Celigo® Cytometer Application Guides

Celigo® Software Version 5.2

8001631 Rev. H

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Celigo® Cytometer

Cell Counting and Growth Tracking Application Guide

Celigo Software Version 5.2

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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.

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: support@nexcelom.com
  phone: +1 978-327-5340
- From Europe:
  e-mail: support@nexcelom.co.uk
  phone: +44 (0) 161 232 4593

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 the application. (see Troubleshooting section 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	2500	ND[1]
6W	Corning	3471	2500	ND[1]
12W	Corning	3512	1000	ND[1]
24W	Corning	3524	≥500	ND[1]
96W	Greiner	655090	≥200	≥100
96W half area	Greiner	675090	50	50
384W low vol	Corning	3542	20	20
384W high vol	Corning	3712	≥40	≥40
384W high vol	Greiner	781091	≥40	≥40
1536W	Corning	3838	8	8
1536W	Greiner	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

- In the Current Application dropdown list (Figure *1*), select **Cell Counting** > **Direct Cell Counting**.

Figure 1. Selecting an Application

Table 2. Selecting an Application

Direct Cell Counting	Identifies and counts individual cells using brightfield or fluorescent 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 Image Acquisition Settings for brightfield or fluorescent 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 **Auto Exposure, Gain if necessary**.
     - In Frequency, select **Every scan area**.
   - For Fluorescent illumination:
     - It is recommended to use the channel type: Custom Channel. 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. Register focus with **Focus Setup.**
   - It is recommended to select Hardware Auto Focus for most routine plate scanning.
   - For instructions on selecting correct focus position for brightfield imaging. See section 3.3.
   - For details on how to set up focus, reference the User Guide.

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.

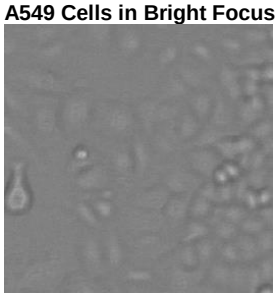
This section describes how to select the correct focus position for brightfield imaging. For instructions on focusing using fluorescent illumination, see the User Guide.

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 see Figure 2.
4. Select **Focus Setup**, then 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.

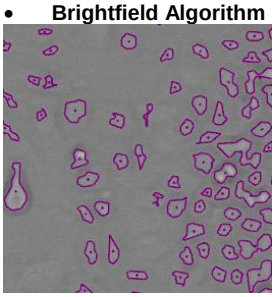
Figure 2. Examples of Proper Bright and Dark Focus

A549 Cells in Bright Focus

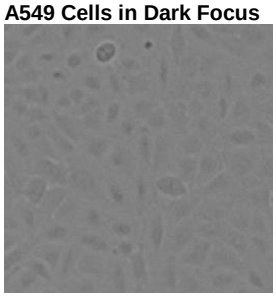


Optimal Identification of A549 Cells Using:

- **Bright Target Focal Plane**
- **Brightfield Algorithm**

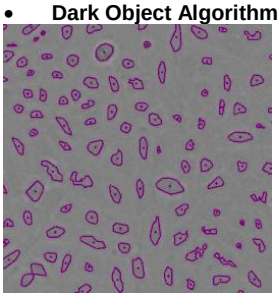


A549 Cells in Dark Focus

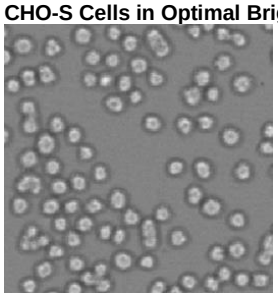


Optimal Identification of A549 Cells Using:

- **Dark Target Focal Plane**
- **Dark Object Algorithm**

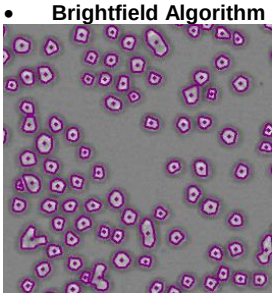


CHO-S Cells in Optimal Bright Focus

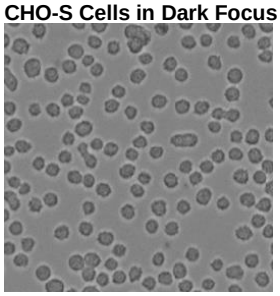


Optimal Identification of CHO-S Cells Using:

- **Bright Target Focal Plane**
- **Brightfield Algorithm**

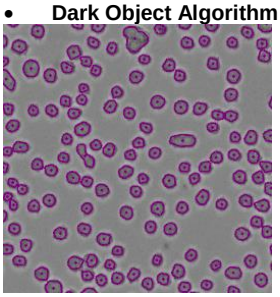


CHO-S Cells in Dark Focus



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.

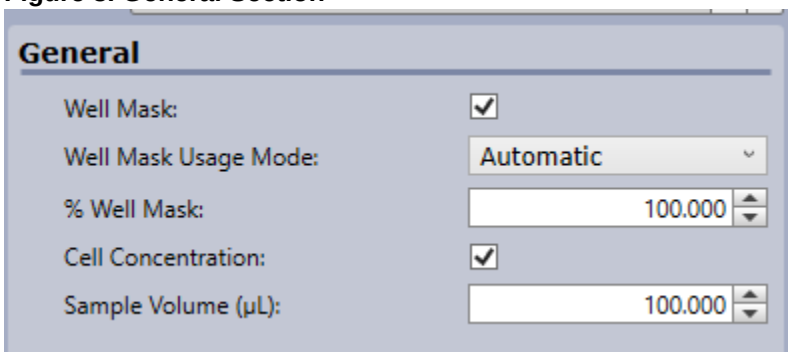
4.1 Analysis Settings for Direct Cell Counting Analysis

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 as well as Figure *3* to Figure *5*. The settings typically provide good image segmentation. For a detailed explanation of the analysis settings, see the User Guide.

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.

   Note: Cell Concentration will become checkable when Well Mask is enabled.

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.

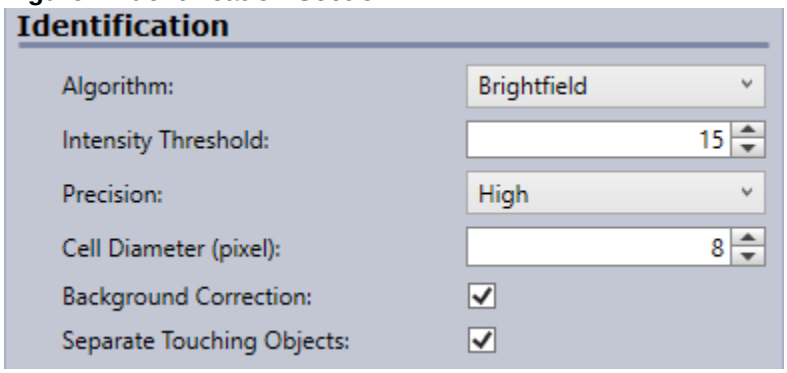
c. % Well Mask – Reduce the well mask to a percentage that is useful for looking at cells toward the center of the well. Decreasing the % Well Mask entry can exclude well edge artifacts that can occur during plate manufacturing.

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

d. Cell Concentration – This measurement calculates the concentration of cells present in the well (cells/mL) per defined Sample Volume (i.e., range is from 0.5 to 20,000 µl).

3. In the Identification section (Figure 4), 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 the background from cells. With an appropriate threshold set, background pixels fall below the threshold while the pixels in cells are above the threshold. Adjust the Intensity Threshold to match cell types:

- Non-adherent cells – Typically, non-adherent cells are rounded with bright centers and dark edges. Higher Intensity Threshold values of ≥10 are recommended for identifying cells and ignoring darker debris.
- Adherent cells – Adherent cells are flatter, with darker centers and have less well-defined edges than non-adherent cells. 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. The High setting is 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:

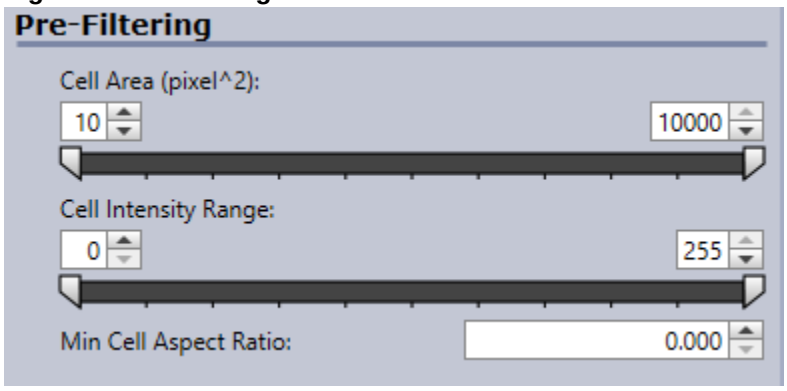
   - Non-adherent or dense cell cultures – Non-adherent cells or dense cells have smaller cell diameters. Lower Cell Diameter values of ≤10 are recommended.
   - Adherent cells – Adherent cells have larger cell diameters. Higher Cell Diameter values of ≥15 are recommended.
   - At full resolution, the Celigo cytometer provides 1 µm/pixel.

e. Background Correction – Select if necessary. See the User Guide for more information.

f. Separate Touching Objects – Select as needed. This selection is used to separate touching cells during segmentation. See the User Guide for more information.

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 are 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 are 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	Check marked	Check marked	Check marked	Check marked	Check marked	Check marked
Separate Touching Objects	Check marked	Not Check marked	Check marked	Not Check marked	Check marked	Not Check marked
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

5. Gating Cells

This section 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.
- 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. For more information reference the User Guide.
- 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 plots, gates, and populations

1. Create a plot in the Plot Populations pane using the Add Plot (+) button and then the Add Plot dialog box.

Table 4 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.

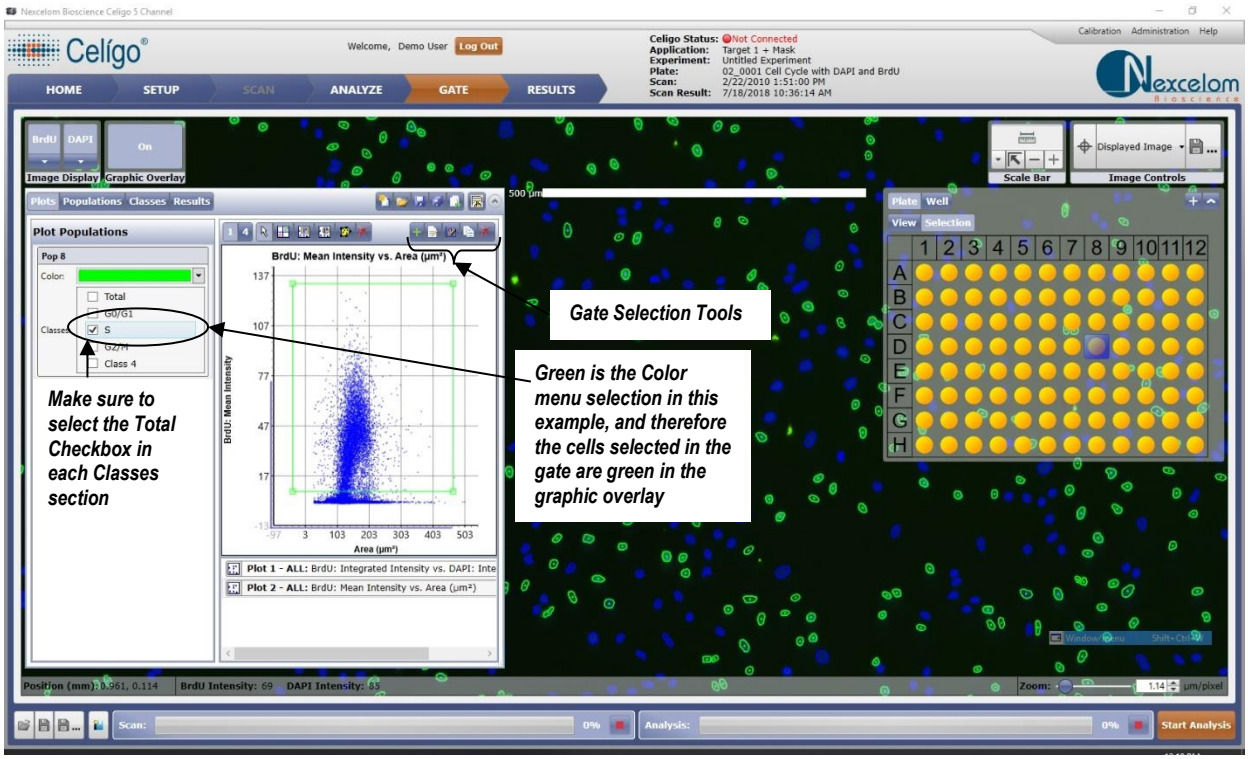
When you create the plot in the Direct Cell Counting Application, all the cells will automatically belong to a population named All. Based on the All population, a class (a class is a population for which data will be reported) is also automatically created, named the Total class.

Table 4. 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 *6*). In each Pop section, make sure to check mark 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.
3. In the Graphic Overlay display, the color displayed for the plot population selected in the gate corresponds to the Color menu selection (Figure *6*). The figure shows the single class (Total) used in this application.

Figure 6. Gating Cells



4. Repeat steps 1 and 2 as needed to refine the population that you want to count.
5. Assign the Total class to the population as follows:
   d. Click the gate.
   e. In the Plots, Populations, or Classes view, make sure that the Total class is check marked.

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

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

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 5 summarizes the outputs of the Cell Counting applications.

Table 5. Cell Counting and Growth Tracking Application Outputs

Parameter	Description
Cell Count	Number of Total cells positive with intensity above a user-defined intensity threshold.
% Well Sampled	Percent of well surface imaged.
Cell Concentration	Displays the cells per mL per well.

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.

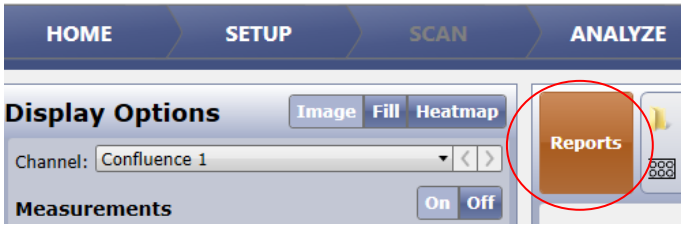
For a detailed explanation of the Identification and Pre-Filtering settings, see the User Guide.

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.**

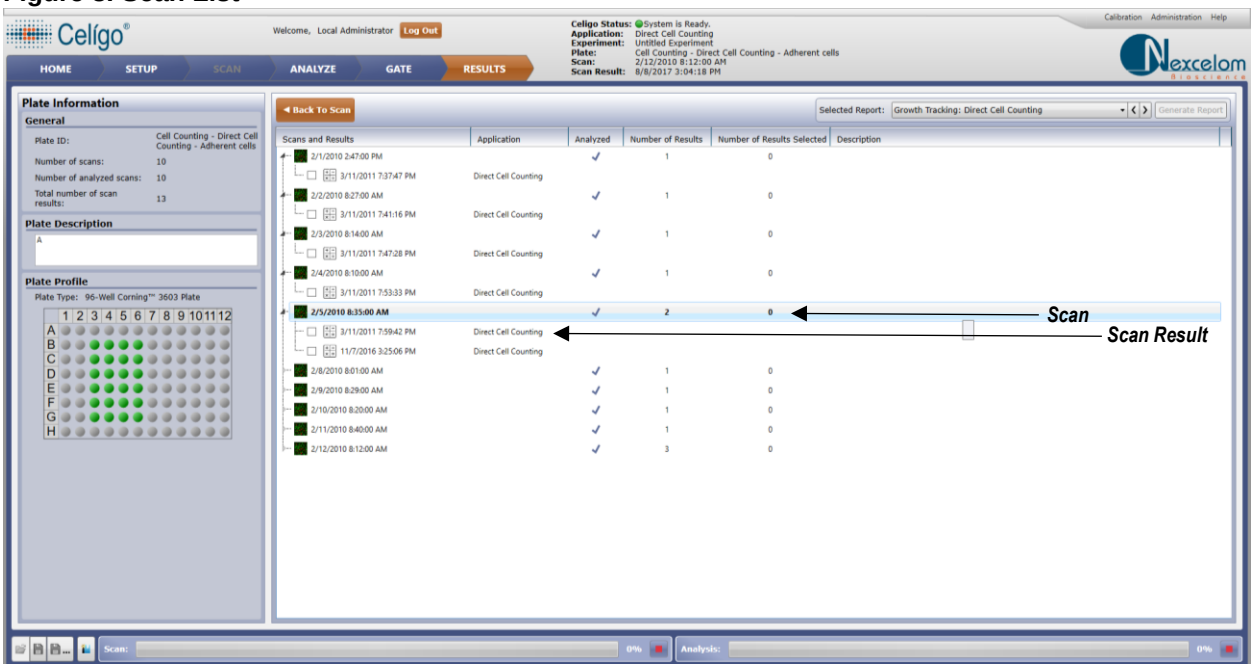
6. In the RESULTS tab, click **Reports** (Figure 7)

Figure 7. Displaying Scans for Reporting



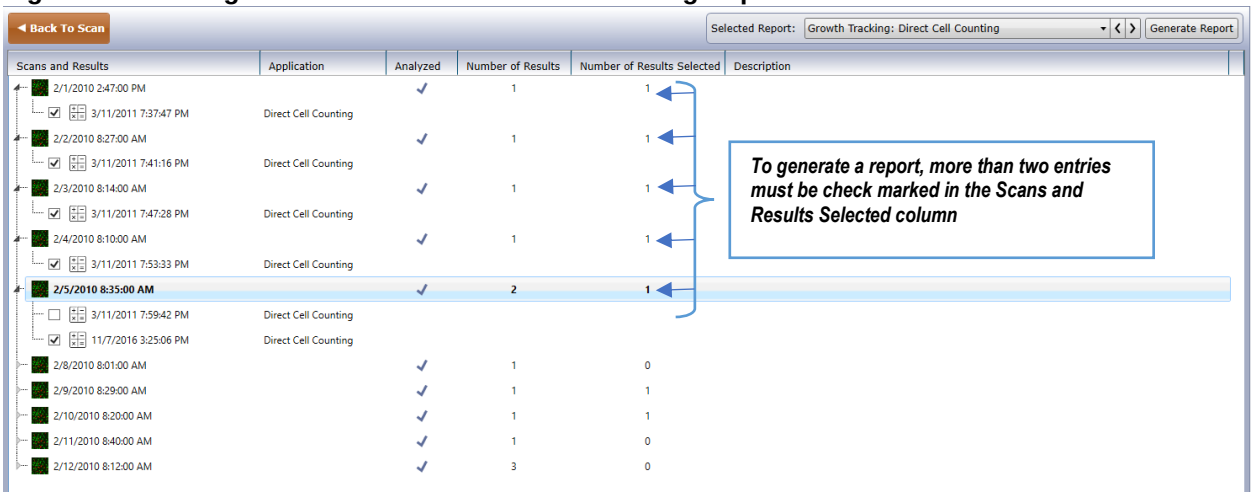
The list of existing scans and associated scan results for the plate ID appears (Figure 8).

Figure 8. Scan List



7. Check mark the checkbox (Figure 9) next to each scan result for which you want to include in the growth tracking report. The Number of Results Selected column will update with a value of 1 to confirm your selection.

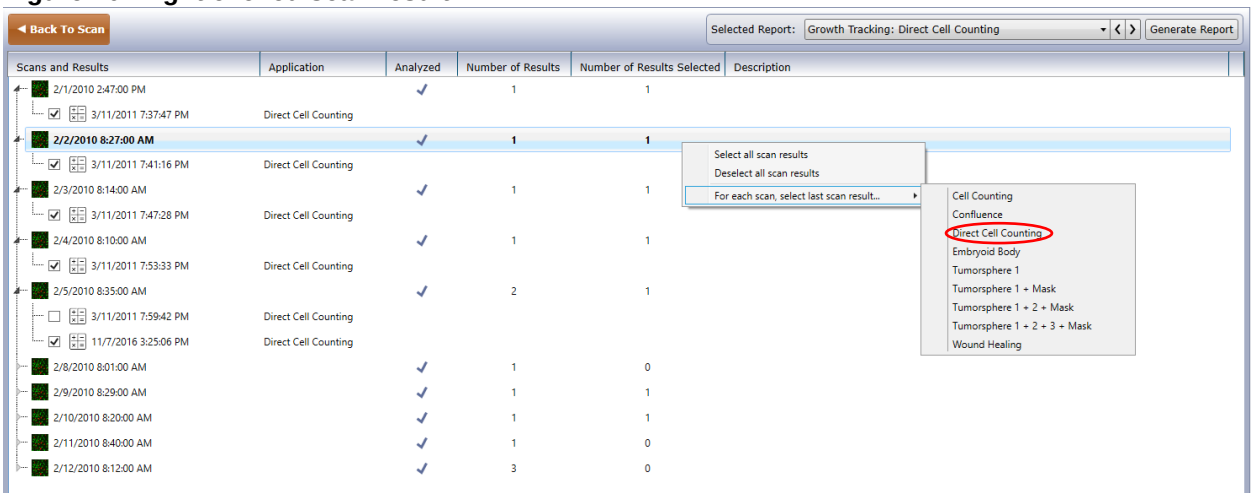
Figure 9. Selecting Scan Results for a Growth Tracking Report



Option: Right-click a scan result and select one of the following (Figure *10*):

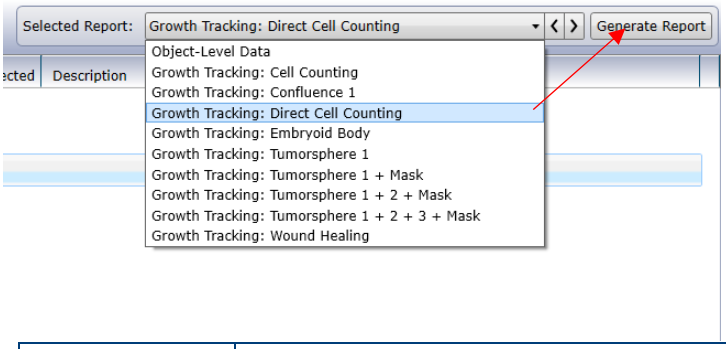
- Select all scan results
- Deselect all scan results
- Select last Direct Cell Counting scan result for each scan

Figure 10. Right clicked scan result



8. In the Selected Report menu, select Growth Tracking: Direct Cell Counting (Figure *11*).

Figure 11. Selected Report Menu



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

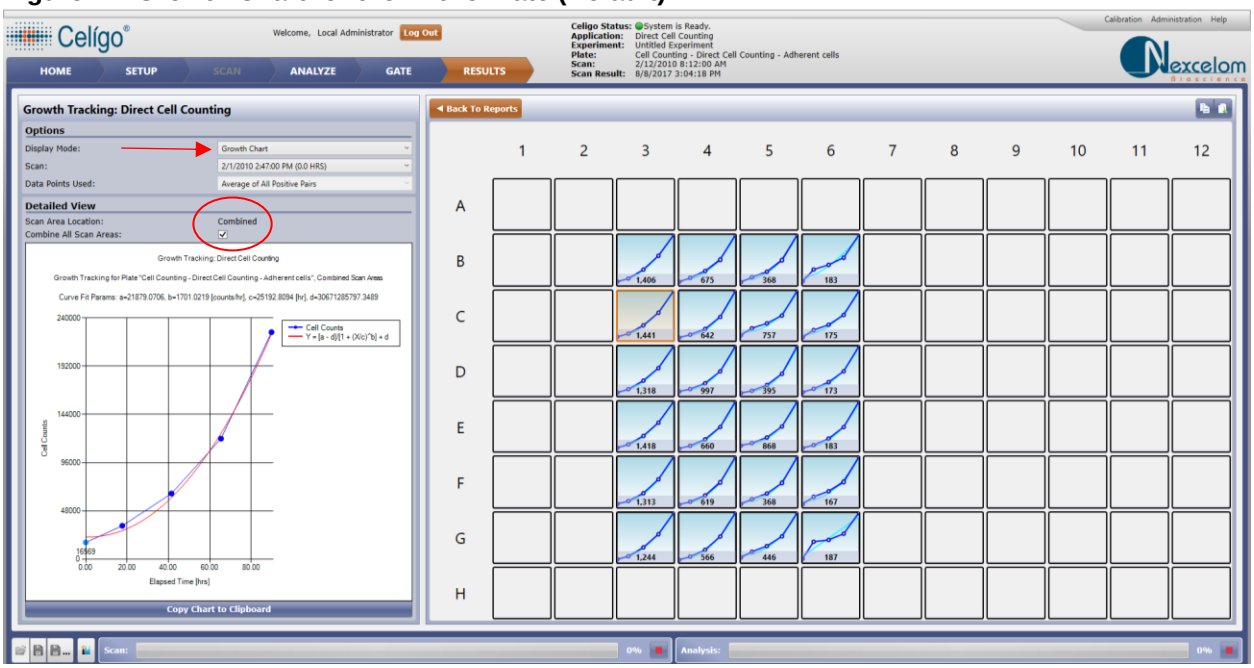
9. Click Generate Report at the top right (Figure *11*).
   - A growth chart (default display mode) for the entire plate, with Combine All Scan Areas selected, appears in the RESULTS tab (Figure 12).

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 12). This option makes calculations based on all wells of the plate.

To get a detailed view of a single well, see section 6.2.2.

For more information about viewing detailed information within the generated report see section 6.2.

Figure 12. Growth Chart for the Entire Plate (Default)



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 Display Mode

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.

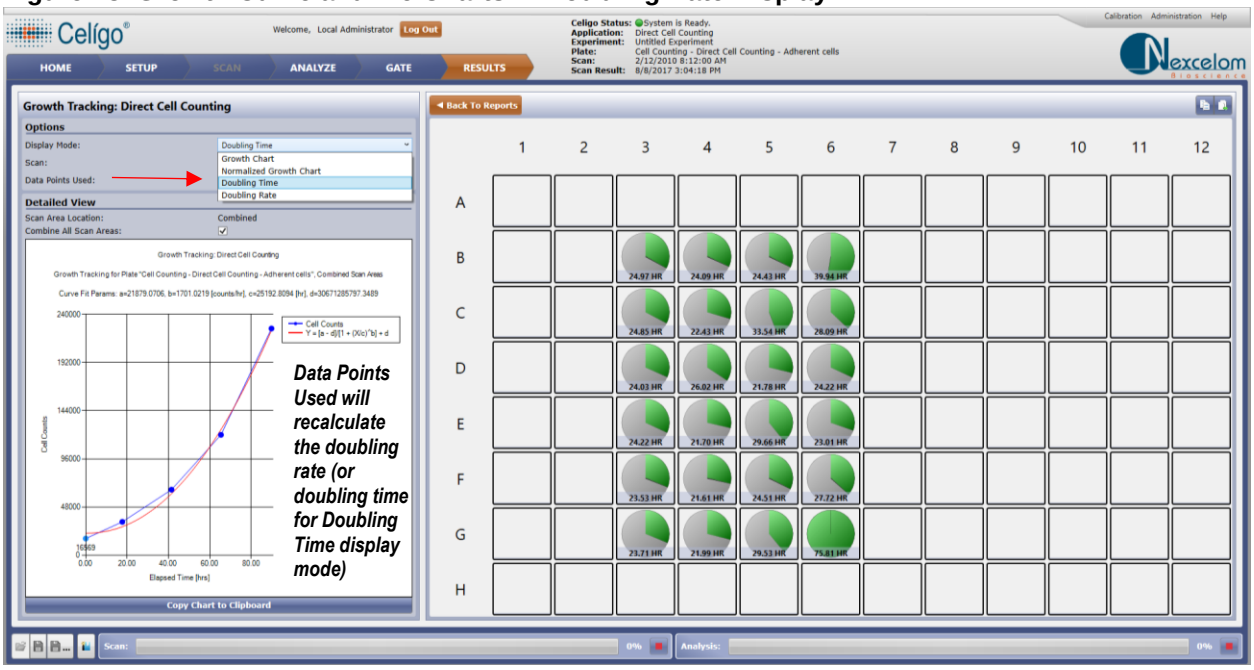
  Growth curves are displayed using the identical Y-axis for each well, determined by the highest count for the plate ID.

- 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 13).

Figure 13. Growth Curve and Pie Charts in Doubling Rate Display



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.

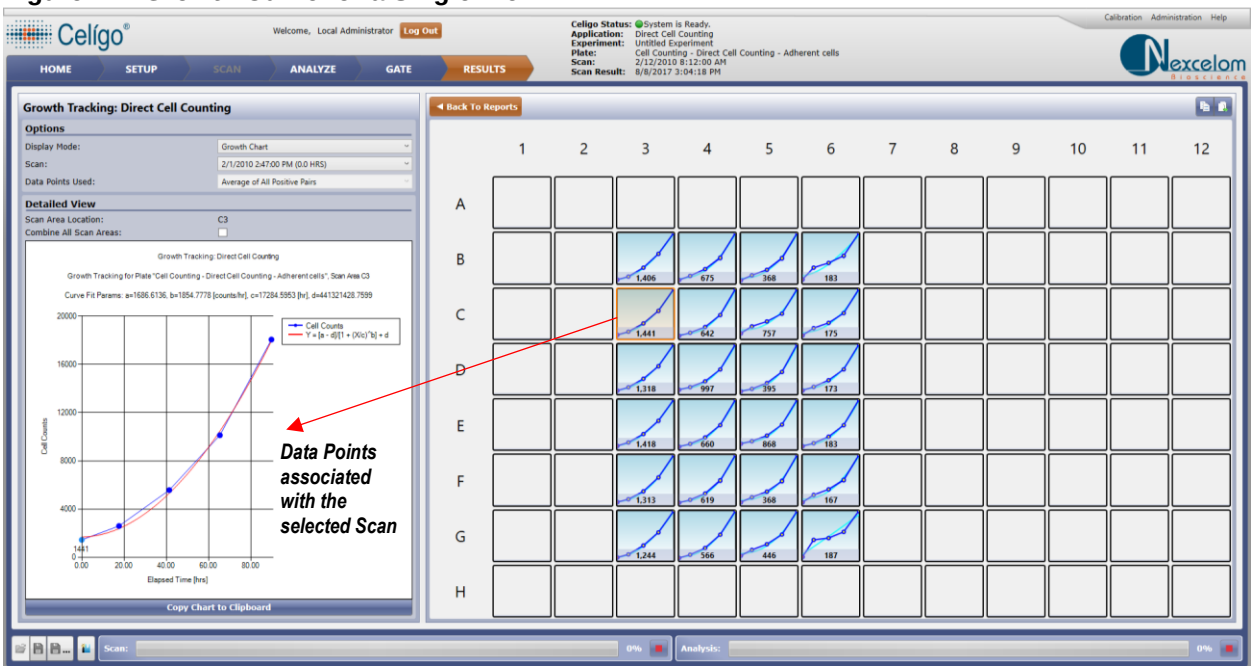
An orange border appears around the selected well (Figure 14).

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 14), select a different scan in the Scan menu.

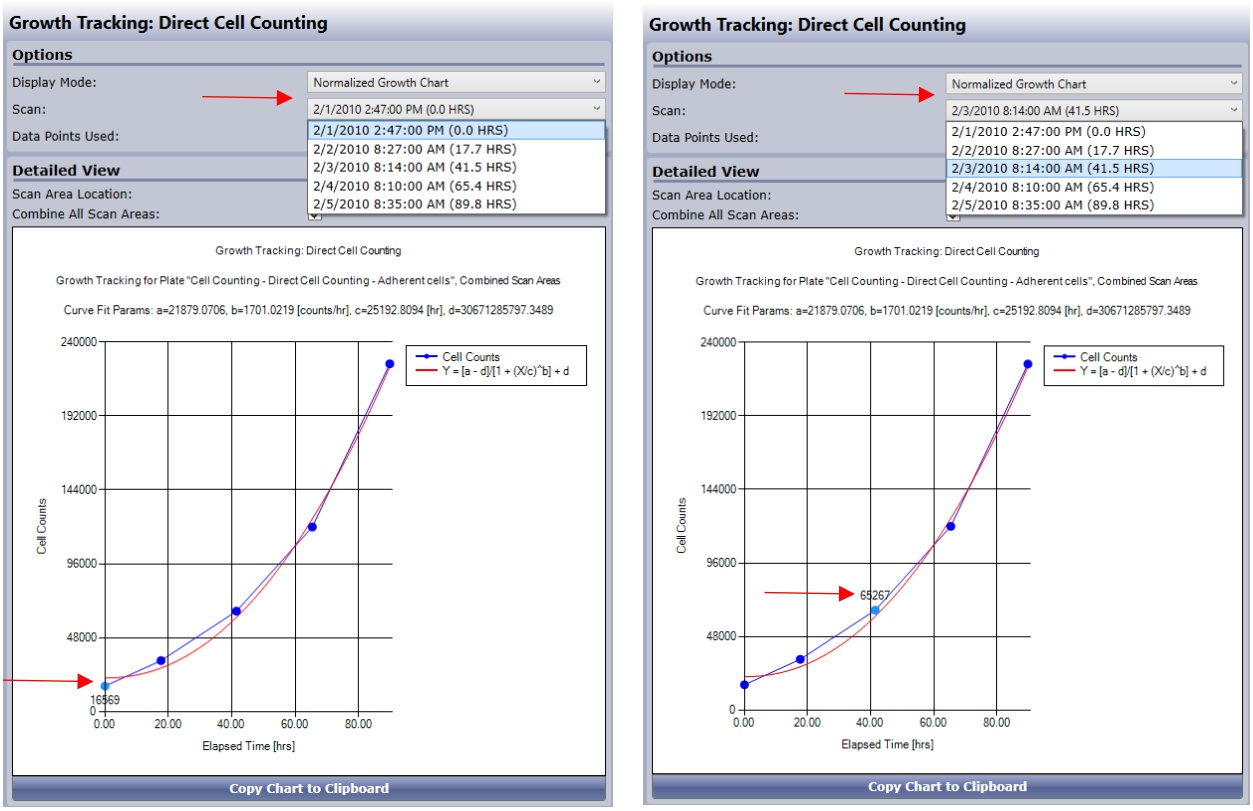
Figure 14. Growth Curve for a Single Well



6.2.3 Displaying a Growth Curve for the Entire Plate

To change the cell count associated with the time indicated at each blue data point on the curve (Figure 15.), select a different scan in the Scan menu.

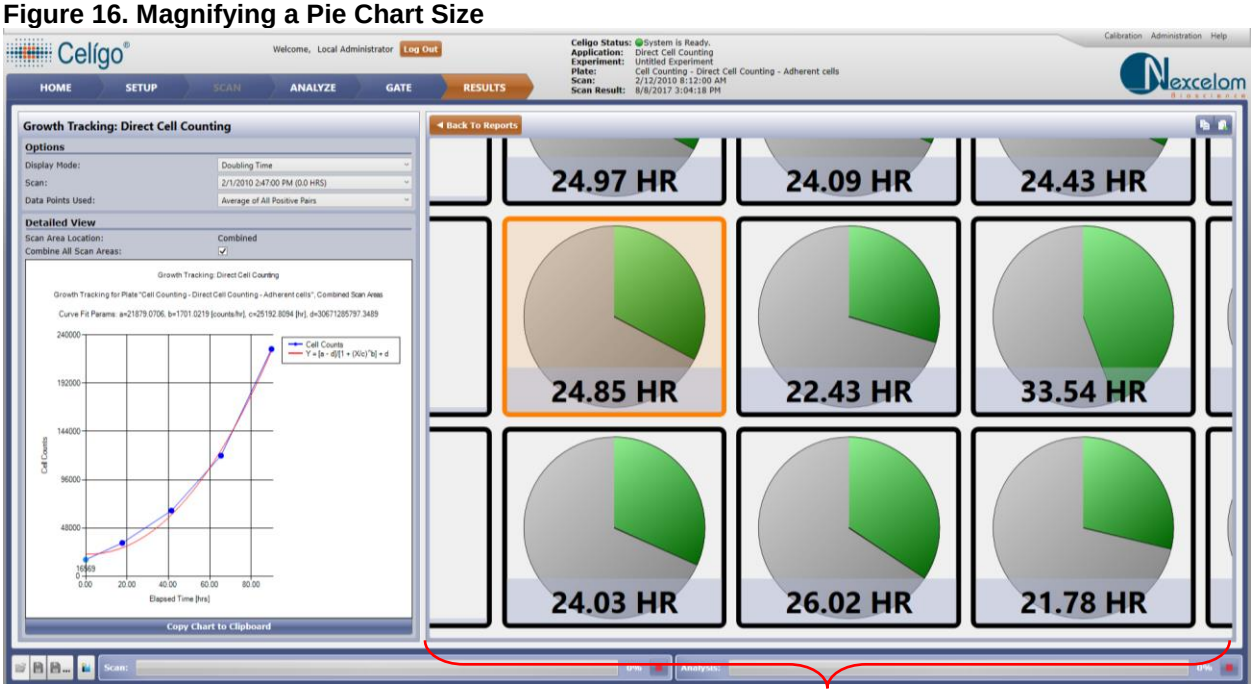
Figure 15.. Growth Curve for the Entire Plate



6.2.4 Magnifying a Pie Chart Size (Zoom)

You can magnify the appearance of a pie chart pane (not a growth curve) (Figure 16.) by hovering the mouse over the pie chart and then scrolling with the mouse scroll wheel.

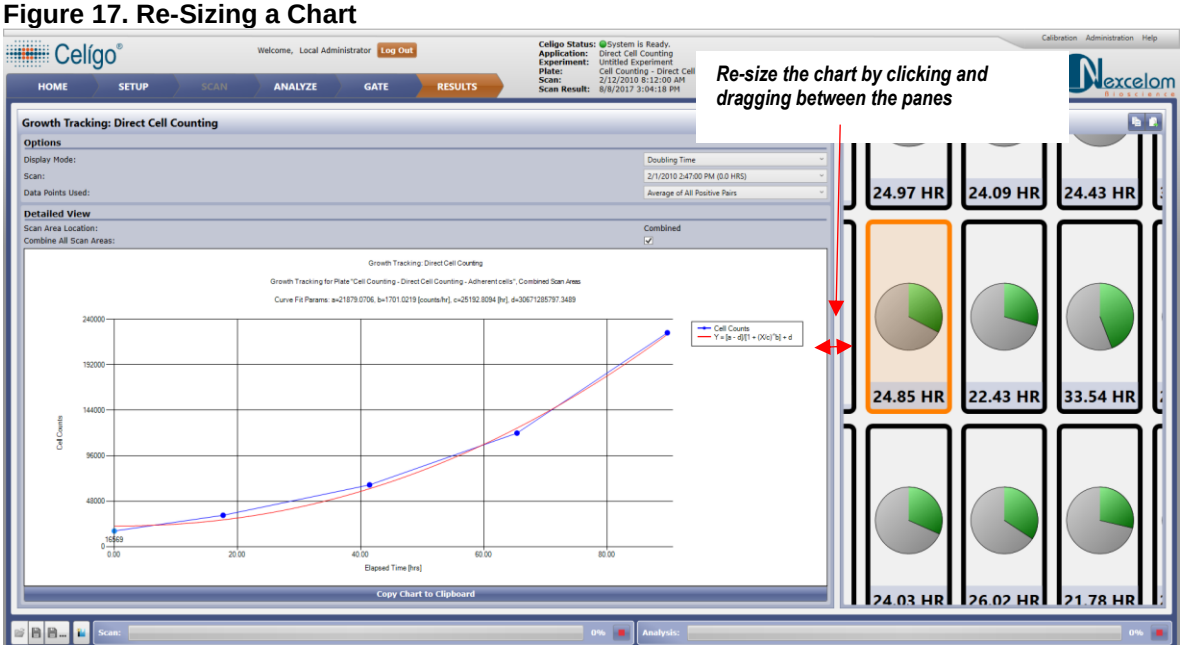
Figure 16. Magnifying a Pie Chart Size



6.2.5 Re-Sizing a Chart

Widen or narrow the size of a chart by clicking and dragging the window pane between the left and right panes (Figure 17.). This action is not to change the chart data, but is only for viewing purposes.

Figure 17. Re-Sizing a Chart



6.2.6 Exporting Report Data

Export growth tracking report data by clicking **Export Growth Tracking Report**. For a summary of outputs, see Table 6.

Table 6. Growth Tracking CSV Report Outputs

Parameter	Description
Cell Count	Number of Total Cells positive with intensity above a user-defined intensity threshold
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 (Figure *18*B) by clicking **Copy Chart to Clipboard** (Figure 18A).

Figure 18. A. Copy Chart to Clipboard

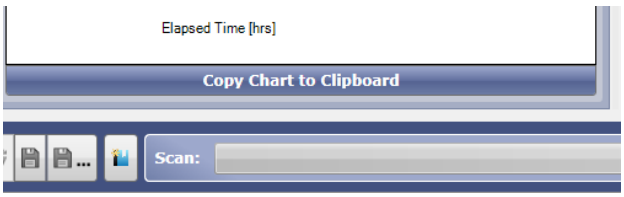
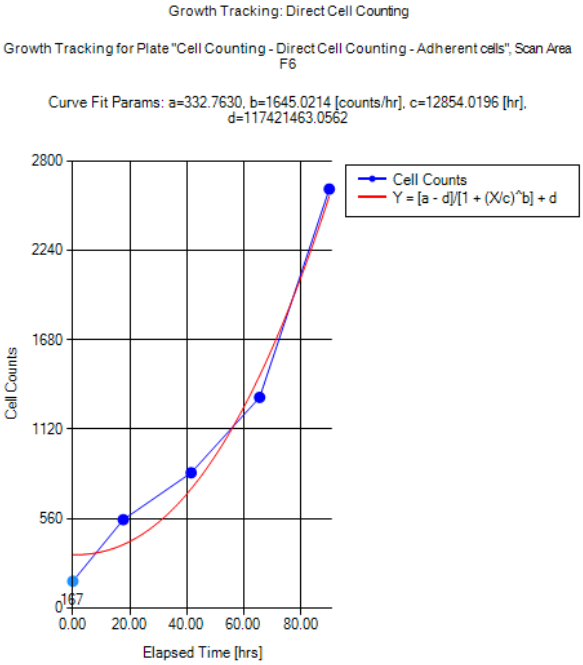


Figure 18 B. Image copied to clipboard



Export an image of the plate grid (Figure 19B) by clicking the double pages in the top right corner of the window (Figure 19 A).

Figure 19. A. Copy Plate Grid to Clipboard

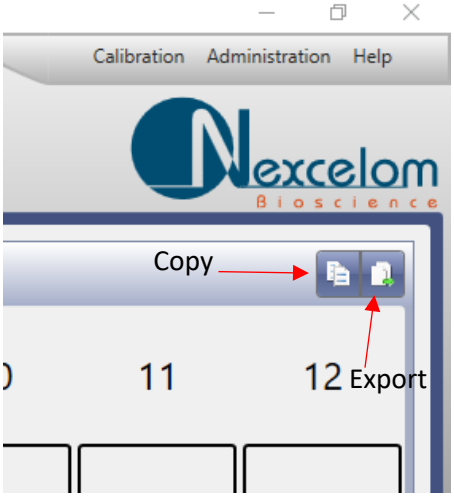
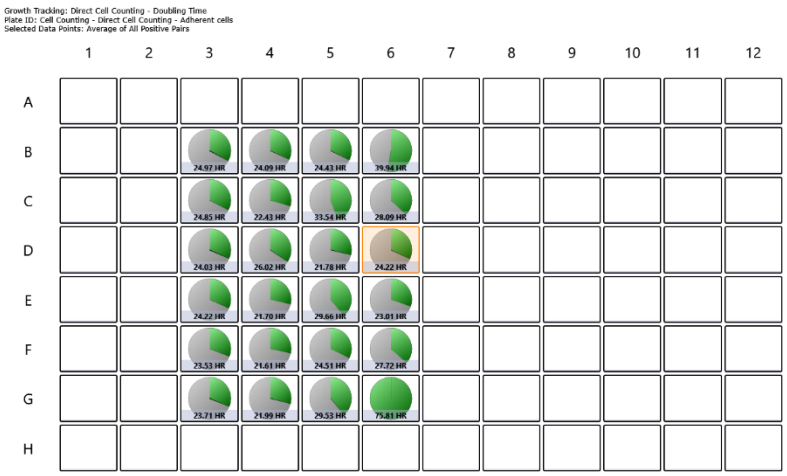


Figure 19 B. Copied plate from Celigo



7. Troubleshooting

This section provides troubleshooting recommendations.

Table 7. 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). 4. Inappropriate gate is applied in Gating screen. • Go to GATE tab and remove gates.
Software identifies debris as cells or confluent areas in brightfield	Often debris have unique properties that can be used to remove them 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	4. Adjust liquid volume level to prevent meniscus-dependent optical effects. • For proper liquid volumes for cell plating, see Table 1. 5. 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. H

Celigo® Cytometer
Cell Secretion
Application Guide

Celigo Software Version 5.2

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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 the secreted protein in the vicinity of each producing cell. Cell bodies and the secreted proteins must also be fluorescently stained. Analysis using the Cell Secretion application provides a variety of intensity- and morphology-related measurements, and can also be used for the 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: support@nexcelom.com
phone: +1 978-327-5340

From Europe:
e-mail: support@nexcelom.co.uk
phone: +44 (0) 131 232 4593

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 the image acquisition parameters. 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 proteins (Figure 1). This guide demonstrates the assay in this configuration, but other combinations of fluorophores and dyes may also be used.

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

Figure 1. Cell Secretion Schematic

(A) *Schematic illustrating both top and side view of a labeled cell and the secreted protein. Cells are labeled with a green fluorescent dye, which can appear yellow due to the mixing of red and green colors. A capture reagent traps the secreted protein around the immediate vicinity of the cell.*

(B) *A 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.*

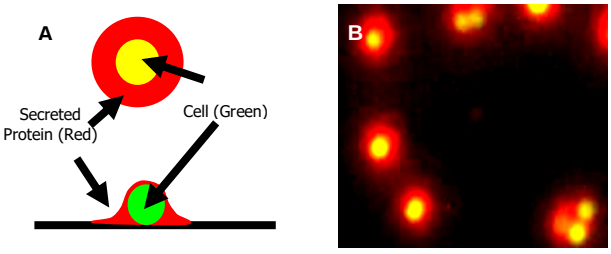


Figure 2. Selecting the Cell Secretion Application

3.2 Image Acquisition Settings

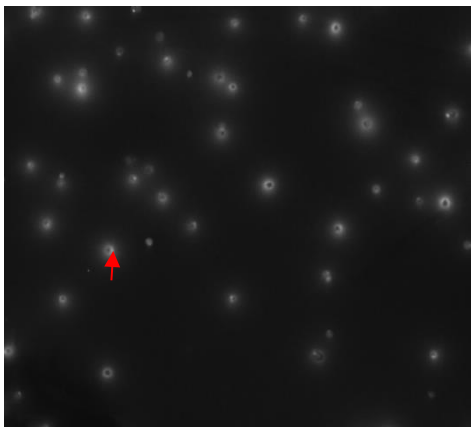
Perform the following steps to select the image acquisition settings.

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 in the 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 the fluorescence channel, e.g., Green 483/536 for a cytoplasmic cell stain or Red 531/629 for a secretion marker.
4. Click **Live** to see a live image.
5. Select Custom for the Type, then determine a desirable exposure and gain settings for the sample being scanned

   Where cells are visible, yet not overexposed, object level pixel intensity should be between 125-175. Avoid over saturation, i.e. overexposure is where pixels intensities are ≥ 255 max camera pixel intensity).
6. If less image resolution is appropriate for acquisition, select **2 µm/pixel in Acquisition Resolution.** For a detailed explanation of image resolution, see the User Guide.
7. Use focus setup to find the focus by selecting **Image Based Auto Focus** or **Hardware Auto Focus** (Hardware Auto Focus is recommended), and then click Register Auto. See the User Guide for more information
8. Switch to the second channel (Secretion) for setup, and repeat steps 2-6.
9. After step 6, select **Find Focus** or manually adjust to find the correct focus, and then select set offset.

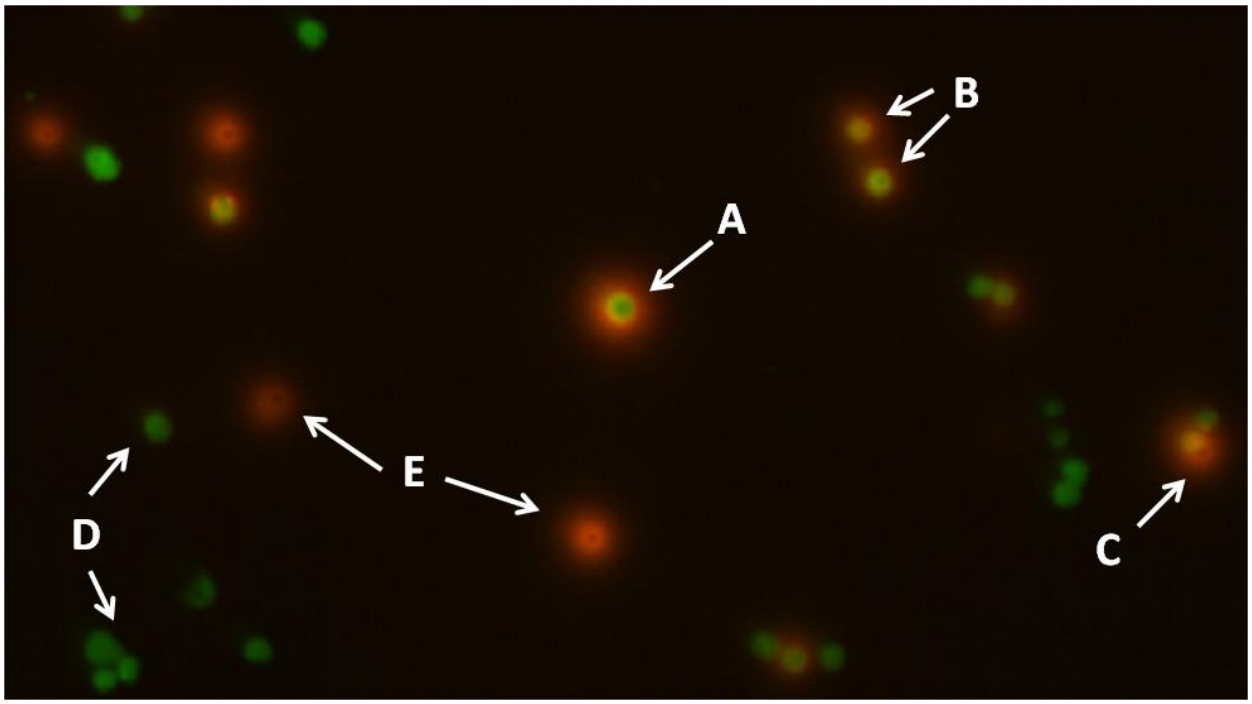
Figure 3. Image Acquisition Settings



Hovering the mouse over the cells will display the Intensity at the bottom of the SCAN tab.

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) *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 of one 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. The recommendation to ensure minimal disturbance of settled cells is to use gentle plate movements, as well as 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) *Significant movement of cells away from their original immediate area of cell secretion indicated by the green cell marker where co-localization is absent from these red secretion rings.*

4. Analyzing Images

This chapter provides information on how to analyze scans from the Cell Secretion: Cell + Secretion application. Perform these tasks in the ANALYZE tab.

4.1 Analysis Settings

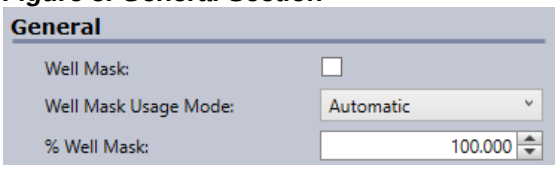
Perform the following steps to select analysis settings. The recommended initial analysis settings for identification and pre-filtering when using the Cell Secretion application are shown in Table 1. The settings typically provide good image segmentation. Alterations should be made as appropriate for 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:

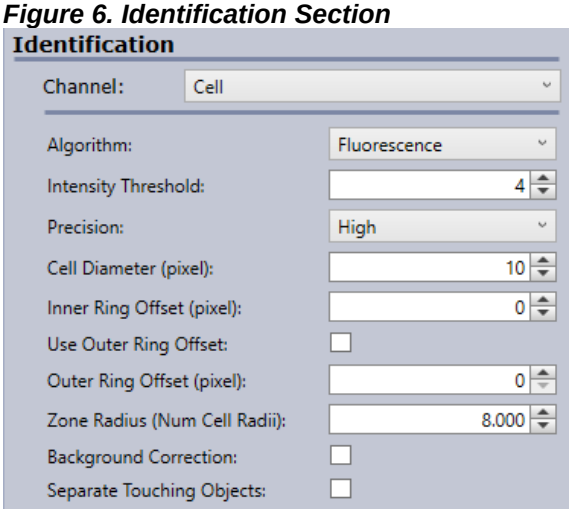
Figure 5. General Section



   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.

2. 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 a more accurate identification of cell clusters. Normal is recommended because Normal is sufficient to provide acceptable results, while High results in a 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.

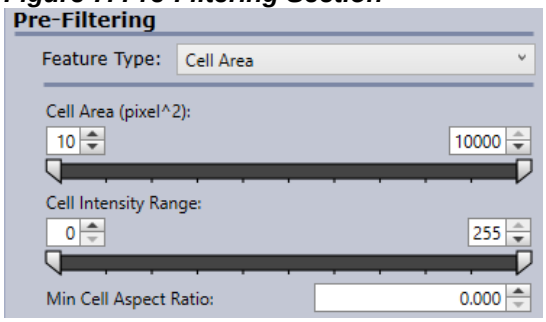
e. Inner Ring Offset, Outer Ring Offset and Zone Radius – These settings are primarily associated with secretion analysis (see section 4.1.2)

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.

3. Pre-Filtering settings are applied to three distinct segmentation areas: Cell Marker, Cell Area and Secretion Area (see Table 1 and Section 4.1.1). In the Pre-Filtering section, make the following selections:

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	Check marked	—
Outer Ring Offset (pixel)	10	—
Zone Radius (number of cell radii)	6	—
Background Correction	Not Check marked	—
Separate Touching Objects	Not Check marked	—

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 8 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 identified by Intensity Threshold, Cell Diameter and Cell Marker Pre-Filtering settings, so as to eliminate any cells that may be undesirably small or low in stain uptake from the analysis (possibly indicative of compromised viability).

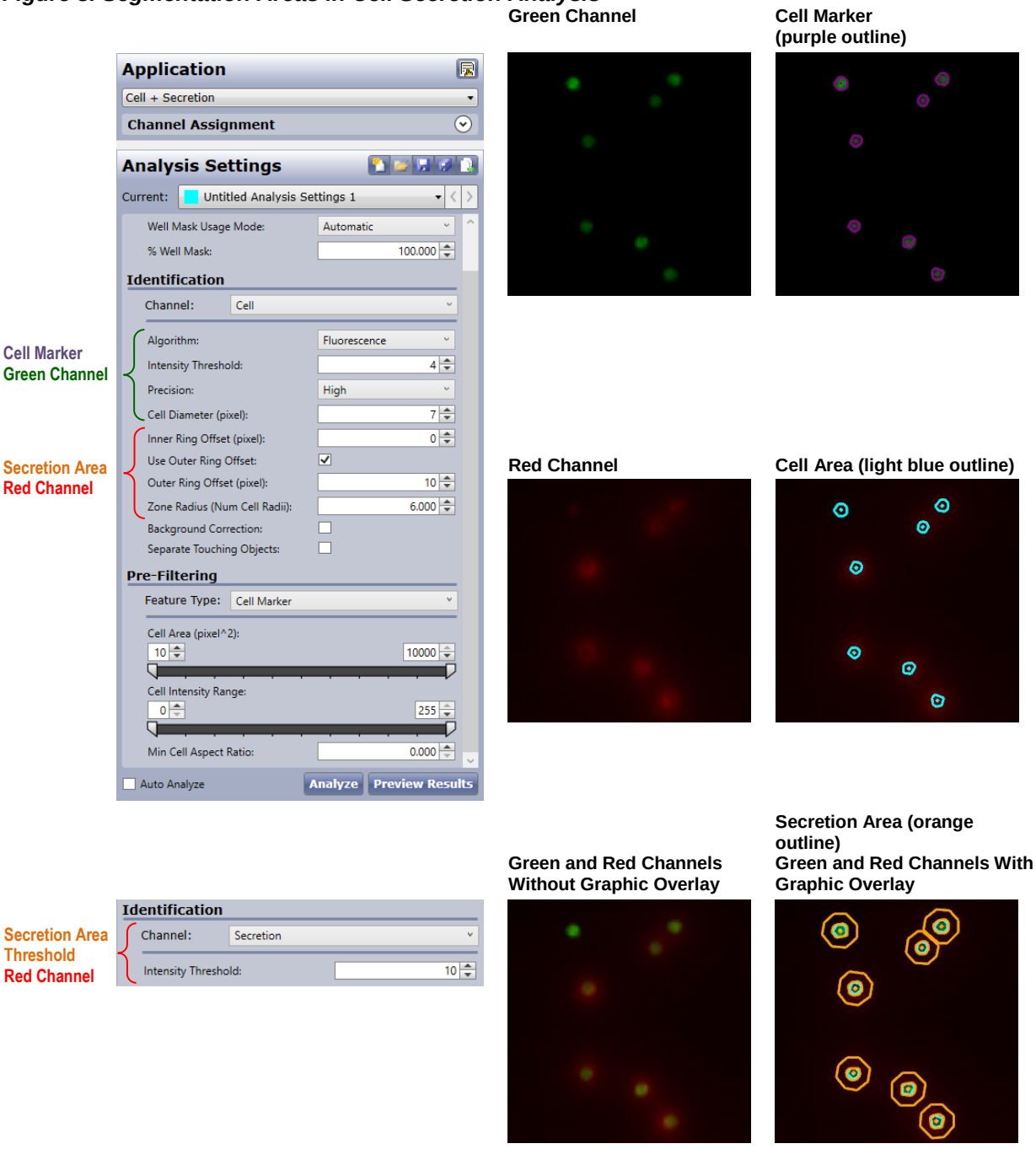
Cell Area refers to an object previously outlined by the Cell Marker segmentation (cell channel), but also displaying co-localized signal in the secretion channel. Segmented cells with both Cell and Secretion channel signal (within identification settings) will appear as light blue outlines; segmented 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 check marked)
- 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 9) 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.

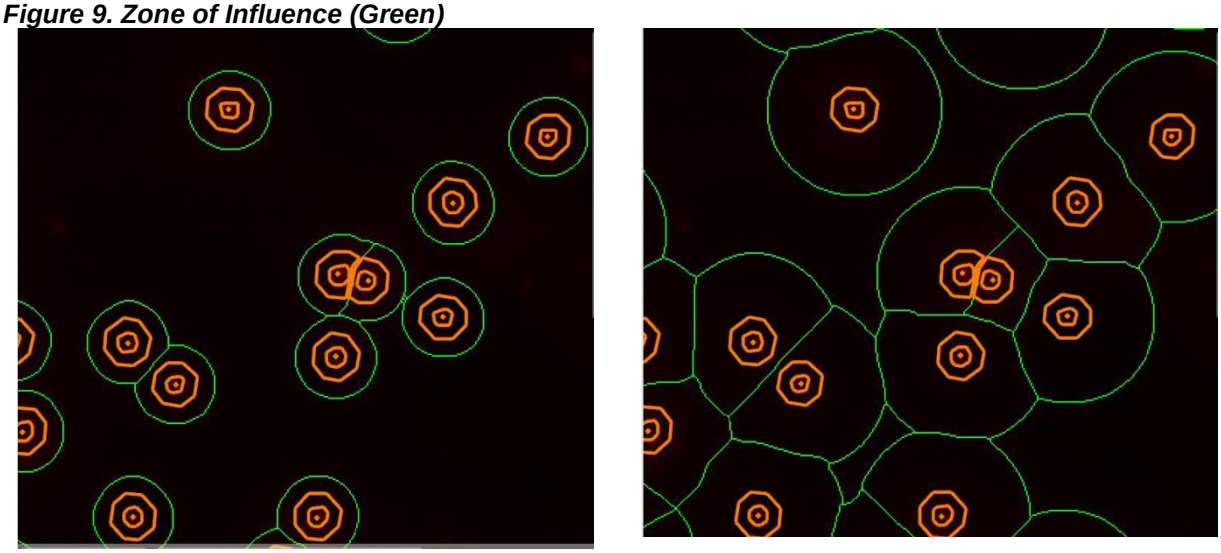
Inner Ring Offset designates how far out (in pixels) from the edge of the Cell Marker to begin the measure of secretion signal. Zero to one pixel is typically sufficient.

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 check marked, 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 10).

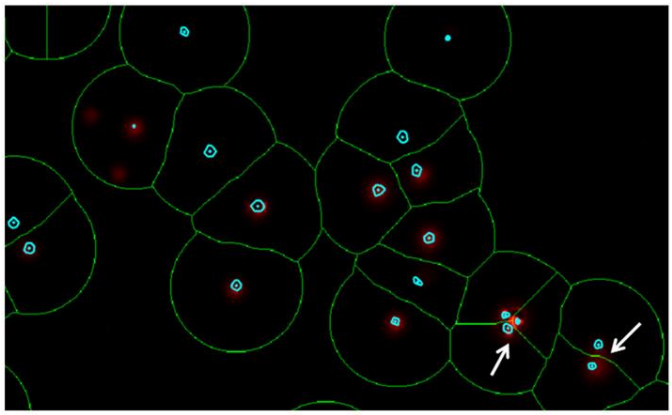
Intensity Threshold (see Figure 11) analysis setting for the Secretion channel designates the lower intensity limit on the secretion signal to be analyzed. Pixels below the Intensity Threshold are excluded from analysis. Low thresholds may result in Secretion Area segmentation that is fragmented in appearance due to inclusion of low intensity pixels (Figure 11 **(A)**); high thresholds will tend to limit analysis to higher intensity pixels (Figure 11 **(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 11 **(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 (Green)

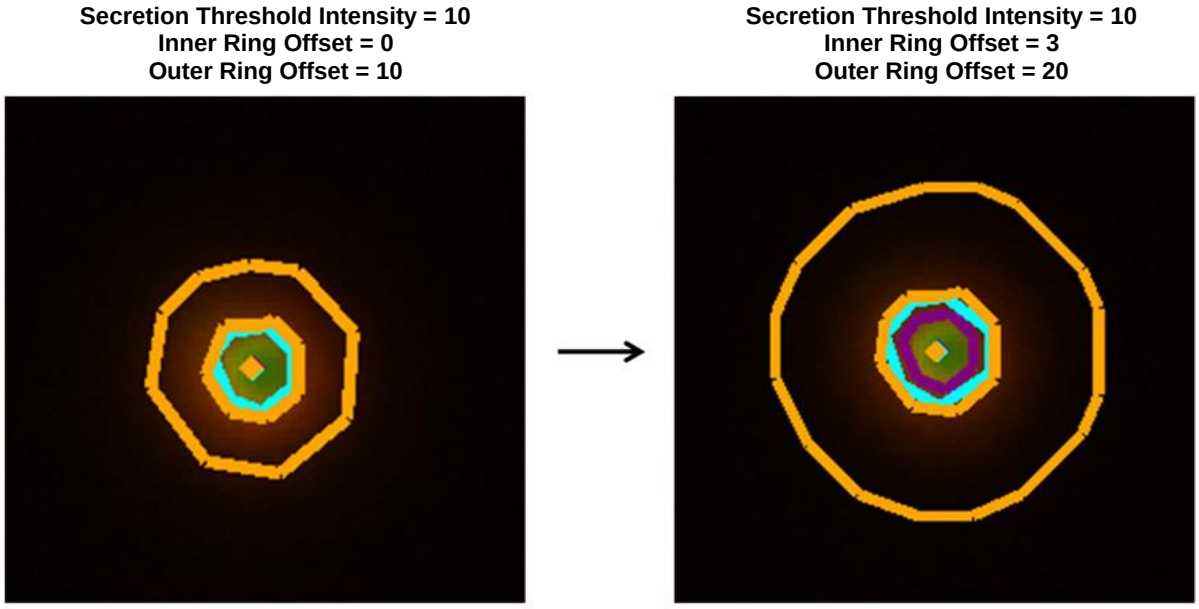


Alterations in Zone Radius affect dimensions of Zone of Influence (outlined in green; detected secretion areas outlined in orange).

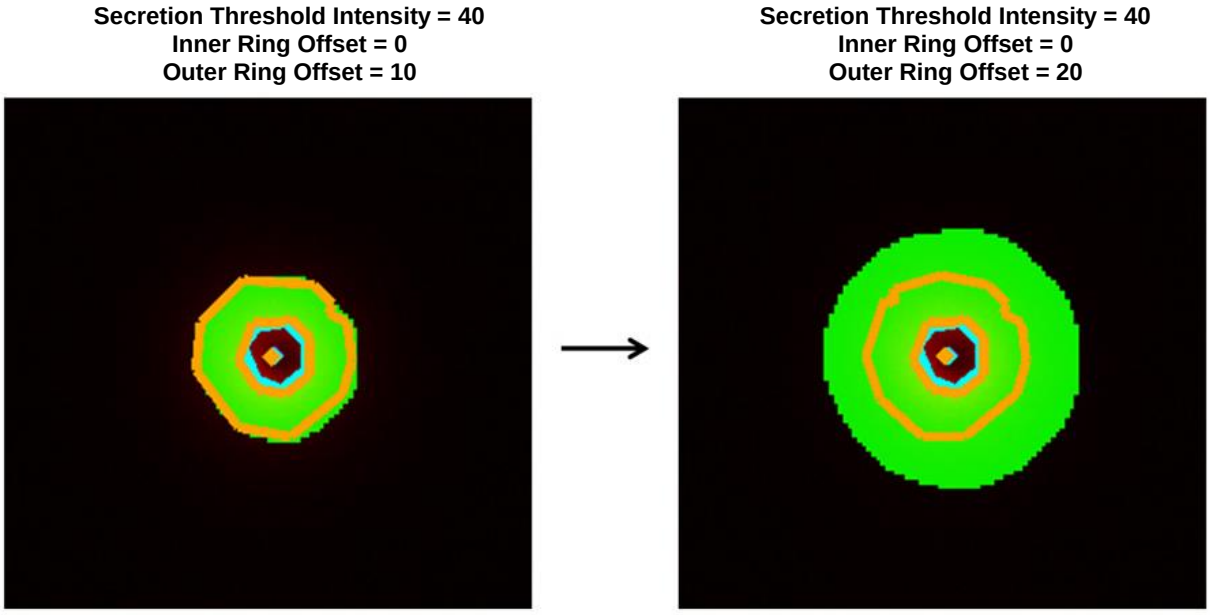


Unlike fixed-dimension Inner and Outer Ring Offsets (see Figure 10), Zones of Influence (outlined in green) can encompass unknown or variable secretion areas. Zones of Influence will also distinguish closely adjacent cells (see white arrows).

Figure 10. Inner and Outer Ring Offsets



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

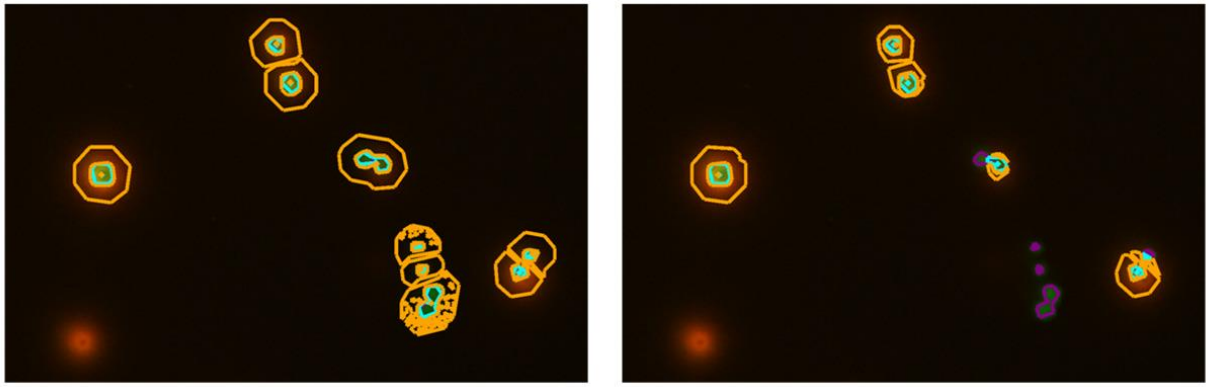


The Cell Marker Secretion Ring Mask (highlighted in green) displays the fixed area bound by the Inner and Outer Ring Offsets. Whereas the Ring Mask can extend past the detected Secretion Area (outlined in orange), the Secretion Area cannot extend past the Ring Mask, as it is bound by it (see section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

Figure 11. Secretion Threshold Intensity

A

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

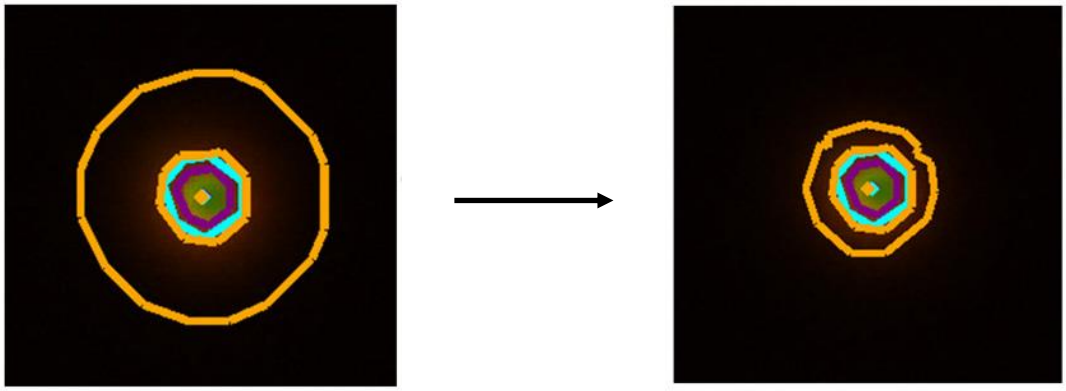


Due to the inclusion of low intensity pixels, a lower Threshold Intensity may result in cells with Secretion Areas (outlined in orange) of segmented appearance.

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. Perform these tasks in the GATE tab.

For optimal analysis using the Cell Secretion application gating should be performed. There are two classes of cells (Total and Gated) which 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

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. 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 Area1: Integrated Intensity2	Sum of all the pixel intensities within the region outside of a Cell Area3 used to measure Secretion Area4 intensities (integrated intensity1 of secreted protein outside of the cell)
Secretion Area1: Mean Intensity	Mean intensity of the region outside of a Cell Area3 used to measure Secretion Area1 intensities (mean intensity of secreted protein outside of the cell)
Secretion Area1: Standard Deviation	Standard deviation of all the individual pixel intensities within the region outside of a Cell Area3, used to measure Secretion Area4 intensities (the standard deviation of secreted protein intensities outside of the cell)
Secretion Area1: Area (μm2)	Area of the region outside of a Cell Area3 used to measure Secretion Area1 intensities (area of the secreted protein outside of the cell)
Secretion Area1: Form Factor	Compactness of the Secretion Area1, 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 of the cell)
Secretion Area1: Smoothness	Evenness of contour of the Secretion Area4, 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 the secreted protein outside of the cell)
Secretion Area1: Aspect Ratio	Ratio of the minor axis to the major axis of the Secretion Area1; a value of 1 is a perfect circle; values less than 1 suggest an oval

Feature	Description
	(elongated) object (roundness of secreted protein area outside of the cell)
Cell Area3: Integrated Intensity2	Sum of all pixel intensities within the cell body displaying signal in the Secretion channel (integrated intensity2 of the secreted protein on the cell body)
Cell Area3: Mean Intensity	Mean intensity of the cell body region in the Secretion channel (mean intensity of the secreted protein on the cell body)
Cell Area3: Standard Deviation	Standard deviation of all individual Secretion channel pixel intensities within the cell body (standard deviation of the secreted protein intensities on the cell body)
Cell Area3: Area (μm2)	Area of the cell body displaying signal in the Secretion channel (area of secreted protein on the cell body)
Cell Area3: Form Factor	Compactness of the Cell Area3, 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 the secreted protein on the cell body)
Cell Area3: Smoothness	Evenness of contour of the Cell Area3, 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 the secreted protein on the cell body)
Cell Area3: Aspect Ratio	Ratio of the minor axis to the major axis of the Cell Area3; 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 Marker4: Integrated Intensity1	Sum of all pixel intensities within the cell body displaying signal in the Cell channel (integrated intensity2 of cytoplasmic dye)
Cell Area Marker4: Mean Intensity	Mean intensity of the cell body region in the Cell channel (mean intensity of cytoplasmic dye)
Secretion Total Area: Area (μm2)	Area of the cell body used to measure Cell Area3 intensities + the region outside of the Cell Area3 used to measure Secretion Area4 intensities (total area of secreted protein on the cell body and outside the cell)
Secretion Total Area: Integrated Intensity2	Sum of all Cell Area3 + Secretion Area4 pixel intensities (total integrated intensity of the secreted protein on the cell body and outside of the cell)
FOOTNOTES: Secretion Area = The region outside of a Cell Area displaying signal in the 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) Integrated Intensity = Sum of all pixel-level intensities within a region Cell Area = The region previously defined by the Cell Marker, but also displaying co-localized signal in the Secretion Channel (secreted protein on the cell body) Cell Area Marker = Region defined by whole cell cytoplasmic stain in the Cell Channel (cell body)	

1. 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 the 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 of the 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 of the cell)
Secretion Area[1]: Standard Deviation	Standard deviation of all the individual pixel intensities within the region outside of a Cell Area[3], used to measure Secretion Area[4] intensities (the standard deviation of secreted protein intensities outside of the 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 the secreted protein outside of the 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 of the 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 the secreted protein outside of the 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 of the 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 the secreted protein on the cell body)

Feature	Description
Cell Area[3]: Mean Intensity	Mean intensity of the cell body region in the Secretion channel (mean intensity of the secreted protein on the cell body)
Cell Area[3]: Standard Deviation	Standard deviation of all individual Secretion channel pixel intensities within the cell body (standard deviation of the secreted protein intensities on the cell body)
Cell Area[3]: Area (μm²)	Area of the cell body displaying signal in the Secretion channel (area of secreted protein on the 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 the secreted protein on the 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 the secreted protein on the 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 (μm²)	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 the secreted protein on the cell body and outside of the cell)

FOOTNOTES**:**

1) Secretion Area = The region outside of a Cell Area displaying signal in the 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 = The region previously defined by the Cell Marker, but also displaying co-localized signal in the Secretion Channel (secreted protein on the cell body)
4) Cell Area Marker = Region defined by whole cell cytoplasmic stain in the Cell Channel (cell body)

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

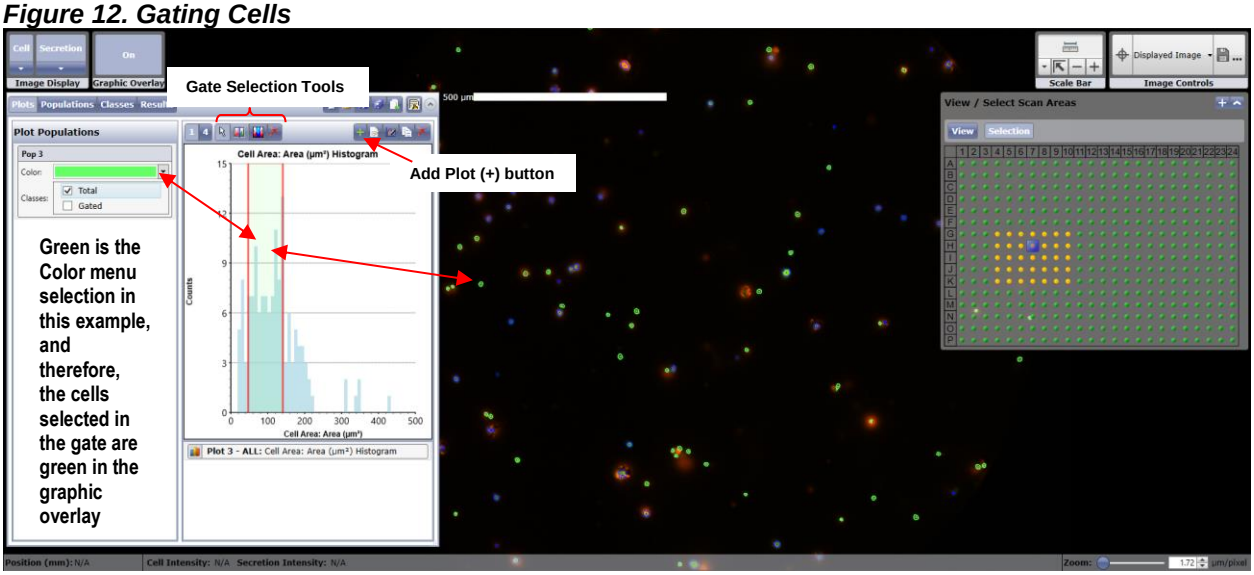
When creating the plot, the system, by default, assigns the class called Total to the population for the entire well, also known as the ALL population.

2. Create a gate on the plot, using the gate selection tools (Figure12).

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 Populations pane corresponds to the Color menu selection (Figure12).

Figure 12. Gating Cells



3. Repeat steps 0 and 2 as needed to refine the population requiring analysis.
4. Assign the Total class to the population as follows:
   d. Click the gate.
   e. In the Plots, Populations, or Classes view, check mark the Total class.

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

   Pick a source population: It is common to select the Total population that was assigned in step 4.

   Pick a plot type: Scatter plot

   Parameter 1: Cell Area Marker: Mean Intensity

   Parameter 2: Secretion Total Area: Integrated Intensity

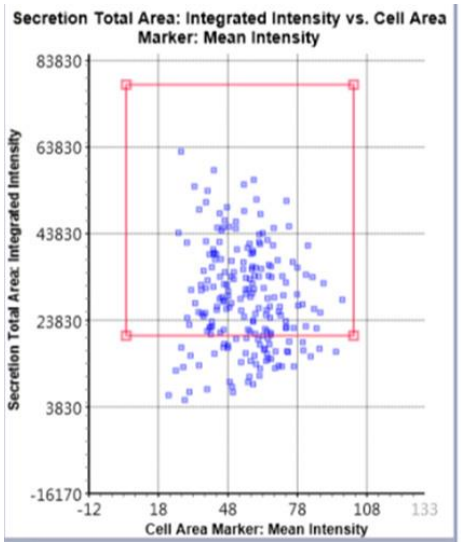
   For more information on creating plots, see User Guide section Creating a Plot.
6. Create a gate on the scatter plot, using the gate selection tools.

   For details, see User Guide section Creating a Gate.

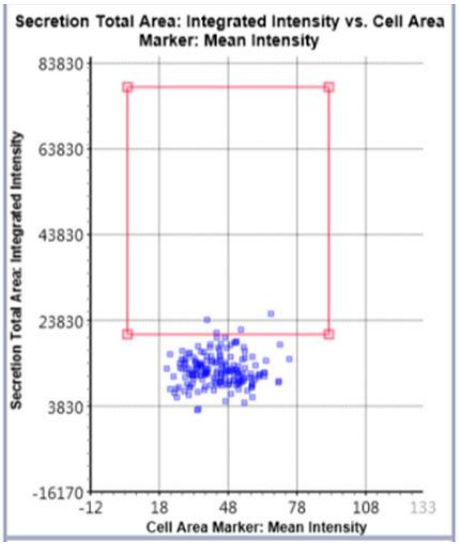
   In the Graphic Overlay display, the color displayed for the plot population selected in the Plot corresponds to the Color menu selection.
7. On the scatter plot (Secretion Total Area: Integrated Intensity vs. Cell Area Marker: Mean Intensity) (Figure 63), drag the gate so that it captures only the cells that are positive for Cell Secretion. Figure 63 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 63. Scatter Plot

Positive Control Wells

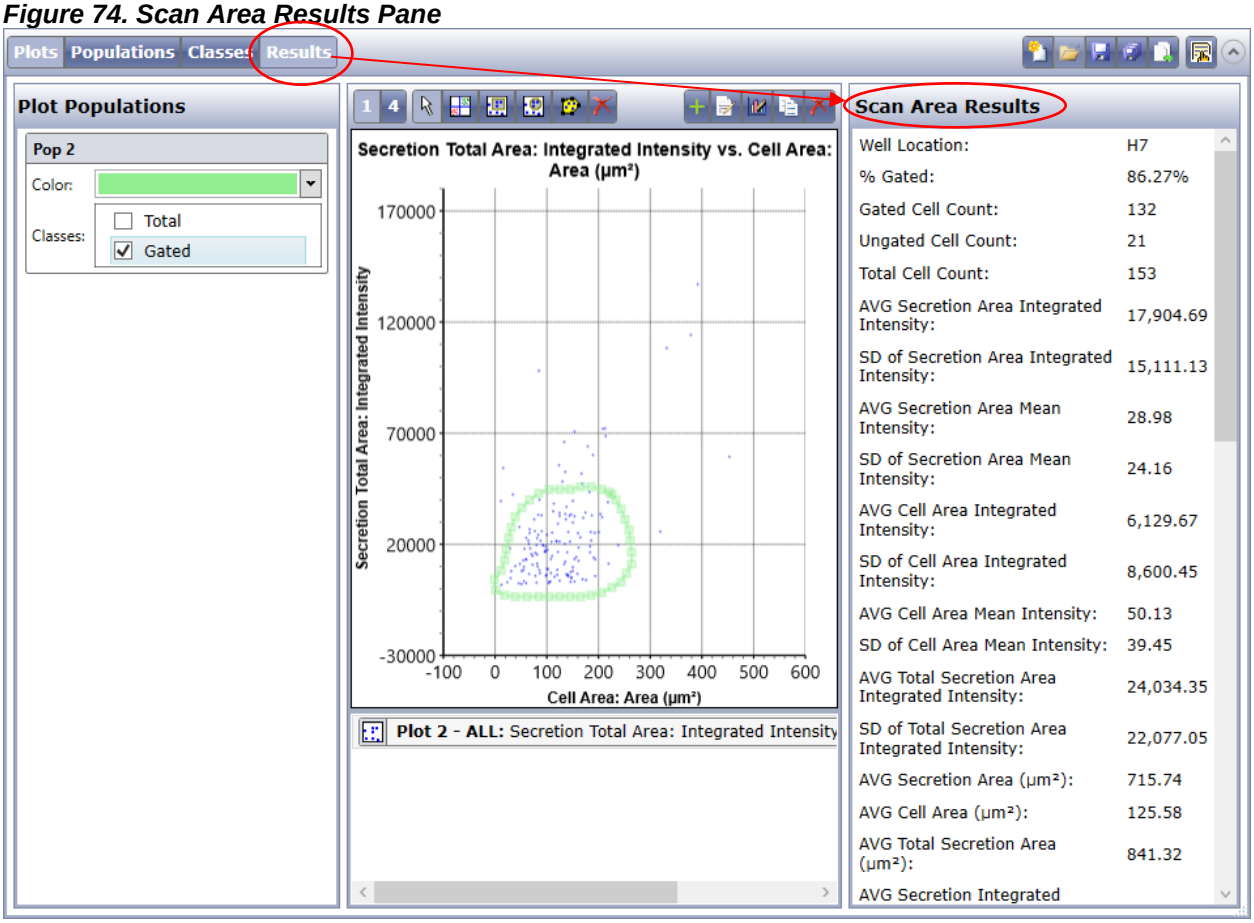


Negative Control Wells



To capture only the positive wells, it is helpful to review the Scan Area Results pane (Figure 74) 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 74. 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 check marked.

6. Viewing Results

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

Table 2. 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	**Description**
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
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

Feature	Description
FEATURE REPORTED FOR EACH CLASS	
Average Secretion Area[4] (μm²)	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] (μm²)	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] (μm²)	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 3. 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. F

Celigo® Cytometer
Cell Viability
Application Guide

Celigo Software Version 5.2

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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 which 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 cell DNA. Multi-channel images are acquired and analyzed providing live, dead and total cell counts, as well as 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 are as 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: support@nexcelom.com
phone: +1 978-327-5340

From Europe:
e-mail: support@nexcelom.co.uk
phone: +44 (0) 161 232 4593

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
- A plate suitable for use with Celigo (Table 1. has suggestions)

Table 1. Recommended Plating Volumes

Plate Type	Vendor	Cat#	Recommended Final Volume (µl) for Single Cell Analysis	
			Brightfield	Fluorescence
6W	Corning	3516	2500	ND[1]
6W	Corning	3471	2500	ND[1]
12W	Corning	3512	1000	ND[1]
24W	Corning	3524	≥500	ND[1]
96W	Greiner	655090	≥200	≥100
96W half area	Greiner	675090	50	50
384W low vol	Corning	3542	20	20
384W high vol	Corning	3712	≥40	≥40
384W high vol	Greiner	781091	≥40	≥40
1536W	Corning	3838	8	8
1536W	Greiner	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 choosing the appropriate Cell Viability application and setting the image acquisition parameters. 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 Application dropdown list, select one of the four Cell Viability applications (Figure 1 and Table 2) based on the dyes used in the assay, as follows:

Figure 1. Selecting a Cell Viability Application

Table 2. 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 the selection of 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 3.

To select image acquisition settings

1. Choose a well for setup by clicking **Navigation** in the Navigate/Select Scan Areas window**,** selecting a representative well, and then selecting Live or Snap.

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

2. In the Channel field under Application, select the first channel to set up (**Live**, **Dead**, or **Total**).

3. For the selected channel, make the following selections:
   a. In Type, select **Custom Channel**.
   b. In Illumination, select the appropriate illumination for the dye (e.g., **Blue 377/447** to visualize the Hoechst signal).
   c. Click **Live** to see a live image.
   d. Use Focus Setup to achieve a clear image of the cells.
   e. Select whether Auto exposure provides optimal exposure for the sample (an object pixel intensity of 125-175) where cells are visible, yet not overexposed (i.e., saturated: pixels intensities >254).
      - Auto exposure: The system will attempt to determine the optimal exposure time and gain setting.
      - Custom: User manually establishes the optimal exposure time and gain by adjustment of Exposure Time (µs). When doing so, adjust the exposure so the majority of cells aren't overexposed.
   f. If less image resolution is appropriate for acquisition, select **2 µm/pixel** in Acquisition Resolution. For a detailed explanation of image resolution, see the User Guide.
   g. Set up autofocus (see the User Guide).
      - Typically, it is recommended to select **Hardware Auto Focus** (because it provides maximum speed) and click **Register Auto**. For manual registration or using a focus offset, see the User Guide.

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

5. For the selected Channel, make the following selections:
   a. In Type, select **Custom Channel**.
   b. In Illumination, select the appropriate illumination for the dye (e.g., **Green 483/536** to visualize the Calcein AM signal, **Red 531/629** 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 3. 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
Illumination	Green 483/536	Red 531/629	Blue 377/447
Acquisition Resolution	1 µm/pixel or 2 µm/pixel	1 µm/pixel or 2 µm/pixel	1 µm/pixel or 2 µm/pixel
Exposure Time (µs)*	6,000-15,000*	10,000-25,000*	50,000-100,000*
Gain	0	0	0
Motion Control and Focus	See User Guide	See User Guide	See User Guide

*For all fluorescent channels, the object pixel intensity should be between 125 and 175 (targeting 150). See the User Guide for more information on appropriate exposure time.

Figure 2a shows an example of a total channel settings and Figure 2b shows the cells at an appropriate object pixel intensity.

Figure 2a. Total Channel Settings

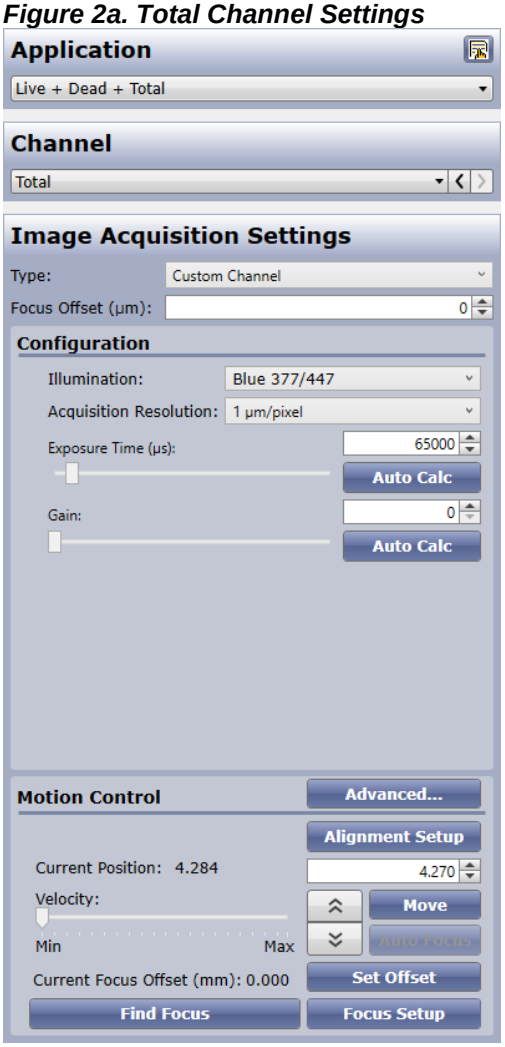


Figure 2b. Cells at an appropriate pixel intensity

Hovering the mouse over the cells will generate a pixel intensity to help determine the correct exposure for each fluorescent channel

4. Analyzing Images

This chapter describes how to set up analysis for the Cell Viability application. 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 objects identified in the Total channel are included in the analysis. This helps eliminate fluorescent objects that are not cells from the analysis.**

4.1 Analysis Settings

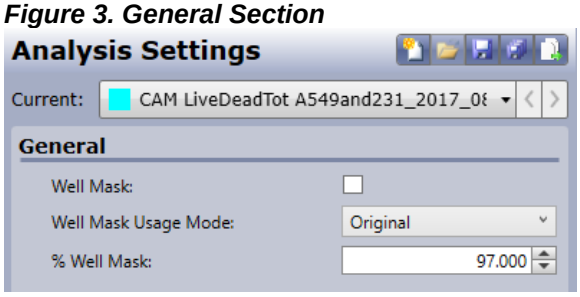
Perform the following steps to select the optimal analysis settings. The recommended initial analysis settings for identification and pre-filtering when using the Cell Viability application are shown in Table 4. The settings typically provide good image segmentation.

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. Otherwise, proceed to step 2.
2. In the General section (Figure 3), make the following selections:

Figure 3. General Section



a. Well Mask – Check mark as needed

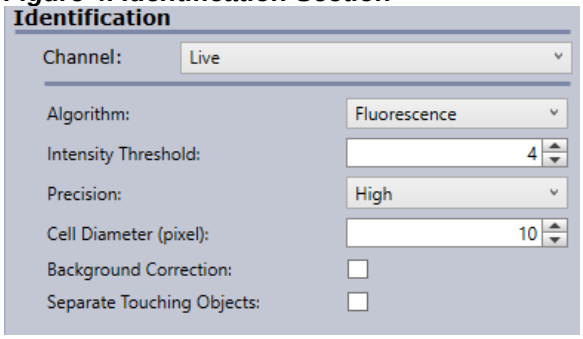
b. Well Mask Usage Mode – Selected as needed.

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

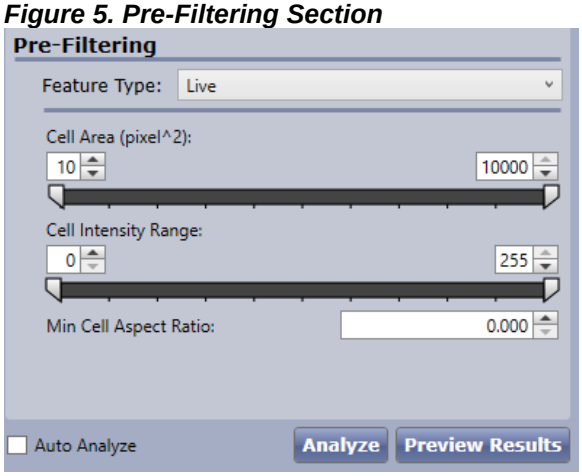


a. Channel – Select one channel to be analyzed.
b. Algorithm – Select **Fluorescence**.
c. Intensity Threshold – Enter the optimal intensity threshold.

   The intensity threshold is the level of intensity that separates the background from the cells. With an appropriate threshold set, background pixels fall below, and the pixels inside cells are above the threshold. Any pixels below the threshold are not considered in the cell counting 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 the cells that are to be analyzed. At full resolution, the Celigo provides 1 µm/pixel. For a detailed explanation on determining cell diameter, see the User Guide.
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 – Automatically updates with the Channel selected in the Identification settings, but can be changed if necessary.

b. Cell Area Range – Enter an appropriate range.

c. Cell Intensity Range – Alter 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 Cell Aspect Ratio – Alter if necessary.

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

5. Repeat this section for each remaining channel.

Table 4. 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)	10	8	10
Background Correction	Not Check marked	Not Check marked	Not Check marked
Separate Touching Objects	Not Check marked	Check marked	Check marked
PRE-FILTERING			
Cell Area (pixel ^2) Range	(35-60) - 1000	(30-50) - 1000	(30-60) - 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 pseudo colored 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

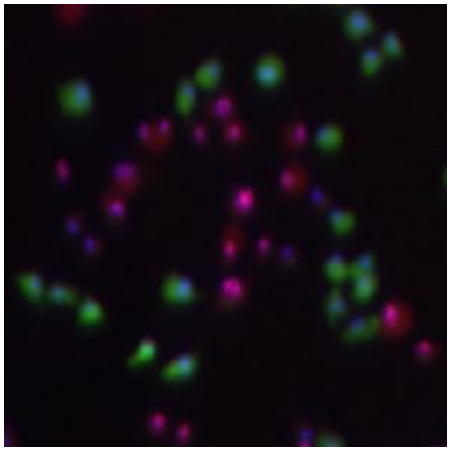
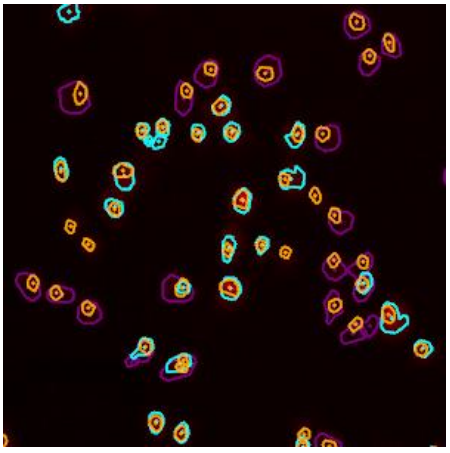


Figure 7. Corresponding Target Overlay



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 5 appear below the Display Options section in the Scan Information pane.

Table 5. 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
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	√	√		√
% Well Sampled	Percent of well surface processed	√	√	√	√

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 6. 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 bleed through 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. F

Celigo® Cytometer Embryoid Body Application Guide

Celigo Software Version 5.2

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1. About this Guide

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

1.1 Introduction

The 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 Embryoid Body 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: support@nexcelom.com
  phone:+1 978-327-5340
- From Europe:
  e-mail: support@nexcelom.co.uk
  phone: +44 (0) 161 232 4593

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 Embryoid Body: Embryoid Body application and setting the image acquisition parameters. Perform these tasks in the SCAN tab.

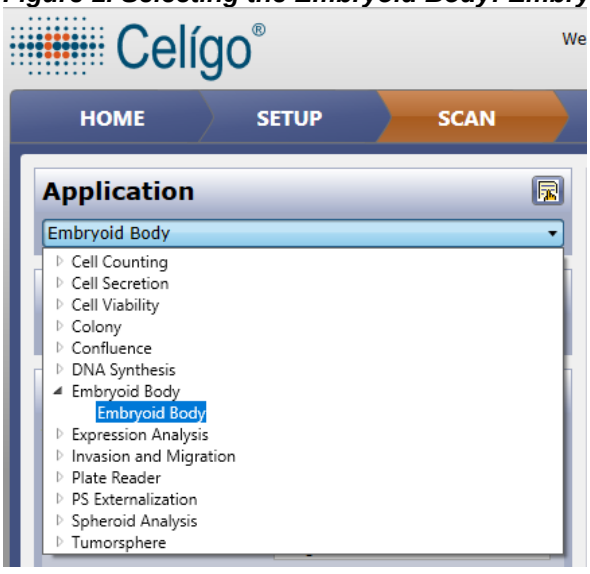
3.1 Embryoid Body Application

Perform the following step to select the Embryoid Body: Embryoid Body application:

To select the Embryoid Body Application

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

Figure 1. Selecting the Embryoid Body: Embryoid Body Application



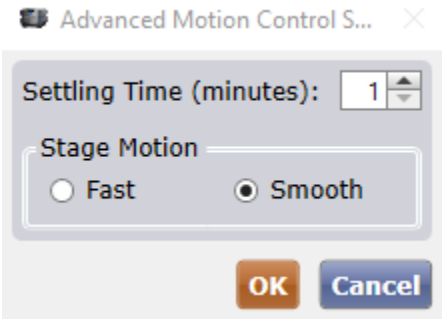
3.2 Acquisition Settings

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 **Auto Exposure/Gain Channel** and click **Apply** in the configuration section of the panel (Recommended). The system will use the preset settings shown in Table 1.
   - In Type, select **Custom Channel** and then do one of the following:
     - Click **Auto Calc**: The system will attempt to determine the optimal exposure time and gain setting for the brightfield illumination.
     - Manually set the optimal exposure time and gain by making an entry in the Exposure Time and Gain fields.
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, a 1-minute Settling Time and Smooth Stage Motion are used in this application.

Figure 2. Recommended Motion Control Settings



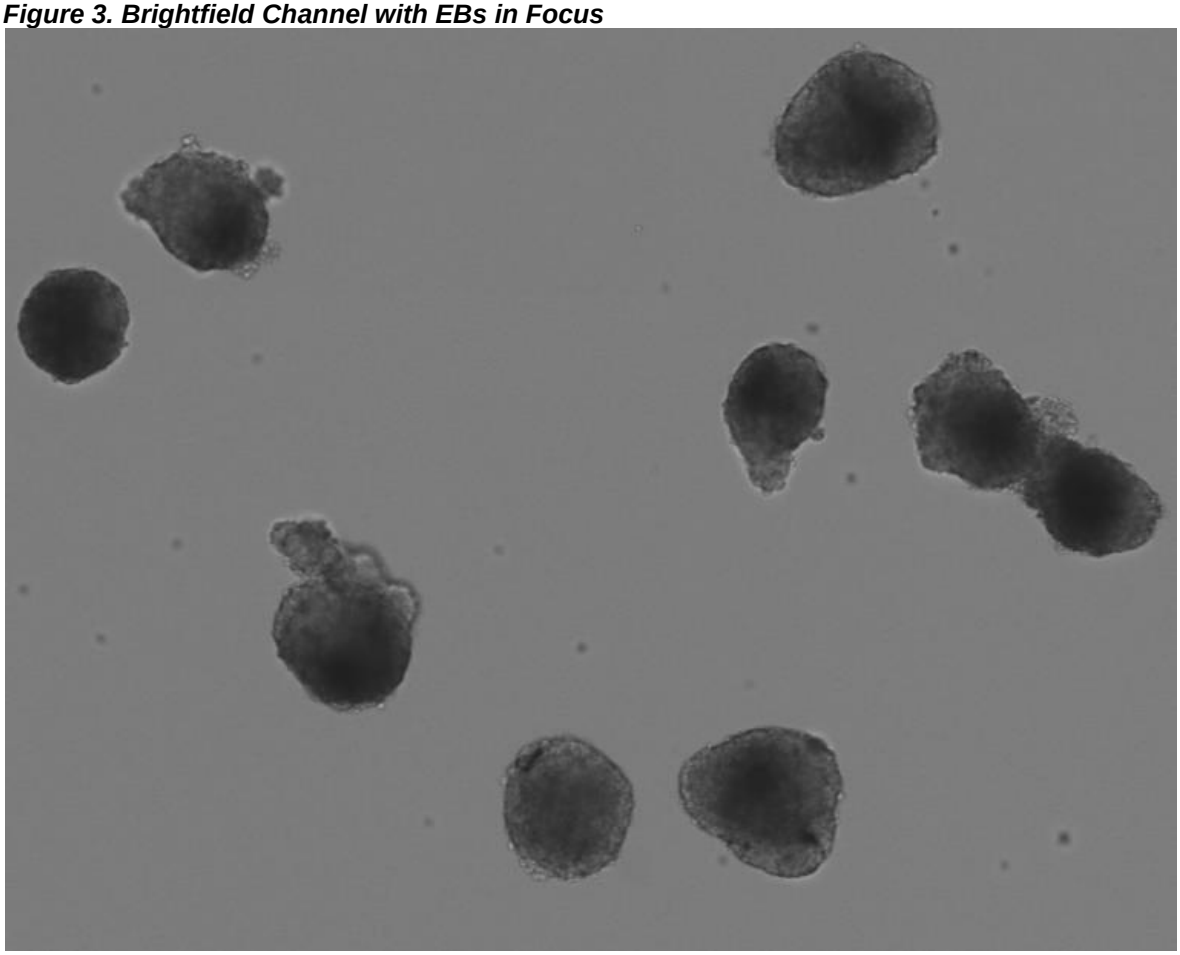
6. Set up Focus. Click the up/down buttons in the Focus section until a crisp focus at the edge of the EBs is visible. Repeat using several EBs. Click **Focus Setup** to register this position with hardware auto focus by then clicking **Manual Register**.

Table 1. Recommended Initial Settings for Image Acquisition

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

Figure 3 shows an example of EBs for the brightfield channel in an appropriate focus position. This figure demonstrates the 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 Embryoid Body application. 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 Embryoid Body application are shown in Table 2. The settings typically provide good image segmentation.

Important guidelines

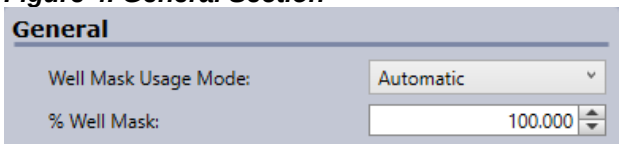
While selecting analysis settings, use the following important guidelines:

- 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 and have unclear (fuzzy) edges 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:

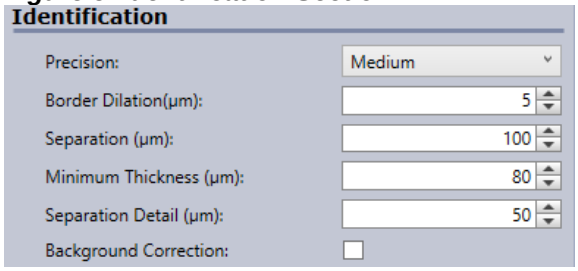
Figure 4. General Section



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

3. In the Identification section (**Error! Reference source not found.**), make the f ollowing selections:

Figure 5. Identification Section



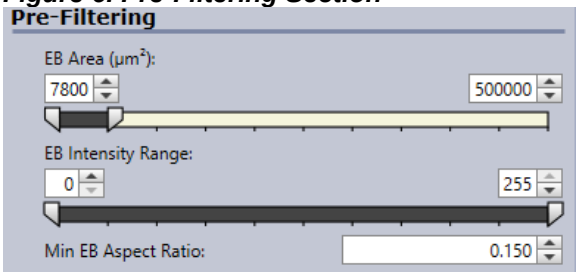
a. Precision – Select the desired level of precision of separation of touching EBs:
   - None – No separation.
   - Low – Minimal separation.
   - Medium – Medium separation.
   - High – Most precise separation.

     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 the EBs to be analyzed.
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 the 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 the amount of EB separation when EBs are touching/fused. Lower value: Less separation of touching/fused EBs. Higher value: Smoothens 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	Uncheck marked	Check marked or Uncheck marked	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 the 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 Embryoid Body: Embryoid Body 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

Precision Low **Precision Medium** **Precision High**

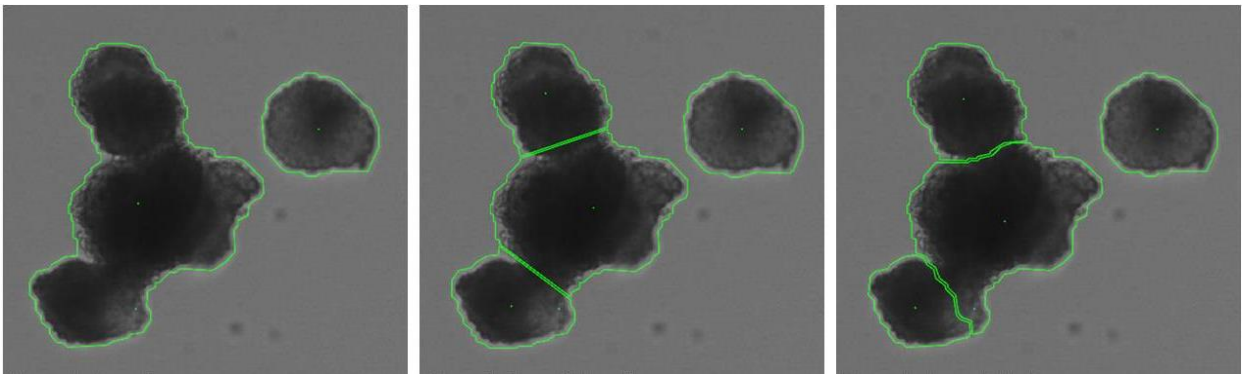


Figure 8 show examples of display results when adjusting border dilation.

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

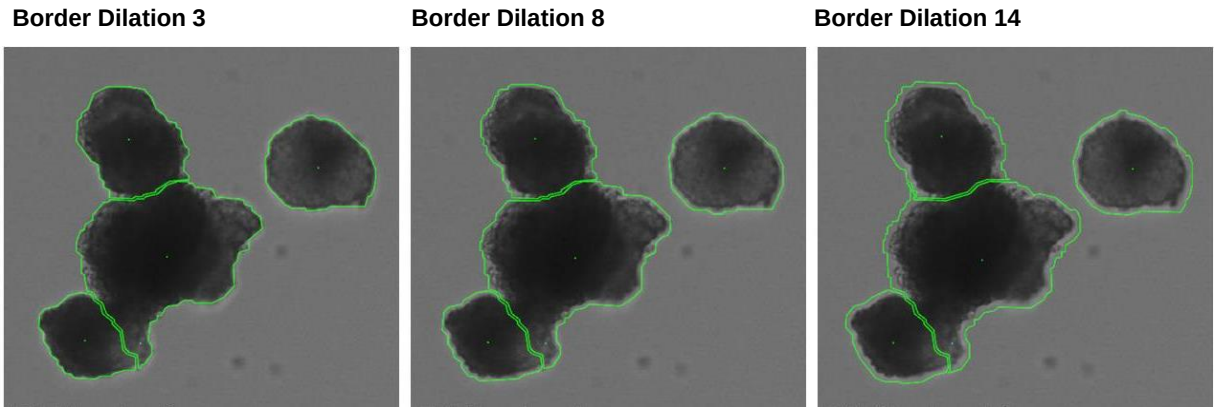


Figure 9 shows an example of display results when adjusting separation.

Figure 9. EBs with Corresponding Target Overlay – Separation Adjustments

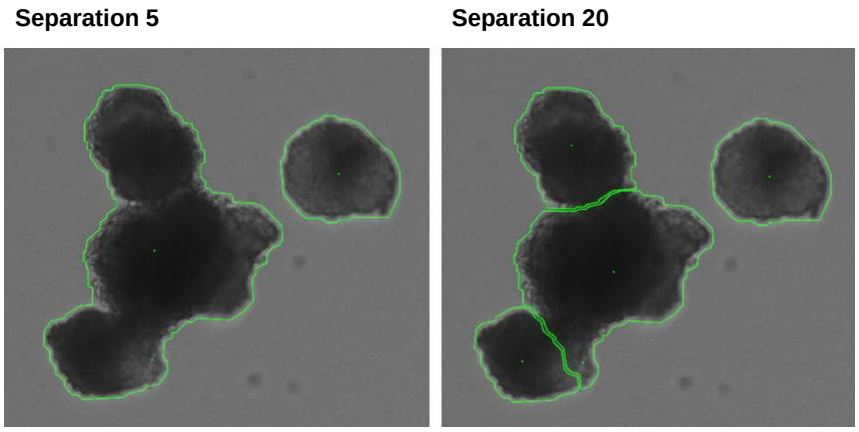


Figure 10 shows an example of display results when adjusting separation detail.

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

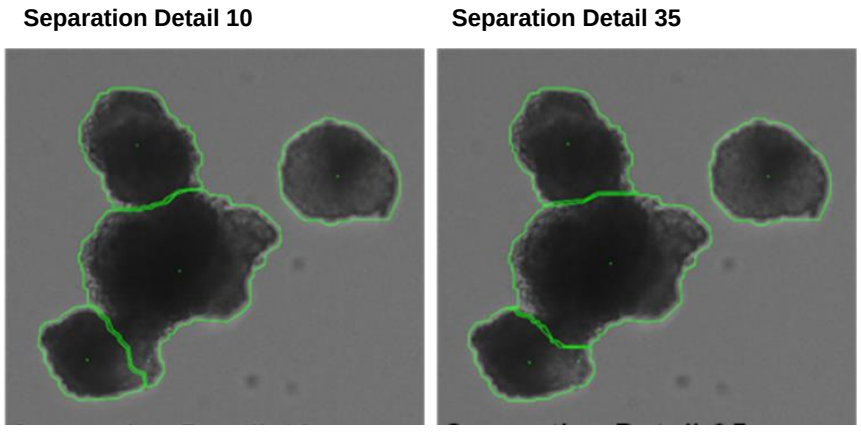


Figure 11 shows an example of display results, with segmentation of small EBs, when adjusting EB area.

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

EB Area 7800 **EB Area 2000**

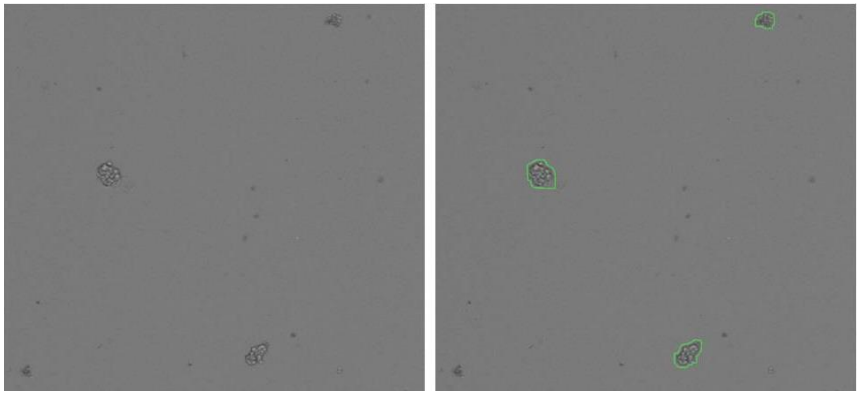


Figure 12 shows an example of display results, with segmentation of single cell debris, when adjusting EB intensity range.

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

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

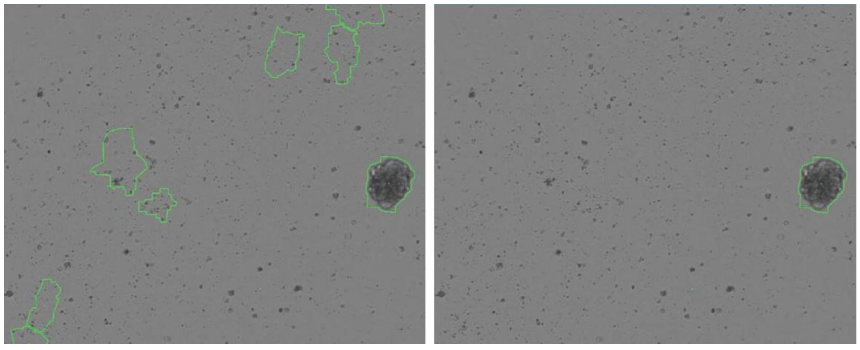
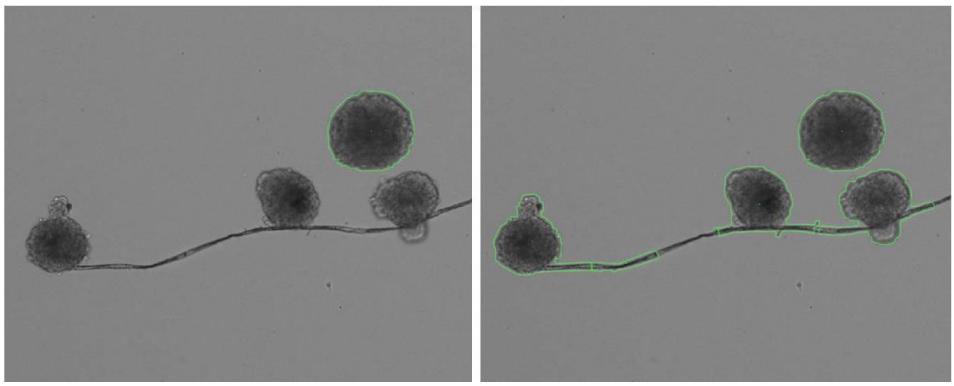


Figure 13 shows an example of display results, with segmentation of debris, when adjusting minimum EB aspect ratio.

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. Perform this task in the GATE tab.

The Embryoid Body: Embryoid Body application relies on the identification of EBs and removal of debris using the EB area, EB intensity, and minimum EB aspect ratio from the ANALYZE tab.

5.1 Working with Gates

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

- If choosing to 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; the assignment of any additional classes to populations is not possible.
- Make sure that a Classes checkbox has been selected to assign a class to the Total population. Check marking 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 check mark would be the same as not using the GATING tab; all data would be reported from the ANALYZE 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 possible 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 Embryoid Body: 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 creating a plot, 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

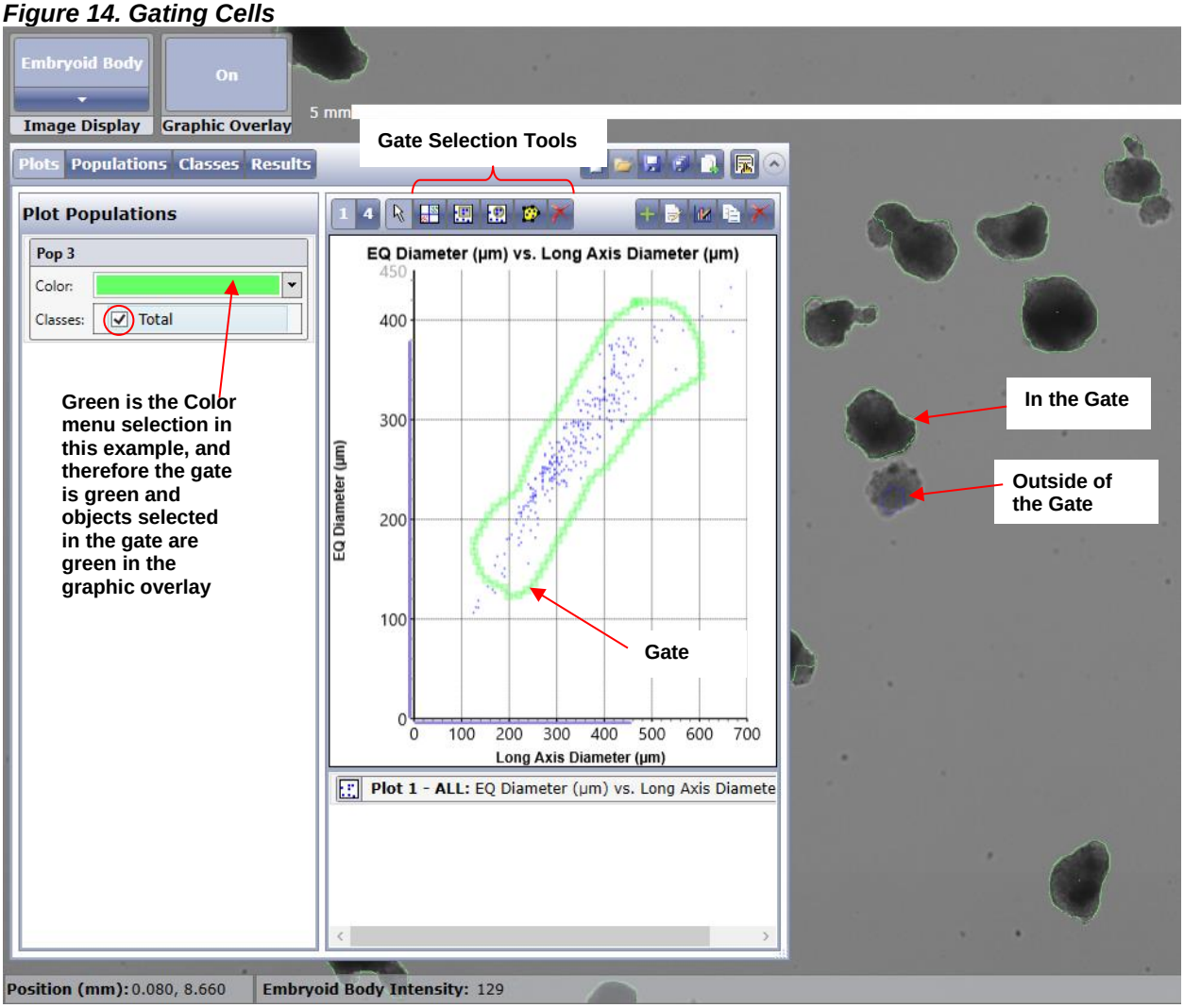
Feature	Definition
X Position (µm)	Location of a cell along the horizontal axis of the well: left (-µm) or right (+µm) of the center (origin of the well).
Y Position (µm)	Location of a cell along the vertical axis of the well: below (-µm) or above (+µm) of the center (origin of the well).
Distance to 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 desired population.
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 check marked.

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

   In this application, assign only the Total class to populations; assigning any additional classes to populations is not possible.

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. Embryoid Body: Embryoid Body 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.	√	√	√	

Parameter	Description	Ave	St Dev	CV	Min/ Max
% 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 non-representative 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 that are 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.

Issue	Recommended Action
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. F

Celigo® Cytometer
Colony: Single Colony Verification Application Guide

Celigo Software Version 5.2

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1. About this Guide

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

1.1 Introduction

The Colony: 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: 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: support@nexcelom.com
  phone: +1 978-327-5340
- From Europe:
  e-mail: support@nexcelom.co.uk
  phone: +44 (0) 161 232 4593

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 high vol	Greiner	781091	40 – 60 µL
384W high vol	Corning	3712	40 – 60 µL
384W low vol	Corning	3542	20 µL
96W	Greiner	655090	≥ 200 µL
24W	Corning	3524	≥ 500 µL
6W	Corning	3516	2.5 mL
6W	Corning	3471	2.5 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: Single Colony Verification application and setting the image acquisition parameters. Perform these tasks in the SCAN tab.

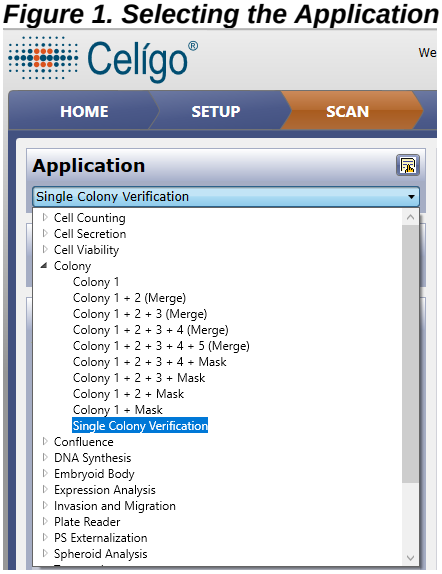
3.1 Colony: Single Colony Verification Application

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

To select the application

In the Application drop down menu, select **Colony: Single Colony Verification** (Figure 1).

Figure 1. Selecting the 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 decreased image resolution, 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. One of the image planes is the Dark Focus image plane in which objects appear dark compared to surrounding background regions. The other image plane is the Bright Focus image plane, which is a higher focal plane in which the cell or object acts as a lens and then 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

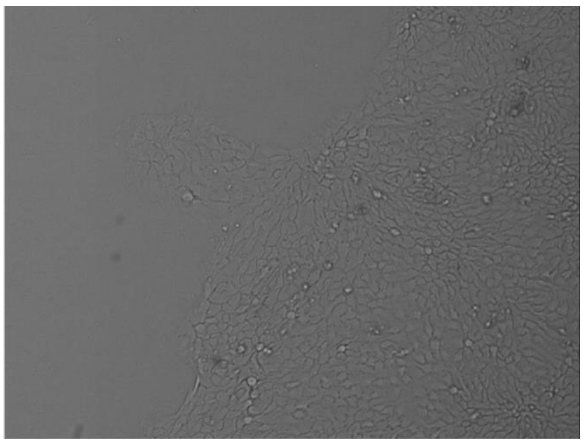
1. Choose a well for setup using the plate map by selecting **Navigation** and a representative well.
2. View a well by selecting **Live** in the Camera Controls field.
3. Adjust the focus until the cells have a large, bright center. For examples of proper focus using the Bright Focus and Dark Focus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

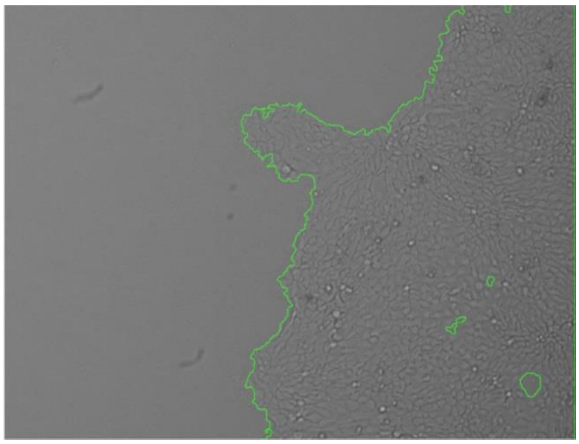
4. Select **Focus Setup** and select **Hardware Auto Focus** in the Focus Type field. Complete the setup by registering the autofocus.
    - Image Based Auto Focus is *not* recommended for brightfield illumination if the wells contain very few cells.
    - Register Manual will register the current focal plane for Hardware Auto Focus. The cell type and its morphology (i.e., how flat a cell adheres to the plate surface), will 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

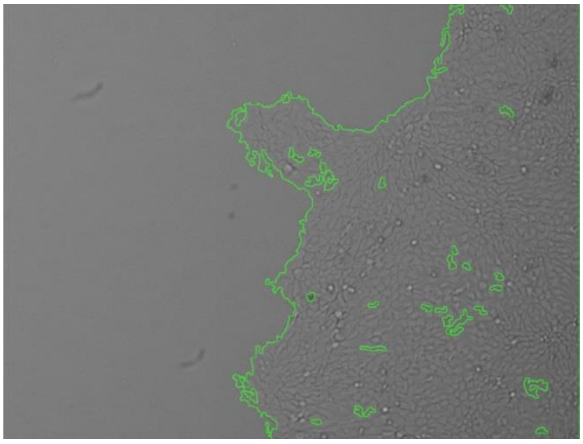
Colony Using Bright Focus Target Focal Plane



Optimal Identification of a Colony Using:
- **Bright Focus Target Focal Plane**
- **Texture Algorithm**

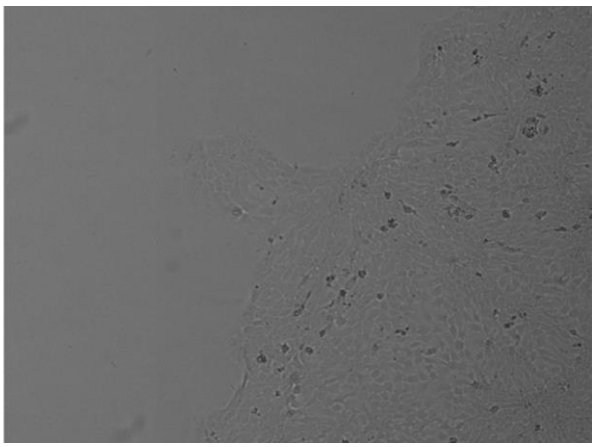


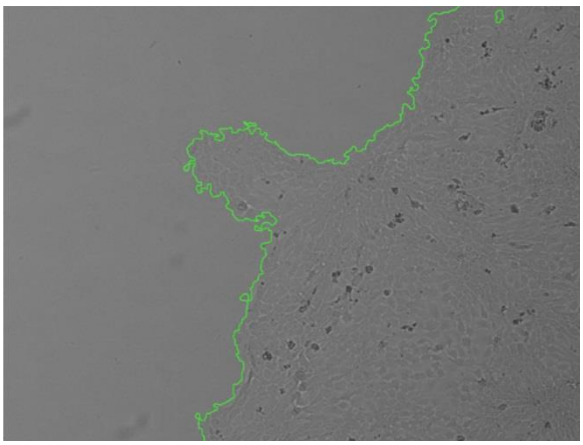
Optimal Identification of a Colony Using:
- **Bright Focus Target Focal Plane**
- **Brightfield Algorithm**



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: Single Colony Verification application. 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.

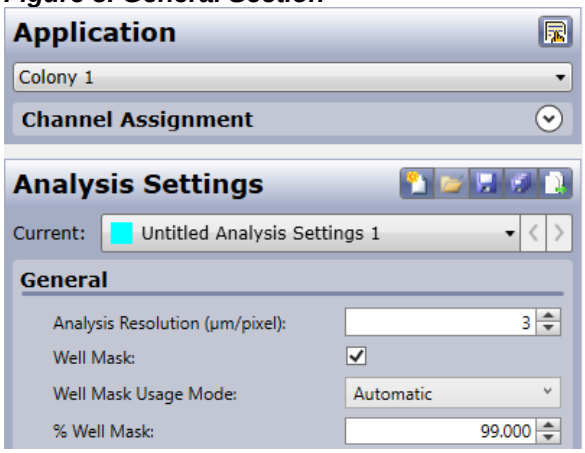
Perform the following steps to select analysis settings. The recommended initial analysis settings for identification and pre-filtering when using the Colony: Single Colony Verification application are shown in Table 2. The settings typically provide good image segmentation.

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.

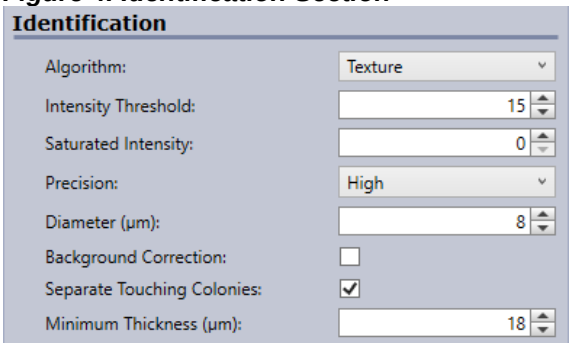
      Well Mask, Well Mask Usage Mode, and % Well Mask. For information about these selections, see the User Guide section Selecting General Analysis Settings.

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: When Dark Focus is selected as the Target Focal Plane, it is recommended to use Texture as the Algorithm in the ANALYZE 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 the background from cells. With an appropriate threshold set, the background pixels fall below and the pixels inside the 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 colony 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 is recommended and is sufficient to provide acceptable results, while High will result 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.

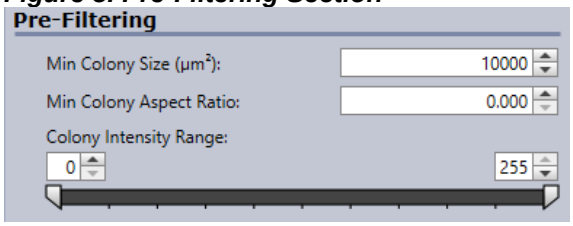
g. Separate Touching Colonies – Select if needed.

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:

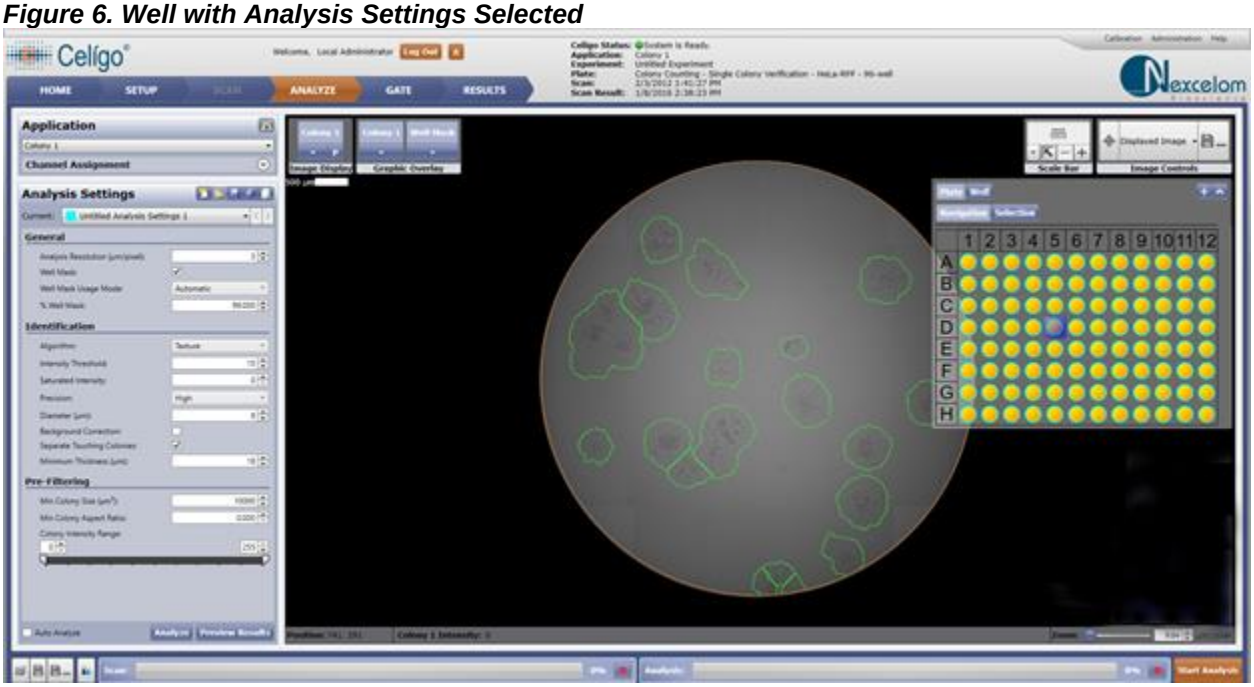
- To update the segmented image and overlays with each selection, check mark **Auto Analyze**.
- To update the segmented image and overlays only when the **Analyze button is clicked**, deselect **Auto Analyze**.

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	Uncheck marked
Separate Touching Objects	Uncheck marked
Minimum Thickness	15
PRE-FILTERING	
Min Colony Size (µm²)	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 Colony 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: Single Colony Verification application.

Table 3. Single Colony Verification Output Descriptions

Parameter	Description
Data for Each Well	
Confluency (%)	Area of the well occupied by cell colonies divided by the total area of a 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 (µm^2) per well
Colony Std Dev Area (µm²)	Standard deviation of colony area (µm^2) per well
Colony Total Area (µm²)	Total area of colonies (µm^2) 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 Results View Options: Image, Fill, and Heatmap Views

This section describes the purpose of the Image, Fill, and Heatmap views in the RESULTS tab for the Colony: Single Colony Verification application. In this application, choose between Image, Fill, and Heatmap view within either the plate view or well detail view, to see the properties and patterns of interest.

Figure 7. Image, Fill and Heatmap Button

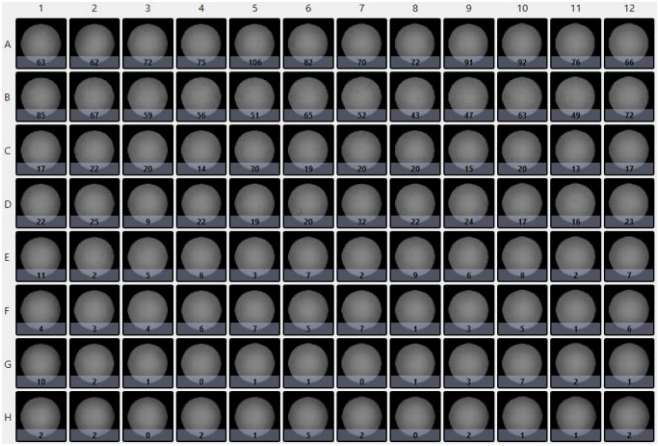
- Image button – Turns on/off the raw image display
- Fill button – Fills/un-fills all identified areas with a selected color
- Heatmap button – Provides color coded identification of data patterns in a high to low format

When the RESULTS tab first appears, the default view is a plate-level Image view (Figure 8a). In this application, the measurement parameters available in the Image view include the colony count for each well. In Figure 8b the Fill View

is shown, which provides a colony overlay of any identified colonies. This display provides an overview of the colony culture results in terms of single or multiple colonies. Figure 8c displays a heatmap of the data to quickly identify patterns.

Figure 8. Plate-Level Image View, Fill View, and Heatmap View

8a. Image View



8b. Fill View

8c. Heatmap View

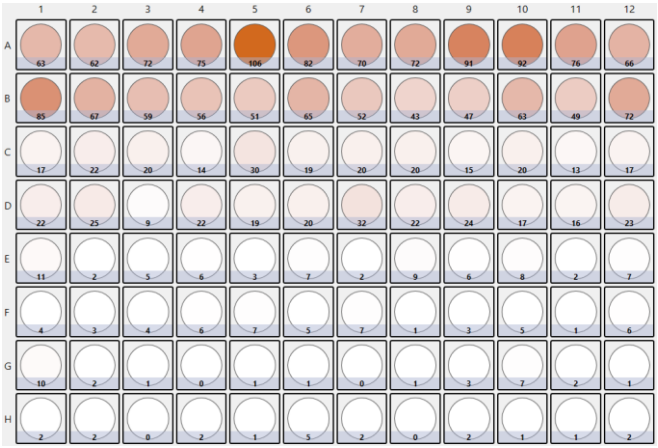


Figure 9 shows a well-level Fill view zoomed out.

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

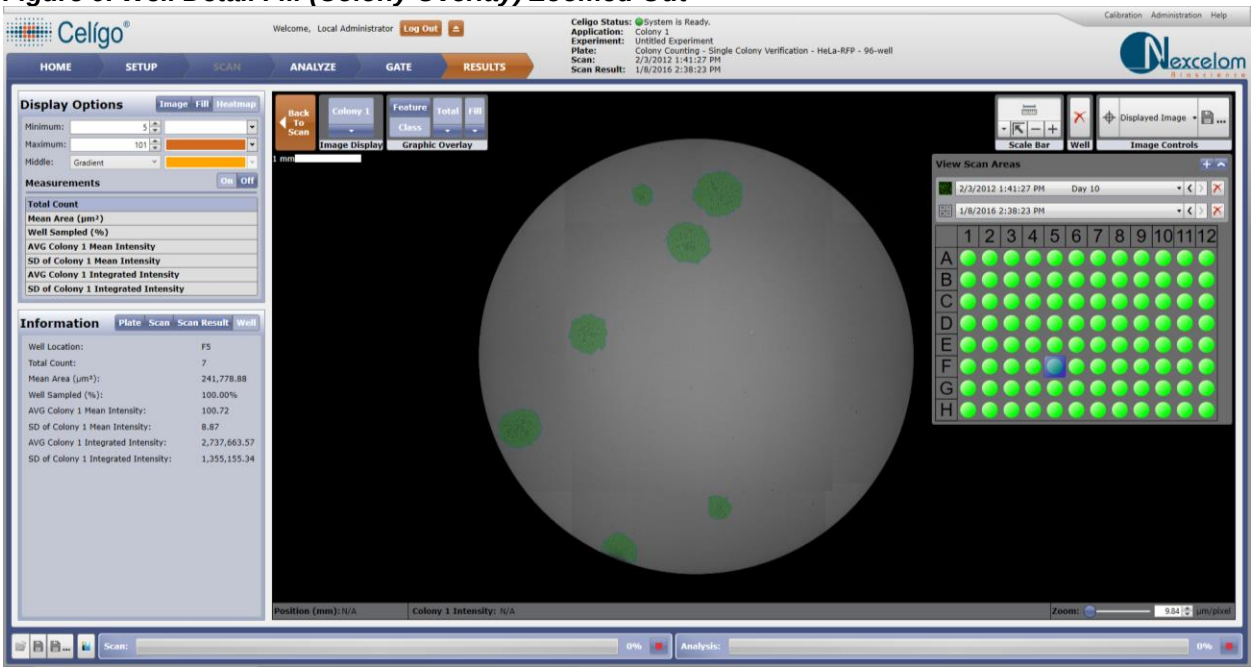
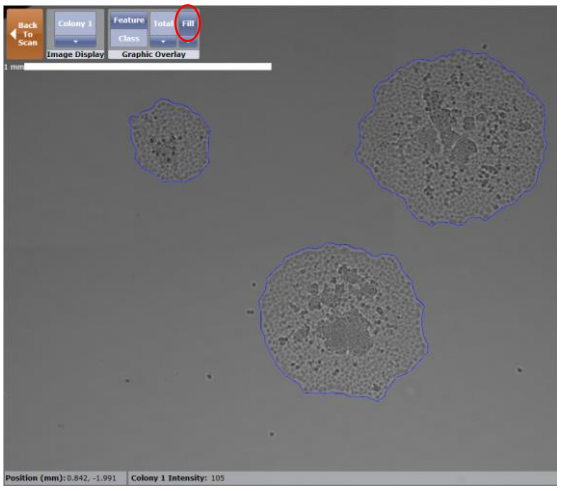


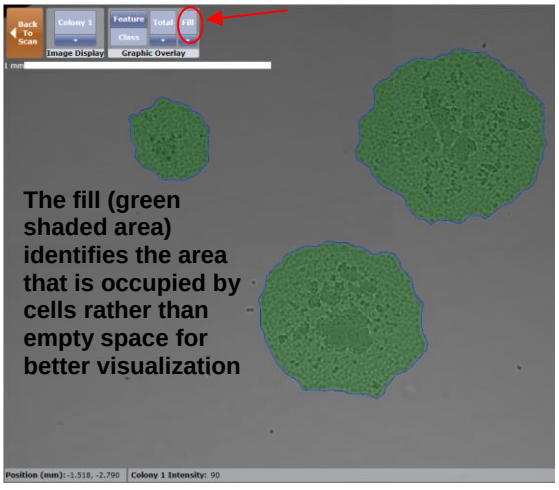
Figure 10 shows a well-level Fill view zoomed in (magnified).

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

Fill Off



Fill On



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 is 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 have unique properties that can be used to remove them from the scan results. Use the Pre-Filtering settings to remove debris.
Improper colony counts on well edges in brightfield	1. Uncheck Separate Touching Colonies in the ANALYZE tab – Identification section. 2. Increase Minimum Thickness. 3. Shrink % Well Mask to less than 100%. 4. Check mark/Uncheck mark Background Correction.
Well edges are too bright or dark	Liquid volume is 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 plating volumes, 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. • Check mark Background Correction.

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Celigo® Cytometer

Tumorsphere Application Guide

Celigo Software Version 5.2

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1. About this Guide

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

1.1 Introduction

The Tumorsphere application identifies and counts individual tumorspheres and small clusters of tumorspheres using brightfield and fluorescent 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 tumorspheres 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 Tumorsphere application. Information that is common to all applications is covered in the *Celigo® Cytometer User Guide (Document 8001619)*, henceforth 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.
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360 Merrimack St. Building 9
Lawrence, MA 01843, USA

- From the United States:
  E-mail: support@nexcelom.com
  Phone: +1 978-327-5340
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  E-mail: support@nexcelom.co.uk
  Phone: +44 (0) 161 232 4593

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 tumorspheres.
  - 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 while 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. Perform these tasks in the SCAN tab.

3.1 Tumorsphere Application

Perform the following step to select the Tumorsphere application:

To select the Tumorsphere Application

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

Figure 1. Selecting the Tumorsphere application

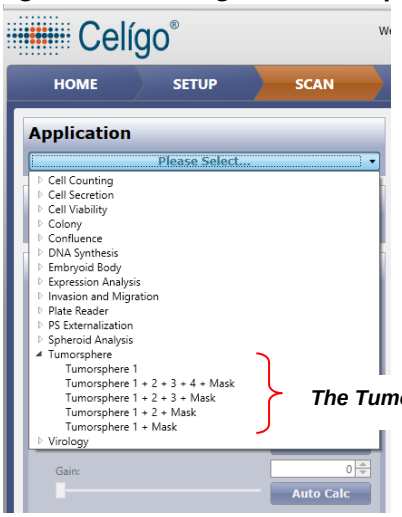


Table 2. Selecting a Tumorsphere Application

Quantity of Channels 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 the tumorspheres over time.
Four FL channels + a BF mask	**Tumorsphere 1 + 2 + 3 + 4 + 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.
Three FL channels + a BF mask	**Tumorsphere 1 + 2 + 3 + Mask**		
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 steps to select image acquisition settings. Use the following guidelines:

– First, select the image acquisition settings for the mask (brightfield) channel (section 3.2.1). Perform 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). Perform this task for any of the following tumorsphere applications:

- Tumorsphere 1+Mask
- Tumorsphere 1+2+Mask
- Tumorsphere 1+2+3+Mask
- Tumorsphere 1+2+3+4+Mask

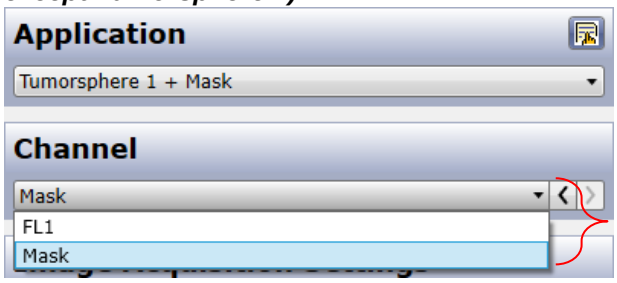
3.2.1 Selecting Acquisition Settings for the Mask (Brightfield) Channel

When performing the following procedure, see Table 3. Recommended Initial Settings for Image Acquisition 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 Tumorsphere 1 is selected as the Current Application, only 1 channel is in use, 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 one of the following applications is selected, select **Mask** in the Channel panel (Figure 62):
     - Tumorsphere 1+Mask
     - Tumorsphere 1+2+Mask
     - Tumorsphere 1+2+3+Mask
     - Tumorsphere 1+2+3+4+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, it is necessary to specify the channel for which settings are being selected.

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. Perform one of the following to set the optimal exposure:
   - If the wells have *different* darkness shadows and the same grey scale background is desired: 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 select either 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.

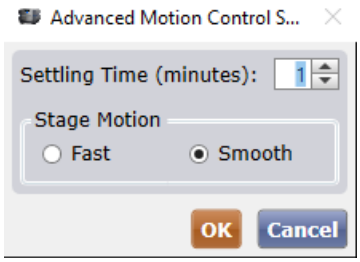
Table 3. Recommended Initial Settings for Image Acquisition

Type	Auto Exposure/Gain Channel
Focus Offset	0
CONFIGURATION	
Illumination	Brightfield
Priority	Auto Exposure, Gain if necessary
Frequency	Every scan area
MOTION CONTROL AND FOCUS	
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.

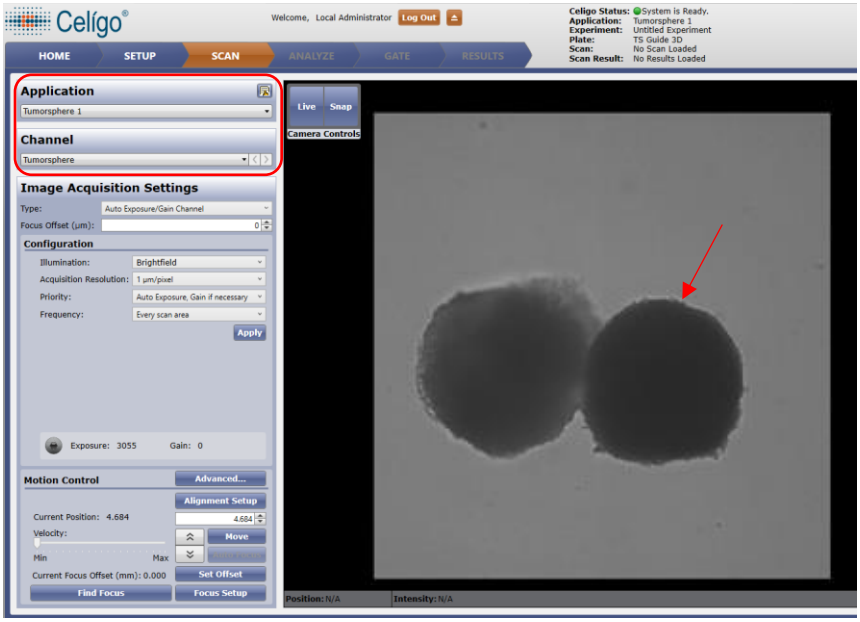
8. Click **Focus Setup**.
9. In the Focus Setup dialog box, make selections as needed. When making the Focus Type selections, use the following guidelines:

- If using a 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**
- If the plate focus map has been calibrated for the plate profile in use, select Plate Focus Map (see User guide for more details).

Figure 4 shows examples of acquisition settings for the bright field 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. In the settings on the right, Tumorsphere 1 + Mask application is selected, which uses 2 channels, where the Mask is the second channel with Brightfield illumination.

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

Tumorsphere 1 Application Mask Settings with Tumorspheres in Focus



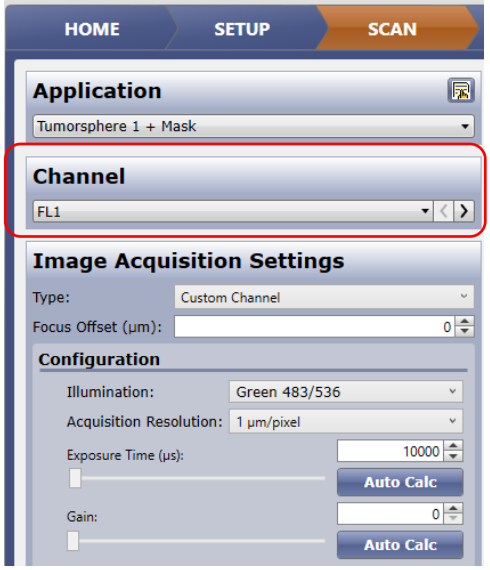
Tumorsphere 1 + Mask Settings

3.2.2 Selecting Acquisition Settings for the Fluorescent Channels

To select image acquisition settings for the fluorescent channels

1. Select an application, depending on the quantity of channels to be acquired (Table 2).
2. In Channel, select a fluorescent channel to set up (for example FL1 or FL2) (Figure 5).

Figure 5. Selecting a Fluorescence Channel



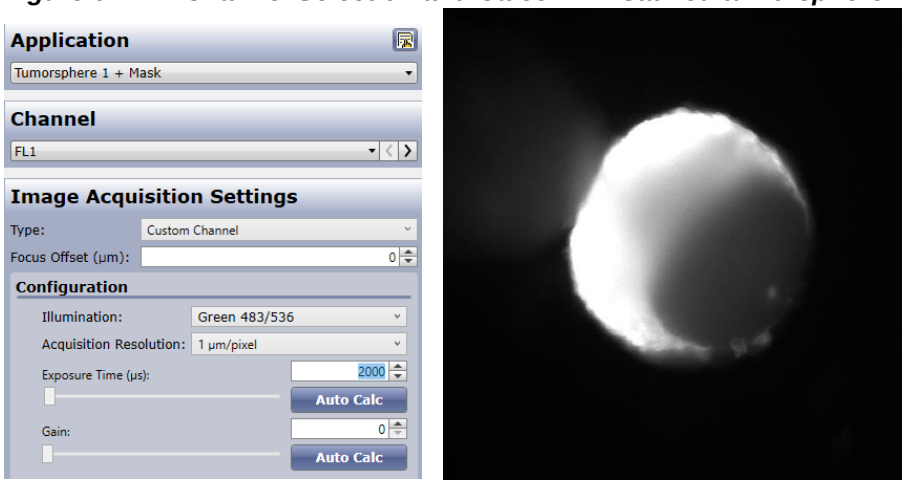
3. For the selected channel in the Channel panel, make the following selections:
   - In Type, select **Custom Channel**.
   - In Focus Offset, keep 0 (default).
   - In Illumination, select the appropriate illumination for the dye (e.g., **Green 483/536** to visualize the calcein AM signal, **Red 531/629** to visualize the propidium iodide signal). **Blue 377/447** to visualize Hoechst.
   - Click **Live** to see a live image or **Snap** to see a snapped image.
     - 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.
4. In Exposure Time and Gain, adjust as needed to correct the displayed live image. Ideal pixel intensity for the highest fluorescent object in the plate should be between 125 and 175, where 255 is the maximum saturated pixel intensity.

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

5. Set up any remaining fluorescent channels as needed by repeating this fluorescent channel setup section (3.2.2).

Figure 6 shows an example of FL 1 channel selections and a CalceinAM stained tumorsphere.

Figure 6. FL 1 Channel Selection and CalceinAM stained tumorsphere



4. Analyzing Images

This chapter provides information on how to analyze scans from the Tumorsphere application. 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 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:

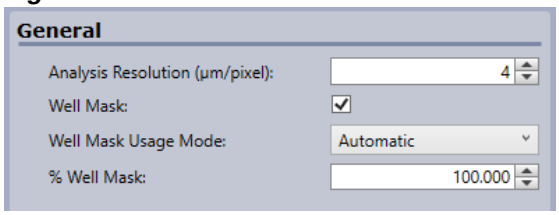
- Test the analysis settings using multiple tumorspheres across at least one well of the plate.
- Tumorsphere border identification is critical for this application. Therefore, images containing tumorspheres with clear, crisp edges will be easy to identify and analyze. Tumorspheres that are out of focus 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. If not, proceed to step 2.

In the General section (Figure 7), make the following selections:

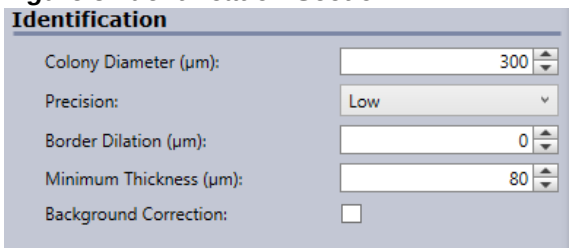
Figure 7. General Section



2. Image Resolution (µm/pixel): Select the image resolution to use for analysis. For the 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.
3. Well Mask – Applies a boundary at the well edge so that the well edge (subject to distortion or plate artifacts) is excluded from segmentation. Uncheck mark for the initial setting.
4. Well Mask Usage Mode – Select Automatic.
5. % 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.

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

Figure 8. Identification Section



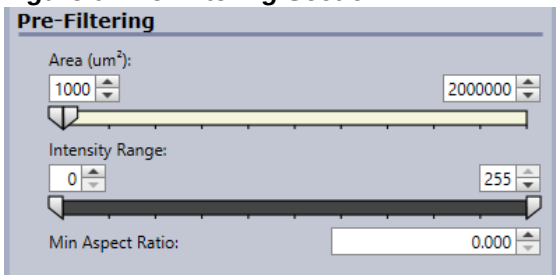
6. Colony Diameter (µm) – Defines the diameter of the tumorsphere. If a range of tumorsphere sizes are present, this should be close to the maximum size.
7. Precision – Select the desired level of precision for separation of touching tumorspheres:
    - None – No separation.
    - Low – Minimal separation.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

8. Border Dilation (µm) – Defines the amount of dilation/erosion for tumorsphere edge segmentation.
9. Minimum Thickness (µm) – Minimizes object artifact extensions.
10. Background Correction – (Use for Brightfield Illumination only) – Minimizes background variations due to the meniscus by correcting an image for segmentation.

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

Figure 9. Pre-Filtering Section



11. Area (µm²) – The tumorsphere area. Enter the tumorsphere area range that corresponds to the scanned tumorspheres.
12. Intensity Range – Make selections to include only tumorspheres and exclude debris/single cells.
13. 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	Uncheck marked	Check marked or Uncheck marked	Minimizes background variations by applying an average value.
Well Mask	Check marked	N/A	Identifies the edge of the well.

Parameter	Initial Setting	Available Range	Description
PRE-FILTERING			
Tumorsphere Area (µm2) 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. Tumorspheres with Corresponding Target Overlay – Minimum Tumorsphere Aspect Ratio Adjustments show examples of Tumorsphere brightfield images with corresponding target overlays.

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

Figure 10. Tumorspheres with Corresponding Target Overlay – Colony Diameter Adjustments

Colony Diameter 100 **Colony Diameter 500**

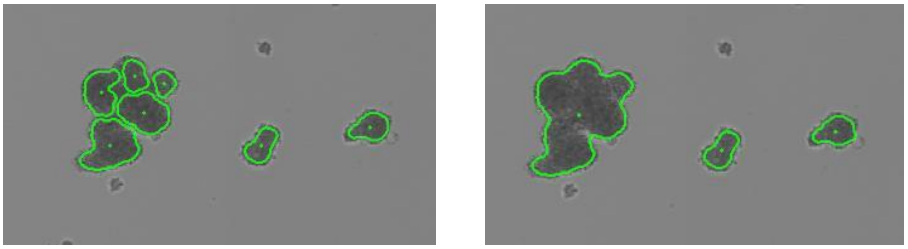


Figure 11 shows an example of the display results when adjusting precision.

Figure 11. Tumorspheres with Corresponding Target Overlay – Precision Adjustments

Precision Low **Precision High**

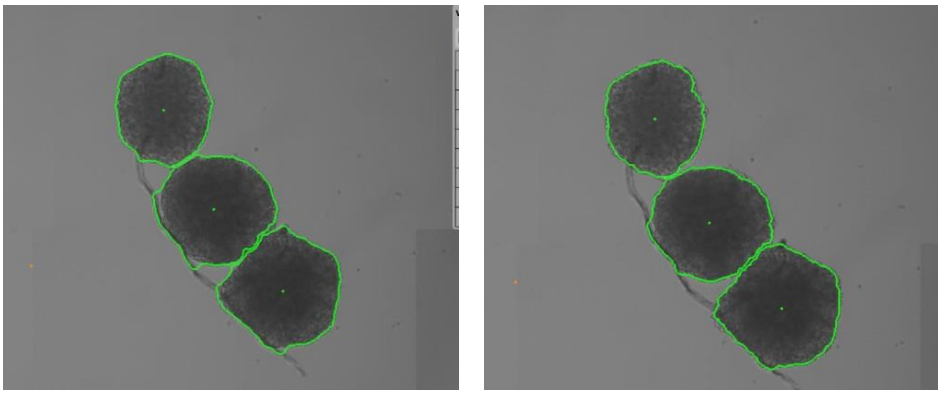


Figure 12 shows an example of display results when adjusting tumorsphere border dilation.

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

Border Dilation 0 **Border Dilation 20**

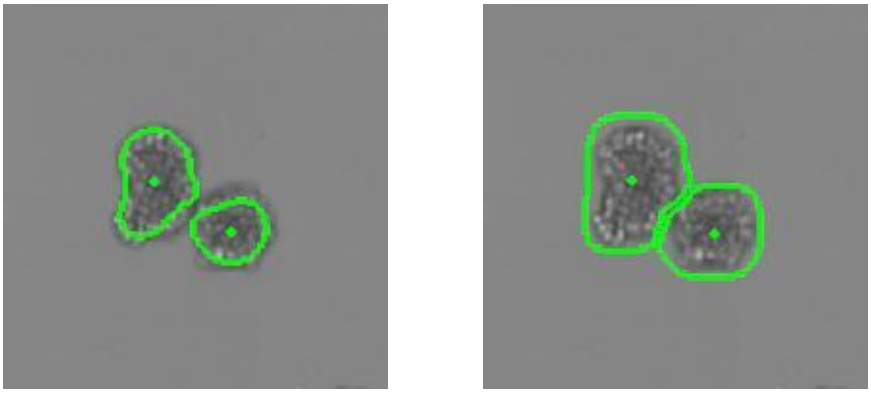


Figure 13 shows an example of display results when adjusting minimum thickness.

Figure 13. Tumorspheres with Corresponding Target Overlay – Minimum Thickness

Minimum Thickness 20 µm **Minimum Thickness 50 µm** **Minimum Thickness 80 µm**

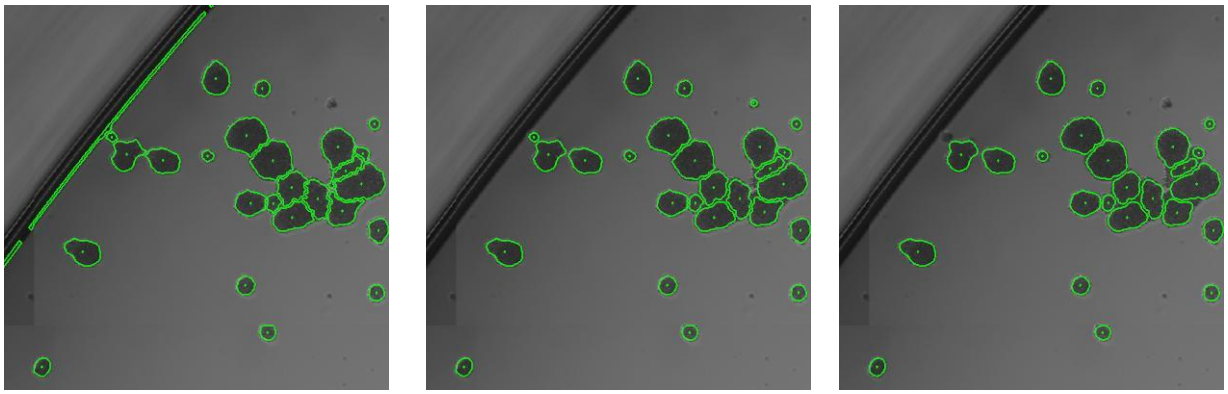


Figure 14 shows an example of display results when adjusting background correction.

Figure 14. Tumorspheres with Corresponding Target Overlay. – Background Correction

Background Correction Off **Background Correction On**

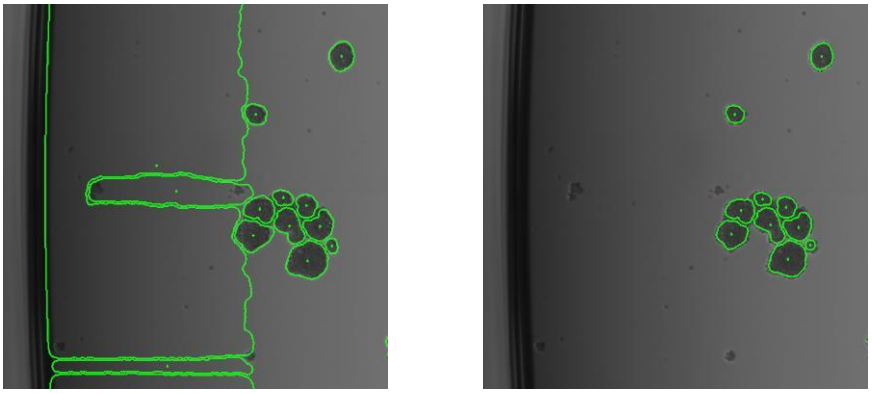
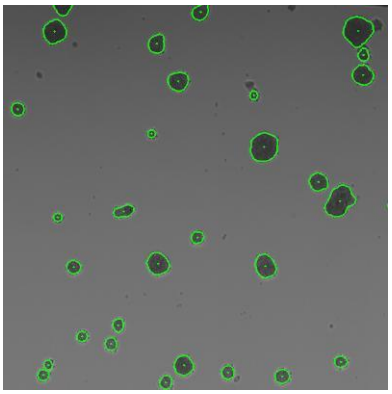


Figure 15 shows an example of display results when adjusting area (µm²) range.

Figure 15. Tumorspheres with Corresponding Target Overlay. – Area (µm²) Range Adjustments

Area (µm²) 500



Area (µm²) 10,000

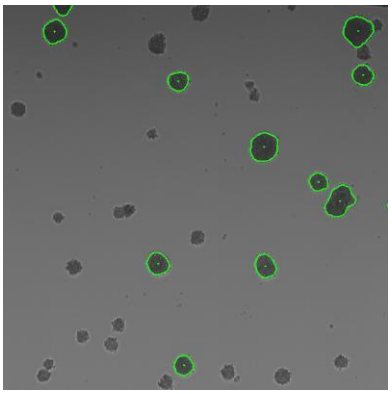
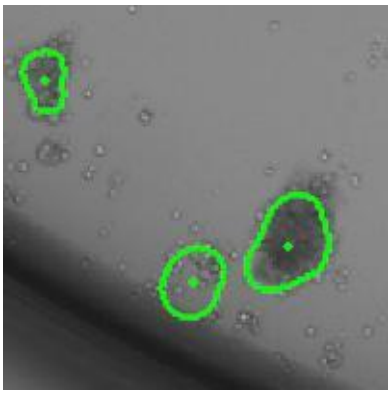


Figure 16 shows an example of display results when pixel intensity range is adjusted.

Figure 16. Tumorspheres with Corresponding Target Overlay – Intensity Range Adjustments

Intensity Range: Max Pixel Intensity 255



Intensity Range: Max Intensity 106

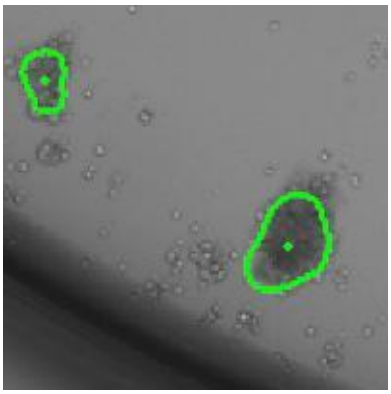
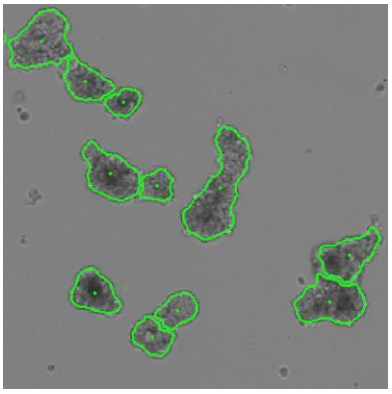


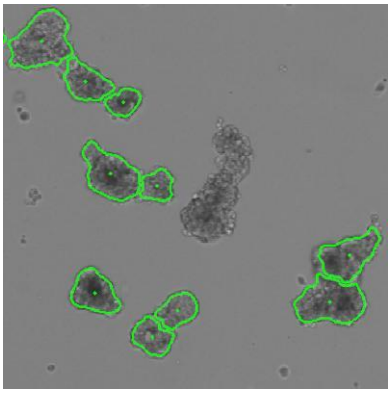
Figure 17 shows an example of display results when minimum tumorsphere aspect ratio is adjusted.

Figure 17. Tumorspheres 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. Perform this task in the GATE tab.

The 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 Tumorsphere application.

- If choosing to 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; it is not possible to assign any additional classes to populations. Reanalysis of the same scan is required to 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. Check marking 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 check mark would be the same as not using the GATING tab; all data would be reported from the ANALYZE tab settings only.**

To create a plot, gate, and populations

1. In the Plot Populations pane, create a plot using the Add Plot (+) button, the Add Plot dialog box, and also by referring to the selections in **Error! R eference source not found.** and Table 6.

   The table lists the possible selections for 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 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 creating a plot and drawing a gate, 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	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) 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.
Area (µm2)	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).

FEATURE	DESCRIPTION
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).
EQ Diameter (µm)	Equivalent diameter of each identified tumorsphere, derived from the area. Equivalent diameter = 2(radius), derived from Area = $\pi(radius^2)$
Est Volume (µm3)	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.

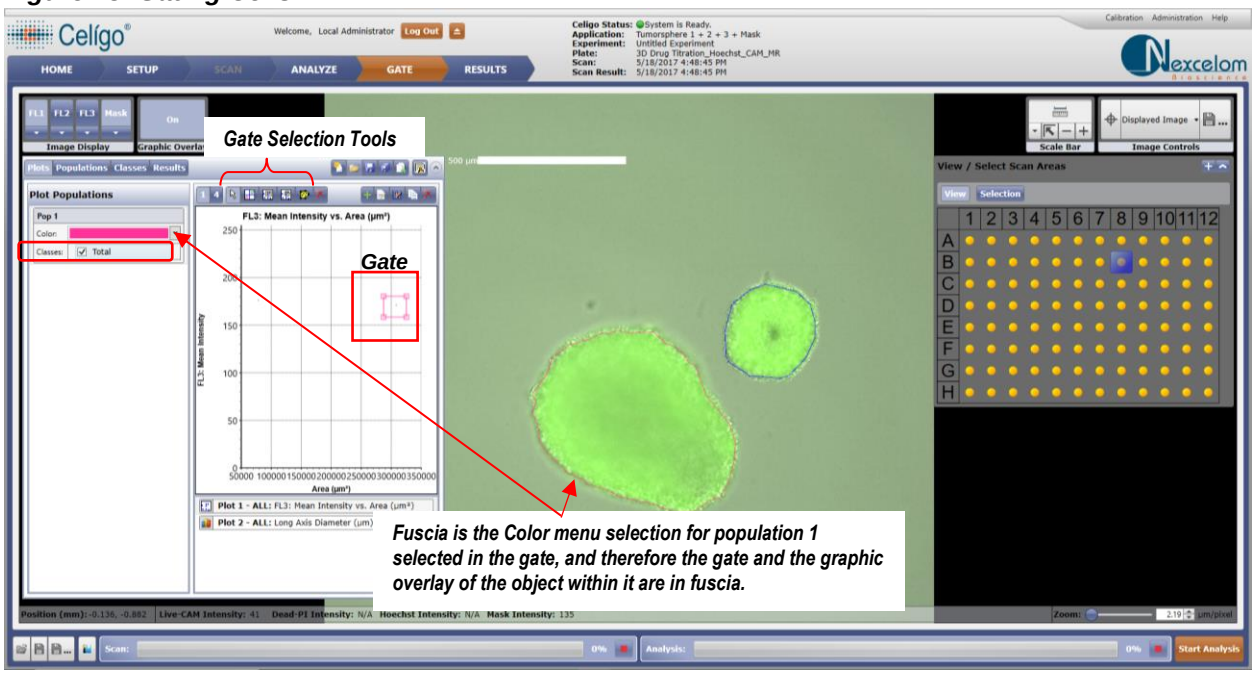
Table 6. Plot Parameter Sections by Channel

For Each Channel	
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 the pixel intensities displaying signal in the segmented objects calculated for each channel.
Inner, ring and outer Area (µm2)	Total area of each identified zone in the tumorspheres (if applicable).

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



2. Repeat steps 1 and 0 as needed to refine the desired population for counting.
3. In the Plots, Populations, or Classes view, make sure that the Total class is check marked. This assigns the Total class to the population.

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

In this application, only assign the Total class to populations; it is not possible to 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 7 and appear below the Display Options section in the Scan Information pane.

Table 7. 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 (µm3)	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 (µm2)	Area of each identified tumorsphere, measured in pixels.	√	√	√	
Perimeter (µm)	The total length of the edge of each identified tumorsphere.	√	√	√	

Parameter	Description	Ave	St Dev	CV	Min/ Max
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 has a smoothness value of 1.0 (the maximum).	√	√	√	
Long Axis Angle	Angle of the long axis of the tumorsphere (longest dimension).	√	√	√	
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 - 4 Mean Intensity	Average of the intensities of the segmented objects. This will differ from the Total Intensity.	√	√	√	
Fluor 1 - 4 Integrated Intensity	Sum of all the pixel intensities displaying signal in segmented objects.	√	√	√	
Mask Mean Intensity	Average of the intensities of the segmented objects. This will differ from Total Intensity.	√	√	√	
Mask Integrated Intensity	Sum of all pixel intensities displaying signal in segmented objects.	√	√	√	
% Well Sampled	Percent of the 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.

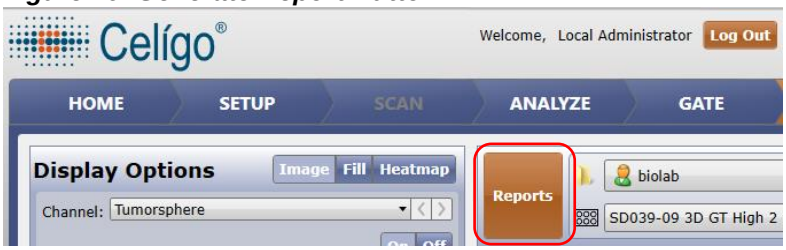
6.3 Generating a Growth Tracking Report

Growth tracking reports calculate the growth characteristics of tumorsphere populations over time. The reports associate tumorsphere counts or area measurements from the time points of multiple scans and determines the 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).

	**NOTE: The Multi-channel Tumorsphere applications will use the Mask channel with Brightfield illumination to create reports.**

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

Figure 20. Display Mode Options include Average Volume Chart

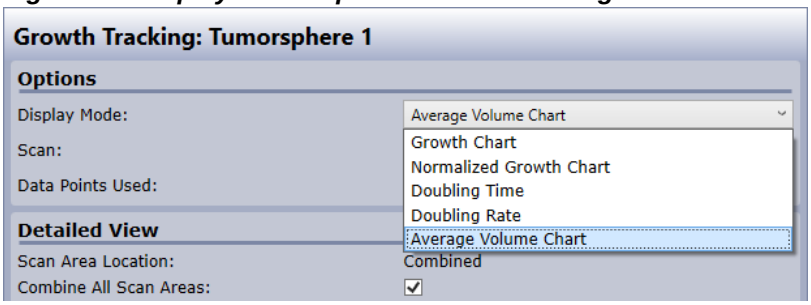
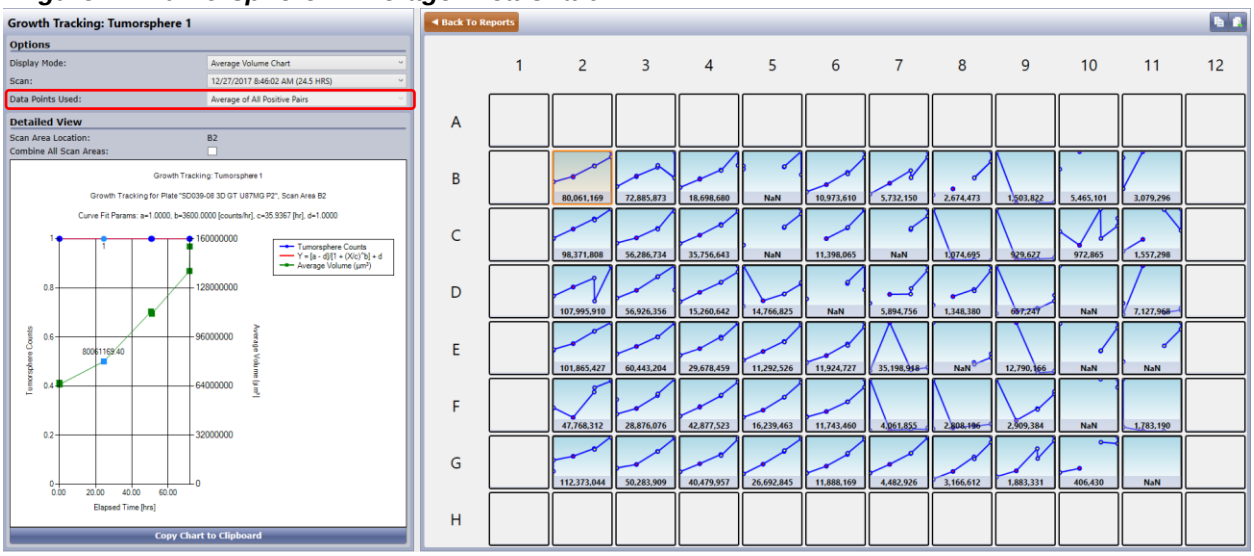


Figure 21. Tumorsphere 1 Average Area Chart



7. Troubleshooting

This chapter provides troubleshooting recommendations.

Table 9. Troubleshooting Recommendations

Issue	Recommended Action
Tumorspheres are out of focus	When the hardware autofocus position is set using only a few non-representative 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 the hardware autofocus when scanning.
Tumorspheres are not being identified	Tumorspheres may not be identified due to: Part of the tumorsphere is out of focus: Adjust tumorsphere border value. Tumorspheres are smaller/larger than the size range selected in the Tumorsphere Area in the ANALYZE tab: Adjust range of Tumorsphere Area. Tumorspheres have a lower/higher intensity than the range selected in the tumorsphere Intensity Range in the ANALYZE tab: Adjust tumorsphere Intensity Range. 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. Single/dead cells can be removed by decreasing the tumorsphere intensity range in the ANALYZE or GATING Tab. Small clumps of cells not considered to be tumorspheres can be removed by adjusting the tumorsphere area range in the ANALYZE or GATING tab. 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. F

Celigo® Cytometer
DNA Synthesis
Application Guide

Celigo Software Version 5.2

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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: support@nexcelom.com
  phone:+1 978-327-5340
- From Europe:
  e-mail: support@nexcelom.co.uk
  phone: +44 (0) 161 232 4593

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. Perform these tasks in the SCAN tab.

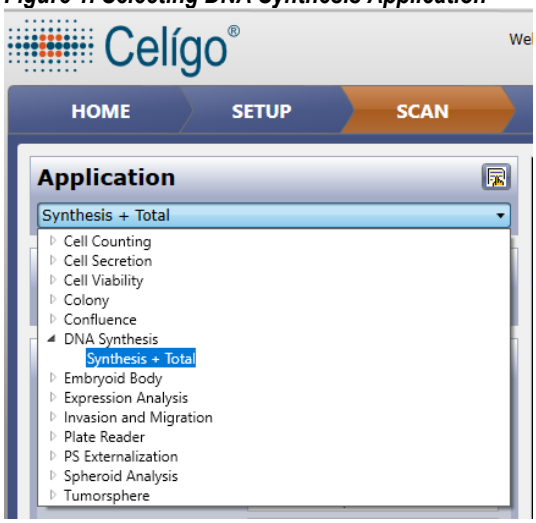
3.1 DNA Synthesis Application

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.
	NOTE: The EdU and DAPI assay can also be used in place of the BrdU and DAPI assay. Both assays work with this application.

3.2 Acquisition Settings

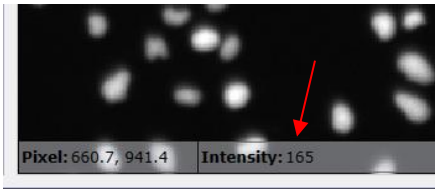
Perform the following steps to select image acquisition settings in the DNA Synthesis application. For the recommended initial settings to use as a guide, see Table 1.

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 Channel select: **Total**.
3. Make the following selections in the Image Acquisition Settings pane:
   a. In Type, select **Custom Channel**.
   b. In Illumination, select the appropriate illumination for the dye (e.g., **Blue** to visualize the DAPI/Hoechst 33342 signal).
   c. Click **Live** to see a live image.
   d. Click **Find Focus** or use manual focus to achieve clear image of cells.
   e. Select appropriate exposure.
   - Custom: (Recommended) User manually establishes optimal exposure time and gain by adjustment of Exposure Time (µs). When doing so, adjust the exposure so the majority of cells aren't overexposed.
   - Auto Calc: The system will attempt to determine the optimal exposure time and gain setting. Not recommended for cell cycle assay as it may over saturate objects.

The exposure time value should provide optimal exposure for your sample (an ideal object pixel intensity of 125-175) where cells are visible, yet not overexposed (i.e. maximum saturated pixels intensities 255). See Figure 2 for where pixel intensity can be observed on screen when you hover mouse over object in image display area.

Figure 2 Pixel Intensity Display



   f. Click **Focus Setup** to set up autofocus registration.
   - It is recommended to use the focus type **Hardware Auto Focus** and select **Register Auto** for most routine plate scanning. For detailed instructions, see the User Guide's section Focusing with automatic registration.
4. In 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**.

The best focal plane for the Green illumination may be slightly higher or lower than the Blue channel. This offset value will acquire the green channel images in that slightly different focal plane.

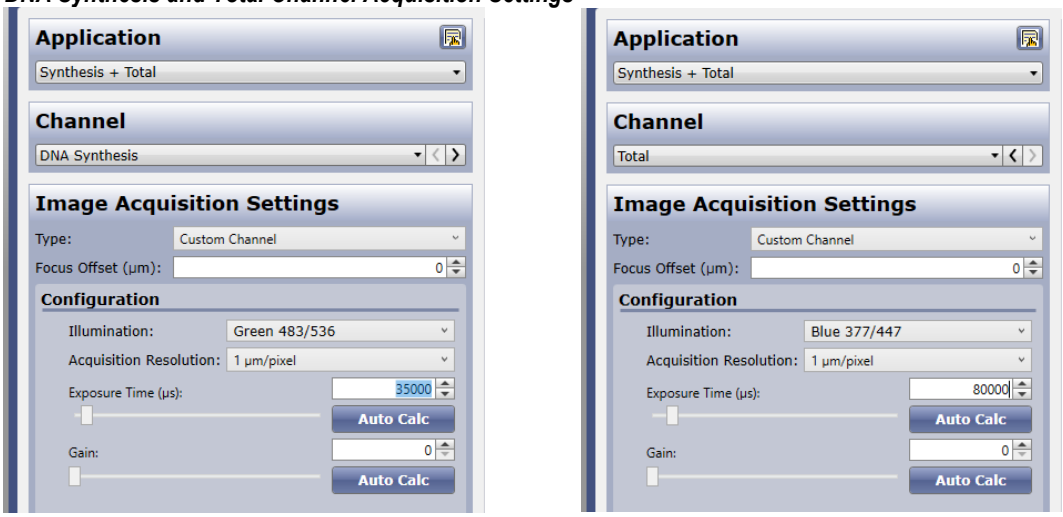
Table 1. Recommended Initial Settings for Image Acquisition

Current Channel	DNA Synthesis	Total
Type	Custom Channel	Custom Channel
Focus Offset	User determined	User determined
Illumination	Green 483/536	Blue 377/447
Acquisition Resolution	1 µm/pixel	1 µm/pixel
Exposure Time (µs)	20,000-80,000*	50,000-100,000*
Gain	0	0
Motion Control and Focus	See User Guide	See User Guide

Select an optimal exposure for your sample (an object pixel intensity of 125-175) where cells are visible, yet not overexposed (i.e., saturated: pixels intensities >254).

Figure 3. shows an example of the DNA Synthesis channel and Total channel.

Figure 3. DNA Synthesis and Total Channel Acquisition Settings



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.

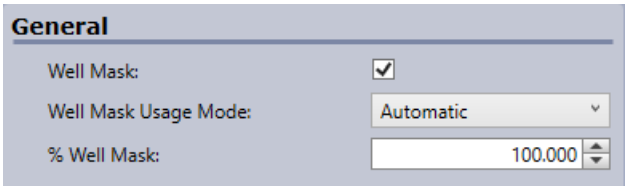
4.1 Analysis Settings

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: **Synthesis + Total** 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, or proceed to next step.
2. In the General section (Figure 4), make the following selections:

Figure 4. 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.**

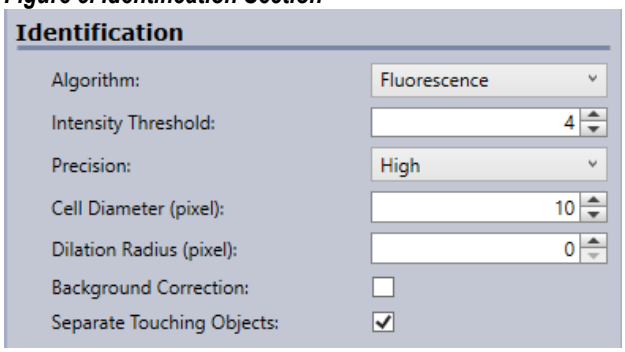
b. Well Mask Usage Mode – Select **Automatic**.

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's section Selecting General Analysis Settings.

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

Figure 5. Identification Section

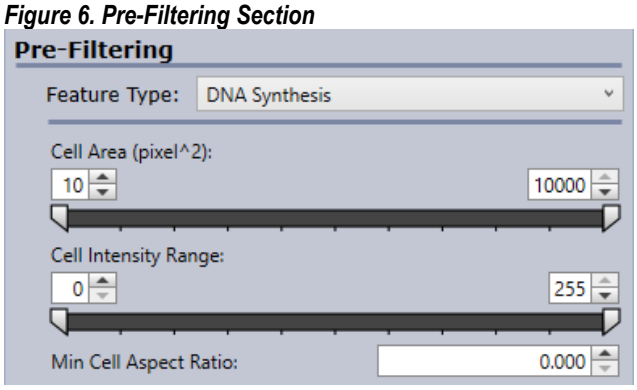


   a. Algorithm – Select **Fluorescence**.
   b. Intensity Threshold – Enter the optimal intensity threshold.

      The intensity threshold is the level of intensity that separates the background from cells. With an appropriate threshold set, background pixels fall below the threshold, while pixels inside the cells fall above. Any pixels below the threshold are not considered in the calculation.
   c. Precision – Higher precision results in more accurate identification of cell clusters. High precision is recommended.
   d. Cell Diameter (in pixels), – Enter the Cell Diameter (pixels) that corresponds to your cells. At full resolution, the Celigo provides 1 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), 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 6. 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 Check marked
Separate Touching Objects	Check marked
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 and Figure show the expected display results for the settings shown in Table 2.

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

Figure 5. DNA Synthesis Application 2-Channel Image of A549

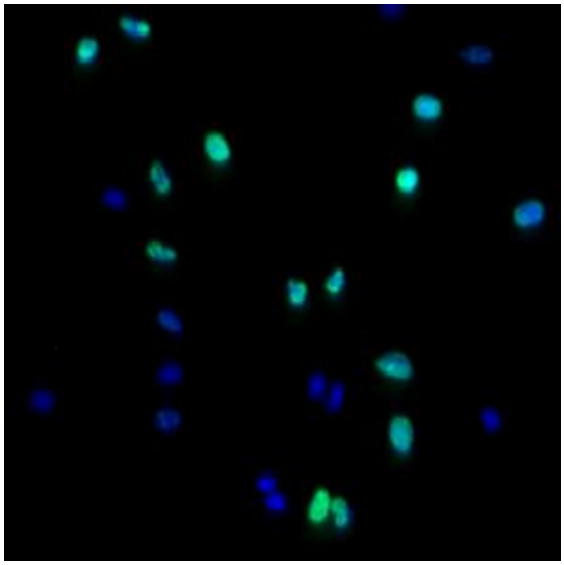
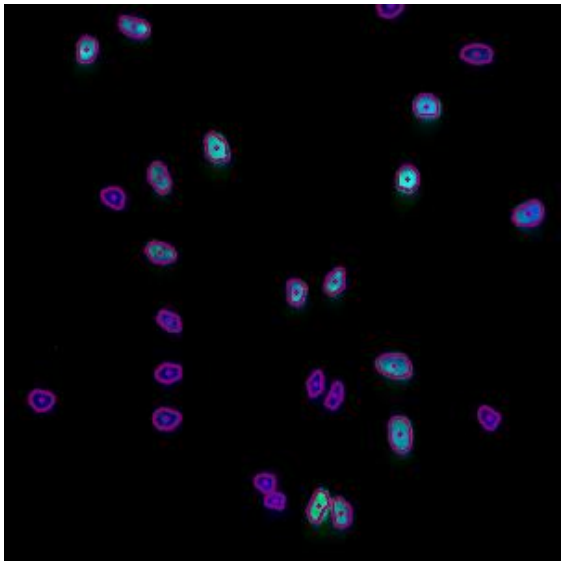


Figure 6. Image + Target Overlays of Image on Left



	NOTE: Antibody staining sometimes leads to the appearance of bright aggregates that should not be considered as positive cells. Bright aggregates do not have corresponding object limits and are eliminated from the analysis with the proper filter settings. These aggregates can be filtered out by lowering the upper limit of the Cell Area Range filter (e.g. 200). 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. Perform this task in the GATE tab.

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

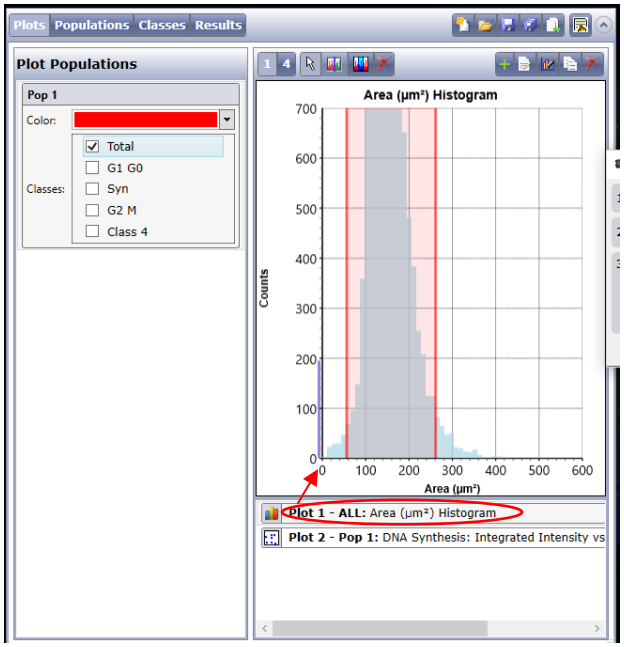
In the DNA Synthesis application, two classes exist (Total and DNA Synthesis). Classes are populations (defined by gates) for which data is reported.

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 (µm^2) Histogram plot (Figure 7) is not in view, display it by clicking its listing in the plot list below the displayed plot, Plot 1.

Figure 7. Histogram



2. On the histogram plot, click and drag the Min/Max gate so that it covers the population you want to analyze. Click and drag the red line to move individual lines individually.

   In the Graphic Overlay display, the color displayed for the plot population selected in the Plot corresponds to the Color menu selection. If Total is red in color, the graphic overlay of cells in Min/Max gate are red.

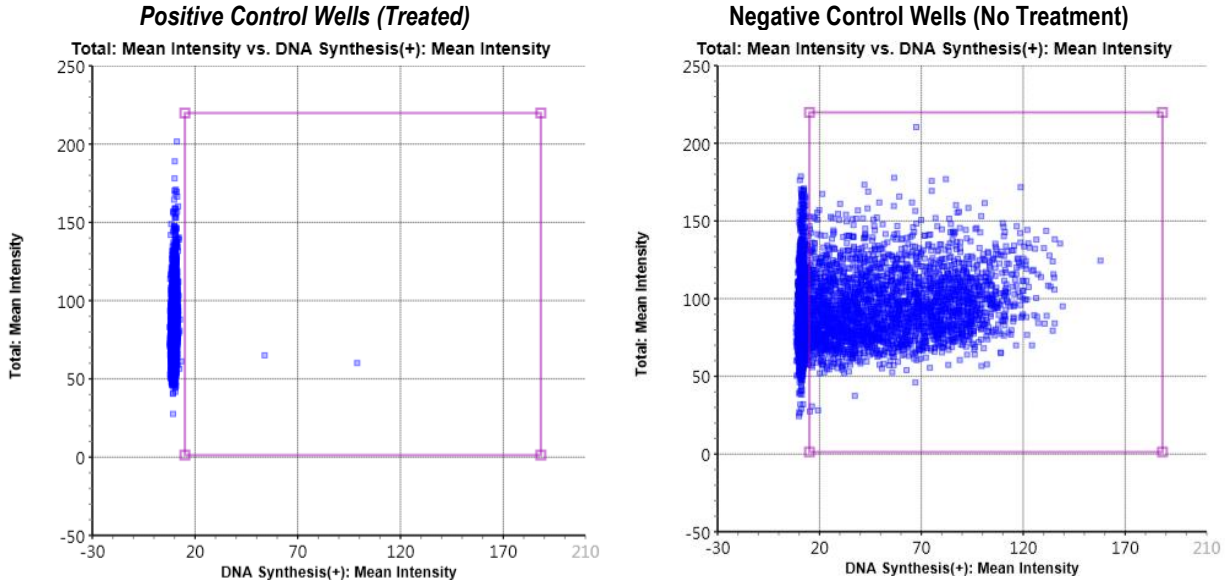
3. On the histogram plot (Plot 1 - ALL), assign the Total class as follows:

a. In the Plots Populations, makes sure that the Total class is check marked.

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

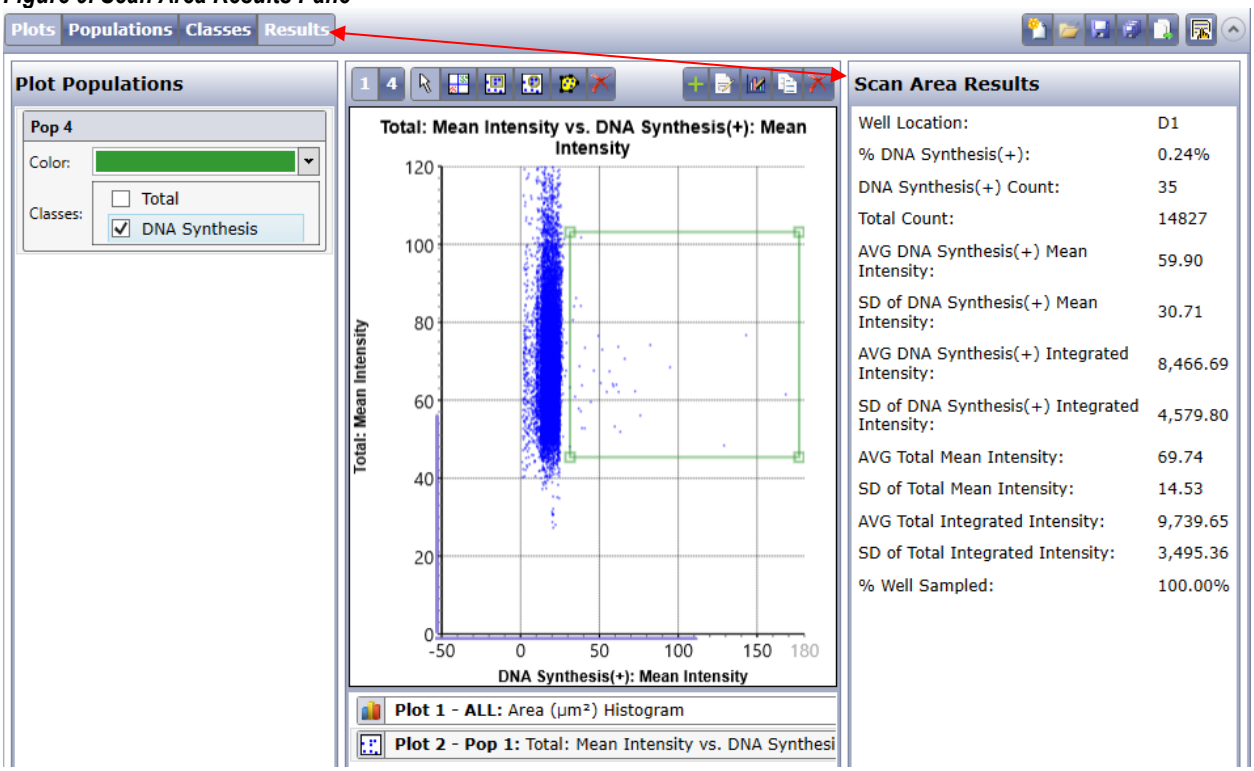
4. On the scatter plot (Plot 2- Pop 1) (Total: Mean Intensity vs. DNA Synthesis(+): Mean Intensity (Figure), drag the gate so that it captures only the cells that are positive for DNA Synthesis. Figure shows examples of a gate capturing positive treated control wells (wells with a low percentage of positive BrdU labelled cells) versus negative untreated control wells (wells with a high percentage of positive BrdU labelled cells).

Figure 8. Scatter Plot



To capture only the positive wells, it is helpful to review the Scan Area Results pane (Figure 9) 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 9. Scan Area Results Pane



5. On the scatter plot, assign the DNA Synthesis class as follows:
   a. On the scatter plot, click the gate (Green rectangle in Figure 10).
   b. In the Plots, Populations, or Classes view, make sure that the DNA Synthesis class is check marked.

For further details on how with work with gates in the GATE tab, refer to the User Guide.

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. • Optimal Pixel intensity range is 125-175, which is in the middle of the camera range.
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 in Analysis tab, some objects are filtered out and no longer part of the analysis in Gate tab. • Expand the lower and upper limits of the filters.

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 8001627 Rev. F

Celigo® Cytometer
Expression Analysis
Application Guide

Celigo Software Version 5.2

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1. About this Guide

The Expression Analysis application is a generic fluorescence protocol for up to four separate channels in addition to a brightfield channel. Cells can be identified label-free in the brightfield channel, while fluorescent signal in four 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).

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:
E-mail: support@nexcelom.com
Phone: +1 978-327-5340

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E-mail: support@nexcelom.co.uk
Phone: +44 (0) 161 232 4593

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. Perform these tasks in the SCAN tab.

3.1 Overview of Application Selection Strategies

The Expression Analysis application provides three types of segmentation (identification) strategies that can be applied to scans.

Identification can be done using either of the following strategies:

1. Single Mask – Identification is performed in one channel, called the Mask channel, and then applied to every channel.
2. Independent Mask – Identification is performed separately in each channel and applied separately to each channel.
3. 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 cells in the channels. 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, select an application based on the expected measurement for the experiment.

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

Single Mask Segmentation

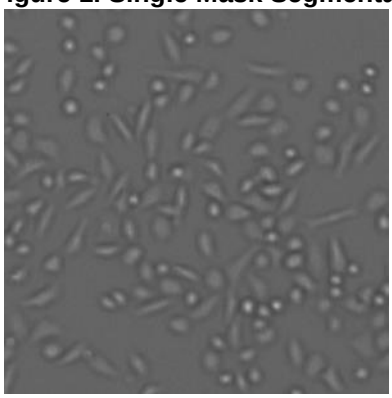
- The single mask segmentation strategy uses only one image to identify objects in the images, and the resulting mask will be used to segment images from all the other channels. Figure 1 shows the Brightfield imaging of cells transfected with either GFP or RFP (imaging using only the Brightfield channel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

from the single mask segmentation while intensity measurements (mean and integrated) will be measured in every channel.

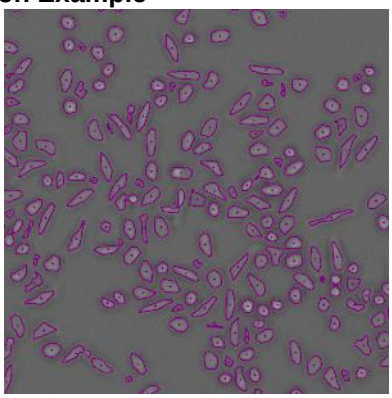
This segmentation method analyzes the images in a similar fashion to flow cytometry, where cells are identified using the forward and side scatter to identify the cells.

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.

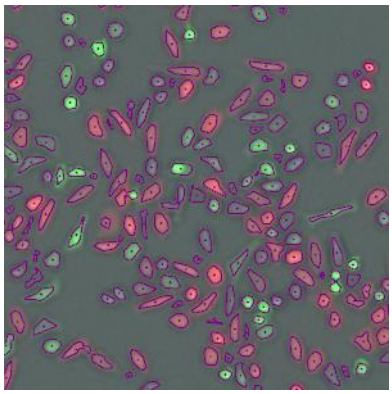
Figure 1. Single Mask Segmentation Example



(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.

3.1.1 Independent Segmentation

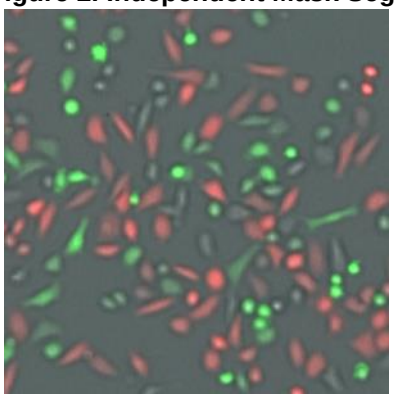
The independent segmentation algorithm will segment images in each channel. Objects will be identified in every channel independently, even if they do not exist in any other channels. Figure 2 shows cells either transfected with GFP or RFP (panel A). GFP positive cells, RFP positive cells and all cells are identified separately by segmenting the green, red and brightfield 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 the 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 non-transfected cells.

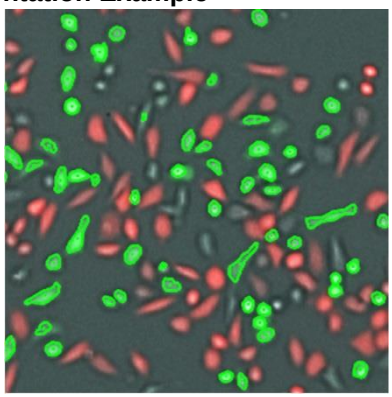
Using the segmentation strategy in this example, green cells will be identified and therefore assigned an intensity value in the green fluorescent channel,

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 histogram representation of these cell populations is recommended (see Gating Cells section 5).

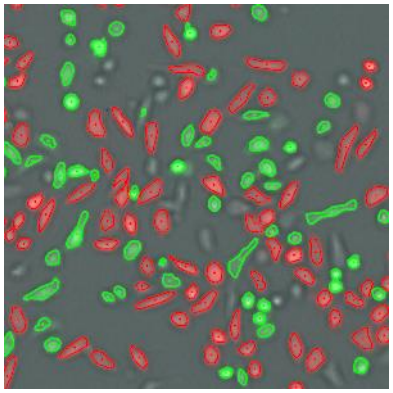
Figure 2. Independent Mask Segmentation Example



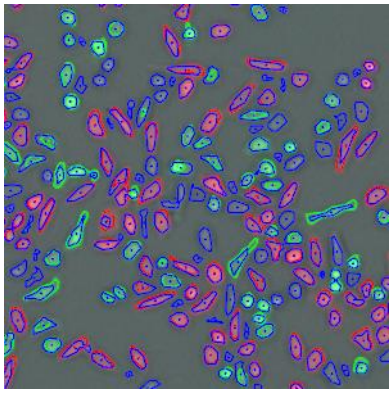
(A) Cells transfected with GFP or RFP – green, red and Brightfield channels combined.



(B) Segmentation of the green objects.



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

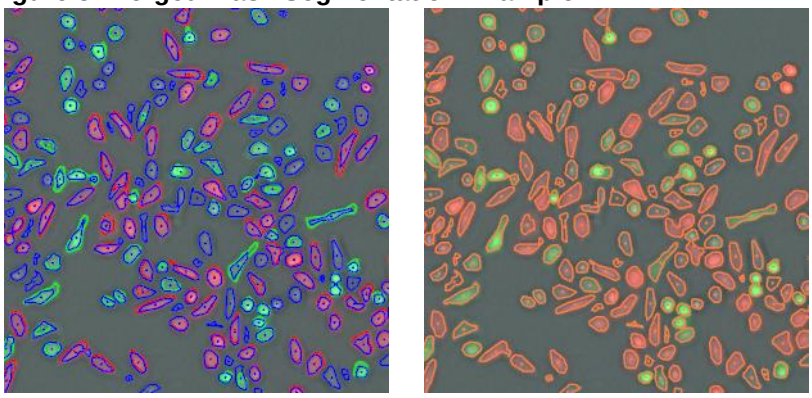


(D) Segmentation of all objects in their respective channels.

3.1.2 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 13 Expression Analysis applications (Figure 4 and Table 1), depending on how many channels (targets) are necessary to acquire, and whether or not an independent, single-mask, or merge segmentation strategy is to be used.

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

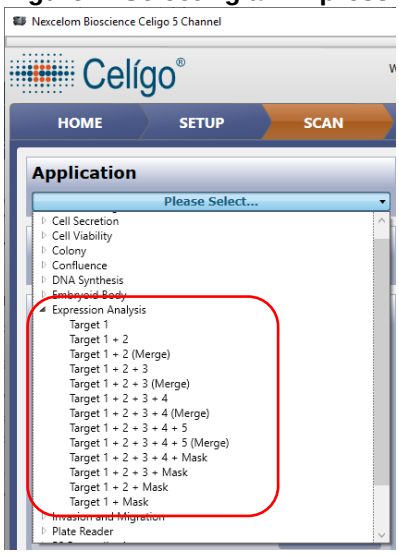


Table 1. Selecting an Expression Analysis Application

Quantity of Channels (Targets) to be Acquired	Select this application	How Fluorescence is Measured	Use Case
Single Mask			
Four channels + a mask	**Target 1 + 2 + 3 + 4 + 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.
Three channels + a mask	**Target 1 + 2 + 3 + Mask**		
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 outlines 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**		
Five channels (Ind*)	**Target 1 + 2 + 3 + 4 + 5**		
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 of the 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)**		
Five channels merged	**Target 1 + 2 + 3 + 4 + 5 (Merge)**		

3.3 Acquisition Settings

Perform the following steps to select image acquisition settings.

To select image acquisition settings

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

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

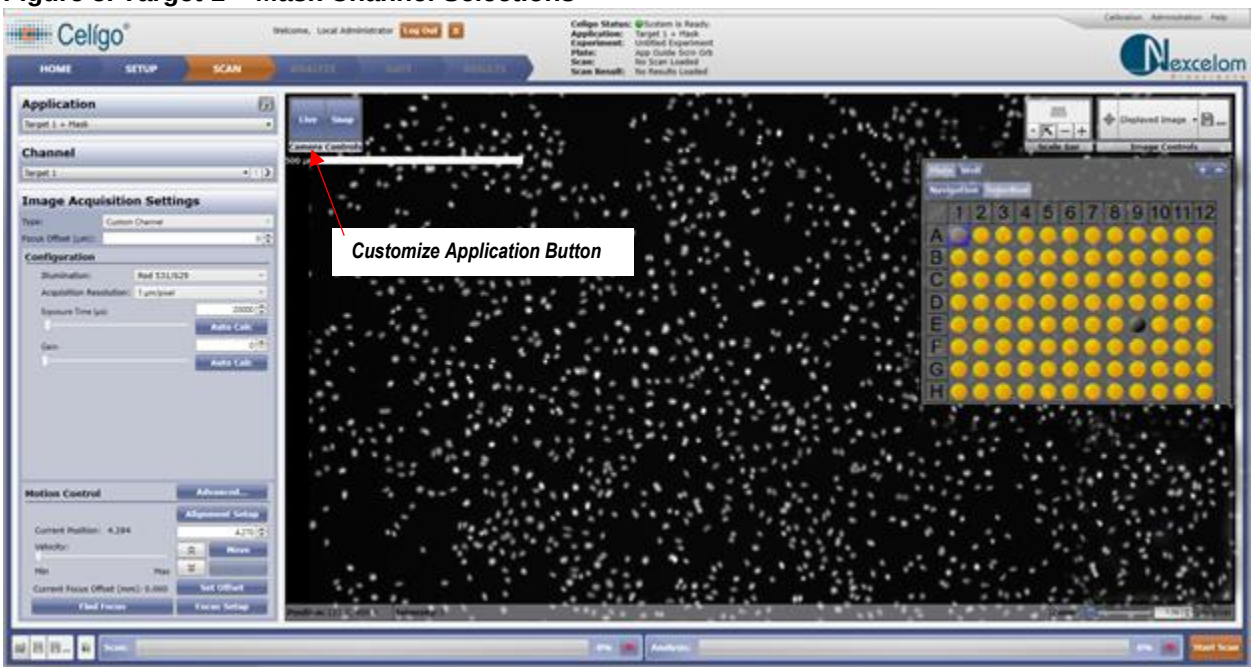
1. In Channel, select the first channel to set up.
2. For mask applications, it is recommended to select the mask channel first.
   - For independent and merge applications, it is recommended to select the channel expected to provide the highest fluorescent signal. It is easier to focus in a fluorescent channel.
3. In Type, select Custom Channel.
4. In Illumination, select the appropriate illumination (e.g., Brightfield or Blue to visualize the Hoechst signal).
5. Acquisition Resolution, select 1 µm/pixel
   - If less image resolution is appropriate for acquisition, select 2 µm/pixel in Acquisition Resolution. For a detailed explanation of image resolution, see the User Guide.
6. Click Live to see a live image.
7. Exposure Time and Gain: User manually establishes optimal exposure time and gain.
   - Adjust the Exposure Time and Gain. When doing so, adjust the exposure so the majority of cells aren't overexposed (object level pixel intensity 125-175, i.e., saturated: pixels intensities ≥255).
8. Set up autofocus (see the User Guide).
   - Typically, it is recommended to select Hardware Auto Focus (because it provides maximum speed), then click Register Auto. For manual registration or using focus offset, see the User Guide.
9. In Channel, select the next channel to set up.
10. In Type, select Custom Channel.
11. In Illumination, select the appropriate illumination for the dye
    - Green 483/536 to visualize the calcein AM signal and Red 531/629 to visualize the propidium iodide signal
12. Click Live to see a live image.
13. Click Auto Calc
    - Determine if Auto Calc provides the optimal exposure for the sample (cells are visible yet not overexposed, i.e., saturated: pixels intensities ≥255).

- If Auto Calc does not provide the optimal exposure, manually set the exposure and gain as in step 7.

14. Click Find Focus to achieve a clear image of the cells.
15. 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.
16. Set up the remaining channel(s) by repeating steps 9 through 15.

Figure 5 shows an example of Target 1 + Mask channel selections.

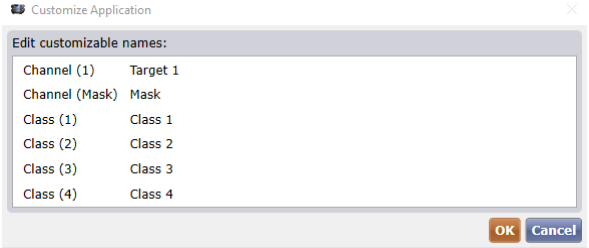
Figure 5. Target 1 + Mask Channel Selections



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



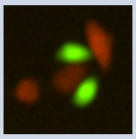
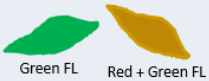
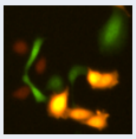
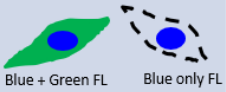
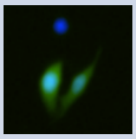
For details, see the User Guide section Changing Channel, Feature, and Class Names in the Scan Tab.

4. Analyzing Images

This chapter describes how to set up analysis for the Expression Analysis application. Perform these tasks in the ANALYZE tab.

In this application, there are three different analysis settings: Independent Mask (Section 4.1), Single Mask (Section 4.2) and Merged Mask (Section 4.3). Images from each channel are segmented according to user-specified analysis settings (see Figure 7).

Figure 7. Expression Analysis Data Analysis Settings Explained

Types of Expression Analysis		Description	
Independent	Red FL Green FL 	**Analysis Tab**: Segment objects for each channel (see Section 4.1). **Results**: Data reports counts and intensities for each channel independent of other channels **When to use**: Just want red counts, and green counts for example	
Merge	Green FL Red FL 	**Analysis Tab**: Segment objects for each channel independently, then check Merge box. Object detected from both channels in same area are merged to create one object or one single mask for that object, when Merged check box is checked (see Section 4.2). **Gate**: Define subpopulations with the objects fluorescence, i.e. "green" vs "green & red" or "red only" as sub populations of the total. **Results Tab**: Data reports objects found in the merged mask. **When to use**: No unique marker to identify all cells. Identify fusion of two fluorescently tagged cell lines.	
Mask	Green FL Blue FL 	**Analysis Tab**: The single mask defines the cell area. Segmentation need only be set up on the mask channel, which is always the last channel. Areas of interest are defined by objects found in the "Mask". It is common to use cells labelled with Hoechst (live) or DAPI (fixed), or brightfield label-free cells as the Total "Mask" image channel (see Section 4.3). **Gate**: Define the objects that are green fluorescent vs non-fluorescent as a sub population of the total. **Results Tab**: Mask channel is reported as Total in Results, unless refined in gate tab. Signal is reported for Channel 1 "Target 1" in areas defined in mask channel. **When to use**: When a percentage of objects have FL signal and total can be defined by nuclei stain or bright field morphology.	

4.1 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 selecting analysis settings for this strategy, perform 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:
   - Algorithm – Select Fluorescence, Brightfield, or Dark Object as appropriate.
   - Intensity Threshold – Enter the optimal intensity threshold.
     - The intensity threshold is the level of intensity that separates the background from cells. With an appropriate threshold is set, the background pixels fall below, and the pixels inside the cells are above the threshold. Any pixels below the threshold are not considered in subsequent calculations.
   - Precision – Higher precision results in more accurate identification of cell clusters. High is the recommended setting for this application.
   - Cell Diameter (pixels) – Enter the cell diameter (in pixels) that corresponds to the size of the cells. At full resolution, the Celigo provides 1 µm/pixel. Images acquired using lower resolutions will provide 2 + µm/pixel.
   - Separate Touching Objects – Select if needed.

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

In the Pre-Filtering section, make selections as follows:

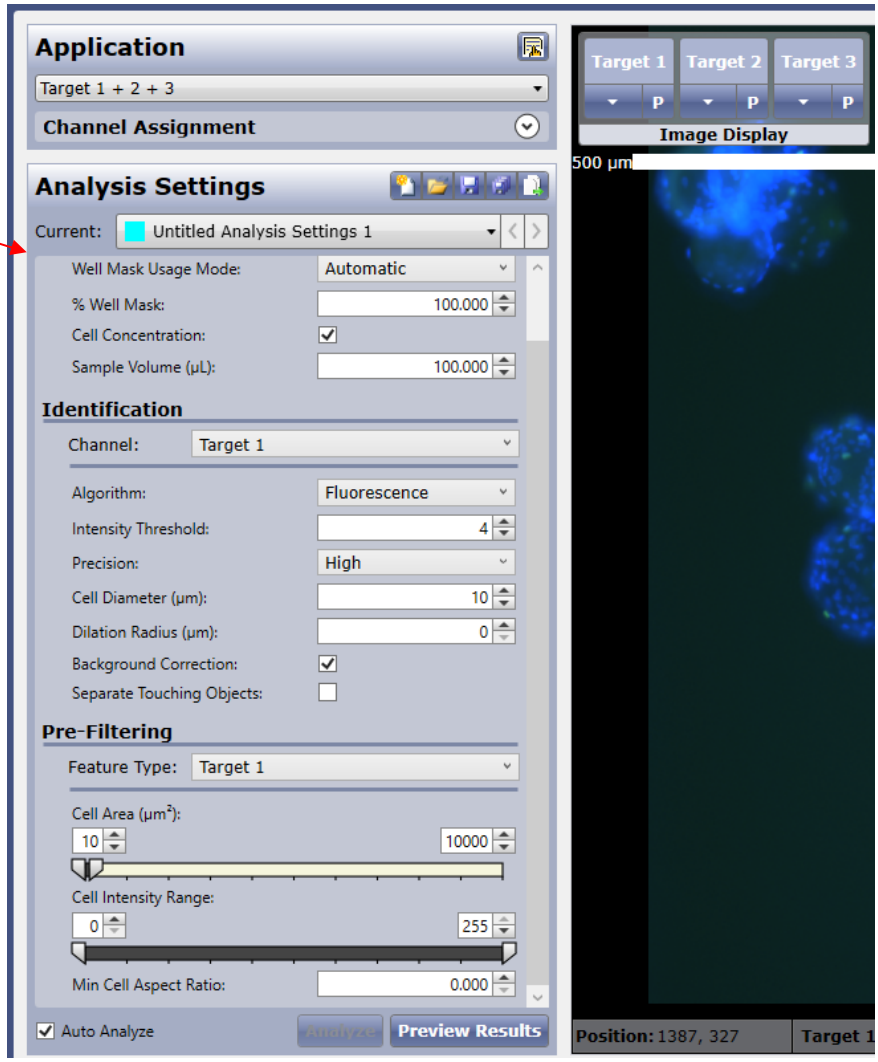
1. Cell Area Range – Enter an appropriate range.
2. Cell Intensity Range – Enter an appropriate range, if necessary.

	**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.**

3. 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.
4. Repeat these steps for each remaining channel.

Figure 7. Independent Mask Identification and Pre-Filtering Example

Channel field is available for independent mask and merge mask segmentation, to allow switching among channels for segmentation and pre-filtering.



4.2 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 selecting analysis settings for this strategy, perform the following:

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

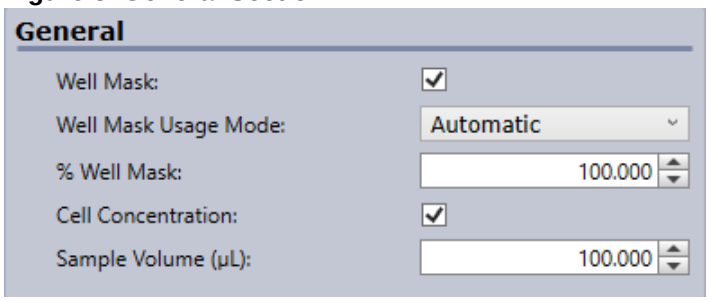
For a detailed explanation of the identification and pre-filtering settings, see the User Guide.

To select analysis settings using a single mask

1. Load prior saved Analysis Settings if available.

   In the General section (Figure 8), make selections as follows:

Figure 8. General Section

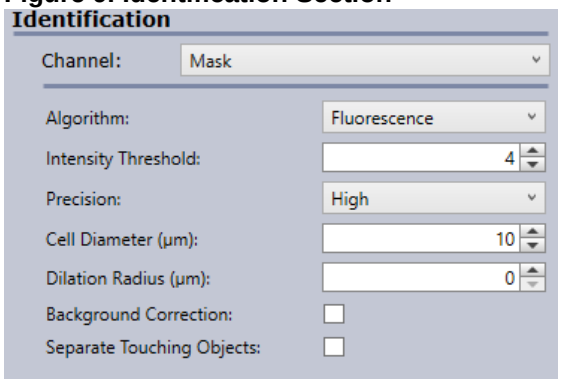


2. Well Mask – Select as needed.
3. Well Mask Usage Mode – Select as needed.
4. % Well Mask – Decrease as needed to exclude well edge artifacts that can occur during plate manufacturing.
   - For information about Well Mask, Well Mask Usage Mode, and % Well Mask, see the User Guide section Selecting General Analysis Settings.
5. Cell Concentration (cells/mL) – The system will display the cells per mL. If unchecked the system will read NaN. In order to activate this option, the % Well Mask must also be selected.

   Note: Cell Concentration is available when Well Mask is enabled.

   In the Identification section (Figure 9), make selections as follows:

Figure 9. Identification Section



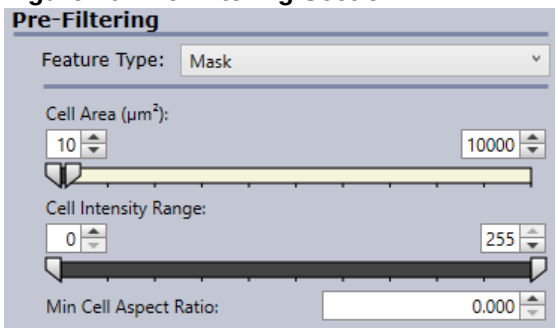
1. Algorithm – Select Fluorescence, Brightfield, or Dark Object, as appropriate.
2. Intensity Threshold – Enter the optimal intensity threshold.
   - The intensity threshold is the level of intensity that separates the background from the cells. With an appropriate threshold set, the background pixels fall below, and the pixels inside the cells are above the threshold. Any pixels below the threshold are not considered in subsequent calculations.
3. Precision – Select the appropriate precision level (High is recommended).

   For a detailed explanation of Precision, see the User Guide.

4. Cell Diameter (pixel) – Enter the cell diameter (in pixels)
   - This should correspond to the diameter of the cells in pixels.
   - At full resolution, the Celigo provides 1 µm/pixel. Decreased image acquisition resolution will provide 2 + µm/pixel.
5. Background Correction: Select if needed.
   - This selection can apply to either brightfield or fluorescent images. The brightfield images will be given an even gray scale pixel intensity while the fluorescent images' intensity values will be corrected for the fluorescent background.
6. 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.

In the Pre-Filtering section (Figure 10), do the following (not recommended if gating is to be performed):

Figure 10. Pre-Filtering Section



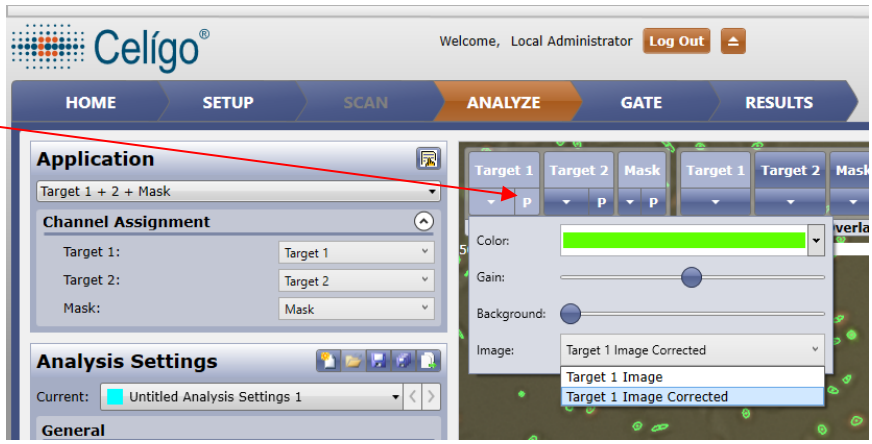
1. Feature Type – Select as needed.
2. Cell Area (pixel^2) – Enter an appropriate range.
3. Cell Intensity Range – Enter an appropriate range, if necessary.
   - The Cell Intensity Range establishes minimum and maximum intensity levels for analysis. This range may be set to filter out abnormally low 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.

4. 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 these steps for each remaining channel.

Figure 11. Single Mask Identification and Pre-Filtering Example

When Fluorescent Background is check marked, select the Image Corrected in the dropdown menu and select P to view corrected image



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 selecting analysis settings for this strategy, perform the same steps as for using an independent mask. The difference being that for this strategy the system will then automatically merge 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

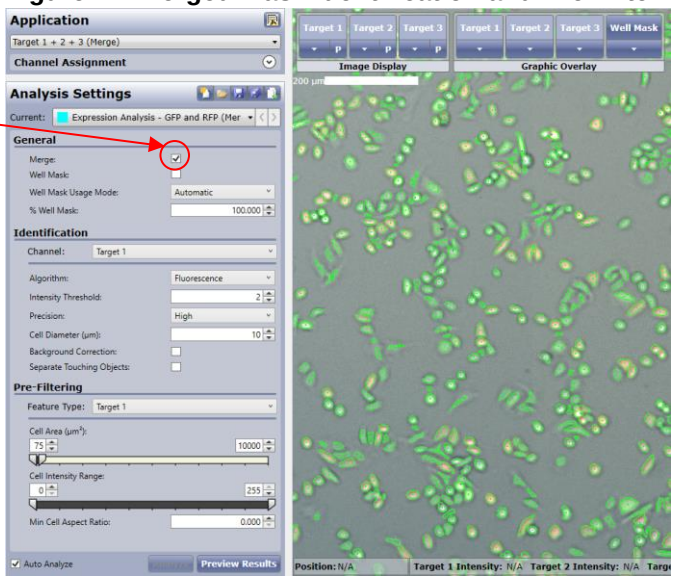
Perform Analysis Settings Using an Independent Mask per section 0.

Because the selected an application has a merge function, such as Target 1 + 2 (Merge), the system will automatically check mark the Merge checkbox (Figure 12).

	**NOTE: It is useful to un-check mark the Merge checkbox to see the independent segmentation of objects for each channel. When satisfied, check mark 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 the Graphic Overlay buttons are toggled ON and OFF, the same objects will be selected when Merge is ON.



5. Gating Cells

This chapter describes how to select filter settings for further scan data analysis. 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, 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.

When 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.

Creation of both types of plots are possible: histograms and scatter plots.

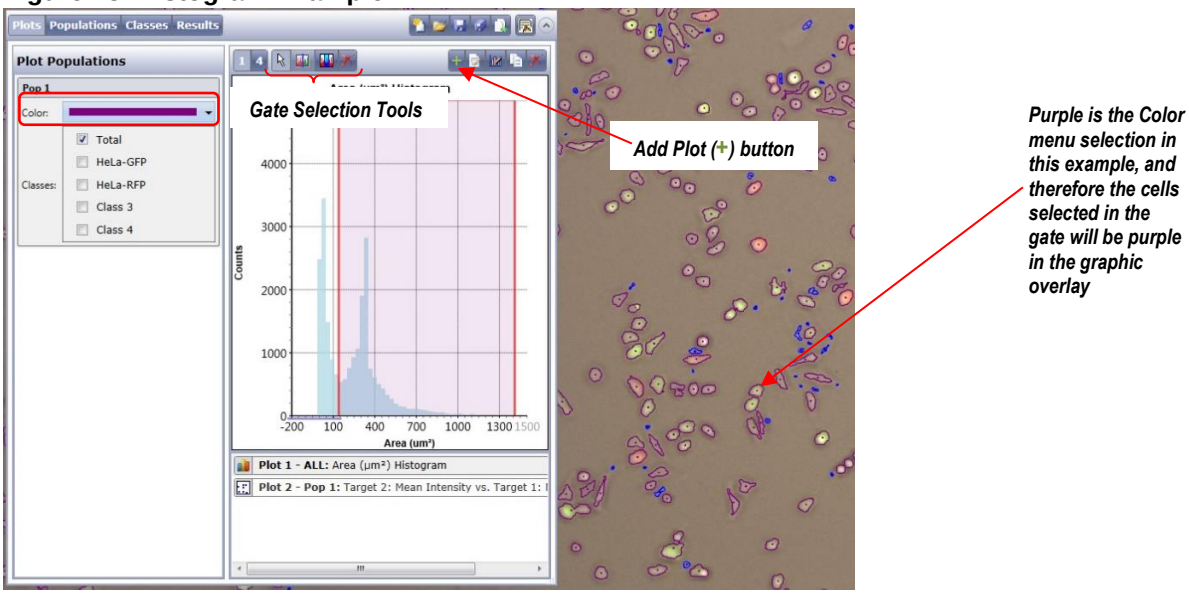
To create a plot, gate, and populations using a single or merged mask

- 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 possible in both Pick plot parameters menus when using a single mask or a merged mask. Different parameters can be combined in scatter plots to enable highly flexible selection criteria.

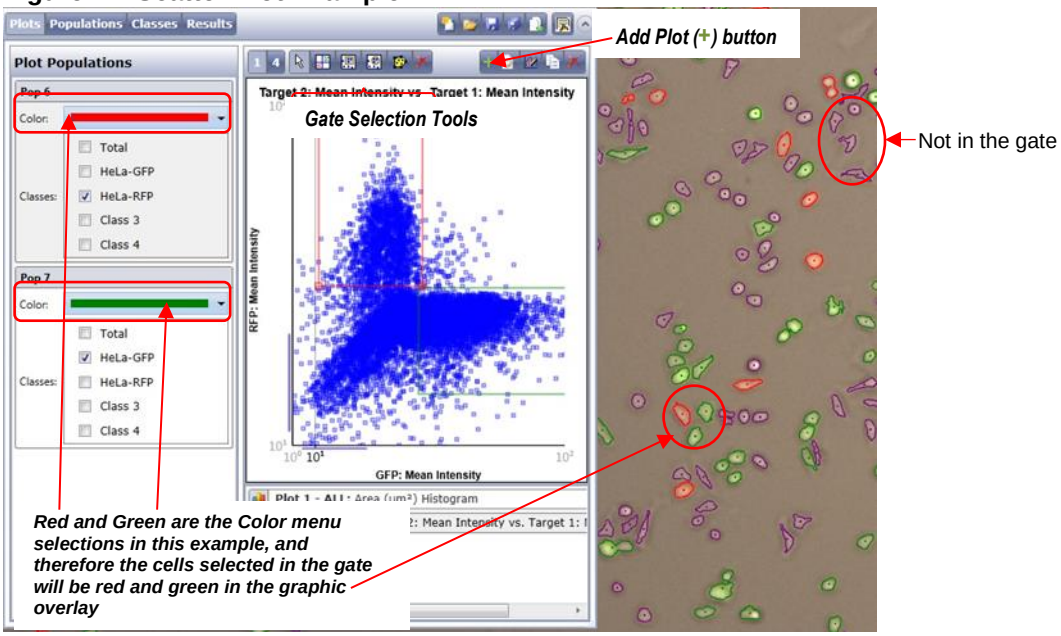
For details, see the User Guide section Creating a Plot.

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 (μm2)	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 the 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.

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.

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 no gating is performed, 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 as a result from the ANALYZE tab.
- It is recommended to 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 (a histogram is recommended for independent mask, not a scatter plot), 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 possible 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 (μm2)	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.

Feature	Description
FOR EACH CHANNEL	
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 the Total Intensity.
Integrated Intensity	Sum of all pixel intensities displaying signal in segmented objects.

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.

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 the 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

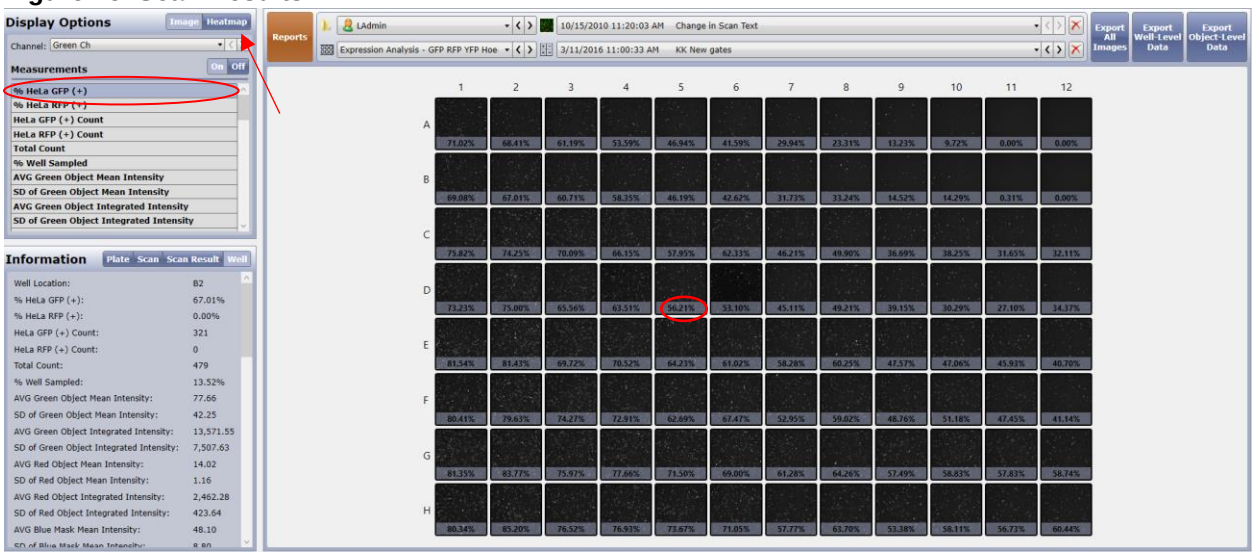
The parameters listed in Table 4 appear in the Measurements Pane on the left-hand side of the RESULTS tab.

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.
Cell Concentration (cells/mL)	Displays the cells per mL per well.
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 Intensity1	Well average integrated intensity of all cells in the specified class.
Standard Deviation of Target Integrated Intensity1	Well average standard deviation of integrated intensity of all cells in the specified class.
FEATURES REPORTED FOR EACH INDEPENDENT CHANNEL	
Total Count	Total number of cells in the well as defined by all the objects identified in all channels. Superimposing objects in multiple channels will be counted as one.
Channel Count	Count of cells in the specified channel.
Channel Count (only)	Count of cells in the specified channel not identified in any other channel.
% Channel	Percentage of cells in the specified channel as compared to the Total Count.
% Channel (only)	Percentage of cells in the specified channel not identified in any other channel as compared to the Total Count.

As analysis progresses, the wells in the plate layout in the RESULTS tab become populated with numbers (Figure 15). Select the number displayed by selecting a parameter in the list on the left. Click the Heatmap icon and 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. Detailed metrics will be reported in the left panel, 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, 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 6. 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 having the 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. G

Celigo® Cytometer
Phosphatidylserine Externalization
Application Guide

Celigo Software Version 5.2

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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: support@nexcelom.com
  phone: +1 978-327-5340
- From Europe:
  e-mail: support@nexcelom.co.uk
  phone: +44 (0) 161 232 4593

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. 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 being used, 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 the selection of 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 to adjust exposure settings using a well where the highest fluorescent signal is expected.
2. In Current Channel, select the first channel to set 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. Select whether Auto exposure provides the optimal exposure for the sample (an object pixel intensity of 125-175) where cells are visible, yet not overexposed (i.e., saturated: pixels intensities >255).
      - Auto exposure: 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 (µs). When doing so, adjust the exposure so the majority of cells aren't overexposed.
   e. If less image resolution is appropriate for acquisition, select **2 µm/pixel in Acquisition Resolution.** For a detailed explanation of image resolution, see the User Guide.
   f. Set up autofocus (see the User Guide).
      - It is recommended to select **Hardware Auto Focus** (because it provides maximum speed) and then select the **Register Auto** option.
4. In Channel, select the next channel to set 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)	6,000-15,000*	10,000-25,000*	50,000-100,000*
Gain	0	0	0
Motion Control and Focus	See User Guide	See User Guide	See User Guide

*For all fluorescent channels, the object pixel intensity should be between 125 and 175 (targeting 150). See the User Guide for more information on appropriate exposure time

Figure 2a shows an example of PS Externalization channel settings and Figure 2b displays an appropriate exposure setting for that channel.

Figure 2a. PS Externalization Channel

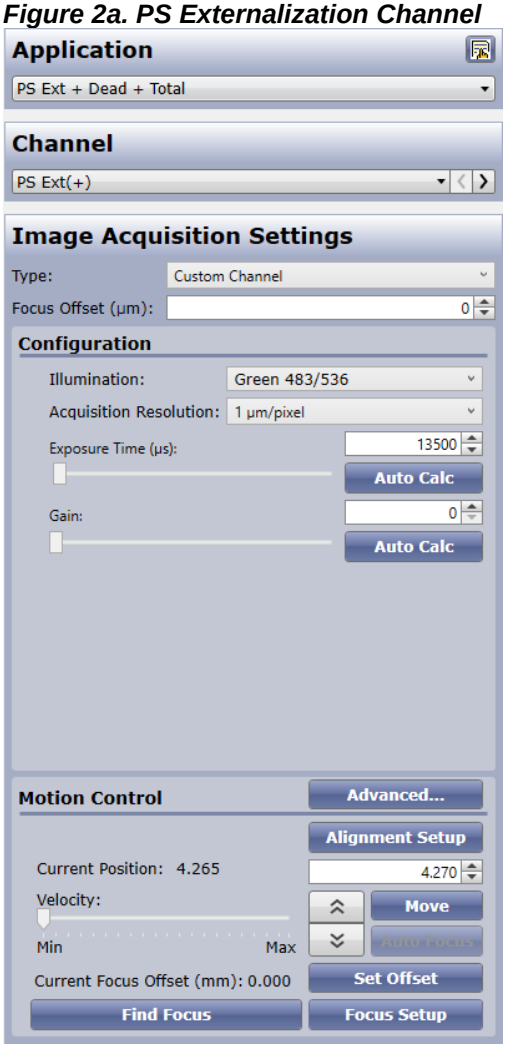
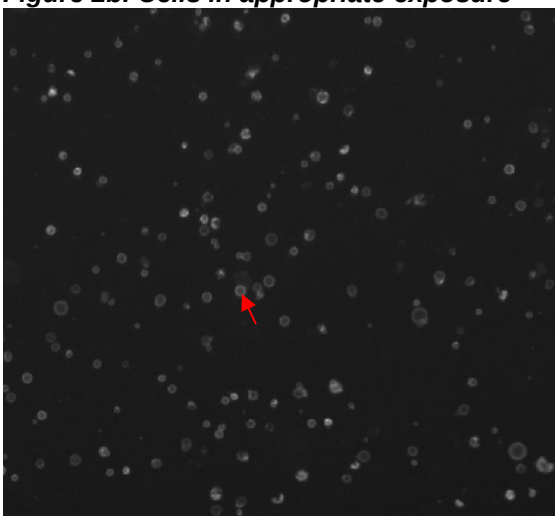


Figure 2b. Cells in appropriate exposure



Hovering the mouse over the cells will display the Intensity at the bottom of the SCAN tab.

4. Analyzing Images

This chapter provides information on how to analyze scans from the PS Externalization application. 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 objects identified in the Total channel are included in the analysis. This allows objects that are fluorescent in the wells and are not cells, to be excluded from the analysis.**

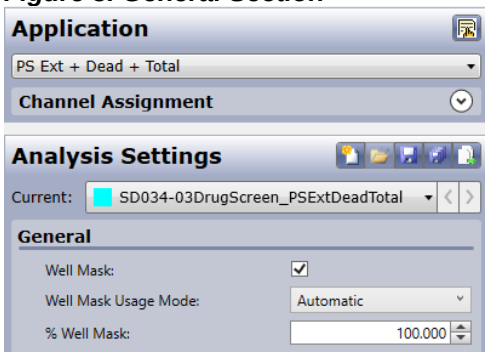
4.1 Analysis Settings

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 the segmentation of cells at the well edge are 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.**

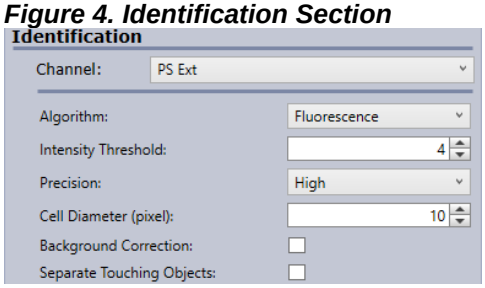
b. Well Mask Usage Mode – Select as needed.

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

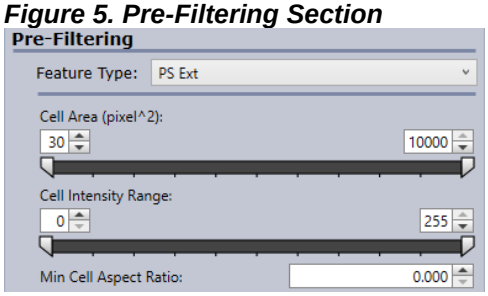


   a. Channel – Select one channel to be analyzed.
   b. Algorithm – Select **Fluorescence**.
   c. Intensity Threshold – Enter the optimal intensity threshold.

      The intensity threshold is the level of intensity that separates the background from cells. With an appropriate threshold, the background pixels fall below and the 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 is recommended.
   e. Cell Diameter (pixel) – Enter the cell diameter (pixels) that corresponds to the cells. At full resolution, the Celigo cytometer provides 1 µm/pixel. Lower resolution acquisition provides 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 is not typically 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 Check marked	Not Check marked	Not Check marked
Separate Touching Objects	Not Check marked	Check marked	Check marked
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

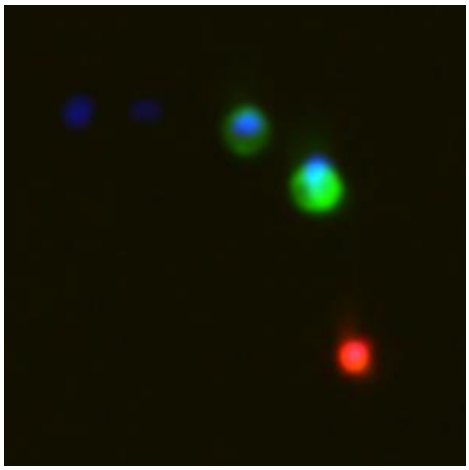
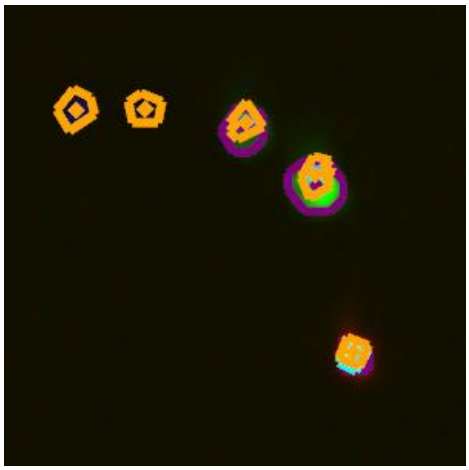


Figure 7. Image + Target Overlays of Image on Left



5. Gating Cells

When using the PS Externalization application, gating is not typically 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	√	√

(continued on the next page)

(continued from previous page)

Parameter	Description	PS Ext (+) + Total	PS Ext (+) + Dead + Total
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		√
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 bleed through 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. F

Celigo® Cytometer
Invasion and Migration: Wound Healing Application Guide

Celigo Software Version 5.2

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1. About this Guide

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

1.1 Introduction

The Invasion and 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 Invasion and Migration: Wound Healing application. Information that is common to all applications is covered in the *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: support@nexcelom.com
  phone:+1 978-327-5340
- From Europe:
  e-mail: support@nexcelom.co.uk
  phone: +44 (0) 161 232 4593

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	Vendor	Cat#	Volume
384-well	Platypus Technologies	PRO384CMA5	≥ 40 µl
384-well high vol	Greiner	781091	≥ 40 µl
96-well	Greiner	655090	≥ 175 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 Invasion and Migration: Wound Healing application and setting the image acquisition parameters. Perform these tasks in the SCAN tab.

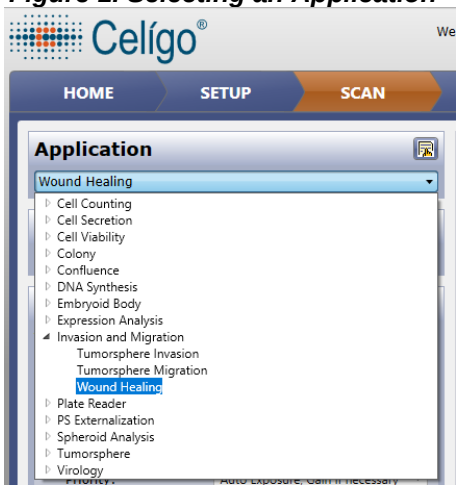
3.1 Invasion and Migration: Wound Healing Application

Perform the following steps to select the Invasion and Migration: Wound Healing application.

To select an application

In the Application menu, select **Invasion and 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**.
     - After these selections, click **Apply**
   - For Fluorescent 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 lower resolution acquisition, see the User Guide.

4. Set up focus per the User Guide.
   - For detailed instructions on selecting the 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

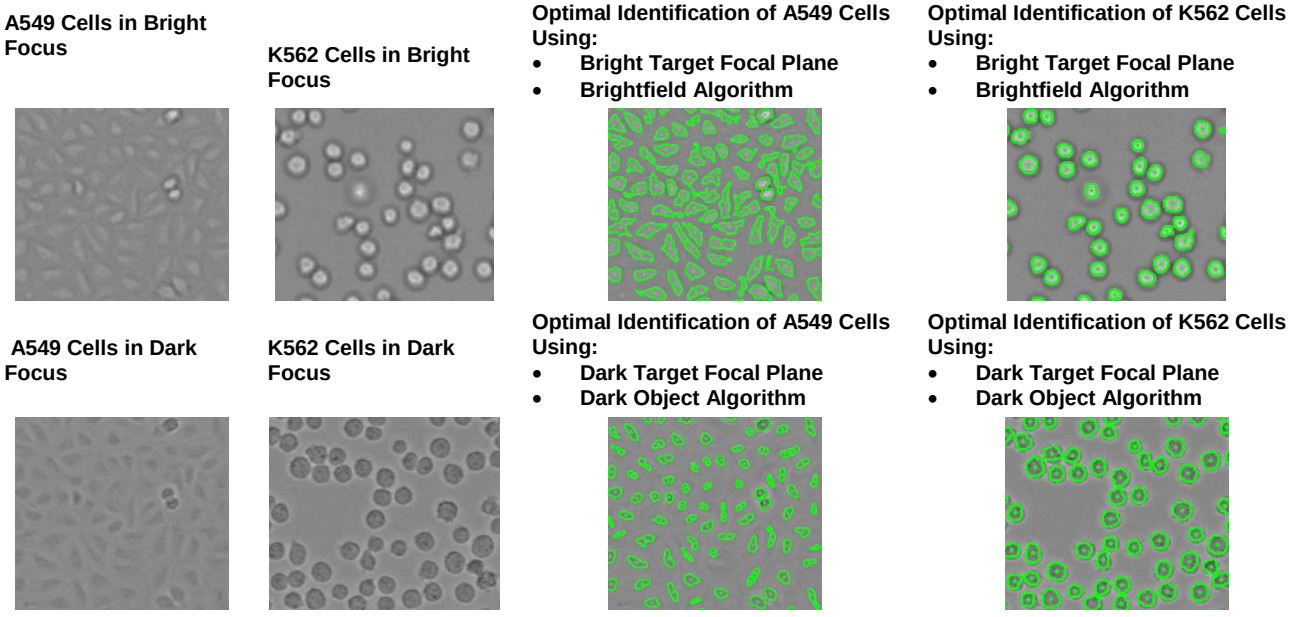
The proper focus for brightfield illumination is important for optimal application performance. There are two image planes visible on the Celigo Cytometer using brightfield illumination. The Dark image plane is the real image plane in which objects appear dark compared to surrounding background regions. 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 fluorescent 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 double clicking 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 see 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, especially 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 Invasion and Migration: Wound Healing application. Perform these tasks in the ANALYZE tab.

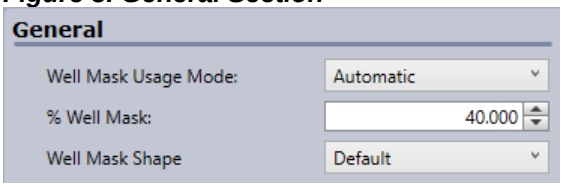
4.1 Analysis Settings

Perform the following steps to select analysis settings. The recommended initial analysis settings for identification and pre-filtering when using the Invasion and 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%**. This allows the reduction of 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.

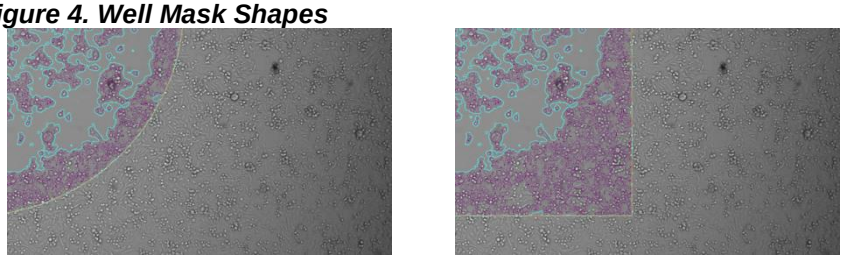
   c. Well Mask Shape – Select one of the following as needed (Default is the well shape) to define the shape of the exclusion zone for the Wound Healing experiment. Typically, a round well mask is used for Platypus Oris plates and a square well mask is used for scratches with a pipette tip.

      – Circle – System uses a circular shape.

      – Square – System uses a square shape.

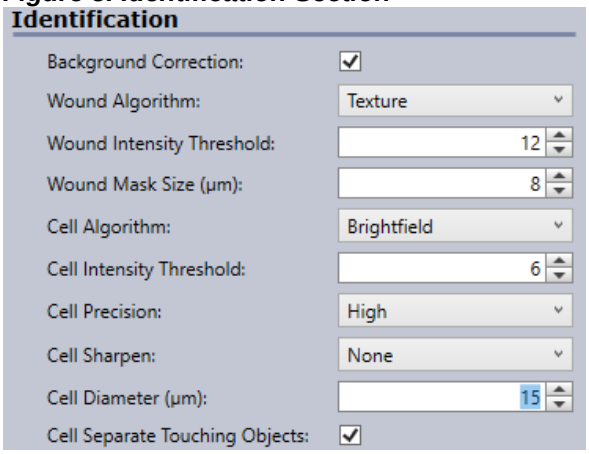
      Figure 4 shows an example of the same well with a Circle (on left) versus a Square (on right) selected in the Well Mask Shape menu.

Figure 4. Well Mask Shapes



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

Figure 5. Identification Section



a. Background Correction – Check mark 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 the background from the area of growth. With an appropriate threshold set, background pixels will fall below and the pixels in the area of growth will fall above the threshold.

d. Wound Mask Size (µm) – Select as follows:
   - If Texture was not selected in the Identification Section's Wound Algorithm field in step 3, fill in Wound Mask Size.
   - If Texture is selected, do not fill in the 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 – This algorithm looks for objects with a bright center and dark edges
   - Fluorescence – This algorithm looks for fluorescent objects (bright pixels over darker background)
   - Dark Object – This 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 the background from cells. With an appropriate threshold set, the background pixels fall below and the pixels inside the cells fall above the threshold.
   - Flat, adherent cells 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 is recommended.

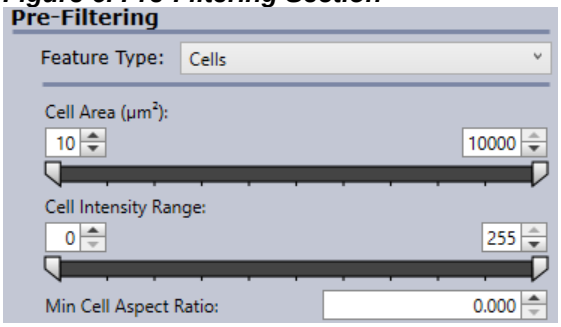
h. Cell Sharpen – Select to enhance the object's edge contrast before image processing.

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 are 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 are often dark (brightfield imaging) or very bright (fluorescent). 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, check mark this selection as needed. For information, see the User Guide.

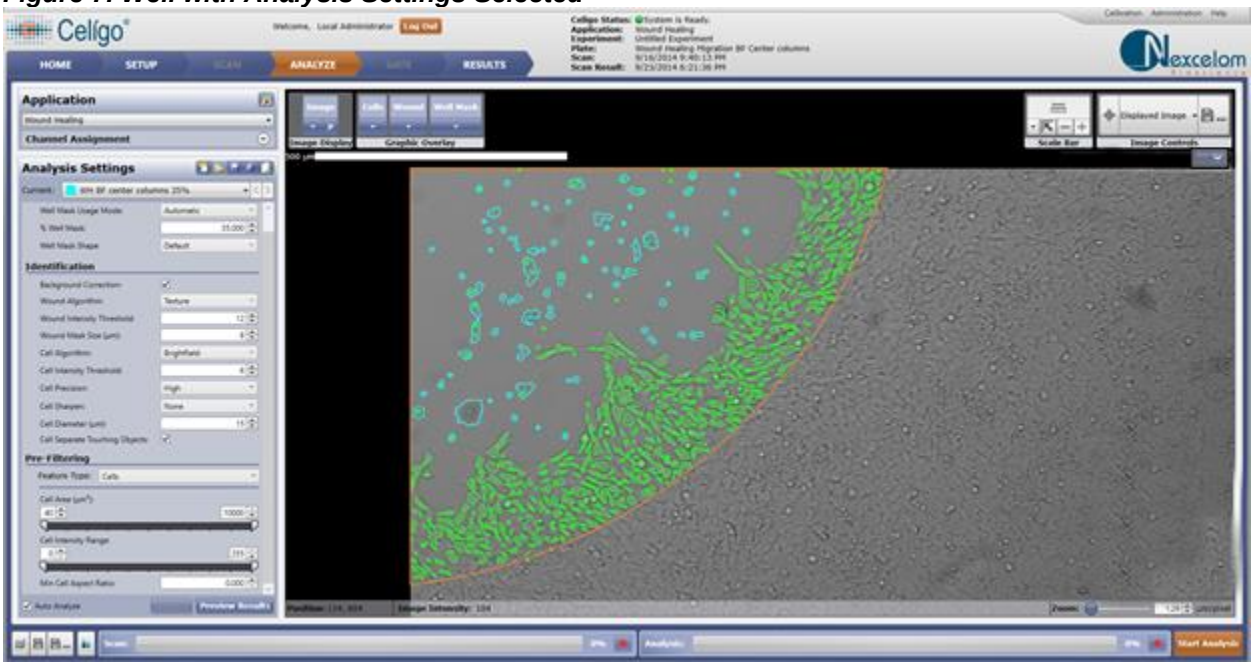
Table 2. Recommended Initial Identification and Pre-Filtering Settings for Analysis

IDENTIFICATION		PREFILTERING	
Background Correction	Check marked	**Feature Type**	40 – 1000
Wound Algorithm	Texture	**Cell Intensity Range**	0 – 255
Wound Intensity Threshold	10	**Min Cell Aspect Ratio**	0
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	Check marked		

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
- Green – The cells growing within the wound area
- Orange – The well mask

Figure 7. Well with Analysis Settings Selected



5. Viewing Results

This chapter describes the feature outputs available from the Invasion and Migration: Wound Healing 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 Invasion and Migration: Wound Healing application.

Table 3. Wound Healing Application Outputs

Parameter	Description
Cell Count	Number of Total positive cells 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 the total area of the scan area or well
Well Sampled (%)	Percent of the well sampled

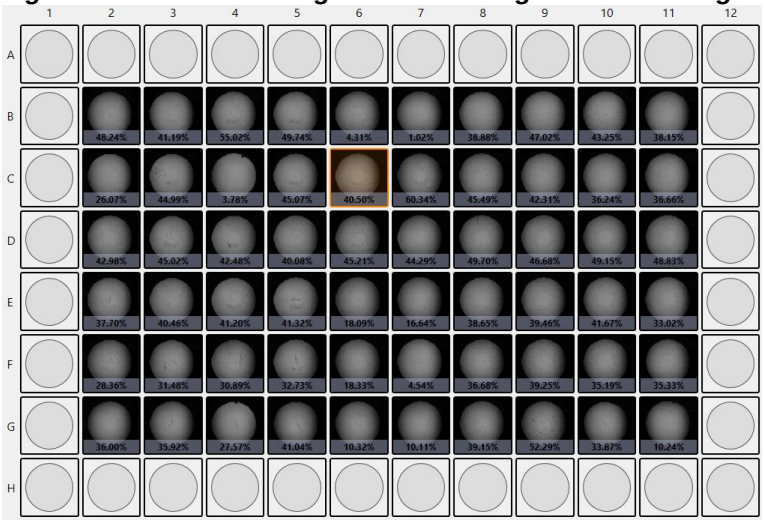
5.1 Image, Fill, and Heatmap Views

This section describes the purpose of the Image, Fill, and Heatmap views in the Invasion and Migration: Wound Healing application. In this application, choose between Image, Fill, and Heatmap view within either the plate view or well detail view. This helps visualize the properties and patterns of interest.

- Image button – Turns on/off the raw image display
- Fill button – Fills all identified objects with a selected color (default is green)
- Heatmap – Allows a visual aide for quick identification of patterns in the results

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). In the examples shown in the below two figures, the user has selected well C6.

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



An alternative view is the plate-level Fill view, which provides a confluence overlay of any identified colonies (Figure9). This display provides an overview of the wound healing progress.

Figure 9. Plate-Level Fill (Confluence Overlay) View

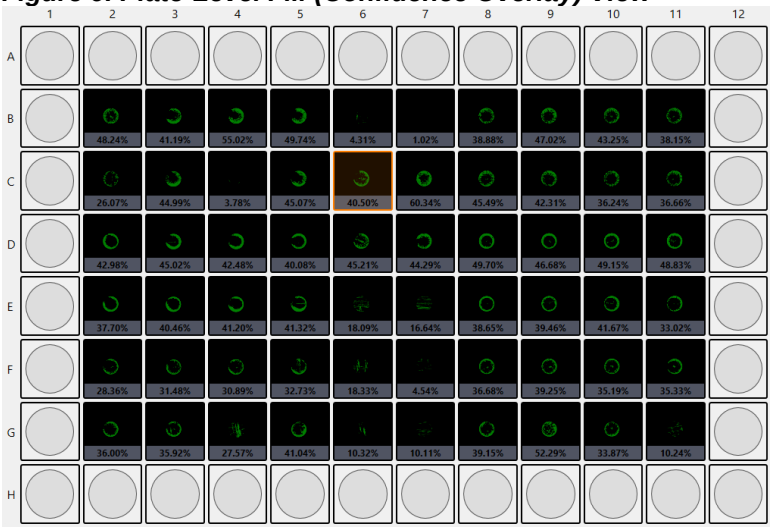


Figure 10 displays the plate-level Heatmap view, which allows users to quickly identify patters in the results provide by the measurements window to the left in the RESULTS TAB

Figure 10. Plate-level Heatmap view in the SCAN tab

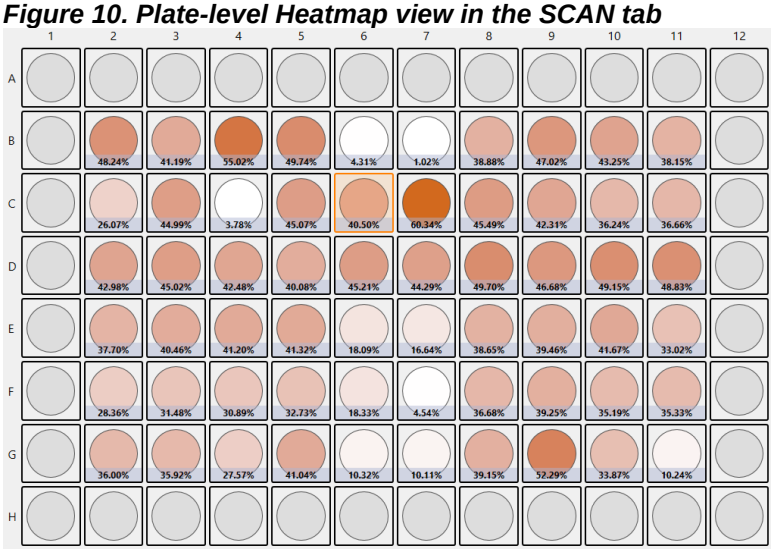
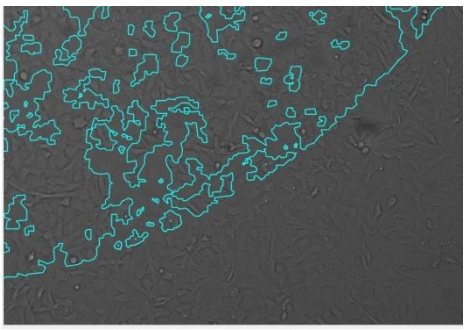


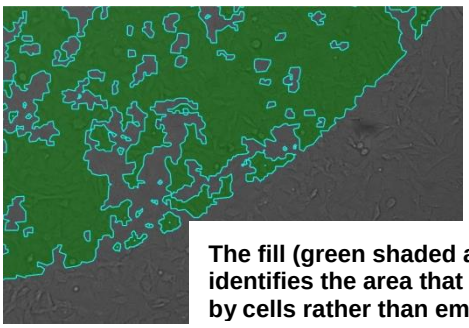
Figure 9 shows well A7 at the well detail view, with fill off versus on.

Figure 9. Well Detail Fill (Confluence Overlay) View Magnified

Fill Off



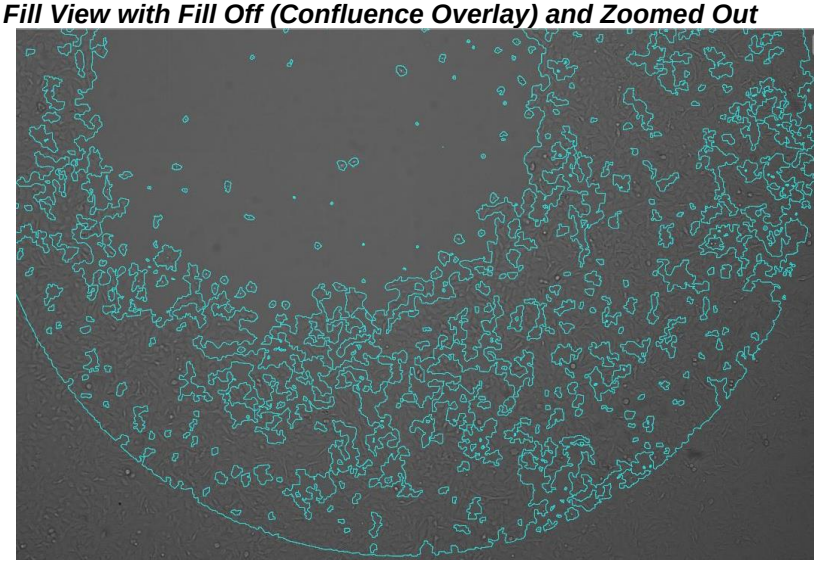
Fill On



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

Figure 10 shows well A7 zoomed out.

Figure 10. 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 determines doubling times and rates for individual wells. The reports are in the forms 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

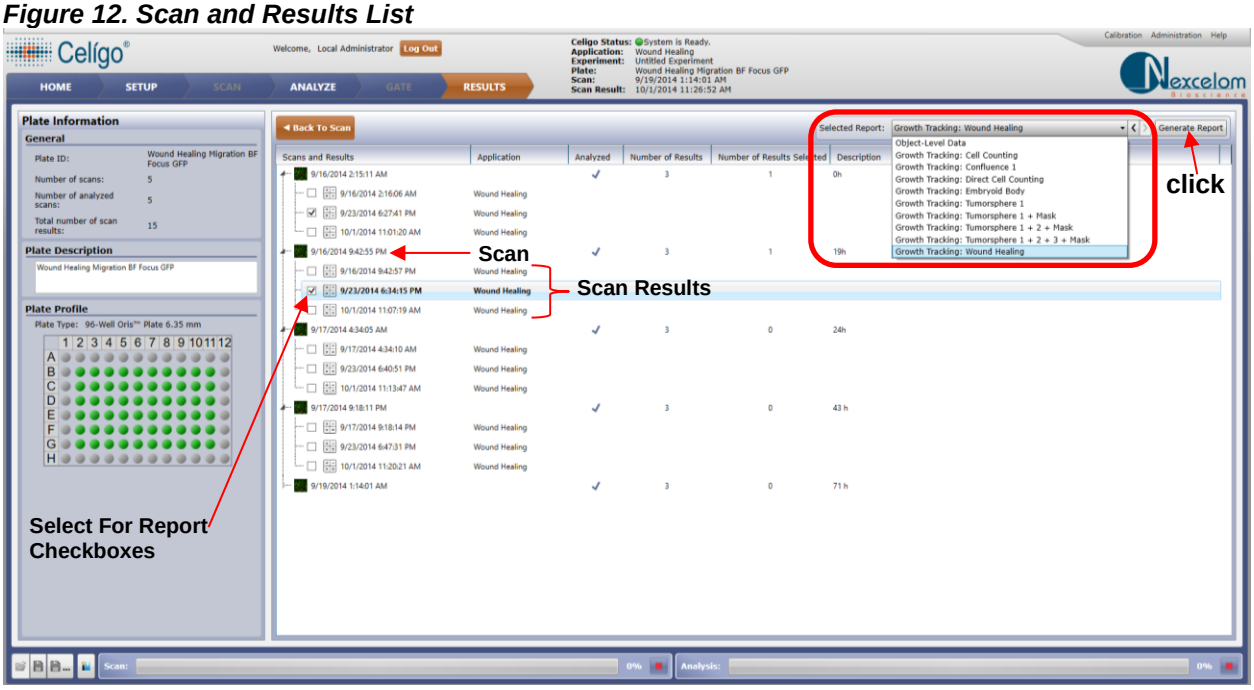
	**NOTE: To generate a pie chart, make sure that 4 or more scans exist for selection. To generate a growth chart (curve), make sure that 2 or more scans exist for selection.**

1. In the RESULTS tab, click **Reports** (Figure 11)

Figure 11. Displaying Scans for Reporting

The list of existing scans and associated scan results for the plate ID appears (Figure 12).

Figure 12. Scan and Results List



2. Click the Scan Time desired for generating a wound healing growth tracking report.

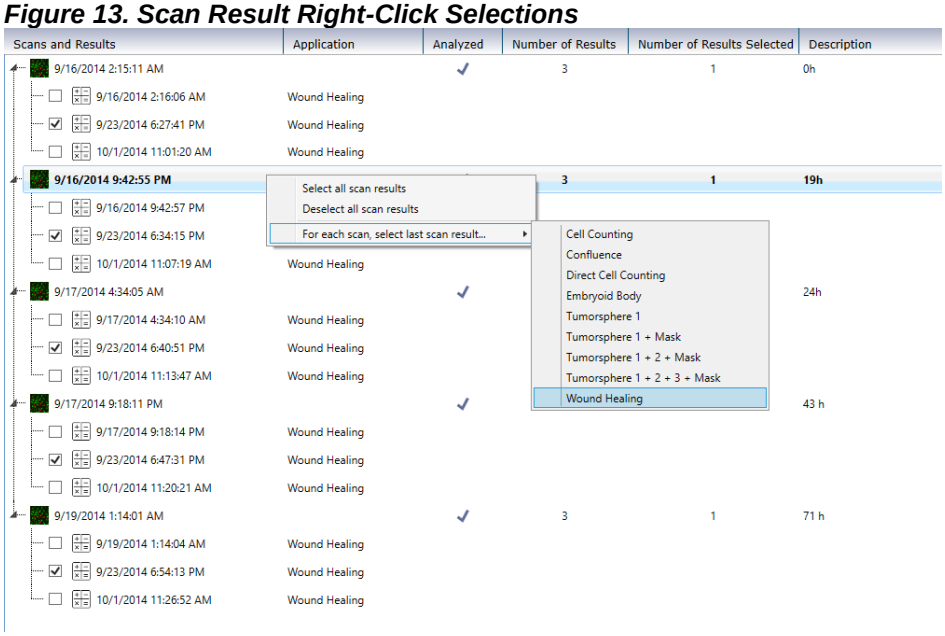
   The Scan Time selection highlights in blue.

3. Checkmark the Select For Report checkbox (Figure 12 above) next to each scan result desired for the report.

   Another option is to right-click a scan result and select one of the following (Figure 15):

   - Select all scan results
   - Deselect all scan results
   - For each scan select last scan result...Wound Healing

Figure 13. Scan Result Right-Click Selections



4. In the Selected Report menu (Figure 12), select **Growth Tracking: Wound Healing**.

	NOTE: Only the scans analyzed using the application selected will be available.

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 of the scanned area of the plate; a well is not selected by default.

5.3 Working with a Generated Report

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 **Reports** is clicked in the RESULTS tab, the Detailed View in the resulting growth chart is a summary view; at this point a well is not yet selected. Then, when a well is selected, a chart specific to that selected well will be displayed 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 14) 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 14) – 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.

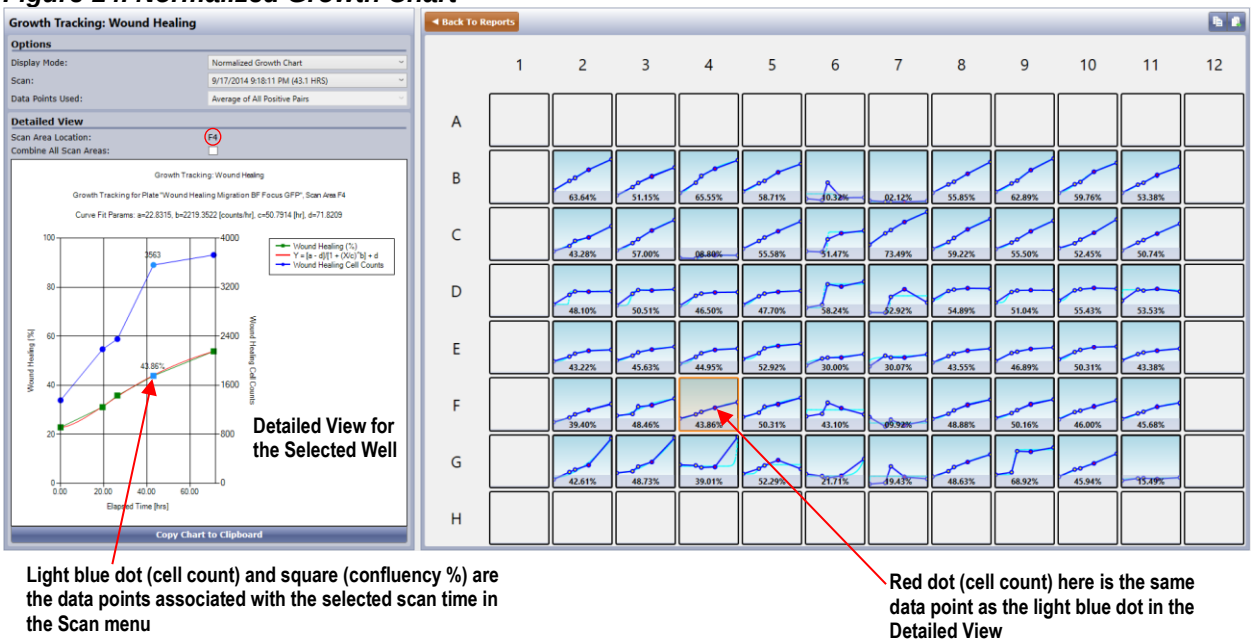
- Growth Chart – Displays the wound healing growth curve for a given well, based on the Wound Healing % measurement, and the fitted wound healing growth curve. Each Scan Area plot is fitted for the display window by varying the Y axis range. The curve is fitted using a standard Four-Parameter Logistic 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 16).

- Count Chart – Displays the well count and growth curve within the well mask area.

Figure 14. Normalized Growth Chart



The difference between the displays from selecting a Display Mode of Growth Chart versus Normalized Growth Chart is illustrated in Figure 15. 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 15. Growth Chart vs. Normalized Growth Chart Thumbnail Images

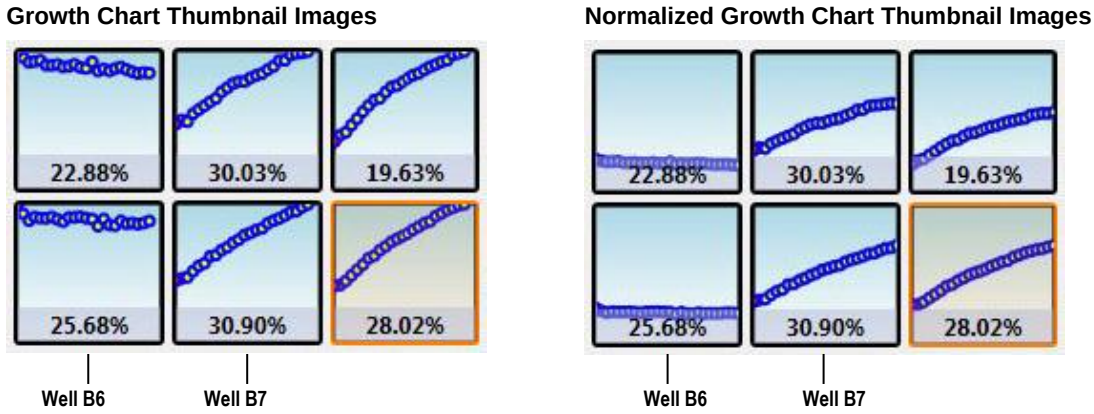
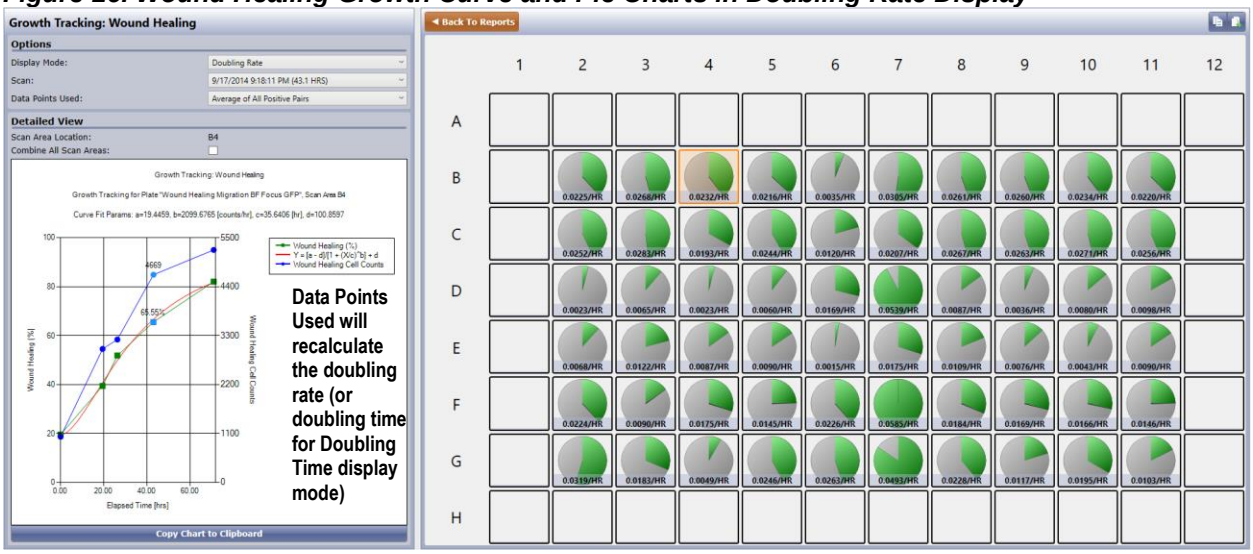


Figure 16. Wound Healing Growth Curve and Pie Charts in Doubling Rate Display



5.3.3 Exporting Report Data

Export wound healing report data by clicking **Export GT Report** (Figure 14 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 a well occupied by individual cells or cell clusters (Brightfield imaging only) divided by total area of scan area or well
% Well Sampled	Surface of the well that was sampled
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 14 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 the *User Guide* for instructions) • Change Pre-Filtering settings to identify desired objects 3. Identification settings do not identify objects. • Recommend following the tips for identifying individual cells (see section 3). 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 have unique properties that can be used to remove them from the scan results. • 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 is 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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 8001630 Rev. F

Celigo® Cytometer

Colony Analysis Application Guide

Celigo Software Version 5.2

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1. About this Guide

The Colony Analysis applications follow a generic fluorescence protocol for up to four separate channels in addition to a brightfield channel. Colonies can be identified label-free in the brightfield channel and fluorescence signal in the four different channels can be quantified. Using an advanced gating interface, calculated colony-based features such as shape, size, and intensities can be used to create gates, or selection criteria, to analyze specific subpopulations of colonies.

1.1 Purpose

The purpose of this guide is to provide the steps and concepts that are unique to the Colony Suite applications. Information that is common to all applications is covered in the *Celigo Cytometer User Guide* (Doc. No. 8001619), henceforth referred to as 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:
E-mail: support@nexcelom.com
Phone: +1 978-327-5340

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E-mail: support@nexcelom.co.uk
Phone: +44 (0) 161 232 4593

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

Samples are 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 the performance of the application. (See Troubleshooting section 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	2500	ND[1]
6W	Corning	3471	2500	ND[1]
12W	Corning	3512	1000	ND[1]
24W	Corning	3524	≥500	ND[1]
96W	Greiner	655090	≥200	≥100
96W half area	Greiner	675090	50	50
384W low vol	Corning	3542	20	20
384W high vol	Corning	3764	≥40	≥40
384W high vol	Greiner	781091	≥40	≥40
1536W	Corning	3838	8	8
1536W	Greiner	789866	8	8
T-25	Corning	43069	ND[1]	ND[1]
T-75	BD Falcon	353136	ND[1]	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 Colony Analysis application, and setting the image acquisition parameters. Perform these tasks in the SCAN tab.

3.1 Segmentation Strategies

The Colony Analysis application provides two types of segmentation (identification) strategies that can be applied to scans ranging from 1 to 5 channels:

Identification can be done using either of the following strategies:

- Single Mask – Identification is performed in one channel, called the section Mask section channel, and then applied to every 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 colony outline defined in a fluorescent or brightfield channel. The mask is used to outline the colony in all channels and define the area where measurements will occur.**

Each of the two segmentation strategies are associated with specific Colony Analysis applications.

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

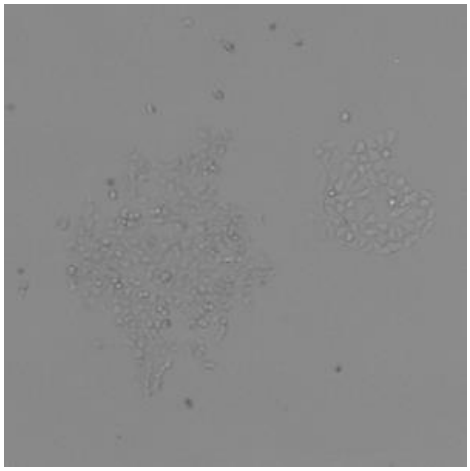
3.1.1 Single Mask Segmentation

The single mask segmentation strategy uses only one channel to identify colonies in the images, and the resulting mask will be used to quantify images from all the other channels.

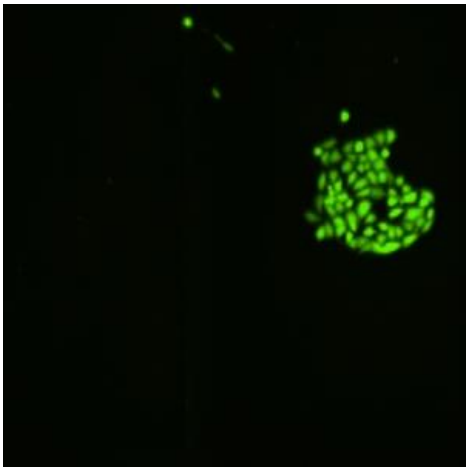
Figure 1 shows the brightfield and fluorescence imaging of colonies expressing GFP and the Single Mask segmentation. Brightfield imaging of two colonies are shown in panel A. The corresponding GFP image is showed in panel B with only one of the colonies being positive for GFP. Panel C shows both channels. The Single Mask is defined by the brightfield channel and the individual colony fluorescence are measured within the Single Mask for all of the colonies. Morphologic measurements such as colony size will all derive from the single mask segmentation.

Figure 1. Colony Single Mask Segmentation Example

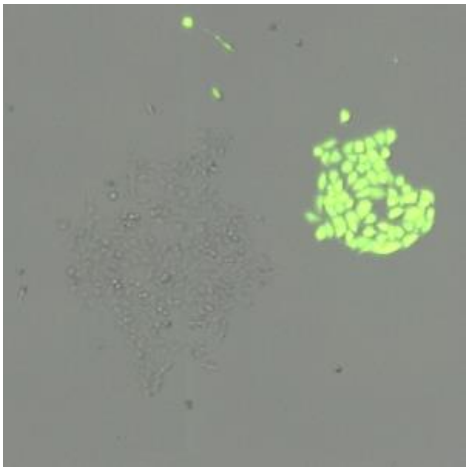
(A) Brightfield image of two adjacent colonies with the segmentation graphic overlay turned off



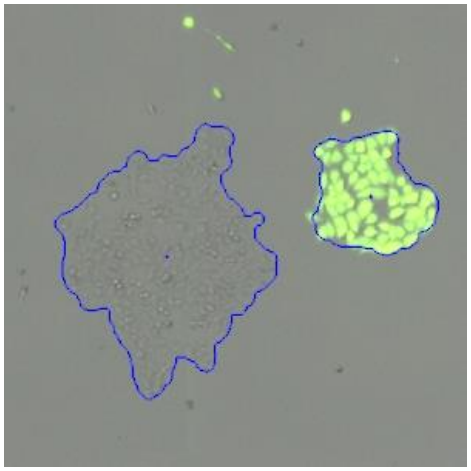
(B) Fluorescent image of the corresponding area showing that only one colony is positive for GFP



(C) Dual color brightfield and fluorescent images of the two colonies.



(D) Segmentation graphic overlays turned on. The resulting brightfield mask will be transferred to the green channel for intensity measurements



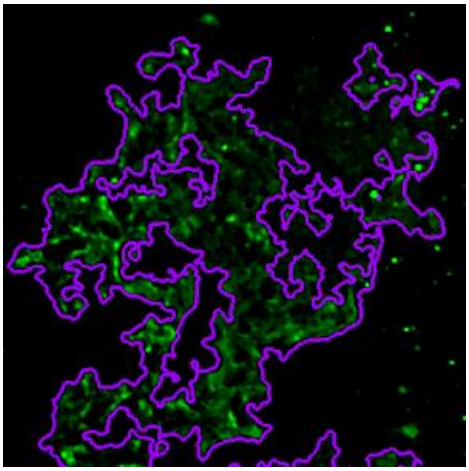
3.1.2 Merge Segmentation

The merge mask segmentation strategy implements an additional step as compared to the single mask segmentation. In brief, colonies are identified in all channels. In this example, iPS colonies stained with SSEA-4 green marker and Hoechst were separately identified in the green and blue channels (Figure 2 panel A, B). A merging step then occurs that will merge the segmentation of all independent channels (Figure 2 panel C) into one merged mask (Figure 2 panel D). As a result, the application will use this single merged mask to measure intensities in all channels. Similar to the single mask, the

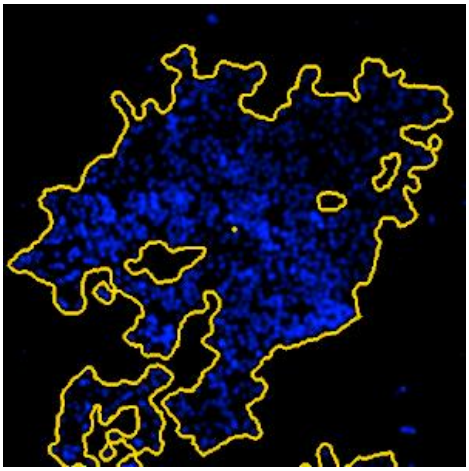
merged strategy reports only one set of morphological measurements for the merged colony mask.

Figure 2. Merged Mask Segmentation Example

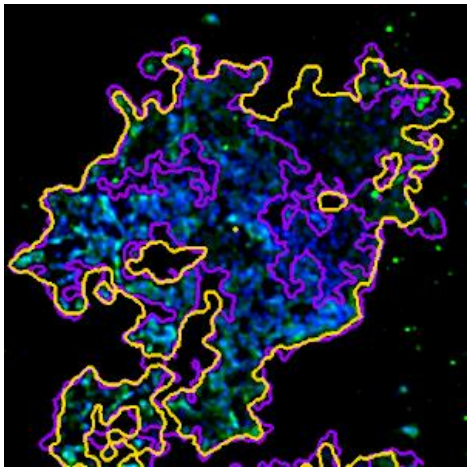
(A) Segmentation of colonies in Green channel.



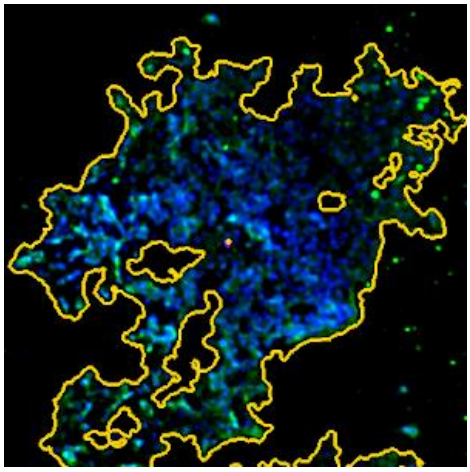
(B) Segmentation of colonies in Blue channel.



(C) Both channels and both overlays independent



(D) Both channels and single overlay Mask Merged



3.2 Colony Analysis Application

Perform the following steps to select a Colony analysis application.

To select an Colony Analysis application

In the Current Application menu, select one of the 7 Colony Analysis applications (Figure 3 and Table 2. Selecting a Colony Application). Selection is dependent on how many channels (targets) are necessary to acquire, and whether or not a single-mask or merge segmentation strategy is going to be used.

The choice of how cells are identified impacts how data is collected, as described in section 3.1. To clarify, Table 2 includes a summary of the differences and gives examples of some use cases.

Figure 3. Selecting a Colony Application

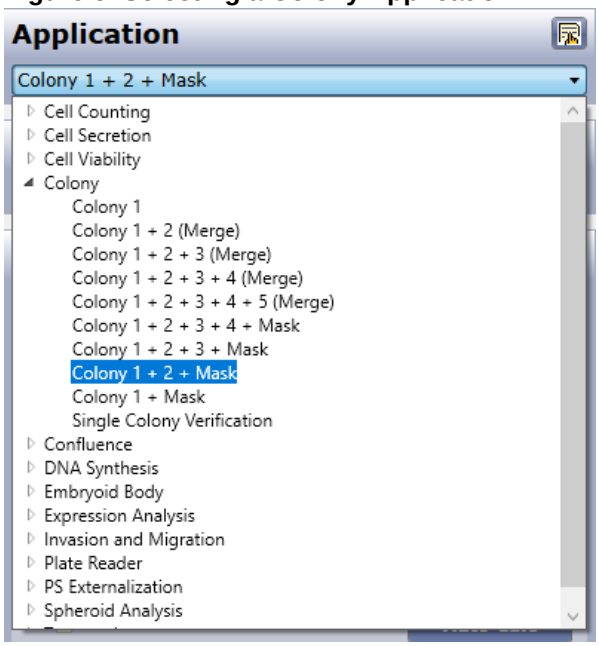


Table 2. Selecting a Colony Application

Quantity of Channels (Targets) to be Acquired	Select this application	How Image Intensity is Measured	Use Case
Single Mask			
Four channels + a mask	**Colony 1 + 2 + 3 + 4 + Mask**	The mask defines the colony. Fluorescence or brightfield signal is measured in the same mask.	Measure the reporter gene expression in colonies.
Three channels + a mask	**Colony 1 + 2 + 3 + Mask**		
Two channels + a mask	**Colony 1 + 2 + Mask**		
One channel + a mask	**Colony 1 + Mask**		
One channel (brightfield or fluorescence)	**Colony 1**	A colony is defined in one channel.	One unique marker to identify all colonies.
Merge			
Two channels merged	**Colony 1 + 2 (Merge)**	A colony 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 colonies, but the assay requires intensity measurements of all the colonies in every channel
Three channels merged	**Colony 1 + 2 + 3 (Merge)**		
Four channels merged	**Colony 1 + 2 + 3 + 4 (Merge)**		
Five channels merged	**Colony 1 + 2 + 3 + 4 + 5 (Merge)**		

3.3 Acquisition Settings

Perform the following steps to select image acquisition settings. The example provided here is for a two channel colony application, Colony 1 + Mask where the first channel is for GFP and the second channel is for brightfield.

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 to adjust exposure settings using a well where the highest fluorescence signal is expected.
2. In Channel, select the **Mask** channel.
   - For Mask applications, it is recommended to select the mask channel first.
   - For merge applications, it is recommended to select the channel that is expected to provide the highest fluorescence signal. It is easier to focus in a fluorescent channel.

For the selected channel, make the following selections:

3. For Brightfield illumination:
   - Select type: **Auto Exposure/Gain Channel**
   - In Illumination, select **Brightfield**.
   - In Priority, select **Auto Exposure, Gain if Necessary**.
   - In Frequency, select **Every Scan Area**.
4. Click Apply
5. Click **Live** to see a live image.
6. If less image resolution is appropriate for acquisition, select **2 µm/pixel in Acquisition Resolution.** For a detailed explanation of image resolution, see the User Guide.
7. Click **Focus Setup** (see the User Guide).
   - Typically, it is recommended to select **Hardware Auto Focus** (because it provides maximum speed), then click **Register Auto**. For manual registration or using focus offset, see the User Guide.

8. In Channel, select **Colony 1**.
9. For Fluorescent illumination:
    - Select channel type: **Custom**
    - In Illumination, select the appropriate illumination (e.g., Green 483/536 for GFP signal).
10. Select whether the fixed exposure time provides the optimal signal (an object pixel intensity of 125-175) where cells are visible, yet not overexposed (i.e., saturated: pixels intensities >255), and do one of the following.
    - Adjust **Exposure** time and **Gain** manually. When doing so, adjust the exposure so the majority of cells aren't overexposed.
    - Apply **Auto Calc** if necessary: the software will calculate an exposure time or a gain so that the signal is visible without saturation of the image.
11. Click **Live** to see a live image.
12. Click **Find Focus** to achieve a clear image of the cells.
13. 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.

Set up the remaining channel by repeating steps 8 through 12.

To change the channel, feature, and class names

Customize the channel, feature, and class names as desired (optional), using the Customize Analysis Application button and the resulting Customize Application dialog box (Figure 4. ***Colony 1+ Mask Channel Customization***).

For details, see the User Guide section sectionChanging Channel, Feature, and Class Names in the SCAN Tab.section

Figure 4. Colony 1+ Mask Channel Customization

4. Analyzing Images

This chapter describes how to set up analysis for the Colony Analysis applications. Perform these tasks in the ANALYZE tab.

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, i.e. Colony 1, Colony 1 + Mask, Colony 1 + 2 + Mask, Colony 1 + 2 + 3 + Mask.

In summary, when selecting analysis settings for this strategy, perform the following:

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

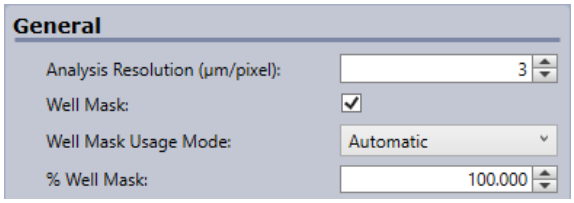
For a detailed explanation of the identification and pre-filtering settings, see the User Guide.

To select analysis settings using a single mask

1. Load prior saved Analysis Settings if available.
   - If not proceed to step 2.

In the General Section (Figure 5), make selections as follows:

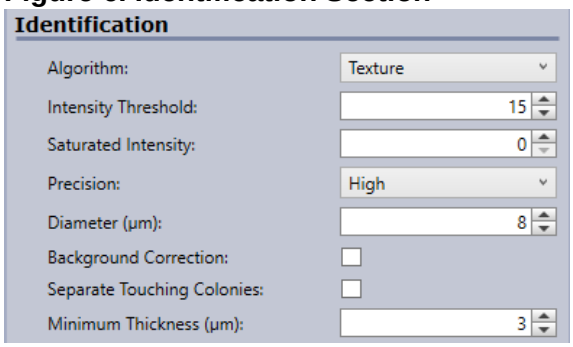
Figure 5. General Section



2. Well Mask – Always on.
3. Well Mask Usage Mode – Select as needed.
4. % 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 sectionSelecting General Analysis Settings.section

In the Identification section (Figure 6), make selections as follows:

Figure 6. Identification Section



5. 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.

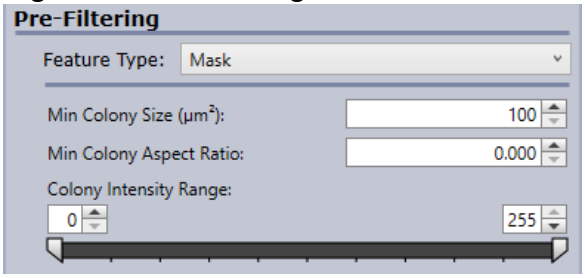
	**NOTE: If Dark Focus is selected as the Target Focal Plane when selecting acquisition settings, it is recommended to select Texture as the Algorithm in the ANALYZE tab.**
	**NOTE: The colony identification success of selecting Brightfield versus Texture will depend on the colony type, shape, and contrast of the colonies being analyzed.**

6. Intensity Threshold – Enter the optimal intensity threshold.
    - The intensity threshold is the level of intensity that separates the background from cells. With an appropriate threshold set, the background pixels fall below, and the pixels inside the cells are above the threshold. Any pixels below the threshold are not considered in subsequent calculations.

7. Saturated Intensity – Select optimal value to fill in objects with holes.
8. Precision – Select the appropriate precision level (High is recommended). For a detailed explanation of Precision, see the User Guide.
9. Diameter (µm) – This parameter only applies when Brightfield or Fluorescent algorithm is selected. Enter the cell diameter (in microns) that corresponds to the cells being analyzed.
10. Separate Touching Colonies – Select if needed.
    - This selection is used to separate objects that are touching, close, or merging.
11. Minimum Thickness (µm) – Increase value to round-out object extensions.
    - This selection is useful when using well edge artifacts that extend long beyond objects. The higher the value will round out the objects detected.

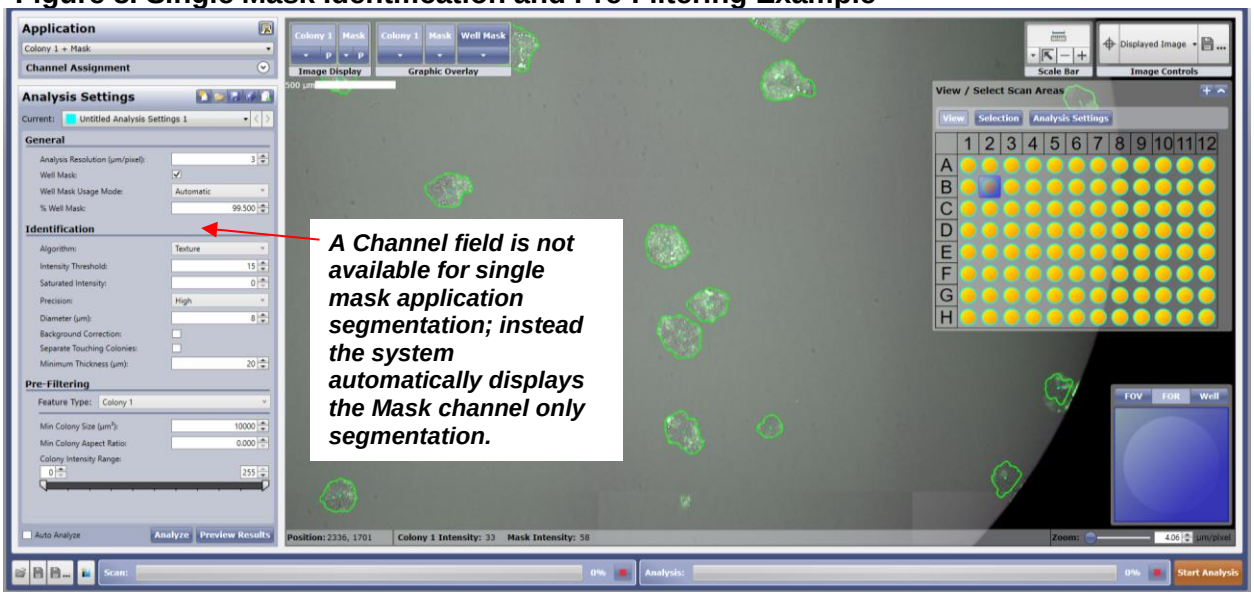
In the Pre-Filtering section (Figure 7), perform the following (not recommended if gating is to be performed):

Figure 7. Pre-Filtering Section



12. 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.
13. Min Colony Aspect Ratio – Enter the appropriate minimum colony aspect ratio.
    - Aspect ratio is the ratio of the minor axis to the major axis of the segmented object. This selection measures an object's elongation and is often used to remove artifacts and debris. A value of 1 is a perfect circle; values less than 1 suggest an oval (elongated) object.
14. Colony Intensity Range – Enter the range of intensity of objects to be included in the analysis. Helps exclude dark artifacts.

Figure 8. Single Mask Identification and Pre-Filtering Example



4.2 Analysis Settings Using a Merged Mask

Perform the following steps to select the optimal analysis settings with a merged mask segmentation strategy. Best use case for this application is when colonies have some partial detection from each channel, but are not entirely identifiable with only one channel.

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

In summary, when selecting analysis settings for this strategy, adjust segmentation parameters for each individual channel, and then check the merge box. All of the overlapping channel objects merge to one segmented colony, creating one mask.

For a detailed explanation of the identification and pre-filtering settings, see the Single Mask Section 3.1.1.

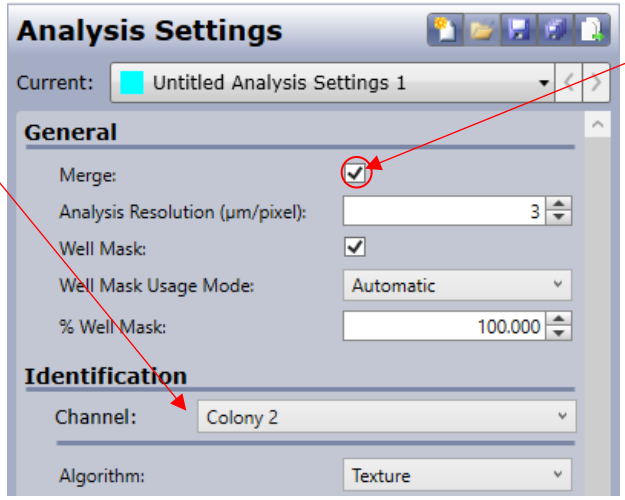
To select analysis settings using a merged mask

1. Unselect the check box Merge.
2. Turn on Image Display and Graphic Overlay for **Colony 1** only.
3. Adjust segmentation parameters to properly segment the colonies in Colony 1 channel image.
4. Repeat steps 2-3 for the other image channels and graphic overlays
5. Select the Merge checkbox to merge the segmentation of the multiple channel overlays together.

Because an application that has a merge function has been selected, the system automatically check marks the Merge checkbox (Figure 9).

	NOTE: Un-checkmark the Merge checkbox to see the independent segmentation of objects for each channel. When satisfied, check mark the Merge checkbox to generate a single merged mask.

Figure 9. Merged Mask Identification and Pre-Filtering Example



Channel field is available for merge mask segmentation, this allows switching among channels for segmentation and pre-filtering.

Merge checkbox combines the segmentation data from all channels into a single display.

Figure 10. Merged Mask Unchecked shows Graphic Overlay Behavior

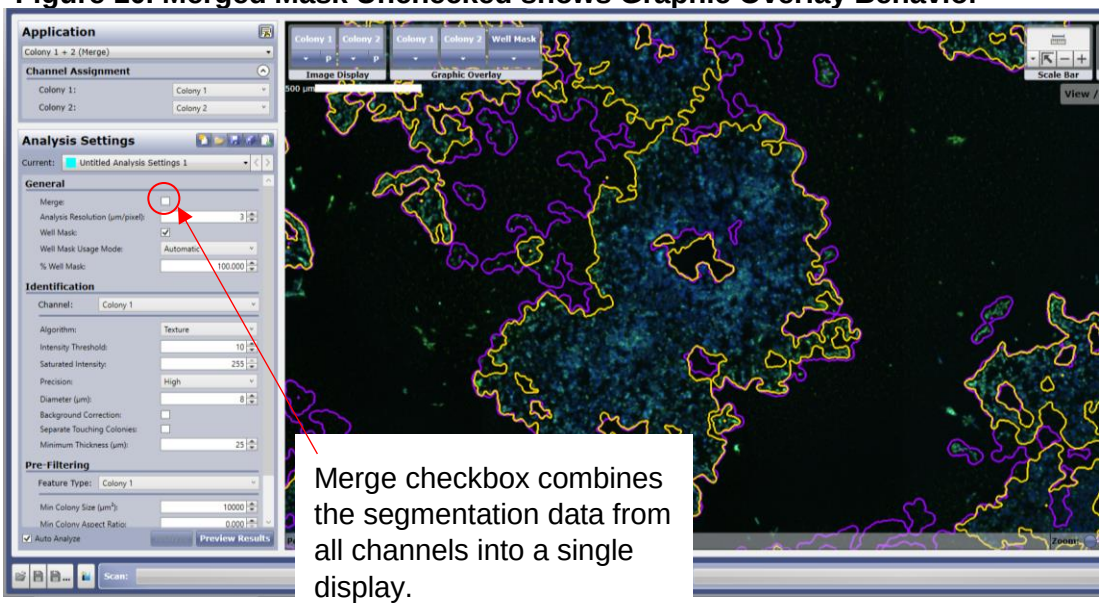


Figure 11. Merged Mask Checked shows Graphic Overlay Behavior

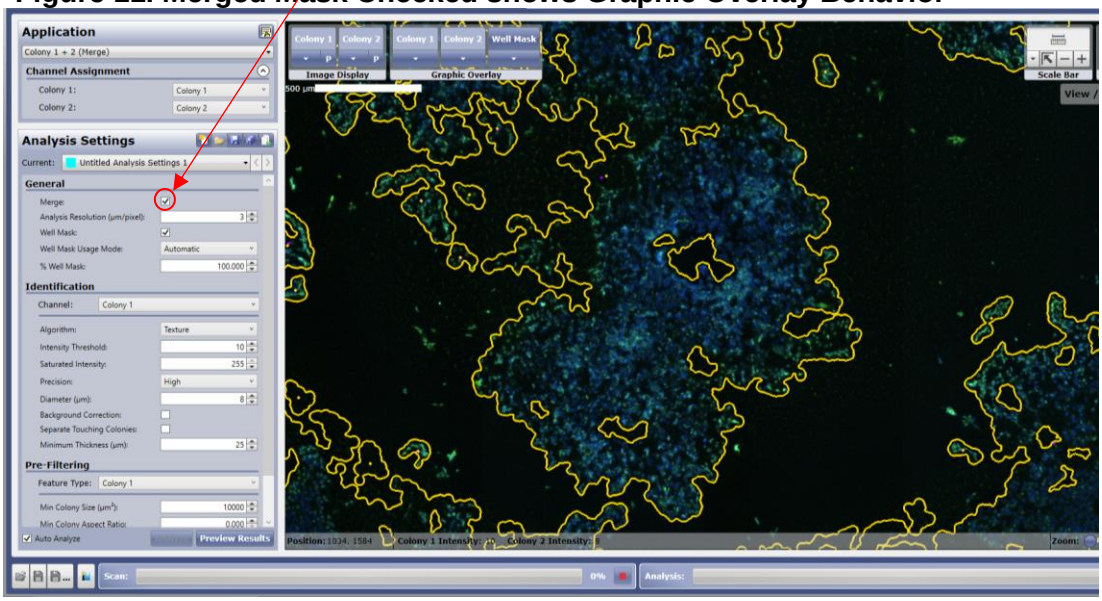


Table 3. Recommended Initial Identification and Pre-Filtering Settings for Colony Analysis Applications

APPLICATION	Colony Merged Mask		Colony Single Mask	
IMAGE ILLUMINATION	Brightfield	Fluorescent	Brightfield	Fluorescent
General				
Merge	Checked	Checked	[1]N/A	[1]N/A
Analysis Resolution	4	4	4	4
Well Mask Usage	Auto	Auto	Auto	Auto
% Well Mask	99	99	99	99
Identification				
Algorithm	Texture	Brightfield	Texture	Fluorescence
Intensity Threshold	15-50	5-20	5-50	5-20
Saturated Intensity	175-255	[1]N/A	0-75	0-75
Precision	High	High		
Diameter (pixel)	[1]N/A			[1]N/A
Background Correction		Checked		[1]N/A
Minimum Thickness (µm)	30	30	30	30
Pre-Filtering				
Min Cluster Size (μm^2)	1000	1000	1000	1000

APPLICATION	Colony Merged Mask		Colony Single Mask	
IMAGE ILLUMINATION	Brightfield	Fluorescent	Brightfield	Fluorescent
General				
Merge	[1]N/A	[1]N/A	[1]N/A	[1]N/A
Analysis Resolution	4	4	4	4
Well Mask Usage	Auto	Auto	Auto	Auto
% Well Mask	99	99	99	99
Identification				
Algorithm	Texture	Brightfield	Texture	Fluorescence
Intensity Threshold	15-50	5-20	15-50	5-20
Saturated Intensity	175-255	[1]N/A	0-75	0-75
Precision	High	High		
Diameter (pixel)	[1]N/A		[1]N/A	[1]N/A
Background Correction		Checked		[1]N/A
Minimum Thickness (µm)	30	30	30	30
Pre-Filtering				
Min Cluster Size (μm^2)	1000	1000	1000	1000

N/A = Not Applicable (selection has no effect)

5. Gating Cells

This chapter describes how to select filter settings for further scan data analysis. Perform this task in the GATE tab.

Gating can be used to analyze subpopulations of colonies that can be discriminated by size, intensity, or morphology. It is possible to use any of the colony-based parameters extracted by the software from the image data to define subsets of colony populations.

5.1 Working with Gates Using a Single or Merged Mask

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

- When NOT perform gating, the system uses the ALL population to analyze the colonies in the wells. ALL is the default population that the system assigns to all the colonies in the wells from the ANALYZE tab.
- Both types of plots are available: 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 12. Histogram Example of gated colony identification and Figure 13).

   Refer to the parameter descriptions in Table 4 for an explanation of the parameters selections available in both Pick plot parameter 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 sectionCreating a Plot.section

Figure 12. Histogram Example of gated colony identification

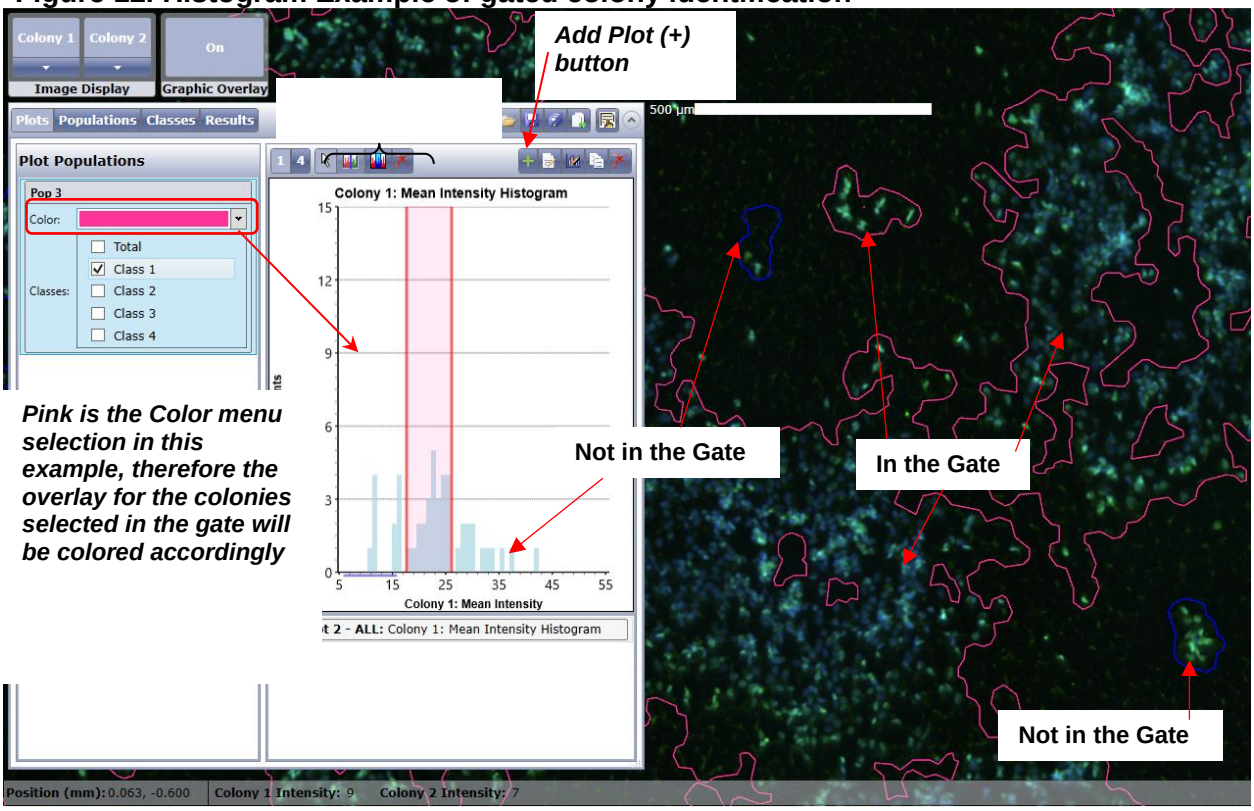
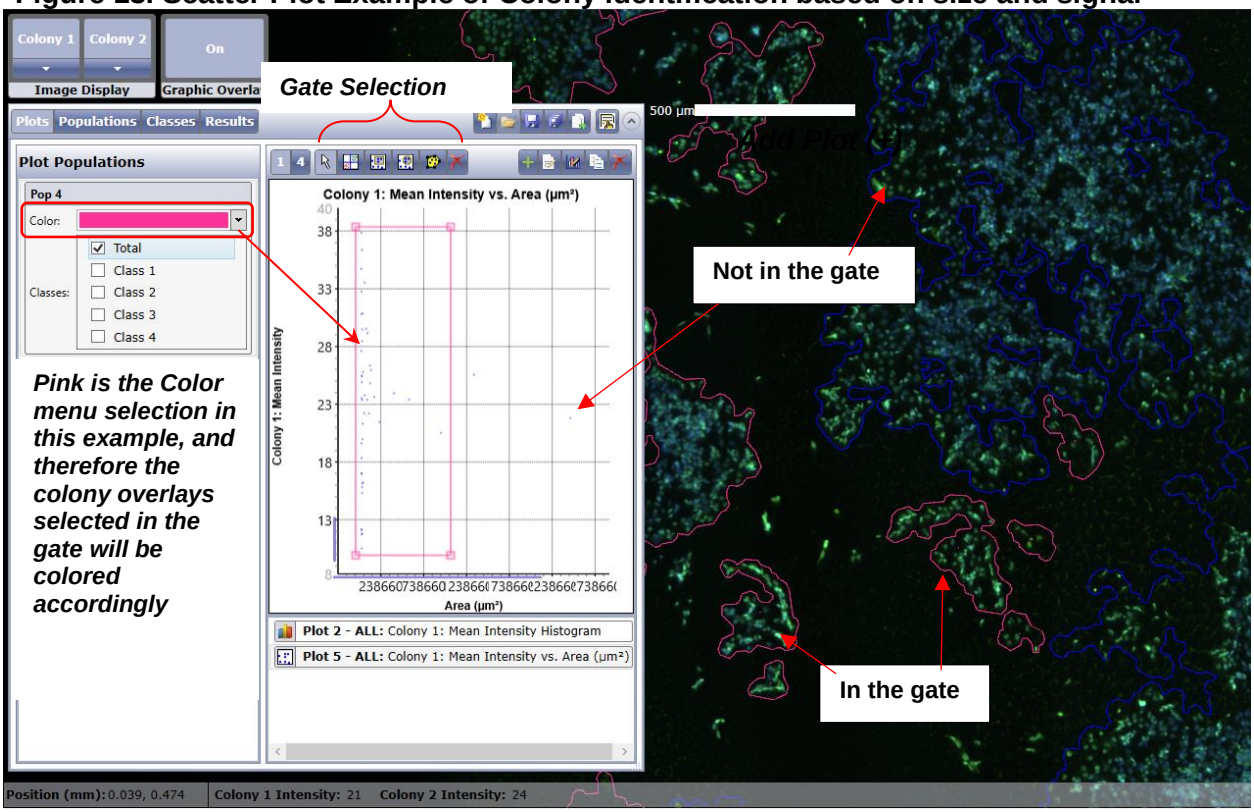


Figure 13. Scatter Plot Example of Colony identification based on size and signal



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

   In the Graphic Overlay display, the color displayed for the plot population selected in the gate corresponds to the Color menu selection. Figure 12. Histogram Example of gated colony identification shows an example of using Class 1 and Figure 13 shows an example of using the Total class.
3. Repeat steps 1 and 2 as needed to define the populations that are desired for analysis.
4. Assign each population to a class, 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 sectionAssigning a Class to a Population.section

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

The following Table 4 provides guidance for initial parameters for the Colony Analysis application gating area.

Table 4. 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 colony 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 colony along the vertical axis of the well: below (-μm) or above (+μm) the center (origin) of the well.
Distance to Nearest neighbor (μm)	Distance from the colony to the closest neighboring colony.
Distance to Well Center (μm)	Distance from the colony to the center (origin) of the well.
Area (μm2)	Total area of the identified colony.
Form Factor	sectionCompactnesssection of the segmented colony, 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	sectionEvenness of contoursection of the segmented colony, 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 colony; a value of 1 is a perfect circle; values less than 1 suggest an oval (elongated) colonies.
FOR EACH CHANNEL	
Mean Intensity	Average of the intensities of the segmented colonies. This will differ from Total Intensity.
Integrated Intensity	Sum of all the pixel intensities displaying signal in segmented colonies.

6. Viewing Results

This section describes the feature outputs available from the application's RESULTS tab.

6.1 Application Outputs

The parameters listed in Table 5 appear below the Display Options section in the Scan Information pane.

Table 5. Well-Level Features Available

Feature	Description
FEATURE REPORTED ONCE FOR EACH WELL	
Total Count	Total number of colonies 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 colonies in the specified class as compared to the Total class.
Count	Count of colonies in the specified class as compared to the Total class.
Average Colony Mean Intensity	Well average mean intensity of all colonies in the specified class.
Standard Deviation of Colony Mean Intensity	Well average standard deviation of mean intensity of all colonies in the specified class.
Average Colony Integrated Intensity	Well average integrated intensity of all colonies in the specified class.
Standard Deviation of Colony Integrated Intensity[1]	Well average standard deviation of integrated intensity of all colonies in the specified class.

As analysis progresses, the wells in the plate layout in the RESULTS tab become populated with numbers (Figure 14). Select the number displayed by selecting a parameter in the Measurements window on the left.

Figure 14. Scan Results with Image View

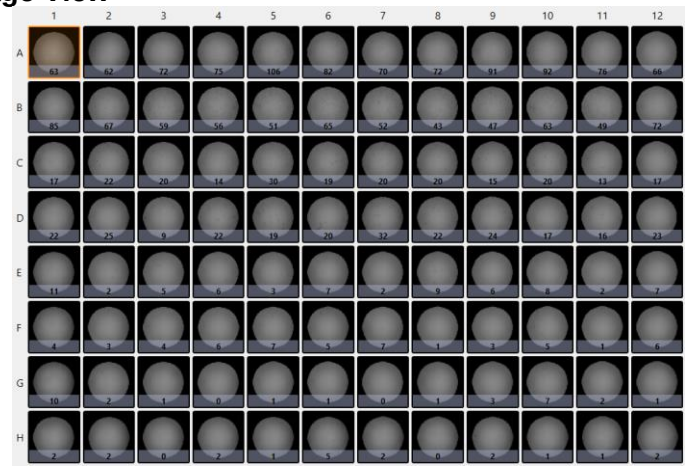


Figure 15. Scan Results with Fill View

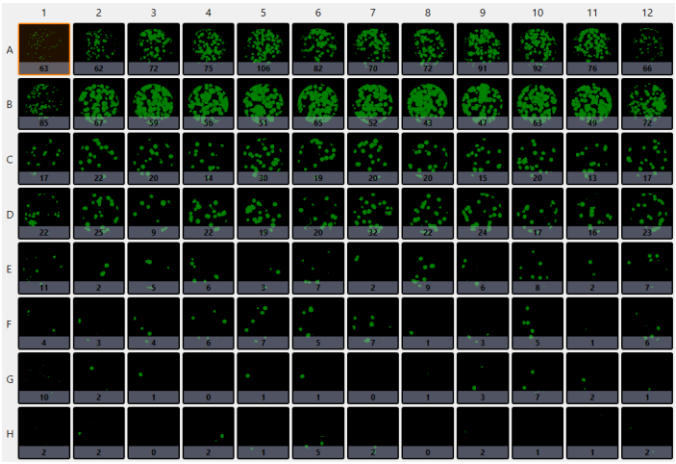
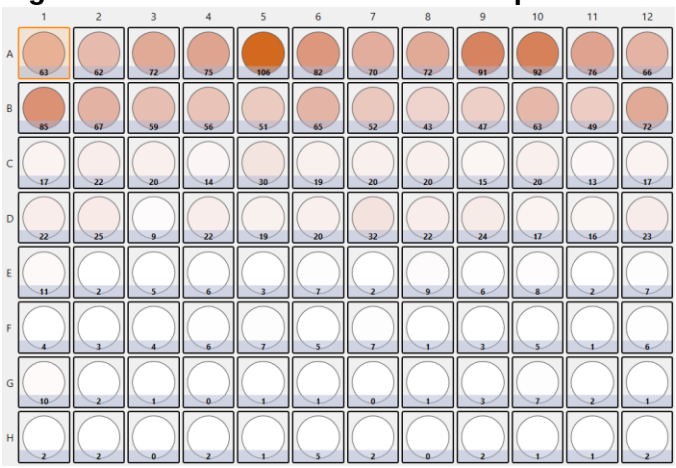


Figure 16. Scan Results with Heatmap view



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, it is possible to 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 6. Troubleshooting

Issue	Recommended Action
Cannot identify individual colonies	1. Inappropriate focus selected. • For brightfield imaging– verify that sectionBrightsection 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. 4. Inappropriate gate is applied in Gating screen. • Go to GATING tab and remove gates.
Colonies are merging together	5. Segmentation is not separating colonies • Image Colonies at an earlier time point.
Software identifies very small colonies	6. Often clusters of cells are not colonies and need to be removed from analysis. • Increase the minimum cluster size value in the 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	7. Adjust liquid volume level to prevent meniscus-dependent optical effects. • For proper liquid volumes for cell plating, see Table 1. • Some small well microplates, such as 1536-well formats, yield meniscus-dependent optical effects that cannot be corrected by liquid volume. Use Pre-Filtering settings in the ANALYZE tab to remove unwanted objects.

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 8001678 Rev. G

Celigo® Cytometer Multichannel Confluence Application Guide

Celigo Software Version 5.2

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1. About this Guide

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

1.1 Introduction

The Multichannel Confluence application is a multiple channel application used to identify the transfection efficiency of cells (or other fluorescently labelled cells) that are very confluent. It acquires in two (or up to five) channels, minimally one fluorescent and one brightfield channel that are used to determine the transfection efficiency. The application identifies the confluence area for each channel, and reports the ratio of the confluence measurements.

1.2 Purpose

The purpose of this guide is to provide the steps and concepts that are unique to the Multichannel Confluence 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: support@nexcelom.com
  phone: +1 978-327-5340
- From Europe:
  e-mail: support@nexcelom.co.uk
  phone: +44 (0) 161 232 4593

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 the performance of the 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 Image Analysis	
			Brightfield	Fluorescence
6W	Corning	3516	2500	ND[1]
6W	Corning	3471	2500	ND[1]
12W	Corning	3512	1000	ND[1]
24W	Corning	3524	≥500	ND[1]
96W	Greiner	655090	≥200	≥100
96W half area	Greiner	675090	50	50
384W low vol	Corning	3542	20	20
384W high vol	Corning	3764	≥40	≥40
384W high vol	Greiner	781091	≥40	≥40
1536W	Corning	3838	8	8
1536W	Greiner	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 Confluence Ratio application and setting the image acquisition parameters. Perform these tasks in the SCAN tab.

3.1 Multichannel Confluence Application

Perform the following steps to select a Multichannel Confluence application. This document will cover a two channel selection, but can be applied to more than two channels.

To select an application

Select the Multichannel Confluence application (Figure 1 and Table 2) as follows:

- In the Current Application dropdown list, select **Confluence** > **Confluence 1 + 2**.

Figure 1. Selecting an Application

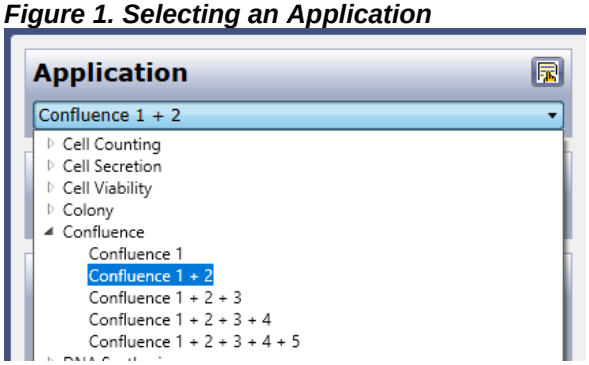


Table 2. Selecting a Multichannel Confluence Analysis Application

Number of Channels to be Acquired	Select this application	Use Case
One channels	**Confluence 1**	Measures the area of a well occupied by individual cells, or cell clusters. For details on single channel confluence image acquisition and analysis, refer to **Confluence and Growth Tracking Application Guide**.
Two channels	**Confluence 1 + 2**	Measures the area of a well occupied by individual cells or cell clusters for each channel. Reports the ratio of the surface occupied by cells and cells clusters relative to every other acquired channel.
Three channels	**Confluence 1 + 2 + 3**	
Four channels	**Confluence 1 + 2 + 3 + 4**	
Five channels	**Confluence 1 + 2 + 3 + 4 + 5**	

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 clicking **Navigation** and selecting a representative well.
   - It is recommended to set up and adjust the exposure settings using a well where the highest fluorescence signal is expected.

	**NOTE: This software application will calculate the ratio of the area of the Confluence 1 channel within the area of the Confluence 2 channel.** **Typically for transfection efficiency, Confluence 1 channels will be a fluorescent channel and Confluence 2 will be the brightfield channel.**
	**NOTE: For all multichannel applications, it is recommended to make the last channel the Brightfield channel. Default parameters for the last channels are for Brightfield channel settings.**
	**NOTE: It is preferred to setup the Brightfield channel first, then any remaining Fluorescent channels.**

2. In Channel, select **Confluence 2**.
3. For the Confluence 2 channel in the Channel window, make the following selections:
   a. For Brightfield illumination:
      - In Illumination, select **Brightfield**.
      - In Acquisition Resolution, select **1 µm/pixel**.

	**NOTE: If less image resolution is appropriate for acquisition, select 2 µm/pixel. For a detailed explanation of image resolution, see the User Guide.**

      - Select channel type: **Auto Exposure/Gain Channel**
      - In Priority, select **AutoExposure, Gain if Necessary**.
      - In Frequency, select **Every Scan Area**.

   b. Click **Apply**
   c. Click **Live** to see a live image.
   d. Click **Focus Setup** (see the User Guide).
      - Typically, it is recommended that to 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 Channel, select **Confluence 1**.
   - It is recommended to select the fluorescent stain for the Confluence 1 channel and Brightfield channel for the last channel, Confluence 2, in this 2-channel selection.

5. For the Confluence 1 channel in Channel, make the following selections:
   - For Fluorescent illumination:
     - Select channel type: **Custom**
     - In Illumination, select the appropriate illumination (e.g., Green for GFP signal, Red for RFP signal or Blue to visualize the Hoechst signal).

   e. Click **Live** to see a live image.

   f. Use manual focus or click **Find Focus** to achieve a clear image of the cells.

   g. Select whether the fixed exposure time provides the optimal signal for the sample (an object pixel intensity of 125-175 is desirable, saturated/overexposed pixel intensity is >255) and do one of the following.
      - Adjust **Exposure time and Gain** manually. When doing so, adjust the exposure so the majority of cells are in an optimal object pixel intensity range and aren't overexposed.
      - Apply **Auto Calc** if necessary: the software will calculate an exposure time or a gain so that the signal is visible without saturation of the image.

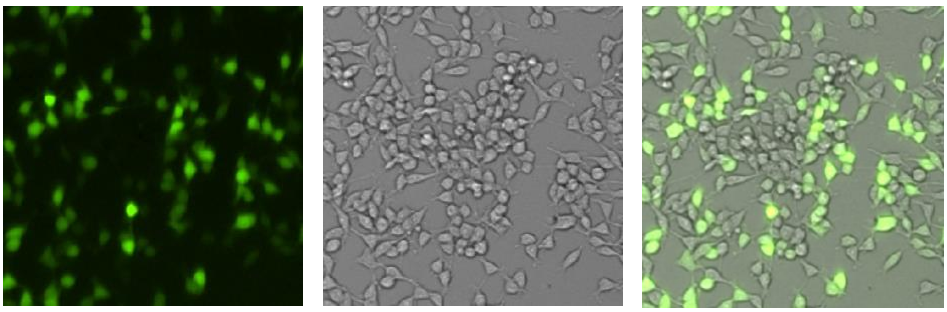
   h. Click **Find Focus** to achieve a clear image of the cells.
      - Cell focus can optionally be adjusted manually by using the **up** and **down** arrows.

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

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

Figure 2 shows a typical example of GFP and Brightfield image acquisition for the Confluence Ratio application

Figure 2. Confluence Ratio typical images



Confluence 1 Channel GFP | **Confluence 2 Channel Brightfield** | **GFP + Brightfield**

4. Analyzing Images

This chapter provides information on how to analyze scans from the Multichannel Confluence application. Perform these tasks in the ANALYZE tab.

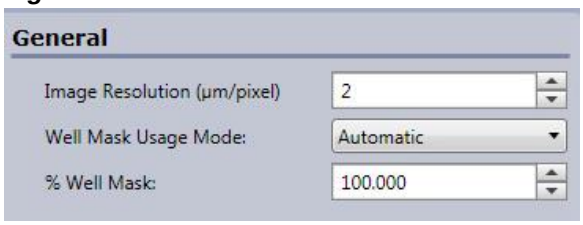
4.1 Analysis Settings

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

To perform Confluence analysis

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

Figure 3. General Section



   a. Image Resolution (µm/pixel) – Select the image resolution to use for analysis. Entering a lower value will result in more defined and longer analysis time. Entering a higher value will result in less defined and shorter analysis time. (Minimum 1 µm/pixel; ≥ 2 µm/pixel recommended)

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

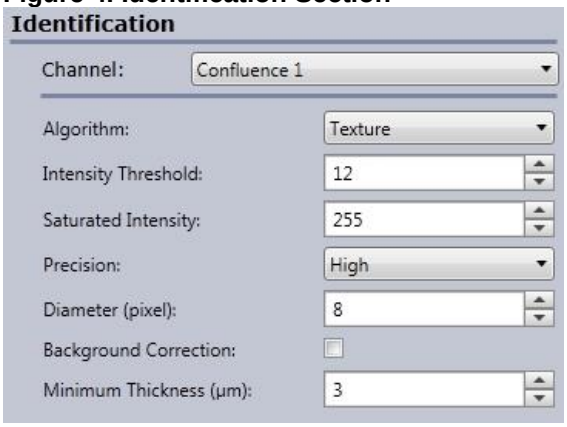
      - **Automatic** (Default) – The system looks at the well image to find the edge of the well.
      - **Original** – The system uses the set mapped position for the edge of the well, specified in the plate profile.

   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 4), make the following selections to be used to identify cells (see Table 3 for recommended parameters):

Figure 4. Identification Section



d. Select **Confluence 1** Channel

e. Algorithm – Select the appropriate algorithm:

- Texture – The algorithm looks for texture differences between the objects found and the background areas (Recommended)
- Brightfield – Do not select for the fluorescent Confluence 1 channel.
- Fluorescence – The algorithm looks for pixel intensity differences between the objects found and the background area's Intensity Threshold

f. Select the optimal intensity threshold.

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

g. 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. Hover mouse over holes to determine a value to enter.

h. Precision – Higher precision results in more detailed identification of cell clusters. Normal is sufficient to provide acceptable results, while High results in longer analysis processing time. Low requires the least processing time.

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

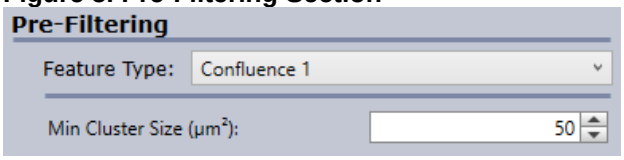
- If the Texture Algorithm is selected, skip to this step. (The entry in Diameter has no effect when Texture is the Algorithm.)
- If the Brightfield Algorithm is selected: In Diameter (pixels), enter the diameter (in pixels) that corresponds to the cell cluster dimensions. At full resolution, the Celigo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

j. Background Correction – Select if necessary (See user guide).

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

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

Figure 5. Pre-Filtering Section



- Min Cluster Size (µm²) – 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.

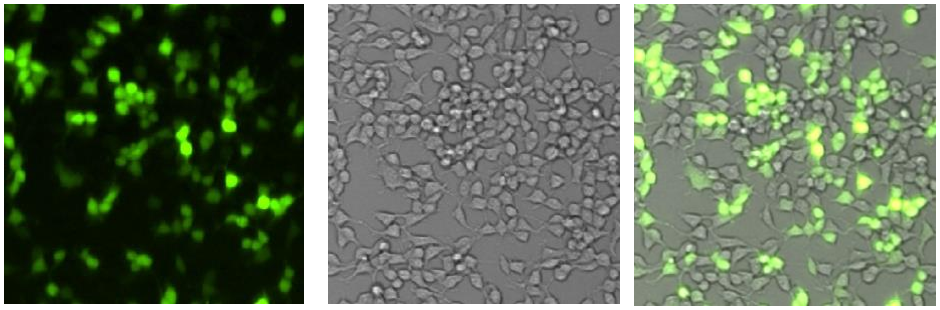
5. Select **Confluence 2** Channel

6. Repeat steps 3.e. to 4 (see Table 3 for recommended parameters).

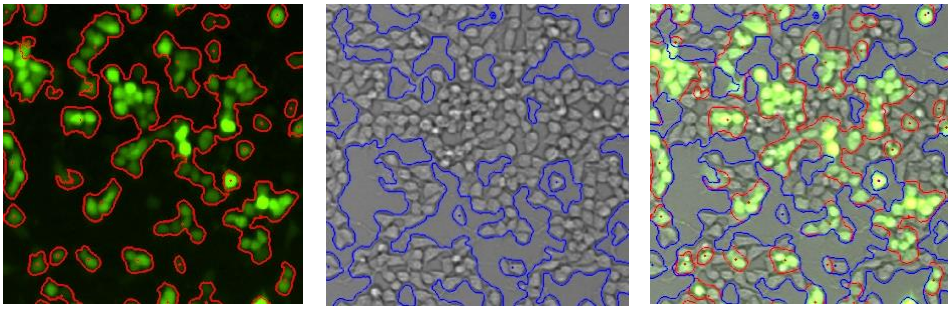
	NOTE: The cell identification success of selecting Brightfield versus Texture will depend on the cell type, shape, and contrast of the cells being analyzed.

Figure 6 shows an example of cells expressing GFP. The total area for the cells is quantified in the brightfield channel and the GFP signal is quantified in the fluorescent channel.

Figure 6. Confluence ratio – GFP and Brightfield segmentation



GFP Image | **Brightfield Image** | **GFP + Brightfield Images**



GFP Confluence 1 Segmentation | **Brightfield Confluence 2 Segmentation** | **Confluence 1 and 2 Segmentation**

NOTE: The segmentation of Confluence 1 channel is confined within the segmentation of Confluence 2 channel. In the example provided above, any GFP signal outside the boundaries of the brightfield segmentation would be ignored.

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

ALGORITHM	BRIGHTFIELD	FLUORESCENCE	TEXTURE	
ACQUISITION ILLUMINATION	**Brightfield**	**Fluorescence**	**Brightfield**	**Fluorescence**
IDENTIFICATION				
Intensity Threshold	10	10	25	10
Saturated Intensity	0	0	0	0
Precision	Normal	Normal	Normal	Normal
Diameter (µm)	10	15	Not Applicable (selection has no effect)	Not Applicable (selection has no effect)
Background Correction	Check marked	Not Applicable (selection has no effect)	Check marked	Not Applicable (selection has no effect)
Minimum Thickness (µm)	3	3	3	3
PRE-FILTERING				
Min Cluster Size (μm^2)	50	50	100	100

5. Gating Cells

When using the Multichannel Confluence application, gating is not performed in the GATE tab. Instead, the application relies on the identification of fluorescent and brightfield cell growth areas using intensity thresholding in the ANALYZE tab. The pre-filtering parameters in the ANALYZE tab (see chapter 4) manage the filtering of debris or very small cell clusters.

6. Viewing Results

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

6.1 Multichannel Confluence Application Outputs

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

Table 4. Multichannel Confluence Application Outputs

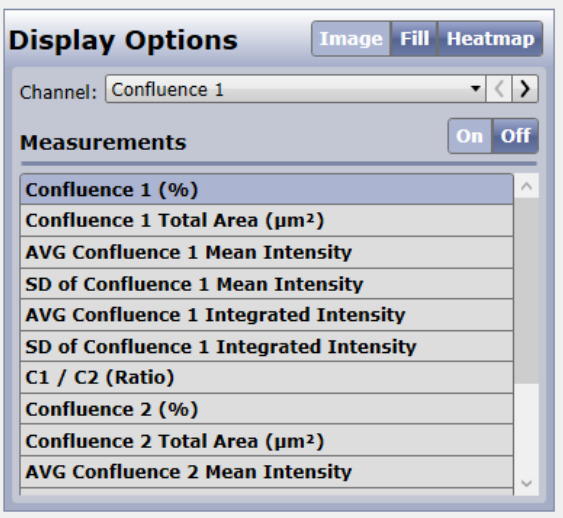
Parameter	Measurement Description for Each Channel Acquired and Analyzed
Confluence (%)	Area of the well occupied by individual cells or cell clusters in a Confluence channel divided by the total area of the scan area or well.
Confluence Total Area	Area of the well occupied by individual cells or cell clusters in a Confluence channel.
AVG Confluence Mean Intensity	Well average and standard deviation mean intensity of all the pixels in a segmented area. Summation of all pixel intensities above the selected threshold divided by the area detected above select threshold.
SD of Confluence Mean Intensity	Well average standard deviation of mean intensity of all pixels in the segmented area.
AVG Confluence Integrated Intensity	Well average integrated intensity of all pixels in the segmented area. Summation of all the pixel intensities above a selected threshold.
SD of Confluence Integrated Intensity	Well average standard deviation of integrated intensity of all the pixels in a segmented area.
Confluence Ratios: (e.g.) Confluence 1/ Confluence 2 (Ratio)	Ratio of the Confluence Total Area of one channel relative to all other channels. Confluence 1 Total Area divided by Confluence 2 Total Area reported as a percentage. Typically represents the proportion of cell area positive for fluorescent signal within the brightfield cell area.
Well Sampled (%)	Percent of well sampled.

Table 5. Multichannel Ratio Output for Different Channel Selections

Application	Displayed Ratio Outputs
Confluence 1+2	C1/C2, C2/C1
Confluence 1+2+3	C1/C2, C1/C3, C2/C1, C2/C3, C3/C1, C3/C2
Confluence 1+2+3+4	C1/C2, C1/C3, C1/C4, C2/C1, C2/C3, C2/C4, C3/C1, C3/C2, C3/C4, C4/C1, C4/C2, C4/C3
Confluence 1+2+3+4+5	C1/C2, C1/C3, C1/C4, C1/C5, C2/C1, C2/C3, C2/C4, C2/C5, C3/C1, C3/C2, C3/C4, C3/C5, C4/C1, C4/C2, C4/C3, C4/C5, C5/C1, C5/C2, C5/C3, C5/C4

As analysis progresses, the wells in the plate layout in the RESULTS tab become populated with numbers (Figure 7. Display Options in the RESULTS Tab for the Confluence 1+2 application). Select the number displayed by selecting a parameter in the Measurements window on the left.

Figure 7. Display Options in the RESULTS Tab for the Confluence 1+2 application



6.2 Data Export

Well-level and object-level data can be exported into CSV (Comma Separated Value) 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 Confluence Percentage and Confluence Total area for each channel. As in the ANALYZE tab, it is possible to zoom in and use the toggles in the Image Display and Graphic Overlays.

7. Troubleshooting

This chapter provides troubleshooting recommendations.

Table 6. 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 a crisp focus was selected for the desired objects. Ensure a good signal to noise ratio by proper illumination, exposure settings and minimizing background auto-fluorescence. 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.
Well edges are too bright or dark	1. 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.
Blank areas are identified in the image	1. This could be due to too much background noise and too little signal. • Increase the texture intensity threshold.

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Celigo® Cytometer
Plate Reader
Application Guide

Celigo Software Version 5.2

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1. About this Guide

The Plate Reader application is a generic fluorescence protocol for up to three separate channels in addition to a brightfield channel. Overall, well signal is measured in the brightfield and/or fluorescence channels. Using a very simple interface, the application reports well mean and integrated intensities for each well.

1.1 Purpose

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

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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
- 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	2500	ND[1]
6W	Corning	3471	2500	ND[1]
12W	Corning	3512	1000	ND[1]
24W	Corning	3524	≥500	ND[1]
96W	Greiner	655090	≥200	≥100
96W half area	Greiner	675090	50	50
384W low vol	Corning	3542	20	20
384W high vol	Corning	3712	≥40	≥40
384W high vol	Greiner	781091	≥40	≥40
1536W	Corning	3838	8	8
1536W	Greiner	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 Plate Reader application and setting the image acquisition parameters. Perform these tasks in the SCAN tab.

3.1 Plate Reader Application

Perform the following steps to select a Plate Reader application.

To select a Plate Reader application

In the Current Application menu, select one of the four Plate Reader applications (Figure 1 and Table 2), depending on how many channels (targets) are necessary to acquire.

Figure 1. Selecting an Application

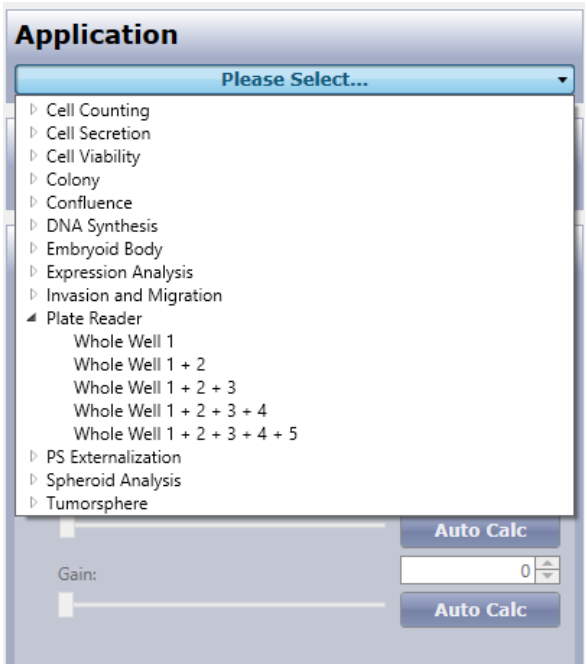


Table 2. Selecting an Application

Whole Well 1	Measures the mean and integrated pixel intensities for 1 fluorescent or brightfield channel for the whole well area
Whole Well 1 + 2	Measures the mean and integrated pixel intensities for 2 fluorescent and/or brightfield channels for the whole well area
Whole Well 1 + 2 + 3	Measures the mean and integrated pixel intensities for 3 fluorescent and/or brightfield channels for the whole well area
Whole Well 1 + 2 + 3 + 4	Measures the mean and integrated pixel intensities for 3 fluorescent and a brightfield channels for the whole well area
Whole Well 1 + 2 + 3 + 4 + 5	Measures the mean and integrated pixel intensities for 4 fluorescent and a brightfield channels for the whole well area

3.2 Image Acquisition Settings

Perform the following steps to select image acquisition settings. The example provided here is for 1 channel Whole Well 1 application.

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 to adjust exposure settings using a well where the highest fluorescent signal is expected.
2. In Channel, select **Channel 1**.
3. For the selected Channel, make the following selections:
   a. For Fluorescent Illumination
      - In Type, select **Custom Channel**.
      - In Illumination, select the appropriate illumination (e.g., Green for GFP signal, Red for RFP signal or Blue to visualize the Hoechst signal).

NOTE: Although provided in the application, a brightfield channel is typically not used for this application.

   b. Click **Live** to see a live image.
   c. Select whether the fixed exposure time provides the optimal signal for scanning (an object pixel intensity of 125-175). Make sure that where cells are visible, they are also not overexposed (i.e., saturated: pixels intensities >255). Now perform one of the following.
      - Adjust **Exposure** time and **Gain** manually. When doing so, adjust the exposure so the majority of cells aren't overexposed.
      - Apply **Auto Calc** if necessary: the software will calculate an exposure time or a gain so that the signal is visible without saturation of the image.
   d. If less image resolution is appropriate for acquisition, select **2 µm/pixel in Acquisition Resolution.** For a detailed explanation of image resolution, see the User Guide.
   e. Click **Focus Setup** (see the User Guide).
      - Typically, it is recommended to 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. If using a Plate Reader application with more than one channel, proceed with the following

a. In Channel, select the next channel to set up.

5. For the selected channel in Current Channel, make the following selections (otherwise, go to the section 4):

   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. Select whether the fixed exposure time provides the optimal signal for scanning (an object pixel intensity of 125-175). Make sure that where cells are visible, they are also not overexposed (i.e., saturated: pixels intensities >255). Now perform one of the following.

      - Adjust **Exposure** time and **Gain** manually. When doing so, adjust the exposure so the majority of cells aren't overexposed.
      - Apply **Auto Calc** if necessary: the software will calculate an exposure time or a gain so that the signal is visible without saturation of the image.

   e. Click **Find Focus** to achieve a clear image of the cells.

   f. Click **Set Offset**.

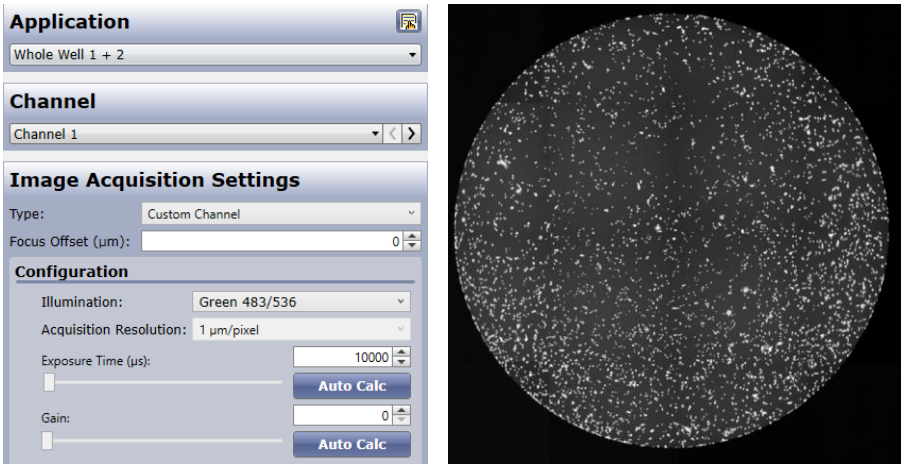
      - Setting focus offsets is important because the optimal focus positions for red, green, and blue wavelengths and brightfield are different. The software will adjust the focus position for each channel during a scan to provide the best focus.

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

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

Figure 2. Whole Well 1 application selections and Whole Well 1 example image shows an example of Whole Well 1 selections and zoomed image of a nuclear stain for a near confluent well.

Figure 2. Whole Well 1 application selections and Whole Well 1 example image



4. Analyzing Images

This chapter describes how to set up analysis for the Plate Reader application. Perform these tasks in the ANALYZE tab.

In this application, images are not being segmented to find individual objects. Instead, the whole well pixels' mean and integrated signal will be reported.

4.1 Analysis Settings

Perform the following steps to select the optimal analysis:

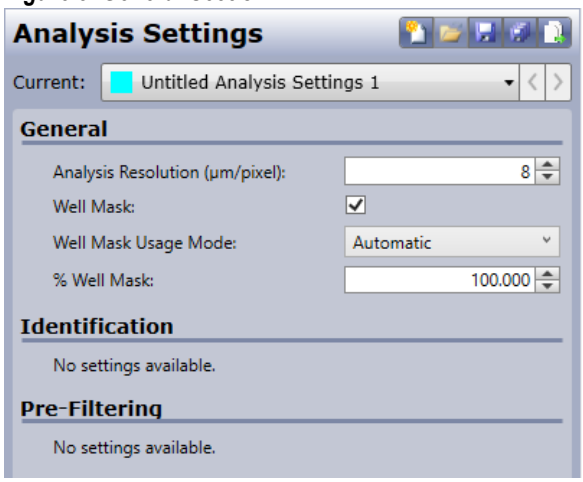
- Select the resolution at which the analysis is performed
- Adjust the well mask size to analyze a fraction of the image only
- No individual cell level data is generated for this application

For the recommended initial identification and pre-filtering settings to use as a guide, see Figure 4.

To perform Plate Reader analysis

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

Figure 3. General Section



a. Analysis Resolution (µm/pixel) -- Select the image resolution to use for analysis. Entering a lower value (minimum 2 µm/pixel) will result in greater segmentation definition and longer analysis time. Entering a higher value will result in less defined and shorter analysis time.

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

- **Automatic** (Default) – The system looks at the well image to find the edge of the well.
- **Original** – The system uses the set mapped position for the edge of the well, specified in the plate profile.

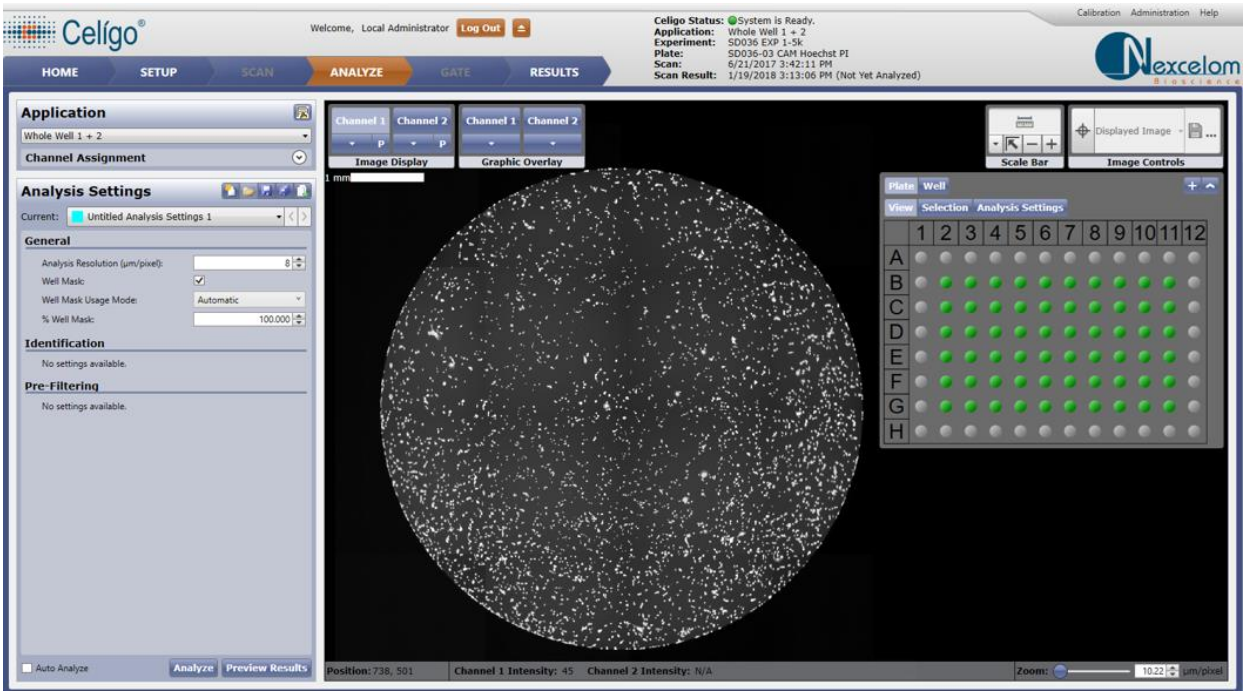
c. % Well Mask – Sets the percentage of the well to be analyzed. Decreasing the % Well Mask entry can exclude well edge artifacts that can occur during plate manufacturing.

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

7. The Identification and Pre-Filtering sections are not used in this application.

Figure 4 shows the typical ANALYZE Tab view for the Whole Well 1 application.

Figure 4. Single Mask Whole Well 1 analysis parameters



	**NOTE: Whether using a single channel or multiple channels in the Plate Reader application, the analysis parameters remain identical and the data will be reported for all channels.**

Table 3. Recommended Initial Settings for Whole Well application

APPLICATION	**ALL PLATE READER APPLICATIONS**
GENERAL	
Analysis Resolution	2
Well Mask	Check marked
Well Mask Usage Mode	Automatic
% Well Mask	100%
IDENTIFICATION	
Not Applicable	
PRE-FILTERING	
Not Applicable	

5. Gating Cells

When using the Plate Reader application, gating is typically not performed in the GATE tab. Instead, the application relies on quantifying mean and integrated intensities as defined in the ANALYZE tab.

6. Viewing Results

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

6.1 Application Outputs

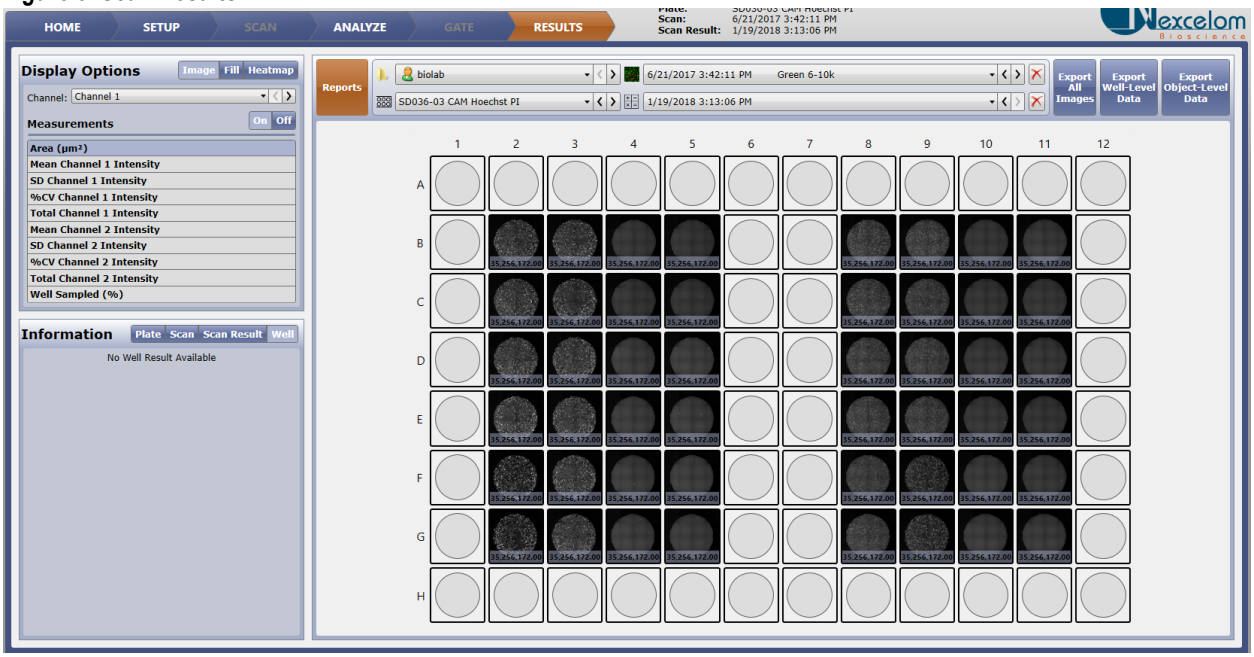
The parameters listed in Table 4 appear below the Display Options section in the Scan Information pane.

Table 4. Well-Level Features Available

Feature	Description
FEATURE REPORTED ONCE FOR EACH WELL	
Area	Area of the well analyzed reported in μm^2.
% Well Sampled	Percent of well surface imaged.
FEATURE REPORTED FOR ALL CHANNELS	
Mean Intensity	Mean intensity of all pixels in the well area.
SD Intensity	Standard deviation of the mean intensity of all pixels in the well area.
%CV Intensity	Coefficient of variation of the mean intensity of all pixels in the well area.
Integrated Intensity	Integrated intensity of all pixels in the well area.

As analysis progresses, the wells in the plate layout in the RESULTS tab become populated with numbers (Figure 5). Select the number displayed by selecting a parameter in the Measurements window on the left. Click heatmap, and the plate layout will be automatically color-coded to rapidly visualize trends.

Figure 5. Scan Results



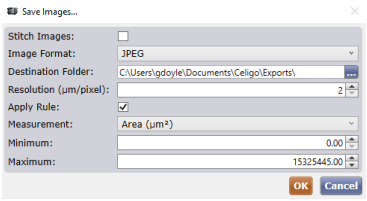
6.2 Data Export

Well-level data can be exported into CSV (Comma Separated Value). Object-level data is not available for this application. For the procedures to perform data export, see the User Guide.

6.2.1 Rule Based Export

The Apply Rule option is available as part of the Export All Images functions. This allows a rule to be established exporting only those images that fit within those defined parameters.

Figure 6. Apply Rule



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 mean and integrated intensity in each channel, and standard deviations. As in the ANALYZE tab, it is possible to 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
Cannot visualize 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.
Well edges are too bright or dark	2. 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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 8001680 Rev. F

Celigo® Cytometer Tumorsphere Migration Application Guide

Celigo Software Version 5.2

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1. About this Guide

The Invasion and Migration: Tumorsphere Migration application is designed to evaluate the growth of cells out of a spheroid. Experiments can be performed in growth media or Matrigel, the analysis will be similar. Overall, well signal is measured in the brightfield channel and is based on the measurement of confluence around the spheroid. Using a very simple interface, the application reports the well mean and integrated intensities.

1.1 Purpose

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

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Customer Service
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- From Europe:
  e-mail: support@nexcelom.co.uk
  phone: +44 (0) 161 232 4593

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
- 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 the performance of the 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)
6W	Corning	3516	2500
6W	Corning	3471	2500
12W	Corning	3512	1000
24W	Corning	3524	≥500
96W	Greiner	655090	≥200
96W ½ Area	Greiner	675090	50
384W Low Volume	Corning	3542	20
384W High Volume	Corning	3712	≥40
384W High Volume	Greiner	781091	≥40

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

3. Scanning Plates

This chapter provides the procedures for selecting the appropriate Tumorsphere Migration application and setting the image acquisition parameters. Perform these tasks in the SCAN tab.

3.1 Invasion and Migration: Tumorsphere Migration Application

Perform the following steps to select a Tumorsphere Migration application.

To select the Invasion and Migration: Tumorsphere Migration application

In the Application menu, select **Invasion and Migration** > **Tumorsphere Migration** Figure 1 and Table 2.

Figure 1. Selecting an Application

Table 2. Selecting an Application

Tumorsphere Migration	Measures the cell growth outside of a spheroid either in growth media or semi-solid media.

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. In Channel, **Sphere** is the only selection available.
3. View a well by selecting **Live** or **Snap** in the Camera Controls field.

Set up acquisition settings for brightfield or fluorescent illumination:

4. For Brightfield illumination:
   - Select channel type: **Auto Exposure/Gain Channel** (recommended)

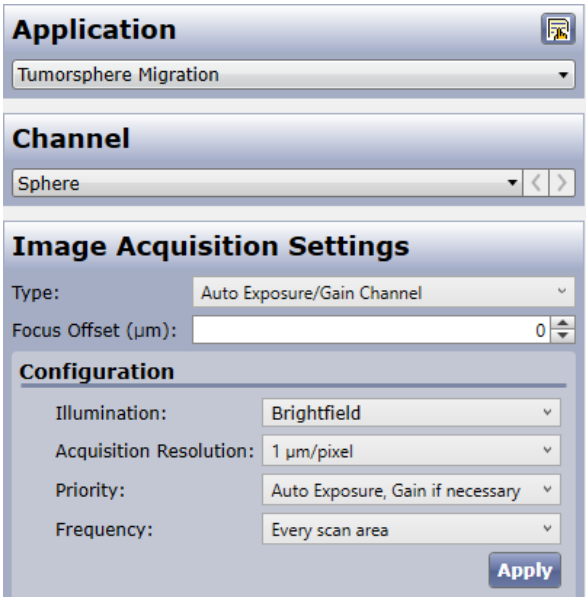
	NOTE: Greater than 1 µm/pixel image resolution is recommended for tumorsphere applications. For a detailed explanation of choosing an image resolution, see the User Guide

   - In Illumination, select **Brightfield**.
   - In Priority, select **AutoExposure, Gain if Necessary**.
   - In Frequency, select **Every Scan Area**.
5. Click **Focus Setup** (see the User Guide).
   - Typically, it is recommended to select **Hardware Auto Focus** (because it provides maximum speed), then click **Register Auto**. For manual registration or using focus offset, see the User Guide.
6. Click **Set Offset**. (see the User Guide).
   - 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.

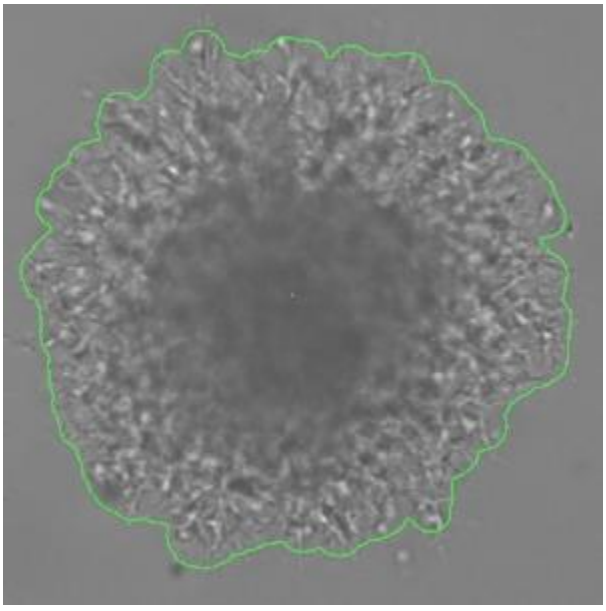
For detailed instructions on other image acquisition options, such as well subsampling, off-axis imaging, and decreased image resolution acquisition, see the User Guide.

Figure 2 shows an example of the Tumorsphere Migration application selections and a zoomed in brightfield image of tumorsphere with cell growth.

Figure 2. Tumorsphere Migration application selections



Tumorsphere Migration example image



3.3 Correct Focus Position for Brightfield Imaging

The proper focus for brightfield illumination is important for optimal application performance. Specifically, the application uses the edge of the cell growth area around the tumorsphere and it is important to set the focus on the cell growth edge that is meant to be analyzed.

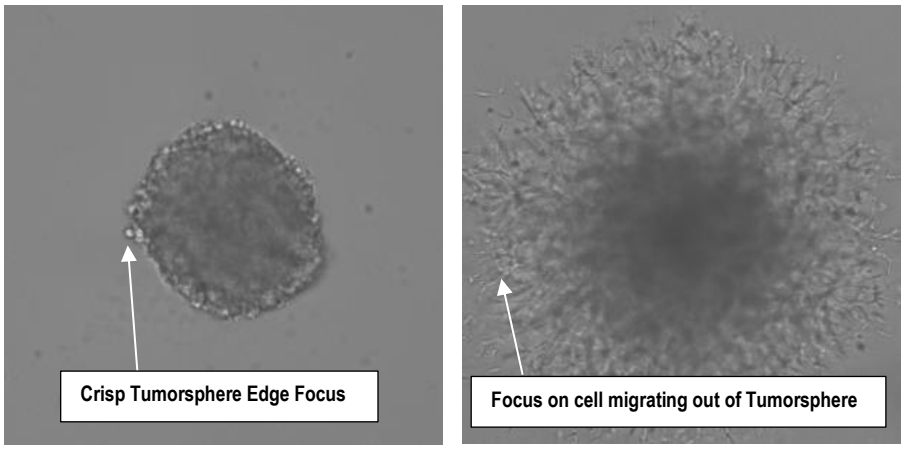
This section describes how to select the correct focus position for brightfield imaging. For instructions on focusing using fluorescent illumination, see the User Guide.

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. Select **Focus Setup** and select **Hardware Auto Focus** in the Focus Type field. Complete the setup by registering the autofocus. Adjust the focus until the cells on the edge of the spheroid have a large, bright center. For examples of proper focus using sharp growth area edge (Figure 3)
   - Image Based Auto Focus is *not* recommended for brightfield illumination. The Celigo will focus on the sphere and not the edge of the growth area.

Figure 3. Examples of Proper cell growth area Edge Focus

Tumorsphere with no cell migration **Tumorsphere with cell migration**



4. Analyzing Images

This chapter describes how to set up analysis for the Tumorsphere Migration application. Perform these tasks in the ANALYZE tab.

4.1 Analysis Settings

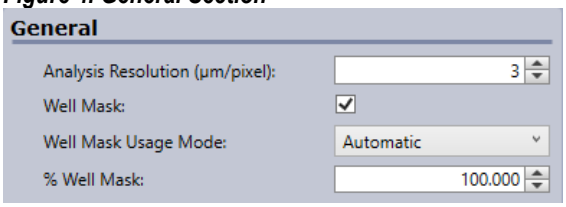
Perform the following steps to select the optimal analysis:

To perform Tumorsphere Migration analysis

1. Load prior saved Analysis Settings, if available.

In the General section (Figure 4), make the following selections:

Figure 4. General Section

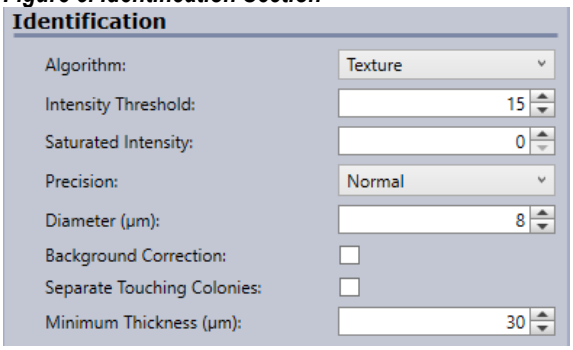


2. Analysis Resolution (µm/pixel) – Select the image resolution to use for analysis. Entering a lower value (minimum1 µm/pixel; ≥ 2 µm/pixel recommended) will result in greater accuracy and longer analysis time. Entering a higher value will result in less accuracy and shorter analysis time.
3. Well Mask 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.
4. % 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.

In the Identification section (Figure 5), make the following selections to be used to identify cells migrating out of the tumorspheres:

Figure 5. Identification Section



5. Algorithm – Select the appropriate algorithm:
   - Texture – The algorithm looks for texture differences between the objects found and the background areas (Recommended)
   - Brightfield – The algorithm looks for objects with a bright center and dark edges (not recommended)

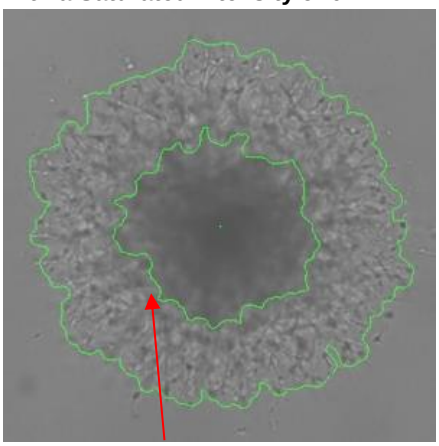
	NOTE: The Brightfield algorithm will detect individual cells, but will not account for the entire tumorsphere. It is recommended to use the Texture algorithm as described above.

6. Intensity Threshold – Select the optimal intensity threshold.
   - This selection is the level of intensity that separates the background from cells. With an appropriate threshold set, the background pixels fall below and pixels inside the cells are above the threshold.
7. Saturated Intensity – Select the optimal saturated intensity threshold to prevent holes in the segmentation of large objects. Figure 7 has an example of properly using the saturated intensity settings.
   - Saturated intensity represents the minimum pixel intensity value that is considered to be saturated (usually, an object pixel intensity value ≥255 is considered saturated). Saturated areas are not detected by the texture segmentation algorithm, and therefore create holes within the confluent areas. Holes consisting of pixels that are all saturated are filled in post-segmentation.

	NOTE: The Saturated intensity value can be used to either detect the whole area corresponding to the tumorsphere and the migration area or the migration area only. See Figure 77 below for details.

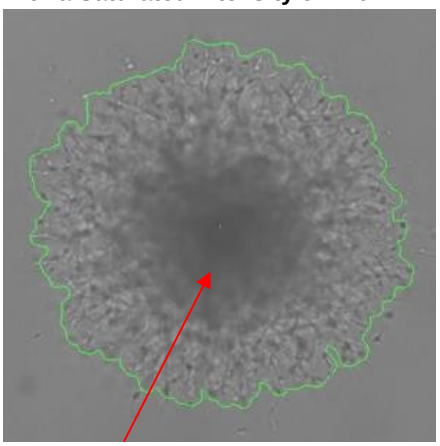
Figure 6. Tumorsphere Migration example of segmentation using saturated intensity

Tumorsphere Migration identification with a Saturated Intensity of 0



When using a low Saturated Intensity, the tumorsphere is excluded from the segmentation and only the migration area will be quantified.

Tumorsphere Migration identification with a Saturated Intensity of 120



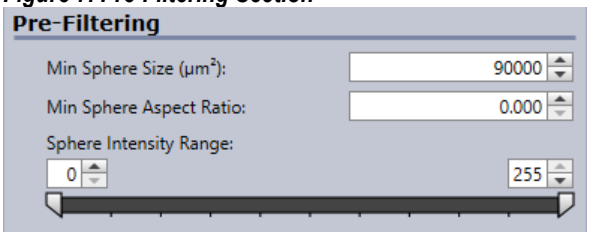
When using a high Saturated Intensity, the tumorsphere is included in the segmentation and both the tumorsphere and the migration area will be quantified.

8. Precision – Higher precision results in more accurate identification of cell clusters. Normal is recommended because this setting is sufficient to provide acceptable results, while High results in longer analysis processing time.
9. Diameter (µm) – Do one of the following:
   - If Texture was selected in the Algorithm field, skip to step 010. (The entry in Diameter has no effect when Texture is the Algorithm.)
   - If Brightfield was selected 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.
10. Background Correction – Select if necessary.
11. Separate Touching Colonies – Select if needed.

    This selection is used to separate objects that are touching, close, or merging.
12. Minimum Thickness (µm) – Minimizes long extensions. Increase this setting if the plate has plate artifacts, such as a plate ring stamp.

In the Pre-Filtering Section, make the following selections:

Figure 7. Pre-Filtering Section



13. Min Sphere Size (µm²) –Enter the minimum tumorsphere size that corresponds to the scanned tumorspheres. This will eliminate small segmentation artifacts.
14. Min. Aspect Ratio – Removes elongated debris and tumorsphere migration areas that are not round.
15. Intensity Range – Make selections to include only tumorspheres and exclude debris/single cells.

Figure 8 shows the typical Analysis Tab view for the Tumorsphere Migration application.

Figure 8. Single Mask Tumorsphere Migration analysis parameters

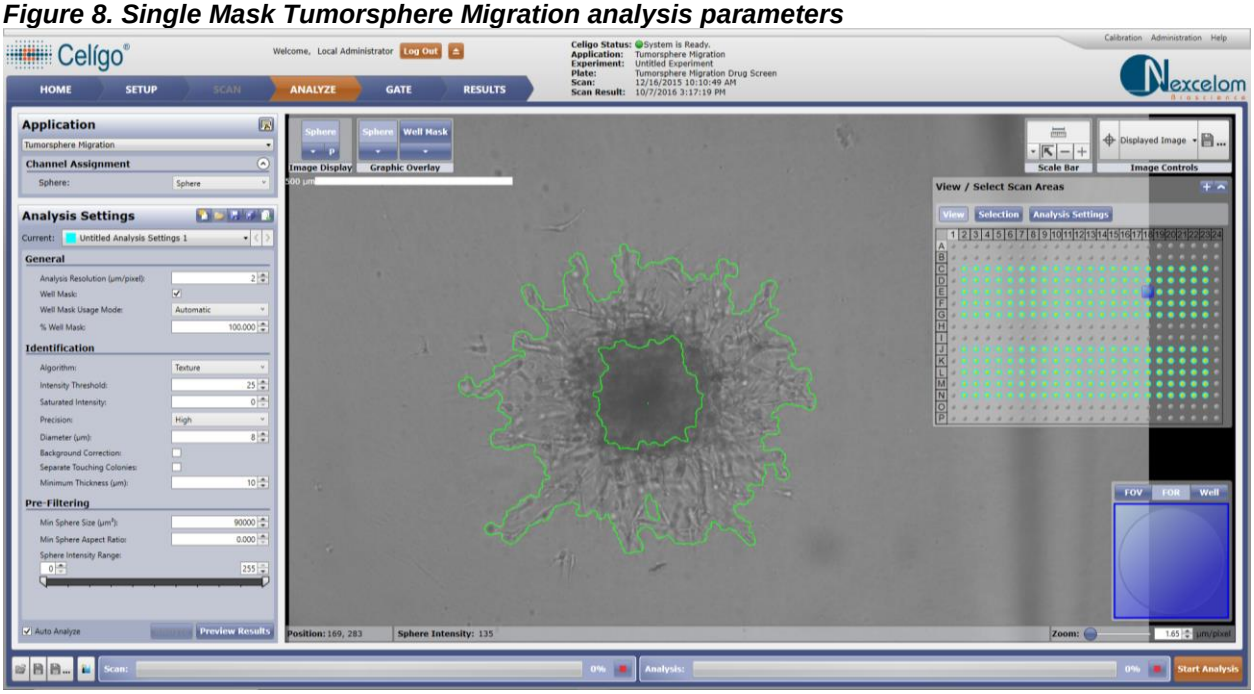


Table 3. Recommended Initial Settings for Tumorsphere Migration application

APPLICATION	TUMORSPHERE MIGRATION APPLICATION	
GENERAL		
Analysis Resolution	2	
Well Mask	Check marked	
Well Mask Usage Mode	Automatic	
% Well Mask	100%	
IDENTIFICATION		
Algorithm	**Brightfield**	**Texture**
Intensity Threshold	3	15
Saturated Intensity	0	0
Precision	High	High
Diameter (µm)	15	Selection has no effect
Background Correction	check marked	check marked
Separate Touching Colonies	Uncheck marked	Uncheck marked
Minimum Thickness (µm)	5	20
PRE-FILTERING		
Min Sphere Size (µm^2)	5000	
Min Sphere Aspect Ratio	0	
Sphere Intensity Range	0-255	

5. Gating Cells

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

The Tumorsphere Migration application relies on the identification of tumorspheres and the removal of debris using the Tumorsphere Migration Algorithm threshold, 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 Tumorsphere Migration application.

- If choosing to not perform gating, the system will use the ALL population to count the tumorsphere 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; assigning any additional classes to populations is not possible. Reanalysis of the same scan and assigning a different class for a different population is possible.
- Make sure that a Classes checkbox has been selected to assign a class to the Total population. Check marking 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 check mark would be the same as not using the GATING tab; all of the data would be reported from the ANALYZE 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 4.

   Table 4 lists the possible selections 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 Tumorsphere Migration 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 creating a plot and drawing a gate, 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 4. Plot Parameter Selections

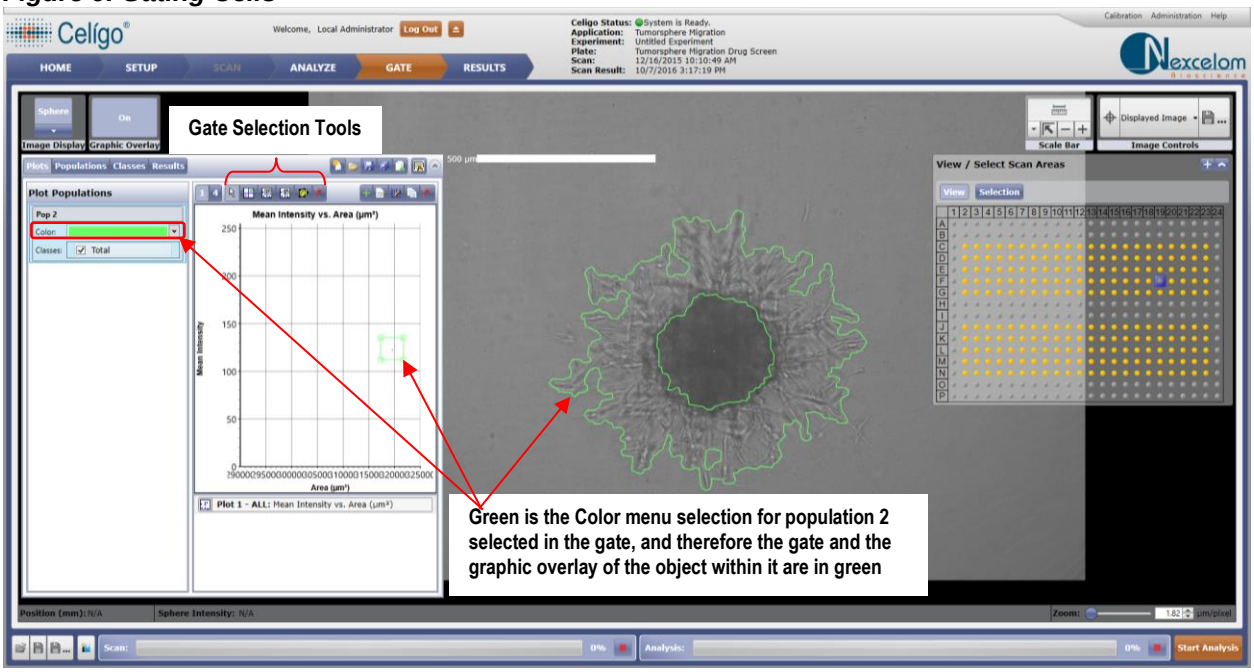
Feature	Definition
X Position (µm)	Location of a cell along the horizontal axis of the well: left (-µm) or right (+µm) of the center (origin of the well).
Y Position (µm)	Location of a cell along the vertical axis of the well: below (-µm) or above (+µm) of the center (origin of the well).
Distance to 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.
Area (μm^2)	Total area 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 has a smoothness value of 1.0 (the maximum).
Aspect Ratio	Measure of the tumorsphere's 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.

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. The figure shows the single class (Total) used in this application.

Figure 9. Gating Cells



3. Repeat steps 1 and 2 as needed to refine the desired population.
4. In the Plots, Populations, or Classes view, make sure that the Total class is check marked. This assigns the Total class to the population.

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

   In this application, assign only the Total class to populations; it is not possible to 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

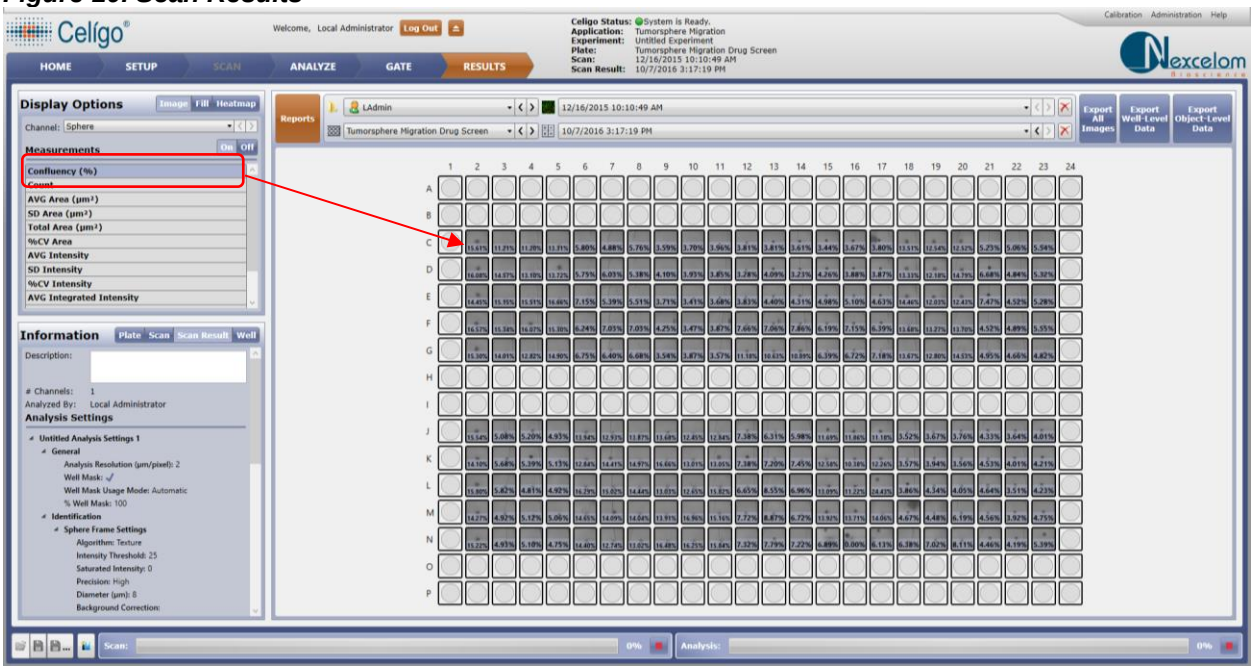
The parameters listed in ***Table 5*** appear in the Measurements pane in the RESULTS tab.

Table 5. Well-Level Features Available

Feature	Description
FEATURE REPORTED ONCE FOR EACH WELL	
Confluency (%)	Area of a well occupied by cell colonies divided by the total area of a scan area or well.
Count	Tumorsphere Migration Colony count identified in the well image.
AVG Area (µm²)	Average Tumorsphere Migration area (µm^2) per well.
SD Area (µm²)	Standard deviation of Tumorsphere Migration area (µm^2) per well.
Total Area (µm²)	Total area of Tumorsphere Migration (µm^2) per well.
%CV Area	Percent CV of area values from the Tumorsphere Migration 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.
AVG Intensity	Well average of mean intensity of all the pixels in the Tumorsphere Migration area.
SD Intensity	Well Standard deviation of the mean intensity of all the pixels in the Tumorsphere Migration area.
%CV Intensity	Well Coefficient of variation of the mean intensity of all pixels in the Tumorsphere Migration area.
AVG Integrated Intensity	Well average of integrated intensity of all pixels in the Tumorsphere Migration area.
SD Integrated Intensity	Well Standard deviation of the integrated intensity of all the pixels in the Tumorsphere Migration area.
%CV Integrated Intensity	Well coefficient of variation of the integrated intensity of all the pixels in the Tumorsphere Migration area.
% Well Sampled	Percent of the well surface imaged.

As analysis progresses, the wells in the plate layout in the RESULTS tab become populated with numbers (Figure 10). To view a measurement to be displayed on the thumbnails, select a parameter in the measurements window on the left.

Figure 10. Scan Results



6.2 Data Export

Well-level and object-level data can be exported into CSV (Comma Separated Value). 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. Detailed metrics will be reported in the left panel, including mean and integrated intensity in each channel, as well as the corresponding standard deviations. As in the ANALYZE tab, it is possible to 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 6. Troubleshooting Recommendations

Issue	Recommended Action
Cannot identify individual Tumorsphere Migration Area	1. Inappropriate focus selected. • For brightfield imaging– verify that the focus is on the edge of the tumorsphere or the cells migrating out of the spheroid. 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.
Improper tumorsphere counts on well edges in brightfield	1. Remove/un-check Separate Touching Colonies in the ANALYZE tab – Identification section. 2. Increase Minimum Thickness. 3. Shrink % Well Mask to less than 100%. Check mark/Un-check mark Background Correction.
Well edges are too bright or dark	Liquid volume is not optimal resulting in a meniscus-dependent effect. 1. For proper liquid volumes for cell plating, see Table 1. • Check mark Background Correction.

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 8001681 Rev. E

Celigo® Cytometer Invasion and Migration: Tumorsphere Invasion Application Guide

Celigo Software Version 5.2

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1. About this Guide

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

1.1 Introduction

The Invasion and Migration: Tumorsphere Invasion application is used to quantify the invasion of a fluorescent spheroid into a non-fluorescent one. It acquires in two channels; one channel is acquired in brightfield illumination and is used to identify the outlines of the two merging spheroids. The other channel is acquired using fluorescent illumination, and is used to quantify which proportion of the merge spheroids comes from the fluorescent one. This application identifies the confluence area for each channel and reports the ratio of the confluence measurements. During the fusion of the two spheroids, the proportion of the merged area will increase over time as the cells from the invading spheroid migrate into the non-fluorescent spheroid.

1.2 Purpose

The purpose of this guide is to provide the steps and concepts that are unique to the Invasion and Migration: Tumorsphere Invasion application. Information that is common to all applications is covered in the *Celigo Cytometer User Guide* (Doc. No. 8001619), henceforth 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: support@nexcelom.com
  phone: +1 978-327-5340
- From Europe:
  e-mail: support@nexcelom.co.uk
  phone: +44 (0) 161 232 4593

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 the performance of the 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	2500	ND[1]
6W	Corning	3471	2500	ND[1]
12W	Corning	376	1000	ND[1]
24W	Corning	3524	≥500	ND[1]
96W	Greiner	655090	≥200	≥100
96W half area	Greiner	675090	50	50
384W low vol	Corning	3542	20	20
384W high vol	Corning	3764	≥40	≥40
384W high vol	Greiner	781091	≥40	≥40
1536W	Corning	3838	8	8
1536W	Greiner	789866	8	8
T-25	Corning	43069	ND[1]	ND[1]
T-75	BD Falcon	353136	ND[1]	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 Invasion and Migration: Tumorsphere Invasion application and setting the image acquisition parameters. Perform these tasks in the SCAN tab.

3.1 Invasion and Migration: Tumorsphere Invasion Application

Perform the following steps to select the Invasion and Migration: Tumorsphere Invasion application.

To select an application

Select the Invasion and Migration: Tumorsphere Invasion application (Figure 1 and Table 2) as follows:

- In the Application dropdown list, select **Invasion and Migration: Tumorsphere Invasion** (henceforth referred to as the Tumorsphere Invasion Application)

Figure 1. Selecting an Application

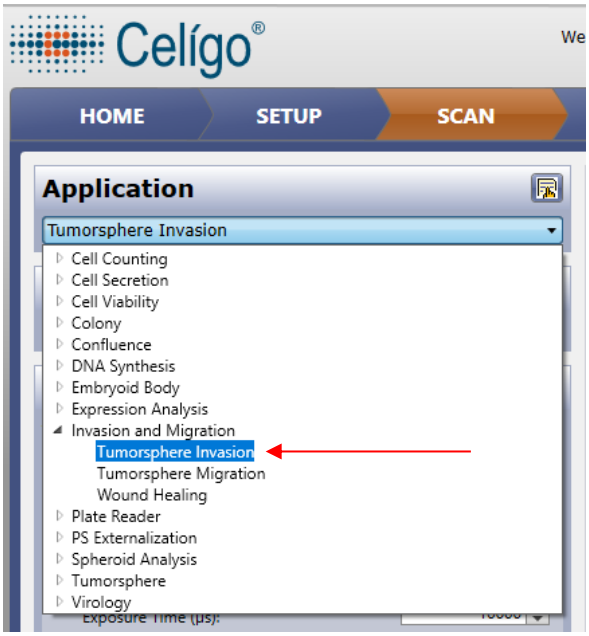


Table 2. Selecting an Application

Tumorsphere Invasion	Measures the invasion of a fluorescent spheroid into a non-fluorescent spheroid. Reports the proportion of the fused spheroid that is invaded by the fluorescent cells.

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 clicking Navigation and selecting a representative well.
    - It is recommended to adjust exposure settings using a well where the highest fluorescent signal is expected.

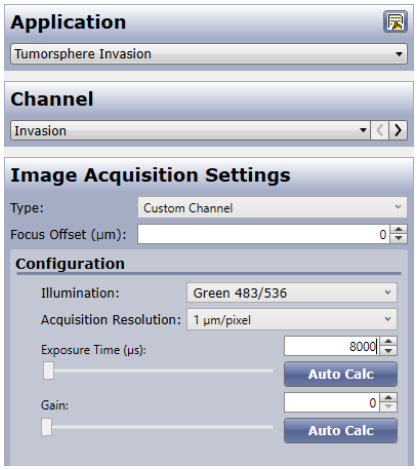
2. In Channel, select **Sphere**.
3. For the Sphere channel, make the following selections:
   a. For Brightfield illumination:
      – In Illumination, select **Brightfield**.
      – Select channel type: **Auto Exposure/Gain Channel**
      – In Priority, select **AutoExposure, Gain if Necessary**.
      – In Frequency, select **Every Scan Area**.
   b. Click **Apply**
   c. Click **Focus Setup** (see the User Guide).
      – Typically, it is recommended to select **Hardware Auto Focus** (because it provides maximum speed), then click **Register Auto**. For manual registration or using focus offset, see the User Guide.
   d. Click **Live** to see a live image.
4. In Channel, select **Invasion**.
   - It is recommended to select the fluorescent stain for the Invasion channel.

	**NOTE: The application will calculate the ratio of the area of the Invasion channel within the area of the Sphere channel. Typically for Tumorsphere Invasion, the Invasion channel will be fluorescent and the Sphere channel will be the brightfield channel.**

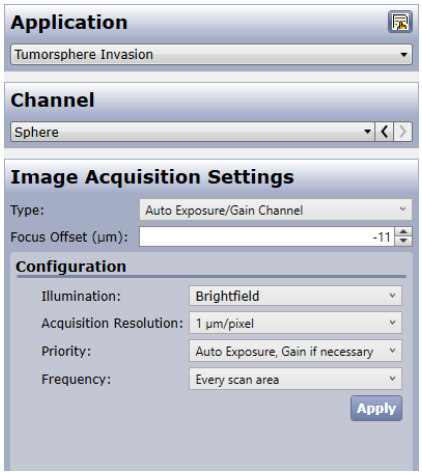
5. For the Invasion channel, make the following selections:
   - For Fluorescent illumination:
      – Select channel type: **Custom**
      – In Illumination, select the appropriate illumination (e.g., Green 483/536 for GFP signal, Red 531/629 for RFP signal or Blue 377/447 to visualize the Hoechst signal).
   a. Click **Live** to see a live image.
   b. Select whether the fixed exposure time provides optimal signal (an object pixel intensity of 125-175) for the sample and do one of the following.
      – Adjust **Exposure** time and **Gain** manually. When doing so, adjust the exposure so the majority of cells aren't overexposed (i.e.: saturated with an object level pixel intensity that is ≥ 255).
      – Apply **Auto Calc** if necessary: the software will calculate an exposure time or a gain so that the signal is visible without saturation of the image.
   c. If a lower image resolution is desired, select 2, or higher µm/pixel. For a detailed explanation of proper image resolution selection, see the User Guide.

Figure 2 shows a typical example of GFP and Brightfield image acquisition parameter selection for the Tumorsphere Invasion application

Figure 2. Tumorsphere Invasion Application Invasion Channel Selections



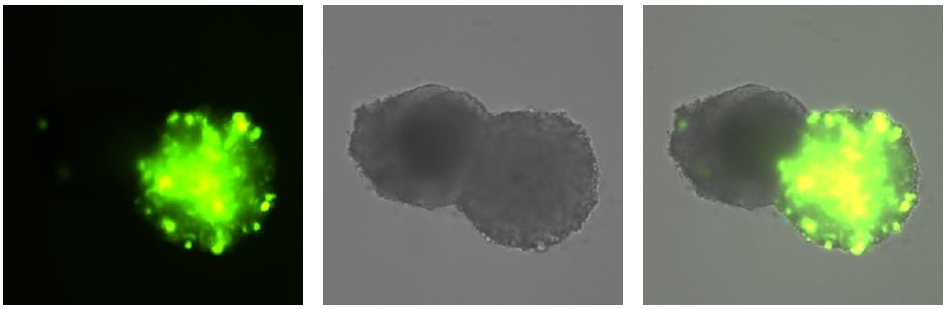
Tumorsphere Invasion Application Sphere Channel Selections



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

Figure 3 shows a typical example of GFP and Brightfield image acquisition for the Tumorsphere Invasion application

Figure 3. Tumorsphere Invasion typical images



Invasion Channel GFP | **Sphere Channel Brightfield** | **Both Channels GFP + Brightfield**

4. Analyzing Images

This chapter provides information on how to analyze scans from the Tumorsphere Invasion application. Perform these tasks in the ANALYZE tab.

4.1 Analysis Settings

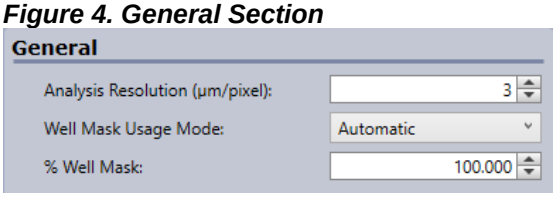
Perform the following steps for Tumorsphere Invasion analysis. For the recommended initial identification and pre-filtering settings to use as a guide, see **Figure 5**.

To perform Tumorsphere Invasion analysis

1. Load prior saved Analysis Settings, if available.

In the General section (Figure 4**), make the following selections:**

Figure 4. General Section

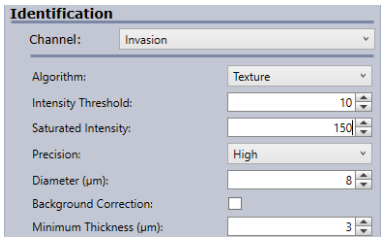


2. Image Resolution (µm/pixel) – Select the image resolution to use for analysis. Entering a lower value (minimum1 µm/pixel; ≥ 2 µm/pixel recommended) will result in a more defined and longer analysis time. Entering a higher value will result in a less defined and shorter analysis time. Analysis of spheroids and tumorspheres performs well with a resolution of 3 µm/pixel.
3. Well Mask Usage Mode – Select one of the following:
   - **Automatic** (Default) – The system looks at the well image to find the edge of the well.
   - **Original** – The system uses the set mapped position for the edge of the well, specified in the plate profile.
4. % 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.

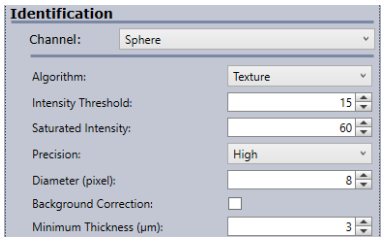
In the Identification section (Figure 5), make the following selections to be used to identify cells (see Table 3 for recommended parameters):

Figure 5. Tumorsphere Invasion Application

Invasion Channel Selections



Sphere Channel Selections



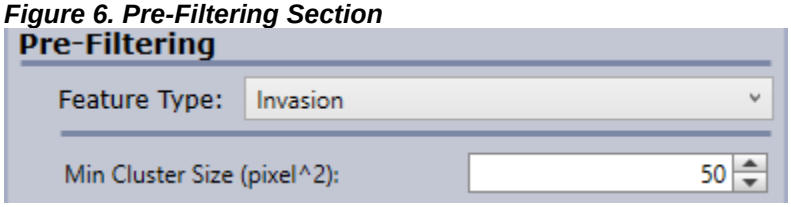
5. Select the **Invasion** Channel
6. Algorithm – Select the appropriate algorithm:
   - Texture – This algorithm looks for texture differences between the objects found and the background areas (Recommended)
   - Brightfield – Do not select for the fluorescent Invasion channel.
7. Intensity Threshold – Select the optimal intensity threshold.

   This selection is the level of intensity that separates the background from cells. With an appropriate threshold set, the background pixels fall below and the pixels inside the cells are above the threshold.
8. Saturated Intensity (0-255) – 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 the confluent areas. Holes consisting of pixels that are all saturated are filled in post-segmentation.
9. Precision – Higher precision results in a more accurate identification of cell clusters. Normal is recommended because this setting is sufficient to provide acceptable results within a shorter time frame, while the High setting results in a longer analysis processing time. For more information on determining the correct precision, see the User Guide.
10. Diameter (µm) – Do one of the following:
    - If **Texture** was selected in the Algorithm field, skip to this step. (The entry in Diameter has no effect when Texture is the selected Algorithm.)
    - If **Brightfield** was selected 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.
11. Background Correction – Select if necessary (See the User Guide).
12. Minimum Thickness (µm) – Minimizes long extensions. Increase if the plate has plate artifacts, such as a plate ring stamp.

In the Pre-Filtering section (Figure 6), make the following selection:

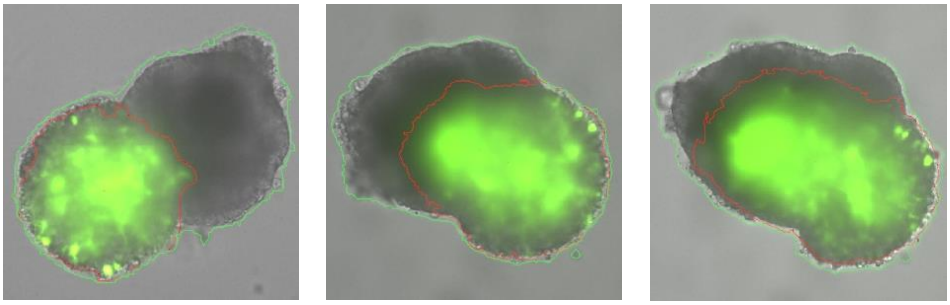
Figure 6. Pre-Filtering Section



13. Min Cluster Size (µm^2) – Enter the appropriate minimum cluster size.
    - To eliminate small debris, adjust the value to be larger than the small debris size and lower than the cell cluster size.
14. Select **Sphere** in the Channel drop down menu
15. Repeat steps 3e to 4 (see Table 3 for recommended parameters).

Figure 7. Tumorsphere Invasion over time – GFP (Invasion-red outline) and Brightfield (Sphere- green outline) segmentation shows an example of a tumorsphere expressing GFP invading an Embryoid body. The total sphere area is identified in the brightfield channel and the GFP invasion is quantified in the fluorescent channel.

Figure 7. Tumorsphere Invasion over time – GFP (Invasion-red outline) and Brightfield (Sphere- green outline) segmentation



t = 24h
Invasion = 47%

t = 48h
Invasion = 63%

t = 72h
Invasion = 70%

	NOTE: The segmentation of the Invasion channel is confined within the segmentation of the Sphere channel. In the example provided above, any GFP signal outside of the boundaries of the brightfield segmentation would be ignored.

Table 3. Recommended Initial Identification and Pre-Filtering Settings for Analysis – Confluence Tumorsphere Invasion

ACQUISITION ILLUMINATION	***Fluorescence***	***Brightfield***
IDENTIFICATION		
Algorithm	*Texture*	*Texture*
Intensity Threshold	*5*	*20*
Saturated Intensity	*200*	*40*
Precision	*Normal*	*Normal*
Diameter (µm)	*Not Applicable (selection has no effect)*	*Not Applicable (selection has no effect)*
Background Correction	*Not Applicable (selection has no effect)*	*Check marked*
Minimum Thickness (µm)	*3*	*3*
PRE-FILTERING		
Min Cluster Size (μm^2)	*50*	*100*

5. Gating Cells

When using the Tumorsphere Invasion application, gating is typically not performed in the GATE tab. Instead, the application relies on the identification of fluorescence and brightfield cell growth areas by quantifying the intensity threshold in the ANALYZE tab. The pre-filtering parameters in the ANALYZE tab (see chapter 4) manages the filtering of debris or very small cell clusters.

6. Viewing Results

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

6.1 Application Outputs

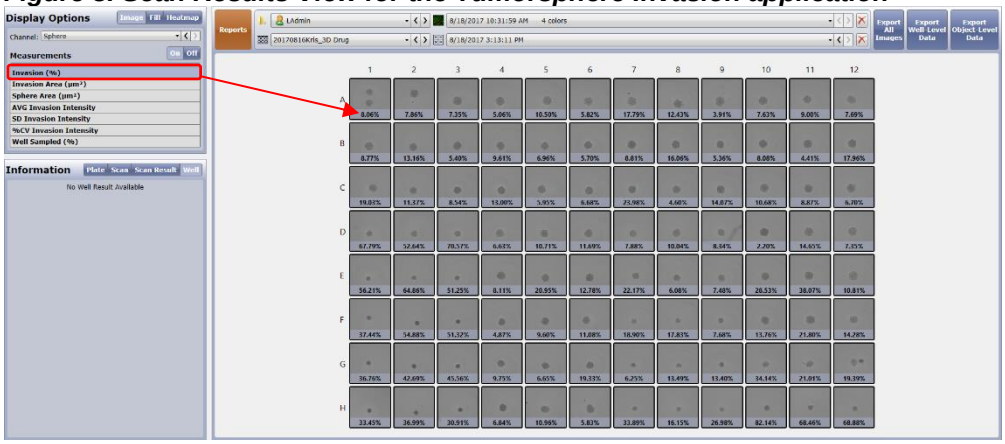
The parameters listed in Table 4 appear in the Measurements section in the Display Options.

Table 4. Cell Counting and Growth Tracking Application Outputs

Parameter	Description
Invasion (%)	Invasion Area divided by Sphere Area reported as a percentage. Typically represents the proportion of fluorescent tumorsphere within the total brightfield spheroid area.
Invasion Area (µm²)	Area of the well occupied by individual fluorescent cells or cell clusters in the Invasion channel (typically fluorescent)
Sphere Area(µm²)	Area of the well occupied by individual spheroids in the Sphere channel (typically brightfield)
AVG Invasion Intensity	Well average of the mean intensity of all the pixels in the Tumorsphere Invasion area.
SD Invasion intensity	Well standard deviation of the mean intensity of all the pixels in the Tumorsphere Invasion area.
Well Sampled (%)	Percent of the well sampled

As analysis progresses, the wells in the plate layout in the RESULTS tab become populated with numbers (Figure 8). To view a measurement to be displayed on the thumbnails, select a parameter in the measurements window on the left.

Figure 8. Scan Results View for the Tumorsphere Invasion application



6.2 Data Export

Well-level and object-level data can be exported into CSV (Comma Separated Value) 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. Detailed metrics will be reported in the left panel, including invasion area percentage and sphere total area for each channel. As in the ANALYZE tab, zoom in and use the toggles in the Image Display and Graphic Overlays.

7. Troubleshooting

This chapter provides troubleshooting recommendations.

Table 5. Troubleshooting Recommendations

Issue	Recommended Action
Cannot identify individual Tumorsphere Area	1. Inappropriate focus selected. • For brightfield imaging– verify that the focus is on the edge of the tumorsphere or the cell migrating out of the spheroid. 2. Intensity threshold value is too high. 3. Desired objects are excluded by the Pre- Filtering settings. • View the segmented image in the ANALYZE tab (see the 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.
Improper sphere counts on well edges in brightfield	1. Increase or decrease intensity threshold. 2. Increase Minimum Thickness. 3. Shrink % Well Mask to less than 100%. • Check mark/Uncheck mark Background Correction.
Well edges are too bright or dark	Liquid volume is not optimal resulting in a meniscus-dependent effect. • For proper liquid volumes for cell plating, see Table 1. • Check mark Background Correction.

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Celigo® Cytometer
Confluence and Growth Tracking
Application Guide

Celigo Software Version 5.2

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1. About this Guide

This chapter provides a brief description of how to use the Nexcelom Celigo Cytometer for determining cell confluence and growth tracking application.

1.1 Introduction

The Confluence and Growth Tracking application is a single channel application and identifies areas with clusters of cells using brightfield or fluorescent imaging. The application includes advanced data analysis functions to determine growth curves and doubling times using the Growth Tracking reporting option.

1.2 Purpose

The purpose of this guide is to provide the steps and concepts that are unique to the Confluence 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:
  E-mail: support@nexcelom.com
  Phone: +1 978-327-5340
- From Europe:
  E-mail: support@nexcelom.co.uk
  Phone: +44 (0) 161 232 4593

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 the application's performance. (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 Image Analysis	
			Brightfield	Fluorescence
6W	Corning	3516	2500	ND[1]
6W	Corning	3471	2500	ND[1]
12W	Corning	3512	1000	ND[1]
24W	Corning	3524	≥500	ND[1]
96W	Greiner	655090	≥200	≥100
96W half area	Greiner	675090	50	50
384W low vol	Corning	3542	20	20
384W high vol	Corning	3764	≥40	≥40
384W high vol	Greiner	781091	≥40	≥40
1536W	Corning	3838	8	8
1536W	Greiner	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 Confluence 1 application, as well as how to set the image acquisition parameters. Perform these tasks in the SCAN tab.

3.1 Confluence 1 Application

Perform the following steps to select a Confluence 1 application.

To select an application

- In the Current Application dropdown list (Figure 1), select **Confluence** > **Confluence 1**.
  For information regarding the differences between the confluence applications see Table 2.

Figure 1. Selecting an Application

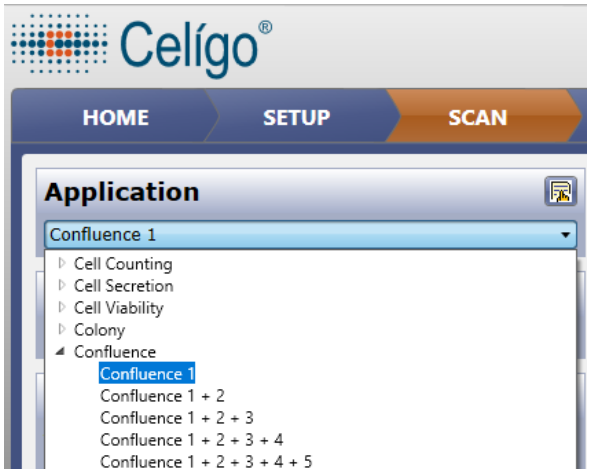


Table 2. Selecting a Confluence Analysis Application

Number of Channels to be Acquired	Select this application	Use Case
One channels	**Confluence 1**	Measures the area of a well occupied by individual cells, or cell clusters. Growth Tracking data can be created with this selection.
Two channels	**Confluence 1 + 2**	Measures area of a well occupied by individual cells or cell clusters for each channel. Reports the ratio of the surface occupied by cells and cells clusters relative to every other acquired channel. Growth Tracking not currently available with these selections. For details on how to acquire and analyze multichannel confluence images, refer to **Multichannel Confluence Application Guide**
Three channels	**Confluence 1 + 2 + 3**	
Four channels	**Confluence 1 + 2 + 3 + 4**	
Five channels	**Confluence 1 + 2 + 3 + 4 + 5**	

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 in the Image Acquisition Settings:
        - Select Type: Auto Exposure/Gain Channel (recommended)
        - Select Focus Offset (µm): 0
        - In Illumination, select **Brightfield**.
        - In Acquisition Resolution: **1 µm/pixel**
        - In Priority: **Auto Exposure, Gain if necessary**
        - In Frequency: **Every Scan Area**
        - Click **Apply** after all settings are selected
    - For Fluorescence illumination:
        - It is recommended to use the Custom channel type, with an appropriate color wavelength selected (Green 483/586, Red 531/629, Blue 377/447, Far Red 632/692). For detailed instructions, see the User Guide.

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

4. Set up focus per the User Guide.
    - For detailed instructions on selecting the 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 the 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 fluorescent illumination, see the User Guide.

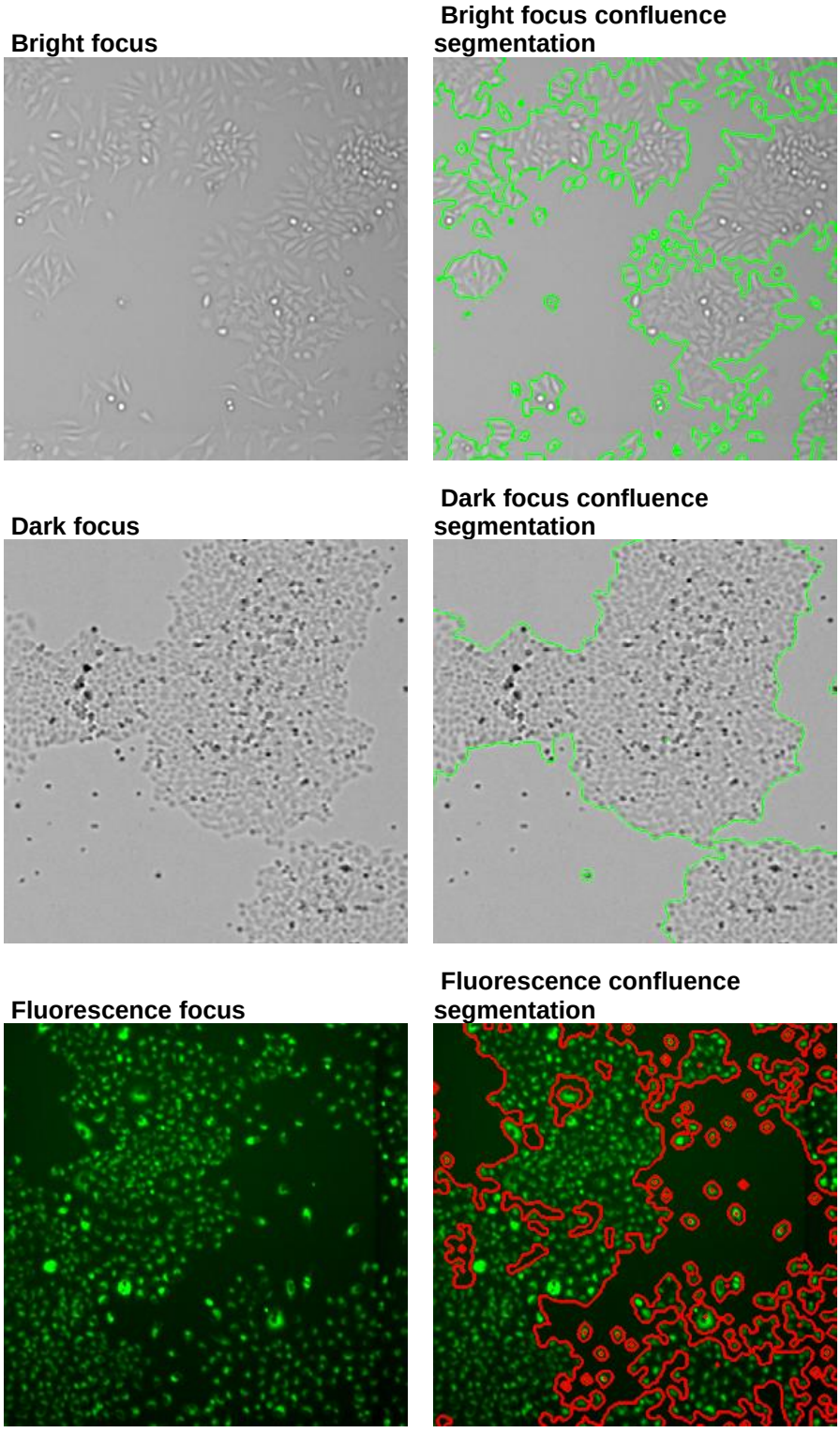
To select the correct focus position for brightfield imaging

1. Choose a well for setup using the plate map by selecting **Navigation** and a representative well.
2. View a well by selecting **Live** in the Camera Controls pane.

	**NOTE: Adjust manually with up/down arrows the focus until the cells have a large, bright center and are lacking high cell details. For examples of proper focus using the Bright and Dark selections in the Target Focal Plane menu, see Figure 2.**

3. Select **Focus Setup** and select **Hardware Auto Focus** in the Focus Type field, then **Register Auto**. The system will 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, Dark and Fluorescence Focus with resulting confluence segmentation



4. Analyzing Images

This chapter provides information on how to analyze scans from the Confluence 1 Application. Perform these tasks in the ANALYZE tab.

4.1 Analysis Settings for Brightfield Confluence Analysis

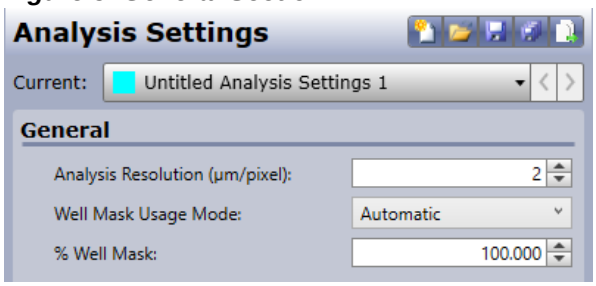
Perform the following steps for Confluence analysis. For the recommended initial identification and pre-filtering settings to use as a guide, see Table 3.

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

To perform Confluence analysis

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

Figure 3. General Section



a. Analysis Resolution (µm/pixel) – Select the image analysis resolution to use for analysis. Entering a lower value (minimum 1 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:

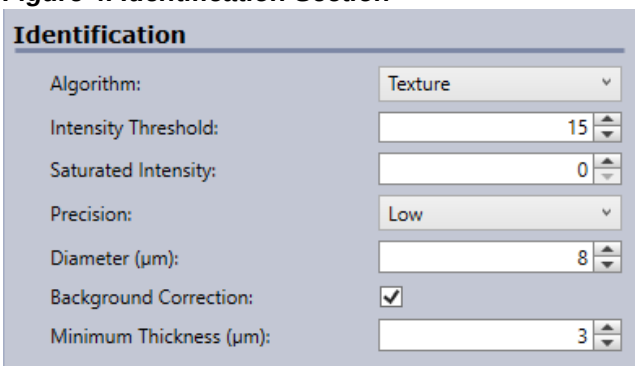
- **Automatic** (Default) – The system looks at the well itself to find the edge of the well (looks for the local minima).
- **Original** – The system uses the set mapped position for the edge of the well, specified in the plate profile.

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 4), make the following selections to be used to identify cells:

Figure 4. Identification Section



a. Algorithm – Select the appropriate algorithm:

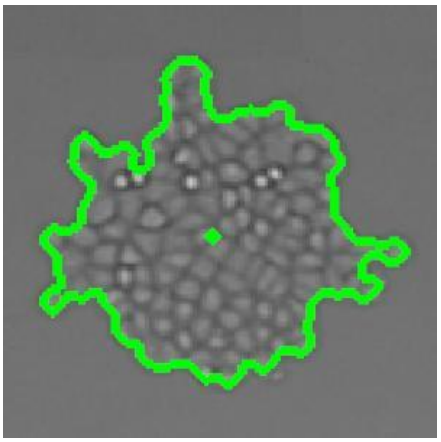
- Brightfield – The algorithm looks for objects with a bright center and dark edges
- Texture – The algorithm looks for texture differences between the objects found and the background areas (Recommended)

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

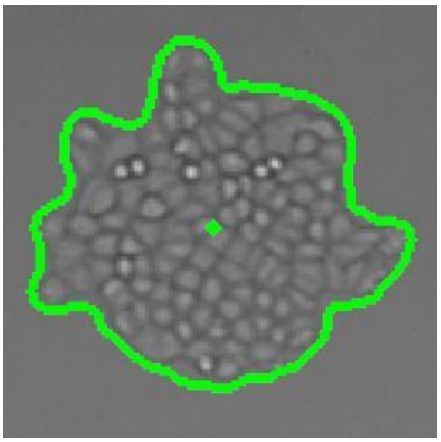
Figure 5 shows an example of selecting Brightfield versus Texture as the Algorithm field.

Figure 5. Confluence Application – Brightfield vs. Texture Algorithm Selected

Brightfield Algorithm Selected for Adherent Cells



Texture Algorithm Selected for Adherent Cells



b. Intensity Threshold – Select the optimal intensity threshold.

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

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 precision is recommended. Normal is sufficient to provide acceptable results, while High results in longer analysis processing time.

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

   - If selecting Texture in the Algorithm field, skip to step 3f. (The entry in Diameter has no effect when Texture is the Algorithm.)
   - If selecting 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.

f. Background Correction – Select if necessary.

g. 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 6), make the following selection:

Figure 6. 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.

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

Table 3. Recommended Initial Identification and Pre-Filtering Settings for Analysis – Confluence 1

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	Check marked	Check marked	Check marked	Check marked
Minimum Thickness (µm)	3	3	3	3
PRE-FILTERING				
Min Cluster Size (µm²)	50	50	500	500

Figure 7 and Figure 8 show examples of analysis settings for Brightfield versus Texture as the Algorithm selection. Texture is Recommended.

Figure 7. Brightfield Analysis Settings

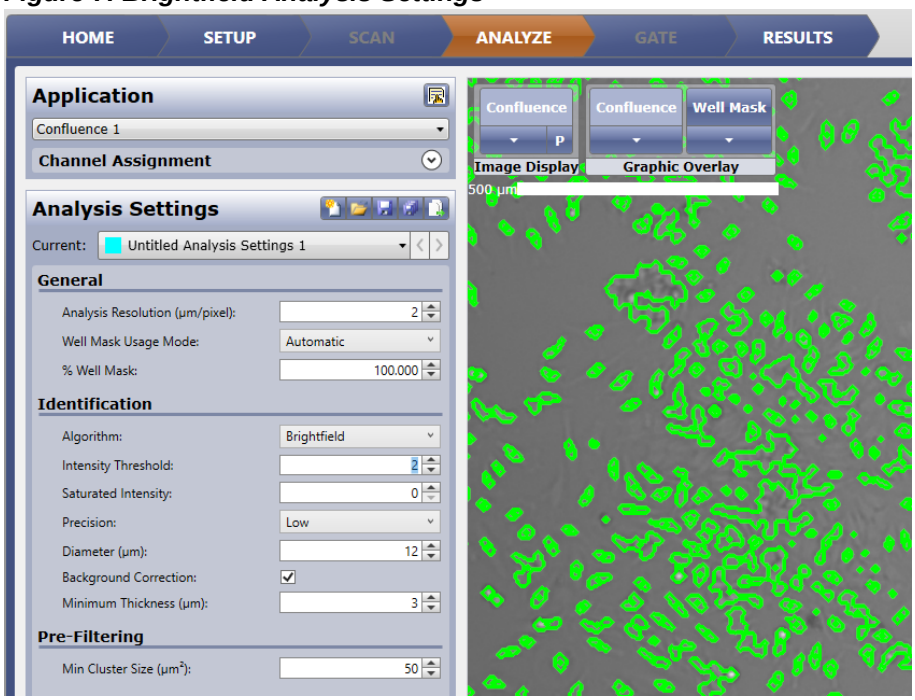
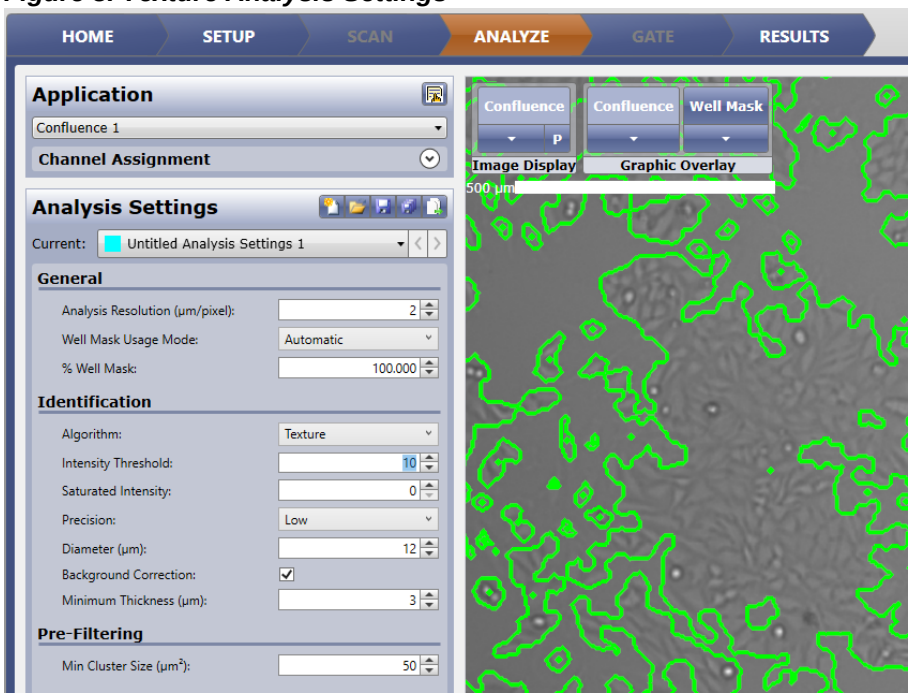


Figure 8. Texture Analysis Settings



6. In the Auto Analyze section: check mark this selection as follows:
    - Check mark Auto Analyze to update the segmented image and overlays with each selection
    - To update the segmented image and overlays only after clicking Analyze, deselect Auto Analyze.

5. Gating Cells

The Confluence applications suite is not compatible with the use of gates and gating is not allowed

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 4. Cell Counting and Growth Tracking Application Outputs

Feature	Description
FEATURE REPORTED ONCE FOR EACH WELL	
% Well Sampled	Percent of well surface imaged.
FEATURE REPORTED FOR EACH CONFLUENCE CHANNEL	
Confluence 1 (%)	Area of the well occupied by individual cells or cell clusters divided by total area of the well.
Confluence 1 Total Area (µm^2)	Area of the well occupied by individual cells or cell clusters.
AVG Confluence 1 Mean Intensity	Well average mean intensity of all pixels in segmented area.
SD of Confluence 1 Mean Intensity	Well average standard deviation of mean intensity of all pixels in segmented area.
AVG Confluence 1 Integrated Intensity	Well average integrated intensity of all pixels in segmented area.
SD of Confluence 1 Integrated Intensity	Well average standard deviation of integrated intensity of all pixels in segmented area.

6.1 Generating a Growth Tracking Report

Growth tracking reports calculate the growth characteristics of cell populations over time. The reports associate confluence measurements from multiple scan time points – using each scan's latest scan result or a default – and then determines the 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

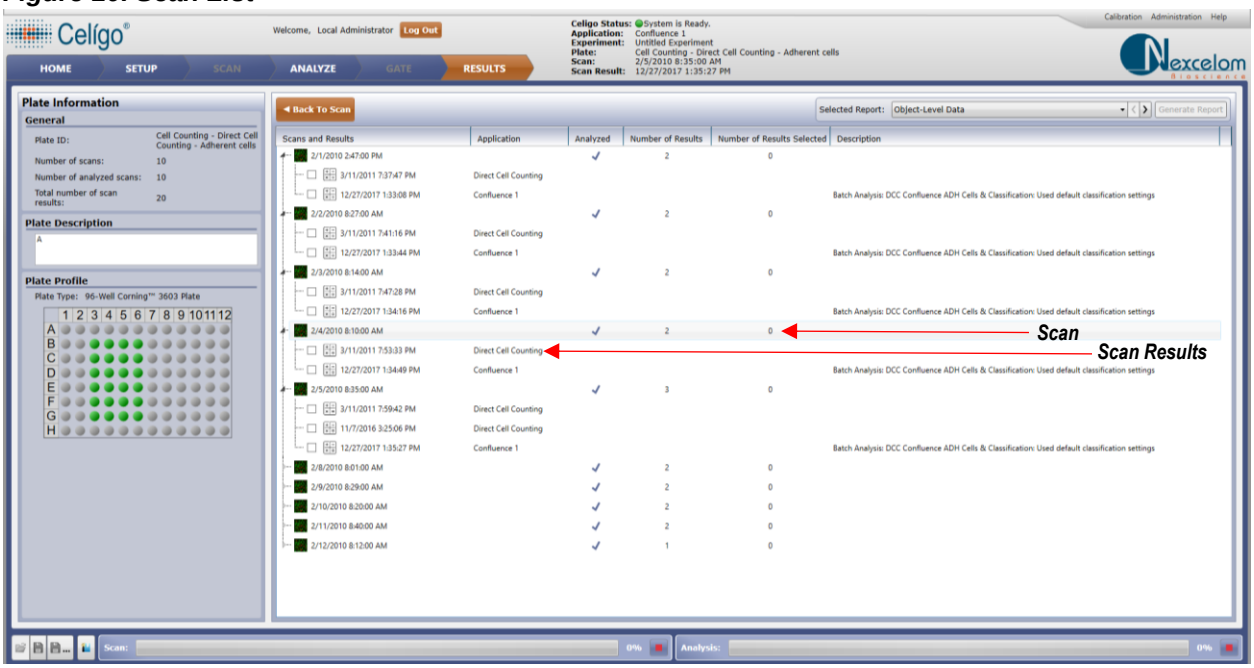
	**NOTE: To generate a pie chart, make sure that 4 or more scans exist for selection. To generate a growth chart (curve), make sure that 2 or more scans exist for selection.**

1. In the RESULTS tab, click **Reports** (Figure 9)

Figure 9. Displaying Scans for Reporting

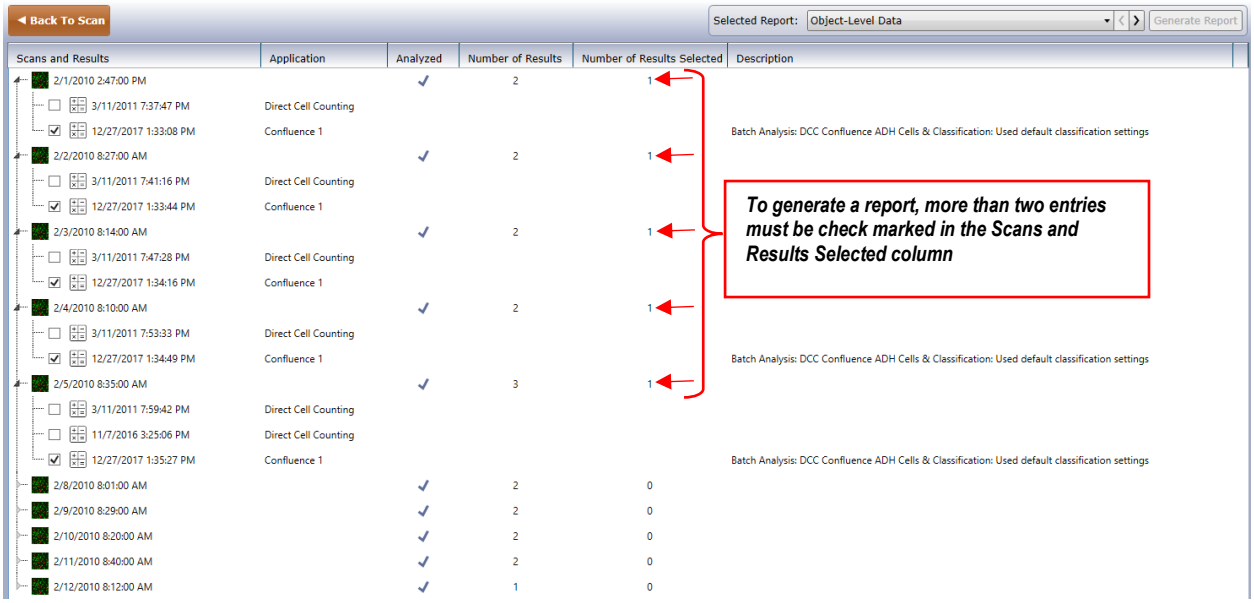
The list of existing scans and associated scan results for the plate ID appears (Figure 10).

Figure 10. Scan List



- Check mark the checkbox (Figure 11) next to each scan result to be included in the growth tracking report. The Number of Results Selected column will update with a value of 1 to confirm selection.

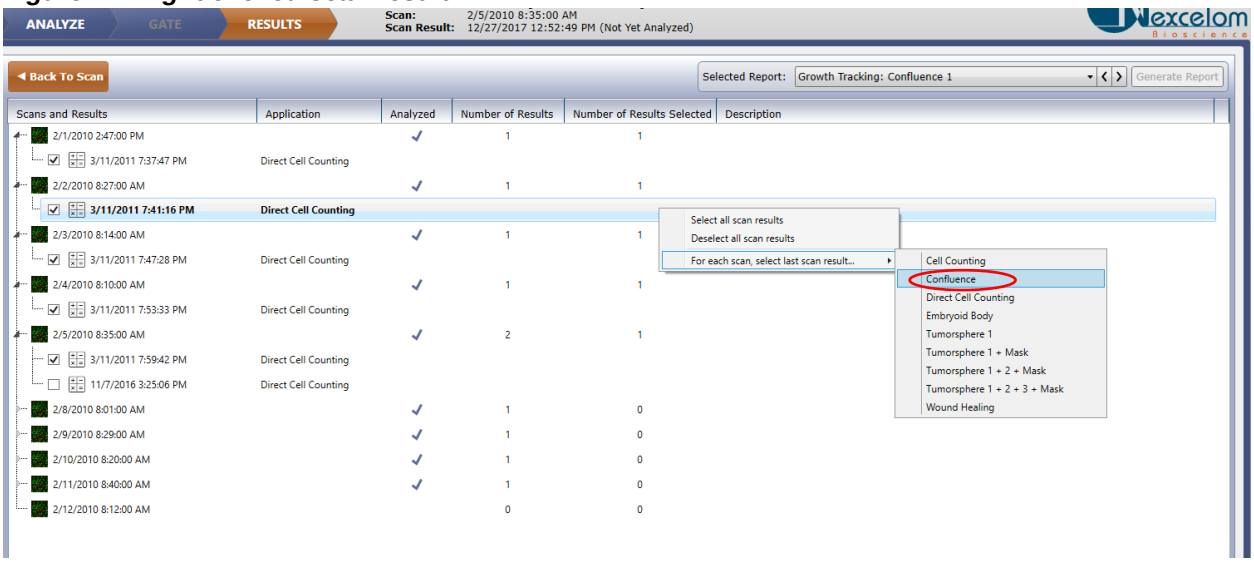
Figure 11. Selecting Scan Results for a Growth Tracking Report



Optional: Right-click a scan result and select one of the following (Figure 12):

- Select all scan results
- Deselect all scan results
- For each scan, select last scan result… Confluence

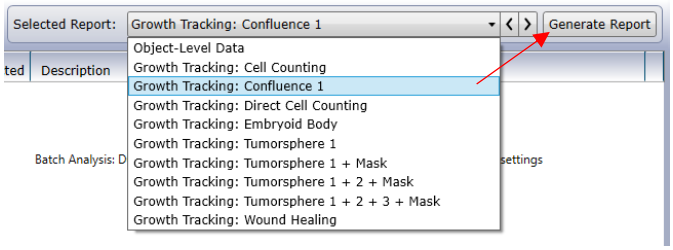
Figure 12. Right clicked scan result



2. In the Selected Report menu, select Growth Tracking: Confluence 1 (Figure 13).

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

Figure 13. Selecting Main Type of Report



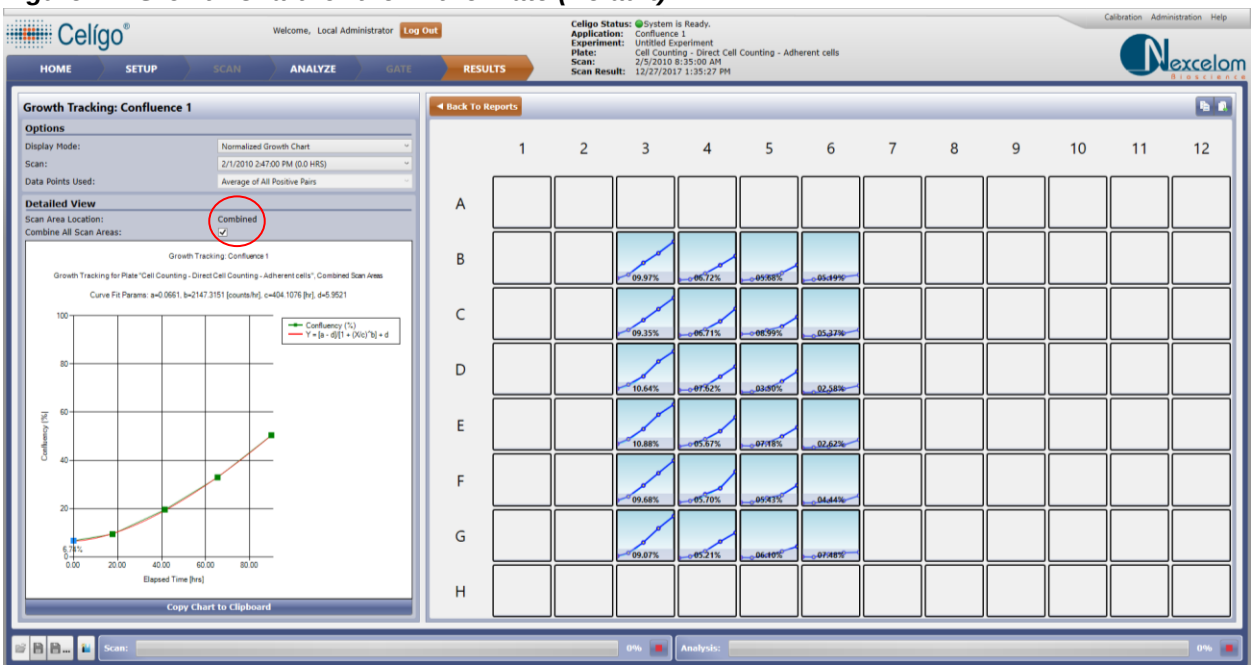
	NOTE: Only the scans analyzed using the application selected for growth tracking reporting will be available for selection.

3. Click **Generate Report** at the top right of the RESULTS tab (Figure 13).

   A growth chart (default display mode) for the entire plate, with Combine All Scan Areas selected, appears in the RESULTS tab (Figure 14).

   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 174). This option makes calculations based on all wells of the plate.

Figure 14. Growth Chart for the Entire Plate (Default)



6.2 Working with a Generated Report

Perform 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 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 15).

Figure 15. Growth Curve and Pie Charts in Doubling Rate Display

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.

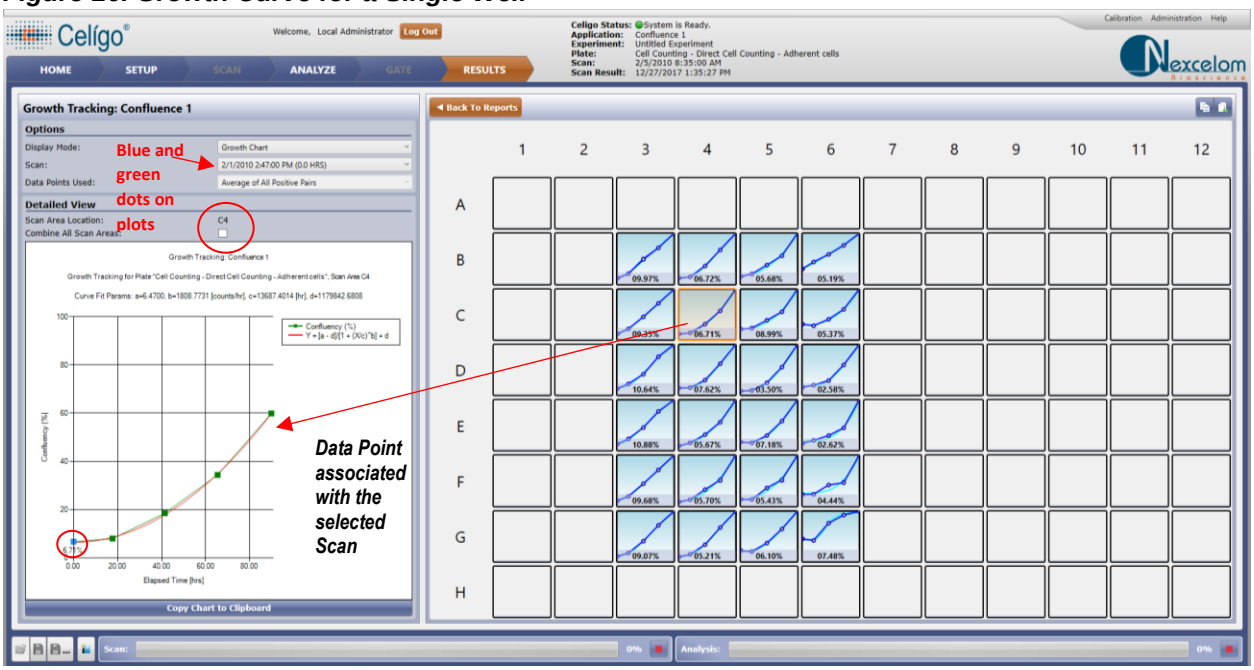
An orange border appears around the selected well (Figure 16).

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 16), select a different scan in the Scan menu.

Figure 16. Growth Curve for a Single Well



6.2.3 Displaying a Growth Curve for the Entire Plate

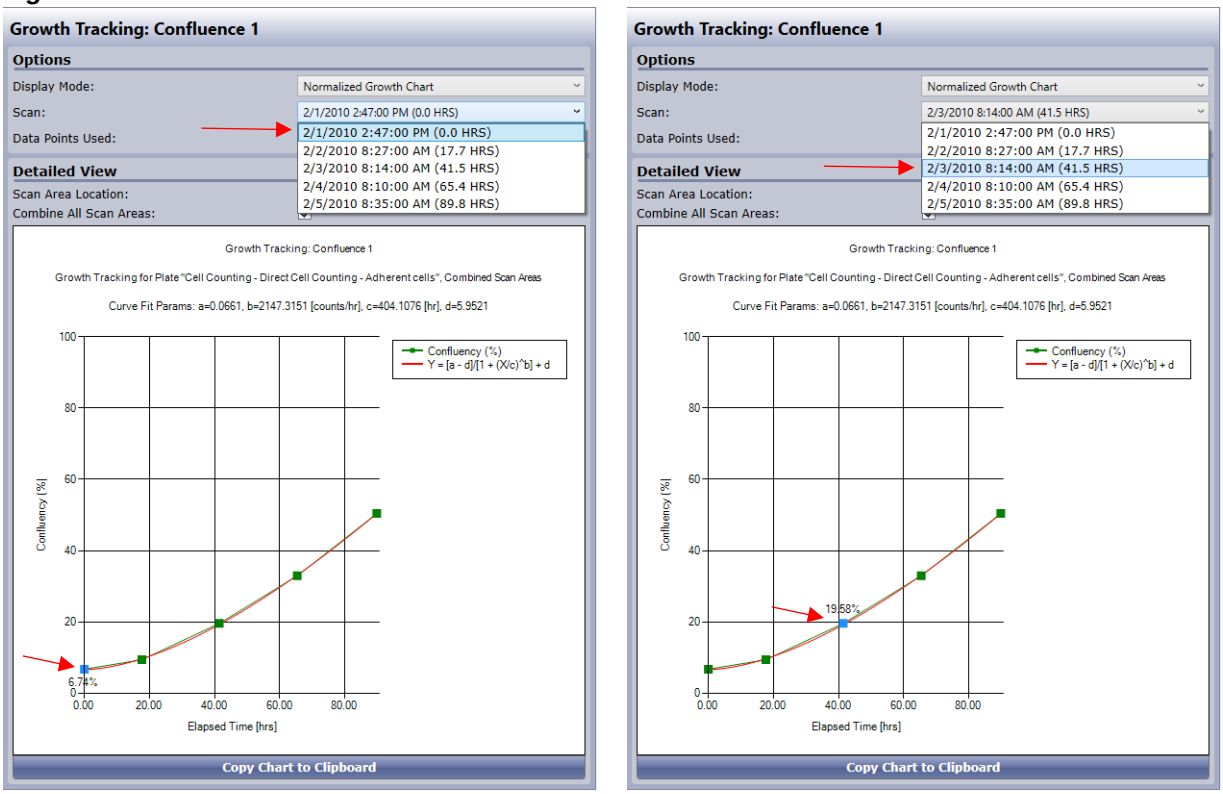
Display the growth curve for the entire plate by clicking the Growth Chart Display Mode and clicking **Combine All Scan Areas** checkbox in the left-hand pane (Figure 17). This option makes calculations based on all wells of the plate.

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 17), select a different scan in the Scan menu.

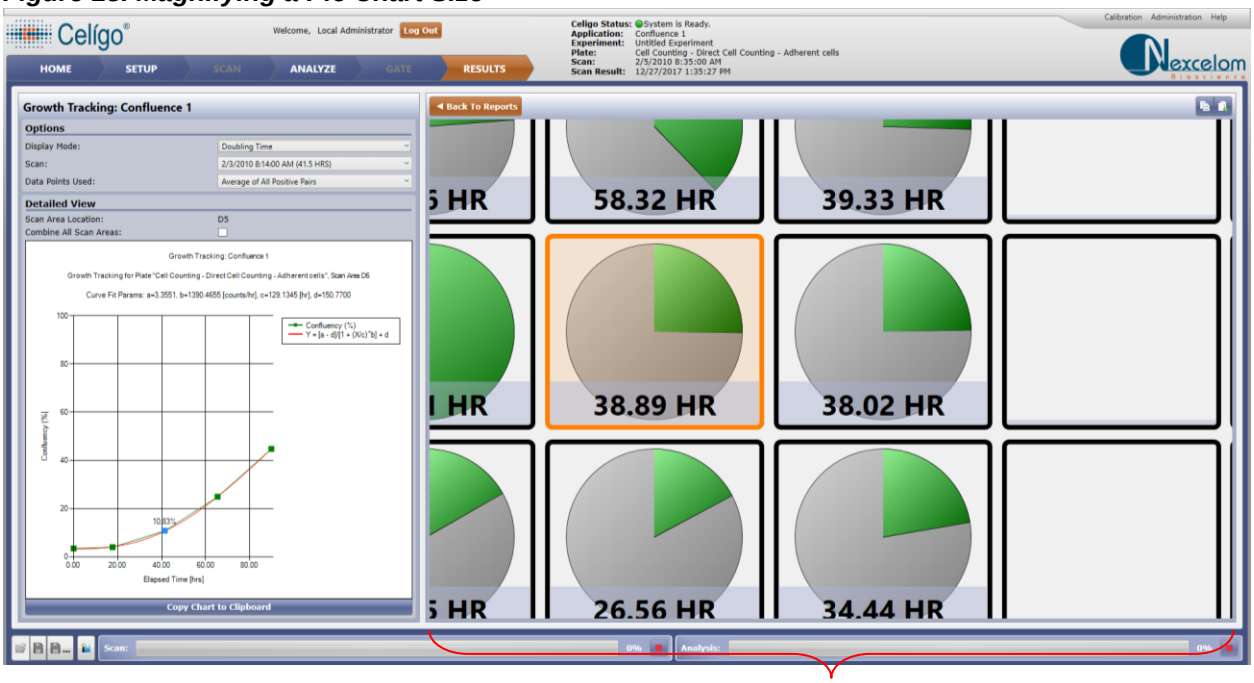
Figure 17. Growth Curve for the Entire Plate



6.2.4 Magnifying a Pie Chart Size (Zoom)

To magnify the appearance of a pie chart pane (not a growth curve) (Figure 18) left-click the pie chart and then scroll with the mouse scroll wheel.

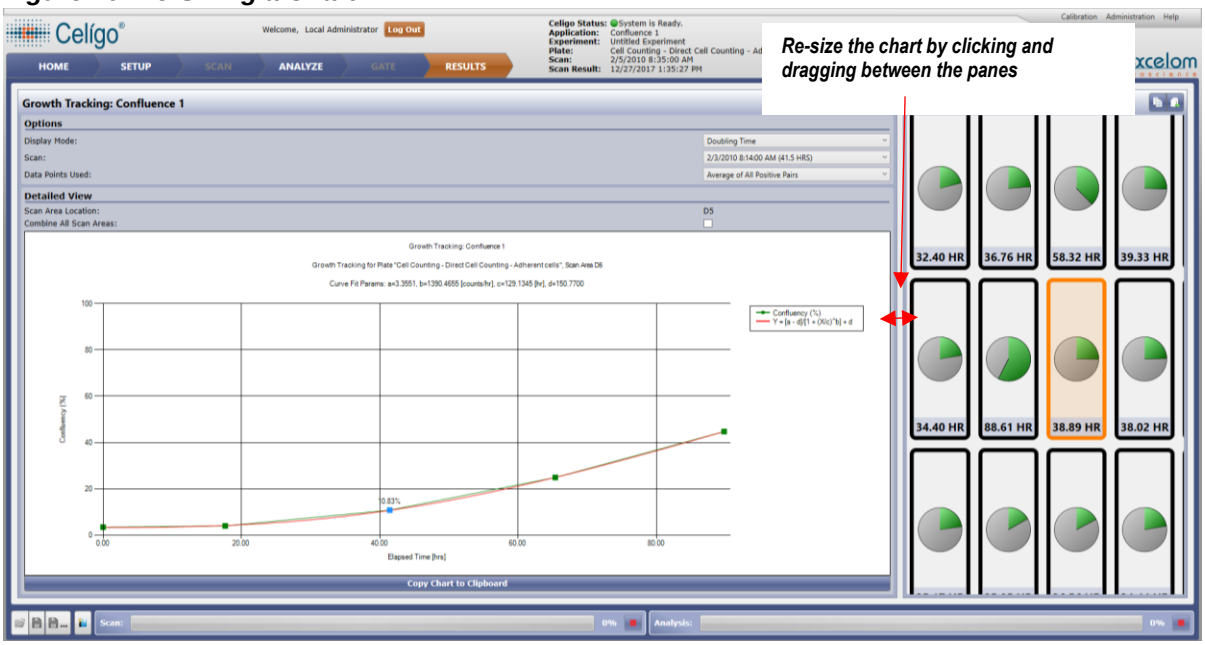
Figure 18. Magnifying a Pie Chart Size



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 19). This action is not to change the chart data, but is only for viewing purposes.

Figure 19. Re-Sizing a Chart



6.2.6 Exporting Report Data

Export growth tracking report data by clicking **Export Growth Tracking Report**. For a summary of outputs, see Table 5.

Table 5. Growth Tracking CSV Report Outputs

Parameter	Description
Confluence 1 (%)	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** (Figures 19 A. and 19 B.).

Figure 19 A. Copy Chart to Clipboard

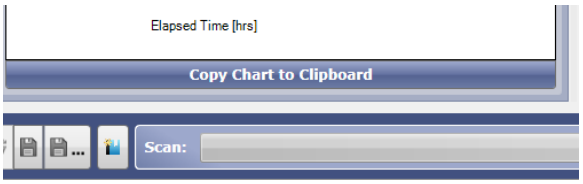
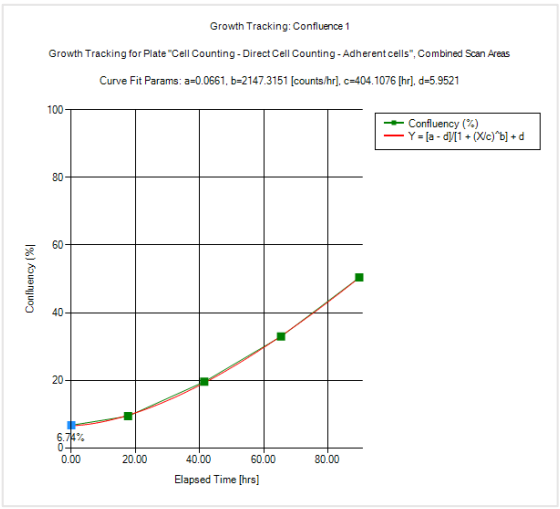
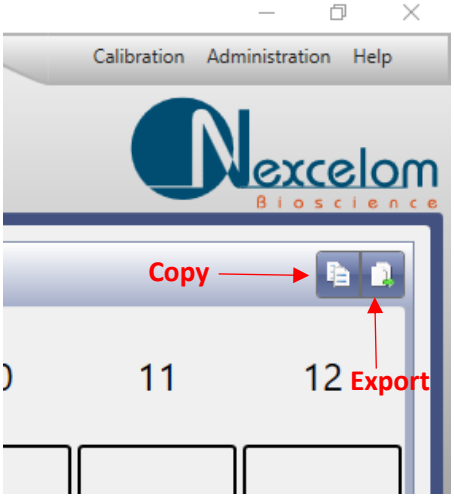


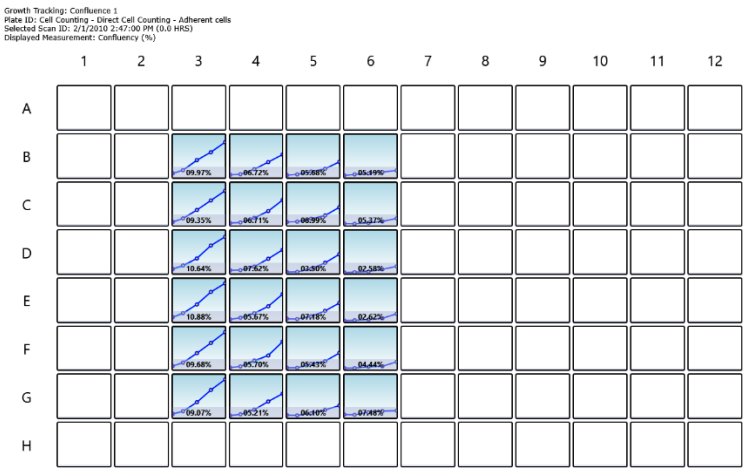
Figure 19 B. Image copied to clipboard



Export or copy an image of the plate grid (Figure 20 B) by clicking the double pages in the top right corner of the window (Figure 20 A).

Figure 20 A. Copy Plate Grid to Clipboard ***Figure 20 B. Image copied to clipboard***





7. Troubleshooting

This chapter provides troubleshooting recommendations.

Table 6. 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 3.14.1, depending on whether Direct Cell Counting or Confluence application is selected).
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. D

Celigo® Cytometer

Spheroid Analysis Application Guide

Celigo Software Version 5.2

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1. About this Guide

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

1.1 Introduction

The Spheroid Analysis application identifies and counts individual tumorspheres and small clusters of tumorspheres using brightfield and fluorescent imaging. Spheroid Analysis application is designed to provide fluorescent measurements in the inner, middle and outer part of the spheroid, zonal. This application significantly reduces the time and effort needed to quantify key aspects of 3D spheres including size, growth, and response to chemotherapeutics. Analysis of tumorspheres 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 Spheroid Analysis application. Information that is common to all applications is covered in the *Celigo® Cytometer User Guide (Document 8001619)*, henceforth 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:
  E-mail: support@nexcelom.com
  Phone: +1 978-327-5340
- From Europe:
  E-mail: support@nexcelom.co.uk
  Phone: +44 (0) 161 232 4593

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 tumorspheres.
  - 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 while 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 Spheroid Analysis application and setting the image acquisition parameters. Perform these tasks in the SCAN tab.

3.1 Spheroid Analysis Application

Perform the following step to select the Tumorsphere application:

To select the Spheroid Analysis Application

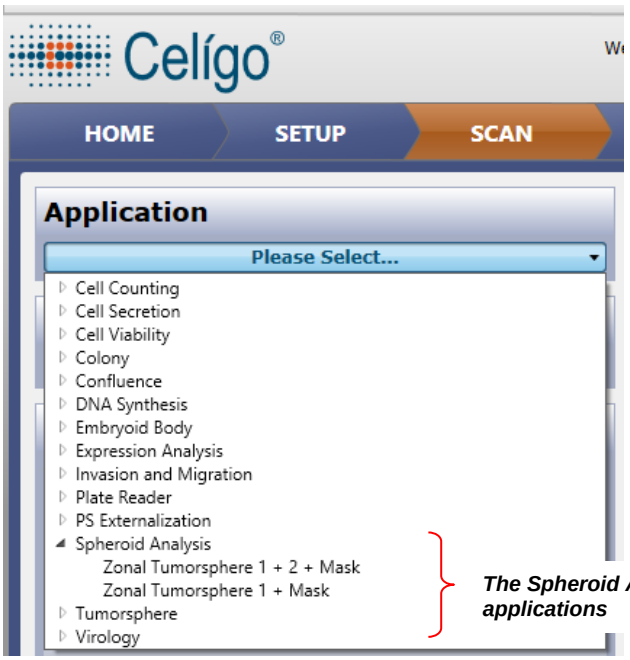
– In the Application dropdown list, select one of the applications (Figure 1), depending on the quantity of channels (targets) to be acquired

– The basic premise of this zonal application is the following:

The brightfield mask defines the tumorsphere area. Fluorescence signal is measured in the same mask area. The application calculates fluorescence signal in tumorspheres identified in inner, middle and outer zonal areas.

– Table 2.

Figure 1. Selecting the Spheroid Analysis application



The basic premise of this zonal application is the following:

The brightfield mask defines the tumorsphere area. Fluorescence signal is measured in the same mask area. The application calculates fluorescence signal in tumorspheres identified in inner, middle and outer zonal areas.

Table 2. Selecting a Tumorsphere Application

Quantity of Channels to be Acquired	Select this application
Three FL channels + a BF mask	**Zonal Tumorsphere 1 + 2 + Mask**
Two FL channels + a BF mask	**Zonal Tumorsphere 1 + Mask**

3.2 Acquisition Settings

Perform the following steps to select image acquisition settings. Use the following guidelines:

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

3.2.1 Selecting Acquisition Settings for the Mask (Brightfield) Channel

When performing the following procedure, see Table 3. Recommended Initial Settings for Image Acquisition for the recommended initial settings to use as a guide.

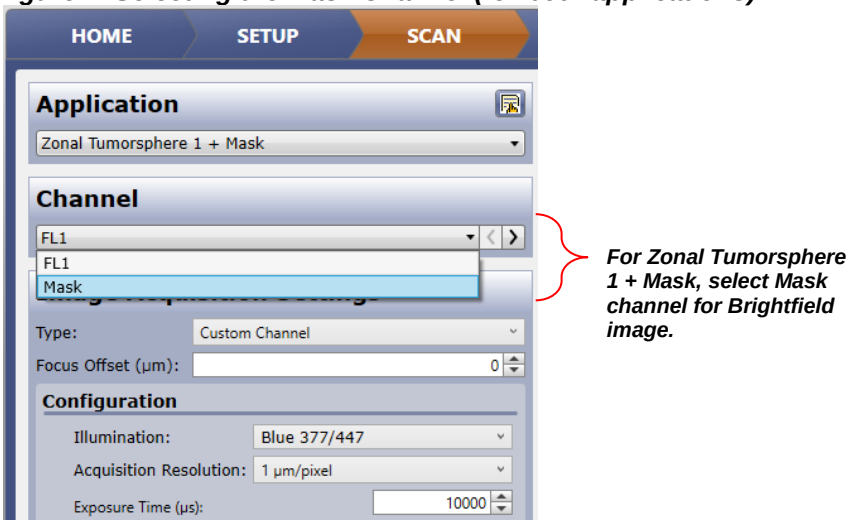
To select image acquisition settings for the mask (brightfield) channel

1. Do one of the following:

   If one of the following applications is selected, select **Mask** in the Channel panel (Figure 6):

   - Zonal Tumorsphere 1+Mask
   - Zonal Tumorsphere 1+2+Mask

Figure 2. Selecting the Mask Channel (for both applications)



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. Perform one of the following to set the optimal exposure:
   - If the wells have *different* darkness shadows and the same grey scale background is desired: 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 select either 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

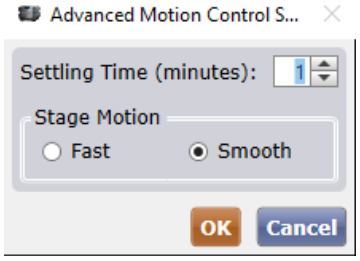
Type	Auto Exposure/Gain Channel
Focus Offset	0
CONFIGURATION	
Illumination	Brightfield
Priority	Auto Exposure, Gain if necessary
Frequency	Every scan area
MOTION CONTROL AND FOCUS	

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 a 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**
   - If the plate focus map has been calibrated for the plate profile in use, select Plate Focus Map (see User guide for more details).

Figure 4 shows examples of acquisition settings for the bright field channel where the Mask is the second channel with Brightfield illumination.

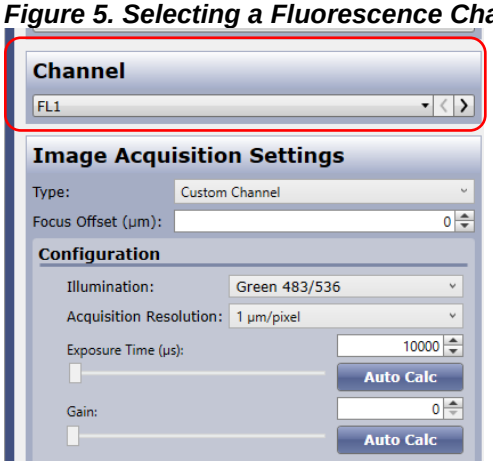
Figure 4. Zonal Tumorsphere 1 +Mask with Brightfield as Mask Channel

3.2.2 Selecting Acquisition Settings for the Fluorescent Channels

To select image acquisition settings for the fluorescent channels

1. In Channel, select a fluorescent channel to set up (for example FL1 or FL2) (Figure 5).

Figure 5. Selecting a Fluorescence Channel



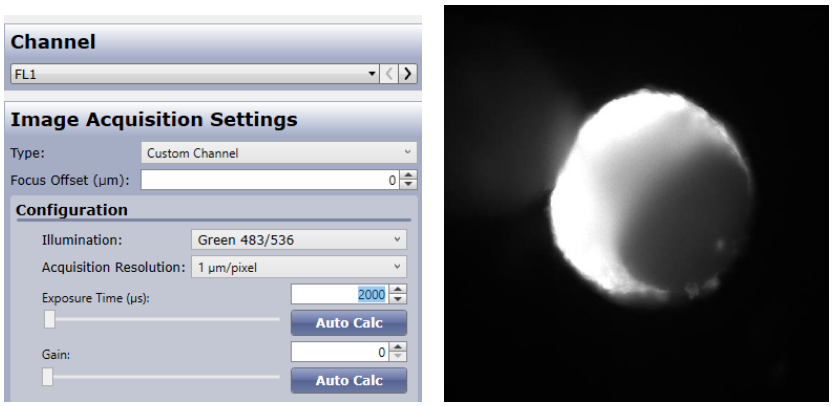
2. For the selected channel in the Channel panel, make the following selections:
   - In Type, select **Custom Channel**.
   - In Focus Offset, keep 0 (default).
   - In Illumination, select the appropriate illumination for the dye (e.g., **Green 483/536** to visualize the calcein AM signal, **Red 531/629** to visualize the propidium iodide signal). **Blue 377/447** to visualize Hoechst.
   - Click **Live** to see a live image or **Snap** to see a snapped image.
     - 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.
3. In Exposure Time and Gain, adjust as needed to correct the displayed live image. Ideal pixel intensity for the highest fluorescent object in the plate should be between 125 and 175, where 255 is the maximum saturated pixel intensity.

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

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

Figure 6 shows an example of FL 1 channel selections and a Calcien AM stained tumorsphere.

Figure 6. FL 1 Channel Selection and CalceinAM stained tumorsphere



4. Analyzing Images

This chapter provides information on how to analyze scans from the Spheroid Analysis application. 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 Spheroid Analysis application are shown in Table 4. The settings typically provide good image segmentation.

Important guidelines

While selecting analysis settings, use the following important guidelines:

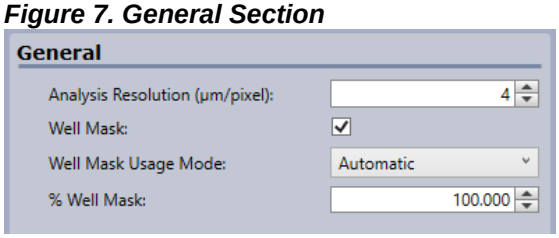
- Test the analysis settings using multiple tumorspheres across at least one well of the plate.
- Tumorsphere border identification is critical for this application. Therefore, images containing tumorspheres with clear, crisp edges will be easy to identify and analyze. Tumorspheres that are out of focus 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.

In the General section (Figure 7), make the following selections:

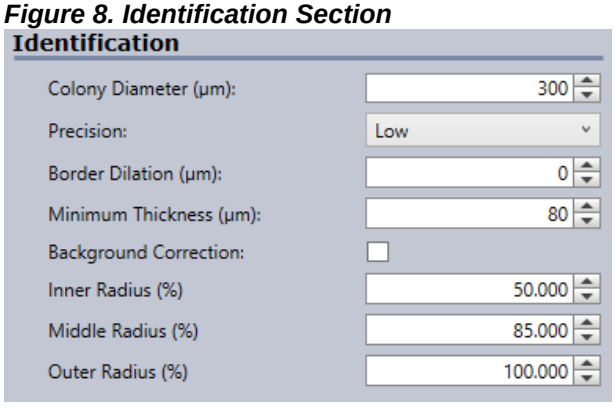
Figure 7. General Section



2. Image Resolution (µm/pixel): Select the image resolution to use for analysis. For the 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.
3. Well Mask – Applies a boundary at the well edge so that the well edge (subject to distortion or plate artifacts) is excluded from segmentation. Uncheck mark for the initial setting.
4. Well Mask Usage Mode – Select Automatic.
5. % 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.

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

Figure 8. Identification Section



6. 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.
7. Precision – Select the desired level of precision for separation of touching tumorspheres:
    - None – No separation.
    - Low – Minimal separation.
    - Medium – Medium separation.
    - High – Most precise separation.

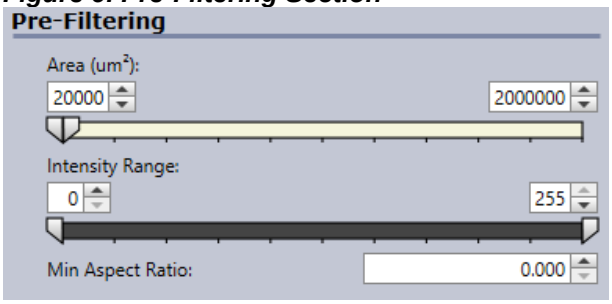
    Higher values will result in the system's attempt to determine the exact boundary between tumorspheres. As the desired precision

increases, so do the chance that intensity artifacts will incorrectly affect the separation borders.

8. Border Dilation (µm) – Defines the amount of dilation/erosion for tumorsphere edge segmentation.
9. Minimum Thickness (µm) – Minimizes object artifact extensions.
10. Background Correction – (Use for Brightfield Illumination only) – Minimizes background variations due to the meniscus by correcting an image for segmentation.
11. Inner Radius (%) – Percent area of center of sphere
12. Middle Radius (%) - Percent area of middle layer of sphere
13. Outer Radius (%) - Percent area of outer area of sphere

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

Figure 9. Pre-Filtering Section



14. Area (µm²) – The tumorsphere area. Enter the tumorsphere area range that corresponds to the scanned tumorspheres.
15. Intensity Range – Make selections to include only tumorspheres and exclude debris/single cells.
16. Min. Aspect Ratio – Removes elongated debris.

Figure 10. Tumorsphere zonal zones identified using 45%, 75% and 100% inner, middle and outer radii respectively.

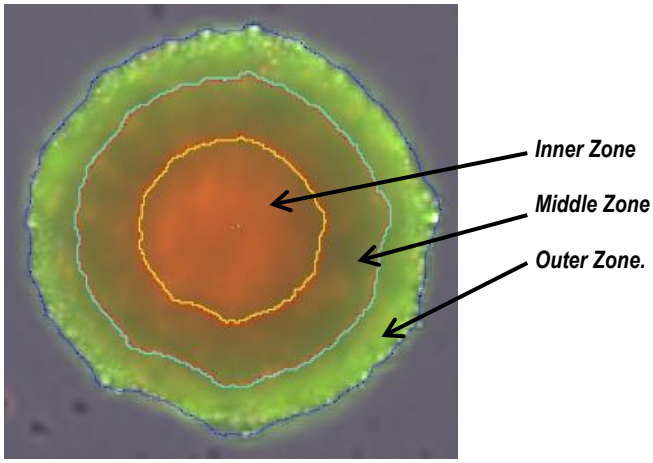


Table 4. Recommended Initial Identification and Pre-Filtering Settings for Analysis

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	Uncheck marked	Check marked or Uncheck marked	Minimizes background variations by applying an average value.
Inner radius (%)	50	0-100	Defines the inner zone of the spheroid from the center of the spheroid to the inner zone boundary. The boundary is defined as a percentage radius of the outside edge of the spheroid identified in the brightfield channel.
Middle Radius (%)	85	0-100	Defines the middle zone of the spheroid from the inner zone of the spheroid to the middle zone boundary. The boundary is defined as a percentage radius of the outside edge of the spheroid identified in the brightfield channel.

Parameter	Initial Setting	Available Range	Description
Outer Radius (%)	100	0-100	Defines the outer zone of the spheroid from the middle zone of the spheroid to the outer zone boundary. The boundary is defined as a percentage radius of the outside edge of the spheroid identified in the brightfield channel.
Well Mask	Check marked	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. Tumorspheres with Corresponding Target Overlay – Minimum Tumorsphere Aspect Ratio Adjustments show examples of Tumorsphere brightfield images with corresponding target overlays.

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

Figure 10. Tumorspheres with Corresponding Target Overlay – Colony Diameter Adjustments

Colony Diameter 100 **Colony Diameter 500**

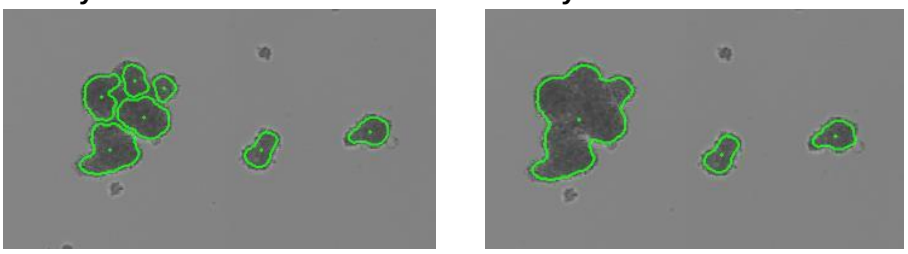


Figure 11 shows an example of the display results when adjusting precision.

Figure 11. Tumorspheres with Corresponding Target Overlay – Precision Adjustments

Precision Low **Precision High**

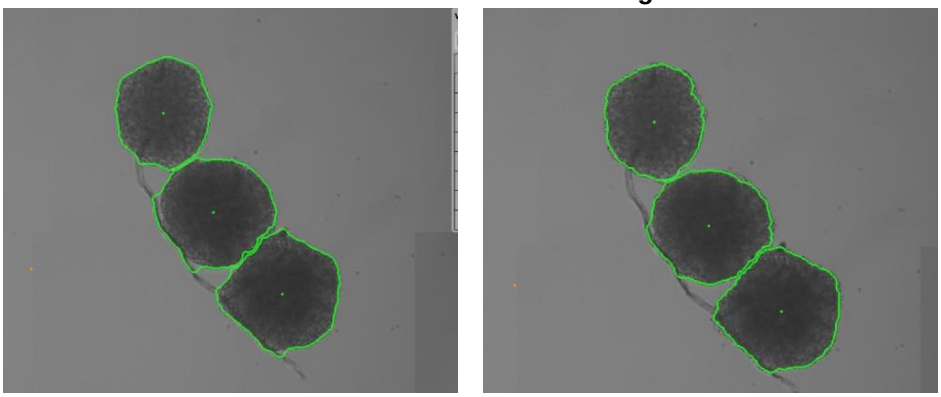


Figure 12 shows an example of display results when adjusting tumorsphere border dilation.

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

Border Dilation 0 **Border Dilation 20**

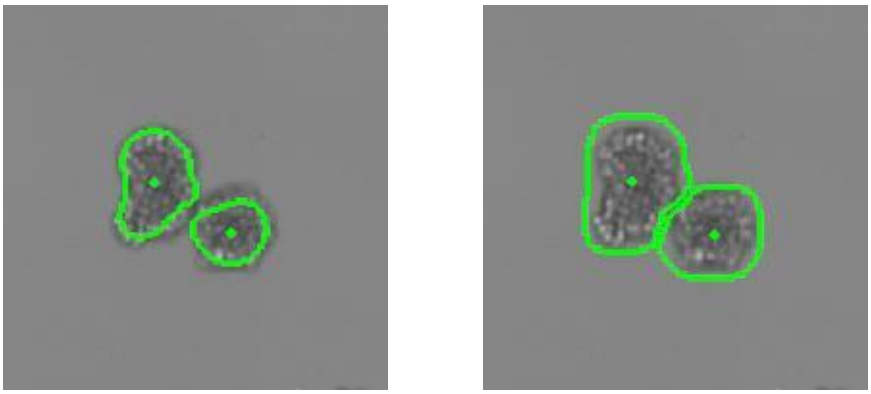


Figure 13 shows an example of display results when adjusting minimum thickness.

Figure 13. Tumorspheres with Corresponding Target Overlay – Minimum Thickness

Minimum Thickness 20 µm **Minimum Thickness 50 µm** **Minimum Thickness 80 µm**

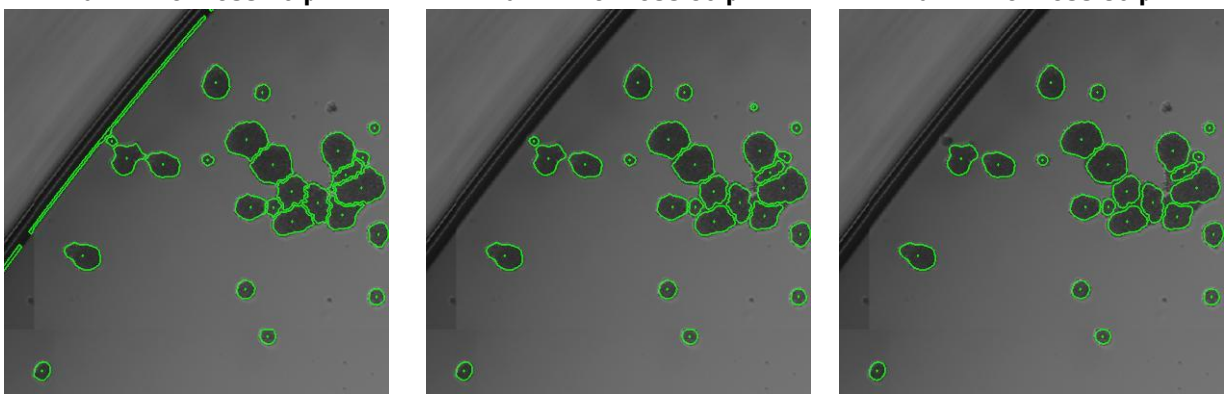


Figure 14 shows an example of display results when adjusting background correction.

Figure 14. Tumorspheres with Corresponding Target Overlay. – Background Correction

Background Correction Off **Background Correction On**

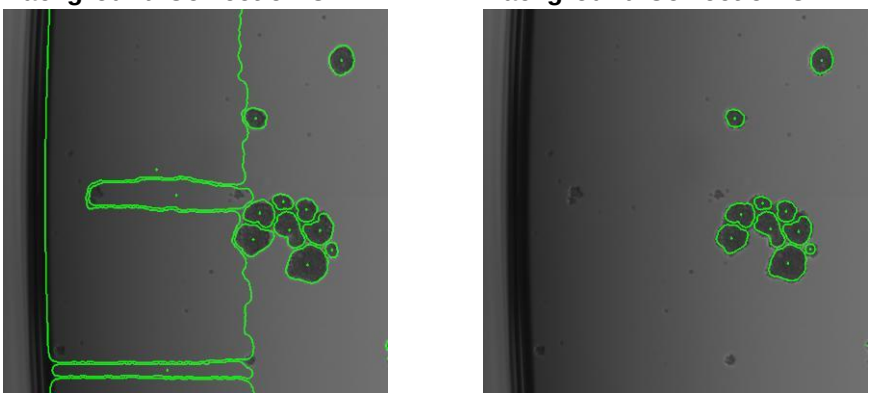
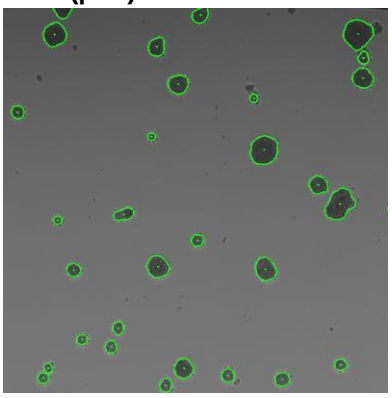


Figure 15 shows an example of display results when adjusting area (µm²) range.

Figure 15. Tumorspheres with Corresponding Target Overlay. – Area (µm²) Range Adjustments

Area (µm²) 500



Area (µm²) 10,000

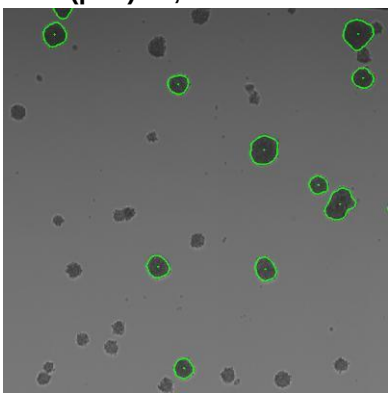
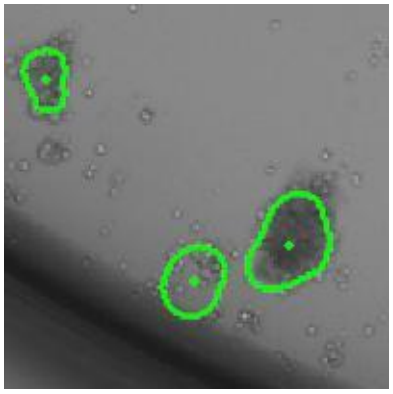


Figure 16 shows an example of display results when pixel intensity range is adjusted.

Figure 16. Tumorspheres with Corresponding Target Overlay – Intensity Range Adjustments

Intensity Range: Max Pixel Intensity 255



Intensity Range: Max Intensity 106

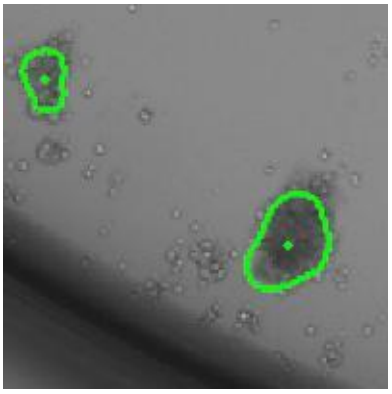
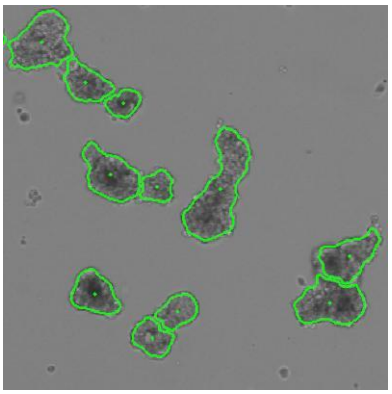


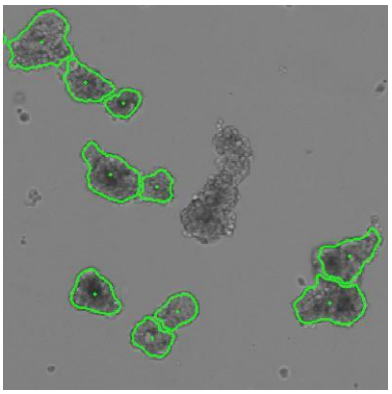
Figure 17 shows an example of display results when minimum tumorsphere aspect ratio is adjusted.

Figure 17. Tumorspheres 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. Perform this task in the GATE tab.

The 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 Tumorsphere application.

- If choosing to 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; it is not possible to assign any additional classes to populations. Reanalysis of the same scan is required to 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. Check marking 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 check mark would be the same as not using the GATING tab; all data would be reported from the ANALYZE tab settings only.**

To create a plot, gate, and populations

1. In the Plot Populations pane, create a plot using the Add Plot (+) button, the Add Plot dialog box, and also by referring to the selections in Figure 18 and Table 6.

   The table lists the possible selections for 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 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 creating a plot and drawing a gate, 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	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) 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.
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).

FEATURE	DESCRIPTION
EQ Diameter (µm)	Equivalent diameter of each identified tumorsphere, derived from the area. Equivalent diameter = 2(radius), derived from Area = π(radius^2)
Est Volume (µm3)	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.

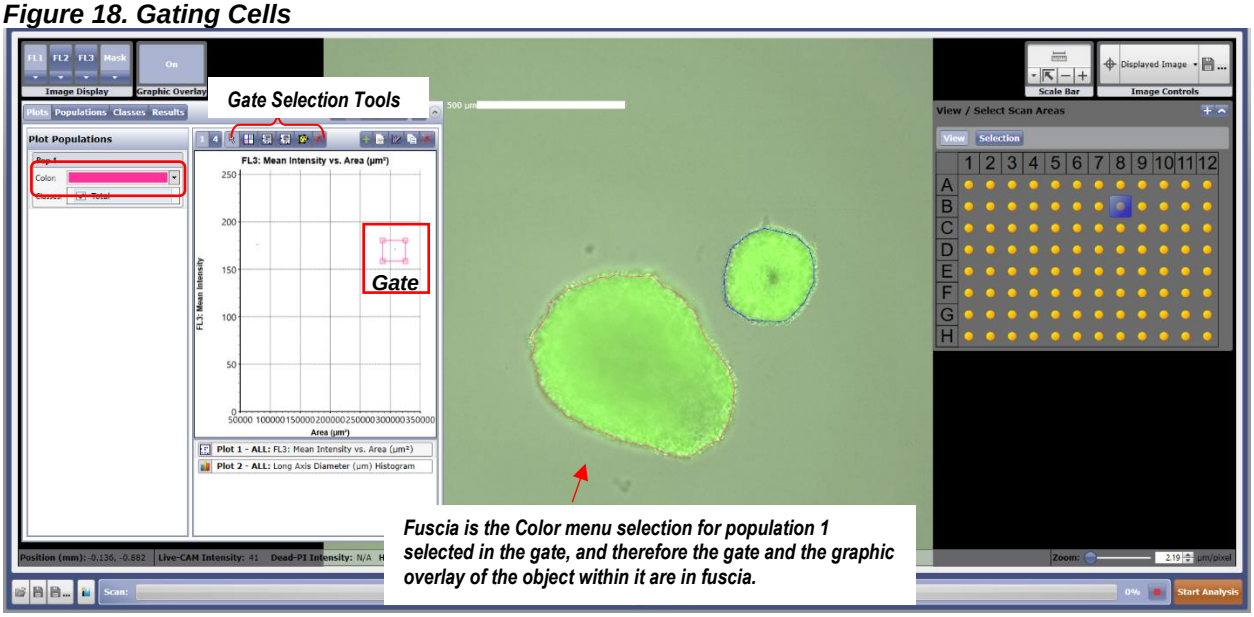
Table 6. Plot Parameter Sections by Channel

For Each Channel	
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 the pixel intensities displaying signal in the segmented objects calculated for each channel.
Inner, ring and outer Area (µm^2)	Total area of each identified zone in the tumorspheres (if applicable).

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



2. Repeat steps 1 and 0 as needed to refine the desired population for counting.

3. In the Plots, Populations, or Classes view, make sure that the Total class is check marked. This assigns the Total class to the population.

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

In this application, only assign the Total class to populations; it is not possible to 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 7 and appear below the Display Options section in the Scan Information pane.

Table 7. Zonal Tumorsphere Application Outputs

Parameter	Description	Channel	Ave	St Dev
Tumorsphere Count	Number of tumorspheres identified and analyzed			
Inner Zone mean intensity	Average of the pixels intensities of the inner zone of the spheroid.	**All**	√	√
Inner Zone integrated intensity	Sum of all pixel intensities in the inner zone of the spheroid.	**All**	√	√
Ring Zone mean intensity	Average of the pixels intensities of the middle zone of the spheroid.	**All**	√	√
Ring Zone integrated intensity	Sum of all pixel intensities in the middle zone of the spheroid.	**All**	√	√
Outer Zone mean intensity	Average of the pixels intensities of the outer zone of the spheroid.	**All**	√	√
Outer Zone integrated intensity	Sum of all pixel intensities in the outer zone of the spheroid.	**All**	√	√
Area (µm^2)	Area of each identified tumorsphere, measured in pixels.	**Bright field**	√	
% 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 9. Troubleshooting Recommendations

Issue	Recommended Action
Tumorspheres are out of focus	When the hardware autofocus position is set using only a few non-representative 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 the hardware autofocus when scanning.
Tumorspheres are not being identified	Tumorspheres may not be identified due to: Part of the tumorsphere is out of focus: Adjust tumorsphere border value. Tumorspheres are smaller/larger than the size range selected in the Tumorsphere Area in the ANALYZE tab: Adjust range of Tumorsphere Area. Tumorspheres have a lower/higher intensity than the range selected in the tumorsphere Intensity Range in the ANALYZE tab: Adjust tumorsphere Intensity Range. 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. Single/dead cells can be removed by decreasing the tumorsphere intensity range in the ANALYZE or GATING Tab. Small clumps of cells not considered to be tumorspheres can be removed by adjusting the tumorsphere area range in the ANALYZE or GATING tab. 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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