Electron microscopy using the ferrocyanide-reduced osmium method for detecting autophagosome-related structures in cultured mammalian cells

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The ferrocyanide-reduced osmium method has been known to well preserve autophagic isolation membranes in electron microscopy. Thus, it is suitable for detecting fine structures of phagophore (or isolation membrane), autophagosome, and autolysosome. Here, we describe our routine method that can be easily applied for electron microscopy (EM) analysis, while doing a conventional immunofluorescence procedure that uses 24-well plates (or 4-well plates).

METHOD

Fixation

1. Grow cells on round glass coverslips of ~12 mm in diameter.
2. Fix the cells in with 2% paraformaldehyde-2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min at room temperature (RT) and then 50 min at 4°C.

NOTE Cells are processed and embedded in a resin with the coverslip, to which ultrathin sections will be cut in parallel. Therefore, obtained images can be compared with immunofluorescence images.

NOTE Cells can be stored for a few days in a refrigerator.

3. Rinse thrice for 15 min each in 0.1 M phosphate buffer (pH 7.4) at RT.

NOTE We did not see any differences in morphology between using 0.1 M phosphate buffer (pH 7.4) and 0.1 M sodium cacodylate buffer (pH 7.4).

4. Post-fix with 1% osmium tetroxide (OsO_4)-1.5% tetrapotassium ferrocyanide in 0.1 M phosphate buffer (pH 7.4) for 60 min at 4°C in a dark chamber (e.g. refrigerator).

NOTE Protect OsO_4 from direct exposure to light during storage and use.

NOTE OsO_4 is volatile and a powerful oxidizing agent, thus it is highly toxic. Careful handling and disposal in a fume hood are required.

5. Rinse twice for 5 min each in 0.1 M phosphate buffer (pH 7.4) at RT.

Dehydration and Resin Embedding

6. Immerse in 50, 70, 80, and 95% ethanol sequentially for 10 min each at RT.

7. Immerse in 100% ethanol thrice for 20 min each at RT.

NOTE Use glass dishes from this step, because propylene oxide that is used in the next step is corrosive for plastic ware. Also, propylene oxide is so volatile that the solution may dry up if it takes times for transfer at the later step.

8. Immerse in propylene oxide twice for 5~10 min each at RT for resin infiltration.
9. Immerse in a mixture of Epon812 resin and propylene oxide (1:1) for 20~60 min at RT.
10. Immerse in Epon812 resin for overnight at RT under weak negative pressure generated with a vacuum pump.
11. Embed in fresh Epon812 resin.
12. Heat in an oven at 80°C for 2 days for polymerization of Epon812 resin.

Sectioning and observation

13. Carefully remove the coverslip while the Epon812 resin is heated appropriately.
14. Excise a small block for sectioning.

NOTE Cells can be observed on the surface of the resin by stereomicroscope.

15. Make ultrathin sections of ~70 nm thickness and pick them up on bar grids.
16. Stain the sections for 5 min with uranyl acetate that is prepared by diluting saturated uranyl acetate 3-fold with acetone.
17. Rinse with distilled water.
18. Stain the sections with lead citrate for 5 min.
19. Rinse with distilled water.
20. Dry the sections and observe with an EM.

MATERIALS

- Paraformaldehyde (MERK)
- Glutaraldehyde (TAAB)
- OsO_4 (MERK)
- Sucrose (Wako)
- Ethanol (Wako)
- Propylene oxide (Nisshin EM)
- Epon812 (TAAB)
- DDSA (TAAB)
- NMA (TAAB)
- DMP-30 (TAAB)

・Tetrapotassium ferrocyanide (Wako)
・Uranyl acetate (Wako)
・Lead citrate (TAAB)

EQUIPMENT

・vacuum pump (ULVAC Kiko, DTC-41)
・Ultramicrotome (Leica, EM UC6)
・80°C-oven (Dosaka EM, TD-230)
・Round cover glass (Matsunami, C012001)
・transfer pipette (BD Biosciences)

REAGENT PREPARATION

0.2 M phosphate buffer (pH 7.4) [stock solution]

400 ml of 0.2 M $Na_2HPO_4 \cdot 12H_2O$ is mixed with 100 ml of 0.2 M $NaH_2PO_4 \cdot 2H_2O$.

10% PFA [stock solution]

10 g of paraformaldehyde is mixed with 80 ml of Milli-Q water that has been heated to 70°C. Add 8 drops of 1N NaOH to the suspension using 3 ml transfer pipette for complete dissolution, then dilute to 100 ml with Milli-Q water. The solution is filtrated with filter paper and stored at 4°C. This solution should be used within a few days.

2% paraformaldehyde–2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4)

0.2 M phosphate buffer (pH 7.4)	5 ml
10% PFA	2 ml
25% GA	0.8 ml

Dilute to 10 ml with Milli-Q water

This fixative solution should be prepared at time of use

7.5% sucrose-0.1 M phosphate buffer (pH 7.4)

0.2 M phosphate buffer (pH 7.4)	50 ml
Sucrose	7.5 g

dilute to 100 ml with Milli-Q water

4% OsO_4 [stock solution]

1 g of OsO_4 is mixed with 25 ml of Milli-Q water and placed at room temperature for overnight. 4% OsO4 should be stored at 4°C.

1% OsO_4–1.5% tetrapotassium ferrocyanide in 0.1 M phosphate buffer (pH 7.4)

0.2 M phosphate buffer (pH 7.4)	0.5 ml
Milli-Q water	0.25 ml
tetrapotassium ferrocyanide	0.015 g
After mix, add 0.25 ml of 4% OsO_4	

Epon 812 resin mixture

MNA	30.14 g
Epon812	54.82 g
DDSA	15.04 g
DMP-30	1.5 g

Mix for 30 min in a disposal plastic beaker using a glass rod or stirring bar and degas under vacuum. Epon resin can be stored at -30°C in a container such as syringe. Note that the frozen resin should be warmed to room temperature before use to prevent dew condensation. For disposal the resin with containers should be polymerized by placing 60°C for 2 days.

Saturated uranyl acetate [stock solution]

1 g of uranyl acetate is dissolved with 50 ml of Milli-Q water. Solution should be stored at 4°C. Supernatant of solution should be used.

0.25～0.30％ lead citrate [stock solution]

25～30 mg of lead citrate is dissolved with 10 ml of Milli-Q water by adding 2 drops of 10N NaOH using 3 ml transfer pipette. After filtration with 0.22 μm filter, solution should be stored at room temperature and used within 1 month.

TROUBLESHOOTING TIPS

1. Presence of precipitates on sections

Under EM observation, we sometimes encounter contamination of dusts, needle-shaped crystal of uranyl acetate, and granular deposits of lead carbonate, especially when specimens are prepared by beginners. Using freshly prepared MilliQ water, centrifugation and/or filtration through 0.22 μm filter for solutions

could reduce such contaminations. It is also necessary to keep the bench clean.

2. Low number of cells in observing fields

Higher confluency of cells is preferable for this method. If lower confluency or floating culture is required, cells should be pelleted and embedded in 10% gelatin, and processed as tissue blocks.

3. Introduction of EM techniques in a laboratory

Each EM laboratory has their own protocols that have been developed for many years to be suitable for their morphology of interest. Even a primary fixative solution varies considerably between groups. Trainings and special cautions are required for sectioning, EM observation, and handling of OsO_4, Epon812 resin, uranium acetate, and lead citrate. Because many of them are somewhat difficult to describe, we strongly recommend doing training at first at a laboratory where EM task is one of routine works.