Analysis Guidelines for the Incucyte® Organoid Analysis Software Module

The Incucyte® Organoid Analysis Software Module is used for real-time quantification of organoids embedded in Matrigel®. Organoid size, count and morphology (see Organoid Quality Control or Organoid Assay Protocols for guidance) can be assessed using brightfield imaging and segmentation to delineate the boundary of objects, enabling label-free analysis. Once analysis parameters are defined, they can easily be applied across selected time points and wells.

This guideline covers the following topics for defining organoid analysis parameters:

- Acquiring images using the Incucyte® Organoid Analysis Software Module
- Analyzing organoid size using label-free brightfield readouts

Please note that it is important to follow Incucyte® protocols for culturing embedded organoids, available at the Incucyte® Resources 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 Organoid Analysis Parameters within the Analysis Wizard.

For new Incucyte® users, it is recommended to review Sections 1, 2 and 3 of the Incucyte® User Manual or to 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® Organoid Analysis Software Module

This module enables acquisition of brightfield and phase images of organoids in 24-well, 48-well or 96-well flat bottom plates. Please see the Incucyte® System User 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 Organoid.
2. In the Scan Settings Window, Phase + Brightfield Image Channels will be selected as default for performing label-free analysis of organoid growth. Only 4x objective is supported.
3. Select appropriate assay plate set-up, either Organoid QC or Organoid Assay.
4. Proceed through the remaining windows referencing the Incucyte® User Manual as needed.

When acquiring transmitted light images of organoids using the Incucyte® Organoid Analysis Software Module, both phase and brightfield images will be acquired. Brightfield images should be used for determining organoid boundaries.

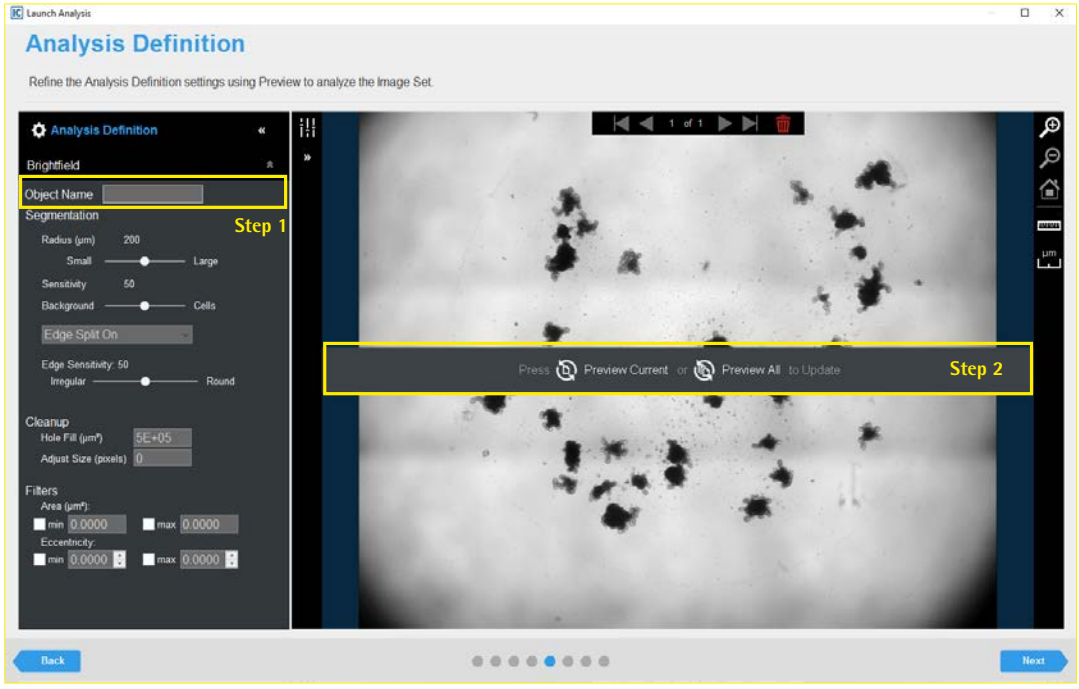
Defining the analysis parameters

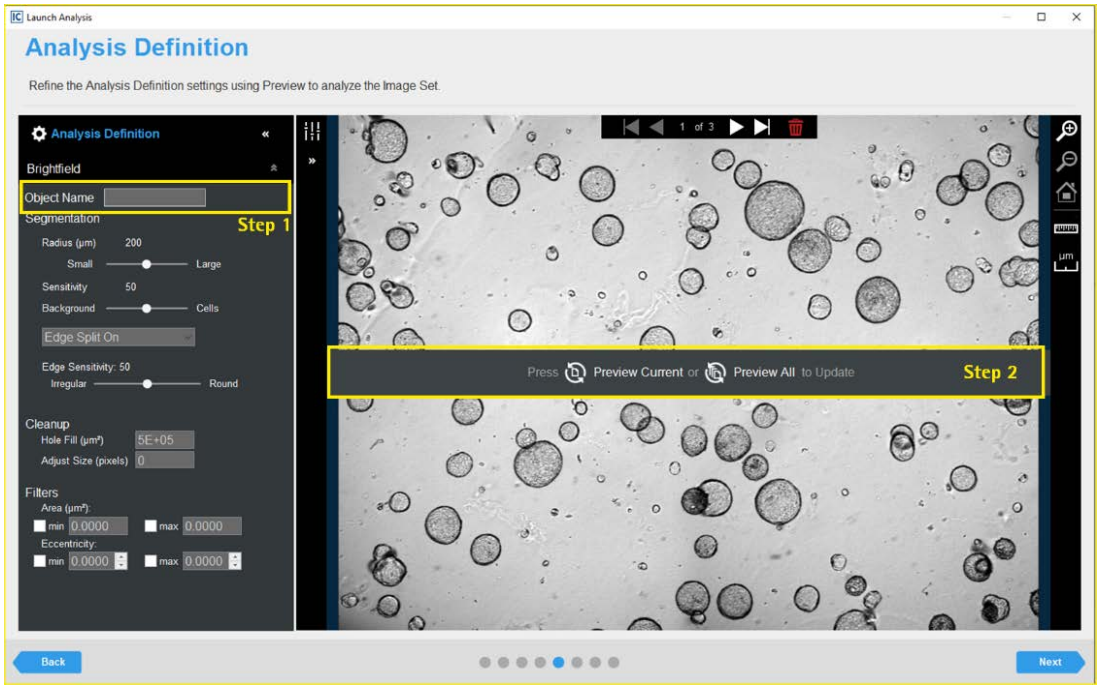
The following section will guide you through creating a new analysis definition to accurately mask brightfield organoid images in order to produce kinetic data tracking label-free organoid size.

1. From an open Vessel View, launch the Analysis Wizard as described in the User Manual.
2. Click Next and select the desired Analysis Type (Organoid).
3. Click next onto the Image Channels window. Phase + Brightfield 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.
5. In the Brightfield Object name field, enter the name of the object(s) that are being analyzed. Figure 1, Step 1.
6. Click Preview Current or All. Figure 1, Step 2.

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, Intestinal organoids.

Figure 1. Organoid Analyzer Image Preview. Example 24W dome (top) and 96W Assay (bottom).





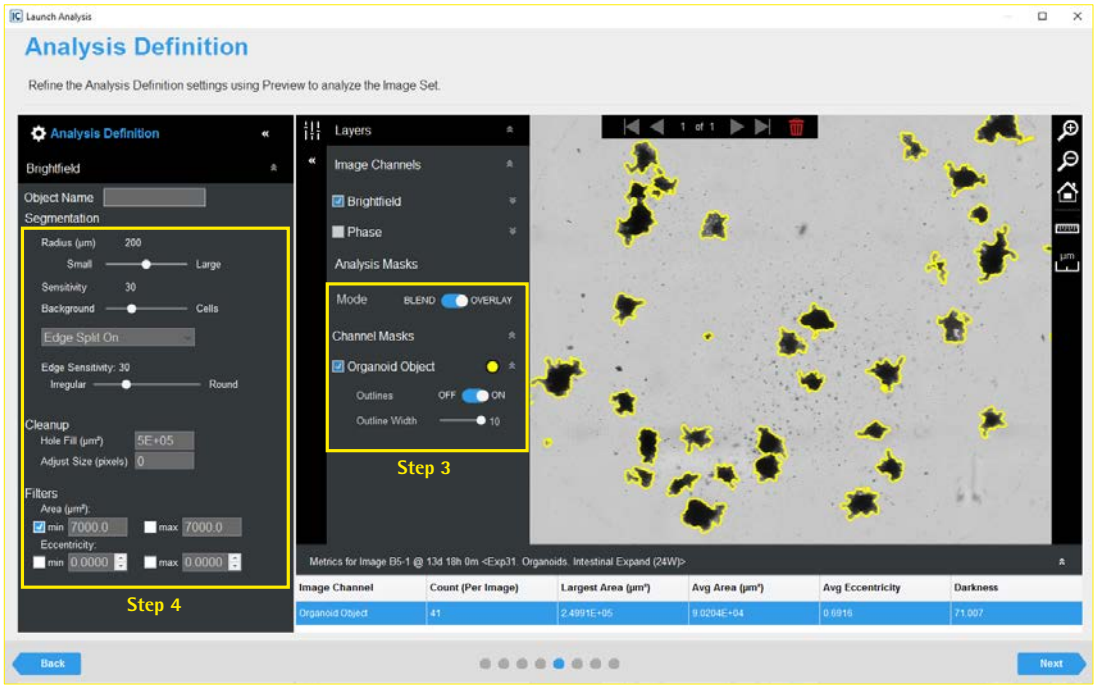
7. Evaluate your Brightfield Mask and refine the parameters accordingly. Figure 2 and Table 1.

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 Editor.

a. Assess the Analysis Mask using the Blend or Overlay Mode (Figure 2, Step 3). 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 the analysis definition.
b. Modify only a single analysis definition parameter at a time (Figure 2, Step 4). 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.

Toggle (on & off) the Brightfield Mask to evaluate if the segmentation appropriately masks the organoids.

Figure 2. Parameter Refinement with Brightfield Mask. Example 24-well dome (top) and 96-well Assay (bottom).



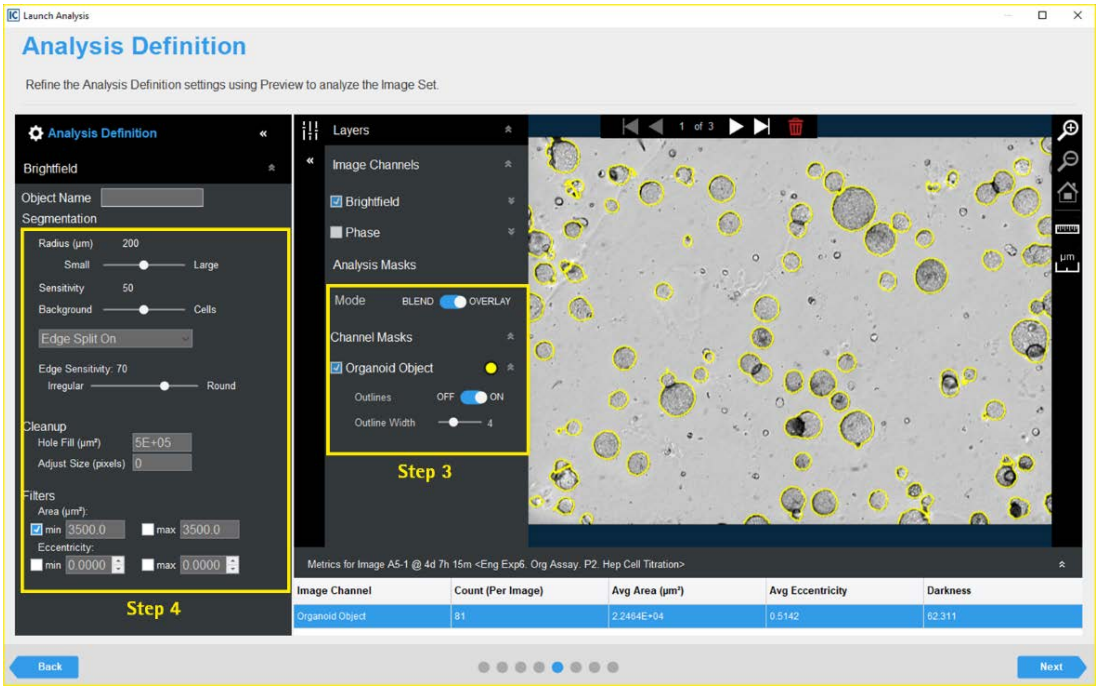


Table 1. Brightfield Analysis Definition Options

Option	Description
Parameters	
Radius	Use this slider to remove background from the object properties. • Default parameter settings recommended. • Radius adjustments mostly required with darker objects. • Adjust the slider bar as needed to remove background. 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 is dynamically updated to reflect the value to which you adjust the Background/Cells slider bar.
Background/Cells	Use the slider bar to adjust the mask. • Moving the slider all the way to the right (towards cells) will pick up the organoids and additional debris and background resulting in over-masking, while moving it all the way to the left may not select entire organoids, resulting in under-masking. Adjust the slider bar as needed to accurately mask the organoids.
Edge Sensitivity	Use the slider bar to adjust the mask. Set slider according to organoid morphology. • Moving the slider towards the right will result in splitting of more spherical shaped objects. • Moving the slider towards the left will result in splitting of more irregular shaped objects. Adjust the slider bar as needed to accurately mask the organoids.
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 organoids. (e.g. cell debris, plate artefacts)	
Area	Defines a range of sizes (in μm^2) for the object and eliminates objects that fall outside this range, e.g., in order 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.

8. Once you have previewed 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® Systems User Manual, Section 3, Chapter 1 for more information on Defining Image Analysis.)

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

Table 2. Brightfield Analysis Metrics

Brightfield Metric	Description
Organoid Object Count (1/image, 1/mm^2 or 1/well)	The number of objects per image, mm^2, or well.
Organoid 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 organoid growth analysis.
Organoid 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.
Organoid Darkness	The average of the darkness of the brightfield objects in the image. Ranges from 0 to 100 with the darkest objects having a value of 100.
Organoid Object Avg Area (µm^2)	The average of the area of the brightfield objects in the image.

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

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