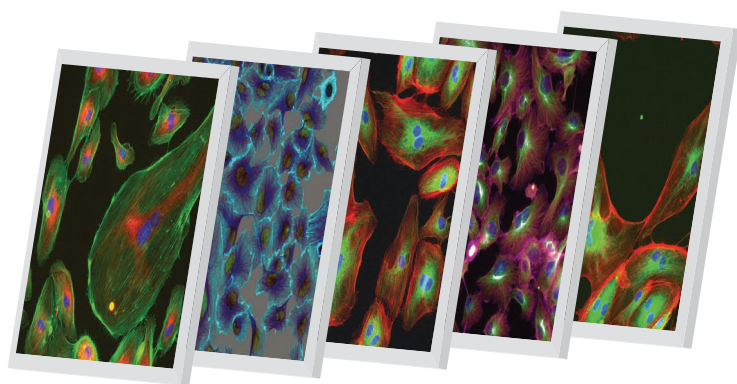


Thermo Scientific Cellomics® Spot Detector V4

BioApplication Guide



Cellomics® Spot Detector BioApplication Guide

V4 Version

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Overview of the Spot Detector BioApplication

High Content Screening (HCS) and High Content Analysis (HCA) use fluorescence-based reagents, advanced optical imaging systems, and sophisticated image analysis software (BioApplications) to quantitatively analyze targets and physiological processes in cells. This guide describes how to use the Cellomics® Spot Detector BioApplication, a versatile and flexible image analysis tool for automatically conducting spot analysis in an HCS environment. This guide contains the following chapters:

Chapter 1 provides an overview of the Spot Detector BioApplication.

Chapter 2 describes how the Spot Detector BioApplication quantitatively analyzes images and provides descriptions of the input parameters and output features.

Chapter 3 describes the use of the Event Wizard with the Spot Detector BioApplication.

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

System Compatibility

The Spot Detector BioApplication is designed to run on the following platforms:

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

NOTE



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

Cell Biology

The Spot Detector BioApplication is a rapid image-analysis algorithm that can be utilized in multiple biological applications. It is designed to provide generic spot analysis that is applicable to a variety of high content assays. Examples include the identification and analysis of intracellular punctate objects such as internalized receptors, multi-protein complexes, lipid globules, specialized endosomes, other organelles, and cellular fragments. This BioApplication can also perform rapid cell counting and analysis of protein expression.

BioApplication Overview and Measurements

The Spot Detector BioApplication is designed to provide generic spot analysis that is independent of any specified biologies. It provides a high degree of flexibility in its analytical methods and is useful in a broad spectrum of assays such as receptor trafficking, vacuolation, steatosis, phospholipidosis, peroxisomal regulation, multi-protein complex regulation, vesicle trafficking, and so on.

The Spot Detector BioApplication supports up to six imaging channels. It can be used for either single-channel or multi-channel assays. For single-channel assays, only Channel 1 is used; it is used for the analysis of spots. Multi-channel assays use Channel 1 for the identification of primary objects (e.g., nuclei or whole cells) and Channels 2-6 for the measurement of spot features and/or the Gating of Channel 1 primary objects. Intra-cellular Target Regions, known as circ (circle) and ring are created from the primary objects and are used to associate spots in Channels 2-4 with Channel 1 objects. The intensities measured in these Target Regions are also used to select Channel 1 objects for analysis (i.e., Gating).

The Spot Detector BioApplication analyzes individual objects and spots. These results are combined at various levels to compute features that are used to characterize response to treatment and are reported as Cell, Field and Well Features, which are summarized in Table 1. Features are reported in Channel 1 for either primary objects or spots, depending on whether Channels 2-6 are present. Spot features are reported for Channels 2-4, including normalized spot features, computed by dividing by the number of objects in the field or well. Finally, well-level statistics are reported on all Cell Features, including the percentages of cells with high and low response to treatment.

Additionally, the Spot Detector BioApplication can be used for live cell analysis. Please refer to your *ArrayScan VTI HCS Reader User's Guide* for more information.

Type	Region	Cell Features	Field Features	Well Features
Cell, Field & Well Features Reported for Channel 1:				
Count	Primary Objects or Spots	Cell Number	Valid Object Count Selected Object Count Selected Object Count %Selected Objects	Valid Object Count Selected Object Count % Selected Objects Field Count Valid Field Count Selected Object Count Per Valid Field MEAN, SD, SE, CV, %LOW, %HIGH for all cell features
Morphology		Object Area & Status Object Shape P2A & Status Object Shape LWR & Status	Object Total Area Object Average Area	Object Total Area Object Average Area MEAN, SD, SE, CV, %LOW, %HIGH for all cell features
Intensity		Object Total Intensity & Status Object Avg Intensity & Status Object Variance Intensity & Status	Object Total Intensity Object Average Intensity Object Total Intensity Per Object	Object Total Intensity Object Average Intensity Object Total Intensity Per Object MEAN, SD, SE, CV, %LOW, %HIGH for all cell features
Cell, Field & Well Features Reported for Channels 2-4:				
Count	Spots (confined by object-derived Circ or Ring Target Regions)	Spot Count & Status	Spot Count Spot Count Per Object	Spot Count Spot Count Per Object MEAN, SD, SE, CV, %LOW, %HIGH for all cell features
Morphology		Spot Total Area & Status Spot Avg Area & Status	Spot Total Area Spot Average Area Spot Total Area Per Object	Spot Total Area Spot Average Area Spot Total Area Per Object MEAN, SD, SE, CV, %LOW, %HIGH for all cell features
Intensity		Spot Total Intensity & Status Spot Avg Intensity & Status Total Intensity & Status Avg Intensity & Status	Spot Total Intensity Spot Average Intensity Spot Total Intensity Per Spot Spot Total Intensity Per Object	Spot Total Intensity Spot Average Intensity Spot Total Intensity Per Spot Spot Total Intensity Per Object MEAN, SD, SE, CV, %LOW, %HIGH for all cell features
Cell, Field & Well Features Reported for Channels 5-6:				
Intensity	Primary Object Area	Total Intensity Avg Intensity	None	None

Table 1. Cell-Level, Field-Level, and Well-Level Features reported by the Spot Detector BioApplication

Example Biology

Example #1: Using Spot Detector for Mitotic Index

Mitotic index is the percentage of cells within a population undergoing mitosis. Mitotic cells are visually identifiable based on the phosphorylation of mitosis-specific proteins which can be labeled with an antibody. The Spot Detector BioApplication can identify mitotic cells by detecting spots corresponding to mitotic nuclei (Figures 1 and 2).

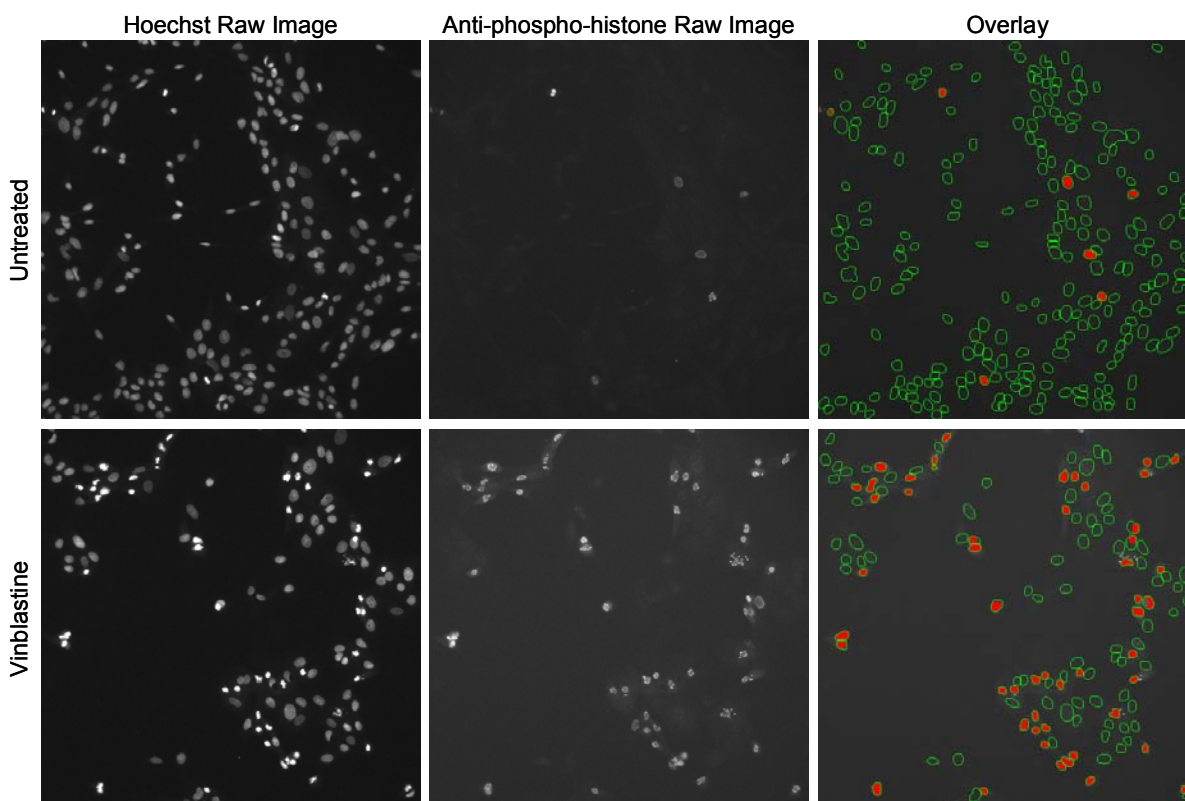


Figure 1. MDCK cell images acquired and analyzed with the Spot Detector BioApplication. Raw (as-acquired) images from Channel 1 (Nuclei, Hoechst), Channel 2 (Mitotic Cells, anti-phospho-histone) and overlays from the analyzed Channel 2 image are shown. The overlays indicate spot identification Target Regions (circ, green) and spots (red). The top images depict untreated control wells and the bottom images depict vinblastine-treated wells.

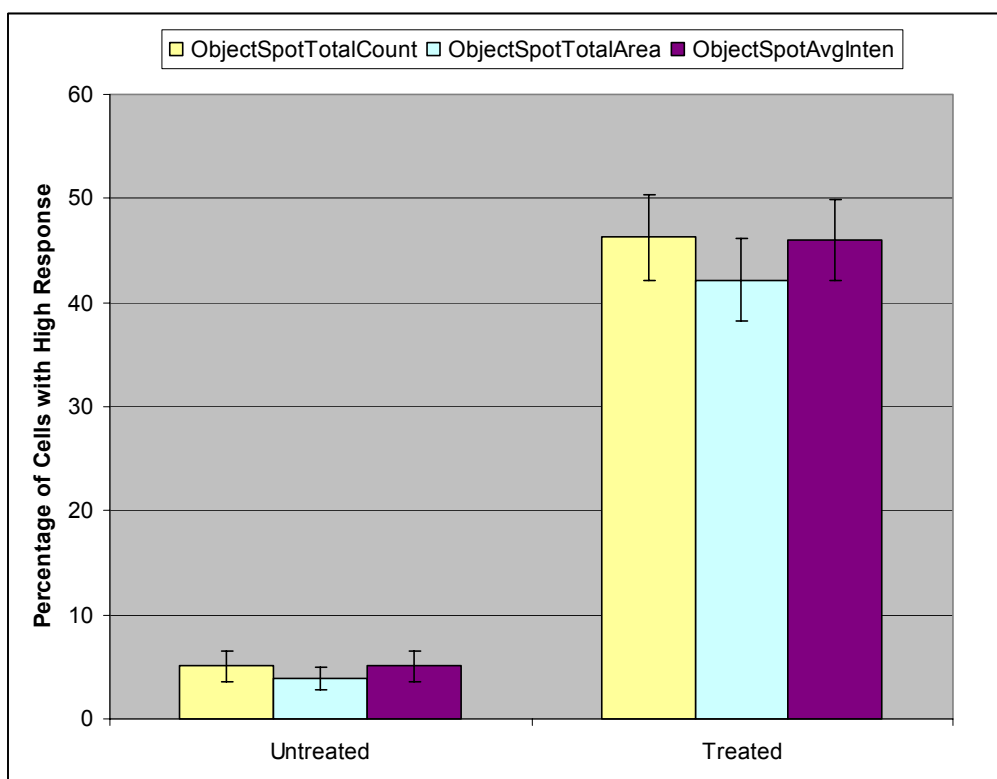


Figure 2. MDCK cells treated with an anti-microtubule drug and stained with the Cellomics® Mitotic Index HCS Reagent Kit and analyzed with the Spot Detector BioApplication using Reference Well Analysis. Measures of Spot Count, Area, and Intensity all show significant differences between treated and untreated cells. The percentage of cells with a high response is an indicator of the number of mitotic cells.

Example #2 Using Spot Detector for Cell Viability

Cells with increased permeability become porous to large macromolecules. Under these conditions, fluorescent stains can enter the cell and be visualized. The Spot Detector BioApplication is able to quickly detect nuclear-size spots of labeled live or dead cells. In Figure 3, cells were treated with Tween-20 which caused the majority of the cells to die. The BioApplication detects and reports the percentage of cells with spots corresponding to live and dead dyes for a rapid population analysis.

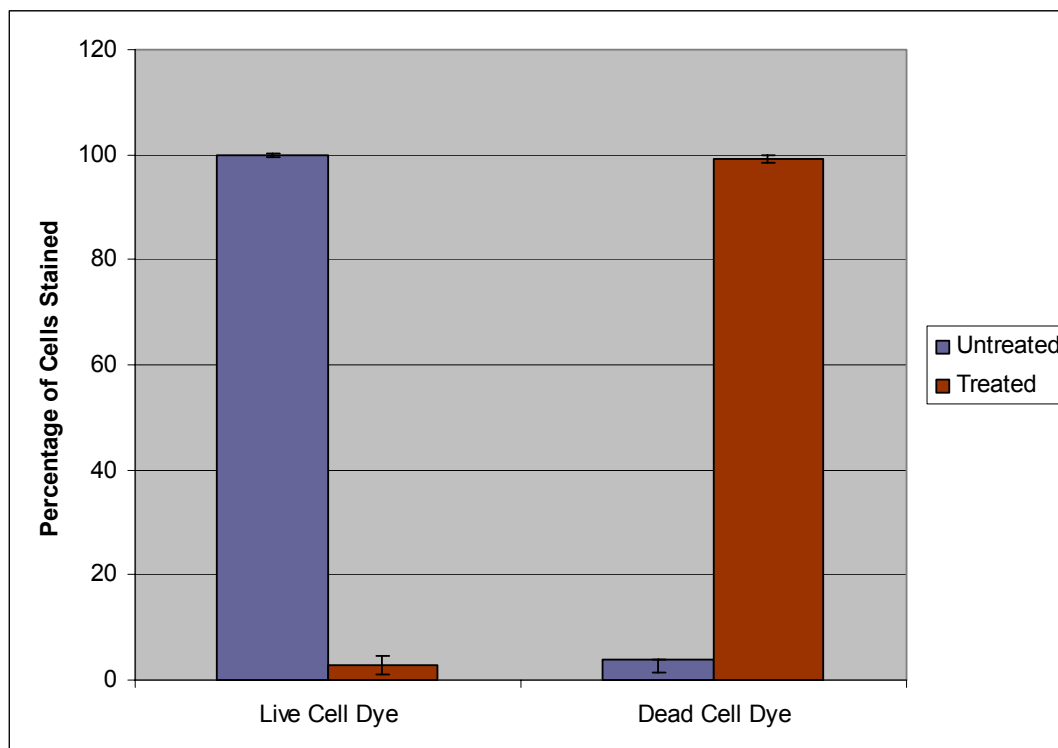


Figure 3. HeLa cells treated with Tween-20, labeled with the Cellomics® Cell Viability HCS Reagent Kit, and analyzed with the Spot Detector BioApplication. The majority of untreated cells stain with the Live Cell Dye while the majority of cells treated with Tween-20 stain with the Dead Cell Dye. The percentage of cells stained reflects %HIGH_ObjectSpotTotalCount Output Feature for cells exhibiting at least one spot for the particular stain of interest.

Example #3 Using Spot Detector for GPCR Analysis

High Content Screening of GPCRs with this BioApplication is possible with the use of cells that stably express GFP labeled β -arrestin (Ghosh et al., 2005). Agonist activation of the V2 Vasopressin GPCR causes GFP-labeled β -arrestin to associate with it, and triggers its internalization. The V2 Vasopressin receptor represents a sub-class of GPCRs (Class B) in which the GFP- β -arrestin remains associated with the receptor during internalization and trafficking into early endosomes, causing it to appear as punctate spots in the cytoplasm.

In one experiment, a Min/Max plate was prepared by treating half of a 96-well plate with a single concentration of [Arg]-Vasopressin for 30 minutes and leaving the other half of the plate untreated. Figure 4 shows images of the GFP- β -arrestin (Channel 2). These images were acquired on the ArrayScan VTI HCS Reader using a 20x objective lens and analyzed with the Spot Detector BioApplication. The established effects of Arg-Vasopressin on the internalization and trafficking of the V2 Vasopressin GPCR are clearly evident in the images by the formation of punctate spots in the cytoplasm.

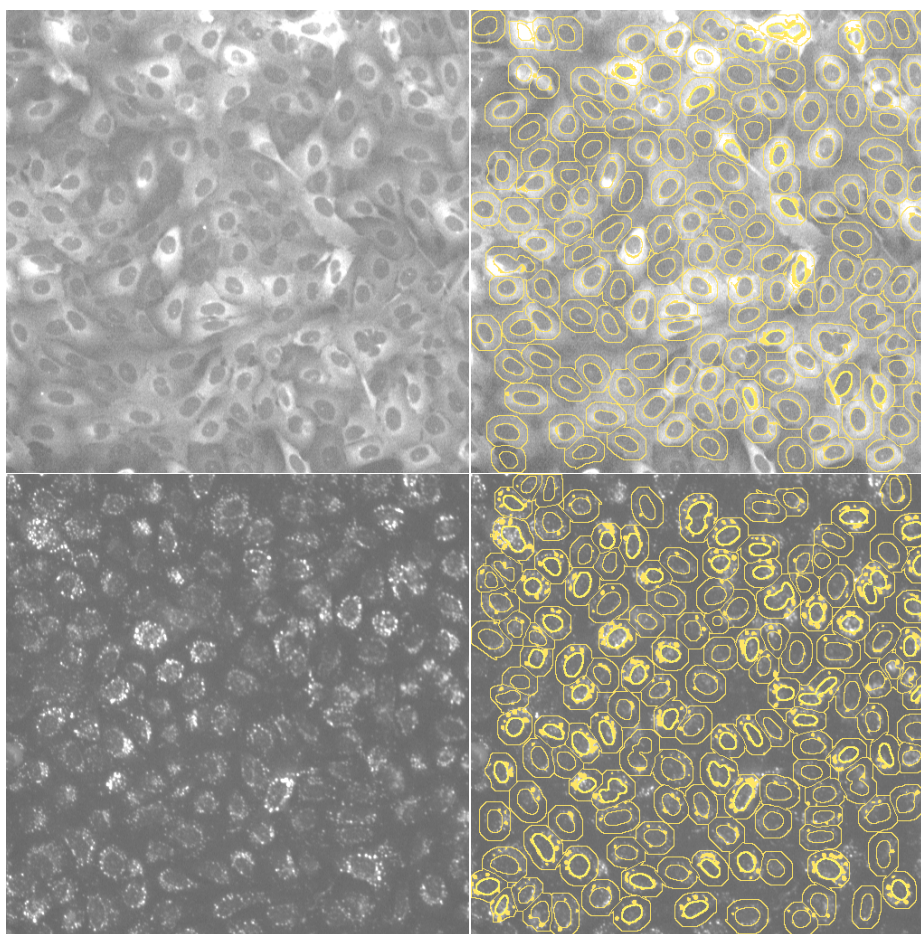


Figure 4. Images acquired in Channel 2 (GFP) using the Spot Detector BioApplication. The images on the left are the raw (as-acquired) images. These are displayed again on the right with overlays indicating spot identification Target Regions (rings) and spots (solid). The top images depict untreated control wells and the bottom images depict treated wells.

Figure 5 shows the difference in response between the Min/Max wells for the well-level feature **SpotTotalIntensityPerObject** in Channel 2. The Z' metric (Zhang, et al. 1999) for these results is greater than 0.5, indicating that this assay would be excellent for screening.

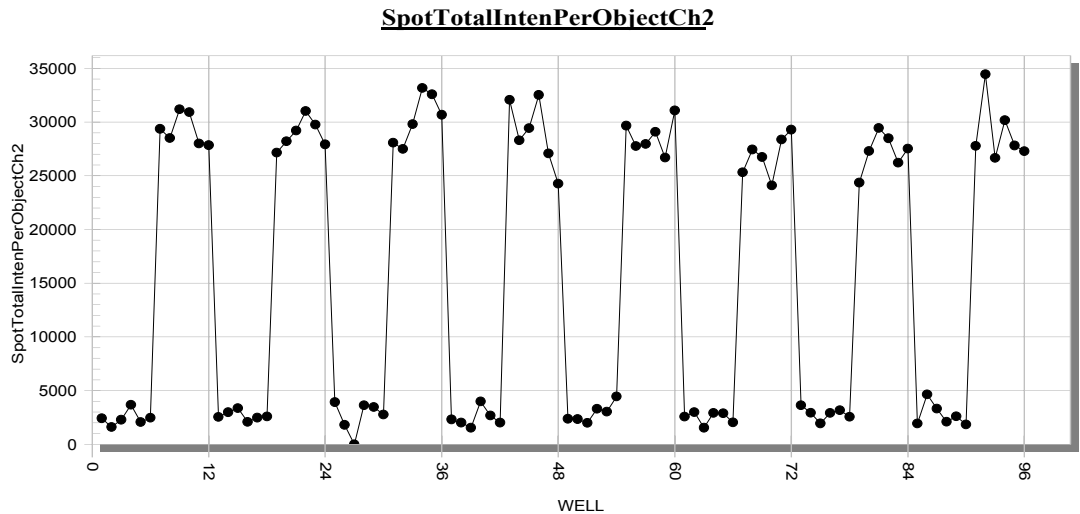


Figure 5. A graph of the well-level Spot Total Intensity Per Object in Channel 2 for a Min/Max plate

In another experiment, the cells were treated with increasing concentrations of Arginine-Vasopressin and incubated under appropriate conditions for 30 minutes. Figure 6 shows the response of the well-level **SpotCountPerObject** in Channel 2 to increasing concentrations of [Arg]-Vasopressin. Each data point is the mean of 8 wells and the error bars represent the standard error of the mean.

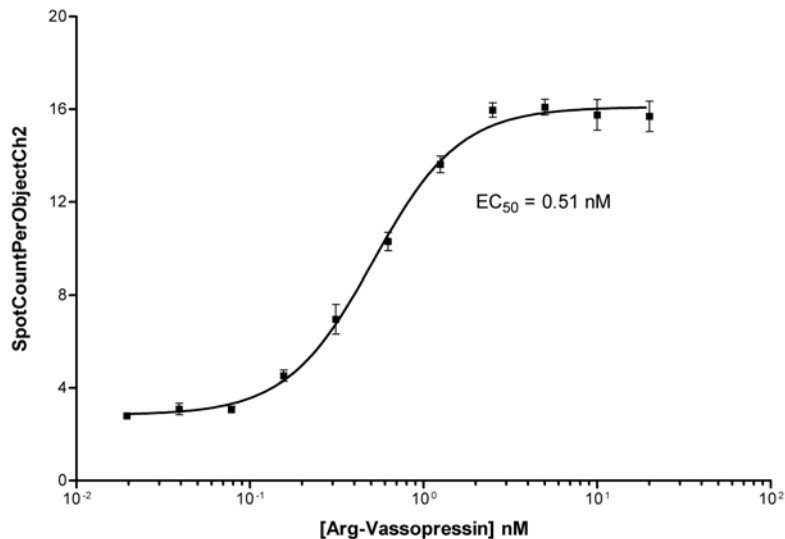


Figure 6. Dose response curve showing the EC50 for the well-level output feature **SpotCountPerObject** in Channel 2

Other Use Cases: Receptor Internalization

Some receptors constitutively internalize and recycle. Cells treated with fluorescent-labeled ligand, internalize the labeled ligand. Internalization can be observed as punctate spots. As ligand-receptor complexes accumulate in the endocytic recycling compartment, spots appear in the perinuclear region and spot size and brightness increases. By adding a receptor antagonist or inhibitor of internalization, a difference in receptor internalization can be easily measured in the Spot Detector BioApplication by readily detecting internalization to the endocytic recycling compartment. The percentage of cells with a minimum spot count is a sensitive measure of these biological changes.

References

- Ghosh, R.N., R. DeBiasio, C.C. Hudson, E.R. Ramer, C.L. Cowan, and R.H. Oakley. 2005. Quantitative Cell Based High-Content Screening for Vasopressin Receptor Agonists Using Transfluor® Technology. *J. Biomol. Screening* 10: 476-484.
- Zhang, J. H., T. D. Chung, and K. R. Oldenburg. 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screening* 4: 67-73.

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 the purpose and measurements of the Spot Detector BioApplication. This chapter describes how the Spot Detector BioApplication works and describes the adjustable input parameters that control the processing and analysis.

Overview

There are three types of input parameters: Assay Parameters, Object Identification Parameters, and Object Selection Parameters. The Assay Parameters control the actual quantitative analysis of the images, the Identification Parameters control the intensity threshold used to identify objects, and the Selection Parameters control which objects are chosen for analysis.

There are several key steps in the Spot Detector algorithm. The BioApplication first performs Reference Well calculations (if necessary), then background correction on all channels, and finally, selection based on size and area. If a single-channel assay is setup, the algorithm will identify spots in Channel 1 (using Assay Parameters **SmoothFactorCh1**, **SmoothMethodCh1**, **SpotDetectRadiusCh1**, and **SpotDetectMethodCh1**) and then select these spots based on morphological and intensity characteristics for Object Selection Parameters. Note that “Object” actually means Spot for a single-channel assay. Upon completion, output features for this channel can be calculated.

For a multi-channel assay, the algorithm will again identify objects in Channel 1, but will first use segmentation to separate touching objects and smooth object boundaries. Object Selection Parameters are then used to select the objects in Channel 1. Object Selection (using average and total intensities under the modified cellular mask, (also called Gating, if it is necessary) is done on objects for Channels 2-6 and spot identification Target Regions are identified (using Assay Parameters **CircModifierCh2-4**, **RingDistanceCh2-4**, and **RingWidthCh2-4**, and Object Selection Parameters for intensity). Channel 1 object features are calculated (as in the single-channel assay). The algorithm then continues to identify spots in Channels 2-4 using Assay Parameters **SmoothFactorCh2-4**, **SmoothMethodCh2-4**, **SpotDetectRadiusCh2-4**, and **SpotDetectMethodCh2-4** and then selects them using the morphological and intensity Object Selection Parameters. From this, all additional spot features are then calculated.

Object Identification Methods

To identify objects and spots, an independent intensity threshold must be set for each channel. In Channel 1, objects or spots are identified; only pixels with intensities above this threshold will be considered as belonging to these structures. Thus the proper setting of an intensity threshold is a key early step in identifying objects and thus configuring the application for the analysis for a particular type of biology. In Channels 2-4, spots within objects are identified, and pixels with intensities above this threshold are used to help identify the individual spots.

There are five different options or methods for intensity thresholds, and a subset of these is available for each channel. The options available for each channel are summarized in Table 2, and the descriptions of the different methods follow in Table 3.

Intensity Threshold Options	Channel Availability		
	Channel 1	Channels 2-4	Channels 5-6
None		✓	✓
Isodata	✓	✓	
Triangle	✓	✓	
3Sigma	✓	✓	
Fixed	✓	✓	

Table 2. Intensity Threshold Options Available for Each Channel

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 2-6), the value must be 0.

Of the five intensity threshold options, **None** means 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 object.

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

The other three threshold methods (**Isodata**, **Triangle**, and **3Sigma**) are known as histogram-derived thresholds because the threshold is chosen from the histogram of pixel intensities in the image (i.e., the image brightness histogram). The schematics in Figure 7 demonstrate how these histogram-derived threshold values are derived. Descriptions can be found in Table 3.

The values entered in the application for the three 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 threshold, T_F , which is finally applied to the image is determined from the user-entered offset, 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, o , of 0.9 will result in a final threshold of 1900 being applied to the

image, whereas entering an offset -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 be between the range 1-32767.

The three histogram-derived methods are dependent on the contents of the image, unlike the **Fixed Threshold** method. For example, for a blank image that contains no cellular objects and only has background pixels with a mean intensity value of 500 and standard deviation of 50, it is unlikely that a Fixed Threshold of 1000 will cause any pixels to be registered. However, all three histogram-derived threshold methods will give thresholds causing pixels in the image to be registered for potential analysis. The reason for this is the histogram is of the pixel intensity distribution of that image, even though there are no cellular objects in the image. 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), a histogram-derived threshold method should be avoided. Rather a **Fixed Threshold** or **Triangle Threshold** with a large offset should be entered.

Threshold Option	Description	Range of Possible Values Entered	Resulting Applied Threshold Range
None	No threshold applied	-----	none
Isodata	Adjusts the object identification threshold relative to the Isodata value. The threshold T is chosen so that it is equal to the average of the mean of the pixel intensities to the left of the threshold (m_L) and the mean of the pixel intensities to the right of the threshold (m_R). A negative value identifies dimmer objects and results in larger object masks. A positive value results in smaller object masks.	-0.999 – 9.999 (offset)	1 - 32767
Triangle	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
3Sigma	The threshold T is at three standard deviations (σ) to the right of the mean.	-0.999 – 9.999 (offset)	1 - 32767
Fixed	A fixed pixel intensity value between 0-32767 is applied.	0 – 32767 (true intensity)	0 - 32767

Table 3. Intensity Threshold Descriptions used in the Spot Detector BioApplication.

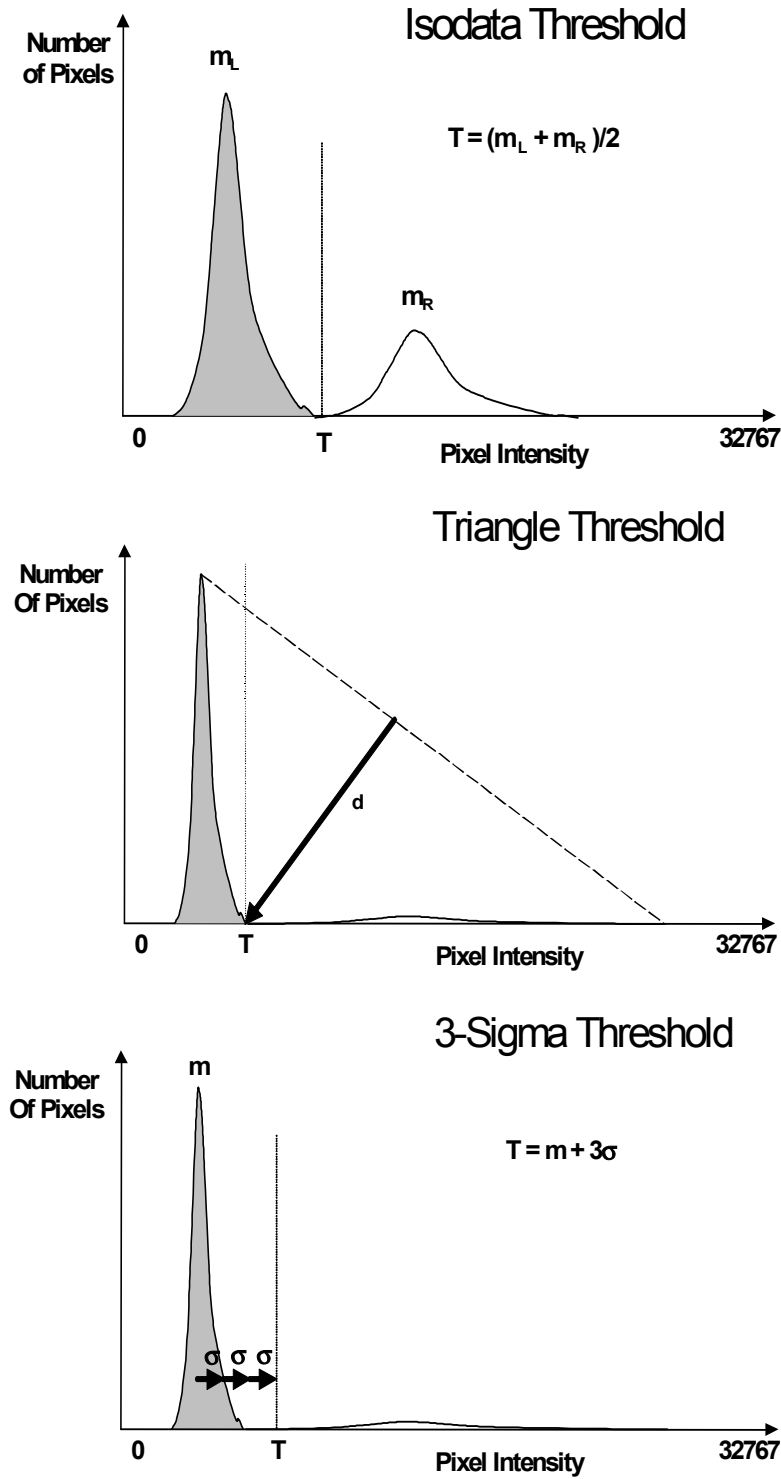


Figure 7. Histogram-derived Intensity threshold methods: Background peak is shown in gray and object peak is shown in white. Top: Isodata; Middle: Triangle; Bottom: 3-Sigma.

Description of Assay Parameters and Settings

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

Assay Parameters for Image Analysis

General Assay Parameters

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

- **Reference Well Control**
- **Units for Morphological Measurements**
- **Object Type**
- **Background Correction**
- **Reject Border Objects**

Reference Well Control

If you would like to have the Spot Detector BioApplication automatically compute the high and low cellular response levels, you must set the Assay Parameters that control the use of Reference Wells. The first of these is **UseReferenceWells**. Set this Assay Parameter to **1** if you want to use Reference Wells or to **0** if you do not want to use Reference Wells. If Reference Wells are not used, the BioApplication will determine cellular response using the manually specified levels. These are channel-specific Assay Parameters and are described later in this chapter.

If Reference Wells are used, the Assay Parameter **MinRefAvgObjectCountPerField** must also be set. This Assay Parameter specifies the minimum average number of objects per field required for acceptance of the high and low levels derived from the Reference Wells. If the average number of objects per field is less than **MinRefAvgObjectCountPerField**, the BioApplication will use the manually specified cellular response levels.

Units for Morphological Measurements

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

Object Type ChN

ObjectTypeCh1, SpotTypeCh2-4

The “Object 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** considers dark areas within an image as potential objects, while leaving it as **0** considers bright areas on a dark background as potential objects (Table 4).

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

Table 4. Binary settings for *ObjectTypeCh1* and *SpotTypeCh2-4*

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

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

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

Background Correction Ch1-6

Prior to image analysis, the non-cellular background can be computed and subtracted from each image in each channel independently, as shown in Figure 8.

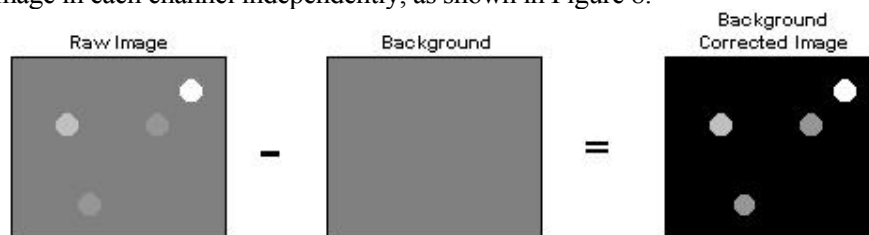


Figure 8. Background Correction that can be applied to all channels.

The background-corrected image is computed by suppressing high frequency components in the image (low pass filtration). You can control the creation of the background image by adjusting the **BackgroundCorrectionCh1-6** Assay Parameter in each channel as needed. This Assay Parameter refers to the radius of the area that is sampled around each pixel in the image to determine its local background. The value of this Assay Parameter should be much larger than the radii of the objects in the image. If the value is set to **0** (zero), background correction is not performed, and analysis is done using the raw, uncorrected images.

If the **BackgroundCorrectionCh1-6** 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. Setting this value to **-1** lets the application decide on the value needed for the optimal background correction. Table 5 provides an overview of the 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 5. Background Correction methods for all channels.

NOTE



In all cases (except where the value = 0), the reported image pixel intensity values will be reduced. Background Correction can be adjusted on each channel separately. The background-corrected image is not stored or shown.

Reject Border Objects

When running the BioApplication, you have the option of not including and analyzing objects that touch the border of your image field. This is controlled by the **RejectBorderObjectsCh1** Assay Parameter. If this Assay Parameter has a value of **1**, objects touching the border are not analyzed. On the other hand, a value of **0** results in all objects in the image field to be eligible for analysis, whether or not they touch the image border.

Channel-Specific Assay Parameters

Channel 1 Assay Parameters

The Assay Parameters that apply specifically to Channel 1 affect the identification of spots or primary objects, depending on whether the assay is single-channel or multi-channel. These Assay Parameters can be divided into the following three groups:

- Object Segmentation
- Object Smoothing
- Spot Detection

The Channel 1 Assay Parameters are listed in Table 6. These Assay Parameters remain the same for both single-channel and multi-channel assays; however, there is a difference in how the algorithm responds to them. The Channel 1 Assay Parameters are described further in the sections that follow.

Ch1 Assay Parameter	Single-Channel Assays (Spots Identified in Ch1)	Multi-Channel Assays (Objects Identified in Ch1)
ObjectSegmentationCh1	Ignored	As Specified
SmoothFactorCh1	As Specified	As Specified
SmoothMethodCh1	As Specified	Ignored
SpotDetectRadiusCh1	As Specified	Ignored
SpotDetectMethodCh1	As Specified	Ignored

Table 6. Channel 1 Assay Parameters for the Spot Detector BioApplication

Object Segmentation

ObjectSegmentationCh1

NOTE



Single-channel assays, which identify spots in Channel 1, ignore the object segmentation Assay Parameter.

Even though you may have chosen an optimal intensity threshold and an appropriate degree of smoothing for object identification, you may encounter situations where individual objects cannot be properly resolved, such as in the case of densely packed objects. Object Segmentation will help resolve and identify individual objects. Two methods are available for object segmentation in the Spot Detector BioApplication: geometric (shape and size) and intensity (intensity peaks). These are illustrated in Figure 9.

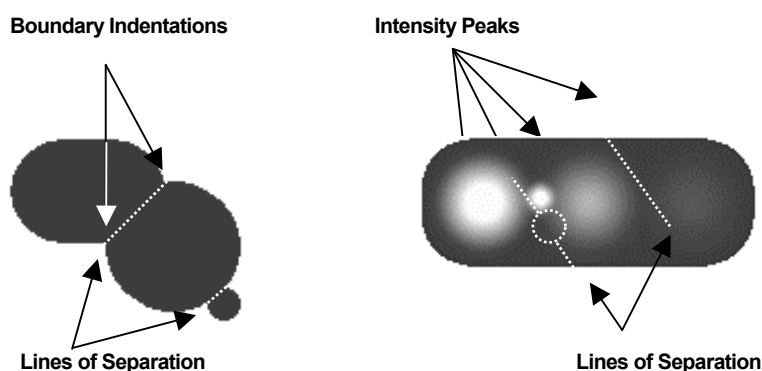


Figure 9: Object Segmentation Options. The image on the left illustrates the Geometric Method while the image on the right illustrates the Intensity Method.

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 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. The peak intensity method can be used to separate objects that have no boundary indentations.

The **ObjectSegmentationCh1** Assay Parameter (see Table 7) controls both segmentation methods. A positive value for this parameter selects the geometric method, and the value is the approximate radius (in pixels) of the smaller of the objects being separated. The value of this parameter for the geometric method depends on the magnifying power of the microscope objective and camera setting (pixel binning).

Setting **ObjectSegmentationCh1** to a negative value selects the peak intensity method. In this case, the absolute value of the parameter specifies the minimum relative height of the intensity peak (image contrast) to be used in the segmentation. Making **ObjectSegmentationCh1** more

negative reduces the number of objects created by the segmentation. In case of noisy and textured objects, the use of object smoothing may be required to reduce over-segmentation. The value of this parameter for the intensity method does not depend on the power of the microscope objective or camera setting (pixel binning).

A value of zero for **ObjectSegmentationCh1** disables the segmentation.

Segmentation Method	ObjectSegmentationCh1 Assay Parameter	Value Range
None	0	0
Geometric	radius (in pixels) of smaller object	1 to 255
Intensity	minimum relative height of intensity peak (local contrast)	-1 to -32767

Table 7: Channel 1 Object Segmentation Options

Geometric Method. Setting the **ObjectSegmentationCh1** value to R1 (Figure 10A) produces object separation shown in Figure 10B and setting the **ObjectSegmentationCh1** value to R3 results in segmentation shown in Figure 10C. Setting the **ObjectSegmentationCh1** value to R2 (the largest radius of all touching objects) produces no segmentation.

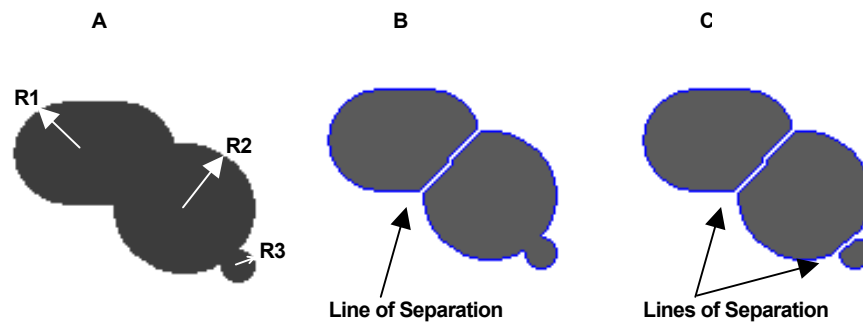


Figure 10: Object segmentation: Geometric Method. A) Radii of touching objects: $R1 = 24$, $R2 = 30$, $R3 = 7$; B) Result of segmentation using **ObjectSegmentationCh1** = R1; C) Result of segmentation using **ObjectSegmentationCh1** = R3.

Intensity Method. Figure 11 shows the intensity profile along the cordial line of an object with four intensity peaks from Figure 9. Relative peak intensity heights measured from the top of a peak to the nearest valley for each of the object are: 1500 (#1), 1250 (#2), 500 (#3), and 200 (#4).

In general terms, these peak intensity heights are nothing but measures of local contrast of an image. The degree of object segmentation can be controlled by setting the **ObjectSegmentationCh1** parameter value equal to the lowest relative height of intensity peaks of objects that need to be separated. The lower the value the more objects will be

segmented and vice versa. In case of over-segmentation, the **ObjectSmoothFactorCh1** > 0 should be used to alleviate the problem.

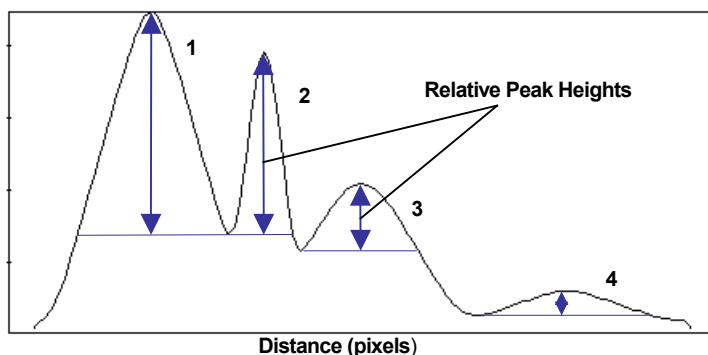


Figure 11: Intensity profile of an object with four intensity peaks. Peak #1 height = 1500, peak #2 height = 1250, peak #3 height = 500, and peak #4 height = 200.

Only two peaks in Figure 11, #1 and #2, have relative intensity > 1000. Thus, setting the **ObjectSegmentationCh1** parameter to -1000 makes only two peaks to be used for segmentation (or marks the two objects that need to be separated) and results in object segmentation as shown in Figure 12A. Setting the **ObjectSegmentationCh1** parameter to -400 or -100 marks three or all four objects that need to be separated. The results of segmentation of three and four objects are shown in Figure 12B and 12C.

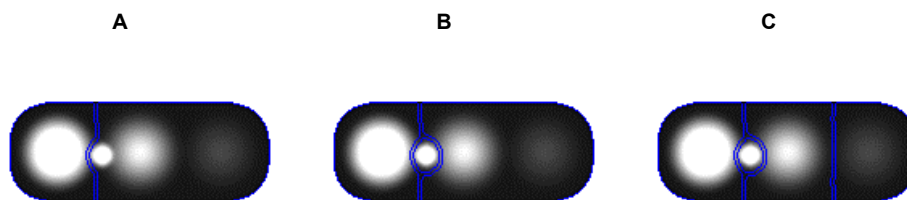


Figure 12: Object segmentation: Intensity Method. A) Result of segmentation using parameter **ObjectSegmentationCh1** = -1000; B) Result of segmentation using parameter **ObjectSegmentationCh1** = -400; C) Result of segmentation using parameter **ObjectSegmentationCh1** = -100.

Object Smoothing

SmoothFactorCh1, SmoothMethodCh1

The Channel 1 image will probably need to be smoothed before either primary objects or spots are identified. In the case of primary objects, smoothing consolidates fragmented objects (e.g., apoptotic nuclei) and rounds their boundaries, giving them a more natural shape. In the case of spot identification, the smoothing removes noise that can be identified as false spots. It also increases the size of the identified spots.

The underlying principle behind smoothing is to sample a square region of the image surrounding a pixel and then to replace that pixel with an “average” value of the pixels in the region. This is done for each pixel in the image. The **SmoothFactorCh1** Assay Parameter specifies the radius (in pixels) of the sampling region. A value of 0 for **SmoothFactorCh1** specifies that the image is not smoothed. The **SmoothMethodCh1** Assay Parameter specifies

the method used to compute the “average” value. Four methods are available when spots are being identified (i.e., single-channel assays). These are listed in Table 8.

SmoothMethodCh1 Assay Parameter Value	Name	Method used to compute “average” value
1	Box	Arithmetic mean
2	Median	Rank ordering (sorted list) Middle value
3	Binomial	Weighted mean using Gaussian-like function
4	Morphological (Tophat)	Rank ordering (sorted list) Pass 1 = Minimum value Pass 2 = Maximum value

Table 8. Image Smoothing Methods used for Spot Identification in a one channel assay

NOTE



Channel 1 smoothing in multi-channel assays is configured internally to use only the **Box** method. In these assays, the **SmoothMethodCh1** Assay Parameter is ignored.

The *Box* method is the fastest and gives good results in most cases. It blurs edges and is thus particularly good at consolidating fragmented objects. The *Binomial* method also blurs edges but does a better job of preserving the roundness of objects. The *Median* and *Morphological* methods are best at removing very small objects (e.g., single-pixel noise) from the image without blurring the edges of the larger objects.

Spot Detection

SpotDetectRadiusCh1, SpotDetectMethodCh1

Spot detection parameters for Channel 1 are only applicable when single-channel assays are performed.

NOTE



Multi-channel assays, which identify primary objects in Channel 1, ignore the spot detection parameters.

Often spots are located within objects, such as cells or nuclei. Since these objects contribute to the fluorescence signal, spots are generally found in image regions that are brighter than the background. Spot detection is an image processing step that removes the underlying brightness of the objects that contain the spots. Applying an object identification threshold without spot detection results in clusters of spots linked together by object pixels.

In the case of bright objects on a dark background (**SpotTypeChN=0**), spot detection is based on two fundamental spot properties: spots are smaller than the containing object and they are brighter than the containing object. These properties lead to two different spot detection approaches. The first is area detection, which identifies spots as areas that can be counted and measured for size, shape, and intensity. The second is peak detection, which identifies spots only as points that can be counted.

The **SpotDetectRadiusCh1** Assay Parameter specifies the radius (in pixels) of the sampling region used for spot detection. Larger radii allow large and small spots to be detected. Smaller

radii allow only small spots to be detected. A value of zero means that no spot detection is used, although you can still use Object Identification Threshold to identify spots.

The **SpotDetectMethodCh1** Assay Parameter specifies the method used for spot detection. These methods are listed in Table 9 and an example of each type of detection method is found in Figure 13. In area detection, a square region of the image surrounding a pixel is sampled and the “average” value of the pixels in the region is computed. The “average” is subtracted from the center pixel, removing its background brightness. This is repeated for each pixel in the image.

SpotDetectMethodCh1 Assay Parameter Value	Detection Method	Detection Type	Description
1	Box	Area	The box method replaces each pixel value in an image with the simple arithmetic average value of its neighbors, including itself.
2	Binomial	Area	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.
3	Median	Area	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.
4	Morphological	Area	The morphological (top hat) method computes the difference between the original grayscale image and the processed image.
5	Local Maxima	Peak	The local maxima method keeps the intensity values of local maxima pixels and sets values of all other pixels within the detection region to zero.
6	Local Range	Peak	The local range method replaces the value of each pixel with intensity gradient value calculated within the detection region.

Table 9. Spot Detection Methods

Area detection is used when you are primarily interested in measuring the area and intensity features (e.g., total area and total intensity) of the spots, and are less interested in spot counts. These counts will only be accurate when the spots are distinct. As mentioned previously, the *Box* method is the fastest method and gives good results in most cases. The other methods are somewhat better at detecting very small spots. The *Median* and *Morphological* methods can be used to detect spots located close to large objects.

The peak detection methods identify spikes in pixel intensity within the spot detection region. The first method identifies local maxima and the second identifies local outliers (i.e., pixels brighter than the estimated “normal” range of pixel values). You should use peak detection if you are most interested in accurate spot counts. This can be done even if the spots are merged together, as long as the intensity peaks remain distinct. However, be aware that peaks are point objects and therefore spot area, shape, and intensity measurements may not be as accurate unless the spot is very small.

NOTE



Area detection is better suited for spot area and intensity features.
Peak detection is better suited for spot counting.

Channels 2-6 Assay Parameters

In multi-channel assays, Channels 2-6 are used for the identification of spots and/or the selection of primary objects. The results derived from the analysis of the spots are also used to characterize the response of the cells (Channel 1 objects) to treatment. The Assay Parameters for Channels 2-4 are associated with the following:

- Spot Identification Target Regions
- Spot Identification and Detection

Spot Identification Target Regions

CircModifierChN, RingDistanceChN, RingWidthChN

Spot identification Target Regions are used in Channels 2-4 to confine the identification of spots to regions associated with each Channel 1 object. You may specify either a Circ or a Ring Target Region independently for each channel (see Figure 13).

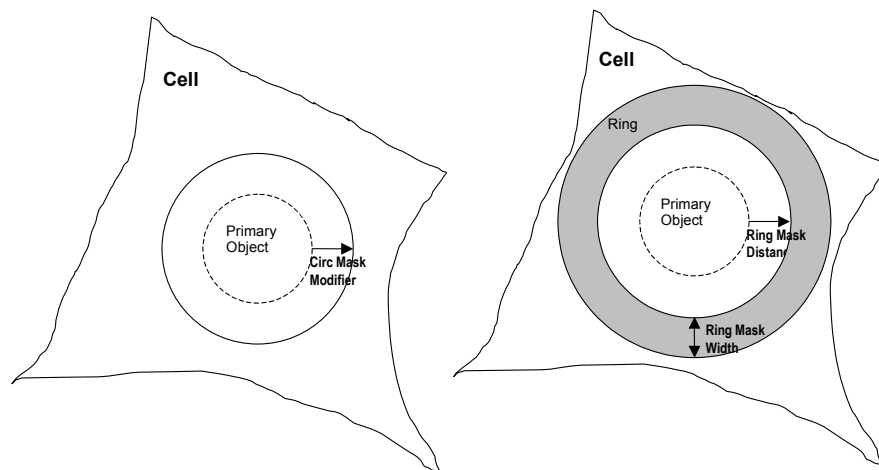


Figure 13. Adjustment of Target Regions in Channels 2-4.

The Circ Target Region is defined by a single boundary, and the location of this boundary is specified relative to the primary object by the **CircModifierChN** Assay Parameter, where N refers to Channels 2-4. A positive value makes the Target Region larger than the primary object, a negative value makes the Target Region smaller, and a value of 0 makes the Target Region the same size.

The Ring Target Region has two boundaries, inner and outer. The inner boundary is controlled by the **RingDistanceChN** Assay Parameter in a fashion identical to the **CircModifierChN** Assay Parameter described above. The location of the outer boundary is specified by the **RingWidthChN** Assay Parameter. The value of this Assay Parameter is never negative. It also serves to specify whether the Target Region is a circ or a ring. Setting the **RingWidthChN** Assay Parameter to 0 specifies a Circ and setting the Assay Parameter to any positive value specifies a Ring. The values of the Target Region Assay Parameters are in pixel units and will need to be adjusted as the magnification of the objective changes.

NOTE



A Circ Target Region is specified by setting **RingWidthChN** to 0.

To save time, special values for **CircModifierChN** and **RingWidthChN** have been set aside to specify the largest possible (i.e., maximal) Target Regions. Setting **CircModifierChN** to **127** (with **RingWidthChN** = 0) creates maximal Circ Target Regions (see Figure 14). Setting **RingWidthChN** to **127** creates maximal ring (see Figure 15). The time saved by these maximal values can be significant. You should use these shortcuts whenever your Target Regions approach their maximum allowed sizes.

NOTE

Maximal Target Regions can be specified by setting **CircModifierChN** or **RingWidthChN** to **127**.

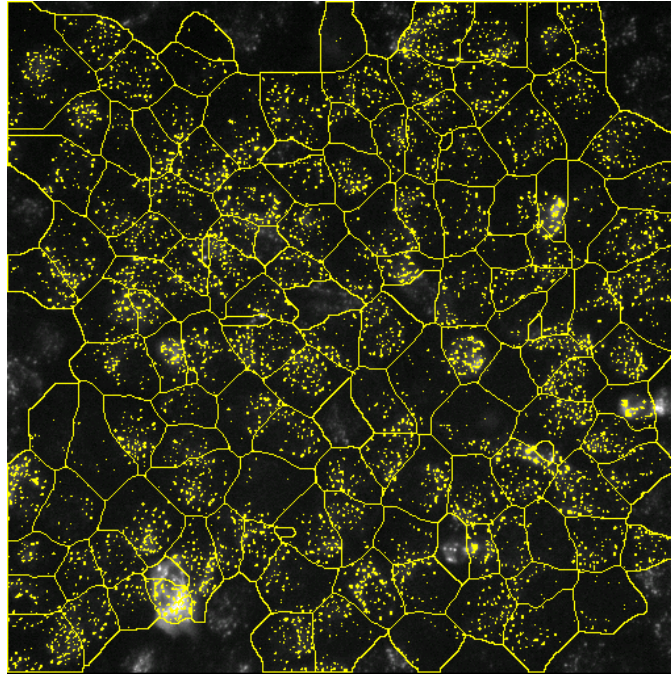


Figure 14. Maximal circ target regions created by setting **CircModifierCh2** to **127** and **RingWidthCh2** to **0**.

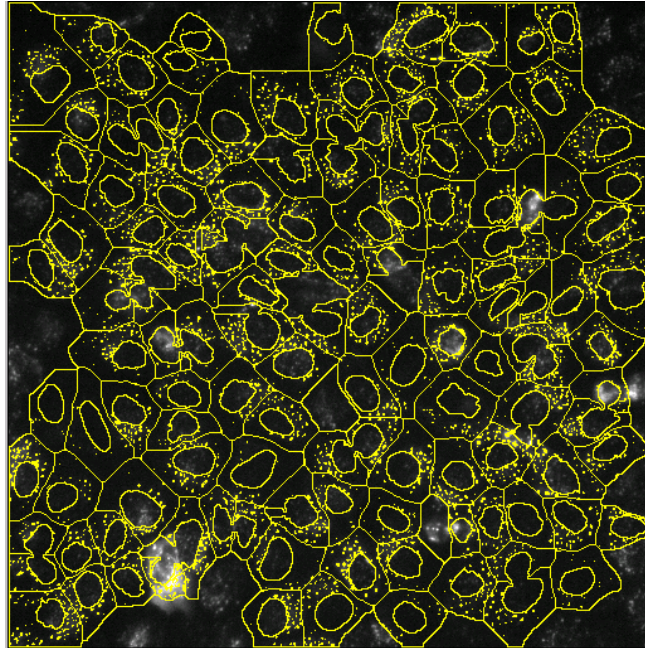


Figure 15. Maximal ring target regions created by setting *RingDistanceCh2* to 0 and *RingWidthCh2* to 127.

It is also possible to select the entire image as a single spot identification Target Region. This is done by setting the Object Identification Method in Channel 1 to **Fixed Threshold** and the Object Identification Value to 0 (see Figure 16).

NOTE



To make the entire image a single spot identification Target Region, select the **Fixed Threshold** object identification method for Channel 1 with a **Value = 0**. This choice affects all Channels 2-4.

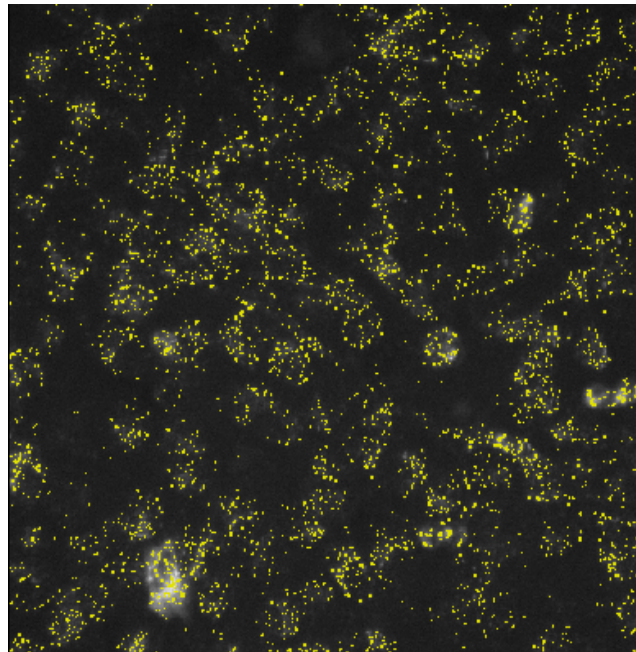


Figure 16. Selecting a **Fixed Threshold** method with a value of 0 for Channel 1 creates a single spot identification zone that encompasses the entire image in Channels 2-4.

The Assay Parameters that define the spot identification Target Regions are summarized in Table 10. Note how the value of the **RingWidthChN** Assay Parameter determines whether the Target Region is a Circ or a Ring, and which Assay Parameters are ignored in either of these cases. If **RingWidthChN** is 0, Circ will be targeted (and **CircModifierChN** values will be applied) and Ring will be ignored (values for **RingDistanceChN** will not be applied). If **RingWidthChN** is any value other than 0, Circ will be ignored (and **CircModifierChN** values will not be applied) and Ring will be targeted (values for **RingDistanceChN** will be applied). Note also the “special” values reserved for creating maximal Target Regions in parenthesis.

Target Region Type	Range of Assay Parameter Values		
	CircModifierChN	RingDistanceChN	RingWidthChN*
Circ	-127 to 127 (127 for Maximal Circ)	Ignored	0
Ring	Ignored	-127 to 127	1 to 127 (127 for Maximal Ring)

Table 10. Values of Circ and Ring Assay Parameters for Spot Identification Target Regions.

* **RingWidthChN** Assay Parameter determines whether the Target Region is a Circ or a Ring. If **RingWidthChN** is 0, Circ will be targeted (and **CircModifierChN** values will be applied) and Ring will be ignored (values for **RingDistanceChN** will not be applied).

Spot Identification and Detection

SpotSmoothFactorChN, SpotSmoothMethodChN, SpotDetectRadiusChN, SpotDetectMethodChN

Spot identification and detection in Channels 2-4 is identical to that described above for Channel 1 (i.e., for single-channel assays).

Optimizing Spot Detection

To optimize spot detection, first adjust the **Object Identification Value** until false spots are eliminated from background (i.e., blank) regions of the image. Use **Run Algorithm** to see the spot overlays. Then adjust the **SpotDetectRadiusChN** and **SpotSmoothFactorChN** Assay Parameters until the spot overlays are big enough to completely cover the spots. Increasing these parameters increases the size of the spot overlays.

When using peak detection to identify spots, the density of detected peaks is controlled by the size of the peak detection region and the degree of smoothing before peak detection. These are controlled by the **SpotDetectRadiusChN** and **SmoothFactorChN** Assay Parameters, respectively. Increasing these parameters decreases the density of peaks.

Basic Assay Parameters

Assay Parameters available in Basic Mode control the morphological identification of the objects in each channel, and control the use of Reference Wells (Table 11). You will not be able to view any Advanced Assay Parameters in this mode (as the **Hide Advanced Parameters** option is checked, uncheck to see a full list of Assay Parameters).

Parameter	Units	Description
UseReferenceWells	Binary	Use reference wells to calculate high and low response levels: 0 = No, 1 = Yes
ObjectTypeCh1	Binary	Type of objects or spots to be identified in Ch1: 0 = Bright objects on dark background, 1 = Dark objects on bright background
BackgroundCorrectionChN	Pixels	Radius (in pixels) of region used to compute background in ChN: Negative value = Use surface fitting, 0 = Do not apply background correction, Positive value = Use low pass filter
SmoothFactorCh1	Number	Degree of image smoothing (blurring) prior to object or spot identification in Ch1: 0 = Do not apply smoothing (Max positive value is 6 when SmoothMethodCh1 = 2 in case of single-channel assay)
SmoothMethodCh1	Number	Method used for smoothing (blurring) before object or spot identification. For single-channel assay: 1 = box, 2 = median, 3 = binomial, and 4 = morphological; For multi-channel assay: only box method is used
SpotDetectionCh1	Binary	Apply small object (spot) detection in Ch1: 0 = No, 1 = Yes
SpotDetectRadiusCh1	Pixels	Radius (in pixels) of region used to detect spots: 0 = Do not apply spot detection. (Max positive value is 6 when SpotDetectMethodCh1 = 2)
SpotDetectMethodCh1	Number	Method used to detect spots. 1 = box, 2 = binomial, 3 = median, 4 = morphological, 5 = local maxima, and 6 = local range
ObjectSegmentationCh1	Pixels	Radius (in pixels) of touching objects that should be separated in Ch1: Negative value = Use intensity peaks method, 0 = Do not apply object segmentation, Positive value = Use shape geometry method
ObjectCleanupCh1	Binary	Clean up object mask and remove small objects by applying erosion followed by dilation procedure: 0 = No, 1 = Yes. It is performed only if ApplyDetectionCh1 = 0.
RejectBorderObjectsCh1	Binary	Reject objects that touch image edges: 0 = No, 1 = Yes
SpotTypeChN	Binary	Type of spots to be identified in ChN: 0 = Bright objects on dark background, 1 = Dark objects on bright background
SpotSmoothFactorChN	Number	Degree of image smoothing (blurring) prior to spot identification in ChN: 0 = Do not apply smoothing
SpotSmoothMethodChN	Number	Method used for smoothing before spot identification in ChN: 1 = box, 2 = median, 3 = binomial, and 4 = morphological
SpotDetectRadiusChN	Pixels	Radius (in pixels) of region used to detect spots: 0 = Do not apply spot detection. (Max positive value is 6 when SpotDetectMethodCh1 = 2)
SpotDetectMethodChN	Number	Method used to detect spots. 1 = box, 2 = binomial, 3 = median, 4 = morphological, 5 = local maxima, and 6 = local range
CircModifierChN	Pixels	Distance (in pixels) from object boundary to edge of circ region in ChN or 127 = use maximal circ
RingDistanceChN	Pixels	Distance (in pixels) from object boundary to inner edge of ring region in ChN
RingWidthChN	Pixels	Width (in pixels) of ring region in ChN: 0 = use circ mask or 127 = use maximal ring
Mask ModifierCh5-6	Pixels	Number of pixels to modify Ch1 object mask in Ch5-6: Negative value = Shrink mask, 0 = Do not modify mask, Positive value = Expand mask

Parameter	Units	Description
MaskCircModifierCh2	Pixels	Distance (in pixels) from object boundary to edge of circ region (used for object selection) in Ch2. Acceptable range= -127 – 127, where 127 uses maximal circ.
MaskRingDistanceCh2	Pixels	Distance (in pixels) from object boundary to inner edge of ring region (used for object selection) in Ch2. Acceptable range= -127 – 127
MaskRingWidthCh2	Pixels	Width (in pixels) of ring region (used for object selection) in Ch2: 0 = use circ mask 127=use maximal ring Acceptable range is 0 to 127
MaskCircModifierCh3	Pixels	Distance (in pixels) from object boundary to edge of circ region (used for object selection) in Ch3. Acceptable range is –127 to 127, where 127 uses maximal circ
MaskRingDistanceCh3	Pixels	Distance (in pixels) from object boundary to inner edge of ring region (used for object selection) in Ch3. Acceptable range is –127 to 127
MaskRingWidthCh3	Pixels	Width (in pixels) of ring region (used for object selection) in Ch3. 0 = use circ mask 127 = use maximal ring Acceptable range = 0 to 127
MaskCircModifierCh4	Pixels	Distance (in pixels) from object boundary to edge of circ region (used for object selection) in Ch4. Acceptable range is –127 to 127, where 127 uses maximal circ
MaskRingDistnaceCh4	Pixels	Distance (in pixels) from object boundary to inner edge of ring region (used for object selection) in Ch4. Acceptable range is –127 to 127.
MaskRingWidthCh4	Pixels	Width (in pixels) of ring region (used for object selection) in Ch4: 0 = use circ mask 127 = use maximal ring Acceptable range = 0 to 127

Table 11. Basic Assay Parameters available for the Spot Detector BioApplication. *Note that “ChN” refers to Channels 1-6 for Background Correction and Channels 2-4 for all other Assay Parameters.

Object Selection Parameters

You can use the Object Selection Parameters to select a subset of the objects or spots that have been identified. If the physical properties (e.g., intensity or morphology) of a spot or object are outside the specified ranges, it is not selected. The Object Selection Parameters can be set either manually, or by using an interactive point-and-click method.

The Object Selection Parameters for objects (Channel 1) and spots (N = Channels 2-4) are shown in Table 12. Recall that for single-channel assays, Channel 1 “objects” are actually spots.

Objects Channel 1	Spots Channels 2-4	Channels 5-6
ObjectAreaCh1	SpotAreaCh2-4	
ObjectShapeP2ACh1	SpotShapeP2ACh2-4	
ObjectShapeLWRCh1	SpotShapeLWRCh2-4	
ObjectTotalIntenCh1	SpotTotalIntenCh2-4	
ObjectAvgIntenCh1	SpotAvgIntenCh2-4	
ObjectVarIntenCh1	SpotVarIntenCh2-4	
Gating →	TargetAvgIntenCh2-4	TargetAvgIntenCh5-6
	TargetTotalIntenCh2-4	TargetTotalIntenCh5-6

Table 12. Object Selection Parameters. The gray region contains the Gating selection parameters for Channels 2-6.

There are two sets of Object Selection Parameters for Channels 2-6. The first is used to select spots identified in Channels 2-4. These parameters are **SpotAreaCh N** , **SpotShapeP2ACh N** , **SpotShapeLWRCh N** , **SpotTotalIntenCh N** , **SpotAvgIntenCh N** , and **SpotVarIntenCh N** . The second set is used for selecting Channel 1 objects for analysis based on their intensity signals in Channels 2-6 (i.e., Gating). The object selection features are **TargetAvgIntenCh2-6** and **TargetTotalIntenCh2-6** and will be explained in the next sub-section.

NOTE



For Channels 2-4, both spot and Target Region overlays are displayed by clicking on **Identify Objects**. To set selection parameters for spots, click on spot overlays. To set selection parameters for Target Regions, click on Target Region overlays. To change from spots to Target Regions, click the **Deselect All** button. **Be sure to click the Deselect All button before switching to a different channel.**

The **SpotAreaCh N** selection parameter is particularly useful in removing noise (1-2 pixel regions) that has been identified as spots. Objects that are too bright or too dim can be excluded by restricting the ranges of **ObjectAvgIntenCh1** or **ObjectTotalIntenCh1**. Objects with odd shapes can be rejected using a combination of **ObjectShapeLWRCh1** and **ObjectShapeP2ACh1**. The **ObjectVarIntenCh1** parameter can be adjusted to restrict the ranges for variation of intensity of the Channel 1 Object.

Channel 1 Parameters

Parameter	Units	Description
ObjectAreaCh1	Pixels or μm^2	Area (in pixels or micrometers) of object or Ch1 spot
ObjectShapeP2ACh1	Number	Shape measure based on ratio of perimeter squared to $4\text{PI} \times \text{area}$ of Ch1 object or spot (ObjectShapeP2ACh1 = 1 for circular object)
ObjectShapeLWRCh1	Number	Shape measure based on ratio of length to width of object-aligned bounding box of Ch1 object or spot
ObjectAvgIntenCh1	Intensity	Average intensity of an object or Ch1 spot
ObjectTotalIntenCh1	Intensity	Total intensity of an object or Ch1 spot
ObjectVarIntenCh1	Intensity	Variation (standard deviation) of intensity of all pixels within Ch1 object or spot

Channels 2-4 Parameters

Parameter	Units	Description
SpotAreaChN	Pixels or μm^2	Area (in pixels or micrometers) of ChN spot
SpotShapeP2AChN	Number	Shape measure based on ratio of perimeter squared to $4\text{PI} \times \text{area}$ of ChN spot (SpotShapeP2AChN = 1 for circular spot)
SpotShapeLWRChN	Number	Shape measure based on ratio of length to width of object-aligned bounding box of ChN spot
SpotAvgIntenChN	Intensity	Average intensity of all pixels within ChN spot
SpotTotalIntenChN	Intensity	Total intensity of all pixels within ChN spot
SpotVarIntenChN	Intensity	Variation (standard deviation) of intensity of all pixels within ChN spot

Channels 2-6 Parameters

Parameter	Units	Description
TargetAvgIntenCh2-6	Intensity	Average intensity of all pixels within ChN Target Region
TargetTotalIntenCh2-6	Intensity	Total intensity of all pixels within ChN Target Region

Table 13. Object Selection Parameters Available for the Spot Detector BioApplication in Channel 1 (top), Channels 2-4 (middle), and Channels 2-6 (bottom).

Object Selection (Gating)

The Spot Detector BioApplication supports object selection on a cell population. This feature provides selective cell processing based on fluorescence intensity. Object selection allows you to specifically identify a subset of Channel 1 objects based on fluorescence intensity and allows you to limit the analysis to this group of cells. Object selection is available in Channels 2-6. If you choose to add object selection channels, an object must pass the criteria in all channels to be selected for analysis. The parameters used for object selection are listed in the gray region of Table 12.

Specifying Intensity Ranges for Object Selection

When working in the Create Protocol or Protocol Interactive View of the ArrayScan Classic software or in the Protocol Optimization task list of the iDev software, you can specify intensity ranges for gating. The Spot Detector BioApplication uses the spot identification

Target Regions for Gating. The intensities in these regions are associated with the **TargetAvgIntenCh2-6** and **TargetTotalIntenCh2-6** Object Selection Parameters. **TargetTotalIntenCh2-6** is a summation of all intensities within the targeted object. **TargetAvgIntenCh2-6** is **TargetTotalIntenCh2-6** divided by the target area.

Specifying Mask Modifiers for Object Selection

The objects identified in Channel 1 may be used to create target masks that can be independently applied to one or more downstream channels (Channels 2-6) to be used for Gating. The sizes of these masks are specified using either the **CircModifierChN** or **RingDistanceChN** Assay Parameters in Channels 2-4 or **MaskModifierCh5-6** for Channels 5-6. As described earlier in this Chapter and with Figure 16 and Table 10, the **RingWidthChN** Assay Parameter determines whether the Target Region is a Circ or a Ring, and which Assay Parameters are ignored in either of these cases. Remember, if **RingWidthChN** is 0, Circ masks will be used (and **CircModifierChN** values will be applied), if **RingWidthChN** is any value other than 0, Ring masks will be used (and values for **RingDistanceChN** will be applied). Therefore, you can focus on the Circ mask as your target in Channel 2 and a Ring mask as your target in Channel 3, and so on.

Once you determine the type and size of the modified masks for each channel, you can then specify the intensity ranges. During the scan, the average and total intensity values within the modified masks are calculated for each channel. If the calculated values do not fall within the specified limits, the entire object is removed from analysis.

Image Overlays

Colored image overlays are available to indicate the objects and spots that were identified and used in the analysis, as well as the spot identification Target Regions. Checkboxes are provided for you to select these overlays on a channel-by-channel basis, and you can also choose their colors. Table 14 lists all overlays available in the Spot Detector BioApplication.

Spot Overlays

Spots that were analyzed are indicated using solid overlays that are exactly the size and shape of each spot. If peaks were detected, their overlays indicate the size of the peak which are typically 1 to 2 pixels in size. The colors of the spot overlays can be selected independently for each channel.

Spot Identification Target Region Overlays

The Target Regions used for spot identification are displayed using one-pixel width outlines. To prevent these lines from hiding spots or peaks, the overlays are enlarged by one pixel. For a Ring Target Region, the radius of the inner boundary of the overlay is one pixel smaller than the Target Region and the radius of the outer boundary is one pixel larger.

Parameter	Description
SelectedObject	Color of the overlay used to display either the primary objects selected for analysis in Channel 1 (multi-channel assays), or the Channel 1 spots selected for analysis in single-channel assays.
RejectedObject	Color of the overlay used to display the rejected primary objects in Channel 1 (multi-channel assays). Overlays are not shown for rejected spots.
ROICh2	Indicates region of interest in Ch2 by outlining the targeted area for spot detection and/or intensity measurements
SpotCh2	Color of the overlay used to display Target Regions and selected spots in Channel 2
ROICh3	Indicates region of interest in Ch3 by outlining the targeted area for spot detection and/or intensity measurements
SpotCh3	Color of the overlay used to display Target Regions and selected spots in Channel 3
ROICh4	Indicates region of interest in Ch4 by outlining the targeted area for spot detection and/or intensity measurements
SpotCh4	Color of the overlay used to display Target Regions and selected spots in Channel 4

Table 14. Overlays available with the Spot Detector BioApplication

Assay Parameters for Population Characterization

Overview of Population Characterization

The Spot Detector V4 BioApplication provides the ability to characterize cells based on their response compared to a control population. For a particular Cell Feature, a range is determined and set by measuring the physiological distribution of feature values in untreated cells. Upper and lower limits (called **FeatureChNLevelHigh** and **FeatureChNLevelLow**) set the upper and lower bounds of this range respectively. The **Status** Cell Feature values indicate whether a given cell is within or beyond this range (Table 15):

Value	Cell Status Definition
0	Feature value within defined range
1	Feature value > Upper Level
2	Feature value < Lower Level

Table 15. Numerical definitions of Cell Feature Status in the Spot Detector V4 BioApplication when using population characterization.

The corresponding Well Features (**%HIGH_FeatureChN**, **%LOW_FeatureChN**) reported are the percentage of cells showing feature values that fall outside this range, i.e. the percentage of cells with Status Cell Feature values = **1** or **2**. Figure 17 illustrates this concept by showing the population distribution for Cell Feature values for a population having a defined physiological state (e.g., untreated) and the shift in this distribution upon compound treatment.

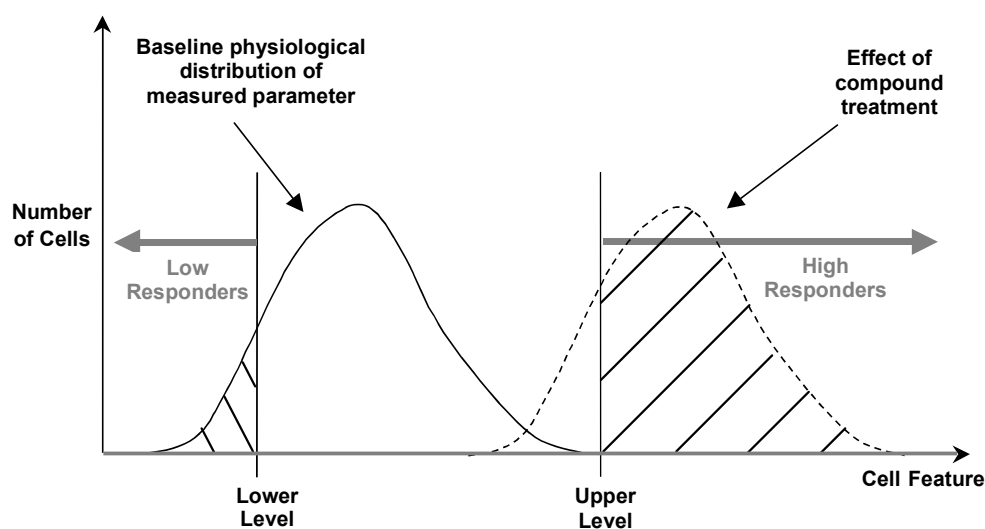


Figure 17. Principle of Population Characterization. %High exhibiting Status Cell Feature value = 1 (High Responders) or %Low exhibiting Status Cell Feature = 2 (Low Responders).

Setting Cellular Response Levels

There are two ways of setting the high and low-response levels to characterize the cell population: manually (`UseRefWells=0`) or automatically (`UseRefWells=1`). To set the levels manually, you specify the values for the ***FeatureChNLevelHigh*** and ***FeatureChNLevelLow*** Assay Parameter that will be used to perform cell population characterization. The Spot Detector BioApplication then uses these levels that you define to calculate the percentage of cells outside the bounds of these levels for the different Cell Features.

To automatically determine the ***FeatureChNLevelHigh***, ***FeatureChNLevelLow*** Assay Parameter values, the BioApplication uses Reference Wells (`UseRefWells=1`). Particular wells on the sample plate are designated as Reference Wells. Typically, Reference Wells contain a control population of cells which displays the normal basal physiological distribution for the Assay Parameter being measured. These wells are analyzed and the population distribution for the different features are determined. The cell population characterization levels are then set by adding to, and subtracting from, the mean of the distribution its standard deviation multiplied by a coefficient. The system then scans the whole plate and applies these levels. For example, if you want to know the percentage of cells that, with compound treatment, have a response beyond the 95th percentile of the response from a control untreated population of cells, then the coefficient by which to multiply the standard deviation would be two.

The advantage of using Reference Wells to automatically calculate the ***FeatureChNLevelHigh***, ***FeatureChNLevelLow*** Assay Parameters is that the levels are determined by a control population of cells and are independent of run-to-run variations when performing the experiment on different days with different illumination conditions, fluorophore amounts, or changes in cell conditions.

Reference Wells Processing Sequence

By setting the ***UseReferenceWells*** Assay Parameter to **1**, the Reference Wells processing is engaged. Specified fields are acquired/analyzed in the wells, and Field, Well, and Plate Features are computed. After this sequence is completed, the computed values will be loaded into the assay processing parameters related to reference features and the regular scanning of the plate will begin. Again, if the feature value for ***MinRefAvgObjectCountPerField*** obtained from the Reference Wells is below the value set for that Assay Parameter, the BioApplication aborts the use of Reference Wells and processes the plate as if ***UseReferenceWells*** is set to **0**. The Spot Detector BioApplication only uses **Known** Reference Wells. The sequence of computation for Reference Wells is as follows:

1. Cell Features are computed for every valid object within a field.
2. For each Cell Feature to be used for population characterization, the distribution's histogram, mean, and standard deviation are computed over all cells in the field.
3. Reference Field Features are determined.
4. Reference Well Features are computed as average values for fields in a well, weighted for the number of cells per field, and then Reference Plate Features are computed as arithmetic averages for all Reference Wells on a plate. Use of a weighted average minimizes the effect of sparse fields.

NOTE



Reference Wells will only work when there is more than one channel and at least one of the targeted channels (Channels 2-6) does not have **None** as the Object Identification Modifier (***UseReferenceWells*** = 1)

Identifying Reference Wells and Control Parameters

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

NOTE



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

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

Specifying and Controlling Reference Wells

There are two general Assay Parameters used to control Reference Wells: **UseReferenceWells** and **MinRefAvgObjectCountPerField**. The **UseReferenceWells** is a binary Assay Parameter that allows you to indicate whether Reference Wells are used to determine the levels necessary to characterize cell populations.

- **UseReferenceWells = 0** (zero) specifies that Reference Wells are not to be used, resulting in the use of manually entered levels for population characterization
- **UseReferenceWells = 1** specifies that Reference Wells are to be used.

If **UseReferenceWells = 1**, there has to be more than one channel and subsequent channels (Channel 2-6) cannot all have **None** as their Object Identification Method. If Reference Wells are to be used, the Assay Parameter **MinRefAvgObjectCountPerField** is applied to specify the minimum average number of selected objects per field that you consider acceptable in the Reference Wells. If the measured cell density is less than or equal to this value, the Reference Features will not be used, rather the manually entered levels for population characterization are applied.

There are four types of Assay Parameters that are used for cell population characterization which are specific to each individual feature for which population characterization is applied and the dependent channels where it is calculated. The Assay Parameter names are of the type:

- **FeatureChNLevelLow**
- **FeatureChNLevelHigh** (when Reference Wells are not used)
- OR
- **FeatureChNLevelLow_CC**
- **FeatureChNLevelHigh_CC** (when Reference Wells are used)

where *Feature* refers to the individual feature and *ChN* refers to the specific channel *N*. Other than the **UseReferenceWells** Assay Parameter, all the Assay Parameters used for population characterization are available only in the Advanced Mode. The **FeatureChNLevelLow**, **FeatureChNLevelHigh** Assay Parameters specify the actual levels and must be manually entered if Reference Wells are not used. If the range is to be set automatically by using Reference Wells, then the appropriate Assay Parameters are those that have the suffix "CC" (i.e., "Correction Coefficient"). The lower and upper bounds for the range for a feature are set by subtracting or adding the distribution's mean from its standard deviation (SD) multiplied by the appropriate CC value respectively:

$$\text{RefFeatureChNLevelHigh} = \text{Mean}(\text{FeatureChN}) + \text{FeatureChNLevelHigh_CC} \times \text{SD}(\text{FeatureChN})$$

OR

$$\text{RefFeatureChNLevelLow} = \text{Mean}(\text{FeatureChN}) - \text{FeatureChNLevelLow_CC} \times \text{SD}(\text{FeatureChN})$$

where *Feature* refers to the name of the Cell Feature. The “CC” values can be different for the upper and lower levels, and the “CC” may be either positive or negative. A positive “CC” value moves the level away from the mean and a negative value moves the level closer to the mean. Since the cellular response features are intrinsically non-negative, the level low Reference Features are set to 0 if their computed values are negative.

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

Advanced Assay Parameters

NOTE



Clear the **Hide Advanced Parameters** checkbox to display all Assay Parameters.

The **Hide Advanced Parameters** checkbox will hide or show all advanced Assay Parameters. When you check the box, only the Basic Assay Parameters are shown; when you clear the box, all Basic and Advanced Assay Parameters are shown. Generally, the Advanced Assay Parameters are related to definition and reporting of responder cells. They control the analysis of the data resulting from image analysis (Table 16).

For each feature undergoing population characterization, there are four advanced Assay Parameters that control the levels: the Levels Low and High that set upper and lower thresholds and the presence of the **_CC** suffix which designates those levels are set using Reference Wells. For example, the Assay Parameters controlling the object's spot count in Channel 2 are:

- **SpotCountPerObjectCh2LevelLow**
- **SpotCountPerObjectCh2LevelHigh**
- **SpotCountPerObjectCh2LevelLow_CC**
- **SpotCountPerObjectCh2LevelHigh_CC**

The following table of Advanced Parameters does not list all four level Assay Parameters for each feature, rather only one entry for the feature is listed giving the four different options. See the following example for the Channel 2 spot count per object:

- **SpotCountPerObjectCh2LevelLow/High, Low/High_CC**

Units will be expressed as what is found with **FeatureChNLevelLow/High**, knowing that **_CC** is expressed as a number.

Assay Parameter	Units	Description
MinRefAvgObjectCountPerField	Number	Minimum average number of objects per field required for acceptance of Reference Well results
UseMicrometers	Binary	Measure lengths and areas in: 0 = Pixels 1 = Micrometers
_PixelSize	µm	Pixel size in micrometers (depends on objective selection)
Type_1_EventDefinition	---	User-defined combination of logic statements involving response features (cannot be edited)
Type_2_EventDefinition	---	User-defined combination of logic statements involving response features (cannot be edited)

Assay Parameter	Units	Description
Type_3_EventDefinition	---	User-defined combination of logic statements involving response features (<i>cannot be edited</i>)
_ObjectAreaCh1Level Low/High,Low/High_CC	Pixel or μm^2	Defines ObjectAreaCh1 population characterization thresholds
_ObjectShapeP2ACh1Level Low/High,Low/High_CC	Number	Defines ObjectShapeP2ACh1 population characterization thresholds
_ObjectShapeLWRCh1Level Low/High,Low/High_CC	Number	Defines ObjectShapeLWRCh1 population characterization thresholds
_ObjectTotalIntenCh1Level Low/High,Low/High_CC	Intensity	Defines ObjectTotalIntenCh1 population characterization thresholds
_ObjectAvgIntenCh1Level Low/High,Low/High_CC	Intensity	Defines ObjectAvgIntenCh1 population characterization thresholds
_ObjectVarIntenCh1Level Low/High,Low/High_CC	Intensity	Defines ObjectVarIntenCh1 population characterization thresholds
_SpotCountChNLevel Low/High,Low/High_CC	Number	Defines SpotCountChN population characterization thresholds
_SpotTotalAreaChNLevel Low/High,Low/High_CC	Pixel or μm^2	Defines SpotTotalAreaChN population characterization thresholds
_SpotAvgAreaChNLevel Low/High,Low/High_CC	Pixel or μm^2	Defines SpotAvgAreaChN population characterization thresholds
_SpotTotalIntenChNLevel Low/High,Low/High_CC	Intensity	Defines SpotTotalIntenChN population characterization thresholds
_SpotAvgIntenChNLevel Low/High,Low/High_CC	Intensity	Defines SpotAvgIntenChN population characterization thresholds

Table 16. Advanced Assay Parameters available with the Spot Detector BioApplication. *Note that "ChN" refers to Channels 2-4.

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

The Spot Detector BioApplication allows simultaneous definition of up to three Events to enable rapid multiparametric analysis at the level of individual cells, across multiple Cell Features. You can use these events to:

- Define a subpopulation by using any combination of up to three Cell Features
- Report characteristics of your subpopulations

Event definitions are created using a stand-alone software tool called the BioApplication Event Wizard. Operation of this software tool for this BioApplication is described in more detail in Chapter 3.

Event definition is achieved through the construction of logic statements employing specific Cell Features and a set of defined logical operators. The Cell Features and Boolean operators available are listed in Table 17. 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 18 using two Cell Features, A and B. The entire cell population analyzed is enclosed within the box in each case. In each case, responder cells (those with a Status = 1 or 2) 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 Cell Features. The Assay Parameters used to store Events are of the type **Type_X_EventDefinition** (X = 1, 2, or 3).

NOTE



Note that the Event Definition Assay Parameters cannot be modified via the Scan software application. Events should only be defined and edited via the BioApplication Event Wizard (see Chapter 3).

Cell Features	Logic Operators
ObjectAreaCh1	NOT
ObjectShapeP2ACh1	AND
ObjectShapeLWRCh1	AND NOT
ObjectTotalIntenCh1	OR
ObjectAvgIntenCh1	OR NOT
ObjectVarIntenCh1	XOR
SpotCountChN	NAND
SpotTotalAreaChN	NOR
SpotAvgAreaChN	
SpotTotalIntenChN	
SpotAvgIntenChN	

Table 17. Cell features and Boolean operators available for Event Definition. Note that ChN refers to Channels 2-4.

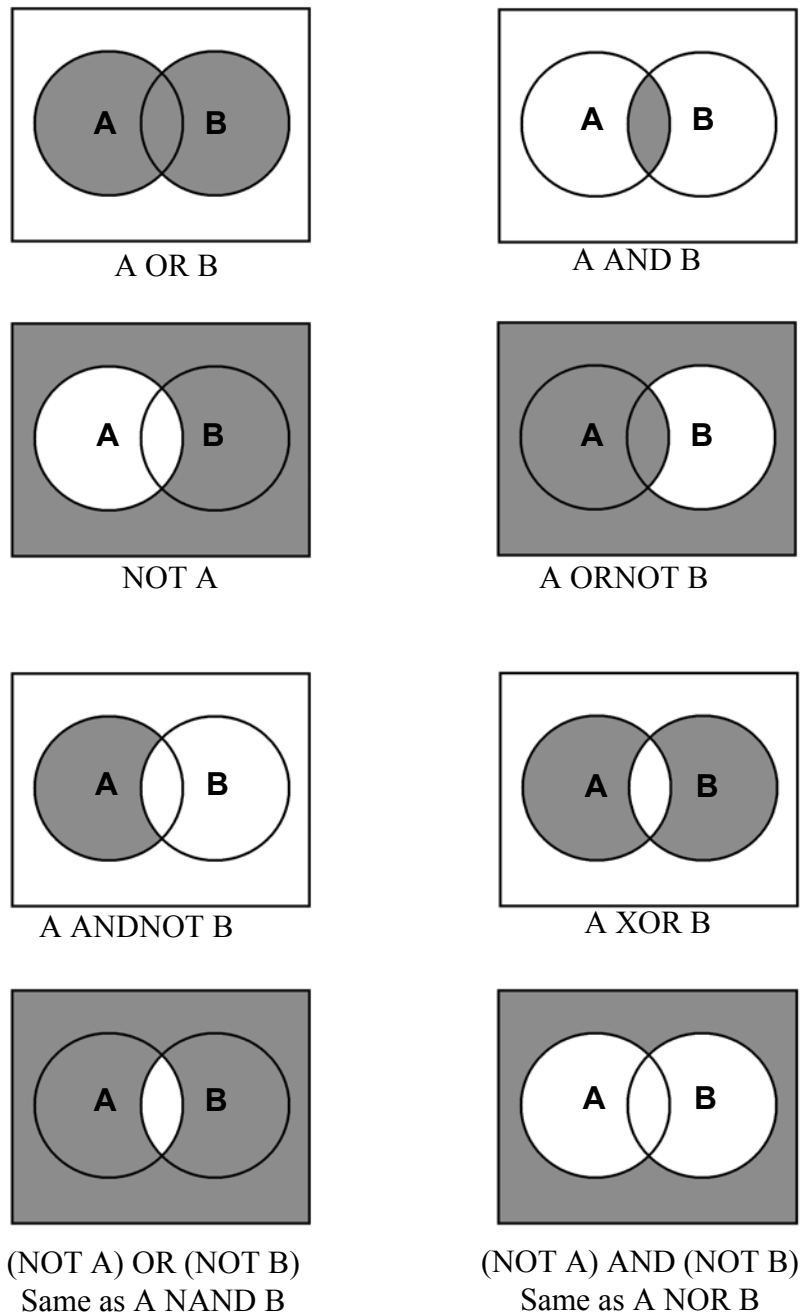


Figure 18. Schematic definition of available Boolean Operators assuming two Cell Features of interest, A and B

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, Field and Well Features, are listed in the Scan Plate View and Create Protocol View 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 Cell, Field, and Well Output Features for the Spot Detector BioApplication are listed and defined below.

For example, the Field-level Spot Total Area is defined as:

Spot Total Area (Field) = Sum of Spot Total Area (Object) in the field

Similarly, at the Well-level, Spot Total Area is defined as:

Spot Total Area (Well) = Sum of Spot Total Area (Object) in the well

From these, average spot intensities at the Field and Well-levels are computed as follows:

$$\text{Average Spot Intensity (Field)} = \frac{\text{Spot Total Intensity (Field)}}{\text{Spot Total Area (Field)}}$$

$$\text{Average Spot Intensity (Well)} = \frac{\text{Spot Total Intensity (Well)}}{\text{Spot Total Area (Well)}}$$

Normalized spot features are also computed by dividing the Field and Well-level totals by the number of objects:

$$\text{Spot Total Intensity Per Object (Field)} = \frac{\text{Spot Total Intensity (Field)}}{\text{Object Count (Field)}}$$

$$\text{Spot Total Intensity Per Object (Well)} = \frac{\text{Spot Total Intensity (Well)}}{\text{Object Count (Well)}}$$

Spot Detector Cell Features

Table 18 shows the output features reported for each selected cell, accessible in the Cell Feature window of the Protocol Interactive View or within the View application. Cell Features shown with a **Status** are those features that indicate if there is a low response (=2), high response (=1), or no response (=0). For Events, 0 = Event did not occur, 1 = Event occurred.

Feature	Units	Description
Cell#	Number	Unique cell body ID
Top	Pixel	Y coordinate (in pixels) of top-left corner of image-aligned bounding box of Ch2 cell body
Left	Pixel	X coordinate (in pixels) of top-left corner of image-aligned bounding box of Ch2 cell body
Width	Pixel	Width (in pixels) of image-aligned bounding box of Ch2 cell body
Height	Pixel	Height (in pixels) of image-aligned bounding box of Ch2 cell body
XCentroid	Pixel	X coordinate (in pixels) of center of Ch2 cell body
YCentroid	Pixel	Y coordinate (in pixels) of center of Ch2 cell body
EventTypeProfile, EventTypeXStatus	Number	Identifies the types of events that occurred: 1, 2, 3, 12, 23, 13, 123
ObjectAreaCh1, Status	Pixel or μm^2	Total area (in pixels or micrometers) of all Ch1 Objects
ObjectShapeP2ACh1, Status	Number	Shape measure based on ratio of perimeter squared to $4\text{PI} \times \text{area}$ of Ch1 object (CellBodyShapeP2ACh2 = 1 for circular object)
ObjectShapeLWRCh1, Status	Number	Shape measure based on ratio of length to width of object-aligned bounding box of Ch1 object
ObjectTotalIntenCh1, Status	Intensity	Total intensity of all pixels within Ch1 object
ObjectAvgIntenCh1, Status	Intensity	Average intensity of all pixels within Ch1 object
ObjectVarIntenCh1, Status	Intensity	Standard deviation of intensity of all pixels within Ch1 object
SpotCountChN, Status	Number	Total number of all selected spots in ChN located within Target Region in ChN
SpotTotalAreaChN, Status	Pixel or μm^2	Total area (in pixels or micrometers) of all spots within Target Region in ChN
SpotAvgAreaChN, Status	Pixel or μm^2	Average area (in pixels or micrometers) of all spots within Target Region in ChN
SpotTotalIntenChN, Status	Intensity	Total intensity of all spots within Target Region in ChN
SpotAvgIntenChN, Status	Intensity	Average intensity of all spot pixels within Target Region in ChN
TotalIntenCh2-6	Intensity	Total intensity of all pixels within Target Region in ChN
AvgIntenCh2-6	Intensity	Average intensity of all pixels within Target Region in ChN

Table 18. Cell Features Available in the Spot Detector v4 BioApplication. *Note that ChN refers to Channels2-4.

Field Features

Table 19 reports the following features at the Field-level in the Field Detail window of the Protocol Interactive View as well as in Field Details within the View application for the Spot Detector BioApplication.

Feature	Description
ValidObjectCount	Number of valid Ch1 objects or spots selected for analysis in the field (Ch1 object selection parameters applied)
SelectedObjectCount	Number of valid Ch1 objects or spots selected for analysis in the field (Ch2-6 object selection parameters applied)
%SelectedObjects	Percentage of valid Ch1 objects or spots selected for analysis in the field
ObjectTotalAreaCh1	Total area (in pixels or micrometers) of all Ch1 objects or spots selected for analysis in the field
ObjectAvgAreaCh1	Average area (in pixels or micrometers) of all Ch1 objects or spots selected for analysis in the field
ObjectTotalIntenCh1	Total intensity of all pixels within Ch1 objects or spots selected for analysis in the field
ObjectAvgIntenCh1	Average intensity of all pixels within Ch1 objects or spots selected for analysis in the field
ObjectTotalIntenPerObjectCh1	ObjectTotalIntenCh1 divided by SelectedObjectCount in the field
SpotCountChN	Number of ChN spots in the field
SpotTotalAreaChN	Total area (in pixels or micrometers) of all ChN spots in the field
SpotAvgAreaChN	Average area (in pixels or micrometers) of all ChN spots in the field
SpotTotalIntenChN	Total intensity of all pixels within ChN spots in the field
SpotAvgIntenChN	Average intensity of all pixels within ChN spots in the field
SpotTotalIntenPerSpotChN	SpotTotalIntenChN divided by SpotCountChN in the field
SpotCountPerObjectChN	Average number of ChN spots per Ch1 object selected for analysis in the field
SpotTotalAreaPerObjectChN	SpotTotalAreaChN divided by SelectedObjectCount in the field
SpotTotalIntenPerObjectChN	SpotTotalIntenChN divided by SelectedObjectCount in the field

Table 19. Field Features available for the Spot Detector BioApplication. *Note that “ChN” refers to Channels 2-4.

Well Features

Spot Detector Well Features

Most of the Well Features are derived from a subset of the Cell Features. Such features are identified by a prefix to the cell feature name (Table 20).

Feature Prefix	Field/Well Feature Description	Units
MEAN_	Average of Feature_X for all objects selected for analysis in the well	Same as cell feature
SD_	Standard deviation of Feature_X for all objects selected for analysis in the well	Same as cell feature
SE_	Standard error of mean of Feature_X for all objects selected for analysis in the well	Same as cell feature
CV_	Coefficient of variation of Feature_X for all objects selected for analysis in the well	Percent
%HIGH	Percentage of selected objects in the well with Feature_X above high-response level	Percent
%LOW	Percentage of selected objects in the well with Feature_X below low-response level	Percent

Table 20. General Well Features available for the Spot Detector BioApplication

Additional Well Features representing counts and percentages calculated for all selected cells are shown below in Table 21.

Feature	Description
ValidObjectCount	Count of valid Ch1 objects or spots identified in the well (Ch1 object selection parameters applied)
SelectedObjectCount	Number of valid Ch1 objects or spots selected for analysis in the well (Ch2-6 object selection parameters applied)
%SelectedObjects	Percentage of valid Ch1 objects or spots selected for analysis in the well
FieldCount	Number of fields imaged in the well
ValidFieldCount	Number of fields in which Ch1 objects or spots were selected for analysis in the well
SelectedObjectCountPerValidField	Average number of Ch1 objects or spots selected for analysis per valid field in the well
EventTypeXObjectCount	Number of objects selected for analysis in the well in which EventTypeX occurred
%EventTypeXObjects	Percentage of objects selected for analysis in the well in which EventTypeX occurred
ObjectTotalAreaCh1	Total area (in pixels or micrometers) of all Ch1 objects or spots selected for analysis in the well
ObjectAvgAreaCh1	Average area (in pixels or micrometers) of all Ch1 objects or spots selected for analysis in the well
ObjectTotalIntenCh1	Total intensity of all pixels within Ch1 objects or spots selected for analysis in the well

Feature	Description
ObjectAvgIntenCh1	Average intensity of all pixels within Ch1 objects or spots selected for analysis in the well
ObjectTotalIntenPerObjectCh1	ObjectTotalIntenCh1 divided by SelectedObjectCount in the well
SpotCountChN	Number of ChN spots selected for analysis in the well
SpotTotalAreaChN	Total area (in pixels or micrometers) of all ChN spots selected for analysis in the well
SpotAvgAreaChN	Average area (in pixels or micrometers) of all ChN spots selected for analysis in the well
SpotTotalIntenChN	Total intensity of all pixels within ChN spots selected for analysis in the well
SpotAvgIntenChN	Average intensity of all pixels within ChN spots selected for analysis in the well
SpotTotalIntenPerSpotChN	SpotTotalIntenChN divided by SpotCountChN in the well
SpotCountPerObjectChN	Average number of ChN spots per object selected for analysis in the well
SpotTotalAreaPerObjectChN	Average area of ChN spots per object selected for analysis in the well
SpotTotalIntenPerObjectChN	SpotTotalIntenChN divided by SelectedObjectCount in the well

Table 21. Well Features available for the Spot Detector BioApplication. *Note that “ChN” refers to Channels 2-4.

Reference Well Features

The Spot Detector BioApplication reports the following Reference Features, whose values indicate the data generated from Reference Wells (Table 22). Reference Features are viewable in the Scan Plate View as well as listed as Plate Features and in the Well Detail View of the View Application.

Note the following table listing of Reference Features does not list both features derived from the previous level parameters, rather only one entry for the feature is listed giving both outputs. See the following example for the object total intensity per object in Channel 1.

- ObjectTotalIntenCh1Level**Low/High**

Feature	Description
RefAvgObjectCountPerField	Average number of objects within fields of reference wells
RefObjectAreaCh1Level Low/High	Low/High-response level for RefObjectAreaCh1 computed from reference well results
RefObjectShapeP2ACh1Level Low/High	Low/High-response level for RefObjectShapeP2ACh1 computed from reference well results
RefObjectShapeLWRCh1Level Low/High	Low/High-response level for RefObjectShapeLWRCh1 computed from reference well results
RefObjectTotalIntenCh1Level Low/High	Low/High-response level for RefObjectTotalIntenCh1 computed from reference well results
RefObjectAvgIntenCh1Level Low/High	Low/High-response level for RefObjectAvgIntenCh1 computed from reference well results
RefObjectVarIntenCh1Level Low/High	Low/High-response level for RefObjectVarIntenCh1 computed from reference well results
RefSpotCountChNLevel Low/High	Low/High-response level for RefSpotCountChN computed from reference well results
RefSpotTotalAreaChNLevel Low/High	Low/High-response level for RefSpotTotalAreaChN computed from reference well results
RefSpotAvgAreaChNLevel Low/High	Low/High-response level for RefSpotAvgAreaChN computed from reference well results
RefSpotTotalIntenChNLevel Low/High	Low/High-response level for RefSpotTotalIntenChN computed from reference well results
RefSpotAvgIntenChNLevel Low/High	Low/High-response level for RefSpotAvgIntenChN computed from reference well results

Table 22. Reference Well Features available for the Spot Detector BioApplication. *Note that “ChN” refers to Channels 2-4.

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 Spot Detector BioApplication. This section provides a detailed description of the operation of the BioApplication Event Wizard. The Wizard should only be used after the Spot Detector BioApplication has been installed on your computer.

NOTE

Note that the Event Definition Assay Parameters **must not** be modified via the Scan software application. Events can only be defined and edited via the BioApplication Event Wizard. Altering the values within the protocol (through the Create Protocol or Protocol Interactive Views) may cause your protocol to become inoperable.

NOTE

It is recommended that you do NOT run the Scan software application and the BioApplication Event Wizard at the same time.

Definition of Events requires that the following steps be followed, in the order listed.

STEP I

- 1) Create a protocol without defining Events. Set optimized parameter values (lower and upper limits) for Cell 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 BioApplication Event Wizard provides the following benefits:

- Can be used with any Spot Detector protocol
- Enables use of standard Boolean operators (NOT, AND, ANDNOT, OR, ORNOT, XOR, NAND, and NOR)
- Provides read-only protocol protection
- Displays previously entered Event Definitions in saved Assay Protocols
- Clears each Event Definition individually
- Provides rapid construction of logical statements using Cell Features and logical operators
- Provides automated validation of each Event Definition when loading or saving protocols
- Saves updated protocol
- Adds Event Definitions to the Protocol Comments field in the Assay Protocol

Steps for Running the Event Wizard

Before Running the Event Wizard...

- 1) Create an Assay Protocol using the Spot Detector BioApplication without defining Events. Set optimized parameter values (upper levels) for detection of responder cells associated with Cell Features to be used for Event Definition.
- 2) Save the Assay Protocol.
- 3) Close the vHCS: Scan software application (recommended).

Starting the BioApplication Event Wizard

NOTE



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

To start the BioApplication Event Wizard,

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



-or-

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

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

Protocol Selection and Loading

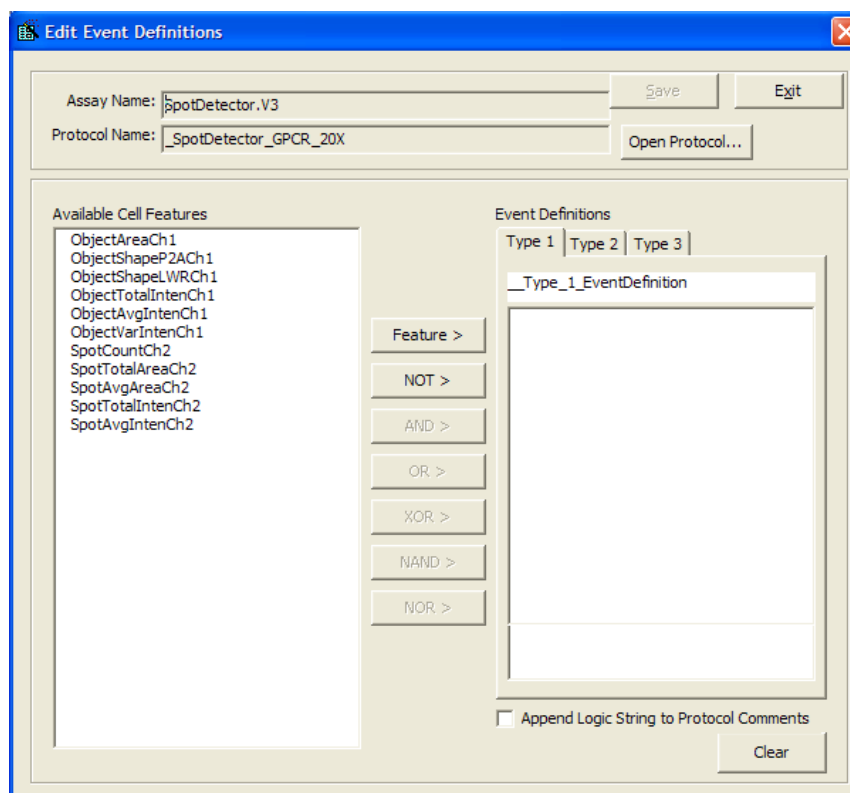
The first window that is displayed (see below) allows selection of any Spot Detector Assay Protocol. Note that Event Definitions can only be specified for pre-existing Assay Protocols.

To select an Assay Protocol,

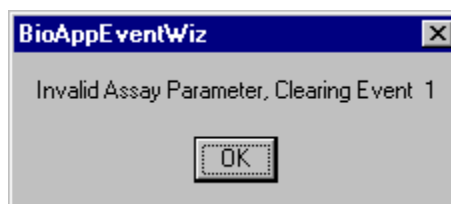
- 1) Select **Open Protocol**.
- 2) Select the desired Assay Protocol from the Protocol Manager.



Once a protocol is selected, the window expands to its full extent as shown below. Cell Features available for Event Definition are listed on the left in the Available Cell 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. A message above the **Save** button will indicate that the protocol cannot be edited.

- 3) Once you have completed the Event Definitions, click the **Save** button and then click the **Exit** button. The screen will close and you can then open the Scan software application.

Defining Events

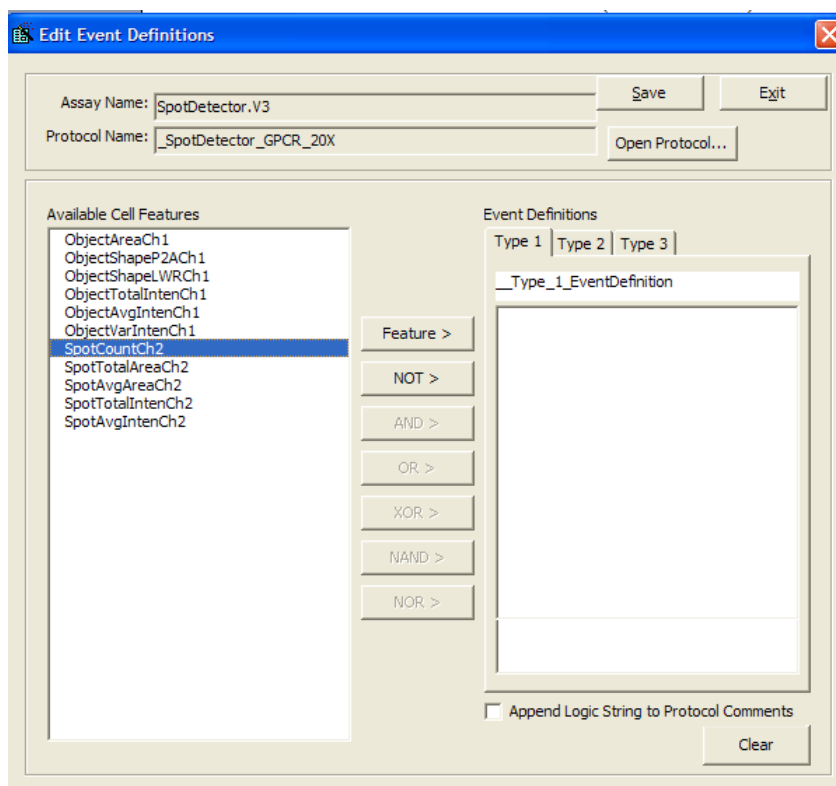
This section describes the steps involved in defining individual Events in a Spot Detector Assay Protocol. Note that logical statements used to define Events can include up to three Cell Features and four logical operators.

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

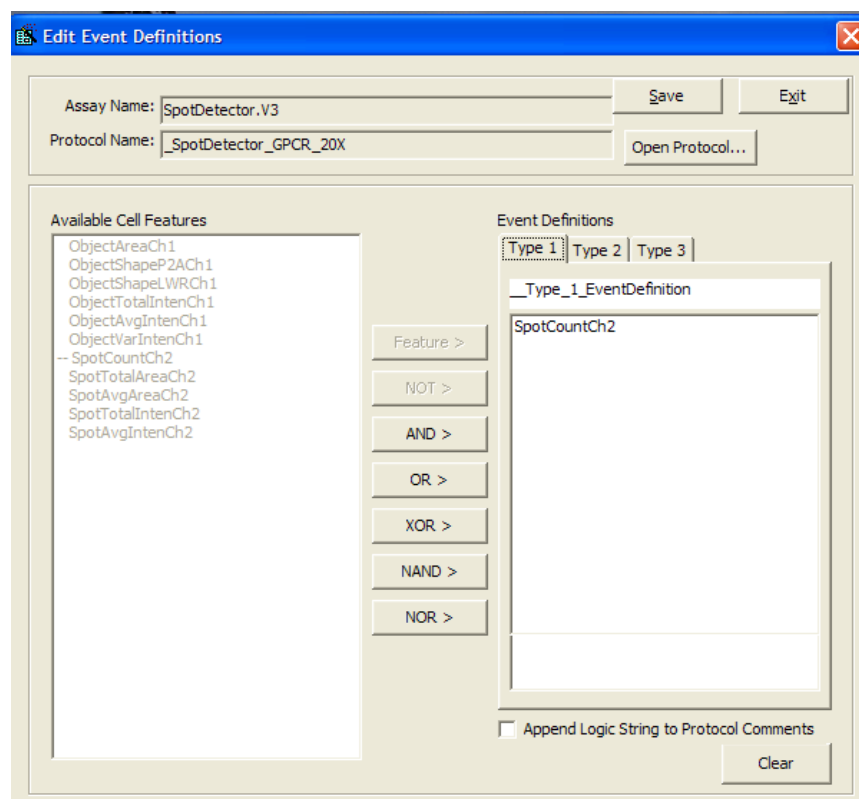
The procedure to be followed when constructing Event Definitions is described in the sequence of screenshots below. Cell Features are combined with Boolean operators to produce Event Definitions. Any Cell Feature can be selected by clicking on the feature name in the Available Cell Features list and 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. Press the appropriate buttons in sequence to build the Event Definition, as shown below.

To build an Event Definition,

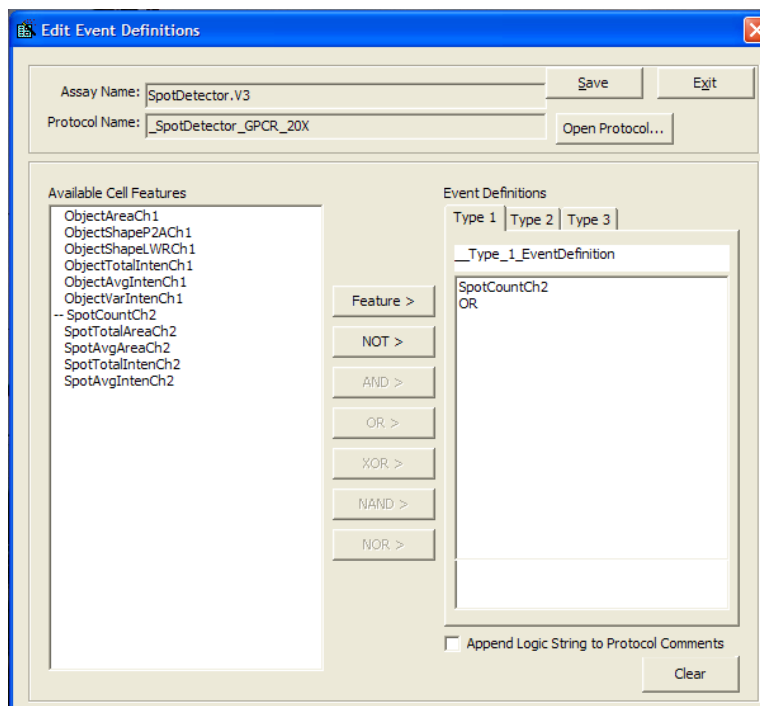
- 1) Select the Event Definition that you want to specify or edit by clicking on the **Type 1**, **Type 2**, or **Type 3** tab.
- 2) Select the desired Cell Feature by clicking on the feature name from the Available Cell Features list. In this example, the **SpotCountCh2** feature is selected. You may also choose to select **NOT >** first (before the Feature) to indicate that you do not want to include this Cell Feature in your event analysis.



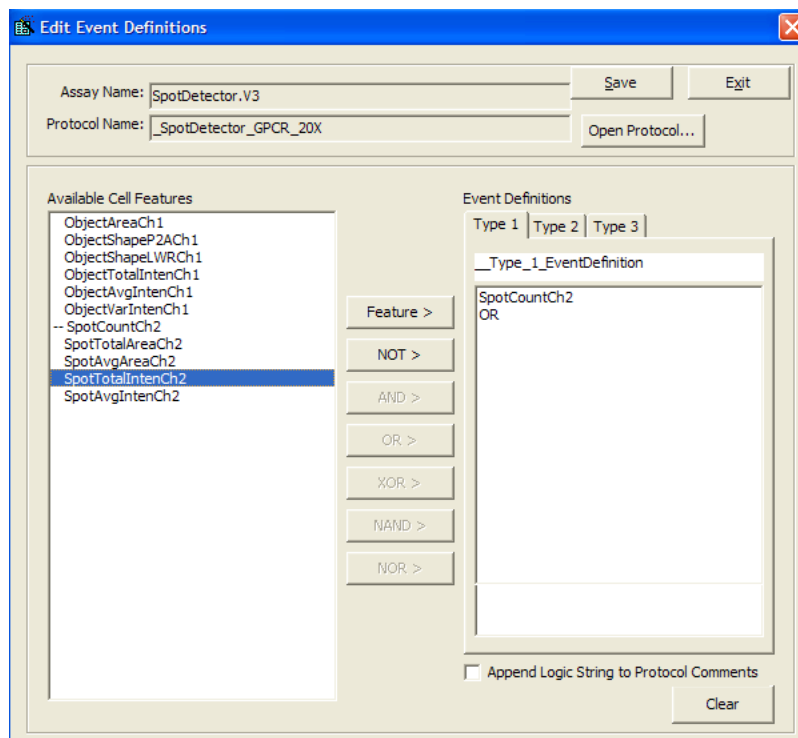
3) Click the **Feature>** button to transfer the Cell Feature into the Event Definition



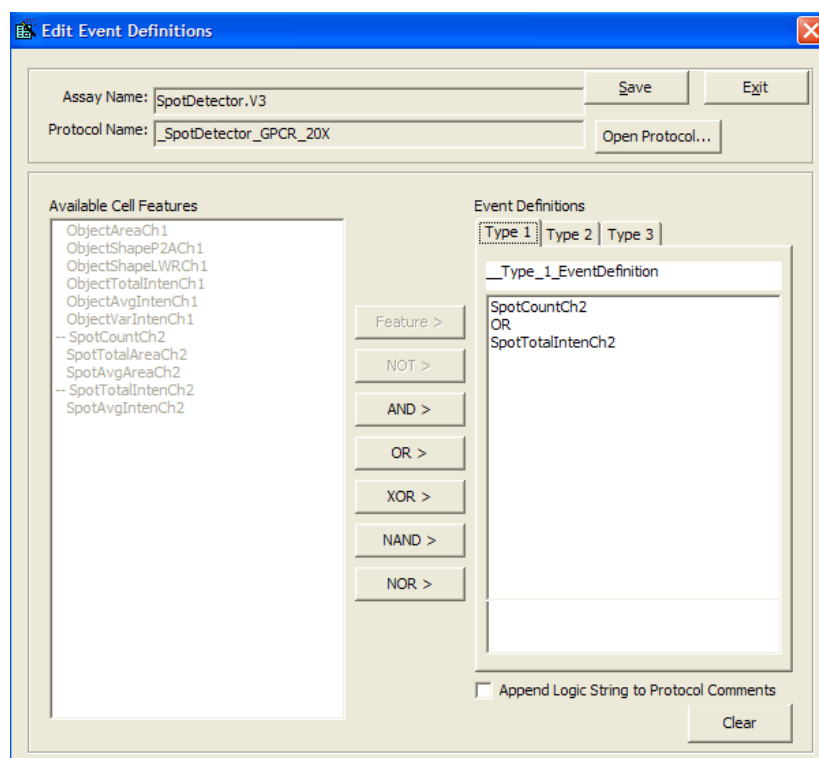
- 4) Click on the desired Operator button to transfer it into the Event Definition. In this example, the **OR** operator is selected (see below).



- 5) Repeat the cycle for adding another Cell Feature to the Event Definition. In this case, the Cell Feature **SpotTotalIntenCh2** has been selected for addition.

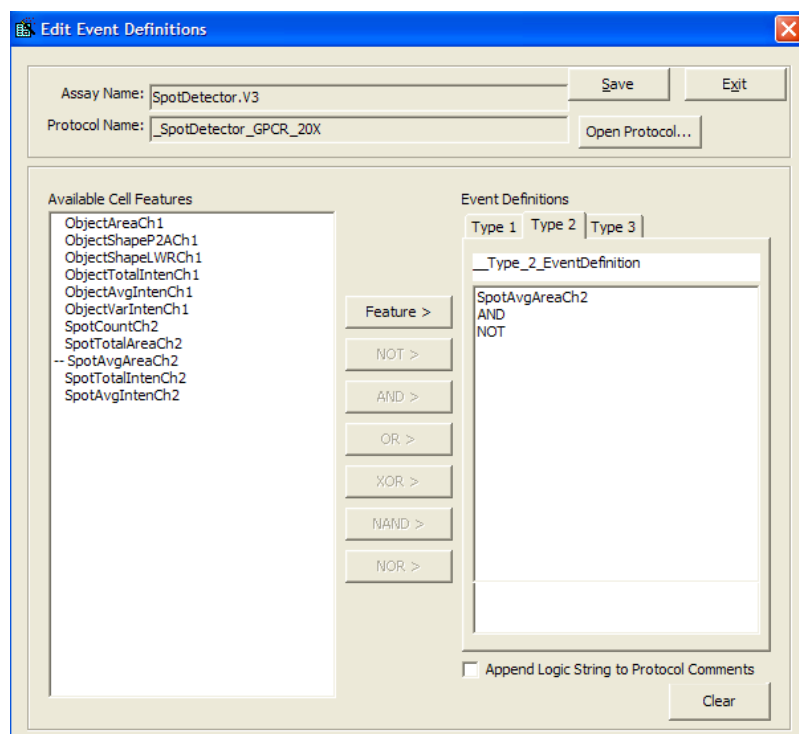
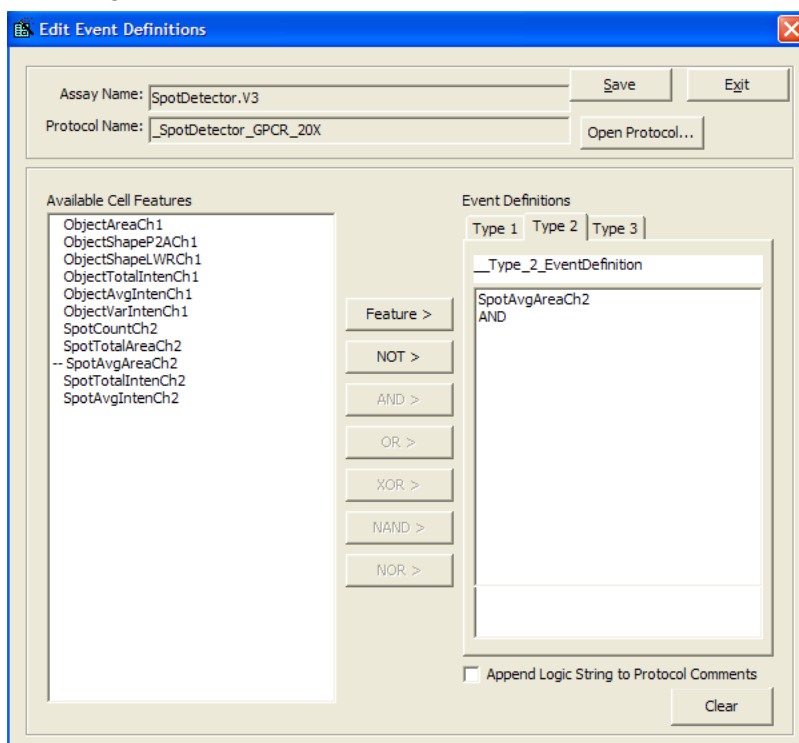


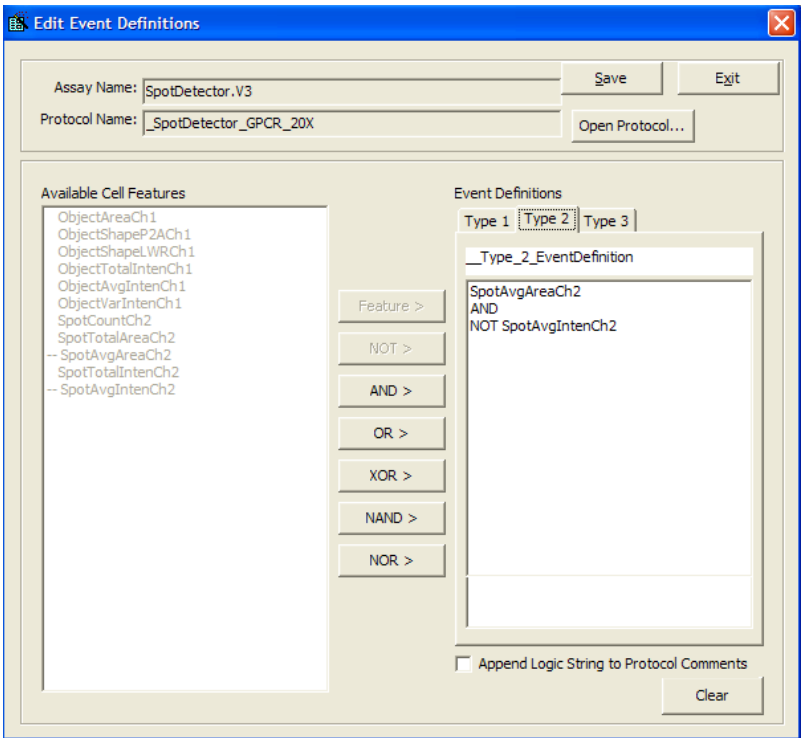
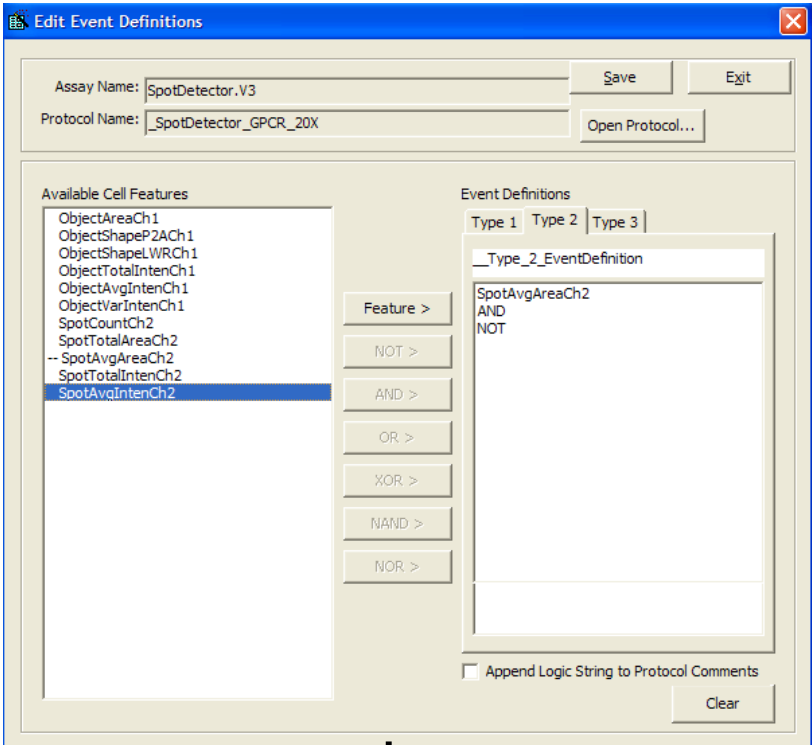
- 6) As before, click on the **Feature >** button to transfer the Cell Feature into the Event Definition (see below).



Type_1_EventDefinition is: **SpotCountCh2 OR SpotTotalIntenCh2**

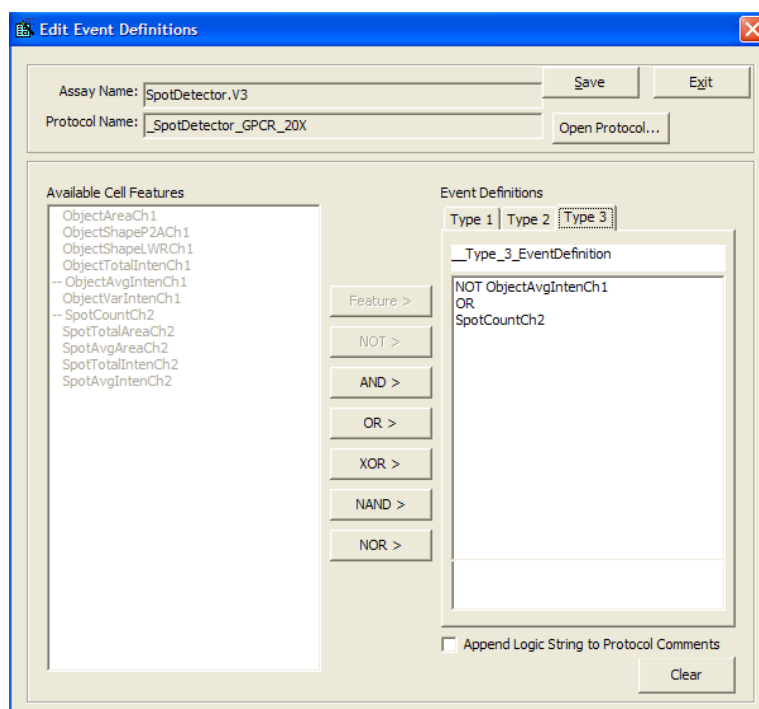
Entry of a logical operator may be followed by entry of another Cell Feature. Alternatively, the operators **AND** and **OR** can be inverted by adding the **NOT** operator by pressing the **NOT >** button prior to selecting the Cell Feature, as shown below.



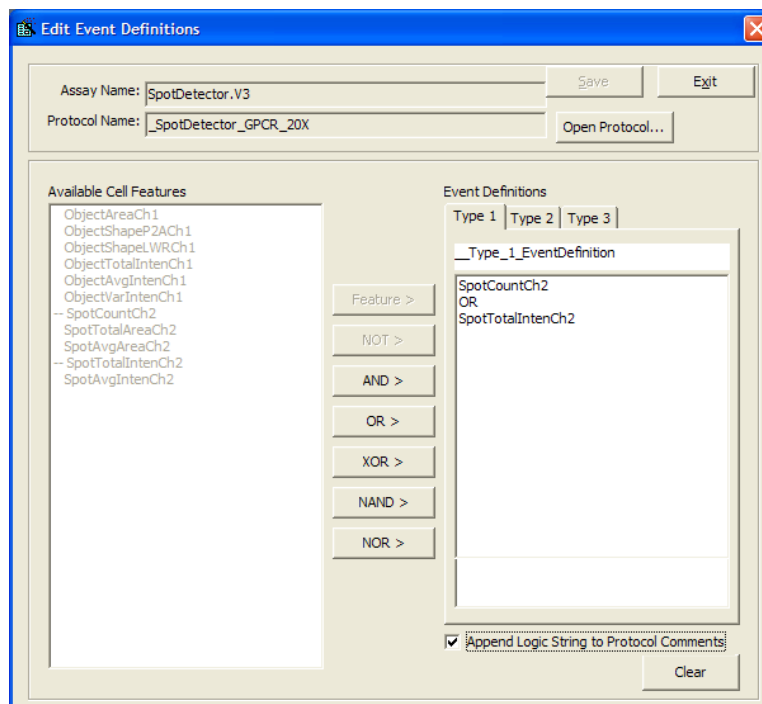


Type_2_EventDefinition is: **SpotAvgAreaCh2** ANDNOT **SpotAvgIntenCh2**

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



If you would like to make the Event Definitions viewable in the comments section of the Assay Protocol, be sure to check the box, **Append Logic String to Protocol Comments**.



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.

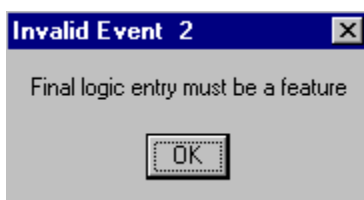
To save the updated Assay Protocol,

- Click the **Save** button. 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.

NOTE

The BioApplication Event Wizard will only make changes to pre-existing Assay Protocols by adding or modifying Event Definitions. If you wish to create a new protocol, use the **Save Assay Protocol As** option under the **File** menu within the Scan software application prior to starting the Event Wizard.

The BioApplication Event Wizard has the capability to 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 exit the BioApplication Event Wizard,**

- Click the **Exit** button at any time to exit the Wizard without saving any changes made to Event Definitions.

If you chose to **Append Logic String to Protocol Comments**, once the Assay Protocol is saved, Event Definitions will be viewable in the **Protocol Comments** field in the **Create Protocol View** when opening the saved Assay Protocol in the Scan software (see arrow).



iDev Software Workflow

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

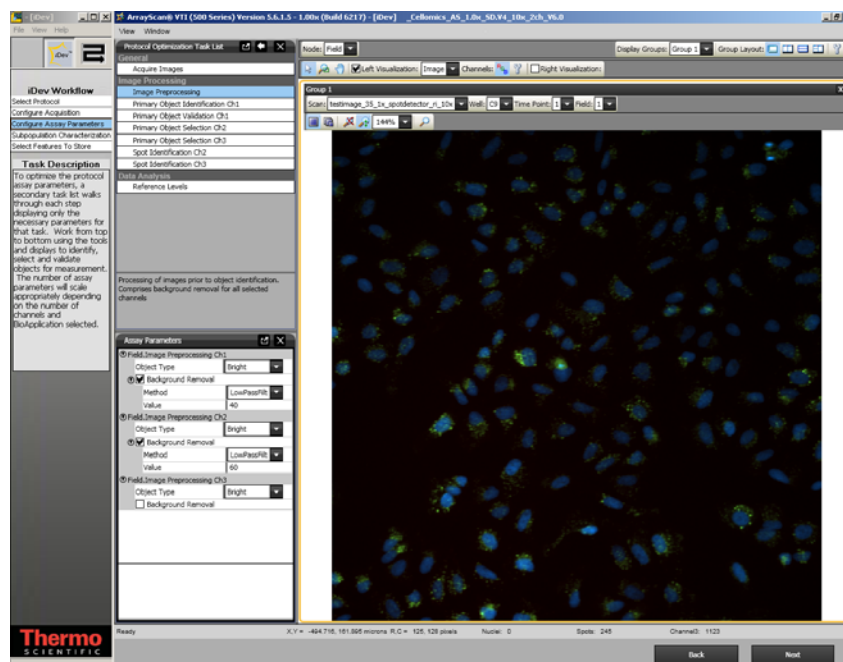


Figure 19. Protocol Optimization Task – Image Preprocessing

Primary Object Identification Ch1

Primary Object Identification is the identification of objects 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. If detection is checked, then spots are identified and treated as primary objects. This configuration is typical for a one channel protocol only.

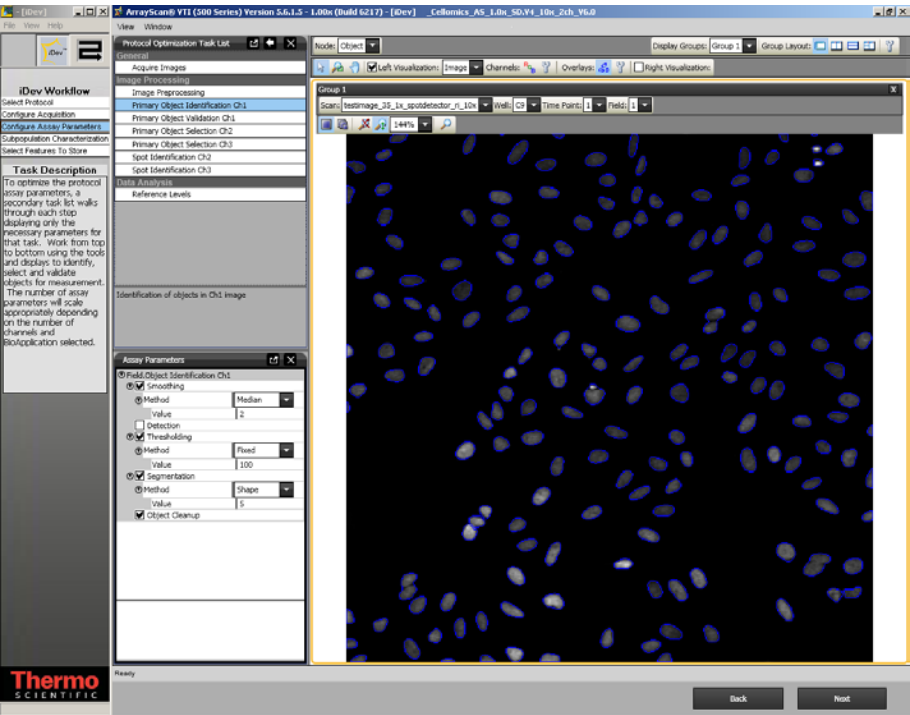


Figure 20. Protocol Optimization Task – Primary Object Identification Ch1

Primary Object Validation Ch1

Primary Object Validation is object selection/rejection based on area, shape, and intensity features calculated for the primary 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) objects in Channel 1 based on object selection features. Additionally in this task, you will determine if objects that are on the border of the field are included or rejected from analysis.

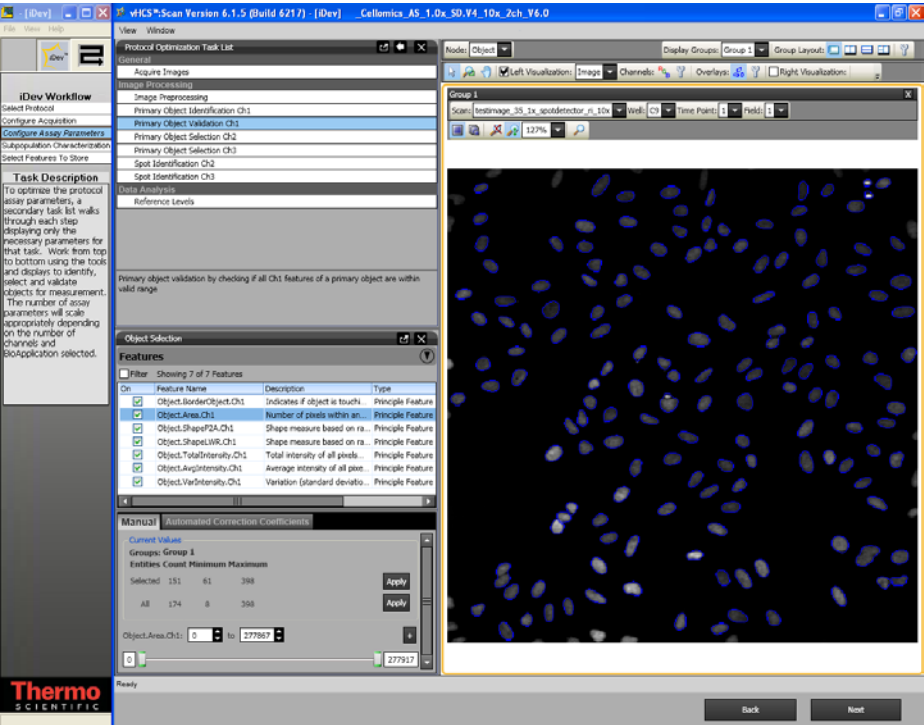


Figure 21. Protocol Optimization Task – Primary Object Validation Ch1

Primary Object Selection Ch2 through ChN

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

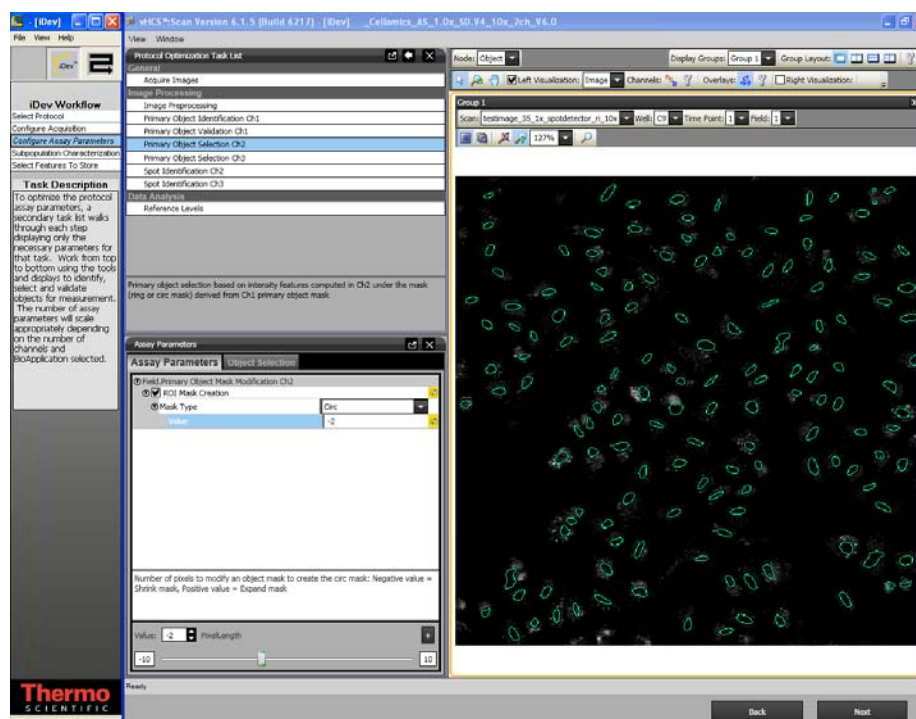


Figure 22. Protocol Optimization Task – Primary Object Selection Ch2 through ChN

Spot Identification Ch2 through Ch4

In this task, you can identify spots in Channel 2 through Channel 4 within the region of interest (ring or circ mask) derived from the Channel 1 primary object mask. In this task, you will define the region of interest (ROI) and set the identification method and threshold for your target in Channel 2. The ROI can be either be a “Circ” or a “Ring”.

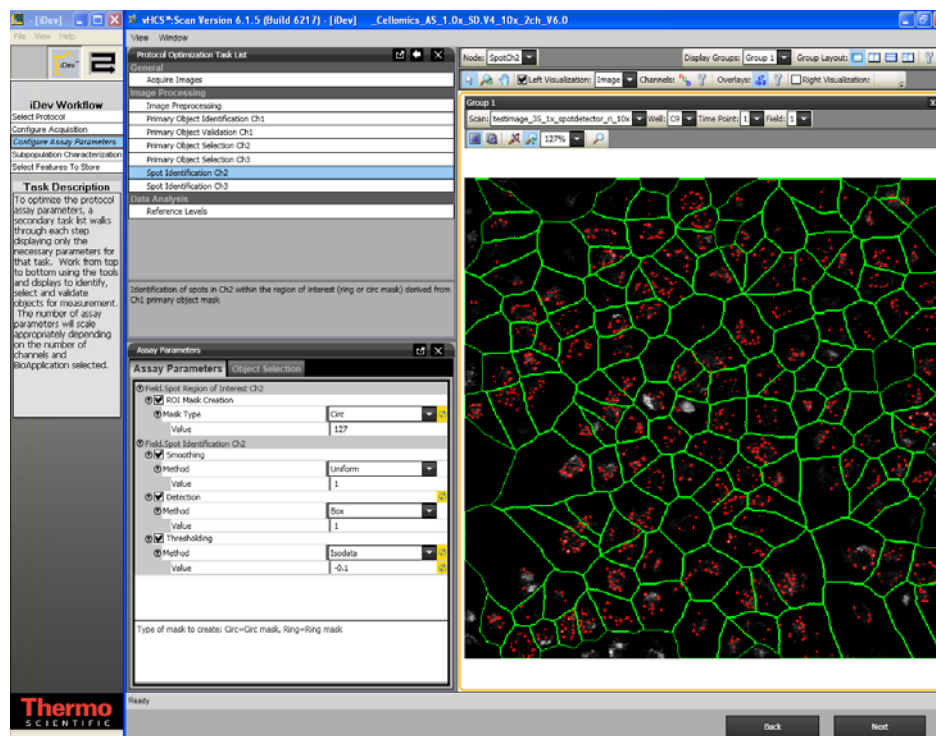


Figure 23. Protocol Optimization Task – Spot Identification

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.

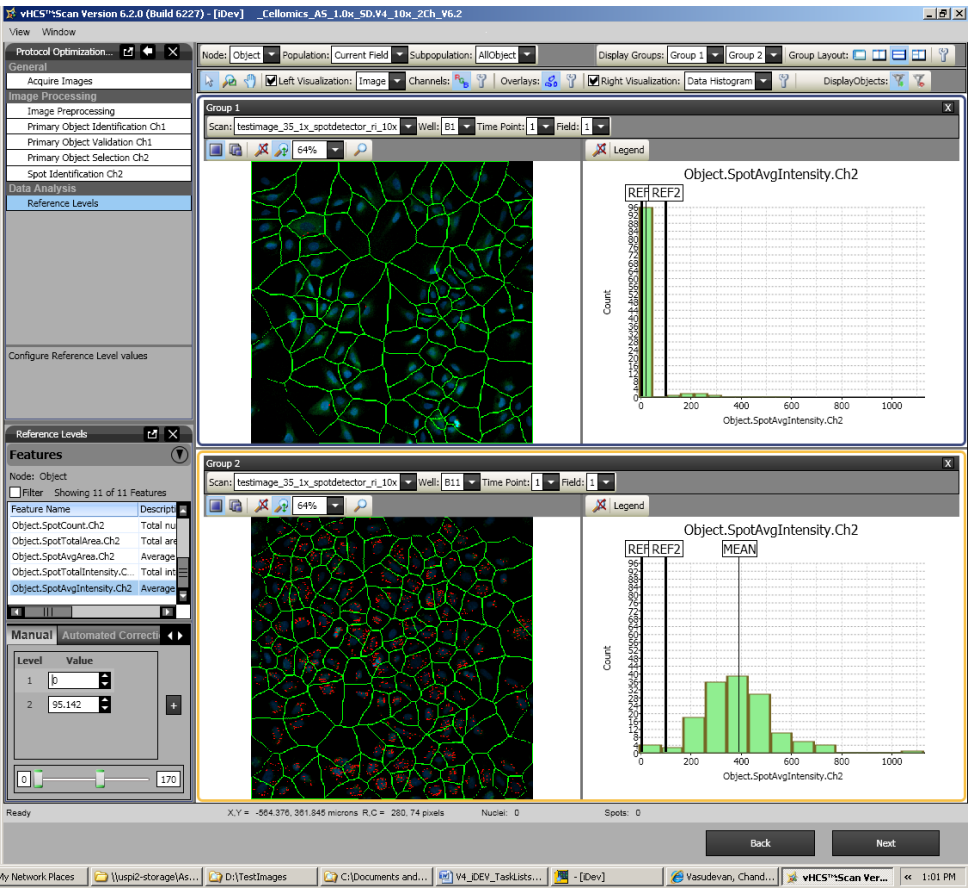


Figure 26. Protocol Optimization Task – Reference Levels

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.

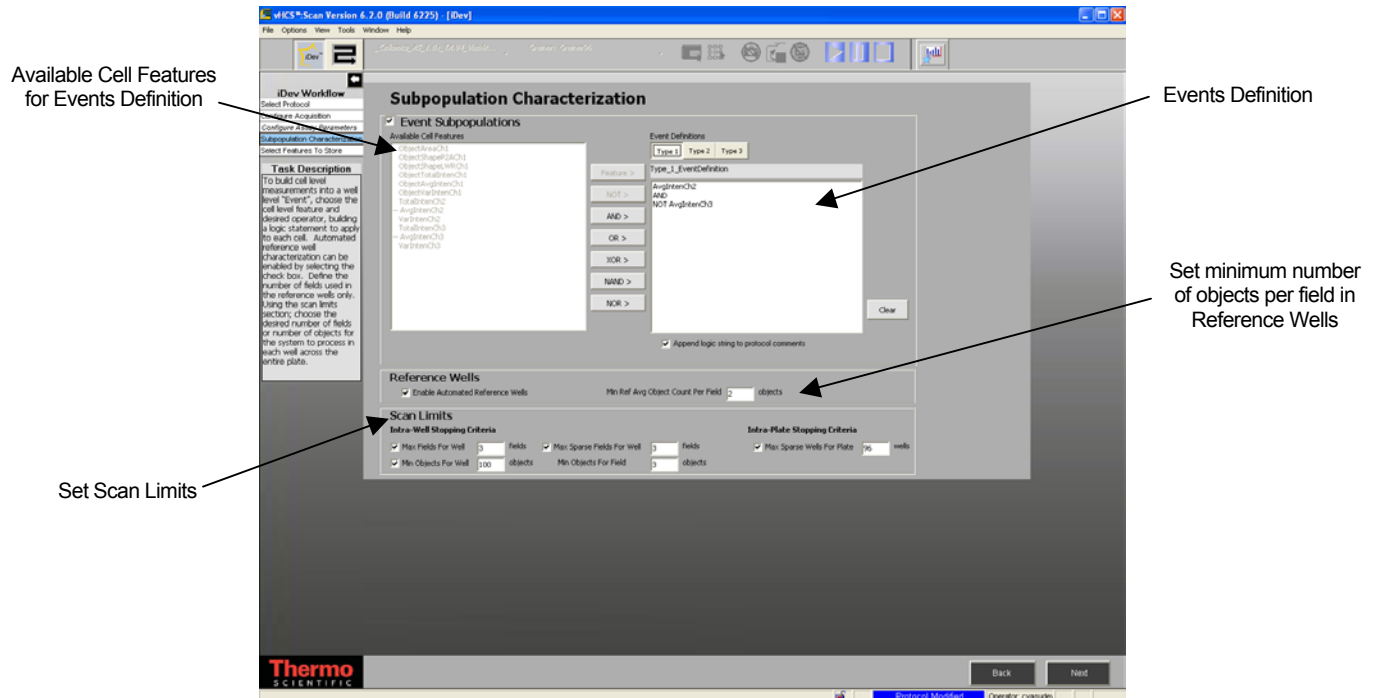


Figure 27. Subpopulation Characterization Task

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