Guidelines for the Incucyte® Spheroid Analysis Software Module

The Incucyte® Spheroid Analysis Software Module enables quantification of spheroid growth and cell health in real-time for both single spheroid and multi spheroids, as well as quantification of single spheroid invasion. Spheroid growth in both single and multi-tumor spheroid models (see protocols linked below for guidance on formation of compatible single or multi spheroids) can be assessed using brightfield imaging with image segmentation to delineate the brightfield boundary, enabling label-free analysis. When used in combination with Incucyte® cell health reagents the mechanisms of cell death can be elucidated by quantification of fluorescence within the brightfield boundary. General cell viability can also be assessed in this way using cells expressing fluorescent proteins. Phase contrast images enable qualitative assessment of morphology. Once analysis parameters are defined, they can easily be applied across selected time points and wells.

This guideline covers the following topics for defining Spheroid Analysis Parameters:

- Acquiring images using the Incucyte® Spheroid Analysis Software Module
- Analyzing size, viability and death of spheroids using label-free or fluorescent readouts

Please note that it is important to follow the Incucyte® protocols for generating single or multi spheroids, available at the Incucyte® Spheroid Growth application page, located here. Software acquisition and analysis tools have been purpose-built, and lab tested with these protocols.

The following procedures are for example purposes only and are designed to provide a frame of reference for defining the Spheroid Analysis Parameters within the Analysis Wizard.

For new Incucyte users, it is recommended to review Sections 1, 2 and 3 of the Incucyte User's Manual or have experience scheduling and acquiring scans, viewing images, and performing image analysis and visualizing results prior to reviewing this technical note.

Acquiring images using the Incucyte® Spheroid Analysis Software Module

This module enables acquisition of brightfield, phase, and fluorescence images for single tumor spheroids grown in 96-well or 384-well round bottom plates and for multiple tumor spheroids grown on a bed of Matrigel® in 96-well flat bottom plates. Brightfield and phase images can also be acquired for multi-spheroids embedded in Matrigel® in 96-well flat bottom plates. Please see the Incucyte® User's Manual Section 1 for a detailed description of how-to login to the Incucyte® and launch the Acquisition Window. Follow instructions for scheduling a scan as described in the User's Manual until the "Scan Type" Window is displayed.

1. In the Scan Type Window, select Spheroid.
2. In the Scan Settings Window, Phase + Brightfield Image Channels will be selected as default for performing label-free analysis of spheroid growth. Additionally, select the appropriate fluorescent channel depending on your chosen reagents, and select Single, Multi or Embedded Multi depending on your chosen 3D culture model.
   NOTE:
   - For the single spheroid model, 4x and 10x objectives are supported.
   - For the multi-spheroid Matrigel® base model, only 10x objective is supported.
   - For embedded multi-spheroids, only Phase + Brightfield channels and the 4x objective are supported.
3. Proceed through the remaining windows referencing the Incucyte User's Manual as needed.

When acquiring transmitted light images of spheroids using the Incucyte Software Module, both phase and brightfield images will be acquired. Brightfield images should be used for determining spheroid boundaries, and phase images can be used for examining morphology.

Defining the analysis parameters using label-free or fluorescent readouts

The following section will guide you through creating a new analysis definition to accurately mask brightfield images of single or multi spheroids, in order to produce kinetic data tracking label-free spheroid size in both single and multi-spheroids as well as invasion for single spheroid assays. Creation of a brightfield mask will also automatically generate data on red or green fluorescence within this boundary (if fluorescence images were acquired). Using this method, separate fluorescence

masks are not required.

1. From an open Vessel View, launch the Analysis Wizard as described in the User's Manual in Section 3, Chapter 1.
2. Click Next and select the desired Analysis Type (e.g. Spheroid or Spheroid Invasion).
3. Click next onto the Image Channels window. Phase + Brightfield and any fluorescence channels will be selected by default.
4. Perform Image Set Selection as described in the User's Manual and click Next where you are presented with the Analysis Definition Window.

For easier identification of the analysis definition, you might want to name the object the same as the cell type that was used in the assay, for example, HT-1080.

5. In the Brightfield Object name field (Spheroid Analysis only), enter the name of the object(s) that are being analyzed. See Figure 1
6. Click Preview Current or All. See Figure 1

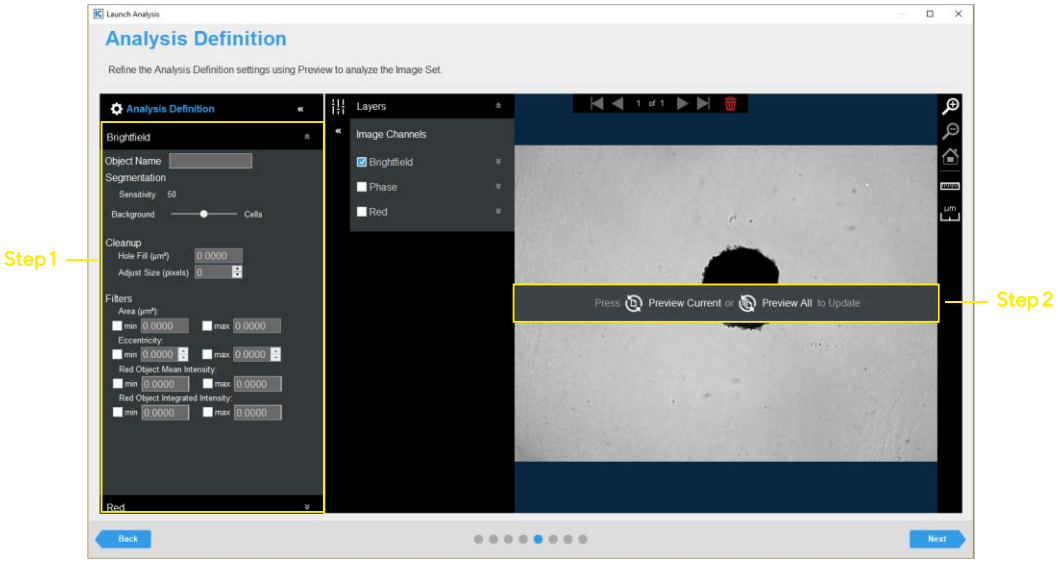


Figure 1a. Spheroid Analyzer Image Preview (brightfield).

The best way to begin setting up the Analysis Definition is to use the preset Segmentation Sensitivity and Cleanup values already contained within the Analysis Definition.

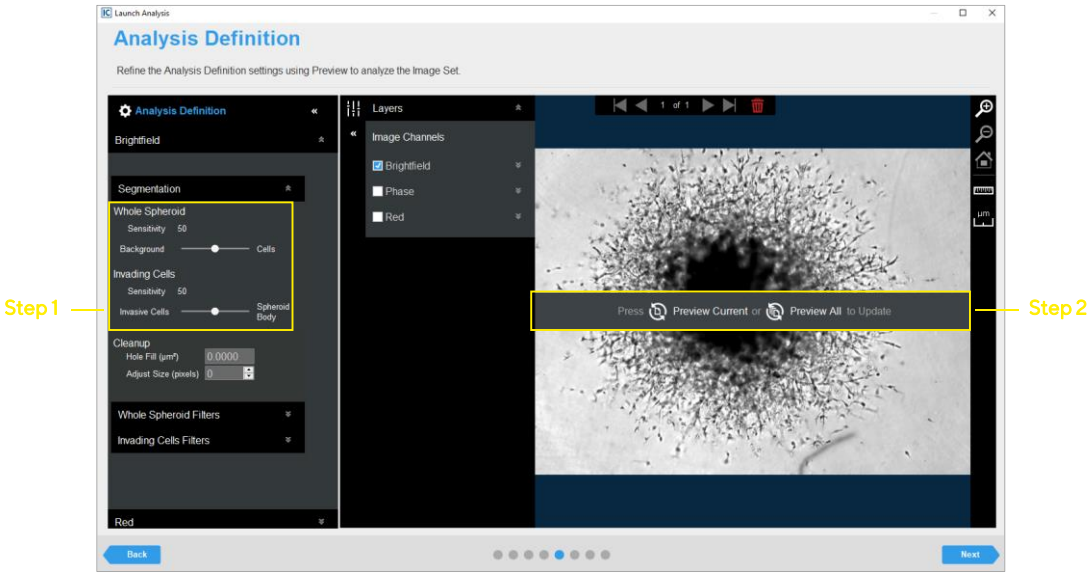


Figure 1b. Spheroid Invasion Analyzer Image Preview (brightfield)

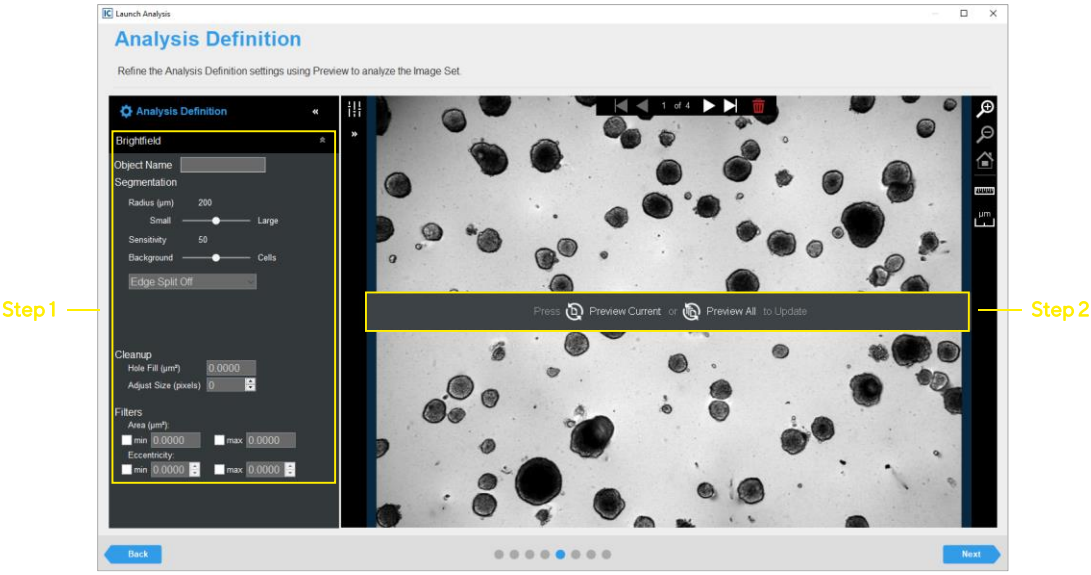


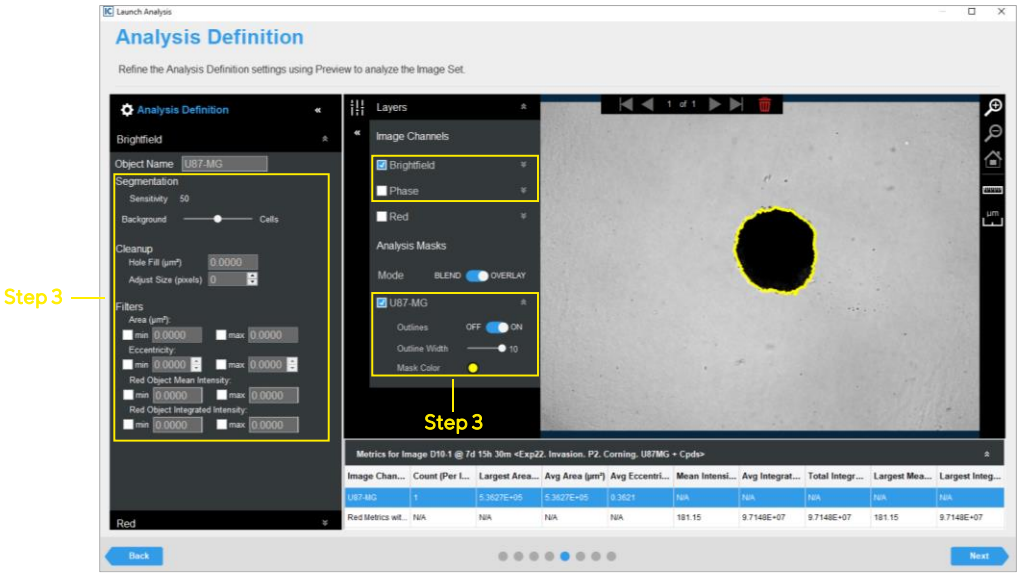
Figure 1c. Embedded Multi-Spheroid Analyzer Image Preview (brightfield)

7. Evaluate your Brightfield Mask and refine the parameters accordingly. See Figure 2 and Table 1.

   a. Assess the Analysis Mask using the Blend or Overlay Mode. A Mask Outline, with slider to adjust the Outline Width, and Color selection options aid in evaluating the Analysis Mask. Changing these parameters will affect the visualization only and not affect the analysis definition.
   b. Modify only a single analysis definition parameter at a time. After you define the value for a parameter, click Preview Current to apply and view the change

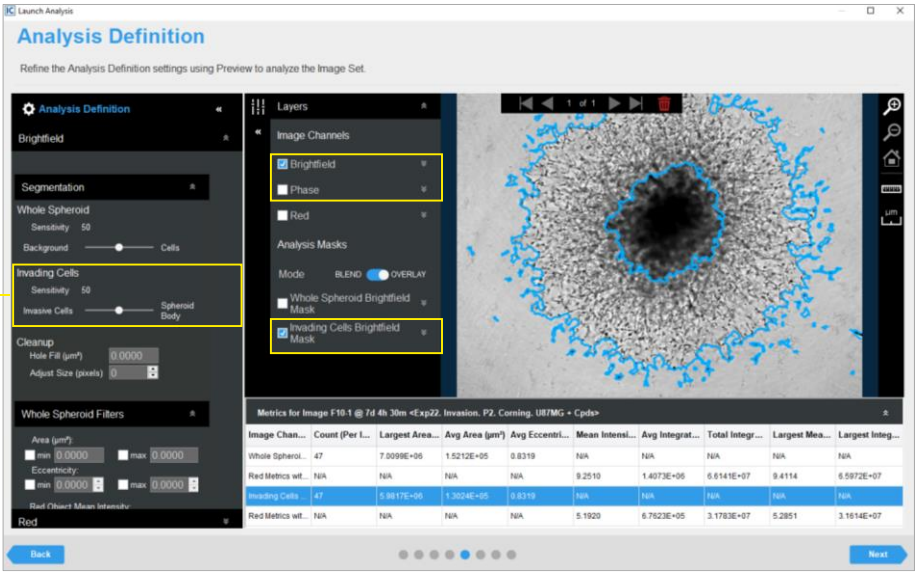
for the image that is currently displayed in the Image panel.

With the Brightfield Mask selected, toggle between the Phase image channel and Brightfield image channel to evaluate if the segmentation appropriately masks the spheroid.



Step 3

Figure 2a. Parameter Refinement with Brightfield Mask (Spheroid Analysis Type)



Step 3

Figure 2b. Parameter Refinement with Invading Cells Mask (Spheroid Invasion Analysis Type)

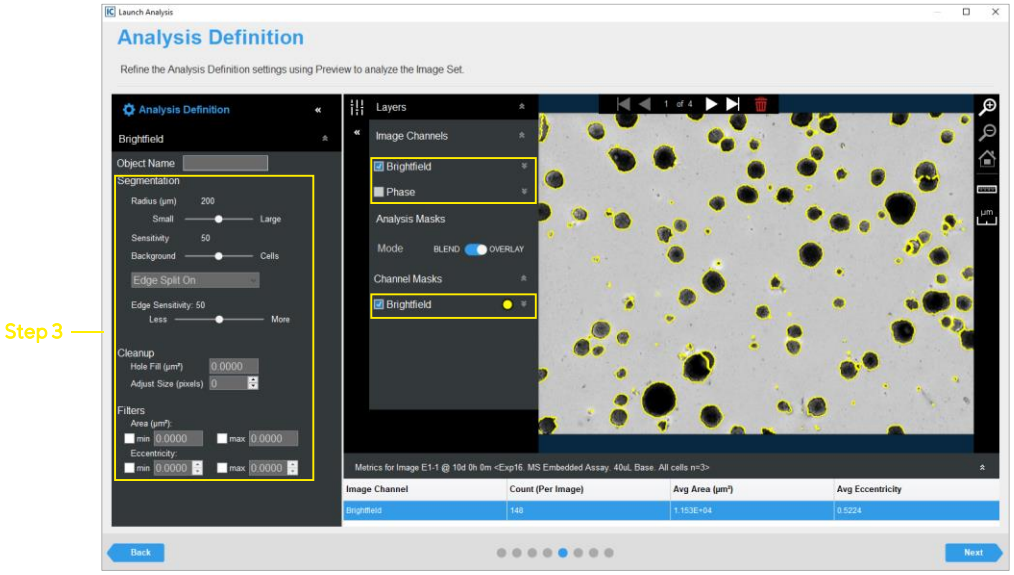


Figure 2c. Parameter Refinement with Brightfield Mask (Embedded Multi-Spheroid Analysis)

Option	Description
Parameters	
Sensitivity	Read-only display that can be used to adjust for the spheroid brightness relative to the background. Move slider towards cells for lower contrast spheroids.
Background/Cells	Use the slider bar to adjust the mask. • Moving the slider bar all the way to the right (towards cells) will pick up the spheroid and additional debris and background resulting in over-masking, while moving it all the way to the left may not select the entire spheroid, resulting in under-masking. Adjust the slider bar as needed to accurately mask the spheroid.
Cleanup	
Hole Fill	Removes any holes in the mask that are smaller than the area that is specified.
Adjust Size	If set to a positive value, this will enlarge the mask by the specified number of pixels. If set to a negative value, the mask will shrink by the specified number of pixels.
Filters - Used to remove any masked objects that are not true spheroids. (e.g. cell debris, plate scratches)	
Area	Defines a range of sizes (in μm^2) for the object and eliminates objects that fall outside this range, e.g., to exclude cell debris. Use the ruler tool to measure debris diameter and calculate an approximate area.
Eccentricity	Defines a range of roundness for the object and eliminates objects that fall outside this range. Eccentricity ranges from 0 to 1 with a perfect circle having a value of 0. This filter can be used to exclude scratches or plate defects.

Green/Red Mean Intensity	Defines a range of green/red mean intensity to further refine the brightfield mask boundary if the green/red channel is enabled. This filter can be used to exclude scratches or non-fluorescent debris.
Green/Red Integrated Intensity	Defines a range of green/red integrated intensity to further refine the brightfield mask boundary if the green/red channel is enabled. This filter can be used to exclude scratches or non-fluorescent debris.

Table 1a. Spheroid (single and multi-spheroids) Analysis Brightfield Analysis Definition Options

Option	Description
Parameters	
Sensitivity (Whole Spheroid)	Read-only display that is dynamically updated to reflect the value to which you adjust the background/cells slider bar. Use the slider bar to adjust the whole spheroid mask (spheroid body and invasive phenotype). • Moving the slider bar all the way to the right (towards cells) will pick up the whole spheroid and additional debris and background resulting in over-masking, while moving it all the way to the left may not select the entire spheroid, resulting in under-masking. Adjust the slider bar as needed to accurately mask the whole spheroid.
Sensitivity (Invading Cells)	Read-only display that is dynamically updated to reflect the value to which you adjust the Invading Cells/Spheroid body slider bar. Use this slider bar to adjust segmentation of the invading cells from the spheroid body. • Moving the slider bar all the way to the right (towards spheroid body) will pick up more of the spheroid body resulting in under-masking of the invasive cells. Moving it all the way to the left (towards invasive cells) will increase segmentation of the invading cells and reduce masking of the spheroid body. Adjust the slider bar as needed to accurately mask the invading cells.
Cleanup	
Hole Fill	Removes any holes in both masks (Whole spheroid and Invading cells) that are smaller than the area that is specified.
Adjust Size	If set to a positive value, this will enlarge the mask by the specified number of pixels. If set to a negative value, the mask will shrink by the specified number of pixels.
Filters (Whole Spheroid & Invading Cells) – As previously described (see Table 1a)	

Table 1b. Spheroid Invasion Brightfield Analysis Definition Options

Option	Description
Parameters	
Radius	Use this slider to remove background from the brightfield image. Default parameter settings recommended. Adjust the slider bar as needed to remove background. • Move to the right for a larger, more coarse correction and left for a smaller, more fine correction. • Note that a radius set too small may result in a loss in object detection. A radius set too large can cause incorrect background estimation.

Sensitivity	Read-only display that can be used to adjust for the spheroid brightness relative to the background. Move slider towards cells for lower contrast spheroids.
Background/Cells	Use the slider bar to adjust the mask. • Moving the slider bar all the way to the right (towards cells) will pick up the spheroid and additional debris and background resulting in over-masking, while moving it all the way to the left may not select the entire spheroid, resulting in under-masking. Adjust the slider bar as needed to accurately mask the spheroid.
Edge Sensitivity	If Edge Split is turned on, then use the slider bar to adjust the Edge Sensitivity. Move the slider towards 'more' to increase the number of objects split. Move the slider towards 'less' to reduce the number of objects split. Adjust the slider bar as needed to accurately delineate multi-spheroids.
Cleanup - as previously described (see Table 1a)	
Filters - as previously described (see Table 1a)	

Table 1c. Embedded Multi-Spheroid Brightfield Analysis Definition Options

8. Since the fluorescence signal within the brightfield boundary is automatically analyzed, it is not necessary to create a mask for the Green or Red channels. The default setting of Top-Hat No Mask for Green or Red channels will enable background subtraction without generation of a mask. Ensure that the Top-Hat radius is set to a value higher than the radius of the largest spheroid to avoid excess background subtraction. See Figure 3.

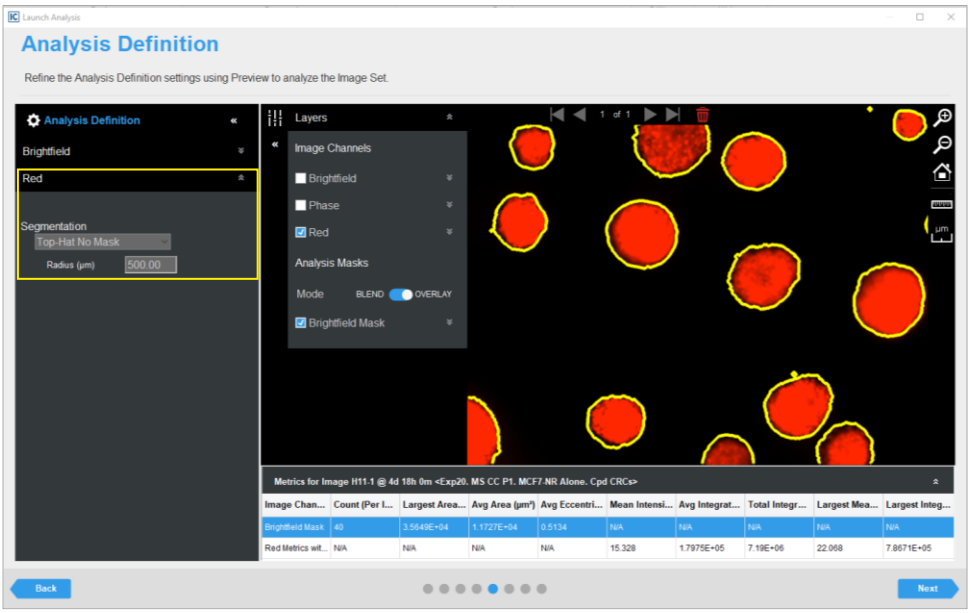


Figure 3a. Red Fluorescence Parameter Refinement with Brightfield Mask (spheroid analysis).

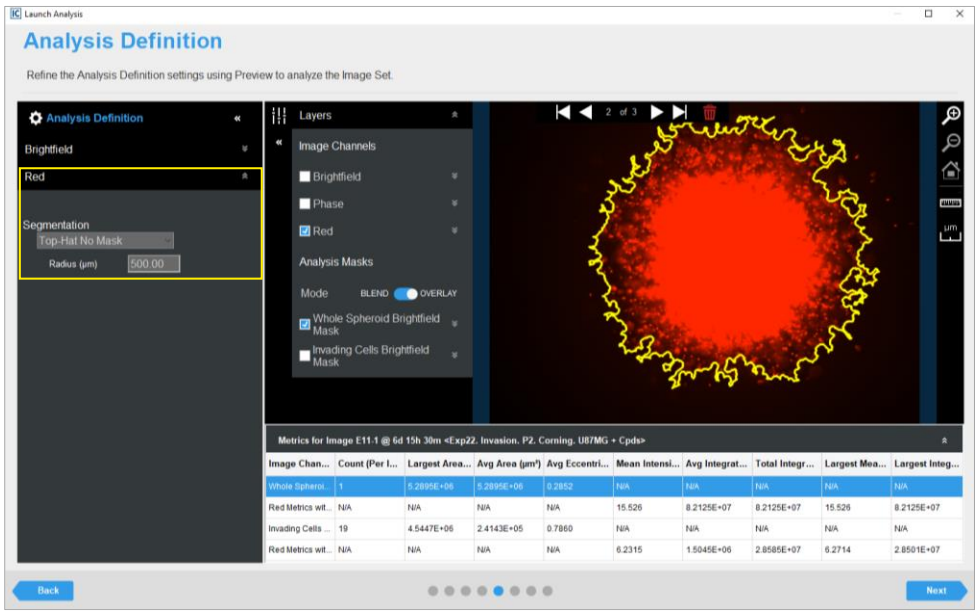


Figure 3b. Red Fluorescence Parameter Refinement with Whole Spheroid Brightfield Mask (spheroid invasion analysis).

9. Once you have previewed all of the images within the wizard image set and are satisfied with the parameters, complete the Launch wizard analysis to select the Scan Times and wells to be analyzed, as well as assigning an analysis definition name. Note that if your experiment is in progress you will have an option to check "Analyze Future Scans" to perform real-time analysis (please see Incucyte User's Manual Section 3 Chapter 1 for more information on Defining Image Analysis.)

After the vessel images have been analyzed using **Brightfield Spheroid Analysis**, the analysis definition can be opened, and the following set of metrics are provided:

Brightfield Metric	Description
Brightfield Object Count (1/image, 1/mm^2 or 1/well)	The number of objects per image, mm^2, or well.
Largest Brightfield Object Area (µm^2)	The area of the largest brightfield object in the image. This is the recommended metric for single spheroid growth analysis.
Brightfield Object Avg Area (µm^2)	The average of the area of the brightfield objects in the image.
Brightfield Object Total Area (µm^2/image or µm^2/well)	The total of the area of the brightfield objects per image or per well. This is the recommended metric for multi-spheroid growth analysis.
Brightfield Object Avg Eccentricity	The average of how round or compact the objects are. Ranges from 0 to 1 with a perfect circle having a value of 0.
Green (or Red) Metrics within Brightfield Object Boundary	
All Brightfield Object Green (or Red) Mean Intensity (CU)	The Green (or Red) Mean Intensities of the all the objects defined by the brightfield mask. The mean intensity is calculated as the sum of all pixel fluorescence divided by the total area of all the objects in pixels.
All Brightfield Object Avg Green (or Red) Integrated Intensity (CU x µm2)	The average of the Green (or Red) Integrated Intensities of the objects defined by the brightfield mask. The integrated intensity is the sum of all red or green fluorescence intensity values within an object multiplied by the pixel area.
All Brightfield Object Total Green (or Red) Integrated Intensity (CU x µm^2/image)	The Total Green (or Red) Integrated Intensity within the boundary defined by the brightfield mask. The integrated intensity is the sum of all red or green fluorescence intensity values within the brightfield mask multiplied by the pixel area. **For multi spheroid applications, this is the recommended metric.**
Largest Brightfield Object Green (or Red) Mean Intensity (CU)	The Mean Green (or Red) Intensity of the largest object in the brightfield mask. **For single spheroid applications, this is the recommended metric.**
Largest Brightfield Object Green (or Red) Integrated Intensity (CU x µm^2/image)	The Green (or Red) Integrated Intensity of the largest object in the brightfield mask. The integrated intensity is the sum of all red or green fluorescence intensity values within the largest object multiplied by the pixel area.

After the vessel images have been analyzed using **Brightfield Spheroid Invasion Analysis**, the analysis definition can be opened, and the following set of metrics are provided:

Brightfield Metric	Description
Largest Whole Spheroid Brightfield Area (µm^2)	The area of the largest brightfield whole spheroid in the image.
Largest Invading Cells Brightfield Area (µm^2)	The area of the largest brightfield invading cells in the image.
Green (or Red) Metrics within Brightfield Object Boundary	
Largest Whole Spheroid Brightfield Green (or Red) Mean Intensity (RCU)	The Mean Green (or Red) Intensity of the largest whole spheroid in the brightfield mask.
Largest Whole Spheroid Brightfield Green (or Red) Integrated Intensity (CU x µm^2/image)	The Green (or Red) Integrated Intensity of the largest whole spheroid in the brightfield mask. The integrated intensity is the sum of all red or green fluorescence intensity values within the largest whole spheroid multiplied by the pixel area.
Largest Invading cells Brightfield Green (or Red) Mean Intensity (CU)	The Mean Green (or Red) Intensity of the largest invading cells in the brightfield mask.
Largest Invading Cells Brightfield Green (or Red) Integrated Intensity (CU x µm^2/image)	The Green (or Red) Integrated Intensity of the largest invading cells in the brightfield mask. The integrated intensity is the sum of all red or green fluorescence intensity values within the largest object multiplied by the pixel area.

Visualization of Analysis Results is described in Section 3, Chapter 2 of the Incucyte User's Manual.

Sales and Service Contacts

For further contacts, visit sartorius.com

Essen Bioscience, A Sartorius Company
sartorius.com/incucyte

Sartorius Lab Instruments
GmbH & Co. KG
Otto-Brenner-Strasse 20
37079 Goettingen
Phone +49 551 308 0

North America
300 West Morgan Road
Ann Arbor, Michigan, 48108
USA
Phone +1 734 769 1600
Email orders.US07@sartorius.com

Europe
Units 2 & 3 The Quadrant
Newark Close
Royston Hertfordshire
SG8 5HL
United Kingdom
Phone +44 1763 227400
Email euorders.UK03@sartorius.com

APAC
Sartorius Japan K.K.
4th Floor Daiwa Shinagawa North Bldg.
1-8-11 Kita-Shinagawa
Shinagawa-ku, Tokyo
140-0001
Japan
Phone +81 3 6478 5202
Email orders.US07@sartorius.com

Specifications subject to change without notice.
© 2021. All rights reserved. Incucyte®, Essen Bioscience® and all names of Essen Bioscience products are registered trademarks and the property of Essen Bioscience unless otherwise specified. Essen Bioscience is a Sartorius Company.
Publication No.: 8000-0525-G00 Status: 09 | 2021