Probes quick guide

Stereo Investigator

AREA FRACTION FRACTIONATOR

Area & volume

Thin/thick

Isotropic/vertical/preferential sections

The Area Fraction Fractionator is essentially a Cavalieri point counting estimate of area and volume performed on a systematically selected fraction of the tissue.

The probe is designed to estimate the fraction of a region occupied by a sub-region. Any number of sub-regions may be examined using this probe.

EXAMPLES

- Compare the ratio of white matter to gray matter in the spinal cord.
- Quantify the ratio of muscle fiber to connective tissue to vasculature in a muscle.

PROCEDURE

1. Place a reference point.
2. Define your sections with the **Serial Section Manager**. The **Section Cut Thickness** is used to calculate the estimates.
3. Trace the region(s) of interest at low magnification (i.e., with low precision).
4. Select a lens suitable to mark the tissue.
5. Go to **Probes>Define Counting Frame** to define the counting frame.
6. Use **Probes>Preview SRS Layout**.
7. Select **Probes>Area Fraction Fractionator** to open the **Area Fraction** window**.**
   The counting frame size and the XY placement of the grid you specified in Step **5** are already entered.
   a. Pick a grid spacing for the distance between points. The grid can also be rotated randomly.
   b. Select marker types:

      Left Button Marker (i.e., left mouse button): Used to mark single points or groups of adjacent points for the sub-region (e.g., plaque, lesion)

      Middle Button Marker (i.e., mouse wheel button): Used to fill the region of interest, that is, area of the contour overlaid by the counting frame (e.g., cortex).
8. Mark areas of interest, and adjust the size of your cursor with the mouse wheel if necessary:
   - To fill the entire area inside the contour with **Middle Button Markers** (cf. Step 7), click the mouse wheel button.
   - To mark a single point, left-click once.
   - To mark several adjacent points, drag the mouse (**Paint** mode).

To measure the area fraction of several types of objects, use additional marker types, one marker type for each region of interest.

9. When you are finished marking, right-click and select **Next Scan Site**.

   To return to the previous site, right-click and select **Previous Scan Site**.

10. Repeat the Steps **8** and **9** until all sites have been visited.

11. If you have multiple sections, move to the next section and repeat Steps **8–12**.

12. To view the results, use **Probes>Display Probe Run List**.

CAVALIERI ESTIMATOR

Area & volume

Preferential sections

Use this probe for a quick and efficient estimation of the area and/or volume of a region of interest.

The software overlays a regular grid of points on the slide/image; you mark the points in the grid that lie over the region of interest.

Volume of a structure
=
(Total number of points marked) * (Distance between points in XY) * (Distance between points in Z)

Area:

You can estimate the area of a 2D structure or the cross-sectional area of a 3D structure. You can determine the estimated area of the region of interest and an estimated CE from the number of points counted and the known spacing between points.

Volume:

To estimate the overall volume of regions contained in serial sections, you need to estimate the volume seen in the individual sections of the serial section sequence.

- The volume of an idividual section is the estimated as the estimated cross-sectional area of the section (as seen at the top or bottom of the section) multiplied by the thickness of the section.
- The estimated volume of the region is the sum of all the individual section volumes.

CAVALIERI ESTIMATOR 2

PROCEDURE

1. Place a reference point.

2. Select the lens that matches the objective from the drop-down menu in the toolbar.

3. Use **Tools>Serial Section Manager** to define sections.

4. Click **Probes>Cavalieri Estimator** to open the **Cavalieri Estimator** window.
   - **Section Cut Thickness**: Defined in the **Serial Section Manager**.
   - **Grid spacing**: Enter a value.

5. Click **OK**; Stereo Investigator displays a grid of points (represented by cross-hairs by default) over the tissue.
   To change the shape and color of the grid points, use the **Options>Stereology Preferences>Colors And Tick Marks** tab.

6. Click a marker type in the toolbar to select it.
   Snap To Grid is the default marking mode. To select another marking mode, place the cursor over the tracing window and right-click to see other options.

7. Mark all the points that overlay the area of interest on the section.
   Use the same unique point for each grid point when determining whether the grid point is over the area of interest; use different markers for different sub-regions.
8. Move to the next section.

> If the grid point is represented by a cross-hair, always approach from the same quadrant.

9. Repeat steps 7**–9** until all regions on all sections have been evaluated.

10. After all of the intersections have been marked, uncheck **Probes>Cavalieri Estimator** to end the probe run.

11. To view the results, use **Probes>Display Probe Run List**.

CAVALIERI ESTIMATOR 3 MARKING MODES

Snap to Grid Default mode for placing markers	To mark a point, click in the vicinity of a grid point(+): your marker is placed exactly on the center of the grid point . To disable **Snap to Grid**: 1. Select the **Options>Stereology Preferences**>**Cavalieri** tab. 2. Uncheck **Allow Snap to Grid for Cavalieri Probe** and click **OK**. To place markers in clusters (rather than one at a time), right-click and select **Paint Cavalieri Markers Mode** or **Marquee Mode**.
Paint Cavalieri Markers Mode	The cursor looks like this .  To mark a cluster of grid points, drag the mouse over the area of interest. To adjust the size of the circular cursor, use the "+" and "-" keys of the numerical keypad on your keyboard OR use the mouse wheel.
Erase Cavalieri Markers	• To delete a cluster of markers, drag the mouse over the area of interest. • To delete markers within the diameter of the cursor, click near the markers.
Marquee Mode	To place a cluster of markers within a rectangular area: 1. Drag from top left to bottom right. 2. Release the mouse button. To delete a cluster of markers within a rectangular area: 1. Drag from bottom right to top left. 2. Release the mouse button.
Paint Markers Into Contours	To fill a contour with markers: 1. A contour is already drawn. 2. Right-click and select **Paint Cavalieri Markers Mode**. 3. Right-click again and select **Paint Markers into Contours**. To remove all the markers from a contour, right-click again and select **Erase Markers into Contours**. *The contour must be accurately traced to the boundaries of the region of interest. If not, manually add or remove markers around the edges of the contour.*
Replace Mode	To replace a marker type: 1. Select a different marker type. 2. Right-click in the tracing window and select **Replace Mode**. 3. Click or paint the markers you want to replace.

COMBINED SLOPE INTERCEPT

	Length & volume	Use this probe to estimate the profile area and profile boundary length. You will mark the intersections with linear features (boundaries) the vertices located within the region of interest.
	Thin sections	

- To estimate boundary length with vertical sections, use L-cycloid or IUR Planes
- To estimate boundary length with thick preferential sections, use Spaceballs or Isotropic Virtual Planes.

PROCEDURE

1. Click **Probes>Combined Point Intercept** to open the C**ombined Point Intercept** window.

   Experiment to determine an appropriate separation so that a statistically significant number of intersections are achieved.

2. Mark each intersection of an object's boundary with the test grid.

   a. Right-click and select **Mark Line.**
   b. Click each line that intersects. Stereo Investigator places a tick mark for each intersection.

   To change the shape and size of the tick marks, use the **Options>Stereology Preferences>Colors and Tick Marks** tab.

3. Mark each grid intersection that falls within the profile of the region of interest.

   a. Right-click and select **Mark Vertices**.
   b. Click each intersection.

4. When done, right-click and click **Exit CPI**.

5. To view results, use **Probes>Display Probe Run List**.

CYCLOIDS FOR LV

Length & volume

Thick sections

Vertical sections

Use this probe to estimate the ratio of total line length to unit volume.

Stereo Investigator displays sets of short test curves (cycloids) in a grid pattern over the material to be examined.

You will place markers where the cycloids cross the projected lineal features of interest.

PROCEDURE

1. Click **Probes>Cycloids for Lv** to open the **Cycloids for Lv** window.
2. Adjust the settings and click **OK**. Stereo Investigator displays a test grid of cycloids and angles.
3. Adjust the vertical axis.
   a. Right-click and select **Define Vertical Axis**.
   b. Drag the head of the arrow to align the white vertical axis with the vertical axis of the tissue.
   c. Right-click and select **Accept**.
4. Click to mark wherever the cycloid curves cross the lineal features of interest.
   a. Mark intersections in all focal planes of the tissue.
   b. Mark the angles wherever they are located within the region of interest.
5. When done marking all intersections, right-click and click **Exit Cycloid**.
6. Results are displayed immediately in the **Sampling Results** window.

CYCLOIDS FOR SV

 Surface area & volume	Use this probe to estimate the surface area per unit volume, sometimes referred to as the surface density.
 Thick/thin sections	Stereo Investigator displays sets of short test curves (cycloids) and test points in a grid pattern over the material to be examined. You will place markers wherever the cycloids cross the boundaries of the surface of interest, or wherever a test-point appears in the region that represents the volume in the length per volume estimate.
 Vertical sections	

PROCEDURE

1. Click **Probes>Cycloids for Sv** to open **Cycloids to Calculate Sv** window.
2. Adjust the values. You may need to experiment to determine an appropriate grid in order to obtain a statistically significant number of intersections.
3. Right-click and select **Define Vertical Axis**.
    a. Drag the head of the arrow to align the vertical axis with the vertical axis of the tissue.
    b. Right-click and select **Accept** to confirm the alignment.
4. Click all the test points (angle marks) inside the region of interest's volume.
5. Click wherever the cycloid curves cross the boundary of the region of interest's surface.
6. To end the probe, right-click and select **Exit Cycloid**.
7. The results are immediately displayed in the **Sampling Results** window.

DISCRETE VERTICAL ROTATOR

Volume

Thin sections

Vertical sections

Use this probe to estimate the volume of small particles in single extremely thin sections (specifically EM samples) obtained through unbiased sampling schemes.

The estimator uses centrioles as the sampling unit and as the vertical axis for the cells being sampled because centrioles are easily recognized and of uniform size between cells. The distance of test points from the centrioles (i.e., vertical axis) is used to estimate the volume of other particles in the cell.

PROCEDURE

1. Click **Probes>Discrete Vertical Rotator**.The **Discrete Vertical Rotator** dialog box appears.
2. Adjust the settings and click **OK**.
3. Click the centriole of the cell. A grid of lines and points ("plus" signs) is displayed. The vertical lines are parallel to the vertical axis and represent the distance classes into which the selected points are sorted.
4. Adjust the vertical axis.
    a. Right-click and select **Define Vertical Axis.**
    b. Drag the head of the arrow until it is in alignment with the desired vertical axis.
    c. Right-click and select **Accept** to set the new vertical axis.
5. Place tick marks by clicking the points that are located within the profiles of particles of interest in the field of view.
    a. You don't need to mark the lines; they are for reference only.
    b. To change the shape and size of the tick marks, use the **Options>Stereology Preferences>Colors and Tick Marks** tab.
6. Once all points are marked within the particles of interest, right-click and click **Exit Discrete Vertical Rotator**.
7. To view results, use **Probes>Display Probe Run List**.

FRACTIONATOR

	Number	This probe is most often used to estimate population counts of 2D specimens such as plated cells for tissue culture analysis.
	Mono-layer	Typically employed to sample populations that are too large to count exhaustively.

PROCEDURE

1. Place a reference point; the point can be placed arbitrarily or at a particular location on the slide.
2. Select a low magnification lens that allows for the efficient and accurate definition of the region of interest (e.g., 4x).
3. Trace a region of interest using the contour tool.
4. Switch to an objective and lens that allow you to accurately identify the cells to be counted (e.g., 40x, or 20x for a sparse population).
5. Click **Probes>Define Counting Frame** to set the size of the **Counting Frame.**

   Set the size of the frame so that it contains a number of cells that you can comfortably mark without errors (i.e., no double count or missing cell).
6. Click **Probes>Preview SRS Layout** to define the **Systematic Random Sampling (SRS) Grid**.
7. Click **Probes>Fractionator** to start **Fractionator**.
   - Stereo Investigator prompts you to open the **Serial Section Manager**. Click **No**.
   - The **Fractionator** dialog box appears, displaying the previously determined counting frame size and grid size **.** Click **OK** move the stage to the first sampling site (the first site is determined randomly).
8. Select a marker from the Markers toolbar and mark all the cells that fall within the counting frame following the counting rules.

   > If the cells are double- or tripled-labeled, use combination markers to count the different cell types.

9. When finished marking, press **F2** to move to the next site.

   When finished with all the sites, the probe ends.

   Optional: if you have multiple regions, move to the next region and repeat steps **7-10** with appropriate sampling parameters for the region.
10. To view the results, click **Probes>Display Probe Run List**.

ISOTROPIC FAKIR

Surface area

Thick

This probe uses a linear probe consisting of three mutually orthogonal lines to estimate surface area in thick sections (slices). This probe can be used on sections cut in any orientation, as the probe lines themselves are isotropic in orientation. The number of intersections between the triplets of probe lines and the surface of the sampled object are counted, and surface area estimated from these counts.

This probe, as implemented in Stereo Investigator, is not a **fractionator** probe. This means that the surface estimate is only valid for the sections sampled—if you skip sections, they are not taken into account. You may, however, keep track of the section fraction yourself.

PROCEDURE

1. Optional: Use the **Serial Section Manager** if you have serial sections. The probe doesn't take the reciprocal of the section sub-fraction and nor does it multiply by the estimate automatically; you need to perform this operation yourself.
2. Click **Probes>Isotropic Fakir Method** to open the **Isotropic Fakir Method** window.
3. Enter the line separation (**Isotropic Lines Separation** refers to the three sets of mutually orthogonal test lines).

   Experiment to determine an appropriate line spacing so that a statistically significant number of intersections are achieved.
4. Click **OK**. The software displays an array of parallel line segments.

   Each line-segment consists of a solid line and a dotted line; the border where the solid line turns into a dotted line describes a border-point.

   As you focus up and down, the line segment moves and the border between dotted and solid shifts. As a result, the combination of border-points will describe a line through the thickness of the tissue.
5. Select a marker type.
6. Place a marker wherever a line intersects the boundary of the particles or sampled region, where that boundary appears in the most clear focus.
7. Focus up and down until all particle boundary intersections in the current field of view are marked.
8. Click the marker to deselect it.
9. Right-click, select **Line Set 2** and switch to the second line orientation.
10. Repeat steps 5–9 for all line sets.
11. Once all line sets have been sampled, move to the next field of view of interest and sample all of the line sets in the new field.
12. Repeat until all fields of interest have been sampled.
13. Right-click and select **Exit Isotropic Fakir** to exit the probe.

ISOTROPIC VIRTUAL PLANES

	Length	Use this probe to estimate the ratio of total line length within an **Optical Fractionator** sampling scheme when isotropic or vertical sections are not practical.
	Thick	Length can be determined for many different types of objects: tubules, nerve fibers, small blood vessels, microvilli, etc.

PROCEDURE

1. Select **Probes>Define Counting Frame**.
   - Define a size that includes several profiles of the fiber(s) on which the length estimation is to be performed.
   - If you are working with acquired images or image stacks, the counting frame needs to be smaller than the image in order to avoid the bias of an edge effect.
2. Select **Probes>Preview SRS Layout** to preview the arrangement of sampling sites within the tissue section.
3. Click in the tracing window to end the **Preview** mode.
4. Start the **Serial Section Manager** and define the first section.
5. At low magnification, draw a contour around the region of interest.
6. Switch to an appropriate high magnification, then click **Probes>Isotropic Virtual Planes** to open the **Isotropic Virtual Planes** window.
7. Select a marker to count transections.
8. Focus through the section until all intersections have been counted.
   More than one plane orientation can be used to count each sampling box.
   - To move to the next orientation, right-click and select **Next Layout** (which lists the layout number). The same sampling box is then resampled from a different orientation.
9. Once all orientations have been completed, right-click and select **Next Scan Site**.
10. Complete all sampling sites within the section.
11. Move the stage to a new section, use the **Serial Section Manager** to designate a new section, and repeat the procedure.

IUR PLANES OPTICAL FRACTIONATOR

Length

Thick

Isotropic sections

Use this probe to estimate the ratio of total line length within an **Optical Fractionator** sampling scheme.

Stereo Investigator displays sets of parallel lines over the material to be examined. These lines represent planes that extend along the Z axis.You will place markers where the lineal features of interest intersect the planes within each **Optical Fractionator** counting frame.

 If you don't have isotropic sections, use **Isotropic Virtual Planes** or **Spaceballs** instead.

PROCEDURE

1. Start the **Serial Section Manager** if multiple sections are to be sampled.
2. At low magnification, trace a contour of the area of interest.
3. At high magnification, define the counting frame.
4. Click **Probes>Preview SRS Layout** to set the desired spacing between counting frames.
5. Switch to a lens that is suitable for viewing the linear structures.
6. Click **Probes>IUR Optical Fractionator** to open the **IUR Planes Fractionator** window. Adjust the settings and click **OK**. We recommend that you select **Refocus to top of sectionat each grid site**. This is the most accurate way to calculate section thickness.
   - Under **Measure Thickness**, if you have very uneven tissue thickness within each section, check **Measure section thickness at each selected grid site**.

     If you use this option, thicknesses are recorded and used to report **Number Weighted Section Thickness** results, which take into account the varying thickness in calculating your final estimated total.
   - **Spacing of Planes**: Allow for 2 to 4 intersections between the planes and the lineal feature of interest per counting frame.
   - **Angle of Sampling Planes**: If no value is entered, the planes appear as horizontal lines. The planes always extend straight down through the section, so the lines do not appear to move as you focus through the section.
7. Stereo Investigator randomly selects the starting point of the scan and moves the stage to the first counting frame with lines.
8. The **Focus Top of Section** dialog box opens. Identify the top and click **OK**.
   - If you selected **Measure section thickness at each selected grid site**, the **Focus Bottom of Section** dialog box opens. Identify the bottom and click **OK**.

     If the Z meter is enabled, it displays the disector's height in green . If you focus above or below the tissue (i.e., outside of the green area displayed in the **Z meter**), the cursor changes.
9. Select a marker for counting intersections.

10. Click to mark wherever the planes (lines) cross the lineal features of interest within the counting frame. Mark intersections in all focal planes of the tissue that fall within the Z boundaries of the counting frame.

L-CYCLOID OPTICAL FRACTIONATOR

	Length	Use to estimate the ratio of total line length to unit volume within an **Optical Fractionator** sampling scheme.
	Thick	Stereo Investigator displays sets of curves (cycloids) in a grid pattern over the objects of interest.
	Vertical sections	You will place markers where the cycloids cross the projected lineal features of interest within the **Optical Fractionator** counting frames.

PROCEDURE

1. At low magnification, trace a contour of the area of interest.
2. Switch to a high magnification lens suitable for viewing the linear structures.
3. Click **Probes>L-Cycloid Optical Fractionator** to open the **Cycloid Lv Fractionator** window. Adjust the settings and click **OK**.

   We recommend that you select **Refocus to top of section at each grid site**. This is the most accurate way to calculate section thickness.

   - Under **Measure Thickness**, if you have very uneven tissue thickness within each section, check **Measure section thickness at each selected grid site**.

     If you use this option, thicknesses are recorded and used to report **Number Weighted Section Thickness** results, which take into account the varying thickness in calculating your final estimated total.
   - **Cycloid Width**: Enter a value that allows for 2-4 intersections between the cycloid and the lineal feature of interest for each counting frame.
4. Click **OK**. Stereo Investigator randomly selects the starting point of the scan and moves the stage to the first counting frame with cycloids.
5. The **Focus Top of Section** dialog box opens. Identify the top and click **OK**.
   - If you selected **Measure section thickness at each selected grid site**, the **Focus Bottom of Section** dialog box opens. Identify the bottom and click **OK**.
   - If the Z meter is enabled, it displays the disector's height in green . If you focus above or below the tissue (i.e., outside of the green area displayed in the **Z meter**), the cursor changes.
6. Right-click and select **Define Vertical Axis**.
   a. Drag the head of the arrow to align its vertical axis with the vertical axis of the tissue (if you can't determine the vertical axis at high magnification, switch to a low magnification lens).
   b. Right-click and select **Accept**.
7. Select a marker for counting intersections
8. Click to mark each point where the cycloid curves cross the lineal features of interest within the counting frame. Mark intersections in all focal planes of the tissue that fall within the Z boundaries of the counting frame.
9. When all intersections in a counting frame have been marked, right-click and select **Next Scan Site** to move to the next site.
10. To view results, use **Probes>Display Probe Run List**.

LINEAR DISECTOR

Number

Use this probe to analyze material that is perpendicular to specific landmarks (the T-square alignment tool is especially useful for this).

This probe combines the **Linear Scan** with a 2D counting frame at each sampling site. You may consider this probe as a one-dimensional fractionator probe.

PROCEDURE

1. At low magnification, trace a contour of the area of interest.
2. Switch to a high magnification lens suitable for counting cells.
3. Click **Probes>Define Counting Frame** and define the size of the counting frame in the XY plane.
4. Click a marker type to use for counting.
5. Click **Probes>Linear Disector**. The **Linear Scan** dialog box appears.
    - Adjust the settings as needed and click **OK**.
    - **Align sampling site with the line**: the counting frame is aligned with the line.
    - **Use T-square alignment aid**: use for objects perpendicular to specific landmarks.
6. Click to set the initial point of the line along which the sampling sites are to be placed.
7. Move your cursor to draw the line then click to set the end point of the line.
8. Stereo Investigator positions you at the first sampling site.
9. Mark cells within the counting frame by clicking them.
    - Use different marker types for different cell populations.
10. Click the **Next Scan Site** icon to move to the next site.
11. Repeat steps **9-10** until you have marked all of the sampling sites.
12. Click **Probes>Display Probe Run List** to see the results for **Linear Scan**.

LINEAR OPTICAL DISECTOR

Number

Use this probe to analyze material that is perpendicular to specific landmarks (the T-square alignment tool is especially useful for this).

This method combines the **Linear Scan** and the **disector probe** into a surveying tool.

It is designed to allow the sampling of sites that are defined by a line (vector) between two locations. An optical disector is applied to each sampling site.

PROCEDURE

1. At low magnification, trace a contour of the area of interest.
2. Switch to a high magnification lens suitable for counting cells.
3. Click **Probes>Define Counting Frame** and define the size of the counting frame in the XY plane.
4. Click a marker type to count cells.
5. Click **Probes>Linear Optical Disector**. The **Linear Scan** dialog box appears .
    - Adjust the settings as needed and click **OK**.
    - **Align sampling site with the line**: the counting frame is aligned with the line.
    - **Use T-square alignment aid**: use for objects perpendicular to specific landmarks.
6. Click to set the initial point of the line along which the sampling sites are to be placed.
7. Move your cursor to draw the line then click to set the end point of the line.
8. Stereo Investigator positions you at a sampling site and prompts you to focus to the top of the section.
    - Focus to the top of the section, then click **OK**.
    - Mark cells within the counting frame by clicking them.
    - Use different marker types for different cell populations.
10. Click the **Next Scan Site** icon to move to the next site.
11. Repeat steps **8-10** until you have marked all of the sampling sites.
12. Click **Probes>Display Probe Run List** to see the results for **Linear Scan**.

MERZ

Surface area/volume

Thin

Isotropic sections

Use this probe to estimate surface density (i.e., surface area per volume). This method uses the curvilinear test system devised by Merz and known as the *Merz Coherent Test System*.

Thin isotropic sections and systematic random sampling are required to perform this probe.

Stereo Investigator draws semi-circles in a square grid over the material to be examined. Tick marks are placed wherever the test lines cross the surface for which you are estimating the density. Tick marks are also placed on points located over the region of interest.

- The more marks for surface, the more surface per volume there is.
- The longer the lines used to probe (with the same amount of tick marks for surface), the less surface per volume there is.

PROCEDURE

1. Click **Probes>Merz** to open the **Merz Coherent Test System** window.
2. Specify the diameter of the semi-circle grid elements. This parameter controls both the distance between the grid of half circles which are drawn on the screen and their length.
   - The smaller the diameter is, the more intersections you get and the more precise the estimate is.
   - Experiment to determine an appropriate grid size so that a statistically significant number of intersections are achieved without "over-counting."
3. Mark each intersection of the surface in the tissue (the surface will appear as a linear structure since the surface is in cross-section) with a semi-circular probe line.
4. Mark the vertices of the "+" signs located over the region of interest.

   To mark multiple points simultaneously, hold down CTRL and draw a marquee around the points.

5. Move to as many fields of view as necessary, using **Meander Scan** or any other technique for moving from one field of view to another.

   To keep track of sections, use the serial section manager.

6. When finished, right-click and select **Exit Merz Grid**.
7. Use **Probes>Display Probe Run List** to view the results.

NUCLEATOR

Volume

Estimate cell volume with isotropic or vertical sections.

Thick

Use the Optical Fractionator for cell selection so that each cell has the same probability of being selected regardless of the shape, orientation, or size of the cell. *If the cells are not chosen in an unbiased manner, the mean of the estimated volumes cannot be unbiased.*

Isotropic or vertical sections

- Use one criterion for cell selection for the optical fractionator, for instance the nucleus top.
- Once that cell is selected, focus to an arbitrary point in the middle of the particle for the nucleator.

PROCEDURE

1. Use a systematic random sampling scheme (e.g., Optical Fractionator) to identify the cells to be sampled.

   If you are using the Optical Fractionator workflow, run the probe until Step 10 - Counting Objects and click the **Start Counting** button.
2. Once the first cell has been identified, but before clicking on it, click **Probes>Nucleator** to open the **Nucleator Parameters** window.
3. In the **Nucleator Parameters** window, select a section orientation (vertical or isotropic) and enter the number of rays desired. To be efficient, use either 2 or 4 rays: 2 rays are oriented in opposite directions; 4 rays form 2 lines that cross.
4. Click a marker type to mark particles.
5. Click a unique point of your choice within the particle or cell. The rays are shown extending out from the central point.
6. For vertical sections only:
   - Right-click and select **Define vertical axis**.
   - Drag the arrowhead to define the axis.
7. Click the location where each of these lines intersects the boundary of the particle.
   - If a line crosses the boundary multiple times, click each intersection. Stereo Investigatorplaces tick marks at each boundary.
   - To delete a tick mark, right-click over the tick mark and select **Delete?**.
8. When finished marking the boundaries of the current particle, right-click and select **Finish Current Nucleator**.
9. Click the next particle to be sampled in the counting frame and repeat steps **4–6**.
10. When done sampling particles in the counting frame, right-click and select **Next Scan Site** to move to the next sampling site.
11. Repeat steps **4–8**.
12. When done marking all the particles of interest, right-click and select **Finish Current Nucleator and Exit**.

13. To view the results, use **Probes>Display Probe Run List**.

OPTICAL FRACTIONATOR

Number

Thick

Preferential sections

The **Optical Fractionator** probe lets you sample a 3D region of interest and estimate the total number of particles. it is an important development in probes involving the estimation of population size.

We recommend that you use the workflow for step-by-step guidance.

INTERPRETING THE RESULTS

Stereo Investigator provides four methods for calculating the **Optical Fractionator Results** for each type of marker used. 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*).

Select the results method to report the measurement 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.

OPTICAL ROTATOR

Surface area, volume

Thick

Vertical or isotropic sections

Use this probe to estimate the volume and surface area of particles, such as cells, in thick, transparent slabs. Isotropic slabs are required to estimate the surface area.

Before using this probe, you need to use Optical Fractionator (or another systematic random sampling scheme) to obtain a systematic random sample of particles.

The term "slab" is used to refer to what is more commonly known as a "section" (or a 3D structure with thickness) according to the authors who first published this method. A "slice" refers to an optical slice, that is, a 2D plane through a tissue slab.

PROCEDURE

1. Start the Probes>Optical Fractionator workflow.
2. Perform steps **1**–**9** in the workflow.
3. At step **10**–Count Objects, click the **Start Counting** button and identify the first particle.
4. Click **Probes>Optical Rotator**. The **Optical Rotator** dialog box appears . Adjust the parameters as needed.
   - **Slab type**: For vertical sections, the vertical axis must be parallel to the screen's vertical axis.
   - **Initial grid orientation**: Refers to the orientation on the first focal plane. The program then alternates between vertical and horizontal for each focal plane.
   - **Focal plane separation**: Refers to the distance between focal planes. The number of focal planes is derived from this number, the optical slice thickness, and the random starting point.
   - **Grid line separation:** Refers to the distance between lines.
   - **Optical slice thickness**: It is centered on the unique point and should be the minimum cell diameter or the mean cell diameter. It can vary from cell to cell.
   - **Number of grid lines**: Refers to the number of grid lines per focal plane.
5. Select a marker to identify a particle.
6. Click the unique point within the particle. The focus position is moved to the first focal plane and a grid is drawn—Don't change the Z-position!
   - If the lines are too short relative to the cell diameter, drag the handles to extend the lines.
7. Click each intersection between the lines and the in-focus boundary of the particle.
   - If a line crosses the boundary multiple times, place a marker at each intersection.
   - All lines don't necessarily need to intersect the particle. A line might intersect a particle multiple times, depending on the shape of the particle and the location of the point associated with the particle. Each intersection should be marked.
   - Only mark intersections if the selected focal plane lies well within the volume of the particle to be marked (i.e., avoid sampling near the extreme top and bottom of cells). Stereo Investigator automatically selects valid focal planes based on **Focal Plane Separation** and **Optical Slice Thickness** entered in step **3**.
8. When done marking the intersections on the current focal plane, right-click and select **Move to Next Focal Plane**. The focus is changed to the next focal plane, and the grid is displayed at a perpendicular orientation to that in the previous focal plane.
   - Mark all of the intersections in this focal plane.
9. If the particle is no longer in focus after moving to a new focal plane, right-click and select **Finish Current Optical Rotator**.
10. Repeat steps **6–9** for the next particles.
11. When done marking all the particles of interest, right-click select **Finish Current Optical Rotator and Exit**.

12. Use **Probes>Display Probe Run List** to see the results.

PETRIMETRICS

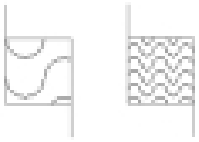
Length

Mono-layer

The **Petrimetrics** probe is used for 2D in vitro studies, such as estimating the length of fibers. It uses the Merz grid in its investigation. It uses the curvilinear test system devised by Merz known as the Merz Coherent Test System.

PROCEDURE

1. Load your image or work with a live image.
2. Trace your region of interest at low magnification.
3. Switch to high magnification (the power at which you will mark the intersections).
4. Use **Probes>Define Counting Frame**.
5. Set up the systematic random sampling with **Probes>Preview SRS Layout**; exit **Preview SRS Layout**.
6. Click **Probes>Petrimetrics**; Stereo Investigator displays a warning dialog box about the **Serial Section Manager**.
    - Click **No** since you are working on a 2D sample; the **Petrimetrics** dialog box opens .
    - **XY Placement of Counting Frames** is based on the SRS Layout.
    - Adjust the **Merz Radius**: A larger number results in larger curvilinear lines spaced farther apart; a smaller number results in smaller curvilinear lines spaced closer together, as seen here:



Example -- Left: Merz radius = 30µm | Right: Merz radius = 10µm.

    - **Optional** Type a value for **Grid Rotation** select **Randomize Rotation**.
7. Select a marker to use for counting.
8. Place a marker wherever the test line of the Merz lines cross a fiber or blood vessel center line, or any other object for which you are estimating length.
    - When you are finished with the first site, right-click and select **Next Scan Site**, and place markers at the next site on all intersections of the probe with a feature.
    - Continue until all sites have been visited.

9. Click **Probes>Display Probe Run List** for results.

PLANAR ROTATOR

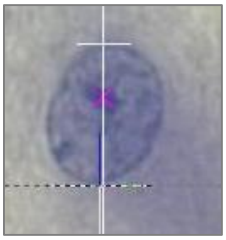
	Volume	Use this probe to estimate the volume of particles such as cells in isotropic and vertical sections.
	Thin	Use Optical Fractionator (or another systematic random sampling scheme) first to obtain a systematic random sample of particles.
	Vertical or isotropic sections	If you have a small population, all cells can be sampled with Planar Rotator.

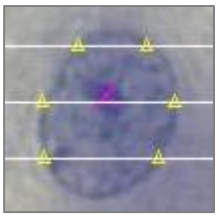
PROCEDURE

1. Start the **Probes>Optical Fractionator workflow**.
2. Perform steps **1**–**9** in the workflow.
3. At step **10**–Count Objects, click the **Start Counting** button and identify the first particle.
4. Click **Probes>Planar Rotator**. The **Rotator Parameters** dialog box appears . Adjust the parameters as needed.
5. Select a marker in the toolbar.
6. Click an object. A single line is drawn through the reference point of the object.
   Use the same relative reference point for each object (e.g., nucleolus).

> **Vertical sections**—the line should be parallel to the vertical axis of the tissue. To change the orientation of the line, position the cursor over one of the small squares on the vertical line. The cursor changes to a hand. Drag the vertical line and align it with the vertical axis for this section.
> **Isotropic sections**—align this vertical line along the major axis of the particle to be measured. This will usually help improve the variance of the estimate.

7. Click the line at each intersection with the boundary of the object (see the vertical line with the top intersection marked and the bottom intersection about to be marked and indicated by the dashed line on the right).
8. Place a tick mark by clicking the point(s) where each horizontal line intersects a boundary of the object (see the tick marks as yellow triangles on the right).
   - If a line crosses multiple boundaries, place a marker at each intersection.
   - To delete a tick mark, right-click over the tick mark and select **Delete?**.
9. When done marking the boundaries of the current object, right-click and select **Finish Current Planar Rotator**.
10. Click the next object to be measured and repeat steps **6–9**.
11. When you are done marking all the objects of interest, right-click and select **Finish Current Planar Rotator and Exit**.
12. Use **Probes>Display Probe Run List** to display the results.





POINT SAMPLED INTERCEPT

Volume

Thin

Vertical or isotropic sections

Use this probe to estimate the volume of cells or other small objects in isotropic or vertically sectioned tissue.

The **Point Sampled Intercept** is more likely to sample a larger cell than a smaller cell, so the results are volume-weighted, that is, the probability of hitting a particle with the probe is proportional to the volume of the particle.

PROCEDURE

1. Click **Probes>Point Sampled Intercept**. Stereo Investigator displays the **Point Sampled Intercept** dialog box .
2. Choose a PSI type and specify the spacing between the lines. Experiment to determine an appropriate line spacing so that approximately 50-100 intercepts are sampled per specimen.
   - If you have vertical sections, right-click and select the direction of the vertical axis.
   - Change the size and shape of the tick marks (the tick marks are essentially markers) with **Options>Stereology Preferences>Colors and Tick Marks**.
3. Click a point on a vertex that falls within the profile of a particle to be sampled. Once the vertex is selected, all other lines and vertices are temporarily hidden; only the line and vertex over the particle are visible.
4. Click to mark the intersections of the line and the particle boundaries.Tick marks are displayed at the selected intersections.

   To delete a tick mark, right-click over the tick mark and select **Delete?.**
5. Once all intercepts for that particle have been marked, right-click and select **Finish Current PSI**. All lines and vertices are visible again.

   The vertex you just marked appears with a tick mark over it to prevent you from selecting it again.
6. Repeat steps 3–5 for each particle. If more than one vertex falls within a given particle, both points should be marked.
7. To end the probe run, right-click and uncheck **Exit PSI**.
8. To view the results, use **Probes>Display Probe Run List**.

SPACEBALLS

Length

Thick

Preferential sections

You can determine length for 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.

Use the **Spaceballs Workflow** instead of the**Spaceballs** probe for greater efficiency.

PROCEDURE

1. Start the Serial Section Manager and define the first section.
2. Draw a contour around the region of interest.
3. Click **Probes>Preview SRS Layout** to set the size of the scan grid.
4. Click **Probes>Spaceballs**. The **Spaceballs** dialog box opens . Adjust the settings as needed.
5. Stereo Investigator drives the stage to the first sampling site. The cursor changes, indicating that the plane of focus is above the counting frame.
    - 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.
6. Select a marker for transections.
7. Place a marker at each location where a fiber crosses the currently visible circle.
    - If multiple circles are visible, only mark the circle in the center, as the other sites will be systematically visited.
8. 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.
    - **Count as 1/2:** This option is available in the right-click menu if Hemispheres are being used and the current focal plane is near the equator of the hemisphere. If a linear feature is at the widest point of the hemisphere, it should be counted as 1/2, since it would also be counted in the lower half of a full sphere.
9. Right-click and select **Next Scan Site.**
10. Complete all sampling sites within the section.
11. Move the stage to a new section, use the **Serial Section Manager** to designate a new section, and repeat steps **2**–**8**.

View Overview Layout
At any time during the probe run, right-click **Preview Spaceballs** to see an overview of the probe layout. **Stereo Investigator** displays the entire file with the sampling boxes overlaid on the tracing.

The current sampling box is indicated by flashing. Click anywhere in the **Preview** window to return to the current sampling site.

SURFACE-WEIGHTED STAR VOLUME

Volume

Use this probe to estimate tissue volume as observed from the boundary-surface of the volume.

Thin

This probe is used for complex, non-convex volumes such as volumes in lung tissue (alveoli as observed from the gas exchange surface) or bone tissue (marrow space as observed from the space's boundary surface)

Isotropic sections

Surface-weighted star volume is the average of the star volumes (un-obscured volumes) calculated from points on the phase interface (surface of the material of interest).

An array of cycloids that interacts isotropically with the tissue is used to sample the surface. At each sampled point, a ray is drawn in an isotropic direction from the surface intersection along the short axis of the cycloid. Mark where the ray intersects the next (and only the next) interface of the phase.

PROCEDURE

1. Click **Probes>Surface Weighted Star Volume** to open the **Surface Star Volume** window.
2. In the **Surface Star Volume** window:
   a. Enter the desired **Cycloid Width**.
   b. Use the **Randomize Angle** button to ensure an isotropic interaction. If the angle was already randomized with another method, enter the angle value in the **Angle of cycloids from vertical** field.
3. The test grid of cycloids appears. Click to mark a point where the surface of the phase of interest intersects with the cycloids.

   Stereo Investigator hides the cycloids and draws a line parallel to the short axis of the cycloid.
4. Click the line once to indicate the distance across the phase (and sample the un-obscured volume).

   Stereo Investigator displays the cycloids again, with a shorter line indicating the marked distance across the phase. If two intercept direction are possible, choose one at random.
5. Repeat steps **3** and **4** until all phases are marked.
6. Right-click and select **Exit Surface Weighted Star Volume** to end the probe. The results are immediately displayed in the **Sampling Results** window.

SURFACTOR

	Surface area of cells	Use this probe to estimate the absolute surface area of particles of arbitrary shape such as cells.
	Thick	Before using this probe, use Optical Fractionator (or another systematic random sampling scheme) to obtain a systematic random sample of particles.
	Isotropic sections	

PROCEDURE

1. Start the **Probes>Optical Fractionator workflow**.
2. Perform steps **1**–**9** in the workflow.
3. At step **10**–Count Objects, click the **Start Counting** button in the workflow and identify the first particle.
4. Click **Probes>Surfactor**. The Surfactor dialog box appears.
   - Enter the number of rays that will be associated with each measurement.
5. Select a marker.
6. Click a particle to be sampled. The selected number of rays is drawn, equi-angular from each other and outward from the arbitrary point.
   - Extend the length of the ray by dragging the white squares delimiting the ray.
7. Click where each ray intersects the boundary of the particle.
   - If a ray intersects the boundary multiple times, mark each intersection.
   - If you placed a marker incorrectly, remove the marker by positioning the cursor over it, right-clicking and selecting **Delete?**.
8. **Stereo Investigator** draws a tangent line perpendicular to the ray crossing the boundary. Change the orientation of the ray by dragging the mouse when the cursor changes to a hand.
   - If you have more than 2 rays, right-click and select **Mark Next Tangent** to mark the tangents on the remaining rays.
9. When done marking the boundary of the current particle, right-click and select **Finish Current Surfactor**.
10. Click the next particle and repeat steps **6–9**.
11. When you're done sampling particles, right-click and select **Finish Current Surfactor and Exit**.
12. To view results, use **Display Probe Run List**.

SV-CYCLOID FRACTIONATOR

Surface area/volume

Thick/thin

Vertical sections

This probe is identical to Cycloids for Sv except that Stereo Investigator guides you throughout the systematic random sampling process.

Instead of marking points and intersections on the whole region, you mark points and intersections in sub-fractions of the region.

PROCEDURE

1. Use **Define Counting Frame**.
2. Use **Preview SRS Layout**.
3. Click **Probes>Sv-Cycloids Fractionator.** The **Cycloids Sv Fractionator** window appears.
    - The **Counting Frame Size** was defined in step 1.
    - The **XY Placement of Counting Frames** reflects step 2 but can be changed manually.
    - Enter a value for the cycloid.
4. Click all the test points (angle marks) inside the counting frame and wherever the cycloid curves cross the boundary of the counting frame.
5. Right-click and select **Next Scan Site** to move to the next site.
6. To view the results, click **Probes>Display Probe Run list**.

VERTICAL SPATIAL GRID

Surface/volume

Thick

Vertical sections

Use this probe to estimate the volume and surface area of objects contained within a vertical uniform random stack of parallel sections, such as a confocal image stack.

Within each section, an array of parallel cycloid segments and grid points are used to estimate the surface area.

PROCEDURE

1. Click **Probes>Vertical Spatial Grid** to open the **Vertical Spatial Grid** dialog box.

2. Adjust the value and click **OK**.

4. Right-click and select **Define Vertical Axis.**

5. Click all of the points where the surface of the object of interest intersects the cycloid array.
   - To move to the next focal plane, right-click and select **Move Up One Plane** or **Move Down One Plane**.
   - To mark intersections within the region of interest (for estimating area), right-click and select **Mark Grid Points**. An array of points is displayed.

     Mark all grid points that fall within the profiles of objects or areas of interest.

6. Once all points are marked within the particles of interest, click **Probes>Vertical Spatial Grid** to exit the probe.

   The results are immediately displayed in the **Sampling Results** window.

WEIBEL

Surface area/volume

Thin

Isotropic sections

Use this probe to estimate the ratio of length to cross-sectional area and surface area to volume.

This method uses a linear test system devised by Weibel. The probe displays a set of short lines laid out in a grid over the material to be examined. Markers are placed wherever the test lines cross the boundaries of the surface.

PROCEDURE

1. Click **Probes>Weibel**. The **Lines Coherent Test System** dialog box appears .
2. Specify the length of the test lines. Based on the length value, Stereo Investigator determines the distance from the end of each line to the beginning of its nearest neighbors.

   Experiment to determine an appropriate grid size in order to obtain a statistically significant number of intersections.
3. Select a marker.
4. Mark each intersection of an object's boundary with the grid.
6. When finished, click **Probes>Weibel** to exit the probe**.**
7. To view results, click **Probes>Display Probe Run List**.