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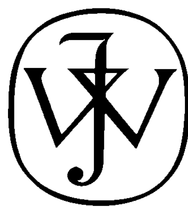

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Visualizing Soft Tissue in the Mammalian Cochlea With Coherent Hard X-Rays

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KEY WORDS hard X-rays; high-resolution imaging; coherence; synchrotron radiation; cochlea; in-line phase contrast; microscopy

ABSTRACT This paper concerns an important aspect of current developments in medical and biological imaging: the possibility for imaging soft tissue at relatively high resolution in the micrometer range or better, without tedious and/or entirely destructive sample preparation. Structures with low absorption contrast have been visualized using in-line phase contrast imaging. The experiments have been performed at the Advanced Photon Source, a third generation source of synchrotron radiation. The source provides highly coherent X-ray radiation with high photon flux ($>10^{14}$ photons/s) at high photon energies (5–70 keV). Thick gerbil cochlear slices have been imaged and were compared with those obtained by light microscopy. Furthermore, intact gerbil cochleae have been imaged to identify the soft tissue structures involved in the hearing process. The present experimental approach was essential for visualizing the inner ear structures involved in the hearing process in an intact cochlea. *Microsc. Res. Tech. 69:000–000, 2006.* © 2006 Wiley-Liss, Inc.

INTRODUCTION

It is important to examine cochlear morphology from many points of view, including comparative anatomy, cochlear developmental changes, malformation caused by genetic defects, changes related to diseases, sensory physiology, and cochlear modeling. Best imaging results were obtained from in situ experiments in the absence of tissue distortion. However, most contemporary imaging methods require invasive specimen preparation and/or are time consuming, or lack sufficient spatial resolution. A method that potentially overcomes these shortcomings is presented in this paper and it uses hard X-ray in-line phase contrast imaging. The cochlea is a particularly challenging system because it consists of bony and soft tissues, which are intimately connected. Bone has strong amplitude contrast, which tends to conceal the anatomy of the adjacent soft tissue.

A common method to investigate cochlear morphology is to fix, decalcify, dehydrate, and embed the tissue. After embedding the cochlea in plastic, paraffin, or sucrose, the specimen is serial-sectioned and placed on glass slides for staining. The procedures are extremely labor intensive and may take several weeks or months. Moreover, tissue dehydration is accompanied with tissue shrinkage and distortion. According to the literature, tissue shrinkage is expected to be 5–25% (Edge et al., 1998). In addition to the "classical" histological techniques, methods have been developed to examine fresh, undisturbed tissue. For example, Lim (1980) studied acutely isolated tectorial membranes from chinchilla. More recently, a different method the hemicochlea was introduced to study fresh, unfixed, and hydrated gerbil cochlear tissue (Edge et al., 1998; Hu et al., 1995; Richter et al., 1998, 2000).

Distortion of the tectorial membrane and the basilar membrane caused by dehydration are generally obvious. The importance of visualizing cochlear tissue in pristine condition is particularly important for studying cochlear mechanics (for a review, see Robles and Ruggero, 2001). Opening the cochlear wall frequently results in either cochlear damage or leads to imaging artifacts (Cooper, 1999; Cooper and Rhode, 1996). To study the micromechanics of the organ of Corti, confocal microscopy has been used (Fridberger et al., 2002). However, the experiments using confocal microscopy also required opening the cochlea and injecting fluorescent dyes.

In addition to classical histology, methods are available that allow visualization of the undisturbed cochlea. High-resolution magnetic resonance microscopy and computer microtomography are powerful tools to study living and preserved tissue (Vogel, 1999). Nuclear magnetic resonance (NMR) studies were reported with a voxel size of 25 μm^3 (Salt et al., 1995; Vogel, 1999) and an intact mammalian cochlea was studied by orthogonal-plane fluorescence optical sectioning with a 16-μm^3 voxel resolution (Voie, 2002). The resolution with these methods is limited when compared to that with light microscopy. The main disadvantage of the aforementioned methods is that they either suffer from poor spatial resolution or they require strong sample modifications prior to imaging.

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Imaging with hard X-rays is an elegant way to overcome the difficulties described earlier. In recent years, important progress has been made in developing X-ray microtomography. To date, table-top systems for biomedical applications, with resolution in the 10 μm range are commercially available (Van Spaendonck et al., 2000). Hard X-ray imaging with micrometer resolution using synchrotron radiation has become a well-established technique.

When imaging soft tissue or very small objects, the contrast due to the absorption of X-ray photons is unfortunately very weak. However, the phase shift that occurs when a light wave propagates through material of different optical density is still significant. Phase contrast imaging methods depend on the transformation of phase information into amplitude information, which becomes measurable with a detector. The contrast of interfering light waves can be tuned and increased (e.g., Zernike phase contrast) to further reduce the necessary radiant exposure. Phase-contrast imaging is fundamentally dose-efficient because the underlying physical principle does not rely on the radiation dose deposited in the tissue. The advantage of phase contrast imaging technologies over methods using absorption contrast becomes progressively greater with increasing photon energy.

Phase contrast techniques have been developed (Fitzgerald, 2000; Momose, 2003) using X-ray interferometers (Beckmann et al., 1997; Bonse and Hart, 1965; Momose et al., 1996), analyzer crystals (Chapman et al., 1997; Davis et al., 1995), or wave propagation techniques (Cloetens et al., 1999; Snigirev et al., 1995; Wilkins et al., 1996). Third generation synchrotron sources are optimized for performing X-ray science. The high coherence of the radiation allows applying in-line phase contrast imaging. Weak phase-shifting features of soft tissue can be visualized by edge enhancement. The plane wave is diffracted by the sample and discontinuities create fringes around the features. By changing the distance between the sample and detector, the contrast of the fringes can be modulated. The experimental setup is simple and straightforward, providing an extremely powerful imaging method.

Moreover, at high photon energies the object's integrity and functionality can be preserved because of the high penetration depth of the radiation. Sample environments can be built specifically for the in situ experimental requirements. Dynamic studies of the cochlea are feasible because of the source's high photon flux ($>10^{14}$). This study extends previous reports on in-line phase contrast soft tissue imaging with hard X-rays (Snigirev et al., 1995; Spanne et al., 1999). For example, frog muscle structures with 2 μm periodicity were imaged at 20 keV photon energy (Kohn et al., 2005).

Important progress in X-ray imaging technology relates to the development of the X-ray microscopes, which are equipped with both X-ray and visible-light optics to work as microscopes in both wavelength ranges. In the soft X-ray regime slices of a cochlea have been resolved with a resolution of 40 nm at a photon energy of 554 eV (Johansson et al., 2004). Again, the tissue was dissected for imaging. The low energy X-ray radiation is absorbed by the cochlear walls and it was not possible to image intracochlear structures without removing the bony shell. Recently, a hard X-ray microscope for sub-100 nm resolution imaging has been built at our beamline (Rau et al., 2004, 2005). It will be used in our future studies.

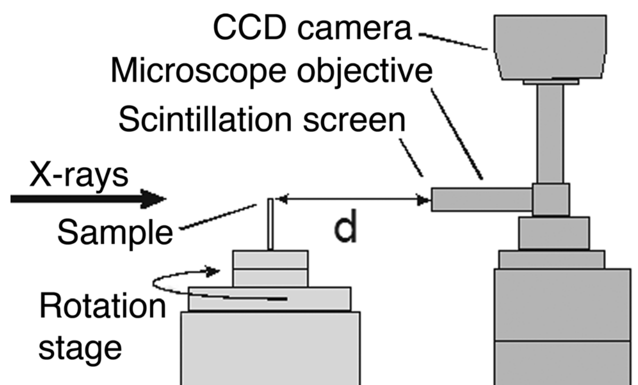

Fig. 1. The detector consists of a CCD camera system, coupled via a microscope to a scintillation screen. By changing the distance *d* between sample and detector, the features of weak absorption contrast can be rendered visible (in-line phase contrast). This is due to the highly coherent radiation of the source.

METHODS

Experiments were made at the Advanced Photon Source (APS), Argonne National Laboratory. The APS is a synchrotron radiation source of the third generation, whose particular characteristic is the highly coherent X-ray radiation. X-rays are generated with an undulator, inserted in a straight section of the storage ring. The full width half maximum (FWHM) source size is 600 by 40 μm (horizontal × vertical) and the beam divergence is 40 by 12 μrad (horizontal × vertical). The energy spectrum of undulator radiation consists of a series of equidistant, relatively narrow ($\Delta E/E = 10^{-2}$) peaks: the undulator harmonics. At 34 ID the beam is shared between two different hutches (Benson and Robinson, 2000). When the settings of the undulator are controlled by the 34 ID-E station, this operation mode is called "parasitic mode." The beam splitting is realized by inserting a platinum-coated silicon single-crystal mirror into the beam, deflecting most of the central cone of the X-ray beam to the C hutch. The liquid nitrogen-cooled mirror rejects higher undulator harmonics above 15 keV at an incidence angle of 5 mrad. The fixed-exit double crystal monochromator is water-cooled and yields an energy bandwidth of $\Delta E/E = 10^{-4}$ using Si (111) crystals over an energy range of 6–30 keV. Optionally, the monochromator can be removed for experiments with pink beam. Pink beam is a single undulator harmonic, cut out of the entire radiation spectrum. The lower part of the energy spectrum is filtered out by the reflecting mirror and the higher part is removed by filter or air absorption. Pink beam is 50–100 times more intense than monochromatic light in a broader energy bandwidth ($\Delta E/E = 10^{-2}$) (Rau et al., 2002).

The beam size is $\sim$1 mm^2 at a distance of 55 m from the source and the monochromatic flux is about 10^{13} photons/s.

The imaging setup was at 55 m from the source, consisting of a sample stage and a CCD-based detector sys-

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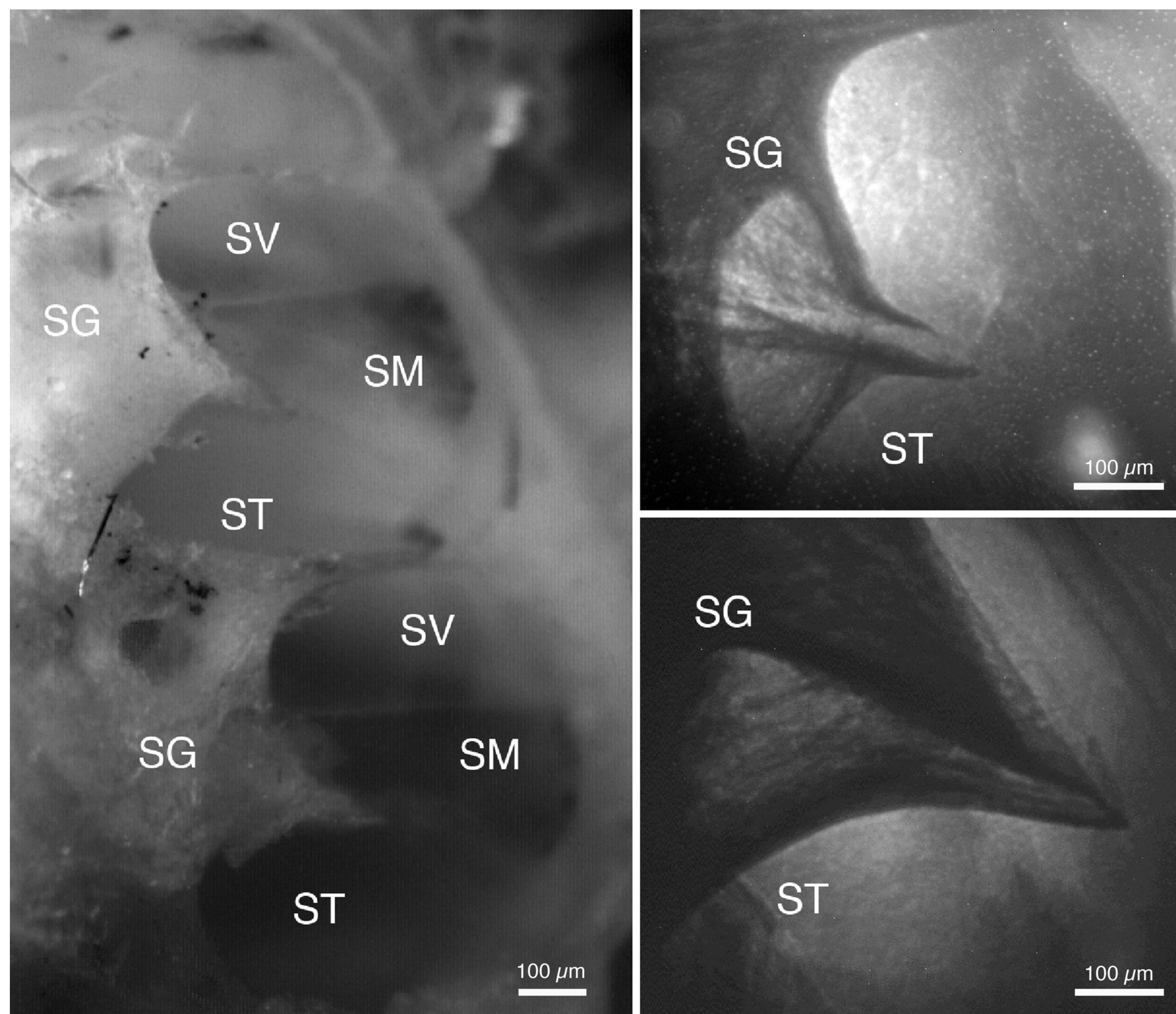

Fig. 2. Gerbil cochlear cross section captured with a light microscope after cutting the cochlea along its mid modiolar plane (left column). Similar cross sections captured with hard X-rays while the cochlea is intact (right column). The labeled structures are the spiral ganglion (SG), scala tympani (ST), scala media (SM), and scala vestibuli (SV). Dark regions correspond to low intensities.

tem both placed on a vibration isolation table. The distance between sample stage and detector can be easily selected for absorption or in-line phase contrast imaging or both. For this study, all images were captured at a distance $d = 40$ mm (Fig. 1). All alignment stages are fully motorized and are also chosen to meet the requirements for high-resolution tomography. The camera system consists of a scintillation screen coupled via an optical microscope to a CCD detector (Fig. 1). The scintillation screen transforms the X-rays into visible light, which was then projected with an optical microscope onto the chip of a CCD camera (Kodak KX2e). The camera has a 14-bit resolution with a 1560×1024 pixel array and 9×9 μm^2 size of each pixel. Using a $20\times$ objective lens (Mitutoyo) the effective pixel size on the screen is 0.45 μm. The scintillation screen was a Yttrium Aluminum Garnet single crystal with an 6-μm-thick europium-doped active layer. With the above elements, the camera system provided a resolution of 2.4 μm. The resolution was determined by taking the FWHM of the line-spread function, which was measured using a sharp edge. The exposure time to capture the images was between 4–5 s. For all projections, additional images were taken: the "flat field" beam profile (without the sample) and a dark count image of the CCD without X-rays on the detector system (dark field). Inhomogeneities of the beam profile were corrected with these additional images.

F1

Light microscopic images were captured with an upright Leitz microscope (Medilux) equipped with 10, 20, and $40\times$ Olympus water-immersion lenses, an optivar ($1\times - 2\times$), an epi-illuminator, and a trinoc viewing head. Pictures of the cochlea preparation were taken with a CCD camera (Pixera, Model PVC 100C), which

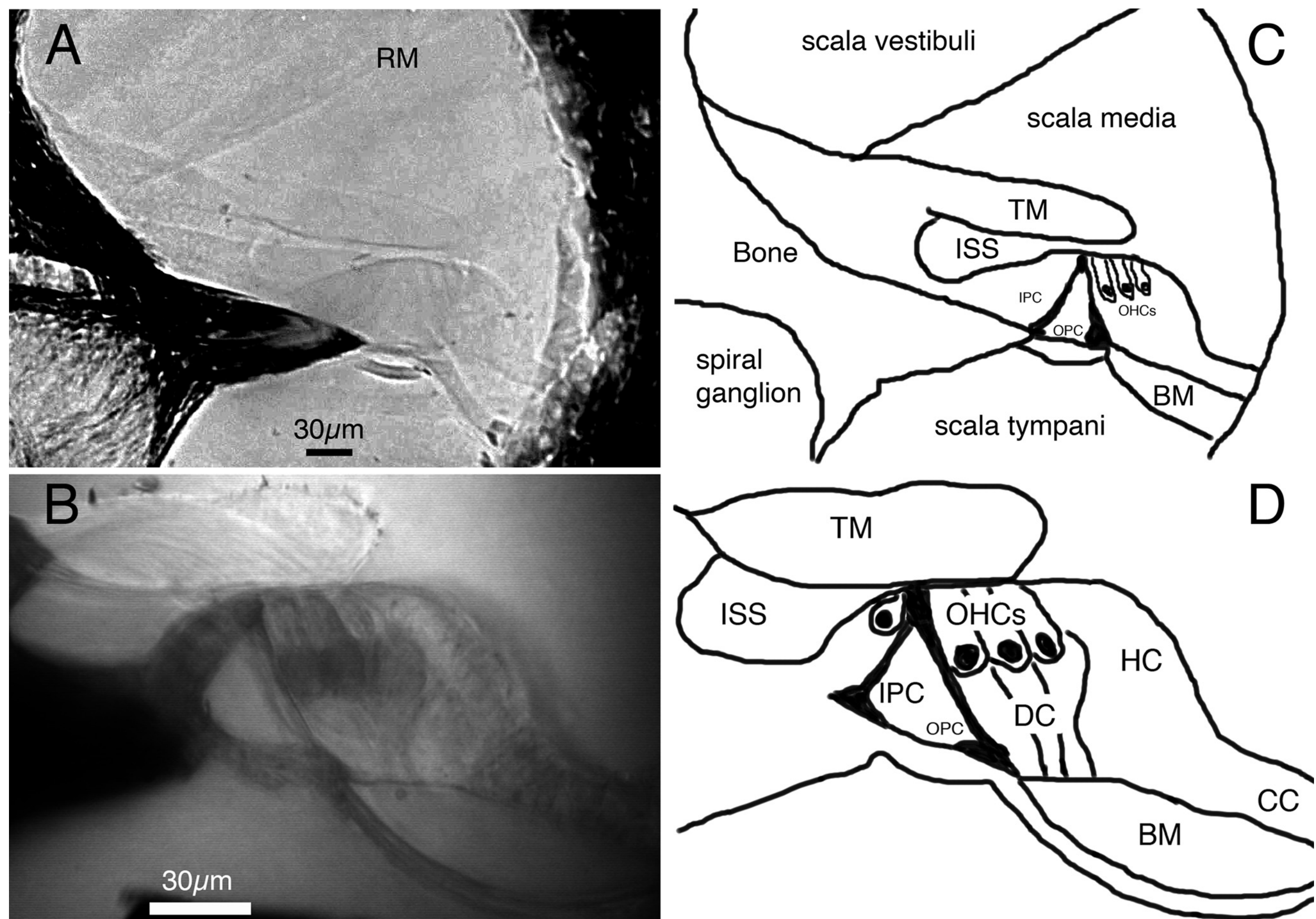

Fig. 3. Soft cochlear tissue structures are shown with hard X-rays (**A**) and light microscopy (**B**). Structures are as shown in the sketch (**C** and **D**). The following soft tissue structures can be identified: outer pillar cells (OPCs), inner pillar cells (IPCs), inner hair cells (IHCs), the inner spiral sulcus (ISS), outer hair cells (OHCs), Hensen's cells (HCs), Deiters' cells (DCs), Claudius cells (CCs), the tectorial membrane (TM), the basilar membrane pectinate zone (BM), and the Reissner's membrane (RM). Dark regions correspond to low intensities.

was connected to one port of the viewing head. The cochlear slice was attached with high vacuum grease (Dow Corning Corp., Midland, MI) to the bottom of a Petri dish on the stage of the microscope and was entirely immersed in Hank's balanced salt solution (HBSS). Despite most of the organ of Corti tissues being light transparent, different translucent structures still could be visualized by oblique illumination (Edge et al., 1998; Kachar, 1985; Richter et al., 1998). In the present experiments the light source was a group of five bright red-light emitting diodes (LEDs) arranged below the microscope's condenser. The position of these diodes was off the optical axis so that the preparation could be illuminated by oblique light.

Hemicochleae and cochlear slices were made as follows. After an intraperitoneal injection of a lethal dose of sodium pentobarbital (180 mg/kg body weight), gerbils were killed. Following decapitation, the head was divided in the medial plane and the bullae were removed. Next, one of the bullae was opened, the cochlea was exposed, and was placed in phosphate buffered saline (0.1 M, pH 7.4) containing 4% paraformaldehyde. After 4 h, the cochlea was transferred into phosphate buffered saline. After fixation of the bony inner ear with acrylic (QuickTite®, Locitite North America, Rocky Hill, CT) to a metal block, the cochlea was cut along its mid modiolar plane into two parts. One of the resulting hemicochleae was used for imaging. In some cases, a second cut was made to produce a thick (>400 μm) cochlear slice. Again, the slice was used for imaging.

RESULTS

Figure 2 shows the comparison of a radiograph (right) and an image obtained with the light microscope (left). The X-ray images (right) were obtained at 9 keV photon energy in an intact cochlea. The left image was obtained from a hemicochlea, which is a cochlea cut in two along its mid modiolar plane. Two turns can be seen. Corresponding structures are labeled in both images. The cochlear images in Figure 2 were taken using different specimens. F2

With higher magnification and appropriate orientation several soft tissue structures, such as Reissner's membrane (RM), tectorial membrane (TM), basilar membrane (BM), inner and outer pillar cells (IPCs and OPCs), supporting cells, and outer hair cells (OHCs), can be identified (Fig. 3). In Figure 3, panel A shows F3

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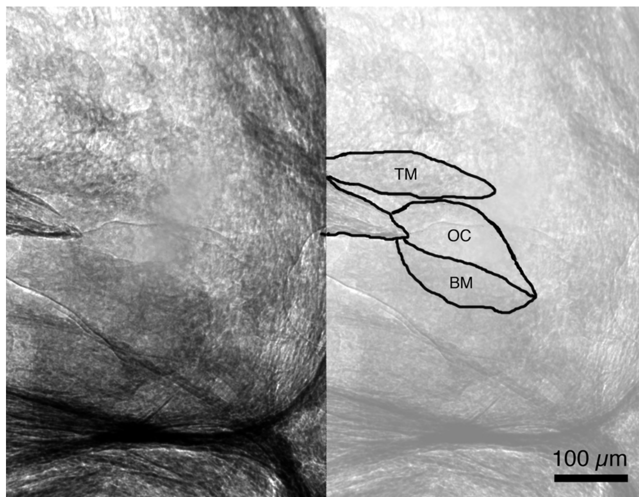

Fig. 4. When largely ossified structures impede the view, fine structures cannot be identified. The outline of the tectorial membrane (TM), the organ of Corti (OC), and the basilar membrane (BM) can still be seen. Further improvements, such as partial tomography, will be used in the future to improve the image quality. Both panels show the same image, the right one with an overlay of the outlines of anatomical details. In particular, studies of cochlear morphology and cochlear physiology benefit from a noninvasive method to visualize tissue. Hard X-ray phase-contrast imaging provides such information.

the X-ray image of a thick (>400 μm) cochlear slice and panel B the light microscopic image of a hemicochlea. At 9 keV bone absorbs X-rays strongly. The edge enhancement of translucent structures such as the basilar membrane, tectorial membrane, or the organ of Corti is caused by the in-line phase contrast.

F4

Figure 4 shows the X-ray image of an entire cochlea captured at 13.8 keV photon energy. This figure represents our first attempt to image the organ of Corti through intact bone. The outline of the tectorial membrane can be identified, however with much more difficulty because the projection of structures in the bony wall overlies the cochlear structure image. The thickness of the gerbil otic capsule is ~85 to 125 μm (Kumar, 2003). For future experiments, the membrane will be revealed without the bony background structure, using microtomography.

DISCUSSION

Soft tissue in a mammalian cochlea has been visualized with hard X-rays. Structures on the micrometer length scale with weak absorption contrast have been imaged using in-line phase contrast. The comparison of radiographs of thick cochlear slices with light microscopic images makes possible the visualization of essential structures involved in the hearing process. An intact cochlea was imaged at 13.8 keV photon energy. The identification of details is difficult but will become clearer in the future by applying tomography. Because of the high intensity of the synchrotron radiation, it will be possible to make dynamic studies on the cochlea. The present approach has permitted the study on soft cochlear tissue at a high spatial resolution without opening the cochlea. Thus, hard X-rays provide an advantage over conventional histological techniques, which can provide good spatial resolution but require dehydration and embedding of the tissue. Hard X-ray phase contrast imaging is advantageous over NMR because of its better spatial resolution. The spatial resolution in the present experiment is in the micrometer range and will be below 100 nm when using the hard X-ray microscope becomes functional. Furthermore, dynamic experiments are enabled with this technique because acquisition times (typically seconds) for capturing high-resolution images with hard X-rays are much shorter when compared with capturing NMR images (several minutes to hours).

CONCLUSION

In the present study using in-line phase contrast, an X-ray imaging method developed over the past 10 years was applied to the mammalian cochlea, a system of genuine medical interest. The results are a demonstration of how X-ray phase contrast can complement other imaging methods used to investigate the inner ear.

In particular, it has been shown that soft-tissue parts of the cochlea, inaccessible to conventional amplitude contrast X-ray imaging, can be seen quite well using phase contrast. This finding is new for the cochlea, and relevant to studies of other organs as well. The comparison with light microscopy images of sectioned samples not only proves the validity of the approach, but it also emphasizes some of the advantages of the new method over conventional techniques.

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