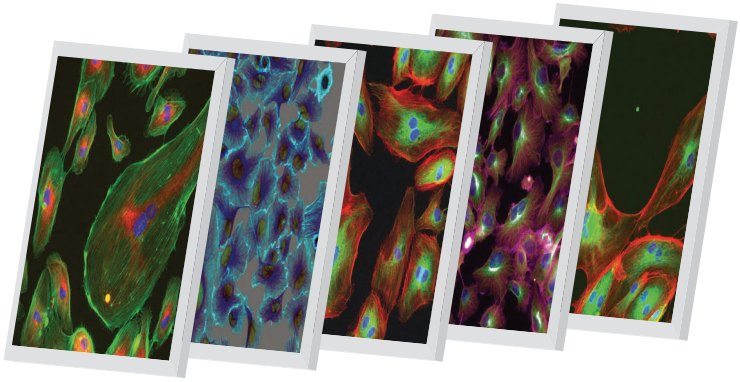
Thermo Scientific Cellomics® Cytoplasm to Cell Membrane Translocation V4

BioApplication Guide



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Cellomics® Cytoplasm to Cell Membrane Translocation BioApplication Guide

V4 Version

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Chapter

1

Overview of the Cytoplasm to Cell Membrane Translocation BioApplication

High Content Screening (HCS) uses fluorescence-based reagents, advanced optical imaging and automated image analysis algorithms (BioApplications) to analyze targets and physiological processes in single cells. The system is optimized for collection of multi-parameter data, using up to six spectrally distinct fluorescent indicators. This BioApplication guide provides a brief description for performing one such HCS assay, the measurement of target translocation between the cytoplasm and the cell membrane. This guide contains the following chapters:

Chapter 1 provides an overview of the BioApplication, such as use cases in which it can be applied.

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

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

System Compatibility

The Cytoplasm to Cell Membrane Translocation (abbreviated Cyto-Cell Mem) BioApplication described in this document is designed to run on the following platforms:

- ArrayScan® HCS Readers, 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

Mechanism of Cytoplasm to Membrane Translocation of Proteins

Cell signaling proteins translocate to different cell compartments in response to specific inducers. The cellular functions and regulation of these proteins, in most part, depend on specific subcellular localization (Cho 2001). Membrane targeting by peripheral proteins is mediated mainly by two conserved cysteine-rich (C1 and C2 domains) and the pleckstrin domains. The C1 domain was first identified as the interaction site for diacylglycerol (DAG) and phorbol esters in protein kinase C (PKC) isoforms (Nishizuka 1988). The domain consists of about 50 amino acids, which occur in tandem repeats (C1A, C1B, etc.) and have been found

in other proteins of diverse functions including: protein kinase D, chimaerin, Ras-GRP, Raf, Unc-13 isoforms and DAG kinases (Cho 2001). The membrane affinity for the C1 domain is dramatically increased upon ligand binding. The C2 domain is composed of about 130 amino acid residues. Several proteins that contain the C2 domain have been identified and include: PKC, cytosolic phospholipase A2, phospholipase C-γ, and phosphatidylinositol 3-kinase. Proteins associated with membrane trafficking have also been shown to contain the C2 domains and include: synaptotagmins, rabphilin-3A, and Unc-13, etc. (Rizo and Sudhof 1998). In the C2 domain, the affinity for negatively charged phospholipids, notably phosphatidylserine, increases upon Ca^{2+} binding. In the case of PKC, the high affinity binding of lipid ligands activates the enzyme by inducing a conformational change that removes an autoinhibitory (pseudosubstrate) domain from the active site (Newton 1996). Other proteins that have been shown to translocate from the cytoplasm to the cell membrane include Glut4, beta-arrestin, and β-adrenergic receptor kinase (Benovic, et al. 1986; Strasser, et al. 1986). Several of these proteins are also involved in G-protein coupled receptor (GPCR) activation and a battery of effector proteins could be used to create a broad orphan GPCR assay.

Different types of interactions are involved in membrane binding of cytosolic proteins and these interactions depend on the physicochemical properties of both the membrane and the binding protein. The bulk lipid composition of the membrane modulates membrane targeting either through specific lipid metabolites or by providing unique microenvironments. Cell signaling proteins localize to the plasma membrane by specific lipid binding domains that attach to specific membrane domains under regulatory control (Hurley and Meyer 2001).

Cytoplasm to Membrane Translocation in Drug Target Identification

Signal transduction, the process of relaying extracellular messages to the cytoplasm and the nucleus, is critical to normal cellular homeostasis. These cell-signaling events provide molecular targets for therapeutic intervention. Redistribution of these targets between cytoplasm and membrane and vice versa in response to agonist binding provides an example of such method for measuring pharmacokinetics (distribution and metabolism) and pharmacodynamics (activity and potency). Proteins that translocate to the cell membrane are involved in a variety of cellular functions.

The protein kinase C family of homologous serine/threonine protein kinases has been shown to play an important role in the processes of cell proliferation, differentiation, cytokine secretion, apoptosis, muscle contraction, and hormone release (Vallentin, et al.1999). Protein kinase B, which also translocates to the cell membrane upon activation by several growth and survival factors, is linked with multiple signaling pathways involved in insulin signaling, cell survival, and cancer (Alessi and Downes 1998). Glut4, another protein that translocates to the cell membrane, plays significant role in insulin-responsive glucose transport (Tavare, et al. 2001). Beta-arrestins and beta-adrenergic receptor kinase (βARK), which mediate GPCR regulation transiently, translocate to the cell membrane, and, in the case of βARK, the process seems to be facilitated by interactions of COOH-terminal regions of the kinase with the G protein βγ subunit where it phosphorylates the GPCR.

BioApplication Overview

The Cytoplasm to Cell Membrane Translocation (Cyto-Cell Mem) BioApplication provides information on net protein translocation between the cytoplasm and the cell membrane within a time interval that you define using images of live or fixed cells. To monitor redistribution of drugs and targets between cell membrane and cytoplasm, a new approach was developed where cell morphology is controlled to improve quantitation of the target in the membrane. This is accomplished by plating cells at high density, creating a tall, cuboidal cell morphology. The intensity of stains or luminescent labels in the membrane depends dramatically on the cell morphology. For example, if the membrane is viewed on edge, it will appear brighter than if viewed on face. More information can be collected from cells that are tall, where a portion of the membrane stands on edge.

The Assay Protocol was developed to maximize the portion of the membrane that can be viewed on edge to improve the dynamic range of the BioApplication. Membrane edges are identified using a membrane specific reference stain. The BioApplication reports how much of the target protein is at the membrane in comparison to how much is in the cytoplasm.

The Cyto-Cell Mem BioApplication is useful for screening compounds that are potential agonists or inhibitors of protein translocation from the cytoplasm to the cell membrane and vice versa. It is a functional BioApplication and measures the efficacy of compounds to induce protein translocation. The BioApplication can be used to eliminate lead compounds that bind to target receptors, but fail to effect translocation of proteins from the cytoplasm to the cell membrane, or, conversely, translocation from the cell membrane to the cytoplasm.

This BioApplication also enables selective measurements of objects in Channels 3-6 that are within an intensity range that you specify. This Gating can be performed based on fluorescence within the whole cell, cytoplasmic ring, or nuclear regions. For example, if measurements need to be made in cells that exhibit a certain expression level of a GFP–protein chimera, then gating could be used to measure cytoplasm to cell membrane translocation only in those cells.

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.

BioApplication Measurements

The Cyto-Cell Mem 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 illustrates the number of Cell and Well Features available based on the number of channels selected.

Measurement	Cell Region Measured	Cell Features	Well Features
Cell & Well Features Reported for Channel 1:			
General		Cell # Top Left Width Height X Centroid Y Centroid	Valid Cell Count Selected Cell Count %Selected Cells Valid Field Count Selected Cell Count Per Valid Field
Morphology	Nucleus	Area Shape P2A Shape LWR	MEAN, SD, SE, CV (All Selected Cells)
Intensity	Nucleus	Total Intensity Average Intensity Variation of Intensity	MEAN, SD, SE, CV (All Selected Cells)
Cell & Well Features Reported for Channels 3 -6:			
Intensity	Membrane	Total Intensity Average Intensity	MEAN (All Selected Cells)
		% Membrane Colocalization	MEAN, SD, SE, CV %RESPONDERS (All Selected Cells)
	Ring	Total Intensity Average Intensity	MEAN (All Selected Cells)
	Membrane/Ring	Average Intensity Ratio and Status	MEAN, SD, SE, CV, %RESPONDERS (All Selected Cells)
	Modified Mask	Total Intensity Average Intensity	none

Table 1. *Cyto-Cell Mem BioApplication Cell and Well Level Measurements*

Example Biology

Freshly passaged cells are plated into standard Packard ViewPlate® microplates and are incubated overnight to allow cells to attach, proliferate, and spread, forming a cuboidal morphology. Cells are treated with chosen compounds to effect the desired protein translocation. The cells are incubated for a time that you define, which is dependent on the compound and target of interest. The cells are fixed, and the membrane edges are stained with a fluorescent membrane marker. The cells are then permeabilized, treated with a specific primary antibody against the target of interest, and subsequently treated with a fluorescently tagged secondary antibody and a nuclear stain. After the appropriate incubation time, the plates are washed and scanned using a Cellomics HCS Reader using the Cyto-Cell Mem BioApplication. The type of compound applied, concentration, time of induction, and protein target of interest affect the net rate of protein translocation.

Protein translocation to the cell membrane can be determined at fixed time intervals by analysis of selected output features for controls (untreated) and stimulated (treated) cells. To validate this BioApplication, the Cellomics PKCα Activation HCS Reagent Kit was used. This reagent kit combines fluorescent dyes, buffers, and validated protocols for sample preparation. In the assay, PKCα translocation from the cytoplasm to the cell membrane was induced with phorbol myristate acetate (PMA). The Cyto-Cell Mem BioApplication works well for detection of PKCα translocation using standard tissue culture treated Packard ViewPlate microplates. However, coated plates such as BD Falcon® and BioCoat® 96-well assay microplates may also be used. The Cyto-Cell Mem BioApplication has been shown to work with HeLa and A549 cells. In general, cells that assume a cuboidal morphology upon packing are compatible with this BioApplication.

Detection of translocation events between the cytoplasm and the cell membrane is optimal when cells are in a closely packed monolayer.

A properly optimized biology protocol is essential for maximal performance of the algorithm. The membrane component of flattened cells is very similar in extent and intensity to the cytosol; hence, its identification in cell colonies is very different in cells that are rounded. Cell packing is, therefore, used to produce tall cells. Tall cells present regions of the cell where the membrane is visible on edge and present a chance to measure membrane localization with sufficient signal to background to produce a robust assay.

It is important to understand what the Cyto-Cell Mem BioApplication reveals about protein translocation. The BioApplication measures the net translocation of a protein (molecular target) from the cytoplasm to the cell membrane and vice versa. It does not measure the subsequent signaling cascade from the cell membrane or to the nucleus. It also does not measure formation of membrane clathrin pits, endocytic vesicles, as seen in some signaling pathways, or cytoplasm to nucleus translocation of proteins.

The image from the reference channel detects membrane edges stained with an appropriate membrane marker for the cell type being used (Figure 1). Analysis of protein translocation to the cell membrane is derived relative to the membrane marker stain on the cell membrane. Therefore, optimal reference staining of the membrane edges is required for best results. The rate of protein translocation to the cell membrane upon agonist induction varies considerably

from one protein to another and depends on the agonist used. Figure 2 shows a dose response curve for PKCα translocation to the cell membrane after induction with PMA.

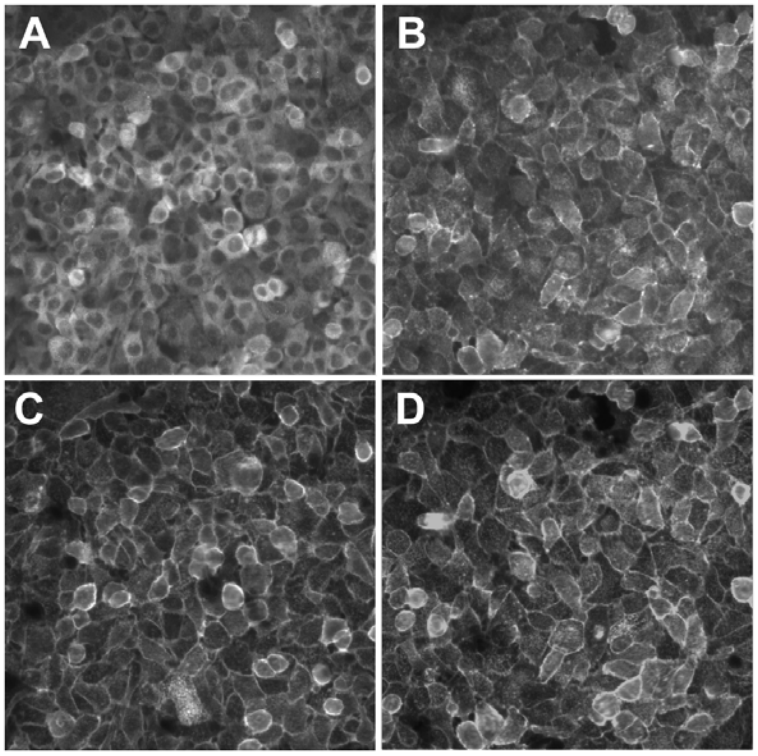

Figure 1. *Demonstration of cytoplasm to membrane translocation. HeLa cells were plated at 40,000 cells /well and incubated overnight. Cells were stimulated with PMA at a concentration of 800 nM. PKCα distribution in untreated cells (A) and cells treated with 800 nM PMA for 10 min. (B). Distribution of Membrane Marker is shown in each case in (C) and (D).*

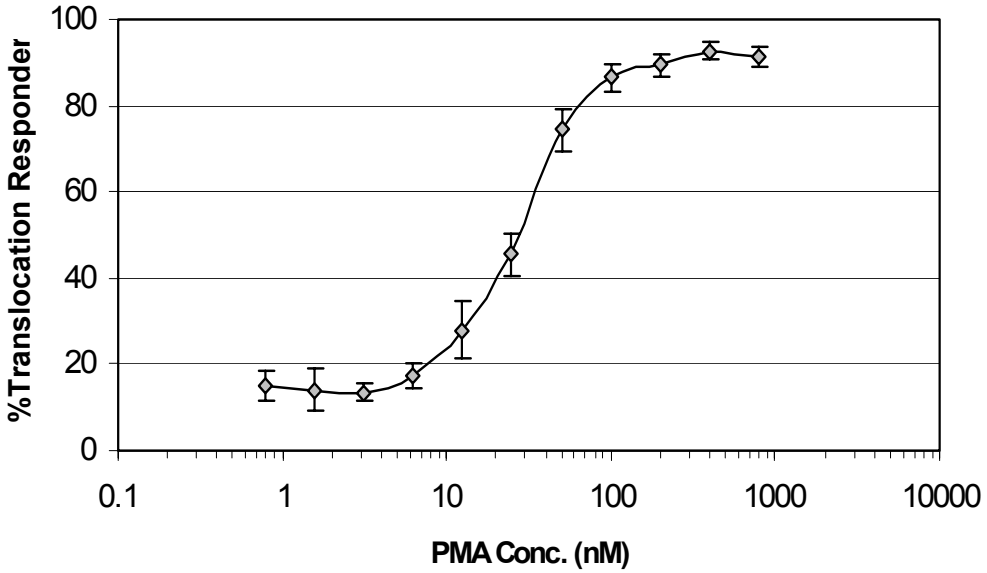

Figure 2. *PKC-α translocation response to PMA. The PKCα activation assay was performed with increasing concentration of PMA (0 - 800 nM). A dose-dependent induction of PKCα translocation from the cytoplasm to the cell membrane was obtained.*

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Chapter

2

Description of the Algorithm

NOTE

The Assay Parameters described in this guide function in the same manner regardless of using the Classic or iDev versions of the ArrayScan software. For descriptions of each task for optimizing a Protocol in the iDev Assay Development Workflow, please refer to Chapter 3 of this guide.

Acquired images are analyzed by an image-processing algorithm. This chapter briefly describes how this algorithm works within the Cyto-Cell Mem BioApplication, the output features, and input parameters that you can adjust.

Overview

The algorithm measures the translocation of proteins from the cytoplasm to the cell membrane and vice versa in single cells when activated by an agonist or inhibitor. The algorithm has three core channels, Channels 1-3 (designated as Ch1, Ch2, and Ch3) to identify and quantitate cell nuclei, membrane, and target, respectively. Additional target channels (Ch4, Ch5 and Ch6) are provided to enable gating.

The algorithm works by locating and validating objects (cell nuclei) in Channel 1 based on size and shape. Using the segmented nuclear mask, a region of interest (ROI) or cell domain is constructed for each valid nucleus. The domains are separated by single pixel lines to minimize unmeasured areas within the image. You can adjust the cell domain size to suit your needs. A value of 0 for the **CellDomainModifier** Assay Parameter removes the limit on the size of the domain.

Membranes associated with each valid nucleus are limited to the defined domain and the membrane intensity is based solely on the membrane detected within the domain. Membranes that are detected under the nuclear mask are rejected. The size of the nuclear mask can be adjusted to allow for a larger or smaller area to be used for membrane rejection. The average and total intensities under the final membrane mask in Channels 3-6 are measured and reported for each domain. The mean of these features for every domain in the well are reported as Well Features. You can set the Assay Parameters to define a cytoplasmic ring to measure the intensity in a perinuclear cytoplasmic region. The extent of coincidence of fluorophore distribution between Channel 2 and Channel 3 is reported for each cell domain analyzed. Ratios of cytoplasmic and membrane intensities are also reported for each domain.

Lastly, upper and lower limits that you define can be used to calculate the percentage of responders associated with each measurement. These limits can be designated manually or automatically determined using Reference Wells, as described in a following section, titled *Assay Parameters for Population Characterization*.

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 identification and thus configuring the application. The membrane is identified in Channel 2, and pixels with intensities above this threshold are used to help estimate target counts. 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	Channels 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 3-6), 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}, ArrayScan X.5 Readers. However, the entire range may be available on images from other sources.

Of the three intensity threshold method options, **None** means that no intensity threshold is applied. This option is not available in Channel 1 or Channel 2 because an intensity threshold is required to define the pixels making up the nuclei and membrane. The choice of the **None** threshold is 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** 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 3 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 Cyto-Cell Mem BioApplication

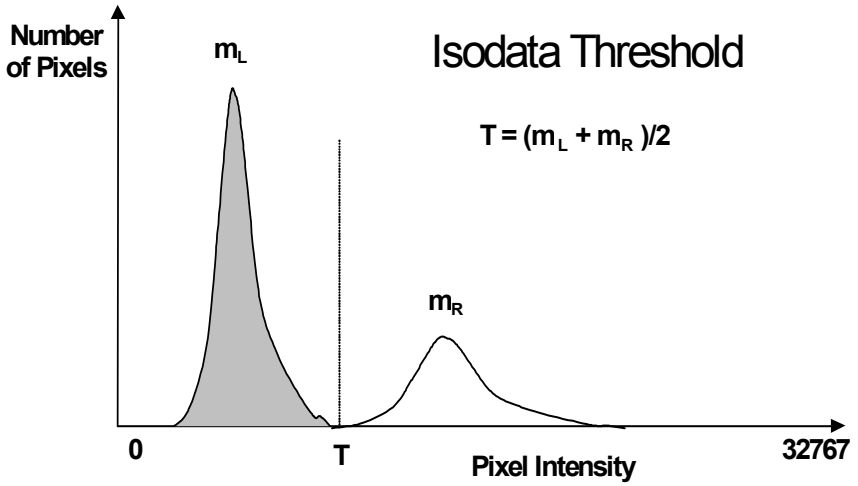

Figure 3. Histogram-derived Intensity threshold method for Isodata Threshold.: Background peak is shown in gray and object peak is shown in white.

Description of Assay Parameters and Settings

The Cyto-Cell Mem 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 protocol. These Assay Parameters are adjustable to allow customization of the algorithm to your own samples and conditions but normally do not have to be changed once they are established and stored in an Assay Protocol unless the sample or its preparation method has been changed significantly.

Input parameters can be found in Create Protocol View of the ArrayScan Classic software or in the Protocol Optimization task list of the iDev software. 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 is used for optimization of the biology, while Advanced Mode is used mainly for population characterization. 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**
- **Object Type**
- **Background Correction**
- **Object Smoothing**
- **Object Segmentation**

Reference Well Control

Population characterization can be performed using manual levels. Please see the *Assay Parameters for Population Characterization* section for more details.

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 levels. If Reference Wells are to be used (value = **1**), then the Assay Parameter **MinRefAvgCellCountPerField** must be set. This is the minimum number of cells/colonies detected per field that are required for acceptance of the data in the Reference Wells. This allows you to enter the minimum number of objects that you feel give a good distribution and, thus, statistical validity to the levels calculated from the Reference Wells. Note that in addition to these general Assay Parameters, there are additional parameters for Reference Well processing that are specific for particular features and channels. These will be described in later sub-sections.

Units for Morphological Measurements

You have the option of either choosing micrometers or pixels as the unit to report morphological measurements, i.e. area and size. This is done via the **UseMicrometers** Assay

Parameter, which when set to **0**, causes the morphological measurements to be reported in pixels. Otherwise, if set to **1**, lengths and areas are reported in μm and μm^2, respectively.

Object Type

NucTypeCh1, MembraneTypeCh*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 **MembraneTypeChN**.*

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

BackgroundCorrectionChN

Prior to image analysis, the non-cellular background can be computed and subtracted from the image separately in each channel, if desired, as shown in Figure 4.

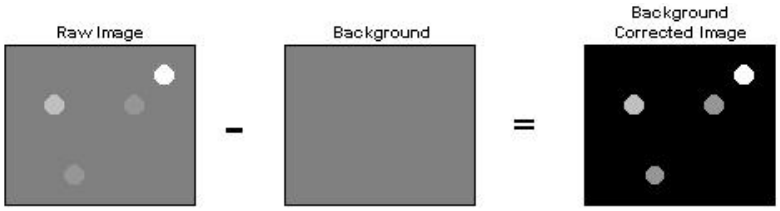

***Figure 4**. Image depicting calculation of Background Correction that can be used in each channel.*

The background-corrected image is computed by suppressing high frequency components in the image (low pass filtration). You can control the creation of the background image by adjusting the **BackgroundCorrectionCh*N*** Assay Parameter. This Assay Parameter refers to the radius of the area that is sampled around each pixel in the image to determine its local background. The value of this 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 Assay Parameter to **–1** lets the application decide on the value needed for the optimal background correction. Table 5 gives an overview of the different Background Correction settings that can be used for each channel.

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

Table 5. *Possible Background Correction Methods used in each channel with the Cyto-Cell Mem BioApplication.*

NOTE

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

Background Correction can be adjusted on each channel separately.

The background-corrected image is not stored or shown.

Object Smoothing

NucSmoothFactorCh1

Channel 1 has an Assay Parameter that enable control over the degree of image smoothing, or blurring, before the identification of objects (Channel 1) . This Assay Parameter is called **NucSmoothFactorCh1** and 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 (Channel 1). To get sharper definition of the shaped edges of objects (i.e., cells), you may want to keep the **NucSmoothFactorCh1** value small, if not **0**. However, if your Channel 1 label is not very homogeneous, the actual object will be erroneously identified as consisting of several smaller sized objects, and then smoothing will result in a homogenizing effect of the Channel 1 label and will help identify the object with its true bounds.

Smoothing reduces the sharpness of intensity variations by redistributing their relative brightness over the immediate vicinity in the image. Nuclear Smoothing can be used to connect fragments of nuclei and can be valuable when measuring morphological changes.

Figure 5 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).

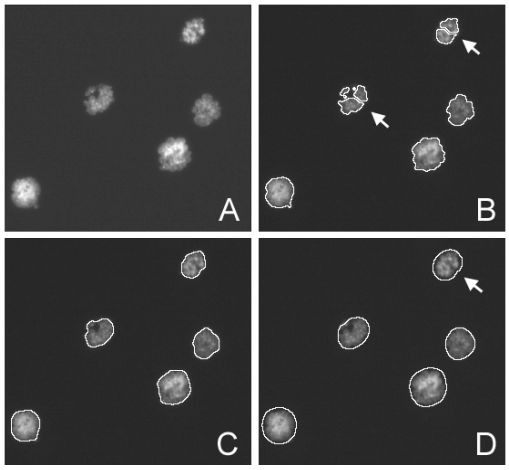

Figure 5. NucSmoothFactorCh1*: Images show the effect of different degrees of smoothing on the same field of apoptotic cell nuclei. A: Raw Image; B:* ***NucSmoothFactorCh1*** *= 0; C:* ***NucSmoothFactorCh1*** *= 3; D:* ***NucSmoothFactorCh1*** *= 8.*

Object Segmentation

NucSegmentationCh1

Even though you may have chosen an optimal intensity threshold and an appropriate degree of smoothing for object identification, you may encounter situations where individual objects cannot be properly resolved, such as in the case of densely packed objects. Object Segmentation will help resolve and identify individual objects. Two methods are available for nuclear segmentation in the Cyto-Cell Mem BioApplication: Geometric (shape and size) and Intensity (intensity peaks). These methods are illustrated in Figure 6.

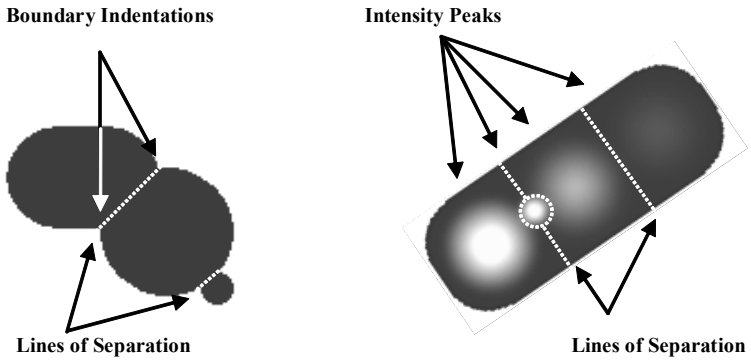

Figure 6. *Object Segmentation Options. The image on the left illustrates the Geometric Method while the image on the right illustrates the Intensity Method.*

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 segmentation methods.

Geometric Method Setting **NucSegmentationCh1** to R1 (Figure 7 A) produces object separation shown in Figure 7B. Setting **NucSegmentationCh1** to R3 results in segmentation shown in Figure 7C. Setting **NucSegmentationCh1** to R2 (the largest radius of all touching objects) produces no segmentation.

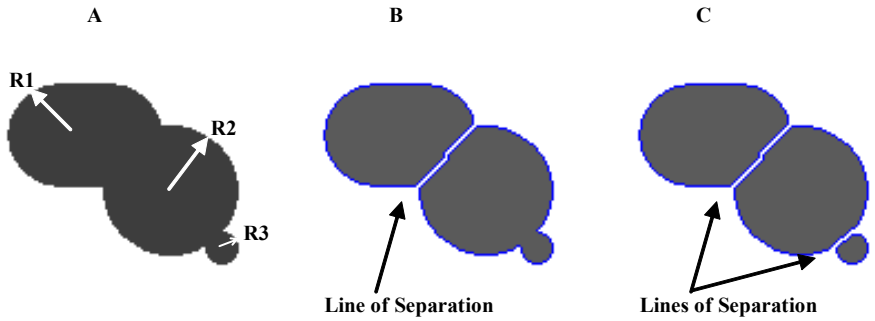

Figure 7. *Object segmentation: Geometric Method. A) Radii of touching objects: R1 = 24, R2 = 30, R3 = 7; B) Result of segmentation using* ***NucSegmentationCh1*** *= R1; C) Result of segmentation using* ***NucSegmentationCh1*** *= R3.*

Intensity Method Figure 8 shows the intensity profile along the cordial line of an object with four intensity peaks from Figure 6. 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 **ObjectSmoothFactorCh1** greater than 0 should be used to alleviate the problem.

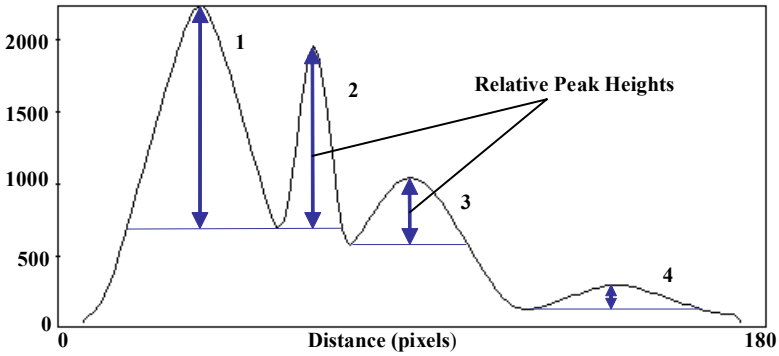

Figure 8. *Intensity profile of an object with four intensity peaks. Peak #1 height = 1500, peak #2 height = 1250, peak #3 height = 500, and peak #4 height = 200.*

Only two peaks in Figure 8, #1 and #2, have relative intensity greater than 1000. Thus, setting **NucSegmentationCh1** 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 9A. Setting **NucSegmentationCh1** 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 9B and 9C.

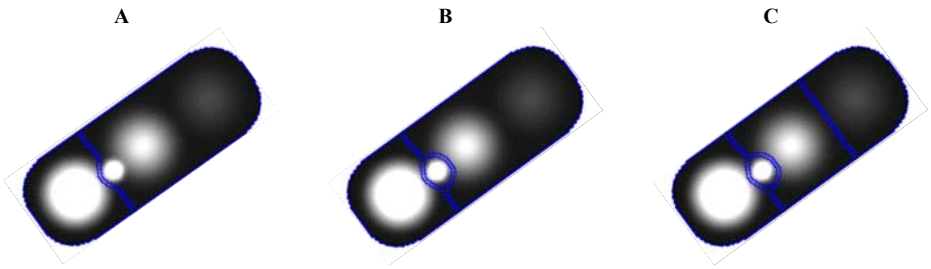

Figure 9. *Object segmentation: Intensity Method. A) Result of segmentation using parameter* ***NucSegmentationCh1*** *= -1000; B) Result of segmentation using parameter* ***NucSegmentationCh1*** *= -400; C) Result of segmentation using parameter* ***NucSegmentationCh1*** *= -100.*

Mask Size Modification

MaskModifierChN (Channels 3-6)

The identified objects in Channel 1 are used to define a mask. Only the pixels that fall under the defined mask are used for the intensity measurements in all channels. The size of the mask can be modified by the **MaskModifierCh*N*** Assay Parameter for Channels 3-6. The mask for Channel 1 cannot be modified. This **MaskModifierCh*N*** Assay Parameter sets the number of pixels for which the object mask will be dilated or eroded based on the parameter being positive or negative respectively (Figure 10).

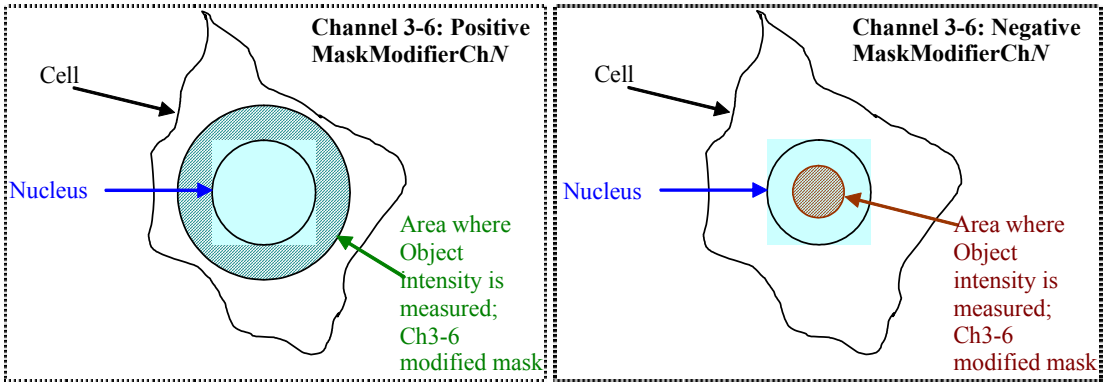

Figure 10. *Cell Mask Modifier Concept.* ***Left Panel****: This mask outlines a dilation that can be applied to Channels 3-6 (outer circle, including the inner circle) to encompass an area larger than the nucleus.*
Right Panel: *This mask outlines an erosion that can be applied to Channels 3-6 to encompass an area smaller than the nucleus.*

Channel-Specific Assay Parameters

Cellular Domain

CellDomainModifier

The BioApplication is able to limit the area of the image that is analyzed based on established cellular domains. The value entered for the **CellDomainModifier** Assay Parameter determines the extent in pixels of this domain as it extends outward from the original position of the object defined in Channel 1. Measurements will only be made within this domain limit for subsequent channels. All areas outside of the defined domains are ignored. In a confluent monolayer, this feature allows cellular domains to be defined even when the cells are in contact with one another.

Membrane Adjustment

Membrane Detect Width Ch2-6

A membrane reference is used to generate the mask in Channel 2.The areas of the image identified for this mask are defined by an image processing filter. This parameter is the expected thickness (in pixels) of the membrane mask found in Channel 2 and duplicated through Channels 3-6. This feature indicates the width of the kernel used by this filter to detect edges. Increasing this value will make edge detection more difficult. After application of the filter, the **Object Identification Methods** adjusted in Channel 2 of the Protocol Interactive View will control areas of the image used to generate the Channel 2 mask.

MembraneRejectionMaskModifierCh2-6

The **MembraneRejectionMaskModifierCh3-6** Assay Parameter is designed to optimize the generation of a membrane mask or rejection of spurious signal in the cell. The value represents the number of pixel dilations or erosions applied to the original object mask defined in Channel 1. Negative values will erode the original mask, whereas positive numbers will dilate it. Areas under this new mask are then ignored in Channel 2 when a final Channel 2 mask is generated. This mask is then duplicated in Channels 3-6. This feature is used to improve the identification of the membrane and eliminate non-specific, spurious labeling of the nucleus and cell interior when using membrane markers and, therefore, refine the final membrane mask that is generated.

UseRejectRingOverMembraneCh3-6

This parameter is used if you would want to remove the membrane mask created in Channel 2 from the Channel 3-6 ring mask. A value of zero will not remove the membrane mask from the ring mask while a value of 1 will remove the mask from the ring mask.

Ring Adjustment

RingDistanceCh*N*, RingWidthCh*N*

The nuclei identified in Channel 1 are used to create a region of interest in Channel 3. Figure 11 shows a schematic representation of the Nucleus with the Ring regions. The Ring region can be described as the sampled cytoplasmic region. You have the ability to vary the width and range of the Ring masks in the target channels. The Ring mask is only applied to objects that pass the object selection criteria based on mean and total pixel intensity that you set in each of the channels used. **RingDistanceCh*N*** is the distance in pixels from the Channel 1 object (nucleus) mask to the inner rim ring mask in Channels 3-6. **RingWidthCh*N*** the width in pixels of the ring mask in Channels 3-6.

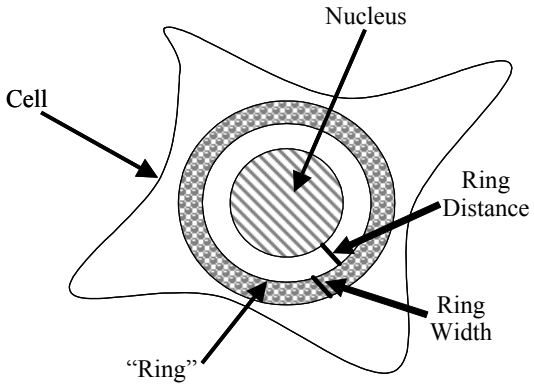

Figure 11. *A schematic representation of the Circ and Ring regions of a cell.*

Basic Assay Parameters

Assay Parameters available in Basic Mode control the morphological identifications of the objects in each channel, and control the use of Reference wells. You will not be able to view and Advanced Assay Parameters in the mode (as the **Hide Advanced Parameters** option is checked). Table 7 represents the Basic Assay Parameters available in this BioApplication.

Parameter	Units	Description
UseReferenceWells	Binary	Use reference wells to calculate high and low response levels: 0 = No, 1 = Yes
CellDomainModifier	Pixels	Number of pixels to modify Ch1 object (nucleus) mask to define the cellular domain within which all measurements are made: 0 = Do not modify mask, Positive value = Expand mask
NucTypeCh1	Binary	Type of nuclei to be identified in Ch1: 0 = Bright objects on dark background, 1 = Dark objects on bright background
BackgroundCorrectionChN	Pixels	Radius (in pixels) of region used to compute background in ChN: Negative value = Use surface fitting, 0 = Do not apply background correction, Positive value = Use low pass filter,
NucSmoothFactorCh1	Number	Degree of image smoothing (blurring) prior to nucleus detection in Ch1: 0 = Do not apply smoothing
NucSegmentationCh1	Pixels	Radius (in pixels) of touching nuclei that should be separated in Ch1: Negative value = Use intensity peaks method, 0 = Do not apply object segmentation, Positive value = Use shape geometry method
NucCleanupCh1	Binary	In Ch 1 image before object identification, clean up nucleus mask and remove small objects by applying erosion followed by dilation procedure: 0 = No, 1=Yes
MembraneTypeChN	Binary	Type of membranes to be identified in ChN: 0 = Bright objects on dark background, 1 = Dark objects on bright background
MembraneDetectWidthCh2-6	Pixels	Expected thickness (in pixels) of membrane mask in Ch2 and target masks in Ch3-6
MembraneRejectionMaskModifierCh2-6	Pixels	Number of pixels to modify Ch1 object (nucleus) mask to remove nuclear region from membrane mask in Ch2 and target masks in Ch3-6: Negative value = Shrink nucleus mask, 0 = Do not modify nucleus mask, Positive value = Expand nucleus mask
UseRejectRingOverMembraneCh3-6	Binary	Remove membrane mask from Ch3-6 ring masks: 0 = No, 1 = Yes
RingDistanceChN	Pixels	Distance (in pixels) from Ch1 object (nucleus) mask to the inner rim of ring mask in ChN
RingWidthChN	Pixels	Width (in pixels) of ring mask in ChN
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 in Cyto-Cell Mem BioApplication. *Note that "ChN" refers to Channels 1-6 for* ***Background Correction****, Channels 2-6 for* ***Membrane Type****, and Channels 3-6 for* ***Mask Modifier, Ring Distance,*** *and* ***Ring Width.***

Object Selection Parameters

Object Selection Parameters available for Channels 1-6 are used to define the cell population to be analyzed. Those found in Channel 1 specify shape criteria for the cells and associated cell nuclei. The object selection parameters for Channel 2 are hard-coded for this BioApplication and cannot be adjusted. Object Selection parameters for Channels 3-6 are intensity measurements used for gating. Table 8 and Table 9 list the Object Selection Parameters available in the Cyto-Cell Mem BioApplication.

Channel 1

Parameter	Units	Description
NucAreaCh1	Pixels or μm^2	Area (in pixels or micrometers) of Ch1 object
NucShapeP2ACh1	Number	Shape measure based on ratio of perimeter squared to 4π*area of Ch1 object (**NuctShapeP2ACh1** = 1 for circular object)
NucShapeLWRCh1	Number	Shape measure based on ratio of length to width of object-aligned bounding box of Ch1 object
NucAvgIntenCh1	Intensity	Average intensity of all pixels within Ch1 object
NucVarIntenCh1	Intensity	Variation (standard deviation) of intensity of all pixels within Ch1 object
NucTotalIntenCh1	Intensity	Total intensity of all pixels within Ch1 object

Table 8. *Object Selection Parameters for Channel 1 available for the Cyto-Cell Mem BioApplication*

Channel N (Channels 3-6)

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

Table 9. *Object Selection Parameters for Channels 3-6 available for the Cyto-Cell Mem BioApplication. Note that "ChN" refers to Channels 3-6.*

Gating

The Cyto-Cell Mem BioApplication supports gating on a cell population. This feature provides selective cell processing based on fluorescence intensity. Therefore, in addition to selecting cells for analysis in Channel 1, you can also select or reject cells based on fluorescence intensity in Channels 3-6. Gating allows you to specifically identify a subset of cells based on fluorescence intensity and allows you to limit the analysis to this group of cells. For example, gating may be used to analyze only those cells showing a certain level of expression of a fluorescent reporter such as green fluorescent protein (GFP). Note that gating is not available in Channel 2.

Specifying Intensity Ranges for Gating

When working in Create Protocol View, you can specify intensity ranges for the modified nucleus mask by entering upper and lower limits for the intensity parameters, **AvgIntenCh*N*** and **TotalIntenCh*N***, for Channels 3-6. **TotalIntenCh*N*** is a summation of all intensities within the cell. **AvgIntenCh*N*** is **TotalIntenCh*N*** divided by the object area. These parameters can also be adjusted interactively in Protocol Interactive View.

You can also adjust the intensity ranges for the ring created in the target channels by entering upper and lower limits for the intensity parameters, **RingAvgIntenCh*N*** and **RingTotalIntenCh*N***, for Channels 3-6. **RingTotalIntenCh*N*** is a summation of all intensities within the adjusted ring region. **RingAvgIntenCh*N*** is **RingTotalIntenCh*N*** divided by the Ring mask area. Although these parameters can also be adjusted interactively in Protocol Interactive View, the rings are not visible until you run the algorithm.

Specifying Mask Modifiers for Gating

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

Likewise, if you would like to gate out areas using the Ring mask region, both the **RingWidthCh*N*** and **RingDistanceCh*N*** Assay Parameter values need to be set. The actual shape of the ring may also be modified if you did not remove the membrane mask from the Channel 3-6 ring masks (using the **UseRejectRingOverMembraneCh3-6** Assay Parameter).

Image Overlays

All Image Display Option Settings are available when running in Basic or Advanced 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 (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.
SelectedNuc	Indicates cell nuclei that have passed all Object Selection Parameters, as well as cells that do not touch the image border.
RejectedNuc	Indicates cell nuclei that are rejected from analysis, either because they touch the image border, or because they do not satisfy Object Selection Parameters. Rejected cells are not counted by the BioApplication and are not analyzed.
CellDomain	Indicates the position of the domain limits within which measurements will be made in all channels. Domains are defined using the nuclear masks generated in Channel 1.
Membrane	The Membrane Mask is determined in Channel 2 and indicates labeled membrane regions identified by the BioApplication. This mask is applied to all downstream channels, Channels 3-6, to measure translocation.
Ring Ch*N* (*N* = 3-6)	Mask can only be applied to Channel N. Indicates the position of the mask used to calculate intensity in Channel N and is derived from the nuclear mask based on the Channel N RingDistanceCh*N* and RingWidthCh*N* Assay Parameters.

Table 10. *Image display options available for the Cyto-Cell Mem BioApplication.*

Assay Parameters for Population Characterization

Overview of Population Characterization

The Cyto-Cell Mem BioApplication provides the ability to characterize cells based on their response compared to a control population. For a particular Cell Feature, a range is determined and set by measuring the physiological distribution of feature values in untreated cells. Upper and lower limits (called ***Feature*ChN LevelHigh** and ***Feature*ChN LevelLow**) set the upper and lower bounds of this range respectively. The **Status** Cell Feature values indicate whether a given cell is within or beyond this range (Table 11):

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

***Table 11**. Numerical definitions of Cell Feature Status when using population characterization.*

The corresponding Well Features (**%RESPONDER_*Feature*ChN**) reported are the percentage of cells showing feature values that fall outside this range, i.e. the percentage of cells with Status Cell Feature values = **1** or **2**. Figure 11 illustrates this concept by showing the population distribution for Cell Feature values for a population having a defined physiological state (e.g., untreated) and the shift in this distribution upon compound treatment.

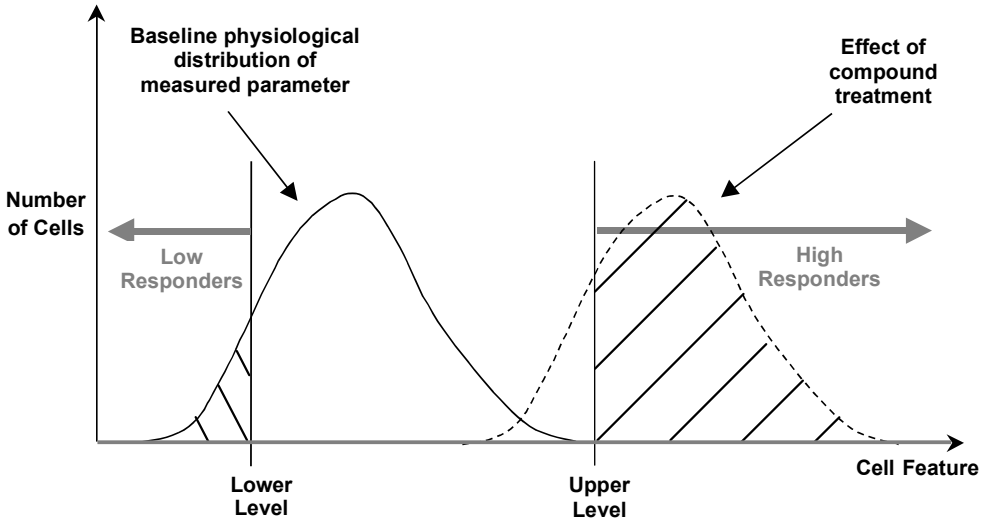

***Figure 11.** Principle of Population Characterization. % Responders exhibiting Status Cell Feature value = 1 (High Responders) or Status Cell Feature = 2 (Low Responders) are highlighted.*

Setting Cellular Response Levels

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

To set the limits (***Feature*Ch*N*LevelHigh** , ***Feature*Ch*N*LevelLow**) manually, you explicitly set the upper and lower limit values for each Cell Feature. This requires prior knowledge of typical feature values. The BioApplication then calculates the percentage of cells outside the range defined by the limits specified for the each Cell Feature.

To automatically determine the limits and set the range, the BioApplication uses 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 analyzed first and then the population distribution for the different features are determined. The population characterization levels are then set by adding or subtracting to the mean of the distribution its standard deviation multiplied by a coefficient that you define, named correction coefficient (_CC) in the Assay Parameters (***Feature*Ch*N*LevelHigh_CC, *Feature*Ch*N*LevelLow_CC**). You can use positive values for the correction coefficient to set the limit above the mean value of the feature and negative values for the correction coefficient to set limits below the mean value for the feature.

Reference Wells Processing Sequence

By setting the **UseReferenceWells** Assay Parameter to **1**, the Reference Wells processing is engaged. The specified Reference Well fields are acquired/analyzed, and Field, Well, and Plate Features are computed. Plate Feature values are directly used for cell population characterization. After this sequence is completed, computed values will be assigned to the Assay Parameters associated with each Reference Feature and regular scanning of the plate will begin. Again, if the feature value for **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 Cyto-Cell Mem BioApplication only uses Known Reference Wells. The sequence of computation for Reference Wells is as follows:

1) Cell Features are computed for every valid object within a field.

2) For each Cell Feature to be used for population characterization, the mean and standard deviation are computed over all cells in the field.

3) The automatically calculated level values for each feature are reported as Reference Features having the name **Ref*Feature*Ch*N*LevelHigh, Ref*Feature*Ch*N*LevelLow**.

4) Well and Plate Features are computed as average values for fields in a well, weighted for the number of cells per field, and then as arithmetic average for wells on a plate. Use of a weighted average minimizes the effect of sparse fields.

Identifying Reference Wells and Control Parameters

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

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

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

You choose whether to manually or automatically set the range by using the **UseReferenceWells** Assay Parameter. The possible values and resulting actions controlled by this parameter are:

- **UseReferenceWells** = 0 – do not use Reference Wells (i.e., set ranges manually)
- **UseReferenceWells** = 1 – use Reference Wells (i.e., the instrument will set ranges automatically using data collected from Reference Wells)

You can specify which wells on the microplate should be used as Reference Wells. These wells typically contain untreated cells that display physiological values for the Cell Features being measured. The instrument first images these wells and determines the population distribution for the different features. The cell population characterization limits are then computed through use of a correction coefficient, or **CC** value, for specific Assay Parameters which is used in combination with the mean and standard deviation of the distribution of feature values.

The calculation performed automatically by the BioApplication to set upper and lower limits (***Feature*Ch*N*LevelHigh** and ***Feature*Ch*N*LevelLow**, respectively) is shown below:

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

Note that the CC value can be different for defining the lower and the upper limits.

The BioApplication then applies the range specified by these limits to the entire plate. For example, if you want to know the percentage of cells that, upon compound treatment, have a response beyond the 95th percentile of the response from a control untreated population of cells, then the coefficient by which the standard deviation would be multiplied would be two (**CC** value = 2), assuming a normal distribution. The advantage of using Reference Wells is that the limits are automatically determined from a control population of cells and are independent of run-to-run variations when doing the experiment on different days that result from different illumination conditions, varying signal, or changes in cell culture conditions.

Advanced Assay Parameters

Advanced Assay Parameters can only be viewed and adjusted when using the BioApplication in Advanced Mode. In Advanced Mode, all basic and advanced input parameteres are adjustable. The **Hide Advanced Features** checkbox will hide or show the advanced Assay Parameters. When you check the box, only the Basic Assay Parameters are shown; when you uncheck the box, all Basic and Advanced Assay Parameters are shown.. The Advanced Mode Assay Parameters pertain to setting the upper and lower thresholds for population characterization.

For each feature undergoing population characterization, there are four advanced Assay Parameters that control its levels: ***Feature*ChN LevelHigh** and ***Feature*ChN LevelLow** that set upper and lower thresholds and the presence of the **_CC** suffix for each, designated when those levels are set using Reference Wells. For example, the four parameters controlling percent membrane colocalization in Channel 3 are:

- **%MembraneColocCh3LevelHigh**
- **%MembraneColocCh3LevelLow**
- **%MembraneColocCh3LevelHigh_CC**
- **%MembraneColocCh3Level_CC**

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

- %MembraneColocCh3Level**Low/High, Low/High_CC**

Units will be expressed with ***Feature*ChN LevelLow, *Feature*ChN LevelHigh**, knowing that *_CC* is expressed as a number.

Feature	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 selection)
MembraneRingAvgIntenRatioChNLevel***Low/High, Low/High_CC***	Number	Defines **MembraneRingAvgIntenRatioChN** population characterization thresholds
%MembraneColocChNLevel***Low/High, Low/High_CC***	Percent	Defines **%MembraneColocChN** population characterization thresholds

Table 12. *Advanced Assay Parameters available for the Cyto-Cell Mem BioApplication. *Note that "ChN" refers to Channels 3-6.*

Description of Output Features

Output features are the biological measurements produced after running the images. All features are categorized and accessible using the View application. There are two main types of output features for this BioApplication. Cell Features represent individual cellular measurements. Well Features are population descriptors for the cell population analyzed and are derived from Cell Features. Well Features, are listed in Scan Plate View and Create Protocol View so that screening results can be viewed during a scan. 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 via Protocol Interactive View once the algorithm has been applied to an image set.

Features That Can Be Used To Measure Translocation

The Cell Features that can be used to measure translocation between the cytoplasm and the plasma membrane are:

- **MembraneRingAvgIntenRatioCh*N*Status** = Ratio of average intensity in membrane region to that in the cytoplasmic ring ("Ch*N*" refers to Channels 3-6). Responders for this feature are quantified by the Well Feature **%RESPONDER_MembraneRingAvgIntenRatioCh*N***.
- **%MembraneColocCh*N*Status** = Extent of colocalization of target in Channel N with the membrane region ("Ch*N*" refers to Channels 3-6). Responders for this feature are quantified by the Well Feature **%RESPONDER_%MembraneColocCh*N***.

Cell Features

Table 13 shows the output features for the Cyto-Cell Mem BioApplication reported for each selected cell, accessible in the Cell Feature window in the Protocol Interactive View or in the View application.

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 object
Left	Pixels	X coordinate (in pixels) of top left corner of image-aligned bounding box of Ch1 object
Width	Pixels	Width (in pixels) of image-aligned bounding box of Ch1 object
Height	Pixels	Height (in pixels) of image-aligned bounding box of Ch1 object
XCentroid	Pixels	X coordinate (in pixels) of center of Ch1 object
YCentroid	Pixels	Y coordinate (in pixels) of center of Ch1 object
NucAreaCh1	Pixels or μm^2	Area (in pixels or micrometers) of Ch1 object
NucShapeP2ACh1	Number	Shape measure based on ratio of perimeter squared to 4π*area of Ch1 object (**NucShapeP2ACh1** = 1 for circular object)

Feature	Units	Description
NucShapeLWRCh1	Number	Shape measure based on ratio of length to width of object-aligned bounding box of Ch1 object
NucAvgIntenCh1	Intensity	Average intensity of all pixels within Ch1 object
NucVarIntenCh1	Intensity	Variation (standard deviation) of intensity of all pixels within Ch1 object
RingTotalIntenChN	Intensity	Total intensity of all pixels within ChN ring mask
RingAvgIntenChN	Intensity	Average intensity of all pixels within ChN ring mask
MembraneTotalIntenChN	Intensity	Total intensity of all pixels within membrane mask in ChN
MembraneAvgIntenChN	Intensity	Average intensity of all pixels within membrane mask in ChN
MembraneRingAvgIntenRatioChN	Number	Ratio of membrane mask average intensity to channel ChN ring mask average intensity in channel ChN
MembraneRingAvgIntenRatioChNStatus	Number	**MembraneRingAvgIntenRatioChN** status: 0 = No response 1 = High response 2 = Low response
%MembraneColocChN	%	Colocalization (in percent) between membrane and ChN target mask
%MembraneColocChNStatus	Number	**%MembraneColocChN** status: 0 = No response 1 = High response 2 = Low response
TotalIntenChN	Intensity	Total intensity of all pixels within modified Ch1 object mask in ChN
AvgIntenChN	Intensity	Average intensity of all pixels within modified Ch1 object mask in ChN

Table 13. *Cell Level output features available for the Cyto-Cell Mem BioApplication. *Note that "ChN" refers to Channels 3-6.*

Well Features

Many Well features are derived from the Cell Features. Such features are identified by a prefix, as listed in Table 14, 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
%RESPONDERS_	The percentage of cells in the well whose feature value is **either above or below** the value specified in the Level Assay Parameters or as calculated by the Reference Wells.	Percent

***Table 14**. General Well Feature prefixes available for the Cyto-Cell Mem BioApplication.*

The algorithm also reports the following additional Well Features (Table 15) in the Scan Plate View of the Scan application in addition to the Well Detail window of the View application.

Feature	Description
ValidCellCount	Count of valid objects (cells) identified in the well (Ch1 object selection parameters applied)
SelectedCellCount	Count of valid cells selected for analysis in the well
%SelectedCells	Percentage of valid cells selected for analysis in the well (Ch2-6 object selection parameters applied)
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 15.** Well level output features available for the Cyto-Cell Mem BioApplication.*

Reference Features

Plate Features provide information about the various Reference Wells. 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 application. The list of reference features and their values are displayed in the Scan Plate View and entered into the plate database in the same way as regular Well Features and can be viewed via the View application. Table 16 lists the following Plate Output Features available for the Cyto-Cell Mem BioApplication.

Feature	Description
RefAvgCellCountPerField	The average number of selected cells within the fields processed for Reference Features.
RefMembraneRingAvgIntenRatioCh*N* Level***Low/High***	Low/High-response level for **MemRingAvgIntenRatioLevelHighCh*N*** when **UseReferenceWells** computed from reference well results
Ref%MemColocCh*N*Level***Low/High***	Low/High-response level for **%MemColocLevelHighCh*N*** when **UseReferenceWells** computed from reference well results

Table 16. *Reference well features available for the Cyto-Cell Mem BioApplication. *Note that "ChN" refers to Channels 3-6. Any* ***RefFeatureChNLevelLow****,* ***LevelHigh*** *Reference Features are grouped as* ***RefFeatureChNLevelLow/High****.*

The feature value for **RefAvgCellCountPerField** must be greater than or equal to the value specified for the **MinRefAvgCellCountPerField** Assay Parameter for Reference Well data to be used for calculation of lower and upper limits for population characterization and identification of responders. If this condition is not met, the BioApplication defaults to using the manually specified values for ***Feature*ChN LevelLow** and ***Feature*ChN LeveHigh**.

Chapter

3

iDev Software Workflow

This chapter describes the tasks in the workflow for Protocol optimization of the Cytoplasm to Cell Membrane 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 background removal methods and values for each of the channels in the Protocol.

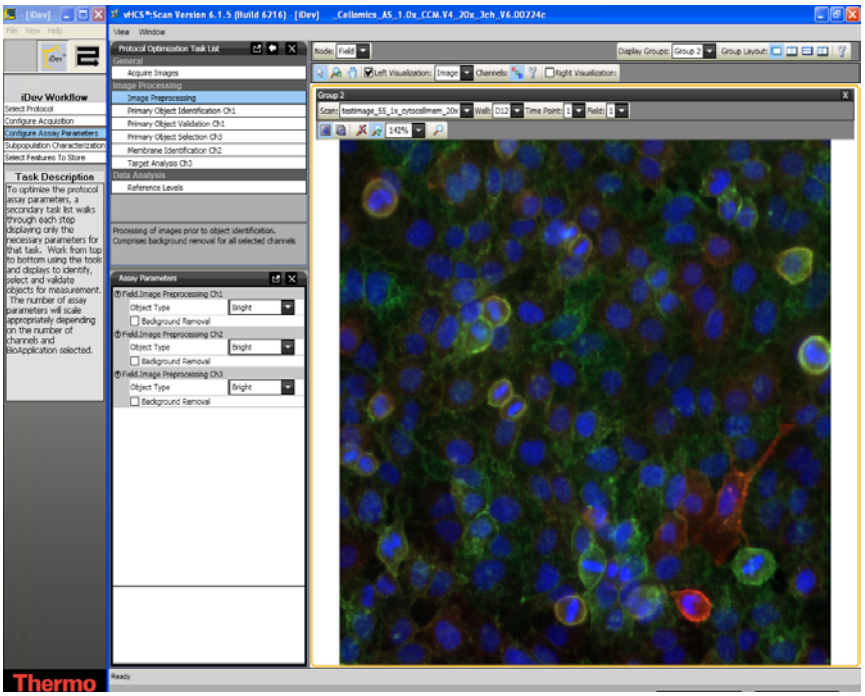

Figure 12. *Protocol Optimization Task – Image Preprocessing*

Primary Object Identification Ch1

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

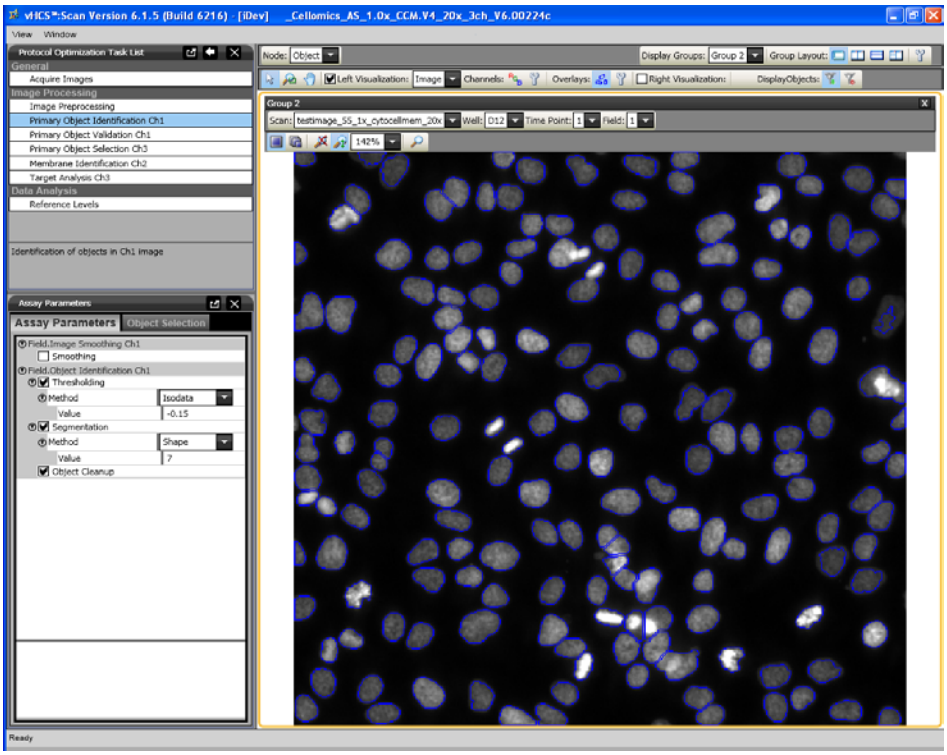

Figure 13. *Protocol Optimization Task – Primary Object Identification Ch1*

Primary Object Validation Ch1

Primary Object Validation is object selection/rejection based on area, shape, and intensity features calculated for the primary object in Channel 1. In this task, you will set minimal and maximal values for validating (equivalent to selecting and rejecting objects in the ArrayScan Classic software) objects in Channel 1 based on object selection features.

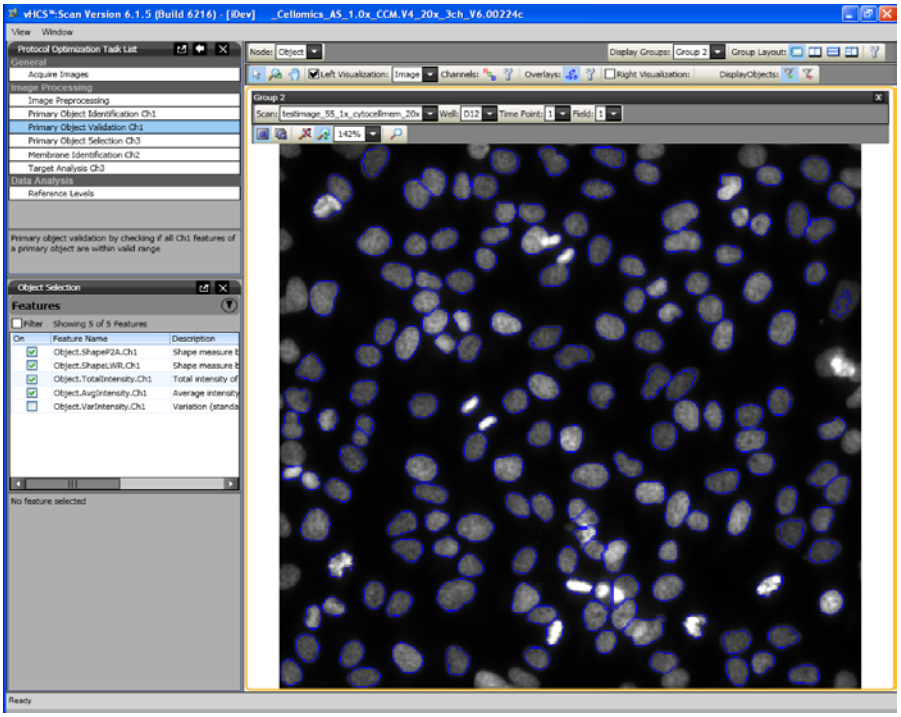

Figure 14. *Protocol Optimization Task – Primary Object Validation Ch1*

Primary Object Selection Ch3 through ChN

Primary Object Selection is object selection based on intensity features computed in Channels 3 through Channel N under the circ mask derived from the Channel 1 primary object mask. In this task, you will set selection / rejection of the primary object based on intensity measurements in Channels 3 through Channel N under a modified primary object mask. This task is similar to setting the Assay Parameter, **MaskModifierCh3,** 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.

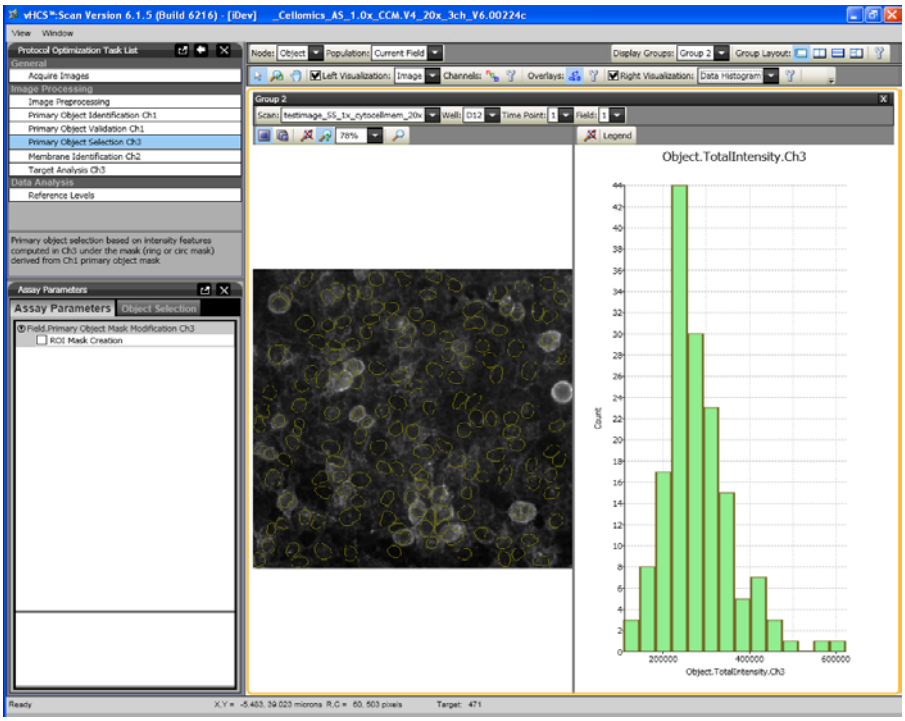

Figure 15. *Protocol Optimization Task – Primary Object Selection Ch3 through ChN*

Membrane Identification Ch2

In this task, you can set the parameters for identifying the membrane in Channel 2. You can set the value for creating a ROI, mask modification, and detection width (including thresholding method and value) for membranes in Channel 2.

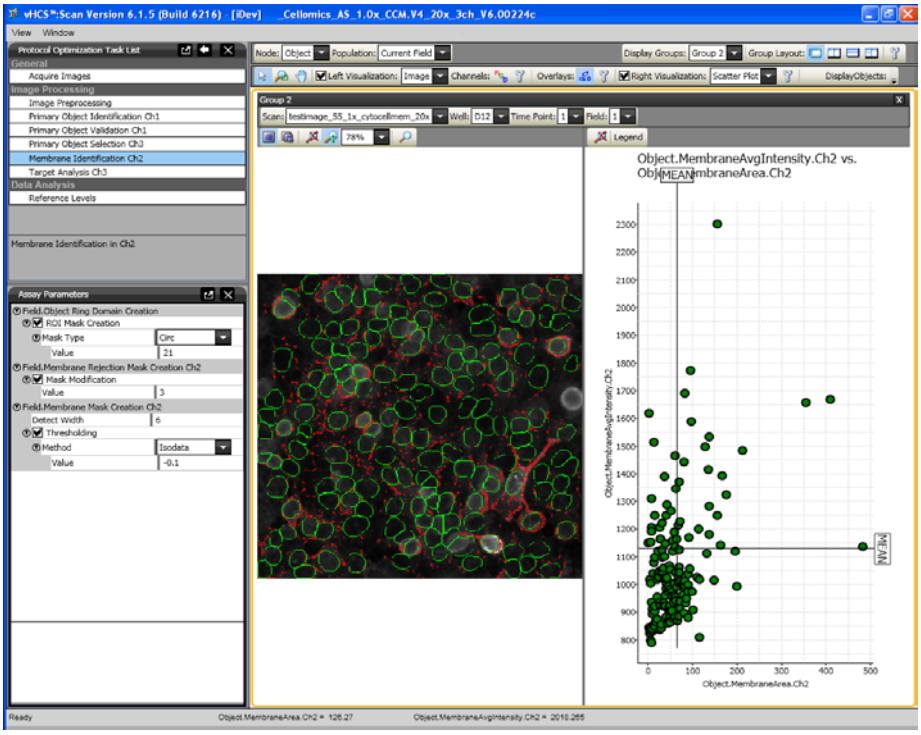

Figure 16. *Protocol Optimization Task – Membrane Identification*

Target Analysis Ch3 through ChN

In this task, you can set the parameters for identifying additional targets (membrane) in Channels 3 through Channel N. You can set the Ring **distance** and **width** and also specify if the membrane mask created in Channel 2 is to be excluded.

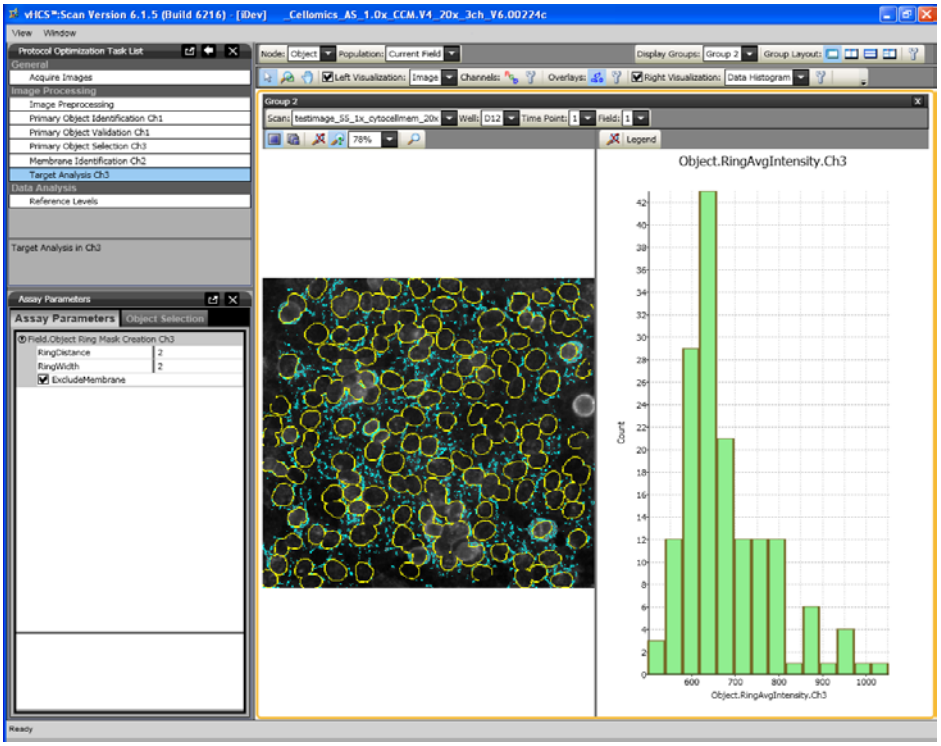

Figure 17. *Protocol Optimization Task – Target Analysis*

Reference Levels

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

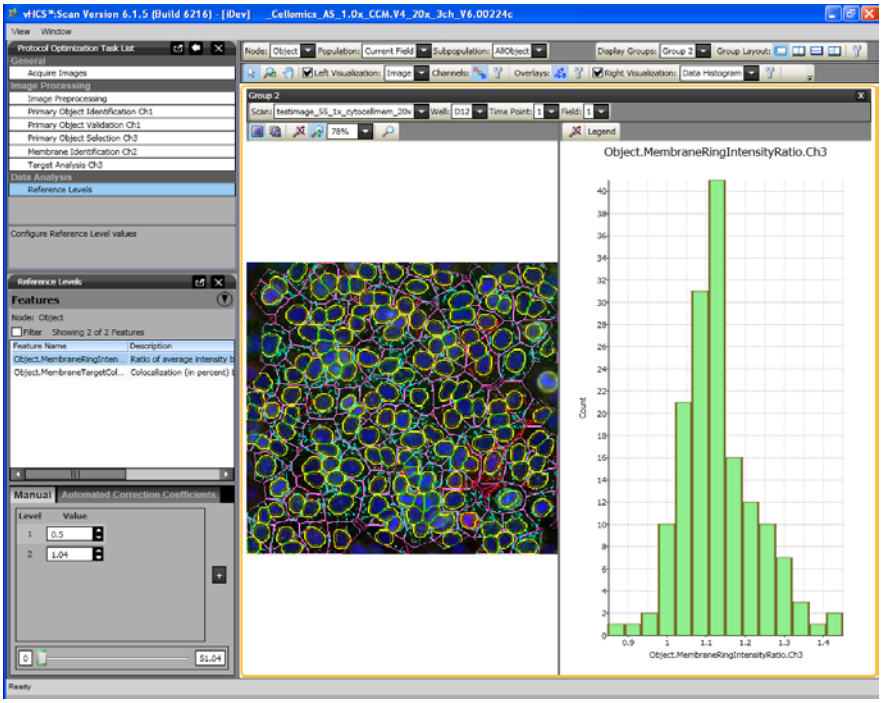

Figure 18. *Protocol Optimization Task – Reference Levels*

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