

EM protocol (Modified on 2006-6-2)

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General idea of tissue processing for EM

- Adequate preservation for ultrastructure requires immediate fixation. This usually means a quick dissection just after sacrifice. The tissue of interest must be rapidly minced into tiny pieces no larger than 1 mm^3 to insure proper penetration of the fixative. (Alternatively perfusion fixation may be a good choice in some case, for example, brain).
- A variety of fixatives and buffers have been used for electron microscopy, and each has its advantage and disadvantage. One thing is certain: it is extremely important that all reagents for EM must be EM grade and freshly prepare.

EM protocol for mouse embryo or P0 diaphragm:

Embryonic diaphragm is thin enough, so you do not mince the tissue. If you know how to dissect diaphragm preparation for electrophysiology, apply the same way. The most important thing is to keep tissue fresh, alive and no damage. If you have no experience on how to prepare the diaphragm for electrophysiology, it may be safe to partially dissect the diaphragm and apply the fixative directly on the muscle and perform the fine dissection with fixative solution cover the tissue throughout the whole dissection process. .

Detail procedures and precautions for dissecting diaphragm for EM

1. Prepare the Ringer's solution (see attachment for recipe) at 4°C , make sure it is well-oxygenated (pump oxygen into the solution for ~20 mins.)
2. Freshly prepare 1% glutaraldehyde in 0.1 M phosphate buffer (see attachment for recipe of phosphate buffer and glutaraldehyde)
3. Dissect the diaphragm quickly in the 4°C Ringer's solution, keep the diaphragm and the phrenic nerve intact, don't touch the diaphragm muscle. Try to leave as much as possible the muscle and phrenic nerves.
4. Pin the diaphragm on the dish (with perfusion of 4°C Ringer solution, oxygenated), we have to stretch the diaphragm until it is flat, use pins to hold the "flat" position of the diaphragm. This is tricky; you will need to stretch the muscle, but not too much. Either way too much will cause damage to the diaphragm.
5. Remove the Ringer's solution quickly by turning the dish upside down, and quickly introduce freshly made 1% glutaraldehyde. Keep diaphragm stretched (do not over stretch).
6. The glutaraldehyde will quickly go into the diaphragm muscle and fix the intracellular structure, avoid any air bubble on the diaphragm during fixation.
7. Keep the diaphragm in 1% glutaraldehyde in 4°C overnight (12-14 hours no

problem).

8. The tissue will be rinsed with washing buffer (see attachment for recipe) for at least 5 times.
9. We can stain the cholinesterase to locate the end-plate location; this helps us to locate nmj. AChE assay should not be over done since resulting crystals may damage the muscle.
10. The tissue will be post-fixed with 1% osmium tetroxide (EM grade, you can get it from EM facility in MCG) in 0.1 M phosphate buffer for 2 hr on ice. **Osmium tetroxide is toxic, make sure everything work inside the flame hood, keep all the osmium-related solution and bring to EM facility for special treatment or you can make appointment with EM facility to perform the step 9**)
11. The tissue was then dehydrated in a graded series of ethanol (usually 10-15 min/step), infiltrated, and polymerized in Epon (keep the solution in step 10, a and b because they still have osmium tetroxide; EM facility of MCG will provide the Epon)
 - a) 50% Ethanol 10 min
 - b) 70% Ethanol 10 min
 - c) 80% Ethanol 10 min
 - d) 90% Ethanol 10 min
 - e) 95% Ethanol 10 min
 - f) 99% Ethanol 10 min
 - g) 100% Ethanol 10 min 2-3 times (avoid any water contamination)
 - h) Propylene Oxide (EM facility of MCG could provide it) 10 min 2 times (this organic solvent is extremely easy to be evaporated. Do not let the tissue dry)
 - i) Mixture of Propylene Oxide : Epon mix (1:1), 2-3 hours
 - j) Mixture of Propylene Oxide : Epon mix (1:2), at least 2-3 hours – overnight
 - k) Pure Epon mix, overnight
 - l) Place the tissue in mold. Decide how to section, cross or longitudinal.
 - m) Put the mold in oven. 45°C for half day, then 60°C for one day.
 - n) Epon blocks should be ready for sectioning.

- Label as Solution B

Mixing Solution A and Solution B:

To make 1 L of 0.2 M Phosphate Buffer, use the following table to add Solution A and Solution B to achieve your desired pH (For the Thompson Lab, a pH of 7.4 is used) You an appropriately sized graduated cylinder with funnel to measure the solutions

<u>Desired pH</u>	<u>Solution A</u>	<u>Solution B</u>
7.0	390mL	610mL
7.2	280mL	720mL
7.4	190mL	810mL
7.6	130mL	870mL

2. 1% glutaraldehyde: EM grade, diluted by 0.1 M phosphate buffer, pH7.4 , we can get 70% glutaraldehyde from EM facility in MCG

3. Perfusion fixative solution: Mixture of 1 % glutaraldehyde and 2-4 % paraformaldehyde is good for the perfusion fixation

4. Ringer's solution : NaCl ,150 mM; KCl, 5.4 mM; CaCl₂.2H₂O, 2mM, MgCl₂, 1mM; NaHEPEs, 10 mM; and freshly add glucose 13 mM, (modified by XM)

5. Washing buffer: 0.9% NaCl in 0.01M phosphate buffer, pH 7.4

6. 1 % Osmium tetroxide: 4% EM grade aqua solution can be purchased. Dilute with 0.1M phosphate buffer to make 1% Osmium tetroxide.