STEREO INVESTIGATOR:

Using the Optical Fractionator workflow

In this guide, you will learn about the optical fractionator probe including: how to use the steps in the workflow, how to read the *Sampling Results* window, and how to interpret the estimates.

OVERVIEW

The Optical Fractionator probe (OF) is used to perform a systematic random sampling of populations distributed within a series of serial sections to estimate the population number in a volume. Properly designed systematic random sampling yields unbiased estimates of population number.

Tissue shrinkage doesn't affect this method and tissue can be sectioned in any preferred orientation.

OF combines the optical disector and the fractionator methods. Objects are counted using optical disectors in a uniform systematic sample with a random start. Systematic random sampling constitutes a known fraction of the volume of the region being analyzed. In practice, this is accomplished by randomly starting and then systematically sampling a known fraction of the section thickness and a known fraction of sectional area of a known fraction of the sections that contain the region of interest.

The theory underlying this sampling methodology also makes it possible to estimate the precision of the population size estimate for a single subject; this estimate of precision is called the Coefficient of Error (CE). This CE should not be confused with the CE of estimates of the populations for the set of subjects that constitutes an entire experiment; the precision of population size estimates that are derived from a set of subjects is discussed later.

To start the workflow:

A. Click *Probes>Recent probes>Optical Fractionator* workflow OR *Probes>Number* drop-down>*Optical Fractionator* workflow

B. You are prompted to start a new subject or to continue with a subject (*Load Subject Data From Existing File).*

   If you Select *Load Subject Data From Existing File*, you are directed to the Count Objects step. Only the relevant steps are displayed and they are re-numbered accordingly (e.g., Step 10 in the complete workflow might be referred to as Step 4 in the streamlined workflow).

C. Follow the steps in the workflow.

STEP 1: SET UP THE SUBJECT

Subject Information

Type your name, the subject name, and any other information that you think will be helpful to someone who may work with this subject in the future.

Sampling Parameters

Choose whether or not to use a previously saved sampling configuration.

a. If **yes**, click on the browse (…) icon.

b. In the Sampling Parameter Chooser window, highlight the parameter set you wish to use.

c. Click OK.

Enter Serial Section Information

Enter the following parameters:

- *# Of Sections To Count*: Number of actual sections you will be sampling (you will be counting objects in the sections)for this subject across all of the slides.
- *Section's Cut Thickness*: Tissue cut thickness or block advance; this is the thickness immediately after sectioning before any artifact such as shrinkage has manifested.
- *Section Evaluation Interval:* Interval of the serially cut sections that you are going to sample.
  - To sample every other section, enter 2.
  - To sample every third section, enter 3, etc.
- *Starting Section Number*: Number of the first section you start from in your serial sections.
  - The first sampling section should be randomly selected. You can use the 'Randomize' button to help with this process. If you have already randomly picked the starting section, you can type in a section number that matches your records.
  - If you don't need to use the 'Starting section number' field to help keep track of the section randomization, you can leave this number as "1".
- *Z-Value of First Section*: This number is automatically adjusted based on the *Starting Section Number* entered before; it reflects an interval of the cut thickness.

Example

Section's cut thickness	Starting section number	Z value of first section
50µm	1	0
50µm	2	50
50µm	3	100

STEP 2: SET MICROSCOPE TO LOW MAGNIFICATION

a. Switch to a low power objective on your microscope, such as 2x or 4x.

b. Select the matching lens from the Select Low Mag Lens drop-down menu.

STEP 3: TRACE YOUR REGION(S) OF INTEREST

Serial Section

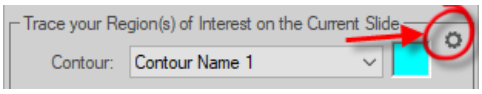
Click a section number in the Working On drop-down menu to select a section.

You can add a new section or edit the section as needed by right-clicking over the name of the section.

Trace your Region(s) of Interest on the Current Slide

Trace contours to delineate regions-of interest (ROIs); modify the tracing options as needed.

a. Select a contour type from the Contour drop-down menu. We recommend that you name the contour after your ROI. To change the name of a contour, click the cog icon:



b. Locate your ROI. Trace a contour to delineate the ROI (use the same contour type for the same region of interest in subsequent sections).

c. Right-click and select Closed Contour to finish the contour.

d. Repeat a-d with different contour names for each ROI.

NOTE 1: If you are using different contours for different anatomical regions within the same animal (e.g., ipsi- and contra-lateral striatum), you can combine these two regions in the analysis, as long as they have been counted with the same sampling parameters.

NOTE 2: You may trace contours one section at a time or trace all the contours for all the sections ahead of time; be sure to pick the appropriate contour in 'Serial Section – Working on' if you do the latter.

Tracing Options

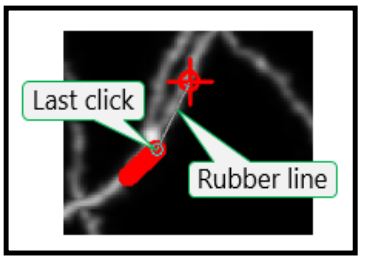
Optional: Use if you're not satisfied with the tracing defaults.

AutoMove moves the stage when you click outside the dotted *AutoMove* area box while keeping your tracing aligned with the tissue.

Tracing methods

There are three tracing methods to trace contours. To change the method while tracing, right-click and select a method from the right-click menu.

- SIMPLE CLICK TRACING (default method): Click to place a point along an object, move the cursor to a new position then click again to draw a line segment between the two points. Continue to place points until the entire object has been traced.
- RUBBER LINE TRACING: This method is similar to simple click tracing. As you move the cursor to a new position, a "rubber line" is drawn from the last point you placed to your current cursor position.
- CONTINUOUS tracing: Trace by dragging the mouse.



STEP 4: SET MICROSCOPE TO HIGH MAGNIFICATION

a. Select a higher objective with a numerical aperture greater than 1.
b. Select the corresponding lens from the Select High Mag Lens drop-down menu.

   We suggest 60x, 63x or 100x oil objectives for counting. These objectives provide a thin depth of field required to count accurately, especially when cells are on top of one another.

   Lower power objectives, such as 10x, 20x, or 40x air objectives do not have a thin enough depth of field to allow you to visualize the top of a cell.

STEP 5: MEASURE MOUNTED THICKNESS

Use the post processing thickness for this step. We recommend that you measure thickness at every sampling site for greater accuracy. In this case, the measured thickness from the counting sites is averaged and used in the calculations of the height sampling fraction.

Choose Your Method(s) for Measuring

The method you choose will affect your results.

Refocus To Top of Section at Each Grid Site (default)

This is turned on by default and should always be used since the top of your section will vary from counting site to counting site.

Measure the mounted thickness while counting (default)

This is turned on by default and recommended for most studies. To measure the mounted thickness while counting, you will need both 'Refocus to top of Section at Each Grid Site' (see above) and 'Measure the Mounted Thickness While Counting', both checked.

NOTE: The Population Estimate By Number-Weighted Section Thickness requires a thickness measurement at each site. Also note that the Resample Disector tool (designed to help you determine the guard zone extent), requires a thickness measurement at each site.

Evaluation Interval is set to "1" by default. If thickness is uniform across sections, increasing the *Evaluation Interval* will save some time, but we still recommend measuring the thickness at every site to improve the accuracy of the estimates.

Choose one of these two options: Manually Enter The Average Mounted Thickness and Measure Mounted Thickness Before Counting:

Manually enter the average mounted thickness

Use this option if you know the average mounted thickness. For example, you are counting from sections that tend to have a uniform thickness (e.g., celloidin), or you have carefully pre-measured the section thicknesses in several locations per ROI.

Measure mounted thickness before counting

Use for accurate measurements using the top and the bottom of each section.

Select this option if, when starting a new study, you have not pre-measured the thickness (see *Manually Enter The Average Mounted Thickness* above).

Take a few measurements of your tissue thickness at this step:

a. Click Start Taking Measurements.
b. Using the joystick, focus on the top of a section; Click *Set Top Of Section*.
c. Focus on the bottom of the section and click *Set Bottom Of Section*.
d. Click *Add Measurement To List*.
e. Continue with steps b through d (a few measures should give you a representative measurement, depending on the variance).
f. Use *Revisit Measurement* to verify and measure again, or *Delete Measurement* if you have made a mistake.
g. Click *Stop Taking Measurements* when finished.

NOTE: You'll need to have an approximation of your tissue thickness when you define the height of your disector and guard zones (described in Step 8).

STEP 6: DEFINE THE COUNTING FRAME SIZE

Counting Frame Display

Optional: Customize the display of the counting frame:

- *Force The Counting Frame To Be Square*: Change the shape of the frame to a square.
- *Snap To Increments Of*: Use if you want the size of the counting frame size to be rounded; a counting frame size set as a whole number is easier to remember and to apply to future experiments.
- *Center on live image*. We recommend checking this box as the counting frame will always be put in the center of the active window to minimize edge effects.

Counting Frame Size

Size the counting frame to fit approximately 5 objects of interest if possible.

a. Choose a unique identifying point that only comes into focus once for your cell such as:
    - Cell top
    - Nucleus top
    - Nucleolus (unless there are multiple nucleoli in your cells of interest)

b. Adjust the counting frame until it is approximately large enough to have, on average, 1 to 5 objects.

> NOTE: Some counting frames may have 0 objects, or more than 5 – this is completely acceptable. The reason for so few cells per counting frame is to minimize user error and fatigue.
> As you focus through the tissue to mark cells, it may be easy to lose track if you try to observe too many cells especially in dense populations.

To move the counting frame's location on the screen, turn off 'center on live image' and hover the cursor over the counting frame, drag the mouse to move the frame. However, it is best to leave 'center on live image' checked.

STEP 7: DEFINE SRS GRID LAYOUT

In this step, the SRS (systematic random sampling) grid size defines how close your counting frames are to each other.

- SMALLER GRID: You visit more sites within the traced contour, and count more cells.
- LARGER GRID: You visit fewer sites; you spend less time but you may be increasing your coefficient of error and making an estimate without enough precision.

Determine grid size based on number of sampling sites

a. Enter the desired number of sites in APPROXIMATE SITES.

Example: If you want to obtain a mean of **10** sites based on this representative section, set the grid size to include approximately **10** counting frames in the region of interest (ROI).

b. Click *Estimate Grid Size* to view an example of the grid.

NOTE: Grid size and counting frame size will stay consistent throughout all of the sections for a given region of interest. Number of sites will change every time because the grid is thrown down randomly every time you start a new probe run.

STEP 8: DEFINE DISECTOR OPTIONS

In this step, you'll set disector height and guard zones.

The **disector** is a 3D stereological probe that samples objects with a probability that is proportional to their number, not their size. [the counting frame is a cross section through a disector]

Guard zones are regions at the top and bottom of your tissue section in which you do not mark cells. They are needed to compensate for the fact that some cells can be damaged, cut in half, or plucked from the tissue when it is cut. It is possible to empirically determine the optimal guard zone height during the parameter determination study (see Defining Parameters For A Pilot Study in the user guide).

Optical Disector Settings

Select *Enter The Guard Zone Heights As Percentages* if you don't want to use absolute values.

The drop-down menu gives you 2 options:

- *Entering Top Guard Zone And Disector Height*: The bottom guard zone is calculated for you.

OR

- *Centering The Disector Between The Guard Zones*: top and bottom guard zones are calculated for you.

Focus Method

We recommend using 'Manual Focus' to ensure enough time to make good decisions about whether the unique point on the cell is in the disector.

STEP 9: SAVE SAMPLING PARAMETERS

To save the sampling parameters determined in the previous steps:

a. Enter a NAME for the parameters.

b. Click SAVE YOUR CURRENT SETTINGS.

You can use the saved sampling parameters again in Step 1 -*Set up the Subject* for subsequent animals.

> NOTE: DO NOT change parameters for every section within an animal! All parameters must be kept constant throughout all the sections of the animal for the calculations to be valid.

STEP 10: COUNT OBJECTS

Under *Regions of Interest*, you can see a list of the sections with the contours that were drawn for each section.

a. Click the name of a contour to select it.
b. Optional: Change the sampling parameters to parameters saved in a previous run.

c. Click START COUNTING**.** The panel displays new options.
d. Focus through the sites (if you selected *Manual Focus* in step 8 – *Define Disector Options*).
    i. The stage moves to the first counting site and, if you selected *Refocus To Top Of Section At Each Grid Site* in step 5 – *Measure Mounted Thickness*, the *Focus Top Of Section* window is displayed.
        a) Focus above the top of the tissue until it is completely out of focus.
        b) Slowly focus back down onto the tissue until something on the tissue (e.g., a cell or interstitial components) comes into focus; this is the top of the section.
        c) Click OK.
    ii. The *Focus Bottom Of Section* window is displayed.
        a) Focus all the way down through and past the bottom of the tissue until it is completely out of focus.
        b) Slowly focus back up until something just comes into focus. This is the bottom of the tissue (use the Z-meter to help you determine the direction that you are focusing).
        c) Click OK.

> NOTE: Determining what point is the top and what point is the bottom of the tissue varies from one researcher to another. This is not an issue as long as you are consistent in your criteria since this bias will be independent of the experimental manipulation.

e. Start counting in the first site:
    i. Select a marker from the *Use Marker* drop-down menu.

ii. Optional: Check the *Enable Middle Mouse Button For Placing Markers* box to mark two populations simultaneously with identical sampling parameters (use for population estimates of two similarly frequent populations).

iii. Focus through the tissue to find the *unique point* of the particle. If you focus above or below the tissue (i.e., outside of the green area displayed in the Z METER), the cursor changes to indicate that you're outside of the appropriate area.

ABOUT THE UNIQUE POINT

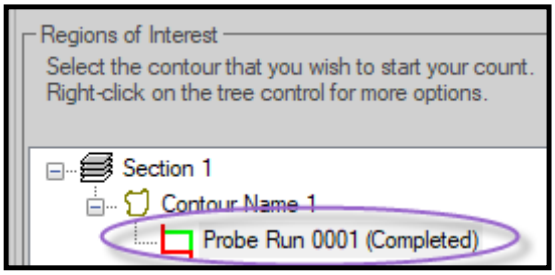
Many researchers use the cell's top, but the top of the nucleus or of the nucleolus (provided that the nucleolus is unique to the cell) is fine. The "unique point" must fall within your disector height (shown as green in the z meter). The rest of the cell may be anywhere relative to the counting frame, but only the unique point matters when deciding whether or not to mark a cell.

If the unique point comes into focus while in the guard zone (shown as red in the z meter), do not count it!

When the unique point comes into focus, place a marker on it in accordance with the rules of the counting frame.

iv. Click the "unique point" to place the marker (see Counting Rules article in the user guide).
v. Repeat steps c-d for the other particles that can be counted in the site.
vi. Once you've identified all the particles in the site, click the NEXT button to move to the next site.

f. Repeat step e for all the sites. Once you have visited all the sites, you are prompted to save your results and the probe run is displayed under *Regions of Interest*.



g. Repeat steps a. through e. for the other contours.
h. Select the next section: Put the slide with the next section on the microscope and 'joy track' to the ROI. Click *the Begin Next Section* button.
i. The workflow redirects you to a previous step.

- The program verifies that you have the correct slide on the microscope to sample, and alerts you that it is redirecting you to the beginning of the workflow.
- Some steps are disabled since the same parameters must be used for all sections in a given region – do not open these steps up to avoid changing parameters.

j. Follow the workflow down to the *Count Objects* step again.
k. Continue counting in each section and adding new sections as needed until you have completed all the sections in this particular specimen.
l. Click *I've Finished Counting*.

One file = one specimen.

DO NOT save each section as a new file.

Use the workflow to add new sections until you've traced and counted all the sections in an animal; this allows you to save these sections as one file, allowing you to view the total cell estimate for the entire structure, not just one section of the structure.

STEP 11: VIEW THE SAMPLING RESULTS

a. Click a set in the PROBE RUNS list to select it then click the *View Results* button OR click *Display Probe Run List* (use after running more than one probe if you want results from multiple probe runs).
b. The *Previous Stereological Runs* dialog box opens. Select all the Probe runs you want results for in the list.
c. Click *View Results*.

INTERPRETING THE ESTIMATES

OF provides four estimates shown in the *Sampling Results* window (described in detail in the next section). The results vary based on the measurement used for the mounted (or post-processing) section thickness.

The mounted section thickness value is divided into the counting frame thickness (or disector height) to calculate the height sampling fraction (*hsf*). We recommend that you report the estimate that best reflects the histological properties of the region of interest.

Estimated population using user-defined section thickness

Calculated using a single value entered manually for the post-processed or “mounted” section thickness.

Because this estimate is generated with only one value for the section thickness, local variations in section thickness are not accounted for. As a result, this estimate should be considered the least accurate of the four available estimates. But if there is no section thickness variation (e.g., embedding protocols such as plastic embedding), reporting the *Estimated Population Using User-Defined Section Thickness* is acceptable.

This value is typically entered manually, or calculated, in Step 5 under the *Manually Enter The Average Mounted Thickness* method.

- If you don't enter a value for *Manually Enter The Average Mounted Thickness*, the estimate equals zero.
- To change the thickness value after the counting procedure, click the *Edit Mounted Thickness* button in the *Sampling Results* window.

Estimated population using mean section thickness

Calculated using the section thickness measurements recorded while counting.

These measurements are recorded in Step 5, after selecting *Measure The Mounted Thickness While Counting* method.

The number of measurements used to calculate this estimate is based on the interval you entered (e.g., if you entered **2**, you are prompted to set the top and bottom of the section at every other counting site). The measured thickness values from these sites are averaged to produce a mean measured thickness value used for the height sampling fraction calculation.

Because this estimate is generated from the mean of all obtained section thickness measurements, it is considered to be the most accurate estimate of the region of interest when measurements are not performed at every site.

You may also choose to use this value for low frequency events (e.g., Brdu+ neurons) with many counting sites containing zero objects when you want to generate an accurate average measured section thickness measurement for the *hsf* using a systematic interval for site measurement.

If you didn't measure the thickness of sections while counting, this estimate is not calculated.

Estimated population using mean section thickness (only using sites with counts)

This estimate is a variation of *Estimated Population Using Mean Section Thickness*.

Calculated using only the section thickness measurements made at counting sites that contain marked objects (in other words, section thickness measurements from counting sites with NO counted objects aren't included in the calculated average).

These measurements are recorded in Step 5, after selecting *Measure The Mounted Thickness While Counting* method.

In many cases, this estimate will be nearly identical to *Estimated Population Using Mean Section Thickness*.

You may choose to use this value when :

- You chose to ignore measuring the section thickness where there were no objects.
- You made errors in section thickness measurement that were not corrected when there were no objects to be marked.

  If you didn't measure the thickness of sections while counting, this estimate is not calculated.

Estimated population using number weighted section thickness

Report this estimate when thickness was measured at every sampling site and when the section thickness varies dramatically across the sections that include the region of interest.

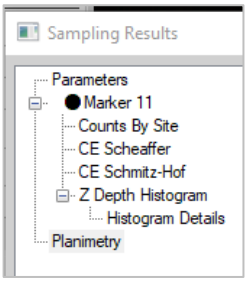
Calculated using only the section thickness measurements from counting sites that contain markers. These measured thickness values are then weighted by the number of objects associated with them to produce a weighted average.

The number weighted mean section thickness is reported in the PARAMETERS section and is used to calculate the height sampling fraction.

NOTE : All estimates calculated from measurements obtained while counting (i.e., all but Estimated population using user-defined section thickness) should return similar results if the measurements were taken correctly.

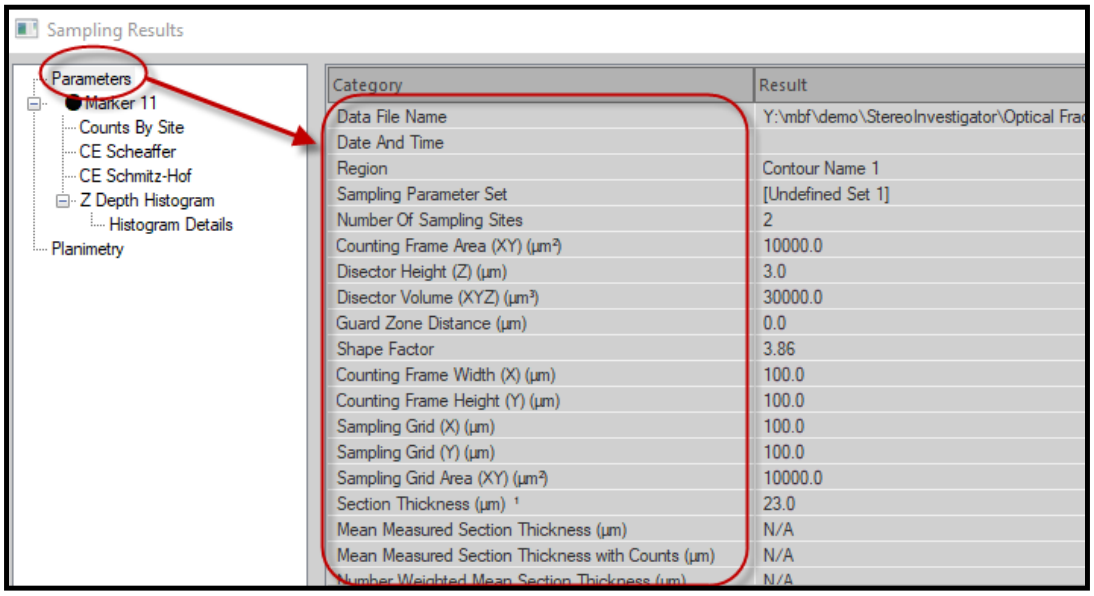
UNDERSTANDING THE RESULTS IN THE SAMPLING RESULTS WINDOW

Left panel



- *Marker X*: Typically, each marker represents one cell type.
- *Counts by Site*: Raw data for each counting frame visited in each of the runs.
- *CE Scheaffer/CE Schmitz-Hof*: See *Coefficients of Error* article in the user guide
- *Z Depth Histogram*: Z depth location for markers placed in each section.
- *Planimetry*: How area and volume are calculated based on the area contained within the traced contour. NOT an unbiased estimate.

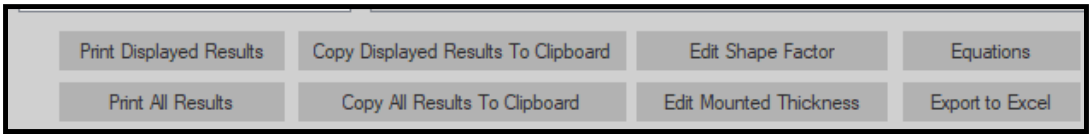
Right panel



- *Data File Name*: File name associated with this data set, if the data was already saved.
- *Date and Time*: When the probe was completed.

- *Region*: Name of the contour type that defines the region of interest. If this is a composite of several runs, displays the contour name used for the first run is shown.
- *Number of Sampling Sites*: Number of sampling sites visited on all selected sections.
- *Counting Frame Area*: Area of a single counting frame.
- *Counting Frame Thickness*: Thickness of the counting frames along the Z-axis.
- *Counting Frame Volume*: Volume of a single counting frame.
- *Counting Frame Width*: X-axis width of each counting frame.
- *Counting Frame Height*: Y-axis height of each counting frame.
- *Sampling Grid Width*: Distance between counting frames (sampling sites) along the X-axis.
- *Sampling Grid Height*: Distance between counting frames (sampling sites) along the Y-axis.
- *Sampling Grid Area*: Area of the region associated with each sampling step.
- *Section Thickness*: Value used for section thickness across all sections that were sampled. This should be the minimum actual section thickness as measured by Stereo Investigator.
- *Number Weighted Mean Section Thickness*: Mean of all sections measured by focusing at the top and bottom of the section. This value should be relatively close to the *Section Thickness* value.
- *Estimated Population Using Number Weighted Section Thickness/ Estimated Population Using Mean Section Thickness / Estimated Population Using Mean Section Thickness (Only Using Sites With Counts) / Estimated Population Using User-Defined Section Thickness*: described in the previous section on Interpreting Estimates.
- *Total Markers Counted*: Actual number of markers of this type counted during the probe run.

Sampling Results buttons/additional options



- *Print All Results*: Prints all the results for the selected probe run.
- *Copy All Results to the Clipboard*: Copies all the selected probe run results to the Windows Clipboard.
- *Edit Shape Factor*: Displays the *Shape Factor* dialog box (see "Shape Factor"). To obtain as accurate an estimate of the Coefficient of Error as possible, use the slider to edit the SHAPE FACTOR which describes the shape of the region of interest.
- *Edit Mounted Thickness*: Use to adjust the section thickness.
- *Equations*: Displays the equations used for the probe run.

ABOUT THE RESULTS EXPORTED TO EXCEL

- SUMMARY tab: Estimates and CEs for each individual marker.
- PARAMETERS tab: Information to be added to the Methods section of a publication to enable other researchers to test the reproducibility of the results.
- COUNTS BY SITE tab: Information about the measured thickness and number of markers at each site. This information can be used to calculate your own CE or to compare thicknesses/number of cells within a section or between sections
- COEFFICIENT OF ERROR tab: Several CEs.
- CE VARIANCE DETAILS tab: Information related to the calculation of the Gundersen and Shaeffer CEs.
- SECTION DETAILS tab: Marker counts by section.
- ALL MARKERS Z HISTOGRAM and INDIVIDUAL MARKERS Z HISTOGRAM tabs: Distribution of cells within the tissue.
    - Ideally, with no sectioning artifact from the microtome blade, there is an equal number of markers placed at each “bin” from the top of the site.
    - In practice, there may be fewer markers at the bottom/top of each site if sectioning has caused cells to be lost, or more markers at the bottom./top if there is compression caused by the knife; set your guard zones so that they cover these regions.
    - A lower number of cells marked in the middle of the histogram is probably due to an incomplete staining penetration.
- Z DEPTH tab: Raw data necessary for your Z depth histogram.
- Z DEPTH DETAILS tab: Actual Z values for each marker (rather than markers per bin).
- SMOOTHNESS tab: Distribution of markers within your ROI.
- PLANIMETRY tab: Area of each section and volume calculated from this area. Note that this information is biased; for an unbiased estimate of area/volume, use the Cavalieri Estimator.
- Z ORDER tab: Z value of the sections and the actual Z at which the contour were drawn in each section.
- RAW REPORT tab: Number of markers and tissue thickness per site.