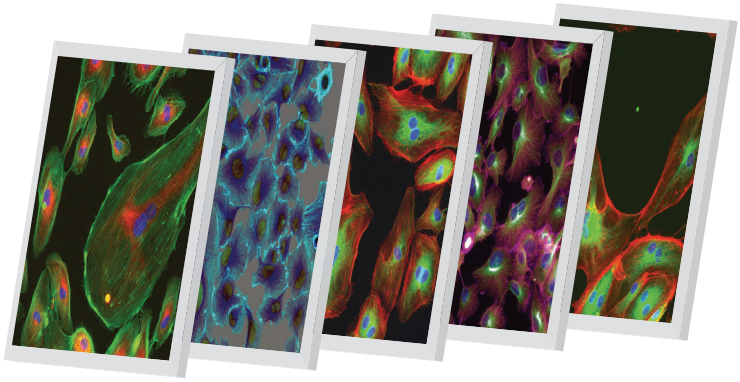
Thermo Scientific Cellomics® Cell Spreading V4

BioApplication Guide



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Cellomics® Cell Spreading BioApplication Guide

V4 Version

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Table of Contents

Chapter

1

Overview of the Cell Spreading BioApplication

An HCS assay combines biological reagents, automated imaging instrumentation, and BioApplication software to automatically extract and quantify the information from acquired cellular images. This guide provides a brief description for performing one such HCS assay with the Cell Spreading BioApplication. A separate protocol detailing the preparation of sample microplates for performing a High Content Screen for Cell Spreading is provided in the Cellomics® Cell Spreading HCS Reagent Kit. This guide contains the following chapters:

Chapter 1 provides an overview of the parts of the assay, such as the cell biological situations where it can be applied.

Chapter 2 describes the image analysis algorithm and provides 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 Cell Spreading BioApplication described in this guide is designed to run on the following platforms:

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

Cancer cells disrupt normal function of local or distant tissues, which leads to organ failure and death. However, cancer can be controlled by inhibiting the growth of tumors and by limiting the dispersion (metastasis) of cells. Tumor cell aggressiveness, metastatic potential, and even tumor regression may be determined in large part by adhesiveness and motility, which are reflected by changes in cell morphology (Klemke et al. 1998; Runger et al. 1994; Taniguchi et al. 1989). Therefore, developing therapeutic interventions that target morphological changes in cells is an important goal. Bone metastasis is a critical clinical problem in patients with breast, prostate, and other cancers. These painful metastases can be inhibited at several steps because formation of these lesions is tissue-specific. Cancers of the breast and prostate selectively metastasize to bone by specific adhesion molecules (integrins, selectins, extracellular matrix proteins, and proteoglycans) in target vascular beds and tissues (Orr et al. 1995; Kostenuik et al. 1996). An outline of this process is as follows:

1. De-adhesion from the primary tumor
2. Adhesion to select vascular beds
3. Attachment and spreading on bone extracellular matrix
4. Stimulation by growth factors and chemotactic agents in bone
5. Tumor-induced morphological changes in bone matrix and osteoclasts

Adhesion and spreading potential control the release of breast cancer cell metastasis. Low integrin expression on breast carcinoma cells promotes detachment of cells from the stromal layers and allows cells to translocate to distant sites (Jones et al. 1997). Promoting adhesion through specific $\alpha_6\beta_4$ integrins helps retain these cells at the primary tumor. Specific signaling cascades are induced by the binding of $\alpha_2\beta_1$ integrins to collagen, which control spreading and movement of breast cancer cells (Coppolino and Dedhar 1999). Preventing adhesion and spreading on collagen may help prevent the metastasis of the primary tumor.

Metastasis depends on attachment to selective substrates, morphological changes that are necessary to remain adherent, and eventually, movement and invasion into the target tissue. Measuring morphological features of cancer cells when they attach and spread provides a functional assay of various regulatory and structural molecules. The efficacy of drugs that target the function or expression of these molecules can be measured in single cells by the rate of attachment and spreading on a tissue-specific substrate.

BioApplication Overview

The algorithm measures the area and perimeter of single cells or colonies of cells during spreading. It is a two-channel assay, where a channel is defined by a unique fluorophore or exposure condition. In this case, Channel 1 identifies cell nuclei and Channel 2 is used to identify the whole cell. Objects in the Channel 2 are identified as monodisperse cells if they contain a single nucleus or as a colony if they contain multiple nuclei. Contiguous cells in a colony are considered a single object, so you can set an upper limit on the number of cells per colony. Although monodisperse cells are best, validation testing indicated that the area of spreading cells is not very different between single cells and colonies of about three cells. However, larger clumps spread more slowly. In addition, if the user is interested in the perimeter of cells, measuring only single cells in the field is important to produce meaningful results (Figure 1).

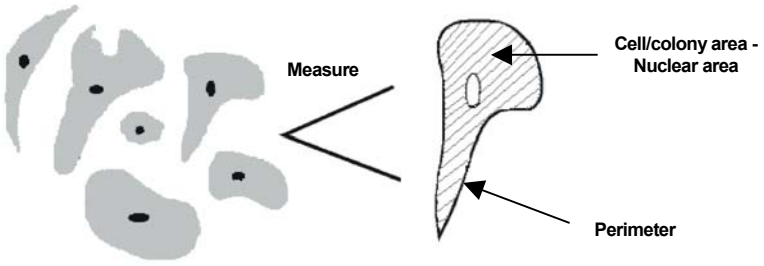

Figure 1. *Cells can be distinguished by measuring cytoplasm area and perimeter as they attach and spread on substrates.*

Cancer cells have a wide variety of morphologies in culture. The normal shape of cells is determined by many variables, not just the kinetics of attachment to the substrate. Therefore, it is important to use standardized conditions for evaluating the effect of drugs on cell attachment and spreading on substrates.

Measuring cell spreading within a few hours of plating is one approach to reduce biological variability. Cells in suspension initially attach and spread at rates that depend more on adhesion molecule binding and signaling to the cytoskeleton and less on movement and cell-cell adhesion. Immediately after plating, cells have a rounded shape, which will increase in diameter as cells attach and spread (Figure 2). This pattern is similar for all cell lines during the initial stages. In addition, factors that influence the attachment of cells will affect the kinetics of this process more than the steady state values. Therefore, the design of this assay is optimized to produce results predictive of cell-substrate interaction and to work for a broad range of cells.

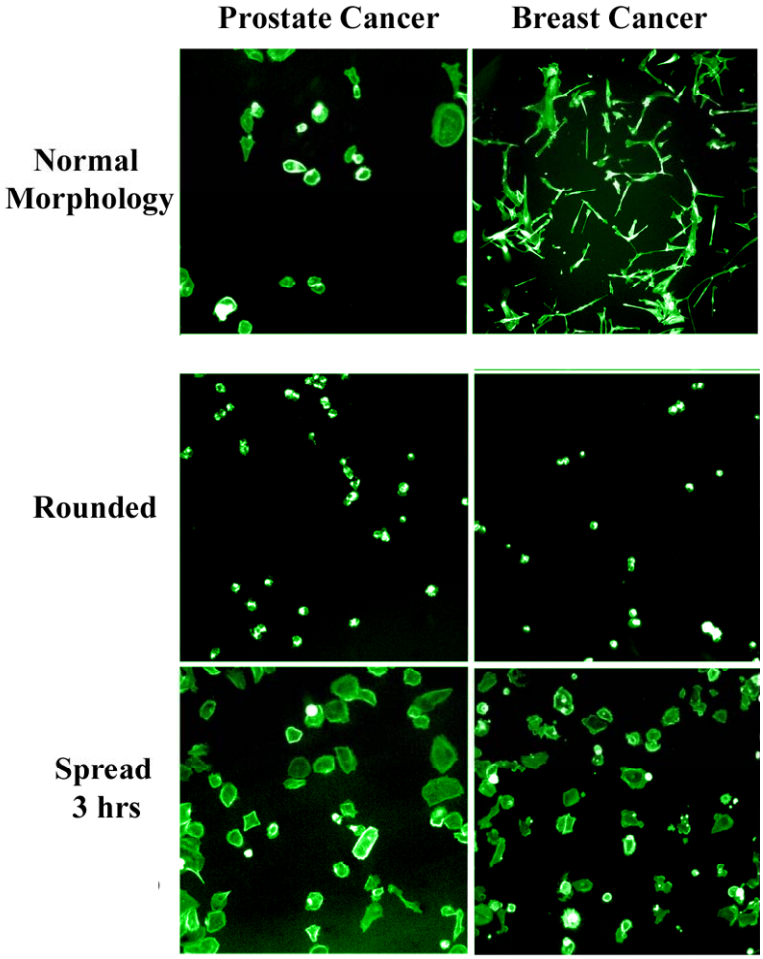

Figure 2. *Progression of cell spreading in culture. Cell spreading depends on multiple variables including cell-cell contacts, substrate adhesion, and unique internal structure. Cells progress through standard morphological changes, including initial attachment of round cells, eventually leading to a fully spread morphology.*

For comparison, most assays apply soluble ligands to immobilized cells, while cell spreading applies cells in solution to immobilized ligands. Collagen I is the ligand used in this assay, but various cell surface receptors and ligands could be assayed if the ligand resulted in increased cell spreading. The number of cells retained after a brief incubation and wash is commonly used as a measure of the avidity of cell-ligand binding.

In contrast, the Cell Spreading BioApplication selectively measures the whole-cell adhesion to substrates. It is a functional assay, not just a ligand binding assay, and so measures the efficacy of compounds on cells rather than the potency of drug binding. From a screening perspective,

the Cell Spreading BioApplication will enable elimination of compounds that bind to target receptors with little effect on cell function. Functional cell assays are more likely to predict drug activity in animal and human trials.

BioApplication Measurements

The Cell Spreading 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.

Measurement	Cell Region Measured	Cell Features	Well Features
Cell & Well Features Reported for Channel 1:			
Morphology	Nucleus within Cell Colony	Average Area Average Shape P2A Average Shape LWR	none
Intensity		Total Intensity Average Intensity	
Cell & Well Features Reported for Channel 2:			
General	Cell Colony	Cell # Top Left Width Height X Centroid Y Centroid	Valid Cell Colony Count Selected Cell Colony Count %Selected Cell Colonies Valid Field Count Selected Cell Colony Count Per Valid Field
	Nucleus within Cell Colony	Count	none
Morphology	Cell Colony	Area Shape P2A Shape LWR Area Per Nucleus	none
		Perimeter, Status	Mean, SD, SE, CV, %HIGH
	Nucleus within Cell Colony	Area Difference Per Nucleus, Status	Mean, SD, SE, CV, %HIGH
		Area Ratio	Mean, SD, SE, CV
Intensity	Cell Colony	Total Intensity Per Nucleus	none
Cell & Well Features Reported for Channels 3-6:			
Intensity	Modified Cell Colony Mask	Total Intensity Average Intensity	none

Table 1. *Cell Spreading BioApplication Cell and Well Feature Measurements*

Example Biology

The assay used to demonstrate this BioApplication involves plating freshly passaged cells into collagen-coated microplates and allowing them to spread for a few hours as described in the Cellomics® Cell Spreading HCS Reagent Kit. PC-3 cells (prostate cancer) are fixed, permeabilized, and then fluorescently labeled for nuclei and actin cytoskeleton. Note that if you would like to track live cells over time, the Cellomics® ArrayScan V^{TI} HCS Reader and the Cellomics® Discovery Toolbox (v1.6) offer a Live Module that can do this. Please see the appropriate user's manuals for more information.

The assay plate is then scanned using the Cell Spreading BioApplication to measure cell spreading. The extent of cell spreading can be assayed at fixed-time intervals after attachment by measuring multiple morphological features cell-by-cell and comparing these values to controls. Drugs that inhibit cell spreading are likely to limit the number of cells that develop a fully spread morphology (Figure 3 and Figure 4). The Cell Spreading reagent kit combines fluorescent dyes, buffers, control compound, and validated Assay Protocols for sample preparation and assays. After fixation, plates are stable for extended periods. Protein coated plates work best for providing a standardized surface for measuring cell spreading. Custom coated plates or Falcon®, Inc. BioCoat® 96 Well Assay Plates coated with Collagen I can be used. Collagen I works with a variety of cell types to promote uniform spreading of cells.

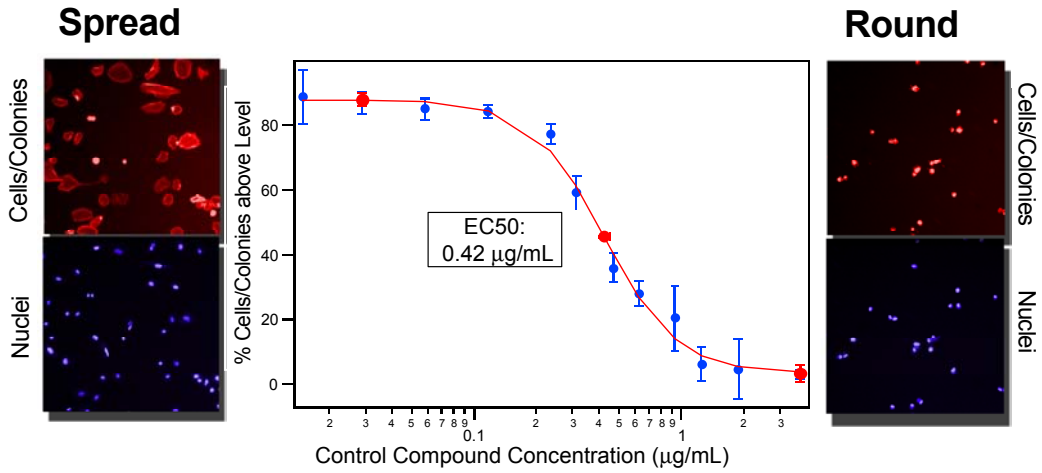

Figure 3. *Dose-dependent inhibition of cancer cell spreading. PC-3 cells spread on a defined extracellular matrix for 3 hr and then were fixed and labeled. The percentage of responder cells/colonies that spread beyond a specified level is plotted as a function of increasing concentration of a control compound. While cells normally spread to a size much larger than the nucleus in this time interval, the control compound maintains cells in a tight rounded morphology, not much larger than the nucleus, without blocking adhesion to the substrate. The concentration of compound effecting half-maximal inhibition is shown.*

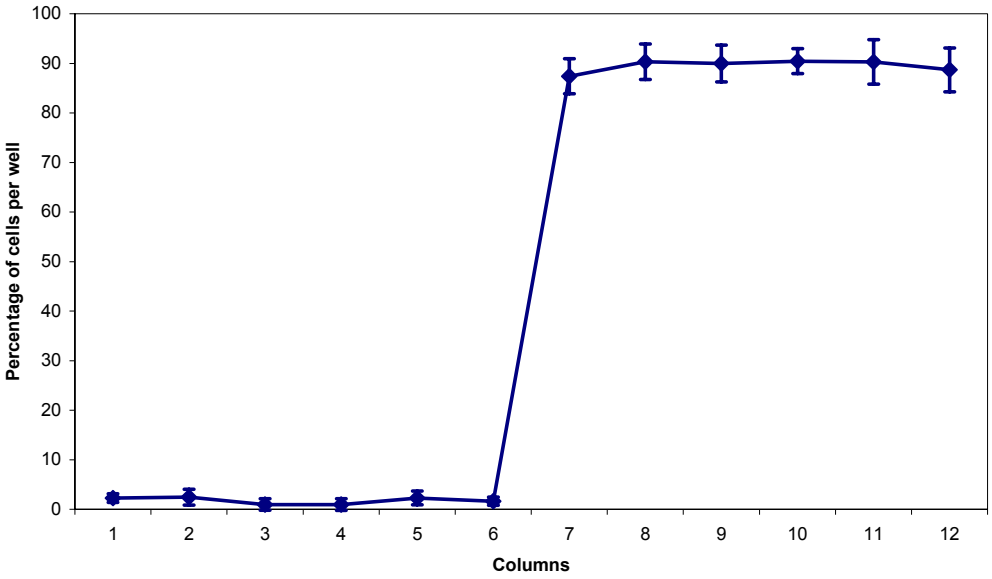

Figure 4. *Prostate cancer cells spread for 3 hr in the presence (Columns 1-6) or absence (Columns 7-12) of a compound that blocks spreading, but not attachment of cells. The number of cells/colonies per well that spread to an extent greater than a pre-defined level was measured. Each data point represents an average of 8 wells, 100 cells/well with error bars indicating standard deviations. These results represent a Z' window = 0.83 and a signal/noise ratio = 22.2.*

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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.

This chapter describes in more detail the assay output features as well as the adjustable input parameters that control the analysis.

Overview

The Cell Spreading assay uses an algorithm and reagents that have 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. Parameters are adjustable to allow customization of the algorithm to your own samples and conditions.

Generally, after images are acquired and Background Correction has been applied, primary objects are identified in Channel 2. Assay Parameters such as **CellColonySeparationCh2** or **RejectBorderObjects** may be applied. Nuclei are then identified in Channel 1 (applying smoothing and segmentation if necessary). Cells/colonies are then validated based on the presence of valid nuclei. Cell Features and Well Features are then calculated.

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. In Channel 2, the cytoplasmic area is identified, and pixels with intensities above this threshold are used to help estimate target counts. Depending on the properties of the objects being identified in Channels 1 and 2, the proper setting of intensity thresholds for the channels is necessary to ensure proper quantitative analysis.

There are four 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	✓	✓	
Triang	-0.99 – 9.99		✓	
Fixed	0 - 32767	✓	✓	

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

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**.

Of the four intensity threshold method options, **None** means that no intensity threshold is applied. This option is not available in Channel 1 or Channel 2 because in these channels an intensity threshold is required to define the pixels making up the cell body and nucleus. 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 other threshold methods (**Isodata** and **Triang**) are known as histogram-derived thresholds in that the threshold is chosen from the histogram of pixel intensities in the image (i.e., the image's brightness histogram). The schematics in Figure 5 demonstrate how these histogram-derived threshold values are calculated.

The values entered for the Object Identification in the application for the two histogram-derived threshold methods are offsets 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 two histogram-derived methods are 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.

The **Triangle Threshold** method is more robust for the situation of blank fields that may contain rare bright objects; this is because the peak is of the background intensity, and the "triangle"-derived offset can be set to always be above the background, yet low enough to pick any bright objects. 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, either a **Fixed Threshold** or the **Triangle 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
Triang	Adjusts the object identification threshold relative to the Triangle value. The threshold *T* which gives the maximum distance *d* 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 Cell Spreading BioApplication*

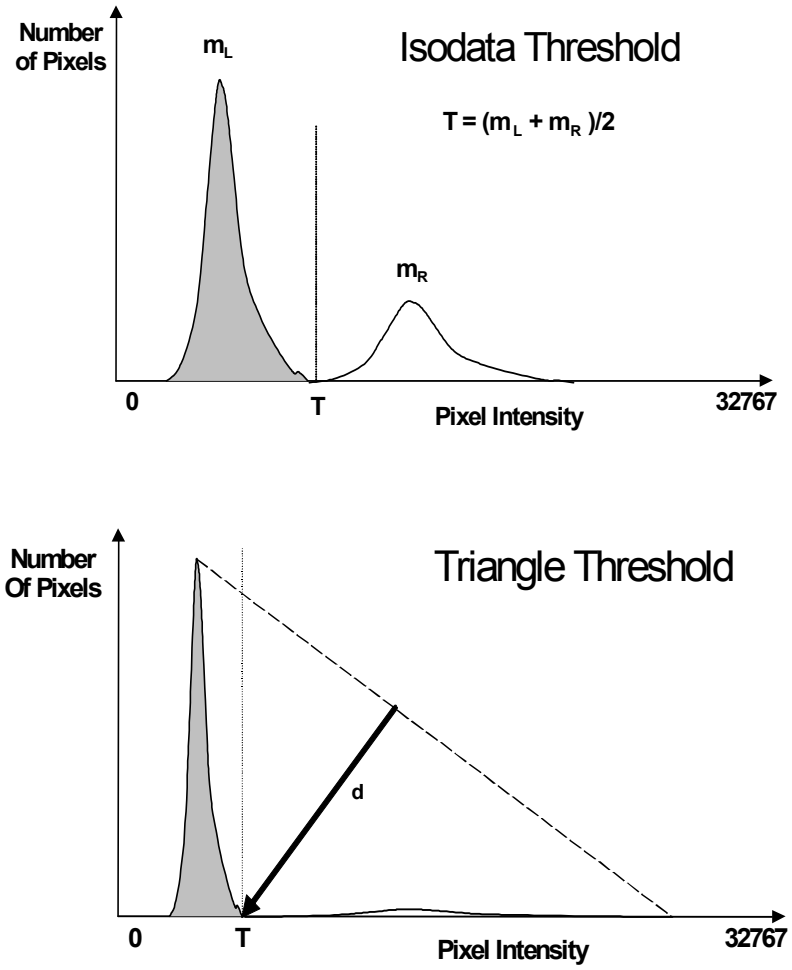

Figure 5. Histogram-derived Intensity threshold methods: Background peak is shown in gray and object peak is shown in white. Methods shown represent Isodata (top) and Triangle (bottom) Thresholding.

Description of Assay Parameters and Settings

The algorithm has Assay Parameters that control the analysis of images and data. The values of these parameters have been determined from demonstration biologies and are supplied as defaults in the standard Assay Protocol. You can adjust these Assay Parameters to allow customization of the algorithm to your own biology and conditions.

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**
- **Reject Border Objects**
- **Object Type**
- **Background Correction**
- **Object Smoothing**

Reference Wells Control

The two General Assay Parameters controlling the use of Reference Wells are: **UseReferenceWells** and **MinRefAvgCellColonyCountPerField**. 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 **MinRefAvgCellColonyCountPerField** 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 gives 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 Assay Parameters for Reference Well processing that are specific for particular features and channels, as described previously. 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.

Reject Border Objects

When running the BioApplication, you have the option of not including and analyzing objects that touch the border of your image field. This is controlled by the **RejectBorderObjects** Assay Parameter. If the value is **1**, objects touching the border are not analyzed. 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. This Assay Parameter is applied to both Channel 1 and Channel 2.

Object Type

NucTypeCh1, CellColonyTypeCh2

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* ***CellColonyTypeCh2****.*

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

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

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

Background Correction

BackgroundCorrectionCh*N*

Prior to image analysis, the non-cellular background can be computed and subtracted from the image separately in each channel, if desired, as shown in Figure 6.

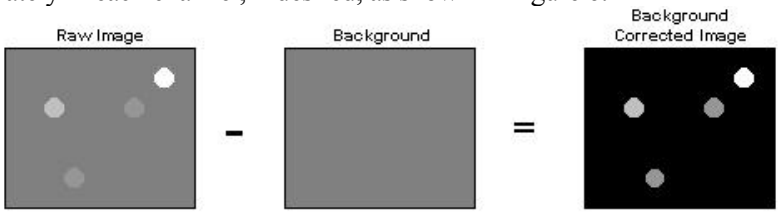

Figure 6*. Image depicting Background Correction that can be used for each channel.*

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

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

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

The **NucSmoothFactorCh1** Assay Parameter enables control over the degree of image smoothing, or blurring, before the identification of objects. This 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 and analysis is performed on the raw, uncorrected images. 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 this 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 7 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).

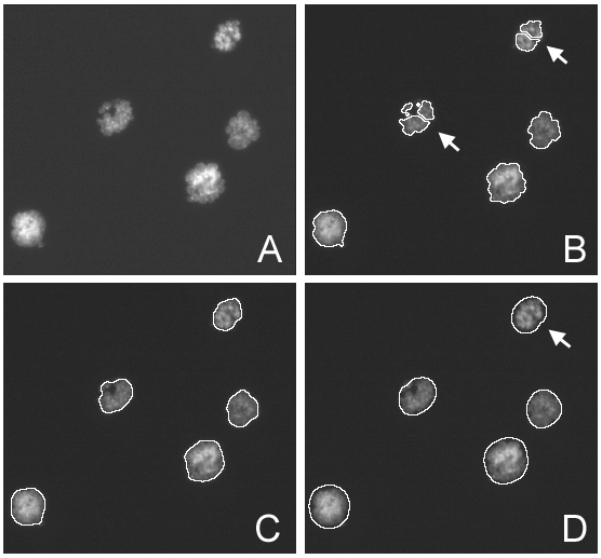

Figure 7. NucSmoothFactorCh1*: Images show the effect of different degrees of smoothing on the same field of apoptotic cell nuclei. A: Raw Image; B:* ***NucSmoothFactorCh1*** *= 0; C:* ***NucSmoothFactorCh1*** *= 3; D:* ***NucSmoothFactorCh1*** *= 8.*

Channel-Specific Assay Parameters

Channel1: 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 Cell Spreading BioApplication: Geometric (shape and size) and Intensity (intensity peaks). These methods are illustrated in Figure 8.

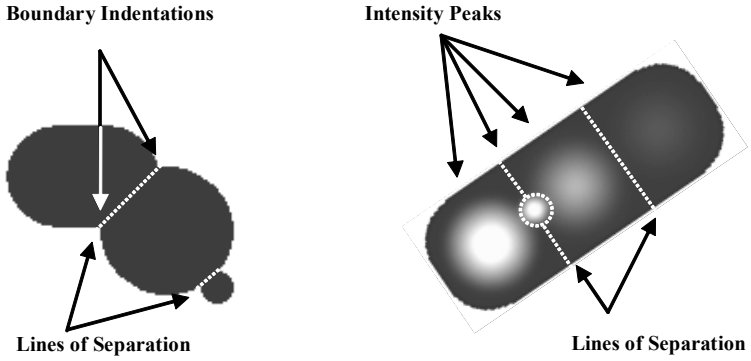

Figure 8: *Object Segmentation Options. The image on the left illustrates the Geometric Method while the image on the right illustrates the Intensity Method.*

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 9A) produces object separation shown in Figure 9B. Setting **NucSegmentationCh1** to R3 results in segmentation shown in Figure 9C. Setting **NucSegmentationCh1** to R2 (the largest radius of all touching objects) produces no segmentation.

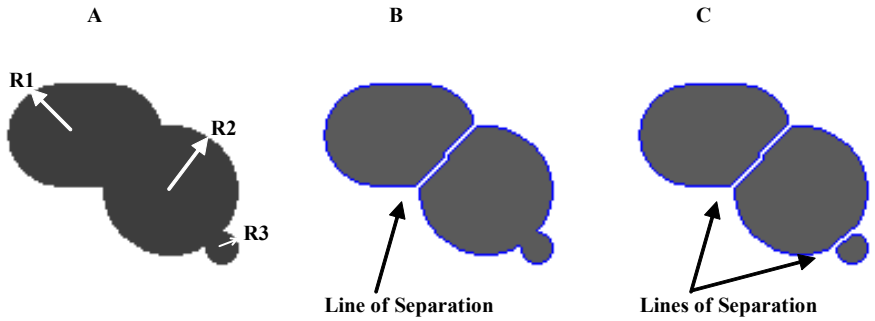

Figure 9: *Object segmentation: Geometric Method. A) Radii of touching objects: R1 = 24, R2 = 30, R3 = 7; B) Result of segmentation using* ***NucSegmentationCh1*** *= R1; C) Result of segmentation using* ***NucSegmentationCh1*** *= R3.*

Intensity Method Figure 10 shows the intensity profile along the cordial line of an object with four intensity peaks from Figure 8. Relative peak intensity heights measured from the top of a peak to the nearest valley for each of the object are:1500 (#1), 1250 (#2), 500 (#3), and 200 (#4).

In general terms, these peak intensity heights are nothing but measures of local contrast of an image. The degree of object segmentation can be controlled by setting the **NucSegmentationCh1** parameter value equal to the lowest relative height of intensity peaks of objects that need to be separated. The lower the value, the more objects will be segmented and vise versa. In case of over-segmentation, setting the **NucSmoothFactorCh1** greater than 0 should be used to alleviate the problem.

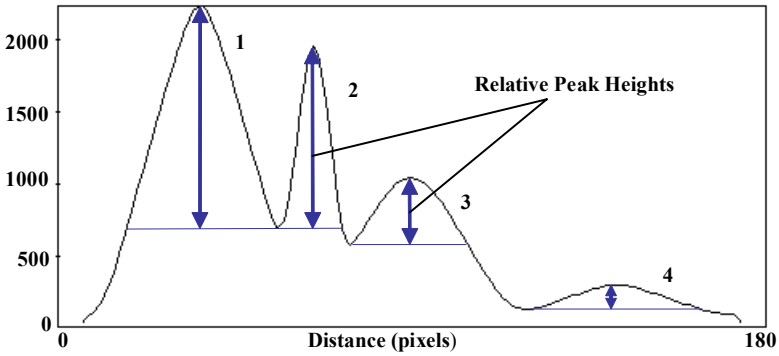

Figure 10: *Intensity profile of an object with four intensity peaks. Peak #1 height = 1500, peak #2 height = 1250, peak #3 height = 500, and peak #4 height = 200.*

Only two peaks in Figure 10, #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 11A. 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 11B and 11C.

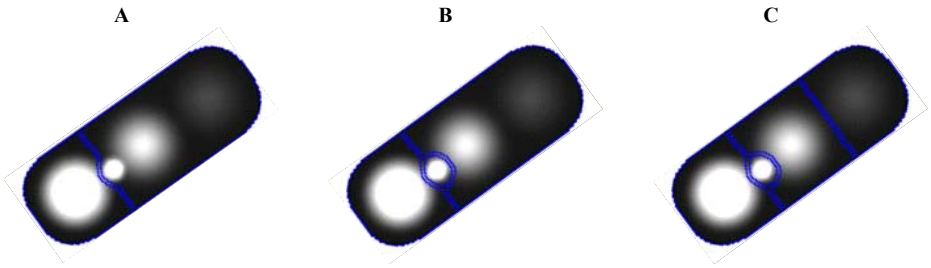

Figure 11: *Object segmentation: Intensity Method. A) Result of segmentation using parameter* ***NucSegmentationCh1*** *= -1000; B) Result of segmentation using parameter* ***NucSegmentationCh1*** *= -400; C) Result of segmentation using parameter* ***NucSegmentationCh1*** *= -100.*

Channel 2: Object Separation

CellColonySeparationCh2

For some applications, it may be valuable to distinguish individual cells within loose aggregates. The **CellColonySeparationCh2** Assay Parameter value specifies the number of erosion steps needed to separate cells that are loosely aggregated. For the assay described in the Cellomics Cell Spreading Reagent Kit, it is recommended that this be set at **0**.

Channels 3-6: Mask Modifier

CellColonyMaskModifierCh*N*

The size of the cell colony mask can be modified in Channels 3-6 via the **CellColonyMaskModifierCh*N***, where Ch*N* is the relevant dependent channel (i.e., Channels 3-6). Its value specifies the number of pixels by which the cell colony mask created in Channel 2 is enlarged (positive value) or reduced (negative value).

In Channels 3-6, the modified cell colony can be used to make intensity measurements for gating. The effect of this parameter is illustrated in Figure 12, where the Assay Parameter value was positive and the cell colony area has been expanded in order to make the intensity measurement.

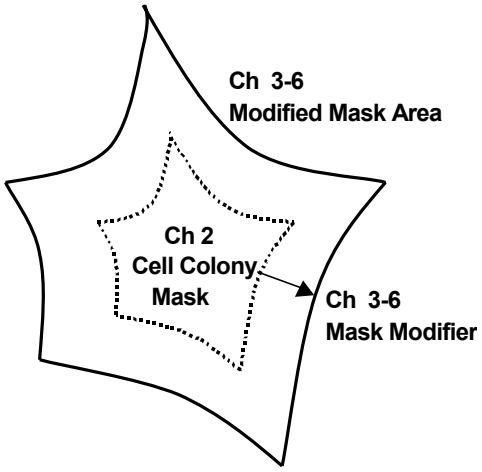

Figure 12. *Adjustment of area using* ***CellColonyMaskModifierChN*** *Assay Parameter. *Note that "ChN" refers to Channels 3-6.*

Basic Assay Parameters

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

The term 'CellColony' in the names of Assay Parameters, Cell Features, Well Features and Plate Features of this BioApplication refers to measurements made on individual cells and/or colonies.

Parameter	Units	Description
UseReferenceWells	Binary	Use reference wells to calculate high and low response levels: 0 = No, 1 = Yes
RejectBorderObjects	Binary	Reject objects that touch image edges: 0 = No, 1 = Yes
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 identification 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
CellColonyTypeCh2	Binary	Type of cells or colonies to be identified in Ch2: 0 = Bright objects on dark background, 1 = Dark objects on bright background
CellColonySeparationCh2	Pixels	Number of pixels to shrink object masks to separate slightly touching cells or colonies in Ch2
CellColonyMaxNucCountCh2	Number	Maximum number of Ch1 nuclei allowed in a valid Ch2 cell or colony
CellColonyMaskModifierChN	Pixels	Number of pixels to modify Ch2 object (cell or colony) mask in ChN: Negative value = Shrink mask, 0 = Do not modify mask, Positive value = Expand mask

Table 7. *Basic Assay Parameters available with the Cell Spreading BioApplication. *Note that "ChN" refers to Channels 1-6 for* ***Background Correction*** *and Channels 3-6 for* ***CellColonyMaskModifier****.*

Object Selection Parameters

Object Selection Parameters available for Channels 1-6 are used to define the cell population to be analyzed. Of these, the parameters in Channel 1 and Channel 2 specify shape criteria for the cells/colonies and associated cell nuclei. The Object Selection Parameters available in the Cell Spreading BioApplication are listed in the Tables 8-10.

Channel 1

Parameter	Units	Description
NucAreaCh1	Pixels or μm^2	Area (in pixels or micrometers) of Ch1 nucleus
NucShapeP2ACh1	Number	Shape measure based on ratio of perimeter squared to 4π*area of Ch1 nucleus (**NucShapeP2ACh1** = 1 for circular nucleus)
NucShapeLWRCh1	Number	Shape measure based on ratio of length to width of 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

Table 8. *Object Selection Parameters Available in Channel 1 for the Cell Spreading BioApplication*

Channel 2

Parameter	Units	Description
CellColonyAreaCh2	Pixels or μm^2	Area (in pixels or micrometers) of Ch2 cell or colony
CellColonyShapeP2ACh2	Number	Shape measure based on ratio of perimeter squared to 4π*area of Ch2 cell or colony (**CellColonyShapeP2ACh2** = 1 for circular cell or colony)
CellColonyShapeLWRCh2	Number	Shape measure based on ratio of length to width of object-aligned bounding box of Ch2 cell or colony
CellColonyTotalIntenCh2	Intensity	Total intensity of all pixels within Ch2 cell or colony
CellColonyAvgIntenCh2	Intensity	Average intensity of all pixels within Ch2 cell or colony

Table 9. *Object Selection Parameters Available in Channel 2 for the Cell Spreading BioApplication*

Channel N (3-6)

Parameter	Units	Description
CellColonyTotalIntenChN	Intensity	Total intensity in ChN of all pixels within modified Ch2 cell or colony mask
CellColonyAvgIntenChN	Intensity	Average intensity in ChN of all pixels within modified Ch2 cell or colony mask

Table 10. *Object Selection Parameters Available in Channels 3-6 for the Cell Spreading BioApplication. *Note that "ChN" refers to Channels 3-6.*

Gating

The Cell Spreading BioApplication supports gating on a cell population. This feature enables you to restrict analysis to a specific population of cells based on fluorescence intensity. This is done by specifying channel-specific intensity ranges of the cells that you would like the algorithm to analyze by adjusting the Object Selection Parameters. Therefore, in addition to selecting cells/colonies for analysis in Channel 1 and Channel 2, you can also select or reject cells/colonies 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).

Specifying Intensity Ranges for Gating

When working in Create Protocol View of the ArrayScan Classic software or the Protocol optimization task list of the iDev software, you can specify intensity ranges in the Object Selection Parameter section by entering upper and lower limits for two intensity parameters, **AvgIntenCh*N*** and **TotalIntenCh*N***, for Channels 3-6. **TotalIntenCh*N*** is a summation of all intensities within the cell/colony. **AvgIntenCh*N*** is **TotalIntenCh*N*** divided by the cell colony area. In the ArrayScan Classic software, these parameters can also be adjusted interactively in Protocol Interactive View.

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 **CellColonyMaskModifierCh*N*** Assay Parameter. Adjusting this Assay Parameter will dilate or contract the mask that identifies each cell/colony (from Channel 2) but will not overlap with other masks from adjacent objects. This setting only impacts the region used to determine whether the cell/colony should be gated in or out, as measured by the **TotalIntenCh*N*** or **AvgIntenCh*N*** Cell Features. Please see the Mask Modifier section earlier in this chapter for more information (Figure 12).

Image Overlays

Image Display Option Settings are used to configure overlay displays used to indicate cellular measurement regions. The colors of these overlays can be changed by choosing the color block beside the overlay name in the Create Protocol or Protocol Interactive View of the ArrayScan Classic software or the Protocol optimization task list of the iDev software. Please refer to Table 11 for additional information. These settings are available when running in Basic or Advanced Mode.

Parameter	Description
Include This Channel In Composite	Determines if image for this channel is included in the composite image.
Composite Color	Determines what color will be assigned to this channel in the composite image.
SelectedNucleus	Indicates nuclei of cells/colonies that have been selected for analysis based on Assay Parameters and Object Selection Criteria in Channels 1 and 2.
ValidNucleus	Indicates nuclei that satisfied selection criteria for Channel1 specified via Object Selection Parameters and Assay Parameters.
RejectedNucleus	Indicates nuclei that did not meet Channel 1 selection criteria.
SelectedCellColony	Indicates cells/colonies that have been selected for analysis based on Assay Parameters and Channel2 Object Selection Parameters.
RejectedCellColony	Indicates cells/colonies that do not meet all selection criteria.

Table 11. *Cell Spreading BioApplication Image Display Option Settings*

Assay Parameters for Population Characterization

Overview of Population Characterization

The Cell Spreading BioApplication provides the ability to characterize cells based on their response compared to a control population. For selected Cell Features, you can specify a upper level value for an input parameter known as ***Feature*ChN LevelHigh** (where ***Feature*** represents the Cell Feature of interest) that best distinguishes treated cells from untreated cells. In all cases, cells having feature values greater than the level specified are defined as 'responder cells'. The corresponding assay output is the percentage of responder cells in the population analyzed. The two features that are characterized are **CellColonyPerimCh2** and **CellColonyNucAreaDiffPerNucCh2**, where

$$\textbf{CellColonyNucAreaDiffPerNucCh2} = \frac{\text{(Cell/colony Area – Total Nuclear Area)}}{\text{number of nuclei}}$$

Figure 13 illustrates this concept by showing the distribution of a normal physiological population of cells and the use of a level to identify responder cells.

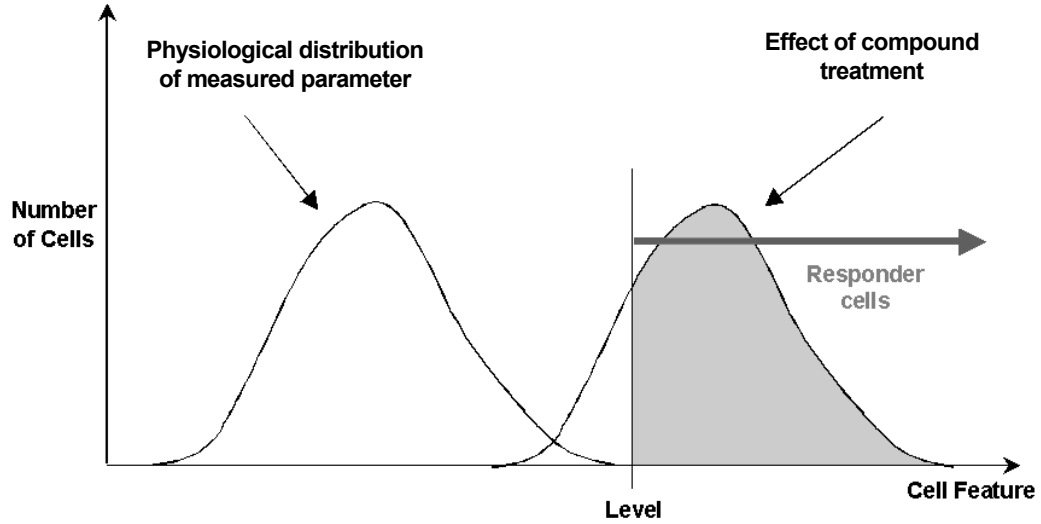

Figure 13. *Cellular response level distinguishing baseline from high subpopulation.*

Setting Cellular Response Levels

There are two ways of setting levels for population characterization. The first is manually entering values for the ***Feature*ChN LevelHigh** Assay Parameters in the Protocol Create or Protocol Interactive Views (Advanced Mode). This requires prior knowledge of typical feature values. The BioApplication then calculates the percentage of cells above the specified ***Feature*ChN LevelHigh** values.

The second is automatically calculating the ***Feature*ChN LevelHigh** Assay Parameters through use of Reference Wells. You designate particular wells on the sample plate as Reference Wells. Reference Wells typically contain an untreated cell population that displays the normal physiological distribution for the feature being measured. These wells are analyzed first and the population distribution for each Cell Feature that can be used for population characterization is determined. Levels are then automatically computed by adding to the mean of the distribution its standard deviation multiplied by a correction coefficient (_CC) that you

specify. The entire plate is then scanned with the calculated levels. For example, if you want to know the percentage of cells that, with compound treatment, have a response beyond the 95th percentile of the response from a control untreated population of cells, then the coefficient by which to multiply the standard deviation would be two, assuming a normal distribution. 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 when doing the experiment on different days such as different illumination conditions, extent of fluorescent labeling, etc.

Reference Wells Processing Sequence

By setting the **UseReferenceWells** Assay Parameter to **1**, the Reference Wells processing is engaged. The specified fields within the wells are acquired/analyzed, and 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 **MinRefAvgCellColonyCountPerField** 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 Cell Spreading 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**.

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

Identifying Reference Wells and Control Parameters

Reference Wells are specified in the Reference Wells Configuration Window (choose **Change Reference Wells** from the **Tools** menu). Select the wells to be set as 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.

NOTE

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

In addition to the two general Assay Parameters used to control Reference Wells that have already been described (**UseReferenceWells** and **MinRefAvgCellColonyCountPerField**), there are two types of Assay Parameters that are used for cell population characterization that are specific to the Cell Feature of interest. These Assay Parameter names are of the type:

- ***Feature*ChN LevelHigh**
- ***Feature*ChN LevelHigh_CC**

where ***Feature*** refers to the individual Cell Feature (e.g. **CellColonyPerimCh2**) and *N* refers to the specific channel. You can manually set a level value that best separates treated cells from untreated populations after reviewing raw data from previously analyzed control wells. Alternatively, the level can be automatically calculated from the distribution of feature values found in Reference Wells. You choose whether the range is set manually or automatically by using the **UseReferenceWells** Assay Parameter. The possible values and resulting actions associated with this parameter are:

- **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 (allowing the instrument to set levels automatically)

If the level value is to be set manually, you have to specify the parameter value for the Assay Parameters of the form ***Feature*ChN LevelHigh** for the Cell Feature of interest.
If the range is to be set automatically by the instrument using Reference Wells, then the level is set by the "_CC" parameters of the type ***Feature*ChN LevelHigh_CC**. In this case, the level is set by adding to the distribution's mean its standard deviation (SD) multiplied by the specified CC value:

$$\textit{Feature}\text{ChN LevelHigh} = \text{Mean} + (\textit{Feature}\text{ChN LevelHigh\_CC} \times \text{SD})$$

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

Advanced Assay Parameters

In Advanced Mode, all basic and advanced input 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 Assay Parameters designated as Basic Parameters. Generally, the Advanced Assay Parameters are related to definition and reporting of responder cells. They control the analysis of the data resulting from the image analysis (Table 12).

For each feature undergoing population characterization, there are two advanced Assay Parameters that control its levels: ***Feature*ChNLevelHigh** that sets upper threshold and the presence of the **_CC** suffix which designates those levels are set using Reference Wells. For example, the Assay Parameters controlling the cell colony's perimeter in Channel 2 are:

- **CellColonyPerimCh2LevelHigh**
- **CellColonyPerimCh2LevelHigh_CC**

In the listing of Advanced Parameters in the following table, instead of listing both level parameters for each feature, one entry for the feature will be listed giving the two different options, as shown in the following example for the Channel 2 cell colony perimeter:

- CellColonyPerimCh2Level***High, High_CC***

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

Parameter	Units	Description
MinRefAvgCellColonyCountPerField	Number	Minimum average number of cells or colonies 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)
CellColonyPerimCh2Level***High, High_CC***	Pixels or µm	Defines **CellColonyPerimCh2** population characterization thresholds
CellColonyNucAreaDiffPerNucCh2Level***High, High_CC***	Number	Defines **CellColonyNucAreaDiffPerNucCh2** population characterization thresholds

Table 12. *Advanced Assay Parameters available for the Cell Spreading BioApplication*

Description of Output Features

Output features are the biological measurements produced by the Cell Spreading BioApplication. Cell Features are measurements of individual cells/colonies. Well Features are population descriptors that are derived directly from individual Cell Features. Well Features and Cell Feature values can be viewed upon running the algorithm interactively on images acquired in Protocol Interactive View. After an assay plate has been scanned, all output features are readily accessible using View.

Cell Features

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

Cell Feature	Units	Description
Cell#	Number	Unique Ch2 cell or colony ID
Top	Pixels	Y coordinate (in pixels) of top-left corner of image-aligned bounding box of Ch2 cell or colony
Left	Pixels	X coordinate (in pixels) of top-left corner of image-aligned bounding box of Ch2 cell or colony
Width	Pixels	Width (in pixels) of image-aligned bounding box of Ch2 cell or colony
Height	Pixels	Height (in pixels) of image-aligned bounding box of Ch2 cell or colony
XCentroid	Pixels	X coordinate (in pixels) of center of Ch1 object
YCentroid	Pixels	Y coordinate (in pixels) of center of Ch1 object
CellColonyNucAvgAreaCh1	Pixels or μm^2	Average area (in pixels or micrometers) of all Ch1 nuclei belonging to Ch2 cell or colony
CellColonyNucAvgShapeP2ACh1	Number	Average shape P2A of all Ch1 nuclei belonging to Ch2 cell or colony
CellColonyNucAvgShapeLWRCh1	Number	Average shape LWR of all Ch1 nuclei belonging to Ch2 cell or colony
CellColonyNucTotalIntenCh1	Intensity	Total intensity of all pixels within all Ch1 nuclei belonging to Ch2 cell or colony
CellColonyNucAvgIntenCh1	Intensity	Average intensity of all pixels within all Ch1 nuclei belonging to Ch2 cell or colony
CellColonyNucCountCh2	Number	Number of all Ch1 nuclei belonging to Ch2 cell or colony
CellColonyAreaCh2	Pixels or μm^2	Area (in pixels or micrometers) of Ch2 cell or colony
CellColonyPerimCh2	Pixels or µm	Perimeter (in pixels or micrometers) of Ch2 cell or colony

Cell Feature	Units	Description
CellColonyPerimCh2Status	Number	**CellColonyPerimCh2** status: 0 = No response, 1 = High response, 2 = Low response
CellColonyShapeP2ACh2	Number	Shape measure based on ratio of perimeter squared to 4π*area of Ch2 cell or colony (**CellColonyShapeP2ACh2** = 1 for circular cell or colony)
CellColonyShapeLWRCh2	Number	Shape measure based on ratio of length to width for object-aligned bounding box of Ch2 cell or colony
CellColonyTotalIntenCh2	Intensity	Total intensity of all pixels within Ch2 cell or colony
CellColonyAvgIntenCh2	Intensity	Average intensity of all pixels within Ch2 cell or colony
CellColonyAreaPerNucCh2	Pixels or μm^2	Area (in pixels or micrometers) of Ch2 cell or colony per Ch1 nucleus in the colony
CellColonyTotalIntenPerNucCh2	Intensity	Total intensity of all pixels within Ch2 cell or colony per Ch1 nucleus in the colony
CellColonyNucAreaDiffPerNucCh2	Pixels or μm^2	Area (in pixels or micrometers) of Ch2 cell or colony cytoplasm per Ch1 nucleus in the colony
CellColonyNucAreaDiffPerNucCh2Status	Number	**CellColonyCytoAreaPerNucCh1Ch2** status: 0 = No response, 1 = High response, 2 = Low response
CellColonyNucAreaRatioCh2	Number	Ratio of Ch2 cell or colony cytoplasm area to total area of Ch1 nuclei in the colony
CellColonyTotalIntenChN	Intensity	Total intensity in ChN of all pixels within modified Ch2 object (colony or cell) mask
CellColonyAvgIntenChN	Intensity	Average intensity in ChN of all pixels within modified Ch2 (colony or cell) object mask

Table 13. *Cell Level Features available for the Cell Spreading BioApplication. *Note that "ChN" refers to Channels 3-6.*

Well Features

Many Well and Field Features are derived from the Cell Features. Such features are identified by a prefix, as listed below in Table 14, to the Cell Feature name. This pertains to **CellColonyPerimCh2** and **CellColonyNucAreaDiffPerNucCh2**, and **CellColonyNucAreaRatioCh2** (excluding %HIGH).

Feature Prefix	Field/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

Table 14. *General Well Features Available in the Cell Spreading BioApplication.*

Table 15 reflects the following additional Well Features found in the Scan Plate View in addition to the Well Detail window of the View application.

Well Feature	Description
ValidCellColonyCount	Number of valid cells or colonies identified in the well (Ch2 object selection parameters applied)
SelectedCellColonyCount	Number of valid cells or colonies selected for analysis in the well (Ch3-6 object selection parameters applied)
%SelectedCellColonies	Percentage of valid cells or colonies selected for analysis in the well
ValidFieldCount	Number of fields in which cells or colonies were selected for analysis in the well
SelectedCellColonyCountPerValidField	Average number of cells or colonies selected for analysis per valid field in the well

Table 15. *Well Level Features available for the Cell Spreading BioApplication*

Reference Well Features

The algorithm reports the following features in the Scan Plate Scan view of the software application as well as Plate Features in the View application (Table 16). These features are computed and reported only when **UseReferenceWells = 1** (enabled).

Feature	Description
RefAvgCellColonyCountPerField	Average number of cells or colonies per field in reference wells
RefCellColonyPerimCh2LevelHigh	High response level for **RefCellColonyPerimCh2**
RefCellColonyNucAreaDiffPerNucCh2LevelHigh	High response level for **CellColonyCytoAreaPerNucCh1Ch2**

Table 16. *Reference Features Available in the Cell Spreading BioApplication.*

Chapter

3

iDev Software Workflow

This chapter describes the tasks in the workflow for Protocol optimization of the Cell Spreading 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.

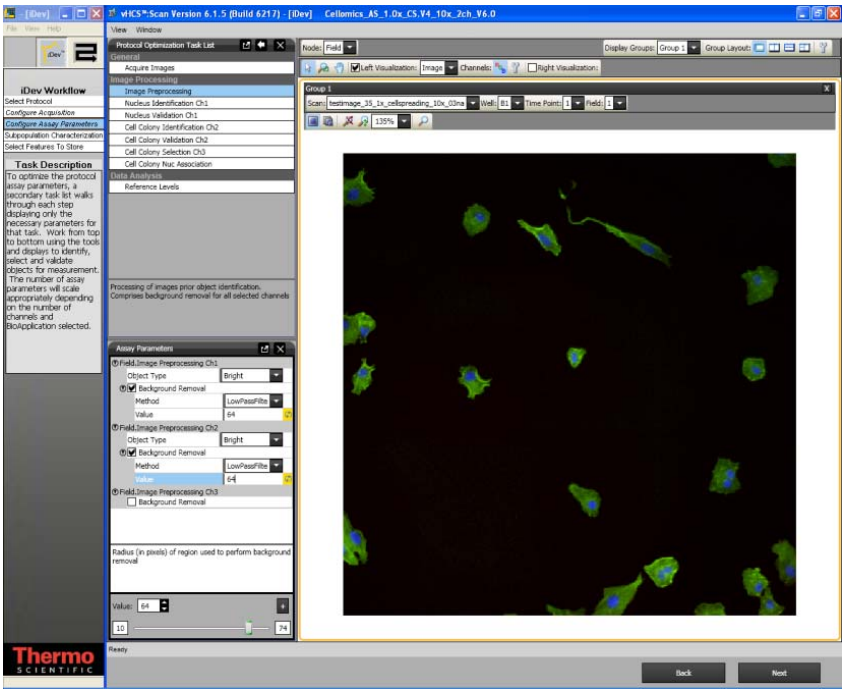

Figure 14. *Protocol Optimization Task – Image Preprocessing*

Nucleus Identification Ch1

Nucleus Identification is the identification of nuclei in the Channel 1 image. Assay Paramteters included in this task are image smoothing, nucleus identification, and segmentation. The task involves setting up methods and values for primary object identification, object smoothing, and object segmentation for Channel 1 objects.

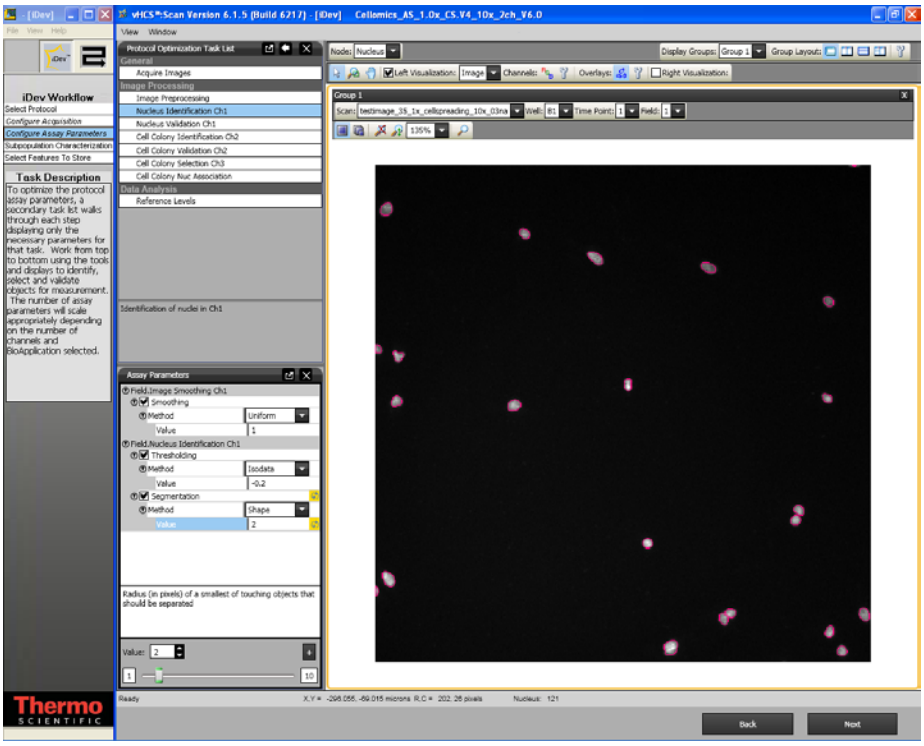

Figure 15. *Protocol Optimization Task – Nucleus Identification Ch1*

Nucleus Validation Ch1

Nucleus Validation is object selection based on area, shape, and intensity features calculated for the nuclei 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) nuclei in Channel 1 based on object selection 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.

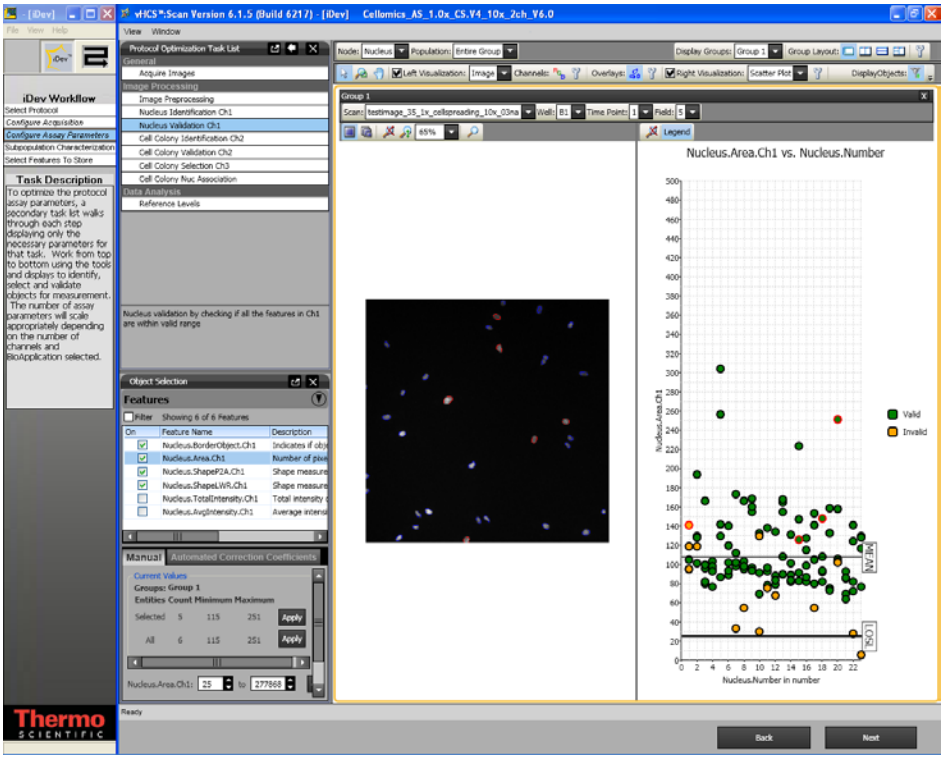

Figure 16. *Protocol Optimization Task – Nucleus Validation Ch1*

Cell Colony Identification Ch2

Cell Colony Identification is the identification of cell colonies in Channel 2. You can set methods and values for thresholding and also values for separating colonies touching one other.

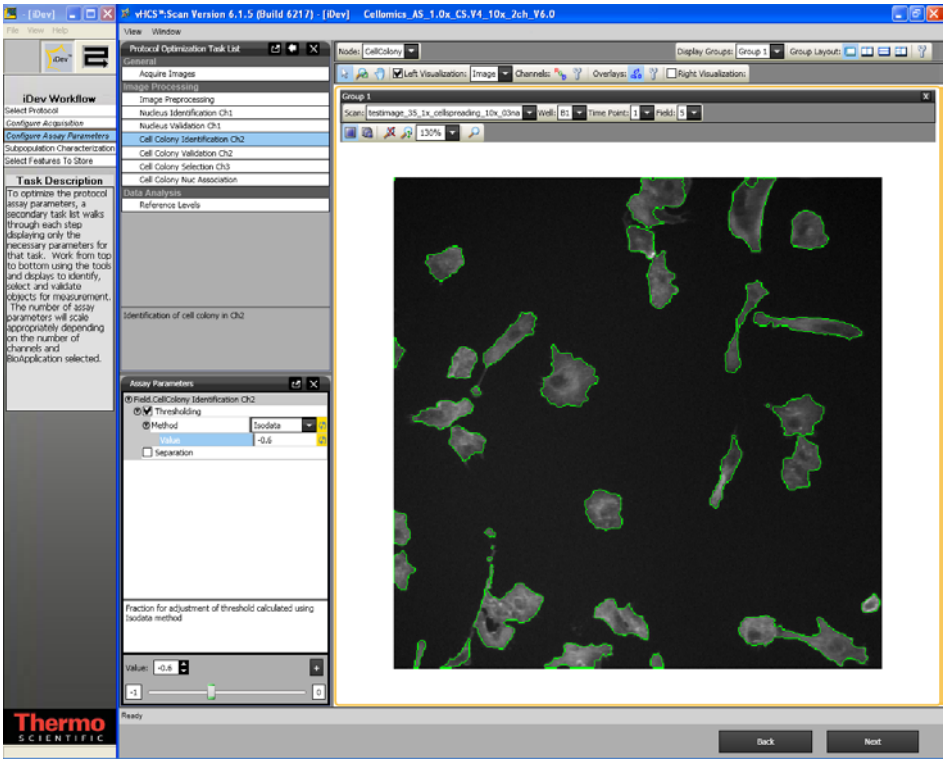

Figure 17. *Protocol Optimization Task – Cell Colony Identification Ch2*

Cell Colony Validation Ch2

Cell Colony Validation is object selection based on area, shape, and intensity features calculated for cell colonies in Channel 2. In this task you will set minimal and maximal values for validating (equivalent to selecting and rejecting objects in the ArrayScan Classic software) cell colonies in Channel 2 based on selection/rejection features. Additionally in this task you will determine if objects that are in the border of the field are included or rejected from analysis.

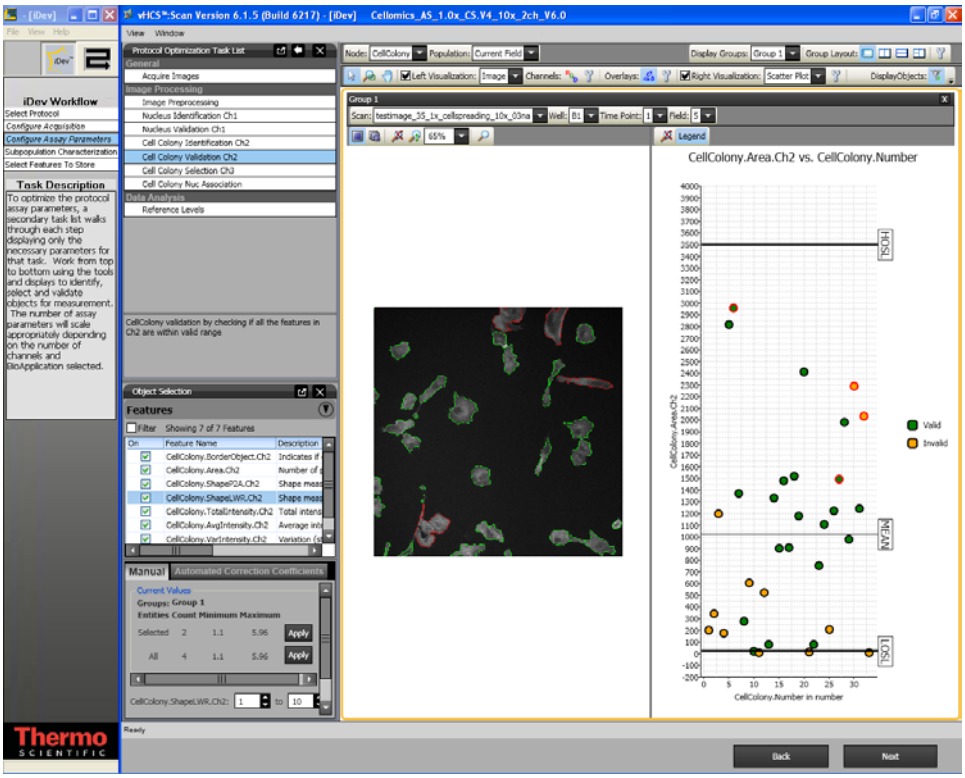

Figure 18. *Protocol Optimization Task – Cell Colony Validation Ch2*

Cell Colony Selection Ch3 through ChN

Cell Colony Selection is the selection of cell colonies based on intensity features computed in Channels 3 through Channel N under the circ mask derived from Channel 2. In this task you will set selection / rejection of the primary object based on intensity measurements in Channel 2 under a modified primary object mask. This task is similar to setting the Assay Parameter **MaskModifierCh2** in the ArrayScan Classic software. The primary object mask can be dilated (**Circ** > 0), or eroded (**Circ** < 0) if the **ROI Mask Creation** box is checked.

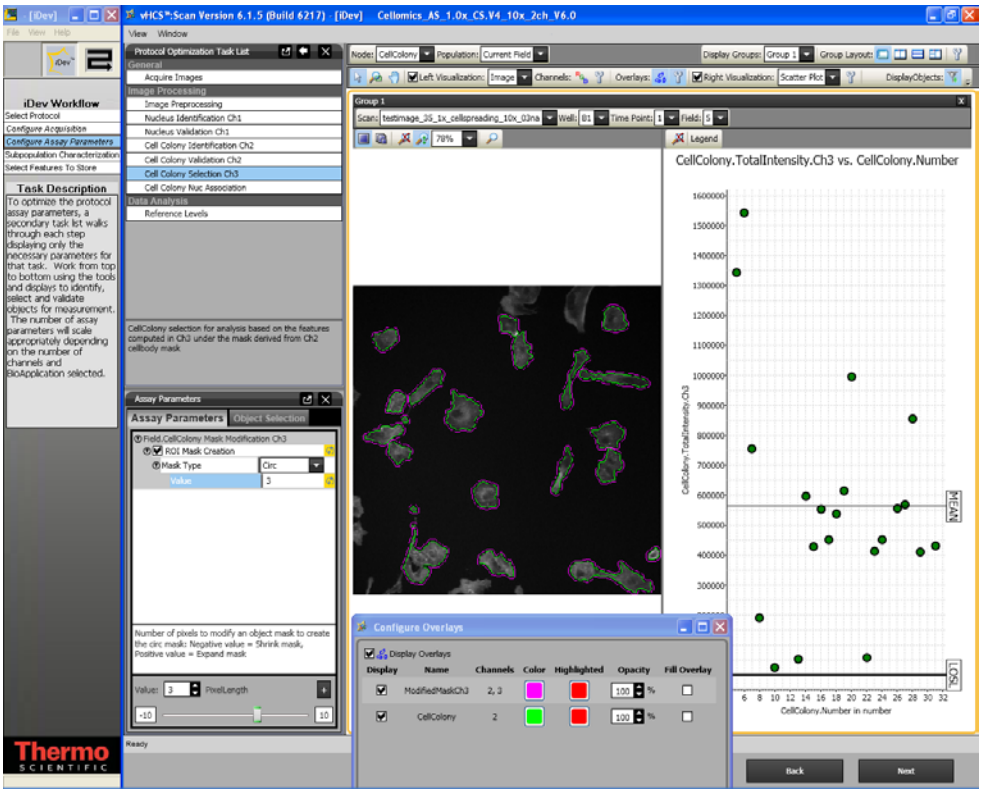

Figure 19. *Protocol Optimization Task – Cell Colony Selection Ch3 through ChN*

Cell Colony Nucleus Association

In this task you can set the range for number of nuclei associated with the cell colony. Colonies whose nuclei count fall outside of this range are rejected from analysis.

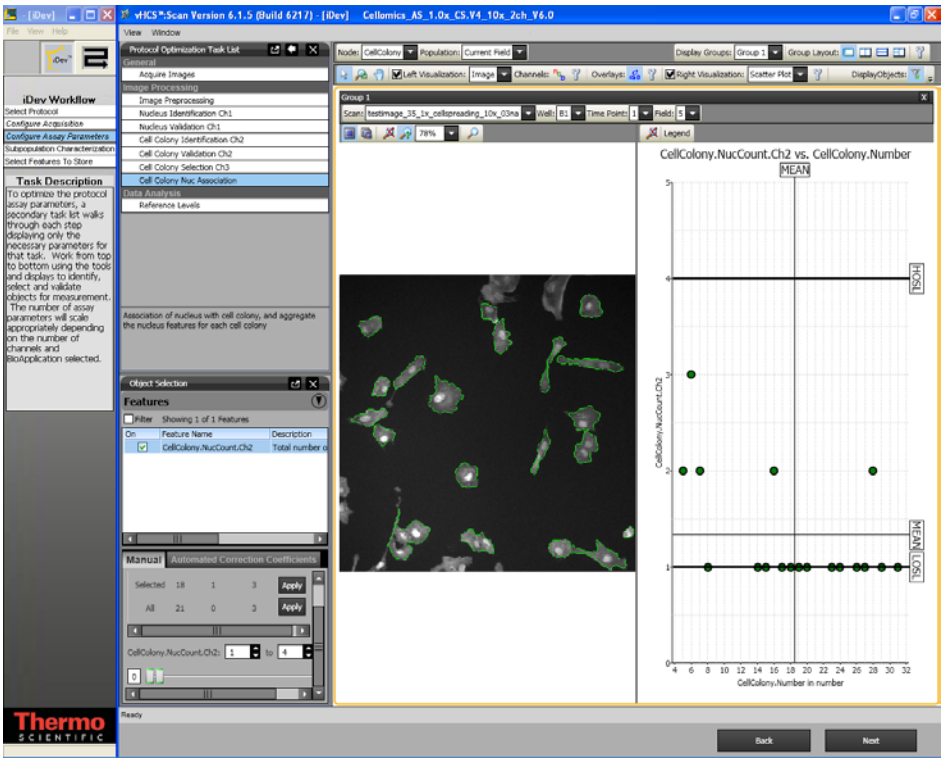

Figure 20. *Protocol Optimization Task – Cell Colony Nucleus Association*

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.

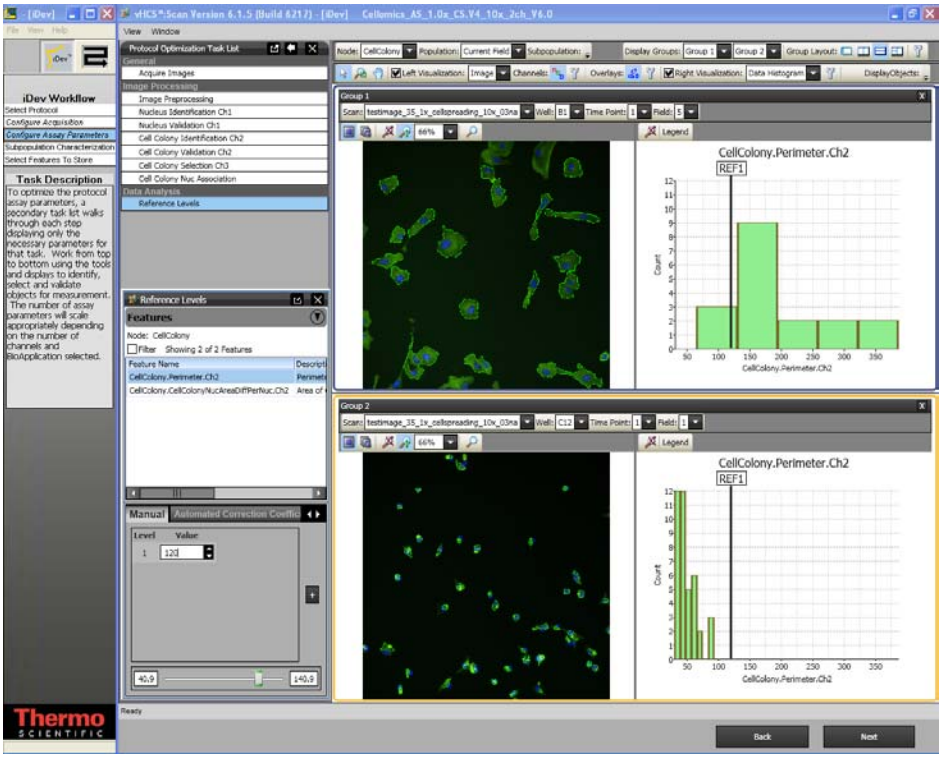

Figure 21. *Protocol Optimization Task – Reference Levels*

Index