Analysis Guidelines for Incucyte® Chemotaxis Analysis Software Module

The Incucyte® Chemotaxis Analysis Software Module allows you to tailor both phase and fluorescent segmentation parameters in order to quantify cell migration and invasion in response to a chemotactic gradient in real-time. Chemotaxis analysis is divided into two types, Chemotaxis Migration (Top/Bot), used to quantify adherent cell migration and invasion between two surfaces, and Chemotaxis Migration (Top Only), used to measure chemotactic migration of non-adherent cells.

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

- Defining the Analysis Parameters for Chemotaxis Migration (Top/Bot)
- Defining the Analysis Parameters for Chemotaxis Migration (Top Only)

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

Defining the Analysis Parameters for Chemotaxis Migration (Top/Bot)

The following section will guide you through refining the analysis definition in order to accurately mask phase and fluorescent images of adherent cell lines, which adhere to the bottom side of the Incucyte® Clearview membrane.

Phase Analysis (Top/Bot)

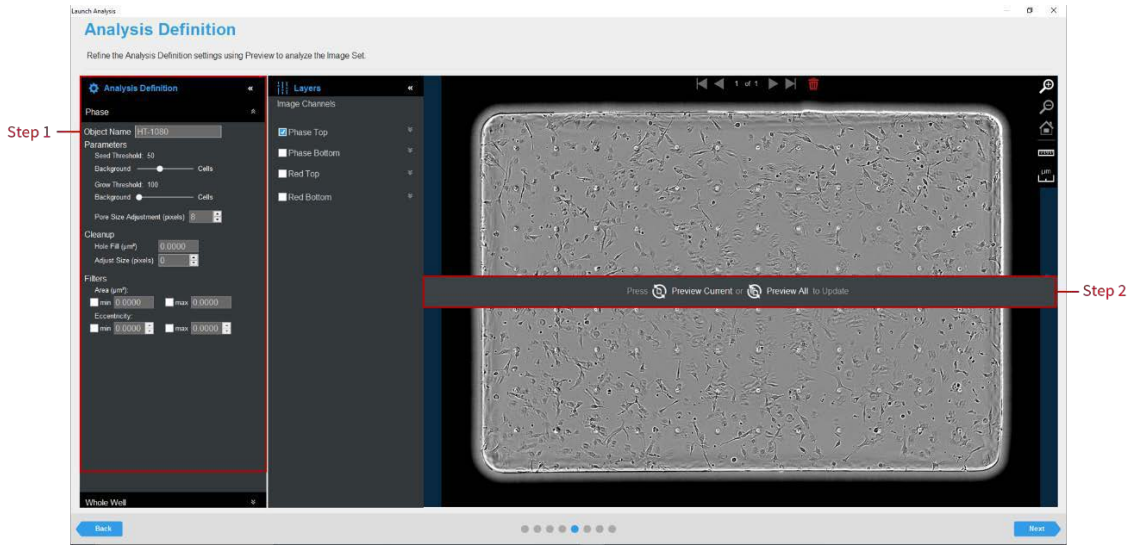
1. In the Object name field, enter the name of the object that is being analyzed. See Figure 1.

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.

2. 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 Parameters, Cleanup, or Filters at this time.

Figure 1. Chemotaxis Migration (Top/Bot) Image Preview (phase analysis)



3. Make sure that the identical image planes (e.g., top or bottom) for the Image Channel and Analysis Mask are checked. 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.

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

Table 1: Analysis Masks

Mask	Description
Keep-out	The mask shows which portion of the membrane was used for analysis. **Note:** The Whole Well Analysis found within the Analysis Definition Pane is used to eliminate the well edges from the phase-contrast analysis mask. We recommend using the default settings.
Top	Analysis mask for the cells that are present on the top-side of the membrane.
Bottom	Analysis mask for the cells that are present on the bottom-side of the membrane.
Phase Pore	Masks the phase pore area, which is excluded from phase analysis of cells.

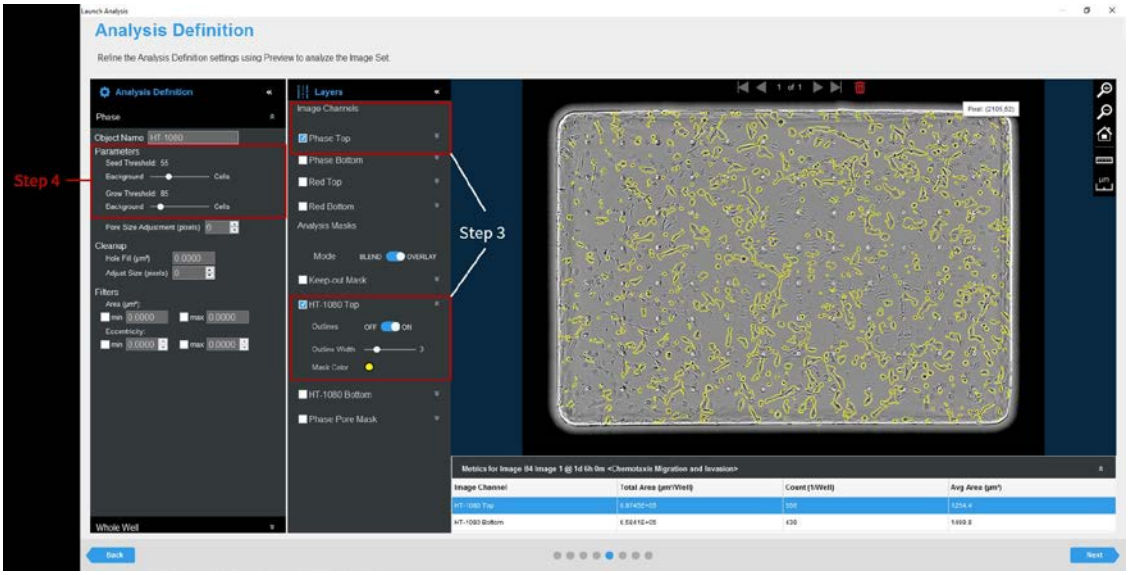
4. Evaluate your phase segmentation masks (top and bottom) and refine the parameters by doing the following:
    - Use the slider bar of the Seed Threshold to find the smallest threshold that masks off portions of the cells without including background.
    - Next, use the Grow Threshold to grow the seed mask to include more cell area. See Figure 2 and Table 2.

The Chemotaxis Migration (Top/Bot) Analysis applies the same set of analysis parameters to both the top-side and bottom side of the membrane.

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.

With the Phase Top Analysis Mask selected, toggle between the Phase-Contrast Top image channel and Phase- Contrast Bottom image channel to evaluate if the segmentation appropriately masks cells located on the membrane as Phase Mask Top objects.

Figure 2. Parameter Refinement with Top Mask



5. Further refine the mask using Cleanup and Filter options, modifying only a single option at a time. Click Preview after defining each value. See Figure 3 and Table 2.

Figure 3. Analysis Refinement with Associated Masks

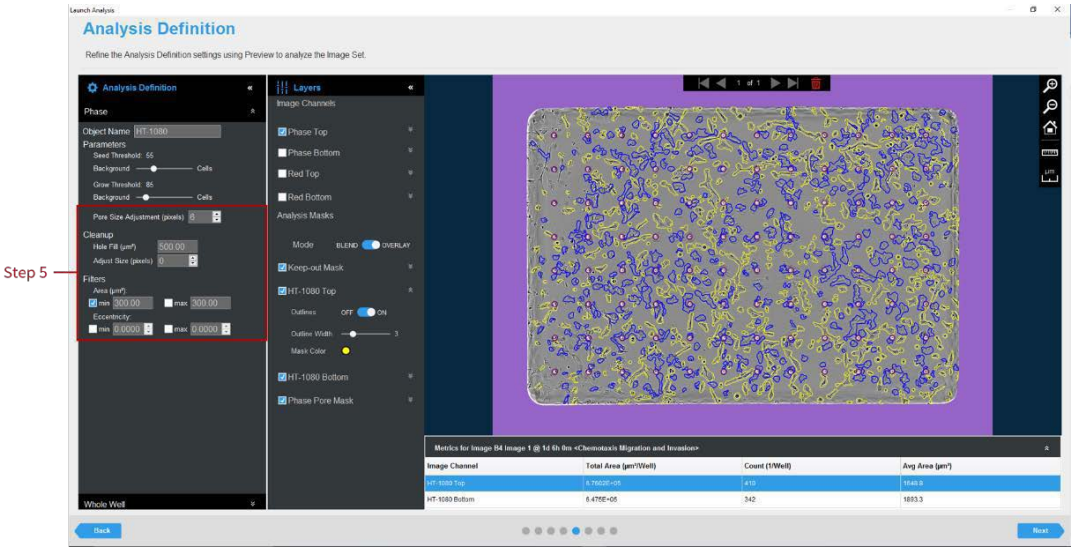


Table 2: Phase image channel analysis definition options

Option	Description
Parameters	
Seed Threshold	Use to find the smallest threshold that masks off portions of the cells without including the background.
Grow Threshold	Use the grow threshold to grow the seed mask to mask off more cell area.
Pore size	Use to adjust the area that is defined as the membrane pore. This are is excluded from phase analysis of cells.
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.

6. 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 Chemotaxis Migration (Top/Bot) Analysis, the following set of metrics are provided:

Phase Metric	Description
Total Phase Object Area (µm²/well)	The total area of the objects in the well
Total Phase Object Area Normalized to Initial Top Value	The total area of the objects in the well normalized to (divided by) the initial area
Phase Object Count (1/well)	The number of objects per well
Total Phase Object Count Normalized to Initial Top Value	The total count of the objects in the well normalized to (divided by) the initial count
Average Area (µm²)	The average of the area of the objects in the image

Fluorescence Analysis (Top/Bot)

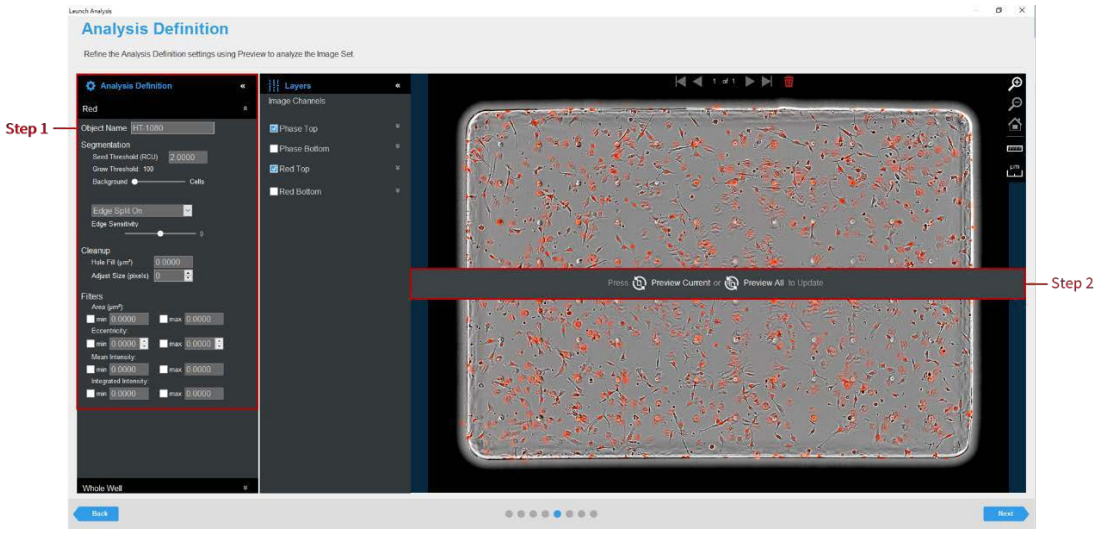
1. In the Object name field, enter the name of the object that is being analyzed. See Figure 4.

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.

2. 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.

Figure 4. Chemotaxis Migration (Top/Bot) Image Preview (fluorescence)



3. Evaluate the fluorescent mask. See Figure 5.
   - Make sure that both the correct fluorescent 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).

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. If necessary, refine the fluorescent segmentation parameters by doing the following:

   - Set the Seed Threshold to find the smallest fluorescence threshold that masks off portions of the cells without including the background.
   - Next, use the slider bar of the Grow Threshold to expand the seed mask to cover more cell area. See Figure 5 and Table 3.

The Chemotaxis Migration (Top/Bot) Analysis applies the same set of analysis parameters to both the top-side and bottom side of the membrane.

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.

With the Top Analysis Mask selected, toggle between the Red Top image channel and Red Bottom image channel to evaluate if the segmentation appropriately masks cells located on the membrane as Red Mask Top objects.

Table 3: Analysis Masks

Mask	Description
Keep-out	The mask shows which portion of the membrane was used for analysis. **Note:** The Whole Well Analysis found within the Analysis Definition Pane is used to eliminate the well edges from the phase-contrast analysis mask. We recommend using the default settings.
Top	Analysis mask for the cells that are present on the top-side of the membrane.
Bottom	Analysis mask for the cells that are present on the bottom-side of the membrane.
Phase Pore	Masks the phase pore area, which is excluded from phase analysis of cells. **Note:** When analyzing fluorescent cells, cells that are located within the pores are defined as positive chemotaxis events and will be masked as a bottom event.

5. Further refine the mask using Cleanup and Filter options, modifying only a single option at a time. Click Preview after defining each value. See Figure 5 and Table 4.

Figure 5. Fluorescent Chemotaxis Analysis Options and Masks

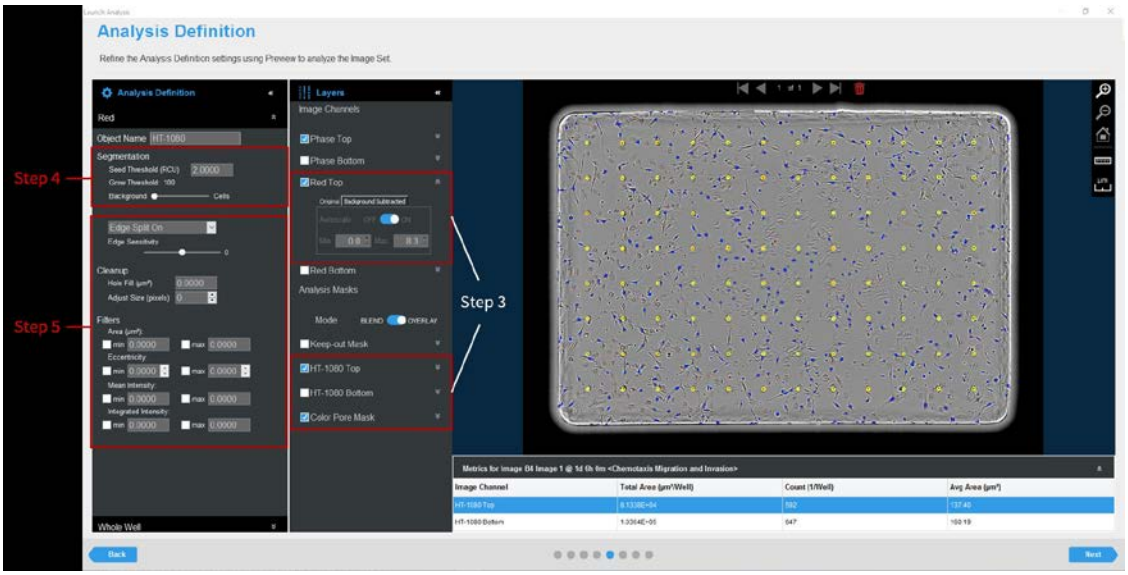


Table 4: Fluorescent Analysis Parameters

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.

6. 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 image sites to be analyzed, as well as assigning an analysis definition name.

After the vessel images have been analyzed using fluorescent Chemotaxis Migration (Top/Bot) analysis, the following set of metrics are provided:

Fluorescent Metric	Description
Object Count (1/well)	The number of fluorescent objects per well
Object Count Normalized to Initial Top Value	The number of fluorescent objects per well normalized to (divided by) the initial object count
Total Object Area (μm^2/well)	The total area of fluorescent objects in the well
Total Object Area Normalized to Initial Top Value	The total area of fluorescent objects in the well normalized to (divided by) the initial area
Avg. Object Area (μm^2)	The average of the area of the fluorescent objects

Defining the Analysis Parameters for Chemotaxis Migration (Top Only)

The following section will guide you through refining the analysis definition in order to accurately mask phase and fluorescent images of non-adherent cell lines. Non-adherent cells transiently adhere to the bottom of the membrane before falling to the reservoir, thus a top-side analysis of the Incucyte Clearview membrane is required.

Phase Analysis (Top Only)

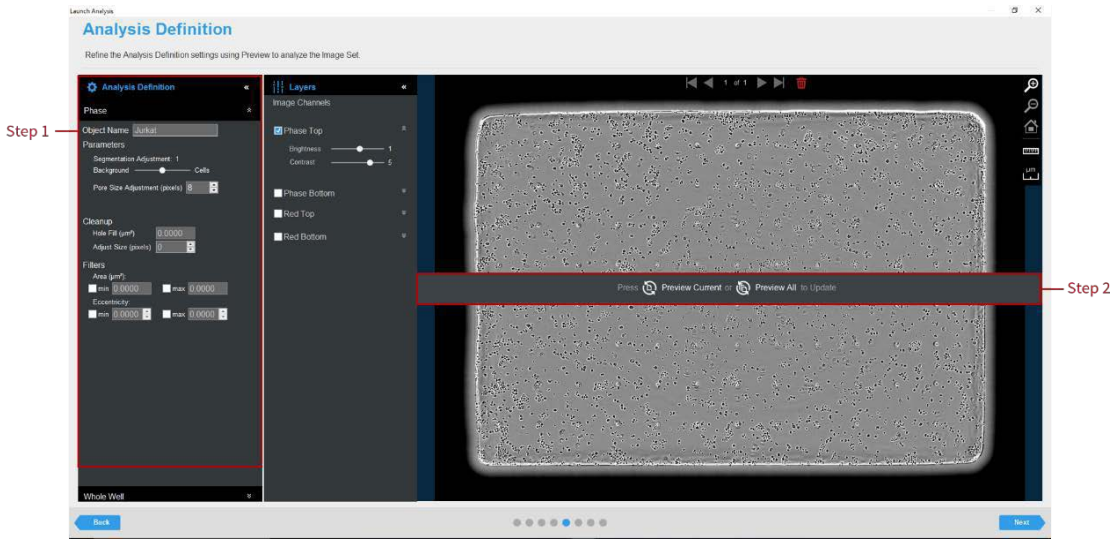
1. In the Object name field, enter the name of the object that is being analyzed. See Figure 6.

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, Jurkat.

2. Click Preview Current or All. See Figure 6.

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 Parameters, Cleanup, or Filters at this time.

Figure 6. Chemotaxis Migration (Top Only) Image Preview (phase analysis)



3. Evaluate your phase segmentation mask
   - 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. See Table 5 and Figure 7.

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

Table 5: Analysis Masks

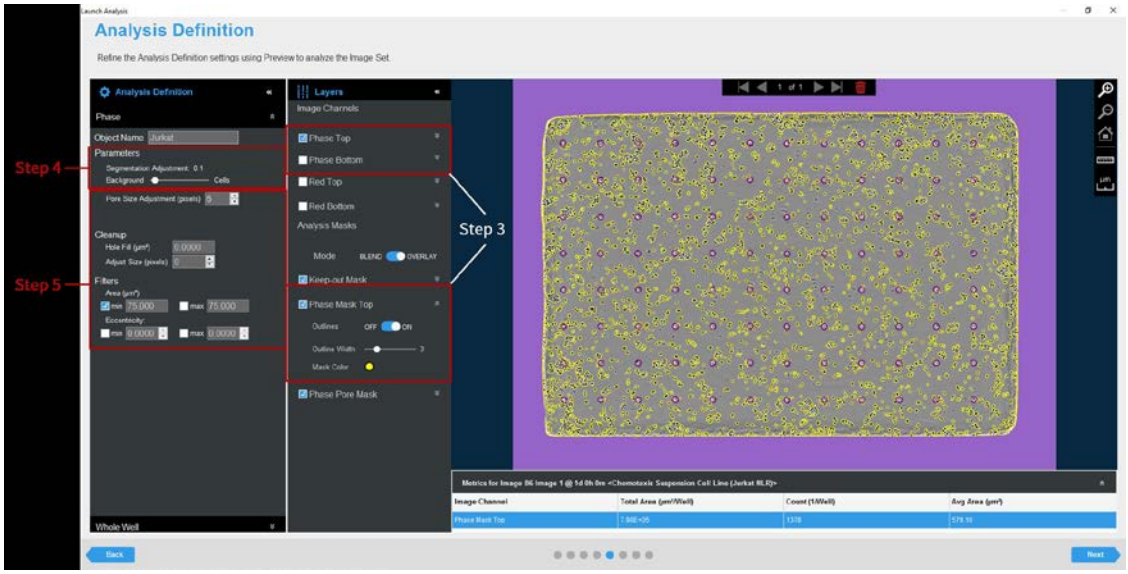
Mask	Description
Keep-out	The mask shows which portion of the membrane was used for analysis. **Note:** The Whole Well Analysis found within the Analysis Definition Pane is used to eliminate the well edges from the phase-contrast analysis mask. We recommend using the default settings.
Top	Analysis mask for the cells that are present on the top-side of the membrane.
Bottom	Analysis mask for the cells that are present on the bottom-side of the membrane.
Phase Pore	Masks the phase pore area, which is excluded from phase analysis of cells.

4. If necessary, refine the segmentation parameters mask by moving the Segmentation Adjustment slider more toward Background to eliminate background texture and debris, or toward Cells to include more cell area. See Figure 7 and Table 6.

After you define a value for the Segmentation Adjustment, click Preview Current to apply and view the change for the image that is currently displayed in the Image pane.

With the Phase Mask Top selected, toggle between the Phase Top image channel and Phase Bottom image channel to evaluate if the segmentation appropriately masks cells located on the top of the membrane, excluding cells that are transiently adhered to the bottom of the membrane.

Figure 7. Analysis Refinement with Associated Masks



5. Further refine the mask using Cleanup and Filter options, modifying only a single option at a time. Click Preview after defining each value. See Figure 7 and Table 6.

Table 6: Phase Image Channel Analysis Definition Options

Option	Description
Parameters	
Segmentation	Use to find the threshold that masks off the cells without including the background. Adjust slider more toward Background to eliminate background texture and debris, or toward Cells to include more cell area.
Pore size	Use to adjust the area that is defined as the membrane pore. This are is excluded from phase analysis of cells.
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.

6. 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 image sites to be analyzed, as well as assigning an analysis definition name.

After the vessel images have been analyzed using phase Chemotaxis Migration (Top Only) Analysis, the following set of metrics are provided:

Phase Metric	Description
Total Phase Object Area (μm^2/well)	The total area of the objects in the well
Total Phase Object Area Normalized to Initial Top Value	The total area of the objects in the well normalized to (divided by) the initial area
Phase Object Count (1/well)	The number of objects per well
Total Phase Object Count Normalized to Initial Top Value	The total count of the objects in the well normalized to (divided by) the initial count
Average Area (μm^2)	The average of the area of the objects in the image

Fluorescence Analysis (Top Only)

1. In the Object name field, enter the name of the object that is being analyzed. See Figure 8.

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.

2. Define the segmentation analysis parameters to segment object. See Table 7 and Figure 8

Table 7: Fluorescent Segmentation 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 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 your 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.

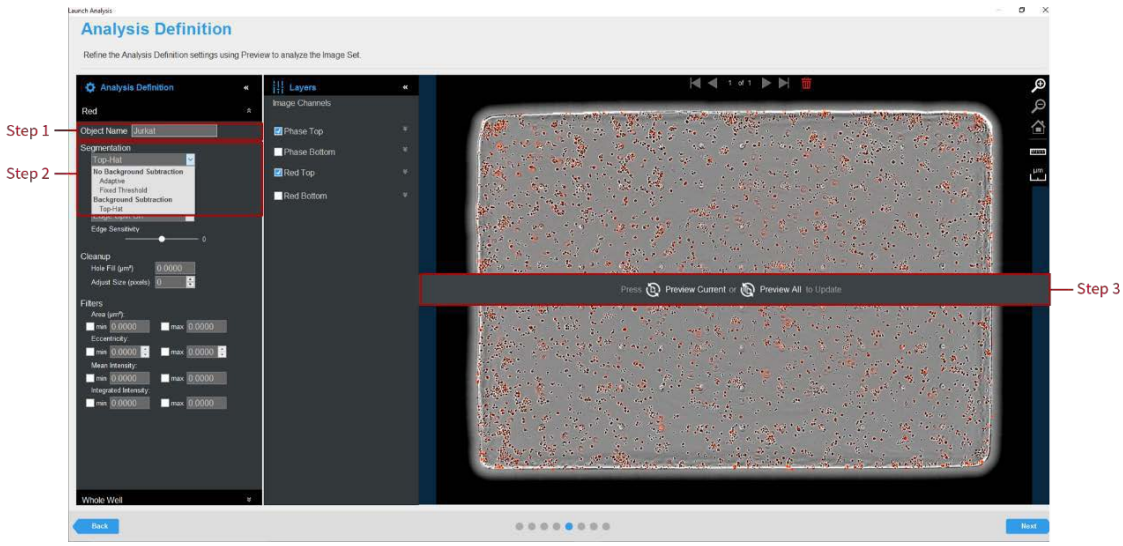
When using Top-Hat segmentation, note that a radius that is set too small may result in a loss in object detection. A radius that is set too large can cause incorrect background estimation.

3. Click Preview Current or All. See Figure 8.

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 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 9.

Figure 8. Chemotaxis Migration (Top Only) Image Preview (fluorescent channel)



4. Evaluate the fluorescent mask. See Figure 9
    - Make sure that both the Phase Top and Red Top image channels 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).

5. If necessary, adjust the segmentation by increasing the threshold to eliminate masking of background or by decreasing the threshold to include dimmer objects. See Figure 9.

With the Red Mask Top selected, toggle between the Red Top image channel and Red Bottom image channel to evaluate if the segmentation appropriately masks cells located on the top of the membrane, excluding cells that are transiently adhered to the bottom of the membrane.

6. Further refine the mask using Cleanup and Filter options, modifying only a single option at a time. Click Preview after defining each value. See Figure 9 and Table 8.

Figure 9. Chemotaxis Migration (Top Only) Refinement with Masks (fluorescent channel)

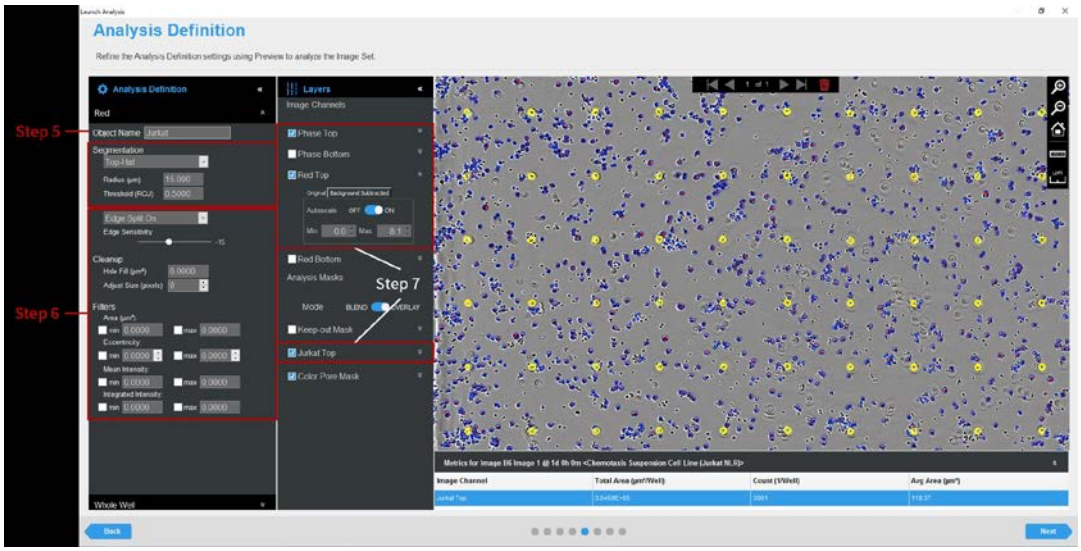


Table 8: Fluorescent Image Channel Analysis Definition Options

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.

7. 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 fluorescent Chemotaxis Migration (Top Only) Analysis, the following set of metrics are provided:

Fluorescent Metric	Description
Object Count (1/well)	The number of fluorescent objects per well
Object Count Normalized to Initial Top Value	The number of fluorescent objects per well normalized to (divided by) the initial object count
Total Object Area (μm^2/well)	The total area of fluorescent objects in the well
Total Object Area Normalized to Initial Top Value	The total area of fluorescent objects in the well normalized to (divided by) the initial area
Avg. Object Area (μm^2)	The average of the area of the fluorescent objects

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