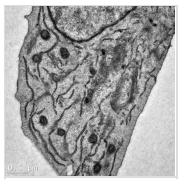

Version 1

Oct 13, 2022

Preparation of cells for transmission electron microscopy ultrastructural analysis V.1

Jillian C Danne[1], Simon A Crawford[1], Rachel Templin[1], Joan Clark[1], Viola Oorschot[2], Georg Ramm[1]

[1]Ramaciotti Centre for Cryo EM, Monash University, Melbourne, Australia;

[2]Electron Microscopy Core Facility, EMBL, Heidelberg, Germany

dx.doi.org/10.17504/protocols.io.j8nlk47ewg5r/v1

Jillian C Danne

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 acquired by a digital camera. Here, a protocol for preparing cells for TEM ultrastructural analysis is described. A cell sample is fixed, dehydrated and infiltrated with resin. The resin is then polymerised, and embedded cell sample 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.j8nlk47ewg5r/v1

PROTOCOL CITATION

Jillian C Danne, Simon A Crawford, Rachel Templin, Joan Clark, Viola Oorschot, Georg Ramm 2022. Preparation of cells for transmission electron microscopy ultrastructural analysis. **protocols.io** https://dx.doi.org/10.17504/protocols.io.j8nlk47ewg5r/v1

KEYWORDS

Transmission electron microscopy, Ultrastructure, Cells, Processing, Epon resin, Contrasting, Sectioning, Fixation, Microwave, Tissue

CREATED

Nov 03, 2021

LAST MODIFIED

Oct 13, 2022

PROTOCOL INTEGER ID

54773

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**

UltraPure™ Agarose 1000 **Thermo Fisher Scientific Catalog # 16550-100**

Ethanol Absolute AR grade 2.5L **AIM Scientific Catalog #AJA214-2.5L**

Molecular sieves 3 Å **Sigma Aldrich Catalog #208574-1KG**

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**

Acetone AR grade 2.5 L **AIM Scientific Catalog #AJA6-2.5L**

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**

Citation: Jillian C Danne, Simon A Crawford, Rachel Templin, Joan Clark, Viola Oorschot, Georg Ramm Preparation of cells for transmission electron microscopy ultrastructural analysis https://dx.doi.org/10.17504/protocols.io.j8nlk47ewg5r/v1

BRAND disposable microcentrifuge tubes, 1.5mL with safety lid

Brand **BR780400-450EA**

Falcon® Centrifuge Tubes

Polypropylene, Sterile, 15 mL

Corning® **352096**

Eppendorf™ 5810R Centrifuge

Centrifuge

Eppendorf **02-262-8187**

Rotary tube mixer

Ratek Instruments **RSM7DC**

Sprout plus mini centrifuge

Sprout **120610**

Falcon 6 well clear flat bottom TC-treated multiwell cell culture plate with lid

Falcon **FAL353046**

Citation: Jillian C Danne, Simon A Crawford, Rachel Templin, Joan Clark, Viola Oorschot, Georg Ramm Preparation of cells for transmission electron microscopy ultrastructural analysis https://dx.doi.org/10.17504/protocols.io.j8nlk47ewg5r/v1

Petri dish 100mm x 20mm
Greiner Bio-One **664160**

Gilson pipetman classic P1000
Gilson **1152009**

Parafilm M
Bemis **IA041**

Corning cell scraper
Corning **CLS3011**

Vortex Mixer
Ratek Instruments **147-VM1**

3ml graduated transfer pipette
Copan **200C**

Block heater
Ratek **DBH20D**

Citation: Jillian C Danne, Simon A Crawford, Rachel Templin, Joan Clark, Viola Oorschot, Georg Ramm Preparation of cells for transmission electron microscopy ultrastructural analysis https://dx.doi.org/10.17504/protocols.io.j8nlk47ewg5r/v1

Black teflon plate

N/A N/A

Fine Forceps

Forceps

Dumont 11251-10

Single edge carbon steel blade

Electron Microscopy Sciences 71960

Bite and boxing wax-500g

Investo (Lordell) WI-BB

Blade scalpel ST #11

Swann Morton 21016SM

Double edge blades

Personna 72000

Tube 5ml 5016 PP yellow cap GS

Pacific Laboratory Products P5016SU

Citation: Jillian C Danne, Simon A Crawford, Rachel Templin, Joan Clark, Viola Oorschot, Georg Ramm Preparation of cells for transmission electron microscopy ultrastructural analysis https://dx.doi.org/10.17504/protocols.io.j8nlk47ewg5r/v1

PELCO BioWave® Pro+ Microwave Processing System, 120VAC

Pelco **36700**

Embedding mould, single ended flat 21 cavities

ProSciTech **RL064**

BEEM® embedding capsules, size 00, PE, Pack/500

BEEM® **RB001-500**

Flat bottom embedding capsules, microwave safe

BEEM® **70021-PPT**

Oven MINO/6/CLAD

Genlab **N/A**

Olympus SZ30 stereo microscope

Olympus **SZ30-PS**

UC7 Ultramicrotome

Leica **EMUC7**

Citation: Jillian C Danne, Simon A Crawford, Rachel Templin, Joan Clark, Viola Oorschot, Georg Ramm Preparation of cells for transmission electron microscopy ultrastructural analysis https://dx.doi.org/10.17504/protocols.io.j8nlk47ewg5r/v1

Glass knife, 45 degree

Leica **N/A**

Ultra Semi diamond knife, 35 degree angle, 3 mm

Diatome **UltraSemi**

Glass knife boat, 6.4mm

Electron microscopy sciences **71008**

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**

Citation: Jillian C Danne, Simon A Crawford, Rachel Templin, Joan Clark, Viola Oorschot, Georg Ramm Preparation of cells for transmission electron microscopy ultrastructural analysis https://dx.doi.org/10.17504/protocols.io.j8nlk47ewg5r/v1

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**

Citation: Jillian C Danne, Simon A Crawford, Rachel Templin, Joan Clark, Viola Oorschot, Georg Ramm Preparation of cells for transmission electron microscopy ultrastructural analysis https://dx.doi.org/10.17504/protocols.io.j8nlk47ewg5r/v1

Filter paper, grade 1, 12.5cm

Whatman **1001-125**

Glass petri dish, 100mm

BRAND **BR455751**

Grid storage box, 100 grid capacity

Gilder **HL065**

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**

Citation: Jillian C Danne, Simon A Crawford, Rachel Templin, Joan Clark, Viola Oorschot, Georg Ramm Preparation of cells for transmission electron microscopy ultrastructural analysis https://dx.doi.org/10.17504/protocols.io.j8nlk47ewg5r/v1

Foil
N/A N/A

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 the appropriate personal protective equipment (PPE). The Material Safety Data Sheet (MSDS) for each chemical must be read before commencing.

Adherent cells grown in a well-plate or petri dish must be submerged in solution at all times to prevent desiccation.

Cells suspended in culture media in 1.5 ml microcentrifuge or 15 ml Falcon® tubes require centrifuging at 1200 rpm for 2 minutes prior to fixation and following Steps 1-4. After removal of the supernatant, cell pellets should be gently resuspended in solution using a pipette.

Adherent cells:
Remove cell culture media from the well-plate or petri dish and gently add Karnovsky's fixative, 2% PFA, 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2). Seal the well-plate or petri dish lid with parafilm, and fix the cells for 2 hours at room temperature or overnight at 4 degrees Celsius, agitating.

Suspended cells:

Citation: Jillian C Danne, Simon A Crawford, Rachel Templin, Joan Clark, Viola Oorschot, Georg Ramm Preparation of cells for transmission electron microscopy ultrastructural analysis https://dx.doi.org/10.17504/protocols.io.j8nlk47ewg5r/v1

Remove cell culture media from the 1.5 ml microcentrifuge or 15 ml Falcon® tube using a pipette and gently resuspend the cell pellet in 1 ml of Karnovsky's fixative, 2% PFA, 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2). Fix cells for 2 hours at room temperature or overnight at 4 degrees Celsius, agitating.

2 Remove fixative and wash with 0.1M cacodylate buffer, 3 x 10 minutes at room temperature, agitating.

3 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 artifactual deposits. Samples must be washed thoroughly (Step 2) prior to post-fixation (Step 3) 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.

Post fix cells in 1% Osmium tetroxide, 1.5% potassium ferrocyanide in 0.1M cacodylate buffer for 1 hour at room temperature, agitating. Well-plate, petri dish and tube lids must be well-secured during fixation.

4 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. If handling suspended cells, proceed to Step 6.

5 Gently detach adherent cells from the bottom of the well-plate/petri dish using a cell scraper.

6 Transfer cells to a 1.5 ml microcentrifuge tube using a 3 ml or 1000 µl transfer pipette, and pellet cells at 1200 rpm for 2 minutes using a benchtop centrifuge.

Agarose embedding

7 Melt 4% aqueous ultra-pure agarose using a Bain Marie water bath containing boiling water or a heat block set to 95 degrees Celsius. Ultra-pure agarose solidifies at ≤ 32.5 - 38 degrees Celsius and melts at ≥ 90 degrees Celsius.

8 Remove the supernatant and add approximately 100 µl (2 drops) of the pre-warmed 4% aqueous agarose to the cell pellet.

Citation: Jillian C Danne, Simon A Crawford, Rachel Templin, Joan Clark, Viola Oorschot, Georg Ramm Preparation of cells for transmission electron microscopy ultrastructural analysis https://dx.doi.org/10.17504/protocols.io.j8nlk47ewg5r/v1

9 Gently vortex the sample to homogeneously resuspend the cell pellet in pre-warmed 4% aqueous agarose.

10 Optionally, to obtain a concentrated cell pellet, return the microcentrifuge tube containing cells in 4% aqueous agarose to the Bain Marie or heat block until the agarose liquefies, and then pellet cells using a benchtop microcentrifuge.

11 Solidify the 4% aqueous agarose with cells by cooling at 4 degrees Celsius for 10 minutes.

12 Cut off the extreme tip of the microcentrifuge tube (avoiding the cells) using a razor blade and push the sample out of the tube using a wooden stick.

13 Place the agarose embedded cell block on a Teflon plate or dental wax sheet and cut the block in half using a scalpel or double edged razor blade. Cut each block half into strips and then cut each strip into cubes no larger than $1mm^3$. Carefully place the agarose-embedded cell cubes into a 5ml tube using forceps.

Dehydration and resin infiltration

14 For Steps 14-27, 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.

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.

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

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

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

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

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

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

Citation: Jillian C Danne, Simon A Crawford, Rachel Templin, Joan Clark, Viola Oorschot, Georg Ramm Preparation of cells for transmission electron microscopy ultrastructural analysis https://dx.doi.org/10.17504/protocols.io.j8nlk47ewg5r/v1

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

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

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

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

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

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

20 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.

21 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 21-25), but is extremely toxic and may dehydrate samples too intensively, causing ultrastructural damage.

22 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.

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

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

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

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

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

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

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

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

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

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

Citation: Jillian C Danne, Simon A Crawford, Rachel Templin, Joan Clark, Viola Oorschot, Georg Ramm Preparation of cells for transmission electron microscopy ultrastructural analysis https://dx.doi.org/10.17504/protocols.io.j8nlk47ewg5r/v1

Residual acetone can adversely impact resin infiltration and polymerisation. A third 100% resin infiltration step may be required to prevent this.

28 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

29 Mount the resin-embedded cell 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 cell block into a trapezoid shape using a double edge razor blade.

30 Transfer the chuck and resin-embedded cell block to a Leica UC7 ultramicrotome and secure in place. Trim the front surface of the block face until a full face of cells has been obtained. Trim manually using a 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.

31 To check the region of interest:

31.1 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.

31.2 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.

31.3 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 cell sections.

31.4 Observe sections with a wide-field light microscope. If the region of interest has not been obtained, trim deeper and repeat Step 31.

32 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 prevent wrinkles.

33 Pick up sections on 50-200 mesh copper or palladium-coated copper grids. 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

Citation: Jillian C Danne, Simon A Crawford, Rachel Templin, Joan Clark, Viola Oorschot, Georg Ramm Preparation of cells for transmission electron microscopy ultrastructural analysis https://dx.doi.org/10.17504/protocols.io.j8nlk47ewg5r/v1

34 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.

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 µl drops for MilliQ water, and 50-100 µl for uranyl acetate and lead citrate.

35 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.

36 Rinse 5 x 1 min in MilliQ water.

37 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.

38 Rinse 5 x 1 min in MilliQ water.

39 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.

40 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.

Citation: Jillian C Danne, Simon A Crawford, Rachel Templin, Joan Clark, Viola Oorschot, Georg Ramm Preparation of cells for transmission electron microscopy ultrastructural analysis https://dx.doi.org/10.17504/protocols.io.j8nlk47ewg5r/v1