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Newsletter of the NIDCD National Temporal Bone, Hearing and Balance Pathology Resource Registry

Inside

APPLICATION OF MODERN MORPHOMETRIC METHODS FOR STUDYING THE HUMAN TEMPORAL BONE

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The goal of our laboratory has been the advancement of temporal bone science, using rapid-fixation and tissue-processing protocols aimed at antigenic and morphological preservation to allow the application of the most modern methodology for quantification and identification of pathological changes.

Application of Unbiased Stereology to Traditional Archival Temporal Bones

The vast majority of archived human temporal bones have been fixed in formalin, and processed using prolonged decalcification, dehydration, and celloidin-embedding. Specimens are sectioned in the mid-modiolar axis at 20 µm thickness, and every tenth section is stained with hematoxylin and eosin (Merchant et al., 1993; Schuknecht,1993). Previous morphometric studies of neuronal number used the Abercrombie (1946) or a related correction method, which is based on assumptions of a uniform size and shape of the object being counted. In the mid 1980s, it was discovered that the traditional assumption-based methods, including the Abercrombie, were inaccurate, creating error rates ranging from 15% to 60% when compared with the true cell number using complete serial reconstruction (Coggeshall and Lekan, 1996; Pakkenberg et al., 1991). The unbiased stereological methods were developed to obtain counts of objects in a 3-dimensional structure by sampling in 2-dimensions, free of sampling and systematic bias (Stereo,1984).

The unbiased stereological - optical fractionator, is an ideal tool to estimate the number of neurons in traditionally processed archival human temporal bones. The optical fractionator represents a combination of the optical dissector and fractionator (Gunderson et al., 1988). The optical dissector is composed of a series of optical planes of a high-power objective (with high NA) as viewed along the z axis of a relatively thick tissue section. The focal plane (optical section) can be moved a known distance through a thick section, producing a continuous series of superimposed sections within which counting can be carried out with dissector counting rules. Our laboratory uses the Stereo Investigator v3.0 software (MicroBrightField, Inc., Colchester, VT). A personal computer and a monitor are connected to a high resolution color

video camera mounted at the top of a Nikon Optiphot microscope, which has differential interference contrast. The motorized stage (LEP Bio-point, Germany) of the microscope is controlled by the software allowing for precise, well-defined movements along the x and y axis. A microcator (Heidenhain, Germany) is attached to the stage of the microscope for precise measurements in the z axis. High image resolution and a thin focal plane are necessary for true stereological technique, which are obtained using oil immersion objective lens with a high numerical aperture (NA = 1.40). Figure 1 demonstrates the optical fractionator to obtain estimates of Scarpa's ganglion neuronal number.
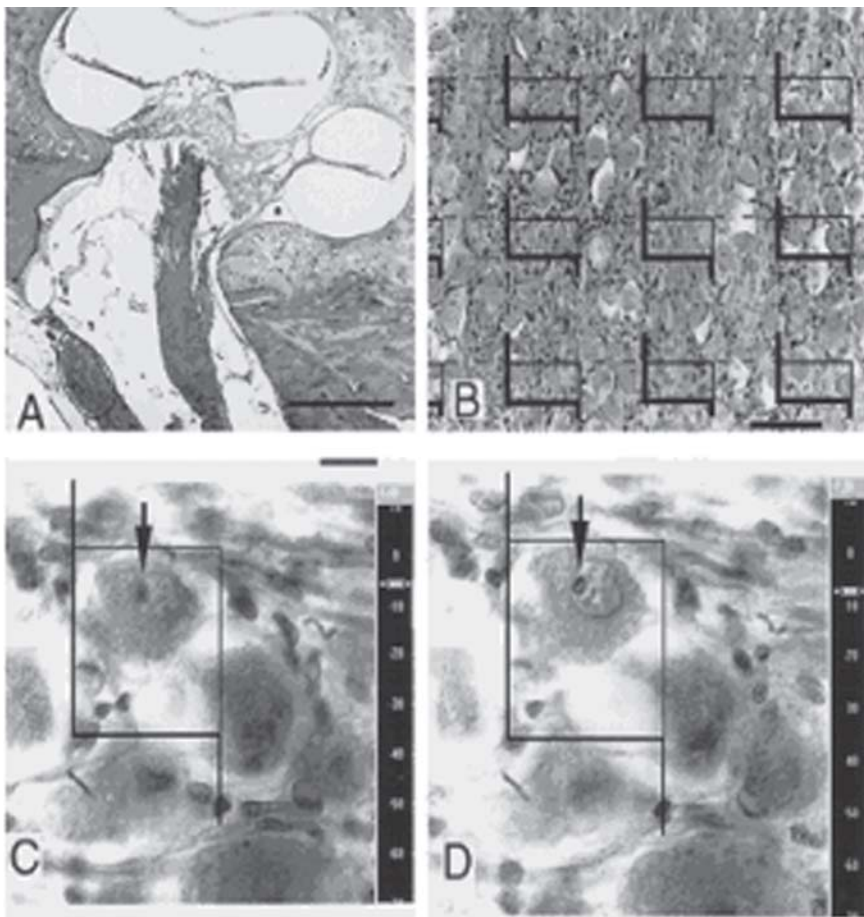
Using the optical fractionator on archival human temporal bones, we estimated the total number of spiral ganglion neurons to be 41,643 from five patients with normal findings on audiogram (Ishiyama et al., 2001; Tang et al., 2002). Previous studies had underestimated the number of spiral ganglion neurons by 16% (Otte et al., 1978), 19% (Hinojosa et al., 1985), and 42% (Pollak et al., 1987). We found that the total number of vestibular ganglion neurons in subjects younger than 60 years of age was 27,635

neurons (Park et al., 2000, 2001; Tang et al., 2002). Previous studies underestimated the number of vestibular ganglion neurons by 36% (Richter, 1980) and 24% (Velazquez-Villasenor et al., 2000). The optical fractionator method was used to obtain unbiased estimates of the vestibular ganglion neuron number in gentamicin and streptomycin ototoxicity, (average post-ototoxicity = 2.2 years), using traditional archival human temporal bones (Ishiyama et al., 2005). The neuronal number was 20,733 in the aminoglycoside, significantly lower than an age-matched normal of 24,902 (p = 0.004). A previous study using the Abercrombie method did not demonstrate significant differences in aminoglycoside ototoxicity (Tsuji et al., 2000), however the post-ototoxicity time was significantly shorter (4–12 months).

Undoubtedly, traditionally processed archival human temporal bones have provided a wealth of information, but this methodology has some limitations. There is no single plane of sectioning that provides equally advantageous exposure for all five vestibular endorgans. Also, prolonged fixation in formalin and celloidin-embedding is associated with poor morphological preservation, impeding immuno-

Figure 1. (A) Representative view of the human temporal bone. A contour has been traced around the vestibular ganglion. (B) View of the computer-generated sampling grid that is placed over the vestibular ganglion in a random fashion. A rectangular counting frame (two-dimensional unbiased counting frame) is seen at the upper left corner of each box of the sampling grid. (C) Counting of nucleoli. Focal depth in µm (measured by the microcator attached to the microscope stage) is indicated on the focal depth bar, where 0 µm represents the top surface of the section. Negative depth values indicate that the microscope is focusing downwards into the section. An unbiased counting frame is put on C and D. The thick border of the frame and its extension is the exclusion line, and the thin border of the frame is the inclusion line. The counting rule of the optical disector is that nucleoli that come into focus within the height of the optical disector and within the counting frame are counted when one moves the focal plane continuously through the section. A nucleolus is considered to be within the counting frame if it is entirely within the counting frame or partially within the counting frame without touching the exclusion line of the counting frame when it first comes into focus. (C) Focal depth is at -5.68 µm. (D) Focusing downward to -7.20 µm brings one nucleolus indicated by arrow into focus. Therefore, one nucleolus is counted. Scale bars: 2000 µm in A; 50 µm in B; 5 µm in C.

histochemical, ultrastructural analysis, and molecular biological studies. Additionally, due to the thickness of the specimens, detailed cytological identification of type I, type II hair cells and supporting cells is subject to error. Lastly, significant postmortem autolysis makes accurate identification of the cellular structures difficult, particularly in the vestibular neuroepithelium.

The Modified Microdissection Technique and Unbiased Stererology of the Vestibular Neuroepitheilium

In order to overcome these limitations, our laboratory has used the modified microdissection technique which allows for rapid fixation because of the immediate removal of the encasing temporal bone, with resultant improved morphological preservation (Hawkins and Johnsson, 1976; Lee et al. 1990; Wright and Meyerhoff, 1989). Also, each of the five vestibular endorgans can be properly oriented to the optimal plane of sectioning for detailed cytological and morphometric studies. Using the traditional processing, only a small fraction (8%) of the utricle is sectioned in a perpendicular plane, predominantly in the superior pole (Merchant, 1999). Thus, we used the modified microdissection technique to obtain unbiased estimates of the type I and type II hair cell number in the human utricle (Gopen et al., 2003) and in the human horizontal crista ampullaris (Lopez et al., 2005b).

The temporal bones are harvested within 3 to 12 hours after death using a modified protocol as described by Schuknecht (1968). A temporal bone plug cutter is used to remove the petrous temporal bone en bloc. Immediately after removal from the skull base, within the autopsy room, the stapes is carefully detached from the oval window and fixative is infused with a micropipette to ensure a uniform distribution of the fixative throughout the vestibule. After removal from the skull base, the temporal bones are immediately immersed in ice-cold 4% paraformaldehyde

Figure 2. A microdissected human utricle (right side) with a 3-mm bar displayed below. Cross-sections using light microscopy were taken from the midportion of the utricle as depicted by the arrows (left side) for comparison (original magnification x2).

plus 0.2% glutaraldehyde in 0.1 mol/L Sorensen buffer at pH 7.4 and are taken immediately to the Morphology Laboratory at UCLA. Within one hour, the vestibular endorgans, nerves, and ganglion are microdissected under the oper-
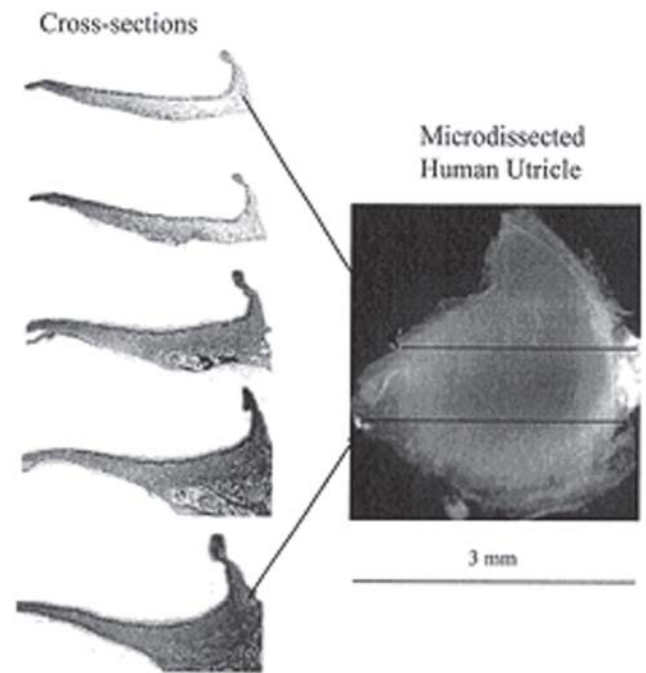
ating microscope. The middle ear is opened, and removal of the encasing temporal bone is continued until the cristae of the three semicircular canals, the utricular and saccular maculae are identified and dissected free by carefully removing the bone around the internal auditory canal. The vestibular nerve (including Scarpa's ganglion cells) is separated from the cochlear and facial nerve. The microdissected tissue is immersed immediately in 4% paraformaldehyde/0.2% glutaraldehyde solution and placed under a shaker rotator for 3 hours, then postfixed with osmium tetroxide for 1 hour, dehydrated in ascending ethyl alcohols, and embedded in epon-Araldite mixture in an oven at 65°C for 48 hours. Serial transverse sections of 2 micron-thickness are made of the entire utricle or crista using a diamond knife with Sorvall MT-2 ultramicrotome, mounted on glass slides, and coun-

Figure 3. Hair cell morphological types I and II. Two consecutive 2 µm sections (sections A and B) taken from the midportion of a human utricle are viewed using light microscopy with oil immersion (original magnification 60). (Section A) Four nuclei of hair cells are labeled as A, B, C, and D, respectively. Nuclei A and D are from type I hair cells. Type I hair cells are identified by their surrounding calyx, flask shape, larger size, and presence in the upper half of the macular neuroepithelium. Nuclei B and C are from type II hair cells. Type II hair cells are identified by their cylindrical shape, smaller size, and presence in the upper half of the macular neuroepithelium. Several supporting cell (SC) nuclei are evident below these hair cells. Supporting cells are identified by their irregular shapes, multiple intensely stained nucleoli, and presence in the lower half of the macular neuroepithelium. When compared with the next consecutive section (section B), nuclei A, C, and D can still be found and therefore are not assigned a count using the physical fractionator technique. A count is assigned for nucleus B (present in section A but absent in section B). Again, supporting cells are evident below the hair cells.

terstained with toluidine blue. Figure 2 shows the microdissected human utricle. Camera lucida drawings are made from the transverse sections.

Unbiased estimates of the total type I and type II hair cell number in the utricular macula were obtained using the physical dissector method on microdissected specimens obtained postmortem (Gopen et al., 2003). The average estimate of hair cells was 27,508, consisting of 17,326 type I hair cells and 10,182 type II hair cells from ten normal subjects of ages ranging from 42 to 96 years old, mean = 82. In the age range of the study, there was no effect of age on hair cell counts. The ratio of type I to type II hair cells in the human utricular macula was 1.70: 1. Figure 3 shows the use of morphological characteristics to identify type I and type II hair cells as described by Merchant (1999).

Regional unbiased estimates of the total type I and type II hair cell number, and supporting cell number in the human horizontal crista ampullaris were obtained using the physical dissector method on microdissected specimens obtained postmortem (Lopez et al., 2005b). The crista regions were derived as described by Fernandez et al.,1995; Lysakowski and Goldberg, 1997. Group 1 averaging 51 years old had 8,066 total hair cells; group 2 averaging 84 years had 7,074 hair cells, and group 3 averaging 94 years had 6,009 hair cells. The type I to type II hair cell ratio was near unity. There was a significant decline in both type I and type II hair cell numbers with increasing age, and there was no significant differences of type I to type II ratio between the different age groups. There was no effect of increasing age on supporting cell counts, and the overall average count was 10,002 supporting cells. The peripheral region had more tightly packed hair cells and supporting cells. Thus, it appears that there is a greater effect of increasing age on hair cell numbers in the horizontal crista ampullaris than in the utricular macula, as has been proposed in previous studies (Merchant et al., 2000; Rauch et al., 2001). Total hair cell counts were in alignment with previous studies using surface preparation hand-counting (Watanuki and Schuknecht, 1976), but our unbiased type I and type II density measurements, and type I: type II hair cell ratios were a significant departure from those obtained using Abercrombie methods (Merchant et al., 2000).

We applied the optical fractionator technique to postmortem microdissected Scarpa's ganglion from five young normal subjects (age ranging from 42 – 49 years old). The ganglion was embedded in paraffin, and cut into 40 micron serial sections. An average of 23,599 neurons was obtained, which is in alignment with estimates obtained using traditional archival human temporal bones (Ishiyama et al., 2004).
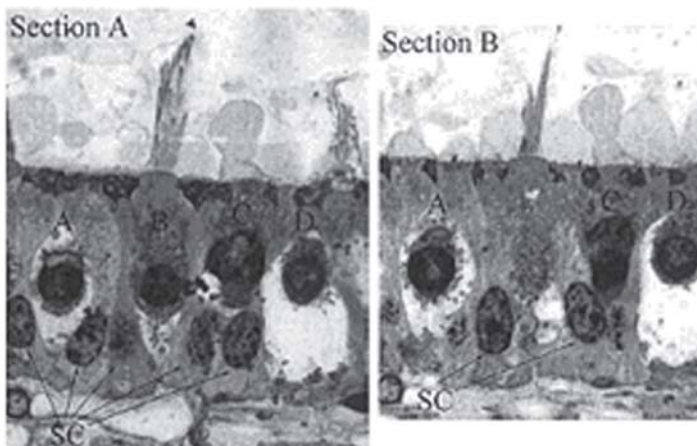
Microdissection Allows for Immunohistochemical Identification and Unbiased Stereology

We obtained estimates of the number and diameter distribution of nerve fibers in the human crista ampullaris and utricular macula from normals. Vestibular endorgans with the attached vestibular nerve stump were microdissected from postmortem temporal bones. The first group was fixed with paraformaldehyde and post-fixed with osmium tetroxide. The second group was fixed with paraformaldehyde, immunoreacted with monoclonal antibodies against neurofilaments, and post-fixed with osmium tetroxide. Estimates of the number of nerve fibers were obtained using an unbiased stereological method, the fractionator. The average number of fibers in the horizontal, posterior and superior cristae of individuals in group 1 was 1424 nerve fibers. In group 2, there was an average of 1792 nerve fibers. In the macula utriculi from group 1, there was an average of 3026 nerve fibers. In the utricular macula from group 2, there was an average of 3715 nerve fibers. The nerve fiber number in both groups was found to be in the range of previous stud-
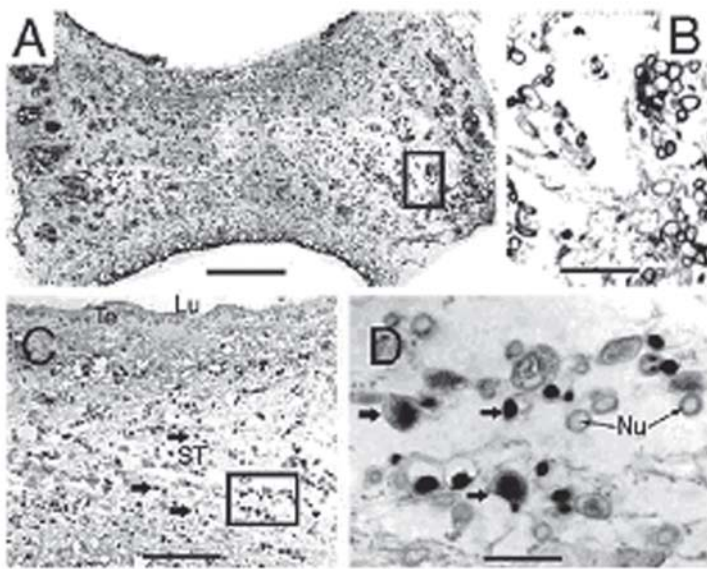

Figure 4. (A) Horizontal 2 µm thick plastic sections from the horizontal crista ampullaris stained with osmium and toluidine blue (group 1). (B) High magnification view from the insert in (A) to illustrate myelinated nerve fibers in the crista stroma. (C) Horizontal section from a crista immunoreacted with antibodies against neurofilaments and thereafter counterstained with osmium and toluidine blue (group 2). (D) Higher magnification view from the insert in (C) to illustrate immunoreactive profiles of nerve terminals (arrows). Note the immunoreactive axoplasm of nerve fibers (arrows). Some nuclei can also be differentiated. St: stroma; Te: transition epithelia; Lu: lumen; Nu: nuclei. Magnification bar in (A) is 300 µm, in (B) is 35 µm, in (C) is 200 µm and in (D) is 25 µm.

ies, however, the number of fibers in group 2 was significantly higher than that in group 1 ($p = 0.01$), indicative that immunohistochemical identification of vestibular nerve fibers increased the sensitivity. Figure 4 shows for the first time the application of unbiased stereology and immunohistochemistry in the postmortem human temporal bone (Lopez et al., 2005a).

In summary, we have applied the modified microdissection technique conducting immediate fixation of the inner ear, and early fixative-infiltration directly onto the neuroepithelium. This enabled us to conduct immunohistochemistry on postmortem specimens to increase the sensitivity to identify small vestibular nerve fibers (Lopez et al., 2005). Also, because of the excellent morphological preservation, we were able to use cytological criteria to apply unbiased stereology to obtain type I and type II vestibular hair cell and supporting cell counts (Lopez et al., 2005b; Gopen et al., 2003). In the future, the use of specific cellular markers may allow the identification of structures and cell types that have altered morphology secondary to otopathological diseases, and may otherwise be difficult to identify. Double- and triple-labeling immunohistochemistry and the use of fluorescent and confocal microscopy can be applied to study the human inner ear anatomy. The immunolocalization of several proteins can be achieved in less than ten days from the temporal bone harvesting time using microdissection, in contrast to traditional protocols that may take up to one year from harvesting to immunostaining. We hope that this modern methodology may potentially open up a new field for future human inner ear research.

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Scientific study of the human temporal bone and related brain tissue is a time-consuming process performed in highly specialized otopathology laboratories by researchers who are dedicated to enhancing our understanding of the pathology underlying disorders of hearing and balance. "Laboratory Spotlight" is a continuing series of articles offering a glimpse inside the laboratories in the United States and abroad conducting temporal bone research.

Temporal Bone Laboratory at Victor Goodhill Ear Center, Los Angeles
Akira Ishiyama, M.D.

In 1966, Ruth Gussen, M.D., a board certified pathologist was recruited by Victor Goodhill, M.D. to direct the UCLA Temporal Bone Laboratory and to conduct histopathological studies. During the early years of the former National Temporal Bone Banks, UCLA was a chapter member under the direction of Paul H. Ward, M.D., Chief of the Division of Head and Neck Surgery. In recent years, the temporal bone laboratory was named the Victor Goodhill Ear Center, a clinical and research center created in honor of Dr. Goodhill, the first otologist at UCLA.

The UCLA Temporal Bone Laboratory houses more than 1000 specimens, many of which have been processed (approximately 400). Information regarding age, gender, race, clinical diagnosis, and histopathology is entered into a database format compatible with the Registry's database. The UCLA Temporal Bone Laboratory contains specimens across a wide range of disease processes affecting the ear. The Temporal Bone Laboratory receives specimens from the western part of the United States, including 13 states.

Under the guidance of Vicente Honrubia, M.D. as the Director of the Victor Goodhill Ear Center, the UCLA Temporal Bone Bank began undertaking the development of new techniques to study the human temporal bones, with many publications and presentations in both clinical and basic science meetings and journals. Under P. Ashley Wackym, M.D., the UCLA laboratory initiated studies using modern molecular biologic techniques, including polymerase chain reaction (PCR) and *in situ* hybridization in the human temporal bone.

Under the current directorship of Akira Ishiyama, M.D., the UCLA Temporal Bone Bank has pioneered the application of unbiased stereological techniques to the human temporal bone, using both traditionally processed archival human temporal bones and microdissected specimens. Collaboration with Yong Tang, M.D., Ph.D., a neuroanatomist with expertise in unbiased stereology, has been instrumental in the development of modern, inno-

vative protocols to quantify and identify pathological changes in the human temporal bone. For more than a decade, Ivan Lopez, Ph.D., Director of the Morphology Laboratory at the Victor Goodhill Center, has been developing protocols aimed at rapid-fixation and antigenic and morphological preservation, leading to the development of the modified microdissection technique which enabled the combined application of immunohistochemistry and unbiased stereology to quantify human vestibular nerve fiber number and diameter distribution.

Robert W. Baloh, M.D. and Gail Ishiyama, M.D., a clinician-scientist from the Department of Neurology at UCLA, have been instrumental in the development of prospective, longitudinal clinicopathological correlations of temporal bone with audiovestibular function in aging and otopathological conditions, and the application of unbiased quantification of morphological changes in varied conditions. The UCLA Temporal Bone Bank continues to be active, with postdoctoral students and residents participating in ongoing research
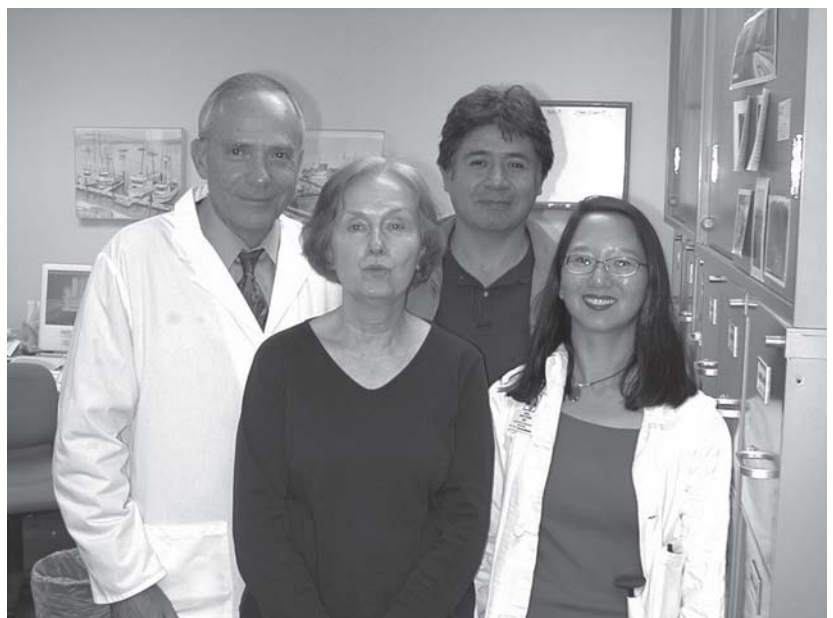
From the left: Robert W. Baloh, M.D., Kate Jacobson, Ivan Lopez, Ph.D., Gail Ishiyama, M.D.

projects with strong support from Gerald S. Berke, M.D., Chief of the Division of Head and Neck Surgery.

Individuals interested in becoming involved in the UCLA temporal bone research projects may contact Akira Ishiyama, M.D., Director of the UCLA Temporal Bone Bank at (310) 206-2041.•

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Upcoming Meetings the Registry will be Attending

2006 AAO-HNSF Annual Meeting & OTO EXPO in Toronto, Ontario, Canada, September 17-20, 2006
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ARO 2007 MidWinter Meeting, at The Hyatt Regency, Denver, CO, February 10-15, 2007
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That Others May Hear is a short brochure which describes briefly the functions of the Registry, and answers commonly asked questions regarding the temporal bone donation process. (Dimensions: 9" x 4")	*The Gift of Hearing and Balance: Learning about Temporal Bone Donation* is a 16-page, full-color booklet which describes in more detail the benefits of temporal bone research. It also answers commonly asked questions regarding the temporal bone donation process. (Dimensions: 7" x 10")

If you are willing to display either or both of these brochures, please complete the form below and return it to the Registry by mail or fax. The brochures will be sent to you **free of charge**. Please circle the amount requested for each brochure or write in amount not listed.

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