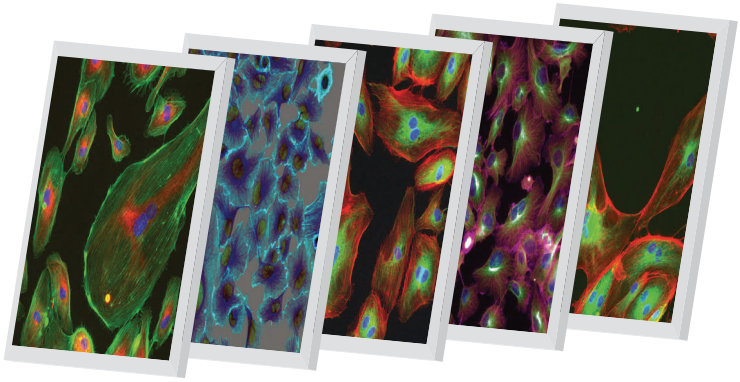
# Thermo Scientific Cellomics® Target Activation V4

## BioApplication Guide



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# Cellomics® Target Activation BioApplication Guide

*V4 Version*

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Chapter

1

# Overview of the Target Activation BioApplication

High Content Screening (HCS) uses fluorescence-based reagents, advanced optical imaging and image analysis software (BioApplications), to analyze targets and physiological processes in single cells. This guide provides a brief description for using a multiparametric HCS BioApplication, Target Activation. It contains the following chapters:

**Chapter 1** provides an overview of the BioApplication, including cell biological situations in which it can be applied.

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

**Chapter 3** provides brief guidance on how to adjust some basic parameter settings as well as using the Events Wizard.

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

## System Compatibility

The Target Activation BioApplication described in this guide is designed to run on the following platforms:

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

Cellular responses to extracellular and intracellular signals are critical to normal cellular functions. Cellular components involved in these cell-signaling events provide molecular targets for the understanding of therapeutic mechanisms. Activation or deactivation of these targets in response to a stimulus allows you to measure how a cell's function is affected by compounds. The responses of a cell to different stimuli or conditions cause it to be in different physiological states. A cell's expression of GFP-tagged protein or whether a cell is alive or dead are two examples of different cell states. Different cell states or conditions can be measured by the fluorescence of different cellular targets. The ability to measure differences in target activation resulting in a variety of cell states using fluorescence is a powerful assay

approach. A wealth of cellular information can be gathered about the activation of a specific target under a range of cellular conditions. This approach of measuring the fluorescent intensity inside the cell can be applied to a wide variety of biological situations requiring the differentiation of cells between two states.

The Target Activation BioApplication analyzes images acquired by a Cellomics HCS Reader and provides measurements of the intracellular fluorescence intensity on a cell-by-cell basis. The BioApplication is capable of measuring fluorescent targets in up to six channels, where a channel represents a fluorophore or a specific exposure condition. Channel 1 is typically used for identifying individual objects, such as cells or organelles, and provides the basis for the intracellular region where intensity measurements in Channels 1-6 are made. The BioApplication allows you to adjust multiple input parameters to control image analysis, such as the cellular region used for measuring the intensity of a fluorescent indicator.

Finally, this BioApplication enables correlation and classification of multiparametric cellular responses through automated detection of cellular Events that you define. Events are biologically meaningful logic statements comprising individual cellular measurements and standard Boolean operators. Event definition and detection will be described in more detail in later sections. A standalone software application, the *BioApplication Event Wizard*, is provided with this BioApplication to enable automatic detection of the occurrence of multiparametric cellular Events. Events are specified as logic statements that query individual cells of a large cell population. A detailed description of the use of the BioApplication Event Wizard is provided in Chapter 3.

## BioApplication Overview

The cell-based Target Activation BioApplication allows you to make measurements of fluorescent indicators of choice on a cell-by-cell basis in up to six channels. The Channel 1 image contains fluorescently labeled objects that may be whole cells or major cellular organelles/compartments such as nuclei or cytoplasm. The Channel 1 objects are used to identify individual cells and define the basis of the area in Channels 1-6 within which intensity measurements are made. You can contract or expand the mask of the primary object, generated in Channel 1, independently in each of the additional channels. This is accomplished using the **MaskModifierCh*N*** Assay Input Parameter that defines the number of pixels for erosion or dilation (see Figure 1).

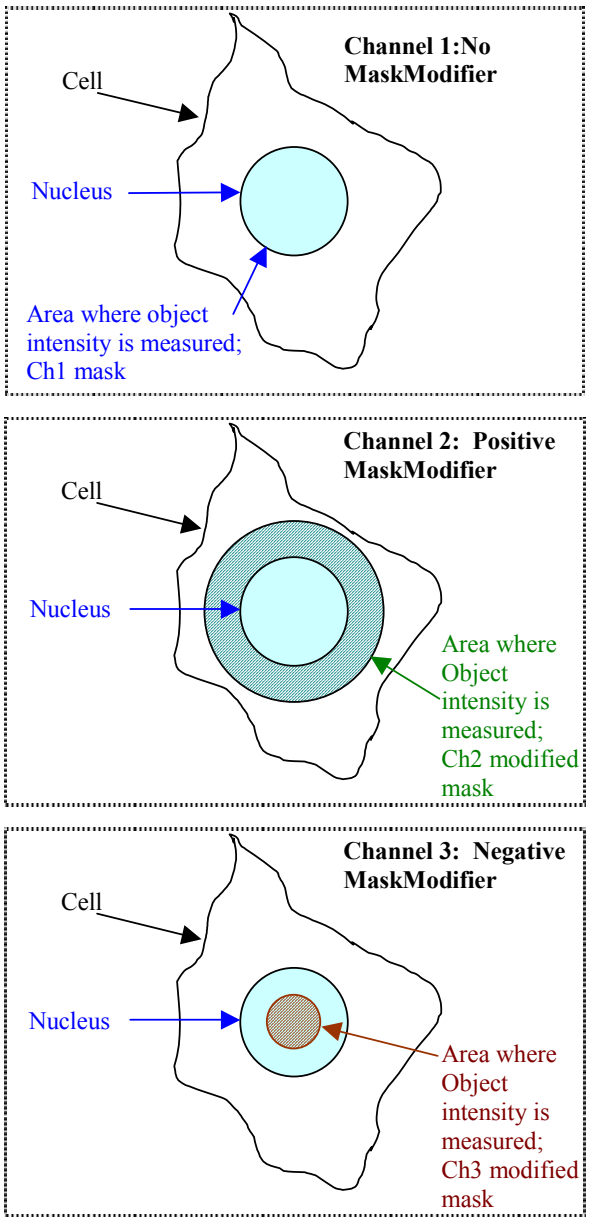



***Figure 1.*** *Schematic of a three-channel assay. Top Panel: Channel 1 measures a fluorescent nuclear stain; the inner circle is the mask. Middle Panel: This mask is dilated for Channel 2 (outer circle, including the inner circle) to encompass much of the cytoplasm without going beyond the cell membrane. Bottom Panel: This mask is eroded for Channel 3 (inner circle) to encompass an area smaller than the nucleus.*

There are two main types: Cell Features, which are measurements of individual cells, and Well Features, which provide well-level data describing the population of cells analyzed. Well Features are derived directly from Cell Features for a particular well.
The main Cell Features of Target Activation, reported in Channels 1-6, are:

- Total fluorescence intensity of each object
- Average fluorescence intensity of each object and
- Variation of fluorescence intensity, a basic quantification of texture, within each object.

This BioApplication also reports the number of cells that are 'responders' for a given Cell Feature. A responder is defined as an object/cell with a Cell Feature value that is outside a range of values that you define. Setting a low and a high threshold specifies this range. The number of responders in a well is compared to the number of all detected objects in the same well. The percentage of responders is then reported as a Well Feature.

The range of values that defines whether a cell is a responder can be set manually or determined automatically using Reference Wells. The Reference Wells feature can be valuable, for instance, when you scan large numbers of plates and want to compare them or when you want to compare plates over time, as it automatically accounts for variations in assay conditions, such as intensity of illumination and brightness of fluorescence.

## Event Definition

Events are patterns of cellular features that indicate the occurrence of a specific biologically meaningful state in an individual cell. The Target Activation BioApplication allows simultaneous definition of up to three Events to enable rapid multiparametric analysis at the level of individual cells, across multiple Cell Features. Events are defined as Assay Parameters and consist of logical statements employing specific Cell Features. These statements are then applied to classify and count responder and non-responder cells for the Cell Features contained in the Event Definition. Figure 2 relays these capabilities and how they interact to enable event definition and detection. Event definitions are created using a stand-alone software tool called the BioApplication Event Wizard. Operation of this software tool is described in more detail in Chapter 3.

Event definition and detection at the level of individual cells of a population requires processing of raw Cell Feature values, i.e., the data extracted from individual cells for all targets being measured. Limits are then applied (manually entered for the ***Feature*Ch*N*LevelHigh** or ***Feature*Ch*N*LevelLow** Assay Parameter or automatically computed via Reference Wells) to identify responder cells for each feature. For the purposes of Event detection, responder cells are defined as those cells showing feature values greater than the upper limit (***Feature*Ch*N*Status** =1) or less than the lower limit (***Feature*Ch*N*Status** = 2) defined initially by ***Feature*Ch*N*LevelHigh** or ***Feature*Ch*N*LevelLow**. It is critical that the upper and lower limits be set accordingly.

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.

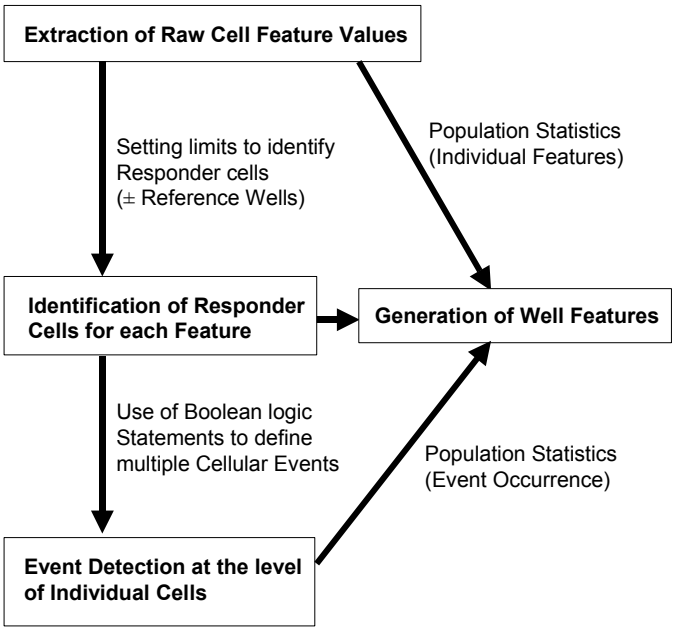



***Figure 2.*** *Schematic showing the process of definition and detection of cellular events.*

## BioApplication Measurements

The BioApplication measures a large number of features in all the dependent channels used. The BioApplication reports both Cell and Well Features. Cell Features are reported for each cell, and Well Features are reported for each well and are derived from the Cell Features for all the cells imaged and analyzed in that well. The following table shows the number of Cell and Well Features available based on the number of channels selected.

Morphological and intensity properties of the primary object in Channel 1, as well as intensity ratios for different regions are also reported. In addition, status information on whether individual cells are beyond a certain range is also reported for certain measured features. The different categories of features measured by the BioApplication for each of the four regions of the cell are summarized in Table 1 and explained in more detail in the sub-sections that follow. Table 1 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.

| Feature Category | Cellular Region/s | Cell Features | Corresponding Well Features |
|---|---|---|---|
| ***Cell and Well-Level Features Reported for Channel 1:*** | | | |
| Cell Counts and Density | Primary Object | Cell Number | ValidObjectCount<br>SelectedObjectCount<br>%SelectedObjects<br>ValidFieldCount<br>SelectedObjectCountPerValidField |
| Morphology and Location | Primary Object | Object Area and Status<br>Object Shape P2A and Status<br>Object Shape LWR and Status | Mean, SD, SE, CV, %Responder |
| | | Top<br>Left<br>Width<br>Height<br>X Centroid<br>Y Centroid | none |
| Intensity: | Primary Object | Total Intensity and Status<br>Average Intensity and Status<br>Variance Intensity and Status | Mean, SD, SE, CV, %Responder |
| Events | Selected Features from Event Wizard | EventTypeProfile<br>EventType1Status<br>EventType2Status<br>EventType3Status | EventType1: CellCount, %<br>EventType2: CellCount, %<br>EventType3: CellCount, % |
| ***Cell and Well-Level Features Reported for Channels 2-6:*** | | | |
| Intensity: | Modified Primary Object | Total Intensity and Status<br>Average Intensity and Status<br>Variance Intensity and Status | Mean, SD, SE, CV, %Responder |

***Table 1.*** *Features reported by the Target Activation BioApplication*

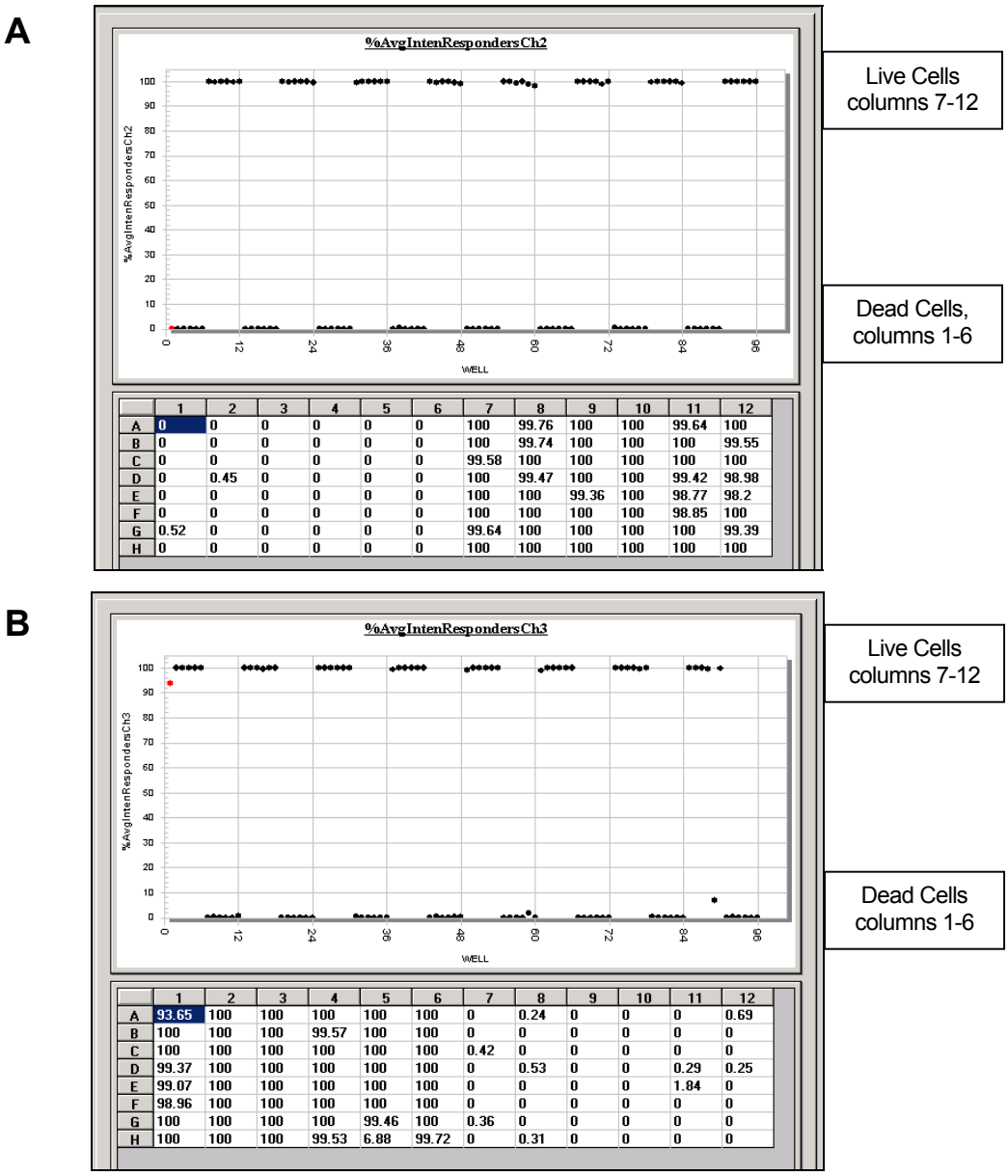
# Example Use Cases

## Example #1: Using Target Activation with the Cellomics Cell Viability HCS Reagent Kit

Live and dead cells represent two physiological states that are well suited to quantitative analysis with this BioApplication. Living, healthy cells will take up and process VitalDye that will then fluoresce in the green channel (Channel 2). In contrast, DeadDye will enter dead cells, but not living cells, and will fluoresce in the red channel (Channel 3). Thus, living healthy cells will exhibit a strong green fluorescent signal and very little red fluorescence. Conversely, dead cells will exhibit a strong red fluorescent signal and very little green fluorescence.

HeLa cells were plated at a density of 7,500 cells/well onto a 96-well poly-L-lysine-coated plate. Half of the plate was treated to kill the cells using the test compound from the Cell Viability HCS Reagent Kit (Columns 1-6), and the other half of the plate (Columns 7-12) was not treated. The same reagent kit product was used to fluorescently label the sample (Please see the *Cellomics Cell Viability HCS Reagent Kit Product Insert* for details). The plate was imaged and analyzed on the ArrayScan HCS Reader using the Target Activation BioApplication. Figure 3 are results of the scan. Dead cells in Columns 1-6 have a strong red fluorescence (Channel 2) and a weak green fluorescence (Channel3), reflected at or near 0 for % Responders in Figure 3A (for Channel 2) and at or near 100 for % Responders in Figure 3B (for Channel 3). In contrast, viable cells in Columns 7-12 have a strong green fluorescence (Channel 3) and a weak red fluorescence (Channel 2), reflected at or near 100 for % Responders in Figure 3A and at or near 0 for % Responders in Figure 3B).

Note that for this biological sample, wells with high values for % Responders in the green channel (Channel 2) always have low values for %Responders in the red channel (Channel 3) and *vice versa*, as expected. The variability between wells is low and there is a large separation between live and dead wells.




| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 99.76 | 100 | 100 | 99.64 | 100 |
| B | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 99.74 | 100 | 100 | 100 | 99.55 |
| C | 0 | 0 | 0 | 0 | 0 | 0 | 99.58 | 100 | 100 | 100 | 100 | 100 |
| D | 0 | 0.45 | 0 | 0 | 0 | 0 | 100 | 99.47 | 100 | 100 | 99.42 | 98.98 |
| E | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 100 | 99.36 | 100 | 98.77 | 98.2 |
| F | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 100 | 100 | 100 | 98.85 | 100 |
| G | 0.52 | 0 | 0 | 0 | 0 | 0 | 99.64 | 100 | 100 | 100 | 100 | 99.39 |
| H | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 93.65 | 100 | 100 | 100 | 100 | 100 | 0 | 0.24 | 0 | 0 | 0 | 0.69 |
| B | 100 | 100 | 100 | 99.57 | 100 | 100 | 0 | 0 | 0 | 0 | 0 | 0 |
| C | 100 | 100 | 100 | 100 | 100 | 100 | 0.42 | 0 | 0 | 0 | 0 | 0 |
| D | 99.37 | 100 | 100 | 100 | 100 | 100 | 0 | 0.53 | 0 | 0 | 0.29 | 0.25 |
| E | 99.07 | 100 | 100 | 100 | 100 | 100 | 0 | 0 | 0 | 0 | 1.84 | 0 |
| F | 98.96 | 100 | 100 | 100 | 100 | 100 | 0 | 0 | 0 | 0 | 0 | 0 |
| G | 100 | 100 | 100 | 100 | 99.46 | 100 | 0.36 | 0 | 0 | 0 | 0 | 0 |
| H | 100 | 100 | 100 | 99.53 | 6.88 | 99.72 | 0 | 0.31 | 0 | 0 | 0 | 0 |

***Figure 3.*** *A: Percent responders in the green channel (viable cells). B: Percent responders in the red channel (necrotic cells). Columns 1-6 were treated with lytic agent while Columns 7-12 served as controls.*

## Example #2: Mitotic Index

The cell cycle in eukaryotic cells consists of alternating phases of cell growth separated by a period of DNA replication, culminating in the production of two daughter cells through a process called mitosis (Johnson and Walker 1999). Mitotic index is defined as the fraction of cells undergoing mitosis within a given population, and is a standard measure used for characterizing proliferative cells. Apart from applications in compound screening and cell cycle research, determination of mitotic index is also important for characterization of cancerous cell lines and prediction of proliferative potential (Sinicrope et al. 1999).

Two of the most dramatic changes that occur during mitosis are the formation of the mitotic spindle and the condensation of DNA and chromatin to form chromosomes. Typically, researchers manually identify and count mitotic cells using these cellular parameters. The Mitotic Index HCS Reagent Kit utilizes a primary antibody specific for a phosphorylated epitope of a core histone protein and a fluorescently labeled secondary antibody to identify dividing cells and calculate the mitotic index for a sample cell population. Mitotic cells are identified by evaluating nuclear size and staining intensity and uniformity of the mitotic-specific antibody. The Target Activation BioApplication can measure these characteristics.

## Example #3: Ligand/Receptor Binding, Internalization or Trafficking

Integral membrane proteins and macromolecules that are endocytosed at the cell surface can have several intracellular fates. The major endocytic trafficking pathways deliver recycling receptors back to the plasma membrane via the early endosomal system and transport soluble volume markers to late endosomes and lysosomes. The macromolecules and integral membrane proteins that are internalized at the cell surface and then recycle back to the plasma membrane traffic through a compartment known as the recycling endosome or the endocytic recycling compartment (Gruenberg and Maxfield 1995; Mukherjee, Ghosh, and Maxfield 1997). The molecules that traffic through or associate with this compartment include the following:

- Receptors that constitutively internalize and then recycle, such as the transferrin receptor (TfR) and the low-density lipoprotein receptor (Mukherjee, Ghosh, and Maxfield 1997).
- Many G-protein-coupled receptors that, upon agonist stimulation, desensitize and internalize into the cell and then recycle back to the cell surface via the recycling compartment as part of their resensitization process (Conway et al. 1999; Drmota, Gould, and Milligan 1998).
- Various lipids and lipid analogs (Mayor, Presley, and Maxfield 1993; Mukherjee, Soe, and Maxfield 1999).
- GPI-anchored proteins (Mayor, Sabharanjak, and Maxfield 1998).
- Cholesterol and its analogs (Mukherjee et al. 1998).
- Other cytoplasmic signaling and trafficking-related proteins (Ghosh et al. 1998; Johnson et al. 1996; Ullrich et al. 1996; Daro et al. 1996).

In many cell types, the recycling compartment, when it contains fluorescently labeled receptors, appears as a bright spot in a perinuclear location (Mayor, Presley, and Maxfield 1993; Ghosh et al. 1998; Dunn, McGraw, and Maxfield 1989). The Target Activation BioApplication is capable of measuring the presence of an internalized, fluorescently labeled receptor in the recycling compartment. It can measure a receptor's internalization by its accumulation in the recycling compartment and the receptor's recycling by its decrease in the recycling compartment (Ghosh et al. 2000). This can be achieved by imaging individual cell nuclei in one channel and a fluorescently labeled ligand/receptor in a second channel. The number of cells that show the internalization/binding event can then be easily quantified.

# Demonstration Data Using the Target Activation BioApplication

## Using the Event Wizard with Cell Viability

For the purposes of illustration, consider the Cell Viability assay configured to allow simultaneous measurement of cell viability/metabolic activity and plasma membrane integrity. Note that fluorescence in the green channel indicates metabolic competence to process VitalDye after internalization in addition to the ability to contain the metabolic activity within the cell. In contrast, fluorescence signal in the red channel is an indicator of membrane integrity only. The possible subpopulations of cells in this assay are shown schematically in Panel A (Figure 4). The entire cell population is contained within the rectangular box in each case. The initial assumption for any set of cellular responses is that individual cells may display effects on a single target or any combination of measured targets.

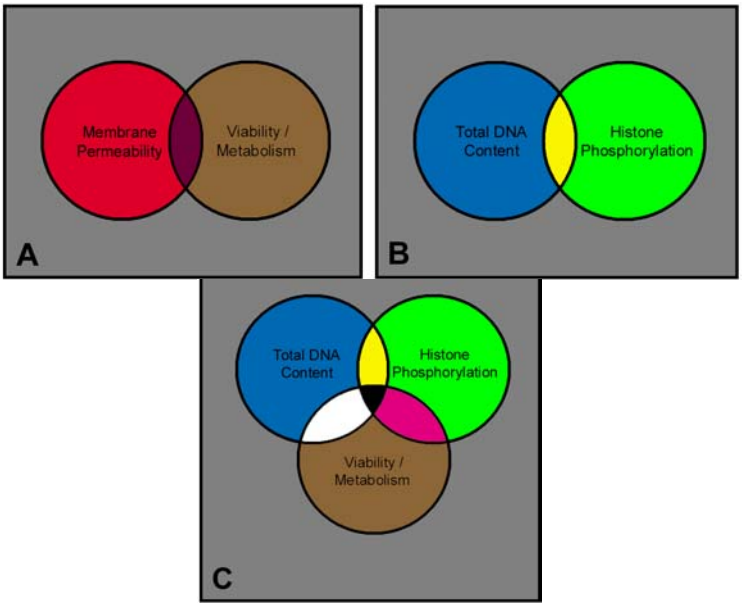



***Figure 4.*** *Classification of individual cells using the Target Activation BioApplication. Circular domains contain responder cells for the target indicated.*

Event Definitions that may be applied to the use case described in Panel A (Figure 4) are:

- *Viability / metabolic activity* NOR *Increased Permeability* – quantifies the percentage of cells having reduced metabolism but intact membrane integrity. These cells are contained within the gray region.
- *Increased Permeability* ANDNOT *Viability / metabolic activity* -quantifies the percentage of cells that show increased membrane permeability and decreased metabolic activity. This population would include the cells in the red region.
- *Viability / metabolic activity* ANDNOT *Increased Permeability*- returns the percentage of viable / metabolically active cells that do not display compromised membrane integrity. These cells occupy the brown region.

Additional use cases are described in Panels B and C in Figure 4. Panel B describes an assay configuration that enables measurement of total DNA content and histone phosphorylation. Examples of Event Definitions that may be applied are:

- *Histone Phosphorylation* AND *Increased DNA content* - identifies cells that have completed S-phase and have begun mitosis. The population displaying this Event would only exclude cells within the yellow region.
- *Increased DNA content* ANDNOT *Histone Phosphorylation* - identifies cells in S-phase that have not yet initiated mitosis. These cells are contained within the blue region.

The definition and detection of Events using this BioApplication is not limited to two output features. Panel C (Figure 4) shows a combination of the use cases shown in Panels A and B. The following Event Definitions may be applied:

- *Histone Phosphorylation* AND *Increased DNA content* AND *Viability / metabolic activity*- identifies viable cells that have initiated S-phase but are in mitosis. These cells would occupy only the central black region.
- *Histone Phosphorylation* OR *Increased DNA content* AND *Viability / metabolic activity*- this includes viable cells that have begun or completed S-phase and/or mitosis. These cells would be contained within the white, black and pink regions.

An actual use case illustrating the definition and detection of Events is provided below. The example used is the two-parameter assay as provided in the Cell Viability reagent kit discussed previously. The Events defined are:

- Event 1: *Viability / metabolic activity* NOR *Increased Permeability* (represented by the gray region in Panel A of Figure 4)
- Event 2: *Increased Permeability* ANDNOT *Viability / metabolic activity* (represented by the red region in Panel A)
- Event 3: *Viability / metabolic activity* ANDNOT *Increased Permeability* (represented by the brown region in Panel A)

Figure 5 shows the frequency of occurrence of the three Events described previously. As expected, Event 2 occurs in nearly every cell that is treated with lytic agent while Event 3 can be detected in almost every untreated cell. Furthermore, the low frequency of occurrence of Event 1 indicates that a loss of fluorescence associated with VitalDye correlates well with increased signal from DeadDye in the same cell in this case. However, there is a small population of cells (< 1%) that show metabolic incompetence (inability to process VitalDye) while maintaining plasma membrane integrity. Examples of cells displaying each of these Events are also shown.

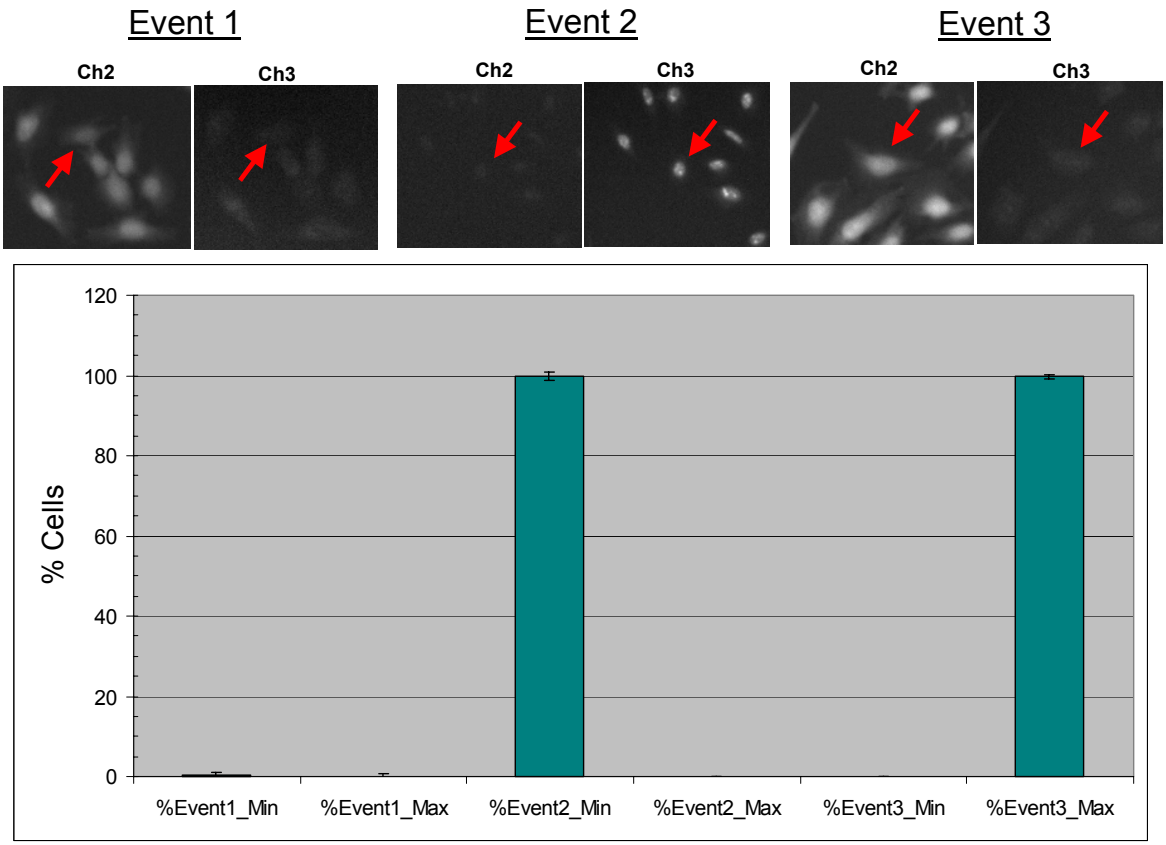



***Figure 5.*** *Use of Event detection to dissect multiparametric responses of individual cells associated with viability. Each condition represents the Mean of 48 wells from a plate in which half the plate was treated with a lytic agent (Min) while the other half was untreated (Max). Error bars indicate standard deviations. Examples of cells showing each Event are shown at the top.*

## Exploring Multi-Drug Resistance

Multiple drug resistance (MDR) biology was used to demonstrate the Live Mode of the vHCS Discovery Toolbox (see ArrayScan V$^{TI}$ HCS Reader or vHCS Discovery Toolbox 1.6 User's Guide). Cells with multiple drug resistance activity continually pump out many drugs and candidate drugs, thus not allowing an effective concentration to build inside cells. Therefore, this biology is important in cancer and infectious disease drug discovery. This activity acts as an efflux pump to prevent the accumulation of MDR substrates into these cells. In cells treated with an MDR1 inhibitor, cyclosporin-A, there will be much greater intracellular accumulation of MDR1 substrates. In control cells, the MDR1 activity will pump much of the MDR substrates out before they can get into the cytoplasm.

This example data was taken from a typical kinetic profiling mode of MDR1 biology. The MDR1 substrate that was used for this demonstration showed no fluorescent activity prior to uptake by healthy cells. Once transported inside a cell, MDR1 substrate fluorescence is activated by intracellular esterases. As it accumulates in the cytoplasm, the fluorescence in the cell increases. If MDR1 substrate is pumped out of the cell via MDR1 activity, it will not accumulate as quickly inside the cell and the fluorescent intensity will be correspondingly less. The membrane proteins responsible for MDR1 are P-glycoprotein (P-gp) and MRP1. They function by actively pumping out the MDR1 substrate as it crosses the cell membrane

(Litman et al. 2001). Thus, MDR1 substrate is unable to accumulate in cytoplasm. When an inhibitor of MDR1 activity is present, the MDR1 substrate is not removed by the pump, but accumulates in the cytoplasm, and cytoplasmic fluorescence increases. Thus, the increase in cytoplasmic fluorescence can be used as an indicator of whether MDR1 activity is inhibited by a test compound.

H69AR cells (available from ATCC, CRL-11351) have high multiple drug resistance activity. H69AR cells are a human small cell lung carcinoma cell line that were grown in the presence of increasing concentrations of adriamcyin (doxorubicin). Cells were plated in a 96-well microtiter plate and placed into the KineticScan HCS Reader (note that this process can also be used with a Cellomics HCS Reader containing the Live Module). Thirty-two wells (Columns 5-8) of a 96-well plate containing H69AR cells were labeled for 10 minutes with Hoechst 33342 dye. This was followed by automated addition of MDR substrate and a period of incubation prior to compound treatment. The experimental conditions for this assay called for automated addition of test compound (cyclosporin-A, an inhibitor of MDR-1) to two of the four columns used (Columns 7 and 8). All 32 wells in the four columns were then repeatedly imaged in sequence for 80 minutes, with a sampling interval of 8 minutes.

Figure 6A depicts the average intensity of intracellular fluorescent MDR1 substrate in cells in each well as a function of time. The intensity of cells with intracellular fluorescent MDR1 substrate is low initially and increases over time. Note the low variability between wells and the separation in intensity between those wells with cyclosporin-A (top traces) and control wells without cyclosporin-A (bottom traces).

Figure 6B is a kinetic output feature value of the rate of intracellular accumulation of the fluorescent MDR1 substrate. The wells with the high initial rates are the same as those depicted in the top traces in Figure 1A (i.e., with cyclosporin-A). Thus, for an assessment of MDR1 activity, you do not have to wait an hour or more for the biology to run its course or stabilize or fix the biology in a particular state. With the Live Module, you only need to examine the MDR1 activity in the live cells approximately 5 minutes after stimulation.

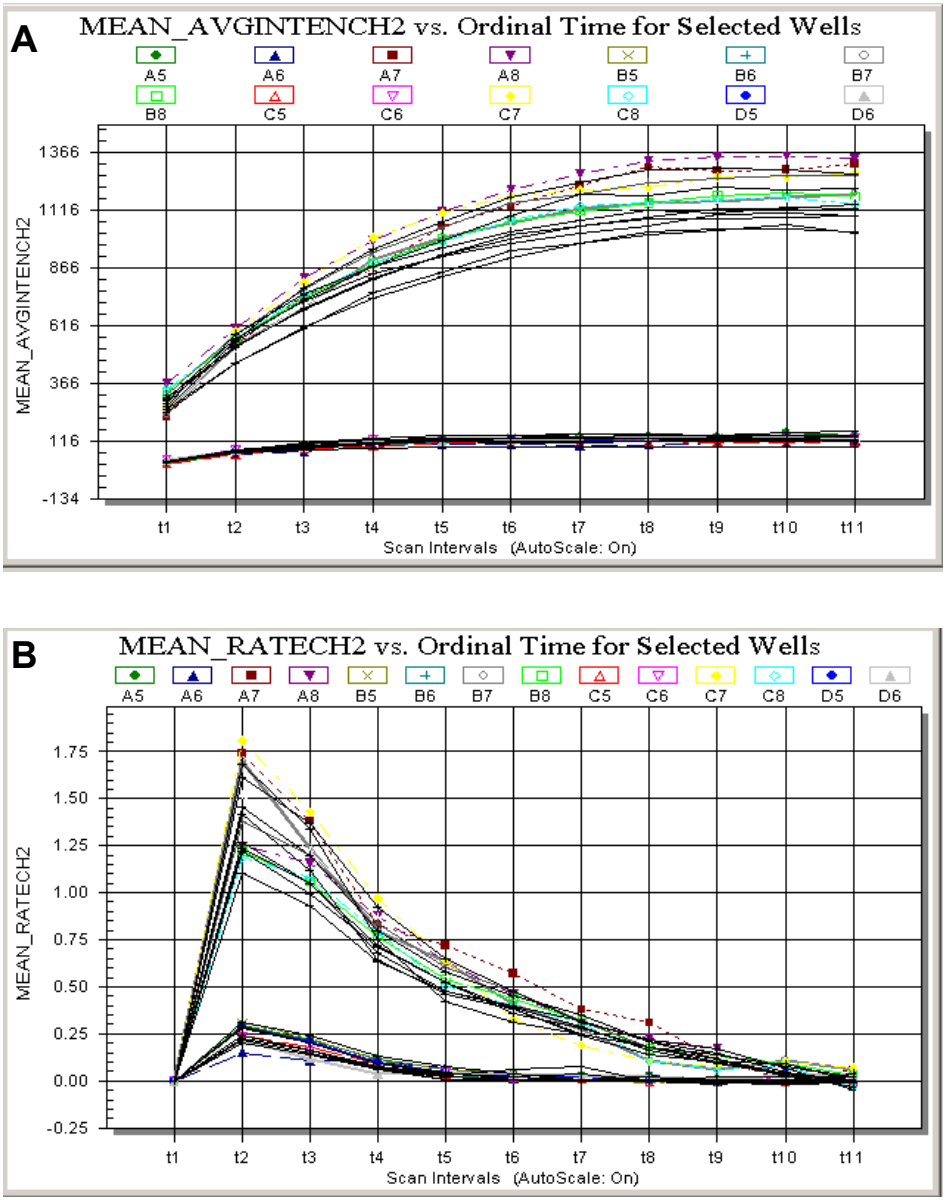



***Figure 6.*** *Kinetic measurement of MDR1 substrate accumulation assayed in four columns of wells using the KineticScan HCS Reader. Columns 5 and 6 were untreated and Columns 7 and 8 were treated with cyclosporin-A. The columns of wells were successively scanned with a sampling interval of 7 minutes. The results are shown as they appear in the View software application. A: The mean average intensity in Channel 2 (MDR1 substrate fluorescence) was selected to be displayed. B: The mean rate of increase of average intensity in Channel 2 (MDR1 substrate fluorescence) is shown.*

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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 briefly describes how the algorithm works, the output features, and input parameters that you can adjust to optimize your analysis.

## Overview

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

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

There are two types of input parameters: Assay Parameters and Object Selection Parameters. The Assay Parameters control the actual quantitative analysis of the images. The Object Selection Parameters control which objects are 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. In Channel 1, primary objects (such as nuclei) are identified; only pixels with intensities above this threshold will be considered as belonging to these structures. Thus the proper setting of an intensity threshold is a key early step in identifying objects and thus configuring the application. Object Identification is not available in Channels 2-6. 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 in Table 3.

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

***Table 2.*** *Intensity Threshold Methods Available for Each Channel in the Target Activation 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 2-6), the value must be **0**

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

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

The value entered for the Object Identification in the BioApplication 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.

The histogram-derived method 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 to the Isodata value.<br>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 ($m_R$).<br>A negative value identifies dimmer objects and results in larger object masks. A positive value results in smaller object masks. | -0.999 – 9.999<br>(offset) | 1 - 32767 |
| Fixed | A fixed pixel intensity value between 0-32767 is applied | 0 – 32767<br>(actual intensity in image) | 0 - 32767 |

***Table 3.*** *Intensity Threshold Descriptions and ranges available for the Target Activation BioApplication*

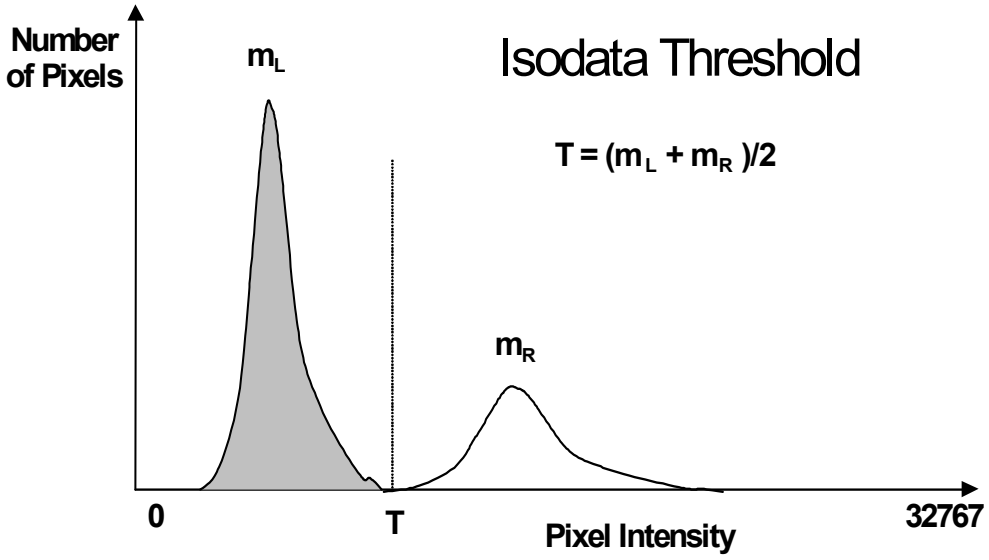



***Figure 7.*** *Histogram-derived Isodata Intensity Threshold Method. Background peak is shown in gray and object peak is shown in white.*

# Description of Assay Parameters and Settings

Understanding the key steps and the principle behind the image processing algorithm will allow you to more effectively adjust the protocol parameter values to analyze your images. All of the key image processing steps occur at the level of the individual object (typically a cell), for which Cell Features are computed. The Cell Features, calculated for every object analyzed, are used to calculate Well Features, or population descriptors for each well.

## Assay Parameters for Image Analysis

### General Assay Parameters

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

- **Reference Well Control**
- **Units for Morphological Measurements**
- **Object Type**
- **Background Correction**
- **Object Smoothing**
- **Object Segmentation**
- **Reject Border Objects**
- **Mask Size Modification**

### Reference Wells Control

The two General Assay Parameters controlling the use of Reference Wells are: **UseReferenceWells** and **MinRefAvgObjectCountPerField**. The **UseReferenceWells** Assay Parameter allows you to choose whether Reference Wells are to be used to determine the population characterization thresholds. If Reference Wells are to be used (value = **1**), then the Assay Parameter **MinRefAvgObjectCountPerField** must be set. This is the minimum number of objects detected per field that are required for acceptance of the data in the Reference Wells. This allows you to enter the minimum number of objects that you feel gives a good distribution and, thus, statistical validity to the thresholds calculated from the Reference Wells. Note that in addition to these general Assay Parameters, there are additional Assay Parameters for Reference Well processing that are specific for particular features and channels. These will be described in later sub-sections.

### Units for Morphological Measurements

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

### Object Type

#### ObjectTypeCh1

The "Object Type" Assay Parameter allows 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***

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

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

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

## Background Correction

### BackgroundCorrectionCh*N*

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

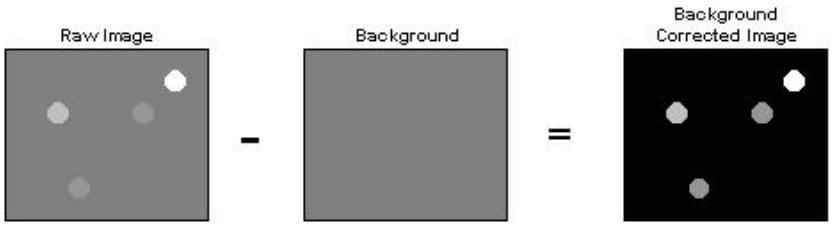



***Figure 8**. Image depicting calculation of positive Background Correction that can be used in each channel.*

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

If the **BackgroundCorrectionCh*N*** Assay Parameter is given a negative value, an optional background correction method based on the 3D surface fitting is applied. The absolute value entered corresponds to the radius of an area used to find local minima across the image. Found minimum values are used to construct a 3D surface of a background which is then subtracted from the original image. The main advantage of the method is that it minimizes the effect of the background correction (removal) procedure on the intensity values of the analyzed objects. In both cases, the larger the absolute value, the larger the sampled region, and the less

subtraction is done. Setting the value to **–1** lets the BioApplication 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**. Possible Background Correction Methods used in each channel with the Target Activation BioApplication.*

**NOTE**

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

Background Correction can be adjusted on each channel separately.

The background-corrected image is not stored or shown.

## Object Smoothing

### ObjectSmoothFactorCh1

Channel 1 has an independent Assay Parameter that enables control over the degree of image smoothing, or blurring, before the identification of objects (**ObjectSmoothFactorCh1**). This Assay Parameter corresponds to the radius in pixels of the area used to smooth the image. For example, a small value, such as **3**, means that a region with a radius of 3 pixels is used to smooth the image (region with dimensions 7×7 pixels, or 49 pixels total). Doubling the value to **6** would mean that a larger region (13×13 pixels or 169 pixels total) is used to smooth the image, and thus the image will be smoothed more than the previous value. A value of **0** means that smoothing is not done.

This Assay Parameter is used to smooth images with a lot of contrast to improve identification of objects. To get sharper definition of the shapes of the edges of objects (i.e., cells), you may want to keep the **ObjectSmoothFactorCh1** small, if not 0. However, if your Channel 1 label is not very homogeneous, the actual object will be erroneously identified as consisting of several smaller sized objects, and then smoothing will result in a normalizing effect of the Channel 1 label and will help identify the object with its true bounds.

Smoothing can be used to connect fragments of objects and can be valuable when measuring morphological changes. Figure 9 shows the effect of smoothing on accurate identification of objects in Channel 1 (white overlay). Note that insufficient smoothing can result in unwanted object fragmentation (arrows in Panel B) while excessive smoothing can result in poor definition of the object perimeter (D).

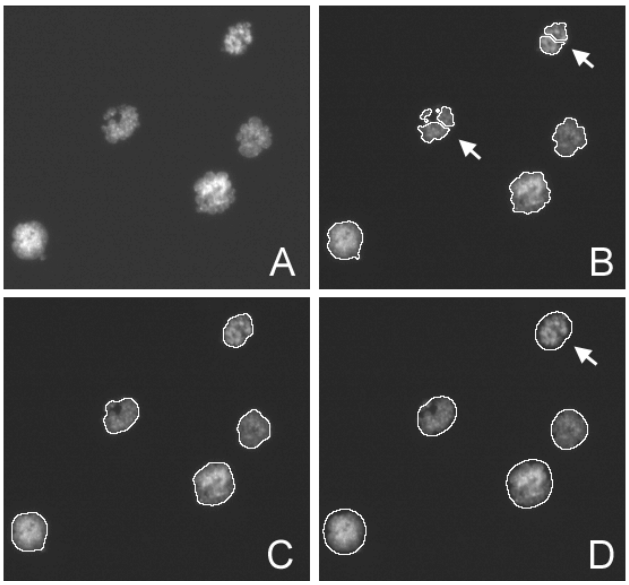



***Figure 9. ObjectSmoothFactorCh1****: Images show the effect of different degrees of smoothing on the same field of apoptotic nuclei. A: Raw Image; B:* ***ObjectSmoothFactorCh1*** *= 0; C:* ***ObjectSmoothFactorCh1*** *= 3; D:* ***ObjectSmoothFactorCh1*** *= 8.*

# Object Segmentation

## 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 Target Activation BioApplication: Geometric (shape and size) and Intensity (intensity peaks). These methods are illustrated in Figure 10.

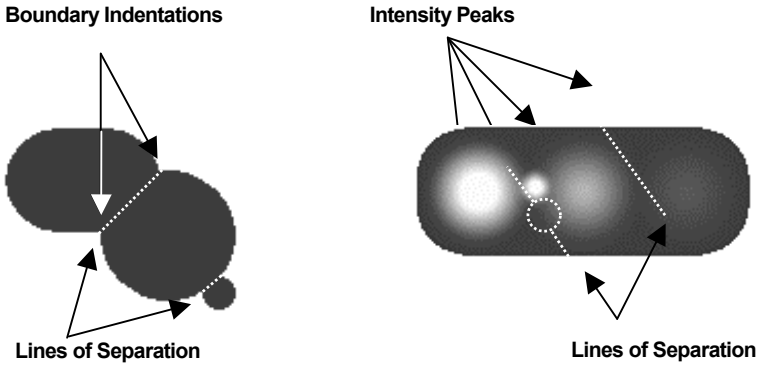



***Figure 10:*** *Object Segmentation Options. The image on the left illustrates the Geometric Method while the image on the right illustrates the Intensity Method.*

The Geometric method splits touching objects on the basis of shape, relying on boundary indentations to locate the line of separation. This method works best if the individual objects have smooth boundaries with pronounced indentations at their point of contact. The Geometric

method can be used to separate objects that are uniform in intensity (i.e., saturated) or that have multiple intensity peaks (i.e., noisy or textured).

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

The **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** value to R1 (Figure 11A) produces object separation shown in Figure 11B. Setting the **ObjectSegmentationCh1** value to R3 results in segmentation shown in Figure 11C. Setting the **ObjectSegmentationCh1** value to R2 (the largest radius of all touching objects) produces no segmentation.

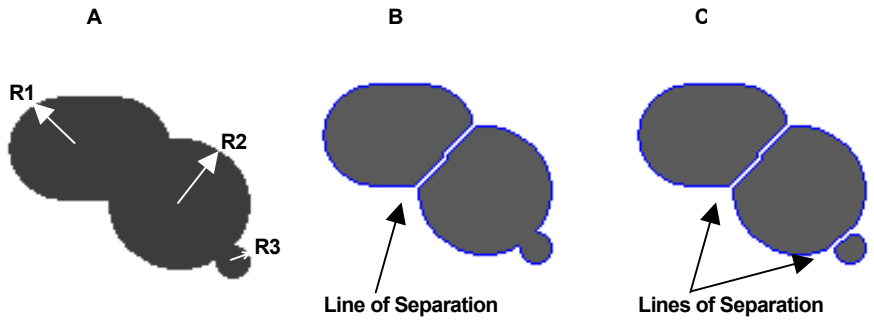



***Figure 11:*** *Object segmentation: Geometric Method. A) Radii of touching objects: R1 = 24, R2 = 30, R3 = 7; B) Result of segmentation using* ***ObjectSegmentationCh1*** *= R1; C) Result of segmentation using* ***ObjectSegmentationCh1*** *= R3.*

**Intensity Method** Figure 12 shows the intensity profile along the cordial line of an object with four intensity peaks from Figure 10. Relative peak intensity heights measured from the top of a peak to the nearest valley for each of the object are:1500 (#1), 1250 (#2), 500 (#3), and 200 (#4).

In general terms, these peak intensity heights are nothing but measures of local contrast of an image. The degree of object segmentation can be controlled by setting the **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** value greater than 0 should be used to alleviate the problem.

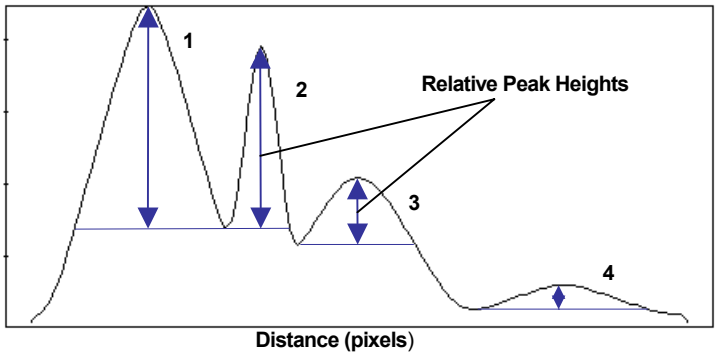



***Figure 12:*** *Intensity profile of an object with four intensity peaks. Peak #1 height = 1500, peak #2 height = 1250, peak #3 height = 500 and peak #4 height = 200.*

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

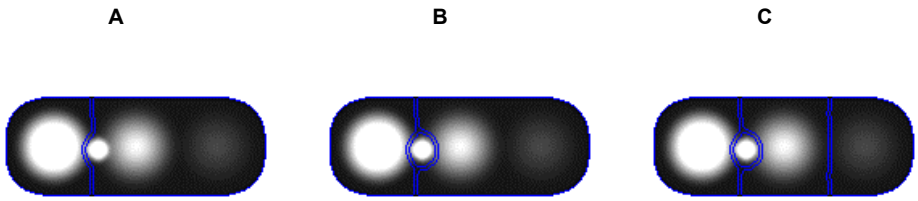



***Figure 13:*** *Object segmentation: Intensity Method. A) Result of segmentation using parameter* ***ObjectSegmentationCh1*** *= -1000; B) Result of segmentation using parameter* ***ObjectSegmentationCh1*** *= -400; C) Result of segmentation using parameter* ***ObjectSegmentationCh1*** *= -100,*

The **ObjectSegmentationCh1** Assay Parameter (see Table 6) controls both methods. Its absolute value should be the approximate radius (in pixels) of the smaller of the two objects being separated, and its sign determines which method is used. The value of this Assay Parameter will depend on the magnification of the objective being used.

### Reject Border Objects

#### RejectBorderObjectsCh1

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 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.

### Mask Size Modification

#### MaskModifierCh*N*

The identified objects in Channel 1 are used to define a mask. Only the pixels that fall under the defined mask are used for the intensity measurements in all channels. The size of the mask can be modified by the **MaskModifierCh*N*** Assay Parameter for Channels 2-6. The mask for Channel 1 cannot be modified. This **MaskModifierCh*N*** Assay Parameter sets the number of pixels for which the object mask will be dilated or eroded based on the Assay Parameter being positive or negative respectively (Figure 1).

## Basic Assay Parameters

Input parameters can be found in Create Protocol View or Protocol Interactive View. Available Assay Parameters will vary depending on whether you are using Basic Mode or Advanced Mode (if **Hide Advanced Parameters** box is checked). Table 7 displays Assay Parameters that can be used in Basic Mode.

| 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 identification 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 |

| Parameter | Units | Description |
|---|---|---|
| RejectBorderObjectsCh1 | Binary | Reject Ch1 objects that touch image edges: 0 = No, 1 = Yes |
| NucCleanupCh1 | Binary | In Ch 1 image before processing, clean up nucleus mask and remove small objects by applying erosion followed by dilation procedure: 0 = No, 1=Yes |
| MaskModifierChN | Pixels | Number of pixels to modify Ch1 object mask in ChN: Negative value = Shrink mask, 0 = Do not modify mask, Positive value = Expand mask |

***Table 7.*** *Basic Assay Parameters found in the Target Activation BioApplication. *Note that "ChN" refers to Channels 1-6 for* ***Background Correction*** *and Channels 2-6 for* ***Mask Modifier***

## Object Selection Parameters

Each channel has an associated set of Object Selection Parameters. If an object has all measured features within the ranges specified by the Object Selection Parameters, it is selected for analysis. Rejected objects are removed from further analysis. Object Selection Parameters for Channel 1 are used for defining valid objects (Table 8) while those for Channels 2-6 define the objects that are selected for analysis (Table 9).

### Channel 1 Parameters

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

***Table 8.*** *Channel 1 Object Selection Parameters available with the Target Activation BioApplication.*

### Channel N (2-6)

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

***Table 9.*** *Channels 2-6 Object Selection Parameters available with the Target Activation BioApplication. *Note that "ChN" refers to Channels 2-6.*

## Gating

The Target Activation BioApplication supports gating on a cell population. This feature provides selective cell processing based on fluorescence intensity. Therefore, in addition to identifying valid objects based on shape and intensity in Channel 1, you can also select or reject cells based on fluorescence intensity in Channels 2-6. Gating allows you to specifically identify a certain group of cells based on labeling intensity and limits the analysis to this group of cells. For example, gating may be used to analyze only those cells showing a certain level of expression of a fluorescent reporter such as green fluorescent protein (GFP). Unlike subpopulation analysis, gating works by rejecting any object that does not meet object selection criteria in all channels. Therefore, if you choose to add gating channels, an object must pass the criteria in all channels to be selected for analysis.

### Specifying Intensity Ranges for Gating

When working in the Create Protocol View, you can specify intensity threshold values by entering upper and lower limits for two intensity parameters, **AvgIntenCh*N*** and **TotalIntenCh*N***, for one or more channels. **TotalIntenCh*N*** is a summation of all intensities within the object of interest. **AvgIntenCh*N*** is **TotalIntenCh*N*** divided by the object area. These parameters can also be adjusted interactively in Protocol Interactive View.

You can view the results of gating in the View software application when viewing Well Details. **ValidObjectCount** displays the total number of objects that pass Channel 1 object selection criteria. **SelectedObjectCount** displays the number of valid objects in each well that satisfied object selection criteria in all channels. To view the intensity values, select Cell Details for a particular well. The View software application also displays the intensity measurements for each cell in each channel to verify gating. Note that analysis is performed only on selected objects.

### Specifying Mask Modifiers for Gating

In addition to specifying intensity threshold ranges for one or more channels, you must also apply a mask to one or more downstream channels using the **MaskModifierCh*N*** Assay Parameter. As described earlier, you may want to adjust this Assay Parameter if you want to dilate the mask of each object from Channel 1 to include other cellular markers. The mask can be dilated or eroded, but will not overlap with other masks from nearby objects. For each additional channel, the average intensity and total intensity values within the modified masks are calculated. If the calculated value does not fall within the specified upper and lower limits, the object is removed from the set of selected objects.

## Image Overlays

All Image Display Option settings are available when running in Basic or Advanced Mode (Table 10). Adjustments to these settings allow you to choose which overlays to display with this BioApplication as well as the colors that will be used for each overlay. These settings affect the Image Display only and not the image analysis and quantification.

| Parameter | Description |
|---|---|
| Selected object | Outlines valid objects (e.g., cells) in the Channel n for analysis. Valid objects are those that have properties in the range specified by the Object Selection Parameters. |
| Rejected object | Outlines objects (e.g., cells) rejected for analysis. Rejected objects have properties outside the range specified by the Object Selection Parameters. Objects that touch the image edge are also rejected. |
| Mask ChN | Outlines the modified mask from Channel 1 used in this Channel. If a positive value for **MaskModifierCh*N*** is used, then the Mask Ch*N* will be larger than the Channel 1 mask. If a negative value for **MaskModifierCh*N*** was used, the Mask Ch*N* will be smaller than the Channel 1 mask. When **MaskModifierCh*N*** is 0, the Mask Ch*N* will be the same as the Channel 1 mask. |

***Table 10.*** *Image Overlays available with the Target Activation BioApplication. Note that "ChN' refers to Channels 2-6.*

# Assay Parameters For Population Characterization

## Overview of Population Characterization

The Target Activation BioApplication provides the ability to characterize cells based on their response compared to a control population. For a particular feature (such as the average intensity in a channel, **AvgIntenCh*N***), a range is determined and set for a control population that has a certain physiological distribution for that feature. Upper and lower thresholds (known as ***Feature*ChNLevelHigh** and ***Feature*ChNLevelLow** 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 (see table below).

| Value | Cell Status Definition |
|---|---|
| 0 | Feature value within defined range |
| 1 | Feature value > upper threshold |
| 2 | Feature value < lower threshold |

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

The corresponding Well Feature that is reported, **%RESPONDER_*Feature*Ch*N***, is the percent of cells that is either greater than the upper threshold or less than the lower threshold (showing feature values that fall outside this range, i.e., the percentage of cells with Status Cell Feature values = **1** or **2**). Figure 14 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.

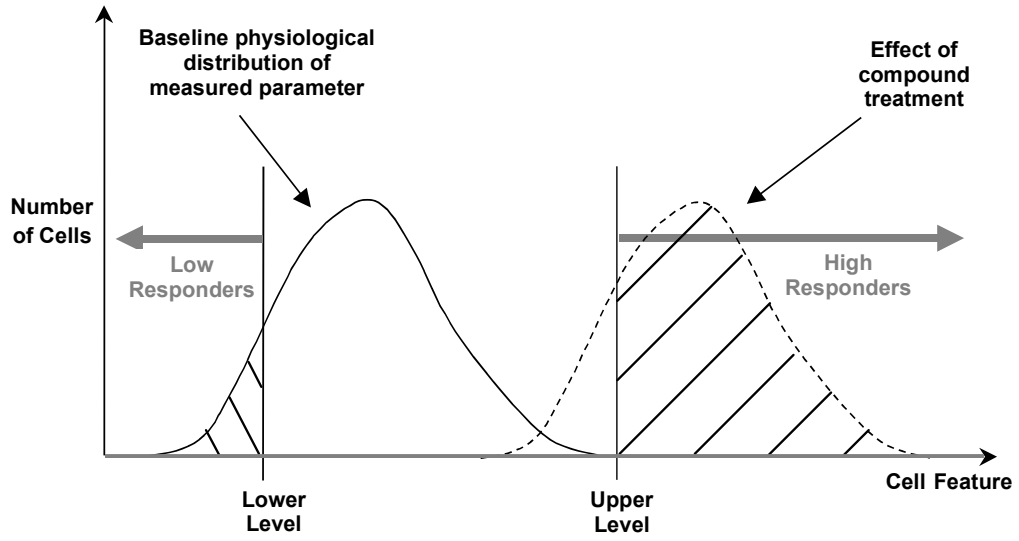



***Figure 14.*** *Principle of Population Characterization. %Responders would constitute those that are less than Lower Level or greater than Higher Level.*

## Setting Cellular Response Levels

There are two ways of setting the upper and lower-response levels to characterize the cell population. The first is manually entering values for the upper and lower ***Feature*Ch*N*Level** Assay Parameters in the Protocol Create or Protocol Interactive Views (Advanced Mode). This requires prior knowledge of typical feature values. The BioApplication then uses the defined levels to calculate the percentage of cells outside the bounds of the specific ***Feature*Ch*N*LevelLow, *Feature*Ch*N*LevelHigh** values.

The second is automatically calculating the ***Feature*Ch*N*Level** Assay Parameters through the use of Reference Wells. You designate which particular wells on the sample plate should be used as Reference Wells. Typically, Reference Wells contain a control (i.e., non-compound treated) population of cells that displays the normal basal physiological response for the Assay Parameter being measured. These wells are first analyzed and the population distribution for the different features are determined. The cell population characterization levels are then set by adding and subtracting from the mean of the distribution its standard deviation multiplied by a coefficient. The instrument then scans the whole plate and applies these levels. For example, if you want to know the percentage of cells that, with compound treatment, have a response beyond the 95$^{th}$ percentile of the response from a control untreated population of cells, then the coefficient by which to multiply the standard deviation would be two. The advantage of using Reference Wells to automatically calculate thresholds is that the thresholds are determined by a control population of cells and are independent of run-to-run variations.

## Reference Wells Processing Sequence

By setting the **UseReferenceWells** Assay Parameter to **1**, the Reference Wells processing is engaged. Specified fields in the Reference Wells are acquired/analyzed, and Field, Well, and Plate Features are computed. After this sequence is completed, computed values will be assigned to the Assay Parameters associated with each Reference Feature and regular scanning of the plate will begin. Again, if the feature value for **MinRefAvgObjectCountPerField** obtained from the Reference Wells is below the value set for that Assay Parameter, the BioApplication aborts the use of Reference Wells and processes the plate as if **UseReferenceWells** is set to **0**. The Target Activation BioApplication only uses **Known** Reference Wells. The sequence of computation for Reference Wells is as follows:

1. Cell Features are computed for every valid object within a field.
2. For each Cell Feature to be used for population characterization, the mean and standard deviation are computed over all cells in the field.
3. Reference Field Features are determined with the following naming convention:
    - **Ref*Feature*Ch*N*LevelLow**
    - **Ref*Feature*Ch*N*LevelHigh**
4. Reference Well Features are computed as average values for fields in a well, weighted for the number of cells per field, and then Reference Plate Features are computed as arithmetic averages for all Reference Wells on a plate. Use of a weighted average minimizes the effect of sparse fields.

## Identifying Reference Wells and Control Parameters

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


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

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

## Specifying and Controlling Reference Wells

Two general Assay Parameters controlling the use of Reference Wells are: **UseReferenceWells** and **MinRefAvgObjectCountPerField**. **UseReferenceWells** is a binary Assay Parameter that allows you to indicate whether Reference Wells are used to determine the levels necessary to characterize neurite populations.

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

If Reference Wells are enabled, the Assay Parameter **MinRefAvgObjectCountPerField** is used to specify the minimum average number of selected neurons per field that you consider acceptable in the Reference Wells. If the measured cell density is less than or equal to this value, the Reference Features will not be used, rather the manually entered levels for population characterization are used.

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

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

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

where *Feature* refers to the name of the Cell Feature (such as **ObjectAreaCh1**) and *N* refers to the specific channel. The ***Feature*LevelLowCh*N*** and ***Feature*ChN LevelHigh** Assay Parameter types specify the actual levels and must be manually entered if Reference Wells are not used. If using Reference Wells, the ***Feature*ChN LevelLow_CC** and ***Feature*ChN LevelHigh_CC** Assay Parameters are correction coefficients (CC) used to derive the ***Feature*ChN LevelLow** and ***Feature*ChN LevelHigh** values from the mean and standard deviation of the reference well population calculated as:

- **Ref*Feature*ChN LevelLow** = Mean – **(*Feature*ChN LevelLow_CC** × SD**)**
- **Ref*Feature*ChN LevelHigh** = Mean + **(*Feature*ChN LevelHigh_CC** × SD**)**

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

responder cells. The number of responder cells, expressed as a percentage of the entire cell population analyzed, is calculated for each assay well.

## Advanced Assay Parameters

When running in Advanced Mode, all basic input as well as advanced input parameters are editable. The **Hide Advanced Parameters** checkbox will hide or show the advanced Assay Parameters. When you check the box, only the Basic Assay Parameters are shown; when you uncheck the box, all Basic and Advanced Assay Parameters are shown. Generally, the Advanced Assay Parameters are related to definition and reporting of responder cells. They control the analysis of the data resulting from the image analysis (Table 12).

For each feature undergoing population characterization, there are four advanced Assay Parameters that control its levels: ***Feature*ChNLevelLow** and ***Feature*ChNLevelHigh** that set upper and lower thresholds and the presence of the **_CC** suffix which designates those levels are set using Reference Wells. For example, the Assay Parameters controlling the object's area in Channel 1 are:

- **ObjectAreaCh1LevelLow**
- **ObjectAreaCh1LevelHigh**
- **ObjectAreaCh1LevelLow_CC**
- **ObjectAreaCh1LevelHigh_CC**

In the listing of Advanced Parameters in the following table, instead of listing all four level parameters for each feature, one entry for the feature will be listed giving the four different options, as shown in the following example for the Channel 1 object size:

- ObjectAreaCh1Level***Low/High,Low/High_CC***

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

| Parameter | Units | Description |
|---|---|---|
| MinRefAvgObjectCountPerField | Number | Minimum average number of objects per field required for acceptance of reference well results |
| UseMicrometers | Binary | Measure lengths and areas in: 0 = Pixels, 1 = Micrometers |
| _PixelSize | µm | Pixel size in micrometers (depends on objective selection) |
| Type1EventDefinition | --- | User-defined combination of logic statements involving response features ***(cannot be edited)*** |
| Type2EventDefinition | --- | User-defined combination of logic statements involving response features ***(cannot be edited)*** |
| Type3EventDefinition | --- | User-defined combination of logic statements involving response features ***(cannot be edited)*** |
| ObjectAreaCh1Level***Low/High, Low/High _CC*** | Pixel or $\mu m^2$ | Defines **ObjectAreaCh1** population characterization thresholds |
| ObjectShapeP2ACh1Level***Low/High, Low/High_CC*** | Number | Defines **ObjectP2ACh1** population characterization thresholds |
| ObjectShapeLWRCh1Level***Low/High, Low/High_CC*** | Number | Defines **ObjectLWRCh1** 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 |
| ObjectVarIntenCh1Leve***lLow/High, Low/High_CC*** | Intensity | Defines **ObjectVarIntenCh1** population characterization thresholds |
| TotalIntenChNLevel***Low/High, Low/High_CC*** | Intensity | Defines **TotalIntenChN** population characterization thresholds |

| Parameter | Units | Description |
|---|---|---|
| AvgIntenChNLevel***Low/High, Low/High_CC*** | Intensity | Defines **AvgIntenChN** population characterization thresholds |
| VarIntenChNLevel***Low/High, Low/High_CC*** | Intensity | Defines **VarIntenChN** population characterization thresholds |

***Table 12.*** *Advanced Assay Parameters Available in the Target Activation BioApplication. *Note that "ChN" refers to Channels 2-6.*

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

The Target Activation BioApplication allows simultaneous definition of up to three Events to enable rapid multiparametric analysis of cellular responses at the level of individual cells, across multiple Cell Features. This capability allows you to rapidly determine mechanism of action associated with treatment, including identification of the initial target or 'trigger' point, and the sequence of cellular responses. You can use these events to:

- Create your own definition for a focused biology
- Define a subpopulation by using any combination of up to four status Cell Features
- Report characteristics of your subpopulations

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

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 15 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. The definitions described in Figure 15 can be directly applied to logic statements that contain more than two Cell Features. The Assay Parameters used to define Events are of the type **Type_X_EventDefinition** (X =1, 2 or 3).

**NOTE**

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

| Cell Features | Logic Operators |
|---|---|
| ObjectAreaCh1 | NOT |
| ObjectShapeP2ACh1 | AND |
| ObjectShapeLWRCh1 | AND NOT |
| ObjectTotalIntenCh1 | OR |
| ObjectAvgIntenCh1 | OR NOT |
| ObjectVarIntenCh1 | XOR |
| TotalIntenChN | NAND |
| AvgIntenChN | NOR |
| VarIntenChN | |

***Table 13.*** *Cell Features and Boolean operators available for Event Definition in the Target Activation BioApplication. *Note that "ChN" refers to Channels 2-6*

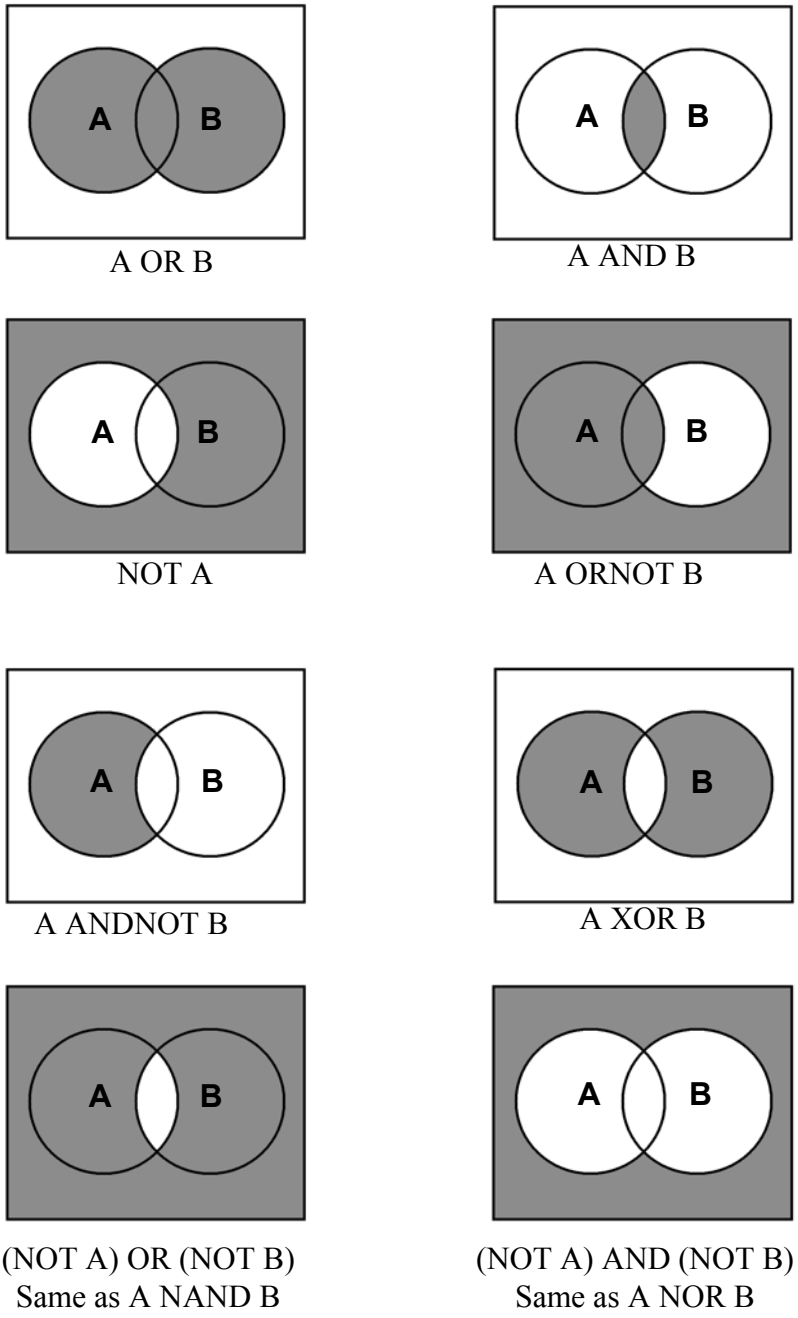



***Figure 15.*** *Schematic definition of available Boolean Operators assuming two Cell Features of interest, A and B.*

## Example of Event Wizard using the Cell Viability assay described in Chapter 1

Consider that the Cell Features of interest are:

1) **AvgIntenCh2**: responder cells of interest display high values indicating viability and/or metabolic competence to process VitalDye
2) **AvgIntenCh3**: responder cells of interest display high values indicating onset of membrane permeability

The upper and lower levels for each feature must be set such that responder cells in Channel 2 and Channel 3 are those that show normal viability/metabolism and increased membrane permeability, respectively (see section on *Assay Parameters for Population Characterization*). 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 | Lower threshold | Upper threshold |
|---|---|---|---|---|---|
| AvgIntenCh2 | Viability | 0-4095 | 50 / 500 | 0 | 350 |
| AvgIntenCh3 | Membrane Permeability | 0-4095 | 20 / 400 | 0 | 200 |

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

**NOTE**

For Event detection, a cell is considered to be a responder if its feature value is greater than the upper threshold or less than the lower threshold 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 definitions given in Chapter 1 (Figure 3) for the Cell Viability assay are provided below as actual logical statements.

***Event 1*: AvgIntenCh2** NOR **AvgIntenCh3 -** identifies cells that show decreased viability/metabolic competence without an increase in membrane permeability (see *Event Detection*, Chapter 1).

***Event 2*: AvgIntenCh3** ANDNOT **AvgIntenCh2 -** identifies individual cells that show both increased membrane permeability and decreased viability/metabolic competence. This definition unequivocally identifies necrotic cells.

***Event 3*: AvgIntenCh2** ANDNOT **AvgIntenCh3** - identifies only those cells that show viability/metabolic competence while maintaining plasma membrane integrity. This unequivocally identifies viable cells.

All logic statements are implemented in sequence from left to right. Therefore, the logical statement: **ObjectTotalIntenCh1** AND **AvgIntenCh2** OR **AvgIntenCh3** is implemented as:

**(ObjectTotalIntenCh1** AND **AvgIntenCh2)** OR **AvgIntenCh3**

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:

- ((**ObjectAvgIntenCh1** AND **AvgIntenCh2**) OR (NOT **TotalIntenCh2**))) XOR **TotalIntenCh3**
- (NOT **AvgIntenCh2**) OR **ObjectAvgIntenCh1**
- **TotalIntenCh2**

## Description of Output Features

Output Features are the biological measurements produced by the Scan software application and are accessible using the View software application and through the Protocol Interactive View. Additionally, a subset of features, the Well Features, are listed in Scan Plate View and Create Protocol View so that screening results can be viewed concurrently with scanning. All features can also be accessed in the Protocol Interactive View once the algorithm has been applied to a set of acquired images, whether that is done manually on the instrument or in a Disk Scan using images acquired from earlier scans.

An overview of both the Cell and Well Output Features can be found in Chapter 1, Table 1. The output features are reported at both the cell and well level. The Cell Features are reported for each object analyzed. The Well Features are derived from Cell Features and reported for the entire population of cells analyzed.

## Cell Features

Table 15 shows the output features reported for each selected cell, accessible in the Cell Feature window of Protocol Interactive View in addition to the View software application.

| Feature | Units | Description |
|---|---|---|
| Cell# | Number | Unique object ID |
| Top | Pixel | Y coordinate (in pixels) of top-left corner of image-aligned bounding box of Ch1 object |
| Left | Pixel | X coordinate (in pixels) of top-left corner of image-aligned bounding box of Ch1 object |
| Width | Pixel | Width (in pixels) of image-aligned bounding box of Ch1 object |
| Height | Pixel | Height (in pixels) of image-aligned bounding box of Ch1 object |
| XCentroid | Pixel | X coordinate (in pixels) of center of Ch1 object |
| YCentroid | Pixel | Y coordinate (in pixels) of center of Ch1 object |
| EventTypeProfile | Number | Identifies the types of 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 |
| ObjectAreaCh1 | Pixels or $um^2$ | Area (in pixels or micrometers) of Ch1 object |
| ObjectAreaCh1Status | Number | **ObjectAreaCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectShapeP2ACh1 | Number | Shape measure based on ratio of perimeter squared to 4PI*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 |
| 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 |
| TotalIntenChN | Intensity | Total intensity in ChN of all pixels within modified Ch1 object mask |
| TotalIntenChNStatus | Number | **TotalIntenChN** status: 0 = No response, 1 = High response, 2 = Low response |
| AvgIntenChN | Intensity | Average intensity in ChN of all pixels within modified Ch1 object mask |
| AvgIntenChNStatus | Number | **AvgIntenChN** status: 0 = No response, 1 = High response, 2 = Low response |
| VarIntenChN | Intensity | Standard deviation of intensity in ChN of all pixels within modified Ch1 object mask |
| VarIntenChNStatus | Number | **VarIntenChN** status: 0 = No response, 1 = High response, 2 = Low response |

***Table15.*** *Cell Features Available in the Target Activation BioApplication. *Note that "ChN" refers to features found in Channels 2-6.*

## Well Features

Many Well and Field Features are derived from the Cell Features. Such features are identified by a prefix, as listed below, to the Cell Feature name (Table 16).

| 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 |
| %RESPONDERS | Percentage of selected objects in the well with Feature_X above high-response level or below low-response level | Percent |

***Table 16.*** *General Well Feature prefixes available in the Target Activation BioApplication.*

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

| 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.*** *Additional Well Features available in the Target Activation BioApplication.*

## Reference Features

The algorithm reports the following Reference Features, whose values indicate the data generated from Reference Wells. Reference Features are viewable in the Scan Plate View as well as in Plate Features in the View software application. These features are computed and reported only when the Assay Parameter **UseReferenceWells = 1** (enabled).

In the Reference Features table below, instead of listing both features derived from the previous level parameters, one entry for the feature will be listed giving both outputs, as shown in the following example for the Channel 1 object area:

- ObjectAreaCh1Level***Low/High***

| Feature | Description |
|---|---|
| RefAvgObjectCountPerField | Average number of objects per field in reference wells |
| RefObjectAreaCh1LevelLow | Low/High-response level for **ObjectAreaCh1** computed from reference well results |
| RefObjectShapeP2ACh1Level***Low/High*** | Low/High-response level for **ObjectShapeP2ACh1** computed from reference well results |
| RefObjectShapeLWRCh1Level***Low/High*** | Low/High-response level for **ObjectShapeLWRCh1** computed from reference well results |
| RefObjectTotalIntenCh1Level***Low/High*** | Low/High-response level for **ObjectTotalIntenCh1** computed from reference well results |
| RefObjectAvgIntenCh1Level***Low/High*** | Low/High-response level for **ObjectAvgIntenCh1** computed from reference well results |
| RefObjectVarIntenCh1Level***Low/High*** | Low/High-response level for **ObjectVarIntenCh1** computed from reference well results |
| RefTotalIntenChNLevel***Low/High*** | Low/High-response level for **TotalIntenChN** computed from reference well results |
| RefAvgIntenChNLevel***Low/High*** | Low/High-response level for **AvgIntenChN** computed from reference well results |
| RefVarIntenChNLevel***Low/High*** | Low/High-response level for **VarIntenChN** computed from reference well results |

***Table 18.*** *Reference Features available in the Target Activation BioApplication. *Note that "ChN" refers to features in Channels 2-6.*

Chapter

3

# Using the Target Activation BioApplication

This chapter focuses on the assay-specific procedures for optimizing the Target Activation BioApplication with your biology as well as a detailed understanding of how to use the Event Wizard.

## Assay-Specific Procedures for Optimizing the BioApplication

The following Assay Parameters are described in more detail in Chapter 2. There are three classes of input parameters that critically influence the image analysis algorithm.

The first set of critical parameters for image analysis influences which objects are identified in Channel 1; this set includes the intensity threshold settings for the Object Identification method (e.g., **Isodata Threshold**). These settings set intensity criteria to determine which image pixels are to be used for measurement. A higher Object Identification Method value results in the intensity of the dimmer pixels being excluded from the intensity calculation. However, a lower value can result in noise being included.

The second set of critical parameters is the Object Selection Parameters. If an object (e.g., Hoechst-labeled nucleus) in Channel 1 has its physical properties (e.g., intensity or morphology parameters) within the range specified by the Object Selection Parameters, then this object (i.e., cell) 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** and **ObjectTotalIntenCh1** parameters. Similarly, non-cellular debris and mitotic cells can be excluded by adjusting the **ObjectArea**, **ObjectShapeP2A**, and **ObjectShapeLWR** parameters.

The third critical parameter is the **MaskModifierCh*N*** Assay Parameter, which sets the size of the mask derived from the object in Channel 1 to apply to Channels 2-6. To restrict the size of the intracellular area from which to intensity is measured, the value of this parameter should be reduced. Conversely, to measure intensity over a larger area of the cell, this Assay Parameter should be increased.

## Event Definition Using the BioApplication Event Wizard

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

The Wizard can be used to enter and edit Event Definitions as values for the **Type_X_EventDefinition** Assay Parameter in the Assay Protocol for the Target Activation BioApplication. This section provides a detailed description of the operation of the

BioApplication Event Wizard. The Wizard should only be used after the Target Activation BioApplication has been installed on your computer.

**NOTE**

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 strongly 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 (lower and upper limits) for 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) Copy Event Definitions from the Protocol Comments field in Create Protocol View into the Scan Comments field in Scan Plate View
3) Scan the assay plate.
4) Close the Scan software application.

**STEP IV (Optional)**

1) Open the saved protocol at a later time using BioApplication Event Wizard.
2) Evaluate and/or modify saved Event Definitions.
3) Close BioApplication Event Wizard and start the Scan software application to scan a new assay plate or analyze previously acquired images using modified Event Definitions.

## Features

The following features are included in the BioApplication Event Wizard:

- Can be used with Any Target Activation protocol
- Enables use of standard Boolean operators (NOT, AND, ANDNOT, OR, ORNOT, XOR, NAND, and NOR) to construct Event Definitions comprised of selected Cell Features
- Read-Only protocol protection
- Displays previously entered Event Definitions in saved Assay Protocols
- Ability to edit 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
- Automatic addition/update of Event Definitions in the Protocol Comments field of the Assay Protocol, viewable in Create Protocol View

## Steps for Running the Event Wizard with Target Activation

### Before Running the Event Wizard...

1) Create a protocol using the Target Activation BioApplication without defining Events. Set optimized parameter values (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

NOTE

We suggest closing the 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**.

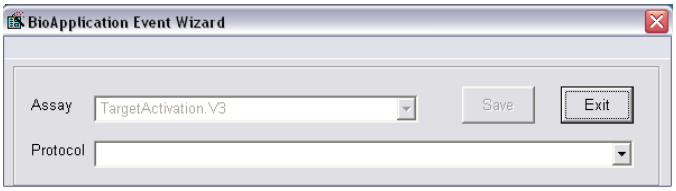
For vHCS Discovery Toolbox computers: From the Windows **Start** menu, select **Programs** > **Cellomics > vHCS Scan > BioAppEventWizard**

## Protocol Selection and Loading

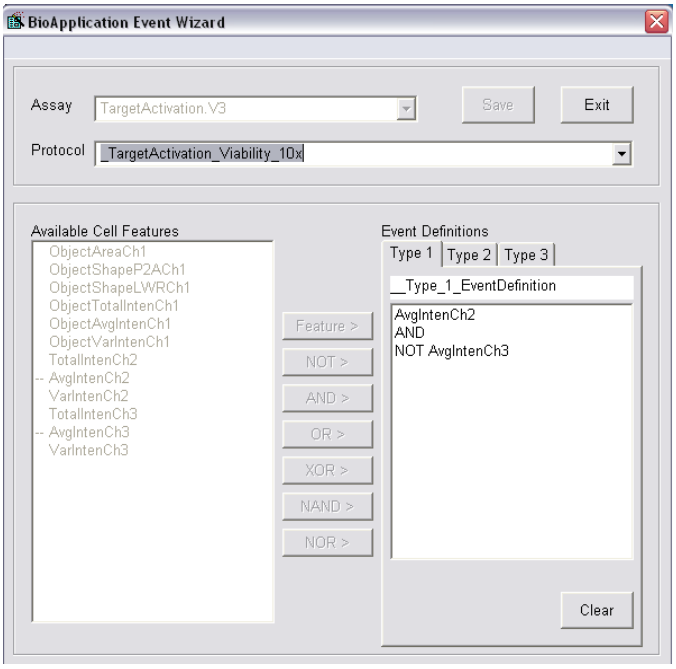
The first window that is displayed (see below) allows selection of any Target Activation Assay Protocol. Note that Event Definitions can only be specified for pre-existing Assay Protocols.

### To select an Assay Protocol,

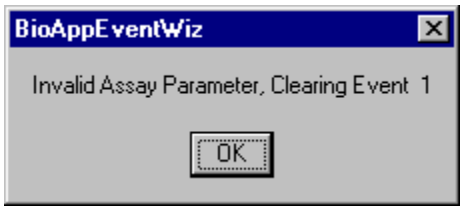
1) From the **Assay** drop-down list, select **TargetActivation.V4**
2) From the **Protocol** drop-down list, click on the arrow to view the list of existing Target Activation Assay Protocols.
3) Select the desired Assay Protocol from the list.




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. If Event Definition is invalid due to unintentional editing from within the Scan software application, the following message will be displayed and the Event Definition will be cleared.



If the 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.

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

## Defining Events

This section describes the steps involved in defining individual Events in a Target Activation 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**).

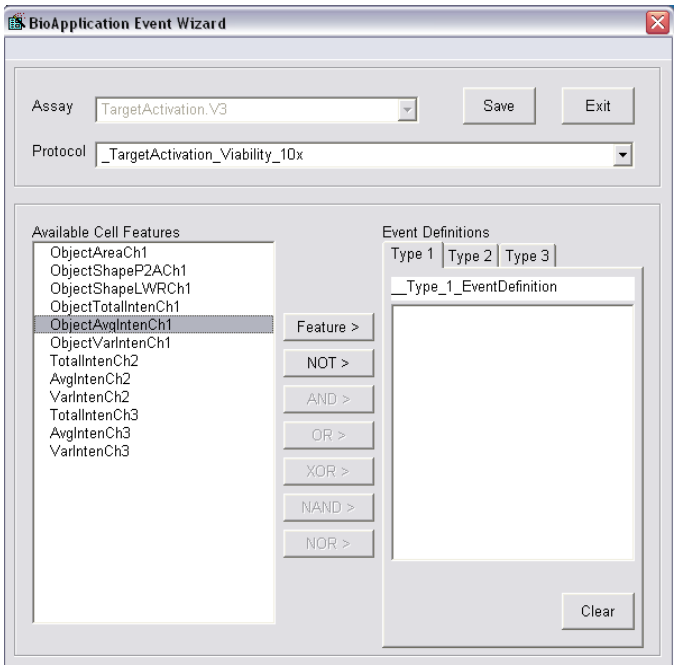
The procedure to be followed when constructing Event Definitions is described in the sequence of screenshots below. Cell Features are combined with Boolean operators to produce Event Definitions. Any Cell Feature can be selected by clicking on the feature name in the Available Cell Features list and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

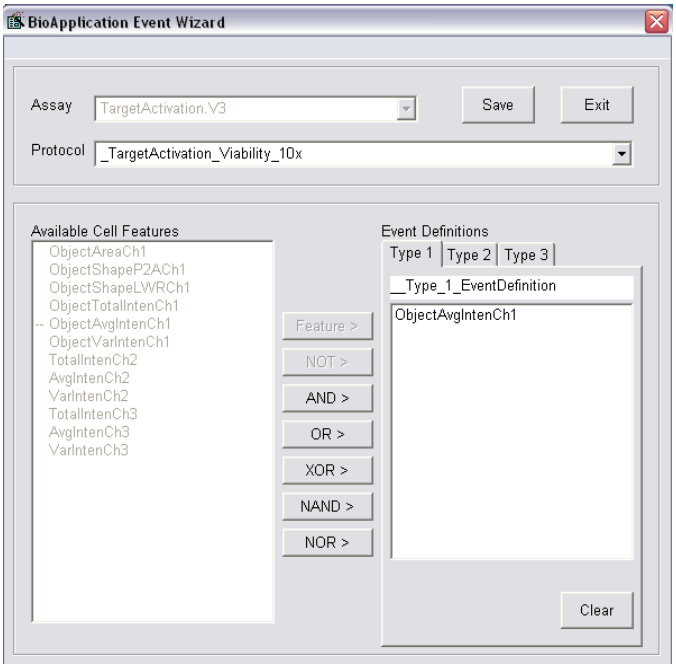
**1)** Select the Event Definition that you want to define by clicking on the **Type 1**, **Type 2**, or **Type 3** tab.

**2)** If you wish to edit a pre-existing Event Definition, click the **Clear** button.

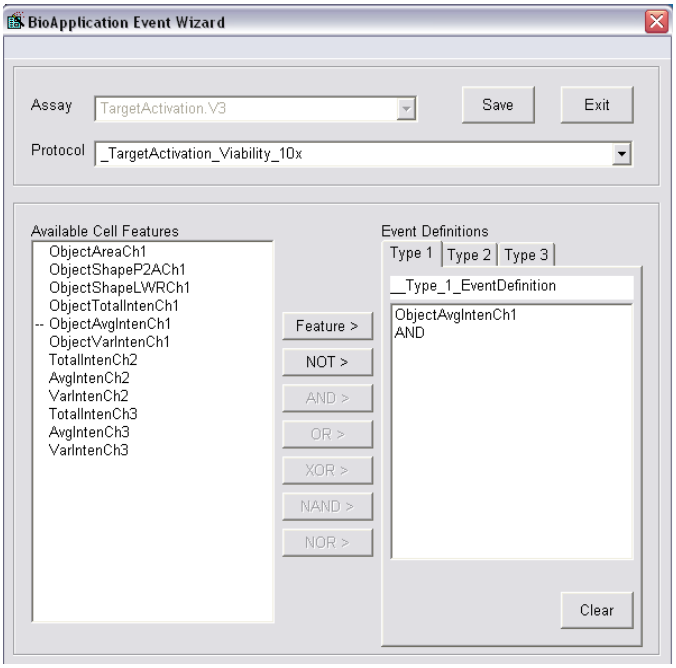
**3)** Select the desired Cell Feature by clicking on the feature name from the Available Cell Features list. In this example, the **ObjectAvgIntenCh1** feature is selected.



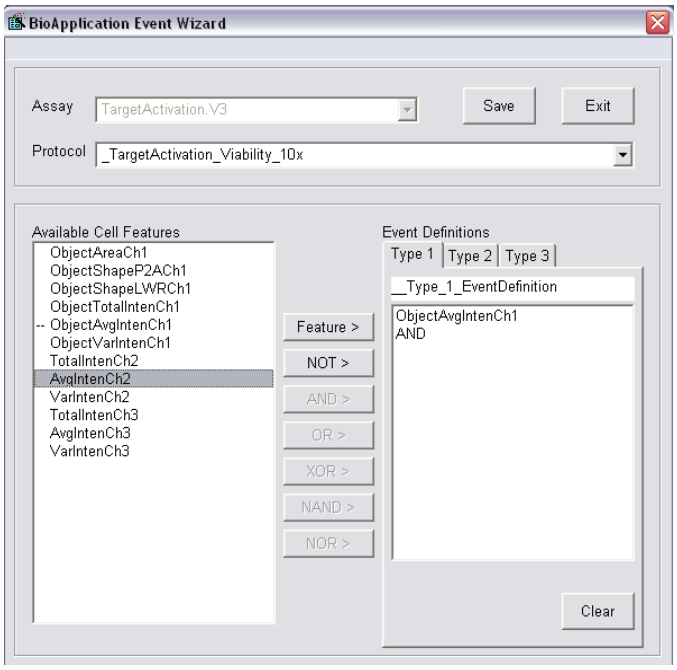
**4)** Click the **Feature>** button to transfer the Cell Feature into the Event Definition (see below).



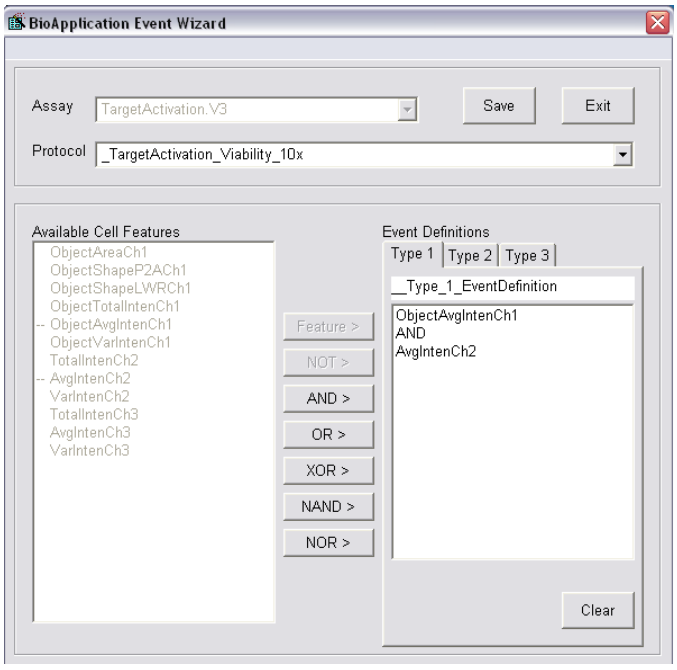

**5)** Click on the desired Operator button to transfer it into the Event Definition. In this example, the **AND** operator is selected (see below).



**6)** Repeat the cycle for adding another Cell Feature to the Event Definition. In this case, the Cell Feature **AvgIntenCh2** has been selected for addition.

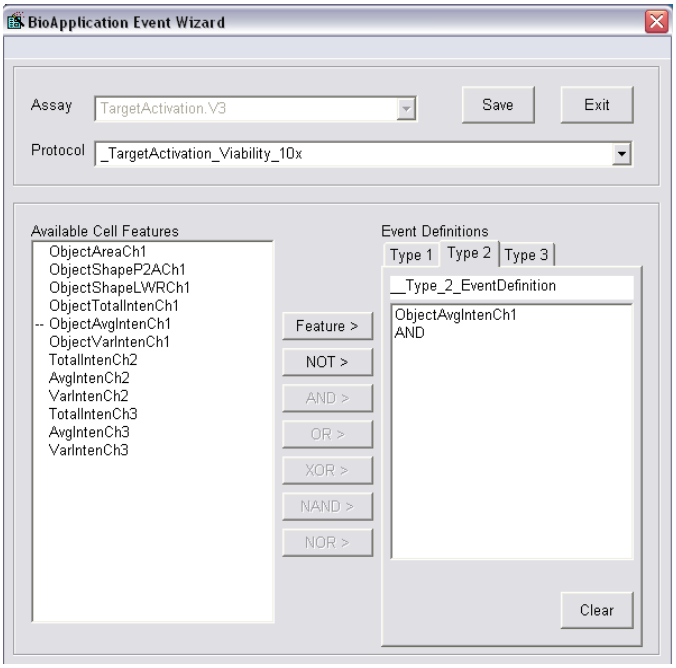


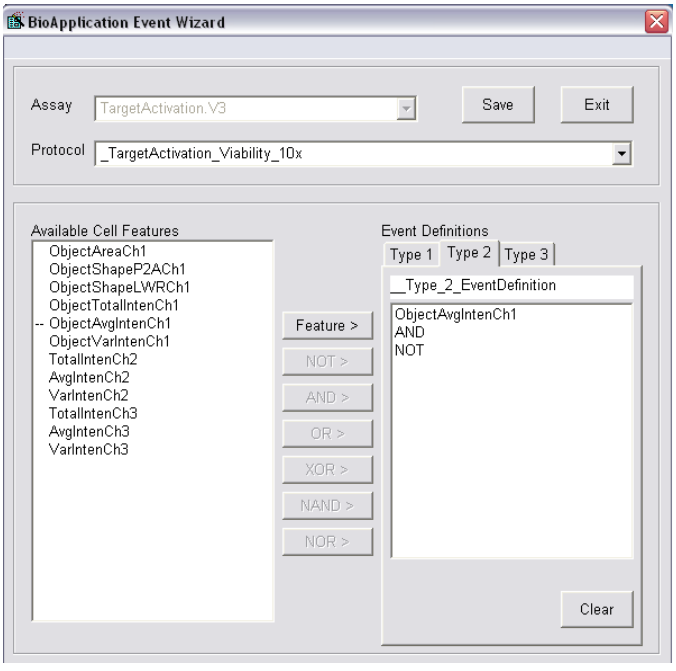

**7)** As before, click on the **Feature >** button to transfer the Cell Feature into the Event Definition (see below).

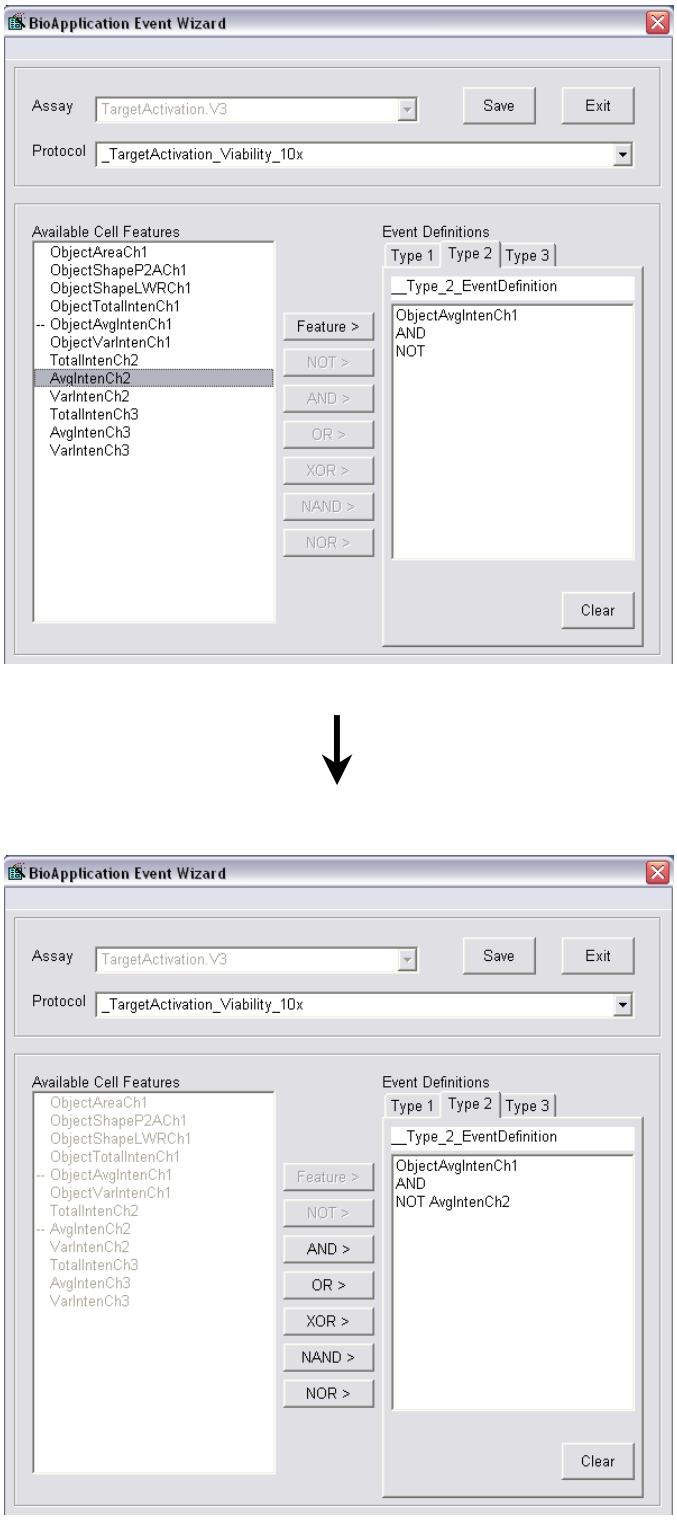



**Type_1_EventDefinition** is: **ObjectAvgIntenCh1** AND **AvgIntenCh2**

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.

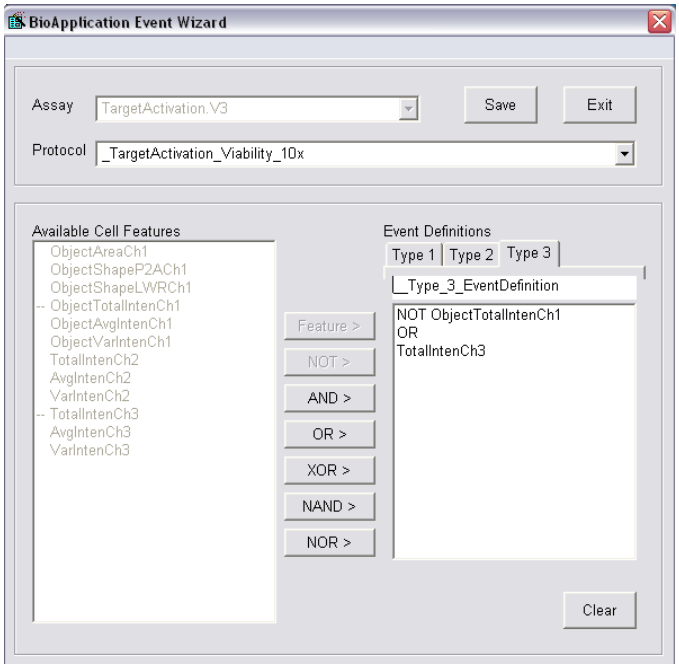





**Type_2_EventDefinition** is: **ObjectAvgIntenCh1** ANDNOT **AvgIntenCh2**

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 **ObjectTotalIntenCh1** OR **TotalIntenCh3**

### To clear an Event Definition,

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

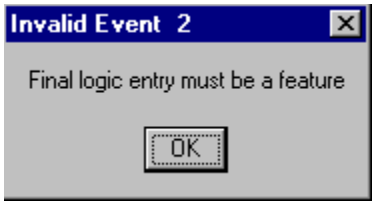
### To save the updated Assay Protocol,

- Click the **Save** button. Note that an Assay Protocol cannot be renamed using the BioApplication Event Wizard. Renaming of protocols can only be done through the Scan software application.

**NOTE**

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

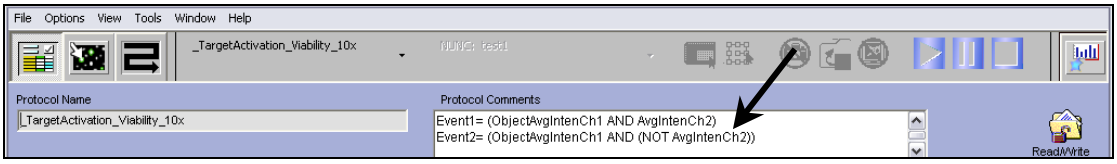
The BioApplication Event Wizard has the capability to automatically validate Event Definitions. If Event Definition validation fails, the protocol will not be saved. For example, Event Definitions are considered invalid if they do not end with a Cell Feature. In this case, clicking on the **Save** button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



**To exit the BioApplication Event Wizard,**

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

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 Target Activation V4 BioApplication using the iDev software. More detailed technical descriptions of each Assay Parameter can be found in Chapter 2 of this guide.

## iDev Protocol Optimization Tasks

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

### Image Preprocessing

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

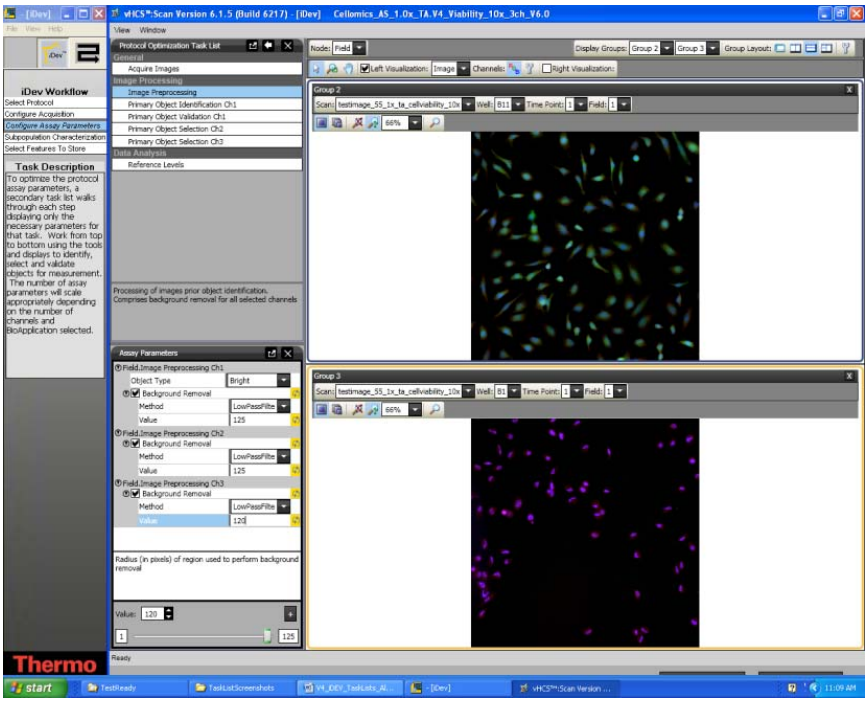



***Figure 16.*** *Protocol Optimization Task – Image Preprocessing*

## Primary Object Identification Ch1

Primary Object Identification is the identification of objects in the Channel 1 image. The task involves setting up methods and values for primary object smoothing, object identification, and object segmentation for Channel 1 objects. You can also remove small artifacts by checking the **Object Cleanup** check box.

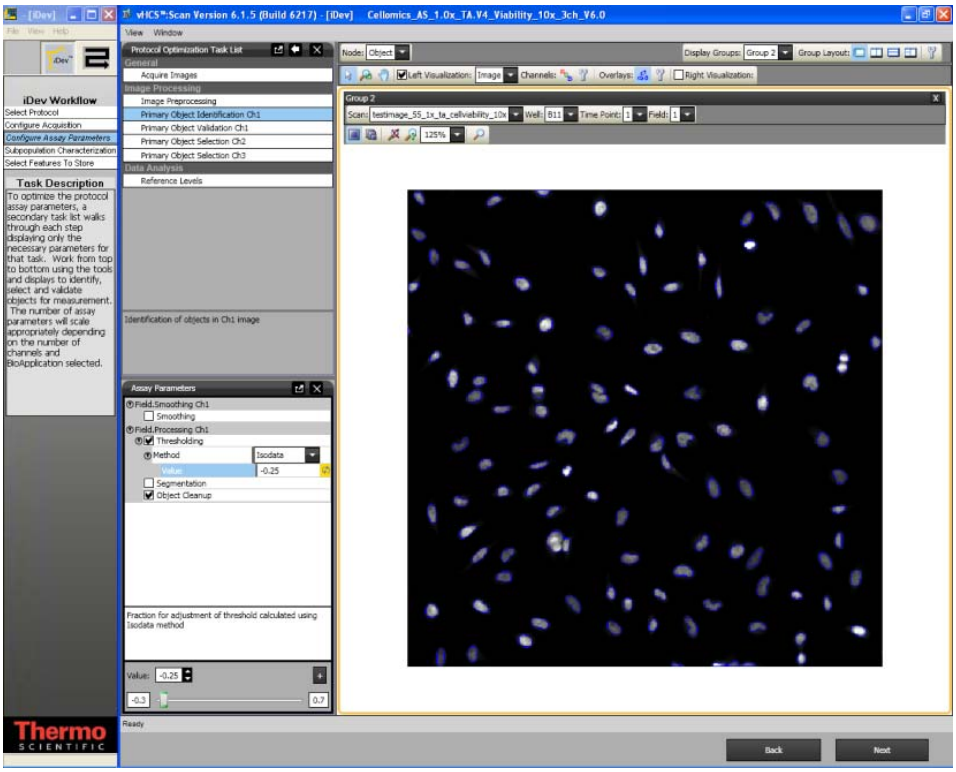



***Figure 17.*** *Protocol Optimization Task – Primary Object Identification Ch1*

## Primary Object Validation Ch1

Primary Object Validation is object selection/rejection based on area, shape, and intensity features calculated for the primary object in Channel 1. In this task, you will set minimal and maximal values for validating (equivalent to selecting and rejecting objects in the ArrayScan Classic software) objects in Channel 1 based on object selection features. Additionally in this task, you will determine if objects that are on the border of the field are included or rejected from analysis.

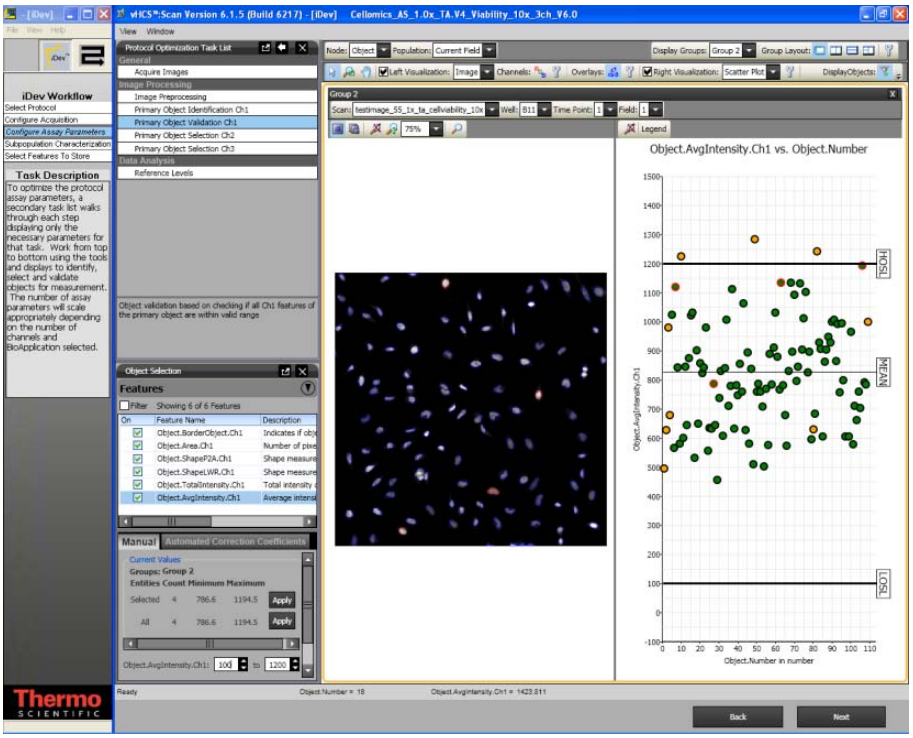



***Figure 18.*** *Protocol Optimization Task – Primary Object Validation Ch1*

## Primary Object Selection Ch2 through ChN

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

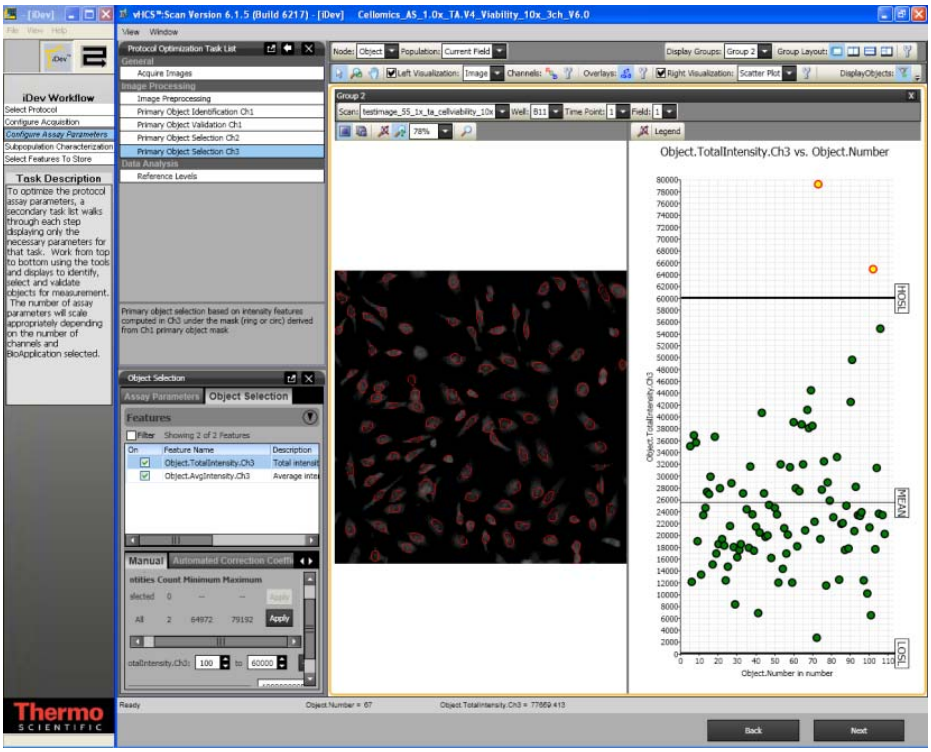



***Figure 19.*** *Protocol Optimization Task – Primary Object Selection Ch2 through ChN*

## Reference Levels

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

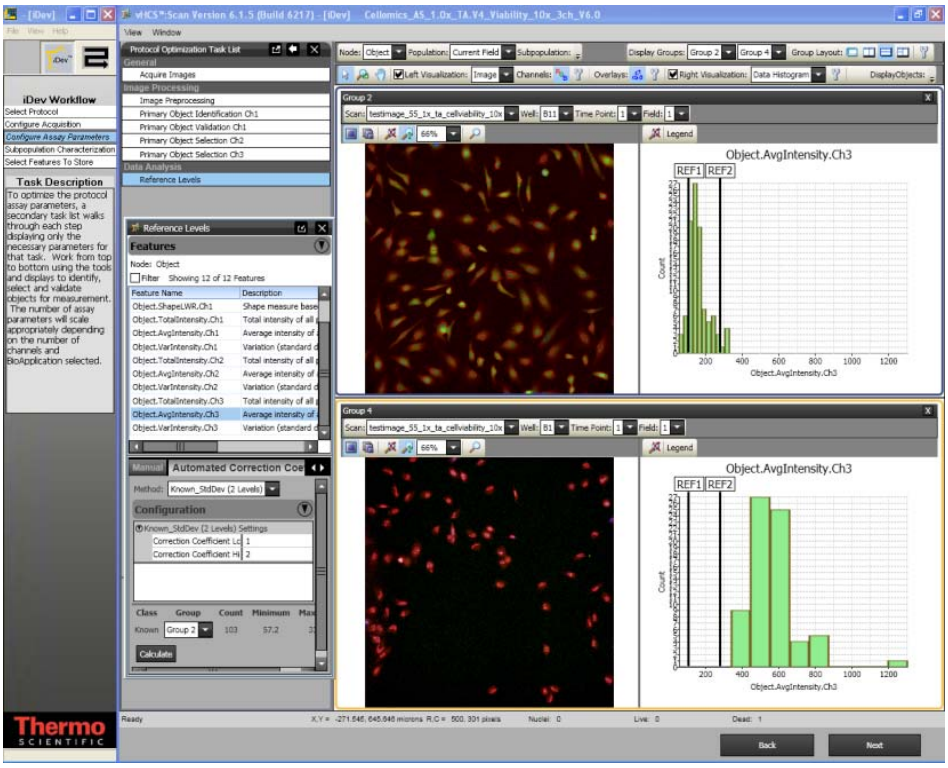



***Figure 23.*** *Protocol Optimization Task – Reference Levels*

## 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.

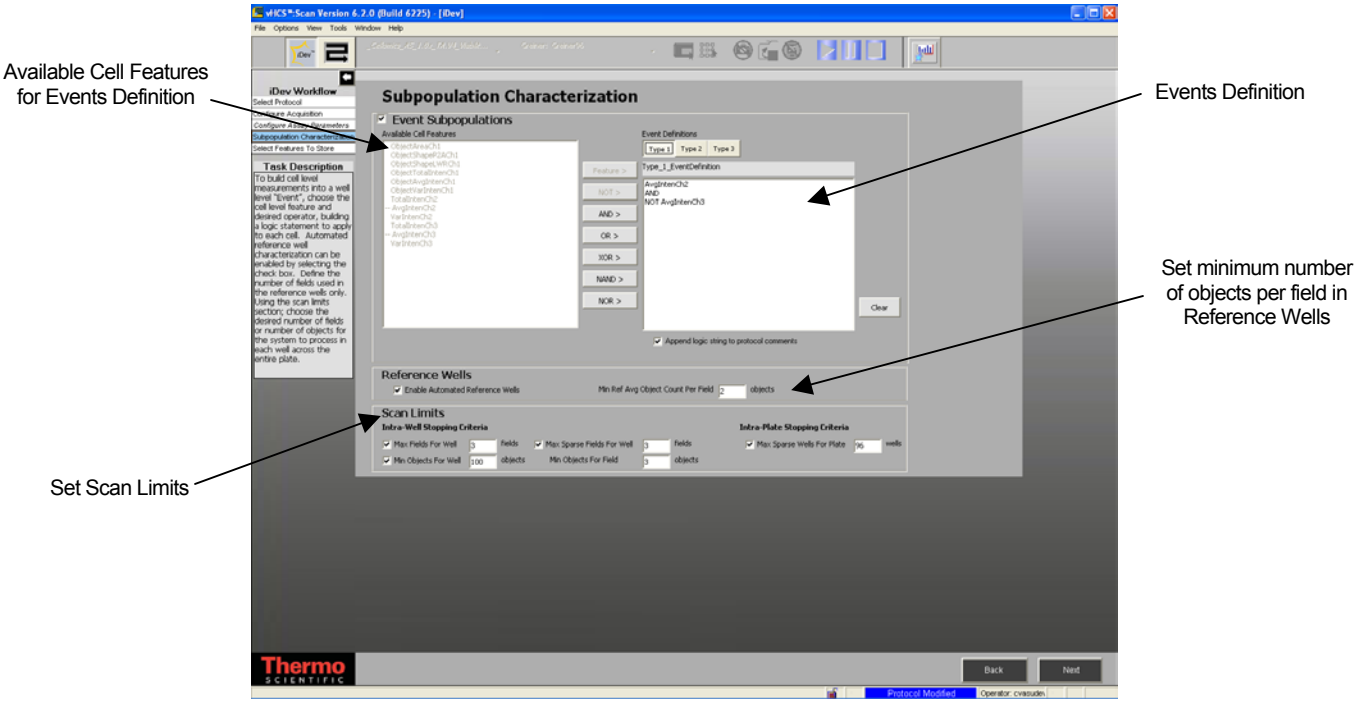



***Figure 24.*** *Subpopulation Characterization Task*

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