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Standardization of experimental animals temporal bone sections

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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

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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

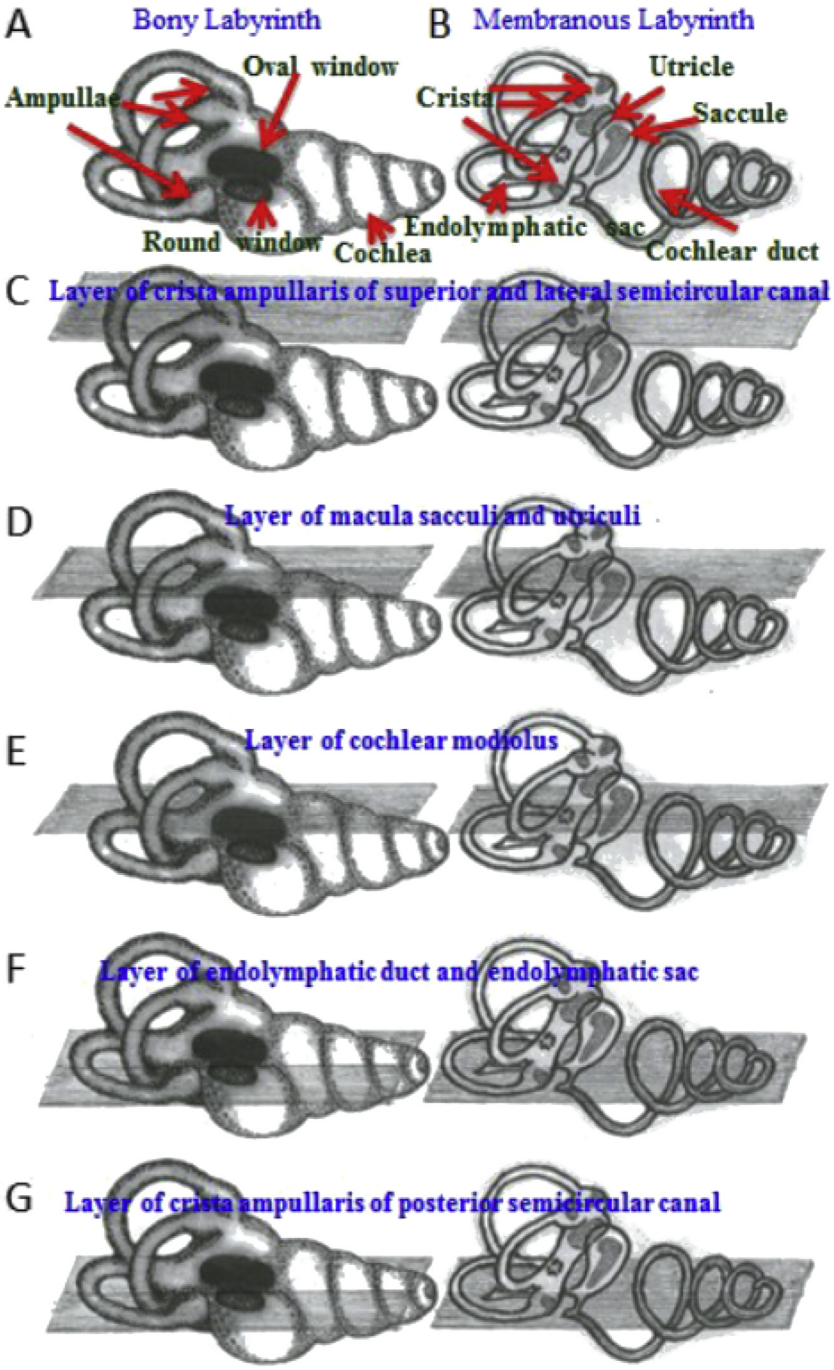

Fig. 1. Depiction of cutting levels in relation to inner ear in standardized temporal bone sections. A. Landmarks of bony labyrinth. B. Landmarks of membranous labyrinth. C. Level 1: parallel to the bottom of epitympanum and cutting through the superior and lateral semicircular canals showing the cupulae. D. Level 2: cutting through the utricle and saccule. E. Level 3: cutting through the modiolus. F. Level 4: cutting through the endolymphatic duct and showing endolymphatic sac. G. Level 5: cutting through cupula of the posterior semicircular canal.

water upon completion of calcification and no sodium sulfate treatment is needed.

Whether dehydration is needed after decalcification depends on what embedding material is to be used. When using water-soluble materials such as gelatin or OCT for frozen sections, no dehydration is needed. On the other hand, if collodion, paraffin or epoxy resin is to be used, water in the specimen must be completed removed through gradient alcohol replacement. The duration of each alcohol gradient step is determined by the size of the temporal bone. For rat or mouse temporal bone, this duration should be about 5–10 min.

2.3. Whole temporal bone staining

Traditionally, in a temporal bone section series, each section is collected and stained separately. This is typically applied to collodion or paraffin sections and time consuming.

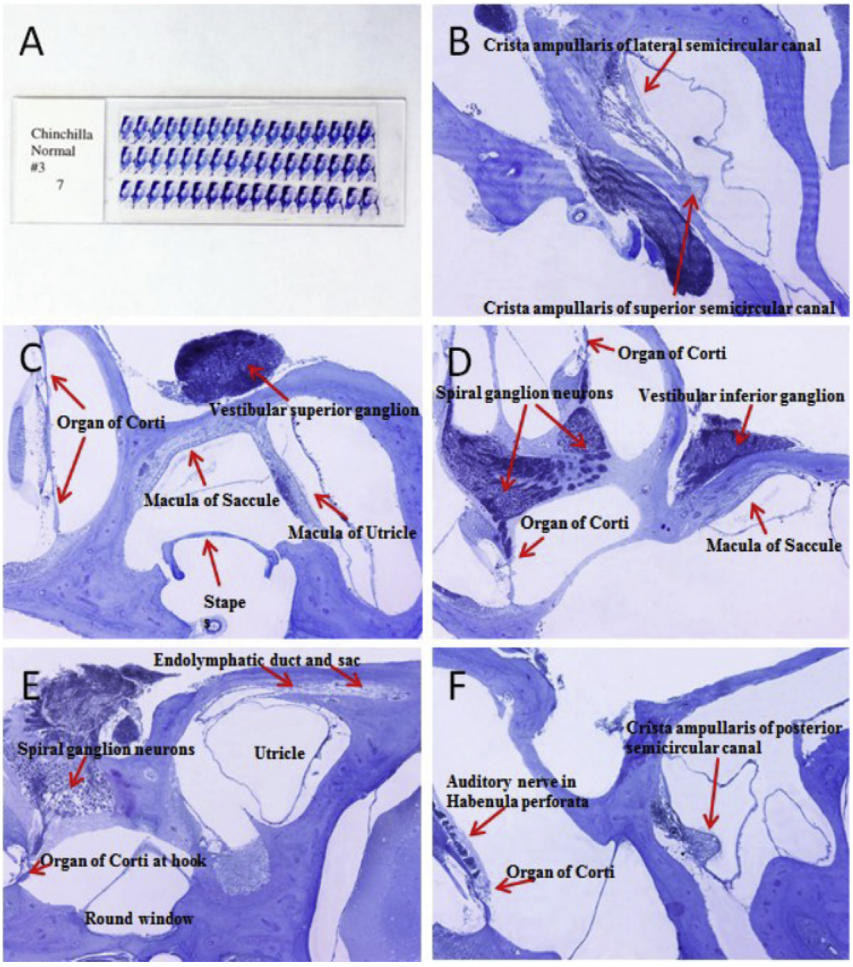

Fig. 2. Important levels on standardized temporal bone sections. A. Serial cochlear sections in chinchilla. B. Sections of cupullae of superior and lateral semicircular canals in mice. C. Sections of utricle and saccule maculae in mice. D. Sections of modiolus, saccule macula and inferior vestibular neurons in mice. E. Sections of endolymphatic duct and sac in mice. F. Sections of cupula of posterior semicircular canal in mice.

Also, this technique is not suitable for temporal bone frozen sections for possible contamination of embedding materials by the routine H.E. staining. To ensure equal staining of every section and to save time, we recommend whole temporal bone staining following decalcification as described below. To prepare Ehrlich's hematoxylin, 2% hematoxylin in ethanol is slowly added into 5% ammonium aluminum sulfate solution. After 5 days, 10% glycerol acetate is added and the complex is left under ample lighting for 50 days for natural oxidation. The stain is ready when showing a deep purple color. Before staining a temporal bone specimen, a diluted staining solution is made by mixing 6 ml of Ehrlich's hematoxylin solution with 5 ml of acetate and 66 ml of 50% ethanol. After decalcification, the temporal bone specimen is immersed in the staining solution at 37 °C for 3 days, followed by flowing water rinsing for 4 h and the specimen is ready for sectioning. In whole temporal bone staining, not only staining is equal throughout the specimen, but microscopic examination is immediately possible after slides collection, which helps minimize contamination of water-soluble embedding materials by staining agents while improving productivity of serial sections (Ding and Jiang, 1989; Ding et al., 2001, 2010).

2.4. Infiltration

Infiltration of the embedding material in cells and into inner ear spaces ensures high quality of temporal bone sections. Of the commonly used embedding materials, gelatin, OCT, collodion and epoxy resin are suitable for collection of large size sections and serial temporal bone sections. Embedding with gelatin, OCT for frozen sections and with epoxy resin for semi-ultra-thin sections are simple and less time consuming, as compared to embedding with collodion which requires a penetration period of weeks to months. Through our experiences, we have established a protocol that can significantly shorten the penetration time for collodion infiltration, as described in the following: Following dehydration, guinea pig or rat temporal bone is placed in 50 ml of alcohol and ether of equal proportion, followed by addition of 2.5 g of collodion. Due to its high density, collodion falls to the bottom of the container while the temporal bone specimen rises up to near surface. After 48 h, as collodion is completely dissolved in the alcohol/ether solution, the temporal bone specimen Sank to the bottom, indicating the concentration of collodion achieve a new balance inside and outside of the temporal bone. Add

another 2.5 g of collodion and the specimen rises again, only to repeat falling to the bottom when concentrations of collodion inside and outside the specimen balance again 48 h later. The secret in this protocol is that the dissolution process of collodion into the alcohol/ether solution parallels the process of its infiltration into temporal bone tissues. After two rounds of dissolution/infiltration, the collodion concentration inside the temporal bone specimen is essentially sufficient for the following step of collodion embedding (Geng and Ding, 1996).

2.5. Orientation embedding

Following infiltration with the embedding material, the temporal bone specimen is to be placed in the embedding frame for orientation and embedding. To ensure that important inner ear structures are included and most inner ear sensory epithelia are in best angles for examination, the embedding must be aligned with the horizontal plane of the temporal bone for sectioning. Using epoxy resin embedding as an example, the temporal bone is laid flat in the embedding frame with the promontory pointing up, petrosal bone down and epitympanum near one end of the frame. This orientation ensures that collection of sections starts from the horizontal plane at the bottom of epitympanum near the end of the frame.

2.6. Selection of embedding agents

There are a number of embedding agents and their selection depends on the goal and needs of the study. In general, for serial sections of the whole temporal bone, due to the large section sizes, collodion should be used as collodion sections are suitable for large tissue specimens as they best preserve tissue structures and morphology through avoiding excessive hardening of fibrous tissues and minimal tissue shrinking and distortion. This is especially true for preparing fetal inner ear sections, temporal bone sections in large animals and specimens of membranous labyrinth hydrops models (Fig. 3). However, as collodion sections are relatively thick, overlaying cells in one section are common as a shortcoming. In contrast to collodion sections, paraffin sections preparation is simple, but not suitable for large size specimens. Tissue shrinking and distortion during high temperature infiltration are also commonly seen problems. Paraffin therefore does not meet the requirements for preparation of temporal bone sections from large animals, nor is it adequate for histological studies of membranous labyrinth hydrops models. Epoxy resin is usually used in preparing ultrathin sections for electron microscopic studies. Under special study conditions, ultrathin sections of hundreds of nanometers can be examined directly under a light microscope. Temporal bone specimens embedded in epoxy resin are therefore considered suitable for studying ultra-structures in inner ear cells. Temporal bone frozen sections are embedded using gelatin or OCT, which are water soluble. These sections are relatively thick and prone to tissue expansion that can lead to tissue or cellular structure distortion. Frozen sections are therefore not suitable for studying cellular ultra-structures or membranous labyrinth hydrops models.

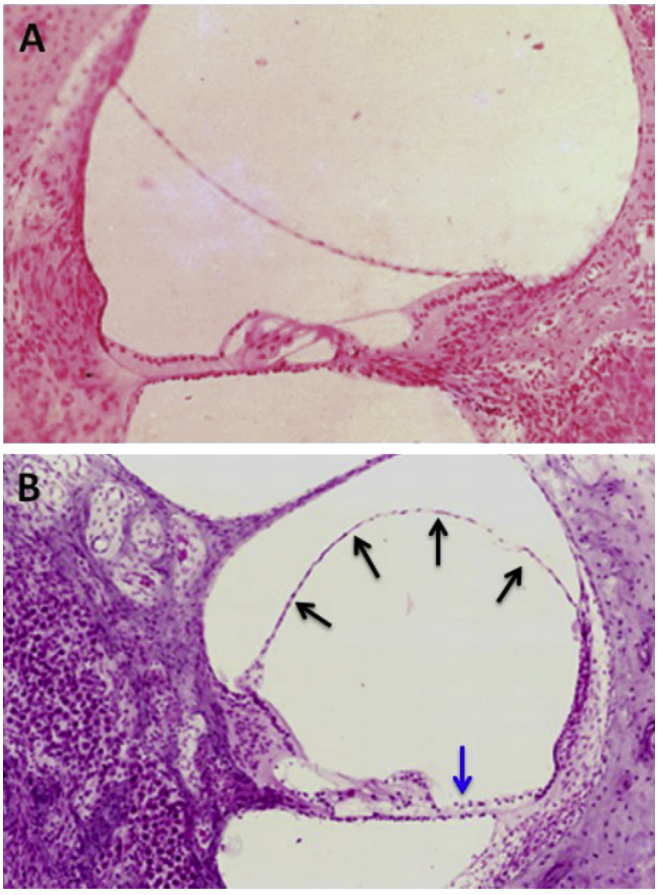

Fig. 3. Collodion sections in a model of obstructive membranous labyrinthine hydrops. A. Normal triangle cochlear duct in a guinea pig. B. Four weeks following endolymphatic sac obliteration via an occipital approach, there is significant distention of the cochlear duct from hydrops with the vestibular membrane bulging into the vestibular scala (black arrowheads) and the basilar membrane shifting downward (blue arrowhead), indicating obstruction of longitudinal endolymphatic circulation that causes expansion of the cochlear duct.

2.7. Key level sections

Unless continuous sections are required by study goals, sections of one or several specific levels can be selectively collected depending on the purpose of the study. Starting from the level of bottom of epitympanum, sections of cupulae of the superior and lateral semicircular canals are first collected (Figs. 1C and 2B), followed by transverse sections of the maculae of utricle and saccule at the level of stapes, as well as sections of the superior vestibular neurons medial to the superior cribrosa and the vestibular nerve branch (Figs. 1D and 2C). As cutting continues, transverse sections of the saccule macula on the medial wall of vestibular cavity and inferior vestibular neurons at the bottom of internal auditory canal, as well as cochlear modiolus, become available (Figs. 1E and 2D). On modiolus sections, not only the organ of Corti in all

cochlear turns and cochlear spiral ganglion cells in Rosenthal's canal of modiolus are visible, the auditory nerve fibers in habenula perforata from all cochlear turns can also be examined (Zheng et al., 2009; Fu et al., 2012; McFadden et al., 2004; Ding and Jiang, 1989; Ding et al., 2001, 2010, 1998a,b,c, 1999c; Fu et al., 2011; Fu et al., 2010). Below the saccule, endolymphatic tube that extends backward and the endolymphatic sac become visible (Figs. 1F and 2E) (Luo and Jin, 1991). At the lowest level of temporal bone sections, transverse sections of cupula of posterior semicircular canal and inferior vestibular nerve fibers going through the inferior cribrosa can be seen (Figs. 1G and 2F).

3. Summary

Standardization of horizontal sections of the temporal bone is important in studying the location and extent of pathologies of inner ear auditory sensory organs, vestibular sensory organs and peripheral cochlear and vestibular neurons. Standardization allows comparison of temporal bone sections across authors and labs, as well as adequate assessment of inner ear pathologies by different authors and their audiences from the same view point or angle. The current paper describes some technical details in preparation of temporal bone sections, based on authors' experiences, which we hope will be helpful for our colleagues engaged in inner ear histopathology research.

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