Published in final edited form as:

J Neurosci Methods. 2007 May 15; 162(1-2): 229–236.

Isolation of Outer Hair Cells from the Cochlear Sensory Epithelium in Whole-Mount Preparation Using Laser Capture Microdissection

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Abstract

Outer hair cells (OHCs) play an important role in frequency selectivity and signal amplification in the mammalian cochlea. Because OHCs are relatively few in number and a minority of the cells in the cochlea, separating and isolating them for applications such as cDNA library creation and proteomic studies is a challenging task. Laser Capture Microdissection (LCM) is designed to capture cells from very thin tissue sections, it can accurately isolate specific cells from large regions of tissue for RNA, DNA, and proteomic studies. Due to the constraints of cochlear anatomy, thin sections of the cochlea contain small numbers of OHCs. Therefore, we adapted the LCM technique to isolate OHCs from organ of Corti whole-mounts, each of which contain hundreds of OHCs that are simultaneously accessible and collectable. For comparison, we also used a more traditional mechanical dissection. The quality of cDNA derived from the OHCs collected with LCM and with the traditional mechanical method are compared and the merits and limitations of the techniques discussed. A similar approach can also be used to isolate large quantities of inner hair cells and selected supporting cells from the whole-mount cochlear preparation.

Keywords

outer hair cells; laser capture microdissection; prestin

INTRODUCTION

Hearing impairment, the most common sensory defect affecting millions of people ranging from newborns to senior citizens, is often associated with damage to outer hair cells (OHC). Because these cells provide the signal amplification and frequency selectivity required for the processing of complex signals like speech (Dallos et al. 1992), OHCs have been the subjects of a wide range of research projects ranging from molecular biology to *in vivo* physiology. In addition to OHCs, the organ of Corti, the sense organ of hearing, contains inner hair cells (IHC) and several varieties of supporting cells, i.e., Dieters and Hensen's cells, pillar cells, etc. Because many studies require large numbers of cells or purified populations, various methods have been developed to isolate individual constituents of the organ of Corti. These techniques have involved enzyme digestion and mechanical trituration of tissues (Brownell 1984; He et al. 2000; Zajic et al. 1987; Zenner et al. 1985). Unfortunately, these traditional methods are inadequate when large numbers of cells are needed. In addition, the enzymes used to separate

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OHCs from their neighbors require long incubation times, thereby decreasing the quality of mRNA and the integrity of protein components. In contrast, Laser Capture Microdissection (LCM) is a newly developed method for accurately isolating specific cells from large regions of tissue for RNA, DNA, and protein studies. In fact, LCM has been used to isolate homogeneous cells from thin sections of the inner ear to explore gene expression profiles (Cristobal et al. 2004; Cristobal et al. 2005; Pagedar et al. 2006). Because LCM is designed to work on very thin sections (5-10 μm), dissection of the bony cochlea, fixation, and decalcification are required before cryosections or paraffin-embedded sections can be made. In addition, a single radial section of the mouse cochlea contains very few total OHCs, making LCM in its present form less desirable when a large quantity of OHCs is required.

As an alternative, we have adapted the LCM technique to isolate large numbers OHCs from their neighboring supporting cells using a cochlear whole-mount preparation. The bony structure surrounding the organ of Corti is quickly removed manually during a cochlear whole-mount preparation; therefore the time required for sample preparation is significantly reduced. In addition, a whole-mount sample contains several hundred OHCs in the same observation frame, making the collection of a large number of OHCs simultaneously from a single sample possible. This approach significantly increases the efficiency of collecting cells. For comparison, we also use a more traditional mechanical dissection to collect OHCs. In this method, modified from He *et al.* (He et al. 2000), a partial organ of Corti is removed from the modiolus followed by the dissection of individual OHCs or groups of OHCs. The mRNA resulting from LCM isolation and from the traditional method was used to create cDNA pools through the non-specific SMART PCR amplification technique. The quality of cDNA derived from the OHCs collected with LCM and the traditional mechanical method are compared by a combination of immunofluorescence experiments and PCR based methods. The merits and limitations of the techniques are discussed.

MATERIAL AND METHODS

Tissue preparation

All surgical and experimental procedures were conducted in accordance with the policies of Northwestern University's Animal Care and Use Committee. After euthanizing the adult mouse with an overdose of anesthetic, the cochlea was removed from the hemi-sected head in DEPC (Diethylpyrocarbonate) treated 0.01M PBS (Phosphate Buffered Saline). In order to preserve the anatomical structure of the organ of Corti during the subsequent dissection, the cochlea was transferred to 4% formaldehyde/DEPC-treated 0.01M PBS. A small section of bone was removed from the apex with fine forceps to allow the fixative access to the organ of Corti. After ~8 minutes in this solution, the cochlea was washed in a large dish containing PBS. After this "light" fixation, the tissue was stable enough to withstand the mechanical stresses of the following procedure. Medium forceps were used to carefully remove the bony wall overlying the stria vascularis from the apical turn. The stria vascularis was "unwound" exposing the organ of Corti without disrupting the anatomy. Fine scissors were then used to cut through the modiolus, perpendicular to its axis, separating the apical turn from the rest of the cochlea. After removing the tectorial membrane with fine forceps, the tissue was mounted on a slide.

Mounting

The tissue was transferred to a 30 μl pool of 10% bovine serum albumin (BSA) in RNase-free PBS on a poly L⁺ coated glass microscope slide (Fisher). This viscous solution was important for adhering the tissue to the slide. Although BSA acted as an adhesive, it did not prevent water from leaving the tissue during dehydration. After ensuring that the organ of Corti was oriented with the hair bundles facing away from the slide, excess BSA was removed using a series of absorbent Whatman paper points. Once all the BSA/PBS solution was removed, the sample

was air dried for approximately three minutes to allow the adhesive to set. A fine scalpel was used to trim away the osseous spiral lamina medial to the IHCs. This step was required to ensure that the LCM film could be successfully positioned and that most of the specimen would reside in the focal plane of the laser. The slide was then stored at -80°C until immediately before the dehydration protocol. The time gap between euthanasia and slide storage in -80°C was 20-30 minutes.

Dehydration

Slides were allowed to reach room temperature (~ 2 minutes) prior to beginning the following dehydration procedure, which is modified slightly from Arcturus' HistoGene Staining Kit procedure (all solutions were RNase-free): 30 seconds in 75% ethanol (EtOH), 15 seconds in dH_2O , < 2 seconds in HistoGene Staining Solution (Arcturus), 30 seconds in dH_2O , 30 seconds in 75% EtOH, 1 minute in 95% EtOH, 1 minute in 100% EtOH, 10 minutes in fresh 100% EtOH, 4 minutes in xylene, and approximately 3 minutes air drying. The first 75% EtOH treatment acted as a coagulating fixative which helped adhere the tissue to the poly-L⁺ coated slide prior to submersion in dH_2O . This protocol was designed to thoroughly dehydrate the tissue so that the film used to collect the cells could bind effectively. Great care was taken to optimize this protocol. Because the tissue was much thicker than a cryosection, the time spent in HistoGene stain was as short as possible. As soon as the stain touched the tissue, the slide was put into the bottle containing the second dH_2O wash. If allowed to stain for too long, it became difficult to identify the location of OHCs. Efficacy of the dehydration protocol was labile. For example, a procedure successful on humid days would leave the tissue too dry on less humid ones, resulting in tissue release from the slide. An adequately dried sample had a punctate appearance in which individual cells were clearly visible when viewed under the microscope. An under-dried sample had a much smoother, shinier look in which individual cells were not distinguishable. Minor adjustments to the time spent in the second 100% EtOH and xylene rinses (more for wet tissue, less for dry) were sometimes required.

Outer hair cell isolation

OHCs were visually identified using anatomical landmarks (primarily the pillar cell demarcation between IHCs and OHCs) and targeted for LCM removal using a PixCell II LCM system and HS Caps (Arcturus). The HS cap is covered with a thin film that, when excited by laser energy, expands and fuses to any dry surface that it touches. This results in spots, i.e., small circles of film fused to the tissue directly beneath them. These spots can be combined to form lines in which the current spot overlaps with the previous spot resulting in a continuously fused film. In this way, complex regions of tissue can be fused to the film. In these experiments, the 7.5-micron spot diameter aperture was used for all tissue. The laser beam power and duration varied but generally stayed between 15-50 mW and 0.7-2.5 ms, respectively. The relatively short duration was chosen to minimize spot diameter. In other words, longer durations tended to increase spot size making it more difficult to target individual OHCs. Due to differences in dehydration and tissue preparation, each slide required slightly different laser parameters and focus. These settings were determined by test firing the laser on a clean region of the slide to assess spot quality and size prior to firing on the OHCs. Once these parameters were established, all accessible OHCs were fused to the cap with the laser. The smallest spot diameter that could be achieved was usually larger than one OHC. But centering the spot on row two of the OHCs generally fused all three rows to the cap. Once the cap was removed, a visual record of the tissue left on the slide, as well as the tissue in the cap, was obtained. It was then possible to determine if more OHCs were available for capture. If OHCs remained in the organ of Corti, these could be acquired using a different area of the same cap. In order to separate OHCs, the cap was incubated in extraction buffer (Arcturus) for 30 minutes at 42°C and spun into a collection tube for 2 minutes at $800 \times g$. Collected OHCs were stored at -80°C .

Traditional Mechanical Method for OHC collection

After the animal was humanely terminated with an overdose of anesthetic, cochleae from mice ranging in age from p11-p23 were dissected in L-15 medium (Sigma). The organ of Corti was exposed by removing the bony cochlear wall and the stria vascularis. Subsequent peeling of the organ away from the modiolus usually resulted in strings of OHCs and their neighboring supporting cells. These strings could be delicately removed using a transfer pipette. All strings collected were visually examined and determined to be relatively free of IHCs and Hensen's cells. Using a wide-mouth pipette, the cells were collected and stored in Dynal lysis buffer (Dynal, Norway) at -80°C . Total time between euthanasia and storage at -80°C was less than 20 minutes. Because OHCs remained in liquid without fixation during the entire process, we refer to the traditional mechanical method as the Wet technique.

Building an OHC-cDNA pool

OHCs collected by LCM were dissolved in extraction buffer. Total RNA was isolated from these cells using PicoPureRNA isolation kit (Arcturus). All eluted RNA was concentrated and used for cDNA synthesis. Messenger RNA (mRNA) of the OHC-rich strip was isolated using 80 μl of oligo-dT magnetic beads in LiCl buffer (Dynal Biotech, Norway). cDNA pools for each source of OHCs were created by reverse transcription using PowerScriptTM reverse transcriptase at 42°C for 90 minutes, followed by 22-26 cycles amplification with 5'Cap and oligo-dT-dependent SMART PCR primers provided by CreatorTM SMART cDNA library Construction kit (Clontech). The OHC cDNA pool was then digested with a *Sfi* I restriction enzyme and separated through a CHROMA SPIN-400 column. cDNA fragments with sizes larger than ~ 200 bp were ligated into a pDL2-xN vector (Dualsystem Biotech, Switzerland) and transformed into XL10-Gold Ultracompetent cells (Stratagene, La Jolla, CA). Plasmid DNA containing different cDNAs was isolated from XL10-Gold using Plasmid Midi kit (Qiagen).

PCR

Different primer sets were used to detect different gene products. The sequences of these primer sets are listed in Table I. PCR reactions contained the following mixture: 40 ng cDNA pool created from collected samples as described above, 0.2 mM of forward primer (A) and reverse primer (B), 200 μM of dNTP, 1X PCR reaction buffer (Eppendorf), and *Taq* DNA polymerase. Cycle parameters were 3 min at 94°C followed by 25-31 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 1-2 min, with a final extension at 72°C for 10 min. 15 μl of PCR product, representing cDNA inserts from the OHC-cDNA pool, was visualized on a 2% agarose/EtBr gel. The intensities of PCR bands were measured using Kodak ID Analysis Software.

Immunofluorescence experiments

Following LCM, mouse cochlear samples were re-hydrated with stepwise decreases in EtOH concentration: 95% EtOH for two minutes, 75% EtOH for two minutes, 25% EtOH for two minutes, 0.01M PBS for two minutes and, finally, fixation for 20 minutes in 4% formaldehyde to tightly adhere samples to the poly-L⁺ slide. After washing five times for 2 minutes each in PBS, samples were incubated in blocking solution (0.001% BSA, 0.5% saponin in PBS) for one hour. Samples were incubated with 1:2000 anti-N-mprestin (Zheng et al. 2005) or anti-C-mprestin antibody (Matsuda et al. 2004) in blocking solution for one hour, followed by either an FITC conjugated anti-rabbit IgG (Pierce) or Alex488 conjugated anti-rabbit IgG (Molecular Probes) secondary antibody solution (1:100 dilution, 0.5% saponin, 1.5% goat serum in PBS). After washing in PBS, samples were cover-slipped with mounting solution (Fluoromount-G) and observed using a Leica confocal system with a standard configuration DMRXE7 microscope. For samples that were not prepared for LCM, the process is similar to that for the

LCM samples save for the omission of re-hydration and the use of a standard epifluorescence microscope for observation.

RESULTS

OHC collection using mechanical dissection

Once the bony wall and the stria vascularis were removed from the cochlea, the spiral basilar membrane and organ of Corti appear as shown in Figure 1A. FITC-tagged anti-N-mprestin was used to label OHCs, the only cells expressing prestin (Zheng et al. 2003). OHC-rich strips were removed mechanically from the modiolus as shown by the arrowhead in Figure 1A. These strips contain OHCs and Deiters cells, as shown in Figure 1B. In other words, they were free of IHCs and Hensen's cells. Each strip contained approximately 100 - 200 OHCs. With the intention of collecting a large quantity of OHCs with great integrity, these OHC-rich strips were not treated with protease, which would usually results in single OHCs isolated from the rest of the tissue. Therefore, every OHC from the Wet technique is possibly accompanied by one or more supporting cell. These supporting cells are not included in the estimated number of OHCs from each strip.

OHC removal from a whole-mount sample using LCM

For OHCs to be collected by LCM, the apical turn was dissected from the rest of the cochlea by cutting through the modiolus perpendicular to its axis. Figure 2A provides an example of the whole-mount preparation. The location of OHCs, IHCs and pillar cells can be identified. As shown in Figure 2B, a clear film binds to dry surfaces when excited by laser energy, allowing OHCs to be "plucked" from the surrounding organ of Corti. In this technique, care was taken to remove all bony tissue medial to the IHCs to allow the film to lie flat over the tissue and to adequately dehydrate the tissue so that the excited film would adhere well. Visual inspection of the cap and the tissue remaining on the slide clearly shows that tissue from the region of the OHCs had fused to the cap. A large number of OHCs can be isolated from a single whole-mount sample preparation and Figure 3 shows an example where approximately 400 OHCs were isolated. On average, each LCM cochlea yielded 200-300 OHCs. Concomitant with the tissue fused to the cap was a loss of tissue from the organ of Corti, i.e. the organ became much lighter when the cells were removed. However, visual inspection alone was not sufficient to conclude that the tissue isolated with LCM was composed of OHCs. Because the OHC has a columnar shape, the basolateral region could have broken away from the cuticular plate and remained in the tissue. To address the question of whether intact OHCs had been isolated from the organ of Corti, prestin immunofluorescence, the hallmark of the OHC (Zheng et al. 2000), was performed. Figure 4 shows a cochlea in which not all of the OHCs were targeted for capture with LCM. In other words, only OHCs between regions I and II in Figure 4B were designated for removal. These targeted cells are shown fused to the film in Figure 4C. The rest of the OHCs were left unmolested and served as an internal control. These confocal images show that the targeted region has a different pattern of prestin immunofluorescence than does the control region. Absence of the characteristic green ring is direct evidence that an OHC has been removed. In this sample, approximately 80% of the OHCs were fused to the film and captured. The other 20% of OHCs remained on the slide as shown in Figure 4B, probably as a result of poor contact with the film. Confocal z-sections of the targeted region showed that green rings were missing from all z planes. This indicates that entire OHCs were missing, i.e. no "half cells" have been left behind. This result indicates that the dehydration process reduces cell-cell adhesion, thereby loosening OHCs from their supporting-cell structures. Hence, intact OHCs can be targeted and removed from the organ of Corti by LCM.

Comparing OHC purity using LCM and Wet cDNA pools

The appeal of LCM is that it offers precise control over which tissue is collected. When using a thin cryosection, the lateral control (x and y axes) of the system provides precise tissue specificity. In contrast, OHCs in the whole-mount preparation are surrounded by a variety of cell types. Even so, LCM was able to remove an entire OHC. Whether other cell types may have been captured during OHC collection is unknown. Because OHC immunofluorescence cannot speak to the presence or absence of other cells collected by LCM, cell-specific gene products in the form of cDNA were analyzed.

PCR analysis of cell markers was used to determine the relative purity of OHCs as compared to other cell types in both cDNA pools. Prestin is an OHC-specific gene product (Zheng et al. 2000) and is used as the OHC marker. Because the cyclin-dependent kinase inhibitor p27^{kip1} is not expressed in OHCs but is found in supporting cells (Chen et al. 1999), it is used as the supporting cell marker. In order to minimize errors associated with variations in the amount of starting material and other artifacts, cyclophilin is used as an internal control to normalize band-intensity levels between the two pools. Cyclophilin is a common housekeeping gene expressed in many different cell types and is often used for gene expression normalization (Bustin 2000; Haendler et al. 1987). As shown in Figure 5, both prestin and cyclophilin were found in LCM and Wet cDNA pools. The presence of prestin indicates that both pools contain mRNA originating from OHCs. The quantity of cyclophilin cDNA is proportional to the number of cells used to make each cDNA pool. Cyclophilin normalized PCR intensities for each cDNA pool are presented as the ratio of the prestin intensity in the selected linear amplification phase divided by the cyclophilin intensity at that phase. Figure 5B shows the comparison between normalized prestin in LCM and Wet cDNA pools. The normalized prestin intensity in LCM cDNA pool is about ~2.5 times that in the Wet cDNA pool. This indicates that there was more prestin cDNA per ng of starting material in the LCM pool than the Wet pool. This would occur if a larger proportion of the cells used to build the LCM pool were OHCs.

Using the exact same PCR mixture, a p27^{kip1} band was found in the Wet cDNA pool after 28 cycles. However, even after 50 cycles of amplification, a p27^{kip1} PCR band was not found in LCM cDNA pool (Figure 5C). Although it is not possible to compare the normalized intensities of the two pools, the absence of the supporting cell marker in the LCM cDNA pool is evidence that there was a very low proportion of supporting cells in the LCM pool compared to the Wet pool. Hence, OHCs make up a larger proportion of the total cells used to make the LCM pool. This is strong evidence for OHC specificity and indicates that the LCM technique only collected OHCs from the whole-mount preparation.

Integrity of OHC-cDNA pool

A major limitation in auditory molecular biology is the difficulty of obtaining enough biological material to study the molecular basis of function. OHCs, for example, make up a small percentage of the cell population in the cochlea. The non-specific SMART PCR amplification technique (Clontech) allows us to overcome this problem. By combining the SMART PCR technique with our newly developed methods for collecting OHCs, we were able to collect thousands of OHCs to create OHC cDNA pools. LCM-collected tissue yielded a cDNA size distribution from 0.1 to 1.2 kb. The Wet-collected tissue had a distribution from 0.1 to 6 kb.

RNA degradation is a common problem associated with RNA isolation and cDNA pool synthesis even though care was taken to prevent it. cDNA quality, i.e., the full-length cDNA in the SMART amplified cDNA pool, is often assayed by analysis of cDNA fragments near the start codon of the 5'mRNA. The better the mRNA template, the more prevalent are regions

of the gene distant from the 3' poly-A⁺ tail. Degraded mRNA templates will be shorter and commonly will not contain sequences near the 5' end of genes. We designed two pair primers for prestin PCR amplification: a 5' pair and a 3' pair. As shown in Figure 6A, the 5'-prestin PCR amplified region is 1.9 kb upstream from the 3'cDNA terminus. As shown in Figure 6B, the 5'-prestin band was found in Wet cDNA but not in the LCM cDNA after 45 cycles of PCR. These data indicate that LCM cDNA does not contain a large number of full-length prestin cDNAs, while Wet cDNA does. Hence, the mRNA obtained by LCM was of poorer quality than that obtained from the Wet technique. However, in contrast to the LCM technique, the tissue collected by the Wet technique was not composed of OHCs only. In order to prevent RNA and protein degradation, the tissue was collected in the shortest period of time. In other words, there is a tradeoff between obtaining high integrity mRNA/cDNA and a highly pure cell population with the Wet technique.

Discussion

The organ of Corti is a highly specialized structure with a high density of different cell types packed into a very small space. Each radial section along the organ of Corti contains one inner hair cell and three outer hair cells. Because a radial thin section of the mouse cochlea contains a maximum of 15 OHCs, this approach is not an attractive tissue preparation method when large quantities of OHCs are required. Consequently, we set out to adapt LCM for OHC collection from a whole-mount preparation because it would allow us to acquire a large number of OHCs from a single cochlea. Evidence from immunofluorescence and PCR gene product analyses suggests that LCM successfully isolates pure populations of OHCs from the organ of Corti in a whole-mount preparation.

OHCs collected by LCM, along with OHCs collected by the Wet technique, were used to build cDNA pools. Non-specific SMART mRNA amplification for full-length cDNA was less successful with fixed LCM tissue than it was with the non-fixed Wet tissue. RNA degradation during LCM sample preparation may contribute to the small sizes of LCM cDNA fragments. We suspect that the light formaldehyde fixation needed for tissue preparation is the most likely cause for short cDNA fragments because formaldehyde links methylol groups to the nucleotides in mRNA. These methylol groups can then go on to bind to another molecule (nucleotide, protein, etc) and cross-link the mRNA, thereby preventing reverse transcription from proceeding past the point of the cross link (Srinivasan et al., 2002). The longer the mRNA template, the more likely it is to have a methylol group somewhere along its length. Because the cDNA is created from the 3' poly-A⁺ tail of the mRNA template, creation of longer full-length cDNA after fixation is less likely. Although the total time in fixative was very short (8 minutes) and steps were taken to uncross link the mRNA prior to cDNA creation (Srinivasan et al. 2002), the bias is still present. It is important to note that this phenomenon is exacerbated by 3'-SMART amplification. It is well known that the poly-A⁺ tail used as the start region for the reverse transcription and amplification is biased to amplify 3'-mRNA. If isolating mRNA is the experimental goal, then adjustments are required to further improve its integrity. Perhaps omitting the fixation or changing to a different fixative that is less deleterious to RNA integrity would be possibilities. For applications in which specific genes can be targeted for amplification or for which random primer pairs can be used, the amplified cDNA should more accurately represent the original mRNA from the tissue.

We have demonstrated that OHCs collected by the LCM method from a whole-mount preparation contain OHC specific mRNA/protein and lack supporting cell specific mRNA. These OHCs can be used to analyze gene expression patterns. This procedure can also be used to collect inner hair cells, pillar cells, Henson's cells, etc. and should prove useful for other whole-mount preparations.

Acknowledgements

Dr. K. Kaul and K. Lannert at Evanston Northwestern Hospital are thanked for their advice and the gracious use of their LCM system. Dr. A. Bennet and M. Bellamy at Arcturus Bioscience are thanked for their technical support. Dr. W. Russin at the Biological Imaging Facility of Northwestern University is thanked for his help in image processing. We also thank Dr. P. Dallos and Dr. M. Cheatham for their comments on the manuscript, and R. Edge and K. Miller for their technical assistance. This work was supported by the National Institutes of Health, NIDCD, Grants DC006412 and DC00089 and The Hugh Knowles Center Leadership Fund.

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Figure 1.
(A). Immunofluorescence image of a wild-type mouse cochlea stained with anti-N-mPrestin. The low-magnification image is taken with an epifluorescent microscope at 25X. The arrow indicates the location of OHCs; the arrowhead, the position where the OHC string was released from the surrounding organ of Corti. (B) A typical string of OHCs collected by the Wet technique. Image was taken at 40X.

Figure 2.
(A) A typical dehydrated whole-mount preparation of the organ of Corti prior to LCM. (B) Cartoon showing the LCM principle for isolating OHCs.

Figure 3.
(A). The stained and dehydrated organ of Corti sample prior to LCM. (B) LCM film containing ~450 OHCs that were removed from the whole-mount preparation shown in 3A.

Figure 4.

(A). The stained and dehydrated organ of Corti whole-mount sample prior to LCM. (B). The organ of Corti sample that remains behind after LCM tissue removal. Note that only the area (I to II) was targeted for LCM. (C). The LCM film showing isolated cells. (D) Prestin immunofluorescence of the organ of Corti whole-mount sample shown in (B). This sample was re-hydrated through a stepwise decrease in EtOH concentrations, followed by fixation, incubation with anti-C-mprestin, and Alexa 488-labeled secondary antibody. Characteristic OHC rings are either present or absent in every confocal plane, indicating that whole OHCs have been collected with LCM.

Figure 5. PCR semi-quantification analysis of prestin and P27^{kip1} contents from LCM and Wet cDNA pools. (A). cDNA was amplified with 3'prestins primer pairs (upper frame) and cyclophilin-specific primers (lower frame) under different amplification cycles. PCR products were observed on 2% agarose gels. (B). A comparison of normalized prestin content from LCM and Wet cDNA pools. (C). PCR amplification of P27^{kip1} from LCM and Wet cDNA. The P27^{kip1} band was detected after 28 cycles in the Wet library. But, even after 50 cycles, there is no P27^{kip1} signal from the LCM cDNA.

Figure 6. (A) PCR amplification of 5'prestins from Wet and LCM cDNA pools. (B) Schematic showing primer location for 3' and 5' prestin PCR.

Table I

primers for PCR

Name of primers	Primer sequence	Size of PCR band
5'-prestin A	gtccacactgtcattctagactttacgcagg	219 bp
5'-prestin B	ccaggactgcatcgtggatactgtggaacag	
3'-prestin A	gacttggtctcgggcataaagc	323 bp
3'-prestin A	ctgaatgattcctgaaagtaagg	
P27 ^{KIP1} A	gagaagcactgccgggatatg	333 bp
P27 ^{KIP1} B	cagcaggtcgttcctcatc	
Cyclophilin A	tggcacaggaggaaagagcatc	301 bp
Cyclophilin B	aaagggttctccacctcgatc	