# Methods of Preparation and Study of Temporal Bones

## Standard light microscopy method

Processing of temporal bones begins with fixation and continues through the steps of decalcification, embedding, sectioning, staining, mounting and labeling.

1. **Fixation and decalcification**. The most common fixative for light microscopy is 10 percent neutral buffered formalin. For electron microscopy (for example, specimens removed within 2 to 4 hours after death), 0.1 percent glutaraldehyde is the preferred fixative. Place each temporal bone in a glass jar with about 300 ml of formalin at 4°C in the refrigerator for 3 to 4 weeks. Follow with decalcification using 0.27 M ethylenediaminetetraacetate (EDTA) at room temperature. Change EDTA weekly, check for calcium and confirm by x-ray.

2. **Embedding.** Remove EDTA by washing the specimen in two changes of distilled water in 24 hours. Then dehydrate the specimen over a period of 10 days using increasing concentrations of alcohol: 50 percent, 70 percent, 80 percent, 95 percent and 100 percent, and finally ether-alcohol in 1:1 ratio. Then, embed the specimen in celloidin over 3 to 4 months beginning with 1.5 percent celloidin and increasing to 3 percent, 6 percent and, finally, 12 percent. After embedding in 12 percent celloidin, allow the specimen to harden for two weeks in a dessicator. Cut away excess celloidin from the sides of the block until a quarter inch border of celloidin remains on all sides of the specimen. Place the block in cedar wood oil for at least one week before cutting.

3. **Sectioning.** Use a special sliding microtome for sectioning. Mount the block on the microtome in the desired place of sectioning by initially softening the inferior surface of the block using ether and alcohol. Proper orientation is critical. Most specimens are sectioned in the axial plane, which is achieved when the following anatomic structures are present simultaneously on the cutting surface: superior canal crista, bony wall of lateral canal, facial nerve genu and superior surface of malleus head and incus body. When cutting vertical sections from medial to lateral, scala tympani and scala vestibuli should appear simultaneously in the basal turn of the cochlea.

4. **Staining.** Use stellite edged knives and a section thickness of 20 microns. Each specimen usually yields 400 to 500 sections in the axial plane and 800 to 1,000 in the vertical plane. Place each section on numbered pieces of onionskin paper. Every tenth section is placed in a dish of 80 percent alcohol and stained with hematoxylin and eosin (H&E). Wrap the remaining sections in gauze and store for long term in 80 percent alcohol.

5. **Mounting.** The stained sections are mounted on glass slides one-by-three inches. Permount mounting medium is used followed by a cover slip. Remove excess mounting medium with Histo-clear. Place lead weights onto the slides and allow them to dry for at least one week.

6. **Labelling.** Label the slides with India ink. A complete set of stained sections from a normal temporal bone is shown to the left.

## Other embedding media: Celloidin, paraffin and polyester Wax

- **Celloidin** is expensive, the time required for its hardening is long, and if re-embedding is necessary, artifacts are seen. The great advantage of celloidin is that it demonstrates the most superior morphologic preservation of the bony and membranous labyrinth.

- Although **paraffin wax** is commonly used in clinical pathology and in animal otopathology, human specimens that have been embedded in paraffin wax often show poor morphologic preservation and artifactual disruptions of the delicate membrane labyrinth. The advantages of paraffin are that it is inexpensive, the time required for its embedment is short, and it facilitates immunostaining.
- Recently, **polyester wax** has been used as an embedding medium for human temporal bones (Merchant et al . 2006). Morphologic preservation is better than in paraffin, but not as good as in celloidin. Polyester wax is also relatively inexpensive and offers ease of re-embedding with minimal artifact. Immunostaining is also feasible in polyester wax. However, its limitations are that the size of the block is small and it is difficult to get polyester wax sections to adhere to the slide.

## Removal of celloidin

In some cases, celloidin has to be removed to facilitate immunostaining, special stains, or re-embedding for electron microscopy. Use slides that have been cleaned with a potassium dichromate solution and coated with gelatin (Wyllie technique), label them with diamond pencil, and coat them with albumin. Place the tissue into a dish of 80 percent ethyl alcohol, float it onto cigarette paper, trim, place on the slide, blot with bibulous paper and press with a roller to remove air bubbles.

Place the tissue section, which is still backed by cigarette paper, on a paper towel, cover it with a piece of bibulous paper that has been dipped in 10 percent formalin and press for 30 to 60 minutes under a wooden block and lead weight. Then, place the tissue section into a solution of 80 percent ethyl alcohol, whereupon the cigarette paper will separate from the tissue and can be removed. Following this, celloidin can be removed by one of four different techniques using clove oil (Portmann et al. 1990), ether alcohol (Miguel-Hidalgo and Rajkowska. 1999), acetone (Keithley et al. 1995) or sodium methoxide (O'Malley et al. Effect of Choice of Fixative and Embedding Medium on Immunostaining of the Cochlea. Association for Research in Otolaryngology Abstracts of the 30th Midwinter Meeting, 2007, #74).

The most complete removal of celloidin is obtained with the sodium methoxide technique and complete removal is critical to succeed with immunostaining. Fifty grams of sodium hydroxide is mixed with 50 ml of methanol vigorously and allowed to settle for 30 minutes at room temperature. The solution (cloudy solution on top of pellets) is diluted 1:2 with methanol and used immediately. Apply a couple of drops of the sodium methoxide to the dried celloidin sections for five minutes, then rinse it with 100 percent methanol. Repeat this twice. Rinse further with 100 percent and 70 percent methanol for 10 minutes each. Then, transfer the slide to distilled water for 10 minutes followed by 0.01 M phosphate-buffered saline for 10 minutes. Proceed with immunostaining or embed in Epon for electron microscopic study.

## Other methods of study of temporal bones

Many other techniques of sectioning temporal bones and of microscopy have been described. Each has pros and cons and is suitable for certain aspects of study.

- The technique of **surface preparation and phase microscopy** is very useful for accurately assessing hair cell population (Engström, et al. 1976).
- **Electron microscopy** can be very useful to elucidate the ultrastructural anatomy. Electron microscopy of the human ear is challenging as the postmortem time has to be very short and tissues must be well preserved.
- Both **transmission and scanning electron microscopy** have been successfully used for studying the human inner ear (recent examples: Thiers et al. 2002, Rask-Andersen et al. 2006).
- Spoendlin devised a **block surface technique** by which the entire cochlea could be studied using both light and electron microscopy (Spoendlin and Brun. 1974, Scholtz et al. 2001).
- Leslie Michaels (Michaels et al . 1985) developed a **microslicing method** whereby the temporal bone is sectioned before decalcification using a high speed diamond saw into sections that are 3 mm in thickness. These 3 mm thick

sections are then further studied by light or electron microscopy after undergoing decalcification, embedding, etc., as needed.

- Investigators have also **micro-dissected the end organs** from the inner ear and used them for light or electron microscopy as well as immunostaining, in-situ hybridization etc (Wright and Hubbard. 1978, Ulualp et al. 2004, Ishiyama et al. 1997, Lopez et al. 2007).
- Functional evaluation of remodeling of bone in the otic capsule can be studied using a **special cutting and grinding technique** to generate tissue sections which are then examined by transmission ultraviolet microscopy. This technique was developed by Sorensen and colleagues (Sørensen et al. 1992).
- Another technique is **orthogonal plane fluorescence optical sectioning (OPFOS)**, developed by Voie and colleagues (Voie and Spelman. 1995). In this technique, the temporal bone specimen is optically sectioned with a special laser and images are acquired for 3-D reconstruction.

## Study of nucleic acids by PCR

The temporal bone offers a significant obstacle in the application of PCR-driven nucleic acid retrieval. Such studies are generally performed using unfixed tissue obtained at biopsy and then frozen at -80°C. In the case of human temporal bones, the bones are removed several hours after death, therefore nucleic acid degradation and autolytic change is inevitable. Temporal bone specimens can be frozen at -80°C but the membranous labyrinth is encapsulated in the dense bone of the otic capsule. One has to section the bone and decalcify it in order to gain access to the membranous labyrinth. There is no practical way of sectioning or decalcification while keeping the bone frozen and preserving the anatomy and morphology of the delicate membranes of the inner ear. Therefore, from a practical point of view, the main source of tissue for genomic studies is temporal bones that have been fixed, decalcified, embedded and sectioned.

Despite these challenges, retrieval of DNA by PCR from archival temporal bone sections has been accomplished. This was first described by Wackym (Wackym et al. 1993) and McKenna and colleagues in 1994 (McKenna MJ, Kristiansen A, Haines J. Isolation and identification of nucleic acid sequences from celloidin and paraffin embedded human temporal bone sections. In: Immunobiology in Otorhinolaryngology, Eds: Mogi G et al, Amsterdam: Kugler Publications 1994; 283-288). Some examples of studies employing such a technique include those used to identify herpes zoster virus DNA in Ramsay Hunt syndrome (Wackym et al. 1993), herpes simplex virus DNA in Bell's palsy (Burgess et al. 1994), measles virus DNA in otosclerosis (McKenna et al. 1996) and investigate the role of mitochondrial DNA mutations in presbycusis (Fischel-Ghodsian et al. 1997; Markaryan et al. 2008).

Archival sections have some limitations for PCR-based DNA studies. Traditional DNA extraction and prior PCR amplification studies have demonstrated that DNA can be extracted, but genetic studies may be limited by small fragment size and contamination from extraneous DNA sources due to processing (McKenna et al. 2002). Ongoing studies are aimed at developing modified extraction protocols and applying next generation sequencing to sequence high value temporal bone studies. Therefore, it is advisable to obtain a clean sample of DNA for each temporal bone case, which can be procured during life using a buccal swab or a blood draw, or at the time of autopsy using a sample of blood, muscle or other tissue. Such clean DNA is extracted and stored at -80°C. Genomic studies from such samples can then be correlated with the otopathology in the temporal bone.

It is difficult to retrieve RNA from archival sections because of degradation that occurs as a result of multiple factors such as postmortem autolysis, the use of fixation and the presence of RNAases during processing (Lee et al. 1997). Nonetheless, several investigators have described retrieval and amplification of RNA from temporal bones including the use of laser capture microdissection (Ohtani et al. 1999, Pagedar et al. 2006, Kimura et al. 2007, Markaryan et al. 2008).

## Immunostaining

The routine processing of temporal bones for H&E morphology can obscure antigens because of fixation, decalcification and use of embedding media. This makes it difficult to perform immunohistochemistry on archival sections. However, with proper techniques, successful immunostaining can be accomplished on archival sections as has been reported by a variety

of investigators under both normal and pathological conditions. Some examples: Wackym et al. (1990), Tian et al. (1999), Fish et al. (2001), Doherty and Linthicum. (2004), Zehnder et al. (2005), Schrott-Fischer et al. (2007), Lopez et al. (2007).

## Study of proteins by mass spectrometry: Proteomics

Hundreds or thousands of proteins can be retrieved from very small samples using mass spectrometry. This has great promise for the application of molecular biologic assays to temporal bones and to gain insight into a variety of otopathologic conditions. Successful application of proteomics has been demonstrated by Palmer-Toy et al. (2005) on archival temporal bone sections using liquid chromatography and mass spectrometry. Additional publications by Robertson et al. (2006) and Merchant et al. (2007) demonstrate the power of this technique.