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Research Report

Gene expression analysis of distinct populations of cells isolated from mouse and human inner ear FFPE tissue using laser capture microdissection – a Technical report based on preliminary findings

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ABSTRACT

Laser Capture Microdissection (LCM) allows microscopic procurement of specific cell types from tissue sections that can then be used for gene expression analysis. We first tested this method with sections of adult mouse inner ears and subsequently applied it to human inner ear sections. The morphology of the various cell types within the inner ear is well preserved in formalin fixed paraffin embedded (FFPE) sections, making it easier to identify cell types and their boundaries. Recovery of good quality RNA from FFPE sections can be challenging, however, recent studies in cancer research demonstrated that it is possible to carry out gene expression analysis of FFPE material. Thus, a method developed using mouse FFPE tissue can be applied to human archival temporal bones. This is important because the majority of human temporal bone banks have specimens preserved in formalin and a technique for retrospective analysis of human archival ear tissue is needed. We used mouse FFPE inner ear sections to procure distinct populations of cells from the various functional domains (organ of Corti, spiral ganglion, etc.) by LCM. RNA was extracted from captured cells, amplified, and assessed for quality. Expression of selected genes was tested by RT-PCR. In addition to housekeeping genes, we were able to detect cell type specific markers, such as Myosin 7a, $p27^{kip1}$ and neurofilament gene transcripts that confirmed the likely composition of cells in the sample. We also tested the method described above on FFPE sections from human crista ampullaris. These sections were approximately a year old. Populations of cells from the epithelium and stroma were collected and analyzed independently for gene expression. The method described here has potential use in many areas of hearing research.

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For example, following exposure to noise, ototoxic drugs or age, it would be highly desirable to analyze gene expression profiles of selected populations of cells within the organ of Corti or spiral ganglion cells rather than a mixed population of cells from whole inner ear tissue. Also, this method can be applied for analysis of human archival ear tissue.

1. Introduction

In order to study gene expression in a particular tissue, it is necessary to obtain and process the tissue in reproducible ways that preserve RNA content. In the inner ear, functionally diverse cell types lie in very close proximity. Therefore, in order for molecular analysis to have even the most basic precision, anatomically and developmentally diverse tissues must be separated, sometimes at the cellular level. The way specimens are processed has significant impact upon the RNA studies that are subsequently possible. To date, formaldehyde as a 10% neutral buffered formalin is the most widely used as a fixative for various human tissues, including human temporal bones. As with DNA, formaldehyde reacts with RNA forming an *N*-methylol (*N*-CH_2OH) followed by an electrophilic attack to form a methylene bridge between amino groups. Of the 4 nucleotides, adenine is the most susceptible to electrophilic attack and it is likely that the adenines within the mRNA sequence and the poly(A) tail at the 3′-end of mRNA will be modified in the FFPE section to varying degrees. This chemical modification renders RNA isolated from FFPE section less suitable for reverse transcription (cDNA synthesis) (Srinivasan et al., 2002). Therefore, formalin fixation results in chemical modification of cellular nucleic acids, effectively limiting the size of cDNAs that can be produced by reverse transcription. Further, RNA isolated from fixed tissues may be degraded to varying degrees depending on the age of the material.

Studies have shown that, compared with formalin fixation, cryofixation and sectioning result in samples with significantly more preserved RNA (Goldsworthy et al., 1999). Until recently, RNA processing from tissue fixed in formalin or other cross-linking fixatives has been considered unworkable since the amount of recoverable RNA is so small (Cancer Research, 2004; Coudry et al., 2004). With the development of modified extraction and detection procedures, it is now possible to 'extract' useful information from fixed material despite hurdles described above. Fixed and archived tissues are an important resource for molecular biological studies and every effort should be made take advantage of this material. It should be noted that in order to detect the expression of genes in a specific cell or tissue type, detection of the entire transcript is not necessary. Detection of small amplicons is sufficient proof that a gene of interest is expressed. Further, quantitative PCR techniques, such as TaqMan PCR (Applied Biosciences, CA), routinely amplify amplicons containing 100 bp or less.

Currently, cryosectioning is the commonly used technique for gene expression analysis on inner ear tissue. The structure of the cochlear duct consists of a thin neuroepithelium and a relatively large volume of aqueous fluid, making it difficult to obtain cryosections in which the morphology is well preserved. Formalin fixation has the advantage of allowing better preservation of tissue architecture during sectioning. Formalin has also been the fixative of choice for human temporal bone archives.

In order to fully characterize inner ear structures consisting of only a few cells, microdissection techniques are required. The laser capture microdissection (LCM) technique may be a useful tool to increase the precision of microdissection (Emmert-Buck et al., 1996). The LCM approach has been successfully used in other fields (Ma et al., 2003). For LCM, tissue sections are mounted on slides and stained. A thermoplastic film "cap" is then placed directly over the tissue and the assembly is viewed with light microscopy. A low-power laser is used to melt the film onto the cells of interest. After cells have been selected, the cap is removed and the cells remain attached to it. Multiple laser captures can be serially performed on an individual tissue section. Thus, LCM represents an effective and precise method to procure specific cell types from tissue sections.

Recent technical advances have significantly improved our understanding of the molecular basis of inner ear development. As an example, the roles played by some members of the protocadherin family of cell adhesion and signaling molecules during differentiation of inner ear receptor cells have been characterized (Alagramam et al., 2001; Curtin et al., 2003; Di Palma et al., 2001). In this respect, the approach described in this report would potentially permit detailed expression analysis of genes in specific functional domains of the inner ear, allowing identification of genes that may be important in inner ear development and function.

This technical report demonstrates the feasibility of gene expression analysis on formalin-fixed mouse and human inner ear tissues using LCM. We successfully use this method to detect expression of housekeeping genes and other cell-type specific markers to confirm the likely composition of cells isolated by LCM.

2. Results

The LCM process is shown in schematic form in Fig. 1. After fixation, embedding, and staining, LCM provided distinct populations of cells from the functional domains of the inner ear. To demonstrate cell capture, cells from the organ of Corti (OC), spiral ganglion (SG) and stria vascularis (SV) were collected from a single cochlear duct from an adult mouse cochlea (P30) onto the same cap (Fig. 2). The border around the captured cells indicates the 'bubble' formed by the thermoplastic film after laser capture (arrows shown in Fig. 2, right panel and Fig. 6 panel B). For gene expression analysis, cells from each functional domain were captured on independent caps. Fig. 3 shows separate captures. For example, each prepared slide typically contained three adjacent sections from a prepared inner ear, and we thus were able to increase

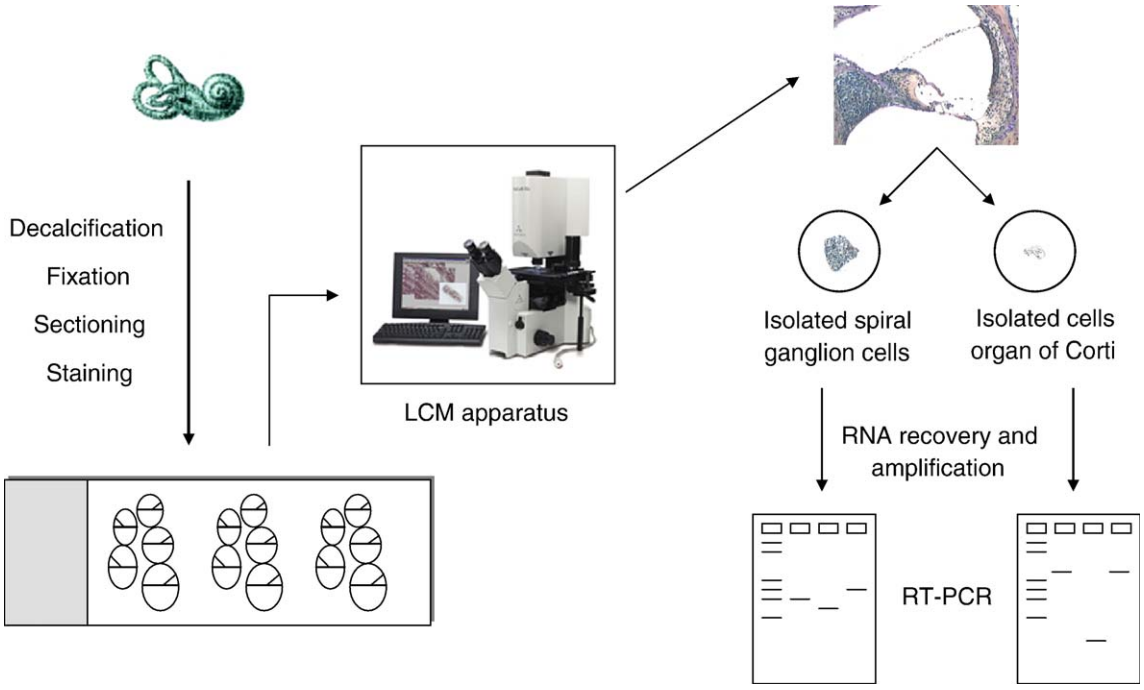

Fig. 1 – Schematic diagram of LCM procedure. LCM physically isolates the cells of interest, which then can be analyzed further. The remaining tissues on the slide are undisturbed, and other cell types can be isolated subsequently by LCM.

cell counts by pooling all cells isolated from OC from several sections. The FFPE sections used were one or two cell layers thick (8 μm sections). By fluorometry, 45 pg total RNA was recovered from a scraped section in 70 μL solution (concentration 0.64 pg/μL). Amplification of 10 μL of this solution or 6.4 pg total RNA yielded ~200 pg RNA after one round of amplification as measured by fluorometry.

We demonstrated the baseline feasibility of the procedure by assaying for a housekeeping gene and markers of cell types with RT-PCR. The expression of hair cell marker *Myosin 7a* (Sahly et al., 1997), supporting cell marker $p27^{Kip1}$ (Chen and Segil, 1999) and spiral ganglion cell marker *Nfl-H* (Nishizaki and Anniko, 1995) were examined. RNA isolated from the cells of the OC was amplified and tested for presence of markers. Fig. 4 shows a photograph of an agarose gel with lanes consisting of the PCR product with primers selected for mouse *GADPH*, *Myo7a*, $p27^{Kip1}$, and *Nfl-H* genes. Clear bands of the expected size for *Myo7a* and $p27^{Kip1}$ were observed. The lack of *Nfl-H* suggests that the neuronal cell population in that capture was low. Genes known to be expressed outside of the organ of Corti, such as connexin 26, were not detected in RNA isolated from the cells of the OC (data not shown). The results shown in Fig. 4 indicate that the LCM and extraction procedures reported here are able to recover mRNA from FFPE inner ear tissue and they confirm the likely composition of cells captured from the OC.

Results of expression analysis from a different batch of laser capture material is shown in Fig. 5 (panel a and b). Cells of the OC and SG were captured on separate caps from 8 sections. Hair cell marker *Myo7a* is detected in the cells from OC (Fig. 5a) and *Nfl-H* expression is detected from the SG cells (Fig. 5b).

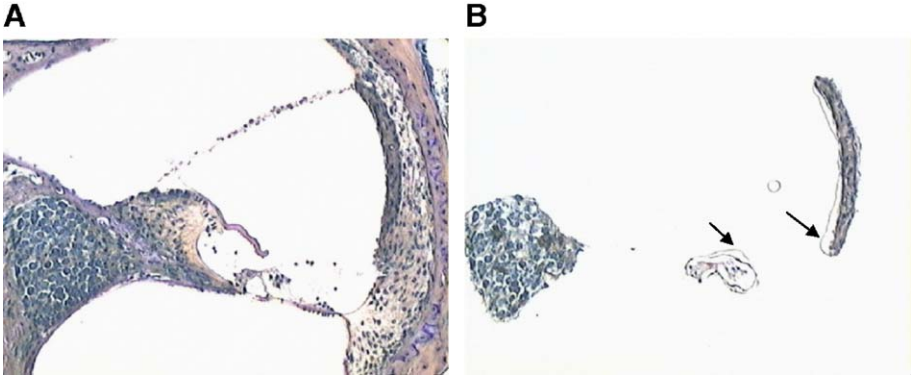

Fig. 2 – Isolation of distinct population of cells from the functional domain in the cochlear duct. Photomicrographs of a tissue section prepared from a P30 mouse illustrating a population of cells captured from the stria vascularis, organ of Corti and spiral ganglion. (A) Pre-capture; (B) Post-capture. Arrowhead in panel B points to 'bubble' formed, by thermoplastic film, around the captured material.

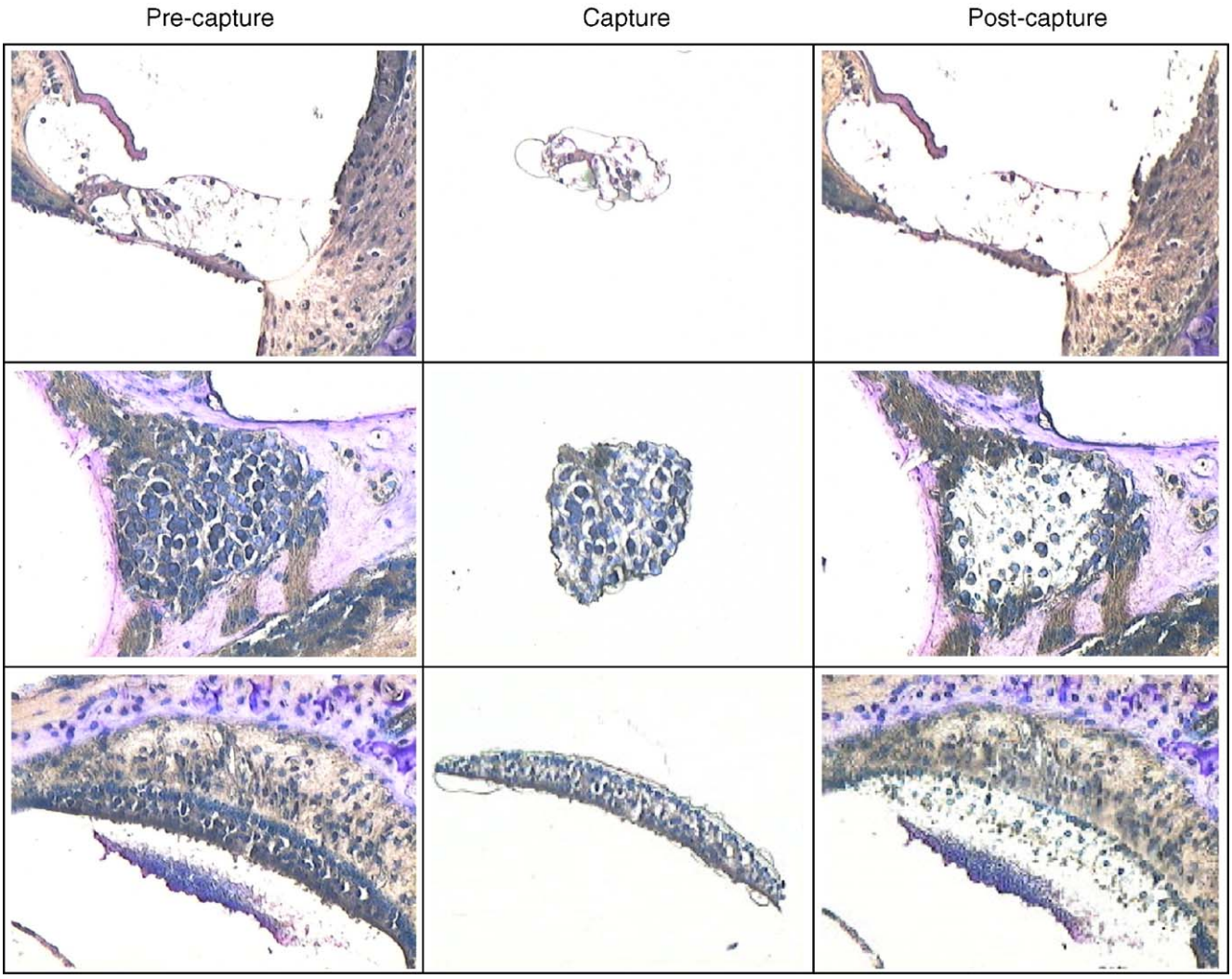

Fig. 3 – LCM process. Left column: stained sections are shown ready for LCM. Center column: cell groups dissected free onto LCM cap and available for independent analysis. Right column: remaining section after removal of LCM cap.

Expression of $p27^{Kip1}$ or *Myo7a* is not detected in the RNA isolated from SG. Once again, these results confirm the likely composition of cells captured from the OC and SG.

One 'signature' we have observed for the small RNA work is the high molecular weight 'smear' seen in each of the RT plus lanes, and it is more likely to be seen when increased amount of RT reaction is used for PCR. Also, the high molecular weight 'smear' is apparent when the ethidium bromide containing gels are exposed longer to UV light. The composition of this 'smear' is not known but we believe it is associated with reverse transcription of amplified RNA. The intensity of the smear varies from one batch of FFPE sections to the other (data not shown).

The protocol used for mouse FFPE sections was applied to human crista ampullaris FFPE sections. Cells of the epithelium and stroma were captured on separate caps. Fig. 6 shows pre-capture and capture of cells of the epithelium. The cells from 4 sections were captured onto a single cap. Fig. 6, panel c shows 4 different segments of epithelium captured onto a single cap. Similarly, cells of the stroma were captured on a separate cap (data not shown). RNA was isolated from captured cells as well as scraped tissue from slides. The protocol for RNA isolation and amplification was carried out as described previously for the mouse FFPE sections. We demonstrate the baseline feasibility of the procedure by assaying for the expression of housekeeping genes with RT-PCR. Fig. 6 (panel d) shows a photograph of an agarose gel with lanes consisting of PCR products with primers selected for human *GAPDH* (Table 1). Each lane contains the expected-size PCR product. Though the PCR is not set to be quantitative, results shown in Fig. 6 (panel d) suggests an increase in signal intensity with increase in cell numbers. The experiment was carried out in duplicate (as described in Methods) and similar results were obtained. The results show that with LCM and extraction procedures reported here we were able to recover mRNA from human FFPE inner ear tissue sections.

3. Discussion

LCM has been previously described and used to demonstrate the presence of specific gene transcripts as well as global

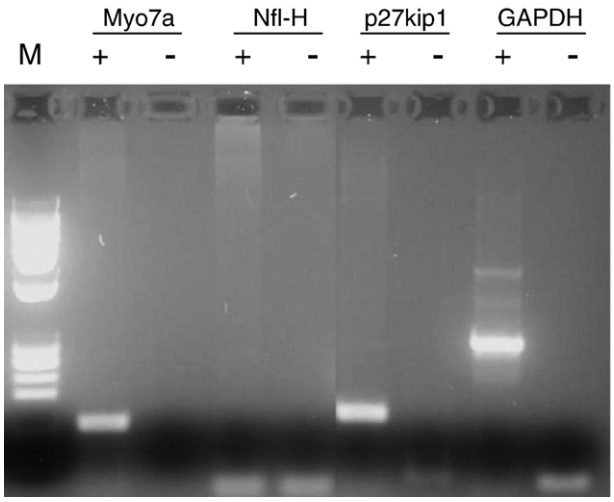

Fig. 4 – Housekeeping and marker gene expression analysis by RT-PCR: Expression of a specific set of genes was evaluated in RNA isolated from cells of the organ of Corti. Gel photograph of PCR directed at mouse *Myo7a*, *Nfl-H*, $p27^{Kip1}$ and *GAPDH*. Expected size product was obtained for *Myo7a*, $p27^{Kip1}$ and *GAPDH*; no amplified product for PCR directed at *Nfl-H*. M = Phix 174 digested with *Hae*III marker; '+' = RT plus; '–' = RT minus.

expression profiling in cryosectioned rat vestibular tissues (Cristobal et al., 2004, 2005). In addition, methods for RNA recovery from various FFPE tissue types have improved to the point that gene expression analysis is now possible (Cancer Research, 2004; Li et al., 2004). Here, we present a technical report describing an LCM-based approach to evaluate gene expression in specific cell types isolated from formalin-fixed paraffin-embedded mouse and human inner ears. Using the present technique, we were also able to show positive evidence of housekeeping gene expression and specific markers that confirm the likely composition of the captured cells from the mouse inner ear sections. In addition, the relative abundance of 3′ and 5′ products was within a range we consider reasonable in order to perform gene expression studies. Furthermore, applying the technique that was optimized using mouse FFPE sections, we were able to detect gene expression in cells microdissected from archival human FFPE sections. Based on the data reported here, we conclude that it is possible to isolate RNA and detect gene expression after laser capture microdissection from FFPE sections. Clearly, further trials and optimization will be necessary to improve yields and consistency for inner ear samples, especially for human archival temporal bone tissue.

The 3′/5′ ratio is a good 'quality control' check for FFPE samples. The 3′/5′ ratio estimates the abundance of 3′ target compared to 5′ target. For good quality RNA, the ratio will be close to 1, because most of cDNA contains both the 3′ and 5′ ends. However, RNA isolated from FFPE sections is more likely to exhibit some level of degradation and 3′/5′ ratios are likely to be > 1. There is no established cut-off for ratios from which no data will be obtained, and there is no prescribed ratio for single gene detection and global expression profiling. Individual experiments will have different tolerance levels. In those studies in which a specific set of genes is being evaluated, ratios in the range of 10–20 could still yield results, as shown in this report. If the FFPE tissue is used to discover genes or in expression profile studies, samples with lower ratios (<10) would be preferred.

We would therefore propose the following procedure to maximize efficiency: first, for each FFPE tissue block, a few sections are prepared and processed for LCM. However, before performing LCM, one or two whole sections from each block are scraped off the slide, followed by RNA extraction and

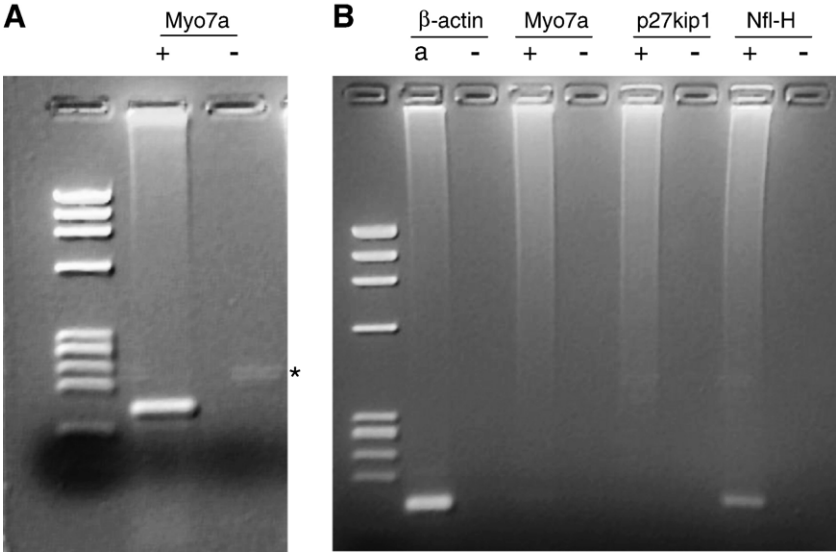

Fig. 5 – RT-PCR analysis of a different batch captured cells from the mouse organ of Corti and spiral ganglion. (A) RNA isolated from cells of the organ of Corti was evaluated for expression of *Myo7a*. Gel photograph of PCR directed at *Myo7a* showing expected size product. Asterisk represent non-specific band. (B) RNA isolated from cells of the spiral ganglion was evaluated for expression of β-actin, *Myo7a*, $p27^{Kip1}$, *Nfl-H*. As expected, β-actin and *Nfl-H*. Gene expression was detected.

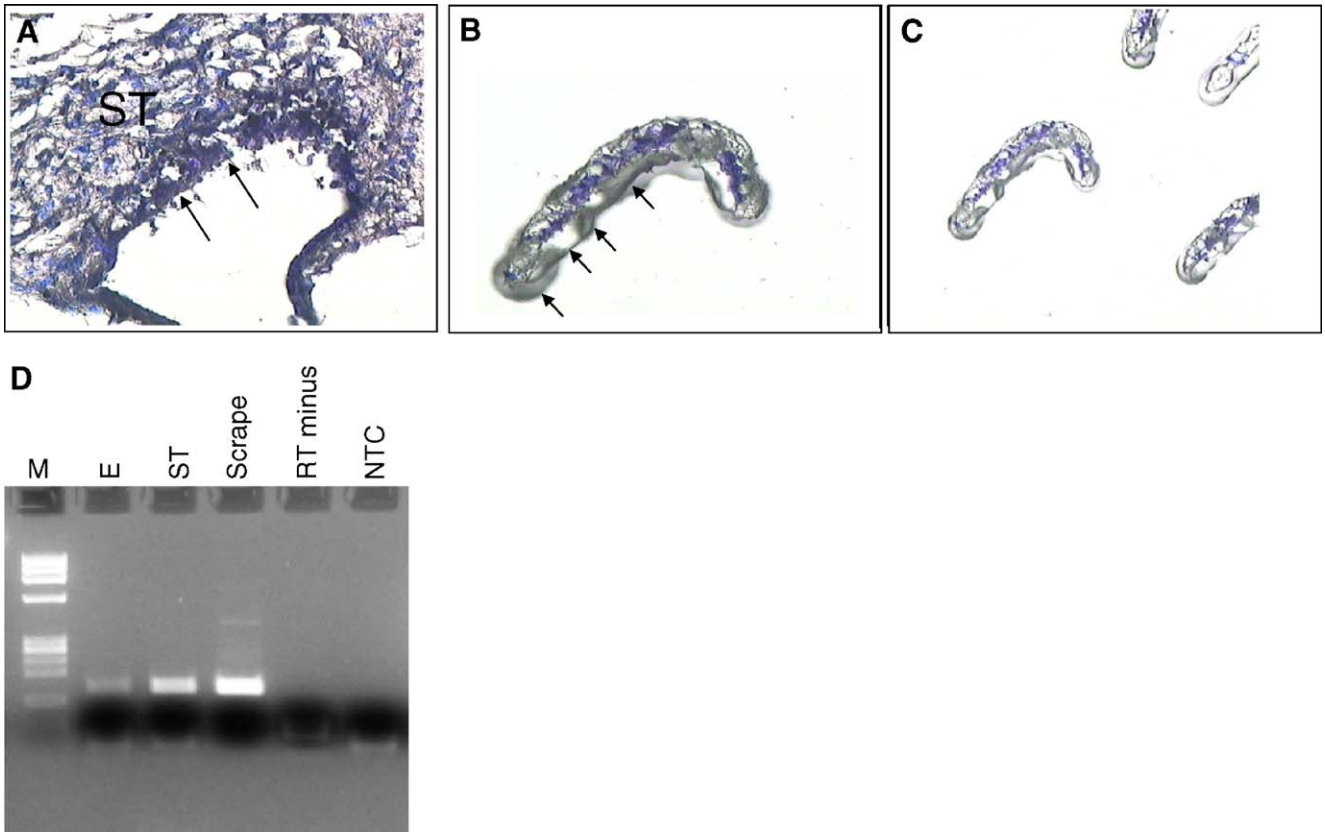

Fig. 6 – LCM and gene expression analysis of human crista ampullaris sections. (A) Pre-capture image of crista ampullaris. This section shows the periphery of the crista, which consists of superficial epithelium (arrows) and underlying connective tissue of stroma (ST). (B) Post-capture image of epithelium from a single section. Arrows points to the 'bubble' formed by the thermoplastic film after laser capture. (C) Shows pooling of epithelium from several sections onto a single LCM cap. D. Housekeeping gene expression. Gel photograph of RT-PCR directed at human *GAPDH*. Lanes: E = epithelium; ST = stroma; Scrape = whole tissue scrape; RT minus = without the addition of reverse transcriptase; NTC = no template control. Expected size produced for *GAPDH* is detected in all but RT minus and NTC lanes.

amplification. RNA quality would have to be measured on these sections by 3′ to 5′ ratio assessment. LCM would then be performed only on sections from blocks with satisfactory RNA quality. It may prove beneficial to measure 3′ to 5′ ratio for more than one housekeeping gene for optimal quality assessment.

The recovery of RNA is likely to vary from one batch of FFPE material to the next. A negative RT-PCR result for a gene in a specific cell type may be due to lack of expression of the target sequence or alternatively from sub-optimal PCR conditions, such as annealing temperature and primer location. It is therefore important to optimize PCR conditions for each gene using control RNA before proceeding with LCM-produced RNA. We anticipate that technological advances in cell processing reagents used downstream from the LCM process will increase the reliability and flexibility of the technique.

Another way to improve the quality of analysis that can be done by RT-PCR would be to optimize primer selection. Formalin fixation results in several chemical reactions with cellular nucleic acids, with adenine residues most strongly affected. This places an effective limit on the size of cDNAs, which can be constructed (Srinivasan et al., 2002). Significant modification of the poly A tail would result in difficulty performing RT with oligo dT primer, and instead points to using random hexamer primers for reverse transcription. In addition, assaying for smaller amplicons at the 3′ end of the transcripts would yield better information. Paska et al. (2004) made an estimate of 225 bp as the upper limit of useful amplicon size in RT-PCR studies of FFPE endometrium. We had success in the current study with larger amplicons, but would generally concur with the finding of better results with smaller amplicons.

Much investigation has centered recently around optimal tissue handling techniques, including comparisons of FFPE with cryosections as well as with other fixatives, such as 70% ethanol. Su et al. (2004) showed that RNA recovery from ethanol-fixed paraffin-embedded brain specimens was comparable to that from cryosections, with histology comparable to FFPE brain. Kim et al. (2003) compared several fixatives on a variety of paraffin-embedded soft tissues and determined that methacarn (a combination of methanol, chloroform, and acetic acid) was the fixative that yielded the highest quality RNA. However, none of these studies addresses preservation of inner ear tissue, which by its particular composition behaves uniquely under the conditions imposed by fixatives, and none of these advances would be of use with archival tissue.

Table 1 – PCR primer pairs[a], annealing temperatures, and amplicon sizes

Gene	Primer sequence (5′–3′)	Annealing temp	Product size
β-actin-5′	(F) GTCCACCTTCCAGCAGATGT (R) TCTGCGCAAGTTAGGTTTTG	60	142
β-actin-3′	(F) AATTTCTGAATGGCCCAGGT (R) TGTGCACTTTTATTGGTCTCAA	60	149
GAPDH	(F) CATCACCATCTTCCAGGAGCGA (R) GTCTTCTGGGTGGCAGTGATGG	53	342
Myo7a	(F) TTA TGG CCT TCC TGG TGT AAG (R) TGG TTG TTC CAA GTA TGC AGA G	50	150
$P27^{Kip1}$	(F) ATT GGG TCT CAG GCA AAC TCT (R) GTT CTG TTG GCC CTT TTG TTT	50	155
Nfl-H	(F) AGC CAA AGA AAG AGG AGA TGC (R) GTC TTG GGT TTG CTA GGC TCT	50	157
Human GAPDH	(F) CATCCTGGGCTACACTGA (R) AGCCAAATTCGTTGTCATAC	60	143

[a] All primers are designed to mouse sequence unless specified.

Certainly, obtaining precise LCM samples from cryosectioned tissue would improve on the present technique by raising RNA yields. Efforts are currently underway to test the efficacy of LCM-based gene expression analysis using cryosections of mouse cochlea. We are using methods for cryoembedding and sectioning that are reported to preserve overall structure and cellular resolution (Whitlon et al., 2001).

Whether it be cryosections or FFPE sections, it is necessary to amplify RNA extracted from captured cells because RNA is extracted from a small number of cells. Does the amplified RNA (aRNA) faithfully reflect the RNA profile of the original sample in terms of quality and quantity (copy number)? Expression of specific target genes can be evaluated from nanogram quantities of RNA using real-time RT-PCR and therefore amplification may not be necessary in these cases. However, in a typical experiment, several or even thousands (microarrays) of genes are evaluated in a single experiment. Small amounts of tissue samples collected by LCM necessitate amplification of extracted RNA in order to have sufficient quantities of RNA for gene expression analysis. This would be the case for cryosections as well as FFPE sections. In this study, T7-based RNA amplification with oligo-dT primer was used. T7-based amplification starting from a small amount of RNA has been shown to be linear by several investigators. Microarray and qPCR methods have been used to assess the fidelity of T7-based RNA amplification Although variations in differential expression between amplified and total RNA hybridizations has been observed, RNA amplification is reproducible, and there is no evidence that it introduces a large systematic bias (Heil et al., 2003; Rudnicki et al., 2004; Schneider et al., 2004; Patel et al., 2005; Zhu et al., 2006). Nevertheless, it is important for each investigator to assess the fidelity of the RNA amplification protocol used in their laboratory.

Though microarrays have been used to compare the amplified versus unamplified RNA, this approach is expensive, time consuming and in many cases a sufficient amount of RNA may not be available. A facile approach would be to use real-time RT-PCR and compare the ratio of the C_t (cycle threshold) values of target-to-housekeeping genes before and after amplification. If the amplification of the target and the housekeeping gene mRNAs is linear, the ratios of the C_ts would be close or equal. Several combinations of target genes and house keeping genes should be tested to gauge the linearity of T7-based amplification used to amplify RNA from inner ear samples.

Several methods are currently used to identify cell type-specific genes in the inner ear. The subtractive hybridization technique has been used to identify genes specific to hair cells, for example. Following manual dissection and separation of the inner and outer hair cells, Zheng et al. (2000) used a subtractive hybridization technique to identify *Prestin*, a motor protein of the outer hair cells. More recently, Zheng et al. (2002) used a PCR subtractive hybridization strategy to enrich for genes predominantly expressed in the outer hair cells. As the first step, they created separate OHC and inner hair cell (IHC) cDNA pools from individually (manually) collected hair cells using a random primed reverse transcription polymerase chain reaction. Here, the IHC cDNA was used as the 'driver' to 'subtract' genes from the OHC cDNA pool, i.e. genes common to both type of hair cells will be eliminated and the resulting product will be enriched for genes preferentially expressed in the OHCs. Though this approach is very useful for identifying genes that are differentially expressed in hair cells in the normal animal, this technique is less suitable for other scenarios. For example, if an investigator wants to study the effect of drug exposure on cells of the organ of Corti or stria vascularis, the LCM technique reported here would permit the investigator to capture cells (on separate caps) and analyze RNA from the various functional domains simultaneously (Fig. 3).

Yet another technique used to obtain cell type-specific genes is the use of fluorescence-activated cell sorting (FACS). FACS allows separation of living cells based on the presence of fluorescent markers, either cell type-specific antibody that is fluorescently tagged or cell type-specific expression of reporters such as green fluorescent protein (GFP). The latter would be derived from transgenic mice expressing GFP under the control of a cell type-specific promoter. An elegant demonstration of how well this technique works was shown by Sigel and colleagues at a recent meeting (Mouse as an Instrument of Ear Research, Oct 1–4, 2005, The Jackson Laboratory, ME). They used FACS to collect supporting cells from the mouse cochlea that express GFP under the control

of a $p27^{Kip1}$ promotor. The FACS-based approach is certainly a powerful technique. Although type-specificity and RNA recovery would likely be very good, FACS requires prior knowledge of cell type-specific markers or expression of reporter genes in specific cell types while LCM does not depend on any existing molecular characterization for capture of cells. Further, the need for transgenic mice poses other problems. For example, if an investigator has to use a 'resistant' CBA/CaJ strain for a noise exposure study and evaluate gene expression profile in hair cells, supporting cells and spiral ganglion cells after exposure, the transgenic strain expressing GFP in the specific cell type may not be available in the CBA/CaJ strain background. Transgenic mice are generated in certain inbred strains and it is time consuming and expensive to make them congenic to the desired genetic background. For the hypothetical experiment above, let us assume that 3 transgenic lines (expressing GFP in hair, supporting and spiral ganglion cells) are available in the CBA/CaJ strain. At the minimum the investigator should: (A) import and maintain 3 different lines, (B) be prepared to handle genotyping protocols for the 3 strains, (C) test to see if GFP is expressed in the desired cell types (and it is not leaky) and at the desired time points, and (D) plan to expose all three lines to noise, which would increases the total number of mice involved in the experiment.

There are different techniques used to identify cell-type specific genes and each method has its advantages and disadvantages as discussed above. The LCM-based approach described in this report has unique advantages and applications. One potential application for the LCM methodology is in screening for genes that may be important for inner ear development and function. RNA isolated from defined populations of cells such as the organ of Corti or saccular macula and reverse transcribed to cDNA would result in a cDNA 'library' representing expressed mRNA from that functional domain. This cDNA library can be used to localize expression of known deafness genes or screen for the presence of yet-uncharacterized genes by PCR within the various functional domains of the inner ear. mRNA in situ hybridization is a tried-and-true method used for spatial localization of candidate genes. However, a PCR-based approach using a cDNA library (described above) has some advantages: it is faster, more sensitive, it allows for screening multiple candidates at the same time, and it is possible to identify domain- specific alternative splice products for a candidate gene. The latter may be difficult to accomplish by mRNA in situ hybridization.

The precision of cell capture in the LCM process is limited largely by the operator's ability to distinguish individual cell types. Morphology of cells and boundaries of the functional domains in the mature inner ear are easy to distinguish compared to immature inner ear, making it more challenging to capture cells from the functional domains during developmental stages. Another issue with LCM is the tedium of sitting for several hours to capture sufficient numbers of cells. With the sensitivity of RT-PCR, we were able to detect *Myo7a* in RNA extracted from a sample that contains ~100 hair cells. *Myo7a* is relatively abundant in the hair cell and it is fairly easy to detect. If the microdissected cells are used to discover genes or expression profile studies, perhaps a greater number of cells would be preferred. Alternatively, more sensitive techniques that can transform picograms of RNA into microgram amounts of amplified RNA (Taylor et al., 2005) or amplify RNA from a single cell (Davis et al., 2004) can be used along with microarrays . In addition, some of the new generation of LCM equipment is designed to address these types of issues (example: 'Veritas' from Arcturus, CA). It is now possible to target laser dissection to labeled cells, fluorescently labeled antibodies or GFP expressing cells, allowing dissection of cells from developmental stages. Also, systems such as the Veritas has a drawing tool that allows the investigator to mark the areas to be cut and the AutoScan feature allows identification and capture of cells of interest from serial sections. These advanced features reduce hands-on time, permitting the user to dissect cells from a large number of sections compared to the PixCell II used in the present study.

In addition, investigators have for decades archived human and nonhuman temporal bones in formalin, the fixative which best allows for long-term storage. There are approximately 17,000 temporal bones in U.S. temporal bone banks (National Temporal Bone Registry); the preliminary studies we report here are encouraging and we anticipate that this new technique will allow analysis of these valuable specimens with the most recent investigational methods. As the next step, we plan to look for cell type specific markers such as MYO7A and NFl-H in the cells captured from human archival samples. Clearly, many more human archival samples need to be tested, and further optimization of the various steps in the protocol will be necessary to improve RNA yields and consistency for human inner ear archival samples.

Based on the data reported here, we conclude that it is possible to isolate RNA and detect gene expression after laser capture microdissection from FFPE sections.

The method described here has potential use in many areas of hearing research. For example, following exposure to noise or ototoxic drugs, it would be highly desirable to analyze gene expression profiles of selected populations of cells within the organ of Corti or spiral ganglion cells rather than a mixed population of cells from whole inner ear tissue. Also, there is hope that this method can be applied to analysis of human archival ear tissue.

4. Experimental procedure

4.1. *Animals and tissue processing*

We used five C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) in this study. The animals were handled according to NIH guidelines, and the Institutional Animal Care and Use Committee at Case Western Reserve University approved all protocols. After euthanizing animals at postnatal day 30 (P30), the middle ears were opened and under the dissecting microscope the stapes footplate was removed. Ten percent-buffered formalin was infused in situ through the oval window to fix the inner ear. Following this procedure, the inner ears were removed and post-fixed by immersing them in 10% buffered formalin for 1 h at room temperature. They were then washed in phosphate buffered saline. The specimens were subsequently decalcified overnight in 0.35 M EDTA at room temperature, dehydrated in ascending ethyl alcohol

solution and embedded in paraffin, and then sectioned at 8 μm thickness in the plane of the long axis of the cochlear modiolus. Sections were mounted on uncharged slides and air-dried at room temperature (3 sections were mounted per slide). Slides were incubated at 50 °C for 2 min to melt the paraffin. They were deparaffinized in xylene and dehydrated in ethanol, then stained with Paradise staining reagent (Arcturus, Mountain View, CA) according to the manufacturer's instructions, after which slides were dipped in ethanol again. Slides were incubated in xylene, then dried at room temperature immediately prior to performing LCM.

4.2. *Human temporal bone acquisition and processing*

FFPE sections of human temporal bone were obtained from the UCLA temporal bone bank. Temporal bones were obtained postmortem from a patient (81 year old female) with no history of auditory or vestibular symptoms. The Institutional Review Board (IRB) of UCLA approved this study. Appropriate informed consent was obtained from subjects before inclusion in the study. The protocol for temporal bone collection is described in detail elsewhere (Lopez et al., 2005a,b). Immediately after harvesting, the temporal bones were immersed for 1 day in ice-cold 4% paraformaldehyde in sodium phosphate buffer (PBS) at pH 7.4. Microdissected vestibular endorgans were immersed in RNAase free solutions. Containers and dissecting tools were autoclaved and RNAzap (from Ambion Inc., TX, USA) spray was used constantly during the microdissection. *Microdissection of vestibular endorgans*: Under the dissecting microscope the middle ear was opened and the ossicles were carefully removed. The oval and round windows were exposed to allow penetration of 4% paraformaldehyde fixative into the inner ear. The tissue then remained immersed in the fixative for 1 day. At the conclusion of fixation, the vestibular endorgans were further dissected to expose the cristae, utricle and saccule. Endorgans were immediately dehydrated in ascending alcohols (70%, 95%, 100% ethyl alcohol). The specimens was cleared with xylene and embedded in Paraplast plus paraffin (Polysciences). Ten micron serial sections obtained and mounted on Superfrost plus glass slides (Fisher Scientific). They were stored at 4 °C until their use.

4.3. *Laser capture microdissection technique*

LCM was performed using the PixCell II system (Arcturus) following manufacturer's instructions. Using this system, we obtained samples containing cells from the spiral ganglion, organ of Corti, saccular and utricular maculae. Each slide contained multiple adjacent sections, and we pooled all cells in each category from individual slides onto a single cap. As a control, we analyzed cells obtained by scraping the whole section from the prepared slide, referred to as 'scrape' tissue.

4.4. *Cell counts*

Metamorph Imaging Analysis Software (Universal Imaging Corporation, PA) was used to count cells within the cochlear duct of the FFPE section. The H&E sections were imaged at 4× on an Olympus BX 60 upright microscope. To image the entire cochlear section four images were obtained (4–5 cochlear duct segments could be seen in the adult mouse cochlea). On each section a color threshold was applied to allow identification of individual pixels that have a given color. For this purpose purple color corresponding to the nuclear staining was selected to estimate the number of nuclei present. The data were further sorted based on the average size of given nuclei, allowing the software to disregard objects that were too small and too large to be considered as nuclei. The spiral ganglion was imaged at 20× and yielded a count of approximately 50 cells per turn on a given section. For the DAPI (4′,6-diamidino-2-phenylindole) staining the same microscope and software were used. DAPI is a fluorescent stain used to visualize nuclear DNA in both living and fixed cells. Cell counts were accomplished with the Cell Counting module. To use this software, the operator enters a range of nuclear sizes and specifies how bright the nuclei are versus the background. The software then analyzes the images based on these parameters and determines a count. Again, 4 sections were needed at 4× to image the entire cochlea. It must be noted that the H&E and DAPI staining were done on different sections. The cell numbers represent averages from the H&E and DAPI staining counts. A total of 4 sections were counted (2 with H&E and 2 with DAPI staining). The total number of cells for one entire cochlear section (includes cells of the OC, SG, spiral lamina and stria vascularis) was ~9000 and the number of spiral ganglion cells per turn is ~50. The number of cells in the utricular macula is ~50 per section.

In the case of the human tissue sample, we had access to one batch of 6 FFPE sections from crista ampullaris, all derived from the same patient. Due to the limited number of sections available, we avoided the DAPI staining/counting step. Instead, we processed the sections for LCM and used an enlarged precapture image to manually count the cells. The epithelium contains a distinct population of cells that are superficially located and darkly stained; the stroma underlying the epithelium is much less dense and shows distinct nuclear staining (see Fig. 6a). By manual counting, we estimate the number of cells in the epithelium to be ~50 cells per section and ~100 cells in the ST (stroma) per section.

4.5. *RNA processing*

4.5.1. *Isolation*

We analyzed cells obtained from LCM and cells obtained from tissue sections scraped from identically-prepared slides. We used the Paradise RNA Extraction and Isolation system (Arcturus, Mountain View, CA) following the manufacturer's instructions, to isolate RNA both from scrape and laser microdissected cells on caps obtained from FFPE sections.

4.5.2. *Cell capture*

For each round, 8–9 FFPE sections from the adult mouse cochlea were used. Cells from 4 or 5 turns of the adult mouse cochlea were captured. From 8 to 9 sections, ~100 hair cells (both IHC and OHC), ~1000 cells of the spiral ganglion and ~200 cells of the utricular macula were captured. It should be noted that some of cells remain attached to the slide after the laser capture procedure; therefore we expect the number of

cells captured per section to be lower than the number of cells estimated by cell counts

4.5.3. *Human crista ampularis*

The experiment was carried out in duplicate. Three slides per set were used for each experiment. For each set four sections were used for LCM and two sections for scrape analysis. A pre-capture image was used to carry out a manual count of the cells in the epithelium and stroma. The pre-capture image was enlarged on a computer monitor and the darkly stained nuclei were counted, to give us an estimate of the number of cells in the epithelium and stroma. We counted cells from three serial sections and took an average of that total. With the approach described above we were able to avoid DAPI staining and counting, saving sections for LCM and RNA extraction. About 200 cells of the neuroepithelia were captured onto a single LCM cap from four sections. On a separate cap, about 400 cells of the stroma were captured for the same four sections.

4.5.4. *Quantification*

After the isolation steps, we quantified RNA using a VersaFluor fluorometer with EX490/10 excitation filter and EM520/10 emission filter (Bio-Rad Laboratories, Hercules, CA) and RiboGreen reagent (Molecular Probes Inc., Eugene, OR) according to the manufacturers' instructions. The dye-based method was preferred over the commonly used method of measuring absorbance at A_{260} for the following reasons: RiboGreen RNA quantification is a sensitive fluorescent stain for quantitating RNA in solution. The dye-based assay can measure as low as 1 ng/mL and it is minimally affected by contamination likely to be found in small nucleic acid preps. The protocol for preparing a low-range standard curve is described in the product literature. Briefly, For the low-range standard curve, make a series of RNA standard solutions at 2× final concentration by diluting a 2 μg/mL RNA (control) solution into disposable cuvettes or nuclease-free plastic test tubes for transfer to minicell cuvettes designed for VersaFluor fluorometer (BioRad, CA). The final RNA Concentration of the standard curve series are 50, 25, 10, 5, 1, 0 (ng/mL).

4.5.5. *Amplification*

RNA amplification was necessary for gene expression analysis given that only a small quantity of total RNA was recovered. We used the Paradise RNA amplification system (Arcturus) according to the manufacturer's instructions to carry out a single round of T7-based RNA amplification with oligo-dT primer. The amplified RNA (aRNA) was used as the template for reverse transcription.

4.5.6. *Quality assessment*

We assessed the quality of the RNA preserved in each batch of the mouse FFPE sections prior to LCM. We used the method described by Li et al. (2004), with the consideration that poor quality RNA would produce cDNAs limited to the 3′ end of the gene. RNA isolation was carried out from scrape and amplified as described above. We designed two pairs of PCR primers against mouse β-actin; the first pair from the 3′ end and the other 250 bp upstream of the first pair (Table 1). We tested total RNA from the scrape by reverse transcribing with an oligo-dT primer into cDNA, as described below, then performing quantitative real-time PCR with both sets of primers using the QuantiTect SYBR green kit (Qiagen, Valencia, CA). The quality of recovered RNA was assessed as the ratio of the C_t values (converted to quantity of RNA) of the two amplicons. There was no strict cut-off for 3′/5′ ratio. If the investigator is screening for expression of a known set of genes, ratios > 20 may be tolerated. However, if the FFPE sections are used to discover new genes, samples with ratios < 20 may be desirable. We selected a 3′ to 5′ ratio of 20 as the maximum allowable value. The 3′ to 5′ ratio of the mouse FFPE sections used in this report ranged between 10 and 20. We did not test the 3′/5′ ratio for the human FFPE sections used in this report. However, the method described for the mouse FFPE section can be used for human FFPE sections.

4.5.7. *RT-PCR*

aRNA was then analyzed using the reverse transcriptase PCR method described previously (Alagramam et al., 2001). We made cDNA utilizing the RNA SuperScript II RNase H-negative reverse transcriptase (Invitrogen, Carlsbad, CA) with RNase inhibitor, random hexamer, oligo-dT primer, dNTP mix, and 4 μL aRNA. Samples were then cycled at 23 °C for 10 min, 42 °C for 45 min, 95 °C for 6 min, then chilled to 4 °C. The cDNA products were amplified with PCR using the Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). Primers were designed with Primer3 (Rozen et al., 2000) using GenBank sequences for mouse genes β-actin, *GAPDH*, *Myo7a*, $p27^{Kip1}$, *Nfl-H* (Neurofilament) and human *GAPDH* genes. Primer sequences and amplicon data are shown in Table 1. In a total volume of 50 μL, we used 2 μL cDNA. The reaction was cycled at 94 °C for 30 s, annealing temperature for 30 s, then 72 °C for 30 s, for 40 iterations, followed by 5 min at 72 °C. PCR products were then run on a 3% agarose gel. Prior to analyzing LCM-produced specimens, we optimized PCR primers and protocol by performing RT-PCR on whole mouse brain RNA at P30 and then whole inner ear RNA at P2. Gene sequencing was done by ABI Inc. after cloning into pCR II-Topo vectors (Invitrogen).

4.5.8. *Repetition*

For the mouse FFPE sections, the entire procedure – laser capture procedure, RNA isolation/ amplification and RT-PCR – was carried out 5 times on sections obtained from independent batches of FFPE blocks. Sections from each block were screened for RNA quality as described above (3′/5′ ratio); the 3′ to 5′ ratio of the mouse FFPE sections that ranged between 10 and 20 were used for LCM and further processing. For the human FFPE tissue specimens, we had access to one batch of 6 slides. Two FFPE sections of human crista were mounted per slide. All of these sections were obtained from the same patient. These sections were ~1 year old. The experiment was carried out in duplicate by using 3 slides per experiment.

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