



Version 6.0

Application Guide: Grain Counting

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Grain Counting

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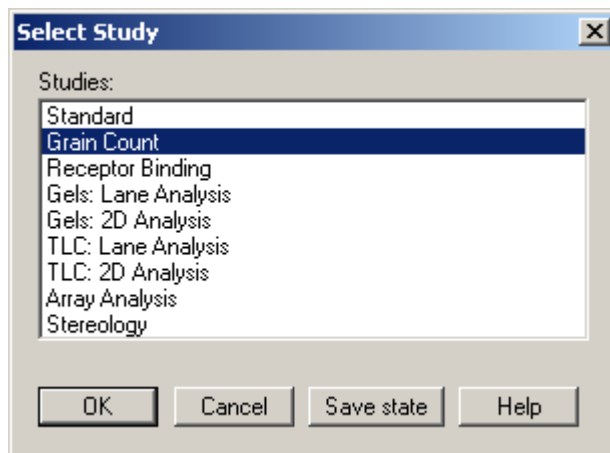
Basics

The **Grain Count** study type is a specialized version of the **AIS** imaging system's automatic target detection feature. It is particularly well-suited to grain and to terminal distributions analyses. Targets (e.g., grains, fluorescent particles) are first defined by their optical density and their spatial characteristics. Sample tools are then used to define a region of interest (e.g., a cell). Finally, **AIS** scans the region of interest for valid targets and reports the target data in the data table.

Selecting the **Grain Count** study type (Figure 1) activates the **AIS** automatic target detection and measurement feature (i.e., **Scan Area**). The following column headings appear in the data table:

- **Scan Area** -- Area of the region of interest
- **Mean Grain Area** -- Mean area of the grains contained in the region of interest.
- **Total Grain Area** -- Total area of grains contained in the region of interest.
- **Proportional Area** -- Proportion of the region of interest that is occupied by grains.
- **Count** -- Number of grains contained in the region of interest.


Figure 1: To enable the Grain Counting study type, select Study > Select.



Establishing Target Criteria

“Target criteria” separate image components into valid targets and background. Target acceptance criteria can be any combination of density, size, and shape.

The *Scan Area* dialog box (Figure 2) is used to define target criteria. Click on the [**Scan Area**] icon while holding down the <Ctrl> key to open the dialog box.

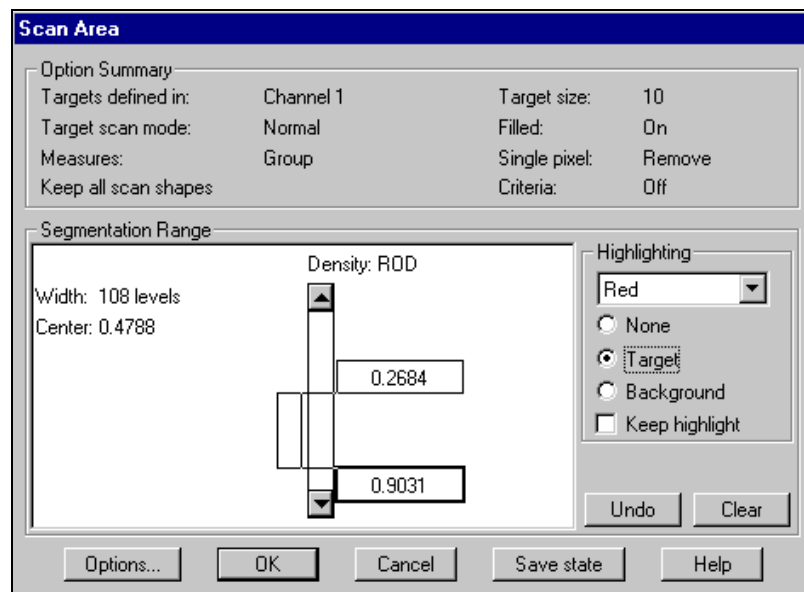
<Ctrl> + 

Use the slider controls in the middle of the dialog box to establish a **Segmentation Range**, between the upper and the lower density limits of your grains. The **Highlighting** feature is a visual aid that helps establish the segmentation range. As an option, you can show targets that fit within the segmentation range in a highlight color. The **Option Summary** displays the current status of various optional features.

Density Criteria

Density thresholds are the simplest criteria for target detection. AIS is given an upper and a lower density threshold (called a **Segmentation Range**). Pixels lying within the segmentation range are regarded as valid targets; pixels lying outside of the range are ignored. In Figure 2, pixels between 0.2684 and 0.9031 ROD are acceptable targets; pixels with ROD values lower than 0.2684 or higher than 0.9031 are ignored.

Figure 2: The Scan Area dialog box is used to define target criteria and to access a variety of target detection options and features.



Set the upper and lower density thresholds with the slider controls. The goal is to establish a **Segmentation Range** that distinguishes grains from background. However, there is a trade-off in setting thresholds.

A range that is too narrow may yield clean targets, but it may also incorrectly define genuine target pixels as background. On the other hand, a range that is too broad may capture all of the genuine targets but it may also include areas of background in the target range and leave halos of background pixels around each target. While determining the segmentation range, it is useful to set the **Highlighting** feature to **Targets** (see below).

You can manipulate the threshold to detect either dark or light targets. AIS is equally capable of detecting “blobs” or “holes”. Blob targets are dark against a light background (e.g., grains) while holes are light against a dark background (e.g., fluorescent terminals).

Highlighting

Targets should be shown in some easily perceived color scheme so that they can be discriminated from background while the segmentation range is being established. Targets may be displayed in their normal appearance against a background filled with any overlay color or alternatively they may be filled with an overlay color against a normal background. In either case, use the drop-down color selection menu in the **[Highlighting]** box to set the desired color.

The **Highlighting** options are:

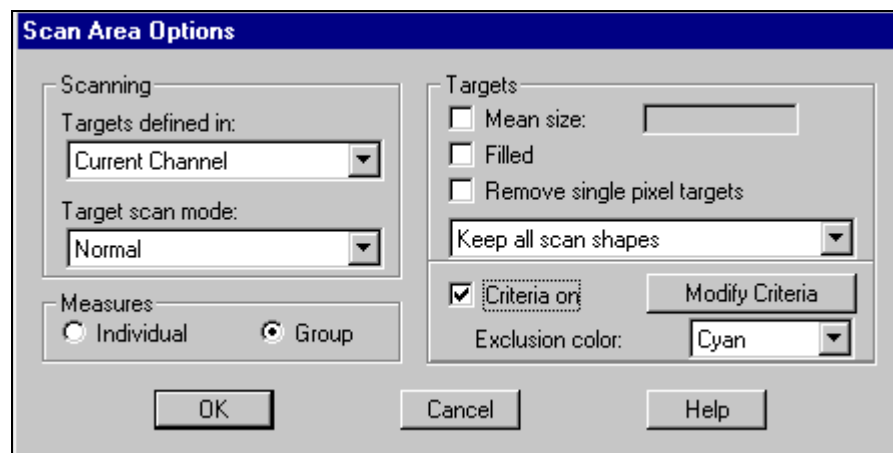
- **None** – Targets and background are shown without alteration.
- **Target** – Color-filled targets are shown against a normal background (the default mode).
- **Background** – Targets are shown in their normal gray levels against a uniform color-filled background.
- **Keep highlight** – You usually want to see targets filled with color during the threshold adjustment only. Once you establish the **Segmentation range**, you can remove the color fill and work with the images. Color fill only re-appears when targets are detected during the automated scan. If you prefer to see targets filled with color at all times, enable the **Keep highlight** checkbox.

Spatial Criteria

A threshold operation rarely succeeds in eliminating all invalid targets from the scan. There are usually bits of background or other image data that have densities similar to those of genuine targets. In a scan based solely on density thresholds, these artifacts become included as valid targets. To prevent this, **AIS** lets you use logical target acceptance criteria as well. Logical criteria include minimum and maximum area, minimum roundness, minimum and maximum chord, and other spatial criteria.

Spatial criteria are defined by pressing the **[Options]** button, which calls up the *Scan Area Options* dialog box.

Figure 3: The *Scan Area Options* dialog box.



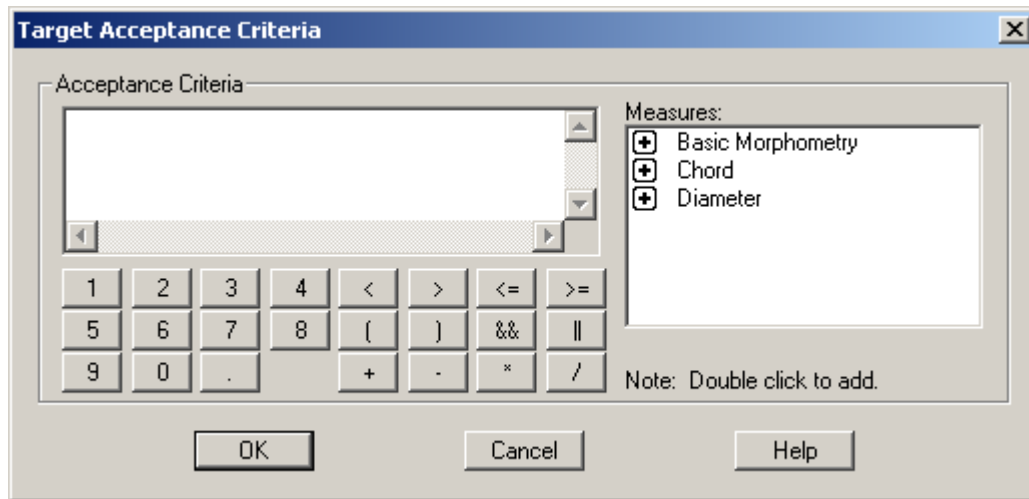
Select **Criteria on** and press [**Modify Criteria**] to display the *Target Acceptance Criteria* dialog box (Figure 4).

This dialog box looks and works much like a calculator. On the right is a list of the spatial measurements that you can use as criteria. The measurements are grouped into categories.

To avoid counting small bits of background detritus as grains, construct a logical criterion that states that a valid target must have an area greater than 2 pixels. If you are working with an uncalibrated image, spatial units are expressed in pixels. Open the **Basic Morphometry** category and select the **Area** measure. Use the **Area** measure and the buttons to construct the statement, “**Area > 2**” (double click on the **Area** term to add it to the statement).

Note, however, that numerical values must be expressed in units that correspond to your current spatial calibration.

Figure 4: The Target Acceptance Criteria dialog box is used to define logical target criteria.



Collecting Data

You can use any sample tool to define a region of interest. Automatic target detection can occur within a box, a circle, a hand-drawn outline, or within regions defined with the **Autoscan** tool. Regions of interest may consist of individual cells, entire fields of view, or entire images.

AIS first scans the region of interest for targets that fall within the segmentation range. If you established logical acceptance criteria as well, **AIS** scans the region a second time and searches for targets that fulfill these criteria. By default, valid targets (i.e., those that meet the density *and* the spatial criteria) are displayed in red and excluded targets (i.e., those that met the density but not the spatial criteria) are displayed in cyan. Only data from valid targets are reported in the data table.

Whole Field Tools



Full-image scan tool



Image view scan tool

Both of the **Whole Field** tools are used to scan large fields. The **Full-image scan** tool scans all of the image, no matter what part of the image appears in the Image View window. The **Image View scan** tool scans only the part of the image that is displayed in the Image View window. To use either tool, first select it and then place the cursor anywhere within the Image View and click the left mouse button.

Editing the Scan

Automated scanning rarely works perfectly. No matter how carefully you define your target criteria, there are bound to be targets that are missed or targets that should or should not have been included. Post-scan data editing is usually necessary. Please note, you can only edit data derived from the last scan.

Open the *Edit* menu and select the **Scan editing tools** option. The **Sample** toolbox becomes the **Scan Edit** toolbox, which contains tools for adding and deleting targets.

Deleting Targets

Select the **Target Delete** tool to delete a target from the scan area. When you click the **Target Delete** tool on any valid target (by default, colored red), the target overlay color disappears and data that correspond to the deleted target are deleted from the table.

Adding Targets

The remaining scan edit tools operate just like sample tools, but instead of defining new regions of interest they add targets to the current region of interest. The data table is updated automatically each time a target is added to the scan.

To add an *excluded* target (by default, colored cyan) to the scan, you must first erase its overlay color. Select the **Overlay eraser** from the toolbox and drag the eraser over an excluded target. Then select another editing tool to add the target to the scan.

To turn off the **Scan editing tools**, return to the *Edit* menu and select the **Scan editing tools** option again, or press <F8>.

The “Keep only last scan shapes” Option

Automatic target detection and measurement is an extremely memory-intensive operation. To avoid making unreasonable demands on system memory, **AIS** is configured so that it only stores the target position and outlines from the most recently scanned region of interest. This means that you can only edit the last scan.

If you wish to override this feature for data auditing purposes, change the **Keep only last scan shapes** option in the *Scan Area Options* dialog box to **Keep all scan shapes**. If you are scanning many targets, the storage requirements become large and you will notice a

drop in system performance. The **Keep no scan shapes** option uses the least amount of system memory, but you cannot edit the scan.

Advanced Details

Maximizing Grain Visibility

When establishing a density segmentation range, it is sometimes difficult to separate grains lying over cells from the cells themselves. There are a number of techniques for maximizing grain visibility.

To make grains more distinguishable from cellular material, you can apply the **Target Accentuate** filter to the image. Although this filter reduces the contrast of all image components, larger targets lose more contrast than smaller targets. Because the counterstain loses more contrast than grains, the grains become more readily detectable. However, filters cannot discriminate between grains and stained granular material, nor can they make grains visible over a very heavily stained cell. To apply the **Target Accentuate** filter, press **<F12>**.

You can enhance grain visibility by applying various optical “tricks” during the image acquisition phase of the study. When combined with re-directed sampling (see below), these techniques improve the accuracy of grain detection.

One trick is to create two images at different focal planes. First, using an objective with small depth of field, focus carefully on the emulsion layer and create an image of the grains at this plane of focus. Then, focus on the cell layer to create an image in which anatomical detail is sharp but grains are not well focused. Process the first image to show maximum grain visibility and then use re-directed sampling to define anatomy on the cell image while counting grains from the emulsion image.

A better option is to use polarized incident light microscopy (DeMey, 1983; Hoefsmit et al., 1986; Ellis, Bell, & Bancroft, 1989). In this procedure, specimens are viewed using an epifluorescence microscope equipped with an immunogold dichroic mirror and polarization filters. Under polarized illumination, metallic grains (including gold and silver compounds) shine brightly. These metallic grains cannot be confused with cellular granules, which lack metal and do not shine. In addition, the epifluorescence illumination comes from the top, hitting the emulsion layer before it enters the stained material. For this reason, the stain has little effect on the illumination of grains.

To use the polarized illumination method, create an image of the grains under epifluorescence. Process this image with the [**Target accentuate**] filter. Then, turn on the substage illumination and create a second image of the cells. Now, use re-directed sampling and define anatomical detail from the cells and count grains from the epifluorescence image. In our experience, this method yields very accurate counts and proportional areas.

Re-directed Sampling With Two Images

One of the most difficult aspects of grain counting is obtaining a *single* image in which grains and cells are both visible and also clearly distinguishable from each other by their respective optical densities. Unfortunately, techniques that make grains more easily detectable often make it more difficult to see individual cells.

You can overcome this problem by using two images from the same field of view -- one in which grains are clearly visible and another in which cells are clearly visible. The “grains” image may be a processed image, an image acquired at a different focal plane, or an image acquired with polarized incident illumination. The “grains” and the “cell” images are each loaded into separate channels; **AIS** uses the “cells” image to define scan areas while counting grains from the “grains” image. We call this two-channel form of data gathering “re-directed sampling”.

TO PERFORM RE-DIRECTED SAMPLING:

1. Place the “grains” image into Channel 1 and the “cells” image into Channel 2.
2. Open the *Scan Area* dialog box (press <Ctrl> + **Scan Area** icon) and press the **[Options]** button to access *Scan Area Options*.
3. Change the **Targets defined in** option from **Current Channel** to **Ch 1**. This “locks” any target criteria that you establish to the image in Channel 1 (i.e., the “grains” image).
4. Establish a segmentation range for grains. If necessary, establish a spatial criterion and a mean target size.
5. Use any sample tool to define a region of interest in Channel 2 (i.e., the “cells” image). **AIS** scans the same region of interest in Channel 1. Data from valid targets contained in Channel 1 are reported in the data table and outlines of valid targets (highlighted in red) are pasted onto the image in Channel 2.
6. If necessary, edit the scan using the image in Channel 2.


Counting ‘Clumps’ of Grains


Grains often appear in “clumps” of two or more. If adjacent grains touch at their edges, **AIS** has difficulty determining whether the clump is a single, large target or many smaller ones. You can overcome this problem by specifying a **Mean size** for targets. **AIS** will then use the **Mean size** to estimate the number of targets contained in clumps.


To set a mean target size, open the *Scan Area* dialog box (press <Ctrl> + **Scan Area** icon) and press the **[Options]** button to access the *Scan Area Options* dialog box. Click on the **Mean size** checkbox and enter the area of a single grain. When **AIS** encounters a large target, it now assumes that this is a clump and uses the specified area to estimate the number of individual targets in the clump. An “Estimated count” column appears in the data display.


Avoiding Solitary Pixels

A valid target usually includes a large clump of pixels whose density values fall within a specified segmentation range *and* whose size falls within a specified range. If you did not specify size criteria, **AIS** may count single pixels as individual targets. For example, a single pixel is counted if it falls within the density segmentation range but adjacent pixels do not. Pixels that are connected diagonally are also counted as distinct targets. The following diagrams summarize **AIS'** counting mechanisms. Pixels that fall within the density segmentation range are shown in black:

 = 1 target

 = 1 target

 = 2 targets

 = 3 targets

Single pixels affect the distribution data (i.e., counts) and mean spatial measurements. If sampling single pixels becomes a problem with your particular application, and you are reasonably certain that an individual grain *is* larger than a single pixel, click on the **[Remove single pixel targets]** checkbox in the *Scan Area Options* dialog box. **AIS** then ignores solitary, unconnected pixels.

Re-using Scan Areas

You may wish to count grains within cells and then count grains within equal areas of background. The simplest way to do this is to first outline a cell and then, before scanning it for grains, make that cell into a template. To do this, select the **Template** tool, place it within the cell outline, and click. You can now position this outline as many times as you wish and read grains within cell and background.

To use a new outline as a template, select the **Outline** tool and trace. Then, press <Ctrl> while clicking the **Template** tool icon. Click the **Template** tool within the new outline. This process of defining templates and then using them for automated scans is tedious. You should avoid it unless it is essential to scan equal areas for comparison.

Counting versus Proportional Area

Machines are very good at estimating area coverage of clearly labeled material. If you are dealing with small, uniform targets such as grains, it may be possible to avoid counting with all of its attendant complexities. Instead, you can measure the proportional area covered by targets.

Proportional area measurements cannot tell you how many targets are present within your tissues but can tell you the area (and based on the area, the volume) occupied by those targets. You must decide whether counts are critical to testing your hypothesis or if proportional area can be substituted instead.

Before you substitute target proportional area measurement for grain counting there are two crucial determinations that you need to make. Both the mean area of all of the discrete targets and the areas scanned for those targets (scan area) must be independent of (i.e., unaffected by) the experimental treatments.

If either mean discrete target area or scan area is affected by treatment, the correlation between proportional area and count will be confounded with treatment condition. That is, any observed differences among the experimental treatments may actually reflect artifactual differences in the mean target area and/or the scan area rather than genuine differences in grain counts. Similarly, any genuine differences in grain counts may be obscured by artifactual differences in the mean target area and/or the scan area. In either case, unbiased counts would be required.

To determine if proportional area and treatment conditions are independent, measure the mean area of all of the discrete targets and the scan area (e.g. discrete cell boundary) across your conditions. Such measurements are easily and quickly performed. If you fail to observe important differences in either measure across experimental treatments, a proportional area measurement may be in order.

What constitutes an important difference can vary across disciplines and is ultimately your decision. Some methodologists have suggested that a liberal statistical probability decision level between .10 and .25 (instead of the more usual .05 or .01) may be preferred in these types of cases (Hays, 1994). That is, it may be preferable to make it relatively easy to *reject* the hypothesis of *no differences* among the treatments (what statisticians call the “null hypothesis”).

This is so because researchers generally want to avoid the chance that a particular analysis is invalid. In the present case, rejecting the null hypothesis would mean that differences among the treatments in mean discrete target area or scan area would call into question the validity of using proportional area as an alternative to the grain count procedure. Consult someone knowledgeable in statistics if you have concerns about these issues.

Other Useful Measures

AIS can also be configured to express a “Count Density” (i.e., number of grains per unit area). The Count Density for any given target is derived by simply dividing the “Count” measure by the “Scan Area” measure. To enable the Count Density measure, select *Sample > Measure selection* and select **Count Density** from the **Scan Area** group of measures. An “Estimated Count Density” measure is also available (**Est. Count Density**) if you have specified a mean grain size to compensate for ‘clumping’.

Please note, given the size of a single silver grain, the Count Density measure requires a spatial calibration (distance or area) in relatively large units. Otherwise, the resulting Count Density values will be far too small to be of any real use.

References

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