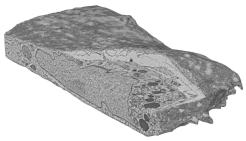
Feb 01, 2024

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



DOI
dx.doi.org/10.17504/protocols.io.n92ldmeq7l5b/v1

Jillian C Danne[1], Rachel Templin[1], Gediminas Gervinskas[1], Denis Korneev[1], Sergey Gorelick[1], Georg Ramm[1]

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

Jillian C Danne
Monash University

DOI: https://dx.doi.org/10.17504/protocols.io.n92ldmeq7l5b/v1

Protocol Citation: Jillian C Danne, Rachel Templin, Gediminas Gervinskas, Denis Korneev, Sergey Gorelick, Georg Ramm 2024. Preparation of Cells for Volume Electron Microscopy using Focused Ion Beam Scanning Electron Microscopy (FIB-SEM). **protocols.io https://dx.doi.org/10.17504/protocols.io.n92ldmeq7l5b/v1**

Protocol status: Working
We use this protocol and it's working

Created: January 17, 2024

Last Modified: February 01, 2024

Protocol Integer ID: 93635

Keywords: Processing, Volume Electron Microscopy, Focused Ion Beam Scanning Electron Microscopy, Epon Resin, Embedding, Fixation, Dehydration, Cells, Ultramicrotomy, cells for volume electron microscopy, using focused ion beam scanning electron microscopy, focused ion beam scanning electron microscopy, volume electron microscopy, electron microscopy, scanning electron microscopy, preparation of cellular specimen, cellular structures at the nanometer scale, dimensional imaging of biological matter, cellular specimen, cell, cellular structure, electron contrast, ion beam, dimensional imaging, imaging, nanometer scale, imaging successive surface, sem

Funders Acknowledgements:
Chan Zuckerberg Initiative DAF, an advised fund of Silicon Valley Community Foundation (funder DOI 10.13039/100014989)
Grant ID: DAF2021-225399 DOI https://doi.org/10.37921/334038myxhsa

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 cellular 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

- Paraformaldehyde, 16% (wt/vol) **Electron Microscopy Sciences Catalog #**15710
- Glutaraldehyde 25% Aqueous Solution 10 × 10 ml ampoules **Electron Microscopy Sciences Catalog #**16220
- Sodium Cacodylate Trihydrate 100g **ProSciTech Catalog #**C020
- 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
- UltraPure™ Agarose 1000 **Thermo Fisher Scientific Catalog #** 16550-100
- 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 2.5 L **AIM Scientific Catalog #**AJA6-2.5L
- Hard epon resin **Catalog #**N/A
- Toluidine blue **Merck MilliporeSigma (Sigma-Aldrich)**
- Liquid nitrogen
- 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

BRAND disposable microcentrifuge tubes, 1.5mL with safety lid
NAME

Brand
BRAND

BR780400-450EA
SKU

Equipment

Falcon® Centrifuge Tubes
NAME

Polypropylene, Sterile, 15 mL
TYPE

Corning®
BRAND

352096
SKU

Equipment

Eppendorf™ 5810R Centrifuge
NAME

Centrifuge
TYPE

Eppendorf
BRAND

02-262-8187
SKU

https://www.fishersci.com/shop/products/eppendorf-5810r-centrifuge-rotor-packages-16/022628187
LINK

Equipment

Rotary tube mixer

NAME

Ratek Instruments

BRAND

RSM7DC

SKU

Equipment

Sprout plus mini centrifuge

NAME

Sprout

BRAND

120610

SKU

Equipment

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

NAME

Falcon

BRAND

FAL353046

SKU

Equipment

Petri dish 100mm x 20mm

NAME

Greiner Bio-One

BRAND

664160

SKU

Equipment

Gilson pipetman classic P1000

NAME

Gilson

BRAND

1152009

SKU

Equipment

Parafilm M

NAME

Bemis

BRAND

IA041

SKU

Equipment

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

Pelco
BRAND

36700
SKU

Equipment

Dry block heater
NAME

Ratek
BRAND

DBH4000D
SKU

Equipment

Corning cell scraper
NAME

Corning
BRAND

CLS3011
SKU

Equipment

3ml graduated transfer pipette

NAME

Copan

BRAND

200C

SKU

Equipment

Vortex Mixer

NAME

Ratek Instruments

BRAND

147-VM1

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	LINK

Equipment

Single edge carbon steel blade	NAME
Electron Microscopy Sciences	BRAND
71960	SKU

Equipment

Bite and boxing wax-500g

NAME

Investo (Lordell)

BRAND

WI-BB

SKU

Equipment

Blade scalpel ST #11

NAME

Swann Morton

BRAND

21016SM

SKU

Equipment

Double edge blades

NAME

Personna

BRAND

72000

SKU

Equipment

Black teflon plate

NAME

N/A

BRAND

N/A

SKU

Equipment

Tube 5ml 5016 PP yellow cap GS

NAME

Pacific Laboratory Products

BRAND

P5016SU

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

Oven MINO/6/CLAD
NAME

Genlab
BRAND

N/A
SKU

Equipment

Petri dish 100mm x 20mm

NAME

Greiner Bio-One

BRAND

664160

SKU

Equipment

Jewellers saw, 125mm cutting depth

NAME

Olson

BRAND

SF63525

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

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

Foil
NAME

N/A
BRAND

N/A
SKU

Equipment

M125 Stereo microscope
NAME

Leica
BRAND

M125
SKU

Equipment

IC80 HD camera

NAME

Leica

BRAND

IC80 HD

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 and 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.

Adherent cells grown in a well-plate or petri dish must be submerged in solution at all times to prevent desiccation. Adherent cells can either be scraped and then processed as an agarose embedded block, or kept intact and processed as a single cell monolayer.

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 1-13. 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% paraformaldehyde (PFA), 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4). 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:
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. 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 × 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 artefactual deposits. Samples must be washed thoroughly (Step 2) prior to post-fixation (Step 3) to avoid this.

The following Steps 3 - 13 and Steps 24-38 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 cells in 2% osmium tetroxide in 0.1M cacodylate buffer for 30 mins at room temperature, agitating. Well-plate, petri dish and tube lids must be well-secured during fixation.

The microwave regime described below (Steps 3, 4, 6, 8, 10, 12) 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.

4 Post fix the cells in 1.5% potassium ferrocyanide in 0.1M cacodylate buffer for 30 mins 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 artifactual precipitates. Potassium ferricyanide can be used as an alternative to potassium ferrocyanide and remains a clear, amber colour if mixed with osmium.

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

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

6 Incubate cells in 1% Thiocarbohydrazide in MilliQ water for 20 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 8, thereby enhancing contrast of lipid membranes. The increase in osmium content also makes cells more conductive.

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

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

8 Post fix cells in 2% Osmium tetroxide in MilliQ water for 30 mins 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

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

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

10 *En bloc* stain cells with 2% uranyl acetate in MilliQ water overnight at 4 degrees Celsius and then for 1 hour at 50 degrees Celsius, agitating the following day.

Alternatively, *en bloc* stain cells 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 cells 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.

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

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

12 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 cells with freshly made lead aspartate for 1 hour 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.

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

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

14 Proceed to Step 15 for embedding of adherent cells into an agarose block, and to Step 17 for embedding of cells in suspension into an agarose block.

Proceed to Step 24 for the processing of intact cell monolayers.

Agarose embedding (optional)

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

16 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.

17 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.

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

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

20 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 the cells using a benchtop microcentrifuge.

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

22 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.

23 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 1 mm^3. Carefully place the agarose-embedded cell cubes into a 5 ml tube using forceps.

Dehydration and resin infiltration

24 For Steps 24-38, 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.

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

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

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

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

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

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

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

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

29 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.

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

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

31 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.

32 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.

33 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.

34 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.

35 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.

36 Infiltrate with 100% hard Epon resin overnight at room temperature, on a rotor.

Biowave settings: 3 minutes, 250W, Vacuum on, at room temperature.

37 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.

38 Infiltrate with 100% hard Epon resin overnight at room temperature, on a rotor.

Biowave settings: 3 minutes, 250W, Vacuum on, at room temperature.

39 Transfer samples to labelled plastic embedding moulds, flat bottom BEEM® capsules or standard tip BEEM® capsules containing 100% hard Epon resin.

For cell monolayers, embed samples in a petri dish by inverting a resin-filled flat bottom BEEM® capsule on top of the cells.

40 Polymerise the resin in an oven set to 60 degrees Celsius for 48 hours.

Sample mounting and trimming

41 Proceed to Step 43 for mounting and trimming of agarose embedded cells.

42 Transfer the petri dish containing the embedded cell monolayer and inverted BEEM® capsule into liquid nitrogen briefly using the appropriate PPE, and snap off the BEEM® capsule from the dish.

43 Cut the cell 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.

44 Mount the resin embedded cell monolayer or agarose cell 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 cell monolayer is facing up.

45 Polymerise the resin overnight in an oven set to 60 degrees Celsius.

46 For cell monolayers, proceed to Step 51.

47 Mount the resin-embedded agarose cell 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 cells using a double edged razor blade.

48 Transfer the chuck and resin-embedded agarose cell block to a Leica UC7 ultramicrotome and secure in place. Trim the top surface of the block face until a smooth surface through the cell sample 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.

49 To check the region of interest:

49.1 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.

49.2 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.

49.3 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 10 and 12).

Toluidine blue stains nucleic acids and proteins, enhancing structural detail of semi thin cell sections.

49.4 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 49.

50 To create an imaging surface perpendicular to the top surface, trim away the right or left side of the cell block to within 5 um 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.

51 For cell monolayers, coat all four sides of the resin block with conductive silver liquid or carbon/graphite paint, taking care not to coat the top imaging surface. Proceed to Step 52.

For agarose embedded cell blocks, coat all uncut surfaces 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 48 and 50). Proceed to Step 53.

52 Optionally, image the block surface using a stereo microscope mounted with a camera. The image can be used to help locate specific cells within the monolayer using a focused ion beam scanning electron microscope (Step 54).

53 Sputter coat the entire cell 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.

54 Place the coated cell block or cell monolayer on specimen pin mount or aluminium rod in a focused ion beam scanning electron microscope for preparation of the cell surface 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

Citation

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 thiocarbohydrazide (TCH). .
The Journal of cell biology.

https://doi.org/10.1083/jcb.30.2.424 LINK

Citations

Walton, J. . Lead asparate, an en bloc contrast stain particularly useful for ultrastructural enzymology
https://doi.org/10.1177/27.10.512319

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

Hua Y, Laserstein P, Helmstaedter M. Large-volume en-bloc staining for electron microscopy-based connectomics.
https://doi.org/10.1038/ncomms8923

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 thiocarbohydrazide (TCH).
https://doi.org/10.1083/jcb.30.2.424