STEREO INVESTIGATOR (CLEARED TISSUE)

Using the Image Volume Fractionator workflow

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

OVERVIEW

The image volume fractionator probe, derived from the Optical Fractionator (OF), is used to perform a systematic sampling of populations distributed within a series of serial sections to estimate the population number in a volume. Properly designed systematic sampling yields unbiased estimates of population number.
Tissue shrinkage doesn't affect this method there are no rigorous definitions of structural boundaries required.
OF combines the optical disector and the fractionator methods. Objects are counted using optical disectors in a uniform systematic sample that constitutes a known fraction of the volume of the region being analyzed. In practice, this is accomplished by systematically sampling a known fraction of the section thickness of 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>Number>Image Volume 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.

Virtual section settings

Total Height Of Image Volume and *Distance Between Image Planes* are automatically identified.

a. Set the top of the ROI: scroll the mouse wheel to focus through the section then click the SET TOP button.
b. Set the bottom of the ROI: scroll the mouse wheel to focus through the section then click the SET BOTTOM button.
c. Enter the *Number Of Virtual Sections* you will be counting for this subject.
d. The *Virtual Section Height i*s calculated based on top and bottom of ROI input.
e. Enter the *Disector Height*: it represents the height of the volume that you are going to count within the virtual section

STEP 2: TRACE YOUR REGION(S) OF INTEREST

Serial section

Select a section from the *Working On* drop-down menu.

- If you have one section per slide, follow the entire workflow for one section at a time up to the **Count Objects** step. When you're done counting for that section, click the *Add New Section* button in the **Count Objects** step and follow the prompts for the next section.
- If you have multiple sections on a single slide, tracing the regions of interest for all the sections in this step may be more efficient.

Trace your region(s) of interest on the current slide

a. Select a contour type from the *Contour* drop-down menu.
Name contours after your area of interest to quickly visualize which contour you should use.
b. Trace a contour to delineate a region of interest (use the same contour type for the same region of interest in subsequent sections).
You can trace more than one contour if you plan on counting different regions of a structure, different structures, or have more than one animal on a slide.

Tracing Options

a. Choose whether you would like to use the AutoMove area; the *AutoMove* function moves the stage when you click outside the dotted *AutoMove* area box while keeping your tracing aligned with the tissue.

b. Choose one of the three tracing methods: SIMPLE CLICK, RUBBER BAND LINE, or CONTINUOUS LINE.

STEP 3: SET ZOOM LEVEL FOR COUNTING

By default, the image is displayed at 100%. Use the buttons to zoom in or out.

STEP 4: DEFINE THE COUNTING FRAME SIZE

Counting Frame Display

Optional: Modify 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.

Counting Frame Size

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

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, hover the cursor over the counting frame, drag the mouse to move the frame.

Verify that:

- The counting frame is in the center of the screen.
- There is a large enough border around the outside of the counting frame so that you can clearly distinguish objects that are on the edge of the counting frame.
- The tails of the counting frame are visible.

STEP 5: DEFINE SRS GRID LAYOUT

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

Define SRS Layout

Select one of the three methods (manual, percentage, or approximate sites).

About Approximate Sites

Enter a value then click *Estimate Grid Size* for a preview.

About manual and percentage

Enter values then click *Display Changes* for a preview.

> 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 6: 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.

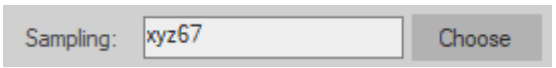
> NOTE: DO NOT change parameters for every section within an animal! All parameters must be kept constant throughout all the sections of the animal for the calculations to be valid.

STEP 7: 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.

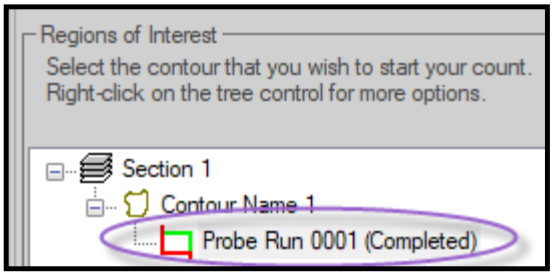
d. Start counting in the first site.
   i. Select a marker from the *Use Marker* drop-down menu in the workflow.
   ii. Optional: Check the *Enable Middle Mouse Button For Placing Markers* box to mark two populations simultaneously with identical sampling parameters (use for population estimates of two 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.
   v. Repeat steps iii-iv 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 in the workflow to move to the next counting site.

e. Once you have visited all the sites, the probe run is displayed under *Regions Of Interest*.
f. Select the next section: Click the BEGIN NEXT SECTION button.
g. Continue counting in each section and visiting new sections until you have completed all the sections in this particular specimen.
h. Click I'VE FINISHED COUNTING.



STEP 8: 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.

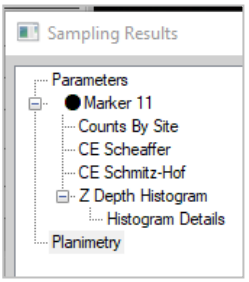
Estimated population using number weighted section thickness

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.

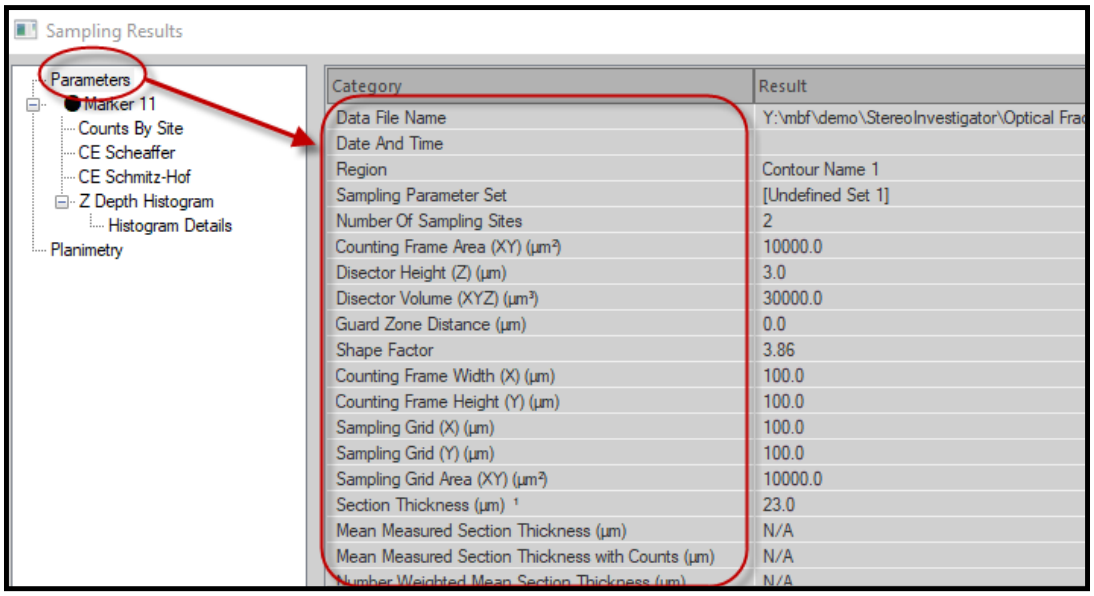
UNDERSTANDING THE RESULTS IN THE SAMPLING RESULTS WINDOW

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.

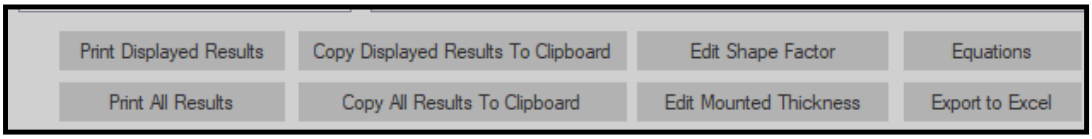
Right panel



- *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 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 are fewer markers at the bottom/top of each site; set your guard zones so that they cover these regions.
    - A spike of cells at the top of the histogram could be caused by focusing through the tissue or counting cell bottoms at the top of the tissue as though they were cell tops (assuming that cell tops are the unique point you're trying to count).

  - 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.