Analysis Guidelines for the Incucyte® Scratch Wound Analysis Software Module

The Incucyte® Scratch Wound Analysis Software Module is used for real-time measurements of both migration and invasion in label-free monoculture or with fluorescence labeling in co-culture models.

This guideline covers the following topics for defining Scratch Wound Analysis parameters:

- Defining the Analysis Parameters for the Phase Image Channel
- Defining the Analysis Parameters for Fluorescence Image Channels

The following procedures are for example purposes only and are designed to provide a frame of reference for defining the Scratch Wound Analysis Parameters (Step 5) within the Analysis Wizard.

For new Incucyte® users, it is recommended to review Incucyte® Live-Cell Analysis Systems User Manual or have experience scheduling and acquiring scans, viewing images, and performing image analysis and visualizing results prior to reviewing this technical note.

Defining the Analysis Parameters for the Phase Image Channel

The following section will guide you through refining the analysis definition in order to accurately mask the migration and invasion of label-free cells in monoculture.

⚠️ *Ensure that images selected for defining the Scratch Wound Analysis Definition (Step 4 of the Analysis Wizard) include an image of cells immediately following wounding, as well as an image showing migration and/or invasion of cell into the wound area between ~10% and ~50% wound closure.*

⚠️ *If multiple cell types are on the same plate, or both invasion and migration conditions are present in the same experiment, representative images of each cell line and condition should be included.*

1. Click Preview Current or All. See Figure 1

💡 The best way to begin setting up the Analysis Definition is to use the preset values already contained within the Analysis Definition Editor, therefore do not change the Segmentation Adjustment, Cleanup, or Filters at this time.

Figure 1. Scratch Wound Analyzer Image Preview (phase analysis)
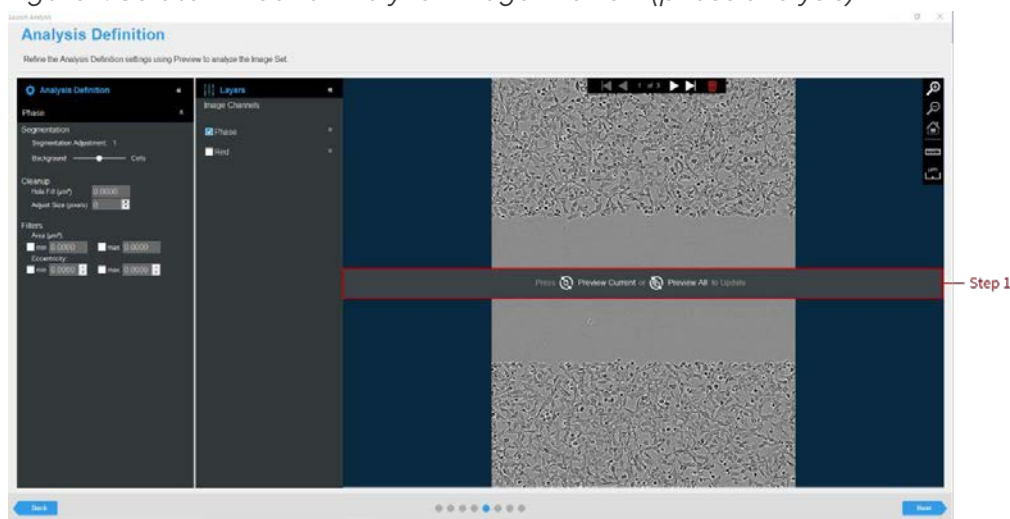
2. Evaluate your Scratch Wound and Confluence Mask and refine the parameters accordingly. See Figure 2 and Table 1

   ▪ 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 will not affect the analysis definition.

⚠️ *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 pane.*

Figure 2. Scratch Wound and Confluence Parameter Refinement with Mask
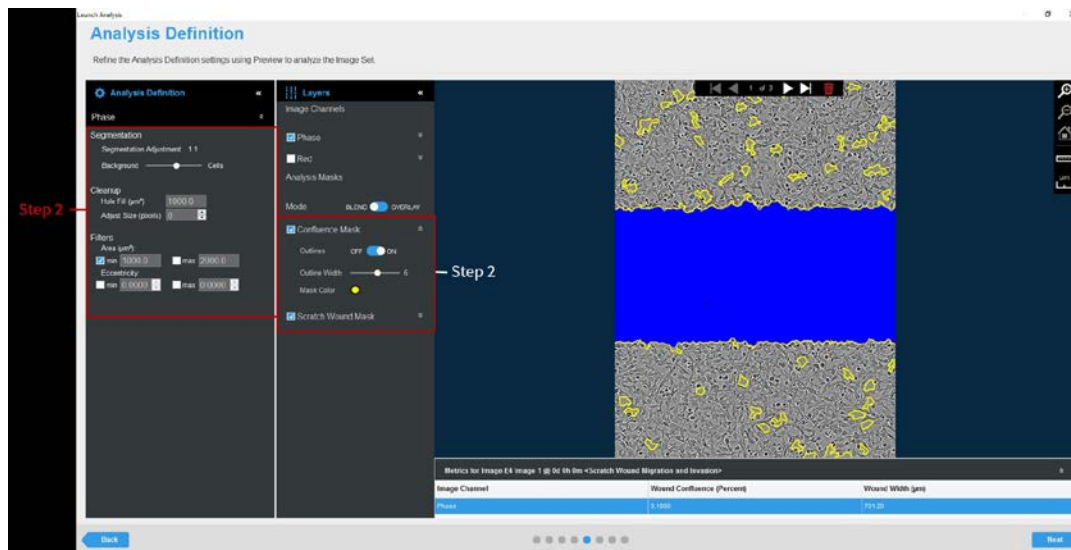

Table 1: Scratch Wound Analysis Definition Options

Option	Description
Parameters	
Segmentation Adjustment	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 to pick up a greater number of cells (move towards Cells) or reduce the image background (move towards Background).
Cleanup	
Hole Fill	Masks any holes smaller than the given size in the cell body cluster mask.
Adjust Size	If set to a positive value, then enlarges the mask by the specified number of pixels. If set to a negative value, then shrinks the mask by the specified number of pixels.
Filters – Used to remove any background that is not a true mask.	
Area	Defines a range of sizes (in μm^2) for the object and eliminates objects that fall outside this range.
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.

3. Once you have previewed all the images within the wizard image set and are satisfied with the parameters, complete the Launch wizard analysis to select the Scan Times and image sites to be analyzed, as well as assigning an analysis definition name.

After the vessel images have been analyzed using phase Scratch Wound Analysis, the following set of metrics are provided:

Phase Metric	Description
Wound Width (μm)	The distance between the migrating/invading edges of the wound.
Wound Confluence (%):	The confluence of cells within the wound region, given as the percentage of the wound region area occupied by cells.
Relative Wound Density (%):	Measures the spatial cell density in the wound area relative to the spatial cell density outside of the wound area at every time point. The metric is self-normalizing for changes in cell density which may occur outside the wound due to cell proliferation and/or pharmacological effects.

Defining the Analysis Parameters for the Fluorescence Channel

The following section will guide you through refining the analysis definition in order to accurately identify fluorescent objects which migrate or invade within the scratch wound.

> ⚠ *All fluorescence scratch wound metrics are dependent on the wound region, which is defined by the HD-Phase images and subsequent Scratch Wound Mask. Therefore, all fluorescence scratch wound assay analysis must include phase analysis in order to analyze fluorescent objects that migrate or invade within the wound.*

> ⚠ *Prior to creating a fluorescence analysis definition, if images were acquired in more than one fluorescence channel, ensure that you have evaluated if spectral unmixing is required to account for unwanted signal contributed from one of the given channels.*

1. In the Object name field, enter the name of the object that is being analyzed. See Figure 3

2. Define the segmentation processing parameters to segment object. See Table 2 and Figure 3

> 💡 *For easier identification of the analysis definition, you might want to name the object the same as the reagent or phenotypic object that was used in the assay, for example HT-1080 NLG.*

Table 2: Fluorescence Analysis Methods

| Option	Description
No Background Subtraction	
Adaptive	A local background level (LBL) across each processed image is automatically determined and the user inputs a Threshold Adjustment value above the LBL. It is advised to preview the default threshold adjustment of 2.0. To include more objects, lower this parameter, to exclude background, increase this parameter.
Fixed Threshold	A single threshold level in calibrated fluorescence units is used across the image. This number can be set as a number near or in between the dimmest positive object and the brightest background area.
Background Subtraction	
Top-Hat	Utilizing the radius of the largest fluorescent object, a background trend across the image is estimated and then subtracted. Objects that are brighter than the specified threshold value are detected in the background-subtracted image.
	Click the Measure image features icon ▦ , and then drag the mouse pointer to measure the radius of the largest object in the selected image channel. The value is displayed in the lower right corner of the image. Enter this value for the Radius.

⚠ *When using Top-Hat segmentation, note that a radius that is set too small may result in a loss in object detection. A radius that is set too large can cause incorrect background estimation.*

⚠ *Surface-Fit background subtraction is not available for Scratch Wound Analysis at this time.*

Figure 3. Scratch Wound Analyzer Image Preview (fluorescence channel)
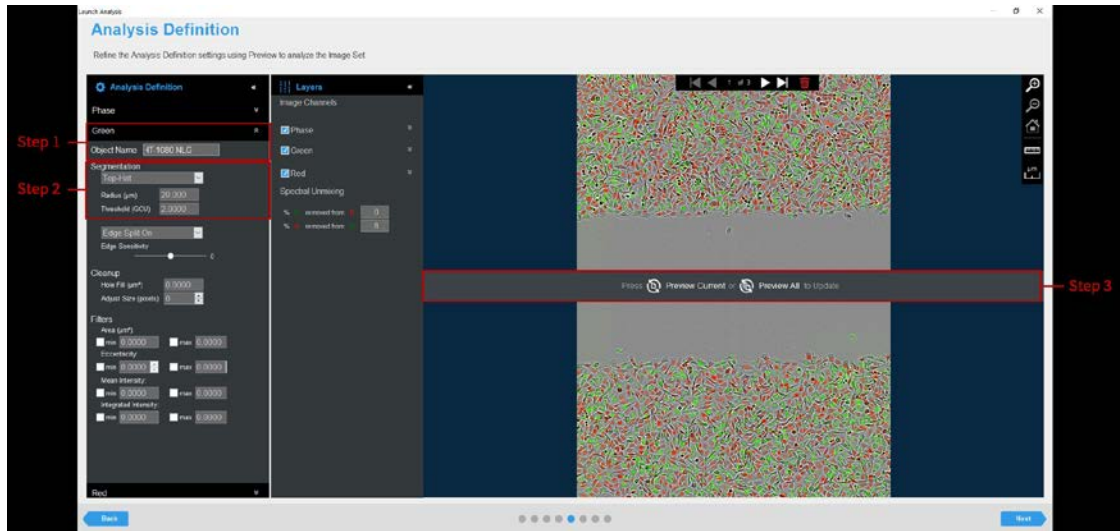
3. Click Preview Current or All. See Figure 3.

The best way to begin setting up the Analysis Definition is to use the preset values, therefore do not change the Segmentation Adjustment, Cleanup, or Filters at this time.

If using Top-Hat segmentation, once the image is previewed, a background subtracted image is formed and displayed in a new tab under the available color channels. Use the Original and Background Subtracted tabs to compare between the two images. Only the Background Subtracted image will be used for segmentation. See Figure 4.

---

4. Evaluate your Fluorescence Channel Object Mask and refine the parameters accordingly. See Figure 4 and Table 3.

    *   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 will not affect the analysis definition.


To assist you with viewing the effects of applying an analysis parameter, use the image navigation functions (zoom in, zoom out, home).

---

5. If necessary, adjust the segmentation by increasing the threshold to eliminate masking of background or by decreasing the threshold to include dimmer objects.

Table 3: Fluorescence Scratch Wound Analysis Definition Options

Option	Description
Edge Sensitivity	If Edge Split is turned on, then use the slider bar to adjust the Edge Sensitivity. The default value is 0. As you increase the Edge Sensitivity, the number of splits are also increased.
Cleanup	
Hole Fill	Removes any holes in the mask that are smaller than the area that is specified.
Adjust Size	Adjusts the size of your mask in pixels by either shrinking the mask (if negative) or growing the mask (if positive).
Filters – Used to remove any undesirable masked objects	
Area	Defines a range of sizes (in μm^2) for the object and eliminates objects that fall outside this range
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.
Mean Intensity	Defines the limits of mean intensity of an object, (the average pixel intensity in calibrated units), and eliminates objects that fall outside this range.
Integrated Intensity	Defines the limits of integrated intensity of an object, (the summed pixel intensity in calibrated units), and eliminates objects that fall outside this range.

Figure 4. Fluorescent Object Refinement with Mask
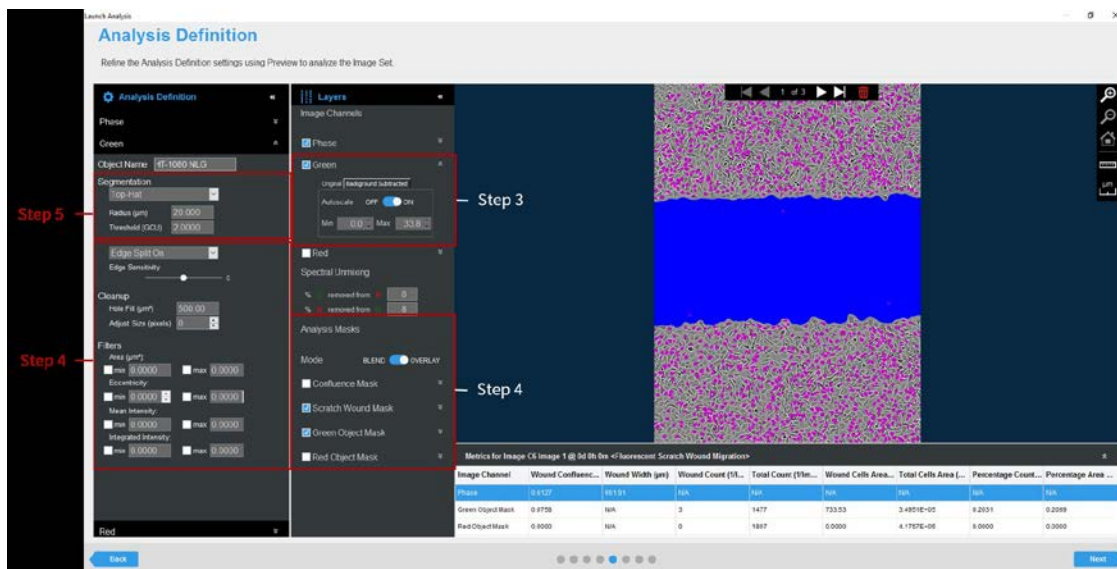
6. Once you have previewed all the images within the wizard image set and are satisfied with the parameters, complete the Launch wizard analysis to select the Scan Times and image sites to be analyzed, as well as assigning an analysis definition name.

After the vessel images have been analyzed using fluorescent object analysis, the following set of metrics are provided:

Color Metric	Description
Wound Confluence (%)	The confluence of objects within the wound region, given as the percentage of the wound region area occupied by the objects.
Wound Count (1/Image)	Number of objects within the wound region.
Total Count (1/Image)	Number of objects inside and outside the wound.
Percentage Count Within the Wound (%)	Number of objects within the wound as a percentage of the total number of objects within the image. (Wound Count / Total Count) x 100.
Wound Cells Area (mm^2)	The total area occupied by the objects within the wound region.
Total Cells Area (mm^2)	The total area occupied by the objects both inside and outside the wound region.
Percentage Area Within the Wound (%)	Area of the objects within the wound as a percentage of the total area of the objects in the image. (Wound Area / Total Area) x 100.

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
E-Mail: AskAScientist@sartorius.com
Telephone +1 734 769 1600

Europe
Units 2 & 3 The Quadrant,
Newark Close, Royston Hertfordshire
SG8 5HL
United Kingdom
E-Mail: euorders.UK03@sartorius.com
Telephone +44 0 1763 227400

APAC
Essen BioScience K.K.
4th floor Daiwa Shinagawa North Bldg.
1-8-11 Kita-Shinagawa
Shinagawa-ku, Tokyo
140-0001 Japan
E-Mail: orders.US07@sartorius.com
Office Telephone: +81 3 6478 5202
Office Fax: +81 3 6478 5499