BIOLOGICAL SAMPLE PREPARATION FOR ELECTRON MICROSCOPY

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Transmission Electron Microscopy (TEM)
Electron microscopy
Specimen requirements

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<td>Stable in the vacuum</td>
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<td>Well preserved internal structure</td>
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<td>Electron dense staining</td>
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<td>Very thin (eg: 70 nm)</td>
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<td>Particulate samples can be stained and viewed quickly</td>
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<td>Cells and tissue require extensive specimen preparation</td>
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TEM of resin-embedded mouse cardiac tissue (scale bar = 2 µm), Tecnai12 TEM, E. Johnson

Specimen Preparation for TEM
Particulate samples

**Negative Staining:**
- Coat grids with plastic film and carbon
- Apply the particulate specimen eg: proteins, viruses, DNA
- Stain with heavy metal solution, eg: uranium salts
- Blot dry and view in the TEM

Bacterial protein stained with uranyl acetate. Tobacco mosaic virus negatively stained with sodium silicotungstate (E. Johnson)
Specimen Preparation for TEM
Cells & Tissue

Specimen Preparation for TEM
Cells & Tissue – Overview

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 resins)
Embedding
Sectioning (0.5 μm for LM, 70 nm for TEM)
Post-stain (uranyl acetate and lead citrate)
Image

C. elegans
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
TEM imaging

A grid mounted on the sample holder for TEM
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

Methods of fixation include:
- Chemical fixation with aldehydes
- Cryo-fixation with liquid nitrogen

Specimen Preparation for TEM
**Cells & Tissue – Chemical Fixation**

**Glutaraldehyde**
irreversible cross-linking of proteins via amino groups

**Paraformaldehyde**: reversible cross-linking, small molecule, penetrates quicker

Standard TEM fix: 2.5% glutaraldehyde + 2-4% PFA for 30 mins to overnight.
Specimen Preparation for TEM
Cells & Tissue – Chemical Fixation artifacts

- 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)
  - Embedding
  - Sectioning (0.5 μm for LM, 70 nm for TEM)
  - Post-stain (uranyl acetate and lead citrate)
  - Image

Specimen Preparation for TEM
Cells & Tissue

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Loss of membranes
Deformed mitochondria

Bad fixation
Good fixation
Specimen Preparation for TEM  
*Cells & Tissue – Cryo-fixation*

- Tissue can be cryo-fixed using LN$_2$ in the High Pressure Freezer and then further processed for TEM (adds 1 week)
- 3-6 mm specimen carriers
- cryo-fixation with LN$_2$ under high pressure (~2000 bar) to prevent damaging ice crystal formation up to 200 μm into the tissue

Samples are then carefully transferred to the AFS and freeze-substituted with solvent (+ osmium and/or glutaraldehyde or uranyl acetate) at sub-zero temperatures.
Specimen Preparation for TEM  
**Cells & Tissue – Cryo-fixation**

- **Pros** of cryo-fixation: best possible ultrastructural preservation, maintains fluorescence and antigenicity

- **Cons** of cryofixation: time consuming, finicky and restrictions on sample size, possible ice crystal issues

Specimen Preparation for TEM  
**Cells & Tissue – Secondary Fixation**

- **Osmium tetroxide** is a heavy metal that fixes unsaturated lipids.
- Used as both a secondary fixative and an electron stain, it significantly improves specimen preservation (especially of membranes) and contrast.
Specimen Preparation for TEM
Cells & Tissue – Dehydration & resin infiltration

- 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.

Resin blocks
Poor resin infiltration

Specimen Preparation for TEM
Microwave-assisted sample processing

Leica EM AMW
From 5 days to 5 hours
Specimen Preparation for TEM

**Cells & Tissue - Ultramicrotomy**

- 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 acryl resin)
- Embedding
  - Sectioning (0.5 μm for LM, 70 nm for TEM)
  - Post-stain uranyl acetate and lead citrate
  - Image

Specimen Preparation for TEM

**Cells & Tissue – Ultramicrotomy artifacts**

- Knife marks
- Chatter, knife marks and compression
- Chatter
- Too thick
- Water contamination

Images by E. Johnson (unfortunately!)
Specimen Preparation for TEM
Cells & Tissue – Post-staining

Contrast can be increased by post-staining sections with salts of heavy metals, specifically uranyl acetate and lead citrate solutions. Uranyl acetate stains protein and DNA and also acts as a mordant for lead citrate, which is a more general stain.

Specimen Preparation for TEM
Cells & Tissue – Post-staining artifacts

No post-staining
Post-staining

Dendritic cells (S Hackett)

No post-staining Post-staining

Dendritic cells (S Hackett)

Images by E Johnson
TEM Specimen Preparation

Critical evaluation of images

Specimen Preparation for TEM

Protein localisation – Immunogold labelling

- Secondary antibody is conjugated to a colloidal gold particle.
- For cells and tissue, post-embedding labelling is usually the best option
- A lighter chemical fixation or cryo-fixation
- The osmium tetroxide step is omitted
- Acrylic resins are used instead of Epoxy resins.
Specimen Preparation for TEM
Protein localisation – EM genetic tags

- Two new **genetically encoded tags** 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

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Scanning Electron Microscopy (SEM)
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.

Sample Preparation for SEM

Drying the sample

Air drying is not recommended, as ethanol evaporation generally causes severe surface tension artifacts.

Ethanol is instead flushed out using transitional fluid of very low surface tension:

- manually, using hexamethyldisilazane (HMDS) – cell monolayers
- in a Critical Point Dryer (CPD) using liquid CO₂ – tissues and whole organisms
Sample Preparation for SEM

**Drying the sample**

- **Good**
- **Bad**

Specimen Preparation for SEM

**Sputter coating**

- Mounting – immobilization and grounding on a conductive backing
- Sputter coating with metal ions
  - continuous conductive layer over the sample
  - increased SE signal (and therefore contrast)
Specimen Preparation for SEM

**Charging artifacts**

- Bright spots (Arabidopsis root)
- Lines in the image (Arabidopsis xylem)
- Focus issues (Leishmania on collagen)
- Image distortion (processed cheese)

Images: E. Johnson

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Specimen Preparation for SEM

**Surface contamination and deformation**

- Bacteria
- Cellular debris
- Tweezer marks
- Blebs or other oddities

Images: E. Johnson
Specimen Preparation for SEM

No problems!

Questions?