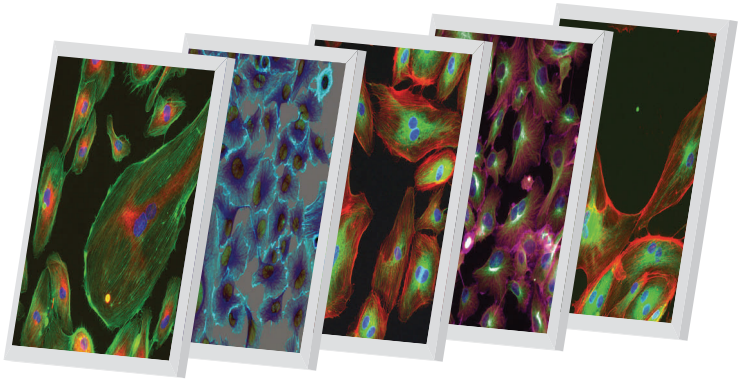
Thermo Scientific Cellomics® Cell Cycle V4

BioApplication Guide



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Cellomics® Cell Cycle BioApplication Guide

V4 Version

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1

Overview of the Cell Cycle BioApplication

High Content Screening (HCS) uses fluorescence-based reagents, advanced optical imaging systems, and sophisticated image analysis software (BioApplications) to quantitatively analyze targets and physiological processes in living and fixed cells. This BioApplication Guide provides a brief description for using one such versatile and flexible BioApplication, the Cellomics® Cell Cycle BioApplication, to automatically conduct multi-parametric HCS assays of the cell cycle in individual cells.

This guide contains the following chapters:

Chapter 1 provides an overview of the Cell Cycle BioApplication.

Chapter 2 describes how the BioApplication quantitatively analyzes the images, along with descriptions of the 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 Cycle BioApplication described in this document is designed to run on the following platforms:

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

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

Cell Biology Background

A cell reproduces by performing an orderly sequence of events in which it duplicates its contents and then divides in two; this cycle of duplication and division is known as the cell cycle (Alberts, 2002). The cell cycle has four sequential phases: G_0/G_1 (first gap phase), S (synthesis), G_2 (second gap phase), and M (mitosis). During a cell's passage through the cell cycle, its DNA is duplicated during the S phase and distributed equally between two daughter cells in the M phase. The two gap phases provide time for the cell to grow and double the mass of their proteins and organelles (Alberts, 2002). They are also used by the cell to monitor internal and external conditions before proceeding with the next phase of the cell cycle (Alberts, 2002). Normal cells in the G_0/G_1 phase of the cell cycle have 2N DNA content (i.e., two copies of each chromosome), and after DNA replication in the S phase, the DNA content doubles to 4N (four copies of each chromosome) in the G_2 phase. The cell's passage through

the cell cycle is controlled by a host of different regulatory proteins, some of whose expression level or state also change during different phases of the cell cycle. The phosphorylation state of one such regulatory protein, the retinoblastoma protein (Rb), and the corresponding DNA content of a cell going through the cell cycle is summarized in the schematic in Figure 1.

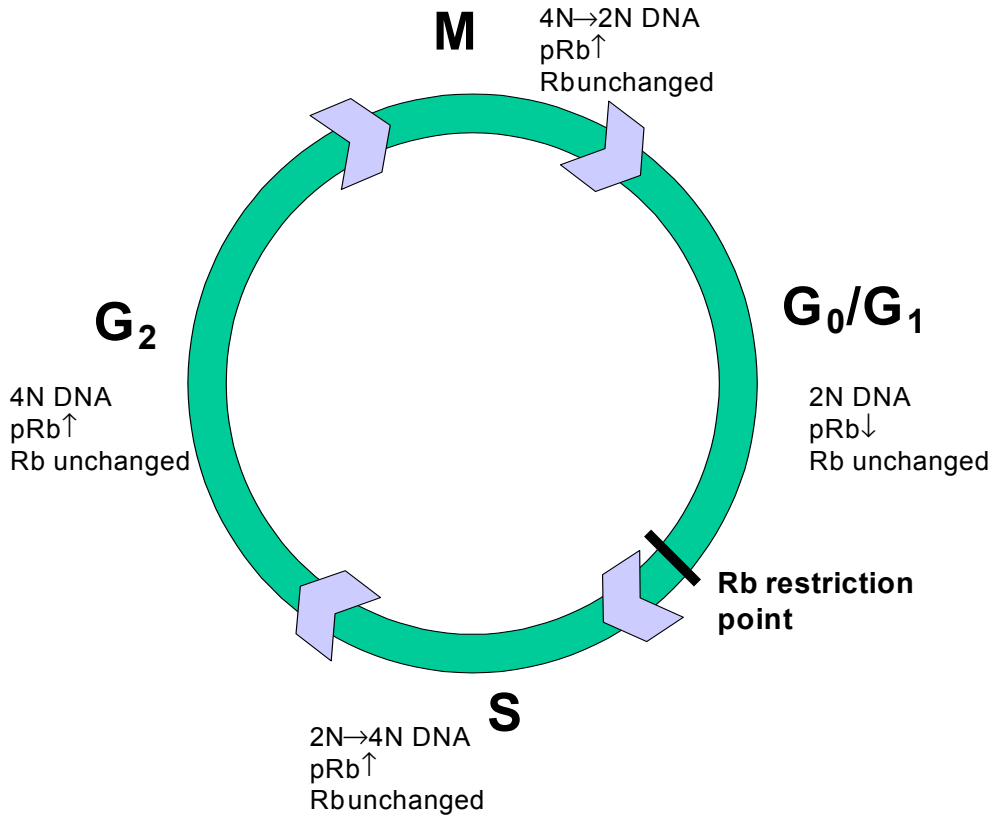

Figure 1. *Schematic of the Cell Cycle showing the relative amounts of DNA content phosphorylated Rb (pRb) and total Rb at the different cell cycle phases. Increased phosphorylation of Rb is required for cells to exit the G_1 phase to the S phase and to proceed with the cell cycle and cell division. Rb is dephosphorylated back to G_1 levels in the M phase.*

A standard method of assessing cell cycle phase is by Fluorescence Activated Cell Sorting (FACS, also known as "flow cytometry") where a cell's DNA is labeled with a fluorescent dye whose intensity is proportional to the cell's DNA content. Some of FACS limitations have been difficulty analyzing adherent cells and immunofluorescence (especially subcellular regions), overall throughput speed and experience, as well as the need for larger cell/reagent volumes. The Cell Cycle BioApplication is complimentary to FACS advantages, but also overcomes some of its limitations.

BioApplication Overview

To address the need for automated multiparametric cell cycle analysis, the Cell Cycle BioApplication was developed and has been designed to:

- Measure up to four cell cycle related targets simultaneously in the same cell.
- Classify the cell cycle phase of each individual cell by measuring the DNA content in a cell's nucleus, from the total intensity of a DNA binding dye in Channel 1.
- Provide the ability to choose up to three additional cell cycle related targets to measure simultaneously such as the different cyclins, cyclin dependent kinases (CDK), Rb, p53, p21/WAF, and others.
- Normalize the intensity between the different cell cycle targets to enable changes in a cell cycle target's state to be monitored (e.g., phosphorylated Rb and Total Rb can be measured in different channels, and normalizing their signal with each other provides a quantitative measure of the degree of Rb phosphorylation).
- Perform automatic cell population characterization and classification to determine whether a cell is a responder for the different targets using Reference Wells or user-defined levels.
- Correlate and report whether the cells in the different cell cycle phases are responders for combinations of different cell cycle targets.

Use of the Cell Cycle BioApplication

The flexibility of this BioApplication will enable a variety of cell cycle measurements. This BioApplication can be run with only a single channel where the DNA content will be measured and the cell cycle phase classifications will be applied to individual cells. Additional cell cycle related targets can be measured simultaneously. For example, measurement of phosphorylated Rb and total Rb in Channels 2 and 3, as well as computation of their intensity ratio, will allow monitoring the degree of Rb phosphorylation, which is an important step in regulating transition from the G_0/G_1 to S phase.

The expression level or state of different protein targets chosen can also vary for different cell cycle phases, so that their correlation with the DNA content can give a better assessment of the cell's passage through the cell cycle. For example, cyclin E expression levels are higher in the late G_0/G_1 phase and early S phases, whereas cyclins A and B have high expression levels during the G_2 phase. Correlation of these cyclins with the DNA content can provide a better characterization of a cell's passage through the cell cycle.

Regulators of DNA damage such as p53 and p21/WAF1 can also be included as targets and correlated with the cell cycle phase determined from DNA content. The nuclear size can be measured simultaneously from the same population of cells and can be used as an indicator of the nuclear fragmentation that results from the apoptosis that may accompany the p53 and p21 activity.

DNA Content and Cell Cycle Phase

The approach taken in the Cell Cycle BioApplication in assessing cell cycle phase is similar to that in FACS, by labeling a cell's DNA with a fluorescent dye having an intensity proportional to the cell's DNA content. The total fluorescence intensity of the dye from the nucleus of each cell typically exhibits a bimodal distribution, as shown by the solid colored distribution in Figure 2. The first peak typically contains cells with 2N DNA content (i.e., that are in the G_0/G_1 phase). The second peak is at an intensity which is the double of the first and contains cells with 4N DNA content (i.e., that are in the G_2/M phase). Under normal conditions, there are more cells in the G_0/G_1 versus the G_2/M phase, resulting in the first peak being higher than the second.

The region between these two peaks represents cells with DNA content between 2N and 4N. These are cells in the process of doubling their DNA and are in the S phase. Thus, based on where a cell's total intensity falls in this distribution due to its DNA content, its cell cycle status can be determined. Occasionally, cells are also found with DNA content less than the 2N distribution or greater than the 4N distribution. Cells with less than 2N DNA content are usually damaged or apoptotic cells with less than a normal total DNA complement. Cells with more than 4N DNA content can occur due to two reasons: they are from clumped cells whose nuclei could not be individually resolved, thus giving a higher apparent DNA content or they are from cells with higher ploidy (i.e., cells with multiple chromosome numbers), which may be natural for certain cells or indicative of an aberrant situation.

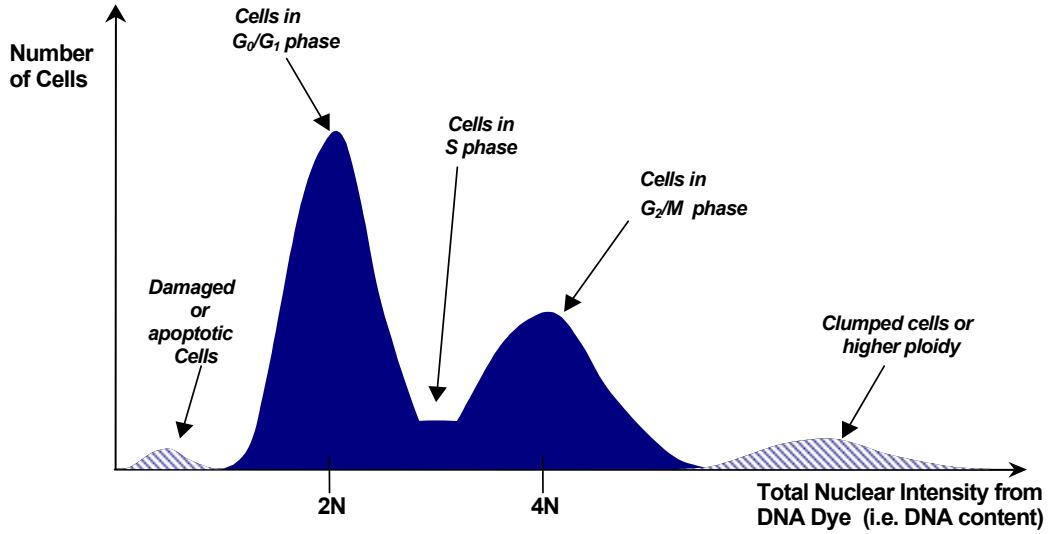

Figure 2. *Schematic of the total fluorescence intensity distribution from the DNA binding dye and the corresponding populations of cells in the different cell cycle phases.*

In the Cell Cycle BioApplication, Channel 1 contains the image of the nucleus stained with a dye with total intensity proportional to the DNA content. Examples of such dyes include: DAPI, Propidium Iodide, and Hoechst 33342. This channel is used to identify the nuclei of individual cells and measure their fluorescence intensities. For each cell, the total intensity of the nucleus is measured. The total intensity is the sum of the intensities of all the individual pixels making up that nucleus. The average intensity of the pixels making up the nucleus is also reported. This average intensity is equal to the total intensity divided by the number of pixels making up the nucleus.

The BioApplication automatically classifies each cell's nuclear total intensity into one of five categories of DNA content. The five categories, schematically depicted in Figure 3, are as follows:

1. DNA<2N
2. DNA~2N
3. 2N<DNA<4N
4. DNA~4N
5. DNA>4N

Cells categorized as having DNA~2N, 2N<DNA<4N, and DNA~4N are assigned the cell cycle phases G_0/G_1, S, and G_2/M respectively. The other two DNA content categories cannot be assigned to specific cell cycle phases.

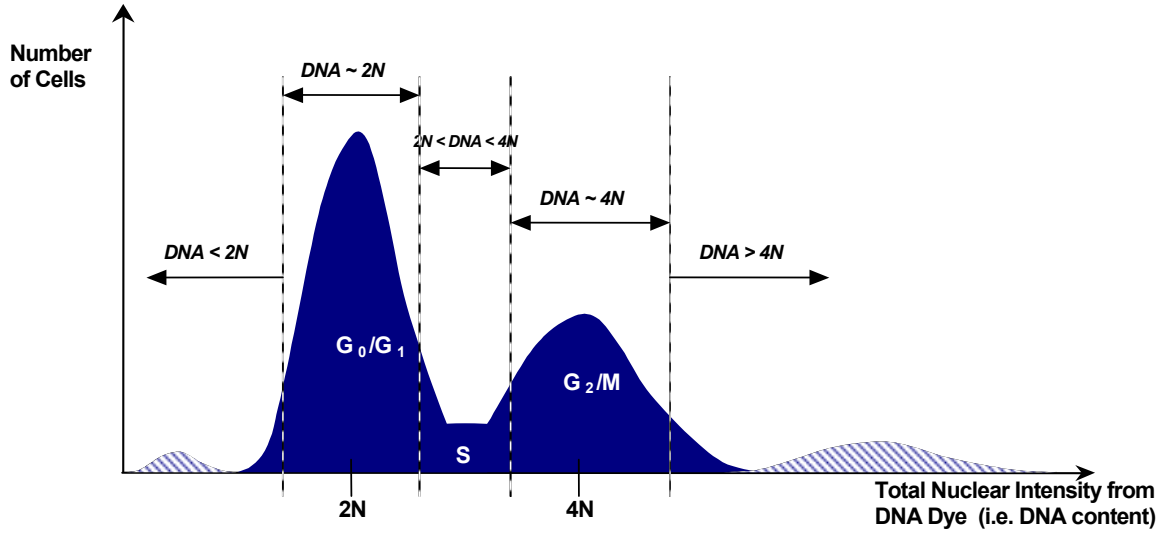

Figure 3. *Assignment of the total fluorescence intensity of cells into the five different DNA content categories.*

For each cell, the BioApplication also reports the total intensity status. This status feature indicates to which of the five different DNA content categories the cell belongs due to its total nuclear intensity, as shown in Table 1:

Total Nuclear Intensity Status	Corresponding DNA Content
1	DNA<2N
2	DNA~2N
3	2N<DNA<4N
4	DNA~4N
5	DNA>4N

Table 1. *Total Nuclear Intensity Status and corresponding DNA content.*

At the well level, the BioApplication reports the mean and related statistics of the total nuclear intensity over all the cells. The BioApplication also reports the intensity where the peak of the total nuclear intensity histogram occurs. For most untreated cells, the distributions will resemble those shown in Figures 2 and 3, and the peak of the total nuclear intensity distribution

would correspond to the location of the 2N DNA content peak. However, if the cells have been treated with a compound that blocks them at the G_2 phase, then the peak of the distribution would correspond to the location of the 4N DNA content peak (see Figure 5B). In the case of cell polyploidy, the distribution's peak may be far right of the 4N DNA location. Thus, the location of the peak of the total nuclear intensity allows you to evaluate the distribution independently of the DNA content categories.

The BioApplication also reports, at the well level, the number and percentage of cells in each of the five DNA content categories (i.e., Population Values). The BioApplication also reports the ratio of the number of cells categorized as having 2N DNA versus those having 4N DNA. This is a ratio of the cells in the G_0/G_1 versus the G_2/M phase and can be used as a simple monitor of the location in the cell cycle of the population of cells in a well.

The boundaries between the different DNA content categories (Figure 3) can be set automatically using Reference Wells, or you can manually set the boundaries (see Chapter 2 subsection on *Population Characterization*).

BioApplication Measurements

The Cell Cycle BioApplication is a multi-channel application where up to six channels can be used. Channels 1-4 are used specifically to assay for cell cycle related targets. Channels 2-6 have the additional capability that allows a subset of cells to be selected for analysis based on their intensity. Thus, you can choose up to four cell cycle related targets to measure. The first target is always the cell's DNA content. Channels 2-4 can be used to simultaneously measure the expression level or state of additional cell cycle associated target proteins in the nucleus. Based on their DNA content, the cells can be classified into their different cell cycle phases, and the BioApplication reports the correlation of the expression level or state of associated proteins with each cell's cell cycle state.

Both Cell and Well Features are reported by the BioApplication. Cell Features are measurements made on a single cell. Well Features are well level averages and other population metrics that are derived from the Cell Features for all the cells imaged and analyzed in that well. Table 2 shows the number of Cell and Well Features for the number of channels selected. This expansive number of features gives the Cell Cycle BioApplication the flexibility to detect and report different cell cycle conditions.

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

FEATURE CATEGORY	CELL-LEVEL FEATURES	CORRESPONDING WELL-LEVEL FEATURES
Cell & Well-Level Features Reported for Channel 1:		
Intensity:	Total Nuclear Intensity	Mean, SD, SE, CV
		Nuclear Total Intensity Peak
	Total Nuclear Intensity Status of Subpopulations	Cell Count and % of cells with DNA content in 5 categories: <2N, 2N, 2N-4N, 4N, >4N
		G_0/G_1 cells (cells with 2N DNA): Mean, SD, SE, CV
		S cells (cells with 2N-4N DNA): Mean, SD, SE, CV
		G_2/M cells (cells with 4N DNA): Mean, SD, SE, CV
		Ratio of cells in 2N/4N (ratio of cells in G_1/G_2)
	Average Nuclear Intensity and Status Variance Nuclear Intensity and Status	Mean, SD, SE, CV, %High, %Low
Morphology & Location	Nucleus Size & Status	Mean, SD, SE, CV, %High, %Low
	Top, Left, Width, Height, X Centroid, Y Centroid	none
	Nucleus Area, Nucleus Shape P2A, Nucleus Shape LWR	Mean, SD, SE, CV
Cell Counts & Density	Cell Number	Valid Cell Count Selected Cell Count % Selected Cells Valid Field Count Selected Cell Count per Valid Field
Cell & Well-Level Features Reported for Channels 2-6:		
Intensity:	Total Nuclear Intensity (Ch 2-6) & Status (Ch 2-4)	All cells: Mean, SD, SE, CV, %High, %Low
		G_0/G_1 cells (cells with 2N DNA): Mean, SD, SE, CV, %High, %Low
		S cells (cells with 2N-4N DNA): Mean, SD, SE, CV, %High, %Low
		G_2/M cells (cells with 4N DNA): Mean, SD, SE, CV, %High, %Low
	Average Nuclear Intensity (Ch 2-6)	none
Intensity Ratios Between Channels:	Ratio with Ch 2: Total Nuclear Intensity Ratio Ch*N*/Ch2 & Status where Ch*N* is either Ch3 or Ch4 (Ch 2-4)	All cells: Mean, SD, SE, CV, %High, %Low
		G_0/G_1 cells (cells with 2N DNA): Mean, SD, SE, CV, %High, %Low
		S cells (cells with 2N-4N DNA): Mean, SD, SE, CV, %High, %Low
		G_2/M cells (cells with 4N DNA): Mean, SD, SE, CV, %High, %Low

Table 2. *Cell and Well Features reported by the Cell Cycle BioApplication. Note: "ChN" refers to the possible numerator channel, which can either be Channel 3 or Channel 4. SD is the standard deviation, SE is the standard error of the mean, and CV is the percent coefficient of variation.*

Demonstration Data Using the Cell Cycle BioApplication

The target chosen to demonstrate the Cell Cycle BioApplication, in addition to the cell's DNA content, is the phosphorylation state of the Retinoblastoma protein (Rb). The DNA content is measured in this assay by binding the DNA with the fluorescent dye DAPI. The DAPI fluorescence intensity measured within the nucleus is proportional to the cell's DNA content.

Rb is phosphorylated (pRb) in a cell cycle dependent manner (Kaelin, 1999; Mittnacht, 1998). It is under-phosphorylated in the G_0/G_1 phase, bound to the transcription factor E2F and prevents the cell from progressing past the G_1/S restriction point (Kaelin, 1999; Mittnacht, 1998; Chellappan et al., 1991; Hiebert et al., 1992). Mitogen signaling causes hyperphosphorylation of Rb through the action of various cyclin dependent kinases (cdks) (Kaelin, 1999; Mittnacht, 1998; Wang, Ghosh, and Chellappan, 1998). The main cdk/cyclin pairs involved are cdk4/cyclinD, cdk6/cyclinD, and cdk2/cyclinE, and these cdk/cyclin pairs phosphorylate at different sites on Rb (Mittnacht, 1998; Connell-Crowley, Harper, and Goodrich, 1997; Zarkowska and Mittnacht, 1997). This phosphorylation causes Rb to release E2F and the cell to pass the G_1/S restriction point and proceed through the cell cycle. Rb is dephosphorylated in late mitosis restoring its activity of blocking passage from G_1 to S (Mittnacht, 1998).

In this example, the Cell Cycle BioApplication's second channel is used to detect total Rb content in the cell's nucleus. This is done by indirect immunofluorescence against total Rb using a secondary antibody conjugated to Alexa Fluor® 555. Phosphorylation of Rb at a particular site is detected by indirect immunofluorescence using a phosphospecific primary antibody against the particular phosphorylation site. Indirect immunofluorescence against phosphorylated Rb uses a secondary antibody conjugated to Alexa Fluor 488, and the intensity detected in the nuclear area of each cell is recorded in the third fluorescence channel of the BioApplication. In this manner, each cell's DNA content, phosphorylated Rb and total Rb are simultaneously detected by three different fluorescence wavelengths, blue, red, and green respectively. Furthermore, the proportional amount of total Rb that is phosphorylated is reported via the pRb/Rb intensity ratio. This corrects for intensity changes in the pRb channel that are due to changes in cell shape (e.g., cell rounding) rather than a true change in the amount of Rb phosphorylation.

Although this demonstration utilizes the second and third channel for monitoring Rb and pRb, other cell cycle associated targets can be substituted. The microtubule depolymerizing drug, nocodazole, which blocks the cell cycle at G_2/M, was used to demonstrate this assay. A549 cells were treated with 200 ng/mL nocodazole for 24 hours before fixation and labeling with DAPI and indirect immunofluorescence against pRb and Rb. Figure 4 shows images taken on a Cellomics HCS Reader from nocodazole-treated and untreated A549 cells.

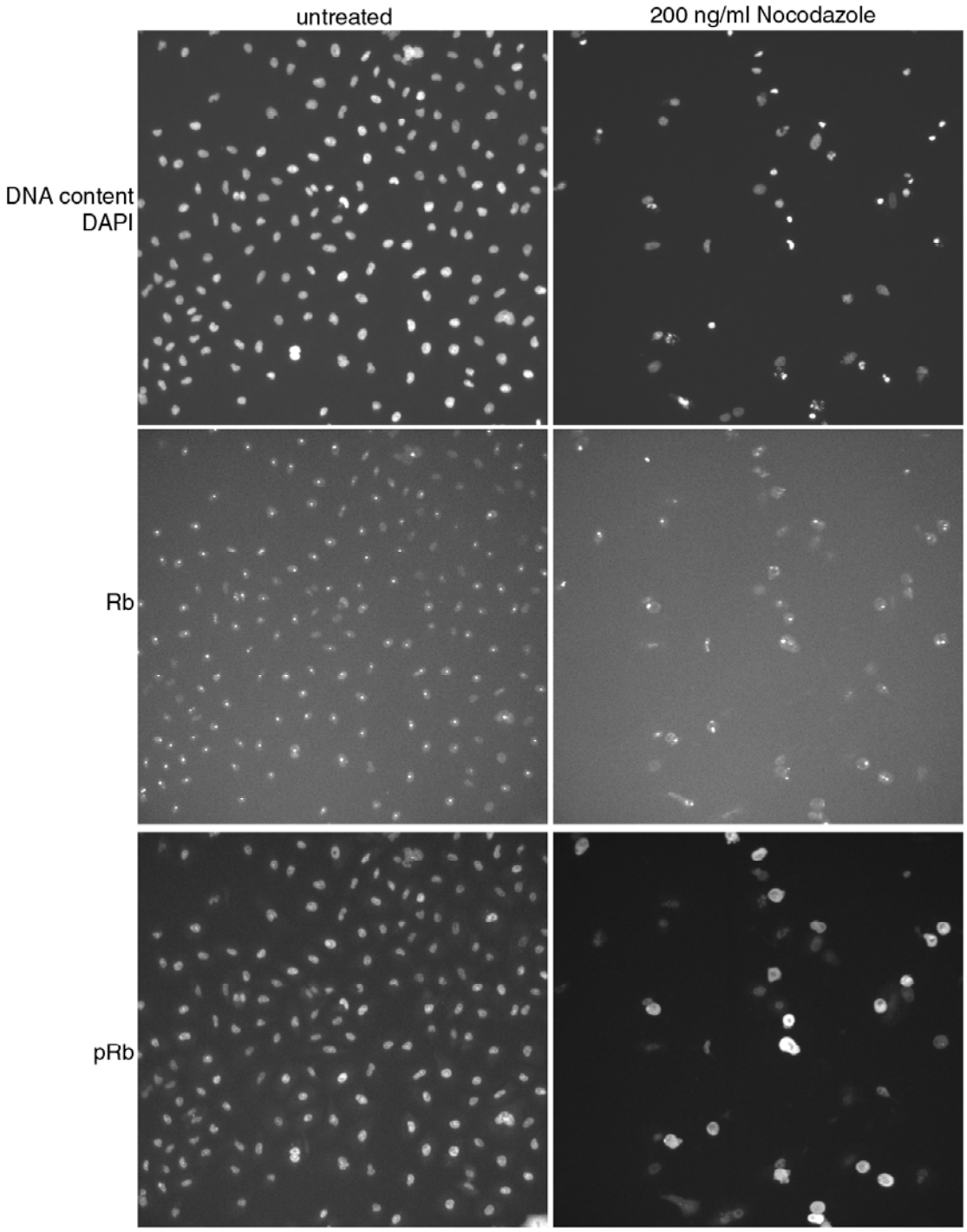

Figure 4. *Control and Nocodazole treated A549 cells imaged on a Cellomics HCS Reader. Cells were labeled with DAPI and stained by indirect immunofluorescence for total Rb with a red conjugated secondary antibody and pRb with a green conjugated secondary antibody.*

There were fewer nocodazole treated cells per imaged field, indicative of blocked cell growth and division. The nocodazole-treated cells had brighter nuclei and some of these cells also had bright pRb staining. The total Rb staining level seemed similar for both control and nocodazole-treated cells. Figure 5 shows the quantitative results from using the Cell Cycle BioApplication on this sample, where the cell details from six wells on the plate for each condition were pooled and plotted. The pRb intensity normalized with the Rb intensity is plotted versus the DNA content (DAPI total intensity) in the scatter plot shown in Figure 5A.

Figure 5B represents the DNA content distributions with control (untreated) cells showing a bimodal distribution, as expected, where the first peak is of cells in the G0/G1 phase and the smaller second peak, at twice the nuclear DAPI intensity, is of cells in the G2/M phase. A bimodal distribution is also seen for nocodazole-treated cells, but the first peak has fewer cells than the second, indicative of most cells having 4N DNA and being trapped at the boundary.

Figure 5C represents the pRb/Rb distribution for the two populations. Most cells in the untreated control well have a low pRb/Rb value. The nocodazole-treated cells show two populations of cells with different pRb/Rb levels when viewed on the log scale of the scatter plot. This is seen as a broad distribution when viewed on the linear scale of the histograms. Thus, nocodazole-treated cells either have low (similar to control) or high levels of pRb/Rb signal (Rb phosphorylation).

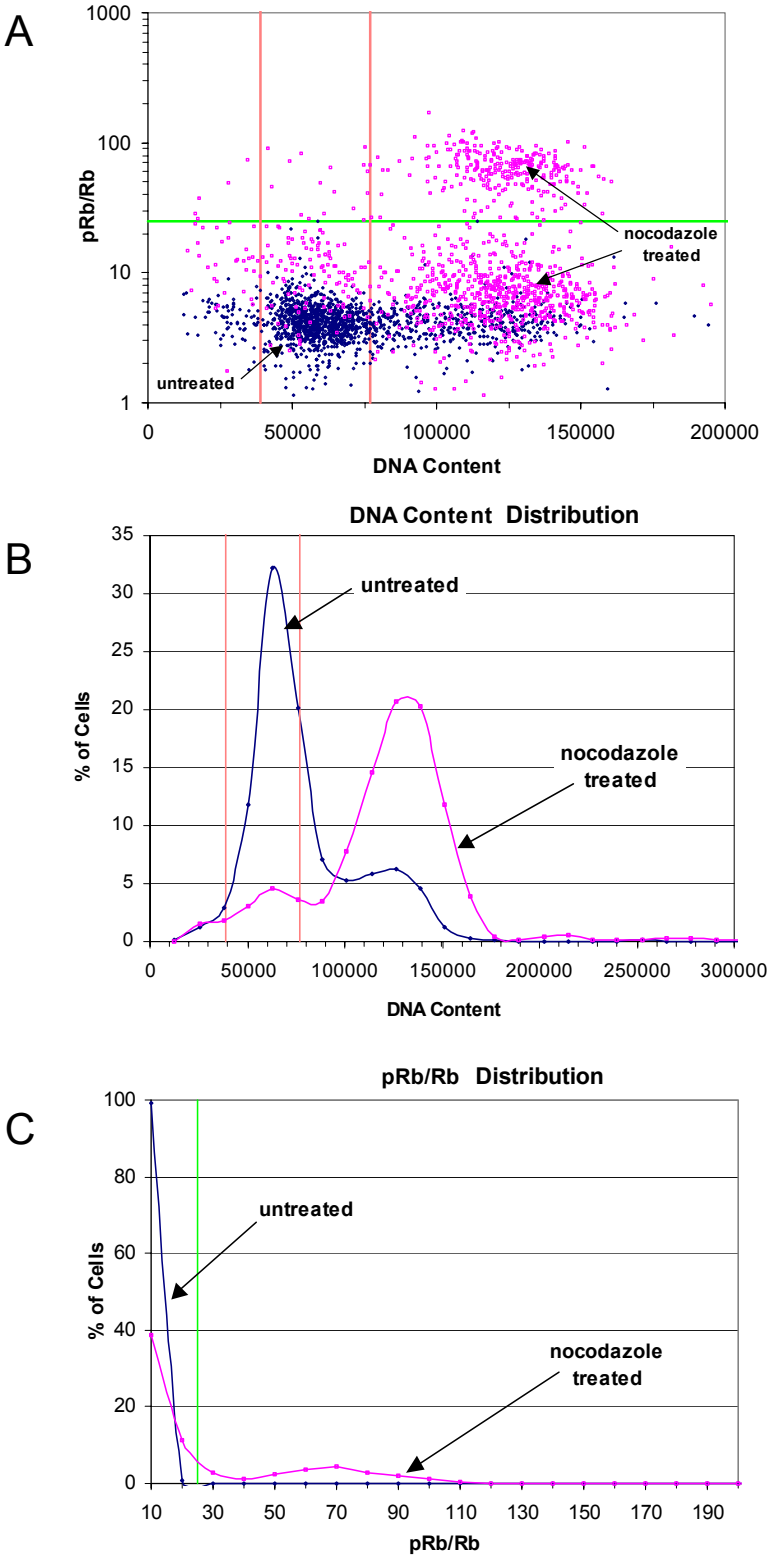

Figure 5. *Analysis of A549 cells treated with either 0 ng/mL or 200 ng/mL nocodazole for 24 hours. (A) The vertical and horizontal lines appearing in the top scatter plot denote user-determined thresholds used to distinguish between the different cell populations. The vertical lines identify the boundaries of the intensity range of cells with 2N DNA to identify cells in the G_0/G_1 phase, and the horizontal line determines cells with low and high amounts of Rb phosphorylation. The same thresholds are shown in the DNA content (B) and pRb/Rb distributions (C).*

Table 3 summarizes the subpopulation values reported by the BioApplication for this sample. Thresholds were manually chosen for this sample after examining the data in scatter plots and histograms similar to those shown in Figure 5. The parameters defining the DNA content thresholds (explained further in Chapter 2) were chosen as follows:

2N_PeakIntenCh1:	58,000
4N_PeakIntenFactorCh1:	2.1
PeakWidthFractionCh1:	0.3

This results in a DAPI total nuclear intensity range for G_0/G_1 cells of 38,860–77,140 and for G_2/M cells of 102,660–140,940. The thresholds binning the G_0/G_1 cells are shown by the two vertical lines in the scatter plot and the DNA content histogram in Figure 5A. A threshold value of 25 was used to differentiate the two pRb/Rb populations seen in Figure 5 (see horizontal line in 5A and vertical line in 5C).

In Table 3, the average number of cells per well is lower for the treated wells, confirming what was seen in Figures 4 and 5; this could be due to the fact that nocodazole blocked cell growth and division. No difference in nuclear size was seen for the two conditions, implying that the nocodazole treatment did not induce apoptosis or necrosis. The results provide evidence and are consistent with the fact that the majority of cells are in the G_0/G_1 and G_2/M phases for the untreated and nocodazole treated cells respectively. The evidence illustrates that the main peak of the DAPI intensity distribution for nocodazole-treated cells is approximately twice that for control cells and that the average DAPI intensity of nocodazole-treated cells is higher than that of untreated cells. With the thresholds chosen, 65% of the untreated cells are in G_0/G_1 versus 12% of the nocodazole-treated cells, and only 17% of the untreated cells are in G_2/M versus 57% of nocodazole-treated cells. The ratio of the number of cells in G_0/G_1 versus G_2/M is 3.9 for untreated cells, but only 0.2 for nocodazole-treated cells. The nuclear intensity variation in the untreated cells is relatively low at 186, while nocodazole-treated cells show a much higher intensity variation at 747 within the nucleus. The difference in the intensity pattern of the DNA binding dye DAPI is an addition indicator of the phase of the cell cycle.

A difference in Rb phosphorylation between the two treatments is also seen in the quantitative results in this table, where the degree of pRb phosphorylation (i.e., pRb/Rb ratio) is much higher for nocodazole treated cells versus control. The mean pRb/Rb value per cell is approximately five times higher for nocodazole treated cells, and, with the thresholds chosen, ~53% of the cells were classified as having a high pRb/Rb level for nocodazole treatment versus only ~3% having this high level for control cells. The percentage of cells with high or low pRb and that are also in the G_0/G_1 or G_2/M phases are also reported, giving a more detailed view of the subpopulation changes due to nocodazole treatment. These results show that the Cell Cycle BioApplication can quantify a change in both nuclear intensity and pRb/Rb as a result of drug treatment.

Parameter	Untreated	+ Nocodazole (200 ng/mL)
Number of cells/well	502 ± 27	219 ± 17
Mean nuclear size (nucleus equivalent diameter in pixels)	11.77 ± 0.05	11.30 ± 0.21
Channel 1 Nuclear Intensity Peak	56,306 ± 311	122,187 ± 3,112
Mean nuclear total intensity Channel 1 (i.e., DNA content)	74,034 ± 1,008	114,802 ± 1,280
% cells with 2N DNA content (i.e., % cells in G_1 phase)	65 ± 2	12 ± 1
% cells with 4N DNA content (i.e., % cells in G_2 phase)	17 ± 1	57 ± 3
Ratio of the number of cells with 2N/4N DNA content (i.e., number of cells in G_1/G_2 phase)	3.86 ± 0.26	0.21 ± 0.02
Nuclear Intensity Variation Ch1 (i.e., variation of the intensity of pixels within the nucleus, which is a texture measurement)	186.5 ± 2.1	747.1 ± 13.5
Mean of Channel 3 to Channel 2 total intensity ratio (i.e., pRb/Rb ratio)	4.4 ± 0.1	21.1 ± 2.1
% Cells with Channel 3 to Channel 2 total intensity ratio > threshold (i.e., % High of pRb/Rb ratio)	2.6 ± 0.6	53.1 ± 3.5
% of cells that are in G_1 phase and with Channel 3 to Channel 2 total intensity ratio > threshold (i.e., % cells in G_1 and have High pRb/Rb ratio)	1.4 ± 0.4	7.8 ± 0.8
% of cells that are in G_1 phase and with Channel 3 to Channel 2 total intensity ratio < threshold (i.e., % cells in G_1 and have Low pRb/Rb ratio)	63.5 ± 1.6	3.8 ± 0.4
% of cells that are in G_2 phase and with Channel 3 to Channel 2 total intensity ratio > threshold (i.e., % cells in G_2 and have High pRb/Rb ratio)	0.5 ± 0.1	29.9 ± 2.8
% of cells that are in G_2 phase and with Channel 3 to Channel 2 total intensity ratio < threshold (i.e., % cells in G_2 and have Low pRb/Rb ratio)	16.6 ± 0.7	27.4 ± 3.3

Table 3. *Effect of nocodazole on DNA Content and Rb phosphorylation on A549 Cells (values are the mean ± standard error over results from six similarly treated wells for each condition). A549 cells were treated with 200 ng/mL of nocodazole for 24 hours. Although mean nuclear size is similar in both treatments, profound differences in cell number, pRb/Rb ratio, and percent cells in phase with varying levels are evident.*

References

Alberts, B., et al. 2002. *Molecular Biology of the Cell*. New York: Garland Science.

Chellappan, S. P., et al. 1991. The E2F transcription factor is a cellular target for the RB protein. *Cell* 65:1053-61.

Connell-Crowley, L., J. W. Harper, and D. W. Goodrich. 1997. Cyclin D1/Cdk4 regulates retinoblastoma protein-mediated cell cycle arrest by site-specific phosphorylation. *Mol. Biol. Cell* 8:287-301.

Hiebert, S. W., et al. 1992. The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. *Genes Dev.* 6:177-85.

Kaelin, W. G., Jr., 1999. Functions of the retinoblastoma protein. *Bioessays* 21:950-8.

Mittnacht, S., 1998. Control of pRB phosphorylation. *Curr. Opin. Genet. Dev.* 8:21-7.

Wang, S., R. N. Ghosh, and S. P. Chellappan. 1998. Raf-1 physically interacts with Rb and regulates its function: a link between mitogenic signaling and cell cycle regulation. *Mol. Cell Biol.* 18:7487-98.

Zarkowska, T. and S. Mittnacht. 1997. Differential phosphorylation of the reinoblastoma protein by G1/S cyclin-dependant kinases. *J. Biol. Chem.* 272:12738-46.

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.

The previous chapter provided an overview of what the Cell Cycle BioApplication does and what it measures. This chapter describes in more detail the adjustable input parameters that control the analysis, and the BioApplication output features.

Overview

The Cell Cycle BioApplication has numerous input parameters that control its image analysis algorithm. 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.

The Cell Cycle BioApplication first identifies the individual nuclei in Channel 1 and measures their Intrinsic Values. The peak nuclear total intensity is also computed. It then computes the Intrinsic Values from the nuclear regions in Channels 2-4. The main nuclei identification is done by the Channel 1 Object Selection Parameters. Identification of the Channel 1 nuclei can be further improved using Assay Parameters that control nuclear smoothing and segmentation.

Input parameters can be found in the Create Protocol View and Interactive View of the ArrayScan Classic software or in the Protocol Optimization task list of the iDev software. The available input parameters are dependent on the number of channels selected; only the input parameters for the selected channels will be displayed. In the ArrayScan Classic software, the available input parameters will also vary depending on the mode in which you are running: Basic Mode or Advanced Mode. Basic Mode enables you to measure the morphology and related properties of cells. Advanced Mode is recommended if you wish to further characterize subpopulations based on the different morphological properties they possess. The Advanced Mode enables you to set criteria that defines responders for various features. Use of both Basic and Advanced Modes are described later in this chapter.

There are two types of input parameters: Object Selection Parameters and Assay Parameters. The Object Selection parameters control which objects are chosen for processing and are specific for each channel. The Assay Parameters control the quantitative analysis of the images.

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

There are three different options or methods for determining intensity thresholds, and sub-sets of these are available for each channel. For each Channel, you must select both a method and a value. The different options and values available for each channel are summarized in Table 4, and the descriptions of the different methods follow in Table 5.

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

Table 4. *Intensity Threshold Methods Available for Each Channel in the Cell Cycle BioApplication*

NOTE

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

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

The effective range for object identification is limited to 0-4095 for the following Cellomics HCS Readers: ArrayScan V^{TI} and ArrayScan X.5 HCS 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 because in this channel an intensity threshold is required to define the pixels making up the nuclei.

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

The **Isodata Threshold** method is known as histogram-derived threshold in that the threshold is chosen from the histogram of pixel intensities in the image (i.e., the image's brightness histogram). The schematic in Figure 6 demonstrates how the histogram-derived threshold value is calculated; Table 5 gives a description of the options.

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 **Isodata Threshold** 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 5. *Intensity Threshold Descriptions available for the Cell Cycle BioApplication.*

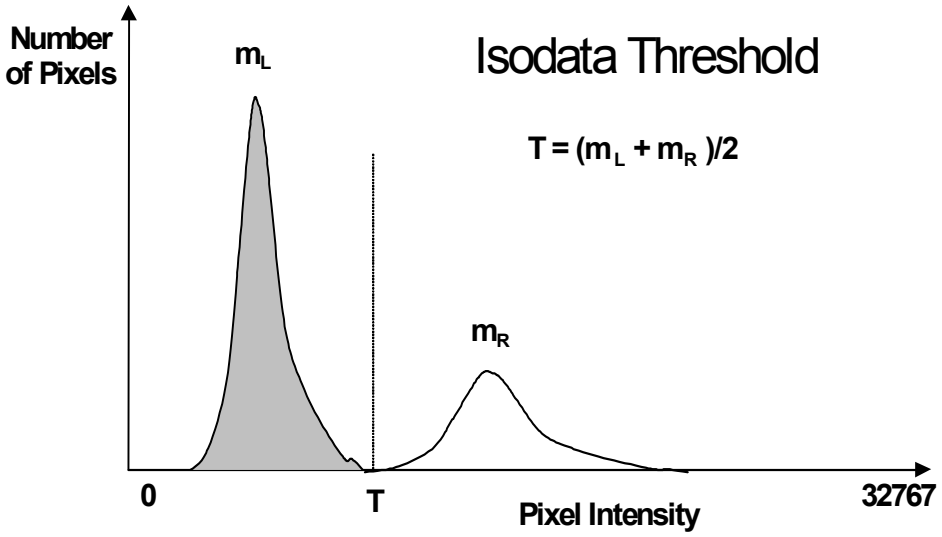

Figure 6. *Isodata Intensity Threshold Method. Background peak is shown in gray and object peak is white.*

Description of Assay Parameters and Settings

Input Parameters Control Either Intrinsic or Population Values

The Cell Cycle BioApplication has two types of outputs: Intrinsic Values and Population Values. Intrinsic Values are the properties derived directly from image processing, such as the Total Nuclear Intensity and Nuclear Size, and represent properties of the cell, or from simple arithmetic operations on other derived Intrinsic Values such as the ratio between the nuclear total intensity measured in two different channels. The Intrinsic Values measured for individual cells allow subpopulations of cells to be identified. For example, the DNA content, which is proportional to the Total Nuclear Intensity (an Intrinsic Value), will be different for cells in different cell cycle phases, and, thus, its distribution may be multi-modal (see Figure 2 and Figure 3). These subpopulations can either be defined from one Intrinsic Value or a combination of Intrinsic Values (e.g., cells that are in a particular cell cycle phase plus have a positive expression level of some other cell cycle associated target).

Population values describe properties of these subpopulations, such as the statistics and cell percentages of the Intrinsic Values that are in a particular subpopulation. To calculate population values, the Cell Cycle BioApplication uses specific calculation and logic steps that are targeted and optimized specifically for cell cycle biology.

The BioApplication's different input parameters can also be thought of as either controlling the measurement and computation of Intrinsic Values or of Population Values. When Reference Wells are used, the BioApplication first goes to Reference Wells and measures the intensity from the nuclear region in all the channels selected for each cell as well as the nuclear size. These measurements (Intrinsic Values) are controlled by the Assay Parameters and Object Selection Parameters that control the Intrinsic Values. The Intrinsic Value properties of cells, measured in these Reference Wells, are used to define the subpopulation ranges. This is done according to criteria specified by the Assay Parameters that control computation of the Population Values. If Reference Wells are not used, you must manually enter the boundaries defining each subpopulation. After the ranges of the different subpopulations have been defined, the rest of the plate is then scanned. For each well, the cell's Intrinsic Values are

measured first, they are then assigned to the different subpopulations based on the subpopulation ranges established earlier, and, finally, the Population Values are reported. Thus, when using this BioApplication, it is important to keep in mind whether an Assay Parameter is used to measure Intrinsic Values or used for controlling subpopulation characterizations and computing Population Values.

Assay Parameters for Image Analysis

General Assay Parameters

Below are Assay Parameters that affect measurement of Intrinsic Values:

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

Reference Well Control

If you would like to have the Cell Cycle BioApplication automatically compute the high and low cellular response levels, you must set the Assay Parameters that control the use of Reference Wells. The first parameter is **UseReferenceWells.**

There are two available methods in which to process Reference Wells. The first method is available when you set **UseReferenceWells** to **1**. Uisng this method, the cell data from Reference field scans are directly aggregated to well data for computing Reference Levels – just as for standard cell and well features.

The second method is accessible when you set **UseReferenceWells** to **-1**. Using this method, cell data is first tabulated to field data for each field in a Reference Well. This reduced data from fields is then aggregated for the whole well (with weighting by cell# for each field). This intermediate, weighted field-tabulation for Reference features differs from how standard cell features are tabulated directly into well features.

The consequence of the second method (setting **UseReferenceWells** to **-1**) is that the Assay Parameters, **2N_PeakIntenCh1** and **4N_PeakIntenFactorCh1** (derived from Min Reference Wells) are based on cell data from separated field tabulations. By the first method, these important peak reference values are computed by tabulating all cell data within a well at once (not field by field, then weighting), so this undivided, larger population sample of cell values may better represent the whole population's peaks.

Set the Assay Parameter **UseReferenceWell** to **0** if you do not want to use Reference Wells. If Reference Wells are not used, the BioApplication will determine cellular response using the manually specified levels. These are channel-specific Assay Parameters and are described later in this chapter.

If Reference Wells are used (value = **1 or -1**), the advanced Assay Parameters **MinRefWellMinAvgCellCountPerField** and **MaxRefWellMinAvgCellCountPerField** must be set. These Assay Parameters specify the minimum average number of cells per field required for acceptance of the high and low levels derived from specific Reference Well types (as MIN or MAX). If the average number of objects per field is less than

MinRefWellMinAvgCellCountPerField or **MaxRefWellMinAvgCellCountPerField,** the BioApplication will use the manually specified cellular response levels.

Units for Morphological Measurements

You have the option of either choosing micrometers or pixels as the unit to report morphological measurements. This is done by the **UseMicrometers** parameter, which when set to **0** causes the morphological measurements to be reported in pixels. If set to **1**, they are reported in micrometers. The different pixel sizes in micrometers are calculated automatically from the magnification of the objective used and camera acquisition mode that was selected.

Object Type

NucTypeCh1

The "Object Type" Assay Parameter allows you to specify whether the objects of interest are brighter or darker than the background of the image. Setting the value to **1** makes dark areas within an image to be considered as potential objects, while leaving it as **0** considers bright areas on a dark background as potential objects (see Table 6).

Setting	Detects
0	Objects that are brighter than the background (most typical)
1	Objects that are dimmer than the background

Table 6. *Binary options for* ***NucTypeCh1****.*

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

Background Correction Ch*N*

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

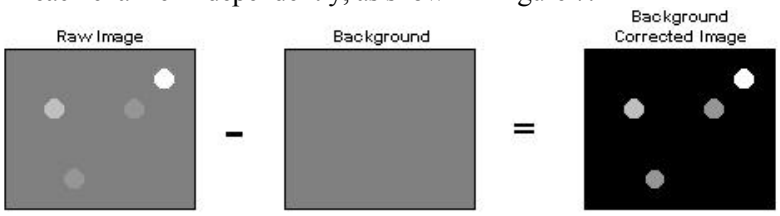

Figure 7. *Background Correction ChN found in each channel*

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 in each channel as needed. 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 larger the absolute value, the larger the sampled region, and the less subtraction is done. 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 determine the value needed for the optimal background correction. Table 7 summarizes the Background Correction methods and settings.

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 7. *Background Correction methods available for all channels.*

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

This BioApplication enables you to control the extent of smoothing to be applied to acquired images by using the **NucSmoothFactorCh1** Assay Parameter. Smoothing is only performed on Channel 1 images. Smoothing reduces the sharpness of intensity variations by redistributing their relative brightness over the immediate vicinity in the image. The value of this Assay Parameter determines the area of the region over which brightness is redistributed. If the **NucSmoothFactorCh1** value is set to **0**, smoothing is not done and analysis is done on raw, uncorrected images.

Nuclear Smoothing can be used to connect fragments of nuclei and can be valuable when measuring morphological changes. Figure 8 shows the effect of smoothing on accurate identification of objects in Channel 1 (white overlay). Note that insufficient smoothing can result in unwanted object fragmentation (arrows in Panel B) while excessive smoothing can result in poor definition of the object perimeter (D).

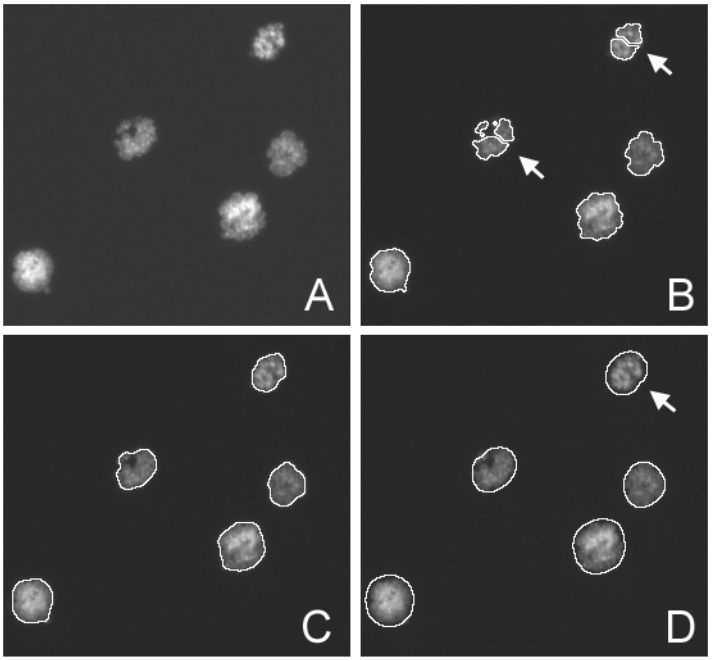

Figure 8. NucSmoothFactorCh1*: Images show the effect of different degrees of smoothing on the same field of apoptotic cell nuclei. A: Raw Image; B:* ***NucSmoothFactorCh1*** *= 0; C:* ***NucSmoothFactorCh1*** *= 3; D:* ***NucSmoothFactorCh1*** *= 8.*

Object Segmentation

NucSegmentationCh1

Even though you may have chosen an optimal intensity threshold and an appropriate degree of smoothing for object identification, you may encounter situations where individual nuclei cannot be properly resolved, such as in the case of densely packed objects. Nucleus Segmentation will help resolve and identify individual objects. Two methods are available for nuclear segmentation in the Cell Cycle BioApplication: Geometric (shape and size) and Intensity (intensity peaks). These methods are illustrated in Figure 9.

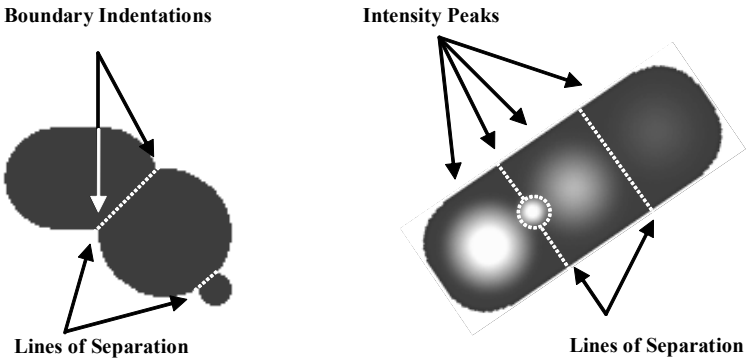

Figure 9: *Nucleus Segmentation Options. The image on the left illustrates the Geometric Method while the image on the right illustrates the Intensity Method.*

The Geometric method splits touching objects on the basis of shape, relying on boundary indentations to locate the line of separation. This method works best if the individual objects

have smooth boundaries with pronounced indentations at their point of contact. The Geometric method can be used to separate objects that are uniform in intensity (i.e., saturated) or that have multiple intensity peaks (i.e., noisy or textured).

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

The **NucSegmentationCh1** Assay Parameter (see Table 8) 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 8: *Channel 1 Object Segmentation Options*

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

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

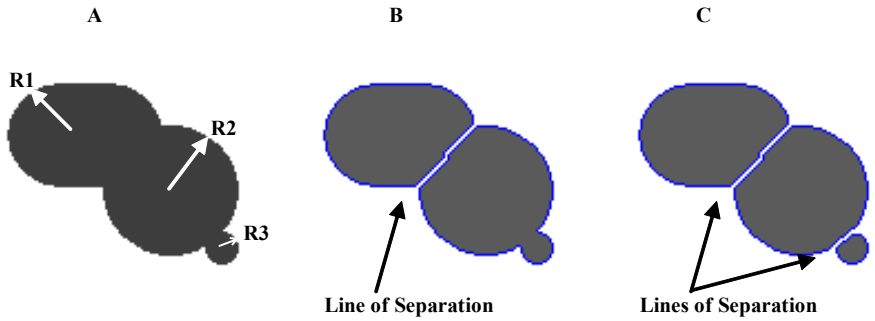

Figure 10: Object segmentation: Geometric Method. A) Radii of touching objects: R1 = 24, R2 = 30, R3 = 7; B) Result of segmentation using NucSegmentationCh1 = R1; C) Result of segmentation using NucSegmentationCh1 = R3.

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

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

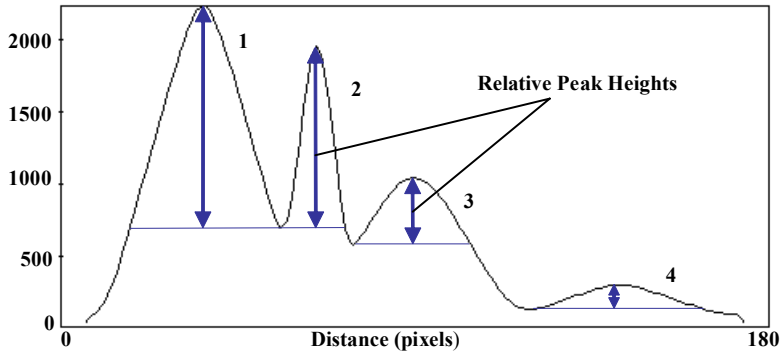

Figure 11: *Intensity profile of an object with four intensity peaks. Peak #1 height = 1500, peak #2 height = 1250, peak #3 height = 500, and peak #4 height = 200.*

Only two peaks in Figure 11, #1 and #2, have relative intensity greater than 1000. Thus, setting the **NucSegmentationCh1** parameter to -1000 marks only two peaks to be used for segmentation (or marks the two objects that need to be separated) and results in object segmentation as shown in Figure 12A. Setting the **NucSegmentationCh1** parameter to - 400 or -100 marks three or all four objects that need to be separated. The results of segmentation of three and four objects are shown in Figure 12B and 12C.

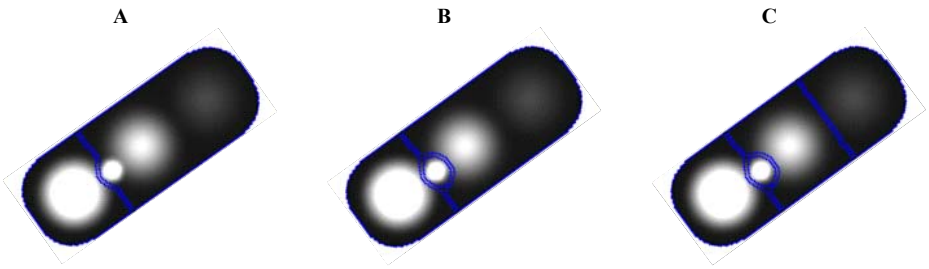

Figure 12: *Object segmentation: Intensity Method. A) Result of segmentation using parameter* ***NucSegmentationCh1*** *= -1000; B) Result of segmentation using parameter* ***NucSegmentationCh1*** *= -400; C) Result of segmentation using parameter* ***NucSegmentationCh1*** *= -100.*

Reject Border Objects

RejectBorderNucsCh1

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

Channel-Specific Assay Parameters: Measuring Intensity from Nuclei in Additional Channels

MaskModifierCh*N*

The Cell Cycle BioApplication can measure the total intensity and the total intensity ratios from the nuclear area in Channels 2-4. Occasionally, the cell cycle targets imaged in Channels 2-4 occupy a slightly different region of the nucleus (i.e., either more or less) than the labeled DNA in Channel 1. You have the option of independently modifying (i.e., expanding or contracting) the nuclear area in which these intensity measurements are made for each dependent channel (Channels 2-4). The Assay Parameter that is used to adjust the area in which to make measurements for Channels 2-4 is the **MaskModifierCh*N***, where Ch*N* is the relevant dependent channel (Channels 2-4). This Assay Parameter is the number of pixels added (or subtracted, depending on the parameter's sign) from the perimeter of the nuclear area identified in Channel 1 (Figure 13). Once intensity measurements have been made, the object will be further analyzed according to the criteria set by the Channel 2-4 Object Selection Parameters. The Channel 2-4 Object Selection Parameters are the Average and Total Intensities in the nuclear area for each specific channel. If an object's Average and Total Intensities in the nuclear region are within the range that you have specified, then the object is selected as a valid object for further analysis.

Please note that this Assay Parameter, not only in Channels 2-4, but also in Channel 5 and Channel 6, can be also used for gating purposes (see Gating section).

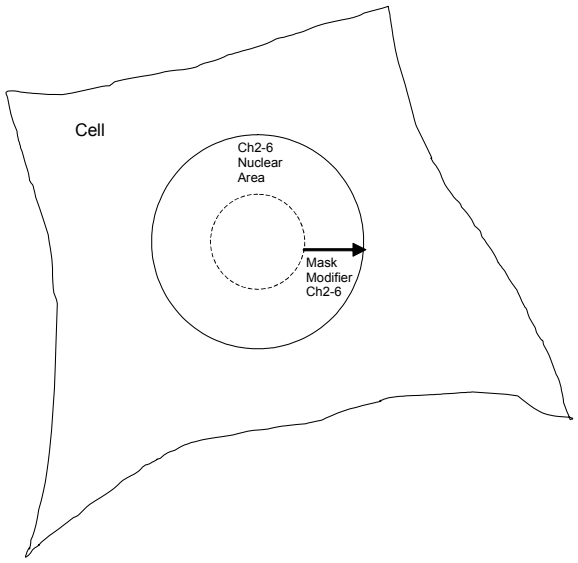

Figure 13. *Adjustment of* ***MaskModifierChN*** *Assay Parameter. In this example,* ***MaskModifierChN*** *is positive and the nuclear area has been expanded to make the intensity measurement. Note that "ChN" refers to Channels 2-6. Channels 2-6 can be adjusted for gating, while Channels 2-4 can be also used for calculating intensity ratios.*

Measuring the Peak Nuclear Total Intensity in Channel 1

Once the nuclear total intensity in Channel 1 is measured for each cell, the BioApplication identifies the peak nuclear total intensity. To do this, the intensities are plotted as a histogram and the intensity is identified where the histogram has the highest peak. In computing the histogram, the BioApplication automatically determines an optimal number of bins to use, with more objects being measured resulting in more bins. The size of each bin is the intensity range divided by the number of bins. However, you have the ability to specify a minimum number of bins to be used with the **MinHistoBinNumberCh1** Assay Parameter. This is useful when a few nuclei are measured, but you a priori know that the total intensity range is large requiring a larger number of bins. If you want to default to the BioApplication automatically for choosing the number of bins, you can enter a low value for the **MinHistoBinNumberCh1** Assay Parameter.

Identifying the 2N and 4N DNA Content Peaks

To identify the ranges of each of the DNA content categories, the BioApplication first identifies the position of the 2N DNA content peak. If you are not using Reference Wells, this is entered manually by specifying the value with the **2N_PeakIntenCh1** Assay Parameter. If you are using Reference Wells, the **Min** wells, wells containing cells that have not been treated with compound, are used for this operation. The nuclear total intensity distribution is assumed to resemble those shown in Figure 2 and Figure 3, where the main peak is from cells containing 2N DNA. This main peak in the reference well is identified in a similar manner as described in the "*Measuring the Peak Nuclear Total Intensity in Channel 1*" subsection above.

Next, the BioApplication identifies the position of the 4N DNA content peak. If you are not using Reference Wells, this is controlled by the **4N_PeakIntenFactorCh1** Assay Parameter. This Assay Parameter is the ratio of the total intensity at the 4N DNA content peak to the 2N DNA content peak. Its default value is 2 because 4N DNA content should have twice the DNA content as 2N DNA content. If you determine that, due to experimental conditions, the peak nuclear total intensity of G_2 cells is not exactly double the peak intensity of G_0/G_1 cells, you can adjust this parameter for the difference. For the example in Figure 5, this Assay Parameter was set to 2.1.

If you are using Reference Wells, the BioApplication looks for a secondary nuclear total intensity peak. If none is found, it also uses the **4N_PeakIntenFactorCh1** Assay Parameter to define where the 4N peak should be.

Identifying the Width of Each DNA Content Category

Once the locations of the 2N and 4N DNA content peaks have been determined, the range (i.e., width) of each of the five DNA content categories must be determined. Figure 14 helps demonstrate how this is done.

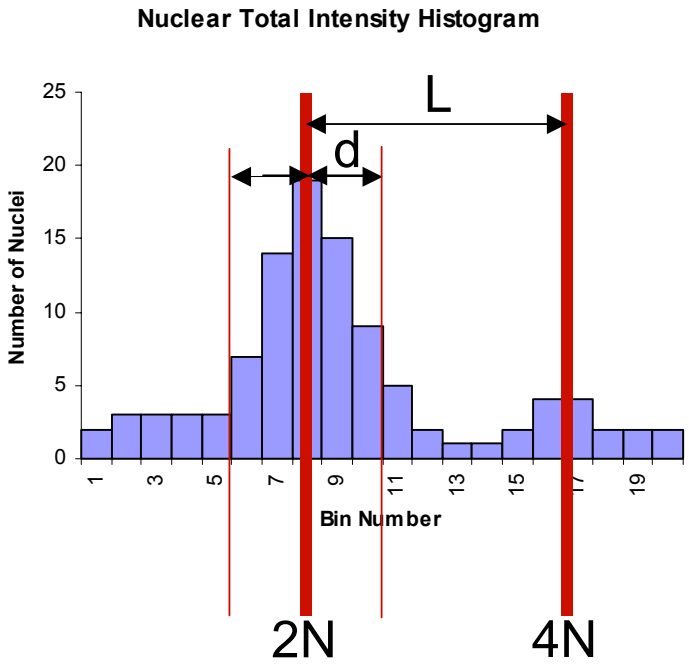

Figure 14. *Nuclear Total Intensity histogram acquired from DAPI labeled nuclei.*

Figure 14 shows a histogram of nuclear total intensities from an image of DAPI-labeled nuclei. The horizontal axis shows the different bin numbers and the vertical axis is the number of nuclei. The cells in this field were untreated and they had the classic bimodal distribution containing the 2N and 4N DNA content peaks. Two intensity ranges are defined in this figure. One is the intensity range between the 2N and 4N peaks, shown as "L" in the figure. The other, shown as "d", is the half-width of the 2N DNA content category, and is what must be determined.

The intensity range of cells containing 2N DNA content is defined as the intensity of the 2N DNA content peak ± d. The intensity range of cells containing 4N DNA content is similarly defined as the intensity of the 4N DNA content peak ± d. The intensity width, "d", is defined as a fraction of the intensity range "L" by the Assay Parameter **PeakWidthFractionCh1**, where: **PeakWidthFractionCh1** = d/L

Thus, entering a value of 0.2 for the **PeakWidthFractionCh1** Assay Parameter means that the half widths of the 2N and 4N DNA content distributions is 20% of the intensity range between these two peaks. The default value for **PeakWidthFractionCh1** is 0.25. This is based on the assumption that the intensity gap "L" between the 2N and 4N peaks contains the right half of the 2N distribution (i.e., "d"), the full intensity range of the S phase (which is assumed to be equal to the range of intensities of the G_0/G_1 or G_2/M phases, which is "2d"), and the left half of the 4N distribution (i.e., "d"). Assuming that the G_0/G_1, S, and G_2/M phases are of equal width, then "d" is 25% of the intensity range between the 2N and 4N peaks.

To summarize, the **PeakWidthFractionCh1** Assay Parameter is used to determine the ranges of each of the DNA content categories and is used whether or not Reference Wells are used.

Once the nuclear total intensity ranges have been determined for each DNA content category, the rest of the plate is scanned, and the total nuclear intensity measured for each cell in each well allows the cell to be assigned to one of these DNA content categories.

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 9). You will not be able to view any Advanced Assay Parameters in this mode (as the **Hide Advanced Parameters** option is checked).

Parameter	Units	Description
UseReferenceWells	Binary	Use reference wells to calculate high and low response levels: 0 = No, 1 = Yes (Well-based analysis directly from cell features), -1 = Yes (Field-based analysis followed by field to well analysis)
NucTypeCh1	Binary	Type of objects (nuclei) to be identified in Ch1: 0 = Bright objects on dark background, 1 = Dark objects on bright background
BackgroundCorrectionChN	Pixels	Radius (in pixels) of region used to compute background in ChN: Negative value = Use surface fitting, 0 = Do not apply background correction, Positive value = Use low pass filter
NucSmoothFactorCh1	Number	Degree of image smoothing (blurring) prior to object detection in Ch1: 0 = Do not apply smoothing
NucSegmentationCh1	Pixels	Radius (in pixels) of touching objects (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
RejectBorderNucsCh1	Binary	Reject objects (nuclei) that touch image edges: 0 = No, 1 = Yes
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
MinHistoBinNumberCh1	Number	Minimum number of bins in the cell NucTotalIntenCh1 histogram
2N_PeakIntenCh1	Intensity	Total intensity that defines the center of 2N histogram peak
4N_PeakIntenFactorCh1	Number	Factor that defines the center of 4N histogram intensity peak in units of 2N peak
PeakWidthFractionCh1	Fraction	Fraction of 2N...4N total intensity range used to define the width of 2N and 4N DNA content peaks

Table 9. *Basic Assay Parameters available for the Cell Cycle BioApplication. *Note that "ChN" refers to Channels 1-6 for* ***Background Correction*** *and Channels 2-6 for* ***Mask Modifier.***

Object Selection Parameters

Each channel has a set of specific Object Selection Parameters associated with it. If an object in a particular channel image has all measured features within the range specified by the appropriate Object Selection Parameters, then it is analyzed; otherwise, it is rejected from the analysis. The Object Selection Parameters for Channel 1 are used to select which of the fluorescently-labeled objects in Channel 1 pass the selection criteria. The objects are identified as individual valid nuclei and are worthy of further analysis. There are two categories for Channel 1 Object Selection Parameters, Intensity and Morphology (see Table 10).

The Average and Total Intensities of the object are the two intensity-related selection parameters. The morphology-related Object Selection Parameters are **NucAreaCh1**, **NucShapeP2ACh1**, and **NucShapeLWRCh1**. If an object's Average and Total Intensities

(for all channels), **NucAreaCh1**, **NucShapeP2ACh1**, and **ShapeLWRCh1** are all within the specified range, then the object is selected as a valid object for further analysis.

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

Table 10. *Object Selection Parameters available for Channel 1 with the Cell Cycle BioApplication*

The Object Selection Parameters for Channels 2-6 qualify whether the object is selected for further analysis (Table 11). Again, only objects whose intensities in Channels 2-6 within the cellular area that you have defined and that fall within the range that you have specified are selected for quantitation.

Object Selection Parameter	Units	Description
TotalIntenCh*N*	Intensity	Total intensity in Ch*N* of all pixels within modified Ch1 object (nucleus) mask
AvgIntenCh*N*	Intensity	Average intensity in Ch*N* of all pixels within modified Ch1 object (nucleus) mask

Table 11. *Object Selection Parameters available for the Cell Cycle BioApplication. Note that "ChN" refers to Channels 2-6.*

Gating

In addition to the measured parameters specific for cell cycle biology, you can also select only a subset of cells for analysis. This selection is done on the basis of the cell's intensity in a cellular area that you have defined, and is not limited to the nucleus. The intensity measurements to select the cells for analysis are done only on cells in Channels 2-6 that are within the total and average intensity range that you have specified. These intensities are measured in an area of the cell that you have defined in relation to the labeled nucleus in Channel 1 and can be made larger or smaller than the nuclear area (see Figure 13). The average and total intensities measured in this cellular area are also reported as Cell Features.

These are useful for monitoring the base intensity range of selected and rejected objects. For example, if you were making measurements in cells that expressed a GFP–protein chimera, and measurements were to be made only in cells that had a certain expression level, then you could use this dependent gating capability. You could set the intensity range to include only cells that had the desired expression level and use the average and total intensities in the primary object area in Channels 2-6 to monitor this. You could then make cell cycle measurements on these selected cells only.

Specifying Intensity Ranges for Gating

When working in Create Protocol View, you can specify intensity ranges by entering upper and lower limits for two intensity parameters, **AvgIntenCh*N*** and **TotalIntenCh*N***, for Channels 2-6. **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.

Specifying Mask Modifiers for Gating

The cellular area for the selection measurements is derived from the area covered by the nucleus and is defined by the Assay Parameter **MaskModifierCh*N***, where Ch*N* is the relevant dependent channel (Channels 2-6). This Assay Parameter is the number of pixels added to, or subtracted from, the perimeter of the nucleus to define the boundary enclosing the cellular region from where the measurements are made (mentioned previously in Figure 13).

Image Overlays

During a scan, various features can be displayed as color overlays on the channel images. 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. All Image Display Option Settings shown in Table 12 are available when running in Basic or Advanced Mode.

Parameter	Description
Include This Channel In Composite	Determines if the image for this channel is included in the composite image.
Selected Cell	Indicates the color of a ring placed on each nucleus identifying accepted objects.
Rejected Cell	Indicates the color of a ring placed on each nucleus identifying rejected objects.
TargetMaskCh*N*	Indicates the color of a ring placed on the selected area on each cell identifying where the intensity measurement for the Channel *N* target is made.

Table 12. *Image Overlays available for the Cell Cycle BioApplication. *Note that "ChN" refers to Channels 2-4.*

Assay Parameters for Population Characterization

Overview of Population Characterization

The Cell Cycle BioApplication can classify cells into subpopulations based on their measured Intrinsic Values. Population values are the cell property descriptions within the subpopulations. This enables you to characterize cells based on their response compared to a control population. Depending on the particular Intrinsic Value being analyzed, this BioApplication divides the cell population being characterized into five, three, or two subpopulation categories. DNA content is an example of the five-subpopulation classification categories of the entire cell population. Table 13 lists the Intrinsic Values that can be used in this BioApplication to characterize the cells into subpopulations.

Intrinsic Value	Reporting Channel(s)	Number of Subpopulations	Cell Populations Characterized
Total Intensity in Nucleus (DNA Content)	1	5	All cells in the well
Nuclear Size Average Intensity in Nucleus Variance Intensity in Nucleus	1	3	All cells in the well
Total Intensity in Nucleus	2, 3, 4	2	• All cells in the well • Cells in G_0/G_1 phase • Cells in S phase • Cells in G_2/M phase
Total Nuclear Intensity Ratio to Channel 2	3	2	
	4		

Table13. *Intrinsic Values that can be used to characterize cell populations in the Cell Cycle BioApplication.*

The boundaries identifying the distinct subpopulations for a particular Cell Feature can be determined and set using a control population that has the normal physiological distribution for that feature. For example, the five different categories of DNA content for cells can be determined from a control (i.e., untreated) population as shown in Figure 2 and Figure 3.

The Total Intensity in the nuclear region measured in Channels 2-4 and their ratios can be divided into two subpopulations (set from the ***Feature*Ch*N*LevelHigh** Assay Parameter); those that are above the defined level and those that are equal to or less than the defined level (see Figure 15). For example, if the cell cycle associated target being measured in Channel 2 is the phosphorylated state of Rb by indirect immunofluorescence with a phospho-Rb specific primary antibody, then cells in the G_0/G_1 phase will have a low Rb phosphorylation level, whereas cells in the S and G_2/M phases will have a high Rb phosphorylation level. An intensity threshold can be set to distinguish those cells with a high versus low Rb phosphorylation signal.

Nuclear size, average nuclear intensity, and variance nuclear intensity can be classified into three subpopulations to distinguish cells. Using nuclear size as an example, populations can be distinguished as those with normal nuclei versus condensed or fragmented nuclei (i.e., smaller or larger nuclear size respectively), and can be used as indicators of apoptosis or necrosis. ***Feature*Ch*N*LevelHigh** and ***Feature*Ch*N*LevelLow** Assay Parameter values set the upper

and lower bounds of the nuclear size, average nuclear intensity, and variance intensity ranges respectively (see Figure 16).

The Cell Status Feature for the two and three-subpopulation cases indicates whether a particular compound treated cell is within or beyond the normal physiological range identified by the level(s), as described in Tables 14 and 15 respectively.

Cell Status	Definition
0	feature within defined threshold(s)
1	feature > (upper) threshold

Table 14. *Cell Status Definitions for Channels 2-4 total intensities and ratios with the Cell Cycle BioApplication.*

Cell Status	Definition
0	feature within defined threshold(s)
1	feature > (upper) threshold
2	feature < lower threshold

Table 15. *Cell Status Definitions for Channel 1 nuclear size, average intensity, and variance intensity with the Cell Cycle BioApplication.*

The corresponding reported Well Features are the percent of cells that are greater or less than the thresholds defining this range **(%HIGH_** or **%LOW_** ***Feature*****Ch*****N*****)**. The following figures illustrate this concept by showing the distribution of a normal physiological population of cells for a particular Cell Feature and the shift in this distribution upon compound treatment for both the two (Figure 15) and three (Figure 16) subpopulation cases.

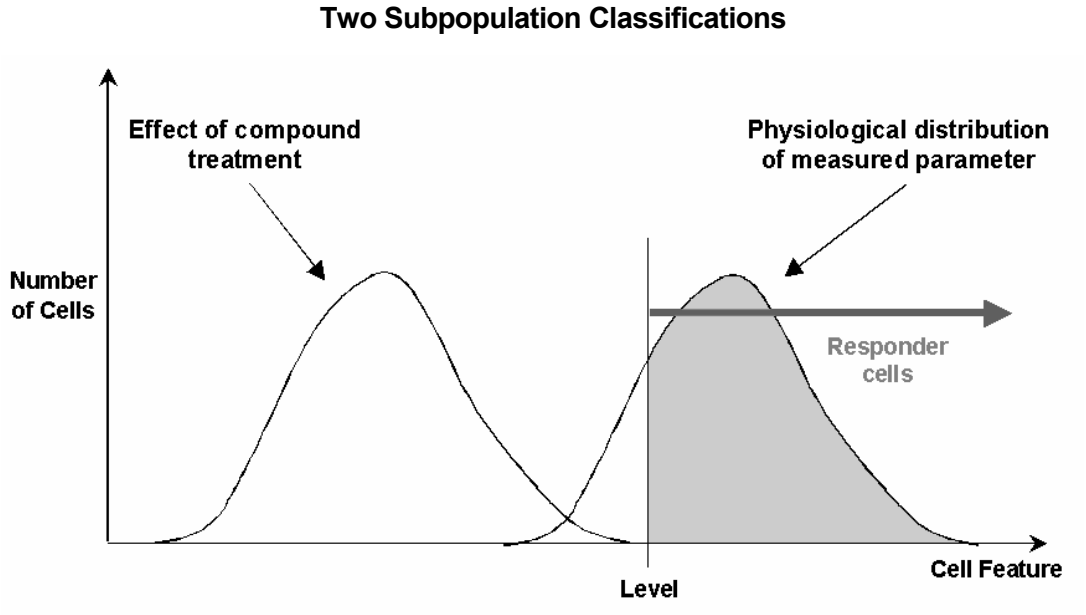

Figure 15. *POPULATION CHARACTERIZATION PRINCIPLE FOR TWO SUBPOPULATIONS. This represents the cell population divided into two subpopulations. Classification requires one level to distinguish baseline from the high level. Compound treatment causes the cell population to shift beyond the level.* ***NOTE:*** *Levels are entered manually or automatically from the Reference Wells.*

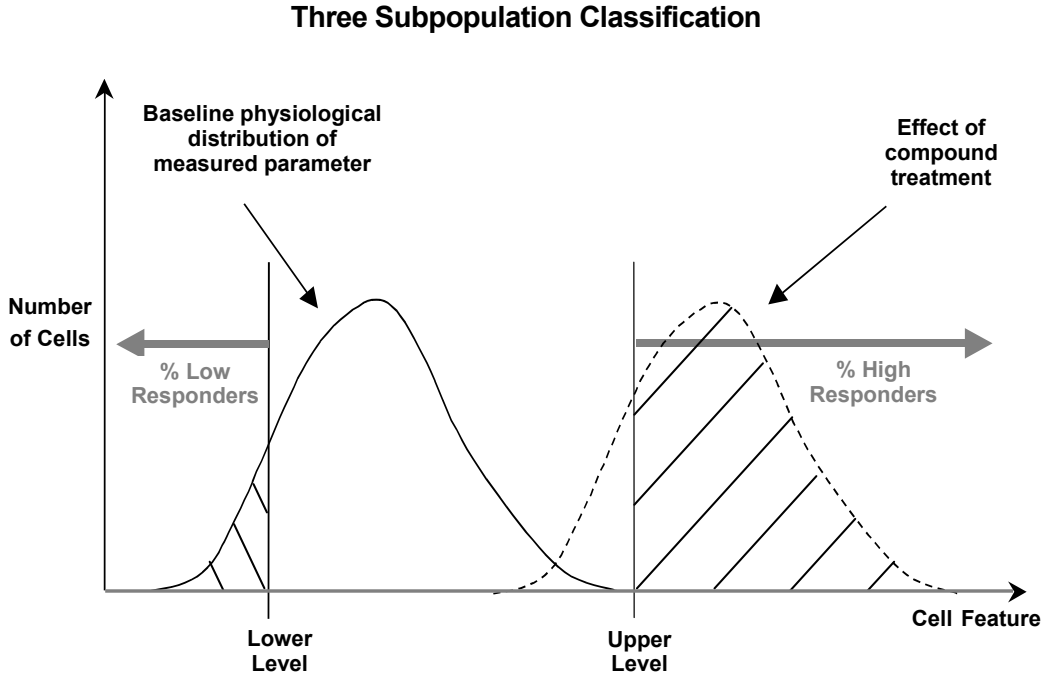

Figure 16. *POPULATION CHARACTERIZATION PRINCIPLE FOR THREE SUBPOPULATIONS. This represents the cell population divided into three subpopulations. Classification requires two separate levels to distinguish the normal subpopulation from the low and high subpopulations. The normal baseline population of the cells is defined by the two levels, and compound treatment causes the cell population to shift beyond these levels.* ***NOTE:*** *Levels are entered manually or automatically from the Reference Wells.*

Characterizing the Cell Population with DNA Content

Classifying a cell according to its DNA content is a specialized form of cell population characterization. In this case, there are five DNA content categories with which to classify the cells (Figure 3), versus the two or three categories used to classify the cells as shown in Figure 15 and Figure 16. Note that this BioApplication combines several of these classifications to further sub-categorize the cells, such as identifying cells that are in a particular cell cycle phase (G_0/G_1, S or G_2/M) and which have a subpopulation above or below a particular feature (such as Channel 2-4 intensities or intensity ratios).

Setting Cellular Response Levels

The Cell Cycle BioApplication offers two ways of setting levels that characterize the different cell population categories: manually or automatically. The first is by manually entering Assay Parameter values distinguishing the different populations (i.e., specifying the values of the upper and lower levels for the listed features and as shown in Figure 15 and Figure 16, or specifying the boundaries for the different DNA content values as shown in Figure 3) for the ***Feature*Ch*N*LevelHigh** (and for some features, the ***Feature*Ch*N*LevelLow**) Assay Parameters in the Protocol Create or Protocol Interactive Views (Advanced Mode). The Cell Cycle BioApplication then uses the levels that you have defined to calculate the population values of the cells in these different subpopulations.

To automatically determine the levels, the BioApplication uses Reference Wells. You can designate particular wells on the sample plate as Reference Wells. Typically, Reference Wells contain a control population of cells which displays the normal basal physiological distribution for the Assay Parameter being measured. These wells are first analyzed and the population distribution for the different features are determined. The cell population characterization levels are then specified through use of a correction coefficient (_CC) value that is calculated using specific rules applied to the distribution of the features measured from all the cells in the Reference Wells. The whole plate is then scanned with the calculated levels applied. The advantage of using Reference Wells to automatically calculate thresholds is that the thresholds are determined by a control population of cells and are independent of run-to-run variations such as different illumination conditions, fluorophore levels, or changes in cell conditions.

Reference Wells Processing Sequence

By setting the **UseReferenceWells** Assay Parameter to **1**, the Reference Wells processing is engaged. Within the specified wells, the listed fields are acquired/analyzed, and Field-, Well-, and Plate-level Reference Features are computed. The BioApplication first determines the ranges of the Channel 1 nuclear total intensities that define the different DNA content categories. Again, this information is either manually computed from Assay Parameters that you entered or automatically determined from **Min** and **Max** type Reference Wells. The computed values will be loaded and regular scanning of the plate will begin. Reference Features are computed on a well basis. The sequence of computation for Reference Wells is:

1. Cell Features are computed for every valid object within every field in a well.
2. For each Cell Feature to be used for population characterization the distribution's mean and standard deviation are computed over all cells in the well.
3. Reference Well features are determined.
4. Reference Plate Features are computed as arithmetic average for wells on a plate.

Setting the **UseReferenceWells** Assay Parameter to **-1** allows to engage an alternative version of the analysis where Reference Features are computed on a field basis. The sequence of computation for Reference Wells using the alternative approach is:

1. Cell Features are computed for every valid object within a field.
2. For each Cell Feature to be used for population characterization the distribution's mean and standard deviation are computed over all cells in the field.
3. Reference Field features are determined.
4. Reference 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

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

NOTE

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

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

The first step in assigning cells to different subpopulations is specifying whether Reference Wells are to be used. The Assay Parameters controlling the use of Reference Wells are: **UseReferenceWells**, **MinRefWellMinAvgCellCountPerField**, and **MaxRefWellMinAvgCellCountPerField**. The **UseReferenceWells** Assay Parameter allows you to choose whether Reference Wells are to be used to determine the population characterization levels.

- **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.

The Cell Cycle BioApplication uses two types of Reference Wells called **Min** and **Max** Reference Well types. If Reference Wells are to be used, then the parameters **MinRefWellMinAvgCellCountPerField** and **MaxRefWellMinAvgCellCountPerField** must be set. These are the minimum number of objects detected per field that are required for acceptance of the data in the **Min** and **Max** Reference Well types respectively. This allows you to enter the minimum number of objects that you feel give an appropriate distribution and, thus, give statistical validity to the levels calculated from the Reference Wells. If the number of objects detected per field for either type of Reference Well is less than the minimum number specified (for **MinRefWellMinAvgCellCountPerField** and **MaxRefWellMinAvgCellCountPerField** Assay Parameters), then the BioApplication uses the manually entered level values for the appropriate population characterization thresholds.

Table 16 summarizes the differences between these two types of Reference Wells as well as the features they influence.

Reference Well Type	Channel & Well Treatment	Features for which Thresholds are Calculated	Rules for Computing Thresholds
Min	Channel 1 Wells are untreated	DNA Content	• The 1st and 2nd Total Intensity peaks that are found are identified as DNA=2N & 4N respectively • User-defined parameter to compute distribution widths to define thresholds for different DNA content categories
		Nuclear Size Nuclear Avg Intensity Nuclear Var Intensity	• Distribution's mean and standard deviation calculated • User specifies a "correction coefficient" (i.e., number of standard deviations from mean to define the population) • Lower and upper thresholds set at the mean plus and minus the standard deviation multiplied by the "correction coefficient"
Max	Channels 2-4 Well is compound Treated	Total Intensity in Nucleus Total Nuclear Intensity Ratio	• Distribution's mean and standard deviation calculated • User specifies a "correction coefficient" (i.e., number of standard deviations from mean to define the population) • Threshold set at mean plus the standard deviation multiplied by the "correction coefficient"; "correction coefficient" sign determines whether threshold is to the right or left of mean

Table 16. *Types of Reference Wells available for the Cell Cycle BioApplication.*

Levels for the nuclei's DNA content, size, average intensity, and variance intensity are obtained from Channel 1 images and are computed from the **Min** Reference Wells. The **Min** Reference Wells must contain a control (i.e., non-compound treated) population of cells that display the normal basal physiological distribution for the cell DNA content and size. Thresholds for the targets measured in Channels 2-4 are computed from the **Max** Reference Wells. The **Max** Reference Wells are typically treated with a compound that restricts the cells to a particular cell cycle phase or causes specific cell cycle associated target proteins to have a particular expression level or state. If needed for the particular targets being measured, you can alternatively designate untreated wells as **Max** Reference Wells. The Cell Features used to characterize various levels are associated with Assay Parameters in the form:

- ***Feature*Ch*N*LevelLow**
- ***Feature*Ch*N*LevelHigh** (when Reference Wells are not used)
  OR
- ***Feature*Ch*N*LevelLow_CC**
- ***Feature*Ch*N*LevelHigh_*CC*** (when Reference Wells are used)

where ***Feature*** refers to the name of the Cell Feature and ***N*** refers to the specific channel. The ***Feature*Ch*N*LevelLow, *Feature*Ch*N*LevelHigh** Assay Parameters specify the actual levels and must be manually entered if Reference Wells are not used. Note that total intensity and

ratios for Channels 2-4 only contain a ***Feature*Ch*N*LevelHigh.** The ***Feature*Ch*N*LevelLow_CC, *Feature*Ch*N*LevelHigh_CC** Assay Parameters are a correction coefficient (CC) used to derive the ***Feature*Ch*N*LevelLow, *Feature*Ch*N*LevelHigh** values from the mean and standard deviation of the reference well population according to the formulas:

- ***Feature*Ch*N*LevelLow** = Mean - ***Feature*Ch*N*LevelLow**_CC × SD
- ***Feature*Ch*N*LevelHigh** = Mean + ***Feature*Ch*N*LevelHigh**_CC × SD

Channel 1 features such as nuclear size, average intensity, and variance intensity are computed from the **Min** Reference Wells and are untreated wells. The other feature levels (total intensity and ratio from Channels 2-4) are computed from the compound-treated **Max** Reference Wells. For these features, the instrument first images these wells and determines the population distribution for the different features. The cell population characterization thresholds are then set by adding and subtracting from the mean of the distribution its standard deviation multiplied by a coefficient known as a Correction Coefficient (_CC). The instrument then scans the whole plate and applies these thresholds. 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.

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.

The total nuclear intensity distribution derived from the images in Channel 1 (from the non-compound treated **Min** Reference Wells) is used to automatically compute the boundaries for the different DNA content categories. To do this, the first two peaks in the distribution are found and identified as being the peaks for cells with 2N and 4N DNA respectively. The ratio of the Total Intensity Ratio of the 4N peak to the 2N peak is also computed. If a second peak is not found, then the center of the distribution of cells with 4N DNA is defined as the 2N peak's intensity multiplied by the value you entered for the 4N to 2N Total Intensity Ratio (typically 2). To define the range and thresholds for the different DNA content categories, the centers of the 2N and 4N peaks are multiplied by a parameter that you defined (manually or from Reference Wells), which computes the widths of the distributions automatically.

Advanced Assay Parameters

When running in Advanced Mode, all basic input as well as advanced input parameters are editable. The **Hide Advanced Features** checkbox will hide or show all 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. Below are further explanations to help understand specific Advanced Assay Parameters available with the Cell Cycle BioApplication.

NOTE

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

For each feature undergoing population characterization, there are either two or four advanced Assay Parameters that control its levels. Two Assay Parameters would consist of the Level High and its _CC designating the level set using Reference Wells. Four Assay Parameters would consist of Level Low and Level High and the presence of their **_CC** suffix designating the levels set using Reference Wells. For example, the Assay Parameters controlling nuclear size in Channel 1 are:

- **NucSizeCh1LevelLow**
- **NucSizeCh1LevelHigh**
- **NucSizeCh1LevelLow_CC**
- **NucSizeCh1LevelHigh_CC**

In the listing of Advanced Parameters in Table 17, instead of listing all two or four level parameters for each feature, one entry for the feature will be listed giving the four different options, as shown in the following example for the Channel 1 nuclear size:

- NucSizeCh1Level***Low/High, Low/High_CC***

If only two advanced Assay Parameters that control its levels are listed (***Feature*Ch*N*LevelHigh** and corresponding correction coefficient), the following will be given (as for Total Intensity in Channel 2):

- TotalIntenCh2Level***High, High_CC***

Assay Parameter	Units	Description
MinRefWellMinAvgCellCountPerField	Number	Minimum average number of cells per field required for acceptance of MIN reference well results
MaxRefWellMinAvgCellCountPerField	Number	Minimum average number of cells per field required for acceptance of MAX reference well results
UseMicrometers	Binary	Measure lengths and areas in: 0 = Pixels, 1 = Micrometers
PixelSize	Number	Pixel size in micrometers (depends on objective selection)
NucSizeCh1Level*Low/High, Low/High_CC*	Number	Defines **NucSizeCh1** population characterization thresholds
NucAvgIntenCh1Level*LowHigh, Low/High_CC*	Number	Defines **NucAvgIntenCh1** population characterization thresholds
NucVarIntenCh1Level*Low/High, Low/High_CC*	Number	Defines NucVarIntenCh1 population characterization thresholds
TotalIntenCh*N*Level*High, High_CC*	Number	Defines **TotalIntenCh*N*** population characterization thresholds
TotalIntenRatioCh3Ch2Level*High, High_CC*	Number	Defines **TotalIntenRatioCh3Ch2** population characterization thresholds
TotalIntenRatioCh4Ch2Level*High, High_CC*	Number	Defines **TotalIntenRatioCh4Ch2** population characterization thresholds

Table 17. *Advanced Assay Parameters available for the Cell Cycle BioApplication. *Note that "ChN" refers to Channels 2-4.*

Description of Output Features

Output Features are the biological measurements produced by the BioApplication. All features are categorized and accessible using the View application (see the appropriate View User's Guide). Additionally, a subset of features (Well Features) are listed in the Scan Plate View and Create Protocol View so that screening results can be viewed concurrently with scanning. The ability to view data during the scanning process allows you to immediately verify success of the run, well by well. The Cell and Well Output Features for the Cell Cycle BioApplication are listed and defined below.

Nuclear Size and Status

Three categories of quantitative measurements are reported for the labeled nuclei in Channel 1:

- Intensity
- Morphology & Location
- Cell Number and Density

Intensity measurements were described previously. In the category of morphology and location measurements, the area of each nucleus is reported in pixels or in micrometers. In addition to the area, shape parameters such as the roundness of the nucleus (P2A), aspect ratio (LWR), and size (defined as its equivalent diameter in this BioApplication) are also reported. The location of the nucleus in the image is reported by the *x* and *y* coordinates of its centroid.

Often, treatment of a cell with a compound that affects the cell cycle will result in apoptosis or necrosis. The nuclear size can indicate whether nuclear condensation or fragmentation, which often accompanies necrosis or apoptosis, has occurred. For each cell, the status of each nucleus size is also reported by the BioApplication. The status parameters indicate whether the cell is within the bounds of the population characterization thresholds for a particular parameter. This can be used to identify cells whose nuclear size is beyond the size range of normal cells (i.e., population values) and whether an apoptotic event has occurred. The use of status parameters was described in the previous section on Population Characterization.

Nuclear Variation Intensity

The Cell Cycle BioApplication is able to provide a measure of the intensity texture in the nucleus. It is an Intrinsic Value based on the standard deviation of the intensities of all pixels that fall under the selected cell mask in Channel 1 only. It is reported as **NucVarIntenCh1.** This parameter and the associated statistics, **MEAN_NucVarIntenCh1**, **SD_NucVarIntenCh1**, **SE_NucVarIntenCh1**, **CV_NucVarIntenCh1**, can give a better understanding of the margination within the nucleus. Intensity variation could be used an additional proof of correct phase identification.

Expression Level or State of Cell Cycle Associated Proteins

You can choose additional channels to evaluate the state or expression level of up to three additional cell cycle associated target proteins.

For each cell, total and average intensity measurements from the nuclear area for each channel (Channels 2-4) are made. The total intensity is the sum of the intensities of all the individual pixels in the nucleus. The average intensity is equal to the total intensity divided by the number of pixels in the region.

$$\text{Average Intensity} = \frac{\text{Total Intensity in the nucleus}}{\text{Number of Pixels in the nucleus}}$$

For Channels 2-4, the total intensities have associated status parameters which can be used to classify and characterize the individual cells in the population (i.e., Population Values - see section on Population Characterization).
At the well-level, the BioApplication reports various statistics of the total nuclear intensity in Channels 2-4 including the mean, standard deviation, standard error, and coefficient of variation. These statistical calculations (i.e., population values) are computed for the following four populations of cells in the well:

- All cells in the well
- Only cells that are in the G_0/G_1 phase
- Only cells that are in the S phase
- Only cells that are in the G_2/M phase

Also computed for each of these four populations is the percentage of all cells that are in the particular population and also have Channel 2-4 intensity higher or lower than the threshold that you have set. Reporting these well-level measurements just for cells within a particular cell cycle phase serves as a quantitative characterization of the dependency and relationship between cells in a particular cell cycle phase and the amount of the cell cycle target being measured.

Intensity Ratios between Channels

If the BioApplication is run with more than two channels, then for each cell, the total intensity measured in the nuclear region for the additional channels (i.e., Channel 3 and Channel 4) is normalized by the total intensity measured in the nuclear region for Channel 2 and reported as a ratio. These intensity ratios also have associated status parameters that can be used to classify and characterize the individual cells in the population (see section on *Population Characterization*).

At the well-level, the BioApplication reports various statistics of the total nuclear intensity ratios including the mean, standard deviation, standard error, and coefficient of variation. These statistical calculations (i.e., population values) are computed for the four populations of cells in the well as listed above.

Also computed for each of these four populations is the percentage of all cells that are in the particular population and have a Channel 2-4 intensity ratio higher or lower than the threshold that you have set. Reporting the normalized total intensity in the nuclear region between different cell cycle targets enables the monitoring of changes in a cell cycle target's state. For example, measurement of phosphorylated Rb and total Rb in Channel 2 and Channel 3, as well as computation of their intensity ratio, will allow monitoring of the degree of Rb phosphorylation, which is an important component in regulating the transition from the G_1 to S phase. Reporting these ratios for cells within a particular cell cycle phase serves as a quantitative characterization of the dependency and relationship between cells in the cell cycle phase and the normalized amount of the cell cycle target being measured.

Cell Features

Table 18 shows the Cell Features reported for the Cell Cycle BioApplication, accessible in the Cell Feature window in the Protocol Interactive and View applications.

Feature	Units	Description
Cell#	Number	Unique object (nucleus) ID
Top	Pixels	Y coordinate (in pixels) of top-left corner of image-aligned bounding box of Ch1 object (nucleus)
Left	Pixels	X coordinate (in pixels) of top-left corner of image-aligned bounding box of Ch1 object (nucleus)
Width	Pixels	Width (in pixels) of image-aligned bounding box of Ch1 object (nucleus)
Height	Pixels	Height (in pixels) of image-aligned bounding box of Ch1 object (nucleus)
XCentroid	Pixels	X coordinate (in pixels) of center of Ch1 object (nucleus)
YCentroid	Pixels	Y coordinate (in pixels) of center of Ch1 object (nucleus)
NucAreaCh1	Pixels or μm^2	Area (in pixels or micrometers) of Ch1 object (nucleus)
NucShapeP2ACh1	Number	Shape measure based on ratio of perimeter squared to 4π*area of Ch1 object (nucleus) (**NucShapeP2ACh1** = 1 for circular object)
NucShapeLWRCh1	Number	Shape measure based on ratio of length to width of object-aligned bounding box of Ch1 object (nucleus)
NucSizeCh1	Pixels or μm	Diameter (in pixels or micrometers) of circle with area equal to area of Ch1 object (nucleus)
NucSizeCh1Status	Number	**NucSizeCh1** status: 0 = No response, 1 = High response, 2 = Low response
NucAvgIntenCh1	Intensity	Average intensity of all pixels within Ch1 object (nucleus)
NucAvgIntenCh1Status	Number	**NucAvgIntenCh1** status: 0 = No response, 1 = High response, 2 = Low response
NucVarIntenCh1	Intensity	Standard deviation of intensity of all pixels within Ch1 object (nucleus)
NucVarIntenCh1Status	Number	**NucVarIntenCh1** status: 0 = No response, 1 = High response, 2 = Low response
NucTotalIntenCh1	Intensity	Total intensity of all pixels within Ch1 object (nucleus)
NucTotalIntenCh1Status	Number	**NucTotalIntenCh1** status: 1 = Below 2N, 2 = Within 2N, 3 = Between 2N and 4N, 4 = Within 4N, 5 = Above 4N
TotalIntenCh*N*	Intensity	Total intensity in Ch*N* of all pixels within modified Ch1 object (nucleus) mask
TotalIntenCh*N*Status	Binary	**TotalIntenCh*N*** status: 0 = No response, 1 = High response
AvgIntenCh*N*	Intensity	Average intensity in Ch*N* of all pixels within modified Ch1 object (nucleus) mask
TotalIntenRatioCh3Ch2	Number	Ratio of **TotalIntenCh3** to **TotalIntenCh2**
TotalIntenRatioCh3Ch2Status	Binary	**TotalIntenRatioCh3Ch2** status: 0 = No response, 1 = High response
TotalIntenRatioCh4Ch2	Number	Ratio of **TotalIntenCh4** to **TotalIntenCh2**
TotalIntenRatioCh4Ch2Status	Binary	**TotalIntenRatioCh4Ch2** status: 0 = No response, 1 = High response

Table 18. *Cell Features available for the Cell Cycle BioApplication. *Note that "ChN" refers to Channels 2-6.*

Well Features

Many general Well Features are derived from the Cell Features. Such features are identified by a prefix, as listed in Table 19, to the Cell Feature name.

Feature Prefix	Well Feature Definition	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 CircSpotCountRatioCh6Ch5 for all objects selected for analysis in the well	Percent
%HIGH_	Percentage of selected objects in the well with Feature_X above high-response level	Percent
%LOW_	Percentage of selected objects in the well with Feature_X below low-response level	Percent

Table 19. *General Well Feature Definitions.*

Specific well features referring to the cell cycle (G_0/G_1, S, and G_2/M) are also given as MEAN, SD, SE, CV for **NucTotalIntenCh1**, and additional %HIGH and %LOW for **TotalIntenChN**, **TotalIntenRatioCh3Ch2**, and **TotalIntenRatioCh4Ch2**. Table 20 lists these specific cell cycle features (without the prefix).

Feature	Description
G1_NucTotalIntenCh1	**NucTotalIntenCh1** for all cells in G1 phase in the well
S_NucTotalIntenCh1	**NucTotalIntenCh1** for all cells in S phase in the well
G2_NucTotalIntenCh1	**NucTotalIntenCh1** for all cells in G2 phase in the well
G1_TotalIntenCh*N*	**TotalIntenCh*N*** for all cells in G1 phase in the well
S_TotalIntenCh*N*	**TotalIntenCh*N*** for all cells in S phase in the well
G2_TotalIntenCh*N*	**TotalIntenCh*N*** for all cells in G2 phase in the well
G1_TotalIntenRatioCh3Ch2	**TotalIntenRatioCh3Ch2** for all cells in G1 phase in the well
S_TotalIntenRatioCh3Ch2	**TotalIntenRatioCh3Ch2** for all cells in S phase in the well
G2_TotalIntenRatioCh3Ch2	**TotalIntenRatioCh3Ch2** for all cells in G2 phase in the well
G1_TotalIntenRatioCh4Ch2	**TotalIntenRatioCh4Ch2** for all cells in G1 phase in the well
S_TotalIntenRatioCh4Ch2	**TotalIntenRatioCh4Ch2** for all cells in S phase in the well
G2_TotalIntenRatioCh4Ch2	**TotalIntenRatioCh4Ch2** for all cells in G2 phase in the well

Table 20. *Specific cell cycle features given for the Cell Cycle BioApplication. *Note that "ChN" refers to Channels 2-4.*

The Cell Cycle BioApplication also reports the following Well Features in the Scan Plate View in addition to the Well Detail window of the View application (Table 21).

Feature	Description
ValidCellCount	Number of valid objects (cells) identified in the well (Ch1 object selection parameters applied)
SelectedCellCount	Number of valid cells selected for analysis in the well (Ch2-6 object selection parameters applied)
%SelectedCells	Percentage of valid objects (cells) selected for analysis in the well

Feature	Description
ValidFieldCount	Number of fields in which objects (cells) were selected for analysis in the well
SelectedCellCountPerValidField	Average number of objects (cells) selected for analysis per valid field in the well
PEAK_NucTotalIntenCh1	Peak value for NucTotalIntenCh1 of 2N peak frequency for all selected cells
CellCount_Below2N_Ch1	Number of cells with total intensity in Ch1 below 2N range
CellCount_2N_Ch1	Number of cells with total intensity in Ch1 within 2N range
CellCount_2N_4N_Ch1	Number of cells with total intensity in Ch1 between 2N and 4N ranges
CellCount_4N_Ch1	Number of cells with total intensity in Ch1 within 4N range
CellCount_Above4N_Ch1	Number of cells with total intensity in Ch1 above 4N range
CellCountRatio_2N_4N_Ch1	Ratio of 2N to 4N cell counts in Ch1
%Cell_Below2N_Ch1	Percentage of cells with total intensity in Ch1 below 2N range
%Cell_2N_Ch1	Percentage of cells with total intensity in Ch1 within 2N range
%Cell_2N_4N_Ch1	Percentage of cells with total intensity in Ch1 between 2N and 4N ranges
%Cell_4N_Ch1	Percentage of cells with total intensity in Ch1 within 4N range
%Cell_Above4N_Ch1	Percentage of cells with total intensity in Ch1 above 4N range

Table 21. *Well Features available for the Cell Cycle BioApplication. *Note that "ChN" refers to Channels 2-4.*

Reference Well Features

The Cell Cycle BioApplication reports the features described in Table 22 in the Scan Plate View as well as the Well Feature window of the View application. These features are computed and reported only when the value of the Assay Parameter **UseReferenceWells** Assay Parameter = **1**.

Feature	Description
RefWellFlag	Flag indicates reference well types (MIN and/or MAX) used
MinRefWellAvgCellCountPerField	Average number of objects (cells) per field in MIN reference wells
MaxRefWellAvgCellCountPerField	Average number of objects (cells) per field in MAX reference wells
RefNucSizeCh1Level*Low/High*	Low/High-response level computed from reference well results for **NucSizeCh1**
RefNucAvgIntenCh1Level*Low/High*	Low/High-response level computed from reference well results for **NucAvgIntenCh1**
RefNucVarIntenCh1Level*Low/High*	Low/High-response level computed from reference well results for **NucVarIntenCh1**
Ref2N_PeakIntenCh1	2N peak center (in intensity units) of **NucTotalIntenCh1** cell histogram computed from reference well results
Ref4N_PeakIntenFactorCh1	Factor that defines the center of 4N intensity peak in units of 2N peak computed from reference well results
RefTotalIntenCh*N*LevelHigh	High-response level computed from reference well results for **TotalIntenCh*N***
RefTotalIntenRatioCh3Ch2LevelHigh	High-response level computed from reference well results for **TotalIntenRatioCh3Ch2**
RefTotalIntenRatioCh4Ch2LevelHigh	High-response level computed from reference well results for **TotalIntenRatioCh4Ch2**

Table 22. *Reference Well Features available for the Cell Cycle BioApplication. *Note that "ChN" refers to Channels 2-4. Listing of* ***RefFeatureLevelHigh, LevelLow*** *is noted as* ***"Low/High"***

Chapter

3

iDev Software Workflow

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

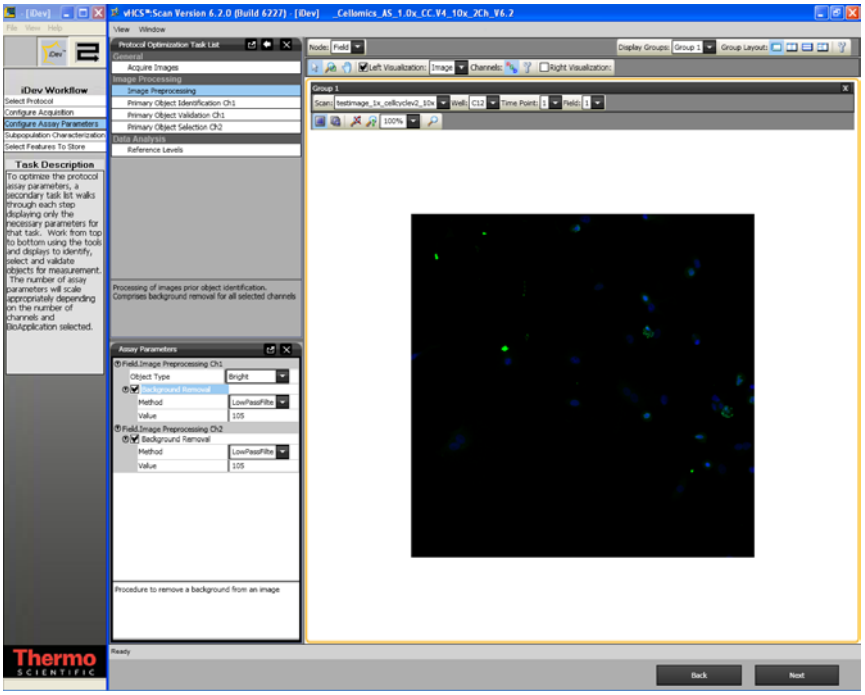

Figure 17. *Protocol Optimization Task – Image Preprocessing*

Primary Object Identification Ch1

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

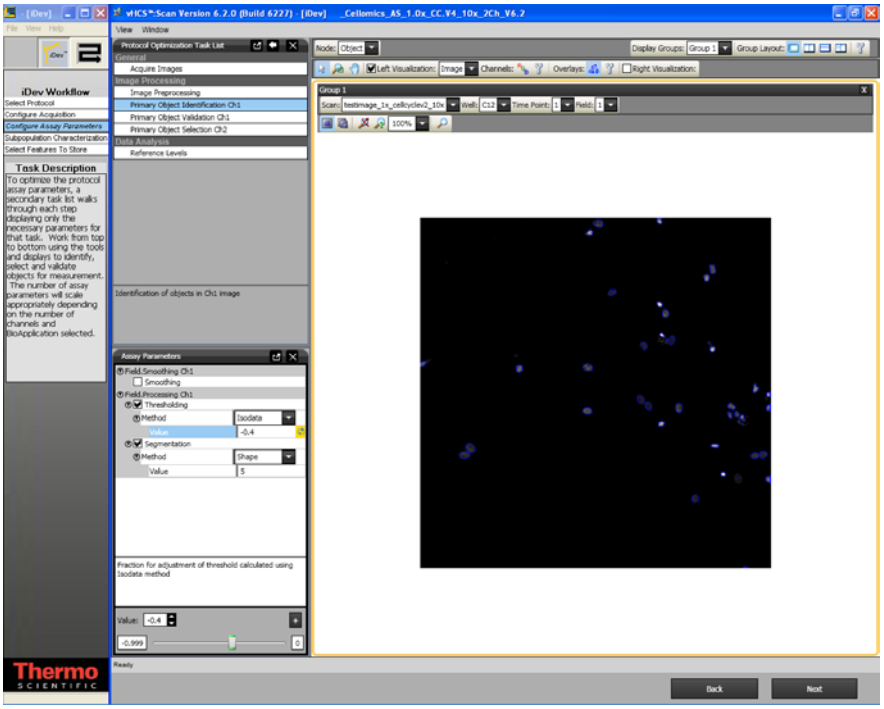

Figure 18. *Protocol Optimization Task – Primary Object Identification Ch1*

Primary Object Validation Ch1

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

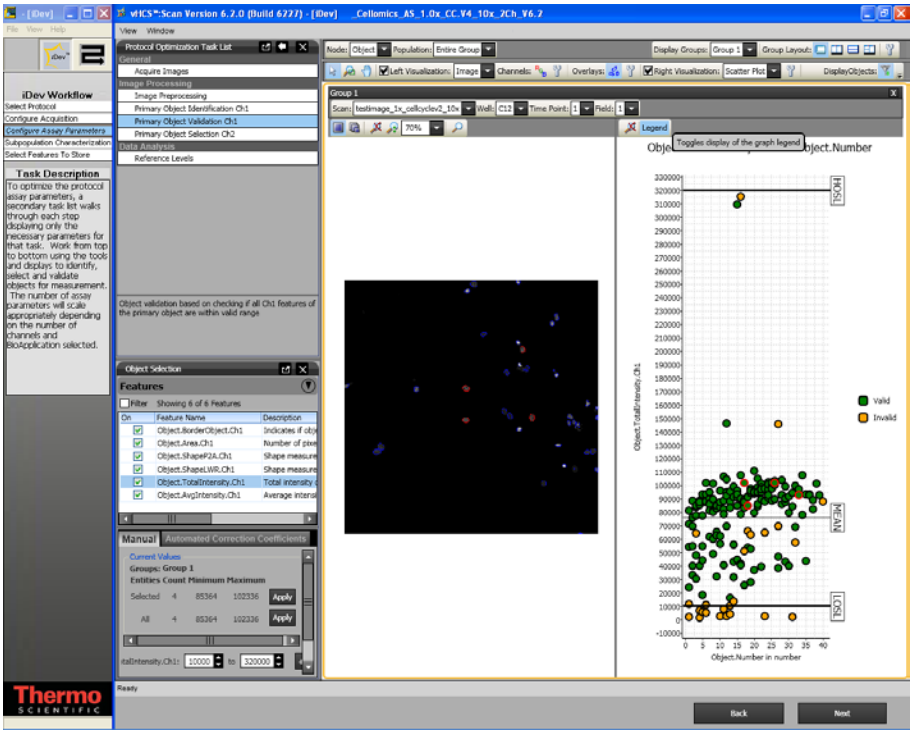

Figure 19. *Protocol Optimization Task – Primary Object Validation Ch1*

Primary Object Selection Ch2 through ChN

Primary Object Selection is object selection based on intensity features computed in Channels 2 through Channel N under the circ mask derived from the Channel 1 primary object mask. In this task, you will set selection / rejection of the primary object based on intensity measuremenst 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.

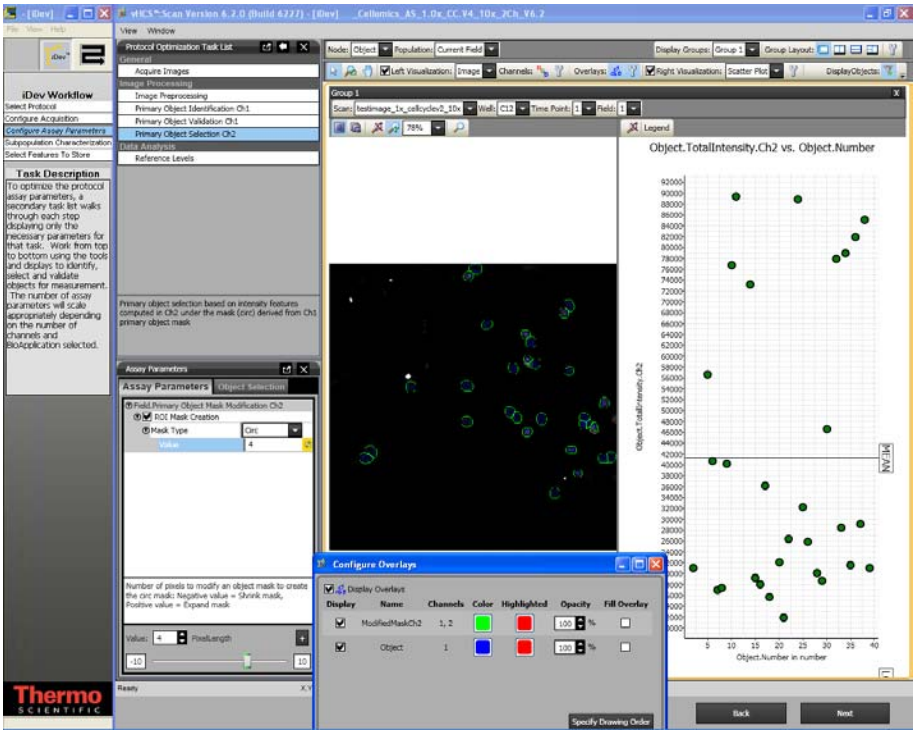

Figure 20. *Protocol Optimization Task – Primary Object Selection Ch2 through ChN*

Reference Levels

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

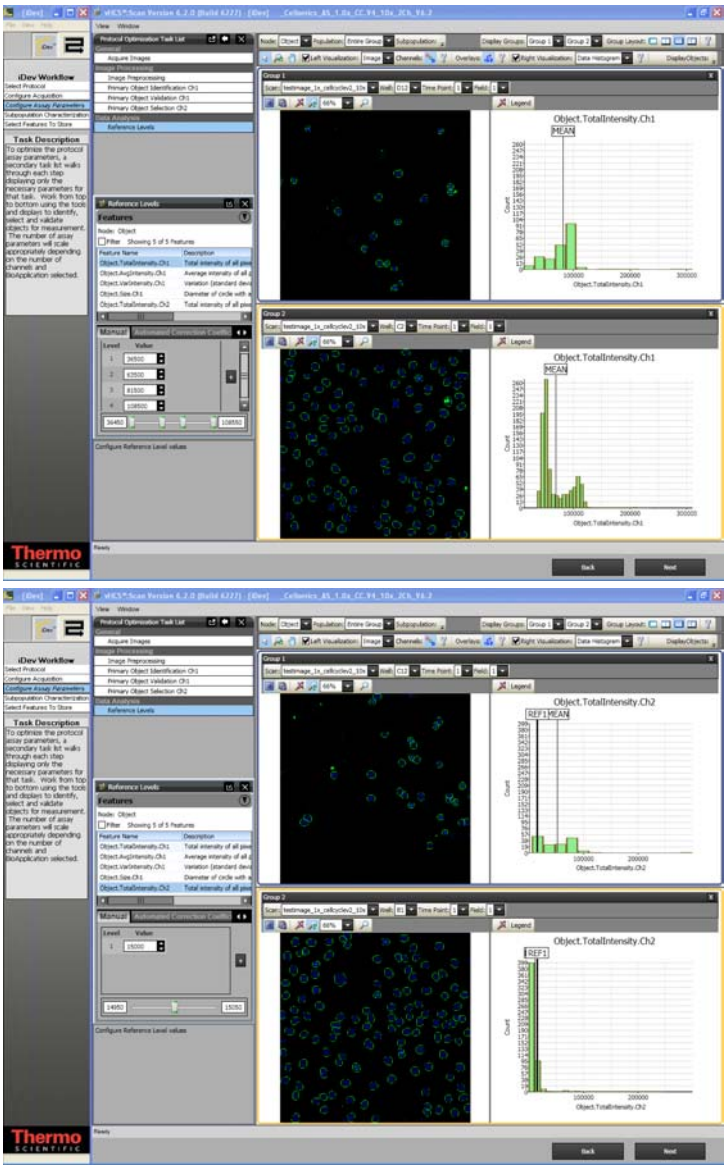

Figure 21. *Protocol Optimization Task – Reference Levels*

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