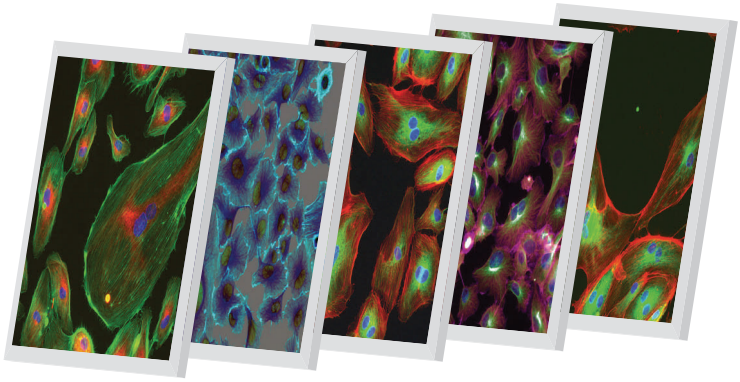
Thermo Scientific Cellomics® Molecular Translocation V4

BioApplication Guide



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Cellomics® Molecular Translocation BioApplication Guide

V4 Version

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Chapter

1

Overview of the Molecular Translocation BioApplication

High Content Screening (HCS) uses fluorescence-based reagents, advanced optical imaging systems and sophisticated image analysis software (BioApplications) to analyze and quantify the status of biologically relevant targets and physiological processes in cells. This Guide provides a brief description for using one such versatile and flexible BioApplication, Molecular Translocation. This BioApplication can be used to study the activation and/or translocation of biomolecules, such as transcription factors and protein kinases, between the nucleus and cytoplasm of a cell. This guide contains the following chapters:

Chapter 1 provides an overview of the Molecular Translocation BioApplication with example biology.

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

Chapter 3 describes the use of the BioApplication with guidance on how to use the BioApplication Events Wizard.

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

System Compatibility

The Molecular Translocation BioApplication described in this document is designed to run on the following versions of the Cellomics® HCS Reader platforms:

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

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

Cell Biology Background

Signal transduction pathways play a fundamental but critical role in almost all cellular physiological processes, such as cell division, differentiation, cell motility, immune system function, neuronal transmission, and apoptosis. The activation of a signaling pathway usually results in the transmission of an extracellular biological signal to the inside of the cell (cytoplasm or nucleus) followed by a physiological response from the

cell such as activation of transcription factors that result in the expression of certain gene products (Karin 1994). This physiological response is often generated by the translocation of macromolecules, such as transcription factors or protein kinases, or smaller molecules, second messengers, from one cellular compartment to another. Cellular signal transduction pathways are very complex by nature and involve multiple regulatory mechanisms with significant crosstalk and redundancy so that critical cellular functions are carried out appropriately (Edwards 1994; Weng, Bhalla, and Iyengar 1999; Jordan, Landau , and Iyengar 2000; Sears and Nevins 2002). Some examples of these complex signaling pathways include, but are not limited to, the MAPK pathways, NFAT mediated pathways, NFκB mediated pathways, FAS pathway, PI3K mediated pathways, and G-protein pathways (Seger and Krebs 1995; Robinson and Cobb 1997; Reiser, Ammerer, and Ruis 1999; Rao, Luo, and Hogan 1997; Mercurio and Manning 1999; Li and Stark 2002; Wajant 2002;Cantley 2002; Neves, Ram, and Iyengar 2002). These signaling pathways have been implicated in a wide range of diseases, and the understanding of these pathways have led to tremendous interest in targeting them to treat certain diseases (Seidel, Lamb, and Rosen 2000; Ethier 2002).

Most biological signaling pathways involve activation and/or translocation of more than one signaling molecule either in parallel or serially and, hence, it is important to study the activation and/or translocation of multiple signaling molecules simultaneously. The Molecular Translocation BioApplication allows researchers to quantify activation and/or translocation of more than one signaling molecule simultaneously between the cytoplasm and nucleus at the single cell level. This BioApplication quantifies the relative distribution of the signaling molecule between the nucleus and cytoplasm of a cell. Additionally, this BioApplication also determines if a cell is a responder to one or more targets, allowing you to delineate complex signal transduction pathways. Figure 1 shows representative images obtained from a Cellomics HCS Reader running the Molecular Translocation BioApplication. Note in Figure 1 that the phospho-c-jun is already present at a basal level in the nucleus and treatment with anisomycin or TNF-α causes an increase in the nuclear levels of phospho-c-jun. The transcription factor NFκB, on the other hand, translocates into the nucleus following treatment with TNF-α only, while anisomycin treatment has no effect on the translocation of NFκB. The Molecular Translocation BioApplication allows you to quantify both types of responses simultaneously.

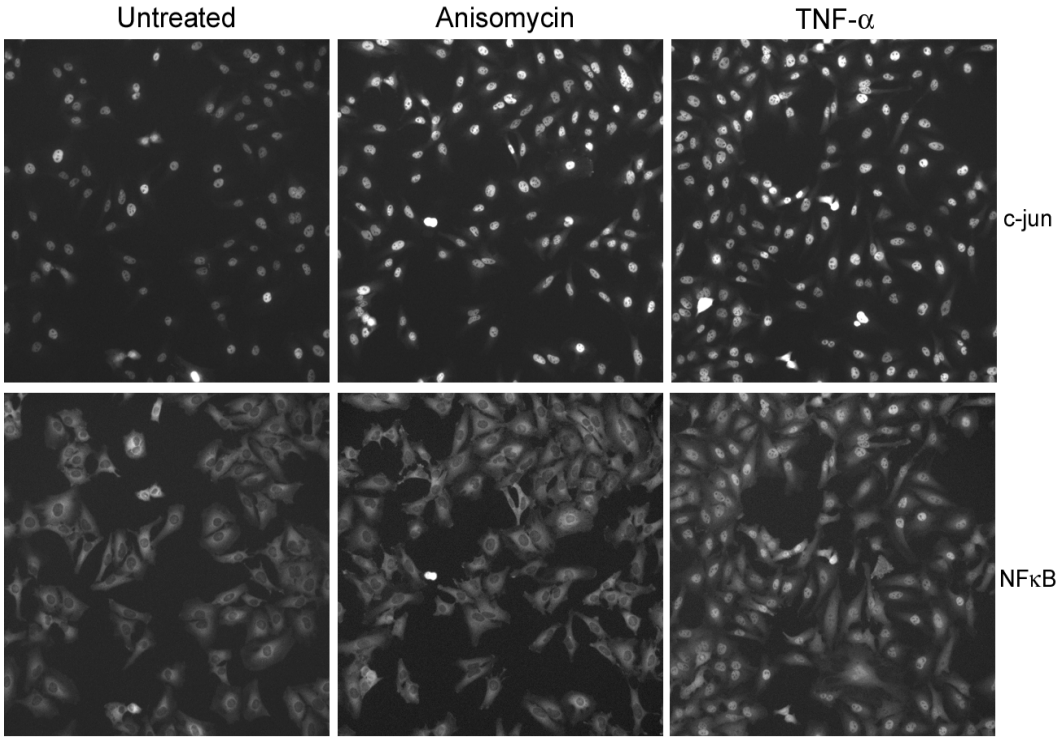


Figure 1. *HeLa cells were treated with vehicle, anisomycin (400 ng/ml) or TNF-α (50 ng/ml) for 25 minutes, fixed in formaldehyde, and stained for phospho-c-jun and NFκB. Cells treated with anisomycin show an increase only in phospho-c-jun staining in the nucleus compared to untreated cells. However, cells treated with TNF- α show an increase in both phospho-c-jun and NFκB staining in the nucleus compared to untreated cells. Images were obtained from Cellomics HCS Reader using the Molecular Translocation BioApplication.*

The Molecular Translocation BioApplication can be used to screen for compounds that can activate or inhibit the translocation of macromolecules to the nucleus from the cytoplasm or from the cytoplasm to the nucleus as a component of various signaling pathways. You are only limited by the choice of primary and secondary antibodies that are available. Because of the unique ability to quantify the percentage of cells in a well that respond to up to three targets simultaneously, the Molecular Translocation BioApplication is a powerful tool that allows you to do pathway analysis involved in biological signal transduction. Additionally, you could also design an assay that would help in determining cross-talk between different signaling pathways.

BioApplication Overview

The Molecular Translocation BioApplication is capable of measuring the activation and/or translocation of multiple targets at the single cell level. It analyzes images and measures differences between and ratios of fluorescence intensities in the nuclear and cytoplasmic regions on a single cell level. The BioApplication is capable of making fluorescence intensity measurements in up to 6 channels, with Channel 1 used for object identification and other channels used for targets. Using Reference Wells, the BioApplication can also automatically set upper and lower limits for nucleus-to-cytoplasm intensity difference and ratio and reports the percentage of cells in each well that is above the upper limit and below the lower limit. Also, this BioApplication is capable of reporting the percentage of cells in each well that are responders to multiple targets (up to three targets), allowing for studying signaling pathways.

Event Definition

The Molecular Translocation BioApplication allows simultaneous definition of up to three Events to enable rapid multiparametric analysis at the level of individual cells across multiple Cell Features. Figure 2 relays these capabilities and how they interact to enable event definition and detection. 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 or automatically computed via Reference Wells as described in Chapter 2) 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 or lower limit defined by ***Feature*Ch*N*LevelHigh** (***Feature*Ch*N*Status =1**) or ***Feature*Ch*N*LevelLow** (***Feature*Ch*N*Status = 2**). It is critical that the upper limits be set accordingly. 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.

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.

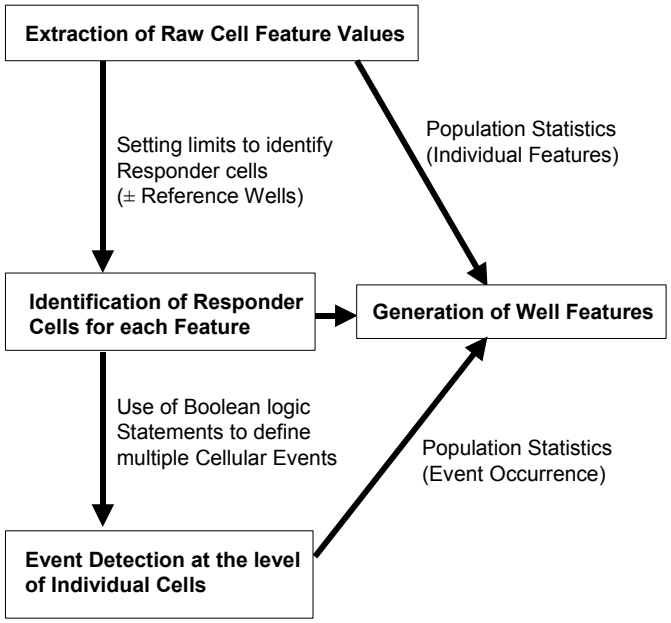


Figure 2. *Schematic showing the process of definition and detection of cellular events*

BioApplication Measurements

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

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

Measurement	Cellular Region Measured	Cell Features	Field/Well Features
Cell & Well Features Reported for Channel 1 :			
General		Cell # Top Left Width Height X Centroid Y Centroid	Valid Cell Count Selected Cell Count %Selected Cells Valid Field Count Selected Cell Count Per Valid Field
Morphology	Nucleus	Area Shape P2A Shape LWR	MEAN, SD,SE,CV All Selected Cells
Intensity	Nucleus	Total Inten Avg Inten	
Cell & Well Features Reported for Channels 2 through 6:			
General		EventTypeProfile EventType1Status EventType2Status EventType3Status	Event Type 1: Cell Count, % Cells Event Type 2: Cell Count, % Cells Event Type 3: Cell Count, % Cells
Intensity	Circ	Avg Inten ChN	MEAN, SD,SE,CV All Selected Cells
	Ring	Avg Inten ChN	
	Circ/Ring	AvgIntenDiffChN & Status AvgIntenRatioChN & Status	MEAN, SD,SE,CV, %HIGH, %LOW - all Selected Cells %HIGH, %LOW for CombinedAvgIntenDiffCh2Ch3, Ch2Ch4, Ch3Ch4, Ch2Ch3Ch4 %HIGH, %LOW for CombinedAvgIntenRatioCh2Ch3, Ch2Ch4, Ch3Ch4, Ch2Ch3Ch4
Gating	Modified nucleus mask	Avg Inten ChN Total Inten ChN	none

Table 1. *Cell and Well Level measurements for the Molecular Translocation BioApplication. *Note that "ChN" refers to Channels 2-6.*

Example Biology

Cellular biosensors are rapidly becoming critical tools in the drug discovery process. A biosensor can be defined as a reporter of a biological process that is typically made up of biologically active molecules, cell fractions, whole cells or even tissues. Biosensors have the ability to transduce biochemical information from a specific biological process into an analytical signal that can be read on various instruments. Fluorescent protein biosensors are finding increasing use in studying various biological processes (Zhang et al., 2002; Meyer and Teruel 2003; Giuliano, Chen and Haskins 2003). These biosensors can be engineered to report on a myriad of biological processes in live cells (Karynov et al., 2000; Kalab, Weis and Heald 2002; Zhang et al., 2001; Sloan-Lancaster et al., 1998; Scott, Malcomber and O'Hare 2001; Marshall et al., 2002). Such fluorescent biosensors can be classified into different types based on how they report about a biological process. These are: (i) intensity based, (ii) wavelength based, (iii) based on molecular mobility such as anisotropy, (iv) chimeras of fluorescent proteins, (v) FRET based, and (vi) positional biosensors.

A positional biosensor changes its cellular location following the biological process that the biosensor is designed to report about. This change in the spatial location of the biosensor is used as the readout to monitor target activity (Giuliano, Chen, and Haskins 2003). The Molecular Translocation BioApplication can be used to kinetically characterize the behavior of fluorescent positional biosensors, provided the biosensor translocates from cytoplasm-to-nucleus or nucleus-to-cytoplasm or can also be used to kinetically characterize fluorescent biosensors whose fluorescent intensity in the nuclear region of cells changes in response to biological processes.

We used a green fluorescent protein (GFP)-tagged positional biosensor that responds to the activation of the caspase 3 apoptosis pathway by the redistribution of the biosensor from a predominantly cytoplasmic localization to nuclear biased localization. Figure 3 shows a schematic representation of the translocation of the caspase 3 biosensor from the cytoplasm to the nucleus, following the activation of the caspase 3 apoptosis pathway. Activation of apoptosis is also usually accompanied by morphological changes to the cell and/or the nucleus. These changes can also be easily monitored using the Molecular Translocation BioApplication.

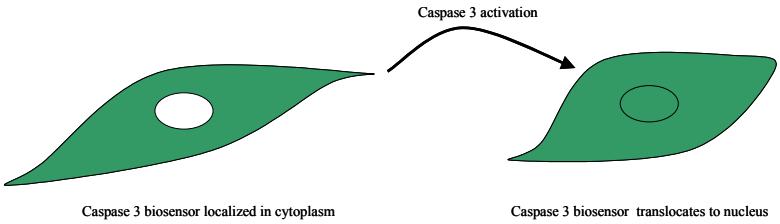


Figure 3. *The activation of a caspase 3 dependent apoptosis pathway results in the translocation of the caspase 3 biosensor from its basal localization in the cytoplasm to a predominantly nuclear localization. The activation of the apoptosis pathway may also lead to morphological changes in the cell.*

Changes in the localization of the caspase 3 biosensor following exposure of cells to staurosporine are shown in Figure 4. These images of live HeLa cells stably expressing the caspase 3 biosensor, either untreated or treated with 4 μM staurosporine, were

acquired with the KineticScan® HCS Reader. As the figure clearly shows, untreated cells show very little translocation of the caspase 3 biosensor to the nuclear region of the cells. However, exposure to staurosporine, a compound known to activate caspase 3 mediated apoptosis (Lopez, and Ferrer 2000; Bijur, De Sarno and Jope 2000), amongst having a variety of biological effects in cells, causes a translocation of the biosensor from the cytoplasm into the nuclear region. Note the corresponding changes in the cell and nuclear morphology (nuclear condensation) of cells exposed to staurosporine.

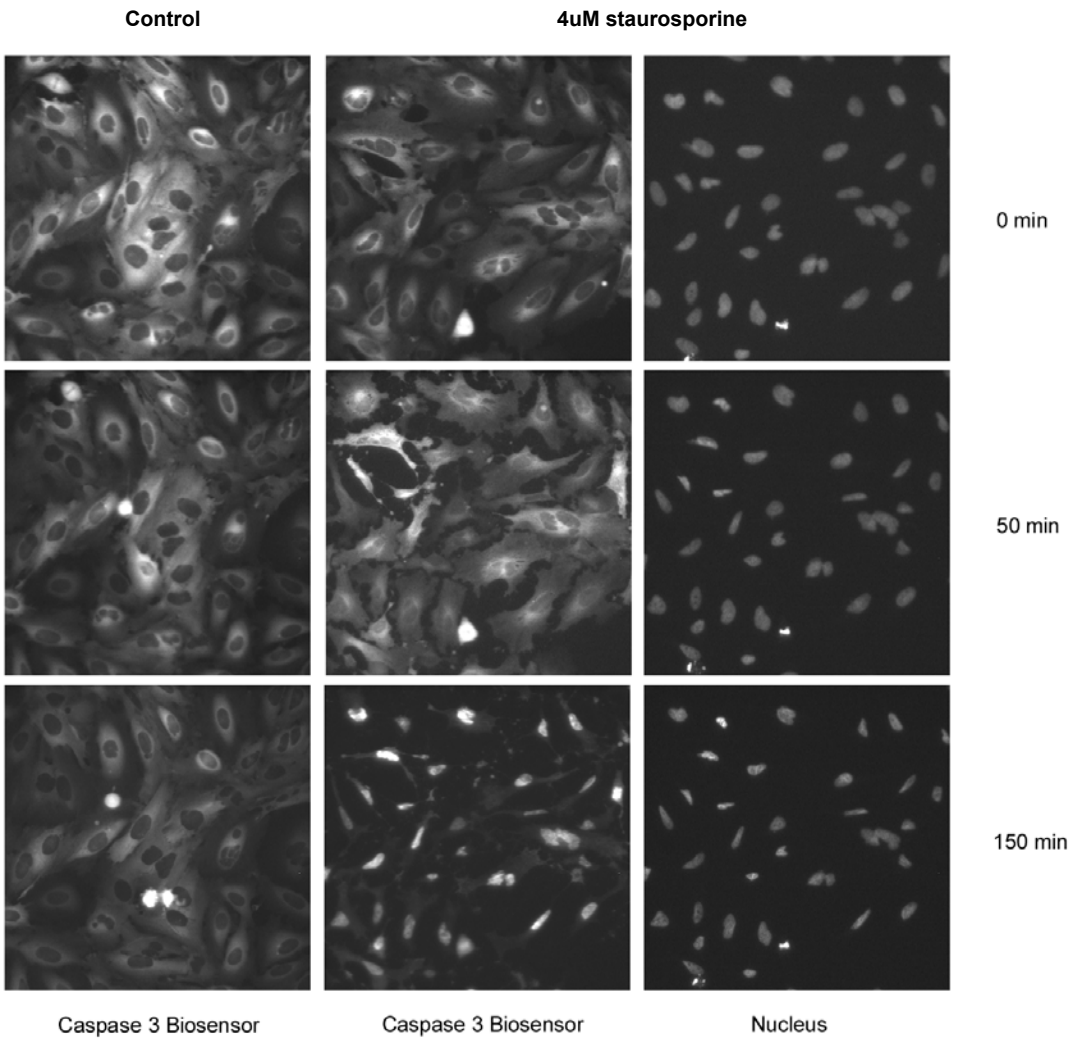


Figure 4. *Time lapse images of live HeLa cells stably expressing the caspase-3 biosensor. Panels in the left and middle columns are images of caspase-3 biosensor in untreated or 4 µM staurosporine treated cells at different time points. Panels in the right most column are Hoechst-33342 stained nuclear images of the cells treated with 4 µM staurosporine. Note that in cells treated with 4 µM staurosporine, the caspase-3 fluorescent protein biosensor has begun to translocate into the nucleus at 50 minutes and by 150 minutes almost all of the fluorescence is located in the nucleus. Untreated cells do not show any translocation of the fluorescent protein biosensor. Also notice that the nuclei have condensed (nuclear morphological change) in the cells treated with 4 µM staurosporine over time. The images were obtained using a 20X, 0.4NA objective on a KineticScan HCS Reader with the Molecular Translocation BioApplication.*

Demonstration Data Using the Molecular Translocation BioApplication

The use of the Molecular Translocation BioApplication to differentiate between the translocation/activation of the transcription factors NFκB and c-jun, by two different compounds, TNF-α and anisomycin, is described here. HeLa cells were plated on to 96-well plates and treated with either TNF-α or anisomycin. Cells were then fixed and stained with a nuclear dye and appropriate primary and secondary antibodies for both NFκB and phospho-c-jun. The cells were then imaged on a Cellomics HCS Reader. Images shown previously in Figure 1 are of untreated, anisomycin or TNF-α treated cells, where only cells treated with TNF-α shows activation/translocation of both NFκB and c-jun.

One of the output features of the Molecular Translocation BioApplication is the percentage of cells in any well that are above an upper limit (user definable) for the nucleus-to-cytoplasm intensity difference and ratio for multiple targets. Figure 5A and Figure 5B show the translocation/activation of NFκB and phospho-c-jun respectively when treated with different doses of TNF-α or anisomycin, as measured by the nucleus to cytoplasm intensity difference. Figure 5C shows the percentage of cells as a function of dosage of TNF-α or anisomycin, whose nucleus to cytoplasm intensity difference for both NFκB and phospho-c-jun are above the upper limit. The data clearly shows that only cells treated with TNF-α have a significant percentage of cells, whose nucleus-to-cytoplasm intensity difference for both NFκB and phosphor-c-jun are above the respective upper limits. This shows that TNF-α can activate both NFκB and c-jun, while anisomycin is capable of activating only c-jun. Thus from one experiment, you can determine the activation/translocation of multiple targets by your test compounds using the Molecular Translocation BioApplication.

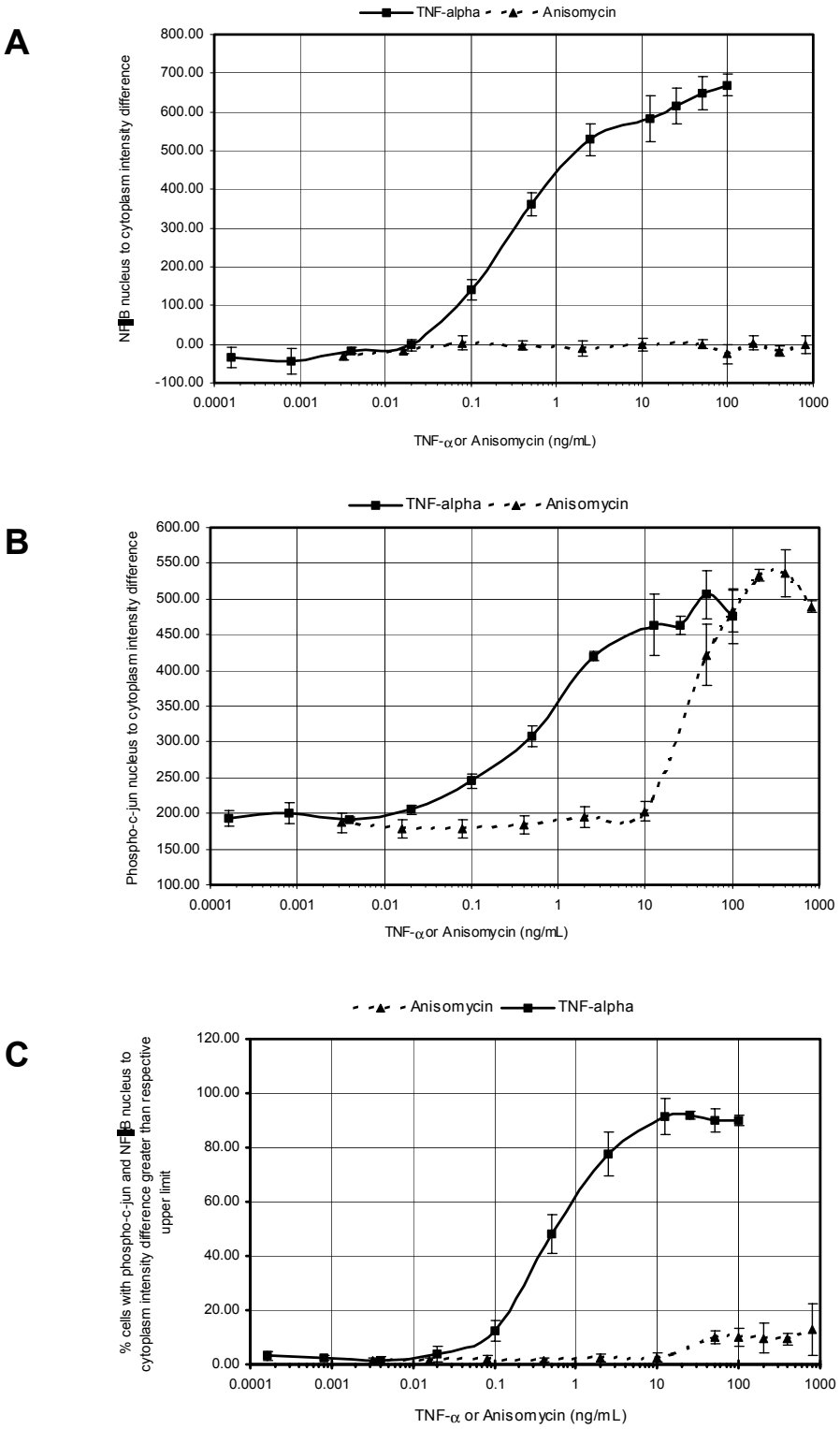


Figure 5. *HeLa cells were treated with varying doses of either anisomycin or TNF-α, fixed and stained for NFκB and phospho-c-jun and scanned on a Cellomics HCS Reader. Data is mean ± sd from 4 wells per concentration of the compound for all the plots. (A) NFκB nucleus to cytoplasm intensity difference in TNF- α or anisomycin treated cells. (B) Phospho-c-jun nucleus to cytoplasm intensity difference in TNF- α or anisomycin treated cells. (C) Percentage of cells whose NFκB and phosphor-c-jun nucleus to cytoplasm intensity difference.*

References

Cantley, L. C., 2002. The phosphoinositide 3-kinase pathway. *Science.* 296:1655-1657.

Edwards, D.R. 1994. Cell signaling and the control of gene transcription. *Trends Pharmacol. Sci.* 15:239-244.

Ethier, S. P. 2002. Signal transduction pathways: the molecular basis for targeted therapies. *Semin. Radiat. Oncol.* 12:3-10.

Giuliano, K. A., Y. T. Chen, and J. R. Haskins. 2003. Positional biosensors: A new tool for high-content screening. *Modern Drug Disc.* 6 (8): 33-37.

Jordan, J. D., E. M. Landau, and R. Iyengar. 2000. Signaling networks: The origins of cellular multitasking. *Cell.* 103:193-200.

Kalab, P., K. Weis, and R. Heald. 2002. Visualization of a Ran-GTP gradient in interphase and mitotic *Xenopus* egg extracts. *Science.* 295: 2452-2456.

Karin, M. 1994. Signal transduction from the cell surface to the nucleus through phosphorylation of transcription factors. *Curr. Opin. Cell Biol.* 6:415-424.

Karynov, V. S., C. Chamberlain, G. M. Bokoch, M. A. Schwartz, S. Slabaugh, and K. M. Hahn. 2000. Localized Rac activation dynamics visualized in live cells. *Science.* 290: 333-337.

Li, X., and G. R. Stark. 2002. NFκB-dependent signaling pathways. *Exp. Hematol.* 30:285-296.

Lopez, E., and T. Ferrer. 2000. Staurosporine and H-7-induced cell death in SH-SY5Y neuroblastoma cells is associated with caspase-2 and caspase-3 activation, but not with activation of the FAS/FAS-L-caspase-8 signaling pathway. *Brain Res. Mol. Brain Res.* 85: 61-67.

Marshall. A. J., A. K. Krahn, K. Ma, V. Duronio, and S. Hou. 2002. TAPP1 and TAPP2 are targets of phosphatidylinositol 3-kinase signaling in B cells: Sustained plasma membrane recruitment triggered by the B-cell antigen receptor. *Mol. Cell. Biol.* 22: 5479-5491.

Mercurio, F., and A. M. Manning. 1999. Multiple signals converging on NF-κB. *Curr. Opin. Cell Biol.* 11:226-232.

Meyer, T., and M. N. Teruel. 2003. Fluorescence imaging of signaling networks. *Trends Cell Biol.* 13: 101-106.

Neves, S. R., P. T. Ram, and R. Iyengar. 2002. G protein pathways. *Science.* 296:1636-1639.

Rao, A., C. Luo, And P. G. Hogan. 1997. Transcription factors of the NFAT family: Regulation and function. *Annu. Rev. Immunol.* 15:707-747.

Reiser, V., G. Ammerer, and H. Ruis. 1999. Nucleocytoplasmic Traffic for MAP kinases. *Gene Expression.* 7:247-254.

Robinson, M. J., and M. H. Cobb. 1997. Mitogen-activated protein kinase pathways. *Curr. Opin. Cell Biol.* 9:180-186.

Scott, E. S., S. Malcomber, and P. O'Hare. 2001. Nuclear Translocation and activation of the transcription factor NFAT is blocked by herpes simplex virus infection. *J. Virol.* 75: 9955-9965.

Sears, R. C., and J. R. Nevins. 2002. Signaling networks that link cell proliferation and cell fate. *J. Biol. Chem.* 277:11617-11620.

Seger, R., and E. G. Krebs. 1995. The MAPK signaling cascade. *FASEB J.* 9:726-735.

Seidel, H. M., Lamb, P., and Rosen, J. 2000. Pharmaceutical intervention in the JAK/STAT signaling pathway. *Oncogene.* 19:2645-2656.

Sloan-Lancaster, J., J. Presley, J. Ellenberg, T. Yamazaki, J. Lippincott-Schwartz, and L. E. Samelson. 1998. ZAP-70 association with the T cell receptor ζ (TCR ζ): Fluorescence imaging of dynamic changes upon cellular stimulation. *J. Cell Biol.* 143: 613-624.

Wajant, H. 2002. The FAS signaling pathway: More than a paradigm. *Science.* 296:1635-1636.

Weng, G., U. S. Bhalla. and R. Iyengar. 1999. Complexity in biological signaling systems. *Science* 284:92-96.

Zhang, J., R. E. Campbell, A. Y. Ting, and R. Y. Tsien. 2002. Creating new fluorescent probes for cell biology. *Nat. Rev. Mol. Cell Biol.* 3: 906-918.

Zhang, J., Y. Ma, S. S. Taylor, and R. Y. Tsein. 2001. Genetically encoded reporters of protein kinase A activity reveal impact of substrate tethering. *Proc. Natl. Acad. Sci. USA.* 98: 14997-15002.

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 Molecular Translocation BioApplication works and provides a description of the adjustable Assay Parameters and Output Features of the BioApplication.

Overview

The Molecular Translocation BioApplication measures the translocation of macromolecules from the cytoplasm to the nucleus and from the nucleus to the cytoplasm in single cells by quantifying the relative amount of fluorescently labeled macromolecules of interest in the nucleus and cytoplasm. The BioApplication measures nucleus-to-cytoplasm or cytoplasm-to-nucleus translocation in up to five target channels. A minimum of two channels is needed to measure nucleus-to-cytoplasmic translocation of a target. Using the intensity and morphological features that you select in Channel 1, the algorithm first identifies each cell's nucleus. The individual nuclei identified in Channel 1 are used to create a mask called "Circ" in the target channels. An annular region called "Ring" is defined in the cytoplasm beyond the nuclear (i.e., Circ) region. You can determine the size of the Circ and Ring masks in the target channels (refer to Figure 7 for a description of the Circ and Ring regions).

The Circ and Ring masks are applied only to objects that pass the object selection criteria based on a mean pixel intensity that you set in each of the target channels. The average intensity is measured within the Circ and Ring masks in each of the channels, and the BioApplication reports both the difference and ratio between the average pixel intensity measured in the Circ and Ring masks for each of the target channels. The difference and ratio between the average pixel intensities in the Circ and Ring masks are two quantitative measures of relative amounts of the molecules of interest in the nuclear and cytoplasmic regions of the cell and the translocation of molecules of interest between these regions.

The BioApplication also determines the status of each cell as to whether the cell is below, within, or above the limits set in the Assay Parameters for the difference and ratio between the Circ and Ring masks. These limits can be set either manually or automatically by the application using Reference Wells. The BioApplication reports the percentage of cells in each well that are below the lower limit (***Feature*Ch*N*LevelLow**) and above the upper limit (***Feature*Ch*N*LevelHigh**) for the difference and ratio between Circ and Ring masks average pixel intensity measurements.

The Molecular Translocation BioApplication allows you to study the effect of test compound(s) on more than one target simultaneously in each well. The BioApplication has the unique ability to report the combined percentage of cells in each well that is either below or above the limit in each target channel for up to three target channels. This unique feature allows you to carry out detailed biological signaling pathway analysis and enables you to classify test compounds as being an activator or an inhibitor of either a single target or multiple targets.

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, 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 tubes and thus configuring the application. In Channels 2-6 intensities of circs and rings are measured. Depending on the properties of the objects being identified in Channel 1, the proper setting of intensity thresholds for the channels is necessary to ensure proper quantitative analysis.

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

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

Table 2. *Intensity Threshold Methods Available for Each Channel*

NOTE

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

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

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

Of the three intensity threshold method options, **None** means that no intensity threshold is applied. This option is not available in Channel 1 because an intensity threshold is required to define the pixels making up the tube and the targets. The choice of the **None** threshold is an option for Channel 2 and the only option for Channels 3-6.

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

The **Isodata Threshold** method is known as a histogram-derived threshold in that the threshold is chosen from the histogram of pixel intensities in the image (i.e., the image's

brightness histogram). The schematic in Figure 6 demonstrates how the histogram-derived threshold value is calculated.

The value entered for the Object Identification in the application for the histogram-derived threshold method is an offset applied to determine the final threshold which is applied to the image. If the histogram-derived threshold is *T*, then its relationship to the actual (final) threshold, T_F, 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. The threshold *T* is chosen so that it is equal to the average of the mean of the pixel intensities to the left of the threshold (m_L) and the mean of the pixel intensities to the right of the threshold (m_R). A negative value identifies dimmer objects and results in larger object masks. A positive value results in smaller object masks.	-0.999 – 9.999 (offset)	1 - 32767
Fixed	A fixed pixel intensity value between 0-32767 is applied	0 – 32767 (actual intensity in image)	0 - 32767

Table 3. *Intensity Threshold Descriptions and ranges available for the Molecular Translocation BioApplication*

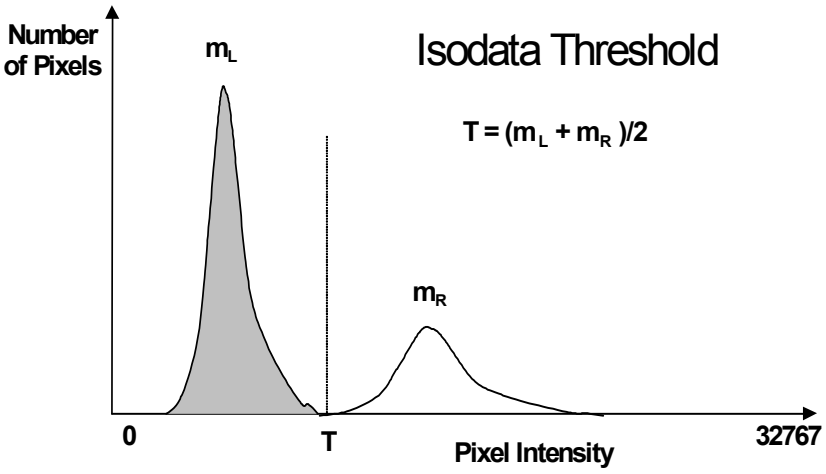


Figure 6. *Histogram-derived Isodata Intensity Threshold Method. Background peak is shown in gray and object peak is shown in white.*

Description of Assay Parameters and Settings

The Molecular Translocation bioassay uses an algorithm and reagents which has been extensively tested and validated for robust screening performance. Assay Parameter values determined from validation plates for representative cell types have been supplied as defaults in the standard Assay Protocol. Assay Parameters are adjustable to allow customization of the algorithm to your own samples and conditions.

Input parameters can be found in the Create Protocol View of the ArrayScan Classic software or in the Protocol Optimization task list of the iDev software. In the ArrayScan Classic software, the available input parameters will 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. These Assay Parameters are described in detail in the following sections.

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**
- **Adjusting the Size of Circ and Ring Masks**
- **Object Type**
- **Background Correction**
- **Object Smoothing**

- **Object Segmentation**
- **Reject Border Objects**

Reference Well Control

The two general Assay Parameters controlling the use of Reference Wells are: **UseReferenceWells** and **MinRefAvgCellCountPerField**. The **UseReferenceWells** Assay Parameter allows you to choose whether Reference Wells are to be used to determine the population characterization limits. If Reference Wells are to be used (value = **1**), then the Assay Parameter **MinRefAvgCellCountPerField** must be set. This is the minimum number of objects detected per field that are required for acceptance of data from that field in the Reference Wells. You can enter a number here that closely matches the average number of selected objects that are needed to obtain statistically valid results for the different output features. 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 parameters will be described in later sections.

Units for Morphological Measurements

You have the option of either choosing micrometers or pixels as the unit to report morphological measurements. This is done with the **UseMicrometers** Assay Parameter, which when set to **0**, causes the area of the objects to be reported in pixels. Otherwise, if the Assay Parameter is set to **1**, the measurements are reported in micrometers. This information is calculated automatically from the chosen magnification and camera resolution settings.

Adjusting Size of Circ and Ring Masks

The size of the Circ and Ring regions can be adjusted and applied for the target channels, and this size is set in relation to the primary object. The same numerical value set for each of these three Assay Parameters (Figure 5) are used for all the target channels. Figure 7 shows a schematic of the three Assay Parameters that control the size of the Circ and Ring masks.

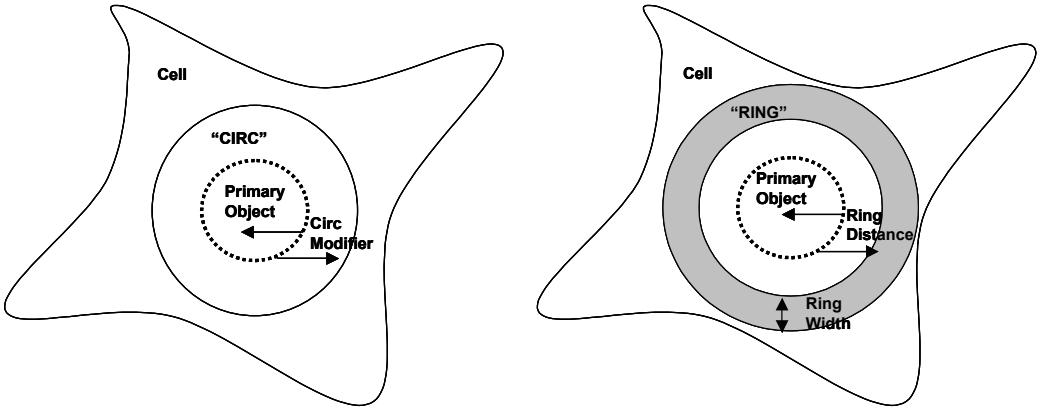


Figure 7. *Schematic representation of the Circ and Ring regions in the Molecular Translocation BioApplication.*

The **CircModifier** Assay Parameter is used to adjust the Circ region for the target channels. The value of this Assay Parameter equals the number of pixels that the primary object mask is dilated (a positive value) or eroded (a negative value) to define the Circ region. In other words, this is the number of pixels added to, or subtracted from, the perimeter of the primary object (nucleus) to define the area covered by the Circ region.

Two Assay Parameters are used to define the area covered by the Ring region for the target channels. These are **RingDistance** and **RingWidth**. The **RingDistance** Assay Parameter is the number of pixels added to the perimeter of the primary object to define the inner boundary of the Ring region. In other words, **RingDistance** is the number of pixels the inner boundary of the Ring region is away from the boundary of the primary object mask. The location of the Ring depends on the sign of the Assay Parameter. For negative values, the inner boundary of the Ring is within primary object mask and closer to the center. For positive values, the inner boundary of the Ring is outside of the primary object mask and farther from the center. The **RingWidth** Assay Parameter defines the width of the Ring region in pixels. Note that since the Ring region is derived from the primary object, the area it covers is defined independently from the area covered by the Circ region.

In addition, the **MaskModifierCh*N*** Assay Parameter works similar to the **CircModifier** Assay Parameter but for gating purposes. This Assay Parameter's value represents the number of pixels to modify the Channel 1 nucleus mask in subsequent channels. Negative values will shrink the mask, positive values will expand the mask, and a value of **0** will not modify the mask.

ObjectTypeCh*N*

NucTypeCh1, TargetTypeCh*N*

The "Object Type" Assay Parameters allow you to specify whether the objects of interest are brighter or darker than the background of the image. Setting the value to **1** 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 **NucTypeCh1** and **TargetTypeChN**.*

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

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

The strategy for object identification/detection is 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 Ch*N*

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

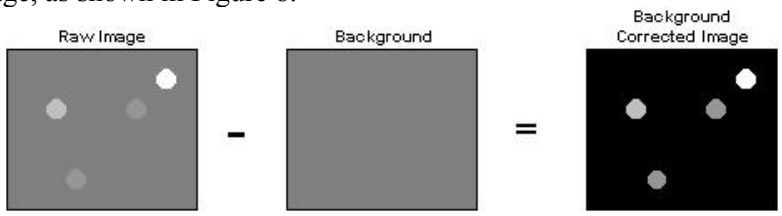


Figure 8. *Background Correction available in each channel.*

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

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

NOTE

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

Background Correction can be adjusted on each channel separately.

The background-corrected image is not stored or shown.

Object Smoothing

NucSmoothFactorCh1

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

Nuclear Smoothing can be used to connect fragments of nuclei 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).

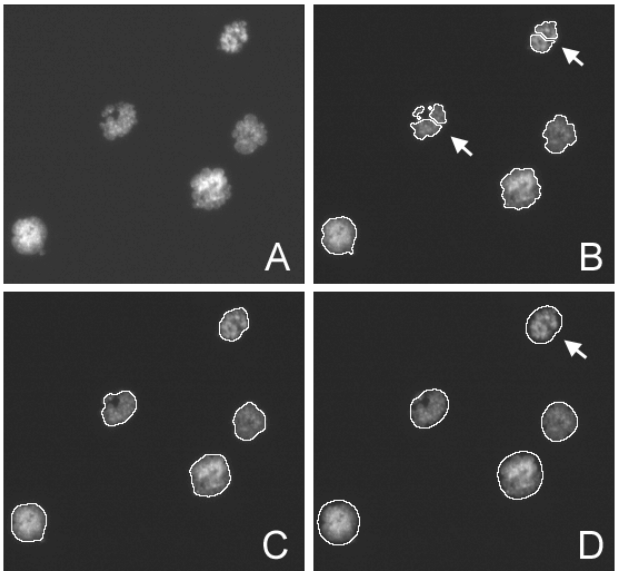


Figure 9. NucSmoothFactorCh1*: Images show the effect of different degrees of smoothing on the same field of apoptotic cell nuclei. A: Raw Image; B:* ***NucSmoothFactorCh1*** *= 0; C:* ***NucSmoothFactorCh1*** *= 3; D:* ***NucSmoothFactorCh1*** *= 8.*

Object Segmentation

NucSegmentationCh1

Even though you may have chosen an optimal intensity threshold and an appropriate degree of smoothing for nucleus identification, you may encounter situations where individual nuclei cannot be properly resolved, such as in the case of densely packed objects. Object Segmentation will help resolve and identify individual objects. Two methods are available for object segmentation in the Molecular Translocation BioApplication: Geometric (shape and size) and Intensity (intensity peaks). These methods are illustrated in Figure 10.

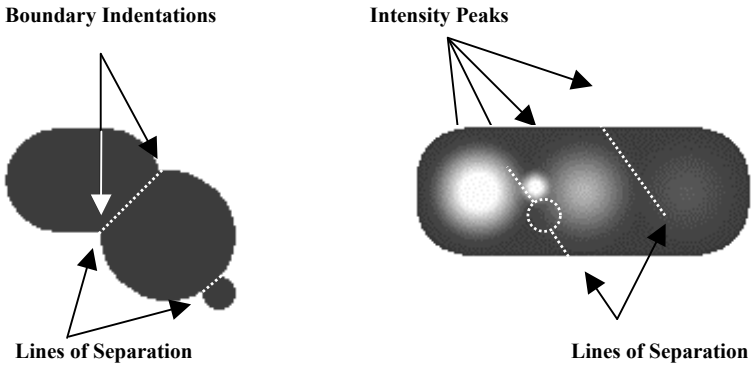


Figure 10: *Object Segmentation Options. The image on the left illustrates the Geometric Method while the image on the right illustrates the Intensity Method.*

The Geometric method splits touching objects on the basis of shape, relying on boundary indentations to locate the line of separation. This method works best if the individual objects have smooth boundaries with pronounced indentations at their point of contact. The Geometric method can be used to separate objects that are uniform in intensity (i.e., saturated) or that have multiple intensity peaks (i.e., noisy or textured).

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

The **NucSegmentationCh1** Assay Parameter (see Table 6) controls both methods. A positive value for this parameter selects the Geometric method, and the value is the approximate radius (in pixels) of the smaller of the objects being separated. The value of this parameter for the Geometric method depends on the magnifying power of the microscope objective and camera setting (pixel binning).

Setting **NucSegmentationCh1** to a negative value selects the peak Intensity method. In this case, the absolute value of the parameter specifies the minimum relative height of the intensity peak (image contrast) to be used in the segmentation. Making **NucSegmentationCh1** more negative reduces the number of objects created by the segmentation. In case of noisy and textured objects the use of object smoothing may be required to reduce over-segmentation. The value of this parameter for the Intensity method does not depend on the power of the microscope objective or camera setting (pixel binning).

A value of zero for NucSegmentationCh1 disables the segmentation.

Segmentation Method	NucSegmentationCh1 Assay Parameter	Value Range
None	0	0
Geometric	radius (in pixels) of smaller object	1 to 255
Intensity	minimum relative height of intensity peak (local contrast)	-1 to -32767

Table 6: *Channel 1 Object Segmentation Options*

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

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

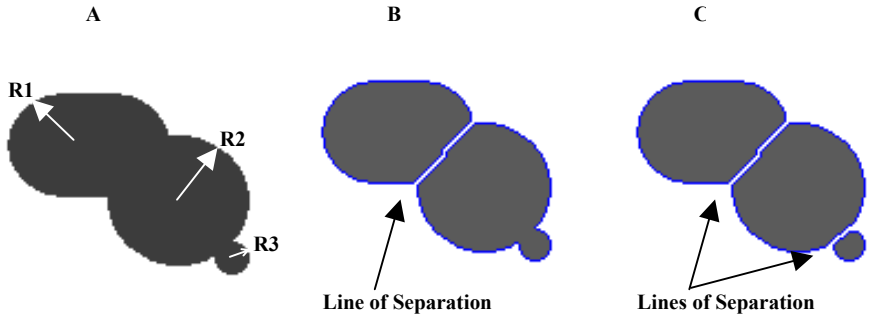


Figure 11: *Object segmentation: Geometric Method. A) Radii of touching objects: R1 = 24, R2 = 30, R3 = 7; B) Result of segmentation using* ***NucSegmentationCh1*** *= R1; C) Result of segmentation using* ***NucSegmentationCh1*** *= R3.*

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

In general terms, these peak intensity heights are nothing but measures of local contrast of an image. The degree of object segmentation can be controlled by setting the NucSegmentationCh1 parameter value equal to the lowest relative height of intensity peaks of objects that need to be separated. The lower the value the more objects will be

segmented and vise versa. In case of over-segmentation, setting the **NucSmoothFactorCh1** value greater than 0 should be used to alleviate the problem.

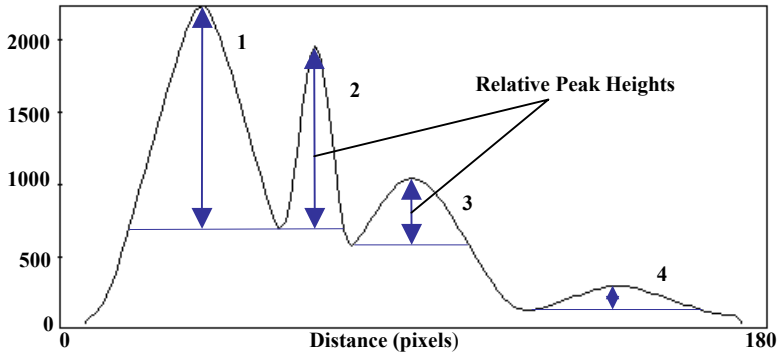


Figure 12: *Intensity profile of an object with four intensity peaks. Peak #1 height = 1500, peak #2 height = 1250, peak #3 height = 500, and peak #4 height = 200.*

Only two peaks in Figure 12, #1 and #2, have relative intensity greater than 1000. Thus, setting the **NucSegmentationCh1** parameter to -1000 marks only two peaks to be used for segmentation (or marks the two objects that need to be separated) and results in object segmentation as shown in Figure 13A. Setting the **NucSegmentationCh1** parameter to - 400 or -100 marks three or all four objects that need to be separated. The results of segmentation of three and four objects are shown in Figure 13B and 13C.

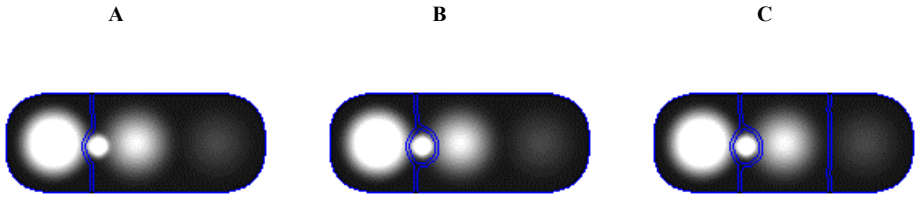


Figure 13: *Object segmentation: Intensity Method. A) Result of segmentation using parameter* ***NucSegmentationCh1*** *= -1000; B) Result of segmentation using parameter* ***NucSegmentationCh1*** *= -400; C) Result of segmentation using parameter* ***NucSegmentationCh1*** *= -100,*

Reject Border Objects

RejectBorderNucsCh1

When running the BioApplication, you have the option of not including and analyzing objects that touch the border of the image field. This is controlled by the **RejectBorderNucsCh1** Assay Parameter. If this Assay Parameter = **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.

Basic Assay Parameters

In Basic Mode, only a subset of the Assay Parameters can be viewed and adjusted. These Assay Parameters mainly pertain to morphological object identification (Table 7).

Parameter	Units	Description
UseReferenceWells	Binary	Use reference wells to calculate high and low response levels: 0 = No, 1 = Yes
CircModifier	Pixels	Number of pixels to modify Ch1 object (nucleus) mask to create circ mask: Negative value = Shrink mask, 0 = Do not modify mask, Positive value = Expand mask
RingDistance	Pixels	Distance (in pixels) from Ch1 object (nucleus) mask to the inner rim of ring mask
RingWidth	Pixels	Width (in pixels) of ring mask
NucTypeCh1	Binary	Type of objects (nuclei) to be identified in Ch1: 0 = Bright objects on dark background, 1 = Dark objects on bright background
BackgroundCorrectionChN	Pixels	Radius (in pixels) of region used to compute background in ChN: Negative value = Use surface fitting, 0 = Do not apply background correction, Positive value = Use low pass filter
NucSmoothFactorCh1	Number	Degree of image smoothing (blurring) prior to object detection in Ch1: 0 = Do not apply smoothing
NucSegmentationCh1	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
RejectBorderNucsCh1	Binary	Reject objects (nuclei) that touch image edges: 0 = No, 1 = Yes
TargetTypeChN	Binary	Type of targets to be identified in ChN: 0 = Bright targets on dark background, 1 = Dark targets on bright background
MaskModifierChN	Pixels	Number of pixels to modify Ch1 object (nucleus) mask in ChN: Negative value = Shrink mask, 0 = Do not modify mask, Positive value = Expand mask

Table 7. *Basic Assay Parameters available for the Molecular Translocation BioApplication. *Note that "ChN refers to Channels 1-6 for* ***Background Correction*** *and Channels 2-6 for* ***MaskModifier*** *and* ***TargetType****.*

Object Selection Parameters

Each channel has a set of specific Object Selection Parameters associated with it (Tables 8 and 9). If an object in the particular channel's image has all measured features within the range specified by the appropriate Object Selection Parameters, then it is analyzed; otherwise, it is rejected from the analysis. Object Selection Parameters can be modified for Channels 1-6. Channel 1 parameters define a valid object and Object Selection Parameters for Channels 2-6 define a selected object within that channel.

Channel 1

Parameter	Units	Description
NucAreaCh1	Pixels or μm^2	Area (in pixels or µm) of Ch1 nucleus
NucShapeP2ACh1	Number	Shape measure based on ratio of perimeter squared to 4π*Area of Ch1 nucleus (**NucShapeP2ACh1** = 1 for circular nucleus)
NucShapeLWRCh1	Number	Shape measure based on ratio of length to width for object-aligned bounding box of nucleus in Ch1. Round objects will have a value close to 1.0
NucTotalIntenCh1	Intensity	Total intensity of all pixels within Ch1 nucleus
NucAvgIntenCh1	Intensity	Average intensity of all pixels within Ch1 nucleus
NucVarIntenCh1	Intensity	Variation (standard deviation) of intensity of all pixels within Ch1 nucleus

Table 8. *Object Selection Parameters available in Channel 1 for the Molecular Translocation BioApplication*

Channel N (2-6)

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

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

Gating

The Molecular Translocation BioApplication supports population gating analysis. This feature provides selective cell processing based on fluorescence intensity. Therefore, in addition to selecting cells based on nuclear area, shape, or intensity in Channel 1, you can also select or reject cells based on fluorescence intensity in a downstream channel of your choice (Channels 2-6). The gating feature allows you to specifically identify a certain class of cells based on labeling intensity in the object mask and limit the analysis to this group of cells.

Specifying Intensity Ranges for Gating

When working in the Create Protocol View, you can specify intensity limit values by entering upper and lower limits for two intensity parameters, **AvgIntenCh*N*** and **TotalIntenCh*N***, for one or more channels. **TotalIntenCh*N*** is the total pixel intensity within the object of interest and **AvgIntenCh*N*** is the average pixel intensity within the object of interest.

After specifying the intensity parameters, the system calculates the average and total intensities for the selected channels. If the value is outside the specified intensity range, the object is removed (rejected) from the selected object set. Processing is performed only on those cells that meet all requirements (selected cells).

You can view the results of the gating process in the View software application when viewing Well Details. The feature **ValidCellCount**, displays the total number of objects found for each well that pass the Channel 1 Object Selection Parameters. The feature **SelectedCellCount**, displays the number of objects in each well that passed all requirements, including the intensity requirements. To view the intensity parameter

values, select Cell Details for a particular well. The View software application displays the intensity parameter values for each selected cell in each channel.

Specifying Mask Modifiers in Assay Channels

In addition to specifying intensity range for one or more channels, you can use the **MaskModifierCh*N*** Assay Parameter setting to adjust the area of the mask within which the intensity-based measurements are made. You may want to use this feature if you want to dilate the mask of each object to include other cellular markers. The mask can be dilated or eroded, but will not overlap with other masks from nearby objects.

Although the Mask Modifier can be set for an individual channel, the modified mask is not displayed in the Protocol Interactive or Scan Plate Views.

To determine the appropriate mask modifier setting in Protocol Interactive View edit the Mask Modifier value, click the **Run Algorithm** button, then the **Identify Objects** button in the channel you are optimizing to determine if the appropriate objects are selected. Once you are satisfied with the object selections made, enter that value in the Mask Modifier parameter.

Image Overlays

All Image Display Option Settings are available when running in Basic Mode. 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. Overlays available for the Molecular Translocation BioApplication can be found in Table 10.

Parameter	Description
Include This Channel In Composite	Determines if image for this Channel is included in composite image.
Composite Color	Determines what color will be assigned to this Channel in the composite image. Can be edited by clicking on the colored box.
SelectedCell	Indicates the cells that pass object selection parameters, as well as cells that do not touch the image border.
RejectedCell	Indicates cells that are rejected from analysis, either because they touch the image border, or because they do not meet object selection criteria set for the different channels. Rejected cells are not analyzed and are never counted by the BioApplication.
CircChN	Identifies the area under the Circ mask for the target channels (Channels 2-6).
RingChN	Identifies the area under the Ring mask for Channels 2-6.

***Table10**. Image Display option settings for the Molecular Translocation BioApplication*

Assay Parameters for Population Characterization

The Molecular Translocation BioApplication provides the ability to characterize cells based on their response compared to a control population. For a particular output feature, a range is determined from a control population (e.g., cells in untreated wells) that has the normal physiological distribution for that feature. An upper and lower limit (known as ***Feature*Ch*N*LevelHigh** and ***Feature*Ch*N*LevelLow**) sets the upper and lower bounds of this range respectively. The Status Cell Feature indicates whether a particular compound treated cell is within or beyond this range. For a particular Cell Feature, a cell with a Status value of **0** means that it is within the defined range, a Status value of **1** means that the feature value is greater than the upper limit, and a Status value of **2** means that the feature value is less than the lower limit (Table 11).

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

Table 11. *Status definitions for the Molecular Translocation BioApplication.*

The corresponding Well Features report the percentage of cells that is either greater or less than the limits defining this range. Figure 14 illustrates this concept by showing the distribution of a normal physiological population of cells for a particular Cell Feature and the shift in this distribution upon compound treatment. Please note that the shift in feature distribution can occur either to above or below the normal physiological distribution.

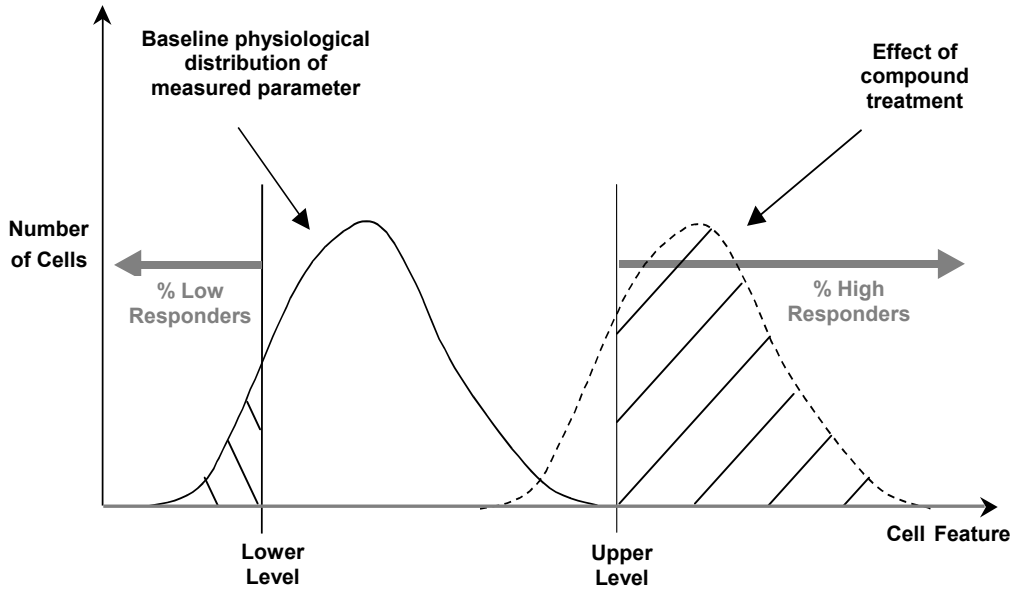


Figure 14*. Schematic population distribution of a measured feature in a population of untreated and compound treated cells. The upper and lower levels correspond to %Low or %High*

Setting Cellular Response Levels

There are two ways of setting the upper and lower limits to characterize the cell population: manually or automatically.

To set the limit (***Feature*Ch*N*LevelLow, *Feature*Ch*N*LevelHigh*)* manually, specify the lower and upper limits for the different features that are to be used to perform cell population characterization in the Assay Parameters of the BioApplication. The BioApplication then uses these limits to calculate the percentage of cells outside the bounds of these limits for the different Cell Features.

To set the limit automatically, designate particular wells on the sample plate as Reference Wells. Typically, Reference Wells contain a control, untreated population of cells that display the normal basal physiological response for the parameter being measured. These wells are first analyzed and the population distribution for the different features is then determined. The cell population characterization limits are then set by adding to, or subtracting from, the mean of the distribution its standard deviation multiplied by a coefficient that you define, named Correction Coefficient (_CC) in the Assay Parameters. The software then applies these limits to all the wells. The advantage of using Reference Wells to automatically calculate limits is that the limits are determined by a control population of cells and are independent of run-to-run variations.

The features measured for Channels 2-6 which can be used to characterize the cells in a population as described above are **MEAN_CircRingAvgIntenDiffCh*N*** and **MEAN_CircRingAvgIntenRatioCh*N***. From these results, **%HIGH/%LOW_CombinedAvgIntenDiffCh2Ch3**, **Ch2Ch4**, and **Ch3Ch4**, as well as **%HIGH/%LOW_CombinedAvgIntenRatioCh2Ch3**, **Ch2Ch4**, and **Ch3Ch4** can be calculated.

Reference Wells Processing Sequence

By setting the **UseReferenceWells** Assay Parameter to **1**, 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, the computed values will be loaded into the Assay Parameters associated with each Reference Feature and regular scanning of the plate will begin. Again, if the feature value for **MinRefAvgCellCountPerField** 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 Molecular Translocation 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.
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

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

NOTE

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

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

Specifying and Controlling Reference Wells

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

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

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

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

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

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

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

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

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

identified. Individual cells having feature values above the set or calculated value are identified as responder cells. The number of responder cells, expressed as a percentage of the entire cell population analyzed, is calculated for each assay well.

Advanced Assay Parameters

In Advanced Mode, all basic and advanced input parameteres are adjustable. The **Hide Advanced Parameters** option provides the ability to either view and adjust all the Assay Parameters or view and adjust the subset of paratmeters designated as Basic Parameters. Generally, these Assay Parameters are realated to definition and reporting of responder cells. They control the ananlysis of the data resulting from the image analysis.

For each feature undergoing population characterization, there are four advanced Assay Parameters that control its levels: ***Feature*ChN*LevelLow** and ***Feature*ChN*LevelHigh** that set lower and upper thresholds and the presence of the **_CC** suffix which designates those levels are set using Reference Wells. For example, the Assay Parameters controlling the average intensity difference of the Circ and Ring in Channel 2 are:

- **CircRingAvgIntenDiffCh2LevelHigh**
- **CircRingAvgIntenDiffCh2LevelLow**
- **CircRingAvgIntenDiffCh2LevelHigh_CC**
- **CircRingAvgIntenDiffCh2LevelLow_CC**

In the listing of Advanced Parameters in Table 12, instead of listing the level parameters for each feature, one entry for the feature will be listed giving the four different options, as shown in the following example:

- **CircRingAvgIntenDiffCh2Level*Low/High, Low/High_CC***

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

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

Parameter	Units	Description
MinRefAvgCellCountPerField	Number	Minimum average number of cells 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 selected)
Type_1_EventDefinition	---	User-defined combination of logic statements involving response feature ***(cannot be edited)***
Type_2_EventDefinition	---	User-defined combination of logic statements involving response feature ***(cannot be edited)***
Type_3_EventDefinition	---	User-defined combination of logic statements involving response feature ***(cannot be edited)***
CircRingAvgIntenDiffChN***LevelLow/High, Low/High_CC***	Intensity	Defines **CircRingAvgIntenDiffChN** population characterization thresholds
CircRingAvgIntenRatioChN***LevelLow/High, Low/High_CC***	Number	Defines **CircRingAvgIntenRatioChN** population characterization thresholds

Table 12. *Advanced Assay Parameters available for the Molecular Translocation BioApplication. *Note that "ChN" refers to Channels 2-6.*

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

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).

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

- Create your own definition for a focused biology
- Define a subpopulation by using any combination of up to four (total) status Cell Features (Two Cell Features: **CircRingAvgIntenDiffCh*N*** and **CircRingAvgIntenRatioCh*N***, are available for Ch*N* [Channels 2-6])
- 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 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. Note that 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 store Events are of the type **Type_X_EventDefinition** (X =1, 2, or 3).

Cell Features	Logic Operators
CircRingAvgIntenDiffChN CircRingAvgIntenRatioChN	NOT AND AND NOT OR OR NOT XOR NAND NOR

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

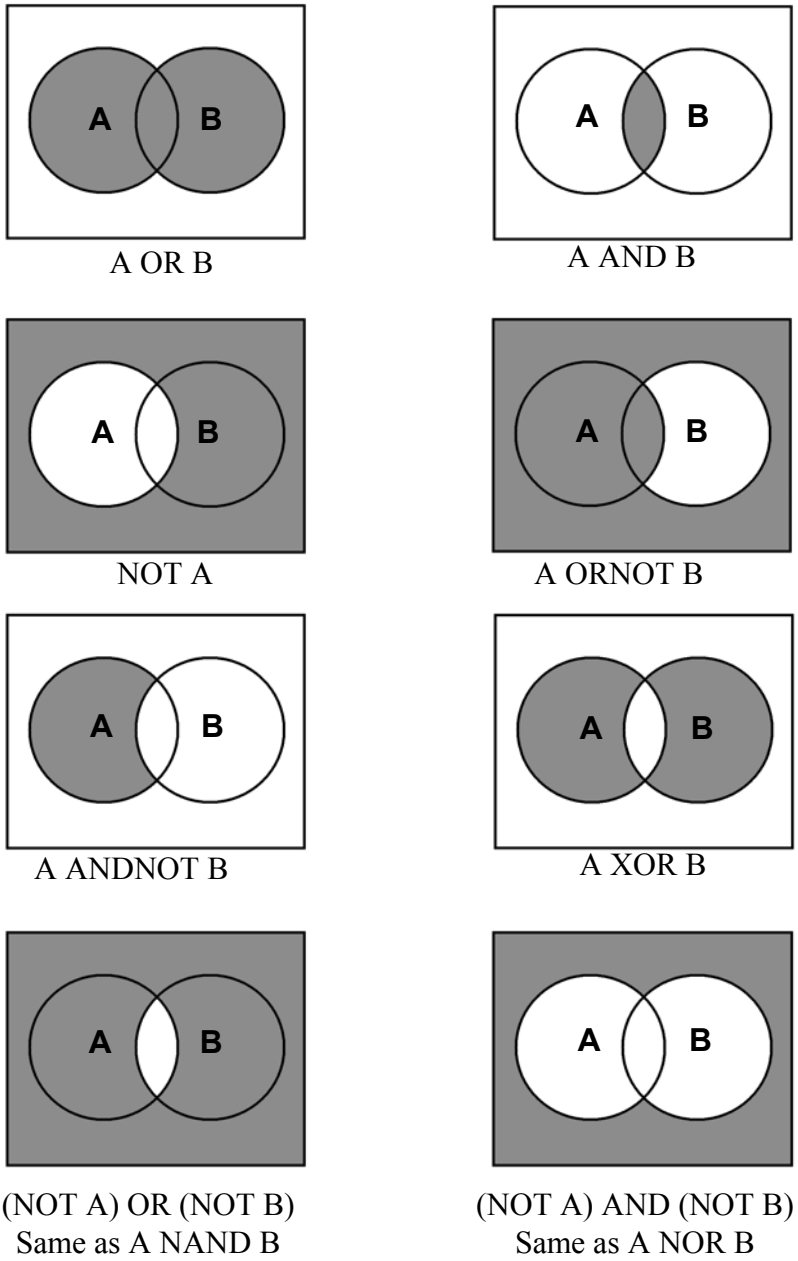


Figure 15. *Schematic definition of available Boolean Operators assuming two Cell Features of interest, A and B*

Description of Output Features

Output features are the biological measurements produced after a scan and are accessible using the View software application. Additionally, a subset of features, the Well Features, is listed in the Scan Plate View and Create Protocol View so that screening results can be viewed concurrently with scanning. The ability to view data during the scanning process allows you to immediately verify success of the run, well by well. All features can also be accessed in the Protocol Interactive View once the algorithm has been applied to the images acquired in that window whether that is done manually on the instrument or running a Disk Scan using images acquired earlier. This section describes Cell, Well, and Reference Well Features available with the Molecular Translocation BioApplication.

Cell Features

Table 14 shows the output features reported for each selected cell, accessible in the Cell Feature window of Protocol Interactive as well as in the View software application.

Cell Feature	Units	Description
Cell#	Number	Unique cell ID
Top	Pixels	Y coordinate (in pixels) of top left corner of image-aligned bounding box of Ch1 nucleus
Left	Pixels	X coordinate (in pixels) of top left corner of image-aligned bounding box of Ch1 nucleus
Width	Pixels	Width (in pixels) of image-aligned bounding box of Ch1 nucleus
Height	Pixels	Height (in pixels) of image-aligned bounding box of Ch1 nucleus
XCentroid	Pixels	X coordinate of center of Ch1 nucleus
YCentroid	Pixels	Y coordinate of center of Ch1 nucleus
EventTypeProfile	Number	Identifies the event types 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
NucAreaCh1	Pixels or μm^2	Area (in pixels or micrometers) of Ch1 nucleus
NucShapeP2ACh1	Number	Shape measure based on ratio of perimeter squared to 4π*area of Ch1 nucleus (**NucShapeP2ACh1** = 1 for circular nucleus)
NucShapeLWRCh1	Number	Shape measure based on ratio of length to width for object-aligned bounding box of Ch1 nucleus
NucTotalIntenCh1	Intensity	Total intensity of all pixels within Ch1 nucleus
NucAvgIntenCh1	Intensity	Average intensity of all pixels within Ch1 nucleus
NucVarIntenCh1	Intensity	Variation (standard deviation) of intensity of all pixels within Ch1 nucleus
RingAvgIntenChN	Intensity	Average intensity of all pixels within ring mask in ChN
RingVarIntenChN	Intensity	Variation (standard deviation) of intensity of all pixels within ring mask in ChN

Cell Feature	Units	Description
CircAvgIntenChN	Intensity	Average intensity of all pixels within circ mask in ChN
CircVarIntenChN	Intensity	Variation (standard deviation) of intensity of all pixels within circ mask in ChN
CircRingAvgIntenDiffChN	Number	Difference between **CircAvgIntenChN** and **RingAvgIntenChN**
CircRingAvgIntenDiffChNStatus	Number	**CircRingAvgIntenDiffChN** status: 0 = No response, 1 = High response, 2 = Low response
CircRingAvgIntenRatioChN	Number	Ratio of **CircAvgIntenChN** to **RingAvgIntenChN**
CircRingAvgIntenRatioChNStatus	Number	**CircRingAvgIntenRatioChN** status: 0 = No response, 1 = High response, 2 = Low response
TotalIntenChN	Intensity	Total intensity in ChN of all pixels within modified Ch1 nucleus mask
AvgIntenChN	Intensity	Average intensity in ChN of all pixels within modified Ch1 nucleus mask

Table 14. *Cell level features for the Molecular Translocation BioApplication. *Note that "ChN" refers to Channels 2-6*

Well Features

The Molecular Translocation BioApplication has a specific and unique set of output features allowing the characterization and pathway analysis of activation and/or translocation of multiple targets simultaneously in a single cell.

- The output feature **MEAN_CircRingAvgIntenDiffCh2-6** is an indicator of activation/translocation of macromolecules between the nucleus and the cytoplasm in target channels at the well level. This feature reports the difference in average pixel intensity between the Circ and Ring mask regions.

- The output feature **MEAN_CircRingAvgIntenRatioCh2-6** is an indicator of activation/translocation of macromolecules between the nucleus and the cytoplasm in target channels at the well level. This feature reports the ratio in average pixel intensity between the Circ and Ring mask regions.

- The output feature **%HIGH (**or **%LOW)_CircRingAvgIntenDiffCh2-6** is the percentage of cells in any well whose **CircRingAvgIntenDiffCh2-6** is higher or lower than the upper or lower limit for **CircRingAvgIntenDiffCh2-6**, obtained automatically from the Reference Wells or entered manually as an Assay Parameter.

- The output feature **%HIGH (**or **%LOW)_CircRingAvgIntenRatioCh2-6** is the percentage of cells in any well whose **CircRingAvgIntenRatioCh2-6** is higher or lower than the upper or lower limit for **CircRingAvgIntenRatioCh2-6**, obtained automatically from the Reference Wells or entered manually as an Assay Parameter.

- The output feature **%HIGH (**or **%LOW)_CombinedAvgIntenDiff (or Ratio)Ch2Ch3** is the percentage of cells in any well whose **CircRingAvgIntenDiff(or Ratio)Ch2** and **CircRingAvgIntenDiff (or Ratio)Ch3** is higher than the upper limits (or less than the lower limits)for these output features. This output feature allows you to classify your compounds of interest as affecting the targets in Channels 2 and 3 simultaneously or only one of the two targets. For example, if a particular well has this output feature at 95% (compared to about 15% in untreated negative control wells), it means that 95% of the sampled cells in that well had both **CircRingAvgIntenDiff (or Ratio)Ch2** and **CircRingAvgIntenDiff (or Ratio)Ch3** above their respective upper limits. This implies that the compound

used to treat this particular well has caused the activation and/or translocation of targets in Channel 2 and 3.

- The output feature **%HIGH (**or **%LOW)_CombinedAvgIntenDiffCh2Ch4** is the percentage of cells in any well whose **CircRingAvgIntenDiffCh2** and **CircRingAvgIntenDiffCh4** is higher than the upper limits (or less than the lower limits) for these output features.
- The output feature **%HIGH (**or **%LOW)_CombinedAvgIntenDiffCh3Ch4** is the percentage of cells in any well whose **CircRingAvgIntenDiffCh3** and **CircRingAvgIntenDiffCh4** is higher than the upper limits (or less than the lower limits) for these output features.
- The output feature **%HIGH (**or **%LOW)_CombinedAvgIntenDiffCh2Ch3Ch4** is the percentage of cells in any well whose **CircRingAvgIntenDiffCh2** and **CircRingAvgIntenDiffCh3** and **CircRingAvgIntenDiffCh4** is higher than the upper limits (or less than the lower limits) for these output features.

Many Well features are derived from the Cell Features. Such features are identified by a prefix, as listed in Table 15, to the Cell Feature name (***Feature*Ch*N*Status**).

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

Table 15. *General Well Feature prefixes available for the Molecular Translocation BioApplication.*

The algorithm also reports the following Well Features (Table 16) in the Scan Plate View in addition to the Well Detail window of the View software application.

Well Feature	Description
ValidCellCount	Number of valid objects (cells) identified in the well (Ch1 object selection parameters applied)
SelectedCellCount	Number of valid cells selected for analysis in the well (Ch2-6 object selection parameters applied)
%SelectedCells	Percentage of valid cells selected for analysis in the well
ValidFieldCount	Number of fields in which cells were selected for analysis in the well
SelectedCellCountPerValidField	Average number of cells selected for analysis per valid field in the well
EventType1CellCount	Number of cells selected for analysis in the well in which EventType1 occurred
%EventType1Cells	Percentage of cells selected for analysis in the well in which EventType1 occurred
EventType2CellCount	Number of cells selected for analysis in the well in which EventType2 occurred
%EventType2Cells	Percentage of cells selected for analysis in the well in which EventType2 occurred
EventType3CellCount	Number of cells selected for analysis in the well in which EventType3 occurred
%EventType3Cells	Percentage of cells selected for analysis in the well in which EventType3 occurred

Table 16. *Additional Well Level Features available for the Molecular Translocation BioApplication.*

Reference Well Features

These values are calculated and displayed only when the **UseReferenceWells** Assay Parameter is ON (=**1**). In addition to Reference Well, the exposure times for various channels are also displayed only when viewing data in the View software application. In the listing of Reference Features in Table 17, instead of showing 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 2 average intensity difference between the Circ and Ring:

- RefCircRingAvgIntenDiffCh2Level***Low/High***

Plate Feature	Description
RefAvgCellCountPerField	Average count of cells per field in reference wells
RefCircRingAvgIntenDiffChNLevel***Low/High***	Low/High-response level for **CircRingAvgIntenDiffChN**
RefCircRingAvgIntenRatioChNLevel***Low/High***	Low/High-response level for **CircRingAvgIntenRatioChN**

Table 17*. Plate level features for the Molecular Translocation BioApplication. *Note that "ChN" refers to Channels 2-6. Instead of listing all features,* ***FeatureChNLevelLow*** *and* ***FeatureChNLevelHigh*** *were combined for corresponding features.*

Chapter

3

Using the Events Wizard in the Molecular Translocation BioApplication

This chapter describes in more detail the use and modification of the BioApplication Event Wizard with the Molecular Translocation BioApplication.

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

NOTE

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

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. Operation of the BioApplication Event Wizard must be consistent with the steps described.

STEP I

1) Create a protocol using the Scan software application without defining Events. Set optimized parameter values (upper and lower 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 upgraded Molecular Translocation protocol (V4 version)
- Enables use of standard Boolean operators (NOT, AND, ANDNOT, OR, ORNOT, XOR, NAND, and NOR)
- Read-Only protocol protection
- Displays previously entered Event Definitions in saved Assay Protocols
- Ability to clear each Event Definition individually
- Rapid construction of logical statements using Cell Features and logical operators
- Automated validation of each Event Definition when loading or saving protocols
- Ability to save updated protocol
- Addition of Event Definitions to the Protocol Comments field in the Assay Protocol

Steps for Running the Event Wizard with Molecular Translocation

Before Running the Event Wizard...

1) Create a protocol using the Molecular Translocation BioApplication without defining Events. Set optimized parameter values (upper levels) for detection of responder cells associated with Cell Features to be used for Event Definition.

2) Save protocol.

3) Close the Scan software application (suggested).

Starting the BioApplication Event Wizard

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**.

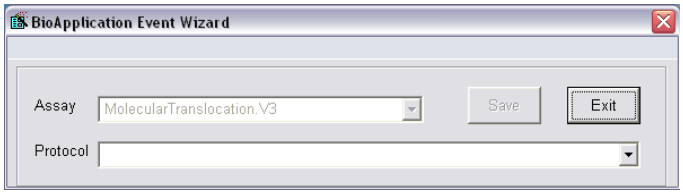
For vHCS Discovery Toolbox computers: From the Windows **Start** menu, select **Programs** > **Cellomics > vHCS Scan > BioAppEventWizard**

Protocol Selection and Loading

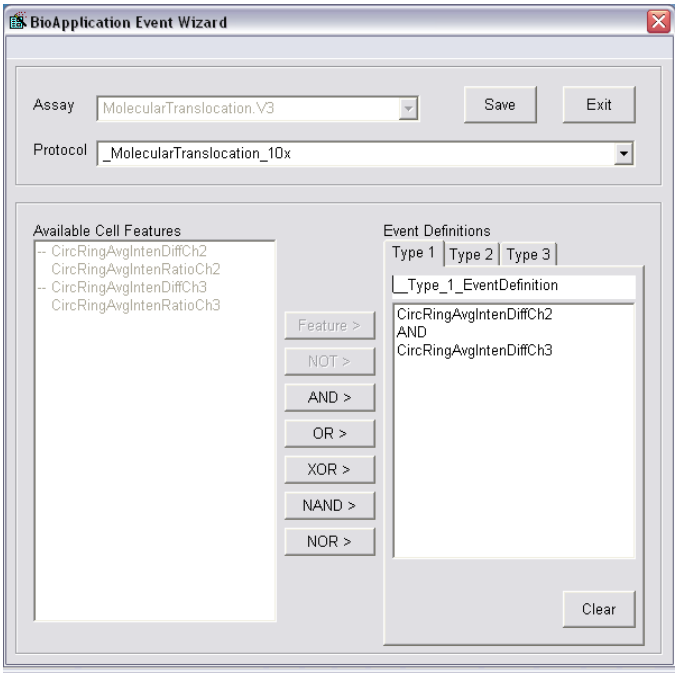
The first window that is displayed (see below) allows selection of any Molecular Translocation Assay Protocol. Note that Event Definitions can only be specified for pre-existing Assay Protocols.

To select an Assay Protocol,

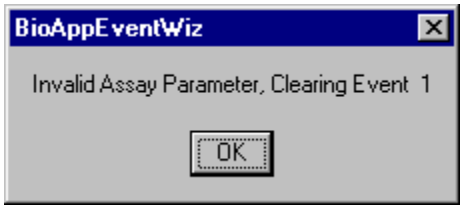
1) From the **Assay** drop-down menu, select **MolecularTranslocation.V4**.
2) From the **Protocol** drop-down menu, click on the drop-down arrow to view the list of existing Molecular Translocation Assay Protocols.
3) Select the desired Assay Protocol from the list.



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



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

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 Molecular Translocation Assay Protocol. Note that logical statements used to define Events can include up to four Cell Features and four logical operators.

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

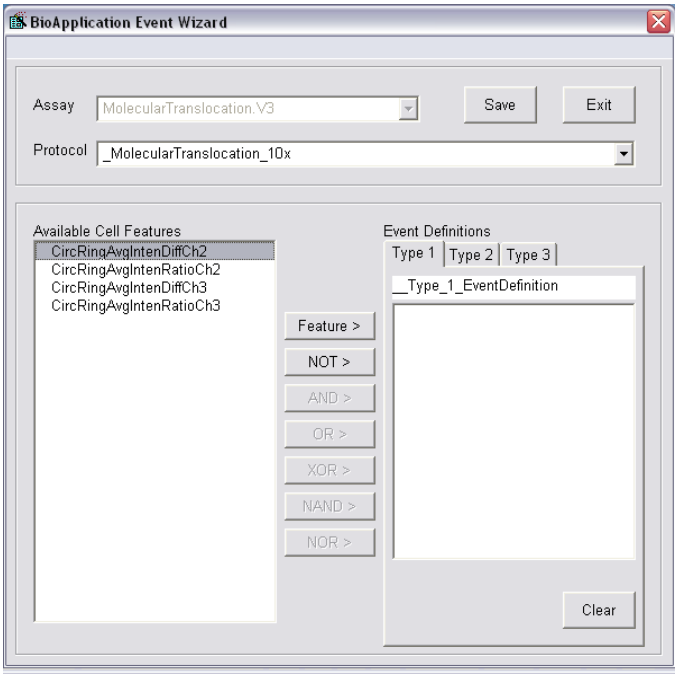
The procedure to be followed when constructing Event Definitions is described in the sequence of screenshots below. Cell Features are combined with Boolean operators to produce Event Definitions. Any Cell Feature can be selected by clicking on the feature name in the Available Cell Features list and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

To build an Event Definition,

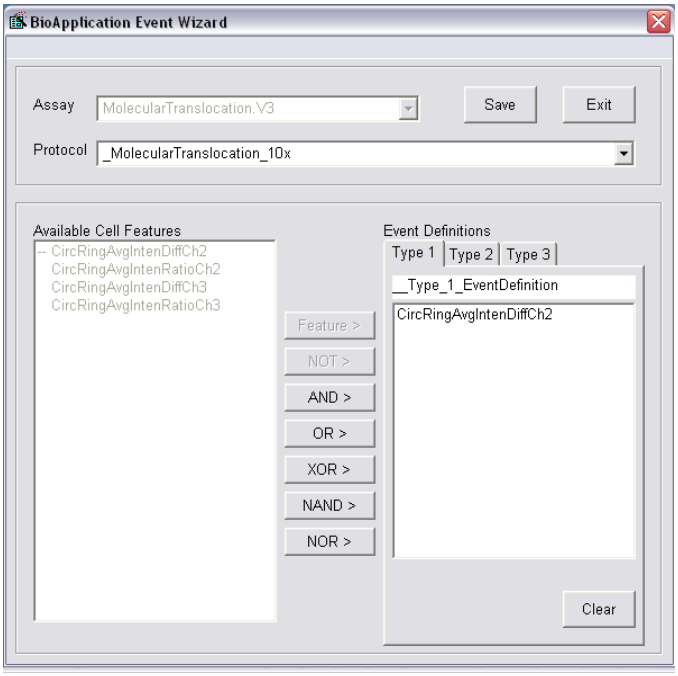
1) Select the Event Definition that you want to specify or edit by clicking on the **Type 1**, **Type 2**, or **Type 3** tab.

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

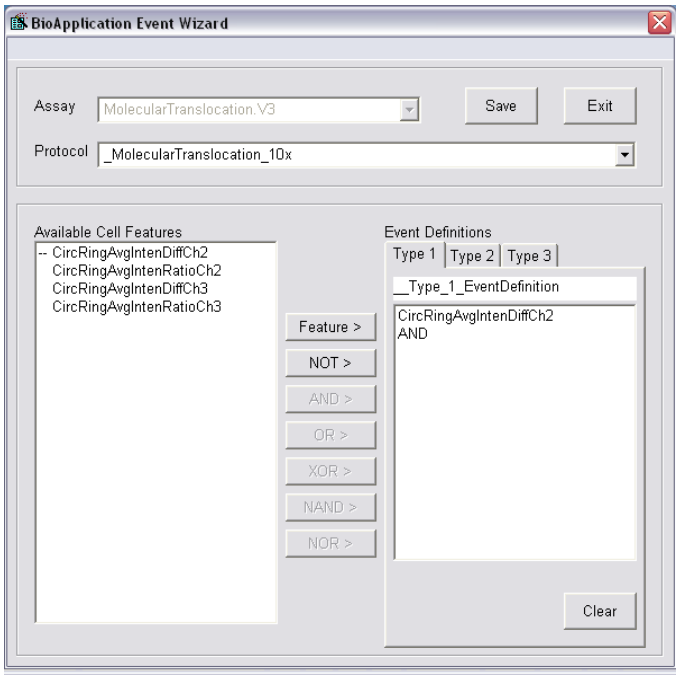
3) Select the desired Cell Feature by clicking on the feature name from the Available Cell Features list. In this example, the **CircRingAvgIntenDiffCh2** feature is selected. You may also choose to select **NOT>** first (before the Feature) to indicate that you do not want to include this Cell Feature in your event analysis.



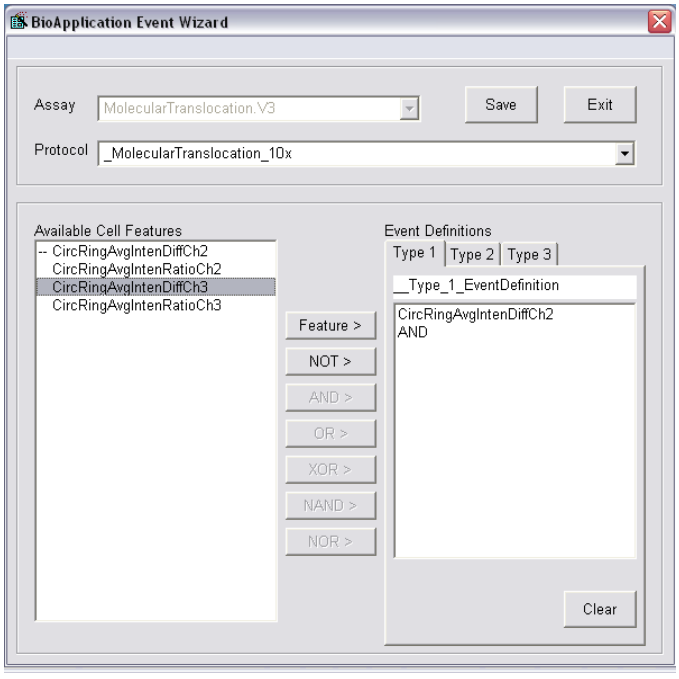
4) Click the **Feature>** button to transfer the Cell Feature into the Event Definition (see below).



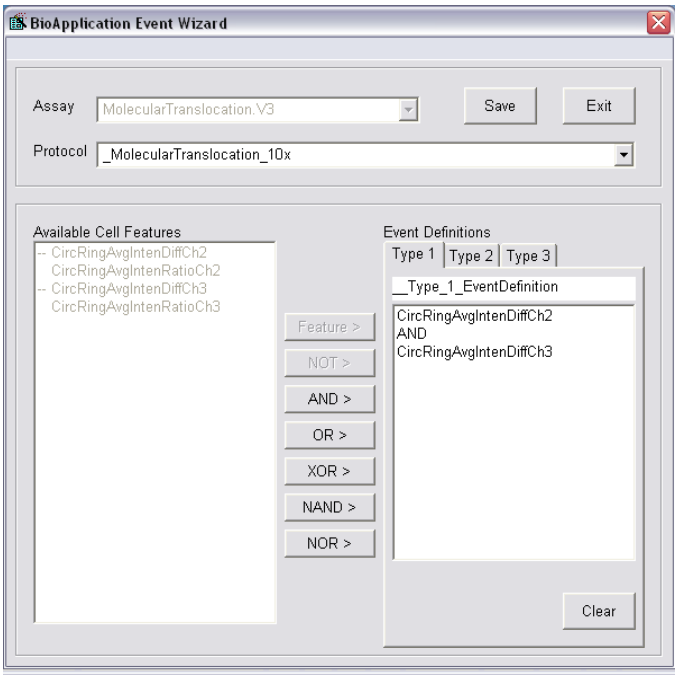
5) Click on the desired Operator button to transfer it into the Event Definition. In this example, the **AND** operator is selected (see below).



6) Repeat the cycle for adding another Cell Feature to the Event Definition. In this case, the Cell Feature **CircRingAvgIntenDiffCh3** has been selected for addition.

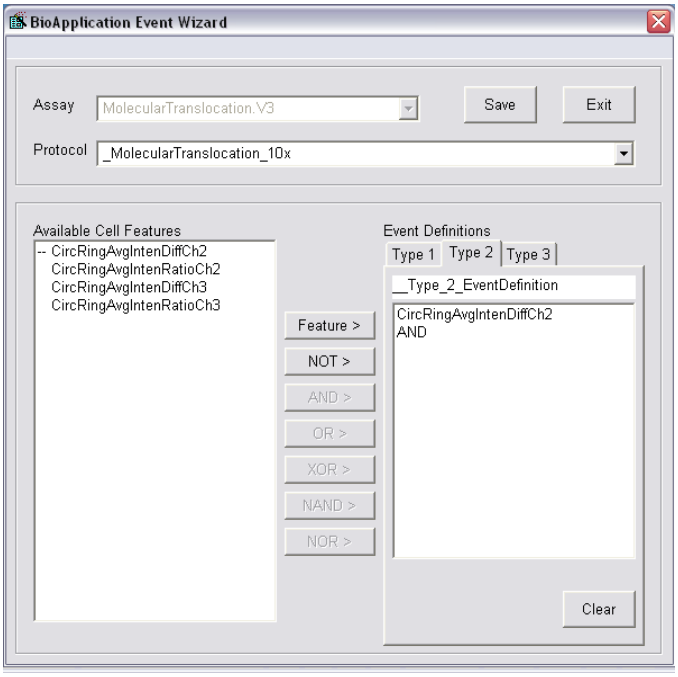


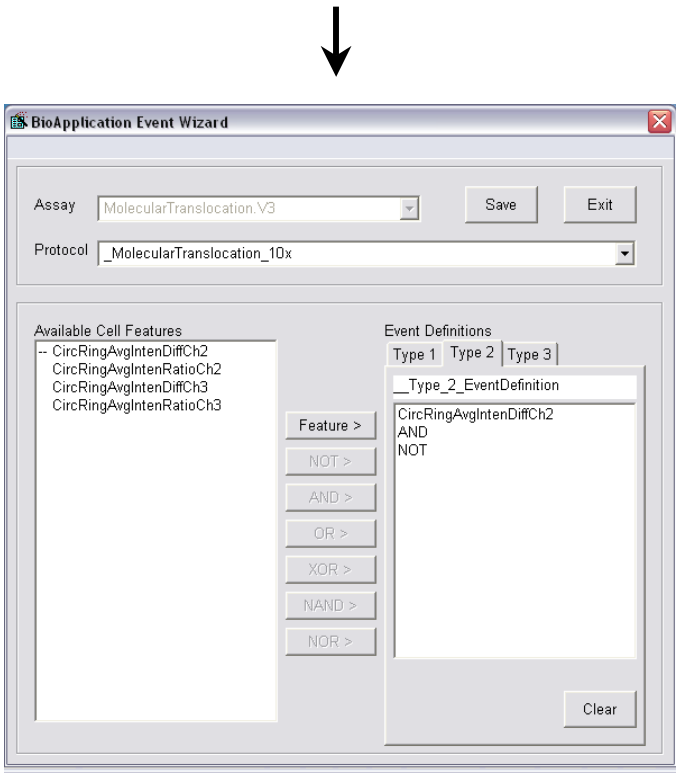
7) As before, click on the **Feature >** button to transfer the Cell Feature into the Event Definition (see below).

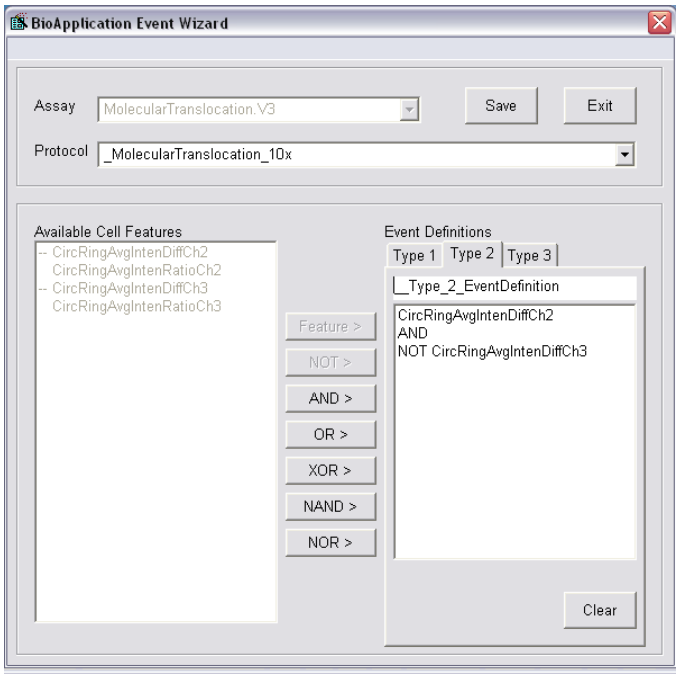


Type_1_EventDefinition is: **CircRingAvgIntenCh2** AND **CircRingAvgIntenCh3**

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.

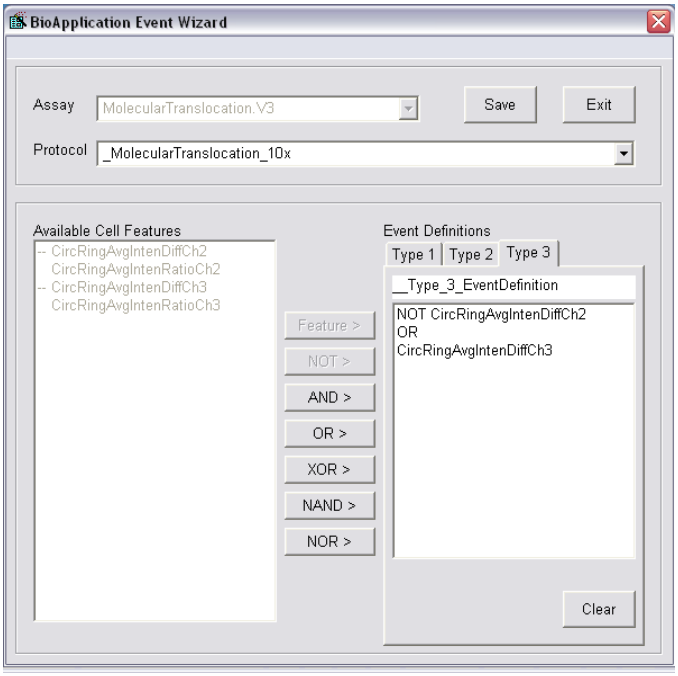






Type_2_EventDefinition is: **CircAvgIntenCh3** ANDNOT **CircRingAvgIntenCh3**

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 **CircRingAvgIntenDiffCh2** OR **CircRingAvgIntenDiffCh3**

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.

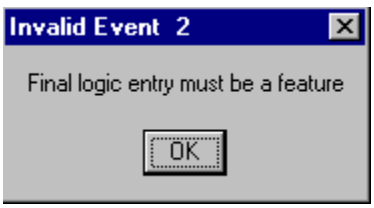
To save the updated Assay Protocol,

- 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.

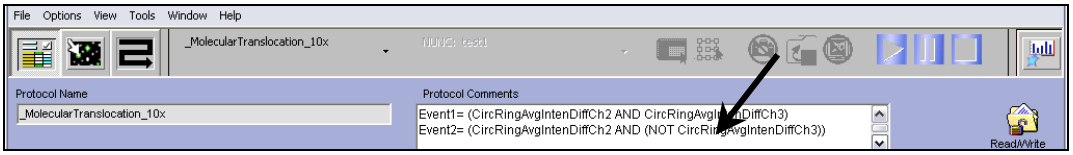
The BioApplication Event Wizard has the capability to automatically validate Event Definitions. If Event Definition validation fails, the protocol will not be saved. For example, Event Definitions are considered invalid if they do not end with a Cell Feature. In this case, clicking on the **Save** button 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 the **Create Protocol View** upon opening the saved Assay Protocol using the Scan software application (see arrow).



Chapter
4

iDev Software Workflow

This chapter describes the tasks in the workflow for Protocol optimization of the Molecular Translocation V4 BioApplication using the iDev software. More detailed technical descriptions of each Assay Parameter can be found in Chapter 2 of this guide.

iDev Protocol Optimization Tasks

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

Image Preprocessing

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

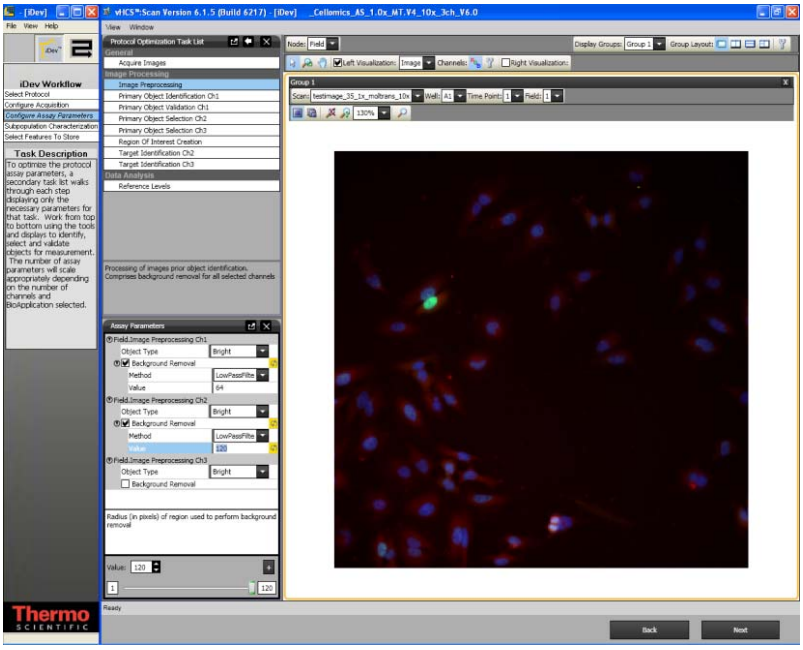


Figure 16. Protocol Optimization Task – Image Preprocessing

Primary Object Identification Ch1

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

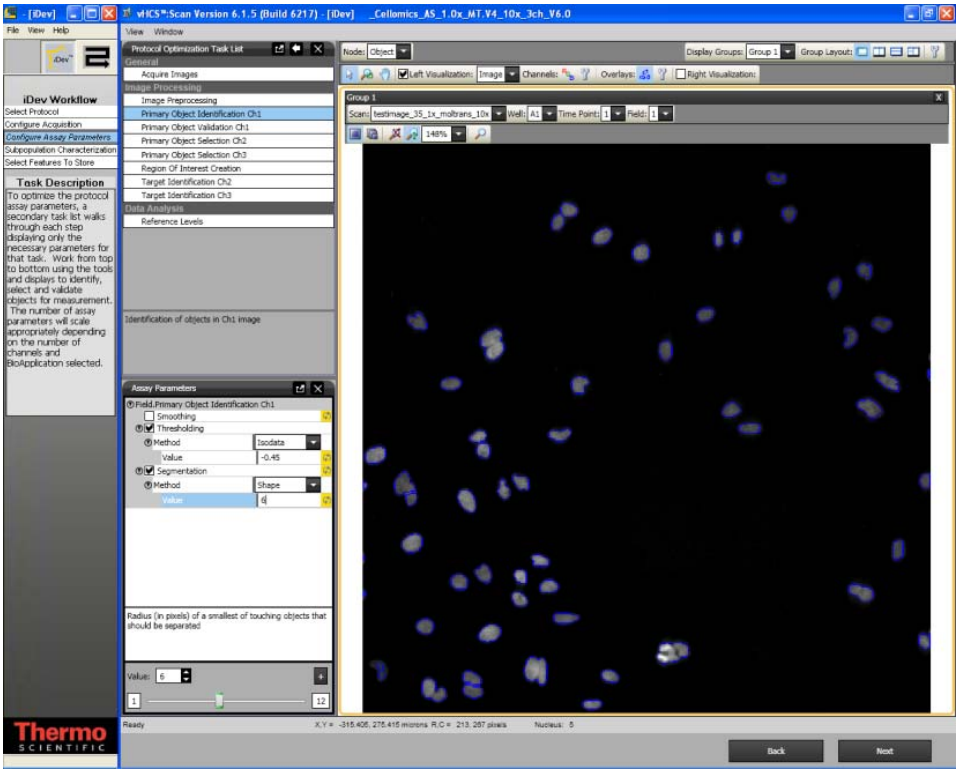


Figure 17. Protocol Optimization Task – Primary Object Identification Ch1

Primary Object Validation Ch1

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

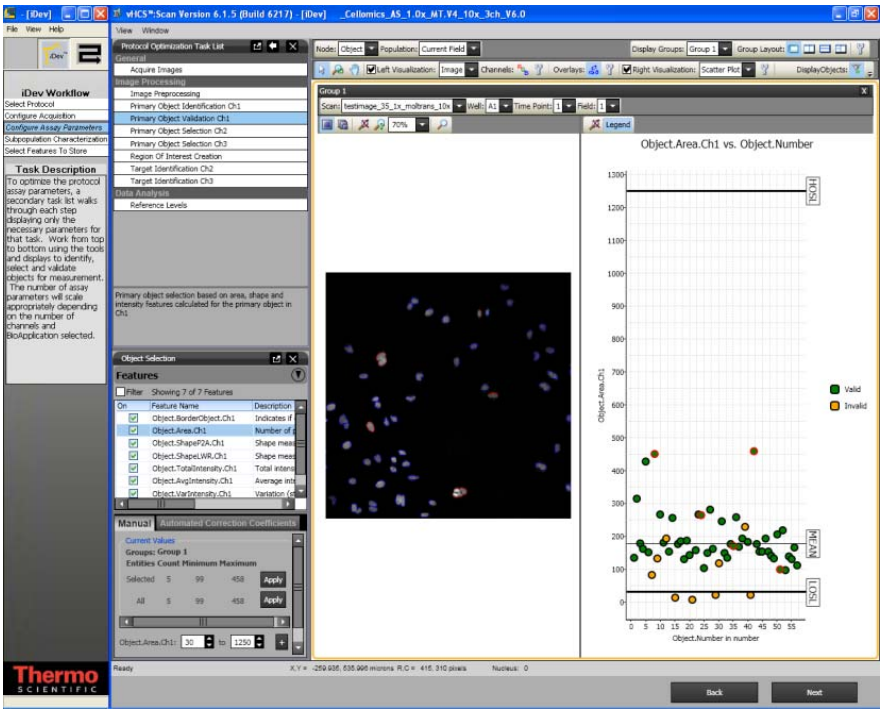


Figure18. Protocol Optimization Task – Primary Object Validation Ch1

Primary Object Selection Ch2 through ChN

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

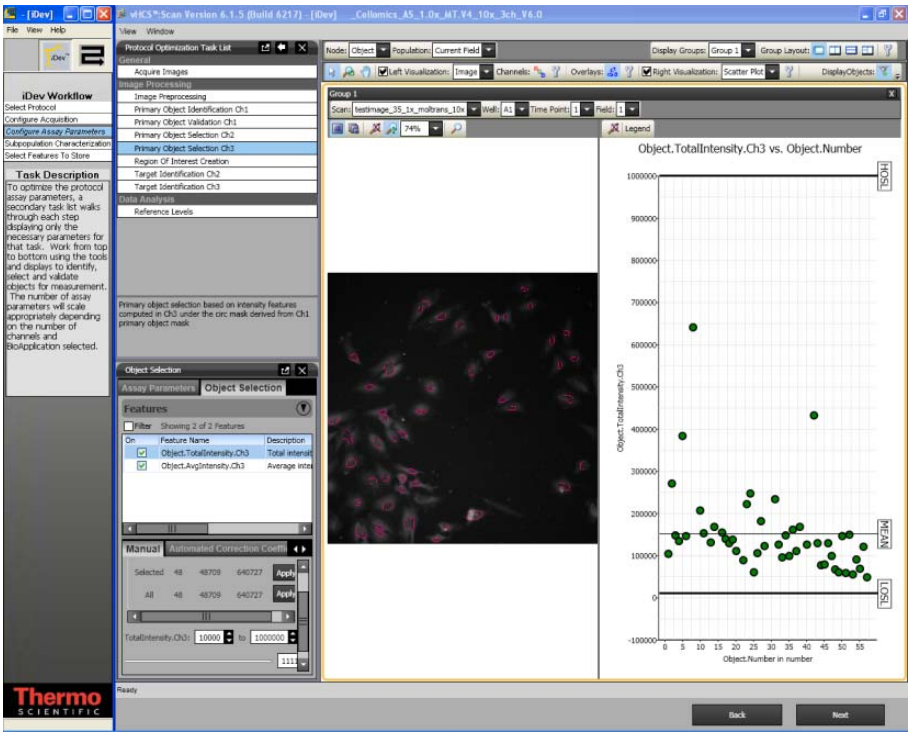


Figure 19. Protocol Optimization Task – Primary Object Selection Ch2 through ChN

Region of Interest Creation

In this task you can create ROIs as Circ and/or Ring for target analysis in Channels 2 to 6. The ROIs are created from the primary object mask in Channel 1.

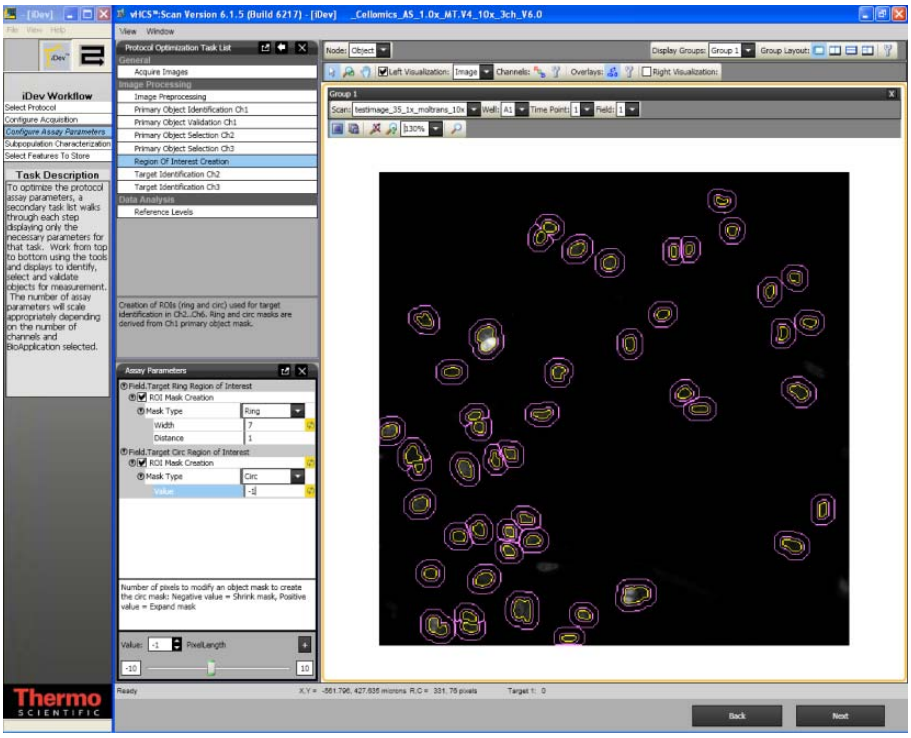


Figure 20. Protocol Optimization Task – Primary Object Selection Ch2 through ChN

Target Identification Ch2 through ChN

In this task you can set the thresholding method and value for identification of targets in Channels 2 through Channel N Ring ROI. For identification of targets in Channels 3 through Channel N, you can also use the same target mask from Channel 2 in all downstream channels by checking the **UseTargetRingMaskCh2** option.

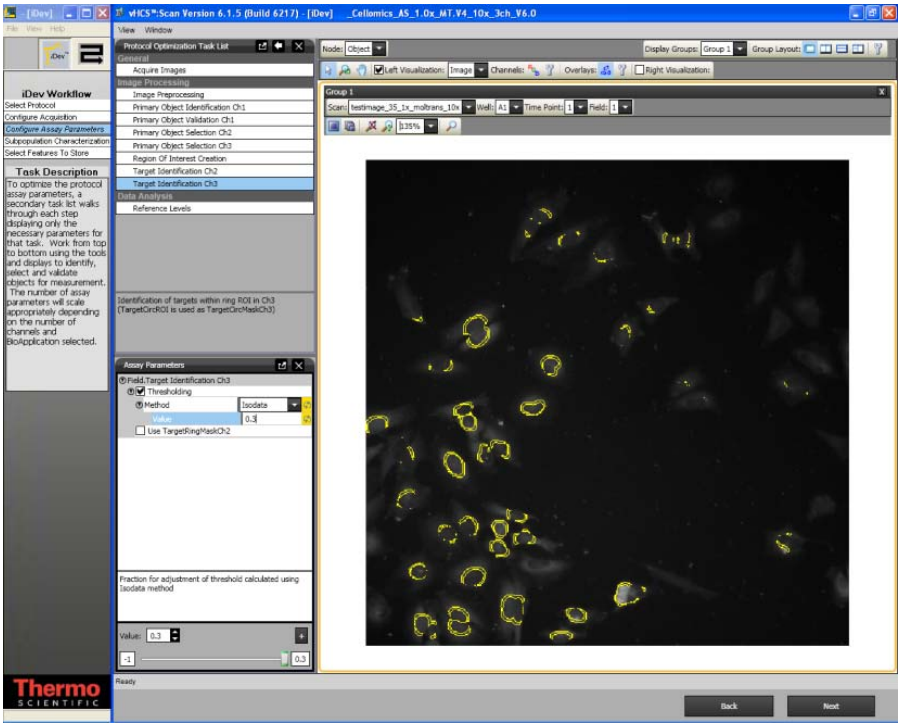


Figure 21. Protocol Optimization Task – Target Identification

Reference Levels

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

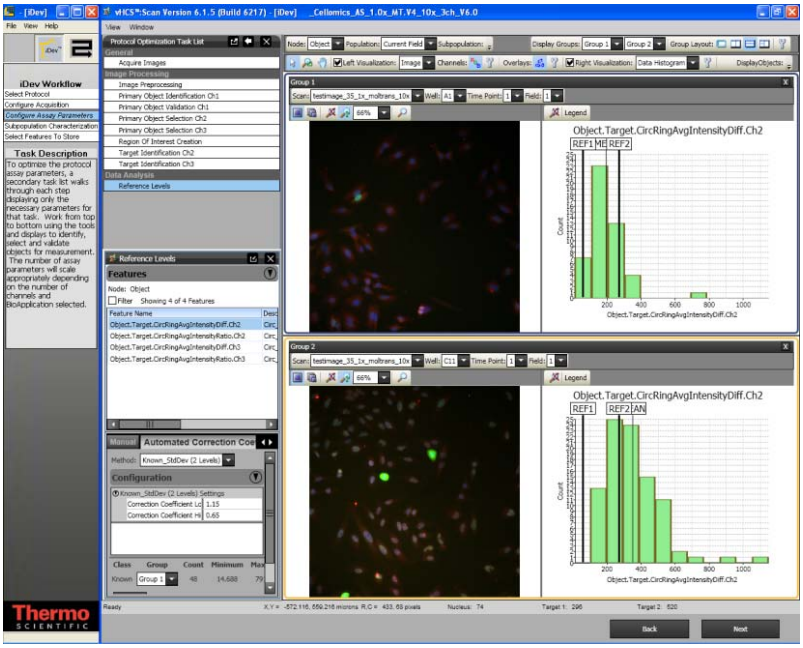


Figure 22. Protocol Optimization Task – Reference Levels

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.

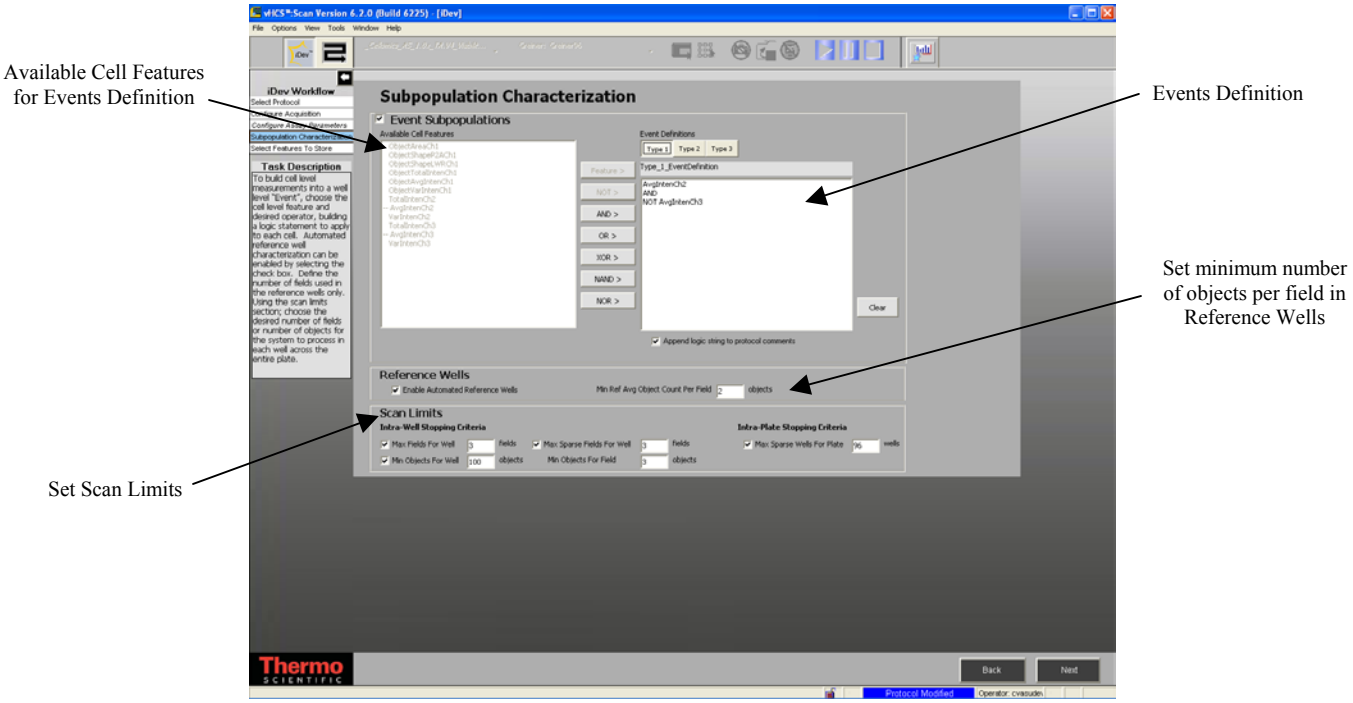


Figure 23. Subpopulation Characterization Task

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