

Imaging of Otoliths for Analysis of Fish Age and Growth

A GUIDE FOR MEASURING DAILY INCREMENTS IN ADULT AND
JUVENILE OTOLITHS USING IMAGE-PRO PREMIER®

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Introduction

A look at a sectioned otolith under magnified light will reveal a pattern of ring-like structures, similar to those found in a tree. These microstructural features represent daily and annual increments, which are formed primarily by the circadian rhythm of the fish (Campana and Neilson 1985). Because they are formed on a consistent and periodic basis they can be used to determine age, growth rate, and size-at-age for individual fish. Counting rings and measuring their widths may seem straight forward, but interpreting otolith increments can be subjective and vary between readers. To minimize error among readers, the following guide has been created to standardize the method for counting and measuring daily increments in Chinook salmon (*Oncorhynchus tshawytscha*) otoliths using Image-Pro Premier[®] software. *This guide starts with a pre-prepared otolith section (sagittal plane), ready for microstructure analysis.*

Terminology

Aragonite: The most common crystalline structure of calcium carbonate in otoliths. Otoliths made of aragonite can be used for aging (e.g. image right).

Check: A band that is thicker and darker than the other daily increments (see image right). Checks are formed during periods of stress in the fish and can be used to identify specific life history events. There are three major checks that we use as landmarks in our work with Chinook salmon: **(1) hatch**, which formed during emergence from the egg; **(2) exogenous feeding**, which formed when the fish use up maternal yolk supply and began to feed in the water column, and **(3) exit**, which sometimes forms when the juvenile outmigrates (*although it is not clear whether it corresponds to the onset of migration – i.e. exit from the natal river, the process of smoltification, or estuary/ocean entry*).

Daily increment: A repeating pattern of light and dark bands in the otolith, where dark bands (under transmitted light) contain higher protein concentrations.

Dorsal: The side of the otolith that faces upwards when inside the fish. Will be smaller and more lobe like than the ventral side in a sagittal section (see image right).

Primordia: These lie in the core of the otolith, and represent the first material deposited while still in the egg. Chinook salmon will have multiple primordia. For

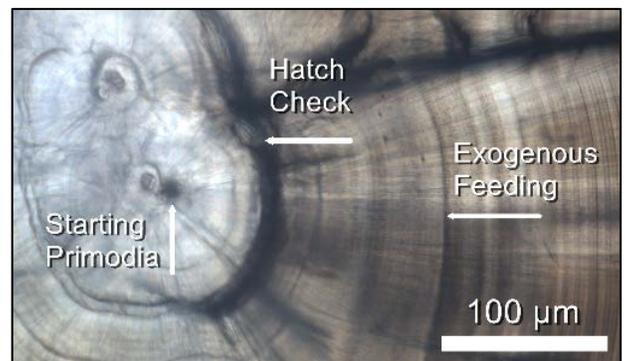


Figure 1: The three main features of the core area of a Chinook salmon otolith.

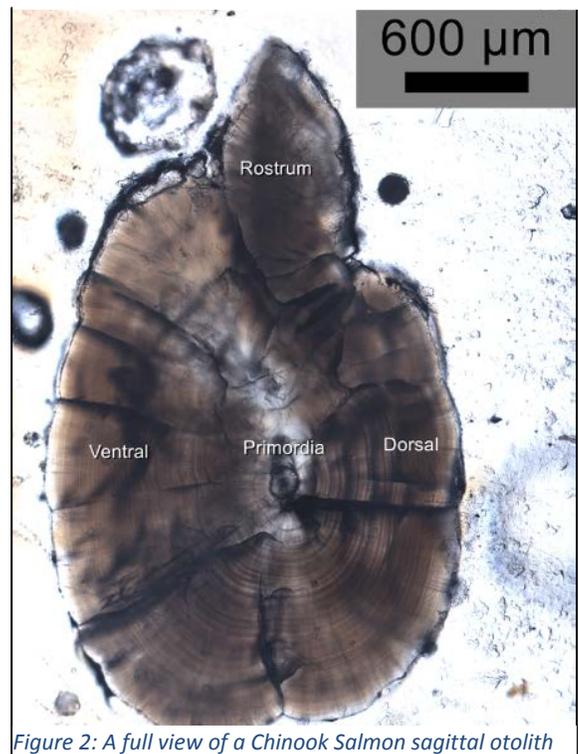


Figure 2: A full view of a Chinook Salmon sagittal otolith with the main regions labeled.

microstructural analyses the axis should be anchored by the dorsal-posterior primordia (identified in figure 1).

Rostrum: Or rostral tip. Will be facing forward (anterior) when inside the fish, forms a point between the ventral and dorsal sides.

Sagitta: One of the three pairs of otoliths present in teleost fish. For many species (Chinook included) the sagitta is much larger than the other two and subsequently the one prepared for analysis.

Vaterite: A different crystal configuration of calcium carbonate. Is clearer, glassier and less dense than the normal aragonite of otoliths. It is not possible to count increments in vateritic material.

Ventral: The side of the otolith that faces downwards inside the fish. Will be the larger of the two sides.

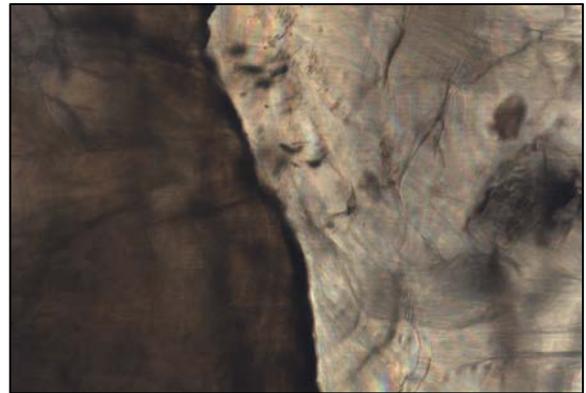


Figure 3: A magnified portion of an otolith under transmitted light that shows aragonite material transitioning into vaterite. Aragonite is the darker material on the left while vaterite the right-side, lighter material.

Initial Positioning

The goal is to position the sagittal otolith section (see XXX guide for how to prepare the otolith in this way) so that you can draw a transect running 90° to the length wise (anterior-posterior) axis. This can be more or less difficult depending on how the otolith is prepped. First, make sure the dorsal side is facing to the right. Second identify the rostrum, **which can point up or down depending on the side of the fish it came from**. Position the otolith until it is possible to draw a straight vertical line from the tip of the rostrum to the joint between the posterior regions of the dorsal and ventral sides. You can do this by moving the sample, or rotating the camera on its mount. A correctly positioned otolith will look like the sample in figure 4.

Positioning of an adult otolith can be more difficult as the features present in the adult and juvenile parts of the otolith don't necessarily align. Try to find the juvenile portion of the otolith in the center of the adult otolith. It will contain the same structures it did while the individual was a juvenile. Use those for positioning.

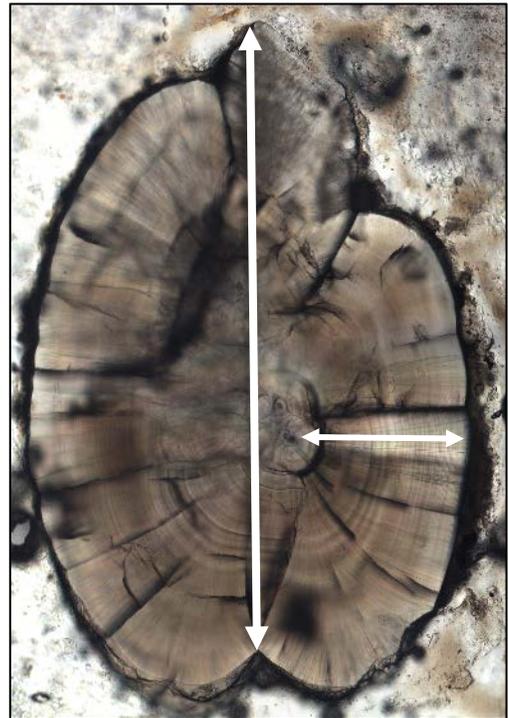


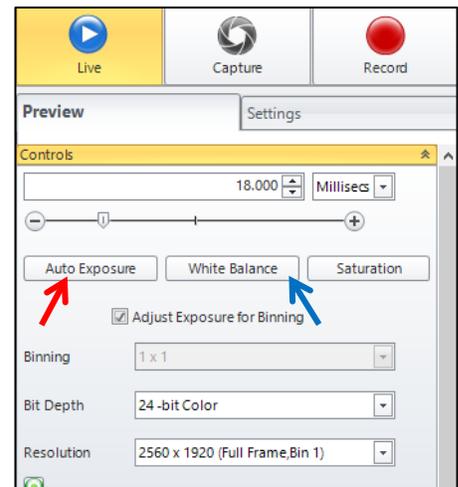
Figure 4: A juvenile Chinook otolith (sagittal section) ready for increment reads. The vertical arrow passes from the rostral tip to the cleft between the dorsal and ventral sides. The horizontal arrow is positioned at 90° to the vertical arrow, and will be used for creating the counting transect.

Photo Capturing

Find the core in your view finder using the 10x objective, then switch to the 20x. Open ImagePro Premier®. Click on the **Capture** menu and click the blue **Live** button (see right). Slide out the metal bar on the right of the microscope eye pieces to redirect the light from the eye pieces to the camera and put the live image on the computer screen. Be sure to readjust the focus when switching from the eyepieces to the camera.

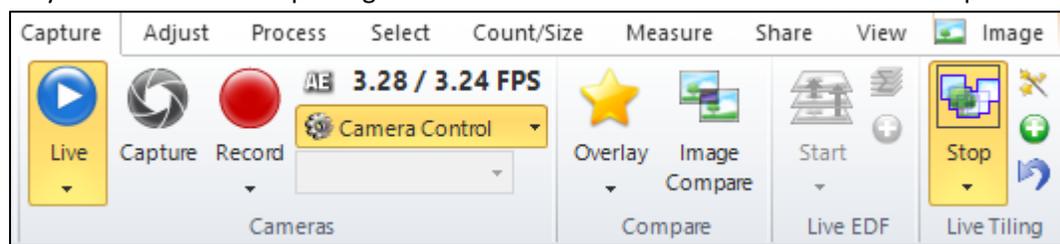


Move the stage until the core and the full area of interest is in view (from core to edge, and in Chinook salmon adults it will typically a distance of $\sim 1000\mu\text{m}$ from the core). Adjust the brightness by either changing the light levels on the microscope power supply, or the exposure time on the camera. To adjust the exposure time, first hit the **Auto Exposure** button in the capture window (see right). The program will automatically select the exposure it finds best. Afterwards the exposure can be changed manually by adjusting the number value that is just above the **Auto Exposure** button. If the sample still appears to be too dark, add water to the surface and allow it to soak for a few minutes. Clean off any excess water and redo the exposure adjustments (above). If the color seems off, press the **White Balance** button (see right) and draw a box over a random area of the otolith surface, and the software will automatically color correct the image.



Focus the scope until the image is clear and the bands are distinct. The increments should be on the surface of the section, but if they are not the otolith may need to be sanded further. Avoid a focus level that causes the increments to form split images that could artificially increase the count number. **Having a well-focused image is extremely important and can have a large effect on the accuracy of the reads.** If focusing is difficult the lens may be dirty. Wet a sheet of lens paper with alcohol; wipe the lens down, then dry off with a fresh sheet. Once you are confident that the image is focused properly and light enough to identify individual increments, hit the **Capture** button (see image below) to take a picture.

If it is not possible to fit the core and area of interest in one photo you can stitch several images together automatically using the **Live Tiling** button in the **Capture** menu (see below). Pressing this button will open up two windows (live view and capture view). Start with the core centered in the live view and very slowly move the microscope stage so that more area of interest is revealed. The capture view will have two yellow boxes, one composed of solid lines, the other



dashes. As the live view reveals more otolith area, the dashed line box will move as well. Keep moving the stage until the box turns green, pause movement briefly and a new image will be taken and stitched on to the composite image. Keep going until the composite image includes the entire area of interest. If you want to image particular areas before the program does it automatically, press the **Add Tile** button, which is the green plus sign (+) in the **Live Tiling** controls (see image on previous page). When the entire area of interest is captured, close the live view window using the “x” button on the top right of the Live View window and the tiled image will be captured. If the live tiling makes a stitching error you can reverse it with the **Undo** button which is the blue arrow in the **Live Tiling** controls (see image on previous page).

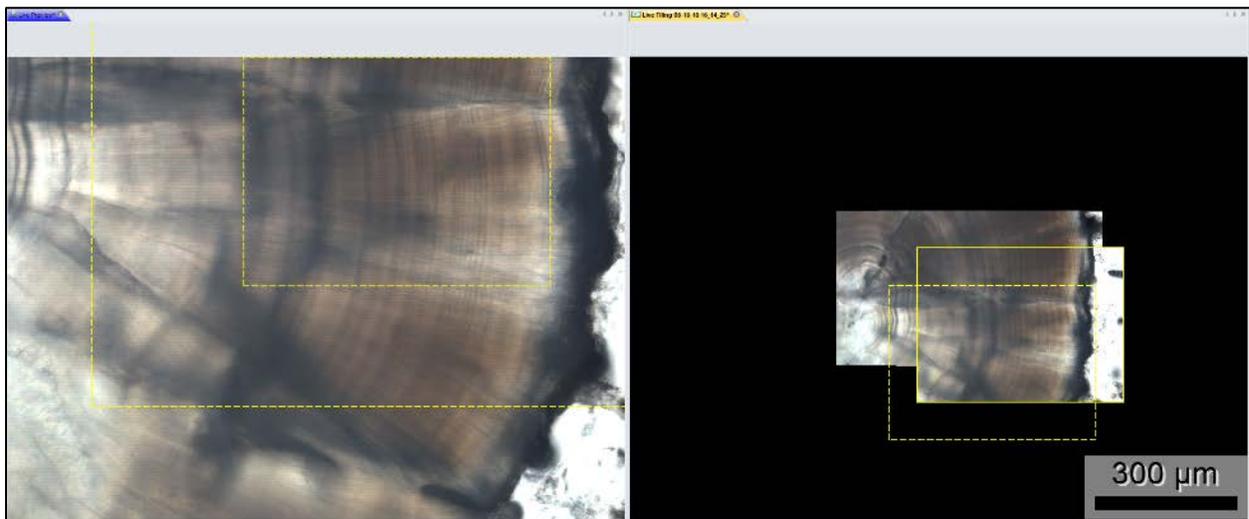
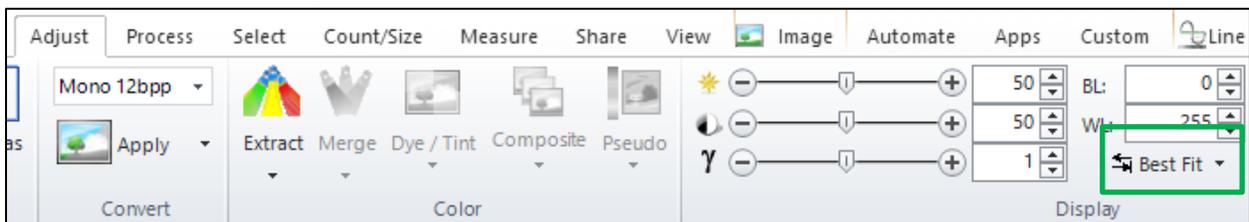


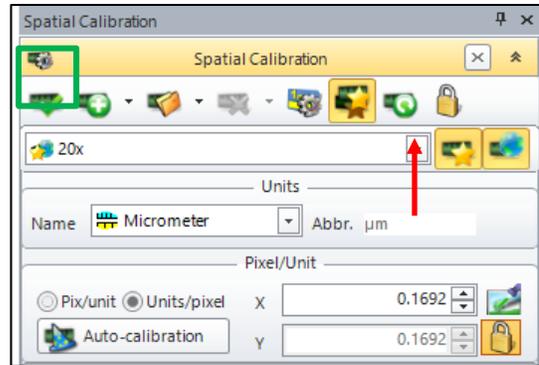
Figure 5: The screen's appearance during Live Tiling. The left window shows the live view, while the right is a composite image consisting of several images stitched together.

When you have captured a usable image, open the **Adjust** menu and press the **Best Fit** button (see below) to automatically adjust the Light, Contrast and Gamma levels. Those levels can also be manually adjusted using the slider bars to the left of the **Best Fit** button. Often a slight increase in the gamma level will optimize the image.

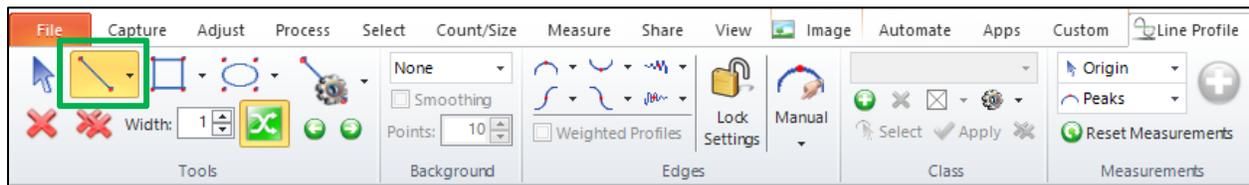


Drawing the Counting Transect

Before counting begins, make sure that image is calibrated for the correct magnification. To do this click the **Spatial Calibration** tab in the right most window and make sure that 20x is selected from the drop down **Calibration Name** menu. Then press the **Apply** button (green check – see right)



Select the **Line Profile** menu a red line will automatically appear on the image. You can also delete that line and redraw it using the **Line** tool.



Starting at the posterior most primordia that sticks out slightly on the dorsal side (identified in figure 1), draw a horizontal line that runs 90 degrees to the vertical line in figure 4 and encompasses your area of interest. If the transect bisects the increments at an angle the width measurements can be artificially increased. As such, adjust the vertical position of the transect (*remaining in line with the primordia and never moved so far that no part of it remains within the hatch check*) and/or its angle (*by no more than 10 degrees*) until it bisects each daily increment perpendicularly. Avoid positioning the transect through areas where the otolith displays shifts in growth planes or the outer increments fan out.



Figure 6: A transect drawn at two different angles, the first bisects the increments while the second passes through at an angle.

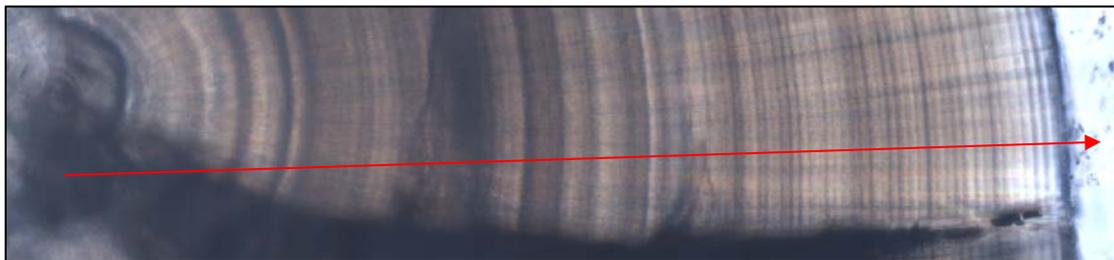
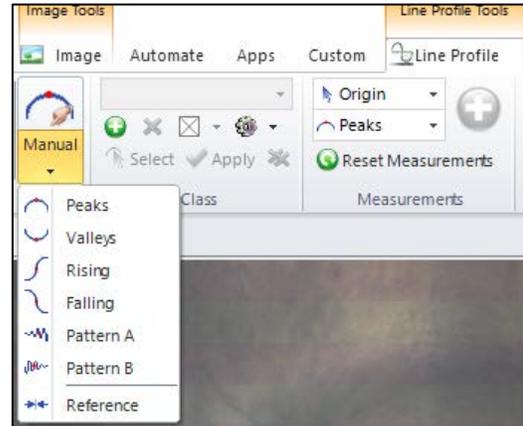


Figure 7: A full transect from primordia to edge that bisects the increments (after exogenous feeding check) perpendicularly.

Counting and Measuring the Daily Increments

Click the **Manual** button in the **Line Profile** menu and make sure “**Valleys**” is selected. Click on the transect where it bisects the hatch check, exogenous feeding check, and outermost point of interest. You should see blue tick marks at these points (see image below). Click on the **Manual** button again and select “**Peaks**”.

Starting with the exogenous feeding check, click on every dark band you see intersecting the transect line (shows up as red tick marks – see image below). To delete a tick, hold shift while clicking on the tick. Sometimes the increments are not completely clear. If you are having trouble look at other areas of the otolith where the increments are clearer and follow them round to the transect line. Sometimes when that isn’t an option, you may want to go back to the original sample and look at that region of the otolith under the microscope to give your best estimation. If large areas are unreadable, try recapturing the image with a better focus and starting again. Continue until you reach the edge of the area of interest. Do not count the outermost edge of the otolith as an increment. When picking increments, be sure to look out for the following common sources of error:



Sub-daily Increments: Not all increments represent an actual day in a fish’s life. In some cases an increment will be placed between two daily bands caused by periods of stress outside the normal day and night cycle. To differentiate a sub-daily from a daily, look at the surrounding increments. Sub-dailies will look both thinner and lighter than their neighbors. The distance between a normal increment and a sub-daily will also be roughly half that of the surrounding increments. The arrows in Figure 6a point out the sub-dailies.

Mirror Increments: Imaging a sample with an optimal focus is paramount to obtaining an accurate age reading. When the focus is off a single band can become split, creating an optical illusion where one increment appears as two. Mirror increments can be identified by two bands having a significantly darker space between when compared to the light zones of surrounding increments. If only a small minority of the total increments contain mirror bands, it is acceptable to count the center of the mirrors as a single band. If a significant portion of the total bands are mirrors, discard the current image and restart. The white arrow in Figure 6b demonstrates a classic mirror increment.

Shadow Increments: While the final image is displayed in two dimensions, the physical sample contains banding that extends below the surface in 3D. When imaging this can appear as a shadow that extends past where a distinct increment, usually towards the direction of the core, or like one long stretched out increment. In these cases, count the center of the one distinct increment and ignore the rest. In the case of long stretched out increments, pick the move edge ward portion to mark. All the increments in figure 6c show some degree of shadow banding.

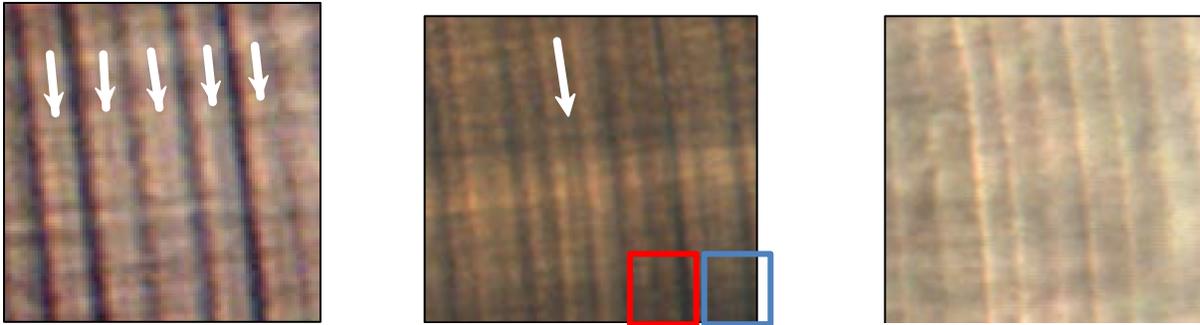


Figure 6: Examples of the three most common types of false increments, Sub-dailies (6a), Mirror (6b) and Shadow (6c).

Difficult to Read Areas: Ideally, every sample will be prepared in a way that makes every increment clear, distinct and easy to read in the optimal transect area. Unfortunately, variation in prep quality will result in a sample that can be pristine in one area and unreadable in the other. Problem areas can be circumvented somewhat via the line and spot tools. When coming across a challenging read area of the transect, follow the microstructure from the bad area to one that is easier to read. First, open up the **Select** tab. Now choose the **line tool** and draw lines that cordon off small sections of the unreadable



portion with their matching readable portion. Next select the **spot tool** and place a mark on each increment between the drawn lines. Count the spot marks to infer how many ticks should be in each section

of the unreadable area and add ticks to the transect accordingly.

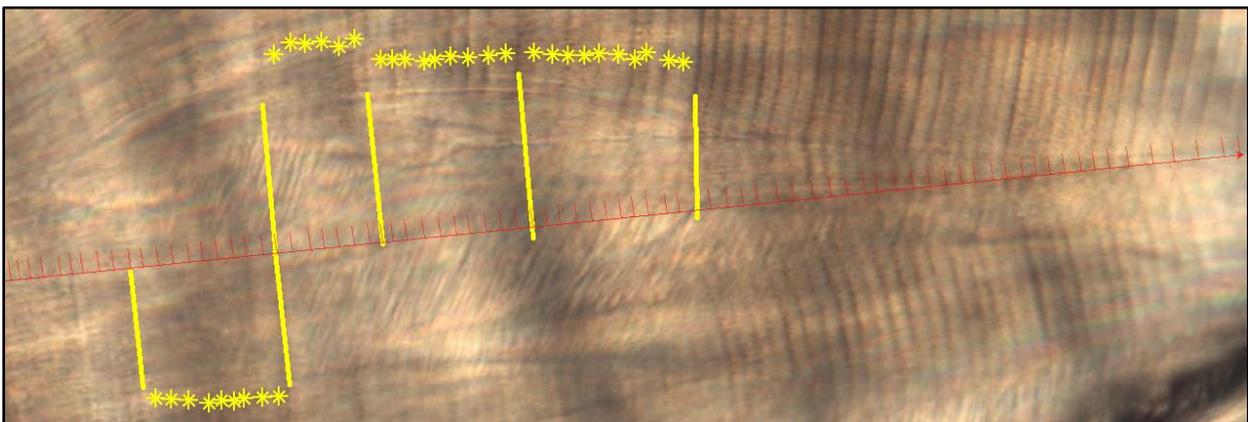


Figure 7: The line and point tools can assist in matching up hard to read areas with those that are easier above and below the transect.

Finally, review your work. Look for any unusually large gaps, places where the pattern breaks, or places where the ticks seem too close together. On average the daily increments tend to be 2-5 microns apart

in Chinook salmon from the California Central Valley. Once all the increments have been counted, the transect should look like Figure 8, below.

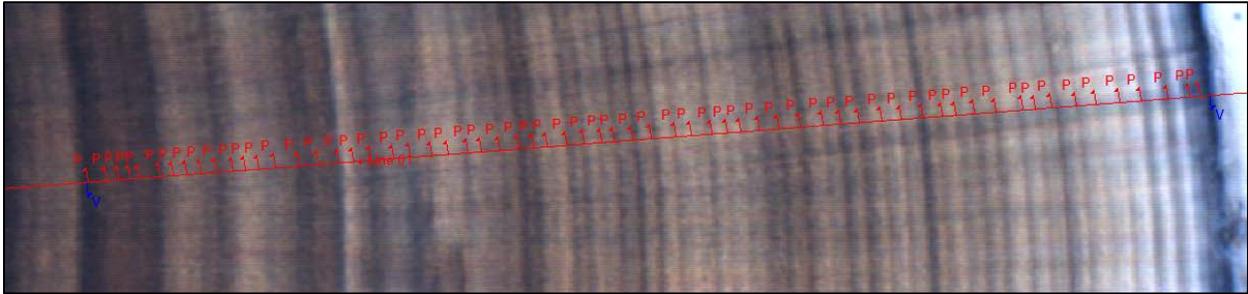
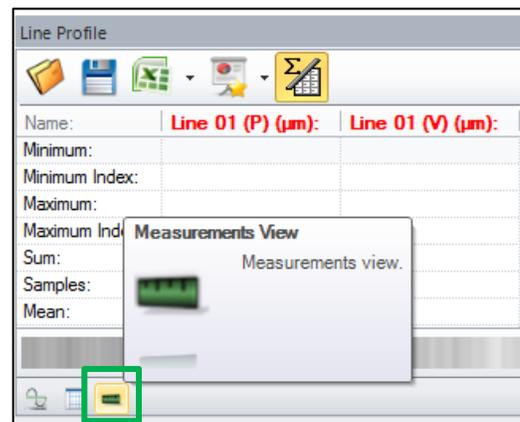
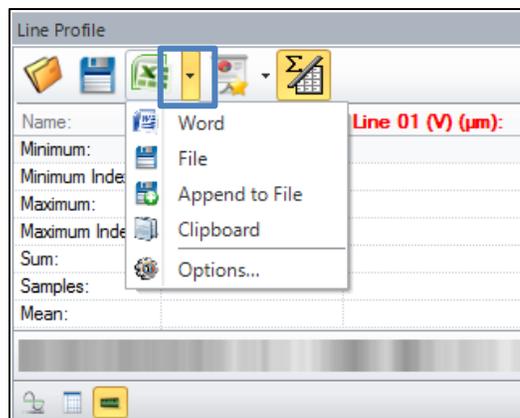


Figure 8: A finished transect. Each daily increment has a red tick mark, while the exogenous feeding check and outer edge are marked with blue.

When you are confident in your transect and selected increments press the **Measurement View** button which is a small green ruler icon on the bottom left corner. This will bring up a table showing the distances between each increment. Press the **small arrow** next to the button that looks like an excel icon to reveal the dropdown menu. Select **Clipboard** to copy the data. Open up an excel file, click on the cell where you want the data to be placed and press ctrl-v to paste.



Measurements for the distances of each individual increment as well as the major features will be recorded for the sample. You have now finished counting and measuring the increments for the otolith sample. Save the picture in **Tiff** format, using **LWZ** compression and name the sample with the following file structure: [Sample Name]_20x_Increments_[Your initials].



References

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