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Original article

Imaging and cell count in cleared intact cochlea in the Mongolian gerbil using laser scanning confocal microscopy

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ABSTRACT

Objectives: To draw up a clearing protocol for Mongolian gerbil cochlea, and to assess the feasibility of quantifying and analyzing 3D cell architecture in the transparent cochleae.
Materials and methods: Freshly dissected inner ears were prepared on a 13-day protocol: fixation, microdissection, post-fixation, decalcification, pretreatment (signal enhancement, permeabilization and blocking), fluorescent labeling (indirect immunolabeling and direct labeling), dehydration, clearing in Spalteholz solution (MSBB: methyl salicylate and benzyl benzoate) and mounting. Image acquisition used laser scanning confocal microscopy. ImageJ software was used to measure the length of the organ of Corti thus available for analysis and to count inner and outer hair cells.
Results: Four cochleas underwent imaging. 3D reconstruction enabled organ of Corti length to be measured, at a mean 1269 ± 346 μm. Mean inner and outer hair-cell count per organ of Corti length was 142 ± 44 and 400 ± 122, respectively.
Conclusion: Cochlear clearing by MSBB was feasible in Mongolian gerbils and provided high-resolution immunofluorescence-labeled inner-ear images. To our knowledge, this was the first application of the technique in this species. Cell count could thus be performed along the organ of Corti length without traumatic dissection.

1. Introduction

The otic capsule, within the temporal bone, is composed of compact lamellar bone which is the densest in the mammalian organism [1–3], through which it is impossible to image the cochlear neuroepithelium. The latter is therefore classically studied by separating the neuroepithelium from the bony structures or on serial cross-sections rather than on the whole organ, but these techniques sacrifice the complex 3D structure. Classic embedded techniques also have limitations: loss of material between cross-sections and trauma to fragile tissue. 3D reconstruction from serial sections is difficult and time-consuming, and may show artifacts [4,5]. To circumvent these issues, a clearing protocol (to render the cochlea transparent) was developed for whole cochlear imaging in mice by fluorescent laser scanning confocal microscopy (LSCM) using the Spalteholz solution [6–8], a blend of 5 parts methyl salicylate and 3 parts benzyl benzoate. The present study sought to extend the protocol to Mongolian gerbils, which are of particular interest in our research program, as their hearing includes low frequencies closer to those of human hearing than in mice [9,10].

The main objective was to evaluate the efficacy of the protocol established by MacDonald and Rubel when used in the gerbil cochlea and its application to hair-cell count, without the limitations inherent to classical tissue preparation techniques.

The secondary endpoint was to measure the length of the organ of Corti made available to assessment, compared to classical dissection techniques.

2. Materials and methods

2.1. Animals

Mongolian gerbils (*Meriones unguiculatus*) were obtained from the Janvier laboratory (Janvier Labs breeding center, Berthevin, France) and hosted in the animal facility of Lille 2 University experimental research center.

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Approval for a project using animals for research purposes was obtained from the regional committee for experimental animal care and use (C2EA-75, Nord-Pas-De-Calais, France; protocol nº 01225.01) Experiments were conducted in compliance with the guidelines of the French Ministry of Agriculture, which regulates animal research in France, in accordance with the EU Directive 2010/63/EU for the protection of animals used for scientific purposes. *Animal Research: Reporting of In Vivo Experiments* (ARRIVE) guidelines were followed [11].

Animals were euthanized under general anesthesia (gas mixture of air 2 L/min and isoflurane 5%) by cervical dislocation according to guidelines.

2.2. Reagents

Reagents comprised: PBS 1 × (phosphate buffered saline, containing 11.9 mM phosphate, 137 mM sodium chloride and 2.7 mM potassium chloride; pH 7.4); ethanol 70%; ethanol 90%; absolute ethanol (100%); and Image iT-FX (Life Technologies, Carlsbad, CA, USA); all other reagents were from Sigma-Aldrich (Saint Louis, MO, USA):

- 4% PFA (paraformaldehyde 4% in PBS 1 × with pH adjusted to 7.4);
- 10% EDTA (ethylene diamine tetraacetic acid disodium salt dihydrate 10%, buffered in PBS 1 × with pH adjusted to 7.4);
- Triton X-100; bovine serum albumin (BSA);
- rabbit polyclonal IgG anti-neurofilament 200 antibodies;
- goat polyclonal IgG anti-rabbit IgG antibodies with Atto 633 fluorophore tag;
- DAPI (4′,-6 diamidino-2-phenylindole dihydrochloride);
- Phalloidin-TRITC (phalloidin-tetramethylrhodamine isothiocyanate);
- methyl salicylate;
- benzyl benzoate.

2.3. Protocol

Freshly harvested inner ears were immersed in PBS 1 × after removal of semicircular canals and vestibule for cochlea preparation. The steps of this 13-day protocol comprised: fixation, microdissection, post-fixation, decalcification, pretreatment (signal enhancer, permeabilization and blocking), indirect immunolabeling, direct labeling, dehydration, clearing and mounting (Fig. 1). All steps were performed at room temperature under continuous gentle agitation (30 rpm), unless otherwise specified. Rinse steps comprised triple 15-min immersion in PBS 1 ×.

The round window membrane was opened and a small hole was pierced in the cochlear apex above the helicotrema with a fine needle (25G). After fixation with 4% PFA in PBS 1 × (slowly infused with a 1 mL syringe and 25G needle) the cochlea was microdissected under a dissecting microscope: i.e., resection of excess bone and the stapedial artery, stapes ablation, enlargement of the burr hole at the apex, and piercing another small hole in the basal turn of the cochlea to facilitate reagent circulation. Samples were post-fixed with PFA 4% in PBS 1 × at 4 °C for 24 hours. Decalcification was achieved by immersion in 10% EDTA in PBS 1 × (4 mL) at 4 °C for 7 days. Specimens were then stored in 70% ethanol at 4 °C until further use.

For pre-treatment, whole cochleae were rinsed, immersed in Image-iT fx solution for 30 minutes and then 3 times 30 min in PBS 1 × containing 0.1% Triton X-100, then incubated in a blocking solution (0.1% Triton X-100, 10% BSA in PBS 1 ×) for 2 hours. They were then exposed to a solution of primary antibodies for 3 days at 4 °C (rabbit polyclonal IgG anti-neurofilament 200 antibodies, diluted 1:80 in 1 mL blocking solution containing 20% BSA), rinsed, and then immersed in a solution of secondary antibodies for 12 hours at 4 °C in darkness (goat polyclonal IgG anti-rabbit IgG antibodies with Atto 633 fluorophore tag, diluted 1:200 in 1 mL blocking solution). Samples were rinsed and incubated in 0.25 μg/mL DAPI solution in PBS 1 × for 30 minutes in darkness, rinsed again, exposed to a 50 μg/mL phalloidin-TRITC solution in PBS 1 × for 30 minutes in darkness, and rinsed again. Then, cochleae were dehydrated with 3 two-hour incubations in incremental ethanol solutions (70%, 90%, and absolute) in darkness. Then, cochleae were transferred to a clearing solution of MSBB (5 parts methyl salicylate and 3 parts benzyl benzoate) diluted 1/1 with absolute ethanol for 4 hours. Then samples were transferred in successive baths of MSBB of 2, 4 and 12 hours in darkness. They were then mounted on μ-Slide 8-Well ibiTreat chambered coverslips (Ibidi, Planegg/Martinsried, Germany).

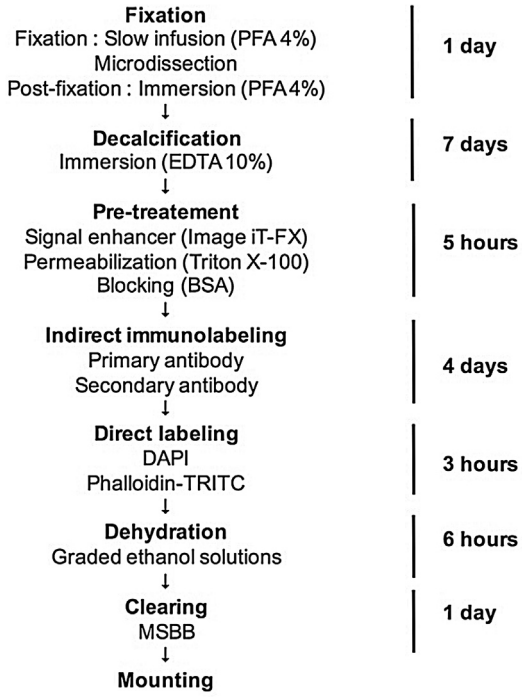

Fig. 1. Flowchart of the intact cochleae labeling and clearing protocol. PFA: paraformaldehyde; EDTA: ethylene diamine tetracetic acid-disodium salt; BSA: bovine serum albumin; DAPI: -4′,6-di-aminido-2-phenyl-indol; Phalloidin-TRITC: phalloidin-tetramethylrhodamine isothiocyanate; MSBB: methyl salycilate benzyl benzoate.

2.4. Laser Scanning Confocal Microscopy (LSCM)

Cleared cochleae were imaged on an LSM 710 inverted confocal microscope with an Objective EC Plan-Neofluar 10 ×/0.3 M27 lens (Carl Zeiss, Jena, Germany). Exposure and optical parameters were set following the manufacturer's recommendations. The laser sources (405 nm UV diode, 561 nm DPSS (diode-pumped solid-state) diode, and 633 nm helium-neon laser) excited DAPI, phalloidin-TRITC and Atto 633, respectively.

2.5. Image processing

3D reconstruction from the optical sections (Z-stacks) used LSM ZEN acquisition software (Carl Zeiss, Jena, Germany). The Z-stack images provided maximum-intensity projections (MIP). The resulting images were analyzed on ImageJ 1.48 v software (National Institutes of Health, Bethesda, MD, USA) using a cell-count plug-in. Organ of Corti lengths were measured and the inner

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