Differentiation 91 (2016) 104–108

Contents lists available at ScienceDirect

Differentiation

journal homepage: www.elsevier.com/locate/diff

Review article

Three-dimensional image analysis of the mouse cochlea

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ARTICLE INFO

Article history:
Received 5 January 2016
Accepted 6 January 2016
Available online 16 January 2016

ABSTRACT

The mouse has proven to be an essential model system for studying hearing loss. A key advantage of the mouse is the ability to image the sensory cells in the cochlea. Many different protocols exist for the dissection and imaging of the cochlea. Here we describe a method that utilizes confocal imaging of whole-mount preparations followed by 3D analysis using the Imaris software. The 3D analysis of confocal stacks has been successfully used for investigating a number of mouse tissues and developmental processes. We propose that this method is also a valuable tool to analyze the cellular and tissue organization of the sensory hair cells in the cochlea.

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1. Introduction

Hearing loss (HL) is a global health problem that affects all ages (Stevens et al., 2013). The elderly and individuals regularly exposed to high noise levels are at particular risk of developing HL, but even in younger populations, HL is on the rise due to noise exposure (Brody, 2013). An etiological feature of noise-induced and age-related HL is the loss of sensory hair cells in the inner ear (Fetoni et al., 2011; Kujawa and Liberman, 2009; Liberman and Dodds, 1984; Schmiedt, 2009). Hair cells line the sensory epithelium of the cochlea and reside in a morphological structure known as the Organ of Corti. They amplify and transduce the mechanical signals generated by sound waves into neural impulses. Since studying HL in humans is restricted to non-invasive procedures, the use of model systems is important to understand changes at the tissue and cell biology levels that accompany HL. The mouse has proven to be an excellent model system for several reasons. Many of the genes that affect hearing in humans also cause HL in mice, often with similar disease progression (Avraham, 2003; Friedman et al., 2007). Structurally, the mouse ear is similar to the human ear, and as in humans, sensory hair cells of the cochlea do not spontaneously regenerate when lost (Forge et al., 1993; Oshima et al., 2010; Warchol et al., 1993). And finally, the genetic tractability of the mouse is invaluable. Some 400 mutations have been identified in the mouse that play a role in hearing

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http://dx.doi.org/10.1016/j.diff.2016.01.002
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0301-4681/

loss (Kikkawa et al., 2012), many of which have been instrumental in promoting our understanding of inner ear development and function.

A distinct advantage of the mouse is that the inner ear is readily isolated and imaged, allowing for the visualization of hair cells at the cellular and subcellular levels. Many different approaches have been developed for visualizing the cochlea, and, with the exception of approaches using MRI (Benveniste and Blackband, 2002), all generally require the isolation of the temporal bone. This procedure has been well-described by several groups (Akil and Lustig, 2013; Bohne and Harding, 2011; Haque et al., 2015; Hertzano and Avraham, 2002; MacDonald and Rubel, 2008), and we will restrict ourselves to highlighting the main points here.

Once the temporal bone is isolated, further preparation to visualize cochlear cells can be divided into three general approaches: (1) whole-mount preparations, (2) cochlear sections, and (3) isolated single cells. Imaging of whole-mount preparations has the potential for giving the clearest overview of the 3-dimensional architecture of the cochlea and the sensory epithelium and can involve either the whole temporal bone with little further manipulation (MacDonald and Rubel, 2008; Voie et al., 1993), sections of the cochlea that require some further dissection (Hardie et al., 2004) or isolation of the sensory epithelium away from the cochlea, which is the most involved in terms of dissection technique, effort, and potential for disruption to the tissue (Akil and Lustig, 2013; Bohne and Harding, 2011; Haque et al., 2015). These tissue preparations allow for a range of resolution, depending on the sample preparation and imaging strategies utilized.

The preparation of cochlear sections has the advantage of allowing access to cells within the Organ of Corti with minimal physical manipulation, though his step does require the decalcification of the temporal bone if examining the ears of older mice. The whole temporal bone is generally processed for fresh–frozen or paraffin sections. In theory, the temporal bone can be sectioned at any angle to give the desired tissue orientation, though the most common orientation is that of the side-on view of the Organ of Corti (e.g. (Kharkovets et al., 2006; Leibovici et al., 2005; Liberman et al., 2002)).

The final commonly used method to prepare cochlear cells for analysis is isolation of single cells. This method is predominantly used for electrophysiological and cell motility studies and involves a combination of enzymatic and physical dissociation steps to generate single sensory hair cells (e.g. (Abe et al., 2007; Homma and Dallos, 2011; Liberman et al., 2002; Zenner, 1986)). However, some groups have also used this approach for immunofluorescent studies (e.g. (Legendre et al., 2008; Yu et al., 2006)).

Confocal microscopy presents a powerful means of imaging and analyzing three-dimensional structures, such as the cochlear hair cells. However, to gain a better understanding of both the cellular and sub-cellular organization of such thick and complex 3D samples, 3D visualization software proves itself extremely useful. Such software takes a series of two-dimensional images (e.g. a confocal *z*-stack) and produces a 3D reconstruction that allows the user to interact with the data by rotating, re-orientating, zooming, cropping and re-slicing the 3D view. Here we made use of the Imaris package from Andor (Oxford Instruments). Other packages are available, both commercial and open source solutions, for example Volocity (PerkinElmer), Amira (FEI), BioImageXD (http://www.bioimagexd.net), ImageSurfer (http://imagesurfer.cs.unc.edu), Vaa3D (http://home.penglab.com/proj/vaa3d/).

2. Isolation of mouse cochlea

The developmental time-point for isolating the cochlea will be experimentally driven. In general, the procedures are similar, but due to the postnatal development and calcification of the cochlea, slight variations are inherent. The head of a sacrificed mouse is bisected sagittaly, and the brain removed while submerged under PBS, exposing the temporal bone (see (Akil and Lustig, 2013; Haque et al., 2015; Hertzano and Avraham, 2002) for detailed procedure on temporal bone isolation). The temporal bone is teased away from the skull and the stapes is removed from the oval window, if still present. A small hole is made at the apical tip of the cochlea by removing some of the cartilage (or bone, depending on the age of the animal) using a #4 or #5 forceps. The entire temporal bone is fixed in freshly-prepared 4% paraformaldehyde (a few hours at room temperature (RT) for less than P5, overnight (O/N) at 4 °C for P5 up).

3. Exposing the sensory epithelium

After fixation, the temporal bones are washed in PBS. All subsequent dissections and manipulations are carried out in PBS. The temporal bone can either be immobilized in a Sylgard® coated dissection dish using mounting pins, or stabilized using another pair of forceps. The outer layer covering the cochlea (otic capsule) is removed, exposing the underlying membranes (Fig. 1). The lateral wall, Reissner's and tectorial membranes are all removed, exposing the sensory epithelium (see Fig. 2 for a schematic cross section of the sensory epithelium). The exposed cochlear sensory epithelium (still attached to the vestibular system) is transferred to a 2 mL Eppendorf tube for immunostaining.

4. Immunostaining

The above-prepared tissues are immunostained according to the following protocol. Care should be taken that the samples do not dry out at any step. To avoid the possibility of drying the samples, it is best to leave a small volume of liquid in the bottom of the tube at the end of each wash step.

1. Permeabilize in 0.2% Triton X-100 in PBS for 1 h at RT. Place

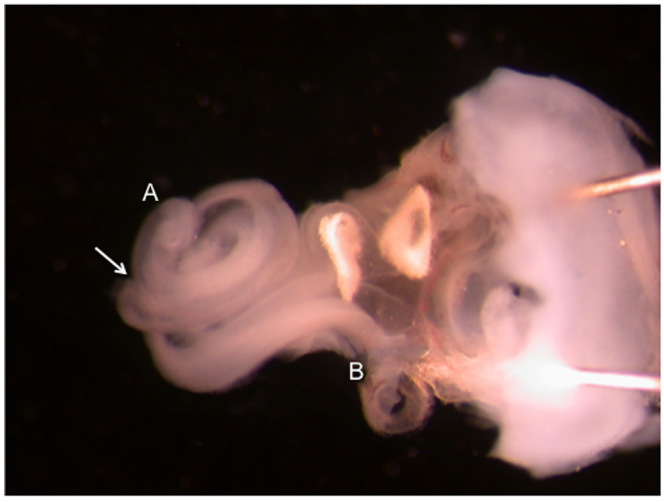

Fig. 1. Dissection of the temporal bone of a newborn (P1) mouse. The outer covering of the cochlea (the otic capsule) has been removed, exposing the turns of the cochlea. The temporal bone is stabilized for dissection by inserting a pair of forceps into the vestibular system, as shown. The lateral wall, Reissner's membrane and tectorial membrane need to be removed before immunostaining can proceed. At the apex of the cochlea, one can see where the lateral wall is beginning to come away from the sensory epithelium (arrow). The base (B) and apex (A) of the cochlea are identified.