STEREO INVESTIGATOR:

Using the Spaceballs workflow

In this guide, you will learn about the spaceballs probe, how to use the steps in the workflow, and how to interpret the estimates.

OVERVIEW

With the Spaceballs probe, you can determine the length of many different types of objects (or fibers): tubules, nerve fibers, small blood vessels, surfaces, microvilli, etc.

Spaceballs provides a length estimate instead of length-density. Reporting length per region instead of length per volume is more effective because a length-density measure can't account for possible concomitant changes in volume along with length.

How it works

The probe is a sphere or hemisphere virtually embedded in the tissue, and the counted parameter is the number of profiles that transect the edge of the sphere within a defined counting region. Since the surface of a sphere is isotropic, the need for isotropic uniform random (IUR) sections is eliminated.

Spaceballs is implemented with a fractionator sampling methodology to return an estimate of total length per region. A series of sites are selected within each section by systematic random sampling. At each site, a 3D sphere of constant volume is superimposed upon the slide to represent the intersection sites to be counted. The item to be counted is a "profile," that is, the point where the fiber transects the sphere boundary. If fibers are thick, you must identify the center point of the fiber, and only count locations where the center point transects the sphere outline.

Prerequisites

- Thick sections (significantly thicker than the diameter of the tubules/fibers to be measured)
- Structure of interest stained through the depth of the tissue section.
- Unstained tissue transparent enough to see the stained structure throughout the depth of the section.
- Thin focal planes achieved with a high magnification, high numerical aperture lens, generally oil immersion.
- A contour traced around the region of interest.

To start the workflow

A. Go to Probes>Spaceballs workflow.

B. You are prompted to start a new subject (*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 6 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

To use a previously saved configuration:

a. Select YES.
b. Click the BROWSE button.
c. In the *Sampling Parameter Chooser* window, highlight the desired parameter set and click OK.

Enter Serial Section Information

a. Enter the number of sections to be counted out of the total number of sections available in # OF SECTIONS TO COUNT.
b. Enter the tissue cut thickness or block advance in SECTION'S CUT THICKNESS.
c. Enter the Interval of the serially cut sections that you are going to count in SECTION EVALUATION INTERVAL:
    - To count every other section, enter 2.
    - To count every third section, enter 3, etc.
d. Enter the number of the first section you will start from in your serial sections in STARTING SECTION NUMBER.
    - The first sampling section from should be randomly selected.
    - If you don't want to keep track of the section count from the microtome, leave this number as "1."
e. The Z-VALUE OF FIRST SECTION is adjusted automatically 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.

The objective should:

- Show a substantial portion of your region of interest (ROI) in the field of view.
- Be suitable for tracing a contour around your ROI.

> If it is difficult to distinguish the anatomical borders of your ROI at a low magnification, use a higher magnification for tracing.

STEP 3: TRACE 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.

> Example: You are counting the Substantia Nigra (SN) and you want to delineate left SN counts separate from the right SN counts. Use a different contour for each side (making sure you use the same contour choices for subsequent sections). The data can then be easily viewed per side or across the entire structure, provided that both sides were counted with the same sampling parameters.

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 4: SET MICROSCOPE TO HIGH MAGNIFICATION

a. Select a higher objective that will be suitable for imaging the objects of interest (e.g. tubules, nerve fibers, small blood vessels, microvilli, etc.).
b. Select the corresponding lens from the SELECT HIGH MAG LENS drop-down menu.

We suggest a high magnification lens with a high numerical aperture (generally oil immersion) to achieve thin focal planes necessary to properly discern intersection points.

Scroll the mouse wheel to adjust the zoom level. We suggest that you zoom in until you can clearly discern intersection points.

The item to be counted is called a “profile;” it corresponds to the point where the fiber transects the sphere boundary.

If the fibers are thick, you must be able to identify the center point of the fiber and only count locations where the center point transects the sphere outline.

STEP 5: MEASURE MOUNTED THICKNESS

The estimates for Spaceballs use the measured post-processing thickness of the tissue. The results will vary depending on the measuring method you use.

We recommend that you measure the tissue thickness at every sampling site for the most accurate estimates.

Preferred method

a. Select MEASURE THE MOUNTED THICKNESS WHILE COUNTING.
b. Set the EVALUATION INTERVAL to **1**.

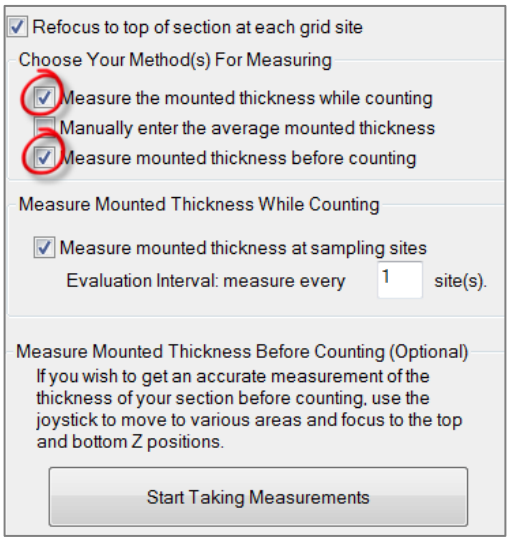
> For some studies, researchers may choose to measure the section thickness at every 3rd sampling site (i.e., EVALUATION INTERVAL=3) to save time. It is acceptable if the thickness of the sections is uniform.

Refocus to top of section at each grid site

This is recommended when the tissue is wavy.

Choose your method(s) for measuring

If you checked *Measure While Counting* **AND** *Measure Before Counting*:



a. Click the *Start Taking Measurements* button.
b. Using the joystick, focus on the top of a section and focus; 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. Repeat b-d (5 to 10 measurements should give you a representative measurement).

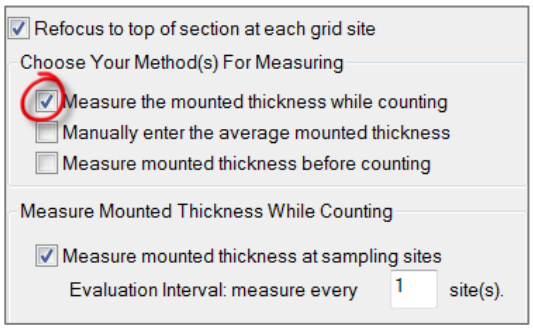
   Use *Revisit Measurement* to check and measure again, or *Delete Measurement* if you've made a mistake.

f. Click *Stop Taking Measurements* when finished.

The *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 increase the accuracy of the estimates.

This method is used to calculate *Estimated Population Using Mean Section Thickness*, *Estimated Population Using Mean Section Thickness(Only Using Sites With Counts), Estimated Population Using Number Weighted Section Thickness* (see **step 10: View the results**).

If you checked *Measure While Counting* **ONLY**:



The *Evaluation Interval* is set to **1** by default.

If thickness is uniform across sections, you can increase the *Evaluation Interval* to be more efficient, but we still recommend measuring the thickness at every site to increase the accuracy of the estimates.

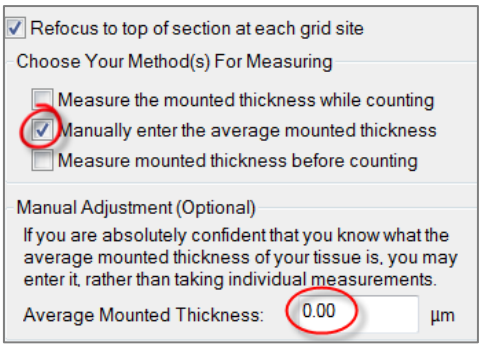
Used to calculate *Estimated Population Using Mean Section Thickness, Estimated Population Using Mean Section Thickness(Only Using Sites With Counts), Estimated Population Using Number Weighted Section Thickness* (see **step 10: View the results**).

If you checked *Manually Enter The Thickness*:

You already know the average mounted thickness and when the thickness is uniform.

This method saves time while counting, but can result in inaccurate estimates, especially if your tissue is somewhat wavy.

It is used to calculate *Estimated Population Using User-Defined Section Thickness* (see **step 10: View the results**).



STEP 6: SPACEBALLS OPTIONS

a. Select *Enter the Guard Zone Heights as Percentages* if you don't want to use absolute values.
b. Optional: *Use Hemisphere*: Uncheck this box to use a sphere instead.
c. Choose one of the 2 options from the drop-down menu.

   Entering Top Guard Zone And Disector Height: With this option, the value of the bottom guard zone is calculated based on these 2 manually entered values.

d. Enter the *Top Guard Zone Height* and *Radius Of Sphere* (i.e., disector height) values.

Focus Method

Manual Focus is most commonly used.

STEP 7: DEFINE SRS GRID LAYOUT

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.

> While the grid size is consistent throughout all of the sections for a given region of interest, the number of sites changes every time because the grid is applied randomly every time you start a new probe run.

While you don't need to define a counting frame for Spaceballs, be aware that the previously defined counting frame size does have an influence on the grid size if the counting frame is larger than the desired grid. To resize the counting frame, go to **Probes>Define Counting Frame**.

If you are performing a parameter determination study, set the grid size so that there are approximately "10" counting frames in the region of interest (ROI). Keep in mind that choosing "10" as the *Approximate Sites* number is just a starting point. You may need to increase or decrease the grid size in order to visit an appropriate number of sites to count more or less events.

STEP 8: SAVE SAMPLING PARAMETERS

Sampling Parameter Set

a. Save the sampling parameters determined in the previous steps by typing in a NAME for the parameters and clicking *Save your Current Settings*.
b. Select the saved sampling parameters in the **Set up the Subject** step for subsequent animals.

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 9: 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 and the first counting site is displayed.

   The stage moves to the first counting site and, if you selected *Refocus To Top Of Section At Each Grid Site* in **step 5**, the *Focus Top Of Section* window is displayed.

   i. Focus above the top of the tissue until it is completely out of focus.
   ii. 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.
   iii. Click OK.

   Then the *Focus Bottom Of Section* window is displayed.

i. Focus all the way down through and past the bottom of the tissue until it is completely out of focus.
ii. 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.
iii. Click OK.
iv. 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 the only one counting a particular experiment according to consistent criteria.

Focus down until a small circle is visible in the center of the field of view; if the grid spacing is small, multiple circles may be visible.

i. Select a marker from the *Use Marker* drop-down menu.
ii. Only mark the circle in the center, as the other sites will be systematically visited.

Select a marker type to count transections then place a marker at each location where a fiber crosses the currently visible circle (shown as green in the Z meter) if the transection is within the disector height/sphere radius (shown as green in the Z meter).

> If this point of a fiber comes into focus while in the guard zone (shown as red in the Z meter), you can't count it.

Focus through the section, marking each intersection between a fiber and the sphere, until all intersections have been counted; the circle will appear larger in each subsequent focal plane to represent the sphere or hemisphere.

> If you are using hemispheres and the current focal plane is near the equator of a hemisphere, the marker is counted as ½.

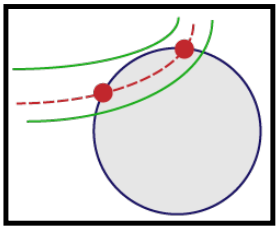
Right-click and select *Next Scan Site*.

d. Once all the sites have been visited, click *Add New Section* or *Begin Next Section* in the panel below the blue arrow button. The workflow redirects you to **the Set microscope to low magnification** step for your next section. Follow the workflow down to the **Counting Objects** step again.
e. Continue marking each section and adding new sections until you have completed all the sections in this particular animal.

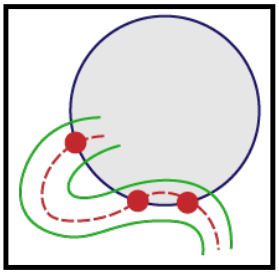
About edge cases

For vessels that appear on the edge of the spaceball, use the “center line” rule:

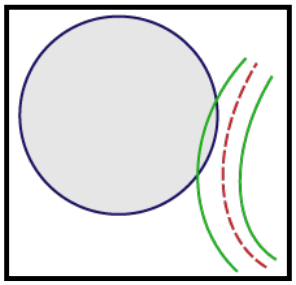
- If the center line enters and then leaves the probe, mark it twice (at the entrance point and at the exit point).



- If the center line weaves in and out, mark the centerline very time it enters or leaves the spaceball.



- If the center line doesn't enter the spaceball, don’t mark it.



One specimen = one file. DO NOT save each section as a new file. By using the workflow to add new sections, as described above, until all the sections in a specimen are traced and counted, you will save all these sections in one file.

STEP 10: VIEW SAMPLING RESULTS

There are 2 methods to view the results:

- Click a set in the PROBE RUNS list to select it and click the VIEW RESULTS button.

  OR

- Click the DISPLAY PROBE RUN LIST button (use after running more than one probe if you want results from multiple probe runs) to open the *Previous Stereological Runs* window. From the list, highlight all the Probe runs you want results for, then click the VIEW RESULTS button.

Results

The program provides four estimates. The results vary based on the measurement used for the mounted (or post-processing) section thickness.

You should report the estimate that best reflects the histological properties of the region of interest.

> 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.

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 4 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 of the workflow 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 of the workflow, 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).

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 with many counting sites containing zero objects.

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 of the workflow, 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.