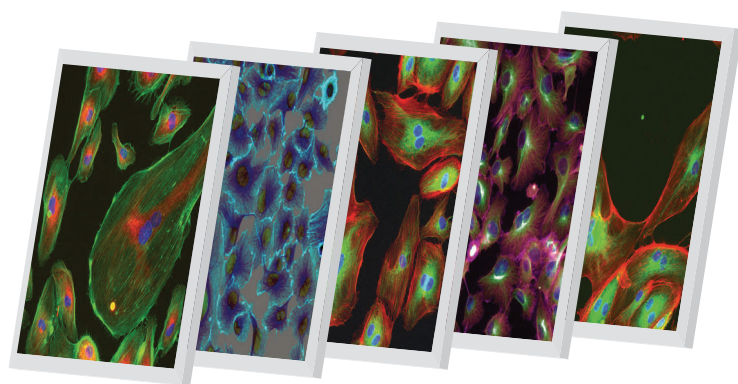


Thermo Scientific Cellomics®
Cytoplasm to Nucleus
Translocation V4

BioApplication Guide



Cellomics® Cytoplasm to Nucleus Translocation BioApplication Guide

V4 Version

PUBLISHED BY

Thermo Fisher Scientific Inc.
100 Technology Drive
Pittsburgh, Pennsylvania 15219
Telephone: (412) 770-2200

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Overview of the Cytoplasm to Nucleus Translocation BioApplication

High Content Screening (HCS) uses fluorescence-based reagents, advanced optical imaging systems and sophisticated algorithms to analyze targets and physiological processes in cells. This BioApplication Guide provides a brief description for performing one such HCS assay, the measurement of Cytoplasm to Nuclear Translocation. This guide contains the following chapters:

Chapter 1 provides an overview of the BioApplication.

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

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

System Compatibility

The Cytoplasm to Nucleus Translocation (Cyto-Nuc Trans) BioApplication described in this document is designed to run on the following platforms:

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

NOTE



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 cross-talk 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 (Seeger and Krebs 1995; Robinson and Cobb 1997; Reiser, Ammerer and Ruis 1999), NFAT mediated pathways (Rao, Luo and Hogan 1997), NF κ B mediated pathways (Mercurio and Manning 1999; Li and Stark 2002), FAS pathway (Wajant 2002), PI3K mediated pathways (Cantley 2002) and G protein pathways (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 a tremendous interest in targeting them to treat certain diseases (Seidel, Lamb and Rosen 2000; Ethier 2002). The canonical regulation of transcription factors, such as c-fos, c-jun, NF κ B, and kinases, such as MAP kinase, is via the translocation of the factor from the cytoplasm to the nucleus (see following figure). This BioApplication provides a direct assessment of the activation of a transcription factor by directly measuring the spatial translocation from the cytoplasm to the nucleus (Figure 1).

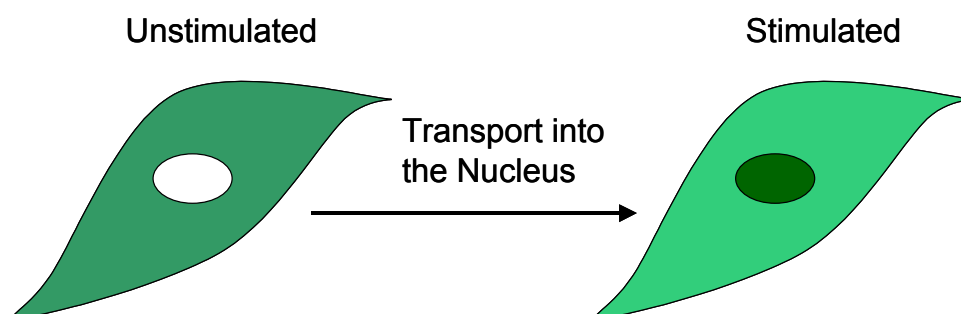


Figure 1. Schematic representation of the Cytoplasm to Nucleus translocation. Upon stimulation, transcription factors in the cell's cytoplasm translocate to the nucleus.

BioApplication Overview and Measurements

The Cytoplasm to Nucleus Translocation BioApplication is capable of measuring the activation and/or translocation of one target at a time 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 six channels. Channel 1 is used for object identification (nuclear channels), Channel 2 is used for the measuring the translocation of macromolecules of interest between the cytoplasm and nucleus, and Channels 3-6 can be used for gating. Using Reference Wells, the BioApplication can also automatically set upper and lower limits for nucleus-to-cytoplasm difference and ratio and reports the percentage of cells in each well that is above the upper limit and below the lower limit.

The Cytoplasm to Nuclear 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 Channel 2:			
Intensity	Circ	Total Inten Avg Inten	MEAN, SD,SE,CV All Selected Cells
	Ring	Total Inten Avg Inten	
		Circ/Ring	AvgIntenDiff & Status AvgIntenRatio & Status
Cell & Well Features Reported for Channel 2-6:			
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

Nuclear factor kappa B (NFκB) is a transcription factor that is activated in response to ligands, such as IL-1α and tumor necrosis factor (Ding et al. 1998), and is associated with the activation of many cellular defense genes (Baldwin 1996). Normally, NFκB is present in the cytoplasm as a complex with members of the IκB inhibitor family. Both the size of this complex and IκB masking of the nuclear localization sequence on NFκB prevents NFκB from entry into the nucleus. Upon phosphorylation and degradation of IκB, the nuclear localization sequence becomes accessible and NFκB translocates to the nucleus. NFκB must be in the nucleus in order to induce specific gene expression. Thus, as with many other transcription factors, translocation from the cytoplasm to the nucleus is a definitive measure of NFκB activation.

Figure 2 shows representative images of cytoplasm to nucleus translocation of NFκB obtained from a Cellomics HCS Reader with the Cytoplasm to Nucleus Translocation BioApplication. Note that in untreated cells, the nuclei are empty, with most of the NFκB staining showing up in the cytoplasm. However, on stimulating the cells with the appropriate growth factor, the NFκB translocates into the nucleus. Figure 3 shows dose response and time course of the cytoplasm to nucleus translocation of NFκB following stimulation of HeLa cells with IL-1α.

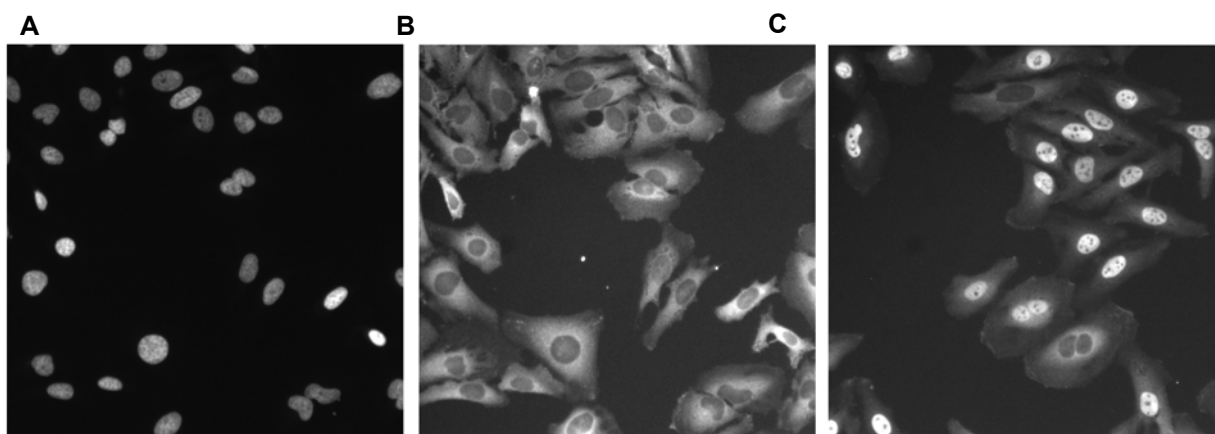


Figure 2. Images of immunofluorescent-stained HeLa cells before and after activation of NF κ B by IL-1 α . Cells were stimulated with 25 ng/mL IL-1 α for 20 minutes. Cells were stained with the Cellomics, NF κ B activation reagent kit. Panel A: Image of the Nucleus Channel (Hoechst). Panel B: Image of the NF κ B channel in unstimulated cells (Alexa Fluor[®] 488). Panel C: Image of the NF κ B channel in stimulated cells (Alexa Fluor 488).

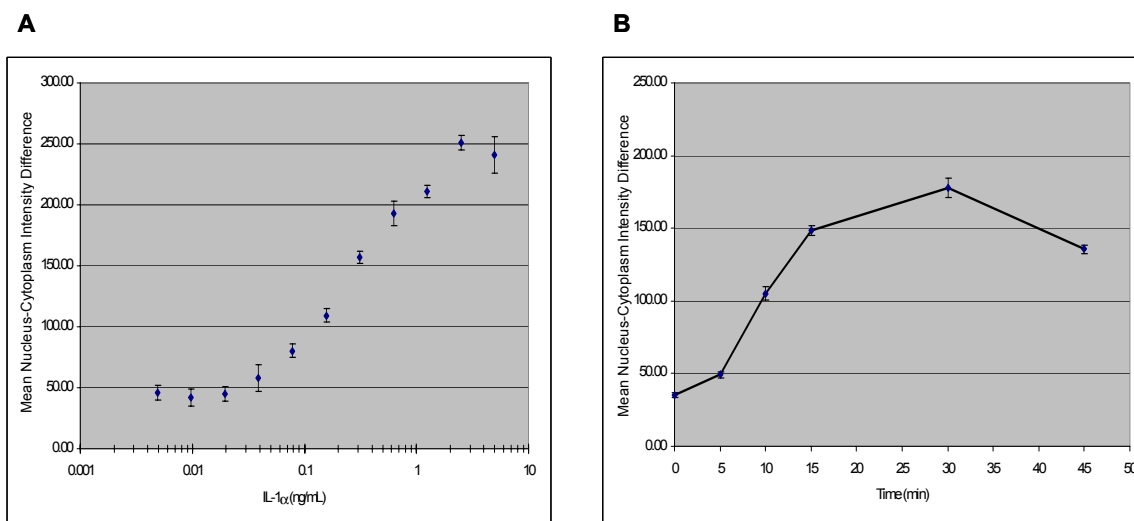


Figure 3. A) Dose Response. HeLa cells were stimulated with IL-1 α for 40 minutes over a concentration range of 0.005 ng/mL to 5 ng/mL and processed with the Cellomics NF κ B activation reagent kit. Data is mean \pm sem from 8 wells per concentration of IL-1 α . **B) Time Course.** HeLa stimulated with 5 ng/mL IL-1 α . Maximal translocation occurred at about 30 minutes. Data is mean \pm sem from 12 wells for each time point. Ding et al. (1998) reported a half maximal stimulation time ($t_{1/2}$) of 7-8 min for IL-1 α and TNF stimulated HeLa cells using a Cellomics HCS Reader.

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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 3 of this guide.

This chapter briefly describes how the Cytoplasm to Nuclear Translocation BioApplication works and provides a description of the adjustable Assay Parameters and Output Features.

Overview

The purpose of the Cytoplasm to Nucleus Translocation BioApplication is to measure the relative distribution of the target protein between two sub-cellular compartments: the cytoplasm and the nucleus. A minimum of two channels is needed to measure the translocation. In Channel 1, nuclei (DNA) stained with the fluorescent dye Hoechst 33342 defines the nuclear region, due to the selective binding of DNA by the dye. This signal is used for both focusing the instrument on the cells and also for identifying and defining the nuclear region. Each individual nucleus identified in Channel 1 is used to create a mask called “Circ” in Channel 2 (the target channel, Figure 4). The Circ region corresponds to the nuclear region of the cell. An annular region called “Ring” is defined in the cytoplasm beyond the nuclear (i.e., Circ) region (Figure 4). You have the ability to determine the size of the Circ and Ring masks in the target channel (see Figure 6 for further descriptions of Circ and Ring regions). The Circ and Ring masks approximate a measure of the nuclear and cytoplasm concentration of the target respectively. This approximation will be adequate if the component being measured is uniformly distributed throughout the cytoplasm. This is the case for a soluble macromolecular complex such as NFκB and other transcription factors and kinases.

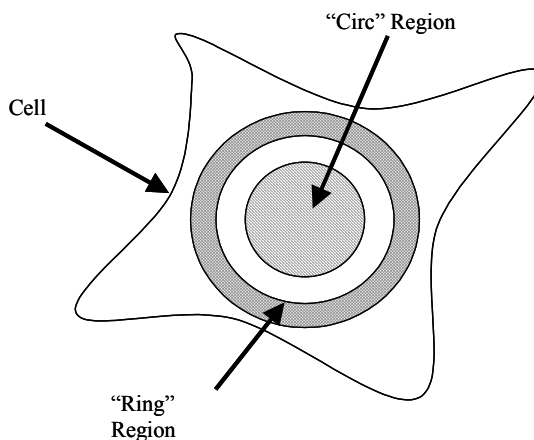


Figure 4. The cell is divided into two regions based on the masks created, the nucleus (**Circ; shaded in lines**) and the cytoplasm (**Ring; shaded in spheres**). The response variable for the assay is the difference between and ratio of the average intensity between the nuclear and the cytoplasmic regions in the target channel (**MEAN_CircRingAvgIntenDiffCh2** and **MEAN_CircRingAvgIntenRatioCh2**).

The total and average pixel intensity within the Circ and Ring masks in the target channel are measured and reported by the BioApplication. The BioApplication also reports 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 Circ and Ring regions measure the relative amounts of the target in the nuclear and cytoplasmic regions of the cell respectively and are good quantitative indicators of the relative amounts of the target between the cytoplasm and the nucleus of a cell.

The BioApplication determines the status of each cell as to whether the cell is below, within, or above the thresholds 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 is below the lower limit (**FeatureChNLevelLow**) and above the upper limit (**FeatureChNLevelHigh**) for the difference and ratio between Circ and Ring masks average pixel intensity measurements.

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. In Channel 2 the Circ and Ring masks are identified. Thus the proper setting of an intensity threshold is a key early step in identification and thus configuring the application. Depending on the properties of the objects being identified in Channel 1 and Channel 2, the proper setting of intensity thresholds for the channels is necessary to ensure proper quantitative analysis.

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

Intensity Threshold Method	Value Range	Channel Availability		
		Channel 1	Channel 2	Channel 3-6
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 Cellomics HCS Readers (ArrayScan V^{TI}, 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 one option for Channel 2 and the only option in 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 5 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	<p>Adjusts the object identification threshold relative to the Isodata value.</p> <p>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).</p> <p>A negative value identifies dimmer objects and results in larger object masks. A positive value results in smaller object masks.</p>	-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

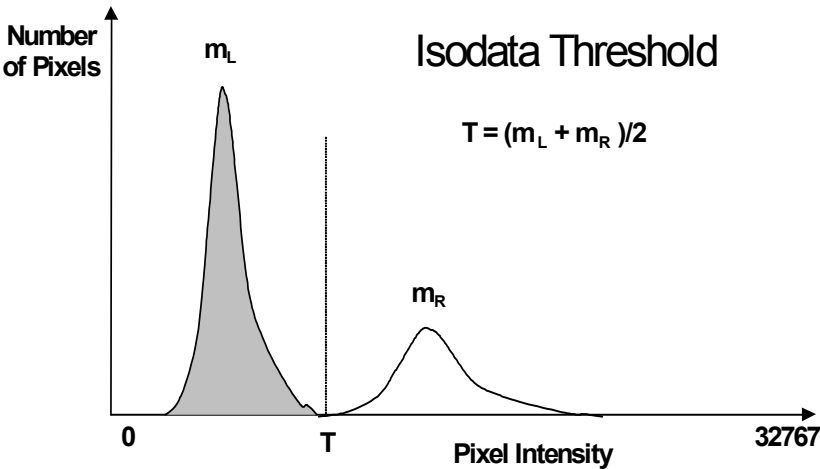


Figure 5. Histogram-derived Isodata Intensity Threshold Method. Background peak is shown in gray and object peak is shown in white.

Description of Assay Parameters and Settings

The Cytoplasm to Nuclear Translocation BioApplication 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

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 the 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 Assay 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 the Circ and Ring Masks

The size of the Circ and Ring regions can be adjusted and applied for the target channel (Channel 2), and this size is set in relation to the primary object. Figure 6 shows a schematic of the three Assay Parameters that control the size of the Circ and Ring masks.

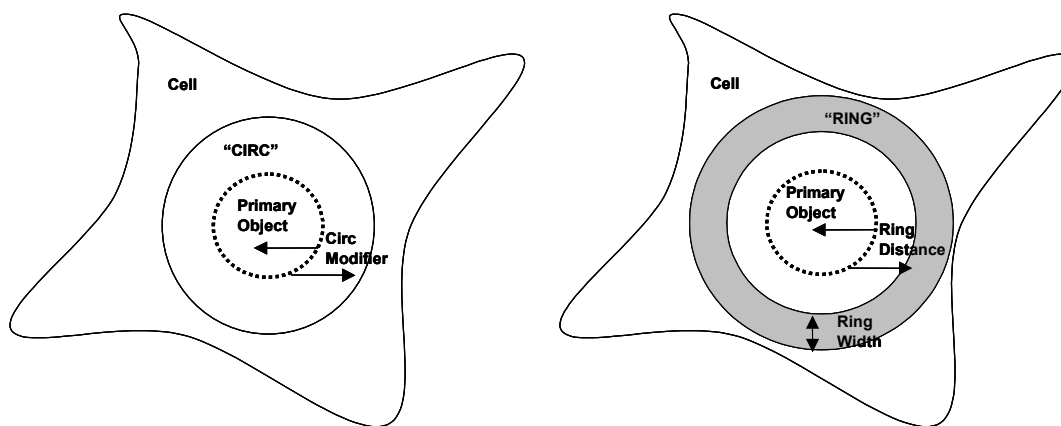


Figure 6. Schematic representation of the Circ and Ring regions in the Cytoplasm to Nucleus Translocation BioApplication.

The **CircModifierCh2** Assay Parameter is used to adjust the Circ region for the target channel. 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 channel. These are **RingDistanceCh2** and **RingWidthCh2**. The **RingDistanceCh2** 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, **RingDistanceCh2** 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 the 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 **RingWidthCh2** 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 **MaskModifierChN** Assay Parameter works similar to the **CircModifierCh2** 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.

ObjectTypeChN

NucTypeCh1, TargetTypeCh2

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 **TargetTypeCh2**.

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 ChN

Prior to image analysis, the non-cellular background can be computed and subtracted from each image, as shown in Figure 7.

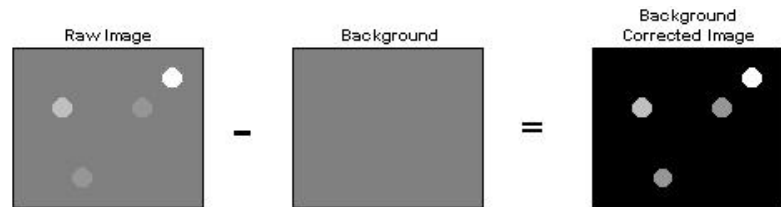


Figure 7. Background Correction available in each channel.

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 **BackgroundCorrectionChN** 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 **BackgroundCorrectionChN** 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 8 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).

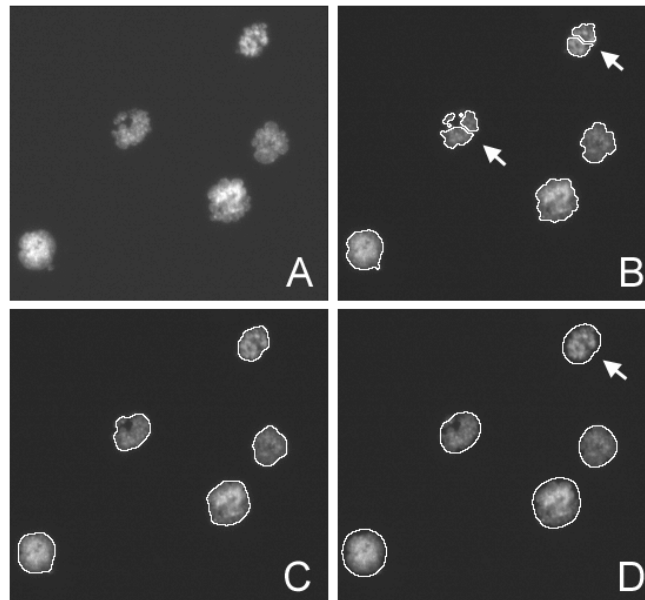


Figure 8. NucSmoothFactorCh1: Images show the effect of different degrees of smoothing on the same field of apoptotic cell nuclei. A: Raw Image; B: **NucSmoothFactorCh1** = 0; C: **NucSmoothFactorCh1** = 3; D: **NucSmoothFactorCh1** = 8.

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 nuclei. Nucleus

Segmentation will help resolve and identify individual nuclei. Two methods are available for nucleus segmentation in the Cytoplasm to Nucleus Translocation BioApplication: Geometric (shape and size) and Intensity (intensity peaks). These methods are illustrated in Figure 9.

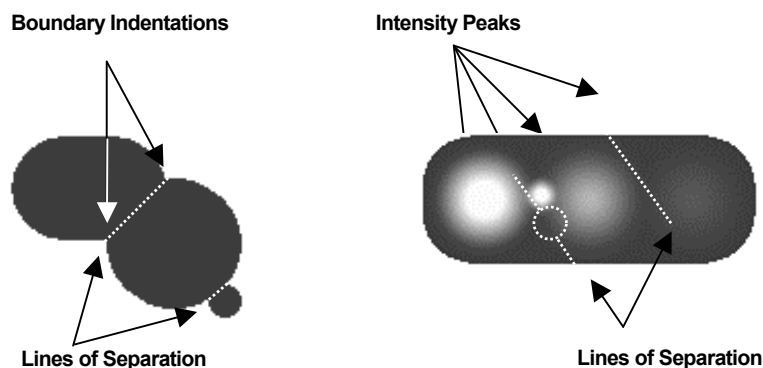


Figure 9: Nucleus Segmentation Options. The image on the left illustrates the Geometric Method while the image on the right illustrates the Intensity Method.

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: Object Segmentation Options

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

Geometric Method Setting the **NucSegmentationCh1** value to R1 (Figure 10A) produces object separation shown in Figure 10B. Setting the **NucSegmentationCh1** value to R3 results in segmentation shown in Figure 10C. Setting the **NucSegmentationCh1** value to R2 (the largest radius of all touching objects) produces no segmentation.

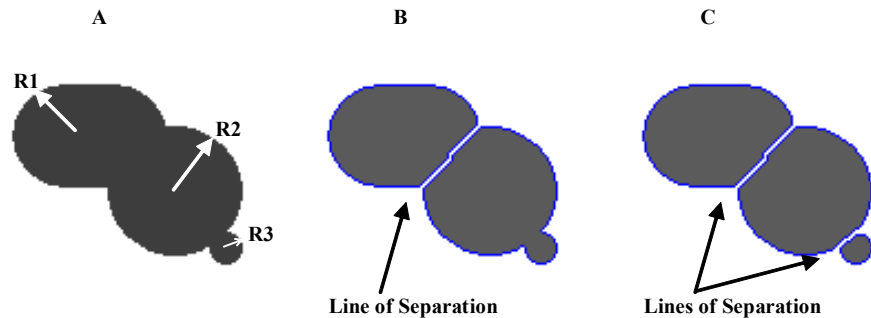


Figure 10: Object segmentation: Geometric Method. A) Radii of touching objects: $R1 = 24$, $R2 = 30$, $R3 = 7$; B) Result of segmentation using **NucSegmentationCh1** = R1; C) Result of segmentation using **NucSegmentationCh1** = R3.

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

In general terms, these peak intensity heights are nothing but measures of local contrast of an image. The degree of object segmentation can be controlled by setting the **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 vice versa. In case of over-segmentation, setting the **NucSmoothFactorCh1** value greater than 0 should be used to alleviate the problem.

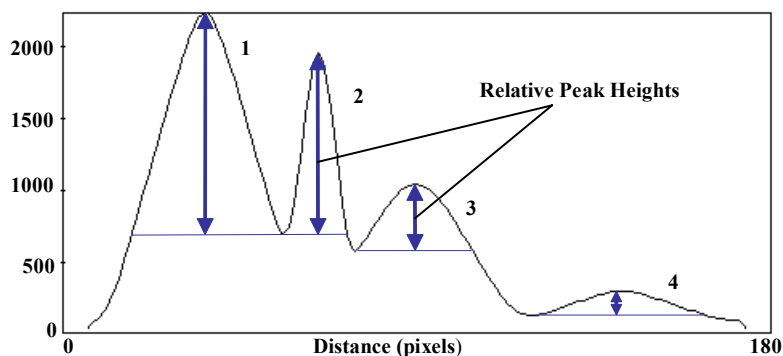


Figure 11: Intensity profile of an object with four intensity peaks. Peak #1 height = 1500, peak #2 height = 1250, peak #3 height = 500 and peak #4 height = 200.

Only two peaks in Figure 11, #1 and #2, have relative intensity 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 12A. 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 12B and 12C.

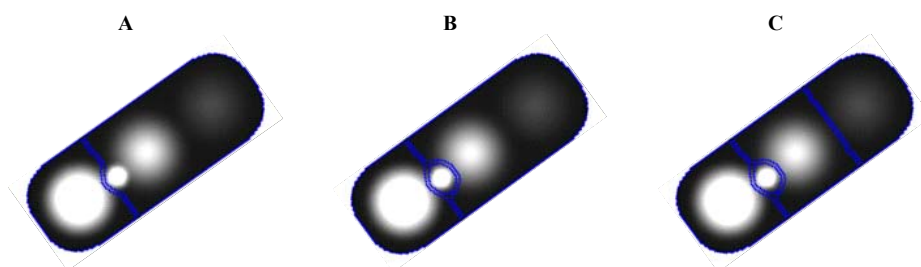


Figure 12: Object segmentation: Intensity Method. A) Result of segmentation using parameter **NucSegmentationCh1** = -1000; B) Result of segmentation using parameter **NucSegmentationCh1** = -400; C) Result of segmentation using parameter **NucSegmentationCh1** = -100,

Reject Border Objects

RejectBorderNucsCh1

When running the BioApplication, you have the option of not including and analyzing objects that touch the border of your image field. This is controlled by the **RejectBorderNucsCh1** Assay Parameter. If this Assay Parameter has a value of **1**, objects touching the border are not analyzed. On the other hand, a value of **0** results in all objects in the image field to be eligible for analysis, whether or not they touch the image border.

Basic Assay Parameters

Assay Parameters control the functioning of the image analysis algorithm. 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
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
TargetTypeCh2	Binary	Type of targets to be identified in Ch2: 0 = Bright targets on dark background, 1 = Dark targets on bright background
CircModifierCh2	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
RingDistanceCh2	Pixels	Distance (in pixels) from Ch1 object (nucleus) mask to the inner rim of ring mask
RingWidthCh2	Pixels	Width (in pixels) of ring mask
MaskModifierChN	Pixels	Number of pixels to modify Ch1 object (nucleus) mask in ChN: Negative value = Shrink mask, 0 = Do not modify mask, Positive value = Expand mask

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

Object Selection Parameters

Each channel has a set of specific Object Selection Parameters associated with it (Tables 8-10). 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. Channel 2 defines the Ring mask, as well as a selected object within Channel 2. Object Selection Parameters for Channels 3-6 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 $4\pi \times \text{Area}$ 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 in Channel 1 available for the Cytoplasm to Nuclear Translocation BioApplication

Channel 2

Parameter	Units	Description
RingAvgIntenCh2	Intensity	Average intensity of all pixels within ring mask in Ch2
TotalIntenCh2	Intensity	Total intensity in Ch2 of all pixels within modified Ch1 nucleus mask
AvgIntenCh2	Intensity	Average intensity in Ch2 of all pixels within modified Ch1 nucleus mask

Table 9. Object Selection Parameters in Channel 2 available for the Cytoplasm to Nuclear Translocation BioApplication

Channel N (3-6)

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

Table 10. Object Selection Parameters in Channels 3-6 available for the Cytoplasm to Nuclear Translocation BioApplication. *Note that "ChN" refers to Channels 3-6

Gating

The Cytoplasm to Nuclear Translocation BioApplication supports 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 select or reject cells based on fluorescence intensity in a downstream channel. The gating feature allows you to specifically identify a certain class of cells based on labeling intensity under the Ring mask (in Channel 2) or modified nuclear mask (in Channels 2-6) 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 threshold values by entering upper and lower limits for the intensity parameters, **RingAvgIntenCh2** (Channel 2), **AvgIntenChN** (Channels 1-6), and **TotalIntenChN** (Channels 1-6). **RingAvgIntenCh2** is the average intensity under the Ring mask in Channel 2 of the object of interest.

TotalIntenChN is the total pixel intensity within the object of interest and **AvgIntenChN** 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 **MaskModifierChN** 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.

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 Cytoplasm to Nuclear Translocation BioApplication can be found in Table 11.

Parameter	Description
Include This Channel In Composite	Determines if image for this Channel is included in composite image.
CompositeColor	Determines the color used for that particular channel in the composite image.
SelectedCell	Cells that are selected and analyzed by the BioApplication. Only cells that pass all the object selection criteria are selected.
RejectedCell	Cells that are rejected and never analyzed by the BioApplication. Only cells that do not pass all the object selection criteria are rejected.
CircCh2	Identifies the area under the Circ mask in Channels 2-6.
RingCh2	Identifies the area under the Ring mask in Channels 2-6.

Table 11. Image Display option settings for the Cytoplasm to Nuclear Translocation BioApplication

Assay Parameters for Population Characterization

The Cytoplasm to Nuclear 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 **FeatureChNLevelHigh** and **FeatureChNLevelLow**) sets the upper and lower bounds of this range respectively. The Status Cell Feature (**FeatureChNStatus**) 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 12).

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 12. Status definitions for the Cytoplasm to Nuclear Translocation BioApplication.

The corresponding Well Features report the percentage of cells that is either greater or less than the limits defining this range. Figure 13 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.

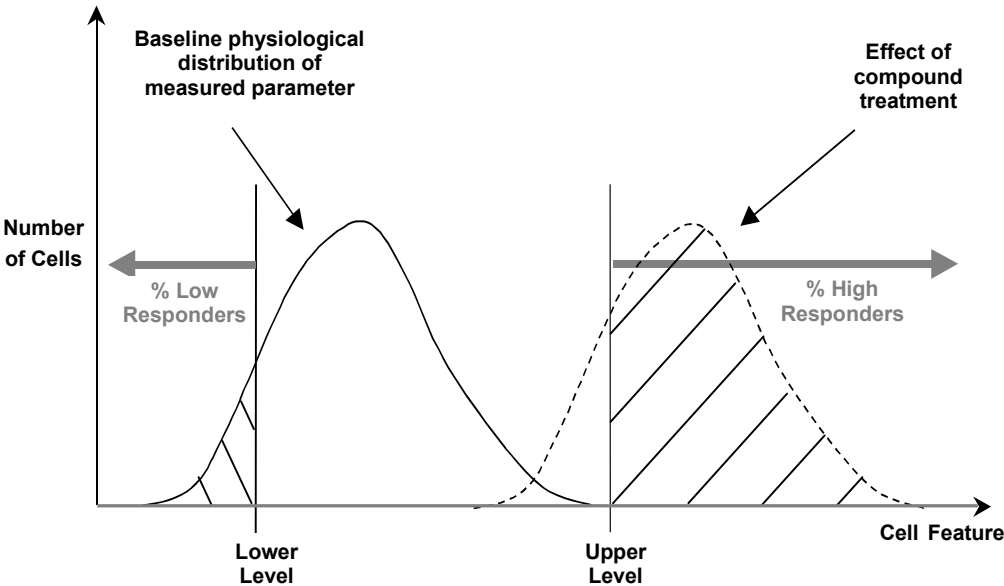


Figure 13. Schematic population distribution of a measured feature in a population of untreated and compound treated cells. The upper and lower levels correspond to %Low or %High

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 (***FeatureChNLevelLow***, ***FeatureChNLevelHigh***) 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. Note that this process can be performed on all Cellomics HCS Readers.

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 advantage of using Reference Wells to automatically calculate levels is that the levels are determined by a control population of cells and are independent of run-to-run variations. The features measured that can be used to characterize the cells in a population as described above are **MEAN_CircRingAvgIntenDiffCh2** and **MEAN_CircRingAvgIntenRatioCh2**. From these results, **%HIGH/%LOW_CircRingAvgIntenDiffCh2** and **%HIGH/%LOW_CircRingAvgIntenRatioCh2** 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 Cytoplasm to Nuclear 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 for the Assay Parameters **CircRingAvgIntenDiffCh2LevelHigh**, **CircRingAvgIntenDiffCh2LevelLow**, **CircRingAvgIntenRatioCh2LevelHigh**, and **CircRingAvgIntenRatioCh2LevelLow**.

- **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:

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

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

- **FeatureChNLevelLow** = Mean - **FeatureChNLevelLow_CC** × SD
- **FeatureChNLevelHigh** = Mean + **FeatureChNLevelHigh_CC** × SD

Thus, from the Reference Wells, if **MEAN_CircRingAvgIntenDiffCh2** = **750** and the **SD_CircRingAvgIntenDiffCh2** = **50** and the Assay Parameters

CircRingAvgIntenDiffCh2LevelLow_CC and **CircRingAvgIntenDiffCh2LevelHigh_CC** both were set at **2**, then calculation for their corresponding Level Assay Parameters would be:

- **CircRingAvgIntenDiffCh2LevelLow** = **750 - (2*50) = 650**
- **CircRingAvgIntenDiffCh2LevelHigh** = **750 + (2*50) = 850**

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

Advanced Assay Parameters

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

For each feature undergoing population characterization, there are four advanced Assay Parameters that control its levels: **FeatureChNLevelLow** and **FeatureChNLevelHigh** 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 13, 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:

- **CircRingAvgIntenDiffCh2LevelLow/High, Low/High_CC**

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

NOTE



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)
CircRingAvgIntenDiffCh2 LevelLow/High, Low/High_CC	Intensity	Defines CircRingAvgIntenDiffCh2 population characterization thresholds
CircRingAvgIntenRatioCh2 LevelLow/High, Low/High_CC	Number	Defines CircRingAvgIntenRatioCh2 population characterization thresholds

Table 13. Advanced Assay Parameters available for the Cytoplasm to Nuclear Translocation BioApplication

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 based scan using images acquired earlier. This section describes Cell, Well, and Reference Well Features that are available as output features from the Cytoplasm to Nucleus 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
NucAreaCh1	Pixels or μm^2	Area (in pixels or micrometers) of Ch1 nucleus
NucShapeP2ACh1	Number	Shape measure based on ratio of perimeter squared to $4\pi \times \text{area}$ 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
RingAvgIntenCh2	Intensity	Average intensity of all pixels within ring mask in Ch2
RingVarIntenCh2	Intensity	Variation (standard deviation) of intensity of all pixels within ring mask in Ch2
CircAvgIntenCh2	Intensity	Average intensity of all pixels within circ mask in Ch2
CircVarIntenCh2	Intensity	Variation (standard deviation) of intensity of all pixels within circ mask in Ch2
CircRingAvgIntenDiffCh2	Number	Difference between CircAvgIntenCh2 and RingAvgIntenCh2
CircRingAvgIntenDiffCh2Status	Number	CircRingAvgIntenDiffCh2 status: 0 = No response, 1 = High response, 2 = Low response
CircRingAvgIntenRatioCh2	Number	Ratio of CircAvgIntenCh2 to RingAvgIntenCh2

Cell Feature	Units	Description
CircRingAvgIntenRatioCh2Status	Number	CircRingAvgIntenRatioCh2 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 Cytoplasm to Nuclear Translocation BioApplication. *Note that “ChN” refers to Channels 2-6

Well Features

The Cytoplasm to Nucleus Translocation BioApplication has a specific and unique set of output features allowing the characterization and pathway analysis of activation and/or translocation of the target in a single cell.

- The output feature **MEAN_CircRingAvgIntenDiffCh2** is an indicator of activation/translocation of macromolecules between the nucleus and the cytoplasm in the target channel 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** is an indicator of activation/translocation of macromolecules between the nucleus and the cytoplasm in the target channel at the well level. This feature reports the ratio of the average pixel intensity between the Circ and Ring mask regions.
- The output feature **%HIGH_CircRingAvgIntenDiffCh2** is the percentage of cells in any well whose **CircRingAvgIntenDiffCh2** is higher than the **CircRingAvgIntenDiffCh2LevelHigh**, obtained automatically from the Reference Wells or entered manually as an Assay Parameter.
- The output feature **%LOW_CircRingAvgIntenDiffCh2** is the percentage of cells in any well whose **CircRingAvgIntenDiffCh2** is less than the **CircRingAvgIntenDiffCh2LevelLow**, obtained automatically from the Reference Wells or entered manually as an Assay Parameter.
- The output feature **%HIGH_CircRingAvgIntenRatioCh2** is the percentage of cells in any well whose **CircRingAvgIntenRatioCh2** is higher than the **CircRingAvgIntenRatioCh2LevelHigh**, obtained automatically from the Reference Wells or entered manually as an Assay Parameter.
- The output feature **%LOW_CircRingAvgIntenRatioCh2** is the percentage of cells in any well whose **CircRingAvgIntenRatioCh2** is less than the **CircRingAvgIntenRatioCh2LevelLow**, obtained automatically from the Reference Wells or entered manually as an Assay Parameter.

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 (**FeatureChNStatus**).

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 Cytoplasm to Nuclear 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

Table 16. Additional Well Level Features available for the Cytoplasm to Nuclear Translocation BioApplication.

Reference Well Features

These values are calculated and displayed only when the **UseReferenceWells** Assay Parameter =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 Ch2 average intensity difference between the Circ and Ring:

- RefCircRingAvgIntenDiffCh2Level**Low/High**

Plate Feature	Description
RefAvgCellCountPerField	Average count of cells per field in reference wells
RefCircRingAvgIntenDiffCh2Level Low/High	Low/High-response level for CircRingAvgIntenDiffCh2
RefCircRingAvgIntenRatioCh2Level Low/High	Low/High-response level for CircRingAvgIntenRatioCh2

Table 17. Plate level features for the Cytoplasm to Nuclear Translocation BioApplication.

iDev Software Workflow

This chapter describes the tasks in the workflow for Protocol optimization of the Cytoplasm to Nucleus 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.

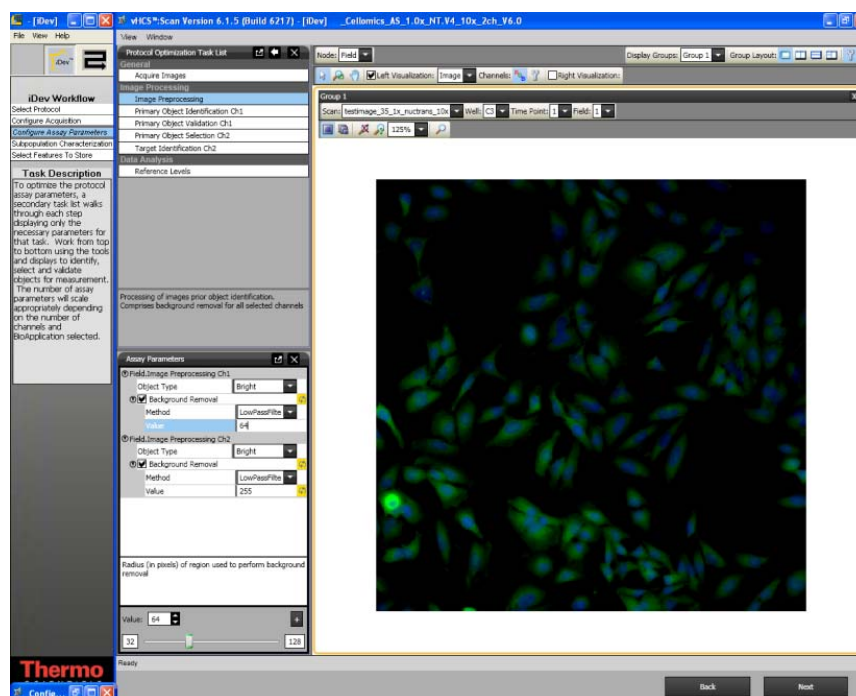


Figure 14. Protocol Optimization Task – Image Preprocessing

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.

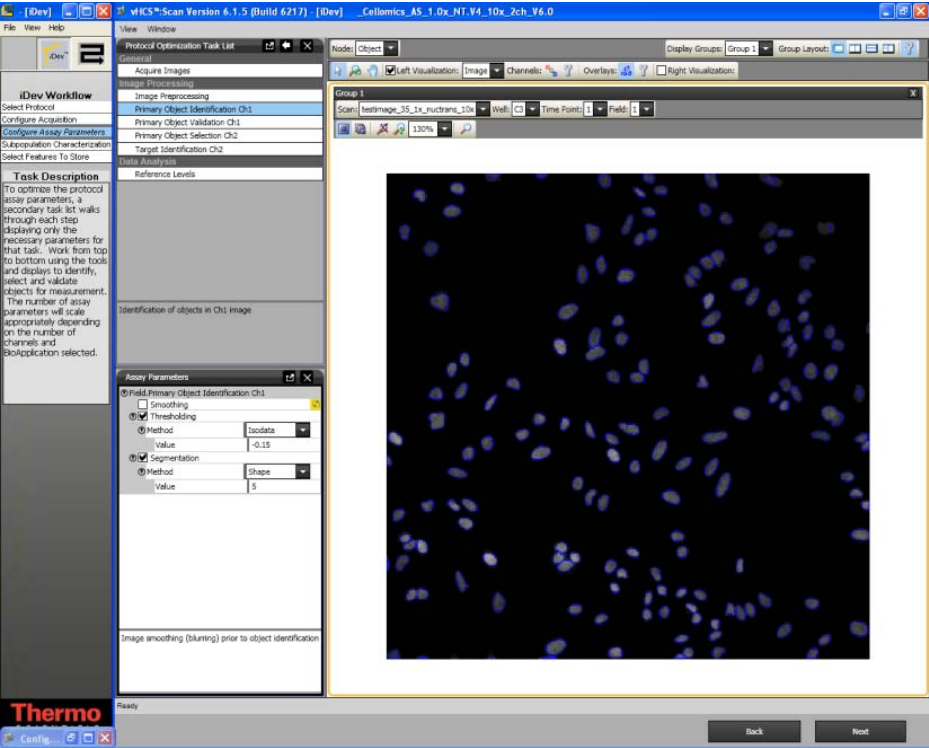


Figure 15. Protocol Optimization Task – Primary Object Identification Ch1

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.

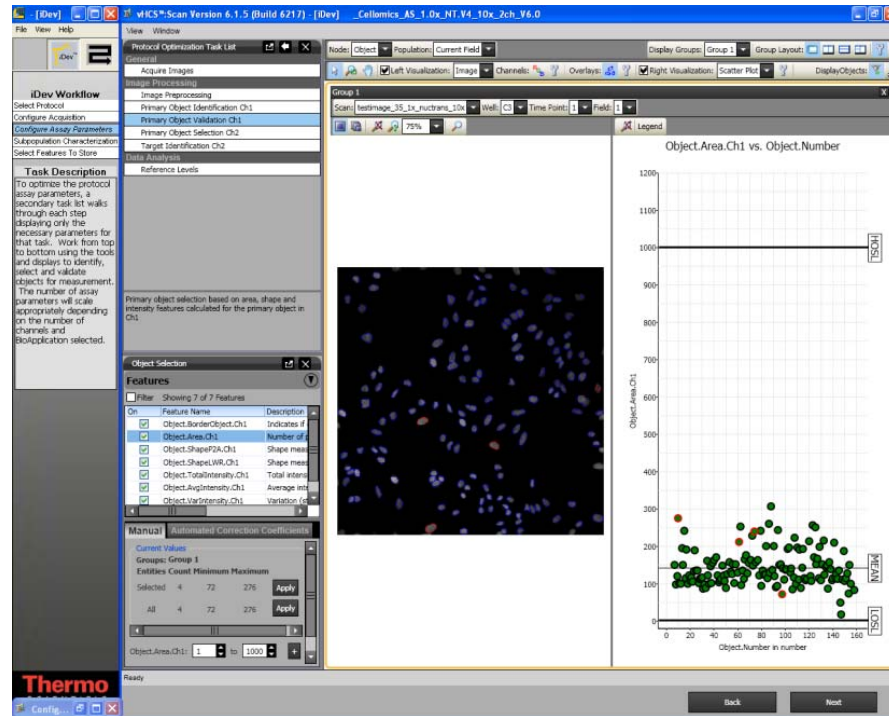


Figure 16. Protocol Optimization Task – Primary Object Validation Ch1

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.

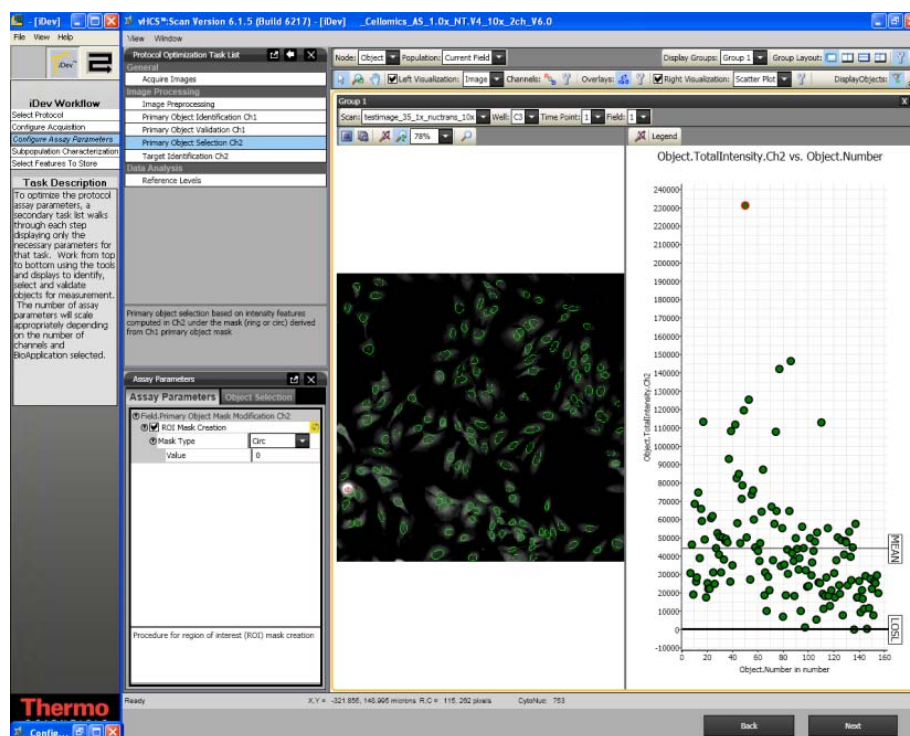


Figure 17. Protocol Optimization Task – Primary Object Selection Ch2 through ChN

Target Identification Ch2

In this task you can identify targets in the ROI (Circ or Ring) in Channel 2. Additionally, you can set thresholding methods to further refine the ring based on the defined Ring or Circ.

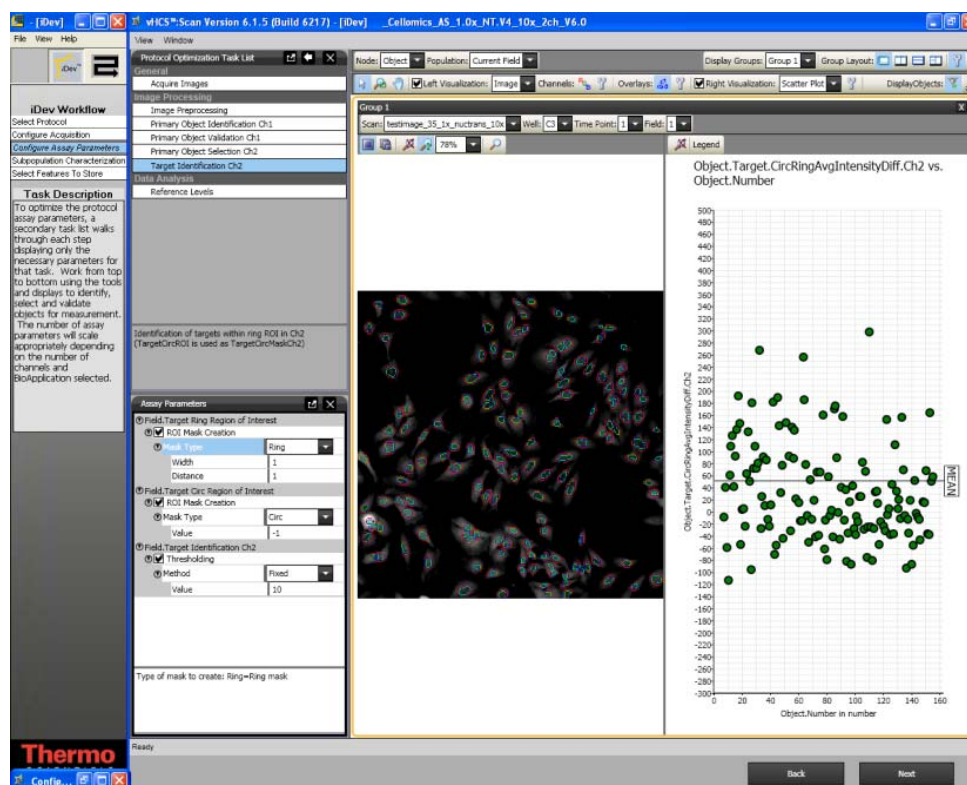


Figure 18. Protocol Optimization Task – Target Identification

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.

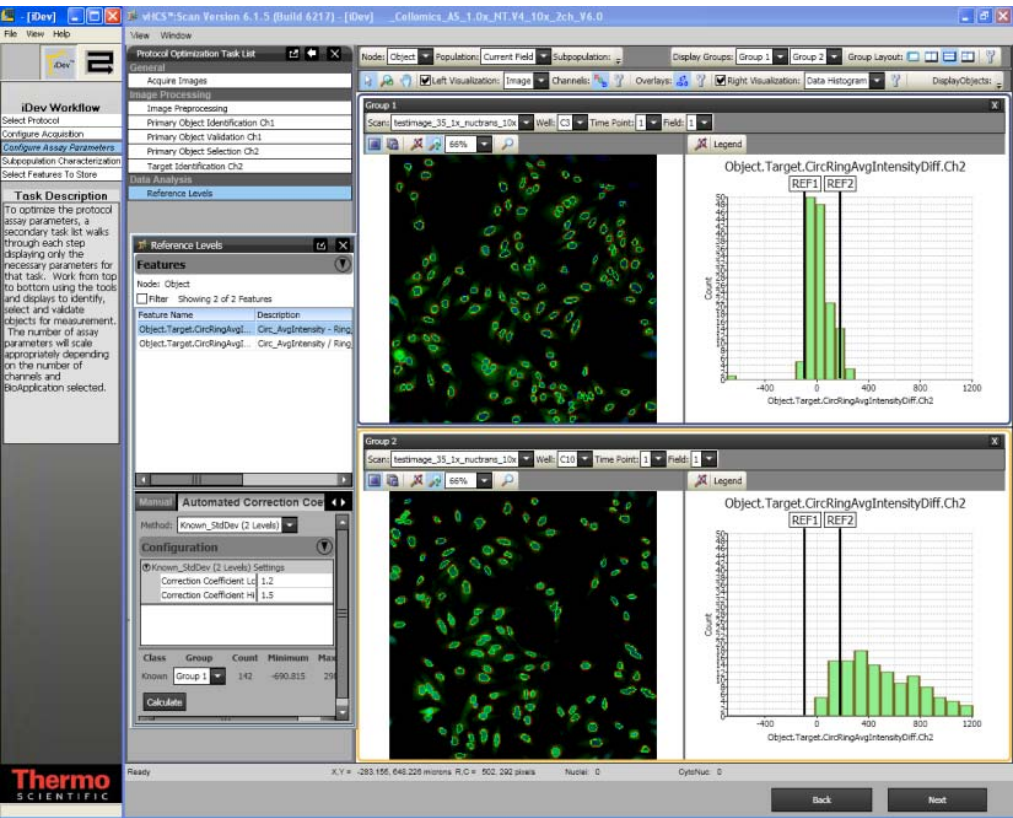


Figure 19. Protocol Optimization Task – Reference Levels

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