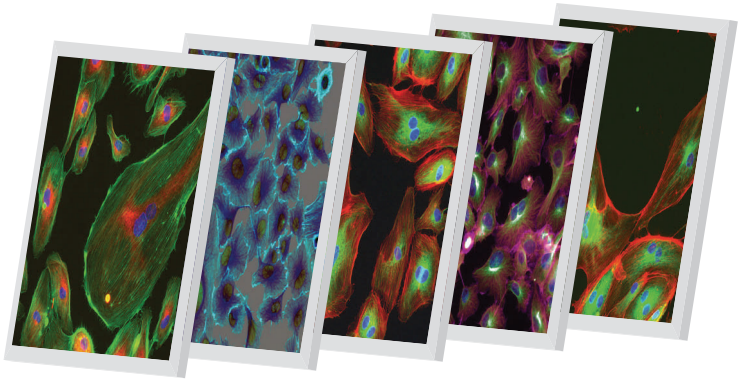
Thermo Scientific Cellomics® Compartmental Analysis V4

BioApplication Guide



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Cellomics® Compartmental Analysis BioApplication Guide

V4 Version

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Chapter

1

Overview of the Compartmental Analysis BioApplication

High Content Screening (HCS) uses fluorescence-based reagents, advanced instrumentation, and sophisticated image analysis software (BioApplications) to analyze and quantify targets and physiological processes in cells. This guide provides a brief description for using one such versatile and flexible BioApplication, Compartmental Analysis, which can be applied to many different biological situations. This guide contains the following chapters:

Chapter 1 provides an overview of the Compartmental Analysis BioApplication with example biology.

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

Chapter 3 provides guidance on how to specifically adjust settings for different biological use cases as well as an overview of the Event Wizard.

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

System Compatibility

The Compartmental Analysis BioApplication described in this guide is designed to run on the following platforms:

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

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

Cell Biology Background

The movements of, or changes in, the presence of macromolecules in or between particular cellular regions, compartments, or organelles are frequent and widespread in the functioning of cells. Important physiological examples of these phenomena include the internalization and intracellular trafficking of macromolecules, the movement of macromolecules between different intracellular regions, such as between the cytoplasm and the nucleus, the expression or accumulation of particular proteins or macromolecules in specific organelles, such as the nucleus or Golgi apparatus, and the change in the concentrations of particular ions such as Ca^{2+}

or H^+ in specific cellular regions. Often, particular events can initiate several of these phenomena either simultaneously or in a cascade.

Compartmental Analysis is an HCS BioApplication that allows the simultaneous quantitation of the presence of macromolecules in or between different cellular regions or compartments. This capability gives this BioApplication great versatility, enabling it to be applied towards a wide range of cell biological situations. Examples of a few such biological situations are explored in further detail in the following sections. Not only is this functionality of value in quantifying the presence of a particular macromolecule in a particular region of the cell, but it also allows the simultaneous quantitation, in distinct cellular regions, of the presence of several macromolecules that are linked by the same signaling or trafficking pathway.

To do this, the Compartmental Analysis BioApplication measures the intensity from a primary cellular object in one channel and then the intensity from four distinct regions of the cell, up to five additional channels, as well as the intensity ratios and differences from these different regions and channels, in addition to performing automatic cell population characterization using Reference Wells.

The Compartmental Analysis BioApplication's features and capabilities provide you the flexibility to design your own assays. It is for scientists who want a versatile tool that they can apply towards many different biological targets and thus want the flexibility in defining the regions of the cell from where they want to make measurements. If you do not want to configure and optimize such a flexible application, you are encouraged to use one of the more specific applications that target particular biologies and thus have been optimized for rapid implementation.

BioApplication Overview

The Compartmental Analysis BioApplication has been designed to do the following:

- Analyze processes within or between different sub-cellular compartments.
- Identify up to six different fluorescence channels, representing different fluorophores or exposure conditions.
- Report, for each cell, the intensity in four regions of the cell in each channel, including sub-cellular regions defined by masks and discrete punctate intracellular objects.
- Report ratios and differences in intensity between particular subcellular regions within a channel, as well as ratios in intensity for subcellular regions between channels.
- Provide the capability to perform automatic cell population characterization using Reference Wells. Measurement Regions

The Compartmental Analysis BioApplication requires that Channel 1 (the primary channel) contain an image of cells where a particular cellular region or organelle of each cell is labeled with a fluorescent marker to define a primary object. The primary object may also be a non-cellular fluorescent object. There are four regions of the cell in each of the dependent channels (Channels 2-6) where measurements are made that are derived from this fluorescently labeled primary object in Channel 1. The primary object is a major constituent of the cell and used to identify the individual cells and then to define the different sub-cellular regions for each cell. Examples of cellular regions or compartments that can serve as the primary object include nuclei, cytoplasm, certain large organelles (golgi apparatus or the endocytic recycling compartment), and other fluorescently labeled non-cellular objects.

The Compartmental Analysis BioApplication can range from a two to six channel assay. Channel 1 is for the primary object, as described above, and the average and total intensity for this object is measured. Channels 2-6 are the dependent channels where the number of channels depend on the number of targets labeled with different colored fluorophores in the sample. You have the ability to define the range covered by the four different regions of the cell independently and separately in each of the five dependent channels (Channel 2-6), and this range is set in relation to the primary object in Channel 1.

Description of the Four Cellular Regions

The four regions of the cell in each of the dependent channels (Channels 2-6), identified using the primary object, are called Circ, Ring, Ring Spots, and Circ Spots. Figure 1 shows a schematic of the four regions of the cell defined for the case where the nucleus in Channel 1 serves as the primary object.

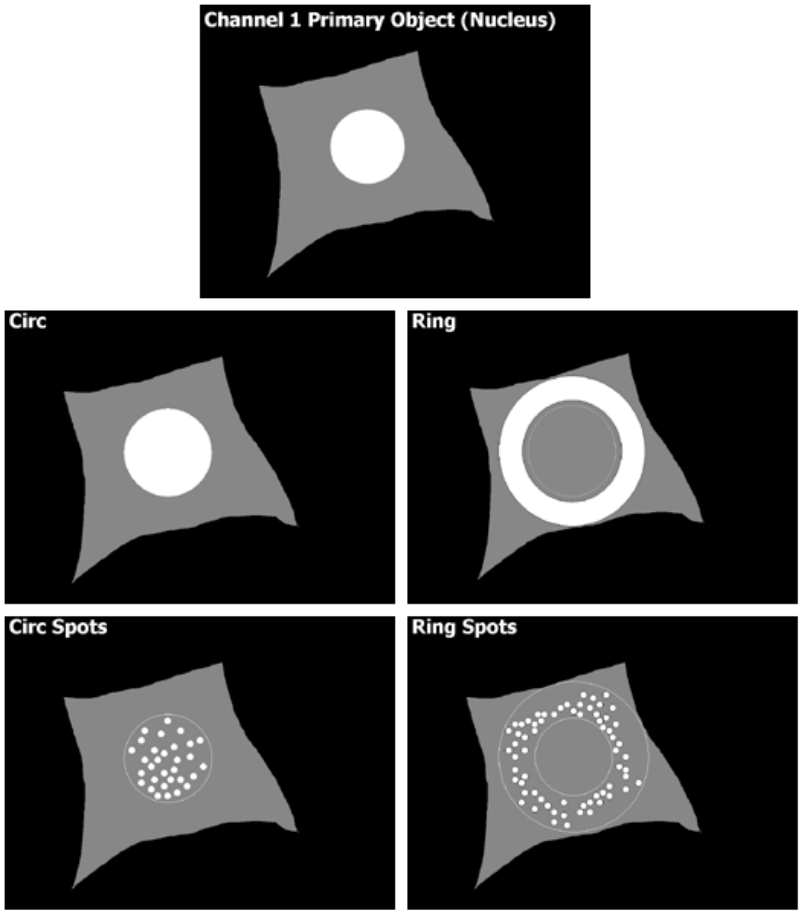

***Figure 1**. Schematic of the primary object and four regions of the cell derived from the primary object for measurement.*

Circ

Circ is a cellular region derived from, and similar to, the area covered by the primary object; you can make the Circ larger or smaller than the primary object. The Circ is used to quantify the presence of a fluorescent macromolecule within the large cellular compartment defined by the primary object. For example, if a nuclear dye was used in Channel 1 to label and identify the individual nuclei of cells, then using a transcription factor label in Channel 2 enables you to quantify how much of it is in the nucleus.

Ring

Ring is an annular region defined beyond the primary object. The position of the rings' inner and outer perimeters can be defined in relation to the primary object's location. If a nuclear label was used to define the primary object in Channel 1, then the Ring area can be used to quantify the presence of a fluorescently labeled macromolecule in an annular region of the cytoplasm outside the nucleus.

Ring Spots

Ring Spots are any discrete punctate objects that fall within the Ring area. Intensity thresholds, which you can adjust, define which punctate objects are to be identified as spots and thus be quantified. These spots can be used to identify discrete organelles or other punctate objects that are located in the cell's cytoplasm. Examples of organelles that can be identified by this feature include: mitochondria, proteasomes, lysosomes, and endosomes.

Circ Spots

Circ Spots are any discrete punctate objects that fall within the Circ area. Intensity thresholds identify these spots in a similar manner as for the Ring Spots. These spots can be organelles similar to those identified in Ring Spots, but in the cytoplasmic region above the nucleus, if the nucleus is the primary channel marker. An alternative possibility is punctate-labeled objects within the primary channel object; examples could be discrete objects within the nucleus if a nuclear marker is used in Channel 1 or discrete cellular objects if a whole cell marker is used as the primary object in Channel 1.

Event Definition

Events are patterns of cellular features that indicate the occurrence of a specific biologically meaningful state in an individual cell. The Compartmental Analysis BioApplication allows simultaneous definition of up to three Events to enable rapid multiparametric analysis at the level of individual cells, across multiple Cell Features. Events are defined as Assay Parameters and consist of logical statements employing specific Cell Features. These statements are then applied to classify and count responder and non-responder cells for the Cell Features contained in the Event Definition. Figure 2 relays this interaction to enable event definition and detection. Event definitions are created using a stand-alone software tool called the BioApplication Event Wizard. Operation of this software tool is described in more detail in Chapter 3.

Event definition and detection at the level of individual cells of a population requires processing of raw Cell Feature values, i.e., the data extracted from individual cells for all targets being measured. Limits are then applied (manually entered for the ***Feature*Ch*N*LevelHigh** or ***Feature*Ch*N*LevelLow** Assay Parameter or automatically computed via Reference Wells as described in Chapter 2) to identify responder cells for each feature. For the purposes of Event detection, responder cells are defined as those cells showing feature values greater than the upper limit (***Feature*Ch*N*Status = 1**) or less than the lower limit (***Feature*Ch*N*Status = 2**), defined initially by ***Feature*Ch*N*LevelHigh** or ***Feature*Ch*N*LevelLow**. It is critical that the upper and lower limits be set accordingly.

Well Features are calculated to provide population statistics for individual Cell Features and frequency of occurrence of a given Event in the cell population. The latter is expressed as the percentage of cells that display a given Event.

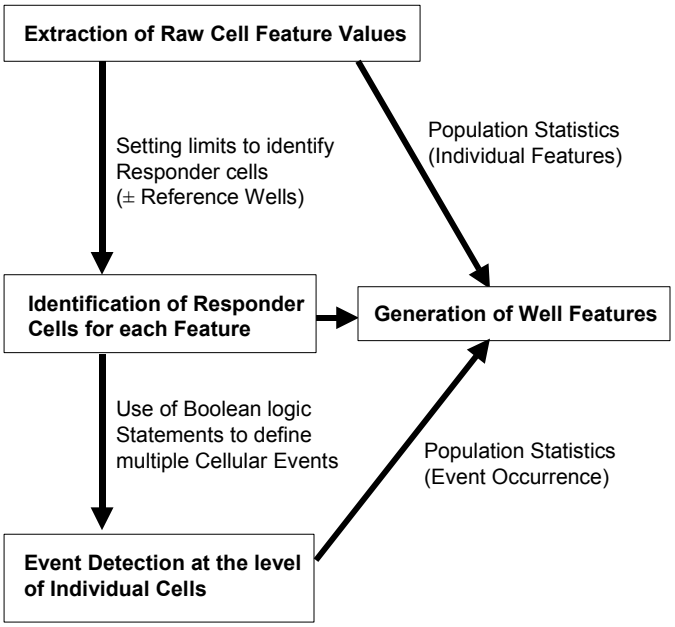

Figure 2. *Schematic showing the process of definition and detection of cellular events*

BioApplication Measurements

The Compartmental Analysis BioApplication measures a large number of features in each of the four cellular regions (Circ, Ring, Circ Spot, and Ring Spot) in all the dependent channels used. The BioApplication reports both Cell and Well Features. Cell Features are reported for each cell, and Well Features are reported for each well and are derived from the Cell Features for all the cells imaged and analyzed in that well.

The measured features include intensities as well as related features such as intensity ratios, differences, spot numbers, and spot areas. Morphological and intensity properties of the primary object in Channel 1 are reported as well as intensity ratios for different regions. Only the Average Intensity from the Circ region in Channels 2-6 is divided by the Average Intensity of the primary object in Channel 1 and reported. Ratio intensities from the other three regions (Ring, Circ Spot, and Ring Spot) are not calculated with Channel 1 and, thus, are not reported. The ratios between the Average Intensities from Channels 2-6 are reported for all four regions. In addition, the ratio of the number of spots in the Circ and Ring regions between different channels are also reported. Although a ratio between each channel from Channels 2-6 is made, not all channel combinations are calculated. Table 1 shows the Channel combinations for which ratios are reported.

Ratio Combinations		**Numerator channel (Ch *N*)**				
		Ch 2	Ch 3	Ch 4	Ch 5	Ch 6
Denominator channel (Ch *D*)	Ch 2		✔	✔	✔	✔
	Ch 3			✔	✔	✔
	Ch 4				✔	✔
	Ch 5					✔
	Ch 6					

Table 1. *Channel ratio combinations reported for the Compartmental Analysis BioApplication.*

In addition, Status information on whether individual cells are responders is given for certain measured features. Table 2 shows all Cell and Well Features available based on the number of channels selected. This large and expansive number of features gives the Compartmental Analysis BioApplication its flexibility by enabling you to apply it towards a wide range of biological situations.

Feature Category	Cellular Region/s	Cell Features	Corresponding Well Features
Cell and Well-Level Features Reported for Channel 1:			
Intensity	Primary Object Area	Total Intensity and Status	Mean, SD, SE, CV, %High, %Low
		Average Intensity and Status	
Morphology and Location	Primary Object Area	Area and Status Shape P2A and Status Shape LWR and Status	Mean, SD, SE, CV, %High, %Low
		Size and Status	
		Top Left Width Height X Centroid Y Centroid	none
Cell Counts and Density	Primary Object Area	Cell Number	Valid Object Count Selected Object Count %SelectedObjects Valid Field Count SelectedObjectCountPerField
Events	Selected Features from Event Wizard	EventTypeProfile EventType1Status EventType2Status EventType3Status	EventType1: Count, % EventType2: Count, % EventType3: Count, %
Cell and Well-Level Features Reported for Channels 2-6:			
Intensity	Circ Ring Circ Spot Ring Spot	Total Intensity and Status	Mean, SD, SE, CV, %High, %Low
		Average Intensity and Status	
	Primary Object Area	Average Intensity Total Intensity	None (gating)
Intensity Ratios Between Channels	Circ	Ratio with Ch 1: Average Intensity Ratio Ch*N*/Ch1 and Status	Mean, SD, SE, CV, %High, %Low
	Circ Ring Circ Spot Ring Spot	Ratio between Ch 2-6: Average Intensity Ratio Ch*N*/Ch*D* and Status	
Circ Ring Arithmetic	Circ Ring	Circ Ring Average Intensity Difference and Status	Mean, SD, SE, CV, %High, %Low
		Circ Ring Average Intensity Ratio and Status	
Spot Area	Circ Spot Ring Spot	Spot Total Area and Status	Mean, SD, SE, CV, %High, %Low
		Spot Average Area and Status	
Spot Number and Ratios	Circ Spot Ring Spot	Spot Count and Status	Mean, SD, SE, CV, %High, %Low
		Spot Count RatioCh*N*/Ch*D* and Status	

Table 2. *Features reported by the Compartmental Analysis BioApplication. *Note: "ChN" refers to Channels 2-6 (numerator) and if ratios, "ChD" refers to the denominator found for Channels 2-6. SD is the standard deviation; SE is the standard error of the mean; and CV is the percent coefficient of variation.*

Example Use Cases

The Compartmental Analysis BioApplication allows a large and varied range of cell biological situations to be quantitatively analyzed. A few examples of such biological situations are described below purely to illustrate the potential of this BioApplication. Configuring this BioApplication to do such measurements on these biological situations is given in Chapter 3.

Example 1: EGF Stimulation of the MAPK Pathway

The Compartmental Analysis BioApplication can monitor the movement of different molecules between different intracellular compartments which are causally linked. This allows different components within the same signal transduction pathway to be measured in the same cell. For example, you can simultaneously monitor the different effects caused by the binding of epidermal growth factor (EGF) to its receptor: receptor internalization and signaling. One effect is that the EGF-receptor complex is internalized upon ligand binding and then traffics to late endosomes and lysosomes where it is degraded (Mukherjee, et al. 1997). The internalized ligand or receptor can be fluorescently detected and appears as intracellular punctate fluorescence, whose intensity initially increases as internalization occurs and then eventually decreases as lysosomal degradation occurs. The other consequence of EGF binding to its receptor is an initiation of the receptor tyrosine kinase signaling cascade resulting in the eventual mitogen activated protein kinase (MAPK) translocation to the nucleus where it causes the expression of proteins related to the cell cycle (i.e., G_1/S cyclins)(Widmann 1999).

Various components of this signaling cascade can be fluorescently detected including the cytoplasm to nuclear translocation of MAPK. The expression of cell cycle gene products and the eventual proliferation of the cells can also be fluorescently detected and measured by this application. Furthermore, additional receptors and macromolecules can also be measured to evaluate cross-talk between different signaling pathways and receptors. Table 3 describes some of the features for the individual targets which can be quantitatively measured and analyzed by the Compartmental Analysis BioApplication. Figure 3 illustrates the types of intracellular distributions these different targets will have for both the EGF-stimulated and unstimulated conditions.

Target	Properties That Can Be Measured by the Compartmental Analysis BioApplication
Phosphorylated MAPK (detected by immunofluorescence)	• Intensity of activated (i.e., phosphorylated) MAPK in the nucleus • The difference and ratio of the activated MAPK signal in the nucleus versus the cytoplasm to indicate the extent of translocation • Indicators of whether a cell's nuclear MAPK intensity or the extent of cytoplasm to nucleus translocation is above a threshold that you set, indicating that the cell is a responder
Internalized EGF (detected by fluorescent EGF)	• Intensity of internalized fluorescent EGF in cells • Intensity of intracellular fluorescent EGF in endosomes • Indicators of whether a cell's internalized fluorescent EGF is above a threshold that you set, indicating that the cell is positive for internalization • Number of resolved distinct endosomes • Indicator of whether the number of distinct endosomes containing fluorescent EGF is above a threshold that you set, indicating that the cell is positive for internalization
Cyclin expression (detected by immunofluorescence)	• Cyclin expression intensity in the nucleus • Indicators of whether the cyclin intensity is above a threshold that you set, indicating that the cell is expressing cyclin
DNA Content (detected by DNA dyes)	• Intensity of DNA in the nucleus to indicate the cell cycle phase
Cell Proliferation (detected by DNA dyes)	• The number of cells to indicate whether cell proliferation has occurred

Table 3. *Cellular properties that can be measured by the Compartmental Analysis BioApplication upon fluorescent EGF stimulation of the MAPK pathway.*

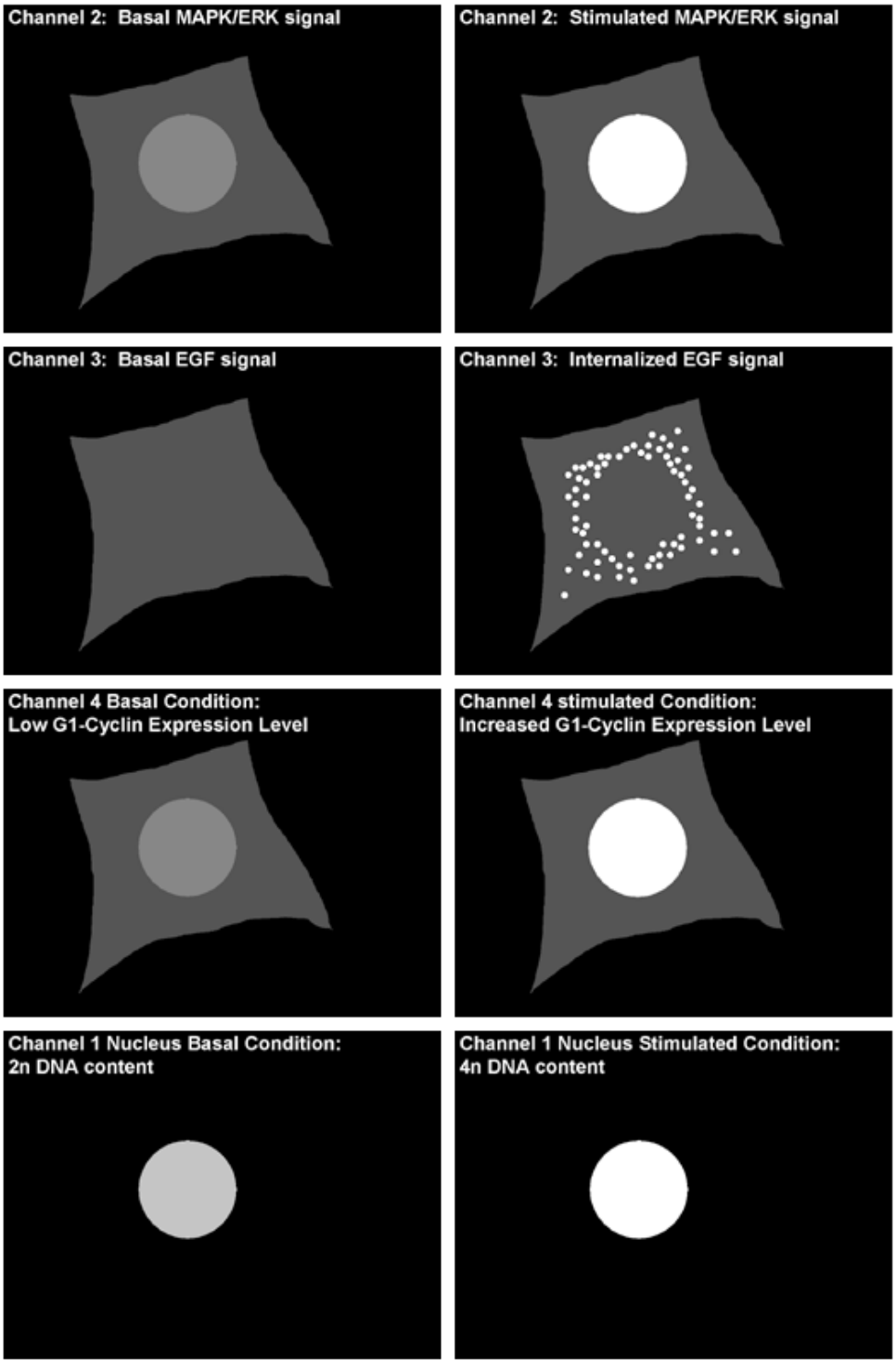

Figure 3. *Schematic of fluorescent EGF stimulation of ERK(extracellular signal-regulated kinases). Unstimulated basal condition (left column) and stimulated condition (right column) are shown.*

Example 2: Intracellular Trafficking and Activation of G-Protein Coupled Receptors

The Compartmental Analysis BioApplication can quantify the internalization of receptors and macromolecules and the movement of such proteins, macromolecules, or fluorescently labeled biosensors between different intracellular compartments. For the purpose of this BioApplication, two types of intracellular compartments exist:

- Smaller compartments, of which multiple copies exist per cell, can often have a punctate appearance (Mukherjee, et al. 1997). Examples include: certain endosomes, lysosomes, mitochondria, peroxisomes, intracellular storage granules, and intracellular vacuoles.
- Large, single compartments, of which only one exists per cell. Examples of these include: the nucleus, the Golgi apparatus, and the endocytic recycling compartment (Mukherjee, et al. 1997; Conway et al. 2001; Ghosh et al. 2000).

The Compartmental Analysis BioApplication can quantify trafficking in and out of both types of compartments. Trafficking to the smaller compartments was shown in biology Example 1, where internalized EGF and its receptor trafficked to punctate late endosomes and lysosomes. The strategy for quantifying trafficking to large compartments is as follows: the compartment under question is fluorescently labeled in Channel 1 and a fluorescently labeled protein or macromolecule of interest is monitored in Channels 2-6. The amount of the different fluorescently labeled proteins or macromolecules in the labeled compartment can be quantified. An example of trafficking to this sort of large compartment occurs when G-protein coupled receptors (GPCRs) are activated. As part of their desensitization step, the GPCRs are internalized and traffic through the endocytic recycling compartment before recycling back to the cell surface (Conway et al. 2001; Ghosh et al. 2000). Table 4 describes some features of the Compartmental Analysis BioApplication that can be used to quantify such internalization and intracellular trafficking. The schematic in Figure 4 shows internalization of GPCRs into the Endocytic Recycling Compartment.

Target	Properties That Can Be Measured by the Compartmental Analysis BioApplication
Compartment Label (Channel 1)	• Intensity of compartment marker
Internalized or intracellular trafficking fluorescent macromolecule (Channel 2)	• Intensity of internalized or trafficking macromolecule in the labeled compartment • Ratio of the macromolecule's intensity to that of the compartment marker as a measure of colocalization • Indicators of whether the intensity of the trafficking macromolecule in the compartment is above a threshold that you set, indicating that the compartment contains the macromolecule. • Indicators of whether the ratio of intensity between the trafficking macromolecule and the compartment marker is above a threshold that you set, as an indicator of colocalization.

Table 4. *Cellular properties measured by the Compartmental Analysis BioApplication that can be used to quantify internalization and intracellular trafficking.*

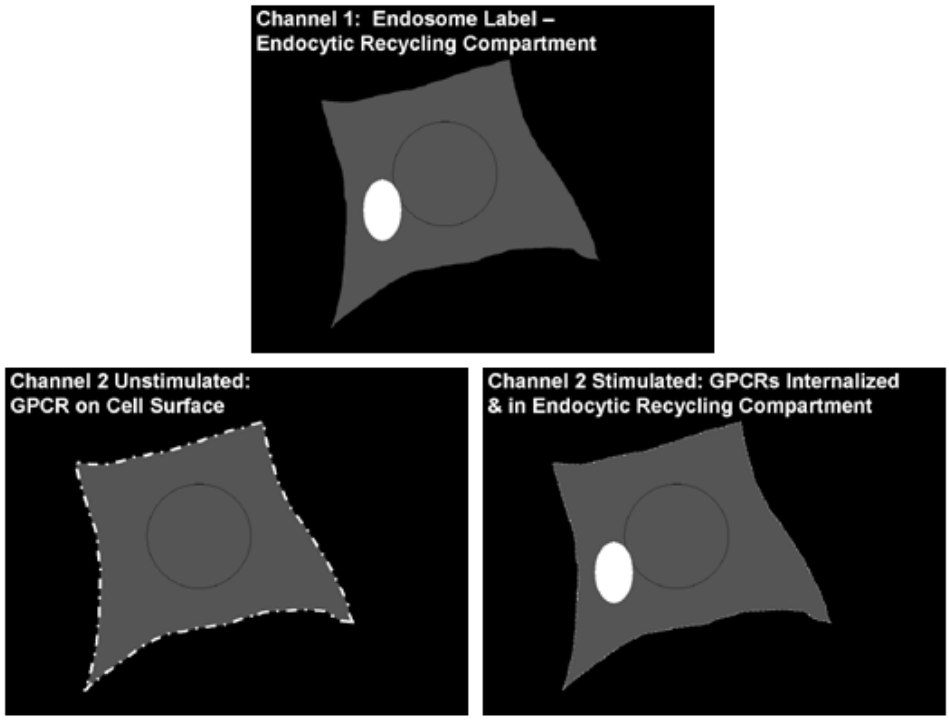

Figure 4. *Schematic of receptor internalization and intracellular trafficking: Activated GPCR internalization into endocytic recycling compartment.*

Example 3: Cytotoxicity

The BioApplication can give an indication of a cell's health by simultaneously measuring different physiological indicators in the same cell. Examples of cellular physiological indicators that can be measured include: nuclear morphology, cell membrane permeability, mitochondrial mass, and membrane potential. Such measurements of a cell's physiological health can be used to monitor cytotoxicity after treating cells with compounds. Table 5 describes some of the features of these physiological indicators that can be quantitatively measured and analyzed by the Compartmental Analysis BioApplication. Figure 5 is an illustration of the types of cellular signals expected from the different physiological monitors of cell health for both normal and cytotoxic conditions. The assay protocol provided with this application is configured to measure nuclear size, mitochondrial transmembrane potential and plasma membrane permeability.

Target	Properties That Can Be Measured by the Compartmental Analysis BioApplication
Nucleus (Channel 1) (detected by DNA binding dyes)	• Nucleus size (i.e., normal nucleus size, nuclear fragmentation, or condensation). • Indicates whether a cell's nuclear size is beyond a range that you set, indicating the nuclear fragmentation or condensation resulting from apoptosis.
Mitochondrial Mass and Potential (detected by MitoTracker® Green in Channel 2 and MitoTracker Red in Channel 3)	• Intensity of MitoTracker Green in mitochondria as a measure of mitochondrial mass. • Number of mitochondria in Channel 2 from MitoTracker Green label. • Intensity of MitoTracker Red in mitochondria as a measure of mitochondrial transmembrane potential ($\Delta\Psi_m$). • Number of mitochondria in Channel 3 from MitoTracker Red staining showing the number of mitochondria that have transmembrane potential that is still at a high value. • Ratio of the MitoTracker Green and Red intensities to normalize for the mitochondrial mass resulting in relative mitochondrial potential. • Ratio of the number of mitochondria detected in Channels 2 and 3 to determine the fraction of mitochondria with decreased transmembrane potential. • Indicates whether a cell's mitochondrial mass and/or potential is beyond a range that you set, indicating cytotoxicity.
Cell Membrane Permeability (Channel 4) (detected by membrane impermeant dyes)	• Intensity of permeability dye in cell – a strong signal indicates that the cell membrane's permeability has been compromised, allowing the dye to enter the cell. • Indicates whether a cell's permeability dye intensity is above a threshold that you set, thus indicating that the cell's membrane permeability has been compromised as a consequence of cytotoxicity.

Table 5. *Cellular properties that can be measured by the Compartmental Analysis BioApplication for different physiological monitors of cell health and cytotoxicity.*

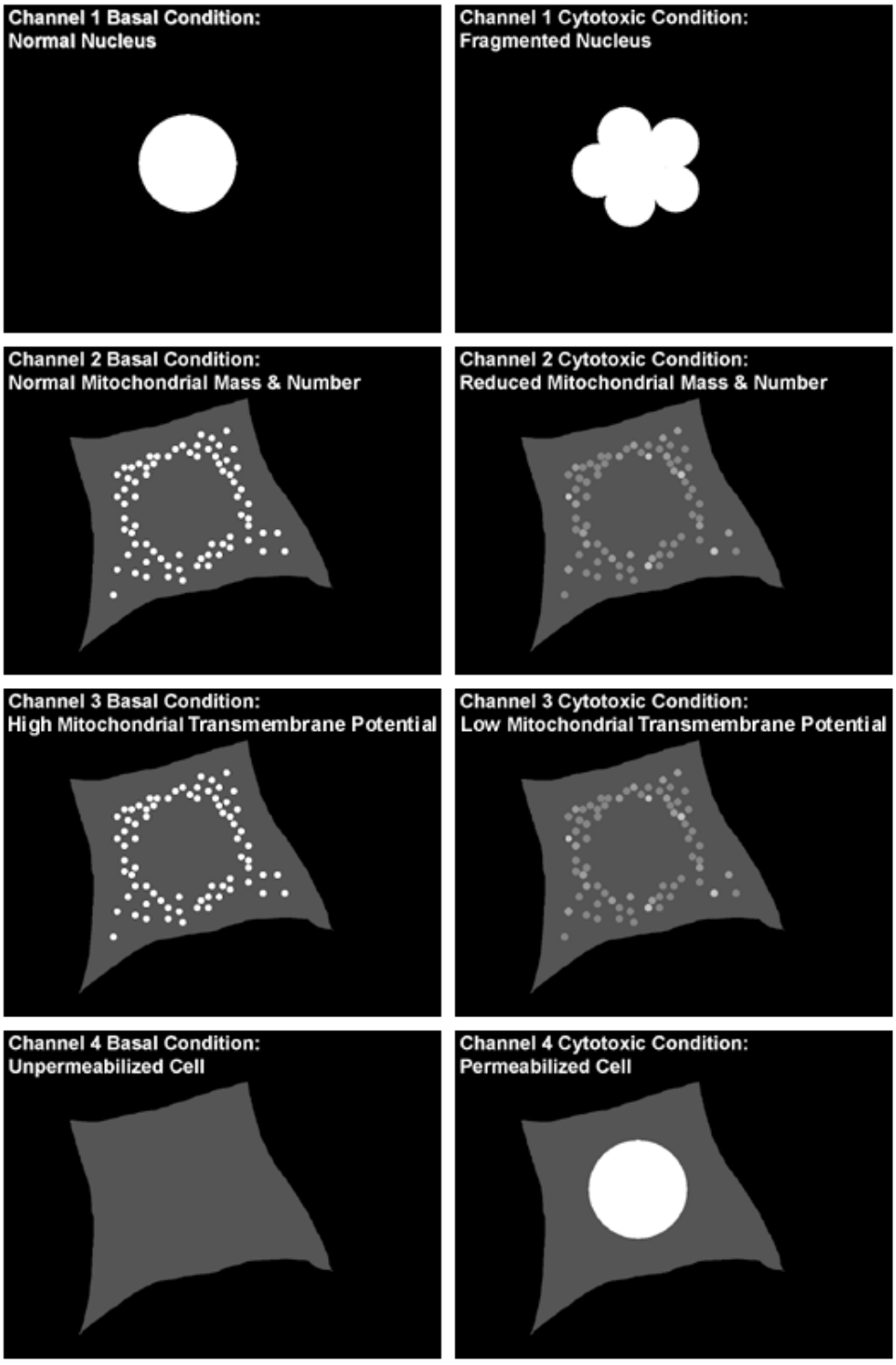

Figure 5. *Schematic of physiological monitors of cell health and cytotoxicity.*

Example 4: Translocation of Multiple Related Kinases

The BioApplication can be used to evaluate compounds that affect the accumulation of a related set of molecules in a particular intracellular location. For example, certain sets of kinases, such as the different MAPKs, will undergo cytoplasm to nuclear translocation upon activation by the correct stimuli (Widmann et al. 1999). It may be desirable to determine and rank the specificity of different compounds among similar kinases. This can be done with the Compartmental Analysis BioApplication. Table 6 describes some of the features that can be quantitatively measured and analyzed by the Compartmental Analysis BioApplication that can be used to rank a compound's effect in such an example. The schematic in the Figure 6 shows three hypothetical kinases that undergo cytoplasm to nucleus translocation upon stimulation by a compound. All three kinases have different levels in the nucleus in the basal unstimulated state. The compound induces the translocation of kinase 1 into the nucleus, a weak effect on kinase 2, and an intermediate effect on kinase 3.

Target	Properties That Can Be Measured by Compartmental Analysis
Multiple Transcription Factors or Kinases in Channels 2-6	• Intensity of each kinase in the nucleus. • The difference and ratio of each kinase signal in the nucleus versus the cytoplasm to indicate the extent of translocation. • For each kinase, indicators of whether a cell's nuclear kinase intensity or the extent of cytoplasm to nucleus translocation is above a threshold that you set, indicating that the cell is a responder. • Ratios of the different kinase intensities in the nucleus to determine the combination of the different kinases that the cell was a responder and the relative strengths of the responses.

Table 6. *Cellular properties measured by the Compartmental Analysis BioApplication that can be used to rank compounds on their effect of cytoplasm to nuclear translocation for related kinases.*

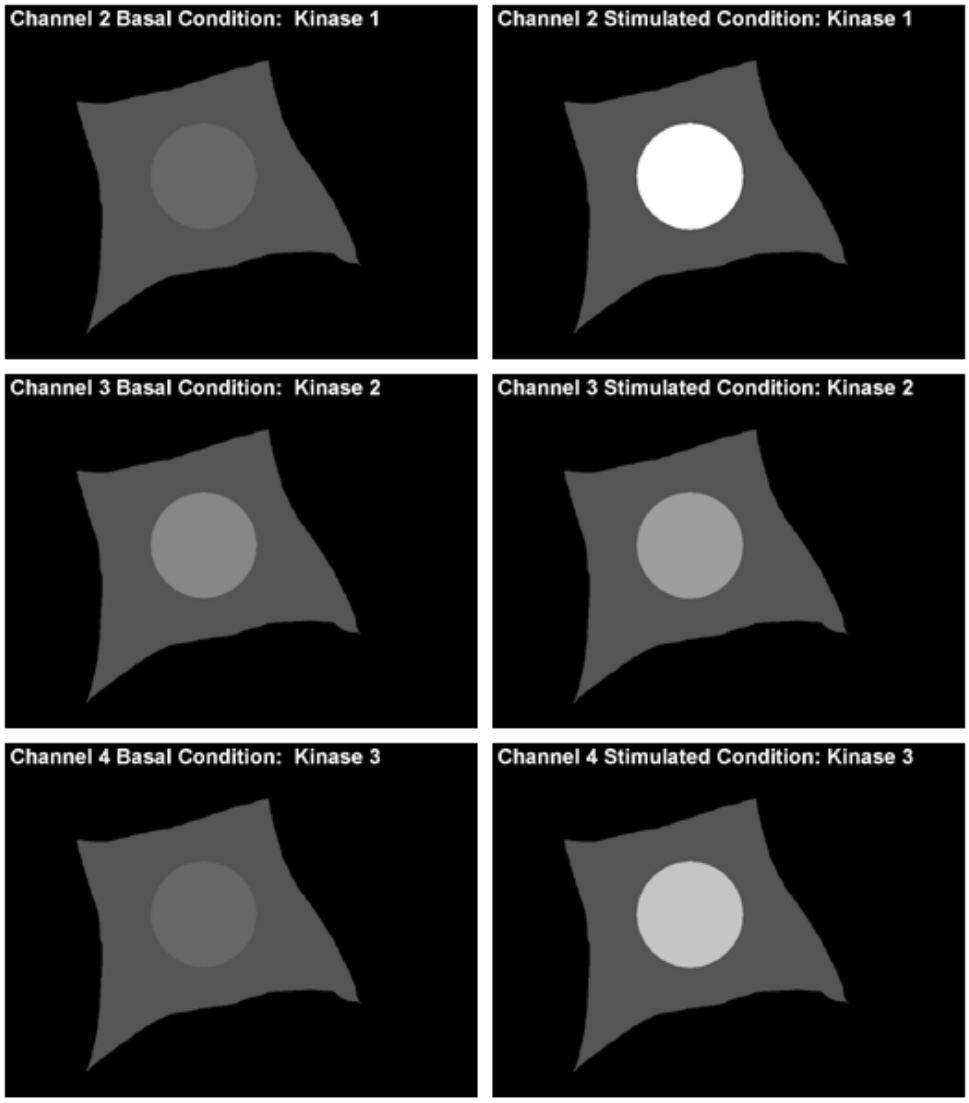

Figure 6. *Schematic of multiple kinase translocation.*

Example 5: Phosphorylation of Macromolecules in Intracellular Locations

Many proteins involved in intracellular signaling (including MAPK used in Example 1) can undergo chemical modifications such as phosphorylation as part of their activation process (Widmann et al. 1999). The Compartmental Analysis BioApplication can be used to quantify the degree of phosphorylation of such a protein in a specific intracellular location by determining the amount of phosphorylated protein as a proportion of the total amount of that protein. The phosphorylated protein can be detected by immunofluorescence using primary antibodies against the phospho-specific form of the protein, and the total protein is similarly measured using primary antibodies that recognize both the phosphorylated and unphosphorylated forms of the protein. The schematic in Table 7 describes some of the Compartmental Analysis BioApplication features that can be used to quantify the protein's phosphorylation. Figure 7 shows a hypothetical transcription factor located in the nucleus that gets activated by phosphorylation.

Target	Properties That Can Be Measured by Compartmental Analysis
Phosphorylated protein (Channel 2)	• Intensity from phosphorylated protein in the nucleus. • Indicators of whether the phosphorylated protein's intensity is above a threshold that you set, indicating that the cell is a responder.
Total protein (Channel 3)	• Intensity from total protein (phosphorylated and unphosphorylated) in the nucleus. • Ratio of phosphorylated protein intensity to total protein intensity as a measure of the degree of the protein's phosphorylation. • Indicators of whether a cell's ratio of phosphorylated protein intensity to total protein intensity is above a threshold that you set, thus indicating whether the cell is a responder and has been activated.

Table 7. *Cellular properties measured by the Compartmental Analysis BioApplication used to measure protein phosphorylation.*

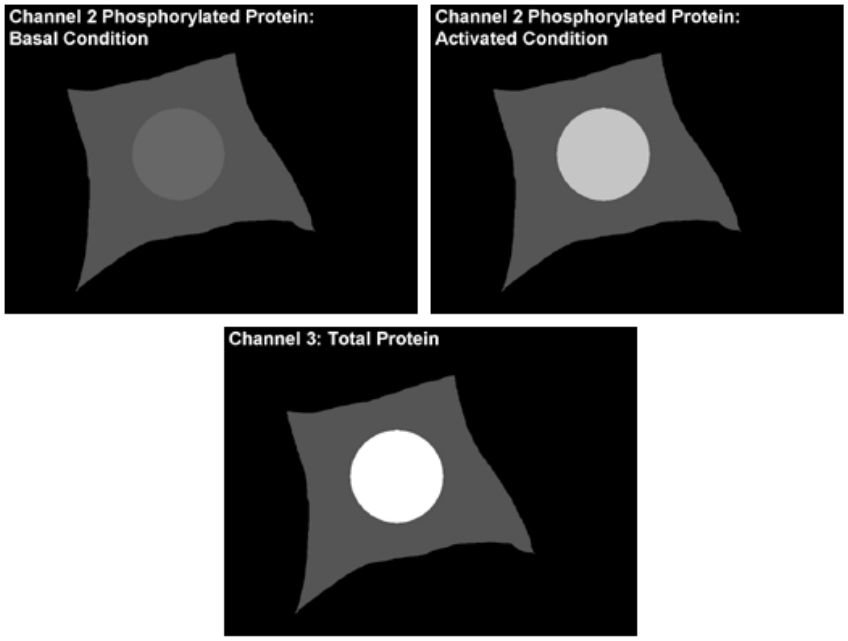

Figure 7. *Cellular properties measured by the Compartmental Analysis BioApplication used to measure protein phosphorylation.*

Demonstration Data Using the Compartmental Analysis BioApplication

EGF Stimulation of the MAPK Pathway

The Compartmental Analysis BioApplication is applicable to a wide range of biological situations, some examples of which were given earlier in this chapter. In this section, the results obtained from applying the Compartmental Analysis BioApplication to one of the examples, EGF stimulation of the MAPK pathway (see Example 1 described previously under *Example Use Cases*). The specific details in configuring the Compartmental Analysis BioApplication input parameters for this and the other biological examples are given in Chapter 3.

Use of the Compartmental Analysis BioApplication is demonstrated by simultaneously measuring in the same cell some of the different elements associated with the MAPK. In this example, EGF induction of the extracellular signal-regulated kinases ERK, also known as p44 and p42 MAPK, is measured (Widmann 1999). EGF binding to its receptor activates the receptor's tyrosine kinase activity, which initiates an intracellular signaling cascade. The signal is relayed by Ras into a serine/threonine phosphorylation cascade, which eventually results in the phosphorylation of the ERK (Widmann 1999). Phosphorylated ERK enters the nucleus and activates the transcription of various genes, including some required for cell proliferation, such as certain cyclins (Widmann 1999). EGF's binding to its receptor also induces the receptor to undergo receptor-mediated endocytosis via clathrin-coated pits. The internalized ligand-receptor complex eventually traffics to late endosomes and lysosomes where they are degraded by enzymes present in these compartments (Mukherjee, Ghosh, and Maxfield 1997). These two parallel events – phosphorylated ERK translocation to the nucleus and the internalization and degradation of the EGF ligand-receptor complex in late endosomes and lysosomes – are initiated by EGF binding to its receptor and both can be simultaneously detected and quantified in the same cell using the Compartmental Analysis BioApplication.

To demonstrate this, HeLa cells were incubated with fluorescent EGF conjugated with the fluorophore Texas Red®, which has a red fluorescence emission. At different time points, the cells were fixed and processed for indirect immunofluorescence using a primary antibody against phosphorylated ERK and a secondary antibody conjugated with the fluorophore Alexa Fluor® 488 (green fluorescence emission). The nuclei of the cells were labeled with Hoechst 33342 and served as the primary object in the analysis by the Compartmental Analysis BioApplication. Thus, Channel 1 contained the nuclear images, Channel 2 contained the ERK images, and Channel 3 contained the fluorescent EGF images, which are shown in Figure 8.

The increase in ERK intensity in the nuclear region is transient, reaching a maximal response by 10 minutes after EGF addition and then decreasing to basal levels within 45 minutes (Figure 9A and 9B). Fluorescent EGF internalization can be measured in the same cells and has a different temporal response than ERK. Internalized fluorescent EGF has a punctate intracellular appearance reflecting its trafficking to, and sequestration in, early and late endosomes and then lysosomes. The internalization followed by the degradation of the fluorescent EGF over time is reflected as an increase and then as a decrease in the Spot Intensity over time. The fluorescent EGF in spots is seen at a maximum around 30 minutes after its addition, and then decreases thereafter. Thus, EGF internalization and degradation has slower kinetics than phosphorylated ERK translocation to the nucleus.

The increase in ERK intensity in the nuclear region can be quantified by the Compartmental Analysis BioApplication, as shown in Figures 9A-9C. For this example, several different features measured in Channel 2 could be used to monitor ERK increase in the nucleus. These include the following:

- Circ Average and Total Intensities
- Difference and Ratio between the Circ and Ring Average Intensities
- Percentage of cells whose Circ Average Intensity is greater than a threshold
- Percentage of cells whose Circ-Ring Average Intensity Difference or Ratio is greater than a threshold

Similarly, the change in the intracellular EGF seen in Figure 8 can also be quantified by the Compartmental Analysis BioApplication, as shown in Figures 9A-9C. The different features measured in Channel 3 that could be used to quantify the presence of fluorescent EGF in the cell and indicate internalization include the following:

- Ring Spot Average and Total Intensities
- Ring Average and Total Intensities
- Percentage of cells whose Ring Spot Average Intensity is greater than a threshold
- Percentage of cells whose Ring Average Intensity is greater than a threshold
- Number of Ring Spots
- Percentage of cells whose Ring Spot number is greater than a threshold

In Figure 9A, the Circ and Ring Spot Total Intensities are plotted to show the ERK and EGF responses, respectively. The Circ Total Intensity is higher as the Circ area is larger than the Ring Spot area. In Figure 9B, the Circ and Ring Spot Average Intensities are plotted to show the ERK and EGF responses respectively. Since the Ring Spots cover a smaller area than the Circ, its Average Intensities are higher. Reference Wells were used to perform population characterization, and the results are in the plot found in Figure 9C.. In this plot, the percentage of cells whose Circ intensity was greater than a threshold represented ERK, and the percentage of cells whose Ring Spot intensity was greater than a threshold represented EGF. All three plots in Figure 9 show a similar temporal response for EGF and ERK, and this reflects the temporal behavior seen in the images in the following figure. This demonstrates that the versatility of the Compartmental Analysis BioApplication allows it to be used to accurately quantify and reproduce the biological behavior in cells of different targets that are seen in fluorescent microscopic images.

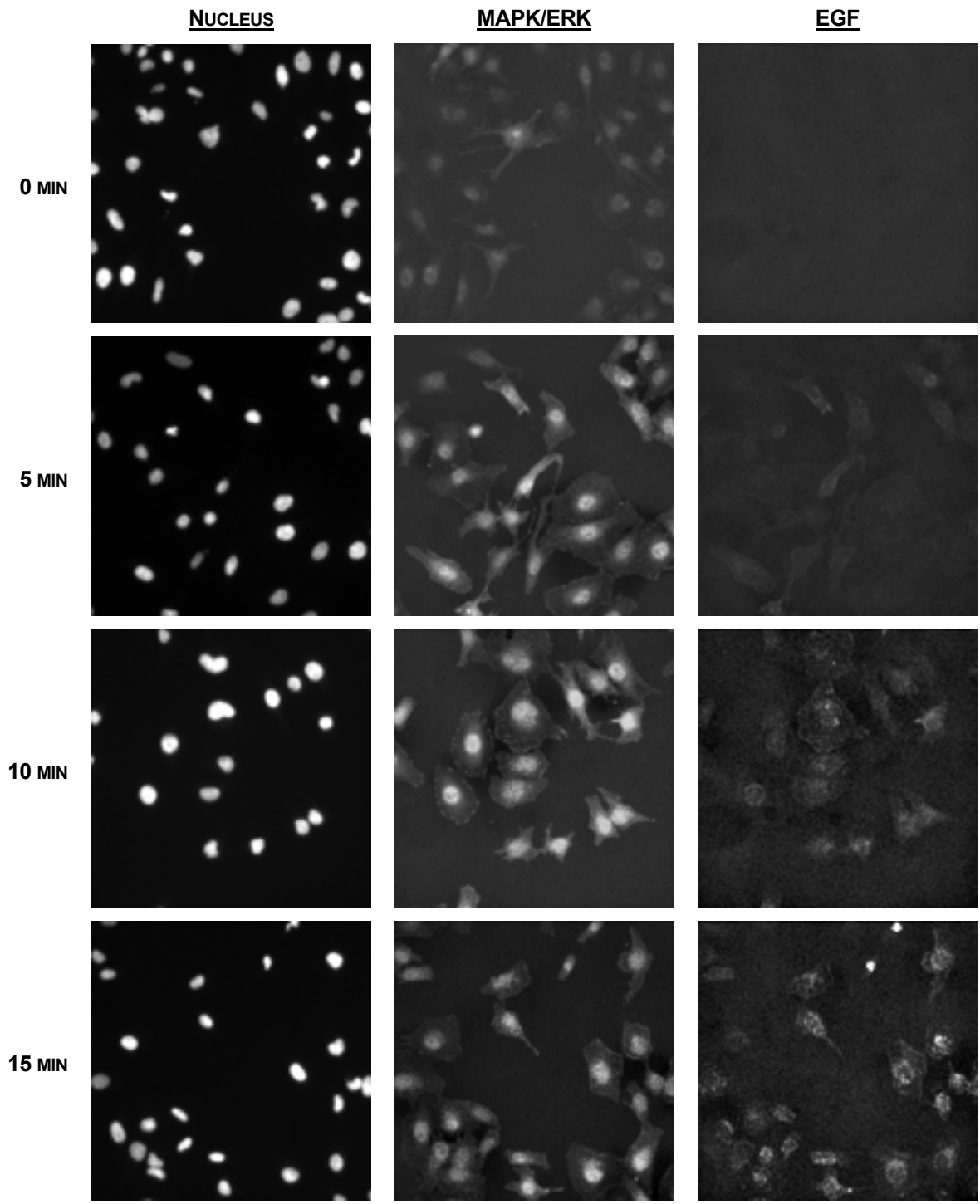

Figure 8. *(continued on next page) Temporal sequence of fluorescent EGF internalization and ERK activation.*

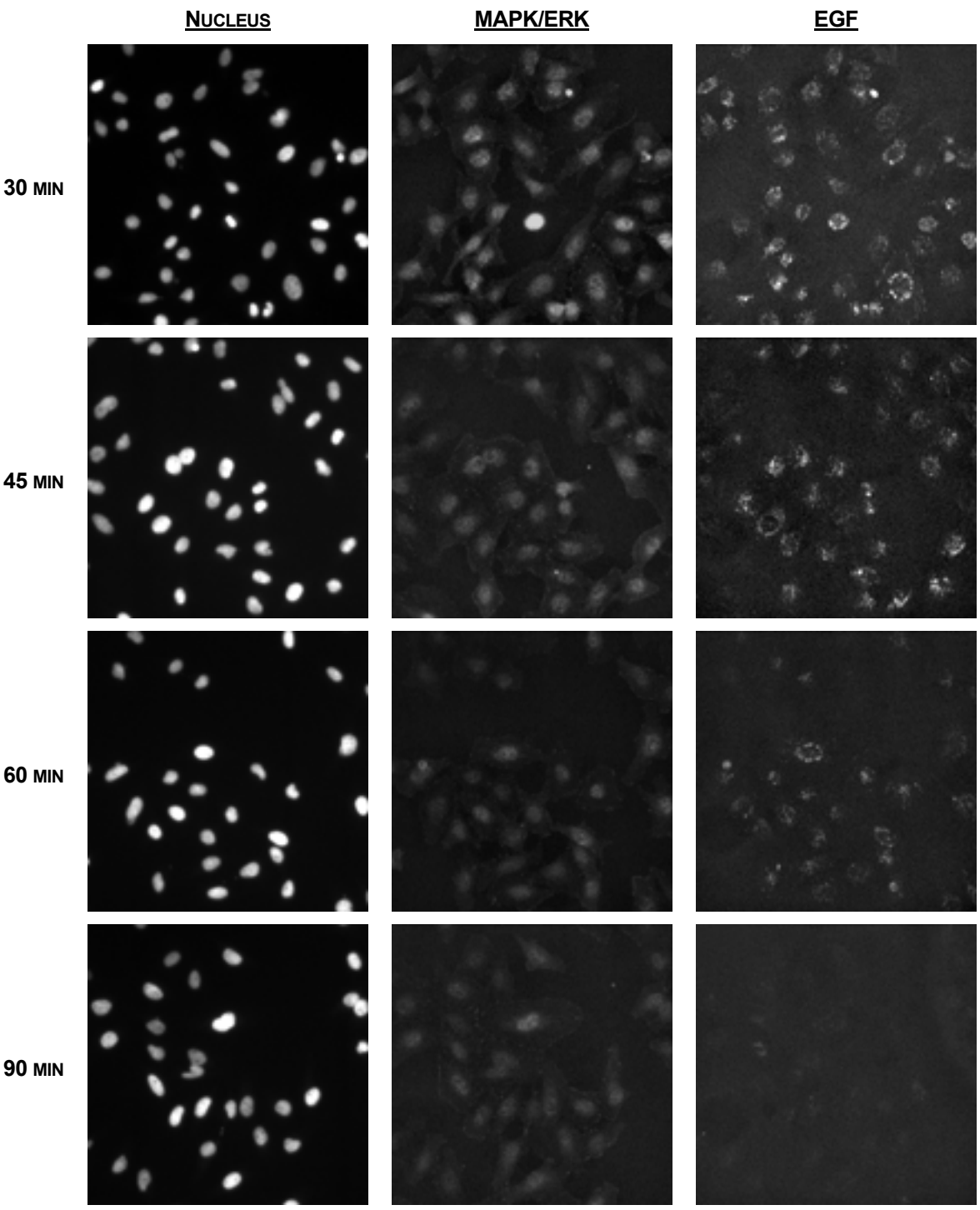

Figure 8. *Temporal sequence of fluorescent EGF internalization and ERK activation. HeLa cells were incubated with Texas Red-conjugated EGF for the times indicated and then fixed. The nuclei were labeled with Hoechst 33342 and the cells were then processed for immunofluorescence against ERK. Images acquired on the ArrayScan HCS Reader were analyzed with the Compartmental Analysis BioApplication. The nuclear intensity of ERK rapidly increased to a maximum at 10 minutes before decreasing to basal levels by 45 minutes. The EGF slowly accumulated in punctate organelles in the cell, reaching a maximum by 30 minutes before decreasing.*

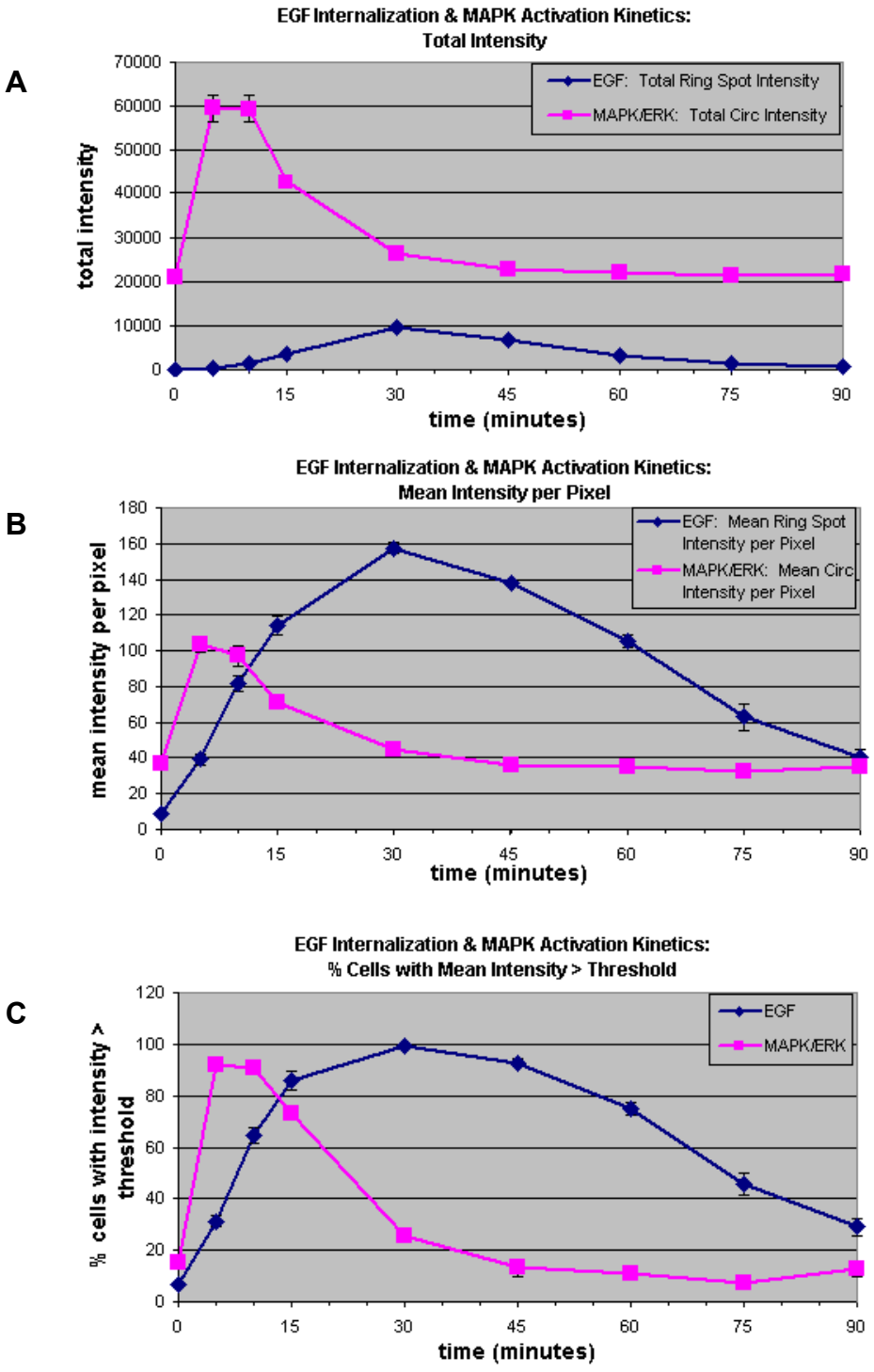

Figure 9. *Results of analyzing images of Figure 8 with the Compartmental Analysis BioApplication. Different output features are used to show MAPK/ERK activation and fluorescent EGF internalization. 9A represents total intensity for RingSpot (EGF) and Circ (MAPK/ERK). 9B represents mean intensity per pixel for RingSpot (EGF) and Circ (MAPK/ERK). 9C represents the percent of cells that have a selected intensity above a calculated threshold. The temporal behavior of the fluorescent EGF internalization and ERK activation is similar in all three plots and is consistent with the observations in Figure 8.*

Kinetic Multiparametric Cytotoxicity Assay

Use of the Compartmental Analysis BioApplication is demonstrated with a four-channel assay that simultaneously measures cytotoxicity indicators and markers of cellular homeostasis in the same cell, using fluorescent vital dyes that concentrate in specific cellular compartments (Haskins et al., 2001; O'brien et al., 2006) . In this example, cell nuclei are labeled with a blue fluorescent DNA binding dye, cytosolic calcium is monitored with a green fluorescent calcium indicator, mitochondrial transmembrane potential is measured with an orange fluorescent dye, and membrane permeability is monitored using a far red fluorophore that labels the nuclei of cells whose plasma membrane permeability has been compromised. The precise assay output features are listed in Table 8.

The cell nucleus was chosen as the Object in Channel 1 for this assay. Note that the image from Channel 1 is used to generate the Object Mask, which is the foundation for definition of remaining cellular regions/masks in Channels 2 through 4. Compartmental Analysis can also be used to monitor changes in the size and shape of nuclei, two morphological features that are often associated with cellular toxicity and apoptosis.

The fluorescent calcium indicator imaged in Channel 2, loads into the cytoplasm where its fluorescence is positively correlated with the concentration of free calcium ions. Calcium homeostasis can be a measure of the overall health of the cell, since healthy, unstimulated, cells maintain a constant level of cytosolic calcium. Calcium signaling is also involved in many cellular processes. In this example, intracellular calcium levels were monitored using a modified mask of the Object (Circ).

Many toxic compounds directly or indirectly compromise cell health by affecting mitochondrial function (Trump and Berezesky, 1998). The mitochondrial transmembrane potential ($\Delta\Psi_m$) indicator, imaged in Channel 3, freely equilibrates across the plasma membrane and, because of its positive charge, concentrates in mitochondria in a Nernstian manner. Healthy, functioning mitochondria accumulate the indicator, whereas inhibition of mitochondrial respiration or dissipation of the mitochondrial electrochemical gradient causes a decrease in mitochondria-associated fluorescence. Detection of discrete spots within the Ring region outside the nucleus identifies mitochondria.

Loss of plasma membrane integrity can be the result of the primary action of a toxic compound (one with detergent-like properties) or may be the final manifestation of cell death. The membrane permeability indicator used is a far-red fluorescent dye that binds nucleic acids and is impermeant to the plasma membrane of healthy cells. However, if plasma membrane integrity is compromised, the indicator can enter the cell and label the nucleus. Internalization of the indicator is monitored in Channel 4 using the Circ mask to identify the nucleus.

Experiment #1

HepG2 cells were incubated with all four fluorescent indicators and placed in the Cellomics V^{TI} HCS Reader with the Live Module enabled. After a baseline image in each channel was acquired, a vehicle control or an aliquot of melittin (at a final concentration of 6 µg/mL) was added. Melittin was delivered to rows B, D, F, and H while vehicle was delivered to rows A, C, E, and G. After a two-hour incubation (within the VTI Live Module), the entire plate was scanned again. Quantifying the percentage of responders in each channel in each well provides a measure of the effect of melittin treatment on each physiological indicator.

Appropriate masks were generated independently for each channel. In order to obtain adequate sub-cellular resolution, the 20x objective was used. Population descriptors in the form of

threshold levels for each channel were determined from previous experiments. These levels allow the classification and tallying of individual cells as being within or outside the range for a healthy population, for each physiological indicator. The setting of these levels is described below (see also Table 8).

Fluorophore	Dye-Filter	Well-features used	Cellular Compartment
DNA binding dye (Blue)	Hoechst - XF93	MEAN_ObjectSizeCh1	Nucleus
Calcium Indicator (Green)	FITC - XF93	MEAN_CircAvgIntenCh2 %HighCircAvgIntenCh2	Entire Cell (Circ)
Mitochondrial Potential sensor (Orange)	TRITC - XF93	MEAN_RingSpotAvgIntenCh3 %LowRingSpotAvgIntenCh3	Mitochondria (RingSpot)
Membrane Permeability indicator (Far Red)	Cy5 - XF93	MEAN_CircAvgIntenCh4 %HighCircAvgIntenCh4	Nucleus (Circ)

Table 8. *Overview of Configuration of Compartmental Analysis for Multiparameter Cytotoxicity assay. Raw output features representing well averages have the prefix "MEAN" while the features that quantify percentage of responding cells have the prefix "%High" or "%Low."*

For Channel 2, the pertinent measurements for calcium homeostasis were gathered from a Circ region that expanded the Object Mask by 10 pixels (**CircModifierCh2 = 10**). This setting typically ensured collection of data from the entire projected area of the cell. In order to monitor responses of individual cells relative to a healthy population, the threshold for **CircAvgIntenCh2LevelHigh** was set to **75**. This enabled the output feature **%HIGH_CircAvgIntenCh2** to be used to show the percentage of cells that had elevated levels of cytosolic calcium at each time point.

The fluorescence associated with punctate mitochondria was measured to monitor $\Delta\Psi_m$ in Channel 3. The Ring mask was set so that it covered the cytoplasmic area of the cell. The default setting of 10 for **SpotDetectRadiusCh3** was optimal for capturing the appropriately sized spots corresponding to mitochondria. Setting **RingSpotAvgIntenCh3LevelLow** to **150** identified responding cells. The percentage of cells with depolarized mitochondria could then be monitored by viewing **%LOW_RingSpotAvgIntenCh3**.

Loss of plasma membrane integrity was measured by measuring the brightness of fluorescence under the Circ mask (nucleus) in Channel 4. The relevant threshold value, **CircAvgIntenCh4LevelHigh**, was set to **50** and the percentage of permeabilized cells was monitored as **%HIGH_CircAvgIntenCh4**.

Image data obtained from this assay performed on HepG2 cells is provided in Figure 10. Note the changes in each physiological indicator observed upon compound treatment. In this case, the compound used is melittin, a cytotoxic peptide from bee venom. Images demonstrate visualization of all four physiological indicators in the same cell population before and after treatment. Note that cytosolic calcium levels appear to increase (Channel 2) while mitochondrial potential (Channel 3) and plasma membrane integrity (Channel 4) appear to decrease.

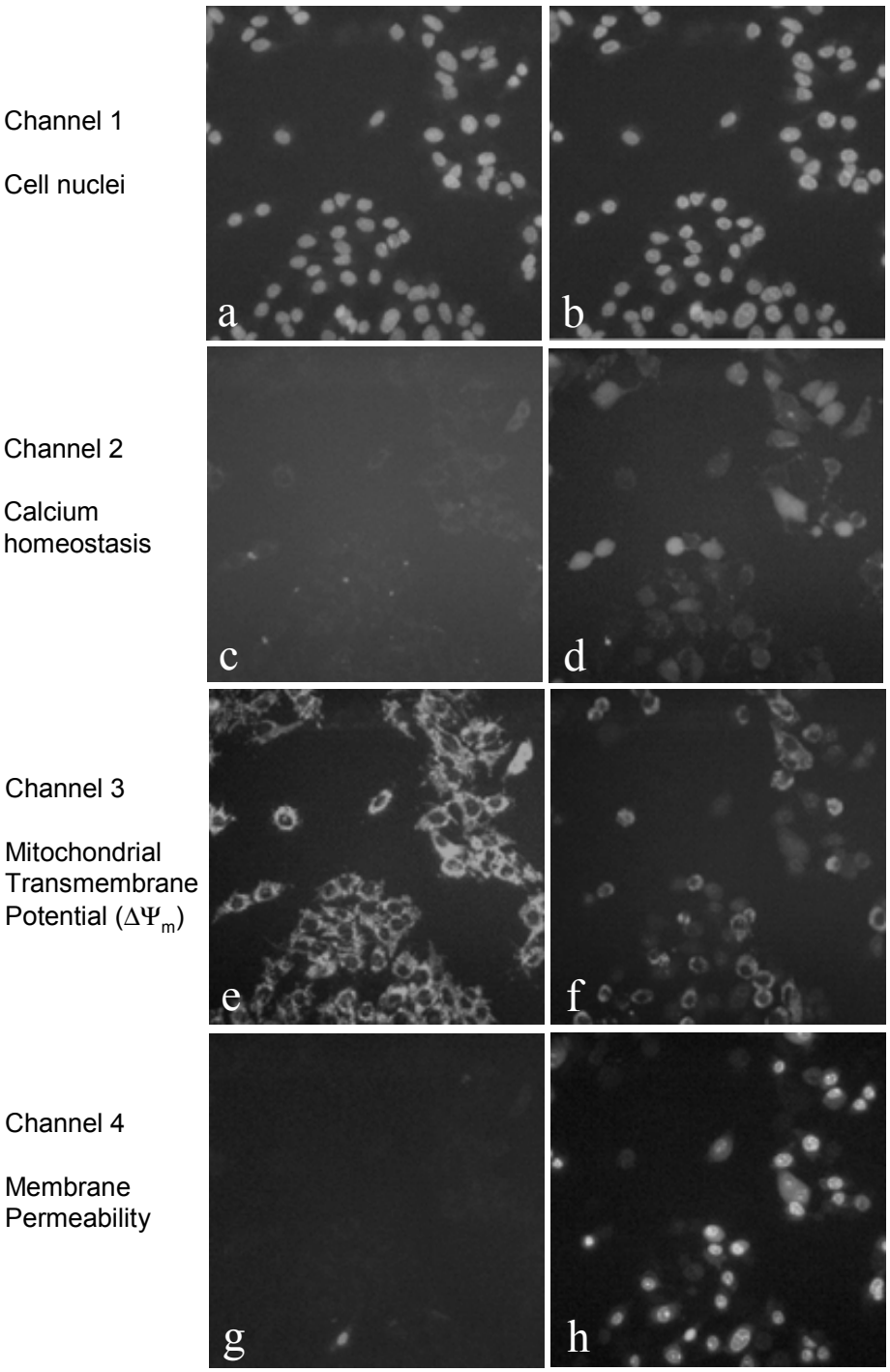

Figure 10. *Representative images demonstrating the employment of Compartmental Analysis for a 4-Channel Cytotoxicity Assay. HepG2 cells were loaded with fluorescent indicators to monitor nuclear morphology, intracellular calcium levels, mitochondrial transmembrane potential, and membrane permeability (see text). The same field of cells was imaged before (a, c, e, g) and after (b, d, f, h) treatment with the cytotoxic peptide, melittin. Field of view = 348 μm.*

The integrity of the plasma membrane was monitored by determining how many cell nuclei showed an increase in brightness of the membrane permeability indicator (Channel 4) above a set threshold using **%HIGH_CircAvgIntenCh4**. It was found that melittin permeabilized between 85 and 100% of cells, while cells treated with vehicle maintained their plasma

membrane integrity (data not shown). Melittin also caused depolarization of mitochondria as indicated by a decrease in average fluorescence within the RingSpot region (Channel 3). About 60-100% of the cells in melittin-treated wells showed a decrease in fluorescence below the set threshold. Cells treated with vehicle maintained their mitochondrial potential and were thus classified as non-responders (data not shown). While there were significant numbers of cells with elevated calcium in wells treated with the peptide, the separation between treated and untreated cells is not as great as was seen for membrane potential and plasma membrane permeability.

Analysis of the images showed that most of the cells in the treated wells had been permeabilized by the time the plate was scanned and it is likely that the calcium indicator had (a) leaked out of these cells and/or (b) been quenched as a result of entry of phenol red into these cells, resulting in a more variable response for calcium homeostasis. In this case, an end point measurement failed to clearly capture an effect which was obvious in the other measurements, demonstrating how monitoring multiple end points allows the gathering of information which may not be apparent by monitoring only one. The above data show only plots of well averages. It cannot be assumed that the same cells showed maximal responses in all four channels upon treatment with melittin. Definitive information on this can only be gathered by observing the multiparametric responses of individual cells.

Experiment #2

MDCK cells were loaded with all four fluorescent indicators as described in the previous section for HepG2 cells. After acquiring a baseline image set from each well, the automated pipetting system was used to deliver an aliquot of three distinct compounds in addition to the vehicle - A23187 (Ca^{2+} ionophore), FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone, mitochondrial poison)) and POESM (polyoxyethylenesorbitan monolaurate, detergent). Subsequently, images were acquired from each well at three-minute intervals to monitor cellular response to each compound. Figure 11 describes the result of this experiment.

Use of Compartmental Analysis allowed differentiation of the effects of these toxins. A23187 caused a transient increase in cytosolic calcium concentration without affecting mitochondrial potential or plasma membrane permeability over this time course. The mitochondrial uncoupler, FCCP, rapidly depolarized mitochondria without affecting calcium or permeability. More complex, multiparametric changes were seen when cells were treated with POESM. As a population, the cells showed an early depolarization of mitochondria, which appeared to be roughly coincident with an elevation of intracellular calcium levels. An increase in membrane permeability followed. In control wells, values for all four output features were steady over the course of the experiment. The successful use of MDCK cells demonstrates the compatiblity of this BioApplication with multiple cell types.

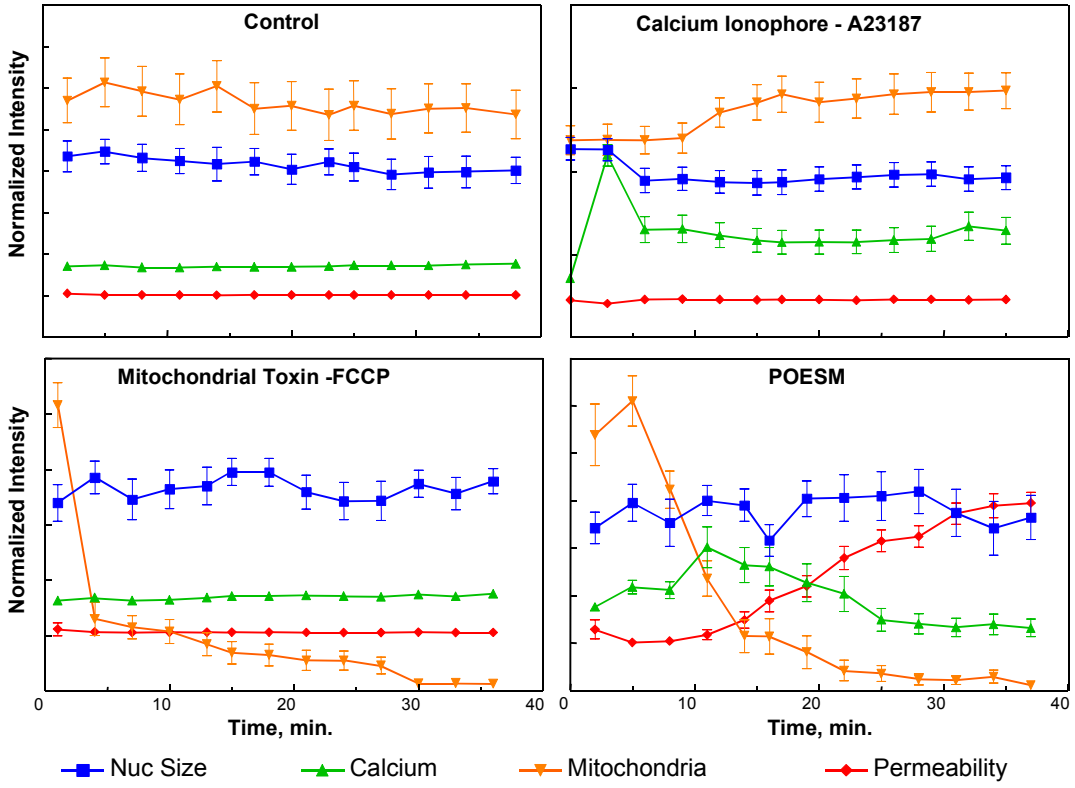

Figure 11. *Well-level Correlation of the kinetics of Multiple Indicators of Cytotoxicity. MDCK cells loaded with all four fluorescent indicators as previously described for HepG2 cells. Image data was acquired in four channels at 3 minute intervals. After the first baseline image, the automated pipetting system delivered vehicle (control), A23187 (10 μM), FCCP (8 μM) or POESM (0.25%), following which the cellular responses were monitored over time. Intensity values associated with the raw output features (Table 8) were normalized to allow visualization of the dynamics of the response in all channels, and plotted vs. time. Error bars indicate standard deviations for the population of cells monitored.*

Figure 12 shows the analysis of results at the cell level for the experiment described in Figure 11. Analysis of responses of individual cells revealed a level of detail not apparent when viewing the averaged well responses. Depolarization of mitochondria and associated release of calcium occurred within 5 minutes of POESM addition in all cells. A secondary correlation between membrane permeability and intracellular calcium was also observed in Cell #24. Note that the onset of increased permeability was always accompanied by a decrease in intracellular Ca^{2+} levels. This may be a result of the leakage of the calcium indicator from permeabilized cells and/or quenching as a result of phenol red entry. Simultaneous kinetic measurements of multiple events in each cell allowed the elucidation of multiple events that were not apparent when examining pooled population responses.

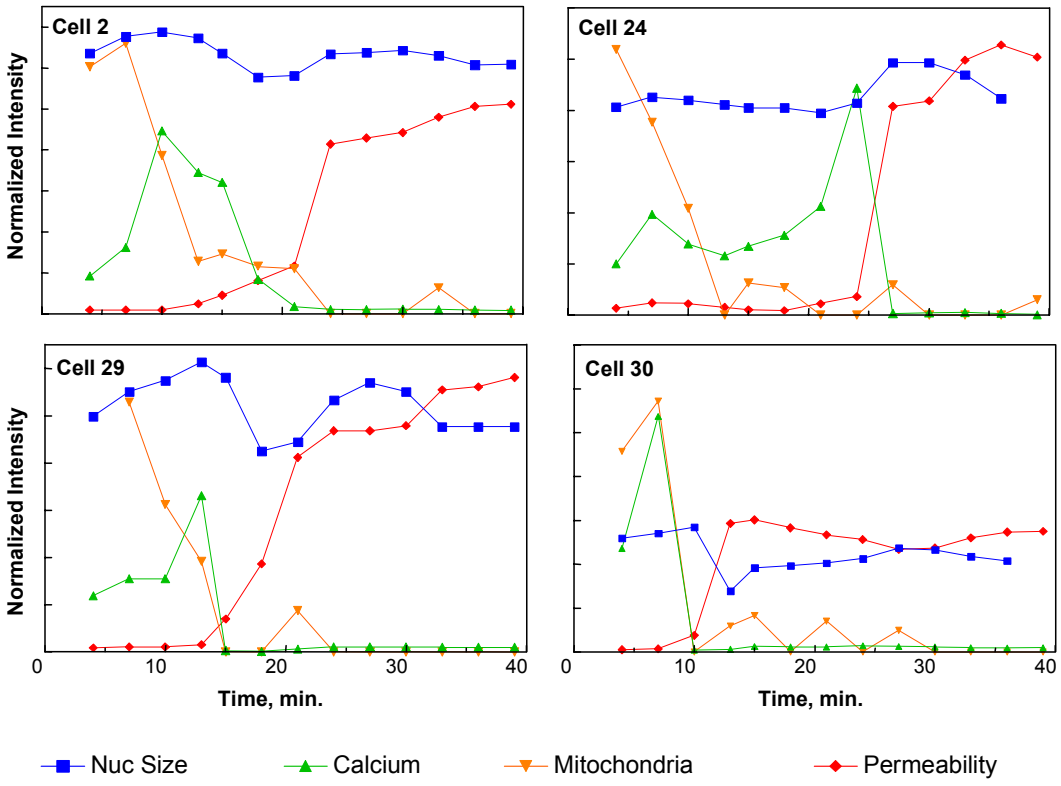

Figure 12. *Cell-level Correlation of the Kinetics of Multiple Indicators of Cytotoxicity in Response to POESM Treatment. Cell-level data was extracted from the well treated with POESM (Figure 11). The multiparametric response from four cells over time is shown. Intensity values associated with the raw output features (Table 8) were normalized to allow visualization of the dynamics of the response in all channels, and plotted vs. time.*

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Chapter
2

Description of the Algorithm

NOTE

The Assay Parameters described in this guide function in the same manner regardless of using the Classic or iDev versions of the ArrayScan software. For descriptions of each task for optimizing a Protocol in the iDev Assay Development Workflow, please refer to Chapter 4 of this guide.

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

Overview

Each biological application uses an image analysis algorithm that has been extensively tested and validated for robust screening performance. The algorithm has input parameters that control its analysis. Parameter values determined from validation plates for representative cell types have been supplied as defaults in the assay protocol provided. Parameters are adjustable to allow customization of the algorithm to your own samples and conditions.

Input parameters can be found in the Create Protocol View of the ArrayScan Classic software or in the Protocol Optimization task list of the iDev software. 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 and enables you to set criteria that defines responders for various features. They will be described in more detail 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 actual quantitative analysis of the images. The following sections provide information on using these parameters.

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, primary objects (such as nuclei) are identified; only pixels with intensities above this threshold will be considered as belonging to these structures. Thus the proper setting of an intensity threshold is a key early step in identifying objects and thus configuring the application. In Channels 2-6, other cellular regions (Circ, Ring, CircSpot, and RingSpot) are identified, and pixels with intensities above this threshold are used to help estimate target counts. Depending on the properties of the

objects being identified, the proper setting of intensity thresholds for the channels is necessary to ensure proper quantitative analysis.

There are three different options or methods for determining intensity thresholds, and sub-sets of these are available for each channel. For each Channel, you must select both a method and a value. The different options and values available for each channel are summarized in the Table 9, and the descriptions of the different methods follow in Table 10.

Intensity Threshold Method	Value Range	Channel Availability	
		Channel 1	Channels 2-6
Isodata	-0.99 – 9.99	✓	✓
Triang	-0.99 – 9.99	✓	✓
Fixed	0 - 32767	✓	✓

Table 9. *Intensity Threshold Methods Available for Each Channel*

NOTE

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

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

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

The other threshold methods (**Isodata** and **Triang**) are known as histogram-derived thresholds in that the threshold is chosen from the histogram of pixel intensities in the image (i.e., the image's brightness histogram). The schematics in Figure 13 demonstrate how these histogram-derived threshold values are calculated.

The values entered for the Object Identification in the application for the two histogram-derived threshold methods are offsets applied to determine the final threshold which is applied to the image. If the histogram-derived threshold is T, then its relationship to the actual (final) threshold, T_F, which is finally applied to the image, is determined from the user-entered offset value, o, as:

$$T_F = (1 + o)T$$

For example, suppose for a particular image, an **Isodata Threshold**, T, of 1000 is obtained. Then entering an offset value, o, of 0.9 will result in a final threshold of 1900 being applied to the image, whereas entering an offset value of -0.9 will result in a final threshold of 100 being applied. The range of possible values for the offset o is –0.999 to 9.999. However, note that the resulting applied threshold, T_F, will be restricted to the range 1-32767.

The two histogram-derived methods are dependent on the contents of the image, unlike the **Fixed Threshold** method. For example, supposing a blank image that contains no cellular objects and only has background pixels with a mean intensity value of 500 and standard deviation of 50, then it is unlikely that a **Fixed Threshold** of 1000 will cause any pixels to be registered as objects. However, the Isodata Threshold method will give thresholds causing pixels in the image to be registered for potential analysis; this is because the histogram is of the pixel intensity distribution of that image, even though there are no cellular objects in the image.

The **Triangle Threshold** method is more robust for the situation of blank fields that may contain rare bright objects; this is because the peak is of the background intensity, and the "triangle"-derived offset can be set to always be above the background, yet low enough to pick any bright objects. Thus, in situations where blank images are expected (e.g., from a loss of signal due to a compound condition, a loss of protein expression, or a lack of label), the **Isodata Threshold** method should be avoided; instead either a **Fixed Threshold** or the **Triangle Threshold** method with a large offset can be entered.

Threshold Option	Description	Range of Possible Values Entered	Resulting Applied Threshold Range
Isodata	Adjusts the object identification threshold relative to the Isodata value. The threshold *T* is chosen so that it is equal to the average of the mean of the pixel intensities to the left of the threshold (m_L) and the mean of the pixel intensities to the right of the threshold (m_R). A negative value identifies dimmer objects and results in larger object masks. A positive value results in smaller object masks.	-0.999 – 9.999 (offset)	1 - 32767
Triang	Adjusts the object identification threshold relative to the Triangle value. The threshold *T* which gives the maximum distance *d* A negative value identifies dimmer objects and results in larger object masks. A positive value results in smaller object masks	-0.999 – 9.999 (offset)	1 - 32767
Fixed	A fixed pixel intensity value between 0-32767 is applied	0 – 32767 (actual intensity in image)	0 - 32767

Table 10. *Intensity Threshold Descriptions and ranges available for the Compartmental Analysis BioApplication*

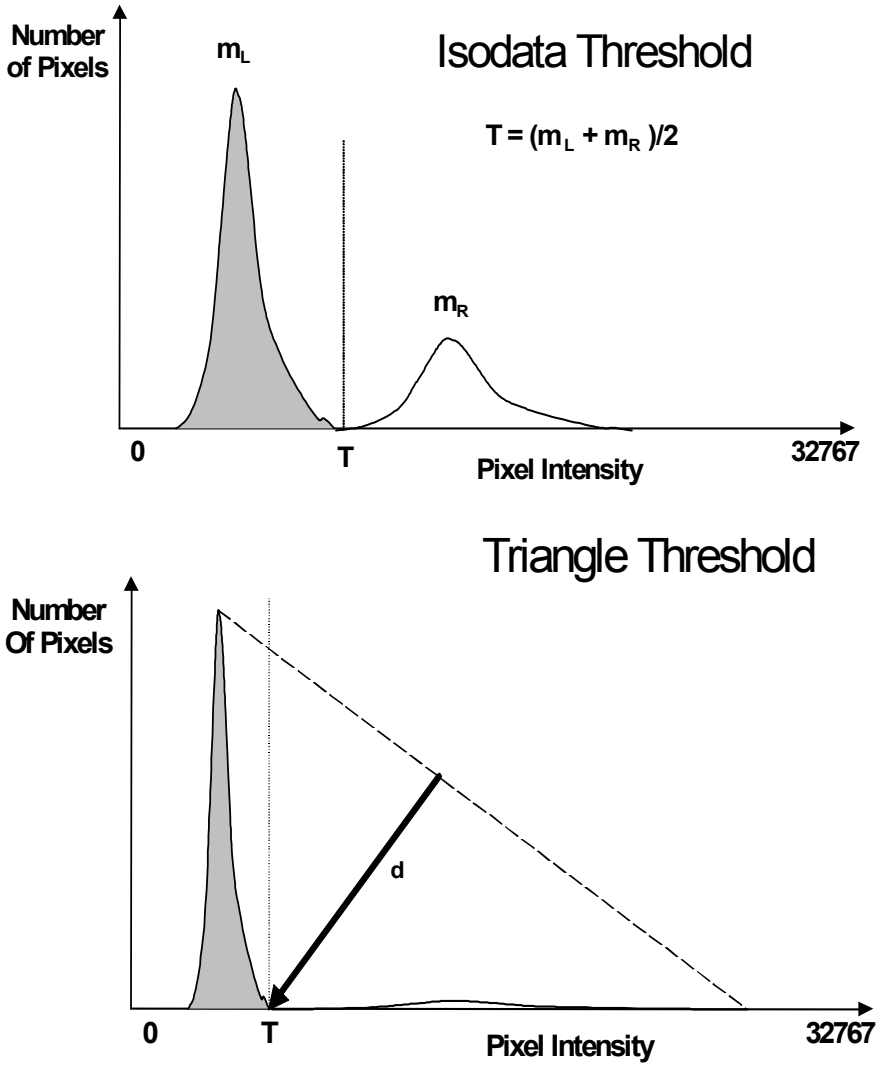


Figure 13. *Histogram-derived Intensity Threshold Methods (top: Isodata, bottom: Triangle). Background peak is shown in gray and object peak is shown in white.*

Description of Assay Parameters and Settings

Understanding the key steps and the principle behind the image processing algorithm will allow you to more effectively adjust the protocol parameter values to analyze your images. All of the key image processing steps occur at the level of the individual object (typically a cell), for which Cell Features are computed. The Cell Features, calculated for every object analyzed, are used to calculate Well Features, or population descriptors for each well.

Assay Parameters for Image Analysis

General Assay Parameters

General Assay Parameters control general aspects of image processing and analysis:

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

Reference Well Control

The two General Assay Parameters controlling the use of Reference Wells are: **UseReferenceWells** and **MinRefAvgObjectCountPerField**. The **UseReferenceWells** Assay Parameter allows you to choose whether Reference Wells are to be used to determine the population characterization thresholds. If Reference Wells are to be used (value = **1**), then the Assay Parameter **MinRefAvgObjectCountPerField** must be set. This is the minimum number of objects detected per field that are required for acceptance of the data in the Reference Wells. This allows you to enter the minimum number of objects that you feel gives a good distribution and, thus, statistical validity to the thresholds calculated from the Reference Wells. Note that in addition to these general Assay Parameters, there are additional Assay Parameters for Reference Well processing that are specific for particular features and channels. These will be described in later sub-sections.

Units for Morphological Measurements

Areas and lengths can be reported in either calibrated units (micrometers) or pixels. This option is controlled by the **UseMicrometers** parameter. Setting this Assay Parameter value to **1** will report results in micrometers and setting it to **0** (zero) reports morphological measurements in pixels. The conversion factor from pixels to micrometers is calculated automatically from the Objective and Camera Acquisition Mode selected.

Object Type

ObjectTypeCh1, SpotTypeCh2-6

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

within an image to be considered as potential objects, while leaving it as **0** considers bright areas on a dark background as potential objects (Table 11).

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

***Table 11**. Binary settings for **ObjectTypeCh1** and **SpotTypeCh2-6**.*

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

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

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

Background Correction

BackgroundCorrectionChN

Prior to image analysis, the non-cellular background can be computed and subtracted from the image separately in each channel, if desired, as shown in Figure 14.

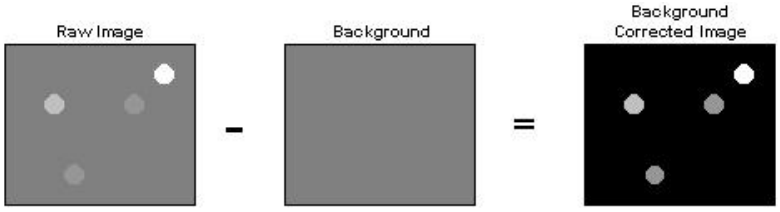


***Figure 14**. Image depicting calculation of positive Background Correction that can be used in each channel.*

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

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

subtraction is done. Setting the value to **–1** lets the BioApplication decide on the value needed for the optimal background correction. Table 12 gives an overview of the different Background Correction settings that can be used for each channel.

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

Table 12. *Possible Background Correction Methods used in each channel with the Compartmental Analysis BioApplication.*

NOTE

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

Background Correction can be adjusted on each channel separately.

The background-corrected image is not stored or shown.

Object Smoothing

ObjectSmoothFactorCh1

Channel 1 has an independent Assay Parameter that enables control over the degree of image smoothing, or blurring, before the identification of objects (**ObjectSmoothFactorCh1**). This Assay Parameter corresponds to the radius in pixels of the area used to smooth the image. For example, a small value, such as **3**, means that a region with a radius of 3 pixels is used to smooth the image (region with dimensions 7×7 pixels, or 49 pixels total). Doubling the value to **6** would mean that a larger region (13×13 pixels or 169 pixels total) is used to smooth the image, and thus the image will be smoothed more than the previous value. A value of **0** means that smoothing is not done.

This Assay Parameter is used to smooth images with a lot of contrast to improve identification of objects. To get sharper definition of the shapes of the edges of objects (i.e., cells), you may want to keep the **ObjectSmoothFactorCh1** small, if not 0. However, if your Channel 1 label is not very homogeneous, the actual object will be erroneously identified as consisting of several smaller sized objects, and then smoothing will result in a normalizing effect of the Channel 1 label and will help identify the object with its true bounds.

Smoothing can be used to connect fragments of objects and can be valuable when measuring morphological changes. Figure 15 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).

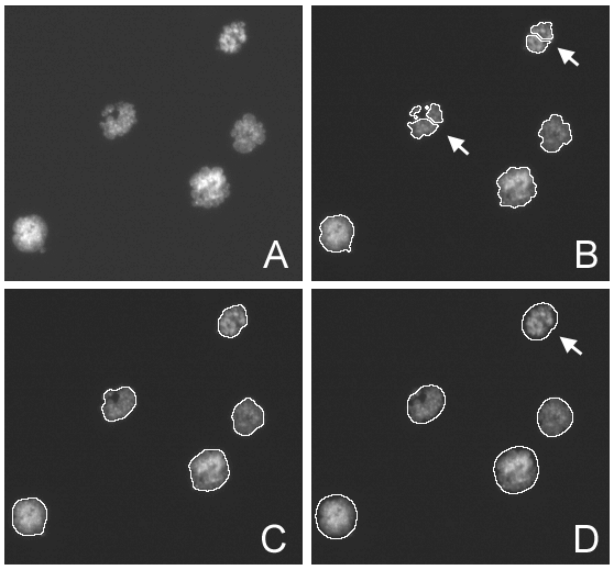


Figure 15. *Images show the effect of different degrees of smoothing on the same field of apoptotic cell nuclei. A: Raw Image; B:* ***ObjectSmoothFactorCh1*** *= 0; C:* ***ObjectSmoothFactorCh1*** *= 3; D:* ***ObjectSmoothFactorCh1*** *= 8.*

Object Segmentation

ObjectSegmentationCh1

Even though you may have chosen an optimal intensity threshold and an appropriate degree of smoothing for object identification, you may encounter situations where individual objects cannot be properly resolved, such as in the case of densely packed objects. Object Segmentation will help resolve and identify individual objects. Two methods are available for object segmentation in the Compartmental Analysis BioApplication: Geometric (shape and size) and Intensity (intensity peaks). These methods are illustrated in Figure 16.

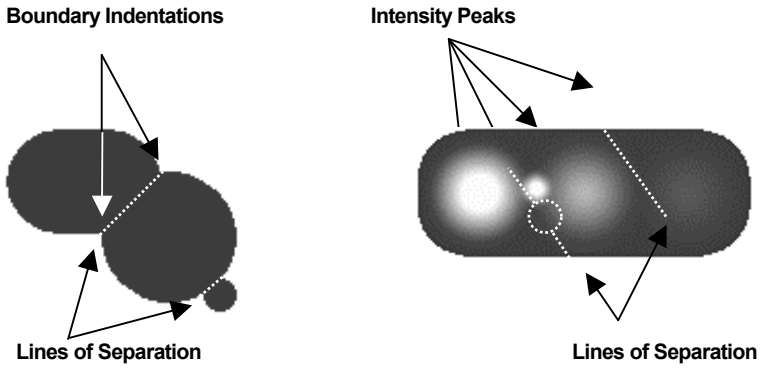


Figure 16. *Object Segmentation Options. The image on the left illustrates the Geometric Method while the image on the right illustrates the Intensity Method.*

The Geometric method splits touching objects on the basis of shape, relying on boundary indentations to locate the line of separation. This method works best if the individual objects have smooth boundaries with pronounced indentations at their point of contact. The Geometric

method can be used to separate objects that are uniform in intensity (i.e., saturated) or that have multiple intensity peaks (i.e., noisy or textured).

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

The **ObjectSegmentationCh1** Assay Parameter (see Table 13) 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 **ObjectSegmentationCh1** 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 **ObjectSegmentationCh1** 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 **ObjectSegmentationCh1** disables the segmentation.

Segmentation Method	ObjectSegmentationCh1 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 13. *Channel 1 Object Segmentation Options*

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

Geometric Method Setting **ObjectSegmentationCh1** to R1 (Figure 17 A) produces object separation shown in Figure 17B. Setting **ObjectSegmentationCh1** to R3 results in segmentation shown in Figure 17C. Setting **ObjectSegmentationCh1** to R2 (the largest radius of all touching objects) produces no segmentation.

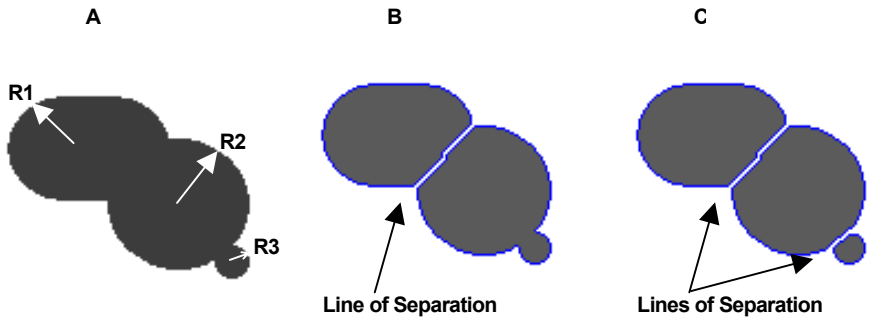


Figure 17. *Object segmentation: Geometric Method. A) Radii of touching objects: R1 = 24, R2 = 30, R3 = 7; B) Result of segmentation using* ***ObjectSegmentationCh1*** *= R1; C) Result of segmentation using* ***ObjectSegmentationCh1*** *= R3.*

Intensity Method Figure 18 shows the intensity profile along the cordial line of an object with four intensity peaks from Figure 16. 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 **ObjectSegmentationCh1** parameter value equal to the lowest relative height of intensity peaks of objects that need to be separated. The lower the value, the more objects will be segmented and vise versa. In case of over-segmentation, setting **ObjectSmoothFactorCh1** greater than 0 should be used to alleviate the problem.

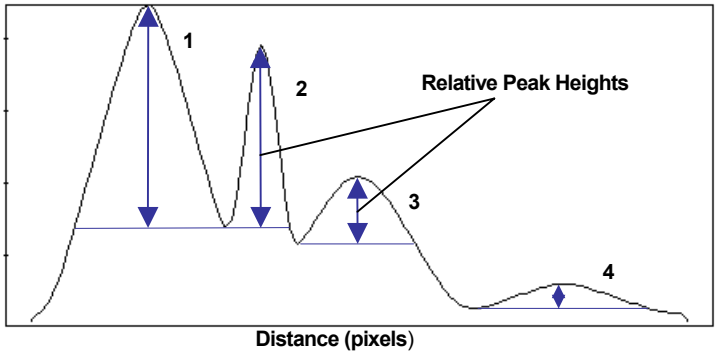


Figure 18. *Intensity profile of an object with four intensity peaks. Peak #1 height = 1500, peak #2 height = 1250, peak #3 height = 500, and peak #4 height = 200.*

Only two peaks in Figure 18, #1 and #2, have relative intensity greater than 1000. Thus, setting the **ObjectSegmentationCh1** 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 19A. Setting the **ObjectSegmentationCh1** 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 19B and 19C.

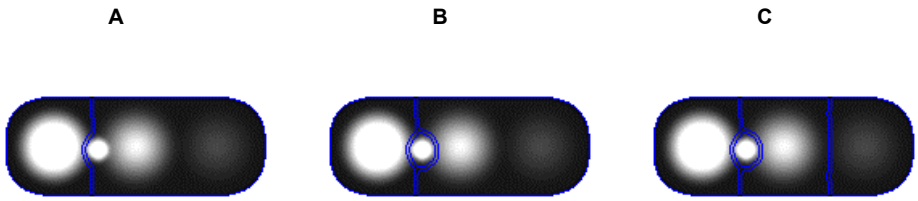


Figure 19. *Object segmentation: Intensity Method. A) Result of segmentation using parameter* ***ObjectSegmentationCh1*** *= -1000; B) Result of segmentation using parameter* ***ObjectSegmentationCh1*** *= -400; C) Result of segmentation using parameter* ***ObjectSegmentationCh1*** *= -100.*

Reject Border Objects

RejectBorderObjectsCh1

When running the BioApplication, you have the option of not including and analyzing objects that touch the border of your image field. This is controlled by the **RejectBorderObjectsCh1** Assay Parameter. If this Assay Parameter has a value of **1**, objects touching the border are not analyzed. On the other hand, a value of **0** results in all objects in the image field to be eligible for analysis, whether or not they touch the image border.

Channel-Specific Assay Parameters

Channels to Derive Cellular Region Overlays

When running the Compartmental Analysis BioApplication, overlays on the displayed image indicate each of the four regions in the cell. However, only one overlay is derived for each of the four regions, and you have to choose from which of the dependent channels each region's overlay is derived. This is done by specifying a dependent channel in each of the following four Assay Parameters: **ChannelToDeriveRingOverlay**, **ChannelToDeriveCircOverlay**, **ChannelToDeriveRingSpotOverlay**, and **ChannelToDeriveCircSpotOverlay**.

The "derived" overlay Assay Parameters only affect the overlay display and do not affect the actual analysis and values of the calculated numbers.

Adjusting Cellular Regions

CircModifierCh*N*, RingDistanceCh*N*, RingWidthCh*N*

The size of the Circ and Ring regions can be independently adjusted for each of the dependent channels being used (i.e., Channels 2-6), and this size is set in relation to the primary object. The schematic in the Figure 20 shows the three parameters that are used to control the Circ and Ring regions.

The **CircModifierCh*N*** Assay Parameter is used to adjust the Circ region in Channels 2-6. The value of this parameter equals the number of pixels that the primary object is dilated (a positive value) or eroded (a negative value) to define the Circ region. In other words, this is the number of pixels added to, or subtracted from, the perimeter of the primary object to define the area covered by the Circ region. In the following figure, this Assay Parameter is positive and the primary object has been dilated to make the Circ region.

There are two Assay Parameters used to define the area covered by the Ring region for Channels 2-6, **RingDistanceCh*N*** and **RingWidthCh*N***. **RingDistanceCh*N*** is the number of pixels added to, or subtracted from, the perimeter of the primary object to define the inner

boundary of the Ring region. **RingWidthCh*N*** defines the width of the Ring region in pixels. Note that since the Ring region is derived from the primary object, the area it covers is defined independently from the area covered by the Circ region.

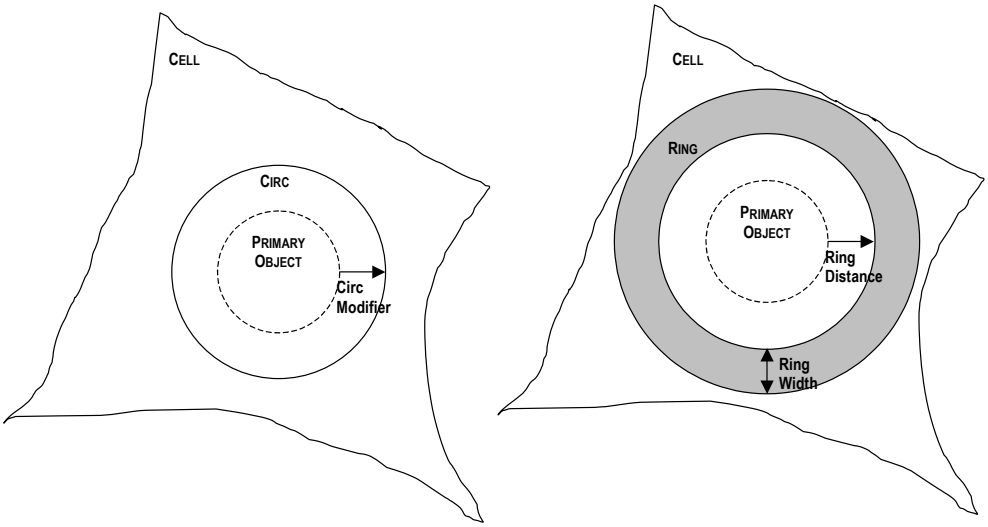


Figure 20. *Adjustment of Circ and Ring Regions found in Channels 2-6 of the Compartmental Analysis BioApplication.*

MaskModifierCh*N*

The Assay Parameter that controls the modification of the primary object area is **MaskModifierCh*N***, where Ch*N* is the relevant dependent channel (Channels 2-6). This Assay Parameter is similar in its functionality to the **CircModifierCh*N*** Assay Parameter used to adjust the Circ region and equal to the number of pixels that the primary object is dilated or eroded to define the mask. However, only objects whose intensities in the dependent channels within a modified area fall within a range that you specify (within the Object Selection Parameter values for Channels 2-6) are selected for quantitation. Please see Gating section for more information.

Identifying Spots

SpotKernalRadiusCh*N*

The Compartmental Analysis BioApplication first identifies all the spots in each of the dependent channels that is used and then identifies those that are Circ Spots or Ring Spots, depending on whether they are in the Circ or Ring regions respectively. Spots in the cell are usually surrounded by background cellular fluorescence, and the area covered by an individual spot first has to be distinguished from this background fluorescence. This is controlled by the **SpotDetectRadiusCh*N*** Assay Parameter found in Channels 2-6. This Assay Parameter controls the removal of local background fluorescence in the cell so that individual spots can be identified; the size of this parameter is similar to the maximum size, in pixels, of the spots to be detected. Once the region covered by each individual spot is identified, then each spot's intensity is measured within its respective region. However, only pixels that are within each spot's region and whose intensity is greater than a threshold are used in that spot's intensity calculation. The higher the intensity threshold is set, the more stringent the criteria are for a pixel to be identified as a spot and for its intensity to be used in the spot's intensity computation. This intensity threshold is an Object Identification Parameter, and three methods of setting the intensity threshold are available: Isodata, Fixed, and Triang (see Table 10).

To summarize, the three steps in identifying Ring or Circ Spots are as follows:

- Spots are distinguished from the local intracellular background fluorescence, and the area covered by each spot is identified.
- Within this area, only those pixels whose intensities are greater than an intensity threshold are selected as belonging to the spot.
- Spots are classified as Ring Spots or Circ Spots, depending on whether they are in the Ring or Circ regions respectively.

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 14). 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
ChannelToDeriveRingOverlay	Channel	Channel from which ring mask overlay will be created
ChannelToDeriveCircOverlay	Channel	Channel from which circ mask overlay will be created
ChannelToDeriveRingSpotOverlay	Channel	Channel from which ring spot mask overlay will be created
ChannelToDeriveCircSpotOverlay	Channel	Channel from which circ spot mask overlay will be created
ObjectTypeCh1	Binary	Type of objects 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
ObjectSmoothFactorCh1	Number	Degree of image smoothing (blurring) prior to object detection in Ch1: 0 = Do not apply smoothing
ObjectSegmentationCh1	Pixels	Radius (in pixels) of touching objects that should be separated in Ch1: Negative value = Use intensity peaks method, 0 = Do not apply object segmentation, Positive value = Use shape geometry method
ObjectCleanUpCh1	Binary	Clean up object mask and remove small objects (debris) by applying erosion followed by dilation; resulting in cleaner masks and overlays: 0 = No, 1 = Yes
RejectBorderObjectsCh1	Binary	Reject objects that touch image edges: 0 = No, 1 = Yes
SpotTypeChN	Binary	Type of spots to be identified in ChN: 0 = Bright spots on dark background, 1 = Dark spots on bright background
SpotDetectRadiusChN	Pixels	Radius (in pixels) of region used for spot detection in ChN: 0 = Do not detect spots
RingDistanceChN	Pixels	Distance (in pixels) from Ch1 object mask to the inner rim of ChN ring mask
RingWidthChN	Pixels	Width (in pixels) of ChN ring mask
CircModifierChN	Pixels	Number of pixels to modify Ch1 object mask to create circ mask in ChN: Negative value = Shrink mask, 0 = Do not modify mask, Positive value = Expand mask
MaskModifierChN	Pixels	Number of pixels to modify Ch1 object mask in ChN: Negative value = Shrink mask, 0 = Do not modify mask, Positive value = Expand mask

Table 14. *Basic Assay Parameters found in the Compartmental Analysis BioApplication. *Note that "ChN" refers to* ***Background Correction*** *in Channels 1-6 and other parameters found in Channels 2-6.*

Object Selection Parameters

Each channel has a set of specific Object Selection Parameters associated with it (see Table 15 and Table 16). If an object in the particular channel's image has all measured features within the range specified by the appropriate Object Selection Parameters, then it is analyzed; otherwise, it is rejected from the analysis. Channel 1 is used to identify valid primary objects. The selection parameters for Channels 2-6 further qualify whether the primary object (or specified area inside the primary object) is analyzed. A **Valid Object** that satisfies Object Selection Parameters for Channels 2-6 is defined as a **Selected Object**. Only the **Selected Objects** are analyzed by the BioApplication.

Channel 1

Parameter	Units	Description
ObjectAreaCh1	Pixels or μm^2	Area (in pixels or micrometers) of Ch1 object
ObjectShapeP2ACh1	Number	Shape measure based on ratio of perimeter squared to 4π*area of Ch1 object (**ObjectShapeP2ACh1** = 1 for circular object)
ObjectShapeLWRCh1	Number	Shape measure based on ratio of length to width for object-aligned bounding box of Ch1 object
ObjectTotalIntenCh1	Intensity	Total intensity of all pixels within Ch1 object
ObjectAvgIntenCh1	Intensity	Average intensity of all pixels within Ch1 object
ObjectVarIntenCh1	Intensity	Variation (standard deviation) of intensity of all pixels within Ch1 object

Table 15. *Channel 1 Object Selection Parameters in the Compartmental Analysis BioApplication.*

Channel N (Channels 2-6)

Parameter	Units	Description
RingAvgIntenChN	Intensity	Average intensity of all pixels within ChN ring mask
CircAvgIntenChN	Intensity	Average intensity of all pixels within ChN circ mask
CircRingAvgIntenDiffChN	Number	Difference between CircAvgIntenChN and RingAvgIntenChN
CircRingAvgIntenRatioChN	Number	Ratio of CircAvgIntenChN to RingAvgIntenChN
RingSpotTotalAreaChN	Pixels or μm^2	Total area of all spot pixels within ChN ring mask
RingSpotAvgIntenChN	Intensity	Average intensity of all spot pixels within ChN ring mask
CircSpotTotalAreaChN	Pixels or μm^2	Total area of all spot pixels within ChN circ mask
CircSpotAvgIntenChN	Intensity	Average intensity of all spot pixels within ChN circ mask
AvgIntenChN	Intensity	Average intensity in ChN of all pixels within modified Ch1 object mask
TotalIntenChN	Intensity	Total intensity in ChN of all pixels within modified Ch1 object mask

Table 16. *Channels 2-6 Object Selection Parameters in the Compartmental Analysis BioApplication. *Note that "ChN" refers to Channels 2-6.*

Gating

The Compartmental Analysis BioApplication supports gating on a cell population. This feature provides selective cellular (and sub-cellular) analysis based on fluorescent intensity and spot area. Therefore, in addition to validating cells based on nuclear area or nuclear shape, you can also select or reject cells based on fluorescent intensity or spot area in all channels. You may want to gate if, for example, cells have been identified in the focus channel, and it is necessary to refine the object selection based on fluorescence intensity of the objects in a second channel.

Specifying Intensity Ranges for Gating

When working in the Create Protocol View or Protocol Interactive View, you can specify intensity ranges by entering upper and lower limits in Channels 2-6 for **AvgIntenCh*N*** and **TotalIntenCh*N***. **TotalIntenCh*N*** is a summation of all intensities within the object of interest. The **AvgIntenCh*N*** parameter is **TotalIntenCh*N*** divided by the object area.

Upper and lower limits can also be set on specific masks within the objects from in Channels 2-6. Average and Total Intensity limits for Ring, Circ, RingSpot, and CircSpot, as well as CircRing intensity differences and ratios can be set in any of these channels. Total Area found within the RingSpot and/or CircSpot can also be used for gating in Channels 2-6.

Specifying Mask Modifiers for Gating

In addition to specifying intensity ranges for one or more channels, you can apply a mask to one or more downstream channels using the **MaskModifierCh*N*** setting. You may want to use this feature if, for example, it is desirable to modify the mask of the primary object to include other cellular markers. The mask functions the same as **CircModifierCh*N*** (which can be dilated or eroded but will not overlap with other masks from nearby objects). Once the mask for each channel is determined, the upper and lower intensity limits can then be specified. For each channel, **AvgIntenCh*N*** and **TotalIntenCh*N*** values within the modified masks are calculated. If the calculated value does not fall within the specified upper and lower limits, the object is removed from set of selected objects.

As mentioned previously (and Figure 17), although **CircModifierCh*N***, **RingDistanceCh*N***, **RingWidthCh*N***, and **SpotKernalRadiusCh*N*** are used to help calculate Cell and Well Output Features for intensity and morphological measurements, these Assay Parameters are also used for gating purposes in order to select the areas for objects to be identified. Adjusted areas are then used in combination with the Object Selection parameters in order to gate out cells or sub-cellular organelles that do not fit intensity or morphological limits.

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. Overlays available for the Compartmental Analysis BioApplication can be found in Table 17.

Parameter	Description
Include This Channel In Composite	If checked, channel image is included in the composite image. Note that in order to view a composite image in Scan or View software applications, at least one box must be checked.
SelectedObject	Outlines valid objects in Channel 1 (e.g., cells) for analysis. Valid objects are those that have properties in the range specified by the Object Selection Parameters.
RejectedObject	Outlines objects rejected for analysis. Rejected objects have properties outside the range specified by the Object Selection Parameters.
Ring	Outlines Ring region. The particular Ring used for the overlay is specified in the **ChannelToDeriveRingOverlay** Assay Parameter.
Circ	Outlines Circ region. The particular Circ used for the overlay is specified in the **ChannelToDeriveCircOverlay** Assay Parameter.
RingSpot	Outlines RingSpot region. The particular RingSpot used for the overlay is specified in the **ChannelToDeriveRingSpotOverlay** Assay Parameter.
CircSpot	Outlines CircSpot region. The particular CircSpot used for the overlay is specified in the **ChannelToDeriveCircSpotOverlay** Assay Parameter.

Table 17. *Image Overlays available in the Compartmental Analysis BioApplication.*

Assay Parameters For Population Characterization

Overview of Population Characterization

The Compartmental Analysis BioApplication provides the ability to characterize cells based on their response compared to a control population. For a particular Cell Feature, a range is determined and set for a control population that has the normal physiological distribution for that feature. Lower and upper thresholds (known as ***Feature*Ch*N*LevelLow** and ***Feature*Ch*N*LevelHigh**) set the lower and upper bounds of this range respectively. The **Status** Cell Feature indicates whether a cell is within or beyond this range (Table 18).

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

***Table 18**. Numeric explanation of Cell Feature Status when using population characterization.*

The corresponding Well Features reported are the percentage of cells that are either less than (**%LOW_*Feature*Ch*N***) or greater than (**%HIGH_*Feature*Ch*N***) the levels defining this value. Figure 21 illustrates this concept by showing the use of lower and upper thresholds to identify low and high responder cells, respectively for a particular Cell Feature.

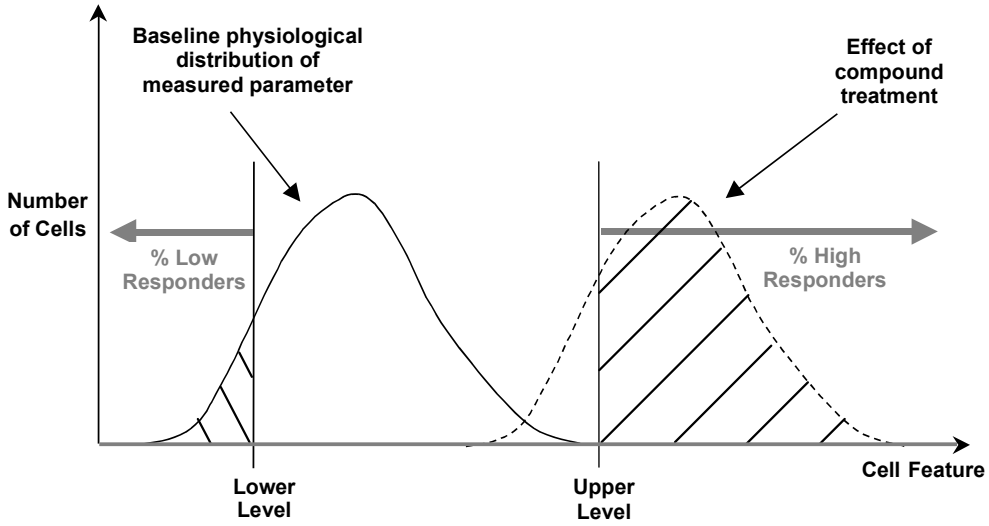


***Figure 21.** Cellular response levels distinguishing baseline from both high and low responders.*

Setting Cellular Response Levels

There are two ways of setting the lower and upper-response levels to characterize the cell population. The first is manually entering values for the lower and upper ***Feature*Ch*N*Level** Assay Parameters in the Protocol Create or Protocol Interactive Views (Advanced Mode). This requires prior knowledge of typical feature values. The BioApplication then uses the defined levels to calculate the percentage of cells outside the bounds of the specific ***Feature*Ch*N*LevelLow, *Feature*Ch*N*LevelHigh** values.

The second is to automatically calculate the ***Feature*Ch*N*LevelLow, *Feature*Ch*N*LevelHigh** Assay Parameters through Reference Wells. You designate which wells on the sample plate should be used as Reference Wells. Typically, Reference Wells contain a control (i.e., non-compound treated) population of cells that displays the normal basal physiological response for the parameter being measured. These wells are first analyzed and the population distribution for the different features is determined. The cell population characterization levels are then set through use of a correction coefficient (_CC) value by adding and subtracting from the mean of the distribution its standard deviation multiplied by a correction coefficient. The whole plate is then scanned with these levels applied. 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 correction coefficient by which to multiply the standard deviation would be two. 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.

Reference Wells Processing Sequence

By setting the **UseReferenceWells** Assay Parameter to **1**, the Reference Wells processing is engaged. Specified fields within the Reference Wells are acquired/analyzed and Well and Plate Features are computed. After this sequence is completed, computed values will be assigned to the Assay Parameters associated with each Reference Feature and regular scanning of the plate will begin. Again, if the feature value for **MinRefAvgObjectCountPerField** obtained from the Reference Wells is below the value set for that Assay Parameter, the BioApplication aborts the use of Reference Wells and processes the plate as if **UseReferenceWells** is set to **0**. The Compartmental Analysis BioApplication only uses **Known** Reference Wells. The sequence of computation for Reference Wells is as follows:

1. Cell Features are computed for every valid object within a field.
2. For each Cell Feature to be used for population characterization, the mean and standard deviation are computed over all cells in the field.
3. Reference Field Features are determined with the following naming convention:
    - **Ref*Feature*Ch*N*LevelLow**
    - **Ref*Feature*Ch*N*LevelHigh**
4. Reference Well Features are computed as average values for fields in a well, weighted for the number of cells per field, and then Reference Plate Features are computed as arithmetic averages for all Reference Wells on a plate. Use of a weighted average minimizes the effect of sparse fields.

Identifying Reference Wells and Control Parameters

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

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

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

Specifying and Controlling Reference Wells

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

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

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

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

- ***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 (such as **ObjectAreaCh1**) and *N* refers to the specific channel. The ***Feature*Ch*N*LevelLow** and ***Feature*Ch*N*LevelHigh** Assay Parameter types specify the actual levels and must be manually entered if Reference Wells are not used. If using Reference Wells, the ***Feature*Ch*N*LevelLow_CC** and ***Feature*Ch*N*LevelHigh_CC** Assay Parameters are correction coefficients (CC) used to derive the ***Feature*Ch*N*LevelLow** and ***Feature*Ch*N*LevelHigh** values from the mean and standard deviation of the reference well population calculated as:

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

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

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

Advanced Assay Parameters

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 the advanced Assay Parameters. When you check the box, only the Basic Assay Parameters are shown; when you uncheck the box, all Basic and Advanced Assay Parameters are shown..

For each feature undergoing population characterization, there are four advanced Assay Parameters that control its levels: the ***Feature*Ch*N*LevelLow** and ***Feature*Ch*N*LevelHigh** that set lower and upper thresholds and the presence of the **_CC** suffix which designates those levels are set using Reference Wells. For example, the Assay Parameters controlling the object's size in Channel 1 are:

- **ObjectSizeCh1LevelLow**
- **ObjectSizeCh1LevelHigh**
- **ObjectSizeCh1LevelLow_CC**
- **ObjectSizeCh1LevelHigh_CC**

In the listing of Advanced Parameters in Table 19, instead of listing all 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 object size:

- ObjectSizeCh1Level***Low/High,Low/High_CC***

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

Parameter	Units	Description
MinRefAvgObjectCountPerField	Number	Minimum average number of objects per field required for acceptance of reference well results
UseMicrometers	Binary	Measure lengths and areas in: 0 = Pixels, 1 = Micrometers
PixelSize	µm	Pixel size in micrometers (depends on objective selection)
Type1EventDefinition	---	User-defined combination of logic statements involving response features ***(cannot be edited)***
Type2EventDefinition	---	User-defined combination of logic statements involving response features ***(cannot be edited)***
Type3EventDefinition	---	User-defined combination of logic statements involving response features ***(cannot be edited)***
ObjectAreaCh1Level***Low/High, Low/High_CC***	Pixel or μm^2	Defines **ObjectAreaCh1** population characterization thresholds
ObjectShapeP2ACh1Level***Low/High, Low/High_CC***	Number	Defines **ObjectShapeP2ACh1** population characterization thresholds
ObjectShapeLWRCh1Level***Low/High, Low/High_CC***	Number	Defines **ObjectShapeLWRCh1** population characterization thresholds
ObjectTotalIntenCh1Level***Low/High, Low/High_CC***	Intensity	Defines **ObjectTotalIntenCh1** population characterization thresholds
ObjectAvgIntenCh1Level***Low/High, Low/High_CC***	Intensity	Defines **ObjectAvgIntenCh1** population characterization thresholds

Parameter	Units	Description
ObjectVarIntenCh1Level***Low/High, Low/High_CC***	Intensity	Defines **ObjectVarIntenCh1** population characterization thresholds
ObjectSizeCh1Level***Low/High, Low/High_CC***	Pixels or µm	Defines **ObjectSizeCh1** population characterization thresholds
RingTotalIntenChNLevel***Low/High, Low/High_CC***	Intensity	Defines **RingTotalIntenChN** population characterization thresholds
RingAvgIntenChNLevel***Low/High, Low/High_CC***	Intensity	Defines **RingAvgIntenChN** population characterization thresholds
CircTotalIntenChNLevel***Low/High, Low/High_CC***	Intensity	Defines **CircTotalIntenChN** population characterization thresholds
CircAvgIntenChNLevel***Low/High, Low/High_CC***	Intensity	Defines **CircAvgIntenChN** population characterization thresholds
CircRingAvgIntenDiffChNLevel***Low/High, Low/High_CC***	Intensity	Defines **CircRingAvgIntenDiffChN** population characterization thresholds
CircRingAvgIntenRatioChNLevel***Low/High, Low/High_CC***	Number	Defines **CircRingAvgIntenRatioChN** population characterization thresholds
RingSpotTotalIntenChNLevel***Low/High, Low/High_CC***	Intensity	Defines **RingSpotTotalIntenChN** population characterization thresholds
RingSpotAvgIntenChNLevel***Low/High, Low/High_CC***	Intensity	Defines **RingSpotAvgIntenChN** population characterization thresholds
RingSpotTotalAreaChNLevel***Low/High, Low/High_CC***	Pixels or μm^2	Defines **RingSpotTotalAreaChN** population characterization thresholds
RingSpotAvgAreaChNLevel***Low/High, Low/High_CC***	Pixels or μm^2	Defines **RingSpotAvgAreaChN** population characterization thresholds
RingSpotCountChNLevel***Low/High, Low/High_CC***	Number	Defines **RingSpotCountChN** population characterization thresholds
CircSpotTotalIntenChNLevel***Low/High, Low/High_CC***	Intensity	Defines **CircSpotTotalIntenChN** population characterization thresholds
CircSpotAvgIntenChNLevel***Low/High, Low/High_CC***	Intensity	Defines **CircSpotAvgIntenChN** population characterization thresholds
CircSpotTotalAreaChNLevel***Low/High, Low/High_CC***	Pixels or μm^2	Defines **CircSpotTotalAreaChN** population characterization thresholds
CircSpotAvgAreaChNLevel***Low/High, Low/High_CC***	Pixels or μm^2	Defines **CircSpotAvgAreaChN** population characterization thresholds
CircSpotCountChNLevel***Low/High, Low/High_CC***	Number	Defines **CircSpotCountChN** population characterization thresholds
CircAvgIntenRatioChNCh1Level***Low/High, Low/High_CC***	Number	Defines **CircAvgIntenRatioChNCh1** population characterization thresholds
RingAvgIntenRatioChNChDLevel***Low/High, Low/High_CC***	Number	Defines **RingAvgIntenRatioChNChD** population characterization thresholds
CircAvgIntenRatioChNChDLevel***Low/High, Low/High_CC***	Number	Defines **CircAvgIntenRatioChNChD** population characterization thresholds
RingSpotAvgIntenRatioChNChDLevel***Low/High, Low/High_CC***	Number	Defines **RingSpotAvgIntenRatioChNChD** population characterization thresholds
RingSpotCountRatioChNChDLevel***Low/High, Low/High_CC***	Number	Defines **RingSpotCountRatioChNChD** population characterization thresholds
CircSpotAvgIntenRatioChNChDLevel***Low/High, Low/High_CC***	Number	Defines **CircSpotAvgIntenRatioChNChD** population characterization thresholds
CircSpotCountRatioChNChDLevel***Low/High, Low/High_CC***	Number	Defines **CircSpotCountRatioChNChD** population characterization thresholds

Table 19. *Advanced Assay Parameters Available in the Compartmental Analysis BioApplication. *Note: instead of showing all options, nomenclature is set so that "ChN" can be Channels 2-6 and if ratios, "ChD" = denominator found for Channels 2-6 (as previously described in Table 1).*

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

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

- Create your own definition for a focused biology (ex. membrane permeability [**CircAvgIntenCh*N***] AND NOT nuclear fragmentation [**ObjectAreaCh1**])
- Define a subpopulation by using any combination of up to three status Cell Features
- Report characteristics of your subpopulations

Event definitions are created using a stand-alone software tool called the BioApplication Event Wizard. Operation of this software tool is described in more detail in Chapter 3.

Event definition is achieved through the construction of logic statements employing specific Cell Features and a set of defined logical operators. The Cell Features and Boolean operators available are listed in Table 20. The operators ANDNOT and ORNOT are obtained by combining AND + NOT and OR + NOT respectively. Definition of each logical operator is provided in schematic form in Figure 22 using two Cell Features, A and B. The entire cell population analyzed is enclosed within the box in each case. In each case, responder cells for each feature are located within the domain for that feature. Note that the definitions described in Figure 22 can be directly applied to logic statements that contain more than two Cell Features. The Assay Parameters used to store Events are of the type **Type_X_EventDefinition** (X =1, 2, or 3).

Note that the Event Definition Assay Parameters cannot be modified via the Scan software application. Events should only be defined and edited via the BioApplication Event Wizard (see Chapter 3).

Cell Features	Logic Operators
ObjectAreaCh1	
ObjectShapeP2ACh1	NOT
ObjectShapeLWRCh1	
ObjectTotalIntenCh1	AND
ObjectAvgIntenCh1	
ObjectVarIntenCh1	AND NOT
ObjectSizeCh1	
RingTotalIntenChN	OR
RingAvgIntenChN	
CircTotalIntenChN	OR NOT
CircAvgIntenChN	
CircRingAvgIntenDiffChN	XOR
CircRingAvgIntenRatioChN	
RingSpotTotalIntenChN	NAND
RingSpotAvgIntenChN	
RingSpotTotalAreaChN	NOR
RingSpotAvgAreaChN	
RingSpotCountChN	
CircSpotTotalIntenChN	
CircSpotAvgIntenChN	
CircSpotTotalAreaChN	
CircSpotAvgAreaChN	
CircSpotCountChN	
CircAvgIntenRatioChNCh1	
RingAvgIntenRatioChNChD	
CircAvgIntenRatioChNChD	
RingSpotAvgIntenRatioChNChD	
RingSpotCountRatioChNChD	
CircSpotAvgIntenRatioChNChD	
CircSpotCountRatioChNChD	

Table 20. *Cell Features and Boolean operators available for Event Definition in Channels 1-6. Nomenclature is set so that "ChN" can be Channels 2-6 and if ratios, "ChD" = denominator found for Channels 2-6 (as previously described in Table 1).*

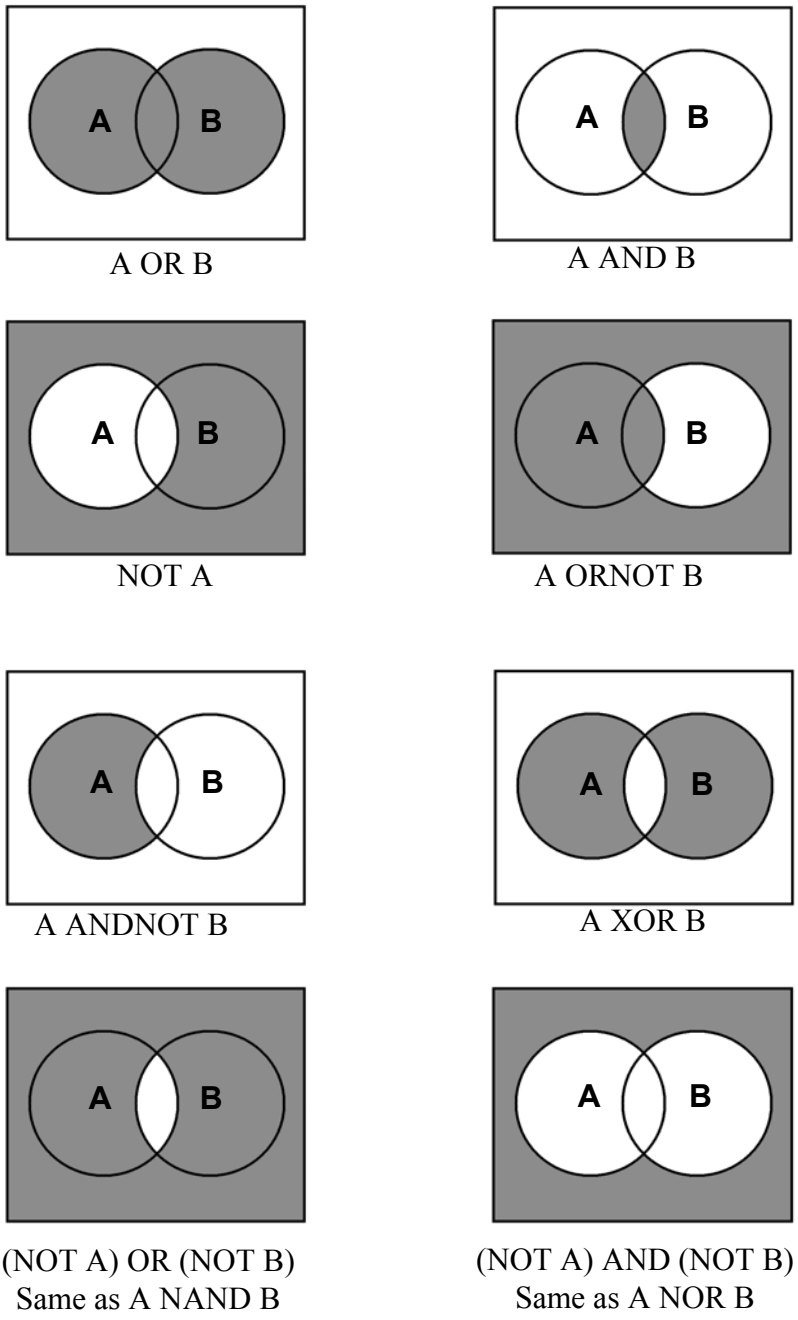


Figure 22. *Schematic definition of available Boolean Operators assuming two Cell Features of interest, A and B*

Description of Output Features

Output features are the biological measurements categorized and accessible using the View software application. Additionally, a subset of features, the Well Features, are listed in the Scan Plate View and Create Protocol View so that screening results can be viewed concurrently with scanning. An overview of both the Cell and Well Output Features can be found in Table 21, 22.

Channel 1 Features

Channel 1 contains the image of the primary labeled objects. Three categories of measurements are reported for the primary objects in Channel 1:

- Intensity
- Morphology and Location
- Cell Counts and Density

For each cell, the primary object's Total Intensity is measured. The total intensity is the sum of the intensities of all the individual pixels making up that object. The average intensity of the pixels making up the object is also reported.

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

In the category of morphology and location measurements, the area of each primary object is reported. This area is proportional to the total number of pixels covered by the object, and the proportionality constant is the pixel size in micrometers. In addition to the area, shape parameters such as the object's roundness (i.e., P2A), aspect ratio (i.e., LWR), and size (i.e., equivalent diameter) are also reported. The object's location in the image is reported by the *x* and *y* coordinates of its centroid. An example is if the cell's nucleus was used as a primary object, its size can indicate whether the nuclear condensation or fragmentation that accompanies apoptosis has occurred.

The status of the total and average intensities and the size are reported for each cell. They 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 and thus identify whether a cytotoxic event has occurred.

Channel 2-6 Intensity Measurements

For each target channel (i.e., Channels 2-6), the four regions (*Circ, Ring, Circ Spot*, and *Ring Spot*) can be defined for each cell and intensity measurements from each of the four regions for each of the channels are made. Both the Total and Average Intensity are measured. The Total Intensity is the sum of the intensities of all the individual pixels in the region. The Average Intensity is equal to the Total Intensity divided by the number of pixels in the region.

For Channels 2-6, any parameter related to the Average Intensity can also be used to classify and characterize the individual cells in the population, and thus have a Status parameter associated with it (see section on Population Characterization).

Intensities in the dependent channels and their status will be the most commonly used output features of this BioApplication. For example, if looking at EGF stimulation of MAPK, the

primary object is the nucleus, and both Total and Average Circ Intensity can be used to measure the amount of MAPK in the nucleus as well as the cyclin expression level in the nucleus. Intensity in the Ring and Ring Spot regions can be used to determine the internalized fluorescent EGF in the cytoplasm and endosomes respectively. If the primary object would be the Endocytic Recycling Compartment, the Circ Intensity is from the internalized macromolecule that has trafficked to this compartment. If focusing on cytotoxicity, cell permeability can be determined from the Circ intensity in one channel, and mitochondrial potential can be obtained from the Ring Spot intensities in additional channels. The Circ intensities can also be used for translocated kinase intensities in the nucleus and the intensities of the phosphorylated and total protein in the nucleus respectively. The status parameter indicates which of these intensities is beyond a range that you define indicating that a particular cell is a responder.

Intensity Measurements for Gating

As mentioned before, this BioApplication has the capability of making intensity measurements only on objects in Channels 2-6 that are within a intensity range that you specify. However, the Average and Total Intensities in the area covered by the primary object are also reported. These are useful for monitoring the base intensity range of selected and rejected objects. For example, if you would like measurements to be restricted to cells that express a GFP–protein chimera and only in cells that had a certain expression level, then this gating capability could be used. The intensity range could be set to include only cells that had the desired expression level, and the Average and Total Intensities in the primary object area in Channels 2-6 could be used to monitor this. Then the intensities in the four cellular regions could be made on these selected cells to measure the intensity in the specific sub-cellular regions as described above.

Intensity Ratios Between Channels

Average Intensity ratios of specific regions from different channels are also reported. There are two types of ratios: ratios against Channel 1 and ratios between Channels 2-6.

Ratios against Channel 1

Only the Average Intensity from the Circ region in Channels 2-6 is divided by the Average Intensity of the primary object in Channel 1 and reported. Intensities from the other three regions (*Ring, Circ Spot*, and *Ring Spot*) are not ratioed with Channel 1 and, thus, are not reported.

An example of this is looking at intracellular trafficking; the ratio of the Average Intensities of the internalized macromolecule with the Endocytic Recycling Compartment label (the primary object) can give a measure of the degree of colocalization of the intracellular macromolecule with the endosome. This is useful when the goal is to monitor trafficking to, and colocalization of, the macromolecule with a specific compartment (which is the primary object) and to distinguish it from the rest of the macromolecule in other locations inside the cell.

Ratios between Channels 2-6

The ratios between the Average Intensities from Channels 2-6 are reported for all four regions. In addition, the ratio of the number of spots in the Circ and Ring regions between different channels are also reported. Although a ratio between each channel from Channels 2-6 is made, not all channel combinations are calculated. Table 1 displayed the Channel combinations for which ratios are reported.

An example of different ratio possibilities is demonstrated with cytotoxicity. The Ring Spot Average Intensity Ratio between MitoTracker Red and Green signals (i.e., between two

channels) is proportional to the mitochondrial transmembrane potential. Note that the measurement regions are identified independently in each channel. The Ring Spot Count Ratio between these channels indicates the fraction of mitochondria with normal transmembrane potential.

Channel 2-6: Circ-Ring Arithmetic

Two calculations using the average intensities of the Ring and Circ regions are reported for Channels 2-6. These calculations include the difference and the ratio between the Average Intensities from the Circ and Ring regions (i.e., Circ – Ring and Circ/Ring respectively). Such Circ-Ring arithmetic can be used to quantify the strength of a cytoplasm to nuclear translocation event, such as those in biology Examples 1 and 4 (MAPK Translocation and Multiple Kinase Translocation).

Channel 2-6: Spot Area, Spot Number, and Spot Ratios

Various additional parameters of the spots in the Circ and Ring regions are reported for each cell. These are the spot total and average areas and the number of spots (i.e., Spot Count) as well as their ratios between channels. Spot Count can be used to count the number of resolvable, discrete, punctate objects, such as the number of distinct endosomes containing internalized receptor, or the number of all mitochondria and the number of mitochondria whose transmembrane potential is at a high value.

For the spots detected in a particular region (i.e., Circ Spots or Ring Spots) each spot has a particular area. The Total Spot Area is the sum of the areas of all the individual spots. The Average Spot Area is the Total Spot Area divided by the number of spots.

$$\text{Average Spot Area} = \frac{\text{Total Spot Area}}{\text{Number of Spots in the region}}$$

Cell Features

Table 21 defines the output features that are reported for each cell, found either in Scan Protocol Interactive View or in the Cell Feature window in the View software application.

Feature	Units	Description
Cell#	Number	Unique object ID
Top	Pixels	Y coordinate (in pixels) of top left corner of image-aligned bounding box of Ch1 object
Left	Pixels	X coordinate (in pixels) of top-left corner of image-aligned bounding box of Ch1 object
Width	Pixels	Width (in pixels) of image-aligned bounding box of Ch1 object
Height	Pixels	Height (in pixels) of image-aligned bounding box of Ch1 object
XCentroid	Pixels	X coordinate (in pixels) of center of Ch1 object
YCentroid	Pixels	Y coordinate (in pixels) of center of Ch1 object
EventTypeProfile	Number	Identifies the types of events that occurred: 1, 2, 3, 12, 23, 13, 123
EventType1Status	Number	**EventType1** status: 0 = Event did not occur, 1 = Event occurred
EventType2Status	Number	**EventType2** status: 0 = Event did not occur, 1 = Event occurred
EventType3Status	Number	**EventType3** status: 0 = Event did not occur, 1 = Event occurred

Feature	Units	Description
ObjectAreaCh1	Pixels or μm^2	Area (in pixels or micrometers) of Ch1 object
ObjectAreaCh1Status	Number	**ObjectAreaCh1** status: 0 = No response, 1 = High response, 2 = Low response
ObjectShapeP2ACh1	Number	Shape measure based on ratio of perimeter squared to 4PI*area of Ch1 object (**ObjectShapeP2ACh1** = 1 for circular object)
ObjectShapeP2ACh1Status	Number	**ObjectShapeP2ACh1** status: 0 = No response, 1 = High response, 2 = Low response
ObjectShapeLWRCh1	Number	Shape measure based on ratio of length to width of object-aligned bounding box of Ch1 object
ObjectShapeLWRCh1Status	Number	**ObjectShapeLWRCh1** status: 0 = No response, 1 = High response, 2 = Low response
ObjectTotalIntenCh1	Intensity	Total intensity of all pixels within Ch1 object
ObjectTotalIntenCh1Status	Number	**ObjectTotalIntenCh1** status: 0 = No response, 1 = High response, 2 = Low response
ObjectAvgIntenCh1	Intensity	Average intensity of all pixels within Ch1 object
ObjectAvgIntenCh1Status	Number	**ObjectAvgIntenCh1** status: 0 = No response, 1 = High response, 2 = Low response
ObjectVarIntenCh1	Intensity	Standard deviation of intensity of all pixels within Ch1 object
ObjectVarIntenCh1Status	Number	**ObjectVarIntenCh1** status: 0 = No response, 1 = High response, 2 = Low response
ObjectSizeCh1	Pixels or µm	Diameter (in pixels or micrometers) of circle with area equal to area of Ch1 object
ObjectSizeCh1Status	Number	**ObjectSizeCh1** status: 0 = No response, 1 = High response, 2 = Low response
RingTotalIntenChN	Intensity	Total intensity of all pixels within ring mask in ChN
RingTotalIntenChNStatus	Number	**RingTotalIntenChN** status: 0 = No response, 1 = High response, 2 = Low response
RingAvgIntenChN	Intensity	Average intensity of all pixels within ring mask in ChN
RingAvgIntenChNStatus	Number	**RingAvgIntenChN** status: 0 = No response, 1 = High response, 2 = Low response
CircTotalIntenChN	Intensity	Total intensity of all pixels within circ mask in ChN
CircTotalIntenChNStatus	Number	**CircTotalIntenChN** status: 0 = No response, 1 = High response, 2 = Low response
CircAvgIntenChN	Intensity	Average intensity of all pixels within circ mask in ChN
CircAvgIntenChNStatus	Number	**CircAvgIntenChN** status: 0 = No response, 1 = High response, 2 = Low response
CircRingAvgIntenDiffChN	Intensity	Difference between **CircAvgIntenChN** and **RingAvgIntenChN**
CircRingAvgIntenDiffHighChNStatus	Number	**CircRingAvgIntenDiffHighChN** status: 0 = No response, 1 = High response
CircRingAvgIntenDiffLowChNStatus	Number	**CircRingAvgIntenDiffLowChN** status: 0 = No response, 2 = Low response
CircRingAvgIntenRatioChN	Number	Ratio of **CircAvgIntenChN** to **RingAvgIntenChN**
CircRingAvgIntenRatioHighChNStatus	Number	**CircRingAvgIntenRatioHighChN** status: 0 = No response, 1 = High response
CircRingAvgIntenRatioLowChNStatus	Number	**CircRingAvgIntenRatioLowChN** status: 0 = No response, 2 = Low response
RingSpotTotalIntenChN	Intensity	Total intensity of all spot pixels within ring mask in ChN
RingSpotTotalIntenChNStatus	Number	**RingSpotTotalIntenChN** status: 0 = No response, 1 = High response, 2 = Low response
RingSpotAvgIntenChN	Intensity	Average intensity of all spot pixels within ring mask in ChN

Feature	Units	Description
RingSpotAvgIntenChNStatus	Number	**RingSpotAvgIntenChN** status: 0 = No response, 1 = High response, 2 = Low response
RingSpotTotalAreaChN	Pixels or μm^2	Total area (in pixels or micrometers) of all spots within ring mask in ChN
RingSpotTotalAreaChNStatus	Number	**RingSpotTotalAreaChN** status: 0 = No response, 1 = High response, 2 = Low response
RingSpotAvgAreaChN	Pixels or μm^2	Average area (in pixels or micrometers) of all spots within ring mask in ChN
RingSpotAvgAreaChNStatus	Number	**RingSpotAvgAreaChN** status: 0 = No response, 1 = High response, 2 = Low response
RingSpotCountChN	Number	Spot count within ring mask in ChN
RingSpotCountChNStatus	Number	**RingSpotCountChN** status: 0 = No response, 1 = High response, 2 = Low response
CircSpotTotalIntenChN	Intensity	Total intensity of all spot pixels within circ mask in ChN
CircSpotTotalIntenChNStatus	Number	**CircSpotTotalIntenChN** status: 0 = No response, 1 = High response, 2 = Low response
CircSpotAvgIntenChN	Intensity	Average intensity of all spot pixels within circ mask in ChN
CircSpotAvgIntenChNStatus	Number	**CircSpotAvgIntenChN** status: 0 = No response, 1 = High response, 2 = Low response
CircSpotTotalAreaChN	Pixels or μm^2	Total area (in pixels or micrometers) of all spots within circ mask in ChN
CircSpotTotalAreaChNStatus	Number	**CircSpotTotalAreaChN** status: 0 = No response, 1 = High response, 2 = Low response
CircSpotAvgAreaChN	Pixels or μm^2	Average area (in pixels or micrometers) of all spots within circ mask in ChN
CircSpotAvgAreaChNStatus	Number	**CircSpotAvgAreaChN** status: 0 = No response, 1 = High response, 2 = Low response
CircSpotCountChN	Number	Spot count within circ mask in ChN
CircSpotCountChNStatus	Number	**CircSpotCountChN** status: 0 = No response, 1 = High response, 2 = Low response
CircAvgIntenRatioChNCh1	Number	Average intensity ratio computed over the colocalized circ mask in ChNCh1
CircAvgIntenRatioChNCh1Status	Number	**CircAvgIntenRatioChNCh1** status: 0 = No response, 1 = High response, 2 = Low response
TotalIntenChN	Intensity	Total intensity in ChN of all pixels within modified Ch1 object mask
AvgIntenChN	Intensity	Average intensity in ChN of all pixels within modified Ch1 object mask
RingAvgIntenRatioChNChD	Number	Average intensity ratio computed over the colocalized ring mask in ChNChD
RingAvgIntenRatioChNChDStatus	Number	**RingAvgIntenRatioChNChD** status: 0 = No response, 1 = High response, 2 = Low response
CircAvgIntenRatioChNChD	Number	Average intensity ratio computed over the colocalized circ mask in ChNChD
CircAvgIntenRatioChNChDStatus	Number	**CircAvgIntenRatioChNChD** status: 0 = No response, 1 = High response, 2 = Low response
RingSpotAvgIntenRatioChNChD	Number	Spot average intensity ratio computed over the colocalized ring mask in ChNChD
RingSpotAvgIntenRatioChNChDStatus	Number	**RingSpotAvgIntenRatioChNChD** status: 0 = No response, 1 = High response, 2 = Low response
RingSpotCountRatioChNChD	Number	Spot count ratio computed over the colocalized ring mask in ChNChD
RingSpotCountRatioChNChDStatus	Number	**RingSpotCountRatioChNChD** status: 0 = No response, 1 = High response, 2 = Low response
CircSpotAvgIntenRatioChNChD	Number	Spot average intensity ratio computed over the colocalized circ mask in ChNChD

Feature	Units	Description
CircSpotAvgIntenRatioChNChDStatus	Number	**CircSpotAvgIntenRatioChNChD** status: 0 = No response, 1 = High response, 2 = Low response
CircSpotCountRatioChNChD	Number	Spot count ratio computed over the colocalized circ mask in ChNChD
CircSpotCountRatioChNChDStatus	Number	**CircSpotCountRatioChNChD** status: 0 = No response, 1 = High response, 2 = Low response

Table 21. *Cell Features Available in the Compartmental Analysis BioApplication. *Note that nomenclature for Cell Features in Channels 2-6 are reported for each of the dependent channels (Ch2-Ch6) for the number of channels selected. Instead of showing all options, nomenclature is set so that "ChN" can be Channels 2-6 and if ratios, "ChD" = denominator found for Channels 2-6 (as previously described in Table 1).*

Well Features

Many Well Features are derived from the Cell Features. Such features are identified by a prefix, as listed in Table 22, added to the Cell Feature name.

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

Table 22. *General Well Feature prefixes available in the Compartmental Analysis BioApplication.*

The algorithm also reports the following additional Well Features (Table 23) in the Scan Plate View in addition to the Well Detail window of the View software application.

Feature	Description
ValidObjectCount	Number of valid objects identified in the well (Ch1 object selection parameters applied)
SelectedObjectCount	Number of valid objects selected for analysis in the well (Ch2-6 object selection parameters applied)
%SelectedObjects	Percentage of valid objects selected for analysis in the well
ValidFieldCount	Number of fields in which objects were selected for analysis in the well
SelectedObjectCountPerValidField	Average number of objects selected for analysis per valid field in the well
EventType1ObjectCount	Number of objects selected for analysis in the well in which EventType1 occurred
%EventType1Objects	Percentage of objects selected for analysis in the well in which EventType1 occurred
EventType2ObjectCount	Number of objects selected for analysis in the well in which EventType2 occurred

Feature	Description
%EventType2Objects	Percentage of objects selected for analysis in the well in which EventType2 occurred
EventType3ObjectCount	Number of objects selected for analysis in the well in which EventType3 occurred
%EventType3Objects	Percentage of objects selected for analysis in the well in which EventType3 occurred

Table 23. *Additional Well Features available in the Compartmental Analysis BioApplication.*

Reference Features

The Compartmental Analysis BioApplication reports the following Reference Features, whose values indicate the data generated from Reference Wells. Reference Features are viewable in the Scan Plate View. In addition, they are also listed as Plate Features and in the Well Detail View of the View software application.

In the listing of Reference Features in Table 24, instead of showing both features derived from the previous level parameters, one entry for the feature will be listed giving both outputs, as shown in the following example for the Channel 1 object area:

- ObjectAreaCh1Level***Low/High***

Reference Feature	Description
RefAvgObjectCountPerField	Average number of objects per field in reference wells
RefObjectAreaCh1Level***Low/High***	Low/High-response level for **ObjectAreaCh1** computed from reference well results
RefObjectShapeP2ACh1Level***Low/High***	Low/High-response level for **ObjectShapeP2ACh1** computed from reference well results
RefObjectShapeLWRCh1Level***Low/High***	Low/High-response level for **ObjectShapeLWRCh1** computed from reference well results
RefObjectTotalIntenCh1Level***Low/High***	Low/High-response level for **ObjectTotalIntenCh1** computed from reference well results
RefObjectAvgIntenCh1Level***Low/High***	Low/High-response level for **ObjectAvgIntenCh1** computed from reference well results
RefObjectVarIntenCh1Level***Low/High***	Low/High-response level for **ObjectVarIntenCh1** computed from reference well results
RefObjectSizeCh1Level***Low/High***	Low/High-response level for **ObjectSizeCh1** computed from reference well results
RefRingTotalIntenChNLevel***Low/High***	Low/High-response level for **RefRingTotalIntenChN** computed from reference well results
RefRingAvgIntenChNLevel***Low/High***	Low/High-response level for **RefRingAvgIntenChN** computed from reference well results
RefCircTotalIntenChNLevel***Low/High***	Low/High-response level for **RefCircTotalIntenChN** computed from reference well results
RefCircAvgIntenChNLevel***Low/High***	Low/High-response level for **RefCircAvgIntenChN** computed from reference well results
RefCircRingAvgIntenDiffChNLevel***Low/High***	Low/High-response level for **RefCircRingAvgIntenDiffChN** computed from reference well results
RefCircRingAvgIntenRatioChNLevel***Low/High***	Low/High-response level for **RefCircRingAvgIntenRatioChN** computed from reference well results

Reference Feature	Description
RefRingSpotTotalIntenChNLevel***Low/High***	Low/High-response level for **RefRingSpotTotalIntenChN** computed from reference well results
RefRingSpotAvgIntenChNLevel***Low/High***	Low/High-response level for **RefRingSpotAvgIntenChN** computed from reference well results
RefRingSpotTotalAreaChNLevel***Low/High***	Low/High-response level for **RefRingSpotTotalAreaChN** computed from reference well results
RefRingSpotAvgAreaChNLevel***Low/High***	Low/High-response level for **RefRingSpotAvgAreaChN** computed from reference well results
RefRingSpotCountChNLevel***Low/High***	Low/High-response level for **RefRingSpotCountChN** computed from reference well results
RefCircSpotTotalIntenChNLevel***Low/High***	Low/High-response level for **RefCircSpotTotalIntenChN** computed from reference well results
RefCircSpotAvgIntenChNLevel***Low/High***	Low/High-response level for **RefCircSpotAvgIntenChN** computed from reference well results
RefCircSpotTotalAreaChNLevel***Low/High***	Low/High-response level for **RefCircSpotTotalAreaChN** computed from reference well results
RefCircSpotAvgAreaChNLevel***Low/High***	Low/High-response level for **RefCircSpotAvgAreaChN** computed from reference well results
RefCircSpotCountChNLevel***Low/High***	Low/High-response level for **RefCircSpotCountChN** computed from reference well results
RefCircAvgIntenRatioChNCh1Level***Low/High***	Low/High-response level for **CircAvgIntenRatioChNCh1** computed from reference well results
RefRingAvgIntenRatioChNChDLevel***Low/High***	Low/High-response level for **RingAvgIntenRatioChNChD** computed from reference well results
RefCircAvgIntenRatioChNChDLevel***Low/High***	Low/High-response level for **CircAvgIntenRatioChNChD** computed from reference well results
RefRingSpotAvgIntenRatioChNChDLevel***Low/High***	Low/High-response level for **RingSpotAvgIntenRatioChNChD** computed from reference well results
RefRingSpotCountRatioChNChDLevel***Low/High***	Low/High-response level for **RingSpotCountRatioChNChD** computed from reference well results
RefCircSpotAvgIntenRatioChNChDLevel***Low/High***	Low/High-response level for **CircSpotAvgIntenRatioChNChD** computed from reference well results
RefCircSpotCountRatioChNChDLevel***Low/High***	Low/High-response level for **CircSpotCountRatioChNChD** computed from reference well results

Table 24. *Reference Features available in the Compartmental Analysis BioApplication. *Note nomenclature for Reference Features in Channels 2-6 are reported for each of the dependent channels (Ch2-Ch6) for the number of channels selected. Instead of showing all options, nomenclature is set so that "ChN" can be Channels 2-6 and if ratios, "ChD" = denominator found for Channels 2-6 (as previously described in Table 1).*

Chapter

3

Using the Compartmental Analysis BioApplication

This chapter describes in more detail the use and modification of the Compartmental Analysis BioApplication Assay Protocol, as well as implementation of the Events Wizard.

The Compartmental Analysis BioApplication is for scientists who want a versatile tool that they can apply towards many different biological targets and want the flexibility in defining the regions of the cell from where they want to make measurements. Thus, the Compartmental Analysis BioApplication is for power users who have the expertise and feel comfortable in configuring and optimizing such an application for their particular biological situation.

Assay-Specific Procedures for Optimizing the BioApplication

This section involves configuring the Compartmental Analysis BioApplication for different use cases and will guide you in ways of modifying these protocols by and using the five biological examples in Chapter 1 (Tables 3-7 and Figures 3-7). This is done to demonstrate different ways of configuring this application to suit different biological situations, so that you can use similar approaches in optimizing the application toward your own particular biology.

For each example below, two tables are given summarizing the assay details for that example. The first table lists a possible labeling strategy with fluorophore examples and a recommendation of the appropriate Cellomics HCS Reader filter choices. The second table is a reproduction of the table summarizing the biology in Chapter 1 along with additional information such as the specific Compartmental Analysis BioApplication output feature corresponding to each measured property (unless otherwise noted, the features are Cell Features and the corresponding Well Features can be determined using Table 2). Each pair of tables is followed by a discussion of the critical input parameters that must be adjusted for the specific output features.

Example #1: EGF Stimulation of MAPK Pathway

Channel	Cellular Target	Detection Strategy	Example Fluorophores
1	DNA Content Cell Proliferation	Blue fluorescent DNA binding dyes to label and count nuclei	DAPI, Hoechst 33342
2	Phosphorylated MAPK	Immunofluorescence using green fluorescent fluorophores	Fluorescein, Alexa Fluor® 488
3	Internalized EGF	EGF conjugated to red fluorescent fluorophores	Rhodamine, Texas Red, Cy3, Alexa Fluor 555
4	Cyclin expression	Immunofluorescence using fluorophores fluorescent in the far red spectrum	Alexa Fluor 660, Cy5
Filter set suggestion: XF53 or XF93 (XF110 if needed)			

Table 25. *Possible fluorescent labeling strategy and example fluorophores for measuring EGF stimulation of the MAPK pathway.*

Target	Cellular Properties	Corresponding Feature Reported by Compartmental Analysis
Channel 1 DNA Content	Intensity of DNA in the nucleus to indicate the cell cycle phase	**ObjectTotalIntenCh1**
	Indicator whether a cell is in the G1 or G2 phase of the cell cycle	**ObjectTotalIntenCh1Status**
Channel 1 Cell Proliferation	The number of cells to indicate whether cell proliferation has occurred (using a fixed # of fields acquired per well)	**SelectedObjectCount** (Well Feature)
Channel 2 Phosphorylated MAPK	Intensity of activated (i.e., phosphorylated) MAPK in the nucleus	**CircAvgIntenCh2** **CircTotalIntenCh2**
	The difference and ratio of the activated MAPK signal in the nucleus versus the cytoplasm to indicate the extent of translocation	**CircRingAvgIntenDiffCh2** **CircRingAvgIntenRatioCh2**
	Indicators of whether a cell's nuclear MAPK intensity, or extent of cytoplasm to nucleus translocation, is above a defined level, indicating that the cell is a responder	**CircAvgIntenStatusCh2** **CircRingAvgIntenDiffCh2Status** **CircRingAvgIntenRatioCh2Status**
Channel 3 Internalized EGF	Intensity of internalized fluorescent EGF in cells	**RingAvgIntenCh3** **RingTotalIntenCh3**
	Intensity of intracellular fluorescent EGF in endosomes	**RingSpotAvgIntenCh3** **RingSpotTotalIntenCh3**
	Indicators of whether a cell's internalized fluorescent EGF is above a defined level, indicating that the cell is positive for internalization	**RingAvgIntenStatusCh3** **RingSpotAvgIntenCh3Status**
	Number of resolved distinct endosomes	**RingSpotCountCh3**
	Indicator of whether the number of distinct endosomes containing fluorescent EGF is above a defined level, indicating that the cell is positive for internalization	**RingSpotCountCh3Status**
Channel 4 Cyclin expression	Cyclin expression intensity in the nucleus	**CircAvgIntenCh4** **CircTotalIntenCh4**
	Indicators of whether the cyclin intensity is above a defined level, indicating that the cell is expressing cyclin	**CircAvgIntenCh4Status**

Table 26. *Compartmental Analysis BioApplication output features reporting the cellular properties measured upon fluorescent EGF stimulation of the MAPK pathway.*

DNA Content and Cell Proliferation (Channel 1)

The first critical step in setting the input parameters for this BioApplication is the correct identification of the primary object in Channel 1. For this particular biological example, the primary object is fluorescently labeled nuclei. A cell's nuclear total intensity is proportional to its DNA content. Thus, the **ObjectTotalIntenCh1** output feature can be used to measure the intensity of the DNA in the nucleus to determine the cell cycle phase. To do so, the primary object (i.e., nucleus) must be correctly identified. This is done by setting the Channel 1 Object Selection Parameters. These Object Selection Parameters control the range of acceptable

intensities (Average and Total Intensity) and morphology (**ObjectAreaCh1**, **ObjectShapeP2ACh1**, and **ObjectShapeLWRCh1**) of the primary object. A recommended method of setting these is to use the Protocol Interactive View to view an image of nuclei and ensure that the range of Object Selection Parameters allow all nuclei to be selected, but any objects which are not single nuclei (e.g., fluorescent debris or multiple unresolved nuclei) to be rejected. Additionally, the intensity threshold used in the Object Identification Method may have to be adjusted (i.e., increased) to resolve multiple nuclei into individual single nuclei.

To separate and characterize the cell population based on DNA content for the specific cell cycle phase, the population characterization High and Low intensity levels (i.e., **ObjectTotalIntenCh1Level*Low/High*, *Low/High_CC***) must be set. This is done by viewing control populations of cells or using Reference Wells where the majority of cells are either in the G_1 or G_2 phases of the cell cycle, and bracketing the total intensity with the thresholds to define them. The number of primary objects reported in the **SelectedObjectCount** Well Feature can be used to count the number of cells and thus monitor whether cell proliferation has occurred.

Phosphorylated MAPK (Channel 2)

Once the settings for identifying the primary object have been optimized, the next critical step is to define the cellular region where the measurements are to be made for the different dependent channels. To measure the translocation of phosphorylated MAPK from the cytoplasm to the nucleus in Channel 2, the cellular areas to measure are the Ring and Circ for these areas respectively. These Assay Parameters **CircModifierCh2**, **RingDistanceCh2,** and **RingWidthCh2** (see Figure 17), must be adjusted to identify the nuclear (Circ) and cytoplasmic (Ring) regions. Since the primary object is the nucleus, the **CircModifierCh2** can be set near 0 (–1 to +1) so that the Circ region is in the nucleus. Similarly, to have the Ring sample the cytoplasmic region, the **RingDistanceCh2** can be set near the primary object (0 or +1) and the **RingWidthCh2** can be set to a somewhat large value (e.g., 10).

Setting the appropriate levels for **CircAvgIntenCh2Level*Low/High_CC*, CircAvgIntenDiffCh2Level*Low/High_CC*,** or **CircAvgIntenRatioCh2Level*Low/High_CC*** based on the appropriate output features gives you an indicator of whether or not the cell is a responder. These can be set on Reference Wells either containing a negative (unstimulated basal) condition or a positive (stimulated) condition.

An example of using the population characterization capability is shown. When interactively viewing samples in the Protocol Interactive View, the following Well Output Features were seen for negative and positive wells:

- Negative Control Well:
  - **MEAN_CircAvgIntenCh2** = 23.895
  - **SD_CircAvgIntenCh2** = 4.880
- Positive Control Well:
  - **MEAN_CircAvgIntenCh2** = 109.147
  - **SD_CircAvgIntenCh2** = 23.277

The intensity threshold Assay Parameters define responders if Reference Wells are not used (**UseReferenceWells** =0), they should be set at:

- **CircAvgIntenCh2LevelLow** = 0.00
- **CircAvgIntenCh2LevelHigh** = 54.00

This means that when the **CircAvgIntenCh2** exceeds a value of 54, that cell's **CircAvgIntenStatusCh2** goes from 0 to 1 indicating it is a responder for MAPK translocation to the nucleus.

To achieve a similar result using Reference Wells (i.e., Reference Wells **ON**) containing unstimulated cells, the corresponding Assay Parameters were set as follows:

- **CircAvgIntenCh3LevelLow_CC** = 5.00 (i.e., 5 standard deviations)
- **CircAvgIntenCh3LevelHigh_CC** = 6.00 (i.e., 6 standard deviations)

This means that for a **MEAN_CircAvgIntenCh2** ~ 24 from the Reference Wells and a **SD_CircAvgIntenCh2** ~ 5, then the lower and upper intensity thresholds respectively are ~ 0 (24 - 5×5) and 54 (24 + 6×5).

Internalized EGF (Channel 3)

The **RingSpotAvgIntenCh3** and **RingSpotTotalIntenCh3** Assay Parameters reflect the internalized fluorescent EGF in intracellular endosomes. Detecting internalized EGF in endosomes means identifying and then measuring the intensity from intracellular spots. This section covers the general principles used in identifying spots which are applicable to this and other biological situations which require measurements from intracellular spots. More details are available in the "Identifying Spots" section in Chapter 2. Note that it is possible to observe the spot overlay for only one channel. In the protocol supplied with the application, the Channel 3 spot overlay is observed on Channel 2 and Channel 3.

The three sets of critical input parameters that must be adjusted to identify spots are the **SpotDetectRadiusCh*N*** (Channel 3 for this biology), the intensity threshold method and value associated with the Object Identification Method, and the Object Selection Parameters. The **SpotDetectRadiusCh3** Assay Parameter is adjusted for the approximate size (radius) of the spots to be measured. This parameter is robust such that if the value entered is a few pixels different from the actual size of the spots in the image, the results will not be significantly altered.

Adjustment of the Object Identification Method and Value (**Isodata Threshold**, **Fixed Threshold**, or **Triang Threshold**) were described in Chapter 2. Since the cytoplasmic spots associated with internalized EGF are relatively dim, the **Fixed Threshold** option is recommended. To use images from an untreated well, adjust the **Fixed Threshold** value to the highest level at which no spots are identified in the Channel 3 window. The lower the threshold, the higher the likelihood of ambiguous spots being identified.

To ensure Spots and Regions are in range, it may be necessary to adjust the Object Selection Parameters. Set a Min or Max for intensity so the cells or spots outside the range are rejected.

To set the population characterization Assay Selection Parameters, compare an untreated well to a positive control well and note the differences in specific output features (i.e., **MEAN_RingAvgIntenCh3, MEAN_RingSpotAvgIntenCh3, MEAN_RingSpotCountCh3)**. Set the **RingAvgIntenCh3Level*Low/High*, RingSpotAvgIntenCh3Level*Low/High*,** and **RingSpotCountCh3Level*Low/High*,** to indicate whether the cell is positive for EGF internalization.

Cyclin Expression (Channel 4)

Cyclin expression intensity in the nucleus is measured by the **MEAN_CircTotalIntenCh4** and **MEAN_CircAvgIntenCh4** output features. A similar strategy for the MAPK example in Channel 1 can be followed in this case. This includes setting the appropriate levels for **CircAvgIntenCh4LevelHigh_CC** to indicate whether the cell is expressing cyclin.

Example #2: Intracellular Trafficking and GPCR Activation

Channel	Cellular Target	Detection Strategy	Example Fluorophores
1	Endosomal Compartment Label (e.g., Endocytic Recycling Compartment)	Red fluorescent marker of Endocytic Recycling Compartment such as fluorescent transferring	Rhodamine, Texas Red, Cy3, Alexa Fluor 555
2	Internalized or intracellular trafficking GPCR	Green fluorescent labeled GPCR either using autofluorescent protein or immunofluorescence	GFP, Fluorescein, Alexa Fluor 488
Filter set suggestion: XF93			

Table 27. *Possible fluorescent labeling strategy and example fluorophores for measuring Intracellular Trafficking of GPCRs.*

Target	Cellular Properties	Corresponding Feature Reported by Compartmental Analysis
Channel 1 Endosomal Compartment Label	Intensity of compartment marker	**ObjectAvgIntenCh1** **ObjectTotalIntenCh1**
Channel 2 Internalized or intracellular trafficking GPCR	Intensity of internalized or trafficking macromolecule in the labeled compartment	**CircAvgIntenCh2** **CircTotalIntenCh2**
	Ratio of the macromolecule's intensity to that of the compartment marker as a measure of colocalization	**CircAvgIntenRatioCh2Ch1**
	Indicator of whether the intensity of the trafficking macromolecule in the compartment is above a defined level, indicating that the compartment contains the macromolecule	**CircAvgIntenCh2Status**
	Indicator of whether the ratio of intensity between the trafficking macromolecule and the compartment marker is above a defined level, as an indicator of colocalization	**CircAvgIntenRatioCh2Ch1Status**

Table 28. *Compartmental Analysis output features reporting the cellular properties measured for the internalization and intracellular trafficking of activated GPCRs.*

Endosomal Compartment (Channel 1)

To identify the endosomal compartment as the primary object, a similar strategy can be followed for identifying primary objects as was given for identifying nuclei in the first biological example.

Internalized or intercellular trafficking GPCR (Channel 2)

The **MEAN_CircTotalIntenCh2**, **MEAN_CircAvgIntenCh2,** and **MEAN_CircSpotAvgIntenCh2** output features measure the intensity of internalized or trafficking macromolecule in the labeled compartment depending on the specific label being

used. It may be necessary to adjust the Object Identification Method and Value for the particular biology.

You can define the levels in the **CircAvgIntenCh2Level*Low/High, Low/High_CC***, using the **MEAN_CircAvgIntenCh2** output feature, to indicate that the compartment contains the macromolecule.

You can set the **CircAvgIntenRatioCh2Ch1Level*Low/High, Low/High_CC*** intensity levels based on the **MEAN_CircAvgIntenRatioCh2Ch1** output feature to indicate colocalization of the trafficking macromolecule and the compartment marker.

Example #3: Cytotoxicity

Channel	Cellular Target	Detection Strategy	Example Fluorophores
1	Nucleus	Blue fluorescent DNA binding dyes that label nuclei	DAPI, Hoechst 33342
2	Mitochondrial Mass	Green fluorescent fluorophore that labels the mitochondrial mass	MitoTracker Green
3	Mitochondrial Transmembrane Potential	Red fluorescent fluorophore that accumulates in mitochondria according to their transmembrane potential	MitoTracker Red
4	Cell Membrane Permeability	Membrane impermeant dye that fluoresces in the far red of the spectrum	TOTO-3
Filter set suggestion: XF93 (XF110 if needed)			

Table 29. *Possible fluorescent labeling strategy and example fluorophores for measuring cell health and cytotoxicity.*

Target	Cellular Properties	Corresponding Feature Reported by Compartmental Analysis
Channel 1 Nucleus	Nucleus size (i.e., normal nucleus size, nuclear fragmentation, or condensation)	**ObjectSizeCh1**
	Indicates whether a cell's nuclear size is beyond a defined range indicating nuclear fragmentation or condensation resulting from apoptosis	**ObjectSizeCh1Status**
Channel 2 Mitochondrial Mass	Intensity of MitoTracker Green in mitochondria in cells to give a measure of mitochondrial mass	**RingSpotAvgIntenCh2** **RingSpotTotalIntenCh2**
	Number of mitochondria in Channel 2 from MitoTracker Green label	**RingSpotCountCh2**
Channel 3 Mitochondrial Transmembrane Potential	Intensity of MitoTracker Red in mitochondria in cells to give a measure of mitochondrial transmembrane potential	**RingAvgIntenCh3Status** **RingSpotAvgIntenCh3Status**
	Number of mitochondria in Channel 3 from MitoTracker Red staining showing the number of mitochondria with a transmembrane potential that is still at a high value	**RingSpotCountCh3**
	Ratio of the MitoTracker Green and Red intensities to normalize for the mitochondrial mass resulting in relative mitochondrial transmembrane potential	**RingSpotAvgIntenRatioCh3Ch2**
	Ratio of the number of mitochondria detected in Channel 2 and Channel 3 to determine the fraction of mitochondria with decreased transmembrane potential	**RingSpotCountRatioCh3Ch2**
	Indicates whether a cell's mitochondrial transmembrane potential is beyond a user defined range indicating cytotoxicity	**RingSpotAvgIntenCh3Status** **RingSpotAvgIntenRatioCh3Ch2Status**
Channel 4 Cell Membrane Permeability	Intracellular intensity of membrane impermeant dye that can only enter cells with compromised membrane integrity	**CircAvgIntenCh4** **CircTotalIntenCh4**
	Indicates whether a cell's permeability dye intensity is above a defined level, thus indicating that the cell's membrane permeability has been compromised as a consequence of cytotoxicity	**CircAvgIntenCh4Status**

Table 30. *The Compartmental Analysis BioApplication output features for the cell-level physiological monitors of cell health and cytotoxicity.*

Nucleus (Channel 1)

Using this biology, the nuclear size may be large and/or have an atypical Object Shape. In this particular case, it is necessary to set the Object Selection Parameters to accept the broadest range of nuclei so all cells can be accurately characterized. Thus, set the **ObjectAreaCh1, ObjectShapeP2ACh1, ObjectShapeLWRCh1, ObjectAvgIntenCh1,** and **ObjectTotalIntenCh1** to their lowest Min and highest Max values.

You can set the Assay Parameter **ObjectSizeCh1LevelHigh, ObjectSizeCh1LevelHigh_CC, ObjectSizeCh1LevelLow,** and **ObjectSizeCh1LevelLow_CC** to define the levels indicating the nuclear fragmentation or condensation differentiating between normal and apoptotic cells.

Mitochondrial Mass and Transmembrane Potential (Channel 2 and Channel 3)

For an accurate measure of the intensity of MitoTracker Green and Red, ensure the Spots and Regions to be measured are in range. For Channel 2 and Channel 3 it may be necessary to adjust the Object Identification Method and Values (**Isodata Threshold**, **Fixed Threshold**, or **Triang Threshold**), the **SpotDetectRadiusCh*N***, **RingDistanceCh*N*,** and **RingWidthCh*N***. General guidelines for identifying spots were given earlier in this section covering the first biological example for internalized EGF.

The intensity of the MitoTracker Green in the mitochondria is reported as **MEAN_RingSpotAvgIntenCh2**. This value serves as an indicator of the mitochondrial mass. The number of mitochondria positive for the MitoTracker Green label is reported by the **MEAN_RingSpotCountCh2** output feature.

The intensity of MitoTracker Red in mitochondria gives a measure of mitochondrial transmembrane potential; this value is reported as the **MEAN_RingSpotAvgIntenCh3** output feature. The **MEAN_RingSpotCountCh3** output feature reports the number of mitochondria maintain a normal transmembrane potential.

Once the parameters are properly set to measure the MitoTracker Green and Red intensities, the **RingSpotAvgIntenRatioCh3Ch2** will give an indication of the normalized mitochondrial potential. The level defined by the **RingSpotAvgIntenRatioCh3Ch2LevelLow** Assay Parameter indicates which cells have physiological normal mitochondrial potential. Cells that have a value lower than that set by **RingSpotAvgIntenRatioCh3Ch2LevelLow** are those with a decreased mitochondrial potential, which indicates cytotoxicity. Similarly, the **RingSpotCountRatioCh3Ch2LevelLow_CC** value can now be set to indicate cells that have mitochondria with decreased potential.

Cell Membrane Permeability (Channel 4)

The intensity of the cell permeability dye will be measured by the **MEAN_CircTotalIntenCh4** and **MEAN_CircAvgIntenCh4** output features. General guidelines for measuring this were given earlier in this section that discussed the measurement of MAPK presence or cyclin expression in the nucleus. The level of cytotoxicity based upon the membrane permeability of the cell can be determined by setting the **CircAvgIntenCh4Level*Low/High, Low/High_CC*** values based on the values in the **MEAN_CircAvgIntenCh4** output feature.

Example #4: Translocation of Multiple Related Kinases

Channel	Cellular Target	Detection Strategy	Example Fluorophores
1	Nucleus	Blue fluorescent DNA binding dyes that label nuclei	DAPI, Hoechst 33342
2	Kinase 1	Immunofluorescence using green fluorescent fluorophores	Fluorescein, Alexa Fluor 488
3	Kinase 2	Immunofluorescence using red fluorescent fluorophores	Rhodamine, Cy3, Alexa Fluor 555
4	Kinase 3	Immunofluorescence using fluorophores fluorescent in the far red spectrum	Alexa Fluor 660, Cy5
Filter set suggestion: XF93 (XF110 if needed)			

Table 31. *Possible fluorescent labeling strategy and example fluorophores for measuring the cytoplasm to nucleus translocation of multiple kinases.*

Target	Cellular Properties	Corresponding Feature Reported by Compartmental Analysis
Channel 1 Nucleus	Number of nuclei (i.e., number of cells)	**SelectedObjectCount** (Well Feature)
Channel 2-4 Kinase 1-3 *nomenclature:* ▪ Ch*N*: *N*=2-4 ▪ ChNChD: Ch3Ch2 Ch4Ch2 Ch4Ch3	Intensity of Kinase in the nucleus	**CircAvgIntenCh*N*** **CircTotalIntenCh*N***
	The difference/ratio of Kinase 1's signal in the nucleus versus that in the cytoplasm to indicate the extent of translocation	**CircRingAvgIntenDiffCh*N*** **CircRingAvgIntenRatioCh*N***
	Indicates whether a cell's nuclear kinase intensity, or extent of cytoplasm to nucleus translocation, is above a defined level, indicating the cell is a responder	**CircAvgIntenCh*N*Status** **CircRingAvgIntenDiffCh*N*Status** **CircRingAvgIntenRatioCh*N*Status**
	Ratios of the different kinase intensities in the nucleus to determine for which combination of the different kinases the cell was a responder and the relative strengths of the responses	**CircAvgIntenRatioCh*N*Ch*D*** **CircAvgIntenRatioCh*N*Ch*D*Status**

Table 32. *The Compartmental Analysis BioApplication output features reporting the cellular properties measured for the cytoplasm to nucleus translocation of multiple kinases.*

Nucleus (Channel 1)

The nucleus is used as a marker for identification as the primary object from which all other channel overlays derive from. The **SelectedObjectCount** can be used as a statistical means for standardizing each well (i.e., decreases in **SelectedObjectCount** may indicate some form of toxicity).

Multiple Transcription Factors or Kinases (Channels 2-4)

The **MEAN_CircTotalIntenCh*N*** and **MEAN_CircAvgIntenCh*N*** features quantitate the nuclear intensity of each kinase. General guidelines for measuring these were discussed earlier for Example 1 (measurement of MAPK presence or cyclin expression in the nucleus). The **MEAN_CircRingAvgIntenDiffCh*N*** and **MEAN_CircRingAvgIntenRatioCh*N*** output features automatically calculate the difference or ratio of the signal in the nucleus versus the cytoplasm to indicate the extent of translocation of the given kinase. The key Assay Parameter to adjust is **CircModifierCh*N*** in order to properly cover the nuclear mask. The **RingDistanceCh*N*** and **RingWidthCh*N*** Assay Parameters may need to be adjusted to accurately calculate the cytoplasmic (Ring) intensity. Since the compartments being measured for all three kinases are the same, the **CircModifierCh*N*** , **RingDistanceCh*N*** and **RingWidthCh*N*** Assay Parameters should be set the same for the three channels (where **Ch*N*** can be Channels 2-4 in this example).

To determine whether or not the cell is a responder, use the intensity measurements in the output features above to set the appropriate levels for the following Assay Parameters: **CircAvgIntenCh*N*Level*Low/High, Low/High_CC*, CircRingAvgIntenDiffCh*N*Level*Low/High, Low/High_CC*,** and **CircRingAvgIntenRatioCh*N*Level*Low/High, Low/High_CC***.

To determine the combination of the different kinases to which the cell was a responder, set the appropriate levels for the **CircAvgIntenRatioCh*N*Ch*D*Level*Low/High, Low/High_CC*** Assay Parameters using the **CircAvgIntenCh*N*** output values from the respective channels (where *D* is the denominator found for Channels 2-4 in this example (*described in Table 1*).

Example #5: Phosphorylation of Macromolecules in Intracellular Locations

Channel	Cellular Target	Detection Strategy	Example Fluorophores
1	Nucleus	Blue fluorescent DNA binding dyes that label nuclei	DAPI, Hoechst 33342
2	Total (phosphorylated + unphosphorylated protein)	Immunofluorescence against the total protein using green fluorescent fluorophores	Fluorescein, Alexa Fluor 488
3	Phosphorylated version of protein	Immunofluorescence against the phospho-specific form of the protein using red fluorescent fluorophores	Rhodamine, Cy3, Alexa Fluor 555
Filter set suggestion: XF93			

Table 33. *Possible fluorescent labeling strategy and example fluorophores for measuring the phosphorylation of a protein in an intracellular location.*

Target	Cellular Properties	Corresponding Feature Reported by Compartmental Analysis
Channel 1 Nucleus	Number of nuclei (i.e., number of cells)	**SelectedObjectCount** (Well Feature)
Channel 2 Total protein	Intensity from total protein (phosphorylated and unphosphorylated) in the nucleus	**CircAvgIntenCh2** **CircTotalIntenCh2**
Channel 3 Phosphorylated protein	Intensity from phosphorylated protein in the nucleus	**CircAvgIntenCh3** **CircTotalIntenCh3**
	Indicates whether the phosphorylated protein's intensity is above a defined level, indicating that the cell is a responder	**CircAvgIntenCh3Status**
	Ratio of phosphorylated protein intensity to total protein intensity as a measure of the degree of the protein's phosphorylation	**CircAvgIntenRatioCh3Ch2**
	Indicates whether a cell's ratio of phosphorylated protein intensity to total protein intensity is above a defined level, thus indicating whether the cell is a responder and has been activated	**CircAvgIntenRatioCh3Ch2Status**

Table 34. *The Compartmental Analysis BioApplication output features used to measure protein phosphorylation.*

Nucleus (Channel 1)

The nucleus again is used as a marker for identification as the primary object from which all other channel overlays derive from. The **SelectedObjectCount** Well Feature can be used as a statistical means for standardizing each well (i.e., decreases in **SelectedObjectCount** may indicate some form of toxicity).

Total Protein (Channel 2)

The intensity of the total protein (phosphorylated and unphosphorylated) in the nucleus is calculated and reported in output features **MEAN_CircTotalIntenCh2** and **MEAN_CircAvgIntenCh2.** The **CircModifierCh2** Assay Parameter may be adjusted to reflect the appropriate nuclear mask as described in the first biological example for MAPK.

Phosphorylated Protein (Channel 3)

The intensity of the phosphorylated protein in the nucleus is calculated and reported in output features **MEAN_CircTotalIntenCh3** and **MEAN_CircAvgIntenCh3.** The **CircModifierCh3** Assay Parameter value should be the same as the value set for Channel 2 (i.e., **CircModifierCh2**) since the same compartment (nucleus) is being evaluated in both channels. To determine whether or not the cell is a responder, use the **MEAN_CircAvgIntenCh3** in the output features to set the appropriate levels for **CircAvgIntenCh3Level*****Low/High, Low/High_CC.***

To assess the degree of the protein's phosphorylation, it is necessary to measure the ratio of phosphorylated protein intensity (**CircAvgIntenCh3**) to total protein intensity (**CircAvgIntenCh2**). This is done with the output feature **MEAN_CircAvgIntenRatioCh3Ch2.** You can set the levels of the **CircAvgIntenRatioCh3Ch2Level*****Low/High, Low/High_CC*** Assay Parameters using the above-mentioned statistics giving an indication as to whether the cell is a responder and has been activated.

Event Definition Using the BioApplication Event Wizard

The BioApplication Event Wizard is a software tool that is designed to allow entry, reading, and modifying Event Definitions as logical statements. Event Definitions are stored in the Assay Protocol as Assay Parameters of the type **Type_X_EventDefinition,** where X can be 1-3, allowing definition of up to three distinct Events.

The Wizard can be used to enter and edit Event Definitions as values for the **Type_X_EventDefinition** Assay Parameter in the Assay Protocol for the Compartmental Analysis BioApplication. This section provides a detailed description of the operation of the BioApplication Event Wizard. The Wizard should only be used after the Compartmental Analysis BioApplication has been installed on your computer.

NOTE

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

Definition of Events requires that the following steps be followed, in the order listed. It is strongly recommended that you do not run the Scan software application and the BioApplication Event Wizard at the same time.

STEP I

1) Create a protocol using the Scan software application without defining Events. Set optimized parameter values (lower and upper limits) for Cell Features to be used for Event Definition.
2) Save protocol.
3) Close the Scan software application.

STEP II

1) Open the BioApplication Event Wizard.
2) Open the protocol saved in Step I using the BioApplication Event Wizard and define up to 3 events at the level of individual cells.
3) Save updated Assay Protocol.
4) Close the BioApplication Event Wizard.

STEP III

1) Restart the Scan software application and open the protocol saved in Step II (Event Definitions will appear as numeric strings in the Assay Parameter window)
2) Copy Event Definitions from the Protocol Comments field in Create Protocol View into the Scan Comments field in Scan Plate View
3) Scan the assay plate.
4) Close the Scan software application.

STEP IV (Optional)

1) Open the saved protocol at a later time using BioApplication Event Wizard.
2) Evaluate and/or modify saved Event Definitions.
3) Close BioApplication Event Wizard and start the Scan software application to scan a new assay plate or analyze previously acquired images using modified Event Definitions.

Features

The following features are included in the BioApplication Event Wizard:

- Can be used with any upgraded Compartmental Analysis protocol (V4 version)
- Enables use of standard Boolean operators (NOT, AND, ANDNOT, OR, ORNOT, XOR, NAND, and NOR) to construct Event Definitions comprised of selected Cell Features
- Read-Only protocol protection
- Displays previously entered Event Definitions in saved Assay Protocols
- Ability to clear each Event Definition individually
- Rapid construction of logical statements using Cell Features and logical operators
- Automated validation of each Event Definition when loading or saving protocols
- Ability to save updated protocol
- Automatic addition/update of Event Definitions in the Protocol Comments field of the Assay Protocol, viewable in Create Protocol View

Running the Event Wizard with Compartmental Analysis

Before Running the Event Wizard...

1) Create a protocol using the Compartmental Analysis BioApplication without defining Events. Set optimized parameter values (upper levels) for detection of responder cells associated with Cell Features to be used for Event Definition.
2) Save protocol.
3) Close the Scan software application (suggested).

Starting the BioApplication Event Wizard

It is suggested to close the instrument or Scan software application before starting the BioApplication Event Wizard and vice versa. Operation of the BioApplication Event Wizard must be consistent with the steps described.

To start the BioApplication Event Wizard,

Double-click the **BioApplication Event Wizard** desktop icon 

-or-

For Cellomics HCS Readers: From the Windows **Start** menu, select **Programs** > **Cellomics** > **BioApp Event Wizard**.

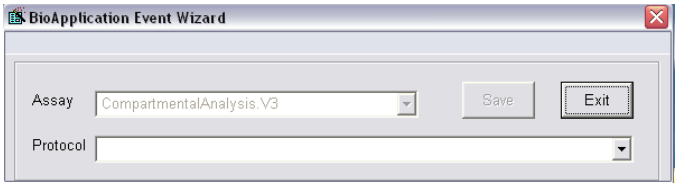
For vHCS Discovery Toolbox computers: From the Windows **Start** menu, select **Programs** > **Cellomics > vHCS Scan > BioAppEventWizard**

Protocol Selection and Loading

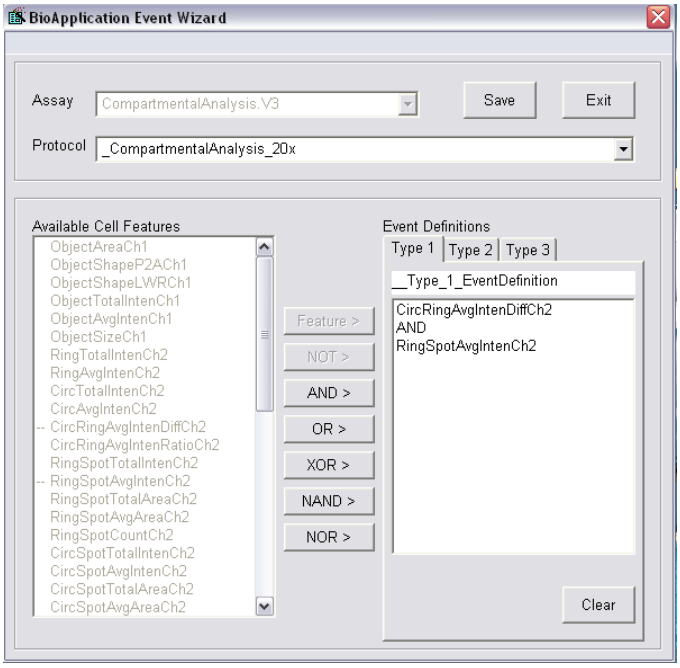
The first window that is displayed (see below) allows selection of any V4 Compartmental Analysis Assay Protocol. Note that Event Definitions can only be specified for pre-existing Assay Protocols.

To select an Assay Protocol,

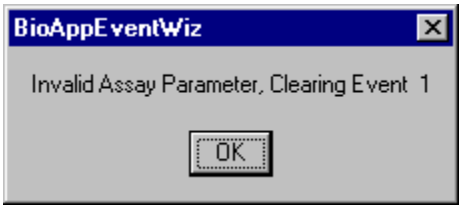
1) From the **Assay** drop-down menu, select **CompartmentalAnalysis.V4**.
2) From the **Protocol** drop-down menu, click on the drop-down arrow to view the list of existing Compartmental Analysis Assay Protocols.
3) Select the desired Assay Protocol from the list.



Once a protocol is selected, the window expands to its full extent as shown below. Cell Features available for Event Definition are listed on the left in the Available Cell Features section.



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



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

4) Once you have completed the Event Definitions, select the **Save** button and then the **Exit** button. The screen will close and you can then open the Scan software application.

Defining Events

This section describes the steps involved in defining individual Events in a Compartmental Analysis Assay Protocol. Note that logical statements used to define Events can include up to three Cell Features and three logical operators.

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

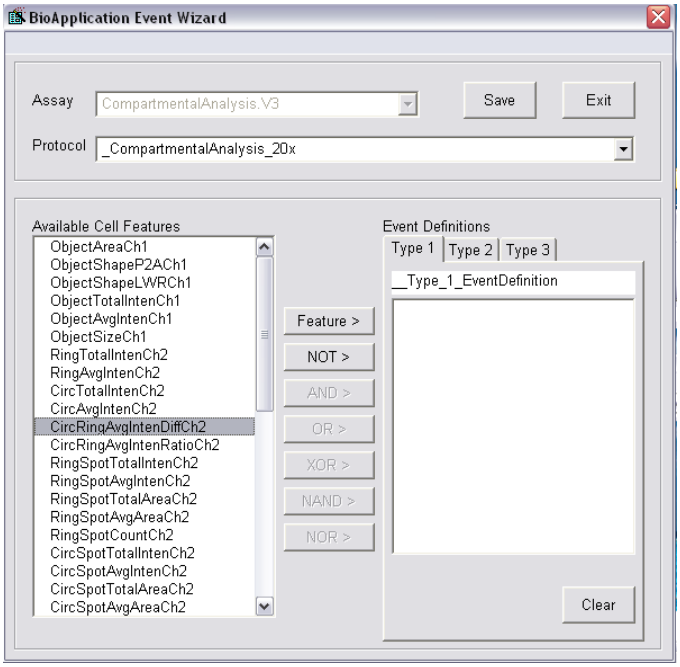
The procedure to be followed when constructing Event Definitions is described in the sequence of screenshots below. Cell Features are combined with Boolean operators to produce Event Definitions. Any Cell Feature can be selected by clicking on the feature name in the Available Cell Features list and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

To build an Event Definition,

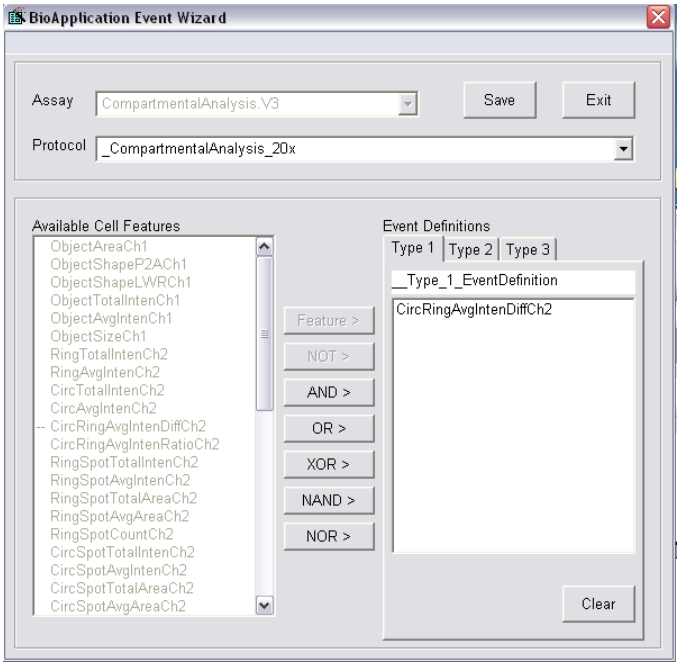
1) Select the Event Definition that you want to specify or edit by clicking on the **Type 1**, **Type 2**, or **Type 3** tab.

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

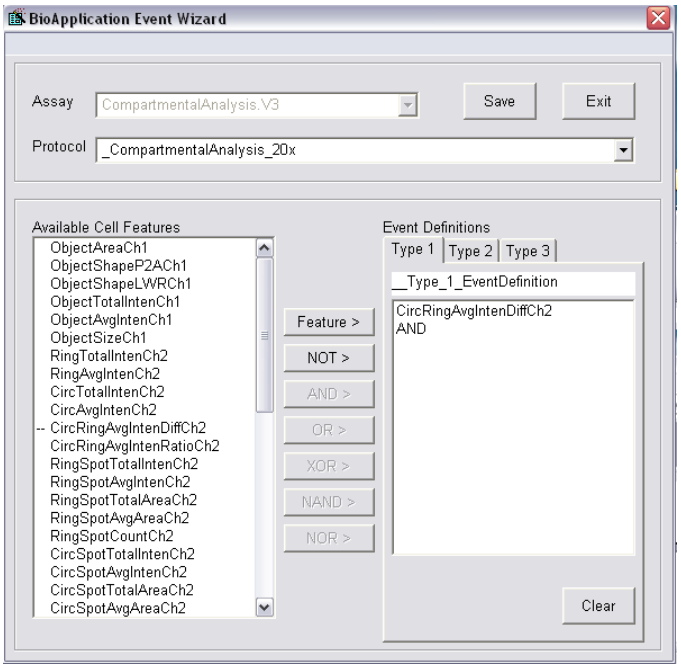
3) Select the desired Cell Feature by clicking on the feature name from the Available Cell Features list. In this example, the **CircRingAvgIntenDiffCh2** feature is selected. You may also choose to select **NOT>** first (before the Feature) to indicate that you do not want to include this Cell Feature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



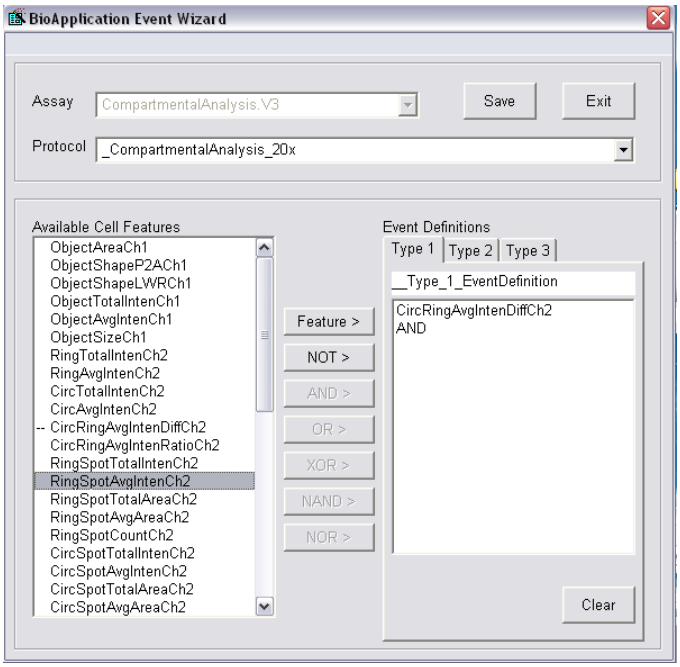
4) Click the **Feature>** button to transfer the Cell Feature into the Event Definition (see below).



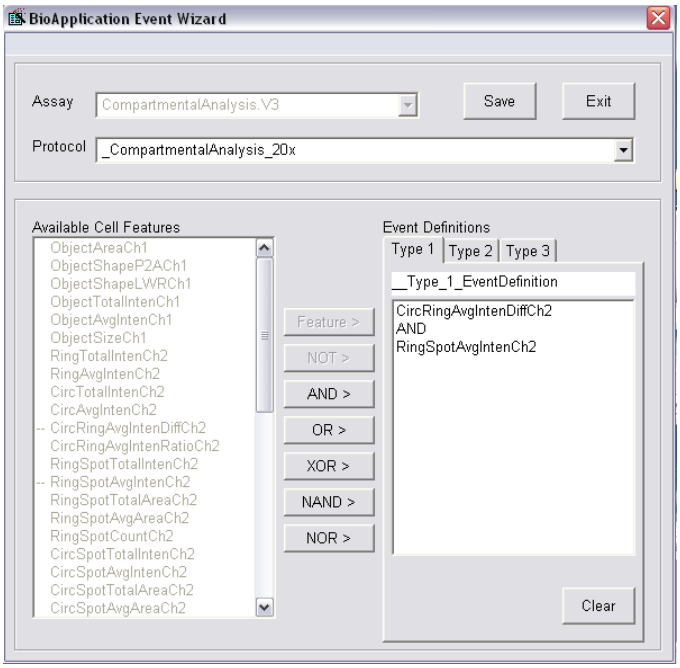
5) Click on the desired Operator button to transfer it into the Event Definition. In this example, the **AND** operator is selected (see below).



6) Repeat the cycle for adding another Cell Feature to the Event Definition. In this case, the Cell Feature **RingSpotAvgIntenCh2** has been selected for addition.

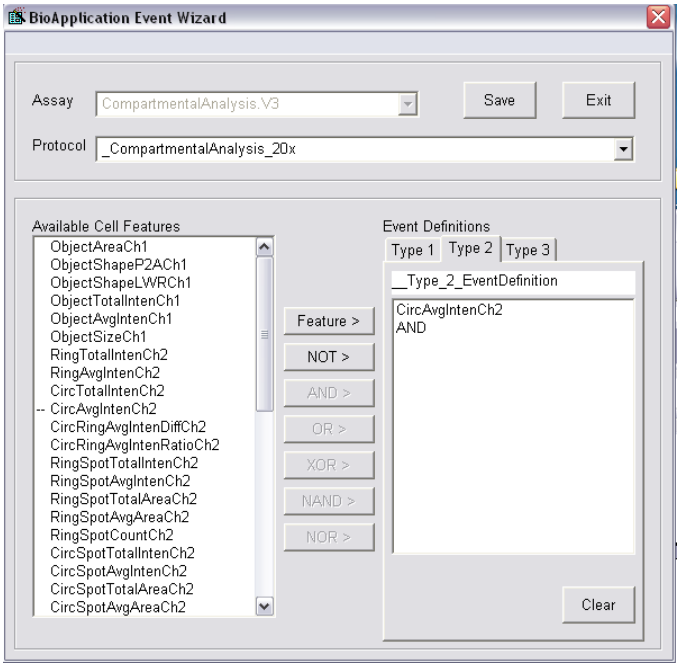


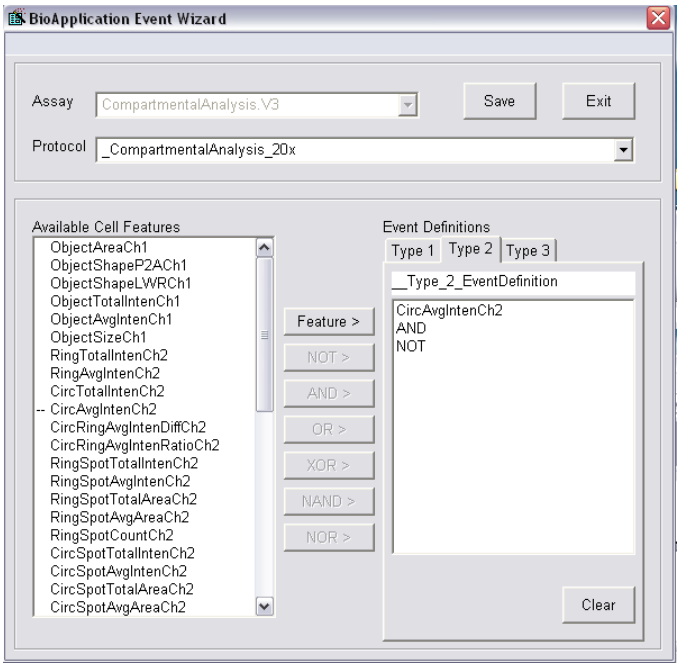
7) As before, click on the **Feature >** button to transfer the Cell Feature into the Event Definition (see below).

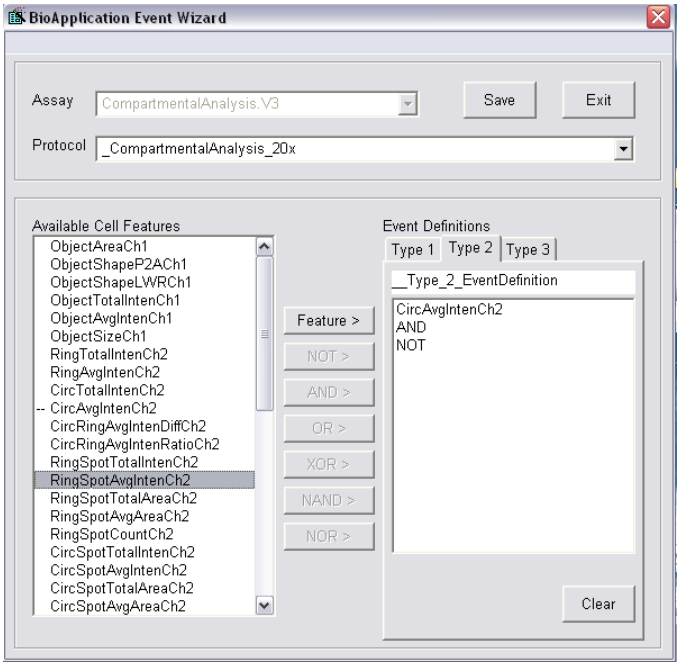


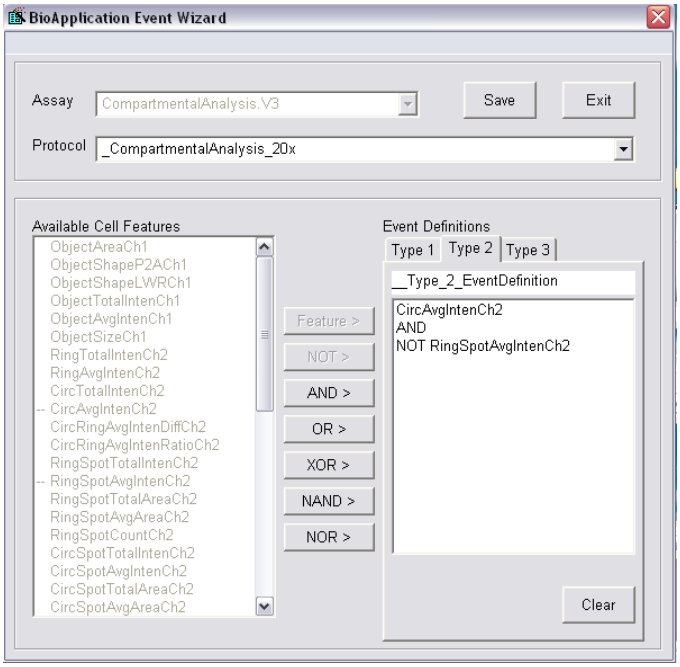
Type_1_EventDefinition is: **CircRingAvgIntenCh2** AND **RingSpotAvgIntenCh2**

Entry of a logical operator may be followed by entry of another Cell Feature. Alternatively, the operators **AND** and **OR** can be inverted by adding the **NOT** operator by pressing the **NOT >** button prior to selecting the Cell Feature, as shown below.



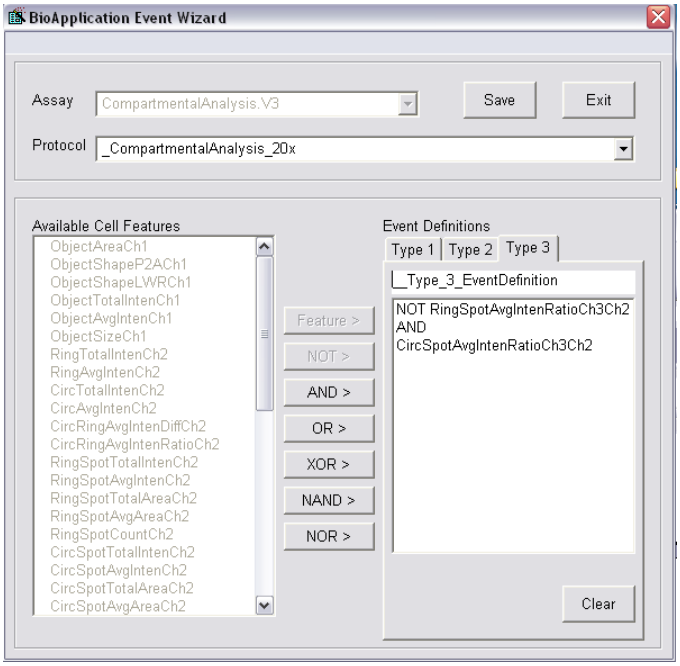

BioApplication Event Wizard
Assay
CompartmentalAnalysis.V3
Save
Exit
Protocol
_CompartmentalAnalysis_20x
Available Cell Features
ObjectAreaCh1
ObjectShapeP2ACh1
ObjectShapeLWRCh1
ObjectTotalIntenCh1
ObjectAvgIntenCh1
ObjectSizeCh1
RingTotalIntenCh2
RingAvgIntenCh2
CircTotalIntenCh2
CircAvgIntenCh2
CircRingAvgIntenDiffCh2
CircRingAvgIntenRatioCh2
RingSpotTotalIntenCh2
RingSpotAvgIntenCh2
RingSpotTotalAreaCh2
RingSpotAvgAreaCh2
RingSpotCountCh2
CircSpotTotalIntenCh2
CircSpotAvgIntenCh2
CircSpotTotalAreaCh2
CircSpotAvgAreaCh2
Feature >
NOT >
AND >
OR >
XOR >
NAND >
NOR >
Event Definitions
Type 1
Type 2
Type 3
__Type_2_EventDefinition
CircAvgIntenCh2
AND
NOT
Clear


BioApplication Event Wizard
Assay
CompartmentalAnalysis.V3
Save
Exit
Protocol
_CompartmentalAnalysis_20x
Available Cell Features
ObjectAreaCh1
ObjectShapeP2ACh1
ObjectShapeLWRCh1
ObjectTotalIntenCh1
ObjectAvgIntenCh1
ObjectSizeCh1
RingTotalIntenCh2
RingAvgIntenCh2
CircTotalIntenCh2
CircAvgIntenCh2
CircRingAvgIntenDiffCh2
CircRingAvgIntenRatioCh2
RingSpotTotalIntenCh2
RingSpotAvgIntenCh2
RingSpotTotalAreaCh2
RingSpotAvgAreaCh2
RingSpotCountCh2
CircSpotTotalIntenCh2
CircSpotAvgIntenCh2
CircSpotTotalAreaCh2
CircSpotAvgAreaCh2
Feature >
NOT >
AND >
OR >
XOR >
NAND >
NOR >
Event Definitions
Type 1
Type 2
Type 3
__Type_2_EventDefinition
CircAvgIntenCh2
AND
NOT
Clear



Type_2_EventDefinition is: **CircAvgIntenCh3** ANDNOT **RingSpotAvgIntenCh2**

Typically logical statements used to define Events begin with a Cell Feature. Alternatively, a logical statement may begin with the logical operator **NOT** as shown below.



Type_3_EventDefinition is: NOT **RingSpotAvgIntenRatioCh3Ch2** AND **CircSpotAvgIntenRatioCh2Ch3**

To clear an Event Definition,

- Once a protocol is loaded, click the **Clear** button to clear any Event Definition. This feature can be used to redefine any event.

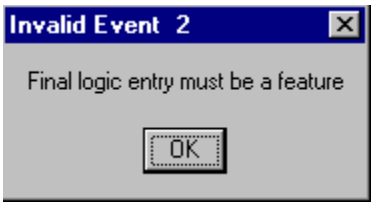
To save the updated Assay Protocol,

- Click the **Save** button. Note that an Assay Protocol cannot be renamed using the BioApplication Event Wizard. Renaming of protocols can only be done through the Scan software application.

NOTE

The BioApplication Event Wizard will only make changes to pre-existing Assay Protocols by adding or modifying Event Definitions. If you wish to create a new protocol, use the **Save Assay Protocol As** option under the **File** menu within the Scan software application prior to starting the Event Wizard.

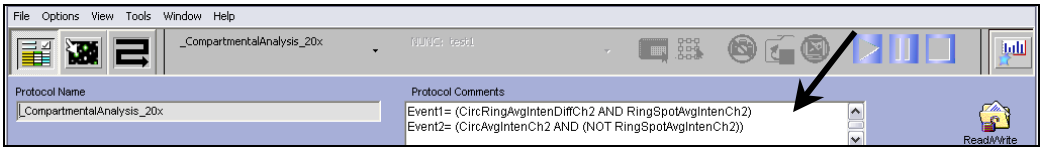
The BioApplication Event Wizard has the capability to automatically validate Event Definitions. If Event Definition validation fails, the protocol will not be saved. For example, Event Definitions are considered invalid if they do not end with a Cell Feature. In this case, clicking on the **Save** button causes the following message to be displayed.



To exit the BioApplication Event Wizard,

- Click the **Exit** button at any time to exit the Wizard without saving any changes made to Event Definitions.

Once the updated Assay Protocol is saved, Event Definitions are viewable in the **Protocol Comments** field in the **Create Protocol View** upon opening the saved Assay Protocol using the Scan software application (see arrow).



Chapter

4

iDev Software Workflow

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

iDev Protocol Optimization Tasks

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

Image Preprocessing

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

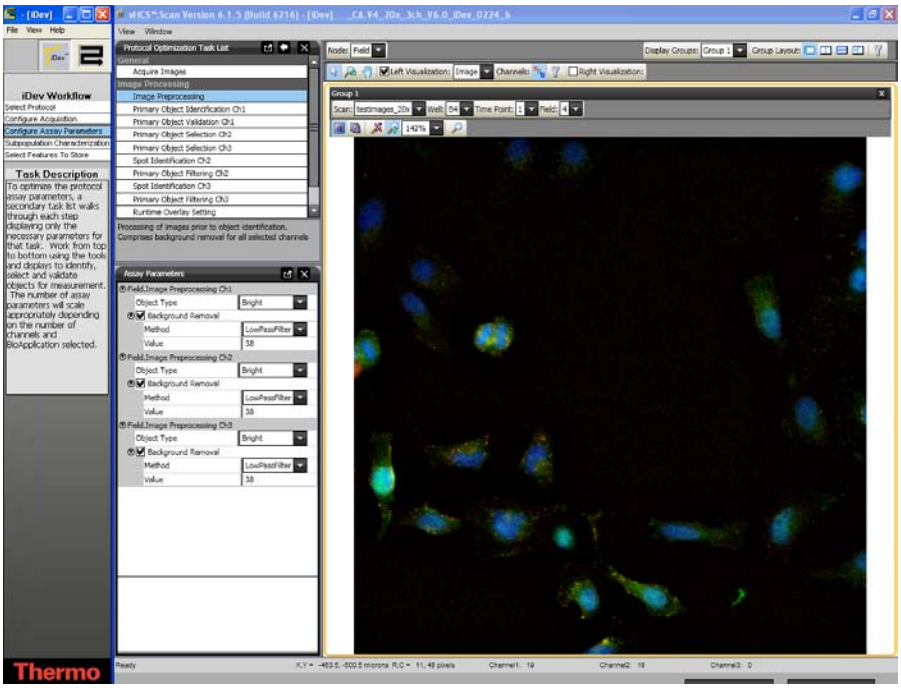


Figure 23. *Protocol Optimization Task – Image Preprocessing*

Primary Object Identification Ch1

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

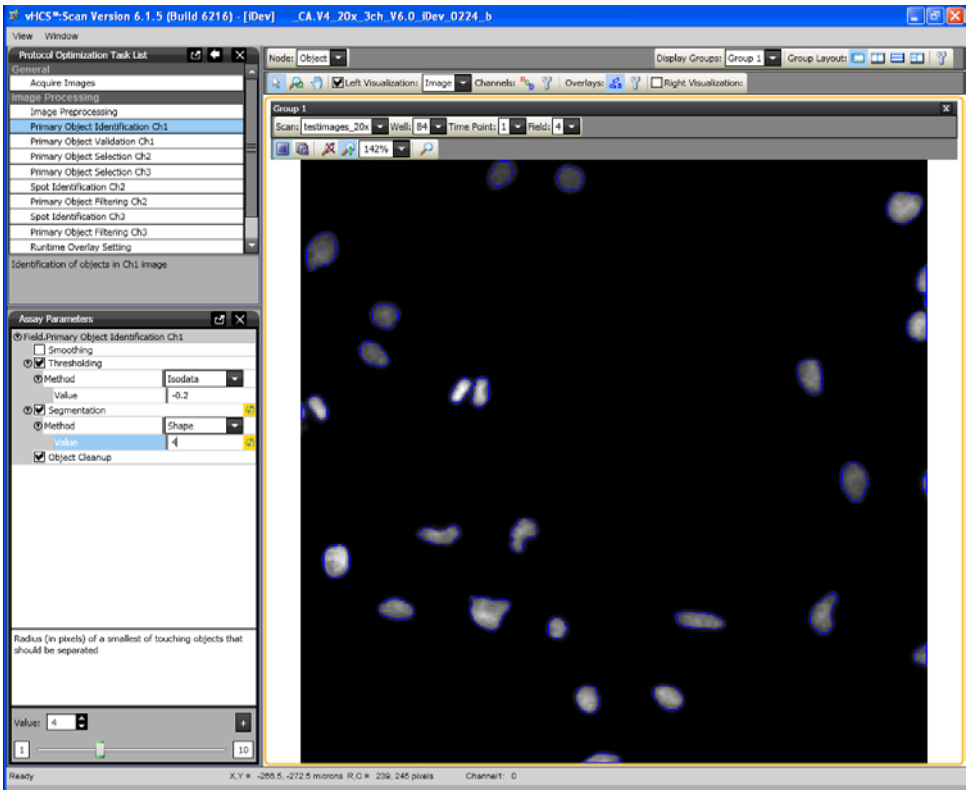


Figure 24. *Protocol Optimization Task – Primary Object Identification Ch1*

Primary Object Validation Ch1

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

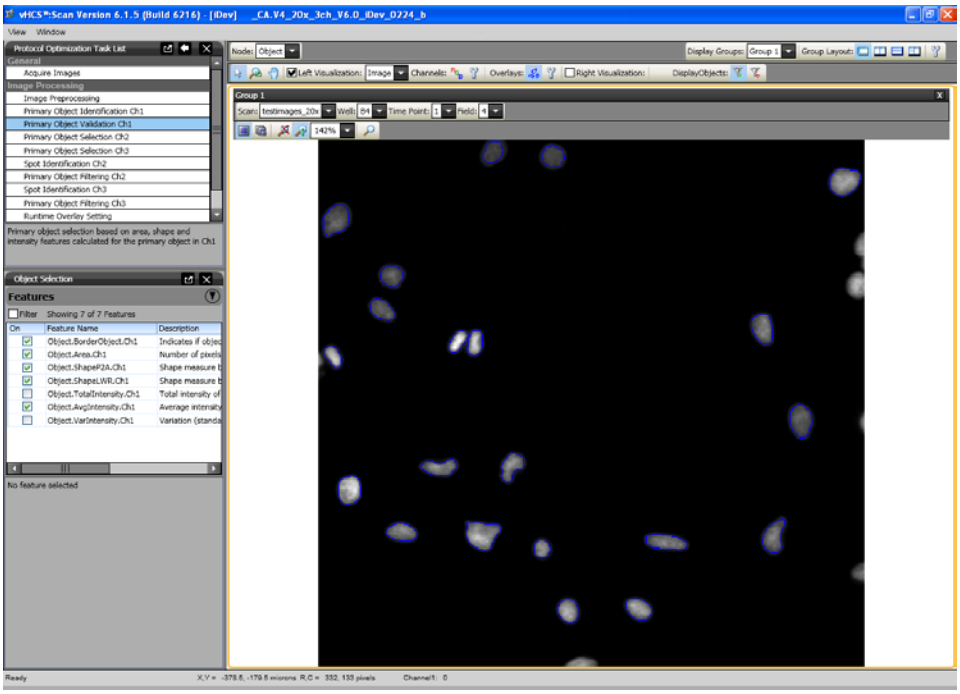


Figure 25. *Protocol Optimization Task – Primary Object Validation Ch1*

Primary Object Selection Ch2 through ChN

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

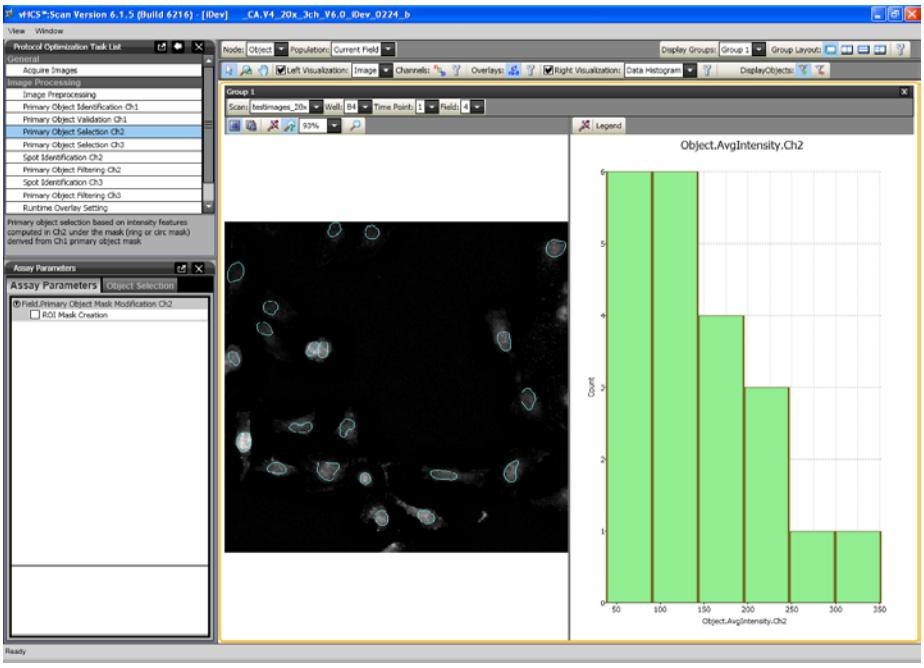


Figure 26. *Protocol Optimization Task – Primary Object Selection Ch2 through Ch6*

Spot Identification Ch2 through ChN

In this task, you can set parameters to identify spots in Channel 2 through Channel N images within the region of interest (ring or circ mask) derived from the Channel 1 primary object mask. In this task, you will define the region of intrest (ROI) and set the identification method and threshold for identifying spots in Channel 2. The ROI can be either be a “Circ” or a “Ring”.

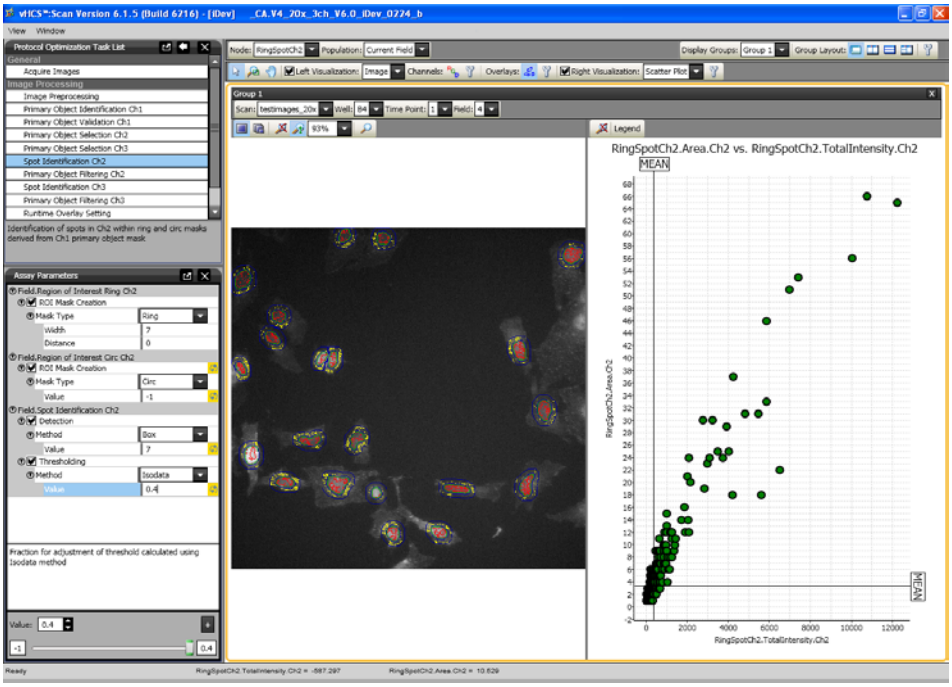


Figure 27. *Protocol Optimization Task – Spot Identification*

Primary Object Filtering Ch2 through ChN

In this task, you can set selection/rejection criteria of primary objects in Channel 1 based on intensity and spot features within the Circ and Ring masks in Channel 2 through Channel N.

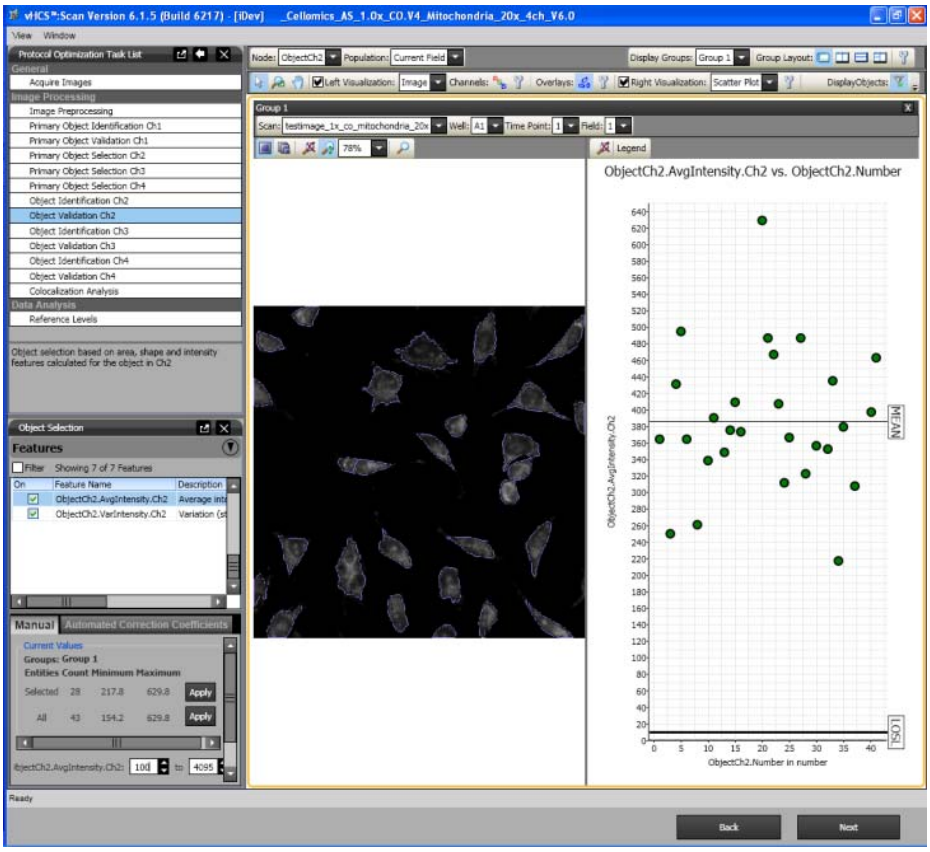


Figure 28. *Protocol Optimization Task – Primary Object Filtering*

Runtime Overlay Setting

In this task, you can select the channels for display of Circ, Ring, CircSpot, and RingSpot during the scan process. These settings will not in any way affect the analysis of your images and are purely for display purposes only.

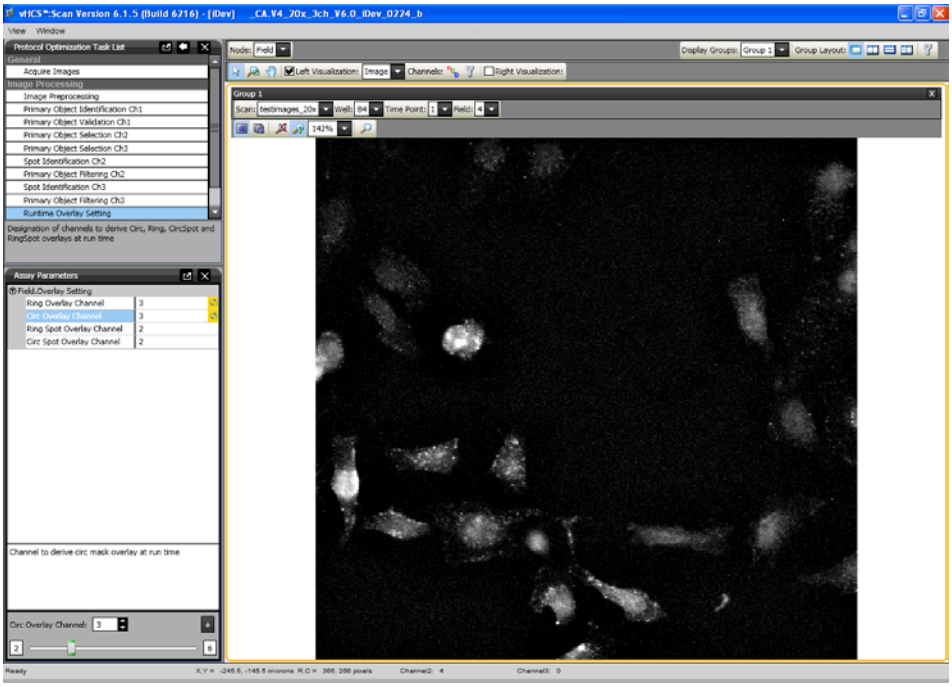


Figure 29. *Protocol Optimization Task – Runtime Overlay Setting*

Reference Levels

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

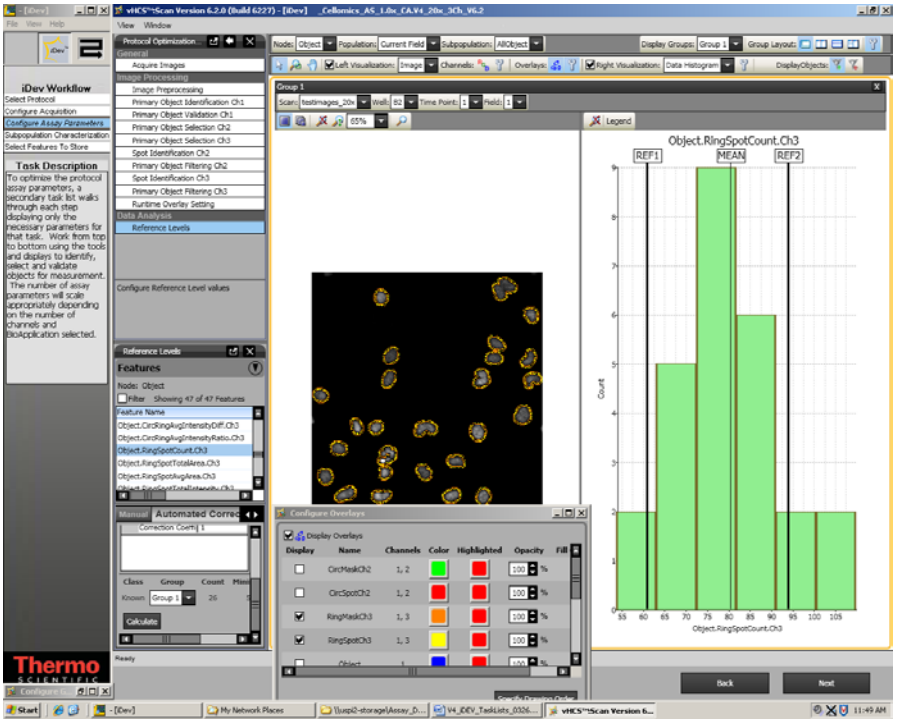


Figure 30. *Protocol Optimization Task – Reference Levels*

Setting Events in the iDev Assay Development Workflow

The process for setting Events in the iDev Assay Development Workflow is identical to that outlined in Chapter 3 of this guide. However, because of the integrated workflow in the iDev product, Events can be set in the Subpopulation Characterization task rather than the BioApplication Event Wizard. Events are based on Reference Levels set in the Protocol optimization task of the Configure Assay Parameters step in the iDev workflow.

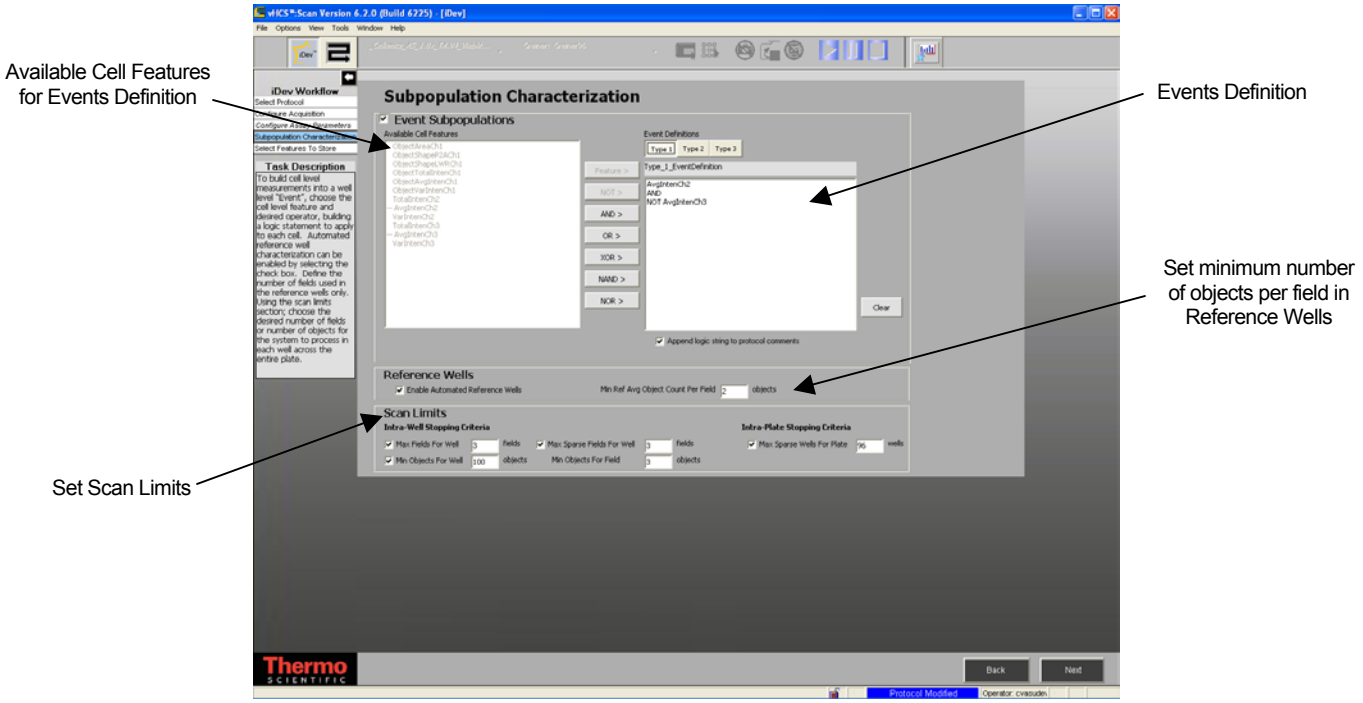


Figure 31. *Subpopulation Characterization Task*

Index