Analysis Guidelines for the Incucyte® Neurotrack Analysis Software Module

The Incucyte® Neurotrack Analysis Software Module is used for real-time measurements of neurite dynamics in monoculture or with fluorescent labeling in co-culture models.

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

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

The following procedures are for example purposes only and are designed to provide a frame of reference for defining the Neurotrack 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.

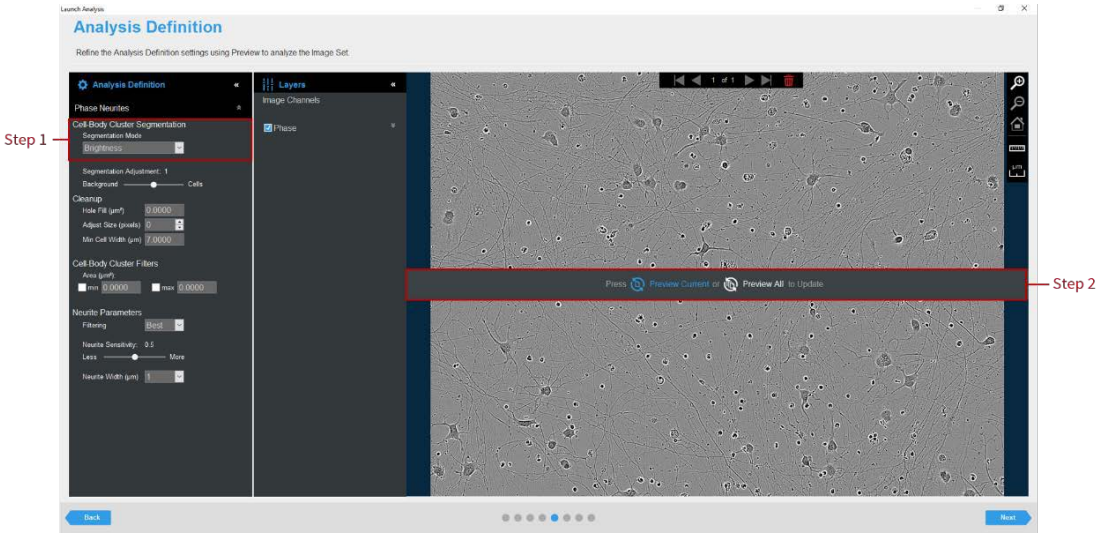
Defining the Analysis Parameters for the Phase Image Channel

The following section will guide you through refining the analysis definition in order to accurately mask the neurites of label-free neurons in monoculture.

1. Select the proper Segmentation Mode.
    - Brightness: use with primary or iPSC derived cells
    - Texture: use with low contrast cell lines such as Neuro-2a
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 the Segmentation Adjustment, Cleanup, or Cell Body Cluster Filters at this time.

Figure 1. Neurotrack Analyzer Image Preview (phase analysis)



3. Evaluate your Cell Body Cluster 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. Cell Body Cluster Refinement with Mask

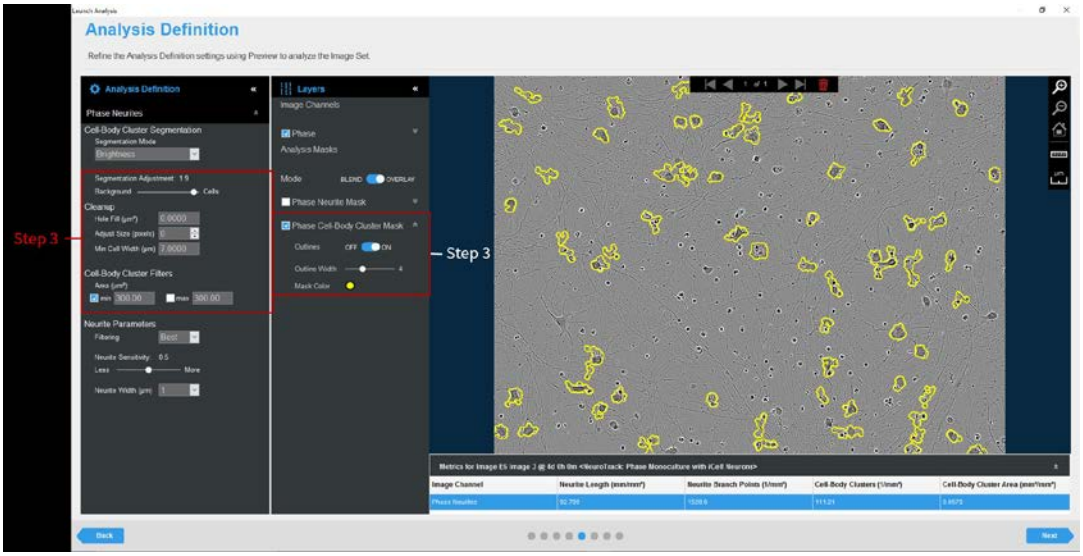


Table 1: Cell Body Cluster Analysis Definition Options

Option	Description
Parameters	
Segmentation Mode	Either Brightness or Texture is prioritized as a means of distinguishing cell bodies from background and neurites.
Background/Cells	Use the slider bar to adjust the mask to pick up a greater number of cell bodies (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. This parameter is never changed from zero (0) for Neurotrack assays.
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.
Min Cell Width	Defines the size at which cell bodies transition into neurites. 7-8 µm is common for primary/iPSC-derived cells, and 14-18 µm for immortalized cell lines.
Cell Body Cluster 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. 60-80 μm^2 is a common minimum filter for primary/iPSC-derived cells, and ~300 μm^2 for immortalized cell lines.

4. Once you are satisfied with the Cell Body Cluster Analysis Mask, evaluate your Phase Neurite Mask and refine the parameters accordingly. See Figure 3 and Table 2
    - 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 3. Neurite Parameter Refinement with Mask

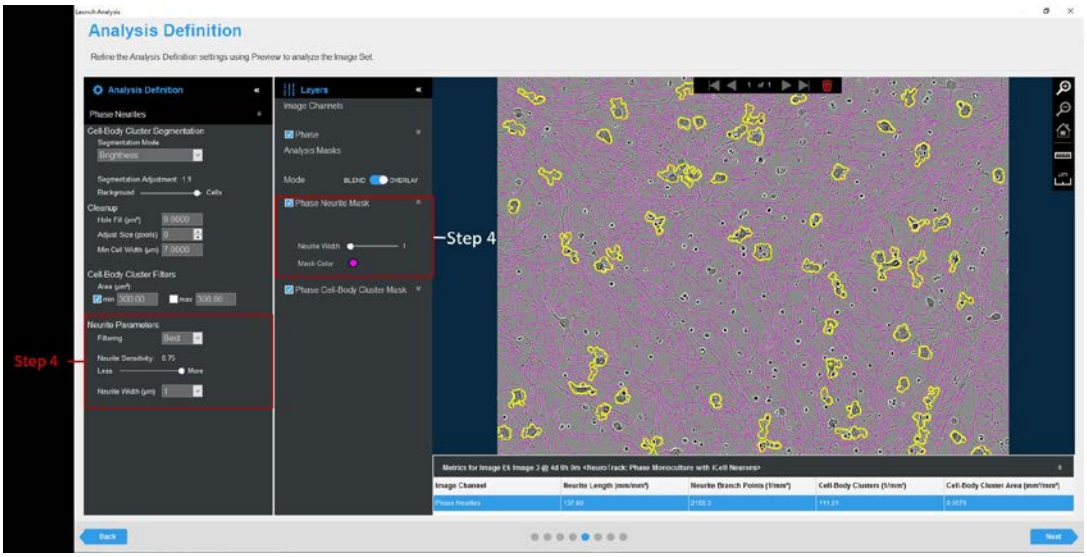


Table 2: Neurite Analysis Parameter Options

Option	Description
Filtering	Reduces the masking of small vessel imperfections and debris • None: Use for very clean cultures and vessels • Better (Fast): Faster processing at the expense of losing detection of very fine neurites; may be sufficient for cells with thick or high-contrast neurites. Also, useful for vessels with many imperfections or debris. • Best (Slower): Longer processing time, but the most sensitive filter setting to ensure detection of very fine neurites.
Neurite Sensitivity	Use the slider bar to adjust the mask to pick up a greater number of neurite structures (move towards More) or reduce the image background (move toward Less).
Neurite Width (µm)	Tunes the detection to the given width of neurite. 1 µm is nearly always a good choice for primary/iPSC-derived neurons while 2 µm is usually preferred for immortalized cell lines.

5. 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 Neurotrack Analysis, the following set of metrics are provided:

Phase Metric	Description
Neurite Length (mm/mm^2, mm/Cell Body Cluster or mm/mm^2)	The summed length of neurites that extend from the cell bodies.
Cell Body Clusters (1/mm^2 or mm^2/mm^2)	An estimation of cell body number or size in an image.
Branch Points (1/mm^2 or 1/Cell Body Cluster)	Branch points- An intersection of two masked neurites in an image.

Defining the Analysis Parameters for the Fluorescence Channel

The following section will guide you through refining the Neurotrack Analysis definition in order to accurately identify fluorescent neurites to measure growth and shrinkage of fluorescence-labeled neurons.

1. Define the Cell Body Cluster Segmentation analysis parameter. See Table 3 and Figure 4.

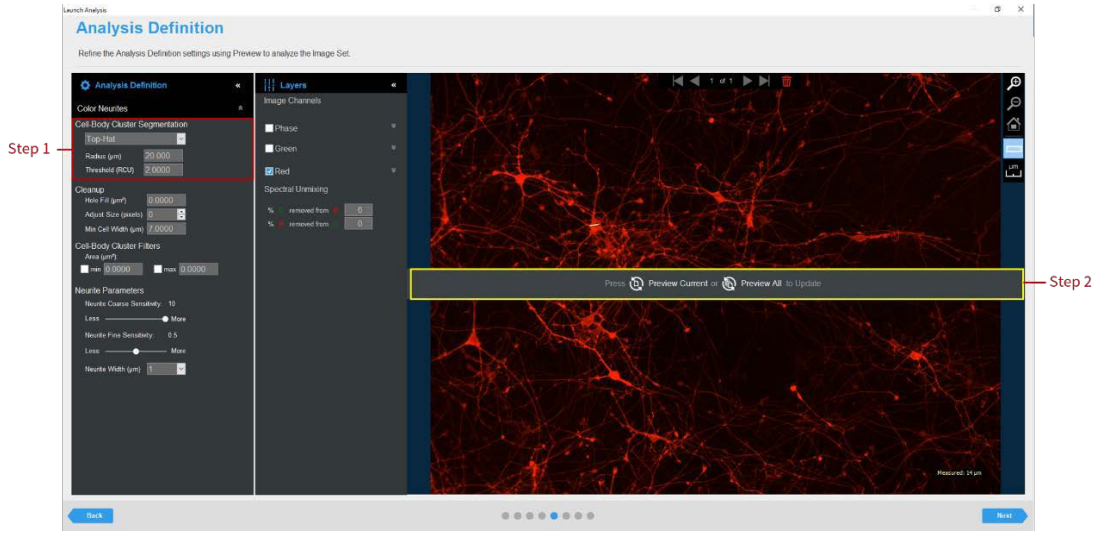
Table 3: Analysis Methods for Fluorescence

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 using 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 between the value of 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 Neurotrack Analysis at this time.

Figure 4. Neurotrack Analyzer Image Preview (fluorescence channel)



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, therefore do not change the Segmentation Adjustment, Cleanup, or Cell Body Cluster 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 5.

3. Evaluate your Cell Body Cluster Mask and refine the parameters accordingly. See Figure 5 and Table 4.
    - 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.

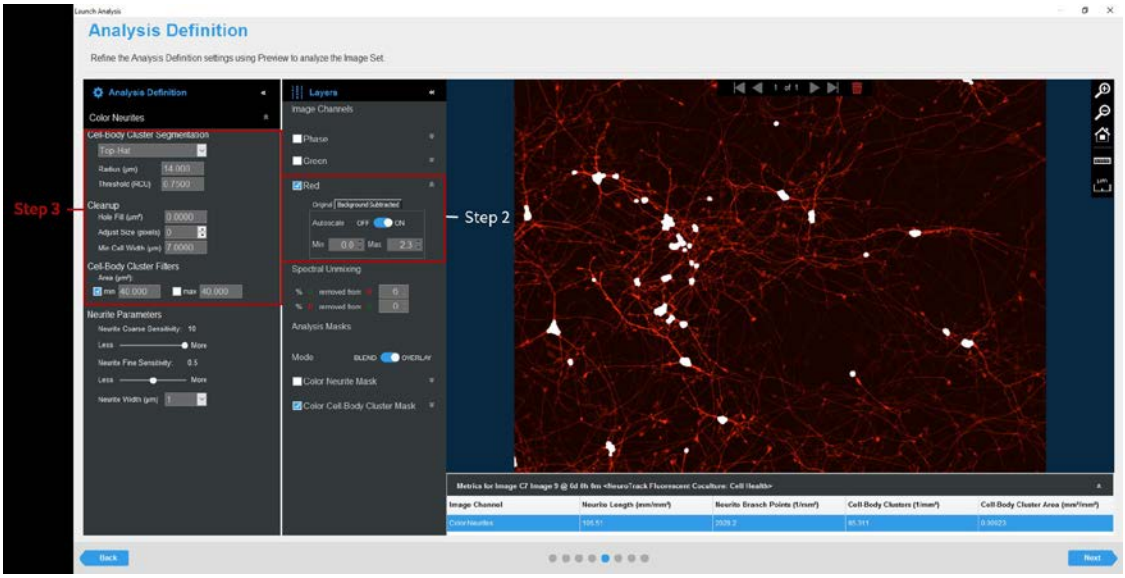
To assist you with viewing the effects of applying an analysis parameter, use the image navigation functions (zoom in, zoom out, home).

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

Table 4: Color Cell Body Cluster Analysis Definition Options

Option	Description
Hole Fill	Masks any holes smaller than the given size in the cell body cluster mask. This parameter is never changed from 0 for Neurotrack assays.
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.
Min Cell Width	Defines the size at which cell bodies transition into neurites. 7-8 µm is common for primary/iPSC-derived cells, and 14-18 µm for immortalized cell lines.
Area	Defines a range of sizes (in μm^2) for the object and eliminates objects that fall outside this range. 60-80 μm^2 is a common minimum filter for primary/iPSC-derived cells, and ~300 μm^2 for immortalized cell lines.

Figure 5. Color Cell Body Cluster Mask Refinement with Mask



5. Click Preview Current or All.

6. Once you are satisfied with the Color Cell Body Cluster Analysis Mask, evaluate your Color Neurite Mask and refine the parameters accordingly. See Figure 6 and Table 5
    - 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.
    - First set the Neurite Coarse Sensitivity slider to adjust for brightness, then adjust the Neurite Fine Sensitivity to detect finer structures.

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 6. Neurite Parameter Refinement with Mask

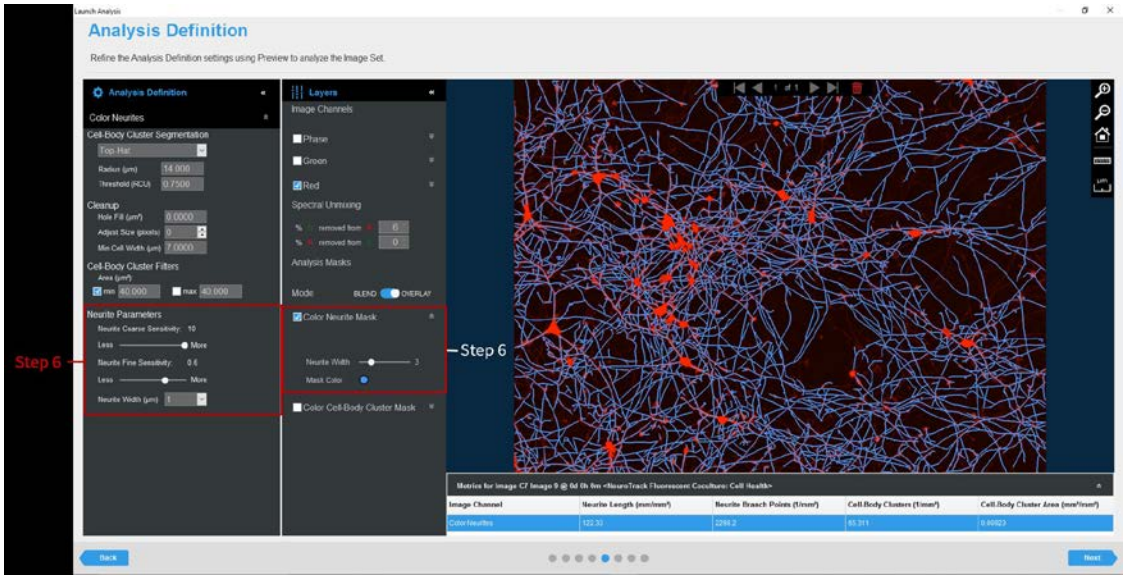


Table 5: Neurite Analysis Fluorescence Parameters

Option	Description
Coarse Sensitivity	Adjust the slider based on the brightness of the fluorescent label used. More sensitivity (slider at or near 10) should be selected when neurite intensity is low (i.e. red fluorescent kinetic labels). Less sensitivity should be used for bright neurites (i.e. green fluorescent antibody staining).
Fine Sensitivity	Adjusts the sensitivity to alter detection of very fine neurite structures.
Neurite Width (µm)	Tunes the detection to the given width of neurite. 1 µm is nearly always a good choice for primary/iPSC-derived neurons while 2 µm is usually preferred for immortalized cell lines.

7. 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, and assign an analysis definition name.

After the vessel images have been analyzed using Neurotrack Analysis for fluorescence assays, the following set of metrics are provided:

Color Metric	Description
Neurite Length (mm/mm^2, mm/Cell Body Cluster or m/mm^2)	The summed length of neurites that extend from the cell bodies.
Cell Body Clusters (1/mm^2 or mm^2/mm^2)	An estimation of cell body cluster number or size in an image.
Branch Points (1/mm^2 or 1/Cell Body Cluster)	A count of the intersections of two masked neurites in an image or per cell body cluster.

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