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Protocol

High-pressure freezing followed by cryosubstitution as a tool for preserving high-quality ultrastructure and immunoreactivity in the *Xenopus laevis* pituitary gland

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Abstract

Subcellular localisation of proteins and peptides yields fundamental information about cell functioning. Immunoelectron microscopy is a powerful tool to achieve this goal, but combining good tissue preservation with strong immunoreactivity is a great challenge in electron microscopy. We have applied a novel approach, using high-pressure freezing (HPF) followed by cryosubstitution, to prepare the pituitary gland of the amphibian *Xenopus laevis* for immunogold-electron microscopy. In this way, we investigated the subcellular distribution of brain-derived neurotrophic factor and the amphibian neurohormone mesotocin in the pituitary neural lobe, and the peptide hormone α-melanophore-stimulating hormone and its protein precursor proopiomelanocortin in melanotrope cells of the pituitary intermediate lobe. In contrast to conventional chemical fixation (followed by cryosubstitution), HPF not only revealed strong immunoreactivity of the secretory products, but also provided excellent ultrastructural preservation of cell organelles, including secretory granule subtypes. We conclude that HPF followed by cryosubstitution provides a preparation technique of choice when both optimal tissue ultrastructure and strong immunoreactivity are required.

Theme: Staining, tracing and imaging techniques, endocrine and autonomic regulation
Topic: Neuroendocrine regulation: other

Keywords: High-pressure freezing; Cryosubstitution; Immunogold labelling; αMSH; BDNF; Melanotrope cell

1. Type of research

Our research aims to elucidate the functioning of the pituitary gland in the amphibian *Xenopus laevis* [2,11,21–24,30,32]. Immunoelectron microscopy is a powerful tool to localise neuropeptides, protein hormones and related factors at the subcellular level, but combining good tissue preservation with strong immunoreactivity is a great challenge. We have applied a novel approach, using high-pressure freezing (HPF) followed by cryosubstitution, to investigate the subcellular distribution of brain-derived neurotrophic factor (BDNF) and the amphibian neurohormone mesotocin in the pituitary neural lobe and the peptide hormone α-melanophore-stimulating hormone (αMSH) and its protein precursor proopiomelanocortin (POMC) in the endocrine melanotrope cells of the pituitary intermediate lobe [34]. It is concluded that HPF followed by cryosubstitution provides a tissue preparation technique of choice when both optimal ultrastructure and strong immunoreactivity are required.

2. Time required

Pituitary gland dissection and cryofixation by high-pressure freezing: 1 day.

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Cryosubstitution, infiltration and polymerisation in Epon 812 or Lowicryl HM20 resin: 5 days.

Ultrathin sectioning: 1–2 days.

Immunogold labelling: 2 days.

Total time required: 10 days for 6 samples.

3. Materials

3.1. Animals

Young-adult male specimens (aged 6 months) of the South-African clawed toad, *X. laevis*, were used. The animals had been reared under standard conditions in our Nijmegen laboratory. They were fed beef heart and trout pellets (Trouvit, Trouw, Putten, The Netherlands) and kept on a grey background under constant light conditions, at 22 °C [6,7,12,34].

3.2. Special equipment needed

Aluminium planchettes (ALU Ø 3 × 0.5 mm and inner cavity Ø 2 × 0.15 mm; Waldner, Uetliburg, Switzerland).

Automatic cryosubstitution unit (CS Auto, Reichert-Jung, now Leica Microsystem, Vienna, Austria).

Jeol 1010 transmission electron microscope (Jeol, Tokyo, Japan).

Leica DMRBE optical system with Leica DC 500 digital camera (Leica Microsystems, Heerbrugg, Switzerland).

Leica EM high-pressure freezing (HPF) machine (Leica HPF, type 7225-01 HPF, Leica Microsystem, Vienna, Austria; now M. Wohlwend, Sennwald, Switzerland).

Microtubes (Saf-T-Seal; Biozym, Landgraaf, The Netherlands).

3.3. Chemical reagents used in this study

1-Hexadecene (822064 Merck, Hohenbrunn, Germany).

Anhydrous glutaraldehyde, 10% in acetone (16530; Electron Microscopy Science, Fort Washington, PA, USA).

BSA (810662; fraction V; Sigma Chemical, St. Louis, MO, USA).

Disodium hydrogenophosphate ($Na_2HPO_4 2H_2O$; K32055480321; Merck KgaA, Darmstadt, Germany).

Formvar (polyvinyl formal) powder (R1201; Agar Scientific, Essex, UK).

Glutaraldehyde 25% EM grade (R1012; Agar Scientific).

Lecithin (3-*sn*-phosphatidyl choline; 61751; Fluka, Buchs, Switzerland).

Lowicryl HM20 KIT (14340-E; Electron Microscopy Science).

Methanol (K25457509 829; Merck KgaA).

Potassium chloride (F515833926; Merck KgaA).

Potassium dihydrogen phosphate (KH_2PO_4; K23956173717; Merck KgaA).

Sodium chloride (K30914004 228; Merck KgaA).

Gelatine (VM 010670 249; Merck KgaA).

Uranyl acetate (R1260A; Agar aids, UK).

Lead nitrate (R1217; Agar aids, UK).

3.4. Antisera

Goat anti-guinea pig IgG 5- and 10-nm gold conjugates (BB International, Cardiff, UK).

Goat anti-rabbit-IgG 5- and 10-nm gold conjugates (BB International).

Polyclonal guinea pig anti-POMC [2].

Polyclonal rabbit anti-αMSH [33].

Polyclonal rabbit anti-BDNF (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Polyclonal rabbit anti-mesotocin (generous gift from Dr. Lutgarde Arckens, Leuven, Belgium).

3.5. Solutions

Formvar solution (0.6%). Dissolve 1.2 g formvar in 200 ml chloroform.

Label buffer (phosphate buffer-gelatin; PBG; pH 7.4). Dissolve 1 g BSA fraction V and 0.09 g gelatin in 200 ml PBS.

Lead citrate (2%). Dissolve 1.33 g lead nitrate and 1.76 g trisodium citrate in 30 ml distilled water. Add 8 ml 1 M sodium hydroxide, dilute up to 50 ml with distilled water and centrifuge for 5 min at 11,600 × *g*. Store supernatant in a dry bottle at 4 °C [34].

Phosphate-buffered saline (PBS; 0.01 M; pH 7.4). Dissolve 4 g NaCl, 0.107 g KCl, 0.575 g $Na_2HPO_4{\cdot}2H_2O$ and 0.102 g KH_2PO_4 in 450 ml distilled water. Adjust pH to 7.4 and make the solution up to 500 ml with distilled water.

Substitution medium. Dissolve 1 g uranyl acetate in 200 ml methanol, add 1.6 ml 25% glutaraldehyde.

Uranyl acetate (2%, aqueous). Dissolve 0.04 g uranyl acetate in 2 ml distilled water, centrifuge for 5 min at 11,600 × *g* and use supernatant.

4. Detailed procedure

4.1. Tissue dissection

Young-adult South-African clawed toad *X. laevis* were taken from holding tanks, rapidly decapitated, and under a dissection microscope, the pituitary gland was exposed. With fine forceps, the distal lobe of the pituitary gland was gently removed from the neurointermediate lobe (NIL, consisting of the neural lobe and the intermediate lobe of the gland). To prevent autolysis, dissection was carried out as fast as possible, within 1 min. All animal procedures were carried out in accordance with the Declaration of Helsinki and the Dutch law for animal welfare.