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Standardization of experimental animals temporal bone sections

Dalian Ding [b,c,e,*], Jintao Yu [a,b], Peng Li [b,c], Kelei Gao [a,b], Haiyan Jiang [b], Wenjuan Zhang [d], Hong Sun [a,b], Shankai Yin [e], Richard Salvi [b]

[a] Department of Otolaryngology Head and Neck Surgery, Central South University Xiangya Hospital, Hunan, 410018, China
[b] Center for Hearing and Deafness, Department of Communicative Disorders and Sciences, University of New York at Buffalo, Buffalo, NY 14214, USA
[c] Department of Otolaryngology Head and Neck Surgery, Sun Ya-sen University Third Hospital, Guangzhou, 510630, China
[d] Department of Otolaryngology Head and Neck Surgery, Union Hospital, Tongji Medical College of Huazhong University of Science & Technology, Wuhan, 430022, China
[e] Department of Otolaryngology Head and Neck Surgery, the Sixth People's Hospital of Shanghai Jiao Tong University, Shanghai, 200233, China

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Abstract

Preparation of the temporal bone for light microscopy is an important step in histological studies of the inner ear. Due to the complexity of structures of the inner ear, it is difficult to measure or compare structures of interest without a commonly accepted standardized measure of temporal bone sections. Therefore, standardization of temporal bone sections is very important for histological assessment of sensory hair cells and peripheral ganglion neurons in the cochlear and vestibular systems. The standardized temporal bone sectioning is oriented to a plane parallel to the outer and internal auditory canals. Sections are collected from the epitympanum to the hypotympanum to reveal layers in the order of the crista ampullaris of the superior and lateral semicircular canals, macula utriculi and macula sacculi, superior vestibular ganglion neurons, macula of saccule and inferior vestibular ganglion neurons, cochlear modiolus, endolymphatic duct and endolymphatic sac, and finally the crista ampullaris of the posterior semicircular canal. Moreover, technical details of preparing for temporal bone sectioning including fixation, decalcification, whole temporal bone staining, embedding penetration, and embedding orientation are also discussed.

Keywords: Experimental animal; Temporal bone section; Temporal bone; Collodion embedding; Decalcification

1. Standard protocols for temporal bone sections in experimental animals

1.1. Importance of standardization

Temporal bond sections are an important means of assessing inner ear pathologies in experimental studies (Jiang, 1999; Dai et al., 2003, 2004; Schuknecht, 1974; Ding et al., 1999a,b, 1997; Zheng et al., 2009; Fu et al., 2012; McFadden et al., 1999, 2004; Ding and Jiang, 1989; Ding et al., 2001, 2010, 1987, 1986, 1998a,b,c, 1991, 1999c, 2002, 1994; Geng and Ding, 1996; Lu et al., 1987; Wu et al., 1993; Xia and Ding, 1992; Fu et al., 2011, 2010; Luo and Jin, 1991; Guo and Jin, 1991; Xu et al., 1991). Although it is possible to quantify changes in cochlear and vestibular hair cells from isolated inner ear terminal organs via whole inner ear membranous labyrinth by surface preparation, however, the technique of surface preparations does not provide the capacity to evaluate the ganglion cells within the Rosenthal's canal, or the superior and inferior vestibular ganglion cells inside the bony wall, nor does it provide the means

* Corresponding author. Center for Hearing and Deafness, Department of Communicative Disorders and Sciences, University of New York at Buffalo, Buffalo, NY 14214, USA.
E-mail address: dding@buffalo.edu (D. Ding).
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to assess changes of neural fibers in the deep layer of the sensory and endolymphatic duct and sac epithelium or changes in deep portions of the stria vascularis, thus limiting its utilization for quantification of peripheral ganglion neurons in the cochlear and vestibular system. Traditional temporal bone sections are useful supplemental approache that cannot be completely replaced by other technologies. The temporal bone houses complex structures, which yield very different histological images when observed from different angles. For example, if the position of cochlear modiolus in mouse was rotated by 45 degrees either in clockwise or counterclockwise, the slice at cochlear axis in each turn will be moved a quarter turn. This means that the hook region of Corti's organ in response to 100kHz at standard horizontal section was changed to corresponding 40kHz, and the region in response to 20 kHz was switched to 4 kHz. Similarly, after turning 45° in the horizontal plane, the position of maculae of saccule and utricle will be completely changed. It is clear from these examples that the temporal bone section must follow a predetermined direction and angle, i.e. the temporal bone horizontal plane (Figs. 1 and 2) This article aims to introduce the standard protocol for temporal bone section in experimental animals, and some technical problems for temporal bone sections were also discussed.

2. Steps and caveats in preparation of temporal bone sections

2.1. Fixation

Inner ear sensory epithelium and cochlear and vestibular peripheral neurons are sensitive to anoxia and even ischemia of a very short time can cause severe anoxic pathological changes in these cells. As these tissues are situated deeply inside the temporal bone, rapid and effective tissue fixation is critical in order to minimize anoxic and ischemic artifacts from sample preparation. Although inner ear perfusion and immersion fixation can effectively fixate inner ear membranous labyrinth in a relatively short time, they are not adequate in timely fixating the spiral ganglion in the modiolus, the superior vestibular ganglion in the petrosal bone, or the inferior vestibular ganglions at the fundus of internal auditory canal. To ensure maintenance of the morphology of inner ear sensory epithelium and peripheral neurons at the time of harvest, cardiac perfusion as well as local perfusion in combination of immersion fixation are required. Briefly, the protocol is as following: 1) The anesthetized animal was put in a supine position; 2) The jugular vein was exposed and clamped via a middle line incision in the neck; 3) The chest is opened via a "U" shape incision to expose the heart; 4) An infusion needle is inserted into the left ventricle and transcardially perfused with 38 °C normal saline with a speed of 0.4 ml per minute, while the clamped jugular vein is opened to form a perfusion circulation from the left ventricle to the draining jugular vein. Some have suggested opening the right atrium for the drain instead of drain from jugular vein. However, it may lead to local perfusion caused by cardiac rupture. In our recommended protocol, the far distance between the left ventricle and jugular vein ensures avoidance of shunting, while maximizing surgical safety and improving the ease of observation. 5) Normal saline perfusion is terminated after 3 min or when the drainage is clear, and replaced by perfusion of 10% formalin in phosphate buffer solution (PBS) or 2.5% glutaraldehyde in PBS for 10 min or until the animal's body stiffens; 6) After harvesting the temporal bone, a hole was drilled on the apex of the cochlea and round window membrane was punctured, the stapes on oval window was also extracted. Then followed by perfusion of fixative via the apical hole or round/oval window opening using a pipette; 7) Immerse the temporal bone into the fixative for 24 h at 4° (Ding et al., 1997; Zheng et al., 2009; Fu et al., 2012; McFadden et al., 2004; Ding and Jiang, 1989; Ding et al., 2001, 2010, 1998c, 2002).

2.2. Decalcification and dehydration

To produce high quality temporal bone sections, not only decalcification must be thorough, the selection of decalcification agent is also important. If the sections are intended for observation of general histological changes of the inner ear sensory epithelium and peripheral neurons, decalcification using 5% nitric acid or 5% hydrochloric acid is sufficient and can be carried out in rats or mice with daily solution replacement for about 3 days. With such agents, a test can be done on the 3rd day by adding a small amount of saturated ammonium oxalate and ammonium to the decalcification solution taken from the specimen container. Presence of chalky sediment indicates incomplete decalcification and more solution replacement and time are needed. Following decalcification by these strong acidic agents, immersion of the specimen in 5% sodium sulfate for 24 h followed by ample rinsing is necessary to remove any residual acidic agent in the tissue. It is worth noting that the double decomposition reaction in strong acidic decalcification solution can convert calcium carbonate into water-soluble calcium chloride which can further break down into carbon dioxide and water. The latter resulting in bubbles in the inner ear cavity. This is the sole step during temporal bone section preparation that produce bubbles in the specimen (Ding and Jiang, 1989; Ding et al., 2001, 2010), which can lead to rupture of sections as they form empty spaces in embedded specimen if not properly removed. Cochlear perfusion or vacuum suction can be used to remove these bubbles, although suction is not needed for each step during specimen preparation. If the specimen is prepared for immunohistochemical studies to examine expression of certain proteins in inner ear cells, 10% (ethylenedinitrilo) tetraacetic acid disodium salt (EDTA) solution should be used for decalcification in rats and mice for 5 days, to minimize damage to proteins and preserve antigen–antibody reactivity in cells. It should be noted that, as a complex with calcium, EDTA solution is not stable and fresh EDTA solution is needed for replacement on a daily basis. Because EDTA is neutral, the specimen can be rinsed in flowing