Three-dimensional guinea pig and pigeon inner ear reconstruction

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Three-dimensional reconstruction of the guinea pig inner ear; comparison of OPFOS and light microscopy; applications of 3D reconstruction

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Abstract
Three-dimensional (3D) reconstruction of anatomical structures can give additional insight in morphology and function of these structures. We compare 3D-reconstructions of the guinea pig inner ear, using light microscopy (LM) and Orthogonal Plane Fluorescence Optical Sectioning (OPFOS) microscopy. Applications of 3D-reconstruction of the inner ear are further explored.
For each method two bullas were prepared for 3D-reconstruction. Both methods are explained. In general the 3D-reconstructions using OPFOS-microscopy are superior to LM.
The exact spiral shape of the cochlea could be reconstructed using OPFOS microscopy and the length of the basilar membrane is measured.
When a resolution of 20 μm is sufficient, OPFOS microscopy is a superior technique for 3D-reconstruction of inner ear structures in animals.
**Introduction**

Three dimensional (3D) reconstruction of the inner ear, giving additional insight in anatomical details, has proven to be useful in morphological and physiological research. Also, inner ear abnormalities can be better investigated in 3D reconstructions than in 2D images (Isono et al., 1997). Extensive 3D-reconstructions of parts of the middle and inner ear have been made using various methods; histological sectioning (Harada et al., 1990; Takahashi et al., 1990; Wada et al., 1998); OPFOS (Voie et al., 1995; Santi, 2002; Hofman et al., 2005, 2008), CT-scanning (Van Spaendonck et al. 2000; Decraemer et al., 2003) and magnetic resonance microscopy (MRM) (Henson et al., 1994; Wilson et al., 1996; Thorne et al., 1999).

Orthogonal-plane fluorescence optical sectioning (OPFOS) is a whole-specimen imaging technique, developed by Voie et al. (1993) to investigate the cochlear architecture. This method turned out to be very suitable for 3D-reconstruction. In OPFOS the specimen can be illuminated in various directions by rotating the specimen. Different series of 2D-images of the same specimen can be made for different orientations of the illuminating beam. This is an advantage compared to the use of histological slides for light microscopy (LM), where the microtome cutting plane is predefined.

In the present study, 3D-reconstructions of the whole vestibular system of the guinea pig, obtained with LM and OPFOS-microscopy, are compared and applications of 3D-reconstruction of the inner ear are further explored.

**Materials and methods**

Two healthy female albino guinea pigs, *Cavia Porcellus* (Harlan Laboratories, UK) weighing 450 g were 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. One guinea pig was used for LM 3D-reconstruction, one for OPFOS 3D-reconstruction.

**Fixation procedure**

The animals were terminated by lethal administration of sodium pentobarbital (hospital farmacist). After decapitation the bullas were dissected and fixated by immersion in a 8% formalin solution, neutral buffered. Then the bullas were rinsed in aqua-dest (Millipore Direct Q 3 UV system). 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 bullas were again rinsed with aqua-dest and dehydrated in a graded seven-step ethanol series (30%, 50%, 70%, 90%, 96%, 100%, and 100%). No difficulties caused by intransparent haemoglobin were encountered, because very little is present in the inner ear capillaries.
**Sequel procedure for LM**

Two bullas were placed in ethanol:2-Hydroxypropyl Methacrylate (HPMA; Polysciences Inc.) (1:1) for 8 hrs. Finally the bullas were embedded in HPMA with addition of a catalyst (N,N-dimethylaniline, PEG 400; 15:1, Fluka Chemie AG). Around the bullas five holes (fiducial markers) were drilled (diameter 0.5 mm) in the HPMA-block, perpendicular to the surface. The holes are references for 3D reconstruction. Sections of 4µm were taken and each 5th section was stained with toluidine blue and contrast-stained with basic fuchsin for reconstruction. Sectioning was not automated (Microm microtome, Heidelberg). A total of 264 sections was used for 3-D reconstruction.

**Sequel procedure for OPFOS**

The other two bullas were placed in Spalteholz fluid, a 5:3 solution of methyl salicylate (Sigma, M-6752) and benzyl benzoate (Sigma, B-6630) (Spalteholz, 1914), to achieve transparency of the specimens. 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 specimens were dyed in a fluorescent dye bath of Rhodamine-B Isothiocyanate (RITC; Sigma, R-1755). RITC absorbs maximally at 570nm and emits at 595nm. 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, 2002, 2003). The specimens were dyed for four days.

**OPFOS imaging system**

A schematic of the OPFOS imaging system is given in figure 1. The beam of a green 100mW Ventus type 532 pulsed diode LASER (wavelength 532nm) is expanded into a 3 cm diameter parallel bundle. The 30x beam expander consists of two independently positionable lenses with focal lengths of 0.7cm and 20cm respectively. A cylindrical lens (Newport Valumax PCX, f = 15cm) focuses the bundle into a line at the centre of the specimen chamber. The illuminated plane in the specimen is projected onto the charge-coupled device (CCD) in the camera (Vosskühler CCD-1300D + IEEE1394 module) by an objective lens (Newport PAC 040 Valumax Achromatic Doublet; f = 5cm). 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 (532nm) 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.
Pictures were taken every 20 µm of the illuminated plane and stored in a personal computer. In total 278 images were used for reconstruction.

Two-dimensional stored images (LM and OPFOS) were processed with an IMOD (http://bio3d.colorado.edu/imod) software package for 3D-reconstruction (Kremer et al., 1996). Input of relevant contours in each 2D-image was manually performed with a writing tablet (Wacom Cintiq 15X). Stacking of the LM-slides was executed with MIDAS software for manual alignment and adjustment (MIDAS is part of IMOD), using the drilled fiducial markers as references. Corrections to be made were at most a few percent of the 2D-image cross section. If after 3D-reconstruction larger irregularities were observed, the MIDAS procedure was repeated for the relevant subsequent 2D-slides.

Figure 1. Schematic of the OPFOS setup.
Results

Figure 2 shows a 2D-image of the endolymphatic system obtained with LM. Figure 3A shows a 2D-image of the endolymphatic system obtained with the OPFOS technique. Figure 3B shows an inverted image of the endolymphatic system obtained with the OPFOS technique, in which the contours of the endolymphatic space are drawn. Figures 4A, B and 5A, B show respectively the resulting LM- and OPFOS-reconstructions of the guinea pig inner ear. The vestibular space, endolymphatic space, tympanic space and stapes are reconstructed. In the OPFOS-reconstruction the endolymphatic sac and part of the endolymphatic duct are not depicted, because the anatomical integrity of the intradural part of the duct and sac is disrupted during the (microscopic) dissecting process, removing all neural tissue. Figure 6 is a 3D-reconstruction from OPFOS images, showing the endolymphatic spaces of the inner ear. Figure 7 shows the area around the vestibular maculas in detail.

X-Y-Z-coordinates of the midpoints of the basilar membrane (fig. 3B) were obtained with the IMOD software package and used to establish the shape of the cochlear spiral (figs. 8A, B, C) and the length of the basilar membrane (20.8 mm), from apex to base.

Figure 2. 2D-image of the cochlear endolymphatic system of the guinea pig made with LM. ES: endolymphatic space, TS: tympanic space, VS: vestibular space. The asterix indicates the rim of one of the drilled reference holes.

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Figure 3.
A. 2D-image of the cochlear endolymphatic system of the guinea pig made with OPFOS. ES: endolymphatic space, TS: tympanic space, VS: vestibular space.
B. Figure 3A inverted. Coloured lines indicate the contours of the inner ear fluid spaces (blue: vestibular space, green: endolymphatic space, red: tympanic space), as they were drawn for the 3-D reconstruction. The white dot indicates the midpoint of the basilar membrane.
Figure 4.

A. Ventral view on 3D-reconstruction of the guinea pig inner ear made with LM (light blue: vestibular space, green: endolymphatic space, purple: tympanic space, dark blue: stapes, red: endolymphatic sac, transparent yellow; perilymphatic space).

B. Dorsal view on 3D-reconstruction of the guinea pig inner ear made with LM (light blue: vestibular space, green: endolymphatic space, purple: tympanic space, red: endolymphatic duct and sac, transparent yellow; perilymphatic space, S: saccule, U: utricle, ED: endolymphatic duct, DR: ductus reuniens).
Figure 5.

A. Ventral view on 3D-reconstruction of the guinea pig inner ear made with OPFOS (light blue: vestibular space, green: endolympathic space, purple: tympanic space, dark blue: stapes, transparent yellow; perilymphatic space).

B. Dorsal view on 3D-reconstruction of the guinea pig inner ear made with OPFOS (light blue: vestibular space, green: endolympathic space, purple: tympanic space, transparent yellow; perilymphatic space, S: saccule, U: utricle, ED: first part of endolympathic duct).
Figure 6. 3D-reconstruction of the endolympathic spaces of the guinea pig inner ear made with OPFOS, showing the spiral configuration of scala media and both vestibular maculas located in perpendicular planes. (SM: saccular macula, UM: utricular macula).

Figure 7. 3D-reconstruction made with OPFOS of a small part of the endolympathic spaces shown in figure 6A. (ED: endolympathic duct, SM: saccular macula, SS: saccular space, UD: utricular duct, UM: utricular macula, US: utricular space).
Figure 8.

A. 3D-plot of the spiral shape of scala media in the guinea pig cochlea, showing its actual size. The plot was obtained by connecting the midpoints of the basilar membrane in subsequent 2D-OPFOS-sections, as shown in figure 3B.

B. Projections of the spiral shown in figure 8A on the XZ- and on the YZ-plane.

C. Projection of the spiral shown in figure 8A on the XY-plane, with its (3D) length measured from top to bottom.
Discussion

Recently good quality 3D-reconstructions of the guinea pig cochlea were published by Liu et al. (2007). These authors used a “photographing while slicing” technique to obtain 2D-images of the cochlea.

As far as we know our figures 4 and 5 are the first good quality 3D-reconstructions of the entire guinea pig inner ear, showing the both cochlea and the vestibular system in their mutual dimensions and orientation.

The reconstructions (figs. 4A and 4B, compared to 5A and 5B) clearly show the better quality of the OPFOS 3D-reconstructions, when about the same number of 2D-slides is used for LM- and OPFOS-reconstructions. Three-dimensional reconstructions with LM are less smooth, due to distortions introduced during material preparation and preparation of the LM-slides. Although the influence of these distortions could be minimized by using the MIDAS alignment and adjustment procedure, small irregularities remain visible in the LM 3D-reconstructions.

Besides this, LM is more time consuming than OPFOS. Serial sectioning and especially superposition of the obtained slides, takes much time in LM. It is not unlikely that the smoothness of the LM-reconstructions could have been improved if more slides were taken (better resolution in the Z-direction), but than the LM-reconstructions would have been even more time consuming.

The guinea pig inner ear is very suitable for the OPFOS technique since all soft tissue around the bulla is completely removable and the bulla itself is relatively easy to decalcify. In other animals, e.g. birds, soft tissue in the proximity of the inner ear (neural tissue) is difficult to dissect without damaging the inner ear.

Henson et al. (1994) mention distortion and shrinkage of the specimen due to hydration as a disadvantage of microscopic techniques other than MRM. This should be kept in mind when absolute dimensions of dehydrated specimens are considered.

With “standard” OPFOS spatial resolution is limited down to around 20 µm (Voie et al., 1993), which is the minimal “thickness” of the plane-focussed laser bundle. This number can be improved to 2 µm with high resolution (HR) OPFOS (Buytaert et al., 2007). Magnetic resonance imaging (MRI), used to measure cochlear fluid space dimensions, has a resolution of 25-27 µm (Thorne et al., 1999; Pettit et al., 2002). In comparison: limited by the wavelength of visible light, the highest resolution that can be obtained with LM is 0.2 µm, which is one to two orders of magnitude better. (When comparing figures 2 and 3a, b it is immediately clear that the X-Y resolution of LM is much better than that of standard OPFOS.)

Wilbrand et al. (1986) produced plastic mouldings of the human inner ear to study the complex anatomy of the temporal bone. Although this method shows the exact topographical relations between the different parts of the inner ear, its resolution is limited to an estimated 100 µm, and the method does not give information about the dimensions of the different fluid compartments.
In stead of the 1.25mW helium-neon LASER (wavelength 543.5 nm) used by Voie et al. (1993), we used a 100 mW pulsed diode LASER (wavelength 532 nm), because of its limited size and its higher and adjustable intensity. Unfortunately, three dimensional reconstructions of the endolymphatic duct, joining the vestibular endolymphatic spaces with the endolymphatic sac, can not be made with the OPFOS technique, because all neural tissue has to be removed by dissection to obtain a fully transparent specimen. Since the endolymphatic sac is located between two layers of the dura mater, it is impossible to keep the sac in its original shape and position when dissected. For this reason we only used LM slides for 3D reconstructions of the endolymphatic sac.

Shinomori et al. (2001) found a cochlear length of 18.17mm (±0.15) for the guinea pig, measured along the apical junctions of the inner and outer pillar cells. This number is close to the value given by Fernandez (1952), determined from rectangular mid-modiolar histological sections across the cochlea. He found a basilar membrane length of 18.8mm (±0.5), measured along points right under the tunnel of Corti. Both the numbers given by Shinomori et al. and by Fernandez are based on histological serial sectioning (LM) of the guinea pig inner ear. Our figure 8C, obtained with OPFOS, gives a 10% larger value for the length of the basilar membrane, measured along points located approximately halfway the basilar membrane (see fig. 3B). More research is needed to decide if this length difference is based on the different techniques to obtain it (LM vs. OPFOS).

OPFOS-microscopy is relatively inexpensive and little time consuming. Once a specimen has been prepared, it can be used over and over again, although due to photo bleaching the fluorescence of the specimen will gradually decrease in a few months time.

Three dimensional reconstructions of the guinea pig inner ear can give a better insight in the relative dimensions and positions of the different parts of the inner ear with respect to each other (figs. 6 and 7).

Three dimensional reconstruction also proved to be useful in obtaining insight in a possible function of Bast's valve (Hofman et al., 2008) and the round window membrane (Hofman et al., 2005) in the inner pressure dependent regulation of fluid transport through the cochlear aqueduct (Feijen et al., 2004).

Conclusion
When a resolution of 20 µm is sufficient, OPFOS microscopy is a superior technique for 3D-reconstruction of inner ear structures in animals.
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