IncuCyte ZOOM™ Angiogenesis Processing Overview

The IncuCyte ZOOM's Angiogenesis Processing Module works in conjunction with the StemKit and PrimeKit to produce a kinetic analysis of angiogenesis networks over the entire course of their formation. By examining fluorescent images at user-defined intervals, the angiogenesis analysis tools identify tube-like structures within the images and automatically computes biologically relevant network properties. Optimizing the algorithm for your PrimeKit or StemKit requires 3 simple steps as described below: 1) creating an **Image Collection**, 2) creating a **Processing Definition**, and 3) **Launching an Analysis Job**. As illustrated in Figure 1, the data processing flow is separated into two distinct phases, the **Assay Development Phase**, and the **Established Assay Phase**.
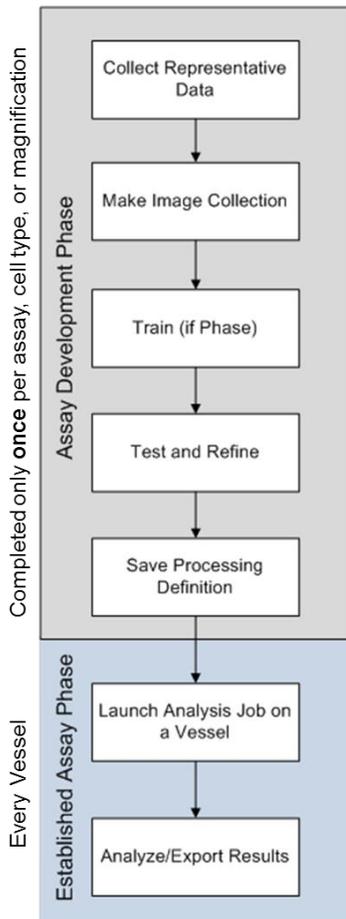

Assay Development Phase: The user defines image analysis parameters that will be used to analyze all images within an established assay. Once completed, the parameters will be applied to all experiments/vessels that use the same experimental conditions (Assay/Magnification).

Established Assay Phase: Once the parameters have been defined and saved in the Assay Development Phase, users can apply those parameters to future experiments with the click of a button.

Assay Development Phase

The first step in the Assay Development Phase is to collect assay data. Next, a small collection of representative images is selected from that data set to be part of an **Image Collection**. This image collection is then used to define and test a **Processing Definition**. It is within this Processing Definition that users set the parameters that will be applied to all images within current and future data sets. One Processing Definition will likely be needed for each angiogenesis co-culture (e.g. PrimeKit and StemKit) in use and/or for each objective being used for data collection. Once the Processing Definition is established, it should not need to be modified again. The tested, finalized and saved Processing Definition is then used in the **Established Assay Phase**.

Established Assay Phase

When the images of an experiment have already been collected, the user can apply the Processing Definition to that experiment by Launching an **Analysis Job**. Alternatively, a user can (and should) apply a Processing Definition to an experiment at the time the experiment is scheduled in the IncuCyte ZOOM Scheduler. In doing so, the IncuCyte ZOOM software will analyze images in real-time as they are acquired. As a result, users can look at, analyze, export, and evaluate their fluorescent angiogenic network data in real-time.

Figure 1: Flow chart illustrating the IncuCyte™ ZOOM assay development strategy.

Image Collection: An Image Collection is a group of images that can be used to train, test, or refine a Processing Definition. Image collections will typically contain 3-6 images that best represent the phenotypes under investigation. Too many images in an Image Collection should be avoided as it can prolong the development of a Processing Definition.

Processing Definition: Processing Definitions are what define the parameters used to analyze all images within an experiment. Each new Assay, Cell Type, and/or magnification will likely require a new Processing Definition, but once established, will not have to be created again. Once finalized and saved, Processing Definitions can be applied to vessels/experiments at the time of scheduling such that images are analyzed in real-time.

Analysis Job: An Analysis Job is launched to analyze images and produce metrics based on the parameters contained within the saved Processing Definition. The scope of the job is specified by the user when the job is launched. This scope includes controls for a single time point, over a defined time frame, or open ended if the Vessel/Experiment is still being actively scanned.

Angiogenesis: Metrics

The IncuCyte ZOOM provides the following metrics relevant to angiogenesis analysis.

- Network Branch Points
- Cell Area
- Average Tube Width Uniformity
- Network (number)
- Network Area
- Network Length
- Average Network Length
- Average Tube Width

To accurately quantify these metrics, a user creates a Processing Definition well-suited to the assay conditions. The following section will guide you through the exact steps you need to complete the Assay Development Phase for your specific assay. Screenshots are included for further clarity, and definitions of all Angiogenesis terms can be found in blue boxes.

Angiogenesis Processing Metrics Defined

Network Branch Points (1/mm²): Sum of the branch point count off all of the networks in the image divided by the image area (mm²).

Cell Area (mm²/mm²): Sum of the area of all the cells in the image divided by the image area (mm²).

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 processing.

Networks (1/ mm²): The number of total networks within an image divided by the image area (mm²).

Network Area (mm²/ mm²): Sum of the areas of all of the networks in the image divided by the image area (mm²).

Network Length (mm/ mm²): Sum of the lengths of all of the networks in the image divided by the image area (mm²).

Average Network Length (mm/ mm²): Average of the length of all the networks in the image divided by the image area (mm²).

Average Tube Width (μm): Average of the tube width of all the networks in the image.

Angiogenesis: Assay Development Phase

Step 1. Creating an Image Collection

1. Collect Fluorescent and HD-Phase Contrast images of your cells every 3-6 hours over the course of the experiment.

2. Open the Vessel View and select 3-6 representative images of your co-culture. Save them in an Image Collection by clicking on the "Create or Add to Image Collection" link **(Screenshot 1)**.

3. Select "Angiogenesis" from the Analysis Job Type drop-down menu.

4. Choose "New" and give a unique name to the collection.

5. Since Network Formation is of primary interest, only the fluorescent image channel (green) is required to be part of the Image Collection.
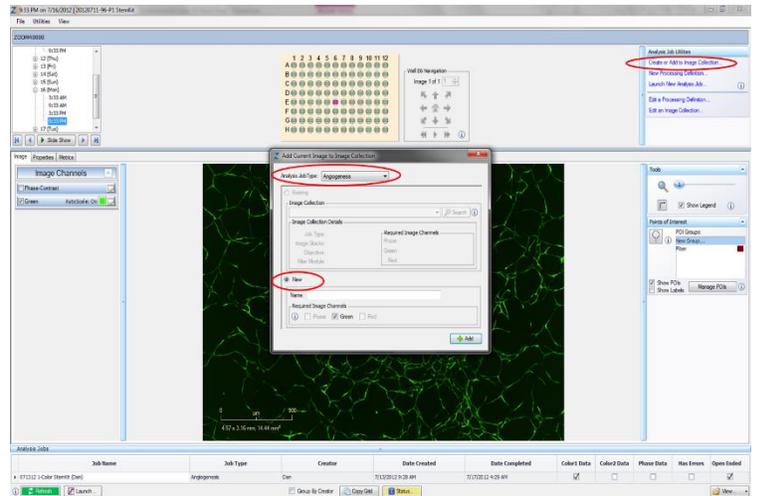
Screenshot 1: Adding Images to Image Collections

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6. Continue to add images to the image collection by clicking on the "Create or Add to Image Collection" link **(Screenshot 1)**. The image collection named in Step 4 will now be listed as an "Existing" image collection. The image collection should consist of images taken at assay initiation, mid-point, and assay completion. The image collection should be limited to 3-6 representative images.

   Note: An Image Collection can ONLY contain images taken at the same magnification. An Image Collection may contain images from multiple tissue culture vessels. The collection should not contain too many images as it will prolong the development of the Processing Definition.
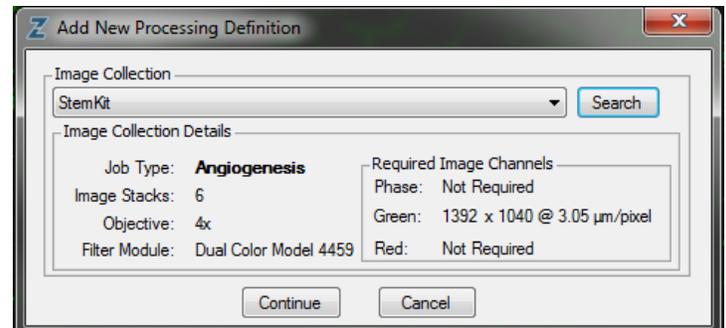
Step 2. Creating a Processing Definition

1. Generate a New Processing Definition by clicking on the "New Processing Definition" link within the Vessel View (found under "Analysis Job Utilities" in **Screenshot 1**).

2. Select the proper Image Collection and click "Continue" **(Screenshot 2)**.

3. The Angiogenesis Processing Definition Editor will open displaying the "Preview Image Collection" drop down menu **(Screenshot 3)**. This will default to the image collection selected in the previous step **(Screenshot 2)**, but it is advised to check before changing parameters. **Note:** You may preview a processing definition on multiple image collections, if desired.

4. Preview the segmentation of your image by clicking the "Preview Current" button.

   Note: The best way to begin setting up the Processing Definition is to use the preset values already contained within the Processing Definition editor, therefore do not change parameters, cleanup, or network filters at this time.

5. Make sure the "Green" image channel box and the "Network Mask" and/or "Skeleton Mask" boxes are checked and evaluate your network segmentation **(Screenshot 4)**.

6. Use the "Blend Mode", "Weight", "Outlines", and "Color" selection options to aid in assessing the network mask. Changing these will not affect the processing definition. To clearly determine the network mask, you can zoom in on the network using the tool slider followed by the weight slider located under the mask section (red circles in **(Screenshot 4)**.

7. Adjust the threshold parameter if necessary. Choose one of the three algorithms available for generating a network mask: the Adaptive Threshold algorithm, the Fixed Threshold algorithm or Top-Hat background subtraction. The Adaptive Threshold algorithm accurately segments images with non-uniform backgrounds. The user simply defines how far above the Local Background Level the image is thresholded. The Fixed Threshold algorithm thresholds all pixels in the image above the user defined threshold. Local background is not considered. The Top-Hat background

Screenshot 2: Starting a New Processing Definition
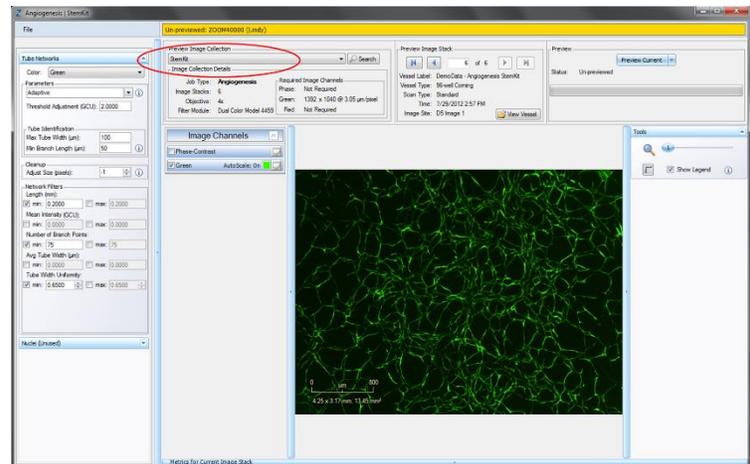
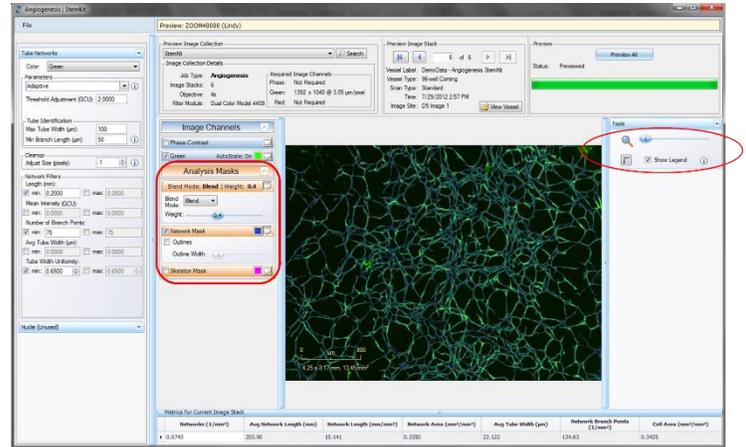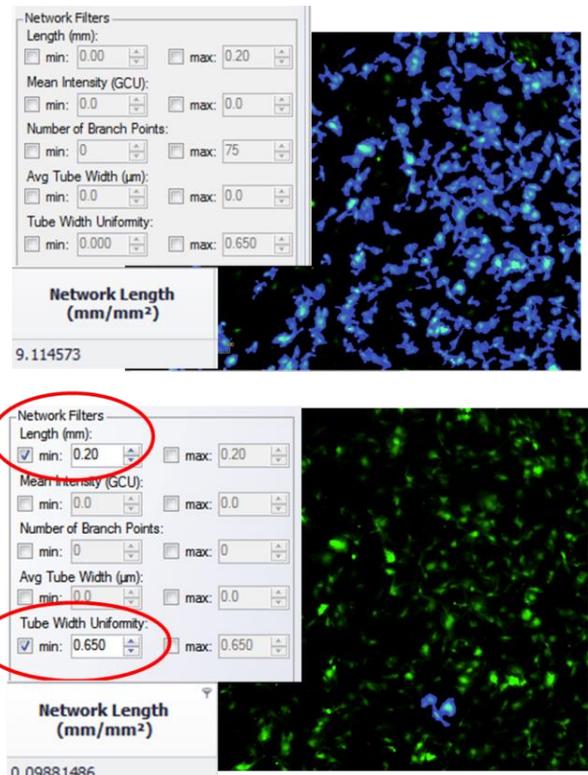**Screenshot 3: Angiogenesis Processing Definition Editor**

subtraction evaluates the background trend and then and subtracts the background from the image. See Fluorescent Processing and Background Fluorescence Technical Notes for more information on these algorithms.

8. If using the Adaptive or Threshold algorithms, adjust the threshold higher to exclude masking background or lower to cover more tubes. When using the Top-Hat background subtraction, use the measuring tool to find the radius of the largest object of interest and add 30% to this value. Input this value as the "Radius" and adjust the threshold based on object intensity.

9. "Preview Current" to preview the current image displayed or use the drop down menu to select "Preview All" to apply the Processing Definition to all Images within the image stack. Depending on the number of images within the image stack, it may be more efficient to preview the current image and make adjustments to the mask prior to applying to all images within the image stack.

10. If necessary, modify the tube identification parameters, which are used to help the algorithm distinguish tubes from other detected cells. The recommended starting values for these parameters are: "Max Tube Width": 100 µm; and "Min Branch Length": 50 µm.

11. To refine the mask, use the Cleanup option "Adjust Size" to shrink the mask (if negative) or grow the mask (if positive).

12. Further refine the angiogenesis mask by setting network filters **(Screenshot 5)**. The important filters to set are "Length" and "Tube Width Uniformity". Our recommended filter settings are: Network Length: 0.2 mm; and Width Uniformity: 0.65.
Note: These filter settings are important to remove groups of cells early on in an angiogenesis experiment that may contribute to overall tube length, but do not constitute mature tube formation. In Screenshot 5, network length was 9.115 mm/mm2 (blue mask) prior to refining the network mask. To exclude this immature tube formation, Length, Branch Points and Tube Width Uniformity filters were used, eliminating small, insignificant networks. The resulting network length is 0.099 mm/mm2.

13. Once you have previewed the complete Image Collection and are satisfied with the parameters, save the Processing Definition (File > Save) and give it a unique name.

14. If you need to modify your processing definition for a new assay (e.g. new cell type or reagent), click on "Edit a Processing Definition" link in the Vessel View. Adjust the necessary parameters, filters, and cleanup, then "Preview" the changes. After you are satisfied with your changes, choose File > Save to replace the original definition, or File > Save As to save a new processing definition and keep the original.

Screenshot 4: Previewing Segmentation

Screenshot 5: Applying Network Filters

Angiogenesis Processing Parameters, Tube Identification, Cleanup, and Filters

Processing Parameters

Adaptive Threshold: A local background level (LBL) of fluorescent intensity is automatically determined across each processed image.

Threshold Adjustment: A threshold is set this far above the LBL in calibrated fluorescence units. Any object that is higher than the background by this value will be included in the mask.

Fixed Threshold: A single threshold level in calibrated fluorescence units is used across the image, so all objects below this level will be excluded.

Top Hat: A background trend across the image is estimated and then subtracted. This method works best for low-density objects.

Radius: A distance from the center of an object to the background pixels plus about 30% of its length. In case of non-circular objects, the smaller distance to the background plus about 30% is measured

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 (mm): 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. Not used.

Number of Branch Points: Defines the limits of the number of branch points in a network and eliminates networks that lie outside this range. Not used.

Avg Tube Width (µm): Defines the limits of the average tube width in a network and eliminates networks that lie outside this range. Not used.

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.

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Angiogenesis: Established Assay Phase

### Option 1. Launch an Analysis Job for an existing vessel

1. Open the Vessel View for the vessel you wish to analyze.
2. Click "Launch New Analysis Job" under "Analysis Job Utilities" (Green circle, Screenshot 6).
3. Select "Angiogenesis" for Job Type
4. Choose the Processing Definition you wish to use from the drop down menu (Red circle, Screenshot 6).
5. Assign a unique name to the Job.
6. You may select a "Time Range", "Single Time", or "Open Ended" to analyze.
7. Select the wells you wish to analyze and click "OK".
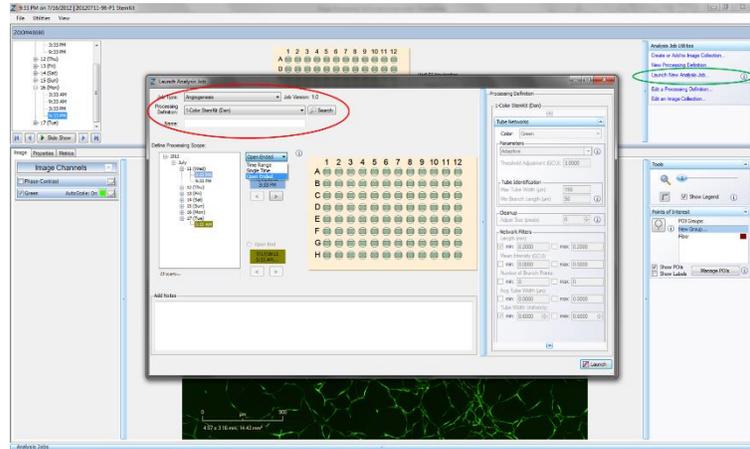
Screenshot 6: Launching an Analysis Job for an existing vessel

Option 2. *(Recommended)* Launch an Analysis Job at time of Vessel Scheduling

1. Add a New Vessel and select the appropriate channels required for imaging.
2. Under Analysis Job Setup, select "Angiogenesis" for Job Type.
3. Under the same heading, select your Processing Definition **(Screenshot 7)**.
4. Name your vessel, add notes, determine the frequency of scans, and click "Apply".
5. Data will be processed following each scan to provide Angiogenesis Metrics in real-time
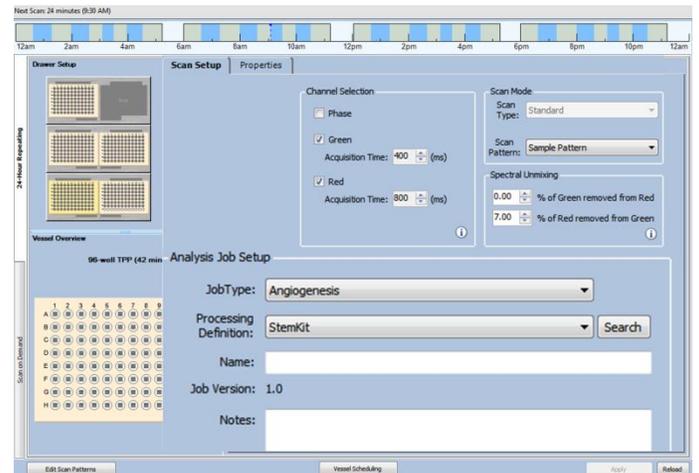
Screenshot 7: Launching an Analysis Job at time of Vessel Scheduling