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Three-dimensional reconstructions from optical sections of thick mouse inner ears using confocal microscopy

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Summary

Three-dimensional (3D) reconstructions of the vertebrate inner ear have provided novel insights into the development of this complex organ. 3D reconstructions enable superior analysis of phenotypic differences between wild type and mutant ears but can result in laborious work when reconstructed from physically sectioned material. Although nondestructive optical sectioning light sheet microscopy may ultimately prove the ideal solution, these technologies are not yet commercially available, or in many instances are not monetarily feasible. Here we introduce a simple technique to image a fluorescently labelled ear at different stages throughout development at high resolution enabling 3D reconstruction of any component of the inner ear using confocal microscopy. We provide a step-by-step manual from tissue preparation to imaging to 3D reconstruction and analysis including a rationale and troubleshooting guide at each step for researchers with different equipment, protocols, and access to resources to successfully incorporate the principles of this method and customize them to their laboratory settings.

Keywords

Confocal microscopy; inner ear; mouse; segmentation; three-dimensional reconstruction; sTSLIM

Introduction

Two-dimensional (2D) visualizations of three-dimensional (3D) structures introduce bias and incompletely characterize the many subtle differences that arise between two paired samples of a mutant and wild type. Furthermore, 2D analyses may not accurately allow quantitative comparisons due to incomplete data or tissue compression. To overcome these limitations, there has been continued effort to produce high quality 3D images of complex structures in specimens of many diverse species. This is especially true with the inner ear that combines six unique sensory epithelia within the complex and delicate labyrinth inside the tiny otic capsule consisting of much harder bone. Due to its size and different hardness, it is difficult to access the intricate structure of the inner ear. Tedious manual sectioning followed by laborious reconstructions of plastic moulds was the state of the art at the turn of the 20th century (Streeter, 1906; Streeter, 1917). In fact, the quality of these early reconstructions arguably took nearly a century to match. In the 1990's, the paint-fill

technique used a dye injection to fill the endolymphatic spaces of the inner ear to generate for the first time, inner ears easily visualized in 3D space (Martin & Swanson, 1993; Bissonnette & Fekete, 1996; Wu & Oh, 1996; Morsli *et al.*, 1998; Fekete, 1999). Despite the advance of the paint-fill technique, the older sectioning method still held its ground with its high quality sections that provided detailed histology in addition to the 3D architecture. What was truly needed was a method that not only provided high quality histologic sections, but also enabled complete resolution of the overall 3D structure.

In 1903, the first description of a rudimentary light sheet fluorescent microscope (LSFM) was presented (for review see Santi, 2011) but this technology was not brought to the mainstream until the 1990's by Voie *et al.* (1993) and Huisken *et al.* (2004). Following the protocols of early scientists such as Spalteholz, scientists of the 1990's and early 2000's modified the light sheet microscopes through iterations such as the Thin Laser Light Sheet Microscope (TLSM) (Fuchs *et al.*, 2002), the HiLo LSFM (Mertz & Kim, 2010), the Thin Sheet Laser Imaging Microscope (TSLIM) (Santi *et al.*, 2009; Schacht *et al.*, 2010) and most recently, the scanning TSLIM (sTSLIM) (Schroter *et al.*, 2012). The last two of these techniques have been used extensively in recent publications detailing the mouse inner ear (Johnson *et al.*, 2011; Kopecky *et al.*, 2011, 2012c; Pan *et al.*, 2011) and were the first publications to use this technology for high quality inner ear digitised 3D reconstruction of mouse mutants. In brief, light sheet microscopy provides very high-resolution optical sections of thick tissues that can be quickly collected and subsequently collated into 3D reconstruction software programs, such as Amira, to produce easily manipulated images that contain valuable quantitative data. In addition to the scientific value of these images, they provide stunning visual impact. Indeed, the past 20 years have seen a boom in the optical imaging field (Eliceiri *et al.*, 2012).

LSFMs and their iterations are the state of the art; however, they are not currently commercially available and although it may be possible to build one (Santi *et al.*, 2009), it would take considerable time and resources. Furthermore, with each new imaging technique, new hardware is accompanied with a dizzying array of commercial software (Eliceiri *et al.*, 2012). Therefore, what is needed is a standardized method to prepare, image and analyse tissue using easily accessible resources. We present here a protocol that produces high quality non-destructive optical sections of the mouse inner ear at various developmental stages with a confocal microscope, an instrument accessible to most researchers. The following describes each step in detail, the rationale behind each step so it can be easily modified, and a troubleshooting guide so that beginners to the 3D world can produce high quality 3D images from not only murine ears but also the species of his/her choice (*i.e.* *Xenopus* or hagfish).

Tissue preparation (Fig. 1)

- 1) Procedure (P): Dissect previously fixed tissue in 0.4% paraformaldehyde (PFA) at room temperature (RT).

Notes (N): It is important to maintain the integrity of the tissues. Crosslinking between proteins may take days and is especially important to withstanding the stresses of dehydration. Also, when dissecting the ear, do not puncture the otic capsule. Extra tissue around the capsule makes the sample easier to handle. You can either continue to the next step immediately or fix any tissue in 4% PFA indefinitely.

Troubleshooting (T): If tissues appear degraded, they may not have been properly fixed. Keep all unprepared tissue or any tissue that has been partially prepared but is not ready for further preparation in at least 4% PFA or equivalent

fixative, preferentially in near isotonic solutions such as 0.1 M phosphate buffer to avoid swelling or shrinking.

- 2) P: For ages greater than postnatal day 0, decalcify with Ethylenediaminetetraacetic acid (EDTA). We use a 10% EDTA solution dissolved in 4% PFA (pH = 7.4) in 0.1 M phosphate buffer (pH 7.4).
 - (a) Decalcify at RT on shaker for at least 3 days with daily solution changes.
 - (b) After decalcification, wash samples three times with 1× phosphate buffered saline (PBS) for at least 1 h at RT on shaker.

*If performing immunochemistry (IC):

- (a) Without disrupting structural integrity, remove a small amount of surrounding capsule, preferentially in a position of lesser interest (vestibular system for cochlea imaging, cochlea for vestibular imaging).
- (b) Perform immunochemistry protocol as normal.

N: Decalcification is an essential step for imaging. EDTA is a chelator that removes calcium from bone. An incompletely decalcified tissues will not allow proper laser penetration. Also, if EDTA is not washed out fully, EDTA crystals will interfere with proper imaging.

***IC:* This step is important for the antibodies to fully penetrate; however, it is better to increase the time of incubation and number of washes than to compromise the integrity of the 3D structure.

T: If there are problems with image quality, either decalcification or proper penetration of the staining may be the issue. To ensure proper decalcification, it is recommended to supersaturate the EDTA solution above 10% weight per volume (EDTA normally dissolves in 4% PFA at RT on shaker). Also, make sure to use fresh EDTA each day. Check the tissue for absence of bone. Tissue should appear more translucent.

***IC:* If penetration is not sufficient, remove more capsular tissue or isolate the area of interest (i.e. if interested in only hair cells in the cochlea, you can remove portions of vestibular system).

- 3) P: Dehydrate tissues through a graded ascending ethanol series.
 - (a) 50% overnight at RT on shaker.
 - (b) 75% two times for at least 2 h at RT on shaker.
 - (c) 100% overnight at RT on shaker.

N: Dehydration is essential for the subsequent clearing step. Take time to dehydrate properly. In the absence of proper dehydration, the clearing solution will not penetrate properly and the imaging will be distorted by the presence of water molecules.

T: If specimens do not clear fully after the completion of Step 6 (they should be nearly invisible except for a very light pinkish coloration), it is likely that this dehydration process was not performed properly. Rehydrate and start Step 3 over.

- 4) P: Stain with Rhodamine B isothiocyanate**

- (a) Using a 0.0005 mg Rhodamine diluted in one milliliter of 100% EtOH solution, stain until very light pink; approximately 1–2 days, at RT on shaker, shielded from light.
- (b) **Staining steps can be skipped if only performing immunochemistry

N: Perhaps the most critical step in tissue preparation. Over-staining will provide unequal intensities of images from the top of the Z-stack to the bottom as well as decreasing overall clarity. Rhodamine dissolves well in 100% EtOH, but only very low concentrations of Rhodamine are needed (we serially dilute). Rhodamine is a nonspecific cellular stain that will stain all cells in the tissue near equally. Remove the 100% EtOH solution and add the Rhodamine-100% EtOH per milliliter of 100% EtOH solution until tissue is very lightly stained. On a white background, dehydrated but not yet cleared ears will have a very weak pinkish appearance, when properly stained.

T: Tissue needs to be perfectly stained for this protocol to work well within the confines of confocal imaging. If upon imaging a prepared ear, the top of the stack is very bright whereas the gain or power needs to be turned up near the bottom of the same tissue, the ear is overstained. It is possible to washout the Rhodamine and restain later. A properly stained ear should have the same intensity at the top and bottom of the stack. Overstained or understained ears will greatly reduce imaging clarity of thick tissue. A clearly understained ear can be restained.

- 5) P: After proper staining, replace the 0.0005 mg per milliliter EtOH solution with fresh 0.0005 mg per milliliter EtOH solution in a 1 to 1 ratio with clearing solution.

N: Clearing is the last preparation step and is easily performed if the tissue was properly dehydrated in Step 3. We have seen best results when we slowly dilute the Rhodamine solution in a 1:1 ratio with fresh MSBB clearing solution overnight.

T: After an overnight of 1:1 Rhodamine and MSBB, the tissue will be more translucent than before but will not yet be transparent.

- 6) P: Replace with fresh MSBB twice for 2 h each

N: If properly performed, tissues become transparent within an hour of adding MSBB.

T: If ears do not become completely transparent (except for their slight pink colour) within a few hours, remove from MSBB and go back to Step 3c and then skip to Step 5.

- 7) P: Use vacuum grease to create a ring on a slide large enough to contain the ear and fill the ring with fresh MSBB. Transfer sample to pool of MSBB inside the ring of grease. Gently place coverslip on top with caution to avoid air pockets from forming. The cover slip may be pressed lightly into the grease to prevent it from sliding, but care should be taken to avoid compressing the sample. Make sure that the ear is held securely in place by the coverslip and the surrounding grease to limit drift while imaging. Since each sample will vary in thickness this step requires skillful adjustment to avoid compression and yet hold the ear in place.

N: It is important to preserve the integrity of the tissue yet to reduce the working distance between the lens and your sample. Carefully use the vacuum grease to create sufficient space for the ear to rest without compression but not too far to limit imaging capacity. The viscosity of the clearing solution and the relative small dimensions in which the ear is placed should reduce the ability of the tissue to move.

T: If clearing solution leaking occurs, use a wider (not higher) application of grease. Air bubbles may shift the specimen during imaging. Take care to slowly apply coverslip at an angle to reduce air bubble formation. Make sure not to compress sample with coverslip to avoid distortions.

Imaging* (Fig. 2)

*The following protocol is written for a Leica SP5 Confocal Microscope, but is applicable to any confocal system.

- 1) *P:* If collecting more than a single channel (i.e. Rhodamine plus immunochemistry or multiple immunochemistry labeling), image sequentially by frame.
N: Rhodamine is maximally excited at 540 nm and has a peak emission at 625 nm.
T: Sequential imaging prevents interference and imaging by frame reduces the risk of the tissue shifting between stacks.
- 2) *P:* Set up confocal microscope for imaging (i.e. set filters, set highest feasible resolution, set appropriate emission collection, set Z-stack).
N: For early embryonic tissue up to mouse embryonic day 12.5 we found that the entire ear could be imaged with either the 10× or 20× objective. Later stages required either a lower power objective 4× or taking multiple images of 10× and stitching them together using confocal software. For late embryonic tissue and all adult tissue, it will be necessary to increase the Z dimension. For the Leica SP5 MP, under configuration is the option 'Super-Z'. The maximum Z dimension for this microscope is 1.5 mm. If properly stained, the gain should not need to be increased as the stack reaches the bottom. In our experience, tissues up to at least 1.5 mm thick can be imaged.
T: Setting up the confocal for optical sections for 3D reconstructions should be performed identical to any Z-stack preparation. If imaging through thick adult tissue it may be necessary to open the pinhole to a larger size or use frame averaging. It also helps to use an objective with a larger working distance (water immersion lenses, for example) to image thicker tissue.
- 3) *P:* Depending on the thickness of tissue, resolution, intended presentation, and whether or not stitching is required, set step size.
N: For embryonic ears, we suggest a step size between 3–5 μm and for adult 6+ μm.
T: Most high resolution Z-stacks will be very large. Adult inner ear files, especially if stitched together, can be upwards of 20 GB. If simply needed for 3D reconstruction, you can reduce resolution and increase step size. Depending on computer hardware, large files sizes can be difficult to work with. Please check the specifications of the software you are using.
- 4) *P:* Image

N: Depending on the size of the ear and the image settings, imaging time may range from several minutes to several hours (longest imaging time for us was 1.5 h).

T: If clarity is low or gain needs to be increased, the staining and/or clearing may not be appropriate. If specimen is overstained, either destain in 100% EtOH or restart entire procedure with a second ear. If specimen came into contact with water, restart entire dehydration and clearing procedure.

3D reconstruction and data analysis* (Figs. 2 and 3)

*The following is written for Amira. There are many programs capable of using the confocal image files to producing 3D reconstructions; therefore, we only provide an overview of the process here.

- 1) *P:* Depending on the size and intended use of the data, it is possible to either downsize the data or remove slices from the original data set using a program such as ImageJ. (You can also resample/resize data within the Amira program.)

N: For segmentation, it is often unnecessary to segment every slice taken (especially if taking very thin sections). Furthermore, in older ears, there may be upwards of 500 sections, making segmentation overly burdensome. However, capturing at smaller Z-steps may be useful if you wish to later count neuronal cell bodies or hair cells using optical dissector approaches.

T: In programs such as ImageJ, load all the files from the confocal stack and convert 'Images to Stack' (follow 'Images' pull down item to 'Stack' tab). You will need to download the 'Slice Remover' plugin from: <http://rsbweb.nih.gov/ij/plugins/slice-remover.html>. Restart ImageJ and you will find 'Slice Remover' under the 'Plugins' tab.

- 2) *P:* Upload the intended image files to the 3D reconstruction program of choice (such as Amira).

N: With Amira, you can either upload an image database (.lif) or you can upload multiple (even hundreds) of single image files (.tif, .gif).

T: When uploading the files, you may need to enter in the dimensions of the images you took. Also, programs such as Amira may be unable to handle or minimally, will be much slower, with large data sizes. This can be alleviated through increasing the available RAM memory of your computer and by closing any other program open on your computer.

- 3) *P:* Create label field from raw data (right click on channel → Labelling → LabelField).

N: Once the images have uploaded, an icon will appear with the image data, right click and select image field. This will allow you to segment.

T: You can perform a number of functions without creating a label field and subsequent segmentation. It is possible to volume render, slice, make measurements, perform neuronal tracing, and much more. However, due to the lack of segmentation, you will be unable to specifically analyse your regions of interest independently of the whole image; furthermore, many quantitative as well qualitative assessments will be unavailable.

- 4) *P:* Segment regions of interest by manually outlining the structure. You will need to segment the region of interest in each section.

******For 3D reconstructions of immunochemistry, you can directly volume render the specific channel of interest or you can segment the label field to produce a 3D image of only labelled tissue within a specific region of interest.

N: Segmentation is a tedious process and the results you obtain are only as good as the quality of the person who segments.

T: If your 3D images are of less quality than anticipated, ensure each image is segmented accurately and consistently. You can touch-up poorly segmented regions after you make the 3D reconstruction by reentering into the label field and saving changes.

- 5) P: After segmentation, there are countless options. For 3D visualization:
- (a) Right click on segmented label field → Compute → Resample.
Resampling will produce a data set with a coarser resolution. This option will decrease the computation time and enhance rendering.
 - (b) Right click on '.resampled' → Compute → SurfaceGen. This will create the polygonal surface model.
 - (c) Right click on '.surf' → Compute → SmoothSurface. The surface of the image may have ragged edges or points where three or more region join. This may be due to low resolution or noise artefacts in the original image data set. This option will add fractional weights to the polygonal surface model to produce smooth boundary connections.
 - (d) Right click on '.smooth' → Display → SurfaceView. This option will now allow you to view the surface model

N: You can experiment with the settings of each of these steps and they will not change the overall value of the volume of your segmented structures. Because the Amira program interpolates between each step in the Z-plane, these four procedural steps (a–d) attempt to smooth out any changes that occur between Z-steps. To measure volume, right click on label field → Measure →

MaterialStatistics. One quick way to measure length, make sure you are in the 3D viewer (icon on the top of the page) and then select the 3D ruler tool. You will be able to click on the segmented structure and produce any measurement of the surface. You can both visualize and measure internal structures using the 'OrthoSlice' feature of the program.

T: By altering 'Resample', 'SurfaceGen' and 'Smooth-Surface' systematically, it will become apparent how you can best represent your 3D image.

Discussion

LSFM and its iterations can non-destructively image, at high resolution, tissues of various species. Scanning TSLIM and its predecessor TSLIM can successfully capture the complex labyrinth of the inner ear both during murine development (Kopecky *et al.*, 2012c) and in later adulthood of both wild types and mutants (Kopecky *et al.*, 2011; Pan *et al.*, 2011). Unfortunately, these technologies are not commercially available. Previous studies have used confocal microscopy, an easily accessible resource, for 3D image reconstruction (Boon *et al.*, 1994; Michael *et al.*, 1997; Scarpa *et al.*, 2007) of various structures in the body, but they either did not follow a universal protocol, or did not deal with thick tissues, or did not image the highly complex inner ear, and often wrote their own software. Ultimately both LSFMs and confocal microscopy is diffraction limited. Here we provide a protocol to use

confocal microscopy (this protocol can also be adapted for LSM) for imaging murine ears during development (Kopecky *et al.*, 2012b) and in adulthood (Kopecky *et al.*, 2012a) as well as both *Xenopus* and hagfish ears at similar resolution as current LSM iterations.

Once images are acquired, they can be quickly and easily manipulated. We have described the use of Amira (Visage Imaging, San Diego, CA, U.S.A.); however, a number of open-source tools such as ImageJ, Fiji (Schindelin *et al.*, 2012), BioimageXD (Kankaanpää *et al.*, 2012), Vaa3D (Peng *et al.*, 2010), the Insight (Yoo *et al.*, 2002; Ackerman & Yoo, 2003; Yoo & Metaxas, 2005) and Farsight (Björnsson *et al.*, 2008) can also be explored and may be useful in expediting the segmentation and data analyses processes. The list is extensive but in general, the features are similar (Eliceiri *et al.*, 2012). Additionally, as the wave of image processing moves forward, many of these software are becoming compatible with one another (Eliceiri *et al.*, 2012). Unfortunately, one deficit of all these programs is the inability to 'autosegment' regions of interest of the inner ear. Unlike some programs such as CellProfiler Analyst (Jones *et al.*, 2009) where researchers can provide active feedback to enhance automatic classifications of cells, we know of no software that can identify the wide range of cell types and structures of the inner ear, making researcher expertise of inner ear anatomy vital. After successful segmentation of regions of interest, 3D reconstruction and analysis are easy and the options available are vast.

With this protocol, various wild type and mutant ears (or many other structures within the organism) of quite disparate species can now be prepared, imaged and reconstructed enabling new discoveries and comparative analyses as we continue through the digital age. Using confocal microscopy, we now have an accessible imaging hardware with multiple channels that can rapidly image a flexible array of specimens in a standardized procedure allowing comparisons between studies and across the research spectrum. Although continued improvement and commercialisation of hardware and software is outpacing most research laboratories, the simple technique presented here will likely be adaptable to all future developments without changing the basis of it; confocal optical sectioning followed by software assisted segmentation of desired structures and their 3D rendering.

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Fig. 1.

Tissue preparation of both embryonic and adult ears. Inner ears must first be removed from the head and as much tissue as possible should be removed, although the otic capsule itself should not be compromised. Additionally, slight excess of tissue increases the ease of ear movement throughout the remainder of steps (A, B, D). For immunochemistry, remove portions of otic capsule but do not compromise the three-dimensional structure (C). After dissection of the ear from the head, it may be necessary to decalcify the inner ear. This may not be necessary at earlier stages such as E15.5 or P0 (A' and B'), but at later stages such as P21, decalcification is essential (D', 4 consecutive days of decalcification). As the tissue becomes decalcified, the opaque bony structures become translucent (B' and B''). After decalcification and dehydration, the ears are very lightly stained in an Ethanol/Rhodamine solution (A'', B'', D''). It is critical with confocal microscopy to not overstain the specimens. The last step of inner ear preparation is to clear the ear with MSBB/Spalteholz solution (Santi *et al.*, 2009). Ears should be transparent except for a very light pink hue (A''', B''', D'''). Scale bar = 10 μ m.

Fig. 2.

Imaging and 3D reconstruction of the inner ear. Prepared ears are imaged through confocal microscopy and ventral images of the reconstructed inner ear are shown at E15.5 (A), P0 (B) and P21 (D). A properly stained ear should be easily visualized at both the top and bottom of the stack without a change in gain or in clarity. After imaging, regions of interest must be segmented (scala media segmentation shown in A', B', D'). Segmentation is the process of manually tracing regions of interest in programs such as Amira. The segmented images can then be easily reconstructed and viewed in a variety of ways, including, reconstruction of the entire endolymphatic space (A''), reconstruction of both the endolymphatic space and the otic capsule (B''), or reconstruction of any specific region of interest, such as the scala media (D''). After segmentation and reconstruction, a number of additional analyses are possible, such as endolymphatic space measurements of WT at different ages or WT comparisons to a mutant (MT), in this case, *Pax2-Cre N-Myc f/f L-Myc f/f* (A'') or length of the scala media at different ages (D''). Additionally, volume rendering of Myosin7a immunochemistry allows visualization of hair cells in the inner ear (C) with original IC in insert. Scale bar = 10 μm . Scale bar (C) volume render = 100 μm .

Fig. 3.

Flexibility of 3D reconstruction of confocal images. In addition to 3D reconstruction of the inner ear endolymphatic space of mice at any age, confocal imaging can be used to reconstruct a variety of tissues in not only mouse but of other species. Shown here are mouse auditory neurons as they enter the hindbrain and bifurcate in the auditory nucleus (red fibres innervate the basal turn of the cochlea; green fibres innervate the apical portion of the cochlea (A), ear of a *Xenopus laevis* embryo (B) and ear of a hagfish (C). Scale bar for A and B = 100 μm . Scale bar for C = 1 mm.