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Title: **Preparation of Samples for Transmission Electron Microscopy.**

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SOP History		
Number	Date	Reason for Change
v1	07/11/2016	Original
V2	27/03/2017	Further additions
V3	27/03/2019	Update
V4	27/03/2021	Update

1.0 Purpose –

This SOP describes the current procedure for preparing Glutaraldehyde / PIPES buffer suitable for fixing samples prior to Transmission Electron Microscopy.

2.0 Scope –

The scope of this document is to describe the step by step procedures for preparing Glutaraldehyde / PIPES buffer.

3.0 Responsibilities –

All staff preparing Glutaraldehyde / PIPES buffer by this protocol are responsible for ensuring that the methods are followed in accordance with this SOP. All staff must have read and signed the relevant risk assessment documents before performing this procedure.

SAFTEY NOTE: Most of the chemicals used for processing specimens for electron microscopy are extremely hazardous. Glutaraldehyde, formaldehyde and osmium tetroxide are volatile and can fix any cells / tissues they contact (they will fix your respiratory epithelium, corneas, and epithelial cells on your hand, etc): it is therefore essential to use them in a fume hood. Although dangerous, these fixatives cannot penetrate more than 1-2 mm into tissues, so exposure to them rarely causes any permanent damage.

The epoxy resins used to embed the specimens are potentially more dangerous than the fixatives. Many of the resin components are known to cause cancer in rats or mice. During the embedding process the resins are dissolved in solvent(s) that can carry the resin into your skin EVEN through plastic gloves. In contrast to fixatives, whose actions are immediate and apparent, the consequences of exposure to the resins are not apparent for years. Therefore be careful with all resins prior to polymerization into hard blocks.

4.0 Procedure –

Preparation of 0.2M PIPES, pH7.4 / 0.5% Glutaraldehyde Fixation Buffer:

This protocol will make up 10ml of Fixative Solution – for structural EM studies where a strong fixation is desired then 0.5–2.5 % glutaraldehyde in 0.2M PIPES would be a good place to start. It is possible to make the fixative up in PBS if you are more interested in looking at membranes but this may compromise the reality of cytoplasmic staining. The sample fixation buffer described in this SOP is therefore used as a 'general fixative' at a final concentration of 0.5% Glutaraldehyde / 0.2M PIPES (pH7.4).

PIPES is purchased in powder form (Sigma; P6757-25G); Glutaraldehyde is purchased as a 25% solution (Agar Scientific; R1010).

The buffer is made up in three steps, namely (i) 0.4M PIPES buffer at 2X concentration, (ii) Glutaraldehyde at 2X concentration, and then (iii) combining the PIPES and Glutaraldehyde in equal volumes.

25% Glutaraldehyde: (A)

Prepare convenient aliquots of Glutaraldehyde (25%) by simply transferring 0.5mL from the main stock bottle to multiple 1.5mL microcentrifuge tubes and freeze at -20°c.

0.4M PIPES, pH7.4 (100mL): (B)

Add 12.096g PIPES to a beaker containing 80-90mL of distilled water. Dissolve the PIPES, adjusting to pH7.4 with concentrated NaOH (in order to get it into solution) and then top up to 100ml. Aliquot into 5mL aliquots and freeze at -20°c.

0.2M PIPES, pH7.4 / 0.5% Glutaraldehyde buffer:

Remove one aliquot of 25% Glutaraldehyde (A) and one aliquot of 0.4M PIPES (B) from the freezer and allow to warm to room temperature. Keep the Glutaraldehyde in the fume cupboard.

Add 200µL of Glutaraldehyde (A) to 4.8mL of DW (total volume will then be 5mL) to give a concertation of 1% Glutaraldehyde (A').

Add the 5ml of 1% Glutaraldehyde (A') to 5mL of 0.4M PIPES, pH7.4 buffer (B), resulting in a fixative solution with final a concentration of 0.5% Glutaraldehyde /

0.2M PIPES. Fixative buffer should be made up as fresh as possible, but can be prepared the evening beforehand if then kept on ice prior to use.

Fixation of Cell Lines for Electron Microscopy:

1. Remove medium from cells.
2. Add 10ml fixative, allow to sit for no more than a minute.
3. Remove 9ml fixative, leaving 1ml in the flask.
4. Gently scrape cells into the remaining 1ml.
5. Remove the cell suspension to a 1.5mL Eppendorf tube, briefly pipette up and down with blue tip to disperse cells slightly (not too clumpy).
6. Spin at maximum in a desktop centrifuge for 20 – 30 minutes at room temperature
7. Evaluate the pellet – there should be a firm, yellowish pellet at bottom of the tube. Do not remove the fixative but leave it in the tube.
8. Store pellet under fixative at 4°C until ready for further processing.

Note 1: Steps 2 to 6 should be performed rapidly to prevent cells from crosslinking too much, as this may make the sample resistant to pelleting.
Note 2: All waste Glutaraldehyde should be disposed of in the 'Aldehyde Waste' container inside the fume hood.
Note 3: For transport (such as taking it to Edinburgh for post-fixation onwards), you can remove fixative, then wash and store the pellet in 0.2M PIPES. For effective washing, pellet can be lifted off the inside of the tube using a long needle.

Fixation of Tissue Specimens for Electron Microscopy:

1. Remove tissue from animal and then immediately transfer to a 6-well tray containing1.5ml (excess) freshly prepared 0.5% Glutaraldehyde / 0.2M PIPES fixative.
2. Using sterile scalpels, chop the tissue into 1mm wide strips and then cut 1mm sections from the ends of each strip to yield 1mm cubes of tissue.
3. Transfer both the 1mm cubes of tissue and the 1.5ml of 0.5% Glutaraldehyde / 0.2M PIPES fixative to a micro-centrifuge tube and ensure the cap is securely snapped shut. Store tubes upright in a sealed container (ideally containing an ice pack) during transit.
4. Store tissue long-term under fixative at 4°C until ready for further processing.

Note: All waste Glutaraldehyde should be disposed of in the 'Aldehyde Waste' container inside the fume hood.

Osmium Tetroxide (post-fixation) staining of samples prior to embedding:

Osmium Tetroxide is traditionally used in electron microscopy both as a fixative and a heavy metal stain. Osmium Tetroxide is a good fixative and excellent stain for lipids in membranous structures and vesicles. Since OsO_4 is toxic and volatile, all work

should be performed in a fume hood using gloves and protective clothing. Handling and waste disposal should be done according to University guidelines.

0.4M Sodium Cacodylate, pH7.1 buffer (stored in fridge):
21.4g of Sodium Cacodylate Trihydrate {$(CH_3)_2AsO_2Na.3H_2O$: eg. Sigma C0250} was weighed out and made up in MilliQ DW. The pH was adjusted to pH7.1 and then the volume was adjusted to 250mL. Label as 'Toxic' due to it containing arsenic and store at 4°c.

0.1M Sodium Cacodylate, pH7.1 buffer (stored in fridge):
Make up sufficient 0.1M Sodium Cacodylate, pH7.1 buffer by diluting the 0.4M Sodium Cacodylate, pH7.1 stock solution 1 in 4 (eg. 5mL + 15mL MilliQ water).

4% Osmium Tetroxide Solution (stored in Lucocq freezer):
Aliquots (0.3mL) of 4% Osmium Tetroxide Solution (Sigma: 75632-10ML) are stored in the -20°c freezer.

6% w/v Potassium hexacyanoferrate(II) trihydrate (Lucocq chemical shelf):
Make up fresh 6% w/v Potassium hexacyanoferrate(II) trihydrate [$K_4Fe(CN)_{6.3}H_2O$] solution (Sigma P3289 / 455989) by weighing out 0.06g (60mg) and then dissolving in 1mL of MilliQ water. The solution should appear yellow in colour.
6% = 6g/100mL = 0.6g/10mL = 0.06g/1mL.

Gently dislodge the hard cell pellet from the side wall of the micro-centrifuge tube using a green syringe needle – the pellet should slide off the wall of the tube without becoming damaged.

Remove the Glutaraldehyde / PIPES buffer from the tube and then wash the cells (x3) with 0.1M Sodium Cacodylate, pH7.1 buffer (approximately 0.5-1mL per wash). The waste material from the first wash should be disposed of in the 'Aldehyde waste' and subsequent washes in the 'Cacodylate waste' bottle.

If the pellets are large, temporarily transfer them to a 6-well or 24-well tray (in 0.1M Sodium Cacodylate, pH7.1 buffer) and then chop up into smaller fragments with a scalpel. Each fragment should be approximately 1mm x 1mm x 1mm in size. Return the fragments to the microcentrifuge tubes before continuing. If the pellets are too large the Osmium Tetroxide will not penetrate fully into the sample.

Make up fresh Osmium Tetroxide / Potassium Ferrocyanide solution as follows:
(1) 1 part 0.4M Cacodylate buffer pH 7.4 - 0.3mL
(2) 1 part 4% Osmium Tetroxide (1% final) – 0.3mL
(3) 1 part MilliQ water – 0.3mL
(4) 1 part of 6% w/v Potassium hexacyanoferrate(II) trihydrate solution in water (1.5% final) – 0.3mL (made up fresh each time).

Add 200µL of Osmium Tetroxide and leave in the hood for 30-60 mins at room temperature.

Remove Osmium Tetroxide and discard to a waste container containing cooking oil.

Wash the pellets in 0.1M Cacodylate buffer (5 minutes x3) – samples can be stopped here in buffer and stored at 4°c over the weekend etc.

Uranyl Acetate staining of samples prior to embedding: *Only put uranyl acetate in the staining protocol if you need more ribosome highlighting.*

> *3% Uranyl Acetate Solution (stored under foil in Lucocq bay at room temp):*
> *3g of Uranyl Acetate powder (Agar Scientific; AGR1260A) was dissolved in 100mL of MilliQ water and then the bottle was covered in tin foil to protect it from the light. Store at room temperature.*

Remove Cacodylate buffer and wash for 5 mins with DW (x2).

Add 300µL of 3% Uranyl acetate and then leave in the hood for 1hr.

Remove 3% Uranyl acetate and transfer to uranyl acetate liquid waste bottle.

Wash with DW (x2).

Resin Preparation in glass vials:

Preparation of Epoxy Resin:

1. Durcupan™ ACM single component A, M epoxy resin (Sigma 44611-100mL).
2. Durcupan™ ACM single component B, hardener 964 (Sigma 44612-100mL).
3. Durcupan™ ACM single component C, accelerator 960 (DY 060) (Sigma 44613-100mL).
4. Durcupan™ ACM single component D (Sigma 44614-100mL)

Epoxy Resin component A (5g) and Hardener component B (5g) were each weighed out on a pan balance into a single glass vial and then thoroughly mixed and stirred in a glass vial using a glass pipette – this may take 15 mins. Try not to create too many air bubbles.

Compound C (6 drops) and component D (7 drops) were then added to the mixed resin and stirred for a further 5-10 mins with the glass pipette prior to use. The solution should be yellow / pale amber in colour.

Epoxy Resin embedding of cell pellets

Transfer the sample pellets to glass vials (approx. 10mL) and then dehydrate in ethanol as follows: NB: Do not allow the pellet to become dry at any point in the procedure. All subsequent steps should be carried out on the rotary mixer.

30% ethanol for 10mins on the rotary mixer
50% ethanol for 10mins on the rotary mixer
70% ethanol for 10mins on the rotary mixer
90% ethanol for 10mins on the rotary mixer
100% ethanol for 20min on the rotary mixer (x2)

Remove the 100% Ethanol and replace with Propylene Oxide for 10mins on the rotary mixer (x2)

In a separate glass vial, add equal volumes of pre-mixed Epoxy Resin and Propylene Oxide and mix thoroughly before adding the 50/50 mixture to each sample. Keep the lid on the glass vial until ready to use so as to prevent the Propylene Oxide evaporating away.

Remove the Propylene Oxide and add the pre-mixed Epoxy Resin and Propylene Oxide. Leave on the rotator with the lids on for 1hr.

Remove the sample from the resin / propylene oxide and place in a fresh glass vial. Add fresh resin alone and leave on the rotator (with the lids off) for 2 hours or overnight allowing the Propylene Oxide to evaporate.

Resin Embedding in beam or gelatin capsules:

Label small pieces of paper (perhaps 4mm x 10mm) with a tube identification ID and then wrap this round the inside of the tube (at the top).

If the samples have not already been cut into small pieces, sample pellets were removed from the glass vials and placed in a small petri dish. Chop the samples into small (1mm square) pieces – use 1 piece per tube.

Add one drop of the pre-prepared resin compound to the bottom of each capsule (try not to get it on the side of the tube as you do so).

Place (drop) one piece of cell pellet in the bottom of each tube (on top of the resin already sitting in the tube). This can be done with a glass pastette or a syringe needle.

Fill the remainder of the capsule to the lid with remaining resin, making sure that the ID tag remains visible. You may find it easier to insert the ID tag just before the tube is filled to the top with resin.

Close the caps and place all of the capsules in an oven to polymerise at 60°c overnight (or over the weekend if necessary). Remove the capsules from the oven and allow to cool to room temperature before cutting.

Preparation of Grids:

Both 150 hexagonal copper mesh (150 hexagonal holes per mrid) and 2 mm slot grids (Agar Scientific, Essex) were coated with pioloform. Pioloform is sometimes preferred to Formvar® because of its higher mechanical and thermal stability, and lower bulk which can help reduce electron scattering.

A clean glass microscope slide was placed inside a glass tube fitted with a quarter-turn tap at the bottom (all held upright in a clamp-stand). The tap was closed and a 2% pioloform in chloroform solution was poured into the tube (at room temperature) until the level of the liquid reached 1cm below the top of the slide.

The tap was opened in one smooth motion to drain the excess solution slowly and smoothly into a waste collection beaker, leaving a thin, homogeneous layer of solution on the slide which dries rapidly to result in a thin film of pioloform plastic.

A razor blade was then run around the four borders of the slide 5 mm from the edge on both sides of the slide to release the films (the coating is obviously on both the front and back of the slides since it was dipped into a solution). The slide was then submerged in a bowl of deionised water, releasing the films which then float on the surface.

Grids were then placed etched-side-down on top of the films using fine forceps – the film will support the weight of the grids sitting on top of the water. The grids / films were removed from the water using parafilm and allowed to dry at room temperature.

Carbon coating (1-2 nm) was conducted as per standard operating procedure using the Leica EM ACE200 carbon coater. Briefly, this involved placing the dried, pioloform coated grids (on top of the parafilm) in a 10 cm petri dish inside the chamber of the coater, selecting the desired thickness of carbon coating (1-2nm) and selecting the “start” option. Once the vacuum was achieved, the coater spread carbon evenly on the surface of the sample in pulses to the desired thickness. Once vented, the petri dish containing the grids was removed and then stored at 4°C prior to being used for section mounting.

Sectioning of Samples:

Following polymerisation, each resin sample plug was removed from the capsule by CAREFULLY cutting down the side of the capsule and then peeling it open. The label should remain stuck to the top of the resin plug.

The resin-embedded sample was placed inside the specimen holder. The specimen holder was then secured to the machine and the sample trimmed using a glass knife until the pellet was exposed (step approach was set at 0.5µm; feed at 200nm).

The sample holder was then removed and the face of the sample was trimmed on all four sides (at 45°) using a razor blade to create a trapezoid face. Ideally the trapezoid should be cut so that the longest edge is the first face to hit the blade when cutting occurs as this will tend to result in cleaner cutting sections. The specimen holder was then returned to the machine and the face recut using the glass knife.

Once happy with the cut, the glass knife was removed and replaced with the diamond knife. The knife holder was filled with MilliQ water until it was level with the cutting blade and a clear reflection could be seen on the surface of the water.

The block face was cut at 0.4mm/sec / feed 80nm and the ribbons allowed to float off onto the water. Ribbons were collected by immersing a pre-coated grid under the water (using fine forceps) and forming a meniscus before lifting the ribbon up with the grid as it is lifted out of the water. Grids were then allowed to air dry on a piece of filter paper inside a petri dish.

Lead Citrate staining of samples prior to TEM:

A small piece of parafilm (approximately 5x5cm) was cut and the placed inside a glass 14cm petri dish (after removing the paper carrier) containing Sodium Hydroxide pellets around the edges.

A drop of 3% w/v Lead Citrate was placed on top of the parafilm and then the grid containing the sample was carefully placed on top of the droplet (facing downwards). Place the lid back on top of the petri dish and leave to incubate for 5mins.

Carefully remove the grid using very fine forceps and wash by dipping (x20) into a 10mL glass vial containing MilliQ water. Perform this washing step in three successive glass vials (x20 each time) before finally leaving the grids to air dry on a piece of filter paper inside a petri dish.

This concludes the preparation of samples which can now be placed inside the TEM for visualisation.

5.0 Personal protection -

A Howie laboratory coat and lab gloves must be worn at all times.

6.0 Spillages -

Always clean up and disinfect any spills immediately after use, only you know what you have spilt and are aware of its hazard.

7.0 Training -

All staff should undergo training in this technique before performing procedure. This is particularly important in view of the nasty chemicals used throughout the procedures. All users MUST electronically sign the appropriate Risk Assessments (after consultation with Dr John Lucocq).

8.0 Related documents –

RA3357: Staining of EM grids using uranyl acetate and lead citrate
RA5956: Osmium Tetroxoid Fixation
RA10097: Embedding of biological Specimens in Epoxy Resin
RA10182: Osmium Tetroxide Fixation

9.0 Approval and sign off –

Author:

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Management Approval:

Name: Peter Mullen

Position: SOP Administrator

Signature:  Date: 22/03/2021

QA release by:

Name: Alex MacLellan

Position: QA Manager

Signature:  Date: 22/03/2021

STANDARD OPERATING PROCEDURE

Please sign below to indicate you have read this S.O.P and understand the procedures involved.

NAME	POSITION HELD	SIGNATURE	DATE