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Research paper

Inner ear tissue preservation by rapid freezing: Improving fixation by high-pressure freezing and hybrid methods

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ABSTRACT

In the preservation of tissues in as 'close to life' state as possible, rapid freeze fixation has many benefits over conventional chemical fixation. One technique by which rapid freeze-fixation can be achieved, high pressure freezing (HPF), has been shown to enable ice crystal artefact-free freezing and tissue preservation to greater depths (more than 40 μm) than other quick-freezing methods. Despite increasingly becoming routine in electron microscopy, the use of HPF for the fixation of inner ear tissue has been limited. Assessment of the quality of preservation showed routine HPF techniques were suitable for preparation of inner ear tissues in a variety of species. Good preservation throughout the depth of sensory epithelia was achievable. Comparison to chemically fixed tissue indicated that fresh frozen preparations exhibited overall superior structural preservation of cells. However, HPF fixation caused characteristic artefacts in stereocilia that suggested poor quality freezing of the actin bundles. The hybrid technique of pre-fixation and high pressure freezing was shown to produce cellular preservation throughout the tissue, similar to that seen in HPF alone. Pre-fixation HPF produced consistent high quality preservation of stereociliary actin bundles. Optimising the preparation of samples with minimal artefact formation allows analysis of the links between ultrastructure and function in inner ear tissues.

1. Introduction

Preservation of biological structure in a state as 'close to life' as possible is best achieved by fixation of all components of the sample at the same time. From this perspective, the use of chemical fixatives is not desirable due to the time taken to diffuse fixatives through the sample and the specificity of the chemical crosslinks formed (Gilkey and Staehelin, 1986). As an alternative to such methods, rapid freeze fixation protocols have been developed and are routinely used in electron microscopy. In rapid freeze fixation, samples are cooled at a rate sufficient for water to be frozen in a vitreous state, without the formation of ice crystals ("vitrification"). Fixation occurs in milliseconds, structures are preserved hydrated, and in as close to their native state as possible. Use of rapid freezing can avoid the common perturbations to structure caused by chemical fixatives. These can include effects on tissue structure caused by shrinkage or swelling of tissue, shrinkage of cellular organelles and extraction or redistribution of cellular constituents such as lipids, proteins and DNA. In addition most chemical fixatives react with proteins and crosslink peptide chains as part of their fixative action. These reactions can be highly deleterious to epitopes and therefore compromise immunohistochemical studies. Rapid freezing can produce superior preservation of epitopes and prevent artificial aggregations of proteins and fixation and processing without the use of crosslinking fixatives can produce samples with higher antigenicity (Kellenberger, 1991; Hayat, 2000; Claeys et al., 2004). The importance of close to life preservation with minimal artefact formation has also increased with the advent of techniques such as electron tomography, which allow visualisation of three-dimensional structures at the macromolecular level (Gilkey and Staehelin, 1986; Studer et al., 2001, 2008; Lucic et al., 2013).

There are a variety of methods in use for rapid freeze fixation of tissues, however the depth of vitrification that can be achieved by most methods is very limited. Methods that involve plunging the

Abbreviations: HPF, High Pressure Freezing; FS, Freeze Substitution; IHC, Inner Hair Cell; OHC, Outer Hair Cell

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sample into liquid cryogen (vitreous thin film/plunge freezing and immersion freezing) are generally limited to a few microns of vitrified sample. Penetration can be improved by methods such as cold metal block freezing ('slam freezing') or freezing by spraying the sample with liquid cryogen (jet freezing) but are still likely to only reach depths of a few tens of micrometres (Gilkey and Staehelin, 1986; Kachar et al., 2000). Slam freezing or immersion freezing combined with freeze fracture and deep etching have been used to produce high quality structural details of hair cells and hair bundles. For examples, details of actin arrangement in stereocilia and cuticular plate, lateral cisternae, lateral links and tip links have been examined using these methods (Hirokawa and Tilney, 1982; Forge et al., 1991; Kachar et al., 2000). However, the depth of good freezing in these methods is severely limited and using freeze-fracture it is difficult consistently to expose regions of interest and only small areas of these regions may be revealed.

Freezing larger volumes of tissue requires high pressures to be exerted on the sample, in combination with cryogenic temperatures. High pressure freezing (HPF) can extend the depth of vitrification of the sample to 200 μm (Studer et al., 1995, 2008). At sufficiently high pressures (210 MPa) the cooling rate required for the vitrification of water is reduced from several 100,000 K/s to a few 1000 K/s making vitrification of relatively thick samples practicable (Studer et al., 2008). Good heat conduction through the sample and between the sample and the carrier upon which it is mounted is vital for effective freezing. Air filled spaces between the tissue and the sample holder used for freezing can reduce the freezing efficiency by acting as insulators. Furthermore, air filled spaces are likely to collapse when high pressures are applied, deforming the tissue. To solve these problems, sample holders are often loaded with cryoprotectant fillers. A filler can act both as a cryoprotectant to improve freezing, and protect the sample against pressure induced shearing (Galway et al., 1995). Fillers are often substances that cannot penetrate the tissue and have low osmotic activity, for example solutions of dextran or the inert solvent 1-hexadecene. Alternatively cryoprotectants that penetrate the tissue, for example glycerol, can be used. The use of penetrating cryoprotectants can be undesirable, as their penetration into the tissue can lead to structural changes. However, in some cases this penetration seems to have no effect, probably due to diffusion barriers preventing entry to the tissue during the short exposure to the cryoprotectant (Dahl and Staehelin, 1989; McDonald, 2007; Studer et al., 2008).

After freezing, samples can be handled in a variety of ways and examined either at cryogenic temperatures or at room temperature after additional processing. Freeze substitution (FS) is a common technique for the low temperature dehydration of samples, before embedding in resin and examination at room temperature. Water in the samples is replaced by a solvent (usually acetone) at a temperature around −90 °C. This temperature is lower than the lowest temperature at which secondary ice crystals have been shown to form in biological samples (−70 °C) (Steinbrecht, 1985). Fixatives and heavy metal, electron dense stains such as osmium tetroxide and uranyl acetate are usually added to the sample to stabilise the frozen structure and improve image contrast. Because chemical fixation occurs when the sample is already frozen, there is no liquid water present and osmotic effects should not occur (Studer et al., 1992). Fixation by osmium tetroxide likely begins at about −70 °C (White et al., 1976). Once dehydration and infiltration of fixatives have occurred, the sample is slowly warmed to a suitable temperature for resin embedding.

Although HPF/FS protocols eliminate many of the artefacts associated with conventional fixation and embedding, the process of rapid freezing and substitution can itself cause artefactual changes to the tissue. Freezing artefacts, such as the growth of small ice crystals within the tissue due to insufficiently rapid freezing or subsequent warming are a common problem. Ice crystals formed during freezing cause dehydration in the surrounding cytoplasm, and solutes are concentrated as water is recruited to the growing ice crystal. After FS these effects are observed as characteristic holes in the sample (from the ice crystal branches) and aggregates of solute in the surrounding cytoplasm. In severe cases these changes in solute concentration can lead to the rupture of membranes (Galway et al., 1995; Dubochet, 2007). Where growth of small ice crystals has occurred a reticulated pattern is also often visible in the sample (Gilkey and Staehelin, 1986). Complex structures containing multiple different cell types such as the organ of Corti can prove difficult to vitrify uniformly due to differing freezing characteristics throughout the tissue. Often, the protocol used for HPF must be tailored specifically to the tissue in question (Koster and Klumperman, 2003).

Despite the potential advantages of freeze fixation for ultrastructural studies, there has been limited use of HPF methods for the examination of inner ear tissues. Work by Triffo et al. (2008) showed that a combination of microaspiration and HPF could be used to preserve isolated strips of guinea pig outer hair cells (OHCs) and work by Meyer et al. showed that good preservation of mammalian inner hair cells (IHCs) and particularly synaptic peripheral processes and morphology could be achieved by HPF but that consistent preservation across the organ of Corti was difficult (Meyer et al., 2009). The aim of this work was to evaluate the preservation of cells and structures in the organ of Corti and vestibular tissue of mammals and amphibians dissected and preserved by HPF and FS and to compare preservation to that achieved by conventional fixation methods. The use of hybrid methods of preservation was also examined.

2. Material and methods

Animals: Inner ear tissues were obtained from adult gerbils, adult tri-colour guinea pigs, C57/Bl6 mice at around P30, and from the red-spotted newt *Notophthalmus viridescens*. Rat tissues were obtained from postnatal or older rats as described in Dumont et al. (2001). All work with animals was conducted in accordance with procedures licenced by the British Home Office and approved by UCL Animal Ethics committee or in accordance with the National Institutes of Health (NIH) guidelines for animal care and use under protocol NIH 1215-11.

2.1. Experimental methods

High Pressure Freezing: For fresh frozen samples from gerbil and guinea pig, the auditory bullae were removed and held on ice. Cochlear and vestibular tissues were dissected from the bullae just prior to freezing. The time between sacrifice and freezing was approximately thirty minutes. Isolated mouse utricular maculae were removed to glutamax minimum essential medium (Life Technologies, UK) with HyClone serum (Thermo Fisher Scientific, USA) and 10 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (pH 7.3) (Sigma–Aldrich, USA) and maintained for 4–6 h prior to freezing. Otic capsules from the newts were removed from the animals into Amphibian Dulbecco's phosphate buffered saline (Taylor and Forge, 2005) and the inner ear samples consisting of the saccular maculae, amphibian papilla, and lagena macula were isolated then transferred to culture medium (Taylor and Forge, 2005) for maintenance at room temperature for 30–90 min prior to freezing.

For fixed tissue, auditory bullae were isolated and opened to expose the cochleae. Fixative was gently injected into the cochlea and vestibular system via an opening made at the apex of the