Guidelines for Defining the Angiogenesis Analysis Parameters

The IncuCyte® Angiogenesis Analysis software allows for the quantification of fluorescent network formation during the angiogenesis process to automatically compute biologically relevant properties in both the IncuCyte® Primekit and IncuCyte® StemKit.

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

- Defining the Analysis Parameters for Angiogenesis

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

Defining the Analysis Parameters for Angiogenesis

The following section will guide you through refining the analysis definition in order to accurately mask fluorescent images of angiogenic network formation overtime.

1. Define the segmentation analysis parameters to segment object. See Table 1 and Figure 1

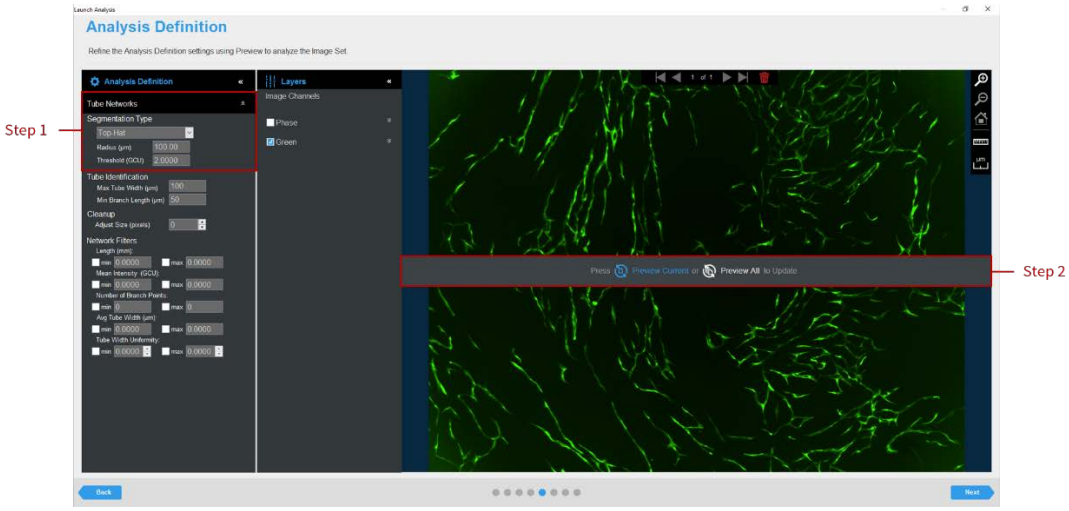
By default, the Top-Hat segmentation is selected. Leave this segmentation selected with the associated preset values prior to making any changes to the analysis method type.

Table 1: 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. See Figure 3

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 1. Angiogenesis Analyzer Image Preview (fluorescent channel)



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 Segmentation Adjustment, Cleanup, or Filters at this time.

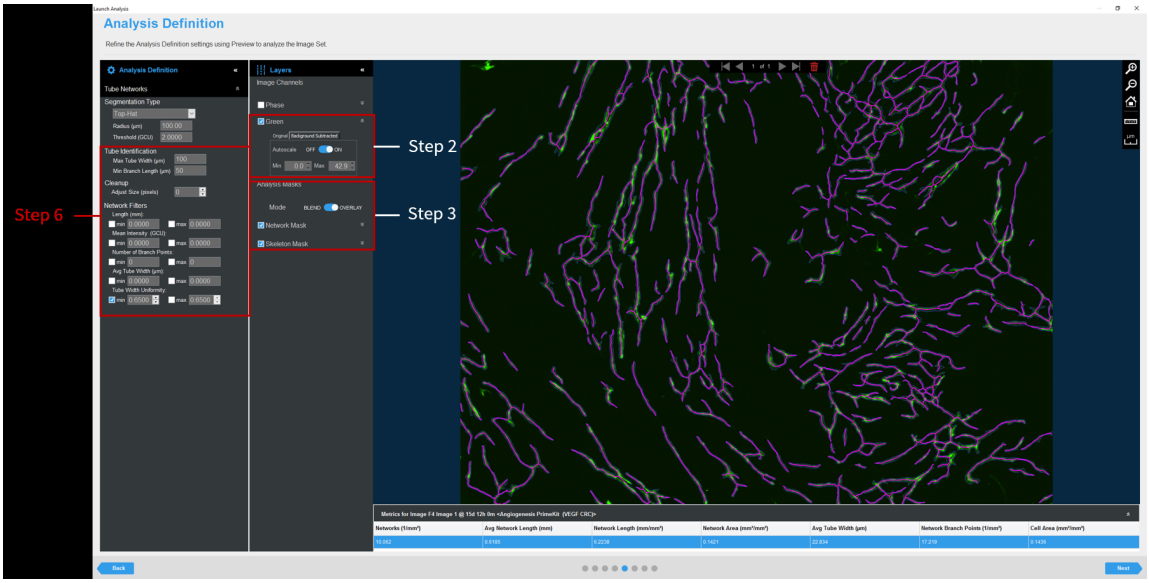
If using Top-Hat segmentation, once the image is previewed, a background subtracted image is formed and displayed in a new tab under the available color channels. Use the "Original" and "Background Subtracted" tabs to compare between the two images. Only the "Background Subtracted" image will be used for segmentation. See Figure 2

3. Evaluate the fluorescent mask network segmenation. See Figure 2
    - Ensure that the Network Mask and/or Skeleton Mask boxes 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).

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

Figure 2. Parameter Refinement of Network Mask



5. Click Preview Current or All.

6. Evaluate your fluorescent network mask and refine the parameters accordingly to help the algorithm distinguish tubes from other detected cells. See Figure 4 and Table 2.

Table 2: Angiogenesis Fluorescent Analysis Parameters

Option	Description
Tube Identification	
Max Tube Width (µm)	Helps to distinguish tubes from other detected cells. Recommended starting value is 100 µm for the PrimeKit and 50µm for the StemKit.
Min Branch Length (µm)	Helps to distinguish tubes from other detected cells. Recommended starting value is 50 µm.
Cleanup	
Adjust Size:	Adjusts the size of your mask in pixels by either shrinking the mask (if negative) or growing the mask (if positive).
Filters	
Length	Defines the limits of total tube length of a network and eliminates networks that lie outside this range. Recommended minimum of 0.2 mm.
Mean Intensity (CU)	Defines the limits of mean intensity of the tube, (the average pixel intensity in calibrated units), and eliminates tubes that lie outside this range.
Number of Branch Points	Defines the limits of the number of branch points in a network and eliminates networks that lie outside this range.
Avg Tube Width (µm)	Defines the limits of the average tube width in a network and eliminates networks that lie outside this range.
Tube Width Uniformity	Defines the limits of the tube width uniformity based on a scale from 0-1 where 0 is completely non-uniform and 1 is of perfect uniformity. Eliminates networks that lie outside this range. Recommended minimum is 0.65.

7. 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 Angiogenesis Analysis, the following set of metrics are provided:

Fluorescent Metric	Description
Network Branch Points (1/mm^2)	Sum of the branch point count off all of the networks in the image divided by the image area (mm^2).
Cell Area (mm^2/mm^2)	Sum of the area of all the cells in the image divided by the image area (mm^2).
Average Tube Width Uniformity	Average of the tube width uniformity of all of the networks in the image. Tube Width Uniformity is a measure of how parallel the edges of the tube are to the skeleton mask that is generated after analysis.
Networks (1/ mm^2)	The number of total networks within an image divided by the image area (mm^2).
Network Area (mm^2/ mm^2)	Sum of the areas of all of the networks in the image divided by the image area (mm^2).
Network Length (mm/ mm^2)	Sum of the lengths of all of the networks in the image divided by the image area (mm^2).
Average Network Length (mm/ mm^2)	Average of the length of all the networks in the image divided by the image area (mm^2).
Average Tube Width (µm)	Average of the tube width of all the networks in the image.