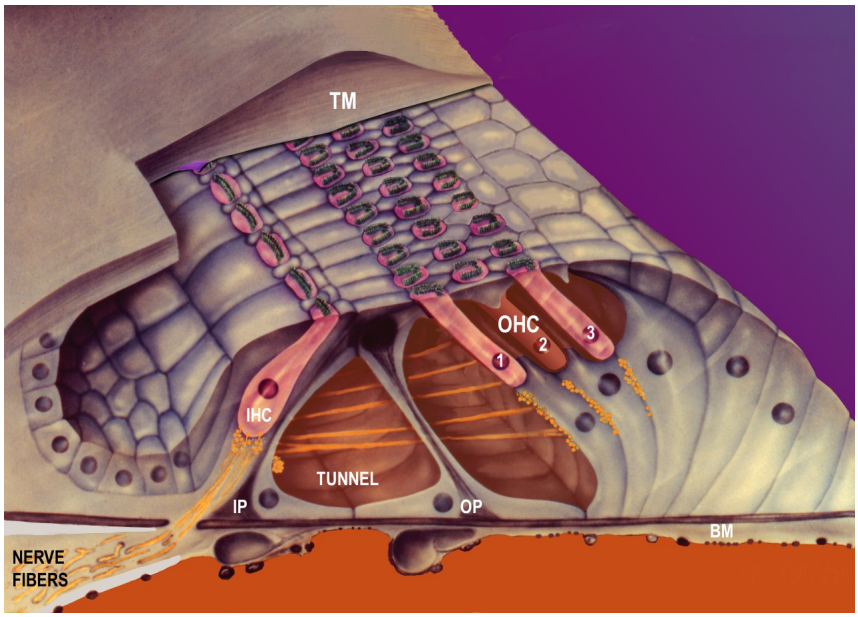
ANALYSIS OF THE NORMAL AND DAMAGED INNER EAR



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INTRODUCTION

After completing the dissection of the cochlea, the specimen is ready for quantitative anatomical evaluation. The evaluation should be done first on the flat preparations (Fig. la), using the phase contrast microscope. Once evaluation of the flat preparation has been completed, areas which are judged to require more detailed evaluation can then be sectioned (Fig. 1b) on an ultramicrotome and examined by light or transmission electron microscopy (TEM). Although dissection techniques may vary somewhat depending on the species under study (see Bohne, 2009), the analysis techniques are nearly identical for all animals.

It is assumed that readers of this manual have some knowledge of biology, in particular, histological techniques and the histological appearance of cells and basic tissue types. If not, the reader should refer to an introductory histology textbook.

This manual will describe the techniques used to: determine the length of the organ of Corti (OC), calculate the percentage of missing sensory cells, evaluate other pathological changes in the cochlear duct and prepare blocks of cochlear duct for semi-thick (1-2.5 µm) and thin sectioning.

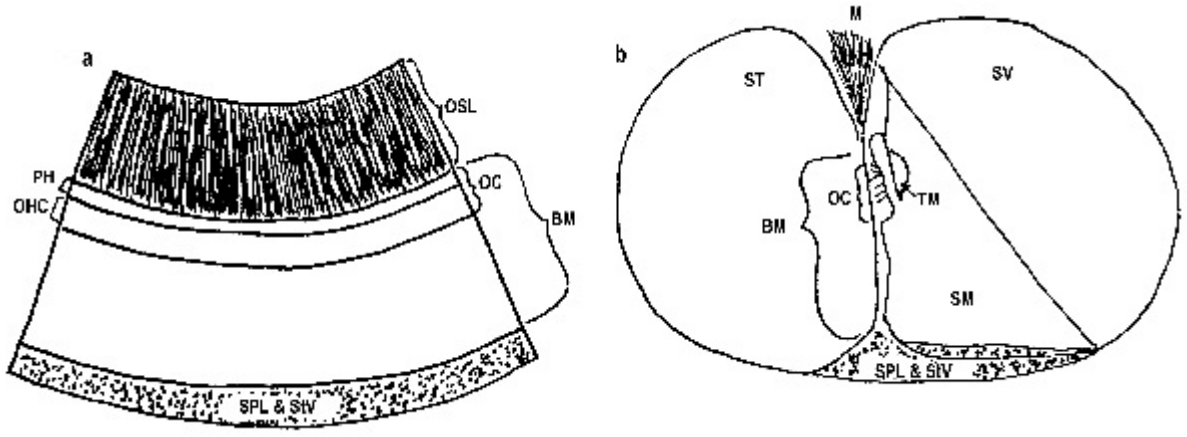


Figure 1: Schematic drawings of a segment of the cochlear duct (a) and cochlear scalae (b) viewed through dissection microscope. BM - basilar membrane; M -modiolus; OHC - lateral portion of organ of Corti (OC) containing outer hair cells, Deiters' and Hensen's cells; OSL - osseous spiral lamina containing myelinated peripheral processes of the spiral ganglion cells; PH - lines indicating inner pillar feet and lateral edge of outer pillar heads; SPL& StV - spiral ligament and stria vascularis; SM - scala media; ST - scala tympani; SV - scala vestibuli; TM - tectorial membrane. a) Horizontal angle; b) Radial angle.

Determination of length of organ of Corti and stria vascularis

In order to analyze quantitatively normal or damaged cochleae, the lengths of the individual segments into which the OC has been dissected must first be measured. Following removal of the blocks of trimmed cochlear duct from the oven after the second dissection, each segment of OC is measured to determine the total length of the specimen.

After cooling to room temperature, the plastic blocks containing the trimmed segments are removed from the peel-away molds by splitting the corners of the molds and bending the molds away from the plastic. Note that the surface which faced the mold (i.e., down) is dull while that which was in contact with air during polymerization (i.e., up) is shiny. The upper edges of each block should be trimmed with a razor blade or ground with a bench grinder until the block sits flat when its upper surface faces down. It is also important to trim the block until it fits into the aluminum holder (Fig. 2) that is used to manipulate the block on the stage of the Nikon zoom and Wild phase contrast microscopes.

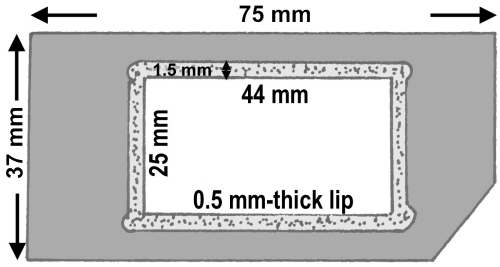


Figure 2: Aluminum holder used to manipulate the plastic block containing trimmed segments of the OC on the stage of the microscope.

Getting started

If not already turned on, boot the Black Knight computer. If not already present, place the digital camera on top of the Nikon microscope with the cable to the left. Tighten the set screw lightly to keep the camera from rotating. If not already present, install the 2X lens on the Nikon microscope. Set the scope magnification to 3.5 for a chinchilla ear or 7.5 for a mouse ear. Load the ImageJ software from the desktop icon, drop down the PLUGINS menu and select ACQUIRECAM. This will open an image window for the camera and a camera settings window. On the latter, adjust the camera exposure to about 10 ms with the slide bar. Turn the microscope light on and adjust the scope's light intensity to a little below maximum. Adjust the camera exposure further so as not to overdrive the camera. Close the camera settings window to reveal the full image window. Zoom the computer's display magnification down 2 steps (button labeled with spyglass and minus sign). Adjust the right and bottom edges of the window to match the size of the image source.

Calibration

Place the Hemocytometer slide on the microscope stage, locate the center area of one of the 2 counting grids with the x, y, and focus controls and adjust the light to get a clear image on the computer's display. This area has a graph paper-like grid which is exactly 1 mm by 1 mm with 5 major subdivisions, each with 4 minor subdivisions. Adjust the camera rotation to square the grid on the computer's display by loosening the set screw and resetting it. Take a picture (button labeled with camera) and minimize the live window. Select the broken line tool from the ImageJ toolbar. Move the cursor to the left edge of the grid at the location of a major horizontal line and left click to initiate the drawing of a line. Draw a horizontal line from the left edge to the right edge of the grid by moving the mouse and right click to terminate the line. A yellow line will indicate the line that you have drawn. Drop down the ANALYSE menu and select MEASURE. This will open a window with the number of pixels in the length of the line that you drew. Drop down the ANALYSE menu again and select SETSCALE. This will open a dialog window. Set the 'number of pixels' to the number that you just determined, leave the 'known distance' at 1.0, change 'units' to mm, and check the 'global' box. Then click the OK button. All subsequent images are now calibrated. However, **if the microscope's magnification is changed, the image will have to be recalibrated.** To do so, close the live image window, reload the ACQUIRECAM plugin and proceed as above.

Setting up

Because the Nikon microscope is non-inverting, place the plastic block in the aluminum holder so that the basilar membrane (dull side) is facing up and the stria vascularis is toward the front of the microscope. A small drop of immersion oil should be placed on each segment to fill in small scratches in the plastic and thereby improve the optical clarity. Place the holder on the microscope stage and adjust the X and Y controls to locate the first segment to be measured (3UU) and position the segment so that the entire OC is visible on the computer monitor. Adjust the focus and light level to obtain a clear image of the segment. Typically, a magnification of 3.5 with the 2X lens is best for chinchilla cochleae.

Measurements

Place the specimen block in an aluminum holder and the holder on the microscope stage with the stria vascularis toward the front of the scope. Maximize the live image window and open the camera settings window (button labeled with a wrench). Set the microscope light to maximum. Adjust the camera exposure so as to get a suitable image without overdriving the camera. Locate the OC segment to be measured using the x, y, and focus controls. Rotate the camera if necessary to orient the segment in the image field. Adjust the focus to get a clear image of the pillar heads. Adjust the camera exposure further to get a clear image. Take a picture (camera button) and minimize the live window. If not already selected, select the broken line tool from the ImageJ toolbar.

Using point and left-click, draw a line along the union of the pillar heads in short increments. Terminate the line with a right click. Drop down the ANALYSE menu and select MEASURE. If not already open, this will open a 'results' window containing line-length measurements in mm. The last entry will be the one just completed. Write this number down on a separate piece of paper, keeping track of which OC segment that you have just measured. Close the picture window (do not save), maximize the live camera window, and repeat the above procedure for the next OC segment.

Occasionally, the OP line may appear thick on the computer monitor. This usually results from the segment being embedded at an angle with respect to the bottom surface of the plastic block. In this case, the line should be made along the inner edge of the OP heads. In segments 3UU and RW_{LL}, the position of the last nerve fiber is a good marker for the apical and basal ends of the organ of Corti, respectively.

Areas of severe damage or total loss of the OC (i.e., OC wipeout), degenerated or atrophied stria vascularis and degenerated myelinated nerve fibers (MNFs) should also be noted in the segments. Atrophied or degenerating stria vascularis appears as an unstained area (Fig. 3) or a region of clumped or granulated material in the stria vascularis band. In these instances, an additional measurement procedure will be required (see below).

If the boundaries of an area of damaged OC are clearly defined when viewed through the microscope, the region of damage or OC wipeout should be measured separately by 'video splitting' the segment (Fig. 3). If the lesion edges are indistinct, measure the entire length of the segment only. If necessary, subdivisions of the segment can be made mathematically (see section on counting sensory cells). Degeneration of MNFs often accompanies damaged OC. This loss of fibers will appear as unstained or lightly stained areas in the osseous spiral lamina (Fig. 3).

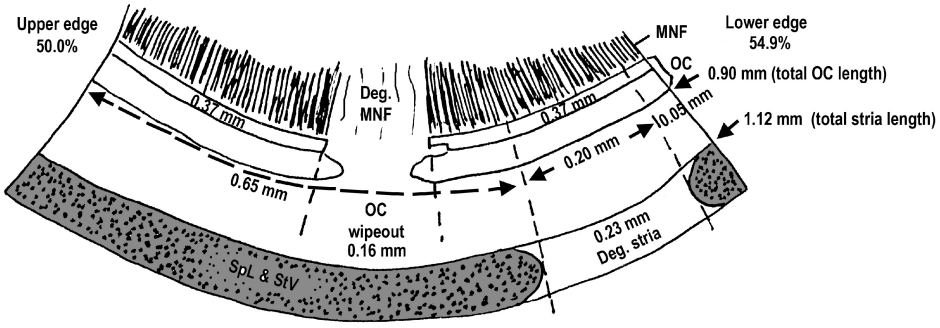


Figure 3: Schematic drawing of a damaged segment of cochlear duct including an OC wipeout, degeneration of MNFs and stria vascularis. Dashed lines have been drawn to indicate measurement subdivisions. See Figure 1 for abbreviations.

Occasional splits may appear in the OC due to artifacts in the plastic and/or the initial dissection. If these gaps are located in the pillar area, they are not included in the length measurements. The easiest way to handle this situation is to draw a line for each split, measure separately then add them together. These procedures insure that the length measurements include only actual OC and not empty space.

Areas of damaged OC that are visible in the dissection microscope must be checked under the phase contrast microscope to determine exactly where the segment should be video split. This area will be measured as a subdivision of the total segment. In order to prevent a mix-up in the location of small regions of damage, care should be taken to note the upper and lower ends of a segment which is to be subdivided. If all segments are oriented in the same fashion (i.e. basilar membrane facing the bottom and MNFs toward the same side of the block), then the upper end of each segment will be on the same end as the apical tip of the OC. Note that the upper ends of the segments from a right and left OC are on opposite sides (i.e., R versus L, respectively). Using the line drawing procedure described above, draw a line for the whole segment and measure its length. Then measure each of the subdivisions separately as described above which reflects the boundaries of the damage. If there are more than two such segments, their sum may be slightly in error due to the difficulty in precisely identifying the endpoints of each segment. Correct for these errors so that the sum of the line segments adds to total length (see below).

In the case of strial degeneration (SV DEG), the distance between the present areas of stria at its endolymphatic surface should be measured to give the millimeter extent of the degeneration. In order to map the region of SV DEG onto the cytocochleogram, the percentage distance of the OC adjacent to the SV degeneration must be determined. Using the same line drawing procedure described above, draw a line along the inner edge of the entire stria of the segment in question and measure its length (e.g., 1.12 mm; Fig. 3). Then mark off and measure the length of the degenerated strial region (e.g., 0.23 mm) and the intact strial region(s) (e.g., 0.81 & 0.08 mm). These results must then be mapped onto the measurement of OC length made previously. The beginning % of the SV DEG is determined by converting the region of organ of Corti corresponding to the stria present at the upper end to a % of the total length of the OC and adding this value to the beginning % of the segment. The ending % of the SV DEG is equal to OC length where stria is present at the upper end plus the OC length where the stria is absent in the middle, converted to % of the total length of the OC and added to the beginning % of the segment. In Figure 3, the beginning % is ((0.65/18.4 mm) x 100) + 50% = 53.5%. The ending % is ((0.65+ 0.20/18.4) x 100) + 50% = 54.6%.

If an OC segment is to be subdivided (Fig. 3), each subdivision needs to be measured, in addition to measuring the length of the whole segment. The total length of all subdivisions of a segment added together should equal the length which was determined when the piece was measured as a whole segment. If the sum of the subdivisions is not equal to the length of the whole segment, re-measure each

subdivision to make sure their lengths are correct. If all lengths are correct then a mathematical formula must be used to adjust the subdivision lengths so that their sum is equal to the length of the whole segment. Re-measure each subdivision to make sure their lengths are correct. If all lengths are correct then a mathematical formula must be used to adjust the subdivision lengths so that their sum equals the total segment length. The lengths of the subdivisions can be measured accurately but their sum can be incorrect because of the errors associated with the resolution of the measurement software. The more subdivisions a segment has, the more likely it is for the summed length to be incorrect. The sum of the subdivision lengths may be larger or smaller than the total length of the segment. The formula described can be used to adjust the lengths of the subdivisions to a larger or smaller value. The length of the whole segment is divided by the sum of the subdivision lengths and the resulting constant is noted. Each subdivision length is multiplied by the constant and recorded to the fifth decimal place. The last three digits of each subdivision length are inspected and the larger number(s) are rounded up until the sum of the subdivisions is equal to the total length of the segment. Sometimes the numbers may have to be rounded up when they are less than 0.005 mm. All new subdivision lengths are recorded, rounded to the second decimal.

For example:

Measured length of the total segment = .90 mm

length of subdivision A = 0.43 mm
length of subdivision B = 0.20 mm
length of subdivision C = 0.26 mm
Total = 0.89 mm

Since the sum of the three subdivision lengths is 0.01 mm less than the total length, new subdivision lengths must be calculated. Therefore:

$$\frac{\text{length of whole segment}}{\text{sum of subdivision lengths}} \quad \frac{0.90 \text{ mm}}{0.89 \text{ mm}} = 1.01123$$

1.01123 x 0.43 = 0.43483 (new length of subdivision A) rounds to 0.44
1.01123 x 0.20 = 0.20224 (new length of subdivision B) rounds to 0.20
1.01123 x 0.26 = 0.26292 (new length of subdivision C) rounds to 0.26
Total 0.90 mm

The last three digits of subdivision A (483) are larger than the last three digits of subdivisions B or C. Since the individual lengths need to be increased by only 0.01 mm, the length of subdivision A should be rounded up to 0.44 so that the sum of the subdivisions will equal 0.90 mm.

Correcting drawing errors

A drawing error can always be corrected by redrawing the line. On the same image, the initiation of a new line automatically removes the old line. In addition, the individual points of a line can be moved by placing the cursor over the point and then click, hold and dragging it to a new location with the mouse.

Finishing up

After measurements have been completed for the day, close the results window (do not save), maximize and close the live image window, and close the ImageJ toolbar. Turn off the computer and microscope.

Counting of sensory cells

On average, there are 1840 inner hair cells (IHCs) and 7500 outer hair cells (OHCs) in the chinchilla cochlea. Counting all of the sensory cells in a particular cochlea is a time-consuming task. In order to expedite counting, a technique for estimating the total number of sensory cells which should have been present in a given cochlea was developed. Hair-cell density per millimeter of the chinchilla organ of Corti was determined by counting the total number of sensory cells in 40 cochleae. These 40 specimens were chosen from our permanent collection so as to be evenly distributed over a wide range in length (i.e., 16.87 to 21.02 mm). From these data, linear equations were developed which describe the relation between hair-cell density (per mm) and total length of the OC. Because densities vary somewhat in the four turns of the cochlea, different equations were developed for each turn. These equations are as follows, where L is the total length of the OC in millimeters:

Third turn (T3) (0-21%)	IHC = 116.952-(0.864 x L) OHC= 454.401-(2.032 x L)
Second turn (T2) (21.1-47%)	IHC=108.315-(0.559 x L) OHC=392.447+(0.307 x L)
First turn (T1) (47.1-79%)	IHC=109.054-(0.754 x L) OHC=396.953-(0.652 x L)
Round-window hook (RW) (79.1-100%)	IHC=120.648-(1.141 x L) OHC=413.932-(2.061 x L)

To select the appropriate equation for estimating total IHCs or OHCs, determine the ending cumulative percentage distance of the segment in question. The cutoff

percentages for the different turns are given above. Make certain that the cumulative percentage of the segment in question falls within the range for that turn. The relevant above equation multiplied by segment length is used to calculate the number of sensory cells (i.e. denominator in the equation to calculate missing IHCs or OHCs) which should have been present in a particular segment of the OC from a given cochlea. For nearly all specimens, these calculations are done by the computer after the raw data are entered using the computer program "CYTOS".

Counts of missing IHCs should be done throughout the organ of Corti using the Wild phase contrast microscope at 500 or 1000X. These data should be recorded on the sensory cell count sheet (Appendix A). Sometimes, a few extra IHCs are present medial to the regular row of IHCs. This information should be noted on the remarks sheet (Appendix C) but it is not used in the calculation of the percentage of missing IHCs. Counts of missing OHCs should be done similarly in all segments except 3UU. It is very difficult and time consuming to count the missing OHCs in 3UU because the rows of hair cells are often irregular. Sometimes scattered cells or partial rows are congenitally absent. Because this region is infrequently damaged by ototraumatic agents, it is deemed acceptable to omit the counting of OHCs in 3UU.

Sometimes the phalangeal scars that replace missing OHCs do not have classical appearances. In some instances it appears that there was insufficient space in the reticular lamina for a hair-cell apex. In this case, the OHCs are considered to be congenitally absent (i.e., never formed during development) and therefore, are not counted as missing on the cell count sheet. A note should be made on the remarks sheet concerning congenital absence of hair cells.

The percentages of missing IHCs and OHCs are determined by dividing the number of missing cells by the calculated total. When hair cell loss is minimal, this calculated percentage missing is nearly identical to the actual percent missing because the most important number in the calculation is numerator not the denominator. In some cases it may be necessary to count both the present and absent sensory cells (see below) and override the computer generated total.

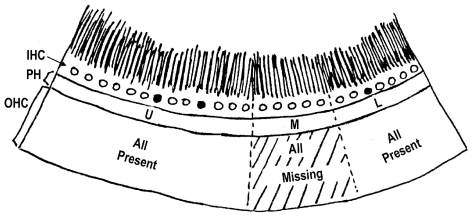
The cell counts which must be done under the phase contrast microscope depend on the degree of damage within the OC. In a particular cochlea, some of the segments may require counts of missing cells only, while others may require both present and absent cells to be counted. At the basal tip of the OC, the rows of OHCs are frequently irregular. Because of this irregularity, total cells cannot be estimated but rather the total number of sensory cells (present plus absent) in all rows must be counted.

In order to compare damage across cochleae, several different techniques have been described in the literature to summarize the extent of sensory cell loss within the cochlea. Each available technique has good and bad features. For example, calculation of the total percentage of missing sensory cells provides an easy way in which to

compare damage across animals. However, these data tell nothing about the pattern of damage along the basilar membrane. On the other hand, cytocochleograms which depict the exact location of every missing cell are too cumbersome to allow easy comparison across cochleae.

The percentages of missing IHCs and OHCs can be calculated for each physical segment (about 0.5 to 1.5 mm in length) into which the OC was divided during the second dissection. In this case, the percentage of missing cells in a given bin will depend upon the dissection. If the loss of cells is scattered throughout the segment, use of the lengths of the segments as bin widths will provide a satisfactory summary of the data. However, if small regions of focal loss of sensory cells are present within a physical segment, calculation of the percentage of missing cells in that segment will not provide an accurate representation of the pattern of damage. For example, if a physical segment was 1 mm in length (average of 400 OHC/mm) and 200 cells were missing, the percentage loss would be 50%. However, if all cell loss was confined to half of the segment, a better summary would be 100% for 0.5 mm and 0% for 0.5 mm.

A more accurate representation of the pattern of sensory cell loss along the basilar membrane is especially important when one is interested in comparing changes in auditory function to structural damage in the OC. In order to provide a better summary of the pattern of damage in the OC, a technique was developed to locate regions of focal loss of sensory cells within physical segments of the OC. This technique is illustrated in Figure 4 and described on subsequent pages.



❍ - cell present; ● - cell missing. Length of segment = 0.24 mm; # IHC = 25. IHC density equals #IHC/segment length or 25/0.24 or 104 IHC/mm.

Figure 4: Schematic drawing of segment that was mathematically subdivided and lengths and percentage missing sensory cells calculated in subdivisions.

Segment Portion	Total # IHCs	Length= #IHC (density)	# missing hair cells				% missing	
			IHC	OHC1	OHC2	OHC3	IHC	OHC
U	13	0.12 mm	2	0	0	0	15.4	0
M	5	0,05 mm	0	all	all	all	0	100
L	7	0.07 mm	1	0	0	0	14.3	0

In order to facilitate counting and to provide a way in which to subdivide mathematically physical segments, eight different patterns of loss (Table I) have been identified that cover all possible combinations of IHC and OHC loss. The different patterns (codes) are listed below where **m** = count phalangeal scars; **t** = count total cells (i.e. missing plus present); **p** = count present cells and c = computer will calculate total that should have been present. See Appendix G for examples of different patterns of hair-cell loss.

After the percentages are calculated by the computer and the data stored on disk, codes 3 and 7 convert to code 1; codes 4, 5 and 6 convert to code 0. Note that code 2 should be reserved for regions where there is a total loss of the organ of Corti (OC wipeout - all sensory and supporting cells missing). If all or most sensory cells have degenerated but some supporting cells remain, use code 5.

Table I: PATTERNS OF SENSORY CELL LOSS AND COUNTING STRATEGIES

		NUMERATORS				DENOMINATORS		
CASE	CODE	mIHC	mOHCl	mOHC2	mOHC3	tIHC	tOHC/row	Comment
Normal	0	**m**	**m**	**m**	**m**	c	c	Count missing cells only.
Actual	1	**m**	**m**	**m**	**m**	**t**	**t**	Count missing & present hair cells. This code is used for math splits (also codes 3 & 7).
Wipeout	2	c	c	c	c	c	c	No OC present. All values calculated by computer. Measure length of wipeout accurately.
m & tIHC pOHC	3	**m**	**p**	**p**	**p**	**t**	c	OHC loss large; only present OHCs can be counted. Count missing & total IHCs. This code can be used for math split.
mIHC & pOHC	4	**m**	**p**	**p**	**p**	c	c	Similar to code 3 but omit total IHC counts. This code **cannot** be used for math split.
pIHC & pOHC	5	**p**	**p**	**p**	**p**	c	c	Cell loss so great that only present hair cells can be counted.
pIHC & mOHC	6	**p**	**m**	**m**	**m**	c	c	High IHC loss. Count present IHCs & missing OHCs.
pIHC & m & tOHC	7	**p**	**m**	**m**	**m**	c	t	Similar to code 6. Count total & missing OHCs. This code can be used for math split.

Technique for mathematic subdivision

Orient the segment under the phase contrast microscope (500X) so that the spiral ligament is away from you when looking at the segment without magnification. If this is done, the upper end of the segment will be at the left in a right cochlea and at the right in a left cochlea. If the segment is to be subdivided on the basis of the IHCs (using codes 1 or 3), the following procedure is used. Count the total number of IHCs (present plus absent) in the segment. Divide this number by the millimeter length of the segment to obtain the IHC density in the segment. The value should be between 85 and 115. If not, recount, re-measure and/or recalculate.

Determine how many parts the segment will be divided into. The number of divisions may vary from 2 to 7 or more, depending on the amount and pattern of cell loss and the length of the segment (see section below on identification of segments which should be mathematically subdivided). Starting at the upper end, count the total number of IHCs (present plus absent) in the first subdivision. This value goes in the column headed tIHC. Make certain that this location can be recognized again. It may be helpful to draw the distinguishing features of the area on a split sheet (Appendix D). Count the total number of IHCs in each of the other subdivisions. Add up the separate counts. If this value does not agree with the original total count of IHCs, recount the number of cells in each subdivision.

Divide the number of IHCs in each of the subdivisions by the previously calculated IHC density to obtain the length of each of the subdivisions. Remember that rounding errors can occur when one length is divided into several parts. Make certain that the sum of the parts does not exceed the total length of the segment. Since the segment was measured to the nearest hundredth of a millimeter, the individual pieces have to be rounded to the nearest hundredth. If the sum of the rounded values differs from the original measurement, redo the calculations and increase or decrease one or more lengths so that the totals will agree. For example: if the total length of a segment is 1.24mm and it contains 120 IHCs, the IHC density is 96.77. Suppose the segment is to be divided into three parts.

The U part contains 50 IHCs = 0.51666 and rounds to 0.52 in length
The M part contains 25 IHCs = 0.25833 and rounds to 0.26 in length
The L part contains 45 IHCs = 0.46500 and rounds to 0.47 in length
1.23999 mm 1.25 mm

Since the sum of the rounded numbers is greater than the original length, only the lengths of the U and M parts should be rounded up because they have the largest values in the third decimal place.

Count the total number of OHCs in the second row in the upper subdivision. Be sure to count only as far as the dividing line. If the second row cannot be counted, count the first

row (and lastly the third row) (all code 1). If none of the OHC rows can be counted, make the piece a code 3. Once the limits for the subdivisions have been set and the total number of IHCs and OHCs in the different subdivisions has been determined, count the number of missing cells in each of the rows.

Note that if IHC loss is great and OHC loss is minimal, code 7 can be used to subdivide. In these instances, density is calculated from OHC counts and the segment subdivided using the OHCs instead of the IHCs.

Identification of segments which should be mathematically subdivided

One should answer the following questions in order to determine whether or not a dissected segment of the OC should be mathematically subdivided:

1. Is the loss of OHCs in the segment equal to or greater than 50% over a distance of at least 0.03mm (Fig. 5a)? If so, this lesion is termed an **OHC focal lesion**.

2. Is pattern of IHC loss such that a minimum of three IHCs in a row are missing or three out of four IHCs are missing in a particular region (Fig. 5b)? If so, this lesion is termed an **IHC focal lesion** and it should be subdivided from the rest of the segment.

3. Are both IHCs and OHCs missing in numbers equal to or greater than 50% over a distance of at least 0.03mm (or 3 IHCs)? If so, the lesion is termed a **combined focal lesion**.

4. Are all sensory and supporting cells missing in a portion of the segment (Fig. 5c)? If so, this lesion is termed an **OC wipeout**.

5. Areas in which remnants of the OC (e.g. a few Deiters' cells) are found should be labeled code 5 (Fig. 5d).

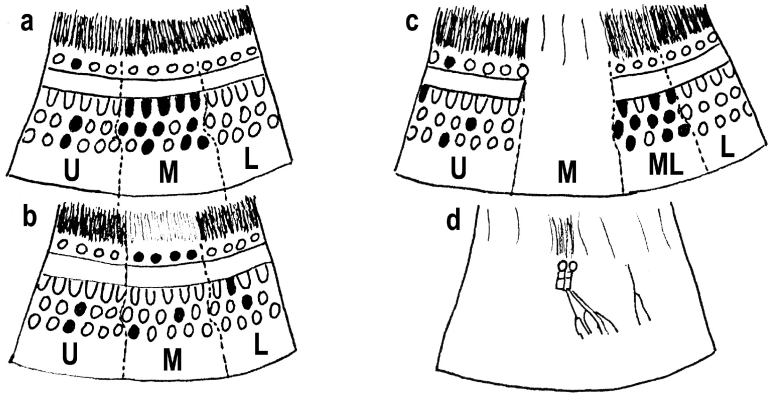


Figure 5: Examples of segments requiring subdivision. a) **Math split** - Focal loss of OHCs in **M** subdivision (i.e. OHC focal lesion); b) **Math split** - Focal loss of IHCs in **M** subdivision (i.e. IHC focal lesion); c) **Math split** - Region of high loss of OHCs in **ML** subdivision adjacent to OC wipeout in M subdivision; d) **Video split** - Remnant of OC (i.e. code 5) in center of dissected segment.

Subdivision Tips:

Look at the segment at a magnification of 500X in order to make a decision about subdivision. If most of the OHCs are gone while most of the IHCs are present in a physical segment, there is no reason to subdivide it.

If there is a wipeout in the segment, the segment will be subdivided into major parts by video splitting (Figs. 3 & 5c). Generally, there is high loss of OHCs adjacent to the OC wipeout. If this is the case, the region of high OHC loss adjacent to the OC wipeout should be split off arithmetically.

Potential artifacts in dissected segments of cochlear duct

With correct use of the plastic-embedding technique, preparation artifacts such as loss or distortion of the sensory cells are relatively rare. Nevertheless, since artifacts can occur, it is important to be able to identify them. Several preparation artifacts and their cause(s) are listed in the following table. Some of the artifacts make it more difficult to conduct the phase contrast microscopic evaluations whereas others can be confused with real pathological changes. If present in excess, either type of artifact may hinder or actually prevent the quantitative evaluation of a specimen.

In order to distinguish between artifacts and real pathology, one or more of the following questions must be answered:

1) Did any unusual events occur during surgery (e.g., excessive bleeding during the removal of the temporal bones (e.g., fracture through the cochlear bone), during the making of the infiltration holes (e.g., bone removed over spiral ligament at basal turn)? Check protocol book for comments.

2) Is the basilar membrane and tectorial membrane disrupted in the same region as the OC is damaged? If so, the OC damage is almost certainly an artifact. The BM is very rarely disrupted by exposure to ototraumatic agents such as noise.

3) Is the osseous spiral lamina present below (scala tympani side) the MNFs? If not, the loss of MNFs is probably due to a trimming artifact.

4) What is the appearance of the edge of the region where the stria is missing? (See drawing above)

5) Can the missing cells or parts of cells be found on the adjacent segment? If so, an angled cut was made through the OC.

6) Does the vertical gap in the OC also pass through the BM and/or TM? If so, this is an extra razor blade cut through the specimen.

7) Are there the same number of missing pillar feet as the suspected number of missing pillar heads? If not, the gap or part of the gap is an artifact (i.e., a pull-apart).

TABLE II: Preparation Artifacts and their Causes

Artifact	Cause
Small region of disruption of BM, OC, osseous spiral lamina &/or StV.	a) Tip of fixation pipette inserted too far into scala; or b) Tissue was torn when infiltrastion holes were made.
Dark-staining droplets in ST &/or SV.	Fixed lipid extracted from tissue entered scalae during processing.
Hazy or distorted appearance of OC.	a) Contaminant in ST (e.g., glass chips from broken pipette tip, bone chips or blood); b) Air bubble trapped in plastic.
Missing region of StV & SpL. 	Stria accidently removed during cochlear dissection because: a) Air bubble was present in SM, ST or SV; b) Plastic too soft; c) Too large a piece of bone was removed at once.
Vertical gap (colorless) through OC & BM.	Extra cut made through OC during 1st dissection.
Missing sensory cells or parts of cells at edge of segment.	Razor blade used to make vertical cut not held perpendicular to BM.
Light zone through OC near edge of segment.	Reflection of SV & SM on BM because of phase-contrast objectives.
Missing region of BM, MNF &/or pillar feet.	Area(s) trimmed away during 2nd dissection.
Gap or open hole in RL in long-term recovery cochlea.	Cells which form scars split apart by forces that were generated during dehydration, embedding &/or polymerization. This artifact is termed a pull-apart.

Estimation of myelinated nerve fiber (MNF) loss

The myelinated nerve fibers visible in the osseous spiral lamina are the peripheral processes of the primary auditory neurons (i.e., spiral ganglion cells). The bodies of these cells are located in Rosenthal's canal that spirals around the modiolus. When

hair cells degenerate, the nerve fibers that innervated these cells undergo retrograde degeneration. This degeneration initially appears as a disruption of myelin at the peripheral ends of the fibers, just medial to the habenulae perforata. Degenerating myelin stains less darkly and forms irregularly shaped ovoids. With longer recovery times, the degeneration of myelin progresses medially towards the ganglion cell bodies in Rosenthal's canal.

In order to determine if there has been any loss of MNFs in a given segment, the staining intensity of the segment in question must be compared to the remainder of the segments in the cochlea, as well as a comparable region of a non-damaged cochlea.

Estimate the percentage loss of MNFs (to nearest 5%) in the segment and then determine dieback distance (i.e., how far towards the modiolus the degeneration has progressed). Dieback distance lateral to the limbus is coded 1 (Fig. 6a); within the region of the limbus is coded 2 (Fig. 6b); medial to the limbus is coded 3 (Fig. 6c). On the cell count sheet where MNF loss is present, record the percentage followed by the dieback distance (e.g., 80, 2; Fig. 6d)

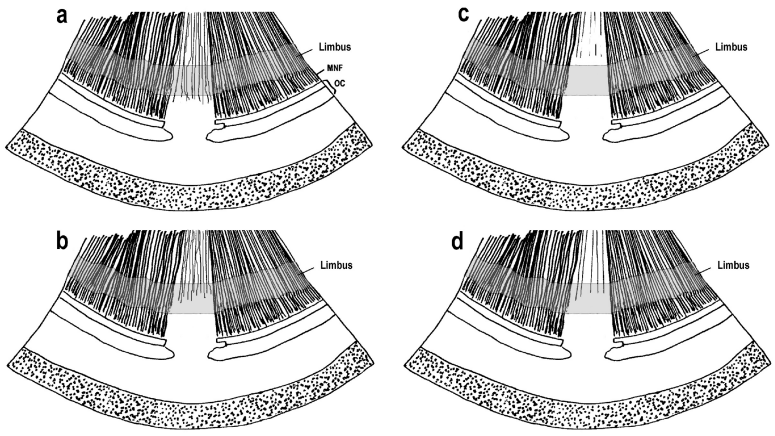


Figure 6: Coding of MNF loss medial to an OC wipeout: a) 75%, dieback 1; b) 75%, dieback 2; c) 95%, dieback 3; and d) 85%, dieback 2. In these hypothetical cases, the loss of MNFs is medial to an OC wipeout. The percentage loss of MNFs and dieback distance depend primarily on the extent of IHC loss and the length of the post-exposure survival time. Case '**a**' would likely be found at 1-2 wks post-exposure whereas case '**c**' would likely be found at least 1 month post-exposure.

Cytocochleogram of pathological changes throughout the cochlea

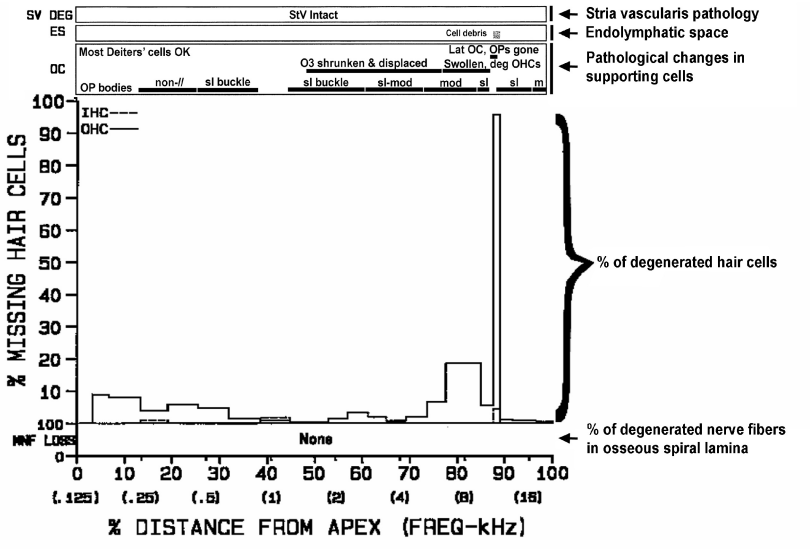


Figure 7: Cytocochleogram from a noise-exposed chinchilla. Data are plotted as a function of percentage distance (x-axis) from the OC apex (0%). A frequency-place scale below the % distance scale was fitted according to the formula of Eldredge et al. (1981); Data plotted from top to bottom of graph are: 1) Status of the stria vascularis; 2) Condition of the endolymphatic space; 3) Qualitative appearance of the hair cells and supporting cells of the OC; 4) Percentage of missing IHCs (dashed line) and OHCs (solid line); and 5) MNF LOSS - Percentage loss and dieback distance of peripheral processes of the spiral ganglion neurons. This cochlea sustained scattered loss of OHCs in the apex and base and a focal OHC lesion at 89% distance from the apex. Cellular debris is visible in the ES where the focal OHC lesion is located. The stria vascularis and MNFs are intact. OHCs are out-of-place and shrunken or swollen throughout most of the basal half of the cochlea. (Exposure: 4-kHz OBN, 92 dB SPL, for 24 h, 1 wk post-exposure recovery).

Data entry into the computer

Once the appropriate cells have been counted in each segment of a given cochlea, the data are entered into the computer in sequential fashion from apex to base. Note that if a segment of the OC was lost during the dissection, the cochlea cannot be used for computer analysis because the program converts length in millimeters to a percentage length. Without the length of the missing piece, the total length of the OC will be incorrect and therefore the calculation of total hair cells will be incorrect.

The data entry and graph program called 'CYTOS' is Java based and it runs under Windows, Mac or Linux. The data sheet summarizing the percentage of missing hair

cells and the cytocochleogram graphically depicting histopathology as a function of percent location in the cochlea can be plotted on any printer installed on the computer.

The CYTOS program performs the following:

● - Calculates % missing hair cells vs. % distance from cochlear apex and plots values on a percentage sheet (Appendix C).

● - Plots cytocochleogram according to data collected by microscopy.

● - Edits raw data (code, length of segment, # of missing hair cells, etc.).

● - Stores data in a text file which has 16 columns and no limit of rows. Some severely damaged cochleas have as many as 110 segments (counting both physical segments and mathematic subdivisions) in which cell loss has been determined.

● - Calculates total # of IHCs and OHCs in the cochlea (based on average densities and/or actual counts).

● - Allows one to average cell loss (integrates area under the curve in a cyto-cochleogram) in any particular region of the cochlea. The user can enter the lower % cutoff and upper % cutoff of region(s) of interest. Following the calculation of the average % missing hair cells, the computer summarizes data on focal lesions in the apical and basal halves of the cochlea and OC wipeouts.

The CYTOS program has 4 main buttons:

1 Enter Data

2 Open File

3 Import File

4 Exit

#1 (Enter Data): It opens a table to enter data from the keyboard. All information needed to fill the table should be available on the cell count sheet (Appendix A; i.e., animal #, exposure, piece #, code, length, m or p cells, total # cells, % MNF loss, Dieback distance).

#2 (Open File): Open existing file for viewing or corrections.

#3 (Import File): Import and convert files from the old MSHC7 program.

#4 (Exit): Exit and close the program.

Both #1 and #2 buttons have sub-buttons: 'INSERT ROW' - to insert extra row in the table; 'CALCULATE' - to calculate total IHCs and OHCs per row, and 'PRINT' - to print the table.

When you have an open file or table, the main panel buttons are changed to:

1 Save File

2 MSG Table

3 Close File

4 Exit

#1 (Save File): Save the data to text file.

#2 (%MSG Table): Plots the % missing sheet (Appendix B) for the cochlea. %MSG Table has two sub-buttons - 'PRINT' to print the table and 'PLOT GRAPH' to plot cytocochleogram. On the graph, the user can change the colors to black and white, to choose only % mIHC, % mOHC or both to display and the regions of interest. That graph can be saved as a png image or printed.

#3 (Close File): Close all tables and file.

#4 (Exit): Exit and close the program.

Pillar counts

Another quantitative evaluation which helps to describe the pattern of OC damage is the counting of missing inner and outer pillar cells (IPs & OPs). Missing pillars are counted and recorded by segment sequentially from apex to base on the remarks sheet (Appendix B). Subdivisions which were made when counting the sensory cells are also observed when counting missing pillars. When observing the segment at 500X with the phase contrast microscope, look for both the pillar heads and the feet. Checking the pillar feet is important since cuticular plate material is sometimes missing in the pillar head. This abnormality gives the appearance of a missing pillar when the body is actually present. In areas of severe damage, there may be too many missing pillars to be able to count them accurately. In this case, count the pillars that are present and record this number in the upper right corner of the appropriate line of the remarks sheet. The number of missing pillars can then be calculated by using the equations below that describe the density of IPs and OPs as a function of OC length for the four turns of the chinchilla cochlea.

Third turn (T3) IP = 249.716-(3.529 x L)
(0-21%) OP = 151.555-(0.586 x L)

Second turn (T2) (21.1-47%)	IP = 253.330-(4.099 x L) OP = 153.683-(1.094 x L)
First turn (T1) (47.1-79%)	IP = 251.282-(4.072 x L) OP = 155.478-(1.443 x L)
Round window hook (RW) (79.1-100%)	IP = 246.221-(3.978 x L) OP = 148.439-(1.223 x L)

Determine which turn the segment is in by checking its cumulative percent distance (see percentage sheet). Determine the pillar density for the segment based on its percentage location and the total length of the OC. Multiply the density by the length of the segment or subdivision being counted. This will give the approximate number of IPs or OPs that should have been present in the segment. Subtract the number of pillars which are present from the calculated total to obtain the estimated number of missing IPs or OPs. This number should be recorded in ink on the count sheet below the number of present pillars.

White blood cells and macrophages

In the whole mount preparations, white blood cells (WBC) within the vessel of the tympanic lip of the osseous spiral lamina (VTL) and the vessel of the basilar membrane (VBM) and macrophages within the OC can be counted and noted on the remarks sheet. It has been noted (unpublished observations) that in control animals, WBCs are rarely seen in the vessels below the basilar membrane and macrophages are never seen within the OC. However, in cochleae hich have been recently injured by exposure to noise, there is a statistically significant increase in WBCs in the VTL and VBM. This increase in WBCs may be indicative of a chemotactic response to the products of cellular degeneration. After the acute stage of degeneration, the number of WBCs in the vessels returns to normal but macrophages appear in and around the damaged OC. After the degeneration has been completed and the OC has healed (i.e., phalangeal and epithelial scars have formed), the macrophages disappear. Thus, the counts of WBCs and macrophages within the cochlea may indicate whether or not the degeneration has been completed.

Qualitative evaluations

Depending on the objective of the experiment, qualitative evaluations may need to be done on sub-cellular components of the sensory and pillar cells as well as other cell types within the OC. These evaluations should be recorded on the remarks sheet (Appendix C). Structures which can be evaluated in whole-mount flat preparations include:

1. **Hair Cells**: Bodies - position, shape [e.g. apoptotic (i.e., shrunken), distorted, oncotic (i.e., swollen); Appendix E]; nuclei - position, shape (e.g. pyknotic, distorted, swollen); stereocilia - orientation (e.g. disarrayed), apparent density and appearance - (e.g. fused,

partly or completely absent - Appendix F); organelles - appearance, relative size, apparent density; lipofuscin - presence or absence;

2. **Pillars**: Bodies - appearance (e.g., non-parallel, slight, moderate or severe buckle); nuclei - position; dark-staining material in head and foot - appearance (e.g., light-staining, deficient); lipofuscin - presence or absence;

3. **Deiters' Cells**: Nuclei - position; bodies - position (e.g., detached from basilar membrane); phalangeal processes - shape; lipofuscin - presence or absence;

4. **Inner Supporting Cells**: Lipofuscin - presence or absence;

5. **Myelinated Nerve Fibers within the Osseous Spiral Lamina**: Condition of myelin (e.g., disrupted; myelin ovoids); loss of % of fibers;

6. **Non-myelinated Nerve Fibers in the OC**: Radial tunnel fibers - apparent density, appearance (e.g., string-like, beaded, attached debris); inner spiral bundle - appearance (e.g,. smooth, vacuolated); outer spiral bundles - appearance;

7. **Tectorial Membrane**: Appearance (e.g., split, shredded); position (e.g., trapped).

The condition of the blood vessels below the basilar membrane can also be evaluated in the flat preparations of the cochlear duct. Relative scales have been developed for this purpose which include: 1) red blood corpuscle (RBC) parameters (e.g., density, columns, aggregations and plasma gaps, and orientation); 2) perivascular cell (PVC) parameters (e.g., PVC frequency, PVC size, and the frequency of PVCs compressing the vessel lumen); and 3) vessel lumen parameters (e.g., lumen irregularity and lumen diameter) (Axelsson and Vertes, 1982).

For most of the structures listed above, quantitative data are either not available or cannot be obtained from the flat preparations. Hence, most of these evaluations are qualitative in nature. Nevertheless, these evaluations are important in order to obtain a comprehensive picture of the type and extent of pathological changes which are present in a particular cochlea. By analyzing as many cells and subcellular features as possible in the whole mount preparations, the sampling errors which are inherent in all studies of sectioned tissue can be largely avoided.

Preparation of samples of cochlear duct for sectioning

Before preparing the blocks of tissue for sectioning, it is important to: 1) determine the location and extent of the pathological change(s) in question; and 2) photograph the change(s) so that their appearance in the whole mounts can be correlated with their appearance in the sections.

The cochlear duct can be sectioned at one of three angles: **radial**, **horizontal**, or **tangential** (Fig.8). Each angle shows different cells or subcellular features to the best advantage.

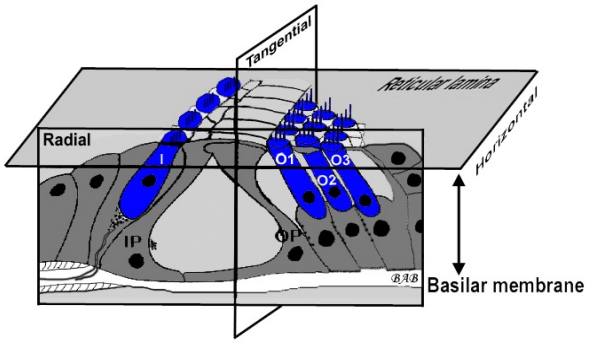


Figure 8: Schematic representation of the angles at which the OC can be sectioned: radial; horizontal; tangential. (Modified from Spoendlin, 1966).

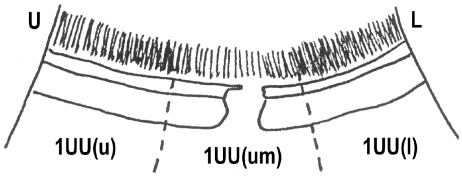
Radial sections, cut perpendicular to the line of pillar heads, are used to determine the cross-sectional area of hair cells and their nuclei. Other structures which can be evaluated in radial sections include: fluid spaces in the OC, including the presence of debris, tectorial membrane, nerve fibers in the inner spiral bundle, tunnel spiral bundle and outer spiral bundles; subcuticular organelles in IHCs and OHCs and infranuclear mitochondria in OHCs and nerve endings on the IHCs and OHCs. **Horizontal** sections, cut parallel to the reticular lamina, are used to count the stereocilia and the rows of cisterns of smooth endoplasmic reticulum in the peripheral membrane system of the OHCs. **Tangential** sections, cut parallel to the pillar lines, are used to examine the junctions between the cells which form the reticular lamina; to determine the number of efferent and afferent radial tunnel fibers; and to evaluate the tectorial membrane and its relation to the OC. Some of these structures can be evaluated in 1-2.5 micrometer thick, stained sections while others require thin sectioning and TEM examination for analysis. In some cases, only representative sections of the blocks of tissue need be cut (e.g., to determine the cross-sectional area of hair cells). However, in other instances, serial sections are required (e.g., to determine the number of nerve endings per hair cell).

Once it has been determined which segment(s) in a cochlea are to be sectioned and at which angle(s), the segments should be evaluated under the dissection microscope to identify any debris or artifacts which may interfere with the sectioning. It is also important to determine the upper (i.e., towards apex) and lower ends (i.e., towards base) of the segment to be sectioned. This can he easily done by comparing the segment to 3UU or RW_{LL}. This identification is necessary in order to determine the appropriate label for blocks and the direction of sectioning (i.e., towards apex or base).

If a quantitative anatomical (i.e., morphometric) analysis of the hair cells or nerve fibers is to be done, the percent location of the region to be sectioned must be determined. If the region is at the edge of the segment, then the percent location can be obtained from the cumulative percentage distance (see percentage sheet; Appendix B). However, in some cases, physical segments must be divided with a razor blade (Fig. 9) in order to begin the sectioning near the region of interest. In order to divide a segment, determine

the percentage length of the whole segment and then decide where the best region to begin sectioning is located. With care and a good eye for judging distance, it is possible to subdivide the segments into halves, thirds, quarters or fifths. The percent location at which sectioning will begin is determined by dividing the percentage length of the piece by the appropriate fraction and adding it to the cumulative percent of the segment apical to it. The exact designation of the segment to be sectioned is carefully noted. If the sectioning is to begin on the edge of a physical segment, add "(u)" to the segment name when the sectioning is in the basal direction; add "(L)" when sectioning is in the apical direction [e.g. 2UL($_u$), lUU($_L$)]. If the block to be sectioned is a division of a physical segment and the sectioning is to begin on the split, precede the "(u)" or "(L)" by an "(m)" for middle [e.g. 3LU($_{mL}$)].

In order to cut radial sections, use a sharpened, stainless steel pick to draw a trapezoid shape around the segment to be sectioned. Draw the narrow side perpendicular to the pillar lines where sectioning is to begin (Fig. 10). Make certain that the wide end of the trapezoid is broad enough to be stable when mounted in LKB vise chuck. Using a jeweler's saw with a #8/0 blade, saw the segment out of the block of plastic, carefully following the outlined trapezoid.



lUU($_u$) - 1.67% (50.0-51.67%)
lUU($_{um}$) - 1.67% (51.67-53.3%)
lUU($_L$) - 1.67% (53.3-55.00%)

Figure 9: Schematic drawing of segment 1UU (5% of total length of OC; located 50.0-55.0% of distance from apex) has been subdivided into three parts. Percentage locations and labels of subdivisions are indicated.

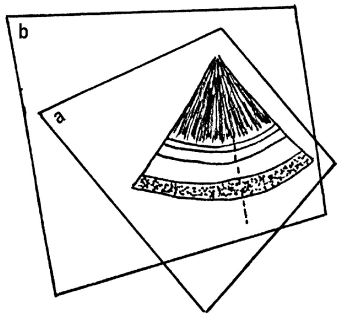


Figure 10: Placement of trapezoid for sawing out segment to be sectioned radially. a) Sectioning to begin at edge of physical segment; b) Sectioning to begin at split edge (dashed line) of physical segment.

Because only small blocks of tissue can be cut on the ultramicrotome, regions which are to be sectioned tangentially or horizontally must be split from the physical segment. Saw the segment to be sectioned out of the block of plastic, leaving a small margin of plastic around the segment. Use a quarter razor blade to split off the appropriate region of the segment to be sectioned (Fig. 11a). Be certain to hold the razor blade perpendicular to the tissue when making the split. Because the plastic tends to fracture when thick cuts

are made, split the block from the basilar membrane side. In this way, if the plastic fractures, the damage will occur in the upper part of scala media rather than through the OC. Since the pieces to be sectioned tangentially or horizontally are too small to be clamped in the vise chuck, re-embed these split segments in a thin layer of plastic. The pieces must be oriented differently, depending on the angle to be used for sectioning (Fig. 11b & c). Once this plastic polymerizes, draw a trapezoid shape around the subdivision and saw it out as described above.

Once the tissue has been sawed out, mount the block in the vise chuck. Orient the block in the chuck under the dissection microscope. For radial sections, the block should be oriented so that the pillar lines are parallel to and centered on the main axis of the chuck. For tangential sections, orient the pillar lines perpendicular to the main axis of the chuck. For horizontal sections, orient both the basilar membrane and the reticular lamina perpendicular to the main axis of the chuck. Once positioned correctly, be certain to tighten the screws of the chuck to hold the specimen securely, otherwise the block may move or vibrate during sectioning. Tighten the three screws of the chuck so that the gap between the jaws of the chuck is equal in width from top to bottom. Tape a label on the front of the chuck noting the segment name and the edge being sectioned.

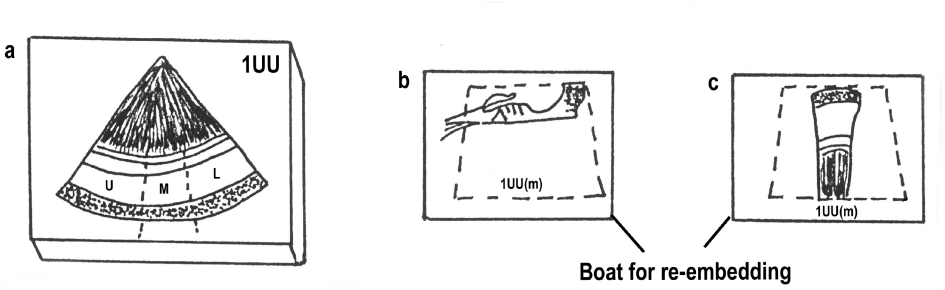


Figure 11: a) Schematic of physical segment sawed out of block of plastic. Dashed lines indicate location of area to be removed for horizontal or tangential sectioning; b) Orientation for re-embedding of split segment for horizontal sectioning; c) Orientation for re-embedding of split segment for tangential sectioning. Dashed lines on b and c indicate placement of trapezoid for sawing tissue out of plastic block.

If there is plastic beyond the tissue on the edge to be sectioned (i.e. face), smooth it using quarter pieces of razor blades under the dissection microscope. This is done by shaving away thin layers of plastic until the tissue can be seen clearly through the plastic. Keep the face of the block as flat as possible. Do not trim so far that tissue is trimmed away. Once the specimen can be clearly seen, the excess plastic on the face which. surrounds the tissue is trimmed away to form a trapezoid. For radial and tangential sections, the trapezoid should be oriented so that the face of the block will have parallel edges running along the basilar membrane (BM) and above the tectorial membrane (TM) and angled sides that are closer together by the TM and wider apart at the BM (Fig. 12a & b). For horizontal sections, the trapezoid should be oriented so that

the face of the block will have parallel edges running parallel to the pillar lines (Fig. 12c).

The objective of this trimming is to make a smooth face close to the tissue so that very little plastic needs to be removed before reaching the tissue and nearly complete sections of the face are made at the start of sectioning.

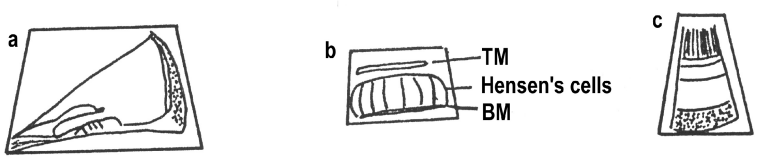


Figure 12: Orientation of trapezoid on face of block to be sectioned. a) Radial angle; b) Tangential angle; c) Horizontal angle.

The faces of the blocks to be sectioned should be made just large enough to include the region of interest. The excess plastic and any extraneous tissue (e.g. spiral ligament in tangential block) should be trimmed away. This procedure will generally make the sectioning much easier. Smaller block faces are usually required if thin sections are being cut for TEM examination. Usually blocks which are being readied for thin sectioning must have important parts of the tissue trimmed away. Thus, before facing a block for thin sectioning it is important to cut one-micron sections first so that the sectioned appearance of all of the cells can be determined before the thin sectioning begins. This enables the investigator to interpret the TEM findings much more easily.

The techniques for the analysis of the normal and traumatized cochlea which are described in this manual appear in part in several publications (e.g., Determination of OC length and the percentage of degenerated sensory cells - Bohne, Bozzay & Harding, 1986; Estimation of the percentage of degenerated MNFs -Bohne, Marks & Glasgow, 1985; Definition and determination of focal hair-cell lesions - Bohne, Zahn & Bozzay, 1985, Bohne, Yohman & Gruner, 1987, and Bohne, Gruner & Harding, 1990; Determination of the base-to-apex location of OC sections - Bohne & Carr, 1985). This manual was prepared in order to provide the beginning student with step-by-step instructions for the different analysis techniques in one convenient source.

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APPENDIX A: Sensory cell count sheet

EAR # _____ TREATMENT _____
RECOVERY _____

Piece	Segment	CODE	LENGTH mm	mIHC	mOHC1	mOHC2	mOHC3	tIHC	tOHC ROW	%MNF LOSS
1			.							
2			.							
3			.							
4			.							
5			.							
6			.							
7			.							
8			.							
9			.							
10			.							
11			.							
12			.							
13			.							
14			.							
15			.							
16			.							
17			.							
18			.							
19			.							
20			.							
21			.							
22			.							
23			.							
24			.							
25			.							

Column headers

Segment - label on dissected segment of OC (e.g. 1UU, RWu)

Code - segments of OC are coded (0-7) on the basis of the pattern and amount of hair cell loss. See Table I 'Patterns of Hair Cell Loss and Counting Strategies' for an explanation of the different codes and which hair cells must be counted.

APPENDIX A: Sensory cell count sheet (cont.)

Column headers (cont.)

Length - length of physical segment (or part of physical segment) in millimeters.

mIHC - missing inner hair cells

mOHCl - missing outer hair cells in first row

mOHC2 -missing outer hair cells in second row

mOHC3 - missing outer hair cells in third row

tIHC - total inner hair cells

tOHC/row - total outer hair cells per row

% MNF loss - % loss of nerve fibers and dieback distance.

Possible entries for missing sensory cells

3rd death pathway - Cell is near normal in shape but lacks an intact basolateral membrane.

a - apoptotic hair cell. Cell is shrunken with dark-staining cytoplasm & pyknotic nucleus.

ALL - written in columns labeled mIHC, mOHCl, mOHC2 and mOHC3. This is used when the entire OC has degenerated in the segment (i.e. OC wipeout).

cong. msg. - congenital missing hair cells. These cells did not degenerate as a result of trauma but rather never formed during development of the ear. This anomaly is generally limited to the extreme apical and basal ends of the OC.

I - immature phalangeal scar replacing missing hair cell

M - mature phalangeal scar replacing missing hair cell

O - open hole, place where hair cell has recently degenerated and not yet been replaced by a phalangeal scar in the reticular lamina.

on - oncotic hair cell. Hair cell is swollen, nucleus has shifted near cell center & organelles have be lost.

rem - remnant cells of OC, usually Deiters' or Hensen's cells. These may be seen in an area in which all sensory cells and nearly all supporting cells have degenerated.. If remnant cells are found the lesion is not a wipeout.

APPENDIX B: Percentage sheet

Ear # _____

PIECE #	SEGMENT	% LENGTH	CUM %	% msg. IHC	% msg. OHC
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					
19					
20					
21					
22					
23					
24					
25					

Column descriptors:

% length - length of segment converted into percentage of total cochlear length.

Cum % - Percentage distance of segment from apex of cochlea.

% msg. IHC - percentage loss of IHCs in segment.

% msg. OHC - percentage loss of OHCs (i.e., 3 rows combined) in segment.

APPENDIX C: Remarks sheet

Ear # _____ Qualitative assessment _____

PIECE #	Segment	mIP	mOP	OHCs	IHCs	Deiters'	OPs	IPs	Remarks
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									
16									
17									
18									
19									
20									
21									
22									
23									
24									
25									

Column headers

Segment - label on dissected segment of OC (e.g. 1UU, RWu)

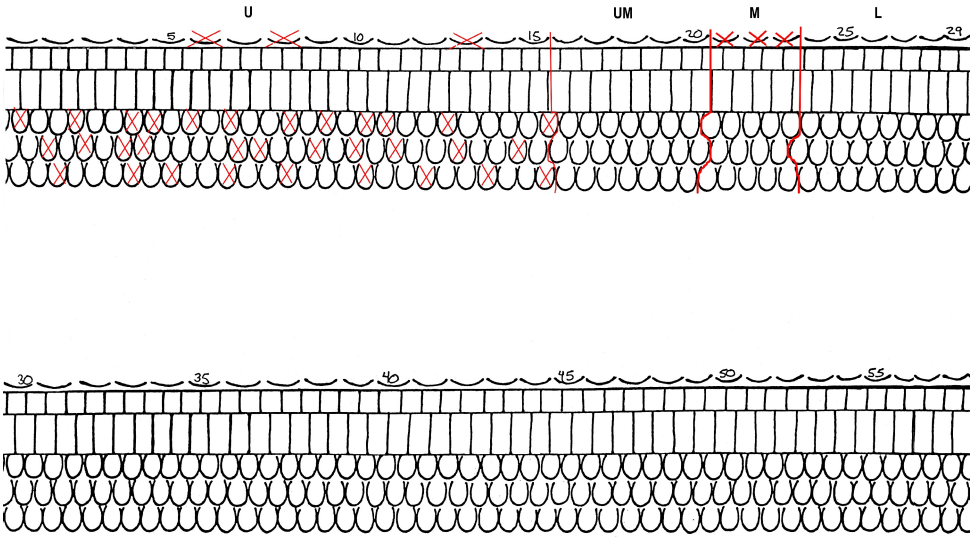
mIP - missing inner pillar cells

mOP - missing outer pillar cells

Qualitative assessment - Shape of remaining IHCs and OHCs; attachment of Deiters' cells to basilar membrane; condition of IP and OP bodies (e.g., moderately buckled).

REMARKS - any other histological findings noted in segment should be recorded in this section. Information such as degenerated stria vascularis, condition of stereocilia, RTFs, dissection artifacts should be recorded here.

APPENDIX D: Sheet for indicating position of math splits



The following structures are indicated on the sheet: numbered lines - IHC stereocilia; small boxes - IP heads; large oblong boxes - OP heads; open ovals - OHCs in 1st, 2nd and 3rd rows. Red 'x's' indicate missing hair cells. To indicate where a split is to be made (e.g., between U and UM) , count the IHCs from the left edge of segment and record the number in the tIHC column (15). Draw a line on the sheet across the pillar heads and OHCs. This line should delineate the border between the two math subdivisions. Count the second row OHCs (present plus missing) and enter this number (27) in the tOHC column. Count the missing IHCs and OHCs in the first subdivision and enter these numbers in the mIHC (3), mOHC1 (12), mOHC2 (11) and mOHC3 (9) columns, respectively. Repeat these operations for the other subdivisions.

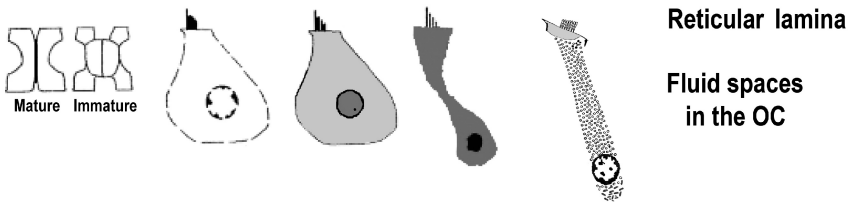
APPENDIX E: Hair-cell pathology

A normal hair cell has a typical shape (i.e., OHC - long and cylindrical; IHC - flask-shaped), the typical arrangement of stereocilia (see Appendix F), an intact plasma membrane and a uniform color and appearance of the nucleus.

Pathological hair cells have been classified primarily on the condition of the plasma membrane and nucleus. These classifications can be distinguished in the flat preparations by phase contrast microscopy. In order to determine mechanisms of cell death, it is important to distinguish among the different death pathways that the different hair cells are following (Bohne et al., 2007).

A missing hair cell has been replaced by a **mature phalangeal scar** (i.e., **M** - dark line between adjacent phalangeal processes), an **immature phalangeal scar** (i.e., **i** - pale, thin line between adjacent phalangeal processes), an **open or partially closed hole** (i.e., **o** - phalangeal processes surrounding the degenerated hair cell had not completely enlarged to form a scar when the cochlea was fixed). Cellular debris may be present on the reticular lamina, in the hole and/or below the reticular lamina.

An **oncotic** (**on**) hair cell has a grossly swollen body, an enlarged, pale-staining nucleus, and may or may not have fused stereocilia. An **apoptotic** (**a**) hair cell has a shrunken, dark-staining body and nucleus (i.e., pyknotic), and may or may not have fused stereocilia. A cell dying by the **3rd death pathway** (**3rd**) has the long cylindrical shape of an OHC but its basolateral membrane is deficient. Its nucleus is deficient in nucleoplasm but is located near the base of the cell. Often the chromatin forms clumps on the nuclear membrane. The stereocilia may be deficient or fused. A **necrotic** (**N**) hair cell is grossly swollen, has an enlarged, pale nucleus and a ruptured plasma membrane. Often, the stereocilia are deficient or fused.



	Cell characteristics		Nuclear characteristics	
Classification	Size	OsO_4-staining	Size	OsO_4-staining
Necrotic	Indeterminate	Very pale	Enlarged	Very pale
Oncotic	Swollen	Pale	Normal	Normal to pale
Apoptotic	Shrunken	Dark	Shrunken	Dark
3rd death pathway	Normal	Stippled	Normal to enlarged	Stippled

APPENDIX F: IHC Stereocilia pathology

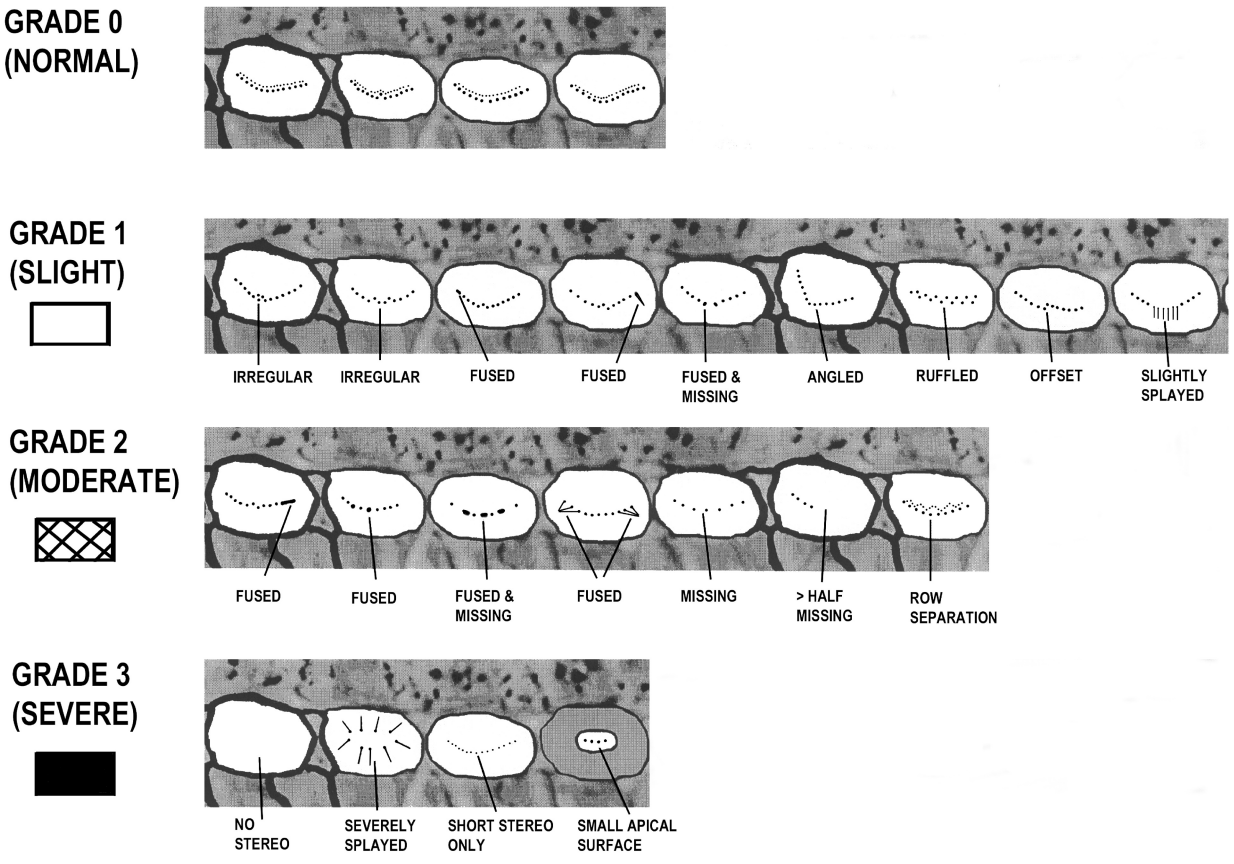
Stereocilia pathology may adversely affect cochlear function. This schematic drawing shows the different types of pathology found in the IHC stereocilia after noise trauma. These alterations have been graded according to their hypothesized effect on hair-cell function.

Normal - Stereocilia not fused. Some may be slightly out-of-line or a few may be missing. These alterations are not thought to have an adverse effect on hair-cell function because most of the stereocilia tip-links will be intact.

Slight - Stereocilia irregular in position; some may be fused, missing or slightly splayed. These alterations are thought to have only a slightly adverse effect on hair-cell function because only a few tip-links are missing or oriented incorrectly.

Moderate - A moderate number of stereocilia are missing, fused or oriented incorrectly. These alterations are thought to have a moderately adverse effect on hair-cell function because a moderate number of tip-links are missing.

Severe - This grade of alteration is uncommon compared to 0, 1 and 2. These alterations are thought to have a profoundly adverse effect on hair-cell function because most or all tip-links are missing.



Appendix G: Photomicrographs of the reticular lamina, phalangeal scars, focal hair-cell lesions & OC remnants

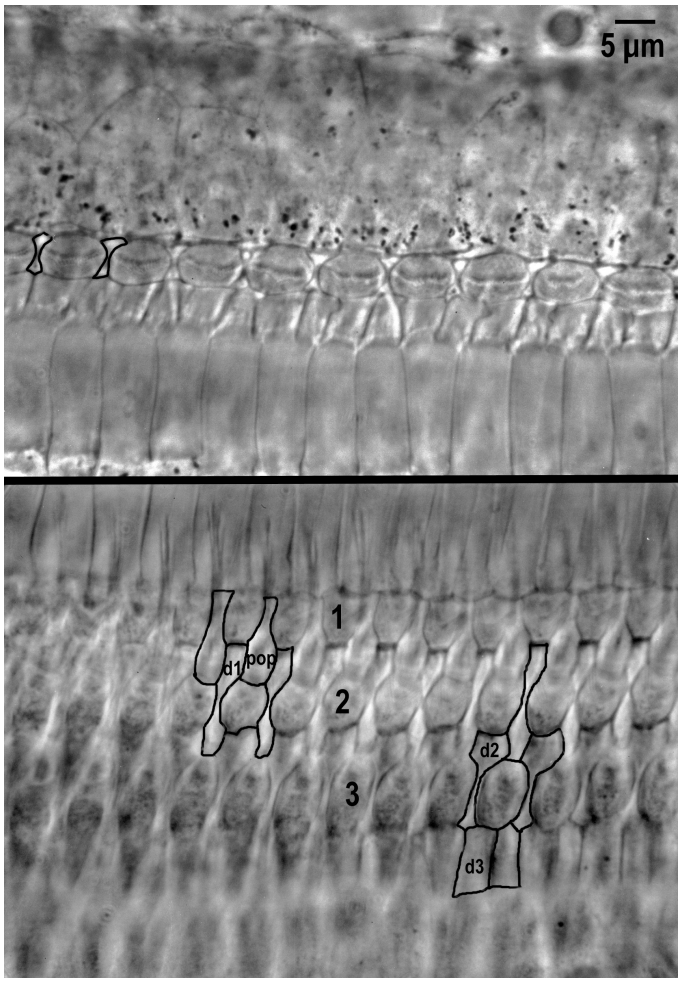
Accurate determination of the number of missing hair cells in damaged organ of Corti requires one to identify present hair cells and phalangeal scars that replace degenerated hair cells. In this Appendix, photomicrographs are included to show the arrangement of cells that form the reticular lamina, and the typical appearance of mature phalangeal scars replacing inner hair cells, and outer hair cells in the first, second and third rows. When several adjacent sensory cells in a particular row are missing, the resulting scars may not have a classical appearance, especially in the second row. Finally, recently formed phalangeal scars, termed "immature", have a distinctly different appearance than mature scars.

In noise-exposed cochleas, damage generally begins with degeneration of scattered OHCs in that region of the cochlea which is maximally stimulated by the exposure. With continued exposure, focal losses of hair cells may develop. Focal lesions have been defined by Bohne and Clark (1982) as a region of the organ of Corti at least 0.03 mm in length (i.e., linear distance of 3 IHCs) in which at least 50% of the IHCs, OHCs or both cell types are missing.

Examples of OHC, IHC and combined focal lesions are shown below. In many instances, focal lesions do not extend the entire length of a dissected segment. Therefore, in order to determine the length of the focal lesion, the segment must be math or video split. If one row of sensory cells can be counted across the entire segment, the segment should be math split. On the other hand, if the stretch of missing hair cells is too long for an accurate count of scars, the segment must be video split.

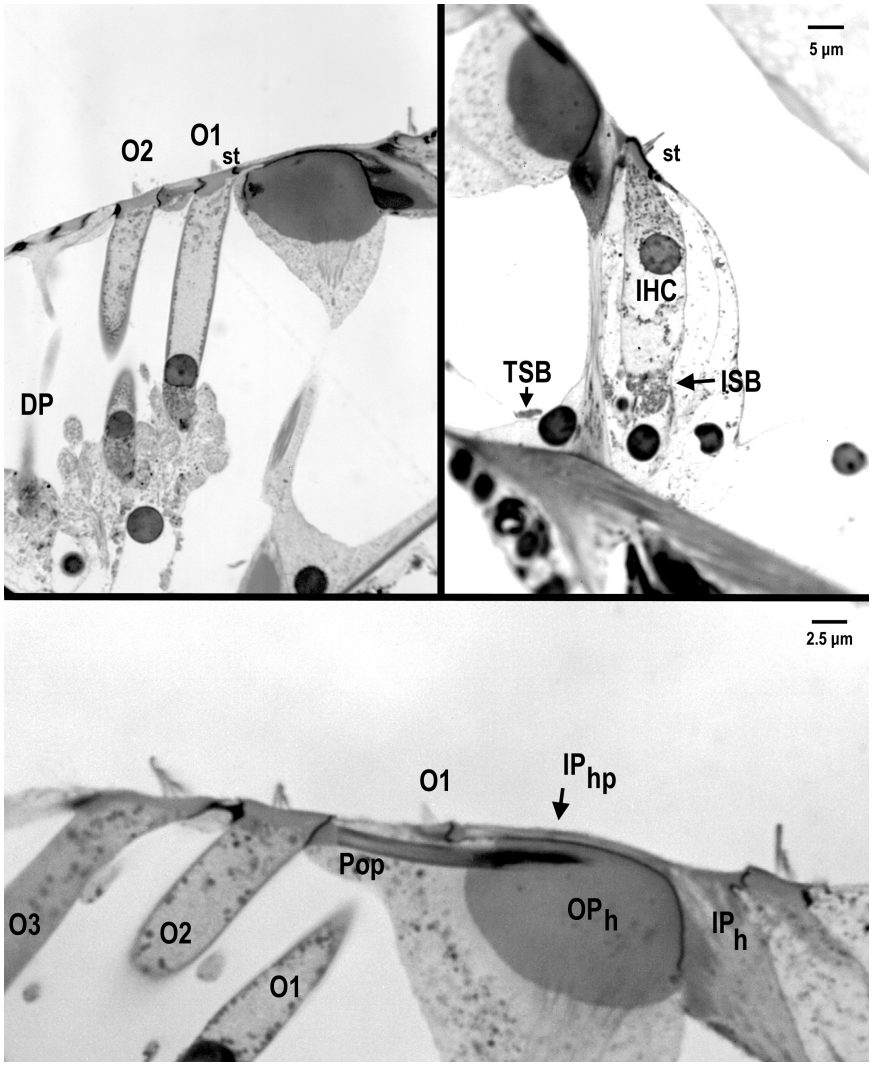
In regions of severe hair cell loss, it is not possible to accurate count missing hair cells. In this case, the segment should coded as 5 and present cells, rather than missing cells, must be counted. Organ-of-Corti remnants that generally contain only a few hair cells should also be coded as 5.

RETICULAR LAMINA



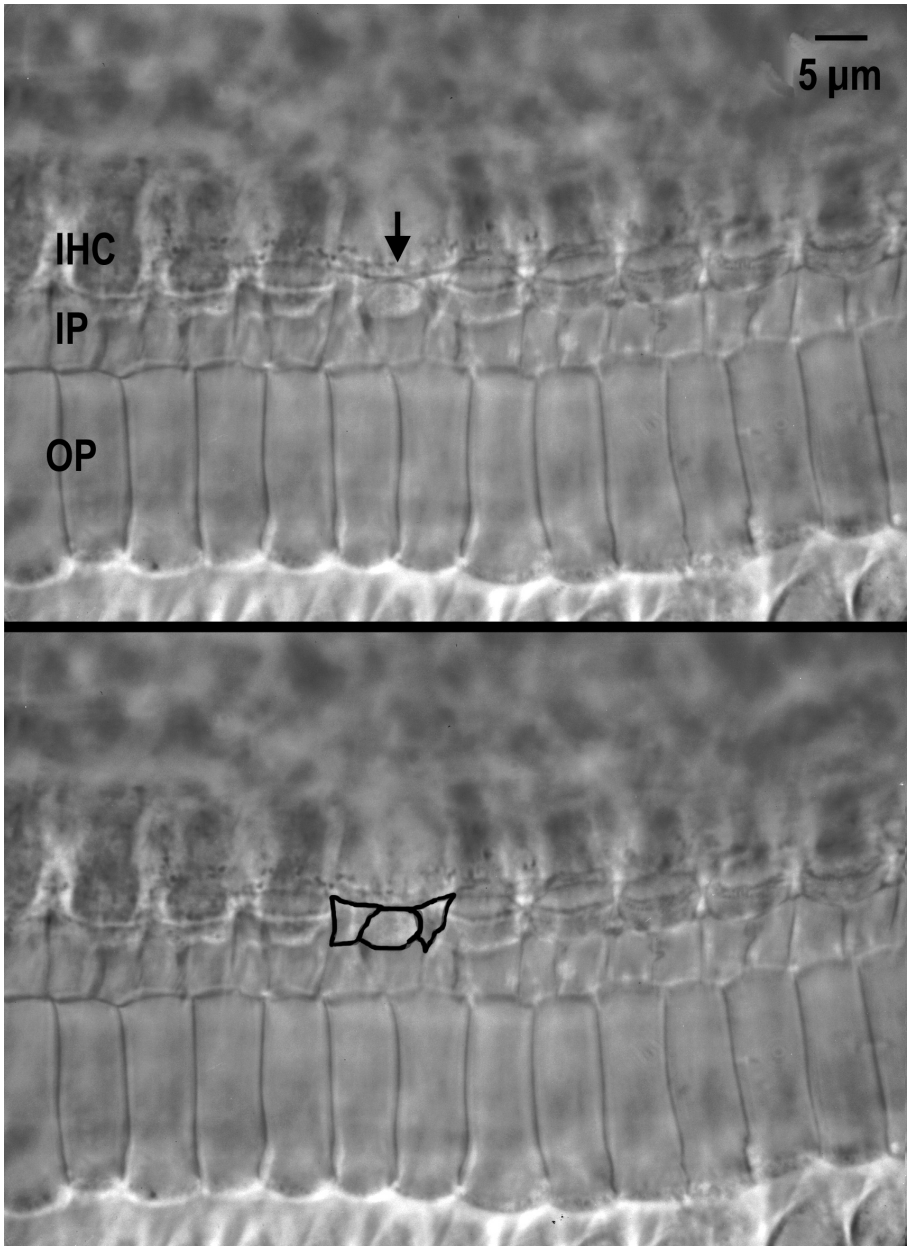
The reticular lamina is composed of the apical surfaces of the hair cells (i.e., inner hair cells; upper photomicrograph) and outer hair cells (1, 2, 3; lower photomicrograph) alternating with phalangeal processes. Between IHCs are processes from the inner phalangeal cells. Between first-row OHCs are processes from the outer pillar heads. Between second-row OHCs are processes from the first-row Deiters cells. Between third-row OHCs are processes from the second-row Deiters cells. Lateral to the third-row OHCs are processes from the third-row Deiters cells. When a hair cell degenerates, the surrounding phalangeal processes enlarge to form a 'phalangeal scar'. By counting phalangeal scars, the extent of hair-cell loss can be determined.

CELL RELATIONS in RETICULAR LAMINA



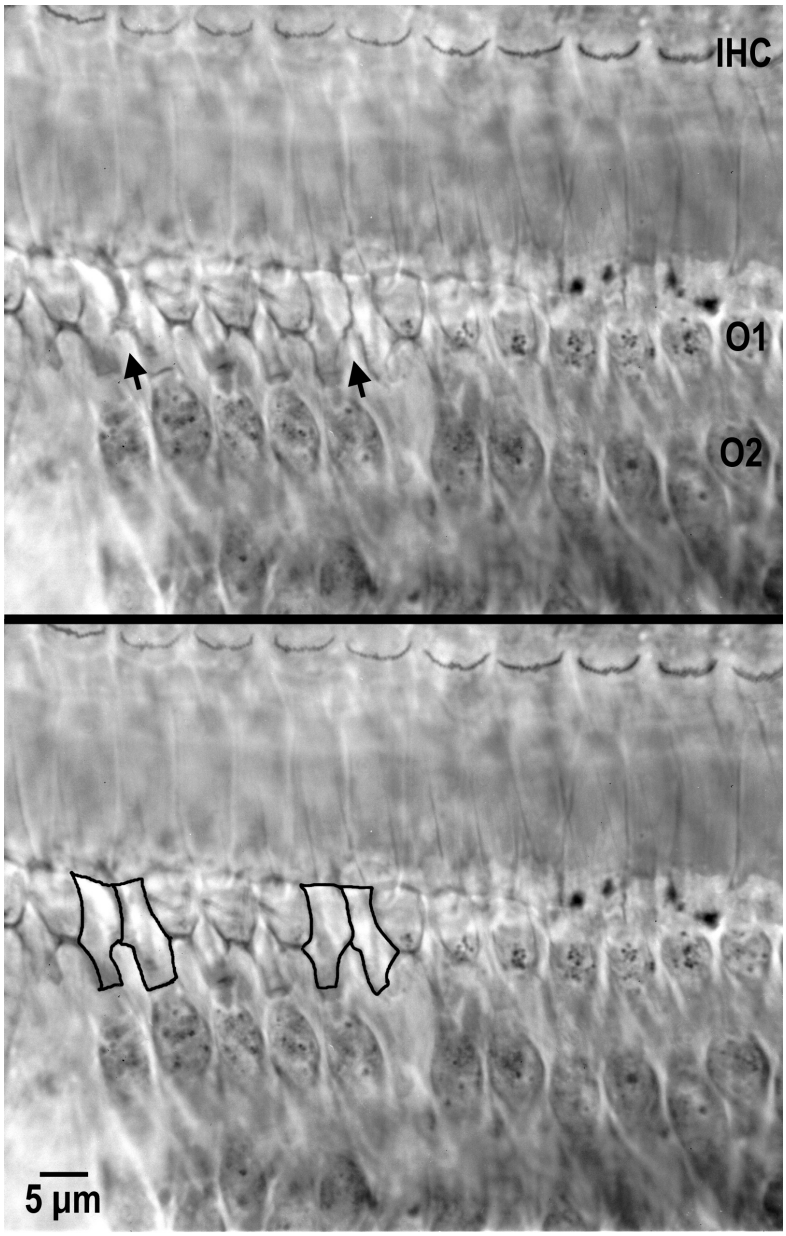
One-micrometer-thick radial sections of the organ of Corti showing the cell junctions that form the reticular lamina. Note that the medial borders of O1 and O2 are contacted by the inner pillar headplate and the phalangeal process from the outer pillar head, respectively. DP - slender process of Deiters' cell; IHC - inner hair cell; IP_h - inner pillar head; IP_{hp} - inner pillar headplate; ISB - inner spiral bundle of nerve fibers; O1 - 1^{st}-row outer hair cell; O2 - 2^{nd}-row outer hair cell; O3 - 3^{rd}-row outer hair cell; OP_h - outer pillar head; P_{op} - phalangeal process from outer pillar; st - stereocilia bundle; TSB - tunnel spiral bundle of nerve fibers (methylene blue-azure II stain).

PHALANGEAL SCAR REPLACING DEGENERATED INNER HAIR CELL



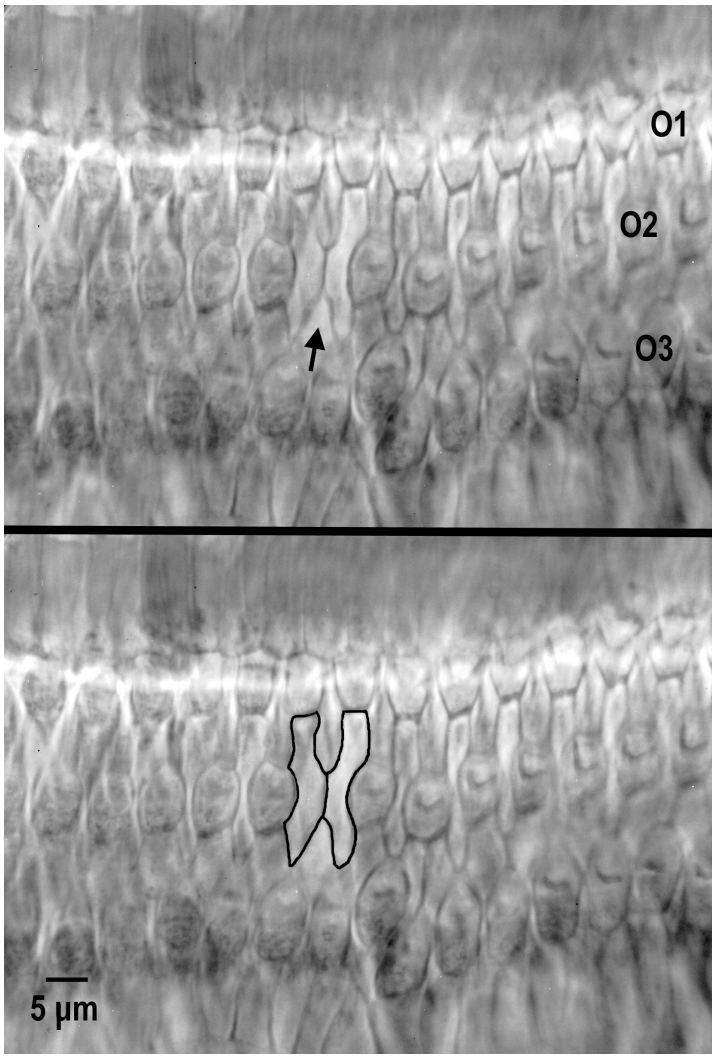
The phalangeal scar (arrow) replacing a missing inner hair cell (IHC) is formed by processes from two inner phalangeal cells and one inner pillar head. The scar is outlined in black in the lower photomicrograph. The dark appearance of the junctions indicates a deposition of dark-staining material in the processes adjacent to the plasma membranes. The presence of this material indicates that the scar is mature.

PHALANGEAL SCARS REPLACING 1ST-ROW OHCS



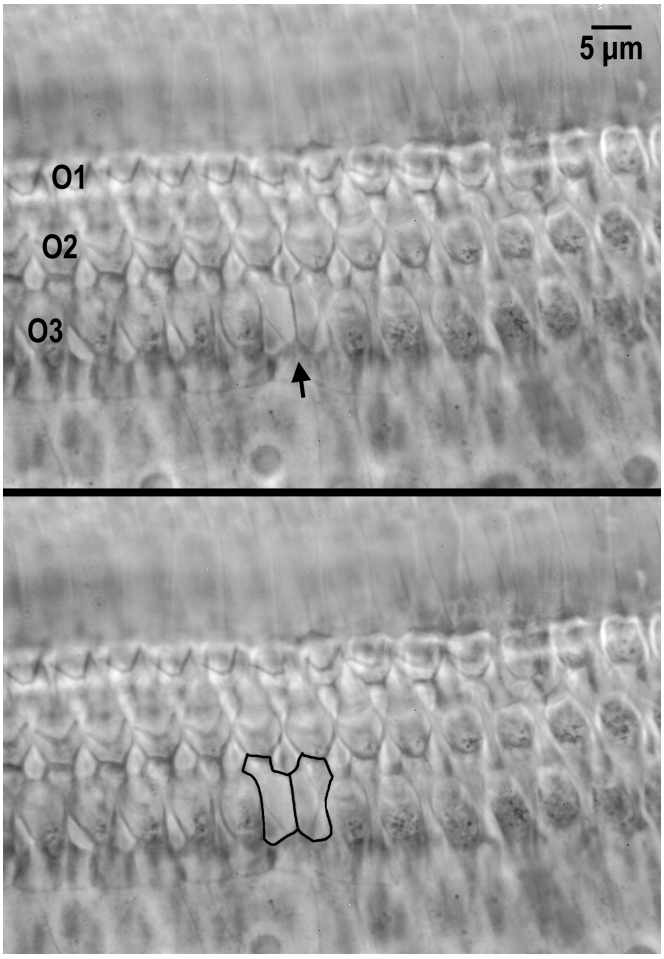
The phalangeal scars (arrows) replacing two missing 1st-row outer hair cells (O1) are each formed by processes from two adjacent outer pillar heads. The scars are outlined in black in the lower photomicrograph. The dark appearance of the junctions indicates a deposition of dark-staining material in the processes adjacent to the plasma membranes. The presence of the dark material indicates that these are mature scars.

PHALANGEAL SCAR REPLACING 2nd-ROW OHC



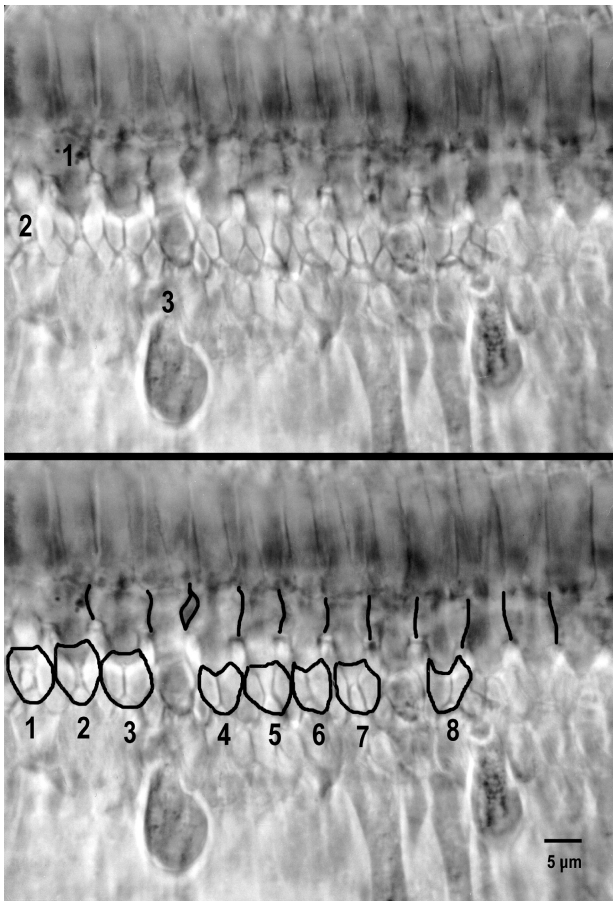
The phalangeal scar (arrow) replacing a missing 2nd-row outer hair cell (O2) is formed by processes from one outer pillar head, two 1st-row Deiters' cells and one 2nd-row Deiters' cell. The scar is outlined in black in the lower photomicrograph. This is a classical-appearing, mature second-row scar.

PHALANGEAL SCAR REPLACING 3rd-ROW OHC



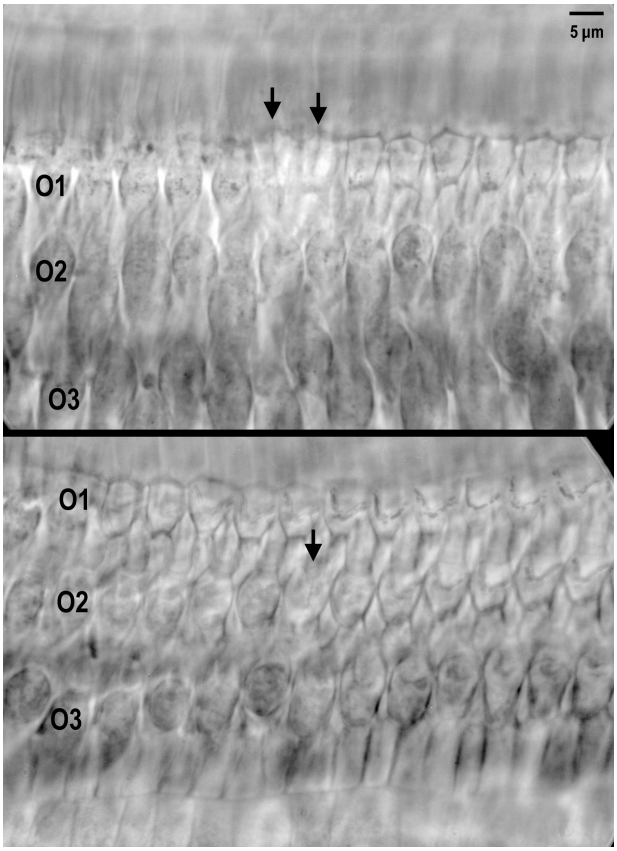
The phalangeal scar (arrow) replacing a missing 3rd-row outer hair cell (O3) is formed by processes from two 2nd-row Deiters' cells. Note that a 'ghost' of the degenerated cell appears in the scar. This ghost is a remnant of the original tight-junction material between phalangeal processes and hair-cell apex. However, because there is a dense line of union between the two phalangeal processes, the scar should be considered mature.

VARIATION in SCAR CONFIGURATION



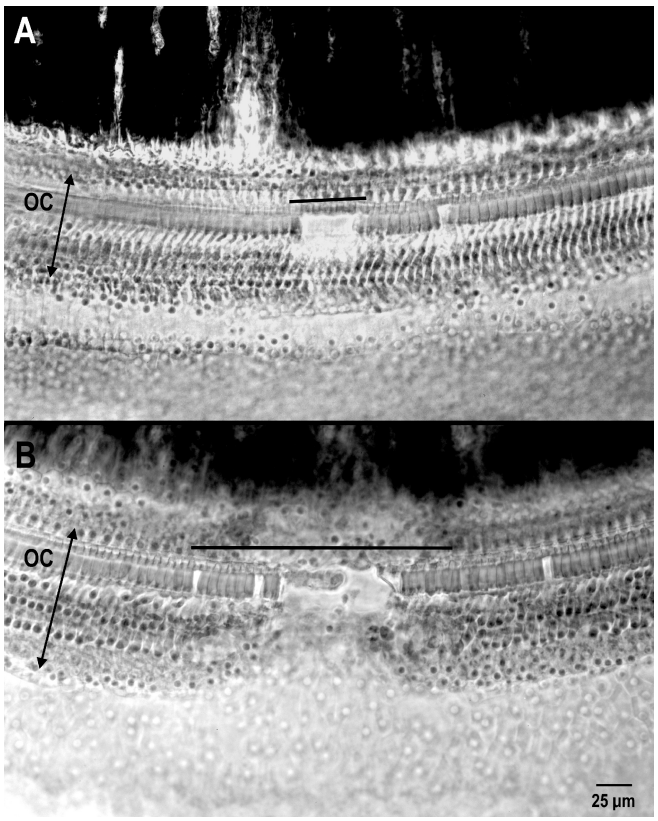
When several hair cells in a row are missing, phalangeal scars may not have the classical appearances as illustrated on the preceding pages. Eleven scars are visible in the 1st row and eight in the 2nd row of OHCs. In the lower photomicrograph, the center line of the 1st-row scars has been blackened while the 2nd-row scars are outlined in black. The 2nd-row scars are quite variable in appearance.

IMMATURE SCARS



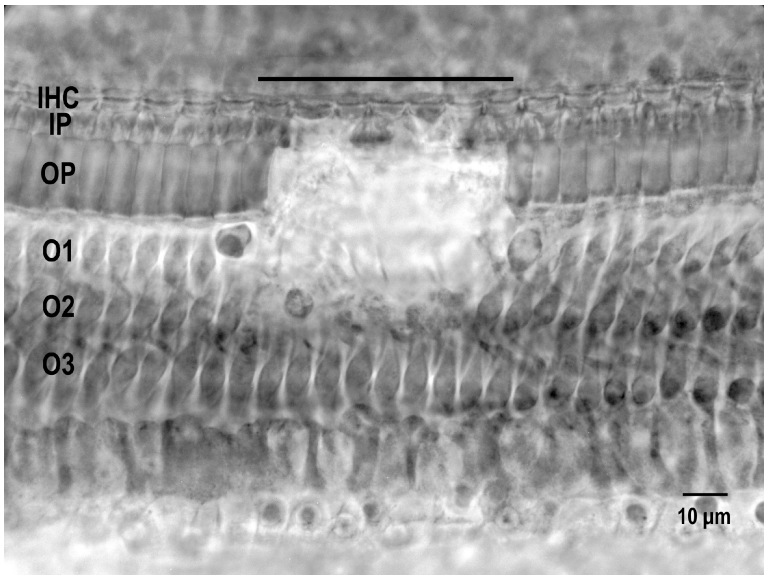
Immature phalangeal scars are those in which the phalangeal processes have come together but the dense junction material has not yet been deposited next to the adjacent cell membranes. Thus, only a faint line is visible between adjacent processes as compared to the dense line seen in mature scars. 'Ghosts' of the degenerated hair cells are visible. The upper photomicrograph shows two immature scars (arrows) in the 1st row of OHCs (O1), and the lower photomicrograph shows one immature scar (arrow) in the 2nd row (O2).

IDENTIFICATION of FOCAL OC LESIONS



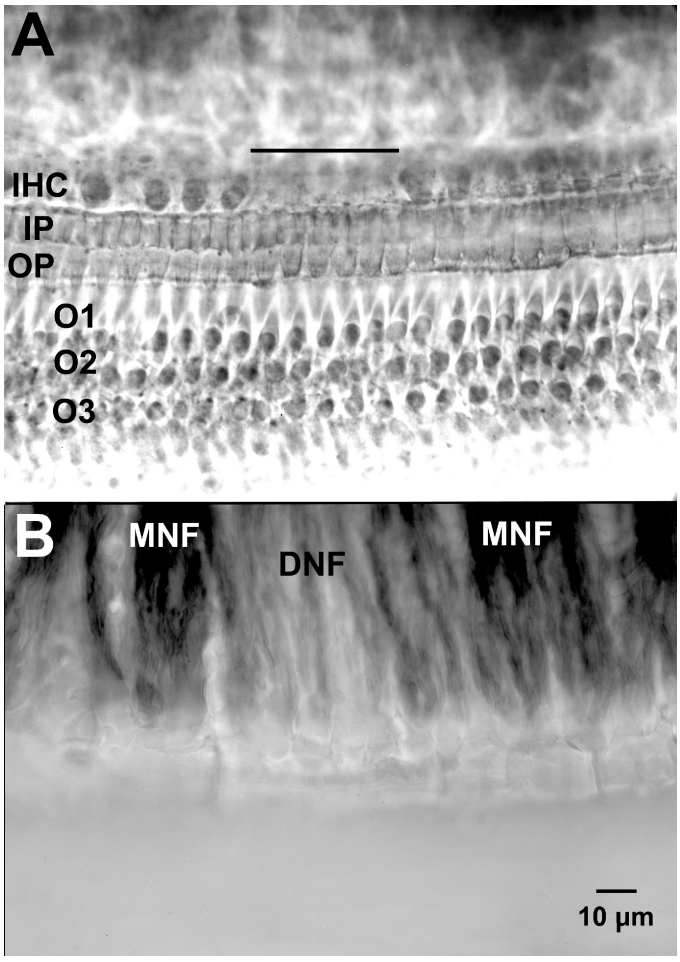
Noise-damaged regions of the organ of Corti (OC) (under black bars) that must be split in order to obtain a better representation of the apex-to-base location of hair-cell loss. A) The segment can be math split because total IHCs can be counted across the entire segment. There is a region of hair-cell and outer pillar loss to the right of the focal lesion that is too small to split (i.e., < 3 IHCs). B) The segment must be video split where the long stretch of outer pillars is missing because most IHCs are also missing in this region. On either side of the stretch of missing pillars, the segment can be math spilt.

OHC FOCAL LESION



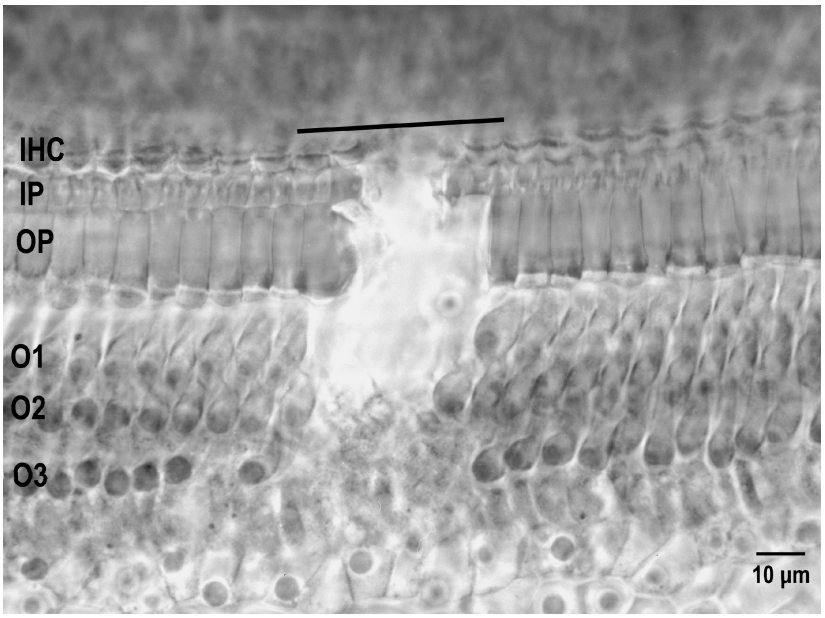
Under the black bar, all OHCs in the 1st (O1) and 2nd (O2) rows have degenerated, while the IHCs and all 3rd-row OHCs (O3) are present. This is an OHC focal lesion because it involves more than 50% loss of OHCs over a linear distance of more than 3 IHCs. In the same region, all OPs and many IPs are also missing.

IHC FOCAL LESION



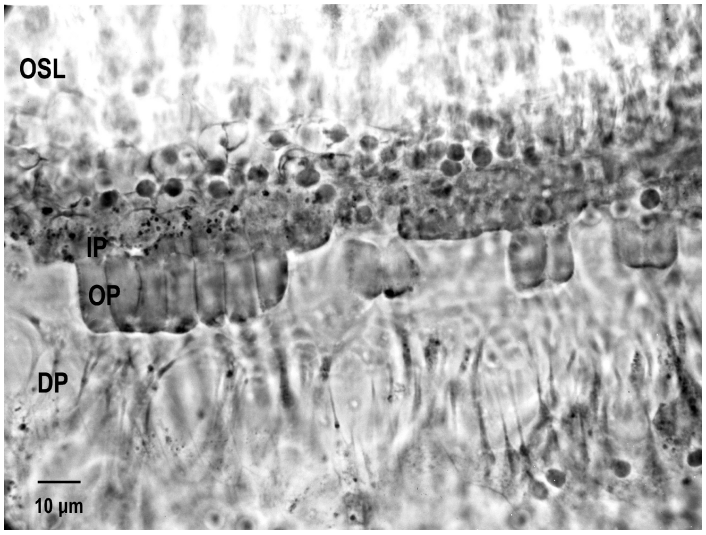
A) Under the black bar, 5 IHCs have degenerated. This is an IHC focal lesion because it involves loss of more than 50% of the IHCs over a linear distance of more than 3 IHCs. Note that the OHCs (O1, O2, O3), inner and outer pillars are intact. B) A number of the myelinated nerve fibers (MNF) medial to the IHC focal lesion have also degenerated (DNF).

COMBINED (IHC & OHC) FOCAL LESION



Under the black bar, three IHCs and all OHCs in the 1st (O1), 2nd (O2) and 3rd (O3) rows have degenerated. This is a combined focal lesion because it involves loss at least 50% of both the IHCs and OHCs over a linear distance greater than 3 IHCs. In the same region, 5 OPs and 4 IPs are also missing.

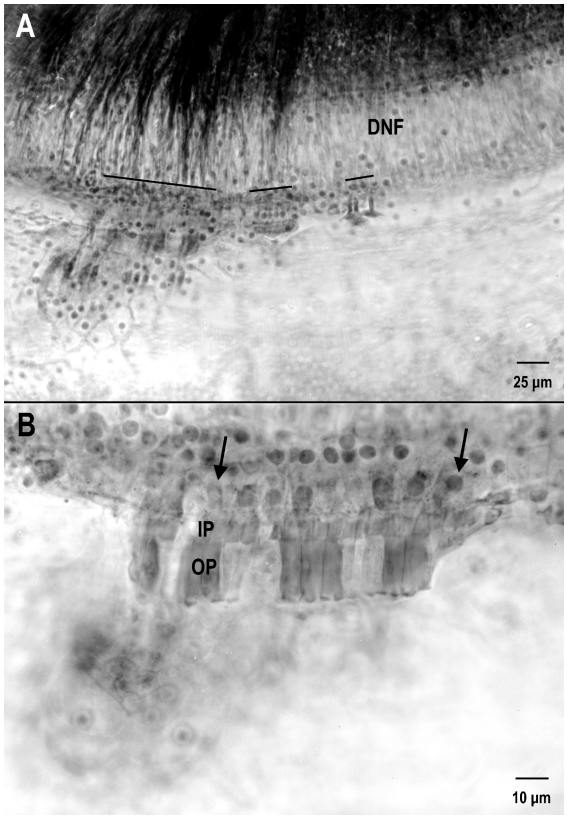
CODE 5 LESION



When hair-cell loss is severe, individual phalangeal scars cannot be easily visualized or counted. In these instances, the segment should be classified as 'code 5', the length of the segment measured, and the remaining IHCs and OHCs counted. On the data sheet, the counts should be entered as present cells. When the data are entered into the computer program that calculates percentage of missing hair cells, the program will calculate how many hair cells should have been present, then subtract present cells from the calculated total to obtain an estimated number missing.

This photomicrograph shows a code 5 region where all OHCs are missing. Only Deiters' processes (DP) and outer pillars (OP) are visible in the lateral organ of Corti. More inner pillars (IP) than outer pillars (OP) are present here, but it cannot be determined if any IHCs are present in this photomicrograph. The area needs to be carefully scanned under the phase-contrast microscope for remaining IHCs. OSL - osseous spiral lamina lacks the myelinated peripheral processes of the spiral ganglion neurons.

OC REMNANTS



OC remnants contain some recognizable cells of the organ of Corti, usually pillars and Deiters cells, but occasionally some hair cells are also present. OC remnants are coded as 5 or 3. A) Low-power photomicrograph of a noise-damaged region that contains three remnants of the organ of Corti (under the horizontal, black bars). If possible, the lengths of these remnants should be determined using the Nikon-Black Knight microscope-computer system; B) High-power photomicrograph of the middle OC remnant shows that seven IHCs (four to right of left arrow & three to left of right arrow), eight OPs and about 20 IPs are present. Most of the lateral organ of Corti is missing.

APPENDIX H: Equipment list

The procedures outlined in this manual were carried out using the following equipment. Equipment from other manufacturers may also produce satisfactory results.

Determination of OC length

Nikon zoom microscope

Computer with ImageJ software (NIH)

Micropublisher camera

Counting of sensory cells, pillar cells & qualitative evaluations

Wild phase contrast microscope with 10X and 20X dry lenses and 50X and 100X oil immersion objective lenses and camera mount.

Data entry into computer and preparation of cytocochleograms

Custom computer (running Windows 3.11) and HP II printer with plotter in a cartridge.

MNF loss

AO stereoscopic dissection microscope with a magnification range from 10X to 40X.
Fiber optic illumination.

Preparation of plastic blocks for sectioning

AO stereoscopic dissection microscope with a magnification range from 10X to 40X.
Fiber optic illumination.

Sectioning tissue blocks

RMC ultramicrotome
Glass knifebreaker

APPENDIX I: ABBREVIATIONS AND TERMINOLOGY USED WITH COCHLEA

ABR (ABRs) - Auditory brainstem response recorded with needle electrodes in response to clicks and tone pips.

ACUTE - specimen preserved for histological study from a few minutes to 1-2 weeks after termination of the experimental treatment.

B - Boettcher's cell

BBN - broad band noise

BM - basilar membrane

C - Claudius' cell

CHRONIC (or LONG-TERM) - specimen preserved for histological study more than three weeks after termination of the experimental treatment.

D1 - Deiters' cell in 1st row

D2 - Deiters' cell in 2nd row

D3 - Deiters' cell in 3rd row

dB - decibel. This is a measure of the intensity of the sound.

DP - Deiters' process which extends from cell base to phalangeal process.

DPOAE (DPOAEs) - Distortion product otoacoustic emission(s) recorded in the external auditory canal in response to two pure tones of different frequencies.

E or ENDO - endolymph. This fluid fills the endolymphatic space which is bounded by Reissner's membrane, the marginal cells of the stria vascularis, the reticular lamina, interdental cells of the limbus, inner sulcus and Claudius cells.

EP - endolymphatic potential. Positive potential (normally +80 to +100 mV) recorded in the endolymphatic space.

H - Hensen's cell

HC - hair cell, either IHC or OHC

IHC (IHCs) - inner hair cell (or cells)

IP (IPs) - inner pillar cell (or cells)

IS - inner sulcus cells

ISB - inner spiral bundle of nerve fibers

kHz - kiloHertz - 1000 Hertz or cycles per second. This is a measure of frequency of sound.

MNF (MNFs) - myelinated nerve fiber (or fibers)

MOD - modiolus

N - Nuel's space (i.e., space surrounding OHC bodies). Filled with perilymph-like fluid.

OBN - octave-wide band of noise

OC - organ of Corti

OC wipeout - region of total loss of all sensory and supporting cells of the OC where the bare basilar membrane has been covered by an undifferentiated squamous epithelium.

OHC (OHCs) - outer hair cell (or cells)

OHC1 or **O1** - 1st-row outer hair cell

OHC2 or **O2** - 2nd-row outer hair cell

OHC3 or **O3** - 3rd-row outer hair cell

OP (OPs) - outer pillar cell (or pillars)

OSB - outer spiral bundle of nerve fibers

OSB1 - 1st outer spiral bundle beneath OHC1

OSB2 - 2nd outer spiral bundle beneath OHC2

OSB3 - 3rd outer spiral bundle beneath OHC3

OSL - osseous spiral lamina

P or **PERI** - perilymph. This fluid fills scalae vestibuli and tympani

RL - reticular lamina

RM - Reissner's membrane

RTF (RTFs) - radial tunnel fiber (or fibers)

T - tunnel of Corti. Fluid space bounded by IPs and OPs in which run TSB and RTFs. Filled with perilymph-like fluid.

TM - tectorial membrane

TSB - tunnel spiral bundle of nerve fibers

SG - spiral ganglion

SGN (SGNs) - spiral ganglion neuron (or neurons)

SL - spiral ligament

SM - scala media

SPL - sound pressure level

ST - scala tympani. This space is bounded by the basilar membrane and the cochlear bone inferior to the basilar membrane.

StV - stria vascularis

SV - scala vestibuli. This space is bounded by Reissner's membrane and the cochlear bone superior to Reissner's membrane.

VBM - vessel of the basilar membrane (located beneath the tunnel space)

VTL - vessel of the tympanic lip of the OSL (located beneath bone of the OSL close to base of IHC and ISB).