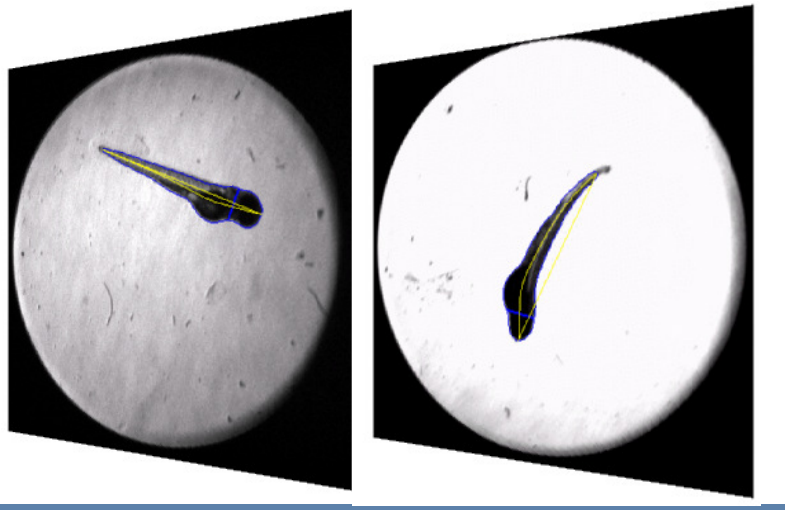
Thermo Scientific Cellomics® Zebratox

BioApplication Guide



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Cellomics® Zebratox BioApplication Guide

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Chapter

1

Overview of the Zebratox BioApplication

This BioApplication Guide provides an in depth description of the Zebratox BioApplication.

This guide contains the following chapters:

Chapter 1 provides an overview of the Zebratox BioApplication and describes biological situations to which it can be applied.

Chapter 2 describes the algorithm used to compute the quantitative results and gives a description of assay input parameters and output measurements.

Chapter 3 describes the use of the Event Wizard

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

System Compatibility

The Zebratox BioApplication described in this document is designed to run on the following platforms:

- Cellomics® ArrayScan® Platforms version VTI
- Cellomics vHCS™ Discovery Toolbox, version 1.6 and higher

Selected images from other sources (e.g., images up to 16-bits) may be imported and used with your platform.

Cell Biology Background

Zebrafish as an *in vivo* Model of Human Disease

More than 20 years ago, zebrafish (*D. rerio*) were first identified as model organisms for human disease. These small vertebrates exhibit many characteristics that make them amenable to a wide variety of research areas, including large numbers of offspring, optically clear embryos, evolutionarily conserved genes, a vulnerability to common environmental toxins, and proven methods of creating mutant embryos and fluorescently tagged proteins of interest. Today, thousands of published articles utilize these fish as models to investigate toxicology, the main focus of this Zebratox BioApplication.

Utilizing Zebrafish in Toxicology

Zebrafish have long been used in toxicology because of their sensitivity to common environmental poisons (1, 2, 6). In fact, standard toxicological tests outlined in organizational guidelines implement zebrafish as a typical model. The relatively large size of the embryos creates a favorable atmosphere for studying the teratogenesis that occurs after exposure to toxins (6). Due to their similarity to mammalian development, zebrafish have become important tools in the investigation of how environmental toxins can affect human growth and development.

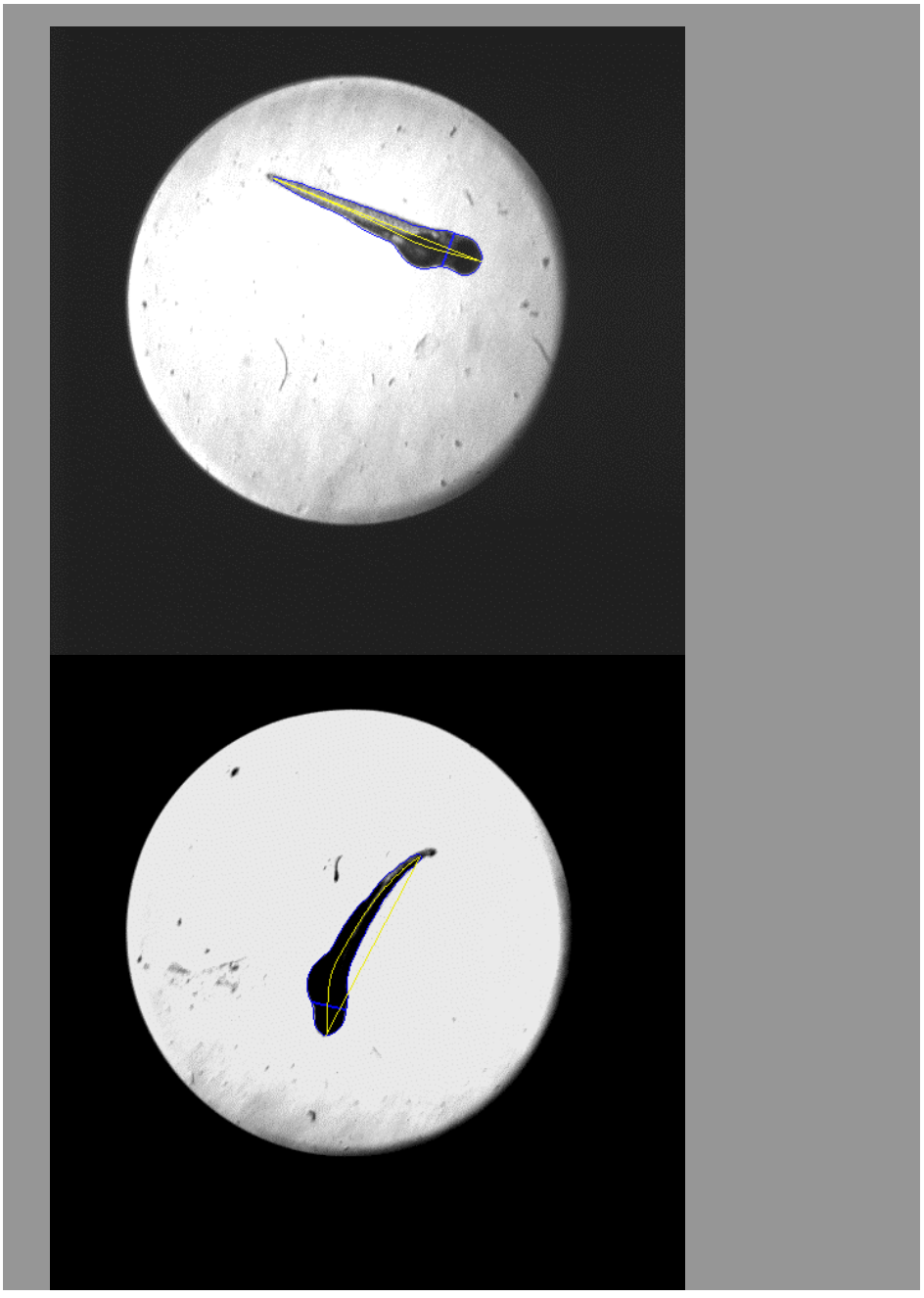

Figure 1. *Brightfield images at 1.25X magnification. Top – untreated embryo. Bottom – embryo treated 18 hours with valproic acid.*

BioApplication Overview

The Zebratox BioApplication is meant to provide automated analysis of zebrafish embryos used for toxicology work. The algorithm is designed to investigate one to five day embryos (post fertilization), with one embryo per well in standard microplates. All channels can be configured as either brightfield or fluorescent channels. In a one channel configuration, morphological measurements are performed on the entire fish, as well as specific calculations on the head and torso. These measurements are useful in the study of compound effects on this model organism because zebrafish exhibit morphology changes with both acute and chronic exposure to toxic stimuli. Additionally, the user has an option to select features related to angiogenic vessel formation in this single channel. By measuring the development of the intersegmental vessel formation, investigation of the early responses in angiogenesis development can be completed. The Zebratox BioApplication is capable of imaging fluorescence in up to 6 individual colors/ channels. A channel refers to the visualization of any fluorescent dye on the ArrayScan HCS Reader by a combination of appropriate excitation, dichroic, and emission filters (please refer to your ArrayScan User's Guide for a detailed description of dye selection). If configured as a two channel assay, Channel 2 can be used to detect spots within vessels or generally within the fish. Channels 3 through 6 are reserved for object selection purposes only. Features are summarized in Table 1 and described further in Chapter 2.

Channel	Features Measured
1 Required	Fish (head & torso) size, shape, and intensity; Angiogenic Vessel count, area, length, width , intensity; Vasculogenic Vessel area, length, width, intensity
2 Optional	Fish (head & torso) intensity; Spot (fish, head, torso, vessel) count, area, intensity
3 – 6 Optional	Object Selection

Table 1. *General description of features and identification per channel within the Zebratox BioApplication*

	Region	Features
Count	Fish	Valid Fish Count Selected Fish Count
	AngioVessel (Angiogenic)	Angio Vessel Total Count
	Spot	Fish Spot Total Count Head Spot Total Count Torso Spot Total Count Vessel Spot Total Count
Size	Fish, head, & torso	Length (curve, straight) Width Area
	Vessel (Angiogenic & Vasculogenic)	Angio_Total Length Vasculo_Total Length Angio_Avg Length Vasculo_Avg Length Angio_Total Area Vasculo_Total Area Angio_Avg Width Vasculo_Avg Width
	Spot	Fish Spot Total Area Fish Spot Avg Area Head Spot Total Area Head Spot Avg Area Torso Spot Total Area Torso Spot Avg Area Vessel Spot Total Area Vessel Spot Avg Area
Shape	Fish, head, & torso	Straightness (Fish only) Shape P2A Shape LWR Convexity (Fish only) Curvature (Fish only)
Intensity	Fish, head, & torso	Total Intensity Avg Intensity Var Intensity Head Total Intensity Head Avg Intensity Torso Total Intensity Torso Avg Intensity
	Vessel (Angiogenic & Vasculogenic)	Angio_Total Intensity Vasculo_Total Intensity Angio_Avg Intensity Vasculo_Avg Intensity Angio_Variance Intensity Vasculo_Variance Intensity
	Spot	Fish Spot Total Intensity Fish Spot Avg Intensity Head Spot Total Intensity Head Spot Avg Intensity Torso Spot Total Intensity Torso Spot Avg Intensity Vessel Spot Total Intensity Vessel Spot Avg Intensity

Table 2. *Zebratox BioApplication Cell and Well Feature Measurements*

Demonstration Data using the Zebratox BioApplication

The experiment carried out for this demonstration data used 24 hour post fertilization wild-type zebrafish embryos. The embryos were dechorionated enzymatically and arrayed by hand with one embryo per well in a black walled 96-well plate. The compounds, dissolved first in DMSO and then in fish water, were added to the wells with the final concentrations described here. The experiment was laid out as follows. The negative control (1% DMSO) was added to two columns, equaling sixteen replicates in all. Valinomycin and valproic acid were added, five replicates per dose, in two fold dilutions ranging from 0 – 200 µM.

The embryos were incubated for 24 hours at 37˚C and were imaged on an ArrayScan VTI HCS Reader with a Brightfield module at 1.25x magnification and a 0.63x coupler. To enable the brightfield imaging at low magnification, all wells were flooded with fish water after the incubation. A glass slide the size of the plate was added on top, with all bubbles removed prior to imaging. A specific form factor was made so that no autofocusing was needed using the large depth of field of the 1.25x objective.

Sample data and images can be seen in the figures below. The standard deviations between the treatments were large, most likely due to the natural variability that exists between embryos whether treated or untreated. That variability is consistent throughout experiments and is a normal factor to consider when using whole organisms models. The data clearly show that valproic acid had a dramatic effect on both FishHeadTailDistance (the shortest distance between the tip of the embryo's head and the tip of the embryo's tail) and FishLWR (the embryo's length to width ratio) compared to DMSO controls. The valinomycin treated embryos showed less morphological change than valproic acid, which was similar to that of the controls. This suggests that valproic acid was more toxic to the embryonic development of the embryos at this timepoint than valinomycin.

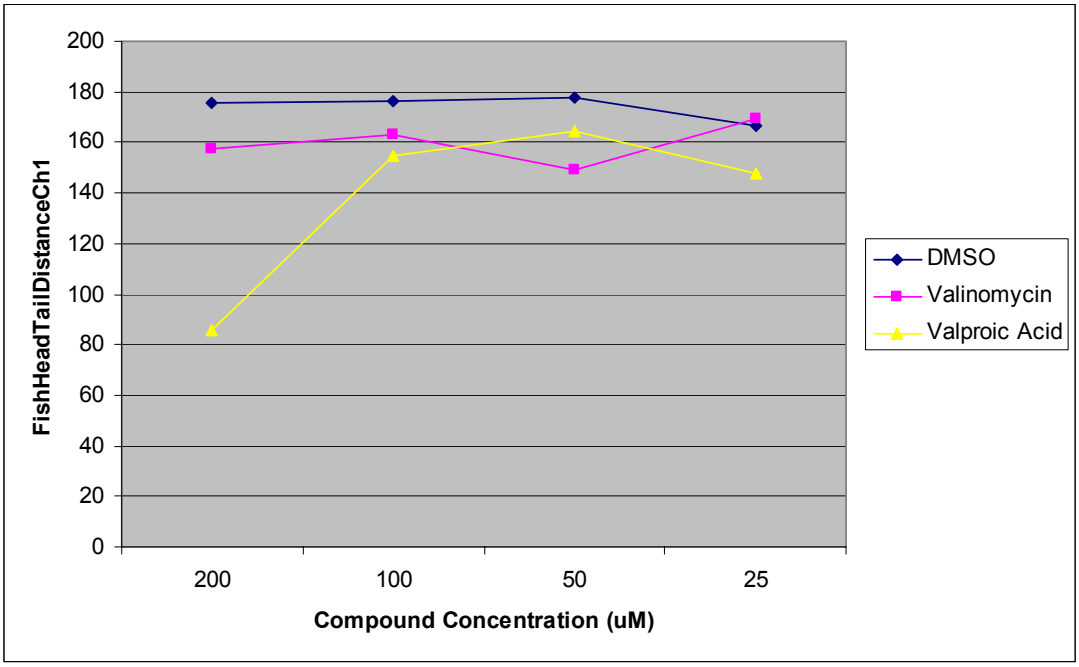

Figure 2. FishHeadTailDistanceCh1 *data showing DMSO and averaged valinomycin and valproic acid data (5 embryos per condition) over the compound doses. DMSO was at 1%.*

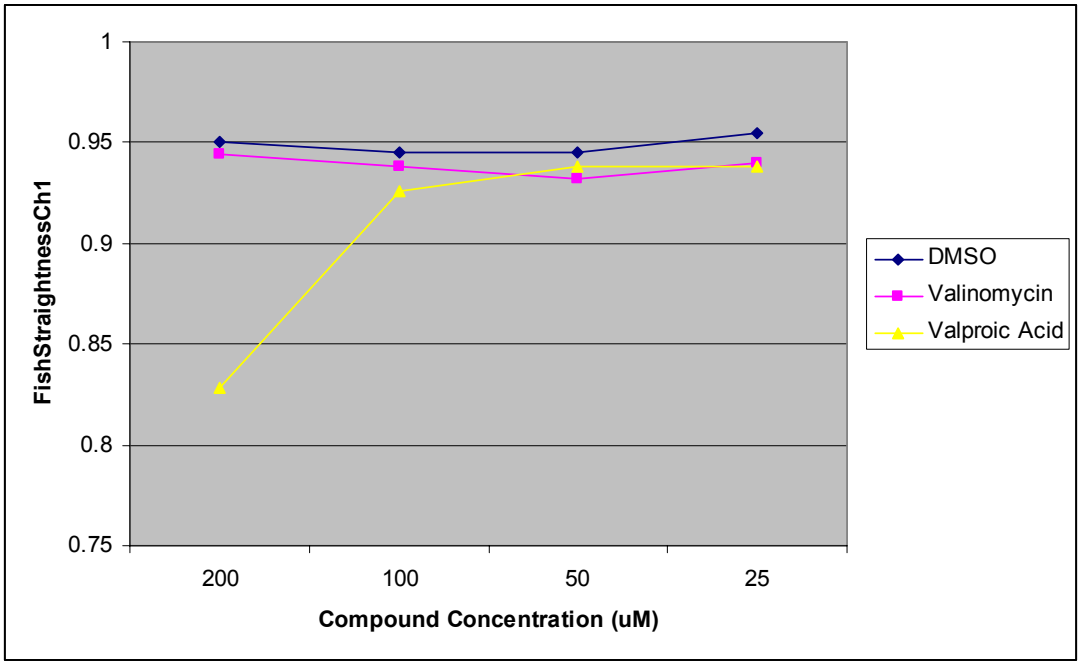

Figure 3. FishStraightnessCh1 *data showing DMSO and averaged valinomycin and valproic acid data (5 embryos per condition) over compound doses. DMSO was at 1%.*

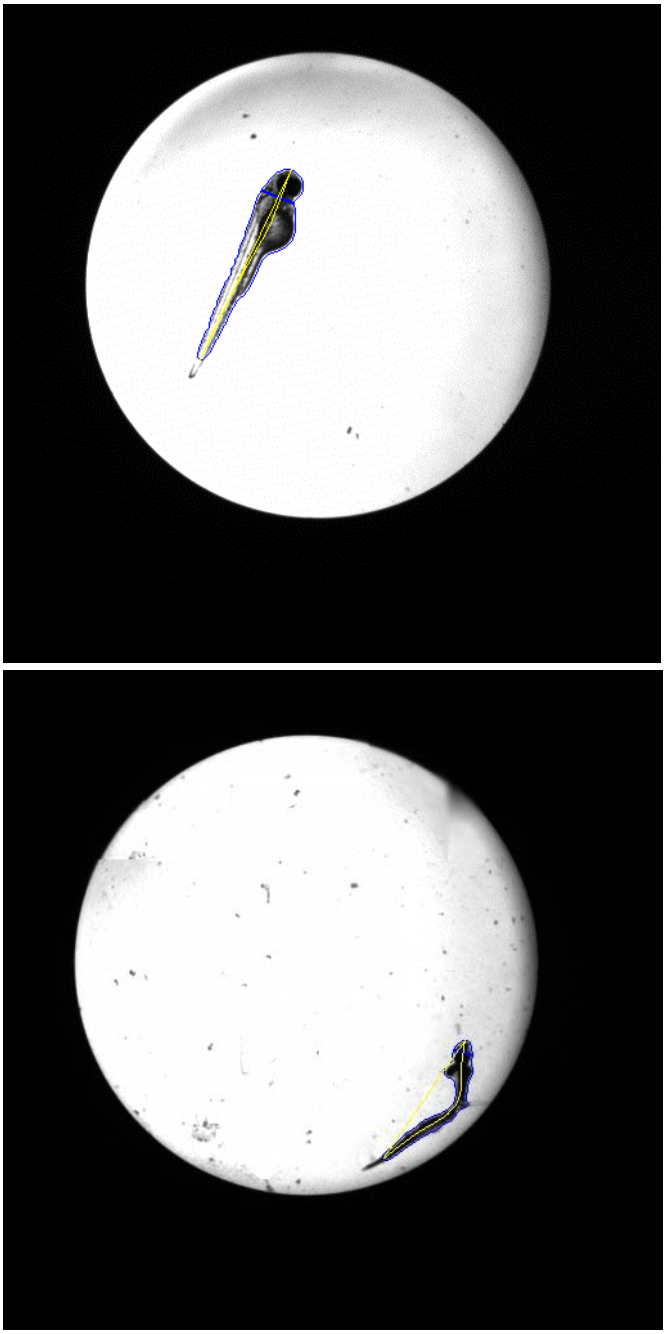

Figure 4. *Images (1.25x) of zebrafish embryos with algorithmic overlay under brightfield after incubation with 1% DMSO (top) or 200 µM valproic acid (bottom) for 24 hours. These images illustrate the difference in Fish LWR and FishHeadTailDistance demonstrated by the data in the figures above.*

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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 this BioApplication. This chapter describes in more detail the assay output features as well as the adjustable input parameters that control the analysis.

Object Identification Methods

To identify objects in each of the images from the different channels, an independent intensity threshold must be set for the first channel. In Channel 1, fish and vessels are identified, while Channel 2 identifies spots; only pixels with intensities above this intensity threshold will be considered as belonging to these structures. If more than one fish is detected within the same image, the fish with the larger value for (total_intensity*sd_intensity) will be the only fish analyzed as only one fish is analyzed per image in this application. 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. If additional channels are desired, Channels 3-6 can be used to select out objects from analysis, but additional structures cannot be identified in these latter channels.

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 options and values available for each channel are summarized in Table 3, and descriptions of the methods follow in Table 4.

Intensity Threshold Method	Channel Availability		
	Channel 1	Channel 2	Channels 3-6
None		✓	✓
Isodata	✓	✓	
Triang	✓	✓	
Fixed	✓	✓	

Table 3. *Intensity Threshold Methods available for each channel in the Zebratox 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 Cellomics HCS Readers, version x.6. However, the entire range may be available on images from other sources.

The intensity threshold method, **None,** indicates that no intensity threshold is applied. This option is not available in Channel 1 because in this channel an intensity threshold is required to define the pixels making up the embryo and targets. If **None** is selected, gating will be performed using the modified object domain (from Channel 1).

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 in that 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.

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, if you have a blank image that contains no 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 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 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 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 4. *Intensity Threshold Descriptions and ranges available for the Zebratox BioApplication*

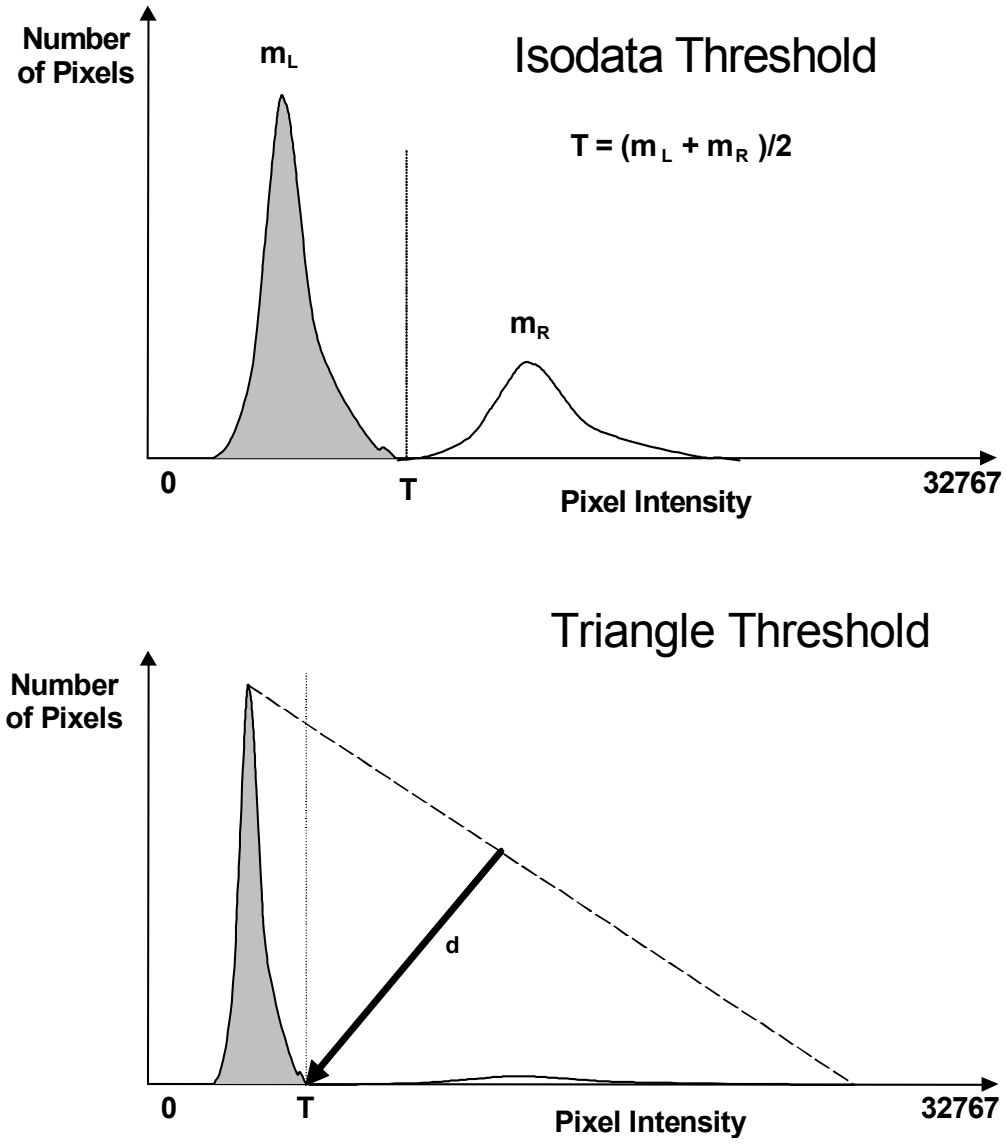


Figure 5. *Histogram-derived Intensity Threshold Methods: Top – Isodata; Bottom - Triangle. Background peak is shown in gray and object peak is shown in white.*

Description of Assay Parameters and Settings

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

Assay Parameters for Image Analysis

General Assay Parameters

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

Reference Well Control

The **UseReferenceWells** Assay Parameter allows you to choose whether Reference Wells are to be used to determine the fish characterization levels. If Reference Wells are to be used, set **UseReferenceWells** to **1**. To disable Reference Wells, set **UseReferenceWells** to **0** (zero).

Units for Morphological Measurements

Areas and lengths can be reported in either calibrated units (micrometers) or pixels. This option is controlled by the **UseMicrometers** Assay Parameter. The default setting of **1** reports the results in micrometers. Setting the Assay Parameter to **0** (zero) reports results in pixels. The conversion factor from pixels to micrometers is calculated automatically based on the Objective and Camera Acquisition Mode selected.

Object Type

FishTypeCh1, SpotTypeCh2

The "Fish Type and Spot Type" Assay Parameters allow you to specify whether the objects of interest are brighter or darker than the background of the image. Setting the value to **1** allows 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 (Table 5).

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

Table 5**. Binary settings for **FishTypeCh1** and **SpotTypeCh2

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 a brightfield image of an embryo. 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 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.

Background Correction

BackgroundCorrectionChN

Prior to image analysis, the non-object background can be computed and subtracted from the image separately in each channel, if desired, as shown in Figure 6.

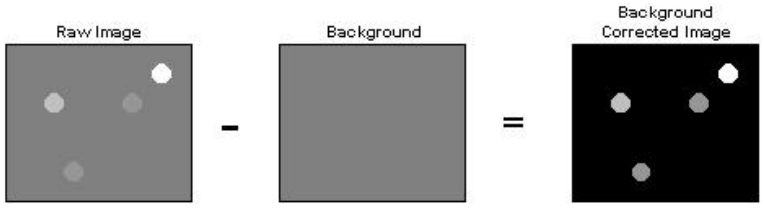


***Figure 6**. Image depicting calculation of positive Background Correction that can be used for each channel*

The background image is computed by estimating low frequency components in the raw image (low pass filtration) and subtracting this background from the raw image. The result is the background corrected image. You can control the creation of the background image by adjusting the **BackgroundCorrectionCh*N*** Assay Parameter. 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. If the value is set to **0** (zero), 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. In both cases, the larger the absolute value, the larger the sampled region, and the less subtraction is done. Table 6 gives an overview of the different Background Correction settings that can be used for each channel.

Setting	Background Correction Method
0	No background correction is performed
Positive	Low pass filter method
Negative	3D surface fitting method

***Table 6.** Possible Background Correction Methods used with the Zebratox BioApplication*

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.

Object Smoothing

FishSmoothFactorCh1, Angio and VasculoVesselSmoothFactorsCh1, SpotSmoothFactorCh2

Object smoothing can be found separately within both channels for fish, vessels, and spots. The image may require smoothing (i.e., blurring) before the specific object can be identified. Smoothing may consolidate fragmented vessels and softens (blurs) their boundaries. The values for **FishSmoothFactorCh1, AngioVesselSmoothFactorCh1, VasculoVesselSmoothFactorCh1,** and **SpotSmoothFactorCh2** specify the degree of smoothing. A value of zero means that smoothing is not applied.

Too much smoothing may cause multiple objects to merge into one object, while an image with jagged appearances in intensity may require smoothing in order to properly detect the objects. Care needs to be taken to ensure that the proper amount of smoothing is used for the biology.

Reject Border Objects

RejectBorderFishCh1

The rejection of border-touching fish is specified by the **RejectBorderFishCh1** Assay Parameter. Setting this Assay Parameter to **1** will reject fish that touch the edge of the field. Setting the value to **0** (zero) will include border-touching fish.

Channel-Specific Assay Parameters

Channel 1: Fish and Vessel Identification

The following Assay Parameters control identification of Fish in Channel 1 or identification of Vessels in Channel 1:

FishMaskModifierCh1

This parameter allows for the modification of the whole fish mask. Negative values shrink the mask. Positive values expand the mask, and entering zero means no mask modification is performed. The value is the number of pixels that modify the mask.

AngioVesselDetectRadiusCh1

This value defines the radius (in pixels) used to detect angiogenic vessels. A value of zero means no vessel detection will be performed.

AngioVesselDetectMethodCh1

The methods listed in Table 7 are used by the algorithm to identify the angiogenic vessels for analysis.

Assay Parameter Value	Name	Description	Advantage
1	Box	The box method replaces each pixel value in an image with the simple arithmetic average value of its neighbors, including itself.	Fastest
2	Median	The median method considers each pixel in the image and looks at its neighbors. Instead of simply replacing the pixel value with the mean of neighboring pixel values, it replaces it with the median of those values.	Minimal edge blurring
3	Binomial	The binomial method replaces each pixel value in an image with the weighted average value of its neighbors, including itself. The weights are set proportional to the binomial coefficients.	Most isotropic
4	Morphological	The morphological (top hat) method computes the difference between the original grayscale image and the processed image.	Minimal edge blurring

Table 7. *Possible AngioVessel Detection Methods used with the Zebratox BioApplication*

VasculoIdentificationModifierCh1

The modifier is an offset used to compute vasculogenic vessel threshold value using the Isodata method. The value can be negative to allow for lower intensities to be identified, or the value can be positive to allow for higher intensities to be identified. A value of zero means no offset is applied.

VasculoVesselMergeFactorCh1

This is the number (in pixels) used to close the gap in the mask of the vasculogenic vessel. The larger the value, the bigger gap removal is possible.

Channel 2: Identification of Spots

Channel 2 is used to identify spots in the head, torso, and vessel (if exist) regions of the fish.

SpotDetectionCh2

This parameter controls the detection of spots in Channel 2. A negative value indicates using the intensity peak method to detect spots, and its absolute value is the intensity range. A positive value indicates using the morphological method, and its value is the radius (in pixels) used to detect spots. Zero value means no detection is applied.

SpotSegmentationCh2

This parameter controls the segmentation of touching spots in channel 2. A negative value indicates the use of the peak intensity method, a positive value indicates the use of the geometry (shape) method, and zero means no segmentation is done.

Methods of Segmentation

Two methods are available for separating spots: geometric (shape) and peak intensity. These are illustrated in the following figure. The geometric method splits touching objects on the basis of shape, relying on boundary indentations to locate the line of separation. This method works best if the individual objects have smooth boundaries with pronounced indentations at their point of contact. The geometric method can be used to separate objects that are uniform in intensity (i.e., saturated) or that have multiple intensity peaks (i.e., noisy or textured).

The peak intensity method separates touching objects using intensity peaks. Successful segmentation requires a single, dominant intensity peak for each object. This method will not work well if the objects are noisy, textured, or uniform in intensity. However, the peak intensity method can be used to separate objects that have no boundary indentations.

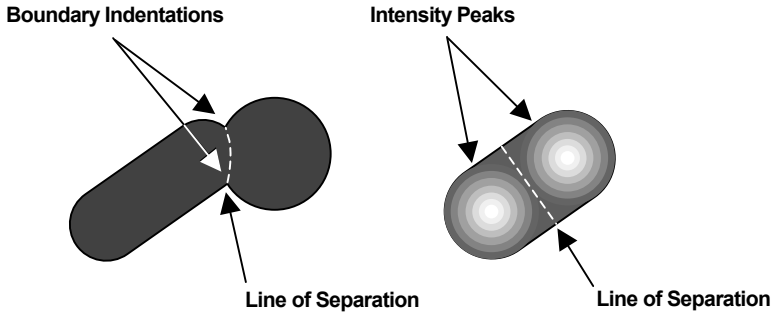


Figure 7. *Object Segmentation Options. The image on the left illustrates the geometric method while the image on the right illustrates the peak intensity method.*

Segmentation Method	SpotSegmentationCh2 Assay Parameter Value
None	0
Geometric	n (radius, in pixels, of the smaller object)
Peak Intensity	-n (minimum height of intensity peaks used in segmentation)

Table 8. SpotSegmentationCh2 *options for the Zebratox BioApplication*

SpotDomainHeadModifierCh2

This parameter controls the Channel 1 fish head mask in Channel 2. The value, in pixels, shrinks the mask when the value is negative, expands the mask when the value is positive, and no modification is performed if the value is zero.

SpotDomainTorsoModifierCh2

This parameter controls the Channel 1 fish torso mask in Channel 2. The value, in pixels, shrinks the mask when the value is negative, expands the mask when the value is positive, and no modification is performed if the value is zero.

SpotDomainVesselModifierCh2

This parameter controls the Channel 1 angio and vasculo vessel masks in Channel 2. The value, in pixels, shrinks the mask when the value is negative, expands the mask when the value is positive, and no modification is performed if the value is zero.

Basic Assay Parameters

Assay Parameters available in Basic Mode control the basic morphological identification of the objects and overlays in each channel, and control the use of Reference Wells (Table 9). You will not be able to view any Advanced Assay Parameters in this mode (as the **Hide Advanced Parameters** option is checked).

Parameter	Units	Description
UseReferenceWells	Number	Use reference wells to calculate high response levels: 0 = No, 1 = Yes
FishTypeCh1	Binary	Type of fish to be identified in Ch1: 0 = Bright fish on dark background, 1 = Dark fish on bright background
BackgroundCorrectionCh1	Pixels	Radius (in pixels) of region used to compute background in Ch1: Negative value = Use surface fitting, 0 = Do not apply background correction, Positive value = Use low pass filter
FishSmoothFactorCh1	Number	Degree of image smoothing (blurring) prior to fish identification in Ch1: 0 = Do not apply smoothing
FishMaskModifierCh1	Number	Number of pixels to expand fish domain mask: 0 = Do not modify mask
RejectBorderFishesCh1	Binary	Reject fish that touch image edges: 0 = No, 1 = Yes
SpotTypeCh2	Binary	Type of spots to be identified in Ch2: 0 = Bright object on dark background, 1 = Dark object on bright background
BackgroundCorrectionCh2	Pixels	Radius (in pixels) of region used to compute background in Ch2: Negative value = Use surface fitting, 0 = Do not apply background correction, Positive value = Use low pass filter
MaskModifierCh2	Pixels	Number of pixels to modify (expand) Ch2 cell mask: 0 = Do not modify mask
SpotSmoothFactorCh2	Number	Degree of image smoothing (blurring) prior to spot identification in Ch2: 0 = Do not apply smoothing
SpotDetectionCh2	Number	Radius (in pixels) or intensity range used to detect spots in Ch2: Negative value = Use intensity peaks method, 0 = Do not apply spot detection, Positive value = Use morphological method
SpotSegmentationCh2	Number	Radius (in pixels) or intensity range of touching spots that should be separated in Ch2: Negative value = Use intensity peaks method, 0 = Do not apply spot segmentation, Positive value = Use shape geometry method

Parameter	Units	Description
SpotDomainHeadModifierCh2	Number	Number of pixels to modify Ch1 fish head mask for spots analysis in Ch2: 0 = Do not modify mask
SpotDomainTorsoModificerCh2	Number	Number of pixels to modify Ch1 fish torso mask for spot analysis in Ch2: 0 = Do not modify mask
SpotDomainVesselModifierCh2	Number	Number of pixels to modify Ch1 vessel (angiogenic and vasculogenic) masks for spot analysis in Ch2: 0 = Do not modify mask
BackgroundCorrectionCh3-6	Pixels	Radius (in pixels) of region used to compute background in Ch3-6: Negative value = Use surface fitting, 0 = Do not apply background correction, Positive value = Use low pass filter
MaskModifierCh3-6	Pixels	Number of pixels to modify mask derived from Ch1 fish mask in Ch3-6: Negative value = Shrink mask, 0 = Do not modify mask, Positive value = Expand mask

Table 9. *Basic Assay Parameters available for the Zebratox BioApplication*

Object Selection Parameters

Each channel has an associated set of Object Selection Parameters. If an object has all measured features within the ranges specified by the Object Selection Parameters, it is selected for analysis. Rejected objects are removed from further analysis.

Channel 1 Object Selection Parameters are used to identify valid fish and vessels. Table 10 is a listing of all Object Selection Parameters available for Channel 1.

Channel 1 Parameters

Parameter	Units	Description
FishAreaCh1	Pixel or μm^2	Area of Ch1 fish
FishShapeP2ACh1	Number	Shape measurement based on ratio of perimeter squared to 4π*area of Ch1 fish
FishShapeLWRCh1	Number	Shape measurement based on ratio of length to width of object-aligned bounding box of Ch1 fish
FishSymmetryCh1	Number	Symmetry measurement of Ch1 fish
FishConvexityCh1	Number	Convexity measurement of Ch1 fish (ratio of fish area to the area of convex hull)
HeadTorsoWidthRatioCh1	Number	Ratio of head width to torso width of Ch1 fish
FishTotalIntenCh1	Intensity	Sum of intensity of all pixels within Ch1 fish
FishAvgIntenCh1	Intensity	Average intensity of all pixels within Ch1 fish

Table 10. *Channel 1 Object Selection Parameters available for the Zebratox BioApplication*

Channel 2 Parameters

Channel 2 Object Selection Parameters are used to identify valid spots.

Parameter	Units	Description
SpotAreaCh2	Pixel or μm^2	Area of Ch2 spots
SpotShapeP2ACh2	Number	Shape measurement based on ratio of perimeter squared to 4π*area of Ch2 spot
SpotShapeLWRCh2	Number	Shape measurement based on ratio of length to width of object-aligned bounding box of Ch2 spot
SpotTotalIntenCh2	Intensity	Total intensity of all pixels within Ch2 spot
SpotAvgIntenCh2	Intensity	Average intensity of all pixels within Ch2 spot
TotalIntensityCh2	Intensity	Total intensity of all pixels in Ch2 within modified Ch1 fish mask
AvgIntenCh2	Intensity	Average intensity of all pixels in Ch2 within modified Ch1 fish mask

Table 11. *Channel 2 Object Selection Parameters available for the Zebratox BioApplication*

Channel *N* (Channels 3-6) Parameters

Channels 3-6 Object Selection Parameters are exclusively for gating purposes. Table 12 provides a listing of all Object Selection Parameters available for Channels 3-6.

Parameter	Units	Description
TotalIntenCh*N*	Intensity	Total intensity of all pixels in ChN within modified Ch1 fish mask
AvgIntenCh*N*	Intensity	Average intensity of all pixels in ChN within modified Ch1 fish mask

Table 12. *Object Selection Parameters Available for the Zebratox BioApplication. *Note that "ChN" refers to Channels 3-6.*

Object Selection

The Zebratox BioApplication supports object selection (commonly referred to as "gating") on fish, vessels, and spots. This feature provides selective object processing based on length, width, shape measurements, and intensity. In addition to selecting fish and vessels for analysis in Channel 1 and spots in Channel 2, you can also select or reject fish based on fluorescence intensity in Channels 2-6. Object selection allows you to specifically identify a subset of fish based on size, shape, and/or intensity, and gating allows you to limit the analysis to a subset of what is imaged. For example, object selection may be used to analyze only those fish showing a certain level of expression of a fluorescent reporter such as green fluorescent protein (GFP) or only those fish that are in proper imaging orientation. Unlike subpopulation analysis, object selection works by rejecting any object that does not meet object selection criteria in all channels. Therefore, if you choose to add object selection channels, an object must pass the criteria in all channels to be selected for analysis.

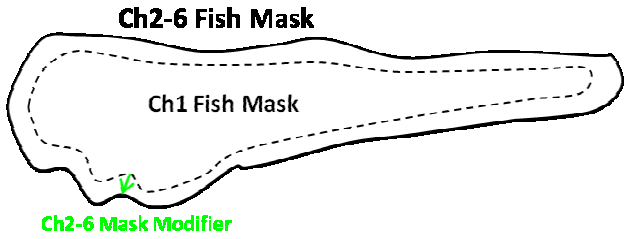
Specifying Intensity Ranges for Object Selection

When working in Protocol Interactive view, you can specify intensity ranges by entering upper and lower limits for two intensity parameters, **AvgIntenCh*N*** (0…32,767) and **TotalIntenCh*N*** (0…32,767), for Channels 2-6. **TotalIntenCh*N*** is a summation of all intensities within the object (fish). **AvgIntenCh*N*** is **TotalIntenCh*N*** divided by the number of pixels within object.

Specifying Mask Modifiers for Object Selection

The mask of a fish identified in Channel 1 may be used to create measurement masks that can be applied to one or more downstream channels (i.e., Channels 2-6) for object selection. The sizes of these masks are specified using the **MaskModifierCh*N*** Assay Parameter where Ch*N* is the relevant dependent channel (i.e., Channels 2-6). The MaskModifier Ch*N* value specifies the number of pixels by which the fish mask created in Channel 1 is enlarged (positive value) or reduced (negative value). You may want to use this feature if, for example, you want to expand the fish mask to include other markers.

Figure 8. *Adjustment of area using* ***MaskModifierChN*** *Assay Parameters for Channels 2-6*



NOTE

Once the fish mask is adjusted (by using the FishMaskModifierCh1), values for **MaskModifierCh*N*** will be based on the *adjusted* mask.

Image Overlays

During a scan, the outlines of objects can be displayed as color overlays on the channel images. The colors of these overlays can be changed by choosing the color block beside the overlay name in the Create Protocol or Protocol Interactive View of the ArrayScan Classic software or in the Protocol Optimization task list of the iDev software. The types of overlays that are displayed depend on the number of channels used.

Table 13 lists the following overlays that can be displayed for this BioApplication.

Parameter	Description
Include This Channel In Composite	If checked, channel image is included in the composite image. Note that in order to view a composite image in Scan or View applications, at least one box must be checked.
Selected Fish	Fish that passes all gating criteria and is therefore included in analysis
Rejected Fish	Fish that does not pass all gating criteria and is therefore excluded from analysis
HeadTailLine	Curve line traverses along fish body (spine) from head to tail; and the straight length is the shortest distance from fish head and tail
Angiogenic Vessel	Overlay of angiogenic vessels, also known as intersegmental vessels
Head Spot	Spots that are identified within the head region of the fish
Torso Spot	Spots that are identified within the torso region of the fish

Table 13. *General set of overlays that can be used with the Zebratox BioApplication*

Assay Parameters for Fish Characterization

Overview of Fish Characterization

The Zebratox BioApplication provides the ability to characterize embryos based on their response compared to a control population. For a particular embryo feature, a range is determined and set by measuring the physiological distribution of feature values in untreated wells. Upper and lower limits defined by Assay Parameters (called ***Feature*Ch*N*LevelHigh** and ***Feature*Ch*N*LevelLow**) set the upper and lower bounds of this range, respectively. The **Status** Feature values indicate whether a given embyro is within or beyond this range (Table 14):

Value	Status Definition
0	Feature value within defined range
1	Feature value > upper level
2	Feature value < lower level

***Table 14**. Numerical definitions of Feature Status in the Zebratox BioApplication when using fish characterization.*

Setting Response Levels

There are two ways of setting the upper and lower limits to define a range of feature values to characterize a fish: manually or automatically.

To set the limits (***Feature*Ch*N*LevelHigh** , ***Feature*Ch*N*LevelLow**) manually, you explicitly set the upper and lower limit values for each feature. This requires prior knowledge of typical feature values. The BioApplication then calculates the status for each feature using the set limits.

To automatically determine the limits and set the range, the BioApplication uses Reference Wells. Typically, Reference Wells contain a control, untreated, population of fish that display the normal basal physiological response for the parameter being measured. These wells are analyzed first and then the population distribution for the different features is determined.

Specifying and Controlling Reference Wells

You choose whether to manually or automatically set the range by using the **UseReferenceWells** Assay Parameter. The possible values and resulting actions controlled by this parameter are:

- **UseReferenceWells = 0 – do not use Reference Wells (i.e., set ranges manually)**
- **UseReferenceWells = 1 – processes Known Reference Wells**

Due to one fish per well, the reference features are calculated directly at plate level. Specifically, we calculate the mean and standard deviation of each feature from all reference wells, and obtain the high and low response feature levels as follows:

$$FeatureLevelHigh = Mean\_Feature + (FeatureLevelHigh\_CC * SD\_Feature)$$

$$FeatureLevelLow = Mean\_Feature - (FeatureLevelLow\_CC * SD\_Feature)$$

where ***Mean_Feature*** and ***SD_Feature*** denote the mean and standard deviation of certain feature from all reference wells on the plate, and ***FeatureLevelHigh_CC*** and ***FeatureLevelLow_CC*** denote the correction coefficient set in the Assay Parameters window, which is used to set high and low response feature levels respectively.

Reference Wells Processing Sequence

By setting the **UseReferenceWells** Assay Parameter to **1**, the Reference Wells processing is engaged. Setting the **UseReferenceWells** Assay Parameter to **0** turns the Reference Wells processing off. Specified Reference Well fields are acquired/analyzed, and Well and Plate Features are computed. After this sequence is completed, computed values will be assigned to the Assay Parameters associated with each Reference Feature and regular scanning of the plate will begin.

The sequence of processing Reference Wells is as follows:

1) Features are computed for valid fish within each well.

2) Mean and standard deviation is computed for each feature from all reference wells on the plate.

3) The high and low response features are automatically calculated for each feature (as described previously), and are reported as Reference Features having the name **Ref*Feature*Ch*N*LevelHigh, Ref*Feature*Ch*N*LevelLow**.

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 Reference Wells and then select the button for the appropriate type of Reference Well (**Known**). Next, enter the Starting Field and set Number of Fields to 1. Click the **Apply** button to save the settings. Please see 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.

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

Advanced Assay Parameters

In Advanced Mode, all Basic and Advanced Assay Parameters are adjustable. The **Hide Advanced Parameters** option provides the ability to either view and adjust all the Assay Parameters or view and adjust the subset of parameters designated as Basic Parameters. For this BioApplication, Advanced Assay Parameters are related to fish charaterization (Table 15).

For each feature, there are four advanced Assay Parameters that control its levels: ***Feature*Ch*N*LevelHigh, LevelLow** that set upper and lower levels and the presence of the **_CC** suffix for each which designates those levels are set using Reference Wells and a correction coefficient. For example, the Assay Parameters controlling the spot total area in Channel 2 are:

- **FishSpotTotalAreaCh2LevelHigh**
- **FishSpotTotalAreaCh2LevelLow**
- **FishSpotTotalAreaCh2LevelHigh_CC**
- **FishSpotTotalAreaCh2LevelLow_CC**

In the listing of Advanced Parameters in the following table, rather than listing both level 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 2 spot total area:

- FishSpotTotalAreaCh2Level***High/Low, High/Low_CC***

Units will be expressed as what is found with ***Feature*Ch*N*LevelHigh/Low**, knowing that ***_CC*** is expressed as a number.

Parameter	Units	Description
FishAreaCh1Level***High/Low, High/Low_CC***	Pixel or μm^2	High/Low response for fish area Ch1 determined manually or by Reference Wells (_CC)
FishShapeP2ACh1Level***High/Low, High/Low_CC***	Number	High/Low response for fish P2A Ch1 determined manually or by Reference Wells (_CC)
FishShapeLWRCh1Level***High/Low, High/Low_CC***	Number	High/Low response for fish LWR Ch1 determined manually or by Reference Wells (_CC)
FishHeadTailDistanceCh1Level***High/Low, High/Low_CC***	Pixel or µm	High/Low response for fish head tail distance Ch1 determined manually or by Reference Wells (_CC)
FishSpineLengthCh1Level***High/Low, High/Low_CC***	Pixel or µm	High/Low response for fish spine length Ch1 determined manually or by Reference Wells (_CC)
FishWidthCh1Level***High/Low, High/Low_CC***	Pixel or µm	High/Low response for fish width Ch1 determined manually or by Reference Wells (_CC)
FishStraightnessCh1Level***High/Low, High/Low_CC***	Number	High/Low response for fish straightness Ch1 determined manually or by Reference Wells (_CC)
FishConvexityCh1***High/Low, High/Low_CC***	Number	High/Low response for fish convexity Ch1 determined manually or by Reference Wells (_CC)

Parameter	Units	Description
FishCurvatureCh1***High/Low, High/Low_CC***	Number	High/Low response for fish curvature Ch1 determined manually or by Reference Wells (_CC)
FishTotalIntenCh1Level***High/Low, High/Low_CC***	Intensity	High/Low response for fish total intensity Ch1 determined manually or by Reference Wells (_CC)
FishAvgIntenCh1Level***High/Low, High/Low_CC***	Intensity	High/Low response for fish average intensity Ch1 determined manually or by Reference Wells (_CC)
HeadAreaCh1Level***High/Low, High/Low_CC***	Pixel or μm^2	High/Low response for fish head area Ch1 determined manually or by Reference Wells (_CC)
HeadShapeP2ACh1Level***High/Low, High/Low_CC***	Number	High/Low response for fish head P2A Ch1 determined manually or by Reference Wells (_CC)
HeadShapeLWRCh1Level***High/Low, High/Low_CC***	Number	High/Low response for fish head LWR Ch1 determined manually or by Reference Wells (_CC)
HeadTotalIntenCh1Level***High/Low, High/Low_CC***	Intensity	High/Low response for fish head total intensity Ch1 determined manually or by Reference Wells (_CC)
HeadAvgIntenCh1Level***High/Low, High/Low_CC***	Intensity	High/Low response for fish head average intensity Ch1 determined manually or by Reference Wells (_CC)
TorsoAreaCh1Level***High/Low, High/Low_CC***	Pixel or μm^2	High/Low response for fish torso area Ch1 determined manually or by Reference Wells (_CC)
TorsoShapeP2ACh1Level***High/Low, High/Low_CC***	Number	High/Low response for fish torso P2A Ch1 determined manually or by Reference Wells (_CC)
TorsoShapeLWRCh1Level***High/Low, High/Low_CC***	Number	High/Low response for fish torso LWR Ch1 determined manually or by Reference Wells (_CC)
TorsoTotalIntenCh1Level***High/Low, High/Low_CC***	Intensity	High/Low response for fish torso total intensity Ch1 determined manually or by Reference Wells (_CC)
TorsoAvgIntenCh1Level***High/Low, High/Low_CC***	Intensity	High/Low response for fish torso average intensity Ch1 determined manually or by Reference Wells (_CC)
FishTotalIntenCh2Level***High/Low, High/Low_CC***	Intensity	High/Low response for fish total intensity Ch2 determined manually or by Reference Wells (_CC)
FishAvgIntenCh2Level***High/Low, High/Low_CC***	Intensity	High/Low response for fish average intensity Ch2 determined manually or by Reference Wells (_CC)
HeadTotalIntenCh2Level***High/Low, High/Low_CC***	Intensity	High/Low response for fish head total intensity Ch2 determined manually or by Reference Wells (_CC)
HeadAvgIntenCh2Level***High/Low, High/Low_CC***	Intensity	High/Low response for fish head average intensity Ch2 determined manually or by Reference Wells (_CC)

Parameter	Units	Description
TorsoTotalIntenCh2Level***High/Low, High/Low_CC***	Intensity	High/Low response for fish torso total intensity Ch2 determined manually or by Reference Wells (_CC)
TorsoAvgIntenCh2Level***High/Low, High/Low_CC***	Intensity	High/Low response for fish torso average intensity Ch2 determined manually or by Reference Wells (_CC)
FishSpotTotalCountCh2Level***High/Low, High/Low_CC***	Number	High/Low response for fish spot total count determined manually or by Reference Wells (_CC)
FishSpotTotalAreaCh2Level***High/Low, High/Low_CC***	Pixel or μm^2	High/Low response for fish spot total area determined manually or by Reference Wells (_CC)
FishSpotAvgAreaCh2Level***High/Low, High/Low_CC***	Pixel or μm^2	High/Low response for fish spot average area determined manually or by Reference Wells (_CC)
FishSpotTotalIntenCh2Level***High/Low, High/Low_CC***	Intensity	High/Low response for fish spot total intensity determined manually or by Reference Wells (_CC)
FishSpotAvgIntenCh2Level***High/Low, High/Low_CC***	Intensity	High/Low response for fish spot average intensity determined manually or by Reference Wells (_CC)
HeadSpotTotalCountCh2Level***High/Low, High/Low_CC***	Number	High/Low response for fish head spot total count determined manually or by Reference Wells (_CC)
HeadSpotTotalAreaCh2Level***High/Low, High/Low_CC***	Pixel or μm^2	High/Low response for fish head spot total area determined manually or by Reference Wells (_CC)
HeadSpotAvgAreaCh2Level***High/Low, High/Low_CC***	Pixel or μm^2	High/Low response for fish head spot average area determined manually or by Reference Wells (_CC)
HeadSpotTotalIntenCh2Level***High/Low, High/Low_CC***	Intensity	High/Low response for fish head spot total intensity determined manually or by Reference Wells (_CC)
HeadSpotAvgIntenCh2Level***High/Low, High/Low_CC***	Intensity	High/Low response for fish head spot average intensity determined manually or by Reference Wells (_CC)
TorsoSpotTotalCountCh2Level***High/Low, High/Low_CC***	Number	High/Low response for fish torso spot total count determined manually or by Reference Wells (_CC)
TorsoSpotTotalAreaCh2Level***High/Low, High/Low_CC***	Pixel or μm^2	High/Low response for fish torso spot total area determined manually or by Reference Wells (_CC)
TorsoSpotAvgAreaCh2Level***High/Low, High/Low_CC***	Pixel or μm^2	High/Low response for fish torso spot average area determined manually or by Reference Wells (_CC)
TorsoSpotTotalIntenCh2Level***High/Low, High/Low_CC***	Intensity	High/Low response for fish torso spot total intensity determined manually or by Reference Wells (_CC)
TorsoSpotAvgIntenCh2Level***High/Low, High/Low_CC***	Intensity	High/Low response for fish torso spot average intensity determined manually or by Reference Wells (_CC)

Table 15. *Zebratox BioApplication Advanced Assay Parameters*

Description of Output Features

Output Features are the biological measurements produced by the BioApplication. All features are categorized and accessible using the View application. Additionally, a subset of features, Well Features, are listed in the Scan Plate View and Create Protocol View of the ArrayScan Classic software or in the Protocol Optimization task list of the iDev software so that screening results can be viewed concurrently with scanning. The ability to view data during the scanning process allows you to immediately verify success of the run, well by well. The Zebratox BioApplication has no Field Features since only one embryo is analyzed per well.

Description of Key Fish Measurements

1. FishStraightness

As shown in Figure 9, the fish mask is shown by blue outline, which consists of two portions: head and torso. Two yellow lines connecting the tip of fish head and tail are shown: (1) *straight line;* and (2) *curve line* that traverses along fish body. The length of these two lines are called **FishHeadTailDistance** and **FishSpineLength** respectively. We define **FishStraightness** as the ratio of FishHeadTailDistance to FishSpineLength. This feature indicates how straight (or curved) the fish is. For a perfectly straight fish, FishStraightness has value of 1. The more curved the fish is, the smaller the value, as shown by examples in Figure 9 (B) and (C).

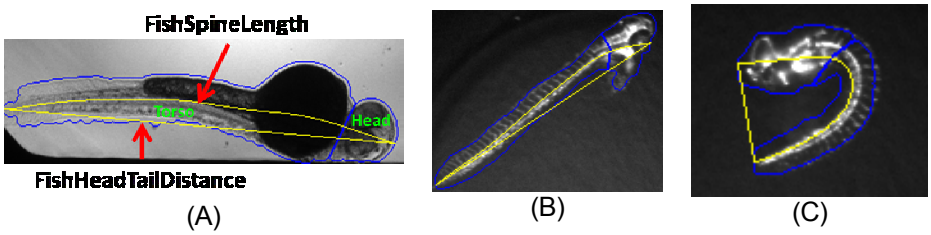


***Figure 9**: (A) Illustrating the FishStraightness measurement; (B) FishStraightness = 0.94; (C) FishStraightness = 0.32.*

2. FishCurvature

FishCurvature is another morphological feature computed based on fish curve line, expressed by the following equation:

$$E = \frac{1}{n}\sum_{i=1}^{n}\left|\Delta\theta_i\right|^2$$

where n denotes the number of points on fish curve line, $\Delta\theta_i$ is the change of tangent angle at i^{th} point of the curve, as illustrated in Figure 10(A). Greater angular difference between adjacent points on the curve, the greater value FishCurvature has, as shown by Figure 10(B)-(D). If the curve line of a fish is perfectly straight, FishCurvature = 0.

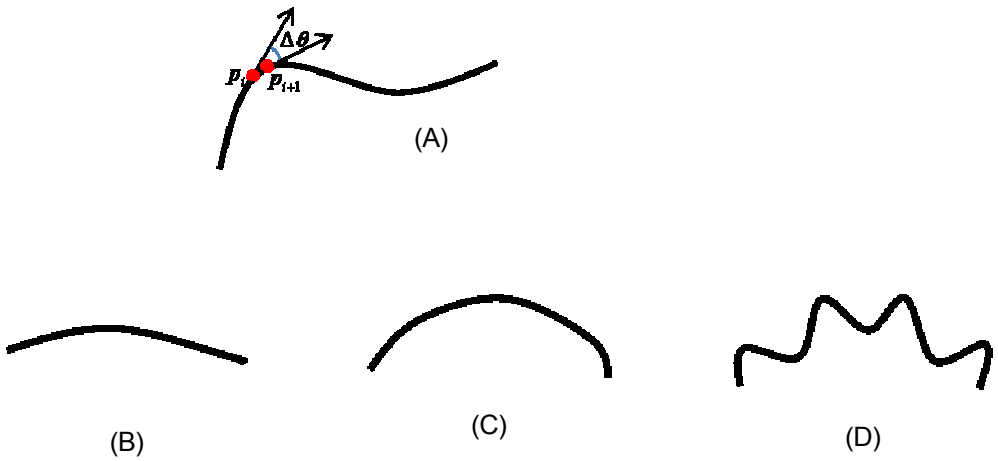


Figure 10. *(A) Illustrating the calculation of FishCurvature; (B-D) The feature value is in increasing order for fish with curve line from (B) to (C) to (D).*

3. FishConvexity

This morphological feature indicates how convex the fish mask is. As shown in Figure 11, the fish mask is outlined in blue, and convex hull of the fish mask is outlined by green dashed line. FishConvexity is defined as the ratio of area of fish mask to the area of its convex hull. For a completely convex fish, the fish area equals the area of its convex hull, thus FishConvexity has the value of 1, as shown by example in Figure 11(B). In general, this feature takes values between 0 and 1, as illustrated by Figure 11(C) and (D), where FishConvexity of (C) is greater than that of (D).

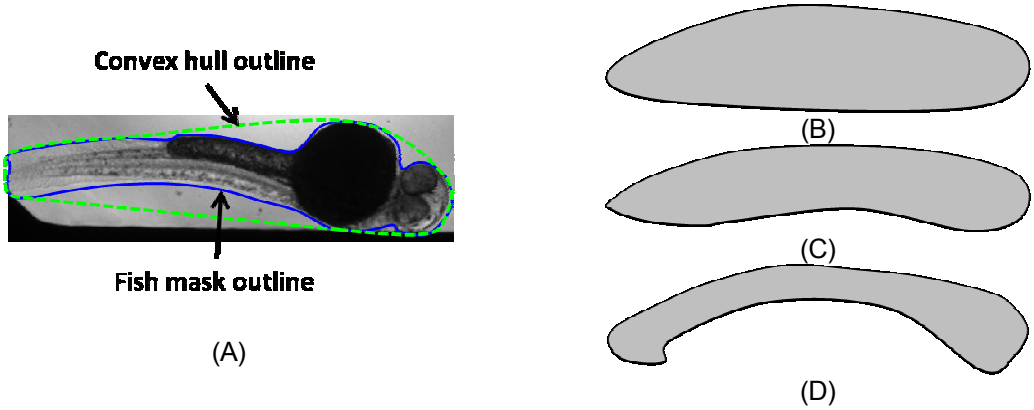


Figure 11*. (A) Illustrating the FishConvexity measurement; FishConvexity value decreases from (B) to (D).*

4. FishSymmetry

This morphological object selection parameter is used in fish rejection/selection to exclude fish with undesirable orientation, which is especially useful for angiogenesis assay, where the fish has to lie on side in order to image and analyze vessels reliably. As shown in Figure 12(A), fish mask is shown by gray region, and its convex hull is outlined by red line. The FishSymmetry feature is calculated based on the resulting mask by subtracting the fish mask from its convex hull, as shown by **c1**, **c2,** and **c3**. The value range of FishSymmetry is between 0 and 1. The greater the value, the more symmetric the fish is. Examples are shown in Figure 12(B) and (C).

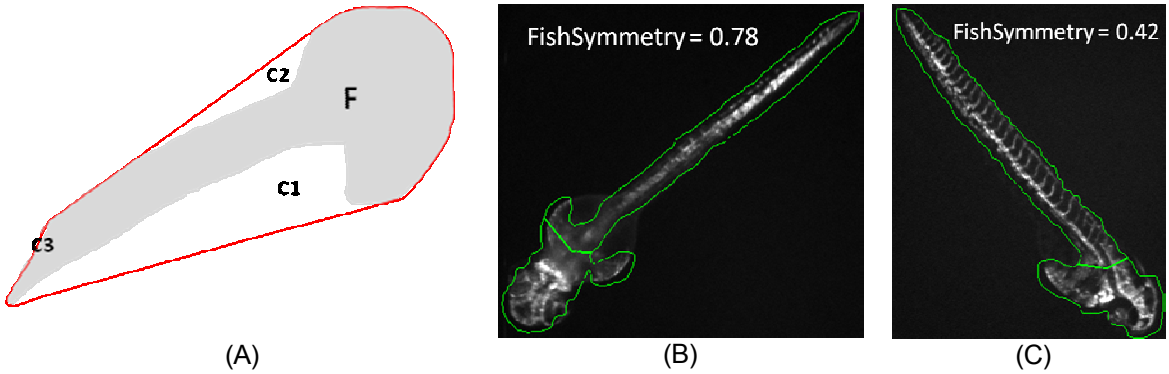


***Figure 12**: (A) Illustrating the FishSymmetry measurement. Two examples are shown in (B) and (C).*

Zebratox Cell/Well Features

The selected embryo is analyzed and results are used to compute the features that are reported for the embryo. Due to one embryo per well in this BioApplication, the Cell and Well Output Features are mostly identical.

Table 16 summarizes the output features reported for each selected embryo. Features shown with a **Status** are those features that indicate in there is a high response (=**1**), low response (=**2**), or no response (=**0**). For Events, **0** = Event did not occur, **1** = Event occurred.

Feature	Unit	Description
Top	Number	Y coordinate (in pixels) of top-left corner of image-aligned bounding box of Ch1 fish
Left	Number	X coordinate (in pixels) of top-left corner of image-aligned bounding box of Ch1 fish
Width	Number	Width (in pixels) of image-aligned bounding box of Ch1 fish
Height	Number	Height (in pixels) of image-aligned bounding box of Ch1 fish
XCentroid	Number	X coordinate (in pixels) of center of Ch1 fish
YCentroid	Number	Y coordinate (in pixels) of center of Ch1 fish
EventTypeProfile	Number	Identifies the types of events that occurred: 1, 2, 3, 12, 23, 13, 123
EventTypeXStatus	Number	EventTypeX status: 0 = Event did not occur, 1 = Event occurred
FishAreaCh1 (and status)	Pixel or μM^2 (binary)	Area (in pixels or micrometers) of Ch1 fish
FishShapeP2ACh1 (and status)	Number (binary)	Shape measure based on ratio of perimeter squared to 4PI*area of Ch1 fish (FishShapeP2ACh1 = 1 for circular object)
FishShapeLWRCh1 (and status)	Number (binary)	Shape measure based on ratio of length to width of object-aligned bounding box of Ch1 fish
FishHeadTailDistanceCh1 (and status)	Pixel or µM (binary)	Distance (in pixels or micrometers) of straight line connecting head and tail of Ch1 fish

Feature	Unit	Description
FishSpineLengthCh1 (and status)	Pixel or µM (binary)	Length (in pixels or micrometers) of Ch1 fish measured along its spine
FishWidthCh1 (and status)	Pixel or µM (binary)	Width (in pixels or micrometers) of Ch1 fish estimated from its area and length
FishStraightnessCh1 (and status)	Number (binary)	Shape measure of Ch1 fish based on ratio of FishHeadTailDistance to FishSpineLength
FishConvexityCh1 (and status)	Number (binary)	Convexity measure of Ch1 fish
FishCurvatureCh1 (and status)	Number (binary)	Bending energy of Ch1 fish
FishTotalIntenCh1 (and status)	Intensity (binary)	Total intensity of all pixels within Ch1 fish
FishAvgIntenCh1 (and status)	Intensity (binary)	Average intensity of all pixels within Ch1 fish
HeadAreaCh1 (and status)	Pixel or μM^2 (binary)	Head area (in pixels or micrometers) of Ch1 fish
HeadShapeP2ACh1 (and status)	Number (binary)	Shape measure based on ratio of perimeter squared to 4PI*area of head of Ch1 fish (HeadShapeP2ACh1 = 1 for circular object)
HeadShapeLWRCh1 (and status)	Number (binary)	Shape measure based on ratio of length to width of object-aligned bounding box of head of Ch1 fish
HeadTotalIntenCh1 (and status)	Intensity (binary)	Total intensity of all pixels within head of Ch1 fish
HeadAvgIntenCh1 (and status)	Intensity (binary)	Average intensity of all pixels within head of Ch1 fish
TorsoAreaCh1 (and status)	Pixel or μM^2 (binary)	Area (in pixels or micrometers) of torso of Ch1 fish
TorsoShapeP2ACh1 (and status)	Number (binary)	Shape measure based on ratio of perimeter squared to 4PI*area of torso of Ch1 fish (TorsoShapeP2ACh1 = 1 for circular object)
TorsoShapeLWRCh1 (and status)	Number (binary)	Shape measure based on ratio of length to width of object-aligned bounding box of torso of Ch1 fish
TorsoTotalIntenCh1 (and status)	Intensity (binary)	Total intensity of all pixels within torso of Ch1 fish
TorsoAvgIntenCh1 (and status)	Intensity (binary)	Average intensity of all pixels within torso of Ch1 fish
FishTotalIntenCh2 (and status)	Intensity (binary)	Total intensity in Ch2 of all pixels within Ch1 fish mask
FishAvgIntenCh2 (and status)	Intensity (binary)	Average intensity in Ch2 of all pixels within Ch1 fish mask
HeadTotalIntenCh2 (and status)	Intensity (binary)	Total intensity in Ch2 of all pixels within Ch1 fish head mask
HeadAvgIntenCh2 (and status)	Intensity (binary)	Average intensity in Ch2 of all pixels within Ch1 fish head mask

Feature	Unit	Description
TorsoTotalIntenCh2 (and status)	Intensity (binary)	Total intensity in Ch2 of all pixels within Ch1 fish torso mask
TorsoAvgIntenCh2 (and status)	Intensity (binary)	Average intensity in Ch2 of all pixels within Ch1 fish torso mask
FishSpotTotalCountCh2 (and status)	Number (binary)	Total number of all Ch2 spots within modified Ch1 fish mask
FishSpotTotalAreaCh2 (and status)	Pixel or μM^2 (binary)	Total area (in pixels or micrometers) of all Ch2 spots within modified Ch1 fish mask
FishSpotAvgAreaCh2 (and status)	Pixel or μM^2 (binary)	Average area (in pixels or micrometers) of all Ch2 spots within modified Ch1 fish mask
FishSpotTotalIntenCh2 (and status)	Intensity (binary)	Total intensity of all pixels within all Ch2 spots within modified Ch1 fish mask
FishSpotAvgIntenCh2 (and status)	Intensity (binary)	Average intensity of all pixels within all Ch2 spots within modified Ch1 fish mask
HeadSpotTotalCountCh2 (and status)	Number (binary)	Total number of all Ch2 spots within modified Ch1 fish head mask
HeadSpotTotalAreaCh2 (and status)	Pixel or μM^2 (binary)	Total area (in pixels or micrometers) of all Ch2 spots within modified Ch1 fish head mask
HeadSpotAvgAreaCh2 (and status)	Pixel or μM^2 (binary)	Average area (in pixels or micrometers) of all Ch2 spots within modified Ch1 fish head mask
HeadSpotTotalIntenCh2 (and status)	Intensity (binary)	Total intensity of all pixels within all Ch2 spots within modified Ch1 fish head mask
HeadSpotAvgIntenCh2 (and status)	Intensity (binary)	Average intensity of all pixels within all Ch2 spots within modified Ch1 fish head mask
TorsoSpotTotalCountCh2 (and status)	Number (binary)	Total number of all Ch2 spots within modified Ch1 fish torso mask
TorsoSpotTotalAreaCh2 (and status)	Pixel or μM^2 (binary)	Total area (in pixels or micrometers) of all Ch2 spots within modified Ch1 fish torso mask
TorsoSpotAvgAreaCh2 (and status)	Pixel or μM^2 (binary)	Average area (in pixels or micrometers) of Ch2 spots within modified Ch1 fish torso mask
TorsoSpotTotalIntenCh2 (and status)	Intensity (binary)	Total intensity of all pixels within all Ch2 spots within modified Ch1 fish torso mask
TorsoSpotAvgIntenCh2 (and status)	Intensity (binary)	Average intensity of all pixels within all Ch2 spots within modified Ch1 fish torso mask

Table 16. *Cell/Well Features Available in the Zebratox BioApplication. *Note that ChN refers to Channels 1-2 for the given features*

Reference Well Features

The algorithm reports the following features in the Scan Plate view of the software application as well as Plate Features in the View application (Table 17). These features are computed and reported only when the Assay Parameter **UseReferenceWells** = **1** (enabled).

Feature	Description
RefFishCount	Number of fish in reference field, well or plate
RefFishAreaCh1LevelHigh/Low	High/Low-response level for **FishAreaCh1** computed from reference well results
RefFishShapeP2ACh1LevelHigh/Low	High/Low-response level for **FishP2ACh1** computed from reference well results
RefFishShapeLWRCh1LevelHigh/Low	High/Low-response level for **FishLWRCh1** computed from reference well results
RefFishHeadTailDistanceCh1LevelHigh/Low	High/Low-response level for **FishHeadTailDistanceCh1** computed from reference well results
RefFishSpineLengthCh1LevelHigh/Low	High/Low-response level for **FishSpineLengthCh1** computed from reference well results
RefFishWidthCh1LevelHigh/Low	High/Low-response level for **FishWidthCh1** computed from reference well results
RefFishStraightnessCh1LevelHigh/Low	High/Low-response level for **FishStraightnessCh1** computed from reference well results
RefFishConvexityCh1LevelHigh/Low	High/Low-response level for **FishConvexityCh1** computed from reference well results
RefFishCurvatureCh1LevelHigh/Low	High/Low-response level for **FishCurvatureCh1** computed from reference well results
RefFishTotalIntenCh1LevelHigh/Low	High/Low-response level for **FishTotalIntenCh1** computed from reference well results
RefFishAvgIntenCh1LevelHigh/Low	High/Low-response level for **FishAvgIntenCh1** computed from reference well results
RefHeadAreaCh1LevelHigh/Low	High/Low-response level for **HeadAreaCh1** computed from reference well results
RefHeadShapeP2ACh1LevelHigh/Low	High/Low-response level for **HeadP2ACh1** computed from reference well results
RefHeadShapeLWRCh1LevelHigh/Low	High/Low-response level for **HeadLWRCh1** computed from reference well results
RefHeadTotalIntenCh1LevelHigh/Low	High/Low-response level for **HeadTotalIntenCh1** computed from reference well results
RefHeadAvgIntenCh1LevelHigh/Low	High/Low-response level for **HeadAvgIntenCh1** computed from reference well results
RefTorsoAreaCh1LevelHigh/Low	High/Low-response level for **TorsoAreaCh1** computed from reference well results
RefTorsoShapeP2ACh1LevelHigh/Low	High/Low-response level for **TorsoP2ACh1** computed from reference well results
RefTorsoShapeLWRCh1LevelHigh/Low	High/Low-response level for **TorsoLWRCh1** computed from reference well results
RefTorsoTotalIntenCh1LevelHigh/Low	High/Low-response level for **TorsoTotalIntenCh1** computed from reference well results

Feature	Description
RefTorsoAvgIntenCh1LevelHigh/Low	High/Low-response level for **TorsoAvgIntenCh1** computed from reference well results
RefFishTotalIntenCh2LevelHigh/Low	High/Low-response level for **FishTotalIntenCh2** computed from reference well results
RefFishAvgIntenCh2LevelHigh/Low	High/Low-response level for **FishAvgIntenCh2** computed from reference well results
RefHeadTotalIntenCh2LevelHigh/Low	High/Low-response level for **HeadTotalIntenCh2** computed from reference well results
RefHeadAvgIntenCh2LevelHigh/Low	High/Low-response level for **HeadAvgIntenCh2** computed from reference well results
RefTorsoTotalIntenCh2LevelHigh/Low	High/Low-response level for **TorsoTotalIntenCh2** computed from reference well results
RefTorsoAvgIntenCh2LevelHigh/Low	High/Low-response level for **TorsoAvgIntenCh2** computed from reference well results
RefFishSpotTotalCountCh2LevelHigh/Low	High/Low-response level for **FishSpotTotalCountCh2** computed from reference well results
RefFishSpotTotalAreaCh2LevelHigh/Low	High/Low-response level for **FishSpotTotalAreaCh2** computed from reference well results
RefFishSpotAvgAreaCh2LevelHigh/Low	High/Low-response level for **FishSpotAvgAreaCh2** computed from reference well results
RefFishSpotTotalIntenCh2LevelHigh/Low	High/Low-response level for **FishSpotTotalIntenCh2** computed from reference well results
RefFishSpotAvgIntenCh2LevelHigh/Low	High/Low-response level for **FishSpotAvgIntenCh2** computed from reference well results
RefHeadSpotTotalCountCh2LevelHigh/Low	High/Low-response level for **HeadSpotTotalCountCh2** computed from reference well results
RefHeadSpotTotalAreaCh2LevelHigh/Low	High/Low-response level for **HeadSpotTotalAreaCh2** computed from reference well results
RefHeadSpotAvgAreaCh2LevelHigh/Low	High/Low-response level for **HeadSpotAvgAreaCh2** computed from reference well results
RefHeadSpotTotalIntenCh2LevelHigh/Low	High/Low-response level for **HeadSpotTotalIntenCh2** computed from reference well results
RefHeadSpotAvgIntenCh2LevelHigh/Low	High/Low-response level for **HeadSpotAvgIntenCh2** computed from reference well results
RefTorsoSpotTotalCountCh2LevelHigh/Low	High/Low-response level for **TorsoSpotTotalCountCh2** computed from reference well results
RefTorsoSpotTotalAreaCh2LevelHigh/Low	High/Low-response level for **TorsoSpotTotalAreaCh2** computed from reference well results

Feature	Description
RefTorsoSpotAvgAreaCh2LevelHigh/Low	High/Low-response level for **TorsoSpotAvgAreaCh2** computed from reference well results
RefTorsoSpotTotalIntenCh2LevelHigh/Low	High/Low-response level for **TorsoSpotTotalIntenCh2** computed from reference well results
RefTorsoSpotAvgIntenCh2LevelHigh/Low	High/Low-response level for **TorsoSpotAvgIntenCh2** computed from reference well results

Table17. *Reference Features Available in the Zebratox BioApplication*

Chapter

3

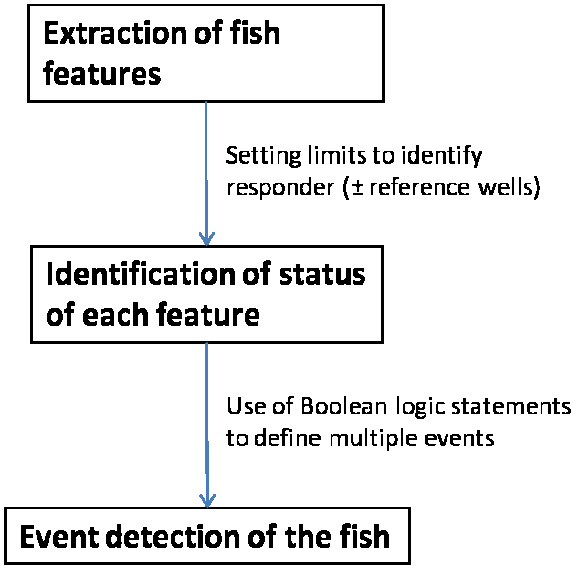
Using the Zebratox BioApplication

The Zebratox BioApplication is provided with Assay Protocols configured to run toxicity and angiogenesis assays described in Chapter 1. Please refer to the appropriate User's Guide for instructions on scanning an individual plate or stacks of plates using an existing Assay Protocol. This chapter describes in more detail the implementation of events and the Events Wizard.

Event Definition

Events are defined as Assay Parameters and consist of logical statements employing specific features. These statements are then applied to classify an embryo as responder or non-responder per feature contained in the Event Definition. The Zebratox BioApplication allows simultaneous definition of up to three events to enable rapid multiparametric analysis across multiple features. The figure below relays these capabilities and how they interact to enable event definition. Operation of the Event Wizard software tool is described in more detail later.

Event analysis requires processing of raw feature values, i.e., the data extracted from the embryo for all targets being measured. Levels are then applied (manually entered or automatically computed via Reference Wells as described in Chapter 2) to identify a responder for each feature. Responder is defined as the embryo showing feature values greater than the upper or smaller than the lower limit defined by ***Feature*ChN LevelHigh** or ***Feature*ChN LevelLow**. It is critical that the limits be set accordingly.



Assay Parameters for Definition of Events at the Level of Single Embryos

The Zebratox BioApplication allows simultaneous definition of up to three events to enable rapid multiparametric analysis of characteristics at the level of individual embryos, across multiple features. You can identify whether each of three events has occurred for a particular embryo.

Event definition is achieved through the construction of logic statements employing specific features and a set of defined logical operators. The features and Boolean operators available are listed in Table 18. The operators ANDNOT and ORNOT are obtained by combining AND + NOT and OR + NOT respectively. Definition of each logical operator is provided in schematic form in Figure 13 using two Features, A and B. In each case, responders for each feature are located within the domain for that feature. Note that the definitions described in this figure can be directly applied to logic statements that contain more than two features. The Assay Parameters used to store events are of the type **Type_X_EventDefinition** (X =1, 2, or 3).

Features	Logic Operators
FishAreaCh1	
FishShapeP2ACh1	
FishShapeLWRCh1	
FishHeadTailDistanceCh1	
FishSpineLengthCh1	
FishWidthCh1	
FishStraightnessCh1	
FishConvexityCh1	
FIshCurvatureCh1	
FishTotalItenCh1	
FishAvgIntenCh1	
HeadAreaCh1	
HeadShapeP2ACh1	
HeadShapeLWRCh1	
HeadTotalIntenCh1	
HeadAvgIntenCh1	
TorsoAreaCh1	
TorsoShapeP2ACh1	
TorsoShapeLWRCh1	
TorsoTotalIntenCh1	NOT
TorsoAvgIntenCh1	
AngioVesselTotalCountCh1	AND
AngioVesselTotalLengthCh1	
AngioVesselAvgLengthCh1	AND NOT
AngioVesselWidthCh1	
AngioVesselTotalAreaCh1	OR
AngioVesselTotalIntenCh1	
AngioVesselAvgIntenCh1	OR NOT
AngioVesselVarIntenCh1	
VasculoVesselTotalLengthCh1	XOR
VasculoVesselTotalAreaCh1	
VasculoVesselWidthCh1	NAND
VasculoVesselTotalIntenCh1	
	NOR

Features	Logic Operators
VasculoVesselAvgIntenCh1	
VasculoVesselVarIntenCh1	
FishTotalIntenCh2	
FishAvgIntenCh2	
HeadTotalIntenCh2	
HeadAvgIntenCh2	
TorsoTotalIntenCh2	
TorsoAvgIntenCh2	
FishSpotTotalCountCh2	
FishSpotTotalAreaCh2	
FishSpotAvgAreaCh2	
FishSpotTotalIntenCh2	
FishSpotAvgIntenCh2	
HeadSpotTotalCountCh2	
HeadSpotTotalAreaCh2	
HeadSpotAvgAreaCh2	
HeadSpotTotalIntenCh2	
HeadSpotAvgIntenCh2	
TorsoSpotTotalCountCh2	
TorsoSpotTotalAreaCh2	
TorsoSpotAvgAreaCh2	
TorsoSpotTotalIntenCh2	
TorsoSpotAvgIntenCh2	
VesselSpotTotalCountCh2	
VesselSpotTotalAreaCh2	
VesselSpotAvgAreaCh2	
VesselSpotTotalIntenCh2	
VesselSpotAvgIntenCh2	

Table 18. *Cell Features and Boolean operators available for Event Definition*

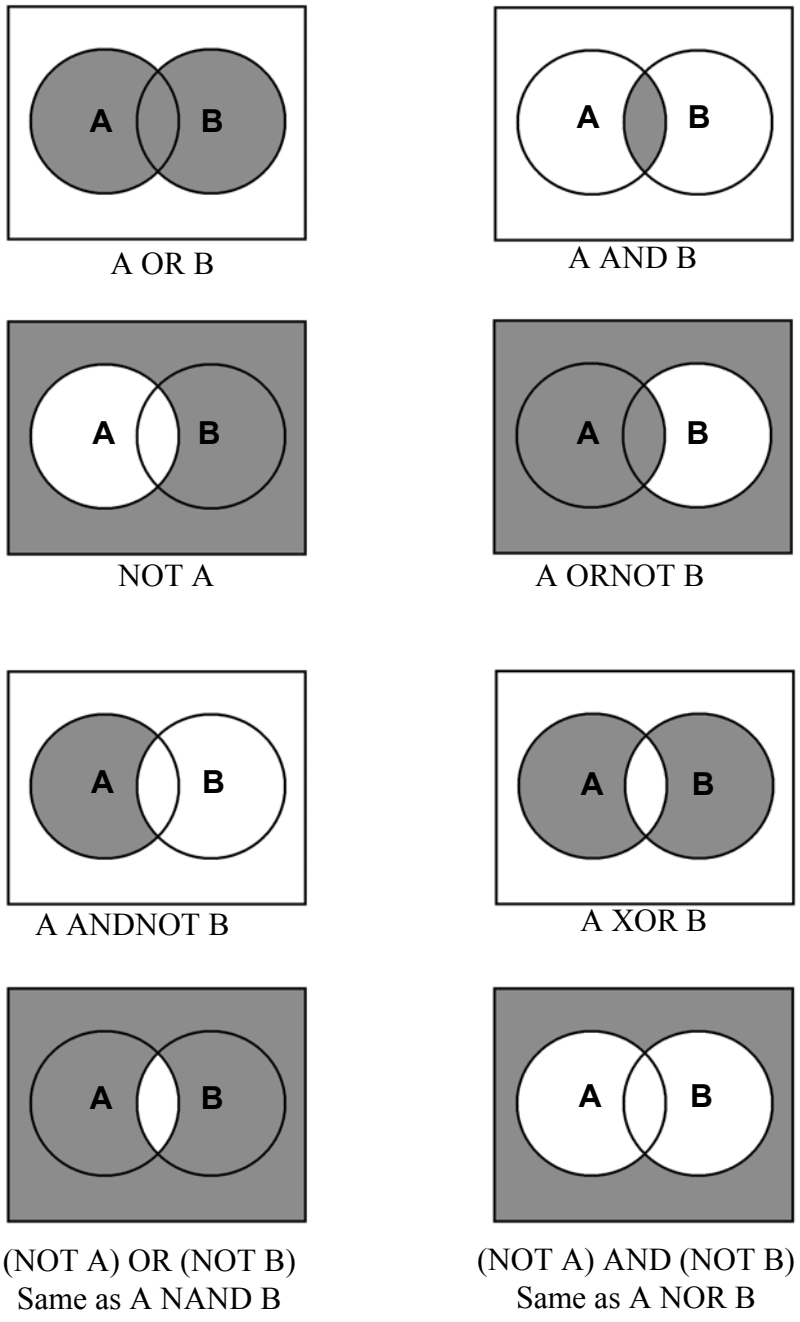


Figure 13. *Schematic definition of available Boolean Operators assuming two Features of interest, A and B*

Event Definition Using the BioApplication Event Wizard

The BioApplication Event Wizard is a software tool that is designed to allow entry, reading, and modifying Event Definitions as logical statements. Event Definitions are stored in the Assay Protocol as Assay Parameters of the type **Type_X_EventDefinition,** where X can be 1-3, allowing definition of up to three distinct events.

The Wizard can be used to enter and edit Event Definitions as values for the **Type_X_EventDefinition** Assay Parameter in the Assay Protocol for the Zebratox BioApplication. This section provides a detailed description of the operation of the BioApplication Event Wizard. The Wizard should only be used after the Zebratox BioApplication has been installed on your computer.

Definition of Events requires that the following steps be followed, in the order listed. **It is recommended that you do not run the Scan software application and the BioApplication Event Wizard at the same time.**

STEP I

1) Create a protocol without defining events. Set optimized parameter values (lower and upper limits) for features to be used for Event Definition.

2) Save protocol.

3) Close the software application.

STEP II

1) Open the BioApplication Event Wizard.

2) Open the protocol saved in Step I using the BioApplication Event Wizard and define up to 3 events at the level of individual cells.

3) Save updated Assay Protocol.

4) Close the BioApplication Event Wizard.

STEP III

1) Restart the Scan software application and open the protocol saved in Step II (Event Definitions will appear as numeric strings in the Assay Parameter window)

2) Copy Event Definitions from the Protocol Comments field in Create Protocol View into the Scan Comments field in Scan Plate View

3) Scan the assay plate.

4) Close the Scan software application.

STEP IV (Optional)

1) Open the saved protocol at a later time using BioApplication Event Wizard.

2) Evaluate and/or modify saved Event Definitions.

3) Close BioApplication Event Wizard and start the Scan software application to scan a new assay plate or analyze previously acquired images using modified Event Definitions.

Features

The following features are included in the BioApplication Event Wizard:

- Can be used with any Zebratox Assay Protocol
- Enables use of standard Boolean operators (NOT, AND, ANDNOT, OR, ORNOT, XOR, NAND, and NOR)
- Read-Only protocol protection
- Displays previously entered Event Definitions in saved Assay Protocols
- Ability to clear each Event Definition individually
- Rapid construction of logical statements using features and logical operators
- Automated validation of each Event Definition when loading or saving protocols
- Ability to save updated protocol
- Addition of Event Definitions to the Protocol Comments field in the Assay Protocol

Steps for Running the Event Wizard with Zebratox BioApplication

Before Running the Event Wizard...

1) Create a protocol using the Zebratox BioApplication without defining events. Set optimized parameter values (upper and lower levels) for detection of responder cells associated with features to be used for Event Definition.
2) Save protocol.
3) Close the vHCS: Scan software application (suggested).

Starting the BioApplication Event Wizard

It is strongly recommended that you close the instrument or software before starting the BioApplication Event Wizard and vice versa. Operation of the BioApplication Event Wizard must be consistent with the steps described.

A. To start the BioApplication Event Wizard from a Saved Protocol:

- Double-click the **BioApplication Event Wizard** desktop icon 

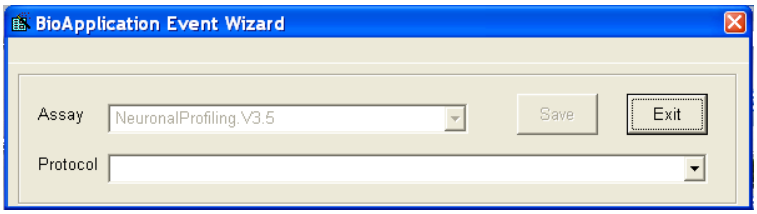
  -or-

  For Cellomics HCS Readers: From the **Start** menu, select **Programs** > **Cellomics** > **BioApp Event Wizard**.

  For vHCS Discovery Toolbox computers: From the **Start** menu, select **Programs** > **Cellomics > vHCS Scan > BioAppEventWizard**

Protocol Selection and Loading

The first window that is displayed (see below) allows selection of any Zebratox Assay Protocol. Note that Event Definitions can only be specified for pre-existing Assay Protocols.



To select an Assay Protocol,

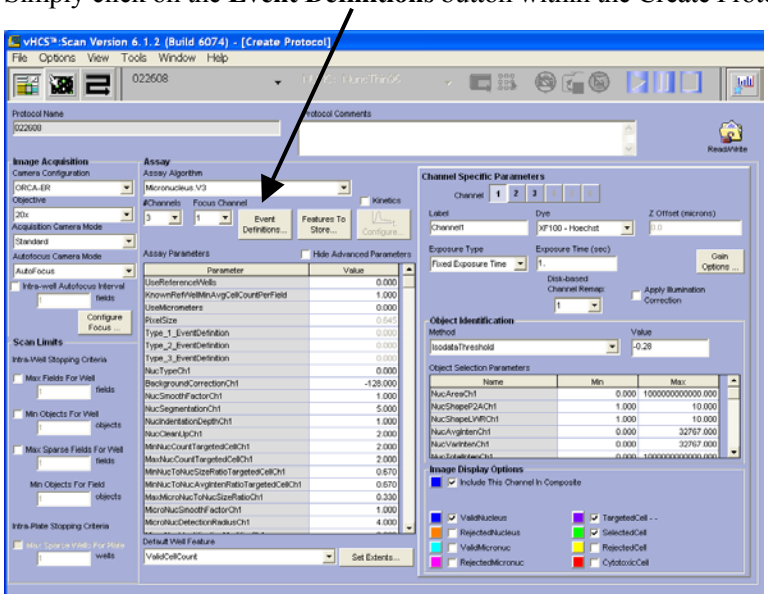
1) From the **Assay** drop-down menu, select **Zebratox.V4.**

2) From the **Protocol** drop-down menu, click on the drop-down arrow to view the list of existing Zebratox Assay Protocols.

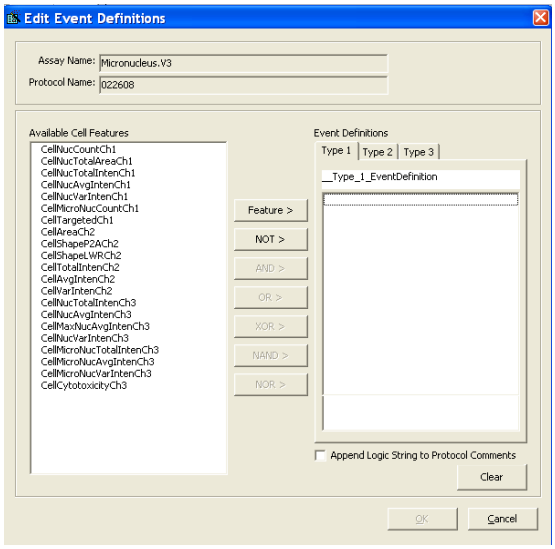
3) Select the desired Assay Protocol from the list.

B. To start the BioApplication Event Wizard from a Currently Used Protocol:

1) Simply click on the **Event Definitions** button within the Create Protocol View.



The Edit Events Definition dialog box appears as shown in the following figure. Features available for Event Definition are listed on the left in the Available Features section.



Once the protocol is loaded, the **Type_X_EventDefinition** Assay Parameters (1-3) are automatically validated. If Event Definition is invalid due to unintentional editing from within the Scan software application, the following message will be displayed, and the Event Definition will be cleared.

If the Assay Protocol is Read-Only, you will only be able to view the Event Definitions and a message above the **Save** button will indicate that the protocol cannot be edited.

2) Once you have completed the Event Definitions, click **OK**. The screen will close and either leave you in the Create Protocol View (if you followed Step B) or an open screen (if you followed Step A).

Defining Events

This section describes the steps involved in defining individual events in a Zebratox Assay Protocol. Note that logical statements used to define Events can include up to three features and four logical operators.

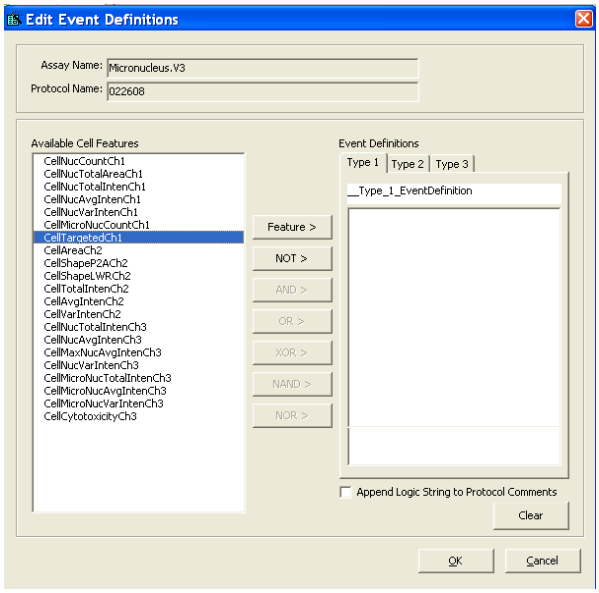
Once the protocol has loaded, each Event Definition can be viewed by clicking on the appropriate tab (Type 1-3).

The procedure to be followed when constructing Event Definitions is described in the sequence of screenshots below. Features are combined with Boolean operators to produce Event Definitions. Any feature can be selected by clicking on the feature name in the Available Cell Features list and then clicking the **Feature** > button. Boolean operators, defined in Chapter 2, are selected by clicking on the Operator buttons (**NOT** >, **AND** >, **OR** >, etc.). Buttons are disabled whenever they cannot be used. Click the appropriate buttons in sequence to build the Event Definition, as shown below.

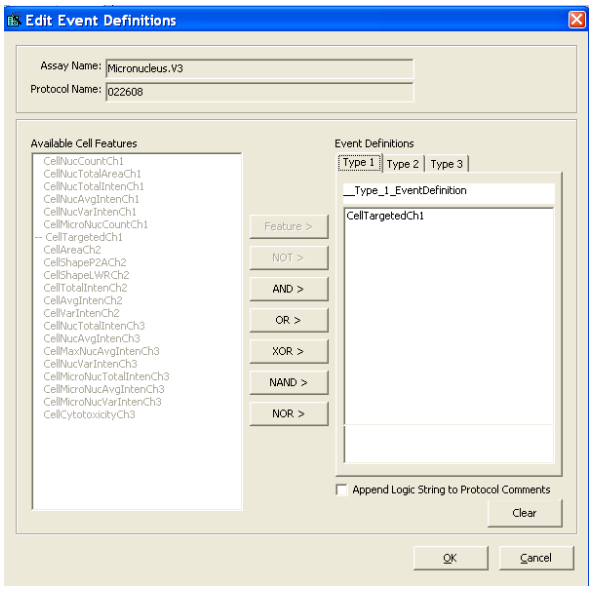
To build an Event Definition

1) Select the Event Definition that you want to specify or edit by clicking on the **Type 1**, **Type 2**, or **Type 3** tab.

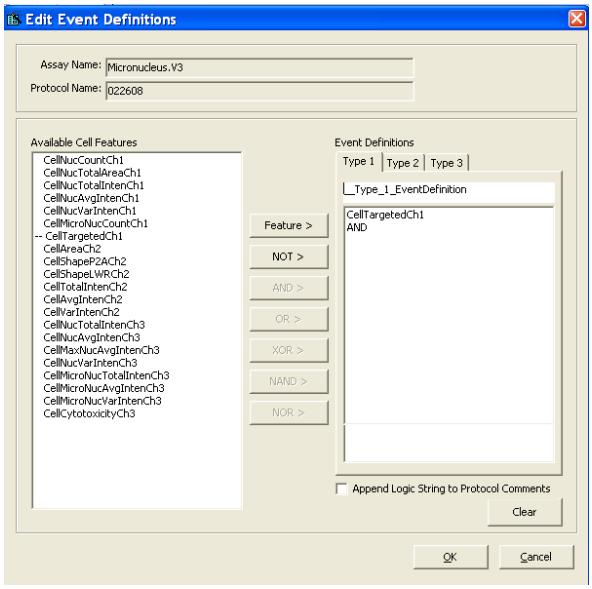
2) Select the desired Feature by clicking on the feature name from the Available Cell Features list. You may also choose to select **NOT>** first (before the Feature) to indicate that you do not want to include this Feature in your event analysis.



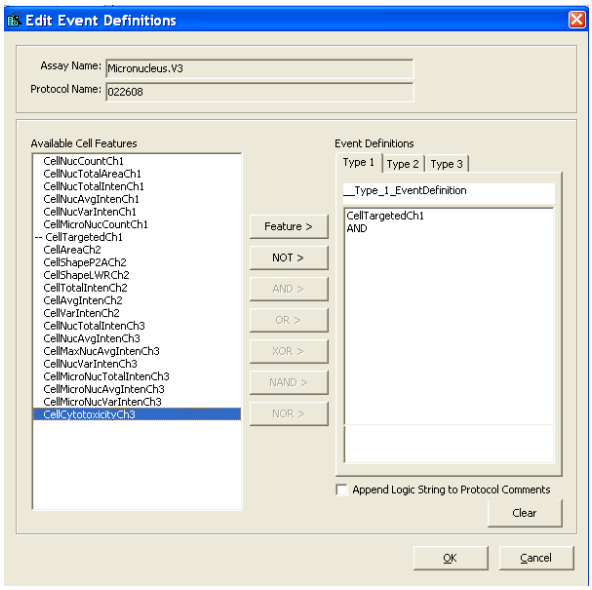
3) Click the **Feature>** button to transfer the Feature into the Event Definition.



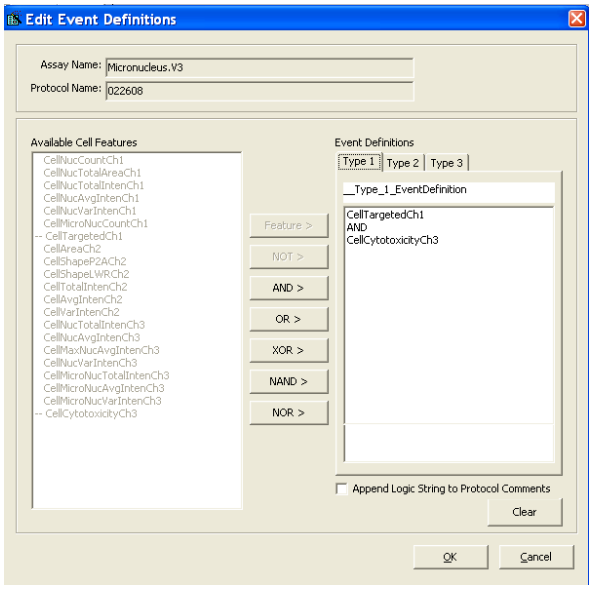
4) Click on the desired Operator button to transfer it into the Event Definition. In this example, the **AND** operator is selected (see below).



5) Repeat the cycle for adding another Feature to the Event Definition.

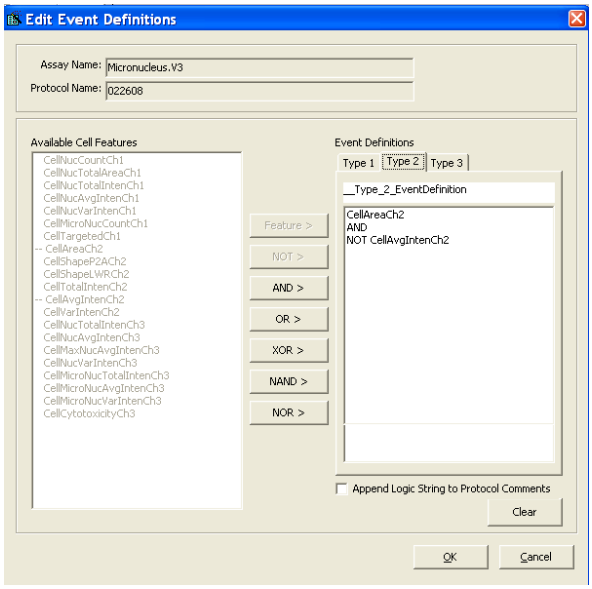


6) As before, click on the Feature > button to transfer the Feature into the Event Definition (see below).



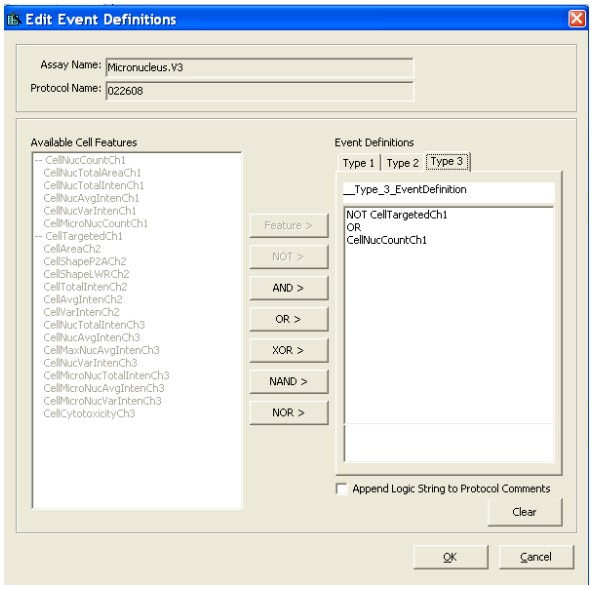
Type_1_EventDefinition

Entry of a logical operator may be followed by entry of another Feature. Alternatively, the operators **AND** and **OR** can be inverted by adding the **NOT** operator by clicking the **NOT >** button prior to selecting the Feature, as shown below.



Type_2_EventDefinition

Typically logical statements used to define Events begin with a Feature. Alternatively, a logical statement may begin with the logical operator **NOT** as shown below.



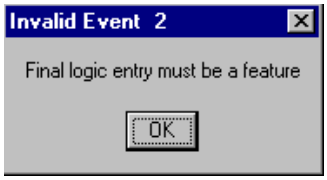
To clear an Event Definition,

- Once a protocol is loaded, click the **Clear** button to clear any Event Definition. This feature can be used to redefine any event.

To save the updated Assay Protocol with Events,

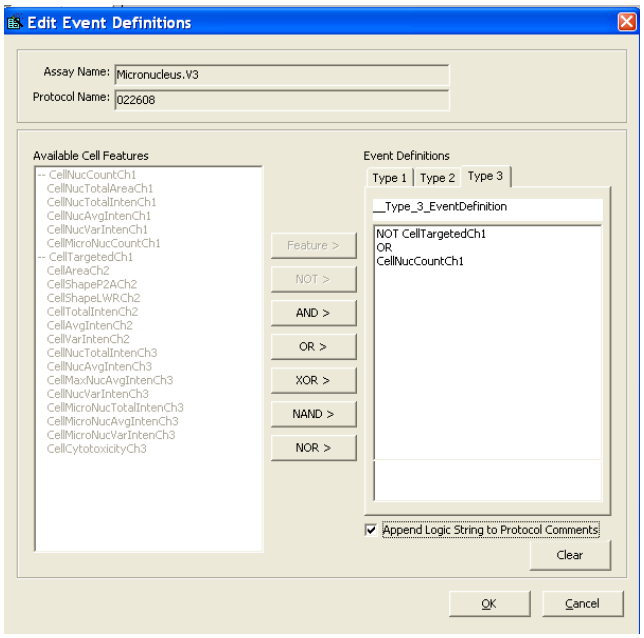
- Click the **OK** button. This will close the Events. From the **File** menu, select **Save Assay Protocol**.
- Note that an Assay Protocol cannot be renamed using the BioApplication Event Wizard. Renaming of protocols can only be done through the Scan software application.

The BioApplication Event Wizard can automatically validate Event Definitions. If Event Definition validation fails, the protocol will not be saved. For example, Event Definitions are considered invalid if they do not end with a Cell Feature. In this case, clicking on the **Save** button causes the following message to be displayed.

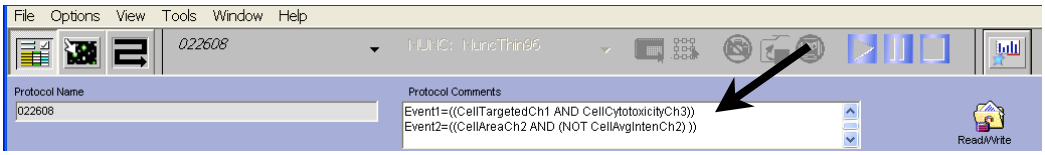


To Show the Events within the Protocol,

- Select the **Append Logic String to Protocol Comments** checkbox. This will transfer the definitions into the protocol.



Event Definitions are then viewable in the **Protocol Comments** field in the **Create Protocol View** upon opening the saved Assay Protocol using the Scan software application (see arrow).



Chapter 4

iDev Software Workflow

This chapter describes the tasks in the workflow for Protocol optimization of the Zebratox 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.

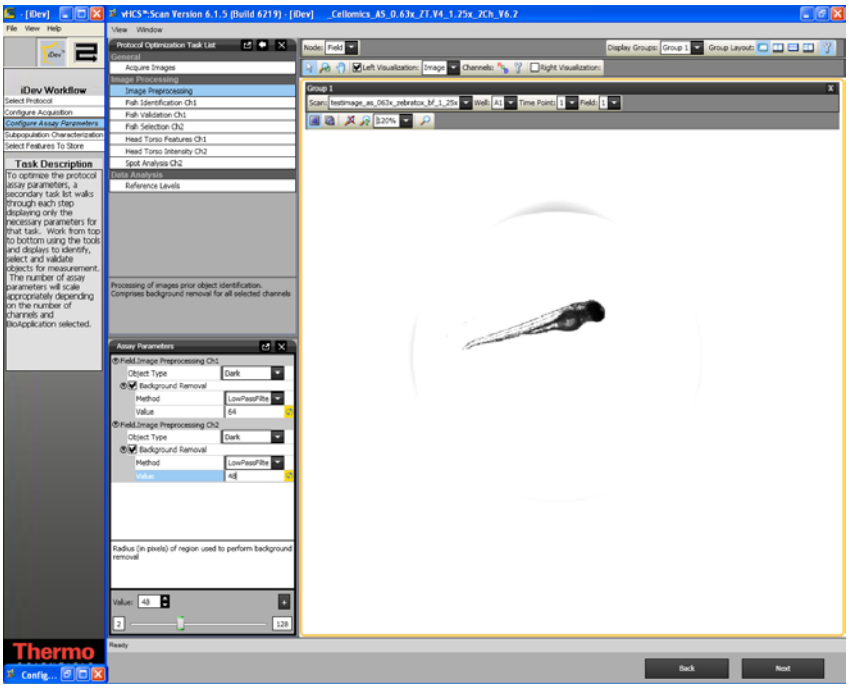


Figure 14. *Protocol Optimization Task – Image Preprocessing*

Fish Identification Ch1

The Fish Identification task is the identification of fish in the Channel 1 image. The task involves setting up methods and values for primary object identification, object smoothing, and object segmentation for Channel 1 objects.

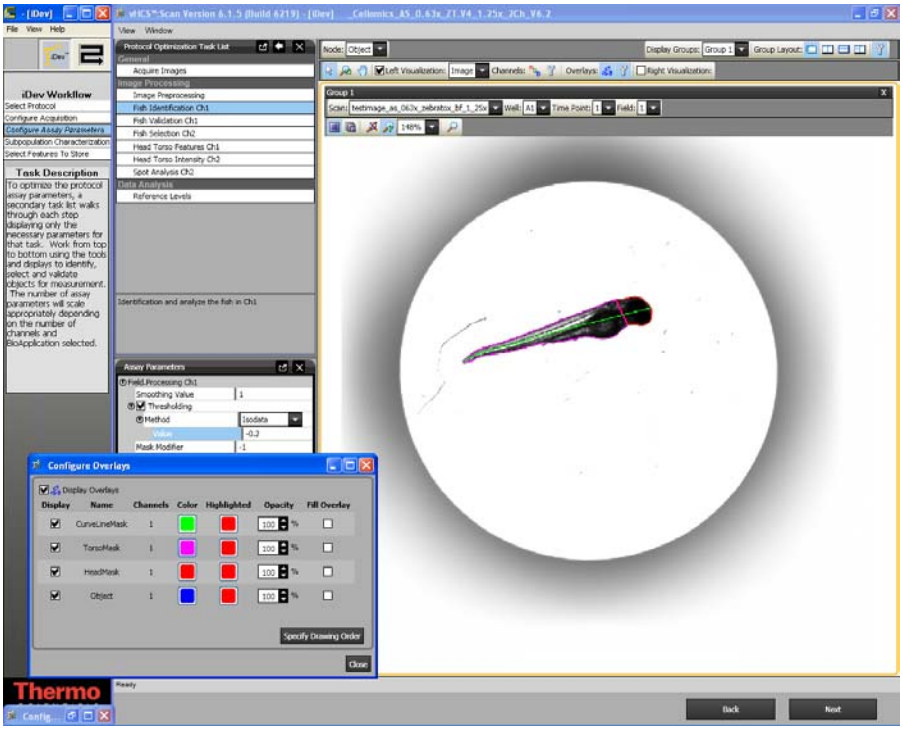


Figure 15. *Protocol Optimization Task – Fish Identification Ch1*

Fish Validation Ch1

The Fish Validation task provides selection and rejection limits for fish identified in Channel 1 based on area, shape, and intensity features calculated for the object 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) the fish in Channel 1 based on selection/rejection features. In this task, you can also determine if objects (fish) that are touching the edges of the field are included or rejected from analysis by checking or clearing the **Object.BorderObjectsCh1** task.

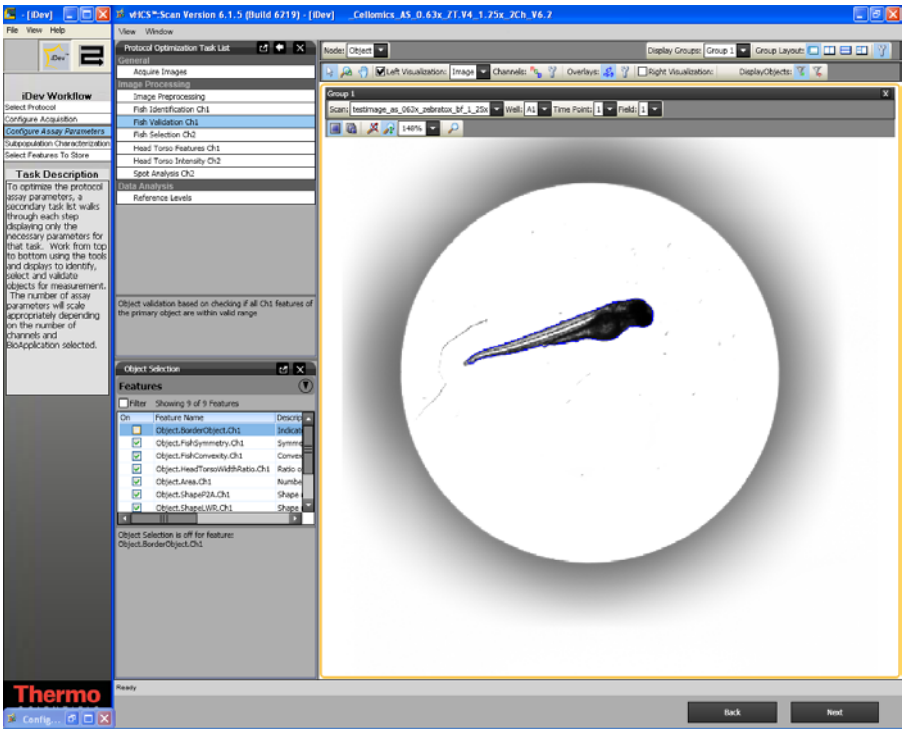


Figure 16. *Protocol Optimization Task – Fish Validation Ch1*

Fish Selection Ch2 through ChN

The Fish Selection task provides object (fish) selection based on intensity features computed in Channels 2 through Channel N under the fish mask derived from Channel 1. 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, **MaskModifierChN,** in the ArrayScan Classic software. The primary object mask can be dilated (> 0), or eroded (< 0) if the **ROI Mask Creation** box is checked.

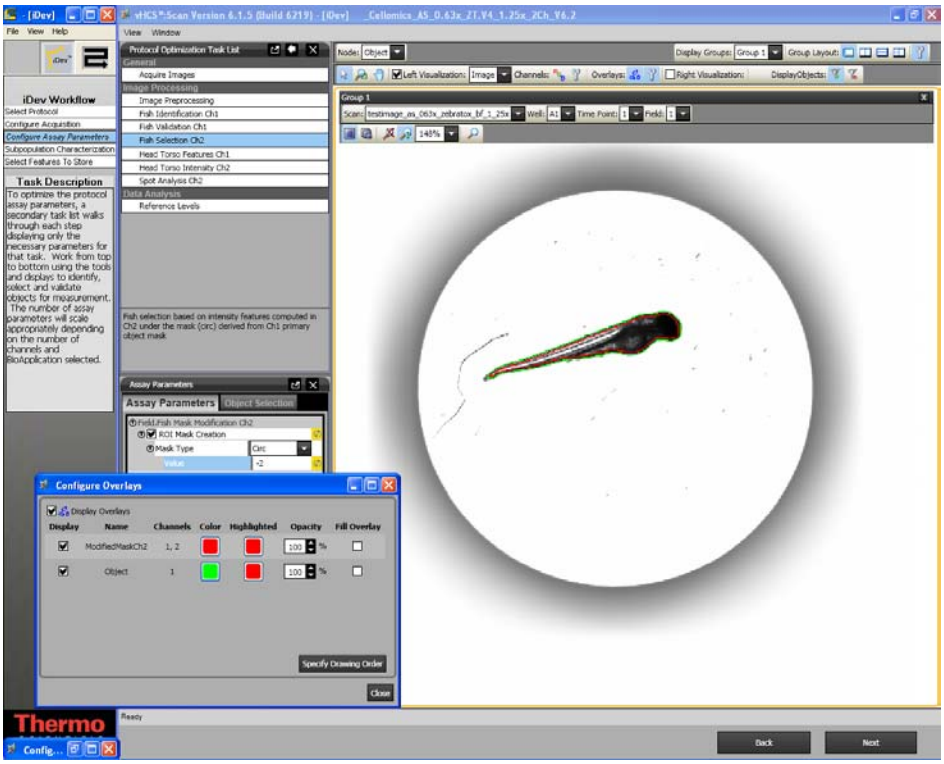


Figure 17. *Protocol Optimization Task – Fish Selection Ch2 through ChN*

Head Torso Intensity Ch2

In this task, you can set parameters for intensity measurements of the fish image in Channel 2.

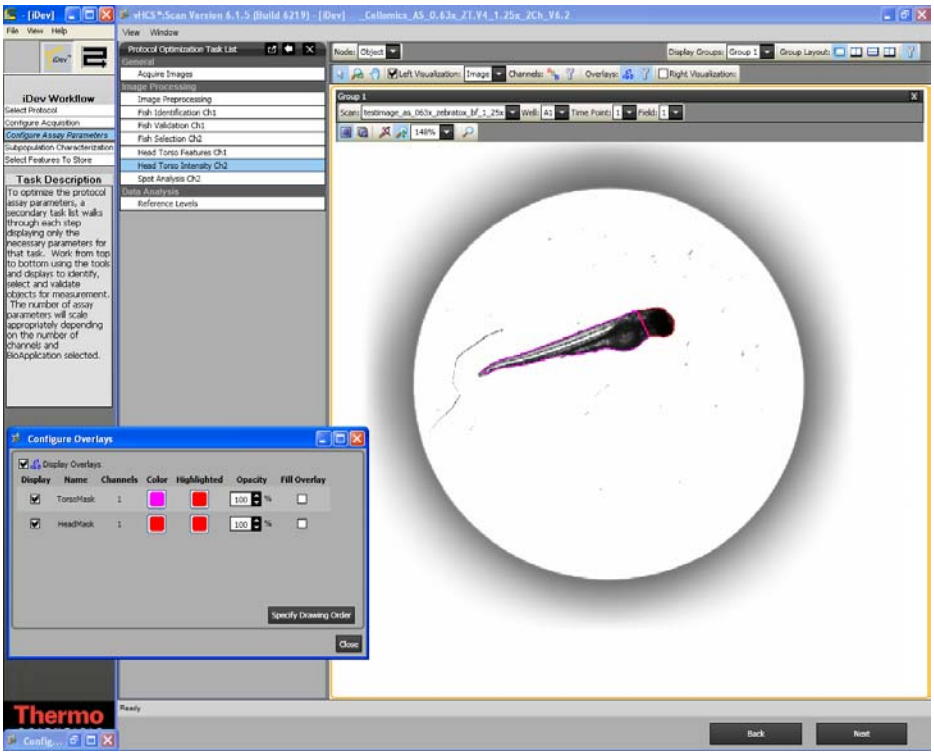


Figure 19. *Protocol Optimization Task – Head Torso Intensity Ch2*

Spot Analysis Ch2

In this task, you will set Assay Parameters (methods and thresholding) including smoothing and segmentation for identifying spots in the head and torso region of the fish image in Channel 2. You can also expand or shrink the fish head and/or torso mask by modifying the values in the tasks **Fish Head ROI Ch2** and **Fish Torso ROI Ch2**.

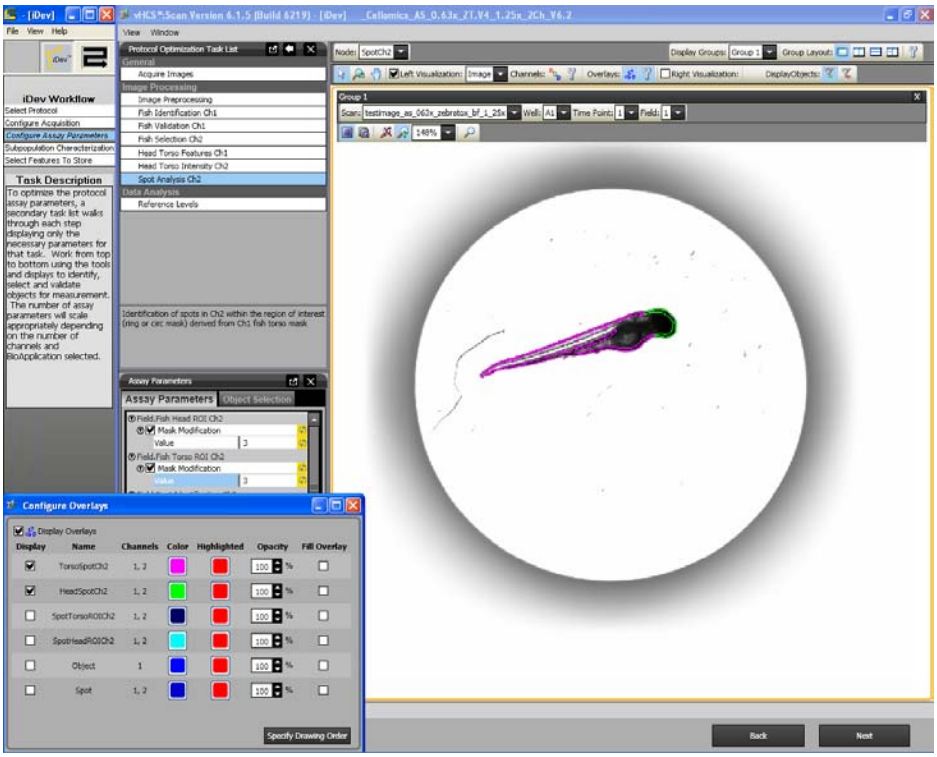


Figure 20. *Protocol Optimization Task – Spot Analysis*

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.

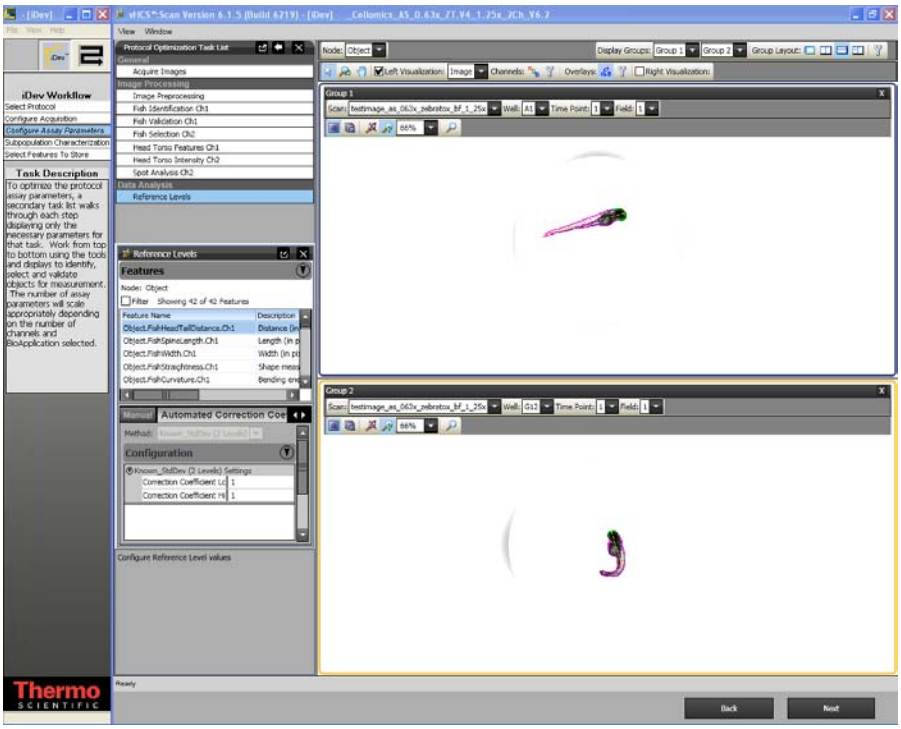


Figure 21. *Protocol Optimization Task – Reference Levels*

Setting Events in the iDev Assay Development Workflow

The process for setting Events in the iDev Assay Development Workflow is identical to that outlined in Chapter 3 of this guide. However, because of the integrated workflow in the iDev product, Events can be set in the Subpopulation Characterization task rather than the BioApplication Event Wizard. Events are based on Reference Levels set in the Protocol optimization task of the Configure Assay Parameters step in the iDev workflow.

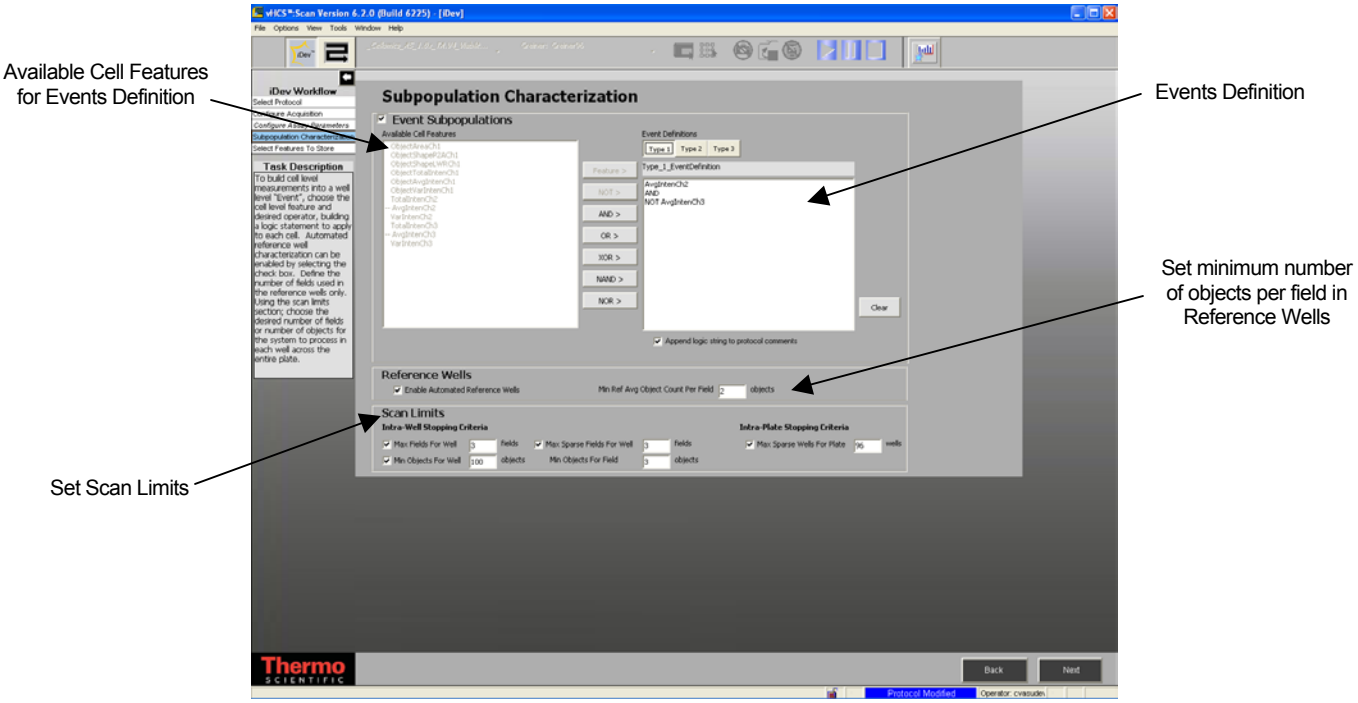


Figure 22. *Subpopulation Characterization Task*

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