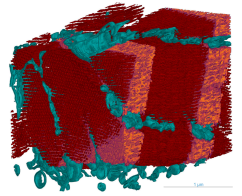




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🌐 Preparation of Tissue for Volume Electron Microscopy using Focused Ion Beam Scanning Electron Microscopy (FIB-SEM)



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Jillian C Danne¹, Rachel Templin¹, Gediminas Gervinskas¹, Denis Korneev¹, Sergey Gorelick¹, Georg Ramm¹

¹Ramaciotti Centre for CryoEM, Monash University, Melbourne, Australia



Jillian C Danne

Monash University

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We use this protocol and it's working

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Abstract

Volume Electron Microscopy (vEM) allows for the three-dimensional imaging of biological matter and to observe cellular structures at the nanometer scale. This protocol details the preparation of tissue specimens for Focused Ion Beam Scanning Electron Microscopy (FIB-SEM). FIB-SEM gives the highest resolution in z direction compared to other vEM techniques. Images are obtained by stepwise slicing of a specimen using the ion beam while imaging successive surfaces by scanning electron microscopy. Good structural preservation and electron contrast are essential, and this protocol details the required preparatory steps for this up to (but not including) the slice and view imaging in the FIB-SEM.




Materials


- ✕ Glutaraldehyde 25% Aqueous Solution 10 × 10 ml ampoules **Electron Microscopy Sciences Catalog #16220**
- ✕ Paraformaldehyde, 16% (wt/vol) **Electron Microscopy Sciences Catalog #15710**
- ✕ 0.2M Sodium Cacodylate Trihydrate Buffer pH 7.4
- ✕ 0.1M Sodium Cacodylate Trihydrate Buffer pH 7.4
- ✕ Osmium tetroxide 10 × 1g ampoule **ProSciTech Catalog #C010-1010**
- ✕ Potassium hexacyanoferrate(II) trihydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P3289-100G**
- ✕ MilliQ Water
- ✕ Thiocarbohydrazide **Merck Catalog #223220-5g**
- ✕ Uranyl acetate **Electron Microscopy Sciences Catalog #22400**
- ✕ L-Aspartic acid **Merck Catalog #A9256-100g**
- ✕ Lead (II) Nitrate **Merck Catalog #228621-100g**
- ✕ Potassium hydroxide ACS reagent, ≥85%, pellets **Merck MilliporeSigma (Sigma-Aldrich) Catalog #221473-500G**
- ✕ 25% Ethanol in MilliQ water
- ✕ 50% Ethanol in MilliQ water
- ✕ 75% Ethanol in MilliQ water
- ✕ 90% Ethanol in MilliQ water
- ✕ Ethanol Absolute AR grade 2.5L **AIM Scientific Catalog #AJA214-2.5L**
- ✕ Molecular sieves 3 Å **Merck MilliporeSigma (Sigma-Aldrich) Catalog #208574-1KG**
- ✕ Acetone AR grade 2.5L **AIM Scientific Catalog #AJA6-2.5LPL**
- ✕ Hard epon resin
- ✕ Toluidine blue **Merck MilliporeSigma (Sigma-Aldrich)**
- ✕ PC-Clear liquid epoxy **Bunnings Catalog #1560386**
- ✕ Conductive silver liquid, SEM adhesive paint **ProSciTech Catalog #I004**
- ✕ Conductive carbon/graphite paint, SEM adhesive 154 **ProSciTech Catalog #I003**

Equipment	
Black teflon plate	NAME
N/A	BRAND
N/A	SKU

Equipment	
Bite and boxing wax-500g	NAME
Investo (Lordell)	BRAND
WI-BB	SKU

Equipment	
Single Edge Carbon Steel Razor	NAME
blade	TYPE
EMS	BRAND
71960	SKU
https://www.emsdiasum.com/c-single-edge-carbon-steel ^{LINK}	
	

Equipment	
Double edge blades	NAME
Personna	BRAND
72000	SKU

Equipment	
Fine Forceps	NAME
Forceps	TYPE
Dumont	BRAND
11251-10	SKU
https://www.finescience.com/en-US/Products/Forceps-Hemostats/Dumont-Forceps/Dumont-5-Forceps/11251-10	LIN K
	

Equipment

Blade scalpel ST #11

NAME

Swann Morton

BRAND

21016SM

SKU

Equipment

Tube 5ml 5016 PP yellow cap GS

NAME

Pacific Laboratory Products

BRAND

P5016SU

SKU

Equipment

Falcon® Centrifuge Tubes

NAME

Polypropylene, Sterile, 15 mL

TYPE

Corning®

BRAND

352096

SKU

Equipment

Falcon 6 well clear flat bottom TC-treated multiwell cell culture plate with lid		NAME
Falcon		BRAND
FAL353046		SKU

Equipment

Rotary tube mixer		NAME
Ratek Instruments		BRAND
RSM7DC		SKU

Equipment

Parafilm M		NAME
Bemis		BRAND
IA041		SKU

Equipment

PELCO BioWave® Pro+ Microwave Processing System, 120VAC^{NAME}

Pelco

BRAND

36700

SKU

Equipment

Embedding mould, single ended flat 21 cavities^{NAME}

ProSciTech

BRAND

RL064

SKU

Equipment

Flat bottom embedding capsules, microwave safe^{NAME}

BEEM®

BRAND

70021-PPT

SKU

Equipment

BEEM® embedding capsules, size OO, PE, Pack/500^{NAME}

BEEM®^{BRAND}

RB001-500^{SKU}

Equipment

3ml graduated transfer pipette^{NAME}

Copan^{BRAND}

200C^{SKU}

Equipment

Oven MINO/6/CLAD^{NAME}

Genlab^{BRAND}

N/A^{SKU}

Equipment

Jewellers saw, 125mm cutting depth	NAME
Olson	BRAND
SF63525	SKU

Equipment

Olympus SZ30 stereo microscope	NAME
Olympus	BRAND
SZ30-PS	SKU

Equipment

UC7 Ultramicrotome	NAME
Leica	BRAND
EMUC7	SKU

Equipment

Glass knife, 45 degree angle

NAME

Leica

BRAND

N/A

SKU

Equipment

Glass knife boat, 6.4mm

NAME

Electron microscopy sciences

BRAND

71008

SKU

Equipment

Trim 90 diamond knife

NAME

DIATOME

BRAND

T1889

SKU

Equipment

Eyelash or Dalmatian hair mounted on a wooden stick NAME

N/A BRAND

N/A SKU

Equipment

Plain glass slides 76mm x 39mm x 1.0-1.2mm NAME

Thermo Scientific BRAND

AGL4222A SKU

Equipment

Mini hot plate NAME

Thermofisher BRAND

HP2310BQ SKU

Equipment

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

Equipment

Dry block heater	NAME
Ratek	BRAND
DBH4000D	SKU

Equipment

Foil	NAME
N/A	BRAND
N/A	SKU

Equipment

SEM specimen pin mount, 12.7mm diameter x 6mm	NAME
ProSciTech	BRAND
GTP16111-9	SKU

Equipment

Aluminium rod, 10 mm x 3 mm	NAME
N/A	BRAND
N/A	SKU

Equipment

EM ACE600 Sputter coater	NAME
Leica	BRAND
EM ACE600	SKU

Equipment

Helios UX/UC/HP G4 focussed ion beam scanning electron microscope	NAME
ThermoFisher Scientific	BRAND
1225989	SKU

Troubleshooting

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.

Thiocarbohydrazide and Osmium tetroxide are 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.

Lead nitrate is acutely toxic, corrosive and may cause damage to internal organs or an unborn child.

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

Epon resin may cause serious skin and eye irritation.

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 7.4). 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 × 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.

The following Steps 4-29 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 tissue in 2% osmium tetroxide in 0.1M cacodylate buffer for 1.5 hours at room temperature, agitating. Tube and well-plate lids must be well-secured during fixation.

The microwave regime described below (Steps 4, 5, 7, 9, 11, 13) cycles between an on and off step, repeating 3 times. The microwave off step prevents overheating of the sample.

Biowave settings: Repeat cycle 3 times:

2 mins microwave 100W, Vacuum on, at room temperature

2 mins microwave off, Vacuum on, at room temperature

Osmium tetroxide stains and fixes lipids by oxidising unsaturated fatty acid bonds. It penetrates deeper into a sample when it is not reduced in solution by potassium ferrocyanide or potassium ferricyanide.

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

Biowave settings: Repeat cycle 3 times:

2 mins microwave 100W, Vacuum on, at room temperature

2 mins microwave off, Vacuum on, at room temperature

Potassium ferrocyanide is a reducing agent that improves contrast of biological samples by enhancing the amount of electron dense osmium deposited in membranes. When used in combination with osmium, osmium becomes reduced and the post fixation solution turns dark brown. Reduced osmium improves membrane and glycogen contrast with limited penetration, and may also prevent the formation of artefactual precipitates. Potassium ferricyanide can be used as an alternative to potassium ferrocyanide and remains a clear, amber colour if mixed with osmium.

- 6 Wash in MilliQ water, 3 × 10 minutes at room temperature, agitating.

Biowave settings: 3 × 40 seconds, 150W, Vacuum off, at room temperature.

- 7 Incubate the tissue in 1% Thiocarbohydrazide in MilliQ water for 45 mins at 40 degrees Celsius, agitating. Tube and well-plate lids must be well-secured during this step.

Biowave settings: Repeat cycle 3 times:

2 mins microwave 150W, Vacuum on, at 40 degrees Celsius

2 mins microwave off, Vacuum on, at 40 degrees Celsius

Thiocarbohydrazide binds to osmium and acts as a bridge for the binding of additional osmium in Step 9, thereby enhancing contrast of lipid membranes. The increase in osmium content also makes tissue more conductive.

- 8 Wash in MilliQ water, 3 × 10 minutes at room temperature, agitating.

Biowave settings: 3 × 40 seconds, 150W, Vacuum off, at room temperature.

- 9 Post fix tissue in 2% osmium tetroxide in MilliQ water for 1.5 hours at room temperature, agitating. Tube or well-plate lids must be well-secured during fixation.

Biowave settings: Repeat cycle 3 times:

2 mins microwave 100W, Vacuum on, at room temperature

- 2 mins microwave off, Vacuum on, at room temperature
- 10 Wash in MilliQ water, 3 × 10 minutes at room temperature, agitating.
- Biowave settings: 3 × 40 seconds, 150W, Vacuum off, at room temperature.
- 11 *En bloc* stain tissue with 2% uranyl acetate in MilliQ water overnight at 4 degrees Celsius and then for 2 hours at 50 degrees Celsius, agitating the following day.
- Alternatively, *en bloc* stain tissue with 2% uranyl acetate in MilliQ water overnight at 4 degrees Celsius and then Biowave using the following settings:
- Biowave settings: Repeat cycle 3 times:
2 mins microwave 150W, Vacuum on, at 50 degrees Celsius
2 mins microwave off, Vacuum on, at 50 degrees Celsius
- Uranyl acetate enhances contrast of tissue 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.
- 12 Wash in MilliQ water, 3 × 10 minutes at room temperature, agitating.
- Biowave settings: 3 × 40 seconds, 150W, Vacuum off, at room temperature.
- 13 Make up a solution of Walton's lead aspartate by dissolving 0.998 g L-aspartic acid into 250 ml MilliQ water and adjust the pH to 3.8 to allow the L-aspartic acid to dissolve more easily. Dissolve 0.066 g lead nitrate in 10 ml L-aspartic acid solution, adjust the pH to 5.5 with potassium hydroxide and stabilise the stain by heating to 60 degrees Celsius for 30 minutes prior to use. L-aspartic acid can be stored for 2 months at 4 degrees Celsius.
- En bloc* stain tissue with freshly made lead aspartate for 2 hours at 50 degrees Celsius, agitating.
- Biowave settings: Repeat cycle 3 times:
2 mins microwave 150W, Vacuum on, at 50 degrees Celsius
2 mins microwave off, Vacuum on, at 50 degrees Celsius
- Lead aspartate increases sample conductivity and enhances contrast of tissue by interacting with nucleic acids, proteins and osmium. Unlike lead citrate, *en bloc* use of lead aspartate does not readily produce electron dense precipitates from exposure to carbon dioxide. Samples contrasted with lead aspartate do not require additional contrasting using lead citrate.
- 14 Wash in MilliQ water, 3 × 10 minutes at room temperature, agitating.

Biowave settings: 3 × 40 seconds, 150W, Vacuum off, at room temperature.

Dehydration and resin infiltration

- 15 For Steps 15-29, 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 25% ethanol in MilliQ water for 15 mins at room temperature, agitating.

Biowave settings: 40 seconds, 150W, No vacuum, at room temperature.

- 16 Dehydrate in 50% ethanol in MilliQ water for 15 mins at room temperature, agitating.

Biowave settings: 40 seconds, 150W, No vacuum, at room temperature.

- 17 Dehydrate in 75% ethanol in MilliQ water for 15 mins at room temperature, agitating.

Biowave settings: 40 seconds, 150W, No vacuum, at room temperature.

- 18 Dehydrate in 90% ethanol in MilliQ water for 15 mins at room temperature, agitating.

Biowave settings: 40 seconds, 150W, No vacuum, at room temperature.

- 19 Dehydrate in 100% ethanol for 15 mins at room temperature, agitating.

Biowave settings: 40 seconds, 150W, No vacuum, at room temperature.

- 20 Dehydrate in 100% anhydrous ethanol for 15 mins 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, at room temperature.

- 21 Dehydrate in 100% acetone for 15 mins at room temperature, agitating.

Biowave settings: 40 seconds, 150W, No vacuum, at room temperature.

- 22 Dehydrate in 100% anhydrous acetone for 15 mins 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, at room temperature.
- 23 Infiltrate with 25% hard Epon resin in 100% anhydrous acetone overnight at room temperature, on a rotor.
- Biowave settings: 3 minutes, 250W, Vacuum on, at room temperature.
- 24 Infiltrate with 50% hard Epon resin in 100% anhydrous acetone for 6 hours at room temperature, on a rotor.
- Biowave settings: 3 minutes, 250W, Vacuum on, at room temperature.
- 25 Infiltrate with 75% hard Epon resin in 100% anhydrous acetone overnight at room temperature, on a rotor.
- Biowave settings: 3 minutes, 250W, Vacuum on, at room temperature.
- 26 Infiltrate with 100% hard Epon resin for 6 hours at room temperature, on a rotor.
- Biowave settings: 3 minutes, 250W, Vacuum on, at room temperature.
- 27 Infiltrate with 100% hard Epon resin overnight at room temperature, on a rotor.
- Biowave settings: 3 minutes, 250W, Vacuum on, at room temperature.
- 28 Infiltrate with 100% hard Epon resin for 6 hours at room temperature, on a rotor.
- Biowave settings: 3 minutes, 250W, Vacuum on, at room temperature.
- 29 Infiltrate with 100% hard Epon resin overnight at room temperature, on a rotor.
- Biowave settings: 3 minutes, 250W, Vacuum on, at room temperature.
- 30 Transfer samples to labelled plastic embedding moulds, flat bottom BEEM® capsules or standard tip BEEM® capsules containing 100% hard Epon resin.
- 31 Polymerise the resin in an oven set to 60 degrees Celsius for 48 hours.

Sample mounting and trimming

- 32 Cut the tissue sample out of the resin block using a jewellers saw or razor blade.
- A fume hood must be used when sawing resin to prevent inhalation of fine resin particles.

- 33 Mount the resin embedded tissue block onto an aluminium SEM specimen pin mount or aluminium rod (10 mm length x 3 mm diameter) using hard Epon resin or fast set liquid epoxy glue. Ensure the region of interest is facing up and the sample is mounted in the correct orientation for imaging.
- 34 Polymerise the resin overnight in an oven set to 60 degrees Celsius.
- 35 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 top face of the block to approach the region of interest using a double edged razor blade.
- 36 Transfer the chuck and resin-embedded tissue block to a Leica UC7 ultramicrotome and secure in place. Trim the top surface of the block face until a smooth surface no more than 5 μm from the region of interest 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 90 degree angle trimming diamond knife.
- 37 To check the region of interest:
 - 37.1 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.
 - 37.2 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.
 - 37.3 Optionally, 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.

Sections may not require staining because they have been *en bloc* stained (Steps 11 and 13).

Toluidine blue stains nucleic acids and proteins, enhancing structural detail of semi thin tissue sections.
 - 37.4 Observe sections with a wide-field light microscope. If you are not within 5 μm of the region of interest, trim deeper and repeat Step 37.
- 38 To create an imaging surface perpendicular to the top surface, trim away the right or left side of the tissue block to within 5 μm of the region of interest, cutting 100 μm deep. Trim using an 0.5 μm feed and a suitable diamond knife, for example a Diatome



90 degree angle trimming diamond knife. This step can be excluded if you plan to prepare your imaging surface using a FIB-SEM.

39 Coat all uncut surfaces of the tissue block with conductive silver liquid or carbon/graphite paint, taking care not to coat the top and perpendicular imaging surfaces trimmed with the diamond knife (Steps 36 and 38).

40 Sputter coat the entire tissue block and specimen pin mount or aluminium rod with 20 nm of gold using a sputter coater.

For example, Leica EM ACE600 high vacuum sputter coater, equipped with 3 axis motorised stage with planetary drive.

41 Place the coated tissue block on specimen pin mount or aluminium rod in a focused ion beam scanning electron microscope for sequential milling and high resolution imaging.

Example: TFS Helios 5 UX FIB-SEM, equipped with slice and view software.



Protocol references

Citation

Walton, J. (1979). Lead asparate, an en bloc contrast stain particularly useful for ultrastructural enzymology. *Journal of Histochemistry & Cytochemistry*.

<https://doi.org/10.1177/27.10.512319>

LINK

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Tapia, J. C., Kasthuri, N., Hayworth, K. J., Schalek, R., Lichtman, J. W., Smith, S. J., & Buchanan, J. (2012) . High-contrast en bloc staining of neuronal tissue for field emission scanning electron microscopy. *Nature Protocols*.

<https://doi.org/10.1038/nprot.2011.439>

LINK

Citation

Hua Y, Laserstein P, Helmstaedter M (2015) . Large-volume en-bloc staining for electron microscopy-based connectomics..

<https://doi.org/10.1038/ncomms8923>

LINK



Citation

Seligman, A. M., Wasserkrug, H. L., & Hanker, J. S.

. A new staining method (OTO) for enhancing contrast of lipid-containing membranes and droplets in osmium tetroxide-fixed tissue with osmiophilic thiocarbonyhydrazide (TCH)..

The Journal of Cell Biology.

<https://doi.org/10.1083/jcb.30.2.424>

LINK

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Walton, J. . Lead aspartate, an en bloc contrast stain particularly useful for ultrastructural enzymology

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Tapia, J. C., Kasthuri, N., Hayworth, K. J., Schalek, R., Lichtman, J. W., Smith, S. J., & Buchanan, J. . High-contrast en bloc staining of neuronal tissue for field emission scanning electron microscopy

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<https://doi.org/10.1083/jcb.30.2.424>