

Oct 19, 2022

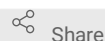
# Preparation of tissue for transmission electron microscopy ultrastructural analysis

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## ABSTRACT

A transmission electron microscope (TEM) enables the magnification and visualisation of cell and tissue ultrastructure that cannot otherwise be resolved using a light microscope. In transmission electron microscopy, a high energy electron beam generated from an electron gun is focused by electromagnetic lenses onto an ultrathin section of plastic embedded specimen. Electrons are transmitted through the specimen onto a fluorescent screen based on sample density, and an image is acquired by a digital camera. Here, a protocol for preparing tissue specimens for TEM ultrastructural analysis is described. A tissue specimen is fixed, dehydrated and infiltrated with resin. The resin is then polymerised, and embedded tissue sectioned using an ultramicrotome. Ultrathin sections are contrasted with heavy metals prior to imaging using a TEM. Options for faster processing using a Microwave processor are described for relevant steps.

## DOI

[dx.doi.org/10.17504/protocols.io.n92ldpz9nl5b/v1](https://dx.doi.org/10.17504/protocols.io.n92ldpz9nl5b/v1)

## PROTOCOL CITATION

Jillian C Danne, Simon A Crawford, Rachel Templin, Joan Clark, Viola Oorschot, Georg Ramm 2022. Preparation of tissue for transmission electron microscopy ultrastructural analysis.

**protocols.io**

<https://dx.doi.org/10.17504/protocols.io.n92ldpz9nl5b/v1>

## KEYWORDS

Transmission electron microscopy, Tissue, Epon resin, Ultrastructure, Processing, Contrasting, Ultramicrotomy, Embedding, Dehydration

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## CREATED

Oct 18, 2022

## LAST MODIFIED

Oct 19, 2022

MATERIALS TEXT

[☒ Paraformaldehyde, 16% \(wt/vol\)](#) **Electron Microscopy**

**Sciences Catalog #15710**

[☒ Glutaraldehyde 25% Aqueous Solution 10 x 10 ml ampoules](#) **Electron Microscopy**

**Sciences Catalog #16220**

[☒ Sodium Cacodylate Trihydrate](#)

**100g ProSciTech Catalog #C020**

[☒ 0.2M Sodium Cacodylate Trihydrate Buffer pH 7.4](#) **Contributed by users**

[☒ 0.1M Sodium Cacodylate Trihydrate Buffer pH 7.4](#) **Contributed by users**

[☒ Osmium tetroxide 10 x 1g](#)

**ampoule ProSciTech Catalog #C010-1010**

[☒ Potassium hexacyanoferrate\(II\) trihydrate](#) **Sigma**

**Aldrich Catalog #P3289-100G**

[☒ Ethanol Absolute AR grade 2.5L](#) **AIM**

**Scientific Catalog #AJA214-2.5L**

[☒ MilliQ water](#) **Contributed by users**

[☒ 30% Ethanol in MilliQ water](#) **Contributed by users**

[☒ 50% Ethanol in MilliQ water](#) **Contributed by users**

[☒ 70% Ethanol in MilliQ water](#) **Contributed by users**

[☒ 90% Ethanol in MilliQ water](#) **Contributed by users**

[☒ 95% Ethanol in MilliQ water](#) **Contributed by users**

[☒ Molecular sieves 3 Å](#) **Sigma**

**Aldrich Catalog #208574-1KG**

[☒ Epon resin](#) **Contributed by users**

[☒ Toluidine blue](#) **Merck Millipore Sigma**

[☒ Uranyl acetate](#) **Electron Microscopy**

**Sciences Catalog #22400**

[☒ Lead \(II\) citrate tribasic trihydrate](#) **Sigma**

**Aldrich Catalog #15326-25g**

[☒ 10M sodium hydroxide solution](#) **Merck Millipore**

**Sigma Catalog #72068**

Black teflon plate

N/A

N/A

Fine Forceps

Forceps

Dumont

11251-10



Bite and boxing wax-500g

Investo (Lordell)

WI-BB

Blade scalpel ST #11

Swann Morton

21016SM

Single edge carbon steel blade

Electron Microscopy Sciences 71960

Tube 5ml 5016 PP yellow cap GS

Pacific Laboratory Products P5016SU

Falcon® Centrifuge Tubes  
Polypropylene, Sterile, 15 mL  
Corning® 352096

Rotary tube mixer  
Ratek Instruments RSM7DC

PELCO BioWave® Pro+ Microwave  
Processing System, 120VAC  
Pelco 36700

Embedding mould, single ended flat 21  
cavities  
ProSciTech RL064

Flat bottom embedding capsules,  
microwave safe  
BEEM® 70021-PPT

BEEM® embedding capsules, size OO, PE,  
Pack/500  
BEEM® RB001-500

3ml graduated transfer pipette

Copan      200C

Gilson pipetman classic P1000

Gilson      1152009

Oven MINO/6/CLAD

Genlab      N/A

Olympus SZ30 stereo microscope

Olympus      SZ30-PS

UC7 Ultramicrotome

Leica      EMUC7

Double edge blades

Personna      72000

Glass knife, 45 degree

Leica      N/A

Glass knife boat, 6.4mm  
Electron microscopy sciences 71008

Ultra Semi diamond knife, 35 degree  
angle, 3 mm  
Diatome                  UltraSemi

Eyelash or Dalmatian hair mounted on a  
wooden stick  
N/A                  N/A

Plain glass slides 76mm x 39mm x 1.0-  
1.2mm  
Thermo Scientific                  AGL4222A

Mini hot plate  
Thermofisher                  HP2310BQ

Olympus widefield microscope, model  
CHK2-F-GS  
Olympus                  N/A

Ultra wet diamond knife, 45 degree angle,  
3mm  
Diatome            UU45-30

Heat pen for ultramicrotomy  
Max Wax            U060

50 Mesh palladium/copper grids  
Gilder            GCU-PD50

50 Mesh copper grids  
Gilder            GCU50

200 Mesh copper grids  
Gilder            GCU200

200 Mesh palladium/copper grids  
Gilder            GCU-PD200

Glass petri dish, 100mm  
BRAND            BR455751

Grid storage box, 100 grid capacity

Gilder HL065

Parafilm M

Bemis IA041

Glass board

N/A N/A

Perfect loop

Diatome 70944

10ml Syringe, Luer lock tip

Terumo 19046TE

Acrodisc syringe filter 25mm, 0.2um pore size

Pall Corporation Z259969

Foil

N/A N/A



Filter paper, grade 1, 12.5cm  
Whatman 1001-125

JEOL JEM-1400 Plus 120keV  
Transmission electron microscope  
JEOL N/A

#### SAFETY WARNINGS

The following chemicals must be handled with extreme care in a fume hood using the appropriate personal protective equipment (PPE):

Paraformaldehyde and glutaraldehyde are toxic, corrosive and potentially carcinogenic.

Cacodylate buffer contains arsenic, and is acutely toxic and carcinogenic.

Osmium tetroxide is extremely toxic. Used osmium should be discarded in a labelled plastic container containing ethanol to reduce and neutralise the osmium.

Potassium ferrocyanide is acutely toxic.

Uranyl acetate is mildly radioactive and extremely toxic if ingested, inhaled or in contact with abraded or cut skin.

#### Fixation

- 1 All fixation and processing steps must be performed in a fume hood wearing appropriate personal protective equipment (PPE). The Material Safety Data Sheet (MSDS) for each chemical must be read before commencing.  
  
Dissect out the tissue of interest on a Teflon plate or dental wax sheet using fine forceps and a scalpel blade at room temperature, and keeping the tissue submerged in Karnovsky's fixative, 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2). Tissue pieces should be no larger than 1 mm cubed to ensure sufficient infiltration of fixative, solvents and resin.
- 2 Place the tissue pieces in 5 ml tubes, 15 ml Falcon® tubes or a well-plate containing 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1M cacodylate buffer and fix overnight at 4 degrees Celsius or for 2-4 hours at room temperature, on a rotor.
- 3 Wash with 0.1M cacodylate buffer, 3 x 10 minutes at room temperature, agitating.

- 4 Osmium tetroxide is extremely toxic and must be handled with care in a fume hood using the appropriate PPE. Used osmium should be discarded in a labelled plastic container containing ethanol to reduce and neutralise the osmium.

Osmium can precipitate out of solution when in the presence of glutaraldehyde to form small electron dense artefactual deposits. Samples must be washed thoroughly (Step 3) prior to post-fixation (Step 4) to avoid this.

Potassium ferrocyanide reduces osmium, turning the post fixation solution black. Reduced osmium may also prevent the formation of artefactual precipitates. Potassium ferricyanide can be used as an alternative in combination with osmium tetroxide. This solution is a clear, amber colour.

The following steps can be performed with or without the use of a PELCO BioWave® Pro+ Microwave Processing System. A Biowave facilitates infiltration of reagents into tissue and cells, and reduces the processing time.

Post fix the tissue in 1% Osmium tetroxide, 1.5% potassium ferrocyanide in 0.1M cacodylate buffer for 2 hours at room temperature, agitating. Tube or well-plate lids must be well-secured during fixation.

Biowave settings: 3x 120 seconds, 100W, Vacuum on.

A two minute pause step without a solution change must be included in between each interval to prevent overheating of samples.

- 5 Wash in MilliQ water, 3 x 10 minutes at room temperature, agitating.

Washed samples can be stored at 4 degrees Celsius for up to 1 week until further processing.

#### Dehydration and resin infiltration

- 6 For Steps 6-19, do not proceed if samples float following ethanol, acetone or Epon resin infiltration. Well-infiltrated samples should sink in solution. If they do not, samples may require extended infiltration times.

Epon resin can be stored in the freezer but must come to room temperature before use.

Dehydrate in 30% ethanol in MilliQ water for 1 hour at room temperature, agitating.

Biowave settings: 40 seconds, 150W, No vacuum.

- 7 Dehydrate in 50% ethanol in MilliQ water for 1 hour at room temperature, agitating.

Biowave settings: 40 seconds, 150W, No vacuum.

- 8 Dehydrate in 70% ethanol in MilliQ water for 1 hour at room temperature, agitating. Tissue can be left at 4 degrees Celsius, overnight if necessary.

Biowave settings: 40 seconds, 150W, No vacuum.

Dehydrate in 90% ethanol in MilliQ water for 1 hour at room temperature, agitating.

9

Biowave settings: 40 seconds, 150W, No vacuum.

10

Dehydrate in 95% ethanol in MilliQ water for 1 hour at room temperature, agitating.

Biowave settings: 40 seconds, 150W, No vacuum.

11

Dehydrate in 100% ethanol for 1 hour at room temperature, agitating.

Biowave settings: 40 seconds, 150W, No vacuum.

12

Dehydrate in 100% anhydrous ethanol for 1 hour at room temperature, agitating. Type 3Å molecular sieves can be added to the ethanol stock solution to dehydrate the solvent prior to use.

Biowave settings: 40 seconds, 150W, No vacuum.

13

Dehydrate in 100% acetone for 1 hour at room temperature, agitating.

Biowave settings: 40 seconds, 150W, No vacuum.

Propylene oxide can be used as an alternative to acetone (Steps 13-17), but is extremely toxic and may dehydrate samples too intensively, causing ultrastructural damage.

14

Dehydrate in 100% anhydrous acetone for 1 hour at room temperature, agitating. Type 3Å molecular sieves can be added to the acetone stock solution to dehydrate the solvent prior to use.

Biowave settings: 40 seconds, 150W, No vacuum.

15

Optionally Infiltrate with 100% anhydrous acetone : epon resin (3 : 1 ratio) overnight at room temperature, agitating.

Biowave settings: 3 minutes, 250W, Vacuum on.

16

Infiltrate with 100% anhydrous acetone : epon resin (1 : 1 ratio) for 6 hours at room temperature, agitating.

Biowave settings: 3 minutes, 250W, Vacuum on.

17

Infiltrate with 100% anhydrous acetone : epon resin (1 : 3 ratio) overnight at room temperature, agitating.

Biowave settings: 3 minutes, 250W, Vacuum on.

18

Infiltrate with 100% epon resin for 6 hours at room temperature, agitating.

Biowave settings: 3 minutes, 250W, Vacuum on.

19

Infiltrate with 100% epon resin overnight at room temperature, agitating.

Biowave settings: 3 minutes, 250W, Vacuum on.

Residual acetone can adversely impact resin infiltration and polymerisation. A third 100% resin infiltration step

may be required to prevent this.

- 20 Transfer samples to labelled plastic embedding moulds, flat bottom BEEM® capsules or standard tip BEEM® capsules containing 100% epon resin. Polymerise in an oven set to 60 degrees Celsius for 24-48 hours.

#### Semi and ultrathin sectioning

- 21 Mount the resin-embedded tissue block in an ultramicrotome chuck, and secure the chuck to a stereo microscope fitted with a chuck mount. Manually trim the front face and all four edges of the tissue block into a trapezoid shape using a double edge razor blade.
- 22 Transfer the chuck and resin-embedded tissue block to a Leica UC7 ultramicrotome and secure in place. Trim the front surface of the block face until a full tissue face has been obtained. Trim manually using an 0.5 µm feed and a 45 degree glass knife (thickness 6.4 mm or 8 mm) or suitable diamond knife, for example a Diatome 35 degree angle ultra semi diamond knife.
- 23 To check the region of interest:
  - 23.1 Cut 500 nm semi thin sections at a speed of 1 mm/sec using a Leica UC7 ultramicrotome and 45 degree glass knife fitted with a filtered water-filled boat or suitable diamond knife.
  - 23.2 Retrieve sections with an eyelash or Dalmatian hair mounted on a wooden stick and float sections in a drop of MilliQ water on a slide before drying on a mini hot plate.
  - 23.3 Stain sections with Toluidine blue solution for 10-20 seconds on a mini hot plate before rinsing with MilliQ water and drying on a mini hot plate.

Toluidine blue stains nucleic acids and proteins, enhancing structural detail of semi thin tissue sections.
  - 23.4 Observe sections with a wide-field light microscope. If the region of interest has not been obtained, trim deeper and repeat Step 23.
- 24 Cut 90 nm gold coloured ultrathin sections at 1 mm/sec using a Leica UC7 ultramicrotome and a Diatome 45 degree angle ultra wet diamond knife. Spread the sections using a Max Wax heat pen to remove wrinkles.
- 25 Pick up sections on 50-200 mesh copper or palladium-coated copper grids using an eyelash or Dalmatian hair mounted on a wooden stick. Place grids section side up on a sheet of filter paper, cover with a glass petri dish and leave to dry for 10 minutes. Store grids in an enclosed grid box until use.

#### Contrasting ultrathin sections

- 26 Place a sheet of parafilm on a clean flat bench or glass board using a small amount of water underneath to keep the film flat.

- 27 For the following steps, use fine forceps or a perfect loop to transfer grids from one drop of solution to another, section side down. Use approximately 100-200  $\mu$ l drops for MilliQ water, and 50-100  $\mu$ l for uranyl acetate and lead citrate.

Stain sections with 0.6% filtered uranyl acetate in MilliQ water for 3 minutes.

Uranyl acetate enhances contrast of sections by interacting with proteins and lipids. The solution must be wrapped in foil and stored in a dark place to prevent precipitation from exposure to UV light.

- 28 Rinse 5 x 1 min in MilliQ water.

- 29 Stain sections with 0.1-0.4% filtered lead citrate in MilliQ water for 3 minutes.

Four drops of 10M sodium hydroxide should be added to the lead citrate stock solution prior to use to prevent lead citrate precipitating to form water-insoluble lead carbonate by exposure to carbon dioxide.

Lead citrate enhances contrast of sections by interacting with proteins and glycogens, and binding to osmium and uranyl acetate.

- 30 Rinse 5 x 1 min in MilliQ water.

- 31 Place grids section side up on a sheet of filter paper, cover with a glass petri dish and dry for 10 minutes. Store grids in a grid storage box.

- 32 Place a grid in a transmission electron microscope grid holder for high resolution imaging using a transmission electron microscope.

Example: JEOL JEM-1400-Plus TEM at 80 keV equipped with a high sensitivity bottom mount CMOS 'Flash' camera.