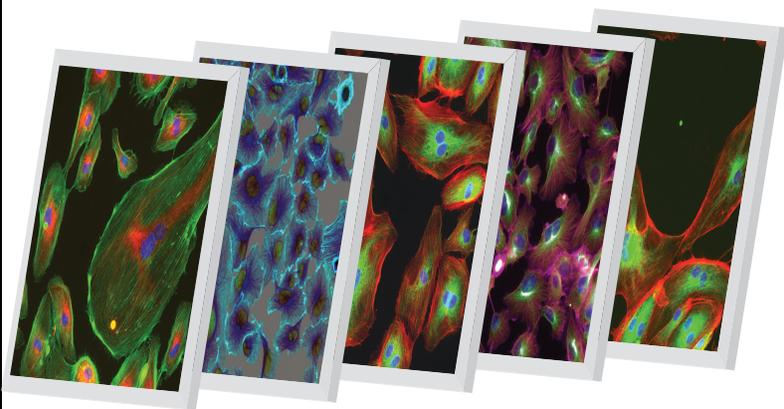


Thermo Scientific Cellomics®
Micronucleus

BioApplication Guide



Cellomics® Micronucleus BioApplication Guide

V4 Version

PUBLISHED BY

Thermo Fisher Scientific Inc.
100 Technology Drive
Pittsburgh, Pennsylvania 15219
Telephone: (412) 770-2200
Fax: (412) 770-2450

Copyright

Copyright © 2008-2010 by Thermo Fisher Scientific Inc. All rights reserved.

Portions of this document are protected by one or more issued patents, patent applications, trade secrets, copyrights, and/or other proprietary material. Use of the Micronucleus BioApplication requires a license from Cellomics, Inc., a subsidiary of Thermo Fisher Scientific Inc., and is entered into in conjunction with the purchase of the software.

Thermo Fisher Scientific Inc. reserves the right to make modifications and additions to the information in this document without notice. No part of this document may be reproduced or transmitted in any form or means, electronic, photographic, mechanical, or otherwise, for any purpose without the express written permission of Thermo Fisher Scientific Inc.

Trademarks

Cellomics, ArrayScan, iDev, and vHCS are trademarks or registered trademarks of Cellomics, Inc.

Microsoft is a registered trademark of Microsoft Corporation. Other products or company names mentioned in this document may be trademarks or registered trademarks of their respective owners and are treated as such. All other trademarks are the property of Thermo Fisher Scientific Inc. and its subsidiaries.

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.

Cellomics, Inc.
Software License Agreement

This Software License Agreement ("Agreement") for the Micronucleus 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) **Licensee'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. 6,416,959 and 6,875,578, Australian Patent No. 730100, European Patent Nos. 0983498 and 1155304, Canadian Patent Nos. 2,282,658 and 2,362,117, and Japanese Patent Nos. 3683591 and 3576491), 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 or 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 Micronucleus BioApplication.....	1
System Compatibility	1
Cell Biology Background	1
Mechanism of Micronucleus Formation	1
Micronucleus Testing for Genotoxicity Detection.....	2
Micronucleus Assay Design (Cytokinesis-Block Method).....	3
BioApplication Overview.....	4
Image Channels	5
BioApplication Workflow	6
Event Definition	7
BioApplication Measurements	9
Demonstration Data using the Micronucleus BioApplication.....	11
Example 1 – Using different cell lines with the Micronucleus BioApplication	11
Example 2 – Using Acridine Orange for Identification of Micronuclei	12
References	14
Chapter 2 Description of the Algorithm.....	15
Overview.....	15
Object Identification Methods	15
Description of Assay Parameters and Settings	19
Assay Parameters for Image Analysis	19
Channel-Specific Assay Parameters	22
Object Selection Parameters	35
Object Selection (Gating).....	36
Image Overlays	38
Assay Parameters for Population Characterization.....	40
Overview of Population Characterization.....	40
Advanced Assay Parameters.....	45
Assay Parameters for Definition of Events at the Level of Single Cells	47
Description of Output Features.....	49
Cell Features.....	49
Field Features	51
Well Features	51

Reference Features	56
Chapter 3 Using the BioApplication Event Wizard	57
Event Wizard Features	58
Steps for Running the Event Wizard with Micronucleus	58
Defining Events.....	60
Chapter 4 iDev Software Workflow	67
iDev Protocol Optimization Tasks	67
Image Preprocessing.....	67
Nuclear Object Identification Ch1	68
Cell Identification Ch2.....	69
Cell Validation Ch2	70
Nucleus and Micronucleus Classification	71
Nucleus Validation Ch1	72
Micronucleus Validation Ch1	73
Cell Targeting	74
Cell Selection Ch3 through ChN.....	75
Reference Levels.....	76
Setting Events in the iDev Assay Development Workflow	77

Overview of the Micronucleus BioApplication

High Content Screening (HCS) and High Content Analysis (HCA) uses fluorescence-based reagents, an advanced optical imaging system (Cellomics® ArrayScan® HCS Reader), and sophisticated image analysis software (BioApplications) to quantitatively analyze targets and physiological processes in cells. This BioApplication Guide provides a brief description for using one such BioApplication, Micronucleus (MN), to automatically quantify micronucleus formation in cells and define specific genotoxic subpopulations.

This guide contains the following chapters:

- Chapter 1** provides an overview of the Micronucleus v4.0 BioApplication and describes biological situations to which it can be applied.
- Chapter 2** describes the algorithm used to compute the quantitative results and gives a description of input parameters and output features.
- Chapter 3** describes the use of the Assay Protocol with the Event Wizard.
- Chapter 4** describes the Protocol optimization tasks that are available in the iDev™ Assay Development workflow.

System Compatibility

The Micronucleus v4.0 BioApplication described in this guide is designed to run on the following platforms:

- ArrayScan Platforms running version VTI
- Cellomics vHCS™ Discovery Toolbox, version 1.6.1.4 and higher

NOTE

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

Cell Biology Background

Mechanism of Micronucleus Formation

In cells of eukaryotic organisms, the nucleus contains DNA that is packaged into chromosomes. Chromosome shape, size, and number are constant for a species. During cell division, the genetic material replicates and then divides equally between the two daughter cells that are produced. If the process is disrupted or the chromosomes are broken or damaged by chemicals or radiation, then the distribution of genetic material between the two daughter nuclei during cell division may be affected and pieces of chromosomes or entire chromosomes

may fail to be included in either of the two daughter nuclei. When this occurs, the genetic material that is not incorporated into a new nucleus may form its own micronucleus which is clearly visible with a microscope. Micronuclei are known to arise from acentric chromosomal fragments and lagging whole chromosomes (Maier and Schmid, 1976; Hayashi et al., 1984). Thus, micronucleus formation may result from clastogens, which cause chromosomal breaks, and/or aneugens, which affect the spindle apparatus and result in the loss of whole chromosomes during anaphase.

Chromosomal mutations of both structure and number are implicated in many human diseases. There is substantial evidence that chromosomal mutations and related events in oncogenes and tumor suppressor genes of somatic cells are involved in the induction and/or progression of some types of cancer in humans and experimental animals. Thus, the purpose of the *in vitro* micronucleus assay is to detect those agents that modify chromosome structure and segregation, leading to formation of micronuclei in interphase cells.

Analysis of the induction of micronuclei in human lymphocyte cultures has indicated that the most convenient stage to score micronuclei is during the binucleate interphase stage (Fenech and Morley, *Solutions*, 1985). Such cells have completed one cell division after chemical treatment and are therefore capable of expressing micronuclei. Treatment of cultures with an inhibitor of actin polymerization, cytochalasin B, prevents cytokinesis and results in trapping cells at the binucleate or multinucleate stage where they can be easily identified (Fenech and Morley, *Measurement*, 1985). The measurement of the relative frequencies of multinucleate to mononucleate cells within a culture also provides a simple method of measuring the toxicity of a treatment, wherein toxic treatments that affect cell cycle progression will result in fewer multinucleate cells (Fenech, 1997).

Micronucleus Testing for Genotoxicity Detection

The most widely used micronucleus assay is an *in vivo* mammalian erythrocyte test. This is used to detect damage to the chromosomes or the mitotic apparatus of erythroblasts through analysis of erythrocytes sampled in the bone marrow and/or peripheral blood cells of animals treated with a test substance. The assay is labor-intensive, requiring animals (rats or mice) with attendant regulatory and cost issues, specialized staff, and an extended time course for completion of a study. Initially, animals are treated with defined doses of chemicals and then at a specified time, the frequency of micronucleate cells is determined in blood/bone marrow samples. Micronuclei can be distinguished by a number of criteria, including identification of the presence/absence of a centromeric DNA in the micronuclei. The centromere or kinetochore is a region of a chromosome with which spindle fibers are associated during cell division, allowing orderly movement of daughter chromosomes to the poles of the daughter cells. The frequency of micronucleate immature erythrocytes is the principal endpoint. If a treated group of animals shows significantly higher frequencies of micronucleate cells than do the untreated control animals, then the chemical is considered to be capable of inducing structural and/or numerical chromosomal damage. Most, if not all, cancers are characterized by chromosomal changes that are frequently specific to a particular tumor type. Also, induction of aneuploidy, an abnormal chromosome count, or chromosomal rearrangements in germ cells can be reasons for birth defects, fetal deaths, and infertility in animals. Therefore, a chemical that can produce chromosomal damage in somatic cells, perhaps presenting a risk of carcinogenicity, may also carry a risk of producing damage in germ cells, resulting in adverse reproductive outcomes.

The *in vitro* micronucleus assay is among a new set of genetic toxicology assays wherein cultured cells are treated and scored for micronucleus induction. The *in vitro* assay employs cultures of established cell lines, cell strains, or primary cell cultures. The cells used are

selected on the basis of their growth ability in culture and their spontaneous micronucleus frequency. The ultimate value of an *in vitro* assay is that screening for indicators of genetic toxicity may be enabled earlier in the development of therapeutic candidates, which will impact attrition rates and yield substantial financial gains. Although many compounds that are positive in this assay are mammalian mutagens and carcinogens, their correlations are dependent on chemical class and there is increasing evidence that there are some carcinogens that are not detected by this test because they appear to act through other mechanisms. Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation, such as activated microsomes. This metabolic activation system cannot entirely mimic *in vivo* conditions. Care should be taken to avoid conditions that would lead to positive results that do not reflect intrinsic mutagenicity and may arise from changes in pH, osmolality, or high levels of cytotoxicity (Miller and Adler 1990; Schriever-Schwemmer et al., 1994).

Micronucleus Assay Design (Cytokinesis-Block Method)

An example of a commonly used approach to performing the micronucleus assay is the cytokinesis-block method. This identifies cells as binucleate, allowing better precision to assess chromosome damage since micronuclei can only be expressed in cells that complete nuclear division. With the cytokinesis-block method, genotoxic agents are added for a designated period of time followed by cytochalasin B treatment (see the figure below). If exogenous metabolic activation, stimulated S9 microsomes, is used in conjunction with a genotoxic agent, stimulation might be for a lesser period of time, followed by cytochalasin B treatment.

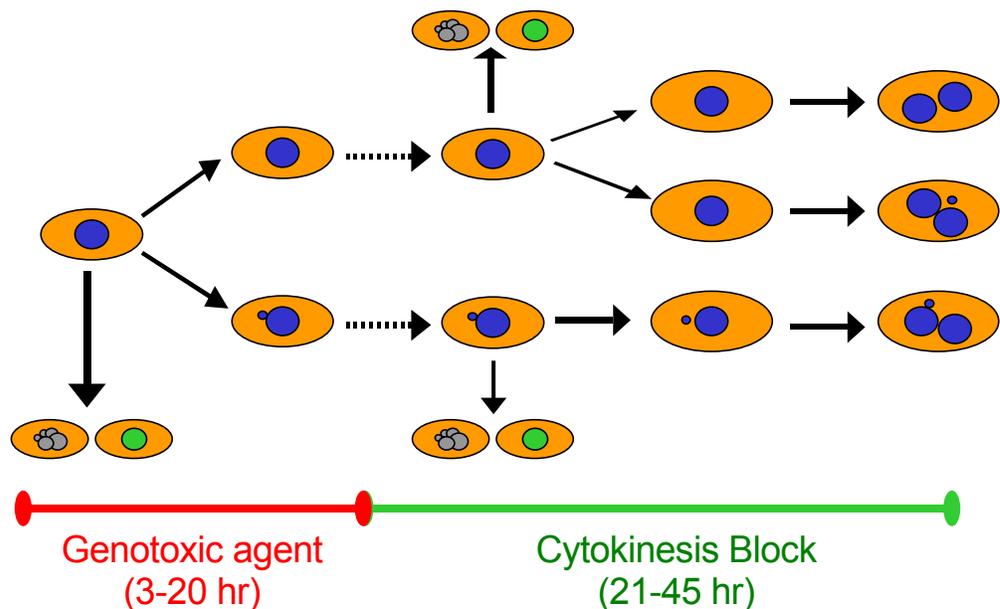


Figure 1. Principle of micronucleus induction with the cytokinesis-block method. Initially cells, upon treatment with a genotoxic agent, may become apoptotic (multiple gray nuclei), cytotoxic (green nuclei), or form spontaneous micronuclei. Upon addition of cytochalasin B, cells may become apoptotic, cytotoxic, blocked in cytokinesis (blue binucleate cells), or blocked in cytokinesis with the induction of micronuclei.

Conventional techniques for quantifying micronucleus formation involve the manual preparation and reading of slides. The micronucleus assay usually involves a scientist using one slide per concentration of genotoxic agent tested. Cells are then fluorescently stained so that the nucleus is visible, and approximately 1000 binucleate cells are visually counted using a microscope, noting cells that contain micronuclei or those that are not binucleate. Micronuclei

in interphase cells can be assessed more objectively than chromosomal aberrations in metaphase cells. Usually more than one trained person will count the cells on a slide to check the accuracy of the data. It is practical to score thousands of cells per treatment, which imparts greater accuracy to the assay. Additionally, as micronuclei may contain whole, lagging chromosomes, there is the potential to detect aneuploidy-inducing agents that are currently very difficult to study in conventional chromosomal aberration tests. Existing *in vitro* assays for micronuclei induction are time-consuming, require multiple trained personnel, can be subjective, and are not automated.

The Cellomics Micronucleus BioApplication was developed to resolve these issues. The biological procedure is similar to the conventional method; however, by using a 96-well plate, more than one agent may be tested and automated analysis takes less time. The Micronucleus BioApplication provides quantitative information of the frequency of micronucleus formation, upon treatment with an agent in question, by analyzing images of fixed cells. Blocking cytokinesis with cytochalasin B helps to easily quantify micronuclei in a population of cells to distinguish micronuclei formation from genotoxicity instead of spontaneous micronucleus formation. The BioApplication has specific parameters to identify micronuclei and distinguish the cells to use in determining micronuclei induction (e.g., size of each nucleus and micronucleus, total number of nuclei within a cellular boundary, etc.). Therefore, the algorithm is selective of which targeted cell is evaluated. Because of this, and the fact that a majority of people manually score 1000 or more cells to determine appropriate micronuclei frequency, it is important to take a large number of fields in order to get a well-defined evaluation of genotoxicity.

The Micronucleus BioApplication is useful for screening purposes in order to find compounds that are potential agents of genotoxicity. It is a functional assay that measures the frequency of micronuclei to determine genotoxicity of a compound, as well as possible cytotoxic effects. It also reduces the time to quantitatively analyze the images, reduces subjectivity, and provides an easy way to score multiple wells with various agents. You can also run this assay to include populations of mononucleate cells.

BioApplication Overview

The BioApplication measures the frequency of micronucleus formation in a population of targeted cells. It can also measure the cytotoxicity of the agent used through nuclear membrane permeability (optional 3rd channel) and comparison of multinucleate to mononucleate cells upon treatment of a genotoxic agent.

The algorithm is primarily set to identify micronucleus induction in binucleate cells treated with genotoxic agents. It is also able to identify agents that permeabilize the nuclear membrane. The algorithm has three core fluorescent image channels, designated as Channel 1 (Ch1), Ch2, and Ch3 that measure the nuclei and micronuclei, cytoplasm, and cells with increased membrane permeability respectively. Additional target channels, Ch4, Ch5, and Ch6, as well as Ch3, have the capability for subpopulation analysis (using average and total intensities under the modified cellular mask).

The algorithm identifies the population to use in determining micronuclei frequency by simultaneously looking at both the cellular and nuclear domains. If the staining on Channel 2 is not optimal, a cell substitute domain can be created from the nuclei. The various distinctions between cell and nuclear type are explained further in Chapter 2.

A properly optimized biological labeling protocol is essential for maximal performance of the algorithm. Cells must be plated so that they are ~70% confluent at the time of fixation. If you would want to assess if the cytoplasm is intact, you should use a whole-cell fluorescent marker to stain the cytoplasm, as well as a fluorescent marker to stain both the nuclei and micronuclei within a cell (such as Hoechst).

Acridine Orange is another staining alternative commonly used to count nuclei and micronuclei. However, bleed-through between channels may occur. Selecting different filter sets for each channel or purchasing alternative filters for the ArrayScan VTI HCS Reader may be necessary for optimum performance for this configuration.

If detection of nuclear membrane permeability is desired, a fluorescent permeability indicator must be used. The Micronucleus HCS Reagent Kit provides a standard procedure including Cellular Dye (TRITC), Permeability Dye (FITC), Hoechst Dye, and cytochalasin B.

Cytochalasin B is used to block cells during cytokinesis in order to monitor cell cycle delay (by evaluating **MultiNucToMonoNucCellRatio** or **ProliferationIndex** Well Features). However, analysis can also be done on mononucleate cells by simply setting the **T_MinNucCountPerCellCh1** and **T_MaxNucCountCellCh1** to **1**.

Image Channels

The Micronucleus v4.0 BioApplication performs cell-based measurements on one to six imaging channels, as described in Table 1. In Channel 1, nuclei and micronuclei are identified using a nuclear stain, such as Hoechst, DAPI, or Acridine Orange. Both nucleus and micronucleus features including size, shape, and intensity, are measured.

If you want to determine if the nucleus is within an intact cytoplasm, the cellular boundary can be identified in Channel 2 by a whole cell marker or by selecting a different filter for Acridine Orange (to evaluate Acridine Orange binding to RNA in the cytoplasm). Features measured include size, shape, intensity, and number of nuclei per cell.

Channel 3 can be used to identify cells with compromised nuclear membranes. Overall nucleus intensity (as well as micronucleus intensity) in this channel can also be calculated. This channel can also be used for object selection by setting the intensity measurements (average and total) within the region of the modified Ch1 or Ch2 cell domain. You also have the option of using Channel 3 to measure nucleus and micronucleus intensity values.

Channels 4-6 can only be used for object selection by setting the intensity measurements (average and total) within the region of the modified Ch1 or Ch2 cell domain.

Table 1 is an example of how each channel could be used for identification, while Figure 2 illustrates how the BioApplication would work for three channels.

Channel	Cellular Features Measured	Method of Identification
1- Required	Nucleus count, shape, intensity, Micronucleus count	Nuclear Label (Hoechst, DAPI, Acridine Orange [DNA])
2 – Optional	Cell size, shape, intensity, nuclear number	Whole cell dye, Acridine Orange [RNA]
3 – Optional	Nucleus intensity Micronucleus intensity or Modified cell domain intensity	Nuclear membrane permeability dye Kinetechore antibody Other preferred marker
4 – Optional	Modified cell domain intensity	Other preferred marker
5 – Optional	Modified cell domain intensity	Other preferred marker
6 – Optional	Modified cell domain intensity	Other preferred marker

Table 1. General description of features and identification per channel within the Micronucleus v4.0 BioApplication

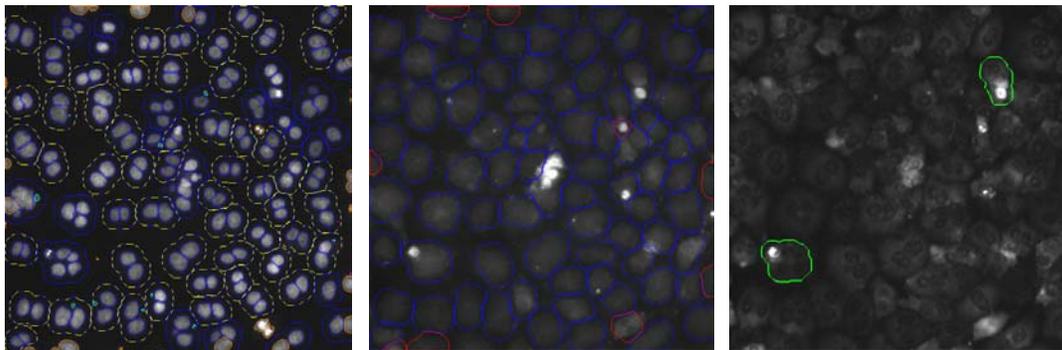


Figure 2. Micronucleus v4.0 BioApplication Overlays: Left: Channel 1 exhibiting nuclei, micronuclei, and Targeted Cells. Middle: Channel 2 exhibiting cytoplasm (optional). Right: Channel 3 exhibiting cytotoxic channel (optional).

BioApplication Workflow

The first task of the BioApplication is to identify all of the valid objects in Channel 1. Then identification of micronuclei from nuclei is through the use of the **MaxMicroNucToNucSizeRatioCh1** Assay Parameter – any objects with their diameter less than the value will then be considered a micronucleus. Object Selection Parameters are used for selecting both types of objects. After all objects are identified and selected in Channel 1, you then either have to identify cells in Channel 2 or create a cell substitute mask from the Channel 1 nuclei through use of the **CreateCellSubstituteMaskCh1** and **CellSubstituteMaskModifierCh1**.

If Channel 2 is used for analysis, Object Selection Parameters may also be used to reject/select a population of cells. If the nuclei are found within the cell mask (per the **MinNucCellAssociationCh1** and **MinNucCellOverlapCh2** Assay Parameters), are not rejected and have the correct count (through **T_MinNucCountPerCellCh1** and **T_MaxNucCountPerCellCh1**), and are similar in size and intensity (through **T_MinNucToNucSizeRatioPerCellCh1** and

T_MinNucToNucAvgIntenRatioPerCellCh1), the Selected Cell will be targeted for analysis.

If Channel 3 is used, nuclear values above the **CellNucMaxAvgIntenCh3LevelHigh** (and, if used, below the **CellNucMaxAvgIntenCh3LevelLow**) Assay Parameter(s) will indicate the cell as cytotoxic and give it the Cytotoxic Cell overlay. If you would also like to target that cell for micronucleus analysis, you must set the **T_CytotoxicCellsCh3** Assay Parameter to **1**. If **T_CytotoxicCellsCh3** is set to **0**, the cell will not be targeted for micronucleus analysis.

If Channels 3-6 are used for additional object selection, the modified cell mask (from Ch1 or Ch2 depending on use) will be applied to this channel and used to measure Average and Total Intensities in Channels 3-6. These intensities can be used to gate, or select, the cells that will be analyzed.

The measurements performed by the BioApplication are summarized in Table 2. In general, nucleus features (based off the selected cell) include count, area, shape, and intensity with micronucleus count. General Channel 2 cell features such as area, intensity, and shape are reported; if the Selected Cell is targeted for micronucleus analysis, a general status feature for targeted cells will be **1**. If Channel 3 is used, both cytotoxic output features (percent, targeting output) as well as general nucleus and micronucleus output features are given.

Event Definition

Events 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. The Micronucleus v4.0 BioApplication allows simultaneous definition of up to three Events to enable rapid multiparametric analysis at the level of individual cells across multiple Cell Features. Figure 3 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 targets being measured. Reference Levels 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 or less than the lower limit defined by **FeatureChNLevelHigh** (**FeatureChNStatus = 1**) or **FeatureChNLevelLow** (**FeatureChNStatus = 2**). It is critical that the upper limits be set appropriately for the Events to properly function when classifying cells.

Well Features are calculated for all selected cells and all cells targeted for micronucleus analysis to provide population statistics for individual Cell Features, as well as 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.

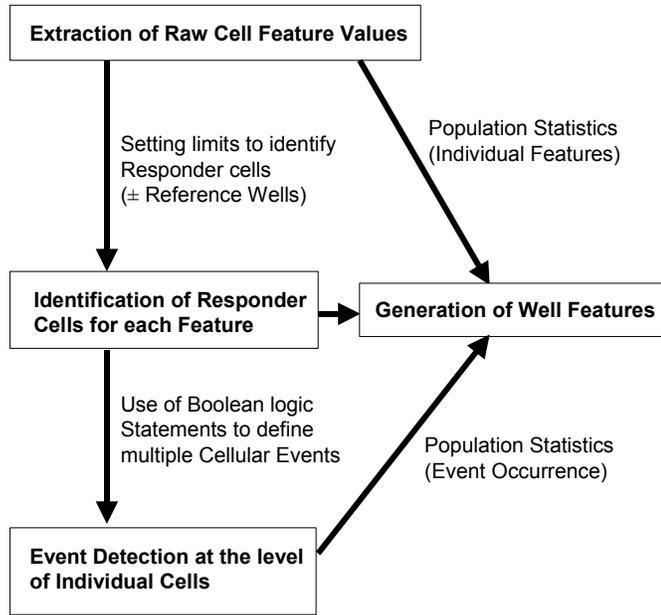


Figure 3. Schematic showing the process of definition and detection of cellular events

BioApplication Measurements

The Micronucleus v4.0 BioApplication reports Reference, Cell, Field, and Well Features. Reference Features are calculated to determine populations relative to set reference levels. Cell Features are measurements made on a single cell. Field features are quantitative descriptors of cells reported for the whole field. Well Features are well-level averages and other population metrics that are derived from the Cell Features for all the cells analyzed in a given well. Overall Cell, Field and Well Features are summarized in Table 2 and described further in Chapter 2.

Additionally, for live cell analysis, the Cellomics ArrayScan VTIHCS Reader supports a Live Module that can track cells over time. Please refer to the appropriate user guides for more information.

Type	Region	Cell Features	Field Features	Well Features
Cell & Well Features Reported for Channel 1:				
Location	Cell	Top Left Width Height X Centroid Y Centroid	None	None
Count	Cell	Cell # TargetedStatus	Valid Cell Count Selected Cell Count Targeted Cell Count MicroNucTargetedCell Count MonoNucCellCount BiNucCellCount MultiNucCellCount	Valid Cell Count Selected Cell Count %SelectedCells Targeted Cell Count %TargetedCells Valid Field Count SelectedCellCountPerValidField TargetedCellCountPerValidField MicroNucTargetedCellCount %MicroNucFrequency %MicroNucFrequencyStatus MonoNucCellCount %MonoNucCells BiNucCellCount %BiNucCells MultiNucCellCount %MultiNucCells MultiNucToMonoNucCellRatio Proliferation Index
	Nucleus	Count & Status	None	MEAN, SD, SE, CV, %LOW, %HIGH for all Targeted and Selected Cells
	Micronucleus	Count & Status	MicroNucCountWithin Targeted Cells	MicroNucCountWithinTargetedCells
			—————→	MEAN, SD, SE, CV, %LOW, %HIGH for all Targeted and Selected Cells
Morphology	Nucleus	Total Area & Status	None	MEAN, SD, SE, CV, %LOW, %HIGH for all Targeted and Selected Cells
Intensity	Nucleus	Total Intensity & Status Avg Intensity & Status Var Intensity & Status MaxAvgIntensity	None	MEAN, SD, SE, CV, %LOW, %HIGH for all Targeted and Selected Cells
Cell & Well Features Reported for Channel 2:				
Morphology	Cell	Area & Status Shape P2A & Status Shape LWR & Status	None	MEAN, SD, SE, CV, %LOW, %HIGH for all Targeted and Selected Cells

Type	Region	Cell Features	Field Features	Well Features
Intensity	Cell	Total Intensity & Status Avg Intensity & Status Variance Intensity & Status	None	MEAN, SD, SE, CV, %LOW, %HIGH for all Targeted and Selected Cells
Cell & Well Features Reported for Channel 3:				
Type	Region	Cell Features	Field Features	Well Features
Count	Cell	CytotoxicCellStatus	CytotoxSelectedCell Count	CytotoxSelectedCellCount % CytotoxSelectedCells CytotoxTargetedCellCount %CytotoxTargetedCells CytotoxMicroNucTargetedCellCount %CytotoxMicroNucTargetedCells NonCytotoxMicroNucTargetedCell Count NonCytotox%MicroNucTargetedCells %CytotoxOnlyMicroNucTargetedCells %NonCytotoxOnlyMicroNucTargeted Cells
Intensity	Nucleus	Total Intensity & Status Avg Intensity & Status Max Avg Intensity & Status Var Intensity & Status	None	MEAN, SD, SE, CV, %LOW, %HIGH for all Targeted and Selected Cells
	Micronucleus	Total Intensity & Status Avg Intensity & Status Var Intensity & Status	None	MEAN, SD, SE, CV, %LOW, %HIGH for all Selected Cells
Object Selection (Intensity)	Modified Cell	Total Intensity Avg Intensity	None	None
Cell & Well Features Reported for Channels 4-6:				
Type	Region	Cell Features	Field Features	Well Features
Object Selection (Intensity)	Modified Cell	Total Intensity Avg Intensity	None	None
Other Cell & Well Features Reported:				
Event (from Cell status features)		EventTypeProfile EventType1 Status EventType2Status EventType3Status	None	Event 1: Count, % Event 2: Count, % Event 3: Count, % for all Targeted and Selected Cells

Table 2. Micronucleus v4.0 BioApplication Cell, Field, and Well Feature Measurements. Note that "Cell" could be the mask created from Ch2 or Cell Substitute Mask in Ch1.

Demonstration Data using the Micronucleus BioApplication

Example 1 – Using different cell lines with the Micronucleus BioApplication

To demonstrate this BioApplication, a549 (human lung), CHO-K1 (Chinese hamster ovary), and CHL (Chinese hamster lung) cells were plated on 96-well collagen-I plates and incubated for approximately 18-22 hours overnight. Cells were then treated with various concentrations of mitomycin C for 4-24 hours, followed by cytochalasin B for 20-28 hours. Permeability and Cellular Dye from the Cellomics HCS Reagent Kit were added to all cells, fixed, and stained with Hoechst. Figure 4 represents RGB images (from all three channels) for each cell type.

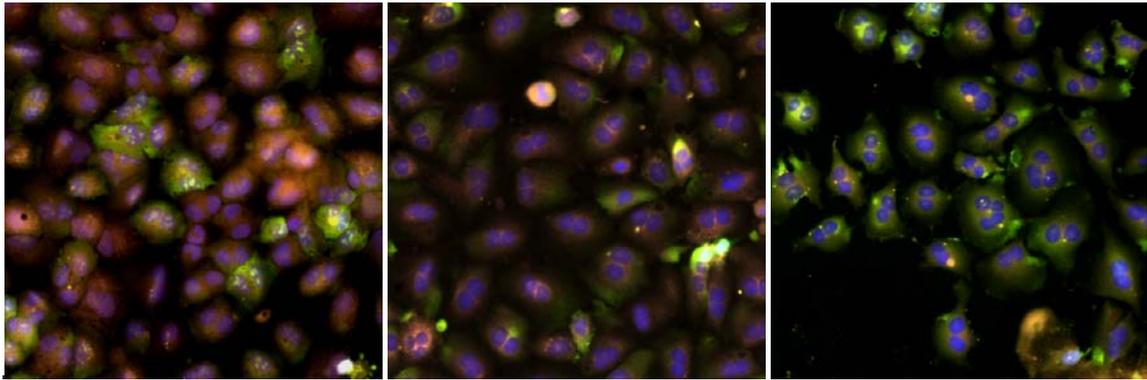


Figure 4. RGB images (20x) of a549 (left), CHO-K1 (middle), and CHL (right) cells

Figure 5 represents the micronucleus frequency and R^2 values computed for each cell type. Dose-responsiveness of mitomycin C was evident for a549, CHO, and CHL cells, exhibiting EC_{50} values of 50, 160, and 105 ng/mL, respectively.

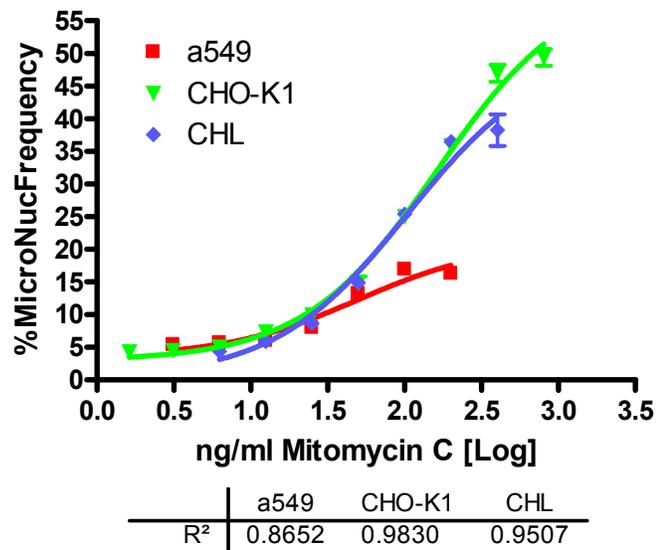


Figure 5. Micronucleus Frequency of a549, CHO-K1 and CHL cells upon treatment with various doses of mitomycin C. Each data point represents an average of six wells. Error bars represent standard deviation.

Example 2 – Using Acridine Orange for Identification of Micronuclei in One- and Two-channel Assays

The Micronucleus BioApplication was used to evaluate Acridine Orange staining in treated CHO-K1 cells as both a one- and two-channel assay. Procedure was the same as with the first example except for the last day with fixation and staining. A fixation solution of methanol: glacial acetic acid was added to the media for 5 minutes and repeated after aspiration. Plates were washed three times with wash buffer and 20 $\mu\text{g}/\text{mL}$ Acridine Orange was added for 3 minutes. Plates were washed three more times and then sealed. With appropriate filters (XF100GFP for nuclei, XF93 Trite for cytoplasm), both Channel 1 and Channel 2 can be seen and analyzed with the BioApplication. Figure 6 shows images acquired with the ArrayScan VTI HCS Reader. Figure 7 shows a graph representing data obtained from running the application as one and two channels. You can see that the EC_{50} values obtained are similar for both one and two channels; however, if you would want to evaluate if the cytoplasm was intact, a 2-channel assay would be preferred.

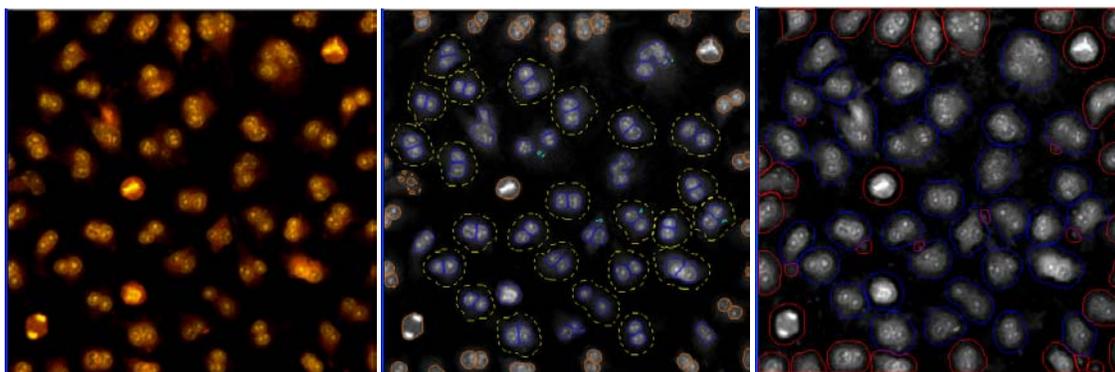
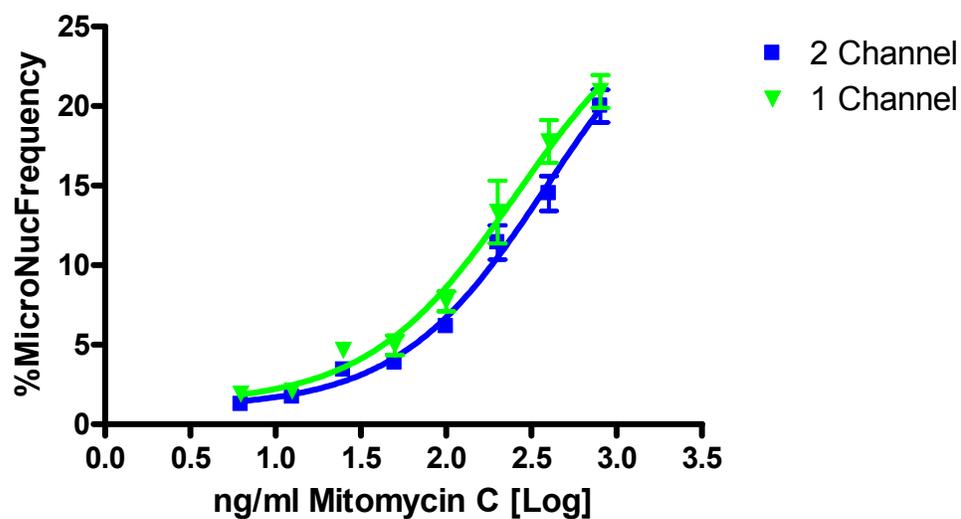


Figure 6. CHO-K1 cells stained with Acridine Orange: RGB image (left), Channel 1 (middle), and Channel 2 (right) images and overlays obtained by utilizing the Micronucleus v4.0 BioApplication



	2 Channel	1 Channel
BOTTOM	1.009	1.241
TOP	28.99	27.77
LOGEC50	2.592	2.415
EC50	390.4	260.0
	2 Channel	1 Channel
R ²	0.9359	0.8977

Figure 7. Dose response and coordinating data after running the Acridine Orange plate using a 1-channel (green) and 2-channel (blue) approach with the Micronucleus.v4.0 BioApplication. Each data point represents an average of six wells. Error bars represent standard deviation.

References

- Bonassi S, M. Fenech, C. Lando, Y. P. Lin, M. Ceppi, W. P. Chang, N. Holland, M. Kirsch-Volders, E. Zeiger, S. Ban, R. Barale, M. P. Bigatti, C. Bolognesi, C. Jia, M. DiGiorgi, L. R. Ferguson, A. Fucic, O. G. Lima, P. Hrelia, A. P. Krishnaja, T. K. Lee, L. Migliore, L. Mikhalevich, E. Mirkova, P. Mosesso, W. U. Muller, Y. Odagiri, M. R. Scarffi, E. Szabova, I. Vorobtsova, A. Vral, and A. Zijno. Human MicroNucleus project: international database comparison for results with the cytokinesis-block micronucleus assay in human lymphocytes: I. Effect of laboratory protocol, scoring criteria, and host factors on the frequency of micronuclei. 2001. *Environ. Mol. Mutagen* 37:31-45.
- Fenech M. 1997. The advantages and disadvantages of the cytokinesis-block micronucleus method. *Mutation Res*, 392:11-8.
- Fenech M., and A. Morley. 1985. Measurement of micronuclei in lymphocytes. *Mutation Res*. 147:29-36.
- Fenech M., and A. Morley. 1985. Solutions to the kinetic problem in the micronucleus assay. *Cytobios* 43:233-46.
- Hayashi M., T. Sofuni, and M. Ishidate. 1984. A pilot experiment for the micronucleus test. The multi-sampling at multi-dose levels method. *Mutation Res*. 141:165-9.
- Kirsch-Volders M., Sofuni T., Aardema M., Albertini S., Eastmond D., Fenech M., Ishidate M. Jr., Kirchner S., Lorge E., Morita T., Norppa H., Surralles J., Canhauwaert A., and A. Wakata. 2003. Report from the in vitro micronucleus assay working group. *Mutation Res*. 540:153-163.
- Lynch A. M., and J. M. Parry. 1993. The cytochalasin-B micronucleus/kinetochore assay in vitro: studies with 10 suspected aneugens. *Mutation Res*. 287:71-86.
- Maier P., and W. Schmid. 1976. Ten model mutagens evaluated by the micronucleus test. *Mutation Res*. 40:325-38.
- Miller B.M., and I. D. Adler. 1990. Application of antikinetochores antibody staining (CREST staining) to micronuclei in erythrocytes induced in vivo. *Mutagenesis* 5:411-415.
- Organization for Economic Co-operation and Development (OECD). 2004. *OECD Guideline for the Testing of Chemicals – Draft Proposal for a New Guideline 487: In Vitro Micronucleus Test*. First Version 1-13.
- Schriever-Schwemmer G., U. Kliesch, and I. D. Adler. 1997. Extruded micronuclei induced by colchicine or acrylamide contain mostly lagging chromosomes identified in paintbrush smears by minor and major mouse DNA probes. *Mutagenesis* 12:201-207.

Description of the Algorithm

NOTE

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

The previous chapter provided a brief overview of this BioApplication. This chapter describes in more detail the assay output features as well as the adjustable input parameters that control the analysis.

Overview

The Micronucleus v4.0 BioApplication focuses on the genotoxic properties of treated versus untreated cells by quantifying the micronucleus frequency, in addition to cytotoxic properties such as nuclear morphology and membrane permeability. The BioApplication measures up to three target channels, with a minimum of two channels needed to measure micronucleus frequency. Using the intensity and morphological features in Channel 1, the algorithm first identifies each cell's nucleus and potential micronucleus. The nuclei are then used to delineate the cytoplasmic boundaries of a cell in order to calculate overall nuclei count within a cell. Channel 2 is used for the cytoplasmic boundaries of the cell, again using intensity and morphological features selected within that channel.

If Channel 3 is used for membrane permeability assessment, the nuclear boundary set in Channel 1 is applied to Channel 3. By using the **CellNucMaxAvgIntenCh3LevelHigh**, **LevelLow** Assay Parameters, nuclei with intensity above the upper (and/or below the lower) threshold will be marked as cytotoxic. This channel can also be used to simply report nucleus and micronucleus intensity within the cell.

If further object selection is desired in Channel 3 (as well as Channel 4-6), the cell mask (created from either Ch1 or Ch2) can be adjusted for intensity purposes (via **MaskModifierCh3-6**).

The BioApplication also determines the status of each cell as to whether the cell is below, within, or above the levels set in the Assay Parameters. These levels can be set either manually or automatically by the application using Reference Wells. The BioApplication reports the percentage of cells in each well that are below the lower limit (**FeatureChNLevelLow**) and above the upper limit (**FeatureChNLevelHigh**).

Object Identification Methods

To identify objects in each of the images from the different channels, an independent intensity threshold must be set for each channel. In Channel 1, nuclei and micronuclei are identified; only pixels with intensities above this threshold will be considered as belonging to these

structures. In Channel 2, cellular domains are identified, and pixels with intensities above this threshold are used to help estimate target counts. Depending on the properties of the objects being identified in Channel 1 and Channel 2, the proper setting of intensity thresholds for the channels is necessary to ensure proper quantitative analysis. Analysis can also be done as a one-channel assay, with cellular domains determined from the nuclear locations using both the **CreateCellSubstituteMaskCh1** and **CreateCellSubstituteMaskModifierCh1**. If additional channels are desired, Channel 3 can be used to help identify cytotoxic cells through evaluation of nuclear membrane permeability.

There are four different options or methods for determining intensity thresholds, and sub-sets of these are available for each channel. For each Channel, you must select both a method and a value. The different options and values available for each channel are summarized in Table 3, and the descriptions of the different methods follow in Table 4.

Intensity Threshold Method	Value Range	Channel Availability		
		Channel 1	Channel 2	Channels 3-6
None	0			✓
Isodata	-0.99 – 9.99	✓	✓	
Triang	-0.99 – 9.99	✓	✓	
Fixed	0 – 32767	✓	✓	

Table 3. Intensity Threshold Methods available for each channel in the Micronucleus v4.0 BioApplication

NOTE



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

When “None” is selected as the Intensity Threshold Method (Channels 3-6), the value must be 0.

The effective range for object identification is limited to 0-4095 for the x.6 Cellomics HCS Readers. However, the entire range may be available on images from other sources.

The **None** intensity threshold method indicates that no intensity threshold is applied. This option is not available in Channel 1 or Channel 2 because in these channels an intensity threshold is required to define the pixels making up the cell and its targets.

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

The other threshold methods (**Isodata** and **Triang**) are known as histogram-derived thresholds in that the threshold is chosen from the histogram of pixel intensities in the image (i.e., the image’s brightness histogram). The schematics in Figure 8 demonstrate how these histogram-derived threshold values are calculated.

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

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

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

The two histogram-derived methods are dependent on the contents of the image, unlike the **Fixed Threshold** method. For example, supposing a blank image that contains no cellular objects and only has background pixels with a mean intensity value of 500 and standard deviation of 50, then it is unlikely that a **Fixed Threshold** of 1000 will cause any pixels to be registered as objects. However, the **Isodata Threshold** method will give thresholds causing pixels in the image to be registered for potential analysis; this is because the histogram is of the pixel intensity distribution of that image, even though there are no cellular objects in the image. The **Triangle Threshold** method is more robust for the situation of blank fields that may contain rare bright objects; this is because the peak is of the background intensity, and the “triangle”-derived offset can be set to always be above the background, yet low enough to select any bright objects. Thus, in situations where blank images are expected (e.g., from a loss of signal due to a compound condition, a loss of protein expression, or a lack of label), the **Isodata Threshold** method should be avoided; instead either a **Fixed Threshold** or the **Triangle Threshold** method with a large offset can be entered.

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

Table 4. Intensity Threshold descriptions and ranges available for the Micronucleus v4.0 BioApplication

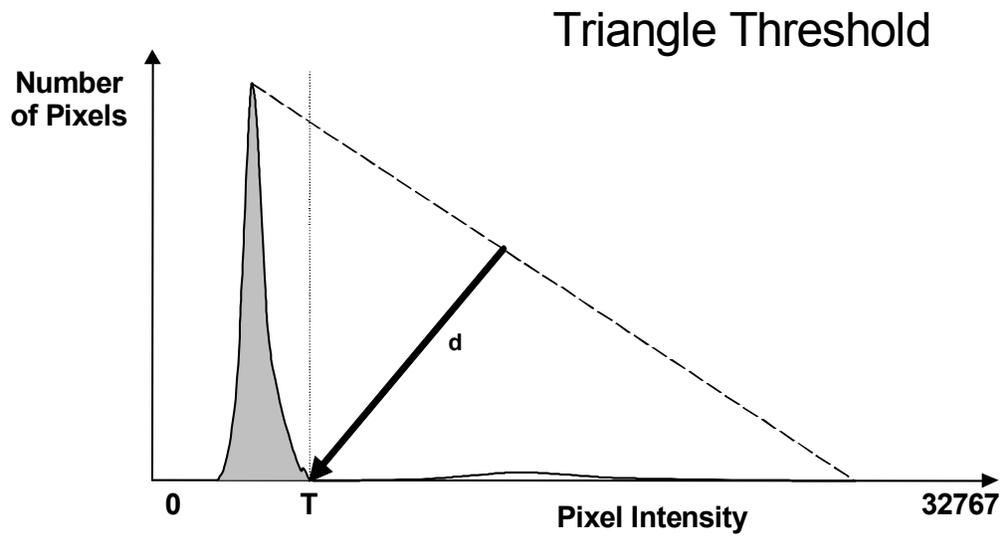
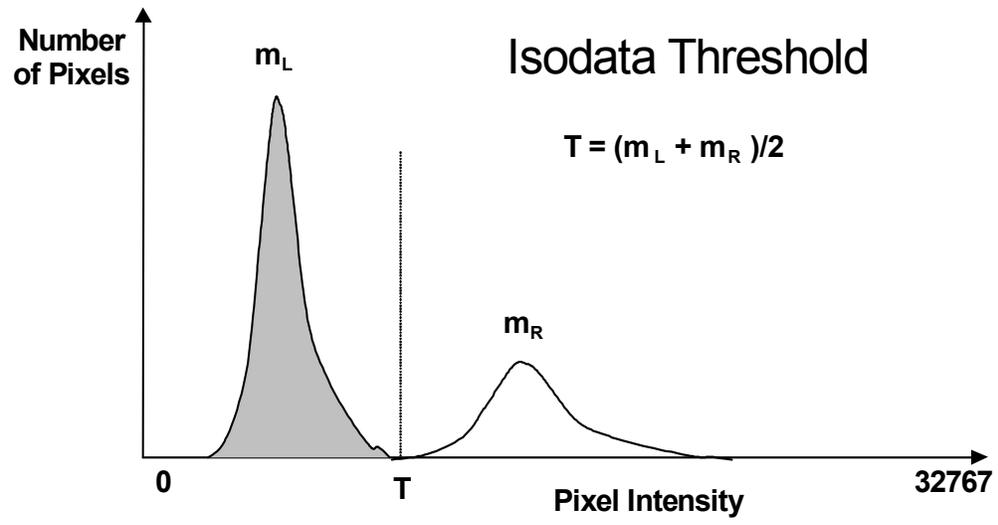


Figure 8. Histogram-derived Intensity Threshold Methods: Top – Isodata; Bottom - Triangle. Background peak is shown in gray and object peak is shown in white.

Description of Assay Parameters and Settings

The algorithm has Assay Parameters that control image analysis. The values of these Assay Parameters have been determined from demonstration biologies and are supplied as defaults in the standard Assay Protocol. Adjusting these Assay Parameters will allow customization of the algorithm to your own biology and conditions.

Assay Parameters for Image Analysis

General Assay Parameters

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

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

Reference Well Control

There are three General Assay Parameters controlling the use of Reference Wells: **UseReferenceWells**, **KnownRefWellMinAvgCellCountPerField**, and **MinRefWellMinAvgCellCountPerField**. The **UseReferenceWells** Assay Parameter allows you to choose whether Reference Wells are to be used to determine the population characterization levels.

If Reference Wells are to be used (**UseReferenceWells** = 1 or -1), then the Assay Parameters **MinRefWellMinAvgCellCountPerField** and **KnownRefWellMinAvgCellCountPerField** must be set. They represent the minimum number of cells (**MinRefWellMinAvgCellCountPerField** = Channel 3 Reference Well statistics and **KnownRefWellMinAvgCellCountPerField** = Channel 1 and Channel 2 Reference Well statistics) detected per field from each Reference Well type that are required for acceptance of the data in the Reference Wells. This allows you to enter the minimum number of objects that you feel will give a good distribution and, thus, statistical validity to the levels calculated from the Reference Wells. Note that in addition to these general Assay Parameters, there are additional Assay Parameters for Reference Well processing that are specific for particular features and channels, as described previously. These will be described in later sub-sections.

Units for Morphological Measurements

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

NOTE



When **UseMicrometers =1**, the Assay Parameter **NucIndentationDepthCh1**, and the Object Selection Parameters, **NucAreaCh1**, **MicroNucAreaCh1**, **MicroNucToNucDistanceCh1**, and **CellAreaCh2** will be specified in μm^2 (area) and μm (distance).

Object Type

NucTypeCh1, CellTypeCh2

The “Object Type” Assay Parameters allow you to specify whether the objects of interest are brighter or darker than the background of the image. Setting the value to **1** allows dark areas within an image to be considered as potential objects, while leaving it as **0** considers bright areas on a dark background as potential objects (Table 5).

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

Table 5. Binary settings for NucTypeCh1 and CellTypeCh2.

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

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

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

Background Correction

BackgroundCorrectionChN

Prior to image analysis, the non-cellular background can be computed and subtracted from the image separately in each channel, if desired, as shown in Figure 9.

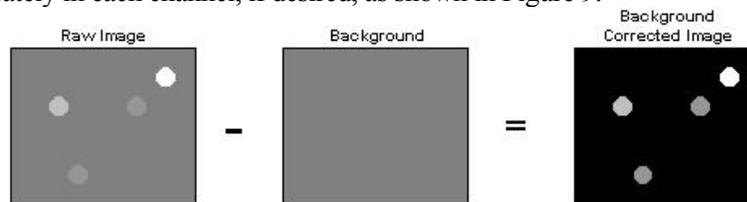


Figure 9. Image depicting calculation of positive Background Correction that can be used for each channel

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

If the **BackgroundCorrectionChN** Assay Parameter is given a negative value, an optional background correction method based on the 3D surface fitting is applied. The absolute value entered corresponds to the radius of an area used to find local minima across the image. Found minimum values are used to construct a 3D surface of a background which is then subtracted from the original image. The main advantage of 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.

Setting	Background Correction Method
0	No background correction is performed
Positive	Low pass filter method
Negative	3D surface fitting method

Table 6. Possible Background Correction Methods used with the Micronucleus v4.0 BioApplication

NOTE



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

Background Correction can be adjusted on each channel separately.

The background-corrected image is not stored or shown.

Object Smoothing

NucSmoothFactorCh1, MicroNucSmoothFactorCh1, CellSmoothFactorCh2

Object smoothing can be applied separately within both channels for nuclei, micronuclei, and cells. The image may require a degree of smoothing (i.e., blurring) before the specific object can be identified. The values for **NucSmoothFactorCh1**, **MicroNucSmoothFactorCh1**, and **CellSmoothFactorCh2** specify the degree of smoothing. A value of zero means that smoothing is not applied.

Smoothing may consolidate fragmented objects and round their boundaries, giving a more natural shape. Too much smoothing may cause multiple objects to merge into one, while an image with jagged appearances in intensity may require smoothing in order to properly detect the objects correctly. For identification of micronuclei, smoothing may increase the overall area measurement and thus cause it to be considered as a nucleus if its size ratio is larger than what is given for the **MaxMicroNucToNucSizeRatioCh1** Assay Parameter. Therefore, care needs to be given to ensure that the proper amount of smoothing is used for the biology.

Reject Border Objects

RejectBorderNucsCh1 and RejectBorderCellsCh2

Rejecting border-touching objects is specified by the **RejectBorderNucsCh1** and **RejectBorderCellsCh2** Assay Parameters. Setting both of these Assay Parameters to **1** will reject nuclei, micronuclei, and cells that touch the edge of the field. Setting the values to **0** (zero) will include border-touching nuclei, micronuclei, and cells as valid objects.

Rejecting border-touching nuclei in Channel 1 will lead to the cell not being targeted for micronucleus analysis (not having the Targeted Cell Overlay). Rejecting border-touching cells in Channel 2 can lead to the rejection of nuclei in Channel 1 (if all nuclei within the cell are rejected, the cell will also be rejected). If the cellular domain touches the edges of the image, both it and the nuclei will be rejected.

Channel-Specific Assay Parameters

The best way to easily identify all of the objects is to first focus on nuclei/micronuclei as one entity, followed by the cytoplasm, and finally cytotoxic cells.

Channel 1: Nucleus Mask Creation/Targeting Cells

The following Assay Parameters for Channel 1 are listed below. Those with a “T” preceding the Assay Parameter name specifically control whether a cell is targeted for micronucleus analysis (to calculate %**MicroNucFrequency**). Additional information for each parameter is detailed in the following sections.

For larger objects (nuclei):

- **NucSmoothFactorCh1**
- **NucSegmentationCh1**
- **NucIndentationDepthCh1**
- **NucCleanupCh1**
- **T_MinNucCountPerCellCh1**
- **T_MaxNucCountPerCellCh1**
- **T_MinNucToNucSizeRatioPerCellCh1**
- **T_MinNucToNucAvgIntensityRatioPerCellCh1**
- **RejectBorderNucsCh1**

For smaller objects (micronuclei):

- **MicroNucSmoothFactorCh1**
- **MicroNucDetectionCh1**
- **MicroNucIdentificationModifierCh1**
- **MicroNucSegmentationCh1**
- **MinMicroNucBackgroundIntensityCh1**
- **MaxMicroNucOverlapCh1**
- **MaxMicroNucToNucSizeRatioCh1**
- **T_MaxMicroNucCountPerCellCh1**

For Cellular Masks (for 1-channel assay or if Ch2 is not used for identification of cellular boundaries):

- **CreateCellSubstituteMaskCh1**
- **CellSubstituteMaskModifierCh1**
- **MinNucCellAssociationCh1**

Nucleus Assay Parameters

NucSmoothFactorCh1

This was previously mentioned on page 21 under *Object Smoothing*. Usually this value is set for a particular cell line and not altered.

NucSegmentationCh1

Two methods are available for separating nuclei: geometric (shape) and peak intensity. These are illustrated in the following figure. The geometric method splits touching objects on the basis of shape, relying on boundary indentations to locate the line of separation. This method works best if the individual objects have smooth boundaries with pronounced indentations at their point of contact. The geometric method can be used to separate objects that are uniform in intensity (i.e., saturated) or that have multiple intensity peaks (i.e., noisy or textured).

The peak 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. However, the peak intensity method can be used to separate objects that have no boundary indentations.

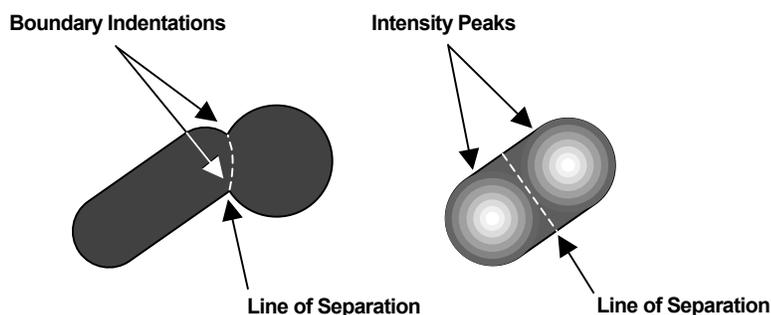


Figure 10. Object Segmentation Options. The image on the left illustrates the geometric method while the image on the right illustrates the peak intensity method.

The **NucSegmentationCh1** Assay Parameter (see Table 7) controls both methods. A positive value for this Assay Parameter selects the geometric method, and the value is the approximate radius (in pixels) of the smaller of the two objects being separated. The value of this Assay Parameter for the geometric method will depend on the magnifying power of the microscope objective and pixel binning.

Setting the Assay Parameter to a negative value selects the peak intensity method. In this case, the absolute value of the Assay Parameter specifies the minimum height of the peaks used in the segmentation. The unit value is the image intensity. For example, if

NucSegmentationCh1 = -100, peaks less than 100 intensity levels above their local background will be ignored in the segmentation. Making **NucSegmentationCh1** 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 objective or pixel binning. A value of zero disables the segmentation function.

Segmentation Method	NucSegmentationCh1 Assay Parameter Value
None	0
Geometric	N [n = radius (in pixels) of smaller object]
Peak Intensity	-n [n = minimum height of intensity peaks used in segmentation]

Table 7. NucSegmentationCh1 options for the Micronucleus v4.0 BioApplication

NucIndentationDepthCh1

If the depth of a nucleus indentation is equal to or greater than the value for the Assay Parameter, the indentation is considered to be used for a secondary segmentation (cutting through indentation dips). This could be the case for a ‘bean-shaped’ nucleus, when three or more nuclei are fused together, or nucleus and micronucleus are tightly connected. Setting the **NucIndentationDepthCh1** to **0** forces secondary segmentation on all detected indentations; setting the value to a larger number reduces the number of additional nucleus cuts to the point where no additional cuts are done. Note that some cells may require use of this Assay Parameter while others may not need to use it at all. This Assay Parameter is not altered a lot once set for a particular cell line. Figure 11 illustrates these examples below.

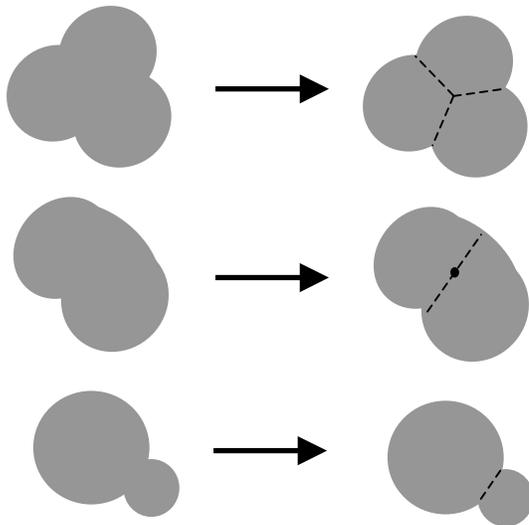


Figure 11. Examples using **NucIndentationDepthCh1** Assay Parameter. Top: separating three nuclei from each other ; Middle: separating one bean-shaped nucleus into 2 separate nuclei ; Bottom: separating a potential micronucleus from its nucleus.

NucCleanupCh1

This Assay Parameter represents the number of erosions followed by the number of dilations in order to clear nuclei of small objects. Figure 12 (left) shows a nuclei with areas that are blebbed out, (**NucCleanupCh1** = 0). Increasing the cleanup factor value caused the blebs to be eliminated from the nucleus (right), however, increasing this value too much may cause nuclei (as well as potential micronuclei) to disappear from analysis.

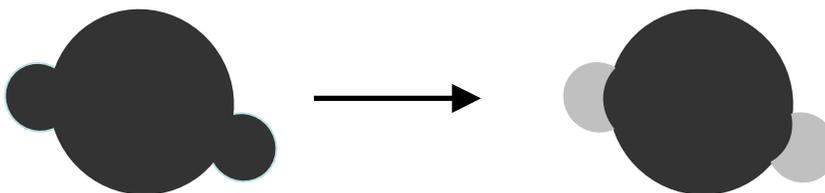


Figure 12. Using **NucCleanupCh1** to eliminate nuclear blebs from analysis. The image on the left is when **NucCleanupCh1** = 0, the image on the right after **NucCleanupCh1** = 3.

T_MinNucCountPerCellCh1

T_MaxNucCountPerCellCh1

OECD guidelines (Organization for Economic Co-operation and Development (OECD). 2004. *OECD Guideline for the Testing of Chemicals – Draft Proposal for a New Guideline 487: In Vitro Micronucleus Test*. First Version 1-13.) suggest the use of cytochalasin B for easy identification of nuclei that underwent nuclear division, forming binucleate cells. Therefore, values for these Assay Parameters represent the lower and upper limit for nuclei count per cell in order for the cell to be targeted for micronucleus processing. Default parameter is set for a minimum and maximum number of two nuclei per cell (corresponding to binucleate cells). If cells have more or less than the appropriate values, the cells can be selected for processing, but not targeted for micronucleus analysis. Once this value is set, it should not need to be altered unless the targeting criteria has changed.

T_MinNucToNucSizeRatioCh1

OECD guidelines also suggest that nuclei within each cell should be similar in size and shape. Values for this Assay Parameter represent a fractional comparison of size (area) from the smallest nucleus to the largest nucleus. As the value increases closer to 1, only cells that have nuclei identical in size will be targeted for processing. Once this value is set, it should not need to be altered unless the targeting criteria has changed.

T_MinNucToNucAvgIntenRatioCh1

If binucleate cells are analyzed, they should be similar in intensity to prove that they came from the same cell. Values for this Assay Parameter represent a fractional comparison of average intensity from the lowest to the highest. As the value increases closer to 1, only cells with nuclei identical in intensity will be targeted for processing. Once this value is set, it should not need to be altered unless the targeting criteria has changed.

RejectBorderObjectsCh1

This Assay Parameter was previously mentioned on page 22 under *Reject Border Objects*. This refers to both nuclei and micronuclei that are touching the field.

Micronucleus Assay Parameters

MicroNucSmoothFactorCh1

This Assay Parameter was previously mentioned on page 21 under *Object Smoothing*. Usually this value is set for a particular cell line and not altered.

MicroNucDetectionCh1

This Assay Parameter controls detection of small, spot-like objects (potential micronuclei) with their identification based on their intensity and morphology. A positive or negative value determines which method of small object detection is applied to the image. See Table 8 below for a brief description of the values and the method used to identify small objects (potential micronuclei).

Setting	Micronucleus Detection Method
0	Micronucleus detection is turned off
Positive	Use morphological method to detect micronucleus
Negative	Use local maxima method to detect micronucleus

Table 8: Micronucleus detection methods used via **MicroNucDetectionCh1** Assay Parameter

Positive value (Detection Radius): The spatial dimension governing which intensity changes are considered is set by the positive value of the Assay Parameter. This is directly related to the radius (half the width) of potential micronuclei. In other words, this Assay Parameter defines the size of a spatial filter that controls the removal of local slow varying background intensities in the object so that individual small, spot like objects can be detected.

Negative value (Intensity Difference): For this detection approach, all cellular components with local maxima are first identified. Then the intensity height of each local peak (local maximum - local minimum) is calculated. Only the components with intensity height equal to or above the value set by the negative Assay Parameter value are considered as potential micronuclei.

Changing the value of the Assay Parameter controls the size and the number of small spot like objects detected. Figure 13 gives an illustration to explain these differences. Using positive **MicroNucDetectionCh1** causes removal of low frequency components, resulting in detecting four small objects (A, bottom image). Using negative **MicroNucDetectionCh1** resulted in detecting three peaks out of four (from matching the intensity difference requirement for small object detection (B, bottom image). Negative **MicroNucDetectionCh1** (intensity-based method) does not require additional intensity thresholding since peaks are selected on the basis of the preset intensity difference (height).

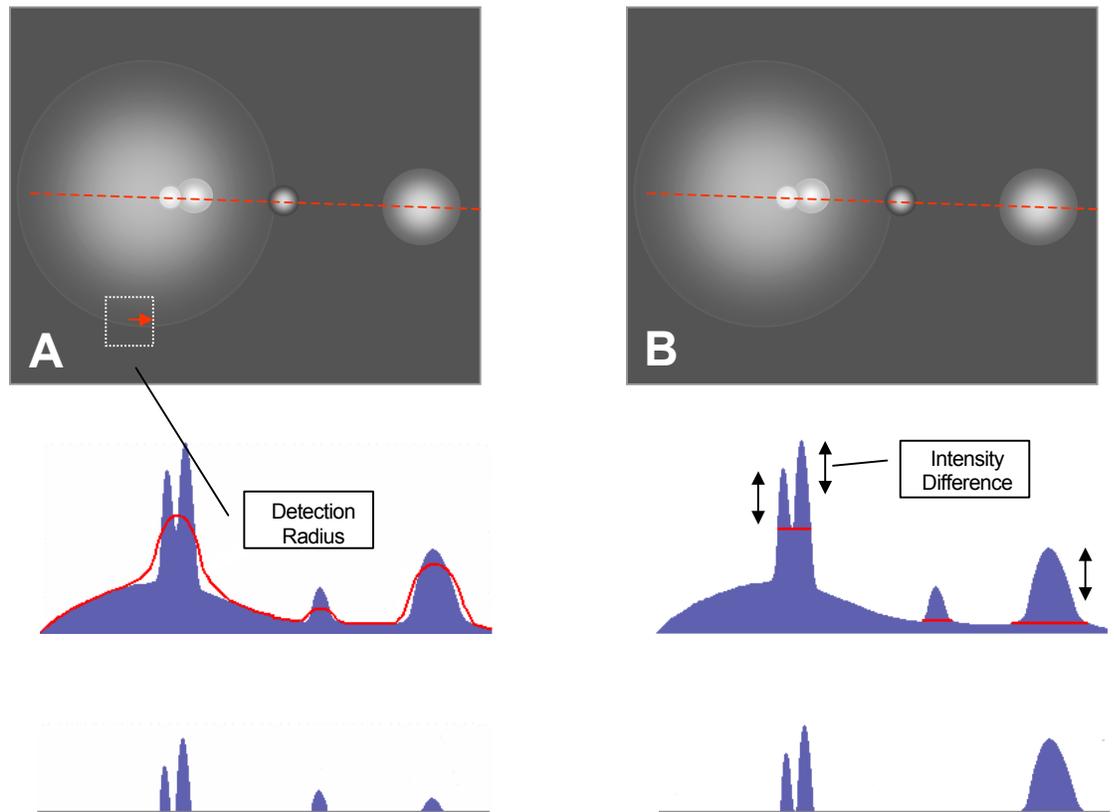


Figure 13. A: Positive **MicroNucDetectionCh1** = Morphological method (removal of low spatial frequency) for small object detection. B: Negative **MicroNucDetectionCh1** = Intensity peaks method (intensity-based) for small object detection.

MicroNucIdentificationModifierCh1

This is a fractional adjustment used to compute micronucleus identification threshold. An initial intensity threshold value is automatically calculated for the image that resulted from the use of a positive detection method to the Channel 1 image (**MicroNucDetectionCh1** is positive). Then the threshold value is adjusted by applying the fraction specified by the Assay Parameter. This Assay Parameter will not be utilized if a negative value for **MicroNucDetectionCh1** is applied. Making this value too negative may cause false nuclei and micronuclei (from background) to appear. Once set, this value should not need to be altered unless the biology has changed.

MicroNucSegmentationCh1

Micronuclei can be separated from each other (and potential micronuclei from a connected nucleus) using the **MicroNucSegmentationCh1** Assay Parameter. This Assay Parameter functions the same as **NucSegmentationCh1**. Once set, this value should not need to be altered unless the biology has changed.

MinMicroNucBackgroundIntensityCh1

This Assay Parameter is used to prevent debris from being considered as potential micronuclei. The value of the parameter is a fraction of the threshold used in nucleus detection. Only small objects with an average intensity above the **MinMicroNucBackgroundIntensityCh1** are considered for micronucleus analysis. Setting this value too low (close to 0) may cause false micronuclei to appear. Likewise, increasing this value too high (close to 1) may cause true micronuclei to not be detected. Once set, this value should not need to be altered unless the biology has changed. Values typically should not be greater than the default unless background staining (such as Acridine Orange) is evident.

MaxMicroNucOverlapCh1

This Assay Parameter is used to prevent counting larger objects identified during micronucleus detection that are touching the nuclei as potential micronuclei. The value is set as a fraction of the overlap of newly detected objects with the initially identified nucleus. Increasing this value too high may cause blebs to be considered for micronucleus analysis. Once set, this value should not need to be altered unless the biology has changed.

MaxMicroNucToNucSizeRatioCh1

This is the main Assay Parameter to separate a micronucleus from a nucleus, calculated as the largest expected ratio of a micronucleus to its nearest main nucleus within its cellular domain. If you set **MaxMicroNucToNucSizeRatioCh1** to 0, small objects will also be identified as nuclei. Default value is set to one-third the size of the nuclear diameter. Once this value is set, it should not need to be altered unless changing the targeting criteria.

T_MaxMicroNucCountCh1

This represents the maximum number of micronuclei within a Targeted Cell. Any cells containing more micronuclei than this value will not be targeted for micronucleus analysis. This Assay Parameter could be used for eliminating apoptotic cells. Once this value is set, it should not need to be altered unless the targeting criteria has changed.

Ch1 Cell and Cell Substitute Assay Parameters**CreateCellSubstituteMaskCh1, CellSubstituteMaskModifierCh1**

If Channel 2 is not desirable, both the **CreateCellSubstituteMaskCh1** and **CellSubstituteMaskModifierCh1** will be used to help create the cellular domain for targeted cells and counting micronuclei.

When **CreateCellSubstituteMaskCh1** is positive, the degree of image blur is intended to fuse nucleus objects together. The **CellSubstituteMaskModifierCh1** represents the number of pixels to further expand this created cell substitute mask. The created cell masks may touch each other but will not overlap.

When **CreateCellSubstituteMaskCh1** is negative, absolute values represent the number of dilations of nuclei in order to fuse them together. **CellSubstituteMaskModifierCh1** acts in the same manner as before.

Increasing the values in either direction may cause separate cells to fuse together. When **CreateCellSubstituteMaskCh1** is zero, analysis of the cellular domain will be done using Channel 2. However, **CreateCellSubstituteMaskCh1** is zero, the mask will be created from the individual nuclei (as mononucleate cells). Table 9 gives an overview of this Assay Parameter.

Processing Method	CreateCellSubstituteMaskCh1 Assay Parameter Value
Will use Channel 2 for analysis of cellular domain	0
Will blur nuclear objects	Positive
Will expand nuclear masks	Negative

Table 9. Processing methods associated with **CreateCellSubstituteMaskCh1**

CellSubstituteMaskModifierCh1 is simply the number of pixels to expand the cell substitute mask. If the value is **0**, the mask will not be modified and will represent the values entered for **CreateCellSubstituteMaskCh1** (if positive, it will show the number of smoothings; if negative, it will show the number of pixels dilated from the nucleus mask).

MinNucCellAssociationCh1

As per OECD Guidelines, nuclei should be within an intact cytoplasm. This Assay Parameter sets the percentage of a nucleus that should be within the cell for the nucleus to be associated with that cell. This situation also refers to micronuclei – If a micronucleus falls between two cells, whichever cell has the higher percentage of that micronuclei within its cell will be associated with it. Figure 14 illustrates both principles.

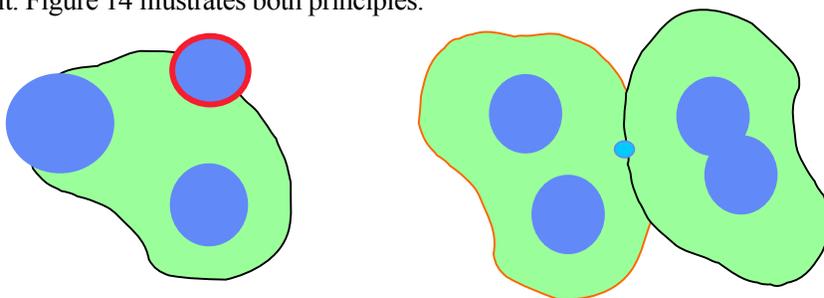


Figure 14. **Left.** Setting the **MinNucCellAssociationCh1** value to 51% will show a cell with 3 nuclei; setting it to a larger value (i.e., 70%) will only show a nucleus count of 2. **Right:** image where micronucleus falls between two cells. If the **MinNucCellAssociationCh1** value is set to 51%, the micronucleus will go to the cell on the left. If it is set to a larger value (i.e., 80%), the micronucleus does not fall at least 80% into either cell and will not be counted at all.

Depending on density, one can increase the cell mask which may help increase the cells overall association percent. This Assay Parameter is tied with **MinNucCellOverlapCh2** and will be explained in more detail with that Assay Parameter.

Channel 2: Cellular Mask Creation

If you want to utilize Channel 2 for creation of the cellular domain, the following Assay Parameters may be used:

- **CellSmoothFactorCh2**
- **CellSegmentationCh2**
- **MinNucCellOverlapCh2**
- **CellMaskModifierCh2**
- **RejectBorderCellsCh2**

CellBodySmoothFactorCh2

This was previously mentioned on page 21 under *Object Smoothing*.

CellSegmentationCh2

CellSegmentationCh2 allows segmentation of the Channel 2 cell based on shape or peak intensity, as was previously described on page 23 for the **NucSegmentationCh1** Assay Parameter.

CellSegmentationCh2 Value	Result
0	No cell segmentation
Positive	Geometric cell segmentation
Negative	Peak intensity cell segmentation

Table10. Overview of different cell segmentation options for the Micronucleus v4.0 BioApplication

MinNucCellOverlapCh2

If the cytoplasmic staining is uneven and areas of the cellular domain are not outside their corresponding nuclei, the areas can be filled by pasting in the nucleus mask so that the nuclei are then engulfed within the cell. This operation is controlled by the **MinNucCellOverlapCh2** Assay Parameter. The **MinNucCellOverlapCh2** value is a percentage of the required overlap between Channel 1 and Channel 2 objects before a Channel 1 object mask can be pasted into Channel 2.

A correlation exists between **MinNucCellAssociationCh1** and is shown in the figure below. If **MinNucCellOverlapCh2** is set low (such as 10%), the **MinNucCellAssociationCh1** may not be used since the majority of the cell will conform to the nuclei present. However, when **MinNucCellOverlapCh2** is set high (100%), **MinNucCellAssociationCh1** is very important in determining if a nucleus is found within the cytoplasm. Figure 15 illustrates this principle.

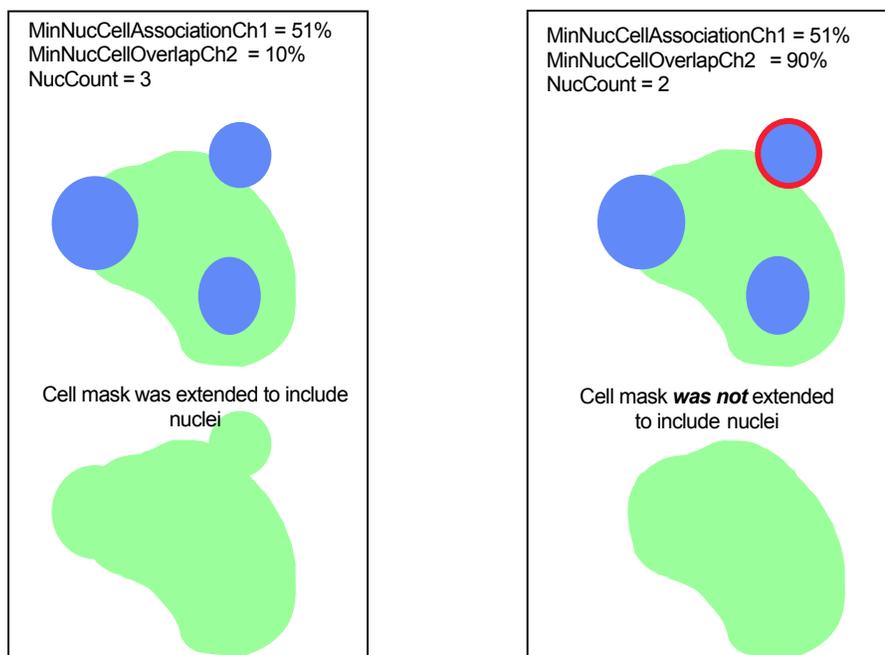


Figure 15. Using *MinNucCellOverlapCh2* and *MinNucCellAssociationCh1* to include nuclei in cells. The image on the left includes three nuclei due to lower value for *MinNucCellOverlapCh2* (bottom left). The image on the right only includes two nuclei due to the increased value for *MinNucCellOverlapCh2* (bottom right).

CellMaskModifierCh2

The size of the cell mask in Channel 2 can be adjusted using the **CellMaskModifierCh2** Assay Parameter. Increasing this mask can help with the overall identification and targeting of the cell to the nuclei. Increasing the mask can also help include other objects (such as micronuclei) into its domain. It is this mask (if **CreateCellSubstituteMaskCh1** is used) that will be the basis for the masks in Channels 3-6 for object selection.

RejectBorderCellsCh2

This was previously mentioned on page 22 under *Reject Border Objects*.

Channel 3 – Cytotoxic Cells

If three channels are desired, you can then look at compromised nuclear membrane integrity via the cytotoxic cell overlay, brought about through the following Assay Parameters:

- **T_CytotoxicCellsCh3**
- **CellNucMaxAvgIntenCh3LevelHigh, LevelLow**

T_CytotoxicCellsCh3

This Assay Parameter is used to evaluate if cells having the cytotoxic overlay should also be targeted for micronucleus analysis. If the value is **0**, cytotoxic cells will not be targeted for micronucleus analysis; a value of **1** will evaluate the cell further to see if it could be targeted for analysis. The cell must then fit the other targeting Assay Parameters in order to be targeted for micronucleus analysis.

CellNucMaxAvgIntenCh3LevelHigh, LevelLow

The **CellNucMaxAvgIntenCh3LevelHigh** and **CellNucMaxAvgIntenCh3LevelLow** Assay Parameters are the only “levels” used to indicate if a cell is cytotoxic (via Cytotoxic Cell Status and overlay) by having the maximal average intensity within the nuclear overlay above the set level (Figure 16). If there are two or more nuclei within a cell, an average intensity that is the highest of the cell’s nuclear average intensities will be compared with the value for **CellNucMaxAvgIntenCh3LevelHigh** to classify the cell’s cytotoxicity status.

Being that these are ‘level’ parameters, it might be difficult to find the correct value to identify cells with increased nuclear permeability. One way is to look at Cell Details under **CellNucMaxAvgIntenCh3** to see the value trend – normally cells with increased membrane permeability will have a much larger intensity value than normal cells. Setting this value above the normal distribution should allow you to identify the cells correctly.

Note that if images are made so that dark objects are on a bright background, **CellNucMaxAvgIntenCh3LevelLow** should then be used. Otherwise, this value should be set to zero since biologically, cells with increased membrane permeability will show increased intensity over baseline.

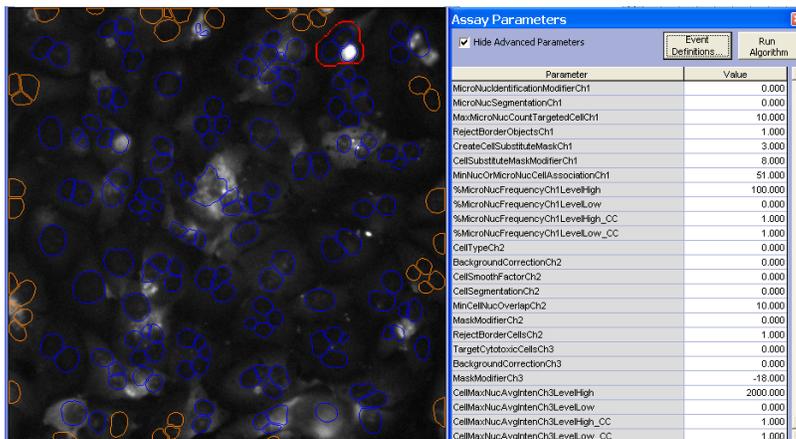


Figure 16. Example of characterizing a cell as being “cytotoxic” (red overlay) by applying the **CellNucMaxAvgIntenCh3LevelHigh** Assay Parameter

Basic Assay Parameters

Assay Parameters available in Basic Mode control the basic morphological identification of the objects and overlays in each channel, and control the use of Reference Wells (Table 11). You will not be able to view any Advanced Assay Parameters in this mode (as the **Hide Advanced Parameters** option is checked).

Parameter	Units	Description
UseReferenceWells	Number (0, 1, -1)	Use reference wells to calculate high response levels: 0 = No, 1 = Yes (V4.0 standard: scan/analyze MIN and KNOWN reference wells without order preference, -1 = Yes (scan/analyze MIN reference wells first and then KNOWN wells
NucTypeCh1	Binary	Type of nuclei and micronuclei to be identified in Ch1: 0 = Bright nuclei and micronuclei on dark background, 1 = Dark nuclei and micronuclei on bright background
BackgroundCorrectionCh1	Pixels	Radius (in pixels) of region used to compute background in Ch1: Negative value = Use surface fitting, 0 = Do not apply background correction, Positive value = Use low pass filter
NucSmoothFactorCh1	Number	Degree of image smoothing (blurring) prior to nucleus identification in Ch1: 0 = Do not apply smoothing
NucSegmentationCh1	Pixels	Radius (in pixels) or Intensity range of touching nuclei that should be separated in Ch1: Negative value = Use intensity peaks method, 0 = Do not apply object segmentation, Positive value = Use shape geometry method
NucIndentationDepthCh1	Pixel or μm	Maximum allowed depth of nucleus indentation (in pixels or micrometers) before the nucleus is cut
NucCleanUpCh1	Number	Removes small objects attached to nuclei. The value (in pixels) controls the size of objects to be removed: 0 = Do not apply clean up
T_MinNucCountPerCellCh1	Number	Minimum number of nuclei per cell for the cell to be targeted for micronucleus analysis
T_MaxNucCountPerCellCh1	Number	Maximum number of nuclei per cell for the cell to be targeted for micronucleus analysis
T_MinNucToNucSizeRatioPerCellCh1	Fraction	Minimum ratio of smaller nucleus area to larger nucleus area in a multi nuclear cell for the cell to be targeted for micronucleus analysis
T_MinNucToNucAvgIntenRatioPerCellCh1	Fraction	Minimum ratio of smaller nucleus average intensity to larger nucleus average intensity in a multi nuclear cell for the cell to be targeted for micronucleus analysis
MaxMicroNucToNucSizeRatioCh1	Fraction	Maximum ratio of smaller nucleus diameter to the closest larger nucleus diameter for the smaller nucleus to be classified as micronucleus
MicroNucSmoothFactorCh1	Number	Degree of image smoothing prior to micronucleus identification in Ch1: 0 = Do not apply smoothing
MicroNucDetectionCh1	Pixels	Radius (in pixels) or intensity range used to detect micronuclei: Negative value = Use intensity peaks method, 0 = Do not detect small objects, Positive value = Use morphological method
MicroNucIdentificationModifierCh1	Fraction	Fractional adjustment used to compute micronucleus identification threshold in Ch1
MicroNucSegmentationCh1	Pixels	Radius (in pixels) or Intensity range of touching micronuclei that should be separated in Ch1: Negative value = Use intensity peaks method, 0 = Do not apply object segmentation, Positive value = Use shape geometry method
MinMicroNucBackgroundIntensityCh1	Fraction	Micronucleus background intensity as a fraction of intensity threshold used for nucleus detection. Average intensity of a small object must be above the computed background for the object to be considered for micronucleus analysis.

Parameter	Units	Description
MaxMicroNucOverlapCh1	Fraction	Maximum fraction of area overlap of potential micronucleus mask with a parent nucleus mask. Prevents debris from entering the analysis field.
T_MaxMicroNucCountPerCellCh1	Number	Maximum number of micronuclei per cell for the cell to be targeted for micronucleus analysis
RejectBorderNucsCh1	Binary	Reject nuclei or micronuclei that touch image edges: 0 = No, 1 = Yes
CreateCellSubstituteMaskCh1	Number	Create cell substitute mask by image blurring or nucleus mask expansion: Negative = Number of pixels to expand nucleus masks until they fuse together, 0 = Do not create cell substitute mask, Positive value = Degree of image smoothing to blur close nuclei until they fuse together
CellSubstituteMaskModifierCh1	Pixels	Number of pixels to expand cell substitute mask: 0 = Do not modify mask
MinNucCellAssociationCh1	Percent	Minimum overlap (in percent) of nucleus or micronucleus mask with a cell mask for the nucleus or micronucleus to be associated with the cell
%MicroNucFrequencyCh1LevelHigh	Percent	High-response level for %MicroNucFrequencyCh1
%MicroNucFrequencyCh1LevelHigh_CC	Number	Correction coefficient (multiplier) used to set high-response level for %MicroNucFrequencyCh1 from reference well results
CellTypeCh2	Binary	Type of cells to be identified in Ch2: 0 = Bright cells on dark background, 1 = Dark cells on bright background
BackgroundCorrectionCh2	Pixels	Radius (in pixels) of region used to compute background in Ch2: Negative value = Use surface fitting, 0 = Do not apply background correction, Positive value = Use low pass filter
CellSmoothFactorCh2	Number	Degree of image smoothing (blurring) prior to cell identification in Ch2: 0 = Do not apply smoothing
CellSegmentationCh2	Pixels	Radius (in pixels) or Intensity range of touching cells that should be separated in Ch2: Negative value = Use intensity peaks method, 0 = Do not apply object segmentation, Positive value = Use shape geometry method
MinNucCellOverlapCh2	Percent	Minimum overlap (in percent) of nucleus with the cell for the nucleus mask to form extended cell mask
CellMaskModifierCh2	Pixels	Number of pixels to modify (expand) Ch2 cell mask: 0 = Do not modify mask
RejectBorderCellsCh2	Binary	Reject Ch2 cells that touch image edges: 0 = No, 1 = Yes
T_CytotoxicCellsCh3	Binary	Consider cytotoxic cells if targeted for micronucleus analysis: 0 = No, 1 = Yes
BackgroundCorrectionChN	Pixels	Radius (in pixels) of region used to compute background in Ch3: Negative value = Use surface fitting, 0 = Do not apply background correction, Positive value = Use low pass filter
MaskModifierChN	Pixels	Number of pixels to modify Ch2 cell mask or Ch1 cell substitute mask in ChN: Negative value = Shrink mask, 0 = Do not modify mask, Positive value = Expand mask
CellNucMaxAvgIntenCh3LevelHigh	Intensity	High-response level for CellNucMaxAvgIntenCh3 . Cells with CellNucMaxAvgIntenCh3 values above the set level are marked as cytotoxic (as indicated with the Cytotoxic overlay)
CellMaxNucAvgIntenCh3LevelHigh_CC	Intensity	Correction coefficient (in standard deviations) used to set high-response level for CellMaxNucAvgIntenCh3 from reference well results

Table 11. Basic Assay Parameters available for the Micronucleus v4.0 BioApplication. Note that ChN represents Channels 3-6.

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.

Table 12 represents Channel 1 Object Selection Parameters used to identify valid nuclei and micronuclei.

Channel 1 Parameters

Parameter	Units	Description
NucAreaCh1	Pixel or μm^2	Area (in pixels or micrometers) of Ch1 nucleus
NucShapeP2ACh1	Number	Shape measure based on ratio of perimeter squared to 4π *area of Ch1 nucleus (NucShapeP2ACh1 = 1 for circular nucleus)
NucShapeLWRCh1	Number	Shape measure based on ratio of length to width of object-aligned bounding box of Ch1 nucleus
NucAvgIntenCh1	Intensity	Average intensity of all pixels within Ch1 nucleus
NucVarIntenCh1	Intensity	Variation (standard deviation) of intensity of all pixels within Ch1 nucleus
NucTotalIntenCh1	Intensity	Total intensity of all pixels within Ch1 nucleus
MicroNucAreaCh1	Pixel or μm^2	Area (in pixels or micrometers) of Ch1 micronucleus
MicroNucShapeP2ACh1	Number	Shape measure based on ratio of perimeter squared to 4π *area of Ch1 micronucleus (MicroNucShapeP2ACh1 = 1 for circular micronucleus)
MicroNucShapeLWRCh1	Number	Shape measure based on ratio of length to width of object-aligned bounding box of Ch1 micronucleus
MicroNucAvgIntenCh1	Intensity	Average intensity of all pixels within Ch1 micronucleus
MicroNucVarIntenCh1	Intensity	Variation (standard deviation) of intensity of all pixels within Ch1 micronucleus
MicroNucTotalIntenCh1	Intensity	Total intensity of all pixels within Ch1 micronucleus
MicroNucToNucDistanceCh1	Number	Distance (in pixels or micrometers) from micronucleus to nearest Ch1 nucleus within Ch2 cell or cellular domain derived from Ch1 nuclei
MicroNucToNucAvgIntenRatioCh1	Number	Ratio of micronucleus average intensity to nearest Ch1 nucleus average intensity within Ch2 cell or cellular domain derived from Ch1 nuclei

Table 12. Channel 1 Object Selection Parameters available for the Micronucleus v4.0 BioApplication.

Channel 2 Object Selection Parameters are used to identify valid cells. If **CreateCellSubstituteMaskCh1** is used, there will be values representing the cell substitute mask. Table 13 is a listing of all Object Selection Parameters available for Channel 2.

Channel 2 Parameters

Parameter	Units	Description
CellAreaCh2	Pixel or μm^2	Area (in pixels or micrometers) of Ch2 cell
CellShapeP2ACh2	Number	Shape measure based on ratio of perimeter squared to 4π *area of Ch2 cell (CellShapeP2ACh2 = 1 for circular cell)
CellShapeLWRCh2	Number	Shape measure based on ratio of length to width of object-aligned bounding box of Ch2 cell
CellAvgIntenCh2	Intensity	Average intensity of all pixels within Ch2 cell
CellVarIntenCh2	Intensity	Variation of intensity (standard deviation) of all pixels within Ch2 cell
CellTotalIntenCh2	Intensity	Total intensity of all pixels within Ch2 cell

Table 13. Channel 2 Object Selection Parameters available for the Micronucleus v4.0 BioApplication

Channels 3-6 Object Selection Parameters are exclusively for object selection purposes. Table 14 is a listing of all Object Selection Parameters available for Channels 3-6.

Channel *N* (Channels 3-6) Parameters

Parameter	Units	Description
AvgIntenCh <i>N</i>	Intensity	Average intensity in Ch <i>N</i> of all pixels within modified Ch2 cell mask or Ch1 cell mask substitute
TotalIntenCh <i>N</i>	Intensity	Total intensity in Ch <i>N</i> of all pixels within modified Ch2 cell mask or Ch1 cell mask substitute

Table 14. Object Selection Parameters available for the Micronucleus BioApplication. *Note that “Ch*N*” refers to Channels 3-6.

NOTE



Object Selection Parameters for Channels 3-6 are **object selection** parameters only.

Object Selection (Gating)

The Micronucleus BioApplication supports object selection on a cell population. This feature provides selective cell processing based on fluorescence intensity. Therefore, in addition to selecting nuclei /micronuclei/cells for analysis in Channel 1 and Channel 2, you can also select or reject cells based on fluorescence intensity in Channels 3-6. Object selection allows you to specifically identify a subset of cells based on fluorescence intensity and allows you to limit the analysis to this group of cells. For example, object selection may be used to analyze only those cells showing a certain level of expression of a fluorescent reporter such as green fluorescent protein (GFP). Unlike subpopulation analysis, object selection works by rejecting any object that does not meet object selection criteria in all channels. Therefore, if you choose to add object selection channels, an object must pass the criteria in all channels to be selected for analysis.

Specifying Intensity Ranges for Object Selection

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

Specifying Mask Modifiers for Object Selection

The cell domains identified in Channel 1 or Channel 2 may be used to create measurement masks that can be applied to one or more downstream channels (i.e., Channels 3-6) for object selection. The sizes of these masks are specified using the **MaskModifierCh*N*** Assay Parameter where Ch*N* is the relevant dependent channel (i.e., Channels 3-6). Its value specifies the number of pixels by which the cell mask created in Channel 2 (or Cell Substitute Mask in Channel 1) is enlarged (positive value) or reduced (negative value). You may want to use this feature if, for example, you want to dilate the mask of each object to include other cellular markers. Masks can be dilated or eroded, but will not overlap with other masks from nearby objects. Figure 17 is a visual representation of increasing the mask modifier.

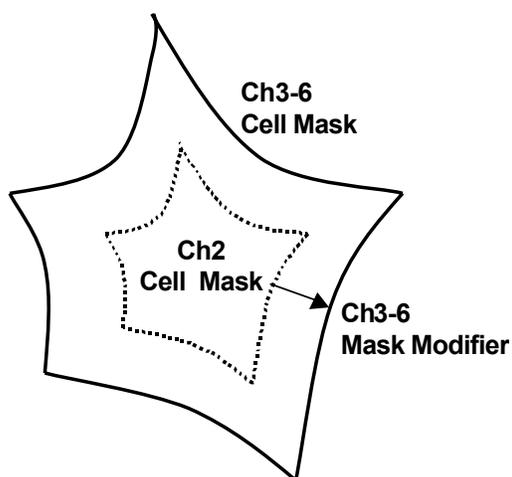


Figure 17. Adjustment of area using **MaskModifierChN** Assay Parameters for Channels 3-6

If the user would like to look at any nuclear markers in Channels 3-6, the modified mask can also be eroded to become a nuclear mask. However, selection of a “nucleus” will also select the entire cell. Again, masks can be dilated or eroded, but will not overlap with other masks from nearby objects.

If **CreateCellSubstituteMaskCh1** was used to identify the cellular region, one can use the **MaskModifierCh3-6** to return to the original nuclear masks by simply taking the negative of the combined values for **CellSubstituteMaskModifierCh1** and **CreateCellSubstituteMaskCh1** (if **CreateCellSubstituteMaskCh1** was negative). If **CreateCellSubstituteMaskCh1** was positive, the combined values will give similar results (but not exact due to the original smoothing operation of **CreateCellSubstituteMaskCh1**).

For example:

If **CreateCellSubstituteMaskCh1** = -3 and **CellSubstituteMaskModifierCh1** = 15, then **MaskModifierCh3** = -18 would give the same nucleus appearance when clicking ‘Identify Objects’ in Protocol Interactive View.

If **CreateCellSubstituteMaskCh1** = +3 and **CellSubstituteMaskModifierCh1** = 15, then **MaskModifierCh3** = -18 would give similar nucleus appearance when clicking ‘Identify Objects’ in Protocol Interactive View.

Figure 18 represents the similarity to the nuclear mask when you make the value negative.

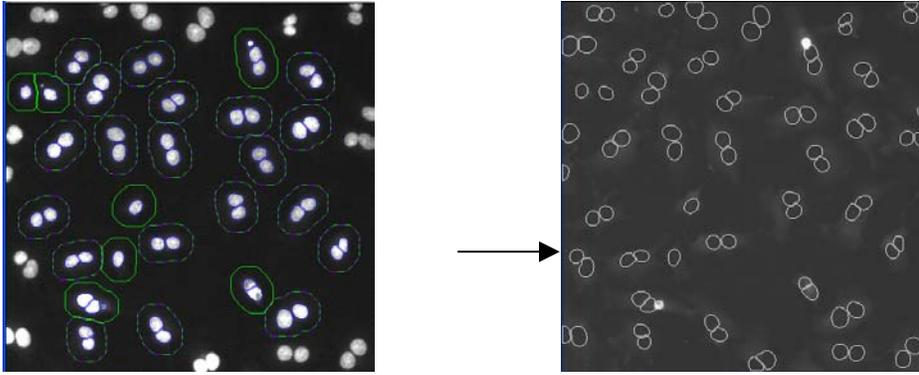


Figure 18. Adjusting **MaskModifierCh3** when **CreateCellSubstituteMaskCh1** and **CellSubstituteMaskModifierCh1** are used. By creating the negative value for **MaskModifierCh1**, the mask in Channel 3 looks similar to the nucleus mask found in Channel 1.

Once you determine the mask for each additional channel, you can then specify the intensity ranges. For each additional channel, the average intensity and total intensity values within the modified masks are calculated. If the calculated value does not fall within the specified upper and lower limits, the object is removed from the set of selected cells.

NOTE



Once the cellular domain is adjusted (either by using the **CellSubstituteMaskModifierCh1** or **CellMaskModifierCh2**), values for **MaskModifierChN** will be based on the *adjusted* cellular domain mask.

Due to cell body density, etc., it is not possible to return to the original, unaltered Channel 2 mask by simply entering the opposite value for **CellMaskModifierCh2** in other channels. One can, however, similarly return to the nuclear mask by making the **MaskModifierChN** the negative value of combining **CreateCellSubstituteMaskCh1** and **CellSubstituteMaskModifierCh1**.

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 of the ArrayScan Classic software or in the Protocol Optimization task list of the iDev software. The types of overlays that are displayed depend on the number of channels used.

Table 15 lists the following overlays that can be displayed for this BioApplication. Figure 19 is an example of the overlays found in a field.

Parameter	Description
Include This Channel In Composite	If checked, channel image is included in the composite image. Note that in order to view a composite image in Scan or View applications, at least one box must be checked.
ValidNucleus	If checked, overlays are displayed for Channel 1 nuclei that passed Channel 1 selection criteria.
RejectedNucleus	If checked, overlays are displayed for Channel 1 nuclei that failed Channel 1 selection criteria or are within rejected cells.
ValidMicronucleus	If checked, overlays are displayed for Channel 1 micronuclei that passed Channel 1 selection criteria.
RejectedMicronucleus	If checked, overlays are displayed for Channel 1 micronuclei that failed Channel 1 selection criteria.
TargetedCell (dashed line)	If checked, overlays are displayed for objects that passed Channel 2-6 selection criteria as well as specified Channel 1 Assay Parameters (T_MinNucToNucSizeRatioPerCell, T_MinNucToNucAvgIntenRatioPerCell, T_MinNucCountPerCell, T_MaxNucCountPerCell, MinNucCellAssociation)
SelectedCell	If checked, overlays are displayed for Channel 2 objects that passed the Channel 2-6 selection criteria
RejectedCell	If checked, overlays are displayed for Channel 2 objects that failed the Channel 2-6 selection criteria or have only rejected/no nuclei within their boundaries.
CytotoxicCell	If checked, overlays are displayed for Channel 3 objects that have a CellNucMaxAvgIntenCh3LevelHigh/LevelLow value above/below the threshold set.

Table 15. List and definition of overlays that can be used with the Micronucleus v4.0 BioApplication

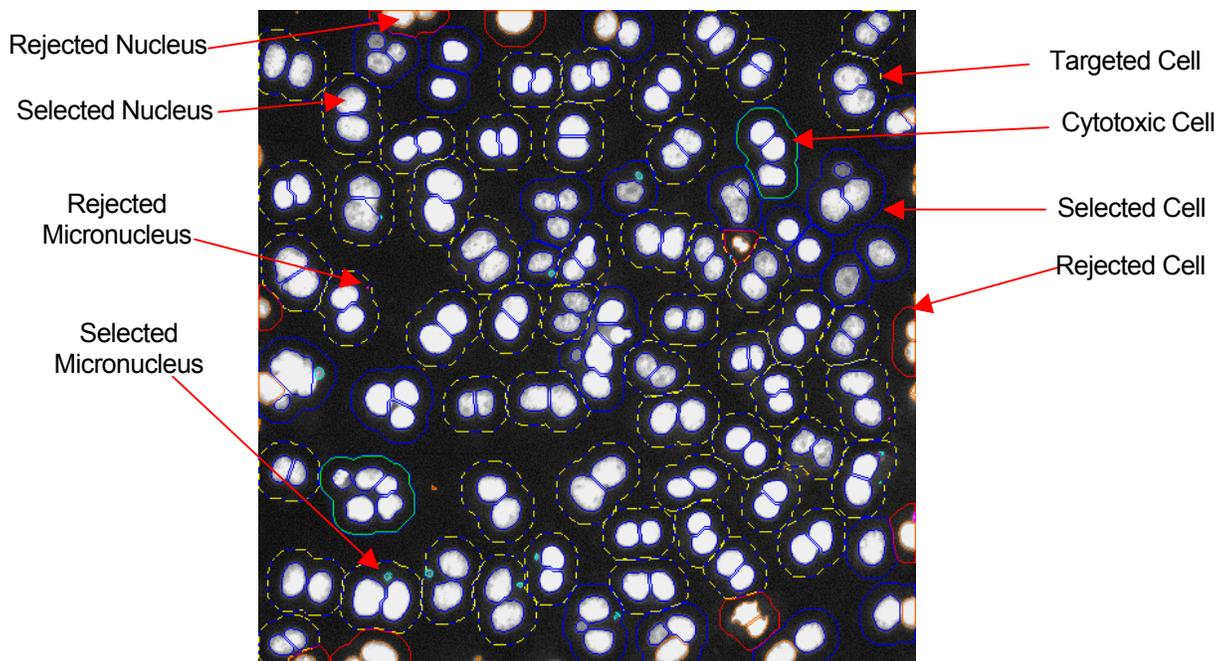


Figure 19. General set of overlays that can be used with the Micronucleus v4.0 BioApplication

Assay Parameters for Population Characterization

Overview of Population Characterization

The Micronucleus v4.0 BioApplication provides the ability to characterize cells based on their response compared to a control population. For a particular Cell Feature, a range is determined and set by measuring the physiological distribution of feature values in untreated cells. Upper and lower limits (called *FeatureChNLevelHigh* and *FeatureChNLevelLow*) set the upper and lower bounds of this range respectively. The **Status** Cell Feature values indicate whether a given cell is within or beyond this range (Table 16):

Value	Cell Status Definition
0	Feature value within defined range
1	Feature value > Upper Level
2	Feature value < Lower Level

Table 16. Numerical definitions of Cell Feature Status in the Micronucleus v4.0 BioApplication when using population characterization

The corresponding Well Features (*%HIGH_FeatureChN*, *%LOW_FeatureChN*) reported are the percentage of cells showing feature values that fall outside this range, i.e., the percentage of cells with Status Cell Feature values = 1 or 2. Figure 20 illustrates this concept by showing the population distribution for Cell Feature values for a population having a defined physiological state (e.g., untreated) and the shift in this distribution upon compound treatment.

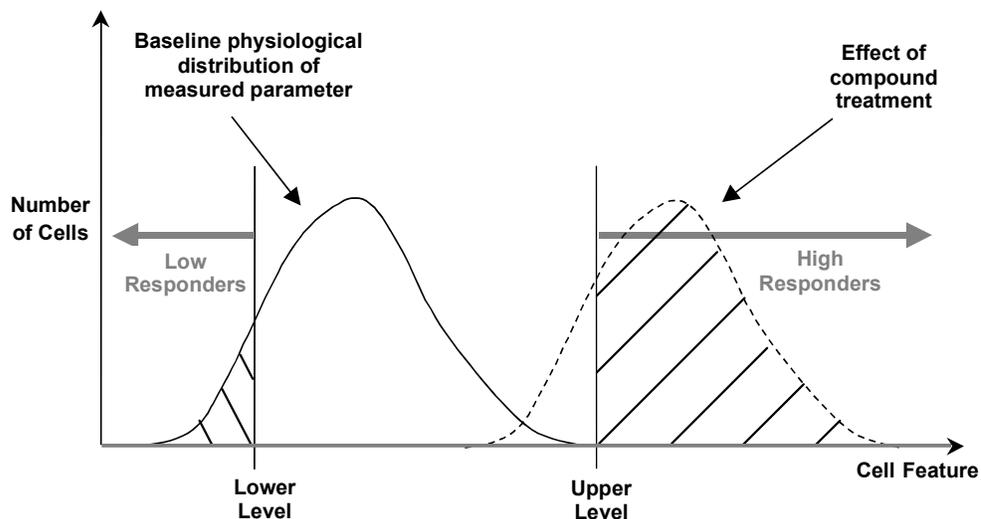


Figure 20. Principle of Population Characterization. %High exhibiting Status Cell Feature value = 1 (High Responders) or %Low exhibiting Status Cell Feature = 2 (Low Responders).

Setting Cellular Response Levels

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

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

To automatically determine the levels and set the range, the BioApplication uses Reference Wells. Typically, Reference Wells contain a control, untreated, population of cells that display the normal basal physiological response for the parameter being measured. These wells are analyzed first and then the population distribution for the different features is determined. The population characterization levels are then normally set by adding or subtracting to the mean of the distribution its standard deviation multiplied by a coefficient that you define, named correction coefficient (*_CC*) in the Assay Parameters (*FeatureChNLevelHigh_CC*, *FeatureChNLevelLow_CC*). You can use positive values for the correction coefficient to set the limit above the mean value of the feature and negative values for the correction coefficient to set limits below the mean value for the feature. For *%MicroNucFrequencyLevelHigh_CC*, *LevelLow_CC*, the correction coefficient is the fold-difference that is multiplied by the level.

Reference Wells Processing Sequence

By setting the *UseReferenceWells* Assay Parameter to **1** or **-1**, the Reference Wells processing is engaged. The specified Reference Well fields are acquired/analyzed, and Well and Plate Features are computed. Plate Feature values are directly used for cell population characterization. After this sequence is completed, computed values will be assigned to the Assay Parameters associated with each Reference Feature and regular scanning of the plate will begin. Again, if the feature values for *MinRefWellMinAvgCellCountPerField* and/or *KnownRefWellMinAvgCellCountPerField* obtained from the Reference Wells are below the value set for that Assay Parameter, the BioApplication aborts the use of Reference Wells and processes the plate as if *UseReferenceWells* is set to **0**. Also, if the Reference Feature *RefKnownWellAvgTargetedCellCountPerField* = 0, the parameters corresponding to the Reference *%MicroNucFrequencyLevelHigh*, *LevelLow* will not be updated despite if the average number of selected cells per field for KNOWN Reference Wells is equal or above the *KnownRefWellMinAvgCellCountPerField*. This Reference Feature is simply to be used as a reference: if you took a lot of reference fields (i.e., 50), the calculated value for *%MicroNucFrequencyLevelHigh* (also *LevelLow* if used) seems higher than expected for untreated wells, and the *RefKnownWellAvgTargetedCellCountPerField* is fairly low, one can expect that the total number of targeted cells for micronucleus analysis was not met. Therefore, one may want to look at how the cells are identified or if they are targeted correctly before running the rest of the plate.

The Micronucleus BioApplication uses both MIN and KNOWN Reference Wells. The sequence of computation for Reference Wells is as follows:

When *UseReferenceWells* = **1**, the standard processing of Reference Wells (as in other V4.0 BioApplications) is performed. The sequence of processing Reference Wells (for both MIN and KNOWN) is as follows:

1. Cell Features are computed for every valid object within a field.
2. For each Cell Feature to be used for population characterization, the mean and standard deviation are computed over all cells in the field.
3. The automatically calculated level values for each feature are reported as Reference Features having the name **RefFeatureChNLevelHigh**, **RefFeatureChNLevelLow**.
4. Well and Plate Features are computed as average values for fields in a well, weighted for the number of cells per field, and then as arithmetic average for wells on a plate. Use of a weighted average minimizes the effect of sparse fields.

When **UseReferenceWells = -1**, a modified processing of Reference Wells takes place (as in the Micronucleus v2 BioApplication). The sequence for this Reference Well process and calculation of Reference Features is as follows:

1. The MIN type Reference Well(s) are processed and the corresponding MIN Reference Well Plate Reference Features are computed
2. The computed values for **CellNucMaxAvgIntenCh3LevelHigh** and **CellNucMaxAvgIntenCh3LevelLow** (Cytotoxicity level) are assigned to the corresponding Assay Parameter and used to help target the population for the KNOWN Reference Wells to help determine **%MicroNucFrequency** (provided that **T_CytotoxicCells = 0**)
3. The KNOWN type Reference Wells are processed and corresponding Reference Field, Well, and Plate features computed

If **T_CytotoxicCells = 1**, the cytotoxic cells (if targeted) will be included into the calculation for **%MicroNucFrequency** independent of setting **UseReferenceWells** to **-1** or **+1**.

To guarantee the described sequence of Reference Well processing when **UseReferenceWells = -1**, the MIN type Reference Wells must be processed sequentially before the KNOWN type Reference Wells. Please see the next section for more information.

NOTE



The reference feature for **%MicroNucFrequency** is computed directly from a set of cell features and not as an average of multiple fields. Correction Coefficient for this feature is multiplied whereas the other reference features use the normal standard deviation.

If **UseReferenceWells = 1**, both the KNOWN and MIN Reference Wells are calculated simultaneously.

If **UseReferenceWells = -1** the MIN Reference Well is calculated before the KNOWN Reference Well.

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 (**MIN** or **KNOWN**). Remember, if **UseReferenceWells = -1**, the **MIN** Reference Wells need to be set above/before the **KNOWN** Reference Wells (see below). Next, enter the Starting Field and Number of Fields. Click the **Apply** button to save the settings. Please refer to the appropriate user guide for details.

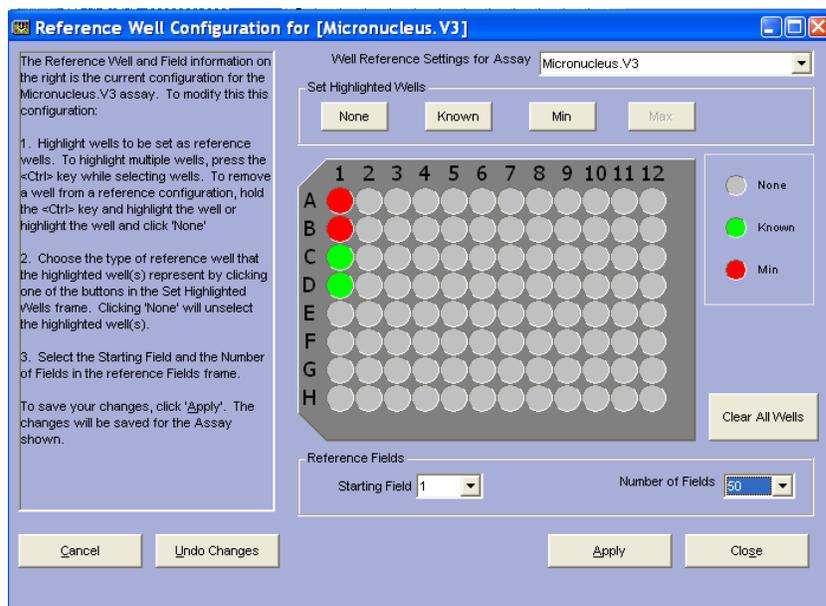


Figure 21. Proper Reference Well setting for the Micronucleus v4.0 BioApplication when **UseReferenceWells = -1**. Note that the **MIN** wells are placed above the **KNOWN** wells so that they are processed first and can be used in determining %**MicroNucFrequency**.

NOTE



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

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

Specifying and Controlling Reference Wells

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

- **UseReferenceWells = 0** – do not use Reference Wells (i.e., set ranges manually)
- **UseReferenceWells = 1** – processes both MIN and KNOWN Reference Wells simultaneously
- **UseReferenceWells = -1** – processes MIN type Reference Wells first followed by the KNOWN type Reference Wells

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

The calculation performed automatically by the BioApplication to set upper and lower limits (**FeatureChNLevelHigh** and **FeatureChNLevelLow**, respectively) is shown below:

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

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

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

The only correction coefficient different for all reference features is **%MicroNucFrequencyLevelHigh_CC**, **LevelLow_CC** which is simply a multiplier that is applied to the computed value for **%MicroNucFrequency**. It is defined as:

$$\text{\%MicroNucFrequencyCh1LevelHigh} = \text{Mean} \times \text{\%MicroNucFrequencyCh1LevelHigh_CC}$$

$$\text{\%MicroNucFrequencyCh1LevelLow} = \text{Mean} \times \text{\%MicroNucFrequencyCh1LevelLow_CC}$$

Advanced Assay Parameters

In Advanced Mode, all Basic and Advanced Assay Parameters are adjustable. The **Hide Advanced Parameters** option provides the ability to either view and adjust all the Assay Parameters or view and adjust the subset of parameters designated as Basic Parameters. For this BioApplication, Advanced Assay Parameters report responder cells and control the analysis of the data resulting from the image analysis (Table 17).

For each feature undergoing population characterization, there are four advanced Assay Parameters that control its levels: **FeatureCh/LevelHigh**, **LevelLow** that sets upper and lower thresholds and the presence of the **_CC** suffix for each which designates those levels are set using Reference Wells. For example, the Assay Parameters controlling the cell total area in Channel 2 are:

- **CellAreaCh2LevelHigh**
- **CellAreaCh2LevelLow**
- **CellAreaCh2LevelHigh_CC**
- **CellAreaCh2LevelLow_CC**

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

- **CellAreaCh2LevelHigh/Low, High/Low_CC**

Units will be expressed as what is found with **FeatureCh/LevelHigh/Low**, knowing that **_CC** is expressed as a number.

Parameter	Units	Description
KnownRefWellMinAvgCellCountPerField	Number	Minimum average number of cells per field required for acceptance of KNOWN reference well results
UseMicrometers	Binary	Measure lengths and areas in: 0 = Pixels, 1 = Micrometers
PixelSize	Number	Pixel size in micrometers (depends on objective selection)
Type_1_EventDefinition	---	User-defined combination of logic statements involving response features
Type_2_EventDefinition	---	User-defined combination of logic statements involving response features
Type_3_EventDefinition	---	User-defined combination of logic statements involving response features
%MicroNucFrequencyCh1LevelLow, Low_CC	Percent	Low-response level for %MicroNucFrequencyCh1
CellNucCountCh1LevelHigh/Low, High/Low_CC	Number	High/Low-response level for CellNucCountCh1
CellNucTotalAreaCh1LevelHigh/Low, High/Low_CC	Pixel or μm^2	High/Low-response level for CellNucTotalAreaCh1
CellNucTotalIntenCh1LevelHigh/Low, High/Low_CC	Intensity	High/Low-response level for CellNucTotalIntenCh1
CellNucAvgIntenCh1LevelHigh/Low, High/Low_CC	Intensity	High/Low-response level for CellNucAvgIntenCh1
CellNucVarIntenCh1LevelHigh/Low, High/Low_CC	Intensity	High/Low-response level for CellNucVarIntenCh1
CellMicroNucCountCh1LevelHigh/Low, High/Low_CC	Intensity	High/Low-response level for CellMicroNucCountCh1
CellAreaCh2LevelHigh/Low, High/Low_CC	Pixel or μm^2	High/Low-response level for CellAreaCh2
CellShapeP2ACh2LevelHigh/Low, High/Low_CC	Number	High/Low-response level for CellShapeP2ACh2
CellShapeLWRCh2LevelHigh/Low, High/Low_CC	Number	High/Low-response level for CellShapeLWRCh2
CellTotalIntenCh2LevelHigh/Low, High/Low_CC	Intensity	High/Low-response level for CellTotalIntenCh2
CellAvgIntenCh2LevelHigh/Low, High/Low_CC	Intensity	High/Low-response level for CellAvgIntenCh2
CellVarIntenCh2LevelHigh/Low, High/Low_CC	Intensity	High/Low-response level for CellVarIntenCh2
MinRefWellMinAvgCellCountPerFieldCh3	Number	Minimum average number of cells per field required for acceptance of MIN reference well results
CellNucTotalIntenCh3LevelHigh/Low, High/Low_CC	Intensity	High/Low-response level for CellNucTotalIntenCh3
CellNucAvgIntenCh3LevelHigh/Low, High/Low_CC	Intensity	High/Low-response level for CellNucAvgIntenCh3
CellNucMaxAvgIntenCh3LevelLow, Low_CC	Intensity	Low-response level for CellNucMaxAvgIntenCh3. Cells with CellNucMaxAvgIntenCh3 above the level are marked as cytotoxic
CellNucVarIntenCh3LevelHigh/Low, High/Low_CC	Intensity	High/Low-response level for CellNucVarIntenCh3
CellMicroNucTotalIntenCh3LevelHigh/Low, High/Low_CC	Intensity	High/Low-response level for CellMicroNucTotalIntenCh3
CellMicroNucAvgIntenCh3LevelHigh/Low, High/Low_CC	Intensity	High/Low-response level for CellMicroNucAvgIntenCh3
CellMicroNucVarIntenCh3LevelHigh/Low, High/Low_CC	Intensity	High/Low-response level for CellMicroNucVarIntenCh3

Table 17. Advanced Assay Parameters available in the Micronucleus v4.0 BioApplication. *Note that “LevelHigh/Low, High/Low_CC” refers to a total of four Assay Parameters per type.

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

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

- **Create your own definition of genotoxicity**
- **Define a subpopulation by using any combination of up to three Cell Features**
- **Report characteristics of your subpopulations**

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

Event definition is achieved through the construction of logic statements employing specific Cell Features and a set of defined logical operators. The Cell Features and Boolean operators available are listed in Table 18. The operators ANDNOT and ORNOT are obtained by combining AND + NOT and OR + NOT respectively. Definition of each logical operator is provided in schematic form in Figure 22 using two Cell Features, A and B. The entire cell population analyzed is enclosed within the box in each case. In each case, responder cells for each feature are located within the domain for that feature. Note that the definitions described in this figure can be directly applied to logic statements that contain more than two Cell Features. The Assay Parameters used to store Events are of the type **Type_X_EventDefinition** (X =1, 2, or 3).

Cell Features	Logic Operators
CellTargetedCh1	
CellNucCountCh1	
CellNucTotalAreaCh1	
CellNucTotalIntenCh1	
CellNucAvgIntenCh1	NOT
CellNucVarIntenCh1	
CellMicroNucCountCh1	AND
CellAreaCh2	
CellShapeP2ACh2	AND NOT
CellShapeLWRCh2	OR
CellTotalIntenCh2	
CellAvgIntenCh2	OR NOT
CellVarIntenCh2	
CellCytotoxicityCh3	XOR
CellNucTotalIntenCh3	NAND
CellNucAvgIntenCh3	
CellNucMaxAvgIntenCh3	
CellNucVarIntenCh3	NOR
CellMicroNucTotalIntenCh3	
CellMicroNucAvgIntenCh3	
CellMicroNucVarIntenCh3	

Table 18. Cell features and Boolean operators available for Event Definition

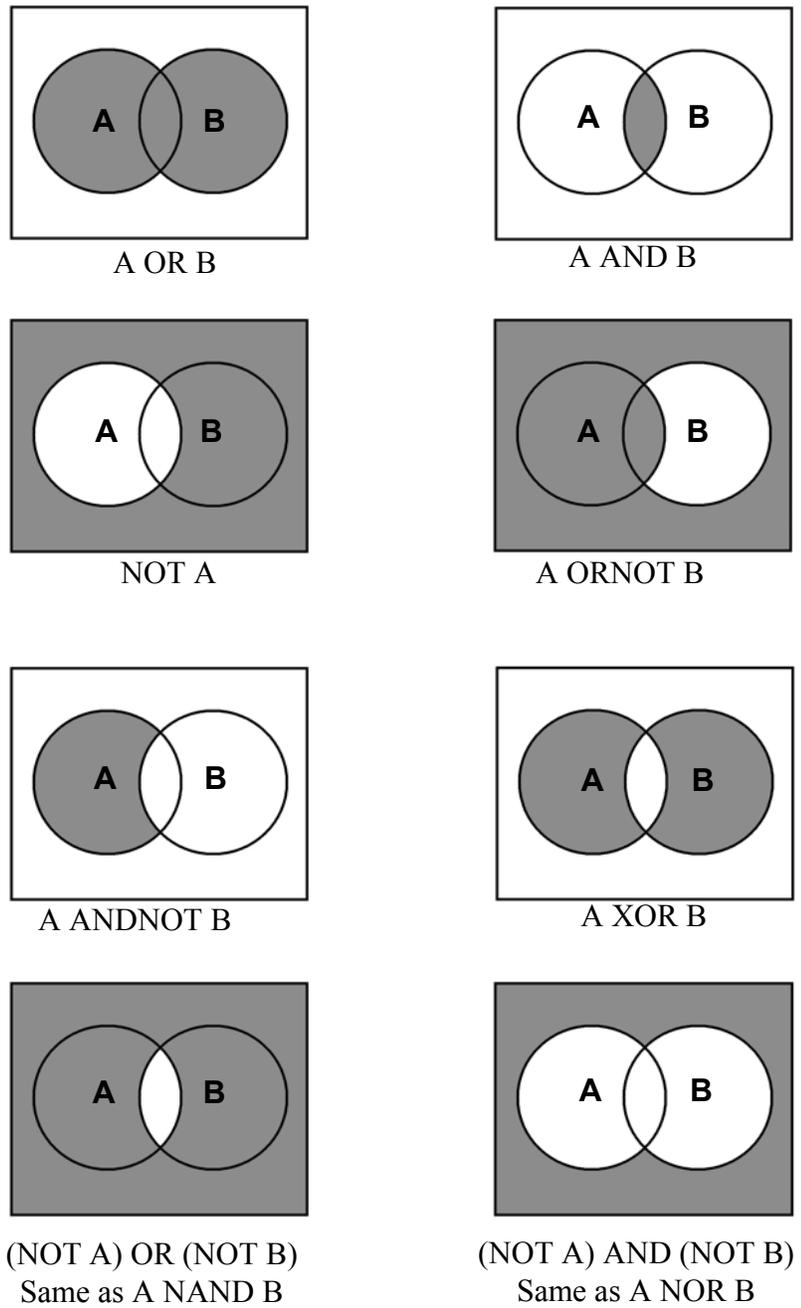


Figure 22. Schematic definition of available Boolean Operators assuming two Cell Features of interest, A and B

Description of Output Features

Output Features are the biological measurements produced by the BioApplication. All features are categorized and accessible using the vHCS:View application. Additionally, a subset of features, Field and Well Features, are listed in the Scan Plate View and Create Protocol View so that screening results can be viewed concurrently with scanning. The ability to view data during the scanning process allows you to immediately verify success of the run, well by well. The Cell, Field, and Well Output Features for the Micronucleus v4.0 BioApplication are listed and defined below.

Cell Features

Selected cells are analyzed and results are used to compute the features that are reported for each cell.

Since a cell may have more than one nucleus, the nuclear results are from all nuclei found within that selected cell. Micronucleus features can also be given for every selected cell; however, only Targeted cells (having **CellTargetedStatus = 1**) will be used for calculation of **%MicroNucFrequency**.

Micronucleus Cell Features

Table 19 shows the output features reported for each selected cell, accessible in the Cell Feature window in the Protocol Interactive View or within the vHCS:View application. Cell Features shown with a **Status** are those features that indicate if there is a high response (=1), low response (=2), or no response (=0). For Events: **0** = Event did not occur, **1** = Event occurred.

Feature	Units	Description
Cell#	Number	Unique cell ID
Top	Pixel	Y coordinate (in pixels) of top-left corner of image-aligned bounding box of Ch2 cell or Ch1 cell substitute
Left	Pixel	X coordinate (in pixels) of top-left corner of image-aligned bounding box of Ch2 cell or Ch1 cell substitute
Width	Pixel	Width (in pixels) of image-aligned bounding box of Ch2 cell or Ch1 cell substitute
Height	Pixel	Height (in pixels) of image-aligned bounding box of Ch2 cell or Ch1 cell substitute
XCentroid	Pixel	X coordinate (in pixels) of center of Ch2 cell or Ch1 cell substitute
YCentroid	Pixel	Y coordinate (in pixels) of center of Ch2 cell or Ch1 cell substitute
EventTypeProfile, EventTypeXStatus	Number	Identifies the types of events that occurred: 1, 2, 3, 12, 23, 13, 123
CellTargetedCh1Status	Binary	Cell targeted status: 0 = Cell is not used for micronucleus analysis, 1 = Cell is used for micronucleus analysis
CellNucCountCh1, Status	Number	Number of all Ch1 nuclei belonging to Ch2 cell or to Ch1 cell substitute
CellNucTotalAreaCh1, Status	Pixels or μm^2	Total area (in pixels or micrometers) of all Ch1 nuclei belonging to Ch2 cell or to Ch1 cell substitute

Feature	Units	Description
CellNucTotalIntenCh1, Status	Intensity	Total intensity of all pixels within all Ch1 nuclei belonging to Ch2 cell or to Ch1 cell substitute
CellNucAvgIntenCh1, Status	Intensity	Average intensity of all pixels within all Ch1 nuclei belonging to Ch2 cell or to Ch1 cell substitute
CellNucVarIntenCh1, Status	Intensity	Standard deviation of intensity of all pixels within all Ch1 nuclei belonging to Ch2 cell or to Ch1 cell substitute
CellMicroNucCountCh1, Status	Number	Number of all Ch1 micronuclei belonging to Ch2 cell or to Ch1 cell substitute
CellAreaCh2	Pixels or μm^2	Area (in pixels or micrometers) of Ch2 cell or Ch1 cell substitute
CellShapeP2ACh2	Number	Shape measure based on ratio of perimeter squared to $4\text{PI} \times \text{area}$ of Ch2 cell or Ch1 cell substitute mask derived from Ch1 nuclei (CellShapeP2ACh2 = 1 for circular cell) or Ch1 cell substitute
CellShapeLWRCh2	Number	Shape measure based on ratio of length to width of object-aligned bounding box of Ch2 cell or Ch1 cell substitute
CellTotalIntenCh2	Intensity	Total intensity of all pixels within Ch2 cell or Ch1 cell substitute
CellAvgIntenCh2	Intensity	Average intensity of all pixels within Ch2 cell or Ch1 cell substitute
CellVarIntenCh2	Intensity	Variation of intensity (standard deviation) of all pixels within Ch2 cell or Ch1 cell substitute
CellCytotoxicityCh3Status	Binary	Cell cytotoxicity status: 0 = Cell is not cytotoxic, 1 = Cell is cytotoxic
CellNucTotalIntenCh3, Status	Intensity	Total intensity in Ch3 of all nuclei pixels belonging to Ch2 cell or Ch1 cell substitute
CellNucAvgIntenCh3, Status	Intensity	Average intensity in Ch3 of all nuclei pixels belonging to Ch2 cell or Ch1 cell substitute
CellNucMaxAvgIntenCh3, Status	Intensity	Maximum average intensity in Ch3 of all nuclei pixels belonging to Ch2 cell or Ch1 cell substitute
CellNucVarIntenCh3, Status	Intensity	Standard deviation of intensity in Ch3 of all nuclei pixels belonging to Ch2 cell or Ch1 cell substitute
CellMicroNucTotalIntenCh3, Status	Intensity	Total intensity in Ch3 of all micronuclei pixels belonging to Ch2 cell or Ch1 cell substitute
CellMicroNucAvgIntenCh3, Status	Intensity	Average intensity in Ch3 of all micronuclei pixels belonging to Ch2 cell or Ch1 cell substitute
CellMicroNucVarIntenCh3, Status	Intensity	Standard deviation of intensity in Ch3 of all micronuclei pixels belonging to Ch2 cell or Ch1 cell substitute
TotalIntenChN	Intensity	Total intensity in ChN of all pixels within modified Ch2 cell mask or Ch1 cell substitute mask
AvgIntenChN	Intensity	Average intensity in ChN of all pixels within modified Ch2 cell mask or Ch1 cell substitute mask

Table 19. Cell Body Features available in the Micronucleus v4.0 BioApplication. *Note that ChN refers to Channels 3-6.

Field Features

Field features for this BioApplication consist only of one group of features: counts calculated for all selected cells, cells targeted for micronucleus analysis, and cytotoxic cells (Table 20). This can be used to get a general idea of overall cell density and count.

Feature	Description
ValidCellCount	Number of valid cells identified in the field (Ch1-2 object selection parameters applied)
SelectedCellCount	Number of valid cells selected for analysis in the field (Ch3-6 object selection parameters applied)
TargetedCellCount	Number of selected cells targeted for micronucleus analysis in the field
MonoNucCellCount	Number of cells that have one nucleus and were selected for analysis in the field
BiNucCellCount	Number of cells that have two nuclei and were selected for analysis in the field
MultiNucCellCount	Number of cells that have two or more nuclei and were selected for analysis in the field
MicroNucCountWithinTargetedCells	Number of micronuclei in all targeted cells in the field
MicroNucTargetedCellCount	Number of targeted cells with at least one micronucleus
CytotoxicSelectedCellCountCh3	Number of cytotoxic cells selected for analysis in the field

Table 20. General Field Features calculated in the Micronucleus v4.0 BioApplication

Well Features

Well-level summary statistics are computed from the Cell-level Features measured for all cells selected or targeted for analysis in the well. Features include mean, standard deviation, standard error, coefficient of variation, and % of responders (**%LOW**, **%HIGH**). To identify if the value came from selected or targeted cells, “_S_” or “_T_” will be inserted after the statistic.

An example of a Well Feature for each type follows:

- **MEAN_S_CellAreaCh2** = average CellAreaCh2 computed over all selected cells in the well
- **SD_T_CellAreaCh2** = standard deviation for CellAreaCh2 computed over all targeted cells in the well

In characterizing events, there are two main output features: the number and percentage of cells where a specific event occurred. Examples of this type are as follows:

- **T_EventType1CellCount** = Number of targeted cells in the well where EventType1 occurred
- **S_%EventType1Cells** = Percentage of selected cells in the well where EventType1 occurred

General Well Features associated with the Micronucleus v4.0 BioApplication (not including the above calculated statistics) are the following:

- **ValidCellCount** = Total number of valid cells identified in the well (applying all Ch2 Object Selection Parameters, or if 1 channel - if nucleus is valid and is not touching the edge of the field (when **RejectBorderNucsCh1** = 1))
- **SelectedCellCount** = Total number of valid cells *selected* for analysis in the well (applying all Channel 3-6 Object Selection Parameters if necessary)
- **%SelectedCells** = $100 \times \frac{\text{SelectedCellCount}}{\text{ValidCellCount}}$

- **BiNucCellCount** – This is the number of Selected Cells that have exactly two nuclei. One can assess the efficiency of the cytochalasin block by using the equation below.

$$(\text{BiNucCellCount}/\text{SelectedCellCount}) * 100\%$$

- **MultiNucCellCount** – This is the number of Selected Cells that have two or more nuclei. If one would like to assess how many cells have *more* than two nuclei, simply subtract this value from the BiNucCellCount:

$$\text{More than 2 nuclei/cell} = \text{MultiNucCellCount} - \text{BiNucCellCount}$$

- **MultiNucToMonoNucCellRatio** – The ratio of Selected Cells that have more than one Selected Nucleus versus Selected Cells that have only one Selected Nucleus. This can be used as an early indicator of cytotoxicity, reporting on potential cell-cycle delay.
- **Proliferation Index** – Cytokinesis-Block Proliferation Index (CBPI) used to determine the number of cell cycles per cell during the period of exposure to cytochalasin B (Kirsch-Volders et al., 2003; Organization for Economic Co-operation and Development 2004).

$$\text{CBPI is calculated as } = \frac{\# \text{ Mononucleate cells} + 2(\# \text{ Binucleate cells}) + 3(\# \text{ Multinucleate cells})}{\text{Total \# cells}}$$

A value of **1** corresponds to all mononucleate cells and 100% cytotoxicity. Another means of calculating cytotoxicity may be done by then using the CBPI with the equation below:

$$\% \text{Cytotoxicity} = 100 - (100 * ((\text{CBPI}_{\text{treated}} - 1) / (\text{CBPI}_{\text{control}} - 1)))$$

- **TargetedCellCount** = Total number of selected cells targeted for micronucleus analysis in the well (applying all necessary Assay Parameters:

T_Min/MaxNucCountPerCellCh1, **T_MinNucToNucAvgIntenRatioPerCellCh1**, **T_MinNucToNucSizeRatioPerCellCh1**, **T_MaxMicroNucCountPerCellCh1**, and **T_CytotoxicCellsCh3** = 1 (if cytotoxic))

- **%TargetedCells** = $100 \times \frac{\text{TargetedCellCount}}{\text{SelectedCellCount}}$
- **ValidFieldCount** = Total number of fields where **TargetedCellCount** = 1 or greater
- **MicroNucCountWithinTargetedCells** = Number of micronuclei in all targeted cells in the well
- **MicroNucTargetedCellCount** = Number of Targeted Cells with at least one micronucleus
- **%MicroNucFrequency** = $\frac{\text{All Targeted Cells with at least one MN}}{\text{All Targeted Cells}}$

Note that some of the features determined from Targeted cells will also be dependent on if **T_CytotoxicCellsCh3 = 1**. Therefore, for explanations below, 'NonCytotoxic' Targeted Cells refer to Targeted Cells without the CytotoxicCell Overlay in Channel 3, while 'Cytotoxic' Targeted Cells are Targeted Cells with the CytotoxicCell Overlay.

Therefore, if **T_CytotoxicCells = 1**, then:

$$\%MicroNucFrequency = \frac{\text{Targeted Cells (Cytotox and NonCytotox) with at least one MN}}{\text{All Targeted Cells (Cytotox and NonCytotox)}}$$

- **NonCytotoxMicroNucTargetedCellCountCh3** – Number of 'NonCytotoxic' Targeted Cells with at least one micronucleus calculated from all Targeted Cells ('NonCytotoxic' and 'Cytotoxic'). If **T_CytotoxicCellsCh3** is set to 0, this value will be the same as **MicroNucTargetedCellCount**.
- **%NonCytotoxMicroNucTargetedCellsCh3** – The percent of 'NonCytotoxic' Targeted Cells with at least one micronucleus calculated from all Targeted Cells ('NonCytotoxic' and 'Cytotoxic'). If **T_CytotoxicCellsCh3** is set to 0, this value will be the same as **%MicroNucFrequency**.

$$\%NonCytotoxMicroNucTargetedCells = \frac{\text{Targeted Cells (NonCytotox only) with at least one MN}}{\text{All Targeted Cells (Cytotox and NonCytotox)}}$$

- **%NonCytotoxOnlyMicroNucTargetedCellsCh3** - The percent of 'NonCytotoxic' Targeted Cells with at least one micronucleus calculated from only 'NonCytotoxic' Targeted Cells. If **T_CytotoxicCellsCh3** is set to 0, this value will be the same as **%MicroNucFrequency**.

$$\%NonCytotoxOnlyMicroNucTargetedCells = \frac{\text{Targeted Cells (NonCytotox only) with at least one MN}}{\text{NonCytotox Targeted Cells only}}$$

- **CytotoxSelectedCellCountCh3** – The number of Selected Cells that also have the *Cytotoxic* overlay.
- **%CytotoxSelectedCellsCh3** – The percent of Selected Cells that have the maximum intensity within the nuclear area above the defined level set for **CellNucMaxAvgIntenCh3LevelHigh** (or **CellNucMaxAvgIntenCh3LevelLow**). This can be used as an indicator of late-stage toxicity.
- **CytotoxTargetedCellCountCh3**– The number of Targeted Cells that also have the *Cytotoxic* overlay. If **T_CytotoxicCellsCh3** is set to 0, this feature value will be 0. **%CytotoxTargetedCells** is also calculated.
- **CytotoxMicroNucTargetedCellCountCh3** - The number of *Cytotoxic* Targeted Cells that have at least one micronucleus. If **T_CytotoxicCellsCh3** is set to 0, this feature value will be 0. **%CytotoxMicroNucTargetedCells** is also calculated.
- **%CytotoxMicroNucTargetedCellsCh3** – The percent of 'Cytotoxic' Targeted Cells with at least one micronucleus calculated from all Targeted Cells ('NonCytotoxic' and 'Cytotoxic'). If **T_CytotoxicCellsCh3** is set to 0, this feature value will be 0.

$$\%CytotoxMicroNucTargetedCells = \frac{\text{Targeted Cells (Cytotox only) with at least one MN}}{\text{All Targeted Cells (Cytotox and NonCytotox)}}$$

- **%CytotoxOnlyMicroNucTargetedCellsCh3**- The percent of ‘Cytotoxic’ Targeted cells with at least one micronucleus calculated from only ‘Cytotoxic’ Targeted Cells. If T_CytotoxicCellsCh3 is set to 0, this feature value will be 0.

$$\%CytotoxOnlyMicroNucTargetedCells = \frac{\text{Targeted Cells (Cytotox only) with at least one MN}}{\text{Cytotox Targeted Cells only}}$$

Micronucleus Well Features

Most of the Well Features are derived from a subset of the Cell Features. Such features are identified by a prefix, as listed below, to the cell feature name (Table 21) as well as a “T” for Targeted Cells and “S” for Selected Cells.

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

Table 21. General Well Features available in the Micronucleus v4.0 BioApplication

Additional Well Features representing counts and percentages calculated for all selected cells, cells targeted for micronucleus analysis, cytotoxic cells, and cellular events are shown below in Table 22.

Feature	Description
ValidCellCount	Number of valid cells identified in the well (Ch1-2 object selection parameters applied)
SelectedCellCount	Number of valid cells selected for analysis in the well (Ch3-6 object selection parameters applied)
%SelectedCells	Percentage of valid cells selected for analysis in the well
TargetedCellCount	Number of selected cells targeted for micronucleus analysis in the well
%TargetedCells	Percentage of selected cells targeted for micronucleus analysis relative to all cells selected for analysis in the well
ValidFieldCount	Number of fields in which cells were selected for analysis in the well
SelectedCellCountPerValidField	Average number of cells selected for analysis per valid field in the well
TargetedCellCountPerValidField	Average number of selected cells targeted for micronucleus analysis per valid field in the well
MicroNucCountWithinTargetedCells	Number of micronuclei in all targeted cells in the well
MicroNucTargetedCellCount	Number of targeted cells with at least one micronucleus
%MicroNucFrequency	Well percentage frequency of targeted cells with at least one micronucleus

Feature	Description
%MicroNucFrequencyStatus	Well status of percentage of targeted cells with at least one micronucleus: 1 = %MicroNucFrequency is above user defined high level, 2 = %MicroNucFrequency is below user defined low level, 0 = %MicroNucFrequency is equal or above user defined low level and equal or below user defined high level
MonoNucCellCount	Number of selected cells that have one nucleus and were selected for analysis in the well
%MonoNucCells	Percentage of selected cells that have one nucleus and were selected for analysis in the well
BiNucCellCount	Number of selected cells that have two nuclei and were selected for analysis in the well
%BiNucCells	Percentage of selected cells that have two nuclei and were selected for analysis in the well
MultiNucCellCount	Number of selected cells that have two or more nuclei and were selected for analysis in the well
%MultiNucCells	Percentage of selected cells that have two or more nuclei and were selected for analysis in the well
MultiNucToMonoNucCellRatio	Ratio of MultiNucCellCount to MonoNucCellCount
ProliferationIndex	Cytokinesis block proliferation index (CBPI): Indication of cell cycle delay during the period of exposure to cytochalasin B.
EventTypeNCellCount	Number of cells targeted for analysis in the well in which EventTypeN occurred
%EventTypeNCells	Percentage of cells targeted for analysis in the well in which EventTypeN occurred
CytotoxSelectedCellCountCh3	Number of cytotoxic (CellNucMaxAvgIntenCh3 is above preset high level or below low level) cells selected for analysis in the well
%CytotoxSelectedCellsCh3	Percentage of cytotoxic cells relative to the number of all cells selected for analysis in the well
CytotoxTargetedCellCountCh3	Number of cytotoxic cells targeted for micronucleus analysis in the well
%CytotoxTargetedCellsCh3	Percentage of cytotoxic cells relative to the number of all cells targeted for micronucleus analysis in the well
CytotoxMicroNucTargetedCellCountCh3	Number of cytotoxic cells that have at least one micronucleus and were targeted for micronucleus analysis in the well
%CytotoxMicroNucTargetedCellsCh3	Percentage of cytotoxic cells that have at least one micronucleus relative to the number of all cells targeted for micronucleus analysis in the well
NonCytotoxMicroNucTargetedCellCountCh3	Number of non cytotoxic cells that have at least one micronucleus and were targeted for micronucleus analysis in the well
%NonCytotoxMicroNucTargetedCellsCh3	Percentage of non cytotoxic cells that have at least one micronucleus relative to the number of all cells targeted for micronucleus analysis in the well
%CytotoxOnlyMicroNucTargetedCellsCh3	Percentage of only cytotoxic cells that have at least one micronucleus relative to the number of all cytotoxic cells targeted for micronucleus analysis in the well
%NonCytotoxOnlyMicroNucTargetedCellsCh3	Percentage of only non cytotoxic cells that have at least one micronucleus relative to the number of all noncytotoxic cells targeted for micronucleus analysis in the well

Table 22. Specific Well Features available in the Micronucleus v4.0 BioApplication. Note that EventTypeN represents all three Events (1, 2, and 3).

Reference Features

The algorithm reports the following features in the Scan Plate View of the software application as well as Plate Features in the vHCS:View application (Table 23). These features are computed and reported only when Reference Well functionality is enabled (**UseReferenceWells = 1** or **-1**).

Note that Reference Wells that have both High and Low levels are listed together as High/Low.

Feature	Description
RefWellType	Type of reference well used: 0 = MIN (permeability), 3 = KNOWN (all other reference features)
RefKnownWellAvgCellCountPerField	Average number of cells selected for analysis per field in KNOWN reference wells
RefKnownWellAvgTargetedCellCountPerField	Average number of cells targeted for analysis per field in KNOWN reference wells
Ref%MicroNucFrequencyCh1LevelHigh/Low	High/Low- response level for %MicroNucFrequencyCh1 computed from reference well results
RefCellNucCountCh1LevelHigh/Low	High/Low- response level for CellNucCountCh1 computed from reference well results
RefCellNucTotalAreaCh1LevelHigh/Low	High/Low- response level for CellNucTotalAreaCh1 computed from reference well results
RefCellNucTotalIntenCh1LevelHigh/Low	High/Low- response level for CellNucTotalIntenCh1 computed from reference well results
RefCellNucAvgIntenCh1LevelHigh/Low	High/Low- response level for CellNucAvgIntenCh1 computed from reference well results
RefCellNucVarIntenCh1LevelHigh/Low	High/Low- response level for CellNucVarIntenCh1 computed from reference well results
RefCellMicroNucCountCh1LevelHigh/Low	High/Low- response level for CellMicroNucCountCh1 computed from reference well results
RefCellAreaCh2LevelHigh/Low	High/Low- response level for CellAreaCh2 computed from reference well results
RefCellShapeP2ACh2LevelHigh/Low	High/Low- response level for CellShapeP2ACh2 computed from reference well results
RefCellShapeLWRCh2LevelHigh/Low	High/Low- response level for CellShapeLWRCh2 computed from reference well results
RefCellTotalIntenCh2LevelHigh/Low	High/Low- response level for CellTotalIntenCh2 computed from reference well results
RefCellAvgIntenCh2LevelHigh/Low	High/Low- response level for CellAvgIntenCh2 computed from reference well results
RefCellVarIntenCh2LevelHigh/Low	High/Low- response level for CellVarIntenCh2 computed from reference well results
RefMinWellAvgCellCountPerField	Average number of cells selected for analysis per field in MIN reference wells
RefCellNucTotalIntenCh3LevelHigh/Low	High/Low- response level for CellNucTotalIntenCh3 computed from reference well results
RefCellNucAvgIntenCh3LevelHigh/Low	High/Low- response level for CellNucAvgIntenCh3 computed from reference well results
RefCellNucMaxAvgIntenCh3LevelHigh/Low	High/Low- response level for CellNucMaxAvgIntenCh3 computed from reference well results
RefCellNucVarIntenCh3LevelHigh/Low	High/Low- response level for CellNucVarIntenCh3 computed from reference well results
RefCellMicroNucTotalIntenCh3LevelHigh/Low	High/Low- response level for CellMicroNucTotalIntenCh3 computed from reference well results
RefCellMicroNucAvgIntenCh3LevelHigh/Low	High/Low- response level for CellMicroNucAvgIntenCh3 computed from reference well results
RefCellMicroNucVarIntenCh3LevelHigh/Low	High/Low- response level for CellMicroNucVarIntenCh3 computed from reference well results

Table 23. Reference Features available in the Micronucleus v4.0 BioApplication

Using the BioApplication Event Wizard

This chapter describes in more detail the use and modification of the Micronucleus v4.0 BioApplication Assay Protocol through implementation of the 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 Micronucleus v4.0 BioApplication. This section provides a detailed description of the operation of the BioApplication Event Wizard. The Wizard should only be used after the Micronucleus v4.0 BioApplication has been installed on your computer. Generally:

STEP I

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

STEP II

- 1) If you closed the application, open the BioApplication Event Wizard by clicking on the icon, login, and select the protocol saved in Step 1. If the application is still open, simply click the Event Definitions button in Create Protocol View within the Scan window.
- 2) Select up to 3 events at the level of individual cells (maximum of 4 features per event).
- 3) Save updated Assay Protocol (either by clicking Save and Exit if in the stand-alone Event Wizard or click OK and then Save Assay Protocol if in the Create Protocol View).

STEP III

- 1) If in stand-alone Event Wizard mode, close the Event Wizard, 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.

Event Wizard Features

The following features are included in the BioApplication Event Wizard:

- Can be used with any Micronucleus v4.0 protocol
- 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 Micronucleus

It is suggested that you first create a protocol using the Micronucleus v4.0 BioApplication without defining Events. Set optimized parameter values (upper levels) for detection of responder cells associated with Cell Features to be used for Event Definition and save the protocol.

A. To start the BioApplication Event Wizard from a Saved Protocol:

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

-or-



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

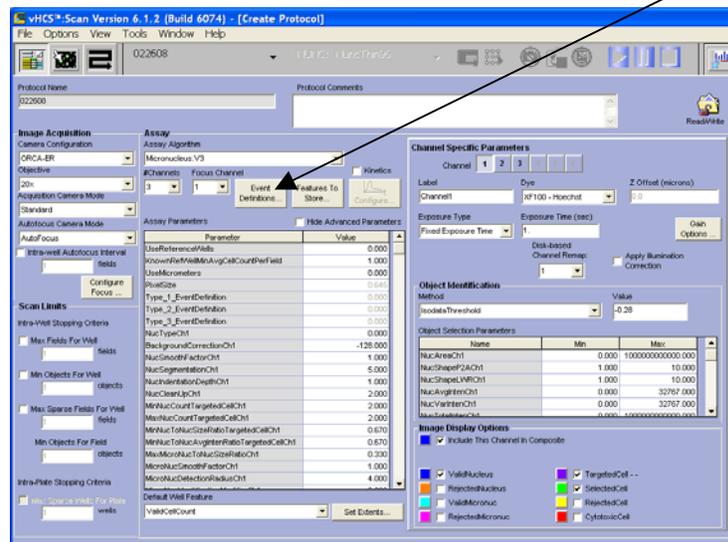
For vHCS Discovery Toolbox computers: From the Windows **Start** menu, select **Programs > Cellomics > vHCS Scan > BioAppEventWizard**

- 2) Login as if you would for the Scan application. The first window that is displayed (see below) allows selection of any protocol that can use events. Note that Event Definitions can only be specified for pre-existing Assay Protocols.
- 3) Select “Open Protocol...” and select the desired Protocol from the list.

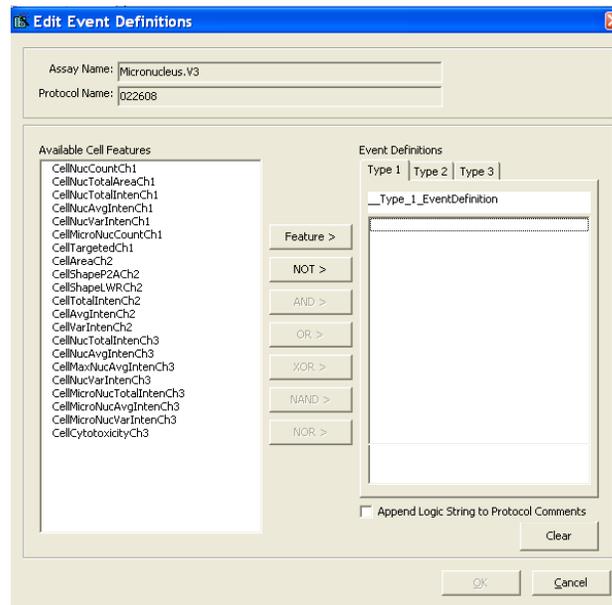


B. To start the BioApplication Event Wizard from an Opened Protocol:

1) Simply click on the Event Definition button within the Create Protocol View.



Either way, 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 the Assay Protocol is Read-Only, you will only be able to view the Event Definitions and a message will indicate that the protocol cannot be edited.

2) Once you have completed the Event Definitions, click **OK**. The screen will close and leave you in the Create Protocol View.

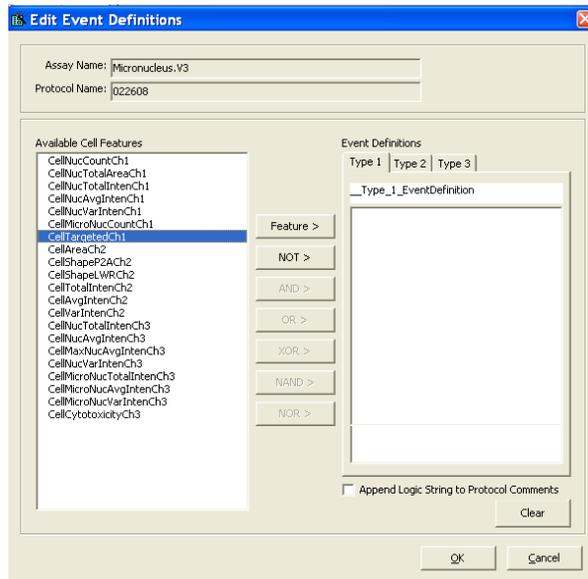
Defining Events

This section describes the steps involved in defining individual Events in a Micronucleus v4.0 Assay Protocol. Note that logical statements used to define Events can include up to four Cell Features and four logical operators. Once the protocol has loaded, each Event Definition can be viewed by clicking on the appropriate tab (Type 1-3).

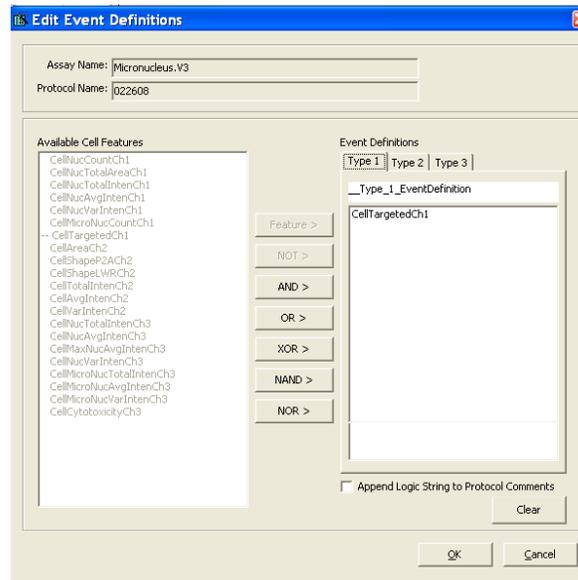
The procedure to be followed when constructing Event Definitions is described in the sequence of screenshots below. Cell Features are combined with Boolean operators to produce Event Definitions. Any Cell Feature can be selected by clicking on the feature name in the Available Cell Features list and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

To build an Event Definition

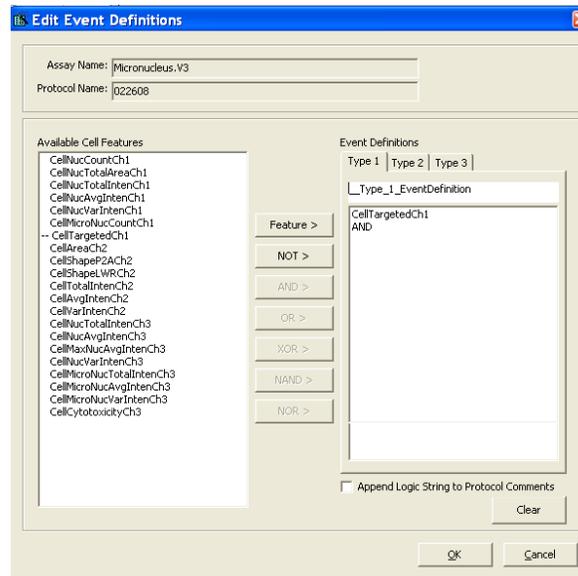
- 1) Select the Event Definition that you want to specify or edit by clicking on the **Type 1**, **Type 2**, or **Type 3** tab.
- 2) Select the desired Cell Feature by clicking on the feature name from the Available Cell Features list. In this example, the **CellTargetedCh1** feature is selected. You may also choose to select **NOT**> first (before the Feature) to indicate that you do not want to include this Cell Feature in your event analysis.



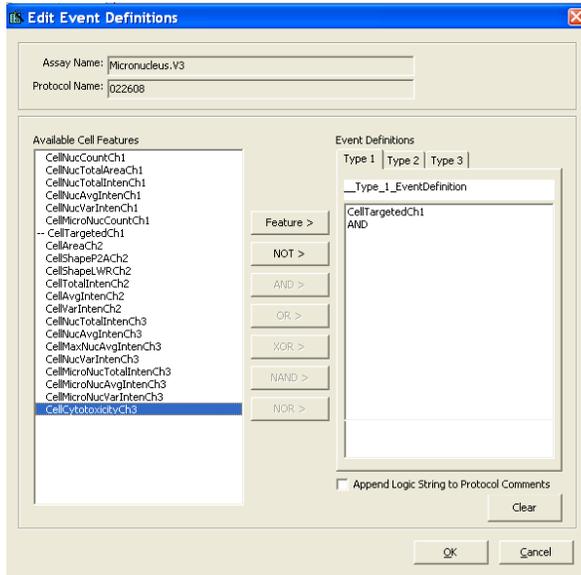
- 3) Click the **Feature>** button to transfer the Cell Feature into the Event Definition.



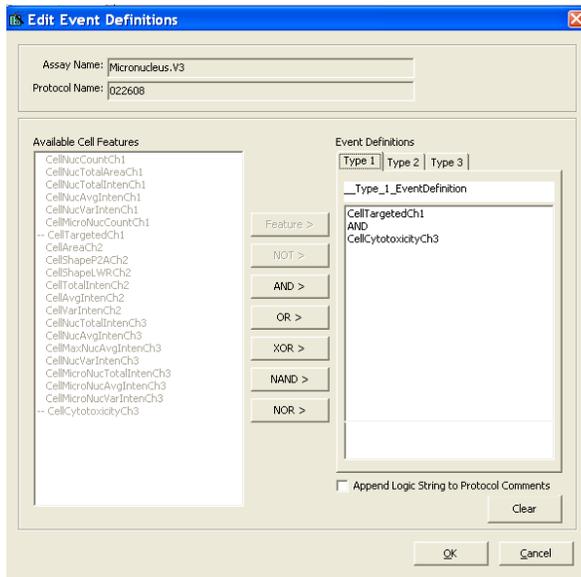
- 4) Click on the desired Operator button to transfer it into the Event Definition. In this example, the **AND** operator is selected (see below).



- 5) Repeat the cycle for adding another Cell Feature to the Event Definition. In this case, the Cell Feature **CellCytotoxicityCh3** has been selected for addition.

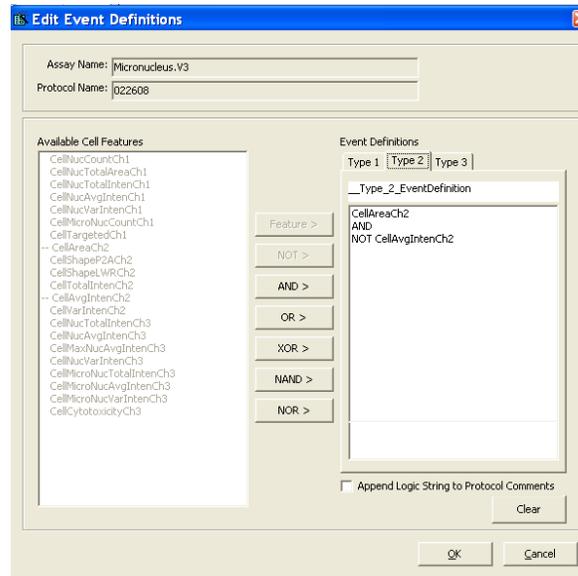


- 6) As before, click on the **Feature >** button to transfer the Cell Feature into the Event Definition (see below).



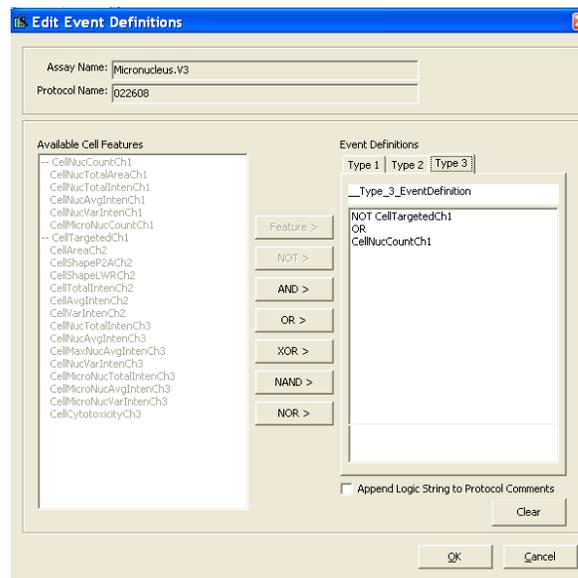
Type_1_EventDefinition is: **CellTargetedCh1 AND CellCytotoxicityCh3**

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 is: **CellAreaCh2 ANDNOT CellAvgIntenCh2**

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.

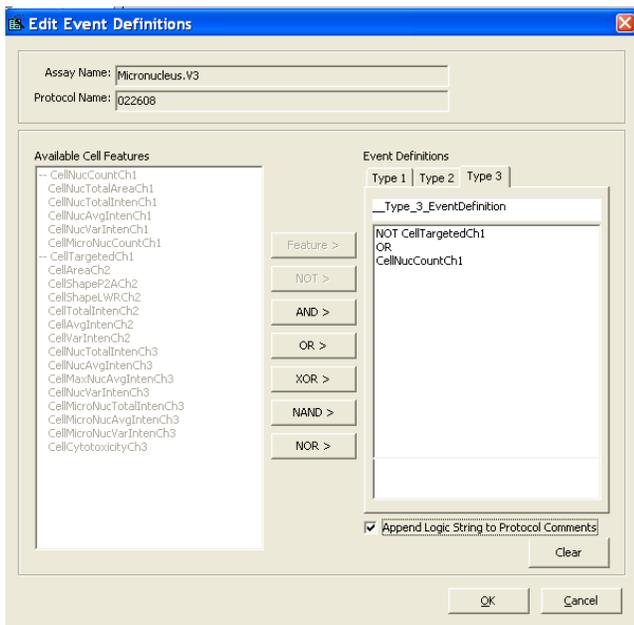


To clear an Event Definition

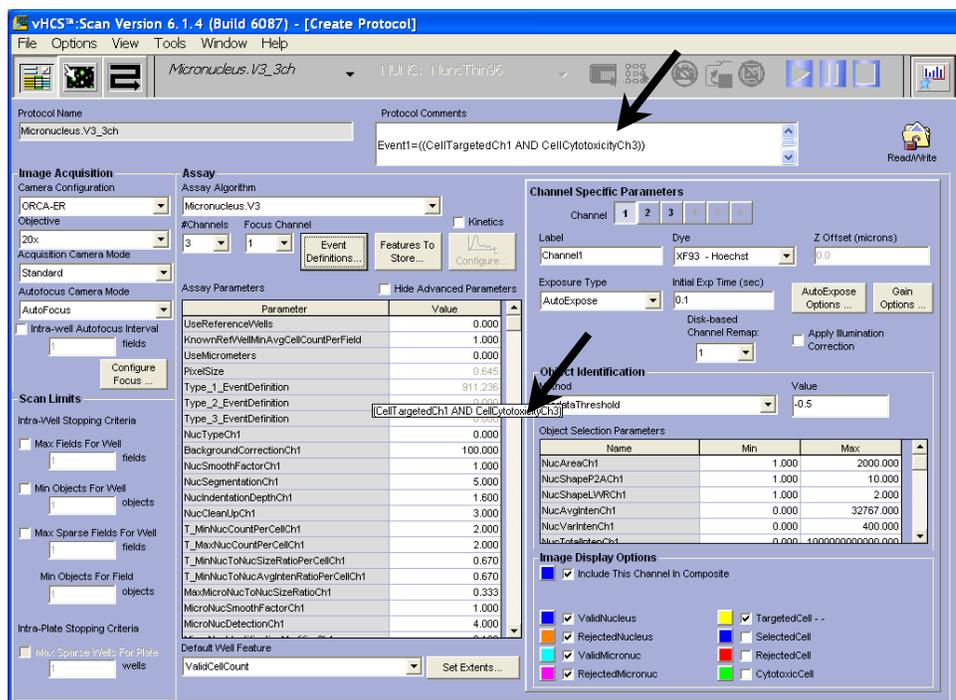
- Once a protocol is loaded, click the **Clear** button to clear any Event Definition. This feature can be used to redefine any event.

To Show the Events within the Protocol

- Click the **Append Logic String to Protocol Comments** option. This will transfer the definitions into the protocol.



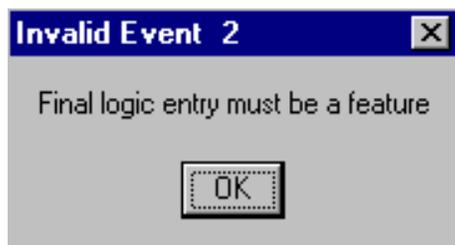
Event Definitions are then viewable in the **Protocol Comments** field in the **Create Protocol View** upon opening the saved Assay Protocol using the Scan software application. Otherwise, definitions are viewable by hovering over the value in the Assay Parameter list.



To save the updated Assay Protocol with Events

- 1) Click the **OK** button. This will close the Events Wizard.
- 2) Click File → Save Assay Protocol in the Scan software application. 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.

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.



iDev Software Workflow

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

iDev Protocol Optimization Tasks

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

Image Preprocessing

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

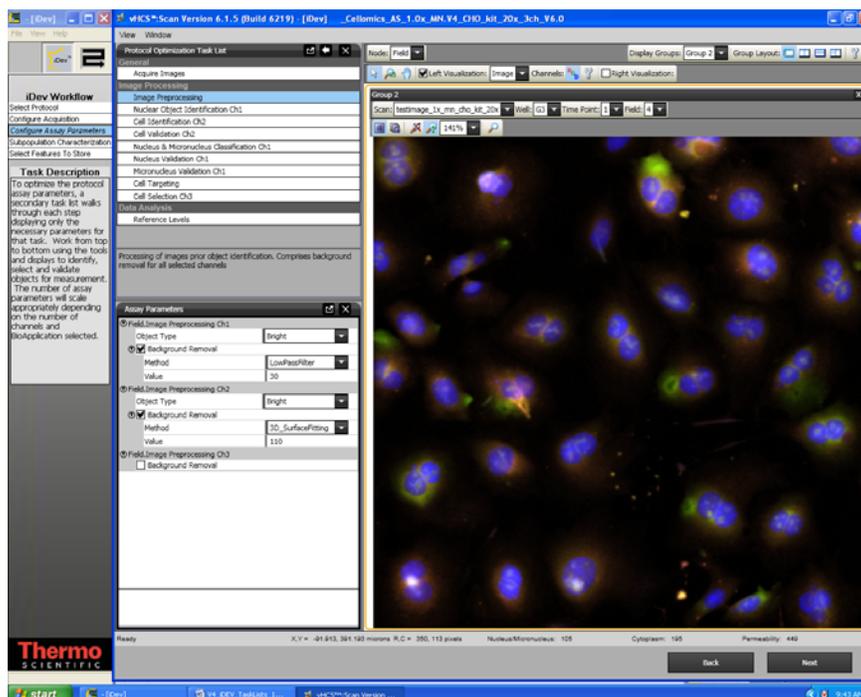


Figure 23. Protocol Optimization Task – Image Preprocessing

Nuclear Object Identification Ch1

Nuclear Object Identification is the identification of nuclei and micronuclei in the Channel 1 image. The task comprises setting methods and values for object smoothing, thresholding/detection, and segmentation. Additionally in this task you can set the maximum area overlap between a micronucleus and its parent nucleus and also the minimum background intensity of the micronucleus as a fraction over the intensity threshold set for nucleus detection.

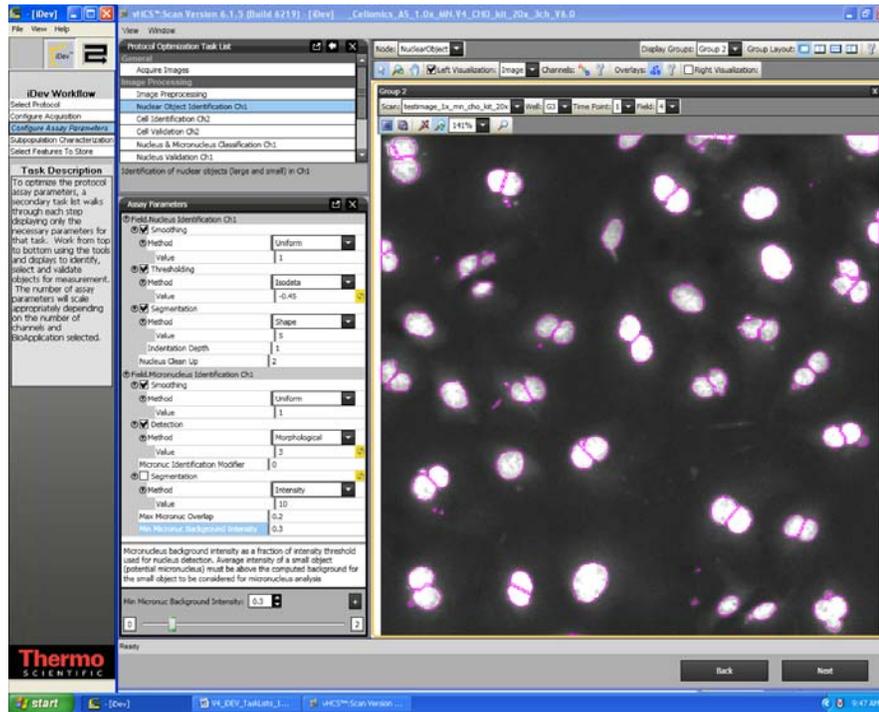


Figure 24. Protocol Optimization Task – Nuclei Object Identification Ch1

Cell Identification Ch2

In this task, you will set the identification methods and value for the cells in Channel 2. This includes methods and values for smoothing, thresholding, and segmentation. In this task, you can also set minimum percent overlap of the nucleus with the cell to extend the cell mask.

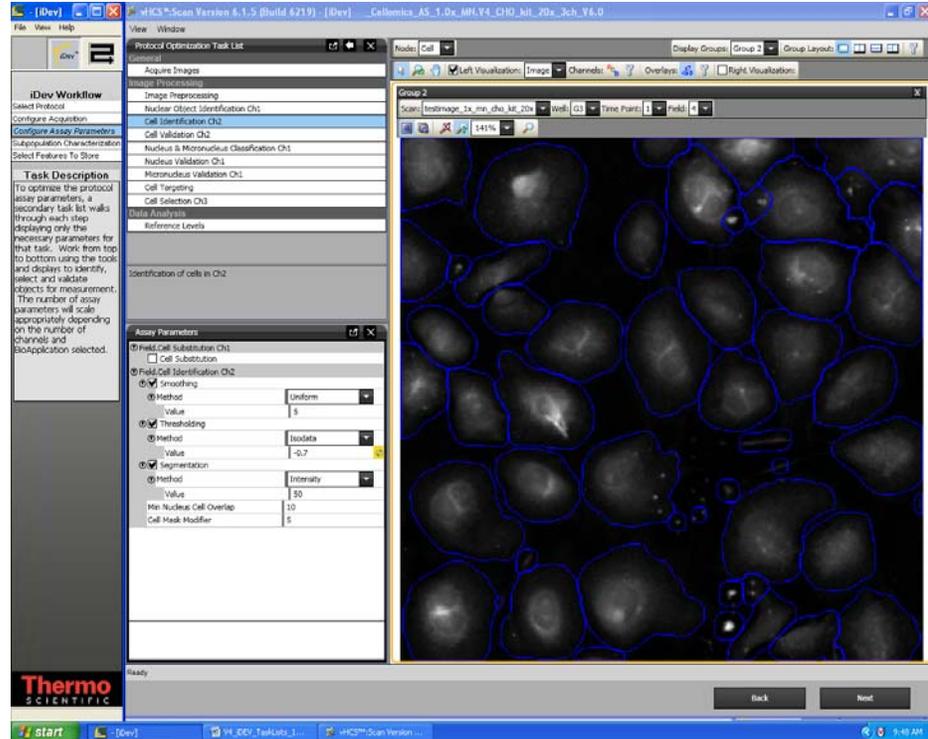


Figure 25. Protocol Optimization Task – Cell Identification Ch2

Cell Validation Ch2

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

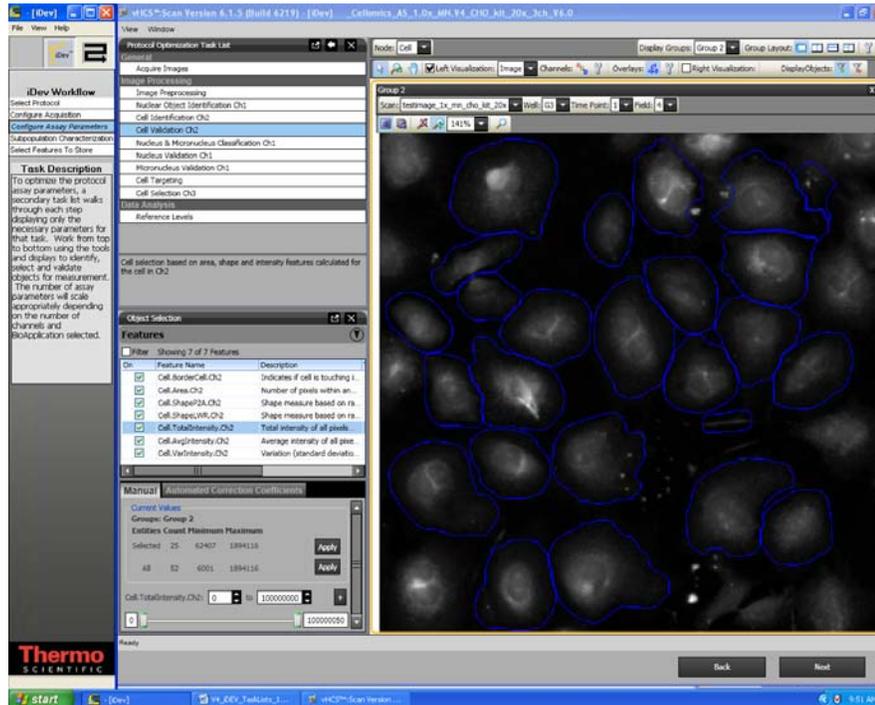


Figure 26. Protocol Optimization Task – Cell Validation Ch2

Nucleus and Micronucleus Classification

In this task, you will classify nuclear objects as either nucleus or micronucleus. This is accomplished by setting the minimum percent of overlap of the nucleus or micronuclei with the cell and setting the maximum ratio of a small nucleus to its closest nuclei to classify the smaller as a micronucleus.

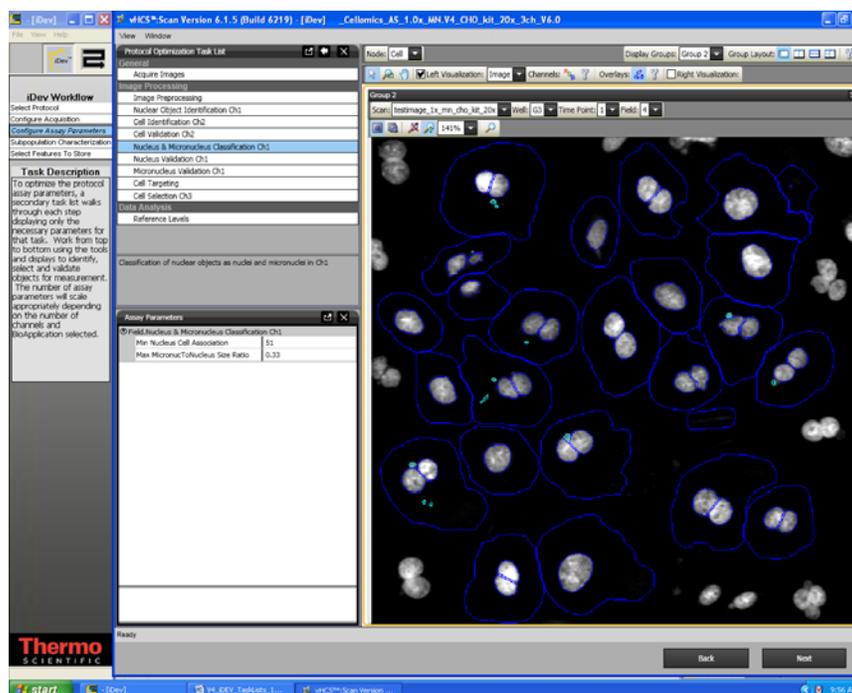


Figure 27. Protocol Optimization Task – Nucleus and Micronucleus Classification

Nucleus Validation Ch1

In this task, you will set minimal and maximal values for validating (equivalent to selecting and rejecting objects in the ArrayScan Classic software) nuclei in Channel 1 based on object selection features. Additionally in this task you will determine if nuclei that are on the border of the field are included or rejected from analysis.

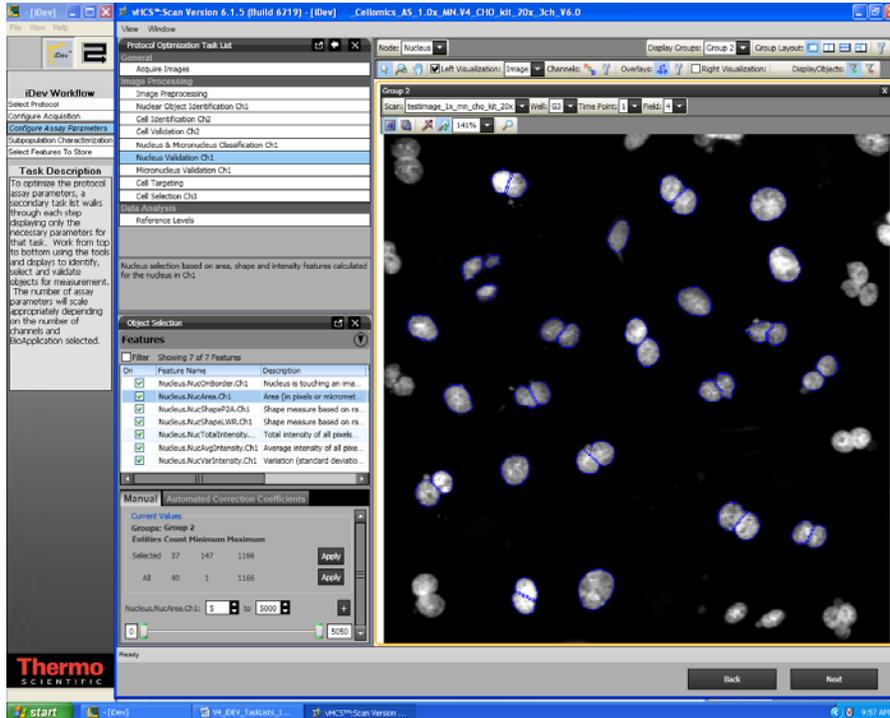


Figure 28. Protocol Optimization Task – Nucleus Validation Ch1

Micronucleus Validation Ch1

In this task, you will set minimal and maximal values for validating (equivalent to selecting and rejecting objects in the ArrayScan Classic software) micronuclei in Channel 1 based on object selection features. Additionally in this task you will determine if micronuclei that are on the border of the field are included or rejected from analysis.

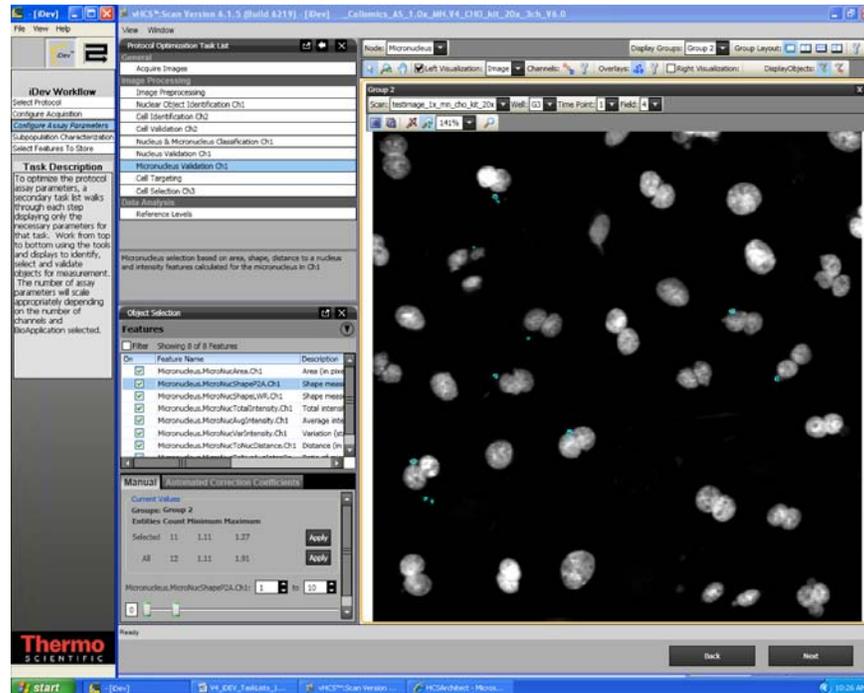


Figure 29. Protocol Optimization Task – Micronucleus Validation Ch1

Cell Targeting

In this task, you will set criteria for the cells based on nucleus count, ratio of small nuclear area to larger nuclei, ratio of small nuclei intensity to large nuclei intensity, and maximum micronuclei count in a cell.

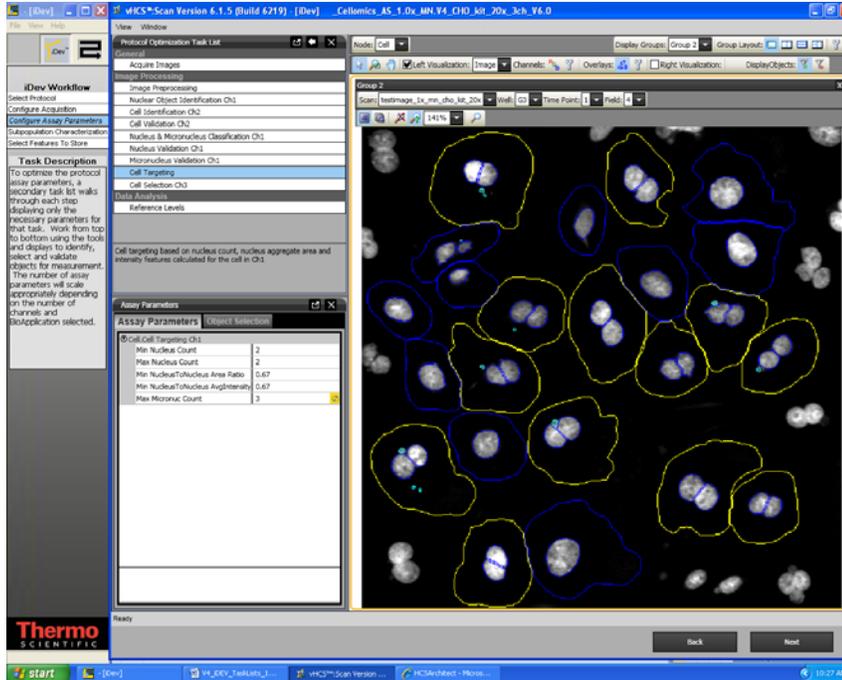


Figure 30. Protocol Optimization Task – Cell Targeting and Filtering Ch1

Cell Selection Ch3 through ChN

In this task, you can set criteria for selection of cells in Channels 3 through Channel N based on an ROI mask derived from the Channel 3 cell mask.

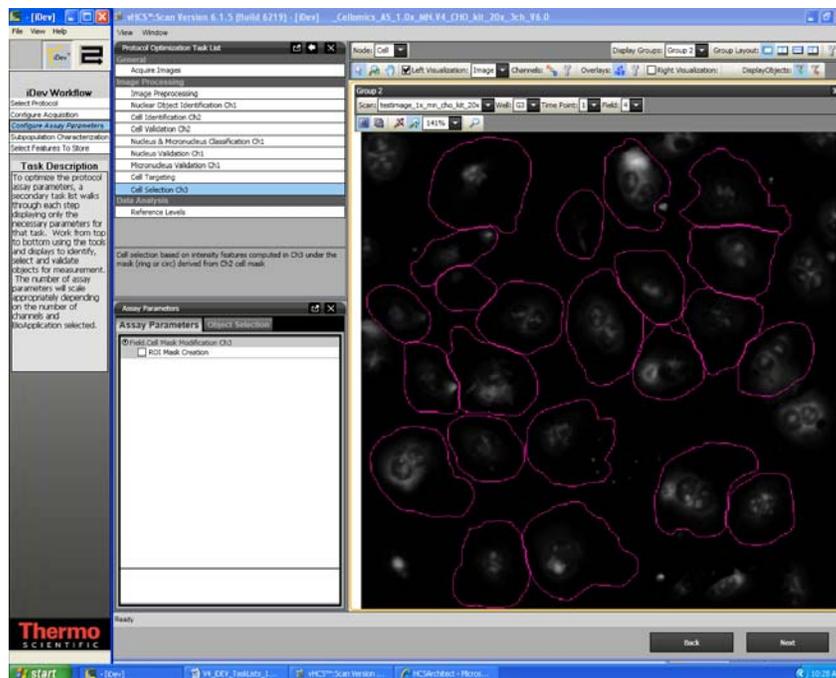


Figure 31. Protocol Optimization Task – Cell Selection Ch3 through ChN

Reference Levels

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

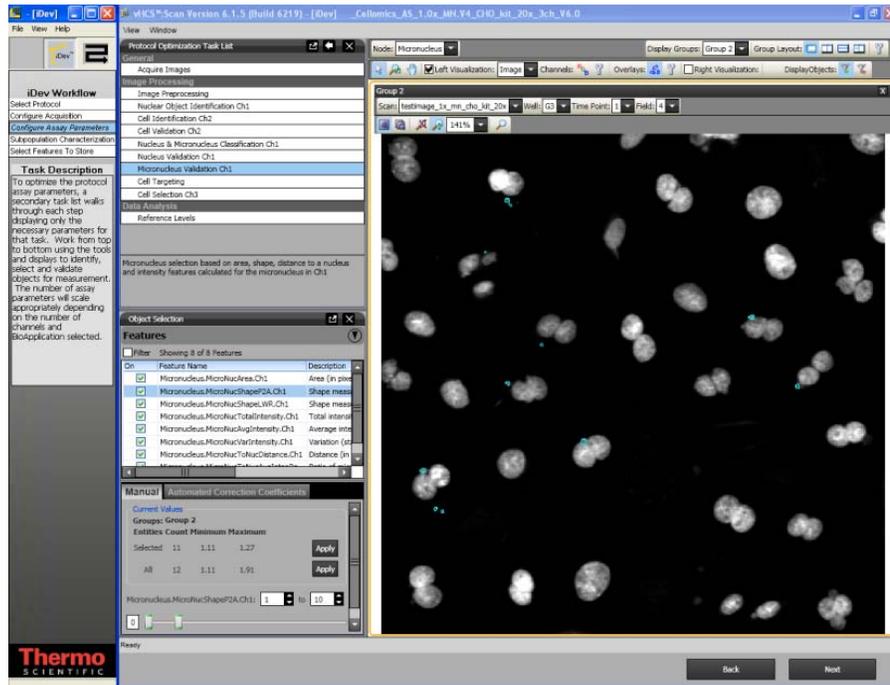


Figure 32. Protocol Optimization Task – Reference Levels

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.

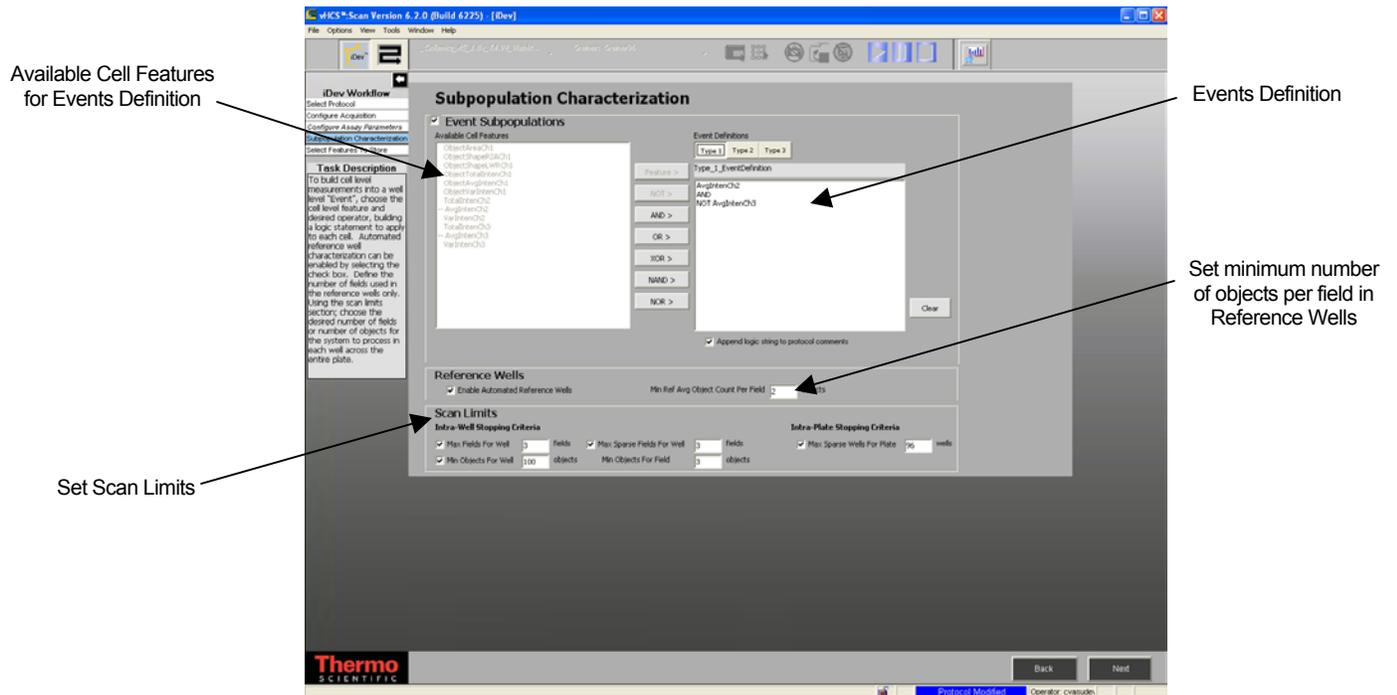


Figure 33. Subpopulation Characterization Task

Index

A

Acridine Orange, 5, 12
Advanced Assay Parameters, 45
Assay Output Features, 49

B

Background correction, 20
Basic Assay Parameters, 33
BioApplication Overview, 4
Boolean Operators, 47, 60

C

Cell Features, 49
CellMaskModifierCh2, 31
CellNucMaxAvgIntenCh3LevelHigh, 32
CellSegmentationCh2, 30
CellSmoothFactorCh2, 21
CellSubstituteMaskModifierCh1, 28
CreateCellSubstituteMaskCh1, 28
Cytokinesis-Block, 3

D

Demonstration data, 11

E

Event Definition and Wizard, 57

F

Field Features, 51
Fixed threshold, 16, 17

G

Gating, 36

I

Identifying Reference Wells, 43

iDev Software, 67
Image Overlays, 38
Intensity thresholds
 Fixed, 16, 17
 Isodata, 16
 None, 16
 Triang, 16
Isodata, 16

L

Limits, 15
Logic statements, 47

M

Mask Modifier, 36
MaxMicroNucOverlapCh1, 28
MaxMicroNucToNucSizeRatioCh1, 28
MicroNucIdentificationModifierCh1, 27
Micronucleus HCS Reagent Kit, 5
MicroNucSegmentationCh1, 27
MicroNucSmoothFactorCh1, 21
MinMicroNucBackgroundIntensityCh1, 28
MinNucCellAssociationCh1, 29
MinNucCellOverlapCh2, 30

N

NucCleanupCh1, 25
NucIdentificationDepthCh1, 24
NucSegmentationCh1, 23
NucSmoothFactorCh1, 21

O

Object Identification Methods, 15
Object Selection
 gating, 36

Object Selection Parameters, 35

Object Smoothing, 21

Object Type, 20

Object validation, 5

P

Population Characterization, 40

R

Reference Features, 56

Reference Well Control, 19

Reference Wells, 56

Reference Wells Processing Sequence

 Reject Border Objects, 22

 RejectBorderCellsCh2, 22

 RejectBorderNucsCh1, 22

Reference Wells Processing Sequence, 41

S

Segmentation

 CellSegmentationCh2, 30

 MicroNucSegmentationCh1, 27

 NucSegmentationCh1, 23

System Requirements, 1

T

T_CytotoxicCellsCh3, 31

T_MaxNucCountPerCellCh1, 25

T_MicroNucDetectionCh1, 26

T_MinNucCountPerCellCh1, 25

T_MinNucToNucAvgIntenRatioCh1, 25

T_MinNucToNucSizeRatioCh1, 25

Triang, 16

U

Use micrometers, 20

W

Well Features, 51

Wizard

 Event Wizard, 57