Analysis Guidelines for the Incucyte® Basic Analysis Software

The Incucyte® Basic Analysis Software allows for the quantification of phase and fluorescent object metrics in real-time. AI-driven Confluence Analysis provides a simple workflow for highly accurate segmentation of cells in Phase Contrast images, adapting to a wide range of cell types and morphologies with minimal user input. The Classic Confluence enables you to tailor phase segmentation parameters to individual cell types and experimental conditions to accurately distinguish between background and positive fluorescent objects across a wide range of intensities, and depending on the optical module, allows for the quantification of overlap between green, orange, red, and/or NIR fluorescent objects within each image.

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

- Defining the Analysis Parameters for the Phase Image Channel with AI Confluence or Classic Confluence
- Defining the Analysis Parameters for the Fluorescence Image Channels
- Coincident object analysis

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

Refining the AI Confluence Analysis Definition Options for the Phase Image Channel

The following section will guide you through refining the analysis definition in order to accurately mask phase images of your experimental conditions (e.g., assay conditions and magnification).

1. In the Segmentation drop-down menu, select AI Confluence. See Figure 1

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

3. Click Preview Current or All. See Figure 1

Figure 1. Basic Analyzer AI Confluence Image Preview (phase only)
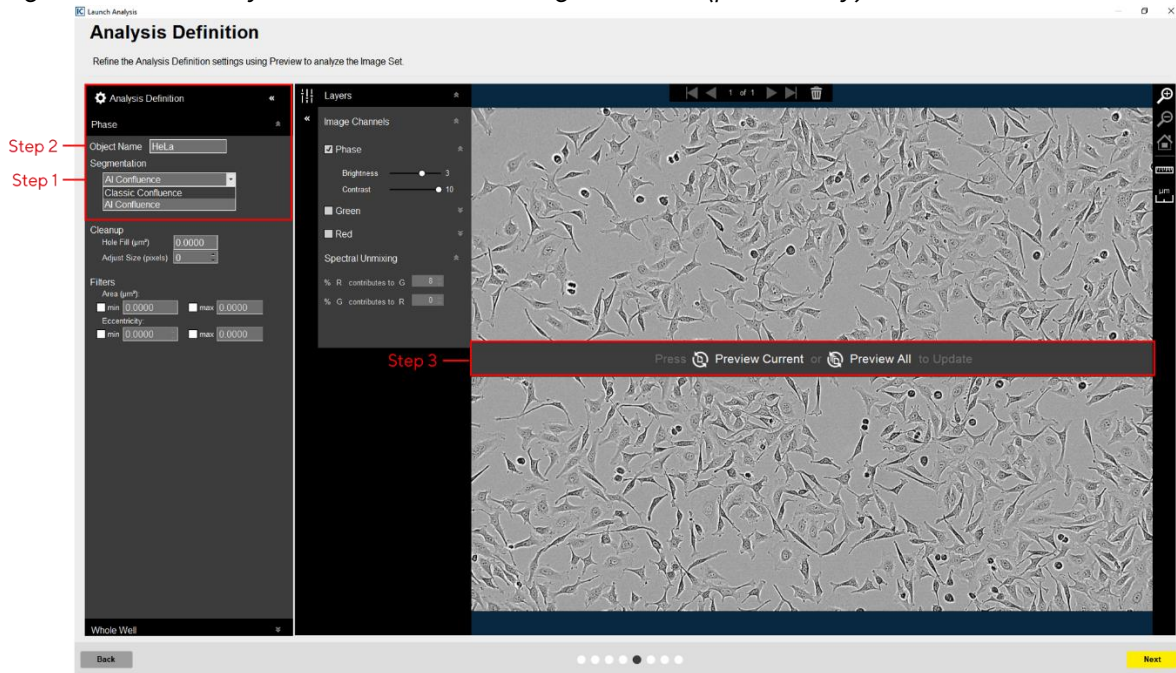
4. Evaluate your Confluence Mask (which is the mask applied to the phase channel) and refine the Cleanup options and Filters accordingly. See Figure 2 and Table 1
Modify only a single analysis definition parameter at a time. After you define the value for a parameter, click Preview Current to apply and view the change for the image that is currently displayed in the Image pane.

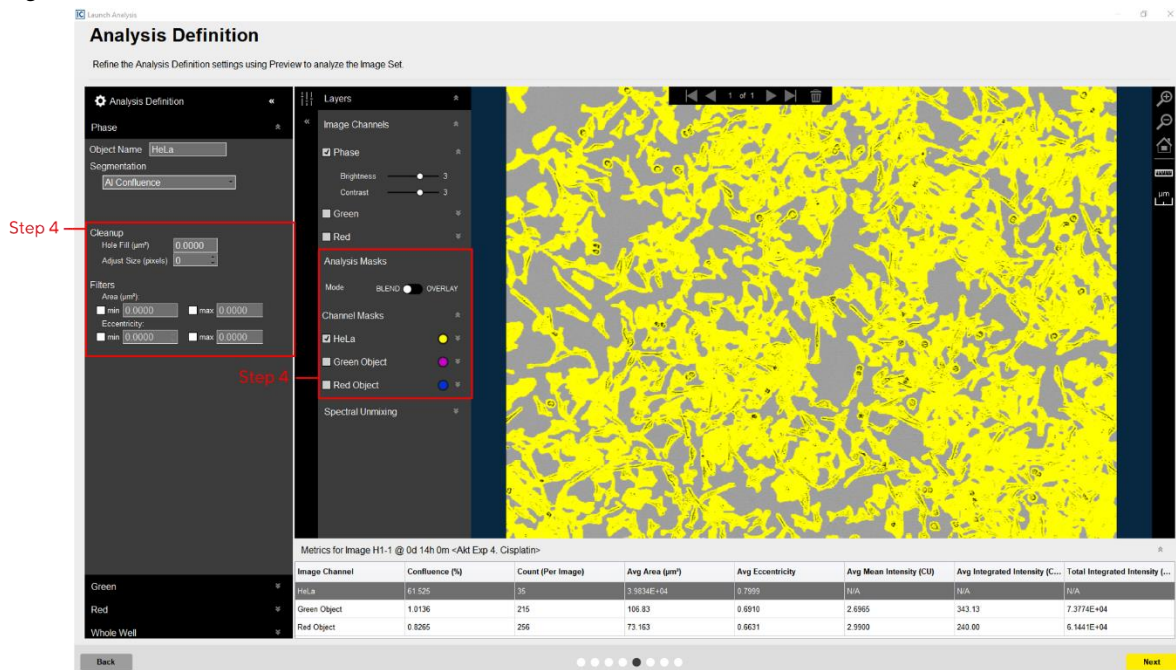*Figure 2. Parameter Refinement with AI Confluence Mask*

Table 1: Phase Image Channel AI Confluence Analysis Definition Options

Option	Description
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, 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.

5. Once you have previewed all images within the wizard image set and are satisfied with the adjustment, 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 object analysis, the following set of metrics are provided:

Phase Metric	Description
Confluence (%)	The percentage of the image area that is occupied by objects.
Count (1/image, 1/mm² or 1/well)	The number of objects per image, mm^2, or well.
Average Area (μm^2)	The average of the area of the objects in the image.
Average 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.

Defining the Analysis Parameters for the Phase Image Channel using Classic Confluence

The following section will guide you through refining the Basic Analyzer analysis definition in order to accurately mask phase images of your experimental conditions (e.g., cell type, assay conditions, and magnification).

1. In the Segmentation drop-down menu, select Classic Confluence. See Figure 1

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

3. 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 Segmentation Adjustment, Cleanup, or Filters at this time.

4. Evaluate your Confluence Mask (which is the mask applied to the phase channel) and refine the parameters accordingly. See Figure 3 and Table 2

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 3. Parameter Refinement with Classic Confluence Mask

Table 2: Phase Image Channel Classic Confluence 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	Removes any holes in the mask that are smaller than the area that is specified.
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 µm²) 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.

5. Once you have previewed all 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 object analysis, the following set of metrics are provided:

Phase Metric	Description
Confluence (%)	The percentage of the image area that is occupied by objects.
Count (1/image, 1/mm² or 1/well)	The number of objects per image, mm², or well.
Average Area (µm²)	The average of the area of the objects in the image.
Average 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.

Defining the Analysis Parameters for the Fluorescence Image Channel

The following section will guide you through refining the analysis definition in order to accurately identify fluorescent objects across a wide range of intensities, in order to produce data that can kinetically measure apoptosis, cytotoxicity, proliferation of fluorescence-labeled cells and other cell biology applications.

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

> *If more than one fluorescence image has been acquired, define the analysis parameters for one channel at a time using the expand and collapse arrows within the Analysis Definition Pane.*

> *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, Annexin V.*

1. In the Object name field, enter the name of the object that is being analyzed. See Figure 4

2. Define the segmentation analysis parameters to segment object. See Table 3 and Figure 4

Table 3: 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 this far 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 parameters.
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	
Surface Fit	The background-subtracted image is calculated using a best-fit polynomial. Objects that are brighter than the specified threshold value are detected in the background-subtracted image.
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 [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. See Figure 4

⚠️ *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.*

Figure 4. Basic Analyzer Image Preview (fluorescence channels)
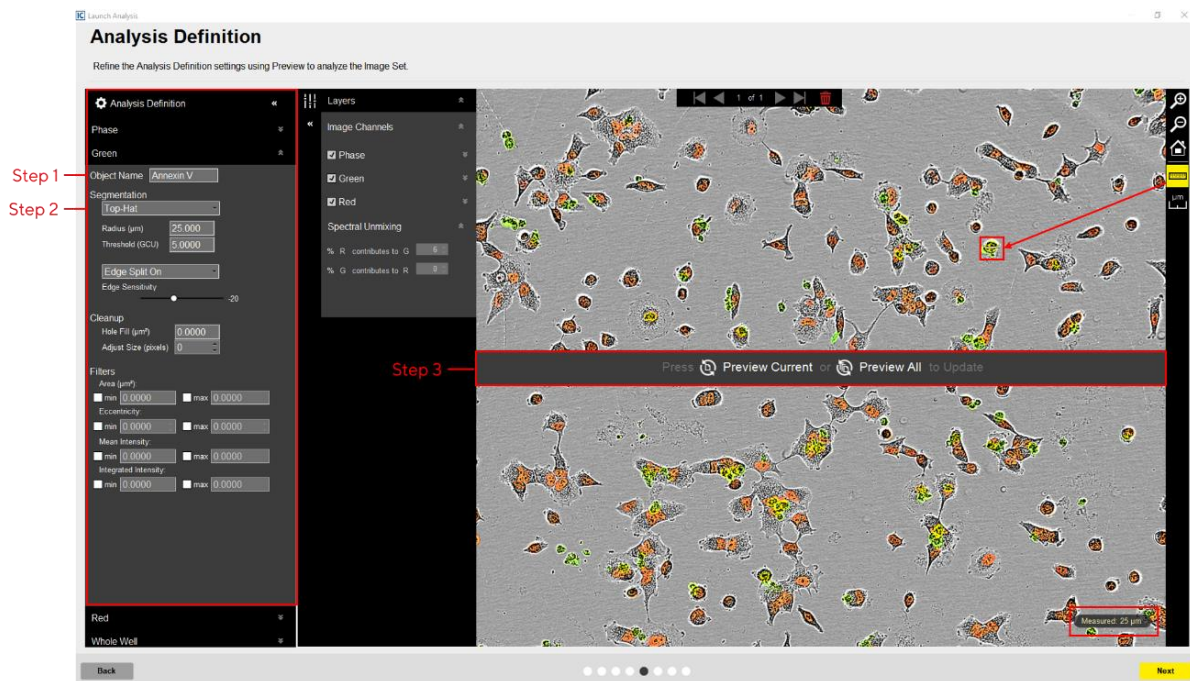
3. Click Preview Current or All. See Figure 4

💡 *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 "Segmentation Adjustment", "Cleanup", or "Filters" at this time.*

👆 *If using Surface Fit or 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 5*

4. Evaluate the fluorescence mask. See Figure 5

- Make sure that both the correct fluorescence image channel box and the Analysis Mask box are checked.

- 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).*

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

Figure 5. Parameter Refinement with Fluorescence Channel Mask

6. Click Preview Current or All.

7. Evaluate your fluorescence mask and refine the parameters accordingly. See Figure 5 and Table 4.

Table 4: Fluorescence Analysis Parameters

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.

8. 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 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:

Fluorescence Metric	Description
Object Count (1/image or $1/mm^2$ or 1/well)	The number of objects per image, per mm^2, or per well.
Avg. Area (μm^2)	The average area of the objects in the image.
Total Object Area (μm^2/Image or μm^2/Well):	The total amount of area within an image or a well containing fluorescent objects.
Confluence (%)	The percentage of the image area occupied by objects.
Avg. Mean Intensity (CU)	The image average of the objects' mean fluorescence intensity.
Avg. Integrated Intensity (CU x μm^2)	The image average of the objects' integrated fluorescence intensity.
Total Integrated Intensity (CU x μm^2/Image)	The total sum of the objects' fluorescence intensity in the image.
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.

Coincident Object Analysis

The following section will guide you through refining the analysis definition in order to accurately quantify the extent of the overlap between fluorescent objects from different channels within each image. The example used below acquired images in the green and red image channels, but the same principles will apply if images have been acquired in the green, orange, red, or NIR channels.

1. Create a suitable mask for the fluorescent objects (refer to Defining the Analysis Parameters for the Fluorescence Image Channels)

2. In the Object name field, enter the name of the object that is being analysed. See Figure 6


For easier identification of the analysis definition, you might want to name the object the same as the biology that is occurring in the assay, for example, G-S (Yellow).

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3. Click Preview Current or All.

4. Evaluate the Overlap Mask and filter for size to eliminate small or large objects, if required. See Figure 6

The Overlap Mask does not consider the intensities of the fluorescence channels, it only quantifies the overlay of the masked areas (area or count).

Figure 6. Overlap Analysis within the Basic Analyzer
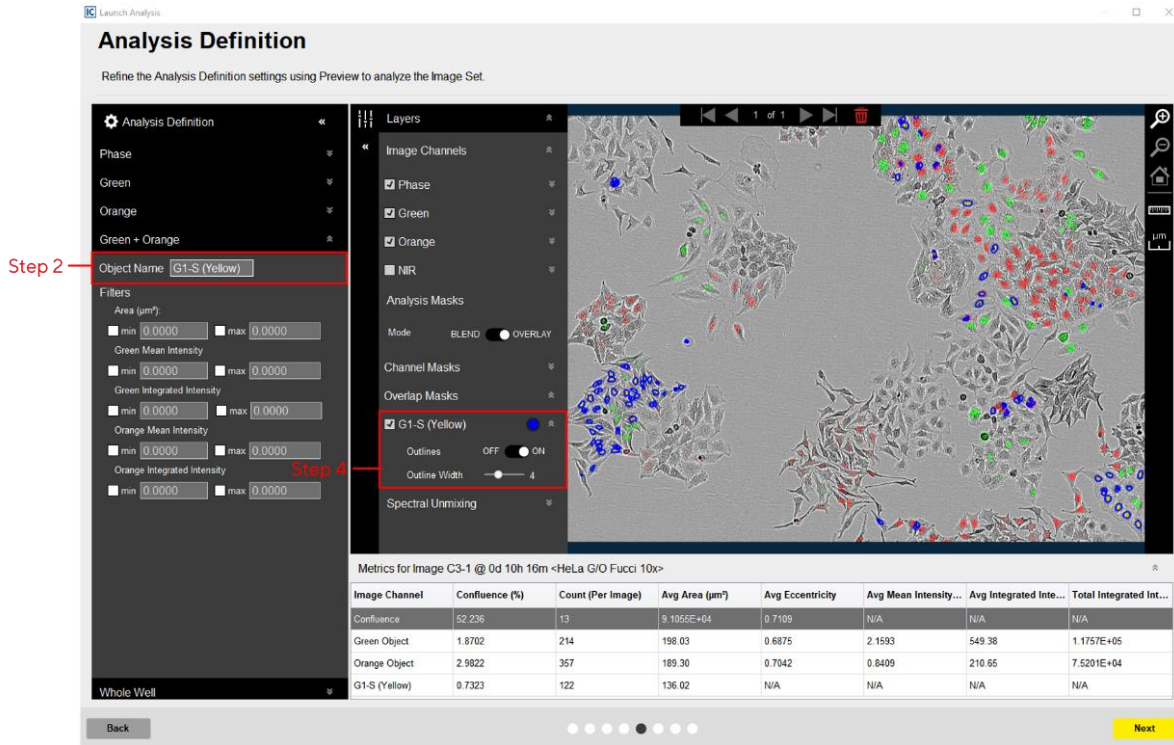
5. Once you have previewed all of the images within the wizard image set and are satisfied with the parameters, complete the Launch wizard analysis to select the Scan Times and Image Sites to be analyzed, as well as assigning an analysis definition name.

After the vessel images have been analyzed using coincident (overlap) analysis, the following set of metrics are provided:

Option	Description
Count (1/image, 1/mm² or 1/well)	The number of objects per image, mm² or well.
Average Area (µm²)	The average of the area of the object in the image.
Nuclear Translocation Ratio (NTR)	The NTR quantifies shuttling of the sensor between the cytoplasm and the nucleus by measuring the green fluorescence coming from the nuclear area (as defined by the overlap mask) divided by that coming from the total cell area (as defined by the green segmentation mask). The formula for the NTR metric is in Incucyte® Kinase Akt Lentivirus Product Guide.

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