



Version 6.0

Application Guide: Stereology

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Stereology

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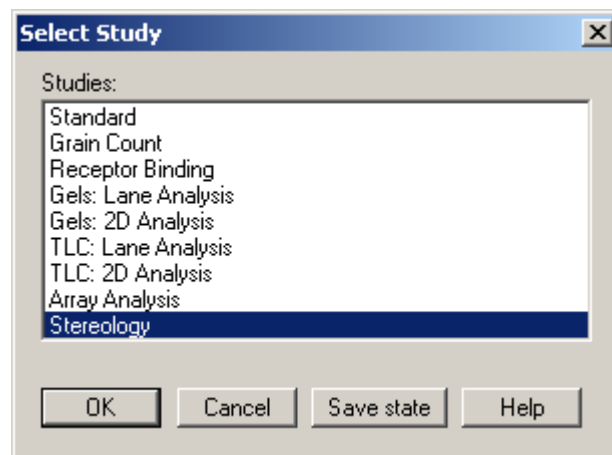
Introduction

Stereology is a set of techniques for extracting 3D information from 2D images. Traditional stereological methods are referred to as *model-based* because they make the assumption that targets are spheres or ellipsoids. Because the mathematics behind model-based methods is complex, it is tempting to disregard these implicit assumptions and simply accept the methods as useful tools. Recently, however, a new set of stereological procedures has been developed. This “new stereology” differs from the traditional forms because it makes no assumptions regarding the shape of the objects to be measured. It is called *unbiased* stereology.

The optional **Stereology** module for **AIS** uses the new, unbiased stereology. This document describes our stereological procedures for counting, sizing, and three dimensional (3D) surface measurements.

The **Stereology** module has a dedicated user interface and set of tools that run within the **AIS** environment. The user interface provides convenient selection of the factors that configure your system for stereology. To enable the **Stereology** module, open the *Study* menu and select the *Stereology* study type.

Figure 1: Enabling the Stereology study type.



Available Measures

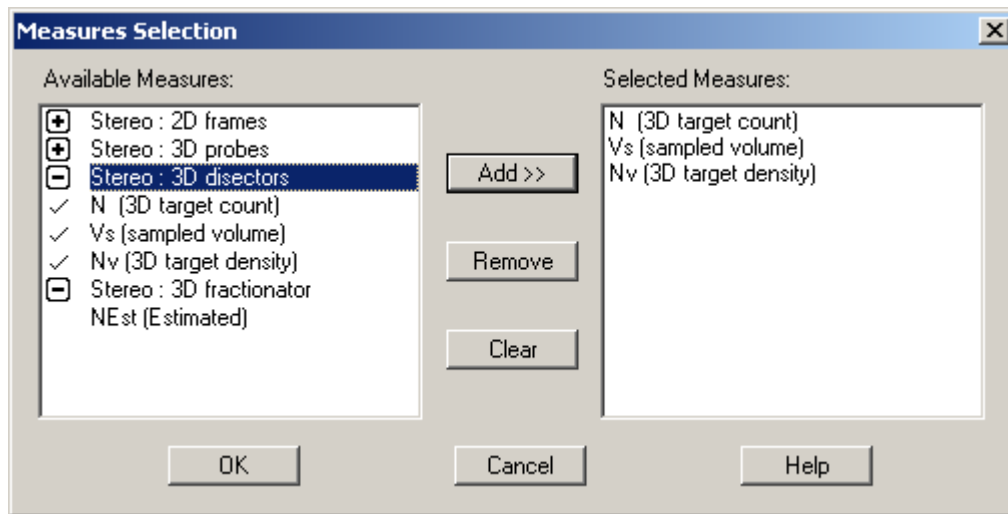
Most stereological measurements are ratios or densities. For example, we measure numerical density (N_a , number of targets per unit area; N_v , number of targets per unit volume) or surface density (S_v , surface area of targets per unit volume). You can also use these core measurements to calculate other parameters. For example, you can derive the total number of targets, or the total surface area, or the average surface area per target.

If stereological measures are to be reported in relevant spatial units (e.g., mm^3 , μm^2), the images must contain the appropriate spatial calibrations (e.g., Distance, Area, Section interval). See the online *AIS Reference Manual, Chapter 7: Calibrating Images* for calibration instructions.

To select the kinds of measurements you wish to make, open the *Sample* menu and select the *Measures selection* option (Figure 2).

AIS's stereological measures are grouped into four categories: **2D Frames**, **3D Probes**, **3D Disectors**, and **3D Fractionator**. These measurements can be selected singly or in combination, allowing multiple stereological measurements to be carried out simultaneously. **3D Disectors** and/or **3D Fractionator** measures, however, cannot be combined with any other measures.

Figure 2: The *Measures Selection* dialog box is used to select the kinds of stereological measures you wish to make.



2D Frames Measures

2D Frames measures are calculated from the 2D dimensions of a “sampling frame” and the target profiles contained within it. A sampling frame (Gundersen, 1977) is a rectangular tool, with two inclusive and two exclusive boundaries. A target is accepted only when it is completely inside the frame, or is touched by the inclusive boundary but not the exclusive boundary. In theory, the sampling frame gives any target an equal chance of being sampled, regardless of its 2D shape or size.

N_s: Number of 2D Targets

Number of target profiles contained within the sampling frame.

A_s: Area Sampled

Sampled area (area of the sampling frame). If a Distance or Area calibration is not in effect, *A_s* is the number of pixels contained in the counting frame.

N_a: 2D Numerical Density

Number of target profiles per unit area. *N_a* is calculated from the area of the sampling frame and the number of targets sampled in the frame. If a Distance or Area calibration is

not in effect, N_a is expressed as the number of target profiles per pixel² (abbreviated in the data table as “pixel⁻²”).

V_v: Volume Ratio

Volume ratio (Equation 1) is also often called *volume fraction*. It is the ratio of target profile volume to sample volume.

Equation 1 $V_v = est. [a / A_s]$

- V_v volume ratio
- A sum of the area of all the target profiles in the sample
- A_s area of the sampling frame

3D Probes Measures

3D Probes measures are calculated from a set of linear test probes oriented around points in a sampling grid. If data are to be expressed in spatial units, images must be calibrated to a reference scale.

A_a: Area Ratio

This is a simplified volume ratio measure, which divides the number of grid points overlaying a target by the total number of grid points measured.

S_v: Mean Surface Density

Surface area per unit volume (Equation 2). If a Distance calibration is not in effect, S_v is expressed as the number of targets per pixel (abbreviated in the data table as “pixel⁻¹”).

Equation 2 $S_v = est. [2 (P / L)]$

- S_v surface area per unit volume of the reference space
- est. is estimated by
- P total number of intersections between probes and profile boundaries
- L total length of probes within the reference space

“Reference space” here is the total containing space. P can be automatically computed from the test probes.

V_m: Mean Volume

Mean volume of sampled target profiles (Equation 3). If a Distance calibration is not in effect, V_m is expressed in “voxels”.

Equation 3 $V_m = mean [(pi/3) x (l^3)]$

- V_m mean volume
- l intercept length - an intercept in a random direction in 3D through a random test point hitting a cell

3D Disectors

The “dissector” was proposed as a direct estimator of the number of 3D objects, unbiased by their shape and size. It consists of two parallel sections, the *reference* section and the *lookup* section, separated by a known distance. On the reference section, target profiles are detected within a sampling frame (as described in 2D Frames Measures, above). To be accepted, targets must be absent from the lookup (i.e., previous) section. This condition results in the counting of cell tops. Use of the lookup section is critical, because the cells seen in the sampling frame are a biased sample in the height dimension. Bias results because the section hits cells with a non-uniform probability proportional to their height. Higher cells are more likely to be sampled than thinner ones. However, the subset of cells not hit by the look-up section is an unbiased, uniform sample (Sterio, 1984).

We use the term unbiased to mean that the probability of having a cell included in the subset is independent of the cell’s shape or size. We use the term uniform to mean that the lack of bias is present in all three spatial dimensions.

3D Disectors measures are derived from aligned serial sections separated by a known distance. When the disector is applied to each section, it identifies new “target tops” seen within the sampling frame and computes both the number of 3D targets and the volume.

Note: Since the first section in the series has no “lookup” section, no data is reported for the first image.

If data are to be expressed in spatial units, images must be calibrated to a Distance reference scale. A *Section interval* must also be specified in the *Distance* calibration.

N: Number of 3D Targets

Number of 3D targets contained in the sample, which are not present in the preceding (i.e., lookup) section.

Nv: Numerical Density of 3D Targets

Number of targets per unit volume (Equation 4). If a Distance calibration is not in effect, and if a Section interval is not specified, *Nv* is expressed as the number of targets per pixel³ (abbreviated as “pixel⁻³”).

Equation 4 $Nv = N / Vs$

<i>Nv</i>	numerical density of 3D targets
<i>N</i>	number of 3D targets counted
<i>Vs</i>	volume sampled (see Equation 5)

Vs: Volume Sampled

Volume sampled (Equation 5). If a Distance calibration is not in effect, and if a Section interval is not specified, *Vs* is expressed in “voxels”.

Equation 5 $V_s = A_s \times h$

V_s	volume sampled
A_s	area of sampling frame
h	section interval (distance between images)

You may be wondering about the difference between the volume sampled (V_s) and the volume of the reference space $V(\text{ref})$. Conceptually, these terms are different, though we simplified our discussion by equating the reference space and the space actually sampled by the disector(s). In this case, $V(\text{ref})$ is numerically equal to V_s , and N is equal to the sum of the “target tops” counted. However, we can set a general rule that we should use Equation 5 when we deal with small, clearly demarcated volumes, that are well sampled by our disectors. This gives us the density of targets in our sampled volume. If our interest is to estimate the total number of targets in a much larger $V(\text{ref})$ than we can sample, Equation 6 should be used. This gives us an extrapolated target number.

The estimate of the total number of 3D targets may be simplified as the following:

Equation 6 $N = \text{est. } [N_v \times V(\text{ref})]$

N	total number of 3D targets
est.	is estimated by
N_v	numerical density of 3D targets
$V(\text{ref})$	volume of the reference space

The volume of the reference space $V(\text{ref})$ generally means a region of interest, such as the cerebral cortex.

3D Fractionator

3D Fractionator measures are a very direct and robust way of estimating a “total” number and the principles involved are very straightforward. Take a known fraction of the whole area/structure of interest, count every particle in that fraction and multiply by the fraction to arrive at an unbiased estimate of the “total”.

The AIS automated optical fractionator integrates with a motorized XYZ stage and the Tiled Field Mapping (TFM) module to perform systematic random sampling over an entire region of interest. The user first specifies the number of sections to be counted, the section thickness, and the sampling frequency. A montage of each section is then created (with TFM) at low-magnification, and an area of interest is outlined on the montage. A higher magnification is then selected, and the stage automatically moves to random locations within the region of interest. At each location the user focuses up or down and uses the mouse to mark targets (i.e., cells) within the counting frame. The computer keeps track of the counts and calculates an estimate of the total number of targets based on the various fractions sampled.

NDet: Determined

This measure does not appear in the *Measures Selection* dialog box for the **3D fractionator**. It is displayed as one of the columns next to the **NEst – count** and contains the actual number of targets counted in all the sampled fractions.

NEst: Estimated

This measure is an unbiased estimate of the total number of targets present within the area/structure of interest. It is based on the total NDet value multiplied by the various fractions sampled.

Summary

Stereological measures are summarized in Table I, below. The table also indicates how each measure is estimated, how the specimen must be sectioned (see below), and which spatial calibration is necessary to express the data in appropriate units.

Table I: *Stereological measurements supported in AIS.*

Measure	Definition	Method Name	Section Type	Calibration
Ns	Number of 2D targets sampled	Unbiased sampling and counting frame	Vertical or IUR	Not required
Na	Numerical density (2D)	Unbiased sampling and counting frame	Vertical or IUR	Distance or Area
As	Sampled 2D area	Unbiased sampling and counting frame	Vertical or IUR	Distance or Area
Vv	Volume Ratio	Cavalieri's principle	Vertical or IUR	Not required
Aa	Area Ratio	Point-sampled-intercept	Vertical or IUR	Not required
Sv	3D surface density	Test probes	Vertical or IUR	Distance
Vm	Mean target volume	Point-sampled-intercept	Vertical or IUR	Distance
N	Number of 3D targets	Disector	Serial	Not required
Nv	Numerical density (3D)	Disector	Serial	Distance, Section interval
Vs	Volume sampled	Disector	Serial	Distance, Section interval
NDet	Number of targets counted	Unbiased sampling and counting frame	Serial	Distance
NEst	Estimated total number of targets	Unbiased sampling and counting frame	Serial	Distance

Section Types

To ensure that stereological estimates are unbiased, tissue sections must be obtained in an appropriate fashion. This ensures that every part of the specimen has the same chance of being included in the sample, and that the sample is representative of the entire specimen.

Estimates based on disectors and/or fractionators (e.g., 3D target count, 3D target density) require *Serial* sections. All other estimates require *Vertical* or *Isotropic Uniform Random* (IUR) sections. Each of these section types has its own set of algorithms for generating test probes, ensuring that the probes are isotropic in 3D space.

Serial Sections

Stereological estimates that are calculated from disectors require serially adjacent sections, separated by a known distance and perfectly registered (aligned) with each other. The sections may be physical (i.e., physically cut) or optical (i.e., different focal planes through a thick specimen). The interval between the sections must be small enough so that no object of interest will escape detection between sections. The most effective interval between sections is about 30% of the smallest feature of interest.

Vertical Sections

A vertical section is a plane perpendicular to a given “horizontal” plane. No special meaning is attached to the “horizontal”, other than as a reference plane which defines the orientation of the sections. There are three ways to define the vertical direction (Baddeley et al. 1986):

1. Find a naturally defined, intrinsic vertical direction in the specimen. In muscle tissue, for example, cylindrical fibers run roughly parallel to the macroscopic longitudinal orientation of the tissue. We could take vertical sections with reference to the fiber orientation, in either the longitudinal or the coronal planes.
2. A uniquely identifiable base plane is set as a horizontal reference plane, and the vertical direction would be defined to be normal to the horizontal. Many synthetic structures in material science and metallurgy, and flat tissues such as skin can be measured using this strategy.
3. Define the vertical direction artificially.

Generate vertical sections using the following rules:

1. All vertical sections must be normal to the pre-defined horizontal plane;
2. Vertical sections must have random positions, and random direction, which is normal to the horizontal plane.

Isotropic Uniform Random (IUR) Sections

IUR sections are not used as frequently as vertical sections, because they are difficult to generate and must satisfy the following conditions:

1. There is no preference in selecting any particular orientation within the region of interest (isotropic selection). That is, the sectioning plane is randomly selected.
2. The location of the section is randomly selected from within the sample (uniform selection). There is no preference in selecting a section from any particular position.

In practice, IU sections are usually generated according to a procedure called *orientator*. see Mattfeldt et al. (1985) for details.

Procedure: 2D Frames, 3D Probes and 3D Disectors

The **Stereology** module works in conjunction with the **AIS** software's automatic target detection and measurement feature (Scan Area) for gathering 2D Frames, 3D Probes and 3D Disectors data. You first define a set of target criteria that distinguish features of interest (i.e., potential target profiles) from other image features, based on any combination of density, size, or shape. Once you have established an appropriate set of criteria, you use one of the **Stereo Sample Tools** (i.e., a 2D sampling frame) to define a region of interest. **AIS** then searches the region for features that satisfy all of the detection criteria, generates the appropriate test probes, and reports the stereological data in the data table.

The basic procedure is as follows:

1. Load the images.
2. Make all necessary calibrations (e.g., Distance)
3. Select the measures you wish to make.
4. Establish detection criteria for target profiles.
5. Where required, adjust the sampling grid and specify the way in which the specimen was sectioned (e.g., Vertical or IUR)
6. Sample the images.
7. Edit the sampled images as required.
8. Compile the results in the Stereology table.

This section describes how to use the **AIS Stereology** functions and features to gather 2D Frames, 3D Probes and 3D Disectors data. At the end of this document is a [Stereology Tutorial](#), which provides an explicit step-by-step guide through the sampling process.

The Stereo Sampling Grid

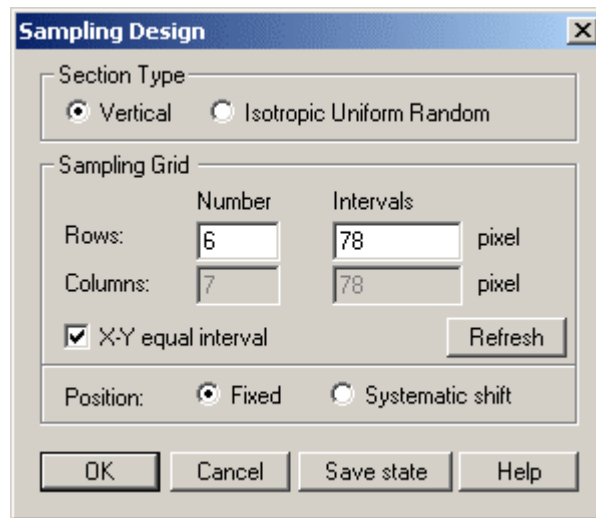
To calculate *3D Probes* measures (S_v , mean surface volume; V_v , mean volume; A_a , area ratio), linear probes are oriented around regularly spaced points in a "sampling grid." Before the specimen is sampled, the sampling grid should be adjusted so that no feature of interest contains more than a single grid point. The section type must also be specified, to

ensure that the appropriate algorithms and test probes are used. For access to these settings, click on the **Stereo Grid** icon:



A sampling grid appears over the image in the current channel. The *Sampling Design* dialog box also appears (Figure 3), as well as a set of **Stereo Grid** tools. The *Sampling Design* dialog box is used to specify a section type and sampling grid size. The **Stereo Grid** tools allow you to adjust the grid with the mouse.

Figure 3: The *Sampling Design* dialog box is used to specify a section type and sampling grid size.



Section Type

Specify whether sections are **Vertical** or **IUR**. Each of these section types has its own set of algorithms for generating test probes, ensuring that the probes are isotropic in 3D space.

Sampling Grid Options

The sampling grid should be adjusted so that no feature of interest contains more than one grid point. The size of the sampling grid is defined by the number of **Rows** and **Columns** and the **Intervals** between them. Values may be changed by typing new values into the appropriate entry fields. Click on the **[Refresh]** button to update the grid after entering new values.

In theory, the position of the sampling grid should be systematically shifted by half of the grid point interval for every image in the stack to achieve better measurement precision. In practice, a fixed grid position will serve just as well. Either a **Fixed** grid position, or an (automated) **Systematic shift** of grid position may be selected.

Stereo Grid Tools

The size and position of the sampling grid may also be adjusted with **Stereo Grid Tools** (Figure 4). Values in the **Rows**, **Columns** and **Interval** fields are automatically updated as the grid is adjusted.

Figure 4: Stereo Grid Tools are used to adjust the size and/or position of the sampling grid.



Shift

The **Shift** tool is used to select a new location for the sampling grid. Move the cross-hair cursor over any grid point. When the cross-hair turns into a pointing finger, drag the grid point to a new XY coordinate and release the mouse button. The entire grid is redrawn using these coordinates as a reference.



Size

The **Size** tool is used to set a new grid point interval for the sampling grid. Move the cross-hair cursor over any grid point and it becomes a pointing finger. Press the left mouse button and the grid point becomes one corner of a variable dimension box, defined by its three neighboring points. Drag the grid point to a new location and release the mouse button. The grid is redrawn using these coordinates as a reference.



Autoscan B

The **Autoscan B** tool automatically establishes an optimal grid point interval based on the size of the largest feature of interest. To use this tool, the target must be clearly distinct from the regions around it. Place the hollow arrow cursor on a background next to a target and click the left mouse button. When the cursor becomes a solid arrow, place it over the largest feature of interest and click again. The target outline is shown in red and its dimensions set the optimum grid size for this sample.



Outline

The **Outline** tool establishes an optimal grid point interval based on the dimensions of a hand-drawn target. Hold down the left mouse button and trace around the border of the largest potential target. Release the mouse button and **AIS** uses the feature's dimensions to set the optimum grid size for this sample.

 **Pixel Info**

The **Pixel Info** tool cursor is a cross hair with a single pixel in the middle. Position the cross hair anywhere on the image and click the left mouse button. The X Y coordinates and the density value for the pixel under the cross hair is displayed at the top left of the image monitor. Use it to have a quick look at pixel density values.

Establishing Target Detection Criteria

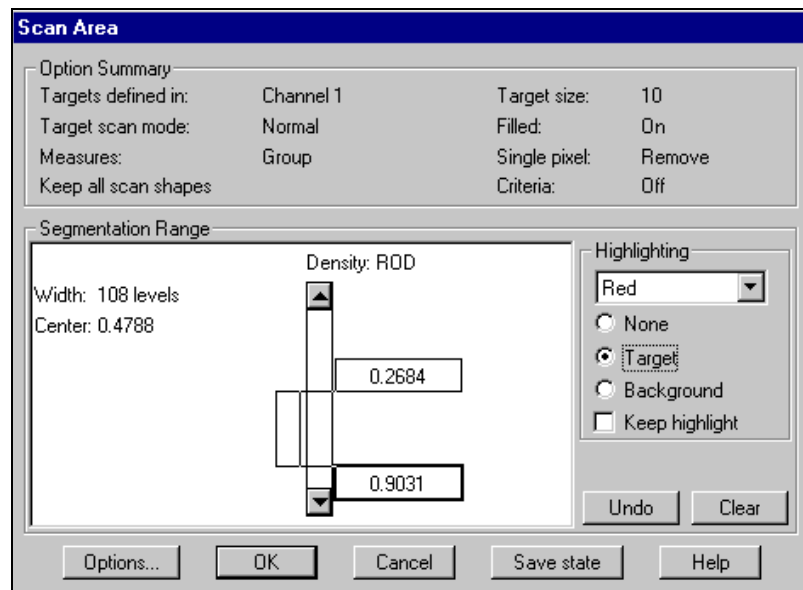
The **Stereology** module works in conjunction with the **AIS** automatic target detection and measurement feature, called “Scan Area” (see *AIS Reference Manual, Chapter 8: Automated Target Detection* for complete details). You first define a set of target criteria that distinguish features of interest from other image features, based on any combination of density, size, or shape. Once you have established an appropriate set of criteria, you use one of the **Stereo Sample Tools** (see below) to define a region of interest. **AIS** then searches the region for features that satisfy all of the target criteria, generates the appropriate test probes, and reports the stereological data in the data table.

Click on the **Scan Area** icon to turn on the automatic target detection feature:



Selecting the **Scan Area** icon turns the automatic target detection feature on and off. Selecting the icon while pressing the <Ctrl> key calls up the *Scan Area* dialog box (Figure 5).

Figure 5: The *Scan Area* dialog box defines the segmentation range and has a variety of target detection options.



The *Scan Area* dialog box is used to define target detection criteria. In the middle of the dialog box there is a set of slider controls for setting the upper and lower target density

limits. Together, these limits are called a “**Segmentation Range**”. The **Highlighting** box is simply a visual aid to help establish the Segmentation Range. The **Option Summary** section displays the current status of optional features that can be modified by clicking on the **[Options...]** button.

Setting a Segmentation Range

Density thresholds (light vs. dark) are the simplest criteria for target detection. You define a segmentation range using the upper and lower thresholds. Pixels having densities that fall within the segmentation range are regarded as valid targets. Pixels that are lighter or darker than the threshold values are ignored. In Figure 4, pixels lying between 0.2684 and 0.9031 ROD are valid targets.

When adjusting the segmentation range, the goal is to set thresholds that distinguish targets from background. A very narrow segmentation range may yield pure targets, but genuine targets go undetected. By contrast, a wide segmentation range may include some background features as targets, or leave halos of background around each genuine target. It is much easier to set the correct threshold range if you have the **Highlighting** drop down list set to **Targets** (see below).

The segmentation range can detect either dark or light targets. **AIS** is equally capable of detecting dark targets against a light background or light targets against a dark background.

Highlighting

It is much easier to discriminate targets from background if they are shown in some easily perceived color. Select a target color from the **Highlighting** drop down list, then click on the **[Target]** radio button. Alternatively, you can color the background instead of the targets. To do this, select a color from the **Highlighting** list then click on the **Background** radio button. If you select **None**, neither target nor background are highlighted (this is **AIS's** default condition).

While you are setting the segmentation range, you usually want to see targets filled with color. The color fill is removed when you work with the images. Color fill only reappears when the automated scan detects targets. If you prefer to see target pixels colored at all times, click on the **Keep highlight** checkbox.

Processing the Image

Before establishing a density segmentation range, you may wish to perform a few image processing operations to make genuine targets stand out more clearly from background or modify the target structure. Image processing operations and filters are available through the Transform operation window (see *AIS Reference Manual, Chapter 13: Processing Images*). Because image processing operations change image data you should limit pre-processing only to the extent needed for setting an adequate segmentation range.

Various image processing features are available. In some instances, you could perform a series of **Max** or **Min** operations to establish more distinct boundaries between closely spaced targets and fill in holes within them. The **Target Accentuate** filter maximizes the

contrast between small image features and larger background regions (to apply the **Target Accentuate** filter, press <F12>). This filter reduces the contrast of all image components but more so for larger targets.

Establishing Spatial and Logical Criteria

A threshold operation rarely eliminates all the invalid targets from the scan. Usually, bits of background or other image features have density values similar to those of valid targets. In a scan based entirely upon density thresholds, these image characteristics may be confused with valid targets. To avoid this problem, **AIS** lets you include additional spatial and logical target acceptance criteria. These target criteria include minimum and maximum area, minimum roundness, minimum and maximum chord, and other parameters.

You set spatial and logical criteria by clicking on the **[Options]** button in the *Scan Area* dialog box. This produces the *Scan Area Options* dialog box. Selecting **Criteria on** and then clicking on **[Modify Criteria]** calls up the *Target Acceptance Criteria* dialog box (Figure 6).

The dialog box looks and works much like a calculator. To construct logical target criteria, use the number, symbol, and mathematical operation buttons in combination with the list of spatial measure categories on the right.

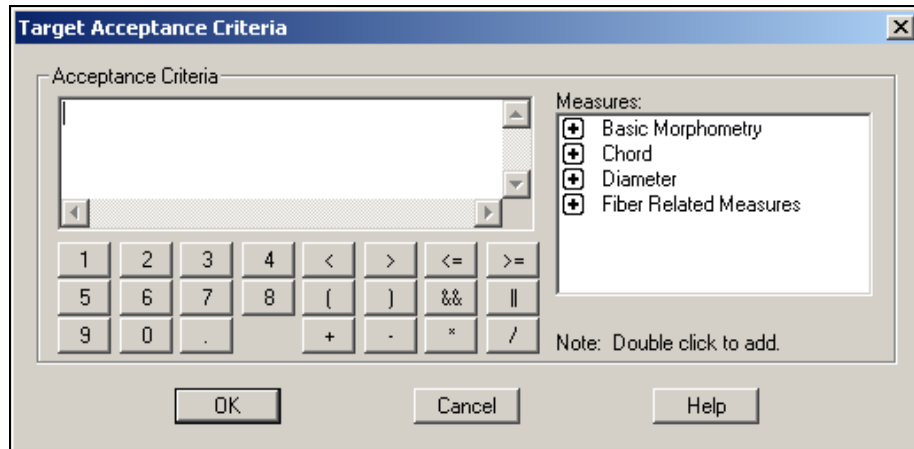
Suppose you only wish to count image features that are between 100 and 200 μm^2 in size. Open the **Basic Morphometry** group of measures to see the **[Area]** measure. Use the **[Area]** measure and the buttons to construct one line that says “**[Area] > 100**” and a second line that says “**[Area] < 200**” (double-click on the **[Area]** measure to add it to the statement). You can use any number of criteria. If targets are round, for example, you could also construct another line that says “**[Form Factor] > .8**”. (See *AIS Reference Manual, Chapter 5: Selecting Measures* for more discussion on how to use these measures).

When you are constructing logical criteria, you should use numerical values that correspond to your current spatial calibration units (see *AIS Reference Manual, Chapter 7: Calibrating Images*). If your image is not calibrated, spatial unit values are expressed in pixels.

When Target Criteria are not Adequate

If it is not possible to establish detection criteria that adequately distinguish features of interest from background, then each feature of interest must be manually defined when you sample the image. See [Manual Target Definition](#), below.

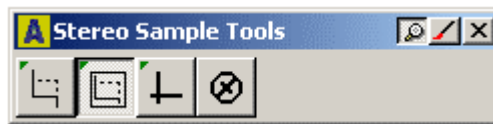
Figure 6: The Target Acceptance Criteria dialog box is used to define spatial and logical target criteria.



Stereo Sample Tools

Stereo Sample tools (Figure 7) are used in conjunction with the **AIS** automatic target detection feature (Scan Area) to define regions of interest and derive stereological measures from the objects (target profiles) found within them.

Figure 7: The Stereo Sample Tool box.



The basic sample tool is the “sampling frame” or “counting frame” (Figure 8), a rectangular box with broken lines for its upper and right boundaries (inclusion lines) and solid lines for its left and lower boundaries (exclusion lines). The sampling frame counts targets that fall completely within its boundaries plus any target, which touches the inclusion lines. Targets that touch the exclusion lines are not counted.

Two sampling frames are available, one that can be dragged to any size and moved about, and one that remains in a fixed position.



Continuous Frame

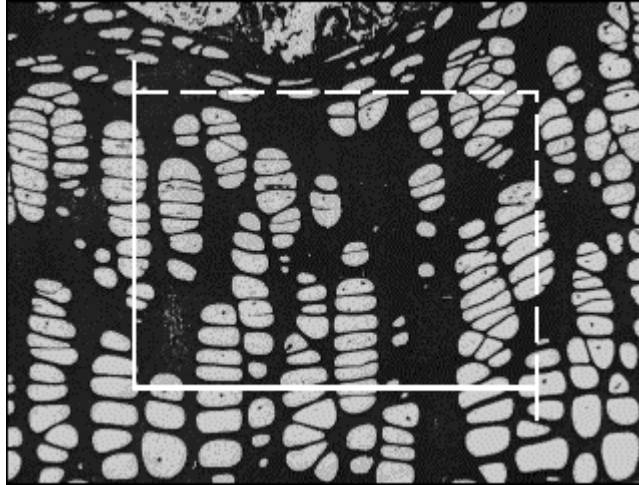
The **Continuous Frame** can be positioned anywhere within the image. To adjust the size of the frame, move the mouse while holding down the <Ctrl> key. Click the left button to sample.



Channel Frame

The **Channel Frame** can be locked into a fixed position within a channel. To adjust the initial size of the frame, move the mouse while holding down the <Ctrl> key. To position the frame, move the mouse while holding down the <Shift> key.

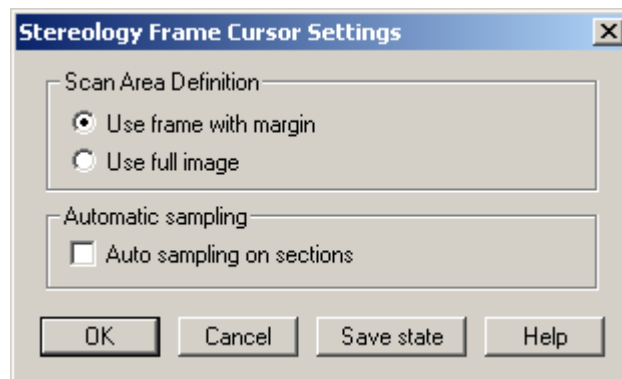
Figure 8: The 2D sampling and counting frame.



Defining Outer Scan Areas

Recall that the sampling frame counts and samples any target that touches its inclusion lines, even if the target extends beyond the frame. AIS must consequently scan a region outside of the frame, adjacent to the inclusion lines. To specify the amount of adjacent image area to scan, double-click on one of the frame tool icons. This will display the *Stereology Frame Cursor Settings* dialog box (Figure 9).

Figure 9: The Frame Cursor Settings dialog box is used to define the amount of adjacent image area to scan, and to enable automatic sampling across an entire image set.



If you select the **Use frame with margin** option, AIS scans the area within the counting frame plus a small region adjacent to the inclusion lines. The adjacent region extends to about one third of the length and width of the sampling frame. If you select the **Use full**

image option, **AIS** scans the entire image area adjacent to the inclusion lines. This option may tax system memory if you are working with very large (e.g., tiled) images.

Labeling Data

AIS tracks stereological data using the following labeling scheme:

- Subject** Animal/Subject used in study.
- Section** Tissue section obtained from subject (image filename or channel number).
- Region** Anatomical area.
- Target** Area of image sampled with a single sampling frame.

Subject, **Section** and **Region** labels are located at the top of the Sample window. Before gathering data, click on the **Subject** and **Region** headings and enter the appropriate labels. The default label is “?”.

AIS automatically creates **Section** labels as each image is sampled. If you are working with image files, **AIS** will use their filenames as labels. If images are digitized into consecutive channels, **AIS** will use channel numbers for **Section** labels.

Collecting Data

To gather data, select one of the sampling frame tools and position it over the image. The general rule is to size the frame to select as many target profiles as possible, while making sure that the broken sides of the frame (inclusion lines) avoid targets that intersect the edges of the image.

Press the left mouse button to sample the image. **AIS** first scans the sampling frame (and the region adjacent to the inclusion lines) for targets that fall within the segmentation range. If you have established spatial criteria, **AIS** scans the frame a second time and searches for targets that also fulfill these additional criteria. As the scan proceeds, **AIS** displays “valid” targets (i.e., those that meet the spatial criteria as well as the density criteria) in one overlay color and “excluded” targets (i.e., those that meet the density criteria but *not* the spatial criteria) in another. Stereological measures are derived from “valid” targets and reported in the data table.

Repeat the sampling procedure for all other images in the series, or use auto-sampling feature (described below) to sample the entire image set in one operation. If you are working with **3D Disectors** (i.e., with aligned serial sections), be sure to sample the same 2D coordinates in each image. Use the **Channel frame** tool or the auto-sampling feature to keep the same coordinates across all sections.

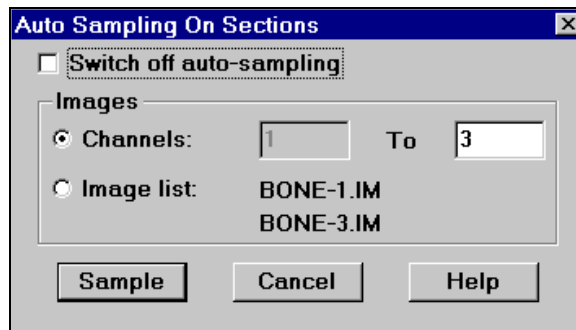
Automatic Sampling Across an Image Set

You can analyze your specimens one section at a time, or as a stack containing any number of sections. Use the *Image – Image set – Retrieve* command to load multiple image files into consecutive channels or into an image file storage list (see *AIS Reference Manual, Chapter 6: Making and Loading Image Files* for complete details). You can then perform an automated analysis across the entire stack with a single operation.

To enable auto-sampling mode, double-click on whichever counting frame tool you will use to gather data (i.e., **Continuous frame** or **Channel frame**). When the *Frame Cursor Settings* dialog box appears (Figure 9), turn on the **Auto sampling on sections** option.

When you are ready to sample the images, click the counting frame in the first image in the series. When the *Auto Sampling On Sections* dialog box appears (Figure 10), specify the range of images to sample.

Figure 10: The *Auto Sampling On Sections* dialog box. If images are loaded into consecutive channels, specify the range of channels to sample. If images files are loaded into a storage list, the filenames of the first and last images are displayed.



Click the [**Sample**] button to begin sampling the image set. AIS generates the appropriate test probes, scans each image for targets that meet the detection criteria, and reports the results in the data table.

Displaying Data

Measurement results are displayed in the **Sample** window in both tabular and graphical forms.

Figure 11: Tabular data display.

Section	Target	Ns - count	As - μm^2	Na - # / μm^2	Vv - ratio	Sv - $\mu\text{m}^2 / \mu\text{m}^3$	Vm - μm^3
SV1	1	17.000	172919.000	9.8312e-005	0.066	0.012	17276.768
SV2	1	17.000	172919.000	9.8312e-005	0.066	0.014	16051.733
SV3	1	17.000	172919.000	9.8312e-005	0.066	0.015	23105.264
SV4	1	17.000	172919.000	9.8312e-005	0.066	0.011	21310.775

Data Tables

AIS provides two data tables for displaying stereological data:



Target table



Stereo table


Only one of the above icons is visible while you are in the **Sample** window (labeled as **Select Table**, in the top right corner of the Sample window). Click on the visible icon to access the other data table.

The default data table is the **Target table** (Figure 11). It displays data from individual sampling frames for the specified Subject, Section and Region label. Use this table to gather and edit data.

The **Stereo table** summarizes data gathered from all counting frames across all sections (images). AIS computes and displays the appropriate summary value for each measure and summarizes the appropriate stereological parameters upon which the measure is based (e.g., # frames, # intercepts). Data may be sorted by Subject and/or Region label.

Please note, although summary statistics (Mean, SD) are displayed at the bottom of the **Target table**, these values should *not* be used to express final results. In order to generate measures that are representative of the entire specimen, we must pool data obtained from individual samples (i.e., across all sections). Always use the **Stereo table** for reporting final results.

Levels of Data Display

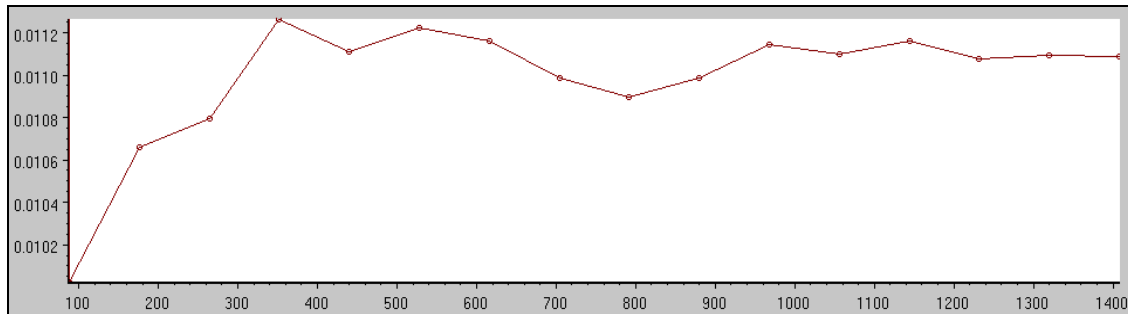
Within each summary table, data may be sorted across or within Subject, Section and/or Region labels. Click on the  button (next to the Subject heading) to select different combinations of Subject, Section and Region display modes.

- Current region** Displays data gathered under the currently selected Region label (default Region display mode). Click on the Region heading to select another label.
- All regions** Displays data gathered under all Region labels. Data are labeled with individual Region names.
- Current section** Displays data gathered under the currently selected Section label.
- All sections** Displays data gathered from all Section labels (default Section display mode). In the Target table, data are labeled with individual Section names (i.e., image filename or channel number).
- Current subject** Displays data gathered under the currently selected Subject label (default Subject display mode).
- All subjects** Displays data from all Subject labels. Data are labeled with individual Subject names.

Graphical Data Display

The graph area in the Sample window displays the incremental calculation of any measure in the data table, over all sampled sections. Figure 12, for example, shows the estimation of mean surface density (S_v) plotted against the number of test probes used.

Figure 12: Graphical display of mean surface density (S_v), plotted against the number of probes.



The graphical display is an effective way to monitor the reliability of particular measures (e.g., S_v) as sampling progresses. If the estimated measure is fairly stable (as in Figure 12, above), it is an indication that enough measurements have been made to reach a reliable result. Further measurement is probably unnecessary. In contrast, if the graph shows variable measurements, this indicates that more data should be taken.

Select which measure to plot by clicking on its column heading in the data table. When a different measure is selected from the data table, the graph is redrawn and the axis labels are updated. The following shows what AIS plots for each of the measures:

Ns -- Number of 2D targets sampled, plotted against the number of counting frames used.

As -- Area sampled, plotted against the number of counting frames used.

Na -- 2D target density, plotted against the number of counting frames used.

Aa -- Area ratio, plotted against the number of intercepts used.

Vv -- Estimated volume ratio, plotted against the number of counting frames used.

Sv -- Mean surface density, plotted against the number of probes used.

Vm -- Mean target volume, plotted against the number of intercepts used.

N -- 3D target count, plotted against the number of disectors used.

Vs -- Volume sampled, plotted against the number of disectors used.

Nv -- 3D number density, plotted against the number of disectors used.

Editing Data

Automated measurements depend upon an accurate discrimination between target and background. Making this discrimination (usually on the basis of a density threshold) is known as *segmentation*. Automated segmentation works very well with clearly stained or

Deleting Targets

Select the **Delete** tool to delete a target profile (filled with red overlay) from the scan area. When you click the **Delete** tool on any sampled target profile, the overlay color disappears and data are updated accordingly.

Adding Targets

The remaining **Scan Edit** tools operate just like standard **Sample** tools. For example, select the **Outline** tool to manually trace around the border of an undetected feature of interest. Click inside the outline and **AIS** adds the feature to the sample. Data are updated accordingly.

Note: **AIS** will only derive data from feature(s) of interest, which lie completely within the boundaries of the sampling frame or touch an inclusion line.

Stereo Editing Tools

Stereo Editing Tools (Figure 15) are used to edit the test probes used to estimate particular stereological measures. These tools are not available until an associated measure (S_v , V_m , N , or N_v) is selected in the data table.

Figure 15: Stereo Editing Tools.



Surface probe

The **Surface probe** tool is used to edit the mean surface density measure (S_v), by adding or deleting intersections between test probes and target profile boundaries. On the sampled image, intersections are shown as small green circles. To delete an intersection point, click on it with the right mouse button. Click the left mouse button to add a new intersection point.



Volume probe

The **Volume probe** is used to edit the mean target volume measure (V_m) measure. It is useful if segmentation is unreliable and you need to manipulate the lengths of the intercepts accurately. Click the cursor on any sampling grid point (displayed in green on the sampled image) and **AIS** creates a linear probe oriented around the point. To extend the probe, place the cursor on one end of the probe and drag it outward. The probe extends to the point where you release the mouse button. To shorten the probe, click the right mouse button on the point where you wish the probe to end. To delete a probe, hold down the <Ctrl> key and click on the probe with the right mouse button.



Area probe

The **Area probe** tool is used to edit the area ratio measure (Aa), by adding or deleting intersections between test probes and targets. On the sampled image, intersections are shown as small yellow circles, which appear over grid crosses. To delete an intersection point, click on it with the right mouse button. Click the left mouse button to add a new intersection point over a grid cross.



Profile pointer

The **Profile pointer** is used to edit 3D target count (N) or 3D target density (Nv) measures. On sampled images (other than the first image), 2D target profiles are displayed in the current cursor color. Profiles that were not present in the preceding section (i.e., the tops of new 3D targets) are outlined in another color. To unmark a 3D target, click on it with the right mouse button. It is then treated as a 2D target profile. To designate a 2D target profile as a 3D target, click on it with the left mouse button.



Pixel Info

The **Pixel Info** tool cursor is a cross hair with a single pixel in the middle. Position the cross hair anywhere on the image and click the left mouse button. The XY location and the density value for the pixel under the cross hair is displayed at the top left of the image monitor. Use it to have a quick look at densities without actually taking data samples.

You can set the **Pixel Info** tool to express pixel location and density in various units. Click on the **Pixel Info** icon while pressing the <Ctrl> key to define the location and the density units.

Manual Target Definition

If it is impossible to establish an automatic target detection criteria that adequately discriminates between features of interest and other image features (i.e., if it is not possible to use AIS's automated target detection feature at all), then each feature of interest must be manually defined.

If the **Scan Area** feature is not turned on when you sample an image, AIS will automatically enter the data editing mode and display the **Scan Edit** toolbox. Use any of the available tools to define all target profiles that fall completely within the boundaries of the counting frame, and all targets, which touch the inclusion boundaries. Data from the sample are updated each time a target profile is defined.

Procedure: 3D Fractionator

When stereology is used with an AIS system it is able to integrate microscope stage control and implement an automated optical fractionator for cell counting procedures. Taking advantage of the optical fractionator requires that the AIS system be equipped with

a motorized XYZ microscope stage (preferably with a linear encoder on the Z-axis control). Below are listed the additional measures available with the automated optical fractionator within stereology.

Parameter	Definition	Method Name
NEst	Estimated total number of targets	Unbiased sampling and counting frame
NDet	Number of targets counted	Unbiased sampling and counting frame

When using the automated optical fractionator method, the user predefines the number of sections to be counted (from the total sections available), the section thickness, and the sampling frequency. A montage of each section is created at low power, and an area of interest is outlined on this montage. A higher power is selected, and the system uses random systematic sampling to move to the first location within the region of interest. Subsequent locations are located by moving the motorized stage in a systematic pattern over a grid of the area of interest. The precise fraction sampled relative to the area between locations is thus known. At each location, interactive sampling is performed.

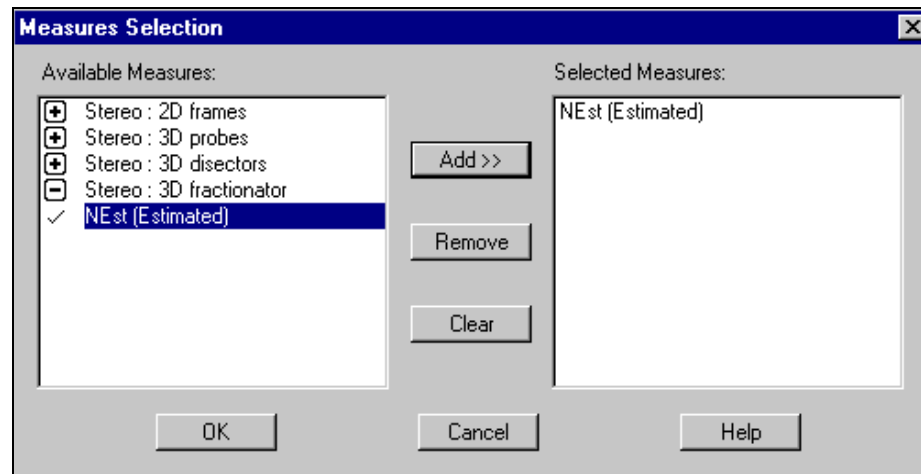
To count targets, the user focuses up and down and marks targets within the counting frame. An on screen indicator displays the current Z position and guard volume locations. The computer keeps track of the counts and calculates an estimate of the total number of targets based on the various fractions sampled.

Setting up the Automated Optical Fractionator

Before entering the **Stereology** study type and selecting the automated optical fractionator, one should be familiar with the configuring and setting up of a motorized stage. This familiarity should also include the stage alignment procedure, objective calibrations and the creation of a nosepiece file. One should also be familiar with the procedure of tiled field mapping (TFM). These topics are explained in detail in the motorized microscope stages and TFM sections of the online *AIS Reference Manual*.

Go to the **Stereology** study type and from the *Sample* menu option choose *Measures selection*. This will display the *Measures Selection* dialog box (Figure 16), listing all the available stereology measures. Open up the **Stereo: 3D fractionator** and select the **NEst (Estimated)** measure.

Figure 16: The Measures Selection dialog box, used for choosing types of stereological measures.



Once you have made your measurement selection, close the *Measures Selection* dialog box and go back to the *Sample* menu option and select *Wizard*, (Figure 17).

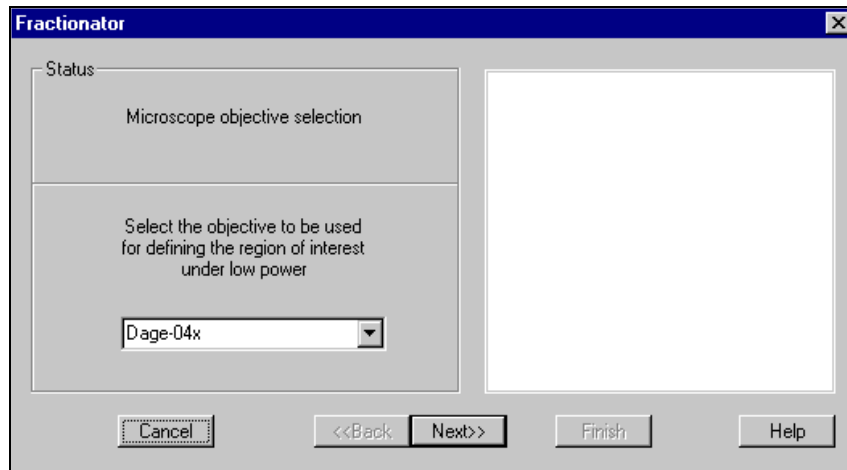
Figure 17: The Wizard option from within the Sample menu option.



This *Wizard* option in the *Sample* menu only appears once you have selected the *NEst* measure from within the **Stereo: 3D fractionators** and allows you to proceed with the automated optical fractionator setup. By selecting *Wizard* you will be guided via a series of dialog boxes, prompting you for various experimental parameters.

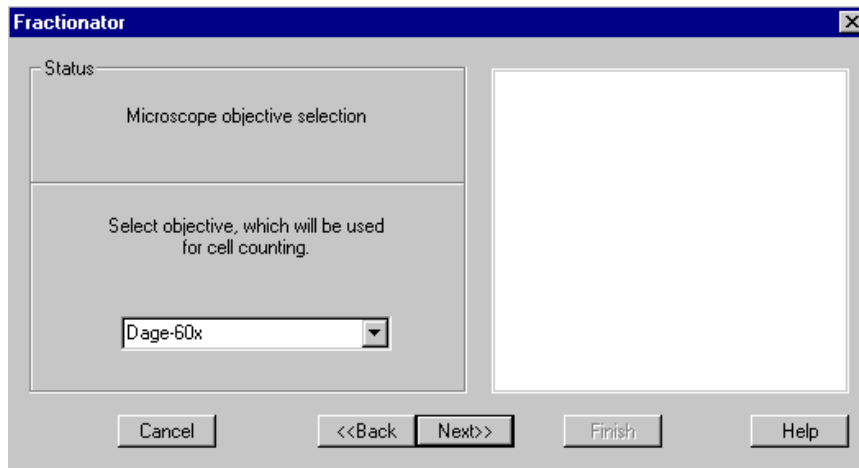
The Optical Fractionator Wizard

Figure 18: The first in a series of the Wizard Fractionator dialog boxes. This one deals with selecting the low power objective.



In the first *Fractionator* Wizard option (Figure 18) you will be asked to select the objective that you plan to use for defining the region of interest at low power. This objective can be selected from a predefined list created during the nosepiece definition procedure. If the objective does not appear on this list, you will have to exit the *Stereology* study type, go into the microscope calibration setup and add the appropriate objective to the nosepiece list. Pressing the [Next] button will move you to the next step in the *Fractionator* wizard. (Figure 19).

Figure 19: Selecting the cell counting objective.



You will now be asked to select the objective you plan to use for counting. This is the end of the objective definition and pressing the [Next] button will take you to the experimental parameters definitions. (Figure 20).

Figure 20: Defining the experimental parameters within the fractionator wizard.

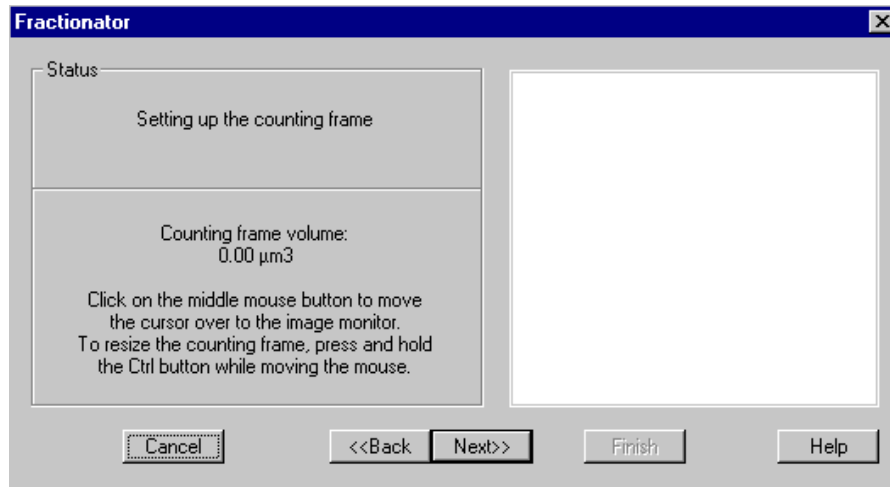
In the **Setting up experiment parameters** section you are asked to describe your experimental protocol. The total number of cut sections, the number of sections you wish to sample and the actual section thickness, are all required. For example, if you have cut 50 sections through your structure of interest then the answer to the total number of sections should be 50. Of those 50 sections you may wish to sample every tenth section so the number of sections to sample should be entered as 5. The actual, measured thickness of the section should be entered and not the thickness the sections may have been cut at.

In the above example we are going to sample 3 sections out of a total of 10, with a measured section thickness of 35 μm . Press [**Next**], once all the parameters have been entered.

Figure 21: Defining the guard volume in the section.

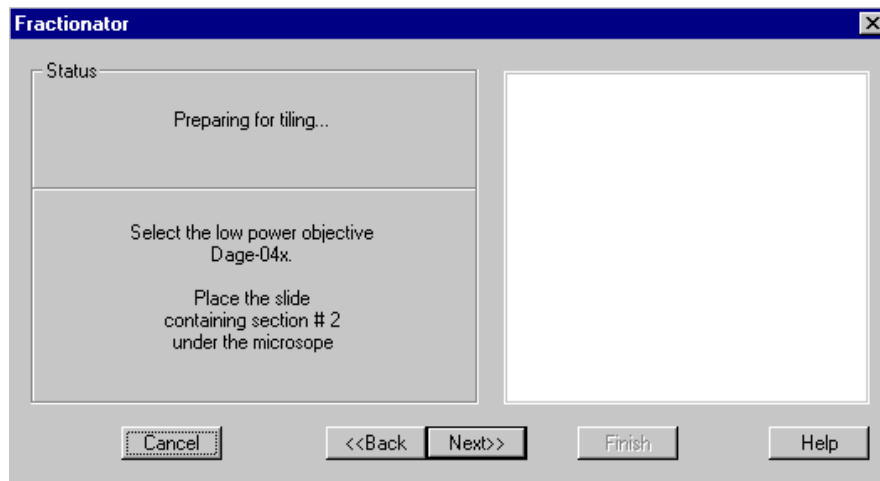
The next input required is the **Guard volume**. This defines the region above and below the counting volume. The guard volume prevents counting errors that maybe associated with artifacts found on the surfaces of the cut planes of your sections. In this example we will define a guard volume of 5 μm . Press [**Next**], once you have defined the Guard volume.

Figure 22: Setting up the counting frame.



After defining the Guard volume the *Fractionator* wizard moves on to allow you to define the size of the counting frame. Initially the counting frame volume is set to $0.00 \mu\text{m}^3$. Move the cursor over to the image view window. The cursor changes to the counting frame cursor with the red solid lines denoting exclusion zones and the green broken lines denoting inclusion zones. To adjust the size of the counting frame, press and hold the **<Ctrl>** key while moving the mouse. Once you are satisfied with the size and position of the counting frame, click on the left mouse button to sample the frame. The *Fractionator* wizard will now update the counting frame volume. Click on the **[Next]** button to move to the next stage in the *Fractionator* wizard.

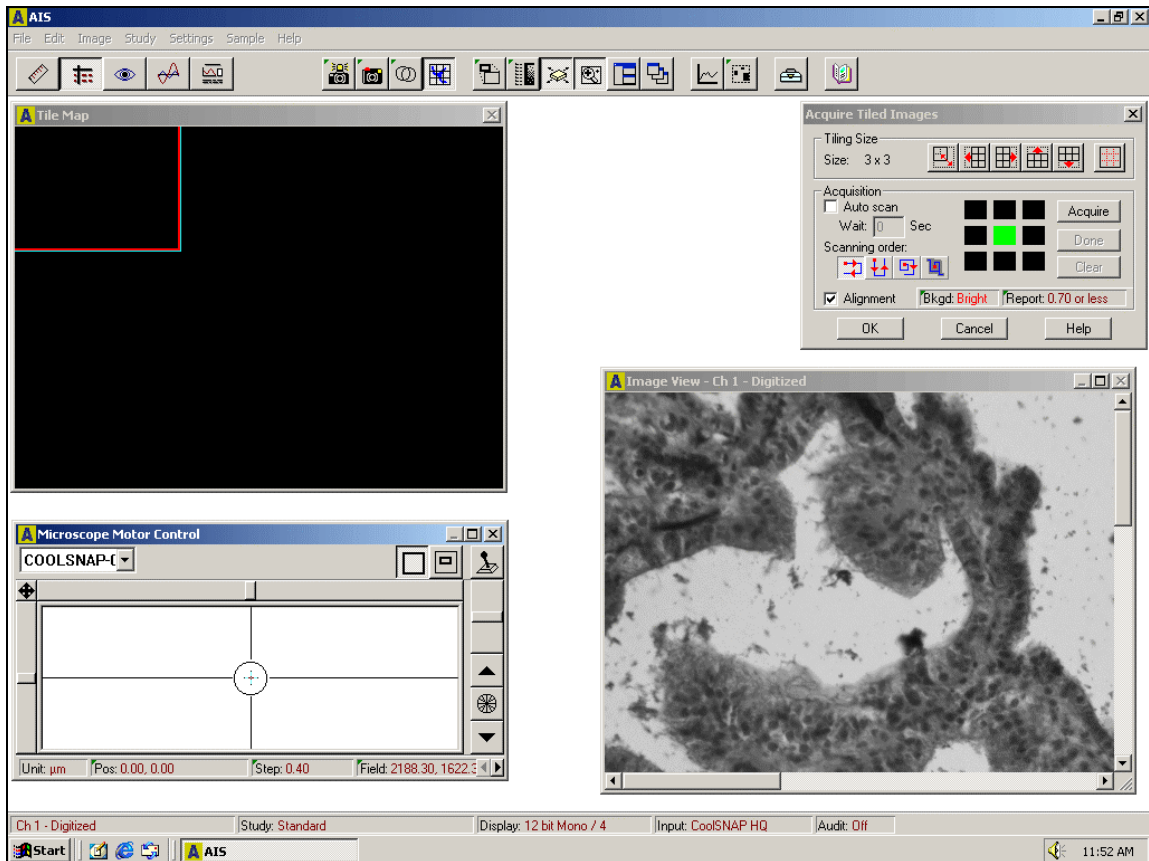
Figure 23: Getting ready to perform the tiling operation.



You are now asked to select the low power objective and place the appropriate slide under the microscope. In the above example you are asked to place the slide containing section #2 under the microscope. Section #2 has been chosen random systematically from the information provided when defining the experimental parameters (Figure 20). Once you

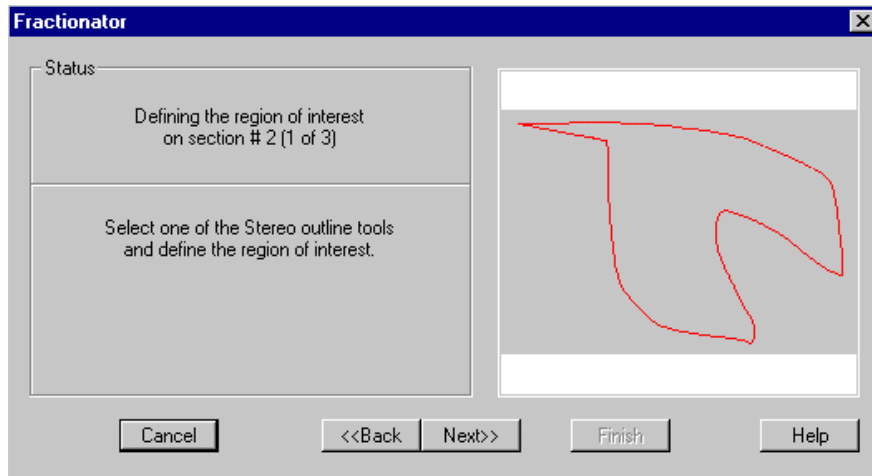
have completed these steps press the [Next] button to continue with the *Fractionator* wizard. You will now be placed into the tiling module part of the system (Figure 24).

Figure 24: Within the Tiling module component of the Optical Fractionator Wizard.



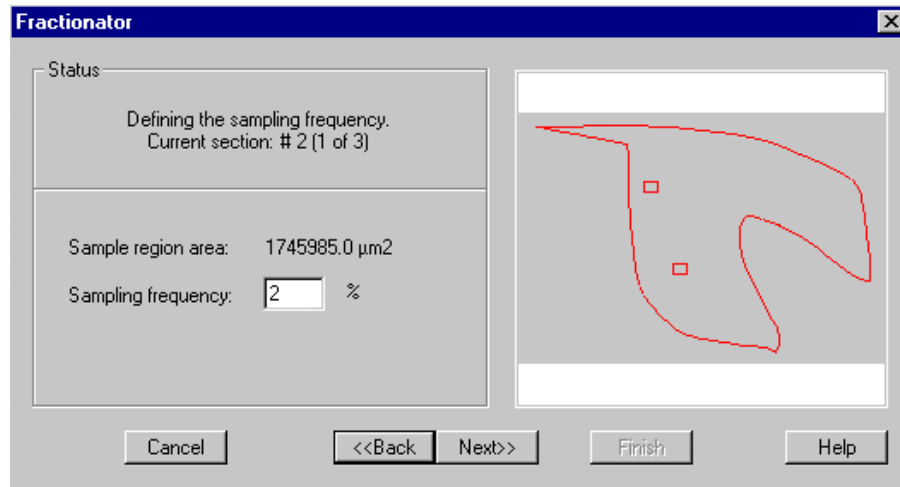
At this point in the proceedings the object of the exercise is to create a tiled image of the area of interest within which, you wish to count targets under high power. You should be familiar with the operation of the tiling module and detailed instructions can be found in the online *AIS Reference Manual, Chapter 17: Tiled Field Mapping*. Once you have completed the tiled image, it will appear in the Image View window.

Figure 25: An outline of the region of interest from the tiled image under low power.



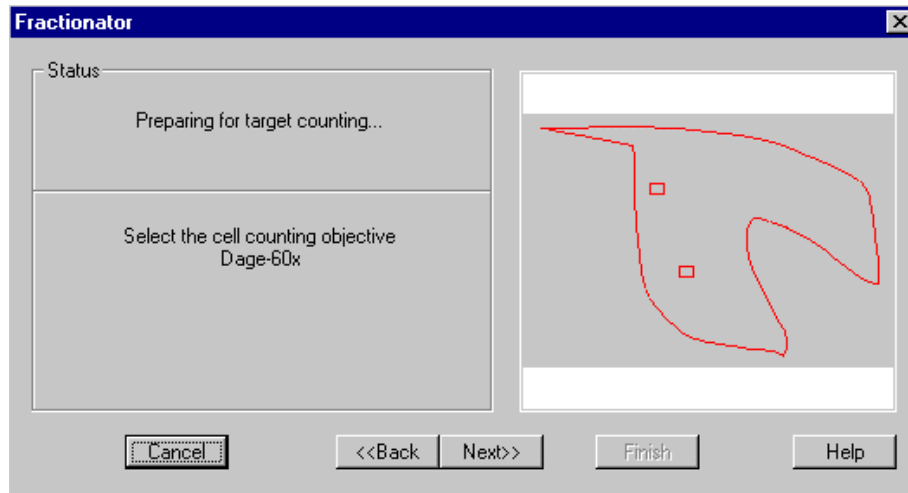
You will be asked to select one of the available **Stereo outline** tools and trace out the region of interest from the tiled image. Sampling this outline will display it within the *Fractionator* dialog box. Click on the **[Next]** button, once you have defined the outline of your region of interest.

Figure 26: Selecting the sampling frequency.



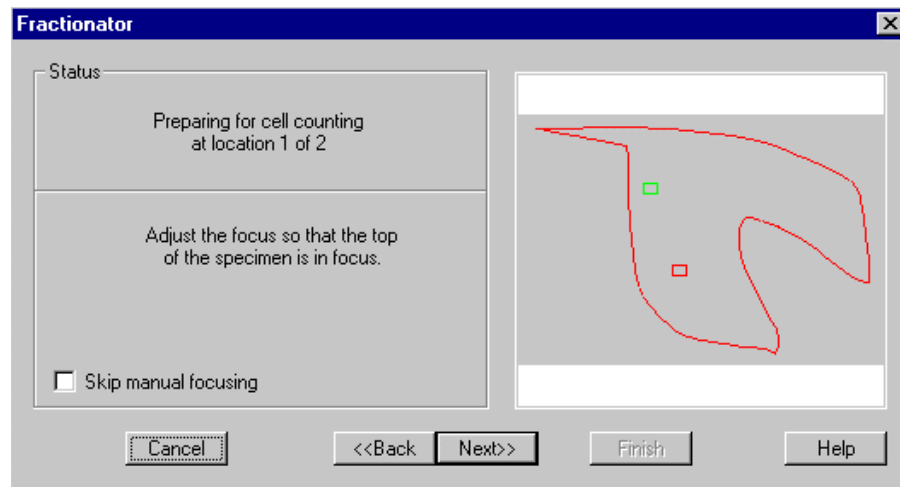
This dialog box displays the total area in calibrated units, of your outlined region of interest. Type a number in the sampling frequency box and a series of small boxes will be displayed over the outline of your defined region of interest. These boxes denote the number and the locations of the counting frames that are to be sampled. The higher the sampling frequency the more sampling points will be displayed. The object here is to set the sampling frequency to a value that will give you a total of between 100 to 200 actual targets counted within the whole experiment, over multiple sections/slides. In this example we have selected a value of 2%, which happens to correspond to 2 locations to be sampled under high power for this region.

Figure 27: Getting ready for target counting under high power.



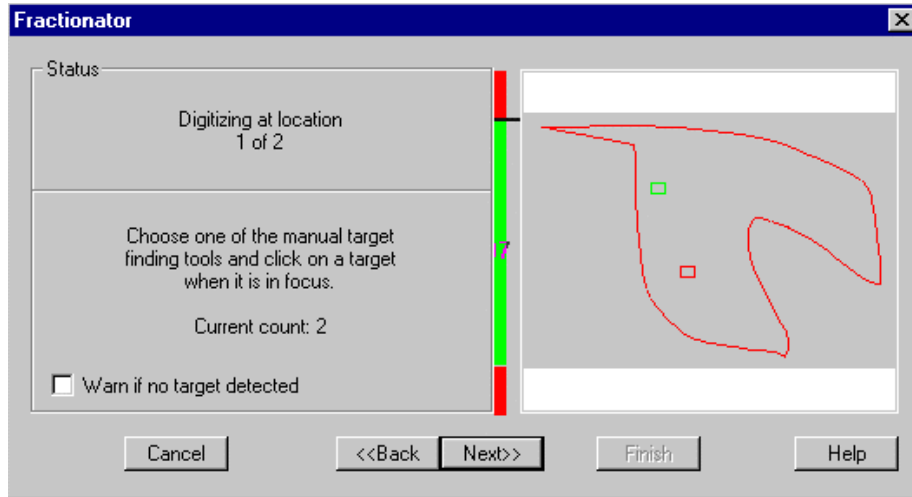
Clicking on the [Next] button will move you to the next *Fractionator* wizard window where you will be asked to select the cell counting objective. In this example (Figure 27), you are asked to select the **Dage-60x** as the cell counting objective. Clicking on the [Next] button will move you to the next *Fractionator* wizard dialog box (Figure 28).

Figure 28: Adjusting the focus under the high power counting objective.



You are now asked to adjust the focus so that the top of the section is just coming into focus. At this point you have the option of checking the **Skip manual focusing** checkbox. Placing a checkmark will cause the system to assume that the first time you adjust the focus for the top of the section, it will now be correct for all subsequent locations within the selected outline. Once you are happy with the focus, press the [Next] button. The system will automatically move down through the sample the distance you have previously specified as the guard volume, in this case 5 μm , see Figure 21. Once the guard volume distance has been adjusted for, the next dialog box appears (Figure 29).

Figure 29: The target counting dialog box. The box outlined in green denotes the current high power location within the outlined region of interest on the first section.



The automated optical fractionator wizard has positioned the section at the first location and placed the counting frame at the top of the section, just below the 5 μm guard volume. You are now ready to begin counting. On the image monitor you will see the counting frame outline and the green bar on the above dialog box denotes the depth of the counting frame. The red bars, above and below the green bar, represent the guard volume. The black line marks your actual Z position within the slice. As you move the focus knob this black line will also move to let you know where you are located within the section. Adjust the focus knob and as a target comes into focus on the image monitor, use one of the **Manual Target Finding** tools (Figure 30), to mark the target on the image monitor screen. If you try and mark a target when the black line is in the red zone or part of the target is over the red solid lines in you counting frame, a beep will sound and the target will not be marked. Once you have marked all the targets, press [Next] to continue.

Figure 30: The Manual Target Finding Tools toolbox.

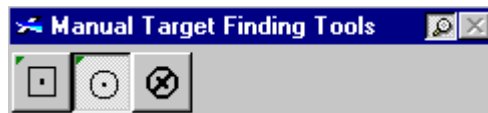
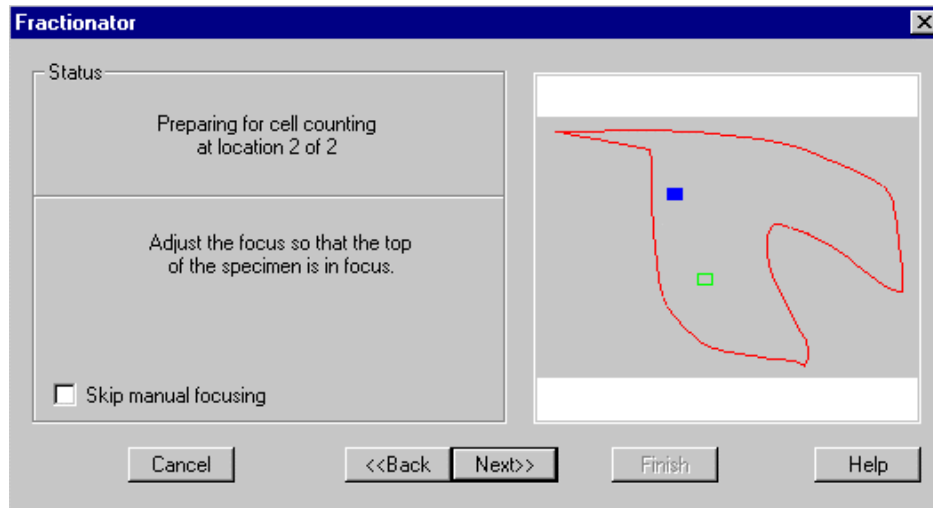
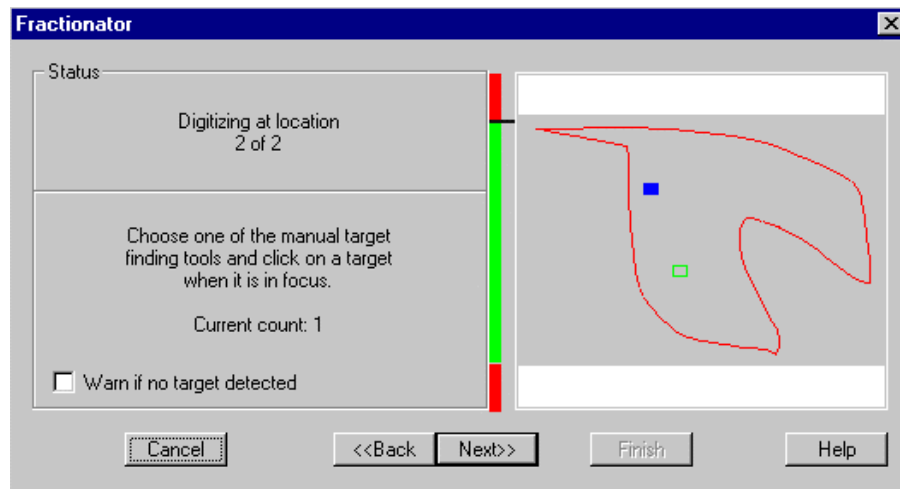


Figure 31: The fractionator wizard for adjusting the focus under the high power, for the next location. The blue box denotes the last location visited and the next location is now outlined in green.



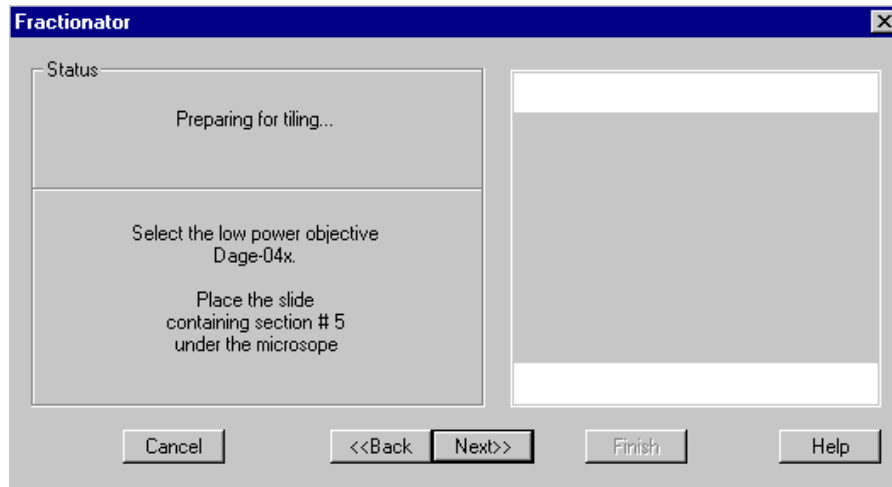
Once again you are now asked to adjust the focus for the new position so that the top of the section is just coming into focus. Once you are happy with the focus, press the [Next] button. Again, the system will automatically move down through the sample the distance you have previously setup as the guard volume, see Figure 21. Once the guard volume distance has been adjusted for, the next dialog box appears (Figure 32).

Figure 32: The target counting dialog box. The blue box denotes the previously sampled location and the box outlined in green denotes the new current location.



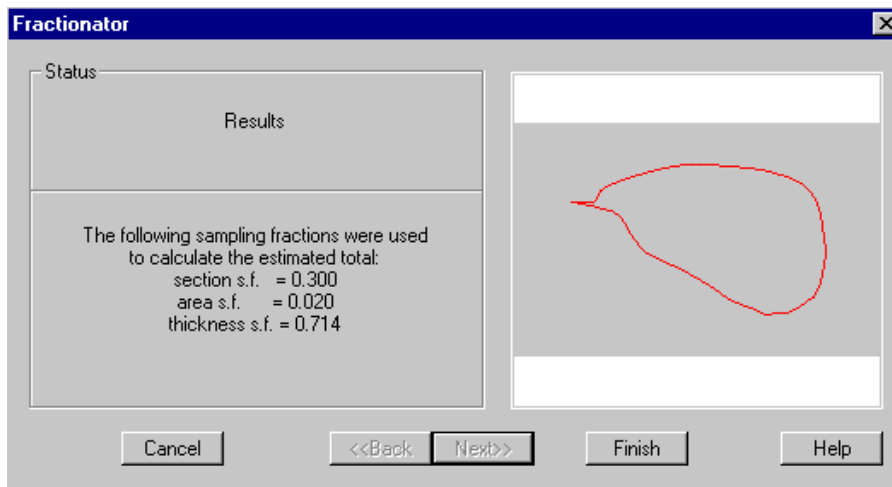
You are now ready to continue counting. Adjust the focus knob and as a target comes into focus on the image monitor, use one of the **Manual Target Finding** tools (Figure 30), to mark the target on the image monitor screen. Once you have marked all the targets press [Next], to continue. As this is the last location for this first section you will be asked to switch to the low power objective and place the next section under the microscope (Figure 33).

Figure 33: The Fractionator, Preparing for Tiling dialog box.



Pressing [Next] will proceed you through the tiling procedure for the second section. In this example you will once again be asked to outline the region of interest, then switch to the high power objective and be moved to the first location in section 2 and asked to adjust the focus for the top of that section etc. Once you have repeated the target counts for locations in section 2 the same procedure would be repeated for the final section. (Remember that in the original sequence we chose to sample three sections from a total of 10 experimental sections, see Figure 20). When the sampling has been finished the various fraction results will be displayed in the final dialog box (Figure 34).

Figure 34: The Fractionator Results dialog box showing the various fractions measured.



Pressing the [Finish] button exits the *Optical Fractionator Wizard* and you are placed back in the **Data Sample** window.

Stereology Tutorial

The following is a step-by-step guide through the **2D Frames**, **3D Probes** and **3D Disectors** data acquisition process. The optical fractionator is not included in this tutorial, as there is already a *Fractionator Wizard* to guide you through the **3D Fractionator** data acquisition process.

Before proceeding with this tutorial, you should know how to carry out the following procedures:

- retrieve and display a set of images
- work with multiple images
- use automatic target detection
- select various measures
- gather data from an image

If you are unfamiliar with any of these operations, please consult the appropriate tutorial lesson in **AIS Tutorials**, and the appropriate sections in the online *AIS Reference Manual*.

The tutorial uses a set of image files contained in the **Stereology Tutorial Data**. The Tutorial Data set is included on the **AIS** installation CD-ROM. If you have not installed the Tutorial Data, please do so now. Select the **Custom** installation option and install the Stereology Tutorial Data component. See the “Software Installation” section in the *AIS Start-Up Guide*, for complete instructions.

In the **Stereology** study type, many stereological parameters (e.g., sampling frames, test probes, intersection points, etc.) are superimposed over images in a variety of different overlay colors. The actual colors in which these features are displayed is determined by the current cursor color. In this tutorial lesson, we refer to particular features by the color they appear when the cursor color is set to red. Before beginning the tutorial, set the cursor color to red. Adhering to this overlay color scheme will help you easily identify particular stereological parameters when they are described in the tutorial.

TO SET THE CURSOR COLOR:

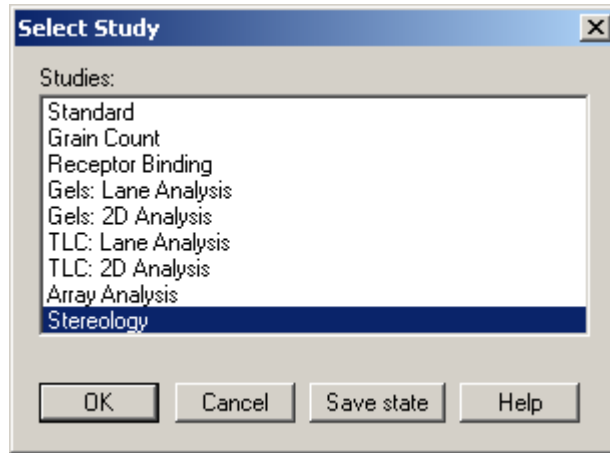
1. Move the cursor to the image monitor.
2. Press <F3> until the cursor turns red.

Selecting the Stereology Study Type

TO SELECT THE STEREOLOGY STUDY TYPE:

1. Open the *Study* menu and click on *Select*.
2. The *Select Study* dialog box containing a list of available “study types” appears on the screen (Figure 35).
3. Select *Stereology* and click [OK].

Figure 35: Select “Stereology” from the list of available study types.



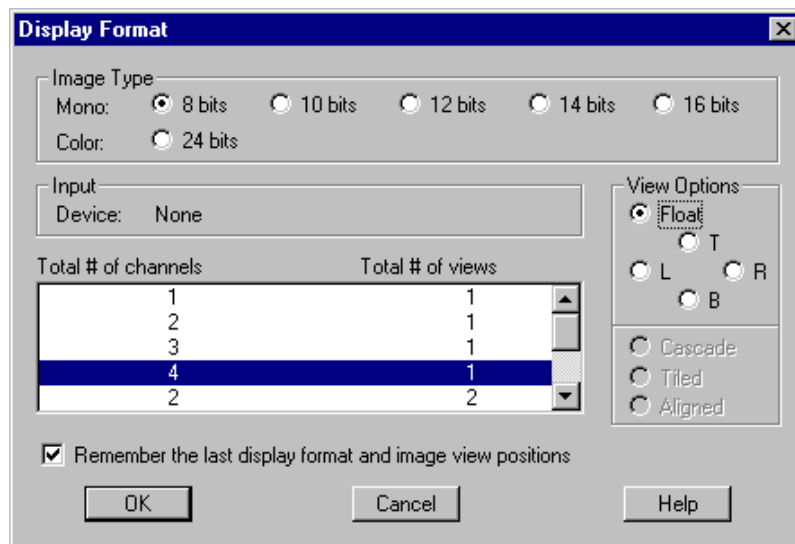
Procedure: 2D Frames and 3D Probes

This section describes the procedures used to derive **2D Frames** and **3D Probes** measures. The procedure for deriving **3D Disector** measures is slightly different, and requires a different set of sections. For these reasons, the **3D Disector** method is described in a separate section.

TO LOAD IMAGES:

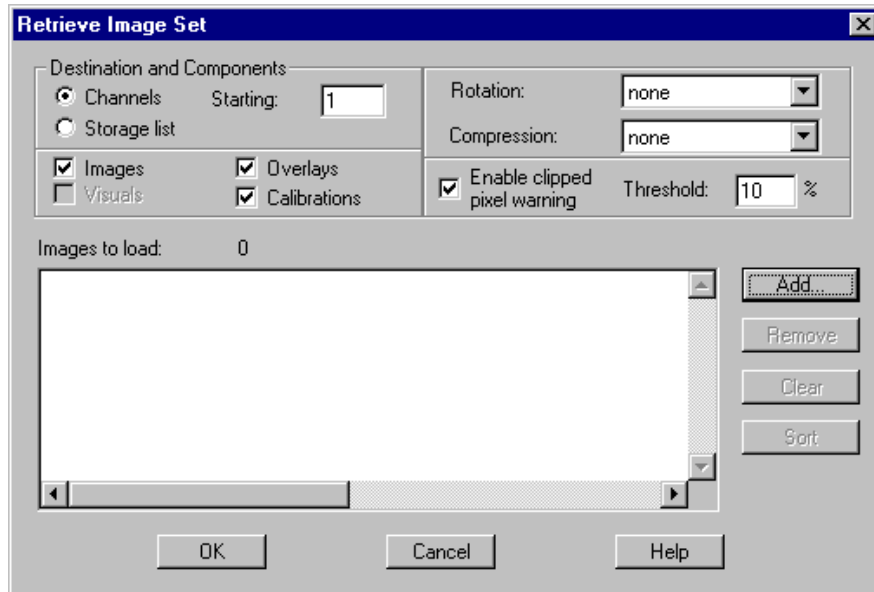
1. Open the *Settings* menu and select the *Display format* option.
2. The *Display Format* dialog box appears on the screen (Figure 36). Select **4** as the total number of channels and **1** as the total number of views.

Figure 36: The *Display Format* dialog box.



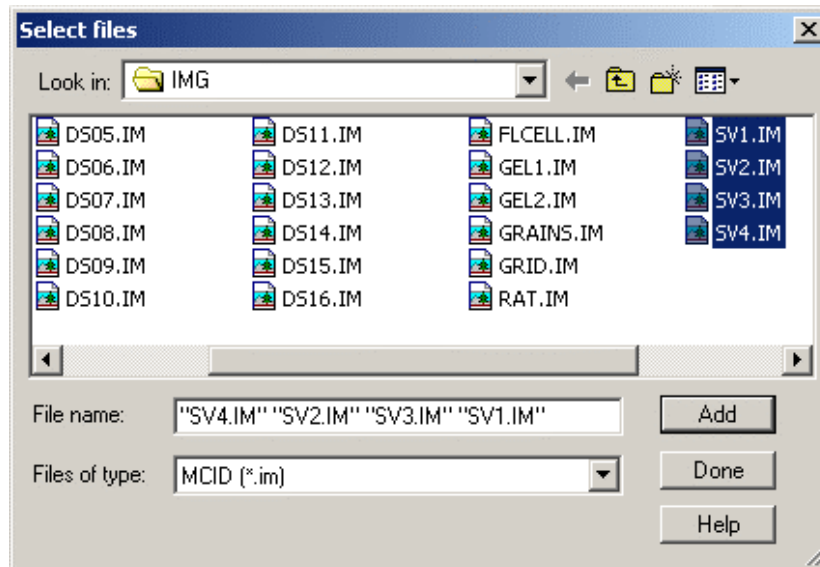
3. Open the *Image* menu and select *Image set – Retrieve*.
4. When the *Retrieve Image Set* dialog box appears (Figure 37), Press the **[Add]** button to specify which image files to load.

Figure 37: The *Retrieve Image Set* dialog box is used to load a set of image files in a single operation.



5. The *Select Files* dialog box will now appear (Figure 38). Navigate to the directory where the sample images are stored (the default directory is \AIS 6.0\IMG) and retrieve the images **SV1.IM – SV4.IM**. To select a file, first click on it then click on the **[Add]** button. The selected file appears in the list of **Images to load** section of Figure 37. There are two ways to select more than one file at a time:
 - i) hold down the **<Ctrl>** key while clicking on the filenames.
 - ii) hold down the **<Shift>** key while clicking on the first file in a consecutive list and then clicking on the last file.

Figure 38: The *Select Files* dialog box is used to select the files that comprise the image set.



6. Press **[Done]** to return to the *Retrieve Image Set* dialog box.
7. Specify retrieval options (overlay, calibrations, rotation) and **[Sort]** the images if necessary.
8. Turn on the **Channels** radio button and enter a **Starting channel** number as channel 1.
9. Click **[OK]** to load the images into consecutive channels. Images load in the order that they appear in the list.
10. The images are now displayed in Channels 1 – 4.

Selecting Measures

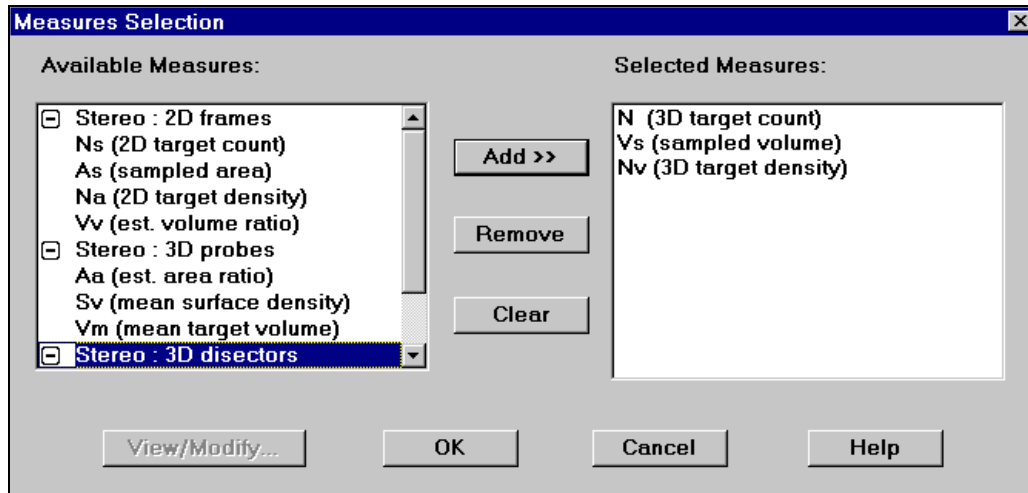
Stereological measures are grouped into four categories: **2D Frames**, **3D Probes**, **3D Disectors** and **3D Fractionators**. These measurements can be selected singly or in combination, allowing multiple stereological measurements to be carried out simultaneously. Because of the different ways in which specimens must be sectioned, however, **3D Disectors** and/or **3D Fractionator** measures cannot be combined with **2D Frames** or **3D Probes** measures.

When you select **Stereology** as your study type, the data table initially contains **3D Disectors** measures (N, Vs, Nv).

TO SELECT ADDITIONAL MEASURES:

1. Open the *Sample* menu and select *Measures selection*.
2. The *Measures Selection* dialog box appears on the screen (Figure 39).

Figure 39: The Measures Selection dialog box is used to select different stereological measures.



3. From the **Available Measures** list, open the **Stereo:2D frames** and **Stereo:3D probes** categories by clicking on the “+” button. Add every measure in these two categories to the list of **Selected Measures**.
4. Click **[OK]** to proceed. The selected measures appear as separate columns in the data table.

Stereo Grid Definition

To calculate **3D Probes** measures (S_v , mean surface volume; V_v , mean volume), linear probes are oriented around regularly spaced points in a “sampling grid.” Before the specimen is sampled, the sampling grid should be adjusted to the appropriate size. The section type must also be specified, to ensure that the appropriate algorithms and test probes are used.

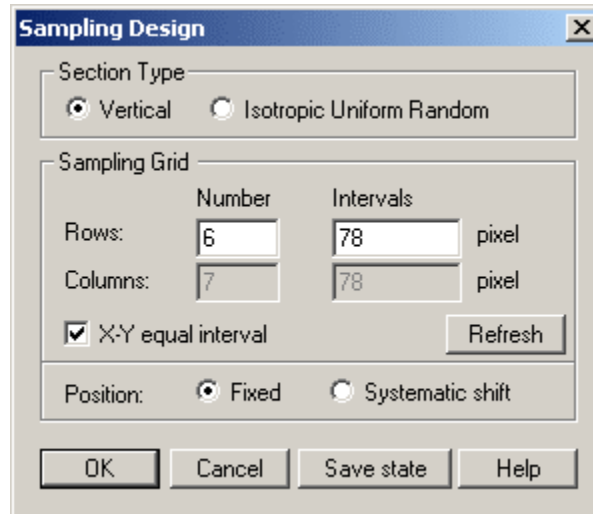
ADJUSTING THE SAMPLING GRID:

1. Click on the **Stereo Grid** icon to access the sampling grid options.



2. A sampling grid appears over the image in the current channel. The *Sampling Design* dialog box also appears (Figure 40), as well as a set of **Stereo Grid** tools. The *Sampling Design* dialog box is used to specify a section type and sampling grid size. The **Stereo Grid** tools allow you to adjust the grid with the mouse.

Figure 40: The *Sampling Design* dialog box is used to specify a section type and sampling grid size.

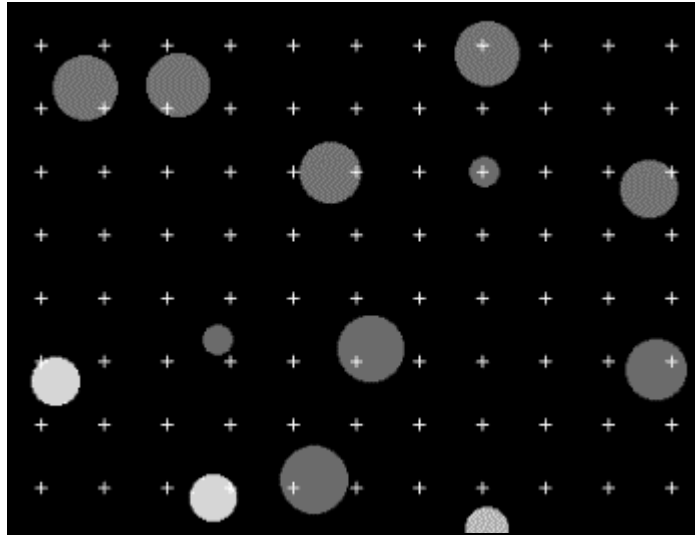


3. Under the **Section Type** heading select **Vertical**. This establishes an appropriate set of algorithms and test probes that will be used to derive the **3D Probes** measures.
4. To adjust the size of the sampling grid, select the **Size** tool from the toolbox:



5. Position the cursor over the image in Channel 1. Move the cross-hair cursor over any grid point and it becomes a pointing finger. Press the left mouse button and the grid point becomes one corner of a variable dimension box, defined by its three neighboring points. Drag the grid point to a new location and release the mouse button. The grid is redrawn using these coordinates as a reference. Note that the grid dimensions are updated in the *Sampling Design* dialog box.
6. The grid size should be chosen with reference to the size of the features of interest. The basic goal is to adjust the grid size so that none of the features of interest contain more than one grid point (as in Figure 41, below). Scroll through all of the images in the set to find the largest feature of interest. Use this feature as a reference when sizing the grid (i.e., adjust the grid size so that the feature contains only one grid point).

Figure 41: The size of the sampling grid should be adjusted so that no feature of interest contains more than one grid point.



7. The simplest way to establish the appropriate grid size is to use the **Outline** or **Autoscan B** tool to define a feature of interest. **AIS** then uses the feature's dimensions to set the grid size. For example, select the **Outline** tool from the **Stereo Grid** tool box:



8. Trace around one of the smaller target profiles with the left mouse button. When you release the mouse button, **AIS** adjusts the grid interval so that it matches the dimensions of the target profile. However, note that larger target profiles contain more than one grid point. Thus, the grid is too small for this sample. For proper grid adjustment, trace around the largest feature of interest.
9. The **Autoscan B** tool may be used in lieu of the **Outline** tool:



Place the hollow arrow cursor on a background region next to a target profile and click the left mouse button. When the cursor becomes a solid arrow, place it over a profile and click again. The target outline is shown in red and its dimensions establish the grid size for this sample. To establish an *optimal* grid size, use the tool to define the largest feature of interest in the sample. This should yield approximately 18 rows of points with an interval of about 36 μm .

10. Return to the **Stereo Grid** toolbox and select the **Shift** icon.



11. Position the cursor over the image in Channel 1. Move the cross-hair cursor over any grid point. When the cross-hair turns into a pointing finger, drag the grid point to a new XY coordinate and release the mouse button. When you release the mouse button, the grid will align itself to a new position.
12. The position of the sampling grid should be random with reference to the image. The **Shift** icon lets you randomize grid positions.
13. Return to the *Sampling Design* dialog box and click **[OK]** to proceed.

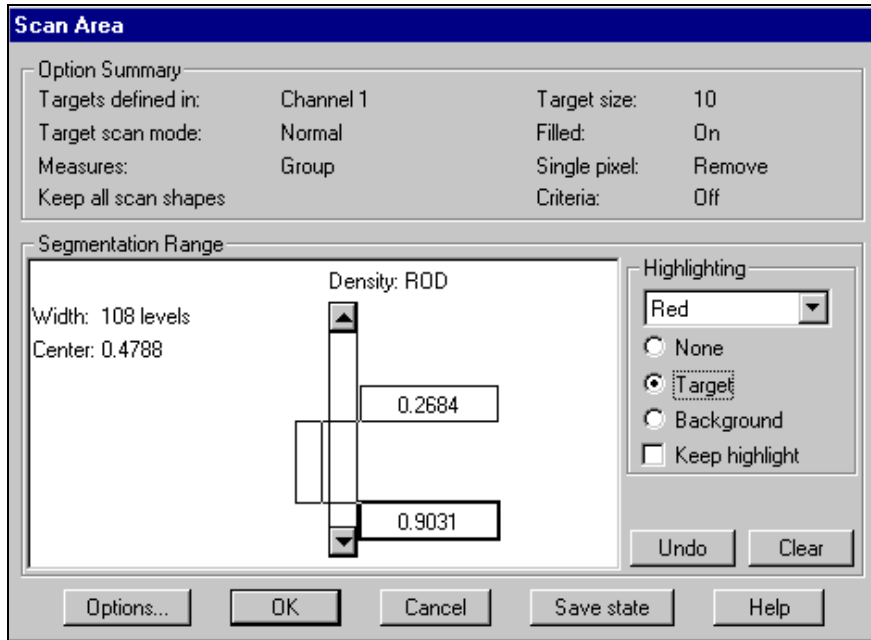
TO ESTABLISH DETECTION CRITERIA:

1. Click on the **Scan Area** icon to turn on the **AIS** automatic target detection and measurement mode:



2. The *Scan Area* dialog box appears (Figure 42). The *Scan Area* dialog box is used to define target detection criteria. In the middle of the dialog box there is a set of slider controls for setting the upper and lower target density limits. Together, these limits are called a “Segmentation Range”. The **Highlighting** box is simply a visual aid to help establish the Segmentation Range.

Figure 42: The *Scan Area* dialog box is used to define criteria used for automatic target detection and measurement.



3. Set the **Highlighting** option to **Target** and set the highlight color to **Red**.
4. To set the density criteria, place the cursor in either of the two flags on the right side of the controls and drag them up or down. The upper flag controls the brightness limit and the lower flag controls the dark limit. The gray level value of

each threshold is displayed in the flag as you drag it. Objects that fall within the range of the upper and lower density limits are filled in red (highlighted). The goal is to adjust the density criteria so that the features of interest are filled with red.

5. Move the upper flag to show about 246 Levels and the lower one to about 20 Levels. With these criteria, all of the target profiles in the sample images are highlighted in red.
6. Click on **[OK]** to exit. The target highlighting disappears when you close the dialog box.

Gathering Data

Images are sampled with a tool called a “sampling frame” or “counting frame”, a rectangular box with broken lines for its upper and right boundaries (inclusion lines) and solid lines for its left and lower boundaries (exclusion lines). Data are derived from target profiles that fall completely within its boundaries plus any target, which touches the inclusion lines. Targets that touch the exclusion lines are not included in the sample.

TO START GATHERING DATA:

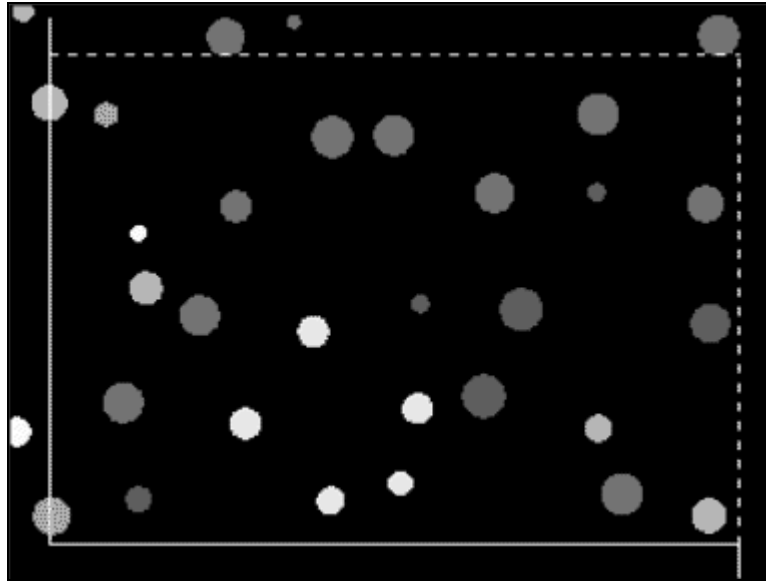
1. Open the **Stereo Sample** tool box and select the **Continuous frame** tool:



2. Move the cursor to the image in Channel 1. The goal is to size the sampling frame to select as many target profiles as possible, while making sure that the broken sides of the frame (inclusion lines) avoid targets that intersect the edges of the image (see Figure 43). To adjust the size of the frame, move the mouse while holding down the **<Ctrl>** key.
3. Press the left mouse button to sample the image. **AIS** scans the sampling frame (and the region adjacent to the inclusion lines) for targets that fall within the segmentation range and gathers data from them. To be included in the sample, a target profile must:
 - i) Satisfy the detection criteria (i.e., fall within the established density segmentation range)
 - ii) Fall within the boundaries of the sampling frame, or
 - iii) Touch an inclusion line

On the image, sampled target profiles are outlined in red.

Figure 43: The sampling frame should be adjusted to include as many profiles as possible. However, make sure that the inclusion lines (broken lines) do not touch targets that also touch the edge of the image.



4. Repeat the sampling procedure for the remaining images in the series. Later, we'll use the auto-sampling feature to sample an entire image set in one operation.

Reporting Data

Data are reported in the data table as each image is sampled. Each line in the table represents data from a single sample. Note that **AIS** automatically uses the image filenames as Section labels. Each column in the data table represents a measure that was selected at the start of the tutorial. Data are defined as follows, in units that reflect the image calibrations.

Ns Number of target profiles sampled

As Area sampled (μm^2)

Na Number of target profiles per μm^2 (abbreviated as μm^{-2})

Vv Ratio of target volume to sample volume.

Aa Ratio of target intercepts to total intercepts.

Sv Mean surface area per μm^3 (abbreviated as μm^{-1})

Vm Mean target volume (μm^3)

Note: Since the images are calibrated in microns, Na is very small. The value of Na would be much larger (10^6 times greater) if it was expressed per mm^2 (i.e., if

the images were calibrated in millimeters instead of microns). However, V_m would then be very small.

Data are also displayed in graphical form. The graph area displays the incremental calculation of any single measure in the data table, plotted against the parameter that is used to calculate it. S_v , for example, is plotted as a function of the number of test probes used in its calculation. It is a useful way to monitor the reliability of particular measures as sampling progresses. To select the measure to plot in the graph area, click on its column heading in the data table.

Editing the Data

Automated target detection rarely works as perfectly as it does with these specially constructed sample images. Inclusion and exclusion errors are bound to occur with real tissue specimens, no matter how carefully you define the target detection criteria. Some post-sampling editing of data is usually necessary to overcome deficiencies in the automated target detection process.

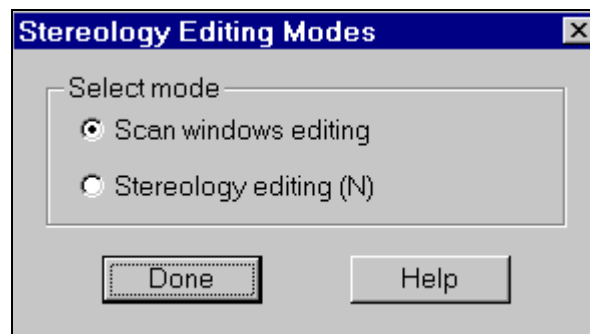
AIS provides two ways to edit stereological measures. One way is to add or delete targets in the sampled image(s). The other way is to edit the stereological probes upon which particular measures (i.e., S_v , V_m) are based.

TO ADD OR DELETE TARGETS IN A SAMPLED IMAGE:

1. Open the *Edit* menu and select the *Stereo editing tool* option (or press <F8>).
2. Select **Scan windows** editing as the editing mode (Figure 44).
3. The **Stereo Sample** toolbox becomes the **Scan Editing** toolbox, which contains tools for adding or deleting targets. Select the **Delete** tool:



Figure 44: AIS provides two editing modes for stereological data. Select “Scan windows editing” to add or delete targets in sampled images. Select “Stereology editing” to edit the probes upon which a measure is based.



4. Move the cursor over the image. The arrow becomes an inverted “V”. Click the cursor on any sampled (i.e., red) target profile and the target fill color disappears.

Data that correspond to the sample are updated in the data table. Delete a few more profiles.

5. Remaining tools are used to add targets to the sample. These tools operate just like sample tools. Select one of the **Autoscan** tools and use it to add one of the targets you deleted in Step 4. To add it, click on background, then click on the target profile. Note again that the data table is automatically updated each time a target is added to the sample.
6. Click **[Done]** to exit **Scan windows** editing.

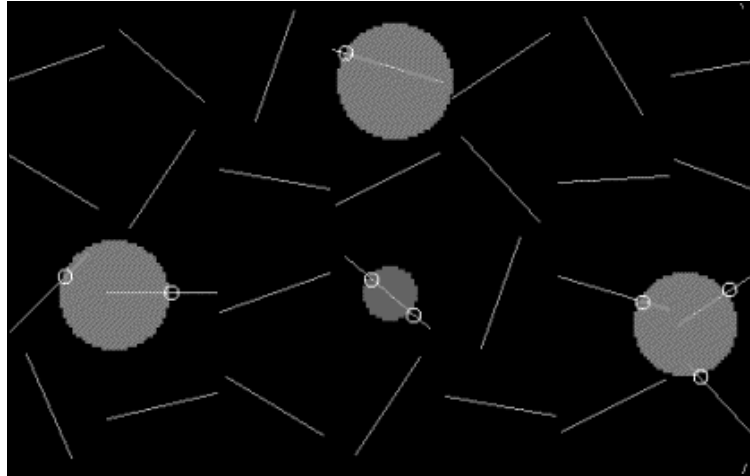
TO EDIT SPECIFIC STEREOLOGICAL PROBES:

1. First select the measure you wish to edit by clicking on its column heading in the data table. Click on the **Sv** column heading. On the images, **AIS** displays the probes and intersection points that were used to derive the Sv measure.
2. Open the *Edit* menu and select the *Stereo editing tool* option (or press <F8>).
3. Select **Stereology editing** as the editing mode (Figure 44).
4. Select the **Surface probe** tool from the **Stereo Editing** tool box:



5. The **Surface probe** tool is used to edit the mean surface density measure (**Sv**), by adding or deleting intersections between test probes and target profile boundaries. On the sampled images, intersections are shown as small green circles (Figure 45).
6. To delete an intersection point, click on it with the right mouse button. Click the left mouse button to add a new intersection point. Data are updated accordingly as each probe is edited.
7. Click **[Done]** to exit editing mode, or press <F8>.

Figure 45: A sampled image showing the probes and intersection points used to estimate the mean surface density measure (S_v).



Compiling Data

AIS provides two summary tables for displaying stereological data:



Target table



Stereology table

Only one of the above summary icons is visible while you are in the **Sample** window (labeled as **Select Table**, in the top right corner of the **Sample** window).

Data is currently displayed in the **Target table**, which contains data from each individual sample. In order to generate measures that are representative of the entire specimen, we must pool data obtained from individual samples (i.e., across all sections).

TO COMPILE DATA ACROSS ALL SECTIONS:

1. Click on the **Select table** icon in the top right corner of the **Sample** window.
2. Select the **Stereology table**.

AIS computes and displays the appropriate summary value (mean, total) for all measures. The table also summarizes the appropriate stereological parameters upon which the measure is based (e.g., # frames, # intercepts).

The data can be printed, saved to disk, or exported to other programs in text or spreadsheet format.

Before proceeding to the next tutorial section, please return to the “Target table” and press the [Clear] button.

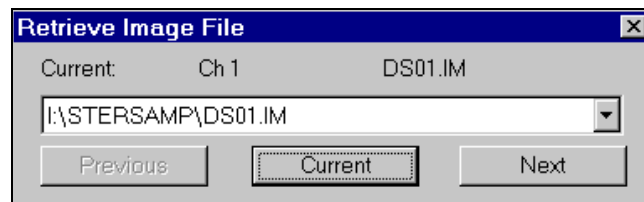
Procedure: 3D Disectors

This section describes the procedures used to derive **3D Disector** measures. Unlike **2D Frames** and **3D Probes** measures, **3D Disector** measures are derived from serially adjacent sections, separated by a known distance and perfectly registered (aligned) with each other.

TO LOAD IMAGES:

1. Press <**Shift+F5**> to clear all of the displayed images. Press <**Shift+F4**> to clear all overlays.
2. Open the *Image* menu and select *Image set > Retrieve*.
3. When the *Retrieve Image Set* dialog box appears (see Figure 37), navigate to the directory where the sample images are stored (the default directory is \AIS 6.0\IMG) and retrieve the images **DS01.IM – DS16.IM**. Retrieve the images into a **Storage list** with their calibrations.
4. Click [**OK**] to proceed.
5. A small *Retrieve Image File* dialog box appears on the screen (Figure 46). Press the [**Current**] button to display the first image file (DS01.IM) in the current channel. Use the [**Previous**] and [**Next**] buttons to navigate through the image set as required.

Figure 46: The image storage list is used to load an image set into the current channel, one file at a time.



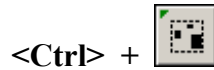
TO SELECT MEASURES:

1. Open the *Sample* menu and select *Measures selection*. The *Measures Selection* dialog box appears on the screen (see Figure 39).
2. Press the [**Clear**] button to remove the previous **2D Frames** and **3D Probes** measures from the list of **Selected Measures**.
3. Open the **Stereo: 3D Disector** group of measures and add every measure to the list of **Selected Measures**.
4. Click [**OK**] to proceed. The selected measures (N, N_v, and V_s) appear as separate columns in the data table.

TO ESTABLISH DETECTION CRITERIA:

1. The segmentation range that was established for the **SV1.IM – SV4.IM** image set is sufficient for the current image set. However, if you wish to establish other

detection criteria, <Ctrl> - click on the **Scan Area** icon to re-display the *Scan Area* dialog box:



2. Click on [OK] to exit.

Gathering Data

These images are also sampled with a “sampling frame”. Data are derived from target profiles that fall completely within its boundaries plus any target, which touches the inclusion lines. Targets that touch the exclusion lines are not included in the sample.

In this example, however, we will use another kind of sampling frame. Given the size of the image set (16 image files), we will also use AIS’s auto-sampling feature to sample the entire set in one operation. Either of these features ensures that the same 2D coordinates are sampled in every image.

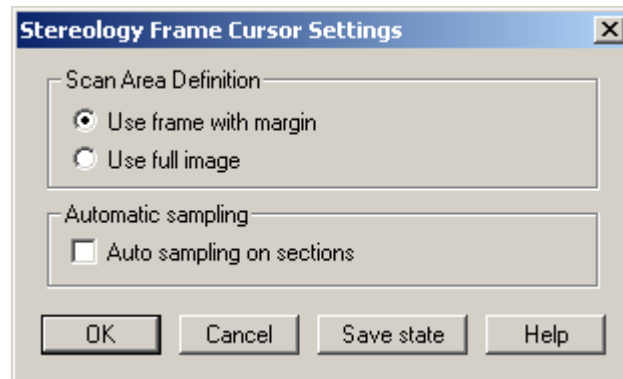
TO GATHER DATA:

1. Open the **Stereo Sample** tool box and double-click on the **Channel frame** tool:



2. When the *Stereology Frame Cursor Settings* dialog box appears (Figure 47), turn on the **Auto sampling on sections** option.
3. Click [OK] to proceed.

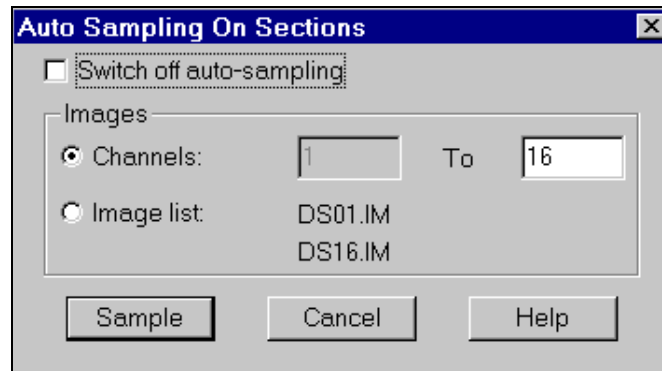
Figure 47: The Frame Cursor Settings dialog box is used enable automatic sampling across an entire image set.



4. Select the **Channel frame** tool and move the cursor to the first image. Again, the goal is to size the sampling frame to select as many target profiles as possible, while making sure that the broken sides of the frame (inclusion lines) avoid targets that intersect the edges of the image (see Figure 43).

5. Unlike the **Continuous frame** tool, the **Channel frame** is locked into a fixed position within a channel. To adjust the initial size of the frame, move the mouse while holding down the <Ctrl> key. To position the frame, move the mouse while holding down the <Shift> key.
6. Click the sampling frame in the first image in the series. When the *Auto Sampling On Sections* dialog box appears (Figure 48), select the **Image list** option.

Figure 48: The Auto Sampling On Sections dialog box.



7. Click the [**Sample**] button to begin sampling the image set. **AIS** generates the appropriate test probes (i.e., disectors), scans each image for targets that meet the detection criteria, and reports the results in the data table.
8. On the images, target profiles that fall within the segmentation range are filled with red overlay. To be counted as a 3D target, a target profile must be absent from the preceding section (the “lookup” section). Profiles that are absent from the lookup section (i.e., new 3D target tops) are outlined in green.
9. Data are reported in the data table as each image is sampled. Each line in the table represents data from a single sample and is designated by the image filename. Since the first image in the series has no corresponding “lookup” section, no data are displayed for this image.
10. Each column in the data table represents a measure that was selected at the start of the tutorial. Data are defined as follows, in units that reflect the image calibrations.
 - N** Number of 3D targets contained in the sample
 - Nv** Number of 3D targets per μm^3 (abbreviated as μm^{-3})
 - Vs** Volume sampled (μm^3)
11. Data are also displayed in graphical form. To select the measure to plot in the graph area, click on its column heading in the data table.

Editing the Data

To overcome deficiencies in the automated target detection process, **AIS** provides two ways to edit **3D Disector** measures. One way is to add or delete 2D target profiles in the sampled image(s). The other way is to add or delete 3D target tops.

TO ADD OR DELETE 2D TARGET PROFILES IN A SAMPLED IMAGE:

1. Select the image you wish to edit by highlighting the corresponding line of data in the data table.
2. Open the *Edit* menu and select the *Stereo editing tool* option (or press <F8>).
3. Select **Scan windows editing** as the editing mode (see Figure 44).
4. Select the **Delete** tool from the **Scan Editing** toolbox:



5. Move the cursor over the image. The arrow becomes an inverted “V”. Click the cursor on any 2D target profile and the target fill color disappears. Data that correspond to the sample are updated in the data table where applicable. Delete a few more profiles.
6. The remaining tools are used to add targets to the sample. These tools operate just like sample tools. Select one of the **Autoscan** tools and use it to add one of the targets you deleted in Step 5. To add it, click on background, then click on the target profile. Note again that the data table is automatically updated where applicable.
7. Click [**Done**] to exit **Scan windows** editing.

TO ADD OR DELETE 3D TARGETS:

1. First select the measure you wish to edit by clicking on its column heading in the data table. Click on the **N** or **Nv** column heading. Select the image you wish to edit by highlighting the corresponding line of data in the data table.
2. Open the *Edit* menu and select the *Stereo editing tool* option (or press <F8>).
3. Select **Stereology editing** as the editing mode (see Figure 44).
4. Select the **Profile pointer** tool from the **Stereo Editing** tool box:



On sampled images (other than the first image), 2D target profiles are displayed in red. Profiles that were not present in the preceding section (i.e., the tops of new 3D targets) are outlined in green. To delete a 3D target from the sample, click on it with the right mouse button. It then becomes a 2D target profile. To add a 2D target profile to the sample, click on it with the left mouse button. Data (**N** and **Nv**) are updated accordingly.

5. Press [**Done**] to exit editing mode, or press <**F8**>.

Compiling Data

Data is currently displayed in the **Target table**, which contains data from each individual sample. In order to generate measures that are representative of the entire specimen, we must pool data obtained from individual samples (i.e., across all sections).

TO COMPILE DATA ACROSS ALL SECTIONS:

1. Click on the **Select table** icon in the top right corner of the **Data Sample** window:



2. Select the **Stereology table**:



AIS computes and displays the appropriate summary value (mean, total) for all measures.

The data can be printed, saved to disk, or exported to other programs in text, Lotus[®] 1-2-3, or Microsoft[®] Excel format.

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