May 13, 2025

Microwave assisted sample processing with cultured cells for 3D FIB-SEM

The Journal of Cell Biology

DOI
https://dx.doi.org/10.17504/protocols.io.36wgq691klk5/v1

Benjamin Padman[1]

[1]UWA

Benjamin Padman
UWA

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DOI: https://dx.doi.org/10.17504/protocols.io.36wgq691klk5/v1

External link: https://doi.org/10.1083/jcb.202411138

Protocol Citation: Benjamin Padman 2025. Microwave assisted sample processing with cultured cells for 3D FIB-SEM. **protocols.io** https://dx.doi.org/10.17504/protocols.io.36wgq691klk5/v1

Manuscript citation:
Padman BS, Lindblom RS, Lazarou M (2025) AI-directed voxel extraction and volume EM identify intrusions as sites of mitochondrial contact. The Journal of Cell Biology 224(10). doi: 10.1083/jcb.202411138

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

Created: March 27, 2025

Last Modified: May 13, 2025

Protocol Integer ID: 125418

Keywords: FIB-SEM, EM sample processing, sem routine procedure for microwave, sample processing of biological tiss, sample processing with cultured cell, biological tiss, sem routine procedure, microwave, 3d fib, cultured cell, sem, assisted sample processing

Funders Acknowledgements:
Aligning Science Across Parkinson's (ASAP)
Grant ID: ASAP-000350

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Abstract

Routine procedure for microwave assisted sample processing of biological tisses for 3D FIB-SEM.

Materials

Ultrapure or MilliQ water
16% paraformaldehyde (EM-grade)
25% Glutraldehyde (EM-grade)
0.2 M Phosphate buffer (pH7.2)
0.2 M sodium cacodylate (pH7.4)
4% (w/v) Osmium Tetroxide (OsO_4; aqueous)
Potassium Ferricyanide ($K_3Fe(CN)_6$; solid)
Thiocarbohydrazide (solid)
4% Uranyl acetate (aqueous)
Walton's Lead Aspartate
Ethanol
Propylene oxide
Araldite 502
Embed 812
NMA (nadic methyl anhydride)
DDSA (dodecenylsuccinic anhydride)
BDMA (benzyldimethylamine)

Equipment

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

Safety warnings

All reagents in this protocol are hazardous; several can kill you.

Before start

This protocol assumes that you have just finished dissecting organ tissues or culturing cells, which you now intend to process for 3D FIB-SEM imaging.

Primary Aldehyde Fixation

4d 4h 24m 40s

1 Primary fix the sample with 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 01:00:00 at 37 °C — 1h

2 Secondary fix with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4 °C Overnight — 1d

3 Rinse twice with 0.1 M sodium cacodylate buffer at Room temperature . — 5m

Tertiary fixation and contrasting

2h 40m

4 Tertiary fix with 2 % OsO_4, 1.5 % (w/v) $K_3Fe(CN)_6$ in 0.1 M cacodylate buffer (pH 7.4) at 4 °C for 02:00:00 , then microwave using three microwave duty-cycles (120 s on, 120 s off) at 100 W under vacuum. — 2h

5 Rinse three times with MilliQ or ultrapure water.

6 TCH crosslink with 1 % (w/v) Thiocarbohydrazide in water, using microwave for three microwave duty-cycles (120 s on, 120 s off) at 100 W under vacuum. — 10m

7 Rinse three times with MilliQ or ultrapure water.

8 Re-osmicate with 2 % OsO_4 (osmium tetroxide) in water, using microwave for three duty-cycles (120 s on, 120 s off) at 100 W under vacuum. — 10m

9 Rinse three times with MilliQ or ultrapure water.

10 *en bloc* stain with 2 % (w/v) aqueous uranyl acetate, using three microwave duty-cycles (120 s on, 120 s off) at 100 W under vacuum. — 10m

Citation

M T Silva, F C Guerra, M M Magalhães (1968)
. The fixative action of uranyl acetate in electron microscopy. Experientia.

10.1007/BF02138757 LINK

11 Rinse three times with MilliQ or ultrapure water.

12 *en bloc* stain with "Walton's Lead Aspartate", using microwave for three microwave duty-cycles (120 s on, 120 s off) at 100 W under vacuum. 10m

Citation

J Walton (1979)
. Lead aspartate, an en bloc contrast stain particularly useful for ultrastructural enzymology .
J Histochem Cytochem.

10.1177/27.10.512319 LINK

13 Rinse three times with MilliQ or ultrapure water.

Microwave assisted dehydration

14 90% (w/v) Ethanol, using microwave at 150 W for 40 s without vacuum.

15 80% (w/v) Ethanol, using microwave at 150 W for 40 s without vacuum.

16 95% (w/v) Ethanol, using microwave at 150 W for 40 s without vacuum.

17 100% (w/v) Ethanol, using microwave at 150 W for 40 s without vacuum.

18 100% (w/v) Ethanol, using microwave at 150 W for 40 s without vacuum.

19 100% (w/v) Propylene oxide, using microwave at 150 W for 40 s without vacuum.

20 100% (w/v) Propylene oxide, using microwave at 150 W for 40 s without vacuum.

Resin infiltration

21 Prepare embedding resin as per manufacturers instructions OR use the following recipe:

- 2.20 g Araldite 502
- 3.10 g Embed 812
- 3.0 g NMA (nadic methyl anhydride)
- 3.05 g DDSA (dodecenylsuccinic anhydride)
- 400 µL BDMA (benzyldimethylamine)

22 25% (v/v) embedding resin in propylene oxide, using the microwave at 250 W for 180s under vacuum.

23 50% (v/v) embedding resin in propylene oxide, using the microwave at 250 W for 180s under vacuum.

24 75% (v/v) embedding resin in propylene oxide, using the microwave at 250 W for 180s under vacuum.

25 100% (v/v) embedding resin in propylene oxide, using the microwave at 250 W for 180s under vacuum.

26 100% (v/v) embedding resin in propylene oxide, using the microwave at 250 W for 180s under vacuum.

27 Position the samples on an SEM stub, then polymerize the resin at 60°C for at least 48 hours.

Final preparation for imaging

28 Using a razor blade, trim away any excess resin. You can also gently expose regions of embedded tissue, provided that the tissue remains attached to the SEM stub.

29 Using an ultramicrotome equipped with a glass knife, slowly cut into the polymerized sample until a sufficient number of cells have been revealed.

> Note
>
> Avoid touching the the top-most surface, so that it remains undamaged and clean.

30 Using a sputtercoater, coat the stub sample with at least 10nm of gold (or your preferred metal substrate).

The sample is now ready for imaging.

Citations

Step 10

M T Silva, F C Guerra, M M Magalhães. The fixative action of uranyl acetate in electron microscopy
10.1007/BF02138757

Step 12

J Walton. Lead aspartate, an en bloc contrast stain particularly useful for ultrastructural enzymology
10.1177/27.10.512319