Three-dimensional guinea pig and pigeon inner ear reconstruction
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2009

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
The relationship of the round window membrane to the cochlear aqueduct shown in three-dimensional imaging

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Hear Res 2005, 209, 19-23
Abstract
The round window membrane and cochlear aqueduct complex in the guinea pig are reconstructed with 3D-imaging, using OPFOS (Orthogonal Plane Fluorescence Optical sectioning). The 3D-images show that the periotic duct and the aqueduct are connected to a pouch-like extension of the round window. The function of this may be regulation of aqueduct flow resistance under the influence of a pressure difference between inner ear fluid and middle ear.
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Introduction

The cochlear aqueduct is a narrow channel between the subarachnoid space and the inner ear. In the inner ear its opening is in scala tympani, close to the round window membrane (Ghiz et al., 2001). The cochlear aqueduct was first noticed by DuVerney (1683), and about one century later Cotugno (1761) described it in detail.

In humans it is about 1 cm long and has a smallest diameter of 0.14 mm. The aperture of the aqueduct at the dural end has the shape of a flat funnel (Gopen et al., 1997). In guinea pigs the aqueduct is much shorter (about 2 mm) but not narrower than in humans (Ghiz et al., 2001).

The cochlear aqueduct is defined as the bony duct passing through the temporal bone from the inner ear on the lateral side to the dura on the medial side. The periotic duct tissue within the lumen of the cochlear aqueduct consists of fibroblasts and loosely arranged connective tissue (Nishimura et al., 1981). Pressure and fluid exchange is possible through the periotic duct (Palva et al., 1969).

The round window membrane is fixated to the temporal bone. It has a pouch-like extension, just adjacent to the opening of the cochlear aqueduct. The membrane is a single cell-layer structure, while the extension is multi cell-layered, about 400 µm in length and 100 µm in diameter (Thalen, 2004). The cochlear aqueduct is thought to play a role in maintaining fluid and pressure balance between the inner ear and the cerebrospinal fluid (CSF). For sudden variations of CSF-pressure it behaves as a low-pass filter with a time constant of about 2 seconds in the guinea pig (Thalen et al., 2002).

Based on the finding that the resistance for fluid flow of the aqueduct is related to the position of the round window membrane, it is assumed that the permeability of the connective tissue (Duckert, 1974; Toriya et al., 1991a) inside the aqueduct changes if the position of the round window membrane changes (Wit et al., 2003). This assumption is supported by the fact that this connective tissue is attached to the pouch-like extension of the round window membrane (Toriya et al., 1991b).

In the present work three-dimensional (3D) reconstructions are presented of the membranous aqueduct and the round window membrane together, with the aim to give insight in the morphology of the round window membrane-cochlear aqueduct region of the inner ear.

Orthogonal-plane fluorescence optical sectioning (OPFOS) microscopy was used to obtain these 3D-reconstructions (Voie et al., 1993; Voie et al., 1995; Voie, 2002).

Materials and methods

One healthy female albino guinea pig (Harlan Laboratories, UK) weighing 450 g was used. Animal care and use were approved by the Experimental Animal Committee of Groningen University, protocol No. 2883, in accordance with the principles of the Declaration of Helsinki.
Fixation procedure

The animal was terminated by lethal administration of sodiumpentobarbital. After decapitation the bulla was dissected and fixated in a 8% formalin solution, neutral buffered. Then the bulla was rinsed in aqua-dest. Decalcification in ethylenediaminetetraacetic acid 10% solution (EDTA; Sigma, ED5SS, pH 7.4) took place at a temperature of 50°C in a microwave oven (T/T MEGA microwave histoprocessor, Milestone) in eight sessions of six hours. After decalcification the bulla was again rinsed with aqua-dest and dehydrated in a graded seven-step ethanol series (30%, 50%, 70%, 90%, 96%, 100%, 100%). Spalteholz fluid, a 5:3 solution of methyl salicylate (Sigma, M-6752) and benzyl benzoate (Sigma, B-6630) (Spalteholz, 1914), was thereafter used to achieve transparency of the specimen. The clearing process consisted of application of a succession of Spalteholz-ethanol solutions, 24 hours each. The Spalteholz fluid fraction in the clearing session was 25%, 50%, 75%, 100%, 100% respectively. Hereafter the specimen was dyed in a fluorescent dye bath of Rhodamine-B Isothiocyanate (RITC; Sigma, R-1755). RITC absorbs maximally at 570 nm and emits at 595 nm. The dye bath was prepared by dissolving 1.0 mg/ml RITC into ethanol, followed by dilution in Spalteholz fluid to a final dye concentration of 5 x 10\(^{-4}\) mg/ml (Voie et al., 1993, 2003). The specimen was dyed for four days.

OPFOS imaging system

An overall schematic of the OPFOS imaging system is given in figure 1. The beam of a green 2.0 mW He-Ne LASER (Research Electro Optics R-30972; wavelength 543 nm) is expanded into a 3cm diameter parallel bundle. The 30x beam expander consists of two independently positionable lenses with focal lengths of 0.7 cm and 20 cm respectively. A cylindrical lens (Newport Valumax PCX, f = 15 cm) focuses the bundle into a line at the centre of the specimen chamber.

![Figure 1. Schematic of the OPFOS setup.](image)
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The illuminated plane in the specimen is projected onto the CCD in the camera (Vosskühler CCD-1300D + IEEE1394 module) by an objective lens (Newport PAC 040 Valumax Achromatic Doublet; \( f = 5 \) cm). Magnification can be adjusted by changing object and image distance. Light intensity can be adjusted with an iris diaphragm (Newport M-ID-1.0). A bandpass filter (Chroma HQ 585/40) blocks scattered laser light (543 nm) from entering the camera and admits fluorescent light (595 nm).

Specimen chambers (2.5 x 2.5 x 7.5) were made of microscope glass slides glued together between anodised aluminium bottom and top covers. The specimen is vertically suspended from a metal wire and can be rotated. The chamber is placed on a motorised linear stage (Newport M-MFN 25 PP), controlled by a Newport ESP-100 driver unit. Photographs of the illuminate plane were taken at 10 \( \mu \)m steps and stored in a personal computer.

Two-dimensional stored images were processed with an IMOD (http://bio3d.colorado.edu/imod) for Linux software package for 3D-reconstruction. Input of relevant contours in each 2D-image was manually performed with a writing tablet (Wacom Cintiq 15X).

Results

Figure 2 shows an example of the 2D-raw data obtained with the OPFOS technique. Figure 3 shows the resulting 3D-reconstruction, as seen from opposite viewing points, of the round window-cochlear aqueduct complex. The distance from medial to lateral is 3.2 mm. For comparison the 3D-images are combined with a photograph of the same structures, obtained with light microscopy in an earlier study (Laurens-Thalen, 2004).

Discussion

In this work we show a 3D-reconstruction of the round window-cochlear aqueduct-complex obtained in one guinea pig. These results are not essentially different from those of pilot studies in other guinea pigs. The inner ear is complicated in anatomy. 3D-reconstruction of the inner ear can give a better understanding of relations between various specific parts of the inner ear.

For 3D-reconstruction OPFOS has the advantage over reconstruction from histological slides that no problems are encountered due to distortions induced by the passage of the microtome blade or to problems with restoring the exact superposition of the 2D-images. The spatial resolution of OPFOS (about 20 \( \mu \)m; Voie, 2002) is sufficient for the present purpose. Beside this, the specimen can be illuminated in various directions by turning the specimen suspension wire.
Figure 2.
B. Same image with the contours, manually drawn to calculate the 3D-reconstruction. Colours are the same as in figure 3.
Figure 3.
B. Medial view on the cochlear aqueduct.
If structures are difficult to decalcify completely, or if partly transparent soft tissue can not be removed, the OPFOS-technique has the limitation that the specimen can not be illuminated completely. The 3D-reconstructions show clearly that the round window membrane of the guinea pig has a pouch-like extension, as suggested before (Wit et al., 2003; Toriya et al., 1991b). The tip of the membrane extension is adjacent to the opening of the cochlear aqueduct (Wit et al., 2003). It is conceivable that the entrance of the aqueduct is obstructed when the inner ear fluid pressure is low and the membrane is moving inwards (with a resulting high flow resistance in the cochlear aqueduct). Vice versa the loose connective tissue inside the aqueduct is stretched when the round window membrane moves outward, resulting in a low flow resistance (Wit et al., 2003). This view is supported by the morphology derived from the 3D reconstructions as shown in this paper. It sustains the findings that the resistance for fluid flow through the aqueduct is dependent on the position of the round window membrane (Feyen et al., 2004).
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