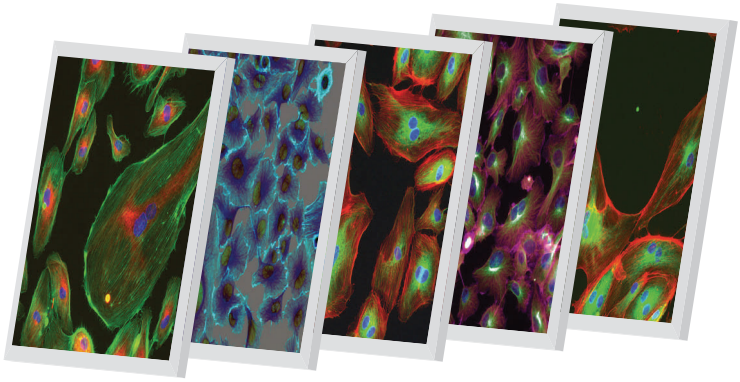
Thermo Scientific Cellomics® Cell Health Profiling V4

BioApplication Guide



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V4 Version

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Chapter

1

Overview of the Cell Health Profiling BioApplication

High Content Screening (HCS) uses fluorescence-based reagents, advanced optical imaging instrumentation and sophisticated image analysis software (BioApplications) to quantitatively analyze physiological processes in cells. This guide provides a brief description for using the highly versatile and flexible BioApplication, Cell Health Profiling, which is designed to enable simultaneous, automatic measurement of a wide range of cell health indicators. This guide consists of the following chapters:

Chapter 1 provides an overview of the Cell Health Profiling BioApplication.

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

Chapter 3 describes the use of the BioApplication with brief guidance on how to specifically adjust settings for different biological use cases and use of the Events Wizard.

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

System Compatibility

The Cell Health Profiling BioApplication described in this document is designed to run on the following platforms:

- ArrayScan® version V^{TI}
- 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

Measurement and characterization of cell health is critical for assessment of toxicity. For example, a given treatment may be considered harmful to cell health because it specifically induces apoptosis (programmed cell death) or because it induces necrosis. These processes and the points of difference between them have been extensively studied (Leist and Nicotera 1997; Zakeri 1998; Lemasters 1999). Additionally, it is often useful to determine the nature of entry into either apoptosis or necrosis to better understand the cellular mechanisms of toxicity.

The dissection of cytotoxic mechanisms can be improved by simultaneously measuring several parameters related to cell health in individual cells; as such analysis enables faster elucidation of mechanism. Some well-known indicators of compromised cell health are as follows:

- Decay in mitochondrial transmembrane potential, or $\Delta\Psi_m$
- Increased plasma membrane permeability
- Disruption of polarity of phosphatidylserine (PS) distribution in the plasma membrane
- Activation of caspases
- Fragmentation and degradation of DNA
- Changes in nuclear morphology
- Release of cytochrome c from mitochondria
- Oxidative stress

Simultaneous measurement of multiple indicators of cell health has been shown to be highly valuable for dissection of toxicity (Haskins et al. 2001). Analysis of apoptosis has also benefited from multiparametric analysis; in particular, cells undergoing apoptotic cell death can be distinguished from those that have undergone necrosis (van Engeland et al. 1998). The power of multiparametric analysis is greatly augmented by correlations between multiple parameters in individual cells. The Cellomics® Cell Health Profiling BioApplication is designed to measure and correlate a wide range of cellular parameters and classify multiparametric cellular responses associated with cell health, at the level of individual cells.

BioApplication Overview

The Cell Health Profiling BioApplication enables measurement of cell health indicators from two basic cellular regions. The definitions of these regions are described in more detail in the following sections.

The features and capabilities of this BioApplication provide you with the flexibility to design your own assays. This BioApplication is for scientists who want a versatile tool that can be applied towards many different cell health-related measurements. This document will guide users in the use of this application.

Measurements made in Distinct Cellular Regions

The Cell Health Profiling BioApplication requires that Channel 1 contain an image of cells in which particular cellular region or organelle is labeled with a fluorescent marker to define a primary cellular object. The measurement regions of the cell in each of the dependent channels (Channels 2-6) are derived from the object in Channel 1. The object should be a major constituent of the cell as it is used to identify individual cells and define the different sub-cellular regions for each cell. Examples of cellular regions or compartments that can serve as the object include the cell nucleus as well as the entire cell.

The Cell Health Profiling BioApplication can accommodate up to six fluorescence channels. A channel is defined as the optical configuration required to acquire/analyze image data for a single cellular target. Channel 1 is reserved for the primary object as described above. The average and total intensities for this object are measured. In addition, several morphological descriptors of the object are measured. Channels 2-6 are the dependent channels; you can choose the number of channels depending on the number of different targets. A high degree of

flexibility is available with regard to definition of the cellular regions used for measurement. In Channels 2-4, you can choose either the Circ region or the Ring region (Figure 1). In Channels 5-6, only the Circ region is available. Description of the Circ and Ring regions is provided in the section below.

Circ

Circ is a cellular region identified from, and similar to, the area covered by the object. You can expand or contract the region defined by the object to occupy a larger or smaller area of the cell. For example, if a nuclear label is used in Channel 1 to label and identify the cell nucleus as the object, the Circ region can be used to measure fluorescence associated with the entire cell by deriving a region that extends symmetrically beyond the object, i.e., from the nucleus to the cell periphery. The cellular region used for measurement of the target(s) of interest can be modified through the application of an intensity threshold to the Circ region to identify punctate fluorescence within the Circ region (Figure 1 and Figure 2). Application of intensity levels also enable measurements of the area occupied by a discrete fluorescent object(s) within the Circ/Ring region. The Circ region is available in Channels 2-6. Targets that could be measured using the Circ region include those that require intensity and/or area measurements of the nucleus or the whole cell.

Ring

Ring is an annular region defined beyond the object. The user defines the position of the annulus' inner and outer perimeters relative to the edge of the object. If a nuclear label is used to define the object in Channel 1, then the Ring region can be used to quantify the presence of a fluorescently labeled macromolecule in the perinuclear cytoplasm. The measurement region used for the targets of interest may be modified by applying an intensity level to the Ring region to identify only punctate fluorescence within the Ring region, which may be used to identify organelles such as mitochondria, lysosomes, etc. (see Figure 1 and Figure 2). The Ring region is available in Channels 2-4 only.

Figure 1 shows a schematic of the two regions of the cell defined for a case where the nucleus in Channel 1 is used as the object. Figure 2 shows the same using actual images of fluorescently labeled cells. Nuclei are labeled in Channel 1 and mitochondria are labeled in Channel 2. The modified Ring and Circ regions are created by using intensity thresholds. Note that measurement regions associated with distinct objects (nuclei in this case) do not overlap.

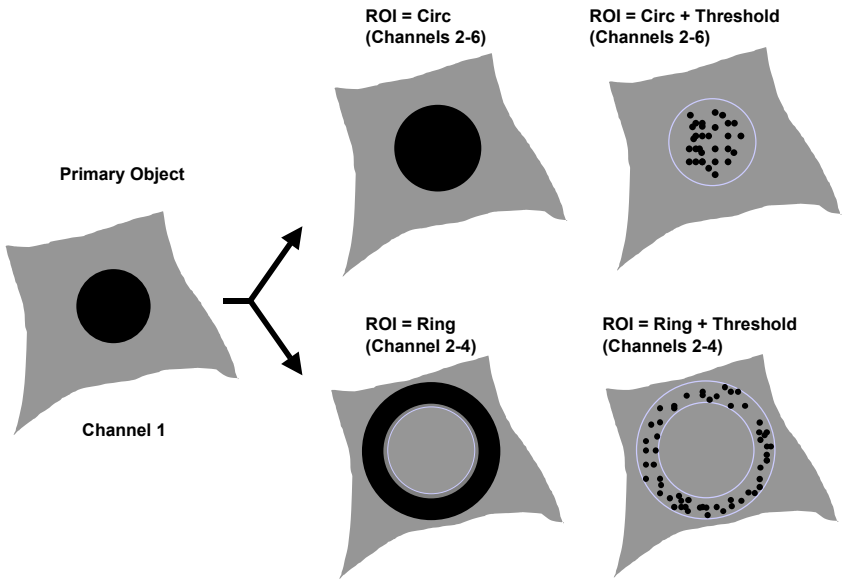

Figure 1. *Cellular regions (shown in black) identified by the Cell Health Profiling BioApplication. ROI = Region of interest used for measurement of cellular targets.*

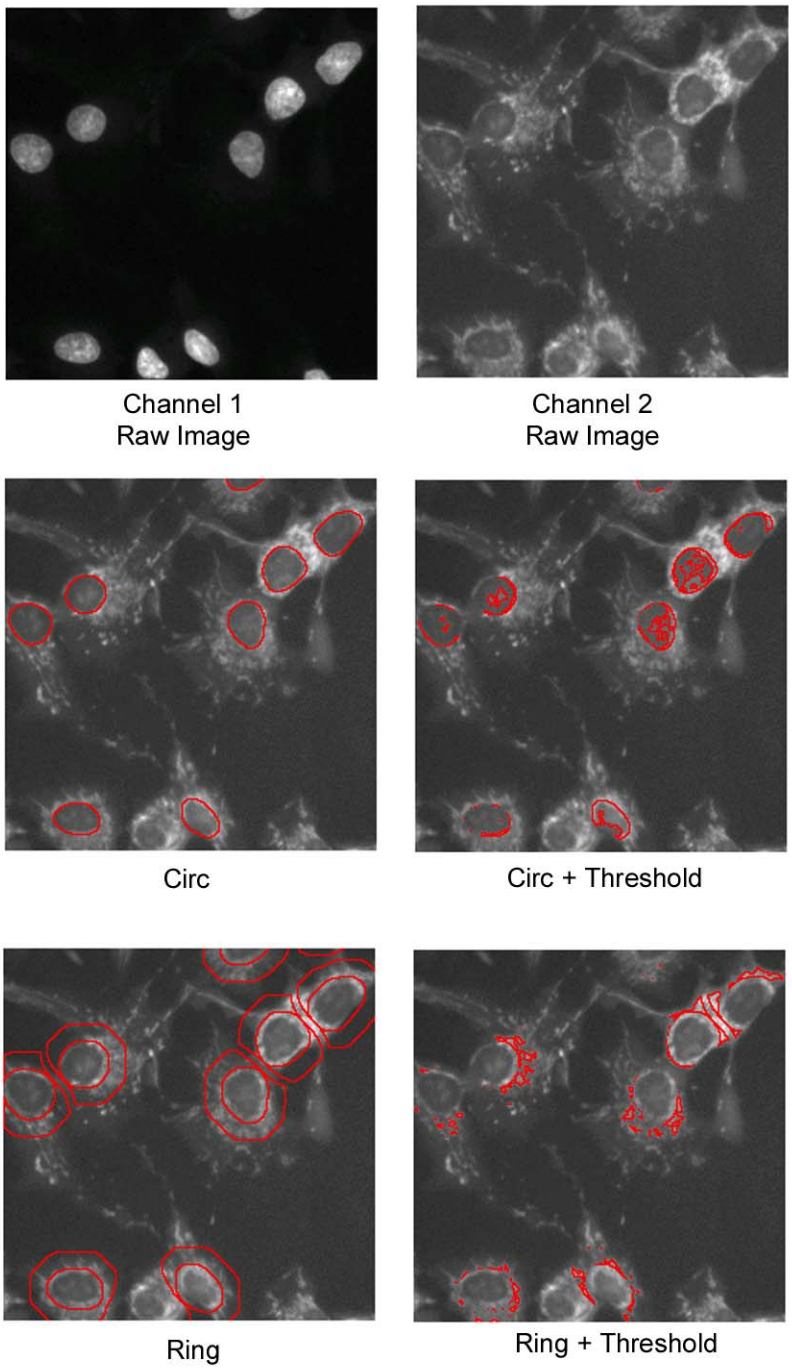

Figure 2. *Cellular measurement regions shown for fluorescently labeled cells. Nuclei are used as primary objects. Measurement regions identified are outlined in red.*

Event Definition

Definition and detection of cellular events are achieved through a combination of multiple BioApplication capabilities (see Figure 3).

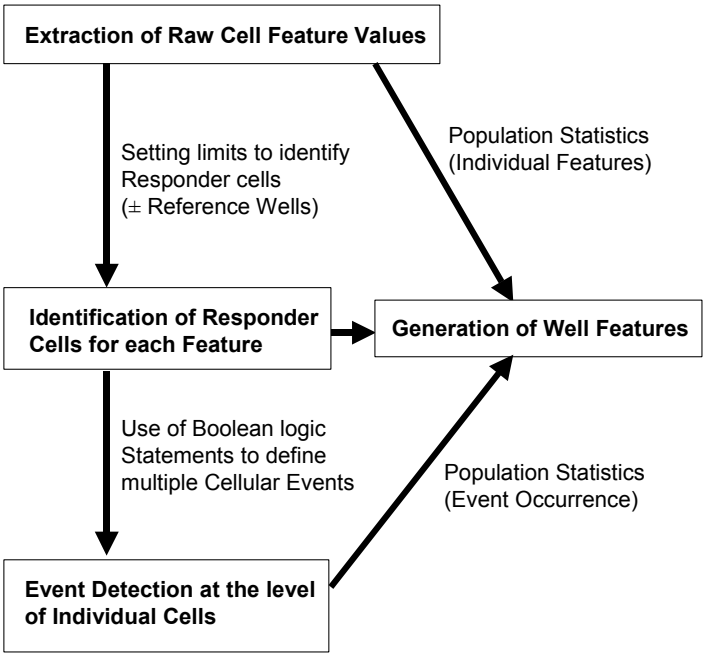

***Figure 3**. Schematic showing the process of definition and detection of cellular events.*

Events are biologically meaningful logic statements comprised of a set of cellular parameters and standard Boolean operators. Actual detection of Event occurrence in individual cells starts with identification of 'responder' cells that show a value outside a level range set for each Assay Parameter. Once responder cells are identified, Event definitions can be generated by combining multiple parameters in a meaningful way using standard Boolean operators. Definitions of the available Boolean operators are provided in Chapter 2.

Well Features are calculated to provide population statistics for individual Cell Features and frequency of occurrence of a given Event in the cell population. The latter is expressed as the percentage of cells that display a given Event. Figure 4 is an example of how different biologies could be set up.

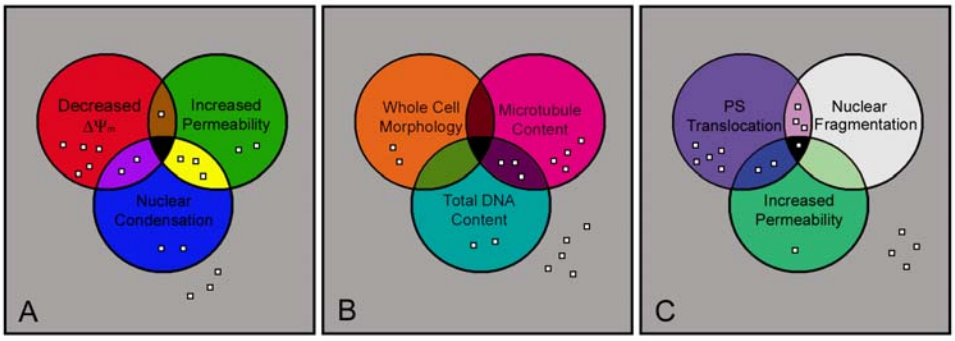

***Figure 4**. Event Definition using multiple cellular targets for measurement of cell health. White squares represent individual cells that are classified according to the combination of targets for which they show a response. Circular domains contain responder cells for the indicated target. In each case, cells in the gray region are those that did not show a response for any of the measured targets. Note that responders can fall within more than one target.*

For the purposes of illustration, consider an assay that is configured to allow simultaneous measurement of the following cellular targets as indicators of cellular injury: $\Delta\Psi_m$, plasma membrane permeability and nuclear morphology. This combination of targets is valuable as $\Delta\Psi_m$ disruption tends to be an early indicator of cellular injury, whereas nuclear shape changes and increase in plasma membrane permeability are indicative of acute toxicity.

Panel A (in Figure 4) is a schematic representation of the occurrence of the above indications of cell injury in a cell population. The entire cell population is contained within the box. The initial assumption for any set of cellular responses is that individual cells may display effects on a single target or any combinations of the measured targets. Therefore, a cell may be classified as belonging to any one of the colored regions in Figure 4A. In this case, cells that belong to the red region would show only a decrease in $\Delta\Psi_m$; cells that belong in the yellow region would exhibit both nuclear condensation and membrane permeability in the absence of $\Delta\Psi_m$ disruption and so on. The following examples of biological Event definitions that may be applied to the use case described in Figure 4A illustrate how this BioApplication can be used:

- ***Nuclear condensation OR Increased Permeability*** – quantifies the percentage of cells that display either nuclear condensation or increased membrane permeability, both of which are indicators of acute cellular injury. This population would exclude cells in the red and gray regions.

- ***Decreased ΔΨm*** **ANDNOT** ***Nuclear condensation*** **ANDNOT** ***Increased Permeability*** – returns the percentage of cells that show only a drop in $\Delta\Psi_m$ without the occurrence of either nuclear condensation or increased membrane permeability. This Event is an indication of an early, possibly reversible stage of cell injury and would include only those cells in the red region.

Figure 4B describes an assay configuration that enables determination of whether the cellular target is cytoskeletal or genomic. Examples of biological Event definitions that may be applied are:

- ***Microtubule polymerization/depolymerization*** **OR** ***Whole Cell Shrinkage*** – identifies one or more cytoskeletal elements as the cellular target. The population displaying this Event would only exclude cells within the turquoise and gray regions.

- ***DNA degradation*** **ANDNOT** ***Microtubule polymerization/depolymerization*** **ANDNOT** ***Whole Cell Shrinkage*** – identifies genomic toxicity in the absence of gross cytoskeletal perturbation. These cells are contained within the turquoise region.

Figure 4C shows a traditional assay for PS translocation to the outer plasma membrane, measured via annexin V labeling, as a marker for apoptosis. Plasma membrane permeability is measured simultaneously to differentiate necrotic cells that also label with annexin V. Lastly, nuclear fragmentation is used to detect late stage apoptosis. The following biological Event definitions enable rapid detection and classification of apoptotic events in this case:

- ***PS Translocation*** **ANDNOT** ***Increased Permeability*** – distinguishes apoptosis from necrosis. These cells would occupy the purple and lavender regions.

- ***PS Translocation*** **ANDNOT** ***Increased Permeability*** **AND** ***Nuclear Fragmentation*** – identifies late-stage apoptosis. The cells displaying this Event would be contained within the lavender region.

BioApplication Measurements

The BioApplication measures multiple features associated with the object (Channel 1) and the measurement region (Circ or Ring) in each dependent Channel used. The BioApplication reports both Cell and Well Features. Cell Features are measurements reported for individual cells. Well Features are reported for each well and represent population statistics for all cells selected for analysis. Therefore, Well Features are derived from Cell Features for all the cells analyzed in that well. Features that report measurements made using the Circ or Ring regions are generically referred to as Target features.

The different categories of features measured by the Cell Health Profiling BioApplication are summarized in Table 1 and explained in more detail in the sub-sections that follow. The table also summarizes the relationship between the different Cell Features and their corresponding Well Features.

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	Cellular Regions	Cell Feature	Corresponding Well Level Features
Cell & Well Level Features Categories Reported for Channel 1:			
Intensity	Primary Object Mask	Total Intensity and Status Average Intensity and Status Variance Intensity and Status	Mean, SD, SE, CV
Morphology & Location	Primary Object Mask	Top Left Width Height X Centroid Y Centroid	none
		ObjectArea and Status ObjectSize and Status ObjectShapeP2A and Status ObjectShapeLWR and Status	Mean, SD, SE, CV, %HIGH, %LOW
Cell & Well Features Categories Reported for Channels 2-6:			
Intensity	Target (Circ or Ring)*	Average Intensity and Status Total Intensity and Status	Mean, SD, SE, CV, %HIGH, %LOW
Morphology	Target (Circ or Ring)*	Target Area and Status	Mean, SD, SE, CV, %HIGH, %LOW
Gating	Modified Mask	Total Intensity Average Intensity	none
Other Cell & Well Features Reported:			
Cell Counts & Density	Primary Object Mask	Cell Number	ValidCellCount SelectedCellCount %SelectedCells ValidFieldCount CellPerFieldCount
Event	Primary Object or Target (Circ OR Ring)	EventTypeProfile EventTypeXStatus	EventTypeXCellCount %EventTypeXCells$^{\otimes}$

Table 1. *Cell Health Profiling BioApplication Measurements.*

* Choice between Circ or Ring regions is available in Channels 2-4. Only the Circ region is available in Channels 5-6.

$^{\otimes}$ X = 1-3 (a total of three Events can be defined using the Cell Health Profiling BioApplication)

Demonstration Data Using the Cell Health Profiling BioApplication

The Cell Health Profiling BioApplication is applicable to a wide range of assays designed to measure cell health, some examples of which were given earlier in this chapter. For the purposes of demonstration, this section describes results obtained by using the BioApplication to rank toxic effects according to acuteness.

Ranking of toxic effects according to acuteness requires selection of targets that indicate varying extents of cellular injury. Given this requirement, the following cell health indicators were selected for measurement: nuclear size, plasma membrane permeability, and $\Delta\Psi_m$. Disruption of $\Delta\Psi_m$ is generally an early indicator of progress along the necrotic/apoptotic pathway and often indicates moderate, sometimes reversible, cellular injury (Lemasters et al. 1999; Minamikawa et al. 1999). In contrast, changes in plasma membrane permeability and nuclear condensation are widely recognized as indicators of later, irreversible stages of necrosis/apoptosis (Zakeri 1998).

The ranking of toxicity is demonstrated by considering the concentration-dependent toxicity of valinomycin (a K^+ ionophore) on HepG2 cells (human liver carcinoma) through the measurement of the above cell health indicators.

Figure 5 below demonstrates distinct cellular responses to two concentrations of valinomycin (24 hour treatment). After drug treatment, a mixture of fluorescent reagents was added, following which the cells were fixed and washed prior to scanning on the Cellomics HCS Reader. Nuclei are labeled in Channel 1 using a cell permeant nucleic acid label. Membrane permeability was assayed with a cell impermeant nucleic acid label that brightly labels the nuclei of cells with increased membrane permeability. $\Delta\Psi_m$ was measured using a cationic fluorophore that accumulates in mitochondria as a result of maintenance of $\Delta\Psi_m$. Control cells display normal nuclear sizes, intact plasma membrane integrity and brightly labeled mitochondria (healthy $\Delta\Psi_m$). Valinomycin at a low concentration (230 nM) is a potent disrupter of $\Delta\Psi_m$, while leaving nuclear size and membrane permeability largely unaffected. Treatment with a higher concentration of valinomycin (120 μM) causes $\Delta\Psi_m$ disruption, but also induces the acute cellular responses of nuclear condensation and increased membrane permeability. This dosage dependence shows that a drop in $\Delta\Psi_m$ is a sensitive indicator of valinomycin toxicity.

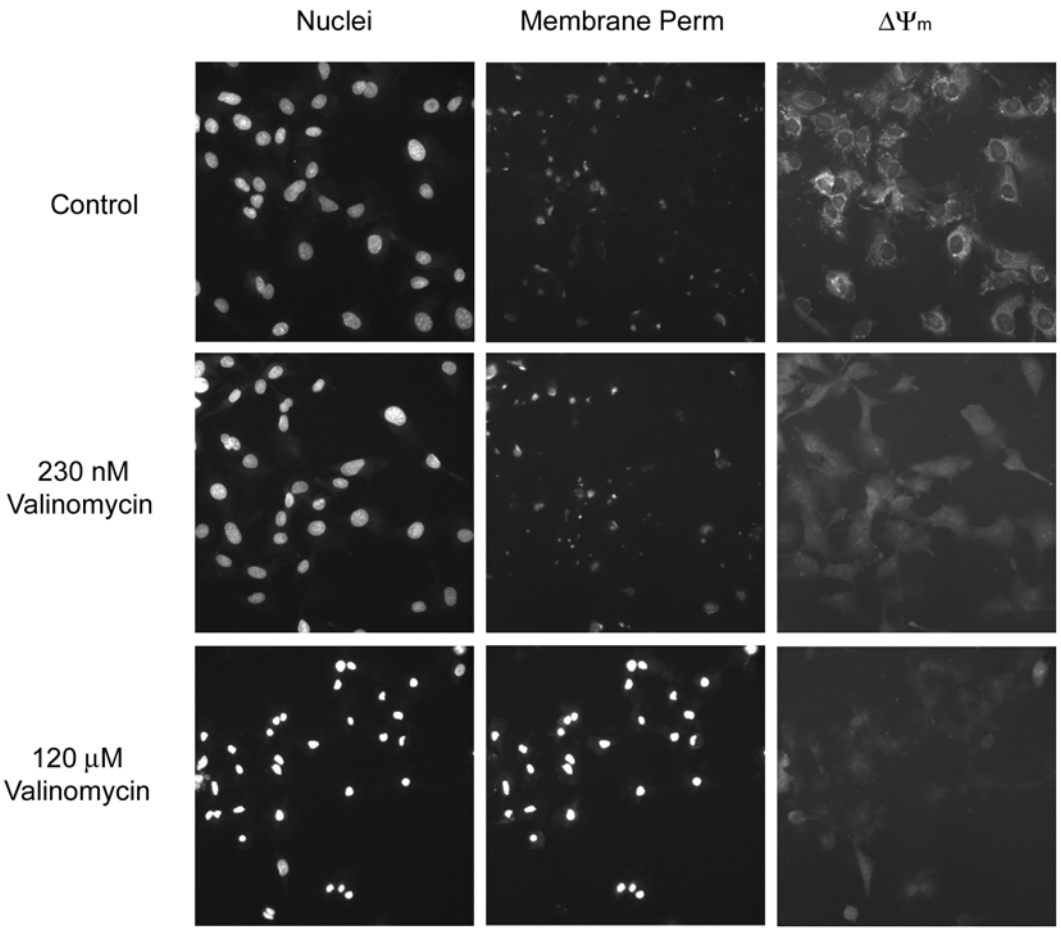

***Figure 5**. Image Data showing Dose-dependent toxicity of valinomycin treatment (24 hr).*

Quantitative data that indicates changes seen in Figure 5 is shown in Figure 6 for all three targets. Cell nuclei were used as objects in Channel 1. Nuclear condensation was determined using an equivalent diameter measurement, membrane permeability was measured as the average intensity within the nuclear region (Circ) in Channel 2, and $\Delta\Psi_m$ was measured in Channel 3 as the average brightness of cytoplasmic spots (mitochondria) using a Ring region modified with an intensity threshold. Panel C in Figure 6 shows a small increase in $\Delta\Psi_m$ at high concentrations (10-100 µM) of valinomycin. Analysis of images showed that the increase is due to a limitation of the $\Delta\Psi_m$ indicator reagent. It nonspecifically labels cytoplasm to a greater extent just prior to the onset of membrane permeability measured in Channel 2 (not shown). Note that the brightness of the indicator decreases again once gross permeabilization has occurred, presumably due to substantial leakage of cytoplasmic components from cells.

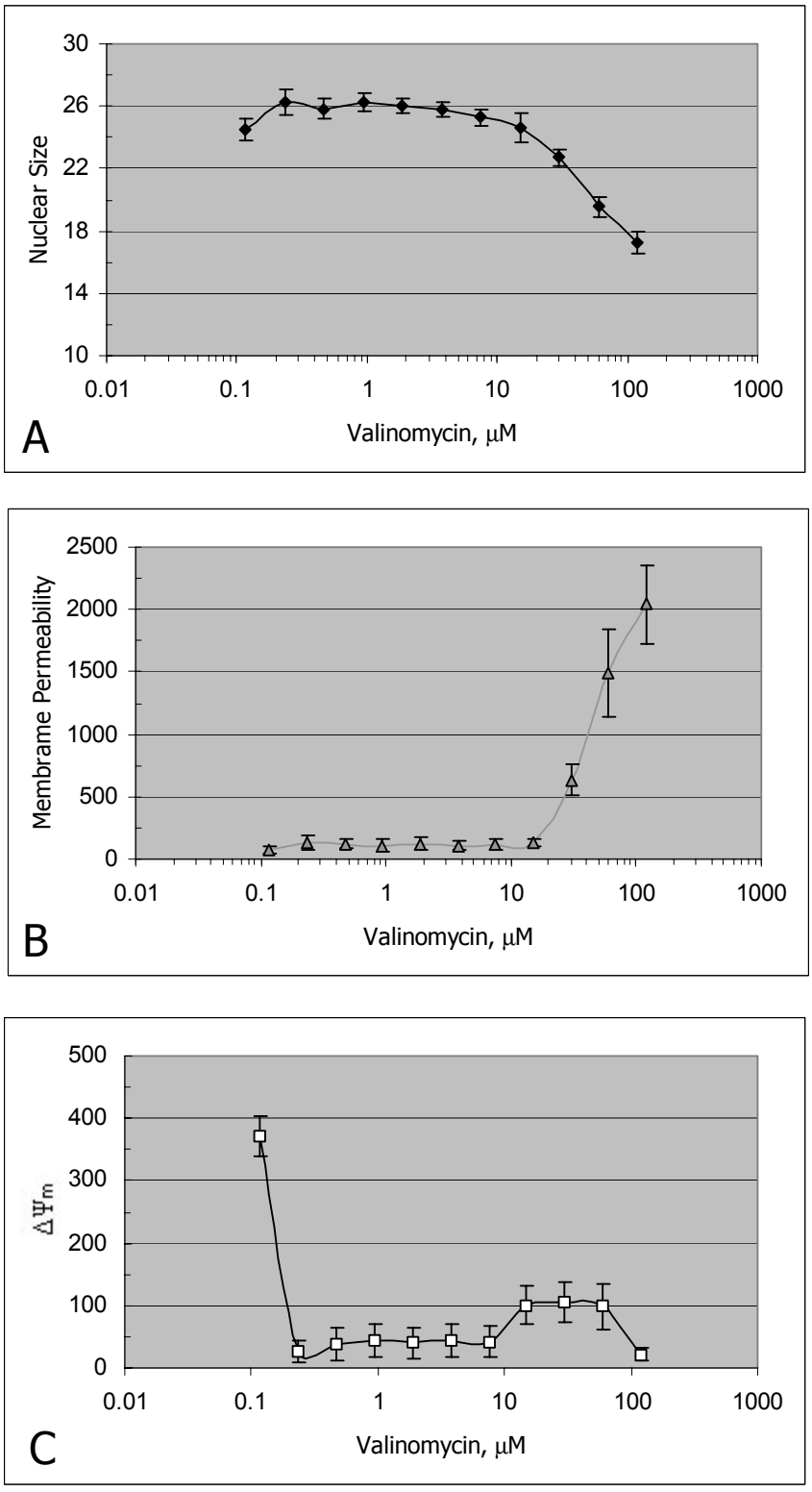

Figure 6. *Quantitation of the dose-dependent effects of valinomycin on HepG2 cells. Error bars indicate the standard deviation from 8 wells.*

The knowledge of the responses of individual cells of a population is often valuable to dissect mechanism of toxicity. Figure 7 shows individual cellular responses for all three targets being measured. The figure was generated using the direct link between the View application and Spotfire® DecisionSite™. Two different perspectives of the same 3D plot are shown to aid interpretation of the data. Treatment with 230 nM valinomycin results in collapse of $\Delta\Psi_m$ only, while 120 µM valinomycin causes decay in $\Delta\Psi_m$, increased membrane permeability and nuclear condensation.

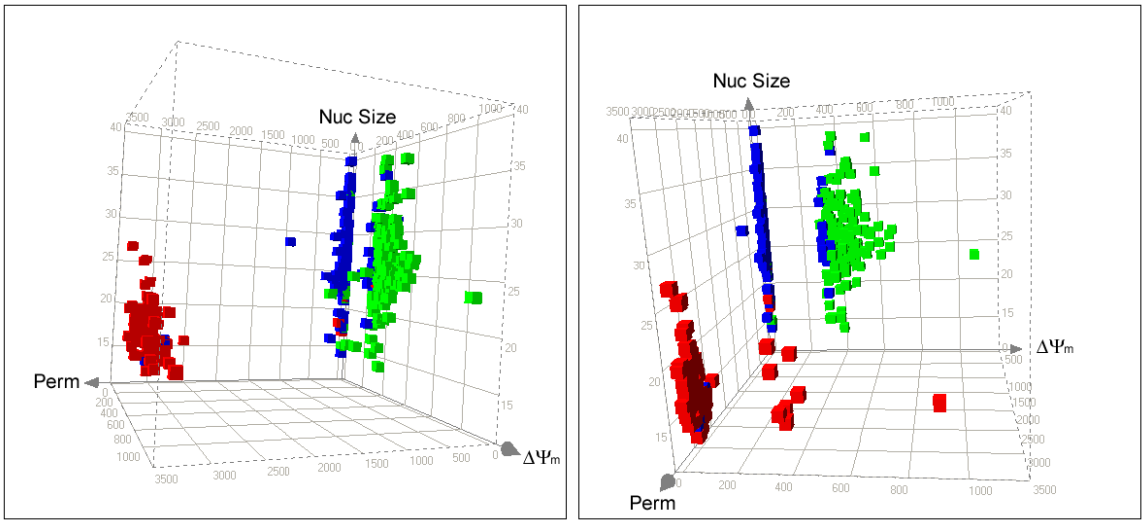

Figure 7. *Spotfire plots showing individual cell responses to valinomycin treatment. Two perspectives of the same 3D plot are shown. Control (green symbols); 230 nM valinomycin (blue symbols); 120 µM valinomycin (red symbols).*

Extraction of individual cellular responses allows calculation of population descriptors that indicate the fraction of responder cells in the population. The numbers of cells in the population showing feature values above the upper limit and below the lower limit are calculated for each target. Typically, a given target requires measurement of one of these subpopulations. For example, detection of decay in $\Delta\Psi_m$ requires determining the number of cells whose $\Delta\Psi_m$ is below the lower limit, while measurement of *increased* membrane permeability requires determination of the number of cells that exhibit membrane permeability greater than the higher limit value. Figure 8 shows the percentage of responding cells for each of the raw target measurements shown in Figure 6.

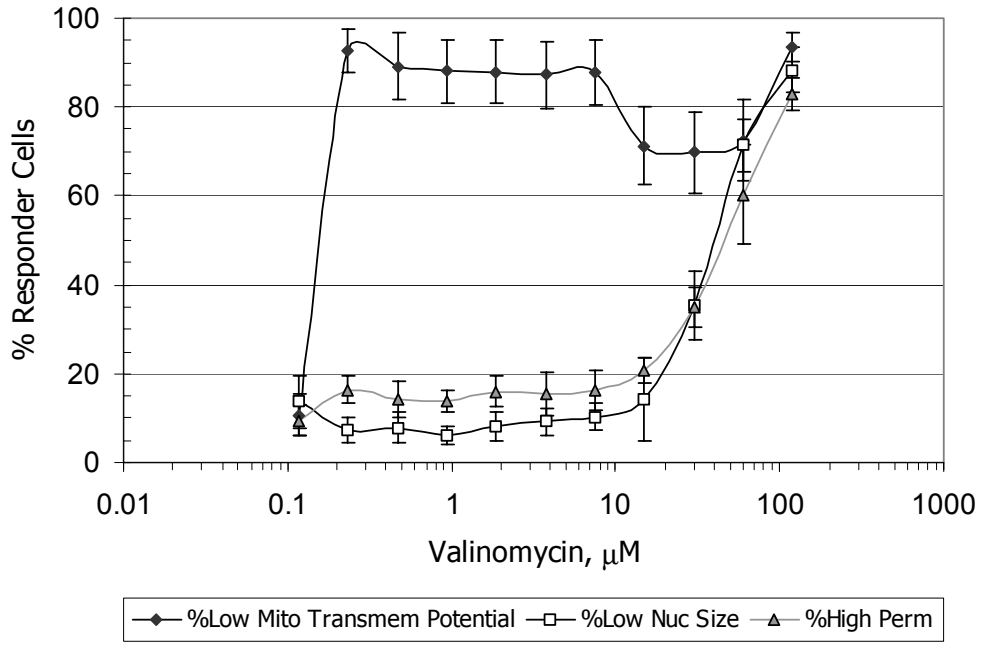

Figure 8. *Quantification of responding cells for decay in ΔΨm (%Low Mitochondrial Transmembrane Potential), nuclear condensation (%Low Nuclear Size) and increased membrane permeability (%High Permeability) induced by valinomycin treatment. Error bars indicate the standard deviation from 8 wells.*

Experiments using the high and low concentrations of valinomycin used typically showed a Z' value of greater than 0.3 for the output features corresponding to each target.
The definition and detection of cellular Events that you defined can also be demonstrated using the dose-dependent response to valinomycin treatment. In this case, three Events were defined to distinguish moderate toxicity from acute toxicity. The logical statements used are described below.

Event 1: *(Decrease in $\Delta\Psi_m$) ANDNOT (Nuclear Condensation) ANDNOT (Increased Permeability)*

This statement is designed to aid detection of moderate /early toxicity where has been $\Delta\Psi_m$ disrupted, while the late stage targets of membrane permeability and nuclear size have not been affected.

Event 2: *(Nuclear Condensation) OR (Increased Permeability)*

Event 3: *(Nuclear Condensation) AND (Increased Permeability)*

Events 2 and 3 are variations of cellular responses indicating acute toxicity. Note that Event 3 represents the most acute toxicity as it is designed to detect simultaneous occurrence of nuclear condensation and membrane permeability in the same cell. Events 1-3 are shown schematically in Figure 9.

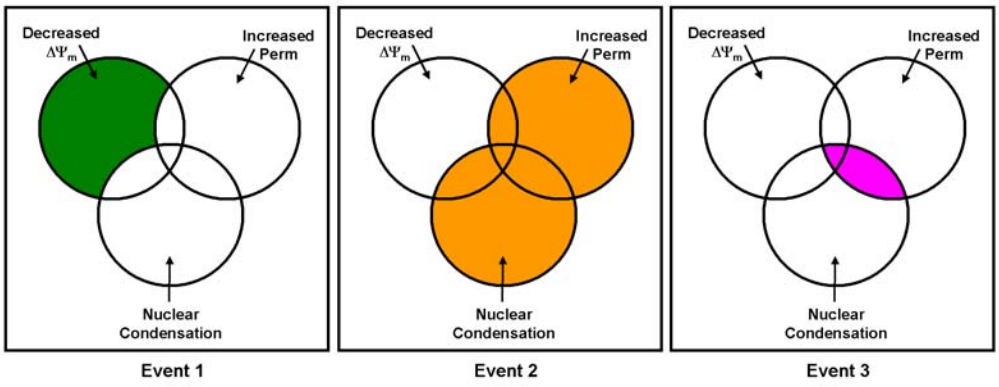

Figure 9. *Schematic descriptions of Event Definitions used to rank toxicity.*

Figure 10 quantifies the occurrence of the above Events for the dose-response experiment. The percentage of cells in which Event 1 occurs increases transiently as only the lower concentrations of valinomycin show lack of effects on nuclear morphology and membrane permeability. Events 2 and 3 show similar responses, except that the fraction of cells displaying Event 2 is consistently higher than that displaying Event 3. This is an expected result, given that Events 2 and 3 apply the OR and AND operators on the same pair of Cell Features respectively. Given these results, the Event Wizard, in conjunction with the Scan application, may be used to define several additional events, such as:

(Nuclear Condensation) ANDNOT (Increased Permeability)

(Increased Permeability) ANDNOT (Nuclear Condensation)

Detection of these events would enable rapid determination of whether nuclear condensation precedes plasma membrane permeability or vice versa.

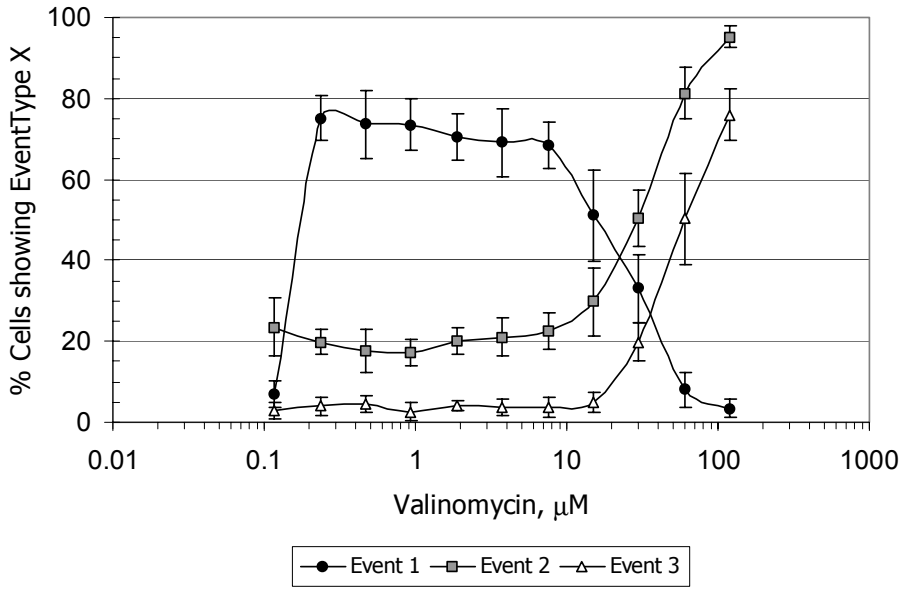

Figure 10. *Event detection to differentiate moderate toxicity from acute toxicity. Error bars indicate the standard deviation from 8 wells.*

References

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Chapter
2

Description of the Algorithm

NOTE

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

This chapter describes the assay output features generated and the adjustable input parameters that control image analysis and extraction of data from the acquired images.

Overview

Every BioApplication provided uses an image analysis algorithm that has been extensively tested and validated for robust screening performance. The algorithm has input parameters that control its analysis. Parameter values determined from validation testing have been supplied in the supplied default Assay Protocol. Parameters are adjustable to allow customization of the algorithm to your own samples and conditions.

Input parameters can be found in the Create Protocol View 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, and only the input parameters for the selected channels will be displayed. In the ArrayScan Classic software, the available input parameters will also vary depending on the mode in which you are running: Basic Mode or Advanced Mode. 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. Both sets of input parameters are described in this chapter.

There are three types of input parameters: Object Identification Methods , Assay Parameters, and Object Selection Parameters. The Object Identification Methods and Assay Parameters directly control the quantitative analysis of the images. The Object Selection Parameters control the selection of objects chosen for processing and are specific for each Channel.

Object Identification Methods

To identify objects in each of the images from the different channels, an independent intensity threshold must be set for each channel. The Object Identification Parameters critically influence the image analysis results because they control the intensity thresholds used to identify objects and spots. Depending on the properties of the objects being identified, 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.

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

Table 2. *Intensity Threshold Methods Available Per Channel in the Cell Health Profiling 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), the value must be **0**.

The effective range for object identification is limited to 0-4095 for the following Cellomics HCS Readers: ArrayScan VTI, ArrayScan X.5 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 because in these channels an intensity threshold is required to define the pixels making up the tube and the targets. The choice of the **None** threshold is the only option in Channels 2-6.

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

The **Isodata Threshold** method 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). The schematic in Figure 11 demonstrates how the histogram-derived threshold value is calculated; Table 3 gives a description of the options.

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, which is finally applied to the image is determined from the user-entered offset value, o, as:

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

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

Isodata Threshold is 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 method with a large offset can be entered.

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

Table 3. *Intensity Threshold Descriptions*

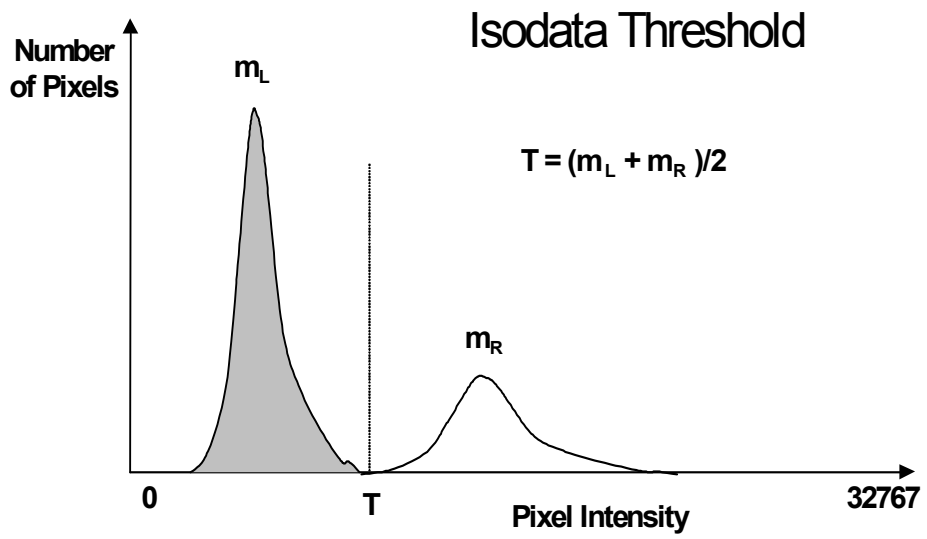


***Figure 11**. Isodata Intensity threshold method: Background peak is shown in gray and object peak is shown in white.*

Description of Assay Parameters and Settings

General Assay Parameters

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

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

Reference Well Control

Please see the *Assay Parameters for Population Characterization* section for more details.

If you would like to have the BioApplication automatically compute the high and low cellular response levels, you must set the Assay Parameters that control the use of Reference Wells. If the **UseReferenceWells** Assay Parameter is set to **1,** Reference Wells will be used. If set to **0,** Reference Wells are not used and the BioApplication will determine cellular responses using manually specified levels. If Reference Wells are used, the advanced Assay Parameter **MinRefAvgObjectCountPerField** must 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. Further explanation of Reference Wells can be found in the *Assay Parameters for Population Characterization* section later in this chapter.

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 was chosen.

Object Type Ch*N*

ObjectTypeCh1, TargetTypeCh*N*

The **ObjectTypeCh1** and **TargetTypeCh*N*** (i.e. Ch 2-6) 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** makes dark areas within an image to be considered as potential objects, while leaving it as **0** considers bright areas on a dark background as potential objects (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* ***TargetTypeChN****.*

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 Ch*N*

Prior to image analysis, the non-cellular background can be computed and subtracted from each image in each channel independently, as shown in Figure 12.

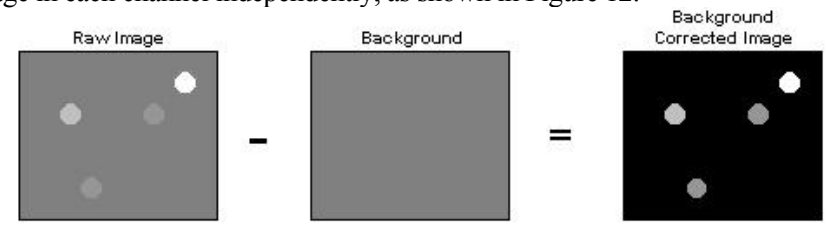


Figure 12. *Background Correction ChN*

The background-corrected image is computed by suppressing high frequency components in the image (low pass filtration). You can control the creation of the background image by adjusting the **BackgroundCorrectionCh*N*** Assay Parameter in each channel as needed. This Assay Parameter refers to the radius of the area that is sampled around each pixel in the image

to determine its local background. The value of this Assay Parameter should be much larger than the radii of the objects in the image. If the value is set to **0**, background correction is not performed, and analysis is done using the raw, uncorrected images.

If the **BackgroundCorrectionCh*N*** Assay Parameter is given a negative value an optional background correction method based on the 3D surface fitting is applied. The absolute value entered corresponds to the radius of an area used to find local minima across the image. Found minimum values are used to construct a 3D surface of a background which is then subtracted from the original image. The main advantage of the method is that it minimizes the effect of the background correction (removal) procedure on the intensity values of the analyzed objects. 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 Method
0	No background correction is performed
Positive	Local minimum in the box specified by the value entered is subtracted from that region of the image
Negative	Local minima detected are used to fit a surface representing the background, which is then subtracted
-1	Automated surface fitting is performed and the computed background is subtracted

Table 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

Object Smooth Factor Ch1

This BioApplication enables you to control the extent of smoothing to be applied to acquired images. Smoothing is only performed on Channel 1 images. Smoothing reduces the sharpness of intensity variations by redistributing their relative brightness over the immediate vicinity in the image. The value of the **ObjectSmoothFactorCh1** Assay Parameter determines the area of the region over which brightness is redistributed. If the value of this Assay Parameter is set to **0**, smoothing is not done and analysis is done on raw, uncorrected images.

Object Smoothing can be used to connect fragments of objects (e.g., nuclei or whole cells), and, therefore, can be valuable when measuring morphological changes. Figure 13 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).

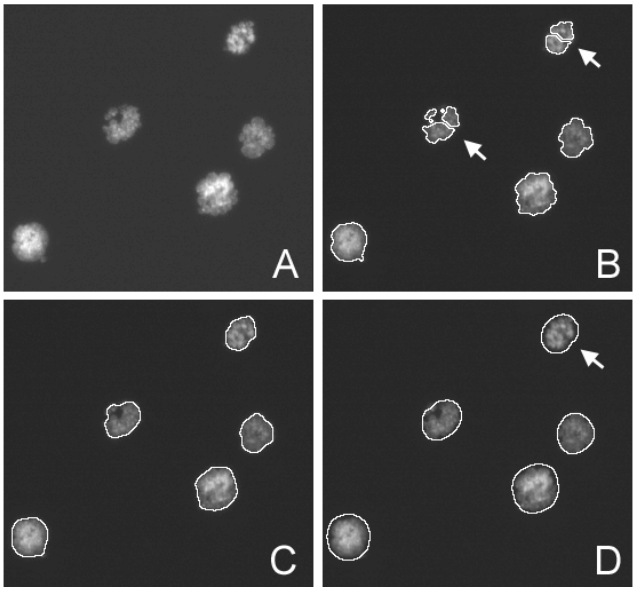


Figure 13. *Object Smoothing. Images show the effect of different degrees of smoothing on the same field of apoptotic cell nuclei. A: Raw Image; B:* ***ObjectSmoothFactorCh1*** *= 0; C:* ***ObjectSmoothFactorCh1*** *= 3; D:* ***ObjectSmoothFactorCh1*** *= 8.*

Object Segmentation Ch1

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 Cell Health Profiling BioApplication: Geometric (shape and size) and Intensity (intensity peaks). These methods are illustrated in Figure 14.

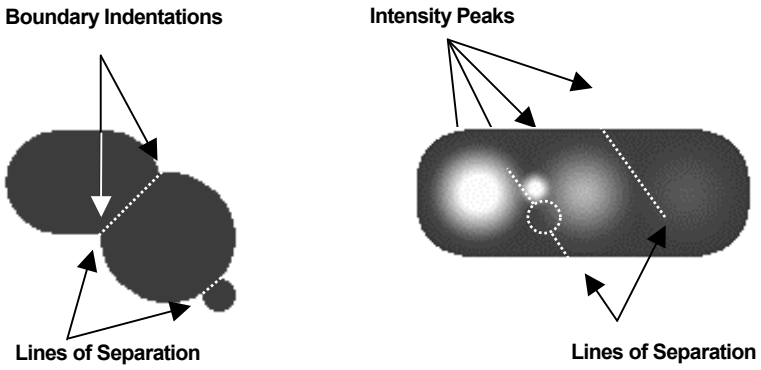


Figure 14. *Object Segmentation Options. The image on the left illustrates the Geometric Method while the image on the right illustrates the Intensity Method.*

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 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 **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 6. *Channel 1 Object Segmentation Options*

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

Geometric Method Setting the **ObjectSegmentationCh1** parameter to R1 (Figure 15A) produces object separation shown in Figure 15B. Setting the **ObjectSegmentationCh1** parameter to R3 results in segmentation shown in Figure 15C. Setting the **ObjectSegmentationCh1** parameter to R2 (the largest radius of all touching objects) produces no segmentation.

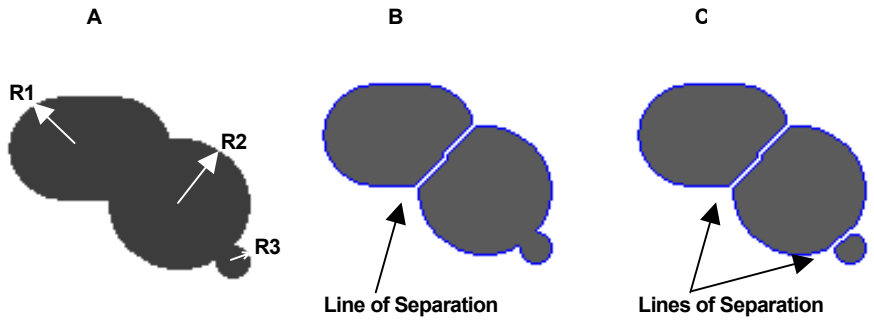


Figure 15: *Object segmentation: Geometric Method. A) Radii of touching objects: R1 = 24, R2 = 30, R3 = 7; B) Result of segmentation using* ***ObjectSegmentationCh1*** *= R1; C) Result of segmentation using* ***ObjectSegmentationCh1*** *= R3.*

Intensity Method Figure 16 shows the intensity profile along the cordial line of an object with four intensity peaks from Figure 14. 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 vise versa. In case of over-segmentation, setting the **ObjectSmoothFactorCh1** greater than 0 should be used to alleviate the problem.

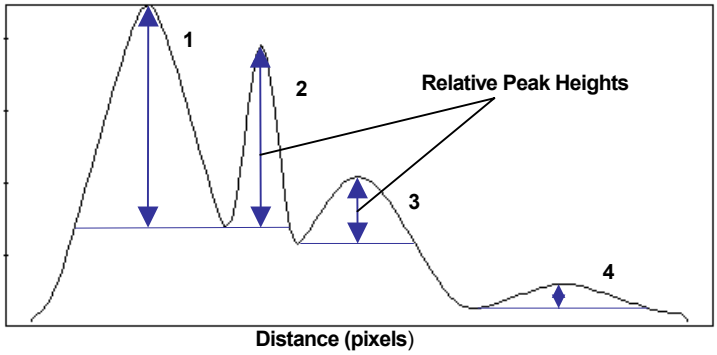


Figure 16: *Intensity profile of an object with four intensity peaks. Peak #1 height = 1500, peak #2 height = 1250, peak #3 height = 500, and peak #4 height = 200.*

Only two peaks in Figure 16, #1 and #2, have relative intensity greater than 1000. Thus, setting the **ObjectSegmentationCh1** 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 17A. 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 17B and 17C.

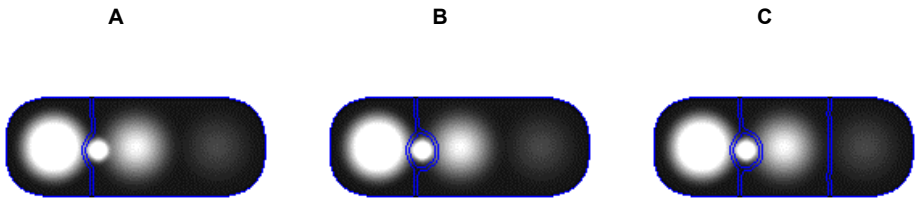


Figure 17: *Object segmentation: Intensity Method. A) Result of segmentation using parameter* ***ObjectSegmentationCh1*** *= -1000; B) Result of segmentation using parameter* ***ObjectSegmentationCh1*** *= -400; C) Result of segmentation using parameter* ***ObjectSegmentationCh1*** *= -100.*

Channel 2-6 Assay Parameters

Adjusting Extents of Measurement Regions

The Cell Health Profiling BioApplication allows flexible definition of the measurement region used for measurement of cellular targets in the dependent Channels 2-6. In Channels 2-4, the measurement region can be set to be Circ or Ring. Only the Circ region is available in Channels 5-6. The size of the Circ or Ring regions can be independently adjusted in each of channel, and this size is set in relation to the object in Channel 1. The schematic in the following shows the three parameters that are used to control the extent of the measurement regions.

The **TargetRingWidthCh*N*** Assay Parameter is used to choose between the Circ and Ring region for the target. Setting the value of this Assay Parameter to **0** automatically results in use of the Circ region. If the Circ region is used, it can be modified using the **TargetCircModifierCh*N*** Assay Parameter. The value of this Assay Parameter equals the number of pixels by which the object is dilated (positive value) or eroded (negative value) to define the measurement region. In other words, this is the number of pixels added to, or subtracted from, the perimeter of the object to define the measurement region. In Figure 18, the **TargetCircModifierCh*N*** has a positive value and the region defining the object has been dilated to define the measurement region.

Setting the value of the Assay Parameter **TargetRingWidthCh*N*** to an integer value greater than **0** specifies use of the Ring region for measurement of the target. In addition, it also sets the width of the Ring region. The **TargetRingDistanceCh*N*** Assay Parameter sets the number of pixels by which the extent of the object should be dilated or contracted to define the inner boundary of the measurement region.

The Assay Parameter that controls the modification of the object mask used for gating is **MaskModifierCh*N***, where *N* is the relevant dependent channel (Channels 2-6). It functions the same as **TargetCircModifierCh*N***, except that it has no impact on defining the regions used for measuring cellular targets and is only used for gating. Please see the Gating section for more information.

NOTE

The **TargetRingWidthCh*N*** Assay Parameter is used to choose between the Circ and Ring regions in Channels 2-4. A finite, non-zero value for this parameter results in employment of the Ring region while a value of zero automatically specifies use of the Circ region.

Both the Ring and Circ regions can be adjusted to include discrete patterns of fluorescence within each region. Examples of such patterns are those observed for labeled organelles such as mitochondria, lysosomes, nucleoli, etc. or supramolecular aggregates such as bundles of actin filaments or microtubules. This is achieved by applying an intensity threshold method (either **Fixed Threshold** or **IsodataThreshold**) for Object Identification. Setting the Object Identification Method to **None** ensures that the measurement region covers the entire Circ or Ring region independent of fluorescence signal (shaded areas in Figure 18).

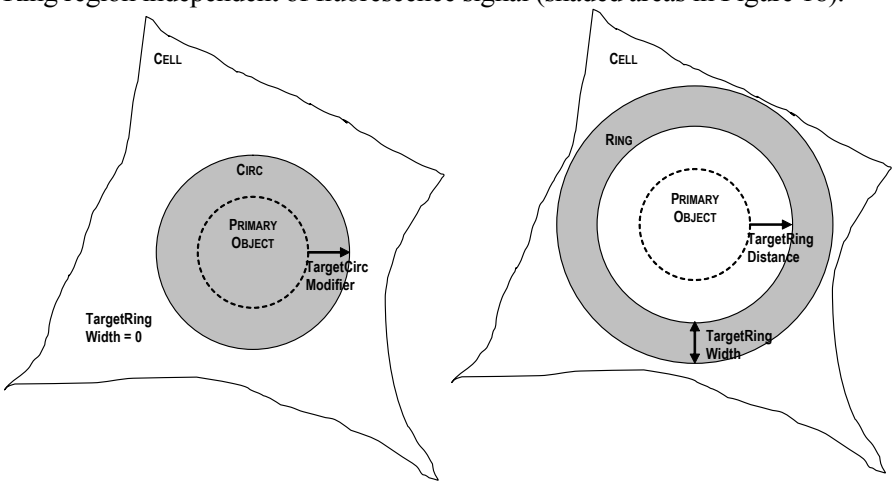


Figure 18. *Adjustment of target areas derived from the primary object.*

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 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
ObjectTypeCh1	Binary	Type of objects to be identified in Ch1: 0 = Bright objects on dark background, 1 = Dark objects on bright background
BackgroundCorrectionChN	Pixels	Radius (in pixels) of region used to compute background in ChN: Negative value = Use surface fitting, 0 = Do not apply background correction, Positive value = Use low pass filter,
ObjectSmoothFactorCh1	Number	Degree of image smoothing (blurring) prior to object detection in Ch1: 0 = Do not apply smoothing
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
RejectBorderObjectsCh1	Binary	Reject objects that touch image edges: 0 = No, 1 = Yes
TargetTypeChN	Binary	Type of targets to be identified in ChN: 0 = Bright targets on dark background, 1 = Dark targets on bright background
TargetRingDistanceChN	Pixels	Distance (in pixels) from Ch1 object (nucleus) mask to the inner rim of ring mask in ChN
TargetRingWidthChN	Pixels	Width (in pixels) of ring mask in ChN
TargetCircModifierChN	Pixels	Number of pixels to modify Ch1 object (nucleus) mask to create circ mask in ChN: 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

Table 7. *Basic Assay Parameters Available with the Cell Health Profiling BioApplication. *Note that "ChN" refers to Channels 1-6 for* ***Background Correction****, Channels 2-4 for "****TargetRing****" Assay Parameters, and Channels 2-6 for all remaining Assay Parameters.*

Object Selection Parameters

Each channel has a set of specific Object Selection Parameters associated with it. If an object in a particular channel image has all measured features within the range specified by the Object Selection Parameters, then it is analyzed; otherwise, it is rejected from the analysis. Object Selection measurements, available in all channels (Channels 1-6), are used to select cells to be used for analysis.

The population of valid cells is first identified in Channel 1 using Channel 1 Object Selection criteria. Channel 1-specific parameters that establish morphological criteria for analyzed objects include **ObjectAreaCh1**, **ObjectShapeP2ACh1,** and **ObjectShapeLWRCh1**. Min and Max values for each parameter define the criteria for object selection. These values can be adjusted to suit your specific needs. Parameter values can be adjusted to reject debris, cell fragments, dividing cells, and multinucleated cells. Gating on this population can then be performed applying adjustable ranges for average and total intensity specified in Channels 2-6.

Channel 1 Object Selection Parameters

The Object Selection Parameters for Channel 1 are used to determine which of the objects identified in Channel 1 are to be analyzed (Table 8). The two categories of Channel 1 Object Selection Parameters are intensity and morphology.

The average and total intensities of the object are the two intensity-related selection parameters. The morphology-related Object Selection Parameters are **ObjectAreaCh1**, **ObjectShapeP2ACh1**, and **ObjectShapeLWRCh1**. If an object's Average and Total Intensities, Area, ShapeP2A, and ShapeLWR are all within the specified range, then the cell represented by the object is considered to be a valid cell. Note that the value entered for the **ObjectAreaCh1** parameter can be entered in μm^2 (**UseMicrometers** set to **1**) or in pixels (**UseMicrometers** set to **0**).

It is important that the Area, ShapeP2A, ShapeLWR, and Total and Average Intensities be set to accommodate changes in morphology of the objects (typically cell nuclei or entire cells). Morphological changes are highly likely when cells are faced with toxic challenges.

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

Table 8. *Object Selection Parameters available in Channel 1 for the Cell Health Profiling BioApplication*

Channel N (2-6) Object Selection Parameters

There are two Object Selection Parameters for every dependent channel (Channels 2-6), which further determine whether the object is to be analyzed (Table 9). All of these parameters are related to intensity. If an object passes all Object Selection criteria in all channels, the cell represented by that object is considered to be selected cell. Cell Features and Well Features are only reported for cells selected for analysis.

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

Table 9. *Object Selection Parameters available in Channels 2-6 for the Cell Health Profiling BioApplication. *Note that "ChN" refers to Channels 2-6.*

Gating

The Cell Health Profiling BioApplication supports gating of a cell population. This feature provides selective cell processing based on fluorescent intensity. You may want to perform gating if cells have been identified in the focus channel and it is necessary to refine the cell selection based on intensity of the objects in a second channel. An example use case is the employment of transient transfection with a fluorescent marker such as GFP. The gating feature enables analysis of only those cells that have a pre-defined expression level of the marker without resorting to physical sorting of the cell population using flow cytometry.

Specifying Intensity Ranges

When working in the Create Protocol View, you can specify intensity ranges in the Object Selection region by entering **Min** and **Max** value ranges for **AvgIntenCh*N*** and **TotalIntenCh*N*** in one or more dependent channels (Channels 2-6). **TotalIntenCh*N*** is a summation of all intensities within the targeted object. **AvgIntenCh*N*** is **TotalIntenCh*N*** divided by the object's area. Alternatively, you can set these values interactively when working in the Protocol Interactive View.

Specifying Mask Modifiers in Assay Channels

In addition to specifying intensity ranges for one or more channels, you can apply a mask to one or more downstream channels using the **MaskModifierCh*N*** setting. The value of this Assay Parameter is equal to the number of pixels that the object is dilated or eroded to define the region which intensity measurements are made for gating purposes; however, the mask will not overlap with other masks from nearby objects. This was previously described in the **MaskModifierCh*N*** section earlier in this chapter. Once the mask for each channel is determined, the intensity ranges can be specified as described above.

For each channel, the Average and Total Intensity values within the modified masks are calculated. If the calculated value does not fall within the specified upper and lower limit values, the object is removed from the object set.

Image Overlays

Colored image overlays are available to indicate the objects and targets that were identified and used in the analysis. Overlays are also available to display the spot identification Target Regions and to indicate the objects that have responded to treatment (Table 10). Checkboxes are provided for you to select these overlays on a channel-by-channel basis, and you can also choose their colors.

Parameter	Description
Include This Channel In Composite	Determines if image for this channel is included in composite image.
SelectedObject	Outlines Valid Objects in Channel 1 (e.g., cells) for analysis. Valid Objects are those that have properties in the range specified by the Object Selection Parameters.
RejectedObject	Outlines Objects rejected for analysis. Rejected Objects have properties outside the range specified by the Object Selection Parameters.
TargetChN	Displays measurement region for Channel N). Measurement region displayed may be Circ or Ring (Channels 2-4) or Circ alone (Channels 5-6)

Table 10. *Overlay displays and descriptions available for the Cell Health Profiling BioApplication. *Note that "ChN" refers to Channels 2-6.*

Assay Parameters for Population Characterization

Overview of Population Characterization

This 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 for a control population that has a defined physiological distribution for that feature. Upper and lower limits (called ***Feature*Ch*N*LevelHigh** and ***Feature*Ch*N*LevelLow** respectively) 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 11):

Value	Cell Status Definition
0	Cell Feature value within defined range
1	Cell Feature value is greater than upper limit
2	Cell Feature value is less than lower limit

***Table 11**. Numeric expression of Cell Feature Status when using population characterization.*

The corresponding Well Features reported are the percentage of cells with feature values that are either greater or less than the specified limits. Figure 19 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.

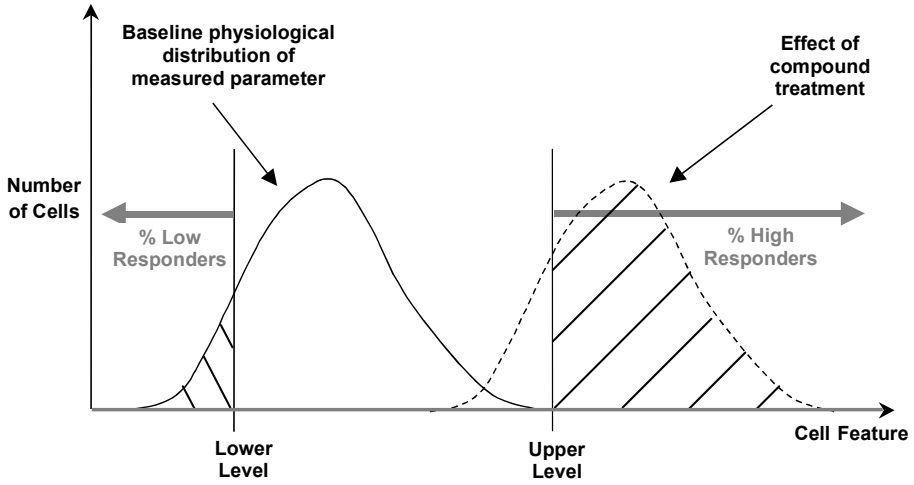


***Figure 19**. Principle of Population Characterization. Responders exhibiting Status = 2 (%Low Responders) or Status = 1 (%High Responders) highlighted.*

Setting Cellular Response Levels

There are two ways of setting the upper and lower limits to characterize a cell population: manually or automatically. To set limits manually, you explicitly specify the upper and lower levels (***Feature*Ch*N*LevelHigh** and ***Feature*Ch*N*LevelLow**, respectively) for each Cell Feature. This requires prior knowledge of typical feature values. The Cell Health Profiling BioApplication then calculates the percentage of cells outside the bounds established by these limits for the different Cell Features.

To automatically determine the limits, the BioApplication uses Reference Wells. You can specify which wells on the sample plate should be used as Reference Wells. Typically, Reference Wells contain untreated cells that display physiological values for the Cell Features being measured. These wells are first imaged and the population distribution for the different features are determined. The cell population characterization limits are then specified through use of a correction coefficient, or **CC** value, that is used in combination with the standard deviation of the distribution of feature values. The limits are then applied and the whole plate is then scanned. 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 the standard deviation would be multiplied would be two (**CC** value = 2). The advantage of using Reference Wells to automatically calculate limits is that the limits are determined from a control population of cells and are independent of run-to-run variations when doing the experiment on different days that result from different illumination conditions, fluorophore amounts, or changes in cell culture conditions.

Reference Wells Processing Sequence

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

NOTE

If the feature value for **MinRefAvgObjectCountPerField** obtained from the Reference Wells is below the value set for the **MinRefAvgObjectCountPerField** Assay Parameter, the BioApplication aborts the use of Reference Wells and processes the plate as if **UseReferenceWells** is set to **0**.

The Cell Health Profiling BioApplication uses only **'Known'** Reference Wells. Reference features are computed on a field basis, as they are field-based features. 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. 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 as arithmetic average for wells on a plate. Use of a weighted average minimizes the effect of sparse fields. The only Reference Feature that is not weighted is **RefAvgObjectCountPerField**.

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 on the **Known** button to define the type of Reference Well. 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 more 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

In addition to **UseReferenceWells** and **MinRefAvgObjectCountPerField**, there are four types of Assay Parameters that are used for cell population characterization that are specific to each individual Cell Feature for which population characterization is applied. These Assay Parameter names are of the type:

- ***Feature*Ch*N*LevelLow**
- ***Feature*Ch*N*LevelHigh**
- ***Feature*Ch*N*LevelLow_CC**
- ***Feature*Ch*N*LevelHigh_CC**

In each case, ***Feature*** refers to the individual Cell Feature and **Ch*N*** refers to the specific Channel number (1-6).

You can manually set the upper and lower limits for each Cell Feature that defines the physiological state (typically untreated) of the cell population in a known physiological state. This is done after reviewing results from previously analyzed plates or via the Protocol Interactive View. Alternatively, limits can be automatically derived from Reference Wells containing cells whose physiological state (untreated or treated) is known. You can choose between manually setting the levels or having it calculated automatically through use of the **UseReferenceWells** Assay Parameter. The possible values and resulting actions controlled by this Assay Parameter are:

- **UseReferenceWells = 0** do not use Reference Wells (i.e., set range manually)
- **UseReferenceWells = 1** use Reference Wells (i.e., let instrument set range automatically)

If the range is set manually, you must specify values for two Assay Parameter types for each output feature to be used for cell population characterization:

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

The values for these Assay Parameters are the lower and higher levels of the range respectively. Therefore, for a given Cell Feature, such as **ObjectSizeCh1**, the upper and lower levels are specified by setting values for both **ObjectSizeCh1LevelLow** and **ObjectSizeCh1LevelHigh** Assay Parameters.

If the range is to be set automatically by the instrument using Reference Wells, the Assay Parameters having the suffix **_CC** (i.e., correction coefficient or CC value) will need to be

adjusted. The lower and upper levels for a specific Assay Parameter are set by multiplying the appropriate CC value by the standard deviation (SD) of the distribution and subtracting or adding from the mean as shown below:

- ***Feature*Ch*N*LevelLow** = Mean – ***Feature*Ch*N*LevelLow _CC** × SD
- ***Feature*Ch*N*LevelHigh** = Mean + ***Feature*Ch*N*LevelHigh _CC** × SD

Note that the _CC value can be different for defining the lower and the upper limits. Also the _CC value may be a positive or negative number.

Therefore for a given feature, such as **ObjectSizeCh1**, Reference Wells are used to set LevelHigh (upper limit) and LevelLow (lower limit) as follows:

- **ObjectSizeCh1LevelLow** = Mean – (**ObjectSizeCh1LevelLow_CC**) × SD
- **ObjectSizeCh1LevelHigh** = Mean + (**ObjectSizeCh1LevelHigh_CC**) × SD

Individual cells having feature values outside the range established by the lower and upper levels 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

The **Hide Advanced Parameters** option enables display of Basic Assay Parameters only or both Advanced and Basic Assay Parameters. The Advanced Assay Parameters available in Cell Health Profiling BioApplication are listed in Table 12.

For each feature undergoing population characterization, there are four advanced Assay Parameters that control its levels: ***Feature*ChN*LevelLow*** and ***Feature*ChN*LevelHigh*** 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 size in Channel 1 are:

- **ObjectSizeCh1LevelLow**
- **ObjectSizeCh1LevelHigh**
- **ObjectSizeCh1LevelLow_CC**
- **ObjectSizeCh1LevelHigh_CC**

Instead of listing all four "level" Assay Parameters for each feature, one entry will be listed, as shown in the following example for the Channel 1 object area:

- ObjectSizeCh1Level***Low/High, LowHigh_CC***

Parameter	Units	Description
MinRefAvgObjectCountPerField	Number	Use reference wells to calculate high and low response levels: 0 = No, 1 = Yes
UseMicrometers	Binary	Measure lengths and areas in: 0 = Pixels, 1 = Micrometers
_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)***
Type_3_EventDefinition	—	User-defined combination of logic statements involving response features ***(cannot be edited)***
ObjectSizeCh1Level***Low/High, Low/High_CC***	Pixels or µm	Defines **ObjectSizeCh1** population characterization thresholds
ObjectAreaCh1Level***Low/High, Low/High_CC***	Pixels 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
TargetTotalIntenChNLevel***Low/High, Low/High_CC***	Intensity	Defines **TargetTotalIntenChN** population characterization thresholds
TargetAvgIntenChNLevel***Low/High, Low/High_CC***	Intensity	Defines **TargetAvgIntenChN** population characterization thresholds
TargetAreaChNLevel***Low/High, Low/High_CC***	Pixels or μm^2	Defines **TargetAreaChN** population characterization thresholds

Parameter	Units	Description
TargetVarIntenChNLevel**Low/High**	Intensity	Low/High-response level for **TargetVarIntenChN**
TargetVarIntenChNLevel**Low/High_CC**	Intensity	Correction coefficient (in standard deviations) used to set low/high-response level for **TargetVarIntenChN** from reference well results

***Table 12**. Advanced Assay Parameters Available for the Cell Health Profiling BioApplication. *Note that "ChN" refers to Channels 2-6.*

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

The Cell Health Profiling BioApplication allows simultaneous definition of up to three Events to enable rapid multiparametric analysis of cell health at the level of individual cells, across multiple Cell Features. This capability allows you to do the following automatically:

- Stage toxic events and determine stage (early/middle/late or mild/moderate/acute) of apoptosis or necrosis
- Rapidly determine mechanism of toxicity, including identification of the initial target or 'trigger' point
- Differentiate apoptosis from necrosis

Event definition is achieved through the construction of logic statements employing upper and lower bounds for specific Cell Features and a set of defined logical operators. The Cell Features and Boolean operators available are listed in Table 13. 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 20 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 for each feature are located within the domain for that feature. Note that the definitions described in Figure 20 can be directly applied to logic statements that contain more than two Cell Features.

The logic statements that comprise Event definitions are specified using a stand-alone software tool called the BioApplication Event Wizard. Operation of this software tool is described in more detail in Chapter 3. The Assay Parameters used to define Events are of the type **Type_X_EventDefinition** (X =1, 2 or 3).

Note that the Event Definition Assay Parameters should not 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
ObjectSizeCh1	NOT
ObjectAreaCh1	AND
ObjectShapeP2ACh1	AND NOT
ObjectShapeLWRCh1	OR
ObjectTotalIntenCh1	OR NOT
ObjectAvgIntenCh1	XOR
ObjectVarIntenCh1	NAND
TargetTotalIntenChN	NOR
TargetAvgIntenChN	
TargetAvgAreaChN	

Table 13. *Cell Features and Boolean operators available for Event Definition with the Cell Health Profiling BioApplication. *Note the "ChN" refers to Channels 2-6.*

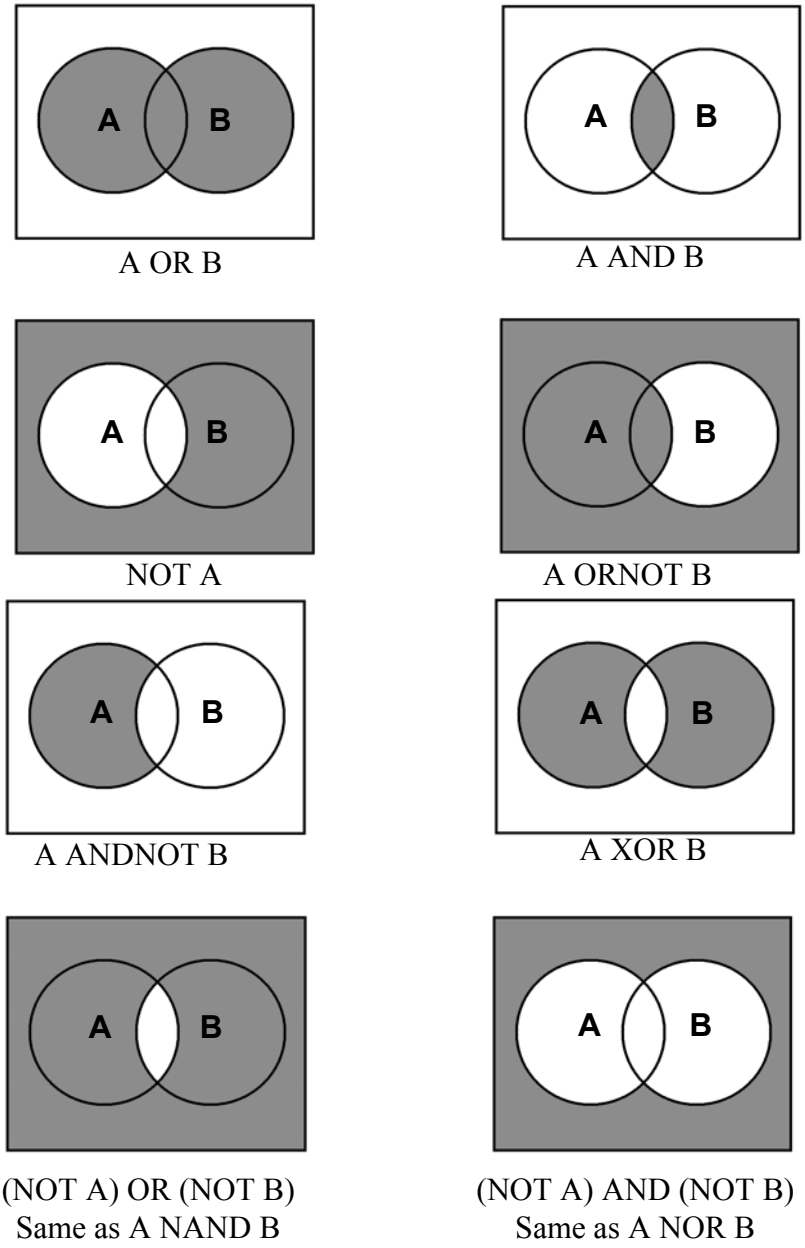


Figure 20. *Schematic definition of available Boolean Operators assuming two Cell Features of interest, A and B.*

Example of Using Events With the Cell Health Profiling BioApplication

Consider that the Cell Features of interest are:

1) **ObjectSizeCh1**: low value indicates nuclear condensation if the cell nucleus is used as the Object
2) **TargetAvgIntenCh2**: high value indicates plasma membrane permeability
3) **TargetAvgIntenCh3**: low value indicates decay of $\Delta\Psi_m$

The upper and lower levels for each feature must be set such that responder cells in Channels 1-3 exhibit nuclear condensation, plasma membrane permeability, and decreased

$\Delta\Psi_m$ respectively (see section on *Assay Parameters for Population Characterization* above). Example values for upper and lower levels that could be used to detect responder cells in this case are shown in Table 14.

Cell Feature	Target	Possible values	Typical values for untreated cells / responder cells	Upper limit (LevelHigh)	Lower limit (LevelLow)
ObjectSizeCh1	Nuclear Morphology	0-1024	24 / 17	1024	21
TargetAvgIntenCh2	Membrane Permeability	0-32767	50 / 1500	200	0
TargetAvgIntenCh3	$\Delta\Psi_m$	0-32767	300 / 10	4095	150

Table 14. *Setting upper and lower limits for Event definition.*

NOTE

For Event detection, a cell is considered to be a responder if its feature value is greater than the upper limit or less than the lower limit for that feature. If only cells that display relatively low feature values are to be used for Event detection, the upper bound should be set to the maximum possible value for that feature. When using Reference Wells, use a large positive value for ***Feature*ChN**LevelHigh_CC**. Conversely, if cells that display only relatively high feature values are to be used, the lower limit should be set to the lowest possible feature value. When using Reference Wells, use a large positive value for ***Feature*ChN**LevelLow_CC**.

The Event defined as:

TargetAvgIntenCh3 ANDNOT **ObjectSizeCh1** ANDNOT **TargetAvgIntenCh2**

identifies individual cells that show disruption of $\Delta\Psi_m$ in the absence of nuclear condensation and plasma membrane permeability. This would identify an early toxic event as a disruption of $\Delta\Psi_m$, typically an early indicator cellular injury, while plasma membrane permeability and nuclear condensation are known to be indicators of acute toxicity (see *Demonstration of the Cell Health Profiling BioApplication*, Chapter 1).

In contrast, the Event defined as:

ObjectSizeCh1 OR **TargetAvgIntenCh2**

identifies an acute toxic event, as cells displaying this event must show either nuclear condensation or plasma membrane permeability.

All logic statements are implemented in sequence from left to right. Therefore, the logical statement:

ObjectSizeCh1 AND **TargetAvgIntenCh2** OR **TargetAvgIntenCh3**

is implemented as:

(ObjectSizeCh1 AND **TargetAvgIntenCh2)** OR **TargetAvgIntenCh3**

This BioApplication allows a logical statement containing a maximum of four Cell Features and four logical operators. Event definitions must end with a Cell Feature to be considered valid. Event definitions may only begin with a Cell Feature, with the exception of the operator NOT, as shown below. A single feature is sufficient to define an Event. Additional examples of valid event definitions are:

- ((**ObjectSizeCh1** AND **ObjectShapeP2ACh1**) OR (NOT **ObjectAvgIntenCh1**))) XOR **TargetAreaCh2**
- (NOT **TargetAvgIntenCh2**) OR **ObjectAvgIntenCh1**
- **TargetAreaCh2**

Description of Output Features

Output Features are the biological measurements produced by the BioApplication. All features are categorized and accessible using the View application. Additionally, a subset of features (Well Features), are listed in the Scan Plate View and Create Protocol View 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.

Cell Features

Table 15 lists the Cell Features available for the Cell Health Profiling BioApplication, viewable in the Protocol Interactive View as well as the View application.

Cell Feature	Units	Description
Cell#	Number	Unique object ID
Top	Pixels	Y coordinate (in pixels) of top-left corner of image-aligned bounding box of Ch1 object
Left	Pixels	X coordinate (in pixels) of top-left corner of image-aligned bounding box of Ch1 object
Width	Pixels	Width (in pixels) of image-aligned bounding box of Ch1 object
Height	Pixels	Height (in pixels) of image-aligned bounding box of Ch1 object
XCentroid	Pixels	X coordinate (in pixels) of center of Ch1 object
YCentroid	Pixels	Y coordinate (in pixels) of center of Ch1 object
EventTypeProfile	Number	Identifies the events that occurred: 1, 2, 3, 12, 23, 13, 123
EventType1Status	Binary	**EventType1** status: 0 = event did not occur, 1 = event occurred
EventType2Status	Binary	**EventType2** status: 0 = event did not occur, 1 = event occurred
EventType3Status	Binary	**EventType3** status: 0 = event did not occur, 1 = event occurred
ObjectSizeCh1	Pixels or µm	Diameter (in pixels or micrometers) of circle with area equal to area of Ch1 object
ObjectSizeCh1Status	Number	**ObjectSizeCh1** status: 0 = No response, 1 = High response, 2 = Low response
ObjectAreaCh1	Pixels or μm^2	Area (in pixels or micrometers) of object in Ch1
ObjectAreaCh1Status	Number	**ObjectAreaCh1** status: 0 = No response, 1 = High response, 2 = Low response
ObjectShapeP2ACh1	Number	Shape measure based on ratio of perimeter squared to 4π*area of Ch1 object (**ObjectShapeP2ACh1** = 1 for circular object)
ObjectShapeP2ACh1Status	Number	**ObjectShapeP2ACh1** status: 0 = No response, 1 = High response, 2 = Low response
ObjectShapeLWRCh1	Number	Shape measure based on ratio of length to width of object-aligned bounding box of Ch1 object
ObjectShapeLWRCh1Status	Number	**ObjectShapeLWRCh1** status: 0 = No response, 1 = High response, 2 = Low response
ObjectTotalIntenCh1	Intensity	Total intensity of all pixels within Ch1 object
ObjectTotalIntenCh1Status	Number	**ObjectTotalIntenCh1** status: 0 = No response, 1 = High response, 2 = Low response
ObjectAvgIntenCh1	Intensity	Average intensity of all pixels within Ch1 object
ObjectAvgIntenCh1Status	Number	**ObjectAvgIntenCh1** status: 0 = No response, 1 = High response, 2 = Low response

Cell Feature	Units	Description
ObjectVarIntenCh1	Intensity	Standard deviation of intensity of all pixels within Ch1 object
ObjectVarIntenCh1Status	Number	**ObjectVarIntenCh1** status: 0 = No response, 1 = High response, 2 = Low response
TargetTotalIntenChN	Intensity	Total intensity in ChN of all pixels within target mask (circ or ring)
TargetTotalIntenChNStatus	Number	**TargetTotalIntenChN** status: 0 = No response, 1 = High response, 2 = Low response
TargetAvgIntenChN	Intensity	Average intensity in ChN of all pixels within target mask (circ or ring)
TargetAvgIntenChNStatus	Number	**TargetAvgIntenChN** status: 0 = No response, 1 = High response, 2 = Low response
TargetVarIntenChN	Number	Variation (standard deviation) of intensity in ChN of all pixels within target mask (circ or ring)
TargetVarIntenChNStatus	Number	**TargetVarIntenChN** status: 0 = No response, 1 = High response, 2 = Low response
TargetAreaChN	Pixels or μm^2	Area (in pixels or micrometers) of ChN target mask (circ or ring)
TargetAreaChNStatus	Number	**TargetAreaChN** status: 0 = No response, 1 = High response, 2 = Low response
TotalIntenChN	Intensity	Total intensity in ChN of all pixels within modified Ch1 object mask
AvgIntenChN	Intensity	Average intensity in ChN of all pixels within modified Ch1 object mask

Table 15. *Cell Features Available with the Cell Health Profiling BioApplication. *Note that "ChN" refers to Channels 2-6.*

Well Features

Most of the Well features are derived from the cell features. Such features are identified by a prefix, as listed in Table 16, to the cell feature name.

Feature Prefix	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 16. *General Well Features available with the Cell Health Profiling BioApplication.*

The algorithm also reports the following additional Well Features found in Table 17 in the Scan Plate View in addition to the Well Detail window of the View application.

Feature	Description
ValidObjectCount	Number of valid objects identified in the well (Ch1 object selection parameters applied)
SelectedObjectCount	Number of valid objects selected for analysis in the well (Ch2-6 object selection parameters applied)
%SelectedObjects	Percentage of valid objects selected for analysis in the well
ValidFieldCount	Number of fields in which objects were selected for analysis in the well
SelectedObjectCountPerValidField	Average number of objects selected for analysis per valid field in the well
EventType1ObjectCount	Number of objects selected for analysis in the well in which EventType1 occurred
%EventType1Objects	Percentage of objects selected for analysis in the well in which EventType1 occurred
EventType2ObjectCount	Number of objects selected for analysis in the well in which EventType2 occurred
%EventType2Objects	Percentage of objects selected for analysis in the well in which EventType2 occurred
EventType3ObjectCount	Number of objects selected for analysis in the well in which EventType3 occurred
%EventType3Objects	Percentage of objects selected for analysis in the well in which EventType3 occurred

Table 17. *Specific Well Features available with the Cell Health Profiling BioApplication*

Reference Well Features

The BioApplication reports the following Reference Features in the Scan Plate View and as Plate Features in the View application. Reference Features are only applied to a scan if **UseReferenceWells = 1** and the value obtained for the **RefAvgObjectCountPerField** Reference Feature is greater than the value set for the **MinRefAvgObjectCountPerField** Assay Parameter. Instead of listing both features derived from the previous level parameters in Table 18, one entry for the feature will be listed giving both outputs, as shown in the following example for the Channel 1 object size:

- RefObjectSizeCh1Level***Low/High***

Reference Feature	Description
RefAvgObjectCountPerField	Average number of objects per field in reference wells
RefObjectSizeCh1Level***Low/High***	Low/High-response level computed from reference well results for **RefObjectSizeCh1**
RefObjectAreaCh1Level***Low/High***	Low/High-response level computed from reference well results for **RefObjectAreaCh1**
RefObjectShapeP2ACh1Level***Low/High***	Low/High-response level computed from reference well results for **RefObjectShapeP2ACh1**
RefObjectShapeLWRCh1Level***Low/High***	Low/High-response level computed from reference well results for **RefObjectShapeLWRCh1**
RefObjectTotalIntenCh1Level***Low/High***	Low/High-response level computed from reference well results for **RefObjectTotalIntenCh1**
RefObjectAvgIntenCh1Level***Low/High***	Low/High-response level computed from reference well results for **RefObjectAvgIntenCh1**
RefObjectVarIntenCh1Level***Low/High***	Low/High-response level computed from reference well results for **RefObjectVarIntenCh1**
RefTargetTotalIntenChNLevel***Low/High***	Low/High-response level computed from reference well results for **RefTargetTotalIntenChN**
RefTargetAvgIntenChNLevel***Low/High***	Low/High-response level computed from reference well results for **RefTargetAvgIntenChN**
RefTargetAreaChNLevel***Low/High***	Low/High-response level computed from reference well results for **RefTargetAreaChN**
RefTargetVarIntenChNLevel**Low/High**	Low/High-response level computed from reference well results for **RefTargetVarIntenChN**

Table 18. *Reference Well Features available with the Cell Health Profiling BioApplication. *Note that "ChN" refers to Channels 2-6.*

Chapter

3

Using the Cell Health Profiling BioApplication

This chapter provides a detailed description of the use and configuration of the Cell Health Profiling BioApplication. The Cell Health Profiling BioApplication is for users who want a versatile tool that they can apply towards many different biological targets and require the flexibility in defining the regions of the cell from where they want to make measurements. Thus, the Cell Health Profiling BioApplication is for users who feel comfortable in configuring and optimizing such an application for their particular biological situation.

The section in this chapter on configuring the BioApplication for different use cases will guide you in modifying the Assay Protocols for four biological examples. This is done to demonstrate configurations of the BioApplication to suit different biological situations, so that you can use similar approaches in optimizing the application toward your own particular biology. Familiarity with use of the Scan software application and adapting protocols for different situations, magnifications, and dyes is assumed. If you are unfamiliar with these procedures, please refer to the appropriate User's Guide. If you do not want the flexibility of this BioApplication, you are encouraged to use a more specific Cellomics BioApplication that is targeted toward specific use cases. Specific BioApplications have been optimized for specific use cases and thus can be more rapidly implemented.

Assay-Specific Procedures for Optimizing the BioApplication

Input Parameter Settings That May Require Adjustment

Assay and Channel Selection Parameters

These Assay Parameters are described in detail in Chapter 2. There are four main classes of parameters that influence the cell-level outputs of the image analysis algorithm: image processing parameters, Object Selection Parameters, region size modifiers, intensity threshold settings, parameters for detection of responder cells, and Event Definition parameters.

Image Processing Parameters

The main Assay Parameters that affect image processing prior to extraction of cellular measurements from images are **BackgroundCorrectionCh*N***, **ObjectSmoothFactorCh1,** and **ObjectSegmentationCh1**. Of these, **BackgroundCorrectionCh*N*** is applied independently to images in any option of channels, while **ObjectSmoothFactorCh1** and **ObjectSegmentationCh1** are only applied to images in Channel 1. The optimal settings for **BackgroundCorrectionCh*N*** and **ObjectSmoothFactorCh1** Assay Parameters are highly dependent on the targets being measured. Changing the **ObjectSmoothFactorCh1** Assay Parameter value can significantly impact object identification and morphological measurements made in Channel 1. If cell nuclei are used as objects, **BackgroundCorrectionCh*N* = 100** and **ObjectSmoothFactorCh1 = 2** are recommended as starting values. **ObjectSegmentationCh1** should only be used for cells that grow in clumps.

Object Selection Parameters

If an object in Channel 1 falls within the range specified by the Object Selection Parameters, then this object is selected for analysis. Altering these parameters will accept or reject objects based on their intensity or shape. Thus, objects that are too bright can be excluded by restricting the range of the **ObjectAvgIntenCh1** or **ObjectTotalIntenCh1** intensity parameters. **ObjectAvgIntenCh1** is particularly useful to eliminate dim, non-cellular objects. Similarly, large or strangely shaped objects can be excluded by controlling the morphological criteria set by the **ObjectAreaCh1**, **ObjectShapeP2ACh1**, and **ObjectShapeLWRCh1** selection parameters. Additionally, selected objects in Channels 2-6 have a range of intensity-based Object Selection Parameters to enable further selection of objects/cells for analysis.

Region Size Modifiers

The next critical set of Assay Parameters includes those that define the measurement region in Channels 2-6. The **TargetRingWidthCh*N*** Assay Parameter is used to choose between the Circ and Ring regions in Channels 2-4 (entering a value of **0** results in use of the Circ region). The **TargetCircModifierCh*N*** Assay Parameter allows alteration of the size of the Circ region if it is selected for the target whereas **TargetRingWidthCh*N*** and **TargetRingDistanceCh*N*** control the extent of the Ring region. The Circ and Ring regions may be modified using an intensity threshold. Note that the measurement regions can be set independently in each of Channels 2-6.

Intensity Threshold Settings for the Object Identification Method

In certain situations, images are better analyzed by one intensity thresholding method versus another. The **Isodata Threshold** method selects a certain percentage of the brightest pixels for analysis. This is well suited for images with clearly defined objects, such as labeled nuclei, and is fairly indifferent to intensity variations between different experiments. Larger **Isodata Threshold** values result in rejection of dimmer pixels in the image. However, for images with low signal/noise, the **Isodata Threshold** method will still choose the required percentage of bright pixels, which may turn out to be non-specific noise. This type of image is better suited to the **Fixed Threshold** method, where only pixels that have an intensity over a specific fixed threshold are chosen for analysis. The **Fixed Threshold** method does not rely on a pixel's relative intensity in comparison to the intensity distribution for all of the pixels in the image, but instead relies only on the absolute intensity of the pixel alone. However, the **Fixed Threshold** method is susceptible to varying illumination conditions between different experiments. The effect of varying illumination can be minimized if the autoexposure method is used to set exposure times and the **BackgroundCorrectionCh*N*** Assay Parameter is used to bring the intensity range of different images within the same range.

Configuring Cell Health Profiling for Different Use Cases

The goal of this section is to demonstrate configuration of this BioApplication for a range of distinct use cases. The examples illustrate the versatility and potential of this BioApplication. For each example, a table summarizing the biology, along with recommended assay output features corresponding to each measured target is provided. In each case, the features shown are Cell Features; corresponding Well Features can be determined using Table 1. Note that upper and lower levels will have to be set (manually or via Reference Wells) for all relevant Cell Features after analysis of magnitude and variation of individual Cell Feature values and assessment of cellular responses to test compounds. A brief discussion on Assay Parameters that will have to be optimized and cellular Event Definitions that may be applied to assay results are also provided. Event Definitions are provided as logical statements employing

Boolean operators and upper and lower limits for values associated with specific Cell Features that can be defined using the BioApplication Event Wizard.

Example 1: Ranking Cytotoxic Events

It is often valuable to rank the toxicity of different chemical entities according to the acuteness of cellular responses displayed upon treatment. Ranking requires selection of cell health indicators that report different levels of toxicity, i.e., from moderate to acute. For example, disruption of $\Delta\Psi_m$ can be chosen as an indicator of moderate toxicity, while plasma membrane permeability and changes in nuclear morphology can be used as indicators of acute toxicity (Haskins et al. 2001). Table 19 lists a set of cell health indicators that can be measured to rank toxic challenges. Combinations of these, defined by logic statements, can be used to further qualify and rapidly analyze individual cellular responses. Also listed are relevant assay output features reported with this BioApplication.

Cellular Target	Channel	Fluorophore Emission	Measurable properties	Relevant Cell Health Profiling Output Feature
Nuclear Morphology	1	Blue	• Nuclear morphology changes such as condensation, fragmentation or swelling • Indicates whether morphological feature value of a cell nucleus is greater/less than user-defined limits • Indicates acute toxicity	**ObjectSizeCh1** **ObjectAreaCh1** **ObjectShapeP2ACh1** **ObjectShapeLWRCh1** **ObjectVarIntenCh1**
Plasma membrane permeability	2	Green	• Decrease in integrity of plasma membrane, allowing entry of fluorophores that are otherwise impermeant to live cells • Indicates whether the membrane permeability of a cell is greater/less than user-defined limits • Indicates acute toxicity	**TargetAvgIntenCh2** (Measurement region = Circ)
Mitochondrial transmembrane potential ($\Delta\Psi_m$)	3	Orange	• Average intensity of mitochondria labeled with a cationic fluorophores that accumulate in mitochondria as a result of $\Delta\Psi_m$ • Indicates whether the average $\Delta\Psi_m$ of a cell's mitochondria is greater/less than user-defined limits • Indicates moderate toxicity	**TargetAvgIntenCh3** (Measurement region = Ring)

Table 19. *Cellular properties measured to rank toxic events according to acuteness with Cell Health Profiling.*

Assay Parameter Optimization

- **Channel 1:** Set morphology and intensity-related object selection criteria for Channel 1 to be as wide as possible to capture changes in nuclear morphology and cell cycle. Use of the **Isodata Threshold** Object Identification Method is recommended if nuclei are brightly labeled.
- **Channel 2:** Use an unmodified Circ region (**TargetRingWidthCh2** = **0**, **TargetCircModifierCh2** = 0) if cell impermeant nucleic acid label that only labels

nuclei of permeabilized cells. If fluorophore labels whole cells, use an expanded Circ to include the entire cell. Use of an intensity threshold is not recommended (set Object Identification Method to **None**).

- **Channel 3:** Use a Ring region (**TargetRingWidthCh3** greater than 0) modified with an intensity threshold set to detect brightly labeled mitochondria in the cytoplasmic region. Use of an intensity threshold is recommended (set Object Identification Method to **Fixed Threshold**).

Suggested Event Definitions

- **ObjectSizeCh1** OR **TargetAvgIntenCh2:** Change in nuclear shape or increase in plasma membrane permeability, indicating acute toxicity
- **TargetAvgIntenCh3** ANDNOT **ObjectSizeCh1** ANDNOT **TargetAvgIntenCh2**: Decrease in $\Delta\Psi_m$ in the absence of both changes in nuclear morphology and plasma membrane permeability, indicating moderate toxicity

Example 2: Toxic Effects on Mitochondrial Activity

Mitochondrial function is well known to be one of the primary targets of agents that negatively impact cell health (Lemasters et al. 1999; Zamzami et al. 1997). A widely accepted indicator of mitochondrial function is maintenance of $\Delta\Psi_m$. However, in some cases, measurement of $\Delta\Psi_m$ can be complicated by mitochondrial proliferation induced by several compounds (Karbowski et al. 2000; Camilleri-Broet et al. 1998). As a change in mitochondrial activity is typically an early indication of toxicity, measurement of mitochondrial mass and $\Delta\Psi_m$ can be supplemented with measurement of nuclear morphology to assess progression through apoptosis or necrosis. Table 20 lists a set of cell health indicators that can be measured to rank toxic challenges. Combinations of these, defined by logic statements, can be used to further qualify and rapidly analyze individual cellular responses. Also listed are relevant assay output features reported with this BioApplication.

Cellular Target	Channel	Fluorophore Emission	Measurable Properties	Relevant Cell Health Profiling Output Feature
Nuclear morphology	1	Blue	• Nuclear morphology changes such as condensation, fragmentation or swelling • Indicates whether morphological feature value of a cell nucleus is greater/less than user-defined limits	**ObjectSizeCh1** **ObjectAreaCh1** **ObjectShapeP2ACh1** **ObjectShapeLWRCh1** **ObjectVarIntenCh1**
Mitochondrial transmembrane potential ($\Delta\Psi_m$)	2	Orange	• Average intensity of mitochondria labeled with a cationic fluorophore that accumulates in mitochondria as a result of $\Delta\Psi_m$ • Indicates whether the average $\Delta\Psi_m$ of a cell's mitochondria is greater/less than user-defined limits	**TargetAvgIntenCh2** (for Ring region)
Mitochondrial mass	3	Green	• Total intensity of fluorophore that labels mitochondria independent of $\Delta\Psi_m$ • Indicates whether a cell's total mitochondrial mass is greater/less than user-defined limits	**TargetTotalIntenCh3** (for Ring region)

Table 20. *Cellular properties measured to analyze toxic effects on mitochondrial activity with Cell Health Profiling.*

Assay Parameter Optimization

- **Channel 1:** See recommendations in Example 1
- **Channels 2-3**: See recommendations in Example 1

Suggested Event Definitions

- **TargetAvgIntenCh2** ANDNOT **TargetTotalIntenCh3**: Decrease in $\Delta\Psi_m$ in the absence of an increase in mitochondrial mass, as shown by protonophores.
- **TargetAvgIntenCh2** OR **TargetTotalIntenCh3**: Increase in mitochondrial mass or decrease in $\Delta\Psi_m$. Toxins such as taxol are known to affect both $\Delta\Psi_m$ and mitochondrial mass

- **TargetTotalIntenCh3** ANDNOT **ObjectVarIntenCh1**: Increase in the mitochondrial mass in the absence of changes in nuclear shape, indicating an early toxic event involving mitochondrial function
- **TargetAvgIntenCh2** ANDNOT **ObjectVarIntenCh1**: Decrease in $\Delta\Psi_m$ in the absence of changes in nuclear shape, indicating an early necrotic/apoptotic event involving mitochondrial function

Example 3: Toxic Effects Impacting Cytoskeletal and Genomic Integrity

Several compounds are known to exert cytotoxic effects by affecting cytoskeletal and/or genomic integrity (Kayalar et al. 1996; Trump and Berezesky 1995). Cytoskeletal components that are often targeted include the microfilament and microtubule networks, while compromised genomic integrity can be manifested as DNA breaks and/or degradation. Table21 outlines a specific set of cell health indicators that can be measured using the Cell Health Profiling BioApplication to analyze the specific pattern of cytoskeletal and/or genomic damage as well as relevant assay output features reported with this BioApplication.

Cellular Target	Channel	Fluorophore Emission	Measurable Properties	Relevant Cell Health Profiling Output Feature
Total DNA content	1	Blue	• Total intensity within nucleus indicating extent of DNA degradation through the enzymatic action of DNases • Indicates whether a cell's total DNA content is greater/less than user-defined limits	**ObjectTotalIntenCh1**
DNA damage	2	Green	• Average or total Intensity within the nuclear region indicating extent of breaks in DNA polymer (TUNEL) • Indicates whether the average number of DNA strand breaks for a given cell is greater/less than user-defined limits	**TargetAvgIntenCh2** (for Circ region) **TargetTotalIntenCh2** (for Circ region)
F-actin content	3	Orange	• Total intensity indicating relative mass of polymerized actin per cell • Indicates whether the total F-actin content for a given cell is greater/less user-defined limits	**TargetTotalIntenCh3** (for Circ region)
Cell morphology	3	Orange	• Area occupied by the mass of polymerized actin per cell • Indicates whether the area occupied by a given cell is greater/less than user-defined limits	**TargetAreaCh3** (for Circ region)
Microtubule content	4	Far Red	• Average intensity from cytoplasmic region indicating density of microtubules in the cytoplasmic region • Indicates whether a cell's microtubule density is greater/less than user-defined limits	**TargetAvgIntenCh4** (for Ring region)

Table 21. *Measurement of cytoskeletal and genomic perturbation with Cell Health Profiling.*

Assay Parameter Optimization

- **Channel 1:** Set intensity-related object selection criteria for Channel 1 to be as wide as possible to capture changes in total DNA content. Use of the **IsodataThreshold** Object Identification Method is recommended if nuclei are brightly labeled.
- **Channel 2:** Use a Circ region that coincides with the nuclear region identified in Channel 1 (**TargetRingWidthCh2 = 0**, **TargetCircModifierCh2** = **0**). Use of an intensity threshold (Object Identification Method = **Fixed Threshold**) may be required to eliminate non-specific background signal.
- **Channel 3:** Modify the Circ region to include the entire cell (**TargetRingWidthCh3 = 0**, **TargetCircModifierCh3** greater than 0). A low intensity threshold is recommended to eliminate any extracellular regions as this could confound the cell morphology measurement. It is recommended that cells be plated sparsely to distinguish individual cells, thus enabling accurate measurement of morphological changes.
- **Channel 4:** Same as for Channel 3. A higher intensity threshold may be applied to eliminate signal from unpolymerized tubulin.

Suggested Event Definitions

In this case such statements may be used to stage progress along the pathway to cell demise or identify the initial cellular target.

- **ObjectTotalIntenCh1** AND **TargetAvgIntenCh4**: Changes in total DNA content and microtubule mass (often correlated because of the importance of microtubule dynamics for mitosis).
- **ObjectTotalIntenCh1** AND **TargetAreaCh3** AND **TargetTotalIntenCh3**: Degradation of DNA and cell shrinkage, and reduction in F-actin content, which are all late stage cytotoxicity indicators.
- **TargetTotalIntenCh3** OR **TargetAreaCh3** OR **TargetAvgIntenCh4:** Changes in F-actin content, cell shrinkage, or microtubule mass (which are all cytoskeletal targets).
- **ObjectTotalIntenCh1** OR **TargetAvgIntenCh2**: DNA degradation or induction of breaks in DNA polymer (both genomic targets).

Example 4: Distinguishing Apoptosis from Necrosis

The Cell Health Profiling BioApplication can also be used to distinguish between apoptotic and necrotic processes at the level of individual cells. For example, consider the translocation of PS into the outer leaflet of the plasma membrane as an indication of progress along the apoptotic pathway. Translocation of PS is traditionally measured via labeling with fluorescently labeled Annexin V (Vermes et al. 1995) and is greatly enhanced by simultaneous measurement of plasma membrane permeability, which allows differentiation of PS translocation from labeling of internal PS of necrotic (permeabilized) cells. In addition, nuclear morphological changes can also be monitored as the nature of nuclear morphological changes can be an indicator of apoptosis, fragmentation or condensation, or necrotic cell death (condensation or swelling). Table 22 lists a set of cell health indicators that can be measured to rank toxic challenges. Combinations of these, defined by logic statements, can be used to further qualify and rapidly analyze individual cellular responses. Also listed are relevant assay output features reported with this BioApplication.

Cellular Target	Channel	Fluorophore Emission	Measurable Properties	Relevant Cell Health Profiling Output Feature
Nuclear morphology	1	Blue	• Nuclear morphology changes such as condensation, fragmentation or swelling • Indicates whether morphological feature value of a cell nucleus is greater/less than user-defined limits	**ObjectSizeCh1** **ObjectAreaCh1** **ObjectShapeP2ACh1** **ObjectShapeLWRCh1** **ObjectVarIntenCh1**
Translocation of phosphatidyl-serine	2	Green	• Total or average intensity of annexin V used as a marker for phosphatidylserine	**TargetAvgIntenCh2** (for Circ region) **TargetTotalIntenCh2** (for Circ region)
Plasma membrane permeability	3	Far Red	• Decrease in integrity of plasma membrane, allowing entry of fluorophores that are impermeant to live cells • Indicates whether the plasma membrane permeability of a cell is greater/less than user-defined limits	**TargetAvgIntenCh3** (for Circ region)

Table 22. *Cell Health Profiling output features for measurement of loss of membrane asymmetry associated with phosphatidylserine distribution.*

Assay Parameter Optimization

- **Channel 1:** See recommendations from Example 1
- **Channel 2:** Use a Circ region that covers the entire cell (**TargetRingWidthCh2** = **0**, **TargetCircModifierCh2** greater than 0). Use of an intensity threshold (Object Identification Method = **Fixed Threshold**) may be required to eliminate non-specific background signal.
- **Channel 3**: Use the Circ region if you are using a cell impermeant nucleic acid label that labels only the nuclei of permeabilized cells. If fluorophore labels whole cells, use an expanded Circ to include the entire cell. Use of an intensity threshold is not recommended (set Object Identification Method = **None**).

Suggested Event Definitions

- **TargetAvgIntenCh2** ANDNOT **TargetAvgIntenCh3**: Identifies apoptotic annexin V-labeled cells while rejecting permeabilized cells, which also label with annexin V.
- **ObjectShapeP2ACh1** OR **TargetAvgIntenCh3**: Identifies cells in late stages of apoptosis/necrosis given the occurrence of changes in nuclear morphology or membrane permeability.
- **TargetAvgIntenCh2** ANDNOT **TargetAvgIntenCh3** AND **ObjectShapeP2ACh1**: Detects cells in late-stage apoptosis (evidenced by changes in nuclear morphology).

Event Definition Using the BioApplication Event Wizard

The BioApplication Event Wizard is a software tool that is designed to allow entry of 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 Cell Health Profiling BioApplication. This section provides a detailed description of the operation of the BioApplication Event Wizard. The Wizard must only be used after the Cell Health Profiling BioApplication has been installed on your computer.

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.

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

STEP I

1) Create a protocol using the Scan software application without defining Events. Set optimized parameter values (upper and lower limits) for detection of responder cells associated with Cell Features to be used for Event Definition.
2) Save protocol.
3) Close the Scan 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) Scan the assay plate.
3) 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 reanalyze previously acquired images using modified Event Definitions.

Features

The following features are included in the BioApplication Event Wizard:

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

Steps for Running the Event Wizard with Cell Health Profiling

Before Running the Event Wizard...

1) Create a protocol using the Cell Health Profiling 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 protocol.
3) Close the Scan software application (suggested).

Starting the BioApplication Event Wizard

It is strongly suggested to close the instrument or Scan software application 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

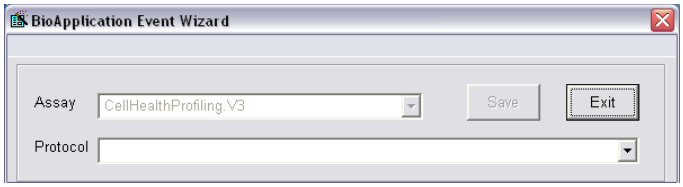
The first window that is displayed (see below) allows selection of any Cell Health Profiling Assay Protocol. Note that Event Definitions can only be specified for pre-existing Assay Protocols.

To select an Assay Protocol,

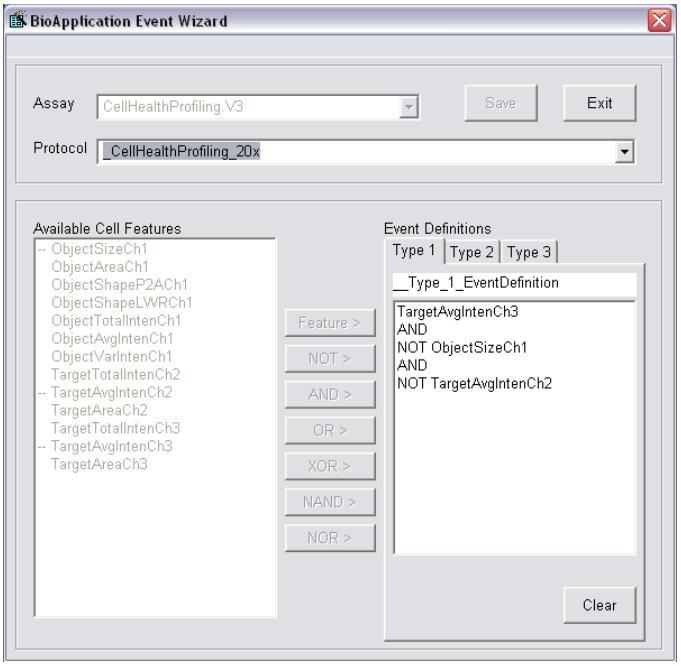
1) From the **Assay** drop-down menu, select **CellHealthProfiling.V4**

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

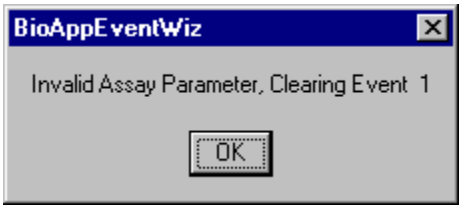
3) Select the desired Assay Protocol from the list.



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 (X=1-3) are automatically validated. For example, if Event Definition is invalidated 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 protocol is Read-Only, you will only be able to view the Event Definitions and a message above the **Save** button will indicate that the protocol cannot be edited.

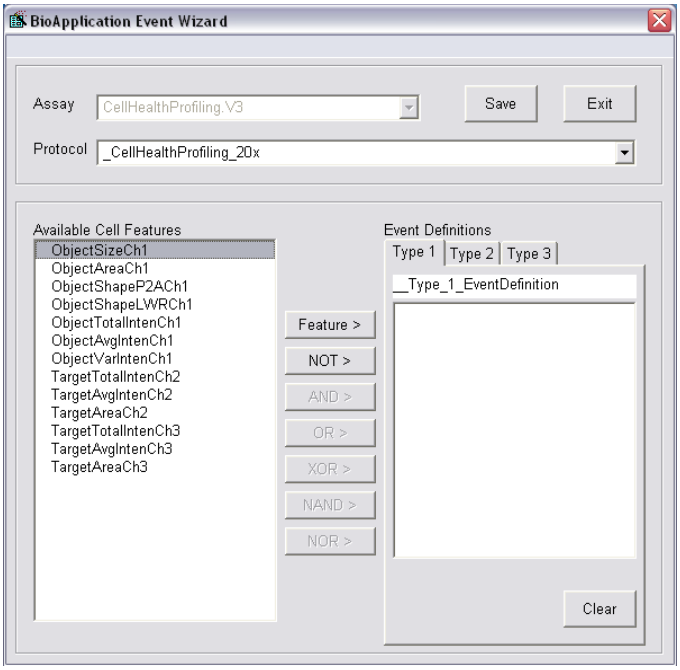
Defining Events

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

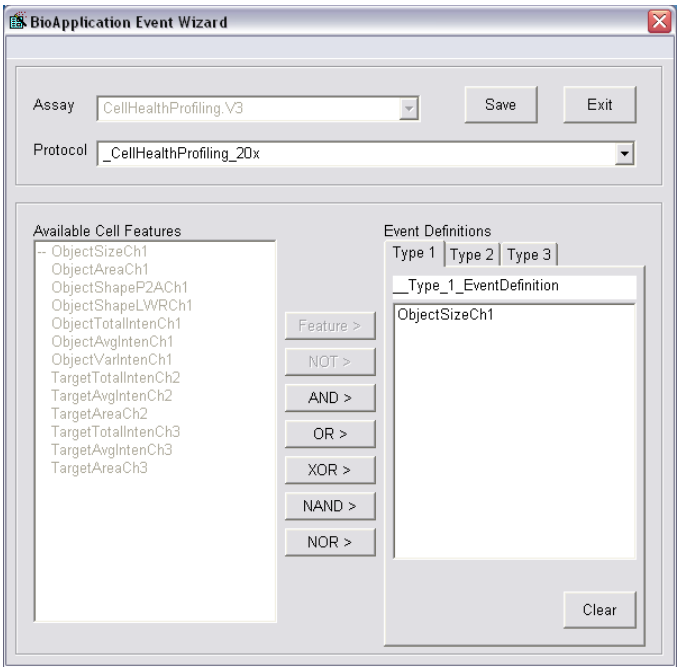
Once the protocol has loaded, each Event Definition can be viewed by clicking on the appropriate tab (Type 1-3). The procedure to be followed when constructing Event Definitions is described in the sequence of screenshots below. Cell Features are combined with Boolean operators to produce Event Definitions. Any Cell Feature can be selected by clicking on the feature name in the Available Cell Features list and then pressing 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,

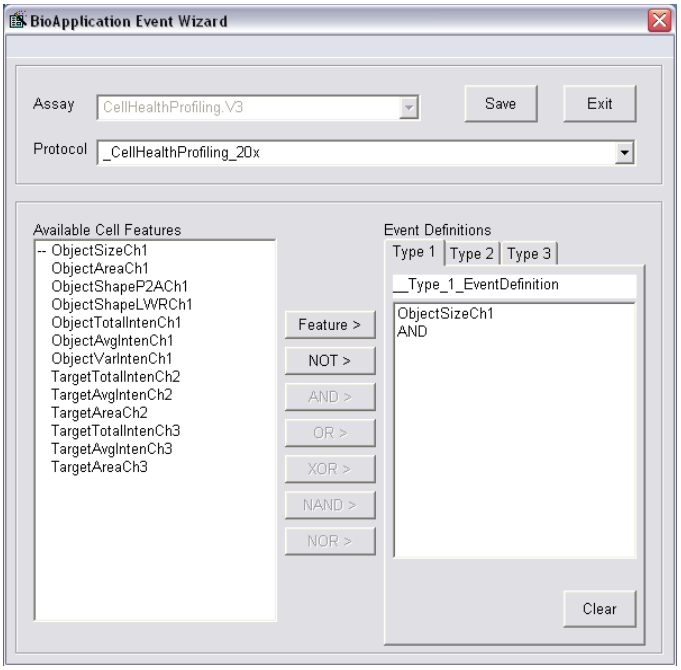
1) Select the Event Definition that you want to specify or edit by clicking on the **Type 1**, **Type 2**, or **Type 3** tab.
2) Select the desired Cell Feature by clicking on the feature name from the Available Cell Features list. In this example, the **ObjectSizeCh1** feature is selected.



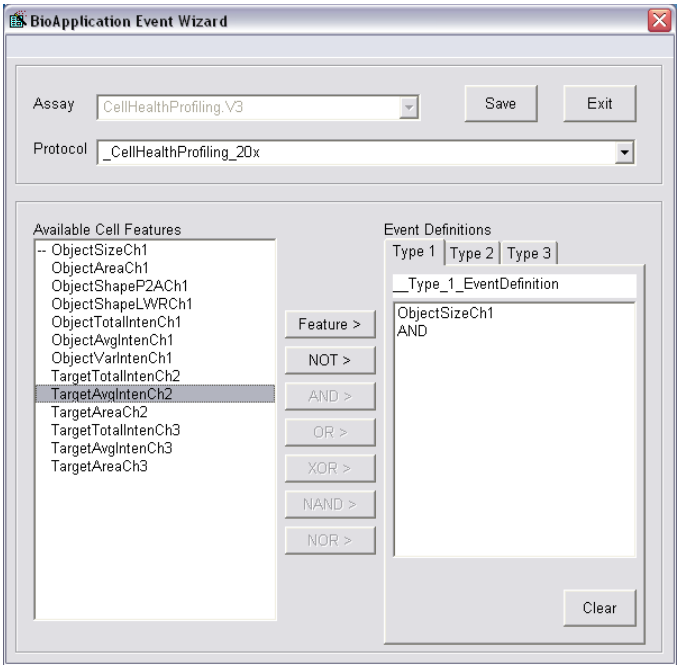
3) Click the **Feature>** button to transfer the Cell Feature into the Event Definition (see below).



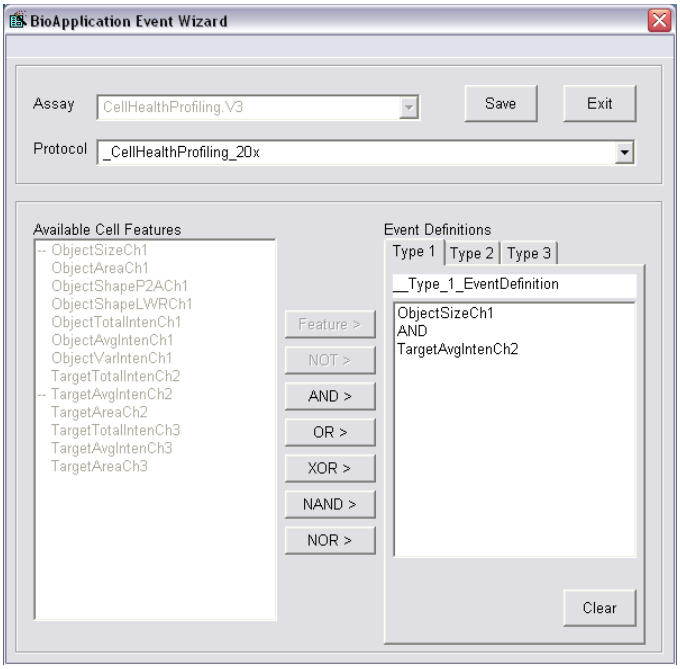
4) Click on the desired Operator button to transfer it into the Event Definition. In this example, the **AND** operator is selected (see below).



5) Repeat the cycle for adding another Cell Feature to the Event Definition. In this case, the Cell Feature **TargetAvgIntenCh2** has been selected for addition.

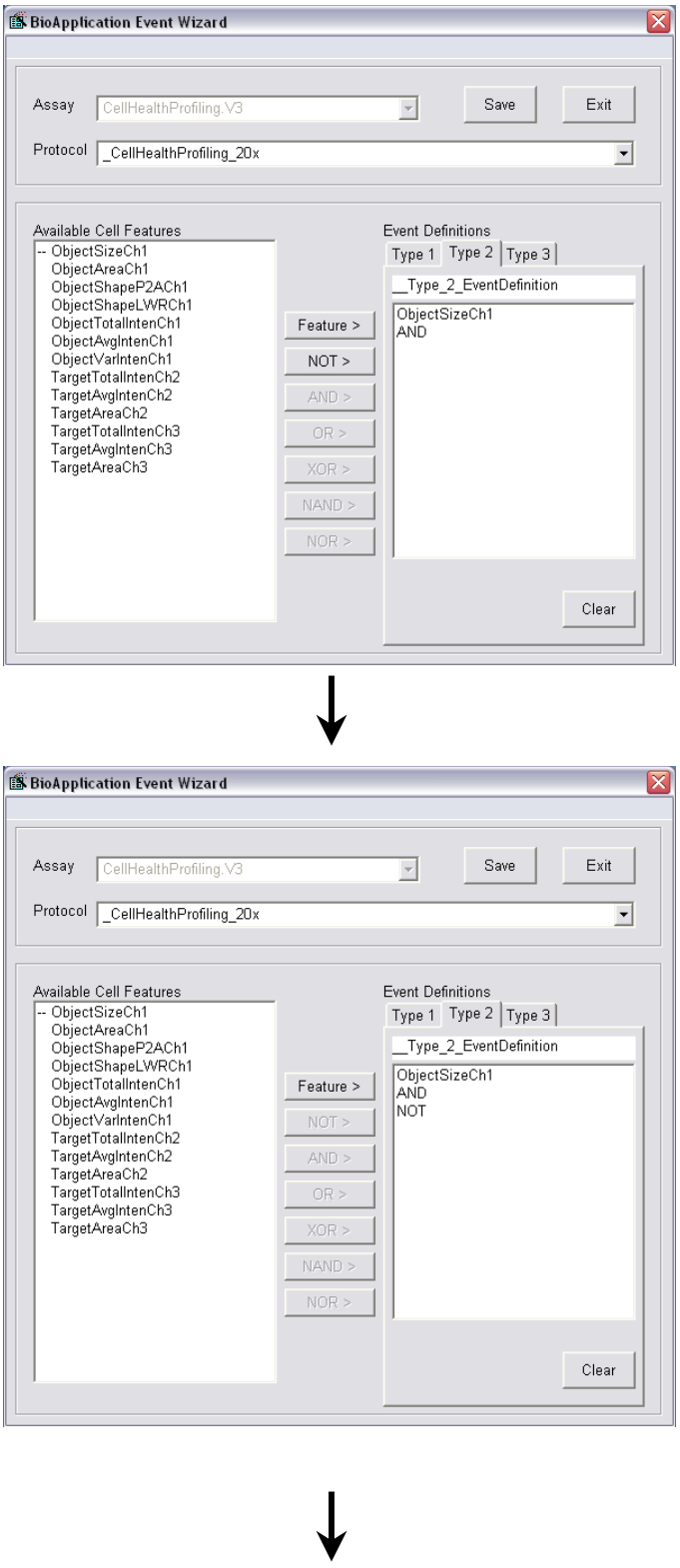


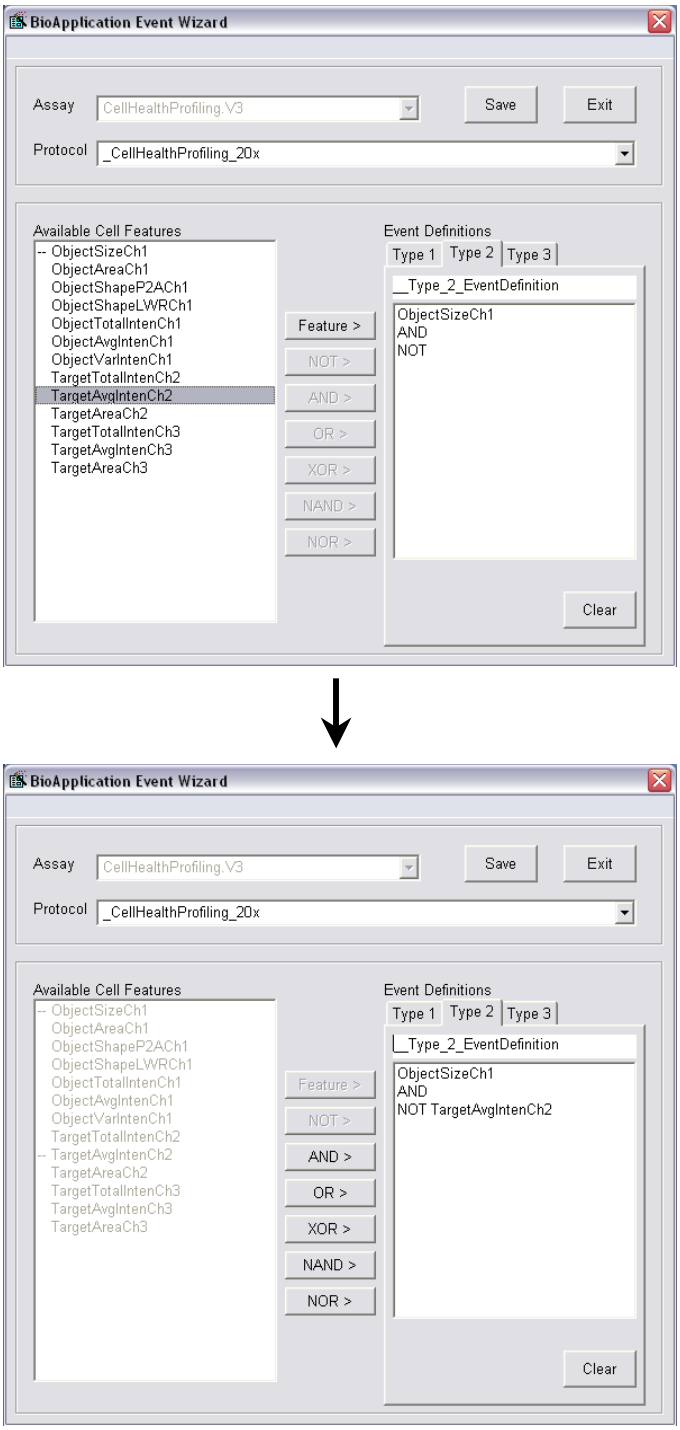
6) As before, click on the **Feature >** button to transfer the Cell Feature into the Event Definition (see below).



Type_1_EventDefinition is: **ObjectSizeCh1** AND **TargetAvgIntenCh2**

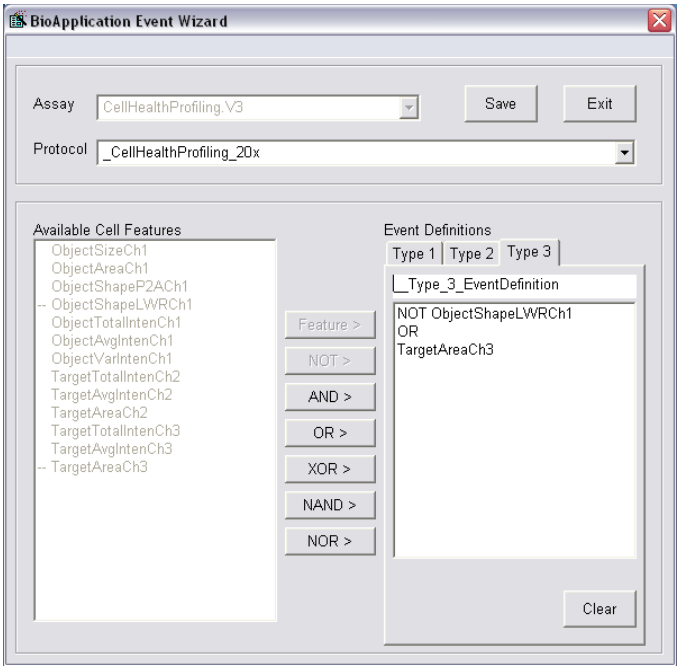
Entry of a logical operator may be followed by entry of another Cell Feature. Alternatively, the operators **AND** and **OR** can be inverted by adding the **NOT** operator by pressing the **NOT >** button prior to selecting the Cell Feature, as shown below.





Type_2_EventDefinition is: **ObjectSizeCh1** ANDNOT **TargetAvgIntenCh2**

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.



Type_3_EventDefinition is: NOT **ObjectShapeLWRCh1** OR **TargetAreaCh3**

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.

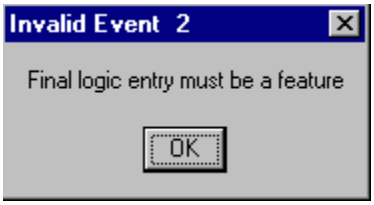
To save the updated Assay Protocol,

- 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

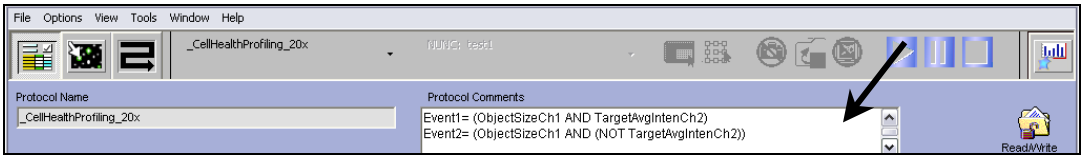
The BioApplication Event Wizard has the capability to automatically validate Event Definitions. If Event Definition validation fails, the protocol will not be saved. For example, Event Definitions are considered invalid if they do not end with a Cell Feature. In this case, clicking on the **Save** button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

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



Chapter

4

iDev Software Workflow

This chapter describes the tasks in the workflow for Protocol optimization of the Cell Health Profiling 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 operations such as background removal methods and values for each of the channels in the Protocol.

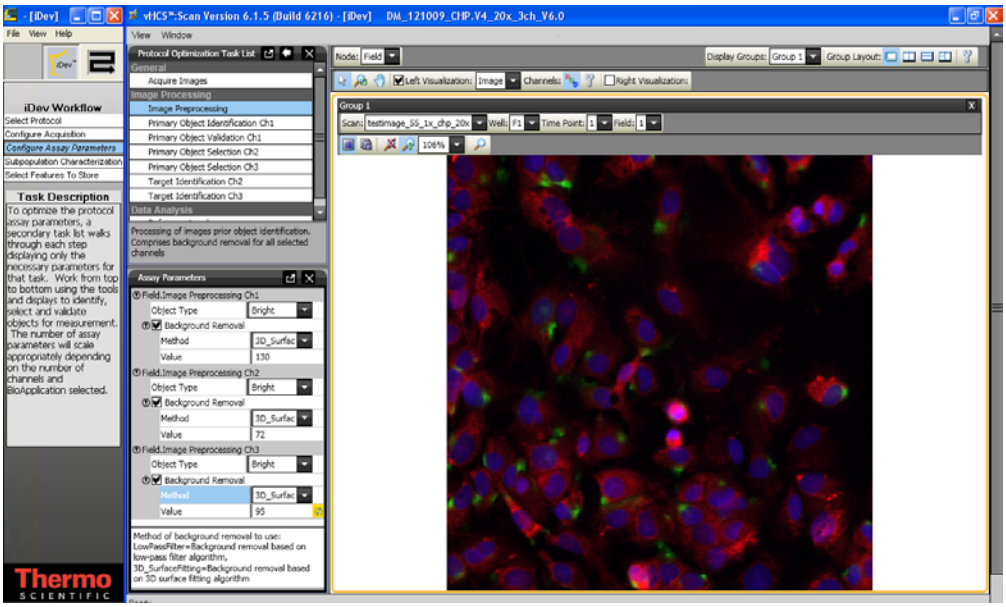


Figure 21. *Protocol Optimization Task – Image Preprocessing*

Primary Object Identification Ch1

Primary Object Identification is the identification of objects in the Channel 1 image. The task, involving setting up methods and values for primary object identification, object smoothing, and object segmentation, is only applied to Channel 1 objects.

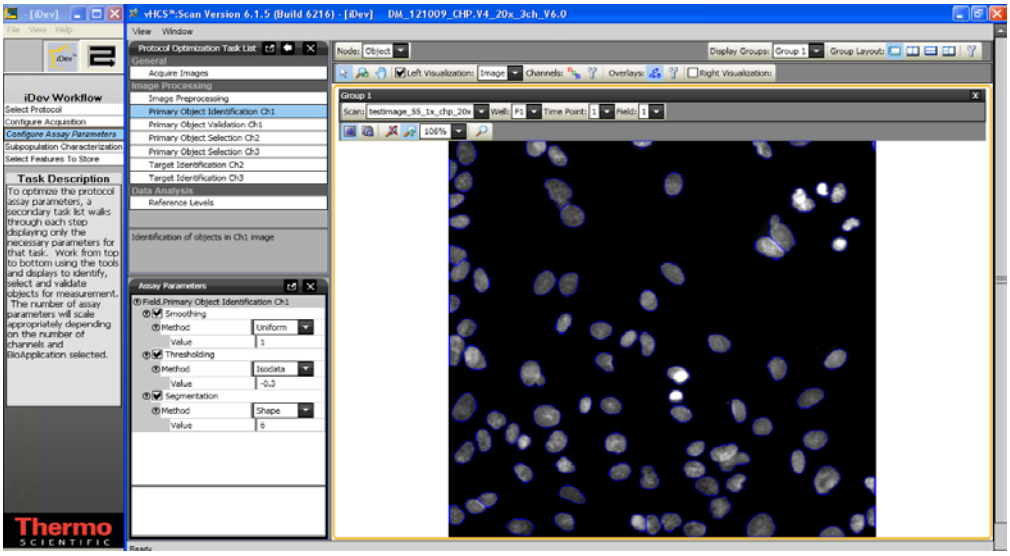


Figure 22. *Protocol Optimization Task – Primary Object Identification Ch1*

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 also determine if objects that are in the border of the field are included or rejected from analysis.

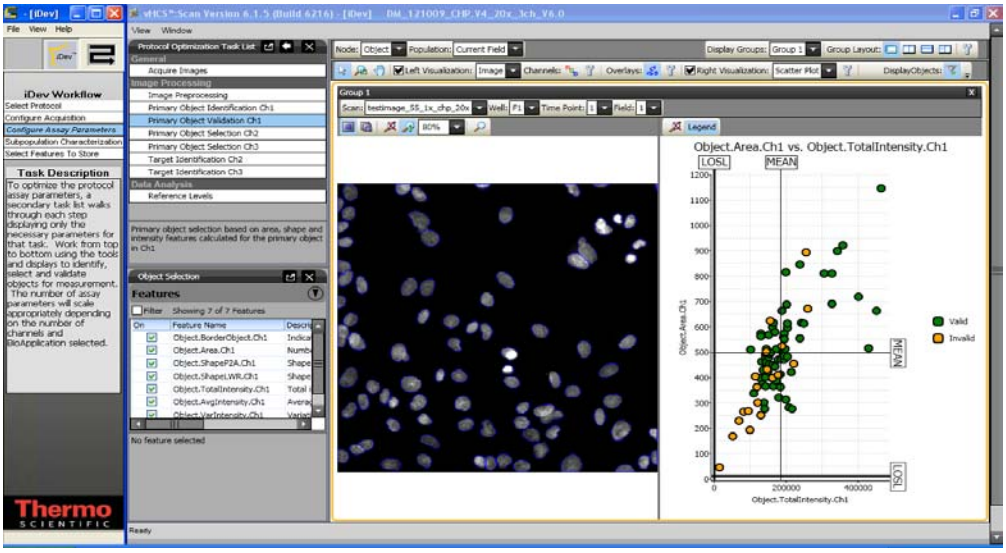


Figure 23. *Protocol Optimization Task – Primary Object Validation Ch1*

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 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.

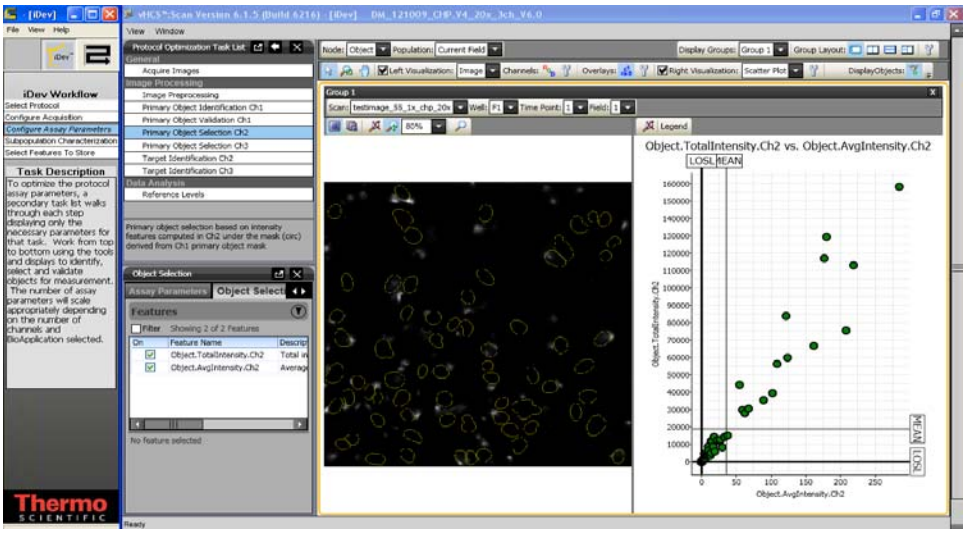


Figure 24. *Protocol Optimization Task – Primary Object Selection Ch2 through ChN*

Target Identification Ch2 through ChN

Target Identification is the identification of targets in Channels 2 through Channel N 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 intrest (ROI) and set the identification method and threshold for your target in Channel 2. The ROI can either be a “Circ” or a “Ring”.

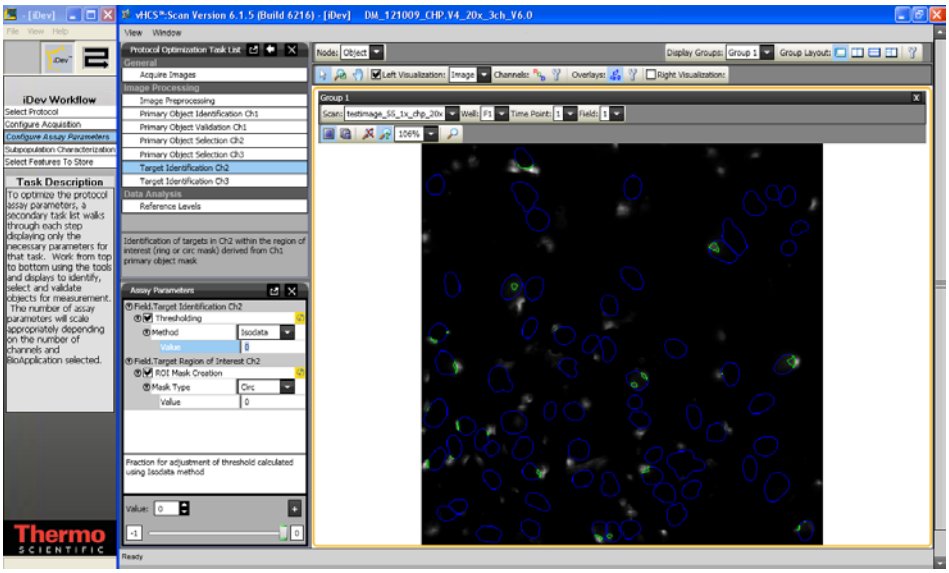


Figure 25. *Protocol Optimization Task – Target Identification*

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.

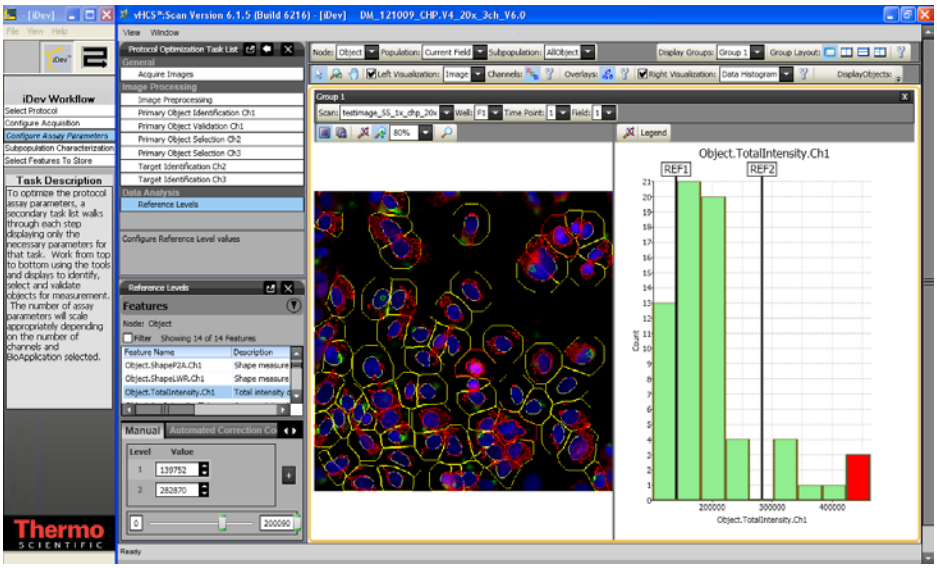


Figure 26. *Protocol Optimization Task – Reference Levels*

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.

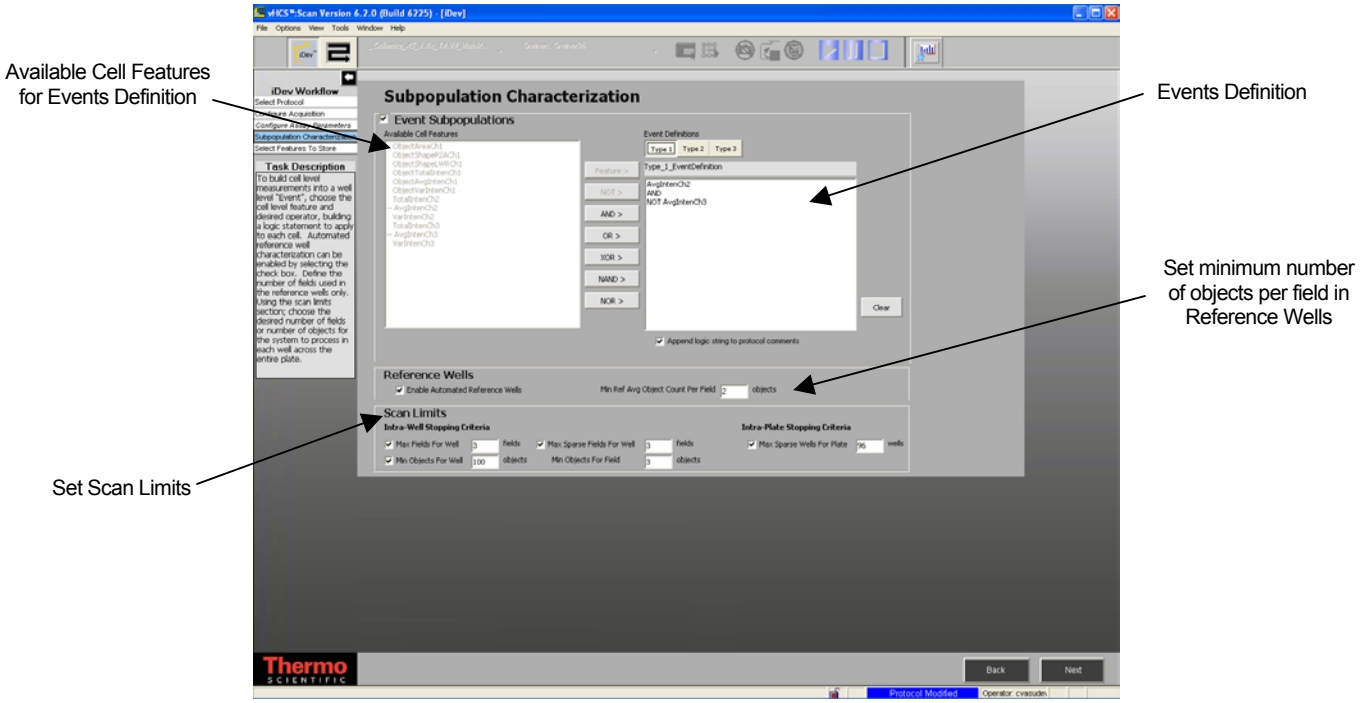


Figure 27. *Subpopulation Characterization Task*

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