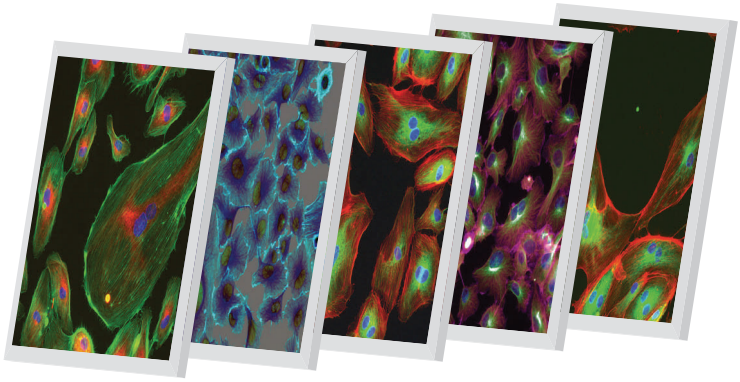
Thermo Scientific Cellomics® Neuronal Profiling V4

BioApplication Guide



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Cellomics® Neuronal Profiling BioApplication Guide

V4 Version

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Chapter

1

Overview of the Neuronal Profiling V4 BioApplication

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

This guide contains the following chapters:

Chapter 1 provides an overview of the Neuronal Profiling V4 BioApplication and describes biological situations to which it can be applied.

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

Chapter 3 describes the use of the Assay Protocol with guidance on how to adjust some of the parameter settings as well as use of the Event Wizard.

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

System Compatibility

The Neuronal Profiling V4 BioApplication described in this guide is designed to run on the following platforms:

- ArrayScan HCS Reader version VTI running software versions x.6.1 and higher
- Cellomics vHCS™ Discovery Toolbox, version 1.6.1 and higher

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

Cell Biology Background

Neurobiology has been an increasingly important research area. Neurons are ever-differentiating entities, and the generation of new neurons in the mammalian brain continues into adulthood. Differentiated neurons assemble into functional networks by developing axons and dendrites (collectively called neurites) that can synapse with other neurons (Ooyen et al., 1995). Growth cones are specialized structures at the terminal ends of neurites, where the

cone's migration elongates the neurites and splitting creates branch points. Many cellular mechanisms are involved in the actual behavior of growth cones, effecting their migration and branching. These mechanisms include modulation of intracellular calcium levels, activation of various signal transduction cascades and cytoskeletal changes.

Neuronal cell morphology, including neurite outgrowth, total neurite count, cell body size, and growth cone behavior, is modulated by a variety of conditions such as trophic factors, electrical activity, synaptogenesis, and functional maturation and differentiation of neurons (Thoenen, 1991; Ooyen et al., 1995; Fields and Nelson, 1992). Identifying changes to the morphological features in vitro can help identify neurotoxins.

Neurogenic regions in the adult central nervous system, such as the spinal cord, contain neural stem cells. Neural stem cells from these regions proliferate in response to factors, such as neurotrophins, epidermal growth factor, or basic fibroblast growth factor, and differentiate into neurons and glial cells in vitro (Gritti et al., 1996). Neurotrophins act to control developmental cell death of neurons. However, neuronal abnormality (such as neurodegeneration, neurotransmitter imbalance, etc) can be implicated in a variety of pathophysiological conditions such as the central and peripheral nervous system diseases including Alzheimer's, Parkinson's, and psychiatric disorders. These disorders affect changes in neuronal cell morphology and/or changes in neurotransmitter release. Mounting evidence implicates neuronal cell death after stroke and in conditions following hypoxia, ischemia, neurodegeneration, etc. Some disease conditions affect outgrowth and elongation of neurites, branching of the neurites, changes in neuronal cell survival, cell body area, and expression of certain genes. It is studying the axonal outgrowth and functional recovery after nerve injury and the altering of various signaling cascades (including Raf/MEK/MAP kinase pathways) (Das et al., 2004), along with multiplexing these changes that help scientists understand the pathways leading to abnormalities and how to treat them.

Synapses allow neurons to communicate with each other via the release of a neurotransmitter that open ion channels or activate second messenger systems. This allows changes in membrane potential and generation of an action potential. Synapse formation is a complex process that requires appropriate projection of the axon to a specific target followed by the synthesis and targeting of multiple ion channels proteins to the synaptic site. Pre-synaptic cells will have accumulation of small vesicles, with post-synaptic sites exhibiting clustering of receptors to receive neurotransmitters released from the pre-synaptic vesicles. Synaptophysin, synapsin, beta-amyloid, microtubule-associated proteins, and PSD95 are a few of the highly researched targets to better recognize their role in synaptogenesis. Understanding the purpose of these targets and their relationship to overall neuronal functioning that can help discover the meaning and possible treatment of neurological disorders.

BioApplication Overview

The Neuronal Profiling V4 BioApplication enables quantification of morphological changes in neurons, allowing you control over selecting neurites based on morphological as well as intensity differences. You are able to select neurons based on nucleus and cell body/neurite object selection parameters, identifying subpopulations of cells due to biology, and through implementation of the Event Wizard. You can identify spots in downstream channels through use of Assay and Object Selection Parameters.

Image Channels

The Neuronal Profiling V4 BioApplication performs cell-based measurements using two to six imaging channels, described in Table 1.

All cells (neuronal and non-neuronal) are identified in Channel 1 using a nuclear stain, such as Hoechst 33342 or DAPI. Nuclear features, including size, shape, and intensity, are measured. Neurons and neuronal-like cells can be identified in Channel 2 by indirect immunofluorescence using a primary antibody against a protein specific to neurons that is present in both neuronal cell bodies and neurites. Cell body features measured include size, shape, and intensity. Neurite features such as, count, size, intensity, and branch and cross point counts, are also measured in Channel 2.

Channel 3 and Channel 4 can be used to identify spots found within the neurons, whether in/along cell bodies or modified neurite masks. If identifying spots in both channels, you can also see if spots are overlapping between the channels (such as with pre-synaptic and post-synaptic vesicles).

Channel 3 and Channel 4 (as well as Channel 5 and Channel 6) can be used for gating by setting the intensity measurements (average and total) within the region of the modified cell body. Please refer to Chapter 2 as well as your appropriate instrument user's guide for more information.

Below is an example of how each channel could be used for identification.

Channel	Cellular Features Measured	Method of Identification
1- Required	Nucleus size, shape, intensity	Nuclear label (e.g., Hoechst 33342)
2 – Required	Cell body size, shape, intensity Neurite count, size, intensity, branch point count, cross point count	Immunofluorescence against a protein in neurons and neurites
3 – Optional	Spots within cell body and/or modified neurite masks Modified cell body region intensity	Pre-synaptic marker or Immunofluorescence against a neurotransmitter
4 – Optional	Spots within cell body and/or modified neurite masks Modified cell body region intensity	Post-synaptic marker **or** Immunofluorescence against another neurotransmitter
5 – Optional	Modified cell body region intensity	Immunofluorescence against another neurotransmitter or another stain
6 – Optional	Modified cell body region intensity	Immunofluorescence against another neurotransmitter or another stain

Table 1. *General description of features and identification per channel within the Neuronal Profiling V4 BioApplication*

Neuron Structure and Analysis

The primary regions within a neuron are nucleus, cell body, and neurites. This is illustrated in the following figure. Also illustrated are where cell body and neurite spots could be found.

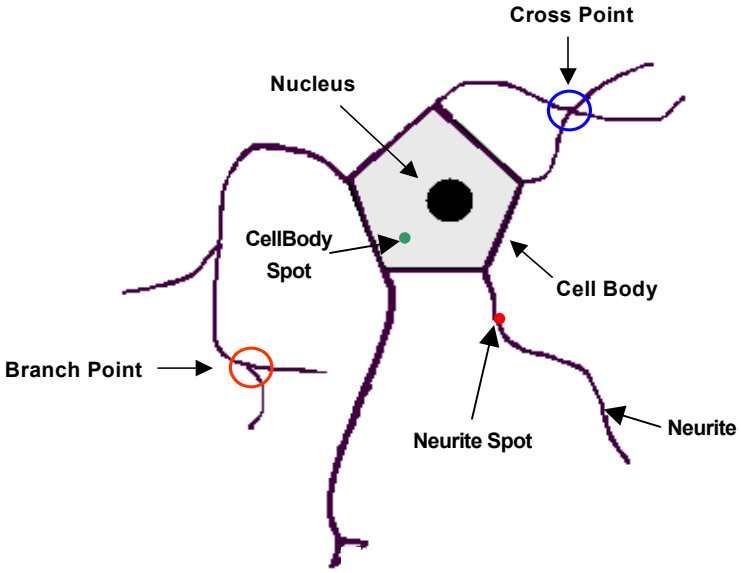


Figure 1. *General structural components of a neuron*

Since a neuron has one cell body, and may have multiple nuclei and neurites, the Neuronal Profiling BioApplication uses the cell body as the primary object. This is the object used to identify the neuron and to collect the features of its parts.

BioApplication Workflow

The first task of the BioApplication is to identify all of the valid neurons in the field. This is done by a combined analysis of Channel 1 and Channel 2. Valid nuclei are identified in Channel 1 and valid neuronal cell bodies are identified in Channel 2. Results are combined to identify valid non-neuronal cells, in which only the nucleus is labeled, and valid neuronal cells, which can have a cell body and corresponding nucleus/nuclei (depending on the Object Selection Parameter **CellBodyNucCountCh2**, which is described in more detail in Chapter 2).

The second task is to select the valid neurons that will be analyzed by the BioApplication. This is done by creating a mask from each valid cell body and using it to measure Average and Total Intensities in Channels 3-6. These intensities can be used to gate, or select, the neurons that will be analyzed.

Neurites are analyzed in the third task. The neurite mask is created from the Channel 2 image (also used to identify the cell bodies). Neurites can split at branch points and cross themselves, or each other, at cross points. They are traced outward from each cell body, analyzed, and validated against object selection parameters (see Figure 1).

Finally spots are analyzed within/along the cell body and modified neurite masks in Channel 3 and/or Channel 4. Spots can be detected using object identification thresholding, spot segmentation, spot smoothing, spot detect method and spot detect radius. Spots can also be selected through size, shape and intensity selection parameters. If spots are identified in both Channel 3 and Channel 4, and if **MinAreaSpotOverlapCh3Ch4** does not equal zero, overlap

analysis is performed based on the spot masks in each channel, and the spot features and overlap features are extracted.

The measurements performed by the BioApplication are summarized in Table 2. Cell body measurements include the area, shape, and intensity, and their features are reported for each individual cell body. The features for nuclei are summed over the cell bodies to which they belong. These include count, total area, and intensity. Neurite features are also grouped per cell body and include total length, average length, maximum length (with and without branches), total area, width, branch point count, cross point count, average branch point distance from the cell body, and intensity. Spot features are based on if they are found within the cell body (CellBodySpot) or modified neurite mask (NeuriteSpot). Spot features are similar to those found in other channels and include area and intensity. Intensity ratios and overall overlap between channels are also calculated.

Event Definition

The Neuronal Profiling V4 BioApplication allows simultaneous definition of up to three Events to enable rapid multiparametric analysis or defining a subpopulation of neurites, neurons, and spots at the level of individual cells, across multiple Cell Features. Events are defined as Assay Parameters and consist of logical statements employing specific Cell Features. These statements are then applied to classify and count responder and non-responder cells for the Cell Features contained in the Event Definition. Figure 2 relays these capabilities and how they interact to enable event definition and detection. Event definitions are created using a software tool called the BioApplication Event Wizard. Operation of this software tool is described in more detail in Chapter 3.

Event definition and detection at the level of individual cells of a population requires processing of raw Cell Feature values, i.e., the data extracted from individual cells for all targets being measured. Limits are then applied (manually entered for the ***Feature*Ch*N*LevelHigh** 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 defined by ***Feature*Ch*N*LevelHigh** (***Feature*Ch*N*Status =1**) or, if placing NOT in front of the feature, those less than the upper limit defined by ***Feature*Ch*N*LevelHigh** (***Feature*Ch*N*Status = 0**). It is critical that the upper limits be set accordingly. Well Features are calculated to provide population statistics for individual Cell and Field Features, as well as frequency of occurrence of a given Event in the cell population. The latter is expressed as the percentage of cells that display a given Event.

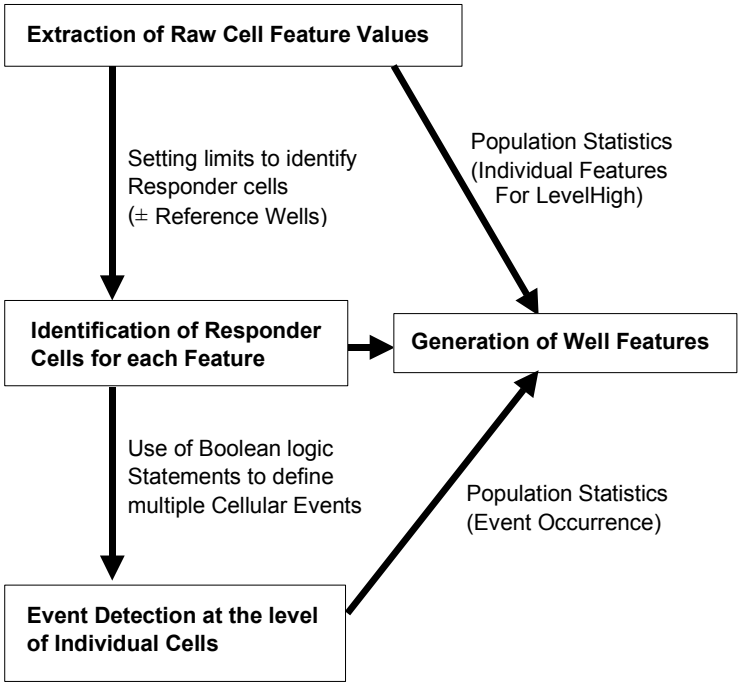


Figure 2. *Schematic showing the process of definition and detection of cellular events*

BioApplication Measurements

The Neuronal Profiling V4 BioApplication reports Reference, Cell, Field, and Well Features. Reference Features will only be given when Reference Wells are selected. Cell Features are measurements made on a single cell. Field features are quantitative descriptors of cells, cellular and/or sub-cellular structures reported for the whole field. Well Features are well-level averages and other population metrics that are derived either from the Cell or Field Features for all the cells analyzed in a given well. Overall Cell, Field, and Well Features are summarized in Table 2 and described further in Chapter 2.

Additionally, for live cell analysis, the Cellomics ArrayScan V^{TI} HCS Reader supports a Live Module that can track cells over time. Please refer to the *Live Module User's Guide* for more information.

Type	Region	Cell Features	Field Features	Well Features
Cell & Well Features Reported for Channels 1 and 2:				
Location	Cell Body	Top Left Width Height X Centroid Y Centroid	None	None
Count	Cell Body	Cell #	Valid Neuron Count Selected Neuron Count %Selected Neurons Neuron Nucleus Ratio Neuronal Nucleus Count %Neuronal Cells AvgSelectedNeuronCountPerField Valid Field Count	Valid Neuron Count Selected Neuron Count %Selected Neurons Neuron Nucleus Ratio Neuronal Nucleus Count %Neuronal Cells AvgSelectedNeuronCountPerField Valid Field Count
	Nucleus	Count & Status (those within Cell Body)	Valid Nucleus Count Non-Neuronal Nucleus Count %NonNeuronal Cells	Valid Nucleus Count Non-Neuronal Nucleus Count %NonNeuronal Cells
			→	MEAN, SD, SE, CV, %HIGH for all Selected Cells
	Neurite	Count & Status	Total Count Per Field Total Count Per Neuron	Total Count Per Well Total Count Per Field Total Count Per Neuron
			→	MEAN, SD, SE, CV, %HIGH for all Selected Cells
	Branch Point	Total Count & Status Avg Count & Status Total Count Per Neurite Length & Status	→	MEAN, SD, SE, CV, %HIGH for all Selected Cells
			Total Count Per Field Total Count Per Neuron Total Count Per Neurite Total Count Per Neurite Length	Total Count Per Well Total Count Per Field Total Count Per Neurite Total Count Per Neurite Length
	Cross Point	Total Count & Status Avg Count & Status	None	MEAN, SD, SE, CV, %HIGH for all Selected Cells
Morphology	Nucleus	Area & Status	None	MEAN, SD, SE, CV, %HIGH for all Selected Cells
	Cell Body	Area & Status Shape P2A & Status Shape LWR & Status	None	MEAN, SD, SE, CV, %HIGH for all Selected Cells
	Neurite	Total Length & Status Avg Length & Status Max Length w/Branches & Status Max Length w/o Branches & Status Width & Status Total Area & Status	→	MEAN, SD, SE, CV, %HIGH for all Selected Cells
			Total Length Per Field Total Length Per Neuron Total Length Per Neurite	Total Length Per Well Total Length Per Field Total Length Per Neuron Total Length Per Neurite
	Branch Point	Avg Distance from Cell Body & Status	→	MEAN, SD, SE, CV, %HIGH for all Selected Cells
Intensity	Nucleus	Total Intensity & Status Avg Intensity & Status	None	MEAN, SD, SE, CV, %HIGH for all Selected Cells
	Cell Body	Total Intensity & Status Avg Intensity & Status	None	MEAN, SD, SE, CV, %HIGH for all Selected Cells
	Neurite	Total Intensity & Status Avg Intensity & Status Variance Intensity & Status	Total Intensity Per Field Avg Intensity Per Field Variance Intensity Per Field	Total Intensity Per Well Total Intensity Per Field Avg Intensity Per Field Variance Intensity Per Field
			→	MEAN, SD, SE, CV, %HIGH for all Selected Cells

Cell & Well Features Reported for Channels 3 and 4:				
Type	**Region**	**Cell Features**	**Field Features**	**Well Features**
Count	Spots confined by CellBody (CellBodySpot)	Total Count & Status	Total Count Per Field Total Count Per Neuron	Total Count Per Well Total Count Per Field Total Count Per Neuron
			→	MEAN, SD, SE, CV, %HIGH for all Selected Cells
	Spots confined by modified Neurite Mask (NeuriteSpot)	Total Count & Status Avg Count & Status	Total Count Per Field Total Count Per Neuron Total Count Per Neurite Total Count Per Neurite Length	Total Count Per Well Total Count Per Field Total Count Per Neuron Total Count Per Neurite Total Count Per Neurite Length
			→	MEAN, SD, SE, CV, %HIGH for all Selected Cells
Morphology	CellBodySpot	Total Area & Status	Total Area Per Field Total Area Per Neuron	Total Area Per Well Total Area Per Field Total Area Per Neuron
			→	MEAN, SD, SE, CV, %HIGH for all Selected Cells
	NeuriteSpot	Total Area & Status Avg Area & Status	Total Area Per Field Total Area Per Neuron Total Area Per Neurite Total Area Per Neurite Length	Total Area Per Well Total Area Per Field Total Area Per Neuron Total Area Per Neurite Total Area Per Neurite Length
			→	MEAN, SD, SE, CV, %HIGH for all Selected Cells
Intensity	CellBodySpot	Total Intensity & Status Avg Intensity & Status TotalIntenRatioCh3Ch2 (or Ch4Ch2 or Ch4Ch3) & Status	Total Intensity Per Field Total Intensity Per Neuron Avg Intensity Per Field	Total Intensity Per Well Total Intensity Per Field Total Intensity Per Neuron
			→	MEAN, SD, SE, CV, %HIGH for all Selected Cells
	NeuriteSpot	Total Intensity & Status Avg Intensity & Status TotalIntenRatioCh3Ch2 (or Ch4Ch2 or Ch4Ch3) & Status	Total Intensity Per Field Total Intensity Per Neuron Total Intensity Per Neurite Total Intensity Per Neurite Length Avg Intensity Per Field	Total Intensity Per Well Total Intensity Per Field Total Intensity Per Neuron Total Intensity Per Neurite Total Intensity Per Neurite Length Avg Intensity Per Field
			→	MEAN, SD, SE, CV, %HIGH for all Selected Cells
Overlap	CellBodySpot overlap of Ch3 and Ch4	Overlap Count & Status % Overlap Count & Status Overlap Area & Status % Overlap Area & Status	Overlap Count Per Field %Overlap Count Per Field Overlap Count Per Neuron Overlap Area Per Field %Overlap Area Per Field Overlap Area Per Neuron	MEAN, SD, SE, CV, %HIGH for all Selected Cells
	NeuriteSpot overlap of Ch3 and Ch4	Overlap Count & Status % Overlap Count & Status Overlap Area & Status % Overlap Area & Status	Overlap Count Per Field %Overlap Count Per Field Overlap Count Per Neuron Overlap Count Per Neurite Overlap Count Per NeuriteLength Overlap Area Per Field %Overlap Area Per Field Overlap Area Per Neuron Overlap Area Per Neurite Overlap Area Per Neurite Length	MEAN, SD, SE, CV, %HIGH for all Selected Cells
Gating Intensity	Modified Cell Body	Total Intensity & Status Avg Intensity & Status	None	MEAN, SD, SE, CV, %HIGH for all Selected Cells

Cell & Well Features Reported for Channels 5 and 6:				
Type	**Region**	**Cell Features**	**Well Features**	**Field Features**
Gating Intensity	Modified Cell Body	Total Intensity Avg Intensity	None	None
Other Cell & Well Features Reported				
Event		EventTypeProfile EventType1 Status EventType2Status EventType3Status	None	Event 1: Count, % Event 2: Count, % Event 3: Count, %

Table 2. *Neuronal Profiling V4 BioApplication Cell, Field, and Well Feature Measurements.*

Demonstration Data using the Neuronal Profiling BioApplication

Example 1 – Agonistic Studies Using Neuroscreen™-1 Cells

The Neuronal Profiling BioApplication was verified using Cellomics® Neuroscreen-1 (NS-1) cells, a sub-clonal cell line of PC-12 cells. PC-12 cells are established as a standard model system for the study of neuronal cells (Greene et al., 1998; Tsuji et al., 2001; Wu and Bradshaw 1996) and acquire a number of properties characteristic of sympathetic neurons (Das et al., 2004). Neuroscreen-1 cells display several significant advantages over the parental PC-12 cell line, including a much shorter doubling time, easier growth and culture, a lower tendency to aggregate, and visible neurite outgrowth in 48 hours, compared to 6-7 days in PC-12 cells. Neurotrophic factors such as nerve growth factor cause the Neuroscreen-1 cells to differentiate into neuronal-like cells with neurites within 3-4 days.

To demonstrate this BioApplication, Neuroscreen-1 cells were plated in 96-well microplates. Half of the plate was treated with a constant amount of nerve growth factor and grown for approximately 72 hours. The cells were then fixed and processed to fluorescently label the cells. Labeled cells were quantitatively analyzed with the Neuronal Profiling BioApplication. The established effects of nerve growth factor on Neuroscreen-1 cells, including an increase in the outgrowth of neurites, neurite elongation, cell body area enlargement, and development of branch points, were clearly evident in the results. A Z'-factor of greater than 0.5 was obtained when responses from the half of a 96-well microplate were compared to controls for parameters and indices of neurite outgrowth such as the percentage of cells displaying a total neurite length or total neurite count greater than a defined response level (**%HIGH_NeuriteTotalLengthCh2** or **%HIGH_NeuriteCountCh2**; data not shown). The Z-factor reflects both the sensitivity and robustness according to Zhang et al., (1999), with values above 0.5 are considered excellent for screening.

Figure 3 shows images from a field of Neuroscreen-1 cells treated with nerve growth factor. These images were acquired on the ArrayScan HCS Reader using a 10x objective lens. Panel A (Channel 1) in Figure 3 shows the cell nuclei labeled with Hoechst 33342. In Figure 3, Panel B (Channel 2), neuronal cells were labeled using the primary and secondary antibodies found within the Cellomics Neurite Outgrowth HCS Reagent Kit. If additional channels were used (e.g., Channel 3 and Channel 4), subpopulations of cells expressing a protein of interest (such as different neurotransmitters) could be identified.

Figure 4 demonstrates neurite outgrowth in response to a range of concentrations of nerve growth factor. The results illustrate the usefulness of the Neuronal Profiling V4 BioApplication for elucidating cellular responses in the form of neurite outgrowth.

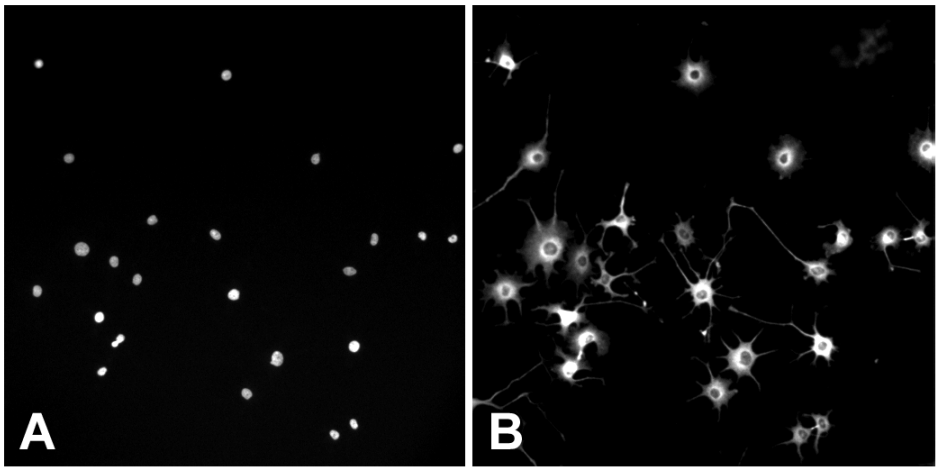


Figure 3. *Raw images (10x) of Neuroscreen-1 cells treated with nerve growth factor (200 ng/mL) acquired with the ArrayScan HCS Reader. Panel A shows the cell nuclei labeled with Hoechst 33342. Panel B shows the neuronal cells and the neurites. Cells were treated for 72 hours to induce neurite outgrowth.*

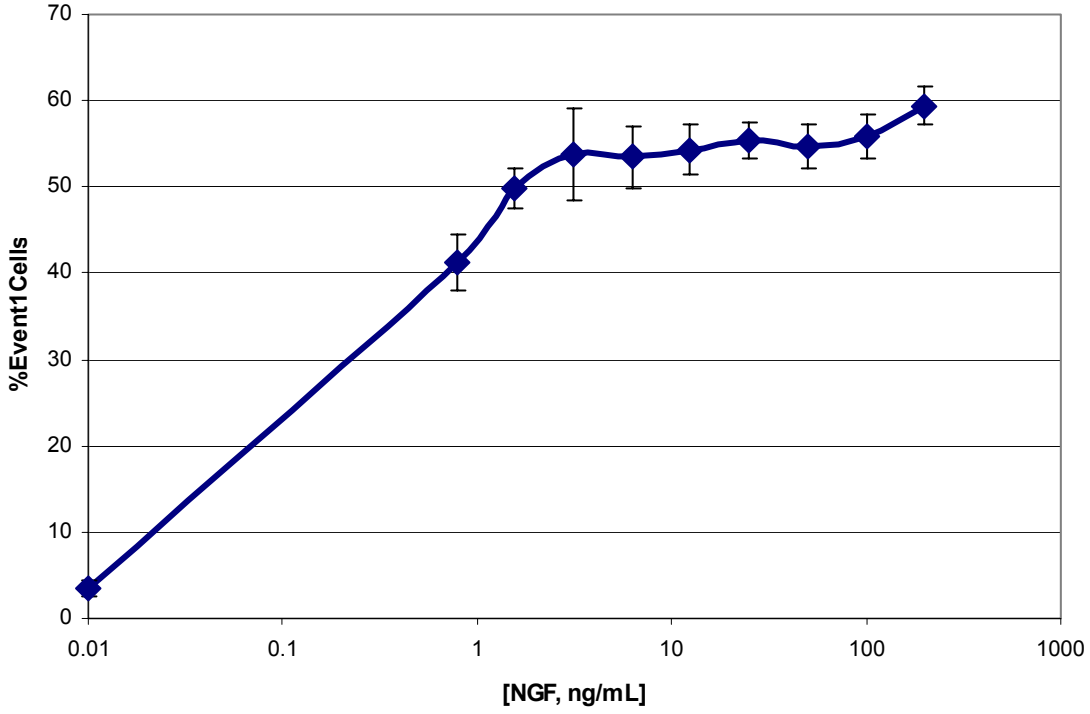


Figure 4. *Neurite Outgrowth induced by nerve growth factor in Neuroscreen-1 cells. The percent of cells exhibiting a total neurite count OR total neurite length above a defined threshold combined together as an event (**%Event1Cells** Well Feature) was plotted against the increasing concentrations of nerve growth factor (NGF). Limits were set through use of Reference Wells. Cells were treated for 72 hours. Error bars represent the standard deviation of six wells.*

Example 2 – Antagonistic Studies Using Primary Neuronal Cells

The Neuronal Profiling BioApplication was validated using cultured primary cells isolated from rat brain cortex (Lonza, Allendale, NJ). Following the suppliers' instructions, neuronal cells were plated at approximately 25,000 cells per well, initially incubated for 4 hours, and then exposed to various doses of SU6656, a known Src-Family of Kinase (SFK) inhibitor. SU6656 is known to inhibit axon outgrowth once induced by Netrin-1 (Meriane et al., 2004) and GDNF (Paveliev et al., 2004). After incubation for approximately 5 days, cells were fixed and stained using the Cellomics Neurite Outgrowth HCS Reagent Kit and run with the Neuronal Profiling BioApplication. With this approach, the non-neuronal cells would not be visible in Channel 2 (since non-neuronal cells do not have associated cell bodies or neurites that would be detected with the reagent kit's Neurite Outgrowth antibody). Figure 5 shows an example of the cells being used with the Neuronal Profiling BioApplication and Figure 6 exemplifies the dose-responsiveness of this inhibitor when evaluating the percentage of non-neuronal cells and the percentage of cells exhibiting a branch point count higher than a defined response (**%HIGH_BranchPointTotalCountCh2**).

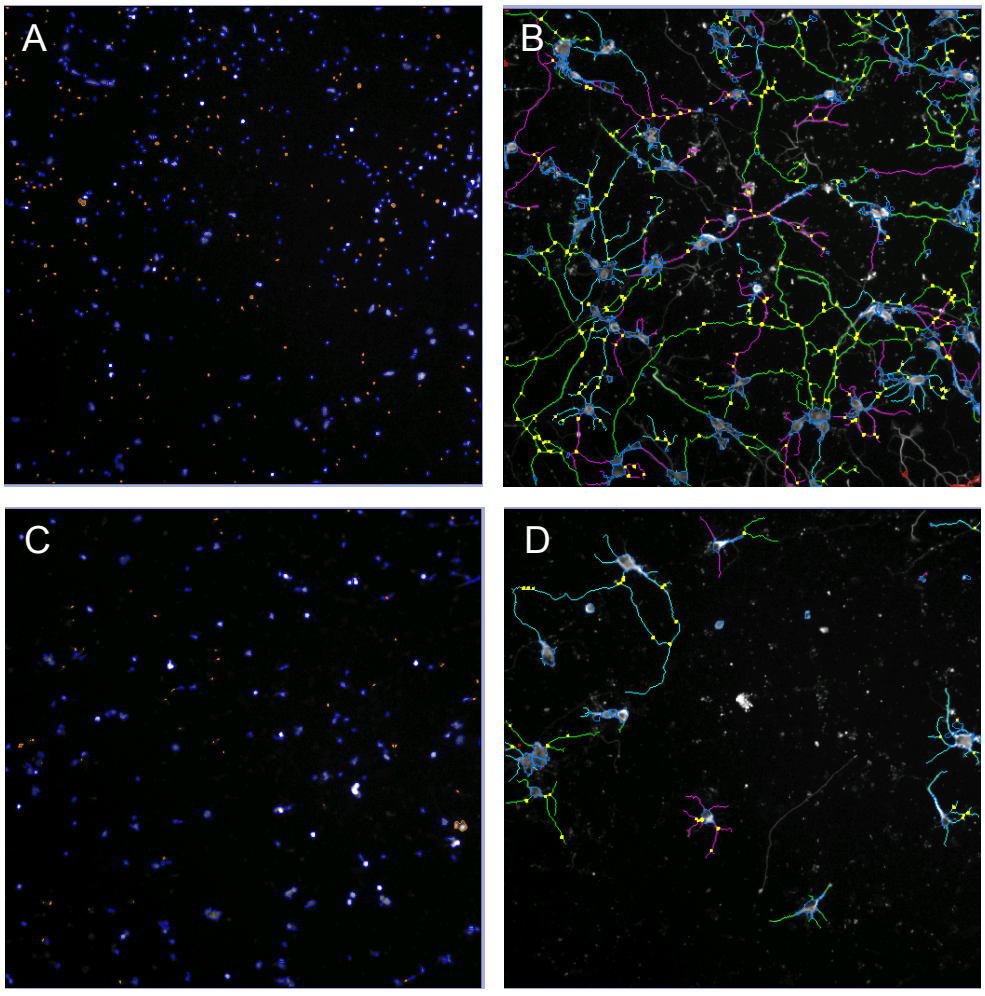


Figure 5. *Images of rat brain cortex neuronal structures captured with the Neuronal Profiling BioApplication. Nuclei can be found in images A and C, with neuronal cell bodies and neurites found in images B and D. Images A and B represent an untreated field and Images C and D represent cells exposed to 4 µM SU6656, which decreased the number of neuronal cells. Cells were stained with the Cellomics Neurite Outgrowth HCS Reagent Kit.*

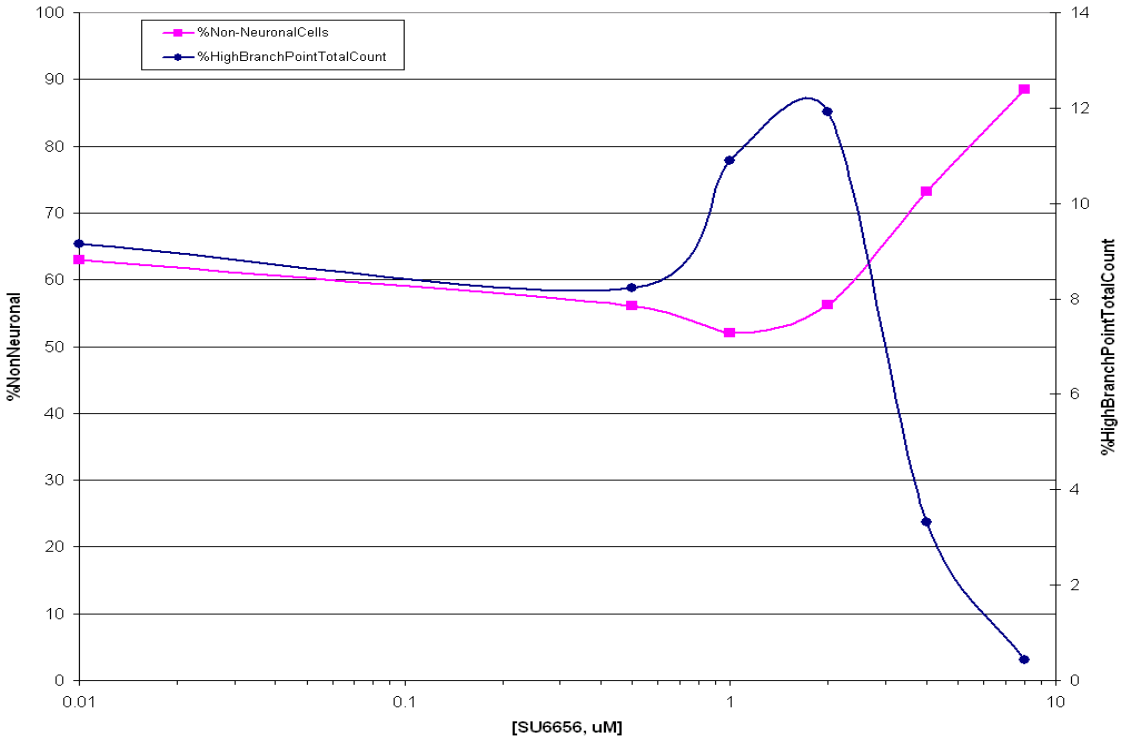


Figure 6. *Neurite outgrowth inhibited by SU6656 in primary cortical cultures. Evaluation was done by looking at the percent of cells above the reference well level set for* ***BranchPointTotalCountCh2*** *(blue), as well as evaluating the percent of non-neuronal cells (pink) over log concentration of inhibitor. An increase in the percentage of non-neuronal cells, with a corresponding smaller percentage of neuronal cells (not shown), was seen with increasing concentrations of SU6656. This was presumably due to toxicity of the compound, with remaining cells having a decrease in the total number of branch points (and total neurites [not shown]). Values around 1.5 µM would be ideal for evaluating branch point and neuronal cells. Cells were treated for 5 days, fixed and stained using the Cellomics Neurite Outgrowth HCS Reagent Kit.*

Example 3 – Identification of Pre-Synaptic Vesicles Using Primary Neuronal Cells

The Neuronal Profiling BioApplication was then used to evaluate antagonistic effects on neurite and pre-synaptic vesicle formation in primary hippocampal cultures (Lonza Allendale, NJ) Neuronal cells were plated at approximately 9,000 cells per well and incubated in NSF-1 –rich media for 4 days. Media was removed and cells were incubated for an additional 3 days with various doses of SU6656. After incubation, cells were fixed with paraformaldehyde. Cells were stained with Hoechst and indirect immunofluorescence detection of beta-III tubulin and syanptophysin was performed. Beta-III tubulin is a neuronal marker that identifies cell bodies and neurites. Synaptophysin is a 38 kDa glycoprotein that is a useful pre-synaptic marker used for the identification of normal neuroendocrine cells and neuroendocrine neoplasms.

Plates were scanned using three imaging channels. Figure 7 shows an example of both a composite and individual images taken with the Neuronal Profiling V4 BioApplication. Figure 8 exemplifies the dose-responsiveness of this inhibitor when evaluating different output features compared to untreated wells. The number of spots found within the modified neurite

mask was greatly decreased when comparing treatment to control. The numbers of branches, as well as the total neurite counts were also decreased with SU6656 (although both features peaked at ~2.2 µM). The number of spots found within the cell body were similar to control values for lower doses.

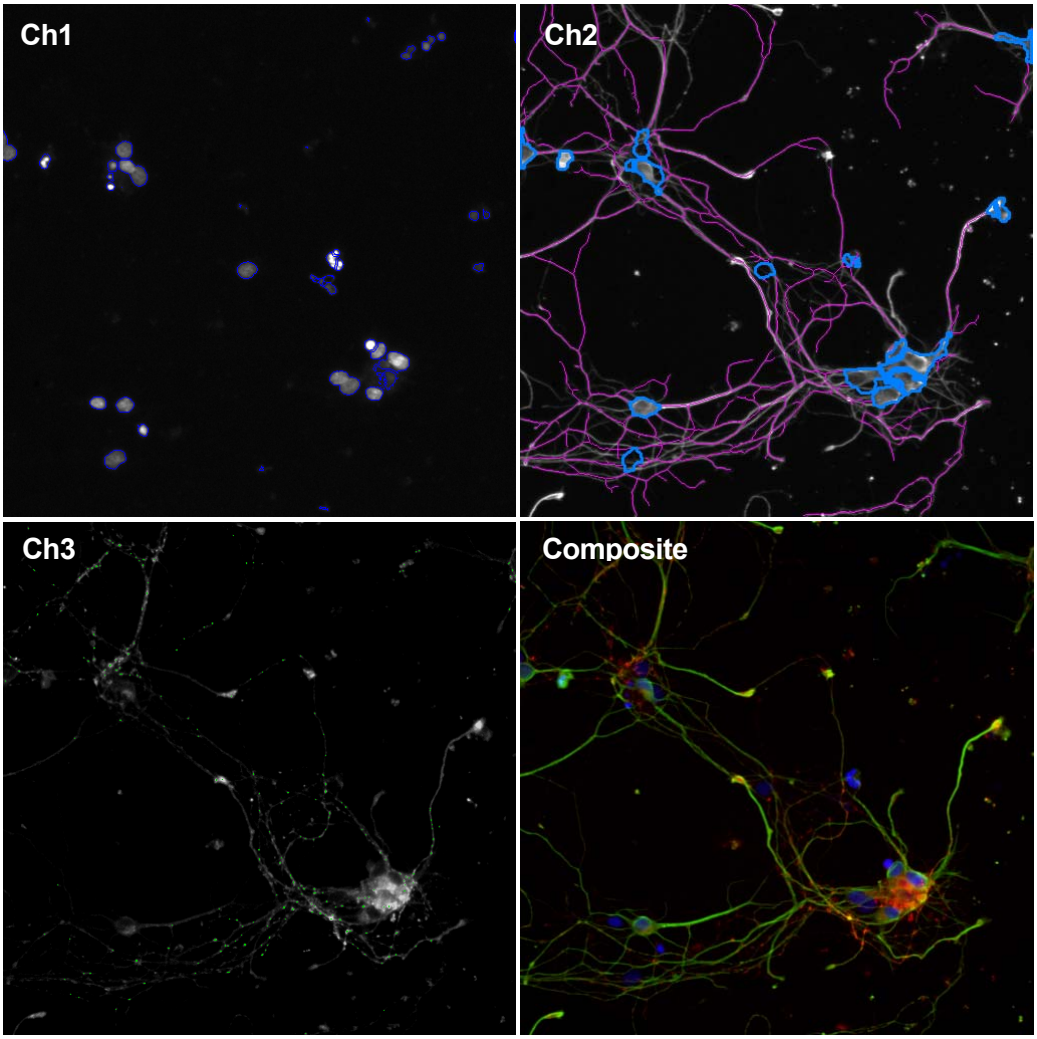


Figure 7*.Utilizing the Neuronal Profiling BioApplication to detect nuclei (Top Left), cell bodies and neurites (Top Right), and spots found within the cell body and modified neurite mask (Bottom Left). Bottom Right: composite image for all three channels (red = synaptophysin staining, green = beta tubulin).*

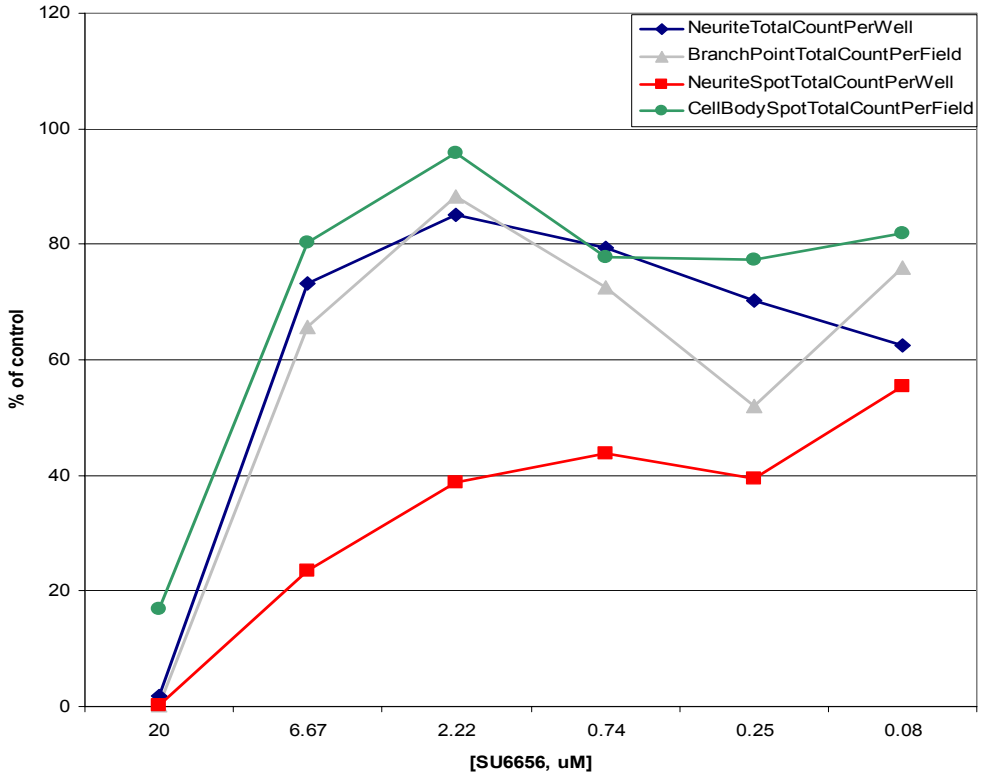


***Figure 8**. Dose-responsiveness of SU6656 as a percent of control in primary hippocampal neurons. Shown are various Well Output Features that were derived from Field Features. Data was taken from a total of 30 fields per well, with each compound concentration in duplicate.*

References

Das, K. P., T. M. Freudenrich, and W. R. Mundy. 2004. Assessment of PC12 cell differentiation and neurite growth: a comparison of morphological and neurochemical measures. *Neurotoxicology and Teratology*. 26: 397-406.

Fields, R. D., and P. G. Nelson. 1992. Activity-dependent development of the vertebrate nervous system. *Int. Rev. Neurobiol.* 34: 133–214.

Greene, L. A., S. E. Farenelli, M. E. Cunningham, and D. S. Park. 1998. Culture and experimental use of the PC-12 rat pheochromocytoma cell line. In *Culturing Nerve Cells,* eds. G. Banker and K. Goslin, 161–187. Cambridge, MA: MIT Press.

Gritti, A., E. A. Parati, L. Cova, P. Frolichsthal, R. Galli, E. Wanke, L. Faravelli, D. J. Morassutti, F. Roisen, D. D. Nickel, and A. L. Vescovi. 1996. Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. *J. Neurosci.* 6: 1091–100.

Meriane, M., J. Tcherkezian, C. A. Webber, E. I. Danek, I. Triki, S. McFarlane, E. Bloch-Gallego, and N. Lamarche-Vane. 2004. Phosphorylation of DCC by Fyn mediates Netrin-1 signaling in growth cone guidance. *J. Cell Biol.*167: 687-698.

Ooyen, A. V., J. V. Pelt, and M. A. Corner. 1995. Implications of activity dependent neurite outgrowth for neuronal morphology and network development. *J. Theor. Biol.* 172: 63–82.

Paveliev, M., M. S. Airaksinen, and M. Saarma. 2004. GDNF family ligands activate multiple events during axonal growth in mature sensory neurons. *Mol. Cell. Neurosci*. 25: 453-459.

Spitzer, N. C. 1991. A developmental handshake: neuronal control of ionic currents and their control of neuronal differentiation. *J. Neurobiol.* 22: 659–763.

Thoenen, H. 1991. The changing scene of neurotrophic factors. *Trends Neurosc.* 14: 165–170.

Tsuji, M., O. Inanami, and M. Kuwabara. 2001. Induction of neurite outgrowth in PC-12 cells by α-phenyl-N-tert-butylnitro through activation of protein kinase C and the ras-extracellular signal-regulated kinase pathway. *J. Biol. Chem*. 276: 32779–32785.

Wu, Y. Y., and R. A. Bradshaw. 1996. Synergistic induction of neurite outgrowth by nerve growth factor or epidermal growth factor and nerve interleukin-6 in PC-12 cells. *J. Biol. Chem.* 271: 13033–13039.

Zhang, J., T. D. Chung, and K. R. Oldenberg. 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomolecular Screening* 4: 67–73.

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

Overview

Often, the nature of the cell body labeling is such that most cell bodies contain a dark region caused by displacement of the Channel 2 fluorophore by the nucleus. Nuclei are identified first in Channel 1, followed by cell bodies in Channel 2. Nuclear masks can be pasted into the cell bodies to fill the corresponding holes. Nucleus and cell body features are computed and these objects are validated independently using the Channel 1 and Channel 2 Object Selection Parameters, respectively. Valid nuclei are associated with the cell bodies to determine the number of nuclei per cell body. This feature is a Channel 2 cell body validation criterion, and a cell body is rejected if it does not have the number of nuclei within the range specified by the **CellBodyNucCountCh2** Object Selection Parameter. Cell bodies are then selected based on intensities in Channels 3-6 (i.e., gating).

The neurite mask is created from the Channel 2 image using a form of neurite detection (**NeuriteDetectMethodCh2** and **NeuriteDetectRadiusCh2**) and then applying an intensity threshold (**NeuriteIdentificationModifierCh2**). The neurites are then analyzed and validated (using Object Selection Parameters). Neurite branch and cross points are counted *after* validation to avoid counting crossings by invalid neurites.

Spots are detected and identified in Channel 3 and Channel 4, and subsequently associated with either the cell body mask or modified neurite mask. Overlap analysis is then performed based on spot masks found between Channel 3 and Channel 4, and the spot features and overlap features are extracted.

Finally overlays are created and displayed depending on the number of channels, whether spot analysis was performed in Channel 3 and/or Channel 4, and if the **MinAreaSpotOverlapCh3Ch4** Assay Parameter has a value other than zero. Cell and Field features are reported. The Cell Features are aggregated over the fields in the well and are reported as Well Features. Select Field Features are also either aggregated or calculated into Well Features.

Object Identification Methods

To identify objects in each of the images from the different channels, an independent intensity threshold must be set for each channel. In Channel 1, nuclei are identified; only pixels with intensities above this threshold will be considered as belonging to these structures. In Channel 2, cell bodies are identified, and pixels with intensities above this threshold are used to help estimate target counts. Depending on the properties of the objects being identified in Channel 1 and Channel 2, the proper setting of intensity thresholds for the channels is necessary to ensure proper quantitative analysis. If additional channels are desired, Channels 3 and 4 are used to help identify spots within the neurite and cell body masks.

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

Intensity Threshold Method	Value Range	Channel Availability			
		Channel 1	Channel 2	Channels 3-4	Channels 5-6
None	0			✓	✓
Isodata	-0.99 – 9.99	✓	✓	✓	
Triang	-0.99 – 9.99	✓	✓	✓	
Fixed	0 - 32767	✓	✓	✓	

Table 3. *Intensity Threshold Methods Available for Each Channel*

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

If "**None**" is selected for Channel 3 and/or Channel 4, spot analysis will NOT be done.

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

The **None** intensity threshold method indicates that no intensity threshold is applied. This option is not available in Channel 1 or Channel 2 because in these channels an intensity threshold is required to define the pixels making up the neuron and the targets. The choice of the **None** threshold is one option for Channel 3 and Channel 4 - if **None** is selected for either/both channel(s), gating will be performed using the modified cell body mask and spot detection will not be performed. The choice of the **None** as the object identification threshold is the only option for Channel 5 and Channel 6.

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

The other threshold methods (**Isodata** and **Triang**) are known as histogram-derived thresholds in that the threshold is chosen from the histogram of pixel intensities in the image

(i.e., the image's brightness histogram). The schematics in Figure 9 demonstrate how these histogram-derived threshold values are calculated.

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

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

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

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

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

Table 4. *Intensity Threshold Descriptions and ranges available for the Neuronal Profiling V4 BioApplication*

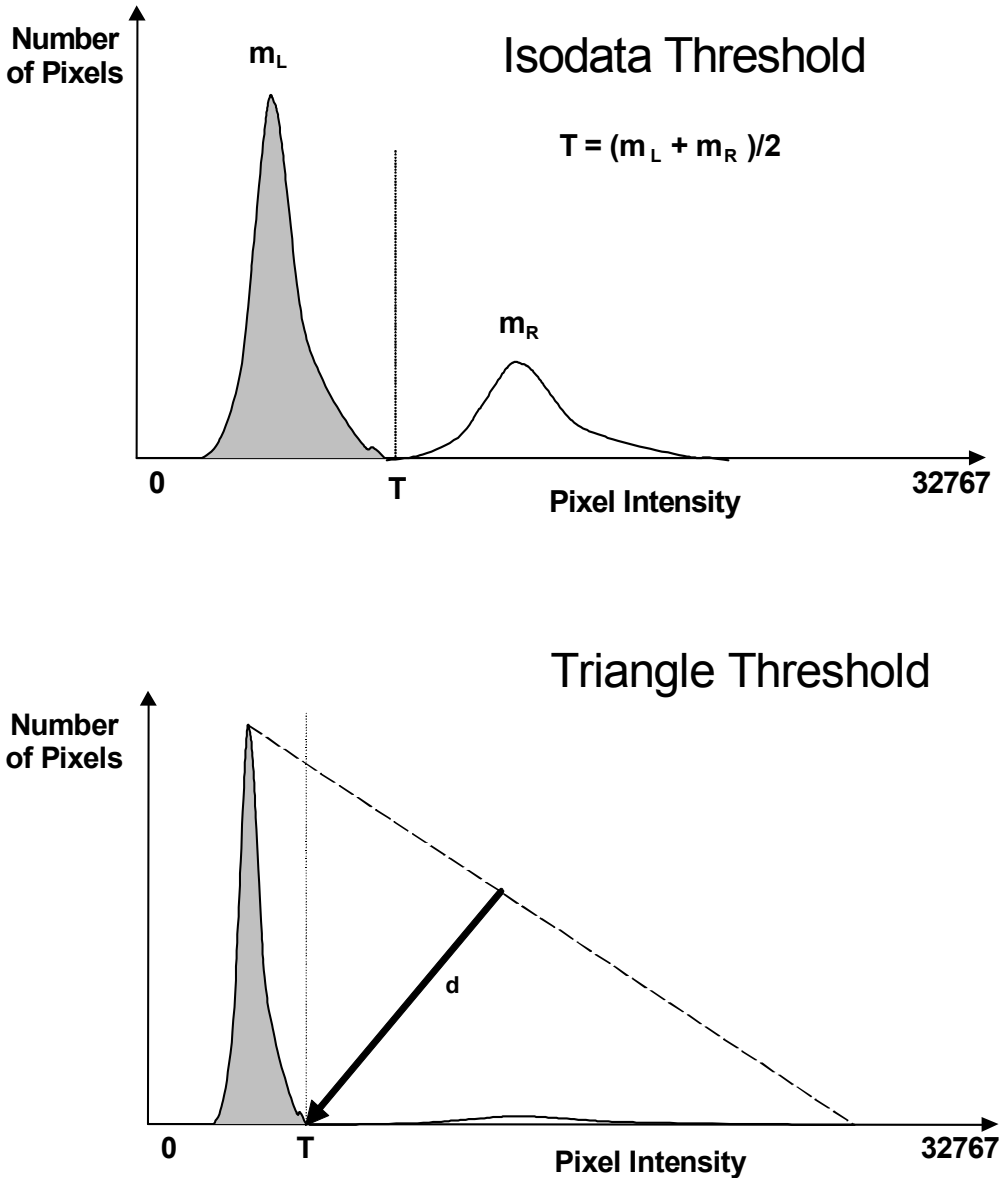


Figure 9. *Histogram-derived Intensity Threshold Methods: Top – Isodata; Bottom - Triangle. Background peak is shown in gray and object peak is shown in white.*

Description of Assay Parameters and Settings

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

Assay Parameters for Image Analysis

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**
- **Reject Border Objects**

Reference Well Control

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

Units for Morphological Measurements

Areas and lengths can be reported in either calibrated units (micrometers) or pixels. This option is controlled by the **UseMicrometers** Assay Parameter. Setting this Assay Parameter to a value of **1** reports results in micrometers, and setting it to **0** (zero) reports results in pixels. The conversion factor from pixels to micrometers is calculated automatically from the Objective and Camera Acquisition Mode selected. It is recommended that **UseMicrometers** be set to **1** for this BioApplication since neurite lengths are easier to compare across experiments if they are expressed in calibrated units.

NOTE

When **UseMicrometers =1**, the Advanced Assay Parameters (LevelHigh) **CellBodyNucTotalAreaCh1, CellBodyAreaCh2**, **NeuriteTotalLengthCh2, NeuriteAvgLengthCh2, NeuriteMaxLengthWithBranchesCh2, NeuriteMaxLengthWithoutBrancesCh2, NeuriteWidthCh2, NeuriteTotalAreaCh2, BranchPointCountPerNeuriteLengthCh2, BranchPointAvgDistanceFromCellBodyCh2, CellBodySpotTotalAreaChN (N = Ch3, Ch4), CellBodySpotOverlapAreaChN, NeuriteSpotTotalAreaChN, NeuriteSpotAvgAreaChN, NeuriteSpotOverlapAreaChN,** and the Object Selection Parameters, **NucAreaCh1, CellBodyAreaCh2, NeuriteLengthCh2**, **NeuriteWidthCh2** and **SpotAreaCh3, Ch4** will be specified in μm^2 or μm.

Object Type

NucTypeCh1, CellBodyandNeuriteTypeCh2 , SpotTypeCh3, SpotTypeCh4

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

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

Table 5**. Binary settings for **NucTypeCh1**, **CellBodyandNeuriteTypeCh2, SpotTypeCh3,** and **SpotTypeCh4.

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 objects and those lower than the threshold as background.

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

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

Background Correction

BackgroundCorrectionCh*N*

Prior to image analysis, the non-cellular background can be computed and subtracted from the image separately in each channel, if desired, as shown in Figure 10.

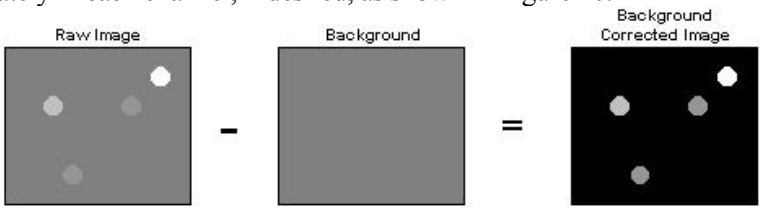


Figure 10. *Image depicting calculation of positive Background Correction that can be used for each channel*

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

If the **BackgroundCorrectionCh*N*** Assay Parameter is given a negative value, an optional background correction method based on the 3D surface fitting is applied. The absolute value entered corresponds to the radius of an area used to find local minima across the image. Found minimum values are be used to construct a 3D surface of a background which is then subtracted from the original image. The main advantage of the method is that it minimizes the effect of the background correction (removal) procedure on the intensity values of the analyzed objects. In both cases, the larger the absolute value, the larger the sampled region, and the less subtraction is done. Setting the value to **–1** lets the application decide on the value needed for the optimal background correction. Table 6 gives an overview of the different Background Correction settings that can be used for each channel.

Setting	Background Correction Method
0	No background correction is performed
Positive	Low pass filter method
Negative	3D surface fitting method
-1	Automated surface fitting is performed and the computed background is subtracted.

Table 6. *Possible Background Correction Methods used with the Neuronal Profiling V4 BioApplication*

NOTE

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

Background Correction can be adjusted on each channel separately.

The background-corrected image is not stored or shown.

Object Smoothing

NucSmoothFactorCh1, CellBodySmoothFactorCh2, NeuriteSmoothFactorCh2, SpotSmoothFactorCh3, SpotSmoothFactorCh4

Object smoothing can be done separately within both channels for nuclei, cell bodies, and neurites. The image may require a degree of smoothing (i.e., blurring) before the specific object can be identified. Smoothing may consolidate fragmented nuclei and rounds their boundaries, giving a more natural shape. The values for **NucSmoothFactorCh1, CellBodySmoothFactorCh2, NeuriteSmoothFactorCh2, SpotSmoothFactorCh3,** and **SpotSmoothFactorCh4** specify the degree of smoothing. A value of zero means that smoothing is not applied.

For identification of neurites, smoothing may increase neurite connection to cell bodies, reconnect fragmented neurites, and smooth any jagged appearances. However, too much smoothing can also merge adjacent neurites into a single neurite.

Reject Border Objects

RejectBorderNucsCh1 and RejectBorderCellBodiesCh2

The rejection of border-touching objects is specified by the **RejectBorderNucsCh1** and **RejectBorderCellBodiesCh2** Assay Parameters. Setting both of these Assay Parameters to **1** will reject nuclei and cell bodies that touch the edge of the field. Setting the values to **0** (zero) will include border-touching nuclei and cell bodies as valid objects.

Rejecting border-touching nuclei can lead to the rejection of cell bodies in Channel 2 (if the user-specified required number of nuclei per cell body [**CellBodyNucCountCh2** Object Selection Parameter] is not met). Also, if any cell bodies in Channel 2 are rejected, nuclei associated with the cell body are also rejected, regardless of the value for **RejectBorderNucsCh1**.

Rejecting border-touching cell bodies will lead to the rejection of their associated nuclei in Channel 1. Also note that all Object Selection criteria for Channel 2 must be met in order to not reject the cell body (regardless of the value for **RejectBorderCellBodiesCh2**).

Channel-Specific Assay Parameters

Channel 1: Nucleus Mask Creation

The following Assay Parameters control creation of the nucleus mask in Channel 1: **NucSmoothFactorCh1** , **NucSegmentationCh1**, and **RejectBorderNucsCh1.**

NucSmoothFactorCh1

This was previously mentioned in the section under *Object Smoothing*.

NucSegmentationCh1

Even though you may have chosen an optimal intensity threshold and an appropriate degree of smoothing for object identification, you may encounter situations where individual 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 nuclear segmentation in the Neuronal Profiling BioApplication: Geometric (shape and size) and Intensity (intensity peaks). These methods are illustrated in Figure 11.

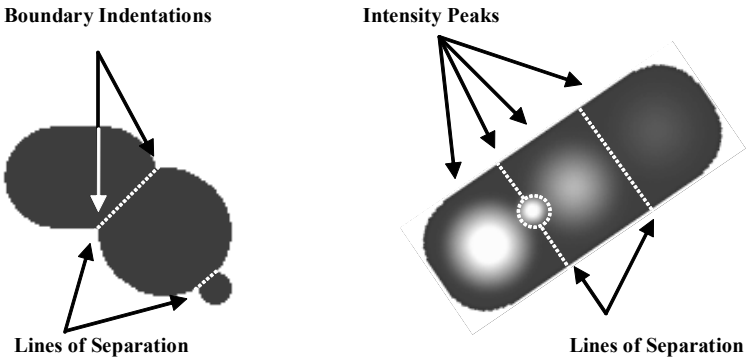


Figure 11: *Object Segmentation Options. The image on the left illustrates the Geometric Method while the image on the right illustrates the Intensity Method.*

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

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

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

Setting **NucSegmentationCh1** to a negative value selects the peak Intensity method. In this case, the absolute value of the parameter specifies the minimum relative height of the intensity peak (image contrast) to be used in the segmentation. Making **NucSegmentationCh1** more negative reduces the number of objects created by the segmentation. In case of noisy and textured objects the use of object smoothing may be required to reduce over segmentation. The value of this parameter for the Intensity method does not depend on the power of the microscope objective or camera setting (pixel binning).

A value of zero for **NucSegmentationCh1** disables the segmentation.

Segmentation Method	NucSegmentationCh1 Assay Parameter	Value Range
None	0	0
Geometric	radius (in pixels) of smaller object	1 to 255
Intensity	minimum relative height of intensity peak (local contrast)	-1 to -32767

Table 7: *Channel 1 Object Segmentation Options*

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

Geometric Method Setting the **NucSegmentationCh1** value to R1 (Figure 12 A) produces object separation shown in Figure 12B. Setting the **NucSegmentationCh1** value to R3 results in segmentation shown in Figure 12C. Setting the **NucSegmentationCh1** value to R2 (the largest radius of all touching objects) produces no segmentation.

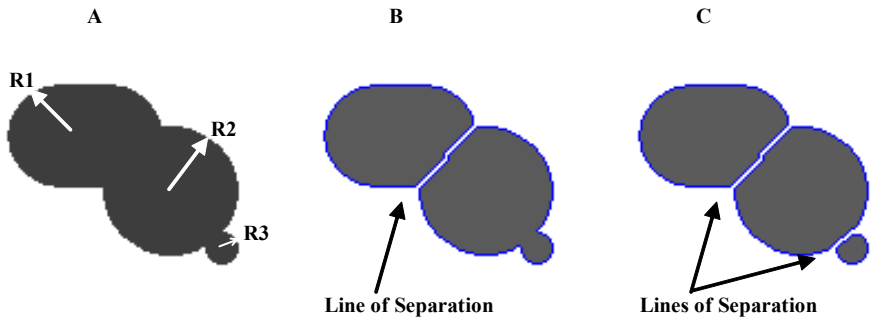


Figure 12: *Object segmentation: Geometric Method. A) Radii of touching objects: R1 = 24, R2 = 30, R3 = 7; B) Result of segmentation using* ***NucSegmentationCh1*** *= R1; C) Result of segmentation using* ***NucSegmentationCh1*** *= R3.*

Intensity Method Figure 13 shows the intensity profile along the cordial line of an object with four intensity peaks from Figure 11. Relative peak intensity heights measured from the top of a peak to the nearest valley for each of the object are:1500 (#1), 1250 (#2), 500 (#3), and 200 (#4).

In general terms, these peak intensity heights are nothing but measures of local contrast of an image. The degree of object segmentation can be controlled by setting the **NucSegmentationCh1** parameter value equal to the lowest relative height of intensity peaks of objects that need to be separated. The lower the value, the more objects will be segmented and vise versa. In case of over segmentation. the setting the **ObjectSmoothFactorCh1** value greater than 0 should be used to alleviate the problem.

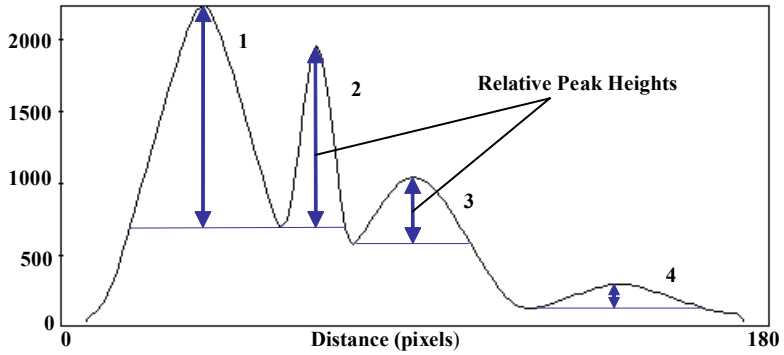


Figure 13: *Intensity profile of an object with four intensity peaks. Peak #1 height = 1500, peak #2 height = 1250, peak #3 height = 500 and peak #4 height = 200.*

Only two peaks in Figure 13, #1 and #2, have relative intensity greater than 1000. Thus, setting the **NucSegmentationCh1** parameter to -1000 marks only two peaks to be used for segmentation (or marks the two objects that need to be separated) and results in object segmentation as shown in Figure 14A. Setting the **NucSegmentationCh1** parameter to -400 or -100 marks three or all four objects that need to be separated. The results of segmentation of three and four objects are shown in Figure 14B and 14C.

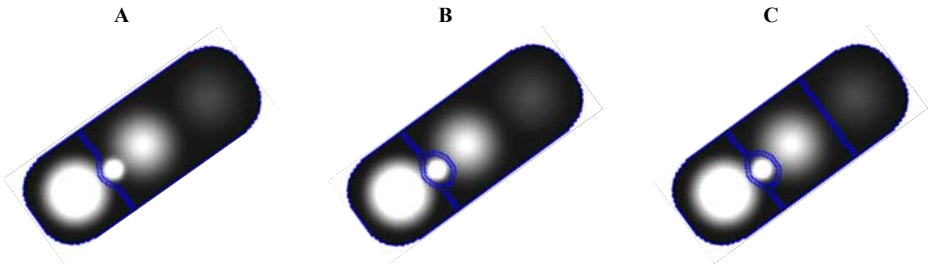


Figure 14: *Object segmentation: Intensity Method. A) Result of segmentation using parameter **NucSegmentationCh1** = -1000; B) Result of segmentation using parameter **NucSegmentationCh1** = -400; C) Result of segmentation using parameter **NucSegmentationCh1** = -100.*

RejectBorderNucsCh1

This was previously mentioned in the section under *Reject Border Objects*.

Channel 2: Cell Body Mask Creation

Creation of the cell body mask has two important aspects: (1) In many cells there is an intensity hole in the cell body region that corresponds to the nucleus, and (2) the neurites attached to the cell body must be removed prior to calculation of cell body features. These features require that several additional Assay Parameters be optimized to detect cell bodies appropriately. The Basic Assay Parameters that affect the creation of the cell body mask in Channel 2 are:

- **CellBodySmoothFactorCh2**
- **MinCellBodyNucOverlapCh2**
- **CellBodyDemarcationCh2**
- **CellBodySegmentationCh2**
- **UseNucForCellBodySegmentationCh2**
- **CellBodyMaskModifierCh2**
- **RejectBorderCellBodiesCh2**

CellBodySmoothFactorCh2

This was previously mentioned in the section under *Object Smoothing*.

MinCellBodyNucOverlapCh2

The openings in the cell body image and mask must be filled before neurite removal and cell body segmentation. This operation is controlled by the **MinCellBodyNucOverlapCh2** Assay Parameter.

The cell body filling process is illustrated in Figure 15. As shown on the left of the figure, objects are first identified in Channel 1. A test ring is constructed around each object. Next, objects are identified in Channel 2, as shown in the right side of Figure 15. These may be cell bodies or neurites, and may be valid, invalid, border-touching, etc. The test rings from Channel 1 are then placed on top of the Channel 2 object mask and are used to measure the overlap between Channel 1 and Channel 2 objects. If this overlap is greater than or equal to the **MinCellBodyNucOverlapCh2** Assay Parameter, the Channel 1 object mask is pasted into the Channel 2 cell body mask.

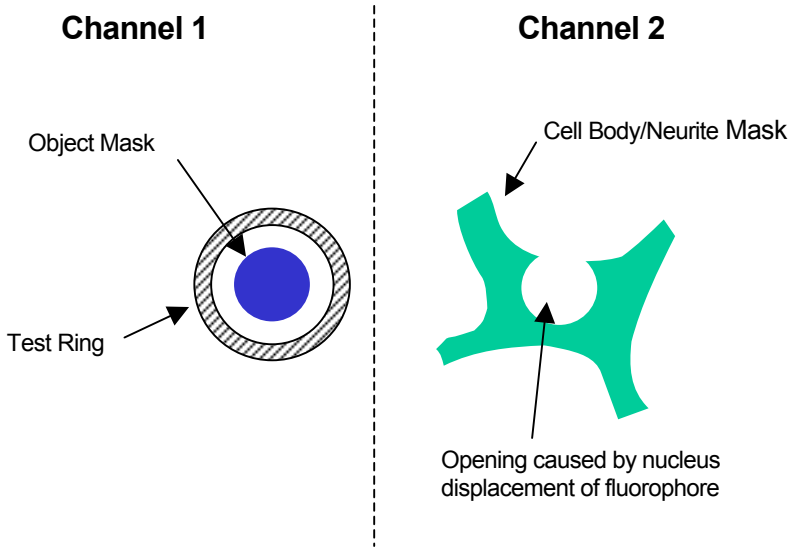


Figure 15. *Process of filling cell body openings*

The value of the **MinCellBodyNucOverlapCh2** Assay Parameter is a percentage and specifies the required overlap between Channel 1 and Channel 2 objects before a Channel 1 object mask can be pasted into Channel 2. The width of the test ring used to measure the overlap is one pixel, and the distance from the Channel 1 object is automatically adjusted by the BioApplication when different microscope objectives and image binning settings are selected. Setting the value of **MinCellBodyNucOverlapCh2** to **0** will result in the pasting of all Channel 1 object masks into Channel 2.

The **MinCellBodyNucOverlapCh2** Assay Parameter is also used later in the analysis process to associate Valid Nuclei with Selected Cell Bodies. This will be described below in the section on Channel 2 Object Selection Parameters.

CellBodyDemarcationCh2

The neurites are processes attached to the cell bodies. For purposes of cell body identification and measurement, a boundary needs to be fixed between the cell body and its neurites. The placement of this boundary is controlled by the **CellBodyDemarcationCh2** Assay Parameter. Smaller values of this Assay Parameter will include more neurite pixels in the cell body and larger values will exclude more neurite pixels from the cell body.

The value of this Assay Parameter is expressed in pixels, and the demarcation boundary will be placed roughly where the half-width of the neurites (in pixels) equals the Assay Parameter value. This Assay Parameter will have to be adjusted for changes in objective and pixel binning. Increasing this value will cause the cell body to round more and help in selecting neurites (refer to Figure 16).

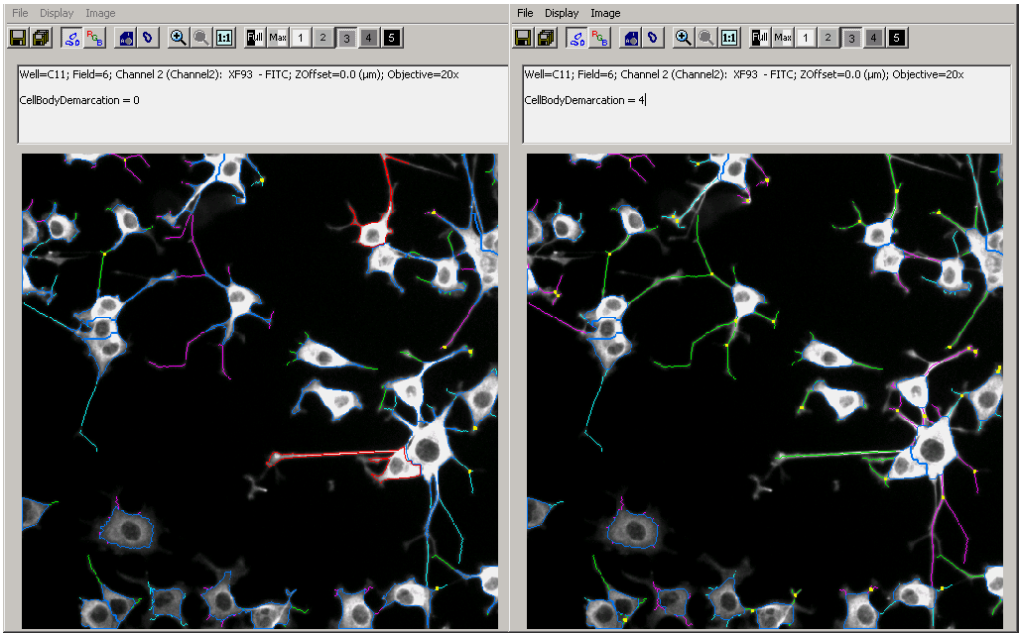


Figure 16. *Adjustment of* ***CellBodyDemarcationCh2****. The image on the left shows* ***CellBodyDemarcationCh2 = 0****, while the image on the right shows the results of increasing this value to* ***4****. Notice that many areas where neurites were misinterpreted as cell body extensions on the left (dark blue or red color) are now correctly selected as neurites on the right. Images taken were 20x, cells were treated with NGF (200 ng/mL) for 72 hours.*

CellBodySegmentationCh2 and UseNucForCellBodySegmentationCh2

Cell Body segmentation is controlled by two Assay Parameters, **CellBodySegmentationCh2** and **UseNucForCellBodySegmentationCh2**. **CellBodySegmentationCh2** allows segmentation of the Channel 2 cell body based on shape or peak intensity, as was previously described for the **NucSegmentationCh1** Assay Parameter.

There are, however, important differences between nucleus and cell body segmentation. Nuclei are convex, circular objects with large, central intensity peaks. Cell bodies are often concave, depending on the degree of neurite removal, and may have a gap in intensity ("hole") within their boundaries, instead of a peak. As a result, it may be difficult to identify good geometric-based or intensity-based seeds to use in segmenting the cell bodies.

A different approach may be to use the validated nuclei identified in Channel 1 to segment the cell bodies. This is controlled by the **UseNucForCellBodySegmentationCh2** Assay Parameter, which has three possible values:

- If **UseNucForCellBodySegmentationCh2 = 0** - Specifies that no nucleus masks are used and that segmentation will proceed solely on the basis of cell body shape and intensity.
- If **UseNucForCellBodySegmentationCh2 = 1** - Specifies that only nucleus masks will be used for segmentation. The cell body is cut along lines determined by dilating the nuclei within a cell body until the cell body is completely filled. The cell body shape and intensity information are ignored, and the **CellBodySegmentationCh2** Assay Parameter has no effect. Note that **CellBodySegmentationCh2** has to be turned on (any value other than **0**) for this to be enabled.
- If **UseNucForCellBodySegmentationCh2 = 2** - Specifies that the nucleus masks will be used for segmentation (**UseNucForCellBodySegmentationCh2**) as well as using cell body shape and intensity information (**CellBodySegmentationCh2**).

Table 8 gives an overview of the segmentation options for this BioApplication.

CellBodySegmentaionCh2	UseNucForCellBodySegmentationCh2	Result
0	0	No cell body segmentation
> 0	0	Cell body segmentation (geometric)
< 0	0	Cell body segmentation (peak intensity)
0	1	No cell body segmentation
Any value other than 0	1	Cell body segmentation using nucleus
0	2	No cell body segmentation
> 0	2	Cell body segmentation (geometric) and nuclei
< 0	2	Cell body segmentation (peak intensity) and nuclei

Table 8. *Overview of segmentation options for the Neuronal Profiling V4 BioApplication*

To prevent segmentation by debris or invalid objects, only validated nuclei are used for Channel 2 segmentation. Results obtained using the cell body segmentation options are illustrated in Figure 17. Note that two different types of cell bodies are included in each situation. The image on the left is a multi-nucleated cell body that the user does not want to segment. It is symmetric in shape and intensity, and does not look like two touching cell bodies. The image on the right illustrates two touching cell bodies, evident from both shape and intensity.

Figure 17A illustrates what happens when cell body segmentation is not used (i.e., **CellBodySegmentationCh2 = 0**). Both cell bodies remain in their original state; the cell on the left remaining as binucleate and the cell on the right not segmented into two separate objects.

Figures 17B and 17C illustrate segmentation obtained by using only cell body shape and peak intensity (i.e., **UseNucForCellBodySegmentationCh2 = 0**). The first case (17B) exemplifies segmentation using shape (i.e., **CellBodySegmentationCh2 = 15**). This method was unable to segment either cell body. The second case (17C) demonstrates segmentation using peak intensity (i.e., **CellBodySegmentationCh2 = –20**). This method allowed segmentation of the second cell body.

In Figure 17D, segmentation is done using only nucleus information (**UseNucForCellBodySegmentationCh2 = 1**). Both cell bodies are segmented, and the results are independent of the value specified for **CellBodySegmentationCh2**, since this Assay Parameter is ignored when **UseNucForCellBodySegmentation = 1**.

Examples for Figures 17E and 17F illustrate the effects of adding cell body shape and intensity to nucleus-based segmentation (i.e., **UseNucForCellBodySegmentationCh2 = 2)**. The first case (17E) adds cell body shape information (i.e., **CellBodySegmentationCh2 = 15**), and the second case (17F) adds cell body intensity (i.e., **CellBodySegmentationCh2 = –20**).

NOTE

If you do not want to analyze multinucleated cells, they can be rejected from analysis by simply setting the Object Selection Parameter **CellBodyNucCountCh2** to a minimum and maximum of 1 and then using **CellBodySegmentationCh2** to separate cells that are touching.

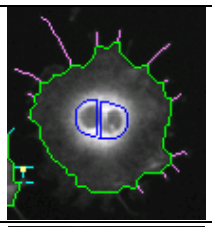
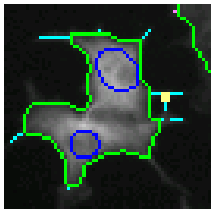
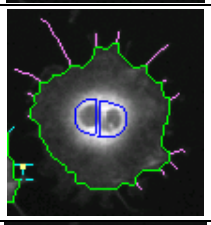
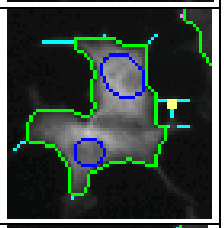
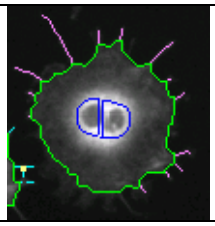
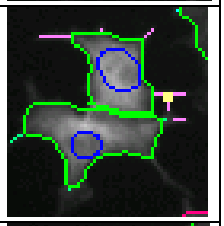
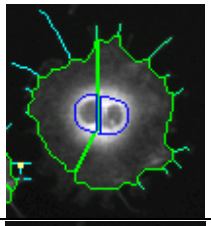
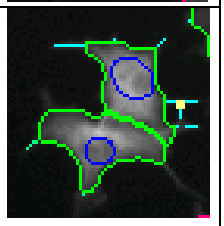
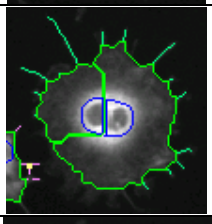
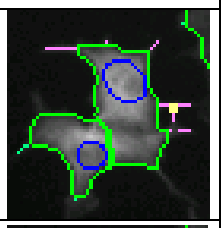
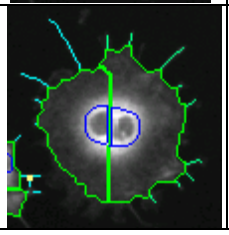
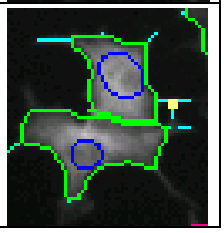
A. **CellBodySegmentationCh2 = 0** **UseNucForCellBodySegmentationCh2 = 0**		
B. **CellBodySegmentationCh2 = 15** **UseNucForCellBodySegmentationCh2 = 0**		
C. **CellBodySegmentationCh2 = -20** **UseNucForCellBodySegmentationCh2 = 0**		
D. **CellBodySegmentationCh2 = 15** ***or*** **CellBodySegmentationCh2 = -20** **UseNucForCellBodySegmentationCh2 = 1**		
E. **CellBodySegmentationCh2 = 15** **UseNucForCellBodySegmentationCh2 = 2**		
F. **CellBodySegmentationCh2 = -20** **UseNucForCellBodySegmentationCh2 = 2**		

Figure 17. *Examples of cell body segmentation options (Nucleus overlay = Blue, Cell Body overlay = Green)*

CellBodyMaskModifierCh2 and CellBodyMaskModifierCh*N*

The size of the cell body mask can be modified for several purposes. The Assay Parameters that control this are **CellBodyMaskModifierCh2** and **CellBodyMaskModifierCh*N***, where Ch*N* is the relevant dependent channel (i.e., Channels 3-6). Its value specifies the number of pixels by which the cell body mask created in Channel 2 is enlarged (positive value) or reduced (negative value).

In Channel 2, one reason for modifying cell body size could be to eliminate the fringe of short neurites that may be identified around the boundary of cell bodies. The cell body needs to be made larger to get rid of this fringe, but doing this by changing the identification threshold may not be an option. In this case, the cell body size can be modified directly using the **CellBodyMaskModifierCh2** Assay Parameter.

In Channels 3-6, the modified cell body is only used to make intensity measurements. Adjustment of the **CellBodyMaskModifier** in Channel 3 or Channel 4 will only be used for gating purposes and will not change spot count features within the cell body or modified neurite masks. When **None** is selected as the Object Identification Threshold in Channel 3 and/or Channel 4, the modified cell body mask will be the only mask present when clicking Identify Objects in the Protocol Interactive View. The modified cell body mask is the only mask present in Channel 5 and Channel 6 and measurements can only be used for gating. The effect of this Assay Parameter is illustrated in Figure 18, where the value was positive and the cell body area has been expanded in order to make the intensity measurement.

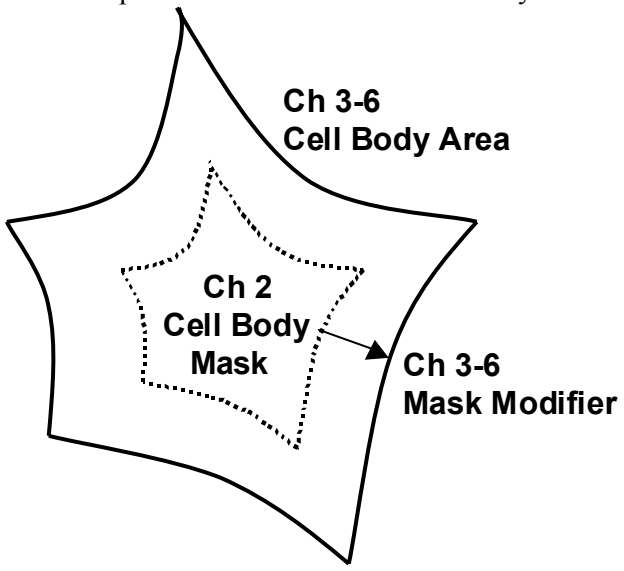


Figure 18. *Adjustment of area using* ***CellBodyMaskModifierChN*** *Assay Parameters for Channels 3-6*

NOTE

Once the Channel 2 mask is adjusted, values for **CellBodyMaskModifierCh*N*** will be based on the *adjusted* Channel 2 mask. Due to cell body density, etc., it is not possible to return to the original, unaltered Channel 2 mask by simply entering the opposite value for **CellBodyMaskModifierCh2** in other channels.

RejectBorderCellBodiesCh2

This was previously mentioned in the section under *Reject Border Objects*.

Channel 2: Neurite Mask Creation

Basic Assay Parameters that affect creation of the neurite mask in Channel 2 are:

- **NeuriteSmoothFactorCh2**
- **NeuriteDetectMethodCh2**
- **NeuriteDetectRadiusCh2**
- **NeuriteIdentificationModifierCh2**
- **NeuritePointResolutionCh2**
- **NeuriteDirectionCh2**
- **RejectMultiplyTracedNeuritesCh2**
- **NeuritePointDisplayModeCh2**
- **UseCellBodyZOIForNeuriteTracingCh2**

NeuriteSmoothFactorCh2

This was previously mentioned in the *Object Smoothing* section.

Neurite Detection

NeuriteDetectMethodCh2, NeuriteDetectRadiusCh2

Neurites are dim, narrow objects that may extend across significant distances over a varying image background. To improve their identification, an imaging processing step, known as detection, is used to remove this background. In the Neuronal Profiling BioApplication, neurite detection is controlled using the **NeuriteDetectMethodCh2** and **NeuriteDetectRadiusCh2** Assay Parameters.

The **NeuriteDetectMethodCh2** Assay Parameter specifies the method used to compute the average value of pixels in the region. There are four methods available in this BioApplication, which are defined in Table 9. The "Box" method (where **NeuriteDetectMethodCh2** = **1**) is recommended for most biologies. This is a general-purpose method that will give good results in most cases. One disadvantage is that this method (and the "Binomial" method) may select an area between multiple neurites if increased too high. You can experiment with the other methods to see if they improve neurite identification for your biologies.

NeuriteDetectMethodCh2 Assay Parameter Value	Name	Description	Advantage
1	Box	The box method replaces each pixel value in an image with the simple arithmetic average value of its neighbors, including itself.	Fastest
2	Binomial	The binomial method replaces each pixel value in an image with the weighted average value of its neighbors, including itself. The weights are set proportional to the binomial coefficients.	Most isotropic
3	Median	The median method considers each pixel in the image and looks at its neighbors. Instead of simply replacing the pixel value with the mean of neighboring pixel values, it replaces it with the median of those values.	Minimal edge blurring
4	Morphological	The morphological (top hat) method computes the difference between the original grayscale image and the processed image.	Minimal edge blurring

Table 9. *Neurite Detection Methods available for the Neuronal Profiling V4 BioApplication*

The **NeuriteDetectRadiusCh2** Assay Parameter specifies the cutoff size of the neurites to be detected (all neurites narrower than the specified size will be detected). The size of the detection region will be related to the width of the neurites you wish to identify. This Assay Parameter may need to be adjusted for different biologies (i.e., different neurite widths), different objectives, and different camera modes. Continuously increasing this value may cause it to pick up false neurites or select areas between two neurites (Figure 19, right).

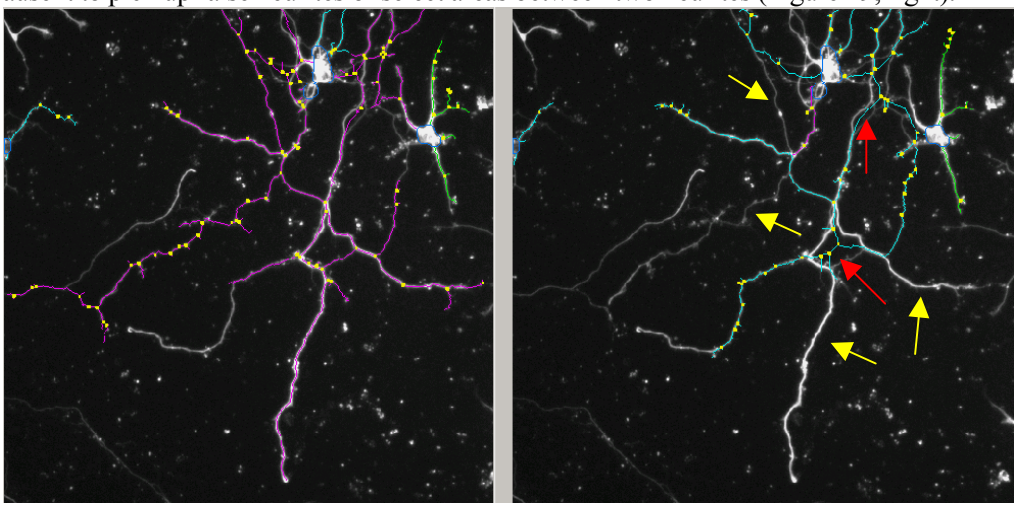


***Figure 19**. Effect of **NeuriteDetectRadiusCh2** values on the identification of primary cortical neurons. The image on the left reflects proper identification whereas the image on the right shows results when the value is too high, causing merging of neighboring neurites (red arrows), or no identification (yellow arrows).*

Neurite Identification

NeuriteIdentificationModifierCh2

After detection, neurites are identified using a fixed threshold. The value of this threshold is determined by the **NeuriteIdentificationModifierCh2** Assay Parameter and the threshold value used to identify cell bodies. The rationale for this approach is that the automated threshold methods used in Channel 2 respond more to the larger objects (i.e., cell bodies), and because the neurites are dimmer than the cell bodies (especially near their ends), a modifier is needed to shift their threshold lower than that of the cell bodies. Negative values for the **NeuriteIdentificationModifierCh2** Assay Parameter will shift the neurite threshold to lower values and include more image pixels in the neurites, as shown in the following figure. However, making a value too negative may actually cause false neurites to appear.

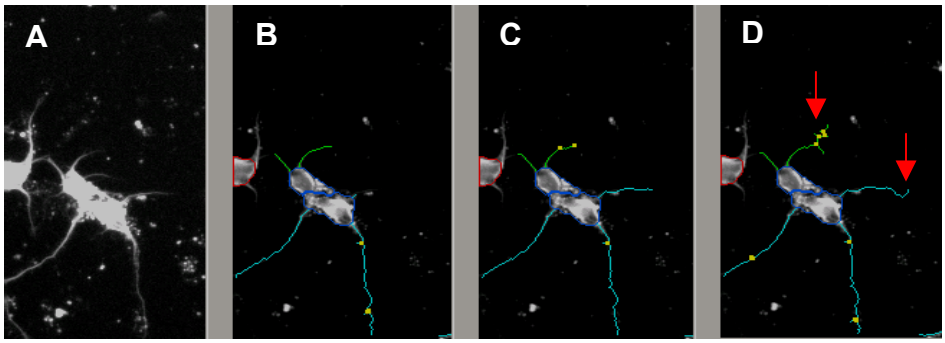


***Figure 20.** Setting the **NeuriteIdentificationModifierCh2** Assay Parameter. This value is varied while the Channel 2 Object Identification value is held constant. As the **NeuriteIdentificationModifierCh2** value is made more negative, dimmer neurites are identified. (A) Raw Image; (B) At less negative values, distal ends of neurites are excluded as they are dimmer than cell bodies; (C) Optimal value will maximize neurite length, but minimize false branch point identification; (D) At more negative values, background pixels are misidentified as neurites/neurite lengths and false branch points are counted (red arrows).*

Neurite Tracing Parameters

Neurite tracing is the process that untangles crossed neurites and assigns them to the correct cell bodies. It begins by skeletonization of the neurites, a process which reduces each neurite to single-pixel-wide lines corresponding to the neurite's medial axis. The skeleton segments are then separated at branch and cross points in the skeleton.

A neurite begins at an initial point where the skeleton segments touch the cell bodies. The process of alternatively adding segments and branch or cross points continues until the end of the neurite is reached. At cross points, the added segment is chosen to minimize the change in direction. For both branch and cross points, the maximum allowed change in direction is 90°.

The Assay Parameters that control neurite tracing are **NeuritePointResolutionCh2**, **NeuriteDirectionCh2** and **RejectMultiplyTracedNeuritesCh2**. The **UseCellBodyZOIForNeuriteTracingCh2** Assay Parameter controls the area to which neurites are traced.

Merging Branch Points

NeuritePointResolutionCh2

During skeletonization, cross points can be converted into pairs of closely spaced branch points, as shown on the left in Figure 21. The value of the **NeuritePointResolutionCh2** Assay Parameter specifies the minimum number of allowed skeleton pixels between adjacent branch points. Branch points that are separated by fewer than this number of pixels are merged into a single cross point.

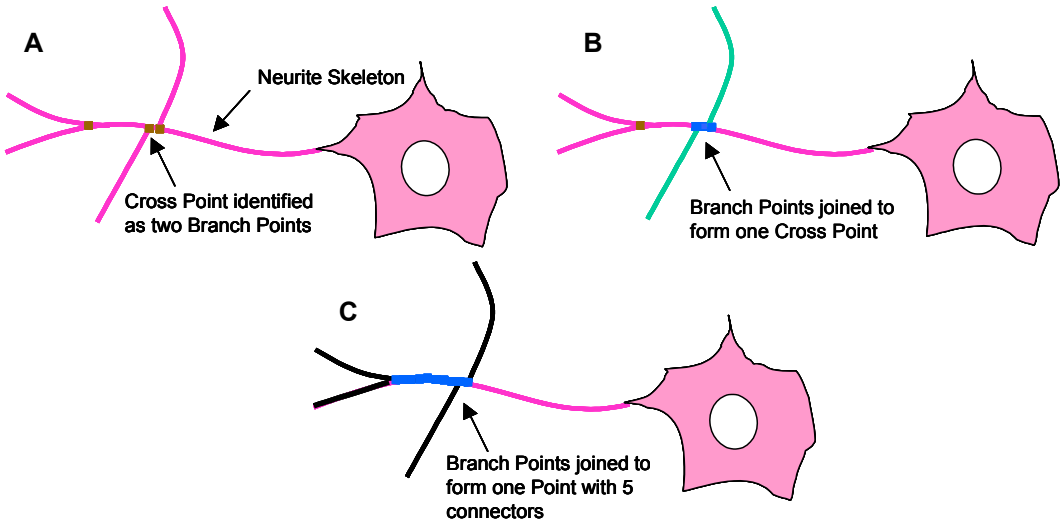


Figure 21. *Joining Branch Points. Image A is an example of two branch points very close to each other that should have been a single cross point (low value used for* ***NeuritePointResolutionCh2****). Image B shows what happens after increasing the* ***NeuritePointResolutionCh2*** *value – the two branches have become a single cross point. Image C illustrates when you make this value too large, multiple branch points can be combined (e.g., five separate connecting points) – if this occurs, this cannot be traced past the initial branch point.*

Tracing Through Cross Points

NeuriteDirectionCh2

The addition of neurite skeleton segment(s) at each critical point requires computing a change in direction. As the following figure illustrates, this is done by fitting the ends of each segment to a straight line and then finding the differences in slopes of these lines. The number of pixels

(i.e., length of the arrows in Figure 22) used to find the direction of each segment is specified by the **NeuriteDirectionCh2** Assay Parameter. This value may need to be adjusted based on the straightness of the neurites in your biology: larger values for straight neurites and smaller values for curved neurites.

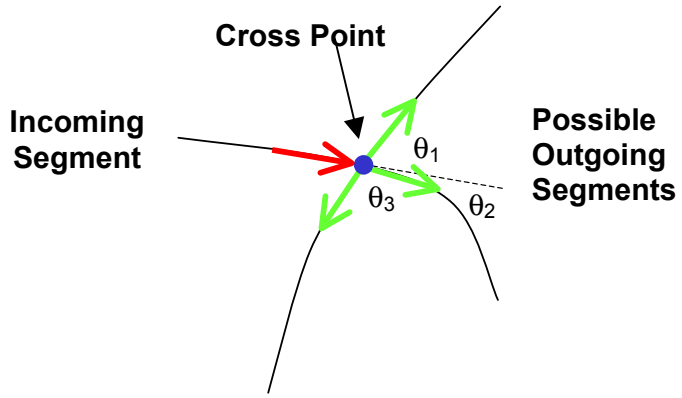


Figure 22. *Directional changes of neurites at critical points (angles are measured between each outgoing segment (green) and the projection of the incoming segment (red)).*

Resolving Multiply Traced Neurites

RejectMultiplyTracedNeuritesCh2

As mentioned above, each initial point results in a traced neurite. Since a neurite may touch several cell bodies, it may be traced several times. This is illustrated in Figure 23. Thus, after neurites are traced, multiply traced neurites must be identified and resolved.

You can specify what happens to multiply traced neurites by setting the **RejectMultiplyTracedNeuritesCh2** Assay Parameter. If **RejectMultiplyTracedNeuritesCh2** = **1**, all multiply traced neurites will be rejected from analysis. In Figure 21 this would mean that neither cell body would have any neurites. If **RejectMultiplyTracedNeuritesCh2** = **0**, one of the multiple neurite copies will be kept and the rest will be rejected. The preserved neurite is assigned to a single cell body.

As Figure 23 illustrates, due to the 90° maximum direction change criterion, multiply traced neurites with critical points will generally be different. In this case, the neurite with the largest length is assumed to be the most complete copy and is kept while the others are rejected (In the following figure, the green neurite would be kept and the orange one would be rejected.). If the multiply traced neurites have the same length, one of them is selected at random and the others are rejected.

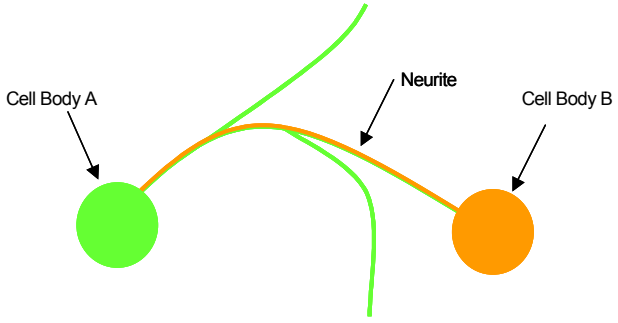


Figure 23*. Resolution of multiply traced neurites. In this example, two cell bodies have a "common" neurite. Because of the maximum 90° direction change criterion, the neurite originating from Cell Body A is longer than the one originating from Cell Body B. If* ***RejectMultiplytracedNeuritesCh2*** *= 0, the neurite is assigned to Cell Body A. If* ***RejectMulitplyTracedNeuritesCh2*** *= 1, neither cell body will have a neurite.*

Merged Neurites

There may be cases where neurites from separate cell bodies merge, making it difficult to determine their individual lengths (Figure 24). Double-counting the neurites will increase statistical error, therefore, a method was designed to decrease overall well length error by reporting the lengths as indicated by the neurite skeleton overlays in the final traced neurite display. In this case, one of the neurites will extend past the merging point, while the other neurite will stop at the merging point, with its total length being less than the neurite extending past the merging point.

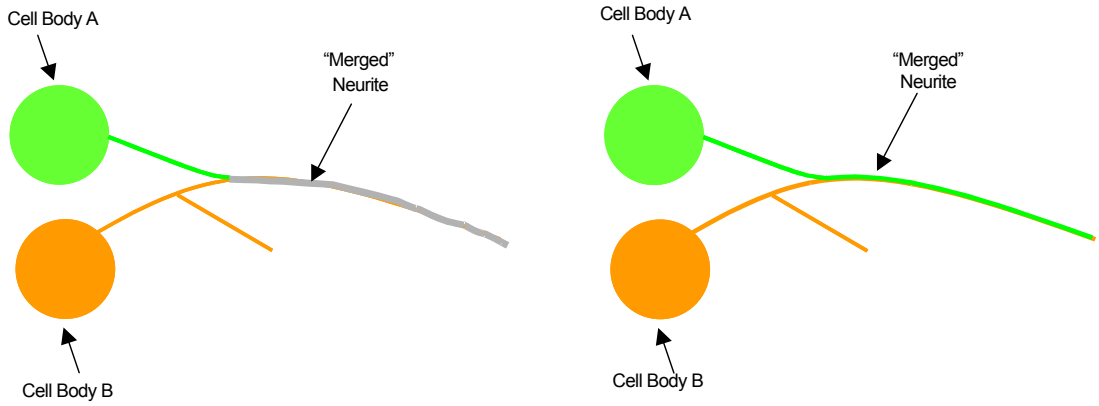


Figure 24. *Determination of merged neurites. The image on the left exhibits two cell bodies where the section in gray is an area that cannot be assigned to either neurite. Instead of double-counting, the algorithm will determine the corrected lengths from the overlays in the final traced neurite display. The order of neurite tracing is based on the cell body positions as left-to-right and bottom-to-top. The neurite originating from Cell Body B will be traced before the neurite originating from Cell Body A. Therefore, the overlay from Cell Body A will overwrite the commonly-traced area from Cell Body B. In the final traced neurite display, the neurite from Cell Body B will stop at the merge point and the neurite from Cell Body A will continue past the merge point. Overall neurite lengths will reflect the final traced neurite display.*

Controlling Neurite Tracing Area

UseCellBodyZOIForNeuriteTracingCh2

It might be difficult to assign ownership of neurites to particular cell bodies when neurites are densely-packed. Therefore, utilization of the **UseCellBodyZOIForNeuriteTracingCh2** Assay Parameter may help alleviate this issue. This Assay Parameter either allows the algorithm to trace the neurites as originally done (value = **0**), or to confine the neurite tracing either equally to all objects (valid and rejected) in Channel 2 (value = **1**) or equally to all valid objects in Channel 2 (Table 10).

UseCellBodyZOIForNeuriteTracing value	Description
0	Tracing of neurites as originally done (no ZOI is incorporated)
1	Use ZOI to confine neurites equally between all objects (rejected and valid)
2	Use ZOI to confine neurites equally between all valid objects

Table 10. *Description for* ***UseCellBodyZOIForNeuriteTracing*** *values.*

Zones of Influence (ZOIs) are created equidistantly from two cell bodies. As seen in Figure 25, the area centrally between the cell bodies has an invisible separation and neurites can only go as far as that separation line. If you have a lot of cell bodies, you can get a good idea as to where the ZOIs are by setting your **CellBodyMaskModifierCh2** to its maximal value (127); see Figure 26. For areas with less cell bodies, the central distance between two cell bodies is created, but the area without any cell bodies will be carried to the end of the field.

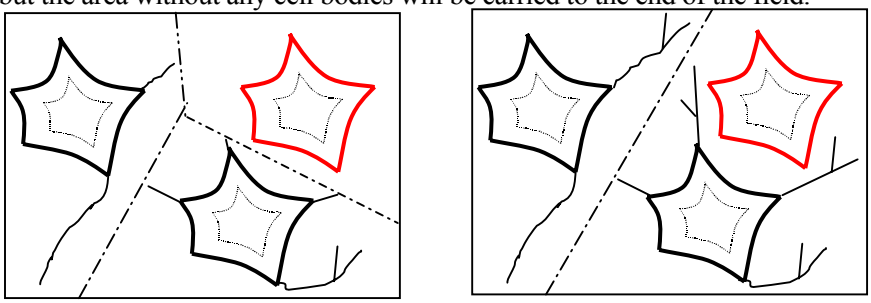


***Figure 25**. Image reflecting how ZOIs are formed between cell bodies using the **UseCellBodyZOIForNeuriteTracingCh2** Assay Parameter. Left: if value = **1**, ZOIs are created from all objects, whether rejected (red) or not. Right: when this value = **2**, only valid objects are used to create ZOIs.*

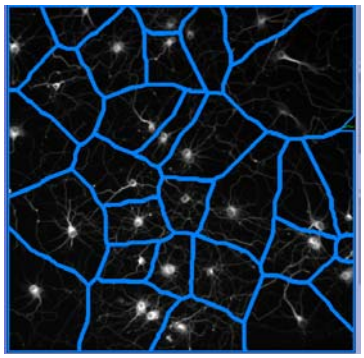


***Figure 26**. Visual representation of ZOI locations by setting **CellBodyMaskModifierCh2** to 127.*

It is up to you to decide which value to select. It is suggested for densely-packed cells that have a lot of debris detected (and therefore rejected), a value of **2** should be most beneficial. It is advisable to use a value of **1** for areas without a lot of debris. If you can easily separate neurites into their respective cell bodies and if you want to try and have as close to a value for length measurements, you should not use this parameter (by setting it to **0**). When using this Assay Parameter, it is important to note that other Assay Parameters may need to be adjusted in order to have a neurite reselected to a particular cell body. If neurites are hard to discern and a value of **2** is selected, it is possible to falsely pick up neurites that might belong to rejected cell bodies but touch other selected cell bodies (Figure 27).

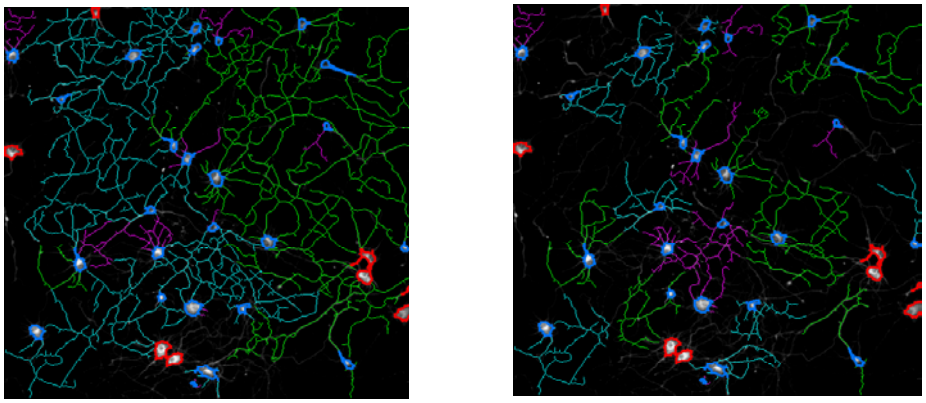


***Figure 27**. Representative image when **UseCellBodyZOIForNeuriteTracingCh2** = 0 (left) vs. = 2 (right). Notice that although the neurites are less on the right, neurites are not all belonging to one neuron and are distributed appropriately.*

Overlay Display Mode for Neurite Branch and Cross Points

NeuritePointDisplayModeCh2

Neurite branch points and cross points (see Figure 1) are collectively called Neurite Points and are displayed as overlays on the channel images controlled by the **NeuritePointDisplayModeCh2** Assay Parameter. As Table 11 illustrates, a setting of **0** displays all Neurite Points; a setting of **1** displays only branch points; and a setting of **2** displays only cross points. If you do not want any neurite points to be displayed, simply uncheck the Neurite Point overlay box in the Image Display Options. Determination of the number of cross points is further explained in Figure 28, based on the number of cell bodies.

NeuritePointDisplayModeCh2 Value	Neurite Point Overlay Representation
0	Branch and Cross Points
1	Branch Points Only
2	Cross Points Only

***Table 11.** Overlay representation for the **NeuritePointDisplayModeCh2** Assay Parameter*

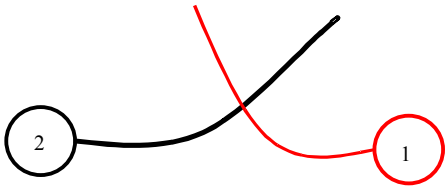
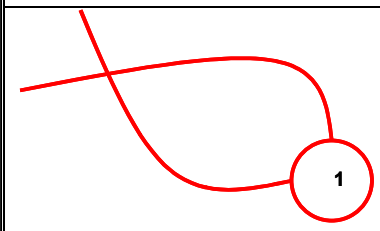
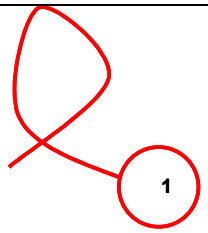
Image Example	Number of Cross Points
 **A**	Cell Body1 = 1 cross point CellBody2 = 1 cross point **Total = 2**
 **B**	Cell Body1 = 1 cross point **Total = 1**
 **C**	CellBody1 = 1 cross point **Total = 1**

Figure 28. *Illustration of counting cross points. (A) Crossing of neurites belonging to different cell bodies =* ***2*** *total cross points (1 per neuron). (B) Crossing of neurites belonging to the same cell body =* ***1*** *cross point. (C) One neurite crossing itself =* ***1*** *cross point.*

Channel 3 and Channel 4 – Spot Detection in Cell Bodies and Neurite Domains

Spots can be detected in Channel 3 and/or Channel 4 within the cell body mask created in Channel 2 (CellBodySpot) or within the neurite mask that can be modified in Channel 3 and/or Channel 4 (NeuriteSpot). Overlays reflect spots in both areas; however, output features keep CellBodySpot and NeuriteSpot values separate. Spots need to be at least 50% within the cell bodies and neurite domains that are selected for analysis.

Since these spots contribute to the fluorescence signal, spots are generally found in image regions that are brighter than the background. Applying an object identification threshold without spot detection may result in clusters of spots linked together by object pixels. Letting **Fixed Threshold = 0** without spot detection (**SpotDetectRadius = 0**) will allow you to detect neurites or cellbodies as spots. This might be beneficial if you have a diffuse stain and want to simply measure this intensity within the neurite or cell body. If it is a punctate stain, it is suggested to use the Basic Assay Parameters below that affect creation of spot overlays in Channel 3 and Channel 4:

- **NeuriteSpotDomainCh3, Ch4**
- **SpotDetectMethodCh3, Ch4**
- **SpotDetectRadiusCh3, Ch4**
- **SpotSmoothFactorCh3, Ch4**
- **SpotSegmentationCh3, Ch4**
- **MinAreaSpotOverlapCh3Ch4**
- **DisplaySpotOverlapCh3Ch4**

NeuriteSpotDomainCh3, Ch4

Although the area encompassing spots in the cell body cannot be adjusted, the area within the neurite mask can be expanded. The **NeuriteSpotDomainModifier** takes the neurites detected in Channel 2 and expands them by the number of pixels set. Any spots found at least 50% within the neurite mask will be selected for analysis. Figure 29 illustrates use of this Assay Parameter.

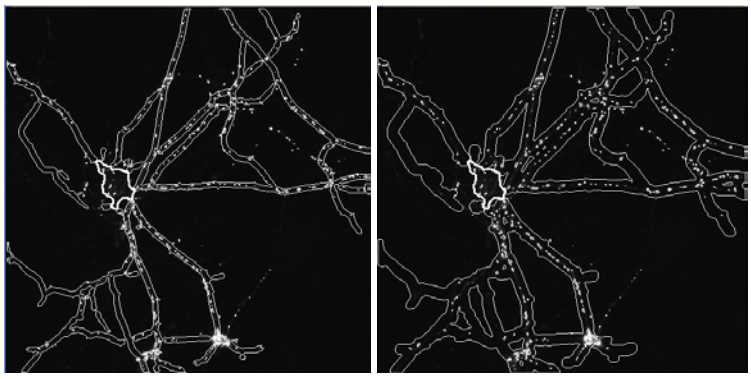


***Figure 29**. Protocol Interactive's Identify Object View where **NeuriteSpotDomainModifierCh3** = 0 vs = 4*

Spots *as* Neurites or Cell bodies vs Spots *in* Neurites or Cell bodies

There is a way to allow the entire neurite or cell body to be detected as a spot (i.e., wanting to look at features for diffuse neurite staining instead of punctate staining in neurite). The procedure to do so is as follows:

1. Set **NeuriteDetectRadius** to **0**. This will turn off detection but will confine spot analysis within the cell body or neuirte domain and allow you to identify spots using Object Identification Threshold, spot smoothing, and background correction.
2. Next, set the Object Identification Method to **Fixed Threshold** and the value to **0**. This will allow the entire neurite and cell body to each be considered a spot.

Figure 30 illustrates this feature.

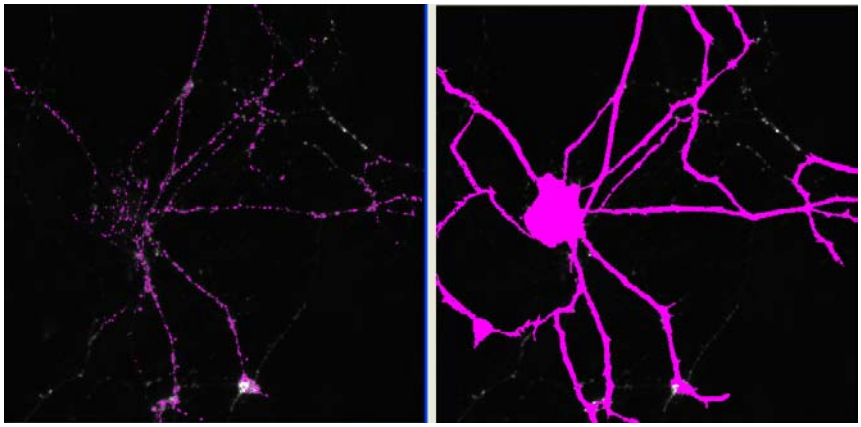


***Figure 30**. Illustration of same image where Fixed Threshold = 40, **SpotDetectRadius** = 1 vs. Fixed Threshold = 0, **SpotDetectRadius** = 0*

Spot Detection

SpotDetectMethodChN, SpotDetectRadiusChN

Spot detection is based on two fundamental spot properties: (1) spots are smaller than the containing object and (2) spots are brighter than the containing object. These properties lead to two different spot detection apporaches. The first is area detection, which identifies spots as areas that can be counted and measured for size, shape, and intensity. The second is peak detection, which identifies spots only as points that can be counted.

The **SpotDetectRadiusChN** Assay Parameter specifies the radius (in pixels) of the sampling region used for spot detection (Table 12). If it is positive, area detection is used; if it is negative, peak detection is used. A value of zero means that no spot detection is used (although you can still use Object Identification Threshold, **BackgroundCorrection**, and **SpotSmoothFactor** to identify spots).

SpotDetectRadiusChN Assay Parameter Value	Method
Positive	Area Detection
0	No Detection
Negative	Peak Detection

***Table 12**. Spot Detection Methods.*

The **SpotDetectMethodChN** Assay Parameter specifies the method used for spot detection. The possible methods are listed in Table 13. In area detection (when **SpotDetectRadius** is positive), a square region of the image surrounding a pixel is sampled and the "average" value of the pixels in the region is computed. The "average" is subtracted from the center pixel, removing the background brightness. This is repeated for each pixel in the image.

Area detection is used when you are primarily interested in measuring the area and intensity features (i.e., total area and total intensity) of the spots, and are less interested in the actual count of the spots (with area detection, counts will only be accurate when the spots are distinct). Of the four types of area detection, the *Box* method is the fastest method and gives good results in most cases. The other methods are somewhat better at detecting very small spots. The *Median* and *Morphological* methods can be used to detect spots located close to large objects.

Peak detection (negative) identifies spikes in pixel intensity within the spot detection region. The first method identifies local maxima and the second identfies local outliers (i.e., pixels brighter than the estimated "normal" range of pixel values). Peak detection should be used for focusing on accurate spot count (and less on actual area measurements). This can be done even if spots are merged together, as long as the intensity peaks reamain distinct. Figure 31 is an example of Peak Detection when **SpotDetectMethod** = 2 with a negative **SpotDetectRadius**.

SpotDetectMethodChN Assay Parameter Value	Area Detection Method (positive)	Peak Detection Method (negative)
1	Box	Local Maxima
2	Binomial	Local Range
3	Median	Local Range
4	Morphological	Local Range

Table 13. *Spot Detection Methods with their corresponding detection obtained from* ***SpotDetectRadius***

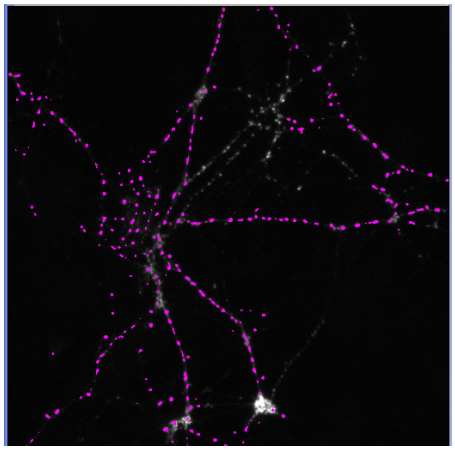


Figure 31. *Illustration of Peak Detection when setting* ***SpotDetectMethod*** *= 1 and a* ***SpotDetectRadius****= -5.*

SpotSmoothFactorCh3, Ch4

This was previously mentioned in the *Object Smoothing* section.

Spot SegmentationCh3, Ch4

Using **SpotSegmentationCh3, Ch4** is the same as what is found for **NucSegmentationCh1**, with positive values selecting the geometric method (approximate radius (in pixels) of the smaller of the two objects being separated) and negative values selecting the peak intensity method (absolute value specifying the minimum height of the peaks used in the segmentation). Figure 32 is a representative image used for segmenting spots.

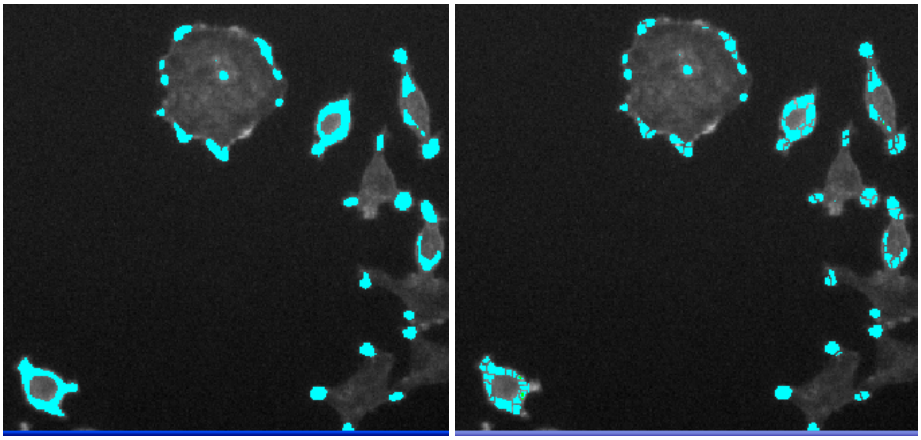


Figure 32. *Illustration of segmenting spots of larger sizes (left) into smaller spots (right). Value of segmentation used was = -2.*

MinAreaSpotOverlapCh3Ch4

It is up to the user to determine if overlap should be analyzed for NeuriteSpots or CellBodySpots between Channel 3 and Channel 4 (i.e., pre- and post-synaptic spots). This is done using the **MinAreaSpotOverlapCh3Ch4** Assay Parameter. If the value is positive, the analysis is based on the spot overlap area (in pixels or micrometers). The larger the value, the larger the area (in pixels or micrometers) that a spot has to overlap between channels (in order for that spot to be considered for overlap analysis). If the value is negative, spot overlap between channels is based on the percentage of spot area overlap relative to the spot area in the current channel. A more negative value constitutes a higher percentage of area that spots have to overlap between channels. When evaluating spot overlap, it is important to set the same value for **NeuriteSpotDomainModifier** in both channels if equal neurite domain areas are desired. Otherwise, the overall percentage may be slightly skewed if the domain was enlarged to include more spots in one channel and not in another.

If you are not interested in analyzing spot overlap, simply set this value to **0**. Turning it off will also give you an additional neurite overlay color in Channel 2. Table 14 and Figure 33 illustrate this principle.

MinAreaSpotOverlapCh3Ch4 Assay Parameter Value	Type of analysis
Positive	Area (in pixels/micrometers) of overlapping spots
0	Do not perform overlap analysis
Negative	Percent of area overlap

Table 14. MinAreaSpotOverlapCh3Ch4 *analysis values.*

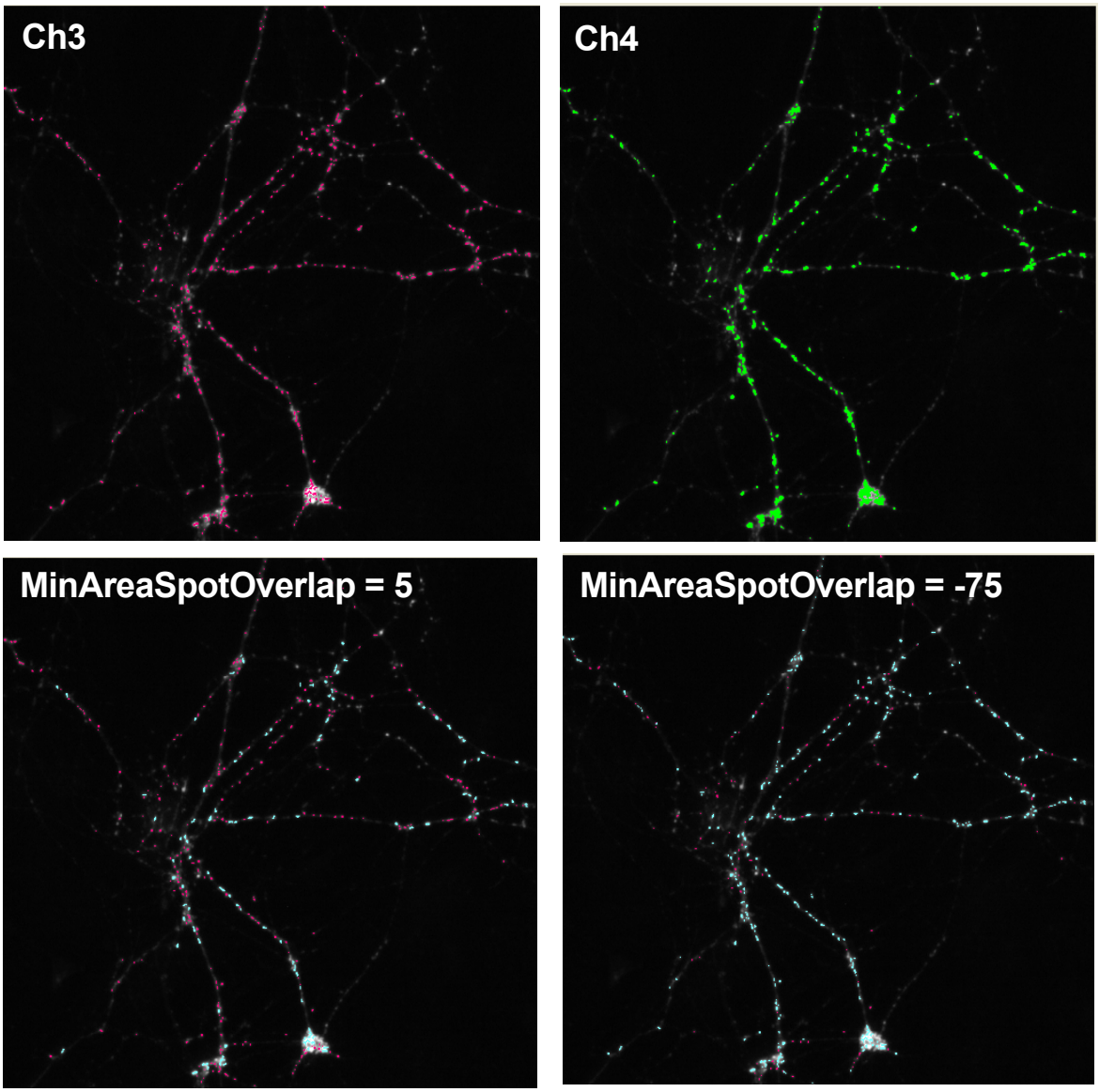


Figure 33. *Representative overlay images of Channel 3 spots (red, top left) and Channel 4 spots (green, top right) and their corresponding overlap from Ch3 (teal, bottom).* ***Bottom Left****: corresponding overlap from Ch3 (teal) when set to 5;* ***Bottom Right****: corresponding overlap from Ch3 (teal) when set to -75, representing 75% or better overlap between channels.*

DisplaySpotOverlapCh3Ch4

Once the **MinAreaSpotOverlapCh3Ch4** is set, the user then must decide which channel to display the derived overlay. For example, if a value of **3** was selected for this Assay Parameter, the overlay will display spot overlap derived from Channel 3 (using the **MinAreaSpotOverlapCh3Ch4** value). Similarly, if a value of **4** was selected for this Assay Parameter, the overlay will display spot overlap derived from Channel 4.

The reasoning for this Assay Parameter is mainly due to the chance of spots being different sizes in each channel, which would account for different overlap values between channels and thus different overlays. Figure 34 illustrates this principle where Channel 4 spots are much larger than Channel 3 and overall interpretation of the **MinAreaSpotOverlap** value can alter the overlay view.

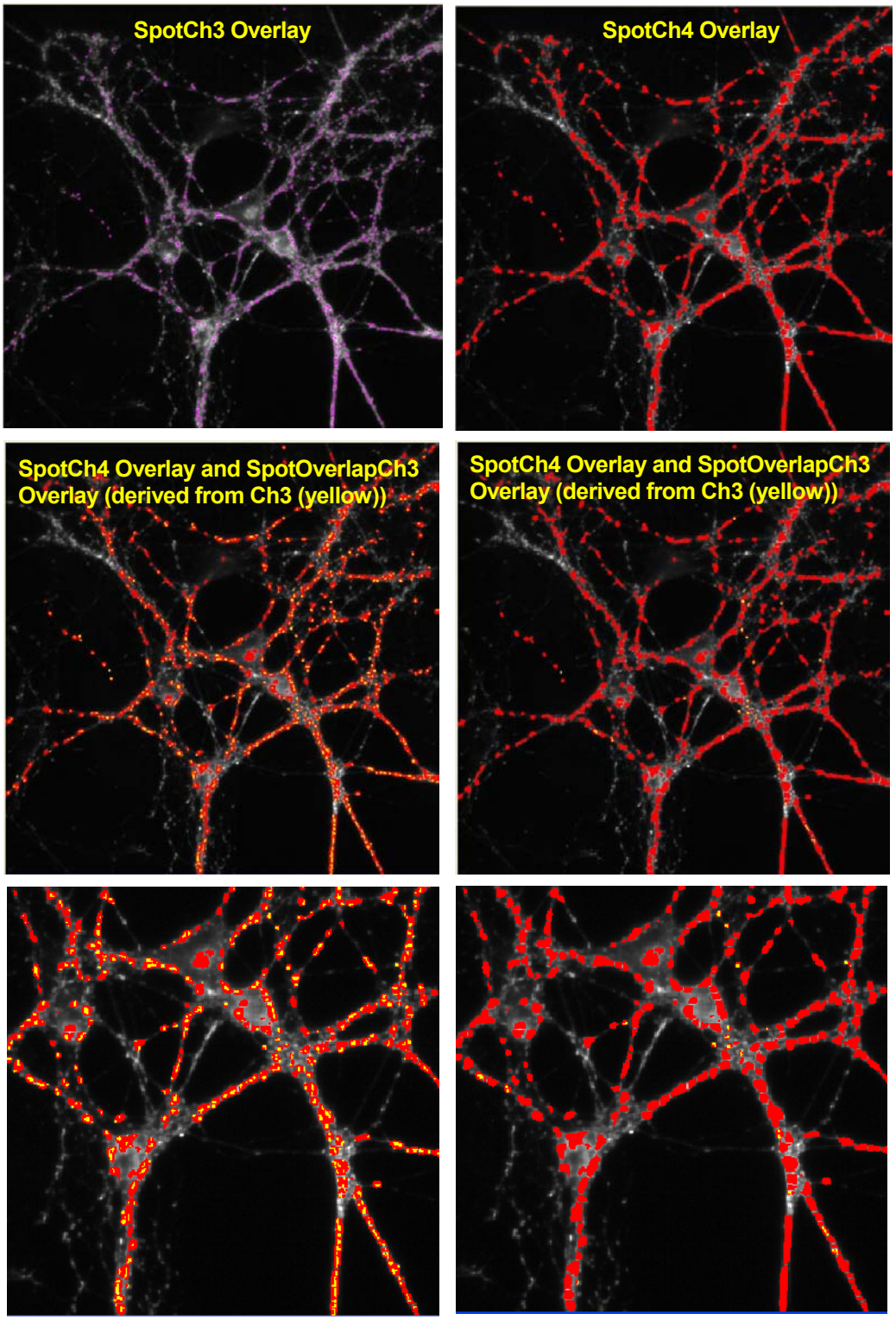


Figure 34. *Illustration of* ***DisplaySpotOverlapCh3Ch4*** *principle.* ***Top Left:*** *SpotCh3 overlay image.* ***Top Right:*** *SpotCh4 overlay image.* ***Middle and Bottom Left:*** *Setting* ***DisplaySpotOverlapCh3Ch4 = 3*** *and viewing the SpotOverlapCh3 overlay on top of the SpotCh4 overlay image (bottom is enlarged inset).* ***Middle and Bottom Right:*** *Setting* ***DisplaySpotOverlapCh3Ch4 = 4*** *and viewing the SpotOverlapCh4 overlay on top of the SpotCh4 overlay image (bottom is enlarged inset). Note that output features for spot overlap are calculated for both channels, but only the SpotOverlap overlay is shown for the selected channel.*

Basic Assay Parameters

Assay Parameters available in Basic Mode control the morphological identification of the objects in each channel, and control the use of Reference Wells (Table 15). You will not be able to view any Advanced Assay Parameters in this mode (as the **Hide Advanced Parameters** option is checked).

Parameter	Units	Description
UseReferenceWells	Binary	Use reference wells to calculate high response levels: 0 = No, 1 = Yes
NucTypeCh1	Binary	Type of nuclei to be identified in Ch1: 0 = Bright nuclei on dark background, 1 = Dark nuclei on bright background
BackgroundCorrectionCh*N*	Pixel	Radius (in pixels) of region used to compute background in Ch*N*: Negative value = Use surface fitting, 0 = Do not apply background correction, Positive value = Use low pass filter
NucSmoothFactorCh1	Number	Degree of image smoothing (blurring) prior to nucleus identification in Ch1: 0 = Do not apply smoothing
NucSegmentationCh1	Number	Controls segmentation of touching nuclei in Ch1: Negative value = Use intensity peaks method, 0 = Do not apply object segmentation, Positive value = Use shape geometry method
RejectBorderNucsCh1	Binary	Reject Ch1 nuclei that touch image edges: 0 = No, 1 = Yes
NucCleanupCh1	Binary	In Ch 1 image before identification, clean up nucleus mask and remove small objects by applying erosion followed by dilation procedure: 0 = No, 1=Yes
MinCellBodyNucOverlapCh2	Percent	Minimum overlap (in percent) between nucleus and cell body required to associate nucleus with neuron
CellBodyAndNeuriteTypeCh2	Binary	Type of cell bodies and neurites to be identified in Ch2: 0 = Bright cell bodies and neurites on dark background, 1 = Dark cell bodies and neurites on bright background
CellBodySmoothFactorCh2	Number	Degree of image smoothing (blurring) prior to cell body identification in Ch2: 0 = Do not apply smoothing
CellBodyDemarcationCh2	Number	Degree to which cell body boundary excludes neurites: 0 = Do not exclude neurites from cell body
CellBodySegmentationCh2	Number	Controls segmentation of touching cell bodies in Ch2: Negative value = Use intensity peaks method, 0 = Do not apply object segmentation, Positive value = Use shape geometry method
UseNucForCellBodySegmentationCh2	Number	Use valid nuclei to segment touching objects in Ch2: 0 = No, 1 = Yes (Use nuclei only), 2 = Yes (Use nuclei with object intensity peaks or shape geometry methods)
CellBodyMaskModifierCh2	Pixel	Number of pixels to modify Ch2 cell body mask: Negative value = Shrink mask, 0 = Do not modify mask, Positive value = Expand mask
RejectBorderCellBodiesCh2	Binary	Reject Ch2 cell bodies that touch image edges: 0 = No, 1 = Yes
NeuriteSmoothFactorCh2	Number	Degree of image smoothing (blurring) prior to neurite identification in Ch2: 0 = Do not apply smoothing
NeuriteIdentificationModifierCh2	Fraction	Fractional adjustment used to compute neurite identification threshold from Isodata threshold in Ch2
NeuriteDetectRadiusCh2	Pixel	Radius (in pixels) of region used for neurite detection in Ch2: 0 = Do not apply neurite detection
NeuriteDetectMethodCh2	Number	Method used to detect neurites in Ch2: 1 = box, 2 = binomial, 3 = median, and 4 = morphological

Parameter	Units	Description
NeuriteDirectionCh2	Pixel	Number of pixels used to compute the direction of a neurite segment at a branch or cross point
NeuritePointResolutionCh2	Pixel	Number of pixels used to merge neighboring branch and cross points into a single neurite point: 0 = Do not merge neighboring branch and cross points
NeuritePointDisplayModeCh2	Number	Display neurite points: 0 = Branch and Cross, 1 = Branch only, 2 = Cross only
NeuriteAggressiveTracingCh2	Binary	Allow aggressive tracing of neurites: 0 = No, 1=Yes
NeuriteTracingWithoutSeedsCh2	Binary	Allow the identification of neurites that do not touch the cell body: 0 = No, 1=Yes
NeuriteGapToleranceCh2	Number	Maximum distance that neurites can be detached from each other. Acceptable range: 1 – 512
RejectMultiplyTracedNeuritesCh2	Binary	Reject neurites that touch more than one cell body: 0 = neurite will be assigned to a cell body, 1 = neurite will be rejected
UseCellBodyZOIForNeuriteTracingCh2	Number	Use cell body ZOI for neurite tracing in Ch2: 0 = Do not use ZOI, 1 = Use ZOI of all cell bodies, 2 = Use ZOI of valid cell bodies
SpotTypeChN	Binary	Type of spots to be identified in ChN 0 = Bright spots on dark background, 1 = Dark spots on bright background
SpotSmoothFactorChN	Number	Degree of image smoothing (blurring) prior to spot identification in ChN = Do not apply smoothing
SpotDetectRadiusChN	Pixel	Radius (in pixels) of region used to detect spots in Ch3: Negative value = Use peak detection, Zero = Do not use detection (and confine spot analysis within cell body or neurite domain), Positive value = Use spot object detection
SpotDetectMethodChN	Number	Method used to detect spots. If SpotDetectRadiusCh3 is negative: 1 = Local maxima, 2-4 = Local range; If SpotDetectRadiusCh3 is positive: 1 = Box, 2 = Binomial, 3 = Median, and 4 = Morphological
SpotSegmentationChN	Number	Controls segmentation of touching spots in ChN: Negative value = Use intensity peaks method, 0 = Do not apply object segmentation, Positive value = Use shape geometry method
NeuriteSpotDomainModifierChN	Pixel	Number of pixels to expand Ch2 neurite mask for spot detection in ChN: 0 = Do not modify mask
MinAreaSpotOverlapCh3Ch4	Area % (neg) or Pixel/µm (pos)	Minimum overlap required for spot overlap analysis in Ch3 and Ch4: Negative = Percentage of overlap area relative to spot area; 0 = Do not perform overlap analysis; Positive = Overlap area (in pixels or micrometers)
DisplaySpotOverlapCh3Ch4	Number	Display spot overlap overlay: 3 = derived from Ch3, 4 = derived from Ch4
CellBodyMaskModifierCh*N*	Pixel	Number of pixels to modify Ch2 cell body mask in Ch*N*: Negative value = Shrink mask, 0 = Do not modify mask, Positive value = Expand mask

Table 15. *Basic Assay Parameters available for the Neuronal Profiling V4 BioApplication. * Note that "ChN" refers to Channels 1-6 for* ***Background Correction****,Channels 3 and 4 for spots, and Channels 3-6 for* ***CellBodyMaskModifier****.*

Object Selection Parameters

Each channel has an associated set of Object Selection Parameters. If an object has all measured features within the ranges specified by the Object Selection Parameters, it is selected for analysis. Rejected objects are removed from further analysis.

Channel 1 Object Selection Parameters are used to identify valid nuclei. Table 16 is a listing of all Object Selection Parameters available for Channel 1.

Channel 1 Parameters

Parameter	Units	Description
NucAreaCh1	Pixel or μm^2	Area (in pixels or micrometers) of Ch1 nucleus
NucShapeP2ACh1	Number	Shape measure based on ratio of perimeter squared to 4π*area of Ch1 nucleus (**NucShapeP2ACh1** = 1 for circular nucleus)
NucShapeLWRCh1	Number	Shape measure based on ratio of length to width of object-aligned bounding box of Ch1 nucleus
NucAvgIntenCh1	Intensity	Average intensity of all pixels within Ch1 nucleus
NucVarIntenCh1	Intensity	Variation (standard deviation) of intensity of all pixels within Ch1 nucleus
NucTotalIntenCh1	Intensity	Total intensity of all pixels within Ch1 nucleus

Table 16. *Channel 1 Object Selection Parameters available for the Neuronal Profiling V4 BioApplication.*

Channel 2 Object Selection Parameters are used to identify both valid cell bodies and neurites. Table 17 is a listing of all Object Selection Parameters available for Channel 2.

Channel 2 Parameters

Parameter	Units	Description
NeuriteLengthCh2	Pixel or μm	Length (in pixels or micrometers) of Ch2 neurite measured along its skeleton
NeuriteWidthCh2	Pixel or μm	Width (in pixels or micrometers) of Ch2 neurite estimated from its area and length
NeuriteAvgIntenCh2	Intensity	Average intensity of all pixels within Ch2 neurite
NeuriteTotalIntenCh2	Intensity	Total intensity of all pixels within Ch2 neurite
CellBodyNucCountCh2	Number	Number of all Ch1 nuclei belonging to Ch2 cell body
CellBodyAreaCh2	Pixel or μm^2	Area (in pixels or micrometers) of Ch2 cell body
CellBodyShapeP2ACh2	Number	Shape measure based on ratio of perimeter squared to 4π*area of Ch2 cell body (**CellBodyShapeP2ACh2** = 1 for circular cell body
CellBodyShapeLWRCh2	Number	Shape measure based on ratio of length to width of object-aligned bounding box of Ch2 cell body
CellBodyAvgIntenCh2	Intensity	Average intensity of all pixels within Ch2 cell body
CellBodyTotalIntenCh2	Intensity	Total intensity of all pixels within Ch2 cell body

Table 17. *Channel 2 Object Selection Parameters available for the Neuronal Profiling V4 BioApplication.*

There are two types of selection parameters for Channel 2; those that analyze the cell body and the nuclei within them, and those that analyze the neurites surrounding the cell bodies. In the Protocol Interactive View, the Identify Objects button will enable all objects to be identified. Three separate objects are displayed in Channel 2: cell bodies, neurites, and a "+" sign as an indicator of the number of nuclei found in each cell body. Placing the cursor over either the cell body or neurites will cause its Object Selection Parameters to be displayed, and these can be selected to set the range

(Figures 35 and 36). Care must be exercised to ensure that the cursor is on the appropriate object so that the appropriate Object Selection Parameters are displayed and can be selected.

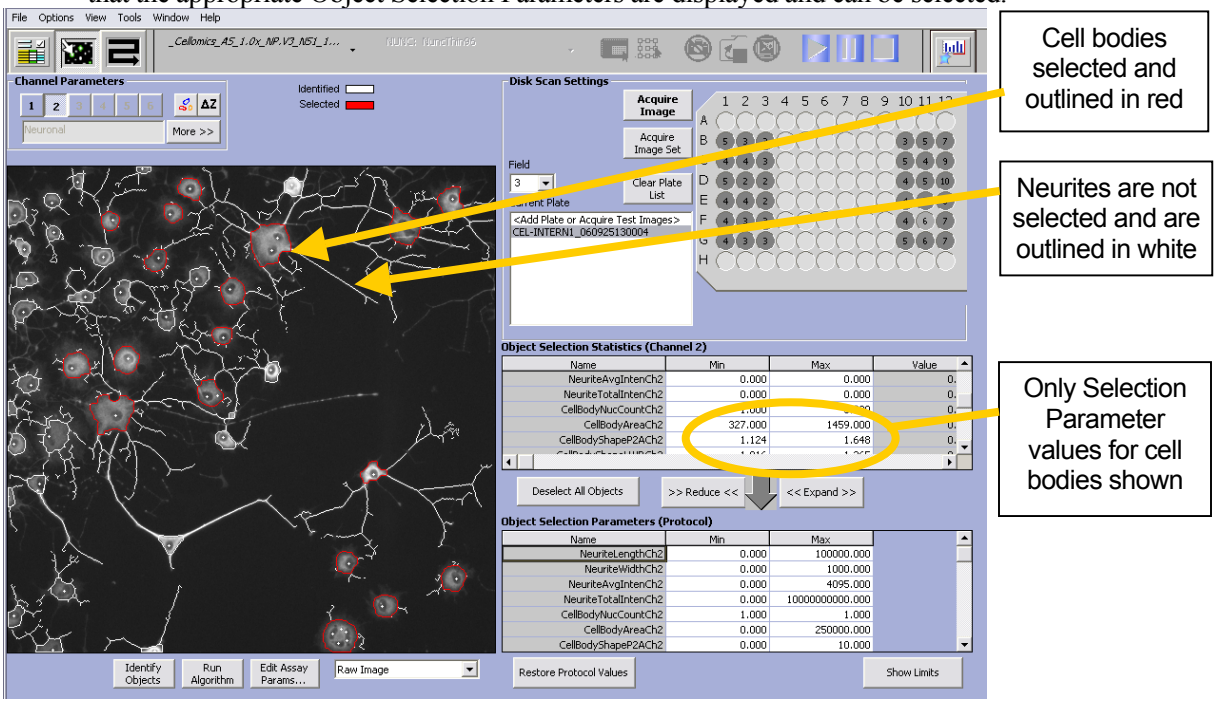


Figure 35. *Selecting only cell bodies in the Protocol Interactive View in Channel 2. The* ***Identify Objects*** *button was enabled and all objects (cell bodies, neurites, and "+" representing nucleus count from Channel 1) were identified. Placing the cursor on a cell body allowed only the cell bodies to be outlined and their Selection Parameter values to be listed.*

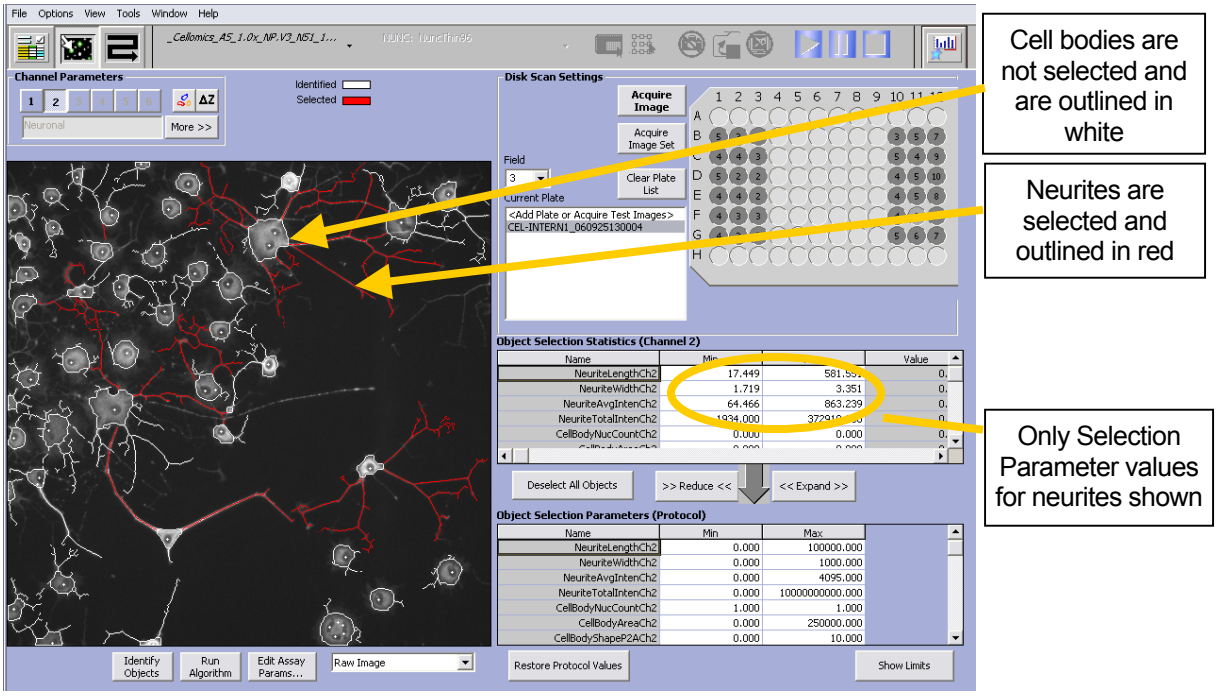
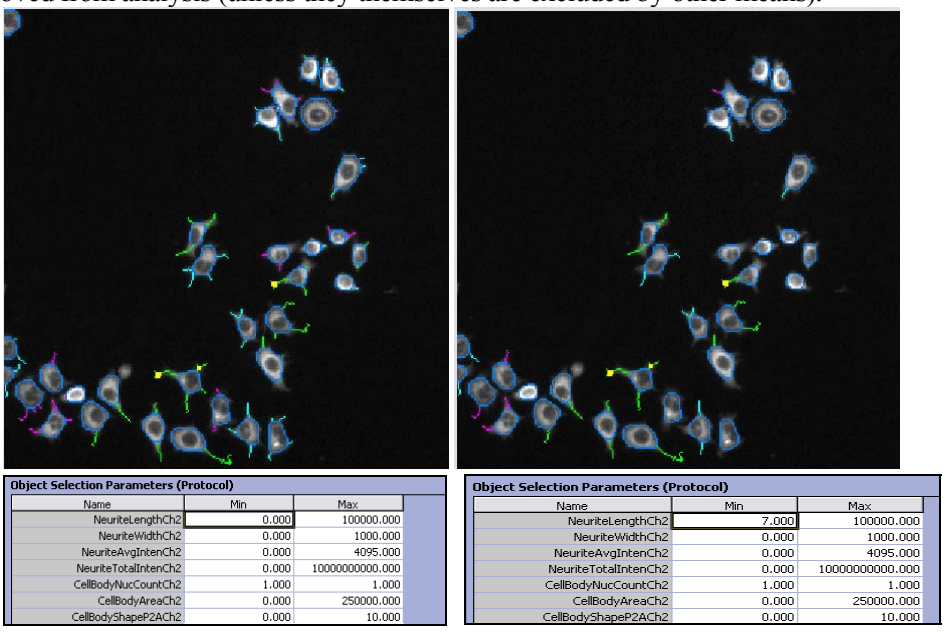


Figure 36. *Selecting only neurites in the Protocol Interactive View in Channel 2. The* ***Identify Objects*** *button was enabled and all objects (cell bodies, neurites, and "+" representing nucleus count from Channel 1) were identified. Placing the cursor on a neurite allowed only the neurites to be outlined and their Selection Parameter values to be listed.*

There are six separate Object Selection Parameters used to distinguish cell bodies. The **CellBodyNucCountCh2** Object Selection parameter requires the association of cell bodies with valid nuclei from Channel 1. If the number of nuclei found within the cell body is not within the range set by the user, the cell body is rejected from analysis (as well as the nuclei within it). If keeping this value at zero, you need to make sure that debris in Channel 1 is not mistaken as part of a neuron for Channel 2 by adjusting appropriate Object Selection parameters in Channel 1. Other cell body Object Selection Parameters, such as intensity, area, and shape may be used to further characterize the biology.

Four separate Object Selection Parameters may be used to distinguish neurites. Intensity, as well as length and width of each neurite can be removed from analysis by setting a minimum and maximum range. The following figure demonstrates the user excluding very small neurites from further analysis by adjusting the **NeuriteLengthCh2** Object Selection Parameter. Although the neurites may be removed from analysis, the selected cell bodies will not be removed from analysis (unless they themselves are excluded by other means).



Object Selection Parameters (Protocol)

Name	Min	Max
NeuriteLengthCh2	0.000	100000.000
NeuriteWidthCh2	0.000	1000.000
NeuriteAvgIntenCh2	0.000	4095.000
NeuriteTotalIntenCh2	0.000	10000000000.000
CellBodyNucCountCh2	1.000	1.000
CellBodyAreaCh2	0.000	250000.000
CellBodyShapeP2ACh2	0.000	10.000

Object Selection Parameters (Protocol)

Name	Min	Max
NeuriteLengthCh2	7.000	100000.000
NeuriteWidthCh2	0.000	1000.000
NeuriteAvgIntenCh2	0.000	4095.000
NeuriteTotalIntenCh2	0.000	10000000000.000
CellBodyNucCountCh2	1.000	1.000
CellBodyAreaCh2	0.000	250000.000
CellBodyShapeP2ACh2	0.000	10.000

Figure 37. *Removing small neurites from analysis using the* ***NeuriteLengthCh2*** *Object Selection Parameter.* ***Left****: overlay image in Protocol Interactive View when the* ***NeuriteLengthCh2*** *range is left open.* ***Right****: same image after increasing the minimum length to 7 microns, thus eliminating a majority of small neurites.*

Channel 3-4 Parameters

Parameter	Units	Description
SpotAreaChN	Pixel or μm^2	Area (in pixels or micrometers) of Ch3, Ch4 spots
SpotShapeP2AChN	Number	Shape measure based on ratio of perimeter squared to 4π*area of Ch3, Ch4 spots (**SpotShapeP2ACh3, Ch4** = 1 for circular nucleus)
SpotShapeLWRChN	Number	Shape measure based on ratio of length to width of object-aligned bounding box of Ch3, Ch4 spots
SpotAvgIntenChN	Intensity	Average intensity of all pixels within Ch3, Ch4 spots
SpotTotalIntenChN	Intensity	Total intensity of all pixels within Ch3, Ch4 spots
AvgIntenChN	Intensity	Average intensity in Ch3, Ch4 of all pixels within modified Ch2 cell body mask
TotalIntenChN	Intensity	Total intensity in Ch3, Ch4 of all pixels within modified Ch2 cell body mask

Table 18. *Object Selection Parameters available for Channel 3 and Channel 4. If* ***None*** *is selected for Object Identification Threshold, spot selection parameters will not be utilized. ChN = Channel 3 and Channel 4*

There are two types of selection parameters for Channel 3 and Channel 4; those that analyze the spots within the cell body mask and neurite spot domain and those that use a modified Channel 2 cell body mask for gating purposes (Table 18). As in Channel 2, placing the cursor over the spots will give appropriate Object Selection Parameters for spot characteristics. If **SpotDetectRadius = 0** and **Fixed Threshold = 0**, the entire cell body and/or neurites will be distinguished as an entire spot. If this is desired, it is important to note that shape factors for neurites as spots will be greater than normally seen on real spots. Therefore, the **SpotShapeP2AChN** and **SpotShapeLWRChN** Object Selection Parameters may need to be expanded so that they are all selected.

Placing the cursor over the cell body or modified cell body mask will give values for **AvgIntenChN** and **TotalIntenChN**. If **None** is selected for Object Identification Threshold, only the cell body mask will be shown for gating purposes and spot features will not be displayed. It is important to note that adjusting the **CellBodyMaskModifier** in Channel 3 and Channel 4 is only used for gating purposes and will not change the **CellBodySpot** statistics for that channel. Adjustment of the mask is simply to increase or decrease the surrounding area. Figure 38 illustrates this principle.

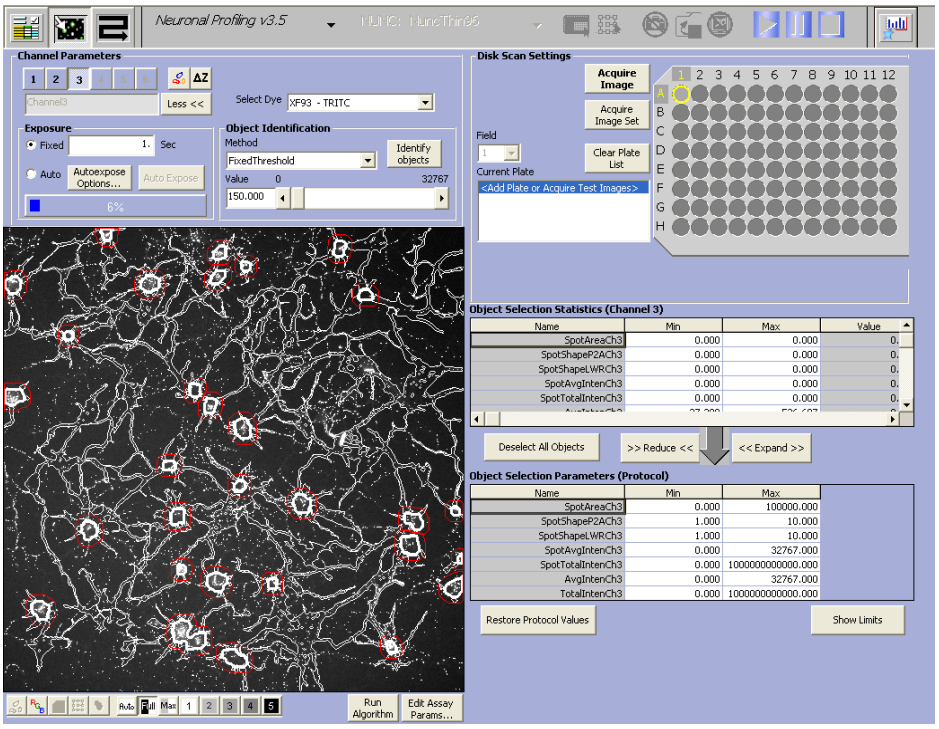


***Figure 38**. Using the Protocol Interactive Identify Objects View to perform gating analysis and adjusting the **CellBodyMaskModifierCh3**. Note that the adjusted mask only affects the AvgIntenCh3 and TotalIntenCh3 and will not affect the **CellBodySpot** statistics.*

Channels 5-6 Object Selection Parameters are used exclusively for gating purposes. Table 19 lists all Object Selection Parameters available for Channels 5-6.

Channel *N* (Channels 5-6) Parameters

Parameter	Units	Description
AvgIntenCh*N*	Intensity	Average intensity in Ch*N* of all pixels within modified Ch2 cell body mask
TotalIntenCh*N*	Intensity	Total intensity in Ch*N* of all pixels within modified Ch2 cell body mask

***Table 19.** Object Selection Parameters Available for the Neuronal Profiling V4 BioApplication. *Note that "ChN" refers to Channels 5-6.*

NOTE

The four Neurite Object Selection Parameters for Channel 2 will select/reject individual neurites and not the neuron.

The six Cell Body Object Selection Parameters for Channel 2 will select/reject the neuron (if rejected, the cell body and any associated nuclei and neurites will be rejected).

In Channel 3 and Channel 4, Object Selection Parameters pertaining to gating (AvgIntenChN, TotalIntenChN and modified cell body mask) will NOT affect the spot Object Selection statistics.

Selecting the **None** Object Identification Threshold for Channels 3-4 will only show the modified cell body mask.

Object Selection Parameters for Channels 5-6 are ***gating*** parameters only.

Gating

The Neuronal Profiling BioApplication supports gating on a cell population. This feature provides selective cell processing based on fluorescence intensity. Therefore, in addition to selecting nuclei /cell bodies/neurites for analysis in Channel 1 and Channel 2, you can also select or reject cells based on fluorescence intensity in Channels 3-6. Gating allows you to specifically identify a subset of cells based on fluorescence intensity and allows you to limit the analysis to this group of cells. For example, gating may be used to analyze only those cells showing a certain level of expression of a fluorescent reporter such as green fluorescent protein (GFP). Unlike subpopulation analysis, gating works by rejecting any object that does not meet object selection criteria in all 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 of the ArrayScan Classic software, you can specify intensity ranges by entering upper and lower limits for two intensity parameters, **AvgIntenCh*N*** and **TotalIntenCh*N***, for Channels 3-6. **TotalIntenCh*N*** is a summation of all intensities within the cell. **AvgIntenCh*N*** is **TotalIntenCh*N*** divided by the object area. These parameters can also be adjusted interactively in the Protocol Interactive View.

Specifying Mask Modifiers for Gating

The cell bodies of the valid neurons identified in Channel 2 may be used to create measurement masks that can be applied to one or more downstream channels (i.e., Channels 3-6) for gating. The sizes of these masks are specified using the **CellBodyMaskModifierCh*N*** Assay Parameter. You may want to use this feature if, for example, you want 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. Masks in other channels are created from the cell body overlay obtained in Channel 2 (after you set your **CellBodyMaskModifierCh2** value, see Figure 18 for a visual representation). Once you determine the mask for each additional channel, you can then specify the intensity ranges. For each additional channel, the average intensity and total intensity values within the modified masks are calculated. If the calculated value does not fall within the specified upper and lower limits, the object is removed from the set of selected objects.

Image Overlays

During a scan, various features can be displayed as color overlays on the channel images. The colors of these overlays can be changed by choosing the color block beside the overlay name in the Create Protocol or Protocol Interactive View. The types of overlays that are displayed depend on different factors:

1. Number of channels used
2. Whether spot analysis is performed in Channel 3 and/or Channel 4
3. Whether a non-zero value is used (for **MinAreaSpotOverlapCh3Ch4**)
4. Which channel the overlay will be derived from (**DisplaySpotOverlapCh3Ch4**)

Table 20 lists the following overlays that can be displayed for this BioApplication. Note that utilization of spots in additional channels will change the overlays represented for the three neurite overlays.

Parameter	Description
Include This Channel In Composite	If checked, channel image is included in the composite image. Note that in order to view a composite image in vHCS:Scan or vHCS:View applications, at least one box must be checked.
ValidNucleus	If checked, overlays are displayed for Channel 1 objects that passed Channel 1 selection criteria.
RejectedNucleus	If checked, overlays are displayed for Channel 1 objects that failed Channel 1 selection criteria or are within rejected cell bodies.
SelectedCellBody	If checked, overlays are displayed for Channel 2 objects that passed the Channel 2-6 selection criteria and the nucleus/cell body coincidence requirement (**MinCellBodyNucOverlapCh2**).
SelectedCellBody+Neurite	Alteration from SelectedCellBody. Visible only when Channel 3 and Channel 4 are used for spot analysis and **MinAreaSpotOverlapCh3Ch4** is not zero. Note that although both are the same color, the cell body overlay will be thicker than the neurite overlays.
RejectedCellBody	If checked, overlays are displayed for Channel 2 objects that failed the Channel 2-6 selection criteria, have only rejected nuclei within their boundaries, or failed the nucleus/cell body coincidence requirement.
NeuritePoint	If checked, overlays are displayed for neurite branch points and/or cross points (specified by the **NeuriteBranchPointDisplayModeCh2** Assay Parameter).
Neurite	If checked, overlays are displayed for neurites. This overlay is present when 2 or 3 channels are selected, if 4 channels are selected and spot analysis is not performed in either or both channels, or if **MinAreaSpotOverlapCh3Ch4** is zero. If found with other Neurite overlays (**Neurite__** and/or **Neurite_**), note that each neuron is assigned one neurite color on a cyclical basis. **PLEASE NOTE THAT ALL THE NEURITE OVERLAYS NEED TO BE SELECTED IN ORDER FOR ALL NEURITES TO BE SHOWN.** You can select the same color for each overlay, but each box MUST be checked so that you can see all of the neurites selected for analysis.
Neurite_	If checked, overlays are displayed for neurites. This overlay is present when 2 channels are selected or if 3 or 4 channels are selected, with spot analysis **NOT** performed in Ch3. If found with other Neurite overlays (**Neurite__** and/or **Neurite**), note that each neuron is assigned one neurite color on a cyclical basis. **PLEASE NOTE THAT ALL THE NEURITE OVERLAYS NEED TO BE SELECTED IN ORDER FOR ALL NEURITES TO BE SHOWN.** You can select the same color for each overlay, but each box MUST be checked so that you can see all of the neurites selected for analysis.

Parameter	Description
Neurite__	If checked, overlays are displayed for neurites. This overlay is present when 2 or 3 channels are selected or if 4 channels are selected, with spot analysis **NOT** performed in Ch4. If found with other Neurite overlays (Neurite_ and/or Neurite), note that each neuron is assigned one neurite color on a cyclical basis. **PLEASE NOTE THAT ALL THE NEURITE OVERLAYS NEED TO BE SELECTED IN ORDER FOR ALL NEURITES TO BE SHOWN.** You can select the same color for each overlay, but each box MUST be checked so that you can see all of the neurites selected for analysis.
SpotCh3	If checked, overlays are displayed for spots in Ch3. This overlay is present when 3 or 4 channels are selected and if spot analysis is desired in Ch3.
SpotCh4	If checked, overlays are displayed for spots in Ch4 when spot analysis is desired in Ch4.
SpotOverlapCh3	If checked, overlays are displayed for spots in Ch3 that have overlapping area equal to/above the value set for **MinAreaSpotOverlapCh3Ch4** and if **DisplaySpotOverlapCh3Ch4** = 3.
SpotOverlapCh4	If checked, overlays are displayed for spots in Ch4 that have overlapping area equal to/above the value for **MinAreaSpotOverlapCh3Ch4** and if **DisplaySpotOverlapCh3Ch4** = 4.

Table 20. *General set of overlays that can be used with the Neuronal Profiling V4 BioApplication.*

1. Number of Channels Used

Selecting the number of channels initially determines the types of overlays that are displayed. For each additional channel added, a Neurite overlay may be removed. If two channels are selected, the following overlays may be used: ValidNucleus, RejectedNucleus, SelectedCellBody, RejectedCellBody, NeuritePoint, and three Neurite overlays. If three channels are selected (for spot analysis), one Neurite overlay will be replaced with a SpotCh3 overlay. If four channels are selected (for spot analysis), another Neurite overlay will be replaced with a SpotCh4 overlay.

2. Whether Spot Analysis is Performed in Ch3 and/or Ch4

If more than 2 channels are desired, then the next factor to determine overlays is whether spots will be analyzed in Channel 3 and/or Channel 4 or if gating only is preferred.

For a 3-channel assay:

- If spots are desired for analysis in Channel 3 (setting Object Identification Threshold in Ch3 as **Isodata**, **Fixed**, or **Triangle**), the difference in overlays will be a replacement of one neurite overlay with a spot overlay for Ch3.
- If spots are not desired for analysis in Channel 3 and gating *only* is preferred, setting the Object Identification Threshold for Ch3 to **None** will allow the overlays to be identical to that found when only 2 channels are used.

For a 4-channel assay:

- If spots are desired for analysis in Ch3 but not in Ch4 (gating *only* in Ch4) – One neurite overlay will be replaced with a spot overlay for Ch3. The overlay in Ch4 will remain as a neurite overlay (providing that Object Identification Threshold for Ch4 is set to **None**).
- If spots are not desired for analysis (with gating *only*) in Ch3 but spots are desired for analysis in Ch4 – One neurite overlay will be replaced with a spot overlay for Ch4. The overlay in Ch3 will remain as a neurite overlay (providing that Object Identification Threshold for Ch3 is set to **None**).
- No spot detection in Ch3 and Ch4 (Gating *only* in both channels) – overlays will be identical to that found when only 2 channels are used (providing that Object Identification Threshold for Ch3 and Ch4 are set to **None**).

3. Utilization of MinAreaSpotOverlapCh3Ch4

If you have a 4-channel assay and spots are desired for analysis in both Ch3 and Ch4, one last overlay is then determined by the value for **MinAreaSpotOverlapCh3Ch4**:

- If **MinAreaSpotOverlapCh3Ch4** is = **0**, one neurite overlay will be displayed along with Ch3 and Ch4 spot overlays.
- If **MinAreaSpotOverlapCh3Ch4** is any value *except* **0**, the neurite overlay will be the same color as the cell body (although the cell body will be thicker), and a spot overlap overlay will be shown. The channel for the SpotOverlap overlay will be determined from the value used for the **DisplaySpotOverlapCh3Ch4** Assay Parameter:

4. Which Channel to Derive Overlay

If you have decided to show the overlay for overlapping spots, the final decision is to state which channel you want the overlay to be derived from

- If **DisplaySpotOverlapCh3Ch4** = **3**, overlay for Spot Overlap will be derived from Ch3 spots that have overlapping area equal to or exceeding the value set for **MinAreaSpotOverlapCh3Ch4**
- If **DisplaySpotOverlapCh3Ch4** = **4**, overlay for Spot Overlap will be derived from Ch4 spots that have overlapping area equal to or exceeding the value set for **MinAreaSpotOverlapCh3Ch4**

Please refer to Table 21 for additional information and images.

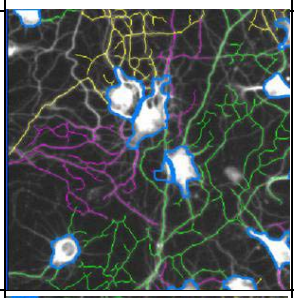
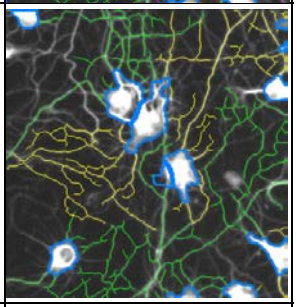
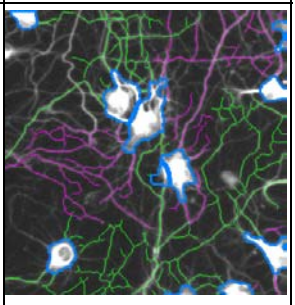
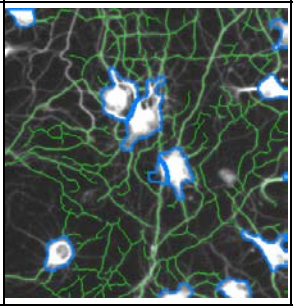
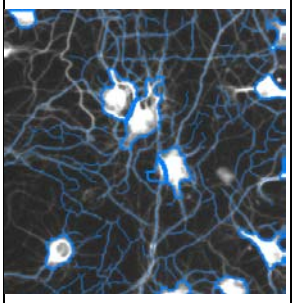
Overlay Choices	Identification Method - Ch3	Identification Method - Ch4	MinAreaSpot OverlapCh3Ch4 Value	Ch2 Example Image
ValidNucleus, RejectedNucleus, Neurite, Neurite_, Neurite__, SelectedCellBody, RejectedCellBody, NeuritePoint **(Or 2-Channel assay)**	= None	= None	NA	
ValidNucleus, RejectedNucleus, Neurite, SpotCh3, Neurite__, SelectedCellBody, RejectedCellBody, NeuritePoint **(Or 3-Channel assay)**	Fixed, Isodata, or Triangle	= None	NA	
ValidNucleus, RejectedNucleus, Neurite, Neurite_, SpotCh4, SelectedCellBody, RejectedCellBody, NeuritePoint	= None	Fixed Isodata or Triangle	NA	
ValidNucleus, RejectedNucleus, Neurite, SpotCh3, SpotCh4, SelectedCellBody, RejectedCellBody, NeuritePoint	Fixed Isodata or Triangle	Fixed Isodata or Triangle	Zero	
ValidNucleus, RejectedNucleus, SpotOverlapCh3, SpotCh3, SpotCh4, SelectedCellBody+Neurite, RejectedCellBody, NeuritePoint If **DisplaySpotOverlapCh3Ch4** = 3 ValidNucleus, RejectedNucleus, SpotOverlapCh4, SpotCh3, SpotCh4, SelectedCellBody+Neurite, RejectedCellBody, NeuritePoint If **DisplaySpotOverlapCh3Ch4** = 4	Fixed Isodata or Triangle	Fixed Isodata or Triangle	NonZero	

Table 21. *Overlay choices, display options, and example Ch2 images available for the Neuronal Profiling V4 BioApplication*

Assay Parameters For Population Characterization

Overview of Population Characterization

This BioApplication provides the ability to characterize cells based on their response compared to a control population. The values of a particular Cell Feature measured over a sample of untreated cells will form a distribution similar to that shown on the left in the following figure. If a sample of these cells is treated and responds positively in a way that is captured by the Cell Feature, the distribution of values for the treated cells will be shifted to the right (shown on the right in Figure 39). Using these two distributions, a high-response level (***Feature*Ch*N*LevelHigh**) for the Cell Feature that will identify responding cells can be set. The **Status** Cell Feature values indicate whether a given cell is within or beyond this range (Table 22).

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

Table 22. *Binary explanation of Cell Feature Status when using population characterization*

The corresponding Well Features reported are the percentage of cells that are greater than the level defining this value (**%HIGH_*Feature*Ch*N***).

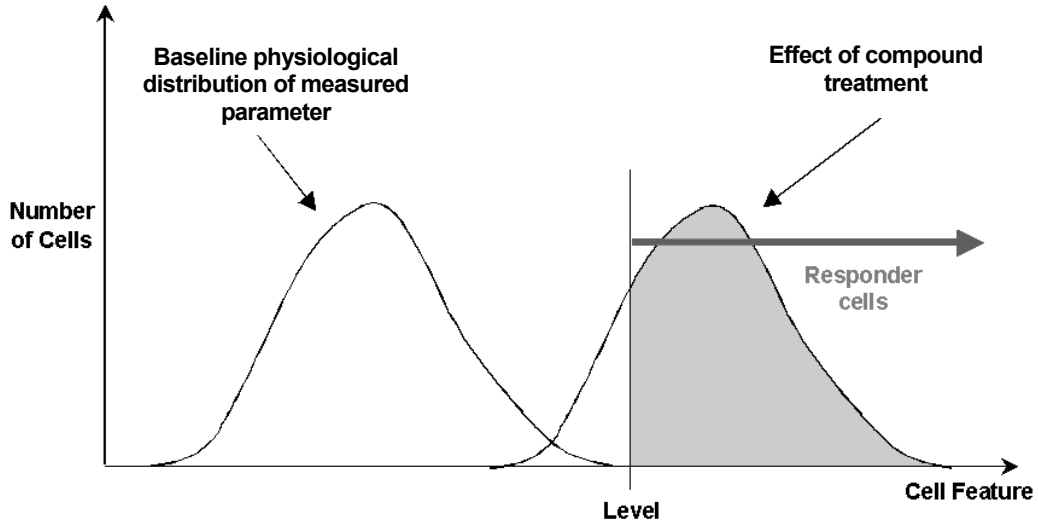


Figure 39. *Cellular response level distinguishing baseline from high subpopulation*

Setting Cellular Response Levels

The Neuronal Profiling BioApplication offers two ways of setting high-response level Assay Parameters. The first is manually entering values for the ***Feature*Ch*N*LevelHigh** Assay Parameters in the Protocol Create or Protocol Interactive Views (Advanced Mode). This requires prior knowledge of typical feature values. The BioApplication then calculates the percentage of cells above the specified ***Feature*Ch*N*LevelHigh** values.

The second is automatically calculating the ***Feature*ChN*LevelHigh** Assay Parameters through use of Reference Wells. Typically, Reference Wells contain a control population of cells which displays the normal basal physiological distribution for the Assay Parameter being measured. You can specify which wells on the sample plate should be used as Reference Wells. These wells are first analyzed and the population distribution for the different features are determined. The cell population characterization levels are then specified through use of a correction coefficient (_CC) value. The correction coefficient specifies the location of the high-response level in terms of standard deviations from the mean. These coefficients are also located in the Advanced Mode for Assay Parameters. The advantage of using Reference Wells to automatically calculate levels is that the levels are determined from a control population of cells and are independent of run-to-run variations.

Reference Wells Processing Sequence

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

1. Cell Features are computed for every valid object within a field.
2. For each Cell Feature to be used for population characterization, the distribution's histogram, mean, and standard deviation are computed over all cells in the field.
3. Reference Field Features are determined.
4. Reference Well Features are computed as average values for fields in a well, weighted for the number of cells per field, and then Reference Plate Features are computed as arithmetic averages for all Reference Wells on a plate. Use of a weighted average minimizes the effect of sparse fields.

Identifying Reference Wells and Control Parameters

The Reference Wells are selected in the Reference Wells Configuration window (choose **Change Reference Wells** from the **Tools** menu). Select the wells to be set as the Reference Wells and then click on the **Known** button to define the type of Reference Well. Next, enter the Starting Field and Number of Fields. Click the **Apply** button to save the settings. Please refer to the appropriate user guide for more details.

NOTE

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

Two general Assay Parameters controlling the use of Reference Wells are: **UseReferenceWells** and **MinRefAvgNeuronCountPerField**. **UseReferenceWells** is a binary Assay Parameter that allows you to indicate whether Reference Wells are used to determine the levels necessary to characterize neurite 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 **MinRefAvgNeuronCountPerField** is used to specify the minimum average number of selected neurons per field that you consider acceptable in the Reference Wells. If the measured cell density is less than or equal to this value, the Reference Features will not be used, rather the manually entered levels for population characterization are used The Cell Features used to characterize various levels are associated with Assay Parameters in the form:

***Feature*ChNLevelHigh** *(when Reference Wells are not used)*

or

***Feature*ChNLevelHigh_CC** *(when Reference Wells are used)*

where *Feature* refers to the name of the Cell Feature (such as **NeuriteTotalLengthCh2**) and Ch*N* refers to the specific channel. The ***Feature*ChNLevelHigh** Assay Parameter specifies the actual levels and must be manually entered if Reference Wells are not used. The ***Feature*ChNLevelHigh_CC** Assay Parameter is a correction coefficient (CC) used to derive the ***Feature*ChNLevelHigh** value from the mean and standard deviation of the reference well population according to the formula:

$$\textbf{\textit{Feature}ChNLevelHigh} = \textbf{Mean} + \textbf{\textit{Feature}ChNLevelHigh\_CC} \times \textbf{SD}$$

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

Advanced Assay Parameters

In Advanced Mode, all Basic and Advanced Assay Parameters are adjustable. The **Hide Advanced Parameters** option provides the ability to either view and adjust all the Assay Parameters or view and adjust the subset of parameters designated as Basic Parameters. 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 two advanced Assay Parameters that control its levels: ***Feature*Ch*N*LevelHigh** that sets upper threshold and the presence of the **_CC** suffix which designates those levels are set using Reference Wells. For example, the Assay Parameters controlling the cell body total area in Channel 2 are:

- **CellBodyTotalAreaCh2LevelHigh**
- **CellBodyTotalAreaCh2LevelHigh_CC**

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

- CellBodyTotalAreaCh2Level***High, High_CC***

Units will be expressed as what is found with ***Feature*ChNLevelHigh**, knowing that ***_CC*** is expressed as a number.

Parameter	Units	Description
MinRefAvgNeuronCountPerField	Number	Minimum average number of neurons 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)***
CellBodyNucTotalAreaCh1Level***High, High_CC***	Pixel or μm^2	Defines **CellBodyNucTotalAreaCh1** population characterization thresholds
CellBodyNucTotalIntenCh1Level***High, High_CC***	Intensity	Defines **CellBodyNucTotalIntenCh1** population characterization thresholds
CellBodyNucAvgIntenCh1Level***High, High_CC***	Intensity	Defines **CellBodyNucAvgIntenCh1** population characterization thresholds
CellBodyNucCountCh2Level***High, High_CC***	Number	Defines **CellBodyNucCountCh2** population characterization thresholds
CellBodyAreaCh2Level***High, High_CC***	Pixel or μm^2	Defines **CellBodyAreaCh2** population characterization thresholds
CellBodyShapeP2ACh2Level***High, High_CC***	Number	Defines **CellBodyShapeP2ACh2** population characterization thresholds

Parameter	Units	Description
CellBodyShapeLWRCh2Level***High, High_CC***	Number	Defines **CellBodyShapeLWRCh2** population characterization thresholds
CellBodyTotalIntenCh2Level***High, High_CC***	Intensity	Defines **CellBodyTotalIntenCh2** population characterization thresholds
CellBodyAvgIntenCh2Level***High, High_CC***	Intensity	Defines **CellBodyAvgIntenCh2** population characterization thresholds
NeuriteTotalCountCh2Level***High, High_CC***	Number	Defines **NeuriteCountCh2** population characterization thresholds
NeuriteTotalLengthCh2Level***High, High_CC***	Pixel or µm	Defines **NeuriteTotalLengthCh2** population characterization thresholds
NeuriteAvgLengthCh2Level***High, High_CC***	Pixel or µm	Defines **NeuriteAvgLengthCh2** population characterization thresholds
NeuriteMaxLengthWithBranchesCh2Level***High, High_CC***	Pixel or µm	Defines **NeuriteMaxLengthWithBranchesCh2** population characterization thresholds
NeuriteMaxLengthWithoutBranchesCh2Level***High, High_CC***	Pixel or µm	Defines **NeuriteMaxLengthWithoutBranchesCh2** population characterization thresholds
NeuriteTotalAreaCh2Level***High, High_CC***	Pixel or μm^2	Defines **NeuriteTotalAreaCh2** population characterization thresholds
NeuriteWidthCh2Level***High, High_CC***	Pixel or µm	Defines **NeuriteWidthCh2** population characterization thresholds
NeuriteTotalIntenCh2Level***High, High_CC***	Intensity	Defines **NeuriteTotalIntenCh2** population characterization thresholds
NeuriteAvgIntenCh2Level***High, High_CC***	Intensity	Defines **NeuriteAvgIntenCh2** population characterization thresholds
NeuriteVarIntenCh2Level***High, High_CC***	Intensity	Defines **NeuriteVarIntenCh2** population characterization thresholds
BranchPointTotalCountCh2Level***High, High_CC***	Number	Defines **BranchPointTotalCountCh2** population characterization thresholds
BranchPointAvgCountCh2Level***High, High_CC***	Number	Defines **BranchPointAvgCountCh2** population characterization thresholds
BranchPointCountPerNeuriteLengthCh2 Level***High, High_CC***	Number	Defines **BranchPointCountPerNeuritelLengthCh2** population characterization thresholds
BranchPointAvgDistFromCellBodyCh2Level***High, High_CC***	Pixel or µm	Defines **BranchPointAvgDistFromCellBodyCh2** population characterization thresholds
CrossPointTotalCountCh2Level***High, High_CC***	Number	Defines **CrossPointTotalCountCh2** population characterization thresholds
CrossPointAvgCountCh2Level***High, High_CC***	Number	Defines **CrossPointAvgCountCh2** population characterization thresholds
CellBodySpotTotalCountChNLevel***High, High_CC***	Number	Defines **CellBodySpotTotalCountChN** population characterization thresholds
CellBodySpotTotalAreaChNLevel***High, High_CC***	Pixel or μm^2	Defines **CellBodySpotTotalAreaChN** population characterization thresholds
CellBodySpotTotalIntenChNLevel***High, High_CC***	Intensity	Defines **CellBodySpotTotalIntenChN** population characterization thresholds
CellBodySpotAvgIntenChNLevel***High, High_CC***	Intensity	Defines **CellBodySpotAvgIntenChN** population characterization thresholds

Parameter	Units	Description
CellBodySpotTotalIntenRatioChNCh2Level***High, High_CC***	Number	Defines **CellBodySpotTotalIntenRatioChNCh2** population characterization thresholds
CellBodySpotOverlapCountChNLevel***High, High_CC***	Number	Defines **CellBodySpotOverlapCountChN** population characterization thresholds
CellBodySpot%OverlapCountChNLevel***High, High_CC***	Percent	Defines **CellBodySpot%OverlapCountChN** population characterization thresholds
CellBodySpotOverlapAreaChNLevel***High, High_CC***	Pixel or μm^2	Defines **CellBodySpotOverlapAreaChN** population characterization thresholds
CellBodySpot%OverlapAreaChNLevel***High, High_CC***	Percent	Defines **CellBodySpot%OverlapAreaChN** population characterization thresholds
NeuriteSpotTotalCountChNLevel***High, High_CC***	Number	Defines **NeuriteSpotTotalCountChN** population characterization thresholds
NeuriteSpotAvgCountChNLevel***High, High_CC***	Number	Defines **NeuriteSpotAvgCountChN** population characterization thresholds
NeuriteSpotTotalAreaChNLevel***High, High_CC***	Pixel or μm^2	Defines **NeuriteSpotTotalCAreaChN** population characterization thresholds
NeuriteSpotAvgAreaChNLevel***High, High_CC***	Pixel or μm^2	Defines **NeuriteSpotAvgAreaChN** population characterization thresholds
NeuriteSpotTotalIntenChNLevel***High, High_CC***	Intensity	Defines **NeuriteSpotTotalIntenChN** population characterization thresholds
NeuriteSpotAvgIntenChNLevel***High, High_CC***	Intensity	Defines **NeuriteSpotAvgIntenChN** population characterization thresholds
NeuriteSpotTotalIntenRatioChNCh2Level***High, High_CC***	Number	Defines **NeuriteSpotTotalIntenRatioChNCh2** population characterization thresholds
NeuriteSpotOverlapCountChNLevel***High, High_CC***	Number	Defines **NeuriteSpotOverlapCountChN** population characterization thresholds
NeuriteSpot%OverlapCountChNLevel***High, High_CC***	Percent	Defines **NeuriteSpot%OverlapCountChN** population characterization thresholds
NeuriteSpotOverlapAreaChNLevel***High, High_CC***	Pixel or μm^2	Defines **NeuriteSpotOverlapAreaChN** population characterization thresholds
NeuriteSpot%OverlapAreaChNLevel***High, High_CC***	Percent	Defines **NeuriteSpot%OverlapAreaChN** population characterization thresholds
CellBodySpotTotalIntenRatioCh4Ch3Level***High, High_CC***	Number	Defines **CellBodySpotTotalIntenRatioCh4Ch3** population characterization thresholds
CellBodySpotTotalAreaRatioCh4Ch3Level***High, High_CC***	Number	Defines **CellBodySpotTotalAreaRatioCh4Ch3** population characterization thresholds
NeuriteSpotTotalIntenRatioCh4Ch3Level***High, High_CC***	Number	Defines **NeuriteSpotTotalIntenRatioCh4Ch3** population characterization thresholds
NeuriteSpotTotalAreaRatioCh4Ch3Level***High, High_CC***	Number	Defines **NeuriteSpotTotalAreaRatioCh4Ch3** population characterization thresholds
TotalIntenChNLevel***High, High_CC***	Intensity	Defines **TotalIntenChN** population characterization thresholds
AvgIntenChNevel***High, High_CC***	Intensity	Defines **AvgIntenChN** population characterization thresholds

Table 23. *Advanced Assay Parameters Available in the Neuronal Profiling V4 BioApplication. *Note that "ChN" refers to Channel 3 and Channel 4.*

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

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

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

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

Event definition is achieved through the construction of logic statements employing specific Cell Features and a set of defined logical operators. The Cell Features and Boolean operators available are listed in Table 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 40 using two Cell Features, A and B. The entire cell population analyzed is enclosed within the box in each case. In each case, responder cells for each feature are located within the domain for that feature. Note that the definitions described in this figure can be directly applied to logic statements that contain more than two Cell Features. The Assay Parameters used to store Events are of the type **Type_X_EventDefinition** (X =1, 2, or 3).

Cell Features	Logic Operators
CellBodyNucAreaCh1 CellBodyNucTotalIntenCh1 CellBodyNucAvgIntenCh1	
CellBodyNucCountCh2 CellBodyAreaCh2 CellBodyShapeP2A Ch2 CellBodyShapeLWRCh2 CellBodyTotalIntenCh2 CellBodyAvgIntenCh2	NOT
NeuriteTotalCountCh2 NeuriteTotalLengthCh2 NeuriteAvgLengthCh2 NeuriteMaxLengthWithBranchesCh2 NeuriteMaxLengthWithoutBranchesCh2 NeuriteWidthCh2 NeuriteTotalAreaCh2 NeuriteTotalIntenCh2 NeuriteAvgIntenCh2 NeuriteVarIntenCh2 BranchPointTotalCountCh2 BranchPointAvgCountCh2 BranchPointAvgDistFromCellBodyCh2 BranchPointCountPerNeuriteLengthCh2 CrossPointTotalCountCh2 CrossPointAvgCountCh2	AND AND NOT OR OR NOT XOR NAND NOR
CellBodySpotTotalCountChN CellBodySpotTotalAreaChN CellBodySpotTotalIntenChN CellBodySpotAvgIntenChN CellBodySpotTotalIntenRatioChNCh2 CellBodySpotTotalIntenRatioCh4Ch3 CellBodySpotTotalAreaRatioCh4Ch3 CellBodySpotOverlapCountChN CellBodySpot%OverlapCountChN CellBodySpotOverlapAreaChN CellBodySpot%OverlapAreaChN NeuriteSpotTotalCountChN NeuriteSpotAvgCountChN NeuriteSpotTotalAreaChN NeuriteSpotAvgAreaChN NeuriteSpotTotalIntenChN NeuriteSpotAvgIntenChN NeuriteSpotTotalIntenRatioChNCh2 NeuriteSpotTotalIntenRatioCh4Ch3 NeuriteSpotTotalAreaRatioCh4Ch3 NeuriteSpotOverlapCountChN NeuriteSpot%OverlapCountChN NeuriteSpotOverlapAreaChN NeuriteSpot%OverlapAreaChN	
TotalIntenChN AvgIntenChN	

Table 24. *Cell features and Boolean operators available for Event Definition. Note that ChN refers to Channel 3 and/or Channel 4*

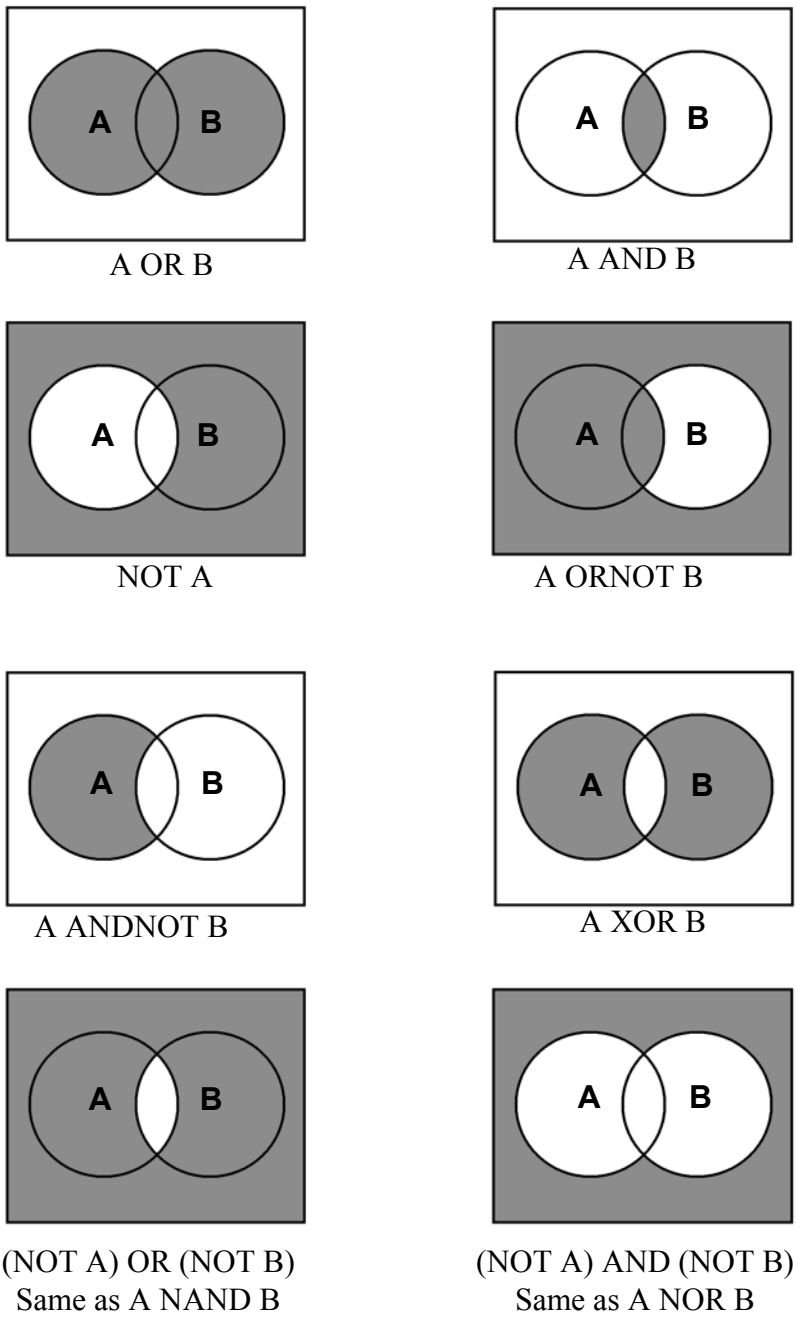


Figure 40. *Schematic definition of available Boolean Operators assuming two Cell Features of interest, A and B*

Description of Output Features

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

Cell Features

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

Since a neuron may have more than one nucleus, the nuclear results are combined and reported for the cell bodies to which they belong. The combined nuclear features are computed as follows:

CellBodyNucTotalAreaCh1 = Total area of all valid nuclei found within a selected cell body

CellBodyNucTotalIntenCh1 = Total intensity of all valid nuclei found within a selected cell body

CellBodyNucAvgIntenCh1 = Average intensity of all valid nuclei found within a selected cell body

CellBodyNucCountCh2 = Number of all valid nuclei found within a selected cell body

The results for the neurite measurements are also combined and reported for the neuron to which they belong. The combined neurite features are computed as follows:

NeuriteTotalCountCh2 = Number of neurites associated with a selected cell body

NeuriteTotalLengthCh2 = Sum of the lengths for all neurites associated with a selected cell body

$$\textbf{NeuriteAvgLengthCh2} = \frac{\textbf{NeuriteTotalLengthCh2}}{\textbf{NeuriteTotalCountCh2}}$$

NeuriteMaxLengthWithBranchesCh2 = Longest individual neurite length associated with a selected cell body (including length of all branches associated with that neurite)

NeuriteMaxLengthWithOutBranchesCh2 = Longest individual neurite length associated with a selected cell body (NOT including length of all branches associated with that neurite)

NeuriteTotalAreaCh2 = Sum of the areas for all neurites associated with a selected cell body

$$\textbf{NeuriteWidthCh2} = \frac{\textbf{NeuriteTotalAreaCh2}}{\textbf{NeuriteTotalLengthCh2}}$$

BranchPointTotalCountCh2 = Number of branch points for all neurites associated with a selected cell body

$$\textbf{BranchPointAvgCountCh2} = \frac{\textbf{BranchPointTotalCountCh2}}{\textbf{NeuriteTotalCountCh2}}$$

$$\textbf{BranchPointAvgDistFromCellBodyCh2} = \frac{\text{Sum of branch point distances for all neurites associated with a selected cell body}}{\textbf{BranchPointTotalCountCh2}}$$

$$\textbf{BranchPointCountPerNeuriteLengthCh2} = \frac{\textbf{BranchPointTotalCountCh2}}{\textbf{NeuriteTotalLenghCh2}}$$

CrossPointTotalCountCh2 = Number of cross points for all neurites associated with a selected cell body

$$\textbf{CrossPointAvgCountCh2} = \frac{\textbf{CrossPointTotalCountCh2}}{\textbf{NeuriteTotalCountCh2}}$$

The results for spot measurements in Channel 3 and Channel 4 are separated based on if they were within the cell body mask (CellBodySpot) or modified neurite mask (NeuriteSpot). Features are reported for the neuron which they belong. General combined features are computed as follows (Note that if *Region* is used below, it corresponds to either CellBody or Neurite and is found only in cells selected for analysis in all channels):

***Region*SpotTotalCountChN** = Total number of all spots within the selected *Region* of a neuron

$$\textbf{NeuriteSpotAvgCountChN} = \frac{\textbf{NeuriteSpotTotalCountChN}}{\textbf{NeuriteTotalCountCh2}}$$

***Region*SpotTotalAreaChN** = Total area of all spots within the selected *Region* of a neuron

$$\textbf{NeuriteSpotAvgAreaChN} = \frac{\textbf{NeuriteSpotTotalAreaChN}}{\textbf{NeuriteTotalCountCh2}}$$

***Region*SpotTotalIntenChN** = Total intensity of all spots within the selected *Region* of a neuron

$$\textbf{\textit{Region}SpotAvgIntenChN} = \frac{\textbf{\textit{Region}SpotTotalIntenChN}}{\textbf{\textit{Region}SpotTotalAreaChN}}$$

Intensity Ratios are also done between Channels (between Channel 3 (or Channel 4) and Channel 2; between Channel 4 and Channel 3). Comparisons with Channel 2 involve the spots found within a *Region* (CellBodySpot or NeuriteSpot) for Channel 3 (or Channel 4) compared to the corresponding entire region (CellBody or Neurite) in Channel 2, while comparisons between Channel 4 and Channel 3 involve the spots found within the same region only:

$$\textbf{CellBodySpotTotalIntenRatioChNCh2} = \frac{\textbf{CellBodySpotTotalIntenChN}}{\textbf{CellBodyTotalIntenCh2}}$$

$$\textbf{CellBodySpotTotalIntenRatioCh4Ch3} = \frac{\textbf{CellBodySpotTotalIntenCh4}}{\textbf{CellBodySpotTotalIntenCh3}}$$

$$\textbf{CellBodySpotTotalAreaRatioCh4Ch3} = \frac{\textbf{CellBodySpotTotalAreaChN}}{\textbf{CellBodyTotalAreaCh2}}$$

$$\textbf{NeuriteSpotTotalIntenRatioChNCh2} = \frac{\textbf{NeuriteSpotTotalIntenChN}}{\textbf{NeuriteTotalIntenCh2}}$$

$$\textbf{NeuriteSpotTotalIntenRatioCh4Ch3} = \frac{\textbf{NeuriteSpotTotalIntenCh4}}{\textbf{NeuriteSpotTotalIntenCh3}}$$

$$\textbf{NeuriteSpotTotalAreaRatioCh4Ch3} = \frac{\textbf{NeuriteSpotTotalAreaCh4}}{\textbf{NeuriteSpotTotalAreaCh3}}$$

Comparison of spot overlap between Channel 3 and Channel 4 are also reported (as determined through the **MinAreaSpotOverlapCh3Ch4** Assay Parameter). Count, area, and corresponding percent values are given for each channel and the corresponding spot region. For example:

NeuriteSpotOverlapCountCh3 = Total number of all spots within the modified neurite region in Channel 3 that overlap with Channel 4

$$\textbf{NeuriteSpot\%OverlapCountCh3} = \frac{\textbf{NeuriteSpotOverlapCountCh3}}{\textbf{NeuriteSpotTotalCountCh3}} \times 100$$

CellBodySpotOverlapAreaCh4 = Total overlapping area of all spots within the CellBody in Channel 4 that overlaps with Channel 3

$$\textbf{CellBodySpot\%OverlapAreaCh4} = \frac{\textbf{CellBodySpotOverlapAreaCh4}}{\textbf{CellBodySpotTotalAreaCh4}} \times 100$$

Figure 41 and Table 25 provide examples to help you better understand these features visually and mathematically. The figure is an illustration of a typical neuron, and the table reports the neurite features for this figure. It also presents how spots within a cell body or on neurites are also calculated.

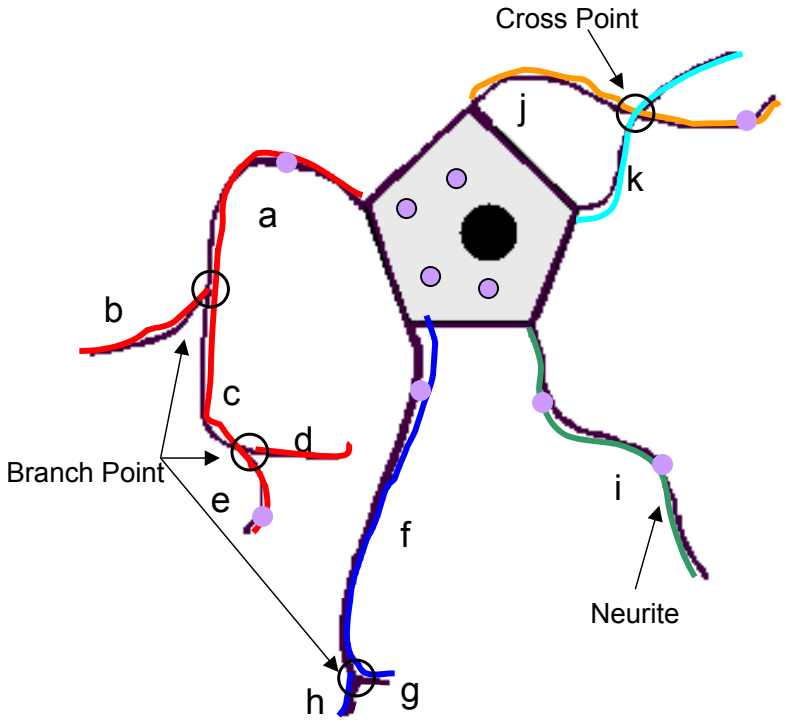


Figure 41. *Illustration of neurite features. Neurite segments are identified with lower-case letters a, b, c, etc.*

Neurite Feature	Figure Result
NeuriteTotalCountCh2	5
NeuriteTotalLengthCh2	a + b + c + d + e + f + g + h + i + j + k + l + m
NeuriteMaxLengthWithBranchesCh2	MAX (a + b + c + d + e, f + g + h, i, j + k, l + m)
NeuriteMaxLengthWithOutBranchesCh2	MAX (a + b, a + c + d, a + c + e, f + g, f + h, i, j + k, l + m)
BranchPointTotalCountCh2	3
BranchPointAvgCountCh2	3/5
BranchPointAvgDistFromCellBodyCh2	(a + (a + c) + f)/3
CrossPointTotalCountCh2	1
CrossPointAvgCountCh2	1/5
BranchPointCountPerNeuriteTotalLengthCh2	3branches/ a + b + c + d + e + f + g + h + i + j + k + l + m
CellBodySpotTotalCountCh3	4
NeuriteSpotAvgCountCh3	6NeuriteSpots/5Neurites

Table 25. *Neurite Features calculated from the example in the previous figure*

Neuronal Profiling Cell Features

Table 26 shows the output features reported for each selected cell, accessible in the Cell Feature window in the Protocol Interactive View or within the vHCS:View application. Cell Features shown with a **Status** are those features that indicate in there is a high response (=**1**) or no response (=**0**). For Events, **0** = Event did not occur, **1** = Event occurred.

Feature	Units	Description
Cell#	Number	Unique cell body ID
Top	Pixel	Y coordinate (in pixels) of top-left corner of image-aligned bounding box of Ch2 cell body
Left	Pixel	X coordinate (in pixels) of top-left corner of image-aligned bounding box of Ch2 cell body
Width	Pixel	Width (in pixels) of image-aligned bounding box of Ch2 cell body
Height	Pixel	Height (in pixels) of image-aligned bounding box of Ch2 cell body
XCentroid	Pixel	X coordinate (in pixels) of center of Ch2 cell body
YCentroid	Pixel	Y coordinate (in pixels) of center of Ch2 cell body
EventTypeProfile, EventTypeXStatus	Number	Identifies the types of events that occurred: 1, 2, 3, 12, 23, 13, 123
CellBodyNucTotalAreaCh1, Status	Pixel or μm^2	Total area (in pixels or micrometers) of all Ch1 nuclei belonging to Ch2 cell body
CellBodyNucTotalIntenCh1, Status	Intensity	Total intensity of all pixels within all Ch1 nuclei belonging to Ch2 cell body
CellBodyNucAvgIntenCh1, Status	Intensity	Average intensity of all pixels within all Ch1 nuclei belonging to Ch2 cell body
CellBodyNucCountCh2, Status	Number	Number of all Ch1 nuclei belonging to Ch2 cell body
CellBodyAreaCh2, Status	Pixel or μm^2	Area (in pixels or micrometers) of Ch2 cell body
CellBodyShapeP2ACh2, Status	Number	Shape measure based on ratio of perimeter squared to 4PI*area of Ch2 cell body (CellBodyShapeP2ACh2 = 1 for circular cell body
CellBodyShapeLWRCh2, Status	Number	Shape measure based on ratio of length to width of object-aligned bounding box of Ch2 cell body
CellBodyTotalIntenCh2, Status	Intensity	Total intensity of all pixels within Ch2 cell body
CellBodyAvgIntenCh2, Status	Intensity	Average intensity of all pixels within Ch2 cell body
NeuriteTotalCountCh2, Status	Number	Number of all neurites belonging to Ch2 cell body
NeuriteTotalLengthCh2, Status	Pixel or μm	Total length (in pixels or micrometers) of all neurites belonging to Ch2 cell body
NeuriteAvgLengthCh2, Status	Pixel or μm	Average length (in pixels or micrometers) of all neurites belonging to Ch2 cell body
NeuriteMaxLengthWithBranchesCh2, Status	Pixel or μm	Maximum length with branches (in pixels or micrometers) of all neurites belonging to Ch2 cell body

Feature	Units	Description
NeuriteMaxLengthWithoutBranchesCh2, Status	Pixel or μm	Maximum length without branches (in pixels or micrometers) of all neurites belonging to Ch2 cell body
NeuriteTotalAreaCh2, Status	Pixel or μm^2	Total area (in pixels or micrometers) of all neurites belonging to Ch2 cell body
NeuriteWidthCh2, Status	Pixel or μm	Estimated width (in pixels or micrometers) of all neurites belonging to Ch2 cell body, computed from NeuriteTotalAreaCh2 and NeuriteTotalLengthCh2
NeuriteTotalIntenCh2, Status	Intensity	Total intensity of all pixels within all neurites belonging to Ch2 cell body
NeuriteAvgIntenCh2, Status	Intensity	Average intensity of all pixels within all neurites belonging to Ch2 cell body
NeuriteVarIntenCh2, Status	Intensity	Standard deviation of intensity of all pixels within all neurites belonging to Ch2 cell body
BranchPointTotalCountCh2, Status	Number	Number of all neurite branch points belonging to Ch2 cell body
BranchPointAvgCountCh2	Number	Average number of all neurite branch points belonging to Ch2 cell body
BranchPointCountPerNeuriteLengthCh2, Status	Number	Total number per neurite length (in pixels or micrometers) of all neurite branch points belonging to Ch2 cell body
BranchPointAvgDistFromCellBodyCh2, Status	Pixel or μm	Average distance (in pixels or micrometers) of all neurite branch points from Ch2 cell body
CrossPointTotalCountCh2, Status	Number	Number of all neurite cross points belonging to Ch2 cell body
CrossPointAvgCountCh2, Status	Number	Average number of all neurite cross points belonging to Ch2 cell body
CellBodySpotTotalCountChN, Status	Number	Total number of all spots in Ch3 belonging to Ch2 cell body
CellBodySpotTotalAreaChN, Status	Pixel or μm^2	Total area (in pixels or micrometers) of all spots in Ch3 belonging to Ch2 cell body
CellBodySpotTotalIntenChN, Status	Intensity	Total intensity of all pixels within all spots in Ch3 belonging to Ch2 cell body
CellBodySpotAvgIntenChN, Status	Intensity	Average intensity of all pixels within all spots in Ch3 belonging to Ch2 cell body
CellBodySpotTotalIntenRatioChNCh2, Status	Number	Ratio of CellBodySpotTotalIntenCh3 to CellBodyTotalIntenCh2
CellBodySpotTotalIntenRatioCh4Ch3, Status	Number	Ratio of CellBodySpotTotalIntenCh4 to CellBodySpotTotalIntenCh3
CellBodySpotTotalAreaRatioCh4Ch3, Status	Number	Ratio of CellBodySpotTotalAreaCh4 to CellBodySpotTotalAreaCh3
CellBodySpotOverlapCountChN, Status	Number	Total number of overlapping spots in Ch3 belonging to Ch2 cell body
CellBodySpot%OverlapCountChN, Status	Percent	Percentage of overlapping spots relative to all spots in Ch3 belonging to Ch2 cell body
CellBodySpotOverlapAreaChN, Status	Pixel or μm^2	Total overlap area (in pixels or micrometers) of all spots in Ch3 belonging to Ch2 cell body
CellBodySpot%OverlapAreaChN, Status	Percent	Percentage of spot overlap area relative to the area of all spots in Ch3 belonging to Ch2 cell body

Feature	Units	Description
NeuriteSpotTotalCountChN, Status	Number	Total number of all spots in Ch3 located within modified Ch2 neurite mask
NeuriteSpotAvgCountChN, Status	Number	Average number of all spots per neurite in Ch3 located within modified Ch2 neurite mask
NeuriteSpotTotalAreaChN, Status	Pixel or μm^2	Total area (in pixels or micrometers) of all spots in Ch3 located within modified Ch2 neurite mask
NeuriteSpotAvgAreaChN, Status	Pixel or μm^2	Average area per neurite (in pixels or micrometers) of all spots in Ch3 located within modified Ch2 neurite mask
NeuriteSpotTotalIntenChN, Status	Intensity	Total intensity of all pixels within all spots in Ch3 located within modified Ch2 neurite mask
NeuriteSpotAvgIntenChN, Status	Intensity	Average intensity of all pixels within all spots in Ch3 located within modified Ch2 neurite mask
NeuriteSpotTotalIntenRatioChNCh2, Status	Number	Ratio of NeuriteSpotTotalIntenCh3 to NeuriteTotalIntenCh2
NeuriteSpotTotalIntenRatioCh4Ch3, Status	Number	Ratio of NeuriteSpotTotalIntenCh4 to NeuriteSpotTotalIntenCh3
NeuriteSpotTotalAreaRatioCh4Ch3, Status	Number	Ratio of NeuriteSpotTotalAreaCh4 to NeuriteSpotTotalAreaCh3
NeuriteSpotOverlapCountChN, Status	Number	Total number of overlapping spots in Ch3 located within modified Ch2 neurite mask
NeuriteSpot%OverlapCountChN, Status	Percent	Percentage of overlapping spots relative to all spots in Ch3 located within modified Ch2 neurite mask
NeuriteSpotOverlapAreaChN, Status	Pixel or μm^2	Total overlap area (in pixels or micrometers) of all spots in Ch3 located within modified Ch2 neurite mask
NeuriteSpot%OverlapAreaCh4, Status	Percent	Percentage of spot overlap area relative to the area of all spots in Ch3 located within modified Ch2 neurite mask
TotalIntenCh*N*, Status	Intensity	Total intensity in Ch3 of all pixels within modified Ch2 cell body mask
AvgIntenCh*N*, Status	Intensity	Average intensity in Ch3 of all pixels within modified Ch2 cell body mask

Table 26. *Cell Body Features Available in the Neuronal Profiling V4 BioApplication. *Note that ChN refers to Channels 3-4 for the given features as well as for Channels 5-6 for AvgInten and TotalInten (Status is not shown for Channel 5 and Channel 6).*

Field Features

When neurite density is high and it is hard to discern which neurites belong to specific cell bodies, the use of Field Features may be more beneficial for overall evaluation. Below is a list of all general Field Features.

Feature	Description
ValidNeuronCount	Number of valid neurons identified in the field (Ch2 object selection parameters applied)
SelectedNeuronCount	Number of valid neurons selected for analysis in the field (Ch3-6 object selection parameters applied)
%SelectedNeurons	Percentage of valid neurons selected for analysis in the field
ValidNucleusCount	Number of valid nuclei identified in the field (Ch1 object selection parameters applied and nucleus not belonging to rejected cell body)
NeuronNucleusRatio	Ratio of selected neurons to valid nuclei in the field
NeuronalNucleusCount	Number of valid neuronal nuclei in the field
NonNeuronalNucleusCount	Number of valid non-neuronal nuclei in the field
%NeuronalCells	Percentage of neuronal cells (selected neurons) relative to total cells in the field (selected neurons + valid non-neuronal nuclei)
%NonNeuronalCells	Percentage of non-neuronal cells (valid non-neuronal nuclei) relative to total cells in the field (selected neurons + valid non-neuronal nuclei)
ValidFieldCount	Number of fields in which neurons were selected for analysis in the field
SelectedNeuronCountPerValidField	Average number of neurons selected for analysis per valid field in the field

***Table 27**. General Field Features calculated in the Neuronal Profiling V4 BioApplication.*

In addition, specific Field Features are calculated as one of 4 factors:

Per*Field* – take total value of feature within a valid field

Per*Neuron* – take total value of feature divided by total number of neurons (cell bodies selected for analysis) in that field

Per*Neurite* – take total value feature divided by total number of neurites (selected for analysis) in that field

Per*NeuriteLength* – take total value feature divided by total neurite length of all neurites (selected for analysis) in that field

Table 28 lists all of the specific Field Features found in the BioApplication. Note that values obtained from these features may differ from overall Well Features when densities are great and it is difficult to discern which neurite belongs to which cell body.

Feature	Description
NeuriteTotalCountPerFieldCh2	Total number of all neurites selected for analysis in the field
NeuriteTotalCountPerNeuronCh2	Total number per neuron of all neurites selected for analysis in the field
NeuriteTotalLengthPerFieldCh2	Total length (in pixels or micrometers) of all neurites selected for analysis in the field
NeuriteTotalLengthPerNeuronCh2	Total length per neuron (in pixels or micrometers) of all neurites selected for analysis in the field
NeuriteTotalLengthPerNeuriteCh2	Total length per neurite (in pixels or micrometers) of all neurites selected for analysis in the field
NeuriteTotalIntenPerFieldCh2	Total intensity in Ch2 of all pixels within all neurites selected for analysis in the field
NeuriteAvgIntenPerFieldCh2	Average intensity in Ch2 of all pixels within all neurites selected for analysis in the field
NeuriteVarIntenPerFieldCh2	Standard deviation of intensity in Ch2 of all pixels within all neurites selected for analysis in the field
BranchPointTotalCountPerFieldCh2	Total number of all branch points belonging to all neurites selected for analysis in the field
BranchPointTotalCountPerNeuronCh2	Total number per neuron of all branch points belonging to all neurites selected for analysis in the field
BranchPointTotalCountPerNeuriteCh2	Total number per neurite of all branch points belonging to all neurites selected for analysis in the field
BranchPointCountPerNeuriteLengthCh2	Total number per neurite length (in pixels or micrometers) of branch points belonging to all neurites selected for analysis in the field
CellBodySpotTotalCountPerFieldChN	Total number of all spots in ChN belonging to all cell bodies selected for analysis in the field
CellBodySpotTotalCountPerNeuronChN	Total number per neuron of all spots in ChN belonging to all cell bodies selected for analysis in the field
CellBodySpotTotalAreaPerFieldChN	Total area (in pixels or micrometers) of all spots in ChN belonging to all cell bodies selected for analysis in the field
CellBodySpotTotalAreaPerNeuronChN	Total area per neuron (in pixels or micrometers) of all spots in ChN belonging to all cell bodies selected for analysis in the field
CellBodySpotTotalIntenPerFieldChN	Total intensity of all pixels within all spots in ChN belonging to all cell bodies selected for analysis in the field
CellBodySpotTotalIntenPerNeuronChN	Total intensity per neuron of all pixels within all spots in ChN belonging to all cell bodies selected for analysis in the field
CellBodySpotAvgIntenPerFieldChN	Average intensity of all pixels within all spots in ChN belonging to all cell bodies selected for analysis in the field
CellBodySpotOverlapTotalCountPerFieldChN	Total number of all overlapping spots in ChN belonging to all cell bodies selected for analysis in the field

Feature	Description
CellBodySpot%OverlapCountPerFieldChN	Percentage of overlapping spots relative to all spots in ChN belonging to all cell bodies selected for analysis in the field
CellBodySpotOverlapTotalCountPerNeuronChN	Total number per neuron of overlapping spots in ChN belonging to all cell bodies selected for analysis in the field
CellBodySpotOverlapTotalAreaPerFieldChN	Total spot overlap area (in pixels or micrometers) of all spots in ChN belonging to all cell bodies selected for analysis in the field
CellBodySpot%OverlapAreaPerFieldChN	Percentage of spot overlap area relative to the area of all spots in ChN belonging to all cell bodies selected for analysis in the field
CellBodySpotOverlapTotalAreaPerNeuronChN	Total spot overlap area per neuron (in pixels or micrometers) of all spots in ChN belonging to all cell bodies selected for analysis in the field
NeuriteSpotTotalCountPerFieldChN	Total number of all spots in ChN belonging to all neurites selected for analysis in the field
NeuriteSpotTotalCountPerNeuronChN	Total number per neuron of all spots in ChN belonging to all neurites selected for analysis in the field
NeuriteSpotTotalCountPerNeuriteChN	Total number per neurite of all spots in ChN belonging to all neurites selected for analysis in the field
NeuriteSpotTotalCountPerNeuriteLengthChN	Total number per neurite length (in pixels or micrometers) of all spots in ChN belonging to all neurites selected for analysis in the field
NeuriteSpotTotalAreaPerFieldChN	Total area (in pixels or micrometers) of all spots in ChN belonging to all neurites selected for analysis in the field
NeuriteSpotTotalAreaPerNeuronChN	Total area per neuron (in pixels or micrometers) of all spots in ChN belonging to all neurites selected for analysis in the field
NeuriteSpotTotalAreaPerNeuriteChN	Total area per neurite (in pixels or micrometers) of all spots in ChN belonging to all neurites selected for analysis in the field
NeuriteSpotTotalAreaPerNeuriteLengthChN	Total area per neurite length (in pixels or micrometers) of all spots in ChN belonging to all neurites selected for analysis in the field
NeuriteSpotTotalIntenPerFieldChN	Total intensity of all pixels within all spots in ChN belonging to all neurites selected for analysis in the field
NeuriteSpotTotalIntenPerNeuronChN	Total intensity per neuron of all pixels within all spots in ChN belonging to all neurites selected for analysis in the field
NeuriteSpotTotalIntenPerNeuriteChN	Total intensity per neurite of all pixels within all spots in ChN belonging to all neurites selected for analysis in the field
NeuriteSpotTotalIntenPerNeuriteLengthChN	Total intensity per neurite length (in pixels) of all pixels within all spots in ChN belonging to all neurites selected for analysis in the field
NeuriteSpotAvgIntenPerFieldChN	Average intensity of all pixels within all spots in ChN belonging to all neurites selected for analysis in the field

Feature	Description
NeuriteSpotOverlapTotalCountPerFieldChN	Total number of all overlapping spots in ChN belonging to all neurites selected for analysis in the field
NeuriteSpot%OverlapCountPerFieldChN	Percentage of overlapping spots relative to all spots in ChN belonging to all neurites selected for analysis in the field
NeuriteSpotOverlapTotalCountPerNeuronChN	Total number per neuron of all overlapping spots in ChN belonging to all neurites selected for analysis in the field
NeuriteSpotOverlapTotalCountPerNeuriteChN	Total number per neurite of all overlapping spots in ChN belonging to all neurites selected for analysis in the field
NeuriteSpotOverlapTotalCountPerNeuriteLengthChN	Total number per neurite length of all overlapping spots in ChN belonging to all neurites selected for analysis in the field
NeuriteSpotOverlapTotalAreaPerFieldChN	Total spot overlap area (in pixels or micrometers) of all spots in ChN belonging to all neurites selected for analysis in the field
NeuriteSpot%OverlapAreaPerFieldChN	Percentage of spot overlap area relative to the area of all spots in ChN belonging to all neurites selected for analysis in the field
NeuriteSpotOverlapTotalAreaPerNeuronChN	Total spot overlap area per neuron (in pixels or micrometers) of all spots in ChN belonging to all neurites selected for analysis in the field
NeuriteSpotOverlapTotalAreaPerNeuriteChN	Total spot overlap area per neurite (in pixels or micrometers) of all spots in ChN belonging to all neurites selected for analysis in the field
NeuriteSpotOverlapTotalAreaPerNeuriteLengthChN	Total spot overlap area per neurite length (in pixels or micrometers) of all spots in ChN belonging to all neurites selected for analysis in the field

***Table 28**. Field features Available in the Neuronal Profiling V4 BioApplication. *Note that ChN refers to Channels 3-4 for the given features.*

Well Features

Well-level summary statistics can be computed either from the Cell-level or Field-level Features measured for all the cells selected for analysis in the well.

For Well Features derived from Cell-level features, these include mean, standard deviation, standard error, coefficient of variation, and % of responders (**%HIGH**). An example of a Well Feature for the selected cells follows:

MEAN_CellBodyAreaCh2 = Average CellBodyAreaCh2 computed over all selected cells in the well

In characterizing events, there are multiple output features. The first type indicate the number and percentage of neurons where a specific event occurred. Examples of this type are:

EventType1NeuronCount = Number of selected neurons in the well where EventType1 occurred

%EventType1Neurons = Percentage of selected neurons in the well where EventType1 occurred

The second type computes the same well-level summary statistics mentioned above for cells with each event (subpopulation) in the well. An example of a Well Feature for a cell event is:

MEAN_ E1CellBodyAreaCh2 = Mean **CellBodyAreaCh2** computed over all event E1 cells in the well

The third type is an "Event-within-an-Event" output feature. Specifically, the number of selected neurons in event X also found in event Y is computed. An example of this type is:

E2_EventType1NeuronCount = Number of **EventType1** neurons in the well in which **EventType2** also occurred

General Well Features associated with the Neuronal Profiling BioApplication (not including the above calculated statistics) are the following:

ValidNeuronCount = Total number of valid neurons identified in the well (applying all Channel 2 cell body Object Selection Parameters)

SelectedNeuronCount = Total number of valid neurons *selected* for analysis in the well (applying all Channel 3-6 Object Selection Parameters)

$$\textbf{\%SelectedNeurons} = 100 \times \frac{\textbf{SelectedNeuronCount}}{\textbf{ValidNeuronCount}}$$

ValidNucleusCount = Total number of valid nuclei identified in the well (applying all Channel 1 Object Selection Parameters). Nucleus can be valid and not be associated with a cell body but it cannot belong to a rejected cell body

NeuronNucleusRatio = $\frac{\textbf{SelectedNeuronCount}}{\textbf{ValidNucleusCount}}$

NeuronalNucleusCount = Total number of valid ***neuronal*** nuclei in the well

NonNeuronalNucleusCount = Total number of valid ***non-neuronal*** nuclei in the well

%NeuronalCells = 100 x $\frac{\textbf{SelectedNeuronCount}}{\textbf{SelectedNeuronCount} + \textbf{NonNeuronalNucleusCount}}$

%NonNeuronalCells = 100 x $\frac{\textbf{NonNeuronalNucleusCount}}{\textbf{SelectedNeuronCount} + \textbf{NonNeuronalNucleusCount}}$

ValidFieldCount = Total number of fields where SelectedNeuronCount = 1 or greater

AvgSelectedNeuronCountPerField = $\frac{\textbf{SelectedNeuronCount}}{\textbf{ValidFieldCount}}$

In addition to the event and general well features, Well Features are also taken from Field Features as PerWell, PerField, PerNeuron, PerNeurite, or PerNeuriteLength. Examples of these are listed below:

BranchPointTotalCountPerWellCh2 = Total (summation) of all branch points of all neurites on all selected neurons in the all of the fields

NeuriteTotalCountPerFieldCh2 = $\frac{\text{Total count of all neurites on all selected neurons in all fields}}{\text{Total number of Valid Fields}}$

NeuriteTotalLengthPerFieldCh2 = $\frac{\text{Total length of all neurites on all selected neurons in all fields}}{\text{Total number of Valid Fields}}$

NeuriteTotalLengthPerNeuriteCh2= $\frac{\text{Total length of all neurites on all selected neurons in all fields}}{\text{Total number of neurites on all selected neurons in all fields}}$

CellBodySpotTotalCountPerNeuronChN = $\frac{\text{Total count of all selected spots within all selected cellbodies in all fields}}{\text{Total number of selected neurons in all fields}}$

NeuriteSpotCountPerNeuriteLengthChN = $\frac{\text{Total count of all selected spots within all selected neurites in all fields}}{\text{Total length of all selected neurites within all fields}}$

Figure 42 and Table 29 provides examples to help you better understand the Well Features visually and mathematically. The figure is an illustration of both non-neuronal cells (nuclei without cell bodies) and neuronal cells (nuclei with cell bodies). The table reports the Well Features for this figure.

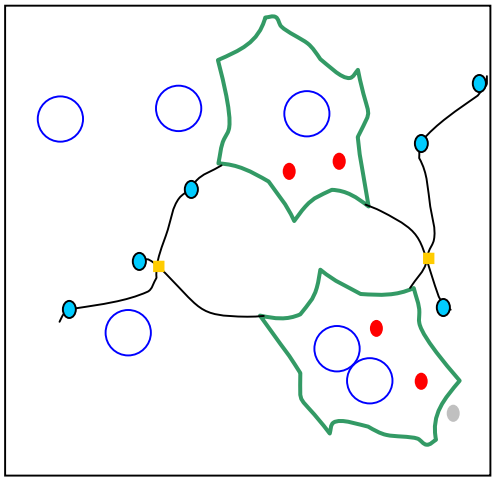


Figure 42. *Illustration of non-neuronal and neuronal cells. Overlays represent cell bodies (green), nuclei (blue), neurites (black), neurite points (yellow), cell body spots (red) and neurite spots (teal).*

Well Feature	Figure Result
SelectedNeuronCount	2
ValidNucleusCount	6
NeuronNucleusRatio	2:6 = 0.333
NeuronalNucleusCount	3
NonNeuronalNucleusCount	3
%NeuronalCells	2/5 = 40%
%NonNeuronalCells	3/5 = 60%
NeuriteTotalCountPerFieldCh2	4
CellBodySpotTotalCount	4
NeuriteSpotTotalCountPerNeurite	6/4

Table 29. *Examples of/Well Features calculated from the example in Figure 42*

Neuronal Profiling Well Features

Most of the Well Features are derived from the Cell Features. Such features are identified by a prefix, as listed below, to the cell feature name (Table 30). Events will be specified by following this prefix as E1, E2, or E3 and then the output feature name and will be shown only if an event is defined.

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

Table 30. *General Well Features Available in the Neuronal Profiling V4 BioApplication*

The following additional Well Features derived from Cell Features in the Scan Plate View in addition to the Well Detail window of the vHCS:View application (Table 31).

Feature	Description
ValidNeuronCount	Number of valid neurons identified in the well (Ch2 object selection parameters applied)
SelectedNeuronCount	Number of valid neurons selected for analysis in the well (Ch3-6 object selection parameters applied)
%SelectedNeurons	Percentage of valid neurons selected for analysis in the well
ValidNucleusCount	Number of valid nuclei identified in the well (Ch1 object selection parameters applied and nucleus not belonging to rejected cell body)
NeuronNucleusRatio	Ratio of selected neurons to valid nuclei in the well
NeuronalNucleusCount	Number of valid neuronal nuclei in the well
NonNeuronalNucleusCount	Number of valid non-neuronal nuclei in the well
%NeuronalCells	Percentage of neuronal cells (selected neurons) relative to total cells in the well (selected neurons + valid non-neuronal nuclei)
%NonNeuronalCells	Percentage of non-neuronal cells (valid non-neuronal nuclei) relative to total cells in the well (selected neurons + valid non-neuronal nuclei)
ValidFieldCount	Number of fields in which neurons were selected for analysis in the well
SelectedNeuronCountPerValidField	Average number of neurons selected for analysis per valid field in the well
EventType1NeuronCount	Number of neurons selected for analysis in the well in which EventType1 occurred
%EventType1Neurons	Percentage of neurons selected for analysis in the well in which EventType1 occurred
EventType2NeuronCount	Number of neurons selected for analysis in the well in which EventType2 occurred
%EventType2Neurons	Percentage of neurons selected for analysis in the well in which EventType2 occurred
EventType3NeuronCount	Number of neurons selected for analysis in the well in which EventType3 occurred

Feature	Description
%EventType3Neurons	Percentage of neurons selected for analysis in the well in which EventType3 occurred
E*N*_EventType1NeuronCount	Number of EventType*N* neurons in the well in which EventType1 occurred
E*N*_%EventType1Neurons	Percentage of EventType*N* neurons in the well in which EventType1 occurred
E*N*_EventType2NeuronCount	Number of EventType*N* neurons in the well in which EventType2 occurred
E*N*_%EventType2Neurons	Percentage of EventType*N* neurons selected for analysis in the well in which EventType2 occurred
E*N*_EventType3NeuronCount	Number of EventType*N* neurons selected for analysis in the well in which EventType3 occurred
E*N*_%EventType3Neurons	Percentage of EventType*N* neurons selected for analysis in the well in which EventType3 occurred

Table 31. *Specific Well Features Available in the Neuronal V4 Profiling BioApplication. *Note that "EN" found in the Event Feature names below signify either Event 1, 2, or 3 and are all shown whether an event is defined or not.*

Also present are Well Features derived from specified Field Features. Table 32 is a general listing of all features and how they are calculated, while Table 33 reflects all field-based well features.

Feature Type	Well Feature Description
*Feature*PerWellChx	Value is totaled for all valid fields in the well
*Feature*PerFieldChx	Average is taken from values calculated for Field output of *Feature*PerFieldChx taken from all valid fields in the well
*Feature*PerNeuronChx	Average is taken from values calculated for Field output of *Feature*PerFNeuronChx taken from all valid fields in the well
*Feature*PerNeuriteChx	Average is taken from values calculated for Field output of *Feature*PerNeuriteChx taken from all valid fields in the well
*Feature*PerNeuriteLengthChx	Average is taken from values calculated for Field output of *Feature*PerNeuriteLengthChx taken from all valid fields in the well

Table 32. *General terms for Well Features derived from Field Features available in the Neuronal Profiling V4 BioApplication.*

Field-derived Well Feature	Well Feature Description
NeuriteTotalCountPerWellCh2	Total number of all neurites selected for analysis in the well
NeuriteTotalCountPerFieldCh2	Total number per field of all neurites selected for analysis in the well
NeuriteTotalCountPerNeuronCh2	Total number per neuron of all neurites selected for analysis in the well
NeuriteTotalLengthPerWellCh2	Total length (in pixels or micrometers) of all neurites selected for analysis in the well
NeuriteTotalLengthPerFieldCh2	Total length per field (in pixels or micrometers) of all neurites selected for analysis in the well
NeuriteTotalLengthPerNeuronCh2	Total length per neuron (in pixels or micrometers) of all neurites selected for analysis in the well
NeuriteTotalLengthPerNeuriteCh2	Total length per neurite (in pixels or micrometers) of all neurites selected for analysis in the well (= average neurite length)
NeuriteTotalIntenPerWellCh2	Total intensity of all pixels within all neurites selected for analysis in the well

Field-derived Well Feature	Well Feature Description
NeuriteTotalIntenPerFieldCh2	Total intensity per field of all pixels within all neurites selected for analysis in the well
NeuriteAvgIntenPerFieldCh2	Average intensity per field of all pixels within all neurites selected for analysis in the well
NeuriteVarIntenPerFieldCh2	Standard deviation of intensity per field of all pixels within all neurites selected for analysis in the well
BranchPointTotalCountPerWellCh2	Total number of all branch points belonging to all neurites selected for analysis in the well
BranchPointTotalCountPerFieldCh2	Total number per field of all branch points belonging to all neurites selected for analysis in the well
BranchPointTotalCountPerNeuronCh2	Total number per neuron of all branch points belonging to all neurites selected for analysis in the well
BranchPointTotalCountPerNeuriteCh2	Total number per neurite of all branch points belonging to all neurites selected for analysis in the well
BranchPointCountPerNeuriteLengthCh2	Total number per neurite length (in pixels or micrometers) of all branch points belonging to all neurites selected for analysis in the well
CellBodySpotTotalCountPerWellChN	Total number of all spots in ChN belonging to all cell bodies selected for analysis in the well
CellBodySpotTotalCountPerFieldChN	Total number per field of all spots in ChN belonging to all cell bodies selected for analysis in the well
CellBodySpotTotalCountPerNeuronChN	Total number per neuron of all spots in ChN belonging to all cell bodies selected for analysis in the well
CellBodySpotTotalAreaPerWellChN	Total area (in pixels or micrometers) of all spots in ChN belonging to all cell bodies selected for analysis in the well
CellBodySpotTotalAreaPerFieldChN	Total area per field (in pixels or micrometers) of all spots in ChN belonging to all cell bodies selected for analysis in the well
CellBodySpotTotalAreaPerNeuronChN	Total area per neuron (in pixels or micrometers) of all spots in ChN belonging to all cell bodies selected for analysis in the well
CellBodySpotTotalIntenPerWellChN	Total intensity of all pixels within all spots in ChN belonging to all cell bodies selected for analysis in the well
CellBodySpotTotalIntenPerFieldChN	Total intensity per field of all pixels within all spots in ChN belonging to all cell bodies selected for analysis in the well
CellBodySpotTotalIntenPerNeuronChN	Total intensity per neuron of all pixels within all spots in ChN belonging to all cell bodies selected for analysis in the well
CellBodySpotAvgIntenPerFieldChN	Average intensity per field of all pixels within all spots in ChN belonging to all cell bodies selected for analysis in the well
CellBodySpotOverlapTotalCountPerWellChN	Total number of all overlapping spots in ChN belonging to all cell bodies selected for analysis in the well
CellBodySpot%OverlapCountPerWellChN	Percentage of overlapping spots relative to all spots in ChN belonging to all cell bodies selected for analysis in the well
CellBodySpotOverlapTotalCountPerFieldChN	Total number per field of all overlapping spots in ChN belonging to all cell bodies selected for analysis in the well
CellBodySpotOverlapTotalCountPerNeuronChN	Total number per neuron of all overlapping spots in ChN belonging to all cell bodies selected for analysis in the well
CellBodySpotOverlapTotalAreaPerWellChN	Total spot overlap area (in pixels or micrometers) of all spots in ChN belonging to all cell bodies selected for analysis in the well
CellBodySpot%OverlapAreaPerWellChN	Percentage of spot overlap area relative to the area of all spots in ChN belonging to all cell bodies selected for analysis in the well

Field-derived Well Feature	Well Feature Description
CellBodySpotOverlapTotalAreaPerFieldChN	Total spot overlap area per field (in pixels or micrometers) of all spots in ChN belonging to all cell bodies selected for analysis in the well
CellBodySpotOverlapTotalAreaPerNeuronChN	Total spot overlap area per neuron (in pixels or micrometers) of all spots in ChN belonging to all cell bodies selected for analysis in the well
NeuriteSpotTotalCountPerWellChN	Total number of all spots in ChN belonging to all neurites selected for analysis in the well
NeuriteSpotTotalCountPerFieldChN	Total number per field of all spots in ChN belonging to all neurites selected for analysis in the well
NeuriteSpotTotalCountPerNeuronChN	Total number per neuron of all spots in ChN belonging to all neurites selected for analysis in the well
NeuriteSpotTotalCountPerNeuriteChN	Total number per neurite of all spots in ChN belonging to all neurites selected for analysis in the well
NeuriteSpotTotalCountPerNeuriteLengthChN	Total number per neurite length (in pixels or micrometers) of all spots in ChN belonging to all neurites selected for analysis in the well
NeuriteSpotTotalAreaPerWellChN	Total area (in pixels or micrometers) of all spots in ChN belonging to all neurites selected for analysis in the well
NeuriteSpotTotalAreaPerFieldChN	Total area per field (in pixels or micrometers) of all spots in ChN belonging to all neurites selected for analysis in the well
NeuriteSpotTotalAreaPerNeuronChN	Total area per neuron (in pixels or micrometers) of all spots in ChN within all neurites selected for analysis in the well
NeuriteSpotTotalAreaPerNeuriteChN	Total area per neurite (in pixels or micrometers) of all spots in ChN belonging to all neurites selected for analysis in the well
NeuriteSpotTotalAreaPerNeuriteLengthChN	Total area per neurite length (in pixels or micrometers) of all spots in ChN belonging to all neurites selected for analysis in the well
NeuriteSpotTotalIntenPerWellChN	Total intensity of all pixels within all spots in ChN belonging to all neurites selected for analysis in the well
NeuriteSpotTotalIntenPerFieldChN	Total intensity per field of all pixels within all spots in ChN belonging to all neurites selected for analysis in the well
NeuriteSpotTotalIntenPerNeuronChN	Total intensity per neuron of all pixels within all spots in ChN belonging to all neurites selected for analysis in the well
NeuriteSpotTotalIntenPerNeuriteChN	Total intensity per neurite of all pixels within all spots in ChN belonging to all neurites selected for analysis in the well
NeuriteSpotTotalIntenPerNeuriteLengthChN	Total intensity per neurite length of all pixels within all spots in ChN belonging to all neurites selected for analysis in the well
NeuriteSpotAvgIntenPerFieldChN	Average intensity per field of all pixels within all spots in ChN belonging to all neurites selected for analysis in the well
NeuriteSpotOverlapTotalCountPerWellChN	Total number of all overlapping spots in ChN belonging to all neurites selected for analysis in the well
NeuriteSpotOverlapTotalCountPerFieldChN	Total number per field of all overlapping spots in ChN belonging to all neurites selected for analysis in the well
NeuriteSpotOverlapTotalCountPerNeuronChN	Total number per neuron of all overlapping spots in ChN belonging to all neurites selected for analysis in the well
NeuriteSpotOverlapTotalCountPerNeuriteChN	Total number per neurite of all overlapping spots in ChN belonging to all neurites selected for analysis in the well
NeuriteSpotOverlapTotalCountPerNeuriteLengthChN	Total number per neurite length (in pixels or micrometers) of all overlapping spots in ChN belonging to all neurites selected for analysis in the well
NeuriteSpotOverlapTotalAreaPerWellChN	Total spot overlap area (in pixels or micrometers) of all spots in ChN belonging to all neurites selected for analysis in the well

Field-derived Well Feature	Well Feature Description
NeuriteSpot%OverlapAreaPerWellChN	Percentage of spot overlap area relative to the area of all spots in ChN belonging to all neurites selected for analysis in the well
NeuriteSpotOverlapTotalAreaPerFieldChN	Total spot overlap area per field (in pixels or micrometers) of all spots in ChN within all neurites selected for analysis in the well
NeuriteSpotOverlapTotalAreaPerNeuronChN	Total spot overlap area per neuron (in pixels or micrometers) of all spots in ChN within all neurites selected for analysis in the well
NeuriteSpotOverlapTotalAreaPerNeuriteChN	Total spot overlap area per neurite (in pixels or micrometers) of all spots in ChN belonging to all neurites selected for analysis in the well
NeuriteSpotOverlapTotalAreaPerNeuriteLengthChN	Total spot overlap area per neurite length (in pixels or micrometers) of all spots in ChN belonging to all neurites selected for analysis in the well

Table 33. *Specific Field-derived Well Features found in the Neuronal Profiling V4 BioApplication. Note that ChN represents both Channel 3 and Channel 4.*

Reference Well Features

The algorithm reports the following features in the Scan Plate view of the software application as well as Plate Features in the vHCS:View application (Table 34). These features are computed and reported only when the Assay Parameter **UseReferenceWells** = **1** (enabled).

Feature	Description
RefAvgNeuronCountPerField	Average number of neurons per field/well in reference wells
RefCellBodyNucTotalAreaCh1LevelHigh	High-response level for **CellBodyNucTotalAreaCh1** computed from reference well results
RefCellBodyNucTotalIntenCh1LevelHigh	High-response level for **CellBodyNucTotalIntenCh1** computed from reference well results
RefCellBodyNucAvgIntenCh1LevelHigh	High-response level for **CellBodyNucAvgIntenCh1** computed from reference well results
RefCellBodyNucCountCh2LevelHigh	High-response level for **CellBodyNucCountCh2** computed from reference well results
RefCellBodyAreaCh2LevelHigh	High-response level for **CellBodyAreaCh2** computed from reference well results
RefCellBodyShapeP2ACh2LevelHigh	High-response level for **CellBodyShapeP2ACh2** computed from reference well results
RefCellBodyShapeLWRCh2LevelHigh	High-response level for **CellBodyShapeLWRCh2** computed from reference well results
RefCellBodyTotalIntenCh2LevelHigh	High-response level for **CellBodyTotalIntenCh2** computed from reference well results
RefCellBodyAvgIntenCh2LevelHigh	High-response level for **CellBodyAvgIntenCh2** computed from reference well results
RefNeuriteTotalCountCh2LevelHigh	High-response level for **NeuriteTotalCountCh2** computed from reference well results
RefNeuriteTotalLengthCh2LevelHigh	High-response level for **NeuriteTotalLengthCh2** computed from reference well results
RefNeuriteAvgLengthCh2LevelHigh	High-response level for **NeuriteAvgLengthCh2** computed from reference well results
RefNeuriteMaxLengthWithBranchesCh2LevelHigh	High-response level for **NeuriteMaxLengthWithBranchesCh2** computed from reference well results
RefNeuriteMaxLengthWithoutBranchesCh2LevelHigh	High-response level for **NeuriteMaxLengthWithoutBranchesCh2** computed from reference well results
RefNeuriteTotalAreaCh2LevelHigh	High-response level for **NeuriteTotalAreaCh2** computed from reference well results
RefNeuriteWidthCh2LevelHigh	High-response level for **NeuriteWidthCh2** computed from reference well results
RefNeuriteTotalIntenCh2LevelHigh	High-response level for **NeuriteTotalIntenCh2** computed from reference well results
RefNeuriteAvgIntenCh2LevelHigh	High-response level for **NeuriteAvgIntenCh2** computed from reference well results
RefNeuriteVarIntenCh2LevelHigh	High-response level for **NeuriteVarIntenCh2** computed from reference well results
RefBranchPointTotalCountCh2LevelHigh	High-response level for **BranchPointTotalCountCh2** computed from reference well results
RefBranchPointAvgCountCh2LevelHigh	High-response level for **BranchPointAvgCountCh2** computed from reference well results
RefBranchPointCountPerNeuriteLengthLevelHigh	High-response level for **BranchPointCountPerNeuriteLengthCh2** computed from reference well results

Feature	Description
RefBranchPointAvgDistFromCellBodyCh2LevelHigh	High-response level for **BranchPointAvgDistFromCellBodyCh2** computed from reference well results
RefCrossPointTotalCountCh2LevelHigh	High-response level for **CrossPointTotalCountCh2** computed from reference well results
RefCrossPointAvgCountCh2LevelHigh	High-response level for **CrossPointAvgCountCh2** computed from reference well results
RefCellBodySpotTotalCountChNLevelHigh	High-response level for **CellBodySpotTotalCountChN** computed from reference well results
RefCellBodySpotTotalAreaChNLevelHigh	High-response level for **CellBodySpotTotalAreaChN** computed from reference well results
RefCellBodySpotTotalIntenChNLevelHigh	High-response level for **CellBodySpotTotalIntenChN** computed from reference well results
RefCellBodySpotAvgIntenChNLevelHigh	High-response level for **CellBodySpotAvgIntenChN** computed from reference well results
RefCellBodySpotTotalIntenRatioChNCh2LevelHigh	High-response level for **CellBodySpotTotalIntenRatioChNCh2** computed from reference well results
RefCellBodySpotTotalIntenRatioCh4Ch3LevelHigh	High-response level for **CellBodySpotTotalIntenRatioCh4Ch3** computed from reference well results
RefCellBodySpotTotalAreaRatioCh4Ch3LevelHigh	High-response level for **CellBodySpotTotalAreaRatioCh4Ch3** computed from reference well results
RefCellBodySpotOverlapCountChNLevelHigh	High-response level for **CellBodySpotOverlapCountChN** computed from reference well results
RefCellBodySpot%OverlapCountChNLevelHigh	High-response level for **CellBodySpot%OverlapCountChN** computed from reference well results
RefCellBodySpotOverlapAreaChNLevelHigh	High-response level for **CellBodySpotOverlapAreaChN** computed from reference well results
RefCellBodySpot%OverlapAreaChNLevelHigh	High-response level for **CellBodySpot%OverlapAreaChN** computed from reference well results
RefNeuriteSpotTotalCountChNLevelHigh	High-response level for **NeuriteSpotTotalCountChN** computed from reference well results
RefNeuriteSpotAvgCountChNLevelHigh	High-response level for **NeuriteSpotAvgCountChN** computed from reference well results
RefNeuriteSpotTotalAreaChNLevelHigh	High-response level for **NeuriteSpotTotalAreaChN** computed from reference well results
RefNeuriteSpotAvgAreaChNLevelHigh	High-response level for **NeuriteSpotAvgAreaChN** computed from reference well results
RefNeuriteSpotTotalIntenChNLevelHigh	High-response level for **NeuriteSpotTotalIntenChN** computed from reference well results
RefNeuriteSpotAvgIntenChNLevelHigh	High-response level for **NeuriteSpotAvgIntenChN** computed from reference well results
RefNeuriteSpotTotalIntenRatioChNCh2LevelHigh	High-response level for **NeuriteSpotTotalIntenRatioChNCh2** computed from reference well results
RefNeuriteSpotTotalIntenRatioCh4Ch3LevelHigh	High-response level for **NeuriteSpotTotalIntenRatioCh4Ch3** computed from reference well results
RefNeuriteSpotTotalAreaRatioCh4Ch3LevelHigh	High-response level for **NeuriteSpotTotalAreaRatioCh4Ch3** computed from reference well results
RefNeuriteSpotOverlapCountChNLevelHigh	High-response level for **NeuriteSpotOverlapCountChN** computed from reference well results
RefNeuriteSpot%OverlapCountChNLevelHigh	High-response level for **NeuriteSpot%OverlapCountChN** computed from reference well results

Feature	Description
RefNeuriteSpotOverlapAreaChNLevelHigh	High-response level for **NeuriteSpotOverlapAreaChN** computed from reference well results
RefNeuriteSpot%OverlapAreaChNLevelHigh	High-response level for **NeuriteSpot%OverlapAreaChN** computed from reference well results
RefTotalIntenChNLevelHigh	High-response level for **TotalIntenChN** computed from reference well results
RefAvgIntenChNLevelHigh	High-response level for **AvgIntenChN** computed from reference well results

Table 34. *Reference Features Available in the Neuronal Profiling V4 BioApplication. *Note that "ChN" refers to Channel 3 and Channel 4.*

Chapter

3

Using the Neuronal Profiling V4 BioApplication

The Neuronal Profiling (NP) V4 BioApplication is provided with Assay Protocols configured to run the assays described in Chapter 1. Please refer to the appropriate user guide for instructions on scanning an individual plate or stacks of plates using an existing Assay Protocol. This chapter describes in more detail the implementation of the Events Wizard.

Event Definition Using the BioApplication Event Wizard

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

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

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

STEP I

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

STEP II

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

STEP III

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

STEP IV (Optional)

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

Features

The following features are included in the BioApplication Event Wizard:

- Can be used with any Neuronal Profiling 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 Neuronal Profiling

Before Running the Event Wizard...

1) Create a protocol using the Neuronal Profiling V4 BioApplication without defining Events. Set optimized parameter values (upper levels) for detection of responder cells associated with Cell Features to be used for Event Definition.

2) Save protocol.

3) Close the vHCS:Scan software application (suggested).

Starting the BioApplication Event Wizard

To start the BioApplication Event Wizard,

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

-or-

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

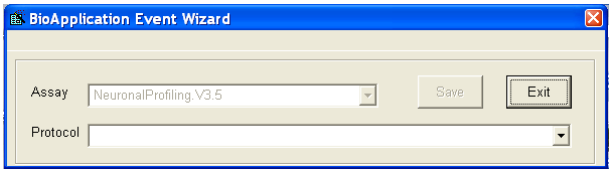
For vHCS Discovery Toolbox computers: From the Windows **Start** menu, select **Programs** > **Cellomics > vHCS Scan > BioAppEventWizard**

Protocol Selection and Loading

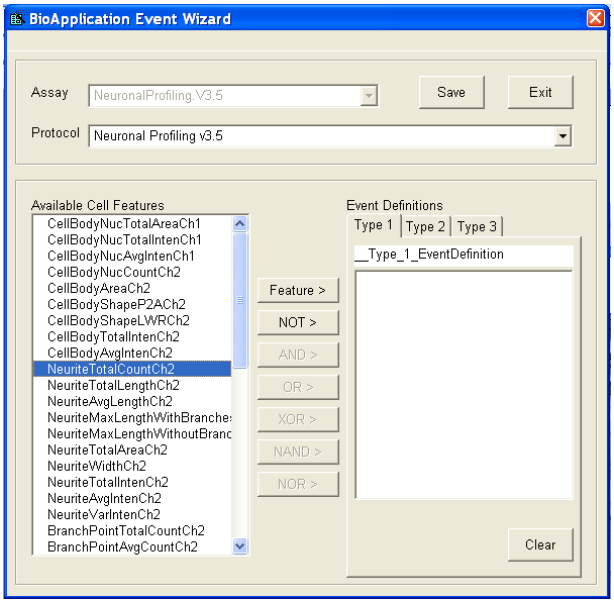
The first window that is displayed (see below) allows selection of any Neuronal Profiling Assay Protocol. Note that Event Definitions can only be specified for pre-existing Assay Protocols.

To select an Assay Protocol,

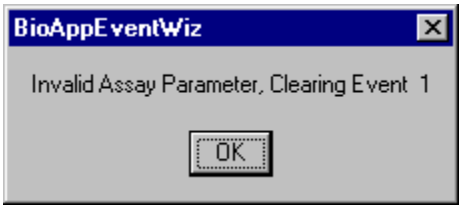
1) From the **Assay** drop-down menu, select **NeuronalProfiling.V4**.
2) From the **Protocol** drop-down menu, click on the drop-down arrow to view the list of existing Neuronal Profiling Assay Protocols.
3) Select the desired Assay Protocol from the list.



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



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



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

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

Defining Events

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

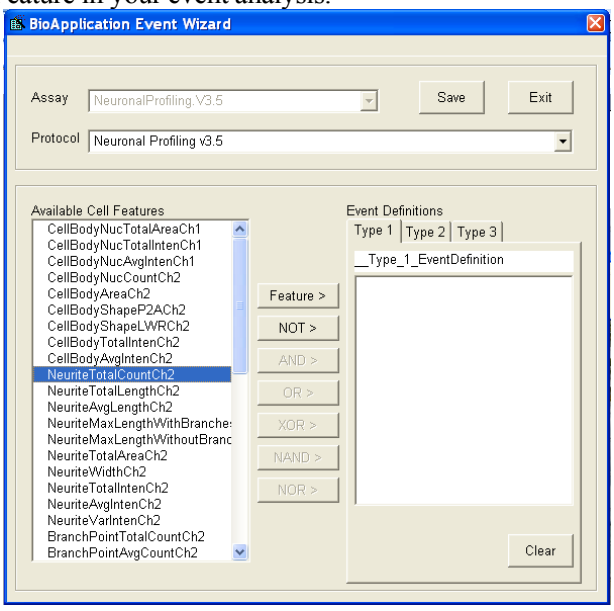
Once the protocol has loaded, each Event Definition can be viewed by clicking on the appropriate tab (Type 1-3).

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.

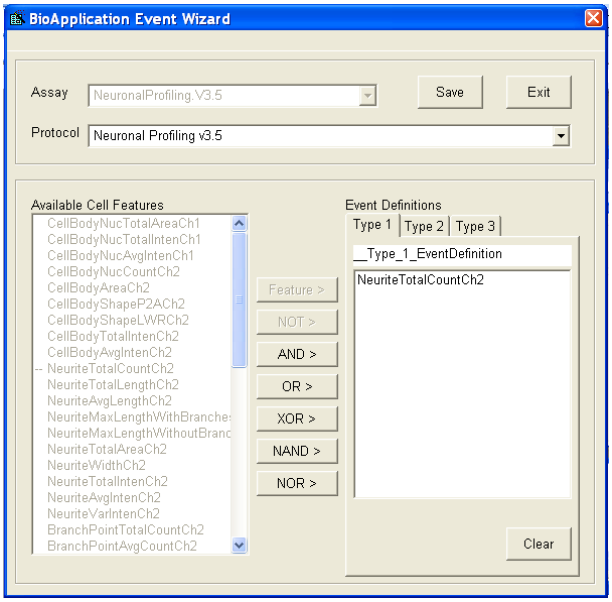
To build an Event Definition,

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

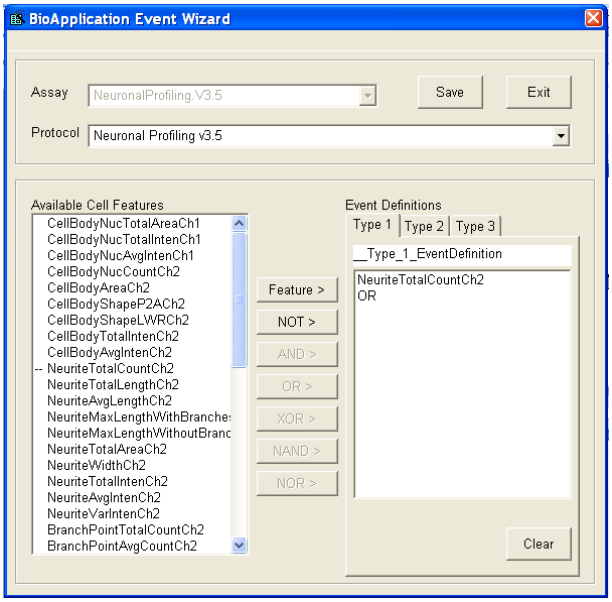
2) Select the desired Cell Feature by clicking on the feature name from the Available Cell Features list. In this example, the **NeuriteTotalCountCh2** feature is selected. You may also choose to select **NOT>** first (before the Feature) to indicate that you do not want to include this Cell Feature in your event analysis.



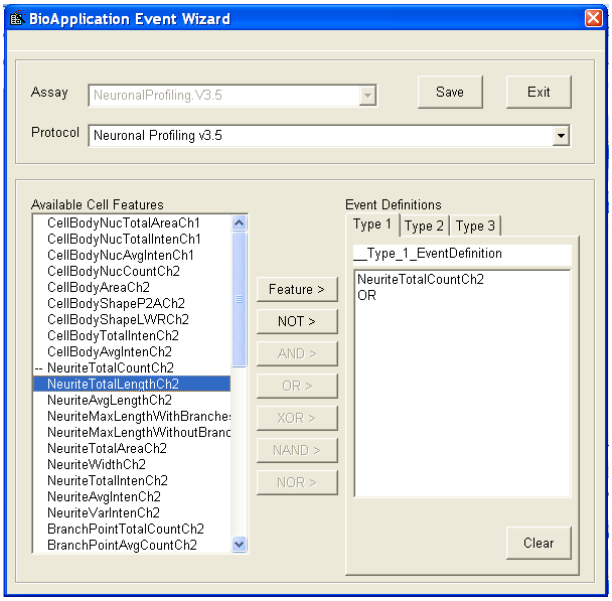
3) Click the **Feature>** button to transfer the Cell Feature into the Event Definition



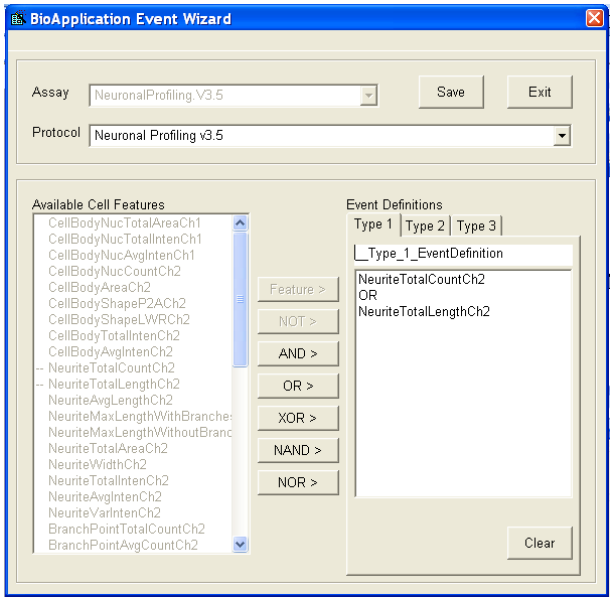
4) Click on the desired Operator button to transfer it into the Event Definition. In this example, the **OR** operator is selected (see below).



5) Repeat the cycle for adding another Cell Feature to the Event Definition. In this case, the Cell Feature **NeuriteTotalLengthCh2** has been selected for addition.

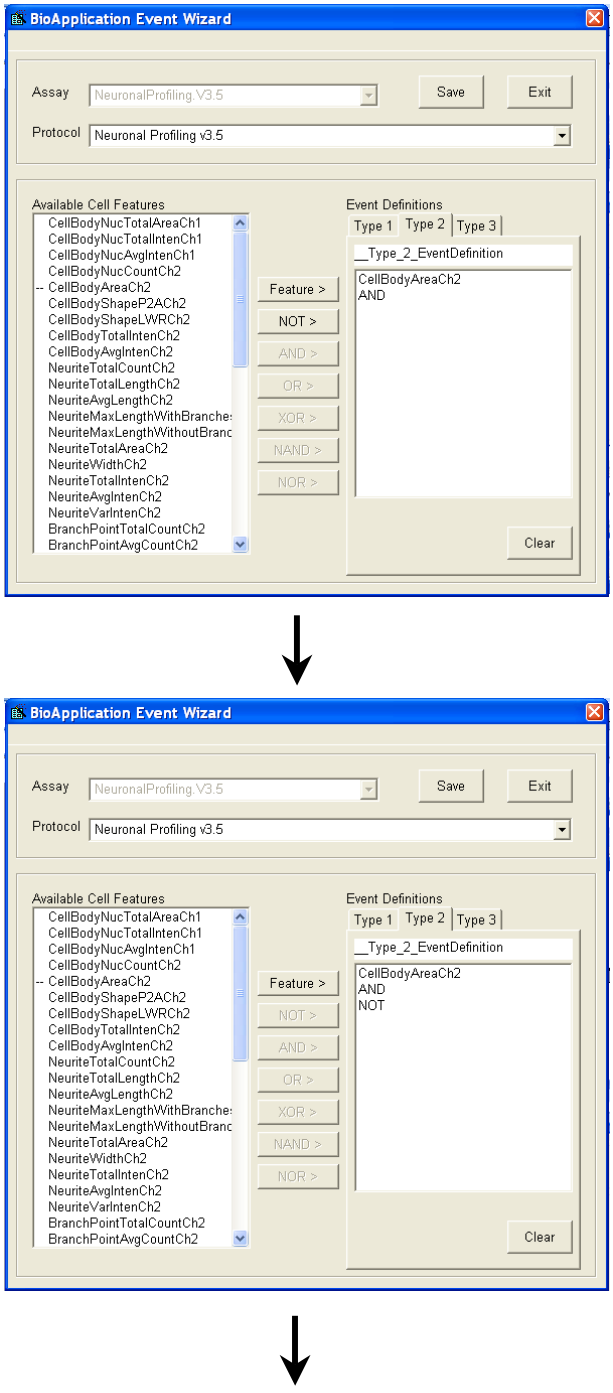


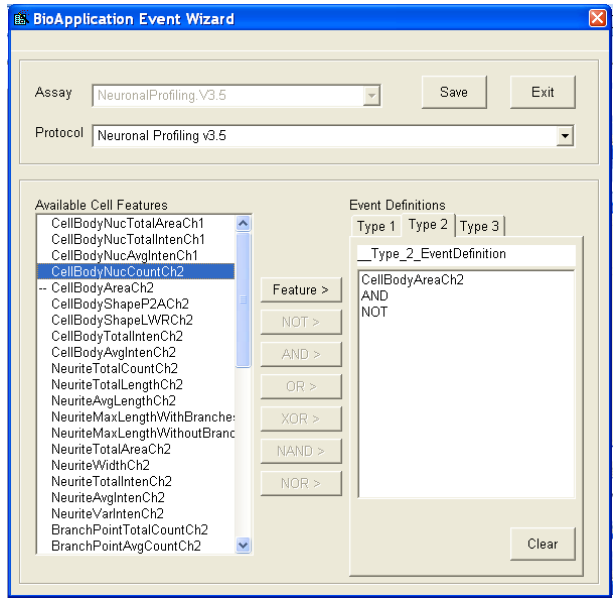
6) As before, click on the **Feature >** button to transfer the Cell Feature into the Event Definition (see below).

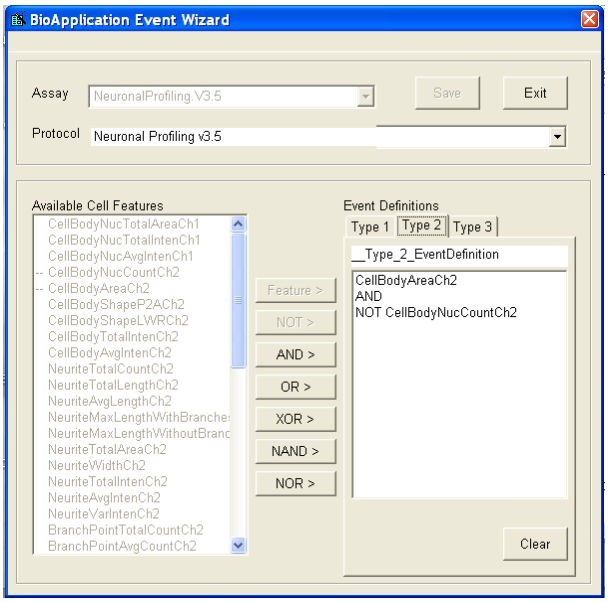


Type_1_EventDefinition is: **NeuriteTotalCountCh2** OR **NeuriteTotalLengthCh2**

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.

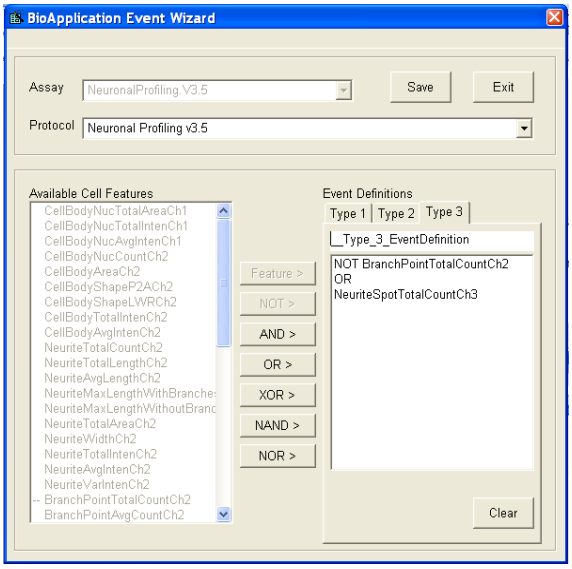






Type_2_EventDefinition is: **CellBodyAreaCh2** ANDNOT **CellBodyNucCountCh2**

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



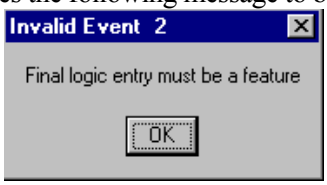
To clear an Event Definition,

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

To 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.

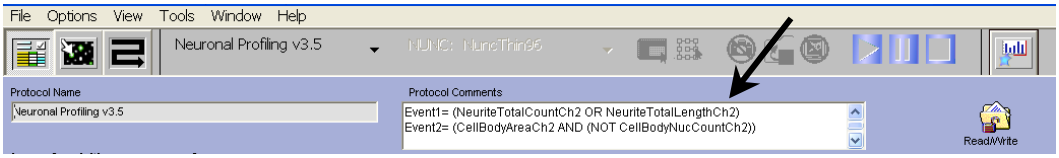
The BioApplication Event Wizard has the capability to automatically validate Event Definitions. If Event Definition validation fails, the protocol will not be saved. For example, Event Definitions are considered invalid if they do not end with a Cell Feature. In this case, clicking on the **Save** button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



To exit the BioApplication Event Wizard,

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

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



Chapter 4

iDev Software Workflow

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

iDev Protocol Optimization Tasks

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

Image Preprocessing

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

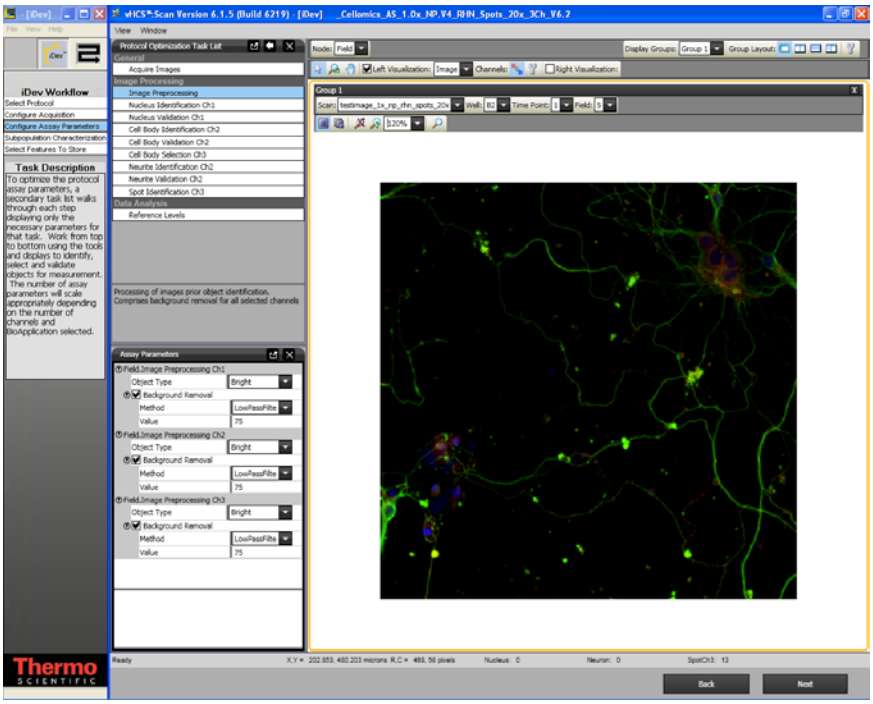


Figure 43. *Protocol Optimization Task – Image Preprocessing*

Nucleus Identification Ch1

Nucleus Identification is the identification of nuclei 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. Additionally you can remove small objects (artifacts) by checking the box for “Object Cleanup”.

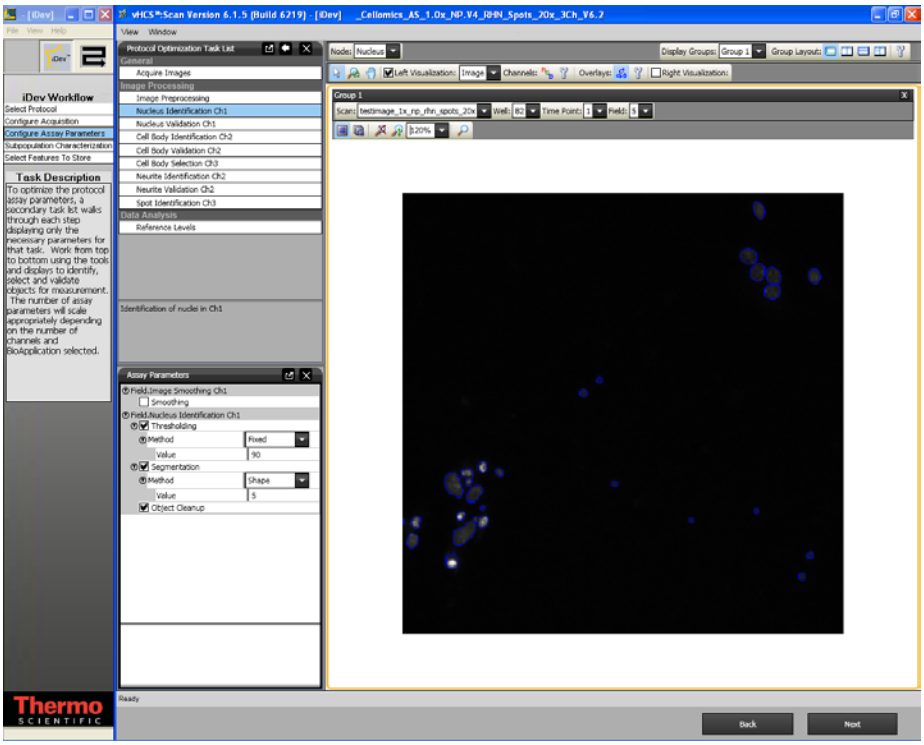


Figure 44. *Protocol Optimization Task – Nucleus Identification Ch1*

Nucleus Validation Ch1

Nucleus Validation is nuclei 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) nuclei in Channel 1 based on object selection features. Additionally in this task, you will determine if nuclei that are on the border of the field are included or rejected from analysis.

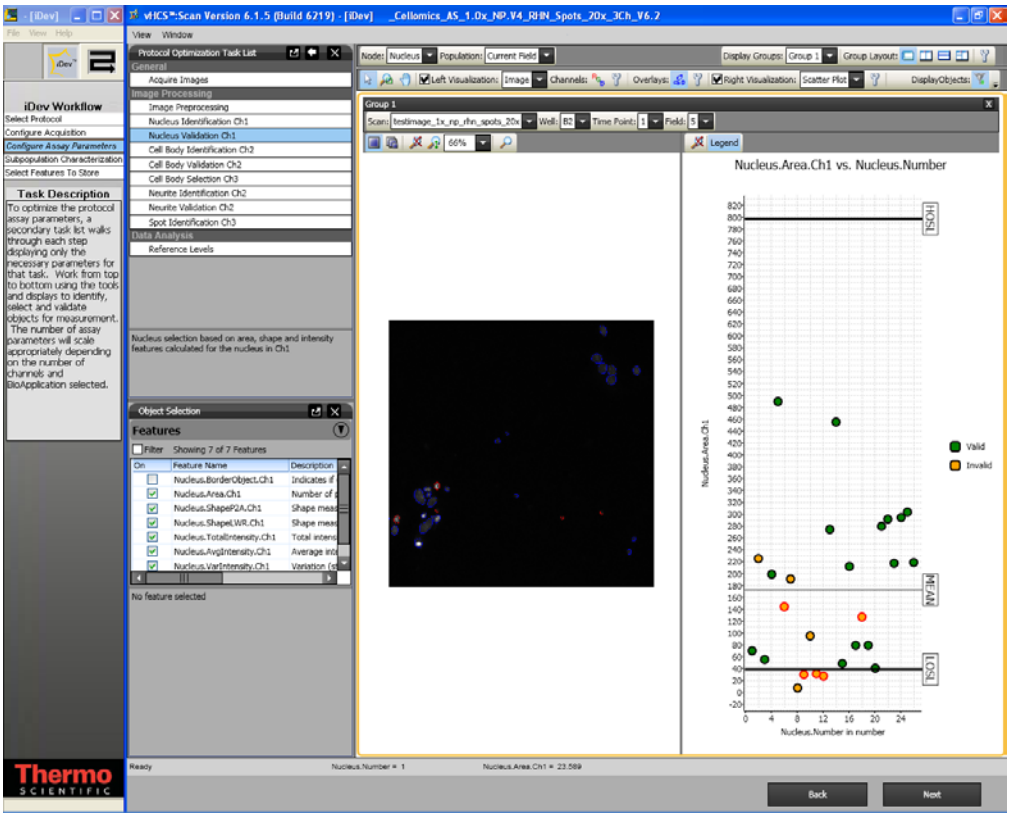


Figure 45. *Protocol Optimization Task – Nucleus Validation Ch1*

Cellbody Identification Ch2

This task is for optimizing methods and values of parameters that allow you to identify the cell body in Channel 2. You can set up methods and values for smoothing, identification, segmentation, and also clean up and modification of the cell body mask in Channel 2.

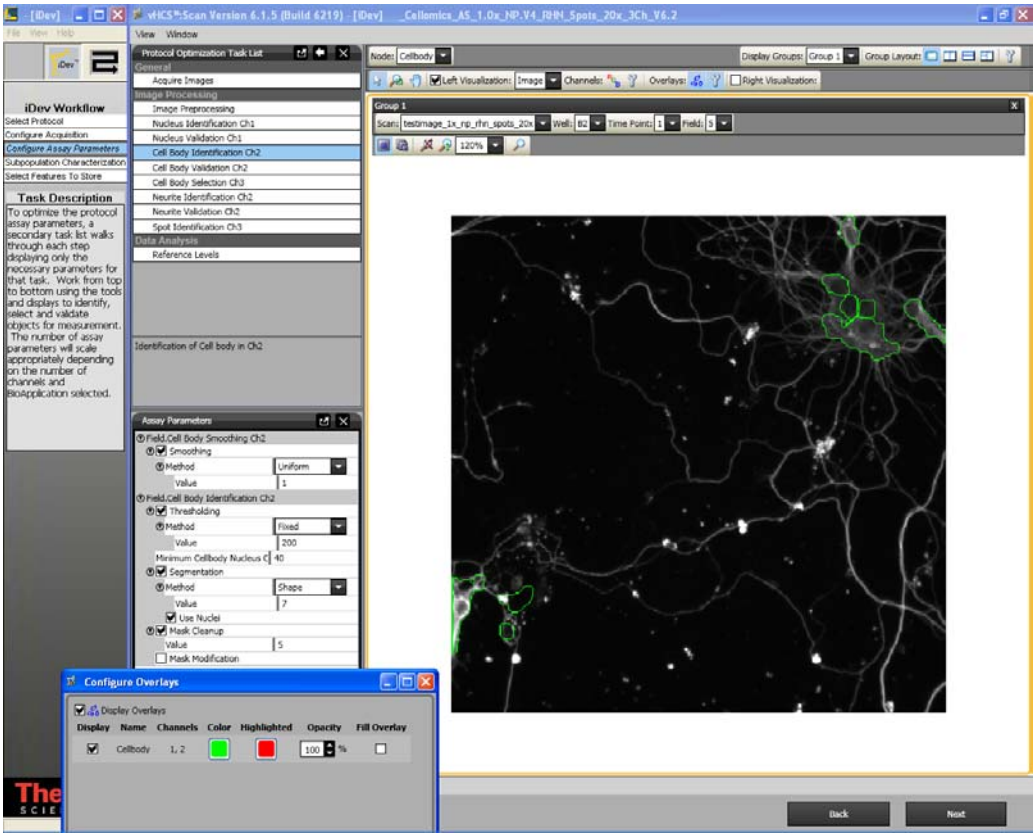


Figure 46. *Protocol Optimization Task – Cellbody Identification*

Cellbody Validation Ch2

In this task, you can set cell body validating criteria based on nuclear count, area, shape, and intenisty features. This is equivalent to setting selection/rejection criteria for the cell body in the ArrayScan Classic software.

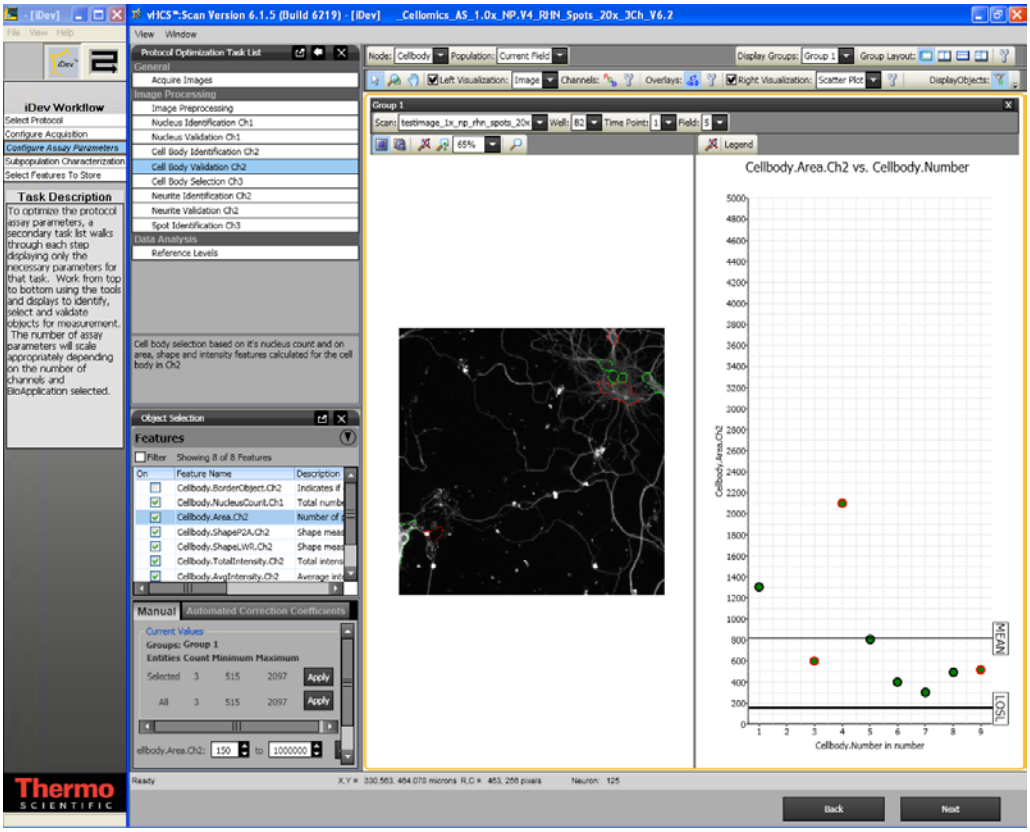


Figure 47. *Protocol Optimization Task – Cellbody Validation*

Cellbody Selection Ch3 through ChN

In this task, you can set criteria for Cell body selection in Channels 3 through Channel N, based on an ROI mask derived from the Channel 2 cell body mask. This task is similar to setting the Assay Parameter, **MaskModifierChN,** in the ArrayScan Classic software. The primary object mask can be dilated (> 0), or eroded (< 0) if the **ROI Mask Creation** box is checked.

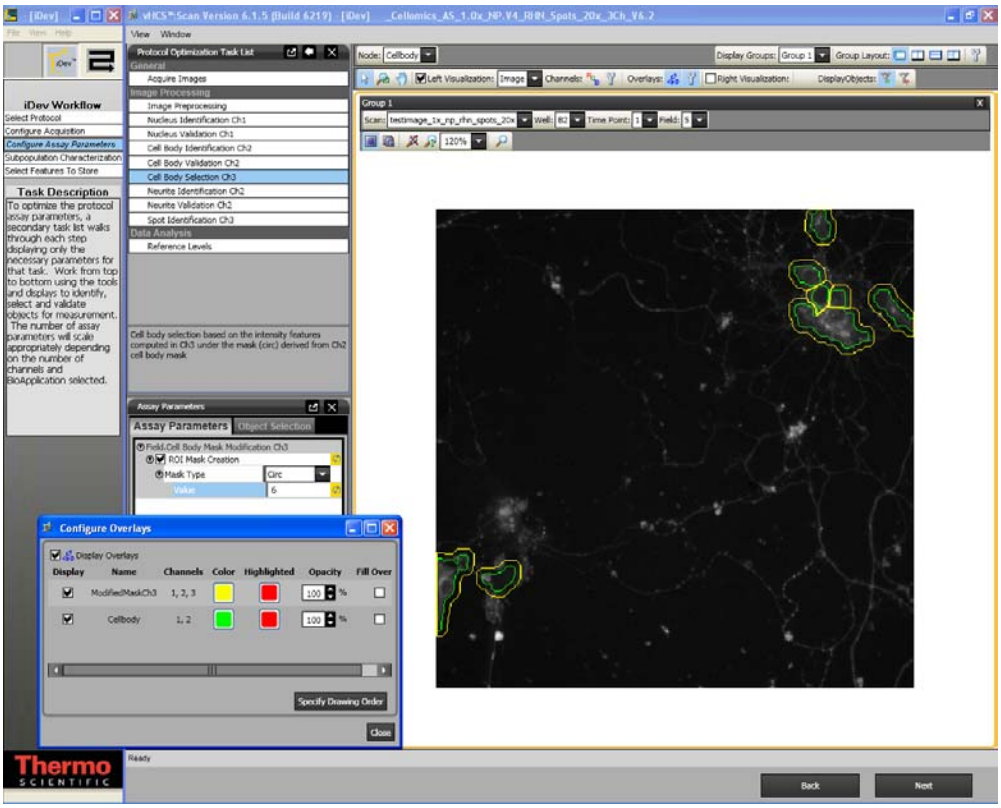


Figure 48. *Protocol Optimization Task – Cellbody Selection*

Neurite Identification Ch2

This task is for optimizing methods and values of parameters that allow you to identify the neurites in Channel 2. You can set up methods and values for smoothing, and detecting and thresholding of neurites in Channel 2. In this task, you can also optimize additional neurite identification parameters such as direction of the neurite, accept or reject neurites that are traced multiple times, identify neurites that are not associated with a cell body, and trace neurites only withn a ROI.

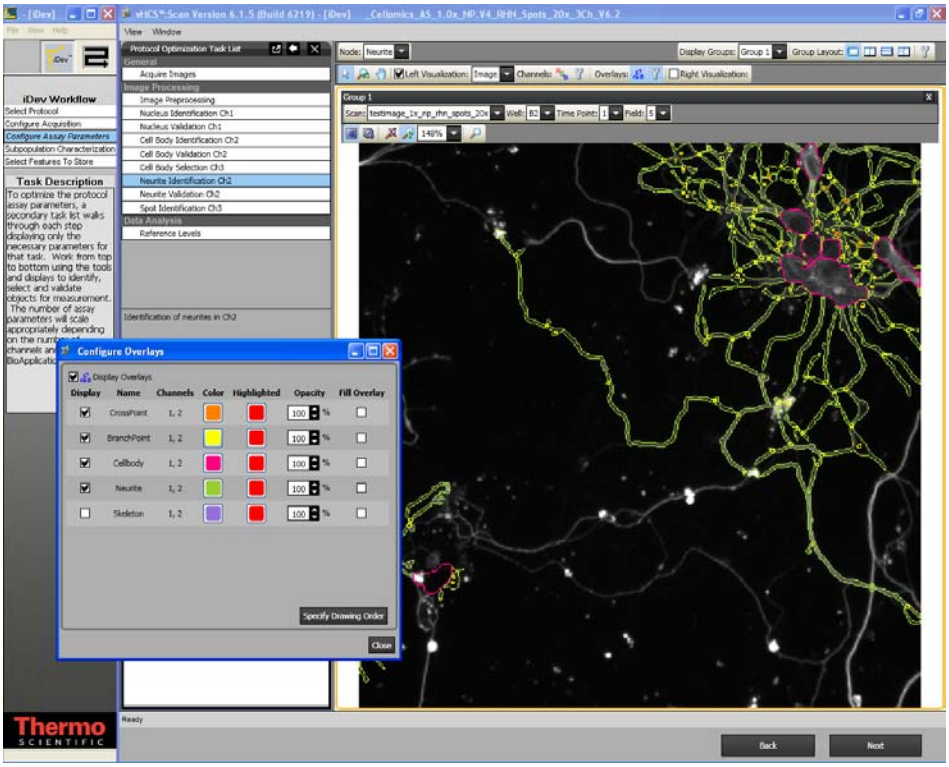


Figure 49. *Protocol Optimization Task – Neurite Identification*

Neurite Validation Ch2

This task allows neurite selection based on dimensions and intensity features calculated for the neurite from Channel 2. In this task, you will set minimal and maximal values for validating (equivalent to selecting and rejecting objects in the ArrayScan Classic software) neurites in Channel 2 based on output features.

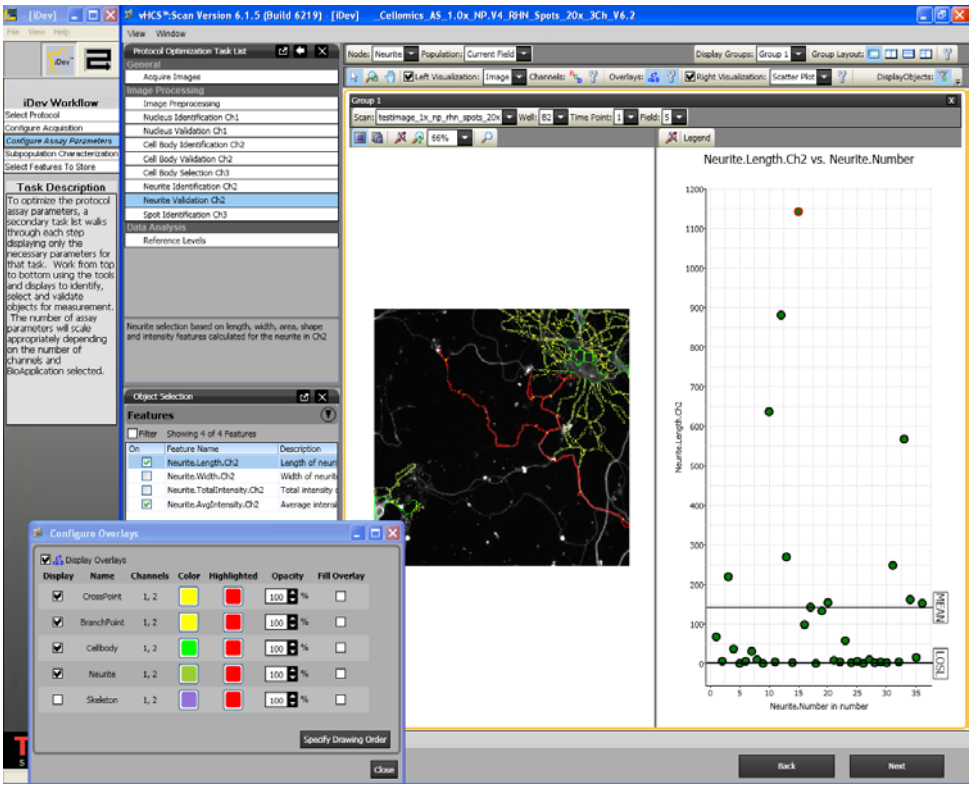


Figure 50. *Protocol Optimization Task – Neurite Validation*

Spot Identification Ch2 and Ch3

This task allows for identification of spots in Channels 3 and 4 within the region of interest (circ mask) derived from the Channel 2 cell body and neurite mask. In this task, you will define method and values for smoothing, detection, thresholding, and segmentation of spots in Channels 3 and 4.

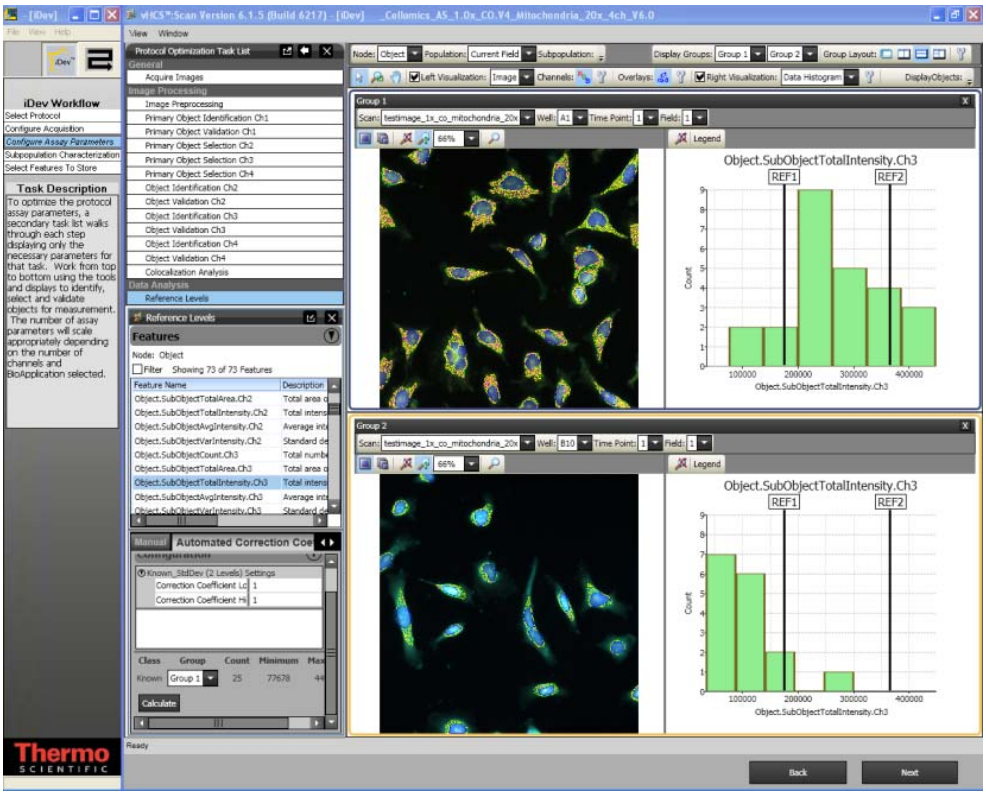


Figure 51. *Protocol Optimization Task – Spot Identification*

Spot Overlap Identification Ch3 and Ch4

In this task, you can set parameters for analysis of spot overlap between Channels 3 and 4 by setting the method and value for the type of overlap analysis.

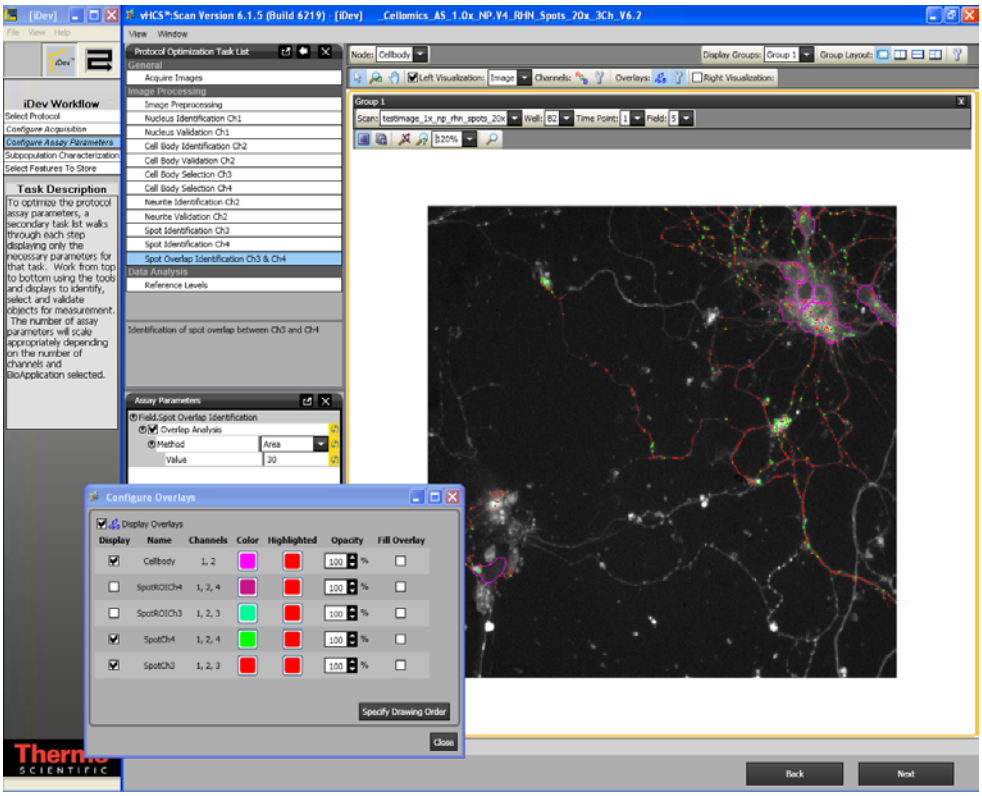


Figure 52. *Protocol Optimization Task – Spot Overlap Identification*

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.

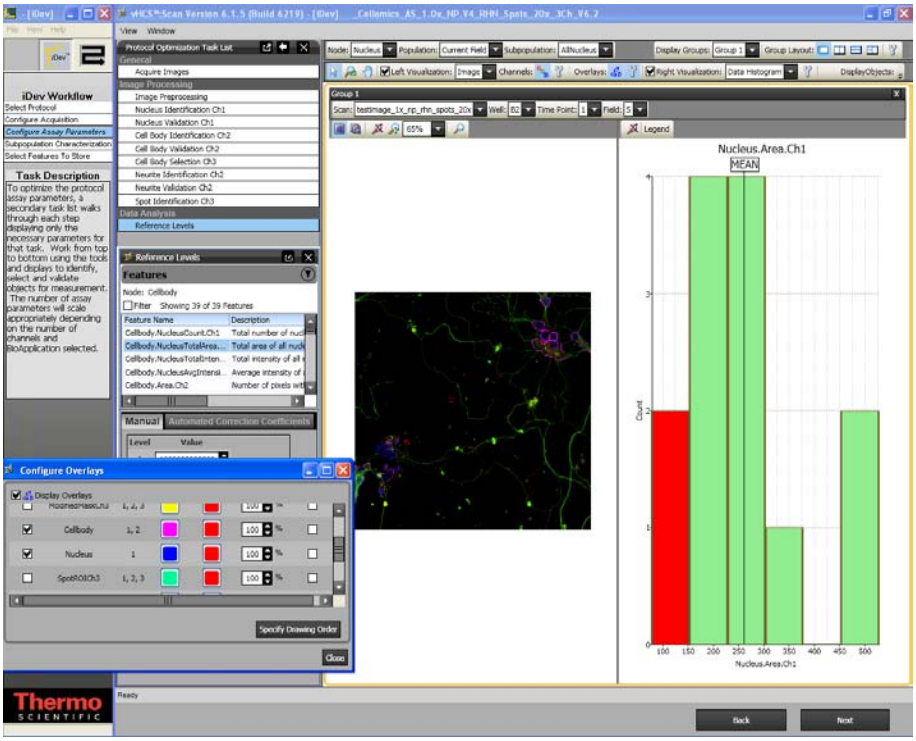


Figure 53. *Protocol Optimization Task – Reference Levels*

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.

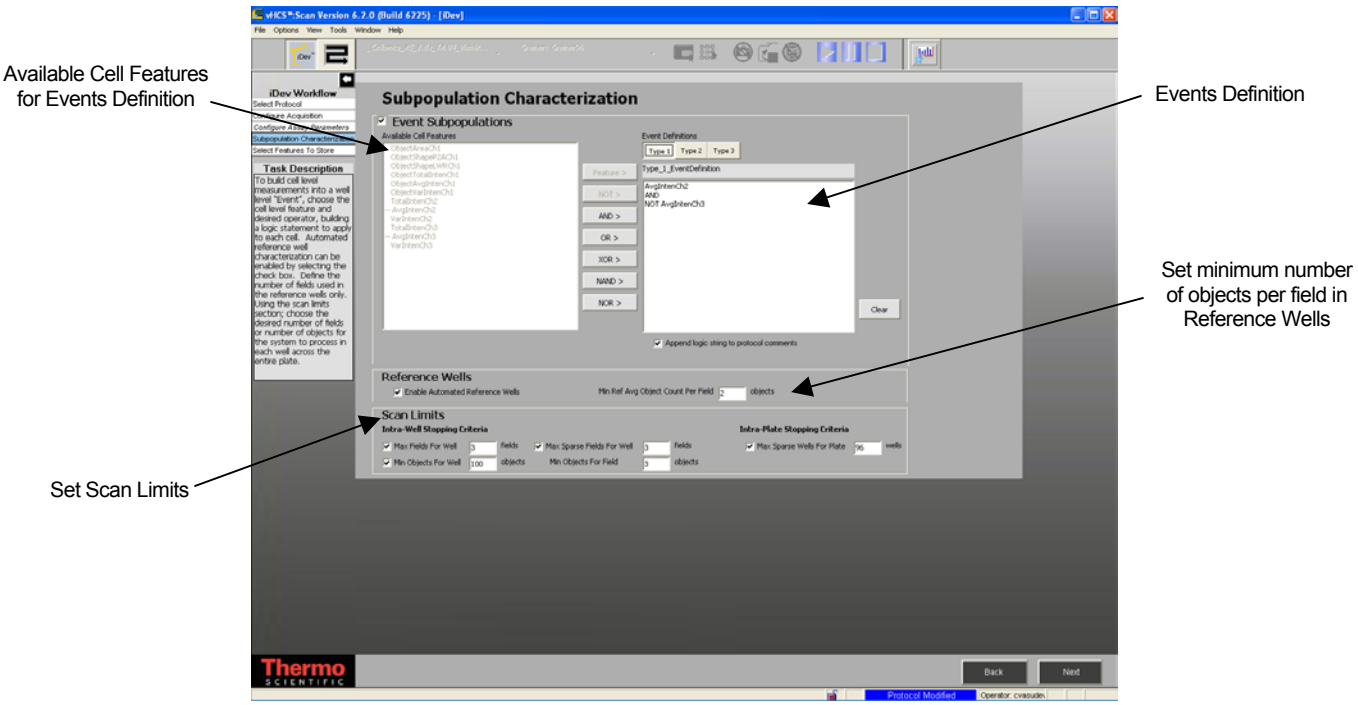


Figure 54. *Subpopulation Characterization Task*

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