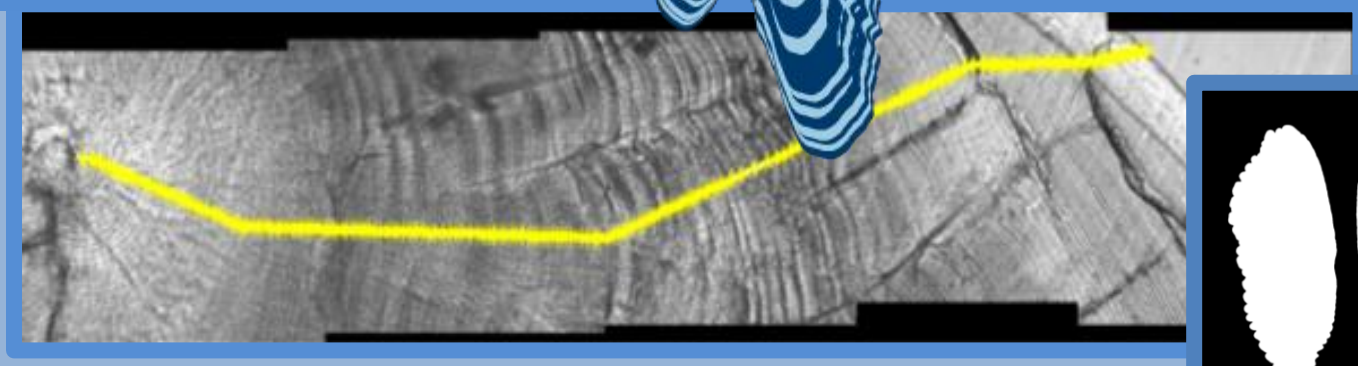
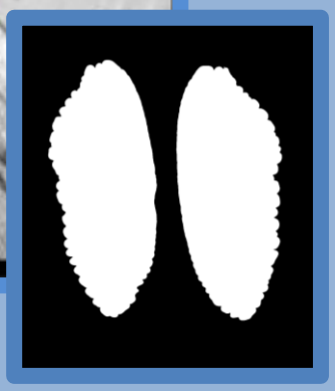
OTO LAB





USER'S GUIDE 2019

UNIDAD DE ESCLEROCRONOLOGÍA IEO

INSTITUTO ESPAÑOL DE OCEANOGRAFÍA 1914

INDEX

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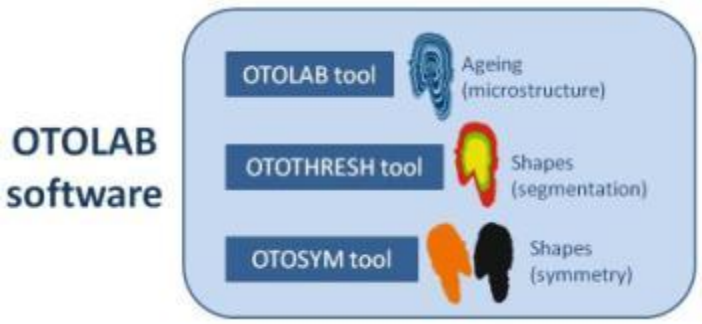
1. INTRODUCTION TO THE OTOLab FREE SOFTWARE

This User Guide has been prepared by the staff of the research group TIC128, University of Málaga (UMA), Málaga, Spain, in cooperation with the staff of the Málaga Oceanographic Center, Spanish Institute of Oceanography (IEO). The Guide describes how to use the open source software OTOLab, which has been jointly developed by the engineers of UMA and the marine scientists of IEO.

The purpose of **OTOLab software** is helping marine scientists in their research activities on otolith structure to estimate: 1) the age of an individual (**OTOLab tool**) and 2) measurements of the otolith morphometry (**OTOTHRESH** and **OTOSYM tools**). The software has been successfully used for hake, sardine and anchovy otoliths, both for ageing (microestructure) and morphometry tasks. Doubtless, it will be also helpful with otoliths of other fish species, as well as other growth structures, namely clams shells and cephalopods beaks.



OTOLab is considered to be Open Source Software since the code is freely available so that every user can modify or improve its performances at his convenience. On the other hand, OTOLab software is a MATLAB script developed with the help of the MATLAB GUIDE and consequently, the MATLAB Software is not Open Software.

Neither the Spanish Institute of Oceanography nor the University of Málaga will be responsible of the wrong use of MATLAB or the script. It is strongly recommended that any interested user will directly download the code from the Repository of the Spanish Institute of Oceanography, and do not use copies delivered by third persons.

Hardware and software requirements

A simple personal computer is needed to run OTOLab. There are no special constraints neither on computational power nor on RAM memory. MATLAB program must NOT be installed in the computer to run the software but for changing the code. To install OTOLab follow the recomendations in readme.txt in the MENU folder.

```
MENU Executable

1. Prerequisites for Deployment

Verify that version 9.3 (R2017b) of the MATLAB Runtime is installed.

If the MATLAB Runtime is not installed, you can run the MATLAB Runtime installer.

To find its location, enter

      >>mcrinstaller

     at the MATLAB prompt.

Alternatively, download and install the Windows version of the MATLAB Runtime for R2017b

from the following link on the MathWorks website:

    http://www.mathworks.com/products/compiler/mcr/index.html

   For more information about the MATLAB Runtime and the MATLAB Runtime installer, see

Package and Distribute in the MATLAB Compiler documentation

in the MathWorks Documentation Center.

NOTE: You will need administrator rights to run the MATLAB Runtime installer.


2. Files to Deploy and Package

Files to Package for Standalone

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-MENU.exe

-MCRInstaller.exe

    Note: if end users are unable to download the MATLAB Runtime using the

    instructions in the previous section, include it when building your

    component by clicking the "Runtime downloaded from web" link in the

    Deployment Tool.

-This readme file

3. Definitions

For information on deployment terminology, go to

http://www.mathworks.com/help and select MATLAB Compiler >

Getting Started > About Application Deployment >

Deployment Product Terms in the MathWorks Documentation

Center.
```

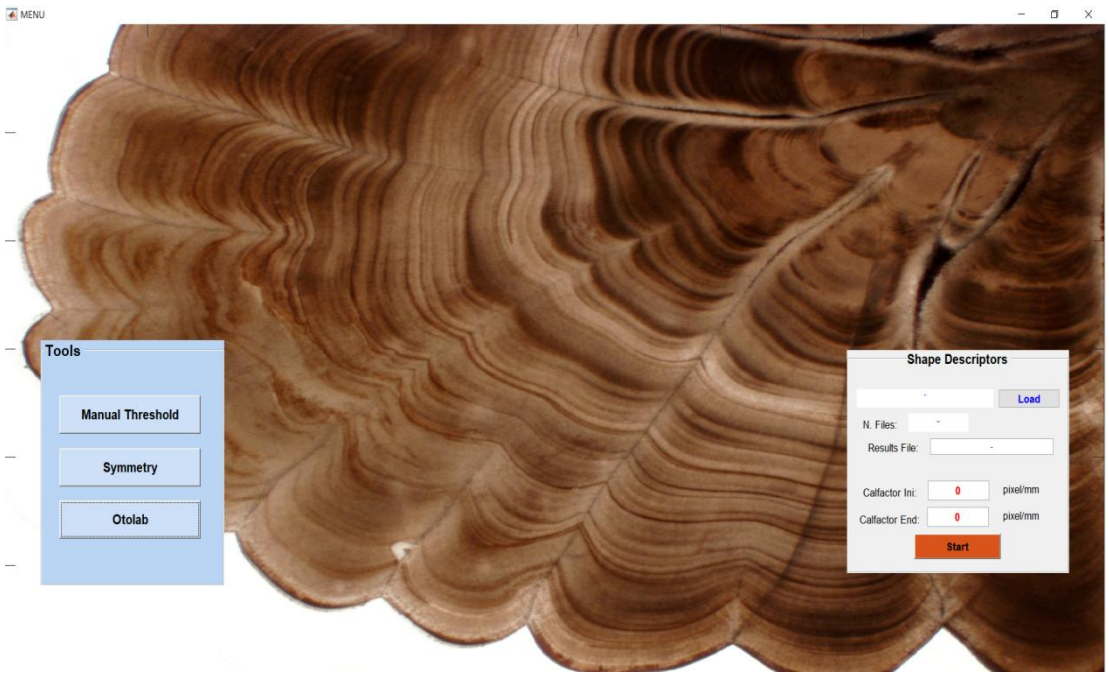


Figure 1.1. The main MENU screen.

2. The ageing tool of OTOLab

The main goal of the OTOLab tool is to facilitate the counting of daily rings (microstructures) in otolith sections and recording track counts. Although it has been designed for assisting otolith microstructure analysis in juvenile and adult fish, it may be used for other measures in otoliths (i.e. annual rings, growth axes, etc.) or even other growth structures (shells, beaks, etc.). The sum of all the daily growth increments (DGI) in a particular otolith is a precise approximation of the individual fish age.

Panoramic views of the whole otolith section (sagittal, transversal, frontal, etc.) are constructed from individual otolith section images to a single picture with the OTOLab program.

Before using the OTOLab tool for ageing, photographs of individual otolith sections must be taken. Because OTOLab will compose the final image output from single images of otolith sections, quality imaging standards must be applied (ANEXE 6.2).

In viewing otolith sections through the microscope, some procedures are strongly recommended for image capture, such as:

1. All image frames from the same sample should have the same magnification.
2. The alignment must be horizontal. The less vertical displacement between adjacent photographs, better vertical overlapping will occur (see below).
3. Photographs should be taken from left to right, doing as many as needed to view entirely the whole otolith.
4. All photographs should have similar luminosity conditions.
5. Maximum care in focusing microstructures is recommended.
6. An horizontal overlapping region between consecutive images must be at least 20%. Vertical overlapping must be at least 90%. Otherwise, the automatic tiling performance will be poor or wrong.
7. Photographs must be all saved in TIFF format without any kind of compression.
8. All the photographs of the same otolith should have the same name, finished by an underscore character and only the last two digits will be different. These two digits are used to number the TIFF files from left to right, starting in 01. Example with 5 photographs: Otolith 1_x400_01.tif, Otolith 1_x400_02.tif,…, Otolith 1_x400_05.tif.

More details in the process have been published recently [1], and a previous version of OTOLab tool has been successfully use to age adult hakes [2].

Once all images throughout a particular otolith section have been saved correctly in the same folder, the OTOLab ageing tool is opened by clicking OTOLab in the general MENU screen. In the OTOLab screen we can distinguish: 1) a central window for the image to examine will appear, 2) 4 different panels containing the OTOLab functions, 3) tool's top menu and 4) CLOSE IMAGE and EXIT buttons.

Figure 2.1. The OTOLab tool MENU screen.

1. **The CALIBRATION FACTOR** panel. The Calibration Factor is used by OTOLab for calibrating all the image views for processing measurements. Calibration Factor refers to the number of microns by pixel, and as such, refers to the optical specifics of each microscope and the magnification used (ANEXE 6.1). Users should estimate the precise Calibration Factor for each magnification in order to obtain accurate results. If no calibration factor is introduced it will remain 0 (in red) and no measurements will be taken.

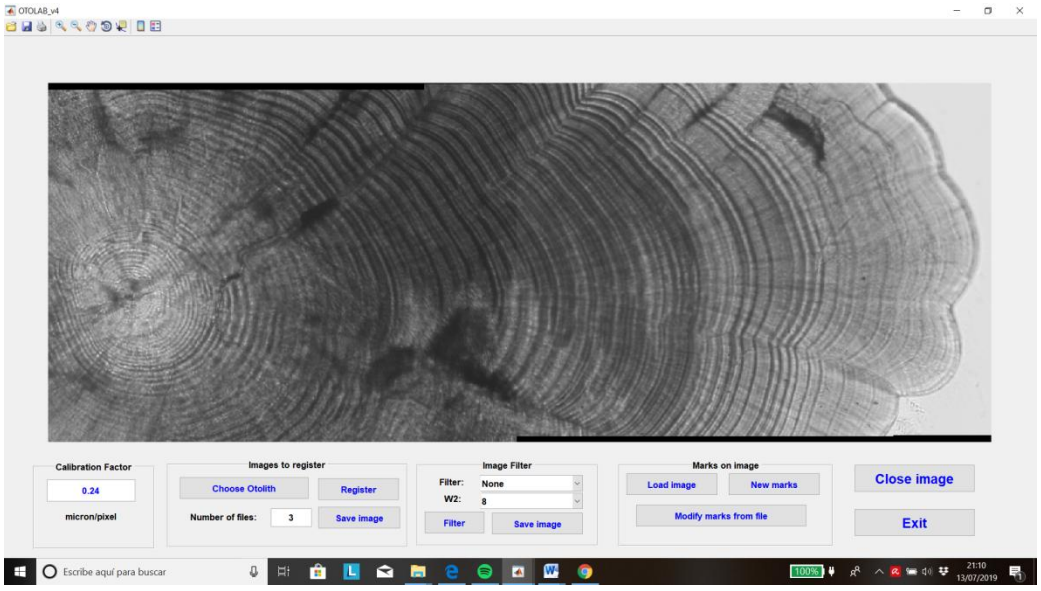


Figure 2.2. Panoramic view after loading a series of 3 successive images of an anchovy otolith sagittal section.

2. **The IMAGES TO REGISTER** panel. This panel refers to the images' tiling process. The user must choose the first otolith image (usually the center of the otolith) in the appropriate PC folder by clicking 'Choose otolith' button. Once selected (only the first one) next step is to indicate the 'Number of files' (photographs) of the otolith section and 'Register' to obtain a panoramic view of the otolith section arranged to age. User could also 'Save image' at this point.

   The number of photographs allowed to register is not prearranged in OTOLab. Nevertheless a maximum of 10 successive photographs have been already analyzed. Increasing the number of photographs involve longer time for the process.

3. **The IMAGES FILTER** panel. In this panel the user find some extra options to enhance image contrast to better visualize DGI. The default option is without any filter, preferable when images are good quality. Filter options are: CLAHE (Contrast Limited Adaptative Histogram Equalization) y FAHE (Fast Adaptative Histogram Equalization)[9]. Also several window sizes are available (8, 16, 32, 64, 128), related with enhanced contrast and less spatial resolution, progressively. The best solution is different for each quality and magnification situation. OTOLab filters work on individual images separately, so individual images (TIFF) and tiled final image (PNG) of the same otolith should be placed in the same folder. Consecuently, filter process is not possible without available individual images. Finally, panoramic view is renewed with the filtering result.

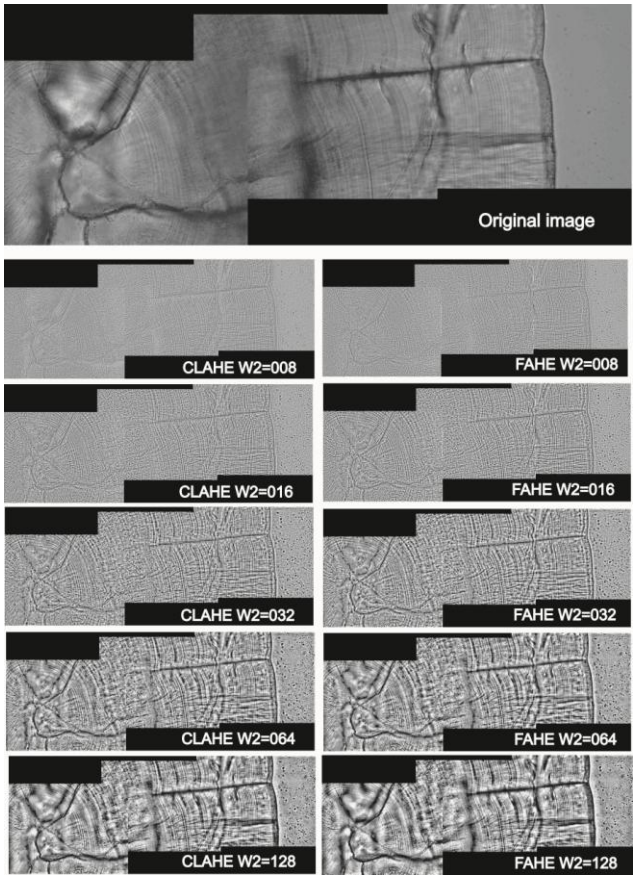


Figure 2.3. Panoramic view, before (original image) and after applying the available filters in the OTOLab tool. Anchovy sagittal otolith section (x400 magnification).

4. **The MARKS ON IMAGE** panel. In this board different tools are implemented to track a reading axis, and also mark/modify DGIs. All these functions are designed to run manually (**work with a mouse is required here**). Tiled .png images are chosen in the working folder and loaded clicking 'Load image' button. Also, the user can bring into play a newly registered image. The mouse pointer can change depending on the function chosen as shown in Figure 2.4.

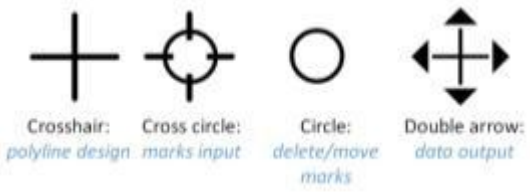
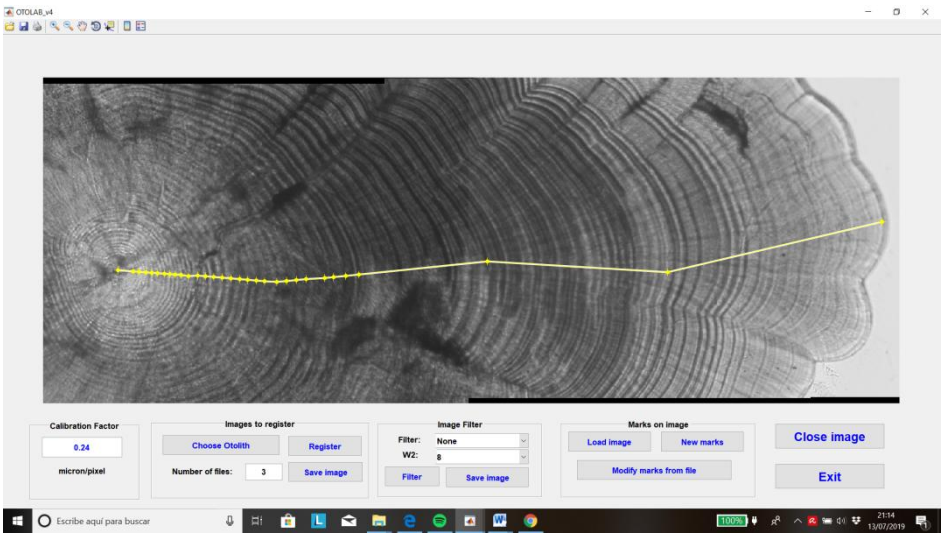


Figure 2.4. The pointer shape image and name (black) and the corresponding function (blue).

To analyze the image displayed in the screen (Figure 2.5 A), either being a newly registered image or a loaded image from a *.png* or *.tiff* file, the user should push the '*New marks*' button. The user will move the pointer on the image, so that it will change to a cross hair. The user can click (left button in mouse) on the image to mark the points of interest to draw a reading axis, where DGIs will be marked later (Figure 2.5 B). Usually, reading axes are not necessarily a straight line, but it should follow a constant direction. A blue polyline will be drawn on the image. When finished, the user should click the left button of the mouse and the polyline will turn yellow.



ADDING MARKS (Figure 2.5 C-E): the user should place the mouse pointer over the polyline, and the single arrow will change to a double arrow. **Be careful:** While the double arrow is on the polyline visible in the screen can be be displaced, and this is usually is not desirable. To mark DGIs on the growth axis the user should press and keep the '**A'** key of the keyboard while clicking (left mouse button). The shape of the pointer will change to cross circle shape. Place this pointer to the desired position and left-click. Release the ***A*** key when finishing the marking

timeline. When finished, place the cursor over the polyline (preferable in a zone without marks), and it will change again to a double arrow shape and double-click (Figure 2.5 F). The marks are stored in an Excel file with the otolith name.xls (i.e. sardine_56_2018_x200.xls).

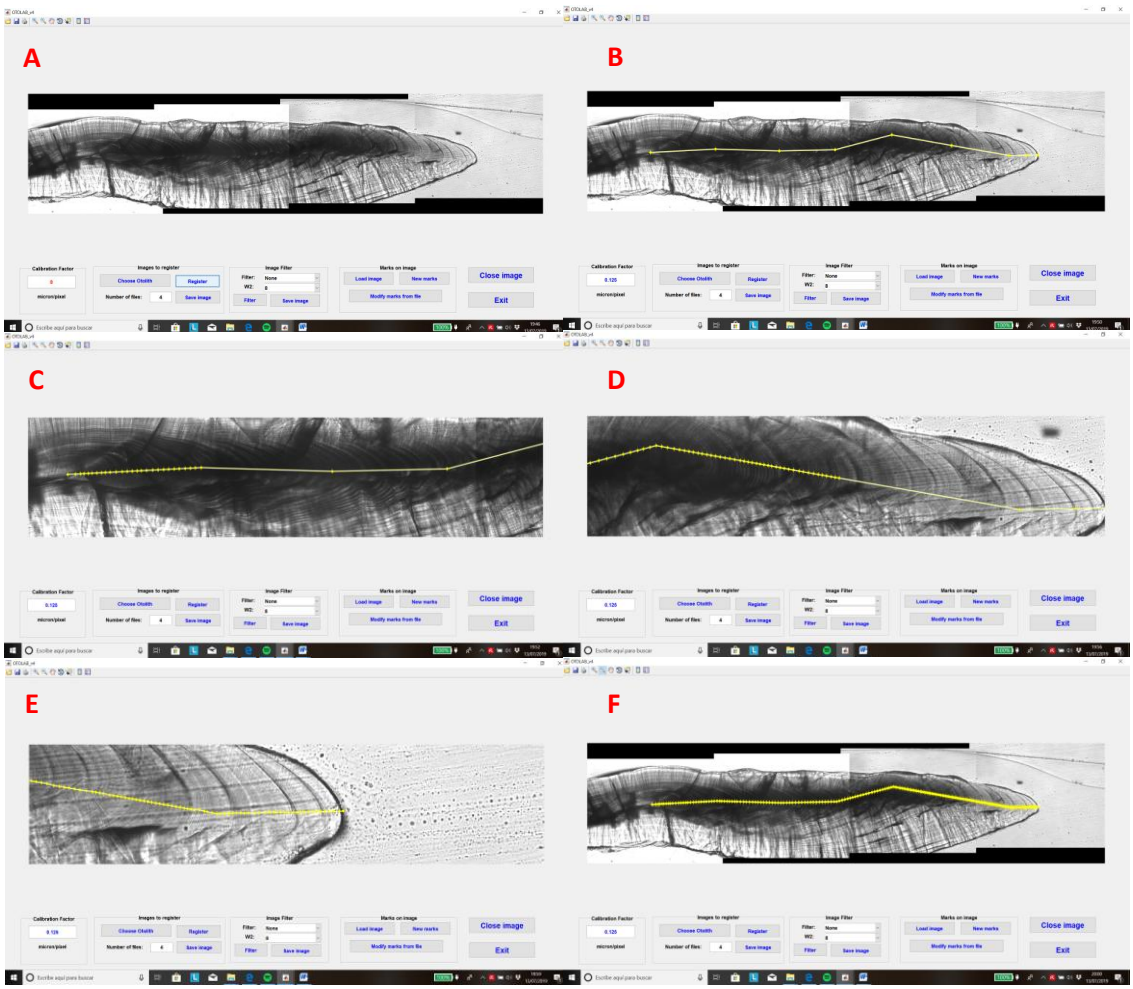
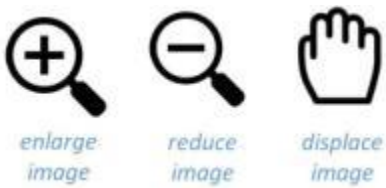


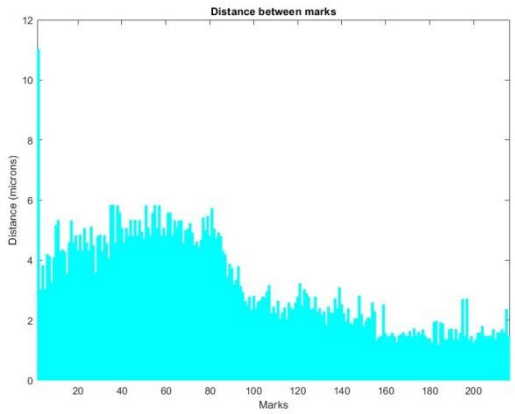
Figure 2.5. Adding marks process in an anchovy otolith (longitudinal section): A) tiling images (4 in this case), B) drawing a polyline, C-E) zooming and growth increments marking and F) marks storing.

New measurements on the same image will be saved again with the same name and the previous one will be overwritten. To avoid losing previous observations it is strongly recommended to change the Excel's file name after every valid reading has been done.

During the reading procedure, the user may need image enlargement to better discriminate DGI's. By using top screen menu tools users can enlarge, reduce or relocate otolith on the screen.



Once finished the image reading task, the user can close the reading stage by pressing 'Close image' and: 1) loading/choosing a new otolith or 2) 'Exit'. A new image shows an histogram of measurements. This plot can be saved manually.



If the user wants to modify a previous observation, he/she should press on *Load image* and chose the .*png* or .*tiff* image of an otolith. Once opened, push the button *Modify marks from file*. A window will emerge and open the Excel file in the working folder, assuring that a file corresponding to the same otolith is chosen. When selected, a red polyline will be drawn on the screen. The possible actions now are moving, deleting or adding marks.

To move or delete a mark, place the pointer over the mark, it changes to a circle. To move it, click and drag the mark to the new position. To delete a mark, user must right-click the mouse and select '*Delete vertex*' from menu. Also, in the same menu the user can choose to change the polyline and the color of marks. To finish the new reading, double-click over the polyline as mentioned before (pointer as double arrow).

To finish the program simply click on the *'EXIT'* button.

3. The OTOTHRESH tool for image segmentation

In computer vision, **image segmentation** is the process of partitioning a digital image into multiple segments (sets of pixels). The goal of segmentation is to simplify and/or change the representation of an image into something that is more meaningful and easier to analyze [3] [4]. Image segmentation is typically used to locate objects and boundaries (lines, curves, etc.) in images. More precisely, image segmentation is the process of assigning a label to every pixel in an image such that pixels with the same label share certain characteristics.

The simplest method of image segmentation is called the **thresholding** method. This method is based on a clip-level (or a threshold value) to turn a gray-scale image into a binary image. There is also a balanced histogram thresholding.

An automatic segmentation for a great amount of otolith images would be a desirable solution. Nevertheless, the high variability among otolith images makes this standardization unfeasible. Eventually, a manual segmentation tool is much more convenient, so we offer here OTOTHRESH tool, a handy tool based on components segmentation HSV (Hue, Saturation, Value), where the user make every choice.

IMPORTANT: Otolith images for morphological studies are referred to the whole otolith and not to an otolith's section. So, for the OTOTHRESH and the OTOSYM tool otolith images are always 'whole otolith' images (Figure 3.1.). Some recommendations on images settings are given in ANEXE 6.3.

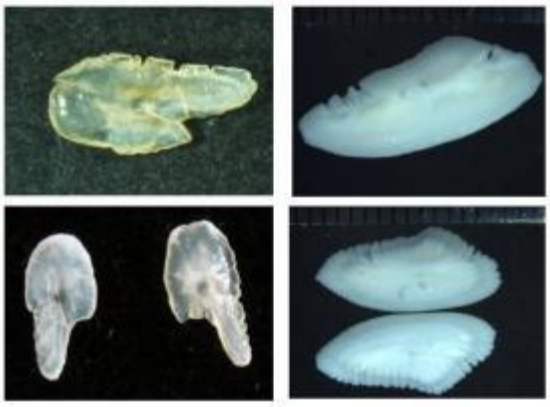


Figure 3.1. Whole otolith images for morphological analysis. Right: sardines, left: hakes. Top: single otolith, bottom: left and right otoliths.

In order to achieve any otolith image segmentation from a wide-ranging sort of sources, a high number of options and filters have been included in the OTOTHRESH tool.

A general view of OTOTHRESH panel before loading an image is shown in Figure 3.2., showing a number of assorted applications (filters, weights, gradients, etc.).

Figure 3.2. The OTOTHRESH tool starting screen.

After selecting an otolith image from a folder (Load image button), we can observe the original image itself in the top left side of the screen (Figure 3.3.). The View mode contains other image options, considering the filters and other selected features (see next section). The Loaded image can show a single otolith or its pair.

In the bottom panel, there is some basic information of the image (Image Info) where a color histogram below shows the Red, Green and Blue components of the otolith color image. This information can help the user set the best options.

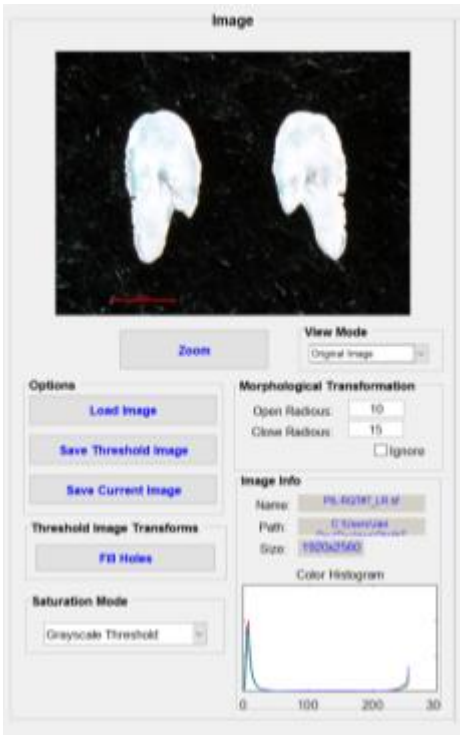


Figure 3.3. OTOTHRESH tool starting screen

The right panel shows automatically some image transformations and their corresponding thresholds. The user can modify each one of the components in several manners (Figure 3.4.).

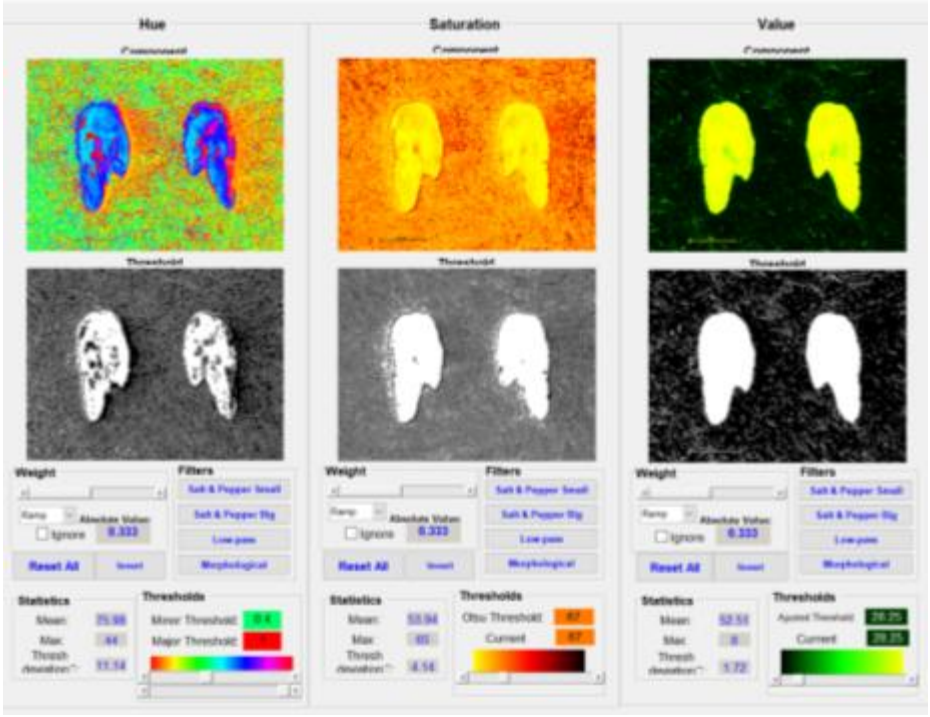


Figure 3.4. The OTOTHRESH tool components right panel.

A broad perspective of the process to obtain the segmentation is showed here. The user can constantly modify the choices so images of components also adjust accordingly as well as the final result. Every modification made manually (filters, weights or thresholds) re-starts the processes.

3.1. Color change

The original images of the otolith are usually color images (RGB). These images are automatically converted into three components HSV images (Figure 3.5.).

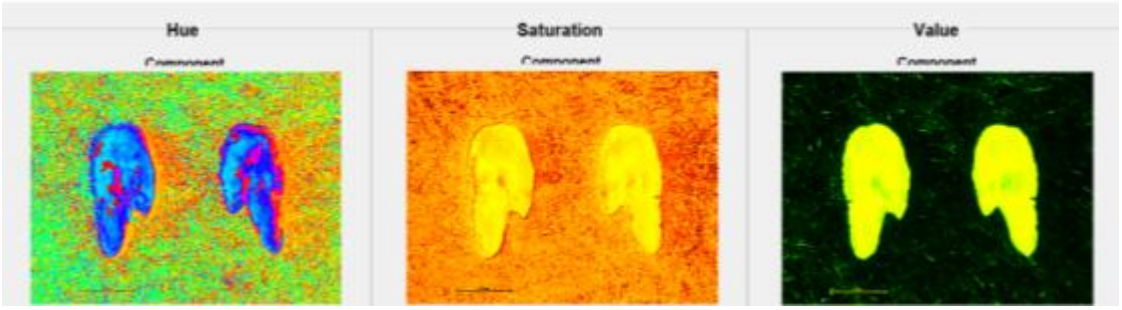


Figure 3.5. HSV converted images from original one.

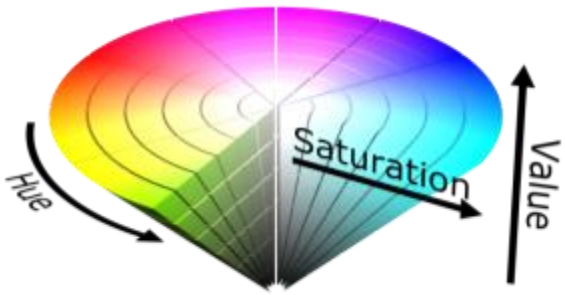


Figure 3.6. HSV cone.

Unlike RGB, which is defined in relation to primary colors, the model HSV is defined in a way that is similar to how humans perceive color. This color space describes colors (hue or tint) in terms of their shade (saturation or amount of gray) and their brightness value (Figure 3.6.)

In the OTOTHRESH tool, the threshold process becomes more instinctive using the color images and the selection bars (Figure 3.7.).

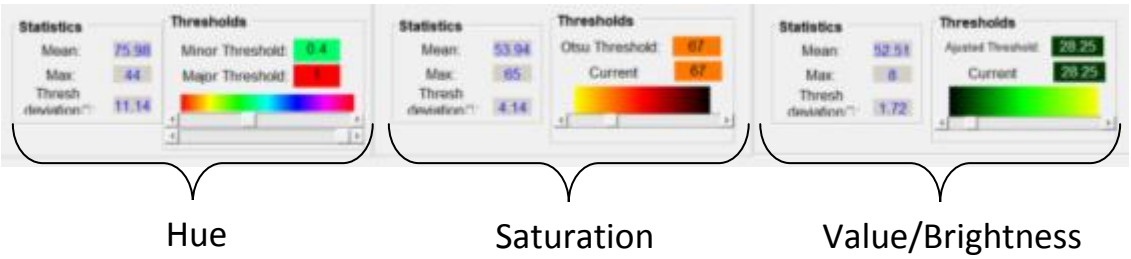


Figure 3.7. Sliding bars and color selection for each HSV component. Left: Hue; Center; Saturation and Right; Value (Brightness).

TONE (HUE): The color selection tool corresponds to the component transformation from RGB to HSV (Figure 3.6.). In most otolith images, the object and background should be of different tones. Dark matt backgrounds are recommended next to pale dry otoliths. Different tones are here transformed into separate colors.

SATURATION: Color selection for saturation ranges from yellow to black, going through red (Figure 3.6.). Low saturation values are shown as high intensity values and vice versa. In this way, the highest saturations are given to the background and image intensity is then visualized with brightness intensities. Here, otoliths appear with high intensity colors while background correspond to low intensity colors. Contrast is amplified within this process, enhancing the perception of the different original values.

BRIGHTNESS: Finally, brightness color selection is performed to increase contrast, as well as saturation. It ranges from black to yellow, going through green (Figure 3.6.).

3.2. The Threshold selection

Thresholding is the main step of the whole process. Each component (H, S and V) is thresholded and results are stored in H_T, S_T and V_T, called **threshold images**. Thresholding methods used here are both automatic and manual. After every image or variable modification, an **automatic thresholding** is completed. The method used in each case depends

in the component, up to four different methods (one per component, and two for saturation). On the other hand, for each component, thresholds could be adjusted using correspondent sliding bars, right through a **manual thresholding**. Color choices in the slide bars are also shown as background of correspondent numeric threshold values just above.

Figure 3.8. sketches the OTOTHRESH tool diagram 1 where some functions for segmentation are represented. The user can change options and preferences until reaching the desired result. Processes are handled by the user and shown in red, in blue for automatic processes and in green for those images visualized in the screen.

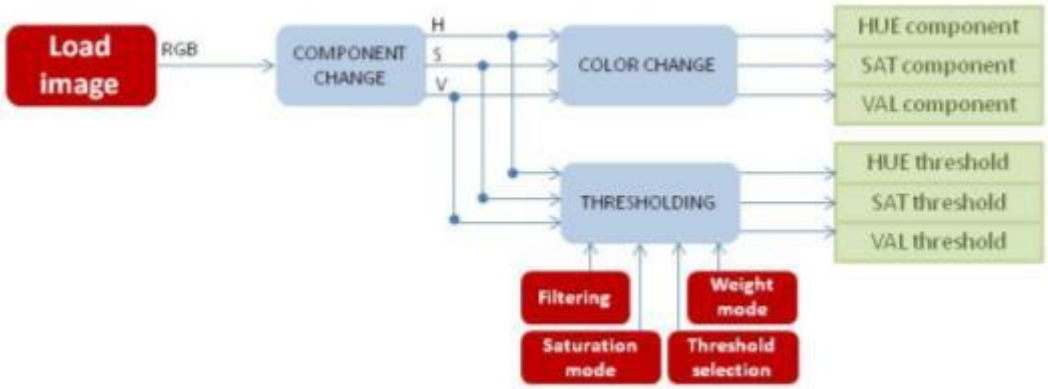
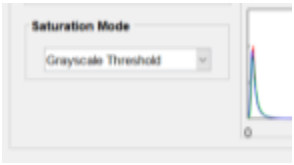


Figure 3.8. The OTOTHRESH tool processes diagram 1. Red: under user control; Blue: automatic processes and Green: screen output.

TONE (HUE): The Tone component does not require threshold calculation, as it does not differentiate the object well and the background is obtained by an automatic process that is not recommended for segmentation. Instead, manual controls help much better in setting threshold levels (Figure 3.6. left).

SATURATION: For saturation thresholding, two 'Saturation Modes' are available (bottom left corner in the screen), although the Otsu method for thresholding is used in both cases:



- Grayscale Threshold: Using saturation component itself.
- Noise Threshold: Using saturation standard deviation.

In both cases, slide bar can be used manually.

BRIGHTNESS: The Brightness or Value component is thresholded by an empirical method (Adjusted threshold). Users can modify thresholds using the sliding bar (Figure 3.6. right).

3.3. Weights sum

After each component has been thresholded following user's criteria, a sum of weights is performed to obtain a final thresholded otolith image. In Figure 3.9, diagram 2 schematizes the rest of processes performed by the OTOTHRESH tool, indicating the user's handling (in red), automatic processes (blue) and screen output (green).

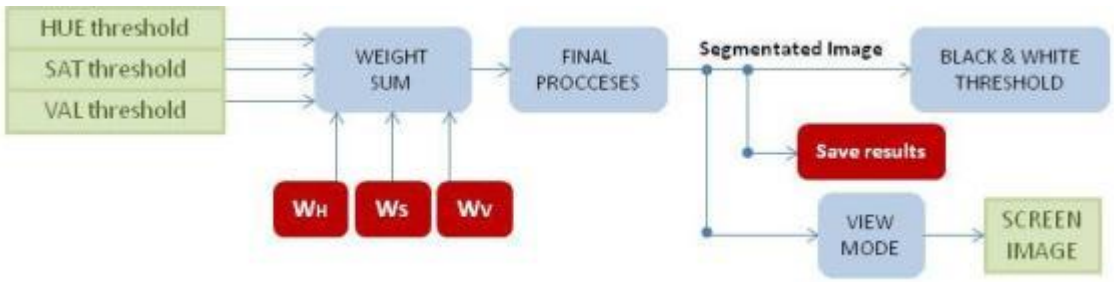


Figure 3.9. The OTOTHRESH tool processes diagram 2. Red: under user control; Blue: automatic processes and Green: screen output. W (weight)

In this process, the contribution of each component to the final image is decided by assigning certain weight, with a value of 0 or higher. The sum of the three components should be 1. By default the contribution of each component is of 0.333. Ignoring any of the components, the contribution of the others automatically increases.

The final image is the sum of the products of each image and its weight, divided by the sum of the weights. So, the 'sum image' provides the results from each component sum raised by a certain weight.

The weights can be manually modified by sliding bars in each component. Below each sliding bar, there is a menu where the user can choose among three kinds of weight to consider, based on different transformations on the thresholded image:

- **Ramp:** this option slightly softens the umbralized image borders to be less abrupt and reduces noise zones considerably. It is the default option in the three components and the mostly recommended.
- **Discrete:** borders are more abrupt and the surrounding noise increases. It is suggested for highly contrasted images.
- **Smooth:** borders are highly softened and consequently noise is reduced but losing object definition. It has been included for occasional circumstances.

This process is linked both with thresholding procedure with the sum of weigths. Once the sum of weights is achieved, we obtain a **segmented final image**. In next section, other tools are explained in detail, in order to modify component profile or the segmentation's final result.

3.4. Component filtering

A practical possibility for users to obtain an optimal segmentation is to apply different spatial filters to each component. Filters are applied on the component image, not on the thresholded one. Its goals range from noise reduction to change border shape. Filter options goes from less to more effect on image shape (Figure 3.10):

- **Salt & Pepper Small:** median filter using a small window size (3x3). It usually cleans some of the surrounding noise.
- **Salt & Pepper Big:** as above with a wider window size (9x9).
- **Low-Pass:** a different filter with same window size than before (9x9).
- **Morphological:** strong filter that usually modifies contour markedly.

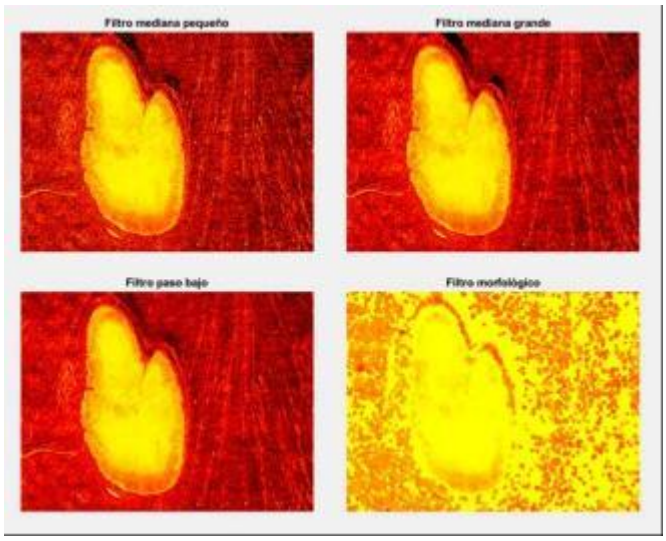


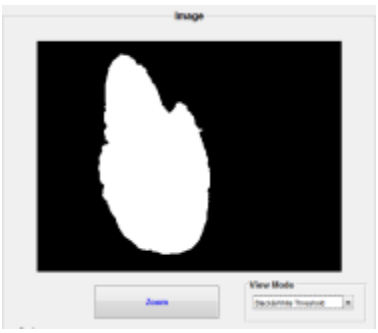
Figure 3.10. Effects of the four filters applied on a saturation component image. Salt and pepper small (top left); Salt and pepper big (top right); Low-pass (bottom left) and Morphological (bottom right).

The user can apply every filter and every time on certain components, as filters work on the current image. When the filtering is not acceptable, the original image can be refreshed clicking 'Reset All'. Also, in each component panel there is a 'Invert' button that inverts the logical values of the thresholded image of the component, before matching weights. Although it is not a filter itself, it can be practical in certain occasions. This function modifies the thresholded image. Filter options offer a wide range of modifications on images.

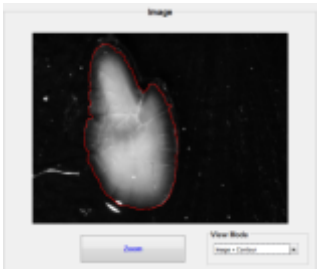
3.5. Views mode and saving

It is essential that user can visualize the results at any time in different ways to adjust modifications progressively until reaching a final adequate solution. The OTOTHRESH tool suggests six different image outputs in the 'View Mode' menu:

- **Original image.** It is the default mode when loading the original color image. This mode allows to visualize otolith's original shape and discern clearly otolith from background.
- **Black & White Threshold.** It is the final segmented image, to be used in further morphological analysis.



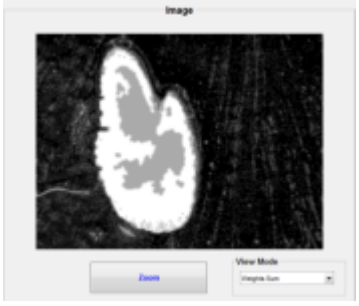
- **Image + Contour**. Image and contour. This mode shows the original image in grey range and on top, the red contour from the segmented image. It is possibly the most useful output, since it illustrates the segmented image if it fits to original one.



- **Original image * Threshold**. It shows the product of original grey and the segmented images. The result is the considered otolith in grey range and a black background. It could be useful to check whether part of the background has been included in the otolith.

- **Original image – Threshold**. This mode shows the difference between the original image and the segmented one. Now, the background is filled in a grey range and the otolith in black. Here we can check whether part of the otolith has been included in the background.

- **Weights Sum**. It is the sum of each component weight. To see the weights sum before applying final algorithms, it may help to better balance weights.



Just below the principal image window, a 'Zoom' button opens a new and bigger window for the current image, practical to identify minor aspects.

For saving images there are two choices, each one with an explicit button:

1) **'Save Threshold Image'**: the segmented image (black and white) is automatically saved in a folder with same name as original folder followed by '_work'. Image will have same name followed by '_T'. This image is essential for morphological analysis.
2) **'Save Current Image'**: in this case current image (at any mode) is saved in a folder and with a name at user's criterion. Although these images will not be used in further OTOLab software analysis, it can be useful for other purposes.

3.6. Final processes

Under 'Final processes', we include some procedures that take place after thresholding and summing routine, namely the 'Morphlogical Transformation' and 'Fill Holes' (Figure 3.11).

Figure 3.11. Final processes in the OTOTHRESH left panel.

- **Morphological Transformation**. These functions include those which change objects profile in binary images (black and white). Here, two functions are included: dilation (opening) and erosion (closure). Usually both processes are used combined, one after the other and vice versa. Both opening and closure require an initial parameter or 'Radius', the value of disc radius that operates in these transformations.

  Wide disc sizes produce an excessive contour smoothness, losing information and deforming the object remarkably. Instead, small disc sizes make smoothness minimum and not appreciable or even counterproductive. Although default values could be manually typed, those chosen produce habitually good results in most images (10 pixels for opening radius and 15 pixels for closing).

  In Figure 3.12, we can appreciate the result of a hake otolith segmentation with and without Morphological transformation. In this case (high quality image and very narrow

inbounds), the segmented image fits better to the original without morphological transformation.

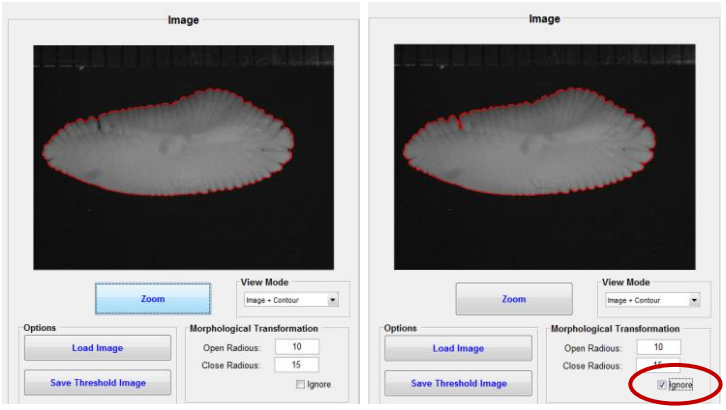


Figure 3.12. Hake otolith image with (left) and without (right) Morphological Transformation.

- **Fill Holes**. The goal of this option is to fill the possible holes (contoured by a red line in Original image and contour option) that could artificially appear inside or outside otolith during segmentation procedure. By clicking 'Fill Holes' button all the holes are filled without affecting the otolith contour, since it is placed at the end of all processes. Conversely, if a modification is made in a previous process, holes will appear again. In this case, 'Fill Holes' should be clicked once more. In Figure 3.13, we can appreciate the 'Fill Holes' result.

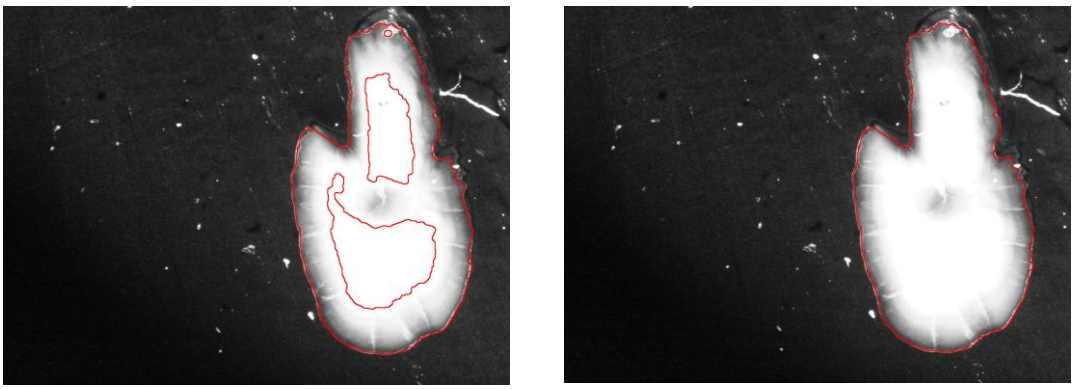


Figure 3.13. An Otolith segmented image before (left) and after (right) filling holes.

4. The OTOSYM tool: Morphometrical analysis

Once images have been segmented, it is time to bring out the information taken from them. The OTOSYM tool makes use of the segmented images obtained with the OTOTHRESH tool for extracting otolith shape parameters, which are extensive data for fish population studies. OTOSYM tool is focused mainly for symmetry analysis, by means of contrasting the left and right otolith measurements (Figure 4.1).

Figure 4.1 The OTOSYM tool initial screen before loading segmented images.

- **The Loading panel.** This panel contains three 'Load images' buttons for the two ways of loading segmented images in the top left corner. First option is to load both otoliths left and right ('Load L' and 'Load R' buttons) separately. Downloading a segmented image containing both otoliths is also possible using 'Load L + R' button. In those cases, the OTOSYM tool automatically will isolate both otoliths. Once loaded, the images will appear in the bottom windows. When trying to load a double otolith image with the left or right button or a single otolith image with 'Load L+R' bottom, an error message will emerge.

- **Calibration.** Just below loading panel calibration factor (pixels per mm) must be typed. Calibration factor is specific for each microscope, camera and magnification. Without a calibration factor, all otolith measurements will be shown in pixels.

- **Otolith information panel.** Once loaded, each otolith has a specific panel with a correspondent segmented image. A 'Rotate 180º' button below each image permits to

adjust both images as specular images (Figure 4.2). Also, next to them, the 'Morphological Features' panel will show some morphologic parameters once the Calibration Factor has been introduced (Figure 4.3).

Figure 4.2 The OTOSYM tool left and right otolith windows and information panels. Top; before rotation and Bottom; after rotation and ready for analysis.

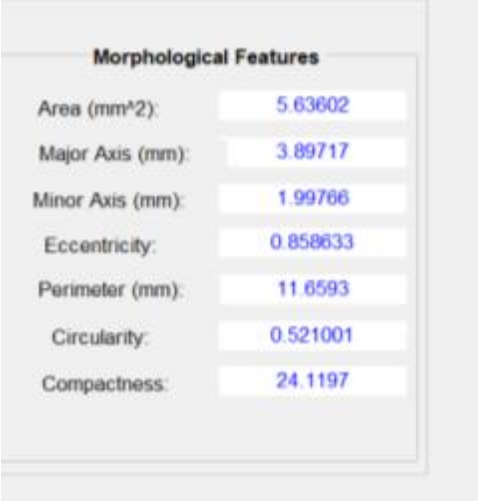
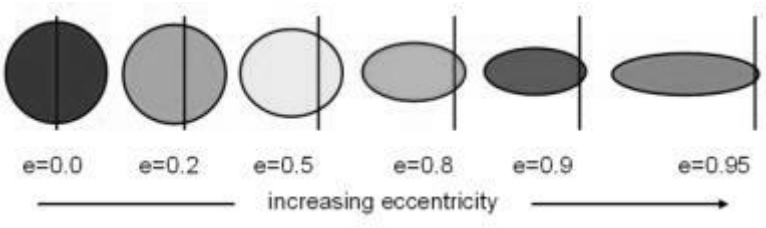


Figure 4.3 The OTOSYM tool Morphological Features panel once images have been loaded and the calibration factor is set.

The following parameters have been selected to automatically measure both otoliths:

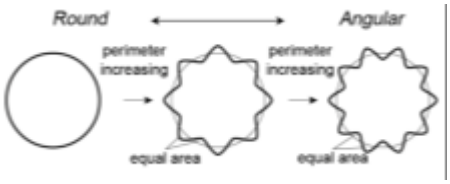
1) **Area.** Area of sagittal otolith plane in mm^2.
2) **Major axis.** Measurement in mm of the largest axis of the otolith, usually taking the antero-posterior axis.
3) **Minor axis.** Measurement in mm of the perpendicular axis through the centroid.

4) **Eccentricity.** A non-dimensional value to express similarity to a circle. Eccentricity is 0 in a perfect circle in an ellipse is higher than 0 but lower than 1. Thus, it measures how elongated an object is.



5) **Perimeter.** Measurement in mm of otolith perimeter (contour).

6) **Circularity (Roundness).** It is a non-dimensional value calculated as $\gamma = 4\pi S/p^2$, where S is the two-dimensional surface of the otolith and p its perimeter.

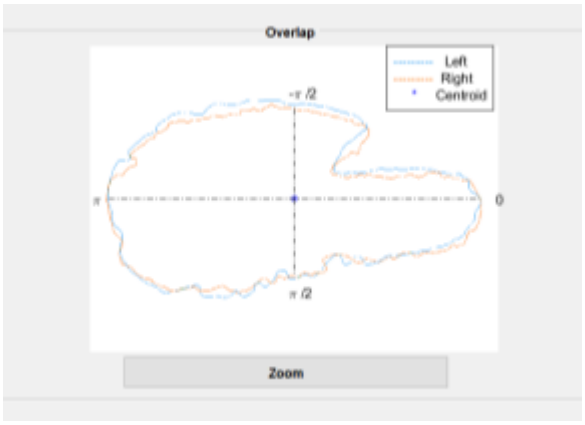


The circle is the two-dimensional object with the largest γ ($\gamma_{\text{circle}} = 1$). Objects with other shapes will have a smaller γ. Circularity is the measure of how closely a shape of an object approaches that of a mathematically perfect circle. More irregularities give more perimeter for the same area. Usually, older individual have more irregular otoliths.

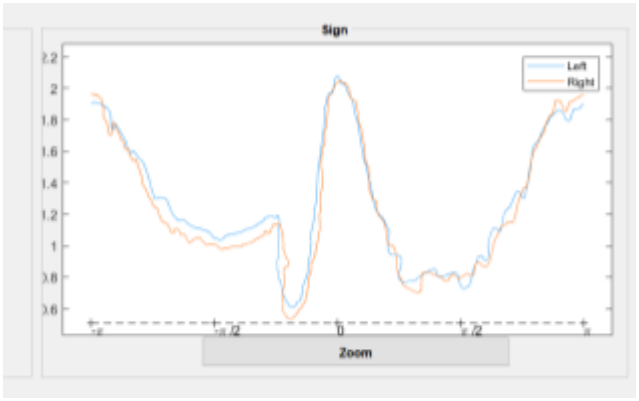
7) **Compactness (Roughness).** It is a non-dimensional value calculated as inverse of circularity: $\kappa = 1/\gamma$. Obviously, $1 \leq \kappa < \infty$.

- **Generate images button.** This button remains dark orange while images are not loaded and no results are available (Figure 4.1). Once the images are loaded, these will turn to pale grey. After clicking it, the overlapped left and right otolith contours and signature plots will show up (Figure 4.3).

- **Overlapping panel.** This window shows both the otolith contours (Left in blue and Right in red) overlapped and centered over otoliths centroid (morphological center). The otolith's biological center (nucleus) is not evident and its calculation may change for each fish species. Instead, a centroid is more evident and therefore preferable. The 'Zoom button' enlarges image in a new window, where the image can be saved in many different formats (JPG, TIFF, MATLAB Figure, etc.).



- **Signature panel.** Finally, this panel shows the signature of each overlapped otolith. Essentially, the signature is the graphic representation of contours in two axes x, y. Exe y represents distances between centroid and contour points as an otolith radius, and exe x the degrees in radians that this radius is turning, from -π rad (-180°) to π rad (180°). The 'Zoom button' enlarges the image in a new window, where the image may be saved in many different formats (JPG, TIFF, MATLAB Figure, etc.).



5. SHAPE DESCRIPTORS: Parameterization of an image collection

In addition to the tools explained above we find in the Main Menu of OTOLab software another panel devoted to Shape Descriptors in the bottom right corner (Figure 5.1).

Figure 5.1 The OTOLab software Main Menu screen.

The Shape Descriptors panel is actually a TOOL itself but it is not considered separately because it has only a few functions when compared with the above described tools. Basically, it takes all the segmented images files from certain folder and generates automatically a unique Excel sheet containing all the parameters concerning shape measurements. Part of these shape parameters have been explained in section 4 (Area, Major axis, Minor axis, Eccentricity, Perimeter, Circularity and Compactness) and other variables will be clarified below.

The user should select the folder containing the segmented images clicking the 'Load' button. Routinely, the number of *.png* files available appears in 'N files' open area. At the same time, a new Excel file will be created within the folder with the same name, adding '_sd', containing results. This name appears in an open area of the panel, and can be manually modified. The software cannot identify whether chosen *.png* files are segmented images or not, but the final results will not be created with non-segmented images. Finally, if two different objects were identified in a file, these objects will be identified as left and right otolith. They will appear in the correspondent Excel file with the same name as original file, adding 'L' for the left and 'R' for the right otolith. It is essential that images are well segmented and that the number of objects is correct (one or two otoliths). Figure 5.2 shows an example of an Excel results file.

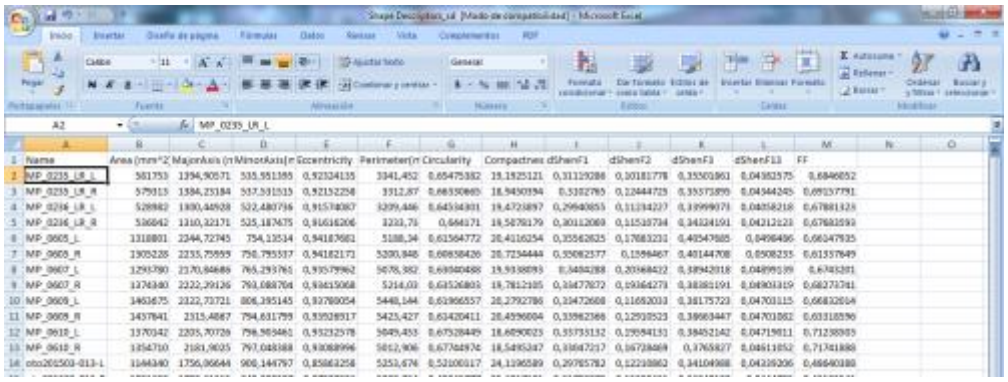


Figure 5.2 Excel result file.

Other shape parameters calculated routinely and included in results are 'Moments of Region Boundaries' [7] and Fourier Descriptors:

1) **sShenF1.** It measures the global irregularity. It is a positive value, normally between 0-1.

$$F'_1 = \frac{\sigma}{mean}$$

2) **sShenF2.** It measures asymmetry (skewness). Positive or negative, normally between -1 and +1.

$$F'_2 = \frac{\sqrt[3]{Skewness}}{mean}$$

3) **sShenF3.** It measures particular irregularity. Positive or negative, normally between -1 and +1.

$$F'_3 = \frac{\sqrt[4]{Kurtosis}}{mean}$$

4) **sShenF13.** It also measures irregularity, more precisely, and quite sensible to small variations along otolith contour (i.e. denticulated edge).

$$F_{13} = F'_3 - F'_1$$

5) **FF.** It measures high frequency contour irregularities. It is also normalized between 0 and 1.

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6. ANEXE. OTOLITH IMAGES: RECOMMENDATIONS

The basic equipment for image acquisition should be a microscope/stereomicroscope with a camera connected to a desktop computer. Each camera works with particular image analysis software. TIFF images could be used in OTOLab while following some recommendations.

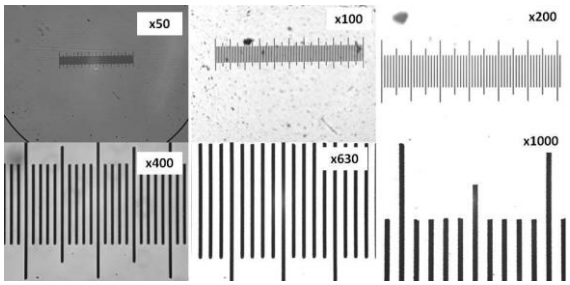
6.1 CALIBRATION

Before any photograph is taken, calibration of the equipment should be made. Each digital image is made with a particular equipment, magnification and quality. The relationship pixels-mm should be known for every combination. This information is essential for OTOLab to work with digital TIFF images.

A micrometre should be used to obtain pixels-mm relationship in every situation (equipment-magnification-image quality). The process is as follows:

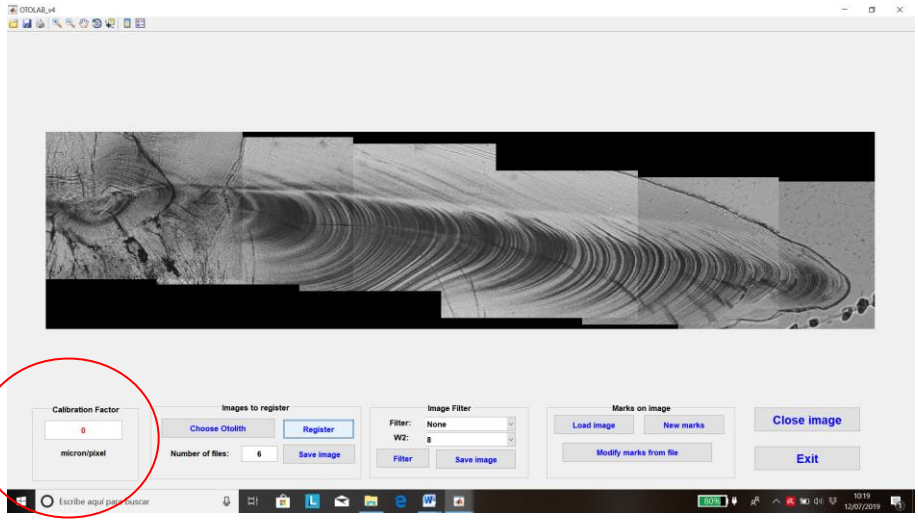
Image of micrometre

1. Select the magnifications that will be used for the whole process.
2. Take micrometer TIFF photographs separately with the scale for each objective.

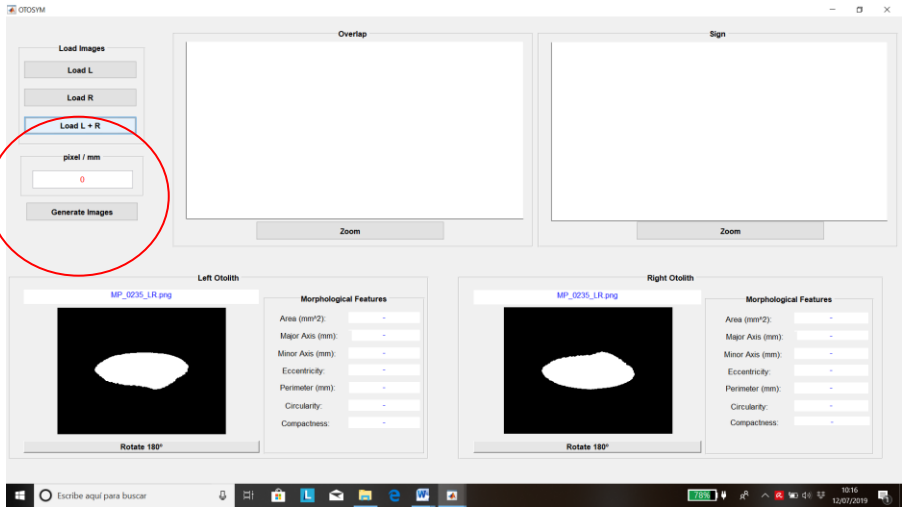


3. Indicate the units that will be used during the photographs acquisition whether microns/pixel or pixel/mm.
4. Write down relationship (micron/pixel in OTOlab or pixel/mm in OTOSYM) using the image analysis system of your equipment. OTOLab does not make this, but requires it in every case (each reading or symmetry exercise). This value remains as 0 (red) after each image loading and has to be changed manually for further working.

In OTOLab tool (micron/pixel):



In OTOSYM tool (pixel/mm):



5. Follow this procedure before the otolith image acquisition starts. Also, after the collection of otolith images haven been taken, it is recommendable to repeat the process, as microscope handling and continuous focussing may change the settings and consequently the calibration factor.

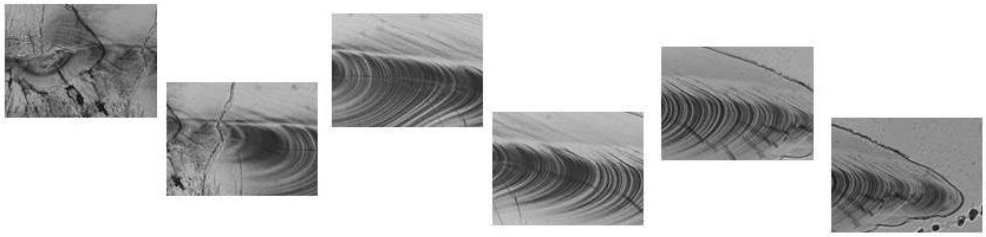
6. It is recommendable to keep a micrometer TIFF photograph in every otolith photographs folder at the same magnification. This avoids future measurement errors.

6.2 OTOLITH SECTIONS FOR AGEING (OTOLab tool)

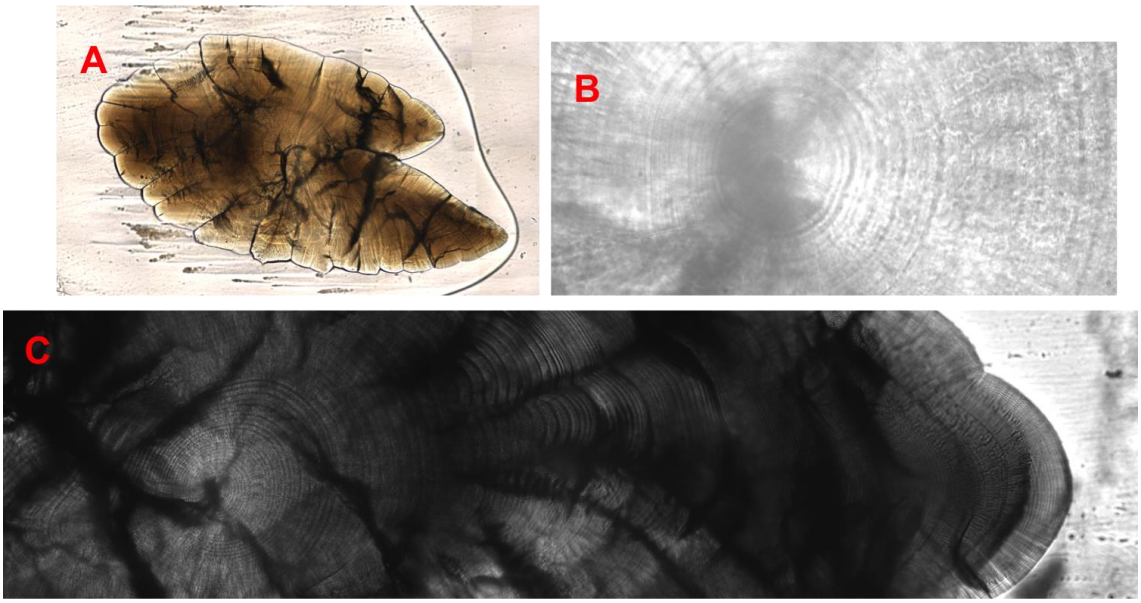
When ageing at seasonal/year level usually a single stereoscope photograph is enough. This section is focused mostly for microstructure analysis (daily increments), where several microscope photographs should be tiled.

In order to obtain high quality images from otoliths for ageing the following steps are recommended:

1. Selection of magnification that will be used to visualize the otoliths and take the photographs. The same magnification will be used during the whole process of a single otolith.

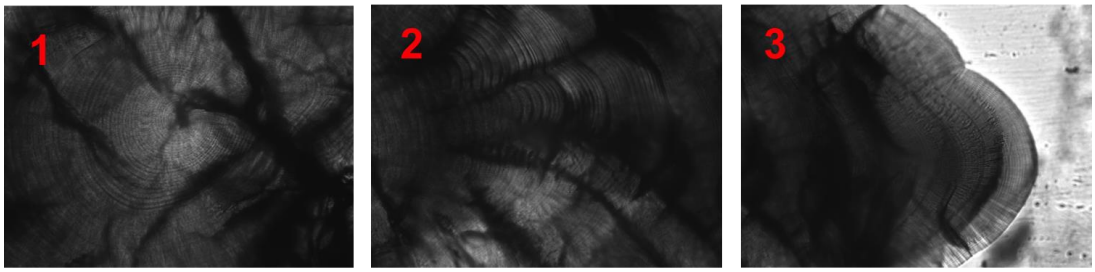


2. *Photograph acquisition:* is it recommended a) to take first a full picture of the whole otolith section in order to have a general view of the full otolith and b) to take several pictures at different magnification of the nucleus (i.e. x200, x400 and x1000).



Images from an anchovy otolith: A) Panoramic view, B) image from the nucleus (x1000 magnification) and C) image from the otolith taken at x200 magnification.

3. *Overlapping images:* the user will have to take more than one picture of the same otolith in order to have a full view of the whole otolith. For this reason it will be needed to overlap images (with 10-20 % overlapping area). It is recommended to search for a reference point to overlap the images and to always follow the same growth axis.



Images taken individually, following the same growth axis and with an overlapping area within 10 – 20 %.

4. *Photographs labelling:* OTOlab **only recognise images that are properly labelled.** The files have to be named with a specific numeric termination. In this way image number one must end in _01; image number two must end in _02 and so on depending on the number of images used to merge into panoramic views from the nucleus to the posterior edge.

   Example: when 3 images have been taken from anchovy (*E. encrasicolus*) otoliths using x200 magnification to merge then into a panoramic view, a proper way to name them would be:

   E.e_x200_01
   E.e_x200_02
   E.e_x200_03

6.3 WHOLE OTOLITHS FOR MORPHOMETRY (OTOTHRESH tool)

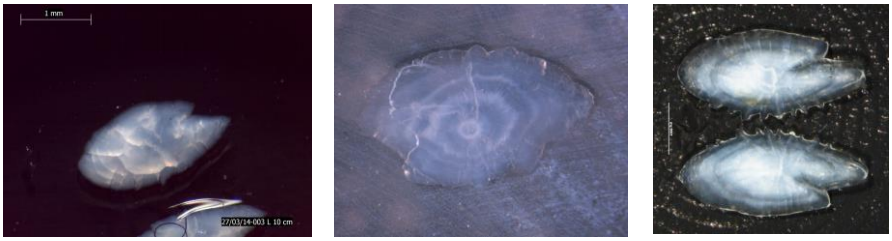
The equipment needed for the image acquisition will be a stereomicroscope with camera connected to a desktop computer. In order to obtain high quality images the following steps are recommended:

1. Selection of magnifications that will be used to visualize the otoliths and to take the photographs. The same magnification will be used during the whole process to homogenize the samples (otolith image collections).

2. A micrometre will be used to calibrate the stereomicroscope before any photographs is taken (See section 6.1).

3. To highlight the otoliths contour/morphology is recommended to use a dark background as for example a velvet fabric or cardboard.

4. Images with bad lighting, low contrast, incomplete or broken otoliths are more difficult to work with. Dry and free (not fixed or embedded) otoliths are preferred, thus avoid images with as resin, glue or water reflections. Here some examples of bad images for morphometry analysis:

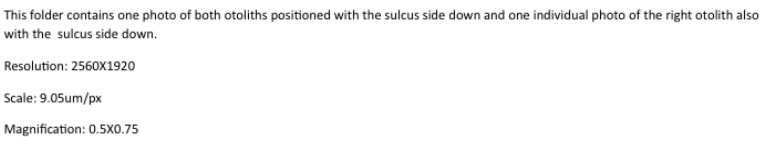


5. For image acquisition it is recommended to always use the same otolith orientation (i.e. sulcus side up or sulcus side down, vertically or horizontally positioned) to homogenize the sample. Otoliths should not be broken or damaged.

6. Light setting is of paramount importance to make sure that the otolith contour can be perfectly distinguished from the background. In this case it is not about differentiating the rings but visualizing the whole otolith morphology.

7. A rational system of image labelling is recommended, as lots of images are usually generated for morphology studies.

8. To finish, it is recommended to add a “readme.txt” file to the folder where images have been saved. This file should contain practical information about the steps previously mentioned (scale, resolution, magnification used…)


This folder contains one photo of both otoliths positioned with the sulcus side down and one individual photo of the right otolith also with the sulcus side down.

Resolution: 2560X1920

Scale: 9.05um/px

Magnification: 0.5X0.75