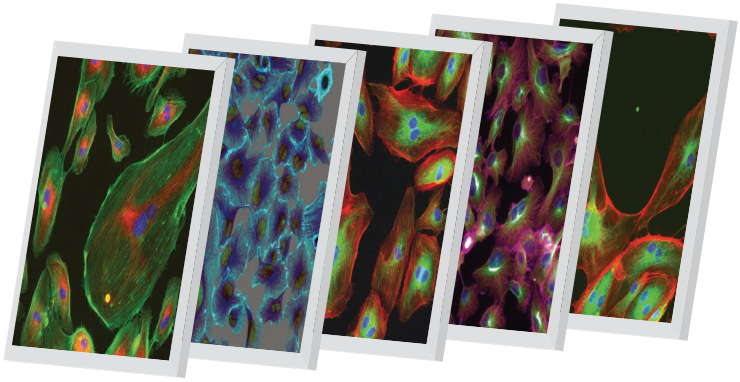
Thermo Scientific Cellomics® Tube Formation V4

BioApplication Guide



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Cellomics® Tube Formation BioApplication Guide

V4 Version

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100 Technology Drive
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Chapter

1

Overview of the Tube Formation BioApplication

High Content Screening (HCS) uses fluorescence-based reagents, an advanced optical imaging system, and sophisticated image analysis software (BioApplications) to quantitatively analyze targets and physiological processes in cells. This guide provides a description for using one such specific BioApplication, the Tube Formation BioApplication, which can be applied to quantitatively analyze fluorescence microscopic images of multi-cellular assemblages with a tube-like morphology such as angiogenic tubes. This guide contains the following chapters:

Chapter 1 provides an overview of the Tube Formation BioApplication and what it measures.

Chapter 2 describes the quantitative algorithm used to analyze results, along with descriptions of input parameters and output features.

Chapter 3 describes the use of the BioApplication to quantitatively analyze a specific biological example: angiogenic tube formation & inhibition by HUVEC cells, and includes guidelines on adjusting settings for this biological use case.

Chapter 4 describes the Protocol optimization tasks that are available in the iDev™ Assay Development workflow.

Appendix A gives the materials and methods for setting up the tube formation assay.

System Compatibility

The Tube Formation BioApplication described in this document is designed to run on the following versions of Cellomics® HCS Readers and software platforms:

- ArrayScan® HCS Reader version VTI
- Cellomics vHCS™ Discovery Toolbox versions 1.5 and 1.6

Selected images from other sources (e.g., images up to 15-bits) may be imported and used with your platform.

Cell Biology Background

Angiogenesis, the formation of new blood vessels, underlies many normal and pathological processes. Discovering drugs that affect the biological processes of angiogenesis offers a novel and promising opportunity in treating a variety of diseases including cancer, rheumatoid arthritis, age-related macular degeneration, heart disease, and diabetes. Investigating whether a compound has any angiogenic ability can also be a part of a drug candidate's *in vitro* toxicity evaluation. The availability of reliable, automated and quantitative functional assays is critical

for the discovery of potent therapeutic drugs. Traditional *in vitro* cell-based assays for screening compounds that affect angiogenic tube formation are time consuming and difficult to quantify. A quantitative, automated High Content Screening assay for angiogenic tube formation will assist this effort. Thus, the Tube Formation BioApplication was developed to provide quantitative measurements related to angiogenic tube formation, and was designed to be used in HCS assays to do rapid, automated, quantitative screening of compounds that inhibit or enhance angiogenic tube formation. To be effectively used in angiogenic tube formation assays, this BioApplication was designed to work with standard cell biological models of angiogenic tube formation. The BioApplication was designed to measure both intensity and morphological properties of angiogenic tubes, and reports quantitative properties related to numerous aspects of tubes, including:

(1) The number of both connected and unconnected tubes

(2) Tube morphology

(3) Branch point properties

(4) Angiogenic Index and Angiogenic status of wells

(5) Number of cells per tube

(6) The presence or expression of specific proteins or targets in the tubes

This BioApplication was designed to be flexible so that it can be optimized to quantify different tube-like structures. It was designed for scientists who want a specific application that they can apply to angiogenic tube formation assays with the flexibility in optimizing and making the measurements relevant for their particular application.

BioApplication Overview & Measurements

Different Channels Have Specialized Outputs

The Tube Formation BioApplication is a multi-channel application where up to six channels can be used. The different channels can represent different fluorophores or exposure conditions. Each channel of the BioApplication is specialized and reports different types of output features.

- **Channel 1:** Channel 1 is the most important channel, and the channel in which tubes are identified and measured. Tubes are identified in this channel by a fluorescent label for the tubes, and thus a whole cell fluorescent label is required to label the entire tube. The analyzed tube is a special type of multi-cellular assemblage containing multiple endothelial cells, which have differentiated and joined up with each other to form a complex tubular network. Dissociation of the tube may also cause detection of objects which are either individual cells or smaller cell clusters; these are typically defined as rejected objects depending on the thresholds set by the user. The BioApplication is able to distinguish between "connected" intact, branched tubes and "unconnected" tubes which could be the disassociated cells, small cell clusters, tube fragments, or unbranched tubes. The criterion for the BioApplication to distinguish between connected and unconnected tubes is the tube's length, and this is specified by the user. Among the properties of the tubes (connected and unconnected) that are measured and reported at the cell level are the tube length, area, and number of nodes (i.e., branch points). In addition to these, at the well level, the angiogenic index and angiogenic status (measures of tubular density within the field) are reported.

- **Channel 2:** In Channel 2, an intracellular target (such as the nucleus) is fluorescently labeled, where typically one copy of the target exists per cell. The tube detected in Channel 1 contains many cells, and the number of Channel 2 targets per tube detected in Channel 1 will be reported – this provides an estimate of the number of cells per tube. Because parts of the tube may be beyond the focal plane of the image (such as parts of tube branch points), some of the nuclei in the tube may not be identified or resolved. Thus, the target count reported is an estimate based only on the identified and resolved targets (i.e., nuclei). Also, since a tube will most likely extend beyond the imaged field, the reported nuclear count is only for the portion of the tube in the image.
- **Channel 3 and Channel 4:** Additional intracellular targets or proteins that are fluorescently labeled are detected in Channel 3 and Channel 4.
- **Intensity Related Measurements:** The tube label's intensity (total and average) and its standard deviation are also measured in the tube for Channel 1. Additionally, the intensity (total and average) and texture (i.e., intensity standard deviation) in a modifiable mask derived from the Channel 1 tube are measured for the fluorescently labeled targets in Channels 2-4.
- **Channel 5 and Channel 6:** Channel 5 and Channel 6 are used for intensity based gating only.

Thus, one can choose up to four targets to make morphology and intensity-based tube-related measurements, and this application can be run as a one-, two-, three-, or four-channel assay where different properties are measured in the different channels. If it is run as a one-channel assay, only whole-tube related morphology measurements are measured. Channels 2-4 can be used to simultaneously make additional measurements within the defined tubular structure. The specialization of the channels imposes requirements on the assay design, the types of properties measured, and the fluorescent probes to label them in each of the different channels. The schematic in Figure 1 illustrates the assay design and the use of the different channels – note that only two connected tubes are shown. Table 1 summarizes the different categories of output features reported by the BioApplication for the different channels.

Additionally, for live cell analysis, the Cellomics ArrayScan V^{TI} HCS Reader and the Cellomics Discovery Toolbox (v1.6) offer a Live Module that can track cells over time. Please see the appropriate user's guides for more information.

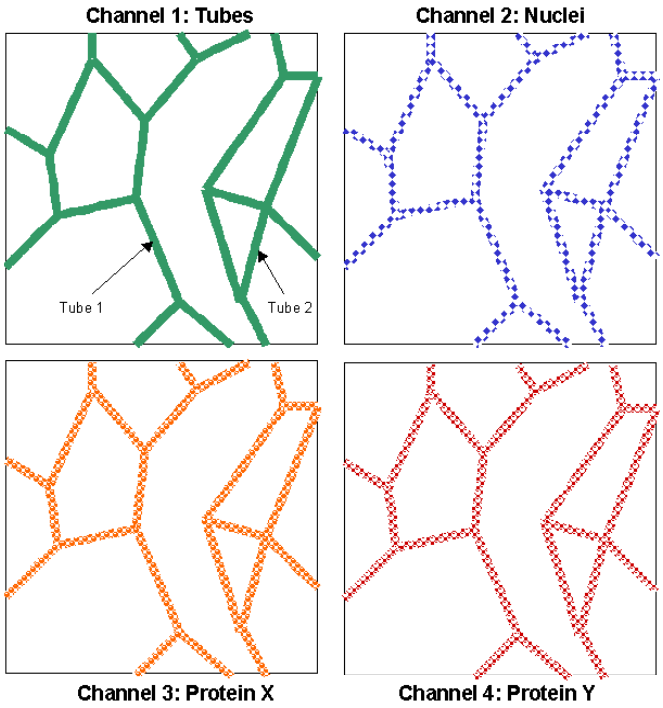

Figure 1. *Tube Formation BioApplication design.*

OUTPUT FEATURE CATEGORIES:			
Channel 1	***Channel 2***	***Channels 3 and 4***	***Channels 5 and 6***
TUBE MORPHOLOGY	INDIVIDUAL CELL IDENTIFIER (E.G., NUCLEUS)	INTRA-TUBE IDENTIFIERS OR LABELS FOR SPECIFIC PROTEINS	GATING
• Tube counts & density • Tube location • Tube connected or unconnected • Tube area • Tube length & width • Tube branching • Angiogenic Index	• Estimated Target Count per tube • Channel 2 label intensity statistics (total, average and standard deviation) inside object or modified Channel 1 tube mask	• Channel 3/4 label intensity statistics (total, average and standard deviation) inside Channel 2 targeted object or modified Channel 1 tube mask	• Average & total intensity within modified Channel 1 tube mask

Table 1*. Categories of Reported Output Features for the Tube Formation BioApplication.*

Example Biology: Evaluating Angiogenic Tube Formation

Angiogenesis plays a key role in tumor growth and spreading. Therefore, anti-angiogenic compounds are promising tools in cancer therapy, and great efforts have been devoted to their identification in recent years (Claesson-Welsh, 1999; Cozzi et al., 2004; Dvorak, 2002; Liekens et al., 2001). Angiogenesis occurs by the differentiation of endothelial cells to form nascent capillary-like structures known as tubes (Auerbach et al., 2003; Bishop et al., 1999;

Lubarsky and Krasnow, 2003; Montesano et al., 1983). This process can also be reproduced *in vitro*, where it occurs within a few hours when endothelial cells are cultured on a layer of reconstituted basement membrane known as Matrigel™. In this system, endothelial cells attach rapidly, cease proliferating, align, and form capillary-like tubular structures (Kubota et al., 1988). Thus, evaluation of the inhibition of endothelial cell differentiation into tubes on Matrigel is a useful and convenient assay approach for testing pro and anti-angiogenic compounds.

The example biology accompanying this BioApplication is to evaluate the effect of an anti-angiogenic compound on inhibiting the formation of tubes by HUVEC cells, a widely used human endothelial cell line, on Matrigel in a standard 96-well clear-bottomed microwell plate. The anti-angiogenic compound used, suramin, is a well-characterized anti-angiogenic agent (Lopez et al., 1992; Takano et al., 1994, Bocci et al., 1999; Firsching et al., 1995; Gagliardi et al., 1998; Meyers et al., 2000). For the experiments, HUVEC cells were plated in a standard 96-well format on Matrigel basement membrane and treated with the known pro-angiogenic compounds, Vascular Endothelial Growth Factor (VEGF) and basic Fibroblastic Growth Factor (FGF) (see Chapter 3 for experimental details). VEGF is a multifunctional growth factor that is synthesized and secreted by the great majority of animal and human tumors. It increases microvascular permeability, induces endothelial cell migration and division, re-programs gene expression, promotes endothelial cell survival, prevents senescence, and induces angiogenesis. FGF is a potent fibrotic agent which seems to be an essential cofactor in angiogenesis; in itself it is insufficient to cause angiogenesis, but works together with VEGF to promote angiogenic tube formation. Tube formation was prevented by the use of suramin.

After fixation, the samples were labeled with components from the Cellomics Cell Spreading HCS Reagent Kit. This assay used two fluorescent labels: the tubes were stained directly with rhodamine conjugated phalloidin (red fluorescence) and the nuclei were labeled with Hoechst 33342 (blue fluorescence), both components of the Cellomics Cell Spreading Reagent Kit. The rhodamine phalloidin is a whole cell stain that labels F-actin, enabling the entire tube to be identified. The nuclei of all cells have Hoechst labeling, enabling an estimate of the number of nuclei per tube.

Figure 2 shows images acquired using a 5X objective on an ArrayScan HCS Reader 4.5 of angiogenic tubes formed by HUVEC cells on Matrigel in complete media supplemented with growth factors VEGF and FGF (top 2 rows), and images where suramin treatment has caused inhibition of angiogenesis (bottom 2 rows). Images of both the rhodamine phalloidin labeled multicellular tube and Hoechst-labeled nuclei are shown. The difference between differentiated, multinucleated, connected, angiogenic tubes versus undifferentiated, unconnected tubes treated with suramin is clear in these images. The well-connected tubes identified by the BioApplication are outlined in blue. The BioApplication can also identify tubes that are "unconnected" in that they are shorter than a minimum length criterion, and these are outlined in aquamarine in the suramin treated case. Nodes, where a tube branches into different segments, are also identified and displayed in the images by a pink spot in the rhodamine phalloidin images. The nuclei that are identified and counted are overlaid by a red dot in the Hoechst images. Objects that are rejected from analysis by not fitting the criteria to be a tube are shown by an orange overlay.

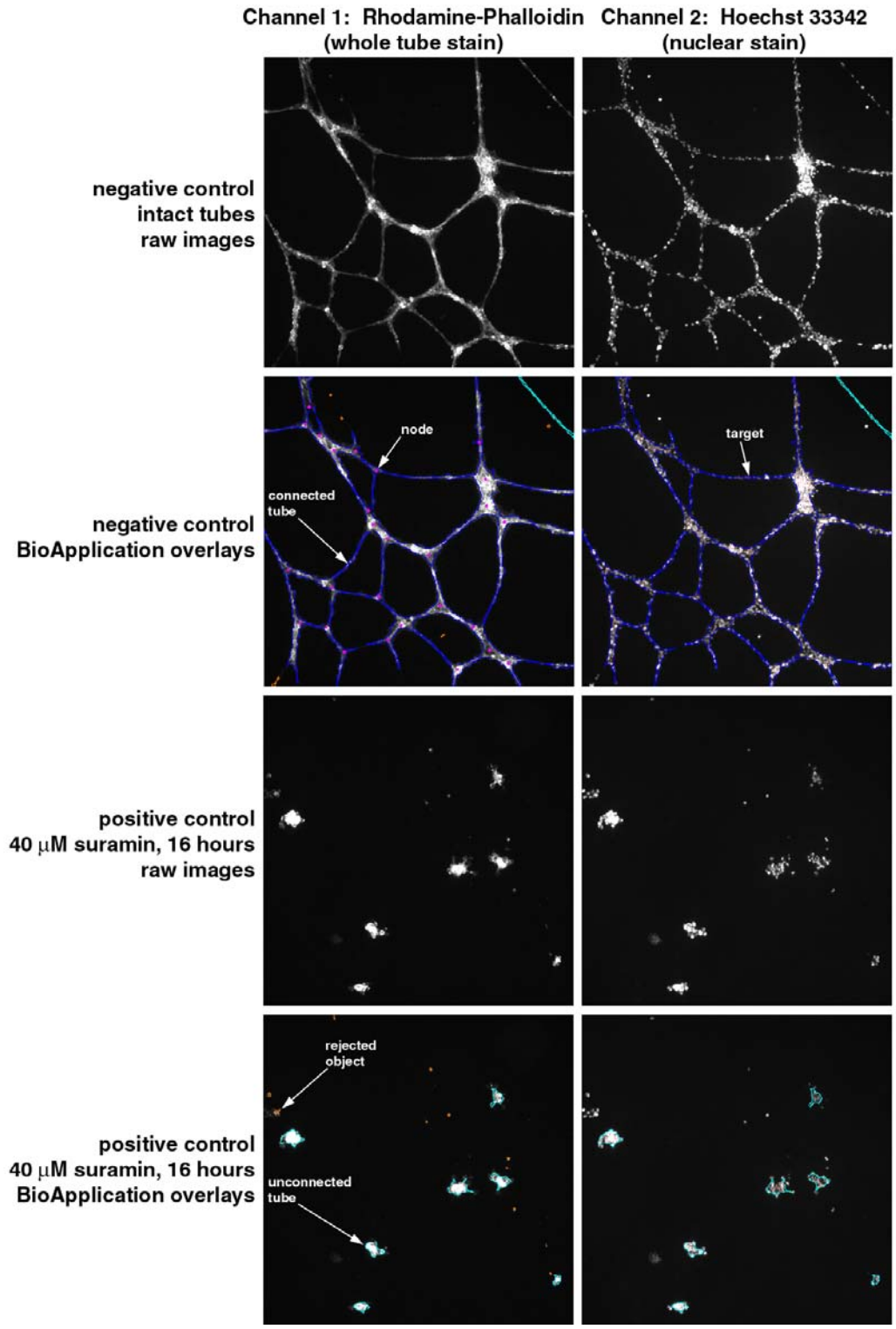

Figure 2. *Angiogenic Tube Formation. The 2 top rows show HUVEC cells grown in the presence of pro-angiogenic compounds VEGF and FGF for 16 hours. In the bottom 2 rows, angiogenic tube formation has been inhibited by 40 μM of the anti-angiogenic compound suramin. The images in the left column are Channel 1 Rhodamine-phalloidin labeled tubes, and the images in the right column are the corresponding tubes' nuclei stained with Hoechst 33342 in Channel 2. The overlay images show what the BioApplication identifies and analyzes (connected tubes outlined in blue, unconnected tubes outlined in aqua, nodes are pink dots, nuclei targets have a red dot, and rejected objects are orange).*

Figure 3 shows results from analyzing a 96-well plate where half the wells were treated with 40 μM suramin for 16 hours (positive control), and half of the plate was left untreated for the same amount of time (negative control). The figure shows the mean values of the positive and negative wells, normalized as a percentage of the negative control, for a range of properties measured by the Tube Formation BioApplication. As a measure of assay robustness, Table 2 lists the Z' values for these same features (Zhang et al., 1999). The Z' values obtained for the different measured properties of the tubes for this sample biology, shown in Table 2, were 0.3 or higher, with several Z' values greater that 0.5; this means this an excellent, robust, assay for screening (Zhang et al., 1999).

Figure 4 represents a suramin dose-response curve, where the IC_{50} for suramin's inhibition of tube formation was measured for different features measured and reported by the Tube Formation BioApplication. The suramin IC_{50}s measured by the different features (summarized in Table 3) are similar, demonstrating the flexibility and robustness of this assay.

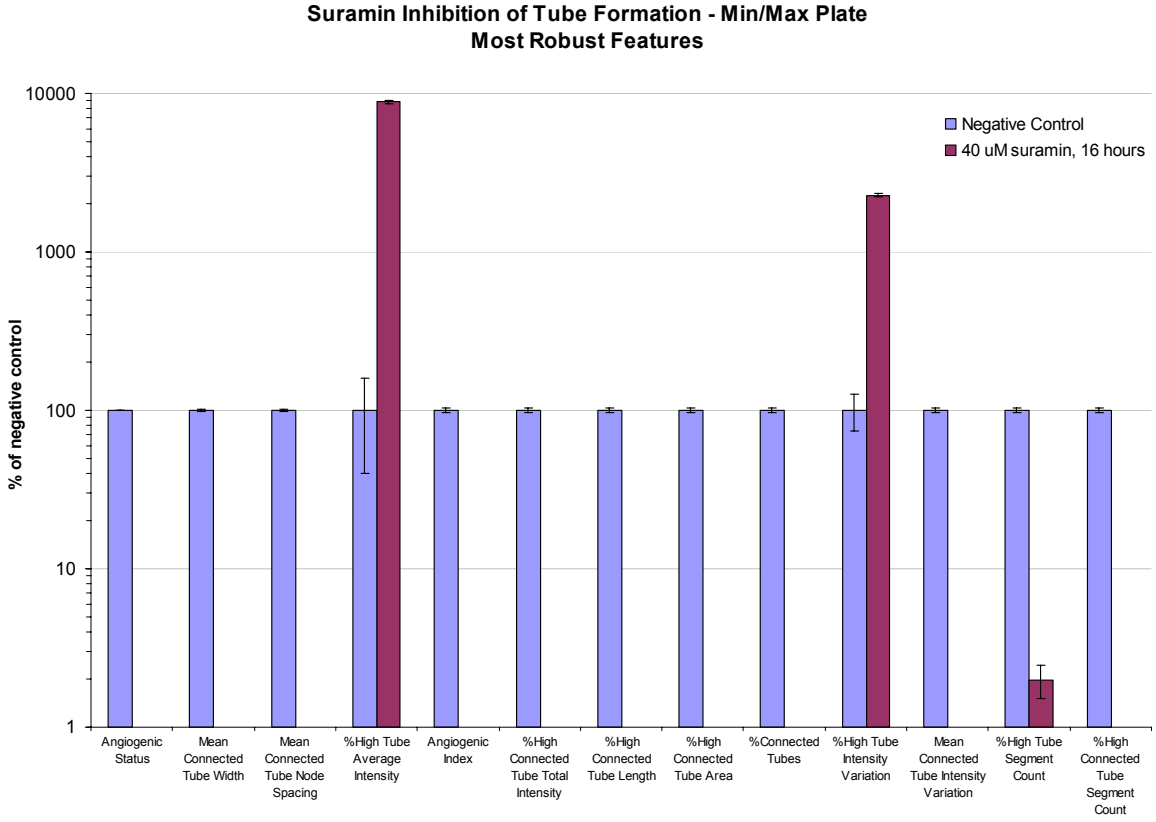

Figure 3. *Different output features from the Tube Formation BioApplication from the analysis of a 96-well min/max plate. Half the wells were treated with 40 μM of the anti-angiogenic compound suramin (positive condition), and the remaining wells contained only the pro-angiogenic compounds VEGF and FGF (negative condition); both treatments were for 16 hours. Output features that gave robust responses between the positive and negative control situations are shown; the data is given as a percentage of the negative control. Each bar is the mean of 48 similar wells from the min/max plate, and the error bars are the standard error of the mean. Table 2 shows the Z' values calculated for these measured features.*

Measured Tube Property	Z' Value
Angiogenic Status of the well (Angiogenic Index > level specified)	1.0
Mean Connected Tube Width	0.8
Mean Connected Tube Node Spacing	0.6
%High Tube Average Intensity (% of all tubes that are responders for Average Intensity criterion)	0.4
Angiogenic Index	0.3
%High Connected Tube Total Intensity (% connected tubes that are responders for Total Intensity criterion)	0.3
%High Connected Tube Length (% of connected tubes that are responders for Tube Length criterion)	0.3
%High Connected Tube Area (% of connected tubes that are responders for Tube Area criterion)	0.3
%Connected Tubes (% of tubes that are connected)	0.3
%High Tube Intensity Variation (% of all tubes that are responders for the internal texture criterion)	0.3
Mean Connected Tube Intensity Variation (mean internal texture of connected tubes)	0.3
%High Tube Segment Count (% of all tubes that are responders for the number of tube segments criterion)	0.3
%High Connected Tube Segment Count (% of connected tubes that are responders for the number of tube segments criterion)	0.3

Table 2. *Z' values for the tube features measured for the suramin min/max plate shown in Figure 3.*

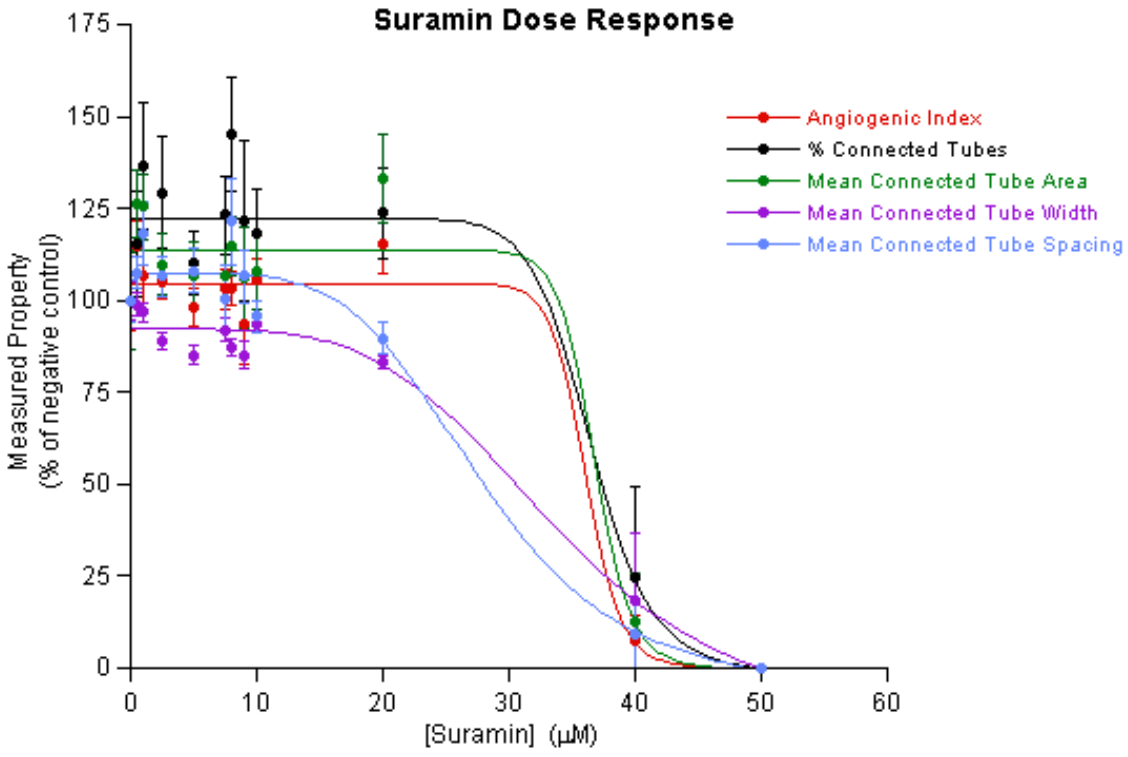

Figure 4. *The IC50 for suramin inhibition of angiogenic tube formation was determined from a 96-well plate containing HUVEC cells on Matrigel treated to eight similar 12-point suramin concentration sequences. For each suramin concentration, the mean and standard error of the mean were calculated over the eight similarly treated wells in the plate. This was done for a variety of different features measured by the Tube Formation BioApplication, and five of them are plotted in this figure. This plot shows that a range of different measured properties can be used to quantitatively demonstrate a compound's anti-angiogenic properties, and all give similar IC_{50} values (Table 3). Although the suramin IC_{50} values for these different properties are similar, the slopes of the curve demonstrate which properties change more gradually than others with increasing doses of the anti-angiogenic compound.*

Measured Tube Property	IC_{50} Value (μM)
Angiogenic Index	36.1
%Connected Tubes (% of tubes that are connected)	36.6
Mean Connected Tube Area	36.8
Mean Connected Tube Width	33.8
Mean Connected Tube Node Spacing	27.9
Mean ± sem:	**34.2 ± 1.7**

Table 3. *Suramin IC_{50} values for these different properties measured are shown in Figure 4. The IC_{50} concentrations for the different measured properties are fairly similar, and the mean (± SEM) of the different IC_{50} concentrations is 34.2 ± 1.7 μM.*

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Chapter
2

Description of the Algorithm

NOTE

The Assay Parameters described in this guide function in the same manner regardless of using the Classic or iDev versions of the ArrayScan software. For descriptions of each task for optimizing a Protocol in the iDev Assay Development Workflow, please refer to Chapter 4 of this guide.

The previous chapter provided an overview of what the Tube Formation BioApplication does and what it measures. This chapter describes in more detail use of the application's input parameters to control its analysis, as well as a summary of the output features.

Overview

Each biological application uses an image analysis algorithm that has been extensively tested and validated for robust screening performance. The algorithm has input parameters that control its analysis. Parameter values determined from validation plates for representative cell types have been supplied as defaults in the standard protocol. Parameters are adjustable to allow customization of the algorithm to your own samples and conditions.

Input parameters can be found in the Create Protocol View window of the ArrayScan Classic software or in the Protocol Optimization task list of the iDev software. The number of available input parameters is dependent on the number of channels selected, and only the input parameters for the selected channels will be displayed. In the ArrayScan Classic software, the available input parameters will also vary depending on the mode in which you are running: Basic Mode or Advanced Mode. The Basic Mode enables you to measure the morphology and related properties of the imaged tubes, and this mode should suffice if this is all you need to measure. The Advanced Mode is recommended if you wish to further characterize the subpopulations of tubes based on the different morphological properties they possess. The Advanced Mode enables you to set criteria that defines responders for various tubular features. Use of both Basic and Advanced Modes are described in this chapter.

There are three types of input parameters: Object Identification Methods, Assay Parameters and Object Selection Parameters. The Object Identification and Selection Parameters control which objects are chosen for quantitative processing. The Assay Parameters control the actual quantitative analysis of the images. This chapter first describes the combined use of these input parameters to control the specific measurements performed in each individual channel. Separate summary tables of the different types of input parameters follow these descriptions.

Object Identification Methods

To identify objects in each of the images from the different channels, an independent intensity threshold must be set for each channel. In Channel 1, tubes are identified; only pixels with intensities above this threshold will be considered as belonging to these structures. Thus the proper setting of an intensity threshold is a key early step in identifying tubes and thus configuring the application. In Channel 2, targets within tubes are identified, and pixels with intensities above this threshold are used to help estimate target counts. Depending on the properties of the objects being identified in Channel 1 and Channel 2, the proper setting of intensity thresholds for the channels is necessary to ensure proper quantitative analysis.

There are four different options or methods for determining intensity thresholds, and sub-sets of these are available for each channel. For each Channel, you must select both a method and a value. The different options and values available for each channel are summarized in the Table 4, and the descriptions of the different methods follow.

Intensity Threshold Method	Value Range	Channel Availability		
		Channel 1	Channel 2	Channels 3 -6
None	0			✓
Isodata	-0.99 – 9.99	✓	✓	
Triang	-0.99 – 9.99	✓	✓	
Fixed	0 – 32767**	✓	✓	

Table 4. *Intensity Threshold Methods Available for Each Channel with the Tube Formation BioApplication.*

NOTE

All Intensity Thresholds are applied to the background-corrected image (when Background Correction is used).

When "**None**" is selected as the Intensity Threshold Method (Channels 3-6), the value must be **0**.

The effective range for object identification is limited to 0-4095 for the following Cellomics HCS Readers: ArrayScan V^{TI} and ArrayScan X.5 HCS Readers. However, the entire range may be available on images from other sources.

Of the four intensity threshold method options, **None** means that no intensity threshold is applied. This option is not available in Channel 1 or 2 because in these channels an intensity threshold is required to define the pixels making up the tube and the targets. The choice of the **None** threshold is the only option in Channels 3-6.

The **Fixed Threshold** method sets an intensity threshold independently of the image data. In this case, you select an intensity level between 0 and 32767, and any pixel above this intensity is retained for the analysis specific to the channel.

The other threshold methods (**Isodata** and **Triang**) are known as histogram-derived thresholds in that the threshold is chosen from the histogram of pixel intensities in the image (i.e., the image's brightness histogram). The schematics in Figure 5 demonstrate how these histogram-derived threshold values are calculated; Table 5 gives a description of the options.

The values entered for the Object Identification in the application for the two histogram-derived threshold methods are offsets applied to determine the final threshold which is applied

to the image. If the histogram-derived threshold is T, then its relationship to the actual (final) threshold, T_F, which is finally applied to the image is determined from the user-entered offset value, o, as:

$$T_F = (1 + o)T$$

For example, suppose for a particular image, an **Isodata Threshold**, T, of 1000 is obtained. Then entering an offset value, o, of 0.9 will result in a final threshold of 1900 being applied to the image, whereas entering an offset value of -0.9 will result in a final threshold of 100 being applied. The range of possible values for the offset o is –0.999 to 9.999. However, note that the resulting applied threshold, T_F, will be restricted to the range 1-32767.

The two histogram-derived methods are dependent on the contents of the image, unlike the **Fixed Threshold** method. For example, supposing a blank image that contains no cellular objects and only has background pixels with a mean intensity value of 500 and standard deviation of 50, then it is unlikely that a **Fixed Threshold** of 1000 will cause any pixels to be registered as objects. However, the **Isodata Threshold** method will give thresholds causing pixels in the image to be registered for potential analysis; this is because the histogram is of the pixel intensity distribution of that image, even though there are no cellular objects in the image. The **Triangle Threshold** method is more robust for the situation of blank fields that may contain rare bright objects; this is because the peak is of the background intensity, and the "triangle"-derived offset can be set to always be above the background, yet low enough to pick any bright objects. Thus, in situations where blank images are expected (e.g., from a loss of signal due to a compound condition, a loss of protein expression, or a lack of label), the **Isodata Threshold** method should be avoided; instead either a **Fixed Threshold** or the **Triangle Threshold** method with a large offset can be entered.

Threshold Option	Description	Range of Possible Values Entered	Resulting Applied Threshold Range
None	No threshold applied	0	none
Isodata	Adjusts the object identification threshold relative to the Isodata value. The threshold *T* is chosen so that it is equal to the average of the mean of the pixel intensities to the left of the threshold (m_L) and the mean of the pixel intensities to the right of the threshold (m_R). A negative value identifies dimmer objects and results in larger object masks. A positive value results in smaller object masks.	-0.999 – 9.999 (offset)	1 - 32767
Triang	Adjusts the object identification threshold relative to the Triangle value. The threshold *T* which gives the maximum distance *d* A negative value identifies dimmer objects and results in larger object masks. A positive value results in smaller object masks	-0.999 – 9.999 (offset)	1 - 32767
Fixed	A fixed pixel intensity value between 0-32767 is applied	0 – 32767 (actual intensity in image)	0 - 32767

Table 5. *Intensity Threshold Descriptions used in the Tube Formation BioApplication.*

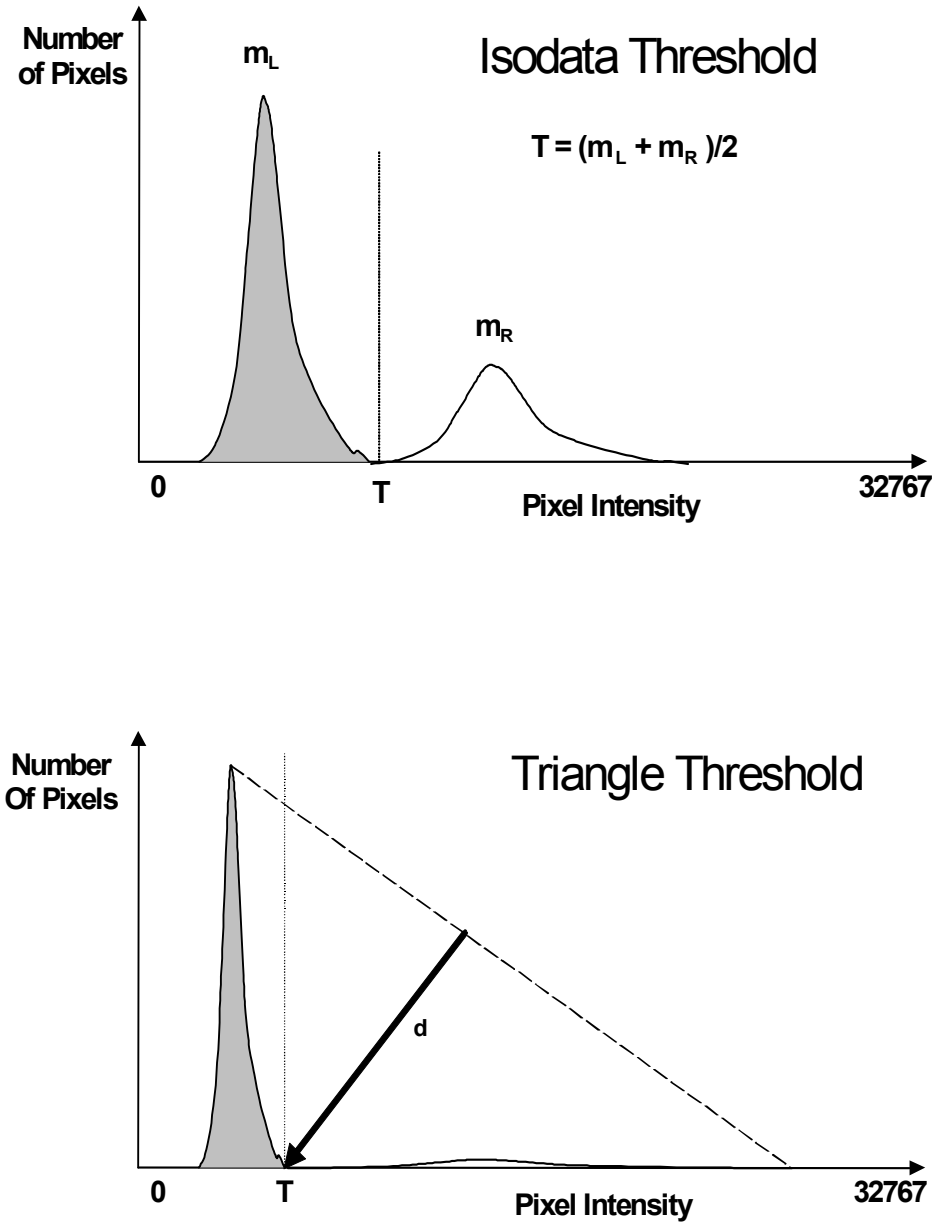

Figure 5. *Histogram-derived Intensity Threshold Methods available for the Tube Formation BioApplication. Top – Isodata; Bottom - Triangle. Background peak is shown in gray and object peak is shown in white.*

Description of Assay Parameters and Settings

The algorithm has Assay Parameters that control the analysis of images and data. The values of these parameters have been determined from demonstration biologies and are supplied as defaults in the standard Assay Protocol. You can adjust these parameters to allow customization of the algorithm to your own biology and conditions.

Assay Parameters For Image Analysis

General Assay Parameters

General Assay Parameters control general aspects of the image processing and analysis:

- **Reference Well Control**
- **Units for Morphological Measurements**
- **Object Type**
- **Background Correction**
- **Smooth Factor**
- **Mask Modification**

Reference Well Control

The two general Assay Parameters controlling the use of Reference Wells are: **UseReferenceWells** and **RefAvgTubeCountPerField**. The **UseReferenceWells** Assay Parameter allows you to choose whether Reference Wells are to be used to determine the population characterization levels. A value of **0** means Reference Wells are not used, whereas a value of **1** engages Reference Wells. If Reference Wells are to be used, then the Assay Parameter **RefAvgTubeCountPerField** must also be set. This is the minimum number of tubes detected per field that are required for acceptance of the data from the Reference Wells. This allows you to enter the minimum number of tubes that you feel gives a good distribution and, thus, statistical validity to the Levels High and Low calculated from the Reference Wells.

Note that in addition to these general Assay Parameters, there are additional Assay Parameters for Reference Well processing that are specific for setting levels for population characterization. They, along with **RefAvgTubeCountPerField**, are available in the Advanced Mode, whereas **UseReferenceWells** is available in the Basic Mode. The population Levels High and Low Assay Parameters available in the Advanced Mode will be described further in later sub-sections.

Units for Morphological Measurements

You have the option of either choosing micrometers or pixels as the unit in which to report morphological measurements. This is done by the **UseMicrometers** Assay Parameter, which when set to **0** causes the morphological measurements to be reported in pixels. If **UseMicrometers** is set to **1**, they are reported in micrometers. The different pixel sizes in micrometers are calculated automatically from the magnification of the objective used, and the camera and camera acquisition mode that is selected.

Object Type Ch*N*

ObjectTypeCh1, ObjectTypeCh2

The "Object Type" Assay Parameters allow you to specify whether the objects of interest are brighter or darker than the background of the image (Table 6). Setting the value to **1** makes dark areas within an image to be considered as potential objects, while leaving it as **0** considers bright areas on a dark background as potential objects.

Setting	Detects
0	Objects that are brighter than the background (most typical)
1	Objects that are dimmer than the background

Table 6. *Binary settings for* ***ObjectTypeCh1*** *and* ***ObjectTypeCh2*** .

Typical fluorescence microscopy images show bright objects on a dark background. The signal that comes from an object is proportional to the amount of fluorescent label in it. Thus, the objects of interest will have intensities that are above the background intensity, and applying the intensity threshold will identify pixels higher than the threshold as object and those lower than the threshold as background.

In some assays dark objects are of interest, such as an unlabelled nucleus within a labeled cell body or cytoplasm. In this case the dimmer areas of the image are considered objects of interest to be identified and measured.

The strategy for object identification/detection is the similar in the two cases; that is, you will always optimize the threshold value so that it separates objects from background based on intensity. If you are detecting dark objects on a bright background, you will need to increase the threshold value to detect more objects or to enlarge them.

Background Correction Ch*N*

Prior to image analysis, the non-cellular background can be computed and subtracted from each image in each channel independently, as shown in Figure 6.

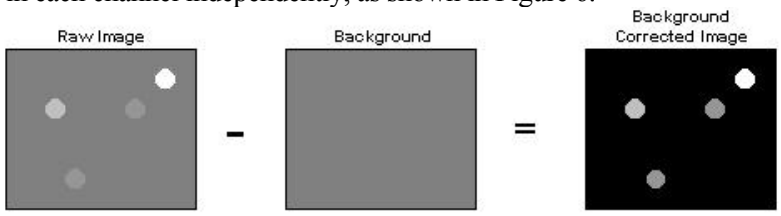

Figure 6. *Background Correction ChN available for each channel*

The background-corrected image is computed by suppressing high frequency components in the image (low pass filtration). You can control the creation of the background image by adjusting the **BackgroundCorrectionCh*N*** Assay Parameter in each channel as needed. This Assay Parameter refers to the radius of the area that is sampled around each pixel in the image to determine its local background. The value of this Assay Parameter should be much larger than the radii of the objects in the image. The larger the absolute value, the larger the sampled region, and the less subtraction is done. If the value is set to **0**, background correction is not performed, and analysis is done using the raw, uncorrected images.

If the **BackgroundCorrectionCh*N*** Assay Parameter is given a negative value an optional background correction method based on the 3D surface fitting is applied. The absolute value entered corresponds to the radius of an area used to find local minima across the image. Found

minimum values are used to construct a 3D surface of a background which is then subtracted from the original image. The main advantage of the method is that it minimizes the effect of the background correction (removal) procedure on the intensity values of the analyzed objects. Setting the value to **–1** lets the application decide on the value needed for the optimal background correction. Table 7 is an overview of these values used with Background Correction

Setting	Background Correction Method
0	No background correction is performed
Positive	Local minimum in the box specified by the value entered is subtracted from that region of the image
Negative	Local minima detected are used to fit a surface representing the background, which is then subtracted
-1	Automated surface fitting is performed and the computed background is subtracted

Table 7. *Background Correction methods for all channels.*

NOTE

In all cases (except where the value = 0) the reported image pixel intensity values will be reduced.

Background Correction can be adjusted on each channel separately.

The background-corrected image is not stored or shown.

Smooth Factor Ch*N*

TubeSmoothFactorCh1, PeakSmoothFactorCh2

Channel 1 and Channel 2 have independent Assay Parameters that control the degree of image smoothing, or blurring, before the identification of tubes (Channel 1) or targets (Channel 2). These Assay Parameters are called **TubeSmoothFactorCh1** and **PeakSmoothFactorCh2** for Channel 1 and Channel 2 respectively. The value represents the degree of image smoothing (blurring) prior to tube detection in Channel. Entering a value of **0** means that smoothing is not applied. These Assay Parameters are used to smooth images with a lot of contrast to improve identification of tubes (Channel 1) or targets (Channel 2). To get sharper definition of the shapes of the edges of tubes, you may want to keep the **TubeSmoothFactorCh1** value small, if not **0**. However, if your Channel 1 label is not very homogeneous, the actual tube could be erroneously identified as consisting of several smaller-sized rejected objects rather than one tube. Smoothing will result in a homogenizing effect of the Channel 1 label and will help identify the actual tube with its true boundaries.

Mask Modifier Ch*N*

The option of independently modifying (i.e., expanding or contracting) the tube area from Channel 1 in which measurements are made for Channels 2-6 is available in the Tube Formation BioApplication. The Assay Parameters that are used to adjust the tube area in which to make measurements for Channels 2-6 are **MaskModifierCh*N***, where Ch*N* is the relevant dependent channel (i.e., Channels 2-6). This Assay Parameter is the number of pixels to modify the Channel 1 object (tube) mask in Channels 2-6: Negative value = Shrink mask, **0** = Do not modify mask, Positive value = Expand mask. Thus, this Assay Parameter is the number of pixels added to, or removed from (depending on the Assay Parameter's sign) the perimeter of the tube area identified in Channel 1 (Figure 7). In the schematic in Figure 7, this value is positive and the tube area has been expanded to make the measurements in Channel *N*. This Assay Parameter is used for intensity gating only in Channels 5-6.

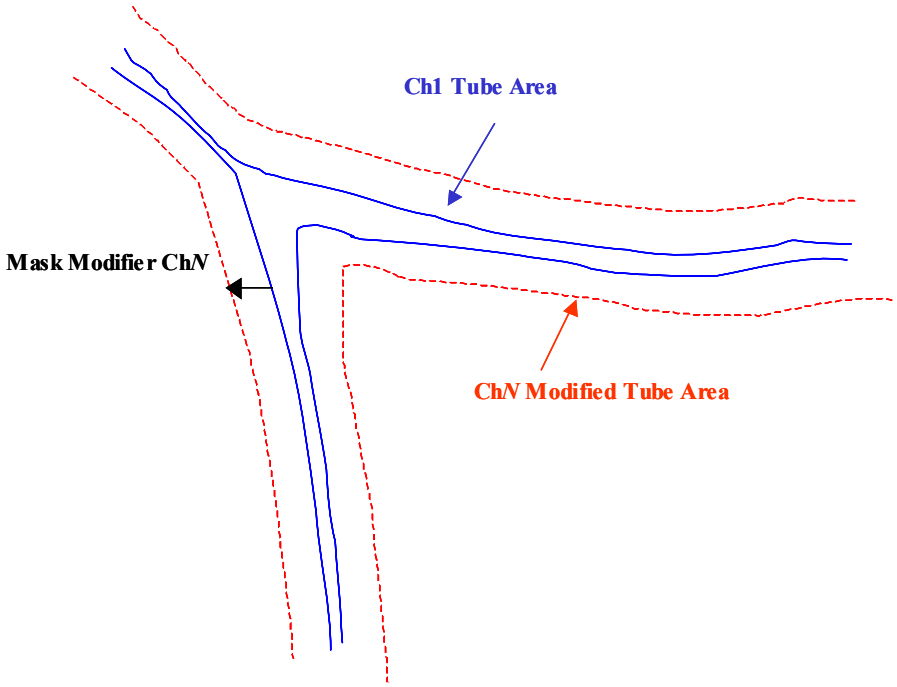

Figure 7. *Adjustment of Measurement Area in Channels 2-6*

Channel Specific Assay Parameters

Connected vs. Unconnected Tubes

The Tube Formation BioApplication distinguishes between two types of tubes: connected versus unconnected tubes. Connected tubes are tubular structures whose length are greater than or equal to a minimum length criterion. Unconnected tubes are defined as objects with lengths less than this criterion. Such objects tend to be disassociated cells, small cell clusters, or short unbranched tubes. The minimum length criterion to distinguish connected tubes from unconnected tubes is set by the Assay Parameter **MinConnectedTubeLengthCh1**. Figure 8 illustrates the differences between connected and unconnected tubes based on setting this Assay Parameter to a value of 300 µm. Sometimes, due to dim staining, parts of a tube may not be visible even though the entire tube appears to be connected. In this case, one can use the Assay Parameter **TubeMergeSizeCh1** to merge tube fragments within specified limits to create a more complete connected tube. Thus, to summarize, there are three types of objects that can be detected and analyzed in Channel 1:

- Connected Tubes
- Unconnected Tubes
- Objects rejected from analysis

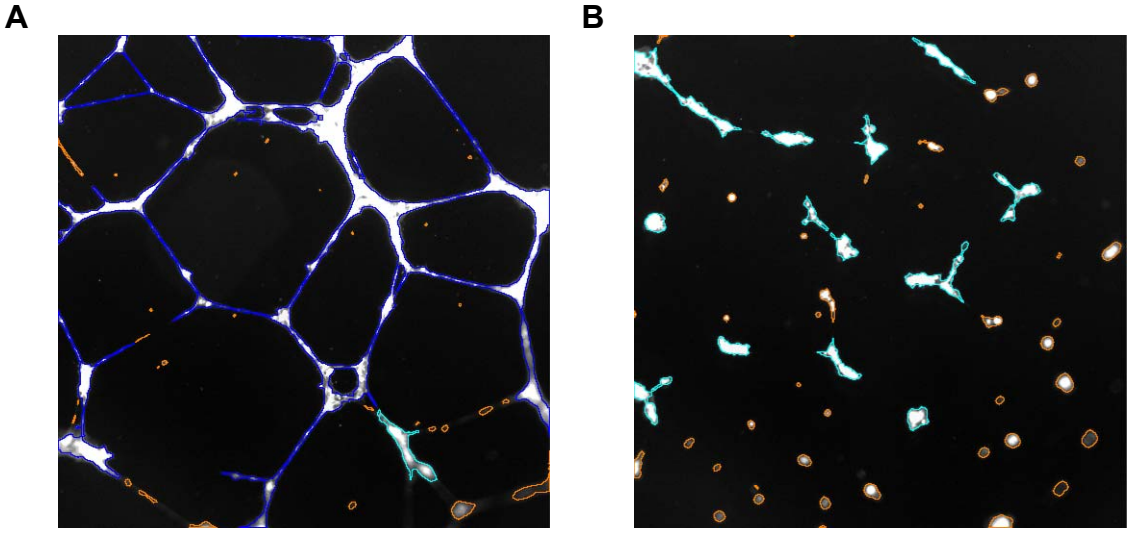

Figure 8. *The differences between typical connected (A) and unconnected tubes (B) based on a* ***MinConnectedTubeLengthCh1 = 300*** *µm and* ***TubeMergeSizeCh1 = 1****. The dark blue overlay in* ***A*** *outlines connected tubes, the light blue overlay in* ***B*** *delineates unconnected tubes, and the orange overlay is for objects rejected from analysis.*

Different Measurement Region Options for Channels 2-4

In Channels 2-4, the total, average and standard deviation of the target intensity in that channel are measured. There are three options for where these measurements are made in each of these channels, and the measurement regions can be set independently in each of these three channels (Figure 9). The three measurement region options for Channels 2-4 are:

- Measurements made in the region covered by the Tube mask identified in Channel 1
- Measurements made in a modified tube mask, where the tube mask from Channel 1 is either expanded or shrunk. The **MaskModifierCh*N*** (where Ch*N* can be Channel 2, Channel 3 or Channel 4) Assay Parameter specifies the number of pixels with which to expand (positive values) or shrink (negative values) the Channel 1 tube mask.
- The target in Channel 2 defines the region where the measurements are to be made. This region is defined by the intensity of the Channel 2 target which is within the Channel 1 tube mask. If the Channel 2 label is a nuclear stain, then the measurement region would be defined by this nuclear stain, and not the overall tube mask identified in Channel 1. In this case, the **MaskModifierCh*N*** Assay Parameter has no effect. Using Channel 2 versus Channel 1 to define the measurement mask is controlled by the **UseTargetMaskCh2** Assay Parameter.

For Channels 2-4, measuring the target intensity in the region defined by the Channel 2 label (versus in the modified tube mask defined in Channel 1) results in any fluorescence in the tube in Channel 2 that is not associated with individual targets not being measured. The target mask for Channel 2 can be carried over to Channel 3 and Channel 4, if the intensities only in the target mask area are of interest.

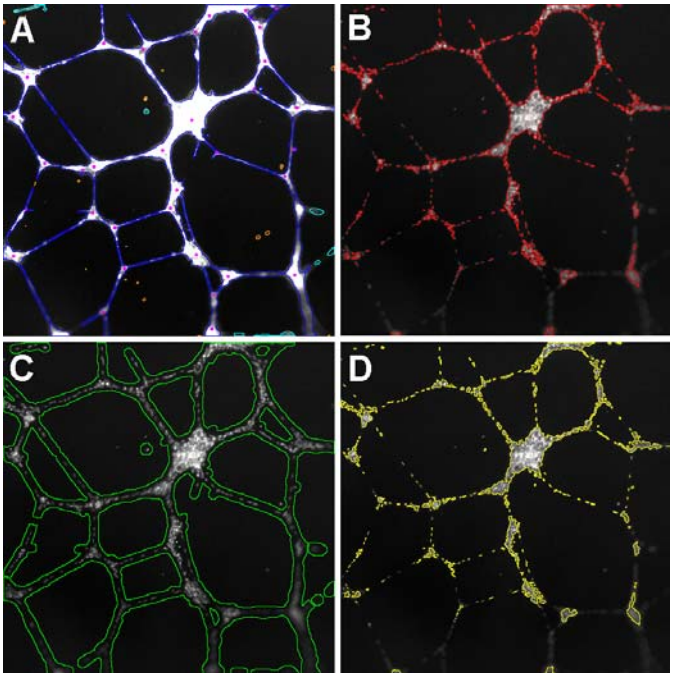

Figure 9. *Demonstration of different measurement region options for Channels 2-4. In this experiment, the tube mask was in Channel 1, Channels 2-4 all had the same image of labeled nuclei, but different measurement regions were chosen for the different channels.* ***A.*** *Channel 1 tube image with connected and unconnected tube mask outlines (blue and light blue respectively), nodes (pink dots), and rejected object outline (orange).* ***B.*** *Channel 2 targets (nuclei) with measurement region defined by its intensity (red).* ***C.*** *Channel 3 measurement region (green) was the Channel 1 tube mask expanded by 5 pixels (**UseTargetMaskCh2inCh3** = **0** and **MaskModifierCh3** = **5**).* ***D.*** *Channel 4 where the measurement region (yellow) was identical to the Channel 2 target mask region by setting **UseTargetMaskCh2inCh4** = **1**, despite setting **MaskModifierCh4** = **5** (mask modifier has no effect in this case).*

Basic Assay Parameters

Assay Parameters available in Basic Mode control calculation of the different properties of each tube (Table 8). Assay Parameters available in Advanced Mode control setting the conditions and levels for population characterization and enable you to identify which tubes are responders for different properties measured. Thus, unless you want to do population characterization for particular features or need to identify responders and, thus, need to set the Levels High and Low, you do not need to use the Advanced Parameters; adjusting the Basic Parameters should suit your needs.

When running in Advanced Mode, all basic input as well as advanced input parameters are editable. The **Hide Advanced Parameters** checkbox will enable you to hide or show all advanced Assay Parameters. When you check the checkbox, only the Basic Assay Parameters are shown; when you clear the checkbox, all Basic and Advanced Assay Parameters are shown.

Note that some of the Assay Parameters function as binary ON/OFF or YES/NO switches that can turn on specific types of processing. A value of **1** is "YES" and turns the function on, whereas a value of **0** is "NO" and turns the function off.

Parameter	Units	Description
UseReferenceWells	Binary	Use reference wells to calculate high and low response levels: 0 = No, 1 = Yes
UseMicrometers	Binary	Measure lengths and areas in: 0= Pixels, 1= Micrometers
ObjectTypeCh1	Binary	Type of objects to be identified in Ch1: 0 = Bright objects on dark background, 1 = Dark objects on bright background
BackgroundCorrectionChN	Pixels	Radius (in pixels) of region used to compute background in ChN: Negative value = Use surface fitting, 0 = Do not apply background correction, Positive value = Use low pass filter
TubeSmoothFactorCh1	Number	Degree of image smoothing (blurring) prior to tube detection in Ch1: 0 = Do not apply smoothing
TubeDetectRadiusCh1	Pixels	Radius (in pixels) of region used for tube detection in Ch1: 0 = Do not detect tubes
TubeMergeSizeCh1	Pixels	Number of pixels to connect (fill the gap between) tube fragments
MinTubeNodeBranchLengthCh1	Pixels or µm	Minimum length (in pixels or micrometers) of a branch to be considered for node identification
MinTubeNodeDistanceCh1	Pixels or µm	Minimum distance (in pixels or micrometers) between nodes
MinConnectedTubeLengthCh1	Pixels or µm	Minimum length (in pixels or micrometers) of a tube to be classified as connected tube
ObjectTypeCh2	Binary	Type of objects to be identified in Ch1: 0 = Bright objects on dark background, 1 = Dark objects on bright background
PeakSmoothFactorCh2	Number	Degree of image smoothing (blurring) prior to target peak detection in Ch2: 0 = Do not apply smoothing
PeakDetectRadiusCh2	Pixels	Radius (in pixels) of region used for target peak detection in Ch2
UseTargetMaskCh2	Binary	Use target mask Ch2 (instead of modified tube mask Ch1) to measure intensities in Ch2 : 0 = No, 1 = Yes
MaskModifierChN	Pixels	Number of pixels to modify Ch1 object (tube) mask in ChN: Negative value = Shrink mask, 0 = Do not modify mask, Positive value = Expand mask
UseTargetMaskCh2inCh3	Binary	Use target mask Ch2 (instead of modified tube mask Ch1) to measure intensities in Ch3 : 0 = No, 1 = Yes
UseTargetMaskCh2inCh4	Binary	Use target mask Ch2 (instead of modified tube mask Ch1) to measure intensities in Ch4 : 0 = No, 1 = Yes

Table 8. *Basic Assay Parameters available for the Tube Formation BioApplication. *Note that "ChN" refers to Channels 1-6* ***for Background Correction*** *and 2-4 for* ***Mask Modifier****.*

Object Selection Parameters

The cells used in a given assay may be different from those developed for the BioApplication. The characteristics of the samples can be determined in the Protocol Interactive View and new protocols can be tailored for particular conditions.

Each channel has a set of specific Object Selection Parameters. If an object in a particular channel's image has all measured features within the range specified by the appropriate Object Selection Parameters, then it is analyzed; otherwise, it is rejected from the analysis.

The Object Selection Parameters for Channel 1 are used to select which of the fluorescently-labeled primary objects in Channel 1 pass the selection criteria and are thus identified as individual, valid tubes and suitable for further analysis.

There are two types of selection parameters for Channels 2-4, those that further select the tube for analysis in that channel and those that determine which targets are selected for analysis. Each channel, for Channels 2-6, has two Object Selection Parameters, Average Intensity and Total Intensity, which further determine whether the tube is to be analyzed. If the tubes' intensity in the specific channels has both average and total intensity in the range specified, it will be selected for analysis.

In the Protocol Interactive View, the Identify Objects button will enable all tubes to be identified. Placing the cursor on an individual tube will cause its properties to be displayed, and these can be selected to set the range for valid tubes (Figure 10).

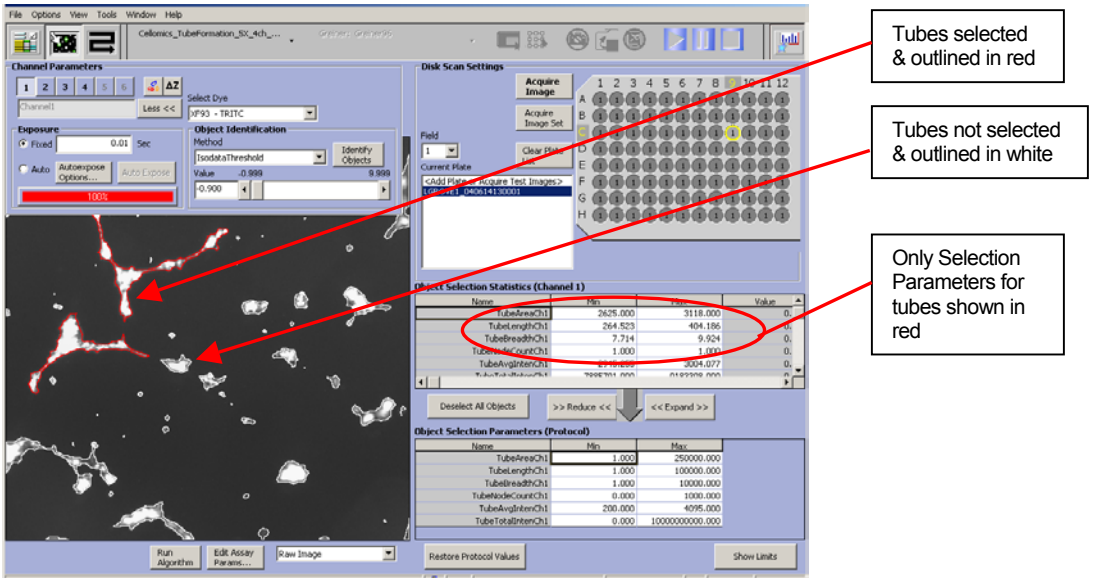

Figure 10. *Selecting only tubes in the Protocol Interactive View in Channel 1. The* ***Identify Objects*** *button was pressed, and all tubes were identified.*

Each channel has a set of specific Object Selection Parameters associated with it (see Tables 9-11). If an object in the particular channel's image has all measured features within the range specified by the appropriate Object Selection Parameters, then it is designated to be a tube and analyzed; otherwise, it is rejected from the analysis. Channel 1 has the largest set of Object Selection Parameters to determine whether an object is valid for analysis. The selection parameters for Channels 2-6 further qualify whether the tube should be selected or rejected from the analysis.

Channel 1 Object Selection Parameters

Parameter	Units	Description
TubeAreaCh1	Pixels or μm^2	Area (in pixels or micrometers) of Ch1 object (tube)
TubeLengthCh1	Pixels or µm	Length (in pixels or micrometers) of Ch1 object (tube) measured along its spine
TubeWidthCh1	Pixels or µm	Width (in pixels or micrometers) of Ch1 object (tube) estimated from area and length
TubeNodeCountCh1	Number	Number of nodes in Ch1 object (tube)
TubeAvgIntenCh1	Intensity	Average intensity of all pixels within Ch1 object (tube) mask
TubeVarIntenCh1	Intensity	Standard deviation of intensity of all pixels within Ch1 object (tube) mask
TubeTotalIntenCh1	Intensity	Total intensity of all pixels within Ch1 object (tube) mask

Table 9. *Channel 1 Object Selection Parameters available for the Tube Formation BioApplication.*

Channel 2 Object Selection Parameters: Selecting Tubes

Parameter	Units	Description
TargetAvgIntenCh2	Intensity	Average intensity in Ch2 of all pixels within modified Ch1 object (tube) mask or Ch2 target mask
TargetTotalIntenCh2	Intensity	Total intensity in Ch2 of all pixels within modified Ch1 object (tube) mask or Ch2 target mask

Table 10. *Channel 2 Object Selection Parameters available for the Tube Formation BioApplication.*

Channel N (3-6) Object Selection Parameters: Intensity Gating

Parameter	Units	Description
TargetAvgIntenChN	Intensity	Target average intensity in ChN of all pixels within modified Ch1 object (tube) mask or Ch2 target mask
TargetTotalIntenChN	Intensity	Target total intensity in ChN of all pixels within modified Ch1 object (tube) mask or Ch2 target mask

Table 11. *Channels 3-6 Object Selection Parameters available for the Tube Formation BioApplication.*Note that "ChN" refers to Channels 3-6.*

Gating

The Tube Formation BioApplication supports gating based on the use of Object Selection Parameters. Therefore, in addition to selecting tubes based on shape parameters, you can also select or reject tubes based on fluorescent intensity. You may want to perform gating if tubes have been identified in the focus channel and it is necessary to refine the object selection based on intensity of the tubes in a second channel.

Specifying Intensity Ranges for Gating

When working in the Create Protocol View, you can specify intensity threshold values by entering upper and lower threshold limits intensity parameters, **AvgIntenCh*N*** and **TotalIntenCh*N*** for one or more dependent channels (Channels 3-6), **TargetAvgIntenCh*N*** and **TargetTotalIntenCh*N*** for Channel 2, and/or **TubeAvgIntenCh1** and **TubeTotalIntenCh1** for parameters in Channel 1. In general, Total Intensity is a summation of all intensities within the object of interest. Average Intensity is the Total Intensity divided by the object area. Alternatively, you can set these parameter values interactively when working in the Protocol Interactive View.

You can view the results of the subpopulation selection in the View application when viewing Well Details. The parameter **ValidTubeCount** displays the total number of objects found for each well. The parameter **SelectedTubeCount** displays the number of objects in each well that passed all requirements, including the intensity requirements. To view the intensity parameter values, select Cell Details for a particular well. The View application displays the intensity parameter values for each cell in each channel.

Specifying Mask Modifiers for Gating

The **MaskModifierCh*N***, controls the area in which measurements are made for gating. The value is the number of pixels to modify Channel 1 object (tube) mask in Channel *N*. For Channel 3 and Channel 4, this parameter will control the dilation or erosion of either the Channel 1 tube mask or Channel 2 target mask defined by the **UseTargetMaskCh2inCh3** or **UseTargetMaskCh2inCh4** Assay Parameters (see Figure 7).

If the value for **UseTargetMaskCh2inCh3** (or **UseTargetMaskCh2inCh4**) is **0**, the **MaskModifierCh3** (or **MaskModifierCh4**) Assay Parameter value is used. If the value for **UseTargetMaskCh2inCh3** (or **UseTargetMaskCh2inCh4**) is **1**, it will ignore the values for

MaskModifierCh3 (or **MaskModifierCh4**) and instead use the value set for **MaskModifierCh2** in Channel 3 (or Channel 4) (see Figure 9).

The final modified masks create the area where average and total intensity is measured and subsequently used for gating purposes when Object Selection Parameters are defined. For Channel 5 and Channel 6, the **MaskModifierCh*N*** value is the number of pixels to modify Channel 1 object (tube) mask in that channel (Channel 5 and/or Channel 6) and is not controlled by the **UseTargetMaskCh2inCh*N*** Assay Parameters.

Image Overlays

All Image Overlay Display Option Settings are available when running in Basic or Advanced Mode as shown in Table 12.

Parameter	Description
Include This Channel In Composite	Determines if image for this channel is included in composite image.
ConnectedTube	Outlines valid connected tube in Channel 1 for analysis.
UnconnectedTube	Outlines valid unconnected tube in Channel 1for analysis.
RejectedObject	Outlines objects rejected for analysis.
TubeNode	Labels nodes within tubes.
TargetCh2	Labels targets in Channel 2.
MaskCh3	Outlines the mask used in Channel 3 to make intensity measurements.
MaskCh4	Outlines the mask used in Channel 4 to make intensity measurements.

Table 12. *Overlay Display Option Settings available for the Tube Formation BioApplication*

Assay Parameters for Population Characterization

Overview of Population Characterization

The Tube Formation BioApplication provides the ability to characterize tubes based on their response compared to a control population. For a particular Cell Feature, a range is determined and set for a control population that has the normal physiological distribution for that feature. Assay Parameters for an upper and lower limit (known as ***Feature*Ch*N*LevelHigh** and ***Feature*Ch*N*LevelLow**) set the upper and lower bounds of this range respectively as found in Table 13. The Status Cell Feature indicates whether a particular tube is within or beyond this range.

Cell Status	Definition
0	feature within defined range (i.e., within limits)
1	feature > Level High
2	feature < Level Low

Table 13. *Cell Status definitions available for the Tube Formation BioApplication*

The corresponding reported Well Features are the percentage of tubes that are either greater or less than the levels defining this range. This concept is shown in Figure 11 for a distribution of a normal physiological population of tubes for a particular Cell Feature and the shift in this distribution upon compound treatment.

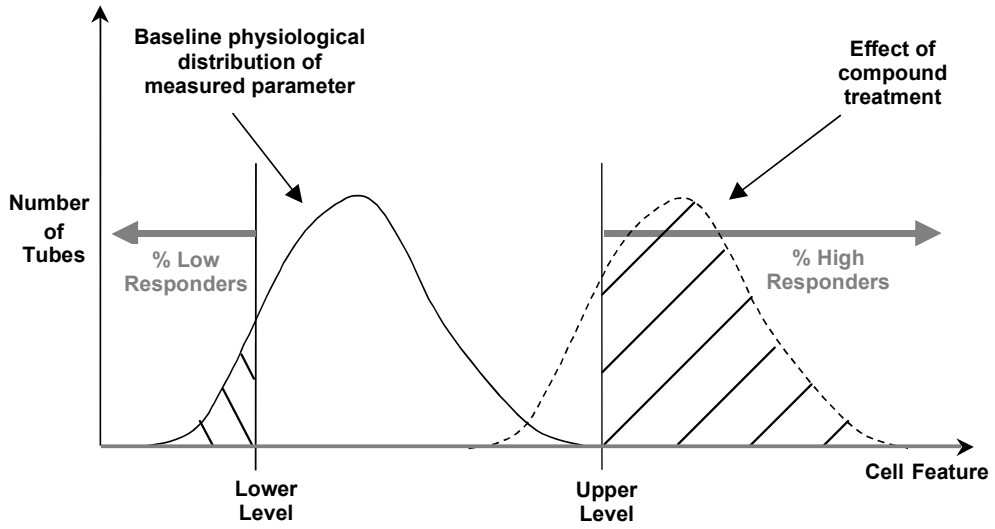

Figure 11. *Population characterization principle. Responders exhibiting Status Cell Feature value = 1 (%High Responders) or Status Cell Feature value = 2 (%Low Responders) are highlighted.*

Angiogenic Status

An exception to two levels defining a responder is in the determination of a well's Angiogenic status. In this case, only one level is used to automatically identify the whole well as either being positive or negative for angiogenesis. Each well's **AngiogenicIndexCh1Status** is reported. If a well's **AngiogenicIndexCh1** is above the specified level, then the well's **AngiogenicIndexCh1Status** is reported as **1**; otherwise it is **0**. The level defining the **AngiogenicIndexCh1Status** can either be automatically determined by the application from reference wells, or can be manually entered by the user, and this Assay Parameter is called:

- **AngiogenicResponseLevelCh1** for manually entry of the level
- **AngiogenicResponseLevelCh1_CC** to determine the value of the level from Reference Wells.

Setting Cellular Response Levels

There are two ways of setting the Levels High and Low to characterize the population: manually or automatically. To set the levels manually, you specify the ***Feature*Ch*N*LevelHigh** and ***Feature*Ch*N*LevelLow** values for the different features that can be used to perform population characterization. The Tube Formation BioApplication then uses these levels that you define to calculate the percentage of tubes outside the bounds of these levels for the different Cell Features.

To automatically determine the ***Feature*Ch*N*LevelHigh** and ***Feature*Ch*N*LevelLow** Assay Parameter values, the BioApplication uses Reference Wells. You designate particular wells on the sample plate as Reference Wells. Typically, Reference Wells contain a control population of tubes that displays the normal basal physiological distribution for the parameter being measured. Images in these wells will be acquired/analyzed and the population distribution for the different features will be computed. The population characterization is then set by adding to, and subtracting from, the mean of the distribution its standard deviation multiplied by a coefficient. The system then scans the whole plate and applies these levels. For example, if you want to know the percentage of tubes that, with compound treatment, have a response beyond the 95th percentile of the response from a control untreated population, then the coefficient by which to multiply the standard deviation would be two. The advantage of using Reference

Wells to automatically calculate ***Feature*Ch*N*LevelHigh** and ***Feature*Ch*N*LevelLow** is that the levels are determined by a control population of cells and are independent of run-to-run variations when doing the experiment on different days such as different illumination conditions, fluorophore amounts, or changes in cell conditions.

Reference Wells Processing Sequence

By setting the **UseReferenceWells** Assay Parameter to **1**, the Reference Wells processing is engaged. Specified fields within the wells will be acquired/analyzed, and Field, Well, and Plate Features will be computed. After this sequence is completed, the computed values will be loaded into the assay parameters related to reference features and the regular scanning of the plate will begin.

Reference features are computed on a field basis. The sequence of computation for Reference Wells is as follows:

1. Cell Features are computed for every valid tube within a field.
2. For each Cell Feature used for population characterization, mean, and standard deviation are computed over all tubes in the field.
3. Reference field features are determined.
4. Reference Well and Plate Features are computed as average values for fields in a well, weighted for the number of tubes per field, and then as an arithmetic average for wells on a plate. Use of a weighted average minimizes the effect of sparse fields. The exception is the tube count feature, whose average is not weighted.
5. Reference Plate Features are computed as simple average values for reference wells in a plate

Identifying Reference Wells and Control Parameters

Reference Wells are specified in the Reference Wells Configuration Window (choose **Change Reference Wells** from the **Tools** menu). Select the wells to be set as the Reference Wells and then select the button for the appropriate type of reference well (**Known**). Next, enter the Starting Field and Number of Fields. Click the **Apply** button to save the settings. Please refer to the appropriate User's Guide for details.

The Reference Well settings are not saved as part of the Assay Protocol, rather they are saved in the Scan software application.

TIP: To document the Reference Wells used in a scan, enter them in the Scan Comments box in the Scan Plate View.

Defining Range for Control Tubes and Population

There are a number of Cell Features that are further classified by the Population Characterization option. There are two general Assay Parameters used to control Reference Wells (**UseReferenceWells** and **MinRefAvgTubeCountPerField**). There are four types of Assay Parameters that are used for population characterization. There is a set for each individual cell feature for which population characterization is applied and the dependent channels where it is calculated. These parameter names are of the type:

- ***Feature*Ch*N*LevelLow**
- ***Feature* Ch*N* LevelHigh**

- ***Feature*Ch*N*LevelLow_CC**
- ***Feature*Ch*N*LevelHigh_CC**

The *Feature* refers to the individual cell feature and Ch*N* refers to the specific channel *N*. Other than the **UseReferenceWells** Assay Parameter, all the Assay Parameters used for population characterization are available only in the Advanced Mode.

You can either manually set the Levels Low and High, defining the physiological population range after reviewing results from previously analyzed control wells or plate or the levels can be automatically derived from Reference Wells containing untreated cells and thus have a normal physiological distribution for the feature measured. You choose whether to manually or automatically set the range by the **UseReferenceWells** Assay Parameter, where the possible values and resulting actions controlled by this Assay Parameter are:

- **UseReferenceWells** = 0: do not use Reference Wells (i.e., set the range manually)
- **UseReferenceWells** = 1 = use Reference Wells (i.e., allow the system to set the range automatically)

If the range is to be set manually, you must enter the appropriate value for the relevant Assay Parameter defining that level. If the range is to be set automatically using Reference Wells, then the Assay Parameters having the suffix "**_CC**" (i.e., "correction coefficient") must be set. The ***Feature*Ch*N*LevelLow** and ***Feature*Ch*N*LevelHigh** for the range for a cell feature are set by subtracting or adding the distribution's standard deviation (SD) multiplied by the appropriate CC value respectively from the distribution's mean:

- ***Feature*Ch*N*LevelLow** = Mean – ***Feature*Ch*N*LevelLow_CC***SD
- ***Feature*Ch*N*LevelHigh** = Mean + ***Feature*Ch*N*LevelHigh_CC***SD

Note that the CC value can be different for defining ***Feature*Ch*N*LevelLow** vs. ***Feature*Ch*N*LevelHigh**.

Advanced Assay Parameters

Other than the **MinRefAvgTubeCountPerField** Assay Parameter, the Advanced Mode Assay Parameters pertain to setting the Levels Low and High for population characterization and enable you to identify which tubes are responders for different properties measured. The **MinRefAvgTubeCountPerField** Assay Parameter is also related to setting these levels as it specifies the minimum number of tubes per field to be counted in Reference Wells for the Reference Well to be valid. Thus, unless you want to do population characterization for particular features or need to identify responders by setting the levels, you do not need to enter values for the Advanced Parameters.

For each feature undergoing population characterization, there are four advanced Assay Parameters that control its levels; two of the four are determined by the presence of the **_CC** suffix designating that levels are set using Reference Wells. For example, the Assay Parameters controlling the tube's area in Channel 1 are:

- **TubeAreaCh1LevelLow**
- **TubeAreaCh1LevelHigh**

} Enter values directly

- **TubeAreaCh1LevelLow_CC**
- **TubeAreaCh1LevelHigh_CC**

} Enter # of SD to offset Reference Well mean

In the listing of Advanced Parameters in Table 14, instead of listing all four level Assay Parameters for each feature, one entry for the feature will be listed giving the four different options, as shown in the following example for the Channel 1 tube area:

- TubeAreaCh1Level***Low/High, Low/High_CC***

Note that **AngiogenicIndexCh1** only has Level High and Level High_CC option and will be listed as AngiogenicIndexCh1Level***High, High_CC***

Parameter	Units	Description
MinRefAvgTubeCountPerField	Number	Minimum average number of tubes per field required for acceptance of reference well results
_PixelSize	µm	Pixel size in micrometers (depends on objective selection)
TubeAreaCh1Level***Low/High, Low/High_CC***	Pixel or μm^2	Defines **TubeAreaCh1** response level
TubeLengthCh1Level***Low/High, Low/High_CC***	Pixel or µm	Defines **TubeLengthCh1** response level
TubeWidthCh1Level***Low/High, Low/High_CC***	Pixel or µm	Defines **TubeWidthCh1** response level
TubeLWRCh1Level***Low/High, Low/High_CC***	Number	Defines **TubeLWRCh1** response level
TubeTotalIntenCh1Level***Low/High, Low/High_CC***	Intensity	Defines **TubeTotalIntenCh1** response level
TubeAvgIntenCh1Level***Low/High, Low/High_CC***	Intensity	Defines **TubeAvgIntenCh1** response level
TubeVarIntenCh1Level***Low/High, Low/High_CC***	Intensity	Defines **TubeVarIntenCh1** response level
TubeNodeCountCh1Level***Low/High, Low/High_CC***	Number	Defines **TubeNodeCountCh1** response level
TubeNodeAvgDistCh1Level***Low/High, Low/High_CC***	Pixel or µm	Defines **TubeNodeAvgDistCh1** response level
TubeSegmentCountCh1Level***Low/High, Low/High_CC***	Number	Defines **TubeSegmentCountCh1** response level
AngiogenicIndexCh1Level***High, High_CC***	Number	Defines **AngiogenicIndexCh1** high response level only
PeakCountCh2Level***Low/High, Low/High _CC***	Number	Defines **PeakCountCh2** response level
TargetTotalIntenCh2Level***Low/High, Low/High_CC***	Intensity	Defines **TargetTotalIntenCh2** response level
TargetAvgIntenCh2Level***Low/High, Low/High_CC***	Intensity	Defines **TargetAvgIntenCh2** response level
TargetVarIntenCh2Level***Low/High, Low/High_CC***	Intensity	Defines **TargetVarIntenCh2** response level
TargetTotalIntenChNLevel***Low/High, Low/High_CC***	Intensity	Defines **TargetTotalIntenChN** response level
TargetAvgIntenChNLevel***Low/High, Low/High_CC***	Intensity	Defines **TargetAvgIntenChN** response level
TargetVarIntenLevelChN***Low/High, Low/High_CC***	Intensity	Defines **TargetVarIntenChN** response level

Table 14.*Advanced Assay Parameters available for the Tube Formation BioApplication. *Note that "ChN" refers to Channels 3-4.*

Description of Output Features

Measurements Reported On Individual Tubes & Per Field/Well

The Tube Formation BioApplication reports Cell, Field and Well Features. Note that for this BioApplication, measurements are made on individual tubes and not individual cells. The term Cell Feature actually refers to measurements made at the individual tube level and not at the individual cell level. Therefore, Cell Feature refers to features that are reported for each individual tube. Field and Well Features are reported for each field and well; they are derived from the Cell Features for the tubes imaged and analyzed in that field or well respectively. The Cell Features are related to the raw morphological properties measured for each tube. In addition, for many of the Cell Features, corresponding status features are also reported at the cell level. The status features indicate whether the tube is outside the bounds of the population characterization limits for a particular morphology feature, and thus can be identified as a responder. The use of status features and population characterization limits is described in more detail in the section on Population Characterization.

The Field and Well Features are statistical measures or population characterization features derived from the distribution of Cell Features. At the field and well level, statistics are reported for all selected tubes (connected + unconnected), as well as for connected and unconnected tubes individually. The statistics reported for all selected tubes at the field and well level are the average, standard deviation of all tubes considered valid by the object selection parameters, as well as the percentage of tubes that are greater or less than some criterion for the feature being measured (percentage greater than a ***Feature*Ch*N*LevelHigh** or less than a ***Feature*Ch*N*LevelLow** – see sub-section on Population Characterization). The latter are determined using the Cell Status Features. Typically, there are only 1-3 highly branched connected tubes per well and a few unconnected tubes. When the values are averaged, the significant statistics for the connected tubes will be diluted out by the unconnected tubes' values. Therefore, statistics are given for all tube categories at the field and well level so the statistics retain their significance.

Channel 1 Features

Channel 1 contains the image of the primary labeled objects. Primary objects are all selected tubes; the tubes are placed into one of two categories, connected tubes or unconnected tubes, depending on their total length.

Number of Fields, Tube Counts, Density & Location

For each well, the **ValidFieldCount** and **TotalFieldCount** are reported. The **TotalFieldCount** is the number of fields imaged per well, and the **ValidFieldCount** is the number of fields that has objects (i.e., tubes) selected for analysis in them. At the field/well level, the number of tubes analyzed in the well is reported (**ValidTubeCount** and **SelectedTubeCount**). The tube density is reported both as the number of selected tubes per valid imaged field (**SelectedTubeCountPerValidField**) and the number of selected tubes per all imaged fields (**SelectedTubeCountPerTotalField**). The number and percentage of connected and unconnected tubes are also reported (**ConnectedTubeCount**, **%ConnectedTubes**, **UnconnectedTubeCount**, **%UnconnectedTubes**). A feature derived from these measurements is the **AngiogenicIndexCh1**, which is defined as:

$$\text{AngiogenicIndexCh1} = \frac{1{,}000 \times \text{Total Area of Connected Tubes}}{\text{Total Image Area}}$$

The **AngiogenicIndexCh1** can be thought of as the percentage of the image area covered by Connected Tubes multiplied by a factor of ten. Note that the image area is the total image area in the well, which is the area of one image multiplied by the **TotalFieldCount**. Each well's **AngiogenicIndexStatusCh1** is also reported. If a well's **AngiogenicIndexCh1** is above some minimum level, then the well's **AngiogenicIndexStatusCh1** is reported as **1**; otherwise it is **0**. The level defining the **AngiogenicIndexStatusCh1** can be automatically determined by the application from reference wells, or can be manually entered via an Assay Parameter. The tube's location in the image is reported by the *x* and *y* coordinates of its centroid.

The distinction between **SelectedTubeCountPerValidField** and the **SelectedTubeCountPerTotalField** may be important when evaluating the dose dependence on the **AngiogenicIndexCh1** of an anti-angiogenic drug. For example, in the experiments shown in Figure 3 and Figure 4, three fields were collected per well. At low suramin concentrations, connected tubes were found in all three fields, whereas at higher suramin doses, only the central field contained a connected tube. If the **AngiogenicIndexCh1** was calculated from the **SelectedTubeCountPerValidField** (i.e., only fields where connected tubes were found), then the **AngiogenicIndexCh1** for both the high and low suramin concentrations would be similar. However, since the **AngiogenicIndexCh1** is actually calculated using the **SelectedTubeCountPerTotalField** (whether or not the field contained tubes) the **AngiogenicIndexCh1** is lower for the higher suramin dose where only one out of the three fields contained a connected tube. This is as expected, since higher concentrations of suramin more strongly inhibit angiogenesis.

Tube Dimensions

The area of each tube is reported. **TubeAreaCh1** is computed as the total pixel count within a tube mask, and reported either as a pixel count, or in units of μm^2. Estimates of tube length and width are also reported. **TubeLengthCh1** is computed as the total length of the medial cord of the tube mask (Figure 12). **TubeWidthCh1** is computed as **TubeAreaCh1** ÷ **TubeLengthCh1**. The **TubeLWRCh1** is computed as the length to width ratio, **TubeLengthCh1** ÷ **TubeWidthCh1**.

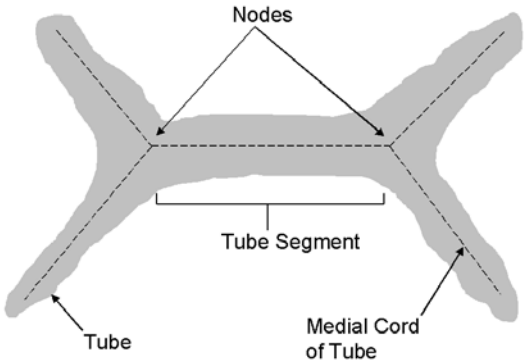

Figure 12. *Tube Length estimation (dotted line – medial cord). Also shown are two nodes and the tube segment between the nodes.*

Tube Branching

Tube branch points are defined as **nodes** (Figures 2, 12 and 13), nodes divide a tube into segments, and segments are the sections of tubes between nodes. For each tube, the number of nodes (**TubeNodeCountCh1**), the mean spacing between nodes (**TubeNodeAvgDistCh1**), and the number of tube segments (**TubeSegmentCountCh1**) are reported. Note that the spacing between the nodes is measured along the length of the tube, and is not just the shortest

Cartesian distance between the two node points. The degree of tube branching can be assessed using **BranchingIndexCh1**, which is defined as "Ratio of 10,0000 * total **TubeNodeCountCh1** to total **TubeAreaCh1** of all tubes selected for analysis in the field"

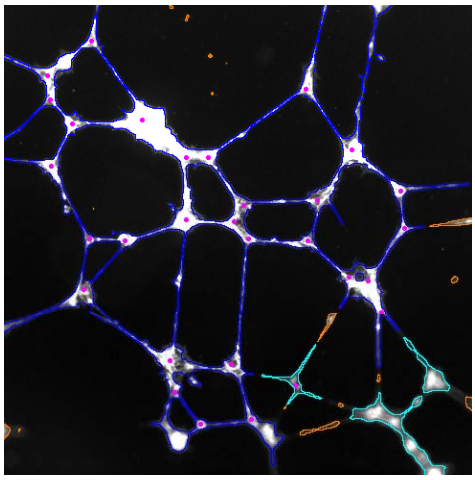

Figure 13. *Tube Nodes (magenta branch points) based on* ***MinTubeNodeBranchLengthCh1 = 0*** *(automatically calculated by algorithm) and* ***MinTubeNodeDistanceCh1 = 0****.*

Tube Intensity & Texture Properties

The tube label's intensity (total and average) and its standard deviation are also reported in Channel 1. The total intensity (**TubeTotalIntenCh1**) is the sum of the intensities of all pixels making up the tube in the image, and the average intensity (**TubeAvgIntenCh1**) is the total intensity divided by the number of pixels (i.e., tube area). The standard deviation of the pixel intensities within the tube (**TubeVarIntenCh1**) is used as a measurement of tube texture.

Channel 2 Features

Channel 2 is designed to identify targets, such as prominent cellular compartments (e.g., nuclei, Golgi), within each tube.

Target Identification

The BioApplication reports an estimate of the number of targets in Channel 2 within each selected object (tube). If the Channel 2 stain labels nuclei and the Channel 1 object is a multi-cellular tube, then this can be used as an estimate of the number of cells in the tube (Figure 2). The nuclear or cell number is an estimate because: (1) tubes contain a large number of cells, (2) the entire tube may not be contained within the image so only nuclei within the tube in the image can be detected, and (3) cells at the nodes are often stacked in three dimensions so all the nuclei in a node may not be individually resolved and identified. Thus, the number of targets in Channel 2 is an estimate rather than an exact number of nuclei in the tube. The number of targets is determined by identifying all the intensity peaks in the target label in Channel 2, and then counting these peaks (**PeakCountCh2**). The estimate of the total area of the targets is also reported (**TargetTotalAreaCh2**).

Intensity & Texture Properties

The total, average and standard deviation of the target intensity are measured in each object (tube) in Channel 2. The user can choose these to be measured either in the modified tube mask defined in Channel 1, or in the region defined by the Channel 2 labeling. The total

intensity is the sum of the intensities of all target pixels in the measurement region (**TargetTotalIntenCh2**). The average intensity is the average pixel intensity for that target, which is the total intensity divided by the number of pixels in the measurement region (**TargetAvgIntenCh2**). The standard deviation of the pixel intensities in the tube (**TargetVarIntenCh2**) is used as a measurement of the tube's texture. The difference between measuring the target intensity in the modified tube mask defined in Channel 1 versus in the region defined by the Channel 2 target's labeling is that if there is any background fluorescence in the object in Channel 2 that is not associated with the individual targets, its intensity will be not be captured by the average and total intensity measurements if the modified Channel 1 mask is chosen as the region in which to make measurements. Similar to Channel 1, well level features are reported for all selected tubes, connected tubes, and unconnected tubes.

Channel 3 and Channel 4 Features

Channel 3 and Channel 4 contain the images of intracellular targets. These could be homogeneous cellular labels so as to distinguish a particular cell type, or discrete entities inside the cell such as organelles, protein clusters, and other structures. The features reported in Channel 3 and Channel 4 can help quantify the different possible targets that can be labeled.

Intensity & Texture Properties

Similar to the Channel 1 and Channel 2 labels, metrics of the pixel intensity distribution for Channel 3 and Channel 4 are reported. These metrics are the total and average intensities as well as the standard deviation. The target mask for Channel 2 can be carried over to Channel 3 and Channel 4 if the user is interested in the intensities only in the target mask area.

Cell Features

For the Tube Formation BioApplication, the Cell Features are the output features reported for each **tube**, not individual cells in Table 15.

Cell Feature	Units	Description
Cell#	Number	Unique tube ID
Top	Pixels	Y coordinate (in pixels) of top left corner of image-aligned bounding box of tube
Left	Pixels	X coordinate (in pixels) of top left corner of image-aligned bounding box of tube
Width	Pixels	Width (in pixels) of image-aligned bounding box of tube
Height	Pixels	Height (in pixels) of image-aligned bounding box of tube
XCentroid	Pixels	X coordinate of tube center
YCentroid	Pixels	Y coordinate of tube center
TubeAreaCh1	Pixels or μm^2	Area (in pixels or micrometers) of Ch1 object (tube)
TubeAreaStatusCh1	Number	**TubeAreaCh1** status: 0 = No response, 1 = High response, 2 = Low response
TubeLengthCh1	Pixels or µm	Length (in pixels or micrometers) of Ch1 object (tube) measured along its spine
TubeLengthStatusCh1	Number	**TubeLengthCh1** status: 0 = No response, 1 = High response, 2 = Low response
TubeWidthCh1	Pixels or µm	Width (in pixels or micrometers) of Ch1 object (tube) estimated from area and length
TubeWidthStatusCh1	Number	**TubeWidthCh1** status: 0 = No response, 1 = High response, 2 = Low response

Cell Feature	Units	Description
TubeLWRCh1	Number	Length to width ratio of Ch1 object (tube)
TubeLWRCh1Status	Number	**TubeLWRCh1** status: 0 = No response, 1 = High response, 2 = Low response
TubeTotalIntenCh1	Intensity	Total intensity of all pixels within Ch1 object (tube)
TubeTotalIntenStatusCh1	Number	**TubeTotalIntenCh1** status: 0 = No response, 1 = High response, 2 = Low response
TubeAvgIntenCh1	Intensity	Average intensity of all pixels within Ch1 object (tube)
TubeAvgIntenStatusCh1	Number	**TubeAvgIntenCh1** status: 0 = No response, 1 = High response, 2 = Low response
TubeVarIntenCh1	Intensity	Standard deviation of intensity of all pixels within Ch1 object (tube)
TubeVarIntenStatusCh1	Number	**TubeVarIntenCh1** status: 0 = No response, 1 = High response, 2 = Low response
TubeNodeCountCh1	Number	Number of nodes within Ch1 object (tube)
TubeNodeCountStatusCh1	Number	**TubeNodeCountCh1** status: 0 = No response, 1 = High response, 2 = Low response
TubeNodeAvgDistCh1	Pixels or µm	Average distance (in pixels or micrometers) between nodes within Ch1 (object) tube
TubeNodeAvgDistStatusCh1	Number	**TubeNodeAvgDistCh1** status: 0 = No response, 1 = High response, 2 = Low response
TubeSegmentCountCh1	Number	Number of segments within Ch1 object (tube)
TubeSegmentCountStatusCh1	Number	**TubeSegmentCountCh1** status: 0 = No response, 1 = High response, 2 = Low response
TubeTypeCh1	Binary	Tube type: 1 = Connected, 0 = Unconnected
PeakCountCh2	Number	Number of peaks within target mask in Ch2
PeakCountStatusCh2	Number	**PeakCountCh2** status: 0 = No response, 1 = High response, 2 = Low response
TargetTotalIntenCh2	Intensity	Total intensity in Ch2 of all pixels within modified Ch1 object (tube) mask or Ch2 target mask
TargetTotalIntenStatusCh2	Number	**TargetTotalIntenCh2** status: 0 = No response, 1 = High response, 2 = Low response
TargetAvgIntenCh2	Intensity	Average intensity in Ch2 of all pixels within modified Ch1 object (tube) mask or Ch2 target mask
TargetAvgIntenStatusCh2	Number	**TargetAvgIntenCh2** status: 0 = No response, 1 = High response, 2 = Low response
TargetVarIntenCh2	Intensity	Standard deviation of intensity in Ch2 of all pixels within modified Ch1 object (tube) mask or Ch2 target mask
TargetVarIntenStatusCh2	Number	**TargetVarIntenCh2** status: 0 = No response, 1 = High response, 2 = Low response
TargetTotalIntenCh3	Intensity	Total intensity in Ch3 of all pixels within modified Ch1 object (tube) mask or Ch2 target mask
TargetAvgIntenCh3	Intensity	Average intensity in Ch3 of all pixels within modified Ch1 object (tube) mask or Ch2 target mask
TargetVarIntenCh3	Intensity	Standard deviation of intensity in Ch3 of all pixels within modified Ch1 object (tube) mask or Ch2 target mask
TotalIntenChN	Intensity	Total intensity in ChN of all pixels within modified Ch1 object (tube) mask or Ch2 target mask
TargetTotalIntenChNStatus	Number	**TargetTotalIntenChN** status: 0 = No response, 1 = High response, 2 = Low response
AvgIntenChN	Intensity	Average intensity in ChN of all pixels within modified Ch1 object (tube) mask or Ch2 target mask
TargetAvgIntenChNStatus	Number	**TargetAvgIntenChN** status: 0 = No response, 1 = High response, 2 = Low response
VarIntenChN	Intensity	Standard deviation of intensity in ChN of all pixels within modified Ch1 object (tube) mask or Ch2 target mask

Cell Feature	Units	Description
TargetVarIntenChNStatus	Number	**TargetVarIntenChN** status: 0 = No response, 1 = High response, 2 = Low response

Table 15. *Cell Features available with the Tube Formation BioApplication. Note that "ChN" refers to Channels 3-6.*

Field/Well Features

The algorithm reports the following Field and Well Features in the Scan Plate View in addition to the Field/Well Detail window of the View application. Most of the Field and Well features are derived from the cell features. Such features are identified by a prefix, as listed in Tables 16 to the cell feature name. In addition, Table 17 gives a list of the Field and Well features that are also reported for Connected (_C_) and Unconnected (_U_) tubes. The Mean_, SD_, %HIGH and %LOW are reported for these features as well.

Feature Prefix	Well Feature Definition	Units
MEAN_	Average of Feature_X for all objects selected for analysis in the well (field)	Same as cell feature
SD_	Standard deviation of Feature_X for all objects selected for analysis in the well (field)	Same as cell feature
%HIGH_	Percentage of selected objects in the well (field) with Feature_X above high-response level	Percent
%LOW_	Percentage of selected objects in the well (field) with Feature_X below low-response level	Percent

Table 16. *Field/Well Features available for Connected and Unconnected Tubes*

Channel	Feature name
Channel 1	TubeArea
	TubeLength
	TubeWidth
	TubeLWR
	TubeTotalInten
	TubeAvgInten
	TubeVarInten
	TubeNodeAvgDist
	TubeSegmentCount
Channel 2	PeakCount
	TargetTotalInten
	TargetAvgInten
	TargetVarInten
Channels 3-4	TargetTotalInten
	TargetAvgInten
	TargetVarInten

Table 17. *Field/Well-Level Output Features available for Connected (_C_) and Unconnected (_U_) Tubes.*

The additional Field and Well Features that are not identical to specific Cell Features are listed in Table 18.

Field/Well Feature	Description
ValidTubeCount	Number of valid tubes identified in the field/well (Ch1 object selection parameters applied)
SelectedTubeCount	Number of valid tubes selected for analysis in the field/well (Ch2-6 object selection parameters applied)
%SelectedTubes	Percentage of valid tubes selected for analysis in the field/well
TotalFieldCount	Total number of fields analyzed in the field/well
SelectedTubeCountPerTotalField	Average number of tubes selected for analysis per field (using **TotalFieldCount**) in the field/well
ValidFieldCount	Number of fields in which tubes were selected for analysis in the field/well
SelectedTubeCountPerValidField	Average number of tubes selected for analysis per valid field (using **ValidFieldCount**) in the field/well
ConnectedTubeCount	Number of connected tubes selected for analysis in the field/well
%ConnectedTubes	Percentage of connected tubes selected for analysis in the field/well
UnconnectedTubeCount	Number of unconnected tubes selected for analysis in the field/well
%UnconnectedTubes	Percentage of unconnected tubes selected for analysis in the field/well
ConnectedTubeAreaCh1	Area of connected tubes selected for analysis in the field/well
UnconnectedTubeAreaCh1	Area of unconnected tubes selected for analysis in the field/well
TotalTubeAreaCh1	Total area of connected and unconnected tubes selected for analysis in the field/well
AngiogenicIndexCh1	Ratio of 1000 * area of connected tubes selected for analysis in the field/well to image area
BranchingIndexCh1	Ratio of 10,0000 * total node count to total area of all tubes selected for analysis in the field/well
TubeAreaPerValidFieldCh1	Average tube area per field of all tubes selected for analysis in the well
TargetTotalAreaCh2	Total area of all Ch2 targets for all tubes selected for analysis in the field/well

Table 18. *Field/Well Features different from cell features available with the Tube Formation BioApplication.*

Reference Well Features

The list of Reference Well Features and their values are displayed in the Scan Plate View in addition to the View application (Table 19). Instead of listing both Reference Well Features derived from the previous level parameters, one entry for the feature will be listed giving both outputs, as shown in the following example for the Channel 1 tube area.

- RefTubeAreaCh1Level***Low/High***

Well Feature	Description
RefAvgTubeCountPerField	Average count of objects (tubes) per field in reference wells
RefTubeAreaCh1Level***Low/High***	Low/High response level for **TubeAreaCh1** computed from reference well results
RefTubeLengthCh1Level***Low/High***	Low/High response level for **TubeLengthCh1** computed from reference well results
RefTubeWidthCh1Level***Low/High***	Low/High response level for **TubeWidthCh1** computed from reference well results
RefTubeLWRCh1Level***Low/High***	Low/High response level for **TubeWidthCh1** computed from reference well results
RefTubeTotalIntenCh1Level***Low/High***	Low/High response level for **TubeTotalIntenCh1** computed from reference well results
RefTubeAvgIntenCh1Level***Low/High***	Low/High response level for **TubeAvgIntenCh1** computed from reference well results
RefTubeVarIntenCh1Level***Low/High***	Low/High response level for **TubeVarIntenCh1** computed from reference well results
RefTubeNodeCountCh1Level***Low/High***	Low/High response level for **TubeNodeCountCh1** computed from reference well results
RefTubeNodeAvgDistCh1Level***Low/High***	Low/High response level for **TubeNodeDistCh1** computed from reference well results
RefTubeSegmentCountCh1Level***Low/High***	Low/High response level for **TubeSegmentCountCh1** computed from reference well results
RefTubeAngiogenicIndexCh1Level***High***	Response level for **AngiogenicIndexCh1** computed from reference well results
RefPeakCountCh2Level***Low/High***	Low/High response level for **PeakCountCh2** computed from reference well results
RefTargetTotalIntenCh2Level***Low/High***	Low/High response level for **TargetTotalIntenCh2** computed from reference well results
RefTargetAvgIntenCh2Level***Low/High***	Low/High response level for **TargetAvgIntenCh2** computed from reference well results
RefTargetVarIntenCh2Level***Low/High***	Low/High response level for **TargetVarIntenCh2** computed from reference well results
RefTargetTotalIntenChNLevel***Low/High***	Low/High response level for **TargetTotalIntenChN** computed from reference well results
RefTargetAvgIntenChNLevel***Low/High***	Low/High response level for **TargetAvgIntenChN** computed from reference well results
RefTargetVarIntenChNLevel***Low/High***	Low/High response level for **TargetVarIntenChN** computed from reference well results

Table 19. *Reference Well Features available for the Tube Formation BioApplication. *Note that* ***RefAngiogenicIndexCh1*** *has only option for Level High and that "ChN" refers to Channels 3-4.*

Chapter

3

Using the Tube Formation BioApplication

The Tube Formation BioApplication comes with example protocols and image set for the use case introduced in Chapter 1. This chapter describes configuring the Tube Formation BioApplication example biology introduced in Chapter 1 and will guide you in optimizing the protocols for this example. This will demonstrate configuring this application for a tube formation biological situation, so that you can use a similar approach in optimizing the application toward your own particular biology.

The protocol supplied with the BioApplication is an example protocol that you are encouraged to modify and optimize toward your own particular biological situation.

The Tube Formation BioApplication is for scientists who want a versatile tool that they can apply specifically to tube formation assays. The BioApplication provides the flexibility to configure protocols to measure one's particular angiogenic tube formation experiment. A familiarity with the use of the Scan software application and optimizing protocols for different situations, magnifications, and dyes is assumed. If you are unfamiliar with these procedures, please refer to the appropriate User's Guide for your system for instructions. Implementation of the Tube Formation BioApplication is meant to be relatively simple since it is a specific BioApplication that has been designed specifically for angiogenic tube formation biologies. This chapter starts with a brief discussion on plating options and fluorescent labels for tube identification, and then is followed by the section describing the biological example. Here we describe the assay design, discuss the choice of labels, and cover special issues for image acquisition. This is followed by a discussion of key issues to consider when setting up the application's protocol for quantitative analysis. Quantitative results from applying the application to the example biology are presented next. Lastly, the key parameter values and protocol settings for the particular biology example, which are supplied with the Tube Formation BioApplication, are listed.

Assay-Specific Procedures for Optimizing the BioApplication

Channel 1 Input Parameters & Object Identification

A critical step in the use of the Tube Formation BioApplication is the proper identification of objects. The Channel 1 fluorescent label defines the tube, and the majority of the Channel 1 input parameters control the correct identification of tubes. Before automated scanning and analysis of plates can be performed, care must be taken to properly set these parameters based on sample images from your plate. Improper object identification will affect the accuracy of the analysis and the results reported from this BioApplication. Thus, the proper functioning of this BioApplication critically depends on proper object identification.

The issues that need to be considered in setting up parameters for proper tube identification, in their correct sequence, are:

- Tube Detect Radius
- Tube Merge Size
- Connected Tube Minimum Length
- Tube Node Minimum Branch Length
- Tube Node Minimum Distance
- Intensity Thresholding
- Object Selection Parameters

The Channel 1 input parameters that control these items are discussed in Table 20.

Channel 1 Input Parameters	
Basic Assay Parameters	**Object Identification & Selection Parameters**
General Assay Parameters	**Channel 1 Object Identification**
UseReferenceWells	Intensity Threshold (method & value)
BackgroundCorrectionCh1	**Channel 1 Tube Selection**
Channel 1 Specific Assay Parameters	TubeAreaCh1
TubeSmoothFactorCh1	TubeLengthCh1
TubeDetectRadiusCh1	TubeWidthCh1
TubeMergeSizeCh1	TubeNodeCountCh1
MinTubeNodeBranchLengthCh1	TubeAvgIntenCh1
MinTubeNodeDistanceCh1	TubeTotalIntenCh1
MinConnectedTubeLengthCh1	

Table 20. *Channel 1 Input Parameters available for the Tube Formation BioApplication.*

Step 1: Smoothing

Channel 1 and Channel 2 have independent Assay Parameters that control the degree of image smoothing, or blurring, before the identification of tubes (Channel 1) or targets (Channel 2). These Assay Parameters are called **TubeSmoothFactorCh1** and **PeakSmoothFactorCh2** for Channels 1 and 2 respectively. The value represents the degree of image smoothing (blurring) prior to tube detection in Channel. A value of **0** means that smoothing is not applied. This parameter is used to smooth images with a lot of contrast to improve identification of tubes (Channel 1) or targets (Channel 2). To get sharper definition of the shapes of the edges of tubes, you may want to keep the **TubeSmoothFactorCh1** small, if not 0. However, if your Channel 1 label is not very homogeneous, the actual tube could be erroneously identified as consisting of several smaller-sized rejected objects rather than one tube. Smoothing will result in a homogenizing effect of the Channel 1 label and will help identify the actual tube with its true boundaries.

Steps 2-4: Tube Detection Parameters

Channel 1 has three parameters that must be set in order for the algorithm to detect the appropriate angiogenic tubes:

- **Step 2: TubeDetectRadiusCh1**
- **Step 3: TubeMergeSizeCh1**
- **Step 4: MinConnectedTubeLengthCh1**

TubeDetectRadiusCh1 is the radius (in pixels) of the maximum region used for tube detection. The parameter should be set to the largest width of an object that you want the algorithm to identify as a tube. Some node (branch point) areas may have a relatively large width; in order to properly define these areas to be part of the tube the **TubeDetectRadiusCh1** must be set to the appropriate size.

TubeMergeSizeCh1 is defined as the width of the gap (in pixels) between tubes that needs to be filled. These gaps may be sections of dim staining in the tube which were not detected, and thus appear as a gap or a break in the tube. This parameter allows gaps between areas of the tube to be automatically filled by the algorithm. **TubeMergeSizeCh1** can be thought of as smoothing for tube length at junctions and narrow points along a tube's length.

MinConnectedTubeLengthCh1 is the minimum tube length (in pixels or micrometers) for the tube to be identified as a connected tube. Any tube whose length is shorter than this value (but still longer than the lower value of the **TubeLengthCh1** object selection parameter) will instead be defined as an unconnected tube, and will be analyzed accordingly. Figure 8 illustrates the differences between connected and unconnected tubes based on a **MinConnectedTubeLengthCh1** = 300 μm and a **TubeMergeSizeCh1** = **1**.

Steps 5 & 6: Tube Branching

Tube branch points are called **nodes**. The **MinTubeNodeBranchLengthCh1** and **MinTubeNodeDistanceCh1** Assay Parameters control what is defined as a node.

MinTubeNodeBranchLengthCh1 is the minimum branch length (in pixels or micrometers) to be used for tube node identification; if an offshoot from the main trunk of the tube is longer than this Assay Parameter, it is defined as a valid branch, and the offshoot point is a valid node. This parameter is used to prevent short offshoots from being defined as valid branches. The user can also let the algorithm automatically identify, in an adaptive manner, what should be defined as a valid branch. This is done by setting the **MinTubeNodeBranchLengthCh1** Assay Parameter to **0**. In this default case, the algorithm automatically compares the potential branch's length with the local width of the trunk of the main tube; if the branch length is longer than this local width, then it is defined as being a valid branch and the point is defined as a node. Otherwise, the user can manually enter a value between 1-512 (pixels or micrometers) to designate the minimum branch length the algorithm will use to designate a node to be valid. Once the number is set manually (1-512) the algorithm will apply this value to all tubes.

MinTubeNodeDistanceCh1 is defined as the minimal distance (in pixels or micrometers) between nodes. The average node distance is computed as the average length of all segments that connect different nodes. Two or more tube nodes are merged into one tube node if:

- the distance between any two tube nodes is less than the **MinTubeNodeDistanceCh1** Assay Parameter, or
- the distance between any two tube nodes is less than tube width at the location of the nodes, in the case of **MinTubeNodeDistanceCh1** = **0** (Figure 14).

This Assay Parameter helps clarify what are nodes in the situation of large nodes with multiple branches.

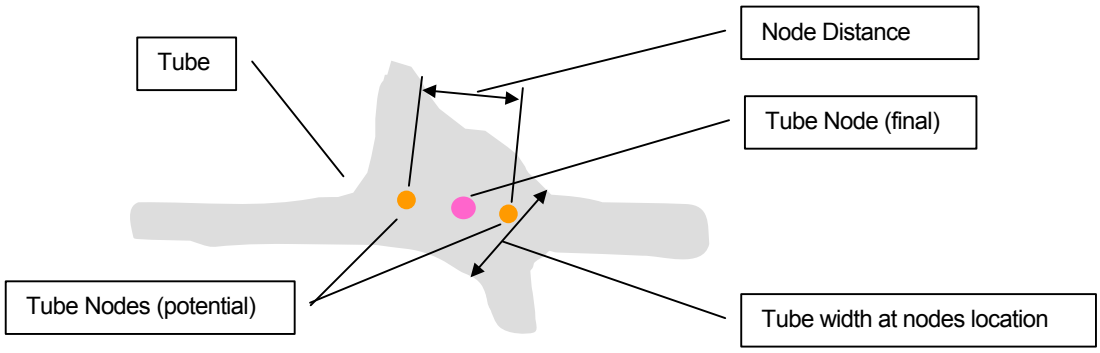

Figure 14. *Tube Node identification.*

Step 7: Intensity Thresholding

Two Channel 1 Object Identification Parameters enable the correct Intensity Threshold to be applied; please refer to Chapter 2 which discussed the available intensity threshold options (Table 4 and Table 5). The intensity threshold must be set so that the pixels making up the object are above the threshold and are thus selected for analysis. If the threshold is set too high, it is possible that some of the dimmer pixels in the object will be excluded. Conversely, setting the threshold too low causes non cell-associated areas that contain background pixels to be included as part of the object.

Step 8: Channel 1 Object Selection Parameters

Once all objects in Channel 1are properly separated and resolved, the final step is to determine which of the many potential objects in Channel 1 are valid and should be quantitatively analyzed and which ones are either junk or otherwise not valid for analysis. This is done by the Channel 1 Object Selection Parameters which are used to select which of the fluorescently-labeled primary objects in Channel 1 pass the selection criteria and are thus identified as individual, cell-associated primary objects and designated for further analysis. There are two categories of Channel 1 Object Selection Parameters:

- Intensity
- Morphology

The average and total intensities of the primary tube are intensity-related criteria used to select tubes and are controlled by the **TubeAvgIntenCh1**, **TubeVarIntenCh1** and **TubeTotalIntenCh1** Object Selection Parameters respectively. The morphology-related Object Selection Parameters are **TubeAreaCh1**, **TubeLengthCh1**, **TubeWidthCh1**, and **TubeNodeCountCh1**. If a tube's average and total intensities and its **TubeAreaCh1**, **TubeLengthCh1**, **TubeWidthCh1**, and **TubeNodeCountCh1** are all within the specified range, then it is selected as a valid tube for further analysis.

For example, suppose the tubes being selected are angiogenic tubes where the Channel 1 label is rhodamine-conjugated phalloidin to label F-actin (Figure 2). Valid objects can be selected on the basis of intensity, where only objects that have a certain average and total intensity are accepted. Furthermore, since connected tubes are long, tubular objects, further object selection can be done on the basis of their morphology. Thus, valid objects are further selected on the

basis of their having a sufficient dimension by being larger than a minimum **TubeAreaCh1** and having an appropriate **TubeLengthCh1**, **TubeWidthCh1,** and **TubeNodeCountCh1** that corresponds to a tubular structure. Based on the **TubeLengthCh1** object selection parameter and the **MinConnectedTubeLengthCh1** assay parameter, you can set the definitions of what are rejected objects, unconnected tubes and connected tubes: See Table 21 for an example.

Object Length	Object Definition
Object Length **<** lower value **TubeLengthCh1** Object Selection Parameter OR Object Length **>** upper value **TubeLengthCh1** Object Selection Parameter	Rejected Object
Lower value **TubeLengthCh1** Object Selection Parameter **<** Object Length **<** **MinConnectedTubeLengthCh1** Assay Parameter **<** upper value **TubeLengthCh1**	Selected Object Unconnected Tube
MinConnectedTubeLengthCh1 Assay Parameter **<** Object Length **<** upper value **TubeLengthCh1**	Selected Object Connected Tube

Table 21. *Defining Rejected Objects, Unconnected Tubes and Connected Tubes*

Channel 2 Input Parameters

The input parameters for Channel 2, shown in Table 22, mainly control identification and quantification of the targets (e.g., nuclei) that are part of the Channel 1 tubes. Issues that need to be considered in setting up parameters for proper identification and quantification of Channel 2 targets, in their correct sequence, are:

- Peak Detect Radius
- UseTargetMaskCh2
- Mask Modification
- Intensity Thresholding
- Object Selection

Channel 2 Input Parameters	
Basic Assay Parameters	**Object Identification & Selection Parameters**
SmoothFactorCh2	**Object Identification**
PeakDetectRadiusCh2	Intensity Threshold (method & value)
UseTargetMaskCh2	**Object Selection Parameters**
MaskModifierCh2	AvgIntenCh2
	TotalIntenCh2

Table 22. *Channel 2 Input Parameters for the Tube Formation BioApplication.*

Step 1: Smoothing

The **SmoothFactorCh2** Assay Parameter allows you to control the degree of image smoothing prior to target identification in Channel 2. This Assay Parameter was discussed in detail in the Smoothing sub-section for Channel 1 Assay Parameters.

Step 2: Peak Detect Radius Channel 2

To detect targets, the BioApplication uses the radius (in pixels) of the largest typical expected target to search for the peaks of the targets. This radius is the **PeakDetectRadiusCh2** Assay Parameter. Use this to count the number of targets (e.g., nuclei) within a tube. It is necessary to set the PeakDetectRadiusCh2 to a reasonable number based on the particular target of interest. Smaller targets should have smaller values for **PeakDetectRadiusCh2**. For example, if Hoechst is being used to identify nuclei as the target in Channel 2 using a 5X objective the **PeakDetectRadiusCh2** should be set to **1**. *Hint*: It is better to have the **PeakDetectRadiusCh2** value set on the low side (i.e., 1) so as not to miss the target of interest.

Step 3: UseTargetMaskCh2

If the **UseTargetMaskCh2** Assay Parameter is turned on (set to 1), it results in the creation of a measurement region mask based on the target's intensity. In this case, the Channel 2 target mask is created using one of object identification methods (i.e., Isodata, Triang, or Fixed). If **UseTargetMaskCh2** is turned off (set to **0**) the features in Channel 2 are measured within the modified object Channel 1 tube mask. Either mask can be carried over for analysis of intensity measurements in Channel 3 and Channel 4. Figure 9 illustrates the difference when using the Channel 1 tube mask versus the **UseTargetMaskCh2**.

Step 4: Mask Modification

The BioApplication needs to define the Channel 2 tube area in which peak detection occurs. This can be derived from the tube area from Channel 1; additionally you have the ability to modify the region covered or it can be created by applying the Object Identification method in Channel 2. You have the option of similarly independently modifying (i.e., expanding or contracting) the tube area in which measurements are made for Channel 3 and Channel 4. The Assay Parameters that are used to adjust the tube area in which to make measurements for Channels 2-4 are **MaskModifierCh*N***, where *N* is the relevant dependent channel (i.e., Channels 2-4). This parameter is the number of pixels to modify the Channel 1 object (tube) mask in Channel 2: Negative value = Shrink mask, **0** = Do not modify mask, Positive value = Expand mask. Thus, this parameter is the number of pixels added to, or removed from (depending on the parameter's sign) the perimeter of the tube area identified in Channel 1.

Step 5: Intensity Thresholding

The intensity threshold in Channel 2 is used only if the **UseTargetMaskCh2** Assay Parameter is turned on (set to **1**) so as to define the measurement region based on the target mask. The correct Intensity Threshold is set by Channel 2 Object Identification Method and Value parameters and the different available intensity threshold options were discussed in Chapter 2 (Table 4 and Table 5). The intensity threshold must be set so that the intensity of the pixels making up the targets is above the threshold. However, setting the threshold too high results in the exclusion of some of the dimmer pixels, and conversely, setting the threshold too low causes non-target-associated areas that only contain background pixels to be included as part of the tube. Note, if using a **Fixed Threshold** method and a value of **0** is entered, then all the pixels within the tube mask will be chosen. If the **UseTargetMaskCh2** Assay Parameter is not turned on (set to **0**), then the measurement region will be derived from the Channel 1 tube mask, and no threshold will be applied to Channel 2. In this case, the tube average and total intensity reported in Channel 2 will be independent of whatever is entered for the Channel 2 intensity threshold; for these features, the intensity for all pixels in the modified tube area is used for analysis, whether or not targets are identified.

Step 6: Object Selection in Channel 2

The last step is to set the Channel 2 Object Selection Parameters to set appropriate tube identification and selection parameters. In many cases, the fluorescent stain used to label the target will also give a background fluorescence staining of the tube, which can be detected in Channel 2. You may use this as a further criterion to select the tube for analysis, and the basis for this selection are the average and total intensities of the tube detected in Channel 2. These are controlled by the **AvgIntenCh2** and **TotalIntenCh2** Object Selection Parameters. The tube fluorescence in Channel 2 must fall within the intensity ranges specified by these two parameters for the tube to be selected for further analysis. These parameters can be used to gate which tubes are chosen for analysis based on the overall tube expression levels of particular targets. Alternatively, if you are satisfied by the tube being selected for analysis by the criteria set in Channel 1 and you do not want to apply any further selection criteria based on the fluorescence in Channel 2, you can leave the gates open for these parameters (i.e., keep the minimum values of these two Object Selection Parameters at their lowest possible level and similarly set their maximum values to the highest possible level).

If the **UseTargetMaskCh2** Assay Parameter is not turned on (set to **0**), then the measurement region will be derived from the Channel 1 tube mask, and the average and total intensity reported in Channel 2 is independent of the Channel 2 intensity threshold and is only controlled by the **AvgIntenCh2** and **TotalIntenCh2** Object Selection Parameters. For these features, the intensity for all pixels in the modified mask of the object area from Channel 1 is used for analysis. If the **UseTargetMaskCh2** Assay Parameter is turned on (set to **1**), the measurement region mask is based on the target's intensity which is controlled by threshold values, and this will also affect the measurements of the average and total intensities in Channel 2.

Channel 3 and Channel 4 Input Parameters

The categories of input parameters controlling quantification in Channel 3 and Channel 4 shown in Table 23 are limited to Target Mask Channel 2 or Channel 1 Mask Modification only in Channel 3 and Channel 4. See the previous section for full description of Channel 2 Target Mask Modification.

Channel 3 and Channel 4 Input Parameters		
Basic Assay Parameters		**Object Identification & Selection Parameters**
Channel 3	**Channel 4**	**Object Selection Parameters (Objects)**
MaskModifierCh3	MaskModifierCh4	AvgIntenCh3
UseTargetMaskCh2inCh3	UseTargetMaskCh2inCh4	TotalIntenCh3

Table 23. *Channel 3and Channel 4 Input Parameters available for the Tube Formation BioApplication.*

Object Selection

The **AvgIntenCh3** and **TotalIntenCh3** Object Selection Parameters control object selection in Channel 3, and an analogous set is available for Channel 4. As in Channel 2, the fluorescent stain used to label proteins in Channel 3 or Channel 4 may be used as a further criterion to select the tube for analysis. The basis for this selection is the average and total intensities of the tube detected and are controlled by the **AvgIntenCh3** and **TotalIntenCh3**. The tube fluorescence in Channel 3 must fall within the intensity ranges specified by these two parameters for the object to be selected for further analysis. These parameters can be used to

gate which objects are chosen for analysis based on the overall object expression levels of particular proteins. Alternatively, if you are satisfied by the object being selected for analysis by the criteria set in Channel 1 and you do not want to apply any further selection criteria based on the fluorescence in Channel 3, you can leave the gates open for these parameters, i.e., keep the minimum values of these two Object Selection Parameters at their lowest possible level and, similarly, set their maximum values to the highest possible level.

UseTargetMaskCh2inCh*N*

UseTargetMaskCh2inCh3 and **UseTargetMaskCh2inCh4** can be turned on (set to **1**) allowing the application of the Channel 2 target mask created in Channel 3 or Channel 4 (Figure 9). If **UseTargetMaskCh2** is turned off (set to **0**) the intensities are measured within the modified object Channel 1 tube mask. Channel 3 and Channel 4 masks are set independently of one another.

Mask Modification

The **MaskModifierCh*N***, where *N* is the relevant dependent channel controls the area in which measurements are made in Channels 3 or Channel 4, as described earlier. The value is the number of pixels to modify Channel 1 object (tube) mask in Channel *N*: Negative value = Shrink mask, **0** = Do not modify mask, Positive value = Expand mask. Thus, this Mask Modification will control the dilation or erosion of either the Channel 1 tube mask or Channel 2 target mask defined by the **UseTargetMaskCh2inCh*N*** Assay Parameter.

Basement Membrane and Plate Options

In Chapter 1 we discussed the need to grow the endothelial cells on a reconstituted basement membrane layer for angiogenesis to occur. We were successful using the Matrigel basement membrane from BD Biosciences to obtain angiogenic tube growth using human endothelial cells. We either coated 96-well plates ourselves with Matrigel obtained from BD Biosciences (Fisher cat# CB-40230), or used their BioCoat™ Angiogenesis System Matrigel coated plates (Fisher cat# 08-774-376). Both types of Matrigel plates performed well and have numerous references to usage in the literature and give a broad range of options for the user. The typical long, branched angiogenic tubes formed on both after 12-16 hours from the time of plating the cells onto the membrane in comparison testing on both plate types. The results shown in Figures 2, 8, and 9 are from experiments done on sample plates that we coated ourselves with Matrigel, so we will describe our protocol for this system. Our sample preparation protocols are similar to the protocols provided by BD Biosciences' protocols for their Matrigel systems, and references for similar experimental protocols were found in the literature (Wu et al., 2000; Sanz et al., 2002). Briefly, a thin Matrigel layer is polymerized to the 96-well plate for 30 minutes. Human endothelial cell lines, HUVEC or HMVEC, were plated at high density (~$2x10^4$ cells/well) onto the polymerized Matrigel membrane in media containing the appropriate pro- or anti-angiogenic compound. Twelve to sixteen hours later the tubes were fixed, permeabilized, and labeled while still attached to the Matrigel. Additional drying of the sample flattened it, enabling more fields per well to be imaged. The plates were then loaded and run on an ArrayScan® HCS Reader.

The Matrigel on the self-coated plates is diluted 1:2 and plated at 35 µL/well giving a final concentration of 12 mg/ml and a depth of 0.92 mm of Matrigel. The BD BioCoat Angiogenesis System plates seem to have a thicker Matrigel coating than our self-coated Matrigel plates. Overall, both plate systems were found to work well and were compatible with the Cellomics Tube Formation BioApplication. Note: The BD BioCoat Matrigel Matrix, thin layer 96-well assay plates (cat #354607) are not well suited for the *in vitro* angiogenesis studies developed for the Tube Formation BioApplication.

Fixation and Labeling Of Angiogenic Tubes

Fixing the delicate tubes on the Matrigel membrane

In our initial attempts to fix the angiogenic tubes, we found it not feasible to use a basic 3.7% formaldehyde fixation method. Intact, healthy tubes observed in live cells disintegrated when subjected to a standard 3.7% formaldehyde fixation method, and 50-60% of the tubular structures were lost when fixed with 3.7% formaldehyde (Figure 15). Because of the tubes' fragile nature and the Matrigel membrane on which they form, it was necessary to find a gentler fixation method that would preserve the tubes' ultrastructure. A variety of different fixation methods were tested (Figure 15). Two methods were found to work well in preserving >90% of the angiogenic tubes on the Matrigel membrane: either fixation with 0.5% buffered paraformaldehyde, or with 0.3% glutaraldehyde. Results are summarized in Table 24.

Fixation with glutaraldehyde preserves cellular ultrastructure better than formaldehyde, and has long been a fixative of choice for electron microscopists. However, it is not popular among fluorescence microscopists due to its broad-spectrum auto-fluorescence. Treating with sodium borohydride prior to fluorescence labeling can quench the auto-fluorescence to allow multiple fluorescent probes to be used. We instead took advantage of glutaraldehyde's auto-fluorescence and used it as the fluorescence label for the angiogenic tubes. This enables a simple, quick, and economic protocol where the fixation and fluorescence labeling steps are combined by the application of a single reagent; i.e., the auto-fluorescence of the glutaraldehyde actually works to the user's advantage. If you only want to visualize the angiogenic tubes and nuclei, the cells can be fixed with glutaraldehyde and Hoechst solution, washed, and the plate can be run for a simple quick assay that takes about 20 minutes to conduct. Although the auto-fluorescence is broad, the intensity of the Hoechst dye is strong enough to supersede the auto-fluorescence in the blue channel. Figure 15 illustrates HUVEC cells, plated at $2x10^4$ cells/well on self-coated Matrigel plates, supplemented with VEGF and hFGF so they have formed angiogenic tubes, then fixed using each method.

Fixation Methods	Advantage	Disadvantage
3.7% formaldehyde	• Easy, standard method	Harsh; >50% tube loss
0.5% buffered paraformaldehyde (RECOMMENDED METHOD)	• Less harsh, • <10% tube loss	none
0.3% glutaraldehyde	• Less harsh, • <10% tube loss, • auto-fluorescence can be used as fluorescent label	If auto-fluorescence is an impediment, it must be suppressed (quench with $NaBO_4$) for multi-color labeling; non-standard wash solution necessary

Table 24. *Fixation Methods*

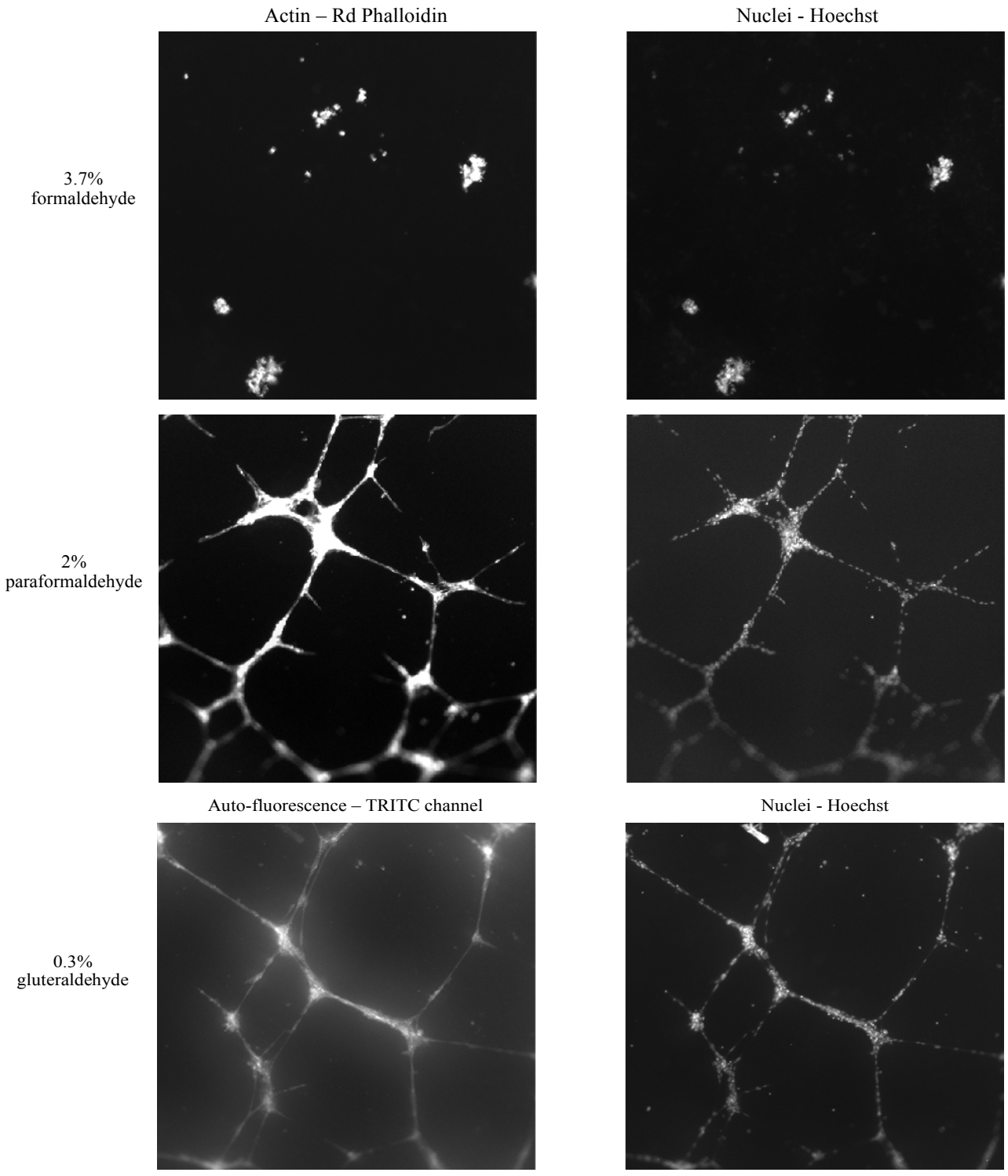

Figure 15. *Fixation method comparison. HUVEC cells were plated at 2x10^4 cells/well on self-coated Matrigel plates in media containing VEGF and FGF. Tubes were then fixed using 3.7% formaldehyde, 2% paraformaldehyde, or 0.3% glutaraldehyde. Hoechst label was added to all wells, Rd-Phalloidin was added to the formaldehyde and paraformaldehyde wells only so the auto-fluorescence of the glutaraldehyde could be assessed. Tubes were visualized using light microscopy prior to fixation; the paraformaldeyde and glutaraldehyde treated wells had similar angiogenic tube formation density. The lack of tubes in the formaldehyde set is due to the harsh fixation method.*

Channel 1 Tube Labeling Options

As discussed in Chapter 2, for the most accurate quantification of your biological problem, the proper identification of tubes in Channel 1 is critical. A key step in the proper identification of tubes is the choice of the optimal fluorescence labeling strategy to allow the tube's extent to be defined. In addition, the tube label may be specific for a certain type or property of the tube to distinguish it from other tube in the image field. Fluorescent labels for tubes can either label the tube's surface, the tube's interior cell volume or cytoplasm, or some major widespread constituent of the cell.

We find that the F-actin label, Rhodamine-conjugated phalloidin (Rd-phalloidin), works well as a fluorescent label for tubes. For this label, the cells need to be fixed and permeabilized, after which a mix of Rd-phalloidin and Hoechst can be placed on the cells for 30 minutes, followed by washing, and plate sealing for analysis. The Rd-phalloidin is a robust fluorescent marker, commonly found, and relatively economical to use. We tested the Rd-phalloidin in combination with the gluteradehyde fix; due to Rd-phalloidin's robust signal we found it enhances the auto-fluorescence signal of the glutaraldehyde, improving identification of the tube rather than labeling with just glutaraldehyde. Cell Spreading HCS Reagent Kit contains the Rd-phalloidin and Hoechst dyes, and is thus suitable for labeling samples in this assay.

If one is looking for a particular quick labeling procedure, see section above Fixation methods to utilize auto-fluorescence of glutaraldehyde fixation method.

Fluorescently conjugated lectins, such as fluorescent concanavalin A or wheat germ agglutinin, can be used to label the cell surface. Lectins give a fairly homogeneous stain over the cell's membrane and a clear definition of the cell's edge is seen. Lectins may also cause capping and aggregation of other cell surface proteins in live cells. Other cell surface stains include fluorescent lipids or lipid analogues; however in live cells, their cell surface labeling is transient as they may eventually get internalized into the cell.

Tips For Optimal Image Acquisition

Objective

Use of a 5x microscope objective for imaging is recommend, in order to get as much of the tube as possible in the field of view.

Scan Limit Settings

There are two approaches for instructing the Scan software to collect the appropriate number of valid tubes per well: well limits can be set such that the number of valid tubes exceeds a fixed number per well and field limits can be set so that a fixed number of fields are collected. In the first approach, the number of fields will vary in each well depending upon how many fields were required to accumulate the number of valid cells that you defined. In the second approach, the actual number of valid tubes will vary depending on the density in each well and the number of fields will be identical. Due to focusing limitations from using Matrigel-like basement membranes, we recommend that the user set scan limits based on number of fields. Scan limits based on collecting a specific number of objects (tubes) is not recommended, because curvature of basement membranes may cause tubes to be out of focus in fields away from the central field, and thus the requisite number of objects may not be identified and collected. The number of in-focus fields in a well is limited by the user's biological design.

Example Biology Details

Assay Design

Capillary-like structures are formed when human endothelial cells differentiate and fuse with each other to form long, multinucleated angiogenic tubes. HUVEC cells are pooled endothelial cells from human umbilical vein samples (available from Cambrex, Inc). We have also successfully used HMVEC cells, another standard endothelial cell line (also available from Cambrex). These two cell types were carried in Endothelial Growth Medium-2 MV (EGM-2MV), also available from Cambrex, which contains the necessary supplements to support their growth. Complete EGM-2MV for our purposes includes the following items from the EGM-2MV Bullet Kit from Cambrex: 2% FBS, hFGF (0.5 mL), R^3-IGF-1 (0.5ml), hydrocortisone (0.2ml), ascorbic acid (0.5 mL), hFGF (0.5 mL), GA-1000 (0.5 mL) (VEGF and hFGF were excluded because of their pro-angiogenic effects). The cells were carried in culture in complete EGM-2, without VEGF and FGF, prior to plating. The cells were plated onto the polymerized Matrigel basement membrane at $2x10^4$ cells/well in complete media containing VEGF/hFGF, suramin, or complete media only. After 16 hours, the angiogenic tube networks had formed and the plates were fixed, permeabilized, and labeled appropriately.

For the 2-color demonstration experiment (Figure 2, 8 & 9), HUVEC cells were stained using Cell Spreading HCS Reagent Kit which contains a Rhodamine conjugated phalloidin to label the F-actin in **Channel 1**, and Hoechst 33342 to label the nuclei in **Channel 2**. All angiogenic tubes were labeled with the Rhodamine conjugated phalloidin. All cell nuclei were labeled with Hoechst.

- ***Control cells****:* HUVEC cells
- ***Anti-angiogenic Compound - Suramin****:* The inhibition of angiogenic tube formation took place when densely plated HUVEC cells were treated with 5 µM suramin for 16 hours. The results were very few complex angiogenic tube networks; mostly individual cells and small clumps were present.
- ***Pro-angiogenic compounds – VEGF & hFGF****:* Angiogenic tube formation was increased when the pro-angiogenic compounds, VEGF and hFGF, were added to the plating medium for 16 hours. The wells treated with these compounds showed a higher tube density and angiogenic index.

Figure 2 has images of the compound treatments acquired with a 5x objective on the ArrayScan HCS Reader 4.0. The assay strategy is summarized in Table 25. The difference between mature, long, multinucleated network of angiogenic tubes grown in VEGF/hFGF versus the suramin treated undifferentiated, unconnected HUVEC cell clusters with limited angiogenic networking is clear in these images.

Channel	Cellular Target	Detection Strategy & Fluorophore	Fluorescence Emission Color
Channel 1	Whole tube marker – F-actin	Rhodamine conjugated phalloidin	Red
Channel 2	Nuclei of all cells	DNA binding dye - Hoechst 33342	Blue

Table 25. *Assay Strategy*

Analysis Strategy and Key Items in Protocol

Channel 1 Tube Identification

The Channel 1 tubes to be identified are the angiogenic tubes (Figure 2). They are visibly different from the undifferentiated cells and clumps in the field. The tubes have a bright fluorescent label, and are long, multicellular (i.e., multi-nucleated) connected tubes, with numerous branch points and nodes. These properties are used to specifically identify them as connected tubes. In wells treated with suramin, angiogenesis was inhibited and there were very few long, connected tubes. Most of the objects were categorized as unconnected tubes or rejected objects, falling outside the ranges specified by the Object Selection Parameters and Assay Parameters (Figure 2).

The Channel 1 Object Selection Parameters are used to specifically identify tubes based on their intensity, shape, and area. For a tube to be positively identified, its tube area, length, width, and tube node count need to be above certain values. Because angiogenic tubes comprise hundreds of cells fused together, their length must be above a certain threshold; individual cells and small clumps of cells would have lengths less than this threshold and would not be selected. Thus, for the sample image sets acquired at 5x magnification a minimum length threshold (**TubeLengthCh1** Object Selection Parameter) of 300 µm was chosen as an to classify an object as a tube, and the **MinConnectedTubeLengthCh1** Assay Parameter was set at 1000 µm to distinguish between unconnected and connected tubes.

Channel 2 Target Identification

The nuclei in Channel 2 are very small in the example images acquired with the 5x objective. The three-dimensional structure of the angiogenic tubes causes the nuclei to be piled on top of one another, particularly at the nodes of the tube. These factors prevent individual nuclei in multi-nucleated angiogenic tubes from being resolved with great accuracy. Therefore, it is only feasible for us to estimate the number of cells (i.e., nuclei) in the tube via the **PeakCountCh2** feature.

Chapter

4

iDev Software Workflow

This chapter describes the tasks in the workflow for Protocol optimization of the Tube Formation V4 BioApplication using the iDev software. More detailed technical descriptions of each Assay Parameter can be found in Chapter 2 of this guide.

iDev Protocol Optimization Tasks

The iDev Assay Development software provides guided workflows for Assay Protocol optimization. Assay Parameters which are user adjustable input parameters that control image analysis are organized in a task list. Each task contains only the Assay Parameters necessary for optimization of that task and has been designed to walk you through a defined workflow for Protocol optimization. Below are basic descriptions of each task and a summary of the Assay Parameters contained within that task. Chapter 2 describes the technical feature of each of the Assay Parameters in greater detail.

Image Preprocessing

Image Preprocessing is the processing of images prior to object identification. The task comprises background removal for all selected channels. In this task, you can set background removal methods and values for each of the channels in the Protocol.

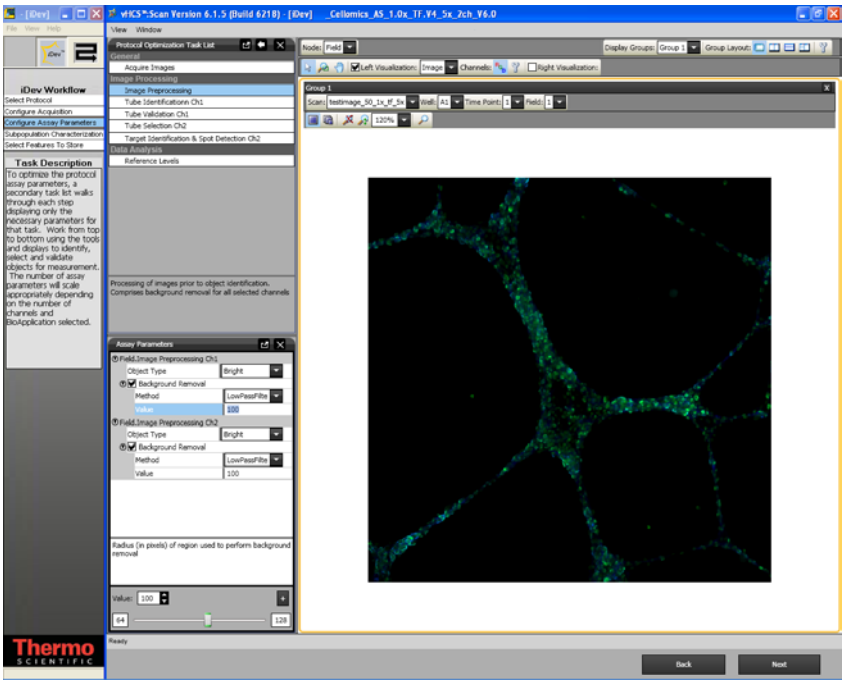

Figure 16. *Protocol Optimization Task – Image Preprocessing*

Tube Identification Ch1

Tube Identification is the identification of tubes in the Channel 1 image. The task involves setting up methods and values for primary object smoothing and object identification for Channel 1. In addition you can set criteria for analysis of connecting tube fragments, number of tube node branches, tube node distance, and connected tube length in this task.

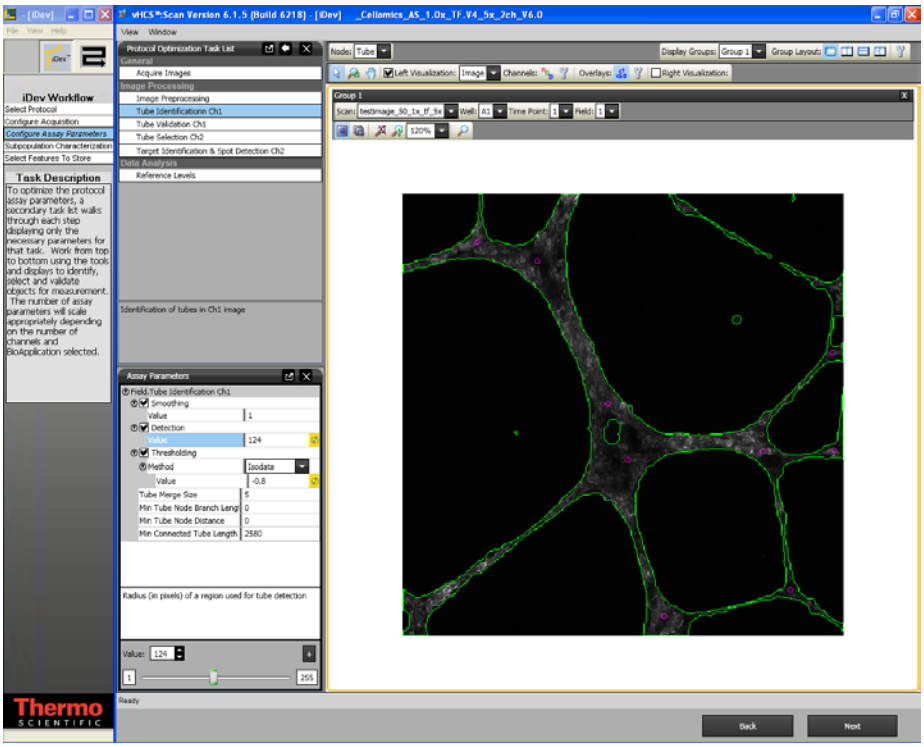

Figure 17. *Protocol Optimization Task – Tube Identification Ch1*

Tube Validation Ch1

Tube Validation is tube selection/rejection based on area, shape, and intensity features calculated for the tubes in Channel 1. In this task, you will set minimal and maximal values for validating (equivalent to selecting and rejecting objects in the ArrayScan Classic software) tubes in Channel 1 based on object selection features.

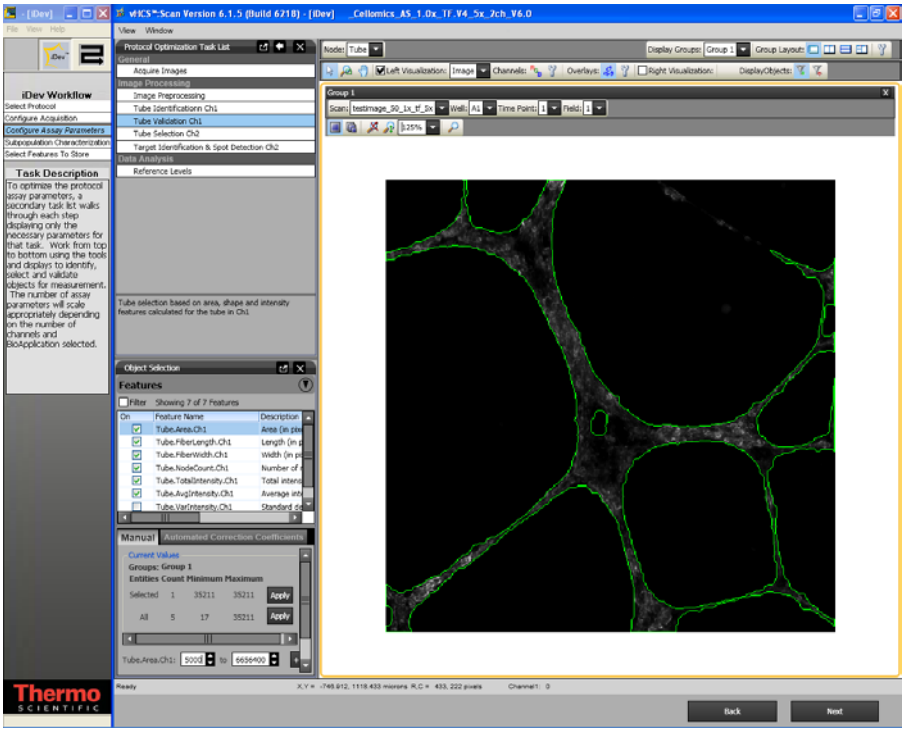

Figure 18. *Protocol Optimization Task – Tube Validation Ch1*

Tube Selection Ch2 through ChN

The Tube Selection task is tube selection based on intensity features computed in Channels 2 through Channel N under the circ mask derived from the Channel 1 primary object mask. In this task, you will set selection / rejection of the primary object based on intensity measurements in Channels 2 through Channel N under a modified primary object mask. This task is similar to setting the Assay Parameter, **MaskModifierCh2,** in the ArrayScan Classic software. The primary object mask can be dilated (**Circ** > 0), or eroded (**Circ** < 0) if the **ROI Mask Creation** box is checked.

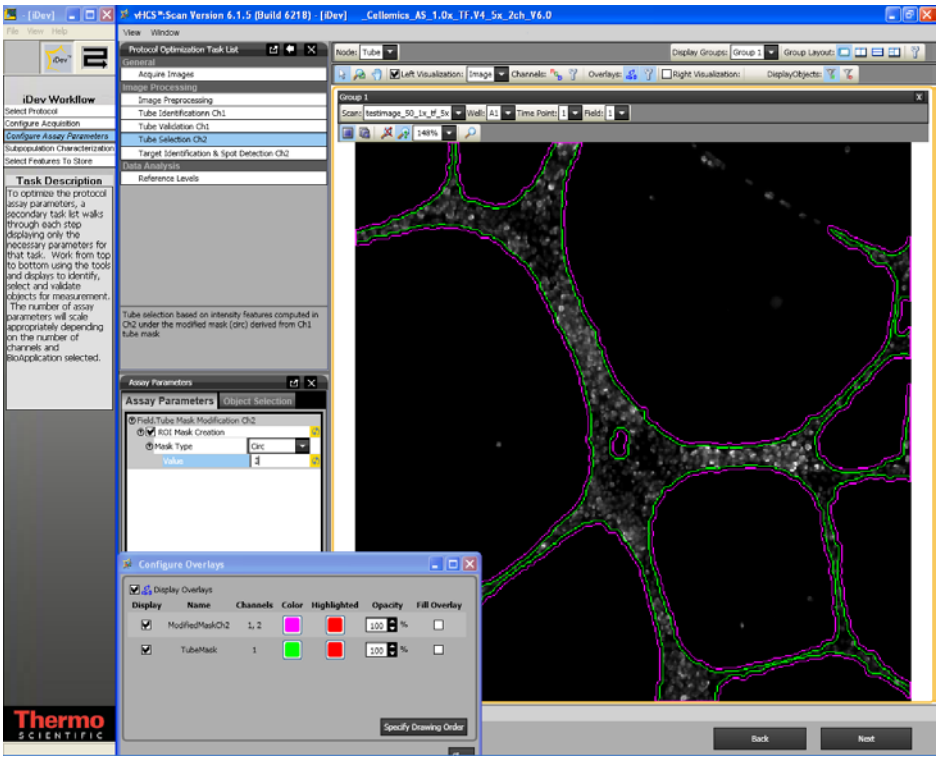

Figure 19. *Protocol Optimization Task – Tube Selection Ch2 through ChN*

Target Identification and Spot Detection Ch2

In this task, you can set parameters for identification and analysis of targets and spots. Modified tube mask from Channel 1 or target mask from Channel 2 can be used. You can set values for smoothing, detection, and thresholding for targets in Channel 2.

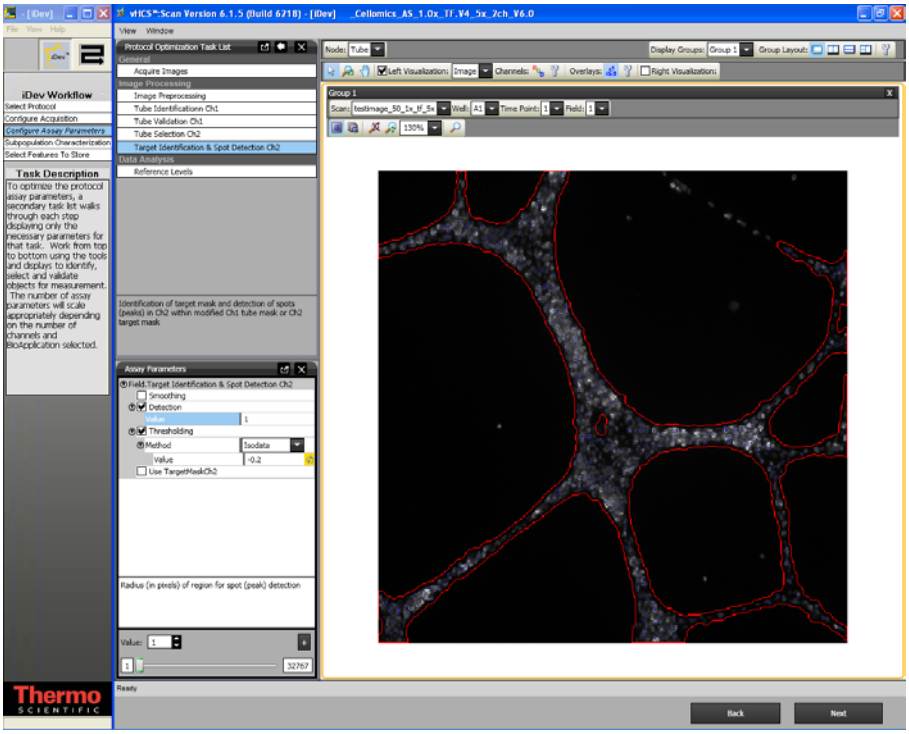

Figure 20. *Protocol Optimization Task – Target Identification and Spot Detection*

Target Analysis Ch3 through ChN

In this task, you can set Assay Parameters for analysis of additional targets in Channels 3 through Channel N under a modified Channel 1 mask or under the Channel 2 target mask (by checking the **UseTargetMaskCh2** button).

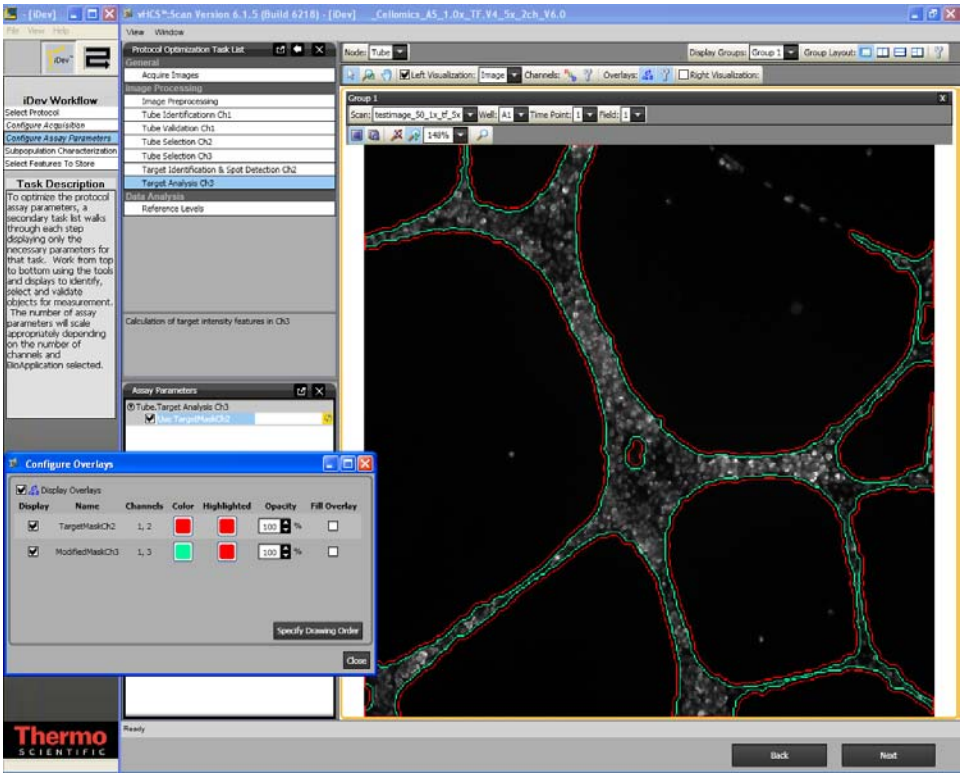

Figure 21. *Protocol Optimization Task – Target Analysis Ch3 through ChN*

Reference Levels

In this task, you can set level high and level low for computing % responders and/or %High and %Low based on several calculated Reference Level features. Reference Levels can be manually or automatically set by adjusting the level value or correction coefficient under the Reference Level task and by checking the **Use Reference Wells** option in the Subpopulation Characterization task.

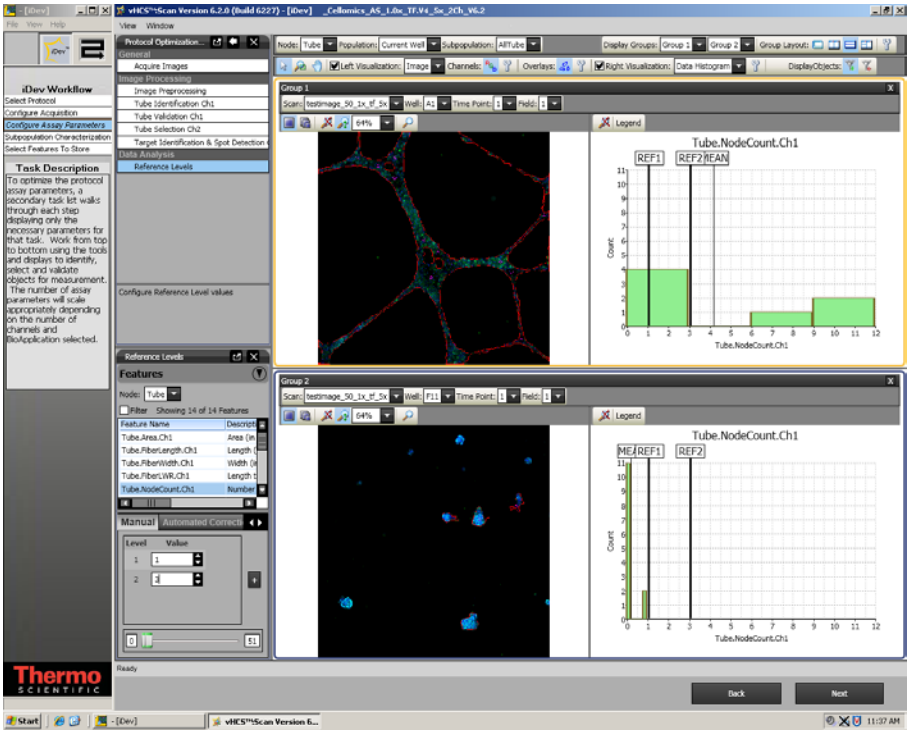

Figure 23. *Protocol Optimization Task – Reference Levels*

Appendix

A

Materials and Methods

Materials and Recommended Suppliers

Basement membrane evaluation:

- BD BioCoat Angiogenesis System: Endothelial Cell Tube Formation – Fisher cat# 08-774-376
- Self coated Matrigel plates – components below
  - 96-well Packard View Plate – Pierce cat# 6005182
  - BD Matrigel Matrix (10ml) – Fisher cat# CB-40230
  - Basel EGM-2 media- Cambrex cat# CC-3156

Endothelial cell lines:

- HUVEC – normal human umbilical vein endothelial cells, pooled, cryopreserved – Cambrex cat# C2519A
- HMVECd – human dermal microvascular endothelial cells – Cambrex cat# CC-2543
- Microvascular Endothelial Growth Medium –2 (EGM-2 MV) Bullet Kit (basal media, growth factors, cytokines, and supplements packaged together) - Cambrex cat# CC-3202

Fixatives:

- Formaldehyde – 37% aq.soln – Sigma cat# F1268
- Glutaraldehyde – 25% aq.soln – Fisher cat# BP2548-1
- Paraformaldehyde – Sigma cat# P6148

Cell markers:

- Cell Spreading HCS Reagent Kit – cat# K06-0001-1 (Evaluation size) and R02-0003-1 (Screening size). Components include:
  - Rhodamine-phalloidin
  - Hoechst

Anti- and Pro-angiogenic factors:

- VEGF – vascular endothelial growth factor – R&D Systems, cat# 2347-VE
- Suramin – Cal Biochem cat# 574625

Buffer:

- HBSS – BioWhittaker
- PBS - BioWhittaker

Solutions

Complete Media

EGM-2 basal media + bullet kit items 2%FBS, hFGF (0.5ml) and R^3-IGF-1 (0.5ml), hydrocortisone (0.2ml), ascorbic acid (0.5ml), hFGF (0.5ml), GA-1000 (0.5ml) (VEGF and hFGF are excluded because of their pro-angiogenic effects)

Collagen Solution

Collagen 1 Rat tail (100mg - BD cat#35-4236) diluted 1:10 in basal EGM-2 medium.

2% Paraformaldehyde Stock Solution

2 g paraformaldehyde in 100 ml PBS, warm to 70°C, pH must be 7.4. Must be made fresh each day. Final solution for fixation is 0.5% buffered paraformaldehyde - use pH 7.4 PBS to dilute the stock solution. **Note:** Buffering all solutions to pH 7.4 is critical. If solutions are not properly buffered nor made fresh daily, the tubes will lift off the Matrigel.

Permeabilization Solution

0.1% Triton X100 (Sigma cat#X100) diluted in PBS

Stain Solution

Rd-phalloidin diluted 1:400 and Hoechst diluted 1:1000 in HBSS (instructions from Cellomics Cell Spreading HCS Reagent Kit).

Protocol

1) Preparing the plates:

a. If using the BD Bioscience BioCoat Angiogenesis plate, prepare plate according to manufacturer's instructions. Briefly thaw plate 4-6 hours at 4°C. Under sterile conditions, remove vacuum plate cover using forceps. Incubate plate at 37°C for 30 minutes to polymerize Matrigel basement membrane.

OR

b. To prepare self-coated Matrigel plates, thaw the appropriate amount of BD Biosciences' Matrigel overnight at 4°C. Working quickly and using pre-cooled tips, pipettes, troughs, and Packard 96-well plates, dilute the Matrigel 1:2 with cold basal EGM-2 media. Immediately add 35 µl/well of the diluted Matrigel to the 96-well plate. Gently rotate the plate to ensure the Matrigel gel membrane covers the entire surface of each well. Place plates at 37°C for 30 minutes for the gel to polymerize. Plates are now ready to use.

2) Grow primary endothelial cells (HUVEC or HMVEC) in EGM-2 MV complete media until 70-80% confluent. Trypsinize, neutralize, and spin cells accordingly. Count cells. Resuspend cells in the appropriate volume of experimental medium with your desired angiogenesis promoters or inhibitors at $4x10^5$ cells/ml.

3) Add 50 µl of the cell suspension ($2x10^4$ cells) to each well. Incubate the angiogenesis assay plate for 16-18 hours at 37°C, 5% CO_2. (For activation assays, the incubation time may be shorter.)

4) Optional: A collagen overlay can be done at the 16-hour time point if necessary. Briefly, aspirate growth medium, add 50 µl/well collagen solution, incubate at 37°C, 5% CO_2 for 1 hour. Aspirate collagen solution, wash with 100 µl basal medium.

5) Warm freshly prepared 0.5% buffered paraformaldehyde solution and HBSS (BioWhittaker) at 37°C to insure angiogenic tube stability. Aspirate medium and wash with 100 µl HBSS.

6) Aspirate, add 50 µl 0.5% paraformaldehyde to each well, and incubate at room temperature for 10 minutes. Aspirate, wash each well with 100 µl HBSS.

7) Add 50 µl permeabilization solution and incubate for 90 seconds. Aspirate, and then wash with 100 µl HBSS.

8) Add 50 µl stain solution to each well. Incubate at room temperature in the dark for 1 hour.

9) Aspirate stain solution. Wash 2X with 100 µl HBSS. Aspirate. Let plate dry in the dark, overnight. Seal plate, image on a Cellomics HCS Reader and analyze with the Cellomics Tube Formation BioApplication.

All aspiration steps should be done as gently as possible with minimal vacuum.

One of the most challenging, yet necessary, components of carrying out the experiment is the need for $2x10^6$ cells per 96-well plate. According to the product data sheet, the HUVEC and HMVEC lines cannot be cryo-preserved (i.e., frozen down) after the initial thaw and can only be carried up to 15 passages once they are thawed before their quality and performance is compromised. The need for an extremely high cell number for plating and the short productive life of the cell lines limits their use.

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