Colon Tissue Immunoelectron Microscopy

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[Abstract] The method described here is intended to study intracellular localization of proteins in colon cells. This protocol was used to localize IRE1β in the endoplasmic reticulum membrane. We used anti-IRE1β antibody raised in guinea pig for this purpose. We also studied the location of BiP (also known as GRP78), with the antibody raised in rabbit. Both antibodies used with appropriate gold particle-conjugated secondary antibodies gave good results. Primary mouse antibodies are not recommended because secondary anti-mouse antibodies also react with the internal mouse epitopes.

Materials and Reagents

1. Mice
2. Paraformaldehyde (TAAB, catalog number: P001/1)
3. Sucrose
4. Glutaraldehyde (Polysciences, catalog number: 111-30-8)
5. Ethanol
6. Propylene oxide
7. LR White (London resin, medium)
8. Hydrogen peroxide
9. ImmunoSaver (Nisshin EM, catalog number: 333)
10. Gelatin
11. Tween 20
12. Uranyl acetate
13. Distilled water or Milli-Q water
14. Primary antibodies
15. 10-nm gold-conjugated secondary antibodies (we purchased from EY laboratories in Tsuru *et al.*, 2013)
16. Phosphate buffered saline (PBS) (see Recipes)
17. 0.1 M phosphate buffer (pH 7.2) (see Recipes)
18. Antigen-retrieval solution (see Recipes)

Equipment

1. Peristaltic pump
2. Refrigerator
3. Freezer
4. UV irradiator (Nisshin EM, model: TUV-100) with 15 W UV light
5. Gold grid (Maxtaform HR25)
6. Ultra microtome (Leica Microsystems, model: Ultracut UCT)
7. Diamond knife (Diatome AG, catalog number: ultra 45)
8. Incubator
9. Microwave (Nisshin EM, catalog number: MWF-2)
10. Electron microscope (Hitachi, model: H-7100)

Procedure

A. Sample preparation
   1. Fix whole mice by perfusion.
      a. Set up perfusion system consisted of needle, tubing, peristaltic pump and beakers with PBS (ice-cold) or 4% paraformaldehyde in PBS (ice-cold).
      b. Anesthetize mice and expose hearts by surgery.
      c. Insert the needle into the left ventricle and make a hole in the right atrium.
      d. Remove blood from mice by perfusion (8 ml/min) with PBS (ice-cold).
      e. Fix mice by perfusion (8 ml/min) with 4% paraformaldehyde in PBS (ice-cold).
   2. Isolate colons and cut them up into small pieces (~1.5 mm thick).
   3. Fix again with 0.1% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h at 4 °C.
   4. Wash samples by 0.1 M phosphate buffer (pH 7.2) containing 8% sucrose.
   5. Dehydrate samples with a series of ethanol as follows.
      a. 25% ethanol, 10 min at 4 °C
      b. 60% ethanol, 30 min at -30 °C
      c. 80% ethanol, 30 min at -30 °C
      d. 99% ethanol, 30 min at -30 °C
      e. 99% ethanol, 10 min at RT
      f. 100% ethanol, two changes, 10 min each at RT
   6. To promote the infiltration of resin, remove ethanol with propylene oxide as follows.
      a. Ethanol-propylene oxide (2:1), 10 min at RT
      b. Ethanol-propylene oxide (1:1), 10 min at RT

c. Ethanol-propylene oxide (1:2), 10 min at RT
d. Propylene oxide, 20 min at RT

7. Make resin infiltrate into samples by immersion as follows.
   a. Propylene oxide-LR White containing accelerator (3:1), 2 h at RT
   b. Propylene oxide-LR White containing accelerator (1:1), 2 h at RT
   c. Propylene oxide-LR White containing accelerator (1:3), 2 h at RT
   d. LR White containing accelerator, 12 h at RT
8. Polymerize resin under UV irradiation for two days at RT.
9. Cut the embedded samples (90 nm thick) with ultra microtome and mount on uncoated gold grids (300 mesh).

B. Immunostaining

1. Treat sections with 3% (vol/vol) hydrogen peroxide for 30 min at RT to unmask epitope.
2. Wash sections with distilled water.
3. Treat sections with antigen-retrieval solution for 15 min under microwave at 95 °C by interval of 2 sec.
4. Block sections with 0.1% gelatin in PBST (PBS containing 0.05% Tween 20) for 30 min at 37 °C.
5. Reacted sections with primary antibodies diluted with PBST overnight at RT.
6. Wash sections three times with PBST for 10 min each at RT.
7. React sections with 10-nm gold-conjugated secondary antibodies diluted with PBST for 2 h at RT.
8. Wash sections twice with PBST for 10 min each at RT.
9. Wash sections twice with distilled water for 5 min each at RT.
10. Stain sections with 4% uranyl acetate for 10 min at RT.
11. Wash sections with distilled water.
12. Observe sections with an electron microscope.

Recipes

1. Phosphate buffered saline (PBS)
   Dissolve 2.89 g of $Na_2HPO_4{\cdot}12H_2O$, 0.2 g of KH_2PO_4, 8.0 g of NaCl, and 0.2 g of KCl in distilled water
   Adjust the final volume to 1,000 ml
   Sterilize by autoclaving
2. M phosphate buffer (pH 7.2)
   Solution A: Dissolve 13.61 g KH_2PO_4 in 1,000 ml distilled water

Solution B: Dissolve 17.8 g $Na_2HPO_4 \cdot 2H_2O$ in 1,000 ml distilled water

Mix appropriate volumes of solution A and solution B to adjust pH to 7.2

3. Antigen-retrieval solution

   Dilute ImmunoSaver 200-fold with distilled water

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