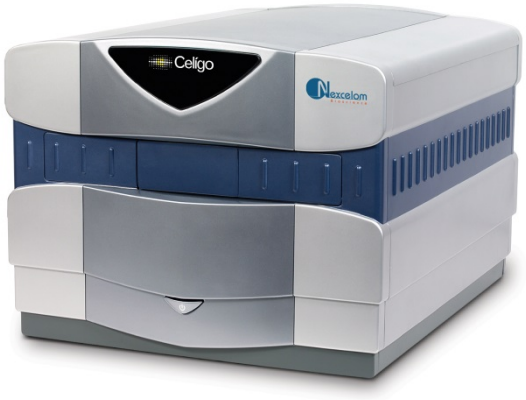
Celigo® Cytometer Application Guides



Celigo® Software Version 2.1

8001631 Rev A

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Celigo® Cytometer
Cell Counting and Growth Tracking Application Guide

Celigo Software Version 2.1

Contents

1. About this Guide

This chapter provides a brief description of this guide and how to use it.

1.1 Introduction

The Cell Counting and Growth Tracking application identifies and counts individual cells or clusters of cells using brightfield or fluorescence imaging. Furthermore, the application includes advanced data analysis functions to determine growth curves and doubling times using the Growth Tracking reporting option. You can choose to identify and count individual cells using the Direct Cell Counting application or determine confluency of cell cultures using the Confluence application.

1.2 Purpose

The purpose of this guide is to provide the steps and concepts that are unique to the Cell Counting and Growth Tracking application. Information that is common to all applications is covered in *Celigo Cytometer User Guide* (Doc. No. 8001619), from here on referred to as the User Guide.

1.3 Safety Precautions

All safety precautions described in the User Guide apply to this guide.

1.4 Technical Assistance

Contact Nexcelom Customer Service for further information:

Nexcelom Bioscience, LLC.
Customer Service
360 Merrimack St. Building 9
Lawrence, MA 01843, USA

- From the United States:
  email: celigosupport@nexcelom.com
  phone: 978-327-5340
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  e-mail: celigosupport@nexcelom.com
  phone: 978-327-5340

2. Prerequisites

The prerequisites for performing the procedures in this document are:

- Familiarity with Celigo operation per the User Guide
- Celigo cytometer is turned on per the User Guide
- Samples prepared as follows:
  - In brightfield imaging, plating liquid volume results in meniscus-dependent brightening or darkening at the well edges. It is recommended to optimize the liquid volumes to optimize performance of application. (see Troubleshooting chapter 7 for details). Table 1 summarizes the recommended plating volumes.

Table 1. Recommended Plating Volumes

Plate Type	Vendor	Cat#	Recommended Final Volume (µl) for Single Cell Analysis	
			Brightfield	Fluorescence
6W	Corning	3516	2000	ND[1]
6W	Corning	3471	2000	ND[1]
12W	Corning	3512	1000	ND[1]
24W	Corning	3524	500	ND[1]
96W	Corning	3603	≥200	≥100
384W	Aurora	1011	40	40
384W	Aurora	1012	40	40
384W	Corning	3712	40	40
1536W	Aurora	1071	6	6
1536W	Aurora	1092	8	8
1536W	Corning	3838	8	8
1536W	Grenier	789866	8	8
T-25	Corning	43069	ND[1]	ND[1]
T-75	BD Falcon	353136	ND	ND[1]

FOOTNOTE:
[1]ND means Not Determined.

For a more complete list of supported plates, see the User Guide.

3. Scanning Plates

This chapter provides the procedures for selecting the appropriate Cell Counting and Growth Tracking application and setting the image acquisition parameters. You perform these tasks in the Scan tab.

3.1 Cell Counting and Growth Tracking Application

Perform the following steps to select a Cell Counting and Growth Tracking application.

To select an application

Select one of the two Cell Counting and Growth Tracking applications (Figure 1 and Table 2) as follows, based on desired use:

- In the Current Application dropdown list, select **Cell Counting** > **Confluence** or **Direct Cell Counting**.

Figure 1. Selecting an Application

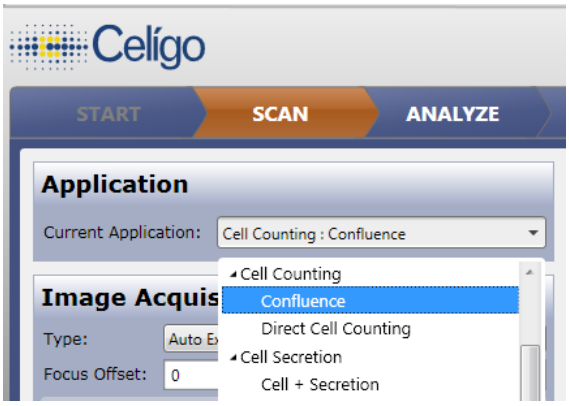


Table 2. Selecting an Application

Confluence	Measures area of well occupied by individual cells or cell clusters (Brightfield imaging only)
Direct Cell Counting	Identifies and counts individual cells using brightfield or fluorescence imaging.

3.2 Image Acquisition Settings

Perform the following steps to select image acquisition settings.

To select image acquisition settings

1. Choose a well for setup using the plate map by selecting **Navigation** and a representative well.
2. View a well by selecting **Live** or **Snap** in the Camera Controls field.
3. Set up acquisition settings for brightfield or fluorescence illumination:
   - For Brightfield illumination:
     - Select channel type: **Auto Exposure/Gain Channel** (recommended)

	NOTE: **2 X 2 Binning is *not* recommended for brightfield direct cell counting. For a detailed explanation of binning, see the User Guide.**

     - In Illumination, select **Brightfield**.
     - In Priority, select **AutoExposure, Gain if Necessary**.
     - In Frequency, select **Every Scan Area**.
   - For Fluorescence illumination:
     - It is recommended to use the Custom channel type. For detailed instructions, see the User Guide.

For detailed instructions on other image acquisition options, such as well subsampling, off-axis imaging, and binning, see the User Guide.

4. Set up focus per the User Guide.
   - For detailed instructions on selecting correct focus position for brightfield imaging. see section 3.3.
   - It is recommended to select Hardware Auto Focus for most routine plate scanning.

3.3 Correct Focus Position for Brightfield Imaging

The proper focus for brightfield illumination is important for optimal application performance. There are two image planes visible on the Celigo Cytometer using brightfield illumination. The “Dark” image plane is the real image plane in which objects appear dark compared to surrounding background regions. The “Bright” image plane is a virtual image plane in which the cell or object acts as a lens and focuses the transmitted light in a secondary plane. In the “Bright” image plane, cells or objects have a bright center and dark edges. The Celigo cytometer identification algorithm is optimized for the “Bright” image plane.

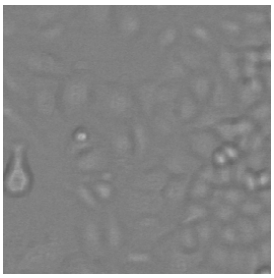
This section describes how to select the correct focus position for brightfield imaging. For instructions on focusing using fluorescence illumination, see the User Guide.

To select the correct focus position for brightfield imaging

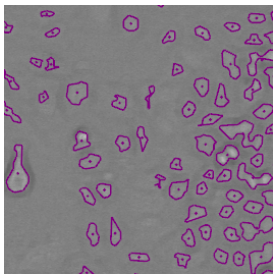
1. Choose a well for setup using the plate map by selecting **Navigation** and a representative well.
2. View a well by selecting **Live** or **Snap** in the Camera Controls field.
3. Adjust the focus until the cells have a large, bright center. For examples of proper focus using the “Bright” and “Dark” selections in the Target Focal Plane menu, (Figure 2).
4. Select **Focus Setup** and select **Hardware Auto Focus** in the Focus Type field. Complete the setup by registering the autofocus.
   - Image Based Auto Focus is *not* recommended for brightfield illumination if the wells contain very few cells.

Figure 2. Examples of Proper "Bright" and "Dark" Focus

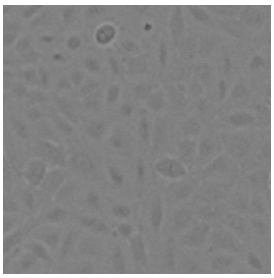
A549 Cells in "Bright" Focus



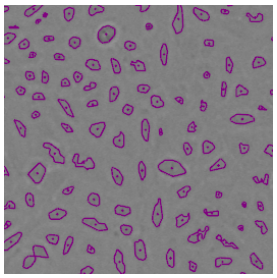
Optimal Identification of A549 Cells Using:
- "Bright" Target Focal Plane
- "Brightfield" Algorithm



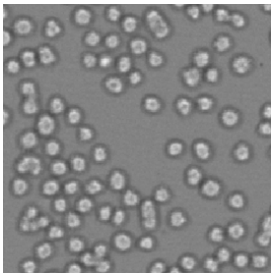
A549 Cells in "Dark" Focus



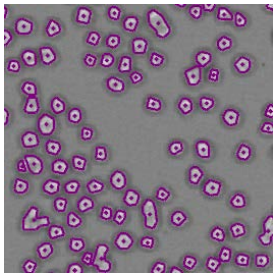
Optimal Identification of A549 Cells Using:
- "Dark" Target Focal Plane
- "Dark Object" Algorithm



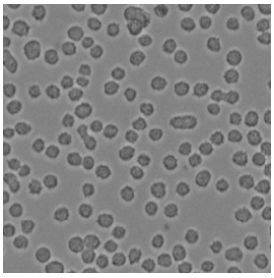
CHO-S Cells in Optimal "Bright" Focus



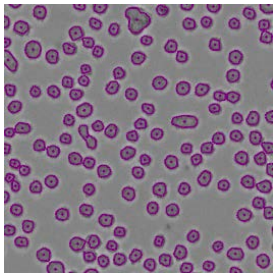
Optimal Identification of CHO-S Cells Using:
- "Bright" Target Focal Plane
- "Brightfield" Algorithm



CHO-S Cells in "Dark" Focus



Optimal Identification of CHO-S Cells Using:
- "Dark" Target Focal Plane
- "Dark Object" Algorithm



4. Analyzing Images

This chapter provides information on how to analyze scans from the Cell Counting and Growth Tracking application. You perform these tasks in the Analyze tab.

4.1 Analysis Settings

To select analysis settings, do one of the following

- For Direct Cell Counting analysis, see section 4.1.1.
- For Confluence analysis, see section 4.1.2.

Perform the following steps to select analysis settings. The recommended initial analysis settings for identification and pre-filtering when using the Cell Counting and Growth Tracking application are shown in Table 3 and Table 4. The settings typically provide good image segmentation. For a detailed explanation of the analysis settings, see the User Guide.

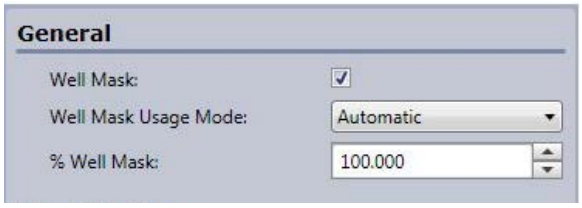
4.1.1 Direct Cell Counting Analysis

Perform the following steps for Direct Cell Counting analysis. For the recommended initial identification and pre-filtering settings to use as a guide, see Table 3.

To perform Direct Cell Counting analysis

1. Load prior saved Analysis Settings, if available.
2. In the General section (Figure 3), make the following selections:

Figure 3. General Section

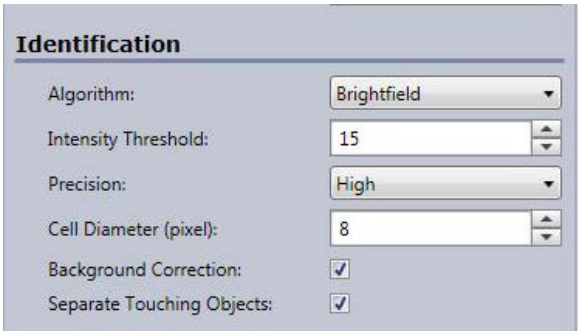


   a. Well Mask – Select this option. Improves segmentation of cells at the well edge and excludes objects outside the well.
   b. Well Mask Usage Mode – Select one of the following:
      - Automatic. The software automatically looks for the mask.
      - Original: The software uses the well dimensions from the plate profile.
   c. % Well Mask – Reduce the well mask to a percentage that is useful for looking at cells toward the center of the well. Decreasing the % Well Mask entry can exclude well edge artifacts that can occur during plate manufacturing.

For information about Well Mask Usage Mode and % Well Mask, see the User Guide section “Selecting General Analysis Settings.

3. In the Identification section (Figure 9), make the following selections to be used to identify cells:

Figure 4. Identification Section



a. Algorithm – Select the appropriate algorithm to be used to identify cells, as follows, matching the algorithm to the illumination source:

   - Brightfield – The algorithm looks for objects with a bright center and dark edges
   - Fluorescence – The algorithm looks for fluorescent objects (bright pixels over darker background)
   - Dark Object – The algorithm looks for dark objects with no bright center

b. Intensity Threshold – Enter the optimal intensity threshold.

   The intensity threshold is the level of intensity that separates background from cells. With an appropriate threshold set, background pixels fall below and pixels in cells are above the threshold. Adjust Intensity Threshold to match cell type:

   - Non-adherent cells – Typically, non-adherent cells are rounded with bright centers and dark edges. Users can use higher Intensity Threshold values of ≥10 to identify cells and ignore darker debris.
   - Flat, adherent cells – Adherent cells are more flat with darker centers and less well defined edges than non-adherent. Lower Intensity Threshold levels of ≤ 6 are recommended.

c. Precision – Select the desired precision level.

   Higher precision results in more accurate identification of individual cells. High recommended, but analysis processing time will be longer.

d. Cell Diameter (pixel) – Enter the cell diameter (in pixels) that corresponds to the cell dimensions.

   Adjust Cell Diameter to match cell type and plating density:

   - Non-adherent or dense cell cultures – Non-adherent cells or dense cells have smaller cell diameters. Recommend users can use lower Cell Diameter values of ≤10.
   - Flat adherent cells – Adherent cells have larger cell diameters. Higher Cell Diameter values of ≥15 are recommended.

- At full resolution, the Celigo cytometer provides 1 µm/pixel.

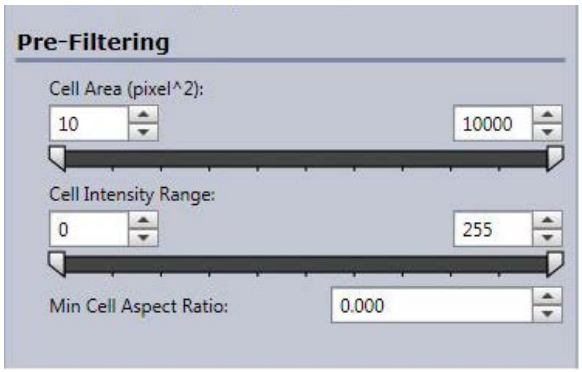
e. Background Correction – Select if necessary.

f. Separate Touching Objects – Select as needed.

This selection is used to separate touching cells during segmentation.

4. In the Pre-Filtering section (Figure 5), make the following selections:

Figure 5. Pre-Filtering Section



a. Cell Area (pixel^2) – Enter the appropriate cell area range.

Debris is often small or very large. Adjust the Cell Area range to >20 and <300 to remove small and/or large objects.

b. Cell Intensity Range – Enter the appropriate cell intensity range, if necessary.

Debris is often dark (brightfield imaging) or very bright (fluorescence). Adjust the Cell Intensity Range to remove dark or bright objects.

c. Min Cell Aspect Ratio – Enter the appropriate minimum aspect ratio, if necessary.

This selection measures an object's elongation and is often used to remove artifacts and debris.

Aspect Ratio is recommended to remove long, oblong objects generally found along bubbles, well edges, and plate imperfections. Increase to >0.10.

Table 3. Recommended Initial Identification and Pre-Filtering Settings for Analysis – Direct Cell Counting

CELL TYPE	Non-Adherent	Adherent	Non-Adherent	Adherent	Non-Adherent	Adherent
IDENTIFICATION						
Acquisition Illumination	Brightfield	Brightfield	Fluorescence	Fluorescence	Brightfield (Dark, Focused Cells)	Brightfield (Dark, Focused Cells)
Algorithm	Brightfield	Brightfield	Fluorescence	Fluorescence	Dark Object	Dark Object
Intensity Threshold	18	6	4	4	6	1
Precision	High	High	High	High	High	High
Cell Diameter (pixel)	10	16	10	15	12	15
Background Correction	Checkmarked	Checkmarked	Checkmarked	Checkmarked	Checkmarked	Checkmarked
Separate Touching Objects	Checkmarked	Not Checkmarked	Checkmarked	Not Checkmarked	Checkmarked	Not Checkmarked
PRE-FILTERING						
Cell Area (pixel ^2) Range	30 - 400	80 - 10000	20 - 300	35 - 10000	30-600	40-1000
Cell Intensity Range	100 - 255	50 - 255	0 - 255	0 - 255	0-255	0-255
Min Cell Aspect Ratio	0.150	0.00	0.150	0.00	0.150	0.000

4.1.2 Confluence Analysis

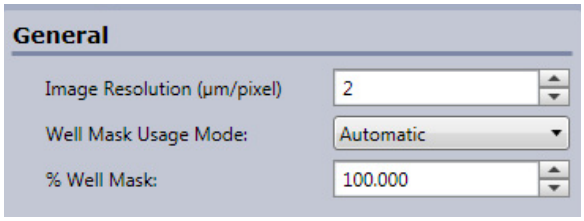
Perform the following steps for Confluence analysis. For the recommended initial identification and pre-filtering settings to use as a guide, see Table 4.

	**NOTE: The confluence relies on the use of the Brightfield or Texture segmentation algorithm.**

To perform Confluence analysis

1. Load prior saved Analysis Settings, if available.
2. In the General section (Figure 6), make the following selections:.

Figure 6. General Section



a. Image Resolution (µm/pixel) – Select the image resolution to use for analysis. Entering a lower value (minimum1 µm/pixel; ≥ 2 µm/pixel recommended) will result in greater accuracy and longer analysis time. Entering a higher value will result in less accuracy and shorter analysis time.

b. Well Mask Usage Mode – Select one of the following:

   – **Automatic** (Default) – The system looks at the well itself to find the edge of the well (looks for the “local minima”).

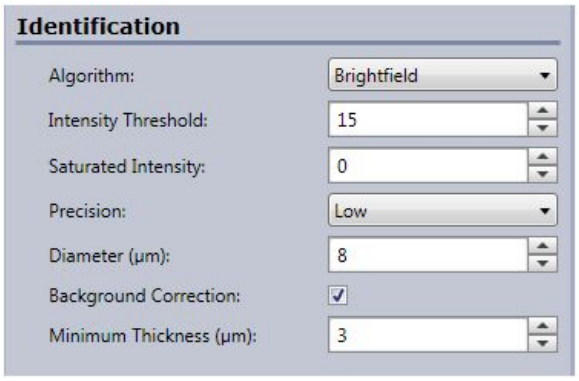
   – **Original** – The system uses the set mapped position for the edge of the well, specified in the plate profile.

c. % Well Mask – Sets the percentage of the well to be analyzed. Decreasing the % Well Mask entry can exclude well edge artifacts that can occur during plate manufacturing.

   For information about Well Mask Usage Mode and % Well Mask, see the User Guide section “Selecting General Analysis Settings.

3. In the Identification section (Figure 7), make the following selections to be used to identify cells:

Figure 7. Identification Section



d. Algorithm – Select the appropriate algorithm:

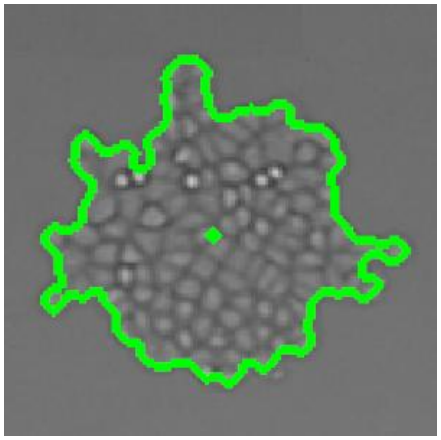
- Brightfield – The algorithm looks for objects with a bright center and dark edges
- Texture – The algorithm looks for texture differences between the objects found and the background areas (Recommended)

	NOTE: The cell identification success of selecting Brightfield versus Texture will depend on the cell type, shape, and contrast of the cells being analyzed.

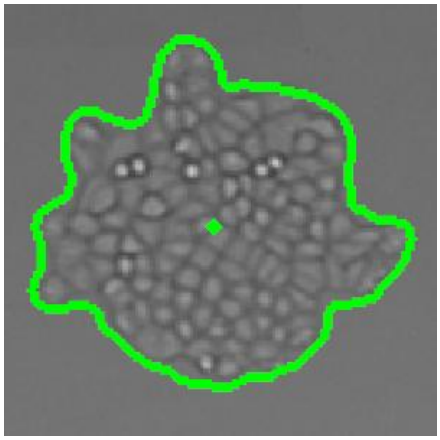
Figure 8 shows an example of selecting Brightfield versus Texture as the Algorithm field.

Figure 8. Direct Cell Counting – Brightfield vs. Texture Algorithm Selected

Brightfield Algorithm Selected for Adherent Cells



Texture Algorithm Selected for Adherent Cells



e. Intensity Threshold – Select the optimal intensity threshold.

   This selection is the level of intensity that separates background from cells. With an appropriate threshold set, background pixels fall below and pixels in cells are above the threshold.

f. Saturated Intensity – Select the optimal saturated intensity threshold to prevent holes in the segmentation of large objects.

   Saturated intensity represents the minimum pixel intensity value that is considered to be saturated. Saturated areas are not detected by the texture segmentation algorithm and therefore create holes within confluence areas. Holes consisting of pixels that are all saturated are filled in post-segmentation.

g. Precision – Higher precision results in more accurate identification of cell clusters. Normal recommended. Normal is sufficient to provide acceptable results, while High results in longer analysis processing time.

h. Diameter (µm) – Do one of the following:

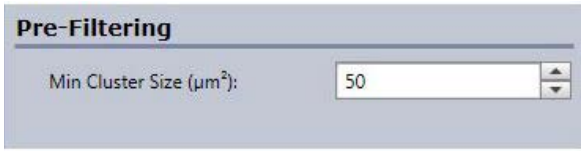
   - If you selected Texture in the Algorithm field, skip to step 3i. (The entry in Diameter has no effect when Texture is the Algorithm.)
   - If you selected Brightfield in the Algorithm field: In Diameter (pixel), enter the diameter (in pixels) that corresponds to the cell cluster dimensions. At full resolution, the Celigo cytometer provides 1 µm/pixel.

i. Background Correction – Select if necessary.

j. Minimum Thickness (µm) – Minimizes long extensions. Increase if the plate has plate artifacts such as a plate ring stamp.

4. In the Pre-Filtering section (Figure 9), make the following selection:

Figure 9. Pre-Filtering Section



- Min Cluster Size (μm^2) – Enter the appropriate minimum cluster size.

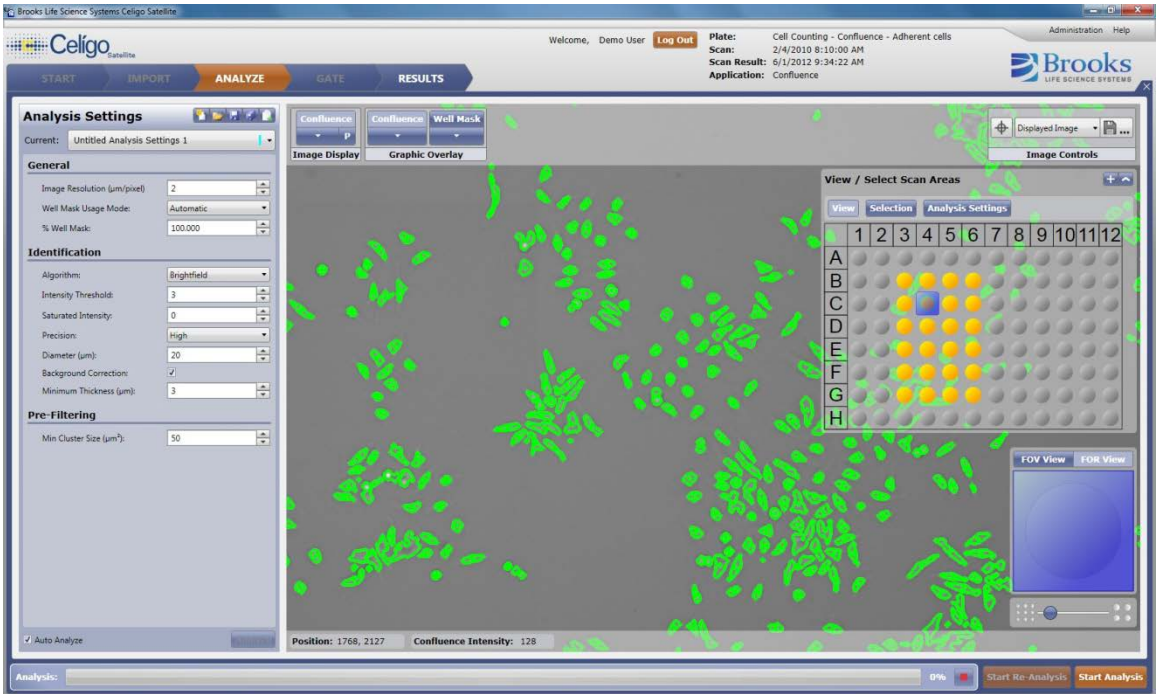
  To eliminate small debris, adjust the value to be larger than small debris size and lower than the cell cluster size.

Table 4. Recommended Initial Identification and Pre-Filtering Settings for Analysis – Confluence

ALGORITHM	BRIGHTFIELD		TEXTURE	
CELL TYPE	**Non-adherent**	**Adherent**	**Non-adherent**	**Adherent**
ACQUISITION ILLUMINATION	**Brightfield**	**Brightfield**	**Brightfield**	**Brightfield**
IDENTIFICATION				
Intensity Threshold	5	6	10	5
Saturated Intensity	0	0	0	0
Precision	High	High	High	Low
Diameter (µm)	8	15	Not Applicable (selection has no effect)	Not Applicable (selection has no effect)
Background Correction	Checkmarked	Checkmarked	Checkmarked	Checkmarked
Minimum Thickness (µm)	3	3	3	3
PRE-FILTERING				
Min Cluster Size (μm^2)	50	50	500	500

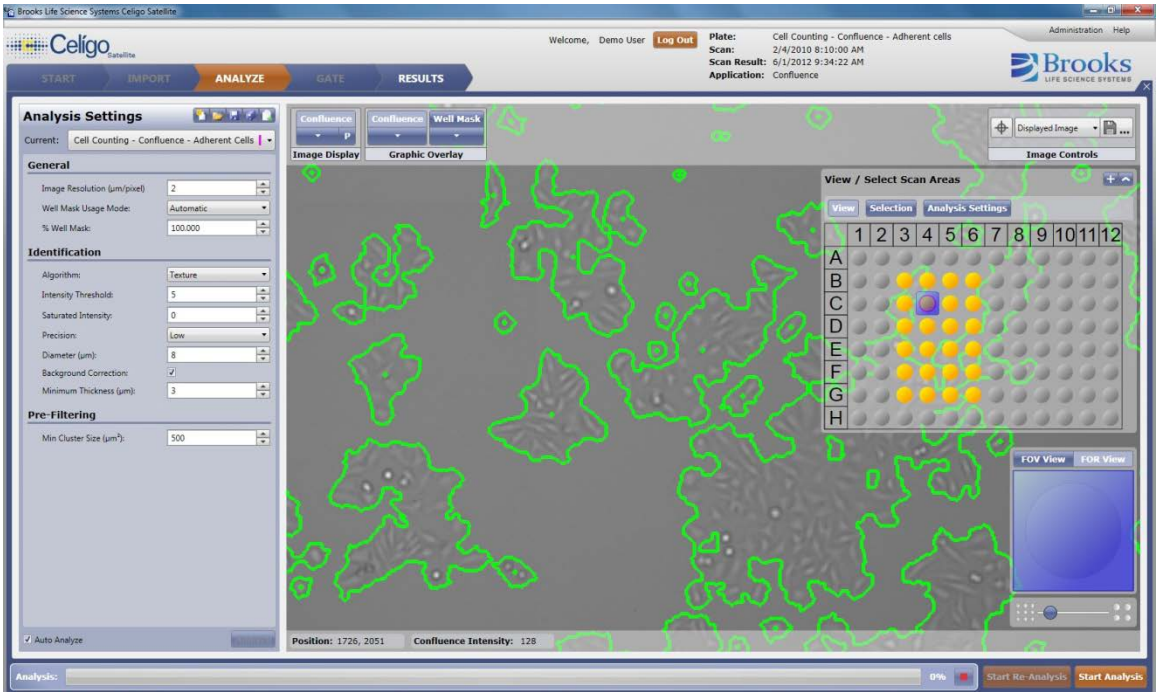
Figure 10 and Figure 11 show examples of analysis settings for Brightfield versus Texture as the Algorithm selection.

Figure 10. Brightfield Analysis Settings



Note: Nexcelom Bioscience acquired the Celigo product line from Brooks Life Science Systems in March 2014. As of the printing of this document, the Celigo software version displays the Brooks logo.

Figure 11. Texture Analysis Settings



5. In the Auto Analyze section, checkmark this selection as follows:
   - To update the segmented image and overlays as you make each selection, displaying a progress wheel after you make each field entry, checkmark Auto Analyze.
   - To update the segmented image and overlays only when you click Analyze, deselect Auto Analyze.

5. Gating Cells

This chapter describes how to select filter settings for further scan data analysis. You perform this task in the Gate tab.

Gating can be used to count and analyze subpopulations of cells that can be discriminated by size, intensity, or morphology.

5.1 Working with Gates

The following are general principles about working with gates in the Cell Counting and Growth Tracking application.

- For optimal analysis using the Cell Counting and Growth Tracking application, you should perform gating.
- Gating is appropriate when using only the Direct Cell Counting application, not the Confluence application. Therefore the Gate tab is available only if you select Direct Cell Counting in the Application menu.
- If you do not perform gating (either because you are using the Confluence application or because you are using Direct Cell Counting but choosing not to perform gating), the system uses the ALL population to count the cells in the wells. ALL is the default population that the system assigns to all the data points (objects) in the segmentation result from the Analyze tab.
- When performing gating in the Direct Cell Counting application, only one class (the Total class) exists; you cannot assign any additional classes to populations.

To create a plot, gate, and populations

1. In the Plot Populations pane, create a plot, using the Add Plot (+) button and Add Plot dialog box.

   Table 5 lists the selections you can make in both Pick plot parameter menus. The Pick plot parameters selections are different from those in the User Guide (which shows selections for the Expression Analysis application).

   For details on creating plots, see User Guide section “Creating a Plot.”

   When you create the plot, in this Application all the cells will automatically belong to a population named “All”. Based on the “All” population, a class (a class is a population for which data will be reported) is also automatically created, named the “Total” class.

Table 5. Plot Parameter Definitions

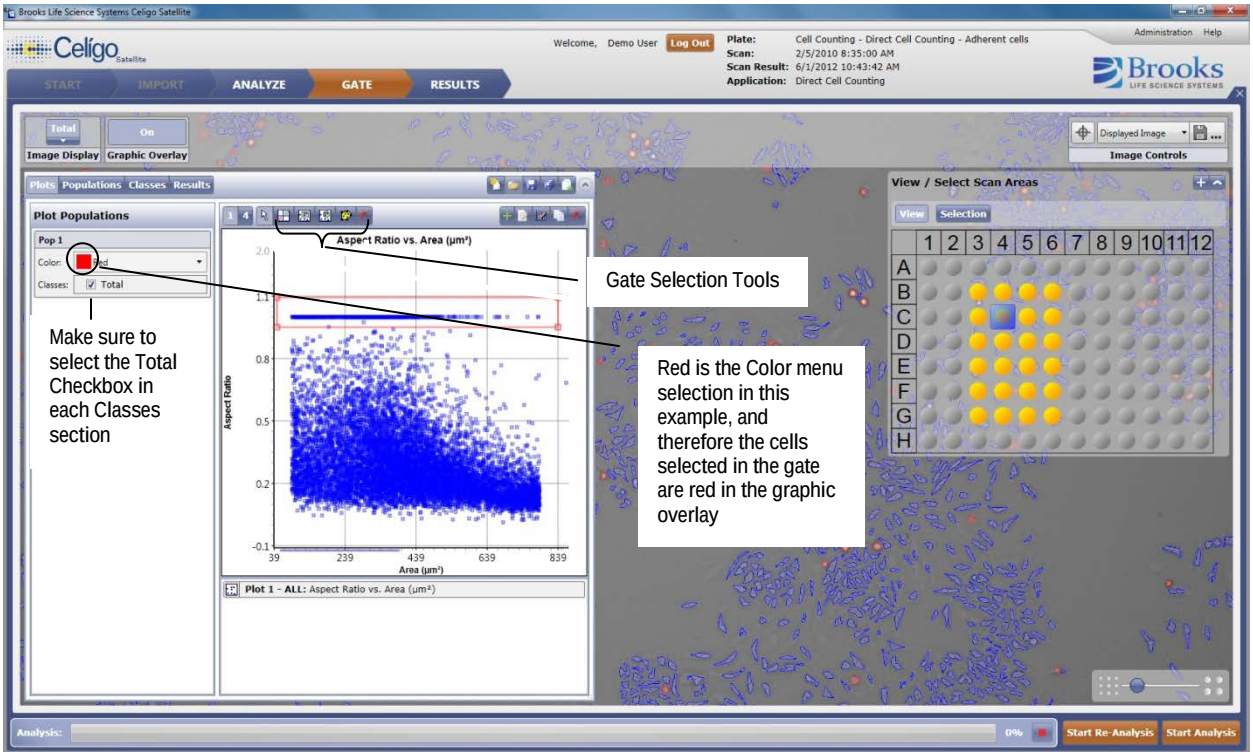
Feature	Description
X Position (μm)	Location of a cell along the horizontal axis of the well: left (-μm) or right (+μm) of the center (origin) of the well.
Y Position (μm)	Location of a cell along the vertical axis of the well: below (-μm) or above (+μm) the center (origin) of the well.
Area (μm^2)	Total area of all the segmented objects' features.
Form Factor	"Compactness" of the segmented object, derived from the ratio of its perimeter to its area; a circle with a value of 1 is most compact; a more convoluted shape with a value less than 1 is less compact .
Smoothness	"Evenness of contour" of the segmented object, derived from the ratio of its convex perimeter to its true perimeter (see the User Guide); a perfectly smooth area has a value of 1; a rough or jagged area has a value less than 1.
Aspect Ratio	Ratio of the minor axis to the major axis of the segmented object. A value of 1 is a perfect circle; values less than 1 suggest an oval (elongated) object.
Mean Intensity	Average of the intensities of the segmented objects.
Integrated Intensity	Sum of all pixel intensities displaying signal in segmented objects.

2. Create a gate on the plot, using the gate selection tools (Figure 12). In each Pop section, make sure to checkmark the Total checkbox in the Classes section. This will allow analysis results to appear for the gated region.

   For details, see User Guide section "Creating a Gate."

   In the Graphic Overlay display, the color displayed for the plot population selected in the gate corresponds to the Color menu selection (Figure 3). The figure shows the single class (Total) used in this application.

Figure 12. Gating Cells



3. Repeat steps 1 and 2 as needed to refine the population that you want to count.
4. Assign the Total class to the population as follows:
   a. Click the gate.
   b. In the Plots, Populations, or Classes view, make sure that the Total class is checkmarked.

   For details, see User Guide section “Assigning a Class to a Population.”

   In this application, you assign only the Total class to populations; you cannot assign any additional classes to populations.

6. Viewing Results

This chapter describes the feature outputs available from the application's Results tab, including how to generate a Growth Tracking report.

Table 6 summarizes the outputs of the Cell Counting applications.

Table 6. Cell Counting and Growth Tracking Application Outputs

Parameter	Description	Direct Cell Counting	Confluence
Cell Count	Number of Total cells positive with intensity above a user-defined intensity threshold	√	
Confluence (%)	Area of the well occupied by individual cells or cell clusters (Brightfield imaging only) divided by total area of scan area or well		√
Well Sampled (%)	Percent of well sampled	√	√

6.1 Generating a Growth Tracking Report

Growth tracking reports calculate the growth characteristics of cell populations over time. The reports associate cell counts or confluence measurements from multiple scans time points – using each scan's latest scan result or a default – and determine doubling times and rates for individual wells. The reports are in the form of plots that can be exported as images or data by the user for documentation and presentation purposes.

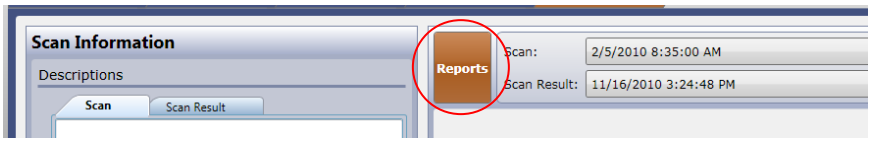
For a detailed explanation of the Identification and Pre-Filtering settings, see the User Guide.

To generate a report

	**NOTE: To generate a pie chart, make sure that 4 or more scans exist for selection. To generate a growth chart (curve), make sure that 2 or more scans exist for selection.**

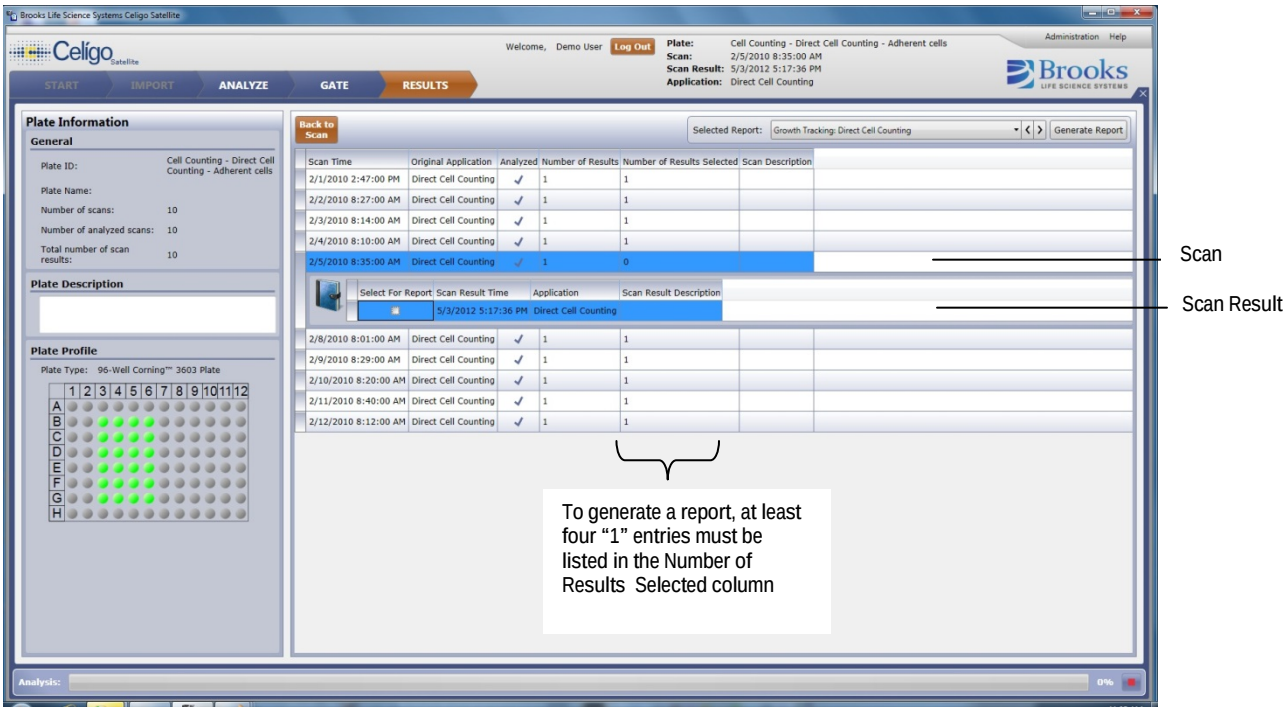
1. In the Results tab, click **Reports** (Figure 13)

Figure 13. Displaying Scans for Reporting



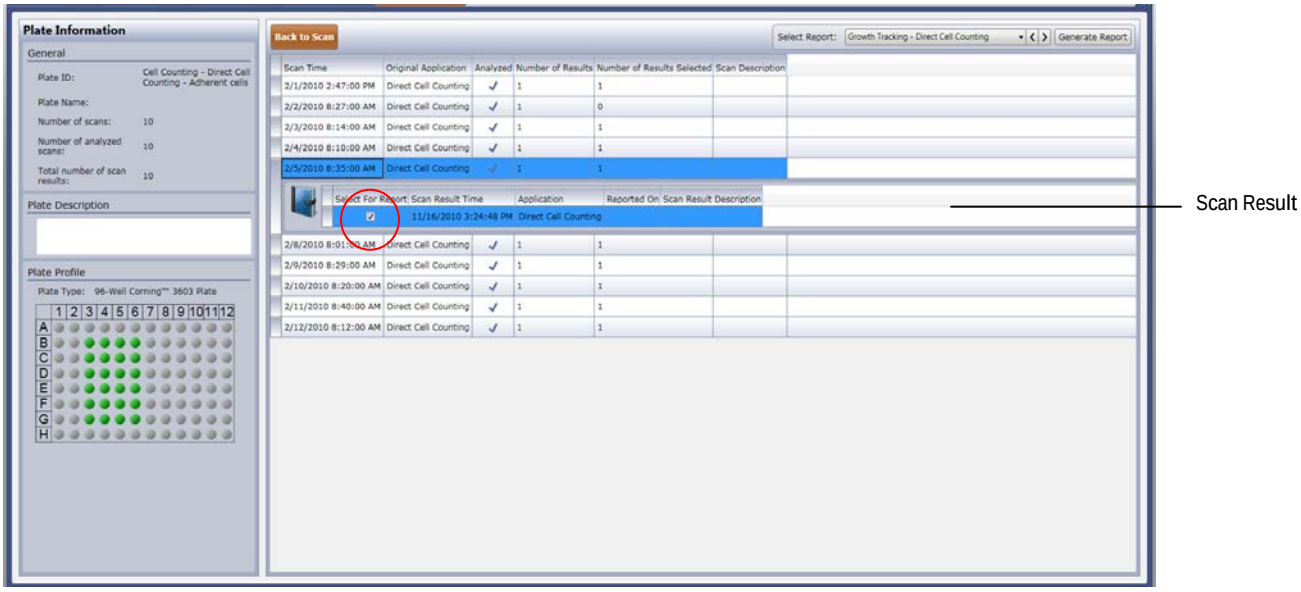
The list of existing scans and associated scan results for the plate ID appears (Figure 14).

Figure 14. Scan List



2. Checkmark the Select For Report checkbox (Figure 15) next to each scan result for which you want to generate a growth tracking report. You can also right-click a scan result and select one of the following:
   - Select all scan results
   - Deselect all scan results
   - Select last Confluence scan result for each scan
   - Select last Direct Cell Counting scan result for each scan

Figure 15. Selecting Scan Results for a Growth Tracking Report

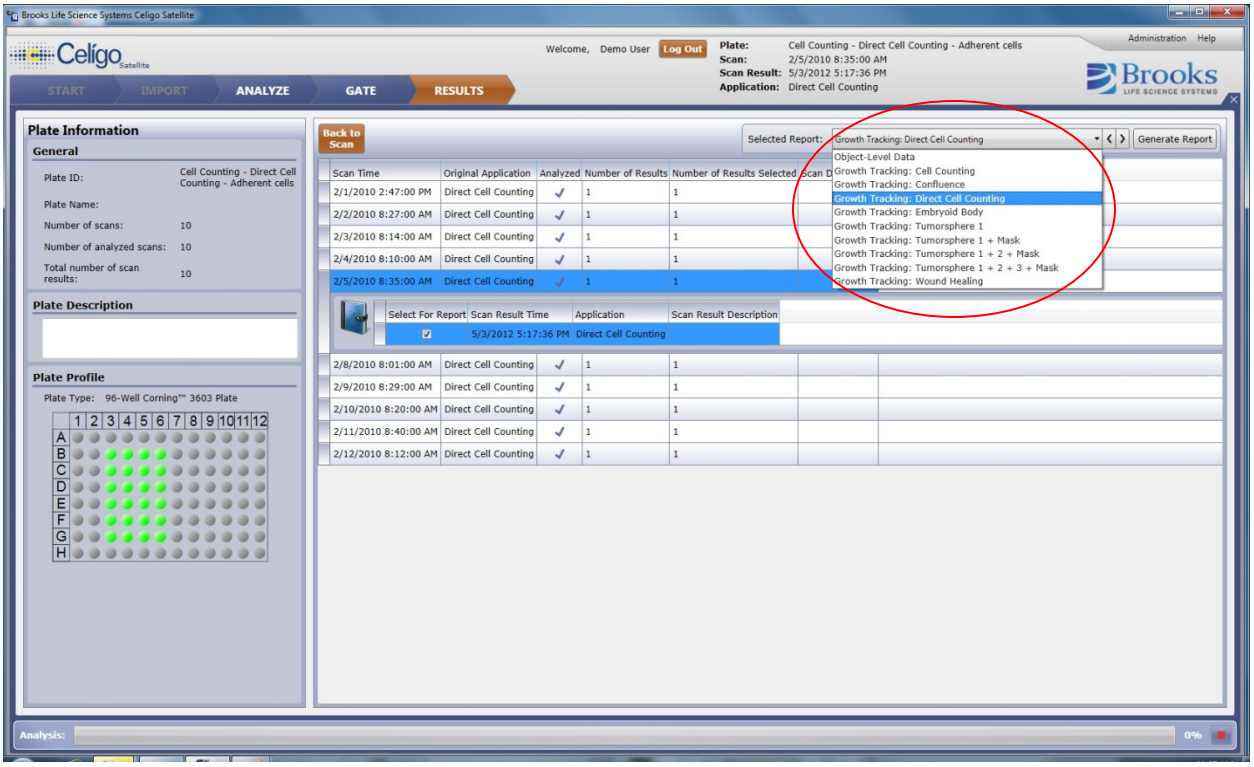


3. In the Selected Report menu, select the main type of growth tracking report you want to generate (Figure 16).

	NOTE: Only the scans analyzed using the application you are selecting for growth tracking reporting will be available for selection.

- To change the selection, use the menu again or click the arrows to the right of the Select Report menu.

Figure 16. Selecting Main Type of Report

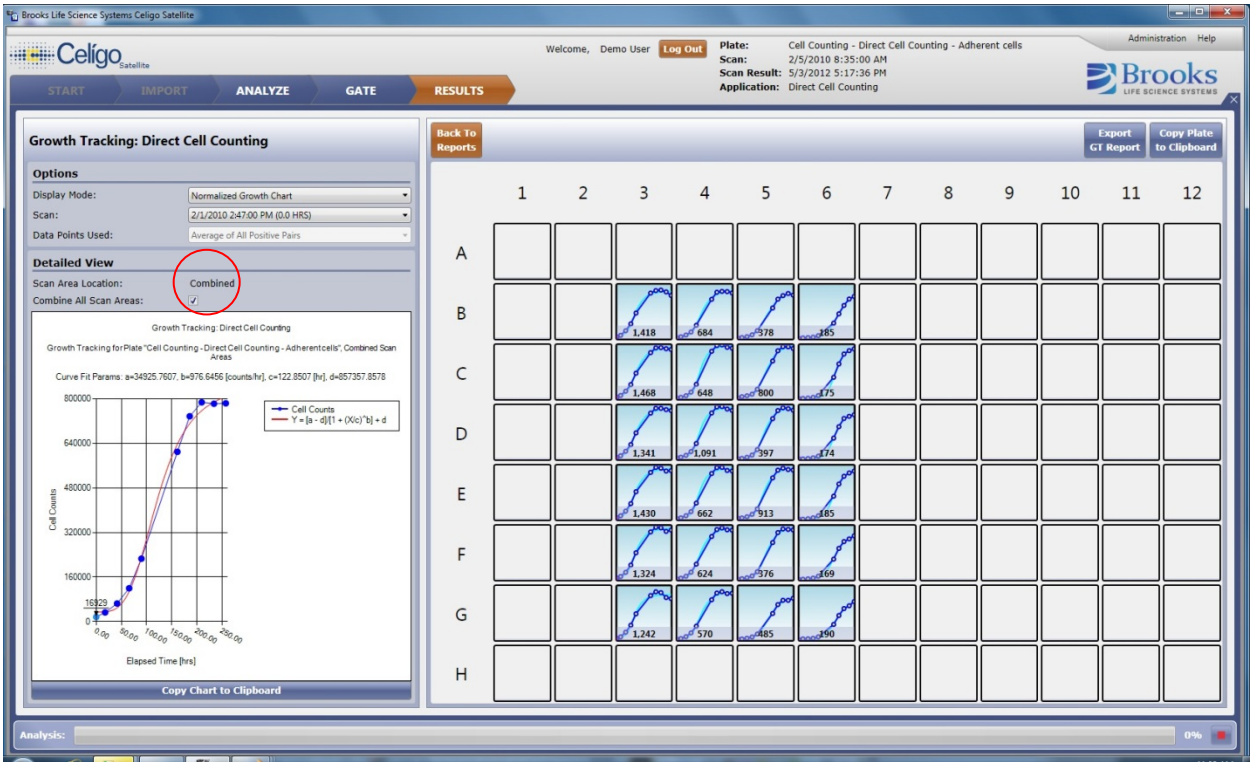


Note: Nexcelom Bioscience acquired the Celigo product line from Brooks Life Science Systems in March 2014. As of the printing of this document, the Celigo software version displays the Brooks logo.

4. Click **Generate Report** at the top right of the Results tab.

   A growth chart (default display mode) for the entire plate, with Combine All Scan Areas selected, appears in the Results tab (Figure 17).

Figure 17. Growth Chart for the Entire Plate (Default)



Note: Nexcelom Bioscience acquired the Celigo product line from Brooks Life Science Systems in March 2014. As of the printing of this document, the Celigo software version displays the Brooks logo.

6.2 Working with a Generated Report

You can take the following actions on a generated growth tracking report (curve or pie chart) as needed.

6.2.1 Changing the Type of Display

Change the type of display by selecting the **Display Mode** menu and then selecting one of the following options:

- Normalized Growth Chart – Growth curve normalized to the well that has the highest number of cells.

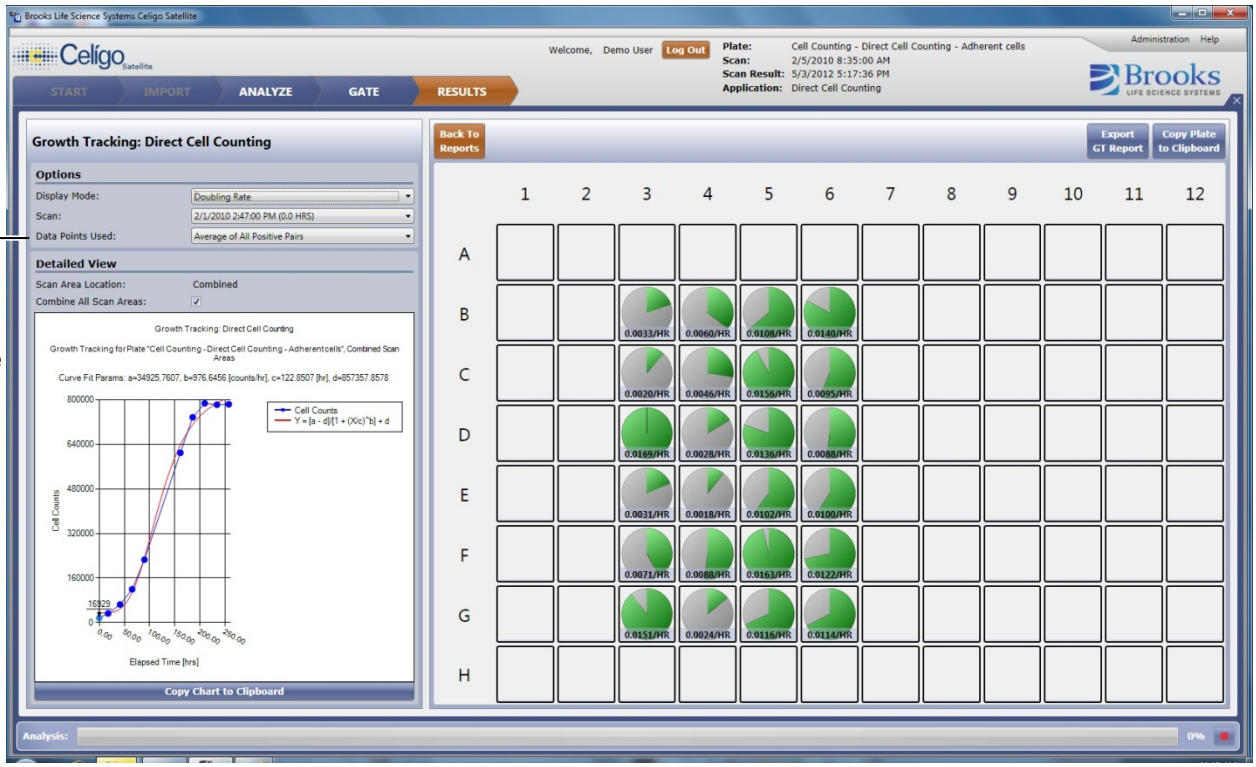
  Growth curves are displayed using the identical Y-axis for each well, determined by the highest count for the plate ID.

- Growth Chart – Displays the growth curve for a given well and the fitted growth curve. Each Scan Area plot is fitted for the display window by varying the Y axis range. Curve is fitted using a standard Four-Parameter Logistic equation: $Y = [a-d]/(1 + X/c^b) + d$.

- Doubling Time – Calculated time in hours for one doubling of the cell count or confluence according to the following equation: doubling time = (time between count 1 and count 2 in hrs) * [ln(2) / ln(count 2/ count 1)]. Displayed with pie charts.

- Doubling Rate – Calculated rate of doubling per hour according to the following equation: 1 / doubling time (hrs). Displays both a growth curve and pie charts (Figure 18).

Figure 18. Growth Curve and Pie Charts in Doubling Rate Display



Data Points Used will recalculate the doubling rate (or doubling time for Doubling Time display mode)

Note: Nexcelom Bioscience acquired the Celigo product line from Brooks Life Science Systems in March 2014. As of the printing of this document, the Celigo software version displays the Brooks logo.

6.2.2 Displaying a Growth Curve for a Single Well

Display the growth curve for a single well by selecting the Growth Chart Display Mode and clicking a well in the right-hand pane.

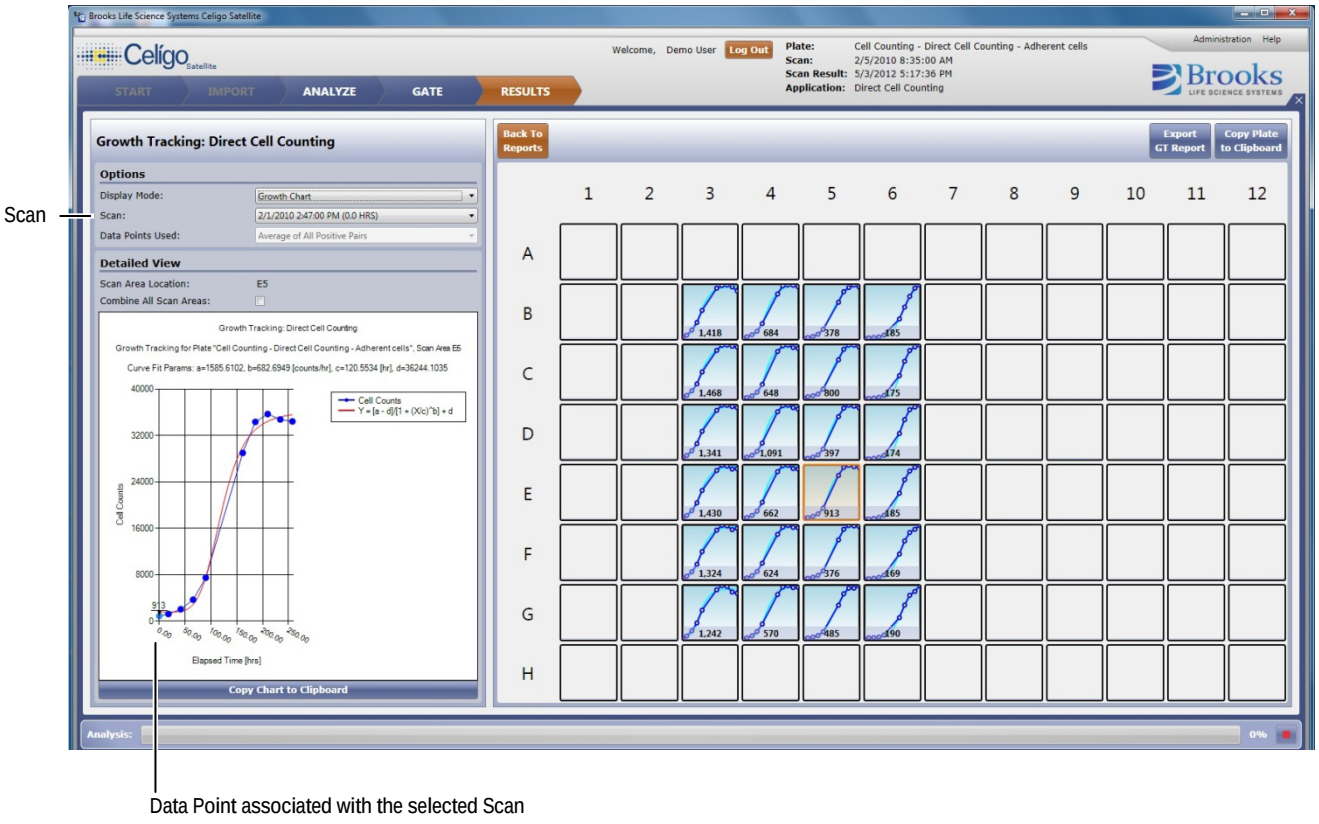
An orange border appears around the selected well (Figure 19).

Each data point and cell count on the curve and in the plate display corresponds to each scan time listed in the Scan menu.

The light blue dot on the curve corresponds to the currently selected scan time in the Scan menu.

To change the cell count associated with the time indicated at each blue data point on the curve (Figure 19), select a different scan in the Scan menu.

Figure 19. Growth Curve for a Single Well



Note: Nexcelom Bioscience acquired the Celigo product line from Brooks Life Science Systems in March 2014. As of the printing of this document, the Celigo software version displays the Brooks logo.

6.2.3 Displaying a Growth Curve for the Entire Plate

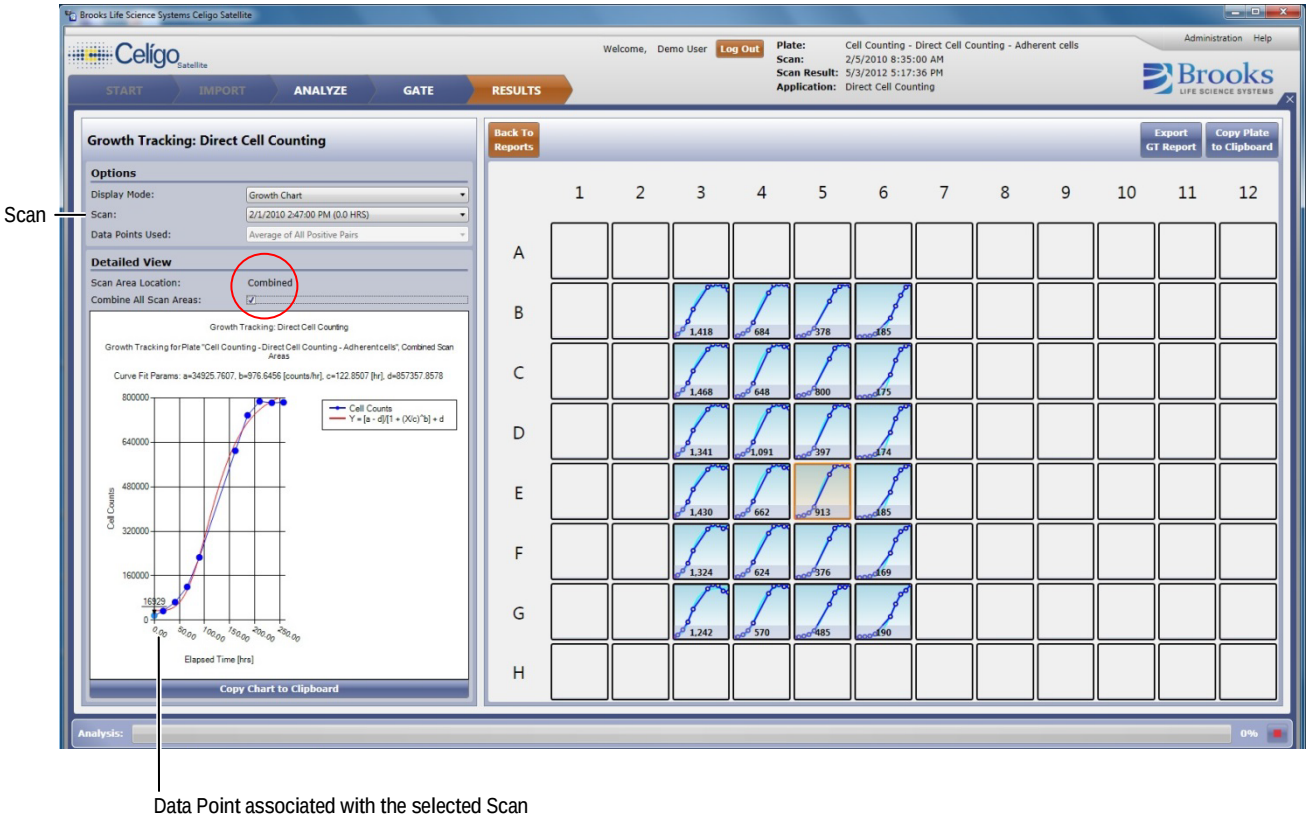
Display the growth curve for the entire plate by clicking the Growth Chart Display Mode and clicking **Combine All Scan Areas** checkbox in the left-hand pane (Figure 20). This option makes calculations based on all wells of the plate.

Each data point and cell count on the curve and in the plate display corresponds to each scan time listed in the Scan menu.

The light blue dot on the curve corresponds to the currently selected scan time in the Scan menu.

To change the cell count associated with the time indicated at each blue data point on the curve (Figure 20), select a different scan in the Scan menu.

Figure 20. Growth Curve for the Entire Plate

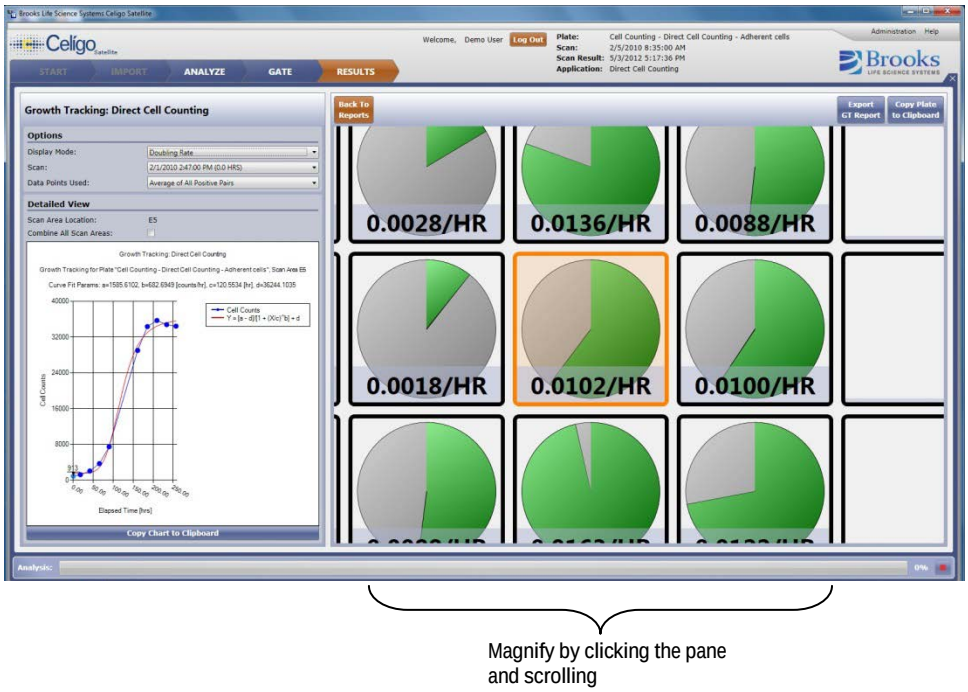


Note: Nexcelom Bioscience acquired the Celigo product line from Brooks Life Science Systems in March 2014. As of the printing of this document, the Celigo software version displays the Brooks logo.

6.2.4 Magnifying a Pie Chart Size (Zoom)

You can magnify the appearance of a pie chart pane (not a growth curve) (Figure 21) by left-clicking the pie chart and then scrolling with the mouse scroll wheel.

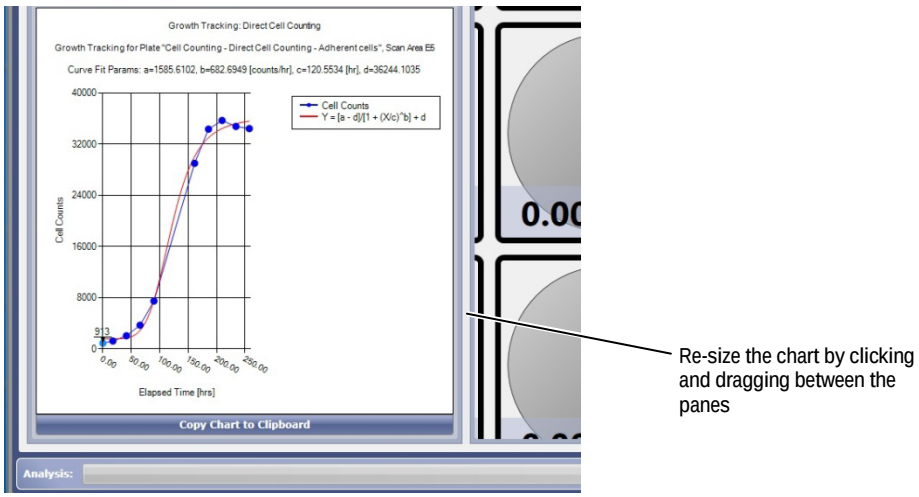
Figure 21. Magnifying a Pie Chart Size



6.2.5 Re-Sizing a Chart

Widen or narrow the size of a chart by clicking and dragging the vertical line between the left and right panes (Figure 22). This action is not to change the chart data, but is only for viewing purposes.

Figure 22. Re-Sizing a Chart



Note: Nexcelom Bioscience acquired the Celigo product line from Brooks Life Science Systems in March 2014. As of the printing of this document, the Celigo software version displays the Brooks logo.

6.2.6 Exporting Report Data

Export growth tracking report data by clicking **Export Growth Tracking Report**. For a summary of outputs, see Table 7.

Table 7. Growth Tracking CSV Report Outputs

Parameter	Description	Direct Cell Counting	Confluence
Cell Count	Number of Total Cells positive with intensity above a user-defined intensity threshold	√	
Confluence (%)	Area of well occupied by individual cells or cell clusters (Brightfield imaging only) divided by total area of scan area or well	–	√
Total Confluent Area (μm^2)	Area of well occupied by individual cells or cell clusters (Brightfield imaging only) divided by total area of scan area or well	–	√
Average of Positive Doubling Time Data (hrs)	Well level average doubling time calculated from all positive two point successive paired doubling times	√	√
Elapsed Time (hrs)	Elapsed time of individual scan from first scan time point	√	√
Date and Time	Date and time of individual scans	√	√
Two Point Doubling Time (hrs)	Doubling time calculated from two successive scans	√	√

6.2.7 Exporting a Chart Image

Export a chart to the clipboard as a jpg image by clicking **Copy Chart to Clipboard**.

7. Troubleshooting

This chapter provides troubleshooting recommendations.

Table 8. Troubleshooting Recommendations

Issue	Recommended Action
Cannot identify individual cells	1. Inappropriate focus selected. • For brightfield imaging– verify that "Bright" focus plane was used to acquire images. • For fluorescence – confirm that crisp focus was selected for desired objects. 2. Desired objects are excluded by the Pre- Filtering settings. • View the segmented image in the Analyze tab (see *Celigo Cytometer User Guide* for instructions) • Change Pre-Filtering settings to identify desired objects 3. Identification settings do not identify objects. • Revisit the steps for identifying individual cells (see section 4.1.1 or 4.1.2, depending on whether Direct Cell Counting or Confluence application is selected). 4. Inappropriate gate is applied in Gating screen. • Go to Gating tab and remove gates.
Software identifies debris as cells or confluent areas in brightfield	Often debris has unique properties that can be used to remove it from the scan results. Use the Pre-Filtering settings to remove debris. • Recommend adjusting Pre-Filtering settings to selectively remove debris.
Improper cell counts on well edges in brightfield	1. Remove/uncheck Separate Touching Objects in the Analyze tab – Identification section. 2. Remove/uncheck Well Mask in the Analyze tab – Identification section. 3. Increase Aspect Ratio in Analyze tab – Pre-Filtering section.
Well edges are too bright or dark	Liquid volume not optimal resulting in a meniscus-dependent effect. • For proper liquid volumes for cell plating, see Table 1.
Bright or dark shadows of cells are identified in brightfield	1. Adjust liquid volume level to prevent meniscus-dependent optical effects. • For proper liquid volumes for cell plating, see Table 1. 2. Some small well microplates, such as 1536-well formats, yield meniscus-dependent optical effects that cannot be corrected by liquid volume. • Use an alternative container. • Use Pre-Filtering settings in the Analyze tab to remove unwanted objects

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 8001621 Rev A

Celigo® Cytometer
Cell Secretion
Application Guide

Celigo Software Version 2.1

Contents

1. About this Guide

This chapter provides a brief description of this guide and how to use it.

1.1 Introduction

The Cell Secretion application provides a means of measuring the magnitude of protein secretion on a per cell basis within a population of cells. This application assumes the use of a capture matrix to sequester secreted protein in the vicinity of each producing cell. Cell bodies and secreted protein must also be fluorescently stained. Analysis using the Cell Secretion application provides a variety of intensity- and morphology-related measures, and can also be used for gating of highly-secreting cells.

1.2 Purpose

The purpose of this guide is to provide the steps and concepts that are unique to the Cell Secretion application. Information that is common to all applications is covered in *Celigo® Cytometer User Guide* (Doc. No. 8001619), from here on referred to as the User Guide.

1.3 Safety Precautions

All safety precautions described in the User Guide apply to this guide.

1.4 Technical Assistance

Contact Nexcelom Customer Service for further information:

Nexcelom Bioscience, LLC.
Customer Service
360 Merrimack St. Building 9
Lawrence, MA 01843, USA

From the United States:
email: celigosupport@nexcelom.com
phone: 978-327-5340

From Europe:
e-mail: celigosupport@nexcelom.com
phone: 978-327-5340

2. Prerequisites

The prerequisites for performing the procedures in this document are:

Familiarity with Celigo operation per the User Guide

Familiarity with the Cell Secretion product insert

Celigo cytometer is turned on per the User Guide

3. Scanning Plates

This chapter provides the procedures for choosing the Cell Secretion application and setting image acquisition parameters. You perform these tasks in the Scan tab.

3.1 Cell Secretion Application

The Cell Secretion application assumes the use of two fluorescent labels: one to serve as a whole-cell marker and the other as a label for plate-bound secreted protein (Figure 1). The Nexcelom Cell Secretion kit for human or mouse antibody secretion provides an antibody capture reagent, a red-fluorescent antibody (secretion) detection reagent, and a green-fluorescent cell detection reagent. Reagent Buffer and 384-well plates are also provided for convenient performance of cell secretion assays. This guide demonstrates the assay in this kit configuration, but other combinations of fluorophores and dyes may also be used.

In the Application section, in the Current Application field, select **Cell Secretion: Cell + Secretion** (Figure 2).

Figure 1. Cell Secretion Schematic

(A) Schematic illustrating both top and side view of labeled cell and secreted protein. Cells are labeled with a green fluorescent dye, which can appear yellow due to mixing of red and green colors. A capture reagent traps the secreted protein around the immediate vicinity of the cell.
(B) Photomicrograph of CHO cells labeled with a cytoplasmic stain (green) and a stain specific for human antibodies (red). The red area around the cells represents the captured secreted antibody.

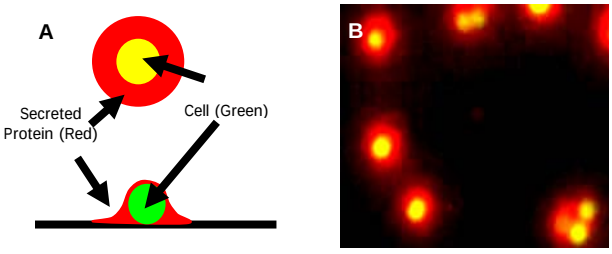


Figure 2. Selecting the Cell Secretion Application

3.2 Image Acquisition Settings

Perform the following steps to select image acquisition settings.

To select image acquisition settings

1. Select a channel to set up. Upon selection of the Cell Secretion application, note that image acquisition settings will be enabled for two fluorescent channels: Cell and Secretion (select on Channel drop-down menu). Typically, it is simplest to begin with Cell channel setup first.
2. Select a well where high fluorescent signal (and cell number) is expected.
3. In Illumination, select the proper Illumination setting for your fluorescence channel, e.g., Green 483/536 for cytoplasmic stain or Red 531/629 for secretion marker.
4. Click **Live** to see a live image.
5. Use manual focus to achieve a clear image of cells.
6. Decide if the Auto Exposure/Gain Channel provides desirable exposure and gain settings for your sample (system will attempt to determine optimal settings). Alternatively, the Custom Channel allows exposure time and gain to be set manually. Obtain an image of acceptable brightness, while minimizing any saturation of pixels (keep intensity <255). Also use an exposure time and gain such as to minimize background fluorescent signal.
7. Select if 2 x 2 Binning (half resolution) is appropriate for acquisition. For a detailed explanation of binning, see the User Guide.
8. Set up auto focus parameters by selecting **Image Based Auto Focus** or **Hardware Auto Focus** (see the User Guide). Cell channel fluorescence is typically used to set Register Focus Position.

9. Switch to the second channel (Secretion) for setup, repeating exposure/gain optimization. Find the proper focus and click “Set Offset” (see the User Guide and Figure 3).

Figure 3. Image Acquisition Settings

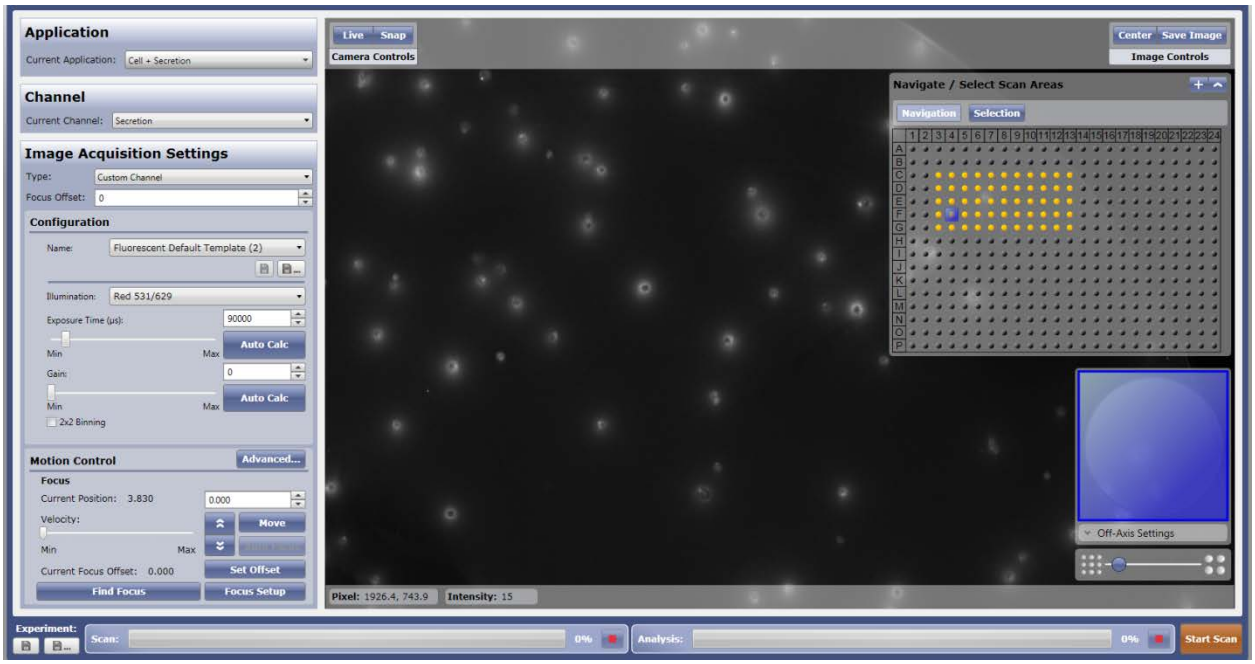
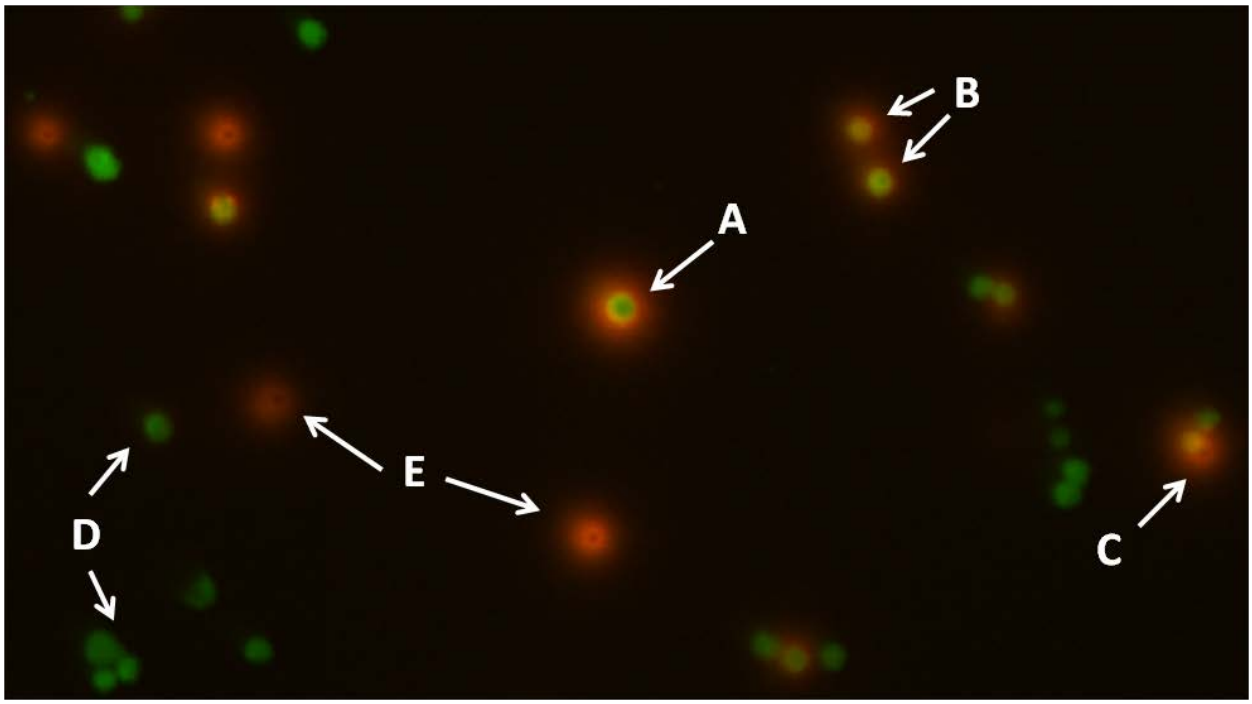


Figure 4 shows an example of two-color fluorescence Cell Secretion imaging and illustrates some cellular features that may be observed in this assay.

Figure 4. Example Cell Secretion Imaging and Features



Suspension CHO cells labeled with a cytoplasmic stain (green) and a stain specific for human antibody secretion (red).
(A) Cell demonstrating central green-yellow (due to color overlay) cell marker surrounded by large red secretion "halo".
(B) Cells demonstrating central green-yellow cell marker with red secretion halos of lower intensity and lesser area than that associated with cell (A). Stain suggests less protein secretion from (B) cells compared to cell (A).
(C) Using suspension cells in the Cell Secretion assay has a risk of cell movement during culture and handling, seen as a slight shift of the cell marker away from the center of the secretion halo. To ensure minimal disturbance of settled cells, we recommend gentle plate movements and low velocity liquid handling.
(D) Bright green cytoplasmic stain associated with either low-secretion cells or cells that have shifted away from their associated secretion halos.
(E) Green cell marker co-localization is absent from these red secretion rings, indicating significant movement of cells away from their original immediate area of cell secretion.

4. Analyzing Images

This chapter provides information on how to analyze scans from the Cell Secretion application. You perform these tasks in the Analyze tab.

4.1 Analysis Settings

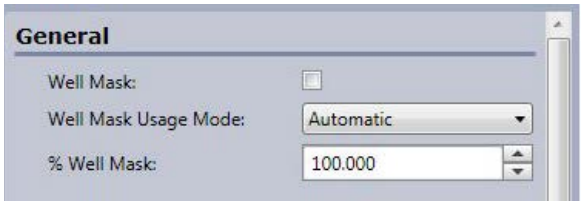
Perform the following steps to select analysis settings. The recommended initial analysis settings for identification and pre-filtering when using the Cell Secretion application are shown in Table 1. The settings typically provide good image segmentation. Alterations should be made as appropriate for scanned image quality, cell types of interest, seeding densities, secretion amounts, etc. More detailed descriptions of analysis settings are provided in subsequent subsections. For more information on the Identification and Pre-Filtering settings, see the User Guide.

To select analysis settings

The Cell Secretion application provides Identification settings for both the cell and secretion channels. Setting of analysis parameters typically begins with the cell channel.

1. In the General section, make the following selections:

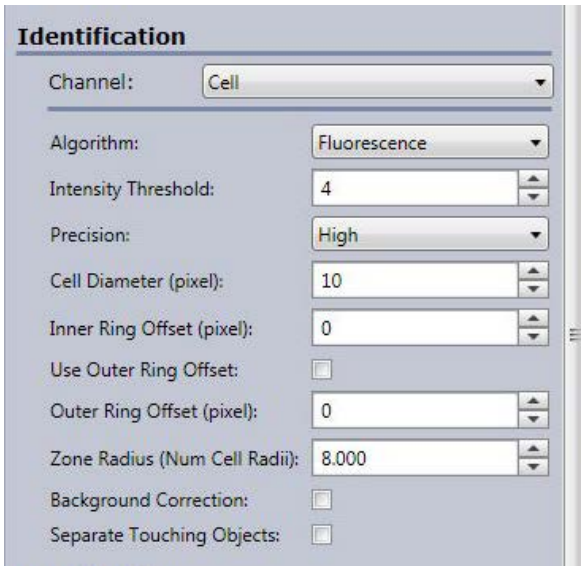
2. *Figure 5. General Section*



   a. Well Mask – Select for potentially improved analysis of cells at the well edge.
   b. Well Mask Usage Mode – Select as needed.
   c. % Well Mask – Select as needed.

3. In the Identification section, make the following selections:

Figure 6. Identification Section



a. Algorithm – Select **Fluorescence**.

b. Intensity Threshold – Enter the optimal Intensity Threshold.

   This selection determines a level of intensity used to distinguish background signal from cells. Pixels below this intensity will be disregarded from analysis. A lower threshold value will enable identification of cells of lower intensity, but may also increase the detection of background “noise”. A higher threshold value will detect only higher intensity cells, and will also decrease the number of analyzed pixels. For Intensity Thresholds in the secretion channel, also see section 4.1.2.

c. Precision – Higher precision results in more accurate identification of cell clusters. Normal recommended. Normal is sufficient to provide acceptable results, while High results in longer analysis processing time.

d. Cell Diameter (pixel) – Enter the cell diameter (in pixels) that corresponds to the cell dimensions.

   At full resolution, the Celigo cytometer provides ~1µm/pixel resolution. A Cell Diameter setting that is too high may segment cell areas that are excessively large; a setting that is too low may exclude some cell area from analysis.

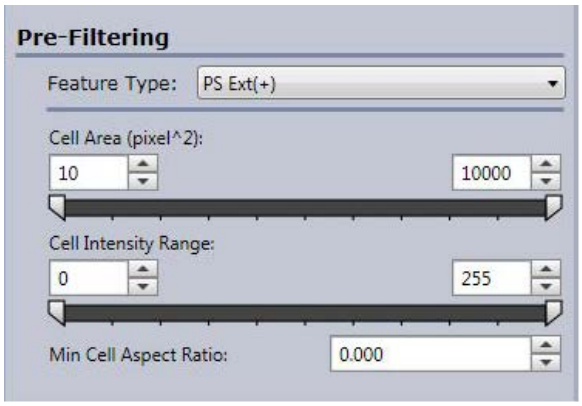
e. Inner Ring Offset, Outer Ring Offset and Zone Radius – These settings are primarily associated with secretion analysis (see section 4.1.2)

f. Background Correction – Select for potentially improved analysis of lower contrast images.

g. Separate Touching Objects – Select if it appears to be difficult to separate closely adjacent cells during segmentation.

4. Pre-Filtering settings are applied to three distinct segmentation areas: Cell Marker, Cell Area and Secretion Area (see section 4.1.1). In the Pre-Filtering section, make the following selections:

5. *Figure 7. Pre-Filtering Section*



   a. Cell Area (pixel^2) – Defines the minimum and maximum size of an analyzed cell, and may be utilized to filter out debris or artifacts from analysis.
   b. Cell Intensity Range – Establishes minimum and maximum intensity levels for analysis. This range may be set to filter out abnormally low or high intensity objects/artifacts.
   c. Min Cell Aspect Ratio – Defined as the ratio of the minor axis to the major axis of a cell. A value of 1 is a perfect circle, while lower values correspond to more oval (elongated) cells. Setting a Min Cell Aspect Ratio can eliminate less round cells from analysis.

Table 1. Recommended Initial Identification and Pre-Filtering Settings for Analysis

Parameter	Cell Channel	Secretion Channel
IDENTIFICATION		
Algorithm	Fluorescence	—
Intensity Threshold	4	40
Precision	High	—
Cell Diameter (pixel)	7	—
Inner Ring Offset (pixel)	0	—
Use Outer Ring Offset	Checkmarked	—
Outer Ring Offset (pixel)	10	—
Zone Radius (number of cell radii)	6	—
Background Correction	Not Checkmarked	—
Separate Touching Objects	Not Checkmarked	—

PRE-FILTERING			
Parameter	**Cell Marker**	**Cell Area**	**Secretion Area**
Cell Area (pixel ^2)	10-10000	10-10000	10-500000
Cell Intensity Range	0-255	0-255	0-255
Minimum Cell Aspect Ratio	0	0	0

4.1.1 Cell Marker, Cell Area and Secretion Area

Figure 5 depicts the three areas of segmentation defined by Identification and Pre-Filtering settings. The following are definitions of terminology used in the figure:

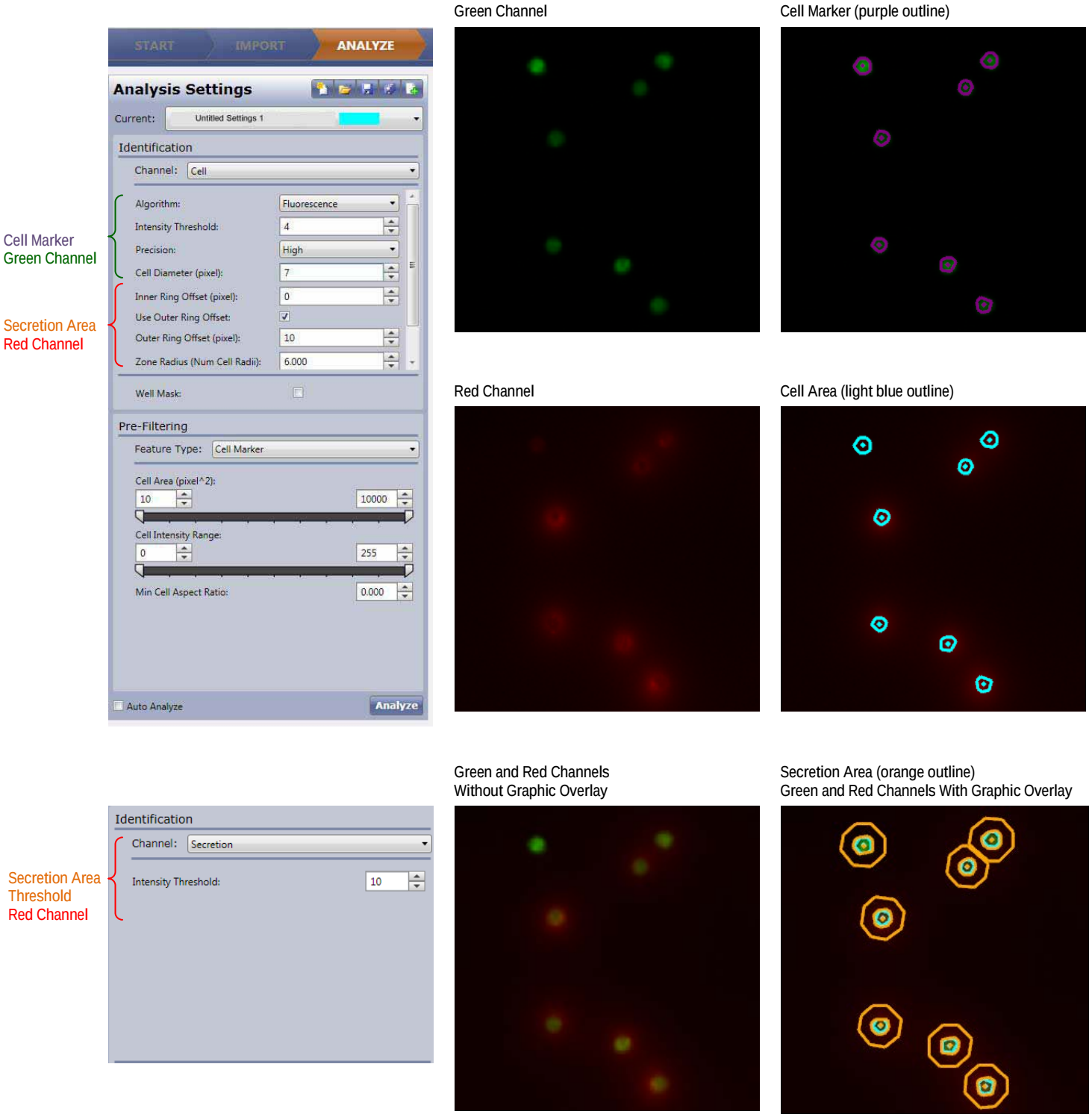
Cell Marker refers to the area defined by the whole cell cytoplasmic stain utilized in the Cell Secretion assay (default outlined in purple). Cells of interest may be thresholded by Intensity Threshold, Cell Diameter and Cell Marker Pre-Filtering settings, so as to eliminate from analysis any cells that may be undesirably small or low in stain uptake (possibly indicative of compromised viability).

Cell Area refers to an object previously outlined by Cell Marker segmentation (cell channel), but also displaying co-localized signal in the secretion channel. Segmented cells with both Cell and Secretion channel signal (within thresholded settings) will appear as light blue outlines; segmented cells with only Cell channel signal will remain outlined in purple.

Secretion Area refers to the region *outside* of a Cell Area demonstrating signal in the Secretion channel (outlined in orange). A detected Secretion Area is bound by three conditions (see section 4.1.2):

- Secretion signal is located within the Zone of Influence as defined by the Zone Radius
- Secretion signal is located within the Inner and Outer Ring Offset boundaries (if Use Outer Ring Offset is checkmarked)
- Secretion signal is higher than the Intensity Threshold value

Figure 8. Segmentation Areas in Cell Secretion Analysis



4.1.2 Detailed Secretion Area Analysis

The Zone of Influence (see Figure 6) associated with any segmented Cell Marker is defined by the Zone Radius, where the Zone Radius input is the number of cell radii desired to form the outermost distance of the Zone of Influence border (e.g., a Zone of Influence border may be up to 20 cell radii from the edge of the Cell Marker). If two or more cells are in close proximity to each other, the algorithm will set the Zone of Influence border equidistant between each cell. The Zone of Influence mask (outlined in green) can be visualized from the Cell button in the Image Display and Graphic Overlay buttons in the Analyze Tab (select the Processing Preview (**P)** button to display the processed image). Utilizing the Zone of Influence to determine secretion area analysis may be useful when appropriate Outer Ring Offsets (see below) are unknown, variable or inappropriate for the given experiment.

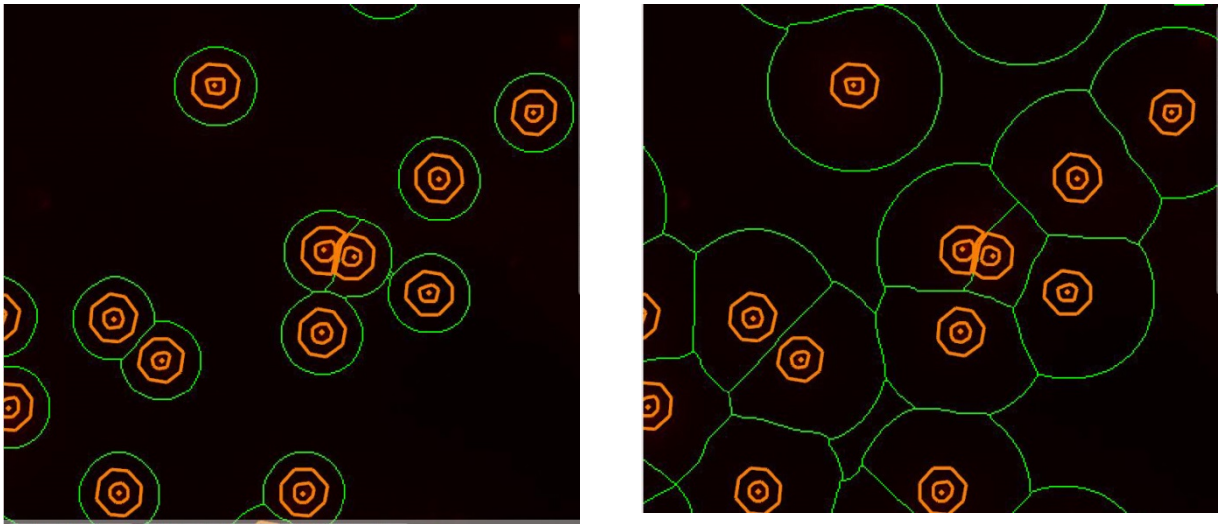
Inner Ring Offset designates how far out (in pixels) from the edge of the Cell Marker to begin the measure of secretion signal. Zero to one pixel is typically sufficient.

Outer Ring Offset, similarly to Inner Ring Offset, designates how far out (in pixels) from the edge of the Cell Marker to end the measure of secretion signal. Four to ten pixels are typically sufficient. If Use Outer Ring Offset is not checkmarked, secretion signal will be measured from the Inner Ring Offset to the Zone of Influence border (see above). The "Cell Marker Secretion Ring Mask", as defined by the fixed-dimension inner and outer rings, can be visualized from the Cell button in the Image Display and Graphic Overlay buttons in the Analyze Tab (also select the **P** button to display the processed image). Whereas the Secretion Ring Mask can extend past a detected Secretion Area, the Secretion Area cannot extend past the Ring Mask, as it is bound within it (Figure 7).

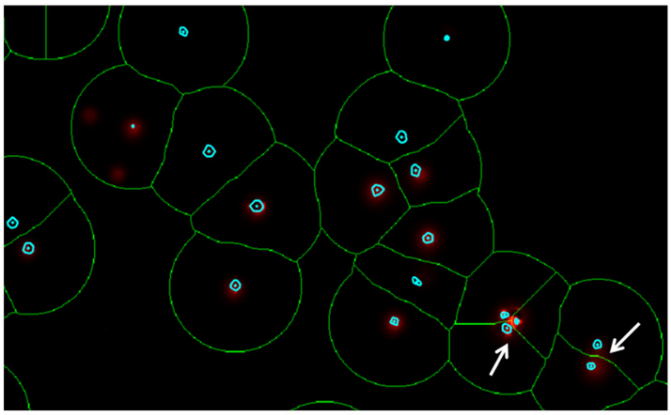
Intensity Threshold (see Figure 8) analysis setting for the Secretion channel designates the lower intensity limit on the secretion signal to be analyzed. Pixels below the Intensity Threshold are excluded from analysis. Low thresholds may result in Secretion Area segmentation that is fragmented in appearance due to inclusion of low intensity pixels (Figure 8 **(A)**); high thresholds will tend to limit analysis to higher intensity pixels (Figure 8 **(B)**). Note that the final extent of the detected Secretion Area can be affected by image signal quality. If the Intensity Threshold is set too low under high background conditions, the Secretion Area may be undesirably extended due to the presence of extraneous, non-eliminated background signal. (Figure 8 **(C)**).

Selection of appropriate Secretion Area analysis settings will depend on many factors, including experimental parameters of interest (e.g., signal intensity over a wide secretion area (Zone of Influence) vs. local intensity peaks (Ring Offsets)), dispersion/distribution of secreted protein and empirical evaluation of acceptable threshold intensities.

Figure 9. Zone of Influence



Alterations in Zone Radius affect dimensions of Zone of Influence (outlined in green; detected secretion areas outlined in orange).

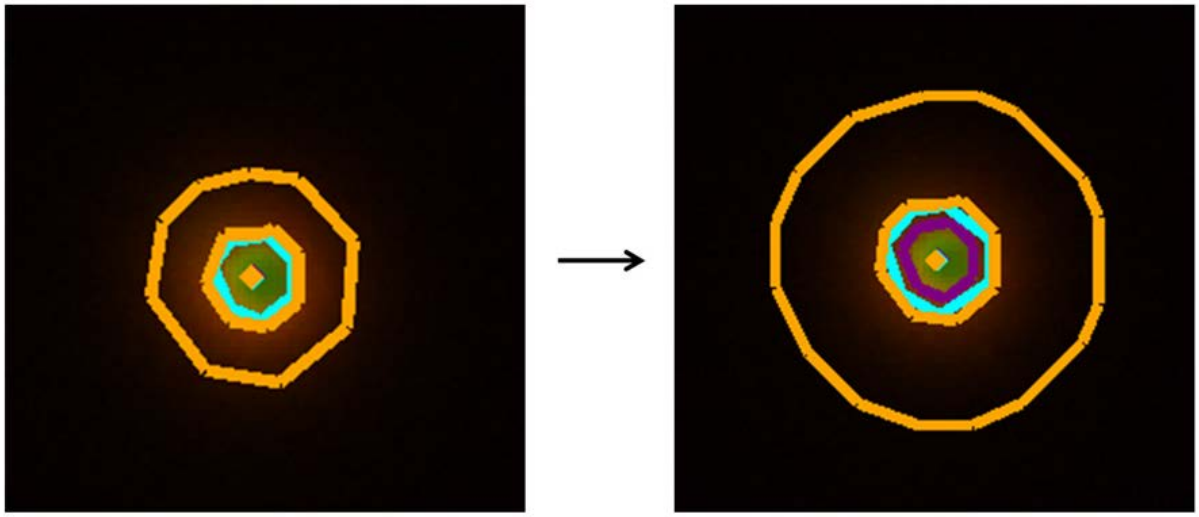


Unlike fixed-dimension Inner and Outer Ring Offsets (see Figure 7), Zones of Influence (outlined in green) can encompass unknown or variable secretion areas. Zones of Influence will also distinguish closely adjacent cells (see white arrows).

Figure 10. Inner and Outer Ring Offsets

Secretion Threshold Intensity = 10
Inner Ring Offset = 0
Outer Ring Offset = 10

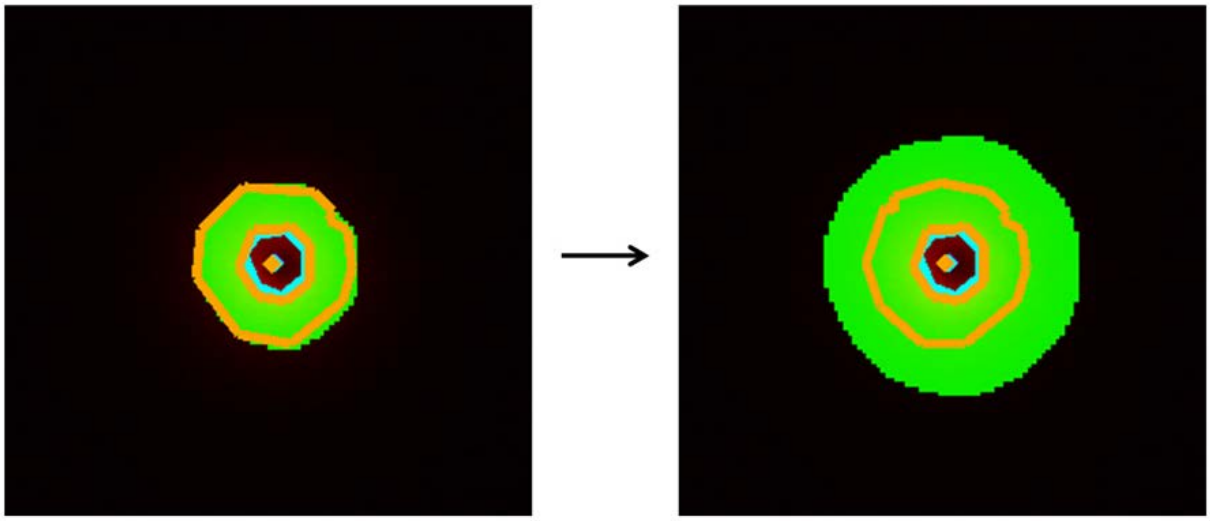
Secretion Threshold Intensity = 10
Inner Ring Offset = 3
Outer Ring Offset = 20



Changes in Inner and Outer Ring Offsets will affect the dimensions of the detected Secretion Area. Increasing the Inner Ring Offset will extend the start of the Secretion Area (inner orange ring) away from the segmented Cell Marker/Cell Area (purple/light blue outlines). Increasing the Outer Ring Offset will further extend the end of the Secretion Area (outer orange ring) away from the cell body, so long as signal still satisfies the Secretion Threshold Intensity

Secretion Threshold Intensity = 40
Inner Ring Offset = 0
Outer Ring Offset = 10

Secretion Threshold Intensity = 40
Inner Ring Offset = 0
Outer Ring Offset = 20

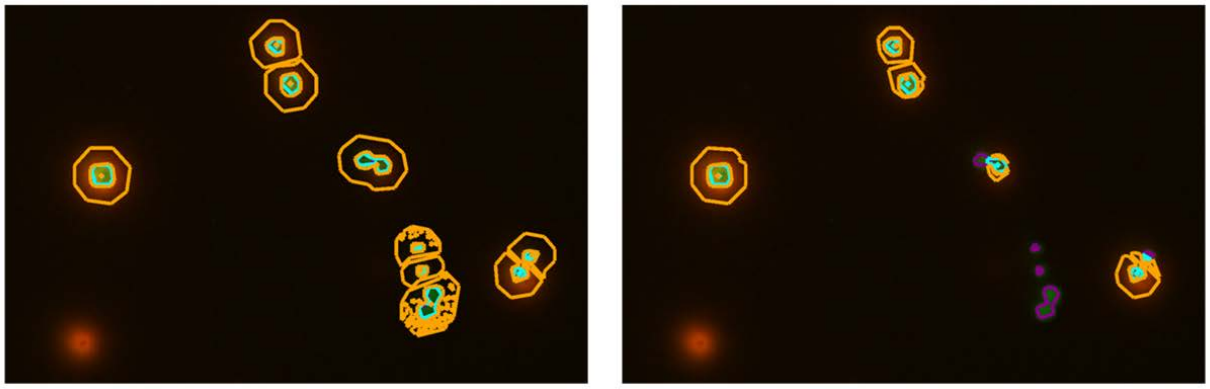


The Cell Marker Secretion Ring Mask (highlighted in green) displays the fixed area bound by the Inner and Outer Ring Offsets. Whereas the Ring Mask can extend past the detected Secretion Area (outlined in orange), the Secretion Area cannot extend past the Ring Mask, as it is bound by it (see section 4.1.1).

Figure 11. Secretion Threshold Intensity

A

Threshold Intensity = 10 **Threshold Intensity = 40**

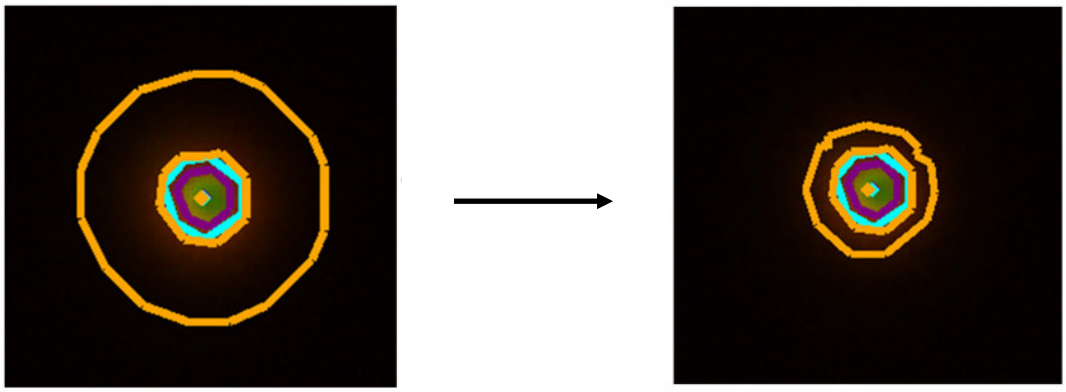


Due to the inclusion of low intensity pixels, a lower Threshold Intensity may result in cells with Secretion Areas (outlined in orange) of segmented appearance.

B

Secretion Threshold Intensity = 10
Inner Ring Offset = 3
Outer Ring Offset = 20

Secretion Threshold Intensity = 40
Inner Ring Offset = 3
Outer Ring Offset = 20



A higher Threshold Intensity will limit analysis to higher intensity pixels, regardless of Inner and Outer Ring Offset definitions

C

Secretion Threshold Intensity = 40
Inner Ring Offset = 3
Outer Ring Offset = 30

Detected Secretion Areas can be dependent upon signal quality. For a given Threshold Intensity, a high background image may result in an inaccurately large Secretion Area, due to non-eliminated background signal.

5. Gating Cells

This chapter provides information on how to select filter settings for further scan data analysis. You perform these tasks in the Gate tab.

For optimal analysis using the Cell Secretion application, you should perform gating.

In the Cell Secretion application, two classes of cells (Total and Gated) are used for calculations. These classes need to be assigned to populations. Populations are generated by selecting cells using a gate in a histogram or scatter plot, or a combination of plots.

To create a plot, gate, and populations

1. In the Plot Populations pane (Figure 9), create a histogram plot, using the Add Plot (+) button and Add Plot dialog box. Refer to the parameter descriptions in Table 2 for an explanation of the parameters that can be used in the histogram plot.

   For details, see User Guide section “Creating a plot.”

   A commonly made selection for Parameter 1 is “Cell Area: Area (um^2).”

   Some parameter selections of particular interest in cell secretion assays include:

   Distance to Neighboring Cell (μm) – selection of isolated secreting cells

   Secretion Area: Mean Intensity

   Secretion Area: Area (μm^2)

   Secretion Total Area: Integrated Intensity

Table 2. Plot Parameter Descriptions

Feature	Description
X Position (μm)	Location of a cell along the horizontal axis of the well: left (-μm) or right (+μm) of the center (origin) of the well
Y Position (μm)	Location of a cell along the vertical axis of the well: below (-μm) or above (+μm) the center (origin) of the well
Distance to Neighboring Cell (μm)	Distance from the target cell to the closest neighboring cell
Distance of Cell to Well Center (μm)	Distance from the target cell to the center (origin) of the well
Secretion Area[1]: Integrated Intensity[2]	Sum of all pixel intensities within the region outside of a Cell Area[3] used to measure Secretion Area[4] intensities (integrated intensity[1] of secreted protein outside cell)
Secretion Area[1]: Mean Intensity	Mean intensity of the region outside of a Cell Area[3] used to measure Secretion Area[1] intensities (mean intensity of secreted protein outside cell)
Secretion Area[1]: Standard Deviation	Standard deviation of all individual pixel intensities within the region outside of a Cell Area[3], used to measure Secretion Area[4] intensities (standard deviation of secreted protein intensities outside cell)
Secretion Area[1]: Area (μm^2)	Area of the region outside of a Cell Area[3] used to measure Secretion Area[1] intensities (area of secreted protein outside cell)
Secretion Area[1]: Form Factor	"Compactness" of the Secretion Area[1], derived from the ratio of its perimeter to its area; a circle with a value of 1 is most compact; a more convoluted shape with a value less than 1 is less compact (compactness of secreted protein area outside cell)
Secretion Area[1]: Smoothness	"Evenness of contour" of the Secretion Area[4], derived from the ratio of its convex perimeter to its true perimeter (see the User Guide); a perfectly smooth area has a value of 1; a rough or jagged area has a value less than 1 (evenness of contour of secreted protein outside cell)
Secretion Area[1]: Aspect Ratio	Ratio of the minor axis to the major axis of the Secretion Area[1]; a value of 1 is a perfect circle; values less than 1 suggest an oval (elongated) object (roundness of secreted protein area outside cell)
Cell Area[3]: Integrated Intensity[2]	Sum of all pixel intensities within the cell body displaying signal in the Secretion channel (integrated intensity[2] of secreted protein on cell body)
Cell Area[3]: Mean Intensity	Mean intensity of the cell body region in the Secretion channel (mean intensity of secreted protein on cell body)
Cell Area[3]: Standard Deviation	Standard deviation of all individual Secretion channel pixel intensities within the cell body (standard deviation of secreted protein intensities on cell body)

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Feature	Description
Cell Area[3]: Area (μm[2])	Area of the cell body displaying signal in the Secretion channel (area of secreted protein on cell body)
Cell Area[3]: Form Factor	"Compactness" of the Cell Area[3], derived from the ratio of its perimeter to its area; a circle with a value of 1 is most compact; a more convoluted shape with a value less than 1 is less compact (compactness of secreted protein on cell body)
Cell Area[3]: Smoothness	"Evenness of contour" of the Cell Area[3], derived from the ratio of its convex perimeter to its true perimeter (see the User Guide); a perfectly smooth area has a value of 1; a rough or jagged area has a value less than 1 (evenness of contour of secreted protein on cell body)
Cell Area[3]: Aspect Ratio	Ratio of the minor axis to the major axis of the Cell Area[3]; a value of 1 is a perfect circle; values less than 1 suggest an oval (elongated) object (roundness of secreted protein area on cell body)
Cell Area Marker[4]: Integrated Intensity[1]	Sum of all pixel intensities within the cell body displaying signal in the Cell channel (integrated intensity[2] of cytoplasmic dye)
Cell Area Marker[4]: Mean Intensity	Mean intensity of the cell body region in the Cell channel (mean intensity of cytoplasmic dye)
Secretion Total Area: Area (μm[2])	Area of the cell body used to measure Cell Area[3] intensities + the region outside of the Cell Area[3] used to measure Secretion Area[4] intensities (total area of secreted protein on the cell body and outside the cell)
Secretion Total Area: Integrated Intensity[2]	Sum of all Cell Area[3] + Secretion Area[4] pixel intensities (total integrated intensity of secreted protein on the cell body and outside the cell)
FOOTNOTE**S:** [1]Secretion Area = Region outside of a Cell Area displaying signal in Secretion Channel, subject to an Intensity Threshold and localization within both the Zone of Influence and the borders set by Inner and Outer Ring Offsets (secreted protein outside of the cell body) [2]Integrated Intensity = Sum of all pixel-level intensities within a region [3]Cell Area = Region previously defined by Cell Marker, but also displaying co-localized signal in Secretion Channel (secreted protein on the cell body) [4]Cell Area Marker = Region defined by whole cell cytoplasmic stain in Cell Channel (cell body)	

For more information on creating plots, see User Guide section "Creating a Plot."

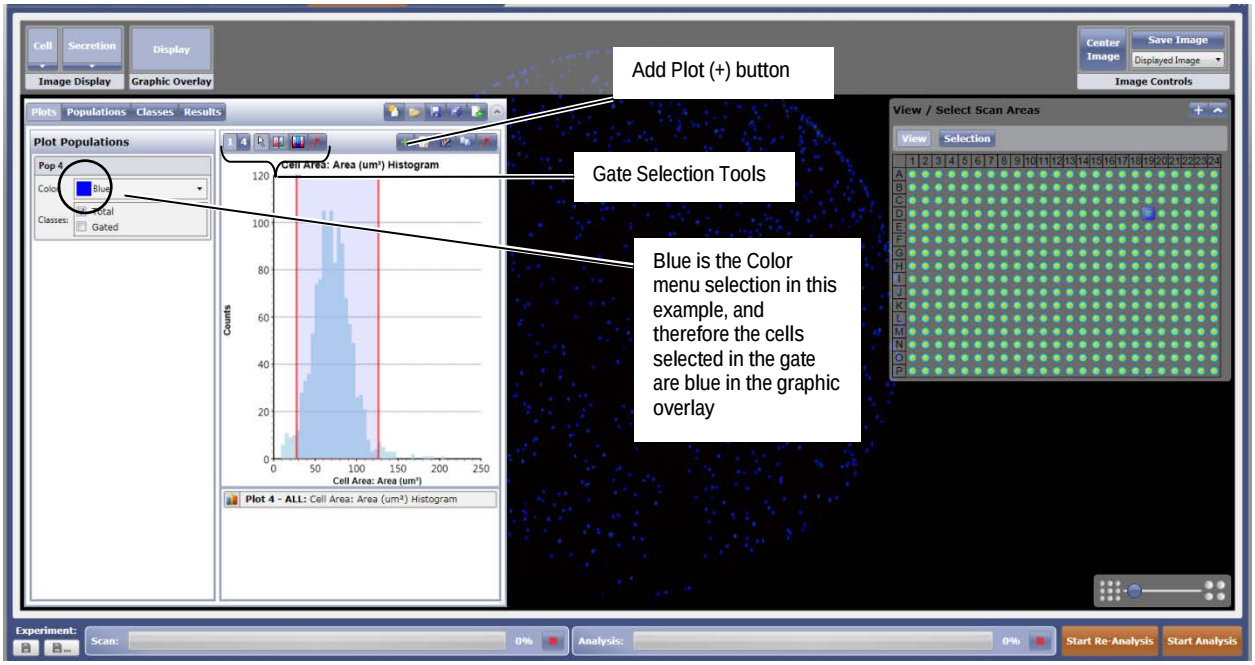
When you create the plot, the system by default assigns the class called "Total" to the population for the entire well, called the "ALL" population.

2. Create a gate on the plot, using the gate selection tools (Figure 9).

   For details, see User Guide section "Creating a Gate."

   In the Graphic Overlay display, the color displayed for the plot population selected in the Plot corresponds to the Color menu selection (Figure 9).

Figure 12. Gating Cells



3. Repeat steps 1 and 2 as needed to refine the population that you want to analyze.
4. Assign the Total class to the population as follows:
   d. Click the gate.
   e. In the Plots, Populations, or Classes view, checkmark the Total class.

   For details, see User Guide section “Assigning a Class to a Population.”
5. In the Plot Populations pane, create a scatter plot based on cell area, using the Add Plot (+) button and Add Plot dialog box. Make the following selections:

   Pick a source population: Typically, you select the Total population that you assigned in step 4.

   Pick a plot type: Scatter plot

   Parameter 1: Cell Area Marker: Mean Intensity

   Parameter 2: Secretion Total Area: Integrated Intensity

   For more information on creating plots, see User Guide section “Creating a Plot.”
6. Create a gate on the scatter plot, using the gate selection tools.

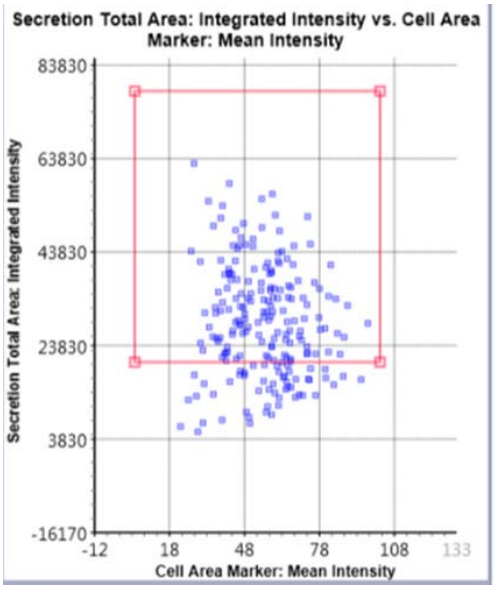
   For details, see User Guide section “Creating a Gate.”

   In the Graphic Overlay display, the color displayed for the plot population selected in the Plot corresponds to the Color menu selection.
7. On the scatter plot (Secretion Total Area: Integrated Intensity vs. Cell Area Marker: Mean Intensity) (Figure 10), drag the gate so that it captures only the cells that are positive for Cell Secretion. Figure 10 shows examples of a gate

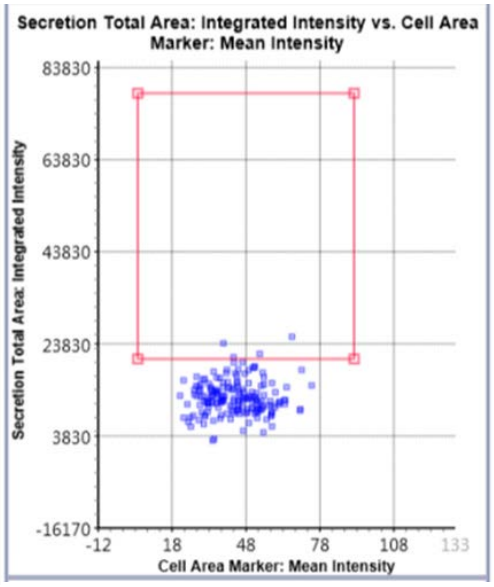
capturing positive control wells (wells with a high percentage of positive cells) versus negative control wells (wells with a low percentage of positive cells).

Figure 13. Scatter Plot

Positive Control Wells

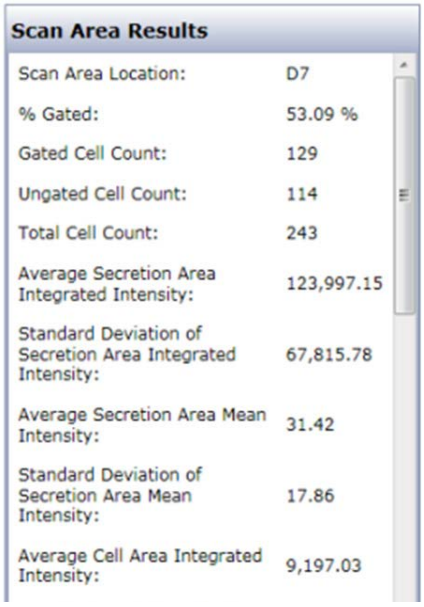


Negative Control Wells



To capture only the positive wells, it is helpful to review the Scan Area Results pane (Figure 11) to see a preview of the well data that the gate is capturing. Adjust the gate position as needed to change the data that the gate is capturing.

Figure 14. Scan Area Results Pane



8. On the scatter plot, assign the Cell Secretion class as follows:

a. On the scatter plot, click the gate.
b. In the Plots, Populations, or Classes view, make sure that the Gated class is checkmarked.

6. Viewing Results

This chapter describes the feature outputs available from the Cell Secretion application.

Table 3. Well-Level Features Available

Feature	Description
FEATURE REPORTED FOR ALL WELLS	
% Gated	Percentage ratio of gated cells count divided by Total cell count
Gated Cell Count	Number of cells kept for well analysis following gating
Ungated Cell Count	Number of cells removed from well analysis following gating
Total Cell Count	Total number of gated and non-gated cells counted within the well (within Cell Marker thresholding)
% Well Sampled	Surface of the well that was sampled
FEATURE REPORTED FOR EACH CLASS	
Average Secretion Area[4] Integrated Intensity[1]	Sum of all Secretion Area[4] pixel intensities divided by the total number of secreting cells in the class
Standard Deviation of Secretion Area[4] Integrated Intensity[1]	Standard deviation of all individual Secretion Area[4] Integrated Intensities within the class
Average Secretion Area[4] Mean Intensity	Mean intensity of each Secretion Area[4], averaged over all the cells in the class
Standard Deviation of Secretion Area[4] Mean Intensity	Standard deviation of all individual Secretion Area[4] Mean Intensities within the class
Average Cell Area[3] Integrated Intensity[1]	Sum of all Cell Area[3] pixel intensities divided by the total number of secreting cells in the class
Standard Deviation of Cell Area[3] Integrated Intensity[1]	Standard deviation of all individual Cell Area[3] Integrated Intensities within the class

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Feature	Description
FEATURE REPORTED FOR EACH CLASS	
Average Cell Area[3] Mean Intensity	Mean intensity of each Cell Area[3], averaged over all the cells in the class
Standard Deviation of Cell Area[3] Mean Intensity	Standard deviation of all individual Cell Area[3] Mean Intensities within the class
Average Total Secretion Area[4] Integrated Intensity[1]	Sum of all Cell Area[3] + Secretion Area[4] pixel intensities divided by the total number of secreting cells in the class
Standard Deviation of Total Secretion Area[4] Integrated Intensity[1]	Standard deviation of all individual Cell Area[3] + Secretion Area[4] Integrated Intensities within the class
Average Secretion Area[4] (μm^2)	Area of the region outside of a Cell Area[3] used to measure Secretion Area[4] intensities, averaged over all the cells in the class
Average Cell Area[3] (μm^2)	Area of the cell body used to measure Cell Area[3] intensities, averaged over all the cells in the class
Average Total Secretion Area[4] (μm^2)	Area of the cell body used to measure Cell Area[3] intensities + the region outside of the Cell Area used to measure Secretion Area[4] intensities, averaged over all the cells in the class
Average Secretion Integrated Intensity[1] Ratio: Cell / Secretion Area[4]	Ratio of the average secretion integrated intensity[1] for the cell and the secretion area[4]
Standard Deviation of Secretion Integrated Intensity[1] Ratio: Cell / Secretion Area[4]	Standard deviation of the ratio of the average secretion integrated intensity[1] for the cell and the secretion area[4]
Average Secretion Mean Intensity Ratio: Cell / Secretion Area[4]	Ratio of the average secretion mean intensity for the cell and the secretion area[4]
Standard Deviation of Secretion Mean Intensity Ratio: Cell / Secretion Area[4]	Standard deviation of the ratio of the average secretion mean intensity for the cell and the secretion area[4]

FOOTNOTES:

[1]Integrated Intensity = Sum of all pixel-level intensities within a region

[2]Cell Marker = Region defined by whole cell cytoplasmic stain in Cell Channel (cell body)

[3]Cell Area = Region previously defined by Cell Marker, but also displaying co-localized signal in Secretion Channel (secreted protein on the cell body)

[4]Secretion Area = Region outside of a Cell Area displaying signal in Secretion Channel, subject to an Intensity Threshold and localization within both the Zone of Influence and the borders set by Inner and Outer Ring Offsets (secreted protein outside of the cell body)

7. Troubleshooting

This chapter provides troubleshooting recommendations.

Table 4. Troubleshooting Recommendations

Issue	Recommended Action
Low signal in either cell channel or secretion channel	1. Low signal intensity may result from inadequate acquisition settings • Increase exposure time or gain • Confirm properly selected fluorescent filters 2. Low signal intensity may also result due to factors in the Cell Secretion assay process • Titrate cytoplasmic dye concentration • Titrate amount of fluorophore/reagent required to detect secreted protein • Confirm secretion from positive-control cell type • Increase secretion duration for low-secreting cells
High image background/ cellular signal	1. High background images can result in difficult image segmentation, and may result from inadequate acquisition settings • Decrease exposure time or gain • Utilize Background Correction during analysis 2. Residual cytoplasmic dye or secretion detection reagent can cause high background • Titrate cytoplasmic dye concentration • Titrate amount of fluorophore/reagent required to detect secreted protein • Increase the number of plate washes to reduce residual fluorophore • Employ multi-step staining strategies to maximize rinsing of residual dye/detection reagent 3. Very high secretion signal or overlapping secretion halos may result from an excessively long secretion duration • Decrease secretion duration for highly secreting cells
Cells/secretion being detected in both fluorescence channels	1. Channel bleed-through can result from inappropriate acquisition settings • Decrease exposure time or gain 2. Excessively high stain concentrations can also cause fluorescence bleed-through between channels • Titrate cytoplasmic dye concentration • Titrate amount of fluorophore/reagent required to detect secreted protein
Cell bodies not associated with secretions halos	Cells (particularly gravity-settled suspension cells) may dissociate from their corresponding secretion halos due to excessive movement • Minimize rapid or jarring plate movement • Minimize disturbance of cells at bottom of plate (e.g., leave residual volumes during washes) • Use low flow velocity liquid handling of plates (automated or manual)

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 8001622 Rev A

Celigo® Cytometer
Cell Viability
Application Guide

Celigo Software Version 2.1

Contents

1. About this Guide

1.1 Introduction

The cell viability assay is a method for studying cytotoxicity, cell health, and cell death. The fluorescent viability assay is based on the simultaneous detection of live and/or dead cells with probes that reflect cellular activities and plasma membrane integrity. Alternatively, either live or dead cells are assayed as a proportion of the total cells present. Live cells are usually distinguished by active enzymatic activity as detected by fluorescent dyes retained within live cells. Late-apoptotic, necrotic, and dead cells are characterized by a compromised plasma membrane and take up membrane-impermeant dyes, such as propidium iodide. All cells can be detected using a stain, such as Hoechst, which stains both live and dead cells. Multi-channel images are acquired and analyzed providing live, dead, and total cell counts and the percentage of live and dead cells.

1.2 Purpose

The purpose of this guide is to provide the steps and concepts that are unique to the Cell Viability application. Information that is common to all applications is covered in *Celigo® Cytometer User Guide* (Doc. No. 8001619), from here on referred to as the User Guide.

1.3 Safety Precautions

All safety precautions described in the User Guide.

1.4 Technical Assistance

Contact Nexcelom Customer Service for further information:

Nexcelom Bioscience, LLC.
Customer Service
360 Merrimack St. Building 9
Lawrence, MA 01843, USA

From the United States:
email: celigosupport@nexcelom.com
phone: 978-327-5340

From Europe:
e-mail: celigosupport@nexcelom.com
phone: 978-327-5340

2. Prerequisites

The prerequisites for performing the procedures in this document are:

Familiarity with Celigo operation per the User Guide

Celigo cytometer is started up per the User Guide

3. Scanning Plates

This chapter provides the procedures for choosing the appropriate Cell Viability application and setting the image acquisition parameters. You perform these tasks in the Scan tab.

3.1 Cell Viability Application

Perform the following steps to select a Cell Viability application.

To select a Cell Viability application

In the Current Application dropdown list, select one of the four Cell Viability applications (Figure 1 and Table 1), based on the dyes you want to use, as follows:

Figure 1. Selecting a Cell Viability Application

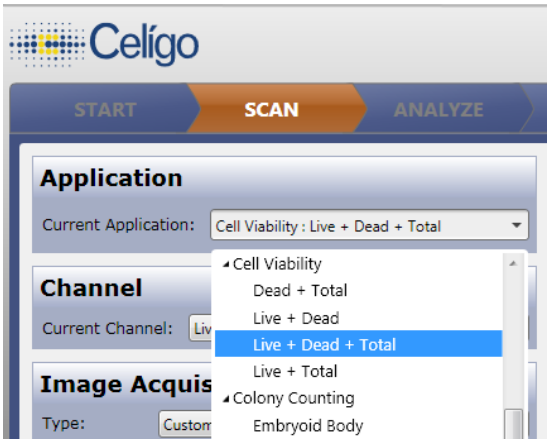


Table 1. Selecting a Cell Viability Application

If using this dye . . .	Select this application . . .
Propidium Iodide and Hoechst 33342	Dead + Total
Calcein AM and Propidium Iodide	Live + Dead
Calcein AM, Propidium Iodide, and Hoechst 33342	Live + Dead + Total
Calcein AM and Hoechst 33342	Live + Total

	**NOTE: Although the Cell Viability application uses three dyes (calcein AM, propidium iodide, and Hoechst 33342), many users prefer to use a combination of only two dyes. This practice allows one channel to be used for running another assay in parallel. The options allow you to select various combinations of two or three dyes.**

3.2 Acquisition Settings

Perform the following steps to select image acquisition settings. For the recommended initial image acquisition settings to use as a guide, see Table 2.

To select image acquisition settings

1. Choose a well for setup using the plate map by clicking **Navigation** and selecting a representative well.

   It is recommended that you adjust exposure settings using a well where the highest fluorescent signal is expected.

2. In Current Channel, select the first channel you are setting up (**Live**, **Dead**, or **Total**).

3. For the selected channel in Current Channel, make the following selections:
   a. In Type, select **Custom Channel**.
   b. In Illumination, select the appropriate illumination for the dye (e.g., **Blue** to visualize the Hoechst signal).
   c. Click **Live** to see a live image.
   d. Use manual focus to achieve clear image of cells.
   e. Select whether Autoexposure provides optimal exposure for your sample (cells are visible yet not overexposed, i.e., saturated: pixels intensities >254).
      - Autoexposure: The system will attempt to determine the optimal exposure time and gain setting.
      - Custom: User manually establishes optimal exposure time and gain by adjustment of Exposure Time and Gain. When doing so, adjust the exposure so the majority of cells aren't overexposed.
   f. If binning (half resolution) is appropriate for acquisition, select **2x2 Binning**. For a detailed explanation of binning, see the User Guide.
   g. Set up autofocus (see the User Guide).
      - Typically, it is recommended that you select **Hardware Auto Focus** (because it provides maximum speed) and click **Register Auto**. For manual registration or using focus offset, see the User Guide.

4. In Current Channel, select the next channel to set up (**Live**, **Dead**, or **Total**).

5. For the selected channel in Current Channel, make the following selections:
   a. In Type, select Custom Channel.
   b. In Illumination, select the appropriate illumination for the dye (e.g., **Green** to visualize the calcein AM signal, **Red** to visualize the propidium iodide signal).
   c. Click **Live** to see a live image.
   d. Click **Find Focus** to achieve a clear image of the cells.
   e. Click **Set Offset**.

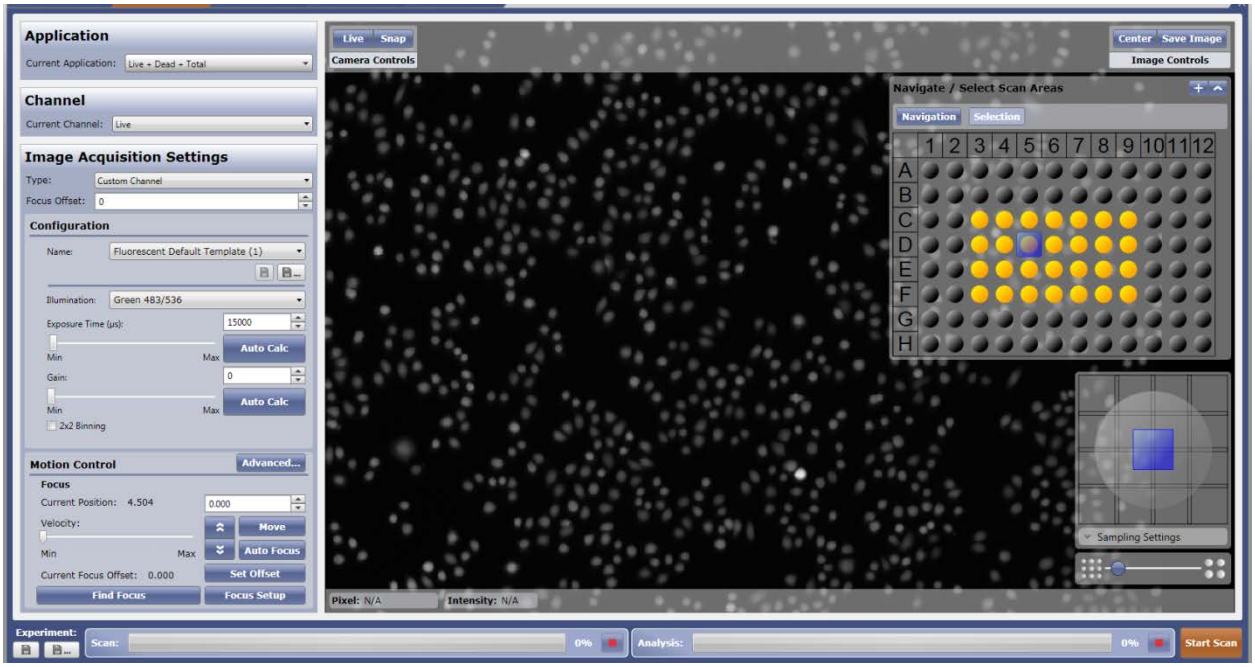
6. Set up the remaining channel (Live, Dead, or Total) by repeating steps 4 through 5e.

Table 2. Recommended Initial Settings for Image Acquisition

Current Channel	Live	Dead	Total
Type	Custom Channel	Custom Channel	Custom Channel
Focus Offset	User determined	User determined	User determined
Name	Fluorescent Default Template (1)	Fluorescent Default Template (2)	Fluorescent Default Template (3)
Illumination	Green 483/536	Red 531/629	Blue 377/447
Exposure Time (µs)	10000	40000	100000
Gain	0	0	0
Motion Control and Focus	See User Guide	See User Guide	See User Guide

Figure 2 shows an example of a Live channel.

Figure 2. Live Channel



4. Analyzing Images

This chapter describes how to set up analysis for the Cell Viability application. You perform these tasks when in the Analyze tab.

In this application, images from each channel are segmented according to user-specified analysis settings and cells are identified in each channel.

	**NOTE: Only objects that spatially merge with objected identified in the Total channel are included in the analysis.**

4.1 Analysis Settings

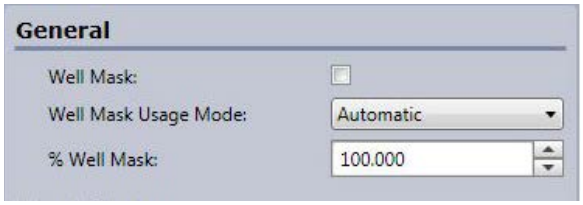
Perform the following steps to select the optimal analysis settings. The recommended initial analysis settings for identification and pre-filtering when using the Cell Viability application are shown in Table 3. The settings typically provide good image segmentation.

For a detailed explanation of the identification and pre-filtering settings, see the User Guide.

To select analysis settings

1. Load prior saved Analysis Settings if available.
2. In the General section (Figure 3), make the following selections:

Figure 3. General Section

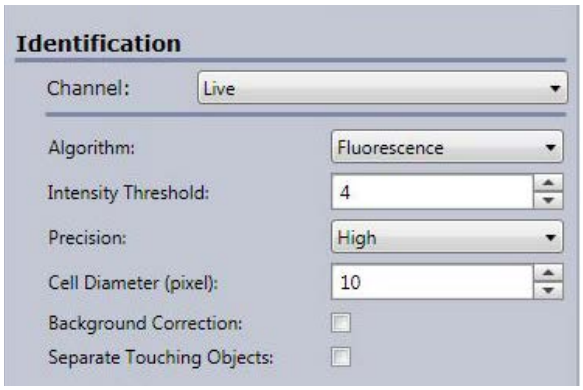


   a. Well Mask – Uncheckmark.
   b. Well Mask Usage Mode – Selected as needed.
   c. % Well Mask – Selected as needed. Decreasing the % Well Mask entry can exclude well edge artifacts that can occur during plate manufacturing.

      For information about Well Mask, Well Mask Usage Mode, and % Well Mask, see the User Guide section “Selecting General Analysis Settings.”

3. In the Identification section (Figure 4), make the following selections,

Figure 4. Identification Section:

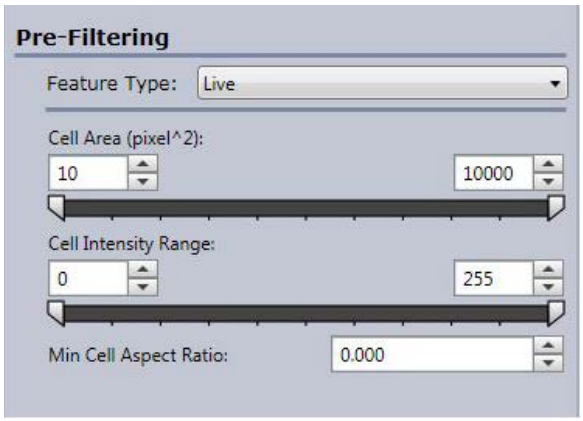


a. Channel – Select one channel to be analyzed.
b. Algorithm – Select **Fluorescence**.
c. Intensity Threshold, – Enter the optimal intensity threshold.

   The intensity threshold is the level of intensity that separates background from cells. With an appropriate threshold set, background pixels fall below, and pixels in cells are above the threshold. Any pixels below the threshold are not considered in calculation.
d. Precision – Select the appropriate precision level (High is recommended). For a detailed explanation of Precision, see the User Guide.
e. Cell Diameter (pixel) – Enter the cell diameter (in pixels) that corresponds to your cells. At full resolution, the Celigo provides 1 µm/pixel. Binned pixels provide 2 µm/pixel.
f. Background Correction – Select as needed.
g. Separate Touching Objects – Recommended for the Total or Dead channel only: Select if it is difficult to separate touching cells during segmentation. For more information on Separate Touching Objects, see the User Guide.

4. In the Pre-Filtering section (Figure 5), make the following selections:

Figure 5. Pre-Filtering Section



a. Feature Type.
b. Cell Area Range – Enter an appropriate range.
c. Cell Intensity Range – Enter if necessary.

	NOTE: Typically, the cell intensity range filter remains set from 0 to 255. Only cell clumps or debris should be eliminated from the analysis using the area filter.

d. Min Aspect Ratio – Enter if necessary.
   - Aspect ratio measures an objects elongation and is often used to remove artifacts and debris.

5. Repeat this section for each remaining channel.

Table 3. Recommended Initial Identification and Pre-Filtering Settings for Analysis

Parameter	Live	Dead	Total
IDENTIFICATION			
Algorithm	Fluorescence	Fluorescence	Fluorescence
Intensity Threshold	4	3	4
Precision	High	High	High
Cell Diameter (pixel)	15	10	10
Background Correction	Not Checkmarked	Not Checkmarked	Not Checkmarked
Separate Touching Objects	Not Checkmarked	Checkmarked	Checkmarked
PRE-FILTERING			
Cell Area (pixel ^2) Range	35 - 1000	30 - 1000	30 - 1000
Cell Intensity Range	0 - 255	0 - 255	0 - 255
Min Cell Aspect Ratio	0	0	0

Figure 6 and Figure 7 show examples of displays after entering analysis settings.

In Figure 6, the calcein AM, propidium iodide, and Hoechst stains are pseudocolored green, red, and blue, respectively.

In the corresponding target overlay in Figure 7, the calcein AM overlay is purple, propidium iodide overlay is light blue, and the Hoechst overlay is orange.

Figure 6. Cell Viability 3-Channel Image of HeLa Cells Treated with Hydrogen Peroxide

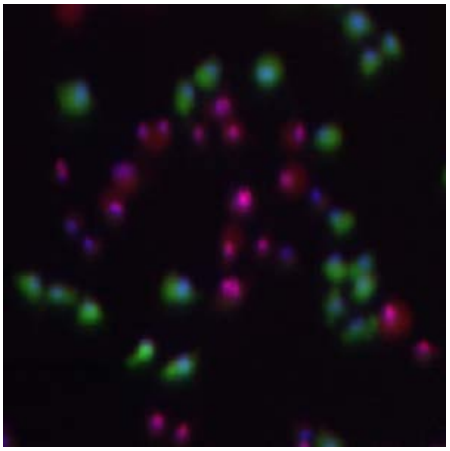
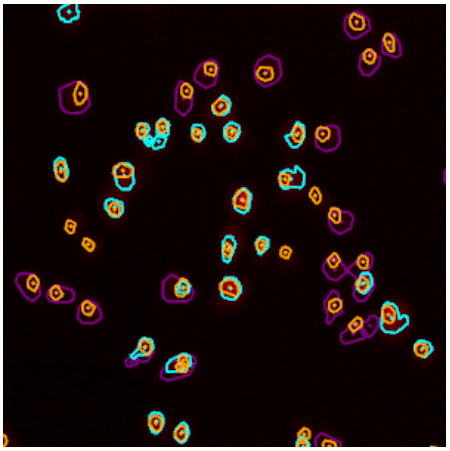


Figure 7. Corresponding Target Overlay



5. Gating Cells

When using the Cell Viability application, gating is typically not performed in the Gate tab. Instead, the application relies on the identification of negative and positive cells using intensity thresholding in the Analyze tab. The pre-filtering parameters in the Analyze tab (see chapter 4) manage the filtering of debris.

6. Viewing Results

This chapter describes the feature outputs available from the application's Results tab.

6.1 Application Outputs

The parameters listed in Table 4 appear below the Display Options section in the Scan Information pane.

Table 4. Cell Viability Application Outputs

Parameter	Description	Application			
		Live + Total	Dead + Total	Live + Dead	Live + Dead + Total
% Live	(Live Count / Total Count) x 100	√		√	√
% Dead	(Dead Count / Total Count) x 100		√	√	√
% Live (corrected)	((Live Count – Live+Dead Count) / Total Count) x 100			√	√
Live Count	Number of Live Cells with stain intensity above a user-defined intensity threshold	√		√	√
Dead Count	Number of Dead Cells with stain intensity above a user-defined intensity threshold		√	√	√
Total Count	Number of Total Cells with stain intensity above a user-defined intensity threshold	√	√		√
Live+Dead Count	Number of cells stained with Live stain and Dead stain above user-defined thresholds.			√	√

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Parameter	Description	Application			
		Live + Total	Dead + Total	Live + Dead	Live + Dead + Total
% Well Sampled	Percent of well surface processed	√	√	√	√
Average Live Mean Intensity	Average of cell-level Live mean stain Intensities above user-supplied threshold	√		√	√
Standard Deviation of Live Mean Intensity	Standard deviation of cell-level Live mean stain Intensities above user-supplied threshold	√		√	√
Average Live Integrated Intensity	Average of cell-level Live integrated stain Intensities above user-supplied threshold	√		√	√
Standard Deviation of Live Integrated Intensity	Standard deviation of cell-level Live integrated stain Intensities above user-supplied threshold	√		√	√
Average Dead Mean Intensity	Average of cell-level Dead mean stain Intensities above user-supplied threshold		√	√	√
Standard Deviation of Dead Mean Intensity	Standard deviation of cell-level Dead mean stain Intensities above user-supplied threshold		√	√	√
Average Dead Integrated Intensity	Average of cell-level Dead integrated stain Intensities above user-supplied threshold		√	√	√
Standard Deviation of Dead Integrated Intensity	Standard deviation of cell-level Dead integrated stain Intensities above user-supplied threshold		√	√	√
Average Total Mean Intensity	Average of cell-level Total mean stain Intensities above user-supplied threshold	√	√		√
Standard Deviation of Total Mean Intensity	Standard deviation of cell-level Total mean stain Intensities above user-supplied threshold	√	√		√
Average Total Integrated Intensity	Average of cell-level Total integrated stain Intensities above user-supplied threshold	√	√		√
Standard Deviation of Total Integrated Intensity	Standard deviation of cell-level Total integrated stain Intensities above user-supplied threshold	√	√		√

For instructions on viewing scan details, see the User Guide.

6.2 Data Export

Well-level and Object-level data can be exported into CSV (Comma Separated Value) or FCS (Flow Cytometric Standard) files. For the procedures to perform data export, see the User Guide.

7. Troubleshooting

This chapter provides troubleshooting recommendations.

Table 5. Troubleshooting Recommendations

Issue	Recommended Action
Cells are being detected in every channels	1. If exposure settings are too long, cells are overexposed and the dynamic range of pixel intensities is reduced. This results in the segmentation improperly detecting cells that should be negative. • Reduce the exposure time. 2. When the concentration of dye for a specific cell type is too high, cell stains are very bright and can cause bleedthrough in the other channels. This also results in the segmentation improperly detecting cells that should be negative . • Titrate and reduce the dye concentrations.
High image background	When images have a very high background, it becomes difficult to properly segment the images. • Wash plate wells more thoroughly and consider reducing dye concentrations.
Improper cell counts on well edges	When cells are plated at high density, they become more difficult to segment accurately, especially on the well edges. • Plate cells at lower density. • An alternative is to use well sampling and acquire only the center of the well where cells can easily be identified and counted accurately.
Cannot Identify cells	If pre-filtering is applied, some objects are filtered out and no longer part of the analysis. • Expand the lower and upper limits of the filters.

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 8001623 Rev A

Celigo® Cytometer
Colony Counting: Embryoid Body Application Guide

Celigo Software Version 2.1

Contents

1. About this Guide

This chapter provides a brief description of this guide and how to use it.

1.1 Introduction

The Colony Counting: Embryoid Body (EB) application identifies and counts individual EBs and small clusters of EBs using brightfield imaging. This application determines the diameter, area, shape, and density of EBs. This application may be used to analyze other spheroid populations.

1.2 Purpose

The purpose of this guide is to provide the steps and concepts that are unique to the Colony Counting: EB application. Information that is common to all applications is covered in the User Guide (Doc. No. 8001619), from here on referred to as the User Guide.

1.3 Safety Precautions

All safety precautions described in the User Guide apply to this guide.

1.4 Technical Assistance

Contact Nexcelom Customer Service for further information:

Nexcelom Bioscience, LLC.
Customer Service
360 Merrimack St. Building 9
Lawrence, MA 01843, USA

- From the United States:
  email: celigosupport@nexcelom.com
  phone: 978-327-5340
- From Europe:
  e-mail: celigosupport@nexcelom.com
  phone: 978-327-5340

2. Prerequisites

The prerequisites for performing the procedures in this document are:

- Familiarity with Celigo operation per the User Guide
- Celigo cytometer is started up per the User Guide

	NOTE: When preparing EBs for image analysis the volume of medium and number of EBs should be considered. For 6-well plates, 3 ml of medium containing up to ~500 EBs is recommended. For 12-well plates, 2 ml of medium containing up to ~200 EBs is recommended. Higher volumes may result in more EB movement during scanning. Higher number of EBs may be more challenging to segment properly. Use of a cell strainer to remove single cells and debris is recommended to obtain images free of debris. **EBs tend to fuse over time during culture in suspension. Therefore older cultures with significant EB fusion will be more difficult to correctly identify. Rocking of cultures daily is recommended to alleviate EB fusion.**

3. Scanning Plates

This chapter provides the procedures for selecting the Colony Counting: EB application and setting the image acquisition parameters. You perform these tasks in the Scan tab.

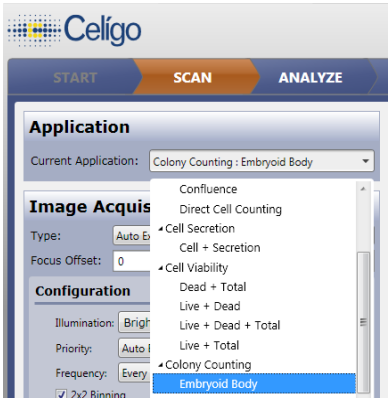
3.1 Colony Counting: EB Application

Perform the following step to select the Colony Counting: EB application:

To select the Colony Counting: EB Application

- In the Current Application dropdown list, select **Colony Counting: Embryoid Body** (Figure 1).

Figure 1. Selecting the Colony Counting: EB Application



3.2 Acquisition Settings

Perform the following steps to select image acquisition settings. For the recommended initial settings to use as a guide, see Table 1.

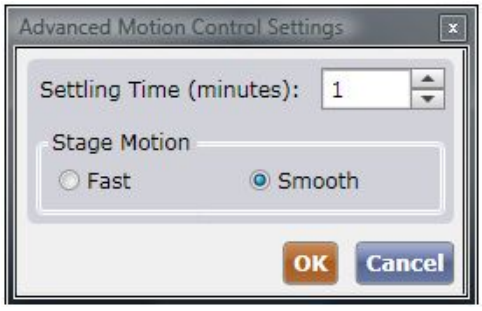
To select image acquisition settings

1. Select a well with a sufficient population of representative EBs.
2. Click **Live** to see the live image.
3. Use manual focus to achieve clear image of EBs
4. Do one of the following to set the optimal exposure:
   - In Type, select **Autoexposure/Gain Channel** and click **Apply Now** (Recommended). The system will use the preset settings shown in Table 1.
   - In Type, select **Custom** and then do one of the following:
     – Click **Auto Exp**: The system will attempt to determine the optimal exposure time and gain setting.
     – Manually set the optimal exposure time and gain by making an entry in the Exposure Time and Gain fields.

	**NOTE: All images are 2 x 2 binned when using this application. For a detailed explanation of binning, see the User Guide.**

5. Set up Motion Control as follows:
   a. Click **Advanced**.
   b. In the Advanced Motion Control Settings dialog box (Figure 2), enter a settling time to allow EBs to settle to the bottom of the plate. Typically 1 minute Settling Time and Smooth Stage Motion are used in this application.

Figure 2. Recommended Motion Control Settings



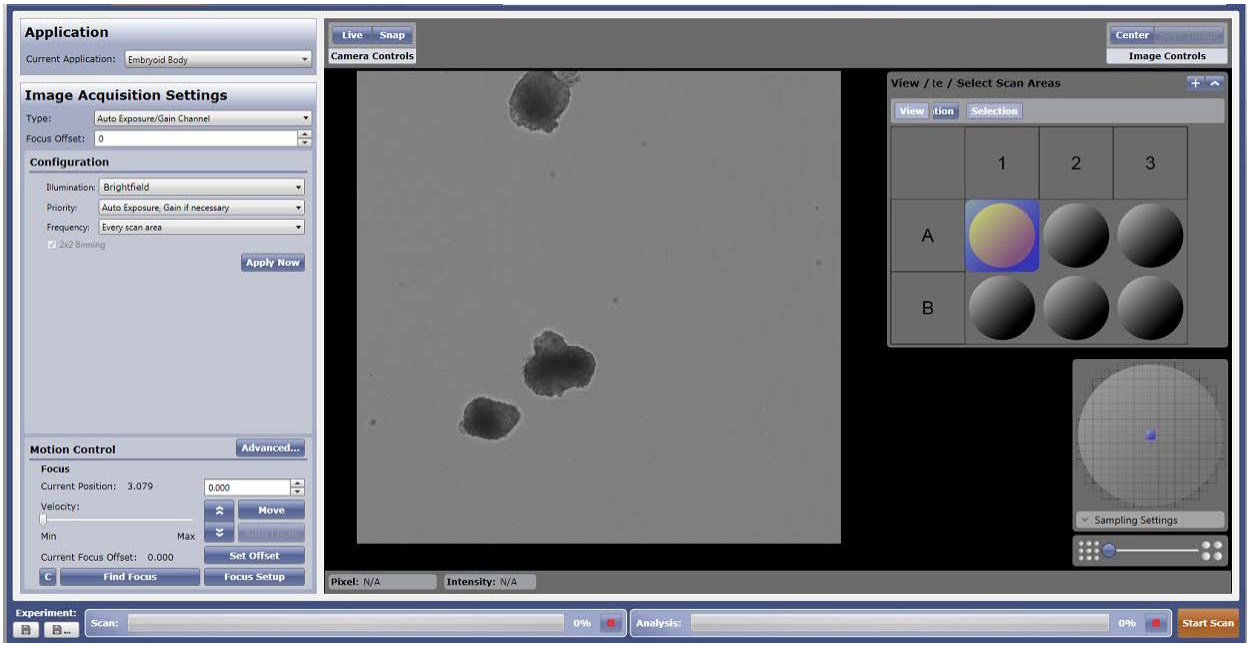
6. Set up Focus. Click the up/down buttons in the Focus section until a crisp focus at the edge of the EBs is visible. Repeat using several EBs. Click **Focus Setup** to register this position as the hardware auto focus position.

Table 1. Recommended Initial Settings for Image Acquisition

Type	Auto Exposure/Gain Channel
Focus Offset	0
CONFIGURATION	
Illumination	Brightfield
Priority	AutoExposure, Gain if necessary
Frequency	Every scan area
MOTION CONTROL AND FOCUS	
Configuration	
Settling Time (minutes)	1
Stage Motion	Smooth
Focus	Focus Setup – Register Hardware Auto Focus Position

Figure 3 shows an example of EBs for the brightfield channel. This figure also demonstrates the appropriate focus position to obtain EBs with clear, crisp edges.

Figure 3. Brightfield Channel with EBs in Focus



4. Analyzing Images

This chapter provides information on how to analyze scans from the Colony Counting: EB application. You perform these tasks in the Analyze tab.

4.1 Selecting Analysis Settings

Perform the following steps to select the optimal analysis settings. The recommended initial settings for identification and pre-filtering when using the Colony Counting: EB application are shown in Table 2. The settings typically provide good image segmentation.

Important guidelines

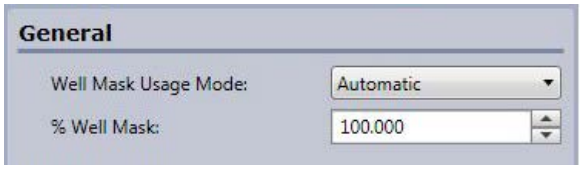
While selecting analysis settings, use the following important guidelines:

- Test the analysis settings using multiple EBs across at least one well of the plate.
- EB border identification is critical for this application. Therefore, images containing EBs with clear, crisp edges will be easy to identify and analyze. EBs that are out of focus have unclear (fuzzy) edges and will require higher Border Dilation values.
- Images will appear in FOR format (4x4 FOV), allowing viewing of a large portion of a well at one time. The FOR format takes more time for the image to appear than viewing a single FOV at one time.

To select analysis settings

1. Load prior saved Analysis Settings if available.
2. In the General section (Figure 4), make the following selections:

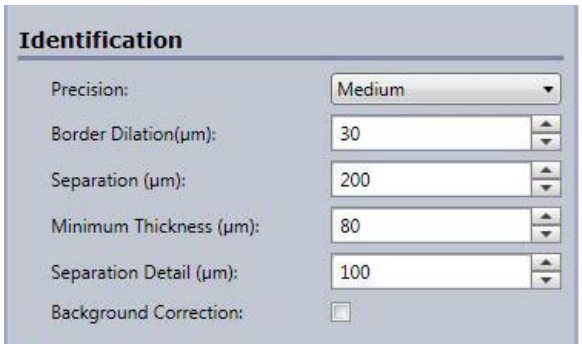
Figure 4. General Section



   a. Well Mask Usage Mode – Select **Automatic** as the initial setting. Select as needed.
   b. % Well Mask – Enter 100% as the initial setting. Select as needed.

3. In the Identification section (Figure 5), make the following selections:

Figure 5. Identification Section

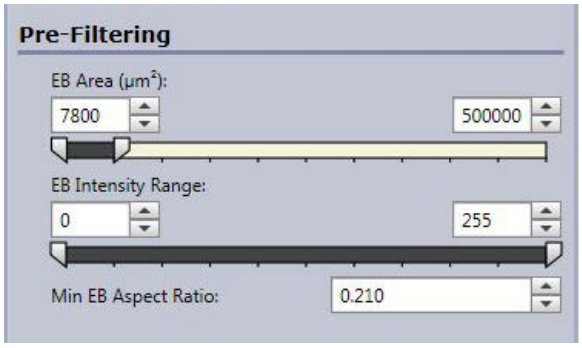


   a. Precision – Select the desired level of precision of separation of touching EBs:
      - None – No separation.
      - Low – Minimal separation.
      - Medium – Medium separation.
      - High – Most precise separation.

        Higher values will result in the system's attempt to determine the exact boundary between EBs. As the desired precision increases, so will the analysis time.
   b. Border Dilation (µm) – Defines the amount of dilation/erosion for EB edge segmentation..
   c. Separation (µm) – Defines the diameter of the EB. If a range of EB sizes are present, this value should be close to the maximum size.
   d. Minimum Thickness (µm) – Minimizes object artifact extensions.
   e. Separation Detail (µm) – Defines the smoothness of EB segmentation separation lines.
   f. Background Correction – Minimizes background variations due to meniscus and applies an even grey background.

4. In the Pre-Filtering section (Figure 6), make the following selections:

Figure 6. Pre-Filtering Section



   a. EB Area (µm) – Enter the EB area range that corresponds to your EBs..

b. EB Intensity Range – Defines pixel intensity range of EBs to include in the analysis.
c. Min EB Aspect Ratio – Removes elongated debris.

Table 2. Recommended Initial Identification and Pre-Filtering Settings for Analysis

Parameter	Initial Setting	Available Range	Description
IDENTIFICATION			
Precision	High	None, Low, Medium, High	Precision – Select the desired level of precision of separation of touching EBs: • None – No separation. • Low – Minimal separation. • Medium – Medium separation. • High – Most precise separation. Higher values will result in the system's attempt to determine the exact boundary between EBs. As the desired precision increases, so will the analysis time.
Border Dilation (µm)	8	2-30	Defines amount of dilation/erosion for EB edge segmentation. Lower value; EB border segmentation will move closer to the edge of EB Higher value: EB border segmentation will move farther from the edge of EB. Can be used to segment EBs with fuzzy edges.
Separation (µm)	20	1-100	Defines amount of EB separation when EBs are touching/fused. Lower value: Less separation of touching/fused EBs. Higher value: Smoothes EB separation segmentation lines.
Minimum Thickness (µm)	80	0 – 500	Minimizes object artifact extensions.
Separation Detail (µm)	10	0-100	Defines the smoothness of EB separation segmentation lines.
Background Correction	Uncheckmarked	Checkmarked or Uncheckmarked	Minimizes background variations by applying an average value.

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Parameter	Initial Setting	Available Range	Description
PRE-FILTERING			
EB Area (µm ^{2}) Range	7800-500000	0-5000000	Defines the range of EBs included in analyses (corresponds to EBs ~100-800 µm in diameter). Lower value: Identification of EBs less than ~100 µm (diameter). Higher value: Identification of EBs more than ~800 µm (diameter).
EB Intensity Range	0-255	0-255	Defines pixel intensity range of EBs included in analyses. Setting a lower limit can exclude dark, black debris. Setting an upper limit can exclude whited-out debris.
Min EB Aspect Ratio	0.21	0-1	Defines the shape of EBs (and debris) included in analysis. Defined as the ratio 1 over the maximum cell elongation. Min EB Aspect Ratio of 1 is a perfect circle. Lower aspect ratios typically remove elongated debris.

Figure 7 through Figure 13 show examples of Colony Counting: EB brightfield images with corresponding target overlays.

Figure 7 shows an example of the display results when adjusting precision.

Figure 7. EBs with Corresponding Target Overlay – Precision Adjustments

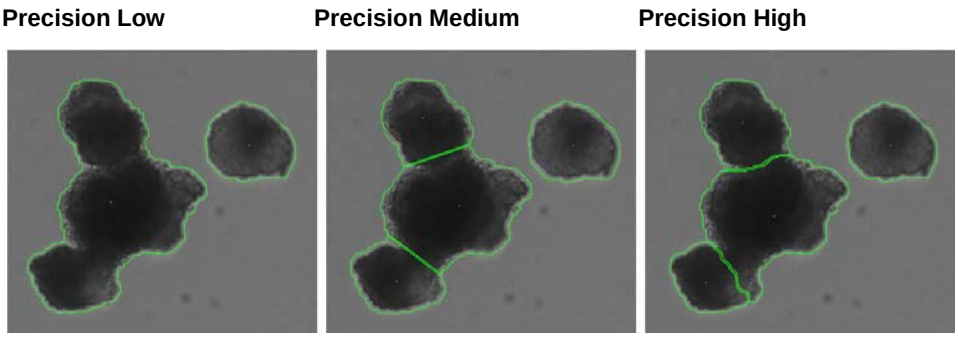


Figure 8 show examples of display results when adjusting border dilation.

Figure 8. EBs with Corresponding Target Overlay – Border Dilation Adjustments

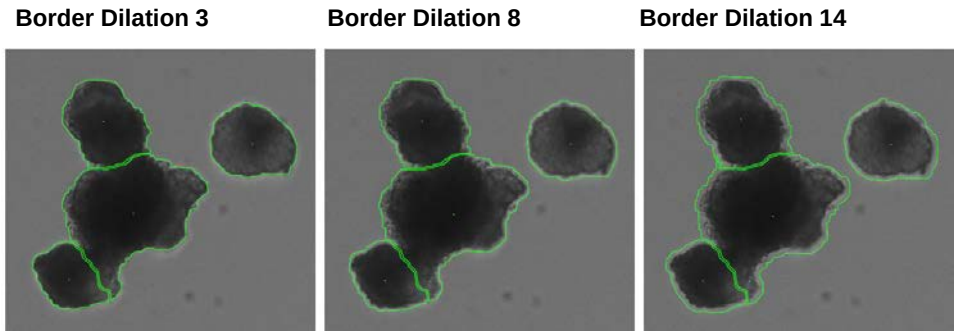


Figure 9 shows an example of display results when adjusting separation.

Figure 9. EBs with Corresponding Target Overlay. – Separation Adjustments

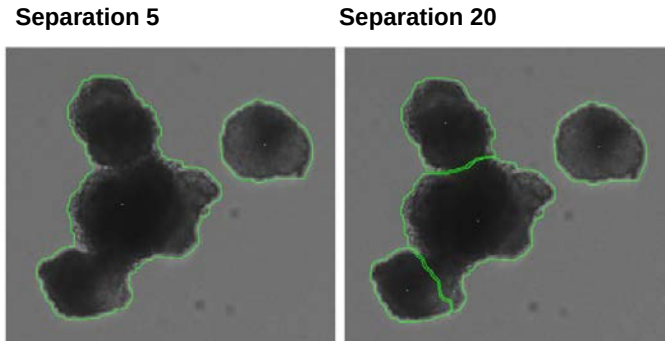


Figure 10 shows an example of display results when adjusting separation detail.

Figure 10. EBs with Corresponding Target Overlay – Separation Detail Adjustments

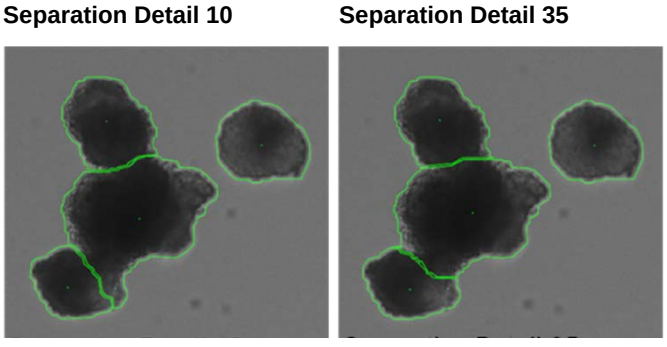


Figure 11 shows an example of display results, with segmentation of small EBs, when adjusting EB area.

Figure 11. EBs with Corresponding Target Overlay – EB Area Adjustments

EB Area 7800 **EB Area 2000**

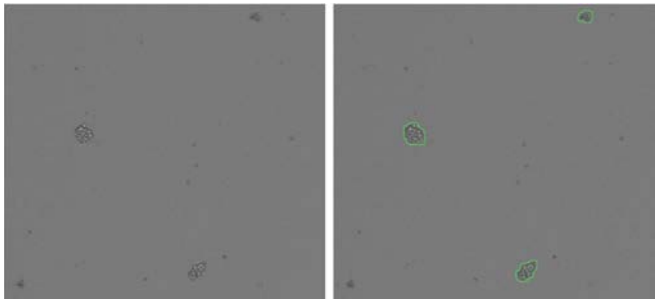


Figure 12 shows an example of display results, with segmentation of single cell debris, when adjusting EB intensity range.

Figure 12. EBs with Corresponding Target Overlay – EB Intensity Range Adjustments

EB Intensity Range 0-255 **EB Intensity Range 0-115**

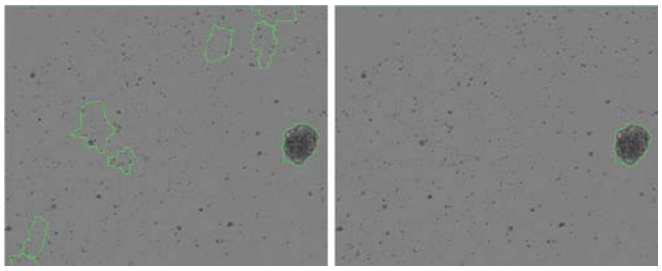
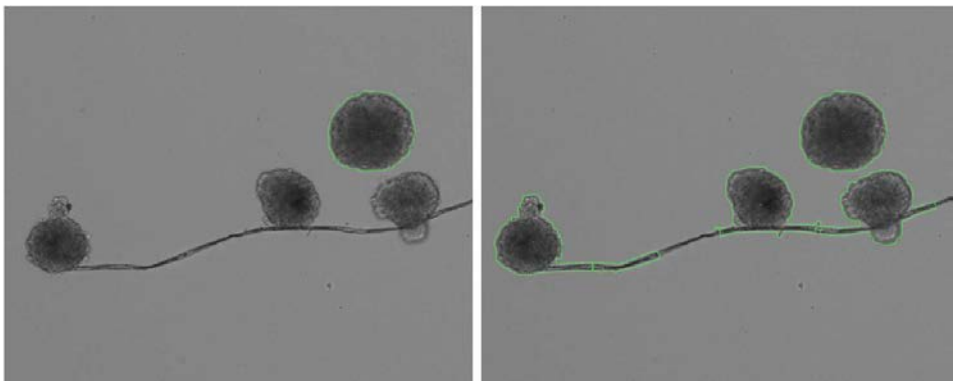


Figure 13 shows an example of display results, with segmentation of debris, when adjusting minimum EB aspect ratio.

Figure 13. EBs with Corresponding Target Overlay – Min EB Aspect Ratio Adjustments

Min EB Aspect Ratio 0.21 **Min EB Aspect Ratio 0.00**



5. Gating Cells

This chapter provides information on how to select filter settings for further data analysis. You perform this task in the Gate tab.

The Colony Counting: EB application relies on the identification of EBs and removal of debris using the EB area, EB intensity, and minimum EB aspect ratio in the Analyze tab.

5.1 Working with Gates

The following are general principles about working with gates in the Colony Counting: Embryoid Body application.

- If you do not perform gating, the system uses the ALL population to count the cells in the wells. ALL is the default population that the system assigns to all the data points (objects) in the segmentation result from the Analyze tab.
- When performing gating in this application, only one class (the Total class) exists; you cannot assign any additional classes to populations.
- Make sure that a Classes checkbox has been selected to assign a class to the Total population. Checkmarking a Classes checkbox will allow the data for the class (the Total class) to appear in the analysis results.

NOTE: Make sure that a Classes checkbox has been selected for the Total population. The absence of a checkmark would be the same as not using the Gating tab; all data would be reported from the analysis tab settings only.

To create a plot, gate, and populations

1. In the Plot Populations pane, create a plot, using the Add Plot (+) button and Add Plot dialog box, and referring to the selections in Table 3.

   The table lists the selections you can make in both Pick plot parameters menus. The Pick plot parameters selections are different from those in the User Guide (which shows displays for the Expression Analysis application) because only one channel is used in the Colony Counting: Embryoid Body application.

   For details, see User Guide section “Creating a Plot.”

   By default, all segmented EBs from the Analyze tab are assigned to the ALL (Total) source population. When you create a plot and draw a gate, then select the Classes checkbox as the new "Total" population of EBs to be analyzed. The new Total class is then assigned to the newly defined population and the data is reported in the Results tab.

Table 3. Plot Parameter Selections

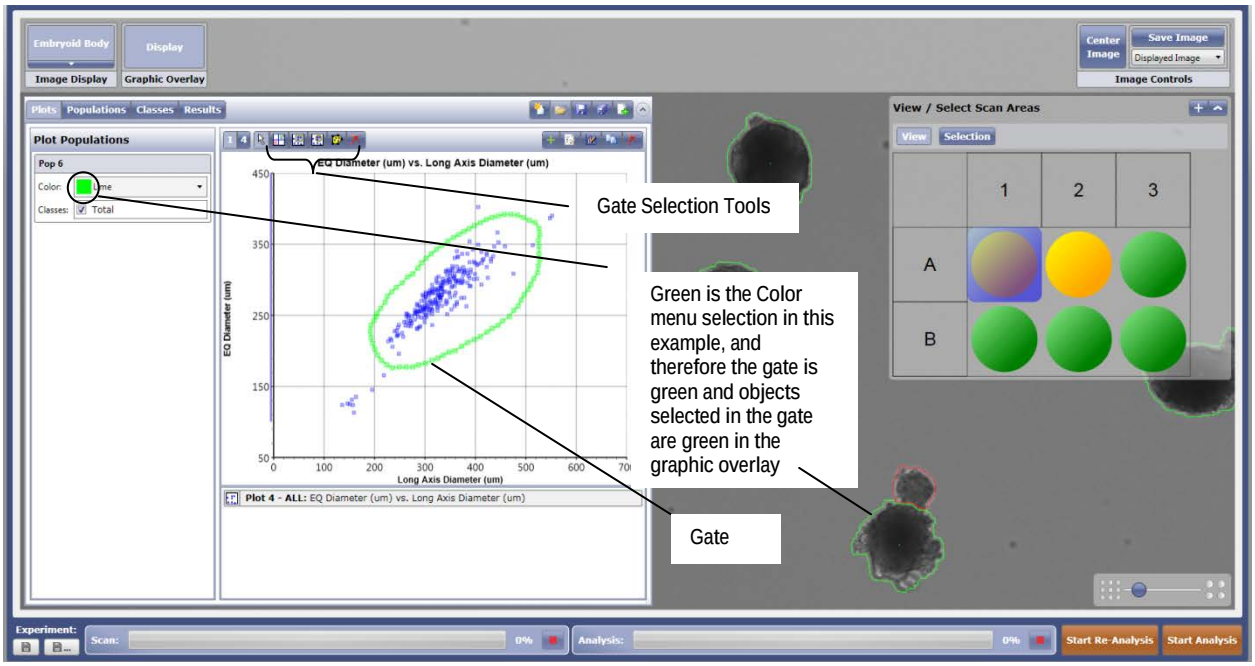
Feature	Definition
X Position (µm)	Location of a cell along the horizontal axis of the well: left (-µm) or right (+µm) of the center (origin of the well).
Y Position (µm)	Location of a cell along the vertical axis of the well: below (-µm) or above (+µm) of the center (origin of the well).
Distance to Nearest Neighbor (µm)	Distance from the target EB to the closest neighboring EB.
Distance to Well Center (µm)	Distance from the target EB to the center (origin) of the well.
Long Axis Angle	Angle of the longest axis of an elliptical approximation of the target EB shape.
Long Axis Diameter (µm)	Measure of the longest diameter of each identified EB.
Short Axis Diameter (µm)	Measure of the shortest diameter of each identified EB.
Area (µm)	Total area of each identified EB.
Perimeter (µm)	The total length of the edge of each identified EB.
Form Factor	Measure of the compactness of each identified EB, derived from the perimeter and area. A circular EB is compact with a form factor of 1.0 (the maximum); a more convoluted shape has a lower value.
Smoothness	Measure of the evenness of an EB’s contour. It is a ratio of the convex perimeter to the true perimeter of an EB. A completely smooth EB is has a smoothness value of 1.0 (the maximum).
Aspect Ratio	Measure of the EBs breadth to the EB’s length. An EB that is a perfect circle has an aspect ratio of 1.0 (the maximum).
EQ Diameter (µm)	Equivalent diameter of each identified EB, derived from the area. Equivalent diameter = 2(radius), derived from Area = $\pi(\text{radius}^2)$

2. Create a gate on the plot, using the gate selection tools (Figure 14).

   For details, see User Guide section “Creating a Gate.”

   In the Graphic Overlay display, the color displayed for the plot population selected in the gate corresponds to the Color menu selection (Figure 14). The figure shows the single class (Total) used in this application.

Figure 14. Gating Cells



3. Repeat steps 1 and 2 as needed to refine the population that you want to count.

4. Assign the Total class to the population as follows:
   a. Click the gate.
   b. In the Plots, Populations, or Classes view, make sure that the Total class is checkmarked.

   For details, see User Guide section “Assigning a Class to a Population.”

   In this application, you assign only the Total class to populations; you cannot assign any additional classes to populations.

6. Viewing Results

This chapter describes the feature outputs available from the application's Results tab.

6.1 Application Outputs

The parameters listed in Table 4 appear below the Display Options section in the Scan Information pane.

Table 4. Colony Counting: EB Application Outputs

Parameter	Description	Ave	St Dev	CV	Min/ Max
EB Count	Number of EBs identified and analyzed				
Diameter (um)	Equivalent diameter of each identified EB, derived from the area. Equivalent diameter = 2(radius), derived from Area = π(radius^2)	√	√	√	√
Short Axis Diameter (um)	Measure of the shortest diameter of each identified EB.	√	√	√	
Long Axis Diameter (um)	Measure of the longest diameter of each identified EB.	√	√	√	
Area (um^2)	Area of each identified EB, measured in pixels.	√	√	√	
Perimeter (um)	The total length of the edge of each identified EB.	√	√	√	
Form Factor	Measure of the compactness of each identified EB, derived from the perimeter and area. A circular EB is compact with a form factor of 1.0 (the maximum); a more convoluted shape has a lower value.	√	√	√	
Smoothness	Measure of the evenness of an EB's contour. It is a ratio of the convex perimeter to the true perimeter of an EB. A completely smooth EB is has a smoothness value of 1.0 (the maximum).	√	√	√	
Aspect Ratio	Measure of the EBs breadth to the EB's length. An EB that is a perfect circle has an aspect ratio of 1.0 (the maximum).	√	√	√	
EB Density (EB count/well area)	The number of EBs divided by total area of well (or scan area).	√			
Nearest Neighbor Distance (µm)	Measure of the distance between EBs.	√	√	√	
Long Axis Angle	The angle at which the maximum diameter is found for each identified EB.	√	√	√	
% Well Sampled	Percent of well surface processed				

6.2 Data Export

Well-level and object level data can be exported into CSV (Comma Separated Value) or FCS (Flow Cytometric Standard) files. For the procedures to perform data export, see the User Guide.

7. Troubleshooting

This chapter provides troubleshooting recommendations.

Table 5. Troubleshooting Recommendations

Issue	Recommended Action
EBs are out of focus	1. When the hardware autofocus position is set using only a few nonrepresentative EBs, the rest of the EB population will likely be out of focus. For example, if the hardware autofocus position is set using only small EBs then larger EBs may be out of focus. • Use a sufficient population of representative EBs to set hardware autofocus when scanning. 2. For analysis of out of focus EBs, increase Border Dilation value (see Figure 9 above).
EBs are not being identified	EBs may not be identified due to: 1. Part of the EB is out of focus: Adjust Border Dilation value 2. EBs are smaller/larger than the size range selected in the EB Area in the Analyze tab: • Adjust range of EB Area. 3. EBs have a lower/higher intensity than the range selected in the EB Intensity Range in the Analyze tab: • Adjust EB Intensity Range. 4. EBs are not circular, therefore have a low aspect ratio. • Decrease aspect ratio (at the expense of increasing debris).
EBs are not being identified on well edges	Turn on/off Background Correction.
Identification of debris	For EBs, debris typically consists of single/dead cells, small clumps of cells that are not spherical, or strings in the medium. 1. Single/dead cells can be removed by decreasing the EB intensity range in the Analyze or Gating Tab. 2. Small clumps of cells not considered to be EBs can be removed by adjusting the EB area range in the Analyze or Gating tab. 3. Strings/artifacts in the medium can be removed by increasing the Min EB Aspect ratio in the Analyze or Gating Tab.
Fused EBs are not being separated properly	EB fusion occurs over time. Therefore the older the culture the more likely EBs will be fused. • Gently rock EB cultures daily to alleviate fusion. • It is likely that parts of fused EBs are out of focus: Increase Border Dilation value in the Analyze tab. • Fused EBs may have a larger size range than the selected range in EB area. Increase range of EB area.

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 8001624 Rev A

Celigo® Cytometer
Colony Counting: Single Colony Verification Application Guide

Celigo Software Version 2.1

Contents

1. About this Guide

This chapter provides a brief description of this guide and how to use it.

1.1 Introduction

The Colony Counting: Single Colony Verification application identifies and counts cell colonies using brightfield imaging. The application is used to determine each well that contains a single colony, for use in cloning it.

1.2 Purpose

The purpose of this guide is to provide the steps and concepts that are unique to the Colony Counting: Single Colony Verification application. Information that is common to all applications is covered in *Celigo Cytometer User Guide* (8001619), from here on referred to as the User Guide.

1.3 Safety Precautions

All safety precautions described in the User Guide apply to this guide.

1.4 Technical Assistance

Contact Nexcelom Customer Service for further information:

Nexcelom Bioscience, LLC.
Customer Service
360 Merrimack St. Building 9
Lawrence, MA 01843, USA

- From the United States:
  email: celigosupport@nexcelom.com
  phone: 978-327-5340
- From Europe:
  e-mail: celigosupport@nexcelom.com
  phone: 978-327-5340

2. Prerequisites

The prerequisites for performing the procedures in this document are:

- Familiarity with Celigo operation per the User Guide
- Celigo cytometer is turned on per the User Guide
- The recommended plates are being used (Table 1)

	NOTE: **Failure to use the recommended plates may result in focus issues or manufacturing particulate debris that will cause difficulty in proper segmentation of colonies.**

Table 1. Recommended Plates

Plate Type	Vendor	Cat#	Volumes
384W	Corning	3712	40 – 60 µl
384W	Aurora	1011	40 – 60 µl
96W	Greiner	655090	150 – 200 µl
24W	Corning	3524	0.5 ml
24W	Cyto One	CC8682-7524	0.5 ml
6W	Corning	3516	2 ml
6W	Cyto One	CC7682-7506	2 ml

- Samples prepared as follows:
  - In brightfield imaging, plating liquid volume results in meniscus-dependent brightening or darkening at the well edges. It is recommended to optimize the liquid volumes to optimize performance of application. (see Troubleshooting chapter 6 for details). See Table 1 for recommended volumes per plate type.

3. Scanning Plates

This chapter provides the procedures for selecting the Colony Counting: Single Colony Verification application and setting the image acquisition parameters. You perform these tasks in the Scan tab.

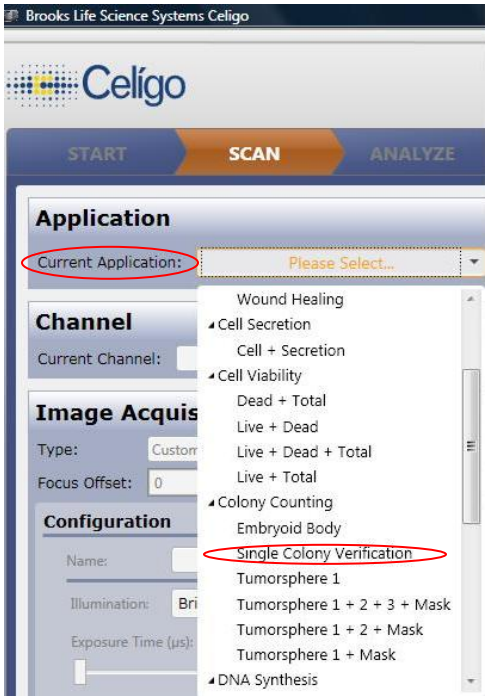
3.1 Colony Counting: Single Colony Verification Application

Perform the following steps to select a Colony Counting: Single Colony Verification application.

To select an application

In Current Application, select **Colony Counting: Single Colony Verification** (Figure 1).

Figure 1. Selecting an Application



3.2 Image Acquisition Settings

Perform the following steps to select image acquisition settings.

To select image acquisition settings

1. Choose a well for setup using the plate map by selecting **Navigation** and a representative well.
2. View a well by selecting **Live** or **Snap** in the Camera Controls field.
3. Set up acquisition settings:
   - Select channel type: **Auto Exposure/Gain Channel** (recommended).
   - In Illumination, select **Brightfield**.
   - In Priority, select **AutoExposure, Gain if Necessary**.
   - In Frequency, select **Every Scan Area**.

     For detailed instructions on other image acquisition options, such as well subsampling, off-axis imaging, and binning, see the User Guide.
4. Click **Apply**.
5. Set up focus per the User Guide.
   - For detailed instructions on selecting correct focus position for brightfield imaging, see section 3.3 below.
   - It is recommended to select **Hardware Auto Focus** for most routine plate scanning.

3.3 Correct Focus Position for Brightfield Imaging

The proper focus for brightfield illumination is important for optimal application performance. There are two image planes visible on the Celigo Cytometer using brightfield illumination. The “Dark Focus” image plane is the focal plane in which objects appear dark compared to surrounding background regions. The “Bright Focus” image plane is a higher focal plane in which the cell or object acts as a lens and focuses the transmitted light in a secondary plane. In the “Bright Focus” image plane, cells or objects have a bright center and dark edges.

For this application, two analysis identification algorithms are available: Brightfield or Texture. The Brightfield algorithm is optimized for the “Bright Focus” image plane. The Texture algorithm will work for either the "Bright Focus" or "Dark Focus" image plane.

This section describes how to select the correct focus position for brightfield imaging.

To select the correct focus position for brightfield imaging

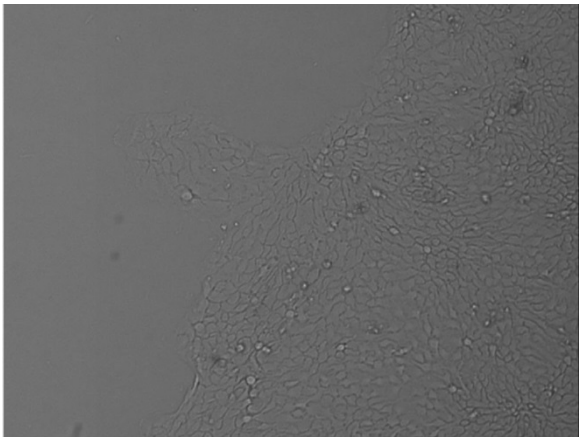
1. Choose a well for setup using the plate map by selecting **Navigation** and a representative well.
2. View a well by selecting **Live** in the Camera Controls field.
3. Adjust the focus until the cells have a large, bright center. For examples of proper focus using the Bright Focus and Dark Focus selections in the Target Focal Plane menu, see Figure 2.

	**NOTE: In this application, selecting Bright Focus as the Target Focal Plane will be appropriate for most cell types. If the segmentation of cell areas is not performing well, select Dark Focus.**

4. Select **Focus Setup** and select **Hardware Auto Focus** in the Focus Type field. Complete the setup by registering the autofocus.
    - Image Based Auto Focus is *not* recommended for brightfield illumination if the wells contain very few cells.
    - Register Manual will register the current focal plane for Hardware Auto Focus. Depending on the cell type and its morphology (i.e., how flat a cell adheres to the plate surface), you can determine which focal plane performs better for analysis – the Bright, lens-like focus or Dark, flat focus.

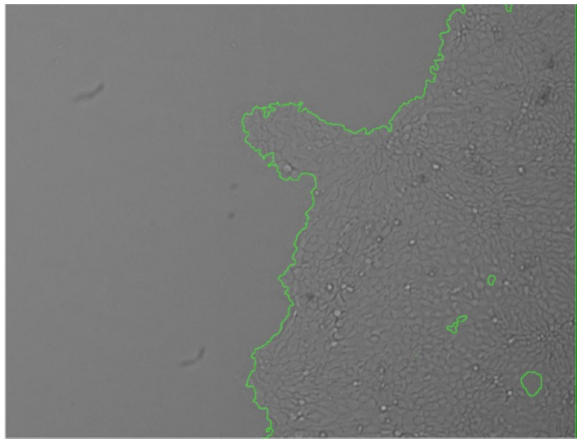
Figure 2. Examples of Proper "Bright" and "Dark" Focus

Colony Using "Bright Focus" Target Focal Plane



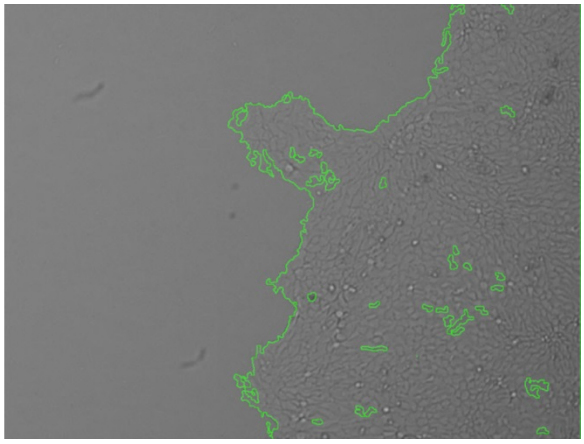
Optimal Identification of a Colony Using:

- "Bright Focus" Target Focal Plane
- "Texture" Algorithm



Optimal Identification of a Colony Using:

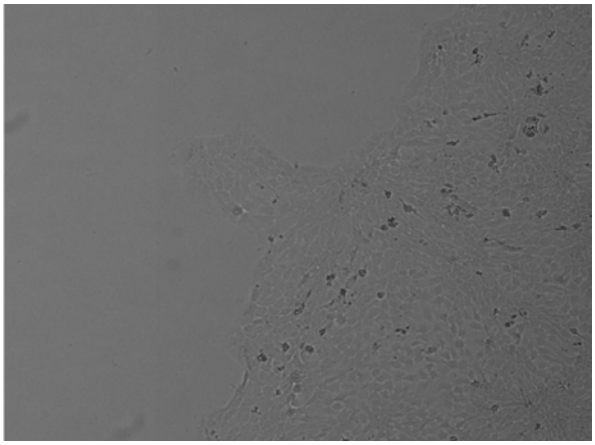
- "Bright Focus" Target Focal Plane
- "Brightfield" Algorithm

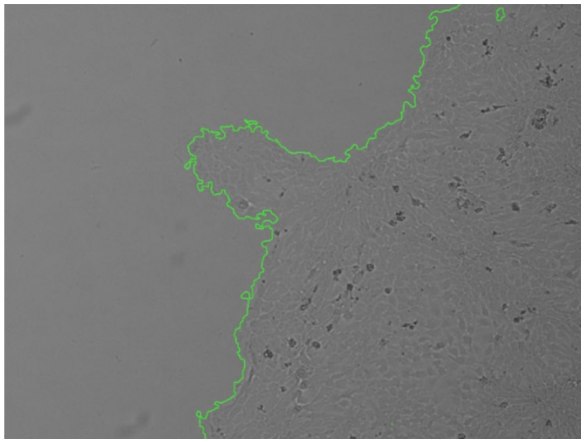


Optimal Identification of a Colony Using:

- "Dark Focus" Target Focal Plane
- "Texture" Algorithm (recommended Algorithm for use with "Dark Focus" Target Focal Plane)

Colony Using "Dark Focus" Target Focal Plane





4. Analyzing Images

This chapter provides information on how to analyze scans from the Colony Counting: Single Colony Verification application. You perform these tasks in the Analyze tab.

4.1 Analysis Settings

Important Guidelines

- Images will appear in FOR format (4x4 FOV), allowing viewing of a large portion of a well at one time. The FOR format takes more time for the image to appear than viewing a single FOV at one time.

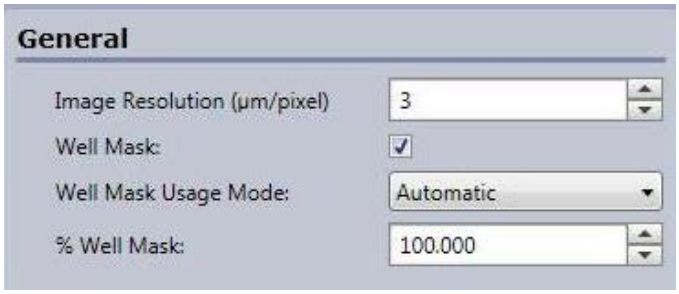
Perform the following steps to select analysis settings. The recommended initial analysis settings for identification and pre-filtering when using the Colony Counting: Single Colony Verification application are shown in Table 2. The settings typically provide good image segmentation.

To select analysis settings

1. Load prior saved Analysis Settings, if available.
2. In the General section (Figure 3), make selections as needed.
   a. Image Resolution (µm/pixel) -- Select the image resolution to use for analysis. Entering a lower value (minimum 2 µm/pixel) will result in greater segmentation precision and longer analysis time. Entering a higher value will result in less accuracy and shorter analysis time.

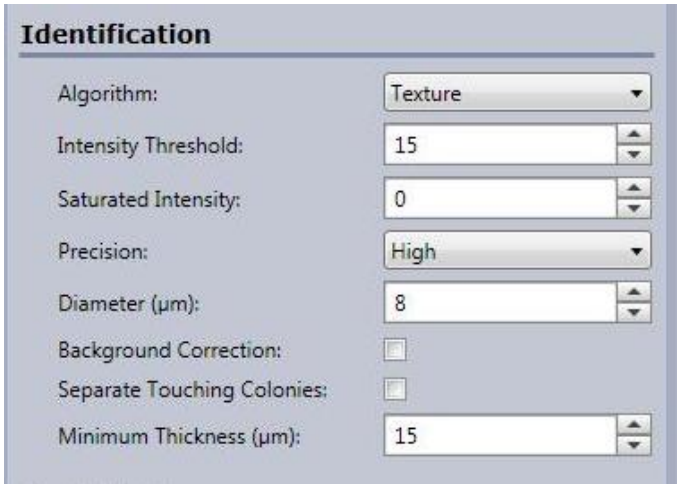
      Well Mask, Well Mask Usage Mode, and % Well Mask. For information about these selections, see the User Guide section “Selecting General Analysis Settings.”

Figure 3. General Section



3. In the Identification section (Figure 4), make the following selections:

Figure 4. Identification Section



a. Algorithm – Select the appropriate algorithm:
   - Brightfield – The algorithm looks for objects with a bright center and dark edges.
   - Texture – The algorithm looks for texture differences between the objects found and the background areas.

	NOTE: If you selected Dark Focus as the Target Focal Plane when selecting acquisition settings, it is recommended that you select Texture as the Algorithm in the Analysis tab.

	NOTE: The colony identification success of selecting Brightfield versus Texture will depend on the colony type, shape, and contrast of the colonies being analyzed.

b. Intensity Threshold – Enter the optimal intensity threshold.

   The intensity threshold is the level of intensity that separates background from cells. With an appropriate threshold set, background pixels fall below and pixels in cells are above the threshold.

c. Saturated Intensity – Select the optimal saturated intensity threshold to prevent holes in the segmentation of large objects.

   Saturated intensity represents the minimum pixel intensity value that is considered to be saturated. Saturated areas are not detected by the texture segmentation algorithm and therefore create holes within confluence areas. Holes consisting of pixels that are all saturated are filled in post-segmentation.

d. Precision – Higher precision results in more accurate identification of cell clusters. Normal recommended. Normal is sufficient to provide acceptable results, while High results in longer analysis processing time.

e. Diameter (µm) (for Brightfield Algorithm only) – Enter the diameter that corresponds to the expected cell dimensions.

f. Background Correction – Select if needed.

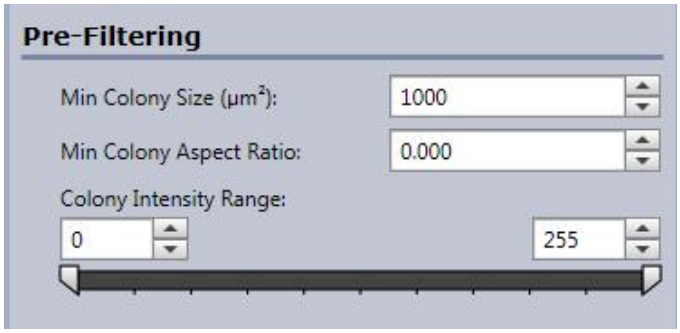
g. Separate Touching Colonies – Select if needed.

This selection is used to separate objects that are touching, close, or merging.

h. Minimum Thickness (µm) – Minimizes object artifact extensions.

4. In the Pre-Filtering section (Figure 5), make the following selections:

Figure 5. Pre-Filtering Section



a. Min Colony Size (μm^2) – Enter the appropriate minimum colony size.

To eliminate small debris or single cells, adjust the value to be larger than small debris size and lower than the colony size.

b. Min Colony Aspect Ratio – Enter the appropriate minimum colony aspect ratio.

This selection measures an object's elongation and is often used to remove artifacts and debris. Aspect ratio is the ratio of the minor axis to the major axis of the segmented object. A value of 1 is a perfect circle; values less than 1 suggest an oval (elongated) object.

c. Colony Intensity Range – Enter the range of intensity of objects to be included in the analysis. Helps exclude dark artifacts.

5. In the Auto Analyze section, checkmark this selection as follows:

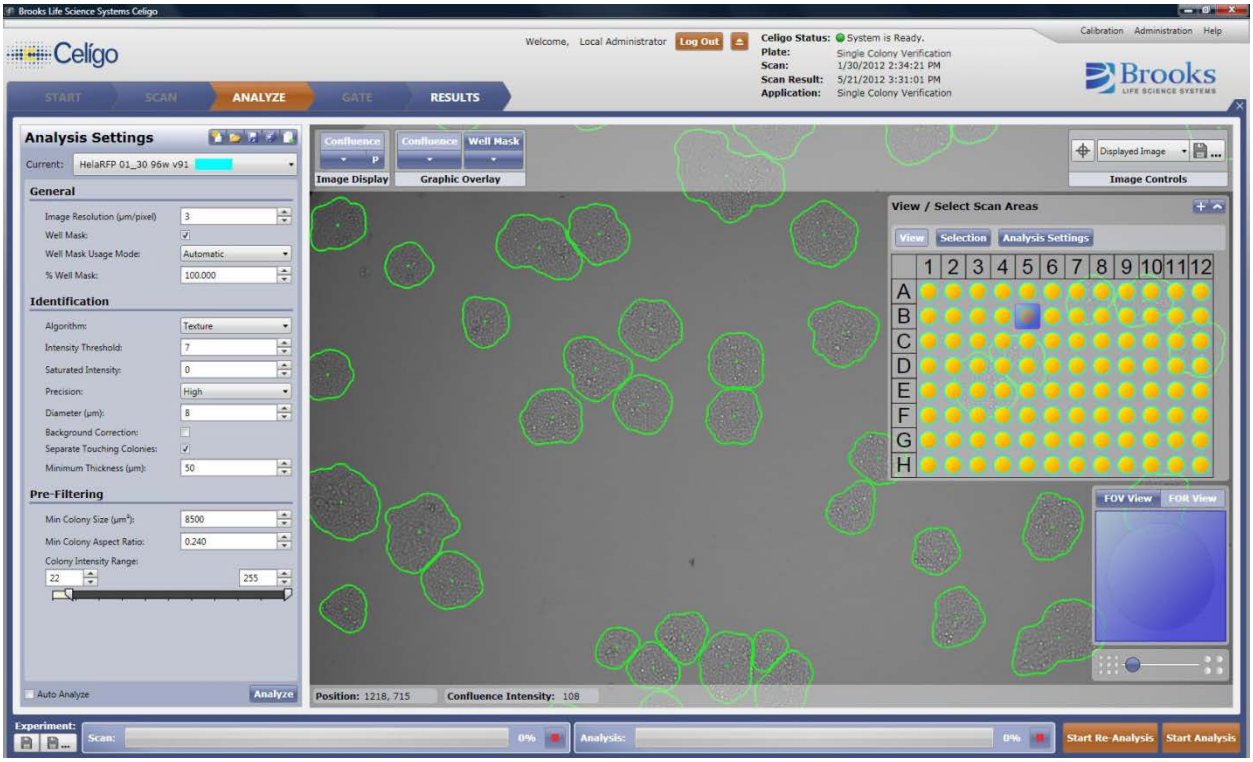
- To update the segmented image and overlays as you make each selection, displaying a progress wheel after you make each field entry, checkmark Auto Analyze.
- To update the segmented image and overlays only when you click Analyze, deselect Auto Analyze.

Table 2. Recommended Initial Identification and Pre-Filtering Settings for Analysis

IDENTIFICATION	
Algorithm	Texture
Intensity Threshold	5
Saturated Intensity	0
Precision	Normal
Diameter (µm)	As Needed
Background Correction	Uncheckmarked
Separate Touching Objects	Uncheckmarked
Minimum Thickness	15
PRE-FILTERING	
Min Colony Size (µm^2)	1000
Min Colony Aspect Ratio	0.100
Colony Intensity Range	10 – 255

Figure 6 shows an example of a well with analysis settings selected and the Confluence Graphic Overlay button turned on (green in this example).

Figure 6. Well with Analysis Settings Selected



5. Viewing Results

This chapter describes the feature outputs available from the application's Results tab.

Table 3 summarizes the outputs of the Colony Counting: Single Colony Verification application.

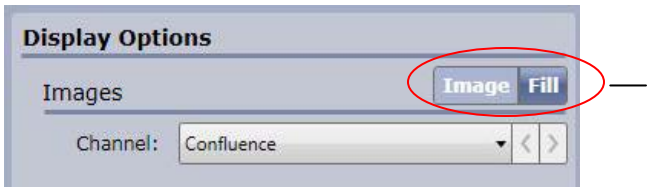
Table 3. Single Colony Verification Output Descriptions

Parameter	Description
Data for Each Well	
Confluency (%)	Area of well occupied by cell colonies divided by total area of scan area or well
Colony Count	Number of Total Colonies positive with intensity above a user-defined intensity threshold
Colony Average Area (µm²)	Average colony area (µm²) per well
Colony Std Dev Area (µm²)	Standard deviation of colony area (µm²) per well
Colony Total Area (µm²)	Total area of colonies (µm²) per well
Colony %CV Area	Percent CV of area values from the colony area average and standard deviation. CV gives the variance of colony areas in a well. Lower value: The colony areas in the well are approximately the same size. Higher value: The colony areas in the well differ in size.
Well Sampled (%)	Percent area of the well that was imaged and analyzed per well

5.1 Image (Colony Count) and Fill (Confluence Overlay) Views

This section describes the purpose of the Image and Fill views in the Colony Counting: Single Colony Verification application. In this application, you can choose between an Image and Fill view within either the plate view or well detail view, helping you see the properties and patterns of interest to you.

Figure 7. Image Button and Fill Button



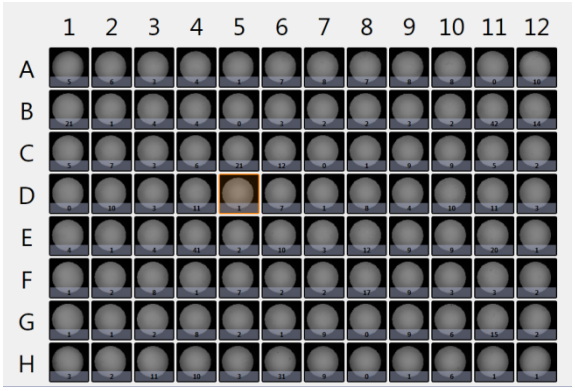
- Image button – Turns on/off the raw image display
- Fill button – Fills/un-fills all identified areas with a selected color

	NOTE: **Clicking the Image button a second time is the same as clicking the Fill button. Clicking the Fill button a second time is the same as clicking the Image button.**

When the Results tab first appears, the default view is a plate-level Image view (Figure 8 left side). In this application, the measurement parameters available in the Image view include the colony count for each well. Figure 8 right side shows the Fill View, which provides a confluence overlay of any identified colonies. This display provides an overview of the colony culture results in terms of single or multiple colonies.

Figure 8. Plate-Level Image View versus Fill View

Image View



Fill View

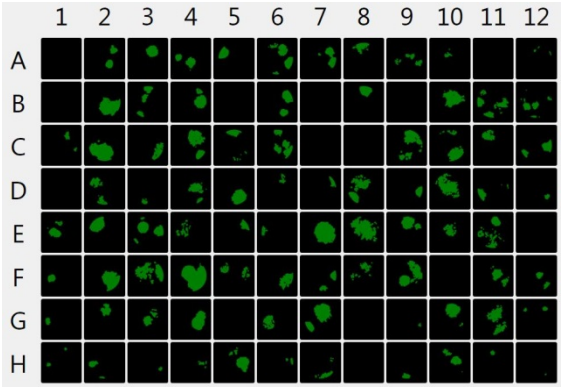


Figure 9 shows a well-level Fill view zoomed out.

Figure 9. Well Detail Fill (Confluence Overlay) Zoomed Out

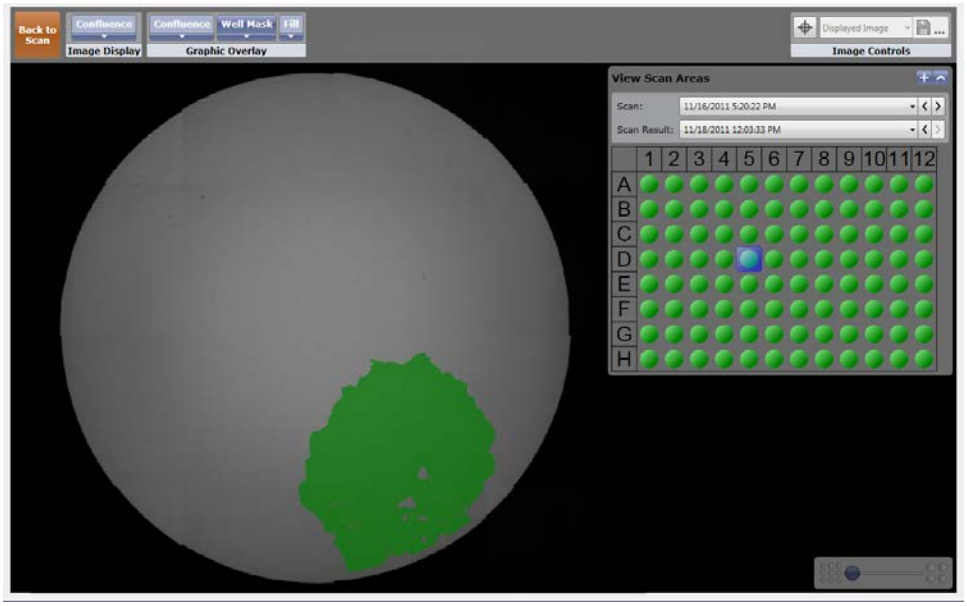
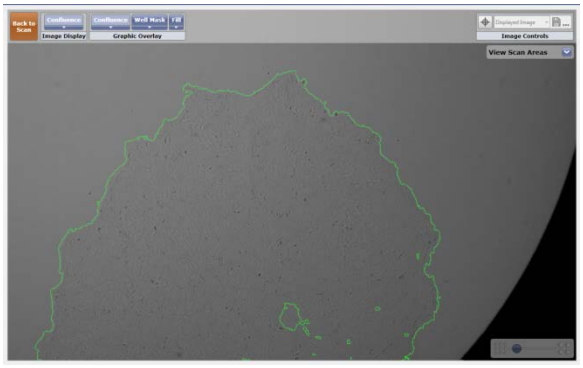


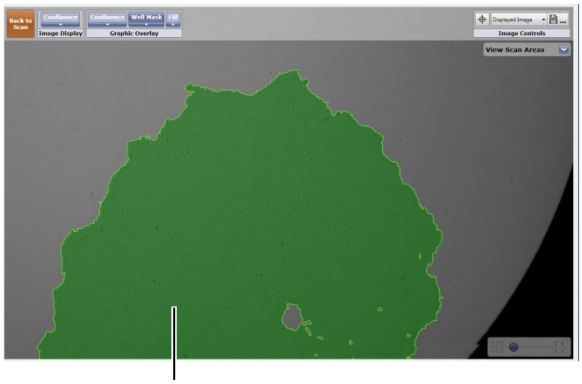
Figure 10 shows a well-level Fill view zoomed in (magnified).

Figure 10. Well Detail Fill (Confluence Overlay) View Magnified

Fill Off



Fill On



The fill (green shaded area) identifies the area that is occupied by cells rather than empty space

6. Troubleshooting

This chapter provides troubleshooting recommendations.

Table 4. Troubleshooting Recommendations

Issue	Recommended Action
Cannot identify individual colonies	1. Inappropriate focus selected. • For brightfield imaging– verify that "Bright Focus" Target Focal Plane was used to acquire images. 2. Intensity threshold value too high. 3. Desired objects are excluded by the Pre- Filtering settings. • View the segmented image in the Analyze tab (see *Celigo Cytometer User Guide* for instructions). • Change Pre-Filtering settings to identify desired objects. 4. Identification settings do not identify objects. • Follow the tips for identifying individual colonies (see section 4.1).
Software identifies debris as cells or confluent areas in brightfield	Often debris has unique properties that can be used to remove it from the scan results. Use the Pre-Filtering settings to remove debris.
Improper colony counts on well edges in brightfield	1. Remove/uncheck Separate Touching Colonies in the Analyze tab – Identification section. 2. Increase Minimum Thickness. 3. Shrink % Well Mask to less than 100%. 4. Checkmark/Uncheckmark Background Correction.
Well edges are too bright or dark	Liquid volume not optimal resulting in a meniscus-dependent effect. • For proper liquid volumes for cell plating, see Table 1. • Checkmark Background Correction.
Bright or dark shadows of cells are identified in brightfield	1. Adjust liquid volume level to prevent meniscus-dependent optical effects. • For proper liquid volumes for cell plating, see Table 1 for proper plating volumes. 2. Some small well microplates, such as 1536-well formats, yield meniscus-dependent optical effects that cannot be corrected by liquid volume. • Use an alternative container. • Use Pre-Filtering settings in the Analyze tab to remove unwanted objects. • Checkmark Background Correction.

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 8001625 Rev A

Celigo® Cytometer
Colony Counting: Tumorsphere Application Guide

Celigo Software Version 2.1

Contents

1. About this Guide

This chapter provides a brief description of this guide and how to use it.

1.1 Introduction

The Colony Counting: Tumorsphere application identifies and counts individual tumorspheres and small clusters of tumorspheres using brightfield and fluorescence imaging. This application significantly reduces the time and effort needed to quantify key aspects of 3D spheres including size, growth, growth tracking over time, and response to chemotherapeutics. Analysis of tumor spheres generated from different cancer cell lines and primary cancer cells can be used to evaluate sphere forming efficiency, tumorigenicity, and self-renewal of cancer stem/tumor-initiating cells.

1.2 Purpose

The purpose of this guide is to provide the steps and concepts that are unique to the Colony Counting: Tumorsphere application. Information that is common to all applications is covered in the User Guide (8001619), from here on referred to as the User Guide.

1.3 Safety Precautions

All safety precautions described in the User Guide apply to this guide.

1.4 Technical Assistance

Contact Nexcelom Customer Service for further information:

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- From the United States:
  email: celigosupport@nexcelom.com
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  phone: 978-327-5340

2. Prerequisites

The prerequisites for performing the procedures in this document are:

- Familiarity with Celigo operation per the User Guide
- Celigo cytometer is started up per the User Guide
- Prepared tumorspheres for image analysis, using the following recommended volume of medium and number of tumorsphere. Higher volumes may result in more tumorsphere movement during scanning. Higher number of tumorspheres may be more challenging to segment properly.

Table 1. Recommended Volumes and Number of Tumorspheres

Plate Format	Volume	Number of Tumorspheres
6 well	3 ml	Up to ~500 tumorspheres
12 well	2 ml	Up to ~200 tumorspheres
24 well	1 ml	Up to ~100 tumorspheres
96 well	200 µl	Up to ~40 tumorspheres

NOTE: Use of a cell strainer to remove single cells and debris is recommended to obtain images free of debris. Tumorspheres tend to fuse over time during culture in suspension. Therefore older cultures with significant tumorsphere fusion will be more difficult to correctly identify. Daily rocking of cultures is recommended to alleviate tumorsphere fusion.

3. Scanning Plates

This chapter provides the procedures for selecting the Colony Counting: Tumorsphere application and setting the image acquisition parameters. You perform these tasks in the Scan tab.

3.1 Colony Counting: Tumorsphere Application

Perform the following step to select the Colony Counting: Tumorsphere application:

To select the Colony Counting: Tumorsphere Application

- In the Current Application dropdown list, select one of the Tumorsphere applications (Figure 1), depending on the quantity of channels (targets) to be acquired (Table 2).

Figure 1. Selecting the Colony Counting: Tumorsphere application

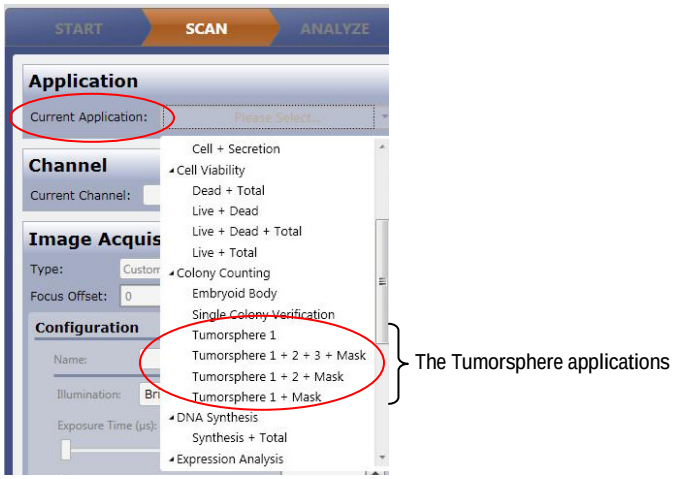


Table 2. Selecting a Tumorsphere Application

Quantity of Channels (Targets) to be Acquired	Select this application	How Fluorescence is Measured	Use Case
One channel (the BF mask channel)	**Tumorsphere 1**	Not Applicable	• Monitor count and size of tumorspheres over time.
Three FL channels + a BF mask	**Tumorsphere 1 + 2 + 3 + Mask**	The brightfield mask defines the tumorsphere. Fluorescence or brightfield signal is measured in the same mask.	• Measure reporter gene expression in tumorspheres identified in brightfield. • Calculate biomarker signal in tumorspheres identified in brightfield.
Two FL channels + a BF mask	**Tumorsphere 1 + 2 + Mask**		
One FL channel + a BF mask	**Tumorsphere 1 + Mask**		

3.2 Acquisition Settings

Perform the following two main steps to select image acquisition settings, using the following guidelines:

- First select the image acquisition settings for the mask (brightfield) channel (section 3.2.1). You do this task for any of the tumorsphere applications (Tumorsphere 1, Tumorsphere 1+2+3+Mask, etc.).
- Next, select the image acquisition settings for the fluorescence channel (3.2.2). You do this task for any of the following tumorsphere applications:
  - Tumorsphere 1+Mask
  - Tumorsphere 1+2+Mask
  - Tumorsphere 1+2+3+Mask

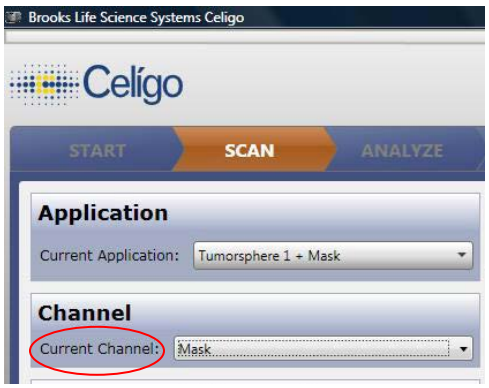
3.2.1 Selecting Acquisition Settings for the Mask (Brightfield) Channel

When performing the following procedure, see Table 3 for the recommended initial settings to use as a guide.

To select image acquisition settings for the mask (brightfield) channel

1. Do one of the following:
   - If you selected Tumorsphere 1 as the Current Application, you are using only 1 channel, which is the mask channel. Therefore a Current Channel field does not display; the system considers all entries as being for the mask channel; skip to step 2.
   - If you selected one of the following applications as the Current Application, select **Mask** in Current Channel (Figure 6):
     - Tumorsphere 1+Mask
     - Tumorsphere 1+2+Mask
     - Tumorsphere 1+2+3+Mask

Figure 2. Selecting the Mask Channel (for all Tumorsphere applications except Tumorsphere 1)



For Tumorsphere 1 + Mask, like all Tumorsphere applications except Tumorsphere 1, you need to specify the channel for which you are selecting settings.

2. Select a well with a sufficient population of representative tumorspheres.
3. Click **Live** to see a live image.
4. Use manual focus to achieve a clear image of the tumorspheres. A clear image is considered an image that has *edges* that are crisp, not blurry. The system looks at the edges to define the sphere boundaries.
5. Do one of the following to set the optimal exposure:
    - If the wells have *different* darkness shadows and you want the same grey scale background: In Type, select **Autoexposure/Gain Channel** and then click **Apply Now**. The system will select the appropriate exposure individually for each well to capture images with the same greyscale background..
    - If the wells have *the same* darkness shadows: In Type, select **Custom** and then either or both of the following:
        - Click **Auto Exp**: The system will attempt to determine the optimal exposure time and gain setting and apply this same exposure value to each well for the entire plate.
        - Manually set the optimal exposure time and gain by making an entry in the Exposure Time and Gain fields.

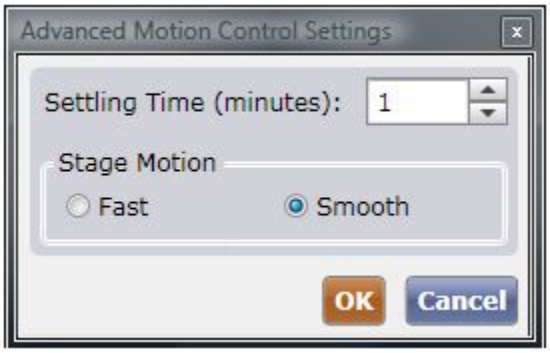
Table 3. Recommended Initial Settings for Image Acquisition

Type	Auto Exposure/Gain Channel
Focus Offset	0
CONFIGURATION	
Illumination	Brightfield
Priority	AutoExposure, Gain if necessary
Frequency	Every scan area
MOTION CONTROL AND FOCUS	
Configuration	
Settling Time (minutes)	1
Stage Motion	Smooth
Focus	Focus Setup – Register Hardware Auto Focus Position

6. Set up Motion Control by selecting **Advanced**. Apply a settling time to allow tumorspheres to settle to the bottom of the plate. Typically 1 minute is used to ensure tumorspheres of all sizes have settled.

   Figure 3 shows the motion control settings that should be used to properly image tumorspheres growing in suspension culture.

Figure 3. Recommended Motion Control Settings



7. Set up Focus. Click the up/down buttons in the Focus section until a crisp focus at the edge of the tumorspheres is visible.
   - Check focus performance by navigating to a different well and viewing the crispness of the tumorsphere edges.
8. Click **Focus Setup**.

9. In the Focus Setup dialog box, make selections as needed. When making the Focus Type selections, use the following guidelines:

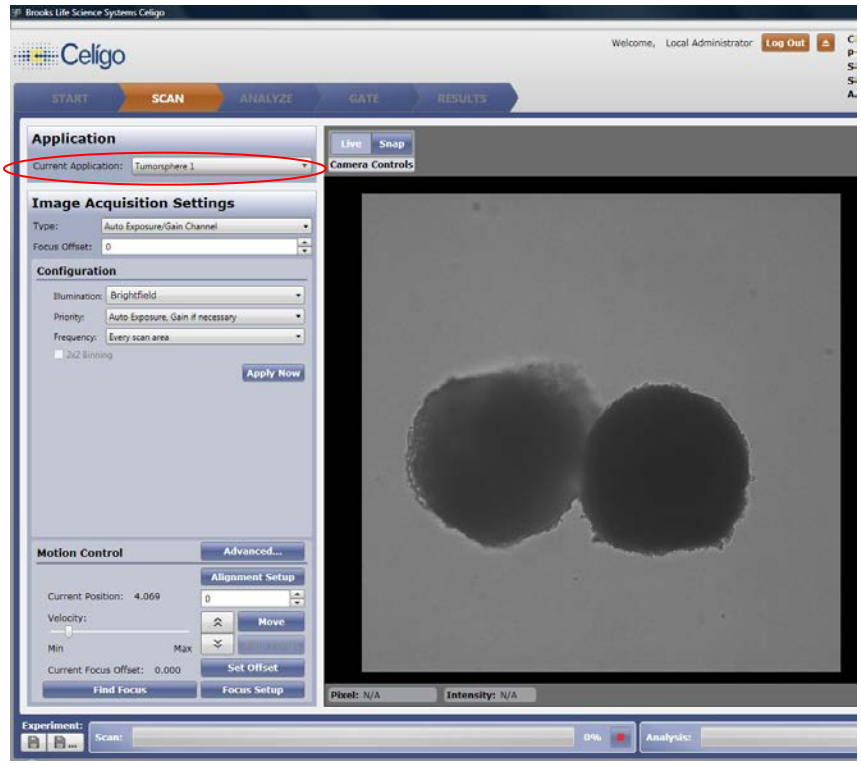
   - If using flat-bottom plate, select **Hardware Auto Focus**.
   - If using a U-bottom plate, select **Image Based Auto Focus**.

   If tumorspheres are large enough for imaging all wells at the same z-position throughout the entire plate, select **None** as the Focus Type.

Figure 4 shows examples of acquisition settings for the brightfield channel with tumorspheres in focus, with clear, crisp edges. In the settings shown on the left, the Tumorsphere 1 application is selected, uses only 1 channel, and therefore a Channel menu does not display. In the settings on the right, Tumorsphere 1+Mask application is selected, which uses 2 channels, and therefore a Channel menu displays.

Figure 4. Tumorsphere 1 Application Mask (Brightfield) Channel with Tumorspheres in Focus

Tumorsphere 1 Application Mask Settings with Tumorspheres in Focus



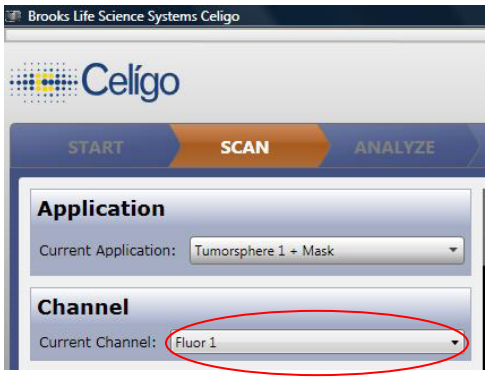
Tumorsphere 1 + Mask Settings

3.2.2 Selecting Acquisition Settings for the Fluorescence Channels

To select image acquisition settings for the fluorescence channels

1. Select an application, depending on the quantity of channels to be acquired (Table 2).
2. In Current Channel, select a fluorescence channel to set up (for example Fluor 1 or Fluor 2) (Figure 5).

Figure 5. Selecting a Fluorescence Channel



3. For the selected channel in Current Channel, make the following selections:
   a. In Type, select **Custom Channel**.
   b. In Focus Offset, keep 0 (default).
   c. In Illumination, select the appropriate illumination for the dye (e.g., **Green** to visualize the calcein AM signal, **Red** to visualize the propidium iodide signal). **Blue** to Hoechst.
   d. Click **Live** to see a live image.
   e. In Exposure Time and Gain, adjust as needed to correct the displayed live image.
   f. To reduce photobleaching, turn off Live view.

      To do this, click **Live** again so that it is deselected. Alternatively, capturing an image by clicking **Snap** only exposes the sample to light for the set exposure time.

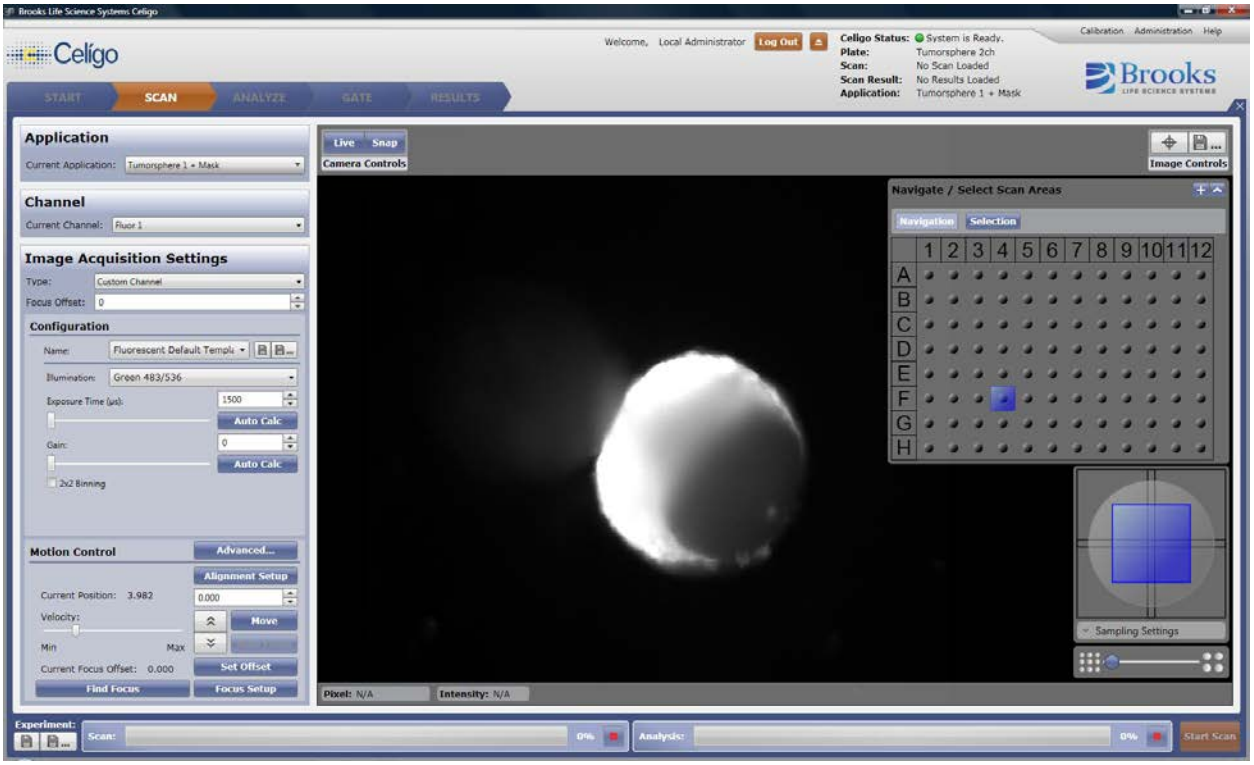
	**NOTE: For scanning with fluorescence illumination, it is recommended that you keep Live on for the shortest period possible. Keeping Live on for an extended period can result in bleached samples.**

   g. Click **Find Focus** to achieve a clear image of the cells.
   h. Click **Set Offset**.
      – Setting focus offsets is important because the optimal focus positions for red, green, and blue wavelengths and brightfield are different from each other. The software will adjust the focus position for each channel during a scan to provide the best focus according to the offset values.

4. Set up any remaining fluorescence channel as needed by repeating this fluorescence channel setup section (3.2.2).

Figure 6 shows an example of Fluor 1 channel selections.

Figure 6. Fluor 1 Channel Selections



Note: Nexcelom Bioscience acquired the Celigo product line from Brooks Life Science Systems in March 2014. As of the printing of this document, the Celigo software version displays the Brooks logo.

4. Analyzing Images

This chapter provides information on how to analyze scans from the Colony Counting: tumorsphere application. You perform these tasks in the Analyze tab.

4.1 Selecting Analysis Settings

Perform the following steps to select the optimal analysis settings. The recommended initial settings for identification and pre-filtering when using the Colony Counting: Tumorsphere application are shown in Table 4. The settings typically provide good image segmentation.

Important guidelines

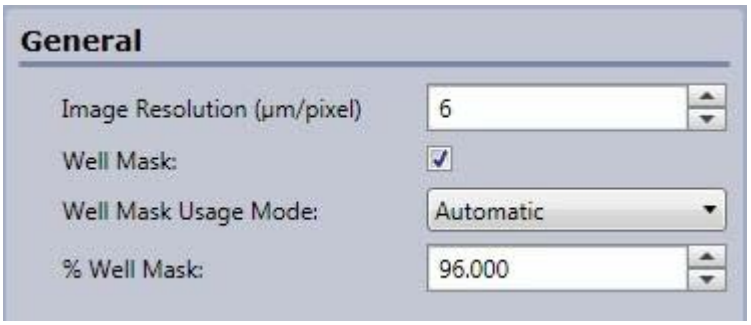
While selecting analysis settings, use the following important guidelines:

- Test the analysis settings using multiple tumorspheres across at least one well of the plate.
- Tumorsphere border identification is critical for this application. Therefore, images containing tumorspheres with clear, crisp edges will be easy to identify and analyze. Tumorspheres that are out of focus have unclear (fuzzy) edges and will be more difficult to define.
- Images will appear in FOR format (4x4 FOV), allowing viewing of a large portion of a well at one time. The FOR format takes more time for the image to appear than viewing a single FOV at one time.

To select analysis settings

1. Load prior saved Analysis Settings if available.
2. In the General section (Figure 7), make the following selections:

Figure 7. General Section



a. Image Resolution (µm/pixel) -- Select the image resolution to use for analysis. For initial setting, enter 4. Available range is 2-100. Entering a lower value will result in greater accuracy and longer analysis time. Entering a higher value will result in less accuracy and shorter analysis time.

b. Well Mask – Applies a boundary at the well edge so that the well edge (subject to distortion or plate artifacts) is excluded from segmentation. For initial setting, uncheckmark.

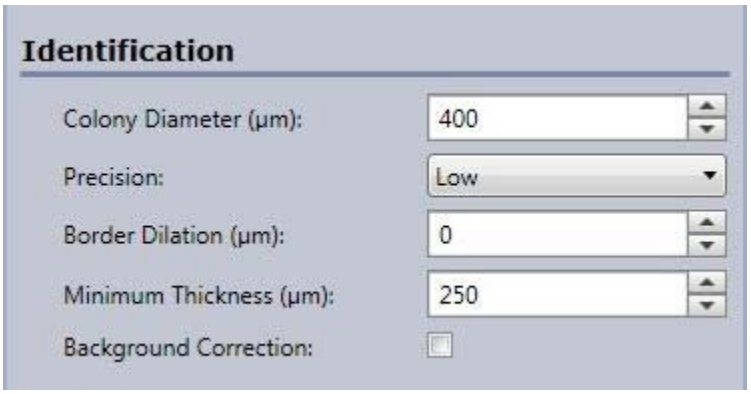
c. Well Mask Usage Mode – Select **Automatic**.

d. % Well Mask – Enter **100%**. Decreasing the % Well Mask entry can exclude well edge artifacts that can occur during plate manufacturing.

For information about Well Mask, Well Mask Usage Mode, and % Well Mask, see the User Guide section “Selecting General Analysis Settings.”

3. In the Identification section (Figure 8), make the following selections:

Figure 8. Identification Section

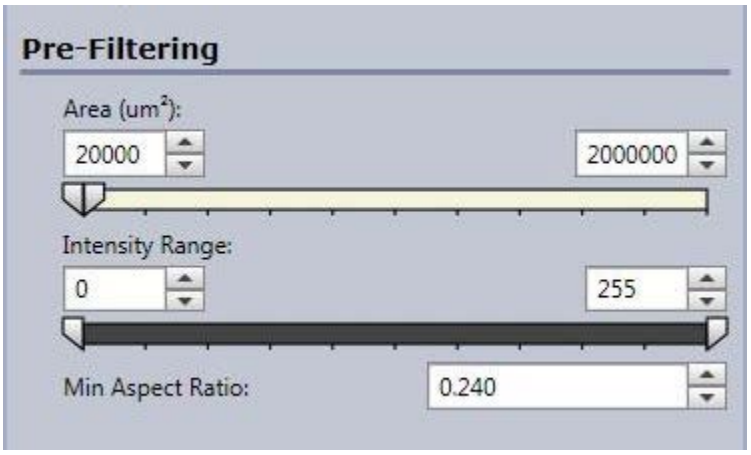


- Colony Diameter (µm) – Defines the diameter of the tumorsphere. If a range of tumorsphere sizes are present, this should be close to the maximum size.
- Precision – Select the desired level of precision of separation of touching tumorspheres:
  - None – No separation.
  - Low – Minimal separation.
  - Medium – Medium separation.
  - High – Most precise separation.

    Higher values will result in the system's attempt to determine the exact boundary between tumorspheres. As the desired precision increases, so do the chance that intensity artifacts will incorrectly affect the separation borders.
- Border Dilation (µm) – Defines the amount of dilation/erosion for tumorsphere edge segmentation.
- Minimum Thickness (µm) – Minimizes object artifact extensions.
- Background Correction – (Use for Brightfield Illumination only) – Minimizes background variations due to meniscus by correcting an image for segmentation.

4. In the Pre-Filtering section (Figure 9), make the following selections:

Figure 9. Pre-Filtering Section



- Area (µm^2) – The tumorsphere area. Enter the tumorsphere area range that corresponds to your tumorspheres.
- Intensity Range – Make selections to include only tumorspheres and exclude debris/single cells.
- Min. Aspect Ratio – Removes elongated debris.

Table 4. Recommended Initial Identification and Pre-Filtering Settings for Analysis

Parameter	Initial Setting	Available Range	Description
IDENTIFICATION			
Colony Diameter (µm)	300	10 – 100,000	Defines the diameter of tumorspheres. Helps determine separation from neighboring or touching spheres. Lower value: Small tumorspheres, or more separation. Higher value: Large tumorspheres, or less separation.
Precision	Low	None, Low, Medium, High	• None – No separation. • Low – Minimal separation. • Medium – Medium separation. • High – Most precise separation. Higher values will result in the system's attempt to determine the exact boundary between tumorspheres. As the desired precision increases, so do the chance that intensity artifacts will incorrectly affect the separation borders.
Border Dilation (µm)	0	- 500 – +500	Defines amount of dilation/erosion for tumorsphere edge segmentation. Lower value: Tumorsphere border segmentation will move closer to the edge of the tumorsphere. Higher value: Tumorsphere border segmentation will move farther from the edge of the tumorsphere. Can be used to segment tumorspheres with fuzzy edges.
Minimum Thickness (µm)	80	0 – 500	Minimizes object artifact extensions.
Background Correction	Uncheckmarked	Checkmarked or Uncheckmarked	Minimizes background variations by applying an average value.
Well Mask	Checkmarked	N/A	Identifies the edge of the well.

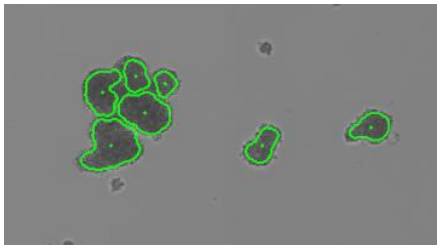
PRE-FILTERING			
Tumorsphere Area (μm^2) Range	20,000-2,000,000	50 – 100,000,000,000	Defines the range of tumorsphere area included in analyses. Setting a lower limit and upper limit can help exclude single cells, small debris and/or large debris.
Tumorsphere Intensity Range	0-255	0-255	Defines pixel intensity range of tumorspheres included in analyses. Setting a lower limit can exclude dark, black debris. Setting an upper limit can exclude whited-out debris. Typically not used for the Tumorsphere Application.
Min Tumorsphere Aspect Ratio	0.000	0-1	Defines the shape of tumorspheres (and debris) included in analysis. Defined as the ratio 1 over the maximum cell elongation. Min tumorsphere Aspect Ratio of 1 is a perfect circle. Lower aspect ratios typically remove elongated debris. Lower value: More debris will be identified. Higher value: Less tumorspheres/debris will be identified.

Figure 10 through Figure 17 show examples of Colony Counting: Tumorsphere brightfield images with corresponding target overlays.

Figure 10 shows an example of the display results when adjusting colony diameter.

Figure 10. Tumorsphere s with Corresponding Target Overlay – Colony Diameter Adjustments

Colony Diameter 100



Colony Diameter 500

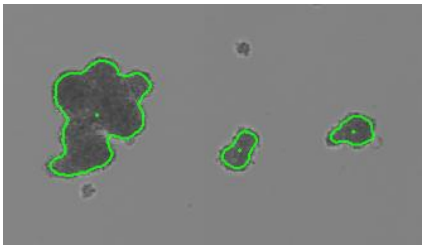
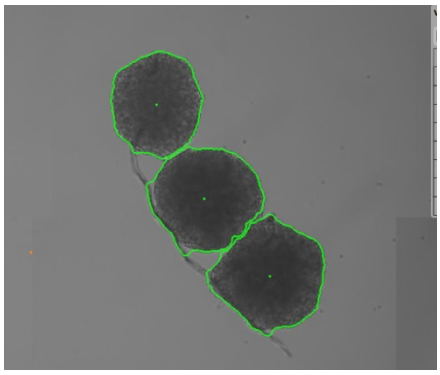


Figure 11 shows an example of the display results when adjusting precision.

Figure 11. Tumorsphere s with Corresponding Target Overlay – Precision Adjustments

Precision Low



Precision High

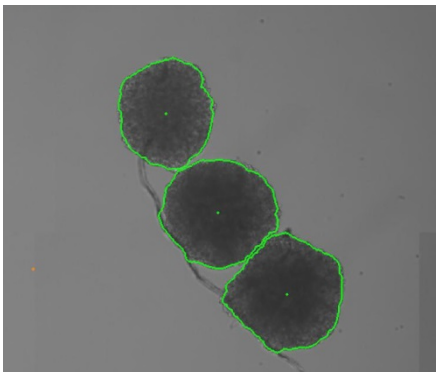
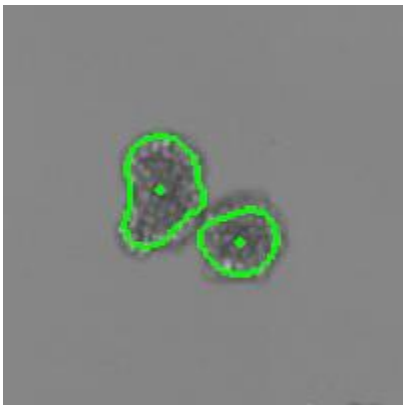


Figure 12 shows an example of display results when adjusting tumorsphere border dilation.

Figure 12. Tumorsphere s with Corresponding Target Overlay – Tumorsphere Border Dilation Adjustments

Border Dilation 0



Border Dilation 20

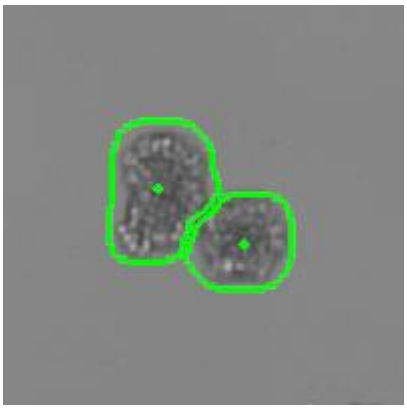
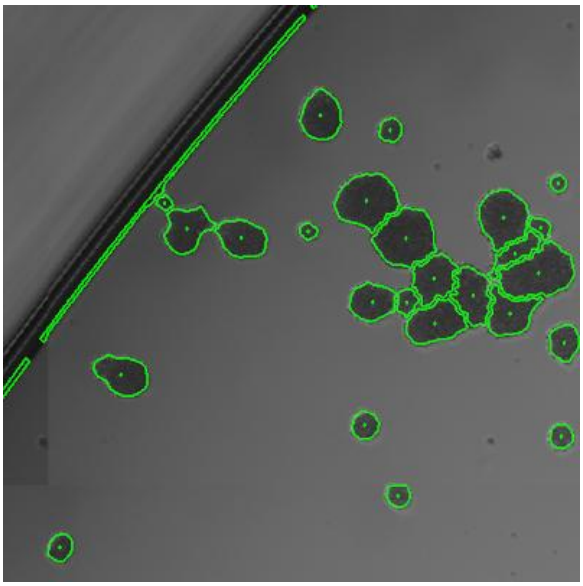


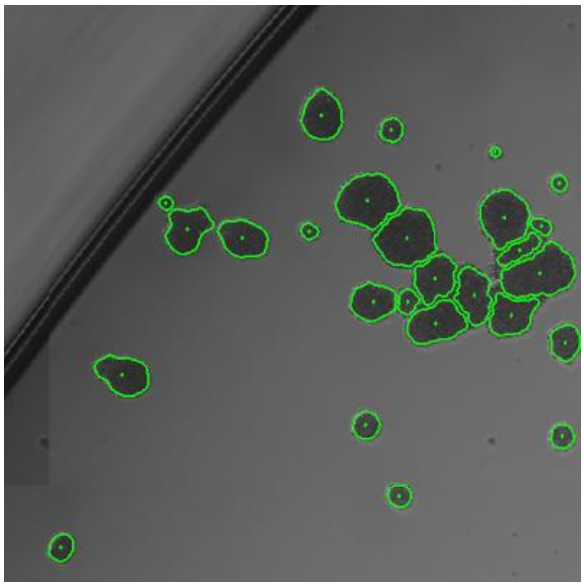
Figure 13 shows an example of display results when adjusting minimum thickness.

Figure 13. Tumorsphere s with Corresponding Target Overlay – Minimum Thickness

Minimum Thickness 20 µm



Minimum Thickness 50 µm



Minimum Thickness 80 µm

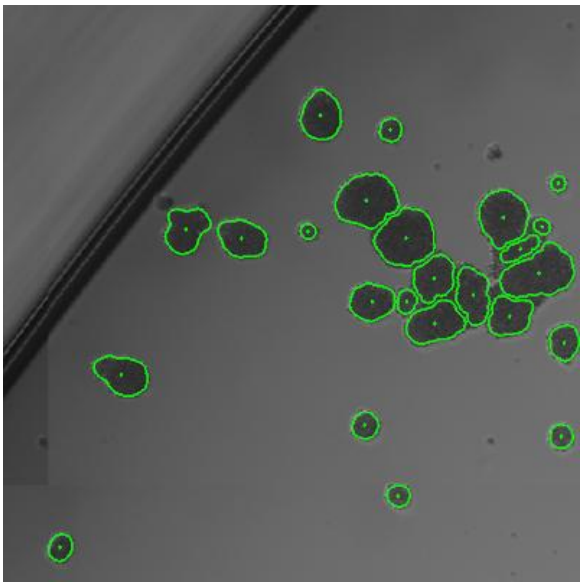


Figure 14 shows an example of display results when adjusting background correction.

Figure 14. Tumorsphere s with Corresponding Target Overlay. – Background Correction

Background Correction Off **Background Correction On**

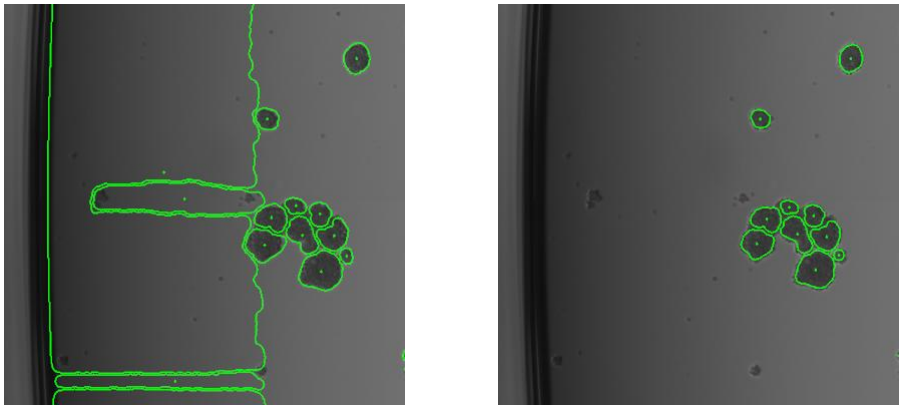


Figure 15 shows an example of display results when adjusting area (µm^2) range.

Figure 15. Tumorsphere s with Corresponding Target Overlay. – Area (µm$^{2)}$ Range Adjustments

Area (µm^2) 500 **Area (µm^2) 10,000**

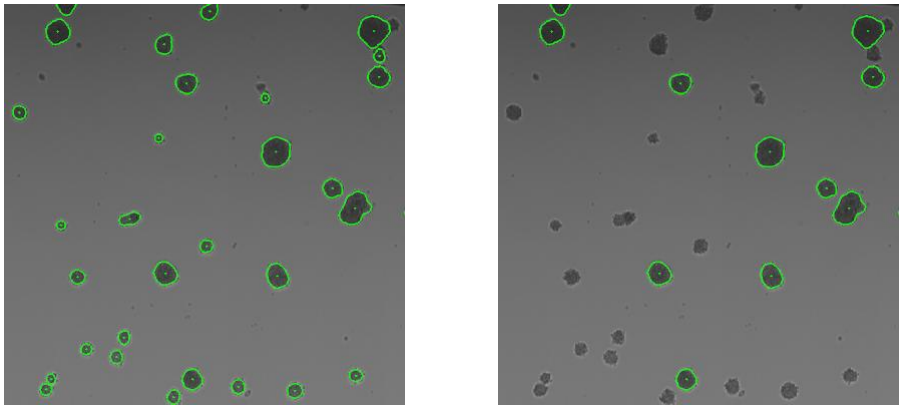
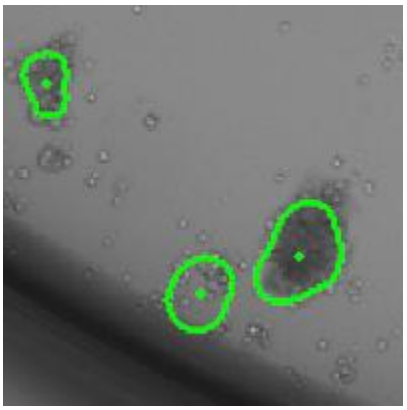


Figure 16 shows an example of display results when pixel intensity range is adjusted.

Figure 16. Tumorsphere s with Corresponding Target Overlay – Intensity Range Adjustments

Intensity Range
Max Pixel Intensity 255



Intensity Range
Max Intensity 106

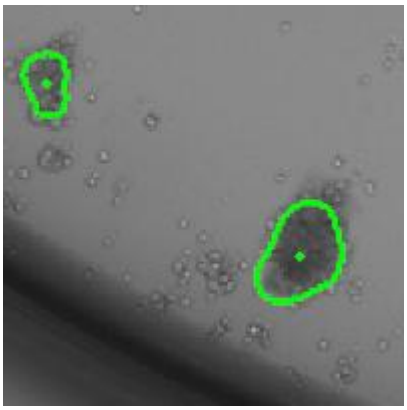
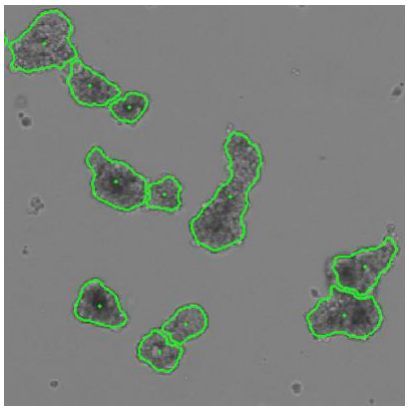


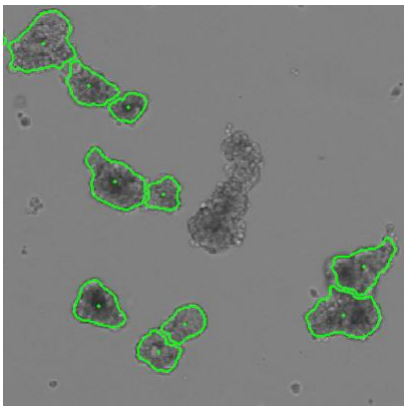
Figure 17 shows an example of display results when minimum tumorsphere aspect ratio is adjusted.

Figure 17. Tumorsphere s with Corresponding Target Overlay – Minimum Tumorsphere Aspect Ratio Adjustments

Aspect Ratio 0.000



Aspect Ratio 0.230



5. Gating Cells

This chapter provides information on how to select filter settings for further data analysis. You perform this task in the Gate tab.

The Colony Counting: Tumorsphere application relies on the identification of tumorspheres and removal of debris using the Colony Diameter, Precision, Minimum Thickness, Area, Intensity Range, and Min Aspect Ratio selections in the Analyze tab.

5.1 Working with Gates

The following are general principles about working with gates in the Colony Counting: Tumorsphere application.

- If you do not perform gating, the system uses the ALL population to count the cells in the wells. ALL is the default population that the system assigns to all the data points (objects) in the segmentation result from the Analyze tab.
- When performing gating in this application, only one class (the Total class) exists; you cannot assign any additional classes to populations. You can reanalyze the same scan and assign a different class for a different population.
- Make sure that a Classes checkbox has been selected to assign a class to the Total population. Checkmarking a Classes checkbox will allow the data for the class (the Total class) to appear in the analysis results.

NOTE: Make sure that a Classes checkbox has been selected for the Total population. The absence of a checkmark would be the same as not using the Gating tab; all data would be reported from the analysis tab settings only.

To create a plot, gate, and populations

1. In the Plot Populations pane, create a plot, using the Add Plot (+) button and Add Plot dialog box, and referring to the selections in Table 5.

   The table lists the selections you can make in both Pick plot parameters menus. The Pick plot parameters selections are different from those in the User Guide (which shows displays for the Expression Analysis application) because only one channel is used in the Colony Counting: Tumorsphere application.

   For details, see User Guide section “Creating a Plot.”

   By default, all segmented tumorspheres from the Analyze tab are assigned to the ALL (Total) source population. When you create a plot and draw a gate, then select the Classes checkbox as the new "Total" population of tumorspheres to be analyzed. The new Total class is then assigned to the newly defined population and the data is reported in the Results tab.

Table 5. Plot Parameter Selections

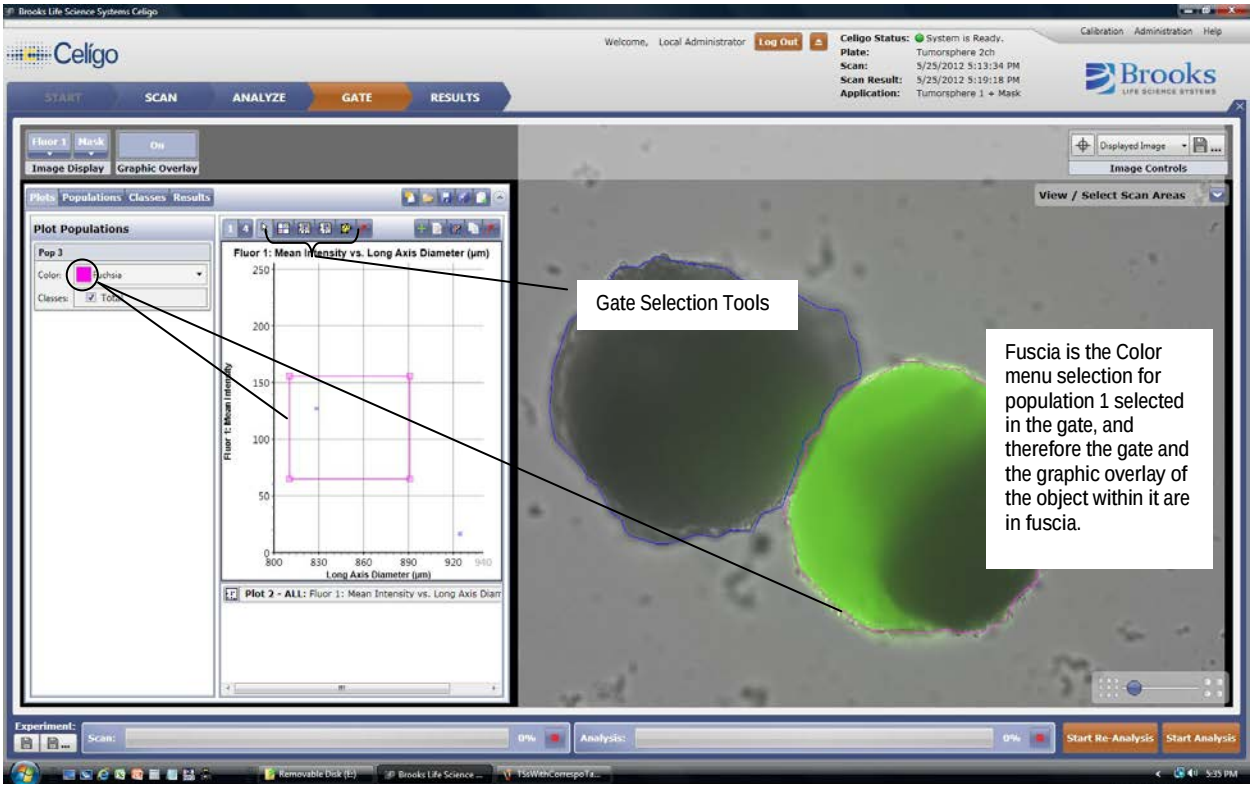
Feature	Definition
X Position (µm)	Location of a cell along the horizontal axis of the well: left (-µm) or right (+µm) of the center (origin of the well).
Y Position (µm)	Location of a cell along the vertical axis of the well: below (-µm) or above (+µm) of the center (origin of the well).
Distance to Nearest Neighbor (µm)	Distance from the target tumorsphere to the closest neighboring tumorsphere.
Distance to Well Center (µm)	Distance from the target tumorsphere to the center (origin) of the well.
Long Axis Angle	Angle of the longest axis of an elliptical approximation of the target tumorsphere shape.
Long Axis Diameter (µm)	Measure of the longest diameter of each identified tumorsphere.
Short Axis Diameter (µm)	Measure of the shortest diameter of each identified tumorsphere.
Est Volume (μm^3)	Estimated volume of the tumorspheres, using the following volume formula for a general ellipsoid: π / 6 x (short diameter x short diameter x long diameter) This assumes that the tumorsphere thickness (Z-axis) is equal to the visible short diameter measurement.
Area (μm^2)	Total area of each identified tumorsphere.
Perimeter (µm)	The total length of the edge of each identified tumorsphere.
Form Factor	Measure of the compactness of each identified tumorsphere, derived from the perimeter and area. A circular tumorsphere is compact with a form factor of 1.0 (the maximum); a more convoluted shape has a lower value.
Smoothness	Measure of the evenness of a tumorsphere's contour. It is a ratio of the convex perimeter to the true perimeter of a tumorsphere. A completely smooth tumorsphere is has a smoothness value of 1.0 (the maximum).
Aspect Ratio	Measure of the tumorspheres breadth to the tumorsphere's length. A tumorsphere that is a perfect circle has an aspect ratio of 1.0 (the maximum).
Mean Intensity	Average of the intensities of the segmented objects calculated for each channel.
Std Dev Intensity	Standard deviation of the intensities of the segmented objects.
Integrated Intensity	Sum of all pixel intensities displaying signal in segmented objects calculated for each channel.
EQ Diameter (µm)	Equivalent diameter of each identified tumorsphere, derived from the area. Equivalent diameter = 2(radius), derived from Area = $\pi(radius^2)$

2. Create a gate on the plot, using the gate selection tools (Figure 18).

   For details, see User Guide section “Creating a Gate.”

   In the Graphic Overlay display, the color displayed for the plot population selected in the gate corresponds to the Color menu selection (Figure 18). The figure shows the single class (Total) used in this application.

Figure 18. Gating Cells



3. Repeat steps 1 and 2 as needed to refine the population that you want to count.

4. In the Plots, Populations, or Classes view, make sure that the Total class is checkmarked. This assigns the Total class to the population.

   For details, see User Guide section “Assigning a Class to a Population.”

   In this application, you assign only the Total class to populations; you cannot assign any additional classes to populations.

Note: Nexcelom Bioscience acquired the Celigo product line from Brooks Life Science Systems in March 2014. As of the printing of this document, the Celigo software version displays the Brooks logo.

6. Viewing Results

This chapter describes the feature outputs available from the application's Results tab.

6.1 Application Outputs

The parameters listed in Table 6 appear below the Display Options section in the Scan Information pane.

Table 6. Colony Counting: Tumorsphere Application Outputs

Parameter	Description	Ave	St Dev	CV	Min/ Max
Tumorsphere Count	Number of tumorspheres identified and analyzed				
Diameter (µm)	Equivalent diameter of each identified tumorsphere, derived from the area. Equivalent diameter = 2(radius), derived from Area = π(radius^2).	√	√	√	√
Short Axis Diameter (µm)	Measure of the shortest diameter of each identified tumorsphere.	√	√	√	
Long Axis Diameter (µm)	Measure of the longest diameter of each identified tumorsphere.	√	√	√	
Est Volume (µm^3)	Estimated volume of the tumorspheres, using the following volume formula for a general ellipsoid: pi / 6 x (short diameter x short diameter x long diameter) This assumes that the tumorsphere thickness (Z-axis) is equal to the visible short diameter measurement.	√	√	√	
Area (µm^2)	Area of each identified tumorsphere, measured in pixels.	√	√	√	
Perimeter (µm)	The total length of the edge of each identified tumorsphere.	√	√	√	
Form Factor	Measure of the compactness of each identified tumorsphere, derived from the perimeter and area. A circular tumorsphere is compact with a form factor of 1.0 (the maximum); a more convoluted shape has a lower value.	√	√	√	
Smoothness	Measure of the evenness of a tumorsphere's contour. It is a ratio of the convex perimeter to the true perimeter of a tumorsphere. A completely smooth tumorsphere is has a smoothness value of 1.0 (the maximum).	√	√	√	
Long Axis Angle	Angle of long axis of the tumorsphere (longest dimension).	√	√	√	

continued on next page

continued from previous page

Aspect Ratio	Measure of the tumorspheres breadth to the tumorsphere's length. A tumorsphere that is a perfect circle has an aspect ratio of 1.0 (the maximum).	√	√	√	
Tumorsphere Colony Density (Col/cm^2)	The number of tumorspheres divided by the total area of the well (or scan area). Provides an estimate of the colony density, or how many colonies are present per unit well area.	√			
Fluor 1 -3 Mean Intensity	Average of the intensities of the segmented objects. Will differ from Total Intensity.	√	√	√	
Fluor 1 -3 Integrated Intensity	Sum of all pixel intensities displaying signal in segmented objects.	√	√	√	
Mask Mean Intensity	Average of the intensities of the segmented objects. Will differ from Total Intensity.	√	√	√	
Mask Integrated Intensity	Sum of all pixel intensities displaying signal in segmented objects.	√	√	√	
% Well Sampled	Percent of well surface processed				

6.2 Data Export

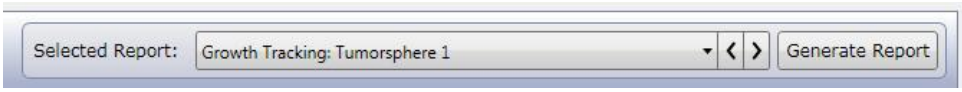
Well-level and object level data can be exported into CSV (Comma Separated Value) or FCS (Flow Cytometric Standard) files. For the procedures to perform data export, see the User Guide.

6.3 Generating a Growth Tracking Report

Growth tracking reports calculate the growth characteristics of tumorsphere populations over time. The reports associate tumorsphere counts or area measurements from multiple scans time points and determine doubling times and rates for individual wells. The reports are in the form of plots that can be exported as images or data by the user for documentation and presentation purposes.

Growth tracking reports for the Tumorsphere Application are generated the same way as for the Cell Counting and Growth Tracking Application, using the Generate Report button (Figure 19).

Figure 19. Generate Report Button



For details on how to generate a Growth Tracking Report, see the *Celigo Cytometer Cell Counting and Growth Tracking Application Guide* (Doc. No. 483584).

The reporting of Average Area is unique to the Tumorsphere Application (Figure 20 and Figure 21).

Figure 20. Display Mode Options include Average Area Chart

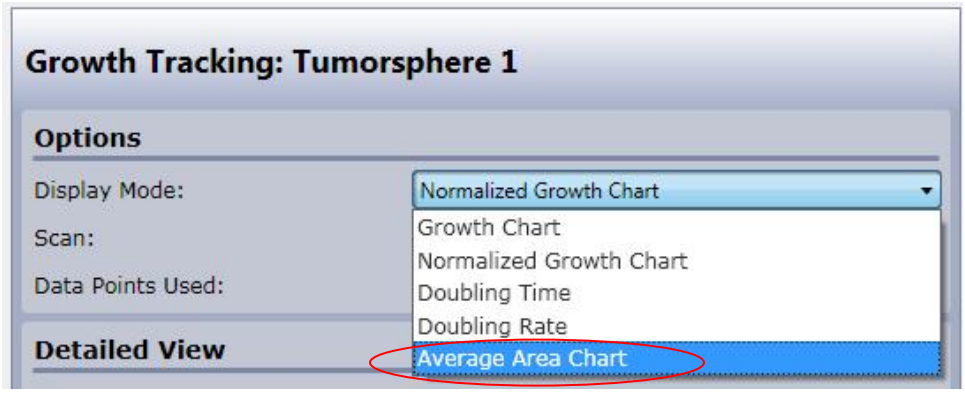
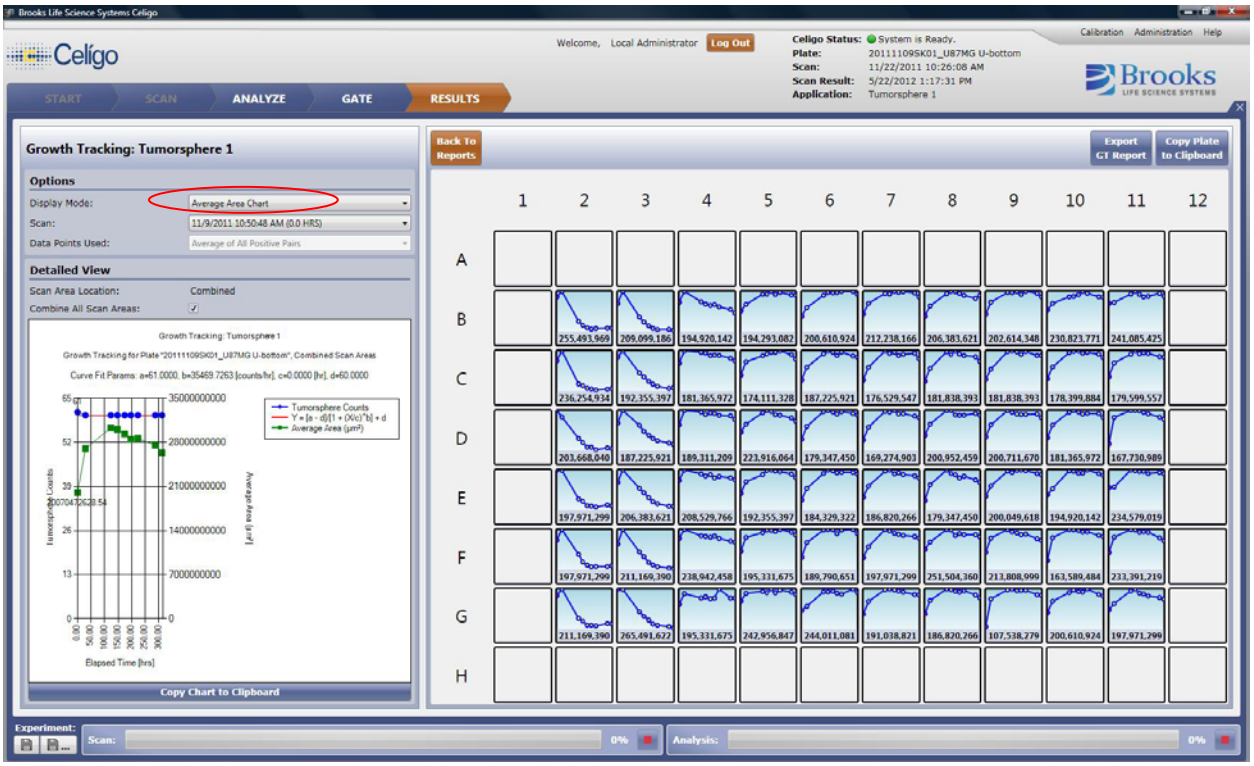


Figure 21. Tumorsphere 1 Average Area Chart



Note: Nexcelom Bioscience acquired the Celigo product line from Brooks Life Science Systems in March 2014. As of the printing of this document, the Celigo software version displays the Brooks logo.

7. Troubleshooting

This chapter provides troubleshooting recommendations.

Table 7. Troubleshooting Recommendations

Issue	Recommended Action
Tumorspheres are out of focus	1. When the hardware autofocus position is set using only a few nonrepresentative tumorspheres, the rest of the tumorsphere population will likely be out of focus. For example, if the hardware autofocus position is set using only small tumorspheres then larger tumorspheres may be out of focus. • Use a sufficient population of representative tumorspheres to set hardware autofocus when scanning.
Tumorspheres are not being identified	Tumorspheres may not be identified due to: 1. Part of the tumorsphere is out of focus: Adjust tumorsphere border value. 2. Tumorspheres are smaller/larger than the size range selected in the Tumorsphere Area in the Analyze tab: • Adjust range of Tumorsphere Area 3. Tumorspheres have a lower/higher intensity than the range selected in the tumorsphere Intensity Range in the Analyze tab: • Adjust tumorsphere Intensity Range 4. Tumorspheres are not circular, therefore have a low aspect ratio. • Decrease aspect ratio (at the expense of increasing debris).
Identification of debris	For tumorspheres, debris typically consists of single/dead cells, small clumps of cells that are not spherical, or strings in the medium. 1. Single/dead cells can be removed by decreasing the tumorsphere intensity range in the Analyze or Gating Tab. 2. Small clumps of cells not considered to be tumorspheres can be removed by adjusting the tumorsphere area range in the Analyze or Gating tab. 3. Strings/artifacts in the medium can be removed by increasing the Min tumorsphere Aspect ratio in the Analyze or Gating Tab.
Fused Tumorspheres are not being separated properly	Tumorsphere fusion occurs over time. Therefore the older the culture the more likely tumorspheres will be fused. • Gently rock tumorsphere cultures to alleviate fusion. • It is likely that parts of fused tumorspheres are out of focus: Increase tumorsphere border value in the Analyze tab. • Fused tumorspheres may have a larger size range than the selected range in tumorsphere area. Increase range of tumorsphere area. • Try a different Precision setting.

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 8001626 Rev A

Celigo® Cytometer
DNA Synthesis
Application Guide

Celigo Software Version 2.1

Contents

1. About this Guide

1.1 Introduction

Cell proliferation and the characterization of agents that either promote or inhibit cell proliferation are important areas of cell biology and drug discovery research. 5-bromo-2´-deoxyuridine (BrdU), a thymidine analog, is traditionally used to detect DNA replication in actively proliferating cells. Cells that have incorporated BrdU into DNA can be quickly detected by using fluorescently-conjugated antibodies directed against BrdU. BrdU staining facilitates the identification of cells that have progressed into, or through, the S-phase of the cell cycle during the BrdU-labeling period.

The Celigo DNA Synthesis application can be used for rapid, full-well imaging and accurate identification and quantification of cells undergoing DNA synthesis. Briefly, live cells are pulse-labeled with BrdU, fixed, and BrdU is detected by immunofluorescence. Images are acquired and analyzed using the Celigo software. Nuclear stain and BrdU stain are acquired in separate fluorescent channels for each well of a microtiter plate. The DNA Synthesis application then automatically reports positive cell counts and their percentages.

1.2 Purpose

The purpose of this guide is to provide the steps and concepts that are unique to the DNA Synthesis application. Information that is common to all applications is covered in the *Celigo® Cytometer User Guide* (8001619), from here on called the User Guide.

1.3 Safety Precautions

All safety precautions described in the User Guide apply to this guide.

1.4 Technical Assistance

Contact Nexcelom Customer Service for further information:

Nexcelom Bioscience, LLC.
Customer Service
360 Merrimack St. Building 9
Lawrence, MA 01843, USA

- From the United States:
  email: celigosupport@nexcelom.com
  phone: 978-327-5340
- From Europe:
  e-mail: celigosupport@nexcelom.com
  phone: 978-327-5340

2. Prerequisites

The prerequisites for performing the procedures in this document are:

- Familiarity with Celigo operation per the User Guide
- Celigo cytometer is started up per the User Guide

3. Scanning Plates

This chapter provides the procedures for selecting the appropriate DNA Synthesis application and setting the image acquisition parameters. You perform these tasks in the Scan tab.

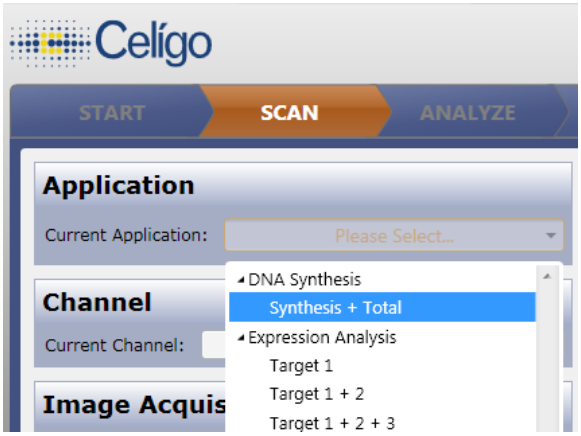
3.1 DNA Synthesis Application

Perform the following steps to select the DNA Synthesis application.

To select the DNA Synthesis application

- In the Current Application dropdown list, **select DNA Synthesis > Synthesis + Total** (Figure 1).

Figure 1. Selecting DNA Synthesis Application



	**NOTE: Although most researchers use DAPI as a counterstain for the cell nucleus, it is possible to use Hoechst 33342 and obtain similar results for the application.**

3.2 Acquisition Settings

Perform the following steps to select image acquisition settings in the DNA Synthesis application. For the recommended initial settings to use as a guide, see Table 1.

To select image acquisition settings

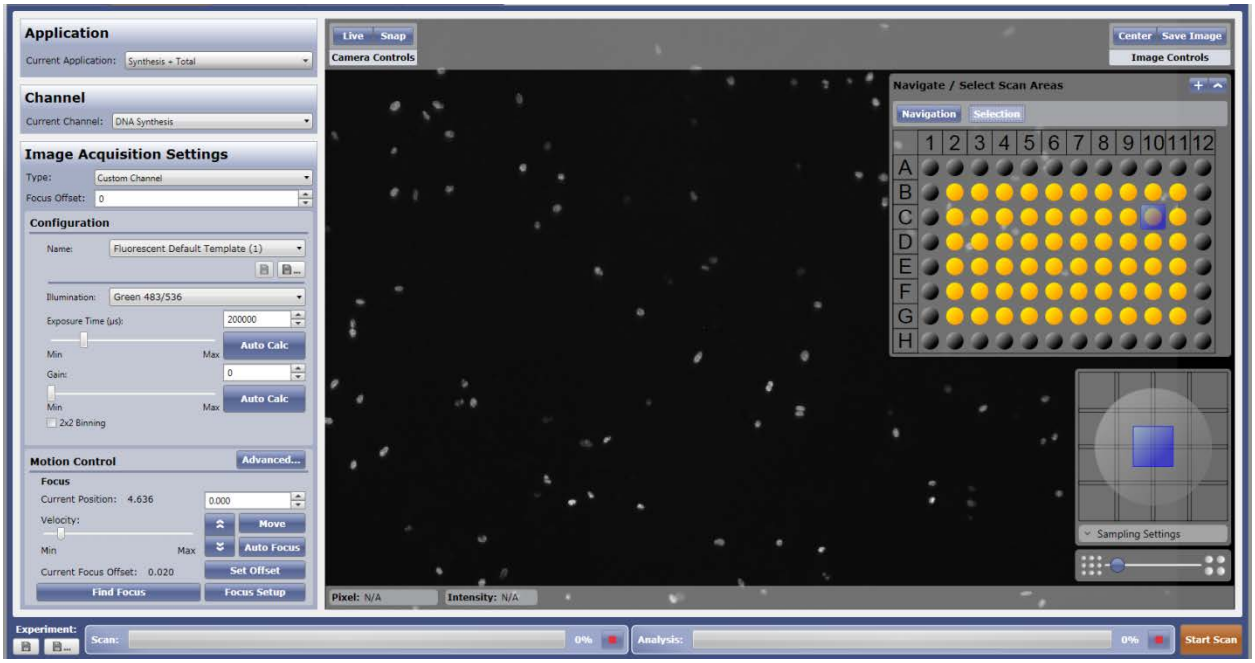
1. Choose a well for setup using the plate map by selecting **Navigation** and selecting a representative well. It is recommended to adjust exposure settings using a well where the highest fluorescent signal is expected.
2. In Current Channel, select **Total**.
3. Make the following selections:
   a. In Type, select **Custom Channel**.
   b. In Illumination, select the appropriate illumination for the dye (e.g., **Blue** to visualize the DAPI signal).
   c. Click **Live** to see a live image.
   d. Use manual focus to achieve clear image of cells.
   e. Select whether Autoexposure provides optimal exposure for your sample (cells are visible yet not overexposed, i.e. saturated: pixels intensities>254).
      - Autoexposure: system will attempt to determine the optimal exposure time and gain setting.
      - Custom: User manually establishes optimal exposure time and gain by adjustment of **Exposure Time** and **Gain**. When doing so, lower/adjust the exposure so the majority of cells aren't overexposed.
   f. If 2x2 binning (half resolution) is appropriate for acquisition, select **2x2 Binning**. For a detailed explanation of 2x2 Binning, see the User Guide.
   g. Set up autofocus.
      - It is recommended that you focus with automatic registration and select the **Hardware Auto Focus** Focus Type for most routine plate scanning. For detailed instructions, see User Guide section "Focusing with automatic registration."
4. In Current Channel, select **DNA Synthesis**.
5. For the DNA Synthesis channel in Current Channel, make the following selections:
   a. In Type, select **Custom Channel**.
   b. In Illumination, select the appropriate illumination for the dye (e.g., **Green** to visualize the DNA Synthesis signal).
   c. Click **Live** to see a live image.
   d. Use **Find Focus** to achieve a clear image of the cells.
   e. Select **Set Offset**.

Table 1. Recommended Initial Settings for Image Acquisition

Current Channel	DNA Synthesis	Total
Type	Custom Channel	Custom Channel
Focus Offset	User determined	User determined
Name	Fluorescent Default Template (1)	Fluorescent Default Template (2)
Illumination	Green 483/536	Blue 377/447
Exposure Time (µs)	20000	60000
Gain	0	0
Motion Control and Focus	See User Guide	See User Guide

Figure 2 shows an example of the DNA Synthesis channel.

Figure 2. DNA Synthesis Channel



4. Analyzing Images

This chapter provides information on how to analyze scans from the DNA Synthesis application. You perform these tasks in the Analyze tab.

In this application, images from only the Total channel are segmented according to user-specified analysis settings. The segmentation logic follows the logic of the two-channel single-mask segmentation, where all the objects are identified using the nuclear mask and the DNA synthesis intensity is measured for the green channel within this mask

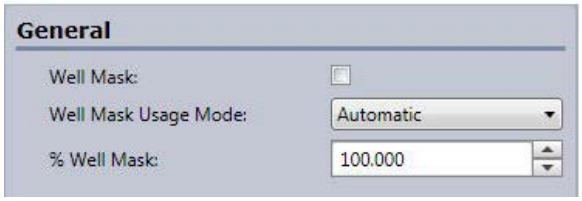
4.1 Analysis Settings

Perform the following steps to select the optimal analysis settings. The initial analysis settings for identification and pre-filtering when using the DNA Synthesis application are shown in Table 2. The settings typically provide good image segmentation. For a detailed explanation of the identification and pre-filtering settings, see the User Guide.

To select analysis settings

1. Load prior saved Analysis Settings if available
2. In the General section (Figure 3), make the following selections:

Figure 3. General Section



a. Well Mask – See Table 2.

	**NOTE: Selecting Well Mask is critical for this application. Failure to select Well Mask in this application will cause the system to identify objects outside the well.**

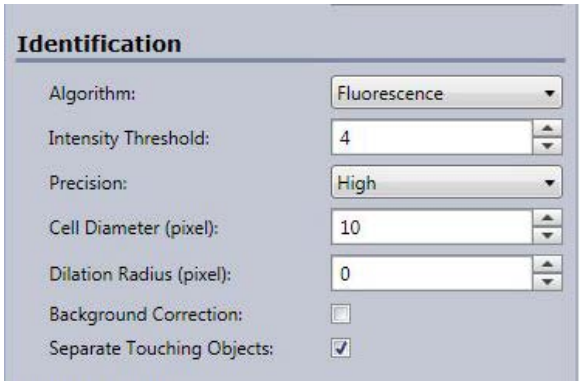
b. Well Mask Usage Mode – Select **Automatic**.

c. % Well Mask – Enter **100%**. Decreasing the % Well Mask entry can exclude well edge artifacts that can occur during plate manufacturing.

For information about Well Mask, Well Mask Usage Mode, and % Well Mask, see the User Guide section “Selecting General Analysis Settings.”

3. In the Identification section (Figure 4), make the following selections:

Figure 4. Identification Section

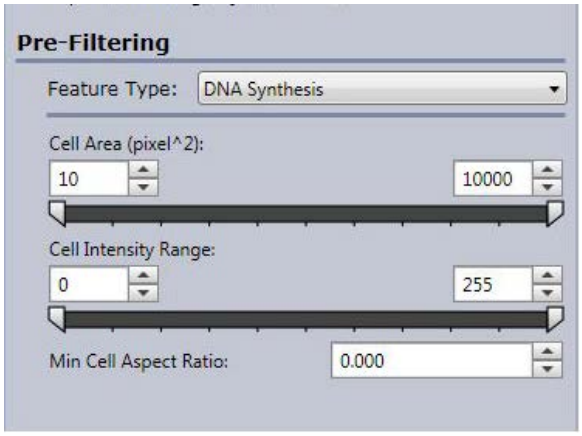


a. Algorithm – Select **Fluorescence**.
b. Intensity Threshold – Enter the optimal intensity threshold.

   The intensity threshold is the level of intensity that separates background from cells. With an appropriate threshold set, background pixels fall below and cell pixels are above the threshold. Any pixels below the threshold are not considered in the calculation.
c. Precision – Higher precision results in more accurate identification of cell clusters. High recommended.
d. Cell Diameter (pixel), – Enter the Cell Diameter (pixels) that corresponds to your cells. At full resolution, the Celigo provides 1 µm/pixel. Binned pixels provide 2 µm/pixel.
e. Dilation Radius (pixel) – Enter the distance in pixels that you want to dilate the object's boundary.
f. Background Correction – Select as needed to minimize background variations by applying an average value.
g. Separate Touching Objects – Select if it is difficult to separate touching cells during segmentation. (Recommended)

4. In the Pre-Filtering section (Figure 5), make the following entries for the DNA Synthesis and Total channel. The purpose of these entries is to eliminate debris from the analysis, not to define positive and negative cells:

Figure 5. Pre-Filtering Section



a. Cell Area (pixel^2) – Enter the appropriate cell area range.
b. Cell Intensity Range – Enter the appropriate cell intensity range if necessary.
   - Typically Cell Intensity Range is not used in the pre-filtering step in the DNA Synthesis application.
c. Min Aspect Ratio – Enter the appropriate minimum aspect ratio if necessary.
   - Aspect ratio measures an objects elongation and is often used to remove artifacts and debris.

Table 2. Recommended Initial Identification and Pre-Filtering Settings for Analysis

Parameter	Setting
IDENTIFICATION	
Algorithm	Fluorescence
Intensity Threshold	3
Precision	High
Cell Diameter (pixel)	12
Dilation Radius (pixel)	0
Background Correction	Not Checkmarked
Separate Touching Objects	Checkmarked
PRE-FILTERING (DNA SYNTHESIS AND TOTAL CHANNELS)	
Cell Area (pixel ^2) Range	30-1000
Cell Intensity Range	0-255
Min Cell Aspect Ratio	0

Figure 6 and Figure 7 show the expected display results for the settings shown in Table 2.

In Figure 6, the DNA Synthesis and Hoechst stains are pseudocolored green and blue, respectively. In the corresponding graphic overlay in Figure 7, the Total channel overlay is purple and is used by the software to identify the cells.

Figure 6. DNA Synthesis Application 2-Channel Image of A549

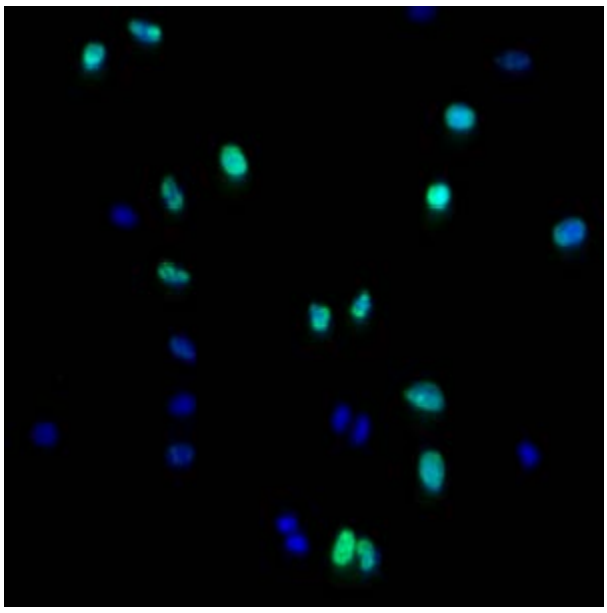
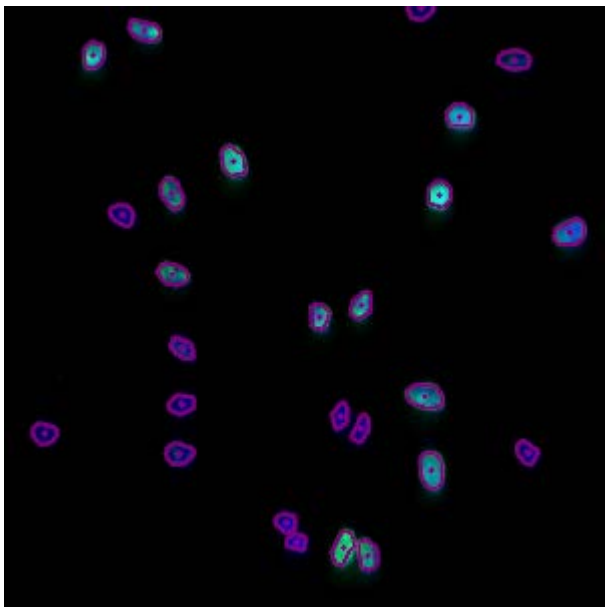


Figure 7. Image + Target Overlays of Image on Left



	NOTE: Antibody staining sometimes leads to the appearance of bright aggregates that should not be considered as positive cells. These aggregates can be filtered out by lowering the upper limit of the Cell Area Range filter (e.g. 200). Antibody staining sometimes leads to the appearance of bright aggregates that should not be considered as positive cells. Bright aggregates do not have corresponding object limits and are eliminated from the analysis. Cell clumps or debris should be excluded from the analysis using this filter as well.

5. Gating Cells

This chapter describes how to select filter settings for further scan data analysis. You perform this task in the Gate tab.

For correct analysis using the DNA Synthesis application, you must perform gating.

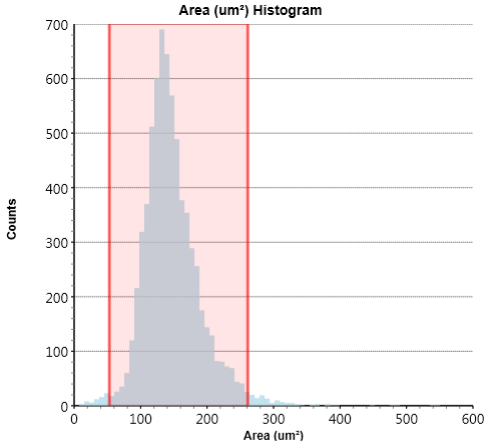
In the DNA Synthesis application, two classes exist (Total and DNA Synthesis). Classes are populations (defined by gates) for which data is reported.

In the DNA Synthesis application, the Gate tab automatically displays a histogram plot based on the population area, with a gate over the cells of expected size. You then adjust the gate position so that mostly cells of a similar area are selected for analysis. A scatter plot, based on the previously selected population, graphs the Total Mean Intensity (in the Blue channel) versus DNA Synthesis (+) Mean Intensity (see Figure 6 on the following page). You adjust the gate position on the scatter plot so that the cells positive for DNA Synthesis are selected. Typically, you can optimize the position of the scatter plot gate by comparing a positive and negative sample for DNA Synthesis.

To adjust a gate on the histogram and scatter plots

1. In the Gate tab, if the Area (um^2) Histogram plot (Figure 8) is not in view, display it by clicking its listing in the plot list below the displayed plot.

Figure 8. Histogram



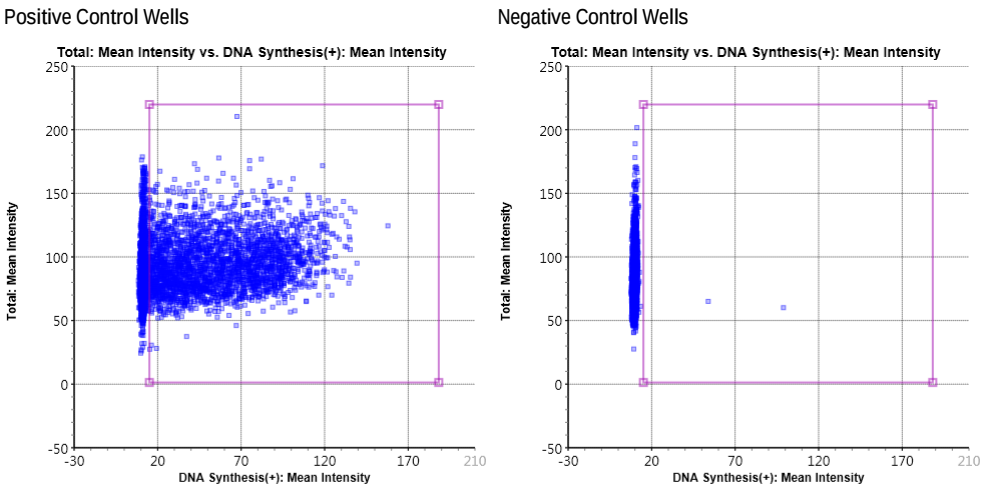
2. On the histogram plot, drag the gate so that it covers the population you want to analyze.

   In the Graphic Overlay display, the color displayed for the plot population selected in the Plot corresponds to the Color menu selection.

3. On the histogram plot, assign the Total class as follows:
   a. Click the gate.
   b. In the Plots, Populations, or Classes view, makes sure that the Total class is checkmarked.

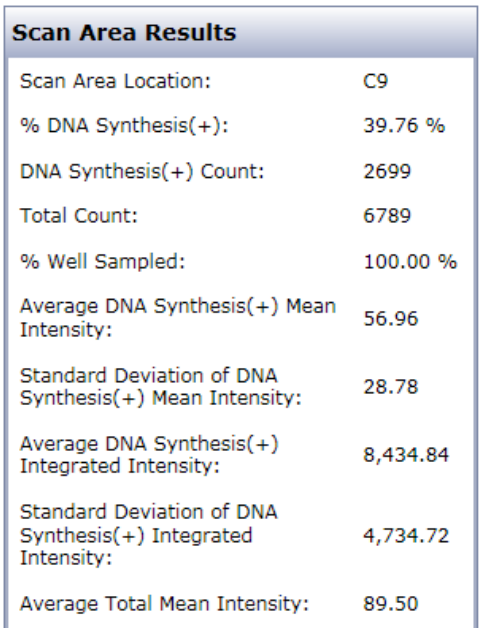
   For details, see User Guide section “Assigning a Class to a Population.”
4. On the scatter plot (Total: Mean Intensity vs. DNA Synthesis(+): Mean Intensity) (Figure 9), drag the gate so that it captures only the cells that are positive for DNA Synthesis. Figure 9 shows examples of a gate capturing positive control wells (wells with a high percentage of positive cells) versus negative control wells (wells with a low percentage of positive cells).

Figure 9. Scatter Plot



To capture only the positive wells, it is helpful to review the Scan Area Results pane (Figure 10) to see a preview of the well data counts that the gate is capturing. Adjust the gate position as needed to change the counts.

Figure 10. Scan Area Results Pane



Scan Area Results

Scan Area Location:	C9
% DNA Synthesis(+):	39.76 %
DNA Synthesis(+) Count:	2699
Total Count:	6789
% Well Sampled:	100.00 %
Average DNA Synthesis(+) Mean Intensity:	56.96
Standard Deviation of DNA Synthesis(+) Mean Intensity:	28.78
Average DNA Synthesis(+) Integrated Intensity:	8,434.84
Standard Deviation of DNA Synthesis(+) Integrated Intensity:	4,734.72
Average Total Mean Intensity:	89.50

5. On the scatter plot, assign the DNA Synthesis class as follows:
   a. On the scatter plot, click the gate.
   b. In the Plots, Populations, or Classes view, make sure that the DNA Synthesis class is checkmarked.

6. Viewing Results

This chapter describes the feature outputs available from the Results tab.

6.1 Application Outputs

The parameters listed in Table 3 appear below the Display Options section in the Scan Information pane.

Table 3. DNA Synthesis Application Outputs

Parameter	Description
% DNA Synthesis	(DNA Synthesis Count / Total Count) x 100
DNA Synthesis Count	Number of DNA Synthesis Cells positive with stain intensity above a user-defined intensity threshold
Total Count	Number of Total cells positive with stain intensity above a user-defined intensity threshold
% Well Sampled	Percent of well surface imaged
Average DNA Synthesis Mean Intensity	Average of cell-level DNA Synthesis mean stain intensities above user-supplied threshold
Standard Deviation of DNA Synthesis Mean Intensity	Standard deviation of cell-level DNA Synthesis mean stain intensities above user-supplied threshold
Average DNA Synthesis Integrated Intensity	Average of cell-level DNA Synthesis integrated stain intensities above user-supplied threshold
Standard Deviation of DNA Synthesis Integrated Intensity	Standard deviation of cell-level DNA Synthesis integrated stain intensities above user-supplied threshold
Average Total Mean Intensity	Average of cell-level Total mean stain intensities above user-supplied threshold
Standard Deviation of Total Mean Intensity	Standard deviation of cell-level Total mean stain intensities above user-supplied threshold
Average Total Integrated Intensity	Average of cell-level Integrated Total stain Intensities above user-supplied threshold
Standard Deviation of Total Integrated Intensity	Standard deviation of cell-level Total Integrated stain intensities above user-supplied threshold

For instructions on viewing scan details, see the User Guide.

6.2 Data Export

Well-level and Object-level data can be exported into CSV (comma separated value) and FCS (flow cytometric standard) files. For instructions on data export, see the User Guide.

7. Troubleshooting

This chapter provides troubleshooting recommendations.

Table 4. Troubleshooting Recommendations

Issue	Recommended Action
Cells are being detected in every channel	If exposure settings are too long, cells are overexposed and the dynamic range of pixel intensities is reduced. This results in the segmentation improperly detecting cells that should be negative. • Reduce the exposure time.
High image background	When images have a very high background, it becomes difficult to properly segment the images. • Wash plate wells more thoroughly and consider reducing exposure times.
Improper cell counts on well edges	When cells are plated at high density, they become more difficult to segment accurately, especially on the well edges. • Plate cells at lower density. • An alternative is to use well sampling and acquire only the center of the well where cells can easily be identified and counted accurately.
Cannot identify cells	If pre-filtering is applied, some objects are filtered out and no longer part of the analysis. • Expand the lower and upper limits of the filters.

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 8001627 Rev A

Celigo® Cytometer Expression Analysis Application Guide

Celigo Software Version 2.1

Contents

1. About this Guide

The Expression Analysis application is a generic fluorescence protocol for up to three separate channels in addition to a brightfield channel. Cells can be identified label-free in the brightfield channel and fluorescence signal in the three different channels can be quantified. Using an advanced gating interface, calculated cell-based features such as shape, size, and intensities can be used to create gates, or selection criteria, to analyze specific subpopulations of cells.

1.1 Purpose

The purpose of this guide is to provide the steps and concepts that are unique to the Expression Analysis application. . Information that is common to all applications is covered in the *Celigo Cytometer User Guide* (Doc. No. 8001619), from here on called the User Guide.

1.2 Safety Precautions

All safety precautions described in the User Guide apply to this guide.

1.3 Technical Assistance

Contact Nexcelom Customer Service for further information:

Nexcelom Bioscience, LLC.
Customer Service
360 Merrimack St. Building 9
Lawrence, MA 01843, USA

- From the United States:
  email: celigosupport@nexcelom.com
  phone: 978-327-5340
- From Europe:
  e-mail: celigosupport@nexcelom.com
  phone: 978-327-5340

2. Prerequisites

The prerequisites for performing the procedures in this document are:

- Familiarity with Celigo operation per the User Guide
- Celigo cytometer is started up per the User Guide

3. Scanning Plates

This chapter provides the procedures for selecting the appropriate Expression Analysis application and setting the image acquisition parameters. You perform these tasks in the Scan tab.

3.1 Segmentation Strategies

The Expression Analysis application provides three types of segmentation (identification) strategies that you can apply to scans ranging from 1 to 4 channels:

Identification can be done using either of the following strategies:

- Single Mask – Identification is performed in one channel, called the “Mask” channel, and then applied to every channel.
- Independent Mask – Identification is performed separately in each channel and applied separately to each channel
- Merged Mask – Identification is performed separately in each channel and applied separately to each channel, and then the segmentation data is merged into a single merged mask that will be applied to all channels

	**NOTE: A mask is a cell outline defined in a fluorescent or brightfield channel. The mask is used to define the outline of the cell in all channels. If no mask is chosen, the software will define the cell outline in each channel. If the outlines in the different channels overlap, they will be considered to belong to the same cell.**

Each of the three segmentation strategies is associated with specific Expression Analysis applications.

When performing image acquisition, you must select an application based on the expected measurement for the experiment.

The types of segmentation strategies are described below in more detail.

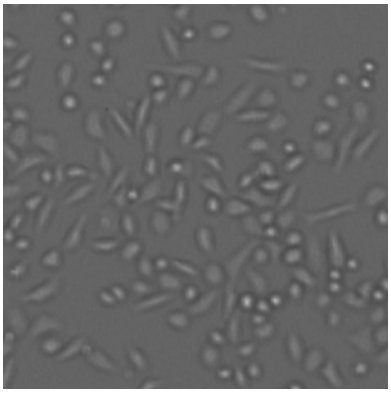
3.1.1 Single Mask Segmentation

The single mask segmentation strategy uses only one image to identify objects in the images, and the resulting mask will be used to segment images from all the other channels. Figure 1 shows the Brightfield imaging of cells transfected with either GFP or RFP (imaging using only the Brightfield channel is shown in panel A). The Brightfield image is segmented in panel B, and the resulting mask will be used to measure the fluorescence intensities of the other two channels (Panel C). Morphologic measurements will all derive from the single mask segmentation while intensity measurements (mean and integrated) will be measured in every channel.

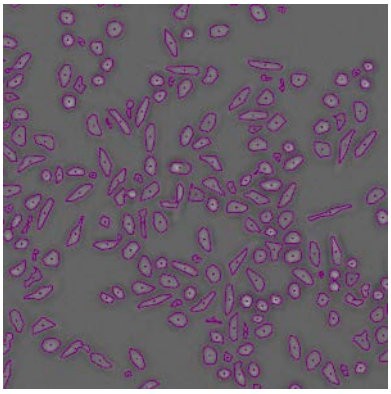
This segmentation method analyzes the images in a similar fashion to flow cytometry, where cells are identified using the forward and side scatter to identify the cells.

An alternative to using the Brightfield channel for the single mask segmentation is to use a nuclear dye, such as Hoechst, in the Blue channel to identify all the cell nuclei.

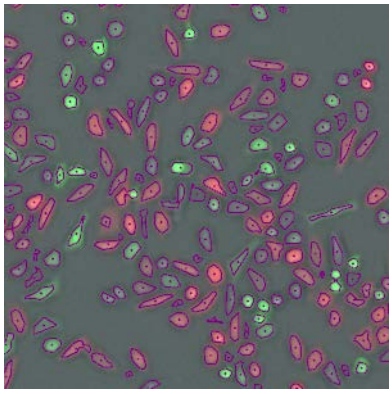
Figure 1. Single Mask Segmentation Example



(A) Brightfield image with the segmentation graphic overlay turned off



(B) Brightfield image with the segmentation graphic overlay turned on. The resulting mask will be transferred to the red and green channels.



(C) The red and green channels. These channels will be measured using the single mask.

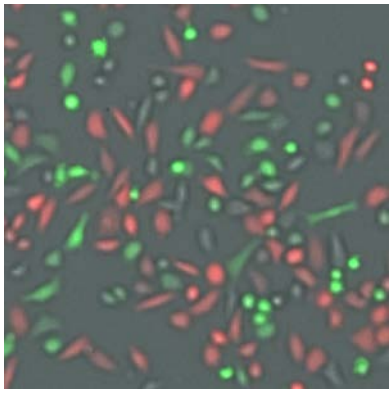
3.1.2 Independent Segmentation

The independent segmentation algorithm will segment images in each channel. Objects will be identified in every channel independently even if they do not exist in any other channels. Figure 2 shows cells either transfected with GFP or RFP (panel A). GFP positive cells, RFP positive cells and all cells are identified separately by segmenting the green, red and BF channels. Panel A shows a superimposition of all 3 channels. Panel B shows the segmentation of the green objects. Panel C shows the separate segmentation of the green and red objects. Panel D shows segmentation of all objects in their respective channels. In addition of intensity measurements (mean and integrated), the independent segmentation strategy reports morphology measurements for objects in each channel. If objects are touching each other, they are considered to belong to the same cell.

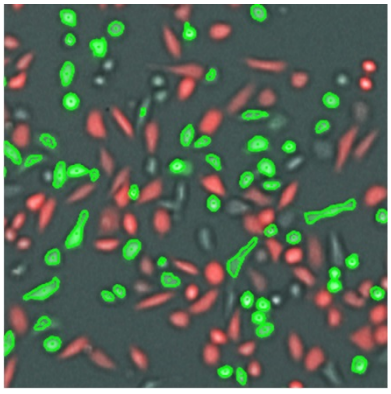
This segmentation method is useful when there is no specific marker that can be used to identify all the cells. In the example provided here, the researcher might want to limit his or her analysis to cells that express a fluorescent marker (GFP or RFP) or to ignore untransfected cells.

Using this segmentation strategy in this example, green cells will be identified and therefore assigned an intensity value in the green fluorescent channel but not in the red channel. Inversely, red cells will be identified and therefore assigned an intensity value in the red channel but not in the green channel. This has important implications for the gating of cells populations using the independent segmentation as the only the histogram representation of these cell population is recommended (see Gating Cells chapter 5).

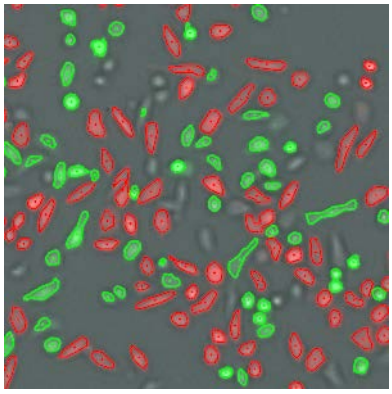
Figure 2. Independent Mask Segmentation Example



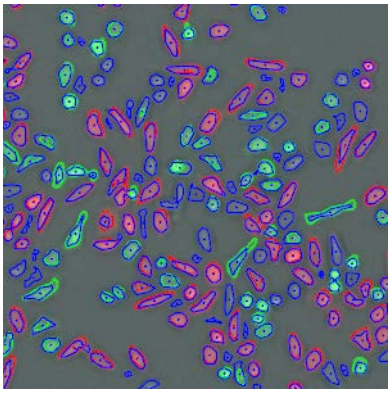
(A) Cells transfected with GFP or RFP – green, red and Brightfield channels combined.



(B) Segmentation of the green objects.



(C) Separate segmentation of the green and red objects.

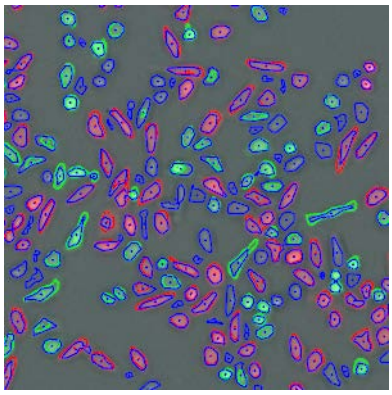


(D) Segmentation of all objects in their respective channels.

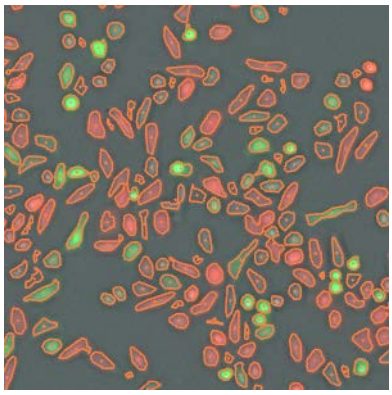
3.1.3 Merge Segmentation

The merge segmentation strategy implements an additional step as compared to the independent segmentation. In brief, objects are segmented in all channels. Continuing with the previous example, cells expressing GFP or RFP were separately identified in the green, red, or brightfield channels (Figure 3 panel A). A merging step then occurs that will merge the mask of all independent channels (Figure 3 panel B). As a result, the application will use a single merged mask to measure intensities in all channels. Similar to the single mask, the merged strategy reports only one set of morphological measurements for the merged mask.

Figure 3. Merged Mask Segmentation Example



(A) Segmentation of all objects in their respective channels.



(B) Merge of all independent channels.

3.2 Expression Analysis Application

Perform the following steps to select an Expression Analysis application.

To select an Expression Analysis application

In the Current Application menu, select one of the 10 Expression Analysis applications (Figure 4 and Table 1), depending on how many channels (targets) you wish to acquire and whether or not you are using an independent, single-mask, or merge segmentation strategy.

The choice of how cells are identified impacts how data is collected, as described in section 3.1. To clarify, Table 1 includes a summary of the differences and gives examples of some use cases.

Figure 4. Selecting an Expression Analysis Application

Table 1. Selecting an Expression Analysis Application

Quantity of Channels (Targets) to be Acquired	Select this application	How Fluorescence is Measured	Use Case
Single Mask			
Three channels + a mask	**Target 1 + 2 + 3 + Mask**	The mask defines the cell. Fluorescence or brightfield signal is measured in the same mask.	• Measure reporter gene expression in cells identified in brightfield. • Calculate transfection efficiency.
Two channels + a mask	**Target 1 + 2 + Mask**		
One channel + a mask	**Target 1 + Mask**		
Independent			
One channel (Brightfield or fluorescence)	**Target 1**	A cell is defined in each channel. If the cell outlines overlap between different channels, all will be considered part of the same cell and the measurements will be associated with the same cell.	• No unique marker to identify all cells. • Identify fusion of two fluorescently tagged cell lines.
Two channels (Ind*)	**Target 1 + 2**		
Three channels (Ind*)	**Target 1 + 2 + 3**		
Four channels (Ind*)	**Target 1 + 2 + 3 + 4**		
Merge			
Two channels merged	**Target 1 + 2 (Merge)**	A cell is defined in each channel. The various masks are merged. Fluorescence or brightfield signal is measured in the same merged mask.	No unique marker to identify all cells but the assay requires intensity measurements of all the cells in every channel
Three channels merged	**Target 1 + 2 + 3 (Merge)**		
Four channels merged	**Target 1 + 2 + 3 + 4 (Merge)**		

3.3 Acquisition Settings

Perform the following steps to select image acquisition settings.

To select image acquisition settings

1. Choose a well for setup using the plate map by clicking Navigation and selecting a representative well.
   - It is recommended that you adjust exposure settings using a well where the highest fluorescent signal is expected.
2. In Current Channel, select the first channel you are setting up.
   - For mask applications, it is recommended that you select the mask channel first.
   - For independent and merge applications, it is recommended that you select the channel that you expect to provide the highest fluorescent signal. It is easier to focus in a fluorescent channel.
3. For the selected channel in Current Channel, make the following selections:
   a. In Type, select Custom Channel.
   b. In Illumination, select the appropriate illumination (e.g., Brightfield or Blue to visualize the Hoechst signal).
   c. Click **Live** to see a live image.
   d. Use manual focus to achieve clear image of cells.
   e. Select whether Autoexposure provides optimal exposure for your sample (cells are visible yet not overexposed, i.e., saturated: pixels intensities >254).
      – Autoexposure: The system will attempt to determine the optimal exposure time and gain setting.
      – Custom: User manually establishes optimal exposure time and gain by adjustment of Exposure Time and Gain. When doing so, adjust the exposure so the majority of cells aren't overexposed.
   f. If binning (half resolution) is appropriate for acquisition, select **2x2 Binning**. For a detailed explanation of binning, see the User Guide.
   g. Set up autofocus (see the User Guide).
      – Typically, it is recommended that you select **Hardware Auto Focus** (because it provides maximum speed) and click **Register Auto**. For manual registration or using focus offset, see the User Guide.
4. In Current Channel, select the next channel to set up.
5. For the selected channel in Current Channel, make the following selections:
   a. In Type, select **Custom Channel**.
   b. In Illumination, select the appropriate illumination for the dye (e.g., **Green** to visualize the calcein AM signal, **Red** to visualize the propidium iodide signal).
   c. Click **Live** to see a live image.
   d. Click **Find Focus** to achieve a clear image of the cells.

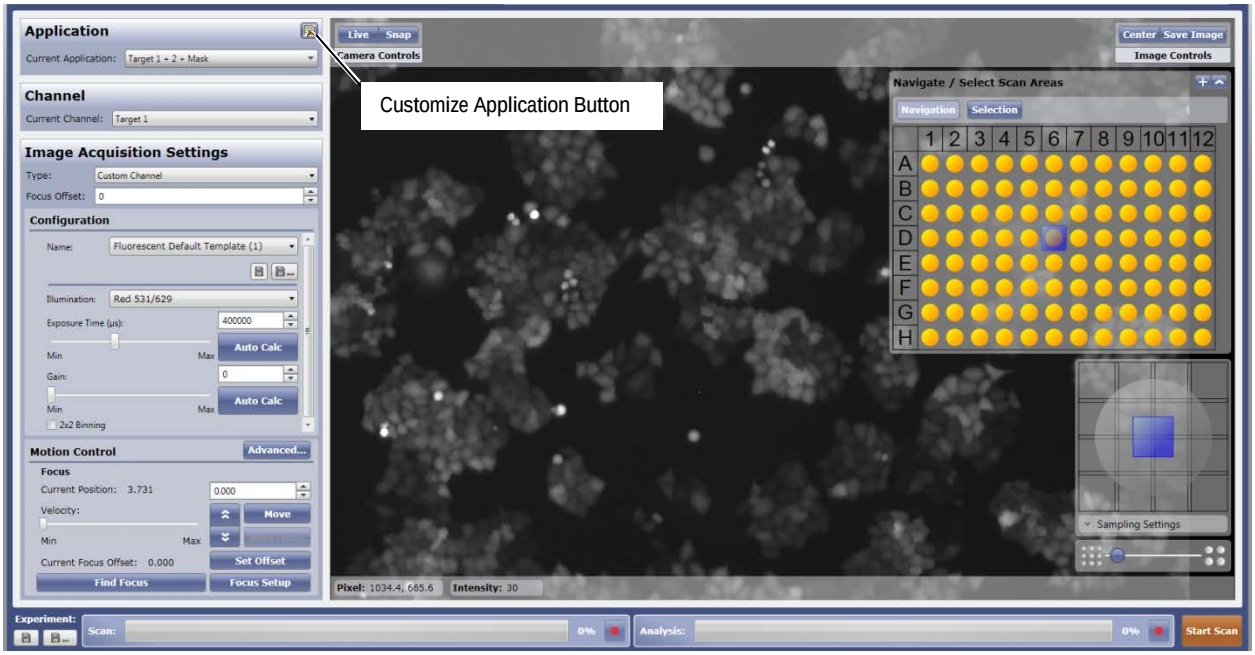
e. Click **Set Offset**.

- Setting focus offsets is important because the optimal focus positions for red, green, and blue wavelengths and brightfield are different. The software will adjust the focus position for each channel during a scan to provide the best focus.

6. Set up the remaining channel by repeating steps 4 through 5e.

Figure 5 shows an example of Target 1 channel selections.

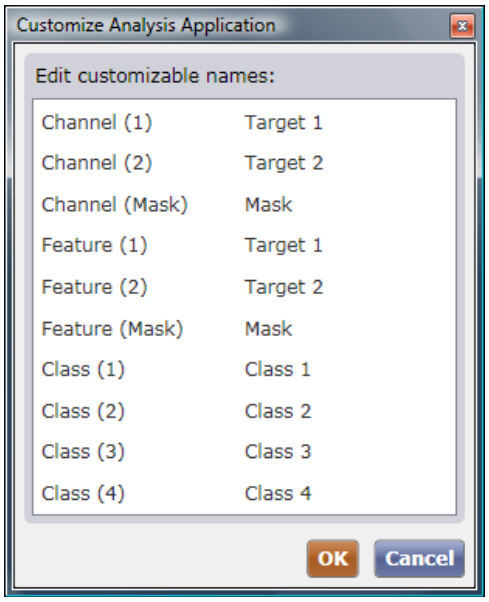
Figure 5. Target 1 Channel Selections



To change the channel, feature, and class names

Customize the channel, feature, and class names as desired (optional), using the Customize Analysis Application button (Figure 5) and the resulting Customize Analysis Application dialog box (Figure 6).

Figure 6. Customize Analysis Application Dialog Box



For details, see the User Guide section “Changing Channel, Feature, and Class Names in the Scan Tab.”

4. Analyzing Images

This chapter describes how to set up analysis for the Expression Analysis application. You perform these tasks in the Analyze tab.

In this application, images from each channel are segmented according to user-specified analysis settings and cells are identified in each channel.

4.1 Analysis Settings Using a Single Mask

Perform the following steps to select the optimal analysis settings for use with a single mask segmentation strategy.

In summary, when you select analysis settings for this strategy, you do the following:

- Identify objects in the mask channel
- Perform pre-filtering on the mask channel
- Repeat the *pre-filtering* for each remaining channel

For a detailed explanation of the identification and pre-filtering settings, see the User Guide.

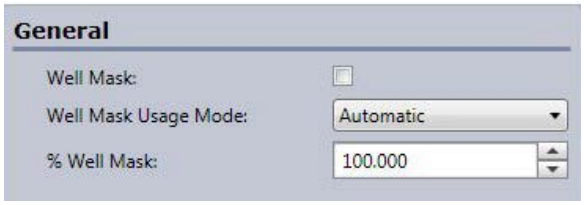
To select analysis settings using a single mask

1. Load prior saved Analysis Settings if available.

   The resulting display shows the channel that you selected for image acquisition in chapter 3.

2. In the General section (Figure 7), make selections as follows:

Figure 7. General Section



   a. Well Mask – Select as needed.

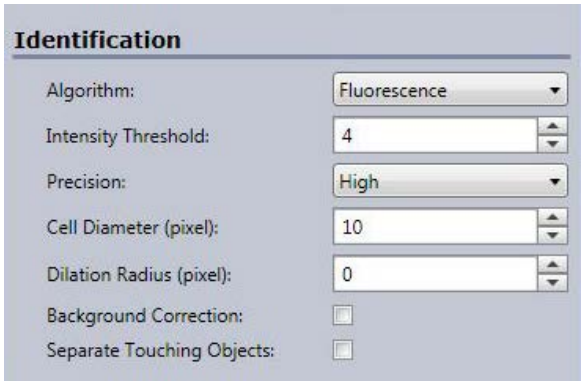
   b. Well Mask Usage Mode – Select as needed.

   c. % Well Mask – Decrease as needed to exclude well edge artifacts that can occur during plate manufacturing.

      For information about Well Mask, Well Mask Usage Mode, and % Well Mask, see the User Guide section “Selecting General Analysis Settings.”

3. In the Identification section (Figure 8), make selections as follows:

Figure 8. Identification Section



   a. Algorithm – Select Fluorescence, **Brightfield**, or **Dark Object**, as appropriate.

   b. Intensity Threshold – Enter the optimal intensity threshold.

      The intensity threshold is the level of intensity that separates background from cells. With an appropriate threshold set, background pixels fall below, and pixels in cells are above the threshold. Any pixels below the threshold are not considered in subsequent calculations.

   c. Precision – Select the appropriate precision level (High is recommended). For a detailed explanation of Precision, see the User Guide.

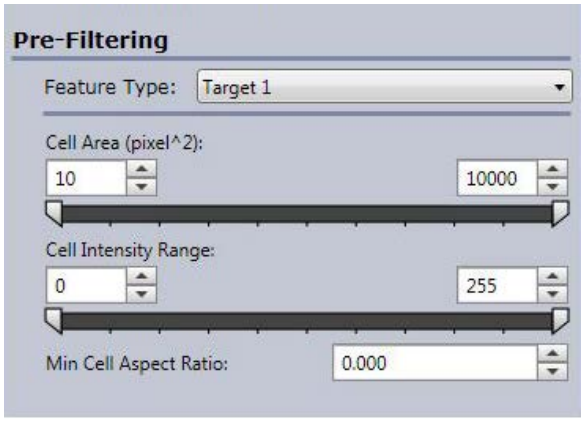
   d. Cell Diameter (pixel) – Enter the cell diameter (in pixels) that corresponds to your cells. At full resolution, the Celigo provides 1 µm/pixel. Binned pixels provide 2 µm/pixel.

   e. Separate Touching Objects – Select if needed.

      This selection is useful when using Fluorescence illumination. For more information on Separate Touching Objects, see the User Guide.

4. In the Pre-Filtering section (Figure 9), do the following (not recommended if you are performing gating):

Figure 9. Pre-Filtering Section



   a. Feature Type – Select as needed.

   b. Cell Area (pixel^2) – Enter an appropriate range.

   c. Cell Intensity Range – Enter an appropriate range, if necessary.

      – The Cell Intensity Range establishes minimum and maximum intensity levels for analysis. This range may be set to filter out abnormally low or high intensity objects/artifacts.

	NOTE: Typically, the cell intensity range filter remains set from 0 to 255. Only cell clumps or debris should be eliminated from the analysis using the area filter.

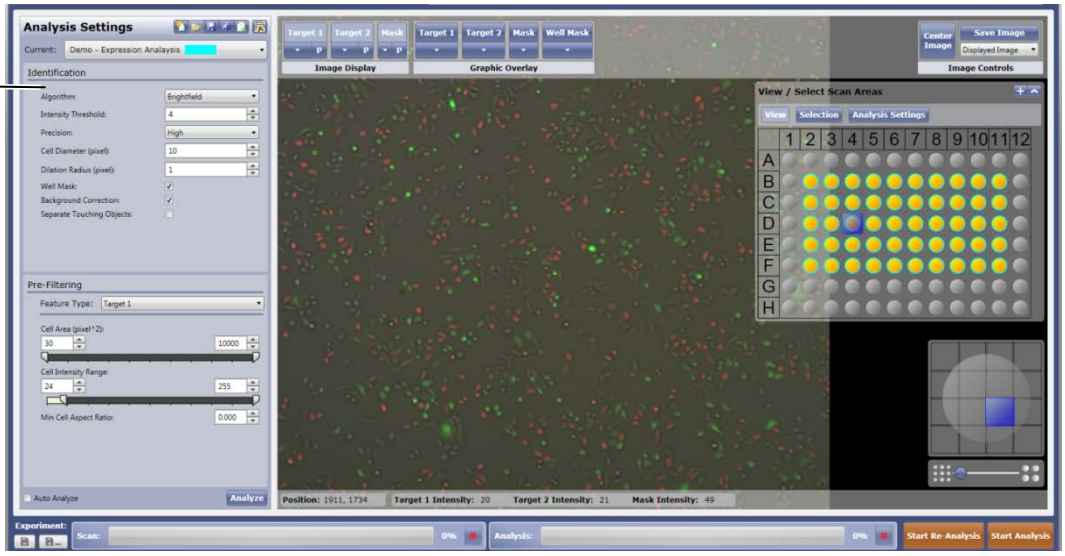
   d. Min Cell Aspect Ratio – Enter an appropriate minimum cell aspect ratio, if necessary.

      – Aspect ratio measures an object's elongation and is often used to remove artifacts and debris.

5. Repeat step 3 through 4d for each remaining channel.

Figure 10. Single Mask Identification and Pre-Filtering Example

A Channel field is not available for single mask segmentation; instead the system automatically displays the channel you have already selected.



4.2 Analysis Settings Using an Independent Mask

Perform the following steps to select the optimal analysis settings with an independent mask segmentation strategy.

In summary, when you select analysis settings for this strategy, you do the following in this order:

- Identify objects and perform pre-filtering in *the first* channel
- Repeat identification and pre-filtering for each remaining channel

For a detailed explanation of the identification and pre-filtering settings, see the User Guide.

To select analysis settings using an independent mask

1. Load prior saved acquisition settings if available.
2. In Current, select one channel to be analyzed.
3. In the General section (), make selections as follows:
   a. Algorithm – Select **Fluorescence**, **Brightfield**, or **Dark Object** as appropriate.
   b. Intensity Threshold – Enter the optimal intensity threshold.

      The intensity threshold is the level of intensity that separates background from cells. With an appropriate threshold set, background pixels fall below, and pixels in cells are above the threshold. Any pixels below the threshold are not considered in subsequent calculations.
   c. Precision – Higher precision results in more accurate identification of cell clusters. High recommended.
   d. Cell Diameter (pixels) – Enter the cell diameter (in pixels) that corresponds to your cells. At full resolution, the Celigo provides 1 µm/pixel. Binned pixels provide 2 µm/pixel.

e. Separate Touching Objects – Select if needed.

This selection is useful when using Fluorescence illumination. For more information on Separate Touching Objects, see the User Guide.

4. In the Pre-Filtering section (), make selections as follows:

a. Cell Area Range – Enter an appropriate range.

b. Cell Intensity Range – Enter an appropriate range, if necessary.

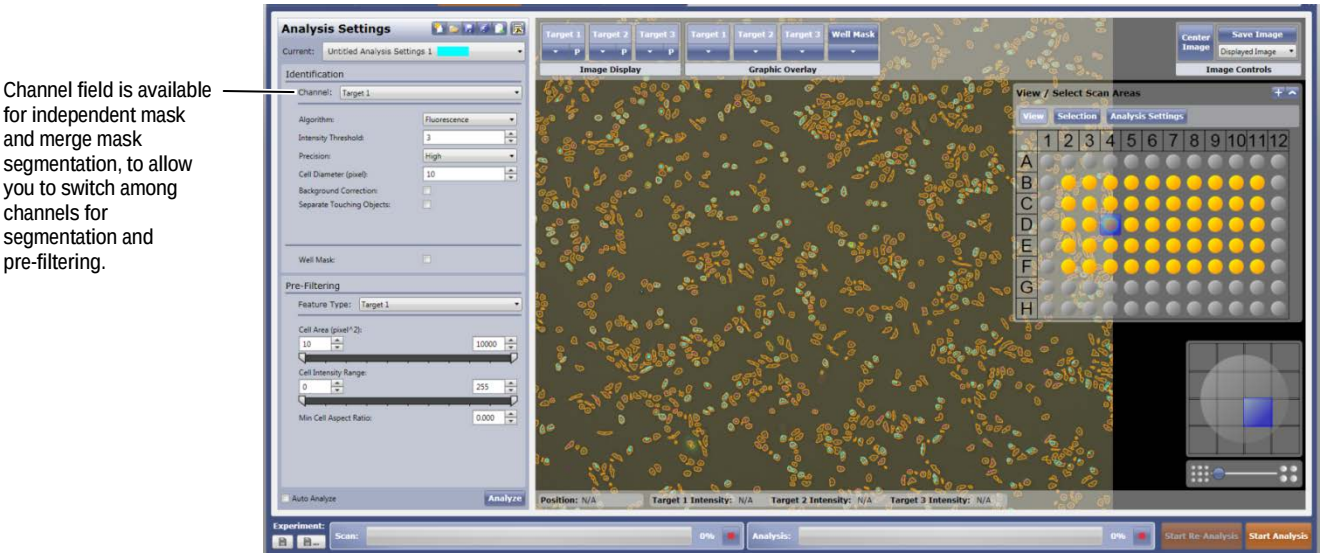
	**NOTE: Typically, the cell intensity range filter remains set from 0 to 255. Only cell clumps or debris should be eliminated from the analysis using the area filter.**

c. Min Aspect Ratio – Enter an appropriate minimum aspect ratio, if necessary.

Aspect ratio measures an objects elongation and is often used to remove artifacts and debris.

5. Repeat steps 2 through 4c for each remaining channel.

Figure 11. Independent Mask Identification and Pre-Filtering Example



4.3 Analysis Settings Using a Merged Mask

Perform the following steps to select the optimal analysis settings with a merged mask segmentation strategy.

In summary, when you select analysis settings for this strategy, you do the same steps as for using an independent mask, except that the system then automatically merges the data, due to the application selected:

- Identify objects and perform pre-filtering in *the first* channel
- Repeat identifying and pre-filtering for each remaining channel

  The system then automatically merges the segmented objects into a single merged mask.

For a detailed explanation of the identification and pre-filtering settings, see the User Guide.

To select analysis settings using a merged mask

Perform “Analysis Settings Using an Independent Mask” per section 4.2.

Because you have selected an application that has a merge function, such as Target 1 + 2 (Merge), the system automatically checkmarks the Merge checkbox (Figure 12).

	**NOTE: It is useful to uncheckmark the Merge checkbox to see the independent segmentation of objects for each channel. When satisfied, checkmark Merge to generate a single merged mask.**

Figure 12. Merged Mask Identification and Pre-Filtering Example

Merge checkbox combines the segmentation data from all channels into a single display. The merging of the segmentation data is illustrated when you toggle the Graphic Overlay buttons on and off and see the same objects selected.

5. Gating Cells

This chapter describes how to select filter settings for further scan data analysis. You perform this task in the Gate tab.

Gating can be used to analyze subpopulations of cells that can be discriminated by size, intensity, or morphology.

In the Gate tab, you can use any of the cell-based parameters extracted by the software from the image data to define subsets of the cell population for analysis.

5.1 Working with Gates Using a Single or Merged Mask

The following are general principles about working with gates in the Expression Analysis application using a single mask or merged mask.

- If you do not perform gating, the system uses the ALL population to analyze the cells in the wells. ALL is the default population that the system assigns to all the objects (cells) in the segmentation result from the Analyze tab.
- You can create both types of plots: histograms and scatter plots.

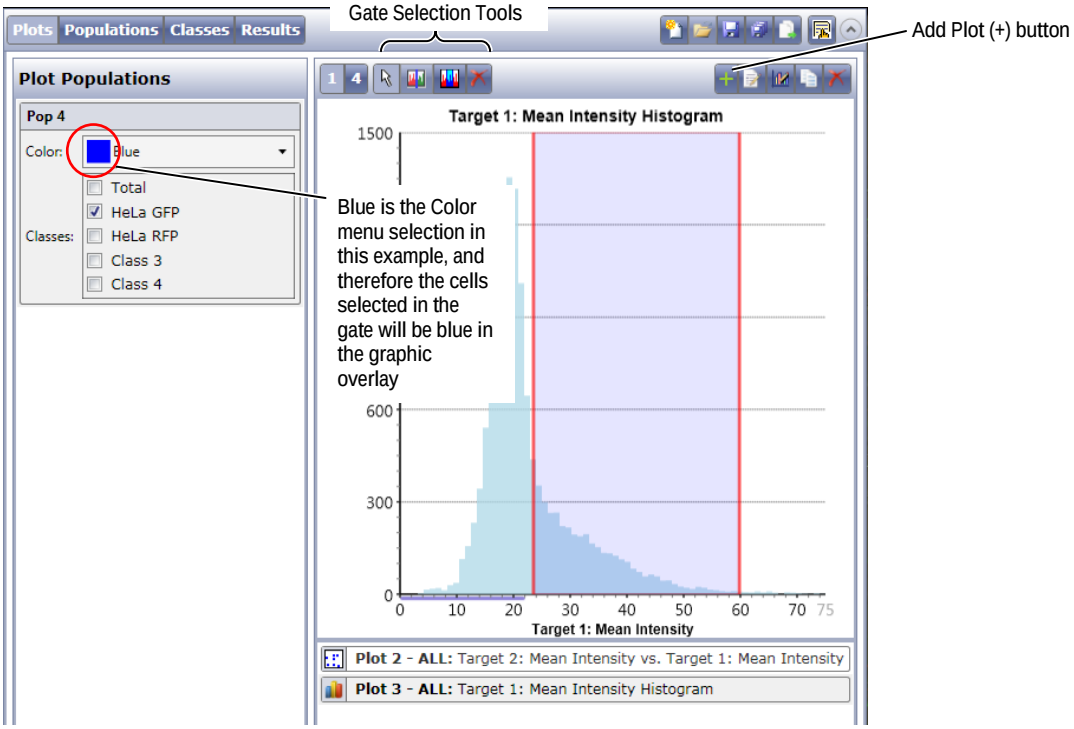
To create a plot, gate, and populations using a single or merged mask

1. In the Plot Populations pane, create a plot (histogram or scatter plot), using the Add Plot (+) button and Add Plot dialog box (Figure 13, Figure 14).

   Refer to the parameter descriptions in Table 2 for an explanation of the parameters selections you can make in both Pick plot parameters menus when using a single mask or merged mask. Different parameters can be combined in scatter plots to enable highly flexible selection criteria.

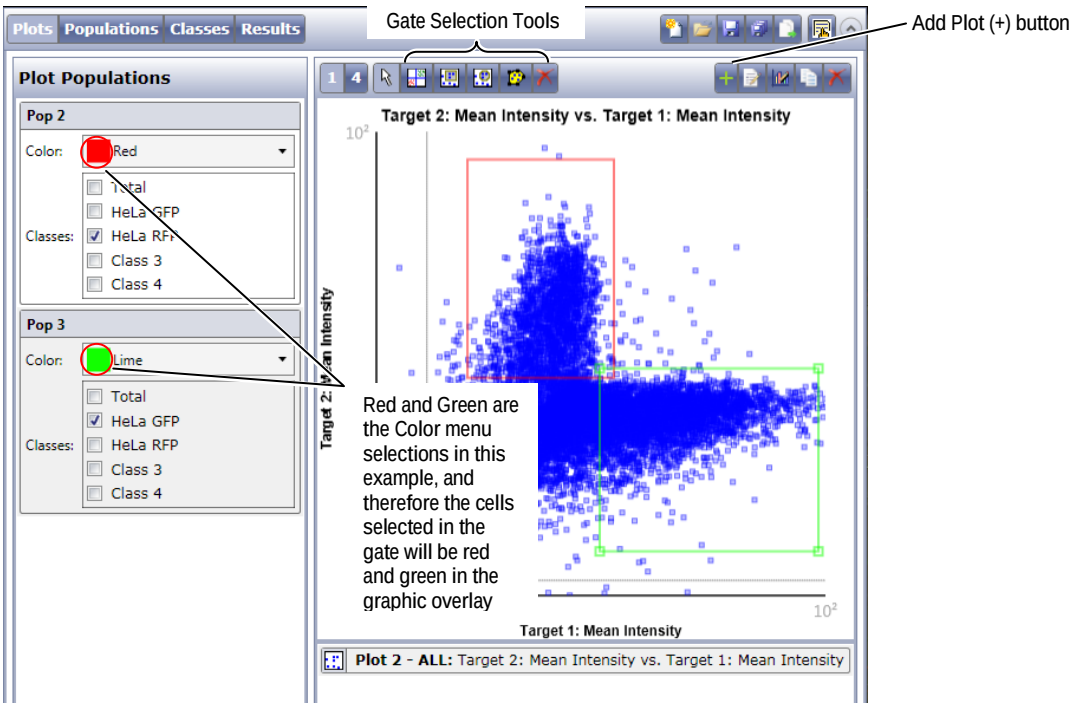
   For details, see User Guide section “Creating a Plot.”

Figure 13. Histogram Example



Histogram plots may be used for any segmentation strategy – single, independent, or merged mask.

Figure 14. Scatter Plot Example



Scatter plots are recommended for use with single mask or merged mask segmentation strategies only (not with the independent mask segmentation strategy).

Table 2. Plot Parameter Definitions for Using a Single Mask or Merged Mask

Feature	Description
FOR THE MASK OR MERGED CHANNEL	
X Position (μm)	Location of a cell along the horizontal axis of the well: left (-μm) or right (+μm) of the center (origin) of the well
Y Position (μm)	Location of a cell along the vertical axis of the well: below (-μm) or above (+μm) the center (origin) of the well
Area (μm^2)	Total area of all the segmented objects' features.
Form Factor	"Compactness" of the segmented object, derived from the ratio of its perimeter to its area; a circle with a value of 1 is most compact; a more convoluted shape with a value less than 1 is less compact.
Smoothness	"Evenness of contour" of the segmented object, derived from the ratio of its convex perimeter to its true perimeter (see the User Guide); a perfectly smooth area has a value of 1; a rough or jagged area has a value less than 1.
Aspect Ratio	Ratio of the minor axis to the major axis of the segmented object; a value of 1 is a perfect circle; values less than 1 suggest an oval (elongated) object .
FOR EACH CHANNEL	
Mean Intensity	Average of the intensities of the segmented objects. Will differ from Total Intensity.
Integrated Intensity	Sum of all pixel intensities displaying signal in segmented objects.

2. Create a gate on the plot, using the gate selection tools (For details, see User Guide section "Creating a Gate."

   In the Graphic Overlay display, the color displayed for the plot population selected in the gate corresponds to the Color menu selection. Figure 13 and Figure 14 show examples using the Total class.

3. Repeat steps 1 and 2 as needed to define the populations that you want to analyze.

4. Assign each class to a population, using one of the following methods:

   - In the Plots view (by clicking Plots button) – Make selections in the Plot Populations pane.
   - In the Populations view (by clicking the Populations button) – Make selections in the All Populations pane.
   - In the Classes View (by clicking the Classes button) – Make selections in the All Classes pane.

   For details, see User Guide section "Assigning a Class to a Population."

5.2 Working with Gates Using an Independent Mask

The following are general principles about working with gates in the Expression Analysis application using an independent mask.

- If you do not perform gating, the system uses the ALL population to analyze the cells in the wells. ALL is the default population that the system assigns to all the objects (cells) in the segmentation result from the Analyze tab.
- It is recommended that you create only one type of plot: histograms, not scatter plots.
- As a result of the independent segmentation, cells identified in one of the channels are not necessarily identified in all the other channels. Therefore, objects will not be assigned intensity values for all channels. When plotting these objects on a scatter plot, only objects identified with intensity values in the two channels are visualized and possibly not all objects will be represented. For example, if GFP- and RFP-expressing cells are mixed and identified independently, GFP positive cells are identified in the green channel and assigned a green intensity value but not a red one. When plotted on a scatter plot representing the red vs. green intensity value of objects, the GFP positive cells in this example will not be plotted.

To create a plot, gate, and populations using an independent mask

1. In the Plot Populations pane, create a plot (histogram, not scatter plot, is recommended for independent mask), using the Add Plot (+) button and Add Plot dialog box (Figure 13 above).

   Refer to the parameter descriptions in Table 2 for an explanation of the parameters selections you can make in the Pick plot parameters menu for Parameter 1 (Parameter 2 is not used for scatter plots, and is not recommended for independent mask strategies).

   For details, see User Guide section “Creating a Plot.”

Table 3. Plot Parameter Definitions for Using an Independent Mask

Feature	Description
FOR EACH OBJECT	
X Position (μm)	Location of a cell along the horizontal axis of the well: left (-μm) or right (+μm) of the center (origin) of the well.
Y Position (μm)	Location of a cell along the vertical axis of the well: below (-μm) or above (+μm) the center (origin) of the well.
FOR EACH CHANNEL	
Area (μm^2)	Total area of all the segmented objects' features.
Form Factor	"Compactness" of the segmented object, derived from the ratio of its perimeter to its area; a circle with a value of 1 is most compact; a more convoluted shape with a value less than 1 is less compact.
Smoothness	"Evenness of contour" of the segmented object, derived from the ratio of its convex perimeter to its true perimeter (see the User Guide); a perfectly smooth area has a value of 1; a rough or jagged area has a value less than 1.
Aspect Ratio	Ratio of the minor axis to the major axis of the segmented object; a value of 1 is a perfect circle; values less than 1 suggest an oval (elongated) object.
Mean Intensity	Average of the intensities of the segmented objects. Will differ from Total Intensity.
Integrated Intensity	Sum of all pixel intensities displaying signal in segmented objects.

2. Create a gate on the plot, using the gate selection tools (Figure 13 above).

   For details, see User Guide section "Creating a Gate."

   In the Graphic Overlay display, the color displayed for the plot population selected in the gate corresponds to the Color menu selection (Figure 13 above). The figure shows an example using the Total class.

3. Repeat steps 1 and 2 as needed to define the populations that you want to analyze.

4. Assign each class to a population, using one of the following methods:

   - In the Plots view (by clicking Plots button) – Make selections in the Plot Populations pane.
   - In the Populations view (by clicking the Populations button) – Make selections in the All Populations pane.
   - In the Classes View (by clicking the Classes button) – Make selections in the All Classes pane.

   For details, see User Guide section "Assigning a Class to a Population."

After determining the gating settings, initiate analysis by clicking **Start Analysis** in the bottom right corner of the Gate tab.

6. Viewing Results

This chapter describes the feature outputs available from the application's Results tab.

6.1 Application Outputs

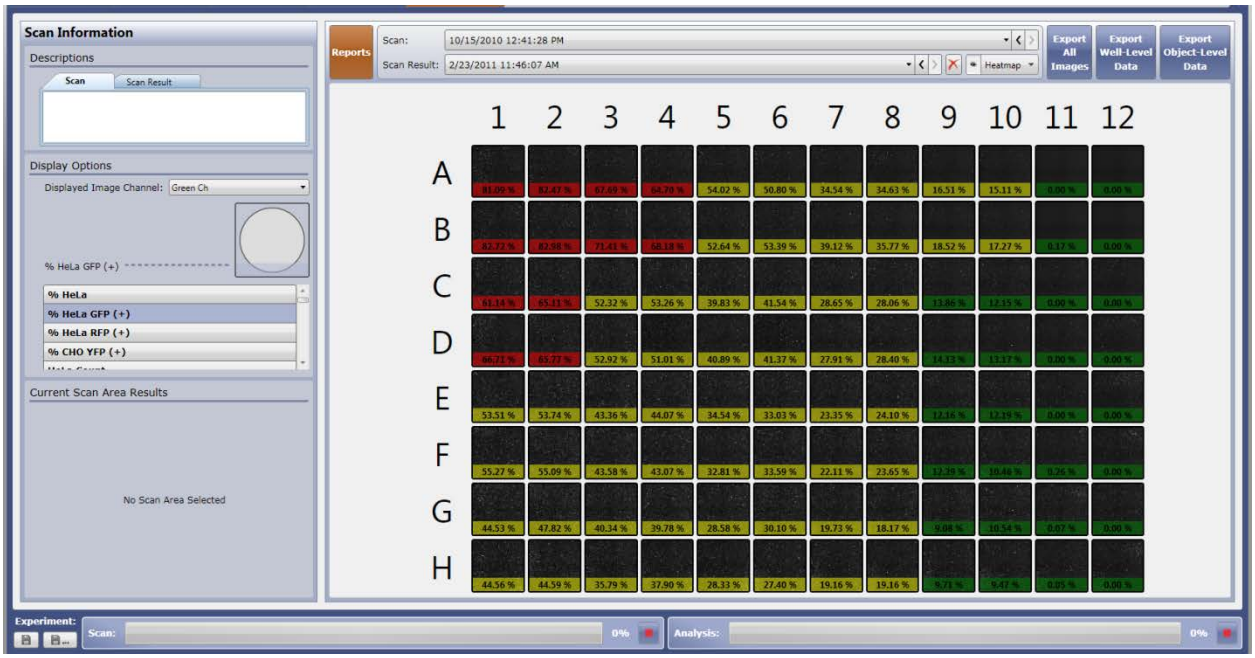
The parameters listed in Table 4 appear below the Display Options section in the Scan Information pane.

Table 4. Well-Level Features Available

Feature	Description
FEATURE REPORTED ONCE FOR EACH WELL	
Total Count	Total number of cells in the well as defined by the Total class.
% Well Sampled	Percent of well surface imaged.
FEATURE REPORTED FOR TOTAL CLASS AND EACH CLASS	
%	Percentage of cells in the specified class as compared to the Total class.
Count	Count of cells in the specified class as compared to the Total class.
Average Target Mean Intensity	Well average mean intensity of all cells in the specified class.
Standard Deviation of Target Mean Intensity	Well average standard deviation of mean intensity of all cells in the specified class.
Average Target Integrated Intensity[1]	Well average integrated intensity of all cells in the specified class.
Standard Deviation of Target Integrated Intensity[1]	Well average standard deviation of integrated intensity of all cells in the specified class.

As analysis progresses, the wells in the plate layout in the Results tab become populated with numbers (Figure 15). You can select the number displayed by selecting a parameter in the list on the left. If you click Threshold, the plate layout will be automatically color-coded to rapidly visualize trends.

Figure 15. Scan Results



6.2 Data Export

Well-level and object-level data can be exported into CSV (Comma Separated Value) or FCS (Flow Cytometric Standard) files. For the procedures to perform data export, see the User Guide.

6.3 Viewing Well Details

To view a well in detail, double-click it in the plate layout and it will open as a full-window image. In the left panel, detailed metrics will be reported, including cell counts, percentages of the whole population, double and triple counts, average mean and integrated intensity in each channel, and standard deviations. As in the Analyze tab, you can zoom in and use the toggles in the Image Display and Graphic Overlay fields to switch the channels, including the identification outlines, on and off.

7. Troubleshooting

This chapter provides troubleshooting recommendations.

Table 5. Troubleshooting Recommendations

Issue	Recommended Action
All Targets are 100% of total population	If using a Mask for cell identification and the pre-filter threshold intensity is set to 0, all cells will be positive in all targets. Reduce the exposure time. Increase the pre-filtering intensity threshold.
Improper cell counts on well edges	When cells are plated at high density, they become more difficult to segment accurately, especially on the well edges. Plate cells at lower density. An alternative is to use well sampling and acquire only the center of the well where cells can easily be identified and counted accurately.
Identification of cells is unreliable in brightfield	Identification of cells in brightfield is critically dependent on correct focus plane, i.e. the “bright” focus. For cells with a very flattened morphology, it may be difficult to achieve this focus plane. Consider using a fluorescent counterstain, such as Hoechst 33342.
Cannot identify cells	If pre-filtering is applied, some objects are filtered out and no longer part of the analysis. Expand the lower and upper limits of the filters.

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 8001628 Rev A

Celigo® Cytometer
Phosphatidylserine Externalization
Application Guide

Celigo Software Version 2.1

Contents

1. About this Guide

1.1 Introduction

The Annexin V-based apoptosis detection assay is a method for studying apoptosis that detects changes in the position of phosphatidylserine (PS) in the cell membrane. In non-apoptotic cells, most PS molecules are localized at the inner leaflet of the plasma membrane. Soon after inducing apoptosis, PS redistributes to the outer layer of the membrane and becomes exposed to the extracellular environment. PS translocation precedes many other apoptotic events, thus allowing early detection of apoptosis. Exposed PS can be easily detected with Annexin V, a 35.8-kDa protein that has a strong affinity for PS.

Following induction of apoptosis, cells are stained with a mixture of fluorescently-conjugated annexin V (a "PS Externalized" cell stain), propidium iodide (a "Dead" cell stain), and Hoechst 33342 (a "Total" cell stain). Images are acquired and analyzed using the Celigo cytometer software. Markers are identified in each fluorescent channel and for each well of a microtiter plate, "PS Externalization" and "Dead" cell counts as well as the percentage of "PS Externalization" and "Dead" cells are automatically reported.

1.2 Purpose

The purpose of this guide is to provide the steps and concepts that are unique to the Phosphatidylserine (PS) Externalization application. Information that is common to all applications is covered in the *Celigo Cytometer User Guide* (8001619), from here on referred to as the User Guide.

1.3 Safety Precautions

All safety precautions described in the User Guide apply to this guide.

1.4 Technical Assistance

Contact Nexcelom Customer Service for further information:

Nexcelom Bioscience, LLC.
Customer Service
360 Merrimack St. Building 9
Lawrence, MA 01843, USA

- From the United States:
  email: celigosupport@nexcelom.com
  phone: 978-327-5340
- From Europe:
  e-mail: celigosupport@nexcelom.com
  phone: 978-327-5340

2. Prerequisites

The prerequisites for performing the procedures in this document are:

- Familiarity with Celigo cytometer operation per the User Guide
- Celigo cytometer is started up per the User Guide

3. Scanning Plates

This chapter provides the procedures for selecting the appropriate PS Externalization application and setting the image acquisition parameters. You perform these tasks in the Scan tab.

3.1 PS Externalization Application

Perform the following steps to select a PS Externalization application.

To select a PS Externalization application

In Current Application, select one of the two PS Externalization applications (Figure 1 and Table 1), based on the dyes you want to use, as follows:

Figure 1. Selecting a PS Externalization Application

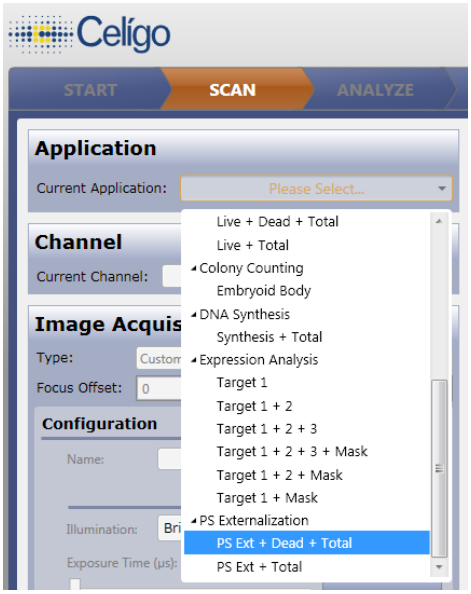


Table 1. Selecting a PS Externalization Application

Using these dyes . . .	Select this application . . .
Green Annexin V, Propidium Iodide, and Hoechst 3342	PS Externalization + Dead + Total
Green Annexin V and Hoechst 33342	PS Externalization + Total

	**NOTE: Although the PS Externalization application uses three dyes (Green Annexin V, Propidium Iodide, and Hoechst 33342), many users prefer to use a combination of only two dyes. This practice allows one channel to be used for running another assay in parallel. The options allow you to select various combinations of two or three dyes.**

3.2 Acquisition Settings

Perform the following steps to select image acquisition settings. For the recommended initial settings to use as a guide, see Table 2.

To select image acquisition settings

1. Choose a well for setup using the plate map by clicking **Navigation** and selecting a representative well.
   - It is recommended that you adjust exposure settings using a well where the highest fluorescent signal is expected.
2. In Current Channel, select the first channel you are setting up (**PS Ext**, **Dead**, or **Total**).
3. For the selected channel in Current Channel, make the following selections:
   a. In Type, select **Custom Channel**.
   b. In Illumination, select the appropriate illumination for the dye (e.g., **Blue** to visualize the Hoechst signal).
   c. Click **Live** to see a live image
   d. Use manual focus to achieve a clear image of the cells
   e. Do one of the following to set the optimal exposure:

NOTE: Cells are visible yet not overexposed at pixel intensities 100–254 (displayed at the bottom of screen).

      – Click **Auto Exp**: The system will attempt to determine the optimal exposure time and gain setting.
      – Manually set the optimal exposure time and gain by making an entry in the Exposure Time and Gain fields.

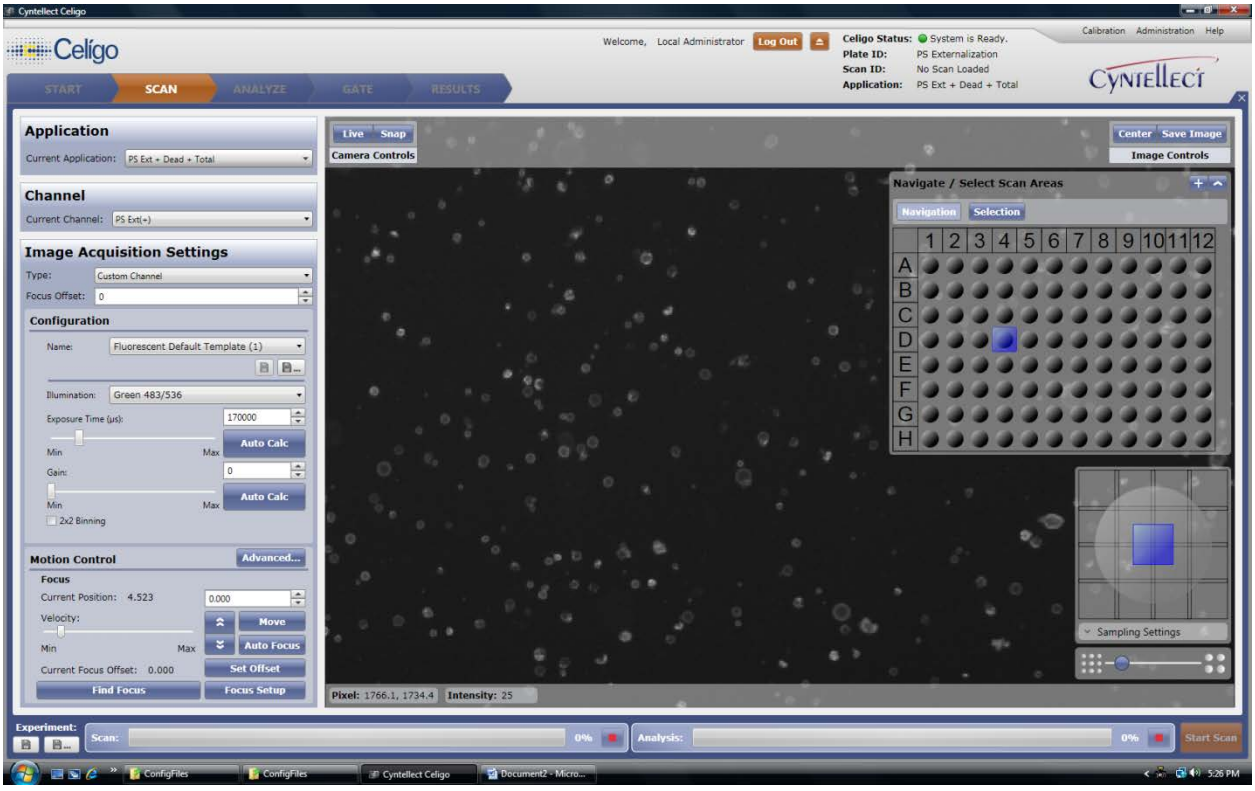
   f. If 2x2 binning (half resolution) is appropriate for acquisition, select **2x2 Binning**. For more information on 2x2 Binning, see the User Guide.
   g. Set up autofocus (see the User Guide).
      – It is recommended that you select **Hardware Auto Focus** (because it provides maximum speed) and select the **Register Auto** option.
4. In Current Channel, select the next channel you are setting up (**PS Ext**, **Dead**, or **Total**).
5. For the selected channel in Current Channel, make the following selections:
   a. In Type, select **Custom Channel**.
   b. In Illumination, select the appropriate illumination for the dye (e.g., **Green** to visualize the PS Externalization signal, **Red** to visualize the propidium iodide signal).
   c. Click **Live** to see a live image.
   d. Click **Find Focus** to achieve a clear image of the cells.
   e. Click **Set Offset**.
6. Set up the remaining channel (**PS Ext**, **Dead**, or **Total**) by repeating steps 4 through 5e.

Table 2. Recommended Initial Settings for Image Acquisition

Current Channel	PS Ext	Dead	Total
Type	Custom Channel	Custom Channel	Custom Channel
Focus Offset	User determined	User determined	User determined
Name	Fluorescent Default Template (1)	Fluorescent Default Template (2)	Fluorescent Default Template (3)
Illumination	Green 483/536	Red 531/629	Blue 377/447
Exposure Time (µs)	100000	50000	100000
Gain	0	0	0
Motion Control and Focus	See User Guide	See User Guide	See User Guide

Figure 2 shows an example of a PS Externalization channel.

Figure 2. PS Externalization Channel



4. Analyzing Images

This chapter provides information on how to analyze scans from the PS Externalization application. You perform these tasks in the Analyze tab.

In this application, images from each channel are segmented according to user-specified analysis settings and objects for each channel are identified.

	**NOTE: Only objects that spatially merge with objected identified in the Total channel are included in the analysis.**

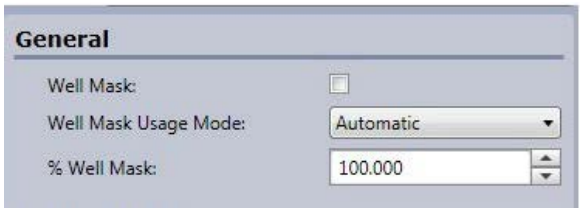
4.1 Analysis Settings

Perform the following steps to select analysis settings. The initial settings for identification and pre-filtering when using the PS Externalization application are shown in Table 3. The settings typically provide good image segmentation. For more information on the identification and pre-filtering settings, see the User Guide.

To select analysis settings

1. Load prior saved Analysis Settings if available.
2. In the General section (Figure 3), make the following selections:

Figure 3. General Section



a. Well Mask – Select if segmentation of cells at the well edge is not satisfactory.

	**NOTE: Selecting Well Mask is critical for this application. Failure to select Well Mask in this application will cause the system to identify objects outside the well.**

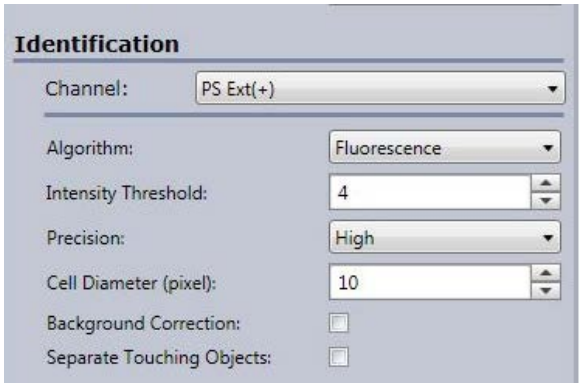
b. Well Mask Usage Mode – Select as needed.

c. % Well Mask – Decrease as needed to exclude well edge artifacts that can occur during plate manufacturing.

For information about Well Mask, Well Mask Usage Mode, and % Well Mask, see the User Guide section “Selecting General Analysis Settings.”

3. In the Identification section (Figure 4), make the following selections:

Figure 4. Identification Section

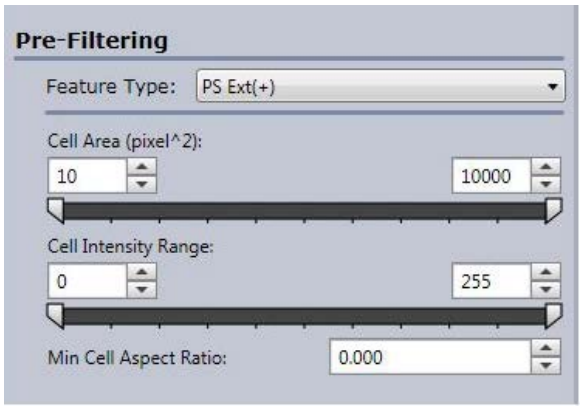


a. Channel – Select one channel to be analyzed.
b. Algorithm – Select **Fluorescence**.
c. Intensity Threshold – Enter the optimal intensity threshold.

   The intensity threshold is the level of intensity that separates background from cells. With an appropriate threshold, background pixels fall below and pixels within cells are above the threshold. Any pixels below the threshold are not considered in the subsequent calculations.
d. Precision – Higher precision results in more accurate identification of cell clusters. High recommended.
e. Cell Diameter (pixel) – Enter the cell diameter (pixels) that corresponds to your cells. At full resolution, the Celigo cytometer provides 1 µm/pixel. Binned pixels provide 2 µm/pixel.
f. Background Correction – Typically not used in this application.
g. Separate Touching Objects – Recommended for the Total or Dead channel only: Select if it is difficult to separate touching cells during segmentation. For more information on Separate Touching Objects, see the User Guide.

4. In the Pre-Filtering section (Figure 5), make the following selections:

Figure 5. Pre-Filtering Section



   a. Feature Type – Select as needed.
   b. Cell Area – Enter the appropriate range.
   c. Cell Intensity Range – Enter the appropriate range, if necessary.

      Typically Cell Intensity Range is not used in the pre-filtering step in the PS Externalization application.
   d. Min Cell Aspect Ratio – Enter the appropriate minimum output ratio, if necessary.

      Aspect ratio measures an object's elongation and is often used to remove artifacts and debris. However, in the PS Externalization application, this function typically is not used.

5. Repeat steps 3 through 4d for each remaining channel to be set up.

Table 3. Recommended Initial Identification and Pre-Filtering Settings for Analysis

Parameter	PS Ext (+)	Dead	Total
IDENTIFICATION			
Algorithm	Fluorescence	Fluorescence	Fluorescence
Intensity Threshold	3	3	3
Precision	High	High	High
Cell Diameter (pixel)	10	10	10
Background Correction	Not Checkmarked	Not Checkmarked	Not Checkmarked
Separate Touching Objects	Not Checkmarked	Checkmarked	Checkmarked
PRE-FILTERING			
Cell Area (pixel ^2) Range	35-1000	30-1000	30-1000
Cell Intensity Range	0-255	0-255	0-255
Minimum Cell Aspect Ratio	0	0	0

Figure 6 and Figure 7 show examples of display results for the settings shown in Table 3.

In Figure 6, the Green Annexin V, Propidium iodide and Hoechst stains are pseudocolored green, red and blue, respectively.

In the corresponding target overlay in Figure 7, the PS Ext (+) overlay is purple, propidium iodide overlay is light blue, and the Hoechst overlay is orange.

Figure 6. PS Ext Application 3-Channel Image of Jurkat cells Treated with Camptothecin

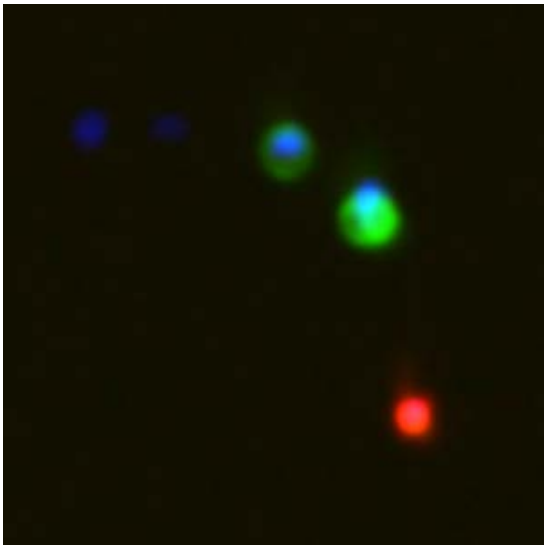
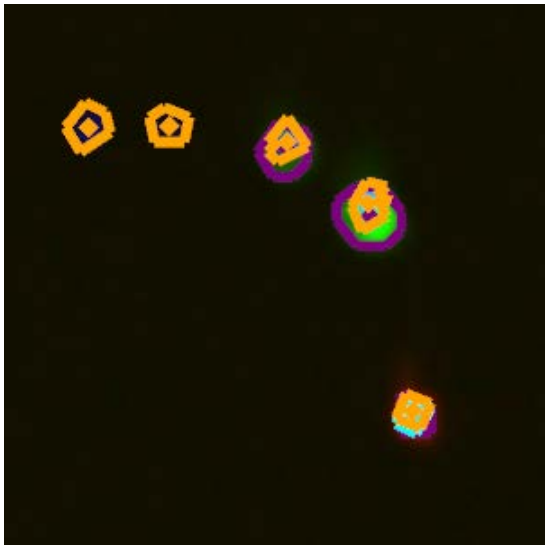


Figure 7. Image + Target Overlays of Image on Left



5. Gating Cells

When using the PS Externalization application, gating typically is not performed in the Gate tab. Instead, the application relies on the identification of negative and positive cells using the intensity threshold in the Analyze tab. The pre-filtering parameters in the Analyze tab (see chapter 4) manage the filtering of debris.

6. Viewing Results

This chapter describes the feature outputs available from the application's Results tab.

6.1 Application Outputs

The parameters listed in Table 4 appear below the Display Options section in the Scan Information pane.

Table 4. PS Externalization Application Outputs

Parameter	Description	PS Ext (+) + Total	PS Ext (+) + Dead + Total
% PS Ext(+)	(PS Ext(+) Count / Total Count) x 100	√	√
% Dead	(Dead Count / Total Count) x 100		√
% PS Ext(+) (corrected)	(PS Ext(+) Count – PS Ext(+)+Dead Count) / Total Count) x 100		√
PS Ext(+) Count	Number of PS Ext(+) cells positive with stain intensity above a user-defined intensity threshold	√	√
Dead Count	Number of Dead cells positive with stain intensity above a user-defined intensity threshold		√
Total Count	Number of Total cells positive with stain intensity above a user-defined intensity threshold	√	√
PS Ext(+) +Dead Count	Number of PS Ext(+) and Dead Cell positive stain intensity above a user-defined intensity threshold		√
% Well Sampled	Percent of well surface imaged	√	√
Average PS Ext(+) Mean Intensity	Average of cell-level PS Ext(+) mean stain intensities above user-supplied threshold	√	√
Standard Deviation of PS Ext(+) Mean Intensity	Standard deviation of cell-level PS Ext(+) mean stain intensities above user-supplied threshold	√	√
Average PS Ext(+) Integrated Intensity	Average of cell-level PS Ext(+) integrated stain intensities above user-supplied threshold	√	√
Standard Deviation of PS Ext(+) Integrated Intensity	Standard deviation of cell-level PS Ext(+) integrated stain intensities above user-supplied threshold	√	√
Average Dead Mean Intensity	Average of cell-level Dead mean stain intensities above user-supplied threshold		√

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Parameter	Description	PS Ext (+) + Total	PS Ext (+) + Dead + Total
Standard Deviation of Dead Mean Intensity	Standard deviation of cell-level Dead mean stain intensities above user-supplied threshold		√
Average Dead Integrated Intensity	Average of cell-level Dead integrated stain intensities above user-supplied threshold		√
Standard Deviation of Dead Integrated Intensity	Standard deviation of cell-level Dead integrated stain intensities above user-supplied threshold		√
Average Total Mean Intensity	Average of Cell-level Total mean stain intensities above user-supplied threshold	√	√
Standard Deviation of Total Mean Intensity	Standard deviation of cell-level Total mean stain intensities above user-supplied threshold	√	√
Average Total Integrated Intensity	Average of Cell-level Integrated Total stain Intensities above user-supplied threshold	√	√
Standard Deviation of Total Integrated Intensity	Standard deviation of cell-level Total Integrated stain intensities above user-supplied threshold	√	√

6.2 Data Export

Well-level and object-level data can be exported into CSV (Comma Separated Value) or FCS (Flow Cytometric Standard) files. For the procedures to perform data export, see the User Guide.

7. Troubleshooting

This chapter provides troubleshooting recommendations.

Table 5. Troubleshooting Recommendations

Issue	Recommended Action
Cells are being detected in every channel	1. If exposure settings are too long, cells are overexposed and the dynamic range of pixel intensities is reduced. This results in the segmentation improperly detecting cells that should be negative. • Reduce the exposure time. 2. When the concentration of dye for a specific cell type is too high, cell stains are very bright and can cause bleedthrough in the other channels. This also results in the segmentation improperly detecting cells that should be negative. • Titrate and reduce the dye concentrations.
High image background	Signal to noise ratio in one or more channels is low. • Wash plate wells more thoroughly and consider reducing dye concentrations.
Improper cell counts at well edges	When cells are plated at high density, they become more difficult to segment accurately, especially at the well edges. • Plate cells at lower density. • An alternative is to use well sampling and acquire only the center of the well where cells can easily be identified and counted accurately.
Cannot Identify cells	If pre-filtering is applied, some objects are filtered out and no longer part of the analysis. • Expand the lower and upper limits of the filters.

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 8001629 Rev A

Celigo® Cytometer
Cell Migration: Wound Healing
Application Guide

Celigo Software Version 2.1

Contents

1. About this Guide

This chapter provides a brief description of this guide and how to use it.

1.1 Introduction

The Cell Migration: Wound Healing application identifies and counts individual cells or clusters of cells using brightfield or fluorescence imaging. Furthermore, the application includes advanced data analysis functions to determine the amount of cell growth and/or migration within the original cleared area from a typical scratch assay or an assay using silicon inserts to create exclusion zones in a well.

The benefit of the Wound Healing application on the Celigo is that it does not require cell staining. The application reports the cell counts and confluence percentages. It is also possible to generate well-level growth curves over time.

1.2 Purpose

The purpose of this guide is to provide the steps and concepts that are unique to the Cell Migration: Wound Healing application. Information that is common to all applications is covered in *Celigo Cytometer User Guide* (Doc. No. 8001619), from here on referred to as the User Guide.

1.3 Safety Precautions

All safety precautions described in the User Guide apply to this guide.

1.4 Technical Assistance

Contact Nexcelom Customer Service for further information:

Nexcelom Bioscience, LLC.
Customer Service
360 Merrimack St. Building 9
Lawrence, MA 01843, USA

- From the United States:
  email: celigosupport@nexcelom.com
  phone: 978-327-5340
- From Europe:
  e-mail: celigosupport@nexcelom.com
  phone: 978-327-5340

2. Prerequisites

The prerequisites for performing the procedures in this document are:

- Familiarity with Celigo operation per the User Guide
- Celigo cytometer is turned on per the User Guide
- The recommended plates are being used (Table 1)

Table 1. Recommended Plates from Platypus Technology

Plate Type	Cat#	Volume
384W	PRO384CMA5	40 µl
96W	CMA1-101	100 µl
96W	PROCMA1	100 µl

Cells can be seeded in Platypus plates. These plates are useful as they generate an exclusion zone in the middle of the well using a silicon plug or a center drop.

Alternatively, cells can be plated confluent in a regular plate and a "scratch" can be generated using a pipette tip, a manifold, or a liquid handler.

Typically, migration assays are run over a period of 24 – 48 hours.

3. Scanning Plates

This chapter provides the procedures for selecting the appropriate Cell Migration: Wound Healing application and setting the image acquisition parameters. You perform these tasks in the Scan tab.

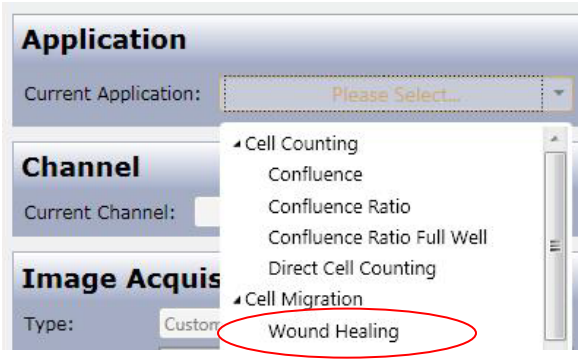
3.1 Cell Migration: Wound Healing Application

Perform the following steps to select a Cell Migration: Wound Healing application.

To select an application

In the Current Application menu, select **Cell Migration: Wound Healing** (Figure 1).

Figure 1. Selecting an Application



3.2 Image Acquisition Settings

Perform the following steps to select image acquisition settings.

To select image acquisition settings

1. Choose a well for setup using the plate map by selecting **Navigation** and a representative well.
2. View a well by selecting **Live** or **Snap** in the Camera Controls field.
3. Set up acquisition settings for brightfield or fluorescence illumination:
    - For Brightfield illumination:
        - Select channel type: **Auto Exposure/Gain Channel** (recommended)
        - In Illumination, select **Brightfield**.
        - In Priority, select **AutoExposure, Gain if Necessary**.
        - In Frequency, select **Every Scan Area**.
    - For Fluorescence illumination:
        - It is recommended to use the Custom channel type. For detailed instructions, see the User Guide.

For detailed instructions on other image acquisition options such as well subsampling, off-axis imaging, and binning, see the User Guide.

4. Set up focus per the User Guide.
    - For detailed instructions on selecting correct focus position for brightfield imaging, see section 3.3 below.
    - It is recommended to select Hardware Auto Focus for most routine plate scanning.

3.3 Correct Focus Position for Brightfield Imaging

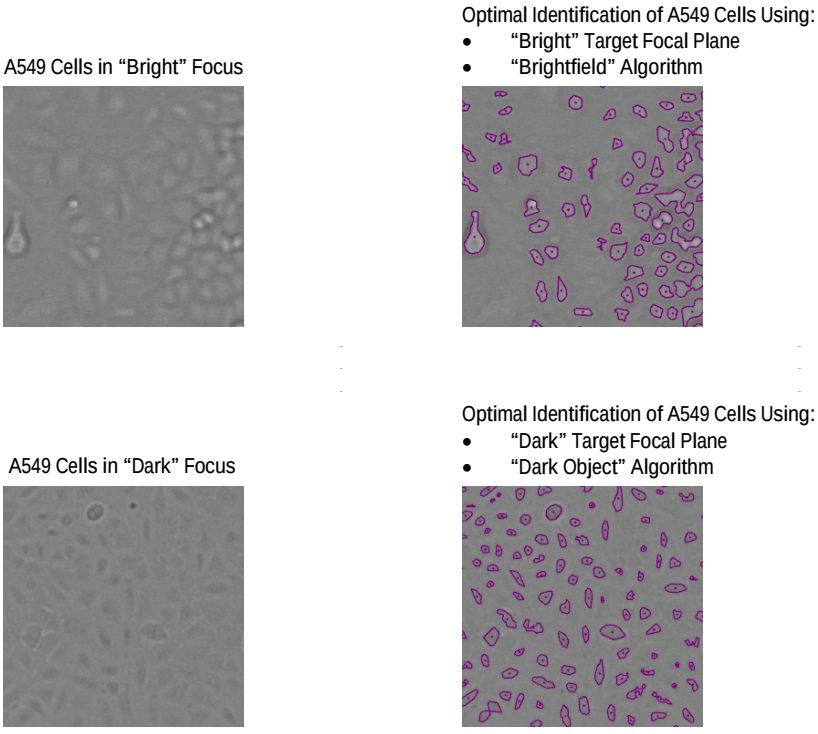
The proper focus for brightfield illumination is important for optimal application performance. There are two image planes visible on the Celigo Cytometer using brightfield illumination. The “Dark” image plane is the real image plane in which objects appear dark compared to surrounding background regions. The “Bright” image plane is a virtual image plane in which the cell or object acts as a lens and focuses the transmitted light in a secondary plane. In the “Bright” image plane, cells or objects have a bright center and dark edges. The Celigo cytometer identification algorithm is optimized for the “Bright” image plane.

This section describes how to select the correct focus position for brightfield imaging. For instructions on focusing using fluorescence illumination, see the User Guide.

To select the correct focus position for brightfield imaging

1. Choose a well for setup using the plate map by selecting **Navigation** and a representative well.
2. View a well by selecting **Live** or **Snap** in the Camera Controls field.
3. Adjust the focus until the cells have a large, bright center. For examples of proper focus using the “Bright” and “Dark” selections in the Target Focal Plane menu, (Figure 2).
4. Select **Focus Setup** and select **Hardware Auto Focus** in the Focus Type field. Complete the setup by registering the autofocus.
    - Image Based Auto Focus is *not* recommended for brightfield illumination if the wells contain very few cells.

Figure 2. Examples of Proper “Bright” and “Dark” Focus



4. Analyzing Images

This chapter provides information on how to analyze scans from the Cell Migration: Wound Healing application. You perform these tasks in the Analyze tab.

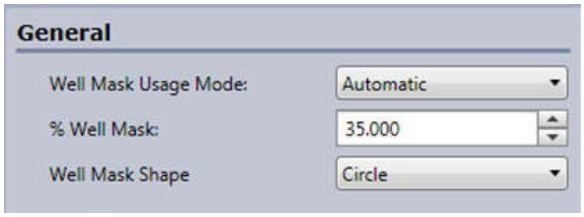
4.1 Analysis Settings

Perform the following steps to select analysis settings. The recommended initial analysis settings for identification and pre-filtering when using the Cell Migration: Wound Healing application are shown in Table 2. The settings typically provide good image segmentation. For a detailed explanation of the analysis settings, see the User Guide.

To select analysis settings

1. Load prior saved Analysis Settings, if available.
2. In the General section (Figure 3), make the following selections:

Figure 3. General Section



a. Well Mask Usage Mode – Select **Automatic**. For information about this selection, see the User Guide.

b. % Well Mask – Select **40%**. Allows you to reduce the area of the well that is analyzed.

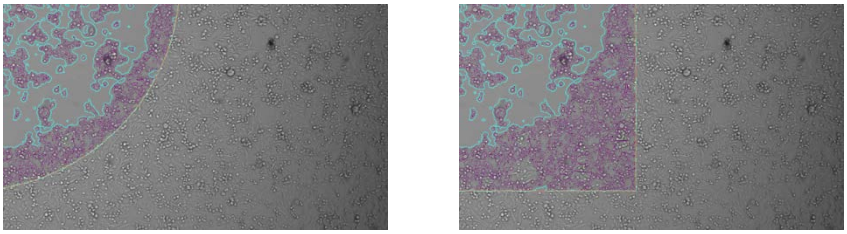
   The Well Mask is critical in the Wound Healing application because the cell exclusion zone does not cover the entire well. Ideally, the % Well Mask needs to be set so that it is near the exclusion zone in control wells at the beginning of the experiment.

c. Well Mask Shape – Select one of the following as needed (Default is the well shape) to define the shape of the exclusion zone for the Wound Healing experiment. Typically, a round well mask is used for Platypus Oris plates and a square well mask is used for scrakhs with a pipette tip.

   - Circle – System uses a circular shape.
   - Square – System uses a square shape.

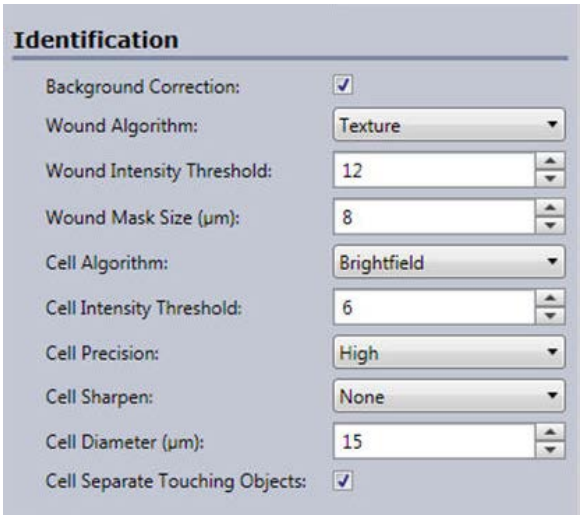
   Figure 4 shows an example of the same well with Circle (on left) versus Square (on right) selected in the Well Mask Shape menu.

Figure 4. Well Mask Shapes



3. In the Identification section (Figure 5), make the following selections:

Figure 5. Identification Section



a. Background Correction – Checkmark as needed (use for Brightfield illumination only). For more information about Background Correction, see the User Guide.

b. Wound Algorithm – Select the appropriate algorithm to be used to determine confluence:

   - Brightfield – The algorithm determines confluence based on the Brightfield cell count algorithm (looks at the cells themselves).
   - Texture – The algorithm determines confluence based on texture variation (the local variation of signal) as opposed to the cells themselves.

	NOTE: The cell identification success of selecting Brightfield versus Texture will depend on the cell type, shape, and contrast of the cells being analyzed.

c. Wound Intensity Threshold – Enter the optimal intensity threshold for the wound area.

   - The wound intensity threshold is the level of intensity that separates background from the area of growth . With an appropriate threshold set, background pixels fall below and pixels in the area of growth are above the threshold.

d. Wound Mask Size (µm) – Select as follows:
   - If you did not select Texture in the Identification section's Wound Algorithm field in step 3, fill in Wound Mask Size.
   - If you selected Texture, do not fill in Wound Mask Size.

e. Cell Algorithm – Select the appropriate algorithm to be used to identify cells, as follows, matching the algorithm to the illumination source:
   - Brightfield – The algorithm looks for objects with a bright center and dark edges
   - Fluorescence – The algorithm looks for fluorescent objects (bright pixels over darker background)
   - Dark Object – The algorithm looks for dark objects with no bright center

f. Cell Intensity Threshold – Enter the optimal intensity threshold for the cells.
   - The cell intensity threshold is the level of intensity that separates background from cells. With an appropriate threshold set, background pixels fall below and pixels in cells are above the threshold.
   - Flat, adherent cells are have darker centers and less well-defined edges than non-adherent cells. Lower Intensity Threshold levels of ≤ 6 are recommended for these cells.

g. Cell Precision – Higher precision results in more accurate identification of cell clusters. High recommended.

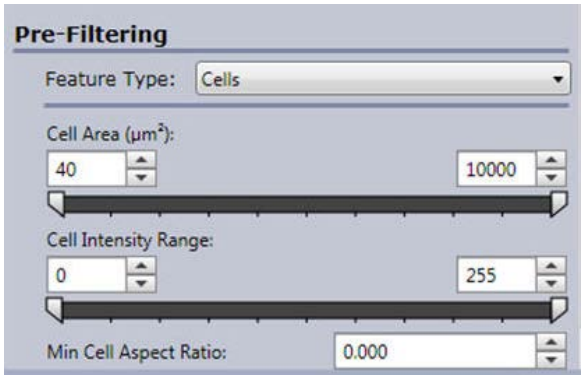
h. Cell Sharpen – Select to enhance the object's edge contrast before image processing.

i. Cell Diameter (µm) – Enter the cell diameter (in pixels) that corresponds to the cell dimensions.
   - At full resolution, the Celigo cytometer provides 1 µm/pixel.
   - Adjust Cell Diameter to match cell type and plating density.
   - Flat, adherent cells have large cell diameters. High Cell Diameter values of ≥15 are recommended for these cells.

j. Cell Separate Touching Objects – Select as needed.
   - Used to separate touching cells during segmentation.

4. In the Pre-Filtering section (Figure 6), make the following selections:

Figure 6. Pre-Filtering Section



   a. Feature Type – Cells or Wound as defined by the application.
   b. Cell Area (µm$^{2)}$ – Enter the appropriate cell area range.
      – Debris is often small or very large. To remove small and/or large objects, adjust the Cell Area range to an appropriate range.
   c. Cell Intensity Range – Enter the appropriate cell intensity range, if necessary.
      – Debris is often dark (brightfield imaging) or very bright (fluorescence). Adjust the Cell Intensity Range to remove dark or bright objects.
   d. Min Cell Aspect Ratio – Aspect ratio measures an object's elongation. Cells outside the minimum value entered will be removed. Enter if necessary.
      – Min. Cell Aspect Ratio is recommended to remove long, oblong objects generally found along bubbles, well edges, and plate imperfections.

5. In the Auto Analyze section, checkmark this selection as needed. For information, see the User Guide.

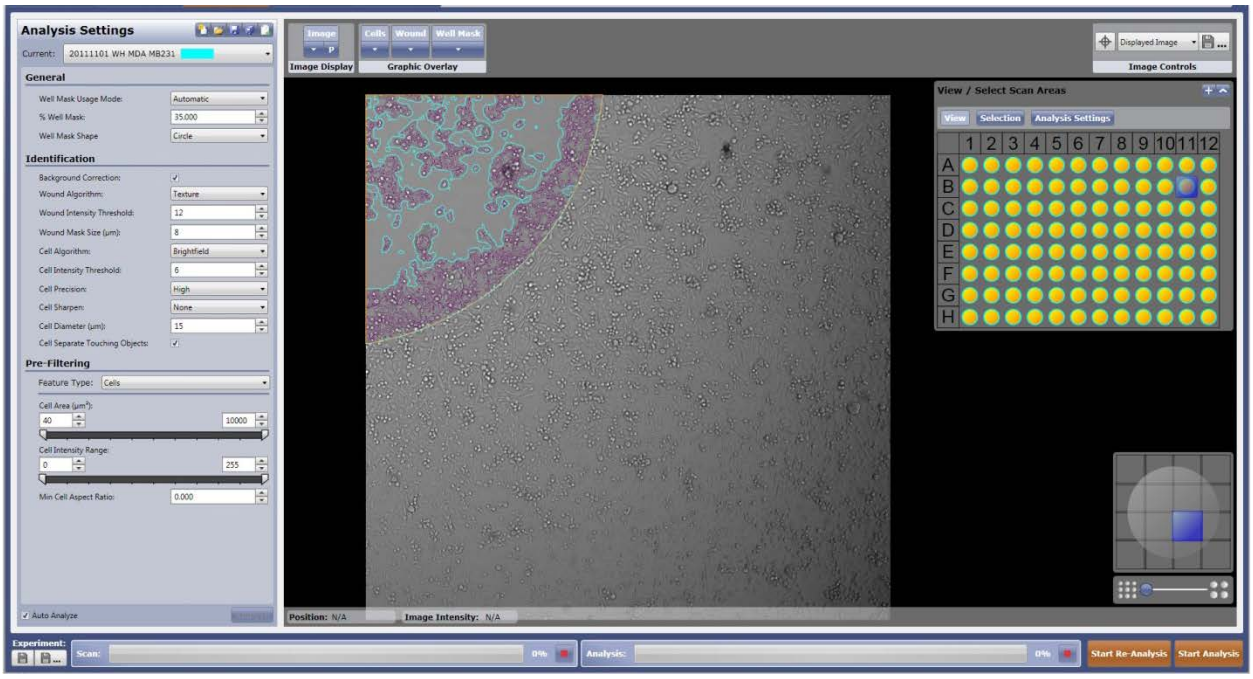
Table 2. Recommended Initial Identification and Pre-Filtering Settings for Analysis

IDENTIFICATION	
Background Correction	Checkmarked
Wound Algorithm	Texture
Wound Intensity Threshold	10
Wound Mask Size (µm)	10
Cell Algorithm	Brightfield
Cell Intensity Threshold	5
Cell Precision	High
Cell Sharpen	None
Cell Diameter (µm)	15
Cell Separate Touching Objects	Checkmarked
PREFILTERING	
Feature Type	40 – 1000
Cell Intensity Range	0 – 255
Min Cell Aspect Ratio	0

Figure 7 shows an example of a well with analysis settings selected and all three Graphic Overlay buttons turned on. The graphic overlay outlines are as follows:

- Blue – Wound area
- Purple – The cells growing within the wound area
- Yellow – The well mask

Figure 7. Well with Analysis Settings Selected



5. Viewing Results

This chapter describes the feature outputs available from the application's Results tab, including how to generate a wound healing growth tracking report.

For instructions on generating a wound healing object level data report, see the User Guide.

Table 3 summarizes the outputs of the Cell Migration: Wound Healing application.

Table 3. Wound Healing Application Outputs

Parameter	Description
Cell Count	Number of Total cells positive with intensity above a user-defined intensity threshold within the healed area
Wound Healing (%)	Area of the well occupied by individual cells or cell clusters (Brightfield imaging only) divided by total area of scan area or well
Well Sampled (%)	Percent of well sampled

5.1 Image and Fill Views

This section describes the purpose of the Image and Fill views in the Cell Migration: Wound Healing application. In this application, you can choose between an Image and Fill view within either the plate view or well detail view, helping you see the properties and patterns of interest to you.

- Image button – Turns on/off the raw image display
- Fill button – Fills all identified cells with a selected color (default is green)

	NOTE: **Clicking the Image button a second time is the same as clicking the Fill button. Clicking the Fill button a second time is the same as clicking the Image button.**

When the Results tab first appears, the default view is a plate-level Image view. In this application, the measurement parameters available in the Image view include the wound healing percentage for each well (Figure 8 and Figure 9). In the examples shown in the below two figures, the user has selected well A7.

Figure 8. Plate-Level Image View Showing Wound Healing % with Heatmap Off

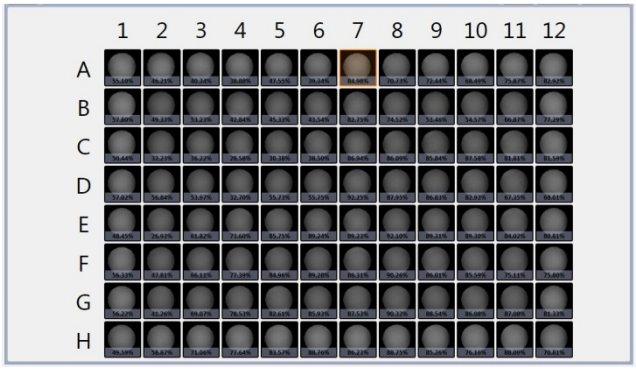
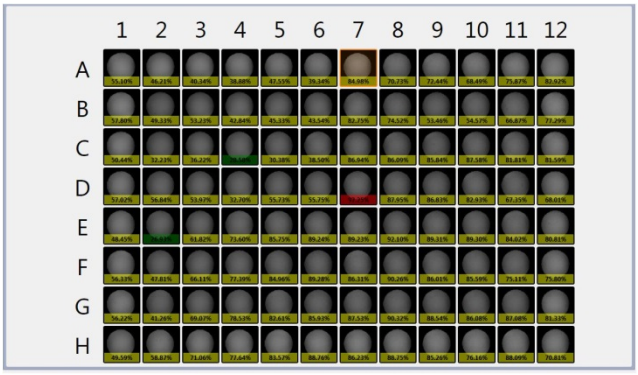


Figure 9. Plate-Level Image View Showing Wound Healing % with Heatmap On



The alternative view is the plate-level Fill view, which provides a confluence overlay of any identified colonies (Figure 10). This display provides an overview of the wound healing progress.

Figure 10. Plate-Level Fill (Confluence Overlay) View

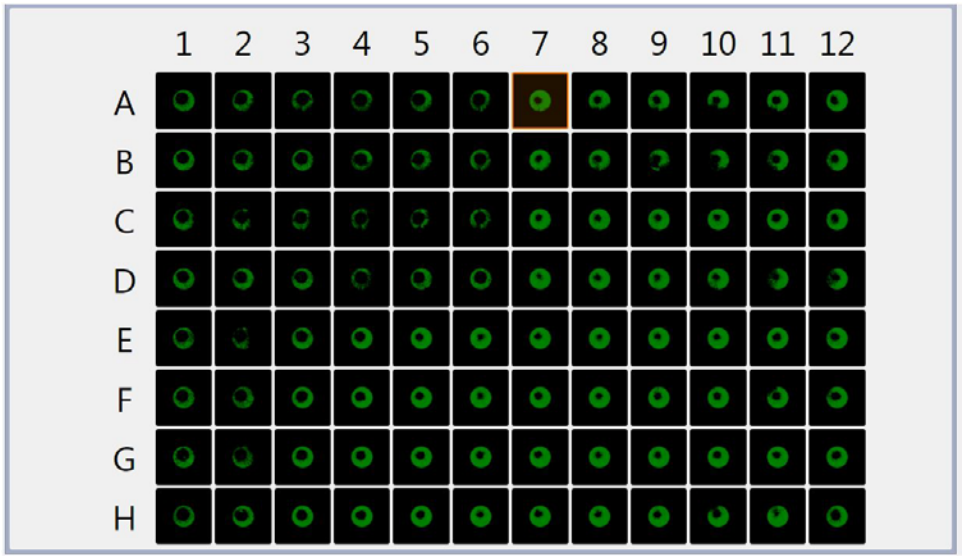
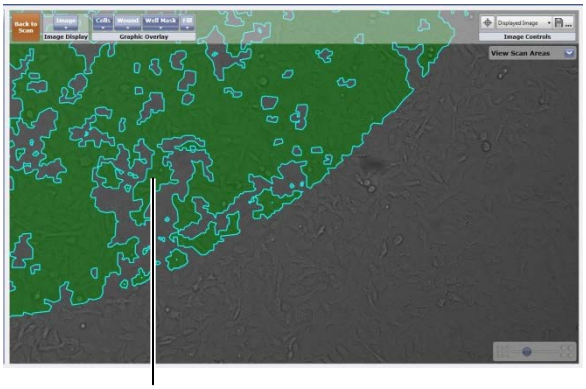


Figure 11 shows well A7 at the well detail view, with fill off versus on.

Figure 11. Well Detail Fill (Confluence Overlay) View Magnified

Fill Off

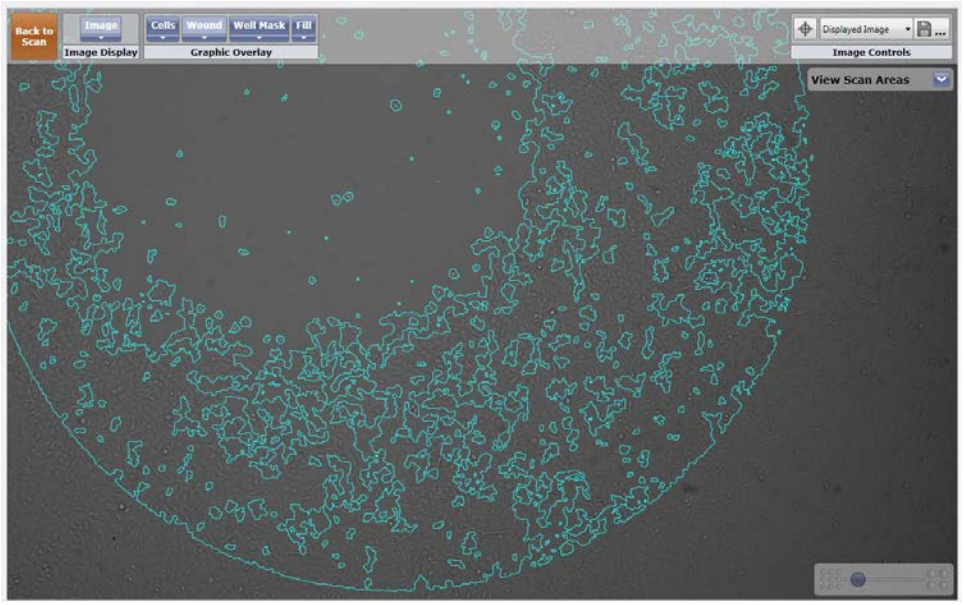
Fill On



The fill (green shaded area) identifies the area that is occupied by cells rather than empty space

Figure 12 shows well A7 zoomed out.

Figure 12. Well Detail Fill View with Fill Off (Confluence Overlay) and Zoomed Out



5.2 Generating a Wound Healing Growth Tracking Report

Wound healing growth tracking reports calculate the growth characteristics of cell populations within a wound area over time. The reports associate cell counts or wound healing % measurements from multiple scans time points – using each scan's latest scan result – and determine doubling times and rates for individual wells. The reports are in the form of curves and pie charts that can then be exported as images or data by the user for documentation and presentation purposes.

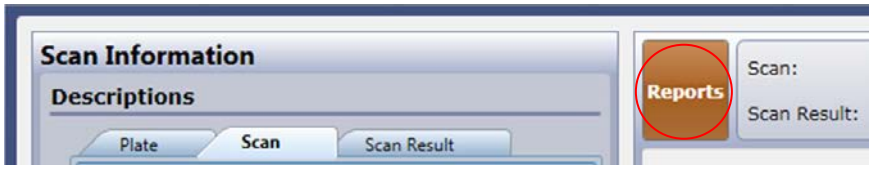
For a detailed explanation of the Identification and Pre-Filtering settings, see the User Guide.

To generate a wound healing growth tracking report

	**NOTE: To generate a pie chart, make sure that 4 or more scans exist for selection. To generate a growth chart (curve), make sure that 2 or more scans exist for selection.**

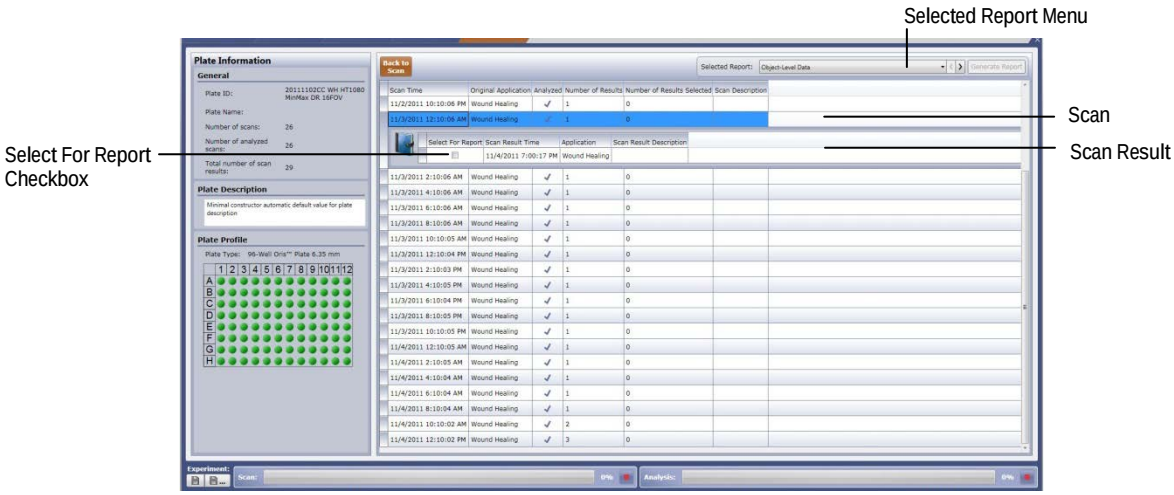
1. In the Results tab, click **Reports** (Figure 13)

Figure 13. Displaying Scans for Reporting



The list of existing scans and associated scan results for the plate ID appears (Figure 14).

Figure 14. Scan List



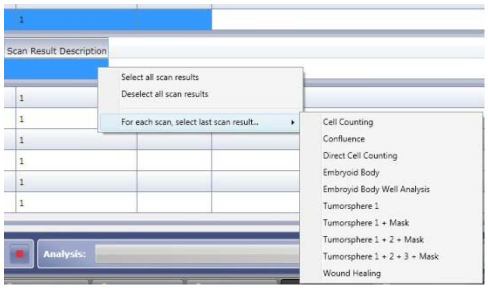
2. Click the Scan Time for which you want to generate a wound healing growth tracking report.

   The Scan Time selection highlights in blue.

3. Checkmark the Select For Report checkbox (Figure 14 above) next to each scan result for which you want to generate a report. You can also right-click a scan result and select one of the following :

   - Select all scan results
   - Deselect all scan results
   - For each scan select last scan result...Wound Healing

Figure 15. Scan Result Right-Click Selections



4. In the Selected Report menu (Figure 14 above), select **Growth Tracking: Wound Healing**.

	NOTE: Only the scans analyzed using the application you are selecting will be available for selection.

5. Click **Generate Report** at the top right of the Results tab.

A growth chart (default display mode) for the entire plate, with Combine All Scan Areas selected, appears in the Results tab. The Detailed View in the left-hand pane is a summary view; a well is not selected by default.

5.3 Working with a Generated Report

You can take the following actions on a generated wound healing growth tracking report (curve or pie chart) as needed. For instructions on magnifying a pie chart size (zoom) and re-sizing a chart, see the User Guide.

5.3.1 Displaying a Chart for a Well

By default, when you click **Reports** in the Results tab, the Detailed View in the resulting growth chart is a summary view; a well is not yet selected. You can then select a specific well for displaying its chart in the Detailed View.

To display a chart for a well

In the right-hand pane, click a well.

An orange border appears around the selected well.

In the right-hand and left-hand panes, each data point and cell count corresponds to each scan time listed in the Scan menu. In the left-hand pane, the light blue dot and square on the curve (Figure 16) indicate the cell counts that correspond to the currently selected scan time in the Scan menu.

5.3.2 Changing the Type of Display

Change the type of display by selecting the **Display Mode** menu and then selecting one of the following options:

- Normalized Growth Chart (Figure 16) – Growth curve normalized to the well that has the highest Wound Healing % value. When this setting is selected, the system converts the data point locations so that the same scale is used among the scan results, for easier comparison.

  Growth curves are displayed using the identical Y-axis for each well, determined by the highest count for the plate ID.

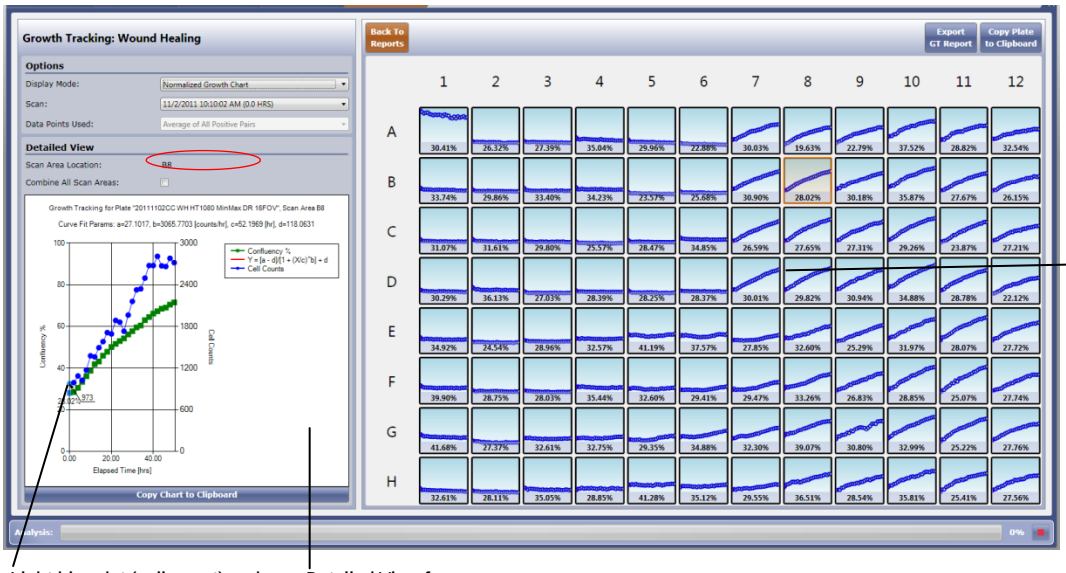
- Growth Chart – Displays the wound healing growth curve for a given well, based on the Wound Healing % measurement, and the fitted wound healing growth curve. Each Scan Area plot is fitted for the display window by varying the Y axis range. Curve is fitted using a standard Four-Parameter Logistic equation: $Y = [a-d]/(1 + X/c^b) + d$.

- Doubling Time – Calculated time in hours for one doubling of the cell count or wound healing % according to the following equation: doubling time = (time between count 1 and count 2 in hrs) * [ln(2) / ln(count 2/ count 1)]. Displayed with pie charts.

- Doubling Rate – Calculated rate of doubling per hour according to the following equation: 1 / doubling time (hrs). Displays both a wound healing growth curve and pie charts (Figure 18).

- Count Chart – Displays the well count and growth curve within the well mask area.

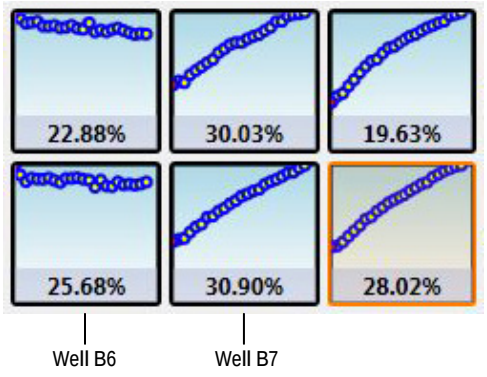
Figure 16. Normalized Growth Chart



The difference between the displays from selecting a Display Mode of Growth Chart versus Normalized Growth Chart is illustrated in Figure 17. The figure shows thumbnail images of the same wells with each of these two Display Mode settings. In this example, with Growth Chart selected, there was a wide disparity between the test result data points for well B6 versus B7. The disparity is better visualized in Normalized view.

Figure 17. Growth Chart vs. Normalized Growth Chart Thumbnail Images

Growth Chart Thumbnail Images



Normalized Growth Chart Thumbnail Images

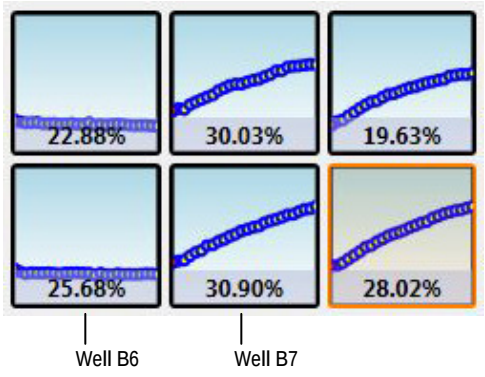
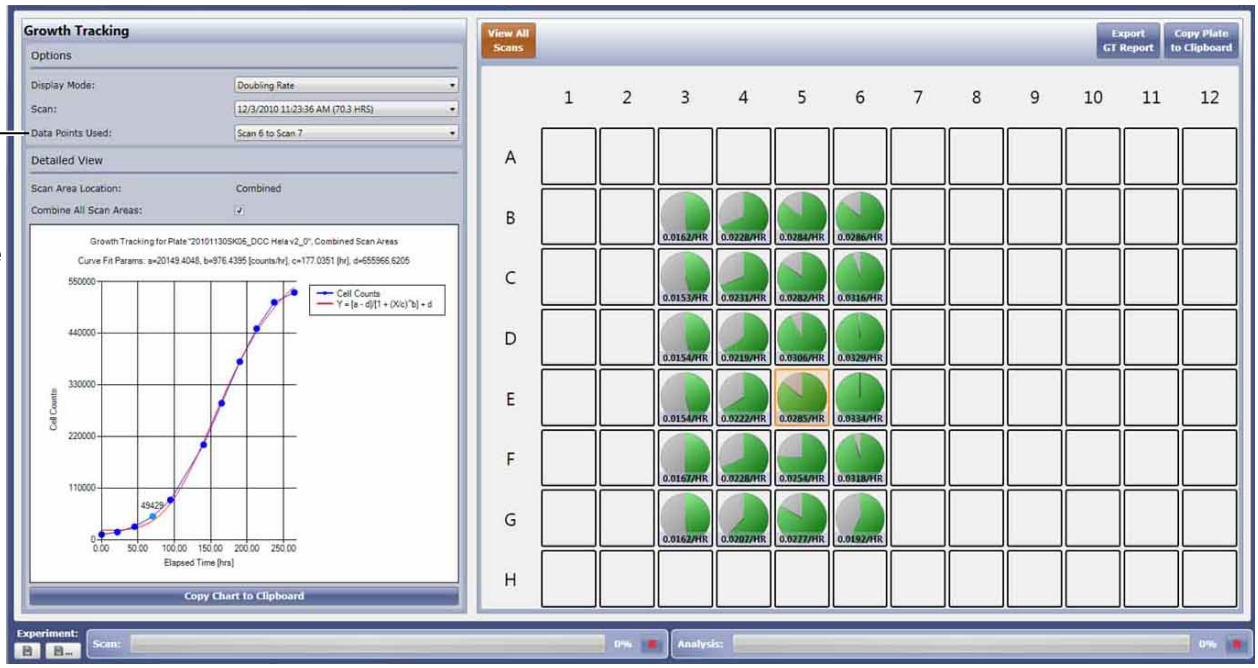


Figure 18. Wound Healing Growth Curve and Pie Charts in Doubling Rate Display



Data Points Used will recalculate the doubling rate (or doubling time for Doubling Time display mode)

5.3.3 Exporting Report Data

Export wound healing report data by clicking **Export GT Report** (Figure 16 above). For a summary of outputs, see Table 4.

Table 4. Wound Healing Growth Tracking CSV Report Outputs

Parameter	Description
Plate-Level Data	
Average of Positive Doubling Time Data (hrs)	Well level average doubling time from all positive two point successive paired doubling times.
Average of Positive Doubling Rate (1/hr)	Well level average doubling rate from all positive two point successive paired doubling times.
Well-Level Data	
Wound Healing Cell Counts	Number of Total Cells positive with intensity above a user-defined intensity threshold within a healed area
Wound Healing (%)	Area of well occupied by individual cells or cell clusters (Brightfield imaging only) divided by total area of scan area or well
% Well Sampled	Area of well occupied by individual cells or cell clusters (Brightfield imaging only) divided by total area of scan area or well.
Two Point Doubling Times (hrs)	Two-point doubling time calculated from two successive scans
Logistics Model Parameters	Logistic 4-parameter fitting curve of the growth curve data.

5.3.4 Exporting a Chart Image

Export a chart to the clipboard as a jpg image by clicking **Copy Plate to Clipboard** (Figure 16 above).

6. Troubleshooting

This chapter provides troubleshooting recommendations.

Table 5. Troubleshooting Recommendations

Issue	Recommended Action
Cannot identify individual cells	1. Inappropriate focus selected. • For brightfield imaging– verify that "Bright" focus plane was used to acquire images. • For fluorescence – confirm that crisp focus was selected for desired objects. 2. Desired objects are excluded by the Pre- Filtering settings. • View the segmented image in the Analyze tab (see *Celigo Cytometer User Guide* for instructions) • Change Pre-Filtering settings to identify desired objects 3. Identification settings do not identify objects. • Recommend following the tips for identify individual cells (see section 0.). 4. Inappropriate gate is applied in Gating screen. • Go to Gating tab and remove gates.
Software identifies debris as cells or confluent areas in brightfield	Often debris has unique properties that can be used to remove it from the scan results. Use the Pre-Filtering settings to remove debris. • Recommend adjusting Pre-Filtering settings to selectively remove debris.
Improper cell counts on well edges in brightfield	1. Remove/uncheck Separate Touching Objects in the Analyze tab – Identification section. 2. Remove/uncheck Well Mask in the Analyze tab – Identification section. 3. Increase Aspect Ratio in Analyze tab – Pre-Filtering section.
Well edges are too bright or dark	Liquid volume not optimal resulting in a meniscus-dependent effect. • For proper liquid volumes for cell plating, see Table 1.
Bright or dark shadows of cells are identified in brightfield	1. Adjust liquid volume level to prevent meniscus-dependent optical effects. • For proper liquid volumes for cell plating, see Table 1. 2. Some small well microplates, such as 1536-well formats, yield meniscus-dependent optical effects that cannot be corrected by liquid volume. • Use an alternative container. • Use Pre-Filtering settings in the Analyze tab to remove unwanted objects

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