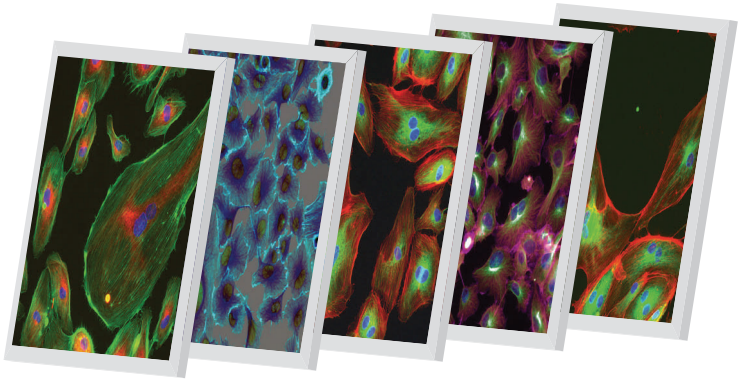
# Thermo Scientific Cellomics® Morphology Explorer V4

## BioApplication Guide



Part of Thermo Fisher Scientific

Thermo SCIENTIFIC

# Cellomics® Morphology Explorer BioApplication Guide

*V4 Version*

PUBLISHED BY

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

## Copyright



## Trademarks



**WARNING**

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

P/N LC06170800

**Cellomics, Inc.**
**Software License Agreement**

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

1) **Licensor's Rights** Cellomics, Inc. ("Licensor") represents that it has all rights necessary to grant the license herein. The Software is protected by patents (including United States Patent Nos. 5,989,835 and 6,716,588, Japanese Patent No. 3683591, European Patent No. 0983498, Canadian Patent No. 2,282,658, and Australian Patent No. 730100), patent applications, and copyrights and includes valuable trade secrets and other proprietary material.

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

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

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

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

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

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

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

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

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

# Table of Contents

Chapter

1

# Overview of the Morphology Explorer BioApplication

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

**Chapter 1** provides an overview of the Morphology Explorer BioApplication and what it measures.

**Chapter 2** describes the quantitative algorithm used to analyze results, along with descriptions of input parameters and output features.

**Chapter 3** describes the use of the BioApplication, guidance on how to generally adjust parameter settings with six different biological examples, and use of the BioApplication Event Wizard.

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


The Morphology Explorer BioApplication is for "power users" who have the expertise and feel comfortable in configuring and optimizing such an application for their particular biological situation.

If you feel that you do not want to develop the expertise in configuring or optimizing such a multi-functional application, you are encouraged to use one of the more specific applications which are targeted toward particular biologies and thus have been optimized for rapid implementation.

## System Compatibility

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

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


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

## Cell Biology Background

Physiological processes in cells are often accompanied by changes in cellular morphology. Such morphological changes can occur in different dimensional scales (Figure 1). For example, the intracellular location, arrangement, or structure of cellular constituents, such as organelles, macromolecular assemblages, or the cytoskeleton, can change due to normal cellular functioning. The morphology of the entire cell, such as its shape or area, may also change as well as the spacing or proximity between different cells. If cells are part of a larger multicellular cluster or assemblage, such as a cell colony or a multinucleated cell such as a myotube, changes in morphology of the entire multicellular assemblage may occur. Some of the changes described may be a part of normal physiological functioning, such as endocytosis, cell motility, mitosis, and differentiation. Changes may also be induced by compound treatment and may have physiological consequences. Often, particular events can initiate several of these morphological changes to occur over different dimensional scales either simultaneously or in a cascade. Quantification of morphological changes over different dimensional scales is a powerful, information-rich method of evaluating compounds and their biological effects and responses.

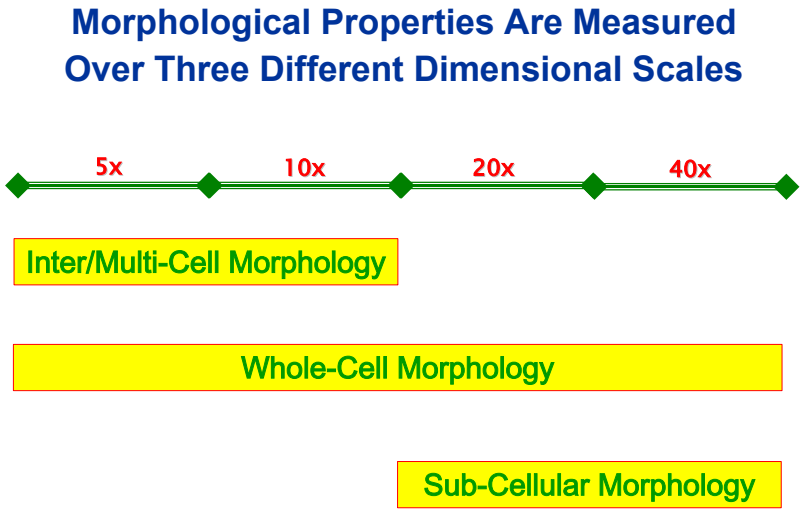



***Figure 1.*** *Dimensional Scales that can be used with the Morphology Explorer BioApplication.*

The Morphology Explorer BioApplication is designed to provide quantitative measurements related to cell morphology. It has the ability to measure morphological properties of individual cells as well as multi-cell assemblages. It enables you to make morphological measurements in three different dimensional scales:

(1) Morphology inside the cell (i.e., intracellular or subcellular morphology)

(2) Whole cell morphology

(3) Multi-cell or inter-cellular morphology, including the morphology of multi-cell assemblages such as colonies, tubes, and multi-nucleated cells.

It is a general BioApplication that can perform a wide range of measurements related to cell morphology. The features and capabilities of this BioApplication provide the flexibility to design your own assays. This application has been designed for scientists who want a versatile application that they can apply towards many different biological targets and thus want the flexibility in choosing and making the morphology-related measurements relevant for their particular biological situation.

## BioApplication Overview

The Morphology Explorer BioApplication identifies up to six different channels which can represent differently colored fluorophores or exposure conditions. In these channels, the application makes quantitative morphology measurements within the following three-dimensional domains in the channels: intracellular (i.e., subcellular) morphology, whole cell morphology, and inter- or multi-cellular morphology. The channels are specialized in what they measure; different features are measured and reported for different channels. Morphology targets are measured in Channels 1-4, and Channels 5-6 are used for gating. The specialization of the channels imposes requirements on assay design, types of cellular entities measured, and fluorescent probes to label them in each of the different channels. Specifically:

- Channel 1 is used to identify individual objects, and a whole cell fluorescent label is required to label the objects. The detected objects are either individual cells, or multi-cell clusters or assemblages, such as colonies, tubes, or multi-nucleated cells (Figure 2). Morphology features pertaining to the whole cell or the entire multi-cellular assemblage are measured and reported for Channel 1. In the nomenclature that will be used for this BioApplication, the individual entities stained by the Channel 1 whole cell stain, whether it is an individual cell or a multi-cellular cluster, will be called an **object**.

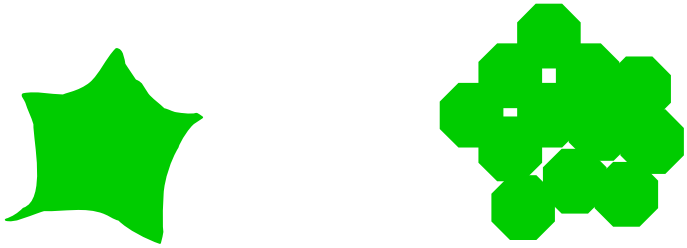


***Figure 2.*** *Channel 1 objects as a whole cell label expressed as (left) individual cells or (right) multi-cell assemblages or clusters (e.g., colonies, tubes, or multi-nucleated cells).*

- In Channel 2, an intracellular compartment or organelle (such as the nucleus or Golgi apparatus) is fluorescently labeled (Figure 3). Typically, only one copy of this compartment exists per cell. If the objects measured in Channel 1 are unresolved multi-cellular assemblages, then based on the Channel 2 measurements, the number of Channel 2 compartments per Channel 1 object can be measured and reported. In addition, different morphology features of the individual compartment or organelles are measured and reported. For objects with multiple organelles, the average of each feature between the different organelles is reported at the cell-level. In the nomenclature that will be used for this BioApplication, the individual compartments or organelles labeled by the Channel 2 fluorescent label are called **members**.

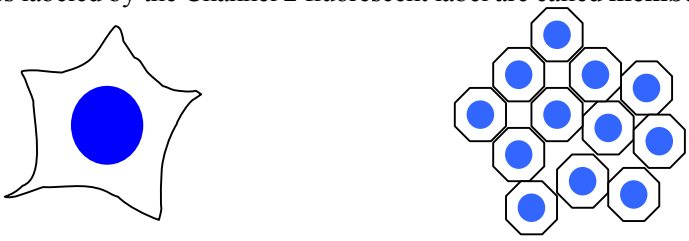


***Figure 3.*** *Channel 2 objects as members expressed as individual organelles (e.g., nucleus, Golgi apparatus) in the object.*

- Two different types of labels, related to different morphological domains, can be used for Channel 3 and Channel 4; morphological parameters relevant for these two domains will be measured and reported for these channels:
    - If you are interested in features between multiple cells or in a mixed-cell population, then the label is a whole cell label identifying different cell types in a mixed cell mixture (Figure 4, top).
    - If you are interested in features with individual cells, then the fluorescent label stains one or more intracellular components of interest such as the cytoskeleton, focal adhesion plaques, specialized macromolecular clusters, and so on (Figure 4, bottom).

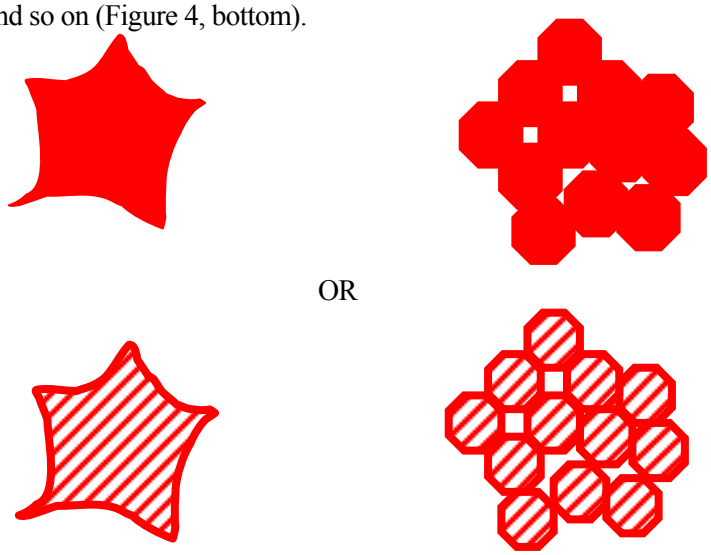



***Figure 4.*** *Whole cell specific stain or labeling of specific intracellular components in Channel 3 and Channel 4. Top panel of images are expressed as Multi/Intercellular measurements where cell-type specific labels are used (e.g., glial specific stain for mixed neuron cultures) and targets are the distinct cells identified by the Channel 3 or Channel 4 label. Bottom panel of images represent Sub/Intracellular measurement where the intracellular component is labeled (e.g., phalloidin for F-actin) and targets are the intracellular components (such as components of the cytoskeleton) identified by the Channel 3 or Channel 4 labels.*

- Channel 5 and Channel 6 are gating channels used for any of the morphological domains (i.e., inter/multi, whole, or intra/sub-cellular domains).

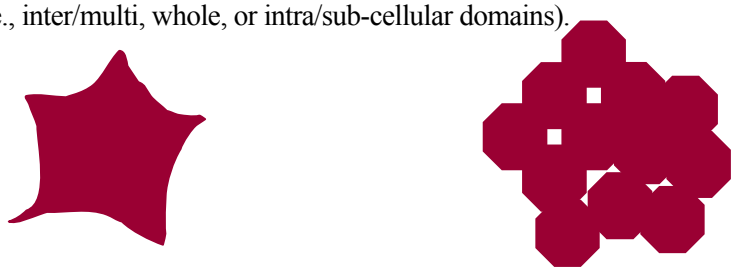


***Figure 5.*** *Other labels for gating.*

## Nomenclature Review

The different input parameters control identification, selection, and analysis of different cellular constituents. To understand the functions of the different input parameters, you need to know how objects and other cellular constituents are defined in this BioApplication's nomenclature. These definitions are covered in depth in the following subsections and are briefly summarized in Figure 6. Basically, the object and any associated processes are

identified in Channel 1, members within the object are identified in Channel 2, and discrete spots and fibers are identified independently in Channel 3 and Channel 4.

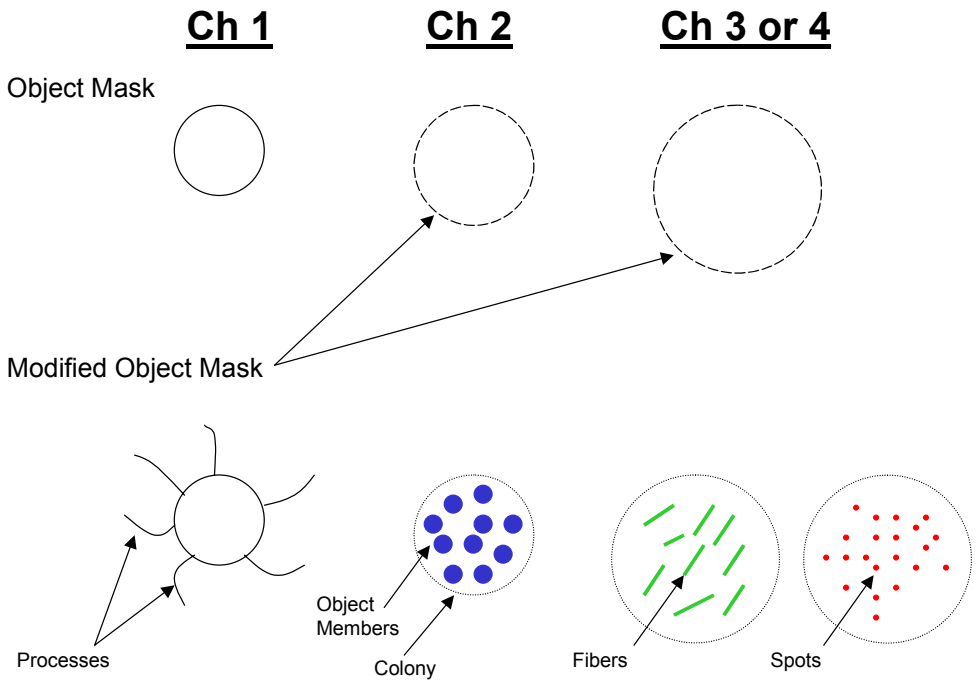



***Figure 6.*** *Definitions of different components analyzed.*

## Event Definition

The Morphology Explorer BioApplication allows simultaneous definition of up to three Events to enable rapid multiparametric analysis or defining a subpopulation at the level of individual cells, across multiple Cell Features. Events are defined as Assay Parameters and consist of logical statements employing specific Cell Features. These statements are then applied to classify and count responder and non-responder cells for the Cell Features contained in the Event Definition. Figure 7 relays these capabilities and how they interact to enable event definition and detection. Event definitions are created using a standalone software tool called the BioApplication Event Wizard. Operation of this software tool is described in more detail in Chapter 3.

Event definition and detection at the level of individual cells of a population requires processing of raw Cell Feature values, i.e., the data extracted from individual cells for all targets being measured. Limits are then applied (manually entered for the ***Feature*Ch*N*LevelLow, *Feature*Ch*N*LevelHigh** Assay Parameters or automatically computed via Reference Wells as described in Chapter 2) to identify responder cells for each feature. For the purposes of Event detection, responder cells are defined as those cells showing feature values greater than the upper limit or less than the lower limit. It is critical that the upper and lower limits be set accordingly. Well Features are calculated to provide population statistics for individual Cell Features and frequency of occurrence of a given Event in the cell population. The latter is expressed as a percentage of cells that display a given Event.

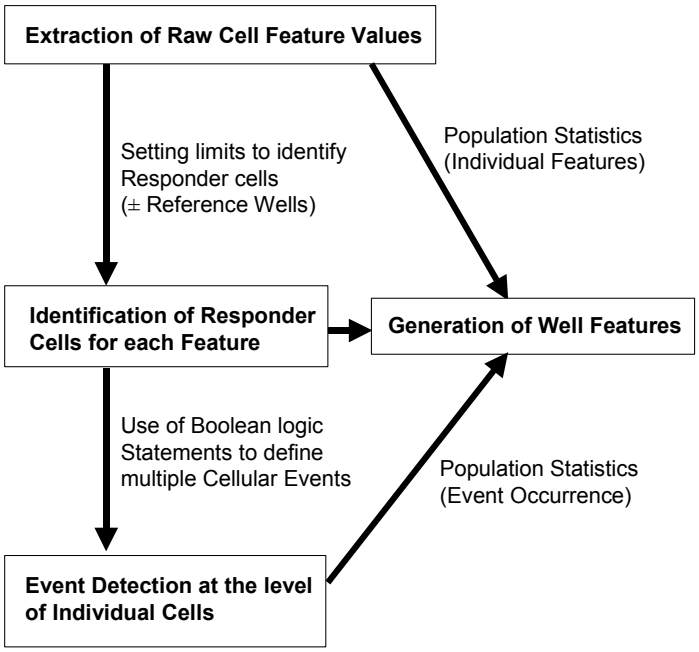



***Figure 7.*** *Schematic showing the process of definition of detection of cellular events.*

## BioApplication Measurements

The Morphology Explorer BioApplication is a multi-channel application where up to six channels can be used. Each channel of the Morphology Explorer BioApplication is specialized and reports different types of output features. Channels 1-4 are used specifically to make specialized morphology-related measurements. Channels 2-6 have the additional capability that allows a subset of cells to be selected for analysis based on their intensity. Thus, you can choose up to four targets to make morphology-related measurements. This application can be run as a one-, two-, three-, or four-channel assay where different properties are measured in the different channels. At a minimum, if it is run as a one-channel assay, only whole-cell related morphology measurements are measured. Channels 2-4 can be used to simultaneously make additional morphology-related measurements. Note that if you would like to track live cells over time, the Cellomics ArrayScan V$^{TI}$ HCS Reader and the Cellomics Discovery Toolbox (v1.6) offer a Live Module that can do this. Please see the appropriate user's manuals for more information. Table 1 summarizes the different categories of output features reported by the application for the different channels.

**NOTE**

The BioApplication measures and reports a wide range of morphology-related features. This provides you with many options of varying sensitivity and robustness for quantifying the biology. For changes seen in images for particular biological situations, the different morphological features will have varying degrees of sensitivity.

For example, a wide range of intracellular texture and arrangement features are measured and reported. Some of these will adequately reflect changes in a specific biological situation, but may not be as sensitive in other situations; a different subset of features may better reflect the phenomenological changes seen in the alternative situations. Thus, you are encouraged to explore the different output features and choose those that are most sensitive and/or robust to the changes seen in the image.

| OUTPUT FEATURE CATEGORIES: | | | | |
|---|---|---|---|---|
| **Channel 1**<br>WHOLE OBJECT MORPHOLOGY | **Channel 2**<br>INTRA-OBJECT COMPARTMENT PROPERTIES | **Channel 3**<br>INTRA-OBJECT IDENTIFIERS OR SPOT/FIBERS | **Channel 4**<br>INTRA-OBJECT IDENTIFIERS OR SPOT/FIBERS | **Channels 5 & 6**<br>GATING |
| • Object counts & field density<br>• Object location<br>• Object area & shape<br>• Object length & breadth<br>• Object orientation<br>• Object extent & perimeter<br>• Object 3-D metrics<br>• Processes extending from object<br>• Ch 1 label intra-object amount, texture & distribution<br>• Object spacing & proximity | • Ch 2 label amount inside object<br>• Degree of multi-cell assemblage<br>• Location of cells in cell cluster<br>• Intracellular compartment shape<br>• Intracellular compartment morphology<br>• Intracellular compartment intensities<br>• Intracellular compartment-whole object comparisons | • Spacing & proximity between similar objects<br>• Ch 3 label amount inside object<br>• Ch 3 label location inside object<br>• Fiber arrangement inside object<br>• Ch 3 label intensity distribution statistics inside object<br>• Ch 3 label texture & intensity arrangement measurements inside object<br>• Discrete object (spot) morphology | • Same output features as in Channel 3<br>• Spacing & proximity between dissimilar objects | • Object average & total intensity |

***Table 1.*** *Categories of Reported Output Features*

The BioApplication provides flexibility in the units for which length, dimension, area, and volume measurements are reported; you can choose to report the features in pixels or micrometers. The BioApplication reports both Cell and Well Features. Cell Features are reported for each individual object, and Well Features are reported for each well and are derived from the Cell Features for the objects imaged and analyzed in that well. The Cell Features are related to the raw morphological properties measured for each object or cell. In addition, for many of the Cell Features, corresponding status features are also reported at the cell-level. The status features indicate whether the cell or object is within the bounds of the population characterization limits for a particular morphology feature, and thus can be identified as a responder. The use of status features and population characterization limits is described in more detail in the section on Population Characterization in Chapter 2. The Well Features are statistical measures or population characterization features derived from the distribution of Cell Features. The statistical measures reported of the cell or object populations are the mean and standard deviation for each of the different Cell Features. The Cell Status Features are used to determine and report at the well-level the percentage of cells in the population which, for each measured Cell Feature, is greater than or less than the population limits for that feature.

The different categories of features and the relationship between the different Cell Features and their corresponding Well Features are summarized in Tables 2-5. The various sections following these tables give detailed descriptions of the various features measured.

| Feature Category | Cell Features | Well Features |
|---|---|---|
| ***Channel 1 Features:*** | | |
| Object Counts & Density | Cell Number | Valid Object Count<br>Selected Object Count<br>% Selected Object Count<br>Valid Field Count<br>Selected Object Count per Valid Field |
| Object Location | Top<br>Left<br>Width<br>Height<br>X Centroid<br>Y Centroid | none |
| Event Profile | EventTypeProfile<br>EventTypeXStatus | EventTypeXObjectCount<br>%EventTypeXCells |
| Object Area & Shape | Area and Status<br>Shape Factor (P2A) & Status<br>Aspect Ratio (LWR) & Status<br>Bounding Box Area Fill Ratio (BFR) & Status<br>Convex Hull Area Ratio & Status | Mean, SD, %HIGH, %LOW |
| Object Length & Breadth | Fiber length & Status<br>Length based on Bounding Box & Status<br>Fiber width & Status<br>Width based on Bounding Box & Status | Mean, SD, %HIGH, %LOW |
| Object Orientation | Angle & Status | Mean, SD, %HIGH, %LOW |
| Object Perimeter & Extent | Perimeter & Status<br>Equivalent circular diameter & Status<br>Equivalent ellipse length/width ratio & Status<br>Convex Hull perimeter ratio & Status | Mean, SD, %HIGH, %LOW |
| Object 3-D Metrics | Equivalent sphere volume & Status<br>Equivalent ellipse oblate volume & Status<br>Equivalent ellipse prolate volume & Status<br>Equivalent sphere surface area & Status | Mean, SD, %HIGH, %LOW |
| Processes Extending from Object | Number of processes & Status<br>Process Length: Total length of all processes & Status<br>Process Length: Average length of all processes & Status<br>Process Length: Length of longest process & Status | Mean, SD, %HIGH, %LOW |
| Amount inside object & intra-object arrangement, distribution and texture | Total intensity inside object & Status<br>Average intensity inside object & Status<br>Variance Intensity inside object & Status<br>Intensity skewness inside object & Status<br>Intensity kurtosis inside object & Status<br>Intensity entropy inside object & Status<br>Intensity difference surface area density & Status | Mean, SD, %HIGH, %LOW |
| Object spacing & proximity | Distance of closest object & Status<br>Average distance of objects & Status<br>Variance distance of objects & Status | Mean, SD, %HIGH, %LOW |

**Table 2.** *Channel 1 Output Features: Whole Object Morphology - Inter/Multi & Intra/Sub-Cellular Modes (Whole cell label; Object is either single cell or multi-cell assemblage). Note that "X" is the number of events selected (1, 2, and 3)*

| Feature Category | Cell Features | Corresponding Well Features |
|---|---|---|
| ***Channel 2 Features:*** | | |
| Amount inside cell | Total Channel 2 intensity inside object & Status<br>Average Channel 2 intensity inside object & Status | Mean, SD, %HIGH, %LOW |
| **Inter/Multi-Cellular Mode:** | | |
| Degree of multi-cell assemblage | Number of members (cells) in object (object = single cell or a multi-cell assemblage) & Status | Mean, SD, %HIGH, %LOW |
| Location of cells in multi-cell assemblage | Number of exterior cells in colony & Status<br>Number of interior cells in colony & Status | Mean, SD, %HIGH, %LOW |
| **Intra/Sub-Cellular Mode:** | | |
| Intracellular Compartment's Shape | Shape Factor (P2A) & Status<br>Aspect Ratio (LWR) & Status<br>Box Fill Ratio (BFR) & Status | Mean, SD, %HIGH, %LOW |
| Intracellular Compartment's Morphology | Average Area & Status<br>Equivalent diameter & Status<br>Equivalent ellipse length/width ratio & Status<br>Convex Hull area ratio & Status<br>Convex Hull perimeter ratio & Status | Mean, SD, %HIGH, %LOW |
| Intracellular Compartment's Intensity | Total intensity per compartment & Status<br>Average pixel intensity per compartment & Status | Mean, SD, %HIGH, %LOW |
| Intracellular Compartment - Whole Cell Comparisons | Compartment/Whole Cell Area Ratio & Status<br>Compartment/Whole Cell Area Difference & Status | Mean, SD, %HIGH, %LOW |

***Table 3.*** *Channel 2 Output Features: Individual Cell Identifiers (Members); (Labeled member is intracellular compartment stain such as nucleus, Golgi apparatus, or endocytic recycling compartment)*

| Feature Category | Cell Features | | Corresponding Well Features |
|---|---|---|---|
| ***Channel 3 and Channel 4 Features:*** | | | |
| **Inter/Multi-Cellular Mode:** | | | |
| Spacing & proximity within specified area between similar object types (i.e., between objects having either Ch3 or Ch4 label) | Distance of closest similar cell & Status<br>Average distance of similar cells & Status<br>Variance distance of similar cells & Status | | Mean, SD, %HIGH, %LOW |
| **Channel 4 only:** Spacing & proximity within specified area between different object types (i.e., between objects with Ch3 & Ch4 label) | Distance of closest different cell & Status<br>Average distance of different cells & Status<br>Variance distance of different cells & Status | | Mean, SD, %HIGH, %LOW |
| **Intra/Sub-Cellular Mode:** | | | |
| Amount inside cell | Total intensity inside cell & Status<br>Average intensity inside cell & Status | | Mean, SD, %HIGH, %LOW |
| Labeled macromolecule's location inside cell (radial intensity distribution statistics) | Average radial intensity distribution & Status<br>Variance radial intensity distribution & Status<br>Skew of radial intensity distribution & Status<br>Kurtosis of radial intensity distribution & Status | | Mean, SD, %HIGH, %LOW |
| Labeled macromolecule's arrangement inside cell | Intensity distribution statistics | Variance intensity inside cell & Status<br>Intensity skewness inside cell & Status<br>Intensity kurtosis inside cell & Status<br>Intensity entropy inside cell & Status<br>Intensity difference surface area density & Status | Mean, SD, %HIGH, %LOW |
| | Texture & intensity arrangement measurements from intensity co-occurrence matrix | Maximum probability of 2D distribution of intensity co-occurrence & Status<br>Contrast of 2D distribution of intensity co-occurrence & Status<br>Entropy of 2D distribution of intensity co-occurrence & Status<br>Angular Second Moment (uniformity) of 2D distribution of intensity co-occurrence & Status | |
| Morphology of discrete objects (i.e., spots or fibers) | Spot or fiber number and areas | Number of spots & Status<br>Total area of spots & Status<br>Average area of spots & Status | Mean, SD, %HIGH, %LOW |
| | Fiber arrangement | SD of fiber alignment angles & Status<br>Fiber alignment autocorrelation & Status | Mean, SD, %HIGH, %LOW |

***Table 4.*** *Channels 3 & 4 Output Features: Inter/Multi-Cellular Mode: whole cell label identifying different cell type; Intra/Sub-Cellular Mode: label stains one or more intracellular components of interest such as cytoskeleton, etc. "SD = Standard deviation.*

| Feature Category | Cell Features | Corresponding Well Features |
|---|---|---|
| **Other: Channels 2-6** | | |
| Cell Intensity | Total Intensity<br>Average Intensity | None (gating purposes) |

***Table 5.*** *Channels 2-6 Output Features: Gating Channels - Inter/Multi & Intra/Sub-Cellular Modes.*

# Example Use Cases

The Morphology Explorer BioApplication can do the following:

- Specify up to six different channels which can represent different fluorophores or exposure conditions.
- Make quantitative morphology measurements within the following dimensional domains in the channels: intracellular (i.e., subcellular) morphology, whole cell morphology, and inter or multi-cellular morphology. Unlike other Cellomics BioApplications, the channels are specialized; different features are measured and reported for each channel. Morphology targets are measured in Channels 1-4. Channels 5-6 can be used for gating.
- Perform automatic cell population characterization and define responders using Reference Wells.

This capability allows the morphology of a large and varied range of cell biological situations to be quantitatively analyzed. The following sections give examples of a range of cell biological situations that can be quantitatively analyzed by the Morphology Explorer BioApplication. The examples are followed by sections that describe the functioning and versatility of this BioApplication in further detail. A guide on how to configure the BioApplication to quantitatively assay these particular biological situations is given in detail in Chapter 3.

## Example 1: Myoblast Differentiation

Muscle fibers are formed when myoblast cells differentiate and fuse with each other to form long, multinucleated cells called myotubes. In these experiments, confluent myoblasts were induced to differentiate into myotubes. Myotube formation was inhibited by blocking calcium channels with nifedipine (Portu, et al. 2002). This experiment is a two-label assay. Cells are stained via indirect immunofluorescence using an anti-myosin heavy chain (MF-20) antibody (secondary antibody with green fluorescence), and the nuclei are labeled with Hoechst 33342 (blue fluorescence). The nuclei of all cells show Hoechst labeling. However, only myotubes will have skeletal myosin (MF-20) staining, and undifferentiated myoblasts will have little or no skeletal myosin staining. Figure 8 shows images acquired using a 5x objective on the ArrayScan HCS Reader of myoblast differentiation into myotubes or their inhibition. The difference between mature, multinucleated myotubes containing MF-20 staining versus undifferentiated myoblasts with little MF-20 is clear in these images. The BioApplication identifies myotubes on the basis of their dimensions, shape, and MF-20 content, and the identified myotubes are outlined in blue in the images on the right.

The BioApplication can measure the following types of properties from the sample images:

- The number of myotubes detected. Their identification is based on their morphology and skeletal myosin content
- Density of myotubes in the image
- The amount of skeletal muscle myosin per myotube
- Myotube dimensions (e.g., length, width, area, aspect ratio)
- Number of nuclei per myotube
- Difference and ratio of the areas of nuclei versus entire myotube

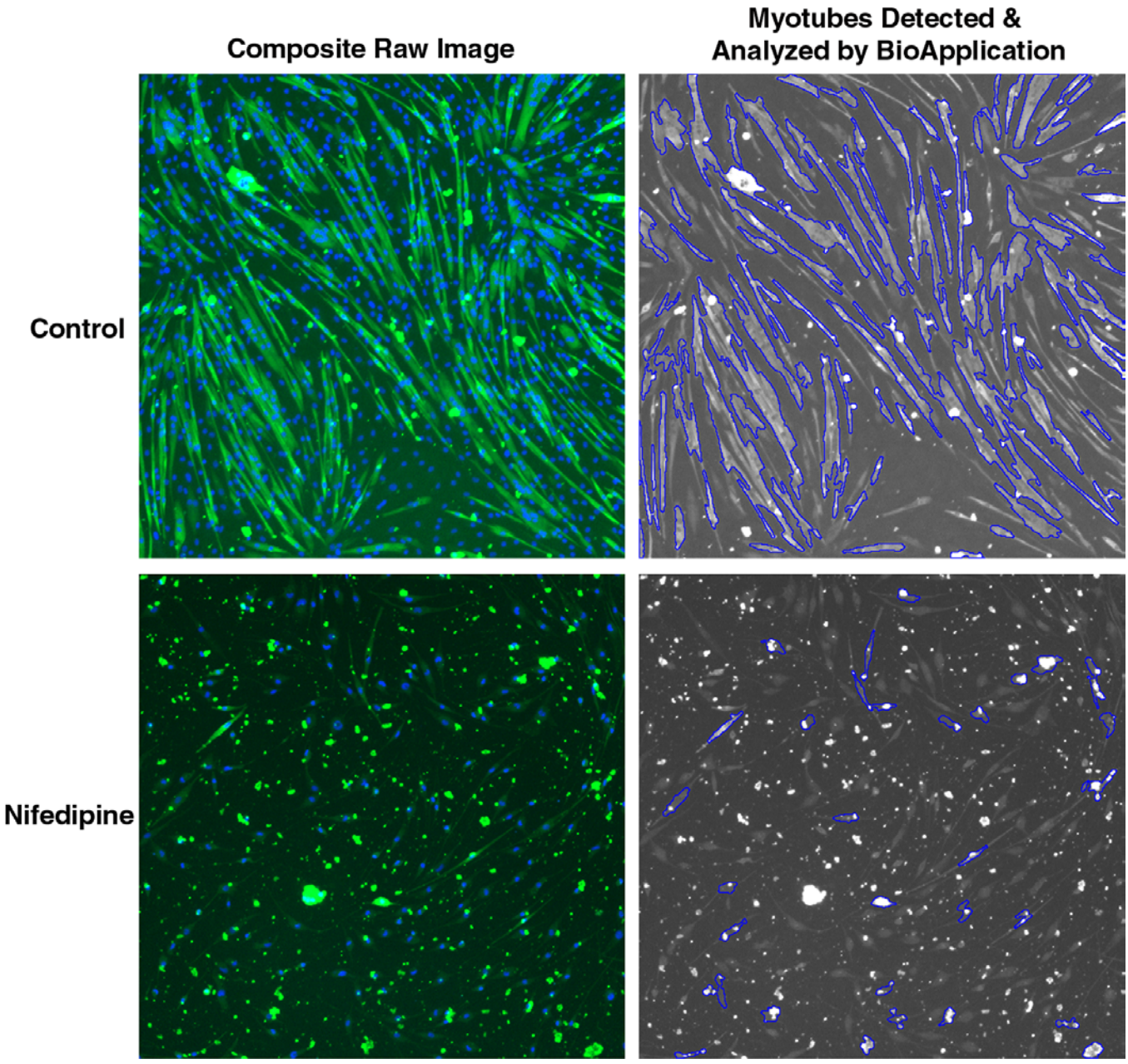



***Figure 8.*** *Myoblast Differentiation. The top row of images shows control untreated cells showing myotube formation after 4 days in differentiation medium. In the bottom images, myoblast differentiation into myotubes has been inhibited by nifedipine. Treatment conditions: confluent C2C12 cells treated with differentiation medium plus 100 μM nifedipine for 4 days. The images in the left column are a psuedocolor composite of the raw images, where the MF-20 skeletal myosin labeling is in green, and cell nuclei are in blue. The images in the right column show what the BioApplication identifies and analyzes as myotubes (outlined in blue). Myotubes are identified on the basis of their shape, dimensions, and MF-20 skeletal myosin expression.*

## Example 2: Cytoskeletal Rearrangement

Compounds or conditions that affect the cytoskeleton can cause cells to have multiple changes in their morphology over all three dimensional scales. The intracellular structure, arrangement, location, and texture of different cytoskeletal fibers and cytoskeletal associated proteins may change. This may result in changes of the whole cell morphology and, sometimes in the cell arrangement, location and morphology over the multi-cellular domain. In this example, NIH 3T3 cells are used to demonstrate changes in different types of cytoskeletal patterns within the cell. Of the three major types of cytoskeletal structures, microfilaments, intermediate filaments, and microtubules, only two of them, microfilaments and microtubules, are simultaneously labeled in the cells of this experiment. The cells are treated with two different compounds that elicit changes in the intracellular structure or shape of the cell to give a broad representation of images which you might be interested in assessing. The compounds used are cytochalasin D, which depolymerizes F-actin, and taxol, which stabilizes microtubules and causes their bundling. Images acquired on the Cellomics HCS Reader showing these effects are shown in Figure 9.

This example biology utilizes four fluorescent probes. Cell nuclei were labeled with Hoechst 33342, and the entire cell was also labeled by the whole cell marker CellTracker™ Green (CMFDA). F-actin is the major component of microfilaments and was labeled with rhodamine-phalloidin. The F-actin filaments in untreated cells typically have two types of patterns: stress fibers, which are long parallel fibers stretching across the cell, and cortical actin filaments, which appear as a line adjacent to the cell's edge. These structures change dramatically when treated with the various drugs. Tubulin, the major component of microtubules, was labeled by indirect immunofluorescence, to visualize microtubules within the cytoplasm as it changes with drug treatment (secondary antibody conjugated to fluorophore that fluoresces in the far red). Microtubules usually radiate out from one or two discrete locations adjacent to the cell's nucleus.

In control cells, long fibers are seen for F-actin and microtubules. Microtubule fibers seem to radiate from a couple of foci near the nucleus. The actin has many parallel fibers spanning the cell. Cytochalasin D depolymerizes F-actin causing the cell to take on a star like shape. F-actin fibers are fewer and condensed with the majority of long fibrous filaments gone and only a bright speckled pattern remaining. In contrast, cells have a more rounded shape when treated with taxol. The cortical actin is more prominent and seems to form an outer circle around the edge of the cell; some parallel stress fibers are also seen inside the cell. Microtubule bundling is seen within the cytoplasm. The microtubule fibers are mostly intact, but they don't have same pattern of radiating from the nuclei as the untreated cells do.

The BioApplication can measure the following types of properties from the sample images:

- Whole cell shape and dimensions
- Number of cytoskeletal fibers
- Cytoskeletal fiber dimensions
- Cytoskeletal fiber alignment
- Intracellular arrangement and location of cytoskeletal labels
- Intracellular texture measurements of cytoskeletal labels

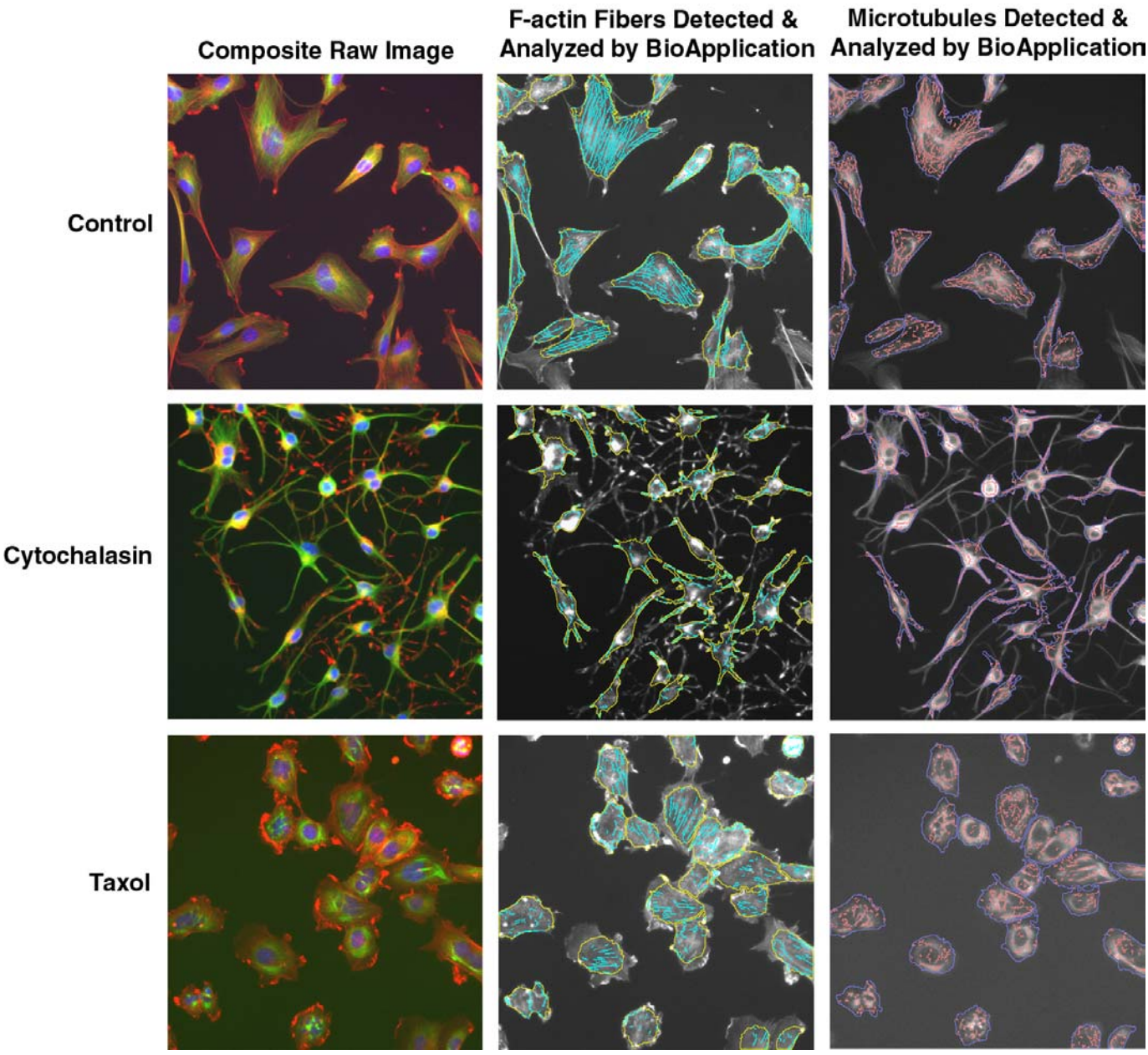



***Figure 9.*** *Cytoskeleton Rearrangement in NIH 3T3 cells. The top row of images shows control untreated cells, the middle row shows cells treated with cytochalasin D, and the bottom row shows taxol-treated cells. The images in the left column are a pseudocolor composite of the raw images, where the rhodamine phalloidin labeling of F-actin is shown in red, the microtubules are shown in green, and the cell nuclei in blue. The middle column of images shows what the BioApplication identifies and analyses as F-actin fibers (outlined in cyan), and the right column of images shows what the BioApplication identifies and analyses as microtubules (outlined in magenta). A close up view of an individual cell's F-actin fibers is in Figure 42.*

## Example 3: Colony Formation

Certain types of cells grow in tight groups or colonies rather than as individual cells. The need for close proximity may affect the cell's growth, cell cycle state, particular protein expression, and appearance. This example demonstrates the ability to quantitatively assay MCF-7 human breast epithelial cells, which normally grow in small clumps or colonies. The clumps are easily dislodged from the surface, but adhere to one another very strongly. Figure 10 shows an image from this colony formation example. This is a simple two-color assay where the whole cell marker CMFDA is used to identify the cell cytoplasm and boundary and Hoechst 33342 is used to label the individual nuclei in the colony. Identification of the individual nuclei enables individual cell identification, easy colony counting, and individual cell separation. In the image, numerous cell clusters are seen throughout the field, each containing multiple cells (nuclei).

The BioApplication can measure the following types of properties from the sample images:

- Number of objects (individual cells or multi-cellular colonies)
- Number of cells per object (i.e., degree of colony formation or disassociation)
- Number of cells that are on the periphery of each colony
- Number of cells that are in the interior of each colony
- Difference and ratio of the areas of the cell nuclei versus the entire colony
- Colony dimensions, shape, and morphology
- Proximity measurements between different colonies (objects) (i.e., density of objects)

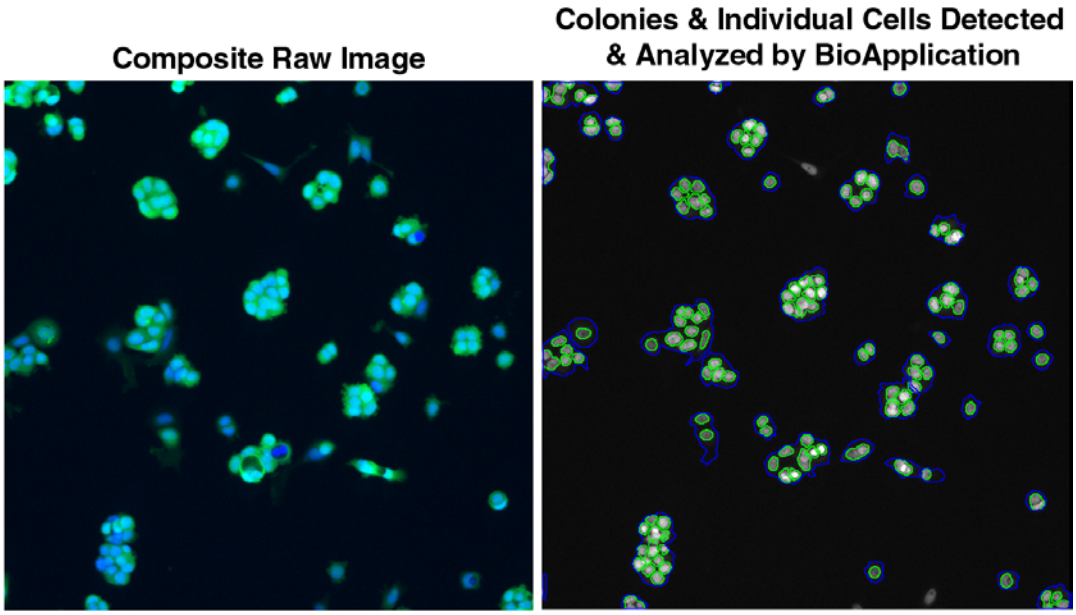



***Figure 10.*** *Colony Formation and Disassociation. The image on the left is a pseudocolor composite of the raw images, where the CMFDA labeling of the entire colony is shown in green and the individual cell nuclei in the colony in blue. The image on the right shows what is identified and analyzed by the BioApplication as colonies (outlined in blue) and the individual cells (i.e., nuclei) within the colony (outlined in green).*

## Example 4: Cell Spreading

Cell spreading can be quantified by the BioApplication by directly measuring the size of flourescently-labeled cells. In the simple two-color example, the F-actin label rhodamine-phalloidin was used to identify the entire cell extent for area measurements and Hoechst 33342 was used to identify the nuclei of the individual cells. In Figure 11, untreated PC-3 cells are spread out nicely on the surface of the type 1 collagen matrix plate and serve as a control for the experiment. The intracellular actin of the cell can be visualized and forms a ring-like boundary around the periphery of the cell. To inhibit spreading, the cells are treated with cytochalasin D for three hours immediately after plating. In this case, the cells resemble bright clumps, there is no visible intracellular (F-actin) structure, and the cell area is decreased compared to control.

The BioApplication can measure the following types of properties from the sample images:

- Cell area, shape, and dimensions
- Difference and ratio of the areas of the cell nucleus versus the entire cell

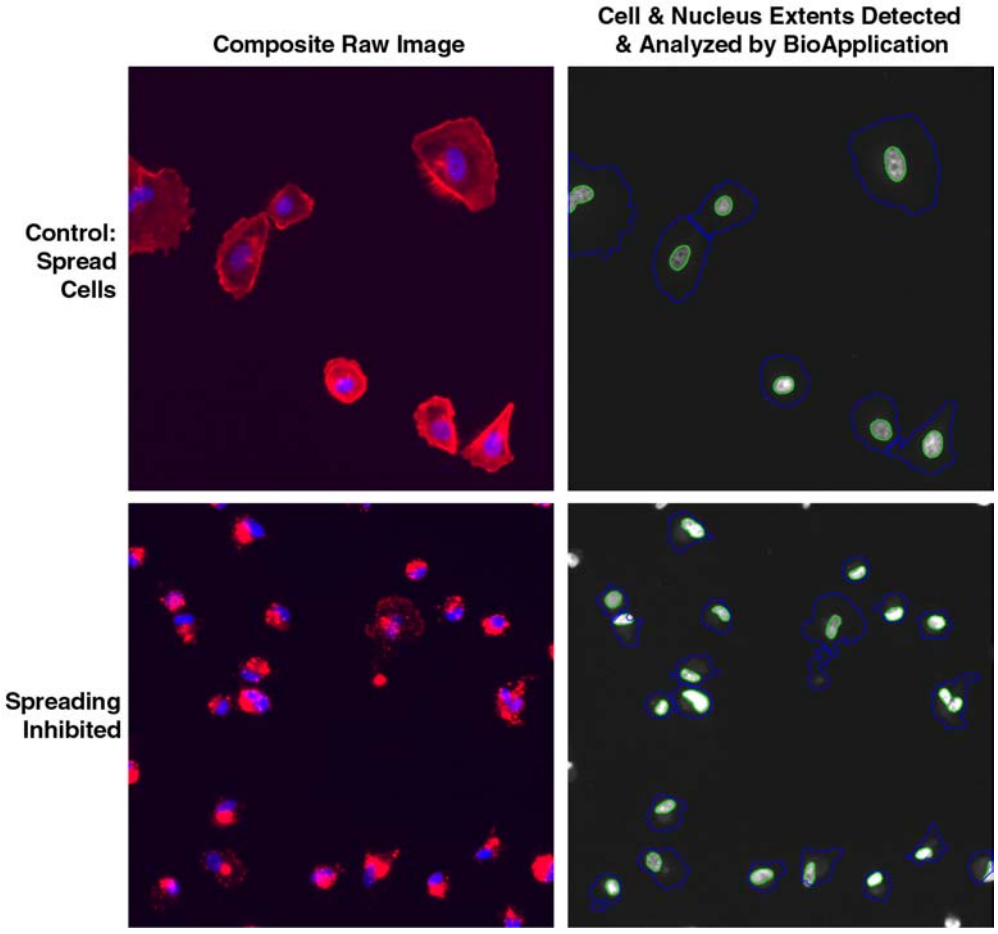



***Figure 11.*** *Cell Spreading. The images in the left column are a pseudocolor composite of the raw images, where the rhodamine-phalloidin labeling of the whole cell is shown in red and the individual cell nuclei are in blue. The images in the right column show what the BioApplication identifies and analyses as individual cells (outlined in blue), and their nuclei (outlined in green). The top images are of control-spread cells, and the bottom images are of cells where cytochalasin D has been used to inhibit spreading.*

## Example 5: Properties of Discrete Intracellular Objects

The Morphology Explorer BioApplication can also quantitate the intracellular distribution and properties of discrete objects. An example of such objects are fluorescently labeled organelles such as endosomes containing internalized fluorescent macromolecules; the location of such internalized fluorescent macromolecules can change during the intracellular trafficking process. Other such objects, focal adhesions, are discrete clusters of specific proteins that mediate the cell's adhesion and attachment to the substrate, whose intracellular location changes as the whole cell moves. A third example is specific cytoskeletal proteins, such as actin-binding proteins, which may change due to drug treatment or cell changes, such as undergone in motile cells. In the example given (Figure 12), the intracellular arrangement and other properties of internalized fluorescent EGF inside HeLa cells is analyzed. The experiment is a three-color assay that utilizes CMFDA to label the cytoplasm, Hoechst dye to label the nuclei, and EGF conjugated to Alexa Fluor® 555 to visualize internalized EGF in endosomes. During internalization, the fluorescent EGF goes from the plasma membrane to peripherally distributed sorting endosomes, to late endosomes and then to the more centrally located lysosomes (Mukherjee, et al. 1997). After 35 minutes incubation, internalized EGF is seen as intracellular punctate spots which correspond to late endosomes and lysosomes.

The BioApplication can measure the following types of properties from the sample images:

- Number, area, and intensity of intracellular spots
- Intracellular arrangement and location of spots
- Intracellular texture measurements

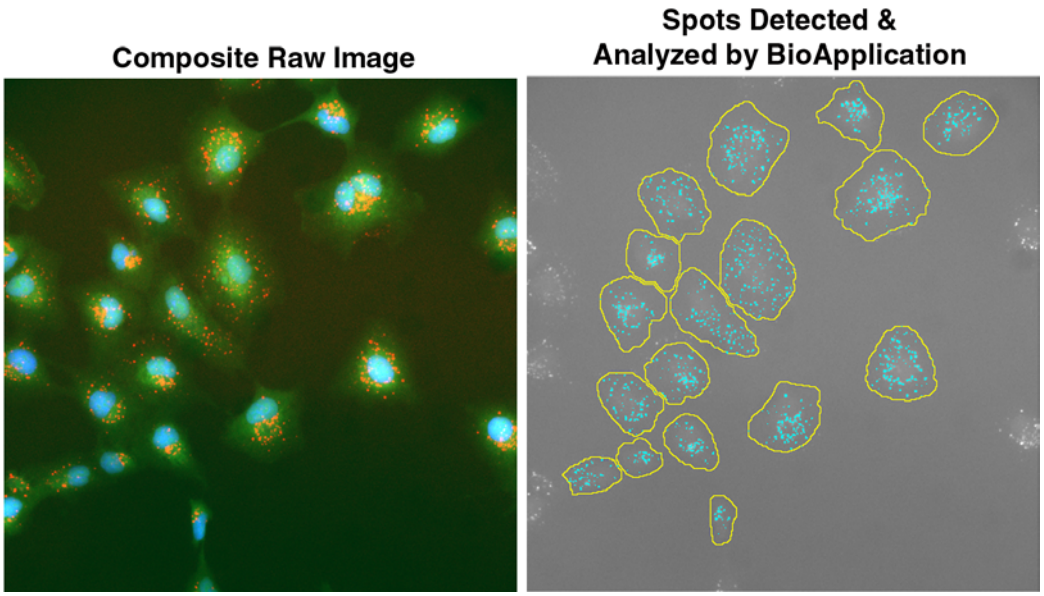



***Figure 12.*** *Discrete Objects. The image on the left is a pseudocolor composite of the raw images, where the CMFDA labeling of whole cells is shown in green, the internalized EGF in endosomes in red, and cell nuclei in blue. The image on the right shows what the BioApplication identifies and analyses as spots within individual cells. A close up view of an individual cell showing its endosomal spots is in Figure 42.*

## Example 6: Mixed Neuronal Population

The images for this particular biology include a mixed population of neuronal cells (Figure 13). Often a primary culture has a mixture of cell types. The Morphology Explorer BioApplication can distinguish between different cell types, quantify the proximity of similar and dissimilar cells in the image, and correlate neurite outgrowth with the presence of different cells nearby. In this example, Neuroscreen™ cells, a sub-clone of PC-12 cells available from Thermo Fisher Scientific., are labeled by indirect immunofluorescence with an anti-neuronal primary antibody and an Alexa Fluor 647 conjugated secondary antibody (long wavelength red fluorescence). Subsets of the cells are labeled either with CMFDA (green fluorescence) or CellTracker Orange (CMTMR - orange fluorescence). Cells are treated with nerve growth factor (NGF) to induce neurite outgrowth. The goal of this example is not only to quantify the neurite outgrowth from the different cells, but also to measure the spacing and proximity between similar and dissimilar cells defined by their specific subpopulation stains (i.e., CMFDA or CMTMR). In control cells (untreated with NGF), very little neurite outgrowth is seen with only few short processes extending from neuronal cells. In cells treated with NFG, neurite outgrowth has occurred and neurites are easily visible extending from the cells. In both cases, subpopulations of cells containing CMFDA or CMTMR are also seen.

The BioApplication can measure the following types of properties from the sample images:

- Total number of cells
- Percentage of cells containing CMFDA or CMTMR
- Number of processes (neurites) extending from each cell body
- Neurite dimensions
- Cell body area, shape and morphology
- Proximity and spacing between all neurons
- Proximity and spacing between similar cells (containing only CMFDA or CMTMR)
- Proximity and spacing between dissimilar cells (between cells containing CMFDA and CMTMR)

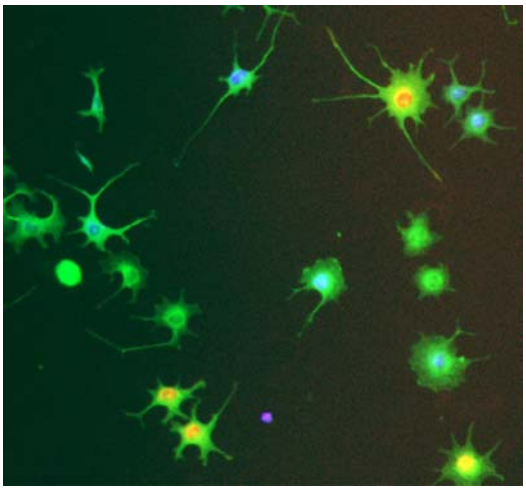


***Figure 13.*** *Mixed Neuronal Population. The image is a pseudocolor composite of neurons treated with NGF. The neurons (in green) show neurite outgrowth of the raw images. A subpopulation of cells has CMFDA (shown in blue in this composite) or CMTMR (red). Figure 34 shows what the BioApplication identifies and analyses as cell bodies and neurites.*

## References

Haralick, R.M., K. Shanmugan, and I. Dinstein, *Texture features for image classification.* IEEE Transactions on Systems, Man, and Cybernetics, 1973. **3**(6): p. 610-621.

Mukherjee, S., R.N. Ghosh, and F.R. Maxfield, *Endocytosis.* Physiol Rev, 1997. **77**(3): p. 759-803.

Porter, G.A., R.F. Makuck, and S.A. Rivkees, *Reduction in intracellular calcium levels inhibits myoblast differentiation.* The Journal of Biological Chemistry, 2002. **277**(32): p. 28942-28947.

Press, W.H., *et al.*, *Statistical Description of Data*, in *Numerical Recipes: The Art of Scientific Computing*. 1986, Cambridge University Press: Cambridge, UK. p. 457-458.

Vaaraniemi, J., *et al.*, *Translocation of MARCKS and reorganization of the cytoskeleton by PMA correlates with the ion selectivity, the confluence, and transformation state of kidney epithelial cell lines.* Journal of Cell Physiology, 1999. **181**(1): p. 83-95.

Chapter

2

# Description of the Algorithm

NOTE

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

The previous chapter provided an overview of what the application does and what it measures. This chapter describes in more detail the use of the application input parameters to control the analysis, as well as a summary of the output features.

## Overview

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

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

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

## 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, objects and any processes are identified; only pixels with intensities above this threshold will be considered as belonging to these structures. Thus the proper setting of an intensity threshold is a key early step in identifying objects and thus configuring the application for the analysis for a particular type of biology. In Channel 2, members within objects are identified, and pixels with intensities above this threshold are used to help identify the individual members. Note the intensity threshold affects only members and does not affect the fluorescence intensity measurements for the whole object in Channel 2. In Channel 3 and Channel 4, pixels above the intensity threshold help to identify discrete spots and fibers. The threshold thus affects measurements pertaining to spots and fibers; other measurements in Channel 3 and Channel 4, such as the different texture, intensity moments, and proximity measures are not affected by the intensity threshold. Thus, depending on the properties or cellular constituents being measured in Channels 2-4 for a particular biology, the proper setting of intensity thresholds for these channels can also have a critical influence on ensuring proper quantitative analysis.

There are five different options or methods for intensity thresholds, and a sub-set of these is available for each channel. The different options available for each channel are summarized in Table 6, and the descriptions of the different methods follow.

| Intensity Threshold Option | Channel Availability | | | |
|---|---|---|---|---|
| | Channel 1 | Channel 2 | Channels 3 & 4 | Channels 5 & 6 |
| None | | ✓ | ✓ | ✓ |
| Isodata | ✓ | ✓ | ✓ | |
| Triang | ✓ | | ✓ | |
| 3Sigma | ✓ | | ✓ | |
| Fixed | ✓ | ✓ | ✓ | |

***Table 6.*** *Intensity Threshold Options Available for Each Channel*

**NOTE**

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

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

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

Of the five intensity threshold options, **None** means that no intensity threshold is applied. This option is not available in Channel 1 because in this channel an intensity threshold is required to define the pixels making up the object. The choice of the **None** threshold option in Channel 2 turns off member identification and analysis. Similarly, the choice of the **None** threshold option in Channel 3 or Channel 4 turns off spot and fiber identification and analysis in these channels. However, the remaining quantitative analysis, which is not dependent on intensity

thresholds, is still done in these channels. In Channels 5-6, **None** is the only option, and is used for gating analysis.

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 three threshold methods (**Isodata**, **Triang** and **3Sigma**) 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 14 demonstrate how these histogram-derived threshold values are derived; and their descriptions are in Table 7.

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

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

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

The three histogram-derived methods are dependent on the contents of the image, unlike the **Fixed Threshold** method. For example, for a blank image that contains no cellular objects and only has background pixels with a mean intensity value of 500 and standard deviation of 50, it is unlikely that a **Fixed Threshold** of 1000 will cause any pixels to be registered. However, all three histogram-derived threshold methods will give thresholds causing pixels in the image to be registered for potential analysis; this is because the histogram is of the pixel intensity distribution of that image, even though there are no cellular objects in the image. Thus, in situations where blank images are expected (e.g., from a loss of signal due to a compound condition, a loss of protein expression, or a lack of label), a histogram-derived threshold method should be avoided; a **Fixed Threshold** with a large offset value should be entered instead.

| Threshold Option | Description | Range of Possible Values Entered | Resulting Applied Threshold Range |
|---|---|---|---|
| None | No threshold applied | ----- | none |
| Isodata | Adjusts the object identification threshold relative to the Isodata value.<br>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 ($m_R$).<br>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.<br>The threshold *T* which gives the maximum distance *d*<br>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 |
| 3Sigma | The threshold *T* is at three standard deviations (σ) to the right of the mean. | -0.999 – 9.999 (offset) | 1 - 32767 |
| Fixed | A fixed pixel intensity value between 0-32767 is applied | 0 – 32767 (true intensity) | 0 - 32767 |

***Table 7.*** *Intensity Threshold Descriptions*

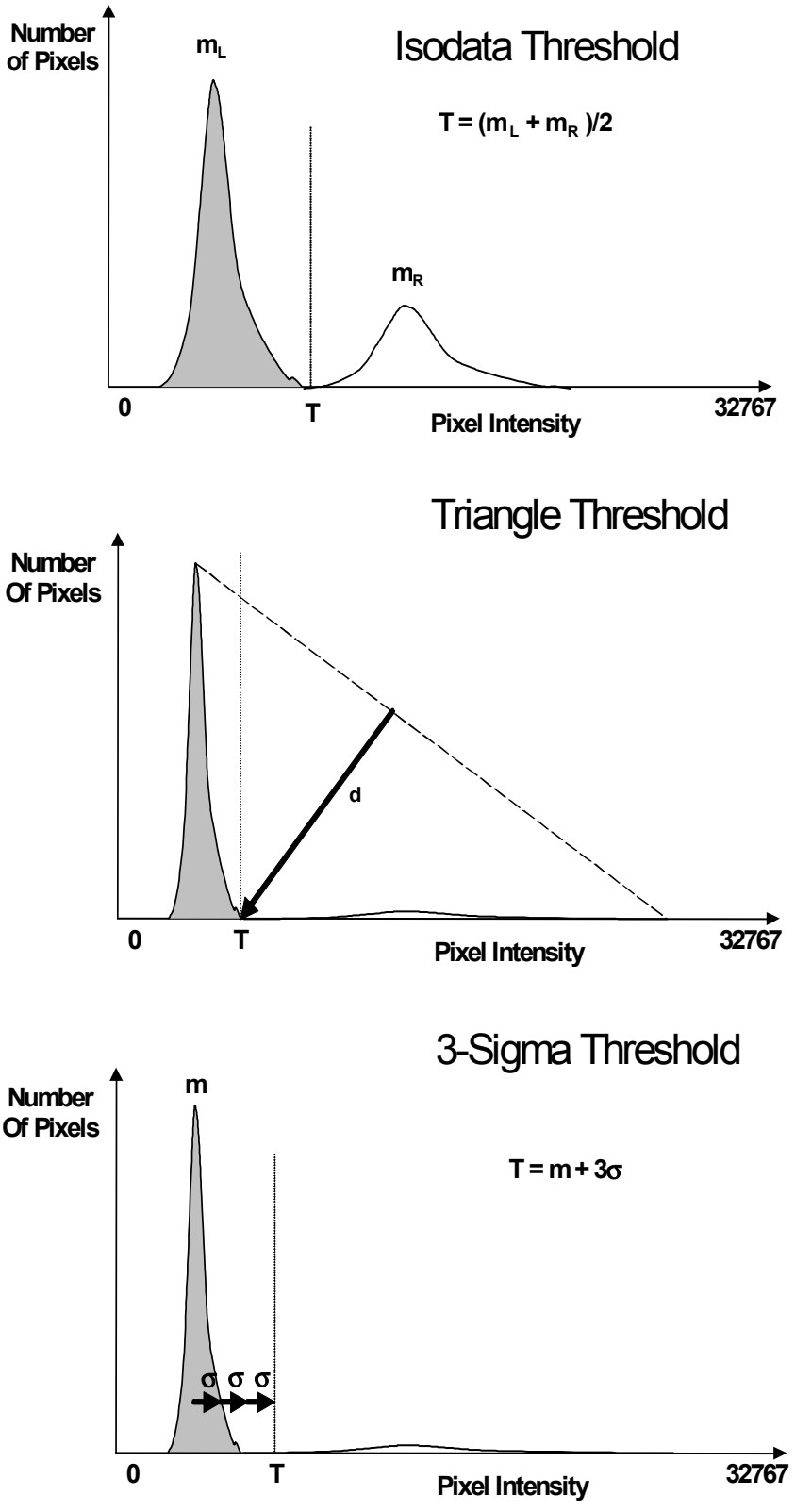



***Figure 14.*** *Histogram-derived Intensity Threshold Methods. Shown are Isodata (top), Triangle (middle) and 3-Sigma (bottom). Background peak is shown in gray and object peak is shown in white.*

# Description of Assay Parameters and Settings

Assay Parameters are one category of input parameters and can be categorized as either general or channel-specific. General Assay Parameters control general aspects of the image processing and analysis and do not pertain to specific tasks done only on images from particular channels. Specific Assay Parameters control the image-processing particular to targets in specific channels. When using the BioApplication as a single channel assay, you have to enter values for the specific Channel 1 Assay Parameters as well as the general Assay Parameters. Adding additional channels to analyze requires you to enter values for the specific Assay Parameters for the new channels.

Assay Parameters are available in the Basic and Advanced Modes. Assay Parameters available in the Basic Mode control calculation of the different morphological properties of each object and target. Advanced Mode Assay Parameters control the conditions and levels for population characterization and enable you to identify which objects are responders for different properties measured. Unless you want to do population characterization for particular features or need to identify responders (and thus need to set the ***Feature*ChN LevelHigh** and ***Feature*ChN LevelLow**), you do not need to use the Advanced Parameters; adjusting the Basic Parameters should suit your needs. The Assay Parameters discussed in the following sections are those that control calculation of the morphological properties of the different objects and targets and thus, unless explicitly mentioned, are available in Basic Mode.

## General Assay Parameters

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

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

## Reference Well Control

The two general Assay Parameters controlling the use of Reference Wells are: **UseReferenceWells** and **MinRefAvgObjectCountPerField**. The **UseReferenceWells** Assay Parameter allows you to choose whether Reference Wells are to be used to determine the population characterization levels. A value of **0** means Reference Wells are not used, whereas a value of **1** engages Reference Wells. If Reference Wells are to be used, then the Assay Parameter **MinRefAvgObjectCountPerField** must also be set. This is the minimum number of objects detected per field that are required for acceptance of the data from the Reference Wells. This allows you to enter the minimum number of objects that you feel gives a good distribution and, thus, statistical validity to the ***Feature*ChN LevelHigh** and ***Feature*ChN LevelLow** 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 setting levels for population characterization. They, along with **MinRefAvgObjectCountPerField**, are available in the Advanced Mode, whereas **UseReferenceWells** is available in the Basic Mode. The population ***Feature*ChN LevelHigh**

and ***Feature*ChN*LevelLow*** Assay Parameters available in the Advanced Mode will be described further in later sub-sections.

## Units for Morphological Measurements

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

## Object Type Ch*N*

### ObjectTypeCh1, MemberTypeCh2, SpotFiberTypeCh3,Ch4

The "Object Type" Assay Parameters allow you to specify whether the objects of interest are brighter or darker than the background of the image. Setting the value to **1** makes dark areas within an image to be considered as potential objects, while leaving it as **0** considers bright areas on a dark background as potential objects (Table 8).

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

***Table 8.*** *Binary characterization of Object Type for each indicated channel.*

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

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

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

## Background Correction Ch*N*

Prior to image analysis, the non-cellular background can be computed and subtracted from each image in each channel independently, as shown in Figure 15.

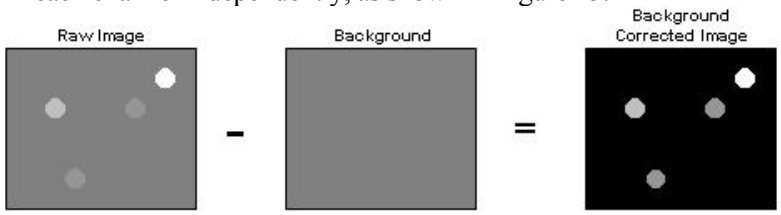



***Figure 15.*** *Background Correction ChN for each channel.*

The background-corrected image is computed by suppressing high frequency components in the image (low pass filtration). You can control the creation of the background image by adjusting the **BackgroundCorrectionCh*N*** Assay Parameter in each channel as needed (Table 9). 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**, background correction is not performed, and analysis is done using the raw, uncorrected images.

If the **BackgroundCorrectionCh*N*** Assay Parameter is given a negative value, an optional background correction method based on the 3D surface fitting is applied. The absolute value entered corresponds to the radius of an area used to find local minima across the image. Found minimum values are used to construct a 3D surface of a background which is then subtracted from the original image. The main advantage of the method is that it minimizes the effect of the background correction (removal) procedure on the intensity values of the analyzed objects. The larger the absolute value, the larger the sampled region, and the less subtraction is done. Setting the value to **–1** lets the application decide on the value needed for the optimal background correction.

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

***Table 9.*** *Possible Background Correction Methods used in each channel with the Morphology Explorer BioApplication.*

**NOTE**

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

Background Correction can be adjusted on each channel separately.

The background-corrected image is not stored or shown.

## Object Smoothing

### ObjectSmoothFactorCh1, MemberSmoothFactorCh2

Channel 1 and Channel 2 have independent Assay Parameters that enable control over the degree of image smoothing, or blurring, before the identification of objects (Channel 1) or members (Channel 2). These Assay Parameters are called **ObjectSmoothFactorCh1** and **MemberSmoothFactorCh2** for Channel 1 and Channel 2 respectively. The Assay Parameter corresponds to the radius in pixels of the area used to smooth the image. A small value, such as **3**, means that a region with a radius of 3 pixels is used to smooth the image (region with dimensions 7×7 pixels, or 49 pixels total). Doubling the value to **6**, means that a larger region (13×13 pixels or 169 pixels total) is used to smooth the image, and thus the image will be more smoothed. A value of **0** means that smoothing is not done. This Assay Parameter is used to smooth images with a lot of contrast to improve identification of objects (Channel 1) or members (Channel 2). To get sharper definition of the shapes of the edges of objects (i.e., cells), you may want to keep the **ObjectSmoothFactorCh1** small, if not 0. However, if your Channel 1 label is not very homogeneous, the actual object will be erroneously identified as consisting of several smaller sized objects, and then smoothing will result in a homogenizing effect of the Channel 1 label and will help identify the object with its true bounds.

## Object Segmentation

Object Segmentation uses either geometric or intensity peaks to separate objects (known as **ObjectSegmentationCh1** and **V3_MemberSegmentationCh2**).

### ObjectSegmentationCh1, V3_MemberSegmentationCh2

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

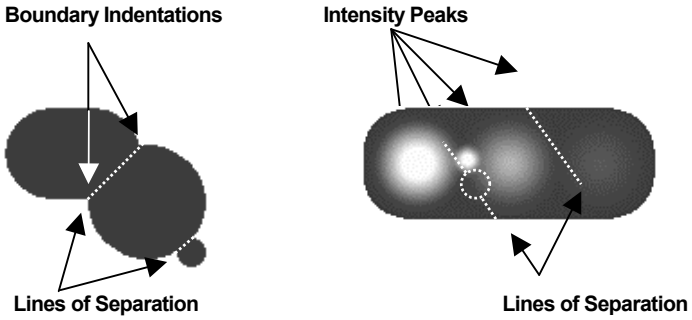



***Figure 16.*** *Current Method Options for Object Segmentation. The image on the left illustrates the Geometric Method while the image on the right illustrates the Intensity Method.*

The Geometric method splits touching objects on the basis of shape, relying on boundary indentations to locate the line of separation. This method works best if the individual objects have smooth boundaries with pronounced indentations at their point of contact. The Geometric method can be used to separate objects that are uniform in intensity (i.e., saturated) or that have multiple intensity peaks (i.e., noisy or textured).

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

The **ObjectSegmentationCh1/ V3_MemberSegmentationCh2** Assay Parameters (see Table 10) control both methods. Positive values for these Assay Parameters will select the Geometric method, and the value is the approximate radius (in pixels) of the smaller of the objects being separated. The value of this Assay Parameter for the Geometric method depends on the magnifying power of the microscope objective and camera setting (pixel binning).

Setting **ObjectSegmentationCh1/ V3_MemberSegmentationCh2** to a negative value selects the peak Intensity method. In this case, the absolute value of the Assay Parameter specifies the minimum relative height of the intensity peak (image contrast) to be used in the segmentation. Making **ObjectSegmentationCh1/ V3_MemberSegmentationCh2** more negative reduces the number of objects created by the segmentation. In case of noisy and textured objects the use of object smoothing may be required to reduce over segmentation. The value of this Assay Parameter for the Intensity method does not depend on power of the microscope objective or camera setting (pixel binning).

A value of **0** for **ObjectSegmentationCh1/ V3_MemberSegmentationCh2** disables segmentation.

| Segmentation Method | ObjectSegmentationCh1/ V3_MemberSegmentationCh2 Assay Parameter | Value Range |
|---|---|---|
| None | 0 | 0 |
| Geometric | radius (in pixels) of smaller object | 1 to 255 |
| Intensity | minimum relative height of intensity peak (local contrast) | -1 to -32767 |

***Table 10.*** *Channel 1 and Channel 2 object segmentation options (Current Method) available for the Morphology Explorer BioApplication*

## Geometric Method

Setting the **ObjectSegmentationCh1** Assay Parameter value equal to the first radius (R1, Figure 17A) produces object separation shown in Figure 17B and setting the **ObjectSegmentationCh1** value to R3 results in segmentation shown in Figure 17C. Setting the **ObjectSegmentationCh1** value to R2 (the largest radius of all touching objects) produces no segmentation.

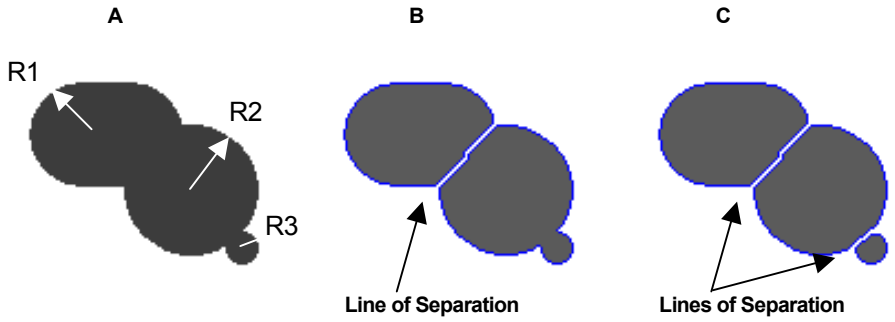



***Figure 17.*** *Current Method for object segmentation using the Geometric Method. A) Radii of touching objects: R1 = 24, R2 = 30, R3 = 7; B) Result of segmentation using* ***ObjectSegmentationCh1*** *= R1; C) Result of segmentation using* ***ObjectSegmentationCh1*** *= R3.*

## Intensity Method

Figure 18 shows the intensity profile along the cordial line of an object with four intensity peaks from Figure 16. Relative peak intensity heights measured from the top of a peak to the nearest valley for each of the object are: 1500 (#1), 1250 (#2), 500 (#3), and 200 (#4).

In general terms, these peak intensity heights are nothing but measures of local contrast of an image. The degree of object segmentation can be controlled by setting the **ObjectSegmentationCh1** Assay Parameter value equal to the lowest relative height of intensity peaks of objects that need to be separated. The lower the value the more objects will be segmented and vise versa. In case of over segmentation, setting the **ObjectSmoothFactorCh1** value greater than 0 should be used to alleviate the problem.

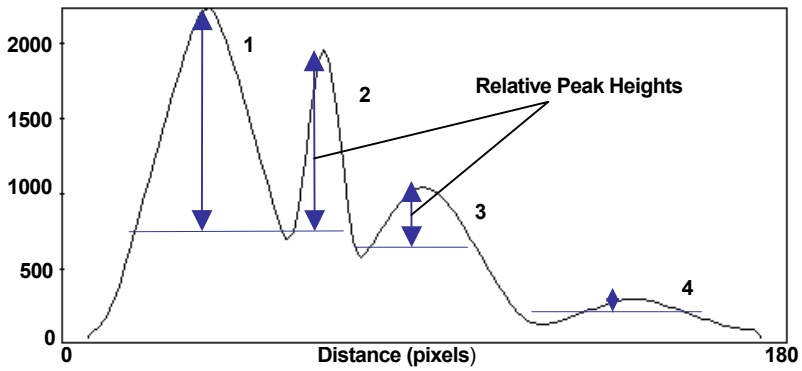



***Figure 18.*** *Intensity profile of an object with four intensity peaks. Peak #1 height = 1500, peak #2 height = 1250, peak #3 height = 500, and peak #4 height = 200.*

Only two peaks in Figure 18, #1 and #2, have relative intensity greater than 1000. Thus, setting the **ObjectSegmentationCh1** Assay Parameter to -1000 marks only two peaks to be used for segmentation (or marks the two objects that need to be separated) and results in object segmentation as shown in Figure 19A. Setting the **ObjectSegmentationCh1** Assay Parameter to -400 or -100 marks three or all four objects that need to be separated. The results of segmentation of three and four objects are shown in Figure 19B and 19C.

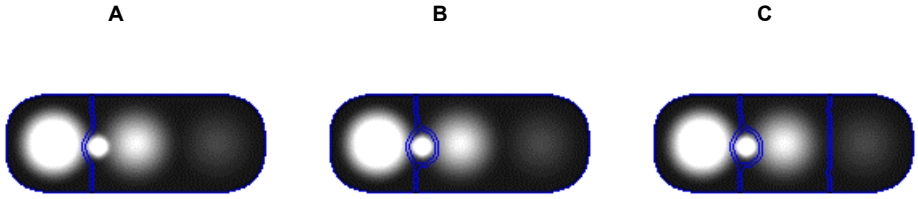



***Figure 19.*** *Current Method for object segmentation using the Intensity Method. A) Result of object segmentation when **ObjectSegmentationCh1** = -1000; B) when **ObjectSegmentationCh1** = -400; C) when **ObjectSegmentationCh1** = -100.*

### Rejecting Border Objects

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

## Channel-Specific Assay Parameters

### Channel 1: Identification of Objects

A critical step in the use of this application is the proper identification of objects. A Channel 1 fluorescent label defines the objects, and the majority of the Channel 1 input parameters control the correct identification of objects. Before automated scanning and analysis of plates can be performed, care must be taken to properly set these parameters based on sample images. Improper object identification will affect the accuracy of the analysis and the results reported from this BioApplication. Thus, the proper functioning of this application critically depends on proper object identification.

The issues that need to be considered in setting up parameters for proper object identification, in their correct sequence, are:

- **Intensity Thresholding**
- **Object Smoothing**
- **Object Segmentation**
- **Identification of Cellular Processes**

Table 12 lists the Channel 1 input parameters that control these items are discussed in the following sections.

| Channel 1 Input Parameters | |
|---|---|
| **Basic Assay Parameters** | **Object Identification & Selection Parameters** |
| **General Assay Parameters** | **Channel 1 Object Identification** |
| UseReferenceWells | Intensity Threshold |
| UseMicrometers | **Channel 1 Object Selection** |
| BackgroundCorrectionCh1 | ObjectAreaCh1 |
| RejectBorderObjectsCh1 | ObjectShapeP2ACh1 |
| **Channel 1 Specific Assay Parameters** | ObjectShapeLWRCh1 |
| ObjectSmoothFactorCh1 | ObjectAvgIntenCh1 |
| | ObjectVarIntenCh1 |
| ObjectSegmentationCh1 | ObjectTotalIntenCh1 |
| ProcessIdentificationModifierCh1 | |

***Table 12.*** *Channel 1 Input Parameters available for the Morphology Explorer BioApplication.*

## Intensity Thresholding

A Channel 1 Object Identification Parameter sets the correct Intensity Threshold; please refer to the Object Identification Methods section earlier in this chapter. The intensity threshold must be set so that the pixels making up the object are above the threshold and are thus selected for analysis. However, setting the threshold too high results in the exclusion of some of the dimmer pixels in the object. Conversely, setting the threshold too low causes non cell-associated areas that only contain background pixels to be included as part of the object.

## Object Smoothing

The **ObjectSmoothFactorCh1** Assay Parameter allows you to control the degree of image smoothing before identification of objects in Channel 1. This Assay Parameter was discussed in detail in the Smoothing sub-section for General Assay Parameters.

## Object Segmentation

The segmentation option available to resolve individual members in Channel 1 is (**ObjectSegmentationCh1**): geometric and intensity.

## Identification of Cellular Processes

A separate issue from object identification is the identification of cellular processes. Usually, processes extending from the cell body, such as neurites extending from a neuronal cell body, are not analyzed. You can turn on/off the ability to identify and analyze such processes. The analysis is turned on and controlled by the **ProcessIdentificationModifierCh1** Assay Parameter, which can have negative, **0**, or positive values. A **0** value for this Assay Parameter results in processes not being identified or analyzed. In this case, the various output features pertaining to processes will be reported as NULL. A non-zero value corresponds to the offset applied to the image threshold to identify objects (i.e., cell body) to arrive at the threshold to specifically identify the processes. This is because the processes may have a different fluorescent intensity than the main object (cell body). In other words, if the threshold applied to identify the processes is $T_P$, then its relationship with the threshold to identify the cell body, $T_F$, using the offset that you entered, *p,* (i.e., **ProcessIdentificationModifierCh1** Assay Parameter) is:

$$T_P = (1 + p)T_F$$

Often in the labeling of neuronal cells with neurite outgrowth, the fluorescence of the neurites is dimmer than that of the cell body. Thus a lower intensity threshold is needed to identify the processes, and the value entered for **ProcessIdentificationModifierCh1** would be negative. For example, if a Fixed Threshold, $T_F$, of 1000 was used to identify the neuronal cell bodies, a value of **–0.1** entered for the **ProcessIdentificationModifierCh1** would result in neurites identified with a threshold, $T_P$, of 900 (90% of 1000). For process identification, a negative value for **ProcessIdentificationModifierCh1** is recommended. In the rare case where the processes are brighter than the cell body, then a positive **ProcessIdentificationModifierCh1** should be entered.

Note if Process Identification is chosen with a non-zero **ProcessIdentificationModifierCh1** and if a particular object has no processes identified, then that object's **ProcessCountCh1** will be **0**. Since the object has no processes, the process morphology features such as its maximum, average and total lengths are irrelevant and will be reported as NULL at the cell-level. In this case, at the well-level the statistical measures (mean and standard deviation) and population characterization features for the process morphology features will not include the object with no processes. Thus the mean, standard deviation and other Well Features for processes will only be computed over a subset of all objects in the field.

# Channel 2: Identification of Members

The input parameters for Channel 2 mainly control identification and quantitation of members (e.g., nuclei) that are part of the Channel 1 objects. Issues that need to be considered in setting up parameters for proper member identification and quantitation, in their correct sequence, are:

- **Intensity Thresholding**
- **Member Smoothing**
- **Member Segmentation**
- **Colony Analysis**
- **Object Mask Modification**

Table 13 lists the Channel 2 input parameters that control these items discussed in the following sections.

| Channel 2 Input Parameters | |
|---|---|
| **Basic Assay Parameters** | **Object Identification & Selection Parameters** |
| MemberSmoothFactorCh2 | **Object Identification** |
| V3_MemberSegmentationCh2 | Intensity Threshold |
| V2_MemberSegmentationCh2 | **Object Selection Parameters (Members)** |
| ColonyAnalysisCh2 | MemberAvgAreaCh2 |
| ObjectMaskModifierCh2 | MemberShapeP2ACh2 |
| | MemberShapeLWRCh2 |
| | MemberAvgIntenCh2 |
| | MemberVarIntenCh2 |
| | MemberTotalIntenCh2 |
| | **Object Selection Parameters (Objects)** |
| | AvgIntenCh2 |
| | TotalIntenCh2 |

***Table 13.*** *Channel 2 Input Parameters available for the Morphology Explorer BioApplication.*

## Intensity Thresholding

The correct Intensity Threshold is set by a Channel 2 Object Identification Parameter; please refer to the Object Identification Methods section earlier in this chapter. The intensity threshold must be set so that the intensity of the pixels making up the members is above the threshold and the members are thus selected for analysis. However, setting the threshold too high results in the exclusion of some of the dimmer pixels in the member. Conversely, setting the threshold too low causes non-member-associated areas that only contain background pixels to be included as part of the member. Selection of the **None** intensity threshold option will result in members not being identified and member analysis not being performed. Note that only this threshold affects output features associated with members. The object average and total intensity reported in Channel 2 is independent of the Channel 2 intensity threshold; for these features, the intensity for all pixels in the object area is used for analysis, whether or not members are identified.

## Member Smoothing

The **MemberSmoothFactorCh2** Assay Parameter allows you to control the degree of image smoothing before identification of members in Channel 2. This Assay Parameter was discussed in detail in the Smoothing sub-section for General Assay Parameters.

## Member Segmentation

Segmentation options available to resolve individual members in Channel 2 are as follows: **V3_MemberSegmentationCh2** (Current Method) and **V2_MemberSegmentationCh2** (Legacy Method) Assay Parameters. They are used to resolve individual members (such as nuclei) in Channel 2 objects. When using the Current Method, a positive value for this Assay Parameter will engage the geometric method of segmentation. The value of this Assay Parameter will depend on the magnification of the objective used. If negative, the

segmentation separates two members based on the intensity peak method. The absolute value of the negative **V3_MemberSegmentationCh2** Assay Parameter defines the intensity range.

When using the Legacy Method, a positive value should approximately be the radius in pixels of typical members (e.g., nuclei) that need to be resolved. Again, the value of this parameter will depend on the magnification of the objective used. If negative, the segmentation separates two members based on the equivalent distance between their peaks. The absolute value of the negative **V2_MemberSegmentationCh2** Assay Parameter defines the radius in pixels of the local area where the search for the object's peak is conducted. If both Assay Parameters (V2 and V3) are set to **0**, segmentation is not performed.

## Colony Analysis

The number of members, such as the nuclei, within a multi-cellular object corresponds to the number of cells within a colony. The Morphology Explorer BioApplication can also identify which of the members (i.e., cells) are on the exterior of the colony (i.e., have some contact with the outside) and which ones are inside the colony (i.e., totally enclosed and surrounded by other cells of the same colony). Since this type of analysis is computationally expensive, you may not want to have this analysis done unless necessary. The Assay Parameter that turns on quantitation of the number of interior and exterior cells in a colony is **ColonyAnalysisCh2**. If it is set to **0**, the further colony analysis of identifying interior and exterior cells is not done. If it is set to **1**, the interior and exterior cells are identified, but the BioApplication runs slower.

## Object Mask Modification

The Morphology Explorer BioApplication needs to define the Channel 2 object area in which all the member identifications take place. This is derived from the object area from Channel 1, but you have the ability to modify the region covered. You also have the option of similarly independently modifying (i.e., expanding or contracting) the object area in which measurements are made for Channel 3 and Channel 4. The Assay Parameters that are used to adjust the object area in which to make measurements for Channels 2-4 are **ObjectMaskModifierCh*N***, where *N* is the relevant dependent channel (i.e., Channels 2-4). This Assay Parameter is the number of pixels added to, or subtracted from, depending on the parameter's sign, from the perimeter of the object area identified in Channel 1 (see Figure 21). This Assay Parameter is also used for intensity gating in Channels 2-6.

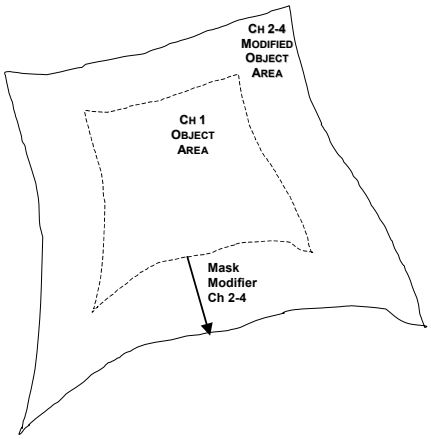



***Figure 21.*** *Adjustment of Measurement Area in Channels 2-4. *Note that this adjustment can also occur in Channel 5 and Channel 6, but for gating purposes only.*

## Channel 3 and Channel 4: Identification of Spots and Fibers

The categories of input parameters controlling Channel 3 and Channel 4 quantitation are:

- **Intensity Thresholding**
- **Spot and Fiber Identification**
- **Fiber Alignment**
- **Object Mask Modification**

Table 14 lists the Channel 3 and Channel 4 input parameters that control these items discussed in the following sections.

<table>
<tr><th colspan="4">Channel 3 and Channel 4 Input Parameters</th></tr>
<tr><td colspan="2">Basic Assay Parameters</td><td colspan="2">Object Identification & Selection Parameters</td></tr>
<tr><td>Channel 3</td><td>Channel 4</td><td colspan="2">Object Identification</td></tr>
<tr><td>SpotFiberDetectRadiusCh3</td><td>SpotFiberDetectRadiusCh4</td><td colspan="2">Separate Intensity Thresholds for Channels 3 and 4</td></tr>
<tr><td>FiberAlign2AnalysisCh3</td><td>FiberAlign2AnalysisCh4</td><td colspan="2">Object Selection Parameters (Spots & Fibers)</td></tr>
<tr><td>ObjectMaskModifierCh3</td><td>ObjectMaskModifierCh4</td><td>SpotFiberAreaCh3</td><td>SpotFiberAreaCh4</td></tr>
<tr><td colspan="2" rowspan="2">Advanced Assay Parameters to Define Responders for Proximity Measurements Between Similar & Dissimilar Objects</td><td>SpotFiberShapeLWRCh3</td><td>SpotFiberShapeLWRCh4</td></tr>
<tr><td colspan="2">Object Selection Parameters (Objects)</td></tr>
<tr><td>AvgIntenLevelLowCh3, AvgIntenLevelHighCh3<br>OR<br>AvgIntenLevelLowCh3_CC, AvgIntenLevelHighCh3_CC</td><td>AvgIntenLevelLowCh4, AvgIntenLevelHighCh4<br>OR<br>AvgIntenLevelLowCh4_CC, AvgIntenLevelHighCh4_CC</td><td>AvgIntenCh3<br>TotalIntenCh3</td><td>AvgIntenCh4<br>TotalIntenCh4</td></tr>
</table>

***Table 14.*** *Channel 3 and Channel 4 Input Parameters available for the Morphology Explorer BioApplication.*

### Intensity Thresholding

The correct Intensity Thresholds for Channel 3 and Channel 4 are independently set by the Channel 3 and Channel 4 Object Identification Parameters; please refer to the Object Identification Methods section earlier in this chapter. The intensity thresholds applied in Channel 3 or Channel 4 control identification of intracellular constituents, such as spots or fibers, and must be set so that the pixels making up these spots or fibers are above the threshold and are thus selected for analysis. However, setting the threshold too high results in the exclusion of some of the pixels of the dimmer spots or fibers. Conversely, setting the threshold too low causes areas that only contain background pixels and noise to be included as part of the spot or fiber, or may cause artificial connections between discrete spots or fibers to be formed thus resulting in their erroneous morphological measurements. Since the correct intensity threshold setting is critical in these channels and because of the nature of discrete intracellular objects such as spots or fibers, the largest number of intensity threshold options is available for these two channels. This includes the sensitive **3Sigma Threshold** method which facilitates the detection of small, discrete objects such as spots and fibers which are distinct from the bulk background of the rest of the object.

### Spot and Fiber Identification

Spots and Fibers are sub-cellular entities (Figure 9 and Figure 12), whose identification is based on their intensity as well as their morphology being within a specific range that you

define. Spots are also distinguished from fibers by their morphology (aspect ratio). Thus Spot and Fiber identification requires specifying the appropriate intensity threshold and morphological ranges and is a two-step process. First, the identification of cellular constituents, which may be potential spots or fibers, is done. Then the specific differentiation of spots versus fibers is done and the size range of the spots or fibers is specified to establish what are valid spots or fibers, versus intracellular noise, for the biological situation being analyzed. The steps required for spot or fiber identification and analysis are described below. Spot and Fiber analysis is done in a similar fashion for both Channel 3 and Channel 4. Thus, the description in this section for Channel 3 also applies for the adjustment of Channel 4 parameters.

In Channel 3, objects typically have background fluorescence associated with them where intensity varies slowly over the span of the object. In contrast, spots and fibers are discrete entities whose intensity varies rapidly over space within the object. These properties, the spot/fiber intensities and how it spatially varies versus the background object fluorescence are used to identify the spots and fibers. Thus, the first step of identifying spots or fibers is done by specifying two criteria: the spot or fiber intensity and the spot or fiber width.

### Spot Fiber Detect Radius

To identify which of the retained pixels with high intensity belong to spots or fibers, the change over space within the object is evaluated. First, any remaining background intensity after thresholding is automatically removed and only discrete cellular constituents whose intensity varies rapidly over space remains, as these may be potential spots or fibers. When removing slowly varying background intensity for spot or fiber identification, the spatial dimension governing which intensity changes are considered slowly varying must be defined. This is set by the Assay Parameter **SpotFiberDetectRadiusCh3** which is related to the typical radius, or half the width, of typical spots or fibers. This Assay Parameter is used as a spatial filter to control the removal of local varying background fluorescence in the object so that individual spots can be identified; the size of this parameter is similar to the size of the spots in pixels that are detected.

For most fluorescence, images of intracellular cytoskeletal fibers, such as F-actin or microtubules), setting the **SpotFiberDetectRadiusCh3** to a value of **1** gives adequate results, and this Assay Parameter rarely needs to be increased. In cases of dim, noisy images which have poor signal to noise ratio, it has been empirically seen that larger values of **SpotFiberDetectRadiusCh3** may improve fiber detection. For spots, the size of the spot or the particular cellular entity governs the value to be set for **SpotFiberDetectRadiusCh3**. For small organelles such as endosomes, a small value would suffice, whereas for larger organelles such as nuclei, the value of this parameter may have to be increased.

### Turning Off Spot Fiber Analysis

If the biology being analyzed does not require the identification and quantitation of spots or fibers, the spot fiber analysis should be turned off so that the application runs faster. The spot fiber analysis can be turned off by either choosing the **None** option for the intensity threshold or choosing a value of **0** for the **SpotFiberDetectRadiusCh3** Assay Parameter. Note that if spots or fibers are not detected in Channel 3 or Channel 4, then the **SpotFiberCount** feature will be **0**. At the cell-level, the **SpotFiberArea** measurements, all radial intensity distribution measurements, and **FiberAlign1** will be reported as NULL, but not **FiberAlign2**, which is independent of whether a spot or fiber is identified using intensity thresholds. For these features, this object will not be used in the doing the well-level calculations.

## Fiber Alignment

The two different metrics that this BioApplication provides for quantifying the degree of intracellular fiber alignment are described in more detail later in this chapter. The simpler method, **FiberAlign1**, is the standard deviation of the angles of all the identified fibers, and is always calculated. The **FiberAlign2** feature is derived from an autocorrelation calculation as described in Chapter 1. It is independent of the intensity threshold; changing the Channel 3 or Channel 4 intensity threshold option or value will not affect this particular calculation. Since this type of analysis is computationally expensive, you may not want to have this analysis done unless necessary. The Assay Parameters which control whether the **FiberAlign2** calculation is done are **FiberAlign2AnalysisCh3** and **FiberAlign2AnalysisCh4**. If it is set to **0**, the **FiberAlign2** calculation for the particular channel (Channel 3 or Channel 4) is not done. If it is set to **1**, the calculation is done, but the BioApplication runs slower. Note the **FiberAlign1** calculation will be done only if the spot fiber analysis is turned on and spots or fibers have been identified. However, since the **FiberAlign2** is a measure of the anisotropy of the image's autocorrelation (i.e., the persistence of any patterns) it will be done even when the spot fiber analysis has been turned off.

## Object Mask Modification

The **ObjectMaskModifierCh*N***, where *N* is the relevant dependent channel controls the area in which measurements are made in Channel 3 or Channel 4, as described earlier (see Figure 21).

## Controlling Proximity Measurements between Similar and Dissimilar Objects

As discussed earlier in this chapter, based on the different fluorescence labels used in Channel 3 and Channel 4, the Morphology Explorer BioApplication can report different distance metrics between similar and dissimilar objects. Similar objects in Channel 3 are those that are positive responders for the Channel 3 fluorescence label and, likewise, similar objects in Channel 4 are those that are positive responders for the Channel 4 fluorescence label. Measurements are done between similar objects in Channel 3 and Channel 4 independently and then between dissimilar objects (i.e., between objects with the Channel 3 label versus the Channel 4 label). These proximity and spacing measurements between similar and dissimilar cells depend on what is defined as a positive responder for the Channel 3 and Channel 4 fluorescence labels. A responder is an object whose average intensity is above a specific threshold. The intensity ranges are controlled by the specific advanced Assay Parameters controlling the population characterization for that intensity.

Theoretically, a responder is defined as an object whose property is greater than the ***Feature*Ch*N*LevelHigh** or less than the ***Feature*Ch*N*LevelLow** Assay Parameter (has Status equal to **1** or **2** – see Table 22 and Figure 25). For example, for an object in Channel 3 to be defined as a responder, its **AvgIntenCh3** Cell Output Feature needs to be either greater than **AvgIntenCh3LevelHigh** or less than **AvgIntenCh3LevelLow**. Practically, since only objects that have the specific label are considered, the ***Feature*Ch*N*LevelLow** value should be set to **0** and responders are those objects with average intensity greater than the ***Feature*Ch*N*LevelHigh** (Status = **1**). If Reference Wells are not used, then the high-response level for **AvgIntenCh3** is controlled by the **AvgIntenLevelHighCh3** advanced Assay Parameter, which is manually entered. If Reference Wells are used, then the high-response level is automatically calculated using the **AvgIntenLevelHighCh3_CC** advanced Assay Parameter. More use of such advanced Assay Parameters to define responders is described in the sub-sections that follow.

To summarize, to measure the spacing and proximity between similar objects in Channel 3, you have to first set the advanced Assay Parameters that control the ***Feature*Ch*N*LevelHigh** limit for the average intensity to enable the program to identify which objects are responders in Channel 3. Responders have average intensities greater than this level. To do the same for similar objects in Channel 4, or to get spacing and proximity measurements between dissimilar objects in Channel 3 versus Channel 4, you have to go through an additional similar procedure to define which objects are responders in Channel 4.

## Basic Assay Parameters

Assay Parameters available in Basic Mode control calculation of the different morphological properties of each object and control the use of Reference Wells. Note that nine of the Basic Parameters function as ON/OFF or YES/NO switches that can turn off specific types of processing. A value of **1** is "YES" and turns the function on, whereas a value of **0** is "NO" and turns the function off. These Assay Parameters are listed in Table 15.

| Basic Assay Parameter | Channel |
|---|---|
| **UseReferenceWells** | 1 |
| **RejectBorderObjectsCh1** | 1 |
| **ObjectTypeCh1** | 1 |
| **ColonyAnalysisCh2** | 2 |
| **MemberTypeCh2** | 2 |
| **FiberAlign2AnalysisCh3** | 3 |
| **SpotFiberTypeCh3** | 3 |
| **FiberAlign2AnalysisCh4** | 4 |
| **SpotFiberTypeCh4** | 4 |

***Table 15.*** *Binary Basic Assay Parameters available with the Morphology Explorer BioApplication.*

Table 16 lists all Assay Parameters available in Basic Mode. You will not be able to view any Advanced Assay Parameters in this mode (as the **Hide Advanced** Parameters option in checked).

| Parameter | Units | Description |
|---|---|---|
| UseReferenceWells | Binary | Use reference wells to calculate high and low response levels: 0 = No, 1 = Yes |
| ObjectTypeCh1 | Binary | Type of objects to be identified in Ch1: 0 = Bright objects on dark background, 1 = Dark objects on bright background |
| BackgroundCorrectionChN | Pixels | Radius (in pixels) of the region used to compute background in ChN: Negative value = Use surface fitting, 0 = Do not apply background correction, Positive value = Use low pass filter |
| ObjectSmoothFactorCh1 | Number | Degree of image smoothing (blurring) prior to object identification in Ch1: 0 = Do not apply smoothing |
| ObjectCleanupCh1 | Binary | Clean up object mask and remove small objects by applying erosion followed by dilation: 0 = No, 1 = Yes |
| ObjectSegmentationCh1 | Pixels | ***Current Method***: Radius (in pixels) or Intensity range of touching objects that should be separated in Ch1: Negative value = Use intensity peaks method, 0 = Do not apply object segmentation, Positive value = Use shape geometry method |
| RejectBorderObjectsCh1 | Binary | Reject objects that touch image edges in Ch1: 0 = No, 1 = Yes |
| ProcessIdentificationModifierCh1 | Fraction | Fractional adjustment used to compute process identification threshold from the object identification threshold in Ch1 |
| MemberTypeCh2 | Binary | Type of members to be identified in Ch2: 0 = Bright objects on dark |

| Parameter | Units | Description |
|---|---|---|
| | | background, 1 = Dark objects on bright background |
| MemberSmoothFactorCh2 | Number | Degree of image smoothing (blurring) prior to member identification in Ch2: 0 = Do not apply smoothing |
| V3_MemberSegmentationCh2 | Pixels | ***Current Method:*** Radius (in pixels) or Intensity range of touching members that should be separated in Ch2: Negative value = Use intensity peaks method, 0 = Do not apply member segmentation, Positive value = Use shape geometry method |
| V2_MemberSegmentationCh2 | Pixels | ***Legacy Method:*** Radius (in pixels) of touching member objects that should be separated in Ch2: Negative value = Use intensity peaks method, 0 = Do not apply member object segmentation, Positive value = Use shape geometry method |
| ColonyAnalysisCh2 | Binary | Count number of peripheral and internal members in Ch2 colonies; 0 = No, 1 = Yes |
| RingDistanceChN | Pixels | Distance (in pixels) from Ch1 object mask to the inner rim of ring mask used for analysis in ChN |
| RingWidthChN | Pixels | Width (in pixels) of ring mask used for analysis in ChN |
| CircModifierChN | Pixels | Number of pixels to modify Ch1 object mask to create circ mask used for analysis in ChN: Negative value = Shrink mask, 0 = Do not modify mask, Positive Value = Expand mask |
| ObjectMaskModifierChN | Pixels | Number of pixels to modify Ch1 object mask in ChN: Negative value = Shrink mask, 0 = Do not modify mask, Positive value = Expand mask |
| SpotFiberTypeCh3 | Binary | Type of spots and fibers to be identified in Ch3: 0 = Bright objects on dark background, 1 = Dark objects on bright background |
| SpotFiberDetectRadiusCh3 | Pixels | Radius (in pixels) of region used for spot and fiber detection in Ch3: 0 = Do not detect spots or fibers |
| FiberAlign2AnalysisCh3 | Binary | Compute fiber alignment2 features in Ch3: 0 = No, 1 = Yes |
| SpotFiberTypeCh4 | Binary | Type of spots and fibers to be identified in Ch4: 0 = Bright objects on dark background, 1 = Dark objects on bright background |
| SpotFiberDetectRadiusCh4 | Pixels | Radius (in pixels) of region used for spot and fiber detection in Ch4: 0 = Do not detect spots or fibers |
| FiberAlign2AnalysisCh4 | Binary | Compute fiber alignment2 features in Ch4: 0 = No, 1 = Yes |
| RadialIntenAnalysisCh4 | Binary | Compute radial distribution of intensity features in Ch4 using spot/fiber masks or ROI (ring or circ) mask: 0=Use ROI mask, 1=Confine analysis to spot/fiber masks |

***Table 16.*** *Basic Assay Parameters available in the Morphology Explorer BioApplication. *Note that "ChN" represents Channels1-6 for Background Correction and Channels 2-6 for Object Mask Modifier.*

## Object Selection Parameters

Each channel has a set of specific Object Selection Parameters associated with it. If an object in the particular channel's image has all measured features within the range specified by the appropriate Object Selection Parameters, then it is analyzed; otherwise, it is rejected from analysis. The Object Selection Parameters for Channel 1 are used to select which of the fluorescently-labeled primary objects in Channel 1 pass the selection criteria and are thus identified as individual, cell-associated primary objects and worthy of further analysis.

There are two types of selection parameters for Channels 2-4: those that further select the object for analysis in that channel and those that select which sub-objects are selected for analysis. For Channels 2-6, there are three Object Selection Parameters, **MembraneCountCh2**, **AvgIntenCh*N*,** and **TotalIntenCh*N***, which further determine whether the object is to be analyzed. If the object's intensity in the specific channels has both average and total intensity in the specified range, it will be selected for analysis. Sub-objects include members (Channel 2) and spots or fibers (Channel 3 and Channel 4) (see Figures 21-23). Object Selection Parameters for these sub-objects are based on their morphology (area, P2A, LWR for members in Channel 2, and area and LWR for spots or fibers in Channel 3 and Channel 4), and the individual sub-objects must be in the range specified for these particular morphological features to be included for analysis.

In the Protocol Interactive View, the **Identify Objects** button will enable all objects and sub-objects to be identified. Placing the cursor on individual objects will cause its Object Selection Parameters to be displayed. Objects can then be selected to set the range (Figure 22). Similarly, in Channels 2-4, placing the cursor on individual sub-objects will cause their Object Selection Parameters to be displayed, and these too can be selected to set the range to select sub-objects (Figures 23-24). Care must be exercised to ensure that the cursor is on the appropriate object or sub-object so that the appropriate and correct Object Selection Parameters are displayed and can be selected.

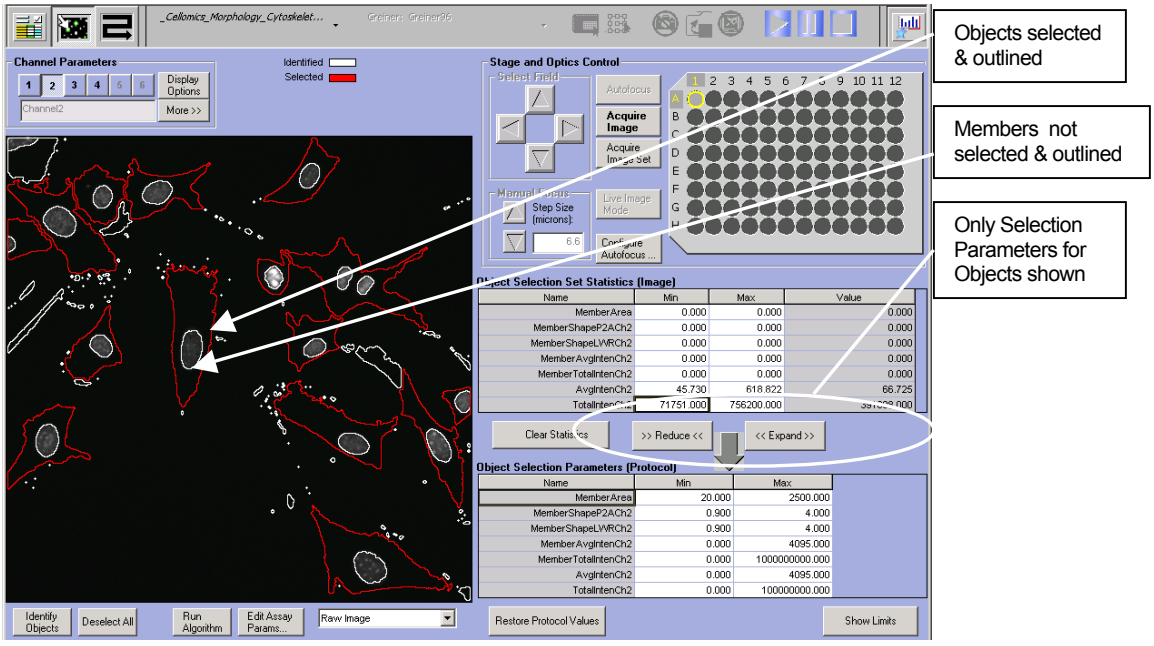



***Figure 22.*** *Selecting only objects in the Protocol Interactive View in Channel 2. The* ***Identify Objects*** *button was clicked and all objects and sub-objects (members in Channel 2) were identified. Placing the cursor on objects only and selecting them causes only the objects to be outlined and their Selection Parameter values to be listed.*

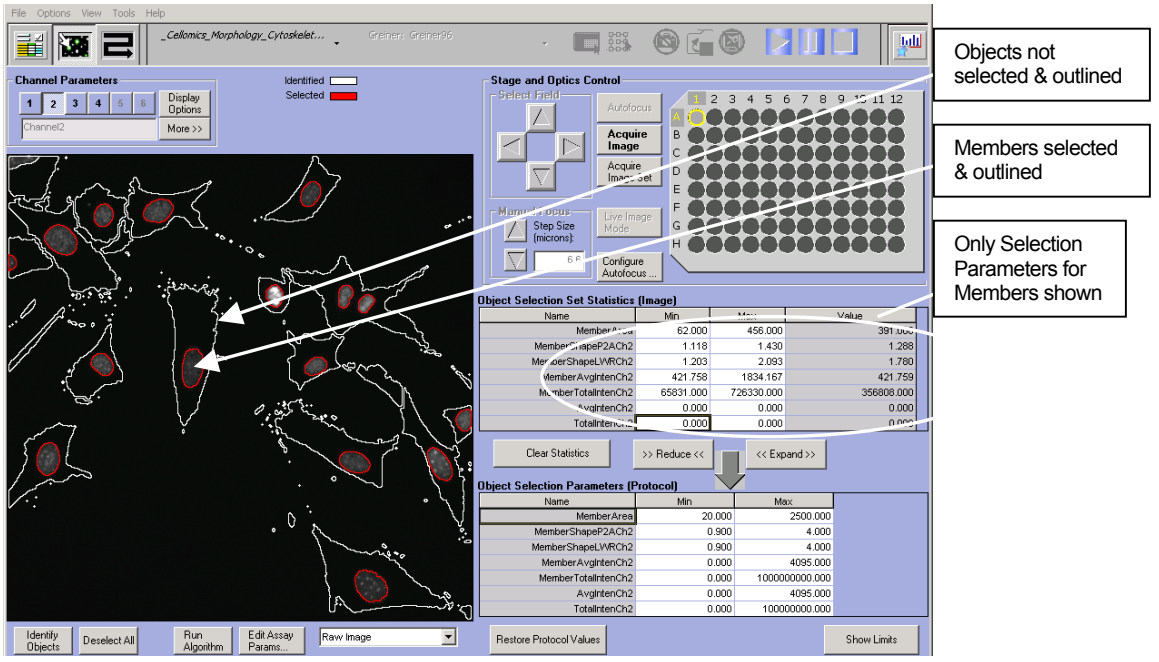



***Figure 23.*** *Selecting only members in the Protocol Interactive View in Channel 2. The* ***Identify Objects*** *button was clicked and all objects and sub-objects (members in Channel 2) were identified. Placing the cursor on members only and selecting them causes only the members to be outlined and their Selection Parameter values to be listed.*

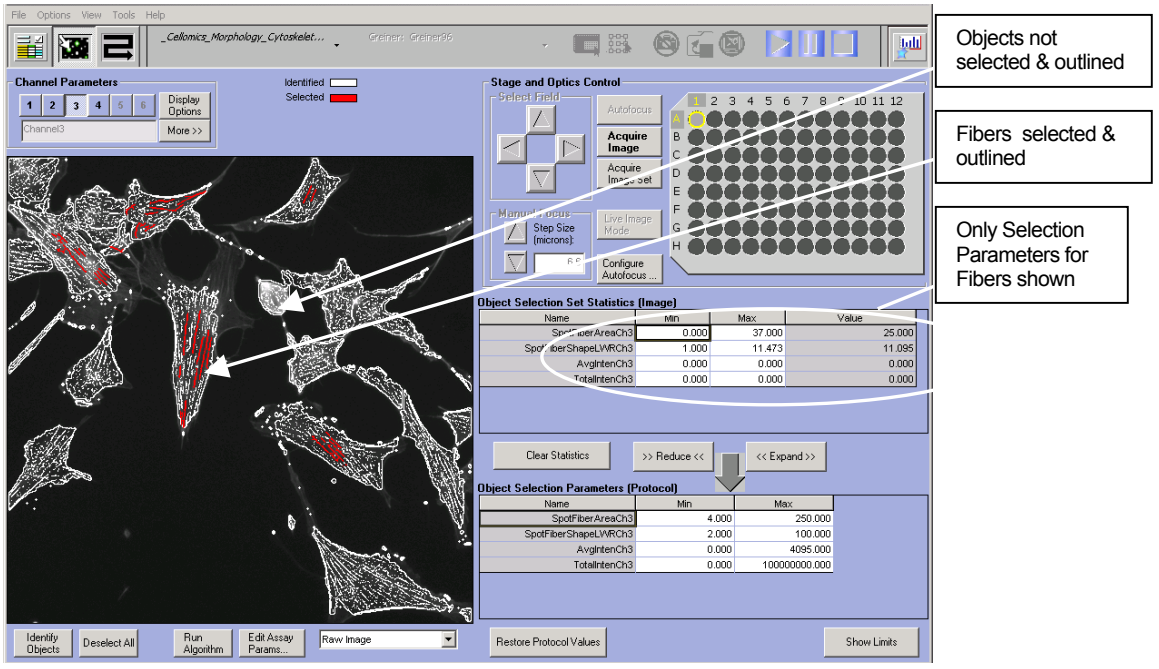



***Figure 24.*** *Selecting only fibers in the Protocol Interactive View in Channel 3. Similar to Channel 2 as shown in Figure 22, sub-objects (spots and fibers) can be selectively identified in Channel 3 and Channel 4. The* ***Identify Objects*** *button was clicked and all objects and sub-objects (fibers in Channel 3) were identified. Placing the cursor on fibers only and selecting them causes only the fibers to be outlined and their Selection Parameter values to be listed.*

## Channel 1 Object Selection Parameters

Once all objects in Channel 1are properly separated and resolved, the final step is to identify which of the many potential objects in Channel 1 are valid and should be quantitatively analyzed and which ones are either junk or not valid for analysis. This is done by Channel 1 Object Selection Parameters which are used to select which of the fluorescently-labeled primary objects in Channel 1 pass the selection criteria and are thus identified as individual, cell-associated primary objects and worthy of further analysis (Table 17). The two categories of Channel 1 Object Selection Parameters are **Intensity** and **Morphology**.

The average, variation and total intensities of the primary object are intensity-related criteria used to select objects and are controlled by the **ObjectAvgIntenCh1, ObjectVarIntenCh1** and **ObjectTotalIntenCh1** Object Selection Parameters respectively. The morphology-related Object Selection Parameters are **ObjectAreaCh1**, **ObjectShapeP2ACh1**, and **ObjectShapeLWRCh1**. If an object's average and total intensities and its **ObjectAreaCh1**, **ObjectShapeP2ACh1**, and **ObjectShapeLWRCh1** are all within the specified range, then it is selected as a valid object for further analysis.

For example, suppose the objects being selected are myotubes where the Channel 1 label is immunofluorescence against the skeletal muscle myosin that only differentiated myoblasts which have formed myotubes express (see Figure 8). Then valid objects can be selected on the basis of intensity, where only objects that have a certain average and total intensity (i.e., express the skeletal muscle myosin) are accepted. Furthermore, since myotubes are long, tubular objects, further object selection can be done on the basis of their morphology. Thus, valid objects are further selected on the basis of their having a sufficient dimension by being larger than a minimum **ObjectAreaCh1** and having a large **ObjectShapeLWRCh1** which corresponds to a tubular structure.

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

***Table 17.*** *Object Selection Parameters available in Channel 1 for the Morphology Explorer BioApplication.*

## Channel 2 Object and Member Selection

The next step is to set the Channel 2 Object Selection Parameters to obtain both correct object and member identification and selection (Table 18). Separate Object Selection Parameters control whether objects and members get selected. In many cases, the fluorescent stain used to label the members will also give a background fluorescence staining of the object, which can be detected in Channel 2. You may use this as a further criterion to select the object for analysis, and the basis for this selection is the average and total intensities of the object detected in Channel 2. These are controlled by the **AvgIntenCh2** and **TotalIntenCh2** Object Selection Parameters. The object fluorescence in Channel 2 must fall within the intensity ranges specified by these two parameters for the object to be selected for further analysis.

These parameters can be used to gate which objects are chosen for analysis based on the overall object expression levels of particular proteins. Alternatively, if you are satisfied by the object being selected for analysis by the criteria set in Channel 1 and you do not want to apply any further selection criteria based on the fluorescence in Channel 2, you can leave the gates open for these parameters (i.e., keep the minimum values of these two Object Selection Parameters at their lowest possible level and similarly set their maximum values to the highest possible level).

In Channel 2, Object Selection Parameters are also used to select or reject members for analysis. However, note that members within an object will be selected only if that object has also been selected for analysis. Members containing the Channel 2 label (i.e., organelles and compartments such as nuclei) are selected based on five criteria: area, shape (P2A), aspect ratio (LWR), average intensity, total intensity, and variation of intensity. For each of these features, a range specifying minimum and maximum values needs to be defined, and the members must have all six properties to fall within these ranges to be selected. Specifically, the **MemberAreaCh2** Selection Parameter sets the range for the member area. Similarly, the range for the member P2A is set by the **MemberShapeP2ACh2** Object Selection Parameter, the range for the member LWR is set by the **MemberShapeLWRCh2** Object Selection Parameter, the range for the member average pixel intensity is set by the **MemberAvgIntenCh2** Object Selection Parameter, the range for the member total pixel intensity is set by the **MemberTotalIntenCh2** Object Selection Parameters, and the range for the member intensity variation is set by the **MemberVarIntenCh2** Object Selection Parameters. Each individual member must fall within the range for all of these features to be analyzed.

Note the identification and analysis of members are affected by both the intensity threshold and the Object Selection Parameters. The object average and total intensity reported in Channel 2 is independent of the Channel 2 intensity threshold and is only controlled by the **AvgIntenCh2** and **TotalIntenCh2** Object Selection Parameters. For these features, the intensity for all pixels in the modified mask of the object area from Channel 1 is used for analysis. Choosing an intensity threshold option of **None** will result in no members being identified and is equivalent to turning member identification off.

If an object has no members detected in it in Channel 2, then all the **MemberCountCh2** Cell Features for that object will be **0**. At the cell-level, all member morphology features for that object will be reported as NULL, its **MemberObjectAreaRatioCh2** will be reported as **0**, and its **MemberObjectAreaDiffCh2** will result in the object area. This object will not be included in the well-level calculations for these member morphology features.

| Parameter | Units | Description |
|---|---|---|
| MemberAvgAreaCh2 | Pixels or $\mu m^2$ | Average area (in pixels or micrometers) of all Ch2 member objects within modified Ch1 object mask |
| MemberShapeP2ACh2 | Number | Shape measure based on ratio of perimeter squared to 4π* area of Ch2 member object (**MemberShapeP2ACh2** = 1 for circular member object) |
| MemberShapeLWRCh2 | Number | Shape measure based on ratio of length to width of object-aligned bounding box of Ch2 member object |
| MemberAvgIntenCh2 | Intensity | Average intensity of all pixels within Ch2 member object |
| MemberVartIntenCh2 | Intensity | Variation (standard deviation) of intensity of all pixels within Ch2 member object |
| MemberTotalIntenCh2 | Intensity | Total intensity of all pixels within Ch2 member object |

| Parameter | Units | Description |
|---|---|---|
| MemberCountCh2 | Number | Number of Ch2 member objects within cell (colony) |
| AvgIntenCh2 | Intensity | Average intensity in Ch2 of all pixels within modified Ch1 object mask |
| TotalIntenCh2 | Intensity | Total intensity in Ch2 of all pixels within modified Ch1 object mask |

***Table 18.*** *Object Selection Parameters available in Channel 2 for the Morphology Explorer BioApplication.*

## Channel 3/Channel 4 Spot Fiber Object Selection Parameters

Intensity thresholding and spatial filtering via the **SpotFiberSizeCh3** (and/or **SpotFiberSizeCh4**) Assay Parameters identify intracellular entities that may be potential spots or fibers. The next step is to differentiate between spots from fibers and to select what should be included for quantitative analysis. This is done by two separate Object Selection Parameters: **SpotFiberAreaCh3 (SpotFiberAreaCh4)** and **SpotFiberShapeLWRCh3 (SpotFiberShapeLWRCh4)**. They specify the range of the valid areas and aspect ratios (LWR) for the potential spots and fibers (Table 19). Increasing the area range means that larger spots or thicker or longer fibers can be selected for analysis; thus the area can be used to differentiate between genuine spots and fibers versus intracellular noise. **SpotFiberShapeLWRCh3 SpotFiberShapeLWRCh4)** is used to differentiate between spots and fibers and set the selection criteria for valid spots and fibers. An aspect ratio close to **1** defines a spot, whereas fibers have a larger aspect ratio. The entity's aspect ratio and area must be within the range set by these two parameters to be identified and selected for analysis.

| Parameter | Units | Description |
|---|---|---|
| SpotFiberAreaChN | Pixels or $\mu m^2$ | Area (in pixels or micrometers) of ChN spot or fiber |
| SpotFiberShapeLWRChN | Number | Shape measure based on ratio of length to width of object-aligned bounding box of ChN spot or fiber |
| AvgIntenChN | Intensity | Average intensity in ChN of all pixels within modified Ch1 object mask |
| TotalIntenChN | Intensity | Total intensity in ChN of all pixels within modified Ch1 object mask |

***Table 19.*** *Object Selection Parameters available in Channel 3 and Channel 4 for the Morphology Explorer BioApplication. *Note that "ChN" refers to Channels 3-4.*

## Channel 5 and Channel 6 Object Selection Parameters

For Channel 5 and Channel 6, the available Object Selection Parameters enable you to gate whether the object is to be analyzed based on its average and total intensity (Table 20).

| Parameter | Units | Description |
|---|---|---|
| AvgIntenChN | Intensity | Average intensity in ChN of all pixels within modified Ch1 object mask |
| TotalIntenChN | Intensity | Total intensity in ChN of all pixels within modified Ch1 object mask |

***Table 20.*** *Object Selection Parameters available in Channel 5 and Channel 6 for the Morphology Explorer BioApplication. *Note that "ChN" refers to Channels 5-6.*

## Gating

The Morphology Explorer BioApplication supports gating on a cell population. Gating allows you to specifically identify a subset of cells in Channels 2-6 based on fluorescence intensity and allows you to limit the analysis to this group of cells. For example, if measurements were being made in cells that expressed a GFP–protein chimera and only in cells that had a certain expression level, then gating could be used such that the intensity range is set to include only cells that had the desired expression level in a specific channel (Channels 2-6). The expression level can be specified by the average and total intensities individually reported for Channels 2-6. For each channel, these intensities are measured in regions that are independently derived from the Channel 1 object, where the region covered by the Channel 1 object can be individually and independently modified by the **ObjectMaskModifierCh*N*** Assay Parameter that is available for each of the other channels. Therefore, if you choose to add gating channels, an object must pass the criteria in all channels to be selected for analysis.

### Specifying Intensity Ranges for Gating

When working in the Create Protocol View, you can specify intensity threshold values by entering upper and lower threshold limits for two intensity parameters, **AvgIntenCh*N*** and **TotalIntenCh*N***, for one or more dependent channels (Channels 2-6) or **ObjectAvgIntenCh1** and **ObjectTotalIntenCh1** for these parameters in Channel 1. **ObjectTotalIntenCh1** is a summation of all intensities within the object of interest. **AvgIntenCh*N*** is **TotalIntenCh*N*** divided by the object's area.

Alternatively, you can set these parameter values interactively when working in the Protocol Interactive View. Results of the subpopulation selection can be observed in the View software application when viewing Well Details. **ValidObjectCount** displays the total number of objects found for each well. **SelectedObjectCount** displays the number of objects in each well that passed all requirements, including the intensity requirements. To view the intensity parameter values, select Cell Details for a particular well. The View software application displays the intensity parameter values for each cell in each channel.

### Specifying Mask Modifiers in Assay Channels

In addition to specifying intensity threshold ranges for one or more channels, you can apply a mask to one or more downstream channels using the **ObjectMaskModifierCh*N*** setting (see Figure 21). You may want to use this feature if, for example, it is desirable to dilate the mask of each object to include other cellular markers. The mask can be dilated or eroded, but will not overlap with other masks from nearby objects.

## Image Overlays

During a scan, various features can be displayed as color overlays on the channel images (Table 21). 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.

| Parameter | Description |
|---|---|
| Include This Channel In Composite | Determines if image for this channel is included in composite image. |
| SelectedObject | Outlines valid objects in Channel 1 (e.g., cells) for analysis. Valid objects are those that have properties in the range specified by the Object Selection Parameters. |
| RejectedObject | Outlines objects rejected for analysis. Rejected objects have properties outside the range specified by the Object Selection Parameters. |
| ExtProcess | Outlines processes extending from the cell body in Channel 1. Processes are identified and quantified only if the **ProcessIdentificationCh1** Assay Parameter ≠ 0. |
| MemberCh2 | Outlines members identified in Channel 2. Multiple members may be identified in Channel 2 for each distinct object (e.g., if Channel 1 label identifies objects as colonies, a Channel 2 nuclear stain will identify individual nuclei as members, with each object having multiple nuclei). |
| SpotFiberCh3 | Outlines Spots or Fibers identified in Channel 3. |
| ObjectCh3 | Outlines region in Channel 3 where measurements are made. **ObjectMaskModifierCh3** Assay Parameter controls region. |
| SpotFiberCh4 | Outlines Spots or Fibers identified in Channel 4. |
| ObjectCh4 | Outlines region in Channel 4 where measurements are made. **ObjectMaskModifierCh4** Assay Parameter controls region. |

***Table 21.*** *Image Overlay Display Option Settings available for the Morphology Explorer BioApplication.*

# Assay Parameters for Population Characterization

## Overview of Population Characterization

The Morphology Explorer BioApplication provides the ability to characterize cells or objects based on their response compared to a control population. For a particular Cell Feature, a range is determined and set for a control population that has the normal physiological distribution for that feature. An upper and lower limit (known as ***Feature*Ch*N*LevelHigh** and ***Feature*Ch*N*LevelLow**) set the upper and lower bounds of this range respectively. The **Status** Cell Feature indicates whether a particular cell is within or beyond this range (i.e., for a particular Cell Feature, a cell with a value of **0** is within the defined range, a value of **1** means that the feature value is greater than ***Feature*Ch*N*LevelHigh**, and a value of **2** means that the feature value is less than ***Feature*Ch*N*LevelLow**; Table 22).

| Cell Status | Definition |
|---|---|
| 0 | feature within defined range (i.e., within limits) |
| 1 | feature > Level High |
| 2 | feature < Level Low |

***Table 22.*** *Cell Status definitions*

The corresponding reported Well Features are the percentage of cells that are either greater or less than the levels defining this range. Figure 25 illustrates this concept by showing the distribution of a normal physiological population of cells for a particular Cell Feature and the shift in this distribution upon compound treatment.

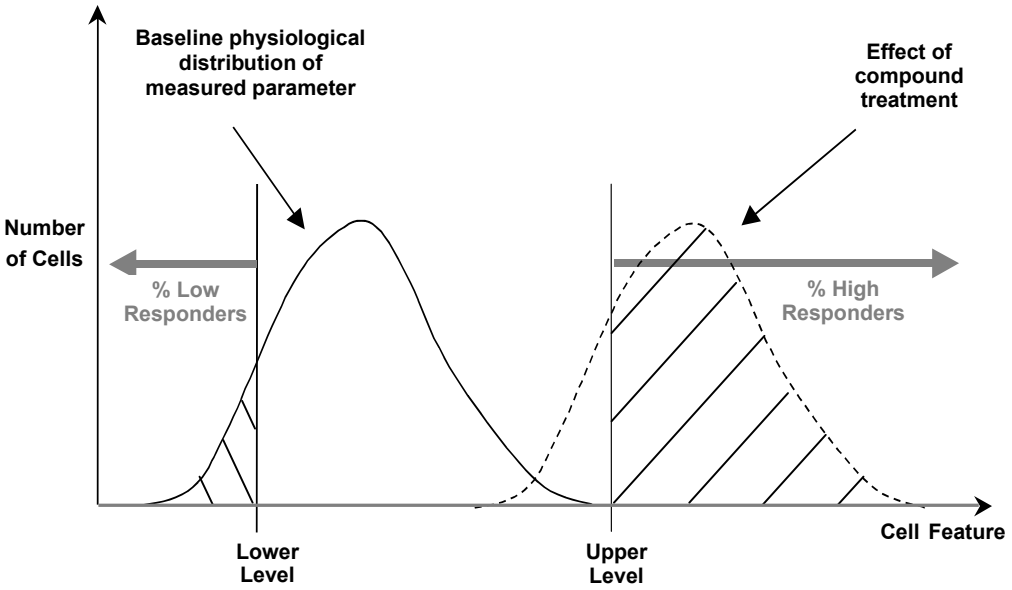



***Figure 25.*** *Schematic population distribution of a measured feature in a population of untreated and compound treated cells. The upper and lower levels correspond to %Low or %High*

## Setting Cellular Response Levels

There are two ways of setting the high and low-response levels to characterize the cell population: manually or automatically. To set the levels manually, you specify the values for

the ***Feature*Ch*N*LevelHigh** and ***Feature*Ch*N*LevelLow** Assay Parameter that will be used to perform cell population characterization. The Morphology Explorer BioApplication then uses these levels that you define to calculate the percentage of cells outside the bounds of these levels for the different Cell Features.

To automatically determine the ***Feature*Ch*N*LevelHigh, *Feature*Ch*N*LevelLow** Assay Parameter value, the BioApplication uses Reference Wells. Particular wells on the sample plate are designated as Reference Wells. Typically, Reference Wells contain a control population of cells which displays the normal basal physiological distribution for the Assay Parameter being measured. These wells are analyzed and the population distribution for the different features are determined. The cell population characterization levels are then set by adding to, and subtracting from, the mean of the distribution its standard deviation multiplied by a coefficient. The system then scans the whole plate and applies these levels. For example, if you want to know the percentage of cells that, with compound treatment, have a response beyond the 95$^{th}$ percentile of the response from a control untreated population of cells, then the coefficient by which to multiply the standard deviation would be two. The advantage of using Reference Wells to automatically calculate the ***Feature*Ch*N*LevelHigh, *Feature*Ch*N*LevelLow** values is that the levels are determined by a control population of cells and are independent of run-to-run variations when doing the experiment on different days such as different illumination conditions, fluorophore amounts, or changes in cell conditions.

## Reference Wells Processing Sequence

By setting the **UseReferenceWells** Assay Parameter to **1**, the Reference Wells processing is engaged. Specified fields in the Reference Wells are acquire/analyzed, and Field, Well, and Plate Features are computed. After this sequence is completed, the computed values will be loaded into the assay processing parameters related to reference features and the regular scanning of the plate will begin. Reference features are computed on a field basis. The sequence of computation for Reference Wells is as follows:

1. Cell Features are computed for every valid object within a field.
2. For each Cell Feature used for population characterization, the mean and standard deviation are computed over all cells in the field.
3. Reference field features are determined.
4. Reference Well and Plate Features are computed as average values for fields in a well, weighted for the number of cells per field, and then as an arithmetic average for wells on a plate. Use of a weighted average minimizes the effect of sparse fields. The exception is the object count, whose average is not weighted.

## Identifying Reference Wells and Control Parameters

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

**NOTE**

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

There are two general Assay Parameters used to control Reference Wells (**UseReferenceWells** and **MinRefAvgObjectCountPerField**). **UseReferenceWells** is a binary Assay Parameter that allows you to indicate whether Reference Wells are used to determine the levels necessary to characterize cell populations.

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

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

There are four types of Assay Parameters that are used for cell population characterization which are specific to each individual feature for which population characterization is applied and the dependent channels where it is calculated. The Assay Parameter names are of the type:

- ***Feature*Ch*N*LevelLow**
- ***Feature*Ch*N*LevelHigh** (when Reference Wells are not used)

  OR
- ***Feature*Ch*N*LevelLow_CC**
- ***Feature*Ch*N*LevelHigh_CC** (when Reference Wells are used)

where *Feature* refers to the individual feature and Ch*N* refers to the specific channel *N*. Other than the **UseReferenceWells** Assay Parameter, all the Assay Parameters used for population characterization are available only in the Advanced Mode.

The ***Feature*Ch*N*LevelLow**/***Feature*Ch*N*LevelHigh** Assay Parameters specify the actual levels and must be manually entered if Reference Wells are not used. If the range is to be set automatically by using Reference Wells, then the appropriate Assay Parameters are those that have the suffix "CC" (i.e., "Correction Coefficient"). The lower and upper bounds for the range for a cell feature are set by subtracting or adding the distribution's mean from its standard deviation (SD) multiplied by the appropriate CC value respectively:

- ***Feature*Ch*N*LevelLow** = Mean – ***Feature*Ch*N*LevelLow _CC** x SD
- **F*eature*Ch*N*LevelHigh** = Mean + **F*eature*Ch*N*LevelHigh _CC** x SD

Note that the **CC** value can be different for defining **F*eature*Ch*N*LevelHigh** versus **F*eature*Ch*N*LevelLow**.

Other than the **MinRefAvgObjectCountPerField** Assay Parameter, the Advanced Mode Assay Parameters pertain to setting the ***Feature*Ch*N*LevelHigh** and ***Feature*Ch*N*LevelLow** values for population characterization and enable you to identify which objects are responders for different properties measured. The **MinRefAvgObjectCountPerField** Assay Parameter is also related to setting these levels as it specifies the minimum number of objects per field to be counted in Reference Wells for the Reference Well to be valid. Thus, unless you want to do population characterization for particular features or need to identify responders by setting the levels, you do not need to enter values for the Advanced Parameters.

## Advanced Assay Parameters

In Advanced Mode, all Basic and Advanced input parameters are adjustable. The **Hide Advanced Parameters** option provides the ability to either view and adjust all the Assay Parameters or view and adjust the subset of parameters (designated as Basic). Generally, the Advanced Assay Parameters are related to definition and reporting of responder cells. They control the analysis of the data resulting from the image analysis (Table 23).

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

- **ObjectAreaCh1LevelLow**
- **ObjectAreaCh1LevelHigh**
- **ObjectAreaCh1LevelLow_CC**
- **ObjectAreaCh1LevelHigh_CC**

In the listing of Advanced Parameters in the following table, instead of listing all four level Assay Parameters for each feature, one entry for the Assay Parameter will be listed giving the four different options, as shown in the following example for the Channel 1 object area:

- ObjectAreaCh1Level***Low/High, Low/High_CC***

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

| Parameter | Units | Description |
|---|---|---|
| MinRefAvgObjectCountPerField | Number | Minimum average number of objects per field required for acceptance of reference well results |
| UseMicrometers | Binary | Measure lengths and areas in: 0 = Pixels, 1 = Micrometers |
| PixelSize | µm | Pixel size in micrometers (depends on objective selection) |
| Type_1_EventDefinition | -- | User-defined combination of logic statements involving response features ***(cannot be edited)*** |
| Type_2_EventDefinition | -- | User-defined combination of logic statements involving response features ***(cannot be edited)*** |
| Type_3_EventDefinition | -- | User-defined combination of logic statements involving response features ***(cannot be edited)*** |
| ObjectAreaCh1Level***Low/High, Low/High_CC*** | Pixels or $\mu m^2$ | Defines **ObjectAreaCh1** population characterization thresholds |
| ObjectPerimCh1Level***Low/High, Low/High_CC*** | Pixels or µm | Defines **ObjectPerimeterCh1** population characterization thresholds |
| ObjectShapeP2ACh1Level***Low/High, Low/High_CC*** | Number | Defines **ObjectShapeP2ACh1** population characterization thresholds |
| ObjectShapeLWRCh1Level***Low/High, Low/High_CC*** | Number | Defines **ObjectShapeLWRCh1** population characterization thresholds |

| Parameter | Units | Description |
|---|---|---|
| ObjectShapeBFRCh1Level***Low/High, Low/High_CC*** | Number | Defines **ObjectShapeBFRCh1** population characterization thresholds |
| ObjectLengthCh1Level***Low/High, Low/High_CC*** | Pixels or µm | Defines **ObjectLengthCh1** population characterization thresholds |
| ObjectWidthCh1Level***Low/High, Low/High_CC*** | Pixels or µm | Defines **ObjectWidthCh1** population characterization thresholds |
| ObjectAngleCh1Level***Low/High, Low/High_CC*** | Degrees | Defines **ObjectAngleCh**1 population characterization thresholds |
| ObjectFiberLengthCh1Level***Low/High, Low/High_CC*** | Pixels or µm | Defines **ObjectFiberLengthCh1** population characterization thresholds |
| ObjectFiberWidthCh1Level***Low/High, Low/High_CC*** | Pixels or µm | Defines **ObjectFiberWidthCh1** population characterization thresholds |
| ObjectConvexHullAreaRatioCh1Level***Low/High, Low/High_CC*** | Number | Defines **ObjectConvexHullAreaRatioCh1** population characterization thresholds |
| ObjectConvexHullPerimRatioCh1Level***Low/High, Low/High_CC*** | Number | Defines **ObjectConvexHullPerimRatioCh1** population characterization thresholds |
| ObjectEqCircDiamCh1Level***Low/High, Low/High_CC*** | Pixels or µm | Defines **ObjectEqCircDiamCh1** population characterization thresholds |
| ObjectEqSphereVolCh1Level***Low/High, Low/High_CC*** | Pixels or $\mu m^3$ | Defines **ObjectEqSphereVolCh1** population characterization thresholds |
| ObjectEqSphereAreaCh1Level***Low/High, Low/High_CC*** | Pixels or $\mu m^2$ | Defines **ObjectEqSphereAreaCh1** population characterization thresholds |
| ObjectEqEllipseLWRCh1Level***Low/High, Low/High_CC*** | Number | Defines **ObjectEqEllipseLWRCh1** population characterization thresholds |
| ObjectEqEllipseProlateVolCh1Level***Low/High, Low/High_CC*** | Pixels or $\mu m^3$ | Defines **ObjectEqEllipseProlateVolCh1** population characterization thresholds |
| ObjectEqEllipseOblateVolCh1Level***Low/High, Low/High_CC*** | Pixels or $\mu m^3$ | Defines **ObjectEqEllipseOblateVolCh1** population characterization thresholds |
| ObjectTotalIntenCh1Level***Low/High, Low/High_CC*** | Intensity | Defines **ObjectTotalIntenCh1** population characterization thresholds |
| ObjectAvgIntenCh1Level***Low/High, Low/High_CC*** | Intensity | Defines **ObjectAvgIntenCh1** population characterization thresholds |
| ObjectVarIntenCh1Level***Low/High, Low/High_CC*** | Intensity | Defines **ObjectVarIntenCh1** population characterization thresholds |
| ObjectSkewIntenCh1Level***Low/High, Low/High_CC*** | Number | Defines **ObjectSkewIntenCh1** population characterization thresholds |
| ObjectKurtIntenCh1Level***Low/High, Low/High_CC*** | Number | Defines **ObjectKurtIntenCh1** population characterization thresholds |
| ObjectEntropyIntenCh1Level***Low/High, Low/High_CC*** | Number | Defines **ObjectEntropyIntenCh1** population characterization thresholds |
| ObjectDiffIntenDensityCh1Level***Low/High, Low/High_CC*** | Intensity | Defines **ObjectDiffIntenDensityCh1** population characterization thresholds |
| ProcessCountCh1Level***Low/High, Low/High_CC*** | Number | Defines **ProcessCountCh1** population characterization thresholds |

| Parameter | Units | Description |
|---|---|---|
| ProcessMaxLengthCh1Level***Low/High, Low/High_CC*** | Pixels or µm | Defines **ProcessMaxLengthCh1** population characterization thresholds |
| ProcessTotalLengthCh1Level***Low/High, Low/High_CC*** | Pixels or µm | Defines **ProcessTotalLengthCh1** population characterization thresholds |
| ProcessAvgLengthCh1Level***Low/High, Low/High_CC*** | Pixels or µm | Defines **ProcessAvgLengthCh1** population characterization thresholds |
| NeighborMinDistCh1Level***Low/High, Low/High_CC*** | Pixels or $\mu m^2$ | Defines **NeighborMinDistCh1** population characterization thresholds |
| NeighborAvgDistCh1Level***Low/High, Low/High_CC*** | $\mu m^2$ or Pixels | Defines **NeighborAvgDistCh1** population characterization thresholds |
| NeighborVarDistCh1Level***Low/High, Low/High_CC*** | Pixels or $\mu m^2$ | Defines **NeighborVarDistCh1** population characterization thresholds |
| MemberCountCh2Level***Low/High, Low/High_CC*** | Number | Defines **MemberCountCh2** population characterization thresholds |
| MemberOutCountCh2Level***Low/High, Low/High_CC*** | Number | Defines **MemberOutCountCh2** population characterization thresholds |
| MemberInCountCh2Level***Low/High, Low/High_CC*** | Number | Defines **MemberInCountCh2** population characterization thresholds |
| MemberAvgAreaCh2Level***Low/High, Low/High_CC*** | Pixels or $\mu m^2$ | Defines **MemberAvgAreaCh2** population characterization thresholds |
| MemberAvgShapeP2ACh2Level***Low/High, Low/High_CC*** | Number | Defines **MemberAvgShapeP2ACh2** population characterization thresholds |
| MemberAvgShapeLWRCh2Level***Low/High, Low/High_CC*** | Number | Defines **MemberAvgShapeLWRCh2** population characterization thresholds |
| MemberAvgShapeBFRCh2Level***Low/High _CC*** | Number | Defines **MemberAvgShapeBFRCh2** population characterization thresholds |
| MemberAvgTotalIntenCh2Level***Low/High, Low/High_CC*** | Intensity | Defines **MemberAvgTotalIntenCh2** population characterization thresholds |
| MemberAvgAvgIntenCh2Level***Low/High, Low/High_CC*** | Intensity | Defines **MemberAvgAvgIntenCh2** population characterization thresholds |
| MemberAvgConvexHullAreaRatioCh2Level***Low/High, Low/High_CC*** | Number | Defines **MemberAvgConvexHullAreaRatioCh2** population characterization thresholds |
| MemberAvgConvexHullPerimRatioCh2Level***Low/High, Low/High_CC*** | Number | Defines **MemberAvgConvexHullPerimRatioCh2** population characterization thresholds |
| MemberAvgEqCircDiamCh2Level***Low/High, Low/High_CC*** | Pixels or µm | Defines **MemberAvgEqCircDiamCh2** population characterization thresholds |
| MemberAvgEqEllipseLWRCh2Level***Low/High, Low/High_CC*** | Number | Defines **MemberAvgEqEllipseLWRCh2** population characterization thresholds |
| MemberObjectAreaRatioCh2Level***Low/High, Low/High_CC*** | Number | Defines **MemberObjectAreaRatioCh2** population characterization thresholds |
| MemberObjectAreaDiffCh2Level***Low/High, Low/High_CC*** | Number | Defines **MemberObjectAreaDiffCh2** population characterization thresholds |
| ROI_TotalIntenCh2Level***Low/High, Low/High_CC*** | Intensity | Defines **ROI_TotalIntenCh2** population characterization thresholds |

| Parameter | Units | Description |
|---|---|---|
| ROI_AvgIntenCh2Level***Low/High, Low/High_CC*** | Intensity | Defines **ROI_AvgIntenCh2** population characterization thresholds |
| SpotFiberCountChNLevel***Low/High, Low/High_CC*** | Number | Defines **SpotFiberCountChN** population characterization thresholds |
| SpotFiberTotalAreaChNLevel***Low/High, Low/High_CC*** | Pixels or $\mu m^2$ | Defines **SpotFiberTotalAreaChN** population characterization thresholds |
| SpotFiberAvgAreaChNLevel***Low/High, Low/High_CC*** | Pixels or $\mu m^2$ | Defines **SpotFiberAvgAreaChN** population characterization thresholds |
| FiberAlign1ChNLevel***Low/High, Low/High_CC*** | Number | Defines **FiberAlign1ChN** population characterization thresholds |
| ROI_FiberAlign2ChNLevel***Low/High, Low/High_CC*** | Number | Defines **ROI_FiberAlign2ChN** population characterization thresholds |
| ROI_TotalIntenChNLevel***Low/High, Low/High_CC*** | Intensity | Defines **ROI_TotalIntenChN** population characterization thresholds. Objects that are responders defined by this range also have their proximity metrics calculated. |
| ROI_AvgIntenChNLevel***Low/High, Low/High_CC*** | Intensity | Defines **ROI_AvgIntenChN** population characterization thresholds. Objects that are responders defined by this range also have their proximity metrics calculated. |
| ROI_VarIntenChNLevel***Low/High, Low/High_CC*** | Intensity | Defines **ROI_VarIntenChN** population characterization thresholds |
| ROI_SkewIntenChNLevel***Low/High, Low/High_CC*** | Number | Defines **ROI_SkewIntenChN** population characterization thresholds |
| ROI_KurtIntenChNLevel***Low/High, Low/High_CC*** | Number | Defines **ROI_KurtIntenChN** population characterization thresholds |
| ROI_EntropyIntenChNLevel***Low/High, Low/High_CC*** | Number | Defines **ROI_EntropyIntenChN** population characterization thresholds |
| ROI_DiffIntenDensityChNLevel***Low/High, Low/High_CC*** | Intensity | Defines **ROI_DiffIntenDensityChN** population characterization thresholds |
| ROI_MaxCoocIntenChNLevel***Low/High, Low/High_CC*** | Number | Defines **ROI_MaxCoocIntenChN** population characterization thresholds |
| ROI_ContrastCoocIntenChNLevel***Low/High, Low/High_CC*** | Number | Defines **ROI_ContrastCoocIntenChN** population characterization thresholds |
| ROI_EntropyCoocIntenChNLevel***Low/High, Low/High_CC*** | Number | Defines **ROI_EntropyCoocIntenChN** population characterization thresholds |
| ROI_ASMCoocIntenChNLevel***Low/High, Low/High_CC*** | Number | Defines **ROI_ASMCoocIntenChN** population characterization thresholds |
| ROI_AvgRadialIntenChNLevel***Low/High, Low/High_CC*** | Number | Defines **ROI_AvgRadialIntenChN** population characterization thresholds |
| ROI_VarRadialIntenChNLevel***Low/High, Low/High_CC*** | Number | Defines **ROI_VarRadialIntenChN** population characterization thresholds |
| ROI_SkewRadialIntenChNLevel***Low/High, Low/High_CC*** | Number | Defines **ROI_SkewRadialIntenChN** population characterization thresholds |
| ROI_KurtRadialIntenChNLevel***Low/High, Low/High_CC*** | Number | Defines **ROI_KurtRadialIntenChN** population characterization thresholds |

| Parameter | Units | Description |
|---|---|---|
| NeighborMinDistChNLevel***Low/High, Low/High_CC*** | Pixels or µm | Defines **NeighborMinDistChN** population characterization thresholds |
| NeighborAvgDistChNLevel***Low/High, Low/High_CC*** | Pixels or µm | Defines **NeighborAvgDistChN** population characterization thresholds |
| NeighborVarDistChNLevel***Low/High, Low/High_CC*** | Pixels or µm | Defines **NeighborVarDistChN** population characterization thresholds |
| NeighborMinDistCh3Ch4Level***Low/High, Low/High_CC*** | Pixels or µm | Defines **NeighborMinDistCh3Ch4** population characterization thresholds |
| NeighborAvgDistCh3Ch4Level***Low/High, Low/High_CC*** | Pixels or µm | Defines **NeighborAvgDistCh3Ch4** population characterization thresholds |
| NeighborVarDistCh3Ch4Level***Low/High, Low/High_CC*** | Pixels or µm | Defines **NeighborVarDistCh3Ch4** population characterization thresholds |

***Table 23.*** *Advanced Assay Parameters available for the Morphology Explorer BioApplication. *Note that "ChN" refers to Channel 3 and Channel 4.*

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

The Morphology Explorer BioApplication allows simultaneous definition of up to three Events to enable rapid multiparametric analysis of cell health at the level of individual cells, across multiple Cell Features. This capability allows you to do the following automatically:

- Create your own definition for a focused biology
- Define a subpopulation by using any combination of up to three status Cell Features
- Report characteristics of your subpopulations

Event definition is achieved through the construction of logic statements employing upper and lower bounds for specific Cell Features and a set of defined logical operators. The Cell Features and Boolean operators available are listed in Table 24. 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 26 using two Cell Features, A and B. The entire cell population analyzed is enclosed within the box in each case. In each case, responder cells for each feature are located within the domain for that feature. Note that the definitions described in Figure 26 can be directly applied to logic statements that contain more than two Cell Features.

The logic statements that comprise Event definitions are specified using a stand-alone software tool called the BioApplication Event Wizard. Operation of this software tool is described in more detail in Chapter 3 . The Assay Parameters used to define Events are of the type **Type_X_EventDefinition** (X =1, 2 or 3).


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

| Cell Features | | Logic Operators |
|---|---|---|
| ObjectAreaCh1 | MemberAvgShapeBFRCh2 | |
| ObjectPerimCh1 | MemberAvgTotalIntenCh2 | |
| ObjectShapeP2ACh1 | MemberAvgAvgIntenCh2 | |
| ObjectShapeLWRCh1 | MemberAvgConvexHullAreaRatioCh2 | |
| ObjectShapeBFRCh1 | MemberAvgConvexHullPerimRatioCh2 | |
| ObjectLengthCh1 | MemberAvgEqCircDiamCh2 | |
| ObjectWidthCh1 | MemberAvgEqEllipseLWRCh2 | |
| ObjectAngleCh1 | MemberObjectAreaRatioCh2 | |
| ObjectFiberLengthCh1 | MemberObjectAreaDiffCh2 | |
| ObjectFiberWidthCh1 | TotalIntenCh2 | |
| ObjectConvexHullAreaRatioCh1 | AvgIntenCh2 | |
| ObjectConvexHullPermRatioCh1 | SpotFiberCountCh3/4 | |
| ObjectEqCircDiamCh1 | SpotFiberTotalAreaCh3/4 | NOT |
| ObjectEqSphereVolCh1 | SpotFiberAvgAreaCh3/4 | AND |
| ObjectEqSphereAreaCh1 | FiberAlign1Ch3/4 | |
| ObjectEqEllipseLWRCh1 | FiberAlign2Ch3/4 | AND NOT |
| ObjectEqEllipseProlateVolCh1 | TotalIntenCh3/4 | |
| ObjectEqEllipseOblateVolCh1 | AvgIntenCh3/4 | OR |
| ObjectTotalIntenCh1 | VarIntenCh3/4 | |
| ObjectAvgIntenCh1 | SkewIntenCh3/4 | OR NOT |
| ObjectVarIntenCh1 | KurtIntenCh3/4 | XOR |
| ObjectSkewIntenCh1 | EntropyIntenCh3/4 | |
| ObjectKurtIntenCh1 | DiffIntenDensityCh3/4 | NAND |
| ObjectEntropyIntenCh1 | MaxCoocIntenCh3/4 | |
| ObjectDiffIntenDensityCh1 | ContrastCoocIntenCh3/4 | NOR |
| ProcessCountCh1 | EntropyCoocIntenCh3/4 | |
| ProcessMaxLengthCh1 | ASMCoocIntenCh3/4 | |
| ProcessTotalLengthCh1 | AvgRadialIntenCh3/4 | |
| ProcessAvgLengthCh1 | VarRadialIntenCh3/4 | |
| NeighborMinDistCh1 | SkewRadialIntenCh3/4 | |
| NeighborAvgDistCh1 | KurtRadialIntenCh3/4 | |
| NeighborVarDistCh1 | NeighborMinDistCh3/4 | |
| MemberCountCh2 | NeighborAvgDistCh3/4 | |
| MemberOutCountCh2 | NeighborVarDistCh3/4 | |
| MemberInCountCh2 | NeighborMinDistCh3Ch4 | |
| MemberAvgShapeP2ACh2 | NeighborAvgDistCh3Ch4 | |
| MemberAvgShapeLWRCh2 | NeighborVarDistCh3Ch4 | |

***Table 24.*** *Cell features and Boolean operators available for Event Definition with the Morphology Explorer BioApplication*

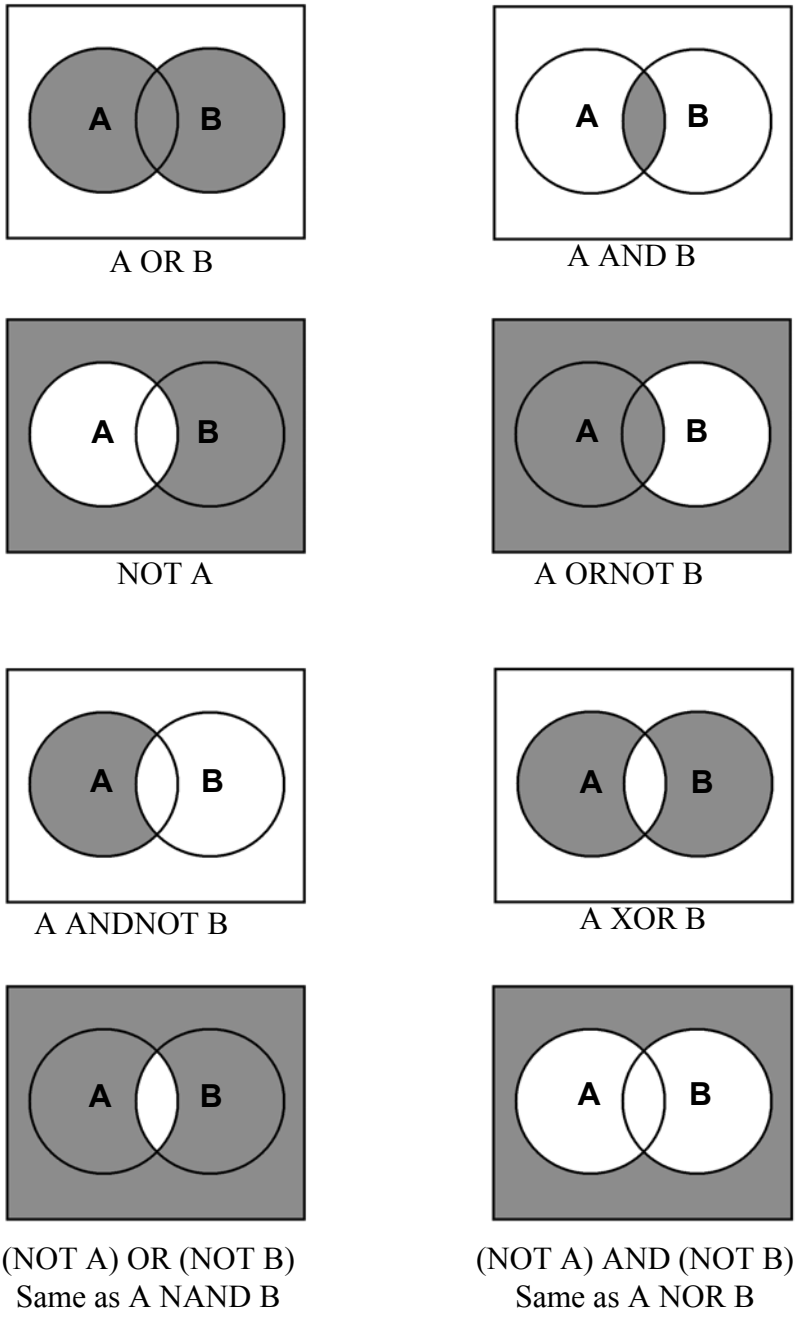



***Figure 26.*** *Schematic definition of available Boolean Operators assuming two Cell Features of interest, A & B.*

# Description of Output Features

Output features are the biological measurements produced by the Morphology Explorer BioApplication. All features are categorized and accessible using the View application software. Additionally, a subset of features, Well Features, is listed in the Scan Plate View and Create Protocol View so that screening results can be viewed concurrently with scanning. The ability to view data during the scanning process allows you to immediately verify success of the run, well by well. An overview of both the Cell and Well Output Features can be found in Chapter 1, Tables 1-5. This section lists these features.

## Channel 1 Features

Channel 1 contains the image of the primary labeled objects.

### Object Counts, Density, and Location

At the well-level, the number of objects analyzed in the well is reported. The object density is reported as the number of selected objects per valid imaged field. The object's location in the image is reported by the *x* and *y* coordinates of its centroid.

### Object Dimensions

Area and perimeter of each object is reported. The area is proportional to the total number of pixels covered by the object, and the proportionality constant is the pixel size in micrometers. Thus, you have a choice for the area to be reported in the number of pixels or $\mu m^2$. Similarly, the perimeter can be reported either in pixels or micrometers. In addition to the area, shape parameters such as the object's roundness (i.e., P2A), aspect ratio (i.e., LWR), and dimensions such as its length and width are also reported. The object's P2A is defined as:

$$\text{P2A} = \frac{\text{Perimeter}^2}{4\pi \times \text{Area}}$$

If the object is a perfect circle, its P2A is equal to 1; as it deviates from roundness, acquires bumps and protrusions, and processes start extending from it, its perimeter gets large relative to its area and thus its P2A becomes larger than 1.

Some dimensions are defined by a rectangular bounding box which encompasses the object (Figure 27). The bounding box is the rectangle of minimum area that includes the object. The length and width of this bounding box is reported, as well as the length to width ratio (i.e., aspect ratio or LWR). A round object's length and width are the same, and thus its LWR is 1. As the object elongates, its length becomes greater than its width and its LWR becomes greater than 1. An additional parameter related to the bounding box is the **Bounding Box Area Fill Ratio** (BFR). This is the ratio of the object's area to the area of its bounding box (i.e., length × width).

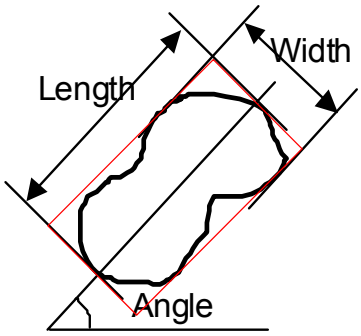



***Figure 27.*** *Bounding Box*

Additional shape and dimension output features are related to an object's **Convex Hull**. The Convex Hull is defined as the smallest convex set containing the points of the original object. The easiest way to think of it is the shape defined by placing a rubber band around the object (Figure 28).

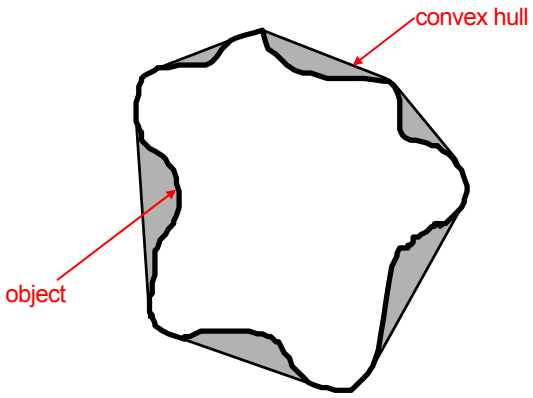



***Figure 28.*** *Convex Hull*

The BioApplication reports the ratio of the area of the Convex Hull to the object's area, and this can be used to assess the degree of bumps or protrusions the object has. A related feature that is reported is the **Convex Hull Perimeter Ratio**, which is the ratio of the perimeter of the Convex Hull to the object's perimeter. For an object with many bumps and protrusions, its area will be less than that of the convex hull, but its perimeter will be larger than the perimeter of the Convex Hull. Thus the **Convex Hull Area Ratio** is always greater than or equal to **1** and becomes larger the more protrusions the object has. Conversely, the Convex Hull Perimeter Ratio is always less than or equal to **1** and decreases with the more protrusions the object has.

$$\text{Convex Hull Area Ratio} = \frac{\text{Convex Hull Area}}{\text{Object Area}} \geq 1$$

$$\text{Convex Hull Perimeter Ratio} = \frac{\text{Convex Hull Perimeter}}{\text{Object Perimeter}} \leq 1$$

In addition to the length and width based on the bounding box of an object, the actual length and width are also reported. In the nomenclature used in this BioApplication, the object's actual length

and width is known as the **Fiber Length** and **Fiber Width**. These measurements are approximations of when an object is elongated, so that it resembles a fiber (see Figure 29). In this approximation, standard formulas for the length and width apply based on the fiber's (i.e., object's) perimeter (P) and area (A), as shown in Figure 29. Note that the meaning of fiber here, which pertains to the shape of an object in Channel 1, is different from its meaning in Channel 3 and Channel 4, where it refers to fiber-like entities inside cells or objects.

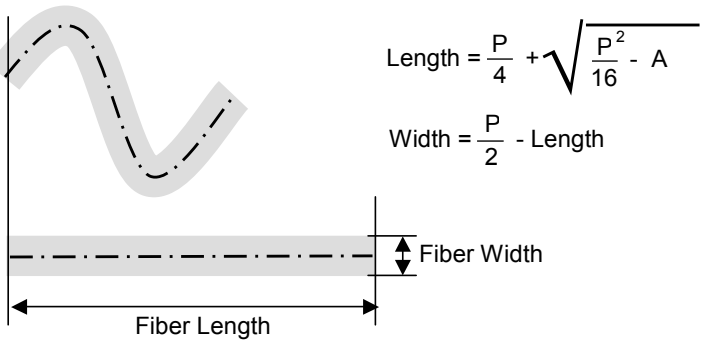



***Figure 29.*** *Fiber Length and Width*

Additional features reflecting the object's dimensions and extent are related to its **Equivalent Circle** and **Equivalent Ellipse**. An object's Equivalent Circle is a circle with the same area as the object (Figure 30A). Similarly, its Equivalent Ellipse is an ellipse with the same area, aspect ratio, and orientation as the object (Figure 30B). The BioApplication reports the object's **Equivalent Diameter**, which is the diameter of its Equivalent Circle. It also reports the ratio of the major to minor diameter of its Equivalent Ellipse.

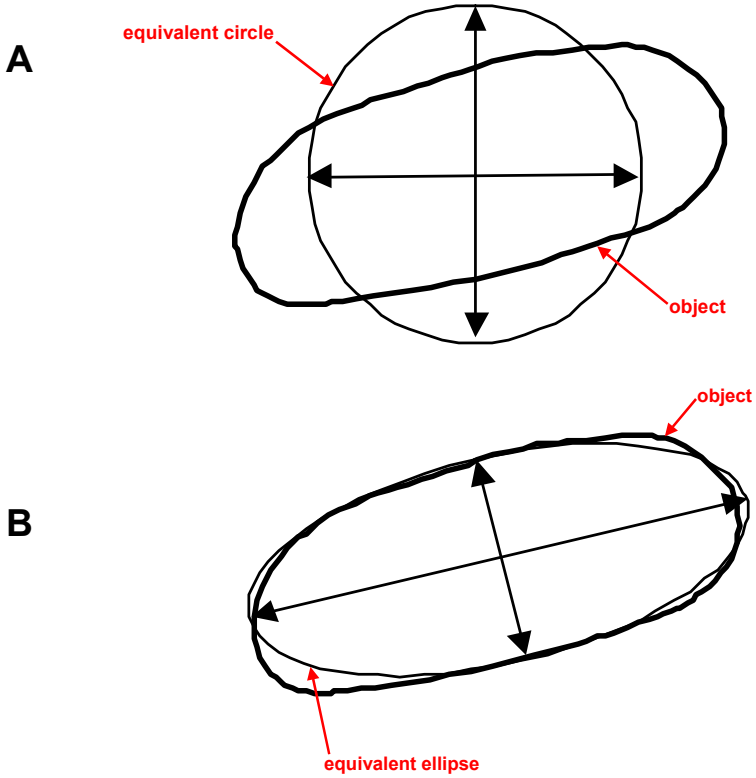



***Figure 30.*** *(A) Equivalent Circle (B) Equivalent Ellipse*

## Object Orientation

The object's orientation in the field is reported by the angle of the bounding box (Figure 27). To determine how well objects in a well are aligned, the standard deviation of the all the objects' angles can be used. Angle measurements are reported in degrees, not radians.

## Object 3-D Metrics

Extrapolations of the object's volume and surface area are made based on its Equivalent Circle and Ellipse. The BioApplication reports the volume and surface area of an **Equivalent Sphere**, which is created by revolving the object's Equivalent Circle around a diameter (Figure 31). These may be useful in assaying round cells, such as different types of blood cells, where changes in their volume and surface area are indicators of their biological response.

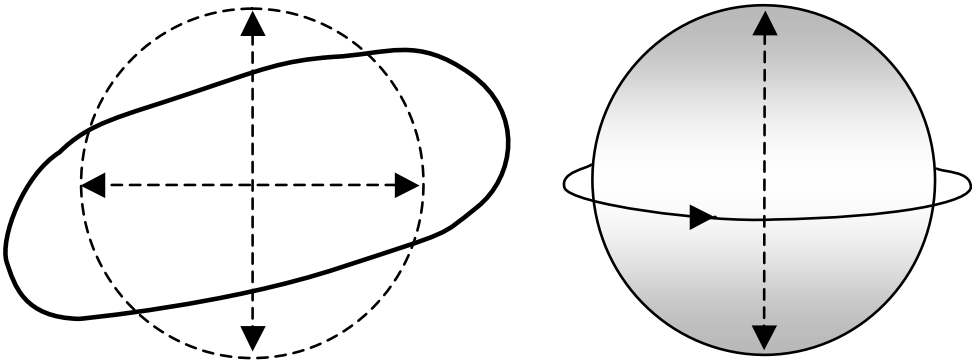


***Figure 31.*** *Equivalent Sphere.*

Similarly, the **oblate** and **prolate** volumes of Equivalent Ellipsoids, which are created by rotating the Equivalent Ellipse around the minor and major axis respectively, are reported. These are illustrated in the schematics in Figure 32 and Figure 33. The prolate volume may be useful in assaying long multicellular objects, such as tubes, where changes in the volume may be sensitive or robust indicators of a biological response.

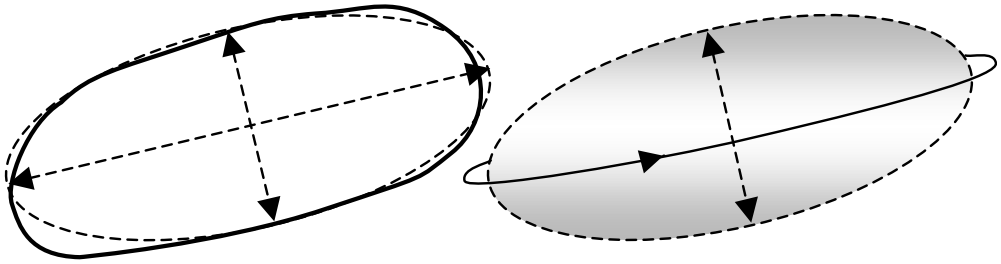


***Figure 32.*** *Equivalent Ellipse Oblate Volume*

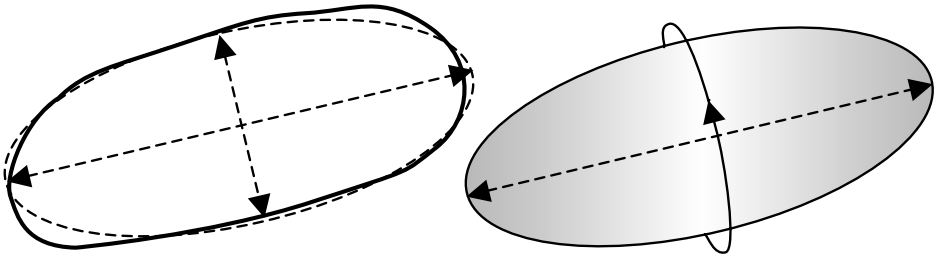


***Figure 33.*** *Equivalent Ellipse Prolate Volume*

## Processes Extending From Object

The Morphology Explorer BioApplication can also identify and analyze processes that extend from the main body of the object. Processes are defined as extensions from the cell body. An example of such a process is a neurite extending from the main cell body of a neuron (Figure 34A and Figure 34B). For each object, the BioApplication reports the following:

- Number of processes
- Length of the longest process
- Average length of all processes
- Sum of the lengths of all the processes

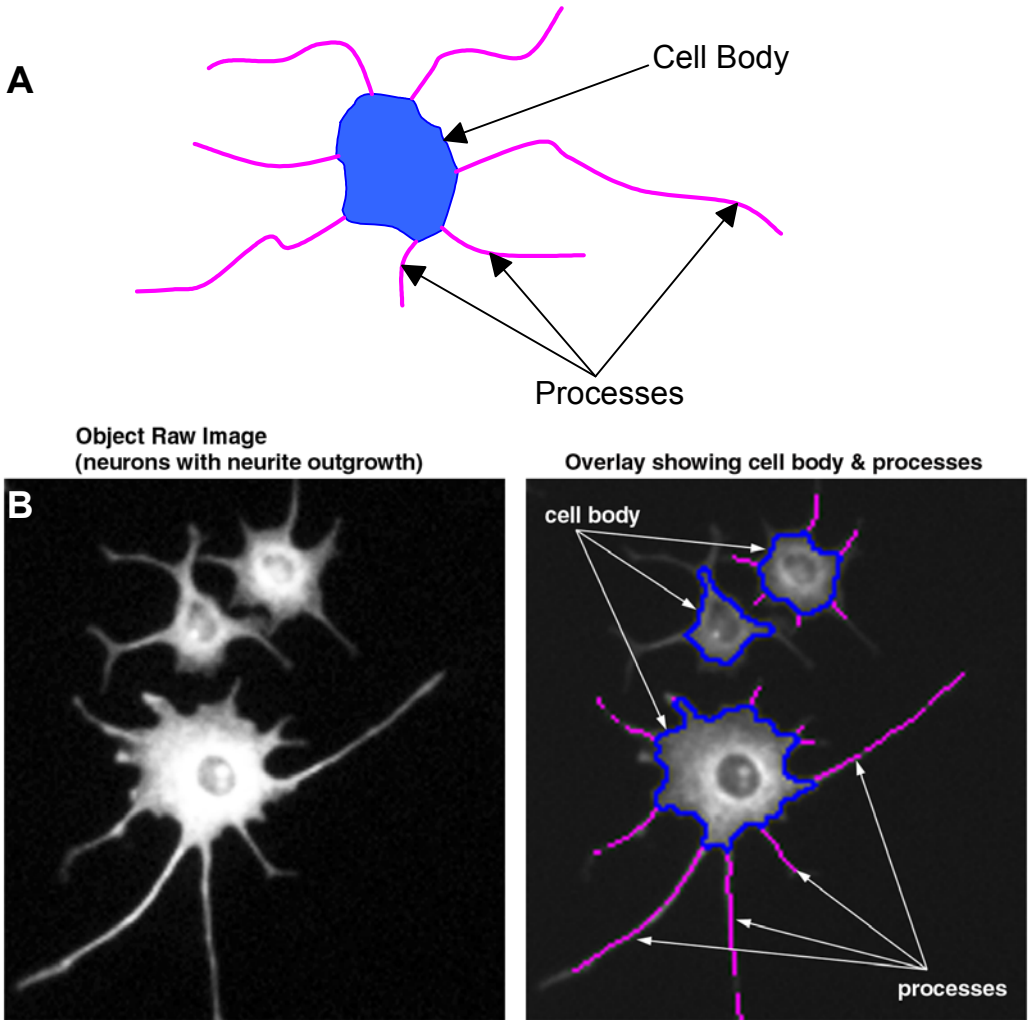



***Figure 34.*** *Neuron/Neurite example. (A) Illustration exemplifying processes extending from a cell body. (B) Raw image (left) of PC-3 cells treated with NGF and its analysis with the Morphology Explorer BioApplication (right). Processes are shown in purple extending from cell bodies (blue).*

## Object Spacing and Proximity

The BioApplication can quantify the spacing and proximity between objects. The proximity features calculated are the distances between the centers of the different objects. Specifically, for each object, the following are measured and reported:

- Minimum distance between it and other objects (i.e., the distance to the nearest object)
- Average distance between it and all objects in the imaged field
- Standard deviation of the distance between it and all objects in the imaged field

The proximity measurements between objects are reported in Channels 1, 3, and 4 (Figure 35). The difference between each channel is that the channel label defines the type of object, and thus different types of proximity measurements are reported for each channel. Since the fluorescent label in Channel 1 identifies all objects of interest, the proximity measurements reported for Channel 1 are between all objects. Specific stains identifying specific cell or object sub-types can be used in Channel 3 and Channel 4. For example, if the sample was a mixture of cells of different types, the Channel 1 stain would label all cells, whereas the Channel 3 and Channel 4 stains would only label cells of particular sub-types (e.g., as in Example 6 of the different biological examples). The proximity measurement reported for Channel 3 would only be between objects that had the Channel 3 stain (i.e., positive responders for the Channel 3 stain). Similarly, proximity measurements between cells, which are positive responders for the Channel 4 stain, would be reported in Channel 4. In addition to these proximity measurements between similar cells, additional proximity measurements are reported in Channel 4 for dissimilar cells (i.e., between cells that have Channel 3 and Channel 4 stains).

This could be used in the study of mixed neuronal cultures if you wanted to quantify the proximity of different cell types on neurite outgrowth. In this case, if the Channel 3 and Channel 4 stains were specific to identifying neurons and astrocytes respectively, not only would proximity measurements between neurons and also between astrocytes be reported, but the proximity between neurons and astrocytes would also be reported.

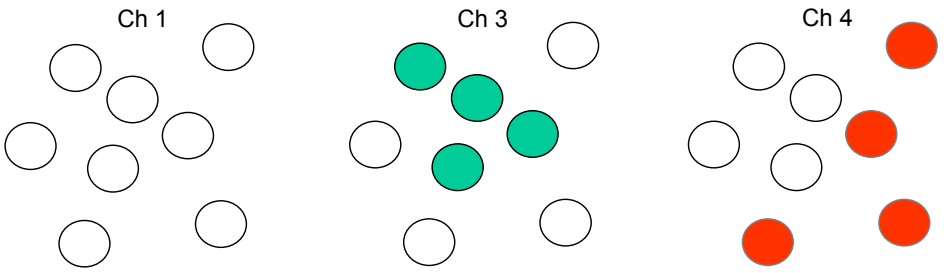



***Figure 35.*** *Cell labels showing similar and dissimilar cells for proximity measurements*

Note, for proximity and spacing measurements between objects in Channel 3 and/or Channel 4, Well Features are computed only for the subset of objects that are identified as responders. If only one responder is identified, then the proximity measurements are reported as NULL. This means that for proximity measurements between similar objects, more than one object needs to be identified as a responder in the same channel. For proximity measurements between dissimilar objects, at least one object needs to be identified in Channel 3 and another distinct object needs to be identified in Channel 4.

# Channel 1 Intracellular Arrangement and Texture

## Intensity Moments

The pixels in an object have a certain distribution of intensity values. The Morphology Explorer BioApplication reports a variety of statistical metrics of the pixel intensity distribution. These can be used as indicators of the intra-object (e.g., intracellular) arrangement and texture of the label used in Channel 1. Several of these metrics are related to the moments of the intensity distribution. The metrics that are reported for the pixel intensity distribution in an object are the total intensity, average intensity (1st moment), standard-deviation (square-root of 2nd moment), skewness (3rd moment), and kurtosis (4th moment). The skewness reflects the asymmetry or bias in any intensity distribution, and the kurtosis reflects the peakedness or flatness of the distribution.

Moments are metrics related to the sums of the integer power of the pixel intensity values in the object's pixel intensity distribution (Figure 36). For the 12-bit images used where pixel intensity values range from 0 to 32767, the moments of the intensity distribution are defined as:

$$M_n = \int_0^{32767} I^n P(I) dI$$

where $M_n$ refers to the n$^{th}$ order moment of the pixel intensity distribution $P(I)$, and $I$ is the pixel intensity which can range from 0 to 32767. The most well known and widely used moments of a distribution are its first and second moments ($n$ = 1 and 2), the mean and the variance respectively. The Morphology Explorer BioApplication reports both the mean and total intensity of the object. The total intensity is the sum of the intensities of all the pixels in the object. The total intensity divided by the number of pixels is the mean or average intensity.

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

The mean intensity estimates the value around which the central clustering of the distribution occurs (Figure 36). Both the total and mean intensities can be used as estimates of the amount of label in the object. The standard deviation is the square root of the variance and estimates the width or the variability of the intensity distribution. The intensity variability, as reported by the standard deviation, can be used as a texture measure. Objects with uniform intensity will have a smaller intensity standard deviation than one that has a variegated staining pattern (Figure 36, top).

The third moment, the distribution's skewness, is related to the degree of asymmetry or bias of the distribution around its mean. Positive skewness indicates an asymmetric distribution with a tail at higher intensities. Similarly, negative skewness indicates an asymmetric distribution with a tail at lower intensities (Figure 36, middle).

The fourth moment, the distribution's kurtosis, is related to the degree of peakedness or flatness of the distribution. Positive kurtosis (leptokurtic distributions) have a more sharp intensity distribution, while negative kurtosis (platykurtic distributions) have a more broad and flat intensity distribution. Both the skewness and kurtosis are non-dimensional parameters and can be used as metrics of intracellular intensity arrangements (Figure 36, bottom).

Since most distributions (even those that are symmetric) will give positive or negative skewness or kurtosis, an estimate is needed as to when the skewness or kurtosis values are meaningful. An estimator of "meaningfulness" can be the standard deviations of the skewness and kurtosis and can be used to determine which values are meaningful. For an ideal case of a normal intensity

distribution, the skewness' standard deviation is $\sqrt{6/N}$ and the kurtosis' standard deviation is $\sqrt{24/N}$, where $N$ is the number of pixels in the object (Press, et al. 1986). In practical situations, it is best to believe in values of skewness or kurtosis when they are many times larger than their standard deviations, or the changed values from compound treatment is much different from the control situation.

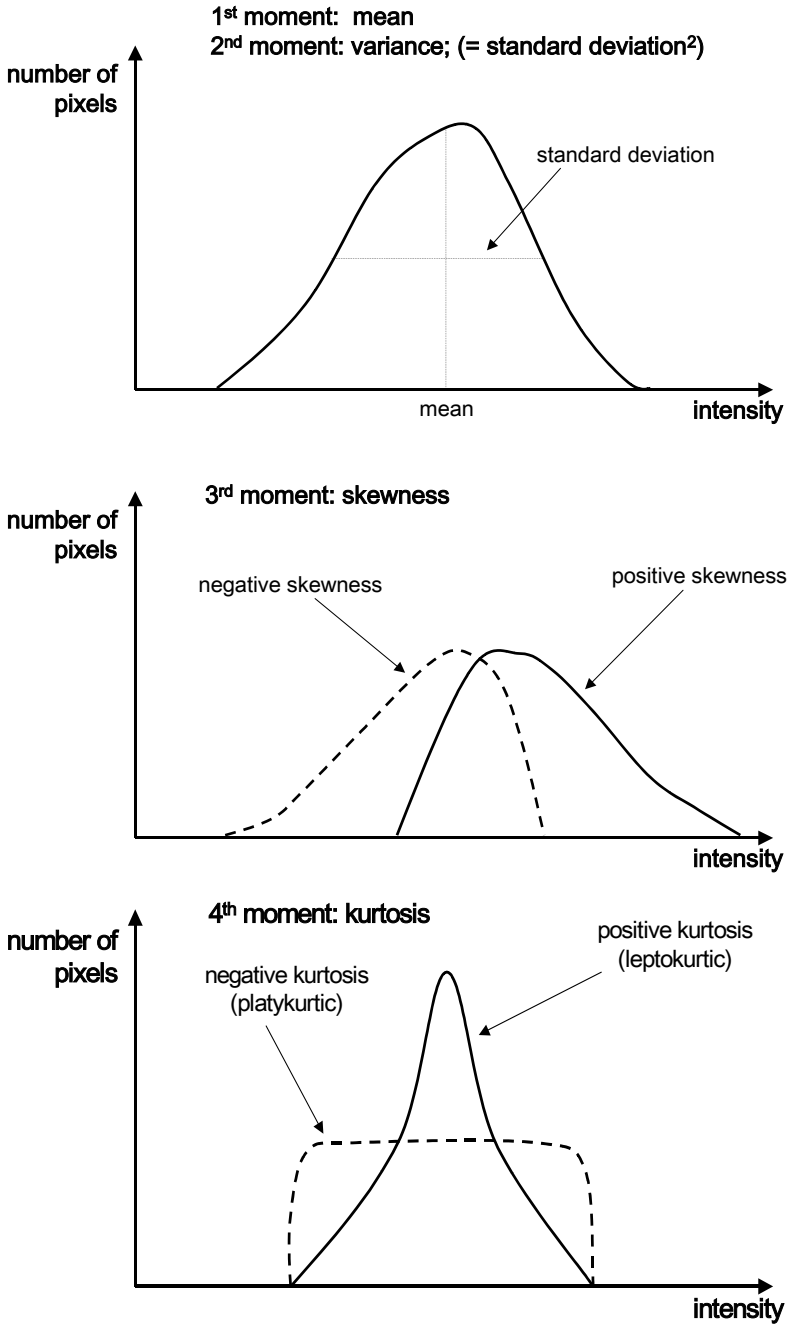



***Figure 36.*** *Object pixel intensity distributions and their moments, used for measuring intracellular amount, arrangement, and texture.*

## Entropy

Additional metrics from the intensity distribution reported by the Morphology Explorer BioApplication are the intensity entropy and the intensity surface area density. Both of these can also be used as further measures of texture. Entropy can be thought of as the information content represented by the pixel intensities present in the object and, thus, can also be used to quantify texture. The entropy quantifies the number of different pixel intensities present in the object and the probability of their occurrence. The more intensity values represented in the object with higher probabilities of occurrence, the higher the entropy. Lower entropy (less information content) occurs when the object has only a few intensity values and those that do exist have a low probability of occurrence (i.e., few intensities, with not many pixels in the objects with those intensities). An object with uniform staining where only a few intensities are represented would have lower entropy than one which has a wide range of intensities present in significant number.

The entropy is defined as:

$$Entropy = -\sum_{i=1}^{N} p_i \log_2 p_i$$

where $N$ is the range of pixel intensities available (32767 for our 12-bit images), $p_i$ is the probability of a particular intensity $i$ occurring in the distribution, and $log_2$ is the logarithm in base 2. Some simple examples are given below to illustrate the concept of entropy.

To help understand entropy, consider a simple image system where only four intensities are possible (with values 1, 2, 3, and 4), instead of the more extensive range of 32767 intensities available in the images analyzed with this BioApplication. In the first example, suppose an equal number of pixels has each of these four intensities; i.e., in a typical image with 512×512 pixels (262, 144 pixels total), 65,536 pixels in the image have an intensity value of 1, and an equal number of pixels have the other three intensities each. Note, the arrangement of the pixels in the image does not matter; this will be taken into account in a following section on co-occurrence matrices. Only the intensities of the pixels in the image are important (i.e., information content). Thus the probability $p_i$ of each of the four possible intensities is ¼:

| $i$ | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| $p_i$ | ¼ | ¼ | ¼ | ¼ |

In this case, the entropy calculated from the formula above is 2.

Instead of each intensity having equal probability of occurrence, suppose the image is uniform with all pixels having the same intensity (e.g., intensity level 3). Then the probabilities are:

| $i$ | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| $p_i$ | 0 | 0 | 1 | 0 |

and the entropy will be 0 (i.e., no information in the image).

In the third example, consider situations where half the pixels have an intensity of 1, the rest of the pixels have an intensity of 2, and no pixels have the remaining two intensities. Then the probabilities are:

| $i$ | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| $p_i$ | ½ | ½ | 0 | 0 |

and the entropy will be 1 (some information content, but less than in the first example).

In the last example, consider an image system where instead of four intensities, eight intensity values are possible (i.e., $N = 8$). If the same number of pixels has each intensity value, then the probability of each intensity value is 0.125, and the entropy will be 3. Similarly, in our image system with 32767 intensity values, if an equal number of pixels (i.e., 64 pixels in an image of 512×512 pixel dimension) have each intensity, then the entropy would increase to 12. Thus by increasing the number of intensity values present in the image (i.e., increasing its information content), its entropy increases.

Figure 37 shows examples of images with dimensions of 8×8 pixels and with entropies of 0-3. For each entropy value, three possible example images are shown. This is to illustrate the point that entropy measures the "information-content" represented by the pixel range and probability of occurrence; entropy is not affected by the spatial arrangement of the pixels of different intensities in the image.

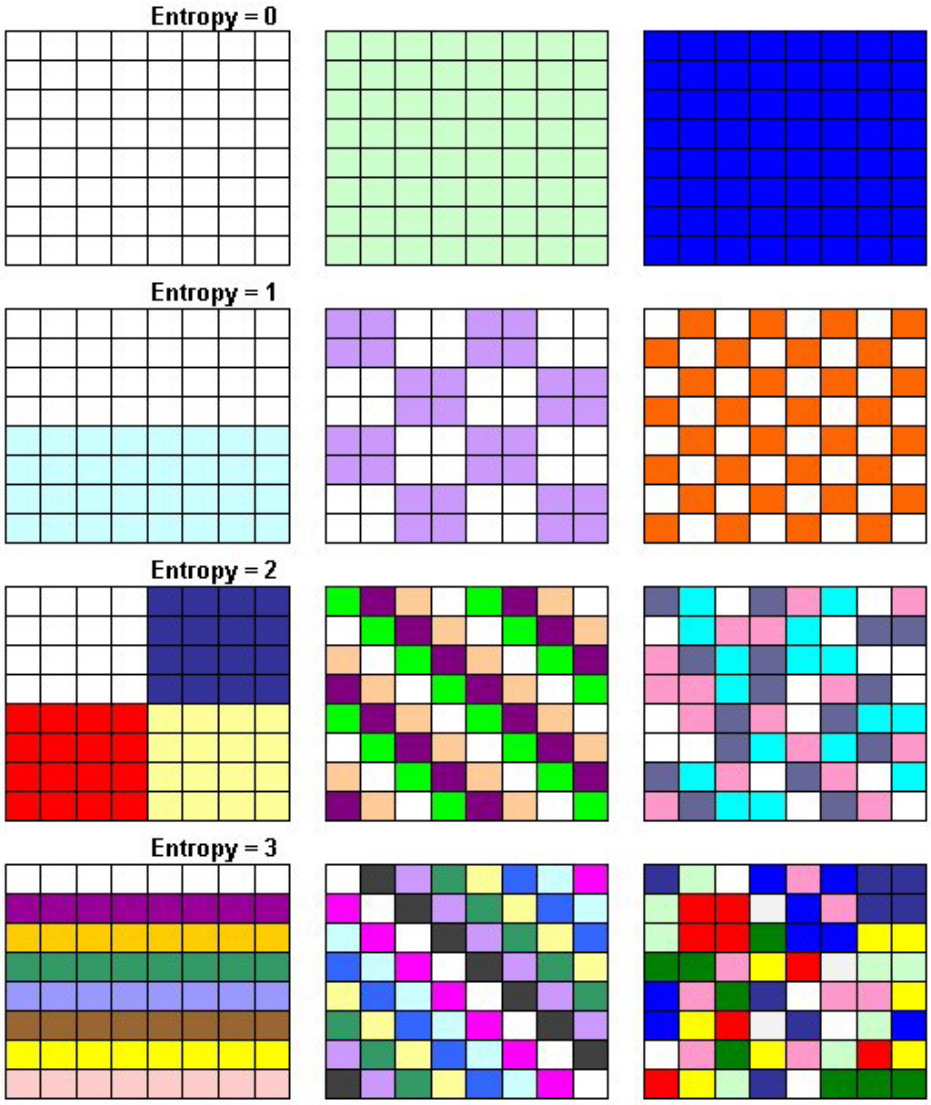



***Figure 37.*** *Examples of images with different entropies. For each entropy value, three example images are shown which have the particular entropy.*

### Intensity Difference Density

The intensity difference density (or surface area density) can be best visualized a three dimensional plot, where, in addition to the two spatial dimensions (*x* and *y*) defining the location of each pixel, the vertical dimension is proportional to the pixel intensity (Figure 38). An analogy would be city skyscrapers, where each building corresponds to a pixel and its height is analogous to the pixel intensity. Imagine a flexible rubber sheet which is draped over the whole city. The intensity difference density (or surface area density) is the area of this sheet divided by the area of the city (i.e., the number of pixels). The less variation in the pixel intensities (i.e., building heights), the less undulations in the sheet and the less the intensity difference density. Note, an image of pixels with uniform high pixel intensity will give a lower intensity difference density than one with lower intensities but with a lot of variability in intensity values between neighboring pixels. The intensity difference density is a texture measure which accounts for both the intensity variation and spatial distribution of the pixels. Additional texture metrics based on the spatial arrangement of pixels is discussed in a following sub-section on co-occurrence matrices.

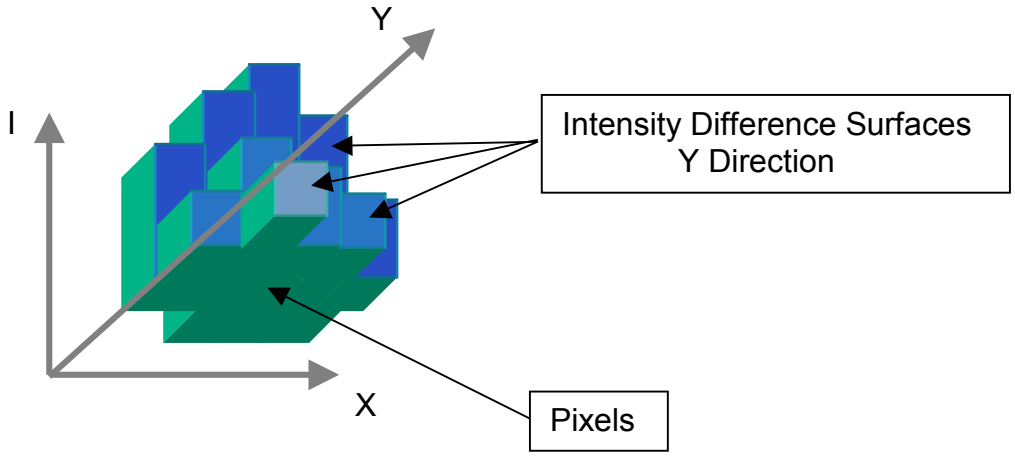



***Figure 38.*** *Intensity difference density.*

## Channel 2 Features

Channel 2 contains the image of the members of objects such as prominent cellular compartments (e.g., nuclei, Golgi apparatus). For objects with multiple members (i.e., multiple compartments of the same type), the cell-level feature reflects the average of each feature among the different members within an object.

### Cell Number and Location in a Colony

The Morphology Explorer BioApplication reports the number of members in each colony. If the Channel 2 stain labels nuclei and the Channel 1 object is a multi-cellular colony, then this can be used to determine the number of cells in the colony (Figure 39). This BioApplication also has the ability to identify which of the cells in the colony are interior cells, that have no contact with the outside and are totally surrounded by other cells of the colony, and which ones are exterior cells, that have contact with the colony's edge in that part of the cell's boundary is the colony's edge. The numbers of such interior and exterior cells (members) in the colony (object) are reported.

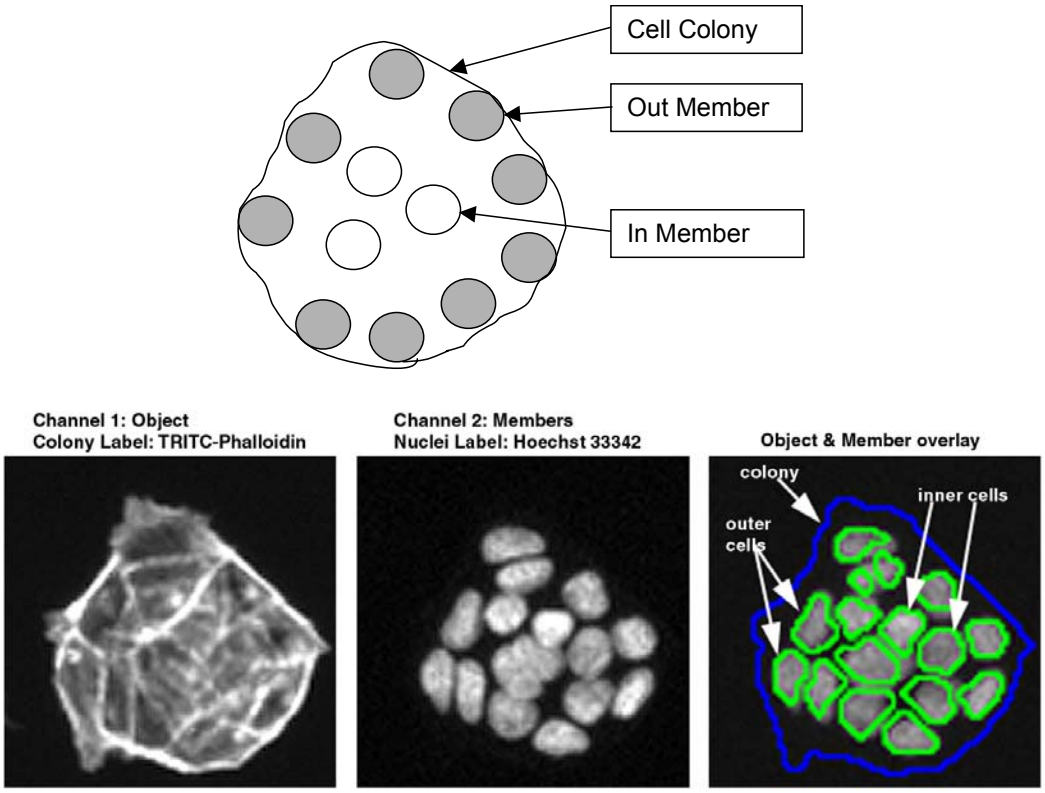



***Figure 39.*** *Colony showing interior and exterior cells. Top – illustration of members within a colony. Bottom - Image is of MCF-7 cells labeled with TRITC-Phalloidin in Channel 1 to label the whole object (colony) and Hoechst 33342 in Channel 2 to label the individual cell nuclei (members).*

## Label Amount

The total intensity and the average intensity of the member in each object are measured. The total intensity is the sum of the intensities of all pixels in that member. The average intensity is the average pixel intensity for that member, which is the total intensity divided by the number of pixels in the object. If the object has multiple members, the average of these over all the members in the object is reported as a Cell Feature. For example, if the object is a cell and the member is the cell's nucleus then the total and average intensity from the Channel 2 fluorophore is reported for the nucleus. If the object is a multi-cellular colony, then the object has many nuclei and the average and total intensity are measured for each of the nuclei in the colony; the average for each of these features over all the nuclei is reported as a Cell Feature.

In addition to the average and total intensity per member, the average and total intensity of the fluorescence in Channel 2 in the object is also reported. Thus, if there is any background fluorescence in the object in Channel 2 that is not associated with individual members, its intensity will be captured by the average and total intensity measurements for the whole object.

## Intracellular Organelle Morphology

A variety of morphological features are measured for each member. These include: area, roundness (P2A), aspect ratio (LWR), Bounding Box Area Fill Ratio (BFR), Equivalent Diameter, the ratio of the major and minor diameters of the equivalent ellipse, the Convex Hull Area Ratio, and the Convex Hull Perimeter Ratio. Note that, similar to the intensity measurements, if the object has multiple members, the average of these over all the members in the object is reported as a Cell Feature.

In addition to these morphological features, two additional features are reported that relate to how much of the whole object area is taken up by all the members in that object. These features are the:

- Ratio of the total member area in the object to the object's area
- Difference of the total member area in the object from the object's area

## Channel 3 and Channel 4 Features

Channel 3 and Channel 4 contain the images of intracellular targets. These could be homogeneous cellular labels so as to distinguish a particular cell type, intracellular fiber-like targets, such as the cytoskeleton, and discrete entities inside the cell such as organelles, protein clusters, and other structures. The features reported in Channel 3 and Channel 4 are to help quantify the different possible targets that can be labeled.

### Spacing and Proximity between Similar and Dissimilar Objects

The spacing between similar and dissimilar objects is reported in these Channels. This was described earlier in the "*Object Spacing and Proximity*" sub-section.

### Label Amount

Metrics of the pixel intensity distribution for Channel 3 and Channel 4 are reported. These metrics are the total and average intensities as well as the standard deviation, skewness, kurtosis, entropy, and surface area density. These metrics were discussed in an earlier sub-section on Channel 1 output features. These measurements also all fit in the larger category of cellular arrangement and texture metrics which is discussed further in a following sub-section.

### Intracellular Location of Label

An important category of morphology measurements is the cellular location of various entities. Many biological functions result in macromolecules, organelles, protein clusters and other cellular entities being translocated from one location of the cell to another. Examples include translocation from the cytoplasm to the nucleus or cell membrane of various macromolecules, the internalization and intracellular trafficking of various ligands, and the change if the location of focal-adhesion plaques in migrating cells. Polarized cells are often characterized by the non-uniform cellular location of specific markers. Thus, the ability to determine the intracellular arrangement of fluorescently-labeled markers would assist in the quantitation of these types of biological situations.

The Morphology Explorer BioApplication determines the radial intensity distribution of the fluorescent marker and then reports various metrics of this distribution as indicators of the marker's cellular distribution and location. Figures 40 and 41 illustrate the concept of the radial intensity distribution. Figure 40 is a schematic of three round cells, each with a different location and arrangement of the fluorescent marker. In the left most cell, the intensity is bright at the cell periphery, but becomes darker towards the center. Both the cell periphery and center is bright in the middle cell, but a dark ring-like area separates the two bright regions. In the right cell, the periphery is dark whereas the center is bright. Below each of the three cells is a plot of the average intensity at various points along the radius. These plots are the radial intensity distributions for each of the three cells.

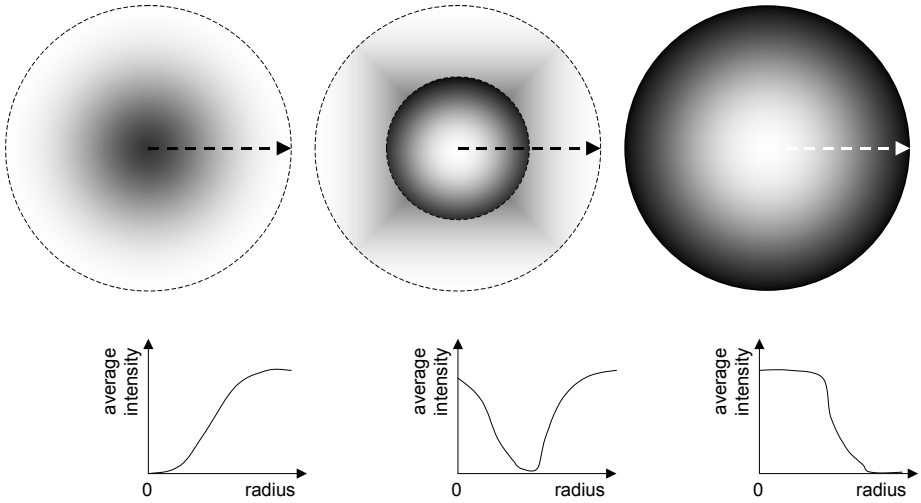



***Figure 40.*** *Three cells with different intensity patterns and radial intensity distributions.*

To determine the radial intensity distributions, the object is divided into a set of concentric rings (Figure 41A) of equal thickness. Each ring has its average pixel intensity measured (i.e., sum of all pixel intensities in the ring divided by the number of pixels in the ring), which is plotted as a function of the ring number to give the radial intensity distribution (Figure 41B). In the scheme used, the rings are numbered consecutively starting from the central ring and moving outwards (Figure 41A). The radial locations are normalized from 0 to 1, where 0 is the cell's center and 1 is the cell's periphery; intensity at location 0 describes intensity in the cell's center. You can choose whether the distribution is from the intensity of all pixels in the object or just from discrete entities such as spots or fibers. If Spot and Fiber Detection is turned off, then all pixels in the object contribute to the radial intensity distribution. If Spot and Fiber Detection is turned on, then the radial intensity distribution is only of the pixels associated with the spots and fibers.

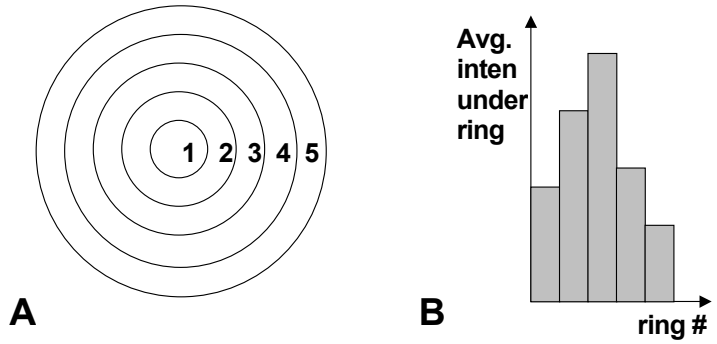



***Figure 41.*** *(A) Target mask (B) Intensity distribution profile along the ring radius*

The metrics reported for the radial intensity distribution are the distribution's mean, standard deviation, skewness, and kurtosis. These can be used as indicators of the cellular location of the fluorescent probe. The mean indicates at which radial position most of the intensity is clustered and the standard deviation can be used to monitor how tight this clustering is. The skewness can be used to monitor the asymmetry in the clustering, and the kurtosis can be used to monitor the sharpness of the distribution. These metrics were described earlier for regular intensity distributions (Figure 36) and the same concepts apply to the radial intensity distributions for the determination of the fluorescent marker's cellular location.

Note, the object area, which is divided into the concentric rings, is the object mask defined in Channel 3; this object mask is derived from the Channel 1 object mask, but then can be further dilated or eroded. Thus, if you are comparing the location of a marker in Channel 3 versus Channel 4, it is extremely important that the same degree of object mask modification has been done for both Channel 3 and Channel 4. Otherwise a comparison of radial intensity distributions between these two channels does not make sense.

If the radial intensity distribution's mean is 0, it signifies that everything is in the center of the object and all other radial intensity distribution metrics are reported as NULL. In this case, the well-level calculations for the other radial intensity distribution metrics will not include objects with such NULL values.

## Fiber or Spot Morphology

The Morphology Explorer BioApplication has the ability to identify discrete intracellular entities as spots or fibers and provide some basic quantitative features describing their morphology and arrangement. This BioApplication treats spots and fibers as similar entities, and you determine which of these are being measured by setting specific morphological criteria, such as the LWR where spots are rounder and have a lower aspect ratio than fibers; these are described in detail later in this chapter. Examples of spots and fibers are shown in Figure 42. The BioApplication reports the number of spots or fibers in the object mask, as well as their total and average areas in the object (total area is the sum of all spot or fiber areas, and the average spot or fiber area is this total divided by the number of spots or fibers).

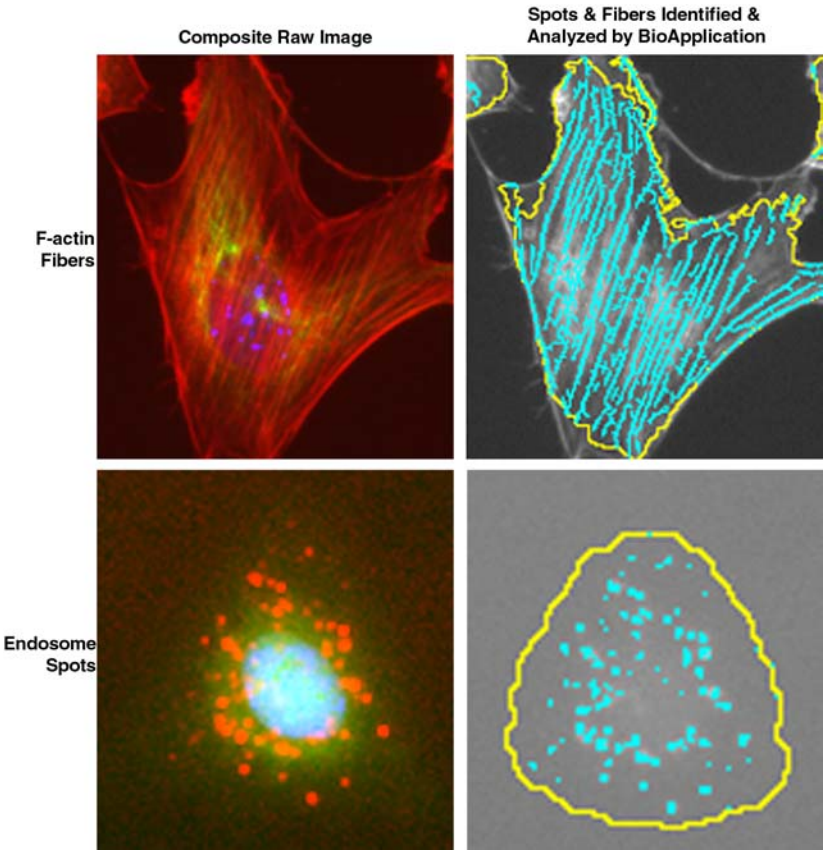



***Figure 42.*** *Spot and Fiber examples. The top images show a cell from Figure 9 where its F-actin cytoskeletal fibers are labeled with rhodamine phalloidin. The bottom images show a cell from Figure 12 of endosomes containing internalized fluorescent EGF. The images on the left are pseudocolor composites of the raw image (spots and fibers in red), and the images on the right show what the BioApplication identifies and analyzes as spots or fibers.*

## Fiber Alignment and Arrangement

The Morphology Explorer BioApplication reports two metrics for the arrangement and alignment of the fibers inside the cell: **FiberAlign1** and **FiberAlign2**. Each individual fiber's orientation has an angle with the axis of the image. **FiberAlign1** is the standard deviation of the angle for all the fibers in an object and is used as a metric for the strength of the fibers' alignment. Fibers that are more parallel to each other (i.e., more aligned) will have a smaller standard deviation of their angles. Fibers, which are either more radially distributed, such as microtubules emanating from a centrosome, or randomly arranged, will have a larger standard deviation in their angles. This feature is computed if spot or fiber analysis is turned on.

A more sophisticated analysis of fiber arrangement is based on the anisotropy of its autocorrelation and is called **FiberAlign2**. This technique measures the strength of any persistence of the labeling pattern in different directions. It scales from **0** to **1**, where a value of **0** reflects totally randomly ordered labeling with no persistence or order, and a value of **1** reflects well-aligned structures that are oriented with each other giving a definite persistence in pattern. Note, the **FiberAlign2** feature computation is done independently of whether spot or fiber analysis is turned on. Since it measures the persistence of any underlying pattern in the object, the pattern does not necessarily need to belong to an identified discrete fiber.

These two metrics, **FiberAlign1** and **FiberAlign2**, vary somewhat inversely with each other and with different sensitivities. In an object containing a lot of parallel fibers, such as F-actin stress fibers, the fibers would have a low standard deviation of their angle and thus a small **FiberAlign1** value. However, the aligned parallel fibers represent a high level of anisotropy, and would result in a larger **FiberAlign2** value. An object containing more radially oriented fibers, such as microtubules radiating from the cell's center would have large standard deviation in their angle, and thus a large **FiberAlign1**. There is still a persistent pattern in the radially oriented microtubules, so the **FiberAlign2** would have an intermediate value.

**FiberAlign2** is based on the anisotropy of the autocorrelation of the object's labeling. The principle of autocorrelation is illustrated in Figure 43 (left), where the image of the fibers is shifted along discrete *x* and *y* positions and then multiplied by the original image. After summing the multiplied intensities in the resulting image and normalizing the results, it can be plotted as a function of the amount of *x* and *y* shift (Figure 43, right). This is the image's autocorrelation function. Any randomly oriented bundle of fibers will have a decrease in the strength of the autocorrelation, whereas any persistence in the image, such as in oriented fibers, will show a corresponding persistence in the strength of the autocorrelation function along the direction of fiber alignment.

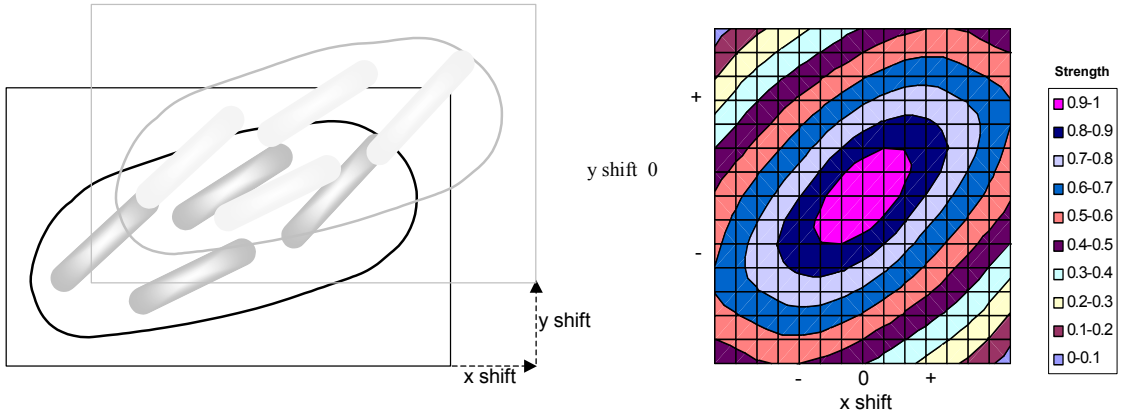



***Figure 43.*** *Autocorrelation of image of fibers*

The metric of the autocorrelation function plot, which reflects the strength of fiber alignment, is the anisotropy or coherency and is called **FiberAlign2** in this BioApplication. It is the ratio of the difference and the sum of the second moments along the principal axes of the correlation (Figure 44). A larger anisotropy means a particular dominance in the strength of the autocorrelation function along a specific direction, implying a stronger fiber alignment.

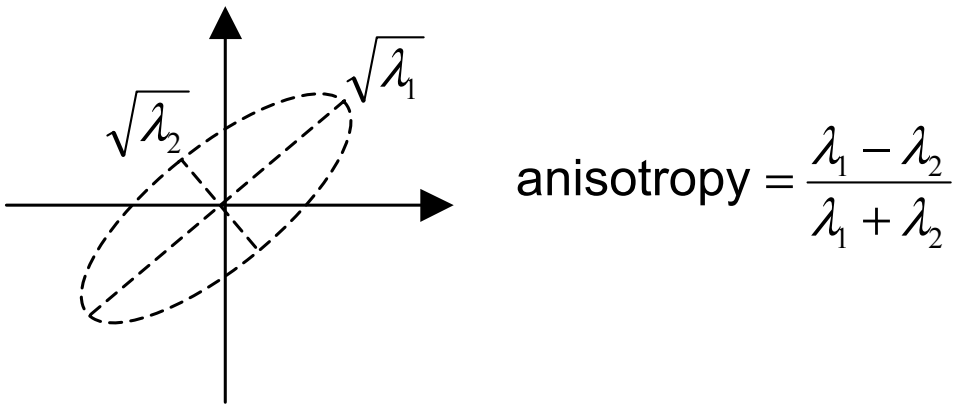



***Figure 44.*** *Anisotropy of autocorrelation is used to measure strength of fiber alignment.*

## Intracellular Texture and Arrangement

The Morphology Explorer BioApplication provides a wide range of texture measurements. Texture gives information about the spatial arrangement of the intensities in an image. There are two levels of texture measurements. The simpler, lower information content measurements are based on the pixel intensity distribution and examples are the moments of this distribution (mean, standard deviation, skewness, and kurtosis; Figure 36) as well as the entropy (Figure 37). These measurements, known as first-order texture measures, only give information on the intensity distribution; no information is provided on the spatial arrangement of the different pixels. This is why some cells can have similar entropy, mean, standard deviation, skewness, and kurtosis, even though their texture may look different.

A more sophisticated level of texture measurements takes into account the spatial arrangements of the different pixels. The surface area density feature is influenced by the spatial arrangement of pixels with different intensities, but a more systematic set of texture metrics is obtained from a classification scheme called the co-occurrence matrix. The co-occurrence matrix is based on the number of occurrences of a pixel with a certain intensity being adjacent (in a specific direction) to a pixel of another specific intensity. A matrix between the number of occurrences of all possible pairs of pixel intensities in the *x* and *y* directions is computed. Various metrics describing this co-occurrence matrix are then reported as texture measurements. In other words, the intensity co-occurrence features are computed from a matrix of probabilities of intensity co-occurrences. These texture measures are known as second-order or Haralick texture measures (Haralick, et al. 1973).

Various properties of the distribution of probabilities within the co-occurrence matrix are calculated, and may serve as quantitative descriptions of texture. The four features that are reported in this BioApplication from the co-occurrence matrix are the maximum probability, the contrast, the entropy, and the angular second moment (also known as the energy or uniformity), and are briefly described below:

**Maximum**: The maximum probability in the co-occurrence matrix indicates whether any particular type of texture or pattern (i.e., intensity co-occurrence) is prevalent in the image. This could range from an area of uniform intensity, which would have a strong probability along the diagonal of the co-occurrence matrix, to an especially prevalent intensity pair in the image, which would have a strong off-diagonal probability in the matrix.

**Contrast:** The contrast measures the strength of occurrences of pixels of different (i.e., disparate) intensities being adjacent to each other. The larger the difference in intensities of adjacent pixels (i.e., salt and pepper pattern), the stronger it is weighted.

**ASM:** The angular second moment or uniformity measures the strength of particular patterns, textures or intensity-pairs prevailing in the image.

**Entropy:** The entropy is, as described before, a measure of information content. In this context, it measures the information content in the co-occurrence matrix. It quantifies the number of different types of intensity co-occurrence pairs present, and the probability (strength) of their occurrence.

The co-occurrence matrix is constructed from the original intensity image after it is subjected to histogram equalization. Histogram equalization results in images with identical sets of intensity values, in this case 0-20; with each intensity having the same probability (Figure 45 depicts the equalization process). This removes the brightness of an image from influencing the texture measurement and only the spatial relation of pixels is computed.

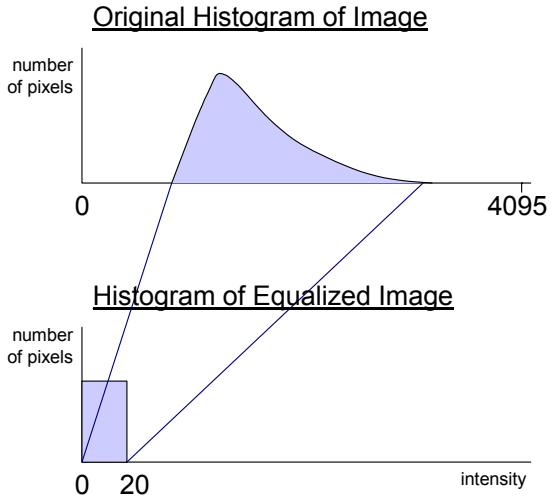



***Figure 45.*** *Histogram equalization: original histogram with 4095 different intensities is mapped into an equalized "uniform" histogram of 20 intensity values.*

The process of constructing the co-occurrence matrix consists of scanning the image line by line and counting the number of times a pixel with intensity "A" neighbors a pixel with intensity "B" (Figure 46 and Figure 47). The total number of possible co-occurrences then normalizes the total number of "AB" co-occurrences in the object. Then the values are entered into a 20×20 co-occurrence matrix (Figure 48). The matrices are created for two directions: horizontal and vertical (Figure 47). The averages of features computed for both directions are reported as co-occurrence features. Thus, the co-occurrence matrix is formed by probabilities of co-occurrences of intensities and is built using the object mask and the intensity equalized gray scale image.

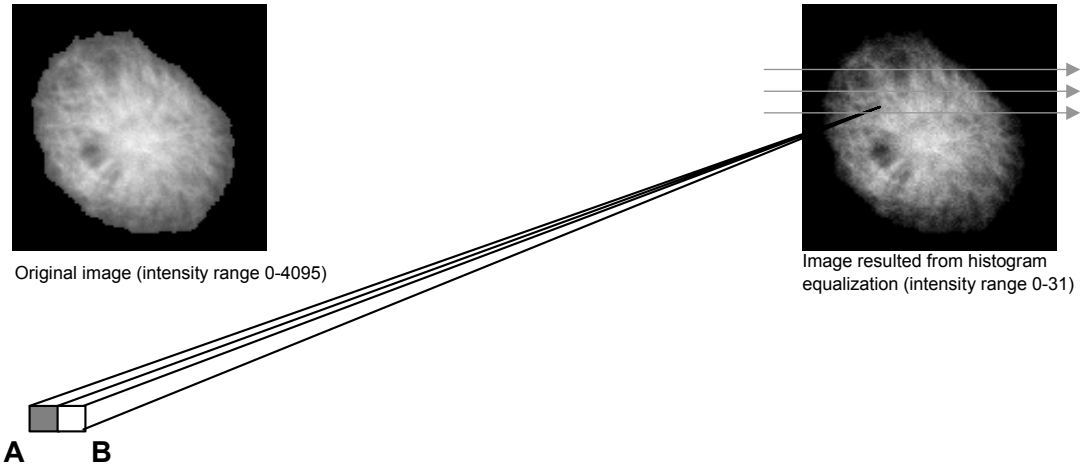



***Figure 46.*** *Equalized image is scanned horizontally to count the occurrences of intensities of neighboring pixels A and B.*

**Image**

| | | | |
|---|---|---|---|
| 1 | 2 | 0 | 3 |
| 1 | 2 | 0 | 3 |
| 1 | 3 | 0 | 2 |
| 1 | 3 | 0 | 2 |

**Horizontal Direction Matrix**

| | **0** | **1** | **2** | **3** |
|---|---|---|---|---|
| **0** | 0 | 0 | 4 | 4 |
| **1** | 0 | 0 | 2 | 2 |
| **2** | 4 | 2 | 0 | 0 |
| **3** | 4 | 2 | 0 | 0 |

**Vertical Direction Matrix**

| | **0** | **1** | **2** | **3** |
|---|---|---|---|---|
| **0** | 6 | 0 | 0 | 0 |
| **1** | 0 | 6 | 0 | 0 |
| **2** | 0 | 0 | 4 | 2 |
| **3** | 0 | 0 | 2 | 4 |

***Figure 47.*** *Examples of a simple horizontal & vertical co-occurrence matrix. The example 4X4 image shown has four possible intensity values, and the un-normalized co-occurrence matrices in the horizontal and vertical directions for this image are shown. To complete construction of the co-occurrence matrix, the matrix values in this figure would have to be normalized by the total number of possible neighbors, and then the probabilities in the horizontal and vertical matrices would be averaged.*

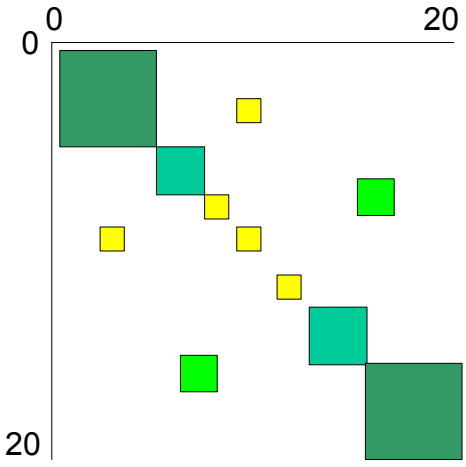



***Figure 48.*** *Co-occurrence matrix: different colors represent different probabilities of intensity co-occurrence. The different colors in this figure stand for different probability values that increase from yellow to green. Increase in matrix values on the main diagonal indicate presence of areas of uniform intensities, while larger matrix values away from the main diagonal indicate sharper contrast areas (i.e., low intensity value are next to high intensity values).*

Computation of the moments of the co-occurrence matrix allows the assessment of the spatial distribution of intensities within an image and may serve as a quantitative description of texture. The features that quantitatively describe the distribution of probabilities within the co-occurrence matrix that are computed are the maximum probability, the contrast, the entropy, and the angular second moment (also known as the energy or uniformity). These are defined as:

$$\text{Maximum} \quad \max_{ij}(C_{ij})$$

$$\text{Contrast} \quad \sum_i \sum_j (i-j)^2 \cdot C_{ij}$$

$$\text{Entropy} \quad -\sum_i \sum_j C_{ij} \cdot \text{Log}(C_{ij})$$

$$\text{Uniformity (ASM)} \quad \sum_i \sum_j C_{ij}^2$$

where $C_{ij}$ is the co-occurrence matrix value at the i,j position.

## Cell Features

Table 25 shows the output features reported for each object (i.e., individual cell or multi-cellular object), accessible in the Cell Feature window in the Protocol Interactive window in addition to the View software application.

| Cell Feature | Units | Description |
|---|---|---|
| Cell# | Number | Unique object ID |
| Top | Pixels | Y coordinate (in pixels) of top-left corner of image-aligned bounding box of Ch1 object |
| Left | Pixels | X coordinate (in pixels) of top-left corner of image-aligned bounding box of Ch1 object |
| Width | Pixels | Width (in pixels) of image-aligned bounding box of Ch1 object |
| Height | Pixels | Height (in pixels) of image-aligned bounding box of Ch1 object |
| XCentroid | Pixels | X coordinate (in pixels) of center of Ch1 object |
| YCentroid | Pixels | Y coordinate (in pixels) of center of Ch1 object |
| EventTypeProfile | Number | Identifies the types of events that occurred: 1, 2, 3, 12, 23, 13, 123 |
| EventType1Status | Binary | Status of EventType1: 0 = Event did not occur, 1 = Event occurred |
| EventType2Status | Binary | Status of EventType2: 0 = Event did not occur, 1 = Event occurred |
| EventType3Status | Binary | Status of EventType3: 0 = Event did not occur, 1 = Event occurred |
| ObjectAreaCh1 | Pixels or $\mu m^2$ | Area (in pixels or micrometers) of Ch1 object |

| Cell Feature | Units | Description |
|---|---|---|
| ObjectAreaCh1Status | Number | **ObjectAreaCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectPerimCh1 | Pixels or μm | Perimeter (in pixels or micrometers) of Ch1 object |
| ObjectPerimCh1Status | Number | **ObjectPerimCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectShapeP2ACh1 | Number | Shape measure based on ratio of perimeter squared to 4π*area of Ch1 object (**ObjectShapeP2ACh1** = 1 for circular object) |
| ObjectShapeP2ACh1Status | Number | **ObjectShapeP2ACh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectShapeLWRCh1 | Number | Shape measure based on ratio of length to width of object-aligned bounding box of Ch1 object |
| ObjectShapeLWRCh1Status | Number | **ObjectShapeLWRCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectShapeBFRCh1 | Number | Shape measure based on ratio of object area to box area of object-aligned bounding box of Ch1 object |
| ObjectShapeBFRCh1Status | Number | **ObjectShapeBFRCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectLengthCh1 | Pixels or μm | Length (in pixels or micrometers) of object-aligned bounding box of Ch1 object |
| ObjectLengthCh1Status | Number | **ObjectLengthCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectWidthCh1 | Pixels or μm | Width (in pixels or micrometers) of object-aligned bounding box of Ch1 object |
| ObjectWidthCh1Status | Number | **ObjectWidthCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectAngleCh1 | Degrees | Orientation (in degrees) of object-aligned bounding box of Ch1 object |
| ObjectAngleCh1Status | Number | **ObjectAngleCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectFiberLengthCh1 | Pixels or μm | Length (in pixels or micrometers) of Ch1 object measured along its spine |
| ObjectFiberLengthCh1Status | Number | **ObjectFiberLengthCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectFiberWidthCh1 | Pixels or μm | Width (in pixels or micrometers) of Ch1 object estimated from area and length |
| ObjectFiberWidthCh1Status | Number | **ObjectFiberWidthCh1** status: 0 = No response, 1 = High response, 2 = Low response |

| Cell Feature | Units | Description |
|---|---|---|
| ObjectConvexHullAreaRatioCh1 | Number | Ratio of convex hull area to area of Ch1 object |
| ObjectConvexHullAreaRatioCh1Status | Number | **ObjectConvexHullAreaRatioCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectConvexHullPerimRatioCh1 | Number | Ratio of convex hull perimeter to perimeter of Ch1 object |
| ObjectConvexHullPerimRatioCh1Status | Number | **ObjectConvexHullPerimRatioCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectEqCircDiamCh1 | Pixels or μm | Diameter (in pixels or micrometers) of circle with area equal to area of Ch1 object |
| ObjectEqCircDiamCh1Status | Number | **ObjectEqCircDiamCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectEqSphereVolCh1 | Pixels or $\mu m^3$ | Volume (in pixels or micrometers) of sphere with diameter = EqCircDiamCh1 |
| ObjectEqSphereVolCh1Status | Number | **ObjectEqSphereVolCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectEqSphereAreaCh1 | Pixels or $\mu m^2$ | Surface area (in pixels or micrometers) of sphere with diameter = EqCircDiamCh1 |
| ObjectEqSphereAreaCh1Status | Number | **ObjectEqSphereAreaCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectEqEllipseLWRCh1 | Number | Ratio of length to width of ellipse with area equal to area of Ch1 object |
| ObjectEqEllipseLWRCh1Status | Number | **ObjectEqEllipseLWRCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectEqEllipseProlateVolCh1 | Pixels or $\mu m^3$ | Volume (in pixels or micrometers) of ellipsoid generated by rotating area-equivalent ellipse about major axis |
| ObjectEqEllipseProlateVolCh1Status | Number | **ObjectEqEllipseProlateVolCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectEqEllipseOblateVolCh1 | Pixels or $\mu m^3$ | Volume (in pixels or micrometers) of ellipsoid generated by rotating area-equivalent ellipse about minor axis |
| ObjectEqEllipseOblateVolCh1Status | Number | **ObjectEqEllipseOblateVolCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectTotalIntenCh1 | Intensity | Total intensity of all pixels within Ch1 object |
| ObjectTotalIntenCh1Status | Number | **ObjectTotalIntenCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectAvgIntenCh1 | Intensity | Average intensity of all pixels within Ch1 object |

| Cell Feature | Units | Description |
|---|---|---|
| ObjectAvgIntenCh1Status | Number | **ObjectAvgIntenCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectVarIntenCh1 | Intensity | Standard deviation of intensity of all pixels within Ch1 object |
| ObjectVarIntenCh1Status | Number | **ObjectVarIntenCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectSkewIntenCh1 | Number | Skewness of intensity of all pixels within Ch1 object |
| ObjectSkewIntenCh1Status | Number | **ObjectSkewIntenCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectKurtIntenCh1 | Number | Kurtosis of intensity of all pixels within Ch1 object |
| ObjectKurtIntenCh1Status | Number | **ObjectKurtIntenCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectEntropyIntenCh1 | Number | Entropy of intensity of all pixels within Ch1 object |
| ObjectEntropyIntenCh1Status | Number | **ObjectEntropyIntenCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ObjectDiffIntenDensityCh1 | Intensity | Total difference of intensity of all pixels within Ch1 object normalized by area of Ch1 object |
| ObjectDiffIntenDensityCh1Status | Number | **ObjectDiffIntenDensityCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ProcessCountCh1 | Number | Number of all processes belonging to Ch1 object |
| ProcessCountCh1Status | Number | **ProcessCountCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ProcessMaxLengthCh1 | Pixels or μm | Length (in pixels or micrometers ) of longest process belonging to Ch1 object |
| ProcessMaxLengthCh1Status | Number | **ProcessMaxLengthCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ProcessTotalLengthCh1 | Pixels or μm | Total length (in pixels or micrometers) of all processes belonging to Ch1 object |
| ProcessTotalLengthCh1Status | Number | **ProcessTotalLengthCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| ProcessAvgLengthCh1 | Pixels or μm | Average length (in pixels or micrometers) of all processes belonging to Ch1 object |
| ProcessAvgLengthCh1Status | Number | **ProcessAvgLengthCh1** status: 0 = No response, 1 = High response, 2 = Low response |

| Cell Feature | Units | Description |
|---|---|---|
| NeighborMinDistCh1 | Pixels or μm | Distance (in pixels or micrometers) from Ch1 object to nearest Ch1 object |
| NeighborMinDistCh1Status | Number | **NeighborMinDistCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| NeighborAvgDistCh1 | Pixels or μm | Average distance (in pixels or micrometers) from Ch1 object to all other Ch1 objects |
| NeighborAvgDistCh1Status | Number | **NeighborAvgDistCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| NeighborVarDistCh1 | Pixels or μm | Standard deviation of distance (in pixels or micrometers) from Ch1 object to all other Ch1 objects |
| NeighborVarDistCh1Status | Number | **NeighborVarDistCh1** status: 0 = No response, 1 = High response, 2 = Low response |
| MemberCountCh2 | Number | Number of all member objects in Ch2 within modified Ch1 object mask |
| MemberCountCh2Status | Number | **MemberCountCh2** status: 0 = No response, 1 = High response, 2 = Low response |
| MemberOutCountCh2 | Number | Number of all peripheral member objects in Ch2 within modified Ch1 object mask |
| MemberOutCountCh2Status | Number | **MemberOutCountCh2** status: 0 = No response, 1 = High response, 2 = Low response |
| MemberInCountCh2 | Number | Number of all internal member objects in Ch2 within modified Ch1 object mask |
| MemberInCountCh2Status | Number | **MemberInCountCh2** status: 0 = No response, 1 = High response, 2 = Low response |
| MemberAvgAreaCh2 | Pixels or $\mu m^2$ | Average area (in pixels or micrometers) of all Ch2 member objects within modified Ch1 object mask |
| MemberAvgAreaCh2Status | Number | **MemberAvgAreaCh2** status: 0 = No response, 1 = High response, 2 = Low response |
| MemberAvgShapeP2ACh2 | Number | Average P2A shape of all Ch2 member objects within modified Ch1 object mask |
| MemberAvgShapeP2ACh2Status | Number | **MemberAvgShapeP2ACh2** status: 0 = No response, 1 = High response, 2 = Low response |
| MemberAvgShapeLWRCh2 | Number | Average LWR shape of all Ch2 member objects within modified Ch1 object mask |
| MemberAvgShapeLWRCh2Status | Number | **MemberAvgShapeLWRCh2** status: 0 = No response, 1 = High response, 2 = Low response |

| Cell Feature | Units | Description |
|---|---|---|
| MemberAvgShapeBFRCh2 | Number | Average BFR shape of all Ch2 member objects within modified Ch1 object mask |
| MemberAvgShapeBFRCh2Status | Number | **MemberAvgShapeBFRCh2** status: 0 = No response, 1 = High response, 2 = Low response |
| MemberAvgTotalIntenCh2 | Intensity | Average total intensity of all Ch2 member objects within modified Ch1 object mask |
| MemberAvgTotalIntenCh2Status | Number | **MemberAvgTotalIntenCh2** status: 0 = No response, 1 = High response, 2 = Low response |
| MemberAvgAvgIntenCh2 | Intensity | Average intensity of all Ch2 member objects within modified Ch1 object mask |
| MemberAvgAvgIntenCh2Status | Number | **MemberAvgAvgIntenCh2** status: 0 = No response, 1 = High response, 2 = Low response |
| MemberAvgConvexHullAreaRatioCh2 | Number | Average convex hull area ratio of all Ch2 member objects within modified Ch1 object mask |
| MemberAvgConvexHullAreaRatioCh2 Status | Number | **MemberAvgConvexHullAreaRatioCh2** status: 0 = No response, 1 = High response, 2 = Low response |
| MemberAvgConvexHullPerimRatioCh2 | Number | Average convex hull perimeter ratio of all Ch2 member objects within modified Ch1 object mask |
| MemberAvgConvexHullPerimRatioCh2 Status | Number | **MemberAvgConvexHullPerimRatioCh2** status: 0 = No response, 1 = High response, 2 = Low response |
| MemberAvgEqCircDiamCh2 | Pixels or µm | Average diameter of area-equivalent circle of all Ch2 member objects within modified Ch1 object mask |
| MemberAvgEqCircDiamCh2Status | Number | **MemberAvgEqCircDiamCh2** status: 0 = No response, 1 = High response, 2 = Low response |
| MemberAvgEqEllipseLWRCh2 | Number | Average LWR of area-equivalent ellipse of all Ch2 member objects within modified Ch1 object mask |
| MemberAvgEqEllipseLWRCh2Status | Number | **MemberAvgEqEllipseLWRCh2** status: 0 = No response, 1 = High response, 2 = Low response |
| MemberObjectAreaRatioCh2 | Number | Ratio of total area of all Ch2 member objects within modified Ch1 object mask to Ch1 object area |
| MemberObjectAreaRatioCh2Status | Number | **MemberObjectAreaRatioCh2** status: 0 = No response, 1 = High response, 2 = Low response |
| MemberObjectAreaDiffCh2 | Pixels or $\mu m^2$ | Difference between area of Ch1 object and total area of all Ch2 member objects within modified Ch1 object mask |

| Cell Feature | Units | Description |
|---|---|---|
| MemberObjectAreaDiffCh2Status | Number | **MemberObjectAreaDiffCh2** status: 0 = No response, 1 = High response, 2 = Low response |
| TotalIntenCh2 | Intensity | Total intensity in Ch2 of all pixels within modified Ch1 object mask |
| TotalIntenCh2Status | Number | **TotalIntenCh2** status: 0 = No response, 1 = High response, 2 = Low response |
| AvgIntenCh2 | Intensity | Average intensity in Ch2 of all pixels within modified Ch1 object mask |
| AvgIntenCh2Status | Number | **AvgIntenCh2** status: 0 = No response, 1 = High response, 2 = Low response |
| ROI_TotalIntenCh2 | Intensity | Total intensity in Ch2 of all pixels within ROI (ring or circ) derived from the Ch1 object mask |
| ROI_TotalIntenCh2Status | Number | **ROI_TotalIntenCh2** status: 0 = No response, 1 = High response, 2 = Low response |
| ROI_AvgIntenCh2 | Intensity | Average intensity in Ch2 of all pixels within ROI (ring or circ) derived from the Ch1 object mask |
| ROI_AvgIntenCh2Status | Number | **ROI_AvgIntenCh2** status: 0 = No response, 1 = High response, 2 = Low response |
| SpotFiberCountChN | Number | Number of all ChN spots and fibers within modified Ch1 object mask |
| SpotFiberCountChNStatus | Number | **SpotFiberCountChN** status: 0 = No response, 1 = High response, 2 = Low response |
| SpotFiberTotalAreaChN | Pixels or $\mu m^2$ | Total area of all ChN spots and fibers within modified Ch1 object mask |
| SpotFiberTotalAreaChNStatus | Number | **SpotFiberTotalAreaChN** status: 0 = No response, 1 = High response, 2 = Low response |
| SpotFiberAvgAreaChN | Pixels or $\mu m^2$ | Average area of all ChN spots and fibers within modified Ch1 object mask |
| SpotFiberAvgAreaChNStatus | Number | **SpotFiberAvgAreaChN** status: 0 = No response, 1 = High response, 2 = Low response |
| FiberAlign1ChN | Number | Standard deviation of orientation of all ChN fibers within modified Ch1 object mask |
| FiberAlign1ChNStatus | Number | **FiberAlign1ChN** status: 0 = No response, 1 = High response, 2 = Low response |
| ROI_FiberAlign2ChN | Number | Alignment of ChN fibers based on autocorrelation of pixel intensity within ROI (ring or circ) derived from Ch1 object mask |
| ROI_FiberAlign2ChNStatus | Number | **ROI_FiberAlign2ChN** status: 0 = No response, 1 = High response, 2 = Low response |

| Cell Feature | Units | Description |
|---|---|---|
| ROI_TotalIntenChN | Intensity | Total intensity in ChN of all pixels within ROI (ring or circ) derived from Ch1 object mask |
| ROI_TotalIntenChNStatus | Number | **ROI_TotalIntenChN** status: 0 = No response,<br>1 = High response, 2 = Low response |
| ROI_AvgIntenChN | Intensity | Average intensity in ChN of all pixels within ROI (ring or circ) derived from Ch1 object mask |
| ROI_AvgIntenChNStatus | Number | **ROI_AvgIntenChN** status: 0 = No response,<br>1 = High response, 2 = Low response |
| ROI_VarIntenChN | Intensity | Standard deviation of intensity in ChN of all pixels within ROI (ring or circ) derived from Ch1 object mask |
| ROI_VarIntenChNStatus | Number | **ROI_VarIntenChN** status: 0 = No response, 1 = High response, 2 = Low response |
| ROI_SkewIntenChN | Number | Skewness of intensity in ChN of all pixels within ROI (ring or circ) derived from Ch1 object mask |
| ROI_SkewIntenChNStatus | Number | **ROI_SkewIntenChN** status: 0 = No response,<br>1 = High response, 2 = Low response |
| ROI_KurtIntenChN | Number | Kurtosis of intensity in ChN of all pixels within ROI (ring or circ) derived from Ch1 object mask |
| ROI_KurtIntenChNStatus | Number | **ROI_KurtIntenChN** status: 0 = No response,<br>1 = High response, 2 = Low response |
| ROI_EntropyIntenChN | Number | Entropy of intensity in ChN of all pixels within ROI (ring or circ) derived from Ch1 object mask |
| ROI_EntropyIntenChNStatus | Number | **ROI_EntropyIntenChN** status: 0 = No response, 1 = High response, 2 = Low response |
| ROI_DiffIntenDensityChN | Intensity | Total difference of intensity in ChN of all pixels within ROI (ring or circ) derived from Ch1 object mask normalized by area of modified Ch1 object mask |
| ROI_DiffIntenDensityChNStatus | Number | **ROI_DiffIntenDensityChN** status: 0 = No response, 1 = High response, 2 = Low response |
| ROI_MaxCoocIntenChN | Number | Maximum probability of 2D co-occurrence intensity distribution in ChN of all pixels within ROI (ring or circ) derived from Ch1 object mask |
| ROI_MaxCoocIntenChNStatus | Number | **ROI_MaxCoocIntenChN** status: 0 = No response, 1 = High response, 2 = Low response |

| Cell Feature | Units | Description |
|---|---|---|
| ROI_ContrastCoocIntenChN | Number | Contrast of 2D co-occurrence intensity distribution in ChN of all pixels within ROI (ring or circ) derived from Ch1 object mask |
| ROI_ContrastCoocIntenChNStatus | Number | **ROI_ContrastCoocIntenChN** status: 0 = No response, 1 = High response, 2 = Low response |
| ROI_EntropyCoocIntenChN | Number | Entropy of 2D co-occurrence intensity distribution in ChN of all pixels within ROI (ring or circ) derived from Ch1 object mask |
| ROI_EntropyCoocIntenChNStatus | Number | **ROI_EntropyCoocIntenChN** status: 0 = No response, 1 = High response, 2 = Low response |
| ROI_ASMCoocIntenChN | Number | Angular second moment of 2D co-occurrence intensity distribution in ChN of all pixels within ROI (ring or circ) derived from Ch1 object mask |
| ROI_ASMCoocIntenChNStatus | Number | **ROI_ASMCoocIntenChN** status: 0 = No response, 1 = High response, 2 = Low response |
| ROI_AvgRadialIntenChN | Number | Mean of radial intensity distribution in ChN of all pixels within ROI (ring or circ) derived from Ch1 object mask |
| ROI_AvgRadialIntenChNStatus | Number | **ROI_AvgRadialIntenChN** status: 0 = No response, 1 = High response, 2 = Low response |
| ROI_VarRadialIntenChN | Number | Standard deviation of radial intensity distribution in ChN of all pixels within ROI (ring or circ) derived from Ch1 object mask |
| ROI_VarRadialIntenChNStatus | Number | **ROI_VarRadialIntenChN** status: 0 = No response, 1 = High response, 2 = Low response |
| ROI_SkewRadialIntenChN | Number | Skewness of radial intensity distribution in ChN of all pixels within ROI (ring or circ) derived from Ch1 object mask |
| ROI_SkewRadialIntenChNStatus | Number | **ROI_SkewRadialIntenChN** status: 0 = No response, 1 = High response, 2 = Low response |
| ROI_KurtRadialIntenChN | Number | Kurtosis of radial intensity distribution in ChN of all pixels within ROI (ring or circ) derived from Ch1 object mask |
| ROI_KurtRadialIntenChNStatus | Number | **ROI_KurtRadialIntenChN** status: 0 = No response, 1 = High response, 2 = Low response |
| NeighborMinDistChN | Pixels or μm | Distance (in pixels or micrometers) from ChN object to nearest ChN object |
| NeighborMinDistChNStatus | Number | **NeighborMinDistChN** status: 0 = No response, 1 = High response, 2 = Low response |
| NeighborAvgDistChN | Pixels or μm | Average distance (in pixels or micrometers) from ChN object to all other ChN objects |

| Cell Feature | Units | Description |
|---|---|---|
| NeighborAvgDistChNStatus | Number | **NeighborAvgDistChN** status: 0 = No response, 1 = High response, 2 = Low response |
| NeighborVarDistChN | Pixels or μm | Standard deviation of distance (in pixels or micrometers) from ChN object to all other ChN objects |
| NeighborVarDistChNStatus | Number | **NeighborVarDistChN** status: 0 = No response, 1 = High response, 2 = Low response |
| NeighborMinDistCh3Ch4 | Pixels or μm | Distance (in pixels or micrometers) from Ch3 object to nearest Ch4 object |
| NeighborMinDistCh3Ch4Status | Number | **NeighborMinDistCh3Ch4** status: 0 = No response, 1 = High response, 2 = Low response |
| NeighborAvgDistCh3Ch4 | Pixels or μm | Average distance (in pixels or micrometers) from Ch3 object to all Ch4 objects |
| NeighborAvgDistCh3Ch4Status | Number | **NeighborAvgDistCh3Ch4** status: 0 = No response, 1 = High response, 2 = Low response |
| NeighborVarDistCh3Ch4 | Pixels or μm | Standard deviation of distance (in pixels or micrometers) from Ch3 object to all Ch4 objects |
| NeighborVarDistCh3Ch4Status | Number | **NeighborVarDistCh3Ch4** status: 0 = No response, 1 = High response, 2 = Low response |
| TotalIntenCh5 | Intensity | Total intensity in Ch5 of all pixels within modified Ch1 object mask |
| AvgIntenCh5 | Intensity | Average intensity in Ch5 of all pixels within modified Ch1 object mask |
| TotalIntenCh6 | Intensity | Total intensity in Ch6 of all pixels within modified Ch1 object mask |
| AvgIntenCh6 | Intensity | Average intensity in Ch6 of all pixels within modified Ch1 object mask |

***Table 25.*** *Cell Features available for the Morphology Explorer BioApplication. *Note that "ChN" refers to Channels 3-4.*

## Well Features

The Well Features are either statistical measures or population characterization features derived from the distribution of Cell Features. Well-level statistical measures reported are the mean and standard deviation. The population characterization features include the percentage greater than a Level High or less than a Level Low.

### Computing Well Features When Cell Features are NULL

In most cases, the statistical measures and population characterization features are computed over all the objects identified and analyzed. However, in certain cases, these features are may be computed for a subset of the objects in the well. The features for which this situation may arise are:

- Process morphology measurements
- Member morphology measurements
- Radial intensity distribution measurements for spots or fibers
- Proximity measurements between similar and dissimilar objects

and occur when specific Cell Features in these categories are reported as NULL.

If Process Identification is chosen and a particular object has no processes identified, then that object's **ProcessCountCh1** will be **0**. Since the object has no processes, the process morphology features such as its maximum and average and total lengths are irrelevant and will be reported as NULL at the cell-level. In this case, at the well-level, the statistical measures and population characterization features for the process morphology features will not include objects with no processes. Thus the mean, standard deviation, and other features will only be computed for a subset of all objects in the well.

Similarly, if an object has no members detected in it in Channel 2, and then all the **MemberCountCh2** Cell Features for that object will be 0. At the cell-level, all member morphology features for that object will be reported as NULL, its **MemberObjectAreaRatioCh2** will be reported as **0**, and its **MemberObjectAreaDiffCh2** will result in the object area. This object will not be included in the well-level calculations for these member morphology features.

A similar action is done for spots or fibers in Channel 3 or Channel 4 if no spots or fibers are detected. In this case, the **SpotFiber** count feature will be **0**, and at the cell-level, the **SpotFiber** area measurements, all radial intensity distribution measurements, and **FiberAlign1** will be reported as NULL. **FiberAlign2**, which is independent of whether a spot or fiber is identified, using intensity thresholds, will not be reported as NULL. For these features, this object will not be used in the doing the well-level calculations.

For proximity and spacing measurements between objects in Channel 3 and/or Channel 4, Well Features are computed only for the subset of objects that are identified as responders. If only one responder is identified, then the proximity measurements are reported as NULL. This means to measure proximity between similar objects, greater than one object needs to be identified as a responder in the same channel, and between dissimilar objects, at least one object needs to be identified in Channel 3 and another distinct object needs to be identified in Channel 4.

Many Well features are derived from the Cell Features. Such features are identified by a prefix, as listed in Table 26, to the Cell Feature name.

| Feature Prefix | Well Feature Definition | Units |
|---|---|---|
| MEAN_ | Average of Feature_X for all objects selected for analysis in the well | Same as cell feature |
| SD_ | Standard deviation of Feature_X for all objects selected for analysis in the well | Same as cell feature |
| %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 26.*** *General Well Feature Definition*

The algorithm also reports the following Well Features in the Scan Plate View in addition to the Well Detail window of the View software application (Table 27).

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

***Table 27.*** *Additional Well Features available for the Morphology Explorer BioApplication*

## Reference Well Features

The algorithm reports the following features in the Plate Scan View of the software application as well as Plate Features in the View software application (Table 28). These features are computed and reported only when the Assay Parameter **UseReferenceWells = 1** (enabled). Rather than showing both features derived from the previous level Assay Parameters, one entry for the feature will be listed giving both outputs, as shown in the following example for the Channel 1 object area:

- RefObjectAreaCh1Level***Low/High***

| Reference Well Feature | Description |
|---|---|
| RefAvgObjectCountPerField | Average number of objects per field in reference wells |
| RefObjectAreaCh1Level***Low/High*** | Low/High-response level for **ObjectAreaCh1** computed from reference well results |
| RefObjectPerimCh1Level***Low/High*** | Low/High-response level for **ObjectPerimCh1** computed from reference well results |
| RefObjectShapeP2ACh1Level***Low/High*** | Low/High-response level for **ObjectShapeP2ACh1** computed from reference well results |
| RefObjectShapeLWRCh1Level***Low/High*** | Low/High-response level for **ObjectShapeLWRCh1** computed from reference well results |
| RefObjectShapeBFRCh1Level***Low/High*** | Low/High-response level for **ObjectShapeBFRCh1** computed from reference well results |
| RefObjectLengthCh1Level***Low/High*** | Low/High-response level for **ObjectLengthCh1** computed from reference well results |
| RefObjectWidthCh1Level***Low/High*** | Low/High-response level for **ObjectWidthCh1** computed from reference well results |
| RefObjectAngleCh1Level***Low/High*** | Low/High-response level for **ObjectAngleCh1** computed from reference well results |
| RefObjectFiberLengthCh1Level***Low/High*** | Low/High-response level for **ObjectFiberLengthCh1** computed from reference well results |
| RefObjectFiberWidthCh1Level***Low/High*** | Low/High-response level for **ObjectFiberWidthCh1** computed from reference well results |
| RefObjectConvexHullAreaRatioCh1Level***Low/High*** | Low/High-response level for **ObjectConvexHullAreaRatioCh1** computed from reference well results |
| RefObjectConvexHullPerimRatioCh1Level***Low/High*** | Low/High-response level for **ObjectConvexHullPerimRatioCh1** computed from reference well results |
| RefObjectEqCircDiamCh1Level***Low/High*** | Low/High-response level for **ObjectEqCircDiamCh1** computed from reference well results |
| RefObjectEqSphereVolCh1Level***Low/High*** | Low/High-response level for **ObjectEqSphereVolCh1** computed from reference well results |
| RefObjectEqSphereAreaCh1Level***Low/High*** | Low/High-response level for **ObjectEqSphereAreaCh1** computed from reference well results |
| RefObjectEqEllipseLWRCh1Level***Low/High*** | Low/High-response level for **ObjectEqEllipseLWRCh1** computed from reference well results |
| RefObjectEqEllipseProlateVolCh1Level***Low/High*** | Low/High-response level for **ObjectEqEllipseProlateVolCh1** computed from reference well results |
| RefObjectEqEllipseOblateVolCh1Level***Low/High*** | Low/High-response level for **ObjectEqEllipseOblateVolCh1** computed from reference well results |
| RefObjectTotalIntenCh1Level***Low/High*** | Low/High-response level for **ObjectTotalIntenCh1** computed from reference well results |
| RefObjectAvgIntenCh1Level***Low/High*** | Low/High-response level for **ObjectAvgIntenCh1** computed from reference well results |
| RefObjectVarIntenCh1Level***Low/High*** | Low/High-response level for **ObjectVarIntenCh1** computed from reference well results |
| RefObjectSkewIntenCh1Level***Low/High*** | Low/High-response level for **ObjectSkewIntenCh1** computed from reference well results |
| RefObjectKurtIntenCh1Level***Low/High*** | Low/High-response level for **ObjectKurtIntenCh1** computed from reference well results |

| Reference Well Feature | Description |
|---|---|
| RefObjectEntropyIntenCh1Level***Low/High*** | Low/High-response level for **ObjectEntropyIntenCh1** computed from reference well results |
| RefObjectDiffIntenDensityCh1Level***Low/High*** | Low/High-response level for **ObjectDiffIntenDensityCh1** computed from reference well results |
| RefProcessCountCh1Level***Low/High*** | Low/High-response level for **ProcessCountCh1** computed from reference well results |
| RefProcessMaxLengthCh1Level***Low/High*** | Low/High-response level for **ProcessMaxLengthCh1** computed from reference well results |
| RefProcessTotalLengthCh1Level***Low/High*** | Low/High-response level for **ProcessTotalLengthCh1** computed from reference well results |
| RefProcessAvgLengthCh1Level***Low/High*** | Low/High-response level for **ProcessAvgLengthCh1** computed from reference well results |
| RefNeighborMinDistCh1Level***Low/High*** | Low/High-response level for **NeighborMinDistCh1** computed from reference well results |
| RefNeighborAvgDistCh1Level***Low/High*** | Low/High-response level for **NeighborAvgDistCh1** computed from reference well results |
| RefNeighborVarDistCh1Level***Low/High*** | Low/High-response level for **NeighborVarDistCh1** computed from reference well results |
| RefMemberCountCh2Level***Low/High*** | Low/High-response level for **MemberCountCh2** computed from reference well results |
| RefMemberOutCountCh2Level***Low/High*** | Low/High-response level for **MemberOutCountCh2** computed from reference well results |
| RefMemberInCountCh2Level***Low/High*** | Low/High-response level for **MemberInCountCh2** computed from reference well results |
| RefMemberAvgAreaCh2Level***Low/High*** | Low/High-response level for **MemberAvgAreaCh2** computed from reference well results |
| RefMemberAvgShapeP2ACh2Level***Low/High*** | Low/High-response level for **MemberAvgShapeP2ACh2** computed from reference well results |
| RefMemberAvgShapeLWRCh2Level***Low/High*** | Low/High-response level for **MemberAvgShapeLWRCh2** computed from reference well results |
| RefMemberAvgShapeBFRCh2Level***Low/High*** | Low/High-response level for **MemberAvgShapeBFRCh2** computed from reference well results |
| RefMemberAvgTotalIntenCh2Level***Low/High*** | Low/High-response level for **MemberAvgTotalIntenCh2** computed from reference well results |
| RefMemberAvgAvgIntenCh2Level***Low/High*** | Low/High-response level for **MemberAvgAvgIntenCh2** computed from reference well results |
| RefMemberAvgConvexHullAreaRatioCh2Level***Low/High*** | Low/High-response level for **MemberAvgConvexHullAreaRatioCh2** computed from reference well results |
| RefMemberAvgConvexHullPerimRatioCh2Level***Low/High*** | Low/High-response level for **MemberAvgConvexHullPerimRatioCh2** computed from reference well results |
| RefMemberAvgEqCircDiamCh2Level***Low/High*** | Low/High-response level for **MemberAvgEqCircDiamCh2** computed from reference well results |
| RefMemberAvgEqEllipseLWRCh2Level***Low/High*** | Low/High-response level for **MemberAvgEqEllipseLWRCh2** computed from reference well results |
| RefMemberObjectAreaRatioCh2Level***Low/High*** | Low/High-response level for **MemberObjectAreaRatioCh2** computed from reference well results |
| RefMemberObjectAreaDiffCh2Level***Low/High*** | Low/High-response level for **MemberObjectAreaDiffCh2** computed from reference well results |
| RefROI_TotalIntenCh2Level***Low/High*** | Low/High-response level for **ROI_TotalIntenCh2** computed from reference well results |
| RefROI_AvgIntenCh2Level***Low/High*** | Low/High-response level for **ROI_AvgIntenCh2** computed from reference well results |
| RefROI_SpotFiberCountChNLevel***Low/High*** | Low/High-response level for **SpotFiberCountChN** computed from reference well results |
| RefROI_SpotFiberTotalAreaChNLevel***Low/High*** | Low/High-response level for **SpotFiberTotalAreaChN** computed from reference well results |

| Reference Well Feature | Description |
|---|---|
| RefROI_SpotFiberAvgAreaChNLevel***Low/High*** | Low/High-response level for **SpotFiberAvgAreaChN** computed from reference well results |
| RefROI_FiberAlign1ChNLevel***Low/High*** | Low/High-response level for **FiberAlign1ChN** computed from reference well results |
| RefROI_FiberAlign2ChNLevel***Low/High*** | Low/High-response level for **FiberAlign2ChN** computed from reference well results |
| RefROI_TotalIntenChNLevel***Low/High*** | Low/High-response level for **TotalIntenChN** computed from reference well results |
| RefROI_AvgIntenChNLevel***Low/High*** | Low/High-response level for **AvgIntenChN** computed from reference well results |
| RefROI_VarIntenChNLevel***Low/High*** | Low/High-response level for **VarIntenChN** computed from reference well results |
| RefROI_SkewIntenChNLevel***Low/High*** | Low/High-response level for **SkewIntenChN** computed from reference well results |
| RefROI_KurtIntenChNLevel***Low/High*** | Low/High-response level for **KurtIntenChN** computed from reference well results |
| RefROI_EntropyIntenChNLevel***Low/High*** | Low/High-response level for **EntropyIntenChN** computed from reference well results |
| RefROI_DiffIntenDensityChNLevel***Low/High*** | Low/High-response level for **DiffIntenDensityChN** computed from reference well results |
| RefROI_MaxCoocIntenChNLevel***Low/High*** | Low/High-response level for **MaxCoocIntenChN** computed from reference well results |
| RefROI_ContrastCoocIntenChNLevel***Low/High*** | Low/High-response level for **ContrastCoocIntenChN** computed from reference well results |
| RefROI_EntropyCoocIntenChNLevel***Low/High*** | Low/High-response level for **EntropyCoocIntenChN** computed from reference well results |
| RefROI_ASMCoocIntenChNLevel***Low/High*** | Low/High-response level for **ASMCoocIntenChN** computed from reference well results |
| RefROI_AvgRadialIntenChNLevel***Low/High*** | Low/High-response level for **AvgRadialIntenChN** computed from reference well results |
| RefROI_VarRadialIntenChNLevel***Low/High*** | Low/High-response level for **VarRadialIntenChN** computed from reference well results |
| RefROI_SkewRadialIntenChNLevel***Low/High*** | Low/High-response level for **SkewRadialIntenChN** computed from reference well results |
| RefROI_KurtRadialIntenChNLevel***Low/High*** | Low/High-response level for **KurtRadialIntenChN** computed from reference well results |
| RefNeighborMinDistChNLevel***Low/High*** | Low/High-response level for **NeighborMinDistChN** computed from reference well results |
| RefNeighborAvgDistChNLevel***Low/High*** | Low/High-response level for **NeighborAvgDistChN** computed from reference well results |
| RefNeighborVarDistChNLevel***Low/High*** | Low/High-response level for **NeighborVarDistChN** computed from reference well results |
| RefNeighborMinDistCh3Ch4Level***Low/High*** | Low/High-response level for **NeighborMinDistCh3Ch4** computed from reference well results |
| RefNeighborAvgDistCh3Ch4Level***Low/High*** | Low/High-response level for **NeighborAvgDistCh3Ch4** computed from reference well results |
| RefNeighborVarDistCh3Ch4Level***Low/High*** | Low/High-response level for **NeighborVarDistCh3Ch4** computed from reference well results |

***Table 28.*** *Reference Well Features available for the Morphology Explorer BioApplication. *Note that "ChN" refers to Channel 3 and Channel 4.*

Chapter
3

# Using the Morphology Explorer BioApplication

The Morphology Explorer BioApplication comes with individual protocols and example image sets for the six different use cases introduced in Chapter 1. This chapter describes configuring the Morphology Explorer BioApplication for the different biologies introduced in Chapter 1 and will guide you in optimizing the protocols for these examples. This will demonstrate different ways of configuring this application to suit different biological situations, so that you can use similar approaches in optimizing the application toward your own particular biology.


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

The Morphology Explorer BioApplication is for scientists who want a versatile tool that they can apply towards many different situations and want the flexibility to configure it to measure their particular biology of interest. Thus, the Morphology Explorer BioApplication is for advanced users who have the expertise and feel comfortable in configuring and optimizing such an application for their particular biological situation. A familiarity with the use of the instrument and optimizing protocols for different situations, magnifications, and dyes is assumed and was briefly described in Chapter 3. If you are unfamiliar with these procedures, please refer to the appropriate User's Guide for instructions. If you do not want to develop the expertise in configuring or optimizing such a multi-functional application, you are encouraged to use a more specific application that is targeted towards a specific biology. These specific applications have been optimized for particular biologies and can be more rapidly implemented.

## Assay-Specific Procedures for Optimizing the BioApplication

This chapter starts with a brief discussion on fluorescent labels for object identification and then is followed by six sub-sections, one for each of the different biological examples. Each sub-section describes the assay design, discusses the choice of labels, and covers any special issues for image acquisition. This is followed by a discussion of key issues to consider when setting up the application's protocol for quantitative analysis of the particular biology and quantitative results from applying the application to the particular biology are presented.

### Channel 1 Object Labeling Options

As discussed in Chapter 2, for the most accurate quantitation of your biological problem, the proper identification of objects in Channel 1 is critical. A key step in the proper identification of objects is the choice of the optimal fluorescence labeling strategy to label and define the object. Fluorescent labels for objects can either label the cell's surface, the cell's interior volume or cytoplasm, some major widespread constituent of the cell that allows the cell's extent to be defined, or a combination of

any of these. In addition, the object label may be specific for a certain type or property of the cell to distinguish it from other cells in the image field. The properties of the label chosen and its cellular distribution will influence the type of object segmentation best suited for the analysis. Figure 49 has examples of several labeling options.

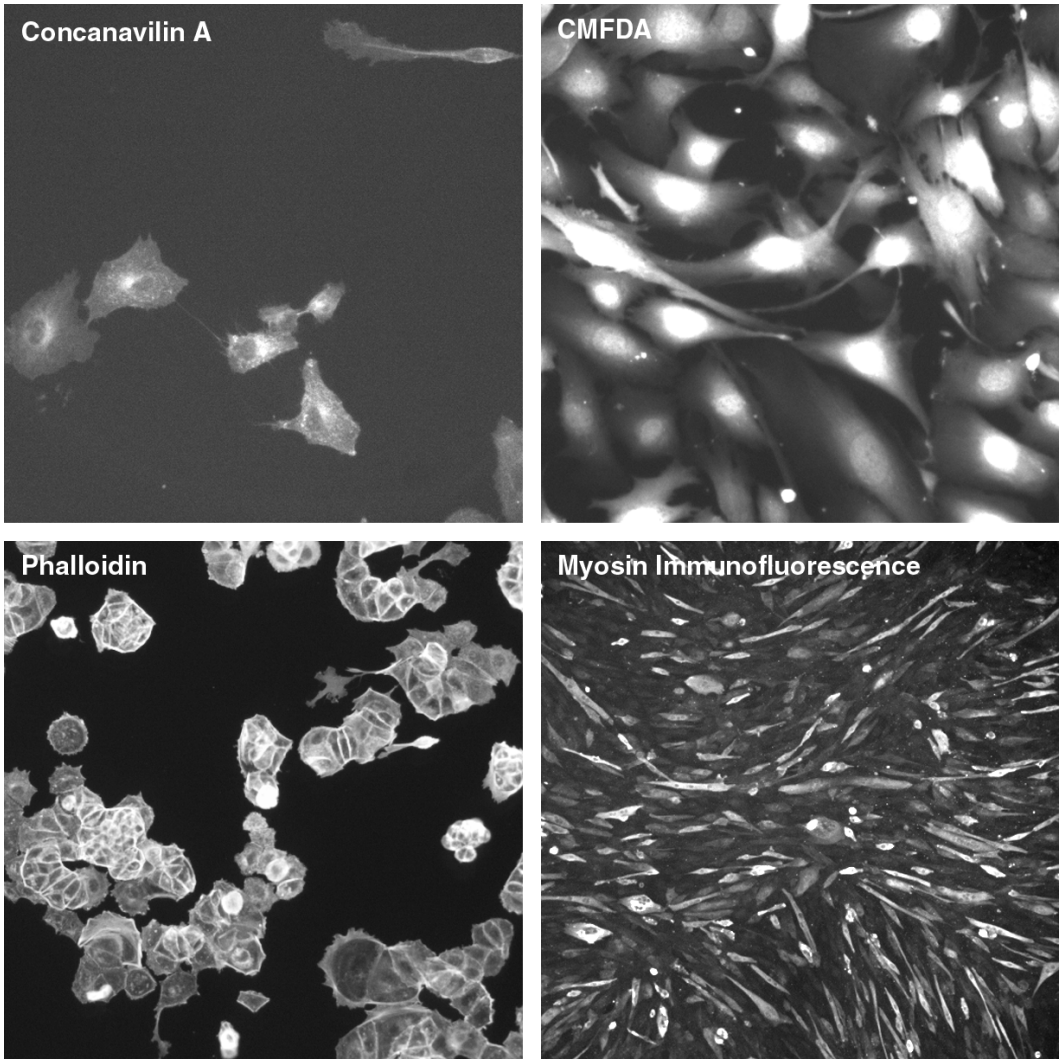



***Figure 49.*** *Four different options for fluorescently labeling objects in Channel 1. Clockwise from the top left, the panels show NIH 3T3 cells labeled with fluorescent concanavilin A, undifferentiated C2C12 myoblasts labeled with CMFDA, myotubes derived from C2C12 cells labeled via indirect immunofluorescence against skeletal muscle myosin, and colonies of MCF-7 cells labeled with rhodamine phalloidin.*

Fluorescently conjugated lectins, such as fluorescent concanavilin A or wheat germ agglutinin, can be used to label the cell surface, as shown in Figure 49. Lectins give a fairly homogeneous stain over the cell's cytoplasm and a clear definition of the cell's edge is seen. However, with the lack of a distinct peak this staining method is not optimal for the separation of labeled objects using the object segmentation peak method (negative value). Lectins may also cause capping and aggregation of other cell surface proteins in live cells. Other cell surface stains include fluorescent lipids or lipid analogues; however in live cells, their cell surface labeling is transient as they may eventually get internalized into the cell.

Fluorescent labels of the entire cell volume or cytoplasm include the Cell Tracker class of reagents available from Molecular Probes Inc. such as CMFDA or CMTMR. As seen in Figure 49, these dyes give a smooth labeling in the cytoplasmic region and a very bright labeling in the nuclear region where the cell's volume is maximal. A drawback of these labels is that because of the low cytoplasmic volume in the lamellipodial region at the cell's edge, the cell's boundary is not well marked. However, the smooth staining with the high nuclear peak allows cells stained with these labels to be well separated with the Current V3 Object Segmentation Intensity Method (Figure 19). Another drawback of these labels is that in some cases they may slowly leak out of cells over the course of several days; this means that samples stained with these labels should be imaged as soon as possible after fixation.

Cellular constituents that are widely distributed in the cell can also be fluorescently labeled to define the cell's extent. In Figure 49, the F-actin of cells in a colony is labeled with rhodamine-phalloidin and the cells' extents are clearly seen. This label clearly identifies the edge of the colonies, but due to the non-smoothness of the intracellular staining, the segmentation and separation of clustered objects based on the object stain is difficult to do.

In Figure 8 and Figure 49, immunofluorescence against skeletal muscle myosin defines differentiated myotubes from undifferentiated myoblasts. This is an example of the object label used to only specifically identify and stain a subset of the cells present. Although the myotubes are specifically labeled, the asymmetrical long shape and inhomogeneous staining (dark holes over the nuclei) prevent any of the current available object segmentation methods from being able to separate and distinguish myotubes when they get densely crowded together.

## Example 1: Myoblast Differentiation

### Assay Design

Muscle fibers are formed when myoblast cells differentiate and fuse with each other to form long, multinucleated cells called myotubes. C2C12 cells are a myoblast cell line that differentiates *in vitro*. In the experiment, C2C12 cells are first grown to confluence. The cells are then induced to differentiate. This is done by replacing complete medium with low serum differentiation medium with or without the calcium channel blocker nifedipine. Blocking calcium channels prevents differentiation and myotube formation.

This experiment is a 2-channel assay; cells are stained using an anti-myosin heavy chain (MF-20) antibody (Alexa Fluor® 488 secondary) in Channel 1 and the nuclei with Hoechst in Channel 2. All cell nuclei will be labeled with Hoechst. Only myotubes will have skeletal myosin staining; undifferentiated myoblasts will have little or no skeletal myosin staining. Table 29 is an overview of the experimental design.

- ***Control cells:*** The C2C12 cells were grown to confluency and then allowed to differentiate into myotubes over four days in differentiation medium. Mature myotubes formed and are long, fiber-like, multinucleated cells.
- ***Drug treated cells (Nifedipine):*** The inhibition of skeletal myoblast formation took place when confluent C2C12 cells were treated with 100 µM nifedipine in differentiation medium over four days. The results were very few cells with myosin stain seen; mostly individual cells present.

Figure 8 shows images of this myoblast differentiation example that were acquired at 5x on the ArrayScan HCS Reader 4.0. The difference between mature, multinucleated

myotubes containing MF-20 staining versus undifferentiated myoblasts with little MF-20 is clear in these images.

| Channel | Cellular Target | Detection Strategy & Fluorophore | Fluorescence Emission Color |
|---|---|---|---|
| **Channel 1** | Skeletal muscle myosin expressed only in differentiated myotubes | Immunofluorescence against MF-20 (skeletal muscle myosin)<br>Alexa Fluor 488 conjugated secondary Ab | Green |
| **Channel 2** | Nuclei of all cells | DNA binding dye - Hoechst 33342 | Blue |

***Table 29.*** *Myoblast Differentiation Experimental Design*

## Analysis Strategy and Key Items in Protocol

### Channel 1 Object Identification

The Channel 1 objects to be identified are the myotubes. They are different from the undifferentiated myoblasts in the field in that they contain skeletal muscle myosin, have a bright fluorescent label, and are long, multicellular (i.e., multi-nucleated) objects (Figure 8 and Figure 49). These properties are used to specifically identify the myotubes as objects.

In wells treated with nifedipine, myoblast differentiation was inhibited and there may not be any cells that have MF-20 labeling. Thus, a histogram-based intensity thresholding approach may give erroneous results. Instead a **Fixed Threshold** method was chosen which would need to be adjusted whenever new samples plates are scanned and analyzed as intensities may differ from the example images supplied for a fresh scan.

The Channel 1 Object Selection Parameters are used to specifically identify myotubes based on their intensity, shape, and area. For an object to be positively identified, its average intensity and total intensity need to be above certain values which mean that only objects with positive MF-20 fluorescence (i.e., above background) will be selected. Because myotubes are comprised of multiple cells fused together, its area must also be above a certain threshold; individual myoblast cells would have an area less than this threshold and would not be selected. Thus, for the sample image sets acquired at 5x magnification, a minimum area threshold of **1200** $\mu m^2$ was chosen as an Object Selection Parameter.

Myotubes are long structures and thus have large LWRs (i.e., aspect ratios). Thus, a large minimum LWR could have been chosen as an Object Selection Parameter. The reality is that although the myotubes are specifically labeled versus myoblasts, the asymmetrical long shape and inhomogeneous staining (dark holes over the nuclei) prevent any of the current available object segmentation methods from being able to separate and distinguish myotubes when they get densely crowded together. When there are a lot of myotubes in the field (e.g., in sample images which were untreated with nifedipine) individual myotubes are hard to resolve from each other and the branched appearance of irresolvable myotubes adjacent to each other necessitated a lower minimum LWR. Thus, an LWR of **1.25** was chosen. You can choose larger values for LWR for less crowded fields.

### Channel 2 Member Identification

The nuclei in Channel 2 are small in the 5x example images. This caused individual nuclei in multi-nucleated myotubes to appear together and unresolved. Segmentation based on the equidistance between peaks method enabled the individual nuclei to be separated and counted. Thus, the **V3_MemberSegmentationCh2** Assay Parameter was empirically found to work well at a setting of **–3**.

## Quantitation Results

The following figures show the ability of the Morphology Explorer BioApplication to quantitate the nifedipine inhibition of myotube formation on numerous levels. Thus clear differences are seen between control and nifedipine treatment in the number of myotubes formed (Figure 50), their morphology (Figure 51), and their skeletal myosin content (Figure 52).

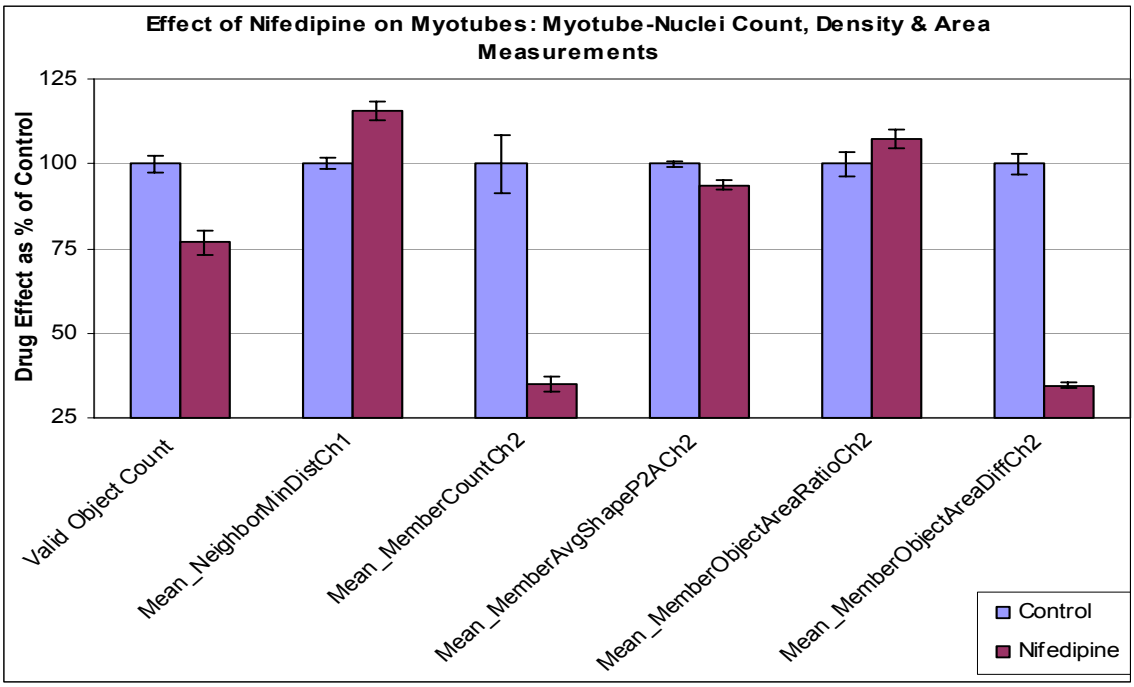



***Figure 50.*** *The inhibition of myotube formation is seen in the lower percentage of objects identified (**ValidObjectCount**) and larger spacing between myotubes identified (**Mean_NeighborMinDistCh1**) in the nifedipine treated wells.*

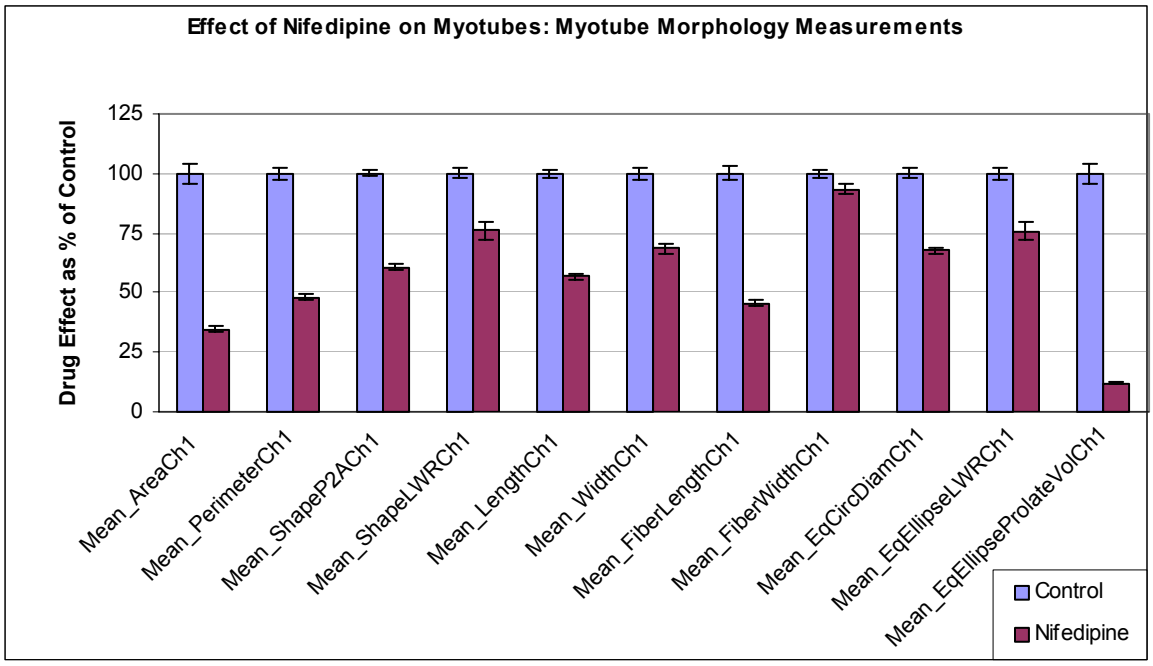



***Figure 51.*** *The myotubes that still form and are identified after nifedipine treatment are smaller. This is seen in the fewer nuclei per myotube (**Mean_MemberCountCh2**) in the previous plot, the difference between nuclear and myotube area (**Mean_MemberObjectAreaDiffCh2**), and all the different Channel 1 object morphology measurements.*

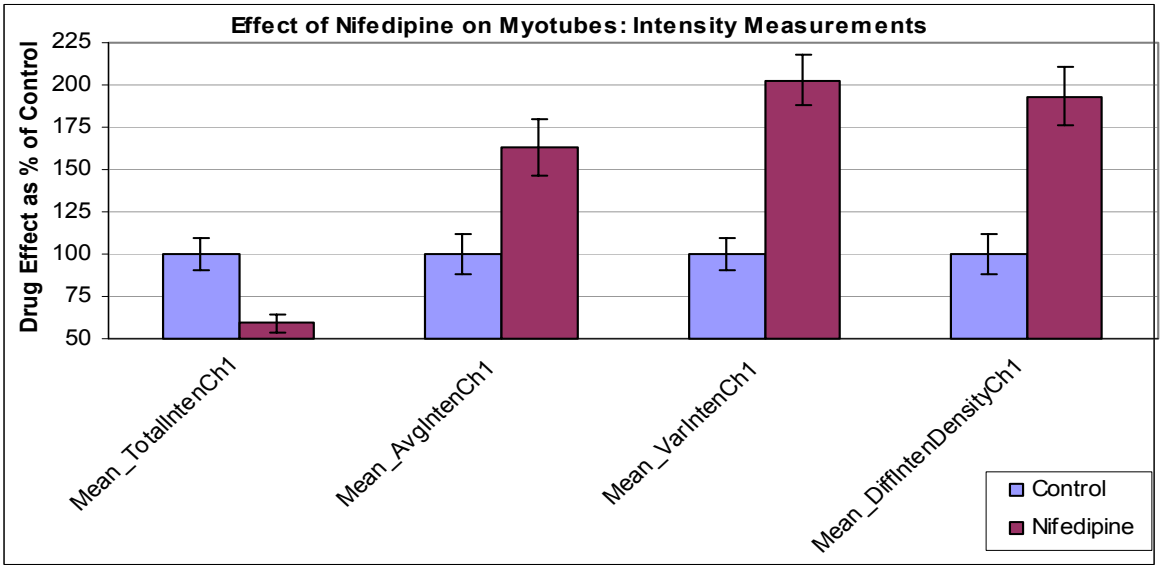



***Figure 52.*** *In addition, the myotubes that still form under nifedipine treatment have less skeletal muscle myosin as seen in the lower **Mean_TotalIntenCh1** measurement. The reduced size results in a higher **Mean_AvgIntenCh1** as well.*

# Example 2: Cytoskeletal Rearrangement

## Description of Biology and Assay

In this experiment, the NIH 3T3 cells are used to demonstrate various types of cytoskeletal patterns within the cell. In addition to control conditions, the cells are treated with two different compounds that elicit changes in the intracellular structure or shape of the cell to give a broad representation of images you might be interested in assessing. Of the three major types of cytoskeletal structures (microfilaments, intermediate filaments, and microtubules), two of them (microfilaments and microtubules) are simultaneously labeled in the cells of this experiment. The experiment was done as a 4-channel assay using a variety of intracellular and cytoskeletal markers, and representative images acquired on the Cellomics HCS Reader are shown in Figure 9 and Figure 42.

Channel 1 uses the whole cell stain CMFDA. CMFDA tends to stain the nucleus rather brightly and the rest of the cytoplasm with less intensity. Channel 2 uses Hoechst 33342 to label the nuclei. F-actin is the major component of microfilaments, and Channel 3 uses Rhodamine-Phalloidin to label the cell's F-actin filaments. The actin filaments in untreated cells typically have two types of patterns: stress fibers which are long parallel fibers stretching across the cell and cortical actin filaments, which appear as a line adjacent to the cell's edge; these structures change dramatically when treated with various drugs. Channel 4 is used to visualize tubulin, which is the major component of microtubules. An anti-tubulin primary antibody is combined with a Cy5® conjugated secondary antibody to visualize microtubules within the cytoplasm as it changes with drug treatment. Microtubules usually radiate out from one or two discrete locations adjacent to the cell's nucleus. Table 30 is an overview of the experimental setup.

- ***Control cells:*** Nuclei and cytoplasm have a normal myoblast size and shape. Long fibers are seen for both Channel 3 (F-actin) and Channel 4 (microtubules). Microtubule fibers seem to radiate from a couple of foci near the nucleus. The actin has a lot of parallel fibers spanning the cell.
- ***Cytochalasin D treatment:*** Cytochalasin D depolymerizes F-actin. In the images, nuclei look normal but the cytoplasm takes on an altered star-like shape radiating from the nucleus, which can be visualized and measured in Channel 2 (CMFDA). NIH 3T3 cells treated with 2 µM cytochalasin D for 18 hours demonstrate F-actin depolymerization; the Channel 3 images show cells with condensing actin fibers. All long fibrous filaments are gone and only a bright speckled pattern remains.
- ***Taxol treatment****:* Taxol inhibits microtubule disassembly and promotes microtubule bundling. In the images, the cytoplasm has a somewhat rounded shape when treated with Taxol for 18 hours at a 10 µM concentration. In Channel 3, the cortical actin is more prominent and the actin seems to form an outer circle around the edge of the cell. Some parallel stress fibers are also seen inside the cell. Channel 4 shows the microtubules bundled within the cytoplasm. The microtubule striations are mostly intact but seem thicker and do not have the same pattern of radiating from the nuclei as in untreated cells.

| Channel | Cellular Target | Detection Strategy & Fluorophore | Fluorescence Emission Color |
|---|---|---|---|
| **Channel 1** | Whole cell volume | CMFDA | Green |
| **Channel 2** | Nuclei of all cells | DNA binding dye - Hoechst 33342 | Blue |
| **Channel 3** | F-actin | Rhodamine conjugated phalloidin | Red |
| **Channel 4** | Microtubules | Immunofluorescence against tubulin<br>Cy5 conjugated secondary Ab | Far red |

***Table 30.*** *Cytoskeletal Rearrangement Experimental Design*

## Analysis Strategy and Key Items in Protocol

### Image Acquisition

In some cells, the cytoskeletal fibers and the nucleus may have different optimal focal planes. Since this assay is used to quantitate cytoskeletal fibers, the images of the cytoskeletal fibers need to be of the best quality. Thus, focusing was done on the F-actin image (Channel 3) and an offset was entered to enable the microtubule image to also be in focus. If focusing had instead been done on the nuclei (Channel 2) or the object (Channel 1), a focal plane may have been chosen where the cytoskeletal fibers in Channel 3 or Channel 4 may have been out of focus.

### Channel 1 Object Identification

Figure 49 has images of cells stained with CMFDA. A smooth staining is seen in the cytoplasmic region, and a very bright staining in the nuclear region where the cell's volume is maximal. The cells are often crowded and adjacent to each other. However, the CMFDA staining enables individual cells to be separated using the **Isodata Threshold** approach with the Current V3 Object Segmentation Intensity Method as demonstrated in Figure 19. For these images, a **ObjectSegmentationCh1** of **-100** was used and the default method for choosing the intensity increments was selected by setting the **ObjectSegmentationCh1** value to **0**. Smoothing with a **SmoothFactorCh1** value of **1** was seen to improve the object identification. Setting minimum thresholds for Average and Total Intensity and Object Area (as Object Selection Parameters) further help select objects and prevent spurious items from being selected.

### Channel 2 Member Identification

Member (nuclei) identification in Channel 2 was straightforward and only necessitated choosing an appropriate minimum area (**20** $\mu m^2$) as an Object Selection Parameter, and an appropriate **Isodata Threshold**. These were both empirically determined from example images.

### Channel 3 Fiber Identification

For the identification of cytoskeletal fibers (Figure 42), the **SpotFiberSizeCh*N*** Assay Parameter works best when set to a value of **1**. In the example images, with an **Isodata Threshold** offset of **–0.9**, F-actin fibers were empirically defined by the Object Selection Parameters to have an area of more than **4** $\mu m^2$ and a LWR of greater than **2** (i.e., a fiber's length had to be more than twice its width). Changing these values cause fibers to be identified with less or more stringency.

Since this assay is about fiber analysis, fiber alignment and persistence of patterns are of interest. Thus, entering a value of 1 turned on the **FiberAlign2AnalysisCh3**. Note that turning this analysis on causes the application to run more slowly.

## Channel 4 Fiber Identification

Similar principles were followed in Channel 4 for the identification and quantitation of microtubules as for F-actin fibers in Channel 3. The **SpotFiberSizeCh4** Assay Parameter was set to a value of **1**. With an **Isodata Threshold** of **–0.8**, microtubules were empirically defined by the Object Selection Parameters to have an area of more than **4** $\mu m^2$. Since microtubules tend to radiate from a focal point rather than be parallel like F-actin, a splay of radiating microtubules could be identified as a single fiber and thus collectively have a lower aspect ratio than an individual fiber. Thus, the minimum LWR was set to a value of **1**. Changing these values causes microtubules to be identified with less or more stringency. To enable quantitation of microtubule alignment and persistence of patterns, **FiberAlign2AnalysisCh4** was turned on by setting this Assay Parameter to a value of **1**.

## Quantitation Results

The effect of the two drugs on cytoskeletal morphology is shown in four different morphological categories: whole cell morphology (Figure 53, nuclear morphology (Figure 54), F-Actin, and microtubules (shown in Figures 55 and 56).

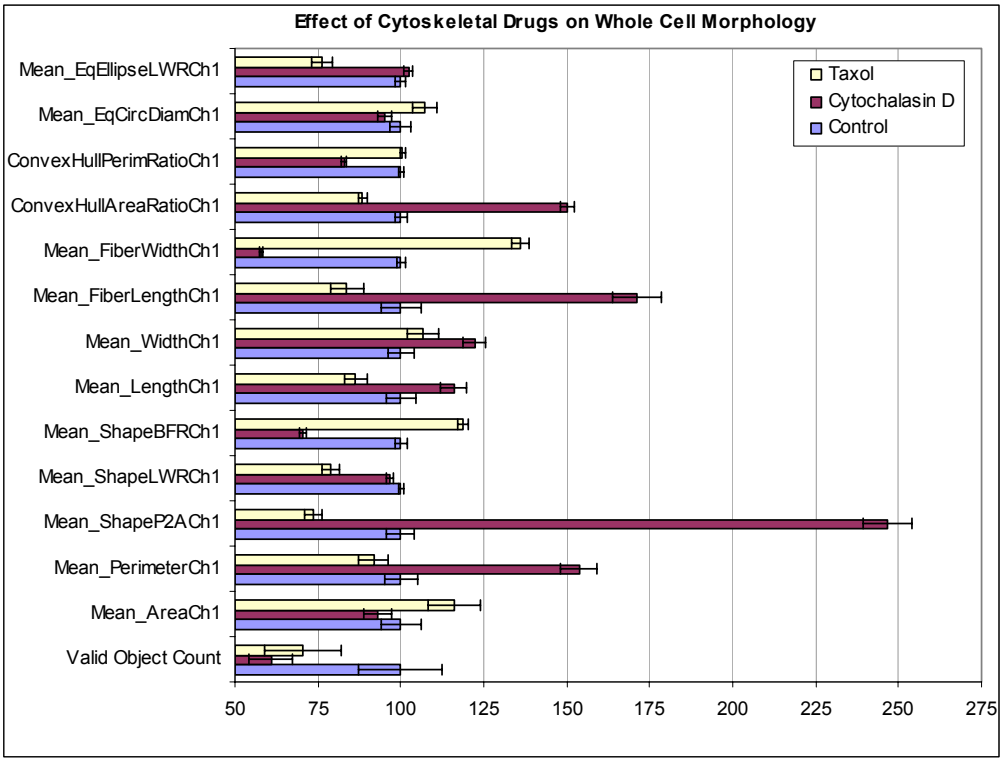



***Figure 53.*** *Effect of Cytoskeletal Drugs on Whole Cell Morphology: There are numerous differences in the whole cell morphology of cells treated with cytochalasin D and taxol versus control cells that can be measured. Some of the most striking differences are seen in the* ***ConvexHullAreaRatioCh1****,* ***Mean_FiberWidthCh1****,* ***Mean_FiberLengthCh1****,* ***Mean_ShapeBFRCh1****,* ***Mean_ShapeP2ACh1****, and the* ***Mean_PerimeterCh1****.*

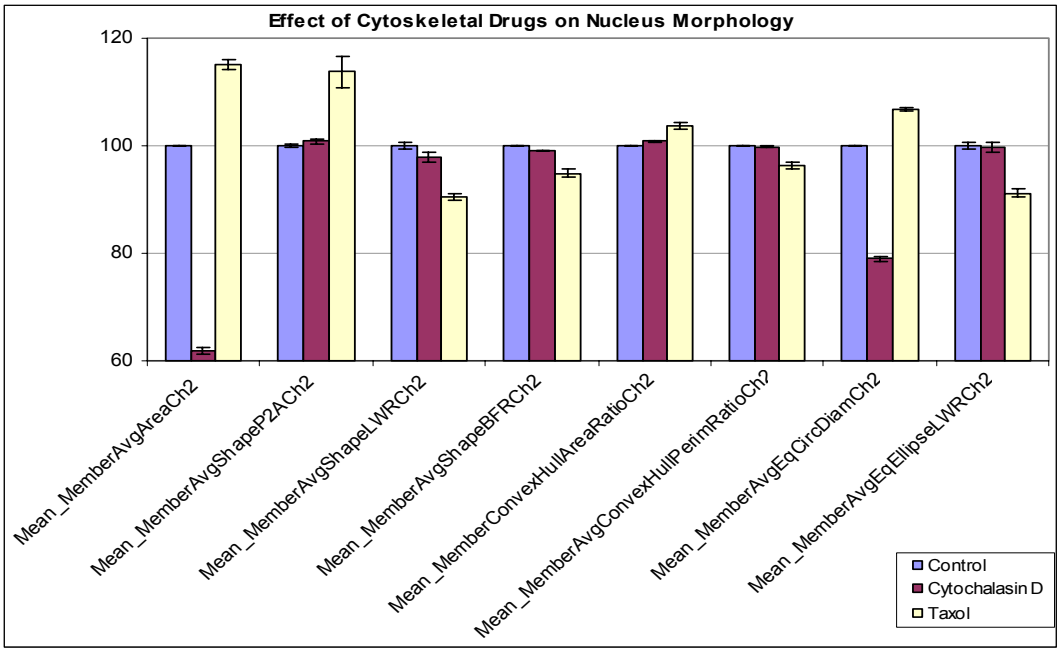



***Figure 54.*** *Effect of Cytoskeletal Drugs on Nucleus Morphology. Some differences between control and the drug treated cells were also seen for their nuclear morphology. The* ***Mean_AvgAreaCh2****,* ***Mean_MemberAvgShapeP2ACh2****, and the* ***Mean_MemberAvgEqCircDiamCh2*** *show the most dramatic effects.*

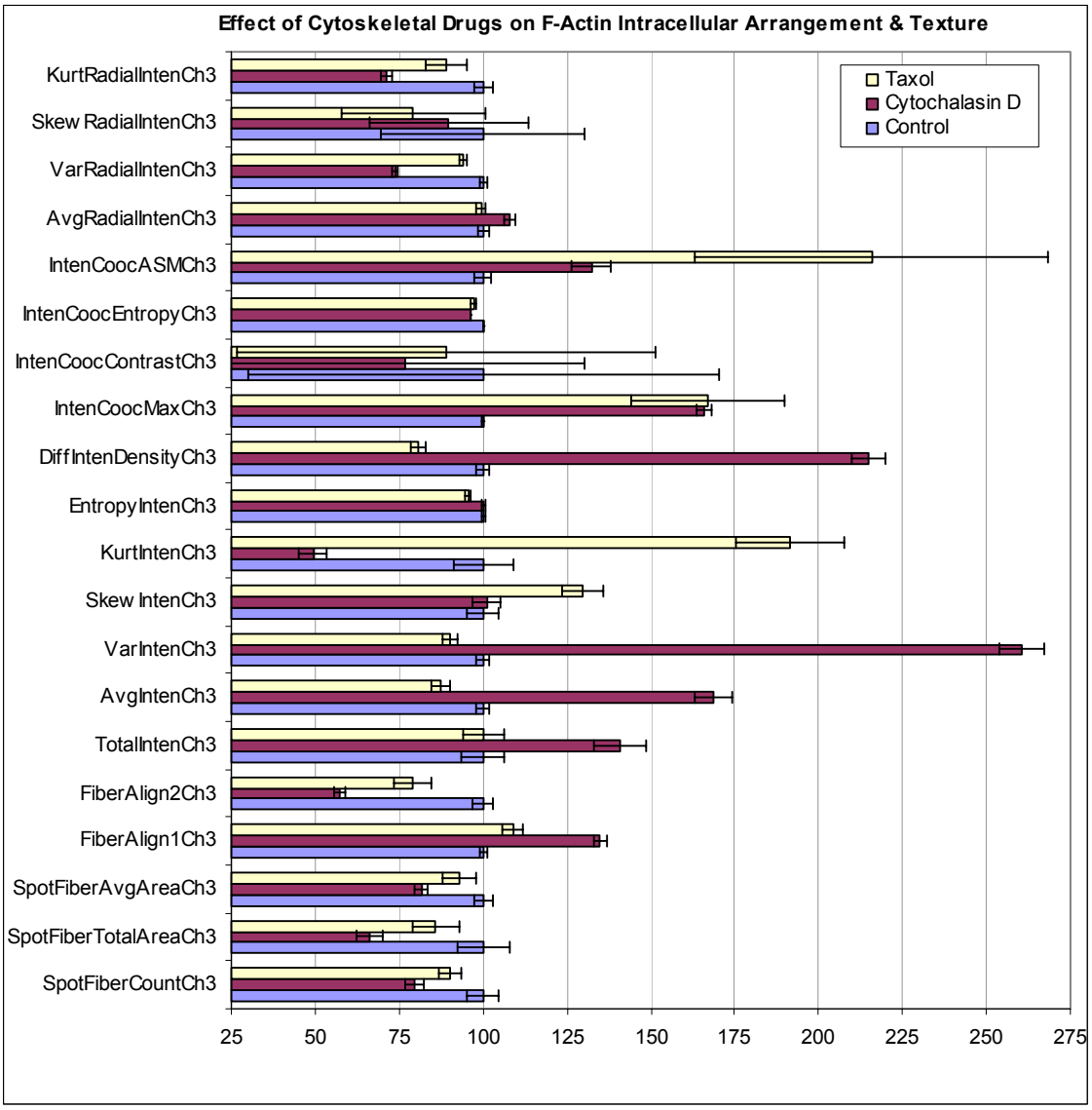



***Figure 55.*** *Effect of Cytoskeletal Drugs on F-Actin Intracellular Arrangement and Texture. A variety of intracellular arrangement, texture and location features in Channel 3 showed differences in F-Actin fibers from controls when treated with the two drugs.*

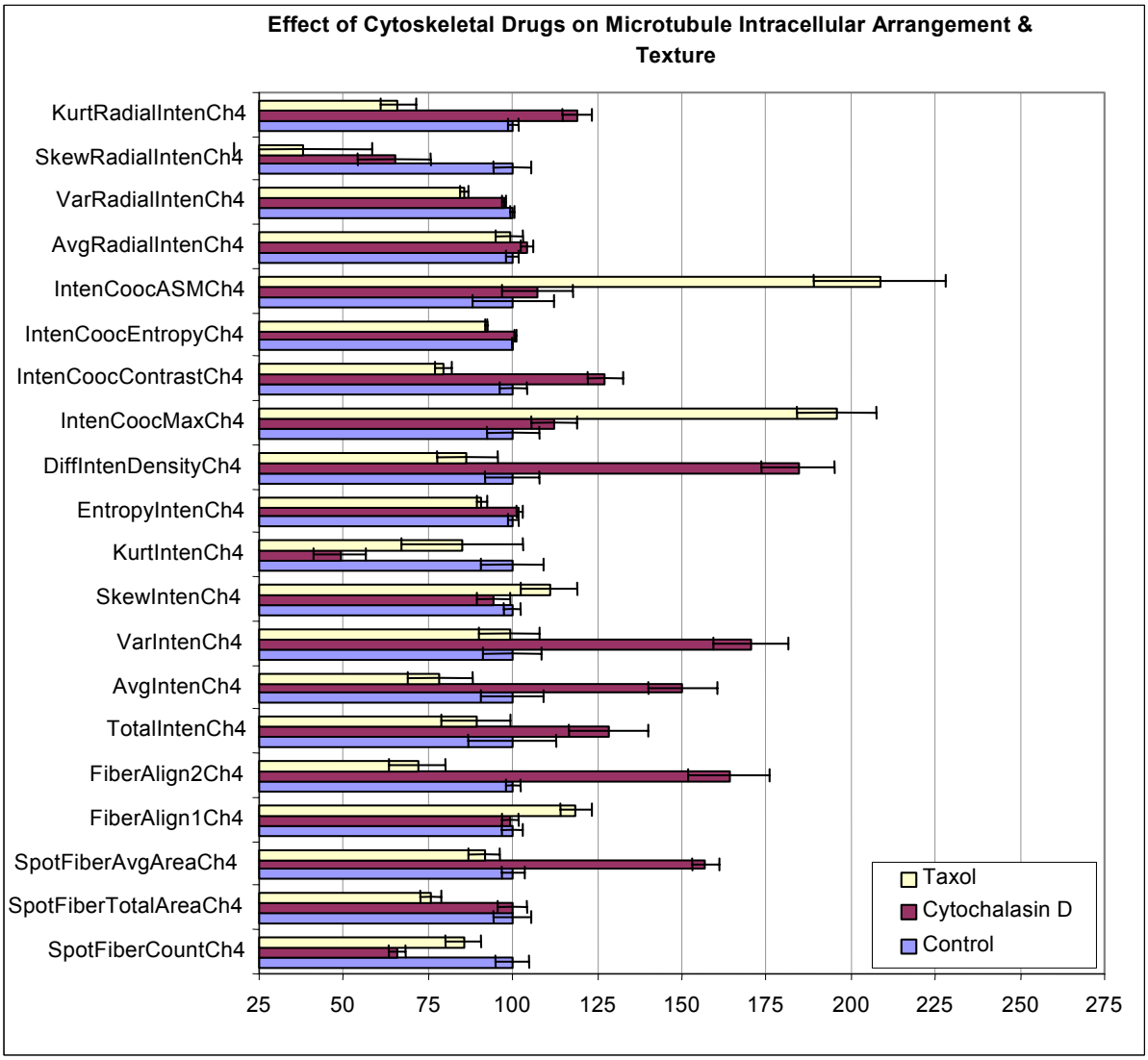



***Figure 56.*** *Effect of Cytoskeletal drugs on Microtubule Intracellular Arrangement and Texture. Several microtubule arrangement and texture parameters show a significant difference between control and drug treated wells.*

# Example 3: Colony Formation and Disassociation

## Description of Biology and Assay

Certain types of cells grow in tight groups or colonies rather than as individual cells. The need for close proximity may affect the cell's growth, cell cycle, specific protein expression, and appearance. In the experiment for this example, the MCF-7 human breast epithelial cell line was used. The cells normally grow in small clumps or colonies. The clumps are easily dislodged from the surface, but adhere to one another very strongly. When cells are treated with the drug Phorbol-12-myristate-12-acetate (PMA) at 2 µM concentration for 8 hours, the cells disassociate from one another while remaining attached to the surface of the plate (Vaaraniemi, et al. 1999). This is a simple two-channel assay where CMFDA is used in Channel 1 to identify the cell cytoplasm and boundary. The Channel 2 stain is Hoechst 33342, which labels the nuclei and is used for individual cell identification, easy colony counting, and cell separation. Note, instead of CMFDA, which was used as the Channel 1 object label as in the example images supplied with the application (Figure 10), rhodamine-phalloidin, which

labels cellular F-actin, can also be used as an object label and to define the extents of the cells and colonies (Figures 39 and 49). Table 31 is an overview of the experimental setup.

- ***Control cells:*** There are numerous cell clumps throughout the field, making it difficult to distinguish one cell from another in Channel 1 and difficult to find an individual cell. The cell boundaries are confined and mostly round in shape but the overall colony shape varies. Channel 2 allows easy counting of nuclei.
- ***Drug treated cells (PMA):*** Cells have disassociated from one another and cell membrane boundaries are now easy to define with fewer cells touching.

| Channel | Cellular Target | Detection Strategy & Fluorophore | Fluorescence Emission Color |
|---|---|---|---|
| **Channel 1** | Whole cell volume | CMFDA | Green |
| **Channel 2** | Nuclei of all cells | DNA binding dye - Hoechst 33342 | Blue |

***Table 31.*** *Colony Formation and Disassociation Experimental Design*

## Analysis Strategy and Key Items in Protocol

### Channel 1 Object Identification

The goal in object identification in this assay is to be able to resolve and distinguish individual colonies from each other. This was made possible with the CMFDA stain and using the **ObjectSegmentationCh1** geometric method. For the example images, the **ObjectSegmentationCh1** value of 5 was used. Setting Object Selection Parameter minimum thresholds for average and total intensity and object area (**40** $\mu m^2$) further helped select objects, which were either colonies or individual cells in the PMA treated wells, and prevented spurious items from being selected.

### Channel 2 Member Identification

For the quantitation of individual cells in colonies, accurate identification and counting of the individual nuclei is needed. This necessitated segmentation to be used to resolve individual nuclei in colonies. Segmentation based on the equidistance between peaks method enabled the individual nuclei to be resolved and counted, and the **V3_MemberSegmentationCh2** Assay Parameter was empirically found to work well at a setting of **–4**. A **MemberSmoothFactorCh2** value of **2** was found to further help member identification. Additional Object Selection Parameters for Channel 2 were not needed and their limits were set to be broad. Since colony analysis was being done, the **ColonyAnalysisCh2** Assay Parameter was set to a value of **1**; this turns on the calculation of the number of interior and exterior cells in each colony (Figure 39).

## Quantitation Results

Three categories of results are plotted for the colony disassociation by PMA: Number of Objects (colonies or individual cells) (Figure 57); Object (colony or individual cells) Morphology (Figure 58); and Object Channel 1 Intensity Measurements (Figure 59).

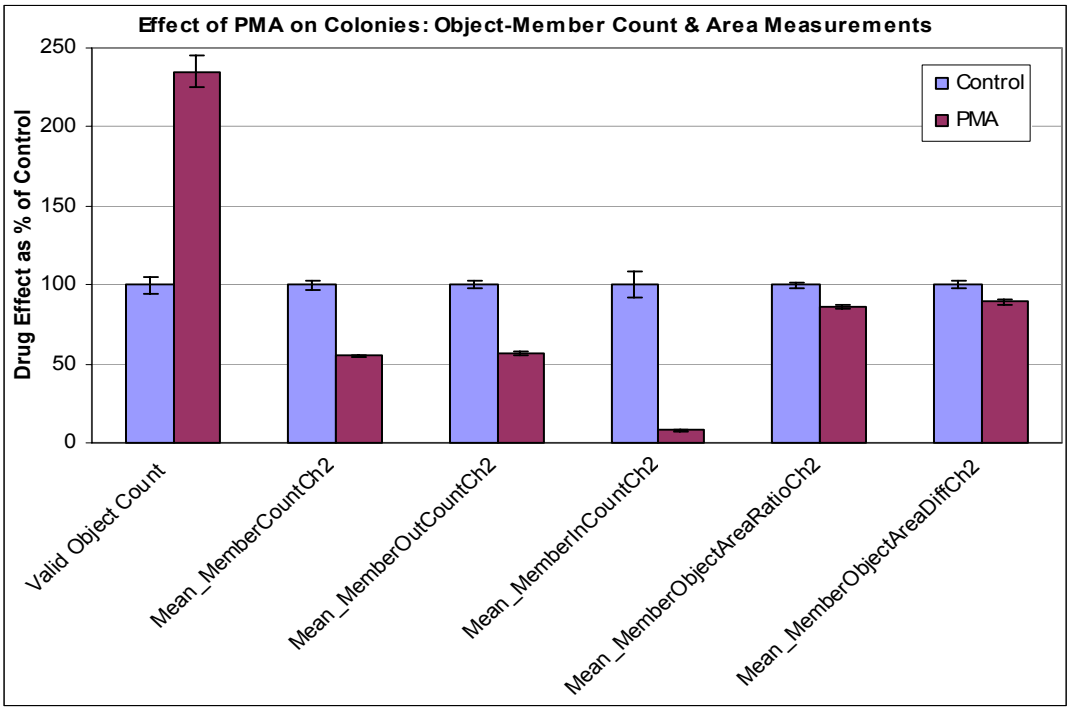



***Figure 57.*** *Effect of PMA on Colonies: Object Member Count & Area Measurements. With PMA, as colony disassociation occurs, the number of objects increase but the number of members per object decrease. Note the large differences in* ***Valid Object Count****,* ***Mean_MemberCountCh2****, and* ***Mean_MemberOutCountCh2****.*

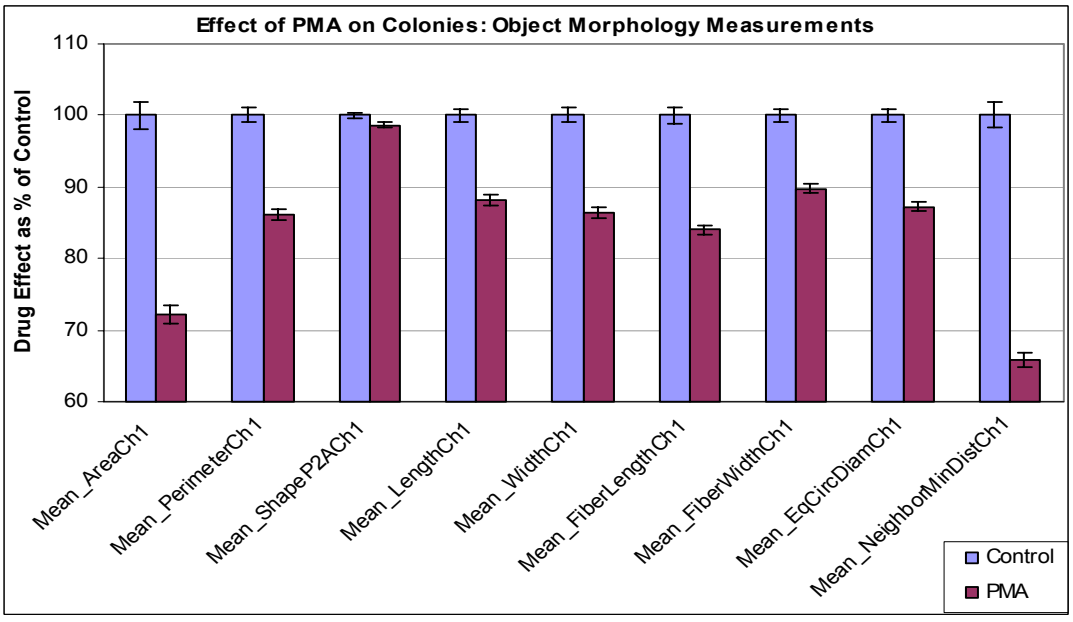



***Figure 58.*** *Effect of PMA on Colonies: Object Morphology Measurements. Most of the object's morphological measurements are significantly decreased with PMA treatment as colony disassociation occurs. The only exception to this effect is in the* ***Mean_ShapeP2ACh1****, where colonies large and small, as well as individual cells, have similar P2A shapes.*

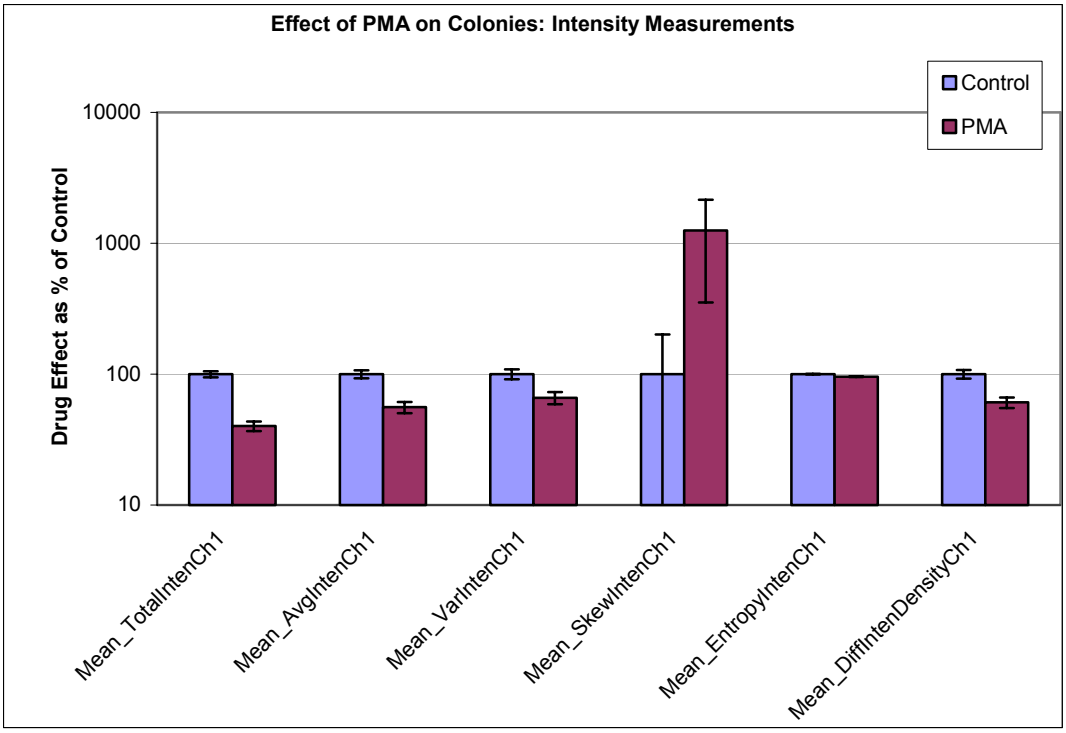



***Figure 59.*** *Effect of PMA on Colonies: Intensity Measurements. Colony disassociation due to PMA also results in a decrease in the Channel 1 label intensity.*

# Example 4: Cell Spreading

## Description of Biology and Assay

In the case of cell spreading, it can be quantified by directly measuring the size of fluorescently labeled cells. The experiment is a two-channel assay and is demonstrated in Figure 11. In Channel 1, Rhodamine-Phalloidin is used to label the entire cell cytoplasm for area measurements. Channel 2 identifies the nuclear region of the cells using Hoechst 33342. Untreated PC-3 cells are spread out nicely on the surface of the type 1 collagen matrix plate and serve as a control for the experiment. The treated cells are dosed with Cytochalasin D for three hours immediately after plating in order to demonstrate inhibition of cell spreading. Table 32 is an overview of the experimental setup.

- ***Control cells****:* Cells are spread out on the plate and their intracellular actin can be visualized. Rhodamine-Phalloidin binding to actin in the PC-3 cell line forms a boundary around the outside of the cytoplasm, which often looks like a bright ring.
- ***Drug treated cells (Cytochalasin D):*** Cells treated with 1 µM cytochalasin D for 3 hours resemble bright clumps in Channel 1 and there are no visible intracellular structures (actin); the cell area is also significantly decreased.

| Channel | Cellular Target | Detection Strategy & Fluorophore | Fluorescence Emission Color |
|---|---|---|---|
| **Channel 1** | Whole cell marker | Rhodamine conjugated phalloidin | Red |
| **Channel 2** | Nuclei of all cells | DNA binding dye - Hoechst 33342 | Blue |

***Table 32.*** *Cell Spreading Experimental Design*

## Analysis Strategy and Key Items in Protocol

### Channel 1 Object Identification

The goal of object identification in this assay is to be able to resolve and distinguish individual cells from each other. This was made possible with the phalloidin stain and using the simple object segmentation method based on the typical radius of objects. For the example images, the geometric method of **ObjectSegmentationCh1** was used at a value of **15**; therefore, a typical object radius was ~15 µm. This is an upper limit for spread cells and thus does not affect spreading, inhibited cells which are smaller. Object identification was further enhanced by smoothing the phalloidin label by setting the **SmoothFactorCh1** value to **2** and setting an Object Selection Parameter minimum threshold for the object area (**300** $\mu m^2$).

### Channel 2 Member Identification

Member (nuclei) identification in Channel 2 was straightforward and only needed smoothing (**MemberSmoothFactorCh2** value set to **2**) to improve the result.

## Quantitation Results

Cytochalasin D inhibited cell spreading. This is seen in a reduction in a wide range of object morphology features and intensity measurements for both Channel 1 and Channel 2 shown in Figure 60 and Figure 61.

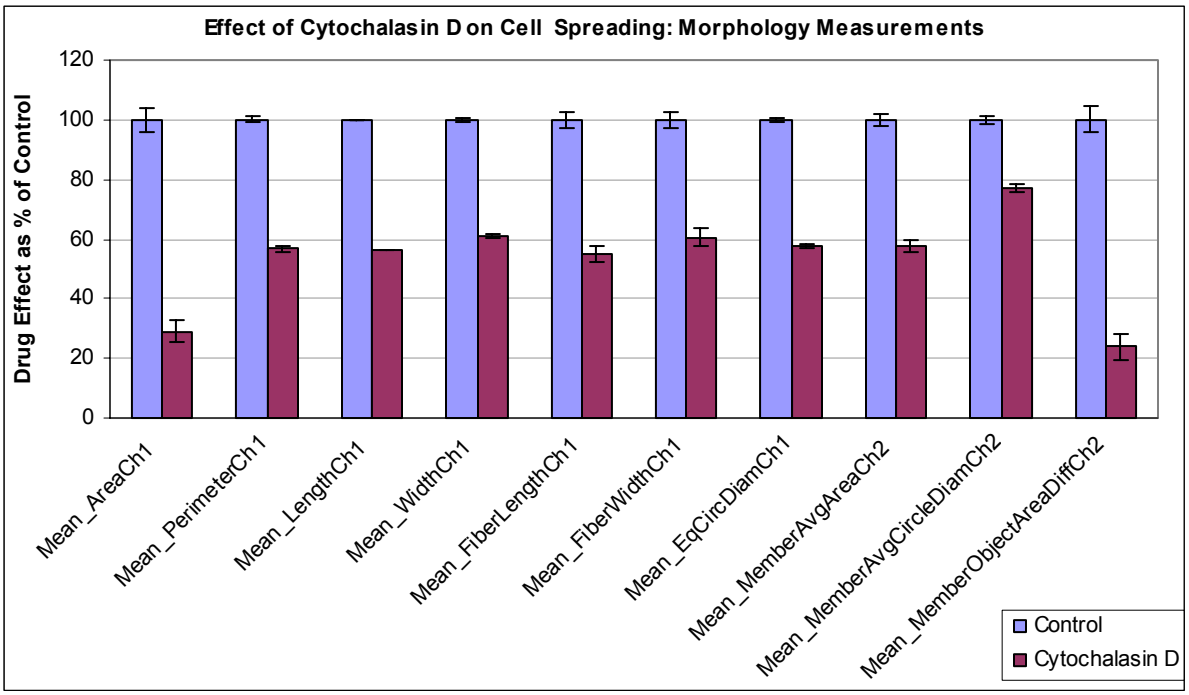



***Figure 60.*** *Effect of Cytochalasin D on Cell Spreading: Morphology Measurements. Numerous object morphology measurements were reduced with cytochalasin D treatment.*

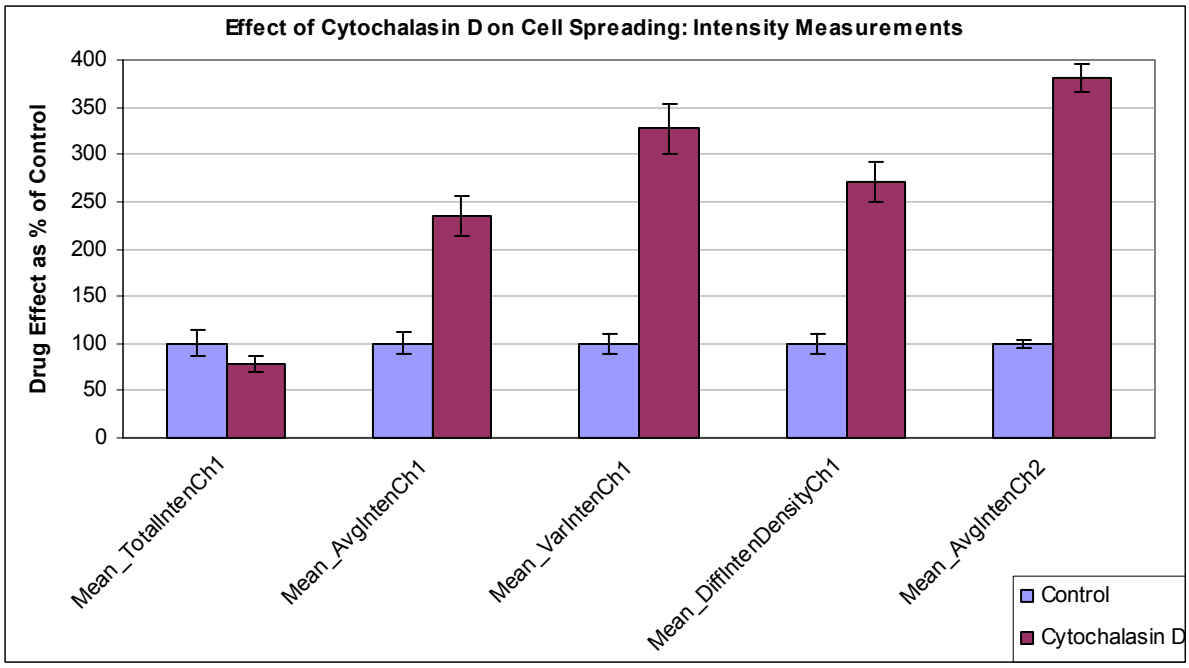



***Figure 61.*** *Effect of Cytochalasin D on Cell Spreading: Intensity Measurements. The intensity measurements in both Channel 1 and Channel 2 were significantly increased when cells were treated with cytochalasin D. The lower* ***Mean_TotalIntenCh1*** *value for the cytochalasin D treated cells is indicative of the reduction in cell spreading.*

# Example 5: Properties of Discrete Intracellular Objects

## Description of Biology and Assay

The Morphology Explorer BioApplication can also quantitate the intracellular distribution and properties of discrete objects. The example of such objects provided are fluorescently labeled endosomes in HeLa cells containing internalized fluorescent EGF. The location of such internalized fluorescent macromolecules can change during the intracellular trafficking process. For the demonstration of this capability of the BioApplication, the intracellular distribution and associated properties of internalized fluorescent EGF inside HeLa cells were analyzed and validated. The experiment is a three-channel assay that utilizes CMFDA to denote the cytoplasm, Hoechst 33342 to label the nuclei, and Alexa Fluor 555-conjugated EGF to visualize internalized EGF in endosomes as small dots within the cytoplasm (Figure 12 and Figure 42). During internalization, the fluorescent EGF goes from the plasma membrane to sorting endosomes, which are peripherally distributed to late endosomes and then lysosomes, which are more centrally located. In the 20x example images (Figure 12 and Figure 42), the cells were incubated with fluorescent EGF for 35 minutes, which would mainly cause a late endosomal and lysosomal staining pattern. Table 33 is an overview of the experimental setup.

- ***Control cells:*** Cells on ice were incubated with fluorescent EGF; no internalized fluorescent EGF is seen in Channel 3.
- ***Treated cells (EGF):*** A time course of EGF internalization was conducted. In Channel 3, the internalized Alexa Fluor 555 EGF (1 µg/mL) after 35 minutes can be visualized as fluorescent punctate spots in the cytoplasm. Channel 1 and Channel 2 look no different from control cells.

| Channel | Cellular Target | Detection Strategy & Fluorophore | Fluorescence Emission Color |
|---|---|---|---|
| **Channel 1** | Whole cell volume | CMFDA | Green |
| **Channel 2** | Nuclei of all cells | DNA binding dye - Hoechst 33342 | Blue |
| **Channel 3** | Internalized EGF in endosomes | Alexa Fluor 555 conjugated EGF | Red |

***Table 33.*** *Discrete Intercellular Object Experimental Design.*

## Analysis Strategy and Key Items in Protocol

### Channel 1 Object Identification

The goal in object identification in this assay is to be able to resolve and distinguish individual cells from each other. Since one of the features of interest is the intracellular location of the internalized EGF, a partial identification of an object, such as from a truncated cell at the border, would give an erroneous result; thus the **RejectBorderObjectsCh1** Assay Parameter was set to **1** so that objects touching the border were not included in the analysis. Similarly, intracellular EGF location measurements will be erroneous if the object is defined as containing more than one cell; individual cells need to be resolved and identified as objects. This was made possible with the CMFDA stain and using the Current V3 Object Segmentation Intensity Method (Figure 19). For the example images, the **ObjectSegmentationCh1** intensity method was used. The **ObjectSegmentationCh1** value of **-200** was found to segment the cells most efficiently. Setting Object Selection Parameter minimum thresholds for Average and Total Intensity and Object Area (**500** $\mu m^2$) further helped object definition and selection.

### Channel 3 Spot Identification

For the identification of fluorescent EGF in endosomes in Channel 3, a **Fixed Threshold** was used. This was because a histogram-based threshold method would give spurious results when there was not much internalized EGF in cells, and the only fluorescence was from background. Thus, the appropriate **Fixed Threshold** needs to be set every time a new set of samples are made and imaged on the ArrayScan HCS Reader. Since the fluorescent endosomes are small spots, a **SpotFiberSizeCh3** value of **1** worked. In the event that there were any underlying persistent patterns, the **FiberAlign2AnalysisCh3** Assay Parameter was also turned on. This further slowed down the application. Although not included in the supplied protocol, occasionally increasing the **ObjectMaskModifierCh3** by one or a few pixels helps further define the cell's boundaries and enhances the cell boundaries identified in Channel 1.

## Quantitation Results

As EGF internalized over the course of 35 minutes at 37 °C, the number of spots inside cells (fluorescently labeled and detected endosomes) and their areas increased. Two categories of plots are reproduced below comparing the internalized EGF (incubation at 37 °C) to the minimized internalization of EGF (incubation on ice). There is a clear difference between the spot count, area, and texture between the two conditions in Figure 62. Figure 63 shows a much less dramatic change in the radial intensity distribution of the spot location, with the more dramatic changes seen at the higher moments of the radial intensity distribution.

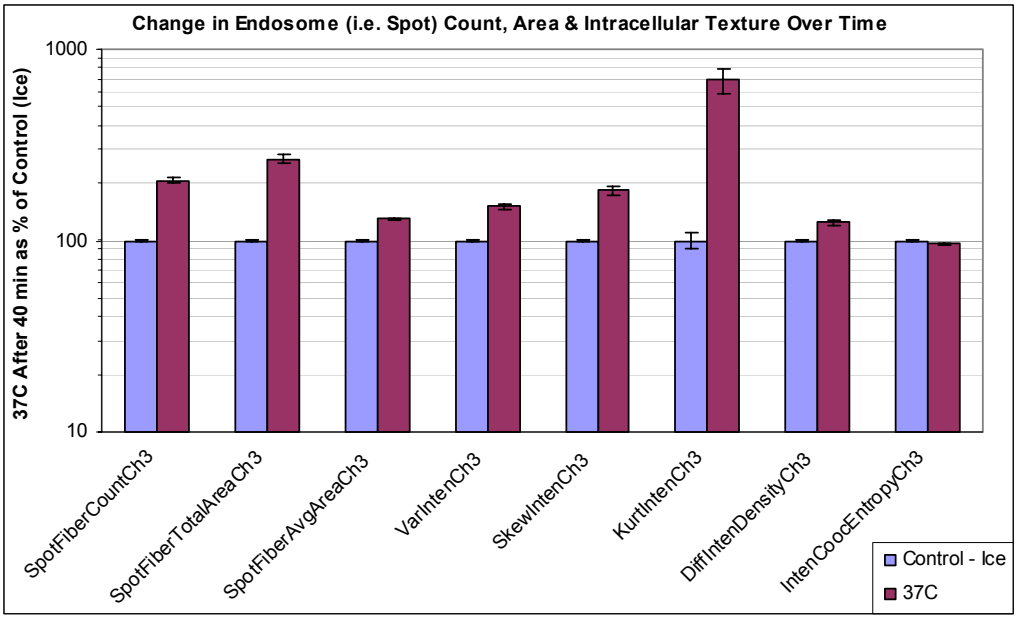



***Figure 62.*** *Change in Endosome (i.e., Spot) Count, Area & Intracellular Texture Over Time. Numerous parameters related to the spot count, area, and texture are increased when EGF is allowed to internalize into the cell.*

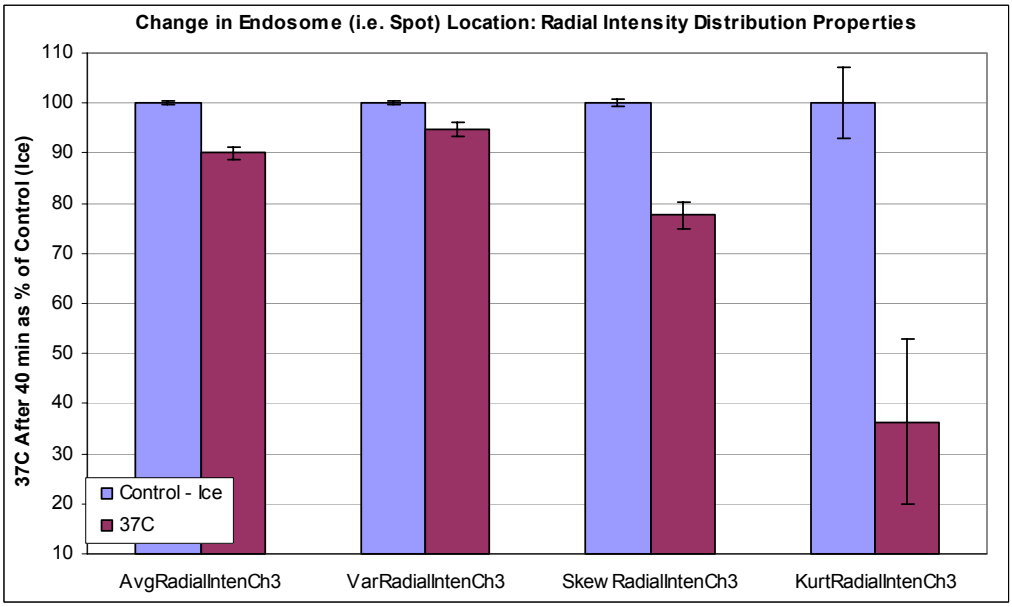



***Figure 63.*** *Change in Endosome (i.e., Spot) Location: Radial Intensity Distribution Properties. A subtle change in the intracellular distribution and location is reflected by changes in the* ***SkewRadialIntenChN*** *and* ***KurtRadialIntenChN*** *of the radial intensity.*

# Example 6: Mixed Neuronal Population

## Description of Biology and Assay

The images for this particular biology include a mixed population of neuronal cells (Figure 13 and Figure 34). Often a primary culture has a mixture of cell types; distinguishing between the cell types and correlating with nearby cells is something the Morphology Explorer BioApplication can do. In Channel 1, all cells are labeled with a cytoplasmic neuronal antibody (Alexa Fluor 647 secondary), which acts as the primary cell mask. In Channel 2, all nuclei are labeled with Hoechst 33342. The Channel 3 and Channel 4 labels are CMFDA and CMTMR respectively; both whole cell stains are used to label subpopulations of the cells. Cells are only labeled with CMFDA or CMTMR but not both. In addition to the overall quantitation of neurite outgrowth from the different cells, these specific subpopulation stains will be used to quantitate cell spacing and proximity between similar and dissimilar cells. Table 34 is an overview of the experimental setup.

- ***Control cells:*** Very little neurite outgrowth is seen with very few short processes extending from neuronal cells in Channel 1.
- ***Treated cells (NGF):*** In NS-1 cells treated with 200 ng/mL NGF for 48 hours, neurite outgrowth has occurred and neurites are easily visible in Channel 1 extending from the cells.

| Channel | Cellular Target | Detection Strategy & Fluorophore | Fluorescence Emission Color |
|---|---|---|---|
| **Channel 1** | Neurons and neurites | Immunofluorescence against neuronal-specific protein<br>Alexa Fluor 647 conjugated secondary Ab | Far red |
| **Channel 2** | Nuclei of all cells | DNA binding dye - Hoechst 33342 | Blue |
| **Channel 3** | Subset of neurons | Cell volume stain - CMFDA | Green |
| **Channel 4** | Subset of neurons | Cell volume stain - CMTMR | Red |

***Table 34.*** *Mixed Neuronal Population Experimental Design.*

## Analysis Strategy and Key Items in Protocol

### Channel 1 Object Identification

For the example 5x images, the neuronal cell bodies were identified by an **Isodata Threshold** value of **–0.7**, applying the Current V3 Object Segmentation based on the Geometric Method (**ObjectSegmentationCh1** Assay Parameter was empirically found to work at a setting of **4**), and Object Selection Parameter minimum thresholds for Average and Total Intensity and Object Area (**250** $\mu m^2$). Since neurites needed to be identified and analyzed, the **ProcessIdentificationCh1** Assay Parameter was turned on and found to work best for these images at a value of **–0.999** as the neurites were much dimmer than the cell bodies. Tweaking this Assay Parameter and the **Isodata Threshold** affected how well the neurites were identified.

### Channel 3 and Channel 4 Object Identification

The different subsets of objects in Channel 3 and Channel 4 that contain the CMFDA and CMTMR labels need to be identified to enable the proximity measurements between similar and dissimilar objects (Figure 35). In these channels, the **None** option for the intensity thresholds were chosen so that spot/fiber analysis was not done and the application ran faster. In the Protocol Interactive View, the average intensities in Channel 3 and in Channel 4 were

examined for the individual objects (**AvgIntenCh3** and **AvgIntenCh4**). A population of cells had low average intensity, showing none or basal-level staining for that label, whereas the cells with positive staining had a high average intensity. Thus for each of the stains, there was a bi-modal distribution for their average intensity with the mean in the middle with a large standard deviation (standard deviation was the size of the mean). To define a responder for each stain, the strategy adopted was to make the lower threshold 0 and the upper threshold above the lower mode of the bi-modal distribution. Since Reference Wells were being used, the **AvgIntenLevelLowCh3_CC** and **AvgIntenLevelLowCh4_CC** values were kept at **1** and the **AvgIntenLevelHighCh3_CC** and **AvgIntenLevelHighCh4_CC** values were set to **–0.5**. This means that the upper threshold is equivalent to the mean of the distribution minus half the standard deviation, i.e., effectively placing the upper threshold to the left of the mean.

## Quantitation Results

Three categories of plots are displayed for the neuron use case biology. Figure 64 shows the changes in the cell body morphology and dimensions due to addition of NGF. Figure 65 shows the increase in neurite outgrowth (measured by neurite number and lengths) due to NGF. Figure 66 demonstrates the ability to do proximity measurements on similarly and dissimilarly labeled cells (i.e., cells labeled with CMFDA and CMTMR); for the proximity measurements, the point is that these measures can be made, and the differences between control and NGF conditions are not significant and occurred purely by chance.

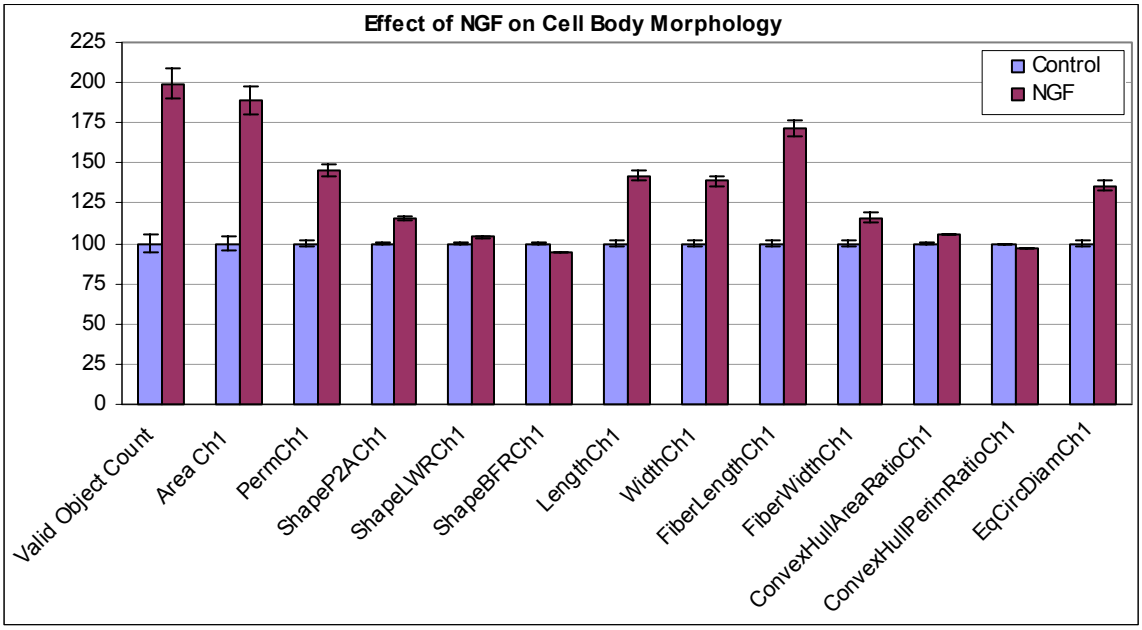



***Figure 64.*** *Effect of NGF on Cell Body Morphology. NGF has a significant effect on numerous Channel 1 features measuring cell body morphology of the neurons.* ***ValidObjectCount****,* ***AreaCh1****,* ***PerimeterCh1****,* ***LengthCh1****,* ***WidthCh1****,* ***FiberLengthCh1****, and* ***EqCircDiamCh1*** *are the most dramatic.*

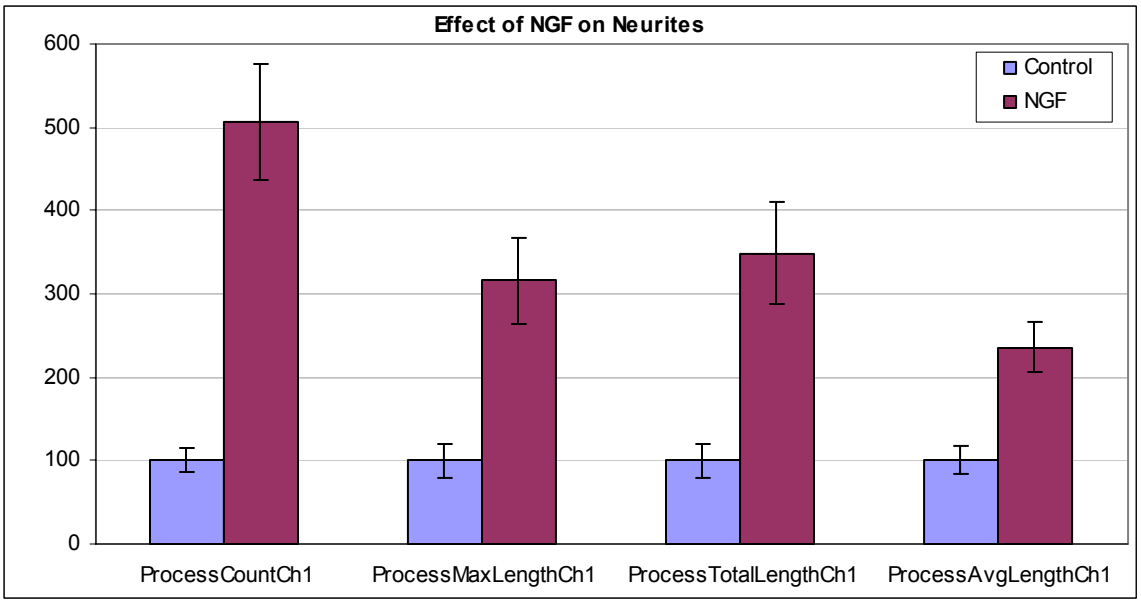



***Figure 65.*** *Effect of NGF on Neurites. All output features defining process attributes are significantly increased with NGF treatment.*

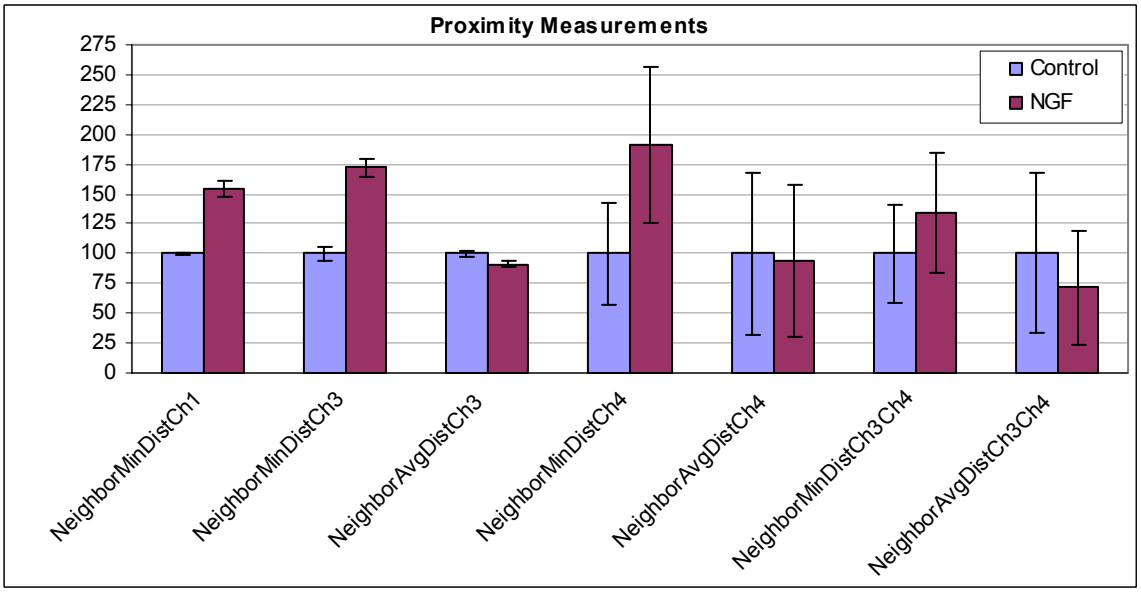



***Figure 66.*** *Proximity Measurements. Demonstrates the ability for the application to quantitate similar and dissimilar cells in a population in Channel 3 and Channel 4.*

# Event Definition Using the BioApplication Event Wizard

The BioApplication Event Wizard is a software tool that is designed to allow entry of 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 BioApplication Event Wizard enables entering, reading and modifying Event Definitions. 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 Morphology Explorer BioApplication. This section provides a detailed description of the operation of the BioApplication Event Wizard. The Wizard should only be used after the Morphology Explorer V3 BioApplication has been installed on your computer.

Definition of Events requires that the following steps be followed, in the order listed. Do not run the Scan software application and the BioApplication Event Wizard at the same time.


Note that the Event Definition Assay Parameters should not be modified via the Scan software application. Events should only be defined and edited via the BioApplication Event Wizard.

**STEP I**

1) Create a protocol using the Cellomics Scan software application without defining Events. Set optimized Assay Parameter values (upper and lower limits) for detection of responder cells associated with Cell Features to be used for Event Definition.
2) Save protocol.
3) Close the Cellomics Scan software application.

**STEP II**

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

**STEP III**

1) Restart the Cellomics Scan software application and open the protocol saved in Step II (Event Definitions will appear as numeric strings in the Assay Parameter window)
2) Scan the assay plate.
3) 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 reanalyze previously acquired images using modified Event Definitions.

## Features

The following features are included in the BioApplication Event Wizard:

- Can be used with any upgraded V3 Morphology Explorer 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 Morphology Explorer

### Before Running the Event Wizard...

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

### Starting the BioApplication Event Wizard


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

**To start the BioApplication Event Wizard,**

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


**-or-**

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

For vHCS Discovery Toolbox computers: From the Windows **Start** menu, select **Programs** > **Cellomics** > **vHCS Scan** > **BioApplication Event Wizard**.

## Protocol Selection and Loading

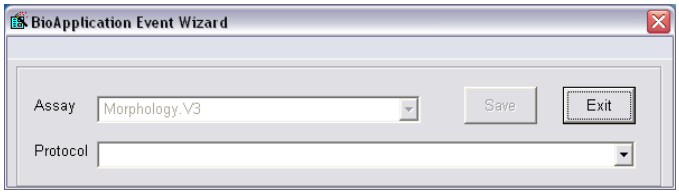
The first window that is displayed (see below) allows selection of any Morphology Explorer Assay Protocol. Note that Event Definitions can only be specified for pre-existing Assay Protocols.

### To select an Assay Protocol,

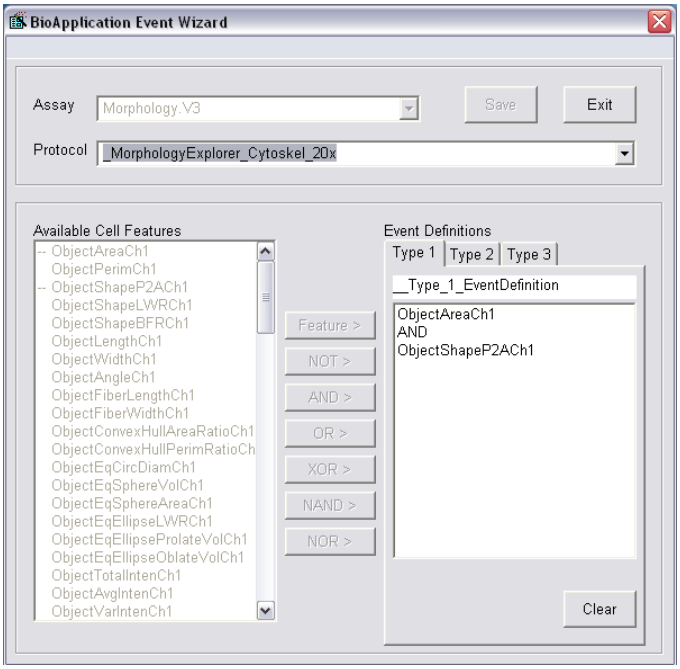
**1)** From the **Assay** drop-down menu, select **Morphology.V3**

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

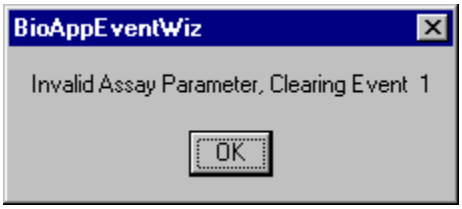
**3)** Select the desired Assay Protocol from the list.




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




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



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

## Defining Events

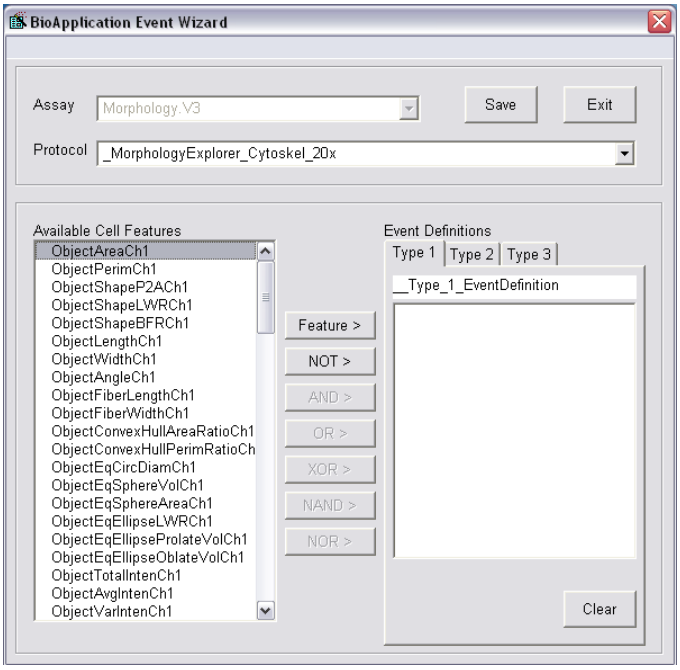
This section describes the steps involved in defining individual Events in a Morphology Explorer Assay Protocol. Note that logical statements used to define Events can include up to four Cell Features and four logical operators.

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

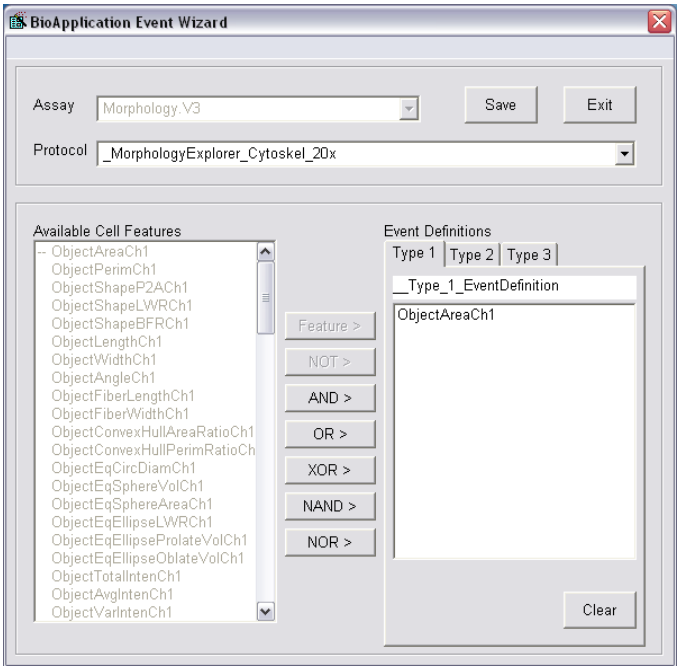
The procedure to be followed when constructing Event Definitions is described in the sequence of screenshots below. Cell Features are combined with Boolean operators to produce Event Definitions. Any Cell Feature can be selected by clicking on the feature name in the Available Cell Features list and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

### To build an Event Definition,

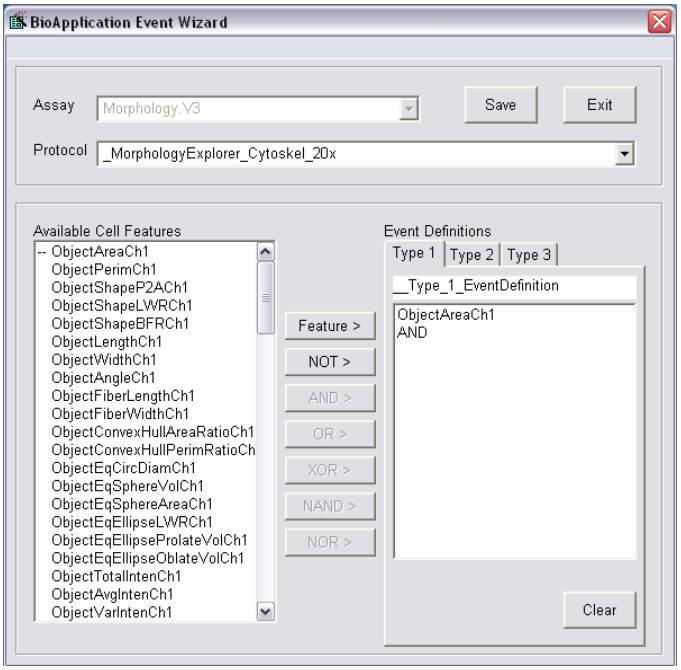
1) Select the Event Definition that you want to specify or edit by clicking on the **Type 1**, **Type 2**, or **Type 3** tab.
2) Select the desired Cell Feature by clicking on the feature name from the Available Cell Features list. In this example, the **ObjectAreaCh1** feature is selected.



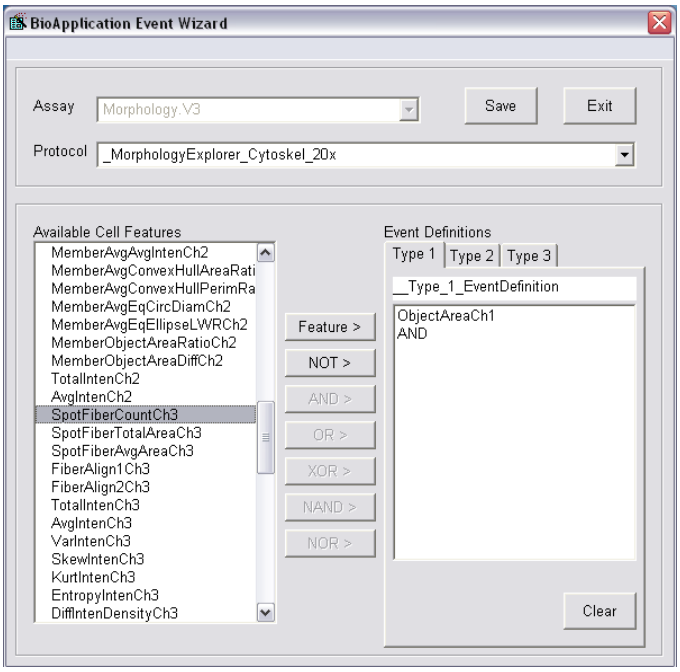
**3)** Click the **Feature>** button to transfer the Cell Feature into the Event Definition (see below).



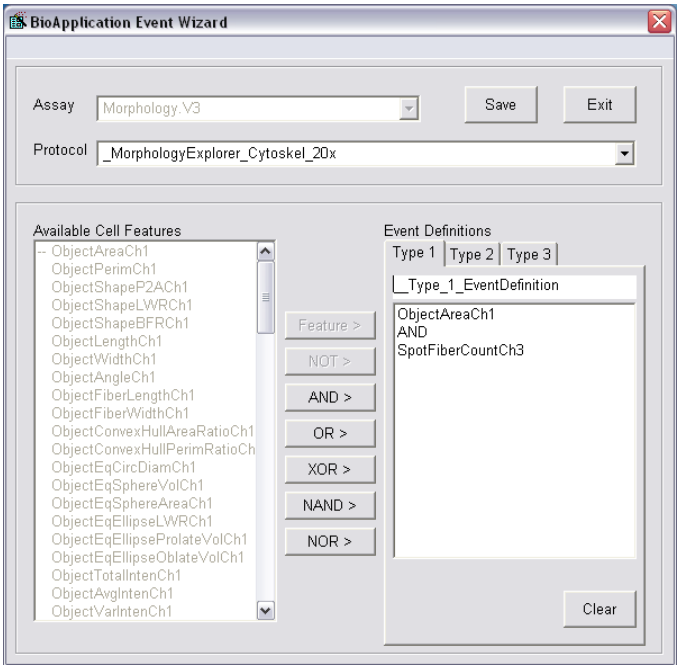

**4)** Click on the desired Operator button to transfer it into the Event Definition. In this example, the **AND** operator is selected (see below).



**5)** Repeat the cycle for adding another Cell Feature to the Event Definition. In this case, the Cell Feature **SpotFiberCountCh3** has been selected for addition.

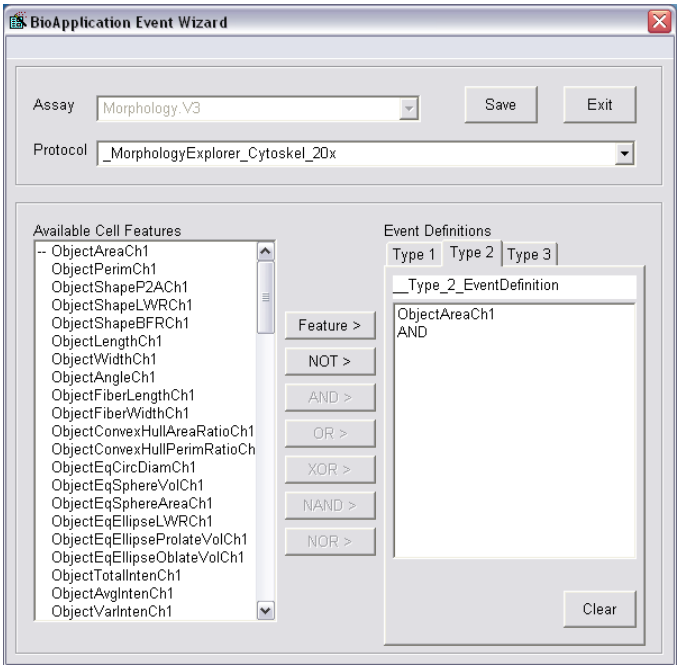


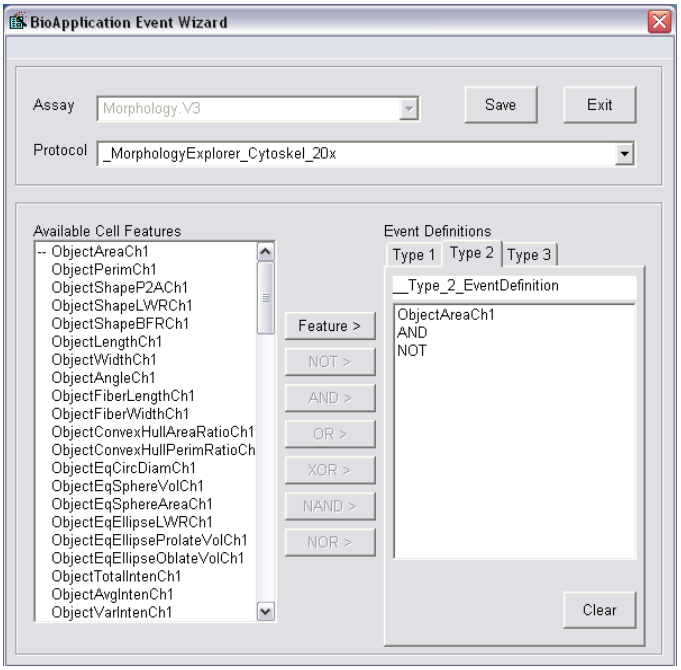

**6)** As before, click on the **Feature >** button to transfer the Cell Feature into the Event Definition (see below).

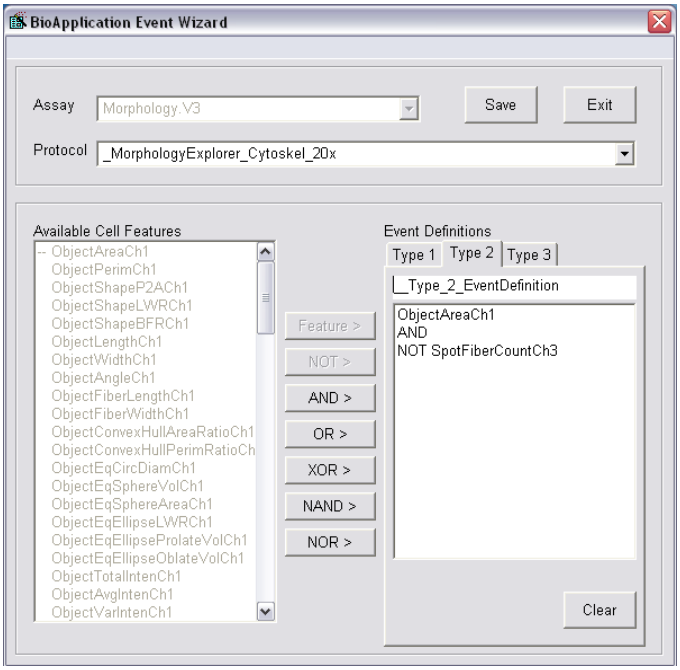



**Type_1_EventDefinition** is:**ObjectAreaCh1** AND **SpotFiberCountCh3**

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.

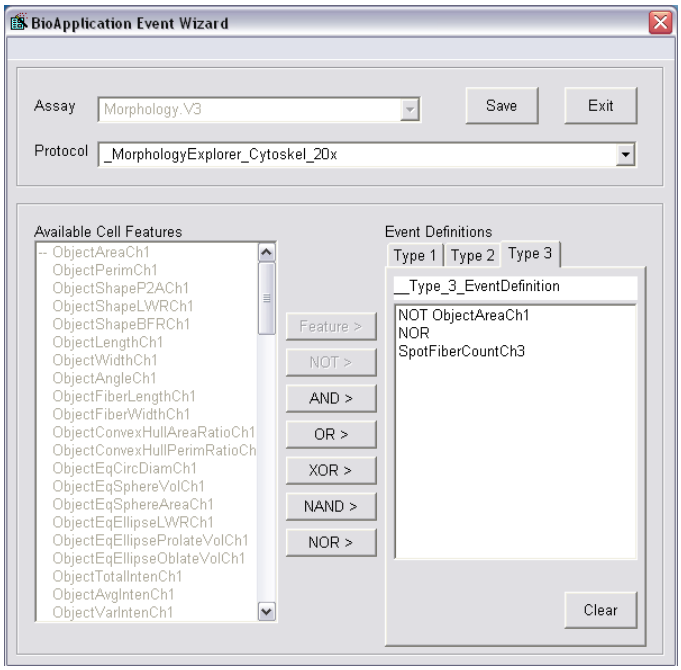





**Type_2_EventDefinition** is:**ObjectAreaCh1** ANDNOT **SpotFiberCountCh3**

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




**Type_3_EventDefinition** is: NOT **ObjectAreaCh1** NOR **SpotFiberCountCh3**

**To clear an Event Definition,**

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

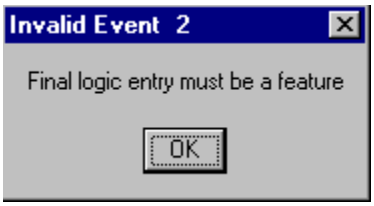
**To save the updated Assay Protocol,**

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

**NOTE**

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

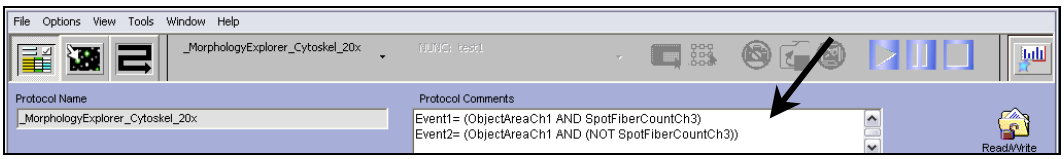
The BioApplication Event Wizard has the capability to automatically validate Event Definitions. If Event Definition validation fails, the protocol will not be saved. For example, Event Definitions are considered invalid if they do not end with a Cell Feature. In this case, clicking on the **Save** button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




**To exit the BioApplication Event Wizard,**

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

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



## Database Limitations

Since the Morphology Explorer BioApplication has a large number of output features, the finite size of the HCS Reader database available to store these features may limit the types of experiments that can be done. This limitation is not expected to be an issue for most uses of the BioApplication, and sufficient database resources should be available to do most experiments. Nevertheless, before each use of this BioApplication, you are advised to check whether the experimental design will cause the available database resources to be exceeded. The following relation must be satisfied if sufficient database resources are available to scan the plate:

$$M > NW \times \left( \left( NC \times CF \right) + WF \right)$$

where:

***M*** is the available database size. For the purposes of this calculation, it is 27,500,500 data points for the ArrayScan HCS Reader 4.0. A single Cell or Well Feature constitutes a single data point.

***NW*** is the number of wells to be scanned in a plate

***NC*** is the typical number of objects (cells) per well expected in a plate

***CF*** is the number of output Cell Features. This varies with the number of channels used, as shown in the following table.

***WF*** is the number of output Well Features. This varies with the number of channels used, as shown in the following table.

The relationship between the number of output Cell and Well Features and the number of channels is as follows in Table 35:

| Number of Channels | Number of Cell Features (*CF*) | Number of Well Features (*WF*) |
|---|---|---|
| 1 | 71 | 133 |
| 2 | 105 | 201 |
| 3 | 151 | 293 |
| 4 | 203 | 397 |
| 5 | 205 | 397 |
| 6 | 207 | 397 |

***Table 35:*** *Database Limitations*

**Example 1:**
The example assay requires scanning an entire 96-well plate using 4 channels and an average of 500 cells/well. Thus, *NW* = 96, *NC* = 500, *CF* = 203 and *WF* = 397, and plugging these numbers into the equation above gives 9,782,112 data points, which is below the database size limit for the ArrayScan HCS Reader 4.0. Thus, this experiment is possible.

**Example 2:**
If a 384-well plate is used instead of a 96-well plate, *NW* = 384, and the equation gives 39,128,448 data points. This plate exceeds the database size limit for ArrayScan HCS Reader 4.0 and cannot be scanned on this platform. The different options to allow scanning on ArrayScan HCS Reader 4.0 would be to either reduce *NW* by not scanning all 384 wells on the plate, reduce *NC* by choosing fewer fields to scan so that a fewer number of cells are expected per well or decrease the number of channels imaged to less than 4 so as to decrease both *CF* and *WF*.

Chapter

4

# iDev Software Workflow

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

## iDev Protocol Optimization Tasks

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

### Image Preprocessing

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

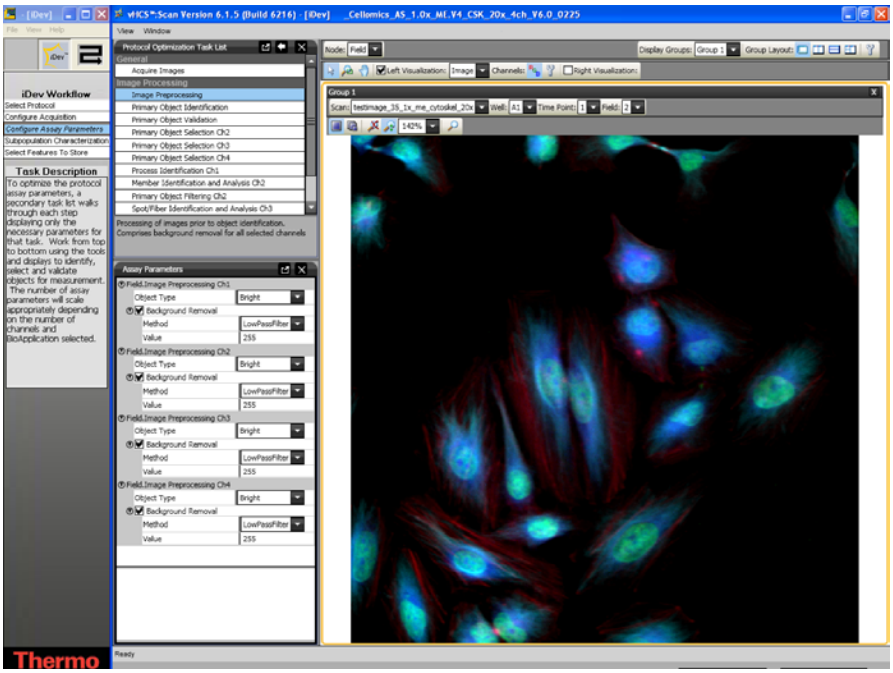



***Figure 67.*** *Protocol Optimization Task – Image Preprocessing*

## Primary Object Identification Ch1

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

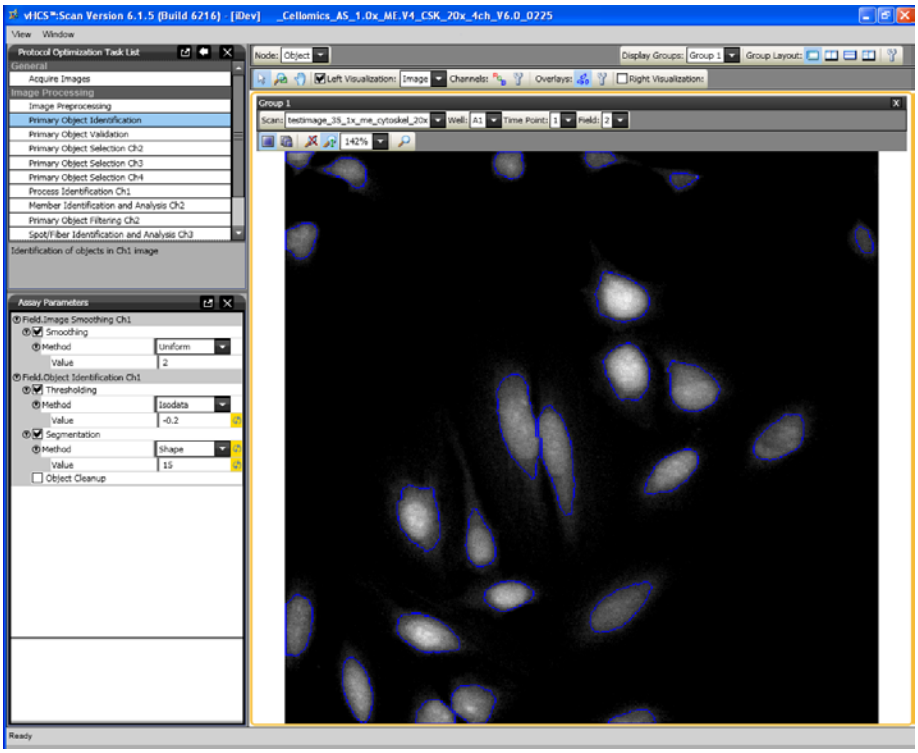



***Figure 68.*** *Protocol Optimization Task – Primary Object Identification Ch1*

## Primary Object Validation Ch1

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

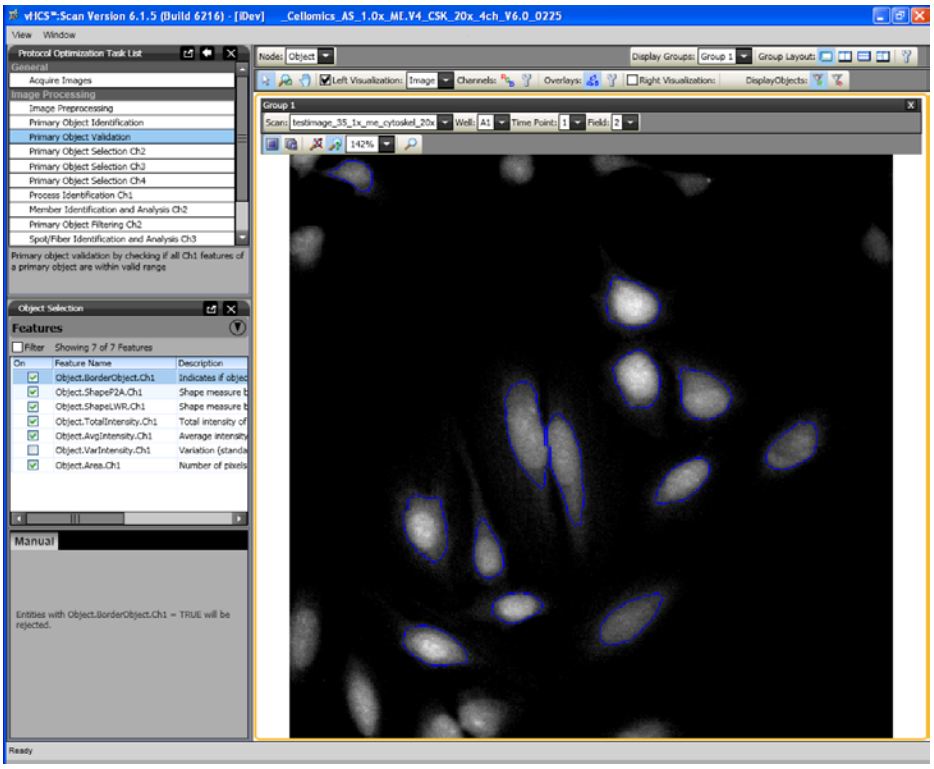



***Figure 69.*** *Protocol Optimization Task – Primary Object Validation Ch1*

## Primary Object Selection Ch2 through ChN

Primary Object Selection is object selection based on intensity features computed in Channels 2 through Channel N under the circ mask derived from the Channel 1 primary object mask. In this task, you will set selection / rejection of the primary object based on intensity measurements in Channels 2 through Channel N under a modified primary object mask. This task is similar to setting the Assay Parameter, **MaskModifierCh2,** in the ArrayScan Classic software. The primary object mask can be dilated (**Circ** > 0), or eroded (**Circ** < 0) if the **ROI Mask Creation** box is checked.

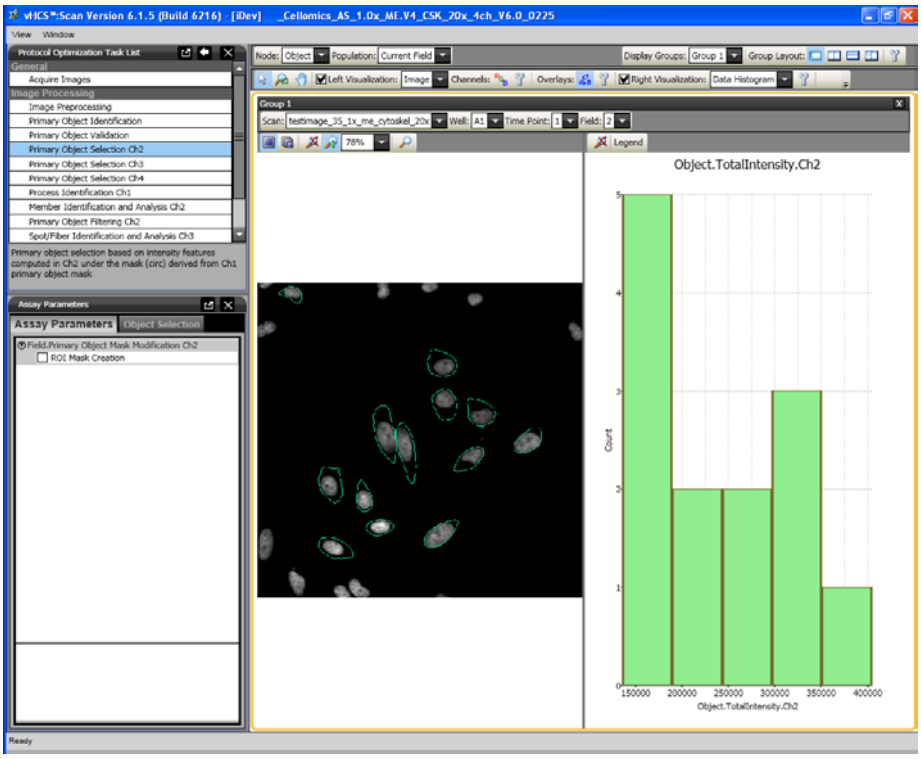



***Figure 70.*** *Protocol Optimization Task – Primary Object Selection Ch2 through ChN*

## Process Identification Ch1

This task is for identification of processes (such as neurites and angiogenic tubes) in Channel 1 based on ZONE OF INFLUENCE from the primary object mask. If the Assay Parameter, Process Identification, is not selected then the algorithm will not detect neurites as primary objects.

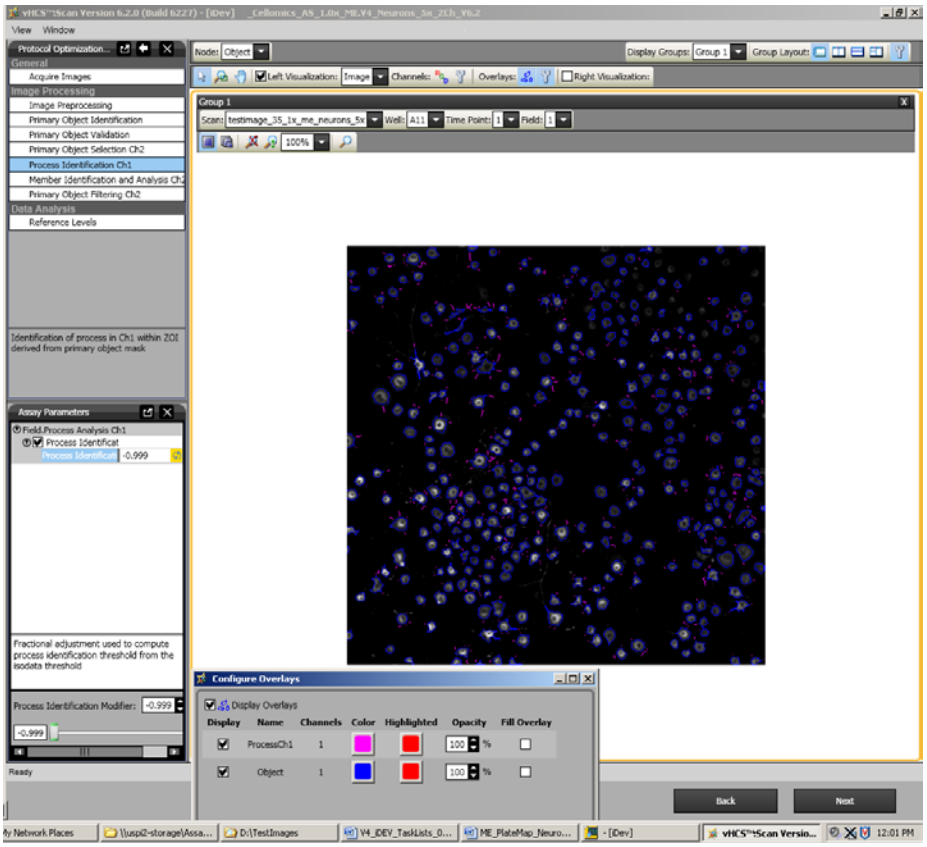



***Figure 71.*** *Protocol Optimization Task – Process Identification*

## Member Identification and Analysis Ch2

This task provides identification of members (such as cell nuclei) in Channel 2 within the region of interest (ROI) derived from the primary object mask. In this task, you can define the ROI as a Circ or Ring, select the method and set a value for object smoothing, identification, and segmentation. You can also specify if colony analysis of objects in Channel 2 is to be performed in this task.

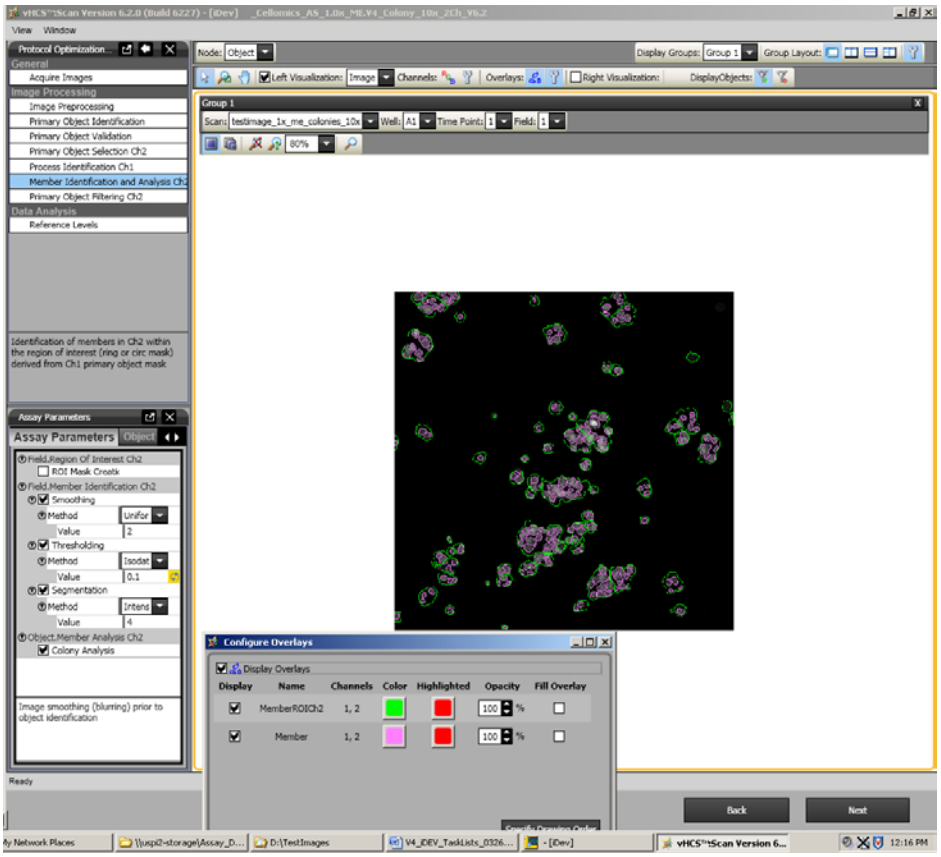



***Figure 72.*** *Protocol Optimization Task – Member Identification and Analysis*

## Primary Object Filtering Ch2

In this task, you can set selection and rejection criteria for primary objects in Channel 1 based on the number of members (Member Count) contained within the ROI in Channel 2.

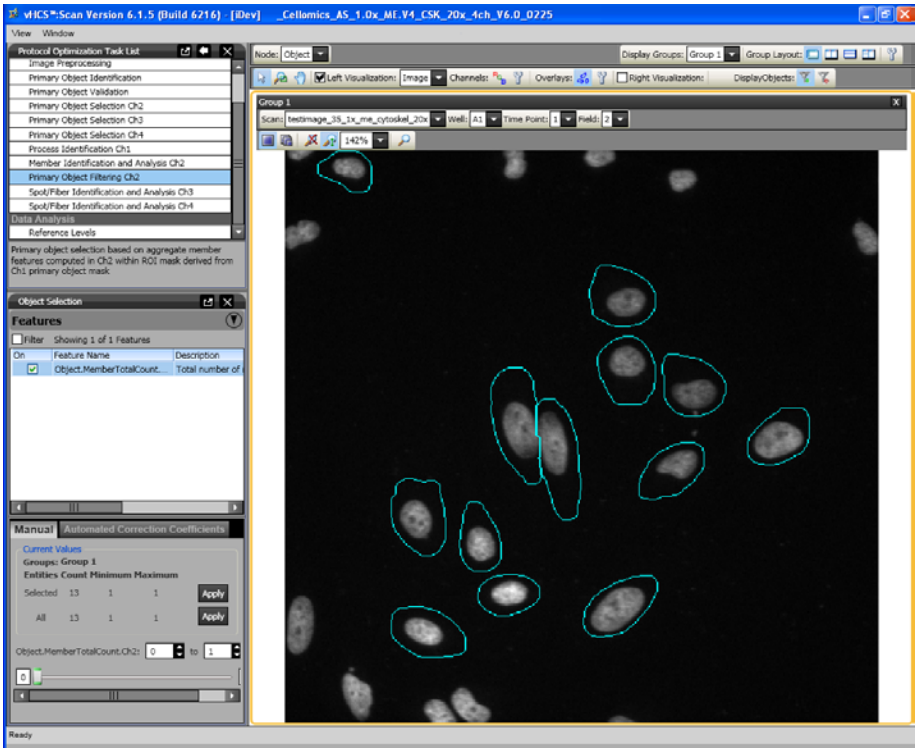



***Figure 73.*** *Protocol Optimization Task – Primary Object Filtering*

## Spot/Fiber Identification and Analysis Ch3 and Ch4

Identification of spots or fibers in Channels 3 and 4 within the region of interest (ring or circ mask) derived from the Channel 1 primary object mask. In this task, you will define the region of interest (ROI) and set the identification method and threshold for your target in Channel 3. The ROI can be either a “Circ” or a “Ring”. You can also specify if fiber alignment measurements are to be made in Channels 3 and 4.

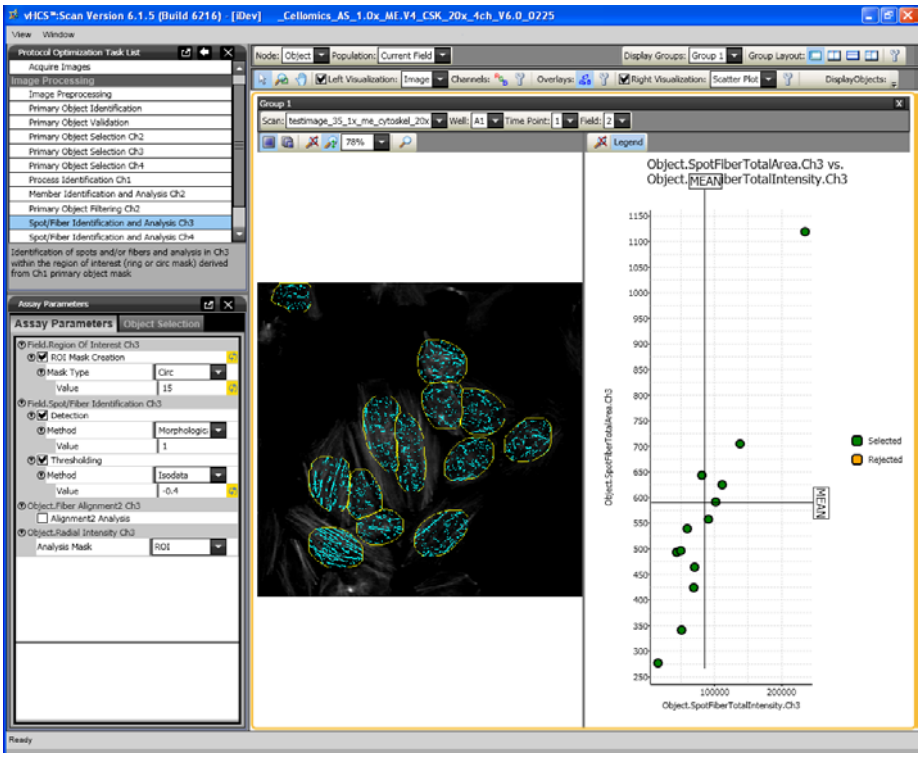



***Figure 73.*** *Protocol Optimization Task – Spot/Fiber Identification and Analysis*

## Reference Levels

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

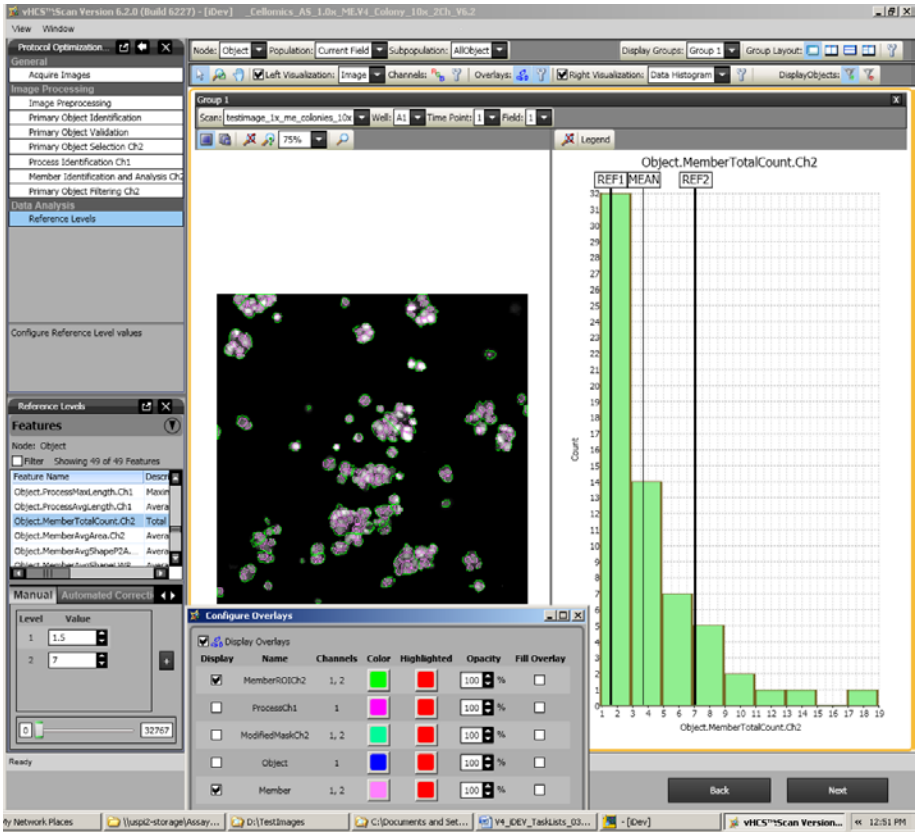



***Figure 74.*** *Protocol Optimization Task – Reference Levels*

## 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.

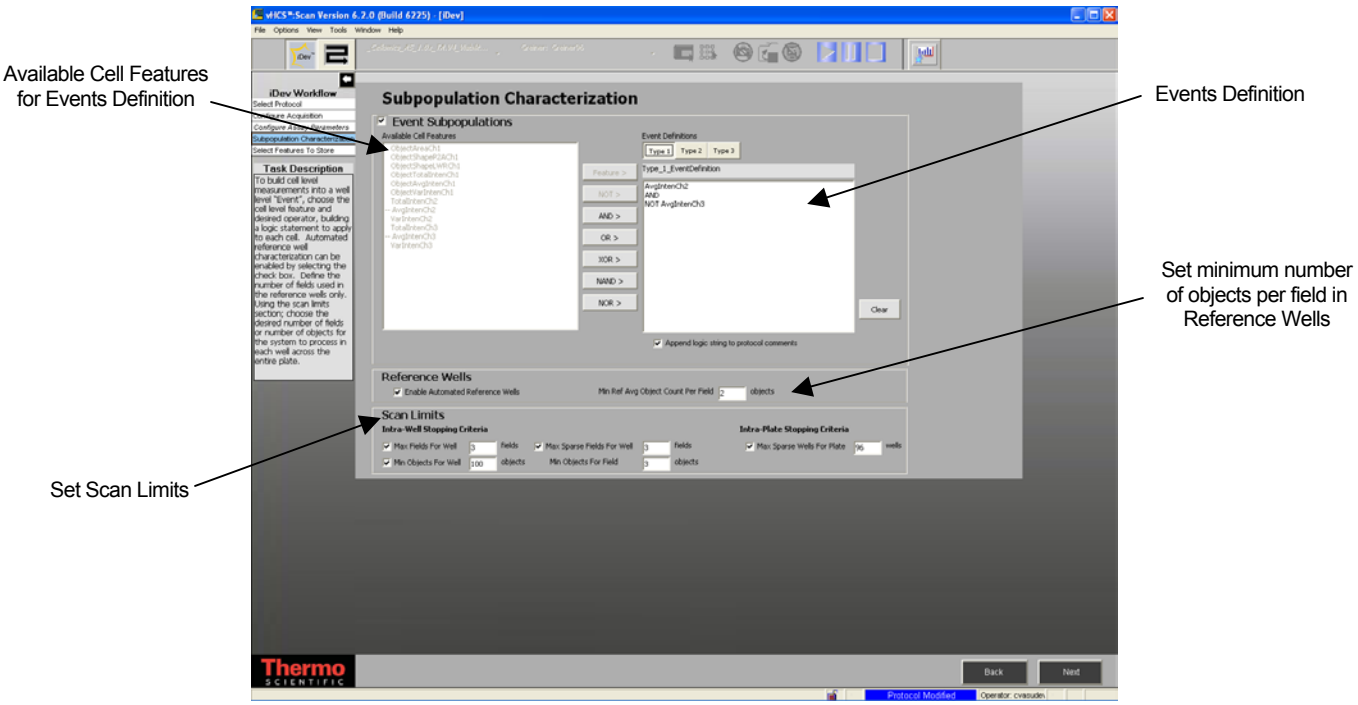



***Figure 75.*** *Subpopulation Characterization Task*

.

# Index

## F



## G



## I



## K



## M



## N



## O

## P



## R



## S



## T



## V



## W