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# RNAlater facilitates microdissection of sensory cell-enriched samples from the mouse cochlea for transcriptional analyses

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## HIGHLIGHTS

- A novel microdissection technique for cochlear tissue collection is described.
- Collected samples contain defined sensory cell and supporting cell populations.
- The RNA integrity of the samples is well preserved.
- Stable reference genes have been identified for noise-traumatized samples.
- The microdissection technique is applicable to both mouse and rat cochleae.

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## ABSTRACT

Molecular analyses of cochlear pathology rely on the acquisition of high-quality cochlear samples. For small rodents, isolating sensory cell-enriched samples with well-preserved RNA integrity for transcriptional analyses poses a significant challenge. Here, we report a microdissection technique for isolating sensory cell-enriched samples from the cochlea. We found that treating the tissue with RNAlater, a RNA preservation medium, alters the physical properties of the tissue and facilitates the dissection. Unlike previous samples that have been isolated from the sensory epithelium, our samples contain defined cell populations that have a consistent ratio of sensory cells to supporting cells. Importantly, the RNA components were well preserved. With this microdissection method, we collected three types of samples: sensory cell-enriched, outer hair cell-enriched, and inner hair cell-enriched. To demonstrate the feasibility of the method, we screened multiple reference genes in the sensory cell-enriched samples and identified stable genes in noise-traumatized cochleae. The method described here balances the need for both quality and purity of sensory cells and also circumvents many limitations of the currently available techniques for collecting cochlear tissues. With our approach, the collected samples can be used in diverse downstream analyses, including qRT-PCR, microarray, and RNA sequencing.



## 1. Introduction

Cochlear hair cells are responsible for converting acoustic stimuli to neural impulses. They are also the major target of common pathological insults (Hu et al., 2002; Keithley and Feldman, 1982; Nakashima et al., 2000). Because hair cells in the mammalian cochlea are unable to regenerate, the loss of these sensory cells can lead to permanent hearing loss. Preventing such functional loss requires a better understanding of the molecular mechanisms that control hair cell pathogenesis.

The success of molecular analyses of cochlear pathology relies on the collection of high-quality cochlear samples. Due to the complexity of the cochlear structure, isolating sensory cell-enriched samples from mammalian cochleae has been a significant challenge for researchers in the field. This challenge is particularly striking for the mouse, which is a widely used animal model for molecular studies of cochlear disorders.

To date, commonly used methods for collecting sensory cell-specific samples involve disassociating the cells from the cochlea using mechanical and/or enzymatic approaches (Harter et al., 1999; He et al., 2000; Towers et al., 2011). The disassociated hair cells are then either manually picked up by a micropipette or automatically sorted via flow cytometry (Hertzano et al., 2011). Although these methods can result in a highly purified population of sensory

*Abbreviations:* OHC, outer hair cell; IHC, inner hair cell; RIN, RNA Integrity Number; ABR, auditory brainstem response.

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cells, their ability to preserve RNA integrity has been a concern because without RNA protection, any disturbance of the cellular environment causes rapid RNA degradation and/or changes in RNA expression. Laser capture microdissection, another method that has been used to collect hair cell-specific samples in the cochlea (Anderson and Zheng, 2007), requires extensive tissue preparation, which can also be problematic in maintaining RNA integrity.

Traditionally, the most effective way to preserve the RNA integrity of cochlear tissues has been to extract the tissues in the shortest time possible in a cold medium (Christensen et al., 2009; Han et al., 2012; Szaumkessel et al., 2012). In recent years, RNA stabilization reagents have been developed to protect RNA (Mutter et al., 2004; Wang et al., 2006). These reagents allow high-quality RNA samples to be obtained from defined cochlear partitions, including the apical and basal segments of the cochlea containing the sensory epithelium and the modiolus (Sato et al., 2009), the lateral wall of the cochlea (Jin et al., 2008), and the sensory epithelium (Hu et al., 2009). Our recent study has further improved the spatial resolution of this partitioning by separating the apical and basal sections of the sensory epithelium (Cai et al., 2012). While the sensory epithelium sample contains a relatively higher percentage of hair cells than the samples from other preparations, the purity of sensory cells in this sample is still unsatisfactory because of the presence of a large quantity of non-sensory cell structures, such as supporting cells, mesothelial cells, blood vessels-derived cells, and extracellular matrix structures including the basilar membrane. Moreover, the precise cell composition of each individual sample is difficult to define. Inconsistent tissue composition can lead to a significant variation in the results of expression analyses conducted across several samples (Hertzano and Elkon, 2012). Because many new technologies, such as whole-transcriptome analysis using RNA-sequencing technology and miniature sample analysis using droplet PCR, are now available for cochlear tissues, there is an urgent need to develop a method to collect high-quality, sensory cell-enriched samples.

Here, we report a microdissection technique that permits the isolation of sensory cell-enriched samples with significantly improved sensory cell purity. Unlike previously obtained sensory epithelium samples, our samples contain defined cell populations with a relatively consistent ratio of sensory cells to supporting cells. Importantly, the RNA of these samples is well preserved. Using this microdissection method, we were able to collect three types of samples from the mouse cochlea: sensory cell-enriched, outer hair cell (OHC)-enriched, and inner hair cell (IHC)-enriched. To demonstrate the feasibility of this method, we screened 12 reference genes in the sensory cell-enriched samples and identified stable genes that are expressed in noise-traumatized cochleae. This method balances the need for both quality and purity of sensory cells and circumvents many of the limitations of the currently available techniques for collecting cochlear tissue.

## 2. Materials and methods

### 2.1. Animals

Both mice (C57BL/6J, 2–4 months old, male and female, the Jackson Laboratory, Bar Harbor, ME) and rats (Sprague Dawley, 2–3 months old, male and female, Charles River Laboratories, Wilmington, MA) were used in this study. All animals received a baseline hearing evaluation using auditory brainstem response testing. Only the subjects that exhibited a normal hearing sensitivity were included in this study. The procedures involving the use and care of the animals were approved by the Institutional Animal Care and Use Committee of the State University of New York at Buffalo.

### 2.2. Sample collection

To better describe the procedures used in this study, we used the following three anatomic distinctions: the cochlear sensory epithelium, the sensory organ partition, and the organ of Corti. The sensory epithelium was defined as the tissue located between the lateral wall and the modiolus, containing all components of the tissue, including cellular components, blood vessels, nerve fibers, the basilar membrane, and the osseous spiral lamina. The sensory organ partition consists of tissue that is similar to the sensory epithelium but lacks the structures medial to the inner border cell. The organ of Corti contains the cells between the Claudius cell and the inner sulcus cell on the top of the basilar membrane. It should be noted that the term "organ of Corti" has been used in some previous investigations to denote the tissue defined as the sensory epithelium in the current investigation. In the current investigation, we adopted the conventional meaning of the term that refers to a much more limited cell population (Iurato, 1961; Santi and Mancini, 1998).

The mouse cochlea has two turns. The first turn, also referred as the basal turn, is a major site of pathogenesis in many disease conditions; therefore, we selected this cochlear location for tissue collection. This region has a relatively wide distance between the inner and outer hair cells than does the basal end of the first cochlear turn, which mitigates the technical difficulty of separating the OHC-enriched and IHC-enriched tissues.

#### 2.2.1. Initial preparation of the cochlea

The animal was decapitated under deep anesthesia with $CO_2$. The cochleae were quickly removed from the skull and placed in an ice cold Dulbecco's phosphate buffer saline solution (DPBS, GIBCO). Under a dissection microscope, the bony shell of the cochlea that faces the middle ear cavity was quickly removed. The modiolus was also removed along with the tissues of the lateral wall and the sensory epithelium; however, the section that was designated for sample collection in the apical portion of the first cochlear turn was left behind. Then, the cochlea was transferred into a PCR tube that contained 0.6 ml of an RNA-stabilizing reagent (RNAlater; Qiagen, Valencia, CA) and stored at 4 °C until further dissection. The initial processing of the cochlea, which took only a few minutes, facilitated the rapid entry of the RNAlater reagent into the tissues. Moreover, the separation of the modiolus from the cochlea generated a smooth cut at the medial edge of the sensory organ partition (Fig. 1), which facilitated the subsequent microdissection.

#### 2.2.2. Microdissection

The microdissection was performed within a period of 1–7 d after the initial cochlear processing. Each cochlea was transferred to a shallow, glass-bottom dish (35-mm Fluorodish with 10-mm glass, FD3510-100, WPI) that had been filled with 200–300 μl of the RNAlater reagent. Under a dissection microscope, the cochlea was oriented to visualize the stripe of the organ of Corti. Because of the differential light transmittance of cells in the fresh tissue that was stored in the RNAlater reagent, the three rows of outer hair cells and one row of inner hair cells were clearly visible under the dissection microscope (Fig. 2A and B).

The medial edge of the sensory organ partition had already been disassociated from the osseous spiral lamina after the bony shelves of the osseous spiral lamina were removed during the initial tissue preparation phase. We therefore focused on separating the lateral edge of the tissue using a custom-made micro-knife. We found that the RNAlater treatment not only hardened the tissue but also weakened the cell-cell attachments, which facilitated the microdissection.

To collect sensory cell-enriched samples, we gently scraped the reticular laminar at the junction between the Deiters cells and the Hensen cells and pushed the tissue away from the basilar