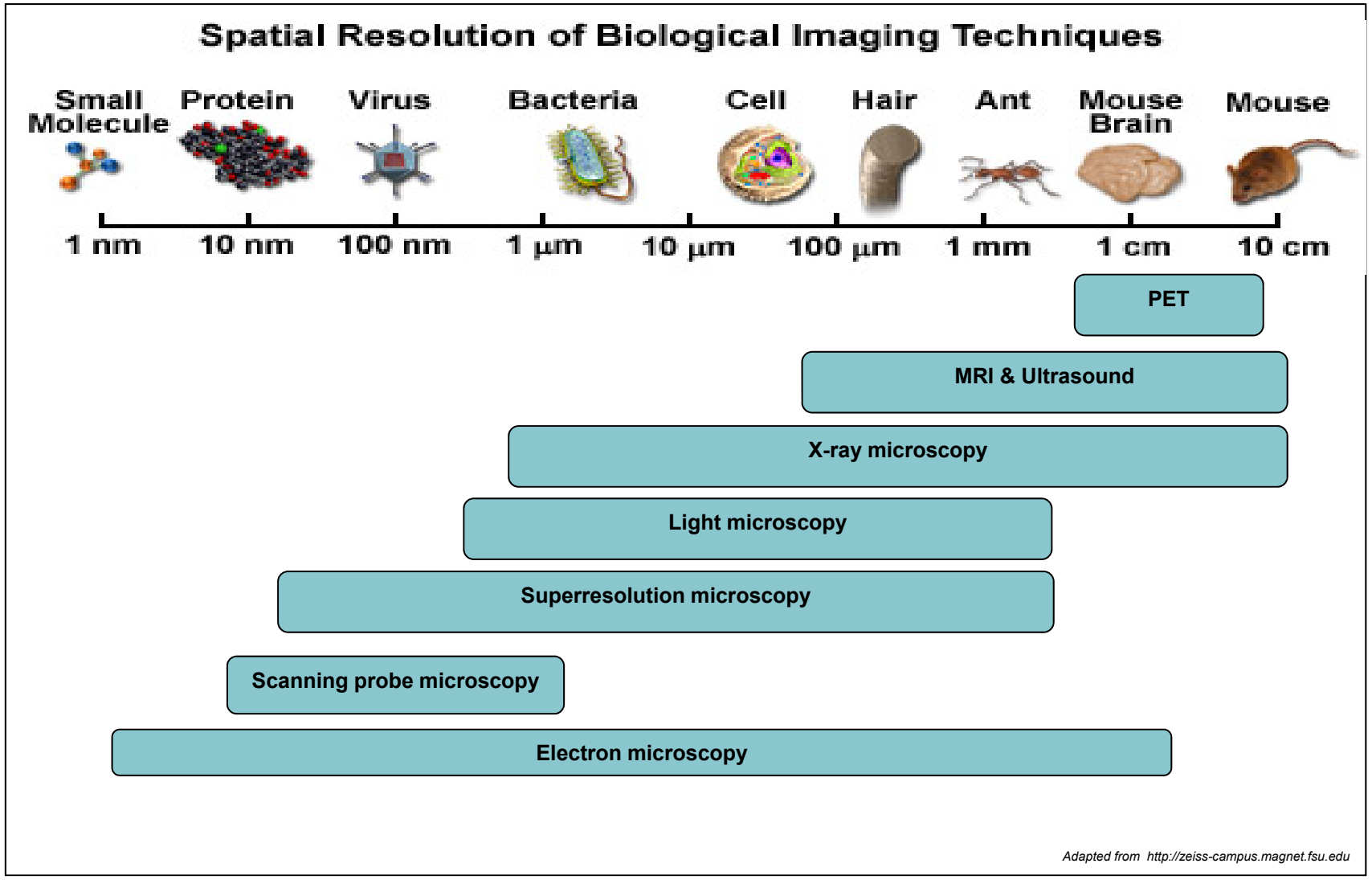
Head of Electron Microscopy

Sir William Dunn School of Pathology





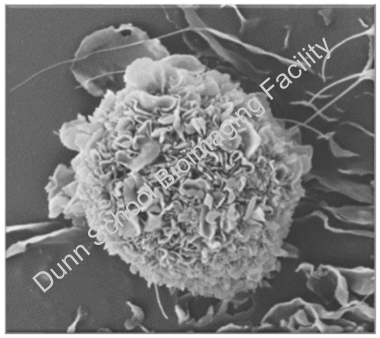
Electron Microscopy – still the king of resolution

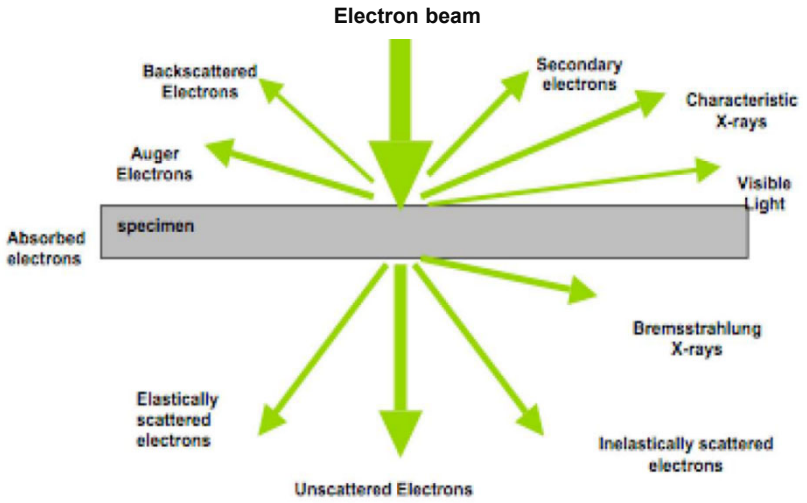


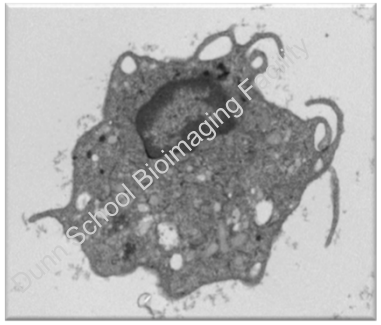
Lecture Overview

- Introduction to Electron Microscopy (EM)
- 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
- Advanced EM techniques
  - Volume EM, protein localization, **correlative microscopy** & cryo-EM
- EM @ the Dunn School

Scanning Electron Microscopy





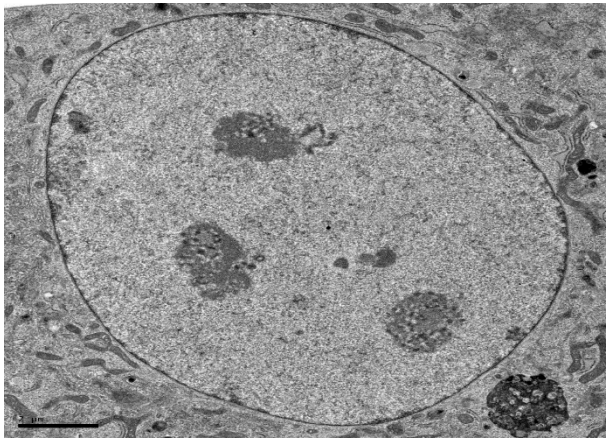


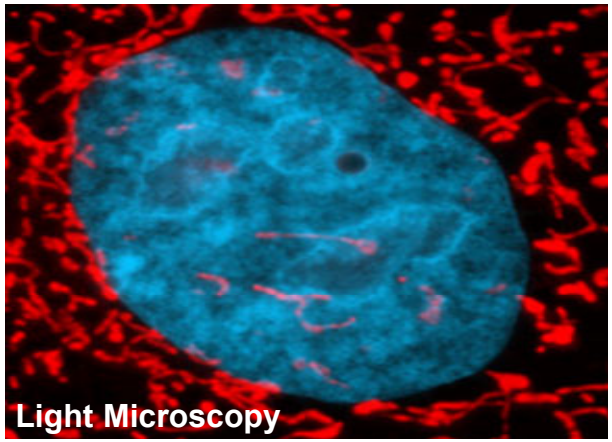
Transmission Electron Microscopy

Electron microscopy

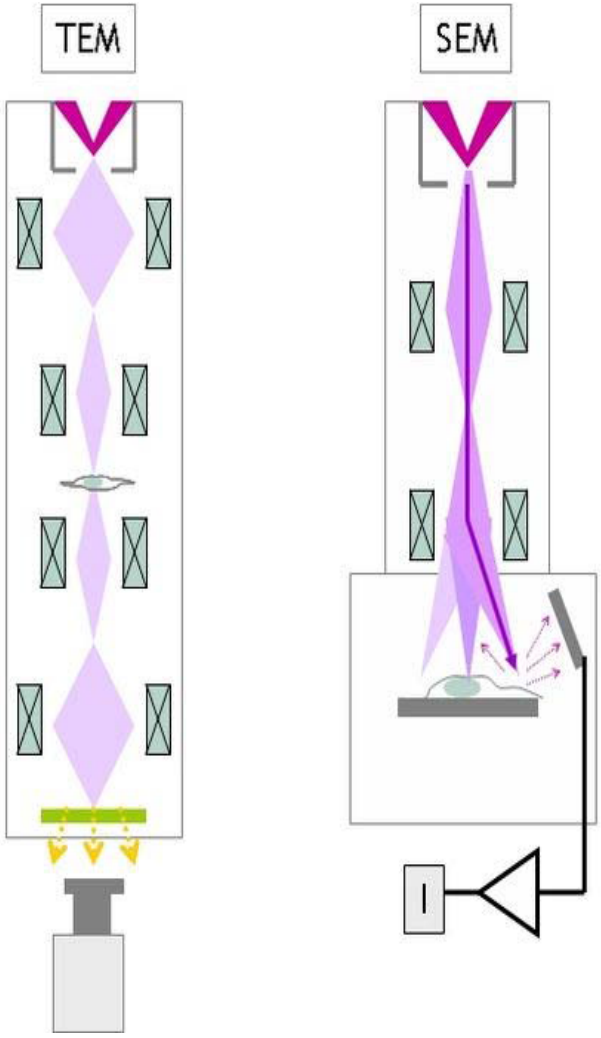
Overview

Transmission Electron Microscopy (TEM)

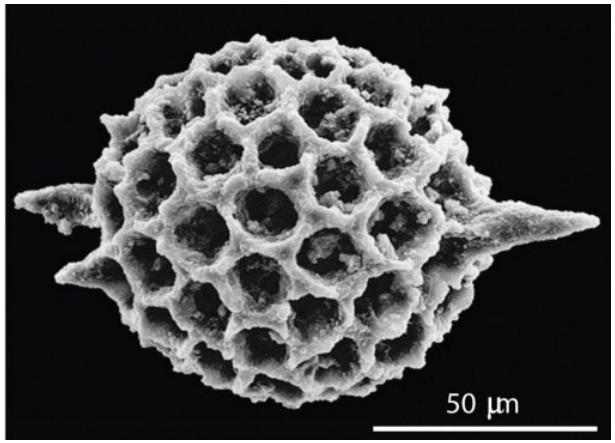


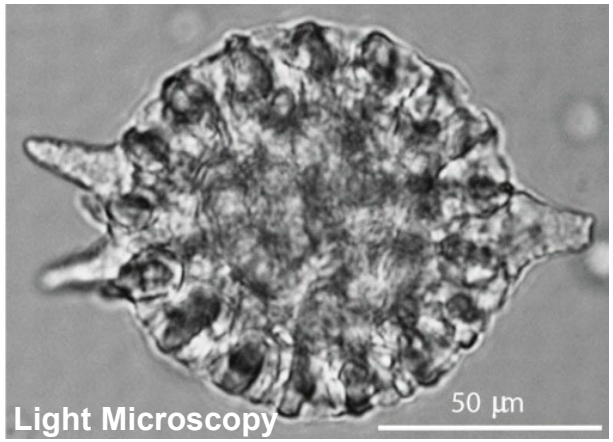


Top: TEM image of fibroblast cell stained with no specific stain (E Johnson, Dunn School). Bottom: Confocal image of a kidney cell stained with DAPI (blue) and MitoTracker (red) (From: Hammamatsu.magnet.fsu.edu)



Scanning Electron Microscopy (SEM)



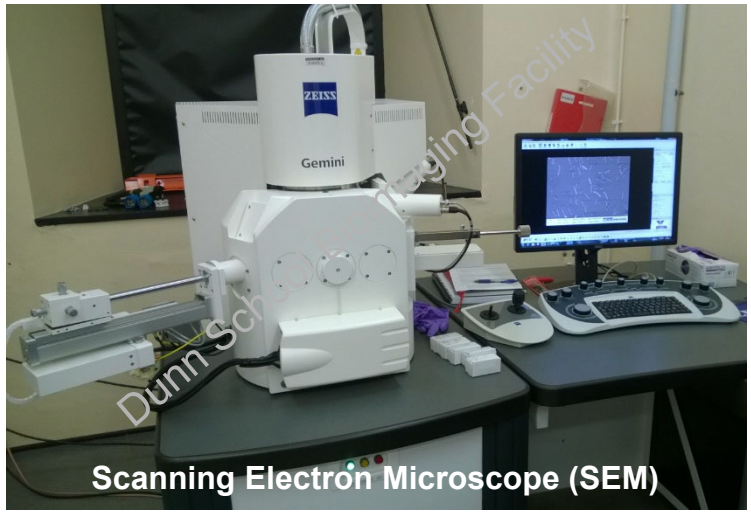


Radiolarian imaged with both SEM (top) and light microscopy (bottom). From: General Chemistry: Principles, Patterns, and Applications, B. Averill & P. Elderege

Introduction to Electron Microscopy
Electron microscopes

- Electron microscopes share the following main components:
  - An electron gun – source of electrons, operated at high voltages to accelerate the electrons
  - Electromagnetic lens system – to manipulate the electron beam
  - Vacuum system – protect beam integrity and prevent electrical discharging
  - Camera/detector – fast and sensitive signal detection
  - Computer – microscope control

Transmission Electron Microscope (TEM)

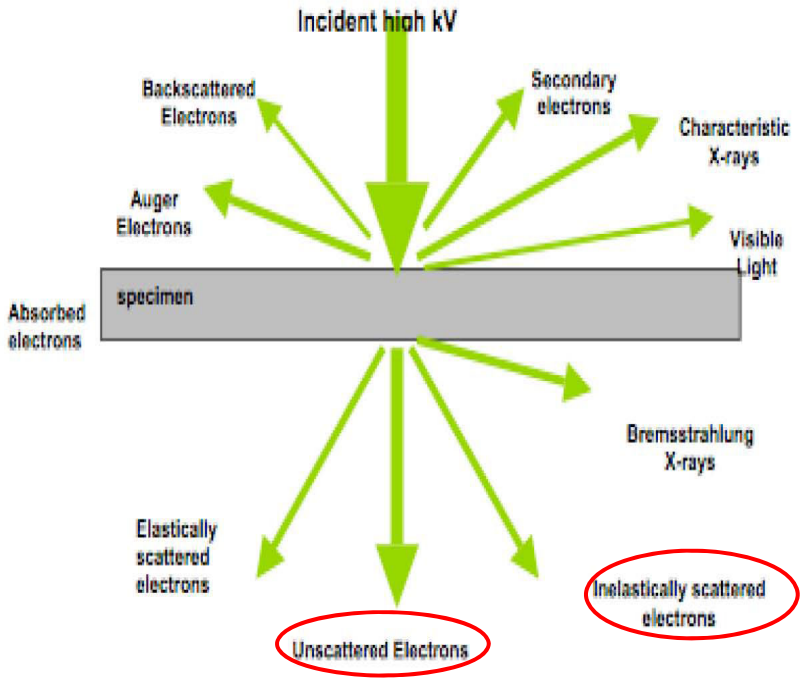


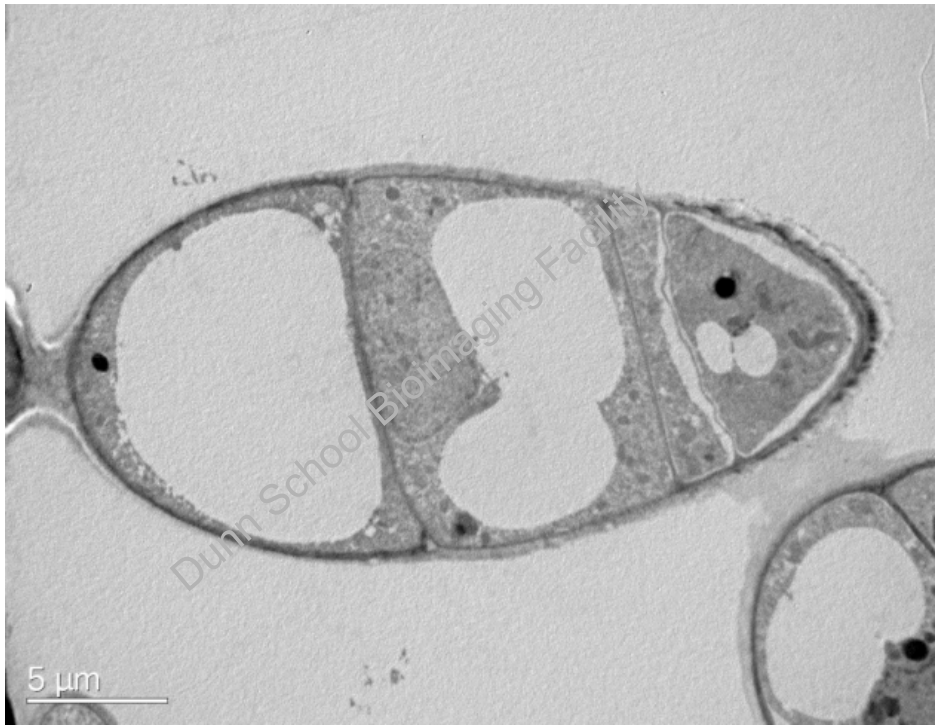
Scanning Electron Microscope (SEM)

Transmission Electron Microscopy (TEM)

Image contrast in the TEM

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





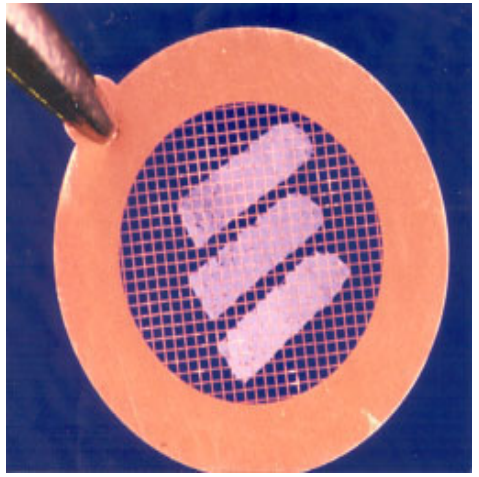
Lavender trichome, E Johnson

Biological TEM

Specimen requirements

TEM
Stable in the vacuum
Well preserved internal structure
Electron dense staining
Very thin (eg: 70 nm)
Particulate samples can be stained and viewed quickly
Cells and tissue require extensive specimen preparation

Mouse heart
~7 mm wide

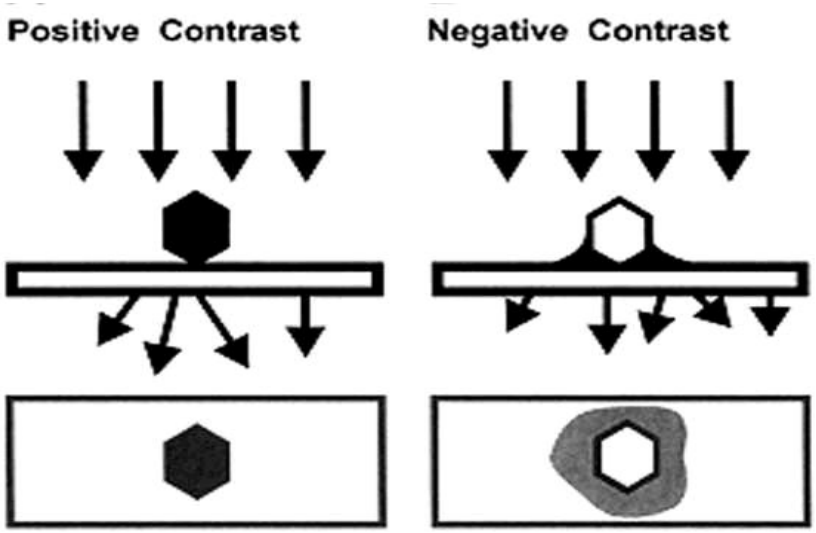


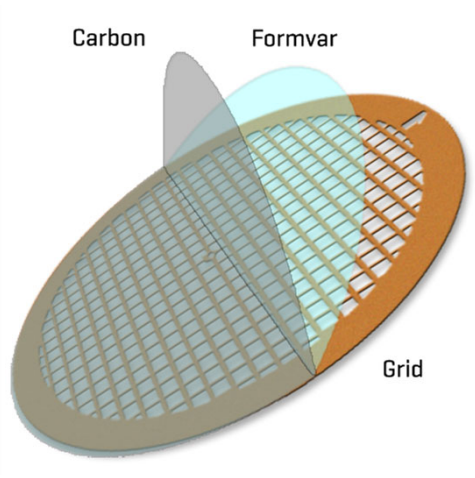
70 nm thick resin-embedded tissue sections on a TEM grid

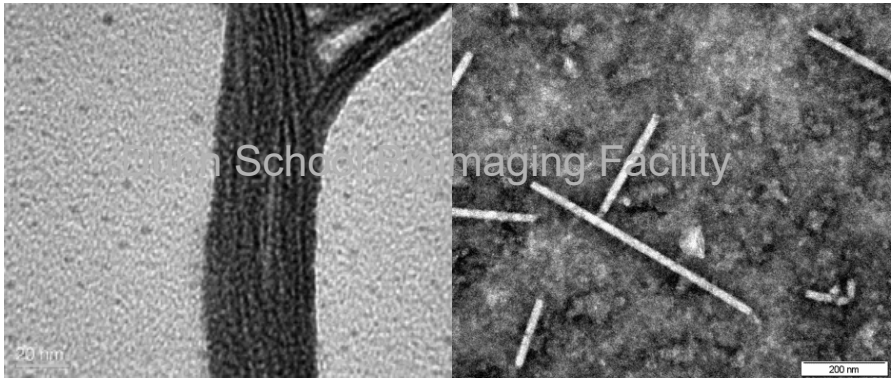
Specimen Preparation for TEM

Negative staining of particulate samples

- For proteins, liposomes, DNA and viruses:
  - Coat grids with plastic film and carbon
  - Apply the particulate specimen
  - (eg: proteins, viruses, DNA)
  - Stain with heavy metal solution – this is most commonly uranyl acetate
  - Blot dry and view in the TEM





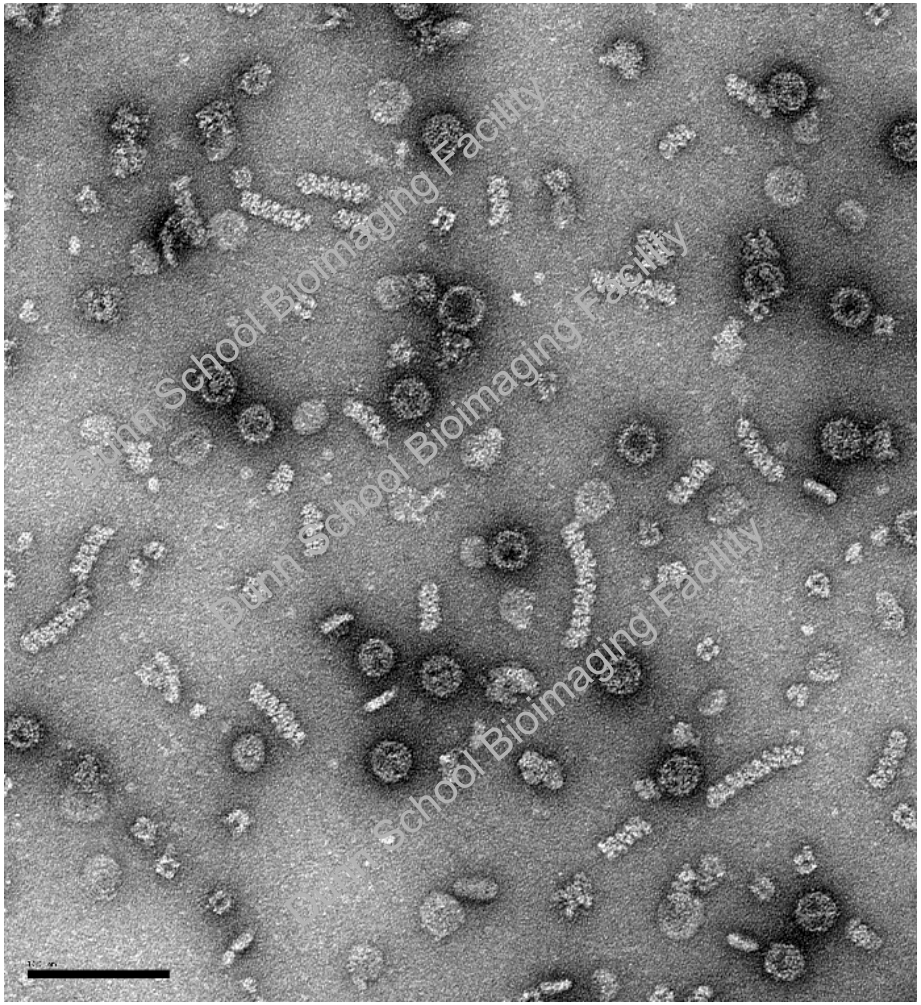


Bacterial protein stained with uranyl acetate; Tobacco mosaic virus negatively stained with sodium silicotungstate (E. Johnson)

Particulate samples

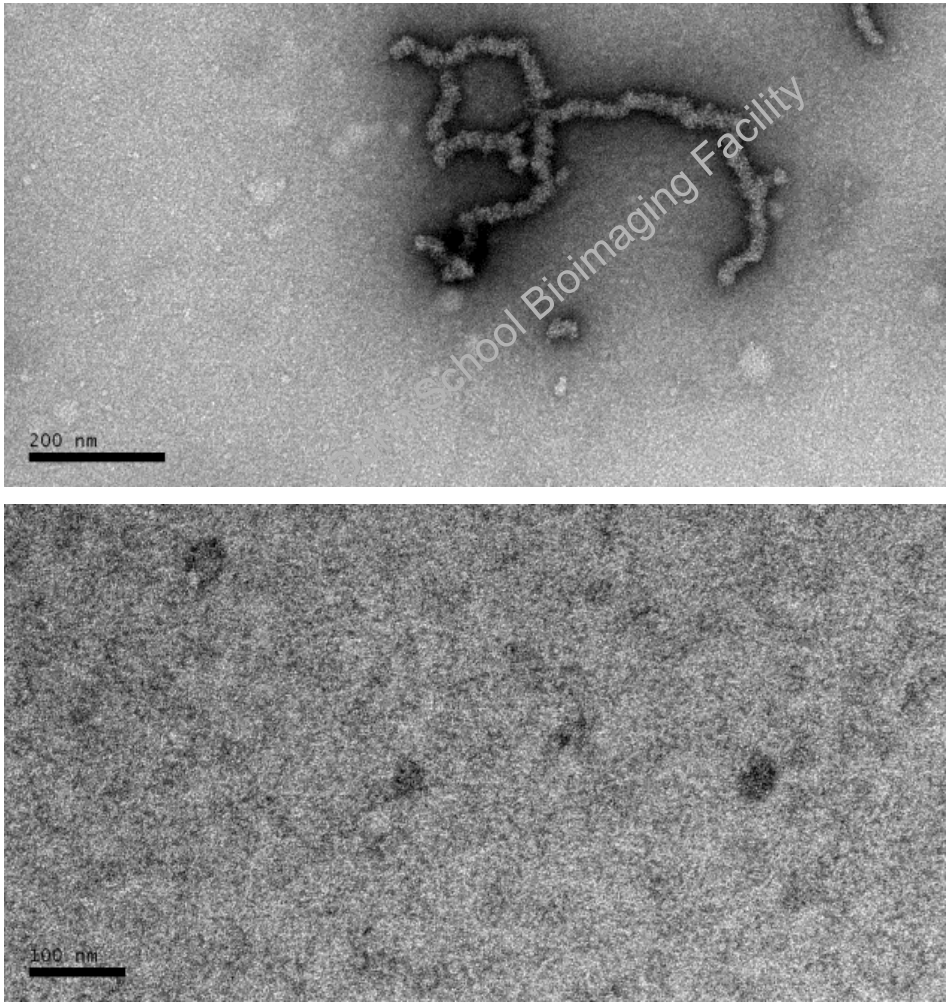
Screening preps using negative staining TEM

Viruses



Negatively stained virus-like particles
(D Leneghan/E Johnson)

Proteins

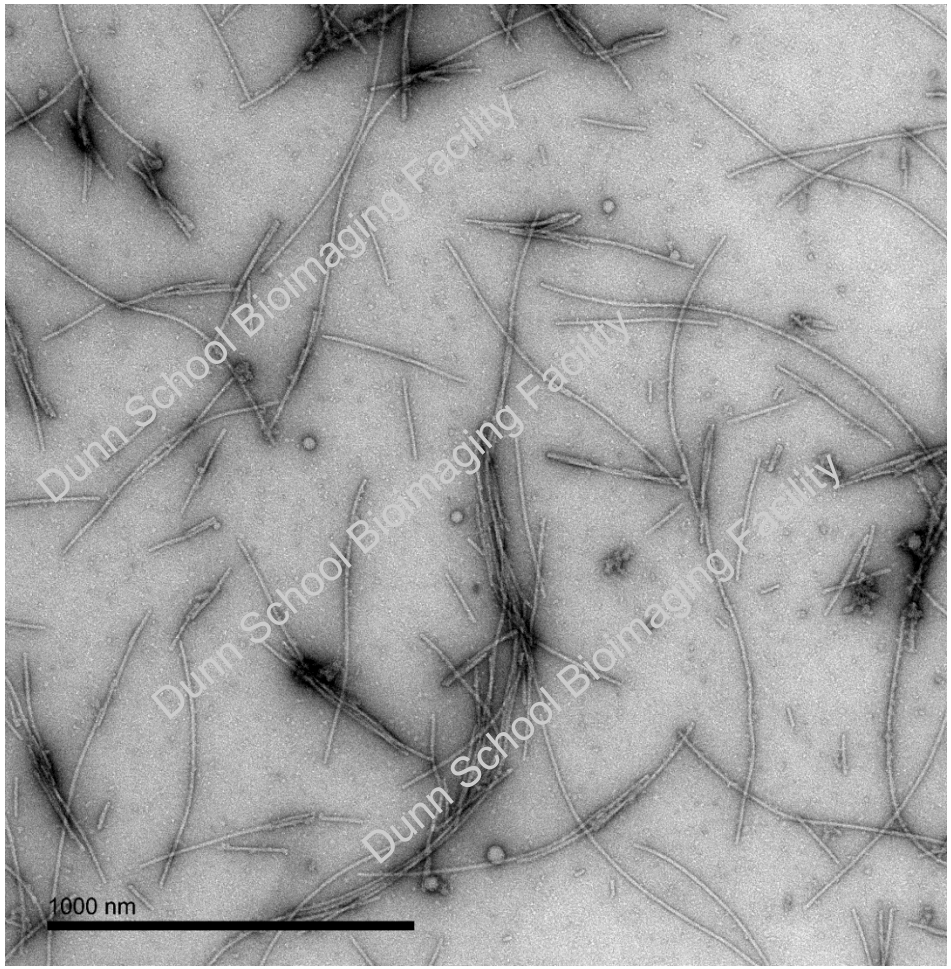


SAS-6 protein WT aggregates (top) & mutant dimers (bottom)
(M Cottee/E Johnson)

Particulate samples

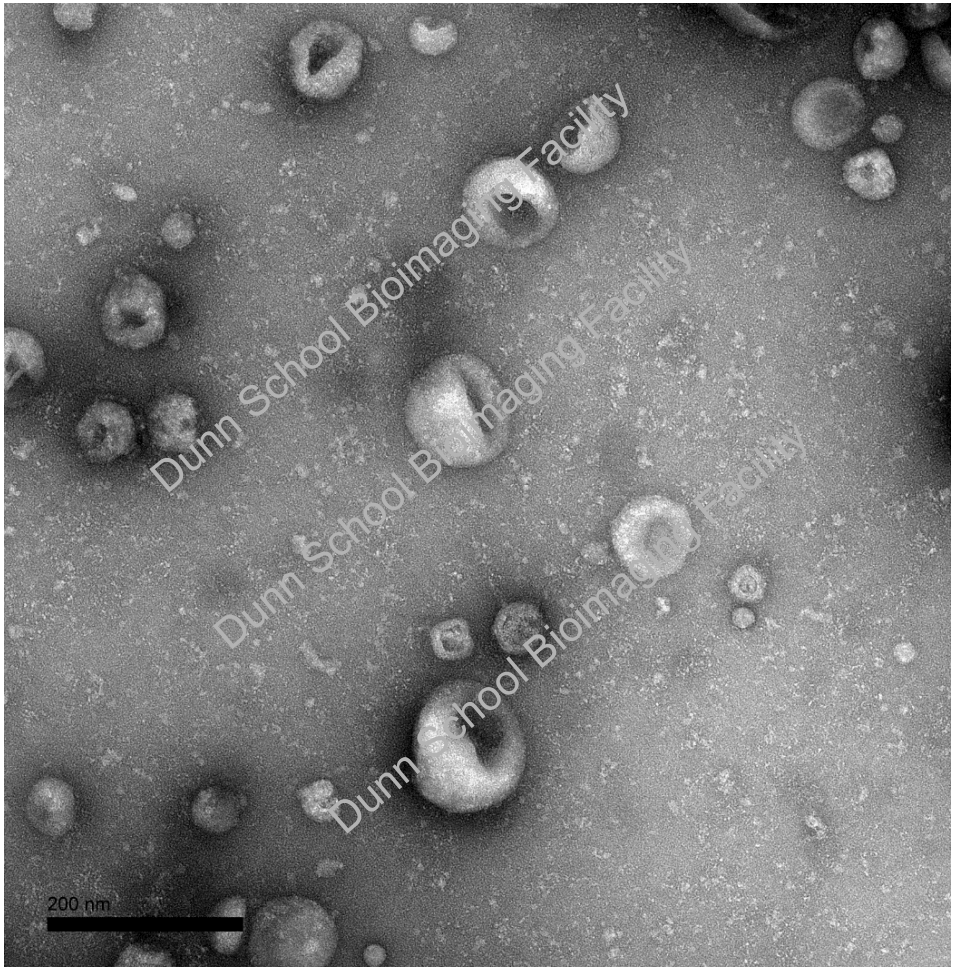
Screening preps using negative staining TEM

Fibrils



Negatively stained a-synuclein fibrils

Vesicles

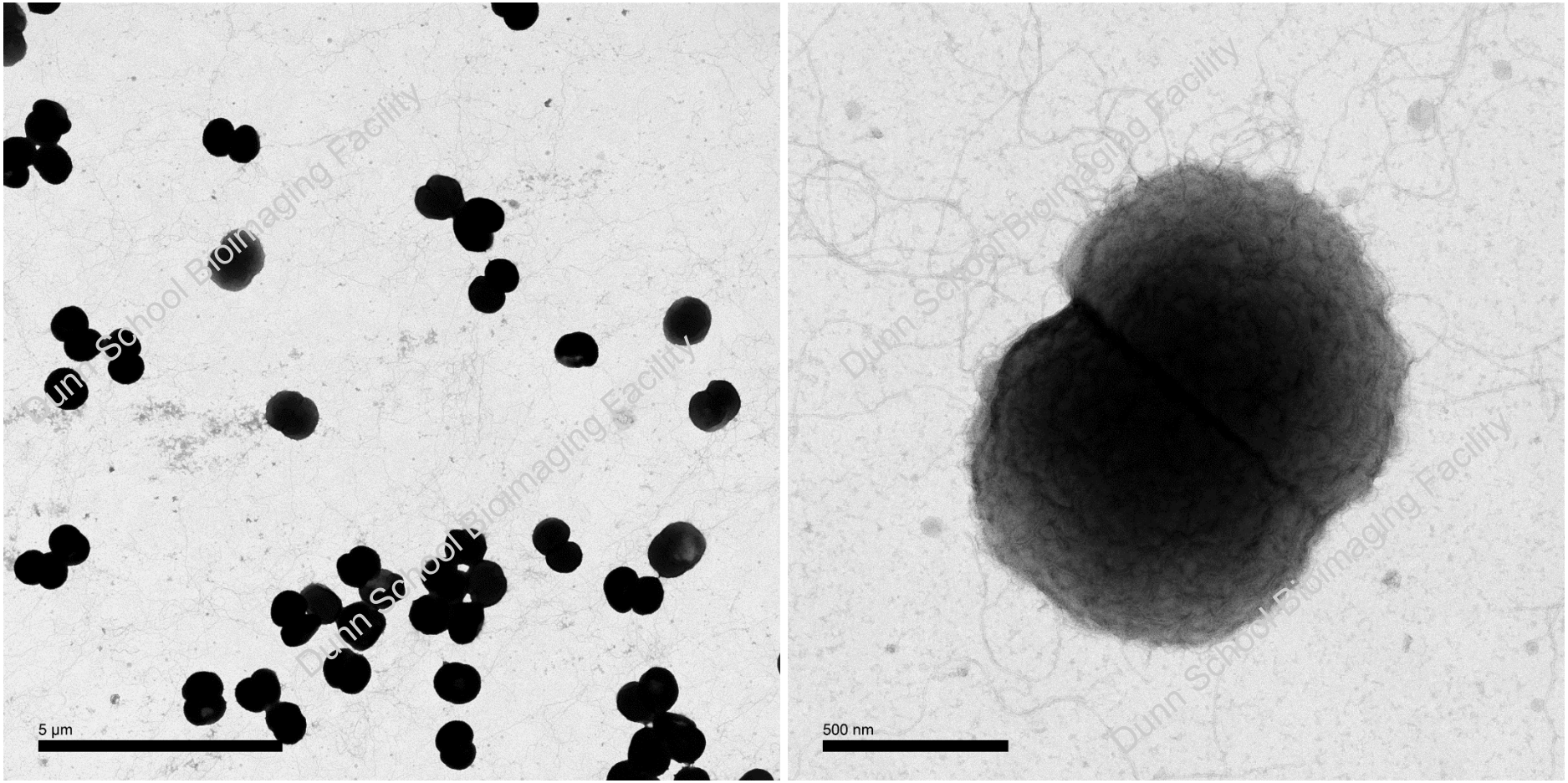


Negatively stained exosomes
*(*H Rodriguez Caro*/E Johnson)*

Particulate samples

Whole-mount negative/positive staining TEM

Bacteria

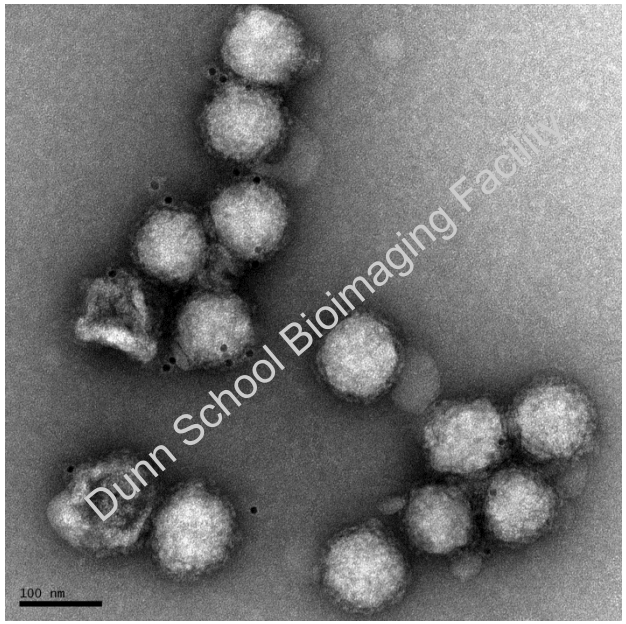


Negatively stained N. meningitidis (Tang lab/EJohnson)

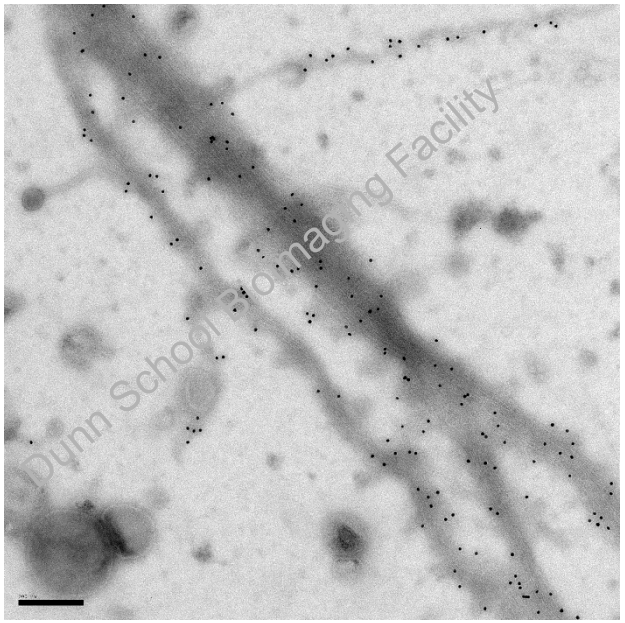
Particulate samples

Protein composition – immuno-negative staining

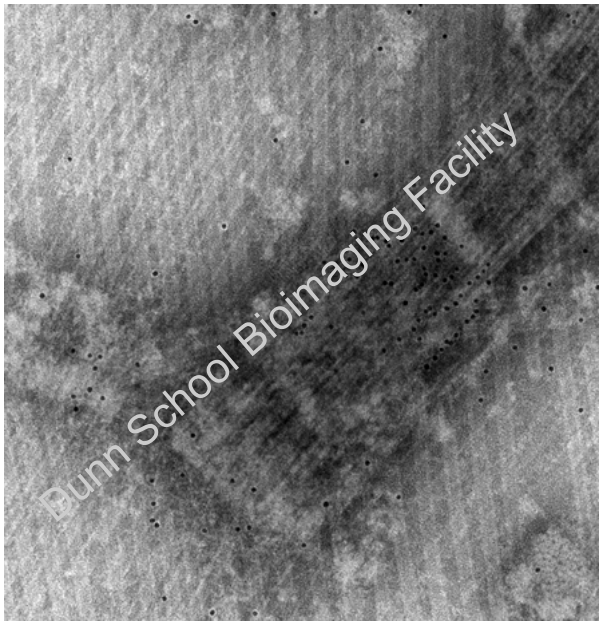
- As for immunofluorescence labelling, but the secondary antibody is conjugated to a small (1-4 nm) collodial gold particle instead of a fluorophore



Immunolabelled influenza (Ed Hutchinson/E Johnson)



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



Whole mount immunolabelled Trypanosome cytoskeleton (S Dean)

Specimen Preparation for TEM

Standard protocol for cells and tissue

- Primary fixation with glutaraldehyde
- Secondary fixation Osmium tetroxide
- Tertiary fixation Uranyl acetate
- Dehydration series Ethanol/acetone
- Epoxy resin infiltration
- Heat polymerisation
- Ultramicrotomy
- Post-staining
- TEM

Estimated time required

Prep: 1 week

Ultramicrotomy: ~3-9 hrs

Microscopy: ~3-9 hrs

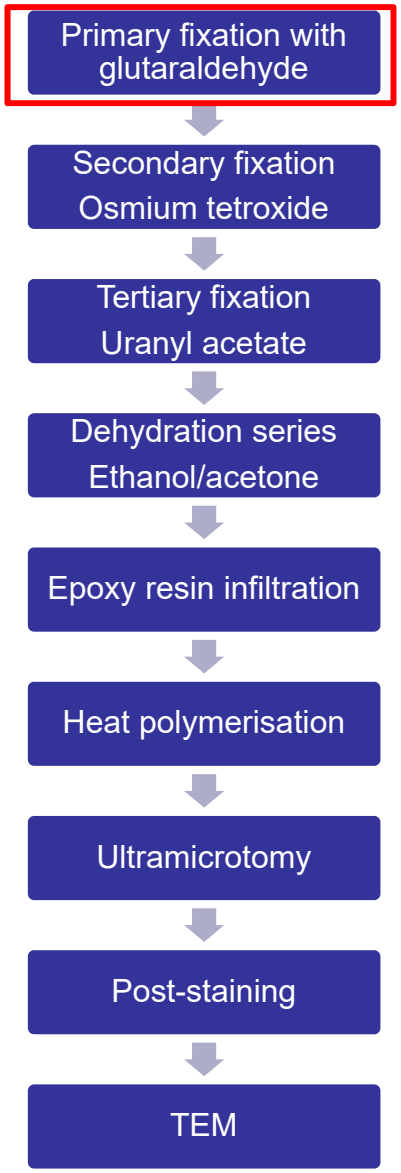
Data analysis: Dependent on number of samples

Factors affecting prep quality

Type of fixative, mode of fixation, type of heavy metals used, tissue size and density (vibratome sections or 1-2 mm^3 pieces)

Specimen Preparation for TEM

Primary Fixation



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

Standard chemical fixation:

2.5% glutaraldehyde +

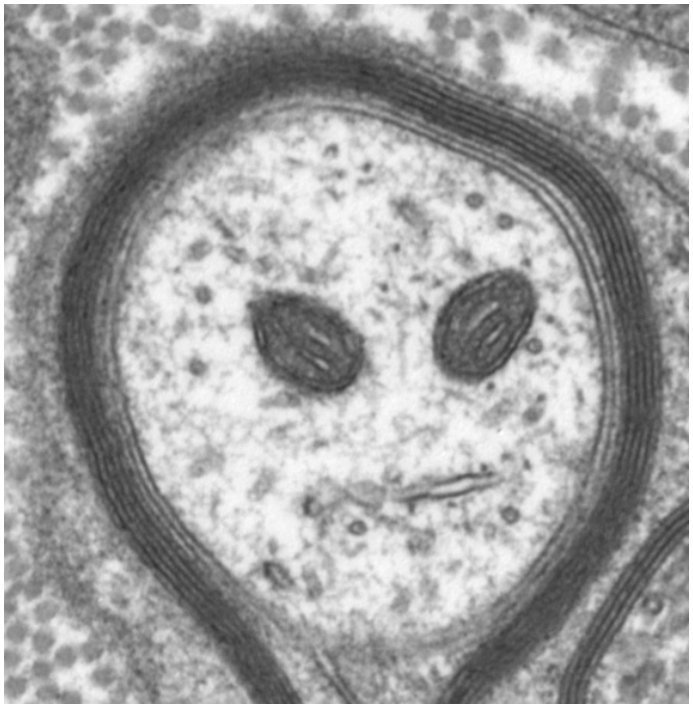
2-4% PFA in 0.1M PIPES or sodium cacodylate buffer

Specimen Preparation for TEM

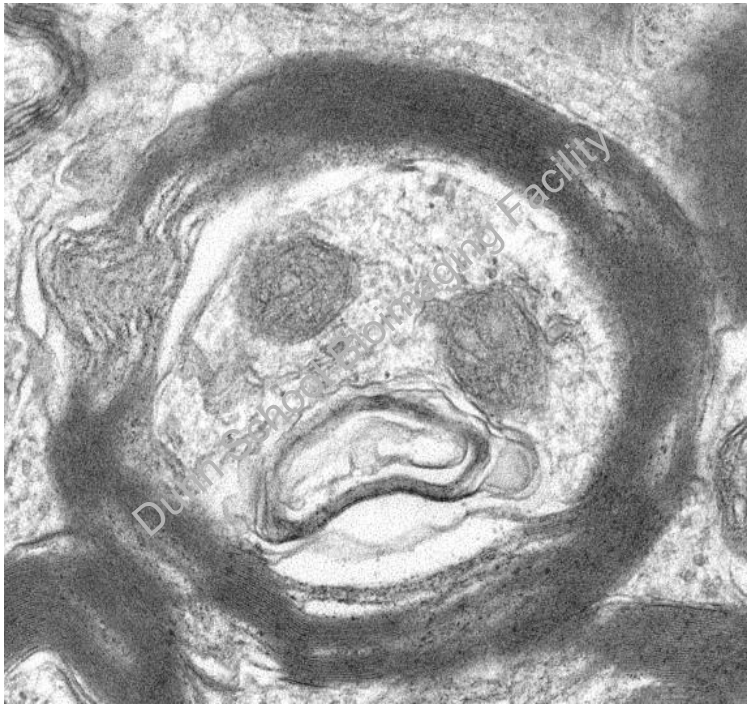
Chemical fixation

- Primary fixation with glutaraldehyde
- Secondary fixation Osmium tetroxide
- Tertiary fixation Uranyl acetate
- Dehydration series Ethanol/acetone
- Epoxy resin infiltration
- Heat polymerisation
- Ultramicrotomy
- Post-staining
- TEM

Good fixation



Bad fixation



Specimen Preparation for TEM

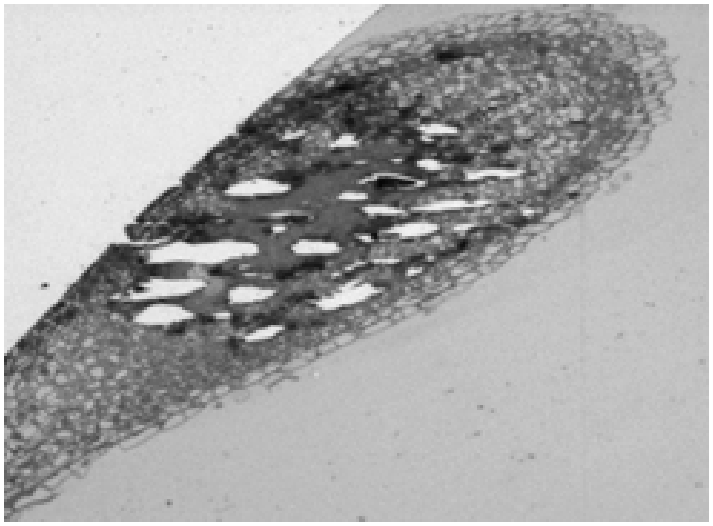
Dehydration & resin infiltration

- Primary fixation with glutaraldehyde
- Secondary fixation Osmium tetroxide
- Tertiary fixation Uranyl acetate
- Dehydration series Ethanol/acetone
- Epoxy resin infiltration
- Heat polymerisation
- Ultramicrotomy
- Post-staining
- TEM

- Dehydration is the process of gradually replacing water in the sample with a solvent (usually acetone or ethanol).
- The solvent is then gradually replaced with resin. This process can be lengthy and depends on both the sample and type of resin used. Dense nerve tissue can be problematic.

Resin blocks

Poor resin infiltration



Specimen Preparation for TEM

Cryo-fixation and freeze substitution

Cryo-fixation
High pressure freezing

↓

Freeze substitution
Osmium tetroxide + Uranyl acetate in acetone

↓

Resin infiltration

↓

UV/heat polymerisation

↓

Ultramicrotomy

↓

Post-staining

↓

TEM

- Tissue can be cryo-fixed using LN_2 in the High Pressure Freezer (Leica EM ICE, Leica EM PACT)

Estimated time req.

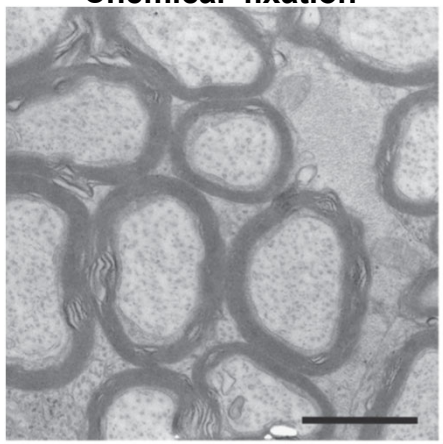
Prep: 1 week

Ultramicrotomy: ~3-9 hrs

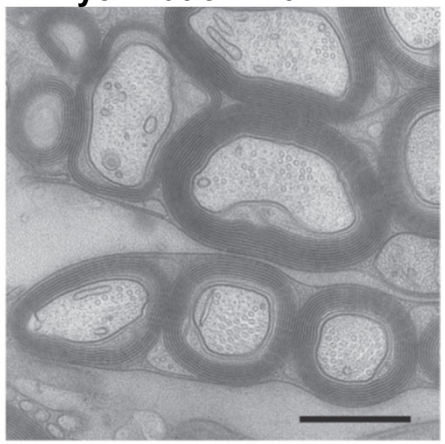
Microscopy: ~3-9 hrs

Data analysis: Variable

Chemical fixation



Cryo-fixation with HPF

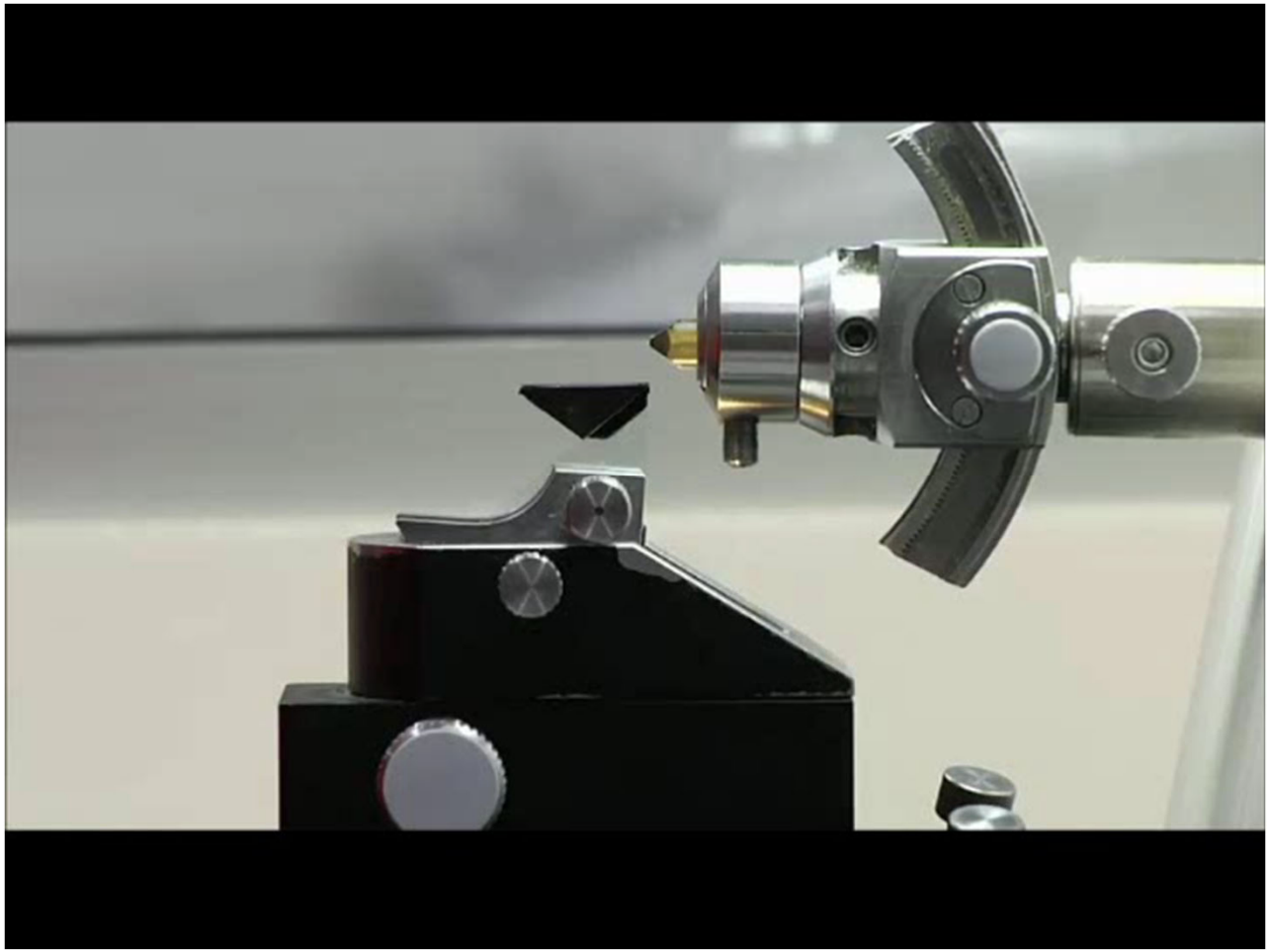


From: Mobius *et al* (2016) *Brain Research* 1641

Specimen Preparation for TEM

Ultramicrotomy

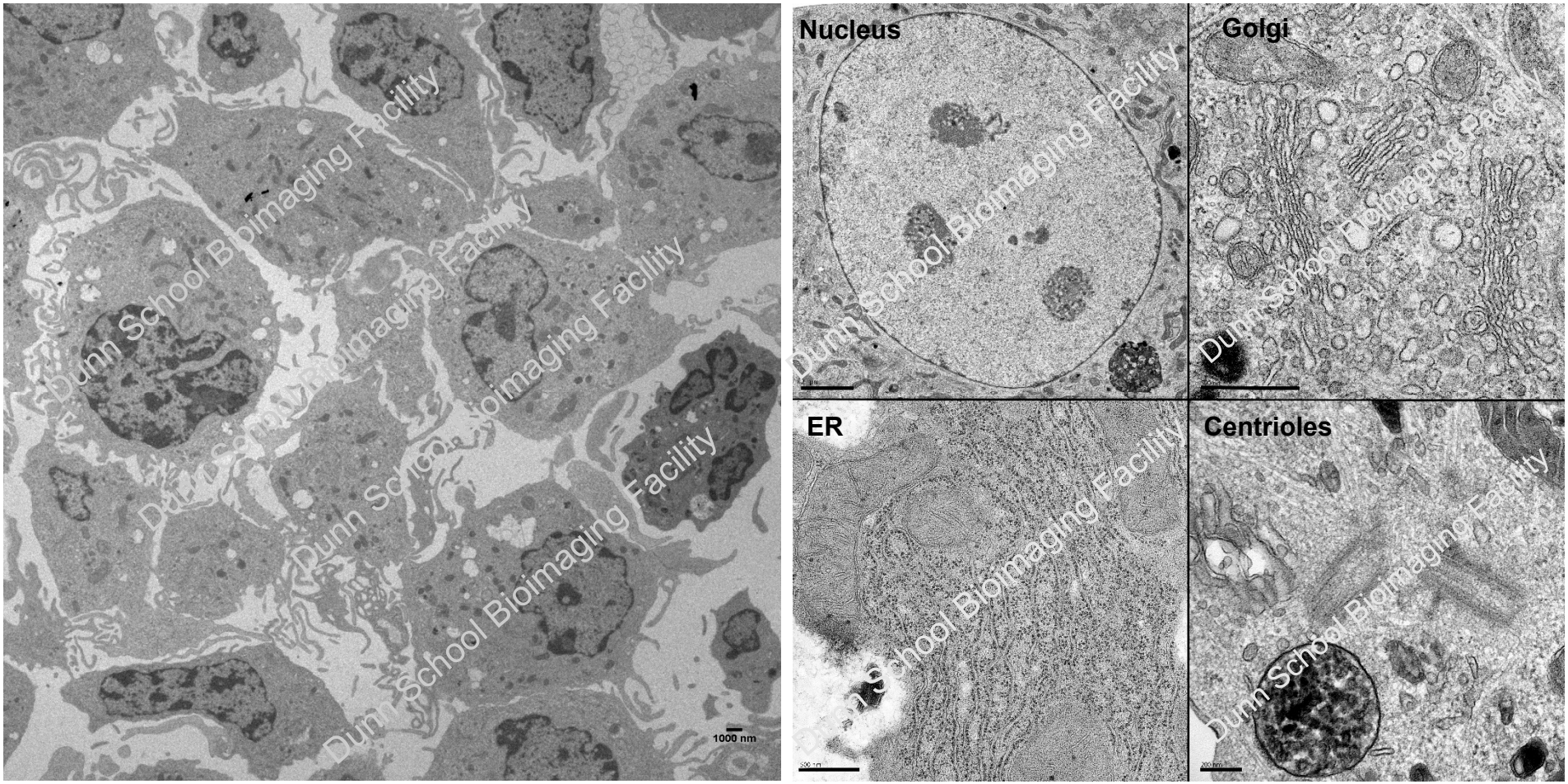
- Primary fixation with glutaraldehyde
- Secondary fixation Osmium tetroxide
- Tertiary fixation Uranyl acetate
- Dehydration series Ethanol/acetone
- Epoxy resin infiltration
- Heat polymerisation
- **Ultramicrotomy**
- Post-staining
- TEM



Introduction to ultramicrotomy video, University of Sydney

TEM Ultrastructure

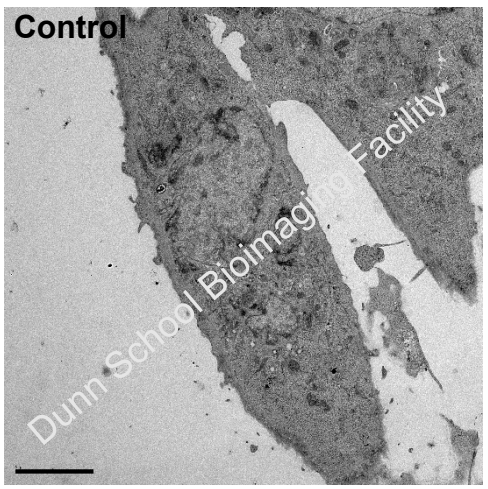
Cells

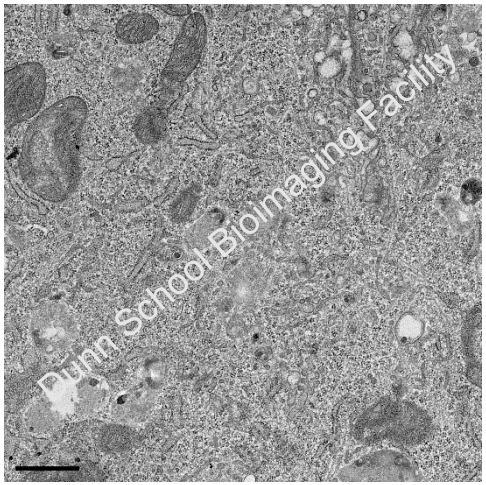


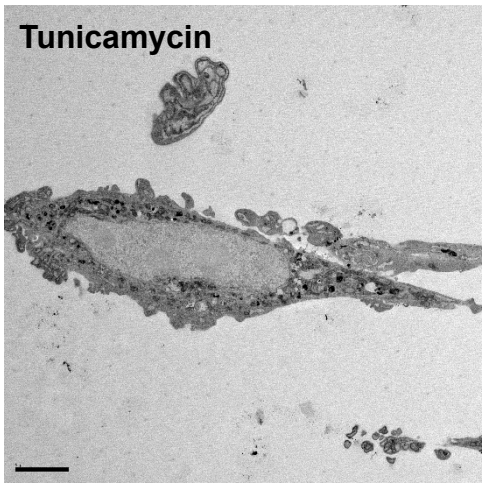
Mammalian culture cells (E Johnson)

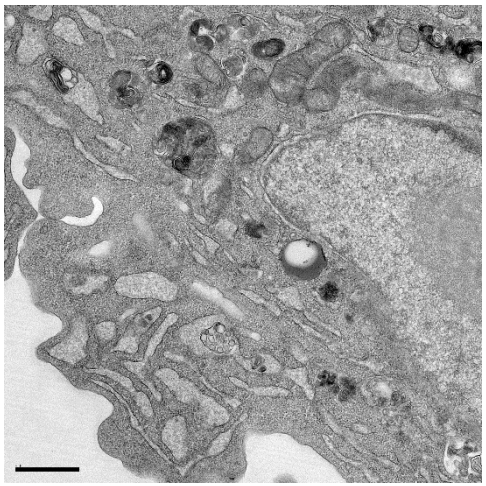
TEM Ultrastructure

Cells

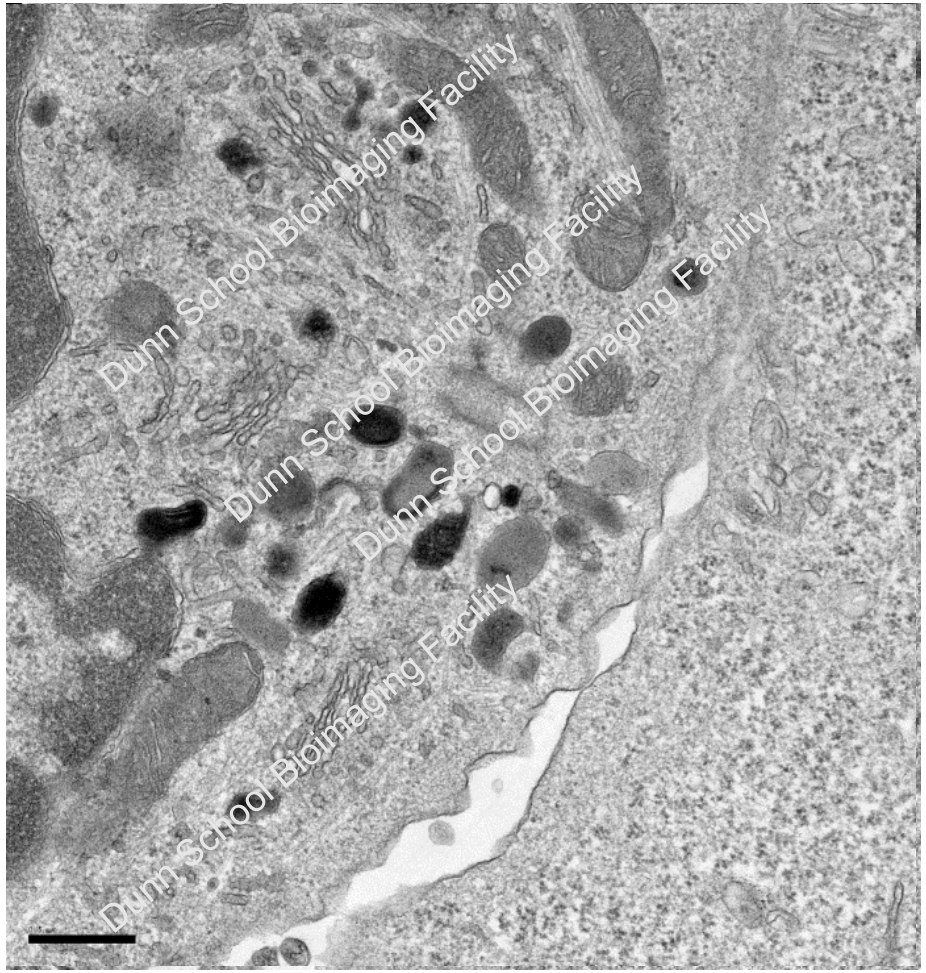








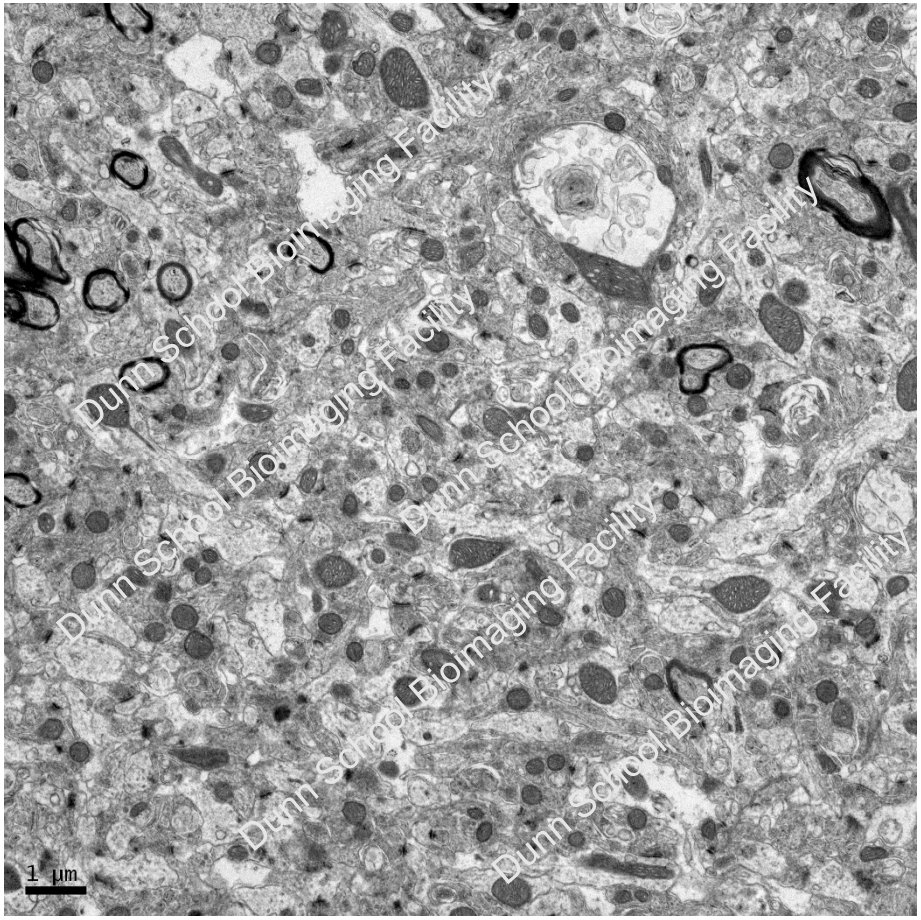
Mouse fibroblasts controls (top) and treated with tunicamycin (bottom)
E Johnson/V Liebe



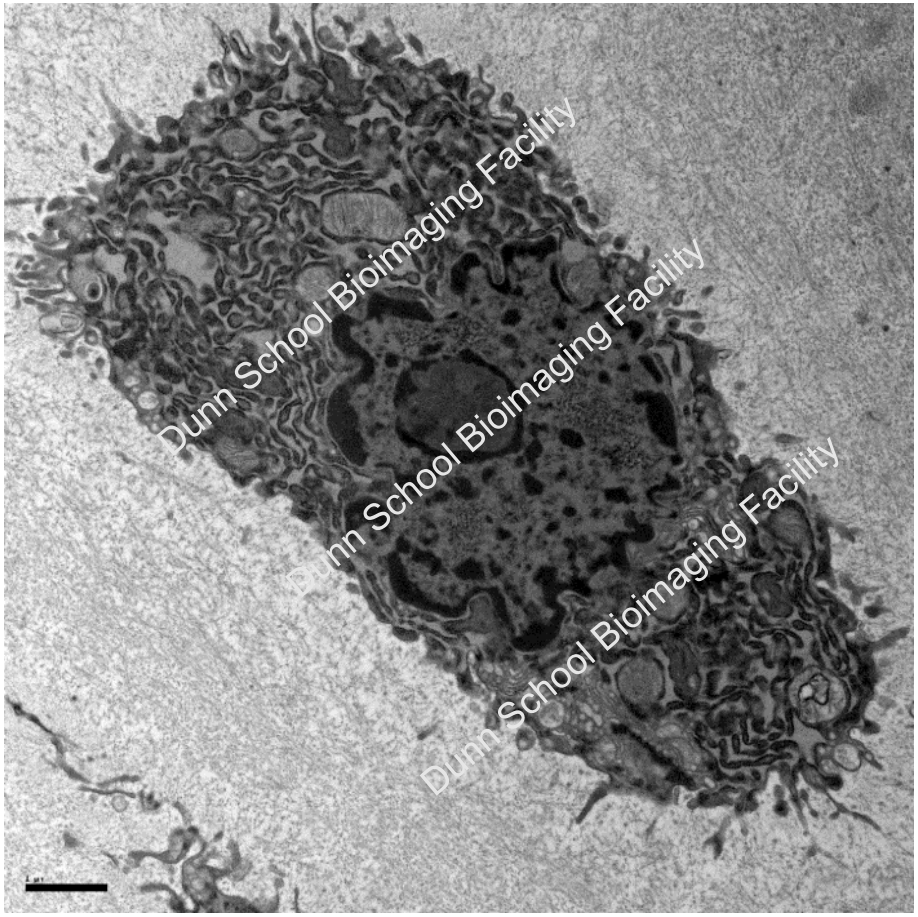
HRP labelled T-cell interacting with a melanoma cell
E Johnson/G Bossi

TEM Ultrastructure

Tissue



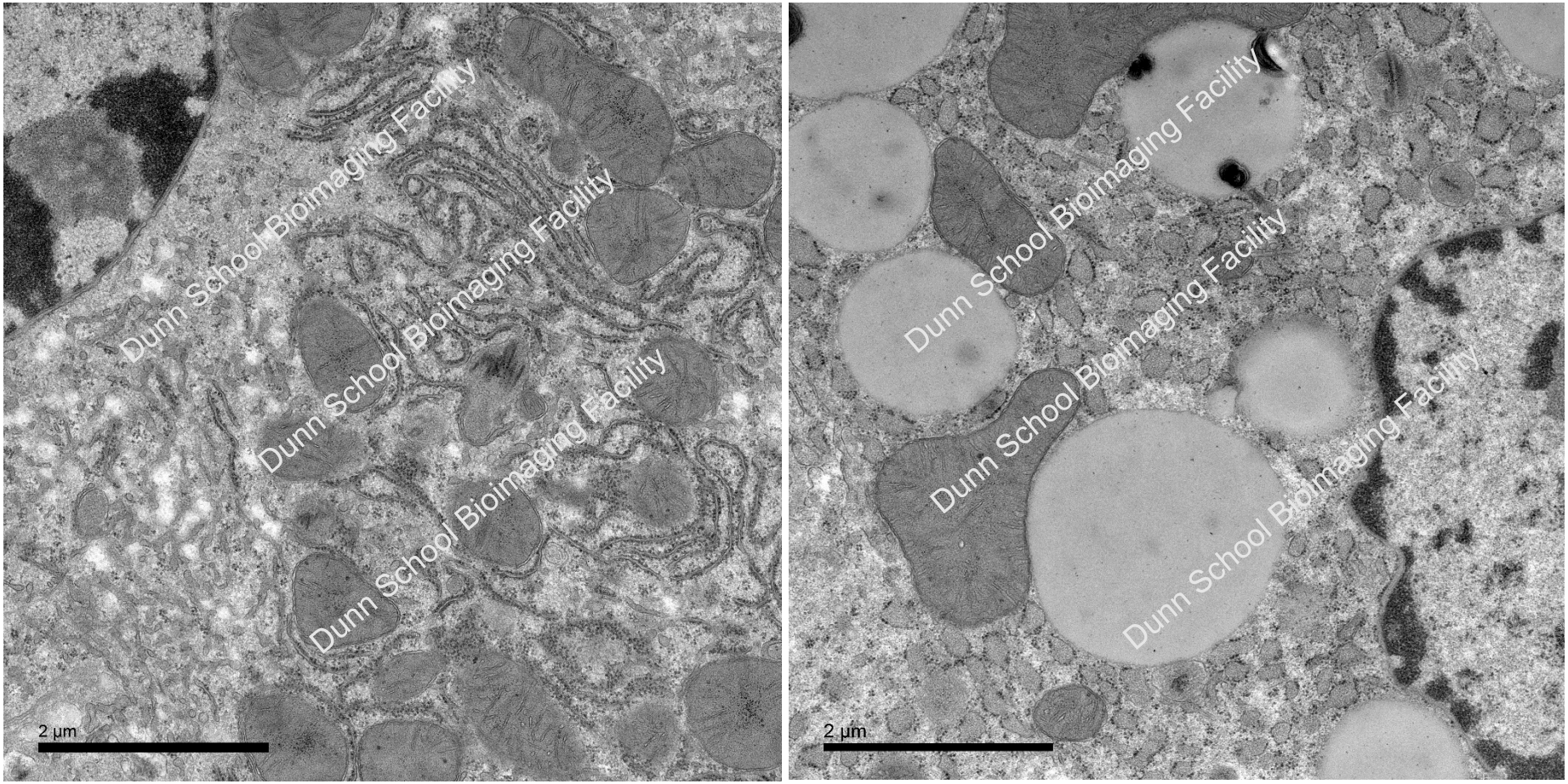
Mouse brain tissue
Tecnai12 TEM, E Johnson



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

TEM Ultrastructure

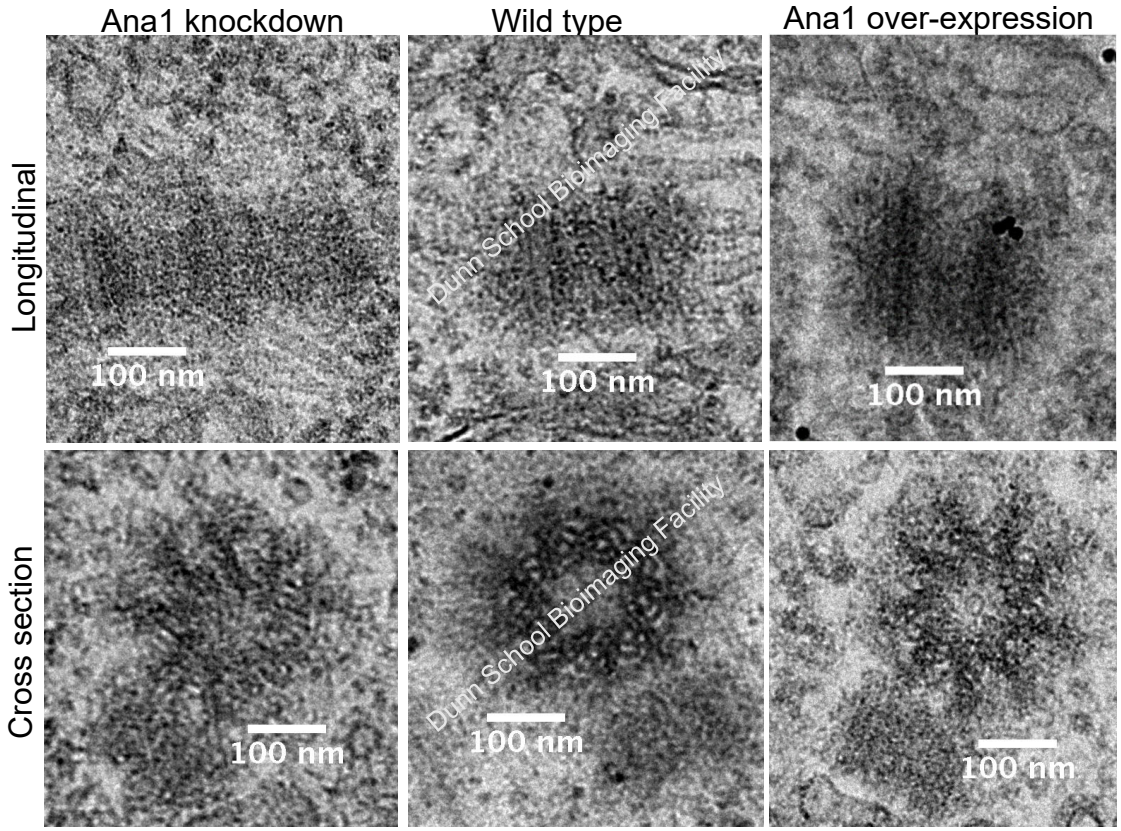
Tissue

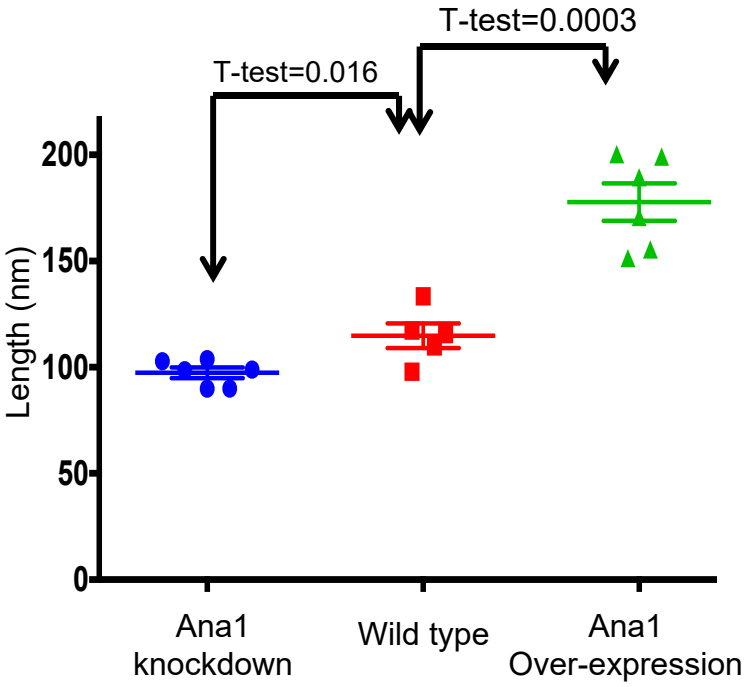


Mouse liver tissue, untreated (left) and under ER stress (right) (V Liebe & E Johnson)

TEM Ultrastructure

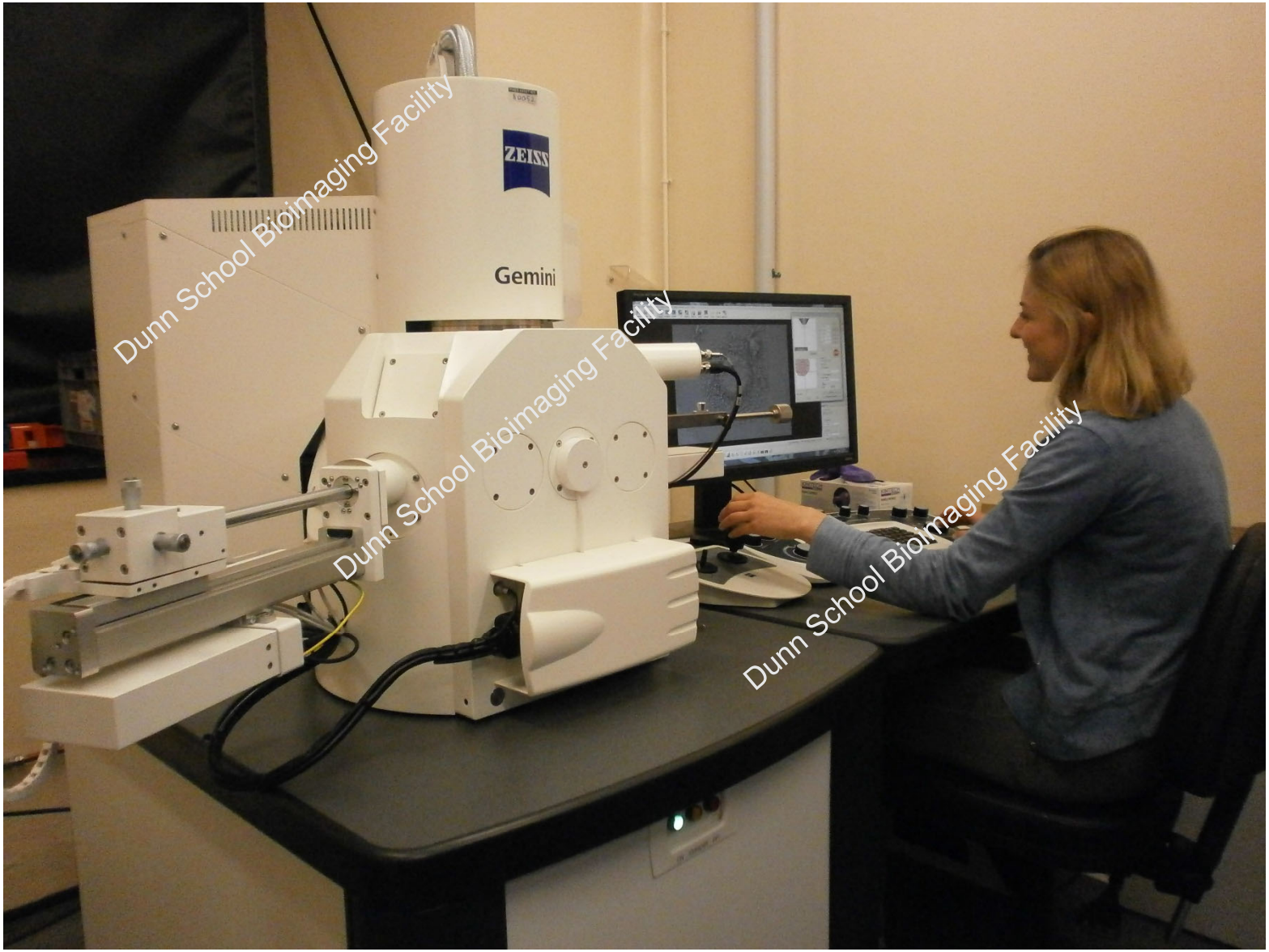
Quantification





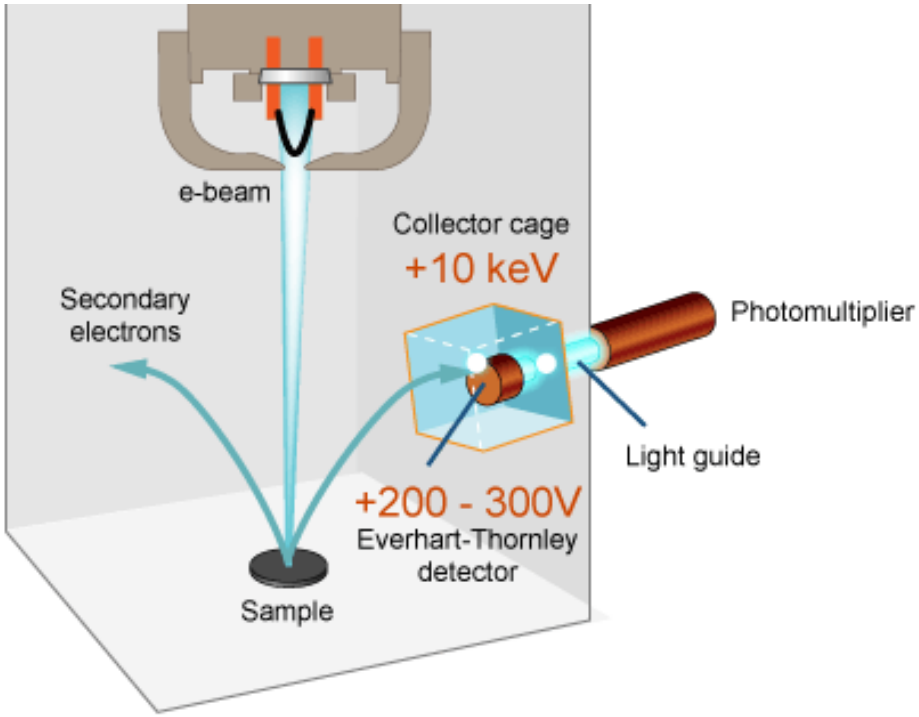
TEM of Drosophila larval wing discs – slide courtesy of Saroj Saurya, Raff lab, Dunn School

Scanning Electron Microscopy (SEM)

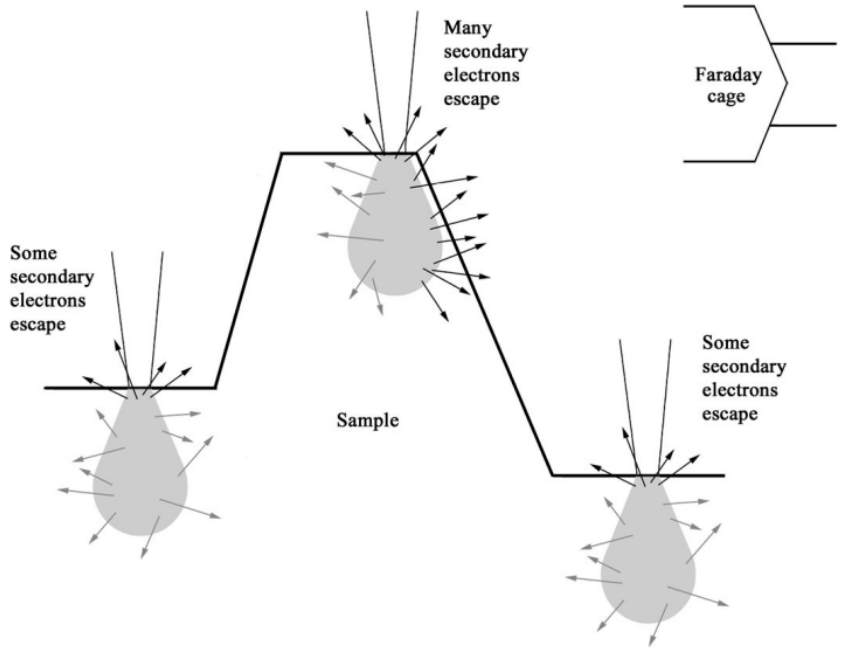


The SEM
Signal detection

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



www.ammrf.org

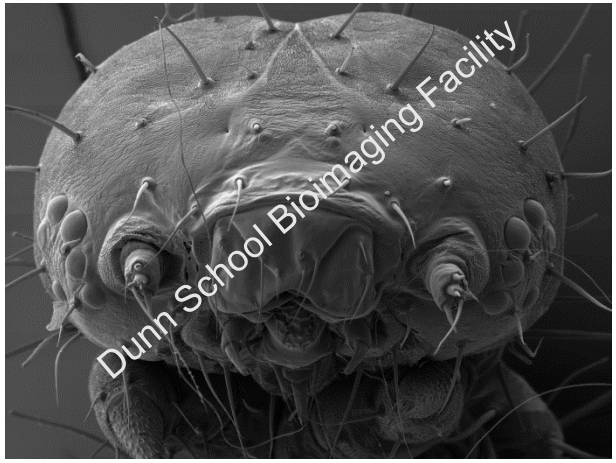


Dept Biological Sciences, Smith College Northampton USA

SEM

Specimen requirements

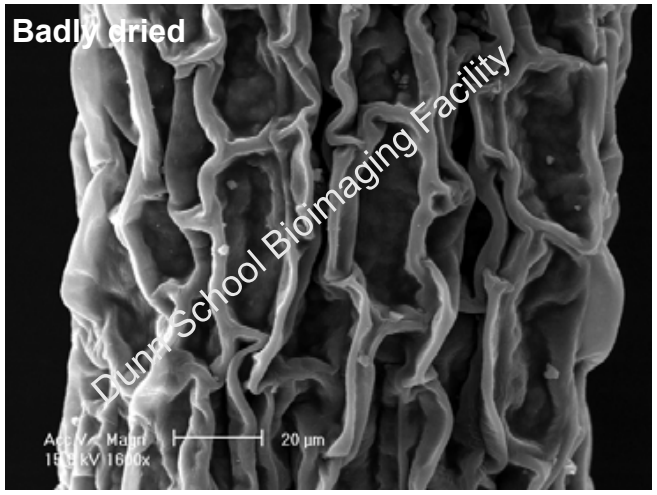
SEM
Stable in the vacuum
Well preserved surface structure
Conductive surface
Whole mount
Particulate samples can be coated and viewed quickly
Cells and whole organisms require some spec prep

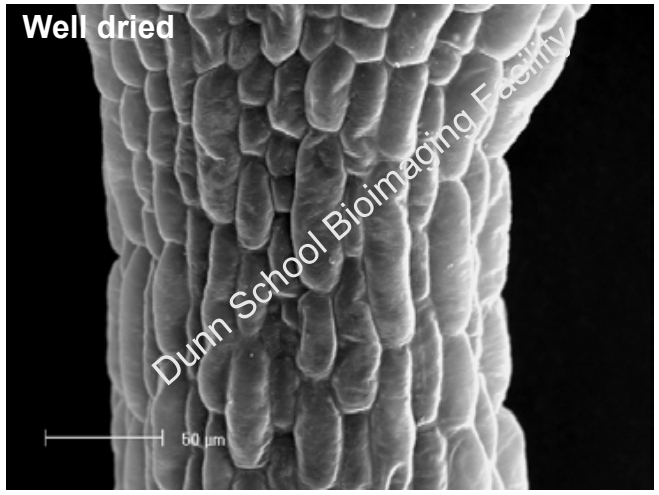


Specimen preparation for SEM

Cells and tissue

- Primary fixation with glutaraldehyde
- Secondary fixation Osmium tetroxide
- Dehydration series Ethanol/acetone
- Dry using HMDS or with the critical point dryer
- Mount & sputter coat
- SEM

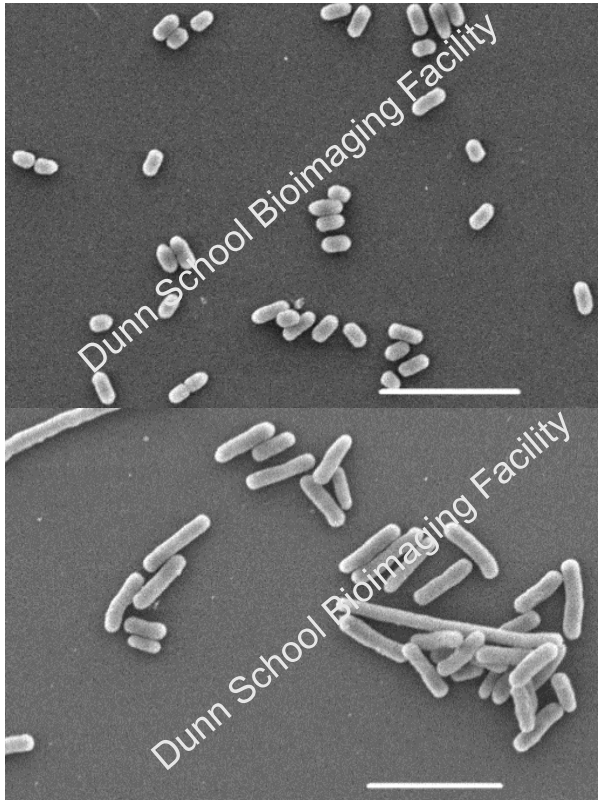




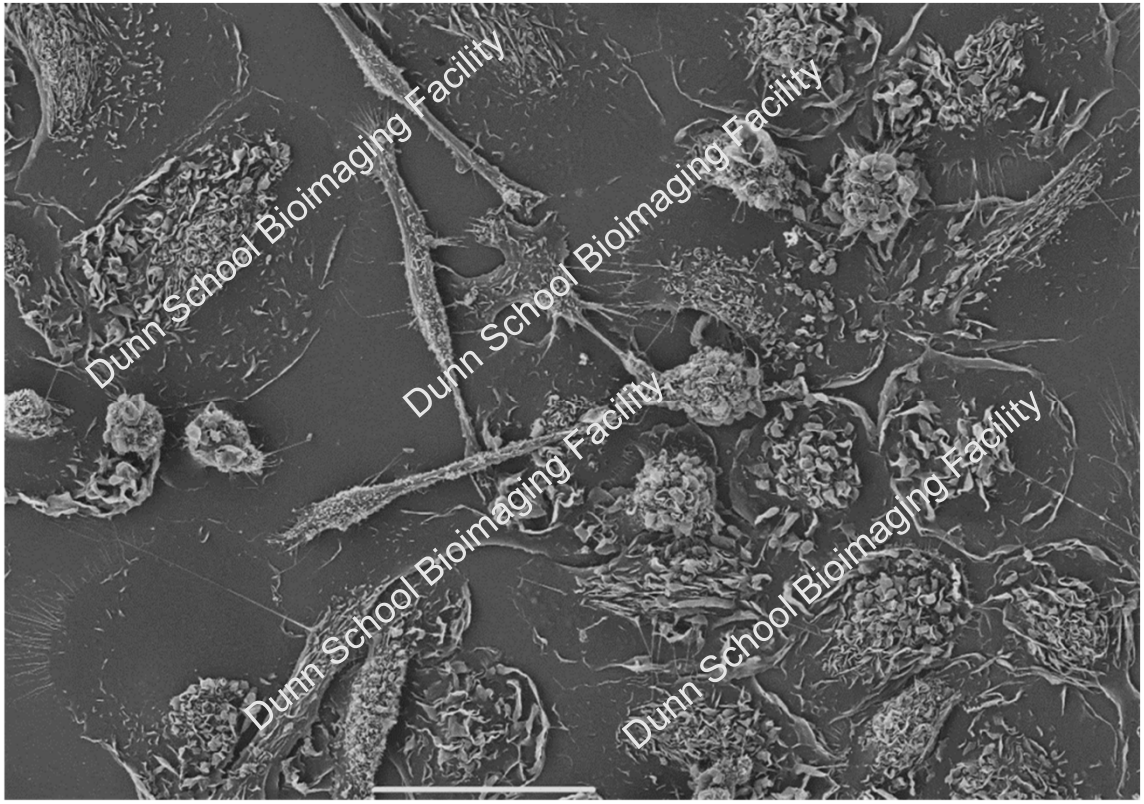
Arabidopsis stem, Phillips XL30 SEM, E Johnson

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

SEM topography
Cells

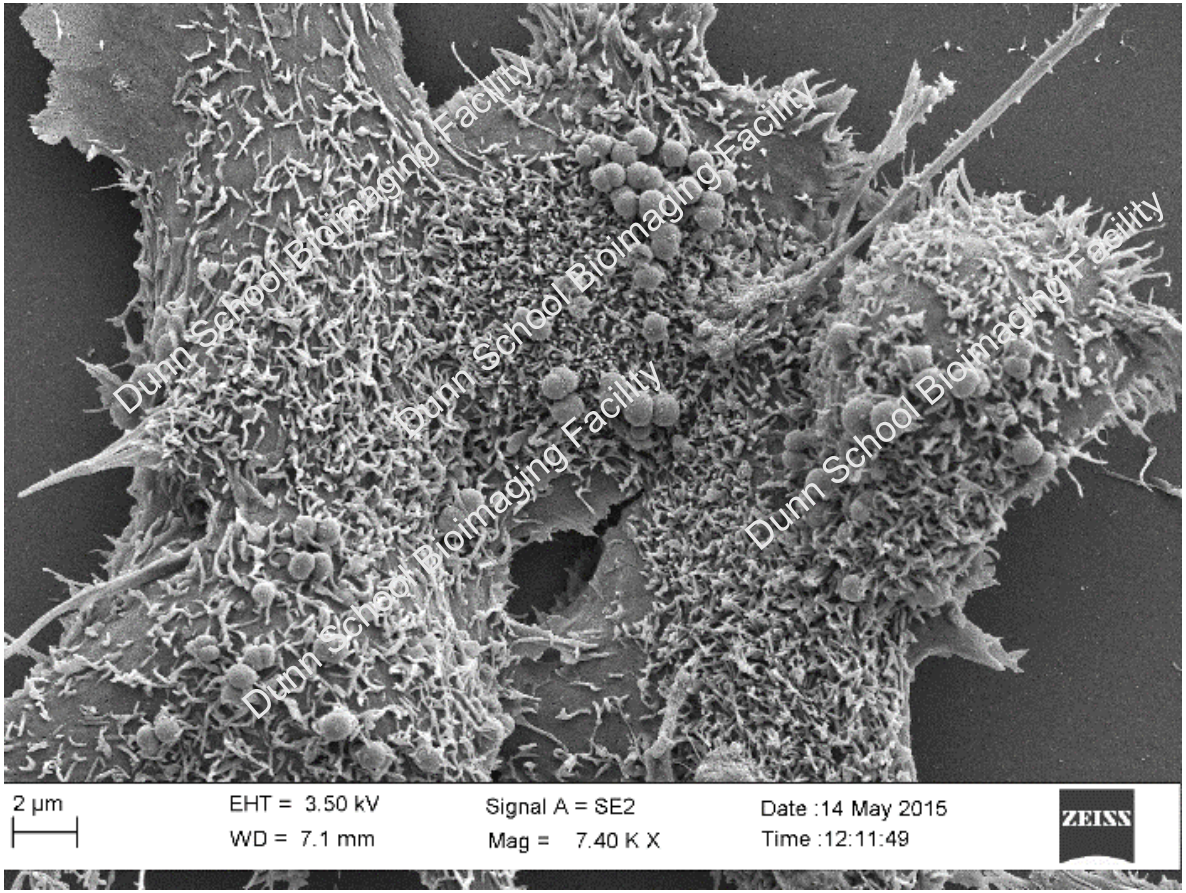


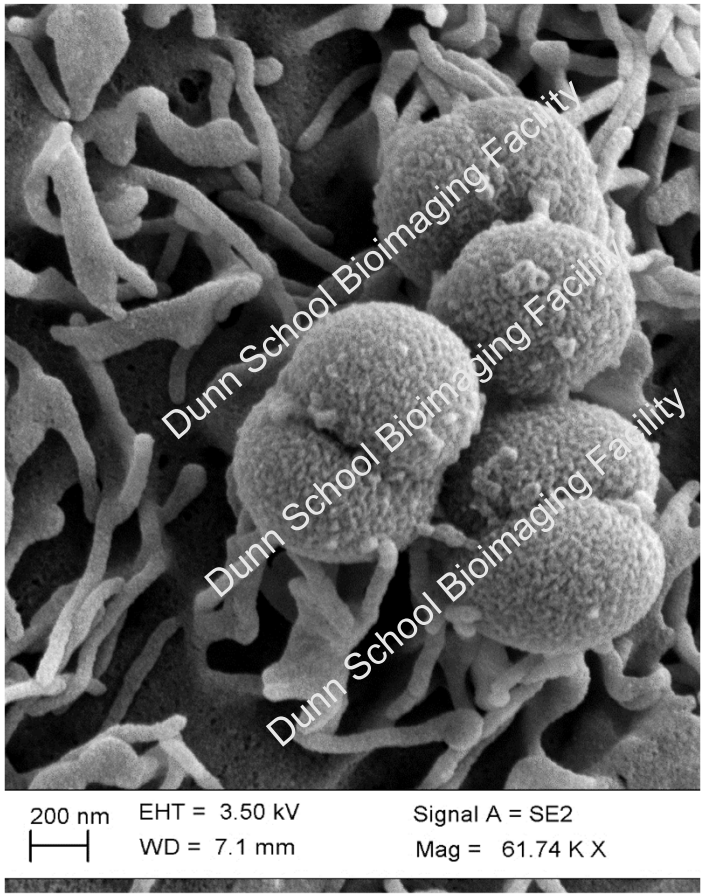
E coli (WT at top, +vector at bottom)
Scale bar 5 µm (R Harding/E Johnson)



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

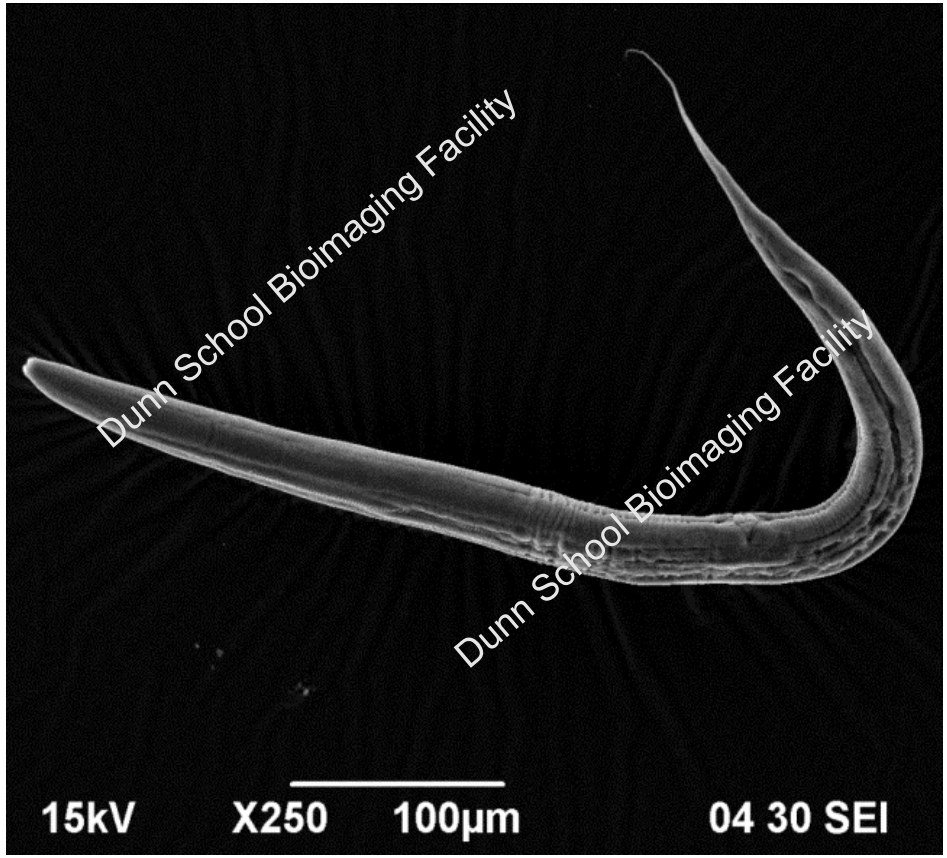
SEM topography
Cells





Neisseria sp. on epithelial cells (R Exley/EJohnson)

SEM topography
Organisms

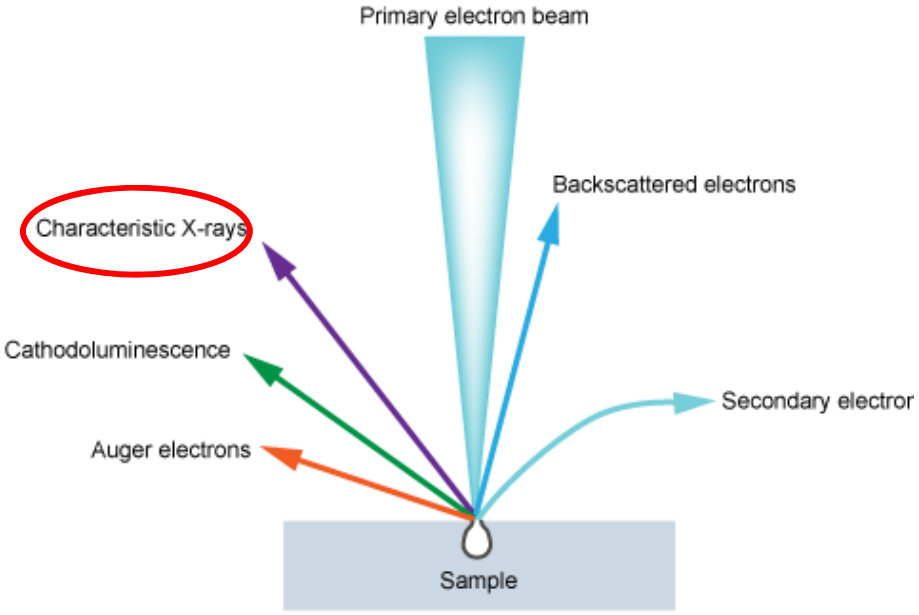


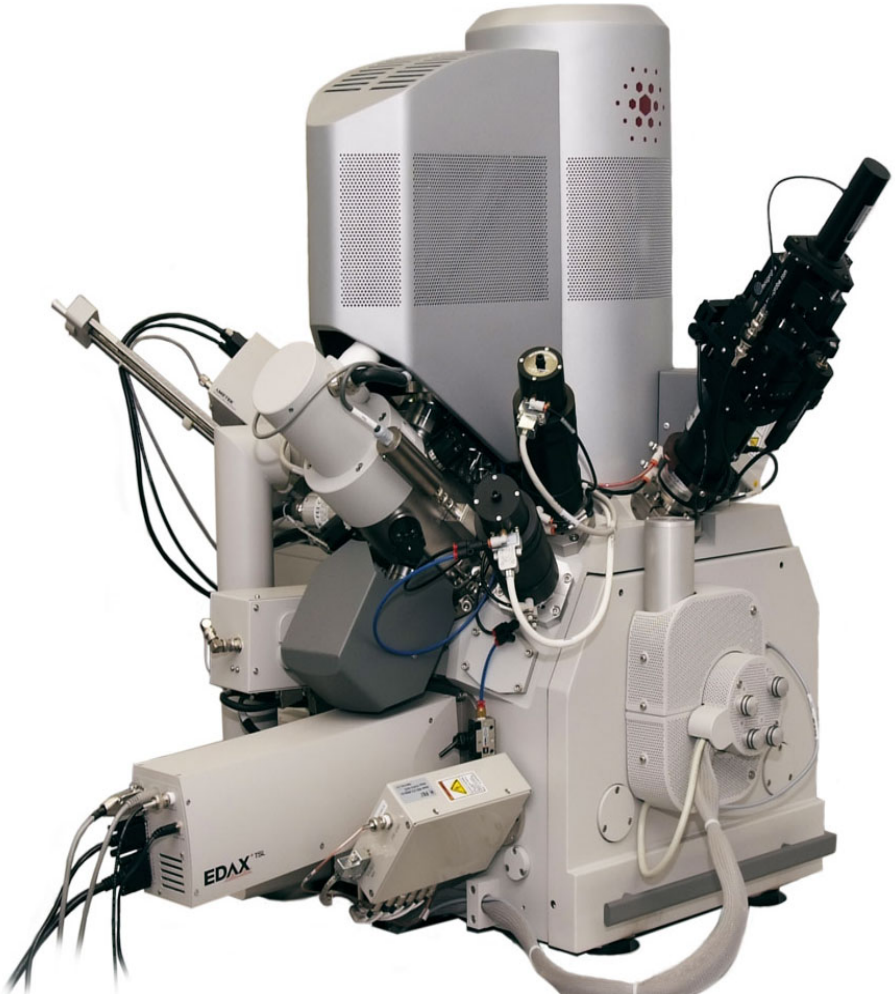
C. elegans
(E Johnson/A Moloney, Dunn School)

Drosophila rough eye phenotype
(M Elschami, NDCN)

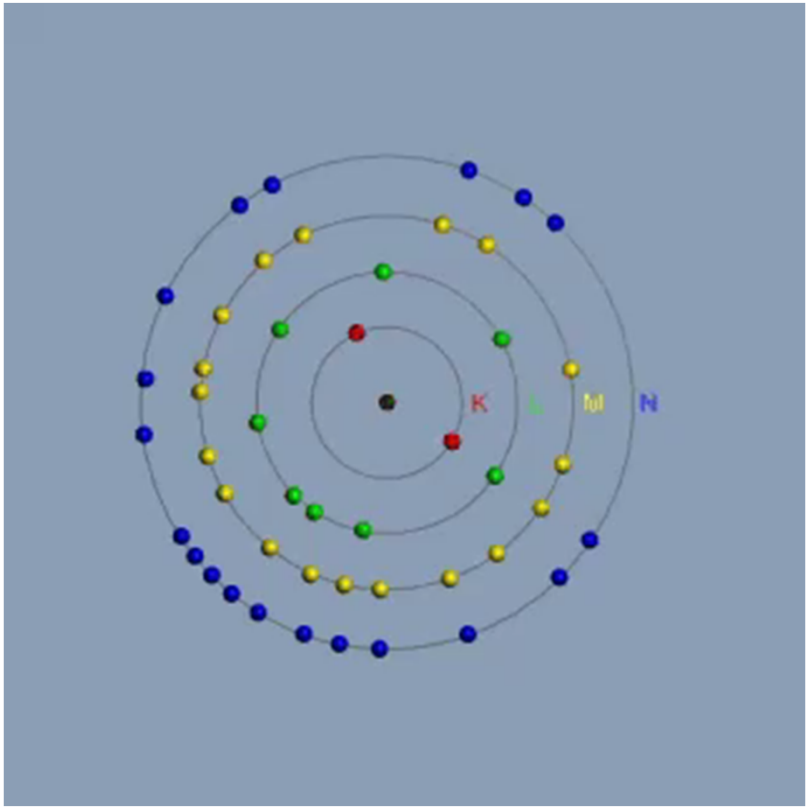
The SEM

Diverse imaging capabilities





Elemental mapping in the SEM
Energy Dispersive X-ray Spectroscopy (EDS)



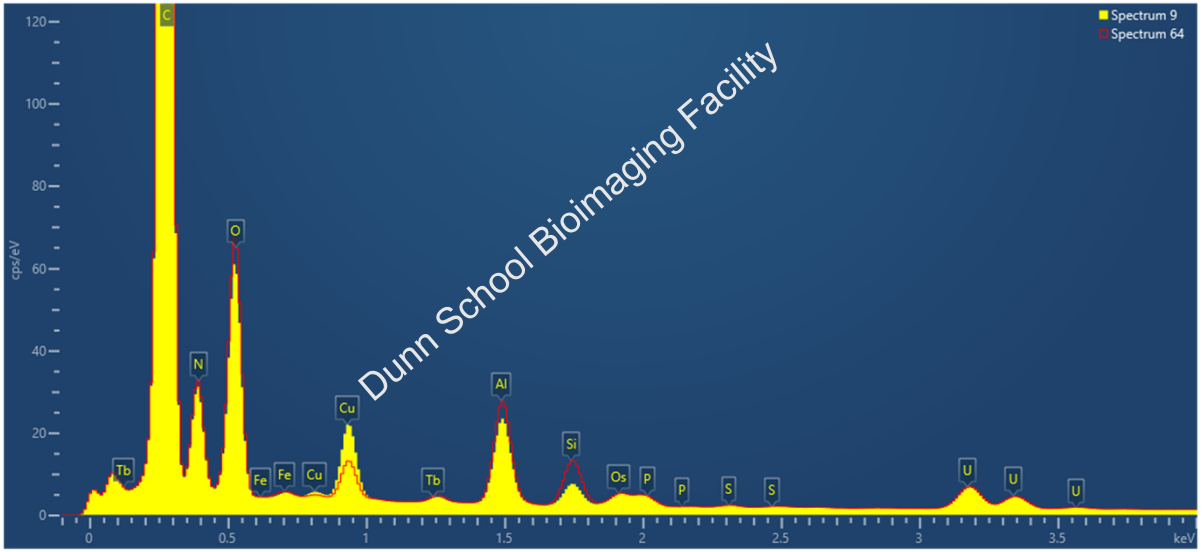
Slide modified from P Trimby, Oxford Instruments

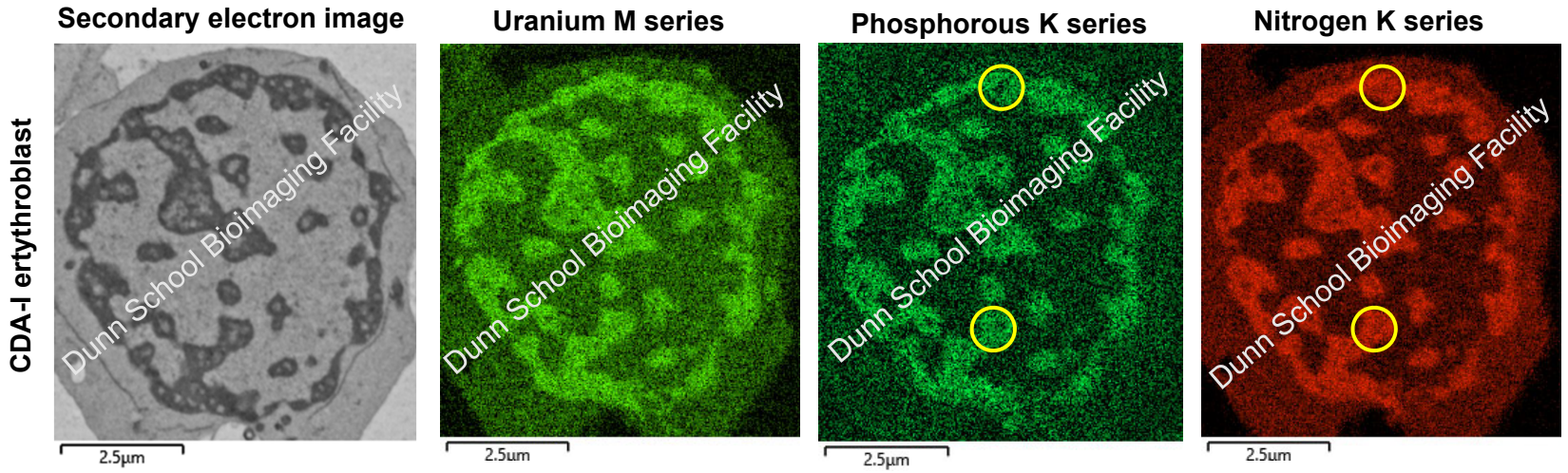
1. Interaction between a high energy beam electron and an atom on the sample's surface
2. Inner orbital electron ejected: ionisation
3. Vacancy filled by outer orbital electron
4. Release of energy as X-ray photon
5. An EDS detector is then used to measure the energy of the X-ray photon which is specific to the atom and therefore identify it

This enables the elements in the sample to be mapped and quantified

Elemental mapping in the SEM

Energy Dispersive X-ray Spectroscopy (EDS)

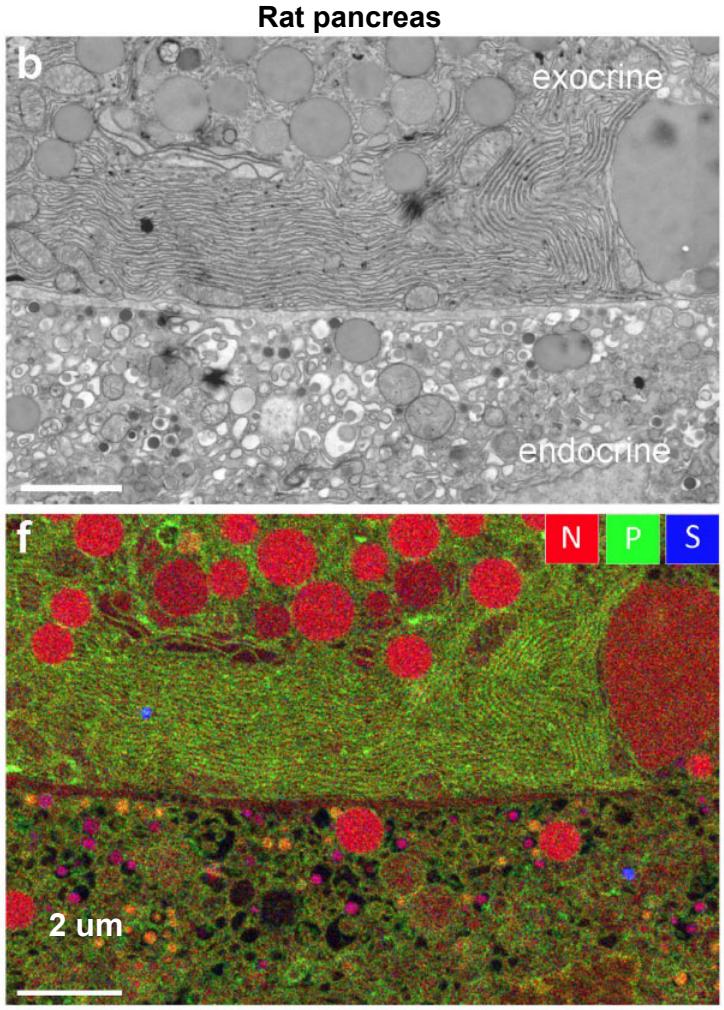




In collaboration with the Buckle Group, WIMM

Elemental mapping in the SEM
Energy Dispersive X-ray Spectroscopy (EDS)

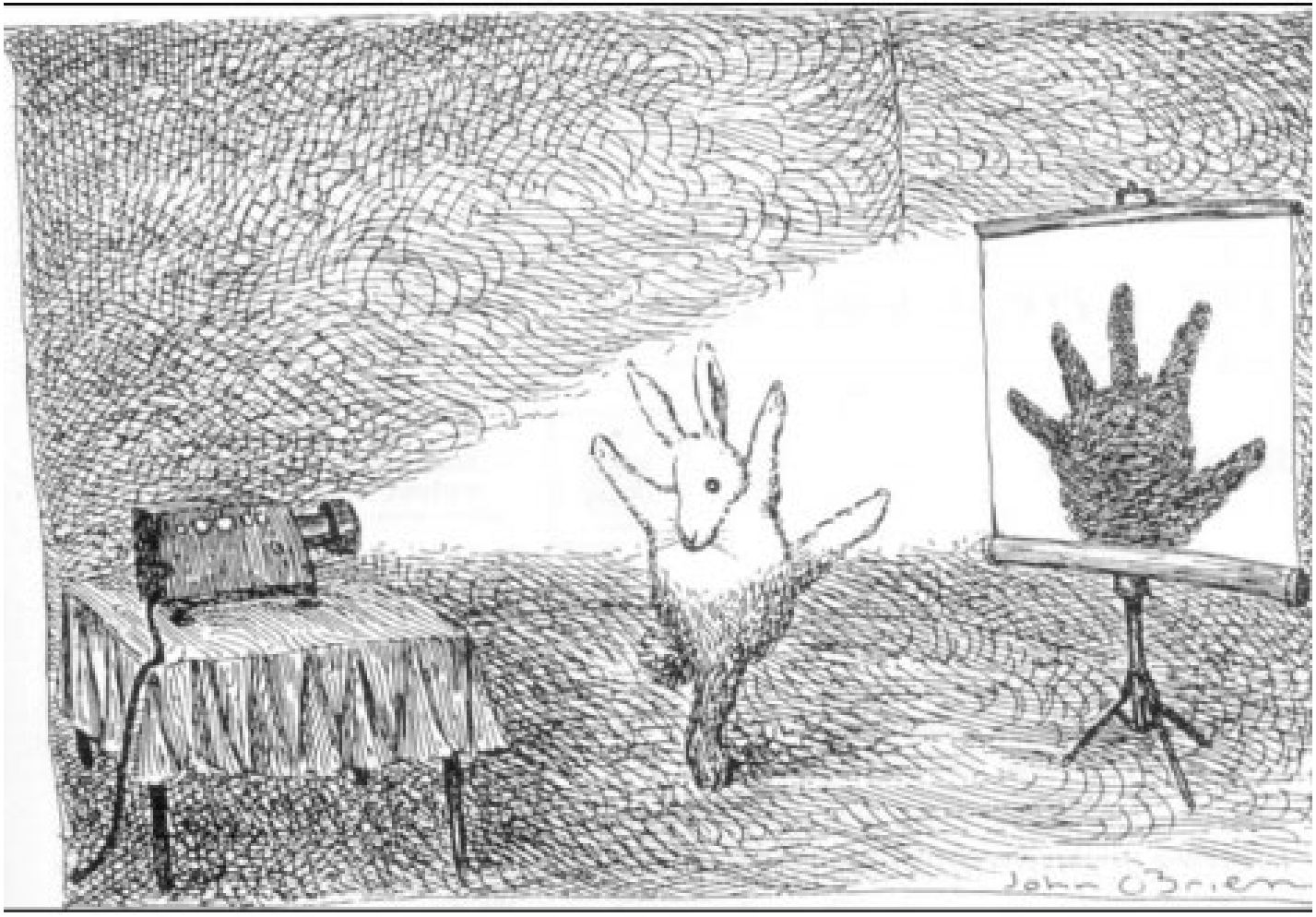
- EDS of ultrathin resin sections from a rat pancreas at the endocrine-exocrine border
- Elemental distributions enable the identification of 3 distinct granules in the endocrine cell:
  - zymogen (N rich: red)
  - glucagen (N+P rich: yellow/orange)
  - insulin (N+S rich: purple)
- Results show potential for elemental fingerprinting of sub-cellular structures
- Quantification is also possible with EDS



From Scotuzzi et al., Nature Scientific Reports 7:45970

Slide modified from P Trimby, Oxford Instruments

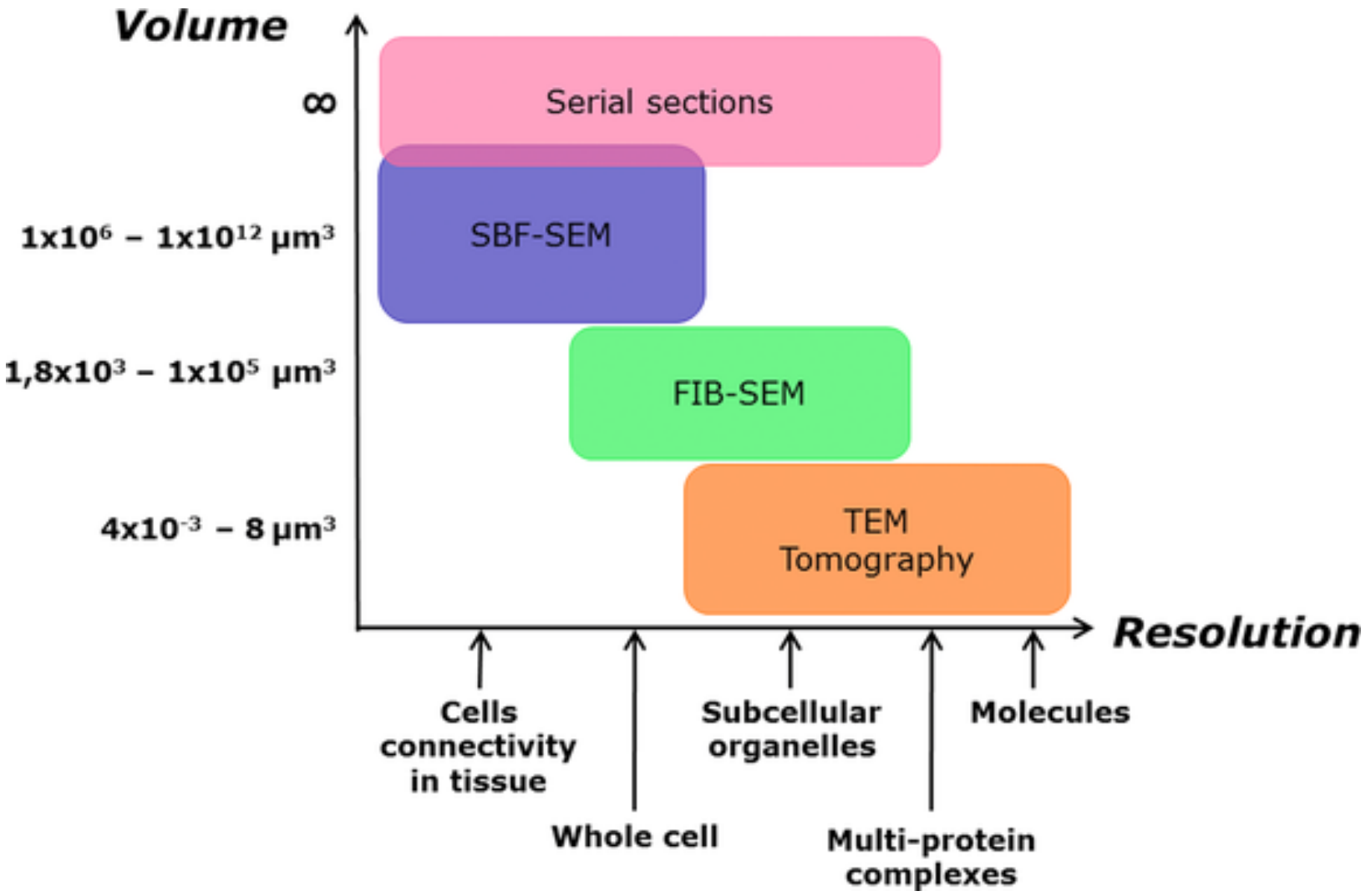
Advanced EM techniques
Volume electron microscopy



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

3D/volume EM techniques

Overview

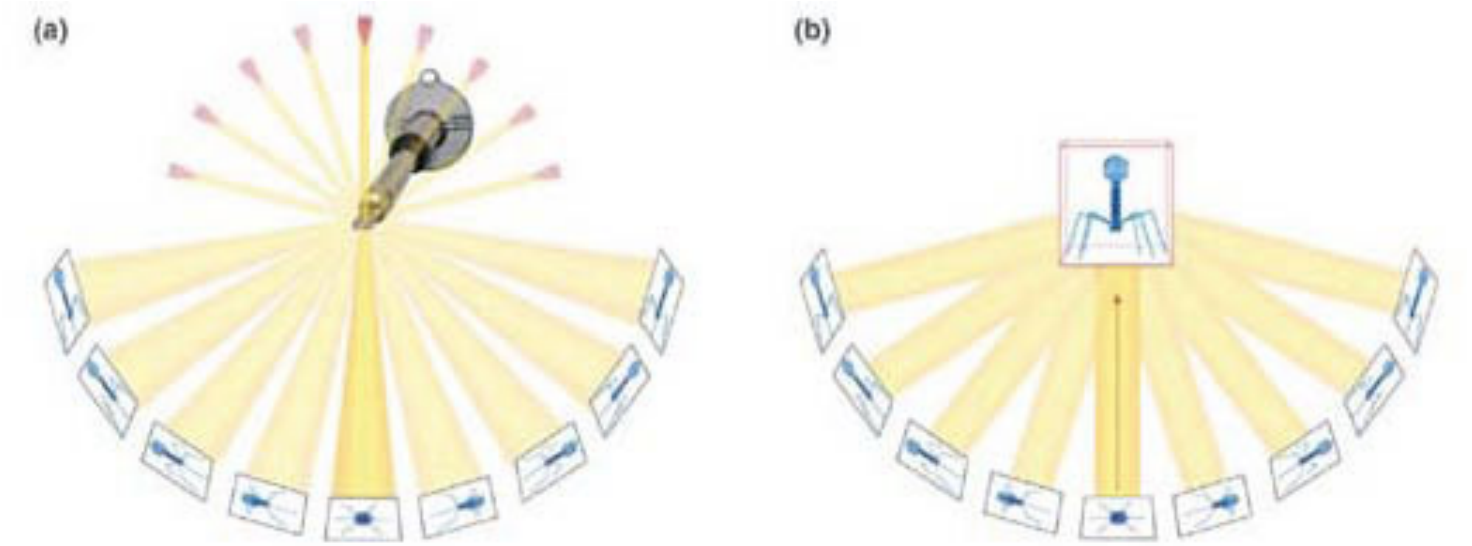


From: Kizilyaprak et al (2014) J Microscopy, 254(3).

3D EM Techniques
TEM - Electron tomography

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

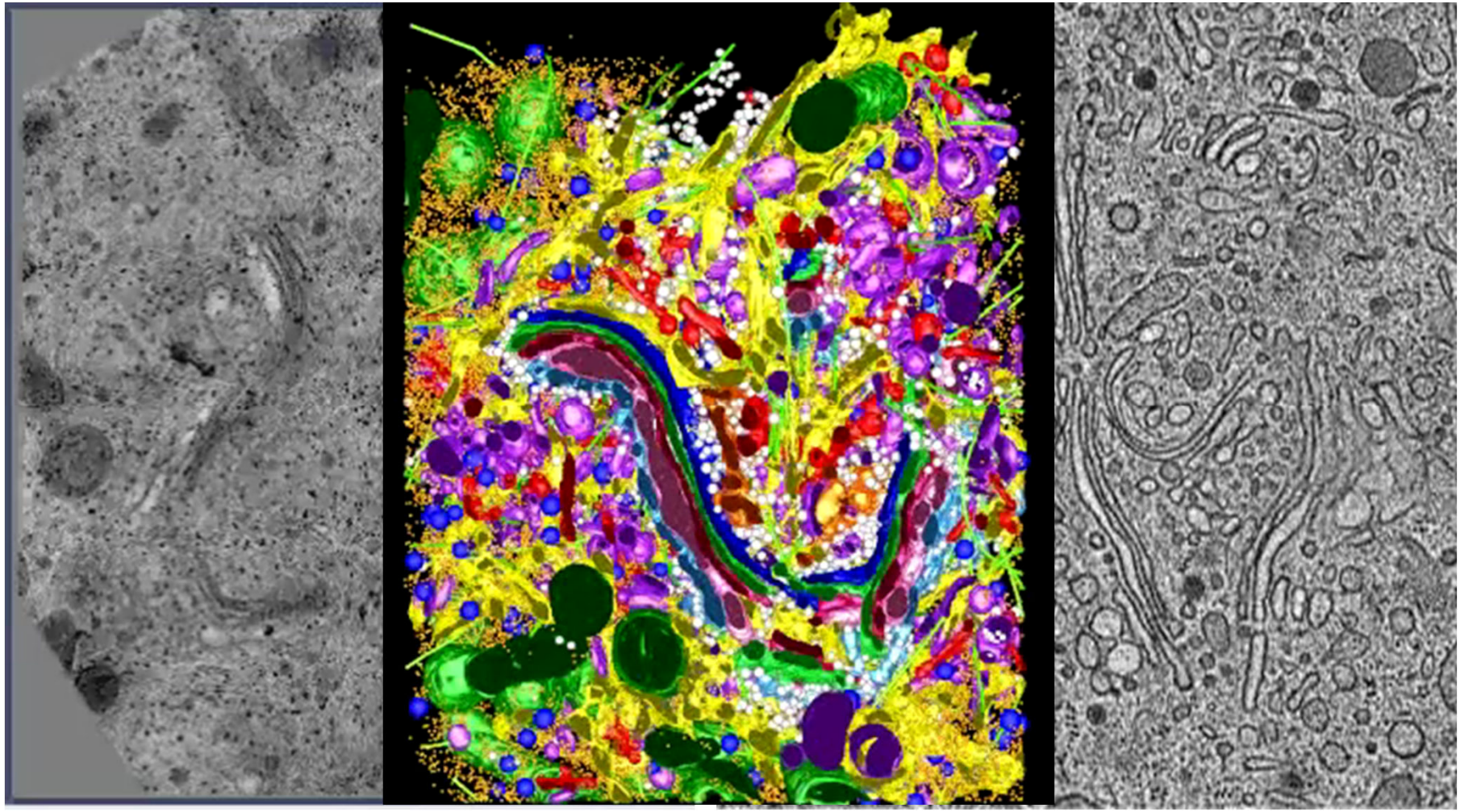
Z resolution: ~2 nm



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].

3D EM Techniques

TEM - Electron tomography



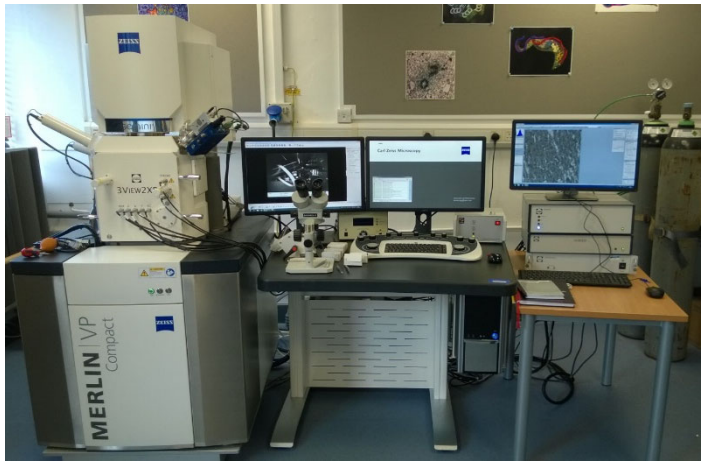
3D ultrastructure in the Golgi region of a pancreatic beta cell line.
Volume: ~3.1 × 3.2 × 1.2 µm, *Marsh et al (2001) PNAS, 98.*

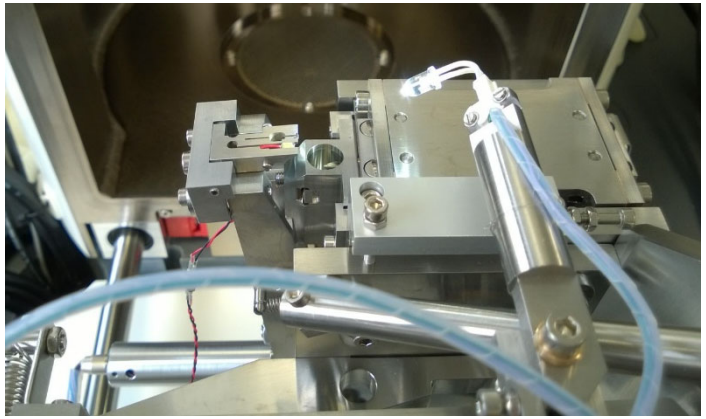
3D EM Techniques

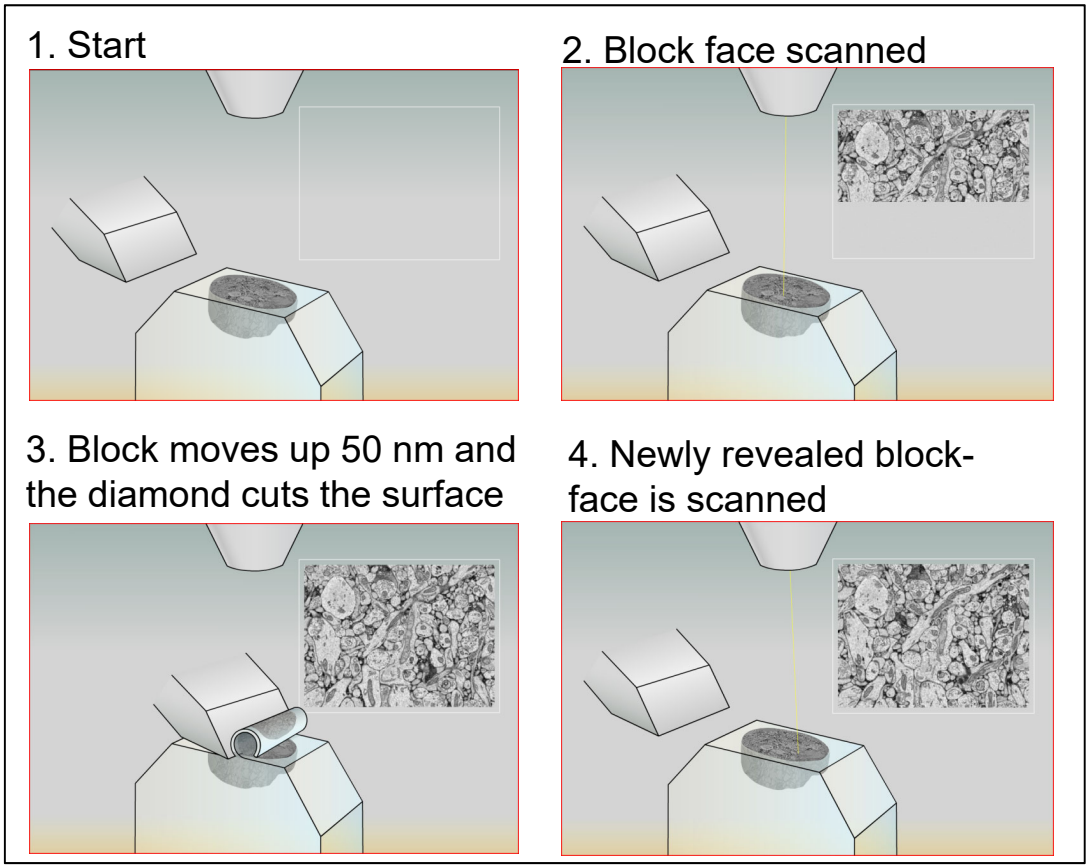
SEM - Serial Block Face Sectioning with Gatan 3View

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

Z resolution: 30-200 nm



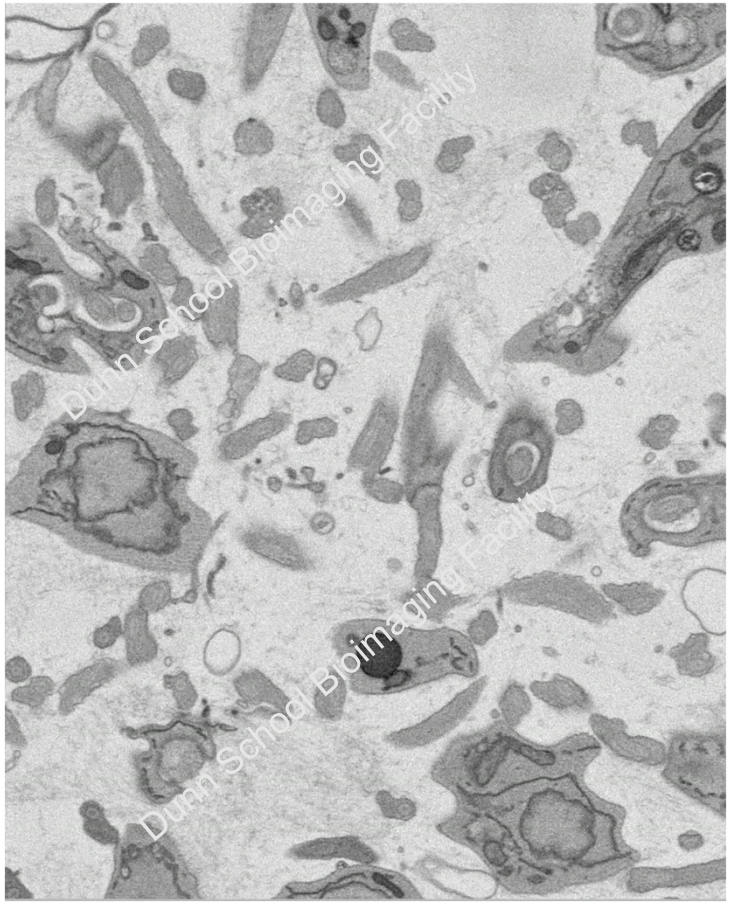


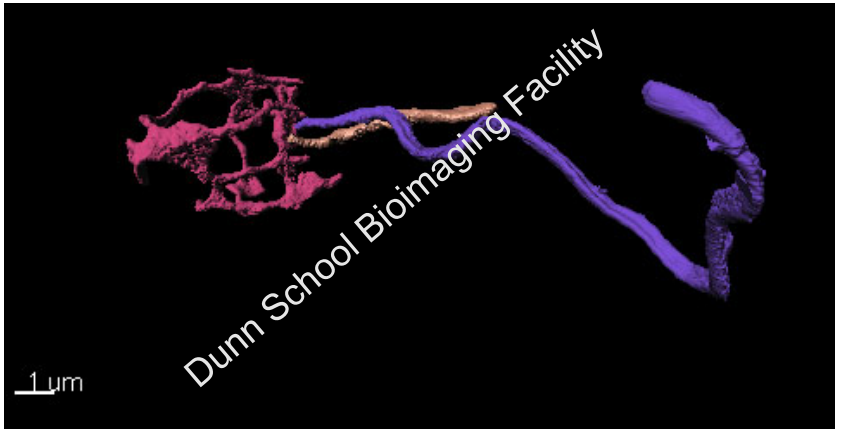


Courtesy Gatan UK

3D EM Techniques

SEM - Serial Block Face Sectioning with Gatan 3View



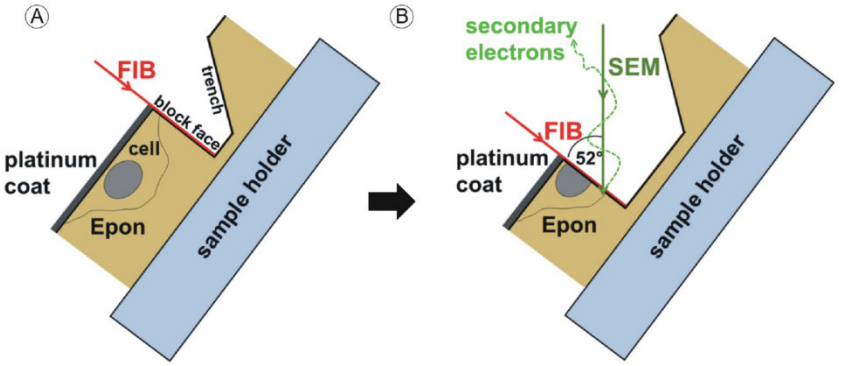


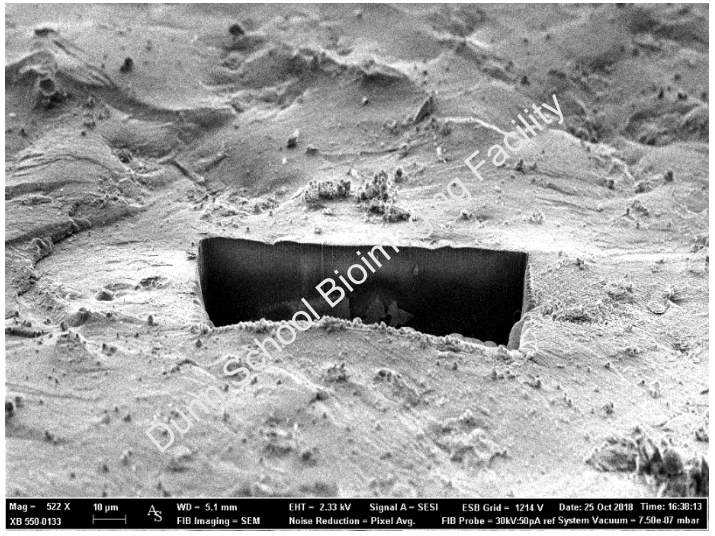
Leishmania Mexicana, promastigote form
Pixel size: 4.6 nm, Slice thickness: 50 nm, Volume: 9.8 µm x 12.2 µm x 16.1 µm, aligned, J Valli & E Johnson

3D EM techniques

SEM - Serial Block Face Sectioning with FIB-SEM

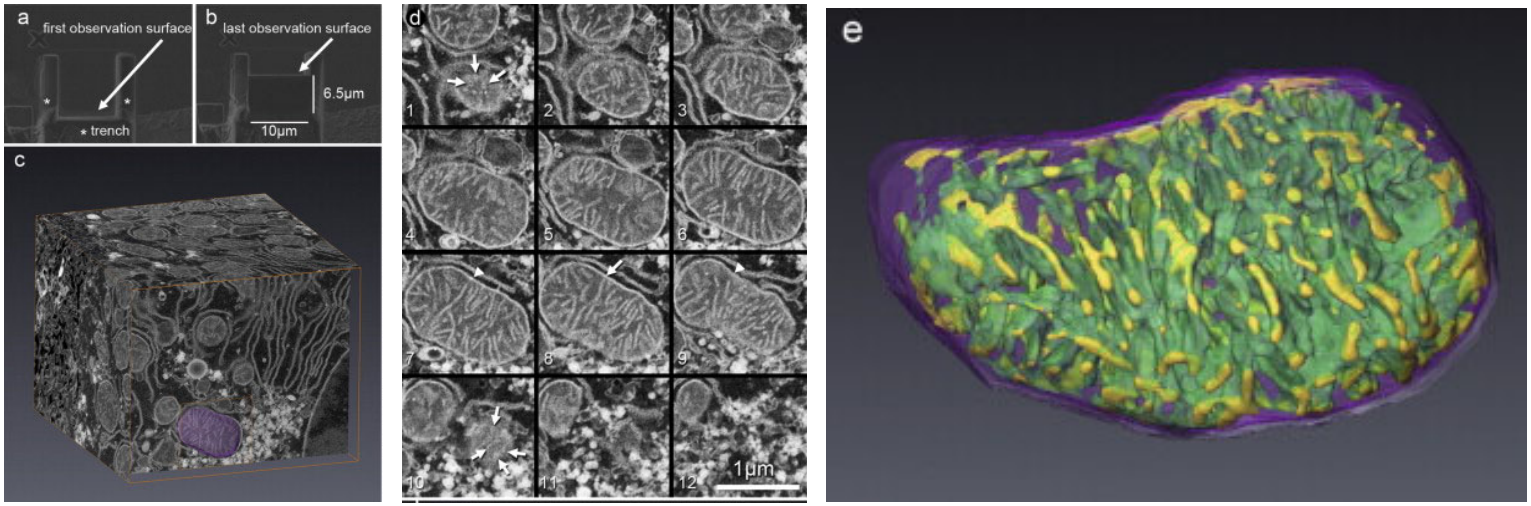
Z resolution: 5-100 nm



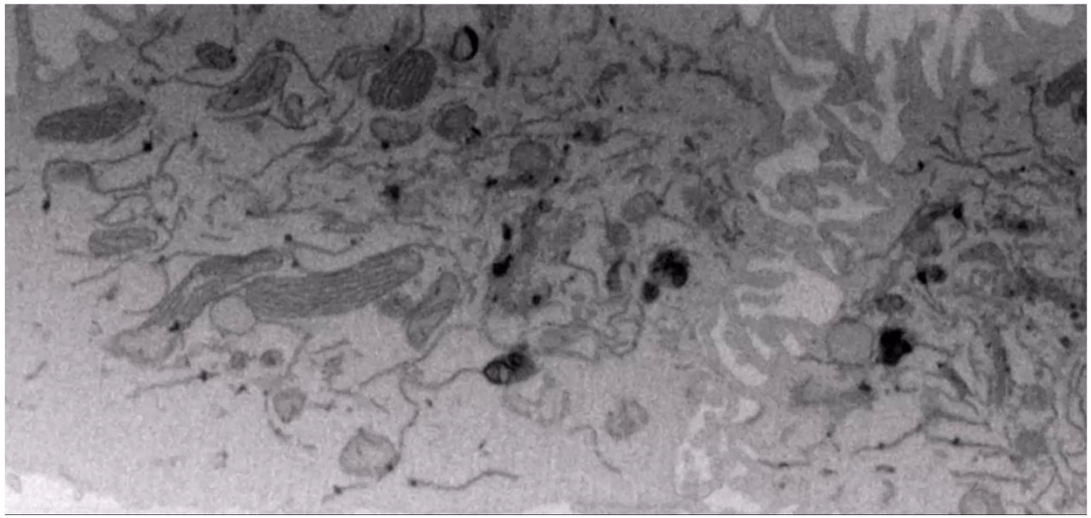


3D EM techniques

SEM - Serial Block Face Sectioning with FIB



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



Top: FIB-SEM of epithelial cells infected with N. cinerea, voxel size = 5x5x15 nm^3, Tang lab/E Johnson

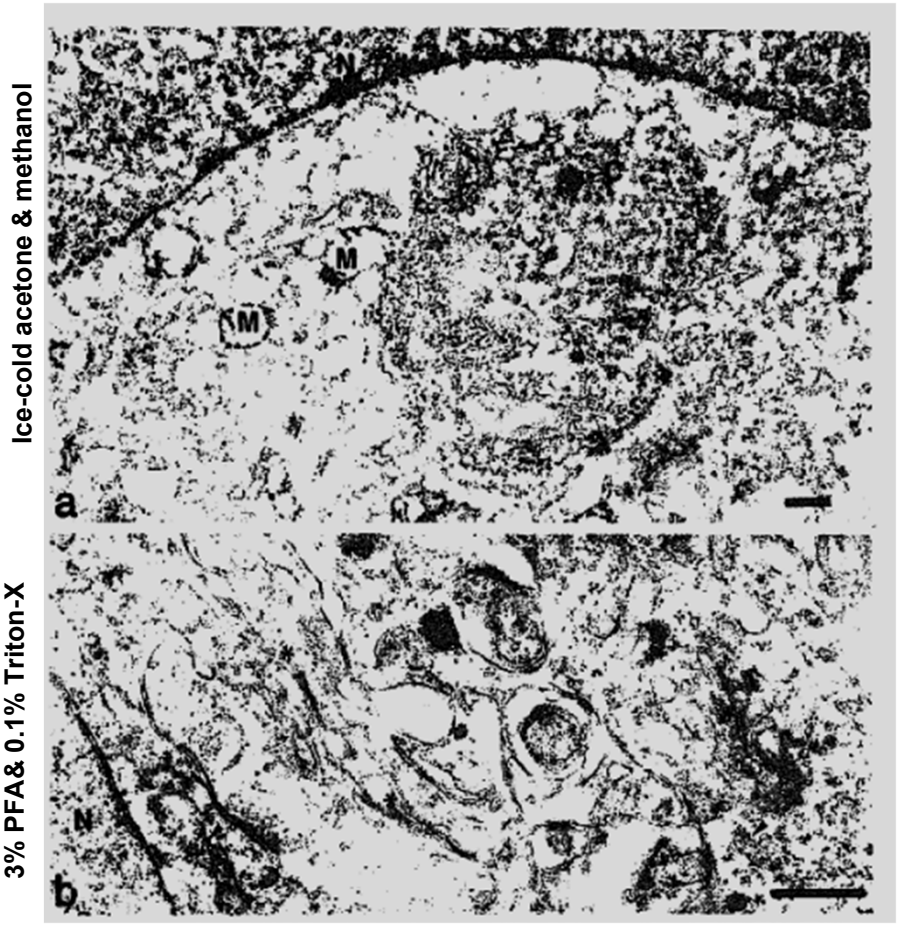
Advanced EM techniques

Protein localisation in cells and tissues

Protein localisation

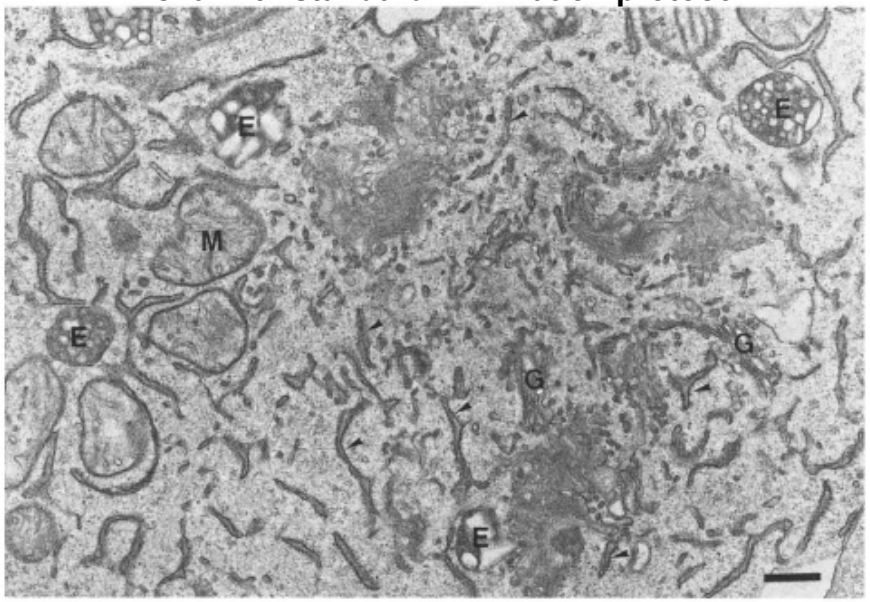
Immunogold labelling without destroying ultrastructure

TEM of HeLa cells following fixation with immunofluorescence protocols

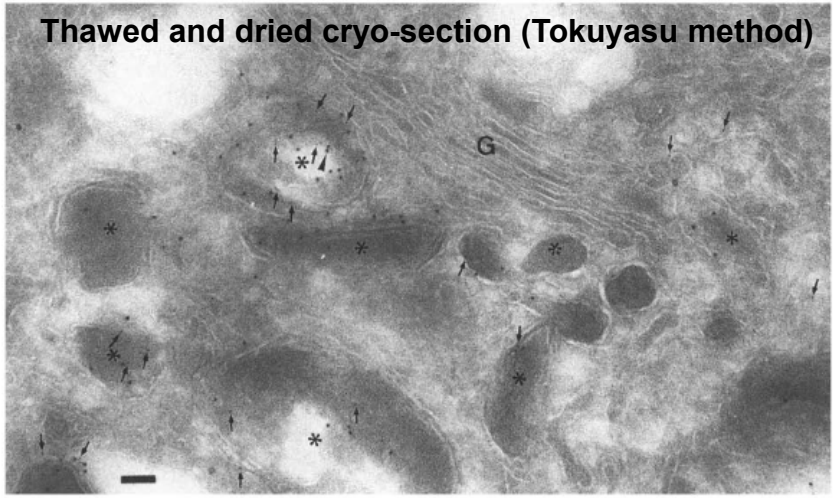


All images from Griffiths et al (1993) Trends in Cell Bio, v3

HeLa with standard EM fixation protocol



Thawed and dried cryo-section (Tokuyasu method)



Protein localisation

Immunogold labelling – Cells & Tissues

Cryo-fixation
High pressure freezing

↓

Freeze substitution
Uranyl acetate/acetone

↓

Acrylic resin infiltration

↓

UV polymerisation

↓

Ultramicrotomy

↓

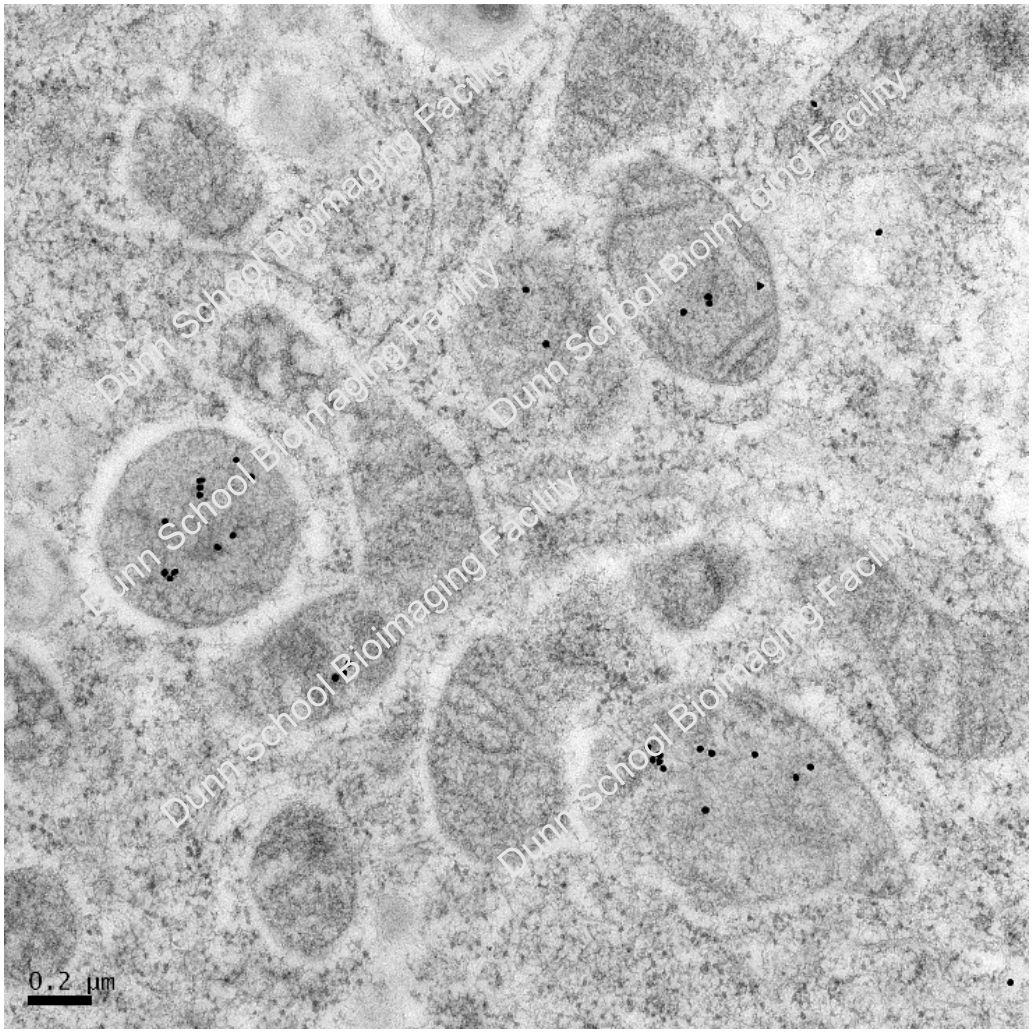
Light microscopy

↓

Post-staining

↓

EM



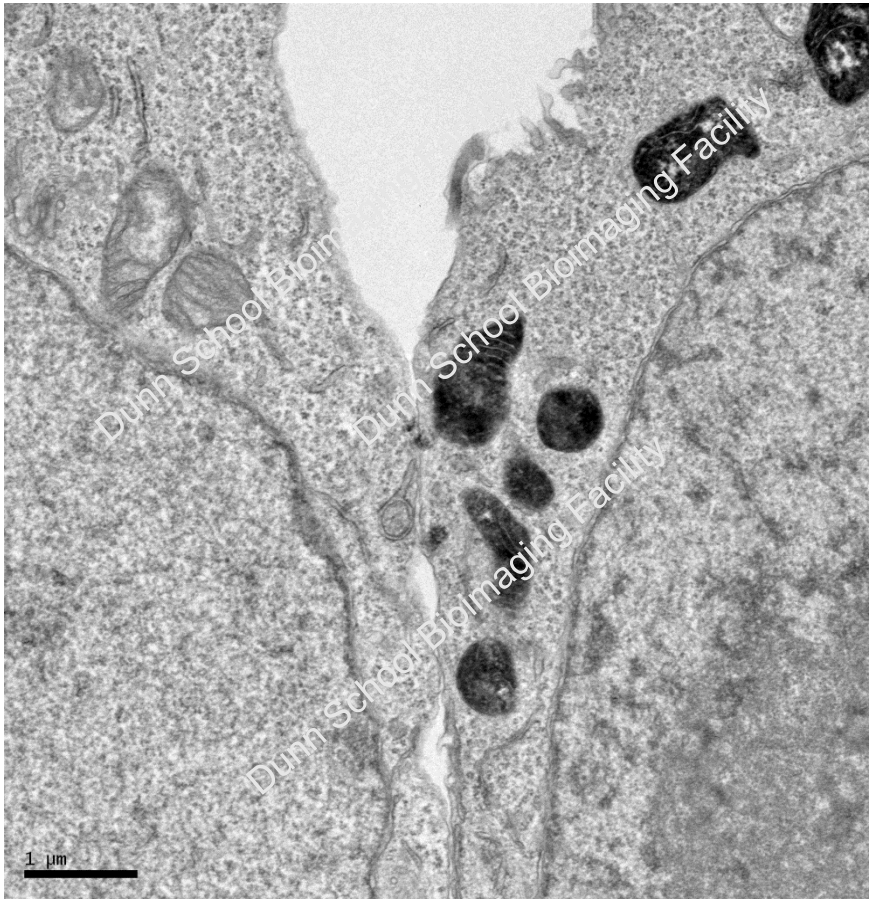
Immunogold labelled mitochondria in a mammalian cell
(A Dhir/EJohnson)

Protein localisation
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

- APEX (Martell et al, Nature Biotech 30, 2012)
  - 28kDa peroxidase that catalyses with DAB (with H_2O_2) to produce a localised osmophilic precipitate

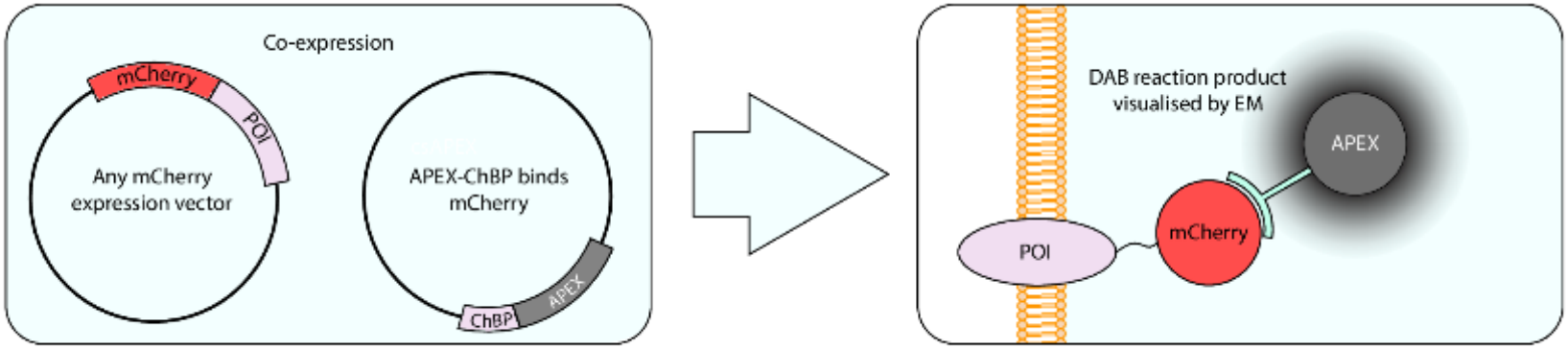
- miniSOG (Shu et al PLOS Biology 9, 2011)
  - Small fluoresecent flavoprotein that can be photo-oxidised to react with DAB to produce a localised osmophilic precipitate - CLEM

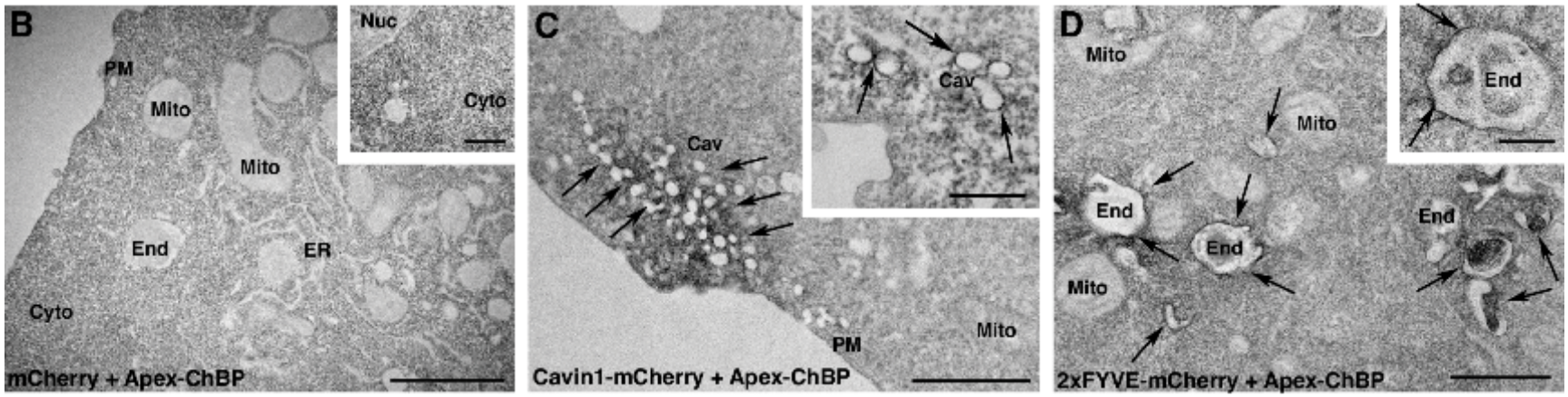


Chemically fixed HEK cells transfected with APEX tagged to a mitochondrial matrix protein (J Long/E Johnson)

Protein localisation

EM genetic tags: APEX2-mCherry/GFP binding protein



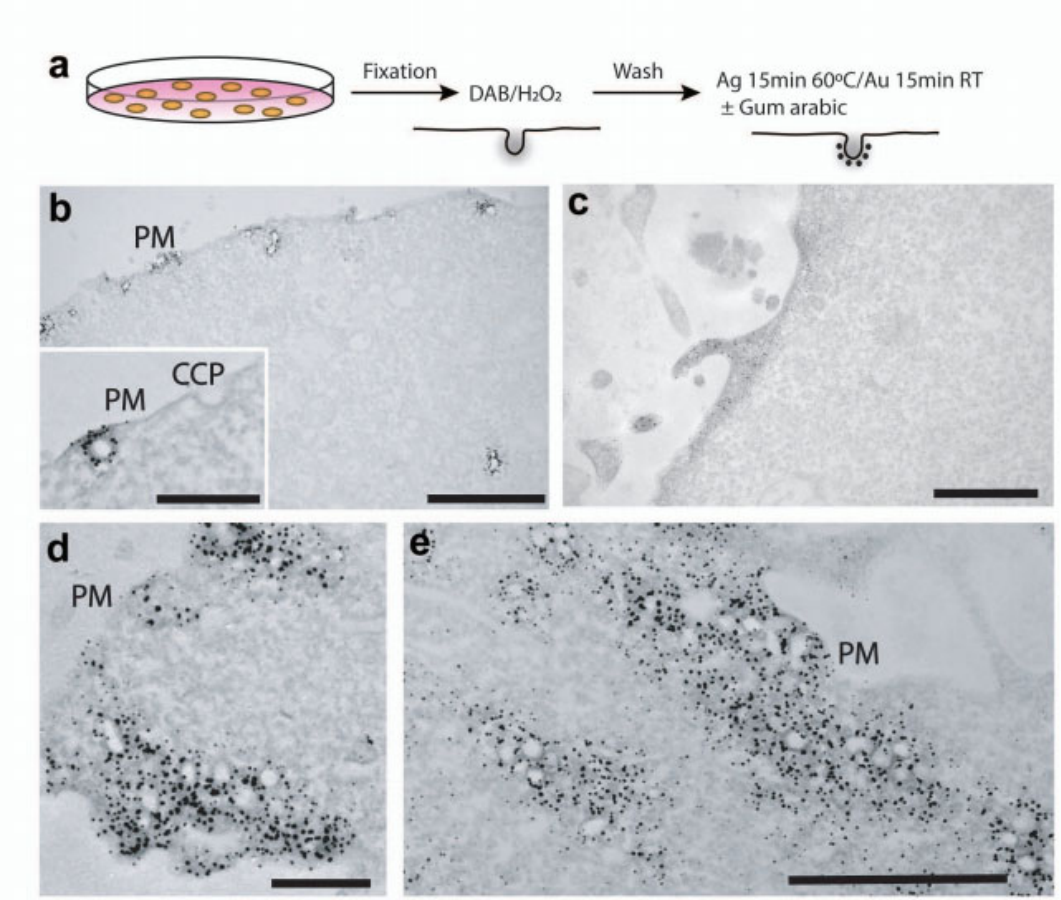


Baby hamster kidney (BHK) cells co-transfected with APEX-ChBP and 3 different subcellular markers: (B) mCherry for the cytoplasm, (C) mCherry-Cavin1 for caveolae on the plasma membrane (PM), and (D) 2xFYVE-mCherry for early endosomes From: Ariotti et al. (2018) PLOS Biology 16(4): e2005473.

Protein localisation

EM genetic tags: APEX-Gold method

- Gold labelling without immunogold!
- Cells transfected with Cavin4-APEX2 were fixed, treated with DAB and incubated with silver nitrate and then gold chloride (in the presence of gum Arabic for uniform nucleation).
- The DAB reaction produce is argyrophilic, such that the metal salts are converted to colloidal particles localised to the APEX fusion.
- This method also enables estimation of the densition of the fusion protein within the cell.



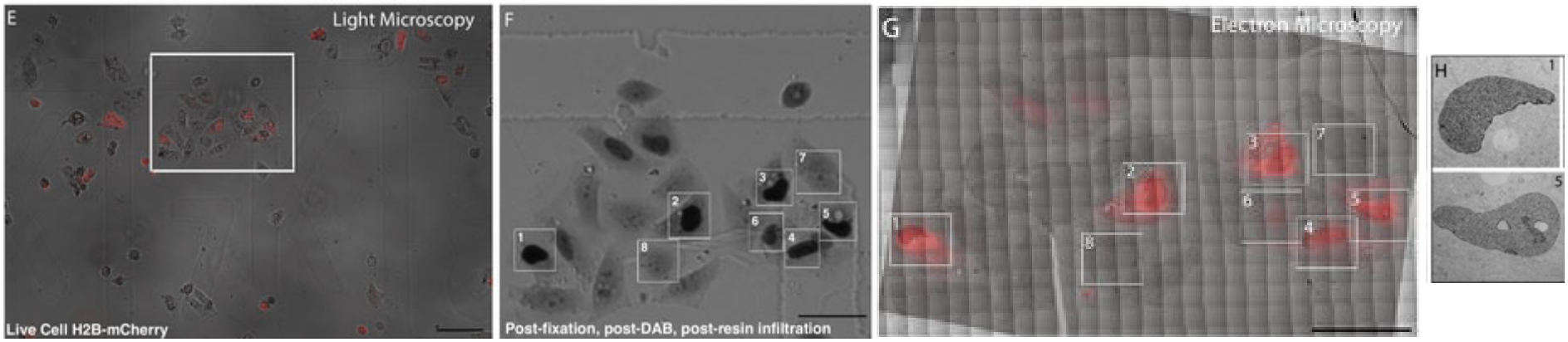
From Rae et al (2020) bioRxiv

Advanced EM techniques
Correlative microscopy

Correlative light & electron microscopy (CLEM)

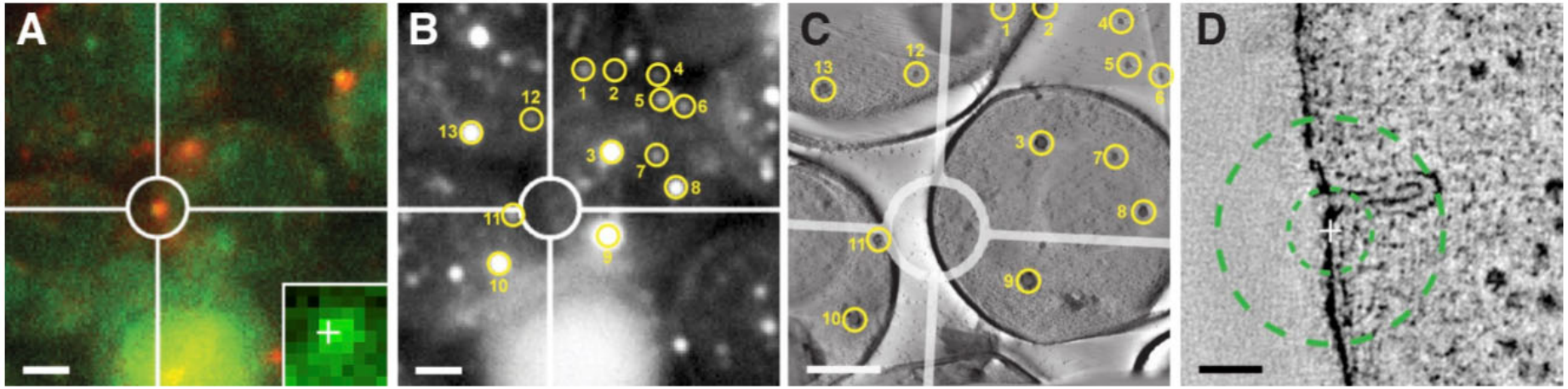
Identifying specific cells/rare events

Specific cells



CLEM of mCherry expressing cells co-transfected with APEX-ChBP (Ariotti et al. (2018) PLOS Biology 16(4): e2005473)

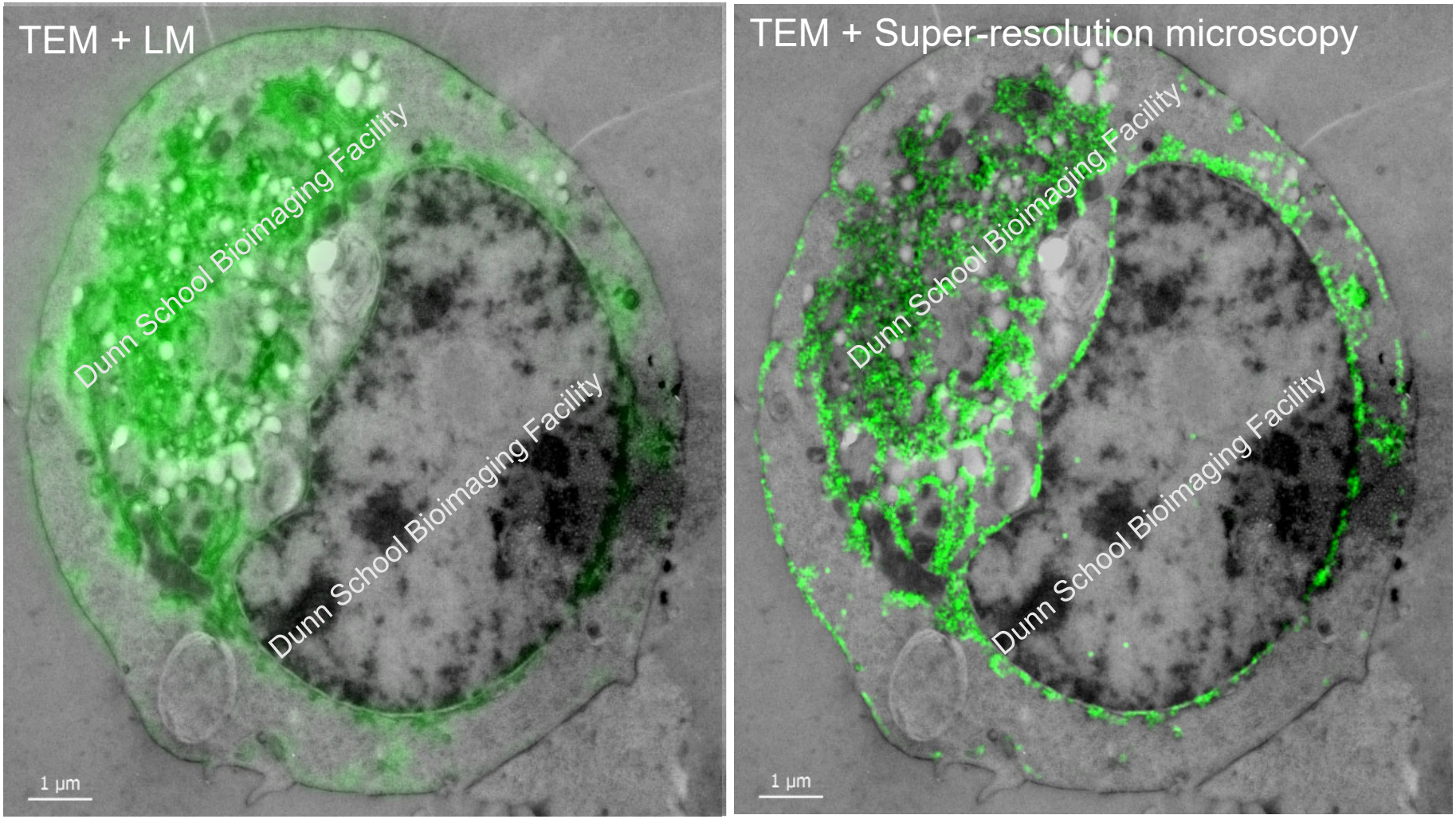
Rare events



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

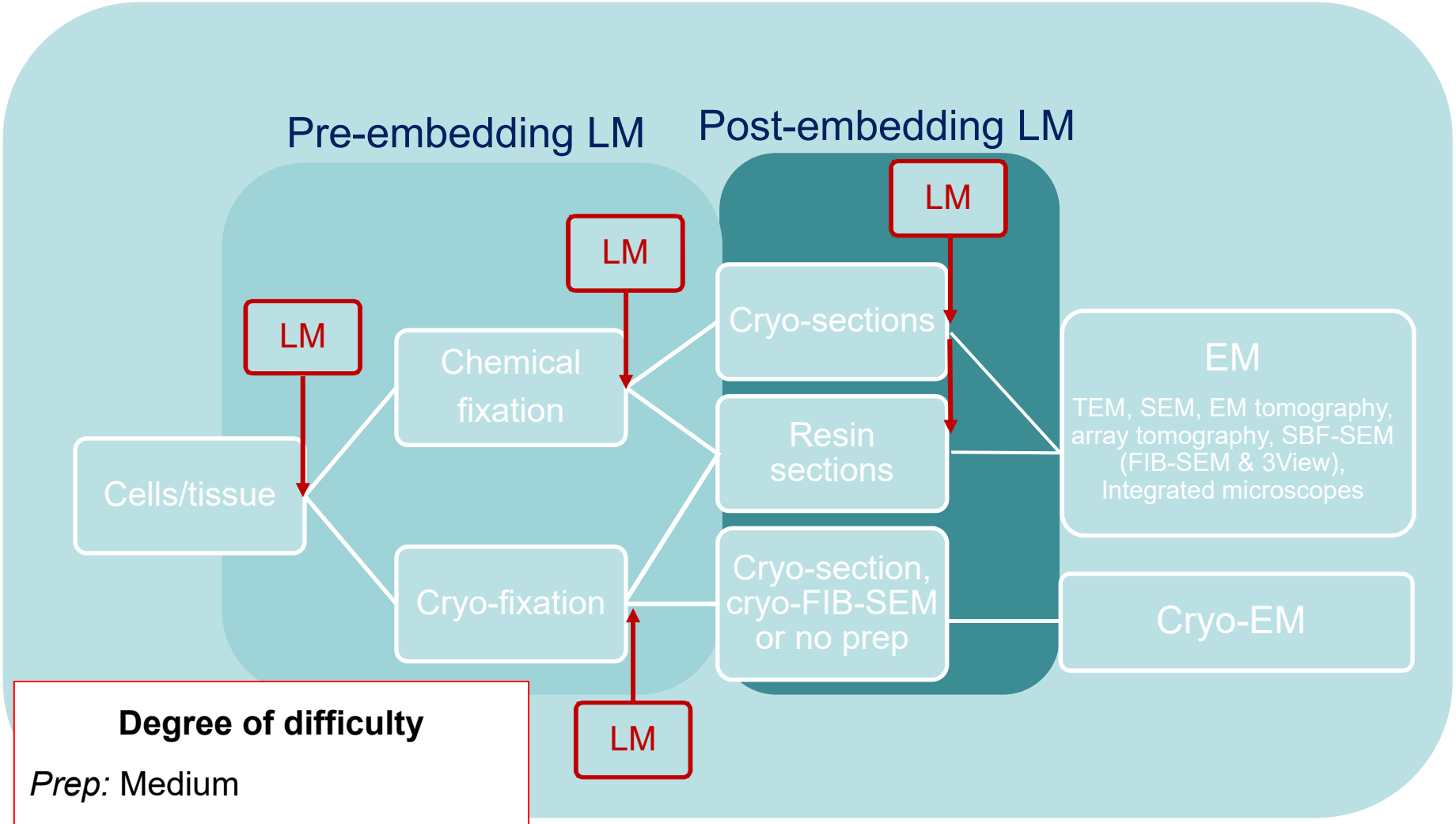
Correlative light & electron microscopy (CLEM)

Protein localisation



CLEM of HEK cells expressing EphA2-mVenus (E Johnson & R Kaufmann)

Simplified overview of CLEM workflows



Degree of difficulty

Prep: Medium

Microscopy: Medium to high

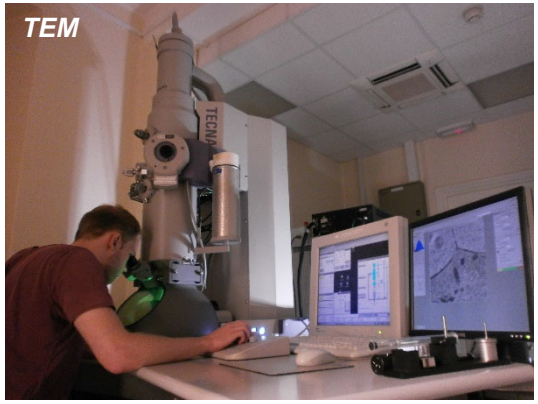
Data analysis: High

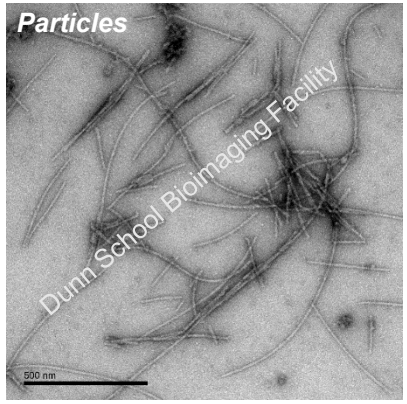
The Dunn School EM Facility

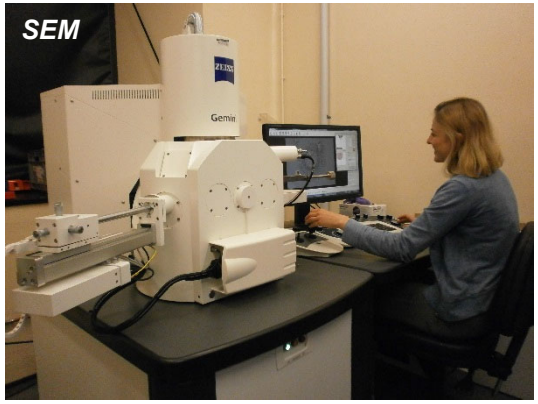
- **Staff:**
  - Errin Johnson (EM Facilities Manager)
  - Raman Dhaliwal (EM support scientist)

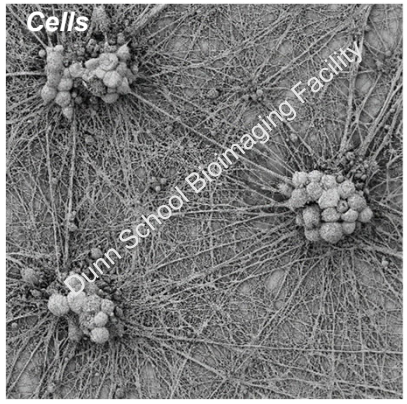
- **Instruments:**
  - FEI Tecnai12 120 kV TEM
  - Zeiss Sigma 300 FEG-SEM
  - Zeiss Merlin compact FEG-SEM + 3View

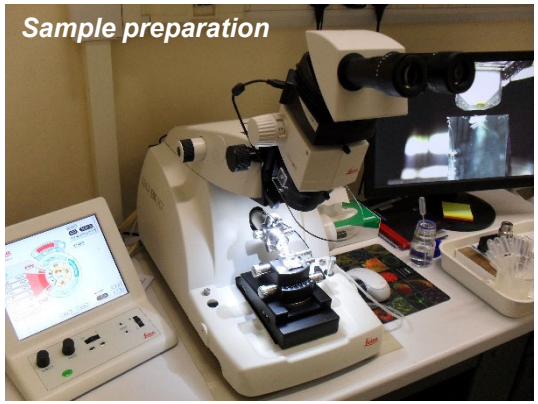
    (sited at & shared with Oxford Brookes)
  - Biological EM specimen preparation lab

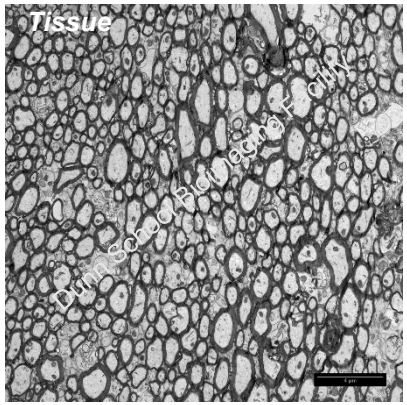






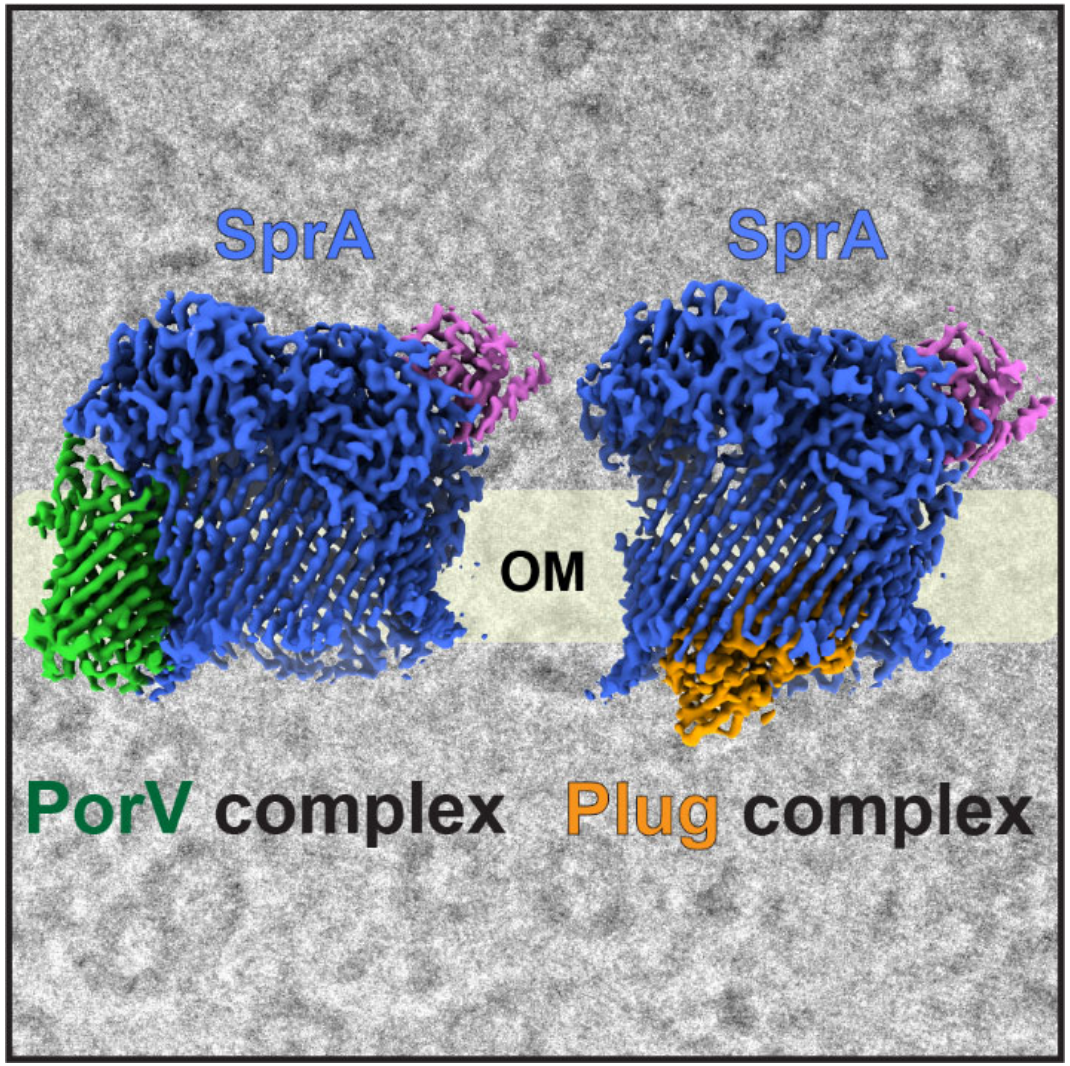






Advanced EM techniques

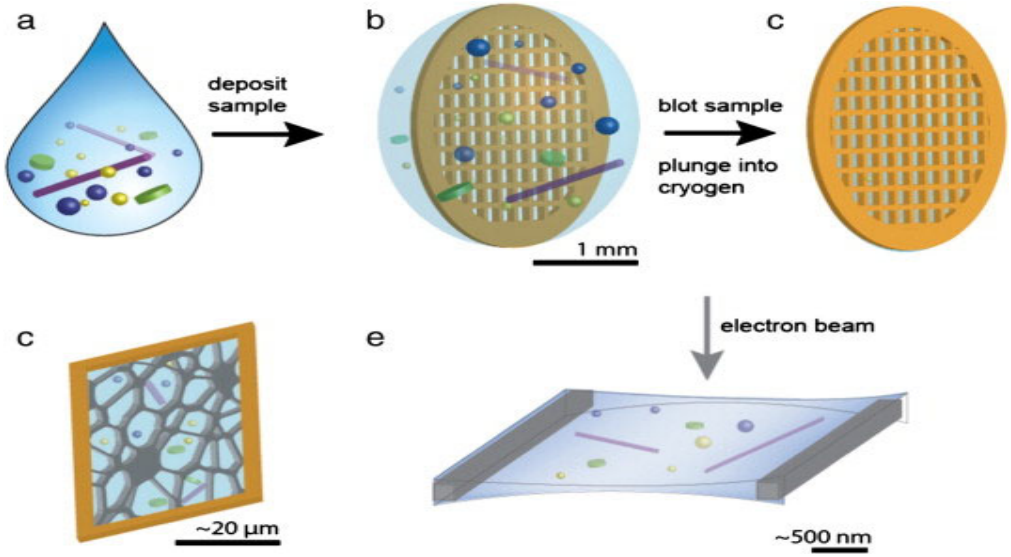
Cryo-Electron Microscopy



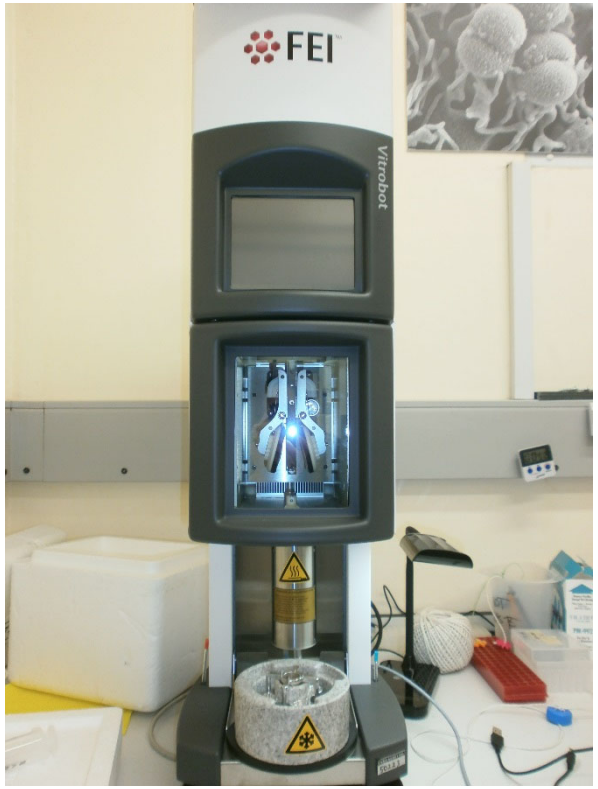
Cryo-EM structures of the type 9 secretion system translocon by Justin Deme and the Lea Lab, data acquired on the Titan Krios TEM

Cryo-TEM
Sample preparation

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



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



FEI Vtrobot for automated plunge freezing of grids

Cryo-TEM

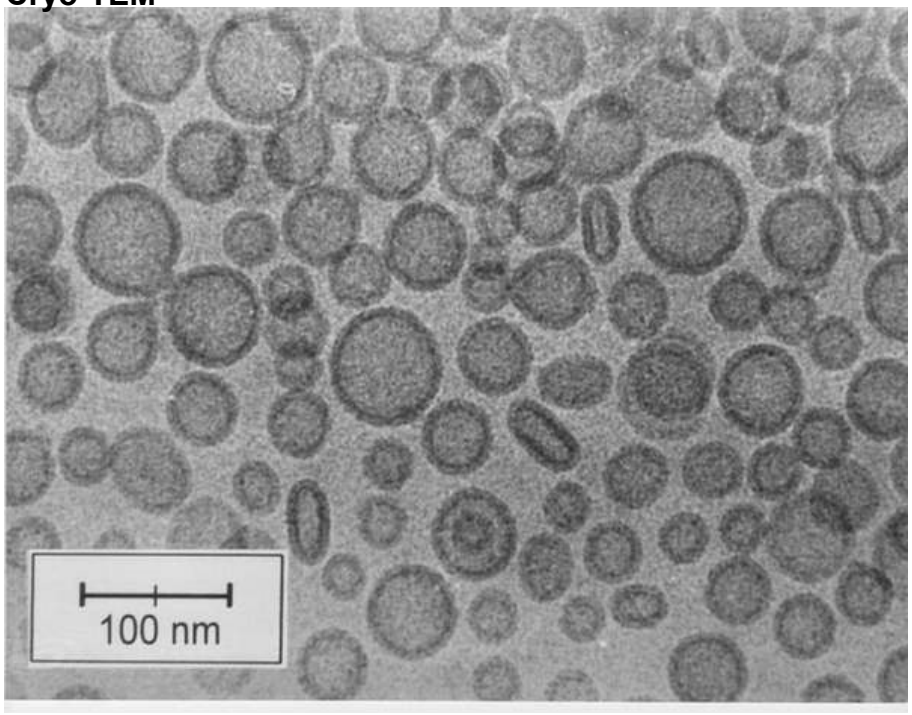
Sample preparation

From: Chen et al (2010) J Vis Exp, 39: 1943

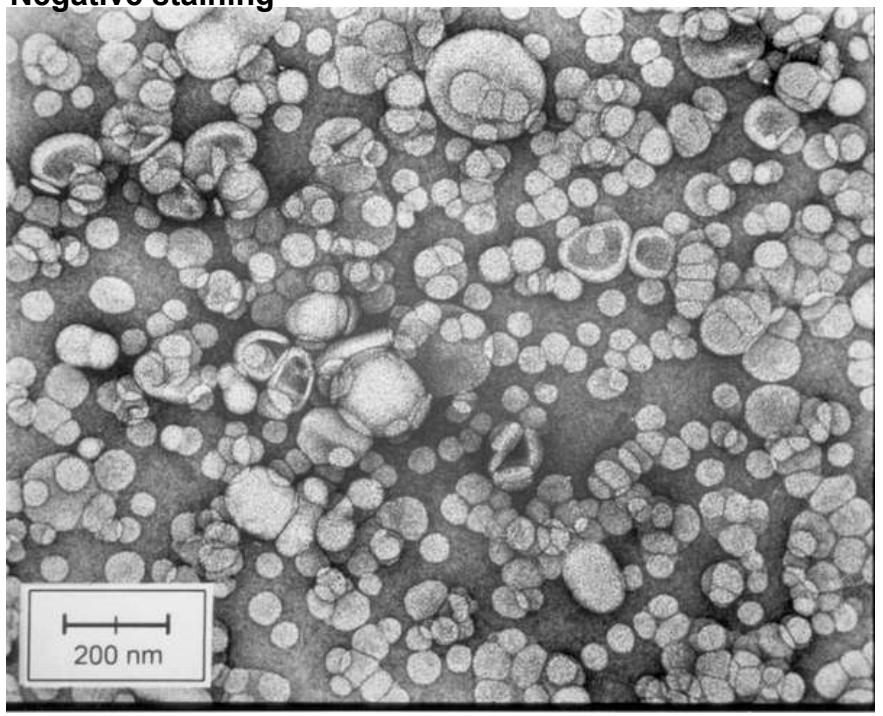
Cryo-TEM

Comparison to negative staining

Cryo-TEM



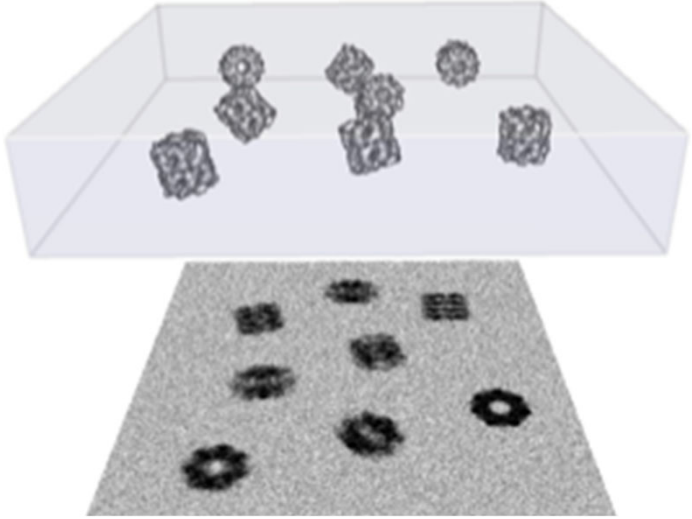
Negative staining

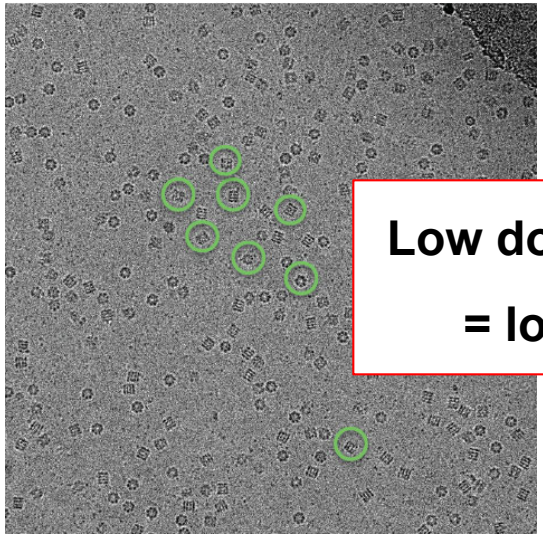


Dispersion of Egg-PC liposomes imaged frozen with Cryo-TEM (left) or negatively stained with uranyl acetate (right)
From: Laboratory for Soft Matter Electron Microscopy, University of Bayreuth

Cryo-TEM

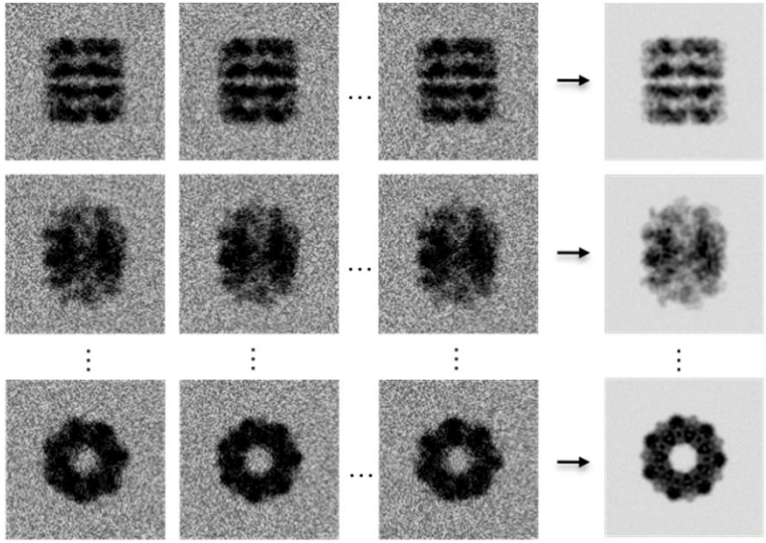
Single particle imaging & reconstruction

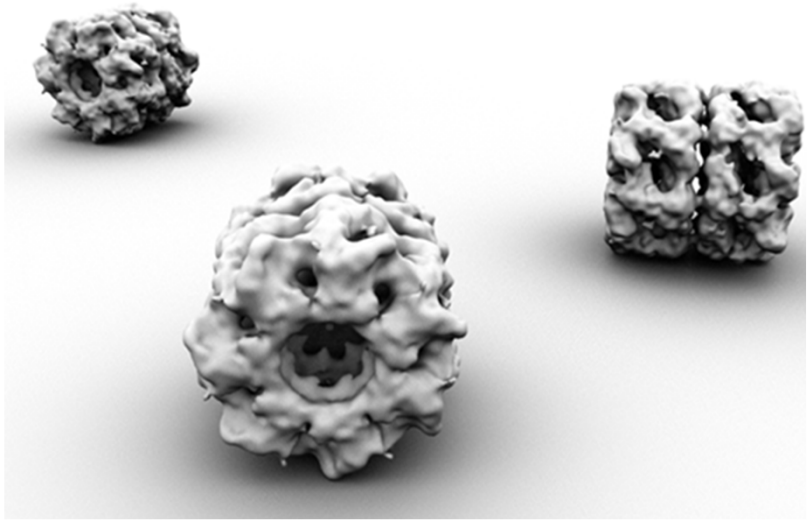




Low dose conditions

= low contrast

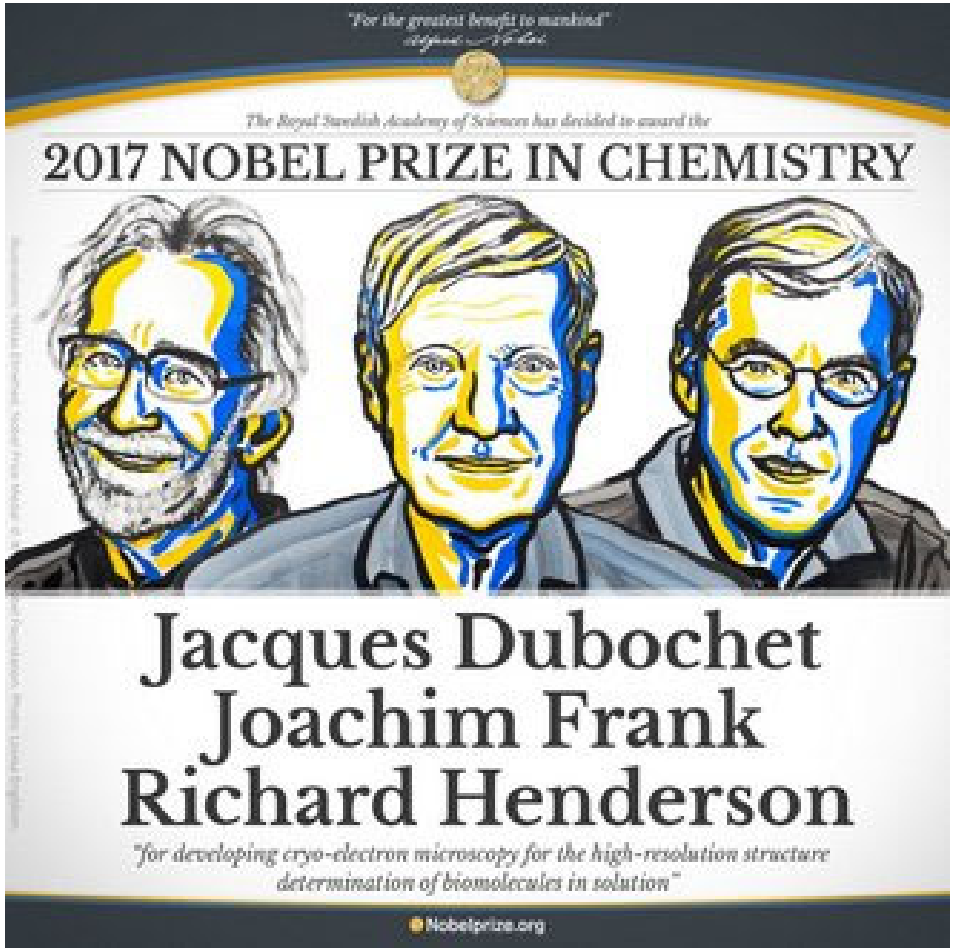




Single particle imaging and reconstruction of the GroEL chaperonin: purified complexes were applied to a grid and vitrified, then imaged with cryo-TEM. Thousands of images are collected and the same orientations are clustered together, averaged and back projected to render the complexes in 3D to 1 nm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

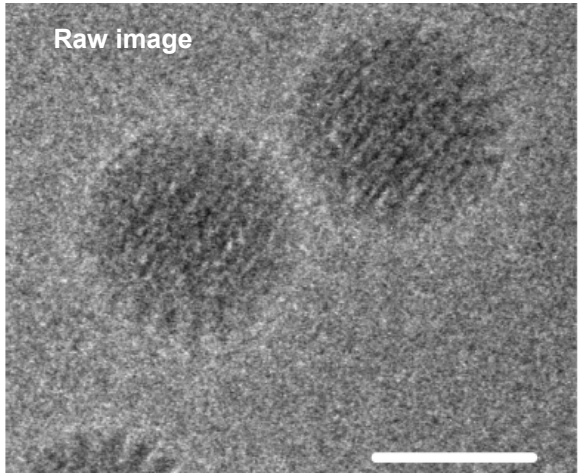
Cryo-Electron microscopy
Advances for structural biology

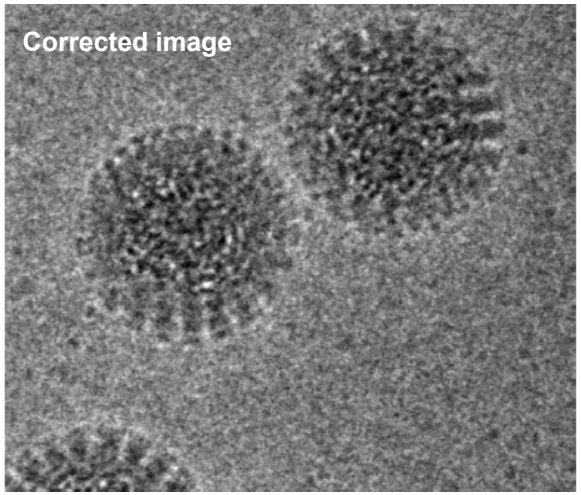
- The recent explosion of cryo-EM as a viable structural biology tool is due to several recent advances which have bought decades of work together



Cryo-Electron microscopy
Advances for structural biology

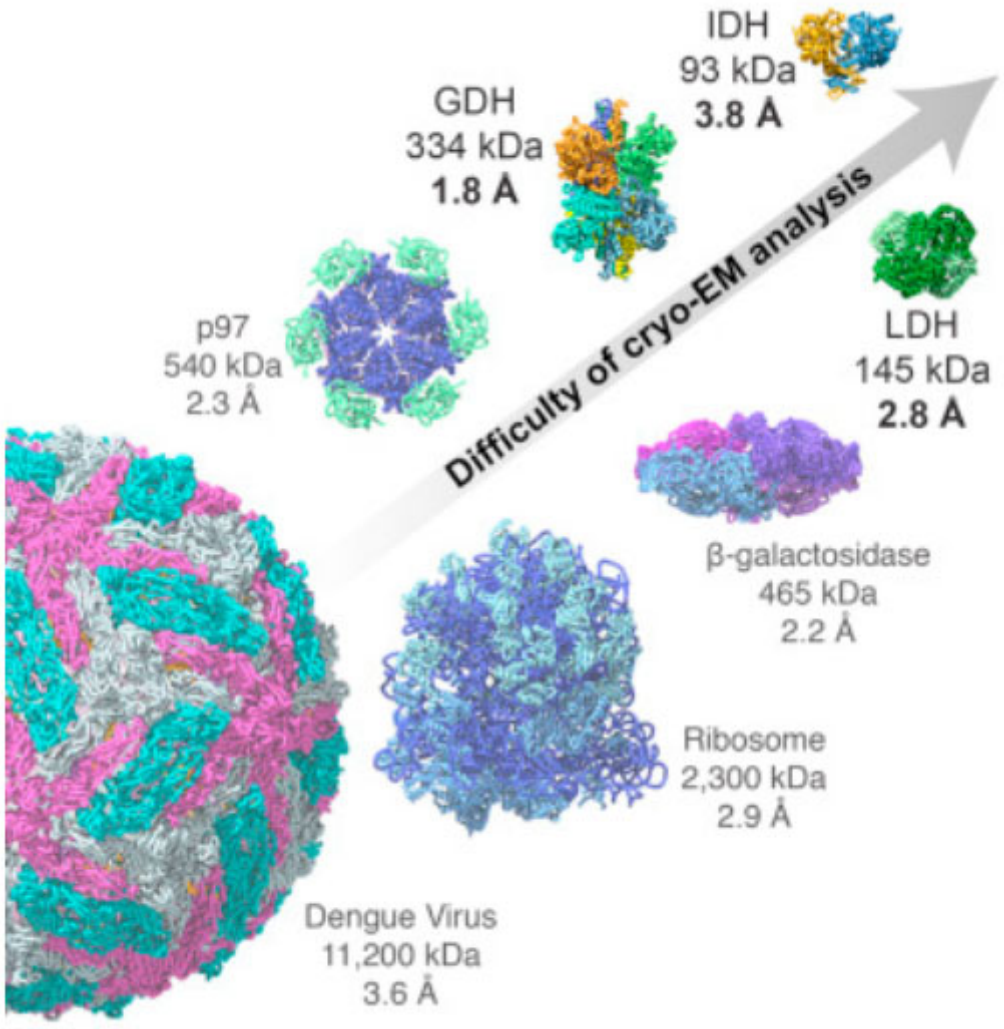
- Greater microscope stability
  - Titan Krios has constant power lenses for greater beam stability, a cube to reduce thermal drift
- Automated data acquisition
  - Allows data to be collected automatically over several days
- Direct electron detectors
  - Unlike CCDs, electrons are directly converted to electric signals.
  - Very sensitive and extremely fast, collecting 40 frames/sec.
  - This allows for dose fractionation and motion correction, which gives ~10x better resolution
- Computational and data processing advances





Brilot et al 2012

Cryo-TEM
Challenges



Merck et al Cell 2016

- Proteins <200kDa are problematic due to low contrast
- Sample preparation optimisation: concentration, purity and stability of protein
- Vitrification optimisation: correct ice thickness, multiple orientations of protein, even distribution in ice

Degree of difficulty

Prep: High

Microscopy: High

Data analysis: High

Central Oxford Single Molecule Imaging Center (COSMIC)

- **Staff:**
  - Matthew Higgins (Director)
  - Tanmay Bharat (Deputy Director)
  - Errin Johnson (EM Facilities Manager)
  - Adam Costin (Cryo-EM support scientist)
  - Joseph Caesar (Computer scientist)

- **Instruments:**
  - FEI Talos 200c cryo-TEM
  - FEI Arctica cryo-TEM with F4 detector
  - FEI Titan Krios cryo-TEM with K3 GIF
  - Vitrobot Mark IV
  - Cryo-EM preparation lab

Thermo Fisher Titan KriosCryo-TEM @ COSMIC

The Dunn School EM Facility &
Central Oxford Single Molecule Imaging Center (COSMIC)

- For more information about our microscopes and the techniques mentioned in this talk, plus recommended papers/books to read and links to instructional videos, please see out website:
- http://web.path.ox.ac.uk/~bioimaging/bioimaginghome.html