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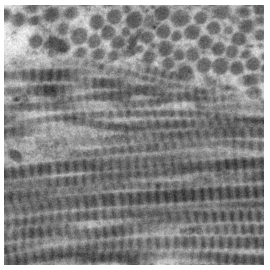
A block staining method using ethanolic phosphotungstic acid for the visualisation of collagens in the TEM



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We use this protocol and it's working

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Abstract

Conventional TEM imaging of biological samples requires contrast enhancement by staining with heavy metal salts. The most widely used stains are uranyl acetate or its non-radioactive lanthanoid replacements, and lead citrate. However, particularly for the visualisation of small fibrillar collagens, these substances proved of limited use. We therefore developed a preparation of ethanolic phosphotungstic acid (E-PTA) as an improvement to overcome this deficiency. Were able to establish a highly effective and time saving block staining procedure that can be integrated in the dehydration steps. The method reliably visualizes fibrillar collagens, prominently including the small collagen VII anchoring fibrils of the human skin, and various other extracellular matrix components. Collagen I/III fibrils are conspicuous in transverse and longitudinal section, accurately showing the characteristic banding patten in the latter. The new E-PTA based block staining method also clearly depicts all relevant intracellular structures, particularly accentuating keratin fibres and
desmosomal and hemidesmosomal plaques. We therefore conclude that beyond the visualization of collagen this method is also a fast, inexpensive and versatile non-radioactive alternative to standard staining methods.

Image Attribution

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Guidelines

This protocol describes the workflow of animal and human tissue sample fixation with aldehydes and osmium tetroxide, followed by an innovative block staining procedure with ethanolic PTA integrated into the dehydration steps, and subsequent epoxy resin embedding. Although originally aimed at the visualization of collagen fibrils, the block staining procedure turned out a much more versatile method. Accordingly, it is possible to adapt fixation steps or incubation times to the specific requirements of the particular type of sample and/or the established procedures of your lab.
The materials section provides a suggested list of chemicals, but the exact lots and vendors of the components listed are not critical. However, it should be made sure that non-denatured ethanol and EM-grade fixatives are used.
If a resin different to the one listed is used, infiltration times must be adapted according to manufacturer's instructions.
Cacodylate buffer can be substituted with PBS, but it should be kept in mind that the fixation quality of structures such as intracellular membranes benefits from cacodylate buffer containing calcium ions.
It is best to use glassware as specimen vials, as glass is resistant to propylene-oxide. If you prefer to use plastic ware, please test its suitability beforehand.
Choose reagent volumes according to sample size and use at least 20x the sample volume to ensure that the sample is fully immersed in and surrounded by the liquid.

Materials

- Dimethylarsinic acid sodium salt trihydrate, molecular weight: 214.03 g/mol, CAS-nr: 6131-99-3, EG-nr.: 204-708-2, Carl Roth 5169.1
- Osmium tetroxide, (4% solution), molecular weight: 254.23 g/mol, CAS-nr: 20816-12-0, EG-nr. 244-058-7, Sigma-Aldrich 251755
- EM-grade glutaraldehyde (1,5 pentanedial) 25 % aqueous solution, molecular weight: 100.12 g/mol, CAS-nr: 111-30-8, EG-nr. 203-856-5, Carl Roth 4157.1
- Paraformaldehyde, molecular weight: 30,03 g/mol, CAS-nr: 30525-89-4, EG-nr. 608-494-5, Carl Roth 0964.1
- Calcium chloride, molecular weight: 110.98 g/mol, CAS-nr: 10043-52-4, EG-nr.233-140-8, Sigma-Aldrich C5670
- Phosphotungstic acid molecular weight: 2880,17 + x H2O g/mol, CAS-nr: 12501-23-4, EG-nr. 603-020-3, Carl Roth 2635.1
- 96% Ethanol undenatured, molecular weight 46,07 g/mol, CAS nr. 64-17-5, EG-nr. 200-578-6, Carl Roth P075.4
- 100% Ethanol undenatured, molecular weight 46,07 g/mol CAS nr. 64-17-5 EG-nr. 200-578-6, Carl Roth 9065.4
- 1,2-Propylene-oxide, molecular weight: 58.08 g/mol, CAS-nr. 75-56-9, EG-nr. 200-879-2, Sigma Aldrich 8.07027
- Agar Low Viscosity Resin (ALVR) Kit, Agar Scientific, AGR1078

Solutions:

10% PFA stock solution 100 mL

10 g paraformaldehyde

Fill to 100 mL with aqua bidest, dissolve by heating to approx. 60°C (do not boil!) and adjust pH with NaOH until the solution appears clear.
Store at 4C° up to one month, or at -20°C for several months.

CCB buffer (100 mM cacodylate buffer containing 2mM $CaCl_2$) 1 L

21.403 g dimethylarsinic acid sodium salt trihydrate (M=214,03g/mol) and

0.222 g $CaCl_2$ (M=110,98g/mol)

Fill to 1 L with aqua bidest. and stir until fully dissolved.

Adjust to pH 7.4

2% PFA, 2.5% GA fixative 50 mL

10 mL 10% PFA stock solution

5 mL 25% glutaraldehyde aqueous solution, EM grade

35 mL 35 ml CCB

Best prepared fresh before use, store at 4°C up to one month

1% OsO_4 solution 10 mL

2.5 ml 4% OsO_4 solution

7.5 ml CCB

Prepare fresh immediately before use. Very toxic, handle with care!

Ethanol dilutions 96 mL each

70% ethanol: 70 mL 96% ethanol, fill to 96 mL with aqua bidest. (26 mL).

80% ethanol: 80 mL 96% ethanol, fill to 96 mL with aqua bidest. (16 mL).

90% ethanol: 90 mL 96% ethanol, fill to 96 mL 96 ml with aqua bidest. (6 mL).

1% ethanolic phosphotungstic acid 10 mL

0.1 g phosphotungstic acid

Fill to 10 mL with 70% ethanol

Prepare fresh immediately before use

Troubleshooting

Safety warnings

Perform all steps under a fume hood and wear protective gear.

Osmium tetroxide is highly reactive, severely toxic and corrosive. Handle with extreme care, avoid any contact with the solution or the fumes. Collect all solutions and the buffer of at least the first two washing steps for special waste disposal according to manufacturer's instructions and local rules.

Glutaraldehyde and paraformaldehyde are toxic, corrosive and environmental hazards. Avoid any contact with the solutions and the fumes. Collect all solutions and the buffer of at least the first two washing steps for special waste disposal according to manufacturer's instructions and local rules.

Cacodylic acid is toxic and an environmental hazard. Avoid any contact with the solution and the fumes.
Collect all solutions for special waste disposal according to manufacturer's instructions and local rules.

Before start

This protocol comprises working steps for 4 lab days, so make sure you plan them accordingly before you start. As some process step times can be altered depending on sample type and other requirements, the number of required days may change.
After the last washing step following fixation with OsO_4, samples may be interim stored in 70% ethanol at 4°C over night or for up to approximately 2 weeks, e.g. enabling to collect samples from several experiments. Caution: Do not store in higher concentrations of ethanol, as this might lead to severe shrinking artefacts. Alternatively, interim storage in buffer is also possible, however, this does not provide against microbial contamination as the washing steps are usually not performed under sterile conditions.

Primary fixation

1d 1h

1 Place samples in a solution of 2% Paraformaldehyde and 2.5% Glutaraldehyde in 100mM cacodylate buffer containing 2mM $CaCl_2$ (CCB) 4 °C Overnight — 1d

2 Wash in CCB (1/4) Room temperature 00:15:00 — 15m

3 Wash in CCB (2/4) Room temperature 00:15:00 — 15m

4 Wash in CCB (3/4) Room temperature 00:15:00 — 15m

5 Wash in CCB (4/4) Room temperature 00:15:00 — 15m

Secondary fixation

4h

6 Fix samples in a freshly prepared solution of 1% OsO_4 in CCB Room temperature 03:00:00 — 3h

Safety information

Highly toxic! Handle with great care.

7 Wash in CCB (1/4) Room temperature 00:15:00 — 15m

8 Wash in CCB (2/4) Room temperature 00:15:00 — 15m

9 Wash in CCB (3/4) Room temperature 00:15:00 — 15m

10 Wash in CCB (4/4) Room temperature 00:15:00 15m

10.1 If applicable store samples in 70% ethanol at 4°C over night or until further use (up to approx. 2 weeks) 4 °C

Block staining 1h

11 Incubate samples in a freshly prepared solution of 1% PTA in 70% ethanol Room temperature 01:00:00 1h

Dehydration 2h 15m

12 80% ethanol Room temperature 00:30:00 30m

13 90% ethanol Room temperature 00:30:00 30m

14 96% ethanol Room temperature 00:30:00 30m

15 100% ethanol (1/3) Room temperature 00:15:00 15m

16 100% ethanol (2/3) Room temperature 00:15:00 15m

17 100% ethanol (3/3) Room temperature 00:15:00 15m

5. Infiltration 3h 45m

18 Propylenoxide (1/3) Room temperature 00:15:00 15m

19 Propylenoxide (2/3) Room temperature 00:15:00 15m

20 Propylenoxide (3/3) Room temperature 00:15:00 15m

21 1:1 mixture of propylenoxide:ALVR resin Room temperature 01:00:00 1h

22 1:2 mixture of propylenoxide:ALVR resin Room temperature 02:00:00 2h

23 ALVR resin Room temperature Overnight

Embedding 1d

24 Place in fresh resin in suitable embedding moulds

25 Cure 60 °C 24:00:00 1d

Protocol references

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