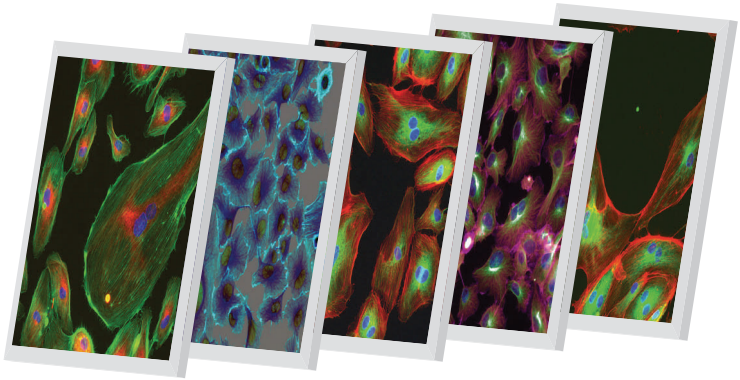
Thermo Scientific Cellomics® Multiparameter Cytotoxicity V4

BioApplication Guide



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Cellomics® Multiparameter Cytotoxicity BioApplication Guide

V4 Version

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Chapter

1

Overview of the Multiparameter Cytotoxicity Application

High Content Screening (HCS) combines biological fluorescent-based reagents, automated optical imaging instrumentation, and an advanced image processing algorithm to automatically extract and quantify the information from the biological system. This guide provides a brief description for performing one such HCS assay, Multiparameter Cytotoxicity. A separate protocol detailing the preparation of samples for performing a High Content Screen with this BioApplication is provided in the Cellomics® Multiparameter Cytotoxicity I HCS Reagent Kit. This guide contains the following chapters:

Chapter 1 provides an overview of the parts of the assay, such as the cell biological situations where it can be applied.

Chapter 2 describes the quantitative algorithm used to analyze the results and gives a brief description of input parameters and output features.

Chapter 3 describes the Protocol optimization tasks that are available in the iDev™ Assay Development workflow.

System Compatibility

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

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

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

Cell Biology Background

In today's drug discovery environment, vast compound libraries are screened in order to identify potential drugs for treatment of different diseases. An essential aspect of the drug discovery and approval process is determining the toxic effects of compounds that are potential drugs. *In vitro* toxicity assessments of compounds are becoming common in the drug discovery industry today and are being applied early in the drug discovery process to identify toxicity issues that may arise (Ohno, Miyajima, and Sunouchi 1998; Ulrich et al. 1995; Horokova 1999). These methods have to be economical, have fast turnaround times, and, ideally, should report toxic effects on several biologically relevant targets. Traditional

toxicity screening methods using animal models are expensive and have slow turn around times. Furthermore, the results obtained from animal models can be misleading due to genetic, biological, and physiological differences between animals and humans. The Cellomics Multiparameter Cytotoxicity BioApplication is an *in vitro* assay tool that will provide comprehensive cytotoxicity information by quantifying changes in nuclear morphology, cell membrane integrity and lysosomal physiology at the single cell level.

Any xenobiotic compound entering an animal is primarily targeted to the liver and kidney for metabolism and excretion (Klaassen 1986). Hence, this assay was developed in models of cell types/lines that have been derived from these organs. In this assay, cells (rat primary hepatocytes or cultured cell lines such as HepG2, BHK, MDCK, etc.) are cultured as required and treated with the compounds being screened for toxicity. Cells are labeled with fluorescent dyes that identify the cellular targets of interest: nuclei, cell plasma membrane integrity, and lysosomes (and other acidic organelles). The biology and the mode of action of these dyes on their targets are briefly described below.

Changes in Nuclear Size/Morphology

Following a toxic insult, cells may undergo either necrosis or apoptosis. Both processes are accompanied by distinguishing changes in nuclear size, morphology, or both (Manjo and Joris 1995; Earnshaw 1995). Hoechst 33342, a dye used in this assay, binds to DNA and emits a blue fluorescence that delineates the nuclear region. The Multiparameter Cytotoxicity BioApplication uses an image processing algorithm to measure changes in nuclear size/morphology and intensity that are brought about by a toxic event. The algorithm extracts measurements of nuclei selectively, objectively, consistently, and automatically. Figure 1 shows a decrease in both nuclear size and cell number due to exposure of HepG2 cells to valinomycin, a potent K^+ ionophore.

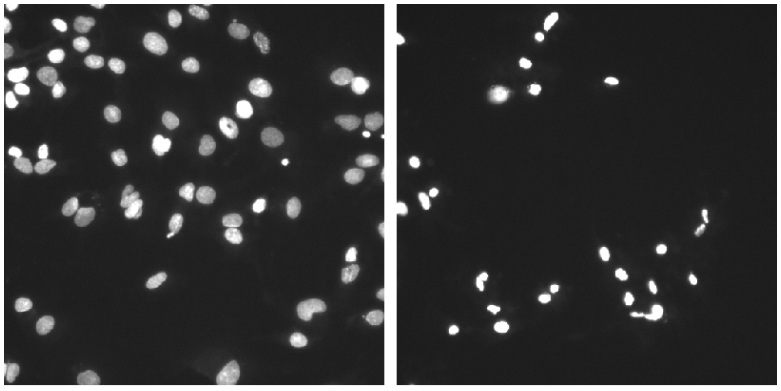

Figure 1. *Changes in nuclear size/morphology induced by exposing HepG2 cells to 200 µM valinomycin for 24 hours. Cells in the left panel are vehicle treated; cells in the right panel have been exposed to valinomycin.*

Changes in Cell Membrane Permeability

The cell membrane maintains cellular homeostasis by holding the various constituents of the cell together providing a specialized environment different from its extracellular surroundings and providing a mechanism for the controlled exchange of its nutrients with its surroundings. Certain cellular toxins affect the integrity of the cell membrane leading to the membrane becoming permeant, eventually causing cell death (Liepins 1989; Spector and Yorek 1985; Mingeot-Leclercq, Brasseur, and Schanck 1995). The membrane permeability indicator dye used in this assay is unable to permeate healthy cells. However, if the cell membrane integrity

is compromised, the dye enters the cell and stains the nucleus with a bright green fluorescence. Figure 2 shows membrane permeability of cells treated with valinomycin.

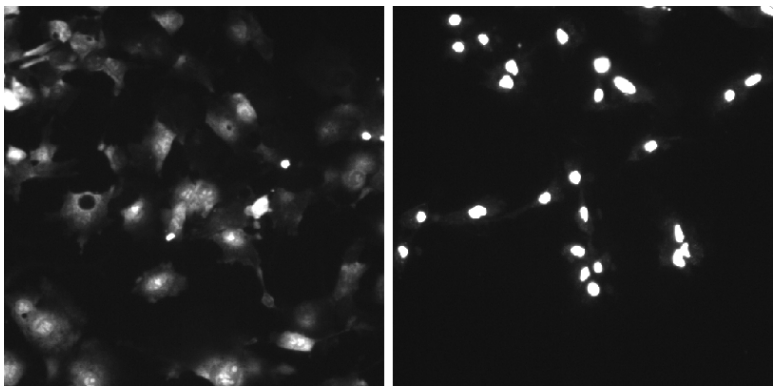

Figure 2. *HepG2 cells treated with 200 µM valinomycin for 24 hours and stained for cell membrane permeability. The panel on left shows vehicle treated cells; the panel on the right shows cells exposed to valinomycin.*

Changes in Lysosomal Mass

A cell's physiological functioning is often mediated by a number of its organelles, such as lysosomes and endosomes, which maintain an acidic pH. Toxins can interfere with cell functionality by affecting the pH of these organelles or by causing an increase in the number of lysosomes (Reasor 1989; Read 1991). The dye used in the assay to determine changes in lysosomal mass is a weak base that accumulates in acidic organelles such as lysosomes and endosomes. A decrease in the pH of these and other acidic organelles or an increase in the number of lysosomes brought about by compound toxicity will be reflected as an increase in the staining intensity of the dye. Conversely, an increase in the pH of these acidic organelles or a decrease in the number of lysosomes due to compound toxicity will be reflected as a decrease in dye staining intensity. Figure 3 exemplifies a decrease in dye staining in HepG2 cells treated with valinomycin.

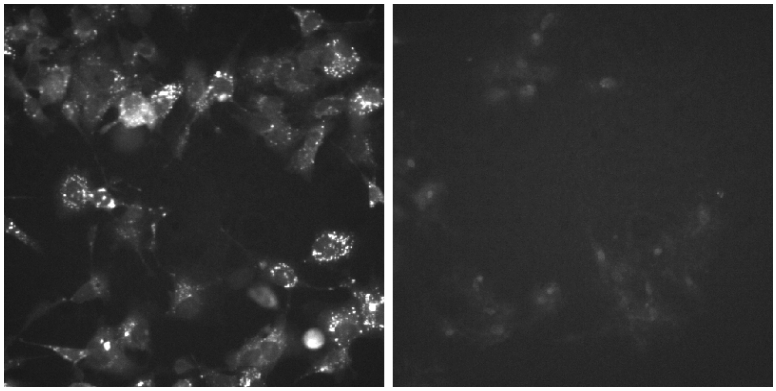

Figure 3. *Changes in lysosomal mass in HepG2 cells treated with 200 µM valinomycin for 24 hours. The panel on the left shows cells treated with vehicle; the panel on the right shows cells exposed to valinomycin.*

BioApplication Overview

The Multiparameter Cytotoxicity BioApplication provides information on multiple cytotoxic related parameters. Utilized with the suggested targets, this assay will specifically provide information on the effects of compounds on individual cells' nuclear morphology, its membrane permeability, and the physiology of its lysosomes and other acidic organelles. In addition, this BioApplication provides information about changes in cell density (number of cells per field) brought about by toxic effects of the compounds that affect cellular attachment to the assay plate matrix.

The protocol is set up so that changes in nuclear morphology, intensity, count and size are measured in Channel 1 along with focusing. These objects (nuclei) are identified using a fluorescent stain, Hoechst 33342. Channel 2 is set to measure membrane permeability via changes in intensity after using a cell permeability stain. Channel 3 measures changes in lysosomal mass/pH using intensity of spots with a stain for lysosomes.

The objects (nuclei) identified in Channel 1 are used to create a region of interest called "Circ**"** in Channel 2 and "Ring" in Channel 3. Figure 4 shows a schematic representation of the Circ and Ring regions of a cell. You have the ability to vary the width and range of the Circ and Ring masks in the target channels. The Circ mask and the Ring mask are only applied to objects that pass the object selection criteria based on mean and total pixel intensity that you set in each of the channels used.

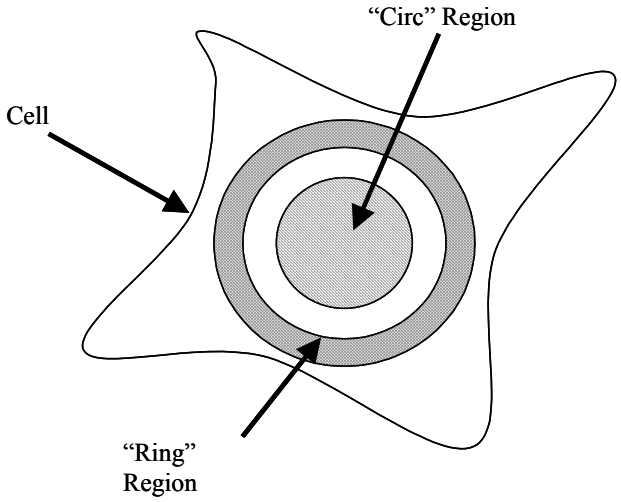

Figure 4. *A schematic representation of the Circ and Ring regions of a cell.*

BioApplication Measurements

The Multiparameter Cytotoxicity BioApplication reports both Cell and Well Features. Cell Features are reported for each cell, and Well Features are reported for each well and are derived from the Cell Features for all the cells imaged and analyzed in that well. Table 1 shows the number of Cell and Well Features available based on the number of channels selected.

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

Measurement	Cell Region Measured	Cell Features	Well Features
Cell & Well Features Reported for Channel 1			
General		Cell # Top Left Width Height X Centroid Y Centroid	Valid Cell Count Selected Cell Count %Selected Cells Valid Field Count Selected Cell Count Per Valid Field %Change Selected Cell Count Per Valid Field Cytotoxicity Index
Morphology	Nucleus	Area Shape P2A Shape LWR	MEAN, SD, SE, CV
		Size & Status	MEAN, SD, SE, CV, %HIGH, %LOW, %RESPONDER
Intensity	Nucleus	Total Intensity Average Intensity	MEAN, SD, SE, CV
Cell & Well Features Reported for Channel 2:			
Intensity	Circ	Total Intensity	MEAN, SD, SE, CV
		Average Intensity and Status	MEAN, SD, SE, CV, %HIGH, %LOW, %RESPONDER
	Modified nuclear mask (gating)	Total Intensity Average Intensity	none
Cell & Well Features Reported for Channel 3:			
Intensity	Spot	Total Intensity	MEAN, SD, SE, CV
		Average Intensity and Status	MEAN, SD, SE, CV, %HIGH, %LOW, %RESPONDER
	Modified nuclear mask (gating)	Total Intensity Average Intensity	none
Cell & Well Features Reported for Channels 4 -6:			
Intensity	Modified nuclear mask (gating)	Total Intensity Average Intensity	none

Table 1. *Cell and Well Features available for the Multiparameter Cytotoxicity BioApplication*

Example Biology

Freshly trypsinized HepG2 cells were plated into collagen-coated microplates and incubated overnight. Following incubation, the cells were exposed to various doses of valinomycin for 24 hours. Prior to fixation, cells were stained with the Multiparameter Cytotoxicity I Cocktail found in the Cellomics Multiparameter Cytotoxicity I HCS Reagent Kit. The Multiparameter Cytotoxicity I Reagent Kit combines fluorescent dyes, buffers, and protocols for sample preparation and measurement by the Multiparameter Cytotoxicity BioApplication. The cells were then fixed using formaldehyde, washed with buffer, and images were collected on a Cellomics HCS Reader and analyzed with the Multiparameter Cytotoxicity BioApplication.

Figure 5 shows as concentration of valinomycin increases, dose-dependent morphological events also occur. Percent of cells with a defined nuclear fragmentation (blue) and lysosomal mass (red) decrease, while cell permeabililty (green) increases, indicating that cytotoxic events have occurred. EC_{50} values for each event could then be calculated from the graphs.

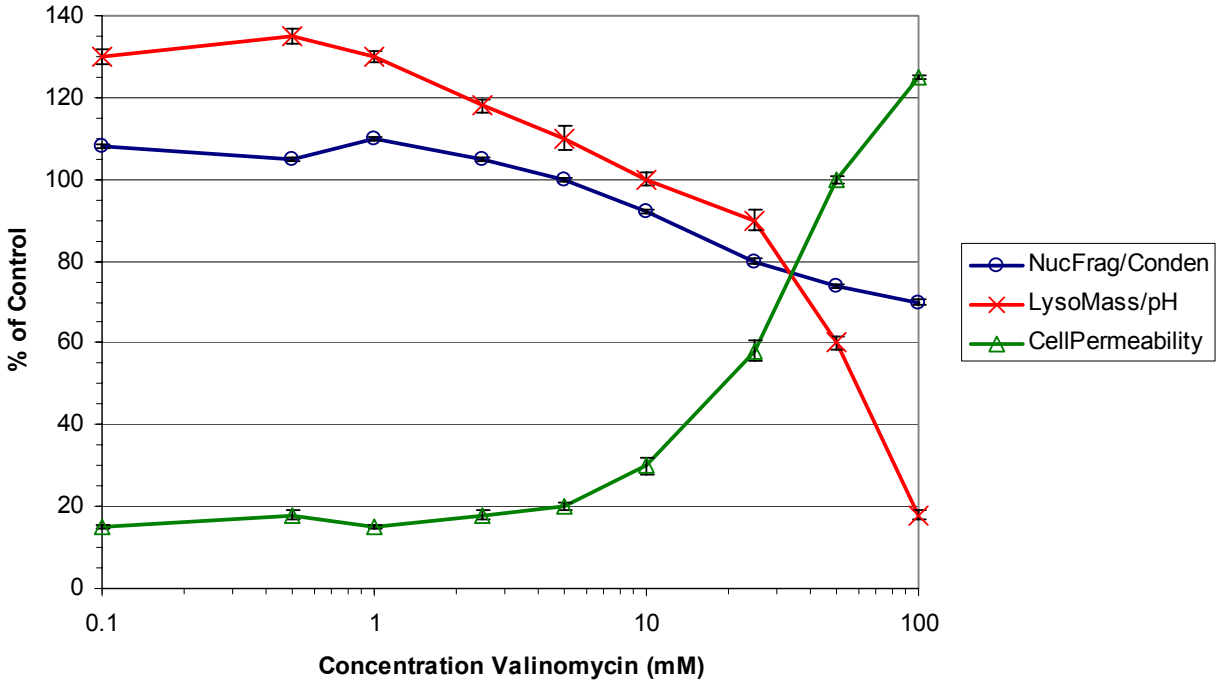

Figure 5. *HepG2 cells were stimulated with Valinomycin for 24 hr, labeled and fixed as described in the Cellomics Multiparameter Cytotoxicty I HCS Reagent Kit. Graph shows the associated dose response curves, where EC_{50} values of about 16 µM (for Nuclear Fragmentation/Condensation), 25 µM (for Cell Permeability), and 43 µM (for Lysosomal Mass/pH) were determined. Response of control (untreated cells) is set as 100%.*

Figure 6 represents a the highest concentration of valinomycin (100 µM) tested over time (in hours), evaluating the same cytotoxic features. Looking all three results at the same time helps in evaluating the overall sequence of events.

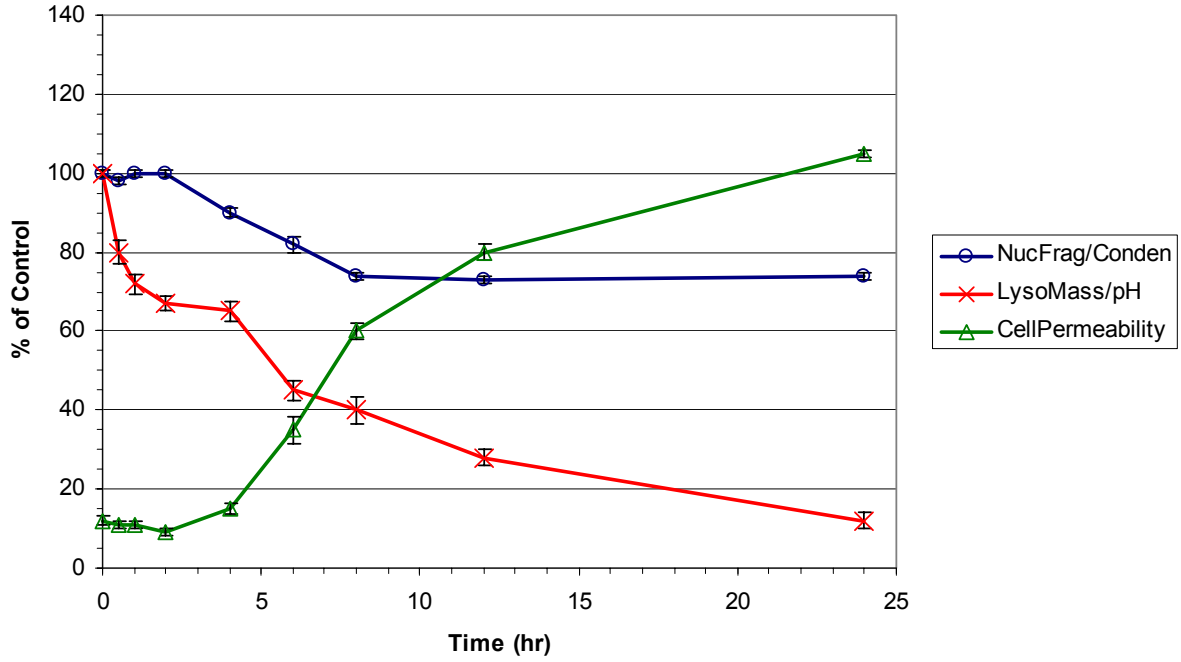

Figure 6. *Time course curves of 100 µM valinomycin from 0-24 hours in HepG2 cells for the three different features (mentioned in Figure 5). Response of control (untreated cells) is set as 100%.*

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Chapter

2

Description of the Algorithm

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 3 of this guide.

Chapter 1 provided a general overview of the Multiparameter Cytotoxicity BioApplication. This chapter briefly describes how the Multiparameter Cytotoxicity BioApplication works and describes its features and adjustable parameters.

Overview

The algorithm is designed to measure changes in three cell parameters related to cytotoxicity: (1) nuclear size/morphology, (2) cell membrane permeability, and (3) lysosomal mass. A minimum of 3 different stains (3 channels or targets) is needed for making the measurements in the aforementioned targets of a cell. In Channel 1 the objects (nuclei) are identified as objects using the fluorescent stain, Hoechst 33342. In Channel 2, a cell permeability stain is used. In Channel 3, a stain for lysosomes is used. The objects (nuclei) identified in Channel 1 are used to create a region of interest called "Circ**"** in Channel 2 and "Ring" in Channel 3.

The BioApplication determines the status of each cell as to whether it is within, below or above limits (***Feature*Ch*N*LevelHigh** or ***Feature*Ch*N*LevelLow**) set for nuclear morphology, cell permeability and lysosomal mass. These limits can be set either manually or automatically by the BioApplication, based on user defined Reference Wells (see later in this chapter). The BioApplication reports percentage of cells that are outside of these limits for each of the three targets listed above. Additionally, the BioApplication also reports the percentage change in the cell density per field in each well, as compared to control wells, with the initial cell density specified manually or measured from untreated Reference Wells.

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

There are two types of input parameters: Object Selection Parameters and Assay Parameters. Object Selection Parameters control which objects are chosen for processing and are specific for each channel. Assay Parameters control the quantitative analysis of the images.

Object Identification Parameters

To identify objects in each of the images from the different channels, an independent intensity threshold must be set for each channel. In Channel 1, nuclei are identified as objects for further measurements; 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 identification and thus configuring the application. In Channel 2, the BioApplication works under a set value and cannot be modified. Channel 3 is used for identification of objects such as lysosomes. Depending on the properties of the objects being identified in Channels 1 and 3, the proper setting of intensity thresholds for the channels is necessary to ensure proper quantitative analysis.

There are three different options or methods for determining intensity thresholds, and sub-sets of these are available for each channel. For each Channel, you must select both a method and a value. The different options and values available for each channel are summarized in the Table 2, and the descriptions of the different methods follow in Table 3.

Intensity Threshold Method	Value Range	Channel Availability			
		Channel 1	Channel 2	Channel 3	Channels 4 -6
None	0		✓		✓
Isodata	-0.99 – 9.99	✓		✓	
Fixed	0 - 32767	✓		✓	

Table 2. *Intensity Threshold Methods Available for Each Channel in the Multiparameter Cytotoxicity BioApplication.*

NOTE

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

When "**None**" is selected as the Intensity Threshold Method (Channels 2, 4-6), the value must be **0**.

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

Of the three intensity threshold method options, **None** means that no intensity threshold is applied. This option is not available in Channel 1 or Channel 3 because in these channels an intensity threshold is required to define the pixels making up the selected objects. The choice of the **None** threshold is the only option in Channels 2, 4, 5, and 6.

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

Isodata Threshold is known as a histogram-derived threshold in that the threshold is chosen from the histogram of pixel intensities in the image (i.e., the image's brightness histogram). Figure 7 demonstrates how this histogram-derived threshold values is calculated.

The value entered for the Object Identification in the application for the histogram-derived threshold method is an offset applied to determine the final threshold which is applied to the image. If the histogram-derived threshold is T, then its relationship to the actual (final) threshold, T_F, that is finally applied to the image is determined from the user-entered offset value, o, as:

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

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

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

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

Table 3. *Intensity Threshold Descriptions and ranges available for the Multiparameter Cytotoxicity BioApplication*

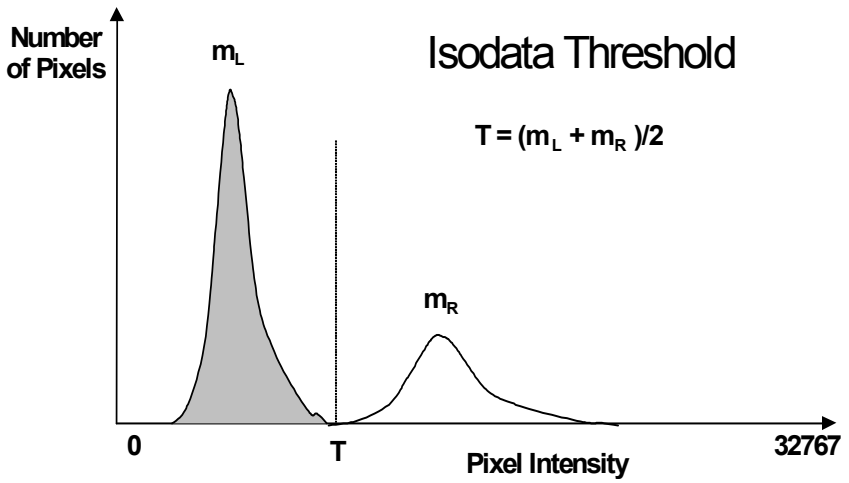

Figure 7. *Histogram-derived Isodata intensity threshold method. Background peak is shown in gray and object peak is shown in white.*

Description of Assay Parameters and Settings

The algorithm has Assay Parameters that control the analysis of images and data which can be adjusted 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**
- **Object Smoothing**
- **Object Segmentation**
- **Rejecting Border Objects**

Reference Wells Control

The two general Assay Parameters controlling the use of Reference Wells are: **UseReferenceWells** and **MinRefAvgCellCountPerField**. The **UseReferenceWells** Assay Parameter allows the user to choose whether reference wells are to be used to determine the population characterization limits. If **UseReferenceWells** = **1**, then the Assay Parameter **MinRefAvgCellCountPerField** must be set. This is the minimum number of objects detected per field required for acceptance of data from that field for calculation of Reference Wells. One can enter a number that closely matches the average number of selected objects needed to obtain statistically valid results for the different output features. There are additional Assay Parameters for Reference Well processing specific for particular features and channels which will be described in later sections.

Units for Morphological Measurements

The user has the option of either choosing Micrometers or Pixels as the unit to report morphological measurements. This is done by the **UseMicrometers** Assay Parameter. When set to **0**, the area of the objects are reported in pixels. Otherwise, if set to **1**, they are reported in micrometers. This information is calculated automatically from the chosen magnification and camera resolution settings. This Assay Parameter does not affect other Assay Parameters such as **CircModifier** or **RingDistance** or **RingWidth**, which are always set in pixels.

ObjectType Assay Parameter

NucTypeCh1, SpotTypeCh3

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** causes dark areas within an image to be considered as potential objects, while leaving it as **0** considers bright areas on a dark background as potential objects (Table 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 NucTypeCh1 and SpotTypeCh3*

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 object 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

BackgroundCorrectionCh*N*

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

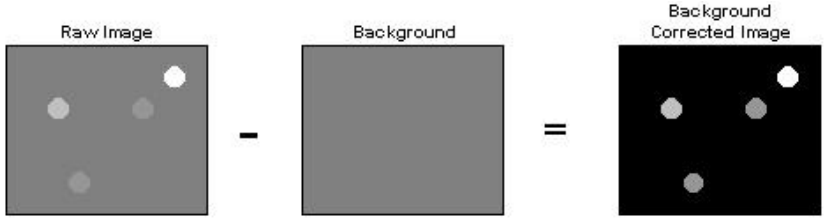

Figure 8. *Image depicting calculation of Background Correction that can be used for each channel.*

The background-corrected image is computed by suppressing high frequency components in the image (low pass filtration). You can control the creation of the background image by adjusting the **BackgroundCorrectionCh*N*** Assay Parameter. 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 larger than the radii of the objects in the image. If the value is set to **0** (zero), background correction is not performed, and analysis is done using the raw, uncorrected images.

If the **BackgroundCorrectionCh*N*** Assay Parameter is given a negative value, an optional background correction method based on the 3D surface fitting is applied. The absolute value entered corresponds to the radius of an area used to find local minima across the image. These minimum values are used to construct a 3D surface of a background which is then subtracted from the original image. The main advantage of this 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 the value to **–1** lets the application decide on the value needed for the optimal background correction. Table 5 gives an overview of the different Background Correction settings that can be used for each channel.

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

Table 5. *Possible Background Correction Methods used in each channel with the Multiparameter Cytotoxicity BioApplication.*

NOTE

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

Background Correction can be adjusted on each channel separately.

The background-corrected image is not stored or shown.

Object Smoothing

NucSmoothFactorCh1

The Channel 1 Assay Parameter that enables control over the degree of image smoothing, or blurring, before the identification of objects is known as **NucSmoothFactorCh1**. This Assay Parameter corresponds to the radius in pixels of the area used to smooth the image. A small value, such as **3**, means that a region with a radius of 3 pixels is used to smooth the image (region with dimensions 7×7 pixels, or 49 pixels total). Doubling the value to **6**, means that a larger region (13×13 pixels or 169 pixels total) is used to smooth the image, and thus the image will be more smoothed. A value of **0** means that smoothing is not done. This Assay Parameter is used to smooth Channel 1 images with a lot of contrast to improve identification of objects. To get sharper definition of the shapes of the edges of objects (i.e., cells), you may want to keep the **NucSmoothFactorCh1** small, if not 0. However, if your Channel 1 label is not very homogeneous, the actual object will be erroneously identified as consisting of several smaller sized objects, and then smoothing will result in a homogenizing effect of the Channel 1 label and will help identify the object with its true bounds.

Smoothing reduces the sharpness of intensity variations by redistributing their relative brightness over the immediate vicinity in the image, connecting fragments of nuclei which can be valuable when measuring morphological changes. Figure 9 shows the effect of smoothing on accurate identification of objects in Channel 1 (white overlay). Note that insufficient smoothing can result in unwanted object fragmentation (arrows in Panel B) while excessive smoothing can result in poor definition of the object perimeter (D).

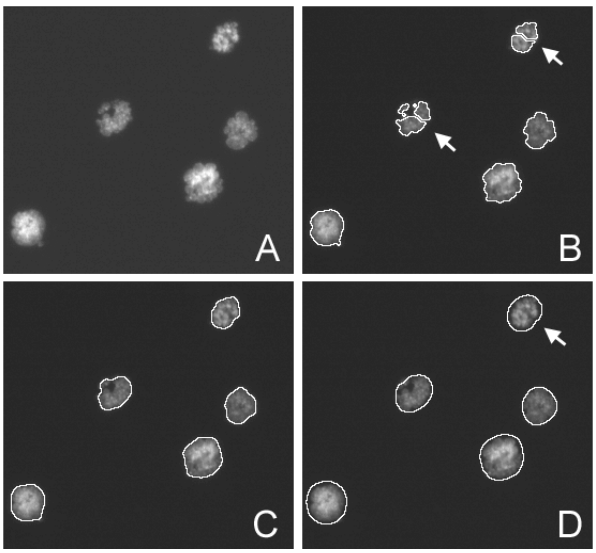

Figure 9. NucSmoothFactorCh1: *Images show the effect of different degrees of smoothing on the same field of apoptotic cell nuclei.* ***A:*** *Raw Image;* ***B:*** *NucSmoothFactorCh1 = 0;* ***C:*** *NucSmoothFactorCh1 = 3;* ***D:*** *NucSmoothFactorCh1 = 8.*

Object Segmentation

NucSegmentationCh1

Even though you may have chosen an optimal intensity threshold and an appropriate degree of smoothing for nucleus identification, you may encounter situations where individual nuclei 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 Multiparameter Cytotoxicity BioApplication: Geometric (shape and size) and Intensity (intensity peaks). These methods are illustrated in Figure 10.

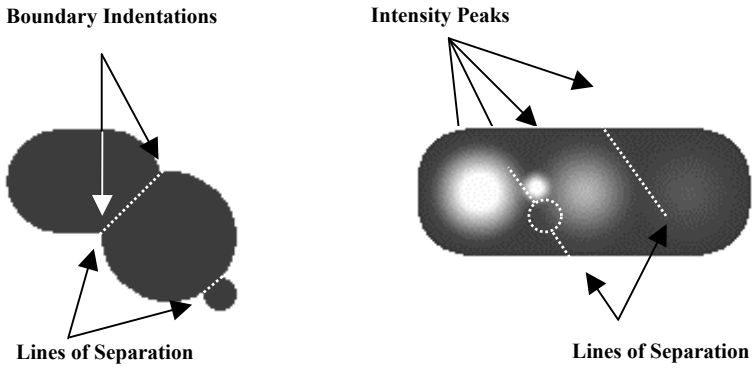

Figure 10: *Object Segmentation Options. The image on the left illustrates the Geometric Method while the image on the right illustrates the Intensity Method.*

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 **NucSegmentationCh1** Assay Parameter (see Table 6) controls both 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 **NucSegmentationCh1** 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 **NucSegmentationCh1** 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 **NucSegmentationCh1** disables the segmentation.

Segmentation Method	NucSegmentationCh1 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 6: *Channel 1 Object Segmentation Options*

The following section describes the functioning of the two object segmentation methods.

Geometric Method: Setting the **NucSegmentationCh1** value to R1 (Figure 11A) produces objects separation shown in Figure 11B. Setting the **NucSegmentationCh1** value to R3 results in segmentation shown in Figure 11C. Setting the **NucSegmentationCh1** value to R2 (the largest radius of all touching objects) produces no segmentation.

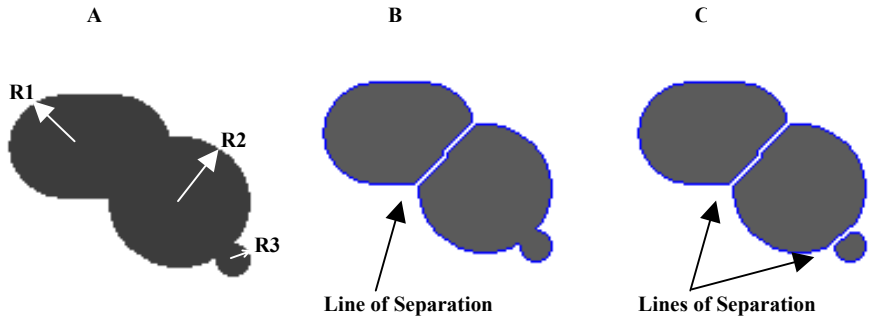

Figure 11: *Object segmentation: Geometric Method. A) Radii of touching objects: R1 = 24, R2 = 30, R3 = 7; B) Result of segmentation using* ***NucSegmentationCh1*** *= R1; C) Result of segmentation using* ***NucSegmentationCh1*** *= R3.*

Intensity Method Figure 12 shows the intensity profile along the cordial line of an object with four intensity peaks from Figure 10. 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 **NucSegmentationCh1** 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 vise versa. In case of over-segmentation, setting the **NucSmoothFactorCh1** greater than 0 should be used to alleviate the problem.

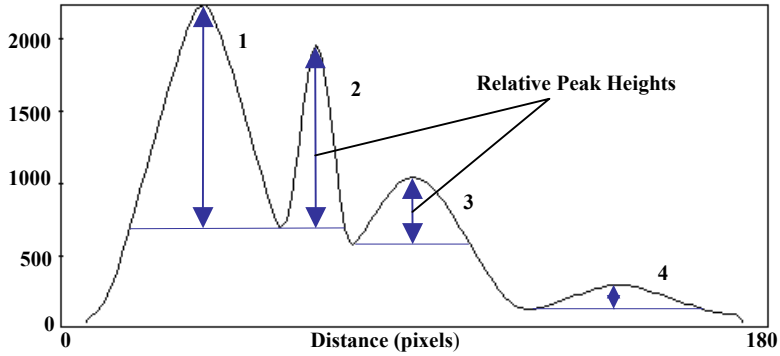

Figure 12: *Intensity profile of an object with four intensity peaks. Peak #1 height = 1500, peak #2 height = 1250, peak #3 height = 500, and peak #4 height = 200.*

Only two peaks in Figure 12, #1 and #2, have relative intensity greater than 1000. Thus, setting the **NucSegmentationCh1** parameter to -1000 marks 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 13A. Setting the **NucSegmentationCh1** 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 13B and 13C.

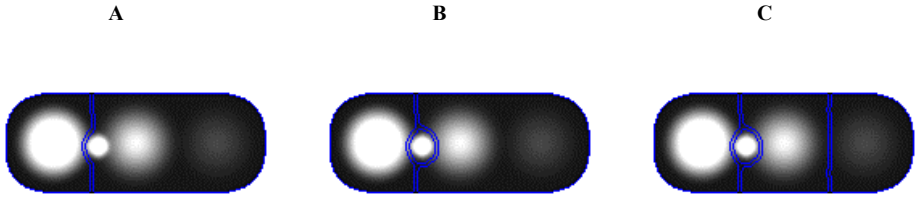

Figure 13: *Object segmentation: Intensity Method. A) Result of segmentation using parameter* ***NucSegmentationCh1*** *= -1000; B) Result of segmentation using parameter* ***NucSegmentationCh1*** *= -400; C) Result of segmentation using parameter* ***NucSegmentationCh1*** *= -100,*

Reject Border Objects

RejectBorderNucsCh1

When running the Multiparameter Cytotoxicity BioApplication, you have the option of not including and analyzing objects that touch the border of the imaged field. This is controlled by the **RejectBorderNucsCh1** Assay Parameter. If this parameter has a value of **1**, objects touching the border are not analyzed. 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

Adjusting Size of Circ and Ring Masks

The sizes of the Circ (sampled nuclear region in Channel 2) and Ring (sampled cytoplasmic region in Channel 3) regions can be adjusted in the Multiparameter Cytotoxicity BioApplication, and these sizes are set in relation to the primary object. Figure 14 shows a schematic of these three Assay Parameters that control the size of the Circ and Ring masks.

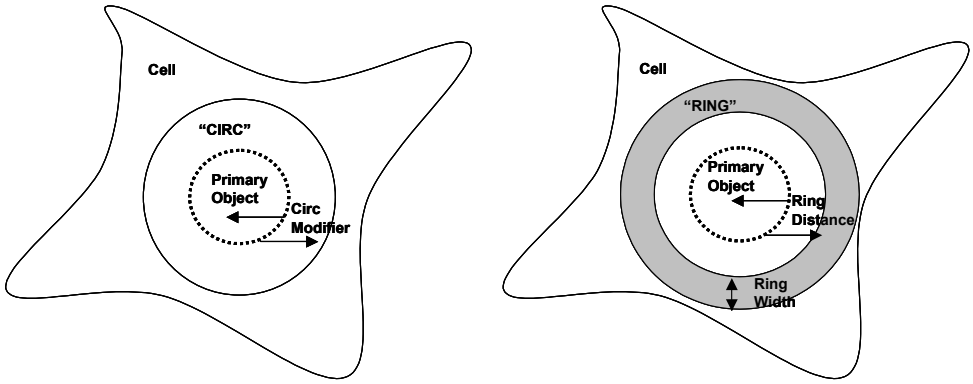

Figure 14. *The sizes of the Circ and Ring mask can be varied in the Multiparameter Cytotoxicity BioApplication, by modifying the appropriate Assay Parameters. The Primary Object in the above diagram refers to the nucleus identified by Hoechst 33342 staining in Channel 1.*

The **CircModifierCh2** Assay Parameter is used to adjust the sampled nuclear region for the cell membrane permeability measurement (Circ) in Channel 2. The value equals the number of pixels that the primary object mask is dilated (positive value) or eroded (negative value) to define the Circ region. This is the number of pixels added (or subtracted) from the perimeter of the primary object (nucleus) to define the area covered by the Circ region.

Two Assay Parameters are used to define the sampled cytoplasmic area covered by the Ring region in Channel 3: **RingDistanceCh3** and **RingWidthCh3**. The **RingDistanceCh3** Assay Parameter is the number of pixels added from the perimeter of the primary object (nucleus) to define the inner boundary of the Ring region. The **RingDistanceCh3** is the number of pixels the inner boundary of the Ring region is away from the boundary of the primary object mask. The location of the Ring depends on the sign of the Assay Parameter: for negative values the inner boundary of the Ring is within primary object mask and closer to the center, for positive values the inner boundary of the Ring is outside of the primary object mask and farther from the center. The **RingWidthCh3** Assay Parameter defines the width of the Ring region in pixels. Both **RingDistanceCh3** and **RingWidthCh3** determine the total size of the cytoplasmic region that is sampled for measuring the lysosomal mass in Channel 3. Note that since the Ring region is derived from the *primary object*, the area it covers is defined independently from the area covered by the Circ region.

In addition, the **MaskModifierCh*N*** Assay Parameter works similar to that of the **CircModifierCh2** Assay Parameter in that it uses the Channel 1 object (nucleus) mask to modify the mask in the selected channel. However, adjustment of this mask is used for gating purposes only and will be described in more detail later in this chapter.

Basic Assay Parameters

Input parameters can be found in the Create Protocol View. The available input parameters will vary depending on the mode in which you are using: Basic Mode or Advanced Mode. Assay Parameters available in Basic Mode control the morphological identification of the objects in each channel, and control the use of Reference Wells (Table 7). You will not be able to view any Advanced Assay Parameters in this mode (as the **Hide Advanced Parameters** option is checked).

Parameter	Units	Description
UseReferenceWells	Binary	Use reference wells to calculate high and low response levels: 0 = No, 1 = Yes
NucTypeCh1	Binary	Type of nuclei 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
NucSmoothFactorCh1	Number	Degree of image smoothing (blurring) prior to nucleus identification in Ch1: 0 = Do not apply smoothing
NucSegmentationCh1	Pixels	Radius (in pixels) of touching nuclei that should be separated in Ch1: Negative value = Use intensity peaks method 0 = Do not separate touching objects Positive value = Use shape geometry method
RejectBorderNucsCh1	Binary	Reject Ch1 nuclei that touch image edges: 0 = No, 1 = Yes
CircModifierCh2	Pixels	Number of pixels to modify Ch1 object (nucleus) mask to create circ mask in Ch2: Negative value = Shrink mask 0 = Do not modify mask Positive value = Expand mask
MaskModifierChN	Pixels	Number of pixels to modify Ch1 object (nucleus) mask in ChN: Negative value = Shrink mask 0 = Do not modify mask Positive value = Expand mask
SpotTypeCh3	Binary	Type of spots to be identified in Ch3: 0 = Bright objects on dark background 1 = Dark objects on bright background
RingDistanceCh3	Pixels	Distance (in pixels) from Ch1 object (nucleus) mask to the inner rim of ring mask in Ch3
RingWidthCh3	Pixels	Width (in pixels) of ring mask in Ch3

Table 7. *Basic Assay Parameters available for the Multiparameter Cytotoxicity BioApplication. * Note that "ChN" refers to Channels 1-6 for Background Correction and Channels 2-6 for Mask Modifier.*

Object Selection Parameters

Each channel has an associated set of Object Selection Parameters. If an object has all measured features within the ranges specified by the Object Selection Parameters, it is selected for analysis. Rejected objects are removed from further analysis. Channel 1 Object Selection Parameters are used to identify valid nuclei. Channels 2-6 Object Selection Parameters are used for gating of objects. Object Selection Parameters for all channels are adjustable in Basic and Advanced modes. Table 8 lists all available Object Selection Parameters for this BioApplication.

Channel 1

Parameter	Units	Description
NucAreaCh1	Pixels or μm^2	Area (in pixels or micrometers) of Ch1 nucleus.
NucShapeP2ACh1	Number	Shape measure based on ration of perimeter squared to 4π*area of Ch1 nucleus (NucShapeP2ACh1=1 for circular nucleus).
NucShapeLWRCh1	Number	Shape measure based on ratio of length to width for object-aligned bounding box of Ch1 nucleus.
NucTotalIntenCh1	Intensity	Total intensity of all pixels within Ch1 nucleus.
NucAvgIntenCh1	Intensity	Average intensity of all pixels within Ch1 nucleus.
NucVarIntenCh1	Intensity	Variation (standard deviation) of intensity of all pixels within Ch1 nucleus.

Channel N (Channels 2-6)

Parameter	Units	Description
TotalIntenChN	Intensity	Total intensity in ChN of all pixels within modified Ch1 nucleus mask.
AvgIntenChN	Intensity	Average intensity in ChN of all pixels within modified Ch1 nucleus mask.

Table 8. *Object Selection Parameters available for the Multiparameter Cytotoxicity BioApplication for Channel 1 (top) and Channels N (bottom). Note that "ChN" refers to Channels 2-6.*

Gating

The Multiparameter Cytotoxicity BioApplication supports gating on a cell population. This feature provides selective cell processing based on fluorescence intensity. Therefore, in addition to selecting objects for analysis in Channel 1, you can also select or reject objects based on fluorescence intensity in Channels 2-6. Gating allows you to specifically identify a subset based on fluorescence intensity and allows you to limit the analysis to this group of cells. For example, gating may be used to analyze only those cells showing a certain level of expression of a fluorescent reporter such as green fluorescent protein (GFP).

Specifying Intensity Ranges for Gating

When working in Create Protocol View, you can specify intensity ranges by entering upper and lower limits for two intensity Object Selection Parameters, **AvgIntenCh*N*** and **TotalIntenCh*N***, for Channels 2-6. **TotalIntenCh*N*** is a summation of all intensities within the cell/object. **AvgIntenCh*N*** is **TotalIntenCh*N*** divided by the object area. These parameters can also be adjusted interactively in Protocol Interactive View.

Specifying Mask Modifiers for Gating

In addition to specifying intensity ranges for one or more channels, you must also apply a mask to one or more downstream channels using the **MaskModifierCh*N*** Assay Parameter. As described earlier, you may want to adjust this Assay Parameter if you want to dilate or contract the mask that identifies each cell; this setting only impacts the region used to determine whether the cell should be gated in or out, as measured by **TotalIntenCh*N*** or **AvgIntenCh*N***. The mask can be dilated or eroded, but will not overlap with other masks from adjacent objects.

Image Overlays

During a scan, various features can be displayed as color overlays on the channel images. The colors of these overlays can be changed by choosing the color block beside the overlay name in the Create Protocol or Protocol Interactive View (Table 9). All Image Display Option Settings are available when running in Basic Mode.

Parameter	Description
Include This Channel In Composite	Determines if the image for this channel is included in the composite image.
Composite Color	Determines what color will be assigned to this channel in the composite image.
Selected Cell	Indicates the color of a ring placed on each nucleus identifying accepted objects.
Rejected Cell	Indicates the color of a ring placed on each nucleus identifying rejected objects.
CircCh2	Indicates the Circ region in Channel 2 of the displayed image.
SpotCh3	Indicates the Ring region in Channel 3 of the displayed image.

Table 9. *Image Display Option Settings available when running the Multiparameter Cytotoxicity BioApplication*

Assay Parameters for Population Characterization

Overview of Population Characterization

The Multiparameter Cytotoxicity BioApplication provides the ability to characterize individual cells based on their response compared to a control population. For a particular output feature, a range is determined from a control population (e.g. cells in untreated wells) that has the normal physiological distribution for that feature. The ***Feature*Ch*N*LevelHigh** and ***Feature*Ch*N*LevelLow** Assay Parameters set the upper and lower bounds of this range respectively. The "**Status**" Cell-level feature indicates whether a particular compound treated cell is within or beyond this range (i.e., for a particular Cell-level feature, a cell with Status **0** means that it is within the defined range, a Status value of **1** means that the feature value is greater than the upper limit, and a Status value of **2** means that the feature value is less than the lower limit; Table 10).

Value	Cell Status Definition
0	Cell Feature is less than or equal to defined value
1	Cell Feature is greater than defined value
2	Cell Feature is less than defined value

Table 10. *Numerical Status values for specified Cell Features*

The corresponding Well-level features are the percent of cells that are either greater or lesser than the limits defining this range. Figure 15 illustrates this concept by showing the distribution of a normal physiological population of cells for a particular Cell-level feature, and the shift in this distribution upon compound treatment (please note that the shift in feature distribution can occur to either above or below the normal physiological distribution).

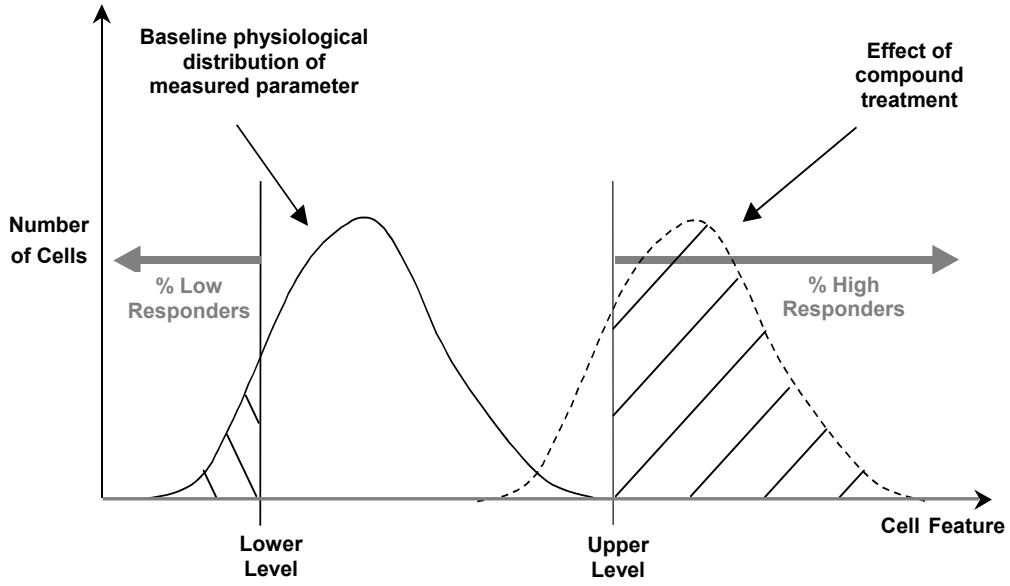

Figure 15. *Schematic of population distribution of a measured feature in a population of untreated and compound treated cells. The upper and lower levels correspond to %Low or %High (or %Responders if combined).*

Setting Cellular Response Levels

The Multiparameter Cytotoxicity BioApplication offers two ways of setting high and low-response level Assay Parameters. The first is manually entering values for the ***Feature*Ch*N*LevelHigh, *Feature*Ch*N*LevelLow** Assay Parameters in the Protocol Create or Protocol Interactive Views (Advanced Mode). This requires prior knowledge of typical feature values. The BioApplication then calculates the percentage of cells above and below the specified ***Feature*Ch*N*LevelHigh, *Feature*Ch*N*LevelLow** values, respectively.

To automatically determine the ***Feature*Ch*N*LevelHigh**, ***Feature*Ch*N*LevelLow** Assay Parameter values, the BioApplication uses Reference Wells. You designate particular wells on the sample plate as Reference Wells. Reference Wells typically contain an untreated cell population that displays the normal physiological distribution for the feature being measured.

These wells are first analyzed and the population distribution for each Cell Feature that can be used for population characterization are determined. The cell population characterization levels are then set by adding or subtracting from the mean of the distribution its standard deviation multiplied by a user defined coefficient (Correction Coefficient (**_CC**)) found as an Advanced Assay Parameter. The software 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, assuming a normal distribution. The advantage of using Reference Wells to automatically calculate levels is that the levels are determined by a control population of cells and are independent of run-to-run variations when doing the experiment on different days such as different illumination conditions, extent of fluorescent labeling, etc.

Reference Wells Processing Sequence

By setting the **UseReferenceWells** Assay Parameter to **1**, the Reference Wells processing is engaged. Specified fields within the Reference Wells are acquired/analyzed, and Well, and Plate Features are computed. Plate Feature values are directly used for cell population characterization, following which regular scanning of the plate begins. Again, if the feature value for **MinRefAvgCellCountPerField** obtained from the Reference Wells is below the value set for that parameter, the BioApplication aborts the use of Reference Wells and processes the plate as if **UseReferenceWells** is set to **0**. The Multiparameter Cytotoxicity 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 mean and standard deviation are computed over all cells in the field.
3. The automatically calculated level values for each feature are reported as Reference Features having the names **Ref*Feature*Ch*N*LevelHigh, Ref*Feature*Ch*N*LevelLow**.
4. Finally, Reference Well and Plate Features are computed as average values for fields in a well, weighted for the number of cells per field, and then as arithmetic average for wells on a plate. Use of a weighted average minimizes the effect of sparse fields.

Identifying Reference Wells and Control Parameters

Reference Wells are specified in the Reference Wells Configuration Window (choose **Change Reference Wells** from the **Tools** menu). Select the wells to be set as Reference Wells and then select the button for the appropriate type of Reference Well (**Known**). Next, enter the Starting Field and Number of Fields. Click the **Apply** button to save the settings. Please see the appropriate 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 Scan software application.

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

Specifying and Controlling Reference Wells

Two general Assay Parameters controlling the use of Reference Wells are: **UseReferenceWells** and **MinRefAvgCellCountPerField**). **UseReferenceWells** is a binary Assay Parameter that allows you to indicate whether Reference Wells are used to determine the levels necessary to characterize neurite 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 Reference Wells are enabled, the Assay Parameter **MinRefAvgCellCountPerField** is used to specify the minimum average number of selected cells 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 used.

The Cell Features used to characterize various levels are associated with Assay Parameters in the form:

- ***Feature*Ch*N*LevelHigh**
- ***Feature*Ch*N*LevelLow** (when Reference Wells are not used)

  OR
- ***Feature*Ch*N*LevelHigh_CC**
- ***Feature*Ch*N*LevelLow_CC** (when Reference Wells are used)

where *Feature* refers to the name of the Cell Feature (such as **CircAvgIntenCh2**) and *N* refers to the specific channel. The ***Feature*Ch*N*LevelHigh, *Feature*Ch*N*LevelLow** Assay Parameter types specify the actual levels and must be manually entered if Reference Wells are not used. The ***Feature*Ch*N*LevelHigh_CC, *Feature*Ch*N*LevelLow_CC** Assay Parameters are correction coefficients (CC) used to derive the ***Feature*Ch*N*LevelHigh** and ***Feature*Ch*N*LevelLow** values from the mean and standard deviation of the reference well population according to the formula:

- ***Feature*Ch*N*LevelHigh** = Mean + ***Feature*Ch*N*LevelHigh_CC** $\times$ SD
- ***Feature*Ch*N*LevelLow** = Mean - ***Feature*Ch*N*LevelLow_CC** $\times$ SD

If the correction coefficient is positive, the level will be greater than the mean, and if it is negative, the level will be less than the mean. A _CC value of **0** generates a level that equals the mean. If you are using Reference Wells, it is important to set the values for the correction coefficient Assay Parameters so that the appropriate subpopulations can be identified. Individual cells having feature values above the set or calculated value are identified as responder cells. The number of responder cells, expressed as a percentage of the entire cell population analyzed, is calculated for each assay well.

Advanced Assay Parameters

In Advanced Mode, all basic and advanced input parameters are adjustable. The **Hide Advanced Parameters** option provides the ability to either view and adjust all the Assay Parameters or view and adjust the subset designated as Basic Parameters. Clear the **Hide Advanced Features** checkbox to display all Assay Parameters. Generally, the Advanced Assay Parameters are related to definition and reporting of responder cells. They control the analysis of the data resulting from the image analysis (Table 11).

For each feature undergoing population characterization, there are four advanced Assay Parameters that control its levels: ***Feature*Ch*N*LevelHigh** and ***Feature*Ch*N*LevelLow** that set upper and lower thresholds and the presence of the **_CC** suffix for each which designate that those levels are set using Reference Wells. For example, the Assay Parameters controlling the nuclear size in Channel 1 are:

- **NucSizeCh1LevelHigh**
- **NucSizeCh1LevelLow**
- **NucSizeCh1LevelHigh_CC**
- **NucSizeCh1LevelLow_CC**

In the listing of Advanced Parameters in Table 11., instead of listing all level Assay Parameters for each feature, one entry for the feature will be listed giving the four different options, as shown in the following example for the above four features:

- NucSizeCh1Level***Low/High, Low/High_CC***

Parameter	Units	Description
MinRefAvgCellCountPerField	Number	Minimum average number of cells per field required for acceptance of reference well results
InitialCellCountPerField	Number	User's estimate of the initial number of cells per field
UseMicrometers	Binary	Measure lengths and areas in: 0 = Pixels, 1 = Micrometers
PixelSize	µm	Pixel size in micrometers (depends on objective selection)
NucSizeCh1Level***Low/High, Low/High_CC***	Pixels or µm	Defines **NucSizeCh1** population characterization thresholds
CircAvgIntenCh2Level***Low/High, Low/High_CC***	Intensity	Defines **CircAvgIntenCh2** population characterization thresholds
SpotAvgIntenCh3Level***Low/High, Low/High_CC***	Intensity	Defines **SpotAvgIntenCh3** population characterization thresholds

Table 11. *Advanced Assay Parameters available for the Multiparameter Cytotoxicity BioApplication. Units will be expressed as what is found with* ***FeatureChNLevelLow/High****, knowing that _CC is expressed as a number.*

Description of Output Features

Output features are the biological measurements produced by BioApplication. All features are categorized and accessible using the View software application. Additionally, a subset of features, the Well Features, is 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. All features can also be accessed in the Protocol Interactive View once the algorithm has been applied to the images acquired in that window whether that is done manually on the instrument or running a disk based scan using images acquired earlier.

This section describes Cell Features, Well Features, and Plate Features that are available as output features from the Multiparameter Cytotoxicity BioApplication. This BioApplication has a specific set of output features allowing the characterization of cytotoxic compounds.

Cell Features

Table 12 shows the output features reported for each selected cell, accessible in the Cell Feature window in Protocol Interactive View in addition to the View software application.

Cell Feature	Units	Description
Cell#	Number	Unique cell ID
Top	Pixels	Y coordinate (in pixels) of top left corner of image-aligned bounding box of object
Left	Pixels	X coordinate (in pixels) of top left corner of image-aligned bounding box of object
Width	Pixels	Width (in pixels) of image-aligned bounding box of object
Height	Pixels	Height (in pixels) of image-aligned bounding box of object
XCentroid	Pixels	X coordinate (in pixels) of nucleus center
YCentroid	Pixels	Y coordinate (in pixels) of nucleus center
NucAreaCh1	Pixels or μm^2	Area (in pixels or micrometers) of Ch1 nucleus
NucSizeCh1	Pixels or µm	Area equivalent diameter (in pixels or micrometers) of Ch1 nucleus
NucSizeCh1Status	Number	**NucSizeCh1** status: 0 = No response, 1 = High response, 2 = Low response
NucShapeP2ACh1	Number	Shape measure based on ratio of perimeter squared to 4π*area of Ch1 nucleus (**NucShapeP2ACh1** = 1 for circular nucleus)
NucShapeLWRCh1	Number	Shape measure based on ratio of length to width of object-aligned bounding box of Ch1 nucleus
NucTotalIntenCh1	Intensity	Total intensity of all pixels within Ch1 nucleus
NucAvgIntenCh1	Intensity	Average intensity of all pixels within Ch1 nucleus
NucVarIntenCh1	Intensity	Standard deviation of intensity of all pixels within Ch1 nucleus
CircTotalIntenCh2	Intensity	Total intensity of all pixels within circ mask in Ch2
CircAvgIntenCh2	Intensity	Average intensity of all pixels within circ mask in Ch2

Cell Feature	Units	Description
CircAvgIntenCh2Status	Number	**CircAvgIntenCh2** status: 0 = No response, 1 = High response, 2 = Low response
TotalIntenChN	Intensity	Total intensity in ChN of all pixels within modified Ch1 object (nucleus) mask
AvgIntenChN	Intensity	Average intensity in ChN of all pixels within modified Ch1 object (nucleus) mask
SpotTotalIntenCh3	Intensity	Total intensity all spot pixels within ring mask in Ch3
SpotAvgIntenCh3	Intensity	Average intensity all spot pixels within ring mask in Ch3
SpotAvgIntenCh3Status	Number	**SpotAvgIntenCh3** status: 0 = No response, 1 = High response, 2 = Low response

Table 12. *Cell Features available for the Multiparameter Cytotoxicity BioApplication.*
****** Note that "ChN" refers to Channels 2-6.*

Well Features

Many Well Features are derived from the Cell Features. Such features are identified by a prefix, as listed below, to the corresponding **Status** Cell Feature name (Table 13). Three Cell Features will have %HIGH, %LOW, and %RESPONDERS: **NucSizeCh1**, **CircAvgIntenCh2**, and **SpotAvgIntenCh3**

Feature Prefix	Well Feature Definition	Units
MEAN_	The arithmetic mean (average) of the Cell Feature value for all selected cells in the well.	Same as Cell Feature
SD_	The standard deviation of the Cell Feature value for all selected cells in the well.	None
SE_	The standard error of the mean of the Cell Feature value for all selected cells in the well. Equal to the standard deviation divided by the square root of the number of selected cells.	None
CV_	The coefficient of variation of the Cell Feature value for all selected cells in the well. Equal to the standard deviation divided by the mean x 100.	Percent
%HIGH	The percentage of cells in the well whose feature value is **above** the value specified in the FeatureChNLevel Assay Parameters or as calculated by the Reference Wells.	Percent
%LOW	The percentage of cells in the well whose feature value is **below** the value specified in the FeatureChNLevel Assay Parameters or as calculated by the Reference Wells.	Percent
%RESPONDERS	The percentage of cells in the well whose feature value is **either above or below** the value specified in the FeatureChNLevel Assay Parameters or as calculated by the Reference Wells.	Percent

Table 13. *General Well Feature definitions*

The algorithm also reports the following Well Features in Scan Plate View in addition to the Well Detail window of the View software application (Table 14).

Well Feature	Description
ValidCellCount	Number of valid objects (cells) identified in the well (Ch1 object selection parameters applied)
SelectedCellCount	Number of valid cells selected for analysis in the well (Ch2-6 object selection parameters applied)
%SelectedCells	Percentage of valid cells selected for analysis in the well
ValidFieldCount	Number of fields in which cells were selected for analysis in the well
SelectedCellCountPerValidField	Average number of cells selected for analysis per valid field in the well
%ChangeSelectedCellCountPerValidField	Percentage decrease in cells selected for analysis per valid field during analysis of the well
CytotoxicityIndex	Maximum value of all well response indices

Table 14. *Well Features available for the Multiparameter Cytotoxicity BioApplication.*

Reference Features

The algorithm reports the following features in the Scan Plate View of the Scan software application as well as the Plate Detail window of the View software application (Table 15). These features are computed and reported only when **UseReferenceWells** = **1**.

Reference Feature	Description
RefAvgCellCountPerField	Average number of selected cells per field as measured from the Reference Wells.
RefNucSizeCh1Level***Low/High***	Low/High-response level for **NucSizeCh1**
RefCircAvgIntenCh2Level***Low/High***	Low/High-response level for **CircAvgIntenCh2**
RefSpotAvgIntenCh3Level***Low/High***	Low/High-response level for **SpotAvgIntenCh3**

Table 15. *Reference Features available for the Multiparameter Cytotoxicity BioApplication. * Instead of listing all features, corresponding* ***FeatureChNLevelLow*** *and* ***FeatureChNLevelHigh*** *were combined as* ***FeatureChNLevelLow/High****.*

Chapter

3

iDev Software Workflow

This chapter describes the tasks in the workflow for Protocol optimization of the Multiparameter Cytotoxicity V4 BioApplication using the iDev software. More detailed 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 operations such as background removal methods and values for each of the channels in the Protocol.

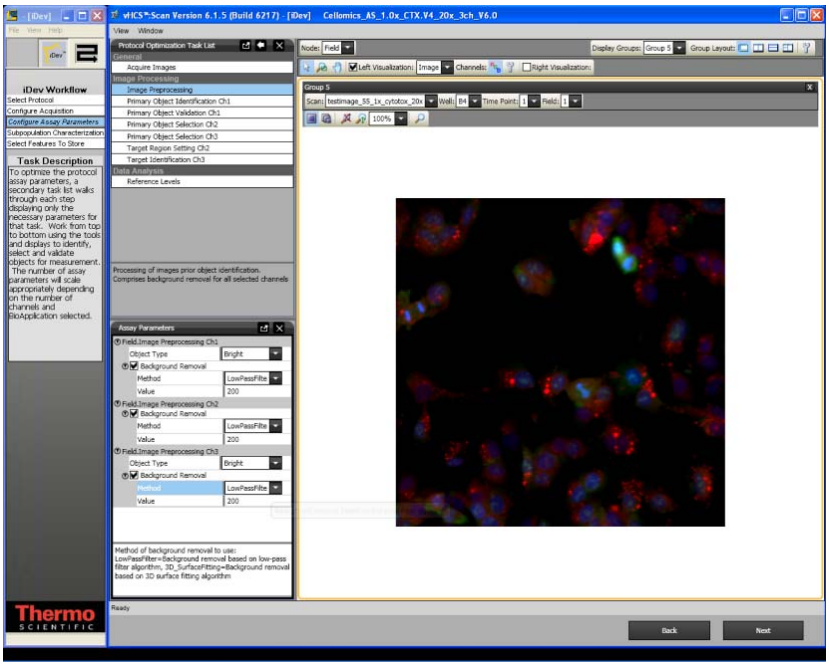

Figure 16. *Protocol Optimization Task – Image Preprocessing*

Primary Object Identification Ch1

Primary Object Identification is the identification of objects in the Channel 1 image. This task involves setting up methods and values for primary object identification, object smoothing, and object segmentation for Channel 1 objects. In addition, **InitialObjectCountperField** can be used to set the number of objects in a non-toxic well to properly calculate the Cytotoxicity Index.

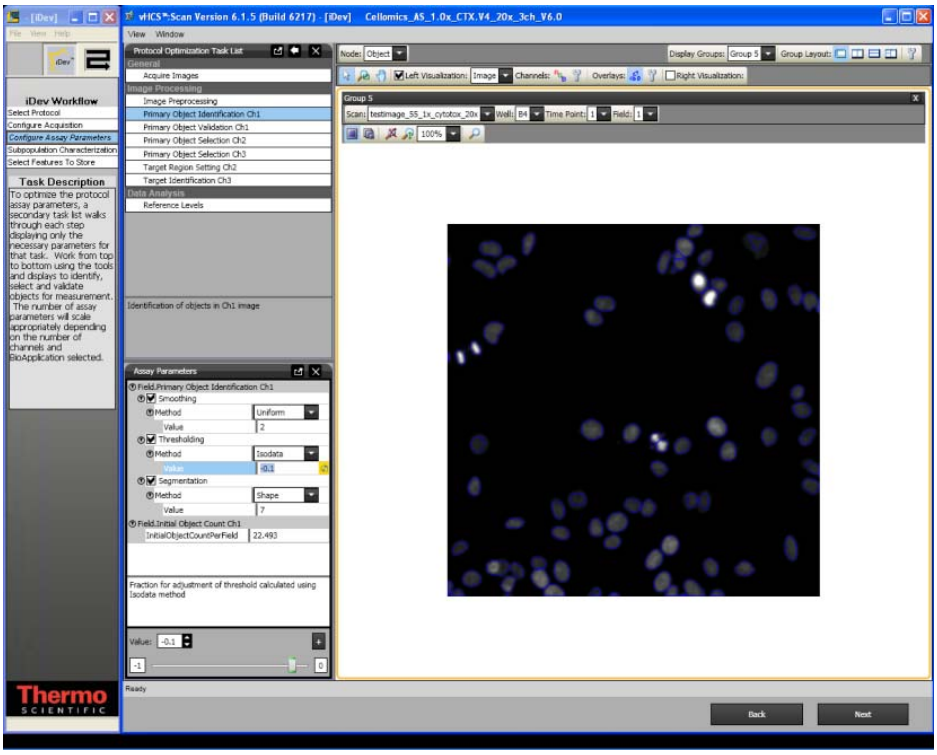

Figure 17. *Protocol Optimization Task – Primary Object Identification Ch1*

Primary Object Validation Ch1

Primary Object Validation is object selection 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 output features. Additionally in this task, you will determine if objects that are on the border of the field are included or rejected from analysis.

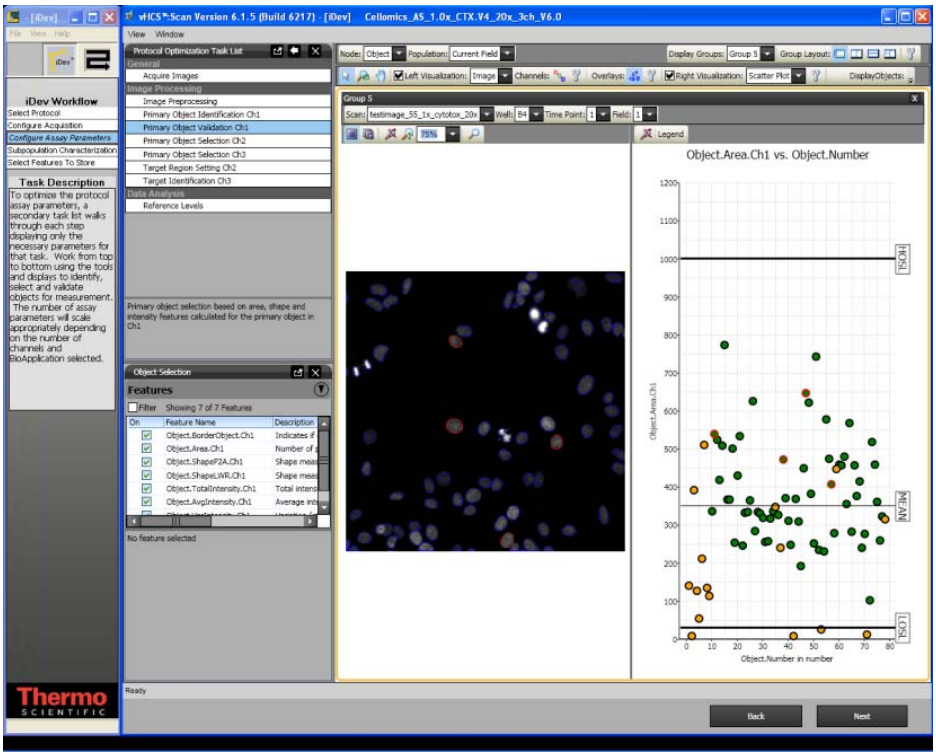

Figure 18. *Protocol Optimization Task – Primary Object Validation Ch1*

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 Channel 2 through Channel N under a modified primary object mask. This task is similar to setting the Assay Parameter, **MaskModifierCh2,** in the ArrayScan Classic software. The primary object mask can be dilated (**Circ** > 0), or eroded (**Circ** < 0) if the **ROI Mask Creation** box is checked.

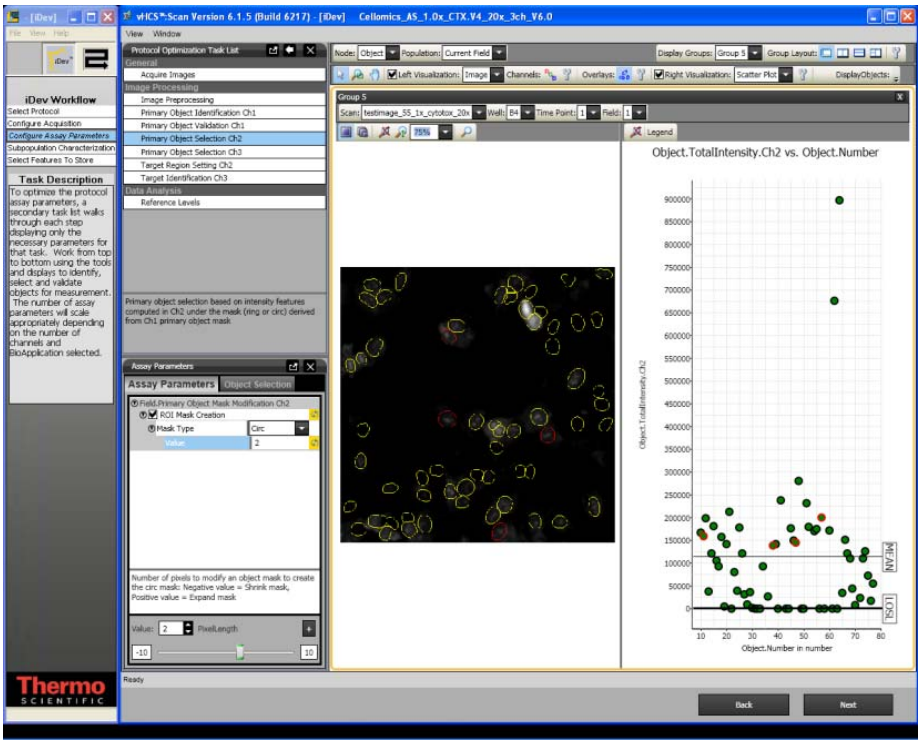

Figure 19. *Protocol Optimization Task – Primary Object Selection Ch2 through ChN*

Target Region Setting Ch2

In this task you can set the ROI (Circ mask) in Channel 2 derived from the primary object mask in Channel 1 by entering a value for ROI mask. The primary object mask can be dilated (**Circ** > 0), or eroded (**Circ** < 0) if the **ROI Mask Creation** box is checked.

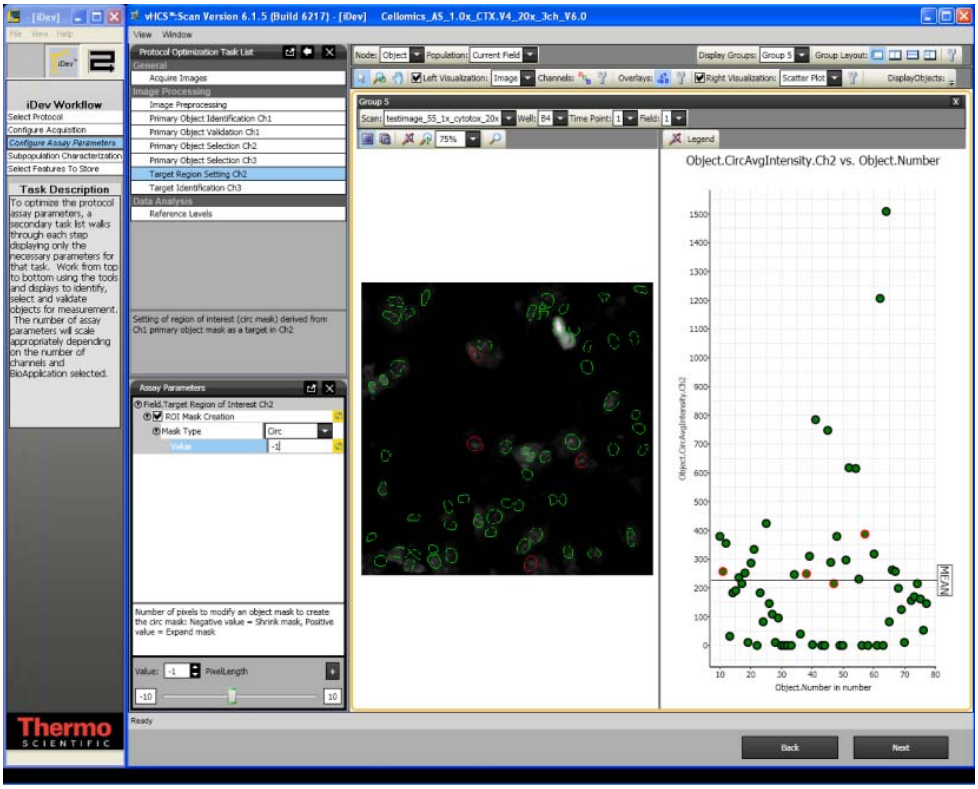

Figure 20. *Protocol Optimization Task – Target Region Setting*

Target Identification Ch3

In this task you can create a ROI (Ring mask) in which the target in Channel 3 is identified. You can also set the thresholding method and values for identifying the target in Channel 3.

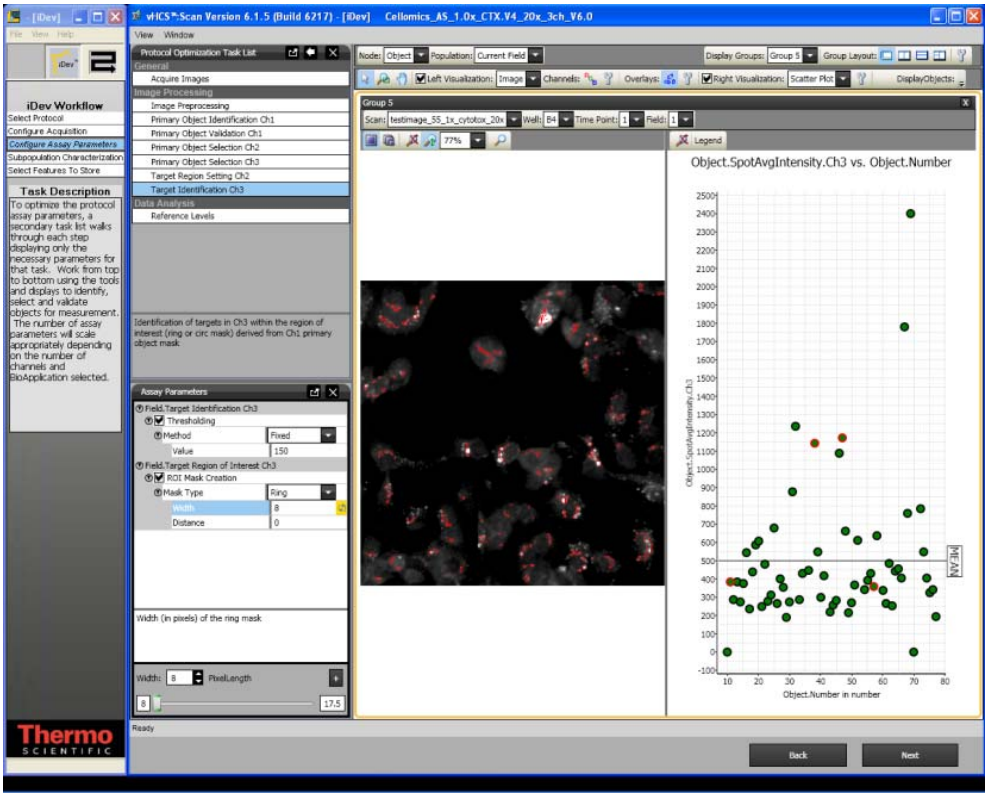

Figure 21. *Protocol Optimization Task – Target Identification Ch3*

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.

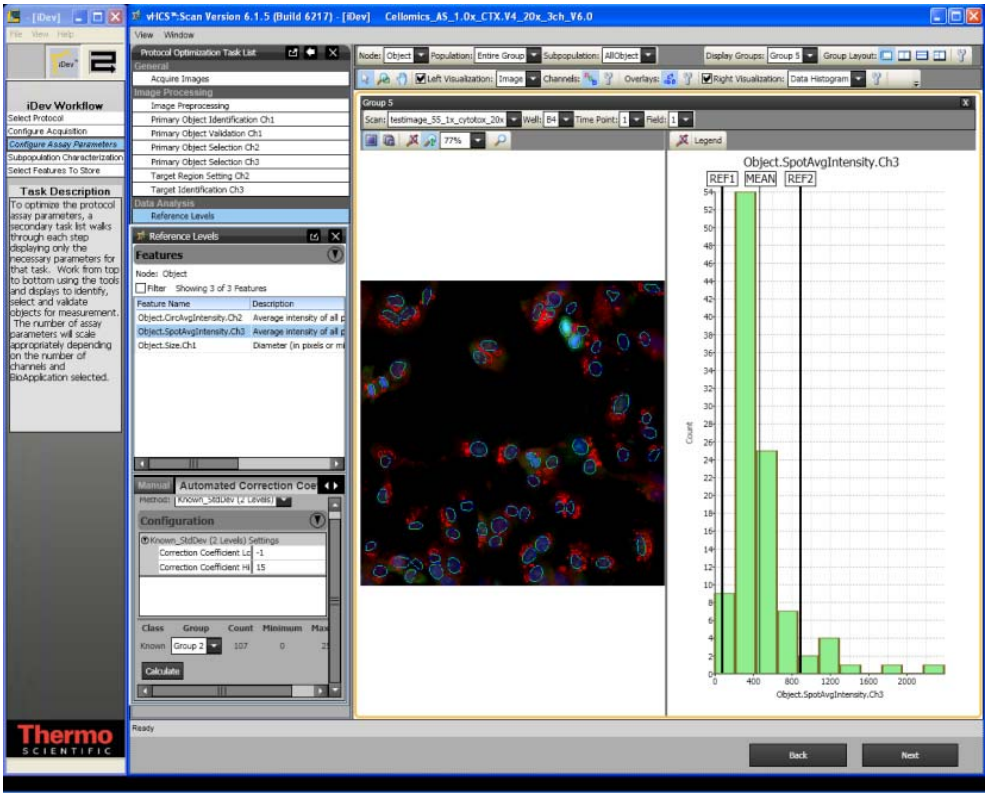

Figure 22. *Protocol Optimization Task – Reference Levels*

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