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DEMONSTRATION OF THE FINE STRUCTURE OF STEREOCILIA IN THE ORGAN OF CORTI OF THE GUINEA PIG BY FIELD EMISSION SCANNING ELECTRON MICROSCOPY

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Abstract

A combined perfusion- and immersion prefixation with glutaraldehyde followed by a tannic acid/arginine/ osmium tetroxide (TAO) treatment of the guinea pig cochlea is described for field-emission gun scanning electron microscopy (FEG-SEM) observation of the fine structure of the stereocilia of the organ of Corti. Conventional osmium tetroxide postfixation methods in combination with a thin conductive coating failed to show the fine structure of the glycocalyx of the epithelial lining in the endolymphatic compartment of the cochlea, in particular, on the stereocilia surface. The antennulae-like glycocalyx covering of the stereocilia surface of the more pronounced rows of outer hair cells has been demonstrated only in ultrathin sections by means of cationic markers. The side- and tip-links connecting the stereocilia have been demonstrated both in scanning and transmission electron microscopy, although at that time these structures often were considered as artificial. However, they can be visualized with FEG-SEM at low accelerating voltage (2-3 kV), and at appropriate working distance and probe current, in combination with a glutaraldehyde perfusion/immersion prefixation and TAO postfixation. Stereo images enhance considerably the three-dimensional appreciation of the stereocilia with glycocalyx lining and side- and tip-links, proving that these connections are a structural part of the hair cell.

Key Words: Field-emission scanning electron microscope (FEG-SEM), cochlea, stereocilia, tannic acid/ arginine/osmium tetroxide (TAO) non-coating, low voltage scanning electron microscopy, glycocalyx, antennulae, cross-links, side- and tip-links.

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Introduction

Structure of the organ of Corti

The internal ear is composed of an outer and an inner labyrinth. The outer labyrinth consists of several small intercommunicating cavities with the temporal bone filled with a fluid, perilymph, which is similar in composition to the cerebro-spinal fluid. The inner membranous labyrinth consists of three semicircular canals, the vestibule and the cochlea. The bony cavities and canals contain thin walled membranous sacs which also intercommunicate; these membranous sacs are suspended in perilymph, but are filled with endolymph. The cochlea is a bony canal that is coiled approximately 2.75 turns around a central core or modiolus. The modiolus is surrounded by a spiral lamina that is divided in a bony and a membranous spiral lamina, the latter is also called basilar membrane. The membranous spiral lamina consists of thin bony ridges that extend laterally from the modiolus. Between the bony ridges blood vessels, nerves and the spiral ganglion are located. The spiral ganglion contains numerous cell bodies of afferent bipolar neurons, from which dendrites extend into the organ of Corti, to contact the base of the hair cells. The membranous cochlea which is surrounded by a bony wall is divided in three compartments: the scala vestibuli, the scala tympani and the scala media; the first two compartments contain perilymph; the scala media contains endolymph. The floor of the scala media contains the organ of Corti which is positioned on the basilar membrane (Kessel and Kardon, 1979).

The organ of Corti consists of special sensory cells as well as supporting cells. A gelatinous tectorial membrane extends from the vestibular lip of the spiral limbus over the internal spiral tunnel to cover the surfaces of the inner and outer hair cells. The hair cells are highly differentiated epithelial cells, which form the mechanoreceptor of the sensory epithelium in the inner ear. From a morphological and physiological point of view the organ of Corti contains two distinct types of sensory cells, namely three rows of outer hair cells and one row of inner hair cells, which are supported by the outer and inner phalangeal cells, respectively.

A characteristic part of the hair cell is the hair bundle, composed of several rows of stereocilia of various lengths. Each stereocilium contains a core of longitudinally arranged actin filaments, which are extensively cross-linked and surrounded by a plasma membrane of the hair cell and anchored in the amorphous structure of the cuticular plate (Friedmann and Ballentyne, 1984). The hair bundles of the outer hair cells (OHC) contain stereocilia of various lengths (short, middle and tall) which are arranged in V- or W-shaped rows. The tallest stereocilia are embedded in the tectorial membrane, which is covering Deiters' cells and the three rows of outer and one row on inner hair cells. The stereocilia of the inner row of hair cells (IHC) are arranged in a single row, and are not embedded in the tectorial membrane. The free luminal membranes of the cochlear epithelium are covered with a surface coat (glycocalyx) which is composed of carbohydrates of glycoproteins and glycolipids and acidic glycosaminoglycans. It has been suggested by Hudspeth (1985), Slepecky and Chamberlain (1986) and Santi and Anderson (1987), among others, that the glycocalyx of the cochlear hair cells plays an important role at the mechano-electrical transduction by creating a specific cationic rich micro-environment along the endolymphatic surfaces of the hair cells, their apical membranes and stereociliary bundles.

The OHC are being considered as active and the IHC as passive receptors of sound modulations. Extensive physiological and morphological studies of the stereociliary structures have been undertaken by Furness and Hackney (1985), Osborne *et al.* (1988) and Osborne and Comis (1990a,b). In transmission electron microscopy (TEM) studies the occurrence of the glycocalyx has been demonstrated by means of cationic markers such as cationized ferritin and colloidal thorium for the glycoconjugates because of their affinity for anionic sites of the cell membrane (Slepecky and Chamberlain, 1986; Lim, 1986; Santi and Anderson, 1987; Osborne *et al.*, 1988). Until now, it has not been possible to observe the glycocalyx scanning electron microscopy (SEM) studies.

Several investigators, e.g., Forge (1985), Furness and Hackney (1985), de Groot and Veldman (1988) and Takumida *et al.* (1989a,b) found that the glycocalyx lining the endolymphatic compartment was affected by application of ototoxic drugs. The reactive groups responsible for the ferritin and thorium reactivity could be identified by treatment with neuraminidase and hyaluronidase. With these enzymes the glycocalyx constituents were digested, so that the cationic markers could not bind anymore (Van Benthem *et al.*, 1992).

Flock *et al.* (1986) demonstrated the presence of lateral connections between the individual stereocilia, while Pickles *et al.* (1984) showed by SEM for the first time the existence of three types of connections between stereocilia: side-to-side, row-to-row and tip-to-side links. Although these cross-links initially were considered as artefacts, several investigators, e.g., Csukas *et al.* (1987) and de Groot and Veldman (1988) considered them as a specialization of the glycocalyx, while Pickles *et al.* (1984), Osborne *et al.* (1988), Takumida *et al.* (1988, 1989a,b) and Osborne and Comis (1990b) consider the cross-links as a separate morphological and functional entities. To provide further structural evidence on the origin of the cross links, we have to investigate the glycocalyx in the guinea pig cochlea with in-lens systems and histochemical derivation of the glycocalyx.

Microscopical technique

Until recently, SEM examination was done with a SEM equipped with a tungsten cathode (resolution about 6-7 nm), while in the early 80's the LaB_6 cathode was introduced (resolution approximately 3-4 nm). These microscopes were operated at accelerating voltages of 15-25 kV in order to obtain the optimal resolution at the smallest working distance and spot size (Soudijn, 1976; Nijdam, 1982). High accelerating voltages result in a large penetration depth of primary electrons in the specimen, which results in a high yield of secondary and backscattered electrons from the bulk of the specimen. The secondary electrons generated in the surface of the sample disappear in the large number of secondary electrons from the bulk. Moreover, an increase in kV leads to more charging, edge effects, and often, to an increase of electron beam damage to the specimen.

A way to improve the resolution is provided by the in-lens SEM systems, which give a high resolution even in the low kV range (0.5-3 kV) but can only examine very small samples. When the specimen is located inside the objective lens, an improvement of the resolution can be expected because lens-faults are minimized. When the secondary electron detector is placed such that almost only secondary electrons emitted in the surface of the specimen are collected, a higher resolution can be obtained. In this case, the influence of scattering electrons inside the bulk of the specimen is eliminated.

Images produced with scanning transmission electron microscopy (STEM) are of high resolution, but require high accelerating voltages (40-60 kV). Dissection of the complete cochlea usually damages the delicate structures of the hair cells, in particular, in brittle non-coated specimens. Surface information can be enhanced by lowering the accelerating voltage, but this often leads to a decrease of resolution and a reduced signal/noise ratio with conventional cathodes.

An improvement of the resolution is only possible with the use of a cathode with higher brightness, like the (cold) field emission (CFE) gun, which combines a small spot size, a large current density (high brightness)

and high resolution (Ansell and Capers, 1989).

The brightness of the CFE-cathode is considerably higher than that of a LaB_6 cathode, producing a high current density spot even at low accelerating voltages. Typical resolutions obtainable with the CFE-cathode are 4-5 nm at 1 kV and better than 1.5 nm at 15-25 kV, compared to 20-25 nm at 1 kV and 3-4 nm at 15-25 kV with the LaB_6 cathode equipped SEM. Thus, the field-emission SEM (FEG-SEM) is useful for low voltage research of surface features.

Preparation methods

In the past, the most common preparation procedure was a glutaraldehyde (GA) prefixation and an osmium tetroxide (OsO_4) postfixation, followed by dehydration in ethanol and critical point drying with Freon or CO_2. Au or Au/Pd coating, produced in the conventional way or by diode sputtering, was necessary to observe the delicate structures at high resolution and without charging. Due to the rather thick conductive coatings (8-10 nm) necessary to avoid charging and the moderate resolution of approximately 7-8 nm at 25 kV, no fine structural details of the hair cells were observable, as can be seen in electron micrographs from that period (Soudijn, 1976; Nijdam, 1982).

The use of non-coating techniques (Murakami, 1974; Murphy, 1978; Chaplin, 1985; Forge *et al.*, 1991; Jongebloed *et al.*, 1992, 1993; Kalicharan *et al.*, 1992) such as the tannic acid/arginine/osmium tetroxide (TAO) and osmium tetroxide/thiocarbohydrazide/osmium tetroxide/thiocarbohydrazide/osmium tetroxide (OTOTO) methods (Jongebloed *et al.*, 1992) has resulted, due to bulk fixation and conductivity improvement, in considerable enhancement of image quality and resolution, even in SEMs equipped with a tungsten cathode. However, the resolution of 3-4 nm at 15-25 kV with a LaB_6 cathode equipped SEM did not really improve, particularly at lower accelerating voltages (1-5 kV).

With the introduction of the FEG-SEM, in particular those with a cold field emission gun, high resolution was possible at low (1-5 kV) and high (5-15 kV) accelerating voltage. Neither cochleas conventionally fixed and not coated with a conductive coating, nor cochleas coated with approximately 2-3 nm Au showed sufficient detail at the apical membrane of the OHC to speak of a breakthrough, not even up in FEG-SEM mode. The non-coating techniques have proven their quality in conventional SEMs (W or LaB_6 cathode) both at low and high kV, therefore, in this investigation, the combination of the TAO non-coating technique and the cold field emission gun SEM was chosen to study the stereocilia fine structure. The purpose of the present study was the visualization of the fine structure of the stereocilia and cross- and tip-links of the hair cells, to determine their origin, distribution and relation to the overlying glycocalyx. A preparation/observation procedure was used which involved a tannic acid/arginine/osmium tetroxide postfixation and a SEM equipped with a high resolution field emission gun with variable accelerating voltage, working distance and beam current.

Materials and Methods

Animal perfusion and prefixation

The animals, 25 healthy female albino guinea pigs with a mean weight of 250 g, were sacrificed by sublethal administration of sodium pentobarbital (60 mg/kg body weight) followed by a transcardial perfusion (flow rate of 15 ml/min) of 0.1 M sodium cacodylate buffer plus 2% polyvinylpyrrolidone (molecular weight = 40 kDa) plus 0.4% $NaNO_2$ (pH 7.4; 400 mOsmol; room temperature (RT); 5 minutes). Subsequently, the animals were fixed by perfusion fixation with a 2% GA solution in the same buffer (pH 7.4; 450 mOsmol; RT; 10 minutes). Then, the bullae were removed. After the perfusion fixation, the specimens were further fixed by immersion for 24 hours in the same fixative at 4°C; therefore, the round window and the apex were opened for a better exchange of the various fixatives and endolymph. Finally, the lateral bony wall and the stria vascularis were removed, the specimens carefully rinsed (2 times) to remove excess GA. Subsequently, the specimens were treated according one of the three postfixation methods: conventional osmium tetroxide, TAO non-coating or OTOTO non-coating.

Postfixation methods

Conventional OsO_4 method Samples were immersed in a 2% OsO_4 solution in 0.1 M sodium cacodylate buffer (pH 7.4; 2 hours; 4°C), and subsequently, carefully rinsed (4 times) in distilled water.

TAO non-coating method Samples were immersed in a mixture of arginine-HCl, glycine, sucrose and sodium glutamate (2%; 16 hours; RT), rinsed (3 times) in distilled water and immersed in a mixture of tannic acid and guanidine-HCl (2%; 8 hours; RT), whereafter the samples were carefully rinsed (3 times) in distilled water. Finally, tissues were immersed in a OsO_4 solution in distilled water (2%; 8 hours; RT), followed by rinsing (3 times) in distilled water.

OTOTO non-coating method Samples were immersed in a 1% OsO_4 solution in 0.1 M sodium cacodylate buffer (pH 7.4; 2 hours; RT), rinsed in buffer solution (3 times), incubated in 1% thiocarbohydrazide in water (30 minutes; RT) and carefully rinsed in water (3 times). Subsequently, samples were immersed in a 1% OsO_4 solution in distilled water (2 hours; RT), rinsed in water (3 times), incubated in 1% thiocarbohydrazide in

water (30 minutes; RT) and rinsed in water (3 times). Finally, the samples were immersed an 1% OsO_4 aqueous solution (2 hours; RT) and rinsed in distilled water again (6 times).

Dehydration/drying and microscopical examination

All samples were dehydrated in a graded ethanol series up till 100%. The ethanol was exchanged for amyl acetate as intermediate liquid. Then, the samples were critical point dried via liquid CO_2. Examination was carried out in a JEOL 6301F SEM (JEOL USA, Peabody, MA) with a field emission gun operated at 2-5 kV, working distance (WD): 6-15 mm; probe current: 1.0-1.5 x 10^{-11} A.

Results

Conventional versus non-coating postfixation

The first attempts in our laboratory to visualize the fine structure of hair cells of the guinea pig were undertaken by Soudijn in 1972 {note: Soudijn (1976) is a published account of Soudijn's till that time}. A micrograph **taken in 1973** by Soudijn at low magnification shows the three outer (OHC I, II and III) and one single row (IHC) of hair cells of the organ of Corti arranged in either V or W-shape or in a straight line, respectively and the Deiters' cells with their microvilli (Fig. 1). The image was taken after a GA-transcardial perfusion and immersion prefixation followed by conventional OsO_4 postfixation and a 6-8 nm Au coating. The image was taken with a conventional SEM with tungsten filament at an accelerating voltage of 25 kV. The image is well in focus, but no real details can be recognized at the hair cell stereocilia surface. Along the outer surface of the stereocilia, no sub-detail is visible; some charging appears despite the conductive Au coating applied.

A comparable image is shown in Figure 2, taken after GA-transcardial perfusion and prolonged immersion prefixation followed by conventional OsO_4 postfixation, but in this case without conductive coating, imaged with a FEG-SEM at 2 kV. The sharpness of the image has increased by using a FEG-SEM, which can be observed even at this moderate magnification. Despite the low kV, some charging appears again, so in the case of conventional postfixation, the bulk fixation is inadequate, and a conductive coating should be applied to avoid charging even at low kV. The image also indicates that artefacts, such as blebbing, precipitation of proteinaceous material or aggregation of coating grains, become more obvious in FEG-SEM.

A moderate magnification of OHC I and II, taken after a GA-perfusion/immersion prefixation but now followed by a TAO non-coating postfixation and imaged with a FEG-SEM at 2 kV, is shown in Figure 3. The

Figure 1. Low magnification of part of basal turn of cochlea with three outer rows of hair cells (OHC I, II and III) and one inner row of hair cells (IHC) with stereocilia (sc), cuticular plate (cp) and Deiters' cells (dc) with microvilli after GA/OsO_4 fixation and 6-8 nm Au conductive coating. No detail of stereociliar surface is apparent. (SEM with W-filament, 25 kV; micrograph taken 1973 by Soudijn).

Figure 2. Similar micrograph with OHC I, II and III with stereocilia (sc), cuticular plate (cp) and Deiters' cells (dc) with microvilli (mi) after GA/OsO_4 fixation without conductive coating. No details of stereociliar surface apparent, although the overall sharpness of the image is increased in comparison to Figure 1; some charging still occurs. (FEG-SEM; 2 kV).

Figure 3. Low magnification of OHC I, II and III with stereocilia (sc), cuticular plate (cp) and Deiters' cells (dc) with microvilli after GA/TAO fixation. Note glycocalyx with filamentous structures (*) at stereocilia and globular structures (gs) as part the cytoplasmic membrane. (FEG-SEM; 2 kV).

Figure 4. High magnification of OHC I; almost no details of the glycocalyx on the stereocilia (sc) surface and cuticular plate (cp) can be seen after GA/OsO_4 fixation and 6-8 nm Au conductive coating. Note the short, intermediate and tall stereocilia (arrowheads) and loss of detail at the cuticular plate surface. (FEG-SEM; 2 kV).

Figure 5. High magnification of IHC stereocilia after GA/TAO fixation, note the rather thick coating of the stereociliary membrane; the cap (*) of the stereocilia seems even more dense, no antennulae present. (FEG-SEM; 2 kV).

Figure 6. Another view of OHC I with stereocilia (sc) covered with a glycocalyx with antennulae (*) after GA/TAO fixation. Note gradient in antennulae covering from apical towards basolateral part of stereocilia. (FEG-SEM; 2 kV).

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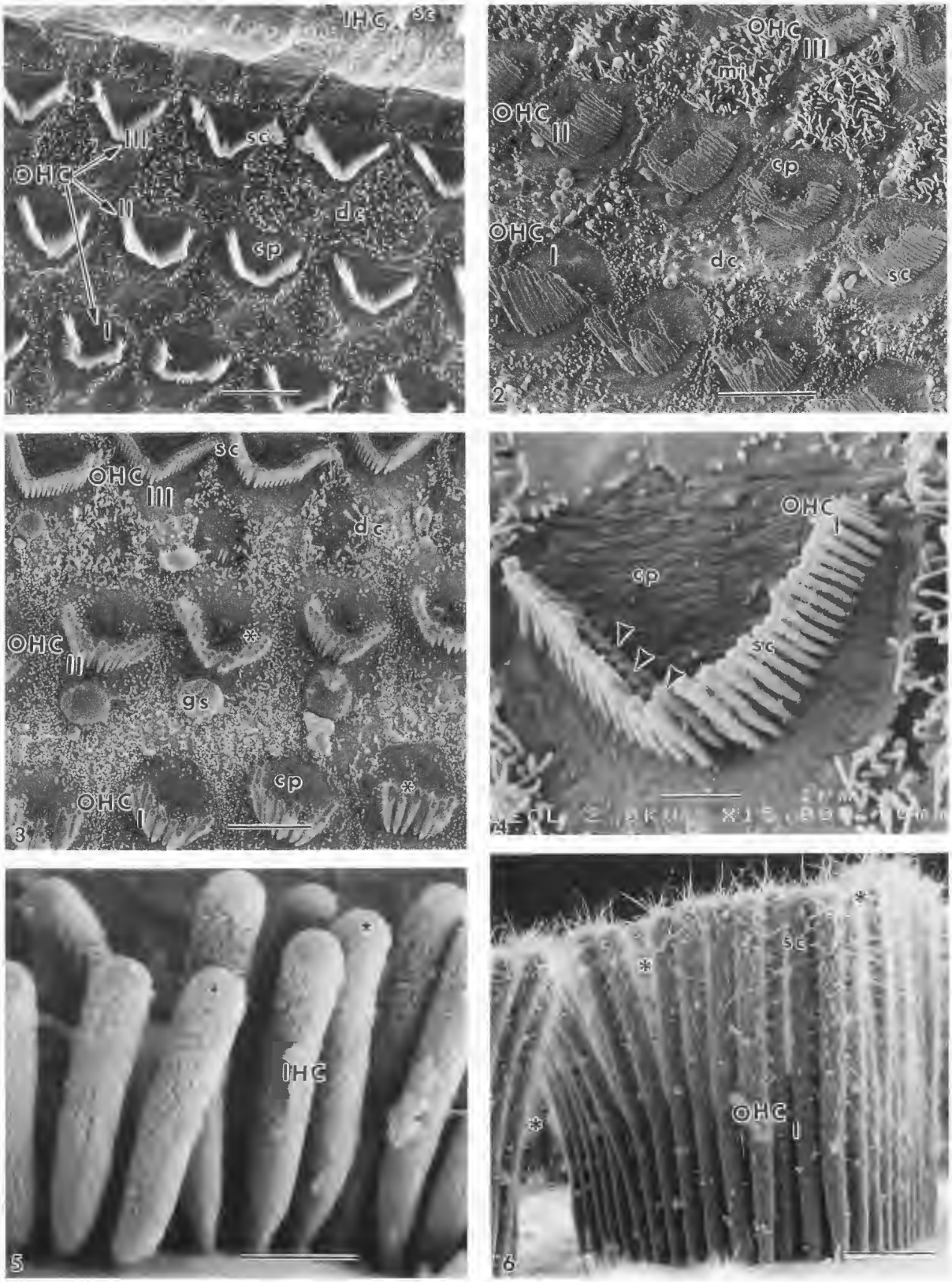
stereocilia of the first and second outer row hair cells (OHC I and II) have a more cylindrical appearance and seem to be covered with a particular fluffy substructure (glycocalyx). They are surrounded by the Deiters' cells. Thin filaments (antennulae) are present in the stereocilia, gradually decreasing towards the cuticular plate. The globular structures seen on the surface of the Deiters' cells are not considered to be an artefact, but are associated with the cytoplasmic membrane of these cells; the outer wall of the globules shows some kind of texture.

Figure 4 represents a higher magnification of a W-shaped arranged OHC-I after GA-perfusion/immersion prefixation, followed by conventional OsO_4 postfixation, but with a 4-6 nm thick Au coating, imaged with the



Figures 1-6. Conventional versus non-coating postfixation. Bars = 5 μm (Figs. 1-3) and 1 μm (Figs. 4-6).

FEG-SEM at 2 kV. The disturbing effect of the conductive Au-layer on the visualization of the fine structure is clearly visible. A close examination shows that only a part of the fine structure at the stereocilia surface can be observed, but elsewhere it has been rinsed away or is hidden under the conductive coating. Not only are the stereocilia covered with the metal coating, but also no fine detail can be seen at the cuticular plate and surrounding area. The edges of the cuticular plate seem to be torn off, while the plate itself seems to have a grated and relative rough substructure, caused by the relative thick Au coating, making magnifications over 10,000x-15,000x not very relevant.

Figure 5 represents a rather high magnification of IHC arranged in a single row (GA-perfusion/immersion prefixation and TAO non-coating postfixation). The image was taken with the FEG-SEM at 2 kV. The surface of the stereociliar membrane seems to be covered with some proteinaceous layer as well, though no antennulae are observable. The stereocilia have a rather rough appearance over most of their outer surface, but their apical end is rather smooth and seems more compact.

Another view of the stereocilia of an OHC I is shown in Figure 6; the image was taken after GA-perfusion/immersion prefixation followed by the TAO non-coating postfixation and taken with the FEG-SEM at 2 kV. The surface of the stereocilia is covered with a rather fluffy and electron dense glycocalyx with a filamentous appearance. From this image, it is obvious that the entire surface of the tallest stereocilia of the OHC I is covered with a glycocalyx decreasing towards the basolateral portion of the stereocilia.

Effect of kV on the glycocalyx fine structure visualization

The accelerating voltage applied is another parameter of great importance for observation of the fine structure of the membranous surface of the hair cell stereocilia. To examine the influence of the accelerating voltage, that means, primary electron beam penetration, we studied with a FEG-SEM the same stereocilia after GA and TAO non-coating fixation at various accelerating voltages, ranging from 0.5 to 10 kV, at a constant working distance and probe current.

Figures 7-11 present (almost) the same area of the stereocilia at 0.5, 1.0, 2.0, 3.0, 5.0 and 10 kV, respectively, at the same magnification. From the micrographs shown, it is obvious that the 0.5 kV image (Fig. 7) lacks resolution compared to the 1.0, 2.0 and 3.0 kV images but mainly shows the very surface of the antennulae. The stereocilia with glycocalyx and antennulae appear with best resolution and focus in Figures 8, 9 and 10, taken at 1.0, 2.0 and 3.0 kV, respectively. The 5.0 kV image of Figure 11 and the 10 kV image of Figure 12

Figure 7. Stereocilia at 0.5 kV, glycocalyx with fibrils not clear due to poor resolution; GA/TAO fixation. (FEG-SEM; d = 2 x 10^{-11} A; WD = 9 mm).

Figure 8. Stereocilia at 1 kV, glycocalyx with clear fibrils; GA/TAO fixation. (FEG-SEM; d = 2 x 10^{-11} A; WD = 9 mm).

Figure 9. Stereocilia at 2 kV, glycocalyx with clear fibrils; GA/TAO fixation. (FEG-SEM; d = 2 x 10^{-11} A; WD = 9 mm).

Figure 10. Stereocilia at 3 kV, glycocalyx with fibrils less clear; GA/TAO fixation. (FEG-SEM; d = 2 x 10^{-11} A; WD = 9 mm).

Figure 11. Stereocilia at 5 kV, glycocalyx and part of fibrils barely visible; GA/TAO fixation. (FEG-SEM; d = 2 x 10^{-11} A; WD = 9 mm).

Figure 12. Stereocilia at 10 kV, high resolution image with flat contrast and almost invisible glycocalyx with fibrils, due to too high bulk signal. GA/TAO fixation. (FEG-SEM; d = 2 x 10^{-11} A; WD = 9 mm).

Figure 13. Stereocilia with fibrillar structure after GA/TAO fixation at 0.5 kV. Despite inferior resolution, the fine fibrillar structure of the stereociliar glycocalyx can be clearly seen. (FEG-SEM; d = 2 x 10^{-11} A; WD = 9 mm).

Figure 14. Stereocilia at 2 kV after GA/OsO_4 fixation and Au-sputtering (1-2 nm). Although the overall sharpness of the image is better, aggregation and coating layer thickness and charging obscure fine details of the glycocalyx on the stereocilia surface. (FEG-SEM; d = 2 x 10^{-11} A; WD = 9 mm).

Figure 15. Stereocilia at 5 kV after GA/OsO_4 fixation and Au-sputtering (4-6 nm). Less charging occurs as result of thicker coating, but fine structure has disappeared under coating. Fibrils at top of cilia seem longer and thicker than at the base. (FEG-SEM; d = 2 x 10^{-11} A; WD = 9 mm).

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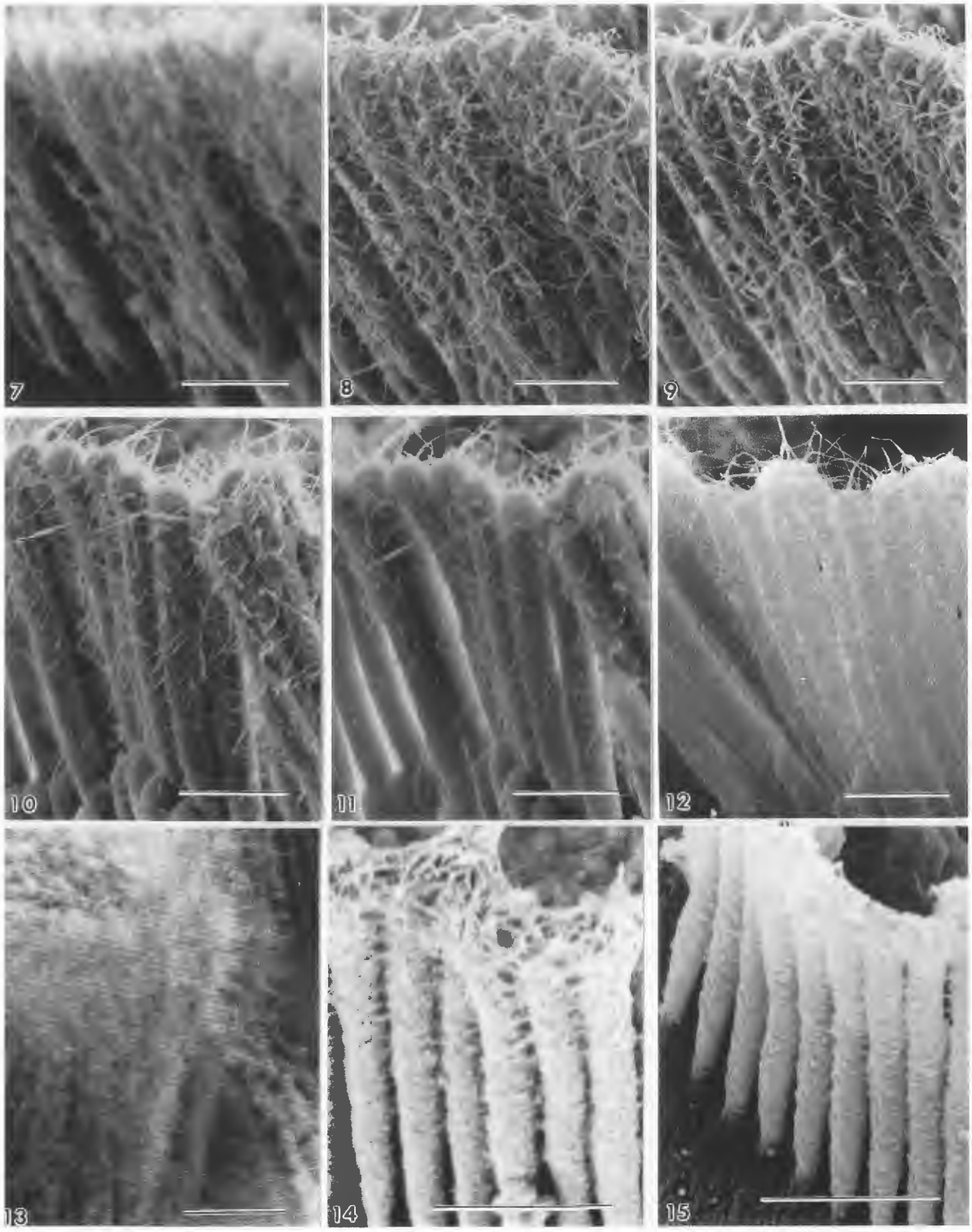
are well in focus, but lack the detailed information of the glycocalyx with antennulae because the signal from the bulk of the stereocilia is superimposed on the surface information. The antennulae are being transmitted by the primary electrons and show insufficient contrast to be seen against a background of secondary electron signal from the bulk.

Effect of conventional osmium postfixation and conductive coating on the glycocalyx fine structure visualization

To investigate the influence of the postfixation, the stereocilia of the OHC were examined after TAO and OsO_4 postfixation, respectively, in the latter case after



Figures 7-15. Influence of accelerating voltage on visualization of the fine structure of glycocalyx. Bars = 1 μm.

application of an Au coating. Figure 13 presents an image taken at 0.5 kV after TAO non-coating postfixation. The lack of resolution is obvious, but details of the fine fibrillar material, forming the antennulae, on the outer surface of the stereocilia are apparent.

Figures 14 and 15 represent OsO_4 postfixed samples with Au coating. A 3-4 nm Au conductive coating was applied in the case of Figure 14. The image was taken at 2 kV, and some charging occurred, decreasing the sharpness of the image.

A 6-8 nm Au conductive coating had to be applied at 5 kV to avoid charging (Fig. 15). The fine structure is obviously obscured by the relative thick conductive Au-coating and the clotting of the fibrillar structure, which had occurred as result of poor preservation of the glycocalyx and conglomeration of Au particles.

Fine structure and location of the glycocalyx

The location and the thickness of the glycocalyx has been another point of study for several investigators. Figure 16 presents an overview at low magnification of the rows of OHC I, II and III and the row of IHC. The glycocalyx with antennulae of the stereocilia is the most abundant at the OHC I, which is indicated by the whitish appearance. The thickness of this layer on the stereocilia of the OHC II obviously is thinner, while the glycocalyx on the stereocilia of OHC III is even less abundant. The stereocilia of the IHC either have a rather rough outer membrane with a dense cap at the apical side, or are covered with a glycocalyx as well but without antennulae.

Tectorial membrane

Figure 17 shows at low magnification the well arranged OHC I, II and III and IHC and part of the undersurface of the tectorial membrane with the W-shaped imprints of the tallest stereocilia of the three rows of OHC. The slightly thicker edge of the tectorial membrane represents the area where the tectorial membrane has been attached with stapes to the phalangeal cells bordering the OHC I.

A detailed image of the undersurface of the tectorial membrane clearly shows the imprints of the tallest stereocilia of the OHC (Fig. 18). In the upper part of the image, the fibrillar layer of the edge of the tectorial membrane (as mentioned in Fig. 17) can be observed. It is clearly different from the tectorial membrane structure found between the imprints.

If the area around the W-shaped imprints of the stereocilia of the OHC I is studied more carefully (Fig. 19), it is obvious that the Hardesty layer at several places is covered by patches of (contrast-rich) fibrillar material (antennulae) originating from the top of the tallest stereocilia of OHC I. The imprint is divided in compartments by means of short connections overlapping the

Figure 16. Low magnification of row of IHC and three rows of OHC (I, II and III). The stereocilia of the OHC I are covered most abundantly with glycocalyx (*) compared to those of OHC II and III; the layer thickness decreases towards OHC III. Note thin coating of Deiters' cells (dc). GA/TAO fixation. (FEG-SEM, 2 kV).

Figure 17. Low magnification of rows of hair cells OHC I, II and III) and IHC, the undersurface of the tectorial membrane (TM) with imprints (short arrows) of the tallest stereocilia of OHC I, II and III. Note slightly thickened edge (*) of tectorial membrane (TM). GA/TAO fixation. (FEG-SEM, 2 kV).

Figure 18. Higher magnification of undersurface of tectorial membrane (TM) with three rows of W-shaped imprints of longest stereocilia of OHC I, II and III. Note difference in structure of tectorial membrane between the imprints and at the slightly thickened edge (*), and fibrillar structure. GA/TAO fixation. (FEG-SEM, 2 kV).

Figure 19. High magnification of imprint of stereocilia of OHC I, note background of Hardesty layer (+) and patches of antennulae material (*), originating from the apical surface of the stereocilia. GA/TAO fixation. (FEG-SEM, 2 kV).

Figure 20. Another view of the imprint of one OHC I, at left background of Hardesty layer with a thin fibrillar network (+) with globular structures. At the right side of the image rather thick fibrils (V) are seen. GA/TAO fixation. (FEG-SEM, 2 kV).

Figure 21. Detail of thickened edge of tectorial membrane (see also Figs. 17 and 18) with relatively long and thick needle-like structures (arrowheads). GA/TAO fixation. (FEG-SEM, 2 kV).

Figure 22. Apical surface of stereocilia of OHC I covered with glycocalyx with long and thick fibrils (*) pointing in different directions, possibly similar to the fibrils observed in Figure 21. GA/TAO fixation. (FEG-SEM, 2 kV).

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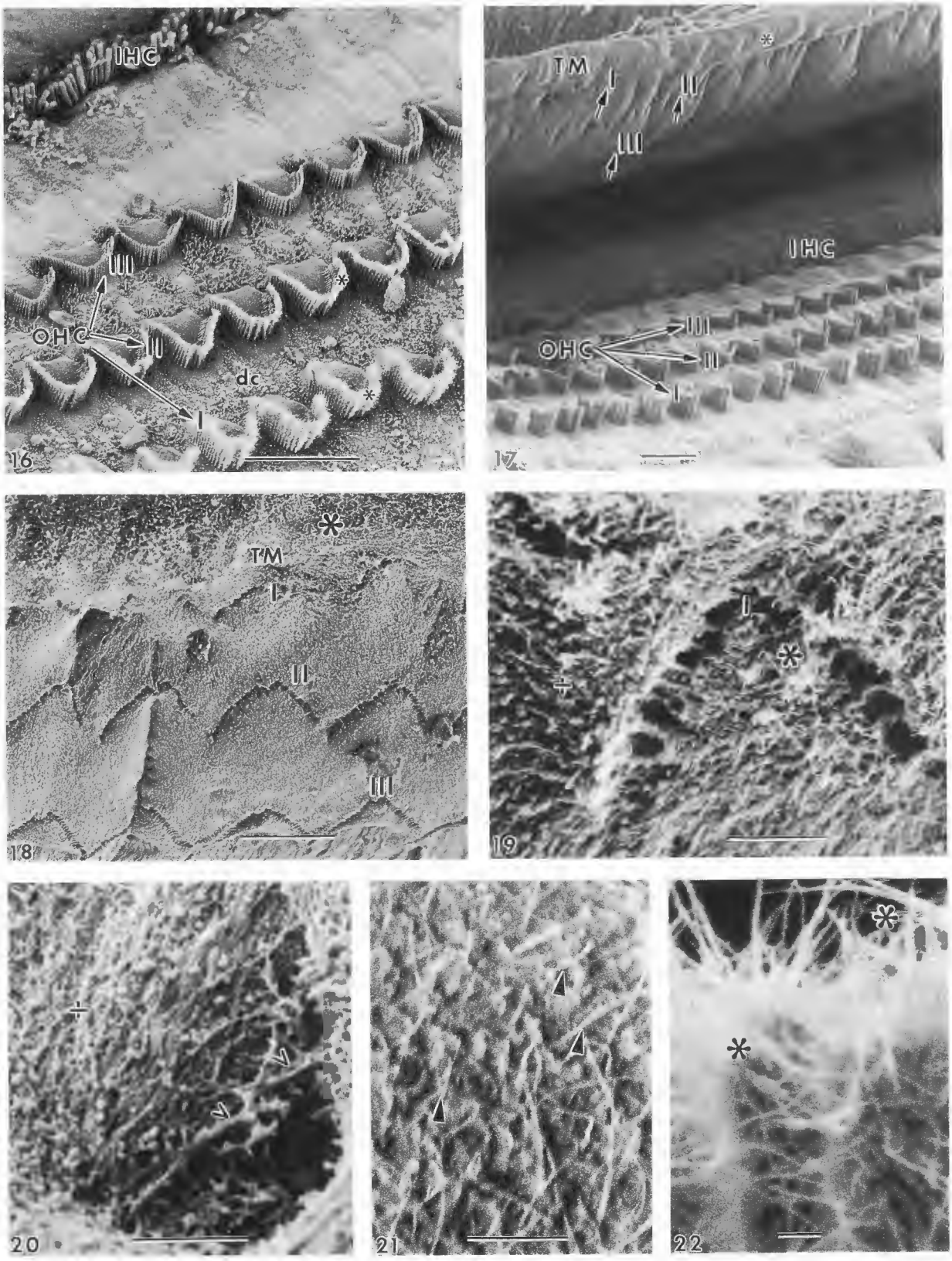
imprint, that correspond with the individually arranged stereocilia.

Observing the stereociliar imprint from another direction at higher magnification reveals part of the Hardesty layer with fibrillar network with very small spherical bodies (Fig. 20). At some places, thicker fibrils are found, which may be part of the connective tissue matrix of the tectorial membrane.

A detail of the thickened edge of the undersurface of the tectorial membrane (Fig. 21) (as observed already in Figs. 17 and 18), reveals large needle-like projections with an average length of 1-1.5 μm and a thickness of



Figures 16-22. Glycocalyx and undersurface tectorial membrane.
Bars = 10 μm (Figs. 16 and 17), 5 μm (Fig. 18), 1 μm (Fig. 19 and 20), 500 nm (Fig. 21) and 100 nm (Fig. 22).

10-20 nm. They only are found at that part of the tectorial membrane which originally was attached to the phalangeal cells bordering the first row of outer hair cells (OHC I).

Figure 22 shows the apical surface with glycocalyx of the tallest stereocilia of the OHC I. At high magnification, rather long fibrils with a diameter of 10-14 nm are seen pointing in all directions covering the top of the stereocilia. As a result of slight charging and aggregation of the fibrils due to the impact of the electron beam, they even appear thicker. They could correspond to the needle-like fibrils seen in Figure 20.

Stereoscopic images

Although the SEM-image already gives a three-dimensional (3-D) impression, false 3-D information can be obtained due to large differences in contrast resulting from edge-effects or charging. A stereoscopic pair of images can overcome these phenomena to a large extent. The reason for this is that tilting the sample away from the detector decreases contrast in the image, while a stereoscopic impression can be obtained from two images with a large difference in contrast.

Figure 23 represents a stereoscopic image at low magnification of three rows of OHC and a single row of IHC with stereocilia, the Hensen cells and the inner pillar cells. The image was taken after a prefixation with a tri-aldehyde mixture (3% GA, 2% formaldehyde, 1% acrolein and 2.5% dimethyl sulfoxide (DMSO) in 0.08 M sodium cacodylate buffer). Complete OHCs are lifted off from their cuticular plate and are clinging to the undersurface of the tectorial membrane. The hyperosmolarity of this mixture could easily cause a premature shrinkage of the tectorial membrane before the stereocilia are sufficiently preserved to provide anchorage.

A stereoscopic image of an OHC I, with a W-shaped arrangement of stereocilia on the relatively smooth cuticular plate, surrounded by Deiters' cells associated with short microvilli, is shown in Figure 24. The relatively thick glycocalyx with antennulae at the outer surface of the stereocilia, in contrast with the much thinner layer on the microvilli surface of the Deiters' cells, is apparent.

Figure 25 presents a stereoscopic image of a more detailed view of an OHC with stereocilia, the glycocalyx with antennulae is predominantly present at the upper half of the stereocilia; the rather long fibrils at the top point into space. The somewhat W-shaped top at most of the stereocilia in this image could be related to the imprint at the undersurface of the tectorial membrane.

In Figure 26, the three rows of stereocilia of a single OHC (short, intermediate and tall) and their arrangement with respect to each other can be observed. The tips of the stereocilia appear to be rounded-off. The glycocalyx has been removed by means of a few extra rinsing steps after the prefixation, prior to the TAO non-coating procedure, in order to visualize the spatial arrangement of the cross-links (side-side and tip). From this image, it is obvious that these cross-links are not artificial structure. Currently, we are investigating cross-links occurrence at all four rows of hair cells.

Discussion

Several investigators (de Groot, 1986; Lim, 1986; Albers *et al.*, 1987; Osborne *et al.*, 1988; Comis *et al.*, 1990; Osborne and Comis, 1990a,b; Pickles *et al.*, 1991; Ruding *et al.*, 1991; Forge *et al.*, 1992; Van Benthem *et al.*, 1992, 1993) have studied the organ of Corti of the guinea pig because of ultrastructural, medical, electrophysiological or audiological reasons. In particular, the structure of the stereocilia, the glycocalyx and the presence of cross-links were subjects of interest. Investigations were carried out with light-(LM) and electron microscopy (SEM and TEM).

Conventional fixation (GA/OsO_4) of the cochlea only preserves the basic structure of the stereocilia, not the structural details of the stereociliar membrane; the glycocalyx with antennulae were observable neither in a conventional SEM nor in a FEG-SEM. Membranes of this type of sensory cells exhibit a glycocalyx, mainly composed of carbohydrates of glycoproteins, glycolipids and glycosaminoglycans which are inadequately preserved by conventional fixation methods as demonstrated. Inferiorly preserved glycocalyx is either washed away in the course of the preparation procedure or leads to aggregation of the fine filamentous material composing the glycocalyx. Moreover, the rather thick Au coating with its relative large grain size applied in the past and necessary for an optimal resolution and sufficient yield of secondary electron at high accelerating voltages (15-25 kV), obscure every detail possibly present. The large penetration depth of the primary electron beam at high kV generates more secondary electrons from the bulk of the specimen and obscures the surface information obtainable at low kV.

In the past, SEMs were equipped with either a W- or a LaB_6 cathode, with at best a resolution of 3-4 nm at 15-25 kV. Improvement has been obtained with a CFE-cathode with a brightness of approximately 10^9 $A/cm^2.sr$ at 10^{-9}-10^{-12} A beam current, a brightness which is approximately 4000 times higher than that of a LaB_6 cathode. An advantage of this high brightness cathode is that 3-4 nm resolution can be obtained at 1-2 kV, which is of interest for the investigation of surface phenomena such as the glycocalyx.

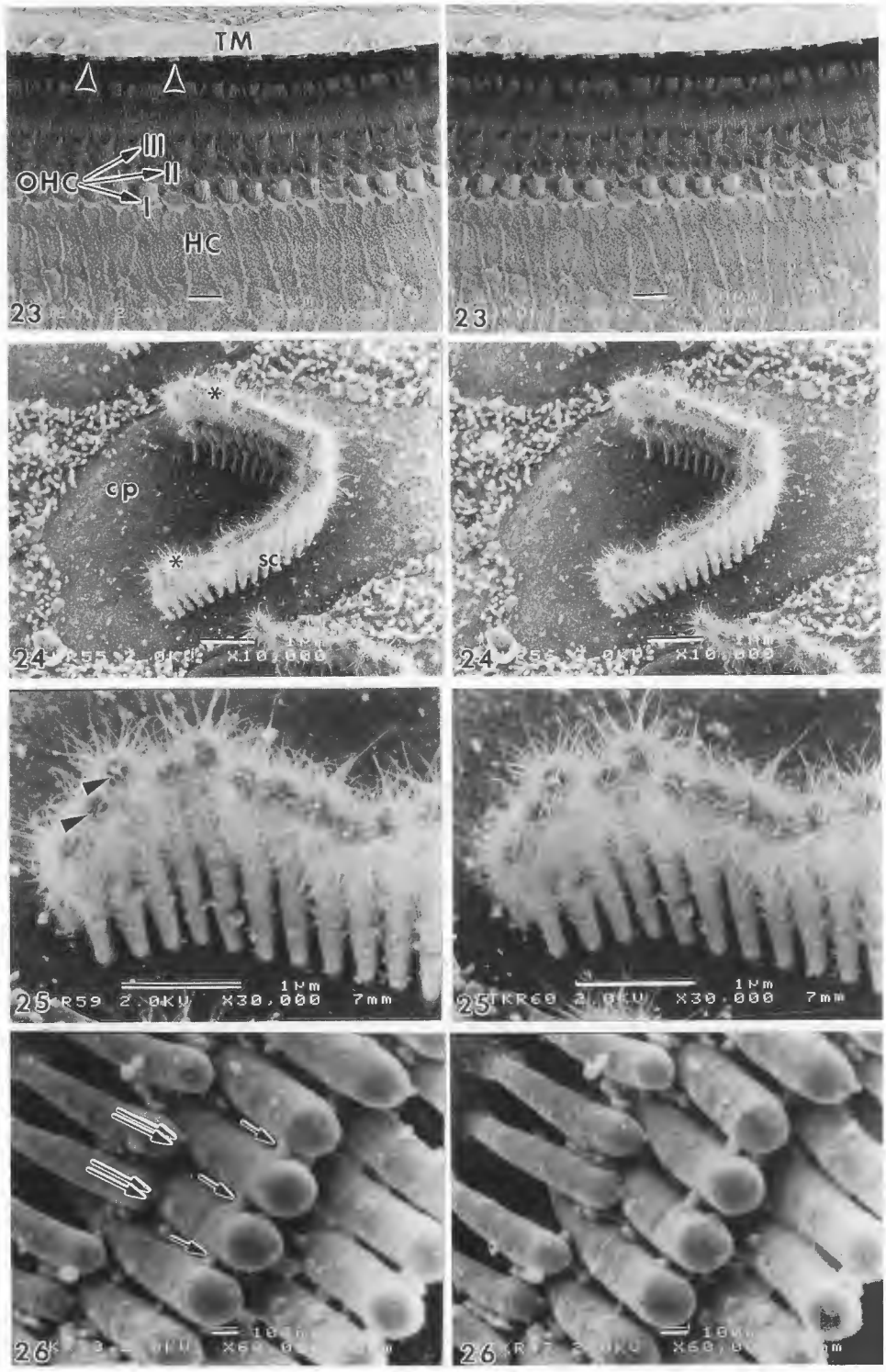


Figure 23. Stereopair of OHC I, II and III and IHC and Hensen's cells (HC) of organ of Corti after tri-aldehyde/TAO fixation; part of hair cells (arrowheads) are attached to the undersurface of the tectorial membrane (TM). (2 kV; FEG-SEM). Bar = 10 μm.

Figure 24. Stereopair of one outer V-shaped hair (OHC) cell with stereocilia (sc) and cuticular plate (cp); note glycocalyx with fibrils (*) on top of stereocilia after GA/TAO fixation. (2 kV; FEG-SEM). Bar = 1 μm.

Figure 25. Stereopair of detail of one OHC I with glycocalyx with abundance of fibrillar material at top of stereocilia; note typical figures on top of stereocilia (arrowheads). (2 kV; FEG-SEM). Bar = 1 μm.

Figure 26. Stereopair of detail of OHC I, three rows of stereocilia with different length and diameter can be observed, GA/TAO fixation, with extra rinsing step in between. Side-links (single arrows) and tip-links (double arrows) between individual stereocilia can be easily observed. (2 kV; FEG-SEM). Bar = 100 nm.

Figures 23-26. Stereoscopic images.

Prefixation

Prefixation consisted of an transcardiac prefixation with GA in cacodylate buffer via the left ventricle at a flow rate of 15 ml/min, as performed in the past on rats in investigations of the ductus submandibularis and of cerebral tissue (Vissink *et al.*, 1992). The upper extremities become pale and rigid within the first minute; the aorta descendens is being closed off, so that not the

whole body is perfused; hence, an optimal prefixation is obtained without a period of oxygen deficiency. The so-called tri-aldehyde prefixation, a mixture of glutaraldehyde, paraformaldehyde, acrolein and dimethyl sulfoxide (de Groot and Veldman, 1988), used as an alternative perfusion/immersion prefixation for TEM instead of GA, gives a rapid penetration of paraformaldehyde and acrolein, causing a high degree of cross-linking of the connective tissue in the tectorial membrane. This extreme cross-linking results in a lift-off of a major part of the stereocilia together with the tectorial membrane. Thus, this method is not well suited for SEM preparation because in the drying step additional shrinkage occurs.

Postfixation: conventional versus non-coating

To improve on conventional SEM osmium tetroxide postfixation, a more appropriate postfixation procedure is necessary, such as the non-coating techniques: the OTOTO or the TAO methods. Although both techniques give better results than conventional OsO_4 fixation, including a conductive layer, the TAO method has been chosen because the samples were less fragile during preparation of the complex membranous system inside the hair cell (Jongebloed *et al.*, 1992, 1993). Davies and Forge (1987), Forge *et al.* (1991, 1992) and Reiss (1992) used thiocarbohydrazide to postfix the organ of Corti with its sub-structure of stereocilia and showed the ciliary bundles with the three rows of stereocilia with various length in SEM and the microfibrillar structure inside the stereocilia and fibrillar extracellular lateral cross-links in TEM images. They did not demonstrate the glycocalyx with filaments in SEM images. Forge *et al.* (1991) reported a rapid freezing method and subsequent fracturing and etching of isolated cochlear hair cells, avoiding chemical fixation, rinsing and physical drying, revealing also internal microfilaments.

With the TAO method, the uptake of osmium is facilitated by the use of the tannic acid/arginine combination. The main constituent of tannic acid is galloyl glucose (Murakami, 1974; Fermin, 1993), which can react with several metal ions such as osmium, uranyl or ruthenium, due to its active COOH, OH and NH_2 groups. The increased uptake of so called heavy metals enhances secondary electron emission, improving the signal/noise ratio and provides an optimal bulk fixation. Moreover, galloyl glucose binds with carboxyl, sulfonic and hydroxyl groups of glycoproteins and glycosaminoglycans present in the stereocilia glycocalyx. The glycocalyx present on top of the microvilli of intestinal epithelial cells is hardly preserved by osmium tetroxide alone, only remnants of fused filamentous material are apparent, as could be observed in GA/OsO_4 fixed samples with conductive coating, viewed with the FEG-SEM. OTOTO and TAO postfixed intestine clearly showed filamentous structures (penicillium) at the microvilli in SEM and TEM images (Kalicharan *et al.*, 1984). Tannic acid/arginine, and to a lesser extent thiocarbohydrazide, preserve glycoproteins and glycosaminoglycans present in the vitreous body of the eye (Los *et al.*, 1992). These structures could be fixed with a single GA immersion fixation and could account for the fact that the glycocalyx was not seen in SEM after conventional fixation. Murakami (1974) described arginine/guanidine as amino acids reacting with an excess of aldehyde groups in the tissue, so they cannot be rinsed away. OsO_4 can react with those amino acids and tannic acid in the tissue, and be reduced to osmium by the osmiophilic compounds and amino acids present.

The immersion in OsO_4 solution involved rather long periods, as compared to the immersion times used by de Groot and Veldman (1988). The penetration rate of OsO_4 is low, particularly in compact tissues. OsO_4 was used in combination with galloyl glucose, arginine and glycine, which bind to specific structures in the organ of Corti, due to the presence of hydroxyl, carboxyl and NH_2 groups. The mixture also can bind heavy metals such as osmium, uranyl and ruthenium. Galloyl glucose penetrates deep into the tissue so the osmium can reach deeper into the tissue, while the number of binding sites for, e.g., osmium, increases considerably. Hence, a longer osmium penetration time is required than in the case of single osmium fixation. We did not find adverse effects of prolonged osmium fixation times as reported by de Groot (1986).

The image of the OHC taken after conventional GA/OsO_4 postfixation by Soudijn in 1973, shows blebbing at the stereocilia as result of the insufficient fixation, as also reported by Forge *et al.* (1992). Examination of such an OsO_4 postfixed sample with FEG-SEM, either coated or not coated with Au, only improves the visualization of those artefacts. The TAO postfixed sample does not show blebbing. The large globular structures are considered as cytoplasmic bulging, too large for blebbing. Moreover, the surface of these bulges still shows a fine texture. Davies and Forge (1987) obtained similar results in OTOTO and TAO postfixed material after freeze drying. Artefacts such as blebbing can be better observed in high resolution FEG-SEM images than in images taken with conventional SEM at an accelerating voltage of 25 kV and a resolution of approximately 6-7 nm, in particular, when examination takes place at low kV. Forge *et al.* (1992) compared various preparation postfixation procedures with a thiocarbohydrazide/osmium tetroxide combination and found a considerable improvement of the preservation of the hair cells in comparison with postfixation with only OsO_4 as used in the past. In all cases, samples were dried either by critical point drying or freeze drying.

Surface features are more pronounced as can be observed by comparing the surface structure of the cuticular plate in TAO postfixed and OsO_4 post-fixed/metal coated samples. Images of conventionally fixed outer hair cells often show this phenomenon (Kessel and Kardon, 1979). In fact, micrographs from the 70's and early 80's should not be compared with FEG-SEM images taken at low kV because the early results were obtained mostly at relatively high accelerating voltage (15-25 kV), needed to obtain optimal resolution, and with metal coating which obscures surface features and possible artefacts.

Tectorial membrane

The tectorial membrane extends into the cochlear duct to cover the stereocilia on the apical ends of the hair cells as a sheet from its attachment to the supporting phalangeal (Deiters') cells or at the boundary between the phalangeal cells and the Hensen cells into the vestibular lip of the limbus. Retraction of the tectorial membrane is the result of both prefixation and dehydration/drying, but mainly of the fixation. It appears that this membrane is firmly attached to the tallest stereocilia of the outer rows of hair cells. We used the GA-perfusion/immersion fixation as described by Soudijn (1976) and Rosenbauer and Kegel (1978). We never removed the stapes for better fixation of the scala vestibuli because we wanted to have a very careful retraction of the tectorial membrane without mechanical damage to the hair cells, the suspension system and the phalangeal cells. As a result of the perfusion and prolonged immersion prefixation, we obtained an optimal morphology already, without need for removal of the stapes. Reiss (1992) used the perilymphatic method instead of the transcardial method for optimal prefixation; after fixation, the undersurface of the tectorial membrane and hair cells were freed by a specially designed micromanipulation unit in the SEM.

The tips of the longest stereocilia of OHC are firmly attached to the undersurface of the tectorial membrane organized in V- or W- patterns. The tectorial membrane consists of a relatively rough network of connective tissue, mainly consisting of bundles of collagen fibers, giving it a fibrillar appearance. The undersurface is covered with a thin layer of tiny fibrils, known as the Hardesty layer. It seems that the moment of retraction of the tectorial membrane is strongly dependent on the state in which the hair cells with glycocalyx and antennulae have been fixed. The tri-aldehyde fixation procedure causes a very strong bonding between stereocilia and bundles of collagen fibers, constituting the tectorial membrane matrix. Complete rows of stereocilia are lifted-off from the cuticular base of the hair cell, damaging stereocilia and its organization. From the images shown, it can be seen that sometimes patches of antennulae stick to the inner side of the tectorial membrane close to the imprints of the longest stereocilia. Particularly, in the first outer row of hair cells, parts of the fibrillar structure of the Hardesty layer at that point stick to the top of the longest stereocilia. This could account for the needle-like appearance of the top of the apical part of the longest stereocilia, particularly of the OHC I row. The dimensions are in agreement with the needle-like structures seen at the attachment area of the undersurface of the tectorial membrane.

Glycocalyx

Until recently, visualization of the stereociliar surface coat (glycocalyx) was possible only with TEM by means of cationic markers such as colloidal thorium and cationized ferritin (de Groot and Veldman, 1988; Van Benthem *et al.*, 1992, 1993). The glycocalyx may be responsible for the creation of a kind of micro-environment around the stereocilia of the hair cells, in particular at the apical top of the stereocilia because of its negative surface charge (Lim, 1986; Slepecky and Chamberlain, 1986). The presence of micro-environments along the length of the endolymphatic duct is very possible and may explain, both in the cochlea and vestibule, the presence of adjacent structures with very different molecular components. In the vestibule, the existence of highly concentrated calcium carbonate components (otoconia) and completely uncalcified components of the cupula at less then 1 mm distance could be very well explained by the existence of micro-environments. Fermin (1993) and Fermin *et al.* (1990) proposed that the differential distributions of various glycosaminoglycans could account for the ability of adjacent calcified and uncalcified substances to coexist (Fermin *et al.*, 1995). In the case of stereociliary bundle fibrillar components on the top 2/3, and their absence on the bottom 1/3 of the stereocilia and the adjacent tectorial membrane, could be explained as well by the presence of differential distribution of glycosaminoglycans as suggested by Fermin. Ruthenium red and Alcian blue have also been used to demonstrate the surface coat of the stereocilia in TEM (Lim, 1986; Slepecky and Chamberlain, 1986; Santi and Anderson, 1987). In TEM, ruthenium red stains the surface coat of the hair cells and the cross-links, inducing a thin uniform coating along the full length of the stereocilia. Staining with Alcian blue demonstrates in TEM two distinctive layers of surface coat material: an electron-dense inner coat, which is present at the surface of the hair cells and connections between the stereocilia, and a loose filamentous outer surface coat, respectively. Alcian blue can stain the tectorial membrane (Lim, 1986) in contrast to ruthenium red (Santi and Andersen, 1987). To stain the carbohydrates,

glycoproteins, glycolipids and glycosaminoglycans in TEM, postfixation with OsO_4 in combination with $K_4Ru(CN)_6$ has been carried out by de Groot (1986), in contrast to the conventional $K_4Fe(CN)_6$, which has been known for years in TEM studies. Ruthenium red, in particular in combination with thiocarbohydrazide, preserves well hyaluronic acid components as associated with the fine collagen fibre network in the vitreous body of the (sheep) eye (Jongebloed *et al.*, 1993). Ruthenium red often is used in combination with GA as prefixation agent, followed by a ruthenium red/OsO_4 postfixation for SEM and TEM.

As result of the reaction of compounds such as thiocarbohydrazide and galloyl glucose (tannic acid) with glycoproteins, glycolipids and glycosaminoglycans, large complexes are formed, so glycocalyx and filaments appear thicker in SEM than in TEM images of ultrathin sections, as demonstrated by Los *et al.* (1992).

From TEM images, it is known that the outer surface coat of stereocilia consists of a loose network of filaments that extends from the cell surfaces into the scala media and can be detected, in particular, along the stereocilia and surfaces of the supporting cells (Santi and Anderson, 1987; Osborne and Comis, 1990a). These studies suggest that the outer filamentous network on the stereocilia and supporting phalangeal cells and the amorphous material of the undersurface of the tectorial membrane could be different molecules. Thus, the presence of more glycocalyx at the tip of the stereocilia than the bottom is reasonable.

Antennulae

The question is: are the tiny fibrillar structures or antennulae seen on the surface of the tallest stereocilia of the OHC real structures or artefacts due to the preparation/fixation procedure? From the micrographs shown, it obvious that there is a gradient in thickness of the glycocalyx with antennulae, the proteinaceous layer is thinner, and less antennulae are found at the base of the stereocilium compared to the apical part. Micrographs of conventionally (GA/OsO_4) fixed OHC, taken at 2.0 and 5.0 kV, respectively, at the same location, show a structure resembling the glycocalyx seen in TAO postfixed samples.

The TAO treated sample taken at 0.5 kV shows, despite the inferior resolution, the structure of the antennulae at the outside, due to scanty penetration of the primary electrons, so almost no bulk signal arrives at the detector. Moreover, there is no disturbing effect of a conductive coating due to the low penetration power.

At 2.0 kV, the thin conductive Au-coating, necessary to minimize charging, is covering the glycocalyx. This image suggests that the fibrillar coat is real. Whether this protein coat is disturbed by an inadequate GA/OsO_4 fixation and/or the presence of the Au layer and the impact of the electron beam is not certain. The image taken at 5 kV of the Au-sputtered sample moreover indicates the presence of a protein layer with thick fibrils. Observation of the image on the screen confirmed that the glycocalyx with antennulae morphologically is changing in time due to the impact of the electron beam and pollution. This effect can be particularly well observed in Au-coated samples, which is understandable since Au particles have the tendency to agglomerate under irradiation by the electron beam. Hence, much thicker fibrils will occur in Au-coated samples compared to samples which have not been made conductive with Au or samples which have been treated according the TAO or OTOTO procedure. The accumulation of Au particles, particularly when applied via diode sputtering, is apparent in FEG-SEM images (Stokroos *et al.*, 1995). The antennulae are seen over the whole surface of the tallest stereocilia of the OHCs, with an obviously lower density at the base. They are seen in GA/OsO_4 fixed, OTOTO and TAO fixed stereocilia as well, so their appearance is not connected with one special postfixation method and in agreement with investigations of de Groot and Veldman (1988). The presence of the glycocalyx with antennulae is not due to the effect of galloyl glucose, nor to the presence of a Au-coating, because even in the absence of Au, the glycocalyx with antennulae is present. This suggests that the binding of osmium to the galloyl glucose, which is bound to the proteinaceous substances of the glycocalyx, improves the secondary electron emission to such a level that low voltages can be used without deterioration of the image.

Due to the binding of thiocarbohydrazide or galloyl glucose to the glycoproteins and glycosamines of the glycocalyx followed by binding of osmium, both the thickness of the layer and that of the antennulae increases. A similar phenomenon has been described for hyaluronic acid components bound to collagen in the vitreous body of the eye (Los *et al.*, 1992; Jongebloed *et al.*, 1993). TEM images of those structures are several sizes of magnitude smaller than their SEM equivalents. In the TEM images, the contrast is obtained by osmium and staining with uranyl acetate/lead citrate. A similar effect could occur with the stereocilia glycocalyx when non-coating SEM images are compared with TEM images of thorium treated samples. The presence of antennulae is not likely the result of crystallization since the different rinsing steps remove the excess of chemicals not bound to the tissue, thus preventing precipitation. X-ray microanalysis demonstrated only the presence of high amounts of osmium. Other tissues, such as bacteria with fimbria postfixed in the same way, never showed any form of salt crystallization or proteinaceous aggrega-

tion. Intestinal microvilli, postfixed with the TAO or OTOTO method, nicely showed a thin glycocalyx with individual fibrils (penicillium) both in SEM and TEM (Kalicharan *et al.*, 1984). Microvilli postfixed with OsO_4 alone showed almost no glycocalyx due to aggregation and washing-out of fibrils. This could indicate an initial rather firm connection between the apical surface of the stereocilium at the undersurface tectorial membrane. When this attachment broke at the tectorial membrane because of shrinkage due to fixation and drying forces, part of the Hardesty layer from the connective tissue matrix from the tectorial membrane was torn off.

Stereoscopic pairs of images provide excellent additional information about the thickness of the glycocalyx layer and the location and arrangement of the filamentous structures. This may contribute to the understanding of findings in earlier studies, such as the blunt appearance of the top of the longest stereocilia and the dense blebs (Lim, 1986; Osborne and Comis, 1990a). These structures may represent filaments of the glycocalyx which have been fused to each other due to the fixation procedure or the conductive coating. Nevertheless, visualization of the stereocilia fine structure was very difficult with a SEM equipped either with a W- or LaB_6- cathode both in the high (15- 25) kV and low (5-10) kV range. In the low kV region, the inferior resolution sets a limit to detailed visualization, while in the high kV region the information from the bulk is superimposed on the surface information; therefore, surface detail gets lost in the bulk information.

Cross-links

The combination TAO non-coating, FEG-SEM and low kV proved to be the solution for observation of the OHC surface coat, filaments and cross- and top-links between the stereocilia of the organ of Corti. Imaging at 2-3 kV gives the optimal result, because the resolution is still good (approximately 2-4 nm) in comparison to 1-2 nm at 10 kV and higher. The penetration of the primary electrons is minimal at these low accelerating voltages, so mainly secondary electrons from the surface contribute to the image. Moreover, the image contrast is better at low kV because the background level due to secondary electrons from the bulk is low (Ansell and Capers, 1989; Jongebloed *et al.*, 1994).

Pickles *et al.* (1984), Hackney and Furness (1986, 1988), Osborne *et al.* (1988), Takumida *et al.* (1988), Osborne and Comis (1990b) and Van Benthem *et al.* (1992, 1993) have reported, in TEM studies, two kinds of stereociliary cross-links: **side-links**, connecting the stereocilia within the same row or from row to row, and **tip-links**, connecting the tips of shorter stereocilia to the flank of an adjacent longer stereocilium. The side-links are perpendicular to the stereociliary surface, consisting of a system of multiple strands. This suggests that the cross-links consist of a proteinaceous core and are surrounded by glycoconjugates. Visualization in FEG-SEM after treatment with galloyl glucose/arginine, which stains glycosaminoglycans, supports this hypothesis.

Side-to-side links and row-to-row links were present between all stereocilia, and were characterized by a strand of fine filaments. Tip-links, which are connections from the tip of the lower stereocilia to the adjacent longer stereocilia, were present. Possibly these tip-links are some kind of row-to-row links. Mechanical stimuli may deflect the stereocilia in several directions (de Groot, 1986; Gitter, 1994). The presence of the tip-links in our study is unlikely to be an artefact. Moreover, the high uptake of osmium in the TAO non-coating treated specimens avoids excessive shrinkage of both the tectorial membrane and cross-links. This may be the reason that many of these delicate structures are found almost undamaged in our samples.

Conclusion

Use of the TAO non-coating technique in combination with low-voltage FEG-SEM provides high resolution images of delicate surface structures, as seen in the stereocilia of the guinea pig in the organ of Corti. The 3-D morphology of fine structures such as cross-links, membrane structures and surface coating reveals interesting findings, which have never been demonstrated three-dimensionally before. The presence of antennulae as part of the glycocalyx of the OHC stereocilia seems a real morphological structure, although its function in relation to its structure is not yet completely understood.

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Discussion with Reviewers

M.P. Osborne: What do you mean by careful washing?
Authors: Careful washing means that, prior to the preservation of the glycocalyx by the TAO method, the number of rinsing steps should be kept to a necessarily minimum and that agitation should be very mild, because the glycocalyx is easily removed when not fixed properly. This is similar to the experience we had with preservation of the glycocalyx on intestinal microvilli.

A. Forge: Can you explain the significance of whole body perfusion versus local cochlea perfusion?
J.C.M.J. de Groot: Please explain the disparity between the results presented here and those in which perilymphatic perfusion was used.
Authors: We have performed a "partial" whole body perfusion, that is at the junction of the aortic arch and the aorta descendens the flow of fixative was blocked, so all fixative went to the head. With this method, we could quite well regulate the flow of fixative, and the preservation of the cochlea is very fast and immediately visible as discoloration of the tissue, while the risk for oxygen shortage is minimal. A local cochlea perfusion after explantation is done by injection of the fixative via a round opening in the bony capsule, and in fact, is a kind of immersion fixation. We think that the method we used gives a better preservation due to the shorter time involved to immobilize the fragile structures, while precipitation of salts is limited.

A. Forge: Why are antenullae not present in the stereocilia of the inner hair cells?
Authors: We did not find antennulae at the stereocilia of the IHC. A possible reason for their absence could be the fact that the stereocilia of the IHC are not touching the tectorial membrane, so no such microenvironments exist at the top of the stereocilia as can be found at the tallest stereocilia of the three rows of OHC.

J.A.M.A. Dormans: Would it be more appropriate to call this method ATO rather than TAO?
Authors: Regarding the sequence of chemicals used: first arginine/glycine/sucrose/glutamate followed by tannic acid/glutamate, you are right. But the basic principle of the method has been known for a long time, and the method is generally referred to as TAO because tannic acid is the principal ligand.

J.A.M.A. Dormans: Can you speculate about the presence of cross-links between cilia on the respiratory tract and how this will optimize movement?
Authors: The movement of the stereocilia of the hair cells is induced by the noise level offered. To defend the system from damages due to extreme movement of the stereocilia of one hair cell, they are interconnected by means of cross-links between stereocilia of one row and between rows of stereocilia with different lengths. The cilia of the epithelial cells of the inner lining of the respiratory system are not arranged in rows, but are equally distributed over the cell surface, so an interconnection would not be helpful; moreover, the role of these cilia is quite different from the stereocilia of the hair cells.

J.C.M.J. de Groot: Are the cross-links demonstrated in this study present in both the inner and outer hair cells stereocilia?
Authors: The cross-links shown are from OHC, presently we are investigating the occurrence of cross-links of the OHC-I, OHC-II and OHC-III.

J.C.M.J. de Groot: Please explain the significance of internal fixation?
Authors: Internal fixation is fixation/preservation of the bulk of the sample.

J.C.M.J. de Groot: Please explain why the cochlea was immersed for 24 hours in the same fixative after vascular perfusion?
Authors: The TAO method is based on the binding of glutaraldehyde (GA) with galloyl-glucose as the principal component of tannic acid. This complex can bind heavy metals as well as various protein groups, a "saturation" with GA therefore is advantageous for galloyl-glucose binding and osmium, and at the end, optimal preservation and conductivity.

J.C.M.J. de Groot: Did the authors notice a reduction in the number of the stereocilia in the middle and the upper turn after TAO fixation as was observed after OTOTO fixation?
Authors: We did not notice such a difference after TAO postfixation, but to be fair, we have to say that we did not investigate this phenomenon in particular.

N. Slepecky: Why were previous references dealing with cross-links between the stereocilia not included in this extensive review?
Authors: Our main interest was focussed on the glycocalyx; we merely have shown cross-links in a stereopair of images. Presently, we are investigating the location of cross-links in the OHC more systematically and will report on that in due time.

N. Slepecky: Why do the authors consider the extensions of glycocalyx rather than the filopodial extensions?
Authors: Images of ultrathin sections of hair cells suggest that the glycocalyx with antennulae is covering the outer surface of the stereocilia without cross-connections with the internal part of the stereocilia. This is proven by the fact that the glycocalyx layer easily is removed when the fixation procedure is not carried out adequately, as discussed in this paper. When the antennulae are considered as filopodia they should have some connection with the internal part of the stereocilium or be some kind of extension of the ciliary membrane, which they are not.