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**LABORATORY PROTOCOL FOR PROCESSING SAGITTAE  
FROM JUVENILE ANCHOVY**

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## Removal , Cleaning and Selection

- 1) Record data from the fish sample (i.e., length and weight)
- 2) Remove otoliths sagittae from fish (Fig.1)



Fig.1 .

- 3) Rinse with distilled water. Place otolith in an tray
- 4) Select the otolith. Discard broken otoliths and those who show no calcification areas(Fig.2 )



Fig.2.

## Sectioning

To obtain sections is important that all include the core to prevent the loss of the sequence of increases and thus avoid underestimation of age.

The most appropriate section plane in the sagittal otolith of anchovy is the sagittal plane (Morales-Nin, 1992).

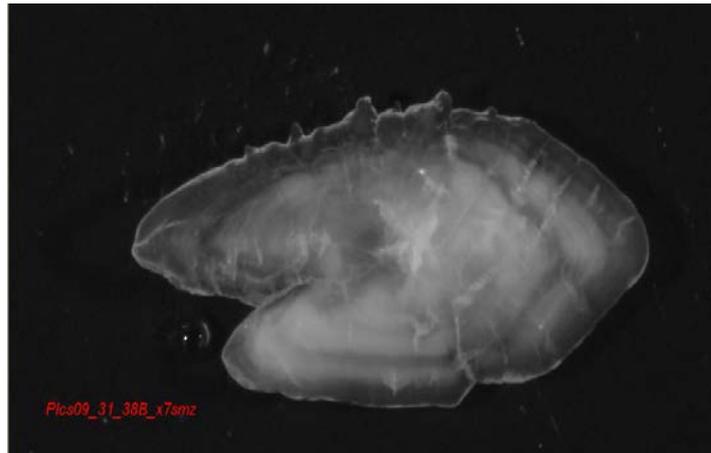


Fig.3 . Anchovy sagitta otolith (*Engraulis encrasicolus*), TL 115 mm. Image taken with stereoscopic magnifier and reflected light.

## Sanding

- 1) Heat a clean slide on a hot plate (SBS-Cpc 135 D) to a temperature of 135 °C .
- 2) Place a small piece of hard Crystal Bond (Mounting wax 40-8150) on the slide and allow it to melt. The thermoplastic glue is a solid substance which melts with heat and we can stick and unstick the otoliths on the slide as many times as necessary in the sanding process (Fig. 4).



Fig.4 .

- 3) Place the otolith on the glue (Fig.5). The face of the otolith that sticks firstly is the proximal face, with the sulcus down. Also make sure the plane you want to polish is parallel to the slide. Write the appropriate coding on the slide.



Fig.5 .

- 4) Check in the otolith has been placed in the correct position (Fig.6).



Fig. 6.

- 5) Adjusting the slide to the grinding workpiece. It is a Teflon clamp that fits the slide (this piece was created in the laboratory of Santander) (Fig. 7)



Fig.7 .

- 6) Otoliths were then sanded on both sides with a Buehler Metaserv rotating wheel covered with a grinding paper (grit 3  $\mu\text{m}$  for smaller otoliths and 5  $\mu\text{m}$  for bigger ones) at 200-500 rpm until nucleus and primary growth increments were visible (Fig.8). A slow and cautious approach should be adopted in order to prevent the over-grinding of the otolith. Regular checks of sanding plane should be made throughout preparation to prevent such an occurrence.



Fig.8 .

- 7) Turn the otolith. Put the slide on the heater and change the otolith to a new slide with Crystal Bond preheated. Observe carefully that there are no air bubbles between the glass and the Crystal Bond, if so try to remove it by moving the otolith or driving it into another slide if it is necessary. The sulcus is impregnated with Crystal Bond so the otolith is not see very clearly. This side is thicker and must be sanded with caution and always cheking the process under the microscope (Fig.9). It is interesting at this point to capture an image with

the image analyzer in case that may be sanded too much and we lose the edge (Fig 10 a, b).



Fig.9 .

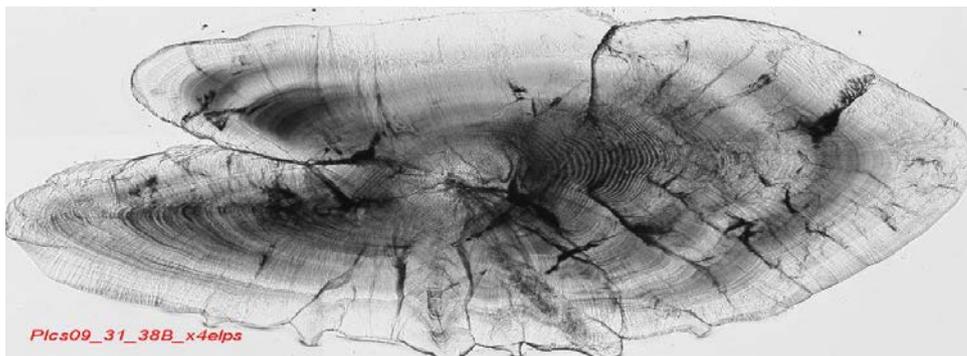


Fig. 10 a.



Fig. 10 b.

Fig.10 a, b. Section images of anchovy otolith (*Engraulis encrasicolus*) sagittal plane. TL 115 mm. Pictures were taken with an electronic microscope at (a) 40x and (b) 100x magnification.

## Storage of sections

Store polished sections (attached to glass slides) in slide boxes (Fig. 11). Label slide boxes with appropriate sample information. Storage of sections on glass slides eases handling for microscopic examination.



Fig. 11.

## Images analysis

It is useful to obtain digital images of the whole otolith preparation at this point as an aid to orientation for analysis.

This acquisition system and image processing consists of three units (Fig. 12.):

- An image acquisition unit (camera): Sony DFW-SX910 microscope connected to a Nikon Eclipse 50i with 5 targets from 4x to 100x magnification
- A central processing unit, storage and visualization: a team with a large random access memory (minimum 2 GB) for digital analysis with 2 screens (Screen 1: display calcified piece, screen 2: Biological parameters or analysis image).
- A software unit: TNPC 4.1 software (VisiLog 6.4) developed by IFREMER with the company Noesis. It is used to acquire and interpret images of objects calcified, particularly otoliths for age determination.

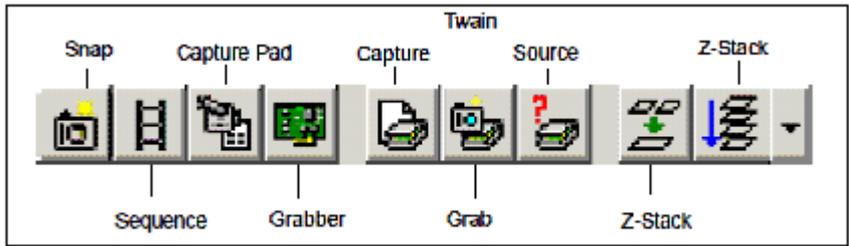


Fig.12. Computer-aided system to estimate the age and growth

## Image acquisition

Image acquisition of the calcified parts is made directly with the software VisiLog 6.4. The full image of the otolith microscopically observed once processed, is too large to be captured at once, so it must be captured in several parts, which are then linked through a macro, to get a picture of complete otolith.

Image acquisition is controlled through the ACQUIRE BAR, which is opened by selecting the VIEW/ACQUIRE BAR option.



**Capture Pad button**



The CAPTURE PAD button opens the CAPTURE PAD, which accesses the LIVE viewer, grabber and camera settings.

**Capture Pad**



The ACQUIRE BAR | CAPTURE PAD button opens the CAPTURE PAD, which consists of a toolbar and an image viewing area.

**Live Option**



When the LIVE button is clicked, the CAPTURE PAD display is constantly refreshed with the output from the camera.

**Snap**



The SNAP button captures a single image according to the camera settings established through the SETTINGS window.

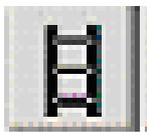
## Stop



The STOP button turns off the LIVE mode.

## Playing a Macro

To play a MACRO, select VBA | MACROS. The MACROS dialog box opens. Select the MACRO from the list, and click the **Run** button.



## Structure interpretation

The structures interpretations are made with TNPC 4.1 software which allows us counting and measuring the distances from the nucleus, the growth rings and the edge of the otolith. Each image before the interpretation must be calibrated.

## Calibrating an Image



Select VIEW | MEASURE BAR to display the MEASURE BAR. The CALIBRATION dialog box is displayed when the CALIBRATION WINDOW button is selected from the MEASURE BAR or by selecting: TOOLS | CALIBRATION

## To define a custom calibration:

- 1) Press the button and enter a name for the new calibration in the calibration name dialog box.
- 2) Enter the INTENSITY CALIBRATION values, if applicable.
- 3) Select the UNITS OF MEASURE.
- 4) Click on the MEASUREMENT TYPE button.

- 5) Select the CALIBRATION TYPE.
- 6) Select the GRAPHIC OBJECT BUTTON.
- 7) Adjust the calibration line(s) in the image.
- 8) Enter the ACTUAL LENGTH(S) of the line(s) next to the PIXEL LENGTH(S).
- 9) Click on the button or just select another calibration.

At the top left is the AVAILABLE CALIBRATIONS list which presents a scrolled list of predefined calibrations. As you select an existing calibration from the list, its name appears in the CALIBRATE NAME field, and the information describing and defining that scale appears in the rest of the dialog box. The contents of the CALIBRATION dialog box are dependent on which calibration you select. The DESCRIPTION area is an optional field to contain a description or comments regarding the current calibration.

### **Calibration Types**

Visilog offers three possible CALIBRATION TYPES. The best TYPE to use depends on your acquisition device and grabber board as well as on what information is known in the image. The contents of the rest of the dialog box will vary depending on which CALIBRATION TYPE you select. The choices are SQUARE, RECTANGULAR 2, and RECTANGULAR 3.

#### **Square Type**

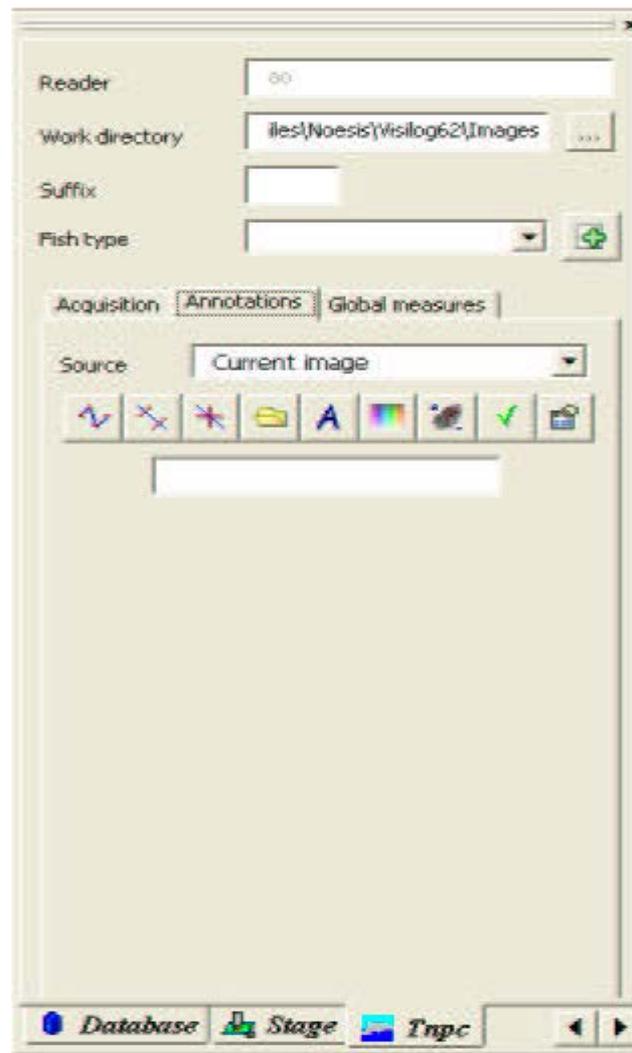


Use the SQUARE CALIBRATION TYPE when any one distance in the image is known (for example, a single dimension of an object) and the X:Y ratio is 1. Use this setting for zero-deformation grabbers.

To calibrate a SQUARE type, select SQUARE as the MEASUREMENT TYPE button and then click on the GRAPHIC OBJECT button. Within the image, click-and drag to resize and reposition the line to define a known element. Once you have drawn the calibration line in the image, the PIXEL LENGTH (line length in pixels) appears within the CALIBRATE MEASURES dialog box. Enter the known REAL SIZE of the line in the field next to the SIZE IN IMAGE and select the UNITS OF MEASURE (ex. feet, cm, etc.) in the field above.

## Image Annotations

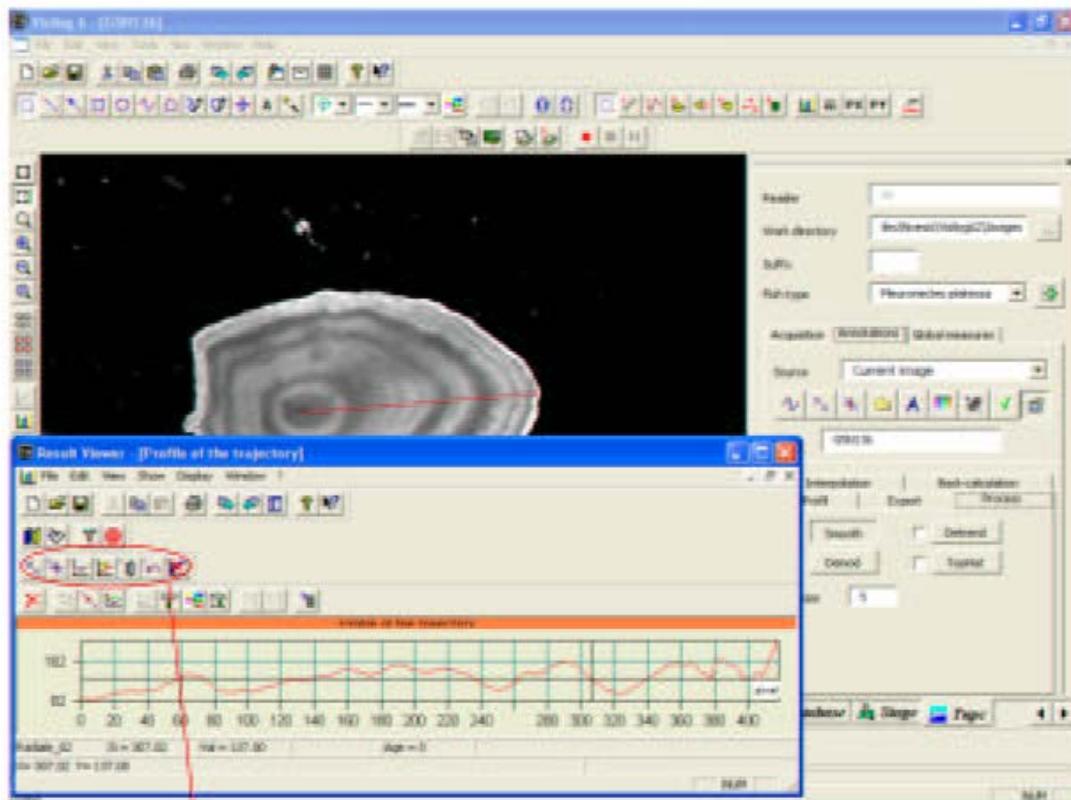
This menu is concerned with the interpretation of calcified structures and data recording. The user can create a radial, set markers or compute markers position using grey levels profiles.



To acquire a radial, click on . To draw a polyline, click one with the left button to set the starting point, and again once to define the end of each segment. To set the end of the polyline, use a double-click.

First, click on  to add GR markers. The result viewer is aimed at visualizing and analysing the intensity profile of a radial and the associated growth measurements.

The TNPC toolbar within the result viewer is used to interact with the intensity profile for its analysis (marker positioning, automatic aging, interpolation,...)



TNPC toolbar in the result viewer

Click on  deletes all unmerged markers from the display. The DELETE ALL button is located on the DISPLAY BAR, normally located to the left of the VIEWING pad.

Click on  to add markers manually. Then click once in the image or in the graphic window to set the positions of the markers. To remove a marker, you need to double-click on it. Once you are confident with your readings, send the results to excel.

