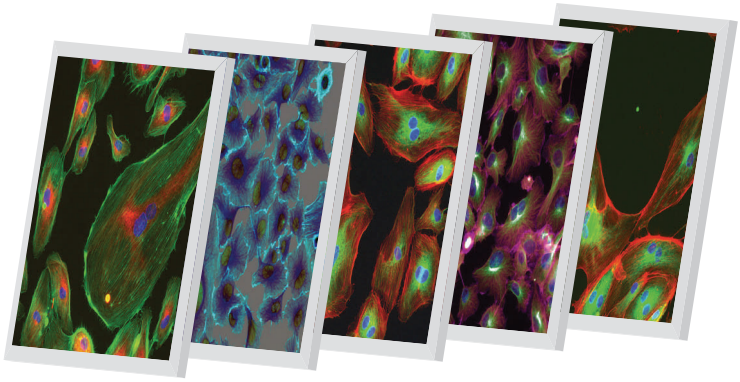
Thermo Scientific Cellomics® Colocalization V4

BioApplication Guide



Part of Thermo Fisher Scientific

Thermo SCIENTIFIC

Cellomics® Colocalization BioApplication Guide

V4 Version

PUBLISHED BY

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

Copyright

Trademarks

WARNING

Use this product only in the manner described in this guide. When used other than as specified, the safety protections may be impaired. Failure to adhere to safety precautions and/or procedures outlined in this document may result in system failure, personal injury, or death. Thermo Fisher Scientific Inc. shall not be held liable under any circumstances.

P/N LC06100800

Cellomics, Inc.
Software License Agreement

This Software License Agreement ("Agreement") for the Colocalization BioApplication software ("Software") is entered into in conjunction with the purchase of the license to the Software. By installing and/or using the Software, Licensee accepts the Software and agrees to be bound by the terms of this Agreement.

1) **Licensor's Rights** Cellomics, Inc. ("Licensor") represents that it has all rights necessary to grant the license herein. The Software is protected by patents (including United States Patent Nos. 5,989,835, 6,573,039, 6,620,591, 6,671,624, 6,902,883, 7,060,445, 7,117,098, and 7,235,373, Japanese Patent No. 3683591, European Patent No. 0983498, Canadian Patent No. 2,282,658, and Australian Patent No. 730100), patent applications, and copyrights and includes valuable trade secrets and other proprietary material.

2) **License Grant**: Licensor grants the purchaser ("Licensee") a non-exclusive, non-transferable license ("License") under the such patent rights and copyrights to use the Software, and associated manuals and documentation, in conjunction with a Cellomics instrument or other system authorized by Cellomics, Inc., at the designated Licensee's business location until this License is terminated in accordance with the terms and conditions specifically set out in the Agreement. This License permits the Licensee to make one copy of the Software in machine-readable form for backup purposes only.

3) **Limitations:** Licensee agrees to treat the Software as confidential and not to copy, reproduce, sub-license, or otherwise disclose the Software or its associated manuals and documentation to third parties, including any parent, subsidiaries, or affiliated entities, without the prior written consent of Licensor. Licensee agrees not to disassemble, decompose, reverse engineer, or otherwise translate the Software. Licensee agrees not to modify the Software, not to allow access to the Software through any terminal located outside of Licensee's location, and that any derivative works and the backup copy are the sole property of the Licensor.

4) **Additional License Rights**: The hardware or software product accompanying this manual (the "Product") and/or the method of use thereof may be covered by one of more of United States Patent Nos. 6,743,576; 6,631,331; 6,738,716; 6,615,141; and 6,651,008 and certain other patents pending owned by Cytokinetics, Inc. (the "Cytokinetics Patent Rights"). In consideration of the purchase of the Product, the direct purchaser (the "Purchaser") hereby acquires a non-exclusive, non-transferable license under the Cytokinetics Patent Rights to use the Product solely for its intended purposes for research and development in the life sciences field, including the discovery of human and animal therapeutics and diagnostics, but not diagnostic testing (the "Research Field"), excluding, however, any rights under the Cytokinetics Patent Rights for that portion of the Research Field in which assays are performed directed toward any mitotic kinesin target (the "Excluded Field"). Purchaser hereby agrees not to use the Product for any application in the Excluded Field. Any use of the Product shall constitute acceptance of and agreement to be bound by the foregoing limitation with respect to the Excluded Field. Except as expressly provided above, the Purchaser receives no rights, express, implied or otherwise, under the Cytokinetics Patent Rights.

5) **Term**: This License is effective at the time the Licensee receives the Software, and shall continue in effect for the period indicated below, based upon the indicated type of License:

- □ **Perpetual** Termination shall occur when Licensee ceases all use of the Software and returns or destroys all copies thereof.
- □ **Annual** Termination shall occur ONE (1) YEAR from the date of installation.
- □ **Beta** Termination shall occur _____ DAYS from the date of installation.
- □ **Trial** Termination shall occur at the end of the evaluation period mutually agreed upon by Licensor and Licensee, but in no event more than _____ DAYS from the date of installation.

This License shall automatically terminate upon the failure of the Licensee to comply with any of the terms of this Agreement. Sections 3, 6, and 7 shall survive the termination of the License for any reason.

6) **LIMITATION OF LIABILITY**: LICENSOR SHALL HAVE NO LIABILITY WITH RESPECT TO ITS OBLIGATIONS UNDER THIS AGREEMENT OR OTHERWISE FOR ANY INDIRECT, INCIDENTAL, SPECIAL, OR CONSEQUENTIAL DAMAGES, HOWEVER CAUSED AND ON ANY THEORY OF LIABILITY, WHETHER FOR BREACH OF CONTRACT, TORT (INCLUDING NEGLIGENCE) OR OTHERWISE, ARISING OUT OF OR RELATED TO THIS AGREEMENT, INCLUDING BUT NOT LIMITED TO LOSS OF ANTICIPATED PROFITS, LOSS OR INTERRUPTION OF USE OF OR CORRUPTION OF ANY FILES, DATA OR EQUIPMENT, EVEN IF LICENSOR HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THESE LIMITATIONS SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY. THE PARTIES AGREE THAT THE FOREGOING LIMITATIONS REPRESENT A REASONABLE ALLOCATION OF RISK UNDER THIS AGREEMENT.

7) **DISCLAIMER OF WARRANTY**: LICENSOR AND ITS DISTRIBUTORS MAKE NO PROMISES, REPRESENTATIONS OR WARRANTIES, EITHER EXPRESS, IMPLIED, STATUTORY, OR OTHERWISE, WITH RESPECT TO THE SOFTWARE, MANUALS, AND DOCUMENTATION, INCLUDING THEIR CONDITION, THEIR CONFORMITY TO ANY REPRESENTATION OR DESCRIPTION, OR THE EXISTENCE OF ANY LATENT OR PATENT DEFECTS, AND LICENSOR AND ITS DISTRIBUTORS SPECIFICALLY DISCLAIM ALL IMPLIED WARRANTIES OF MERCHANTABILITY, NONINFRINGEMENT AND FITNESS FOR A PARTICULAR PURPOSE. NOTWITHSTANDING, LICENSOR REPRESENTS THAT THE SOFTWARE IS Y2K COMPLIANT. LICENSEE ACKNOWLEDGES THAT THE LOADING OF THIS OR ANY THIRD PARTY SOFTWARE ON A COMPUTER SYSTEM MAY RESULT IN SYSTEM DAMAGE, DATA LOSS, DATA CORRUPTION OR SOFTWARE INCOMPATIBILITY.

8) **Miscellaneous**: Licensee may not assign this Agreement or any rights or obligations hereunder, directly or indirectly, by operation of law or otherwise, without the prior written consent of Licensor. Subject to the foregoing, this Agreement shall inure to the benefit of and be binding upon the parties and their respective successors and permitted assigns. No waiver or modification of this Agreement shall be valid unless in writing signed by each party. The waiver of a breach of any term hereof shall in no way be construed as a waiver of any other term or breach hereof. If any provision of this Agreement is held to be unenforceable, such provision shall be reformed only to the extent necessary to make it enforceable. This Agreement shall be governed by Pennsylvania law (except for conflict of laws principles). This Agreement contains the complete agreement between the parties with respect to the subject matter hereof, and supersedes all prior or contemporaneous agreements or understandings, whether oral or written. All questions concerning this Agreement shall be directed to: Cellomics, Inc., 100 Technology Drive, Pittsburgh, PA 15219, Attention: Director, Licensing.

Table of Contents

Chapter

1

Overview of the Colocalization BioApplication

High Content Screening (HCS) combines biological fluorescent-based reagents, automated optical imaging instrumentation, and an advanced image processing algorithm to automatically extract and quantify useful information from biological systems. This guide provides a description of the Colocalization BioApplication, a HCS application that provides a method to quantify colocalization of targets of interest in different cellular regions identified by appropriate markers. This guide contains the following chapters:

Chapter 1 provides an overview of the Colocalization BioApplication and provides examples of biological processes where the BioApplication can be used.

Chapter 2 describes the algorithm used to analyze the results and gives a brief description of input Assay Parameters and Output Features.

Chapter 3 describes the use of the BioApplication on different biological examples and guidance on how to use the BioApplication Events Wizard.

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

System Compatibility

The Colocalization BioApplication described in this document is designed to run on the following platforms:

- ArrayScan® HCS Reader version VTI
- Cellomics® vHCS™ Discovery Toolbox versions 1.6.1

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

Cell Biology Background

Colocalization of proteins or other macromolecules is a fairly common phenomenon in biological processes. In biological terms, colocalization means that two or more macromolecules are in the same location in a specimen, such as tissues, cells or intracellular organelles. Typically when any biological process takes place, several different macromolecules are brought together to the sight of the biological process. Examples of biological processes that include such colocalization include gene transcription, translation of RNA sequences to proteins, internalization of a ligand bound to its cell surface receptor, activation of second messenger pathways by the coming together of different regulatory

subunits of an enzyme, apoptosis, formation and disruption of cytoskeletal proteins/ fibers for cell division and movement.

Within the context of fluorescent digital imaging, colocalization means that the two or more macromolecules that are tagged fluorescently, share the same pixels and have a certain degree of overlap. The Cellomics Colocalization BioApplication will allow you to specifically measure the extent of overlap of the various fluorescent targets, in terms of area overlap and intensity overlap. The Colocalization BioApplication also measures colocalization coefficients such as Pearson's and Mander's coefficient.

The Colocalization BioApplication can be used for screening compounds that affect localization of targets of interest with different cellular organelles or locations. It can also be used to identify the location of newly discovered proteins in a cell, using appropriately stained or tagged marker proteins to identify various cellular locations and organelles.

BioApplication Overview

The Colocalization BioApplication is a multi-featured application that can quantify cell/field/well level features in up to 6 fluorescent channels, each of which represents a combination of a fluorescent dye and a filter set used to visualize the dye with a particular exposure time. Channel 1 is dedicated for primary object identification and target identification and colocalization measurements, Channels 2-4 are dedicated for target identification and colocalization measurements. Channels 5 and 6 are used as gating channels with gating related output features only. The Colocalization BioApplication is a flexible application in which the user can measure colocalization of targets in any of the Channels 1-4 with a marker in any of these channels. The application also allows users to designate wells as "Reference Wells" and automatically set thresholds known as "LevelHigh" and "LevelLow" (levels) for several output features including colocalization coefficients. These limits allow you to automatically compute and report % responders (that is percentage of cells in any well that are above the "LevelHigh" and below the "LevelLow") for the various output features. Users can also enter these limits manually without defining "Reference Wells". All of this is described in greater detail in the next chapters.

The Colocalization BioApplication allows users to define up to 2 regions of interest (ROI) , identified as ROI_A and ROI_B. Each ROI is user defined and is based on selected objects in any of channels 1-4. Quantitative measurements relevant for colocalization are made on "targets" within or across the ROIs. The ROIs can be any cellular organelle or area that is visualized using a fluorescent marker. Some examples of ROIs include nuclear region, entire cytoplasmic region, whole cell, cell membrane region, organelles such as mitochondria, Golgi or ER. User's can also remove certain regions of cells from their ROIs, such as removing the nuclear region from a whole cell stain to give a true cytoplasmic ROI. For each ROI the user can assign up to 2 targets (identified as ROI_A_Target _I, ROI_A_Target_II, ROI_B_Target_I and ROI_B_Target_II). With this setup, the user also has the freedom to identify two completely different sets of targets stained with the same fluorescent stain, by simply duplicating the channel settings (filter settings) and setting different sets of object identification and selection/ rejection criteria in each of the two duplicated channels. The application measures changes in intensity and area of validated objects within each ROI in each of the target channels. More importantly, the application also measures and reports output features such as correlation coefficients and overlap coefficients, based on intensity or area of "Targets" within each ROI, that are relevant to studying colocalization of biological targets (1-4). For example, you can define an entire cell (visualized by a whole cell stain) as ROI_A (see Figure

8 in Chapter 2 for schematic of a ROI), define ROI_A_Target_I as mitochondrial staining (using a mitochondrial stain such as MitoTracker Orange) in the cytoplasm and define a mitochondrial resident protein such as cytochrome-c (visualized using a combination of anti-cytochrome-c and fluorescent secondary antibodies) as ROI_A_Target_II. You can now measure the effect of various compounds that affect mitochondria or induce apoptosis on the colocalization of cytochrome-c with the mitochondrial stain. Figure 1 below shows a schematic of a ROI (panel A, entire cytoplasmic region shaded in pale orange and bounded by red mask) and ROI_A_Target_I (bright orange lines representing mitochondria) and images of ROIs and targets identified within those ROIs (panels B-D).

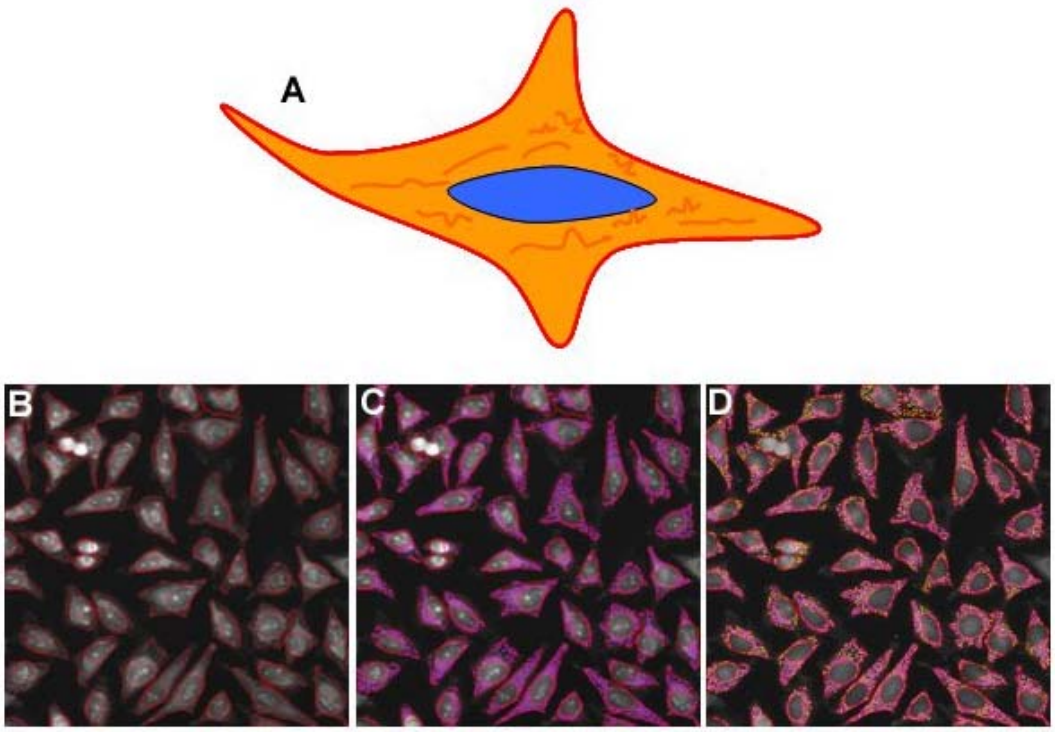


Figure 1. *Schematic showing a ROI and Target_I (panel A) and images showing various ROIs and Targets identified within those ROIs (panels B-D). The ROI in panel A is shaded in pale orange (cytoplasmic region only) and the Targets are in bright orange (mitochondria like organelles). Panel B shows ROI_A, the cytoplasmic region of each cell (identified by red mask surrounding each cell), panel C shows ROI_A_Target_I (pink masks, identifying the mitochondria) and panel D shows ROI_A_target_II (yellow masks identifying cyt-c) overlayed with ROI_A_Target_I.*

Event Definition

The Colocalization BioApplication, like several of our other BioApplications, allows simultaneous definition of up to three Events to enable rapid multiparametric analysis at the level of individual cells across multiple Cell Features. Figure 2 relays these capabilities and how they interact to enable event definition and detection. 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 features being measured. Limits are then applied (manually entered 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 (LevelHigh) or lower limit (LevelLow) defined by ***Feature*Ch*N*LevelHigh** (***Feature*Ch*N*Status =1**) or ***Feature*Ch*N*LevelLow** (***Feature*Ch*N*Status = 2**). It is critical that the limits be set appropriately for meaningful computation of Events.

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. 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.

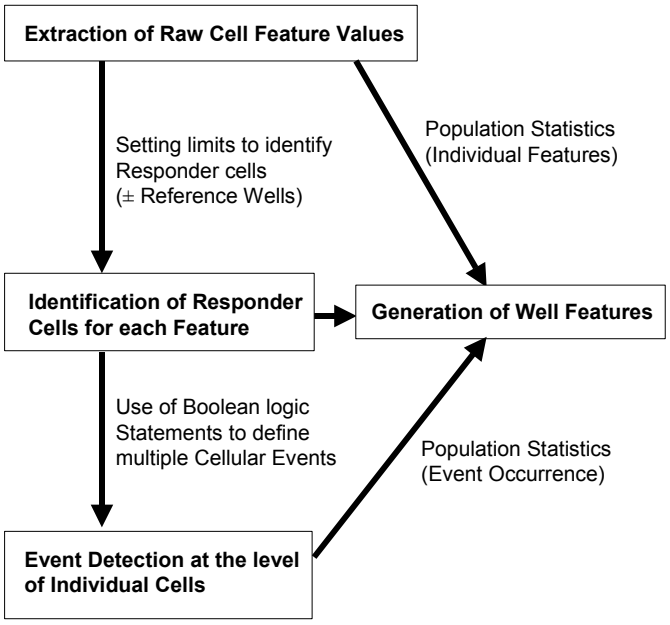


***Figure 2.**. Schematic showing the process of definition and detection of cellular events*

BioApplication Measurements

The Colocalization BioApplication measures and outputs several cell and well level features that are relevant for measuring colocalization of targets with markers. Additionally the BioApplication also measures and reports a few field level features that are useful to ascertain the general performance of the BioApplication, such as identify wells or fields that are out of focus, fields that are sparse, etc. Table 1 below lists the types of features that are computed by the Colocalization BioApplication. A more detailed description of the features are provided in later chapters of this guide.

TYPE	OBJECT	OBJECT LEVEL FEATURES	WELL LEVEL FEATURES
Cell & Well Level Features Reported for Channel 1			
Count	Cell Cluster, Cell	Object Number	Valid Object Count, Selected Object Count, %Selected Objects Valid Field Count %Selected Objects Per Valid Field
Morphology		Area, ShapeP2A, ShapeLWR	MEAN, SD, SE, CV, %HIGH, %LOW
Intensity		Total Intensity, Average Intensity, Variation of Intensity (StdDev)	MEAN, SD, SE, CV, %HIGH, %LOW
Cell& Well Level Features Reported for Channels 1-4 (Features identified as belonging to ROIs and Targets and not to a specific Channel)			
Intensity	Cell Cluster, Cell, Cellular compartment	Total Intensity, Average Intensity, Variation of Intensity (StdDev)	MEAN, SD, SE, CV, %HIGH, %LOW
Colocalization		Pearson's Correlation coefficient Mander's overlap coefficient, Overlap coefficients (k1, k2) Colocalization coefficients based on intensity (m1,m2) Colocalization coefficients based on area (a1,a2) Area Overlap	MEAN, SD, SE, CV, %HIGH, %LOW

TYPE	OBJECT	OBJECT LEVEL FEATURES	WELL LEVEL FEATURES
Translocation		Total intensity ratio between ROI_A and ROI_B Total intensity ratio log between ROI_A and ROI_B Average intensity difference between ROI_A and ROI_B Average intensity ratio between ROI_A and ROI_B Average intensity ratio log between ROI_A and ROI_B	MEAN, SD, SE, CV, %HIGH, %LOW
Cell & Well Level Features Reported for Channels 2-4			
Count	Other Cellular Compartments	Object Count	MEAN, SD, SE, CV, %HIGH, %LOW
Morphology		TotalArea	MEAN, SD, SE, CV, %HIGH, %LOW
Intensity		Total Intensity, Average Intensity, Variation of Intensity (StdDev)	MEAN, SD, SE, CV, %HIGH, %LOW
Cell & Well Level Features Reported for Channels 5-6			
Intensity	Other Cellular Compartments	Total Intensity Average Intensity	None

Table 1. *Types of features computed and reported by the Colocalization BioApplication*

Example Use Cases

Colocalization is a biological phenomenon that occurs in several biological situations. Some common examples of colocalization include internalization of a receptor bound to its ligand, association of kinases and phosphatases with their respective effectors in signaling pathways, colocalization of different subunits of an enzyme complex to make a functional enzyme complex or to regulate an enzyme's activity, colocalization of proteins that are involved in the regulation of apoptosis with the mitochondria, colocalization of transcription factors with DNA, colocalization of ubiquitins with proteins destined for degradation via the ubiquitin pathway and colocalization of focal adhesion with actin and microtubulin cytoskeletal proteins. The Colocalization BioApplication will allow for the quantitative analysis of several of these biological situations. The following examples show the use of the Colocalization BioApplication in 3 such situations.

Example 1: Changes in Colocalization of Cytochrome-c with Mitochondria

The mitochondria of cells are not only the center of energy metabolism but is also involved in other critical biological processes of the cellular system. One such process is apoptosis and its regulation. There are several proteins that are localized to the mitochondria that are involved in regulating apoptosis. One such protein is cytochrome-c (cyt-c). In normal healthy cells cyt-c is localized in the mitochondria. However, in cell undergoing apoptosis, when there are gross changes in the mitochondrial organization, cyt-c leaks out of the mitochondria and redistributes itself into the cytoplasm, prior to cell death (5, 6). In healthy cells, the mitochondria are polarized (high transmembrane potential, $\Delta\psi_m$). These polarized mitochondria can be fluorescently stained and visualized using several fluorescent stains. However, the staining intensity decreases when the cells are exposed to mitochondrial poisons that depolarize the mitochondria such as valinomycin, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), carbonyl cyanide 3-chlorophenylhydrazone (CCCP), etc.

Thus by utilizing a strategy, where we stained cells with a mitochondrial dye that stains mitochondria with high $\Delta\psi_m$, and visualizing cyt-c with a polyclonal antibody, we were able to quantify change in colocalization of cyt-c with mitochondria when cells were treated with FCCP, a compound that depolarizes mitochondria.

HeLa cells growing in 96 well micro-plates were treated with FCCP, a mitochondrial poison. Cells were then stained with MitoTracker Orange, fixed, permeabilized and stained for cyt-c using a sheep ploYclonal anti-cyt-c combined with Alexa 488 donkey-anti-sheep secondary antibody. Increasing doses of FCCP caused a dose dependent decrease in the number of mitochondria and the intensity of the mitochondrial stain.

In this example, we defined ROI_A as the entire cell visualized by the mitochondrial stain MitoTracker Orange in Channel 2. We also removed the nuclear region from ROI_A to give a true cytoplasmic region in which we measured the two targets. The targets were defined as follows: ROI_A_Target_I is the mitochondria in Channel 3 (XF93 TRITC) and ROI_A_Target_II is the cyt-c staining in Channel 4 (XF93 FITC). See Figure 3 below for a schematic of the ROI and Targets for this use case.

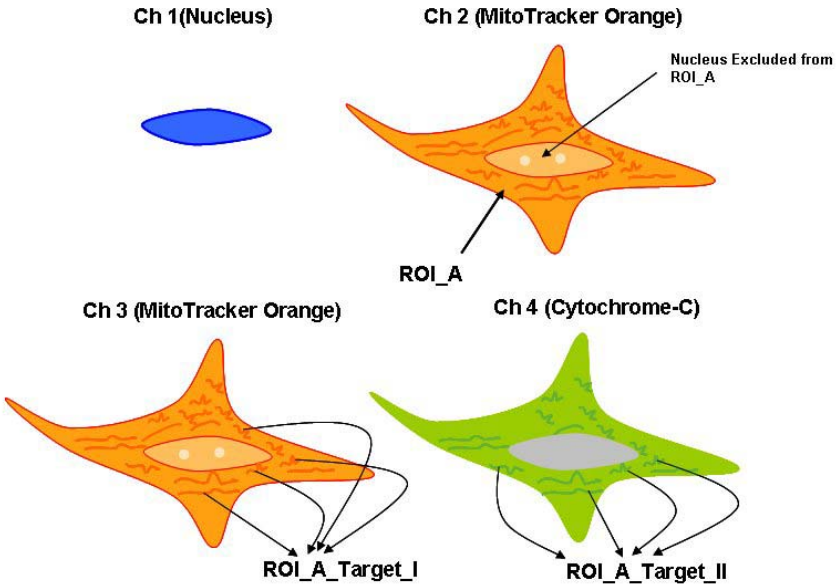


Figure 3. *Schematic of ROI_A, ROI_A_Target_I and ROI_A_Target_II for cyt-c colocalization with mitochondria for cells stained with Hoechst-33342, MitoTracker Orange and anti-cytochrome-c/Alexa 488 IgG secondary antibody. ROI_A visualized with MitoTracker Orange is the cytoplasmic region minus the nuclear region. ROI_A_Target_I are the individual mitochondria from Ch3 and ROI_A_Target_II is the cyt-c stain in the mitochondria from Ch4.*

The decrease in colocalization of cyt-c with mitochondria was captured and reported by the "MEAN_ ROI_A_CorrelationCoef". The decrease in colocalization was further quantified by other output features such as those quantifying the degree of area or intensity overlap between the two targets. Figure 4 below shows the decrease in Pearson's correlation coefficient of cyt-c to the mitochondria with increasing doses of FCCP.

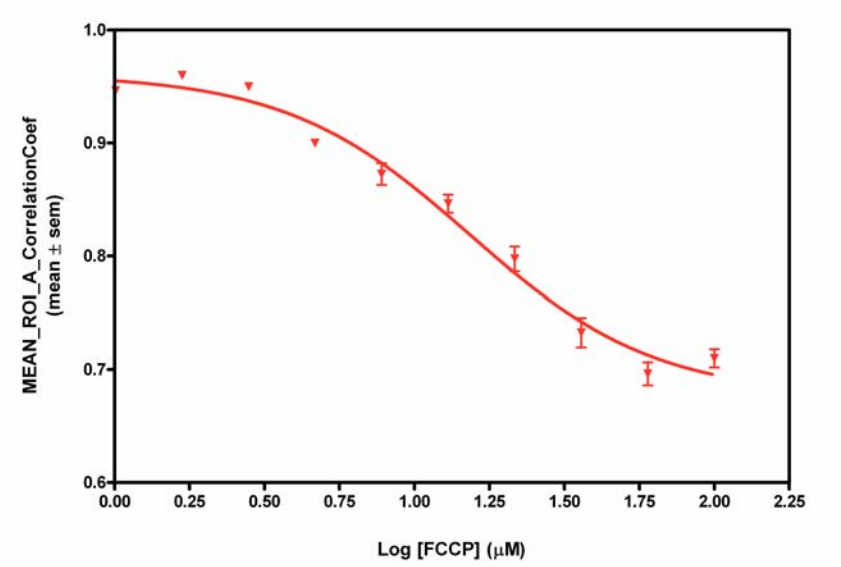


Figure 4 : *Dose response of cyt-c colocalizing with mitochondria in HeLa cells. The plot shows the decrease in the colocalization of cytochrome-c with mitochondria with increasing concentrations of FCCP.*

Example 2: Colocalization of a Transcription Factor to the Nucleus

In this use case we have looked at the activation of the NFκB and c-jun pathways following the stimulation of cells with TNF- α. The binding of TNF-α to its receptor leads to the activation of several biological signaling pathways that eventually lead to the nuclear translocation of NFκB from the cytoplasm. TNF-α also causes the activation of c-jun pathway by the phosphorylation and activation of c-jun in the nuclei of cells, leading to an elevation of phosphorylated-c-jun in the nucleus. The activation of these pathways lead to the transcription of genes that are regulated by NFκB and c-jun (7, 8). Thus there is a colocalization of the NFκB with phosporylated c-jun in the nuclear region. Using the Colocalization BioApplication we have measured the colocalization of these targets into the nuclear region.

A549 cells growing in 96 well micro-plates were incubated with TNF-α for about 25 minutes. Cells were then fixed, permeabilized and stained for NFκB and phospho-c-jun using rabbit polyclonal anti-NFκB and mouse monoclonal anti-phospho-c-jun along with fluorescent dye tagged anti-rabbit or anti-mouse secondary antibodies.

In this case, we defined ROI_A as the nuclear region, using Hoechst-33342 staining of the nucleus. We then defined ROI_A_Target_I as the fluorescent staining in Channel 2 (XF93 FITC- phospho-c-jun) and ROI_A_Target_II as the fluorescent stain in Channel 3 (XF93 TRITC-NFκB). See Figure 5 below, for a schematic of the ROI and targets for this biology. The colocalization of NFκB with phospho-c-jun in the nuclear region was captured and reported by the feature "MEAN_ROI_A_CorrelationCoef" of the Colocalization BioApplication. An increase in this correlation coefficient (see Figure 6) with increasing concentrations of TNF-α, shows the increasing colocalization (translocation) of NFκB with the phospho-c-jun in the nucleus.

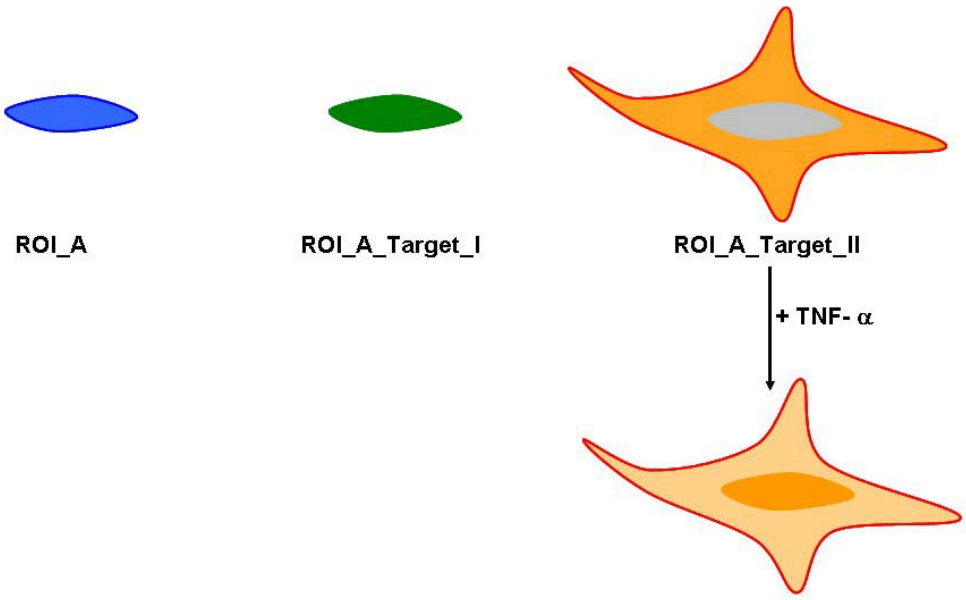


Figure 5. *Schematic showing ROI_A (blue nuclear region), ROI_A_Target_I (green) and ROI_A_Target_II (orange) as defined to measure colocalization of NFκB (orange stain) with phospho-c-jun (green stain) in the nuclear region.*

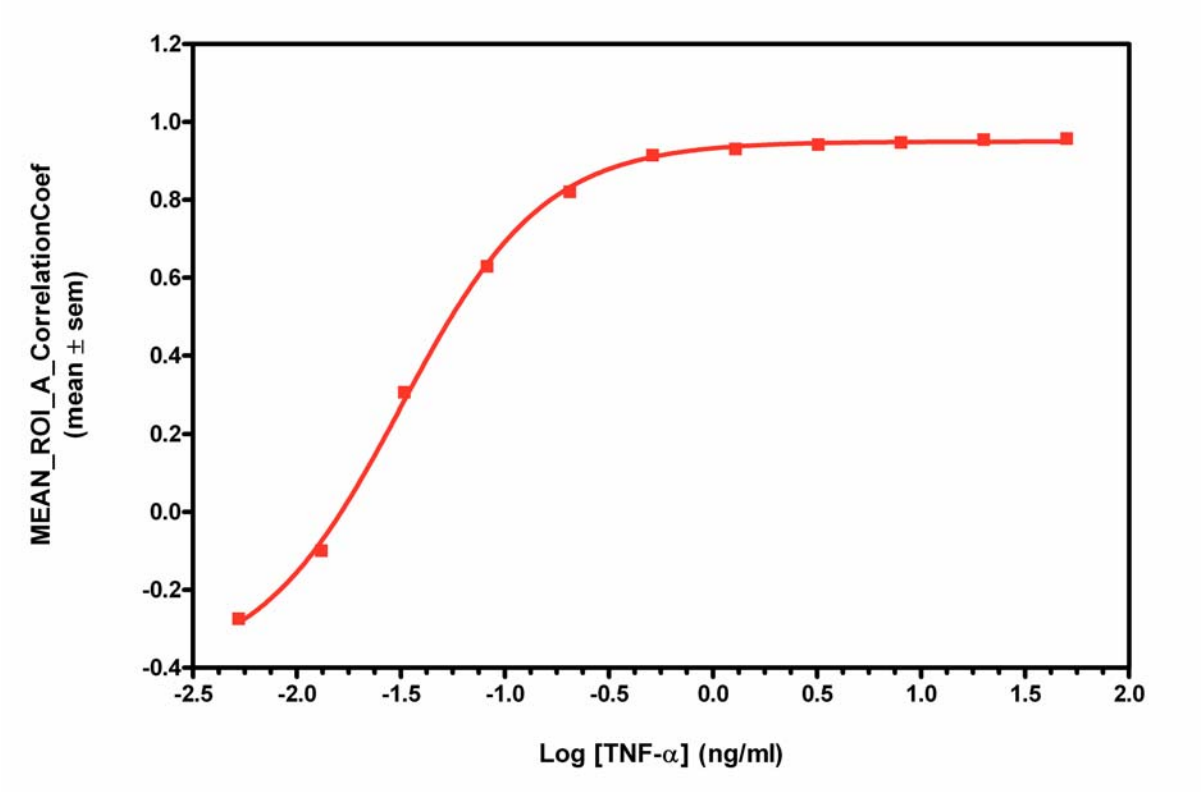


Figure 6. *Dose response of NFκB colocalizing with phospo-c-jun in nuclear region in A549 cells treated with TNF-α. The plot shows the increase in the Pearson's correlation coefficient as the NFκB translocates into the nuclear region from the cytoplasm and colocalizes with the phospho-c-jun that is already present in the nucleus.*

Example 3: Changes in Colocalization of Focal Adhesions to Microtubules, with Microtubule Disrupting Agents

The cytoskeleton of cells composed of actins and microtubules provide the structural integrity to cells. Microtubules and actin along with focal adhesion proteins are critically involved in various cellular process that require constant remodeling of cytoskeleton, such as cell division, motility and regulation of certain intracellular signaling pathways. Several drugs that are effective against cancer are disruptors of cytoskeletal integrity in cells. In these experiments we used a double antibody staining approach to visualize phospho-paxillin (p-pax) a focal adhesion protein and its colocalization with microtubules (9). In healthy cells with an intact microtubular structure, most of the p-pax is found along the periphery of the cell, where the cell attaches to the substrate. However, in cells that were treated with microtubule disruptors, the p-pax staining became more prevalent through the entire cell. This causes a change in the extent of colocalization of microtubules with p-pax., which was measured using the Colocalization BioApplication.

HeLa cells growing in 96 well micro-plates were treated with colchicine, a microtubule disruptor, for 4 hrs. Cells were then fixed, permeabilized and probed with mouse monoclonal anti-tubulin and rabbit polyclonal anti-p-paxillin. These targets were then visualized with fluorescently tagged anti-mouse and anti-rabbit secondary antibodies.

In this example we defined ROI_A to be the entire cell as stained by the anti-tubulin antibody in Channel 2 (XF93 FITC). From this we removed the nuclear region to give an ROI_A that is

truly cytoplasmic. We then identified ROI_A_Target_I, by duplicating the anti-tubulin staining in Channel 3 (XF93 FITC) and identified ROI_A_Target_II as the p-pax staining in Channel 4 (XF93 TRITC). The change in colocalization of p-pax with microtubules was captured and reported by "MEAN_ROI_A_Target_I_and_II_OverlapArea". Figure 7 below shows the decrease in the overlap area of p-pax with microtubules with increasing concentration of colchicine.

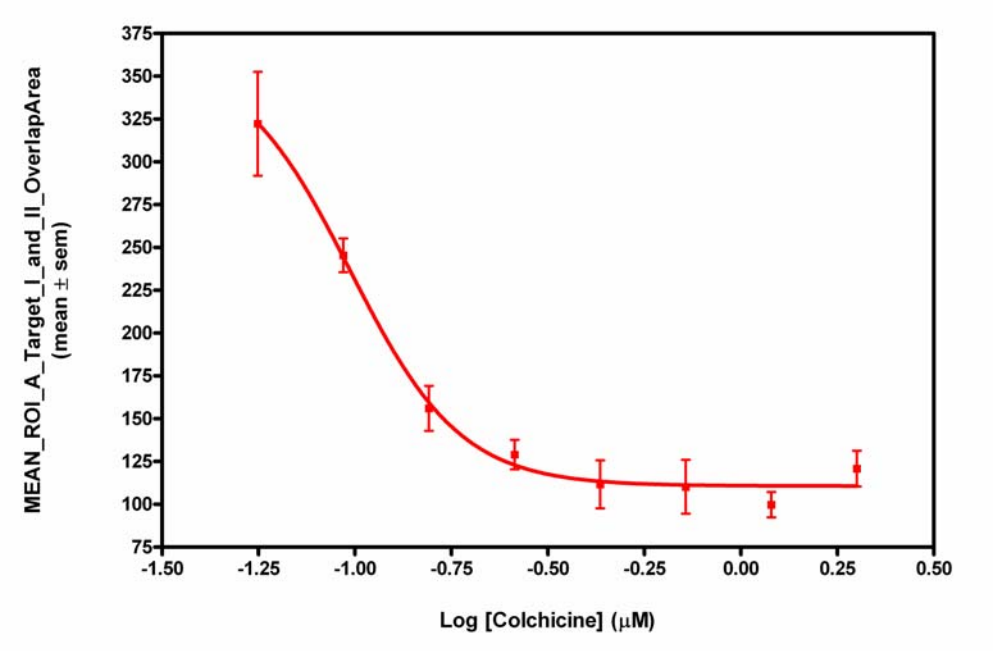


Figure 7. *Dose response of p-pax colocalization with microtubules in HeLa cells. The plot shows the decrease in the colocalizing area of microtubulin with phospho-paxillin with increasing concentrations of colchicine.*

References

1. Manders, E. M. M., J. Stap, G. J. Brakenhoff, R. Van Driel and J. A. Aten. (1992). Dynamics of three-dimensional replication patterns during the S phase, analysed by double labelling of DNA and confocal microscopy. *J. Cell Sci*. **103**, 857–862.
2. Manders, E. M. M., F. J. Verbeek and J. A. Aten. (1993). Measurement of co-localization of objects in dual-colour confocal images. *J. Micros*. **169,** 375-382.
3. Bolte, S. and F. P. Cordelieres. (2006). A guided tour into subcellular colocalization analysis in light microscopy. *J. Micros*. **224,** 213-232.
4. Zinchuk, V., O. Zinchuk and T. Okada. (2007). Quantitative colocalization analysis of multicolor confocal immunofluorescence microscopy images: Pushing pixels to explore biological phenomena. *Acta Histochem. Cytochem.* **40,** 101-111.
5. Ravagnan, L., S. Gurbuxani, S. A. Susin, C. Massie, E. Daugas, N. Zamzami, T. Mak, M. Jaattela, J. M. Penninger, C. Garrido and G. Kroemer. (2001). Heat-shock protein 70 antagonizes apoptosis-inducing factor. *Nat. Cell Biol.* **3,** 839-843.
6. Ito, Y., N. C. Mishra, K. Yoshida, S. Kharbanda, S. Saxena and D. Kufe. (2001). Mitochondrial targeting of JNK/SAPK in th ephorbol ester response of myleoid leukemia cells. *Cell Death and Differ.* **8,** 794-800.
7. Karin, M. (1994). Signal transduction from the cell surface to the nucleus through phosphorylation of transcription factors. *Curr. Opin. Cell Biol.* **6,** 415-424.
8. Mercurio, F., and A. M. Manning. (1999). Multiple signals converging on NF-κB. *Curr. Opin. Cell Biol.* **11,** 226-232.
9. Herreros, L., J. L. Rodriguez-Fernandez, M. C. Brown, J. L. Alonso-Lebrero, C. Cabanas, F. Sanchez-Madrid, N. Longo, C. E. Turner and P. Sanchez-Mateos. (2000). Paxillin localizes to the lymphocyte microtubule orgainzing center and associates with the microtubule cytoskeleton. *J. Biol. Chem.* **275,** 26436-26440.

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.

Chapter 1 provided a general overview of the Colocalization BioApplication. This chapter briefly describes how the Colocalization BioApplication works and describes its adjustable Assay Parameters and Output Features.

Overview

The Colocalization BioApplication is designed to measure the extent or degree of colocalization of targets with marker proteins or stains in various cellular regions. The extent of colocalization is measured in terms of intensity of the target with respect to the marker and area of the target with respect to the marker. The BioApplication also provides Correlation Coefficients as a measure of colocalization. The application is capable of making intensity measurements in 6 channels. However, only Channels 1-4 can be used for measuring colocalization of targets, while Channels 5 and 6 can be used as gating channels. Output features from Channels 5 and 6 are related to gating only.

Users can define up to 2 regions of interest (ROI), identified as ROI_A and ROI_B. The ROIs can be any cellular organelle or area that can be visualized using a fluorescent marker. Within each ROI the user can define up to 2 targets (identified as ROI_A_Target I, ROI_A_Target_II, ROI_B_Target_I and ROI_B_Target_II). Users can also remove certain regions of cells from their ROIs, such as removing the nuclear region from a whole cell stain to give a true cytoplasmic ROI. The creation and use of ROIs and Targets are defined in greater detail later in this chapter.

The analysis of images obtained are governed by two sets of input parameters; Assay parameters and Object Selection parameters. These input parameters are available for every Channel in the BioApplication. A combination of Assay Parameters and Object Selection Parameters with other settings such as objective type, filter set, minimum number of objects per well, provides an Assay Protocol. While Assay Parameters control the quantitative analysis of the images, the Object Selection Parameters control which objects are chosen for processing. These input parameters can be found in the Create Protocol View and Interactive View of the ArrayScan Classic software or in the Protocol Optimization task list of the iDev software. The available input parameters are dependent on the number of channels selected; only the input parameters for the selected channels will be displayed. In the ArrayScan Classic software, the available input parameters will also vary depending on the mode in which you are running: Basic Mode or Advanced Mode. Basic Mode enables you to modify only a few basic input parameters and uses default protocol settings for other parameters in the Assay Protocol, to make Colocalization measurements. Advanced Mode setting allows you to modify all input parameters that govern the Colocalization BioApplication.

Description of Assay Parameters and Settings

The algorithm has Assay Parameters that control the analysis of images, which can be adjusted to allow customization of the algorithm to your own biology and conditions. The Assay parameters can themselves be described in two distinct groups- (i) specifically designed for the Colocalization BioApplication and (ii) general assay parameters that control general aspects of image processing.

Assay Parameters for Colocalization

The following assay parameters are specifically designed for the Colocalization BioApplication, so the user can specify the Channels to be used for ROIs and Targets for the ROIs within which colocalization features will be computed. The application allows the user to define 2 ROIs (ROI_A and ROI_B), modify the size and area covered by these ROIs and two targets (Target_I and Target_II) within each ROI. The BioApplication also allows users to remove certain cellular regions from their ROIs and options for dealing with overlapping regions (if any) of the two ROIs, if the user has defined both ROI_A and ROI_B.

- ROI_A(B) * _Channel
- ROI_A(B)_CircModifer
- ROI_A(B)_RingWidth
- ROI_A(B)_RingDistance
- ROI_A(B)_Exclude
- ROI_A(B)_Overlap
- ROI_A(B)_Target_I_Channel
- ROI_A(B)_Target_II_Channel

*- These parameters are available separately for ROI_A and ROI_B. They are combined here to avoid repetition of parameter names for each ROI. Thus **ROI_A(B)_Channel** refers to 2 separate assay parameters, ROI_A_Channel and ROI_B_Channel, that are available in the BioApplication

ROI_A(B)_Channel

This assay parameter defines the Channel number (1-4) from which the ROI_A (or ROI_B) will be defined. The region of interest is defined as any region of a fluorescently stained cell or image, including subcellular regions, that can be identified using object identification methods and validated using object selection parameters. All measurements related to colocalization are made within the ROIs. If the ROI_A(B)_Channel assay parameter is set equal to 0 (e.g.,- ROI_B_Channel=0), then that ROI is not defined and no measurements related to that ROI are made. Figure 8 below, shows a schematic of two ROIs, ROI_A and ROI_B obtained from two different channel images from a cell. In the example shown below, ROI_A represents a whole cell stain and ROI_B represents a nuclear stain. The limits of the ROIs itself are shown in red color for clarity in the user's guide only.

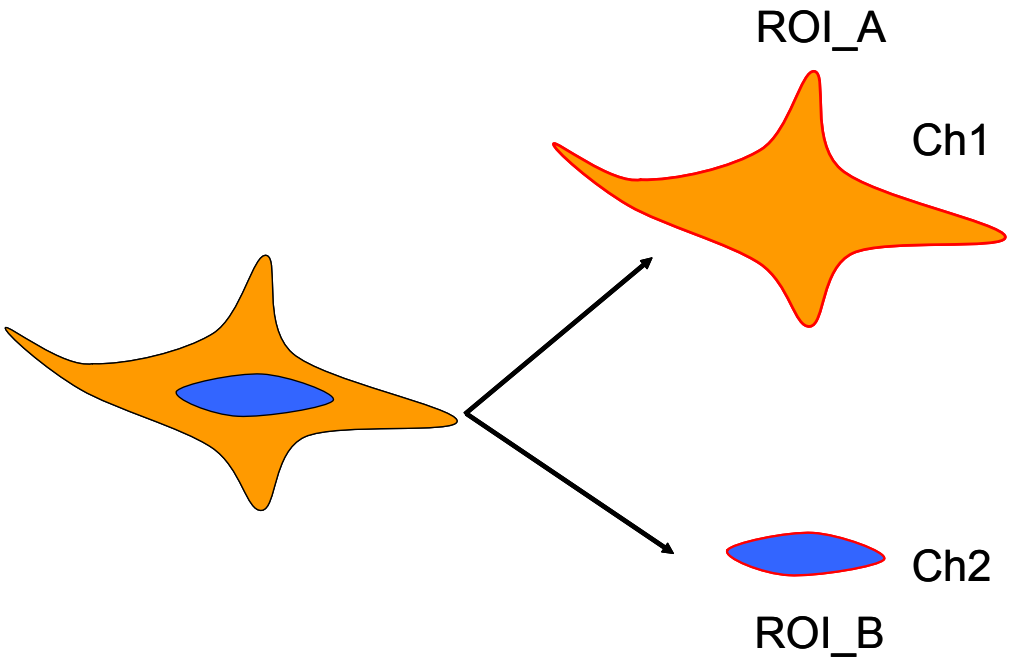


Figure 8: *Schematic representing ROI_A and ROI_B, derived from the a whole cell stain (Ch1, orange) and nuclear stain (Ch2, blue) are shown. The limits of the ROIs itself is shown with a red line around the edge of the ROIs.*

ROI_A(B)_CircModifier, ROI_A(B)_RingWidth and ROI_A(B)_RingDistance

These three assay parameters allow the user to modify the size and shape of the ROI itself. The parameter "**ROI_A_CircModifier**"allows the user to either increase (positive values) or decrease (negative values) the size of ROI_A, in its entirety, based on the object identified and validated in that Channel. Thus, in figure 9 below, a positive value increases the size of the ROI_A, while a negative value decreases the size of the ROI_A, compared to the original object that is identified and validated. The ROI_A itself is shaded in gray for clarity. In both these cases the parameters "**ROI_A_RingWidth**" and "**ROI_A_Ring Distance**" are set to "0". If "**ROI_A_CircModifier**" is set to "0" then the outer edges of ROI_A will be defined by the outer edge of the object identified and validated in that Channel. If the user wants to define an ROI_A(B) that resembles a membrane or a thin ring like section then the parameters "**ROI_A(B)_RingWidth**" and "**ROI_A(B)_Ring Distance**" should have non-zero values associated with them. In figure 10 below, the ROI_B with different "**ROI_B_RingWidth**" and "**ROI_B_Ring Distance**" are shown. In these cases, the ROI_B itself I shown as a thin ring (membrane like) and shaded in gray for clarity. In both these cases the parameter "**ROI_B_CircModifier**" is set to "0". The Assay Parameters "**ROI_A(B)_RingWidth**" and "**ROI_A(B)_Ring Distance**" take precedence over the Assay Parameter "**ROI_A_CircModifier**". Thus if non-zero values are entered for "**ROI_A(B)_RingWidth**" or "**ROI_A(B)_Ring Distance**", then a ring like ROI_A(B) is created.

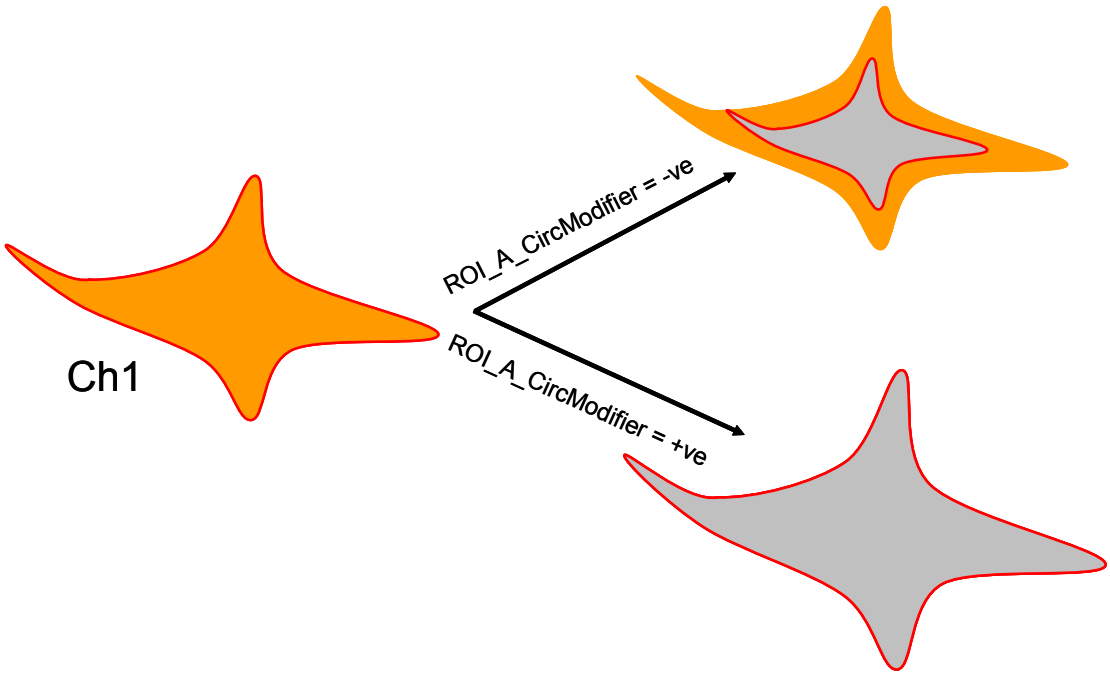


Figure 9. *Schematic representing ROI_A derived from the a whole cell stain (Ch1) and how the size of ROI_A can be changed by using a negative or positive value to the parameter "**ROI_A_CircModifier**". The ROI_A is shaded in gray for clarity.*

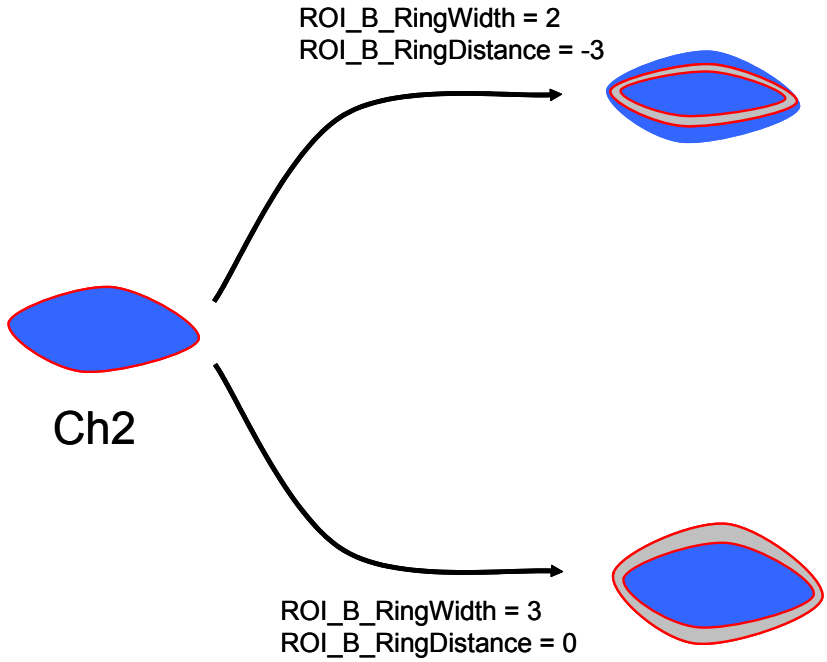


Figure 10. *Schematic representing ROI_B derived from the a nuclear stain (Ch2) and how the size of ROI_B can be modified into a membrane like ring by using appropriate values for the parameters "**ROI_B_RingWidth**" and "**ROI_B_RingDistance**". The ROI_B is shaded in gray for clarity.*

ROI_A(B)_Exclude

This parameter determines the channel from which object masks are excluded from ROI_A(B). If the value for this parameter is "0", then no region is excluded from the ROI_A(B) mask. For example if you desire your ROI_A to be cytoplasmic, excluding the nuclear region, then you can use the image from a whole cell stain to define ROI_A and then use the image from a nuclear channel to be excluded from the whole cell stain, to have an ROI_A that is just cytoplasmic. This is shown in figure 11 below, where the parameter "**ROI_A_Channel**" = 1 (orange whole cell stain) and "**ROI_A_Exclude**" = 2 (blue nuclear stain), giving an ROI_A that is truly cytoplasmic without any interference from the nuclear region.

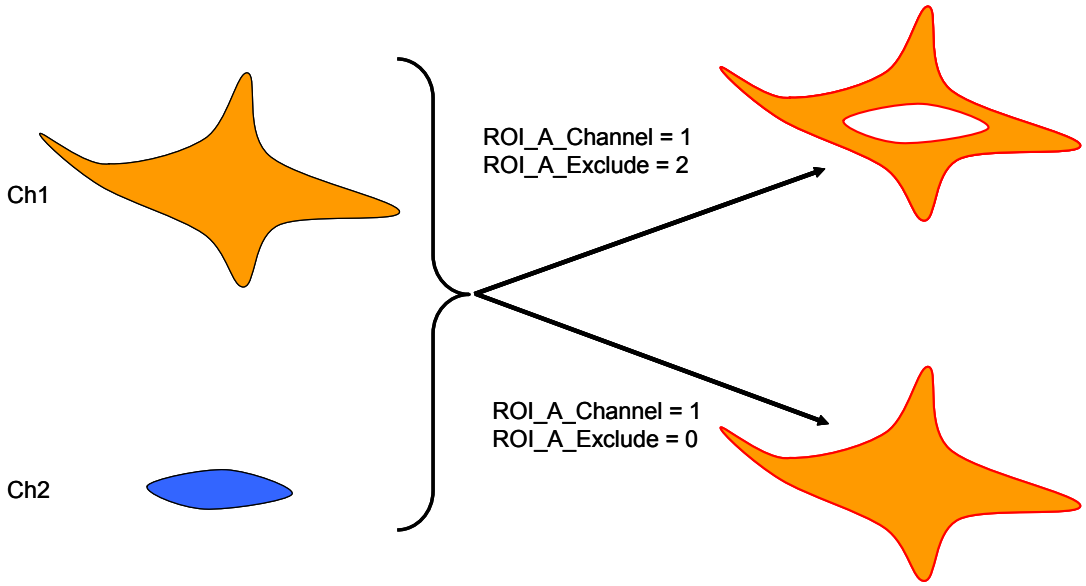


Figure 11. *Schematic representing ROI_A with nuclear region excluded (top right) and nuclear region included (bottom right). The edges of ROI_A are shown in red in the two drawings on the right.*

ROI_A(B)_Overlap

This parameter is applicable only in situations when the Channels for both ROI_A and ROI_B are defined and there is some overlap between these two ROIs. This parameter can have one of 3 values: 0, 1, or 2. If the parameter "**ROI_A_Overlap**" = 0, then any overlap of ROI_A with ROI_B is ignored; if the value is "1", then any overlap with ROI_B is removed from the final ROI_A mask and measurements for colocalization (for ROI_A targets) are made only in regions that do not include ROI_B; if the value is "2", then only the overlapping region of ROI_A with ROI_B is included in the final ROI_A mask and all colocalization measurements (for ROI_A targets) are measured only under this common region. Figure 12 below represents schematically the situations that could arise when ROI_A and ROI_B have overlapping regions.

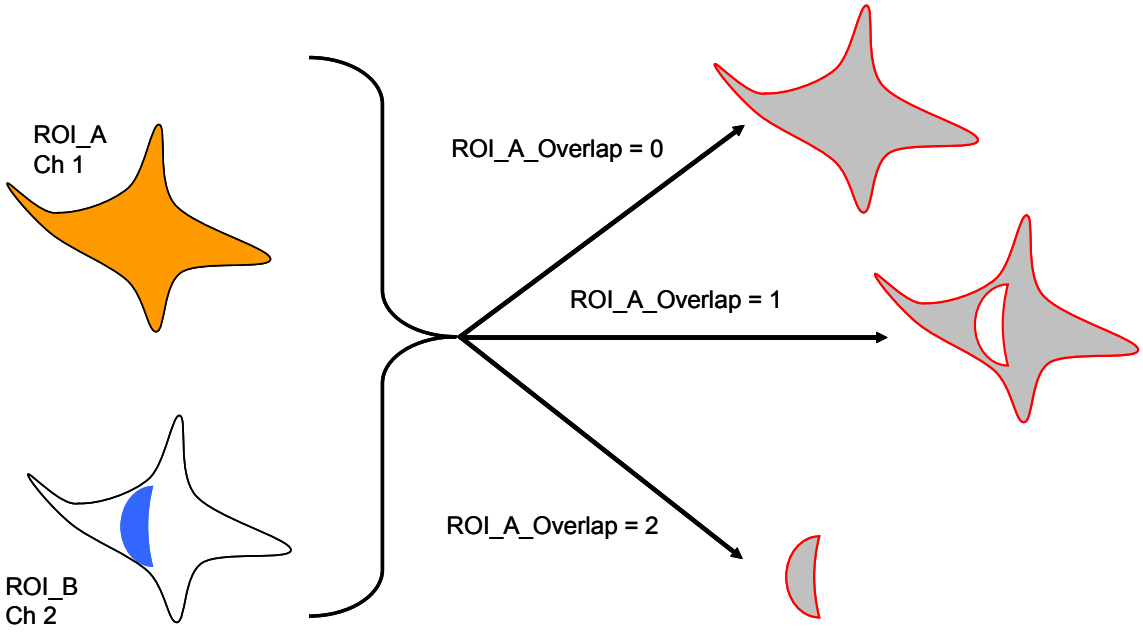


Figure 12. *Schematic representing overlapping regions of ROI_A (orange, top left) and ROI_B (blue, bottom left). The figures on the right represent the final ROI_A after overlap with ROI_B is ignored (top right) or is excluded (middle right) or is only used (bottom right) with different setting for the assay parameter "**ROI_A_Overlap**". The final ROI_A is shaded in gray color.*

ROI_A(B)_Target_I_Channel and ROI_A(B)_Target_II_Channel

This parameter defines the Channels from which identified and validated objects are defined as being "Target_I" and "Target_II" for ROI_A. Only those objects from these Channels that fall within the mask of ROI_A are validated and all measurements are made only for these objects. In many cases of measuring colocalization, "Target_I" usually refers to the fluorescently stained marker whose cellular localization is well characterized and "Target_II" refers to the biological target, whose colocalization is with the marker (Target_I) is being studied. However, the Colocalization BioApplication can also be used to study the colocalization of two biological target proteins whose cellular localization is not known. Under these circumstances, "Target_I" and "Target_II" will refer to two different targets whose cellular localization is not previously characterized. Figure 13 below shows two targets under ROI_A in HeLa cells. ROI_A_Target_I refers to the mitochondrial staining by MitoTracker Orange and ROI_A_Target_II is cytochrome-c visualized using an antibody based staining approach.

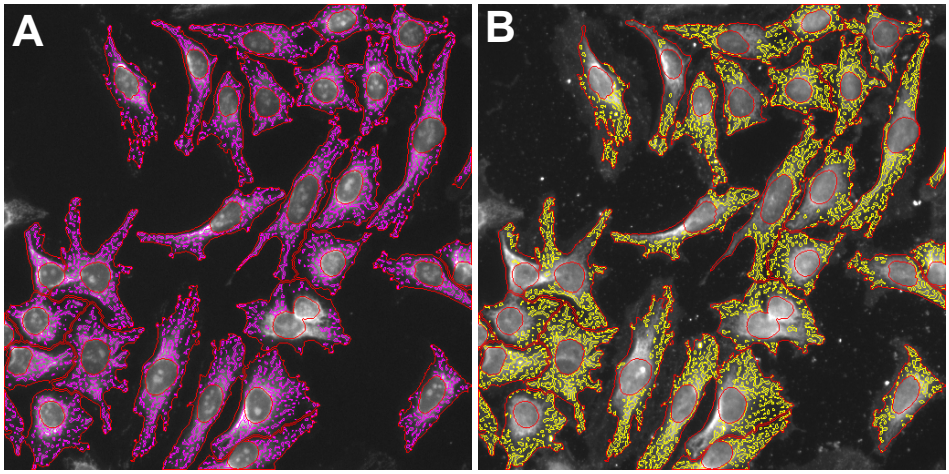


Figure 13. *ROI_A_Target_I (A) and ROI_A_Target_II (B) in HeLa cells. HeLa cells were stained with MitoTracker Orange (A) to visualize mitochondria and a combination of sheep polyclonal anti-cytochrome-c with Alexa 488 conjugated donkey-anti-sheep secondary antibody to visualize cytochrome-c. ROI_A is outlined by a red mask in each cell, Target_I is identified by a pink mask inside ROI_A and Target_II is identified by an yellow mask inside ROI_A.*

General Assay Parameters

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

- Reference Well Control
- Units for Morphological Measurements
- Object Type
- Background Correction
- Smoothing Factor
- Spot or Fiber Detection Radius
- Object Segmentation
- Rejecting Border Objects

Reference Wells Control

The two general Assay Parameters controlling the use of Reference Wells are: **UseReferenceWells** and **MinRefAvgObjectCountPerField**. The **UseReferenceWells** Assay Parameter allows the user to choose whether reference wells are to be used to determine the population characterization limits. If **UseReferenceWells** = **1**, then the Assay Parameter **MinRefAvgObjectCountPerField** must be set. This is the minimum number of objects detected per field required for acceptance of data from that field for calculation of Reference Wells. You can enter a number that closely matches the average number of selected objects needed to obtain statistically valid results for the different output features. There are additional Assay Parameters for Reference Well processing specific for particular features and channels which will be described in later sections.

Units for Morphological Measurements

The user has the option of either choosing Micrometers or Pixels as the unit to report morphological measurements. This is done by the **UseMicrometers** Assay Parameter. When set to **0**, the area of the objects are reported in pixels. Otherwise, if set to **1**, they are reported in micrometers. This information is calculated automatically from the chosen magnification and camera resolution settings. This Assay Parameter does not affect other Assay Parameters such as **ROI_A(B)_CircModifier** or **ROI_A(B)_RingWidth** or **ROI_A(B)_RingDistance** , which are always set in pixels.

Object Type

The "**ObjectTypeCh*N***" Assay Parameters allow you to specify whether the objects of interest are brighter or darker than the background of the image. This parameter is available for Channels 1-4 only. This setting allows users to combine fluorescent images with Brightfield images. The parameters can have a value of either "**0**" or "**1**". Setting the value to **0** (typical setting) lets the application consider bright objects on a dark background as potential objects, while a value of **1,** causes dark areas within an image to be considered as potential objects (Table 2).

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

Table 2. *Binary settings for NucTypeCh1 and SpotTypeCh3*

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 object 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 fluorescently labeled cell body or cytoplasm, or in images obtained with the Brightfield module on the ArrayScan V^{TI}. In this case the dimmer areas of the image are considered objects of interest to be identified and measured.

Irrespective of whether you are analyzing bright objects on a dark background or dark objects on a bright background, the strategy for object identification/detection is similar. You will always optimize the threshold value so that it separates objects from background based on intensity.

Background Correction

The Assay Parameter that accomplishes correcting background from images before they are analyzed is "**BackgroundCorrectionCh*N***". Prior to image analysis, the non-cellular background can be computed and subtracted from the image separately in each channel, if desired, as shown in figure 14, below. This parameter is available for all channels (Ch1- Ch6) and can be set independently. That is depending on the image in each channel, a separate value can be used in every channel. If the value is set to "**0**", for any channel then no background correction is applied to that channel and image analysis is done on raw uncorrected images.

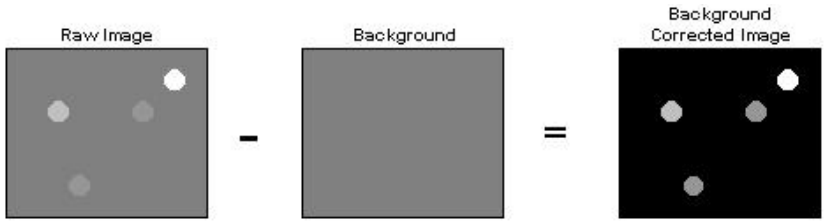


Figure 14. *Image depicting calculation of Background Correction that can be used for each channel.*

The background image is computed by suppressing high frequency components in the image (low pass filtration). You can control the creation of the background image by adjusting the **BackgroundCorrectionCh*N*** Assay Parameter. This Assay Parameter refers to the radius of the area that is sampled around each pixel in the image to determine its local background. The value of this Assay Parameter should be larger than the radii of the objects in the image.

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. These minimum values are used to construct a 3D surface of a background which is then subtracted from the original image. The main advantage of this 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. Table 3 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	Low pass filter method
Negative	3D surface fitting method

Table 3. *Possible Background Correction Methods used in each channel with the Colocalization 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

Object smoothing is used to blur or smooth the images before objects are identified. This is done sometimes to prevent a jagged object from being fragmented into multiple objects. The Assay Parameter that controls object smoothing is '**SmoothFactorCh*N***". This parameter is available in channels 1-4 only.

This parameter corresponds to the radius in pixels of the area used to smooth the image. A small value, such as **3**, means that a region with a radius of 3 pixels is used to smooth the image (region with dimensions 7×7 pixels, or 49 pixels total). Doubling the value to **6**, means that a larger region (13×13 pixels or 169 pixels total) is used to smooth the image, and thus the image will be more smoothed. A value of "**0**" means that smoothing is not done. To get sharper definition of the shapes of the edges of objects (i.e., cells), you may want to keep the **SmoothFactorCh*N*** small, if not 0. For example, if your nuclear label is not very homogeneous (see Figure 13 below), the actual object will be erroneously identified as consisting of several smaller sized objects. Then smoothing will result in a homogenizing effect of the nuclear label and will help identify the object with its true bounds.

Smoothing reduces the sharpness of intensity variations by redistributing their relative brightness over the immediate vicinity in the image, connecting fragments of nuclei which can be valuable when measuring morphological changes. Figure 15 shows the effect of smoothing on accurate identification of objects in the nuclear channel (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).

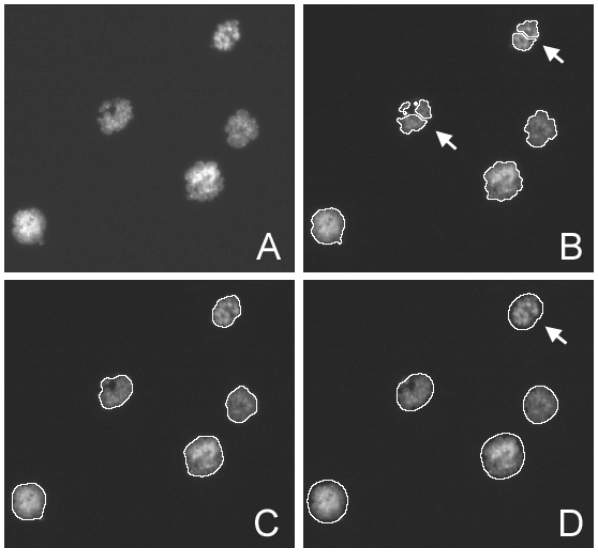


Figure 15: SmoothFactorCh1: *Images show the effect of different degrees of smoothing on the same field of apoptotic cell nuclei.* ***A:*** *Raw Image;* ***B:*** *SmoothFactorCh1 = 0;* ***C:*** *SmoothFactorCh1 = 3;* ***D:*** *SmoothFactorCh1 = 8.*

Spot or Fiber Detection Radius

The Assay Parameter that controls the detection of spots and fiber like objects is "**DetectRadiusCh*N***". This parameter is available in channels 2-4 only. Spots and fibers are usually sub-cellular entities, whose identification is based on their intensity as well as their morphology. Spots can also be distinguished from fibers by their shape (length-width ratio). Thus spot and fiber identification requires specifying the appropriate intensity threshold and morphological ranges and is a two-step process. First, the identification of cellular constituents, which may be potential spots or fibers, is done. Then the specific differentiation of spots versus fibers is done and the size range of the spots or fibers is specified to establish what are valid spots or fibers, versus intracellular noise. This parameter can have negative, positive or "0" values. If the parameter is set to "0", then spot or fiber detection is turned off. A positive or negative value determines what method of spot or fiber detection is applied to the image to identify the spots and fibers. See table 4 below for a brief description of the values and the method used to identify spots and fibers.

Setting	Detection Method
0	Spot and Fiber detection is turned off
Positive	Use morphological method to detect spots and fibers
Negative	Use local peak method to detect spots and fibers

Table 4: Spot and fiber detection methods used by the "**DetectRadiusCh*N***" in the Colocalization BioApplication.

To identify which of the retained pixels with high intensity belong to spots or fibers, the change over space within the object is evaluated. First, any remaining background intensity after thresholding is automatically removed and only discrete cellular constituents whose intensity varies rapidly over space remains, as these may be potential spots or fibers. When removing slowly varying background intensity for spot or fiber identification, the spatial dimension governing which intensity changes are considered slowly varying must be defined. This is set by the Assay Parameter **DetectRadiusCh*N*** which is related to the typical radius, or half the width, of typical spots or fibers. This Assay Parameter is used as a spatial filter to control the removal of local varying background fluorescence in the object so that individual spots can be identified; the size of this parameter is similar to the size of the spots in pixels that are detected.

For images of intracellular cytoskeletal fibers, (F-actin or microtubules or even mitochondria), setting the **DetectRadiusCh*N*** to a value of **1** or **2** gives adequate results. In cases of dim, noisy images which have poor signal to noise ratio, it has been empirically seen that larger values of **DetectRadiusCh*N*** may improve fiber detection. For spots, the size of the spot or the particular cellular entity governs the value to be set for **DetectRadiusCh*N***. For small organelles such as endosomes, a small value would suffice, whereas for larger organelles such as nuclei, the value of this parameter may have to be increased.

Object Segmentation

Usually, the Colocalization BioApplication can identify and resolve individual objects (typically whole cells or cell nuclei) fairly robustly. However, in the case of densely packed or clumped cells, or spot and fibers in cells individual objects may not be adequately resolved. The Assay Parameter "**ObjectSegmentationCh*N***" is useful in resolving these objects that are too close to each other that may not be resolved by other means. This assay parameter is available for channels 1-4 only. An additional Assay Parameter "**ObjectSegmentationCh*N*_UseCh1ObjectMasks**" is also available to segment objects in channels 2-4 only. The functioning of this parameter is described later in this section.

The Assay Parameter "**ObjectSegmentationCh*N***", can have a positive, negative or "0" value (see table 5 below). If parameter is set to "0", the segmentation is turned off in that channel (see below for exception). If the parameter has a positive value, the geometric shape method is used to segment objects, while if the value is negative, then a peak intensity method is used. When a positive value is used, the value should be the approximate radius (in pixels) of the smaller of the two objects being separated. When a negative value is used, the value should be close to the intensity range of the objects that are to be segmented. These methods are illustrated in Figures 16, 17 and 18.

Positive values for these Assay Parameters will select the geometric method, and the value is the approximate radius (in pixels) of the smaller of the objects being separated. The value of this Assay Parameter for the geometric method depends on the magnifying power of the microscope objective and camera setting (pixel binning). This 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 and pronounced indentations at their point of contact. Since the geometric method works on the basis of shape, it 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). See figure 17 for a description of the working of the geometric method.

A negative value selects the peak intensity method. In this case, the absolute value of the Assay Parameter specifies the minimum relative height of the intensity peak (image contrast) to be used in the segmentation. Making assay parameter more negative reduces the number of objects created by the segmentation. The value of this Assay Parameter for the intensity method does not depend on power of the microscope objective or camera setting (pixel binning). 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, in which case they may require object smoothing to reduce over segmentation. The peak intensity method can be used to separate objects that have no boundary indentations. See figures 16 and 19 for a description of the working of the geometric method.

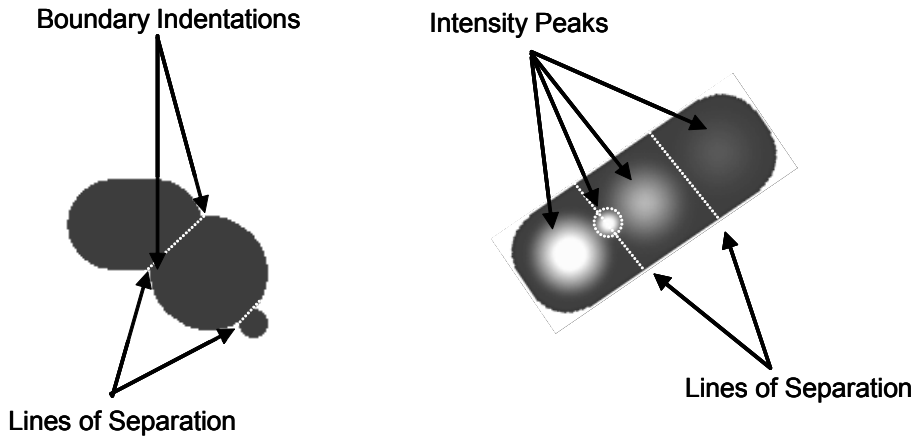


Figure 16 *Positive (geometry method, schematic on left) or Negative (intensity method, schematic on right) values for the Assay Parameter "***ObjectSegmentationCh*N***"allows different methods of segmentation to be applied to objects that are touching each other.*

Parameter Value	Segmentation Method	Value Range
0	0	0
Positive	Geometric method	1 to 255
Negative	Intensity method	-1 to -32767

Table 5: *Options and methods used for Assay Parameter "***ObjectSegmentationCh*N***".*

Geometric Method

Setting the Assay Parameter value equal to the first radius (R1, Figure 17A) produces object separation shown in figure 5B and setting the Assay parameter value = R3 results in segmentation shown in Figure 17C. Setting the Assay parameter value = R2 (the largest radius of all touching objects) produces no segmentation.

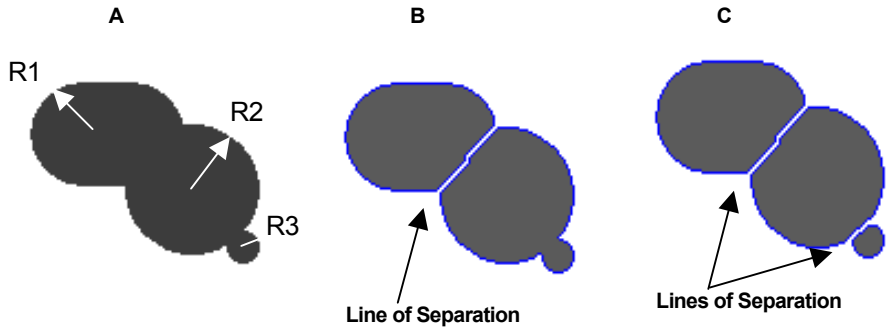


Figure 17: *Current Method for object segmentation using the Geometric Method. A) Radii of touching objects: R1 = 24, R2 = 30, R3 = 7; B) Result of segmentation using "***ObjectSegmentationCh*N** *= R1; C) Result of segmentation using "***ObjectSegmentationCh*N** *= R3.*

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 Assay Parameter value equal to the lowest relative height of intensity peaks of objects that need to be separated. The lower the value the more objects will be segmented and vice versa. In case of over segmentation of objects, setting the **SmoothFactorCh*N*** > 0 should alleviate the problem.

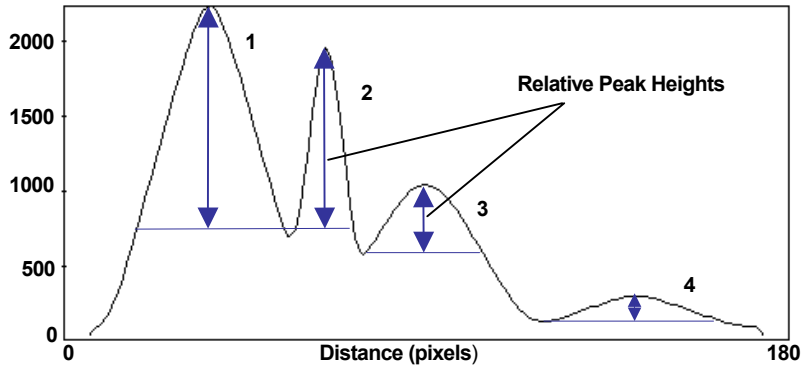


Figure 18: *Intensity profile of an object with four intensity peaks. Peak #1 relative height = 1500, peak #2 relative height = 1250, peak #3 relative height = 500 and peak #4 relative height = 200.*

Only two peaks in Figure 18: #1 and #2 have relative intensity > 1000. Thus, setting Assay Parameter to the value = -1000 makes only two peaks to be used for segmentation (or marks the two objects that need to be separated) and results in object segmentation is shown in Figure 19A; setting the Assay Parameter to the value = - 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 figures 19B and 19C.

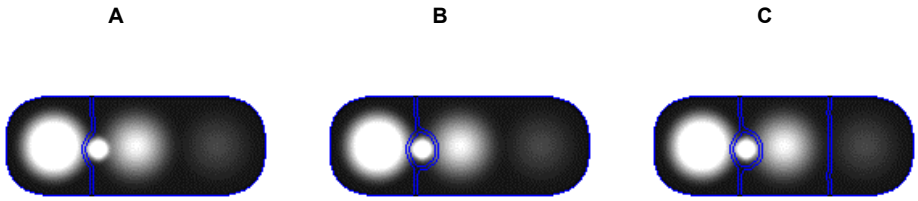
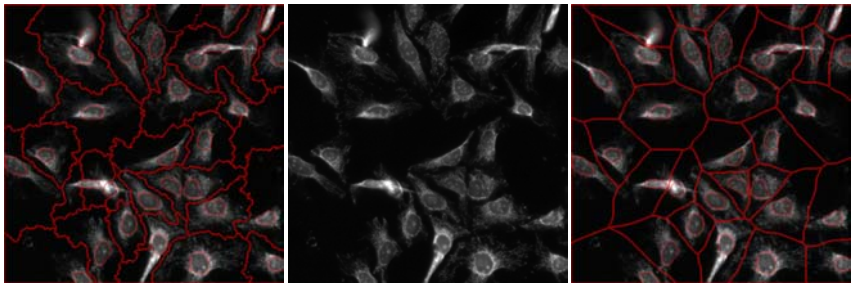


Figure 19: *Current Method for object segmentation using the Intensity Method. A) Result of object segmentation when "***ObjectSegmentationCh*N*** = *-1000; B) when "***ObjectSegmentationCh*N*** = *-400; C) when "***ObjectSegmentationCh*N*** = *-100*

A second object segmentation method available for channels 2-4 is the parameter **ObjectSegmentationCh*N*_UseCh1ObjectMasks**" . This Assay Parameter can have values of "0" or "1". If set to "0", this option is turned off and when set to "1", it is turned on and used. When used, segmentation of objects in Channel *N*, is driven by objects in Channel 1 (primary object channel). This method of segmentation is useful if the earlier described method is unsuccessful or if additional segmentation is needed. Typically, if Channel 1 images are nuclei

images, then the segmentation provided by turning this method on, results in objects in Channel *N* being segmented along Channel 1 images. Thus, this method of segmentation may not be suitable for segmenting spot or fibers or other objects that are relatively small compared to objects from Channel 1. Figure 20 below shows the segmentation of cells stained with MitoTracker Orange to stain mitochondria. It is recommended that only one method be used at a time to effect segmentation of desired objects.



Assay Parameter			
ObjectSegmentationCh2	**-350**	**0**	**0**
ObjectSegmentationCh2_ UseCh1ObjectMask	**0**	**0**	**1**

Figure 20: *Segmentation of cells as effected by different settings to the two segmentation parameters- "***ObjectSegmentationCh*N***" and "***ObjectSegmentationChN_UseCh1ObjectMask***".

Reject Border Objects

With the Colocalization BioApplication, you have the option of not including and analyzing objects that touch the border of the imaged field. This is controlled by the "**RejectBorderObjectsCh*N***" Assay Parameter. If this parameter has a value of "**1"**, objects touching the border are not analyzed. A value of " **0"** results in all objects in the image field to be eligible for analysis, whether or not they touch the image border.

Basic Assay Parameters

Input Assay Parameters can be found in the Create Protocol View. They can also be accessed from the Protocol Interactive View by clicking on the "Edit Assay Parameters" button. The number of available input parameters will vary depending on the number of Channels chosen for the Assay Protocol and also the mode in which you are using the BioApplication: Basic Mode or Advanced Mode (see the ArrayScan User's Guide for a description of switching between Basic and Advanced Mode). In the Basic Mode all of the Advanced Assay Parameters are hidden and none of the Assay Parameters can be altered by the user, irrespective of whether the protocol is write protected or not. In the Advanced Mode, all of the advanced assay parameters are hidden by default. But they can be revealed, by unchecking the "Hide Advanced Parameters" box (see the ArrayScan User's Guide for a description of this). In the Advanced Mode all of the parameter settings can be adjusted, if the Assay Protocol is not write protected. Table 6 below lists the Basic Assay Parameters for the Colocalization BioApplication.

Assay Parameter	Units	Value	Description
UseReferenceWells	Binary	0 or 1	Use Reference Wells to automatically set "LevelHigh" and "LevelLow" parameters 0= No; 1 = Yes
ROI_A(B)_Channel		0 to 4	Channel image from which ROI_A(B) mask is created
ROI_A(B)_CircModifier	Pixels	-127 to 127	Modifies ROI_A(B) mask by either increasing (positive), decreasing (negative) or use as is (0)
ROI_A(B)_RingWidth	Pixels	0 to 127	Width of ring mask created in ROI_A(B)
ROI_A(B)_RingDistance	Pixels	-127 to 127	Distance of ring mask created in ROI_A(B) from the edge of the object identified in that channel
ROI_A(B)_Exclude	Channel	0 to 4	Channel image from which object masks will be excluded from ROI_A(B)
ROI_A(B)_Overlap	Channel	0, 1 or 2	Determines how the overlapping region between ROI_A and ROI_B, if both are defined, will be used in the final ROI_A or ROI_B masks0= ignore overlap from the other ROI 1= exclude overlap region from the ROI 2= use only overlap region as ROI
ROI_A(B)_Target_I_Channel	Channel	0-4	Channel image from which Target_I for ROI_A(B) will be analyzed
ROI_A(B)_Target_II_Channel	Channel	0-4	Channel image from which Target_II for ROI_A(B) will be analyzed
ObjectTypeCh*N*	Binary	0 or 1	Determine type of object in an image 0= bright on dark background 1= dark on bright background

Assay Parameter	Units	Value	Description
BackgroundCorrectionCh*N*[*]	Pixels	-255 to 255	Radius (in pixels) of region used to compute background in ChN 0 = Do not apply background correction positive = Use low pass filter negative = Use surface fitting,
SmoothFactorCh*N*	Pixels	0-127	Degree of image smoothing (blurring) prior to object detection in ChN 0 = Do not apply smoothing
ObjectSegmentationCh*N*	Pixels	-32767 to 255	Radius (in pixels) of touching objects that should be separated 0 = Do not apply object segmentation positive = Use shape geometry method negative = Use intensity peaks method
RejectBorderObjectsCh*N*	Binary	0 or 1	Determines if objects touching the border are included or excluded from analysis 0 = include 1 = exclude
DetectRadiusCh*N*[**]	Pixels	-127 to 127	Radius in pixels of typical spots or fibers 0= do not detect spots or fibers positive = use morphological method negative = use intensity peak method
ObjectSegmentationCh*N*_UseCh1 ObjectMasks[**]	Binary	0 or 1	Determine if object boundaries from Channel 1 are used to segment objects in Channel *N* 0 = No 1= Yes
MaskModifierCh*N*[***]	Pixels	-127 to 127	Number of pixels to modify Ch1 object mask in Ch*N* 0 = Do not modify mask, positive = expand mask, negative= shrink mask
ObjectCleanUpCh1	Binary	0 or 1	Clean up object mask and remove small objects by applying erosion followed by dilation procedure 0 = No 1 = Yes

Table 6: *Basic Assay Parameters available for the Colocalization BioApplication.* [*] *Note that "ChN" refers to Channels 1-6 for BackgroundCorrection,* [**] *Channels 2-4 for DetectRadius and ObjectSegmentationCh*N*_UseCh1ObjectMasks, and* [***] *Channels 2-6 for Mask Modifier.*

Object Identification Parameters

To identify objects in each of the images from the different channels, an independent intensity threshold must be set for each channel. Object Identification methods are available for Channels 1-4. Channels 5 and 6, which are gating channels do not have any object identification methods. If you create a an assay protocol with 5 or 6 channels, then you must choose the method "**None**" as Object identification method for Channels 5 and 6. In Colocalization BioApplication, primary objects are identified in Channel 1 for further measurements; only pixels with intensities above this threshold will be considered as belonging to these objects. The proper setting of an intensity threshold is a key early step in identification and configuring the application. In Channels 2-4 also, only objects whose intensities are above the set threshold are identified and used for analysis. Thus, it is improtant to set an appropriate threshold for object identification in each of Channel 1-4. There are four different options including a "**None**" option, for determining intensity thresholds in each of Channels 1-4. For each Channel, you must select both a method and a value. The different options and values available for each channel are summarized in Table 7, and the descriptions of the different methods follow in Table 8.

Intensity Threshold Method	Value Range	Channel Availability				
		Channel 1	Channel 2	Channel 3	Channel 4	Channel 5,6
None	0					✓
Isodata	-0.99 – 9.999	✓	✓	✓	✓	
Triangle	-0.99-9.999	✓	✓	✓	✓	
Fixed	0 - 32767	✓	✓	✓	✓	

Table 7:Object Identification Methods *Intensity Threshold Methods Available for Each Channel in the Colocalization BioApplication.*

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}, ArrayScan X.5, and KineticScan HCS Readers. However, the entire range may be available on images from other sources.

Of the three intensity threshold method options, **None** means that no intensity threshold is applied. This option available only in Channels 5 and 6, as these are only gating channels. 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 two threshold methods (**Isodata**, and **Triangle**) 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 21 demonstrate how these histogram-derived threshold values are derived; and their descriptions are in Table 8.

The values entered in the application for the three 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 threshold, T_F, which is finally applied to the image is determined from the user-entered offset, 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, *o,* of 0.9 will result in a final threshold of 1900 being applied to the image, whereas entering an offset -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 be between the range 1-32767.

The two histogram-derived methods are dependent on the contents of the image, unlike the **Fixed Threshold** method. For example, for 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, it is unlikely that a **Fixed Threshold** of 1000 will cause any pixels to be registered. However, the histogram-derived threshold methods will give thresholds causing pixels in the image to be registered for potential analysis; this is because the histogram is of the pixel intensity distribution of that image, even though there are no cellular objects in the image. Thus, in situations where blank images are expected (e.g., from a loss of signal due to a compound condition, a loss of protein expression, or a lack of label), a histogram-derived threshold method should be avoided; a **Fixed Threshold** with a large offset value should be entered instead.

Threshold Option	Description	Range of Possible Values Entered	Resulting Applied Threshold Range
None	No threshold applied	0	none
Isodata	Adjusts the object identification threshold relative to the Isodata value. The threshold *T* is chosen so that it is equal to the average of the mean of the pixel intensities to the left of the threshold (m_L) and the mean of the pixel intensities to the right of the threshold (m_R). A negative value identifies dimmer objects and results in larger object masks. A positive value results in smaller object masks.	-0.999 – 9.999 (offset)	1 - 32767
Triang	Adjusts the object identification threshold relative to the Triangle value. The threshold *T* which gives the maximum distance *d* A negative value identifies dimmer objects and results in larger object masks. A positive value results in smaller object masks.	-0.999 – 9.999 (offset)	1 - 32767
Fixed	A fixed pixel intensity value between 0-32767 is applied	0 – 32767 (true intensity)	0 - 32767

Table 8. *Intensity Threshold Descriptions*

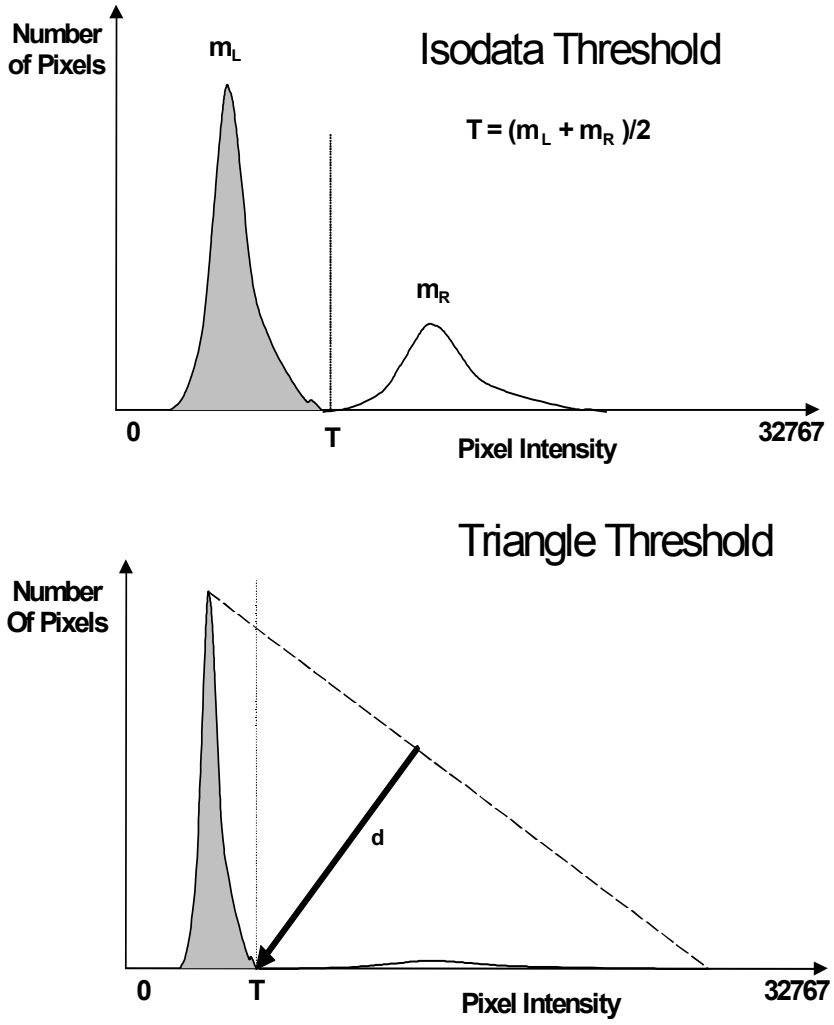


Figure 21. *Histogram-derived Intensity Threshold Methods. Shown are Isodata (top) and Triangle (bottom) Threshold methods. Background peak is shown in gray and object peak is shown in white.*

Object Selection Parameters

Each channel has an associated set of Object Selection Parameters. If an object has all measured features within the ranges specified by the Object Selection Parameters, it is selected for analysis. Rejected objects are removed from further analysis. Object Selection Parameters for all channels are adjustable only in Advanced modes. Table 8 lists all available Object Selection Parameters for this BioApplication.

Parameter	Units	Description
ObjectAreaCh*N*	Pixels or μm^2	Area (in pixels or micrometers) of an object identified in Channel *N*.
ObjectShapeP2ACh*N*	Number	Shape measure based on ratio of perimeter squared to 4π*area of objects identified in Channel *N*. (NucShapeP2ACh1=1 for circular objects).
ObjectShapeLWRCh*N*	Number	Shape measure based on ratio of length to width for object-aligned bounding box of an object identified in Channel *N*.
ObjectAvgIntenCh*N*	Intensity	Average intensity of all pixels within objects identified in Channel *N*.
ObjectVarIntenCh*N*	Intensity	Standard deviation in intensity of all pixels within objects identified in Channel *N*.
ObjectTotalIntenCh*N*	Intensity	Total intensity of all pixels within an object identified in Channel *N*.
AvgIntenCh*N**	Intensity	Average intensity of all pixels within modified Channel 1 object mask in Channel *N*
TotalIntenCh*N**	Intensity	Total intensity of all pixels within modified Channel 1 object mask in Channel *N*

Table 9: *Object Selection Parameters available for the Colocalization BioApplication.* * *The object selection parameters "*AvgIntenCh*N" and "* TotalIntenCh*N" are available only for Channels 2-6.*

Gating

The Colocalization BioApplication supports gating on a cell population. This feature provides selective cell processing based on fluorescence intensity. Therefore, in addition to selecting objects for analysis in Channel 1, you can also select or reject objects based on fluorescence intensity in Channels 2-6. Gating allows you to specifically identify a subset based on fluorescence intensity and allows you to limit the analysis to this group of cells. For example, gating may be used to analyze only those cells showing a certain level of expression of a fluorescent reporter such as green fluorescent protein (GFP).

Specifying Mask Modifiers for Gating

The assay parameter **"MaskModifierCh*N*"**is used to adjust the size of a primary object mask when it is applied to make measurements in channels 2-6. Changing this assay parameter only impacts the region used to determine whether the cell should be gated in or out, as measured by **TotalIntenCh*N*** or **AvgIntenCh*N***. The mask can be dilated or eroded, but will not overlap with other masks from adjacent objects.

Specifying Intensity Ranges for Gating

Intensity ranges for the gating parameters for channels 2-6 can be set in Create Protocol View by entering upper and lower limits for two intensity Object Selection Parameters, AvgIntenChN and TotalIntenChN. These parameters can also be adjusted interactively in Protocol Interactive View.

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 (Table 9). All Image Display Option Settings are available when running in Basic Mode or Advanced Mode.

Parameter	Description
Include This Channel In Composite	Determines if the image for this channel is included in the composite image.
Selected Object	Indicates selected objects by placing a colored ring around the object. Only selected objects are included in analysis.
Rejected Object	Indicates rejected objects by placing a colored ring around the object. Rejected objects are not included in analysis.
ROI_A	Indicates ROI_A by highlighting around ROI_A in each selected object.
ROI_B	Indicates ROI_B by highlighting around ROI_A in each selected object.
ROI_A_Target_I	Indicates Target I for ROI_A by highlighting around each selected target.
ROI_A_Target_II	Indicates Target II for ROI_A by highlighting around each selected target.
ROI_B_Target_I	Indicates Target I for ROI_B by highlighting around each selected target.
ROI_B_Target_II	Indicates Target II for ROI_B by highlighting around each selected target.

Table 10. *Image Display Option Settings available when running the Colocalization BioApplication*

Assay Parameters for Population Characterization

Overview of Population Characterization

The Colocalization BioApplication provides the ability to characterize individual cells based on their response compared to a control population. For a particular output feature, a range is determined from a control population (e.g., cells in untreated wells) that has the normal physiological distribution for that feature. The ***Feature*ChN LevelHigh** and ***Feature*ChN LevelLow** Assay Parameters set the upper and lower bounds of this range respectively. The "**Status**" Cell-level feature indicates whether a particular compound treated cell is within or beyond this range (i.e., for a particular Cell-level feature, a cell with Status **0** means that it is within the defined range, a Status value of **1** means that the feature value is greater than the upper limit, and a Status value of **2** means that the feature value is less than the lower limit; Table 11).

Value	Cell Status Definition
0	Cell Feature is less than or equal to defined Level
1	Cell Feature is greater than defined Level
2	Cell Feature is less than defined Level

Table 11. *Numerical Status values for specified Cell Features*

The corresponding Well-level features are the percent of cells that are either greater or lesser than the limits defining this range. Figure 22 illustrates this concept by showing the distribution of a normal physiological population of cells for a particular Cell-level feature, and the shift in this distribution upon compound treatment (please note that the shift in feature distribution can occur to either above or below the normal physiological distribution).

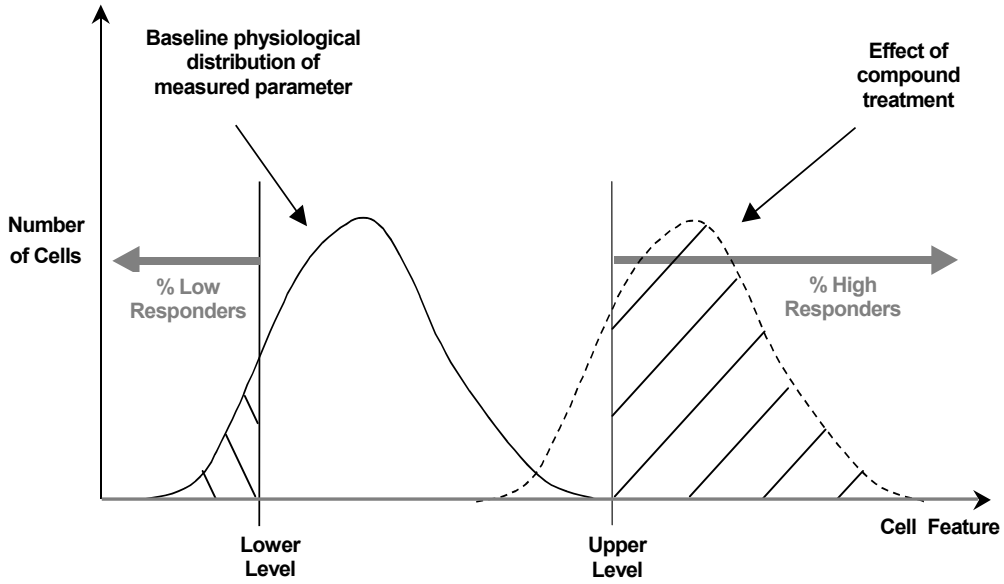


Figure 22: *Schematic of population distribution of a measured feature in a population of untreated and compound treated cells. The upper and lower levels correspond to %Low or %High (or %Responders if combined).*

Setting Cellular Response Levels

The Colocalization BioApplication offers two ways of setting high and low-response level Assay Parameters. The first is manually entering values for the ***Feature*Ch*N*LevelHigh, *Feature*Ch*N*LevelLow** Assay Parameters in the Protocol Create or Protocol Interactive Views. This requires prior knowledge of typical feature values. The BioApplication then calculates the percentage of cells in each well that are above and below the specified ***Feature*Ch*N*LevelHigh, *Feature*Ch*N*LevelLow** values, respectively.

To automatically determine the ***Feature*Ch*N*LevelHigh**, ***Feature*Ch*N*LevelLow** Assay Parameter values, the BioApplication uses Reference Wells. You designate particular wells on the sample plate as Reference Wells. Reference Wells typically contain an untreated cell population that displays the normal physiological distribution for the feature being measured. These wells are first analyzed and the population distribution for each Cell Feature that can be used for population characterization are determined. The cell population characterization

levels are then set by adding or subtracting from the mean of the distribution its standard deviation multiplied by a user defined coefficient (Correction Coefficient (**_CC**)) found as an Advanced Assay Parameter. The plate is then scanned and the BioApplication then calculates percentage of cells that are above and below these levels for each well.

For example, if you want to know the percentage of cells that, with compound treatment, have a response beyond the 95th percentile of the response from a control untreated population of cells, then the coefficient by which to multiply the standard deviation would be two, assuming a normal distribution. The advantage of using Reference Wells to automatically calculate levels is that the levels are determined by a control population of cells and are independent of run-to-run variations when doing the experiment on different days such as different illumination conditions, extent of fluorescent labeling, etc.

Reference Wells Processing Sequence

By setting the **UseReferenceWells** Assay Parameter to **1**, the Reference Wells processing is engaged. Specified fields within the Reference Wells are acquired/analyzed, and Well, and Plate Features are computed, following which regular scanning of the plate begins. Plate Feature values are directly used for cell population characterization. Again, if the feature value for **MinRefAvgCellCountPerField** obtained from the Reference Wells is below the value set for that parameter, the BioApplication aborts the use of Reference Wells and processes the plate as if **UseReferenceWells** is set to **0**. The sequence of computation for Reference Wells is as follows:

- Cell Features are computed for every valid object within a field.
- For each Cell Feature to be used for population characterization, the mean and standard deviation are computed over all cells in the field.
- The automatically calculated level values for each feature are reported as Reference Features having the names **Ref*Feature*Ch*N*LevelHigh, Ref*Feature*Ch*N*LevelLow**.
- Finally, Reference Well and Plate Features are computed as average values for fields in a well, weighted for the number of cells per field, and then as arithmetic average for wells on a plate. Use of a weighted average minimizes the effect of sparse fields.

Identifying Reference Wells and Control Parameters

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

NOTE

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

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

Reference Wells are not available for the KineticScan Reader; please use the manual level settings to define subpopulations.

Specifying and Controlling Reference Wells

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

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

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

- FeatureChNLevelHigh
- FeatureChNLevelLow (when Reference Wells are not used)

  -or-

- FeatureChNLevelHigh_CC
- FeatureChNLevelLow_CC (when Reference Wells are used)

where *Feature* refers to the name of the Cell Feature (such as **CircAvgIntenCh2**) and *N* refers to the specific channel. The ***Feature*ChN LevelHigh, *Feature*ChN LevelLow** Assay Parameter types specify the actual levels and must be manually entered if Reference Wells are not used. The ***Feature*ChN LevelHigh_CC, *Feature*ChN LevelLow_CC** Assay Parameters are correction coefficients (CC) used to derive the ***Feature*ChN LevelHigh** and ***Feature*ChN LevelLow** values from the mean and standard deviation of the reference well population according to the formula:

- FeatureChNLevelHigh = Mean + FeatureChNLevelHigh_CC $\times$ SD
- FeatureChNLevelLow = Mean - FeatureChNLevelLow_CC $\times$ SD

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

Advanced Assay Parameters

In Advanced Mode, all basic and advanced input parameters are adjustable by the user, provided the Assay Protocol is not write protected. The **Hide Advanced Parameters** option provides the ability to either view and adjust all the Assay Parameters or view and adjust the subset designated as Basic Parameters. Clear the **Hide Advanced Parameters** checkbox to display all Assay Parameters. Generally, several of the Advanced Assay Parameters are related to definition and reporting of responder cells. They control the analysis of the data resulting from the image analysis (Table 12). In addition there are 3 Assay parameters that are related to the Events definition (see next chapter for detailed description of how to set Events, using the Events Wizard).

For each feature reporting population characteristics, there are four advanced Assay Parameters that control its levels: ***Feature*Ch*N*LevelHigh** and ***Feature*Ch*N*LevelLow** that set upper and lower thresholds and the presence of the **_CC** suffix for each which designate that those levels are set using Reference Wells. For example, the Assay Parameters controlling the ROI_A_Target_I Object Count are:

- **ROI_A_Target_I_ObjectCountLevelHigh**
- **ROI_A_Target_I_ObjectCountLevelLow**
- **ROI_A_Target_I_ObjectCountLevelHigh_CC**
- **ROI_A_Target_I_ObjectCountLevelLow_CC**

In the listing of Advanced Parameters in Table 12, instead of listing all level Assay 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 above four features:

- ROI_A_Target_IObjectCountLevel***Low/High, Low/High_CC***

Parameter	Description
MinRefAvgObjectCountPerField	Minimum average number of objects per field required for acceptance of reference well results
UseMicrometers	Measure lengths and areas in 0 = Pixels and 1 = Micrometers
Pixel Size	Pixel size in micrometers (depends on objective selection and is not user adjustable)
Type_1_EventDefinition	User-defined combination of logic statements involving response feature. ***Can be changed only by using Events Wizard***
Type_2_EventDefinition	User-defined combination of logic statements involving response feature. ***Can be changed only by using Events Wizard***
Type_3_EventDefinition	User-defined combination of logic statements involving response feature. ***Can be changed only by using Events Wizard***
ROI_A_Target_I_ObjectCount***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_A_Target_I_ObjectCount***

Parameter	Description
ROI_A_Target_I_ObjectTotalArea***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_A_Target_I_ObjectTotalArea***
ROI_A_Target_I_ObjectTotalInten***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_A_Target_I_ObjectTotalInten***
ROI_A_Target_I_ObjectAvgInten***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_A_Target_I_ObjectAvgInten***
ROI_A_Target_I_ObjectVarInten***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_A_Target_I_ObjectvarInten***
ROI_A_Target_II_ObjectCount***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_A_Target_II_ObjectCount***
ROI_A_Target_II_ObjectTotalArea***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_A_Target_ II _ObjectTotalArea***
ROI_A_Target_II_ObjectTotalInten***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_A_Target_ II _ObjectTotalInten***
ROI_A_Target_II_ObjectAvgInten***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_A_Target_ II _ObjectAvgInten***
ROI_A_Target_II_ObjectVarInten***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_A_Target_ II _ObjectVarInten***
ROI_A_Target_I_and_II_OverlapArea***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_A_Target_I_and_II_OverlapArea***
ROI_A_Target_I_%OverlapArea***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_A_Target_I_%OverlapArea***
ROI_A_Target_II_%OverlapArea***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_A_Target_II_%OverlapArea***
ROI_A_CorrelationCoef***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_A_CorrelationCoef***
ROI_A_OverlapCoef***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_A_OverlapCoef***
ROI_A_OverlapCoefTarget_I_***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_A_OverlapCoefTarget_I***
ROI_A_OverlapCoefTarget_II_***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_A_OverlapCoefTarget_II***
ROI_A_ColocCoefIntenTarget_I_***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_A_ColocCoefIntenTarget_I***
ROI_A_ColocCoefIntenTarget_II_***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_A_ColocCoefIntenTarget_II***
ROI_A_ColocCoefAreaTarget_I_***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_A_ColocCoefAreaTarget_I***
ROI_A_ColocCoefAreaTarget_II_***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_A_ColocCoefAreaTarget_II***
ROI_B_Target_I_ObjectCount***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_B_Target_I_ObjectCount***
ROI_B_Target_I_ObjectTotalArea***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_B_Target_I_ObjectTotalArea***

Parameter	Description
ROI_B_Target_I_ObjectTotalInten***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_B_Target_I_ObjectTotalinten***
ROI_B_Target_I_ObjectAvgInten***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_B_Target_I_ObjectAvgInten***
ROI_B_Target_I_ObjectVarInten***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_B_Target_I_ObjectVarInten***
ROI_B_Target_II_ObjectCount***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_B_Target_II_ObjectCount***
ROI_B_Target_II_ObjectTotalArea***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_B_Target_II _ObjectTotalArea***
ROI_B_Target_II_ObjectTotalInten***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI ROI_B_Target_II _ObjectTotalInten***
ROI_B_Target_II_ObjectAvgInten***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_B_Target_II _ObjectAvgInten***
ROI_B_Target_II_ObjectVarInten***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_B_Target_II _ObjectVarInten***
ROI_B_Target_I_and_II_OverlapArea***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_B_Target_I_and_II_OverlapArea***
ROI_B_Target_I_%OverlapArea***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_ B _Target_I_%OverlapArea***
ROI_B_Target_II_%OverlapAreav***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_ B _Target_II_%OverlapArea***
ROI_B_CorrelationCoef***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_ B _CorrelationCoeff***
ROI_B_OverlapCoef***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_ B _OverlapCoef***
ROI_B_OverlapCoefTarget_I_***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_ B _OverlapCoefTarget_I***
ROI_B_OverlapCoefTarget_II_***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_ B _OverlapCoefTarget_II***
ROI_B_ColocCoefIntenTarget_I_***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_ B _ColocCoefIntenTarget_I***
ROI_B_ColocCoefIntenTarget_II_***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_ B _ColocCoefIntenTarget_II***
ROI_B_ColocCoefAreaTarget_I_***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_ B _ColocCoefAreaTarget_I***
ROI_B_ColocCoefAreaTarget_II_***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_ B _ColocCoefAreaTarget_II***
ROI_ AB _Target_I_TotalIntenRatio***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_ AB _Target_I_TotalIntenRatio***
ROI_AB_Target_I_TotalIntenRatioLog***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_ AB _Target_I_TotalIntenRatioLog***
ROI_AB_Target_I_AvgIntenDiff***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_ AB _Target_I_AvgIntenDiff***

Parameter	Description
ROI_AB_Target_I_AvgIntenRatio***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_ AB _Target_I_AvgIntenRatio***
ROI_AB_Target_I_AvgIntenRatioLog***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_ AB _Target_I_AvgIntenRatioLog***
ROI_AB_Target_II_TotalIntenRatio***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_ AB _Target_II_TotalIntenRatio***
ROI_AB_Target_II_TotalIntenRatioLog***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_ AB _Target_II_TotalIntenRatioLog***
ROI_AB_Target_II_AvgIntenDiff***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_ AB _Target_II_AvgIntenDiff***
ROI_AB_Target_II_AvgIntenRatio***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_ AB _Target_II_AvgIntenRatio***
ROI_AB_Target_II_AvgIntenRatioLog***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ROI_ AB _Target_II_AvgIntenRatioLog***
ObjectAreaCh1***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ObjectAreaCh1***
ObjectShapeP2ACh1***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ObjectShapeP2ACh1***
ObjectShapeLWRCh1***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ObjectShapeLWRCh1***
ObjectTotalIntenCh1***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ObjectTotalIntenCh1***
ObjectAvgIntenCh1***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ObjectAvgIntenCh1***
ObjectVarIntenCh1***Level/High, Low/High_CC***	Defines population characterization levels for the feature ***ObjectVarIntenCh1***
ObjectCountCh***NLevel/High, Low/High_CC***	Defines population characterization levels for the feature **ObjectCountCh*N***
ObjectTotalAreaCh***NLevel/High, Low/High_CC***	Defines population characterization levels for the feature **ObjectTotalAreaCh*N***
ObjectTotalIntenCh***N***L***Level/High, Low/High_CC***	Defines population characterization levels for the feature **ObjectTotalIntenCh*N***
ObjectAvgIntenCh***NLevel/High, Low/High_CC***	Defines population characterization levels for the feature **ObjectAvgIntenCh*N***
ObjectVarIntenCh***NLevel/High, Low/High_CC***	Defines population characterization levels for the feature **ObjectVarIntenCh*N***

Table 12. *Advanced Assay Parameters available for the Colocalization BioApplication.*

Description of Output Features

Output features are the biological measurements produced by BioApplication. All features are categorized and accessible using the vHCS:View software application. Additionally, a subset of features, the Well Features, is listed in the Scan Plate View and Create Protocol View so that screening results can be viewed concurrently with scanning. The ability to view data during the scanning process allows you to immediately verify success of the run, well by well. All features can also be accessed in the Protocol Interactive View once the algorithm has been applied to the images acquired in that window, irrespective of whether the images are acquired with a plate in the instrument or running a disk based scan using images acquired earlier.

This section describes Cell Features, Field Features, Well Features, and Plate Features that are available as output features from the Colocalization BioApplication.

Colocalization Features

The Colocalization BioApplication computes and reports several output features that fall into different categories (see Table 1 in this user's guide). However, there are some key cell, well and reference output features that provide a direct measure of the degree or extent of colocalization of the targets within the ROIs (see Refs. 1-4). These output features will be described in this section.

- ROI_A(B) Correlation Coefficient
- ROI_A(B) Overlap Coefficient
- ROI_A(B) Overlap Coefficient Target_I(II)
- ROI_A(B) Intensity Colocalization Coefficient Target I(II)
- ROI_A(B) Area Colocalization Coefficient Target I(II)

Tables 13, 14 and 15 provides a list of all cell, well and reference output features respectively, along with a brief description of each of these features.

The Colocalization BioApplication computes and reports Colocalization specific output features such as correlation coefficients , intensity and area based colocalization measurements only when both Targets (Target_I and Target_II) are specified for each selected ROI (ROI_A and/or ROI_B).

ROI_A(B) Correlation Coefficient

The output feature that provides the correlation coefficient between the two targets for each ROI is called "**ROI_A(B)_CorrelationCoef**". This output feature reports the Pearson's Correlation Coefficient of Target_I and Target_II in ROI_A(B). This features describes the degree of overlap of the two targets with each other, independent of the intensity differences of the two targets. This feature can have a value from -1 to 1. This feature can have a 0 value if one of the two target channels is not defined (i.e., 0) or if the ROI itself is not defined .

$$r_p = \frac{\sum (x - \bar{x})(y - \bar{y})}{\sqrt{\sum (x - \bar{x})^2 \sum (y - \bar{y})^2}}$$

where r_p is the Pearson's correlation coefficient, *"x"* and *'y"* are pixel intensities of each pixel in Target_I and Target_II respectively, and $\bar{x}$ and $\bar{y}$ are average pixel intensities of object identified as Target _I and Target _II respectively.

ROI_A(B) Overlap Coefficient

This output feature provides the overlap coefficient of the two targets under each ROI and is called "**ROI_A(B)_OverlapCoef**". This feature is the Mander's coefficient of colocalization, where the average pixel intensity of each object identified as Target_I or Target_II is ignored from the calculation. This feature can have values from 0 to 1. This feature is also not computed if either of the two target channels or the ROI itself is not defined.

$$r = \frac{\sum xy}{\sqrt{\sum x^2 \sum y^2}}$$

where r is the Mander's Coefficient, *x* and *y* are the pixel intensities of Target_I and Target_II respectively.

ROI_A(B) Overlap Coefficient Target_I(II)

This feature provides information about the degree of intensity overlap of the two targets with respect to each target. The features are called "**ROI_A(B)_OverlapCoef_Target_I**" and "**ROI_A(B)_OverlapCoef_Target_II**", which report the extent of colocalization with respect to Target I (k_1) and Target II (k_2) respectively.

$$k_1 = \frac{\sum xy}{\sum x^2} \qquad k_2 = \frac{\sum xy}{\sum y^2}$$

where *x* and *y* are pixel intensities of Target_I and Target_II respectively.

ROI_A(B)_Intensity Colocalization Coefficient Target_I(II)

This feature provides information about the contribution of each target's intensity to the colocalized intensity in each ROI. The features that report this are called "**ROI_A(B)_ColocCoefInten_Target_I**" and "**ROI_A(B)_ColocCoefInten_Target_II**" respectively for contribution from Target _I (m_1) and Target _II (m_2) respectively.

$$m_1 = \frac{\sum x_{coloc}}{\sum x} \qquad m_2 = \frac{\sum y_{coloc}}{\sum y}$$

where x_{coloc} and y_{coloc} are the colocalized pixel intensities of Target_I and Target_II respectively and *x* and *y* are the pixel intensities of these targets.

ROI_A(B) Area Colocalization Coefficient Target I(II)

This feature provides information on the contribution of each target's area to the colocalized area of the two targets within each ROI. The features that report this are called "**ROI_A(B)_ColocCoefArea_Target_I**" and "**ROI_A(B)_ColocCoefArea_Target_II**" respectively for contribution from Target_I (a_1) and Target _II (a_2) respectively.

$$a_1 = \frac{a_{coloc}}{A_x} \qquad a_2 = \frac{a_{coloc}}{A_y}$$

where a_{aoloc} is the area of colocalized pixels of Target _I and Target _II and A_x and A_y are the total area of Target_I and Target_II respectively.

Cell Features

Table 13 shows the output features reported for each selected cell, accessible in the Cell Feature window in Protocol Interactive View in addition to the vHCS: View software application. In the table below Cell Features that are a measure of the colocalization (intensity or area based) are shaded in gray.

Feature	Unit	Description
Cell#	Number	Unique primary object ID
Top	Pixels	Y coordinate (in pixels) of top-left corner of image-aligned bounding box of primary object
Left	Pixels	X coordinate (in pixels) of top-left corner of image-aligned bounding box of primary object
Width	Pixels	Width (in pixels) of image-aligned bounding box of primary object
Height	Pixels	Height (in pixels) of image-aligned bounding box of primary object
XCentroid	Pixels	X coordinate (in pixels) of center of primary object
YCentroid	Pixels	Y coordinate (in pixels) of center of primary object
EventTypeProfile	Number	Identifies the types of events that occurred: 1, 2, 3, 12, 23, 13, 123
EventType1Status	Binary	EventType1 status: 0 = Event did not occur, 1 = Event occurred
EventType2Status	Binary	EventType2 status: 0 = Event did not occur, 1 = Event occurred
EventType3Status	Binary	EventType3 status: 0 = Event did not occur, 1 = Event occurred
ROI_A_Target_I_ObjectCount	Number	Number of objects belonging to ROI_A_Target_I
ROI_A_Target_I_ObjectCountStatus	Number	ROI_A_Target_I_ObjectCount status: 0 = No response, 1 = High response, 2 = Low response
ROI_A_Target_I_ObjectTotalArea	Pixels or micrometers	Total area (in pixels or micrometers) of each object belonging to ROI_A_Target_I
ROI_A_Target_I_ObjectTotalAreaStatus	Number	ROI_A_Target_I_ObjectTotalArea status: 0 = No response, 1 = High response, 2 = Low response

Feature	Unit	Description
ROI_A_Target_I_ObjectTotalInten	Intensity	Total intensity of all pixels within all objects belonging to ROI_A_Target_I
ROI_A_Target_I_ObjectTotalIntenStatus	Number	ROI_A_Target_I_ObjectTotalInten status: 0 = No response, 1 = High response, 2 = Low response
ROI_A_Target_I_ObjectAvgInten	Intensity	Average intensity of all pixels within all objects belonging to ROI_A_Target_I
ROI_A_Target_I_ObjectAvgIntenStatus	Number	ROI_A_Target_I_ObjectAvgInten status: 0 = No response, 1 = High response, 2 = Low response
ROI_A_Target_I_ObjectVarInten	Intensity	Standard deviation of intensity of all pixels within all objects belonging to ROI_A_Target_I
ROI_A_Target_I_ObjectVarIntenStatus	Number	ROI_A_Target_I_ObjectVarInten status: 0 = No response, 1 = High response, 2 = Low response
ROI_A_Target_II_ObjectCount	Number	Number of objects belonging to ROI_A_Target_II
ROI_A_Target_II_ObjectCountStatus	Number	ROI_A_Target_II_ObjectCount status: 0 = No response, 1 = High response, 2 = Low response
ROI_A_Target_II_ObjectTotalArea	Pixels or micrometers	Total area (in pixels or micrometers) of each object belonging to ROI_A_Target_II
ROI_A_Target_II_ObjectTotalAreaStatus	Number	ROI_A_Target_II_ObjectTotalArea status: 0 = No response, 1 = High response, 2 = Low response
ROI_A_Target_II_ObjectTotalInten	Intensity	Total intensity of all pixels within all objects belonging to ROI_A_Target_II
ROI_A_Target_II_ObjectTotalIntenStatus	Number	ROI_A_Target_II_ObjectTotalInten status: 0 = No response, 1 = High response, 2 = Low response
ROI_A_Target_II_ObjectAvgInten	Intensity	Average intensity of all pixels within all objects belonging to ROI_A_Target_II
ROI_A_Target_II_ObjectAvgIntenStatus	Number	ROI_A_Target_II_ObjectAvgInten status: 0 = No response, 1 = High response, 2 = Low response
ROI_A_Target_II_ObjectVarInten	Intensity	Standard deviation of intensity of all pixels within all objects belonging to ROI_A_Target_II
ROI_A_Target_II_ObjectVarIntenStatus	Number	ROI_A_Target_II_ObjectVarInten status: 0 = No response, 1 = High response, 2 = Low response
ROI_A_Target_I_and_II_OverlapArea	Number	Total area (in pixels or micrometers) of overlap belonging to ROI_A_Target_I_and_II
ROI_A_Target_I_and_II_OverlapAreaStatus	Number	ROI_A_Target_I_and_II_OverlapArea status: 0 = No response, 1 = High response, 2 = Low response
ROI_A_Target_I_%OverlapArea	Number	Percentage of overlap area relative to total area of ROI_A_Target_I
ROI_A_Target_I_%OverlapAreaStatus	Number	ROI_A_Target_I_%OverlapArea status: 0 = No response, 1 = High response, 2 = Low response
ROI_A_Target_II_%OverlapArea	Number	Percentage of overlap area relative to total area of ROI_A_Target_II
ROI_A_Target_II_%OverlapAreaStatus	Number	ROI_A_Target_II_%OverlapArea status: 0 = No response, 1 = High response, 2 = Low response
ROI_A_CorrelationCoef	Number	Pearson's correlation coefficient r_p computed between ROI_A_Target_I_and_II
ROI_A_CorrelationCoefStatus	Number	ROI_A_CorrelationCoef status: 0 = No response, 1 = High response, 2 = Low response
ROI_A_OverlapCoef	Number	Overlap coefficient (Mander's) r computed between ROI_A_Target_I_and_II

Feature	Unit	Description
ROI_A_OverlapCoefStatus	Number	ROI_A_OverlapCoef status: 0 = No response, 1 = High response, 2 = Low response
ROI_A_OverlapCoefTarget_I	Number	Overlap coefficient (k_1) quantifies the contribution of Target_I into intensities overlap between ROI_A_Target_I_and_II
ROI_A_OverlapCoefTarget_I_Status	Number	ROI_A_OverlapCoefTarget_I status: 0 = No response, 1 = High response, 2 = Low response
ROI_A_OverlapCoefTarget_II	Number	Overlap coefficient (k_2) quantifies the contribution of Target_II into intensities overlap between ROI_A_Target_I_and_II
ROI_A_OverlapCoefTarget_II_Status	Number	ROI_A_OverlapCoefTarget_II status: 0 = No response, 1 = High response, 2 = Low response
ROI_A_ColocCoefIntenTarget_I	Number	Intensity colocalization (m_1) coefficient quantifies the contribution of ROI_A_Target_I
ROI_A_ColocCoefIntenTarget_I_Status	Number	ROI_A_ColocCoefIntenTarget_I status: 0 = No response, 1 = High response, 2 = Low response
ROI_A_ColocCoefIntenTarget_II	Number	Intensity colocalization (m_2) coefficient quantifies the contribution of ROI_A_Target_II
ROI_A_ColocCoefIntenTarget_II_Status	Number	ROI_A_ColocCoefIntenTarget_II status: 0 = No response, 1 = High response, 2 = Low response
ROI_A_ColocCoefAreaTarget_I	Number	Area colocalization (a_1) coefficient quantifies the contribution of ROI_A_Target_I
ROI_A_ColocCoefAreaTarget_I_Status	Number	ROI_A_ColocCoefAreaTarget_I status: 0 = No response, 1 = High response, 2 = Low response
ROI_A_ColocCoefAreaTarget_II	Number	Area colocalization (a_2) coefficient quantifies the contribution of ROI_A_Target_II
ROI_A_ColocCoefAreaTarget_II_Status	Number	ROI_A_ColocCoefAreaTarget_II status: 0 = No response, 1 = High response, 2 = Low response
ROI_B_Target_I_ObjectCount	Number	Number of objects belonging to ROI_B_Target_I
ROI_B_Target_I_ObjectCountStatus	Number	ROI_B_Target_I_ObjectCount status: 0 = No response, 1 = High response, 2 = Low response
ROI_B_Target_I_ObjectTotalArea	Pixels or micrometers	Total area (in pixels or micrometers) of each object belonging to ROI_B_Target_I
ROI_B_Target_I_ObjectTotalAreaStatus	Number	ROI_B_Target_I_ObjectTotalArea status: 0 = No response, 1 = High response, 2 = Low response
ROI_B_Target_I_ObjectTotalInten	Intensity	Total intensity of all pixels within all objects belonging to ROI_B_Target_I
ROI_B_Target_I_ObjectTotalIntenStatus	Number	ROI_B_Target_I_ObjectTotalInten status: 0 = No response, 1 = High response, 2 = Low response
ROI_B_Target_I_ObjectAvgInten	Intensity	Average intensity of all pixels within all objects belonging to ROI_B_Target_I
ROI_B_Target_I_ObjectAvgIntenStatus	Number	ROI_B_Target_I_ObjectAvgInten status: 0 = No response, 1 = High response, 2 = Low response
ROI_B_Target_I_ObjectVarInten	Intensity	Standard deviation of intensity of all pixels within all objects belonging to ROI_B_Target_I
ROI_B_Target_I_ObjectVarIntenStatus	Number	ROI_B_Target_I_ObjectVarInten status: 0 = No response, 1 = High response, 2 = Low response
ROI_B_Target_II_ObjectCount	Number	Number of objects belonging to ROI_B_Target_II

Feature	Unit	Description
ROI_B_Target_II_ObjectCountStatus	Number	ROI_B_Target_II_ObjectCount status: 0 = No response, 1 = High response, 2 = Low response
ROI_B_Target_II_ObjectTotalArea	Pixels or micrometers	Total area (in pixels or micrometers) of each object belonging to ROI_B_Target_II
ROI_B_Target_II_ObjectTotalAreaStatus	Number	ROI_B_Target_II_ObjectTotalArea status: 0 = No response, 1 = High response, 2 = Low response
ROI_B_Target_II_ObjectTotalInten	Intensity	Total intensity of all pixels within all objects belonging to ROI_B_Target_II
ROI_B_Target_II_ObjectTotalIntenStatus	Number	ROI_B_Target_II_ObjectTotalInten status: 0 = No response, 1 = High response, 2 = Low response
ROI_B_Target_II_ObjectAvgInten	Intensity	Average intensity of all pixels within all objects belonging to ROI_B_Target_II
ROI_B_Target_II_ObjectAvgIntenStatus	Number	ROI_B_Target_II_ObjectAvgInten status: 0 = No response, 1 = High response, 2 = Low response
ROI_B_Target_II_ObjectVarInten	Intensity	Standard deviation of intensity of all pixels within all objects belonging to ROI_B_Target_II
ROI_B_Target_II_ObjectVarIntenStatus	Number	ROI_B_Target_II_ObjectVarInten status: 0 = No response, 1 = High response, 2 = Low response
ROI_B_Target_I_and_II_OverlapArea	Number	Total area (in pixels or micrometers) of overlap belonging to ROI_B_Target_I_and_II
ROI_B_Target_I_and_II_OverlapAreaStatus	Number	ROI_B_Target_I_and_II_OverlapArea status: 0 = No response, 1 = High response, 2 = Low response
ROI_B_Target_I_%OverlapArea	Number	Percentage of overlap area relative to total area of ROI_B_Target_I
ROI_B_Target_I_%OverlapAreaStatus	Number	ROI_B_Target_I_%OverlapArea status: 0 = No response, 1 = High response, 2 = Low response
ROI_B_Target_II_%OverlapArea	Number	Percentage of overlap area relative to total area of ROI_B_Target_II
ROI_B_Target_II_%OverlapAreaStatus	Number	ROI_B_Target_II_%OverlapArea status: 0 = No response, 1 = High response, 2 = Low response
ROI_B_CorrelationCoef	Number	Pearson's correlation coefficient r_p computed between ROI_B_Target_I_and_II
ROI_B_CorrelationCoefStatus	Number	ROI_B_CorrelationCoef status: 0 = No response, 1 = High response, 2 = Low response
ROI_B_OverlapCoef	Number	Overlap coefficient (Mander's) r computed between ROI_B_Target_I_and_II
ROI_B_OverlapCoefStatus	Number	ROI_B_OverlapCoef status: 0 = No response, 1 = High response, 2 = Low response
ROI_B_OverlapCoefTarget_I	Number	Overlap coefficient (k_1) quantifies the contribution of Target_I into intensities overlap between ROI_B_Target_I_and_II
ROI_B_OverlapCoefTarget_I_Status	Number	ROI_B_OverlapCoefTarget_I status: 0 = No response, 1 = High response, 2 = Low response
ROI_B_OverlapCoefTarget_II	Number	Overlap coefficient (k_2) quantifies the contribution of Target_II into intensities overlap between ROI_B_Target_I_and_II
ROI_B_OverlapCoefTarget_II_Status	Number	ROI_B_OverlapCoefTarget_II status: 0 = No response, 1 = High response, 2 = Low response

Feature	Unit	Description
ROI_B_ColocCoefIntenTarget_I	Number	Intensity colocalization (m_1) coefficient quantifies the contribution of ROI_B_Target_I
ROI_B_ColocCoefIntenTarget_I_Status	Number	ROI_B_ColocCoefIntenTarget_I status: 0 = No response, 1 = High response, 2 = Low response
ROI_B_ColocCoefIntenTarget_II	Number	Intensity colocalization (m_2) coefficient quantifies the contribution of ROI_B_Target_II
ROI_B_ColocCoefIntenTarget_II_Status	Number	ROI_B_ColocCoefIntenTarget_II status: 0 = No response, 1 = High response, 2 = Low response
ROI_B_ColocCoefAreaTarget_I	Number	Area colocalization (a_1) coefficient quantifies the contribution of ROI_B_Target_I
ROI_B_ColocCoefAreaTarget_I_Status	Number	ROI_B_ColocCoefAreaTarget_I status: 0 = No response, 1 = High response, 2 = Low response
ROI_B_ColocCoefAreaTarget_II	Number	Area colocalization (a_2) coefficient quantifies the contribution of ROI_B_Target_II
ROI_B_ColocCoefAreaTarget_II_Status	Number	ROI_B_ColocCoefAreaTarget_II status: 0 = No response, 1 = High response, 2 = Low response
ROI_AB_Target_I_TotalIntenRatio	Number	Ratio ROI_A_Target_I_ObjectTotalInten and ROI_B_Target_I_ObjectTotalInten
ROI_AB_Target_I_TotalIntenRatioStatus	Number	ROI_AB_Target_I_TotalIntenRatio status: 0 = No response, 1 = High response, 2 = Low response
ROI_AB_Target_I_TotalIntenRatioLog	Number	Log of ratio ROI_A_Target_I_ObjectTotalInten and ROI_B_Target_I_ObjectTotalInten. The Log is natural logarithm (base *e*)
ROI_AB_Target_I_TotalIntenRatioLogStatus	Number	ROI_AB_Target_I_TotalIntenRatioLog status: 0 = No response, 1 = High response, 2 = Low response
ROI_AB_Target_I_AvgIntenDiff	Intensity	Difference between ROI_A_Target_I_ObjectAvgInten and ROI_B_Target_I_ObjectAvgInten
ROI_AB_Target_I_AvgIntenDiffStatus	Number	ROI_AB_Target_I_AvgIntenDiff status: 0 = No response, 1 = High response, 2 = Low response
ROI_AB_Target_I_AvgIntenRatio	Number	Ratio ROI_A_Target_I_ObjectAvgInten and ROI_B_Target_I_ObjectAvgInten
ROI_AB_Target_I_AvgIntenRatioStatus	Number	ROI_AB_Target_I_AvgIntenRatio status: 0 = No response, 1 = High response, 2 = Low response
ROI_AB_Target_I_AvgIntenRatioLog	Number	Log of ratio ROI_A_Target_I_ObjectAvgInten and ROI_B_Target_I_ObjectAvgInten. The Log is natural logarithm (base *e*)
ROI_AB_Target_I_AvgIntenRatioLogStatus	Number	ROI_AB_Target_I_AvgIntenRatioLog status: 0 = No response, 1 = High response, 2 = Low response
ROI_AB_Target_II_TotalIntenRatio	Number	Ratio ROI_A_Target_II_ObjectTotalInten and ROI_B_Target_II_ObjectTotalInten
ROI_AB_Target_II_TotalIntenRatioStatus	Number	ROI_AB_Target_II_TotalIntenRatio status: 0 = No response, 1 = High response, 2 = Low response
ROI_AB_Target_II_TotalIntenRatioLog	Number	Log of ratio ROI_A_Target_II_ObjectTotalInten and ROI_B_Target_II_ObjectTotalInten. The Log is natural logarithm (base *e*)
ROI_AB_Target_II_TotalIntenRatioLogStatus	Number	ROI_AB_Target_II_TotalIntenRatioLog status: 0 = No response, 1 = High response, 2 = Low response
ROI_AB_Target_II_AvgIntenDiff	Intensity	Difference between ROI_A_Target_II_ObjectAvgInten and ROI_B_Target_II_ObjectAvgInten

Feature	Unit	Description
ROI_AB_Target_II_AvgIntenDiffStatus	Number	ROI_AB_Target_II_AvgIntenDiff status: 0 = No response, 1 = High response, 2 = Low response
ROI_AB_Target_II_AvgIntenRatio	Number	Ratio ROI_A_Target_II_ObjectAvgInten and ROI_B_Target_II_ObjectAvgInten
ROI_AB_Target_II_AvgIntenRatioStatus	Number	ROI_AB_Target_II_AvgIntenRatio status: 0 = No response, 1 = High response, 2 = Low response
ROI_AB_Target_II_AvgIntenRatioLog	Number	Log of ratio ROI_A_Target_II_ObjectAvgInten and ROI_B_Target_II_ObjectAvgInten. The Log is natural logarithm (base *e*)
ROI_AB_Target_II_AvgIntenRatioLogStatus	Number	ROI_AB_Target_II_AvgIntenRatioLog status: 0 = No response, 1 = High response, 2 = Low response
ObjectAreaCh1	Pixels or micrometers	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
ObjectCountCh*N*	Number	Number of all Ch*N* objects belonging to primary object
ObjectCountCh*N*Status	Number	ObjectCountCh*N* status: 0 = No response, 1 = High response, 2 = Low response
ObjectTotalAreaCh*N*	Pixels or micrometers	Total area (in pixels or micrometers) of all Ch*N* objects belonging to primary object
ObjectTotalAreaCh*N*Status	Number	ObjectTotalAreaCh*N* status: 0 = No response, 1 = High response, 2 = Low response
ObjectTotalIntenCh*N*	Intensity	Total intensity of all pixels within all Ch*N* objects belonging to primary object
ObjectTotalIntenCh*N*Status	Number	ObjectTotalIntenCh*N* status: 0 = No response, 1 = High response, 2 = Low response
ObjectAvgIntenCh*N*	Intensity	Average intensity of all pixels within all Ch*N* objects belonging to primary object
ObjectAvgIntenCh*N*Status	Number	ObjectAvgIntenCh*N* status: 0 = No response, 1 = High response, 2 = Low response

Feature	Unit	Description
ObjectVarIntenCh*N*	Intensity	Standard deviation of intensity of all pixels within all Ch*N* objects belonging to primary object
ObjectVarIntenCh*N*Status	Number	ObjectVarIntenCh*N* status: 0 = No response, 1 = High response, 2 = Low response
TotalIntenCh*N*	Intensity	Total intensity in Ch*N* of all pixels within modified Ch1 object mask
AvgIntenCh*N*	Intensity	Average intensity in Ch*N* of all pixels within modified Ch1 object mask

Table 13. *Cell Features available for the Colocalization BioApplication. * Note that "ChN" refers to Channels 2-6.*

Field Features

The Colocalization BioApplication provides two "Field Level" output features. The features provided are:

i. Valid Object Count- which is the number of valid primary objects in a field. These values are obtained from the image acquired in Channel 1.

ii. Selected Object Count- which is the number of selected primary objects in a field. This value is also obtained from the image acquired in Channel 1.

These two features are essentially provided as means of quickly assessing success of focus, or effect of compounds that are toxic to the cells or incorrect object identification. A numerical value for either one or both of these features in any field that is much different from the values seen in other fields can be a quick indicator of poor focus or near total cell loss, or artifacts being identified and selected as objects due to poor object identification thresholds or non-optimal background correction settings.

Well Features

Many Well Features are derived from the Cell Features. Such features are identified by a prefix, as listed below, to the corresponding Cell Feature name (Table 14).

Feature Prefix	Well Feature Definition	Units
MEAN_	The arithmetic mean (average) of the Cell Feature value for all selected cells in the well.	Same as Cell Feature
SD_	The standard deviation of the Cell Feature value for all selected cells in the well.	None
SE_	The standard error of the mean of the Cell Feature value for all selected cells in the well.	None
CV_	The coefficient of variation of the Cell Feature value for all selected cells in the well.	Percent
%HIGH	The percentage of cells in the well whose feature value is **above** the value specified in the FeatureChNLevelHigh Assay Parameters or as calculated by the Reference Wells (i.e., % of cells with status = 1).	Percent
%LOW	The percentage of cells in the well whose feature value is **below** the value specified in the FeatureChNLevelLow Assay Parameters or as calculated by the Reference Wells (i.e., % of cells with status = 2).	Percent

Table 14. *General Well Feature definitions for Colocalization BioApplication*

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

Well Feature	Description
ValidObjectCount	Number of valid primary objects identified in the well (Ch1 object selection parameters applied)
SelectedObjectCount	Number of valid primary objects selected for analysis in the well (Ch2-6 object selection parameters applied)
%SelectedObjects	Percentage of valid primary objects selected for analysis in the well
ValidFieldCount	Number of fields in which primary objects were selected for analysis in the well
SelectedObjectCountPerValidField	Average number of primary objects selected for analysis per valid field in the well
EventType1ObjectCount	Number of primary objects selected for analysis in the well in which EventType1 occurred
%EventType1Objects	Percentage of primary objects selected for analysis in the well in which EventType1 occurred
EventType2ObjectCount	Number of primary objects selected for analysis in the well in which EventType2 occurred
%EventType2Objects	Percentage of primary objects selected for analysis in the well in which EventType2 occurred
EventType3ObjectCount	Number of primary objects selected for analysis in the well in which EventType3 occurred
%EventType3Objects	Percentage of primary objects selected for analysis in the well in which EventType3 occurred

Table 15. *Additional Well Features available for the Colocalization BioApplication.*

Reference Features

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

Feature	Description
RefAvgObjectCountPerField	Average number of objects per field in reference wells
RefROI_A(*B*)_Target_I(*II*)_ObjectCountLevelHigh/*Low*	High/*Low* response level for ROI_A(*B*)_Target_I(*II*)_ObjectCount
RefROI_A(*B*)_Target_I(*II*)_ObjectTotalAreaLevelHigh/*Low*	High/*Low* response level for ROI_A(*B*)_Target_I(*II*)_ObjectTotalArea
RefROI_A(*B*)_Target_I(*II*)_ObjectTotalIntenLevelHigh/*Low*	High/*Low* response level for ROI_A(*B*)_Target_I(*II*)_ObjectTotalInten
RefROI_A(*B*)_Target_I(*II*)_ObjectAvgIntenLevelHigh/*Low*	High/*Low* response level for ROI_A(*B*)_Target_I(*II*)_ObjectAvgInten
RefROI_A(*B*)_Target_I(*II*)_ObjectVarIntenLevelHigh/*Low*	High/*Low* response level for ROI_A(*B*)_Target_I(*II*)_ObjectVarInten
RefROI_A(*B*)_Target_I_and_II_OverlapAreaLevelHigh/*Low*	High/*Low* response level for ROI_A(*B*)_Target_I_and_II_OverlapArea
RefROI_A(*B*)_Target_I(*II*)_%OverlapAreaLevelHigh/*Low*	High/*Low* response level for ROI_A(*B*)_Target_I(*II*)_%OverlapArea
RefROI_A(*B*)_CorrelationCoefLevelHigh/*Low*	High/*Low* response level for ROI_A(*B*)_CorrelationCoef
RefROI_A(*B*)_OverlapCoefLevelHigh/*Low*	High/*Low* response level for ROI_A(*B*)_OverlapCoef
RefROI_A(*B*)_OverlapCoefTarget_I(*II*)_LevelHigh/*Low*	High/*Low* response level for ROI_A(*B*)_OverlapCoef_Target_I(*II*)
RefROI_A(*B*)_ColocCoefIntenTarget_I (*II*)_LevelHigh/*Low*	High/*Low* response level for ROI_A(*B*)ColocCoefIntenTarget_I(*II*)
RefROI_A(*B*)_ColocCoefAreaTarget_I (*II*)_LevelHigh/*Low*	High/*Low* response level for ROI_A(*B*)_ColocCoefAreaTarget_I(*II*)
RefROI_AB_Target_I(*II*)_TotalIntenRatioLevelHigh/*Low*	High/*Low* response level for ROI_A(*B*)_Target_I(*II*)_TotalIntenRatio
RefROI_AB_Target_I(*II*)_TotalIntenRatioLogLevelHigh/*Low*	High/*Low* response level for ROI_A(*B*)_Target_I(*II*)_TotalIntenRatioLog
RefROI_AB_Target_I(*II*)_AvgIntenDiffLevelHigh/*Low*	High/*Low* response level for ROI_A(*B*)_Target_I(*II*)_AvgIntenDiff
RefROI_AB_Target_I(*II*)_AvgIntenRatioLevelHigh/*Low*	High/*Low* response level for ROI_A(*B*)_Target_I(*II*)_AvgIntenRatio
RefROI_AB_Target_I(*II*)_AvgIntenRatioLogLevelHigh/*Low*	High/*Low* response level for ROI_A(*B*)_Target_I(*II*)_AvgIntenRatioLog
RefObjectAreaCh1LevelHigh/*Low*	High/*Low* response level for ObjectAreaCh1
RefObjectShapeP2ACh1LevelHigh/*Low*	High/*Low* response level for ObjectShapeP2ACh1
RefObjectShapeLWRCh1LevelHigh/*Low*	High/*Low* response level for ObjectShapeLWRCh1
RefObjectTotalIntenCh1LevelHigh/*Low*	High/*Low* response level for ObjectTotalIntenCh1

Feature	Description
RefObjectAvgIntenCh1LevelHigh/*Low*	High/*Low* response level for ObjectAvgIntenCh1
RefObjectVarIntenCh1LevelHigh/*Low*	High/*Low* response level for ObjectVarIntenCh1
RefObjectCountCh*N*LevelHigh/*Low*	High/*Low* response level for ObjectCountCh*N*
RefObjectTotalAreaCh*N*LevelHigh/*Low*	High/*Low* response level for ObjectTotalAreaCh*N*
RefObjectTotalIntenCh*N*LevelHigh/*Low*	High/*Low* response level for ObjectTotalIntenCh*N*
RefObjectAvgIntenCh*N*LevelHigh/*Low*	High/*Low* response level for ObjectAvgIntenCh*N*
RefObjectVarIntenCh*N*LevelHigh/*Low*	High/*Low* response level for ObjectVarIntenCh*N*

Table 16. *Reference Features available for the Colocalization BioApplication. * Instead of listing all Reference Well features, corresponding features that are repeated for ROI_A and ROI_B are listed as "*ROI_A(*B*)", *those that are repeated for Target_I and Target_II are listed as "*Target_I(*II*)" *FeatureLevelHigh and FeatureLevelLow are listed as "*FeatureLevelHigh/*Low"*.

Chapter
3

Using the Colocalization BioApplication

The Colocalization BioApplication can be used in a variety of biological situations, where colocalization of targets is thought to occur with markers (references) in various cellular regions. The markers and targets can be visualized by using organic dyes that localize to specific cellular regions or organelles, primary and secondary combination antibody against both the marker protein and the target protein or even engineered cells expressing fluorescent protein biomarkers.

Irrespective of how the marker and target are visualized, the application requires that you specify one or two regions of interest (ROI_A and ROI_B) and two targets per ROI- i.e., Target_I and Target_II. You have the flexibility to choose either one of these "Targets" as your reference marker and the other as the target of interest, whose colocalization you are interested in. This chapter describes briefly the biological experimental design and assay protocol configuration used to quantify colocalization in the 3 different biological examples described in Chapter 1.

The protocols supplied with the BioApplication are example protocols that you are encouraged to modify and optimize toward your own particular biological situation.

This chapter also describes the use of the BioApplication Events Wizard to describe "Events" and quantify cellular responses. 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.

Use Case Biology Examples for Colocalization BioApplication

The Colocalization BioApplication was used to quantify colocalization of targets in the following biological situations.

Colocalization of Cytochrome-c with Mitochondria

Cytochrome-c (cyt-c) is a protein that normally resides in the mitochondria in healthy cells. However, when cells are induced to undergo apoptosis, cyt-c leaks out of the mitochondria and into the cytoplasm. The colocalization of cyt-c with mitochondria can be visualized using a mitochondrial transmembrane potential ($\Delta\psi_m$) dependent dye to stain healthy mitochondria and primary-secondary antibody combination to visualize the cyt-c.

Biological Experimental Design

For this biology we chose HeLa cells, which showed good mitochondrial staining with MitoTracker Orange in healthy cells. The cyt-c was visualized using sheep polyclonal anti-cytochrome-c combined with Alexa 488 donkey-anti-sheep IgG secondary antibody. Cells were also stained with Hoechst-33342 to visualize nuclei. Cells were treated with FCCP, a mitochondrial poison to decrease $\Delta\psi_m$, thereby decreasing mitochondrial staining with MitoTracker Orange, leading to a decrease in colocalization of cyt-c with mitochondria. A 4 channel Assay Protocol was set up with the Colocalization BioApplication with the following dye filter combinations- (i) Channel 1- Nuclei-Hoechst 33342 (XF93 Hoechst filter), (ii) Channel 2- MitoTracker Orange (XF93 TRITC filter) to identify whole cells (iii) Channel 3- MitoTracker Orange (XF93 TRITC filter) to identify individual mitochondria and (iv) Channel 4- cyt-c (XF93 FITC filter) to visualize cytochrome-c.

The duplication of the MitoTracker Orange image in Ch2 and Ch3 allows the use of a single dye to define both, an ROI and a Target.

Assay Protocol Configuration

The standard protocol provided with this BioApplication, for quantifying colocalization of mitochondrial proteins with mitochondria provides general settings for this biology. This section only provides a description of some of the protocol settings that are relevant for measuring colocalization, such as ROIs and the targets associated with the ROIs. Table 17 below lists the major assay parameter settings and the reasoning behind that setting. Figure 23, shows the dose response of the effect of FCCP on cyt-c colocalization with mitochondrial stain.

Assay Parameter	Value	Reason
ROI_A_Channel	2	Measure colocalization of cyt-c with mitochondria in entire cytoplasm. Used the MitoTracker Orange stain itself to identify the entire cells as ROI_A
ROI_A_CircModifier	0	Did not want to modify the cytoplasmic region in any way
ROI_A_RingWidth	0	No need to make any measurement outside of the cytoplasmic region or in a membrane like region
ROI_A_RingDistance	0	No need to make any measurement outside of the cytoplasmic region or in a membrane like region
ROI_A_Exclude	1	Since Ch1 was generating the nuclear mask, and measurements in ROI_A were to be truly cytoplasmic excluding the nuclear region
ROI_A_Target_I	3	Identify individual mitochondria using MitoTracker Orange stain in channel 3. Set object (mitochondria) identification and selection parameters so as to identify mitochondria
ROI_A_Target_II	4	Target is cyt-c (Channel 4) and the protocol is designed to measure the colocalization of this with the Target_I (mitochondrial marker/stain). Set object (cyt-c in mitochondria) identification and selection parameters so as to identify mitochondria for this channel also
ROI_B_Channel	0	No ROI_B was defined
SmoothFactorCh2	2	This parameter was set at “2” to better define the outer edges of the cell (mitochondrial staining)
ObjectSegmentationCh2	-400	Segment the cells (objects) based on intensity method.
SmoothFactorCh3	1	Smooth Factor was set to “1” to better identify mitochondria. A larger value would have resulted in smaller individual mitochondria being merged together.
DetectRadiusCh3	1	Detect Radius was set at “1”. A larger value would have resulted in some of the smaller mitochondria being left out unidentified.

Assay Parameter	Value	Reason
ObjectSegmentationCh3	0	No segmentation was desired with the mitochondria staining, as the values set in the previous two assay parameters were able to segment the mitochondria successfully.
SmoothFactorCh4	1	Smooth Factor was set to "1" to better identify mitochondria. A larger value would have resulted in smaller individual mitochondria being merged together.
DetectRadiusCh4	1	Detect Radius was set at "1". A larger value would have resulted in some of the smaller mitochondria being left out unidentified.
ObjectSegmentationCh4	2	Segmentation was set to "2" to better segment the cyt-c staining. The antibody used to visualize cyt-c had some cytoplasmic background, hence this value for segmentation successfully identified the cyt-c in mitochondria.

Table 17. *Settings for major Assay Parameters in the Colocalization BioApplication, used to quantify colocalization of cytochrome-c with mitochondria.*

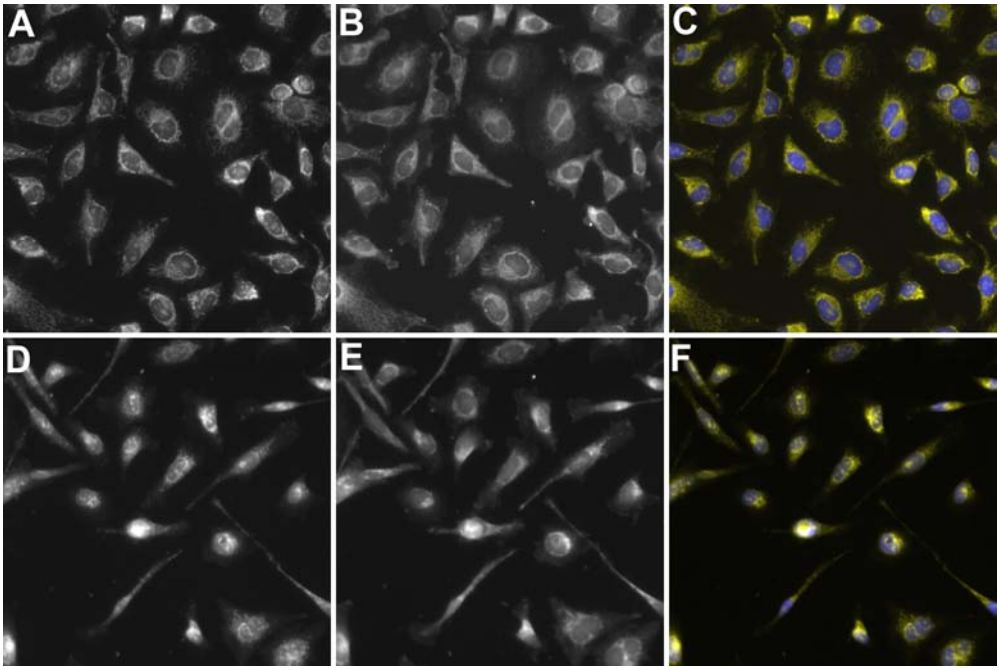


Figure 23. *Effect of FCCP, a mitochondrial poison on the colocalization cytochrome-c with mitochondria. HeLa cells were either untreated (panels A-C) or treated with 4.7 μM FCCP for about 17 hours. Cells were stained with MitoTracker orange (A, D) and anti-cytochrome C visualized with Alexa 488 donkey-anti-sheep(B, E). Panels C and F show an RGB composite of the MitoTracker Orange staining and cytochrome-c staining along with Hoechst-33342 staining (blue nuclei). The images show well defined mitochondrial staining with MitoTracker Orange in untreated cells when compared to cells treated with FCCP.*

Colocalization of Transcription Factor with the Nucleus

Translocation of a transcription factor from the cytoplasm to the nucleus- this biology can be quantified as the colocalization of a transcription factor with a nuclear stain or a nuclear marker. As an example, the translocation of NFκB and phospho-c-jun into the nucleus of HeLa cells treated with TNF-α, was quantified using the Colocalization BioApplication.

Biological Experimental Design

TNF-α treated cells were fixed and stained with a anti-phospho-c-jun which was visualized using a Dy488 goat-anti-mouse IgG secondary antibody and anti-NFkB, which was visualized using a Dy549 goat-anti-rabbit secondary. Additionally cells were also stained with Hoechst-33342 to visualize nuclei. A 3 channel assay protocol was set up with the Colocalization BioApplication with the following combination of dyes and channels- (i) Channel 1- Nuclei- Hoechst 33342 (XF93 Hoechst filter), (ii) Channel 2- c-jun- Dy488 (XF93 FITC filter) and (iii) Channel 3- NFκB- Dy549 (XF93 TRITC filter).

Assay Protocol Configuration

The standard protocol provided with this BioApplication, for quantifying colocalization of transcription factor with the nuclear region provides general settings for this biology. This section only provides a description of some of the protocol settings that are relevant for measuring colocalization, such as ROIs and the targets associated with the ROIs. Table 18 below lists the major assay parameter settings and the reasoning behind that setting. Figure 24, shows the dose response of NFκB and phospo-c-jun colocalization into the nuclear region in A549 cells that were treated with TNF-α. The assay parameters were set up in such a way that not only allows for measurement of NFκB colocalization with phospho-c-jun in the nucleus, it also allows to measure average intensity difference between the nuclear (ROI_A) and cytoplasmic region (ROI_B) for NFκB. This difference in average intensity is reported by the output feature "**ROI_AB_Target_II_AvgIntenDiff**", where "Target_II" is defined as Ch3 (NFκB) for both ROI_A and ROI_B.

Assay Parameter	Value	Reason
ROI_A_Channel	1	Measure colocalization of transcription factor with the nucleus, hence quantify changes in the nuclear region (ROI)
ROI_A_CircModifier	0	Did not want to modify the nuclear region in any way and measure all changes within this ROI
ROI_A_RingWidth	0	No need to make any measurement outside of the nuclear region
ROI_A_RingDistance	0	No need to make any measurement outside of the nuclear region
ROI_A_Target_I_Channel	2	Defining phospho-c-jun as the reference marker
ROI_A_Target_II_Channel	3	Target is NFκB (Channel 3) and the protocol is designed to measure the colocalization of this target with phospho-c-jun
ROI_B_Channel	3	By defining ROI_B as the cytoplasm in Channel 3 (NFκB), allows to measure a change in average intensity between the nucleus (ROI_A_Target_II) and cytoplasm (ROI_B_Target_II)

Assay Parameter	Value	Reason
ROI_B_CircModifier	0	Did not want to modify the nuclear region in any way and measure all changes within this ROI
ROI_B_RingWidth	0	No need to make any measurement outside of the nuclear region
ROI_B_RingDistance	0	No need to make any measurement outside of the nuclear region
ROI_B_Exclude	1	Remove the nuclear region from ROI_B
ROI_B_Target_I_Channel	2	Defining phospho-c-jun as the reference marker
ROI_B_Target_II_Channel	3	Target is NFκB (Channel 3) and the protocol is designed to measure the colocalization of this target with the phospho-c-jun

Table 18.: *Settings for major Assay Parameters in the Colocalization BioApplication, used to quantify colocalization of transcription factor in the nuclear region.*

There are other methods by which this biology can be quantified using the Colocalization BioApplication. You can redefine ROI_A, ROI_B, Target_I and Target_II for each of the ROIs to exactly allow you to measure changes/colocalization in different cellular regions. If you are only measuring the translocation of a single transcription factor, you can define ROI_A only and targets associated with ROI_A in a 2 Channel Assay Protocol.

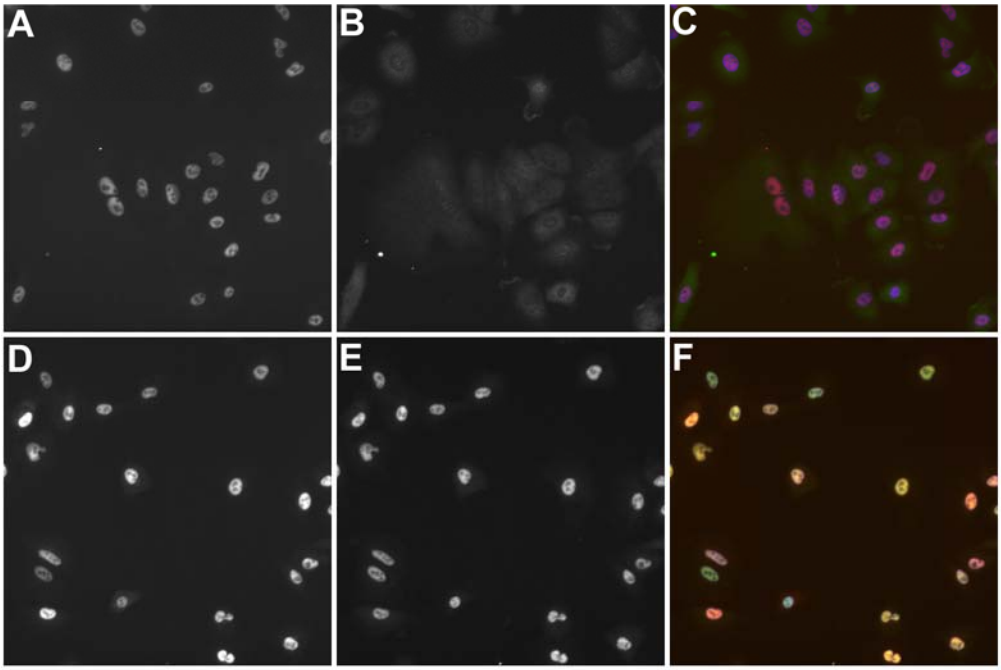


Figure 24. *Colocalization of NFκB and phospho-c-jun with nuclear region in A549 cells treated with different doses of TNF-α.. Untreated cells are shown in panels A-C and cells treated with TNF-α (100 ng/ml) are shown in panels D-F. Phospho-c-jun (panels A, D) was visualized using an DyLight 549 goat-anti-mouse IgG secondary and NFκB was visualized using a DyLight 488 goat-anti-rabit IgG secondary. Panels C and F show an RGB composite of the images from individual channels. In untreated cells (panel C) the nuclear region is purple/red because of the phospho-c-jun only while in treated cell (panel F) the nuclear region is yellow, due to the colocalization of the DyLight 488 stained NFκB with DyLight 549 stained phospho-c-jun.*

Colocalization of phospho-Paxillin with Microtubules

Phosphorylated Paxillin (p-pax) is a focal adhesion protein and microtubules are cytoskeletal proteins. These proteins (p-pax and microtubules) are involved in several critical cell functions including adhesion, motility, cell division, etc. We looked at the colocalization of p-pax with microtubules and the effect of microtubule disrupting agents such as colchicine on this colocalization.

Biological Experimental Design

For this biology also we chose HeLa cells, which showed good microtubule and p-pax staining. Both these proteins were visualized using a antibody based approach. Microtubules were visualized with a mouse monoclonal anti-tubulin combined with DyLight 488 goat-anti-mouse secondary antibody. Phospho-paxillin was visualized with a rabbit polyclonal anti-p-paxillin combined with DyLight 549 goat-anti-rabbit secondary antibody. Cells were also stained with Hoechst-33342 to visualize nuclei. Cells were treated with colchicine, a microtubule disrupting compound. A 4 channel Assay Protocol was set up with the Colocalization BioApplication with the following dye filter combinations- (i) Channel 1- Nuclei-Hoechst 33342 (XF93 Hoechst filter), (ii) Channel 2- microtubules (XF93 FITC filter) to identify the whole cell with the microtubule staining (iii) Channel 3- microtubules (XF93 FITC filter) to identify individual microtubules (iv) Channel 4- p-pax (XF93 TRITC filter) to visualize p-paxillin.

For Channels 3 and 4 the Apotome was used to obtain sectioned images to better visualize and identify the microtubules and p-paxillin.

Assay Protocol Configuration

The standard protocol provided with this BioApplication, for quantifying colocalization of p-paxillin with microtubules provides the settings required to quantify this colocalization. This section provides a description of some of the protocol settings that are relevant for measuring colocalization of focal adhesion with cytoskeletal protein, such as ROIs and the targets associated with the ROIs. Table 19 below lists the major assay parameter settings and the reasoning behind that setting. Figure 25, shows the dose response of the effect of colchicine on the colocalization of p-pax with microtubules.

Assay Parameter	Value	Reason
ROI_A_Channel	2	Measure colocalization of p-pax with microtubules in the entire cytoplasm. Used the microtubule stain to identify the entire cell as ROI_A
ROI_A_CircModifier	6	The ROI_A was expanded by 6 pixels so that p-pax that resides at the periphery of the cell was also identified.
ROI_A_RingWidth	0	No need to make any measurement in a membrane like region
ROI_A_RingDistance	0	No need to make any in a membrane like region
ROI_A_Exclude	1	Since Ch1 was generating the nuclear mask, and measurements in ROI_A were to be truly cytoplasmic excluding the nuclear region
ROI_A_Target_I_Channel	3	Identify individual microtubules in channel 3 (with Apotome sectioning). Set object (microtubules) identification and selection parameters so as to identify microtubules

Assay Parameter	Value	Reason
ROI_A_Target_II_Channel	4	Target is p-pax (Channel 4, with Apotome sectioning) and the protocol is designed to measure the colocalization of this with the Target_I (microtubules). Set object (p-pax) identification and selection parameters so as to identify individual p-pax spots on the cytoskeleton
ROI_B_Channel	0	No ROI_B was defined
SmoothFactorCh2	2	This parameter was set to "2" to better define the edges of the cell as stained by the microtubule staining
DetectRadiusCh2	0	Since the entire cell was identified, and no spots or fibers were identified, this parameter was set to "0"
ObjectSegmentationCh2	0	No segmentation was needed as the cells were generally well separated from each other.
SmoothFactorCh3	1	A value of "1" helped in identifying the long microtubules successfully, without segmenting them into smaller fragments
DetectRadiusCh3	1	This parameter was set to "1" to help identify the fibers. A larger value resulted in bundling of the individual fibers together, which was not desired.
ObjectSegmentationCh3	3	This parameter was set to "3" to segment the microtubules properly.
SmoothFactorCh4	2	This was set to "2" to help in proper identification of the p-pax stain. A smaller value resulted in undesirable fragmentation of the p-pax staining and a larger value merged individual p-pax spots together.
DetectRadiusCh4	2	This parameter was set to "2" to successfully identify the p-pax staining.
ObjectSegmentationCh4	0	No segmentation was desired as the p-pax were well segmented along the edge of the cell.

Table 19. *Settings for major Assay Parameters in the Colocalization BioApplication, used to quantify colocalization of p-paxillin with microtubules.*

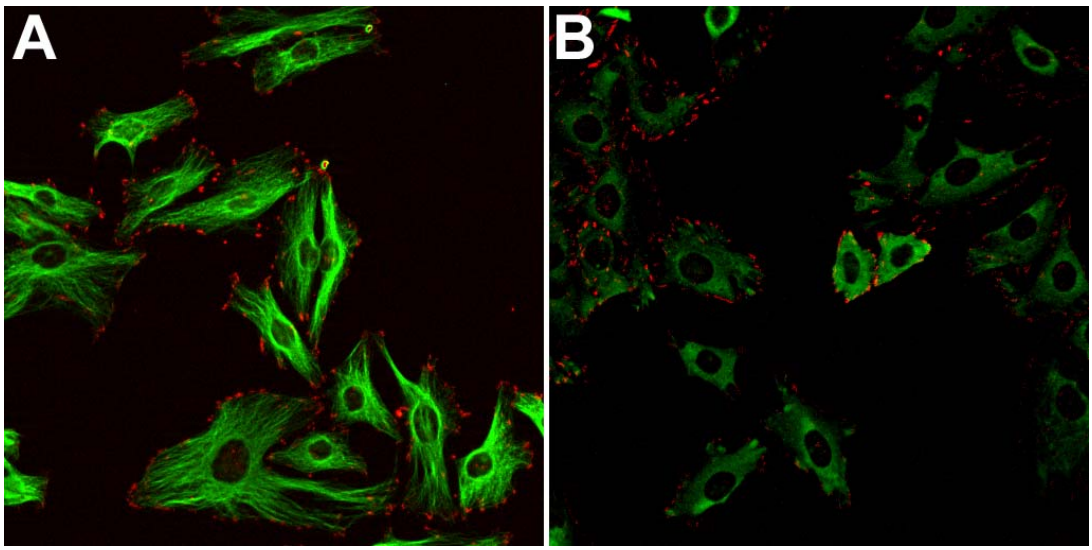


Figure 25. *Colocalization of p-pax with microtubules- effect of microtubule disrupting agent colchicine. Panel A shows untreated HeLa cells stain for microtubulin (green)and phospho-paxillin (red). Panel B shows cells that were treated with colchicine (0.43 μM) for 8 hours.*

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 Colocalization BioApplication. This section provides a detailed description of the operation of the BioApplication Event Wizard. The Wizard should only be used after the Colocalization BioApplication has been installed on your computer.

NOTE

A **maximum of 3 Cell Features** can be combined using logical operators to describe an Event.

Note that the Event Definition Assay Parameters **must not** be modified via the Scan software application. 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. Operation of the BioApplication Event Wizard must be consistent with the steps described.

STEP I

1. Create a protocol using the Scan software application without defining Events. Set optimized parameter values (upper and lower 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.

NOTE

Assay Protocols edited with the BioApplication Events Wizard are not saved as new versions of the protocol.

Features

The following features are included in the BioApplication Event Wizard:

- Enables use of standard Boolean operators (NOT, AND, ANDNOT, OR, ORNOT, XOR, NAND, and NOR)
- 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
- Addition of Event Definitions to the Protocol Comments field in the Assay Protocol

Steps for Running the Event Wizard with the Colocalization BioApplication

1. Create a protocol using the Colocalization BioApplication without defining Events. Set optimized parameter values (LevelHigh and LevelLow) 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

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

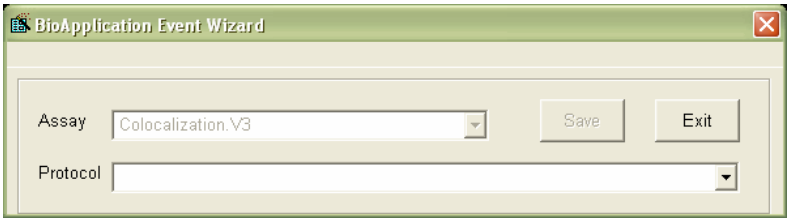
The first window that is displayed (see below) allows selection of any Colocalization Assay Protocol. Note that Event Definitions can only be specified for pre-existing Assay Protocols.

To select an Assay Protocol,

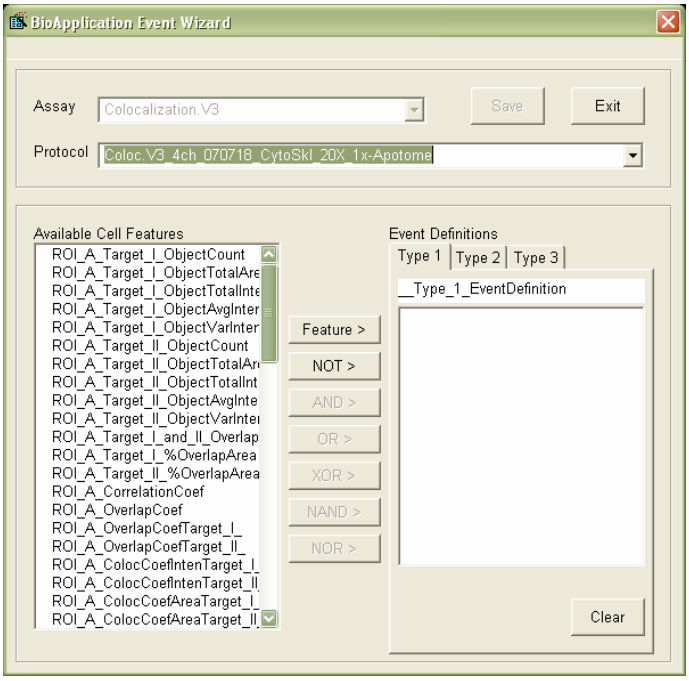
1) From the Assay drop-down menu, select Colocalization.V4.

2) From the Protocol drop-down menu, click on the drop-down arrow to view the list of existing Colocalization Assay Protocols.

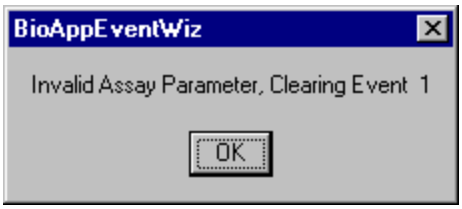
3) Select the desired Assay Protocol from the list.



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 Colocalization BioApplication 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, are selected by clicking on the Operator buttons (**NOT** >, **AND** >, **OR** >, etc.). Figure 26 below, shows a schematic description of the Boolean operators. The operator buttons are disabled whenever they cannot be used. Press the appropriate buttons in sequence to build the Event Definition, as shown below.

Addition of a Cell Feature to the Events means that the cell must be a responder (Status =1 or Status = 2) to meet the definition of the event.

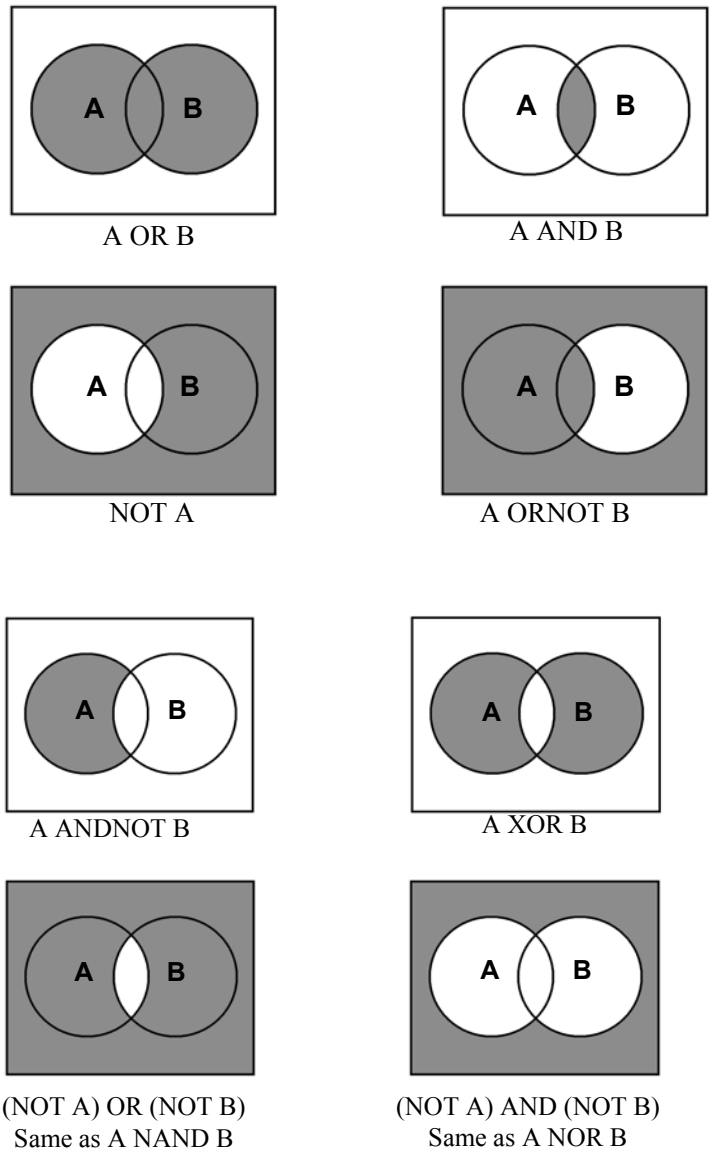


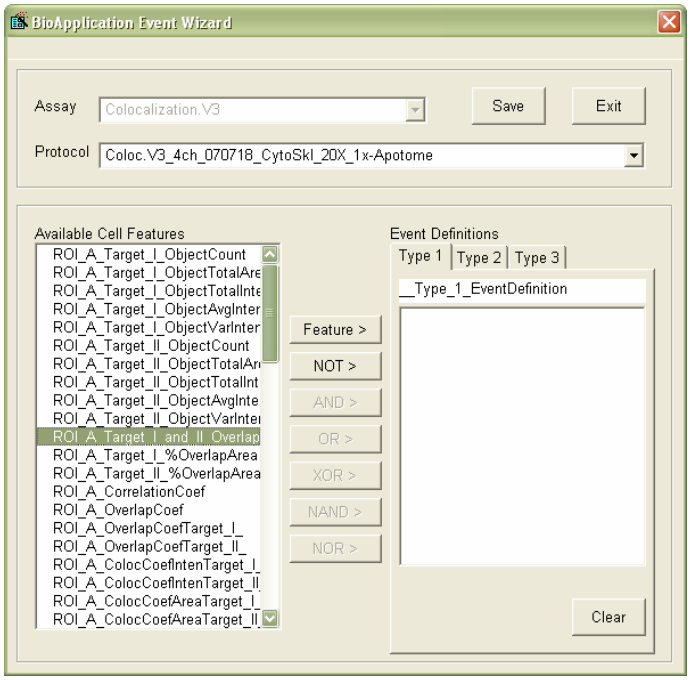
Figure 26. *Schematic definition of available Boolean Operators assuming two Cell Features of interest, A & B.*

To build an Event Definition,

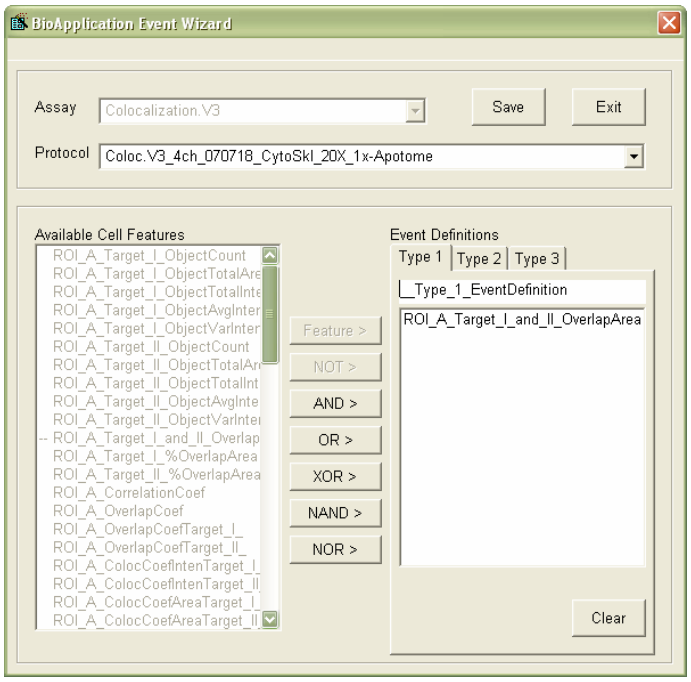
1) Select the Event Definition that you want to specify or edit by clicking on the **Type 1**, **Type 2**, or **Type 3** tab.

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

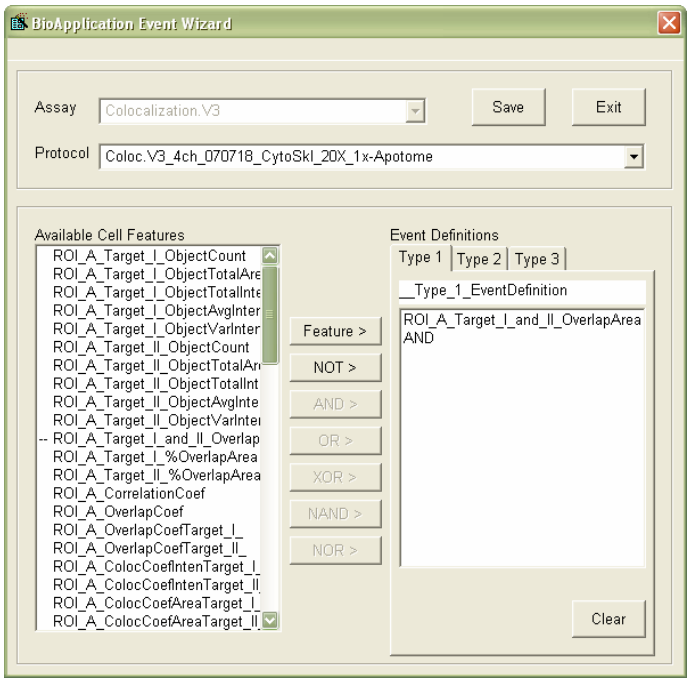
3) Select the desired Cell Feature by clicking on the feature name from the Available Cell Features list. You may also choose to select **NOT>** first (before the Feature) to indicate that you want to include only non-responders (Status = 0) in the definition of the event. In the example shown below the feature "**ROI_A_Target_I_and_II_OverlapArea**" is selected.



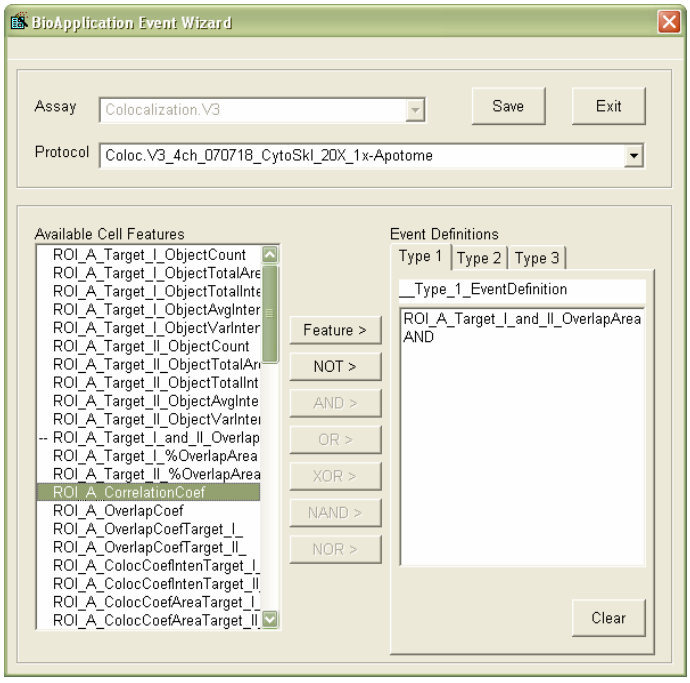
4) Click the **Feature>** button to transfer the Cell Feature into the Event Definition (see below).



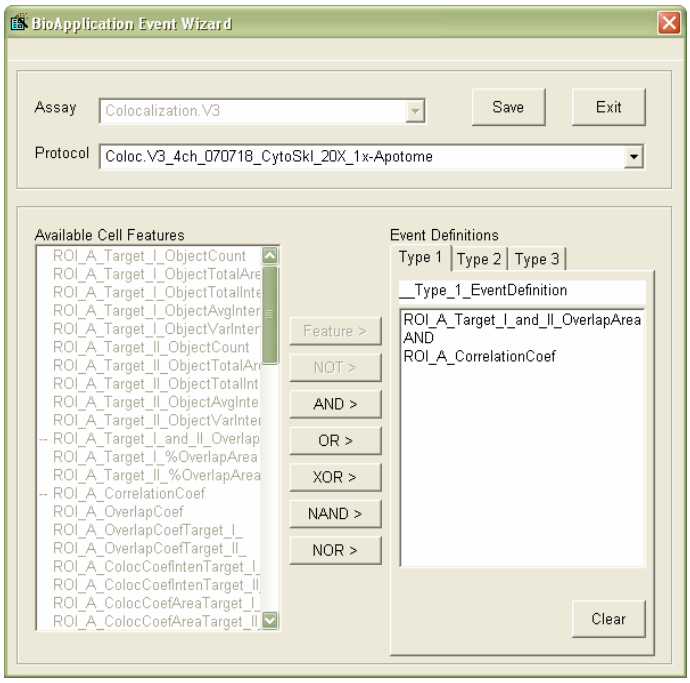
5) Click on the desired Operator button to transfer it into the Event Definition. In this example, the **AND** operator is selected (see below).



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



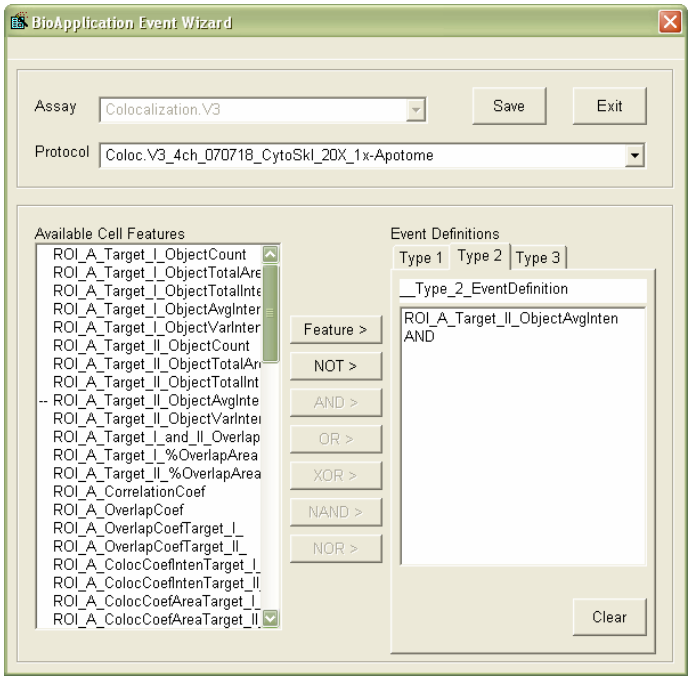
7) As before, click on the **Feature >** button to transfer the Cell Feature into the Event Definition (see below).

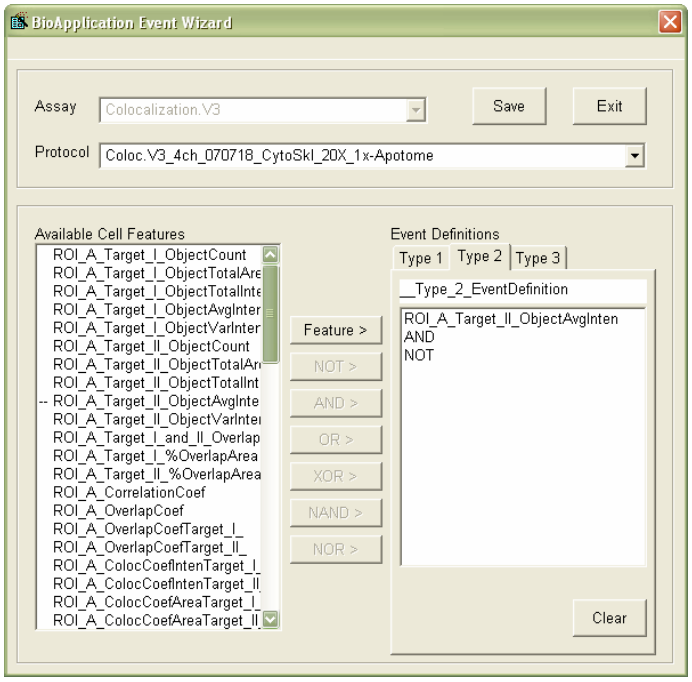


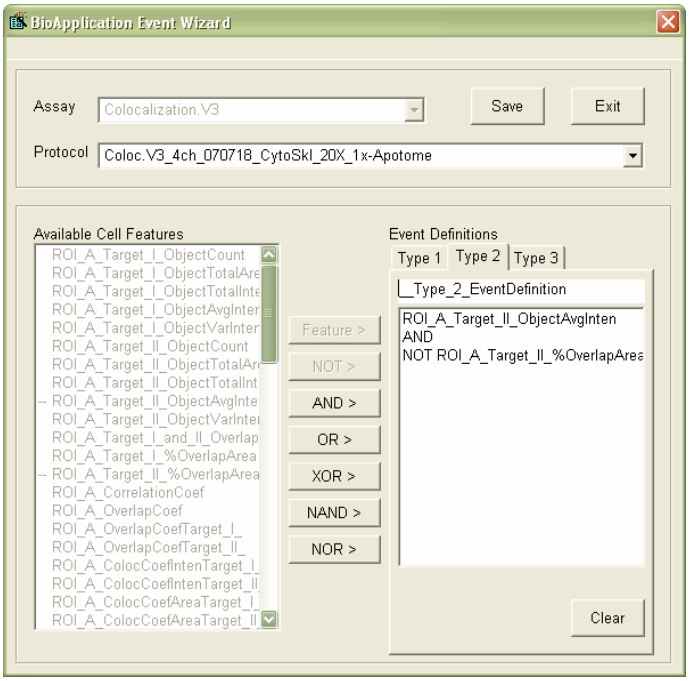
Type_1_EventDefinition in the previous case is:

ROI_A_Target_I_and_II_OverlapArea AND ROI_A_CorrelationCoef. In other words cells meeting the Event 1 criteria must be responders for both features.

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.



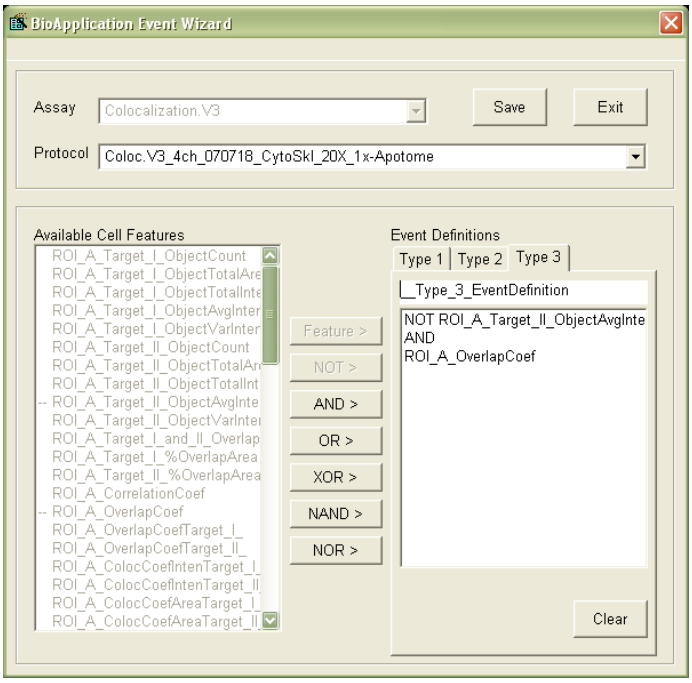




Type_2_EventDefinition in the above example is:

ROI_A_Target_I_ObjectAvgInten AND NOT ROI_A_Target_II_%OverlapArea. Thus for type 2 Event cells must be a responder for ROI_A_Target_1_ObjectAvgInten and not a responder for ROI_A_Target_II_OverlapArea..

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 in the above example is:

NOT ROI_A_Target_II_ObjectAvgInten AND ROI_A_OverlapCoef. Thus cells meeting criteria for Event 3 are only those cells that are non-responders for ROI_A_Target_II_ObjectAvgInten and responders for ROI_A_OverlapCoef.

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.

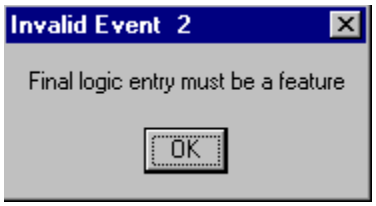
To save the updated Assay Protocol,

- 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 Colocalization V4 BioApplication using the iDev software. More detailed technical descriptions of each Assay Parameter can be found in Chapter 2 of this guide.

iDev Protocol Optimization Tasks

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

Image Preprocessing

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

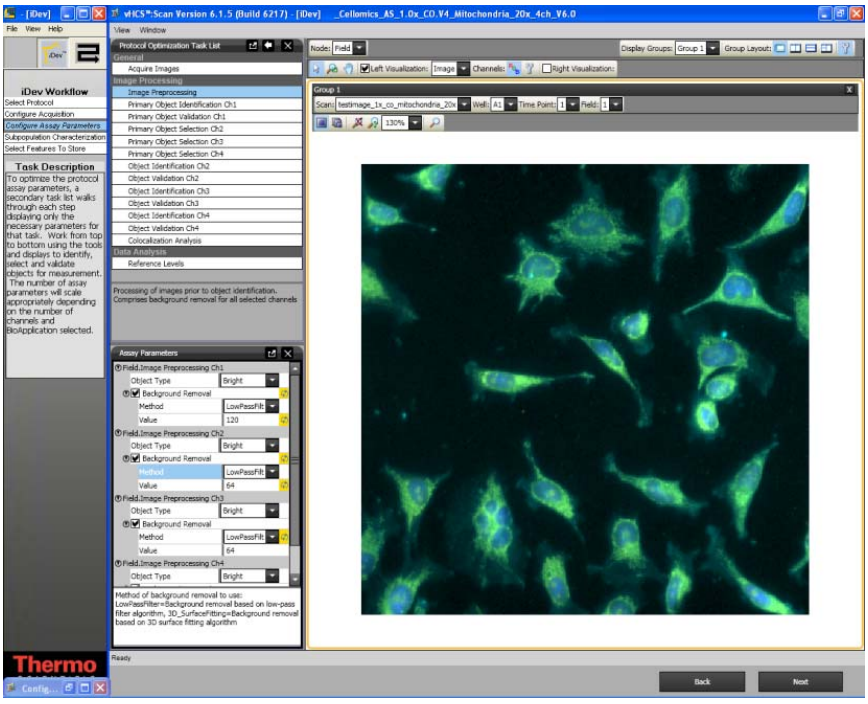


Figure 27. *Protocol Optimization Task – Image Preprocessing*

Primary Object Identification Ch1

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

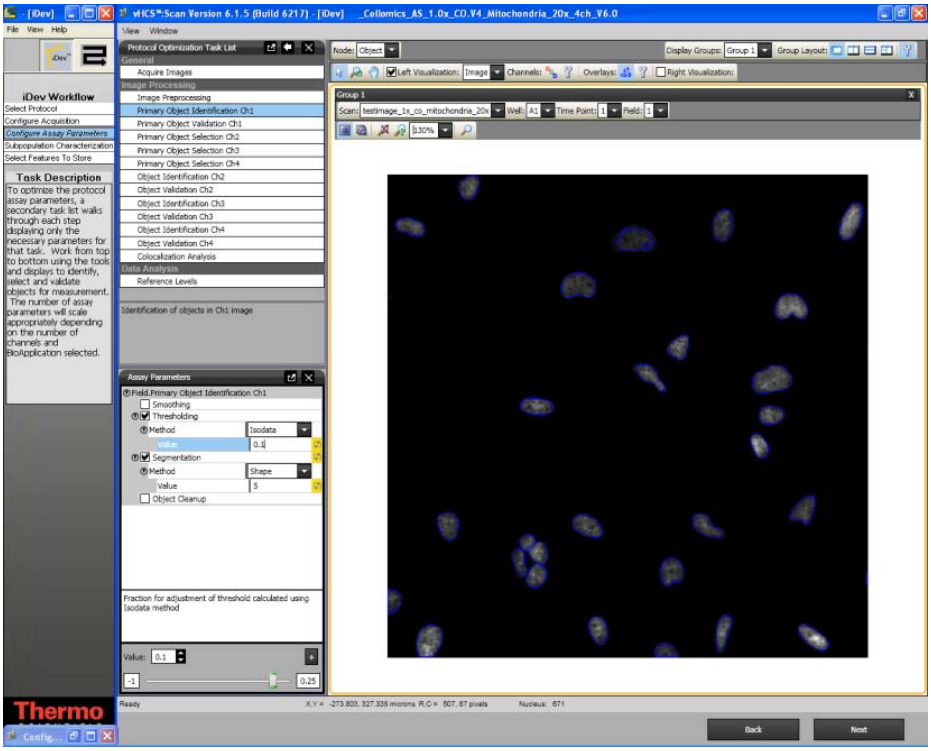


Figure 28. *Protocol Optimization Task – Primary Object Identification Ch1*

Primary Object Validation Ch1

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

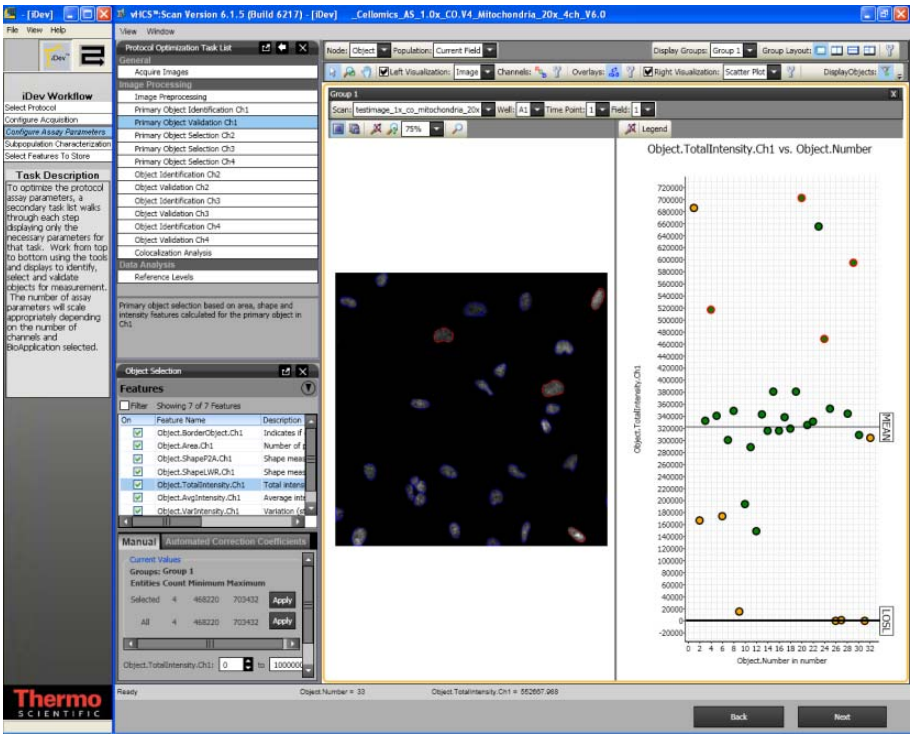


Figure 29. *Protocol Optimization Task – Primary Object Validation Ch1*

Primary Object Selection Ch2 through ChN

Primary Object Selection is object selection based on intensity features computed in Channels 2 through Channel 6 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 6 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.

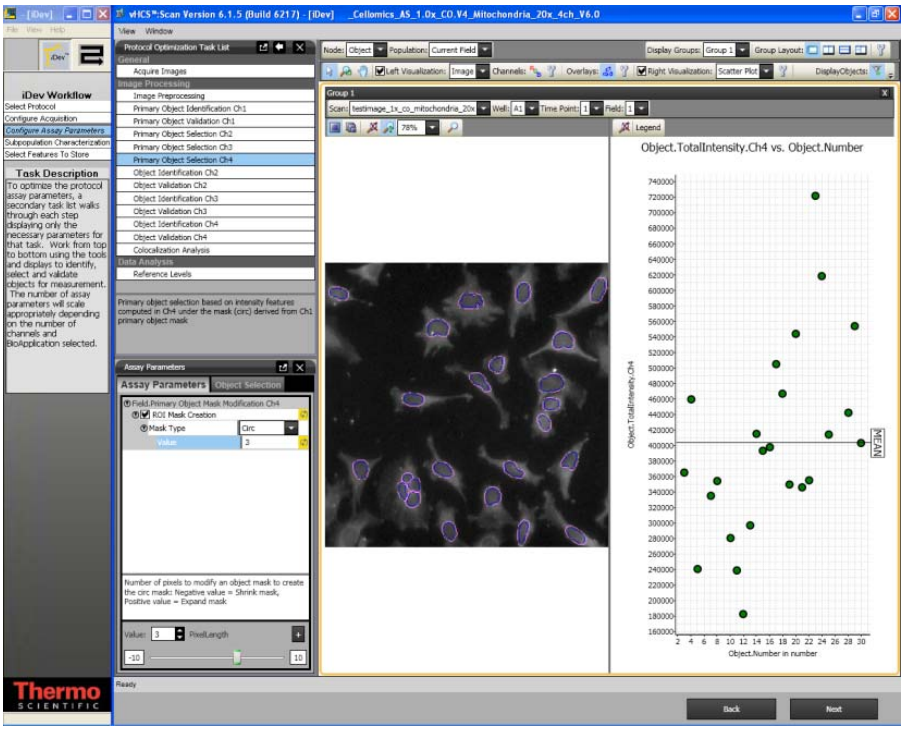


Figure 30. *Protocol Optimization Task – Primary Object Selection Ch2 through Ch6*

Object Identification Ch2 through Ch4

In this task you can identify targets in the ROI (Circ or Ring) in Channel 2. You can set the thresholding method and value and define the ROI as a Circ or Ring.

In this task, you can identify objects in Channel 2 through Channel 4 images. The task comprises Assay Parameters for object smoothing, detection of small objects, identification, and segmentation. The task involves setting up methods and values for identification, smoothing, and segmentation for Channel 2 through Channel 4 objects. You can also set the method and value for small object detection and also determine if the primary object mask from Channel 1 is to be used for segmenting objects in Channel 2 through Channel 4. When this option is chosen the other segmentation method and value is ignored.

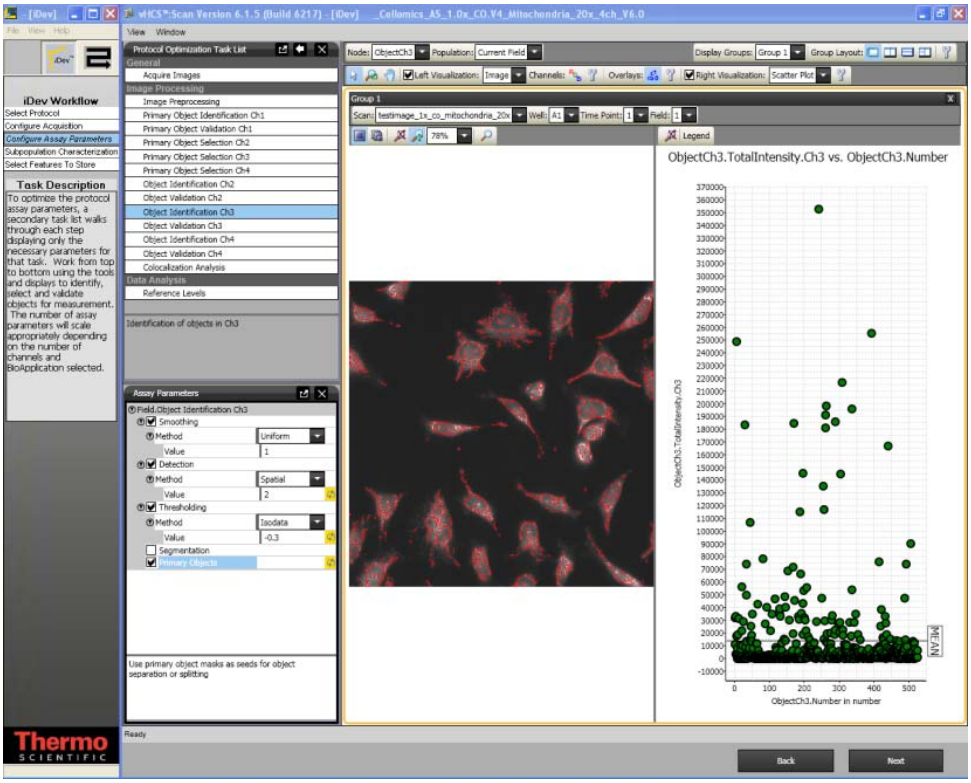


Figure 31. *Protocol Optimization Task – Object Identification*

Object Validation Ch2 through Ch4

This task provides object selection based on area, shape, and intensity features calculated in Channel 2 through Channel 4. 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 2 through Channel 4 based on output features.

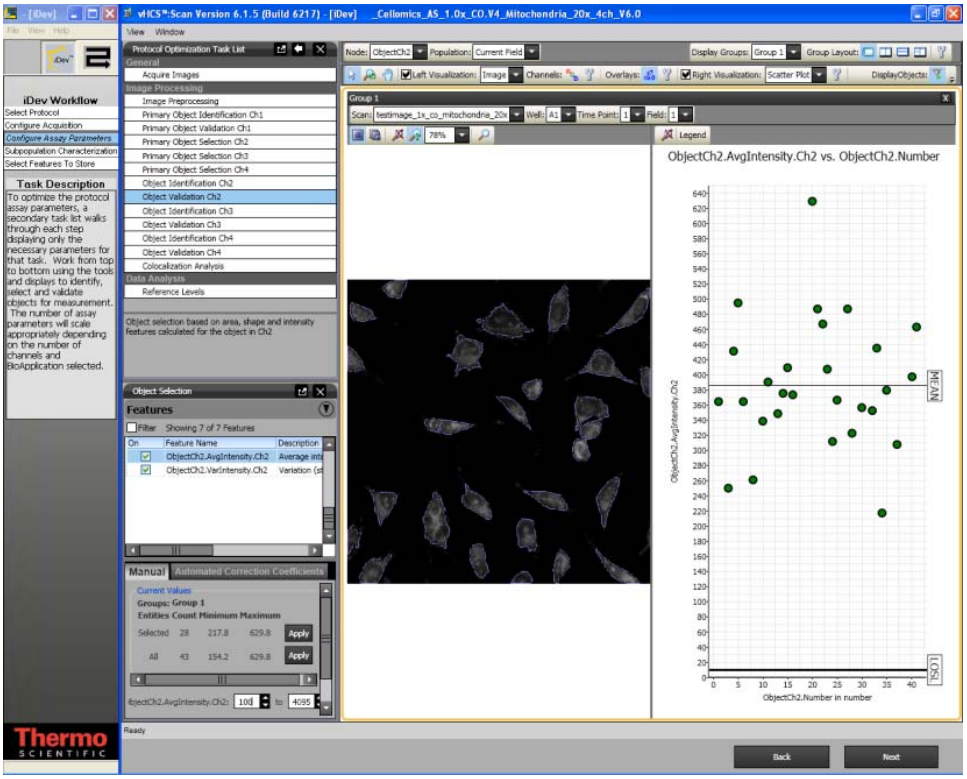


Figure 32. *Protocol Optimization Task – Object Validation*

Colocalization Analysis

In this task you can set Assay Parameters that allow you to perform colocalization analysis of two targets (Target I and Target II) in one or two ROIs (ROI_A and/or ROI_B). You set the channel from which images are to be used to create ROI_A and ROI_B, the type of mask to be used for these ROIs (Circ or Ring). You can also set which Channel object to be excluded from the ROIs, whether to remove overlapping regions of two ROIs from analysis and also the Channels for Target I and Target II within each ROI.

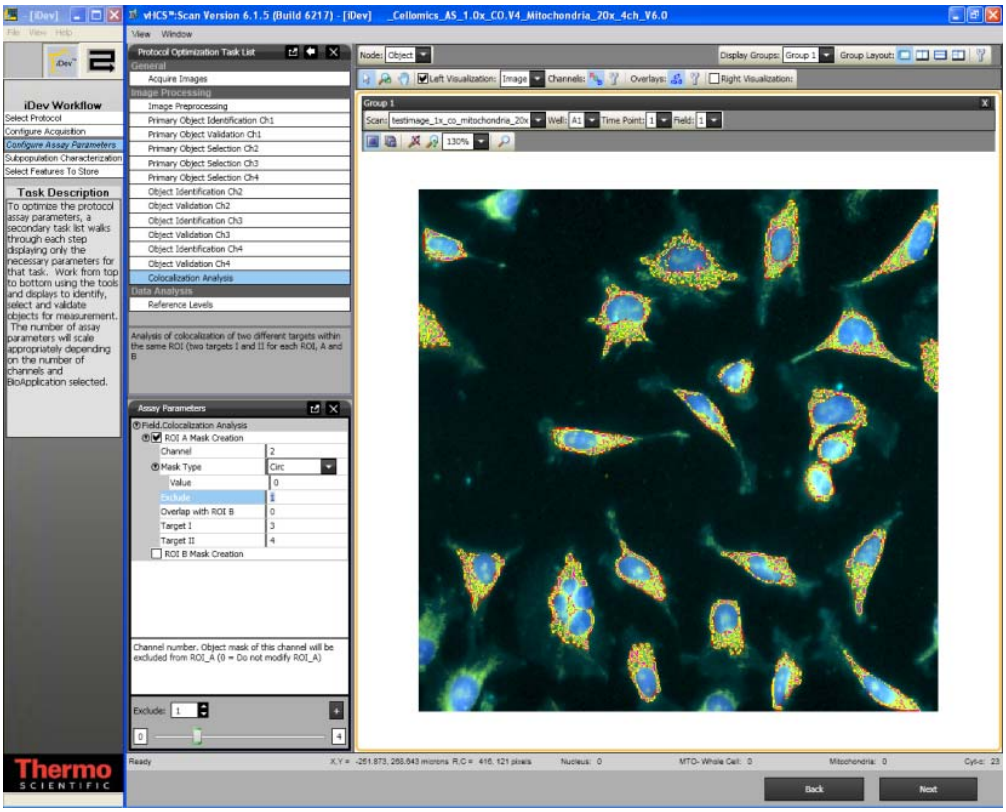


Figure 33. *Protocol Optimization Task – Colocalization Analysis*

Reference Levels

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

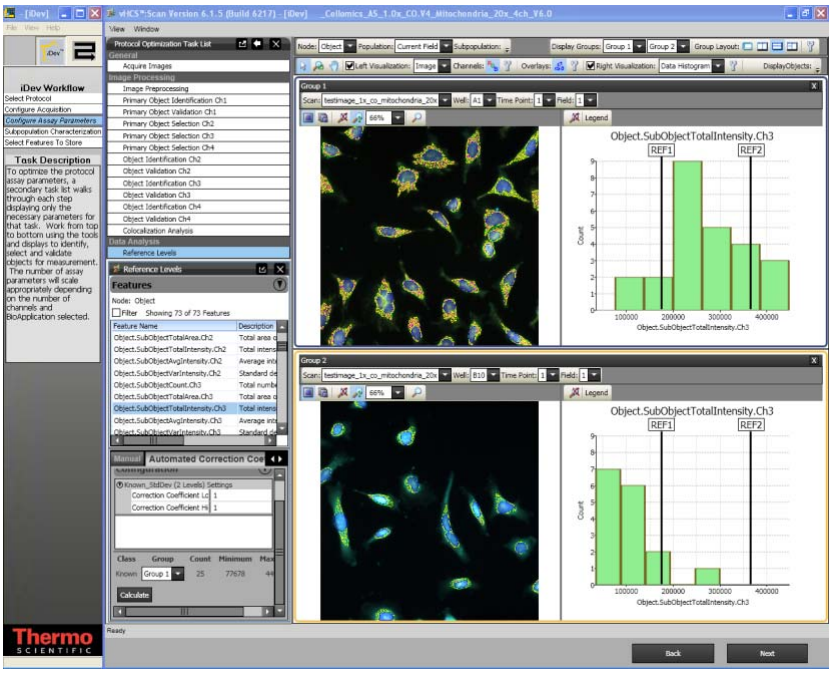


Figure 34. *Protocol Optimization Task – Reference Levels*

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.

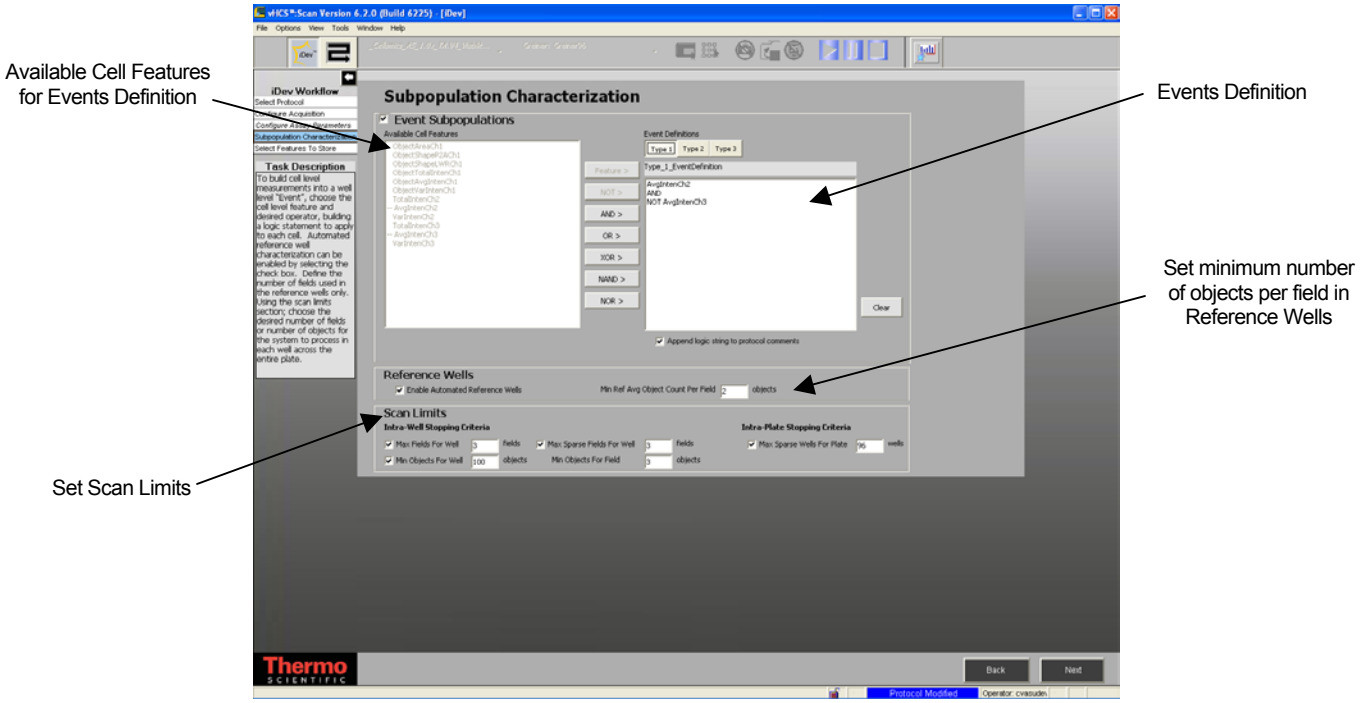


Figure 35. *Subpopulation Characterization Task*

Index

P

R

S

T

U

W