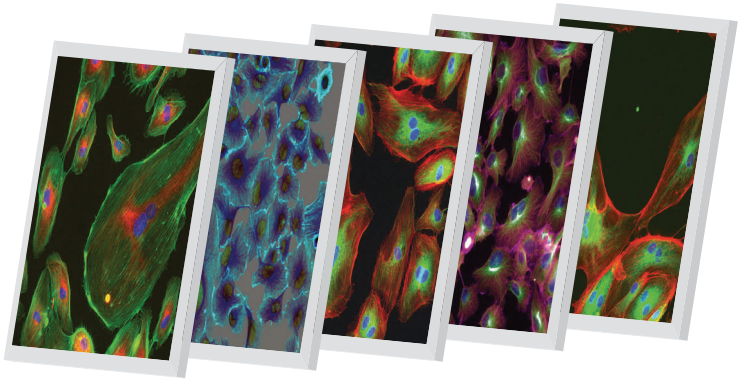
Thermo Scientific Cellomics® GPCR Signaling V4

BioApplication Guide



Part of Thermo Fisher Scientific

Thermo SCIENTIFIC

Cellomics® GPCR Signaling BioApplication Guide

V4 Version

PUBLISHED BY

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

Copyright

Trademarks

WARNING

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

P/N LC06140800

Cellomics, Inc.
Software License Agreement

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

1) **Licensor's Rights** Cellomics, Inc. ("Licensor") represents that it has all rights necessary to grant the license herein. The Software is protected by patents (including United States Patent No. 6,759,206, Japanese Patent Nos. 3683591 and 3466568, European Patent Nos. 0983498 and 1095277, Canadian Patent Nos. 2,282,658 and 2,328,194, and Australian Patent No. 730100), patent applications, and copyrights and includes valuable trade secrets and other proprietary material.

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

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

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

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

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

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

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

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

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

Table of Contents

Chapter

1

Overview of the GPCR Signaling BioApplication

High Content Screening (HCS) uses fluorescence-based reagents and advanced imaging systems and algorithms (BioApplications) to analyze targets and physiological processes in single cells. This guide provides a brief description for performing one such HCS assay by measuring intracellular translocation events involved in G Protein-coupled Receptor (GPCR) signaling events. This BioApplication has been specifically tested and validated for analysis of GPCR signaling events monitored using cells that stably express GFP (Green Fluorescent Protein) labeled β-arrestin. This guide contains the following chapters:

Chapter 1 provides an overview for the BioApplication, such as the biological situations in which it can be applied.

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

Chapter 3 provides detailed description of when and how to adjust Assay Parameter settings.

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

System Compatibility

The GPCR Signaling BioApplication described in this document is designed to run on the following versions of the Cellomics® HCS Reader platforms:

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

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

Cell Biology Background

GPCR Signaling

GPCRs represent a broad class of cell surface receptors involved in a wide range of biological signaling events. As cell surface receptors, they respond to ligand-induced stimuli within the environment and transmit specific signals within the cell through distinct cascades involving a variety of signaling molecules. Examples of GPCRs include: adrenergic receptors, receptors for somatostatin, dopamine, angiotensin, and a variety of other ligands. The genome is

thought to contain hundreds of genes within this class, only fractions of which have been isolated and fully characterized.

GPCRs are widely studied within the pharmaceutical, biotechnology, and biological research communities due to their important roles in vital physiological processes, and they are important targets in drug discovery for this reason. The GPCR Signaling BioApplication was specifically designed for monitoring the activity of GPCRs in a drug-screening environment. The BioApplication can be used to monitor GPCR signaling events in single cells by monitoring target intensity and distribution within distinct sub-cellular compartments: nucleus, cytoplasm, plasma membrane, and small sub-cellular vesicles such as endosomes. The BioApplication can be used in conjunction with a variety of fluorescent labeling methods targeted against specific receptors of interest. It has been developed and validated using cells that stably express GFP labeled β-arrestin.

Figure 1 shows the steps involved in β-arrestin translocation in the course of GPCR signaling. As indicated, β-arrestin moves from a diffuse cytoplasmic distribution to the plasma membrane in response to GRK (G protein-coupled receptor kinase) phosphorylation following ligand-induced GPCR activation. Subsequent to this event, the protein is targeted to clathrin-coated vesicles, from which it either (1) undergoes rapid recycling back to the membrane or (2) undergoes slow recycling through the endosome recycling compartment.

Each GPCR will likely translocate in a unique time and dose-dependent manner, and the GPCR Signaling BioApplication is able to automatically quantitate intracellular fluorescence at the membrane, in the cytoplasm, within punctate vesicles, and within the nucleus. These intensity values are then used to classify each cell at a particular phase in this general pathway. Additionally, for live cell analysis, the Cellomics ArrayScan V^{TI} HCS Reader and the Cellomics Discovery Toolbox (v1.6) offer a Live Module that can track cells over time. Please see the appropriate user's guides for more information.

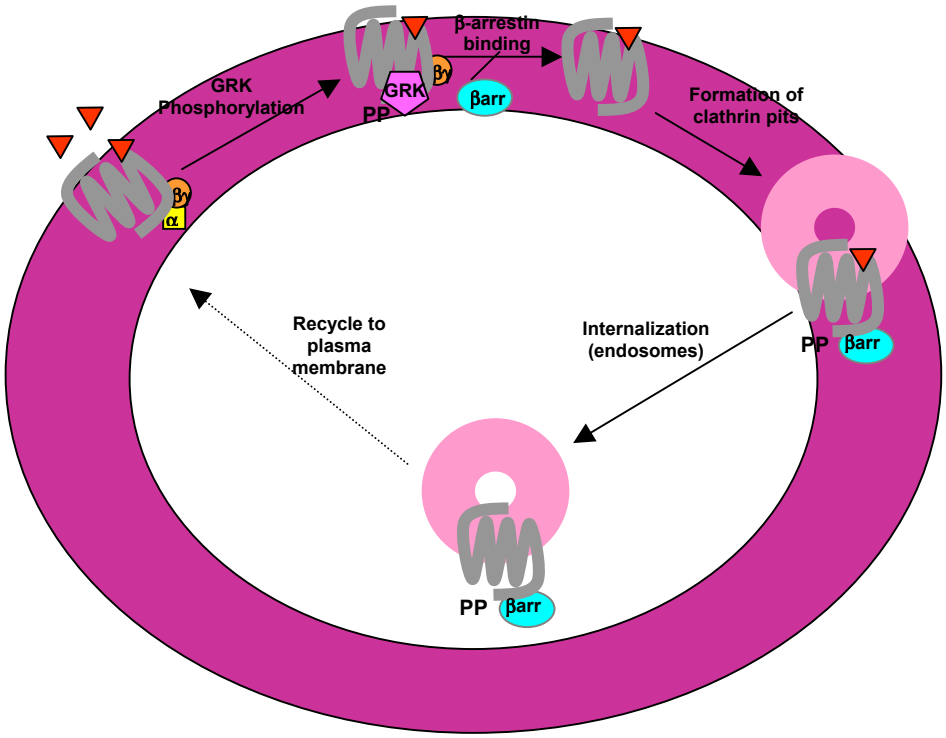


Figure 1. *The events involved in GPCR signaling and β-arrestin translocation. The Cellomics GPCR Signaling BioApplication is able to detect fluorescence within distinct sub-cellular domains and automatically classify the GPCR activation state depending upon the localization of GFP β-arrestin.*

BioApplication Overview

Image Channels

The GPCR Signaling BioApplication is a three-channel assay (Figure 2) designed for detailed analysis of target distribution between four distinct sub-cellular regions within individual cells: plasma membrane, nucleus, small vesicles, and a cytoplasmic ring that you define. These domains are defined by creating two independent reference masks. The first mask is generated from an image obtained using a specific fluorescent dye for the nucleus. The second mask is generated using a membrane-specific fluorescent marker. These two measurement masks are subsequently applied to a third image obtained for the target molecule of interest, i.e., the GFP-β arrestin protein.

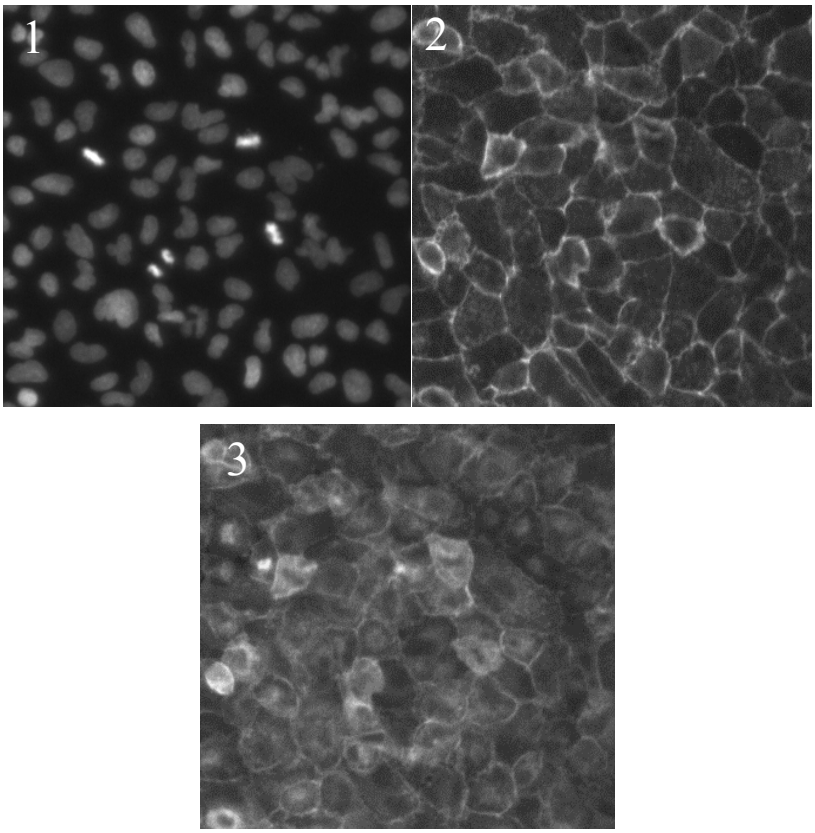


Figure 2. *Example images acquired with the GPCR Signaling BioApplication.* ***Panel 1*** *shows nuclei labeled using a specific indicator dye.* ***Panel 2*** *shows membrane labeling.* ***Panel 3*** *shows a cell line expressing pit-forming receptor. Note that the signal is distributed non-uniformly in single cells between the membrane, the cytoplasm, and the nucleus.*

Once the images have been acquired, the GPCR Signaling BioApplication will apply a series of overlays indicating the regions of the cells that are being measured. A raw image and an image with overlays are shown in Figure 3, as acquired in Channel 3 showing GFP β-arrestin distribution following stimulation.

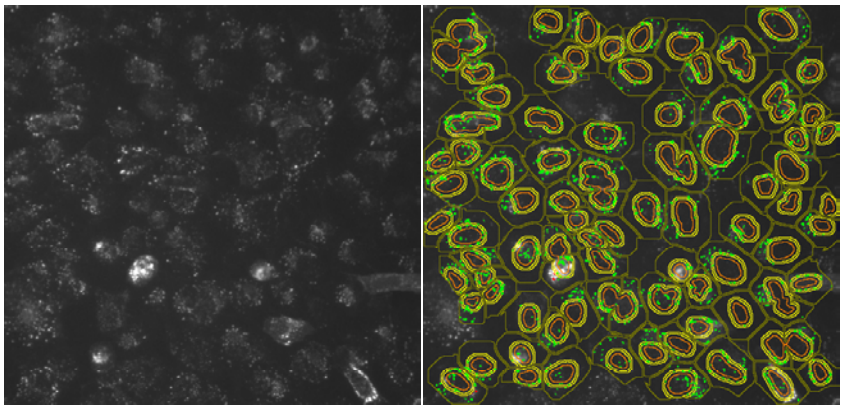


Figure 3. *Images shown were acquired using a vesicle-forming receptor cell line. Cells were stimulated with an agonist for 15 minutes, and the majority of cells show a Phase 3 distribution for GFP β-arrestin, indicative of receptor activation. Left: raw image. Right: image after applying GPCR Signaling BioApplication. Orange overlays indicate the position of nuclei selected for analysis, the yellow overlays the region of the cytoplasm that you defined, and the green overlays the position of vesicles. Cellular domain limits are shown in olive.*

The BioApplication uses the overlay areas to measure raw intensity and shape features for each image set. You have access to all of these raw numerical calculations on a cell-by-cell and well-averaged basis. These measurements are also used to generate the phase classifications.

Phase classification is based on each cell having a certain response characteristic that you defined. Each cell can be designated as a responder based upon exceeding minimum values that you defined for: (1) a ratiometric measurement of intensity at the cytoplasm/nuclear junction, (2) the spot area per cell, (3) the percent co-localization with the reference mask from Channel 2 , membrane in this case, and (4) textured, non-uniform target distribution. The minimum values for each of these criteria are defined in the Assay Parameters for this BioApplication, which are described in detail in Chapter 2 and further in Chapter 3.

Each cell is subjected to the classification scheme (Figure 4, Figure 8). Since it is possible for individual cells to show different distribution patterns in response to expression of different receptors or in different types of analyses, the ability of the BioApplication to characterize the percentage of each phase represented in a population is a valuable benefit.

For example, if you were interested in conducting an antagonist screen for a given receptor and ligand, the application might monitor increases in the percentage of Phase 0 cells representing the inactive state of GFP β-arrestin. Alternatively, you might find a completely different distribution pattern for similar agonist screens against different receptors. This is documented for both vesicle-forming and pit-forming receptors. Activation of vesicle-forming receptors results in an increase in the percentage of Phase 3 cells, whereas the pit-forming receptors result in an increase in the percentage of Phase 2 cells. Cells exhibiting distribution within the membrane would show an increase in the percentage of Phase 1 cells.

Phase Classification

The GPCR Signaling BioApplication offers a unique reporting feature referred to as phase classification, based on the localization of the GFP β-arrestin. This BioApplication has been tested and validated using both a vesicle-forming receptor cell line and a pit-forming receptor cell line. Examples of these cells showing the various distribution patterns for GFP β-arrestin are shown in the Figure 4.

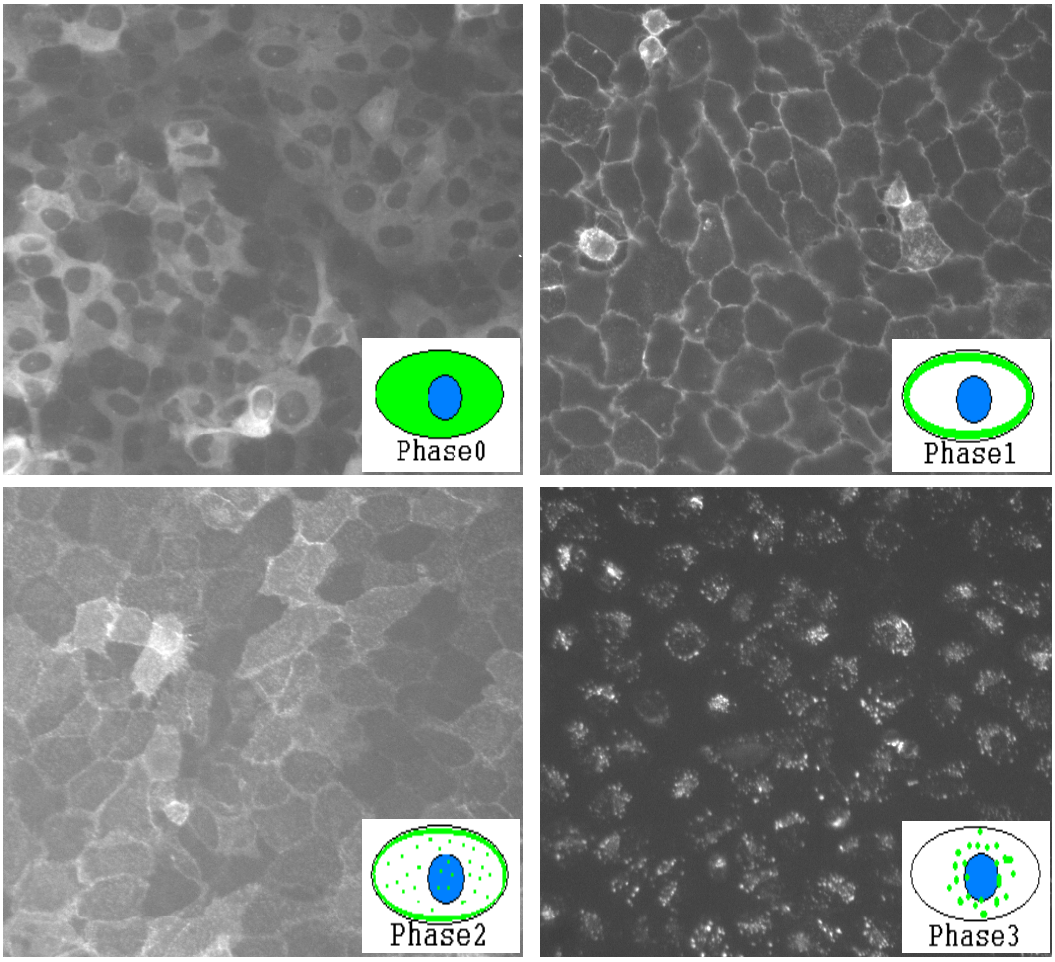


Figure 4. *Examples of GFP β-arrestin distribution at different stages of GPCR activation and desensitization. Phase 0 represents the resting-state distribution observed in unstimulated cells. Phase 1 represents cells with a membrane-labeling pattern. Phase 2 is observed in cells that undergo rapid recycling and show both membrane and vesicle labeling. Phase 3 is seen in cells with slow recycling in which the GFP β-arrestin is found in late endosomes. Although not shown, Phase 4 classification is assigned to cells that do not fit any of the above criteria. Phase 4 might indicate toxicity and/or decreased transfection efficiency.*

Each cell is individually classified based upon intracellular distribution of the GFP β-arrestin defined by a set of characteristics, including the signal contrast between the cytoplasm and the nucleus, the total vesicle area in the cell, localization of target to the plasma membrane, and a texture measurement. At the well level, the percentage of cells in each phase is reported, and the well is given a phase designation based on the most abundant individual phase represented in the population. The classification criteria is described in more detail in Table 1 and also in Figure 8 in the next chapter.

Phase Classification	Target Distribution
Phase 0	Diffuse cytoplasmic distribution of the target, with exclusion from the nucleus
Phase 1	Exclusive co-localization of the target with the cell membrane
Phase 2	Target localized to the membrane, coated-pits and small vesicles near the cell
Phase 3	Targets localized to the endocytic vesicles with an intracellular, peri-nuclear

Table 1*. Phase classification of cells due to various distributions of target.*

BioApplication Measurements

The GPCR BioApplication reports Cell and Well Features. Cell Features are measurements made on a single cell. Well Features are well-level averages and other population metrics that are derived from the Cell Features for all the cells analyzed in a given well. Overall Cell and Well Features are summarized in Table 2 and described further in Chapter 2.

Feature Category	Cellular Region/s	Cell Features	Corresponding Well Features
Cell and Well-Level Features Reported for Channel 1:			
General	Nucleus	Cell# Top Left Width Height XCentroid YCentroid Phase	ValidCellCount SelectedCellCount %SelectedCells ValidFieldCount SelectedCellCountPerValidField Phase PhaseCellCount %Phase0Cells %Phase1Cells %Phase2Cells %Phase3Cells OutOfPhaseCellCount %OutOfPhaseCells
Intensity	Nucleus	NucTotalIntenCh1 NucAvgIntenCh1 NucVarIntenCh1	MEAN, SD, SE, CV (Cell Features)
Morphology	Nucleus	NucAreaCh1 NucShapeP2ACh1 NucShapeLWRCh1	MEAN, SD, SE, CV (Cell Features)
Cell and Well-Level Features Reported for Channel 3:			
Intensity	Nuclear	NucContrastRatioCh3	None
	Circ	CircTotalIntenCh3 CircAvgIntenCh3	MEAN, SD, SE, CV (Cell Features)
	Ring	RingTotalIntenCh3 RingAvgIntenCh3	MEAN, SD, SE, CV (Cell Features)
	Membrane	MembraneTotalIntenCh3 MembraneAvgIntenCh3	MEAN, SD, SE, CV (Cell Features)
	Membrane and Ring	MembraneRingAvgIntenRatioCh3	MEAN, SD, SE, CV (Cell Features)
		MembraneRingAvgIntenRatioStatusCh3	%RESPONDER
	Spot	SpotAvgIntenCh3	MEAN, SD, SE, CV- Phase (Cell Features) **Calculated from Phase cells 0-3 Only**
Morphology	Nuclear	NucTextureCh3	None
	Ring	RingAreaCh3	None
	Spot	SpotTotalAreaCh3	MEAN, SD, SE, CV - Phase (Cell Features) **Calculated from Phase cells 0-3 Only**
Other	Spot	SpotCountCh3	MEAN, SD, SE, CV - Phase (Cell Features) **Calculated from Phase cells 0-3 Only**
	Membrane	%MembraneColoc	MEAN, SD, SE, CV - Phase (Cell Features) **Calculated from Phase cells 0-3 Only**
Cell-Level Features Reported for Channels 4-6			
Gating	Modified Nuclear Mask	TotalIntenChN AvgIntenChN	None

Table 2. *GPCR Signaling BioApplication Cell and Well Feature Measurements. *Note that "ChN" refers to Channels 4-6.*

Demonstration Data: Phase Analysis for Class A Receptors

The GPCR Signaling BioApplication was utilized to validate a screen of validated beta-adrenergic agonists and antagonists in a Class A receptor cell line co-expressing GFP β-arrestin and a β2 adrenergic receptor. Also tested were compounds known not to act on this receptor (such as the Substance P fragment which is a known agonist for a Class B receptor). Focus was on Phase 2 responders since the overall biology for Class A receptor cell lines is clathrin-coated pits. Figure 5 shows the initial time-course of this Class A receptor cell line when treated with Isoproterenol, with cells transitioning from an homogeneous cytoplasmic distribution (Phase 0) to clathrin-coated pits (Phase 2) within two minutes and remaining there for the duration of testing (Peters et al, 2002).

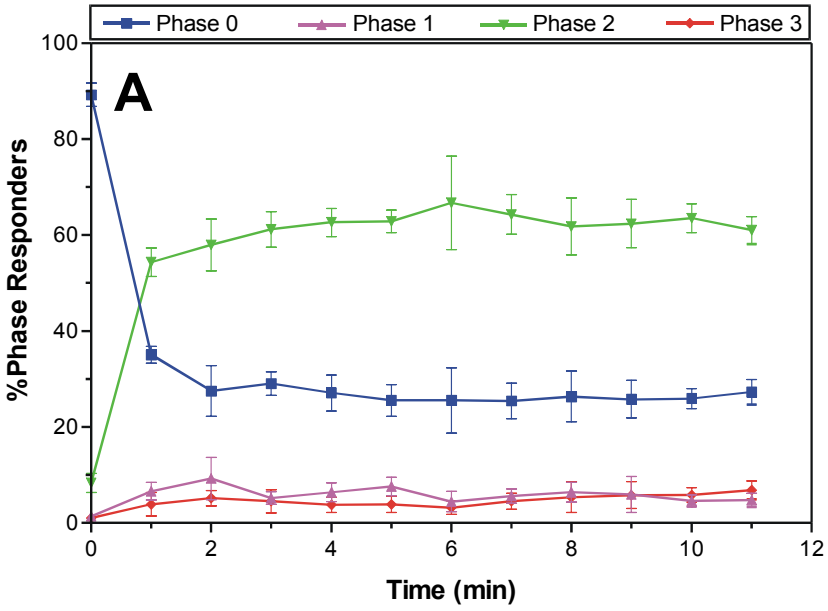


***Figure 5**. Demonstration of various phases over time when treating a Class A receptor cell line with isoproterenol. Error bars represent standard deviation and are from three separate runs.*

Various concentrations (in μM) of the agonists were screened in triplicate against the cells. Initial testing determined dose dependency in six of the nine compounds, and little/no stimulation in 3 of 9 compounds. Figure 6 represents the percent responders exhibiting the Phase 2 biology for all compounds tested, and Table 3 is the EC_{50} values (in μM) for each compound. Note that Substance P does not show any activity for this target biology (Vasudevan and Haskins, 2003).

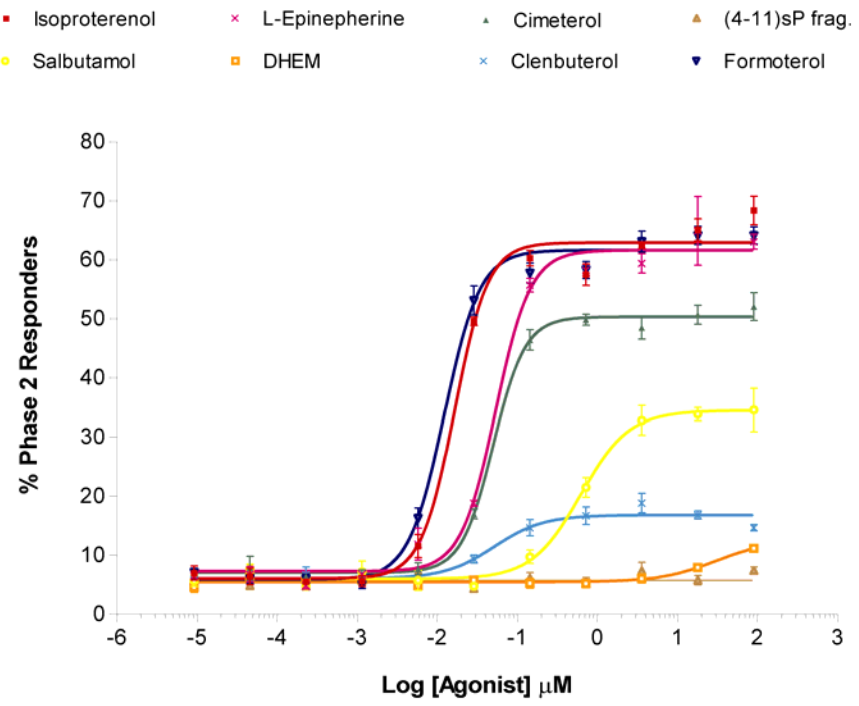


Figure 6. *Graph representing % of cells expressing the Phase 2 biology (clathrin-coated pits). Error bars represent standard deviation and are from three separate runs.*

Agonists	EC_{50} (µM)
Isoproterenol	0.011
Formoterol	0.012
Cimaterol	0.050
Clenbuterol	0.051
L-Epinephrine	0.054
Salbutamol	0.619
Dihydroergocristine Mesylate (DHEM)	30.61
Nylidrin	224.6
Substance P (4-11) fragment	No Activity

Table 3. *EC_{50} concentrations of various compounds tested for agonistic activity on selected Class A receptor cell line. EC_{50} values were calculated from graph in Figure 6.*

Isoproterenol, Formoterol, Cimaterol, and L-Epinephrine were then selected and a series of tests were performed with six suggested antagonists. Figure 7 shows the response of these cells as %Phase 2 (clathrin-coated pits) when cells, pre-incubated with antagonists, were treated with Cimeterol (Vasudevan and Haskins, 2003). Table 4 shows IC_{50} values determined for the antagonists against this agonist with their potency ranked (ranking in parenthesis).

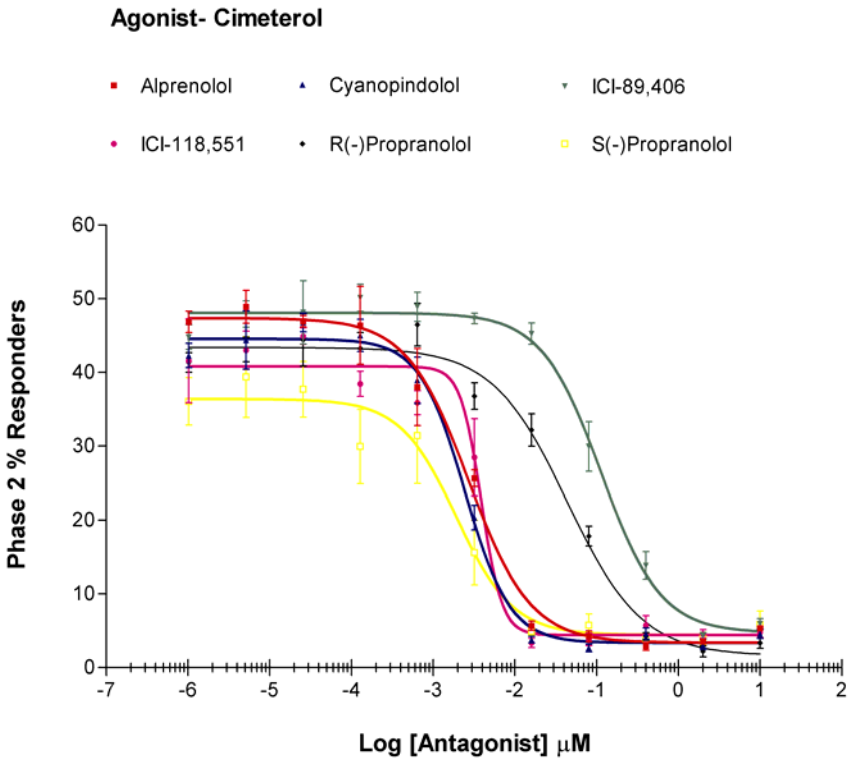


Figure 7. *Graph representing % of cells expressing the Phase 2 biology (clathrin-coated pits) when treated with Cimeterol and selected antagonists. Error bars represent standard deviation and are from a total of three separate runs.*

Antagonist	IC_{50} μM (Rank)			
	Agonist			
	Isoproterenol	Formoterol	L-Epinephrine	Cimaterol
Alprenolol	0.004 (2)	0.040 (4)	0.004 (1)	0.003 (2)
Cyanopindolol	0.003 (1)	0.015 (1)	0.004 (1)	0.002 (1)
ICI-89,406	0.554 (4)	ND	0.102 (3)	0.114 (5)
ICI-118,551	0.004 (2)	0.025 (3)	0.010 (2)	0.004 (3)
(R)-Propranolol	0.156 (3)	ND	0.041 (4)	0.044 (4)
(S)-Propranolol	0.003 (1)	0.018 (2)	0.004 (1)	0.002 (1)

Table 4*. Rank order potency (in parentheses) of four agonists treated against six antagonistic compounds with the Class A receptor cell line. Values were calculated from the graph in Figure 7.*

References

Barak, L. S., S. S. Ferguson, J. Zhang , and M. B. Caron. 1997. A β-arrestin/Green Fluorescent Protein Biosensor for Detecting G Protein-coupled Receptor Activation. *J. Biol.Chem*. 272: 27497-27500.

Ferguson, S. S., and Caron M.B. 1998. G Protein-couple Receptor Adaptation Mechanism. *Semin. Cell Dev. Bio.* 9:119-127

Ferguson S. S. 2001. Evolving Concepts in G Protein-coupled Receptor Endocytosis: The Role in Receptor Desensitization and Signaling. *Pharmaco. Rev.* 53:1-24.

Howard, A. D., G. McAllister, S. Feighner, Q. Liu, R. L. Nargund, L. H. T. Van der Ploeg, and A. A. Patchett. 2001. Orphan G-protein-coupled receptors and natural ligand discovery. *Trends Pharmacol. Sci.* 22:132-140.

Innamorati, G., C. Le Gouill, M. Balamotis, and M. Birnbaumer. 2001. The Long & Short Cycle: Alternative Intracellular Routes for Trafficking of G-Protein Coupled Receptors. *J. Biol. Chem.* 276:13096-13108.

Kallal, L., and J. L. Benovic. 2000. Using Green Fluorescent Proteins to Study G Protein-coupled Receptor Localization and Trafficking. *Trends Pharmacol. Sci.* 21:175-180.

Luttrell, L. M., and R. J. Lefkowitz. 2002. The role of β-arrestins in the termination and transduction of G-protein-coupled receptor signals. *Jour. Cell Sci.* 115:455-466.

Oakley, R.H., S. A. Laporte, J. A. Holt, L. S. Barak, and M. G. Caron. 2001. Molecular Determinants Underlying the Formation of Stable Intracellular G Protein-coupled Receptor-β-Arrestin complexes After Receptor Endocytosis. *J. Biol. Chem.* 276:19452-19460.

Peters, A.M., J. Peterson, A.Stouman, M.Cuda, J.Strauss, B.Miller, K.Olson, and J.R.Haskins. 2002. Analysis of GPCR Signaling Events Using HCS Technology. Proceeds of the Society for Biomolecular Screening, Netherlands.

Tsao, P.I., and M. von Zastrow. 2001. Diversity and Specificity in the Regulated Endocytic Membrane Trafficking of G Protein-coupled Receptors. *Pharmacol. Ther.*89:139-147.

Vasudevan, C. and J.R. Haskins. 2003.Ranking of β2 Adrenergic Receptor Agonists and Antagonists Using a Cell-Based GPCR Signaling Application. Proceeds of the International Society for Analytical Cytology First International Conference on Cytomics, Wales, UK.

Zhang, J., L. S. Barak, P. H. Anborgh., S. A. Laporte., M. G. Caron, and S. S. Ferguson. 1999. Cellular Trafficking of G-Protein Coupled Receptor/β-Arrestin Endocytic Complexes. *J. Biol. Chem.* 274:10999-11006.

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 acquired images are analyzed by an image-processing algorithm. This chapter briefly describes how this algorithm works within the GPCR Signaling BioApplication and describes the user-adjustable parameters and output features.

Overview

As discussed in Chapter 1, this BioApplication was optimized for analysis of GPCR signaling events detected using cells that stably express GFP labeled β-arrestin. The biological events involved in this process are complex, particularly the intracellular translocations observed with GFP β-arrestin. In the absence of GPCR activation, the protein is diffusely distributed throughout the cytoplasm and is excluded from the nucleus. Once a GPCR is activated by a ligand, GFP β-arrestin will translocate to the membrane, which is followed by complex recycling events. The GPCR Signaling BioApplication has been designed to characterize the localization pattern of GFP
β-arrestin within individual cells in response to receptor stimulation/inhibition events and classify the cells as being in different phases based on the localization of the GFP β-arrestin. Figure 8 shows a flowchart depicting the classification of the cells into one of the 3 phases or being out of phase.

Each cell is interrogated against a set of Assay Parameters which you can adjust as needed. The first step in the process assesses the extent of coincidence between the target molecule (GFP β-arrestin) and the mask generated in Channel 2 for the membrane. If the minimum value for this parameter is exceeded, the cell is formally classified as Phase 1. If not, the minimum spot area is analyzed to determine if the cell is Phase 3. If these criteria are also not met, the contrast ratio at the cytoplasm/nucleus interface is determined, and if the minimum value is exceeded, the cell will be classified as Phase 0. The final decision point is made by analysis of the cellular texture. Exceeding this minimum will result in Phase 2 classification. If all points fail to exceed the minimum values that you set in the Create Protocol View, the cell is classified as Out of Phase and is dropped from the selected object count (it is still counted as a valid object at this point).

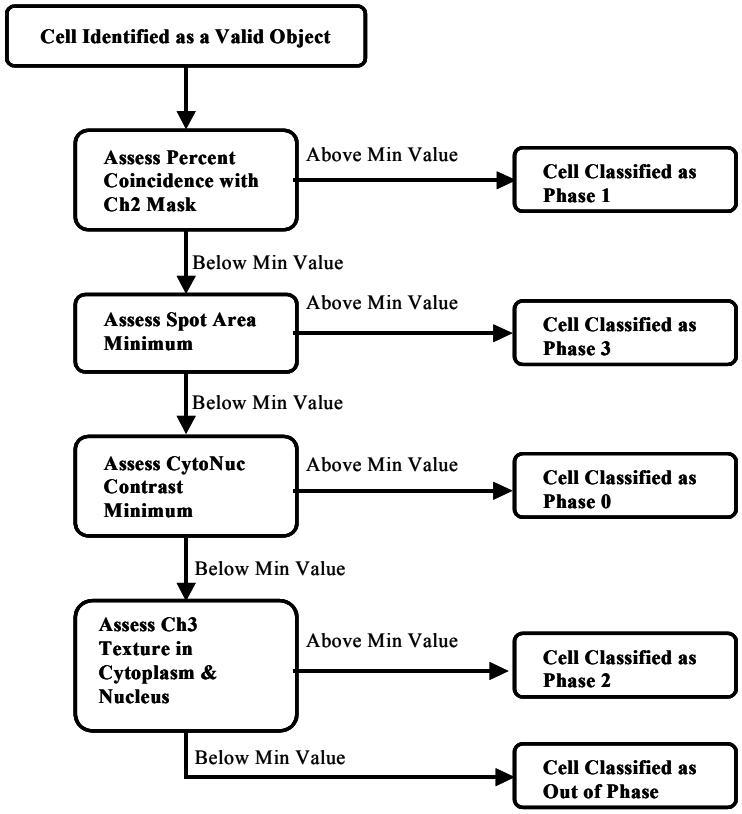


Figure 8. *Flow diagram describing the phase classification approach used in the GPCR Signaling BioApplication.*

Object Identification Methods

The Object Identification Parameters are used to identify objects in all channels of this BioApplication. 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 5, with the descriptions of the different methods following in Table 6.

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

Table 5. *Intensity Threshold Methods Available for the GPCR Signaling BioApplication.*

NOTE

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

When "**None**" is selected as the Intensity Threshold Method (Channels 4-6), the value must be **0**.

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

Of the four intensity threshold method options, **None** means that no intensity threshold is applied. This option is not available in Channels 1-3 because an intensity threshold is required to define the pixels making up the tube and the targets. The choice of the **None** threshold is the only option in Channels 4-6.

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, Table 6 gives a description of the options.

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

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

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

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

Threshold Option	Description	Range of Possible Values Entered	Resulting Applied Threshold Range
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	Value indicates absolute intensity threshold to be used. A fixed pixel intensity value between 0-32767 is applied	0 – 32767 (actual intensity in image)	0 - 32767

Table 6. *Intensity Threshold descriptions and ranges available for the GPCR Signaling BioApplication.*

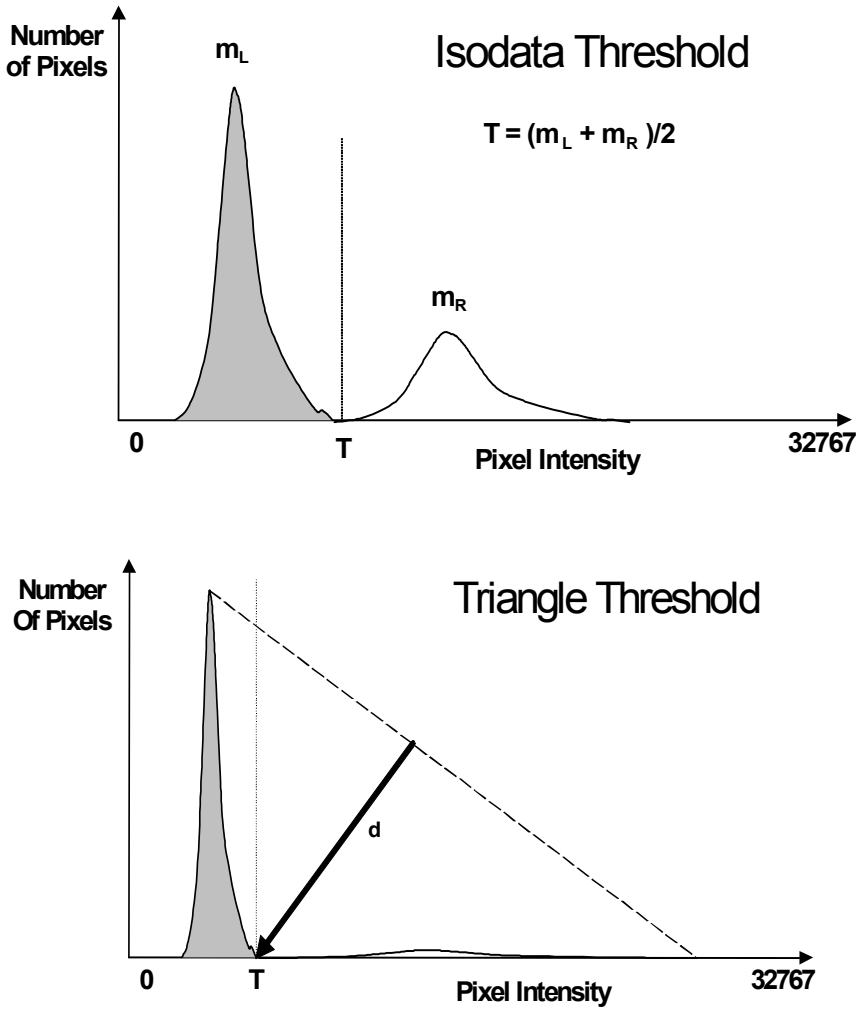


Figure 9. *Histogram-derived Intensity threshold methods: Background peak is shown in gray and object peak is shown in white. Methods shown represent Isodata (top) and Triangle (bottom) Thresholding.*

Description of Assay Parameters and Settings

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

As shown in Figure 10, the BioApplication has been optimized for both ease of use and flexibility, allowing you to precisely define the Assay Parameters required for a cell to be classified as a responder in any of the catagories mentioned previously. The images indicate some of the points at which you can interact directly with the BioApplication. The color overlays show the regions of the cell defined for analysis in yellow. Each valid nucleus is identified with a blue overlay, and those that touch the edge of the field or do not pass object selection criteria that you defined have a red overlay. You can choose to accept or reject nuclei based on object selection parameters for area, shape, or intensity.

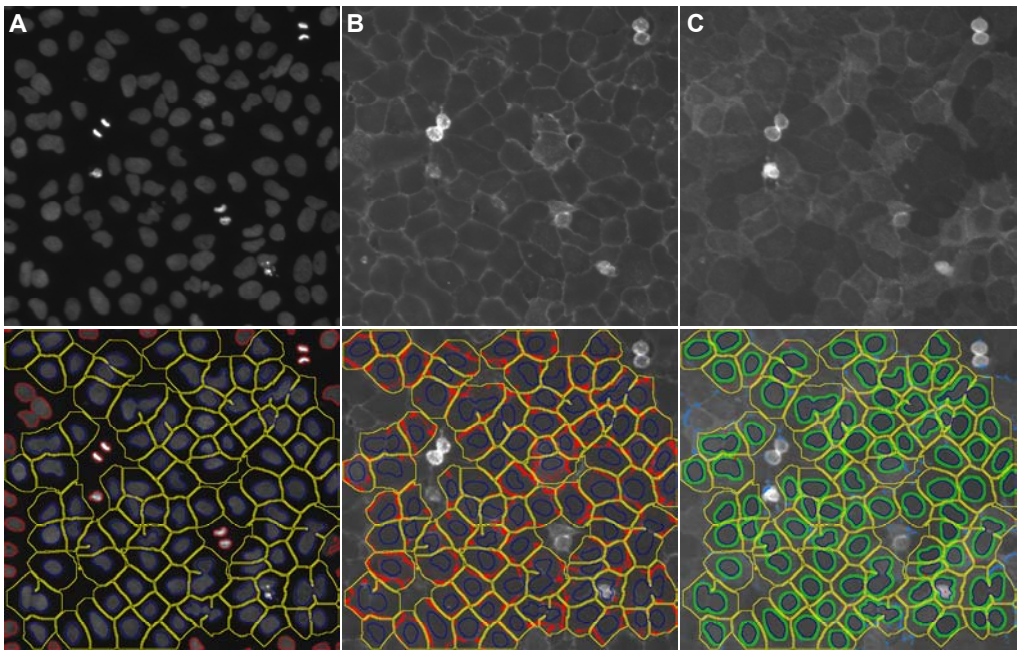


Figure 10. *The upper panels above show the raw images acquired with the Cellomics HCS Reader for nuclei (A), plasma membrane (B), and GFP-β-arrestin (C). The lower panels show the same images coupled with the algorithmic overlays described below. You can define the colors and presence of overlays as desired.*

The center panels in the Figure 10 display the membrane marker (Channel 2), and a red overlay indicates the region of the membrane in each cell. This region is identified based on labeling intensity, and you can precisely define the threshold used for selecting membrane regions using an **Isodata Threshold** selection. In order to improve the identification of membrane labeling, spurious labeling over the cell interior can be ignored using a masking Assay Parameter called **MembraneRejectionMaskModifierCh2**, which is described in more detail in the following section.

The panels on the right in Figure 10 show the target channel (Channel 3), which in this case represents the distribution of GFP β-arrestin. This is the primary channel where quantitative measurements are made for the target molecule of interest. As with Channel 1, the cellular domains are indicated with a yellow overlay and the accepted nuclei are marked with a blue overlay. The region of the cytoplasm used to measure general cytoplasmic intensity is shown

in green. The light blue overlay corresponds to areas in the target channel that are co-incident with the membrane mask that was generated in Channel 2. This gives you an indication of cytoplasm to membrane translocation. Finally, when spots are present in the target channel they are identified with a green overlay.

Assay Parameters for Image Analysis

General Assay Parameters

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

- **Units for Morphological Measurements**
- **Object Type**
- **Background Correction**
- **Object Smoothing**
- **Object Segmentation**

Units for Morphological Measurements

The user has the option of either choosing Micrometers or Pixels as the unit to report morphological measurements. This is done by the **UseMicrometers** Assay Parameter, which when set to **0**, causes the area of the objects to be reported in pixels. If the value is set to **1**, they are reported in micrometers. This information is calculated automatically from the chosen magnification and camera resolution settings. This Assay Parameter does not affect other Assay Parameters such as **CircModifierCh3** or **RingDistanceCh3** or **RingWidthCh3**, which are always set in pixels.

Object Type

NucTypeCh1, MembraneTypeCh2, SpotTypeCh3

The "Object Type" Assay Parameters allow you to specify whether the objects of interest are brighter or darker than the background of the image. Setting the value to **1** makes dark areas within an image to be considered as potential objects, while leaving it as **0** considers bright areas on a dark background as potential objects (Table 7).

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

***Table 7**. Binary settings for **NucTypeCh1**, **MembraneTypeCh2**, and **SpotTypeCh3**.*

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

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

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

Background Correction Ch*N*

Prior to image analysis, the non-cellular background can be computed and subtracted from the image separately in Channels 1, 3, 4, 5, and 6, if desired, as shown in Figure 11.

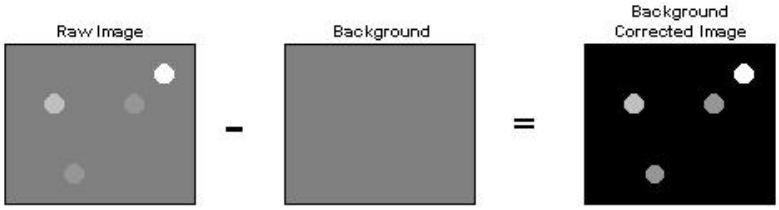


***Figure 11**. Image depicting calculation of Background Correction that can be used for each channel.*

Note that Background correction is not required in Channel 2. This channel is used only as a reference for membrane localization.

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

If the **BackgroundCorrectionCh*N*** Assay Parameter is given a negative value, an optional background correction method based on the 3D surface fitting is applied. The absolute value entered corresponds to the radius of an area used to find local minima across the image. Found minimum values are used to construct a 3D surface of a background which is then subtracted from the original image. The main advantage of the method is that it minimizes the effect of the background correction (removal) procedure on the intensity values of the analyzed objects. In both cases, the larger the absolute value, the larger the sampled region, and the less subtraction is done. Setting the Assay Parameter value to **–1** lets the application decide on the value needed for the optimal background correction. Table 8 gives an overview of the different Background Correction settings that can be used for each channel.

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

***Table 8.** Possible Background Correction Methods used in each channel with the GPCR Signaling 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 (but is not found in Channel 2).

The background-corrected image is not stored or shown.

Object Smoothing

NucSmoothFactorCh1

The **NucSmoothFactorCh1** Assay Parameter corresponds to the radius in pixels of the area used to smooth the image. A small value, such as **3**, means that a region with a radius of 3 pixels is used to smooth the image (region with dimensions 7×7 pixels, or 49 pixels total). Doubling the value to **6**, means that a larger region (13×13 pixels or 169 pixels total) is used to smooth the image, and thus the image will be more smoothed. A value of **0** means that smoothing is not done.

This Assay Parameter is used to smooth images with a lot of contrast to improve object identification. To get sharper definition of the shapes of the edges of objects (i.e., cells), you may want to keep the **NucSmoothFactorCh1** small, if not **0**. If your Channel 1 label is not very homogeneous, the actual object will be erroneously identified as consisting of several smaller sized objects, and then smoothing will result in a homogenizing effect of the Channel 1 label and will help identify the object with its true bounds.

Smoothing reduces the sharpness of intensity variations by redistributing their relative brightness over the immediate vicinity in the image. Nuclear Smoothing can be used to connect fragments of nuclei and can be valuable when measuring morphological changes. Figure 12 shows the effect of smoothing on accurate identification of objects in Channel 1 (white overlay). Note that insufficient smoothing can result in unwanted object fragmentation (arrows in Panel B) while excessive smoothing can result in poor definition of the object perimeter (D).

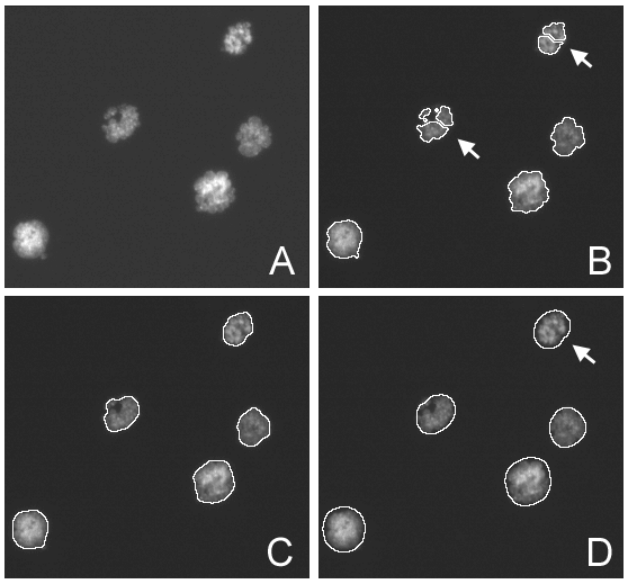


Figure 12. NucSmoothFactorCh1*: Images show the effect of different degrees of smoothing on the same field of apoptotic cell nuclei. A: Raw Image; B:* ***NucSmoothFactorCh1*** *= 0; C:* ***NucSmoothFactorCh1*** *= 3; D:* ***NucSmoothFactorCh1*** *= 8.*

Object Segmentation

NucSegmentationCh1

Even though you may have chosen an optimal intensity threshold and an appropriate degree of smoothing for nucleus identification, you may encounter situations where individual nuclei cannot be properly resolved, such as in the case of densely packed nuclei. Nucleus Segmentation will help resolve and identify individual nuclei. Two methods are available for nuclear segmentation in the GPCR Signaling BioApplication: Geometric (shape and size) and Intensity (intensity peaks). These methods are illustrated in Figure 13.

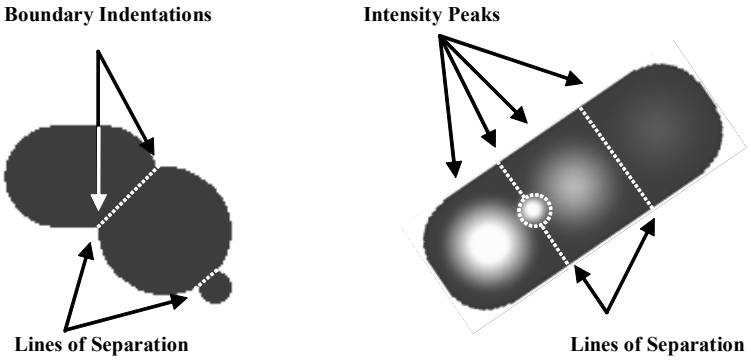


***Figure 13**: Nucleus Segmentation Options. The image on the left illustrates the Geometric Method while the image on the right illustrates the Intensity Method.*

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 9) 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 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 9: *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 14 A) produces object separation shown in Figure 14B. Setting the **NucSegmentationCh1** value to R3 results in segmentation shown in Figure 14C. Setting the **NucSegmentationCh1** value to R2 (the largest radius of all touching objects) produces no segmentation.

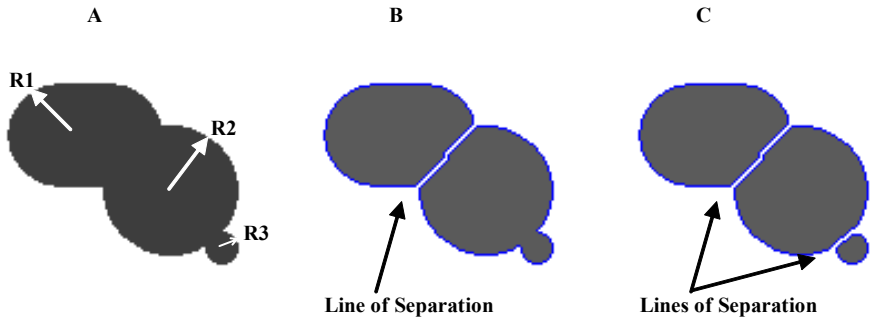


Figure 14: Object segmentation: Geometric Method. A) Radii of touching objects: R1 = 24, R2 = 30, R3 = 7; B) Result of segmentation using **NucSegmentationCh1** = R1; C) Result of segmentation using **NucSegmentationCh1** = R3.

Intensity Method Figure 15 shows the intensity profile along the cordial line of an object with four intensity peaks from Figure 13. 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, setting the **NucSmoothFactorCh1** greater than 0 should be used to alleviate the problem.

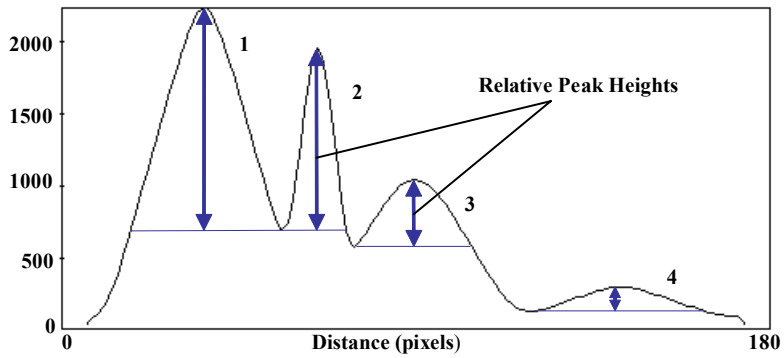


Figure 15: *Intensity profile of an object with four intensity peaks. Peak #1 height = 1500, peak #2 height = 1250, peak #3 height = 500, and peak #4 height = 200.*

Only two peaks in Figure 15, #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 16A. 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 16B and 16C.

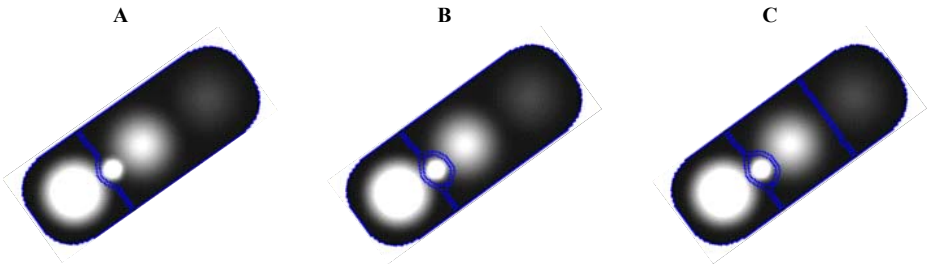


Figure 16: Object segmentation: Intensity Method. A) Result of segmentation using parameter **NucSegmentationCh1** = -1000; B) Result of segmentation using parameter **NucSegmentationCh1** = -400; C) Result of segmentation using parameter **NucSegmentationCh1** = -100.

Channel-Specific Assay Parameters

Cell Domain Modifier

The BioApplication is able to limit the area of the image that is analyzed based on established cellular domains. The domains are defined using a proprietary method of image segmentation. The value entered for this Assay Parameter determines the extent in pixels of this domain as it extends outward from the original position of the object defined in Channel 1. Measurements will only be made within the domain limit in Channel 3. All areas outside of the defined domains are ignored. In a confluent monolayer, this feature allows cellular domains to be defined even when the cells are in contact with one another.

NucCleanupCh1

This Assay Parameter is used to clean up the nuclear mask and remove very small objects and noise from the Channel 1 image prior to object identification and selection.

MembraneDetectWidthCh2

A membrane reference is used to generate the mask in Channel 2, which is used to define the Phase 1 cellular phenotype. The areas of the image identified for this mask are defined by an image processing filter. This Assay Parameter indicates the width of the kernel used by this filter to detect edges. Increasing this value will make edge detection more difficult. After application of the filter, the **Isodata Threshold** adjusted in Channel 2 of the Protocol Interactive View will control areas of the image used to generate the Channel 2 mask.

MembraneRejectionMaskModifierCh2

This Assay Parameter only affects the generation of a mask in Channel 2 and is designed to optimize the generation of a membrane mask in particular. The value represents the number of pixel dilations or erosions applied to the original object mask defined in Channel 1. Negative values will erode the original mask, whereas positive numbers will dilate it. Areas under this new mask are then ignored in Channel 2 when a final Channel 2 mask is generated. This feature is used to eliminate non-specific labeling of the nucleus and surrounding areas when using membrane markers and, therefore, refine the final membrane mask that is generated.

SpotDetectRadiusCh3

This Assay Parameter is used to determine the number of iterations of a filter applied to the Channel 3 image for characterization of spots. A spot is defined as an area of high labeling intensity surrounded by an area of lower intensity. The lower the value, the more granular the detection level.

Phase0_MinNucContrastRatioCh3

This Assay Parameter value determines whether cells are classified as Phase 0. The Phase 0 phenotype is defined as a cell that shows high contrast between the cytoplasm and nucleus in typical examples where the nucleus is the Channel 1 object. A minimum value is defined for this Assay Parameter, and all cells exceeding this minimum will be classified as Phase 0.

Phase1_Min%MembraneColocCh3

This Assay Parameter value determines whether cells are classified in this assay as Phase 1. The Phase 1 phenotype is defined as a cell showing a high level of co-localization with the mask generated in Channel 2 (representing the plasma membrane). You define a minimum value for this Assay Parameter, and all cells exceeding this minimum will be classified as Phase 1.

Phase2_ MinNucTextureCh3

This Assay Parameter value determines whether cells are classified as Phase 2. Phase 2 is defined as a cell with diffuse target distribution throughout the cytoplasm and the nucleus with an uneven, textured pattern. The texture measurement is represented by a value between 0-5000, and the number increases as more brightly labeled edges are detected in the Channel 3 image. Increasing the minimum value will result in classifying the cells as Phase 2, only when there are high amounts of textured edges within the cell.

Phase3_SpotTotalAreaCh3

This Assay Parameter value determines whether cells are classified as Phase 3. The Phase 3 phenotype is defined as a cell that contains a number of cytoplasmic spots representing

endosomal vesicles. You define a minimum value for this Assay Parameter, and all cells exceeding this minimum will be classified as Phase 3.

MembraneRingAvgIntenRatioCh3LevHigh

This Assay Parameter defines a minimum value for the intensity ratio between the cytoplasm and the membrane in Channel 3. All cells that exceed this minimum value are included to calculate the %**RESPONDER_MembraneRingAvgIntenRatioCh3** Well Output Feature. This value is determined using the ratio of the Channel 3 intensity under the membrane mask and the cytoplasmic intensity measured within the Channel 3 Ring mask. This feature is most useful for target translocation from the cytoplasm to the area of the cell identified by the Channel 2 mask (representing the plasma membrane).

Adjusting the Size of Circ and Ring Masks

The nuclei identified in Channel 1 are used to create a region of interest called "Circ**"** and "Ring" in Channel 3. Figure 17 shows a schematic representation of the Circ and Ring regions of a cell. The Circ region can be described as the sampled nuclear region and the Ring region can be described as the sampled cytoplasmic region.

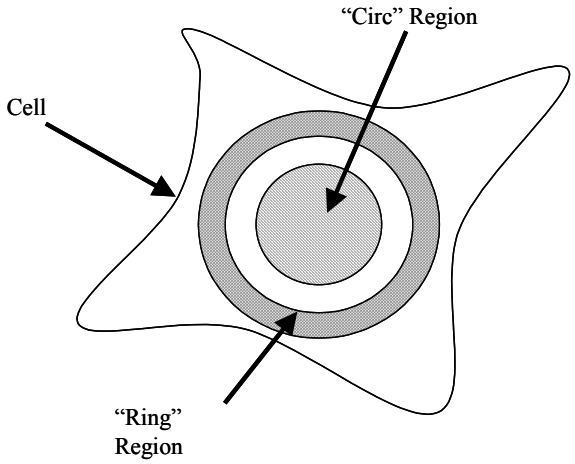


Figure 17. *A schematic representation of the Circ and Ring regions of a cell.*

The size of the Circ and Ring regions can be adjusted in the GPCR Signaling BioApplication within Channel 3, and this size is set in relation to the primary object (nucleus) identified in Channel 1. Figure 18 shows a schematic of the three Assay Parameters that control the sizes of the Circ and Ring masks: **CircModifierCh3**, **RingDistanceCh3**, and **RingWidthCh3**. The Circ mask and the Ring mask are only applied to objects that pass the object selection criteria based on mean and total pixel intensity that you set in each of the channels used.

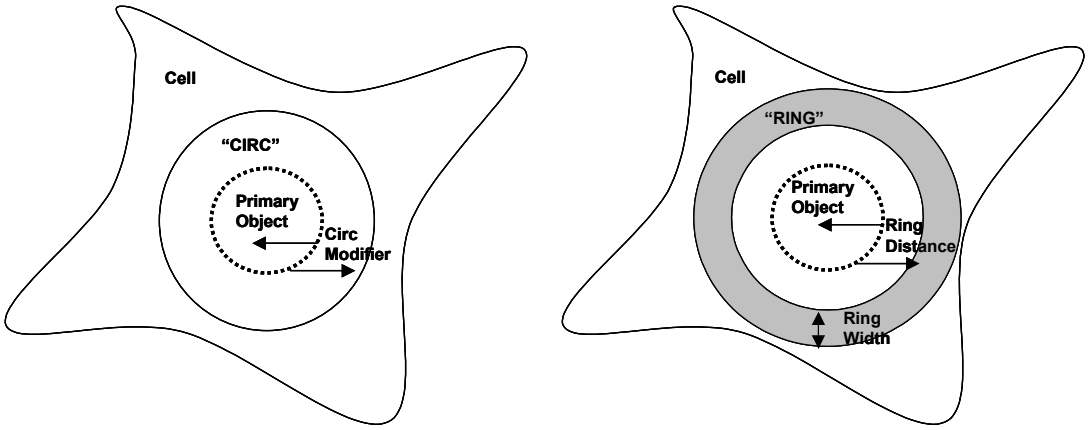


Figure 18. *The sizes of the Circ and Ring mask can be varied in the GPCR Signaling BioApplication by modifying the appropriate Assay Parameters. The Primary Object in the above diagram refers to the nucleus identified by Hoechst 33342 staining in Channel 1.*

The **CircModifierCh3** Assay Parameter is used to adjust (dilate or erode) the sampled region (object mask) in Channel 3. The value of this Assay Parameter equals the number of pixels that the primary object mask is dilated (parameter has a positive value) or eroded (parameter has a negative value) to define the Circ region.

Two Assay Parameters are used to define the area covered by the Ring region in Channel 3. They are **RingDistanceCh3** and **RingWidthCh3**. **RingDistanceCh3** is the number of pixels the inner boundary of the Ring region is away from the boundary of the primary object mask. The location of the Ring depends on the sign of the Assay Parameter: for negative values the inner boundary of the Ring is within primary object mask and closer to the center of the primary object mask and for positive values the inner boundary of the Ring is outside of the primary object mask and farther from its center. **RingWidthCh3** defines the width of the Ring region in pixels. Thus **RingDistanceCh3** and **RingWidthCh3** determine the total size of the Ring region sampled in Channel 3. Note that since the Ring region is derived from the *primary object*, the area it covers is defined independently from the area covered by the Circ region.

Specifying Mask Modifiers in Assay Channels

In addition to specifying intensity threshold ranges for one or more channels, you can apply a mask to one or more downstream channels (Channels 4-6) using the **MaskModifierCh*N*** Assay Parameter. The mask can be dilated or eroded but will not overlap with other masks from nearby objects. Please see the Gating section for more information.

Basic Assay Parameters

Input parameters can be found in the Create Protocol View of the ArrayScan Classic software or in the Protocol Optimization task list of the iDev software. In the ArrayScan Classic software, the available input parameters will depend on the mode in which you are running: Basic Mode or Advanced Mode. Assay Parameters available in Basic Mode control the morphological identification of the objects in each channel (Table 10). 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
CellDomainModifier	Pixel	Number of pixels to modify Ch1 nucleus mask to define the cellular domain within which all measurements are made: 0 = Do not modify mask Positive value = Expand mask
NucTypeCh1	Binary	Type of nuclei to be identified in Ch1: 0 = Bright nuclei on dark background 1 = Dark nuclei on bright background
BackgroundCorrectionChN	Pixel	Radius (in pixels) of region used to compute background in ChN: Negative value = Use surface fitting 0 = Do not apply background correction Positive value = Use low pass filter
NucSmoothFactorCh1	Number	Degree of image smoothing (blurring) prior to nucleus detection in Ch1: 0 = Do not apply smoothing
NucSegmentationCh1	Pixel	Radius (in pixels) of touching nuclei that should be separated in Ch1: Negative value = Use intensity peaks method 0 = Do not apply object segmentation Positive value = Use shape geometry method
NucCleanupCh1	Binary	In Ch 1 image before identification and selection, clean up nucleus mask and remove small objects by applying erosion followed by dilation procedure: 0 = No, 1=Yes
MembraneTypeCh2	Binary	Type of membranes to be identified in Ch2: 0 = Bright membranes on dark background 1 = Dark membranes on bright background
MembraneDetectWidthCh2	Pixel	Expected thickness (in pixels) of membrane mask in Ch2
MembraneRejectionMaskModifierCh2	Pixel	Number of pixels to modify Ch1 nucleus mask to remove nuclear region from membrane mask in Ch2: Negative value = Shrink nucleus mask 0 = Do not modify nucleus mask Positive value = Expand nucleus mask
SpotTypeCh3	Binary	Type of spots to be identified in Ch3: 0 = Bright spots on dark background 1 = Dark spots on bright background
CircModifierCh3	Pixel	Number of pixels to modify Ch1 nucleus to create circ mask in Ch3: Negative value = Shrink mask 0 = Do not modify mask Positive value = Expand mask
RingDistanceCh3	Pixel	Distance (in pixels) from Ch1 nucleus mask to the inner rim of Ch3 ring mask
RingWidthCh3	Pixel	Width (in pixels) of Ch3 ring mask
SpotDetectRadiusCh3	Pixel	Radius (in pixels) of region used for spot detection in Ch3

Parameter	Units	Description
MaskModifierChN	Pixel	Number of pixels to modify Ch1 nucleus mask in ChN: Negative value = Shrink mask 0 = Do not modify mask Positive value = Expand mask

Table 10. *Basic Assay Parameters available with the GPCR S****Table 10.*** *Basic Assay Parameters available with the GPCR Signaling BioApplication. *Note that "ChN" refers to Channel 1 and Channels 3-6 for* ***Background Correction****, and Channels 4-6 for* ***Mask Modifier****.*

Object Selection Parameters

Channel 1 Object Selection Parameters are used to identify valid nuclei. Channel 2 is used is to only identify the membrane mask and use it as a reference for determining colocalization with other objects in Channel 3 (such as spots) and a ring mask created from the nuclear mask in Channel 1. Therefore, there will not be any Object Selection Parameters in Channel 2. Channel 3 Object Selection Parameters are used to identify spots. Object Selection Parameters in Channels 4-6 are used for gating and are derived from the Channel 1 nucleus mask.

Object Selection Parameters are adjustable in Basic and Advanced Mode (Table 11). Any objects with values falling outside of the specified ranges will be excluded from analysis.

Channel 1

Parameter	Units	Description
NucAreaCh1	Pixels 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 object)
NucShapeLWRCh1	Number	Shape measure based on ratio of length to width for object-aligned bounding box of Ch1 nucleus
NucTotalIntenCh1	Intensity	Total intensity of all pixels within 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

Channel 3

Parameter	Units	Description
SpotAreaCh3	Pixels or μm^2	Area (in pixels or micrometers) of spot in Ch3
SpotShapeLWRCh3	Number	Shape measure based on ratio of length to width for object-aligned bounding box of spot in Ch3

Channel N (Channels 4-6)

Parameter	Units	Description
TotalIntenCh*N*	Intensity	Total intensity in ChN of all pixels within modified Ch1 nucleus mask
AvgIntenCh*N*	Intensity	Average intensity in ChN of all pixels within modified Ch1 nucleus mask

Table 11. *Object Selection Parameters available with the GPCR Signaling BioApplication. Note that "ChN" refers to Channels 4-6.*

Gating

The GPCR Signaling BioApplication supports gating on a cell population. This feature provides selective cell processing based on fluorescence intensity. Therefore, in addition to selecting cells based on nuclear area or nuclear shape in Channel 1, you can also select or reject cells based on fluorescence intensity in Channels 4-6. Gating allows you to specifically identify a subset of cells based on labeling intensity and 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, you can specify intensity limits by entering upper and lower threshold limits for two intensity parameters, **AvgIntenCh*N*** and **TotalIntenCh*N***, for Channels 4-6. **TotalIntenCh*N*** is a summation of all intensities within the object of interest. **AvgIntenCh*N*** is the object total intensity (**TotalIntenCh*N***) divided by the object area. These parameters can also be adjusted interactively in Protocol Interactive View.

Specifying Mask Modifiers for Gating

The nuclei identified in Channel 1 may be used to create measurement masks that can be applied to one or more downstream channels (i.e., Channels 4-6) for gating. The sizes of these masks are specified using the **MaskModifierCh*N*** Assay Parameter. You might 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 nucleus overlay obtained in Channel 1. Once you determine the mask for each additional channel, you can then specify the intensity ranges. For each additional channel, the average 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 feature name in the Create Protocol View. For a composite (RGB) image to be displayed, at least one of the "Include This Channel in Composite" boxes must be selected. Please refer to the Table 12 for additional information.

Parameter	Description
Include This Channel In Composite	Determines if image for this channel is included in composite image.
SelectedNucleus	Indicates the cells that pass Object Selection Parameters, as well as cells that do not touch the image border.
RejectedNucleus	Indicates cells that are rejected from analysis, either because they touch the image border or because they do not meet object selection criteria. Rejected cells are not analyzed, and are never counted by the BioApplication.
Spot	Mask can only be derived in Channel 3. The overlay indicates valid spots that pass Channel 3 object selection criteria.
MembraneColoc	Mask can only be derived in Channel 3. Indicates the position of pixels that are coincident with the mask generated in Channel 2.
Membrane	Mask can only be derived in Channel 2. The overlay indicates labeled membrane that has been positively selected by the BioApplication.
Circ	Mask can only be derived in Channel 3. Indicates the position of the mask used to calculate intensity in Channel 3 under the primary object mask defined in Channel 1.
Ring	Mask can only be derived in Channel 3. Indicates the position of the mask used to calculate intensity in Channel 3 under the cytoplasmic ring mask.
CellDomain	Indicates the position of the domain limit area within the zone of interest where measurements will be made in Channels 2 and 3. Domains are defined by the BioApplication using the primary object masks generated in Channel 1.

Table 12. *Image Overlays available with the GPCR Signaling BioApplication.*

Advanced Assay Parameters

In Advanced Mode, all basic and advanced input parameters are adjustable (Table 13). The **Hide Advanced Parameters** option provides the ablility to either view and adjust all the Assay Parameters or view and adjust the subset designated as Basic Parameters.

Parameter	Units	Description
UseMicrometers	Binary	Measure lengths and areas in: 0 = Pixels 1 = Micrometers
_PixelSize	µm	Pixel size in micrometers (depends on objective selection)
Phase0_MinNucContrastRatioCh3	Number	Minimum outer to inner nucleus contrast ratio in Ch3 required for a cell to be classified as a Phase0 phenotype
Phase1_Min%MembraneColocCh3	Percent	Minimum colocalization (in percent) between membrane and Ch3 target mask required for a cell to be classified as a Phase1 phenotype
Phase2_MinNucTextureCh3	Number	Minimum nucleus texture in Ch3 required for a cell to be classified as a Phase2 phenotype
Phase3_MinSpotTotalAreaCh3	Pixel or μm^2	Minimum total spot area required for a cell to be classified as a Phase3 phenotype
MembraneRingAvgIntenRatioCh3LevHigh	Number	High-response level for MembraneRingAvgIntenRatioCh3

Table 13. *Advanced Assay Parameters available with the GPCR Signaling BioApplication.*

Description of Output Features

Output features are the biological measurements produced by the BioApplication. All features are categorized and accessible using the View application (see the appropriate User's Guide). Additionally, a subset of features, the Well Features, is listed in the Scan Plate View and Create Protocol View so that screening results can be viewed concurrently with scanning. The ability to immediately view data allows you to verify the success of the screening run.

This BioApplication does not have Reference Well Features.

Cell Features

The BioApplication reports the following features at the cell level in the Cell Detail window of the Protocol Interactive View and the View application (Table 14).

Feature	Units	Description
Cell#	Number	Unique cell ID
Top	Pixel	Y coordinate (in pixels) of top left corner of image-aligned bounding box of Ch1 nucleus
Left	Pixel	X coordinate (in pixels) of top left corner of image-aligned bounding box of Ch1 nucleus
Width	Pixel	Width (in pixels) of image-aligned bounding box of Ch1 nucleus
Height	Pixel	Height (in pixels) of image-aligned bounding box of Ch1 nucleus
XCentroid	Pixel	X coordinate (in pixels) of center of Ch1 nucleus
YCentroid	Pixel	Y coordinate (in pixels) of center of Ch1 nucleus
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 for object-aligned bounding box of Ch1 nucleus
NucTotalIntenCh1	Intensity	Total intensity of all pixels within 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
NucTextureCh3	Number	Texture within the inner nuclear region in Ch3
NucContrastRatioCh3	Number	Ratio of texture in the outer nuclear region to the inner nuclear region in Ch3
CircTotalIntenCh3	Intensity	Total intensity of all pixels within circ mask in Ch3
CircAvgIntenCh3	Intensity	Average intensity of all pixels within circ mask in Ch3

Feature	Units	Description
RingAreaCh3	Pixel or μm^2	Area (in pixels or micrometers) of Ch3 ring mask
RingTotalIntenCh3	Intensity	Total intensity of all pixels within Ch3 ring mask
RingAvgIntenCh3	Intensity	Average intensity of all pixels within Ch3 ring mask
%MembraneColocCh3	Percent	Colocalization (in percent) between membrane and Ch3 target mask
MembraneTotalIntenCh3	Intensity	Total intensity of all pixels in Ch3 within the membrane mask
MembraneAvgIntenCh3	Intensity	Average intensity of all pixels in Ch3 within the membrane mask
MembraneRingAvgIntenRatioCh3	Number	Ratio of membrane mask average intensity to channel Ch3 ring mask average intensity in channel Ch3
MembraneRingAvgIntenRatioStatusCh3	Binary	MembraneRingAvgIntenRatioCh3 status: 0 = No response 1 = Response
SpotCountCh3	Number	Number of Ch3 spots within the cell domain
SpotTotalAreaCh3	Pixel or μm^2	Area (in pixels or micrometers) of all Ch3 spots within the cell domain
SpotAvgIntenCh3	Intensity	Average intensity of all pixels within Ch3 spots in the cell domain
Phase	Number	Cell phase classification: 0 = Control 1 = Membrane induced 2 = Pits on membrane induced 3 = Vesicle induced 4 = Unknown
TotalIntenChN	Intensity	Total intensity in ChN of all pixels within modified Ch1 nucleus mask
AvgIntenChN	Intensity	Average intensity in ChN of all pixels within modified Ch1 nucleus mask

Table 14. *Cell Features Available with the GPCR Signaling BioApplication. *Note that "ChN" refers to Channels 4-6.*

Well Features

Many Well Features are derived from Cell Features. For those output features that have statistics calculated, a prefix (as listed in Table 15), is added to the Cell Feature name.

Feature Prefix	Well Feature Description	Units
MEAN_	Average of Feature_X for all objects selected for analysis in the well	Same as cell feature
SD_	Standard deviation of Feature_X for all objects selected for analysis in the well	Same as cell feature
SE_	Standard error of mean of Feature_X for all objects selected for analysis in the well	Same as cell feature
CV_	Coefficient of variation of Feature_X for all objects selected for analysis in the well	Percent

Table 15. *Common Well Features found in the GPCR Signaling BioApplication.*

Well Features that start with "Phase" (***FeaturePrefix*_ Phase%MembraneColocCh3**, **PhaseSpotCountCh3**, **PhaseSpotTotalAreaCh3**, and **PhaseSpotAvgIntenCh3**) are calculated from cells that are involved with Phases 0-3, while all other above well features involve all cells in any phase (including "**OutOfPhase**" cells).

The GPCR BioApplication also reports the following additional Well Features in the Scan Plate View as well as the Well Detail window of the View application (Table 16).

Feature	Description
ValidCellCount	Number of valid cells identified in the well (Ch1 object selection parameters applied)
SelectedCellCount	Number of valid cells selected for analysis in the well (Ch2-6 object selection parameters applied)
%SelectedCells	Percentage of valid cells selected for analysis in the well
ValidFieldCount	Number of fields in which cells were selected for analysis in the well
SelectedCellCountPerValidField	Average number of cells selected for analysis per valid field in the well
Phase	Mode of phase classification of cells selected for analysis in the well; 0 = Control 1 = Membrane induced 2 = Pits on membrane induced 3 = Vesicle induced 4 = Unknown
PhaseCellCount	Number of Phase0-3 cells selected for analysis in the well
%Phase0Cells	Percentage of Phase0-3 cells selected for analysis in the well classified as Phase0 phenotype
%Phase1Cells	Percentage of Phase0-3 cells selected for analysis in the well classified as Phase1 phenotype
%Phase2Cells	Percentage of Phase0-3 cells selected for analysis in the well classified as Phase2 phenotype
%Phase3Cells	Percentage of Phase0-3 cells selected for analysis in the well classified as Phase3 phenotype
OutOfPhaseCellCount	Number of Phase4 cells selected for analysis in the well
%OutOfPhaseCells	Percentage of all cells selected for analysis in the well classified as Phase4 phenotype
%RESPONDER_MembraneRingAvgIntenRatioCh3	Percentage of cells selected for analysis in the well with **MembraneRingAvgIntenRatioCh3** below low-response level or above high-response level

Table 16. *Additional Well Features available with the GPCR Signaling BioApplication.*

Chapter

3

Using the GPCR Signaling BioApplication

This chapter describes in more detail the use and modification of the GPCR Signaling BioApplication. The BioApplication is supplied with a standard GPCR Signaling Assay Protocol. This is the Assay Protocol generated during validation and should remain on the system for reference purposes as a read-only version. Please refer to the appropriate User's Guide for instructions on scanning an individual plate or stacks of plates using an existing Assay Protocol.

Assay-Specific Procedures for Optimizing the BioApplication

Each biological application (BioApplication) uses an algorithm that has been extensively tested and validated for robust screening performance. The default Assay Parameters have been set and defined during product validation to optimize performance of the GPCR Signaling BioApplication. However, the following Assay Parameters (initially described in Channel 2) are explained in more detail below in order to allow customization of the algorithm to your own samples and conditions.

CellDomainModifier: Measurements will only be made within the domain limit in Channel 3. All areas outside of the defined domains are ignored. In a confluent monolayer, this feature allows cellular domains to be defined even when the cells are in contact with one another. You may need to modify this parameter when you adopt a new cell type with a unique morphology or if the seeding density is reduced to a sub-confluent situation.

NucCleanupCh1: The default value is set to 1 meaning that the clean up procedure will be performed. This procedure will clean up the nucleus mask and remove small objects from the Channel 1 image before processing.

MembraneDetectWidthCh2: Increasing this value will make edge detection more difficult. After application of the filter, the **Isodata Object Threshold** adjusted in Channel 2 of the Protocol Interactive View will control areas of the image used to generate the Channel 2 mask.

MemRejectionMaskModifierCh2:. This feature is used to eliminate non-specific labeling of the nucleus and surrounding areas when using membrane markers and, therefore, refine the final membrane mask that is generated.

SpotDetectRadiusCh3:. The lower the value, the more granular the detection level. Increasing the value will merge small, adjacent spots into a larger spot or will allow the identification of large spots (>100 pixel area) more effectively.

Phase0_MinNucContrastRatioCh3: You may need to change this value when the assay is not accurately defining cells as Phase 0 when they show a diffuse, non-nuclear target distribution.

%MembraneColocCh3: You may need to change this value when the minimum percent coincidence with the Channel 2 mask does not meet your criteria for defining a cell as Phase 1.

MinNucTextureCh3: The texture measurement is represented by a value between 0-5000, and the number increases as more brightly labeled edges are detected in the Channel 3 image. Increasing the minimum value will result in classifying the cells as Phase 2, only when there are high amounts of textured edges within the cell.

SpotTotalAreaCh3: You may need to change this value when the assay is not accurately defining cells with the appropriate amount of vesicles as Phase 3. The **SpotTotalAreaCh3** for each cell is also a feature accessible in View, which can be used to determine how to set the minimum value for a given assay.

MembraneRingAvgIntenRatioCh3LevHigh: This feature is most useful for target translocation from the cytoplasm to the area of the cell identified by the Channel 2 mask.

Chapter

4

iDev Software Workflow

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

iDev Protocol Optimization Tasks

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

Image Preprocessing

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

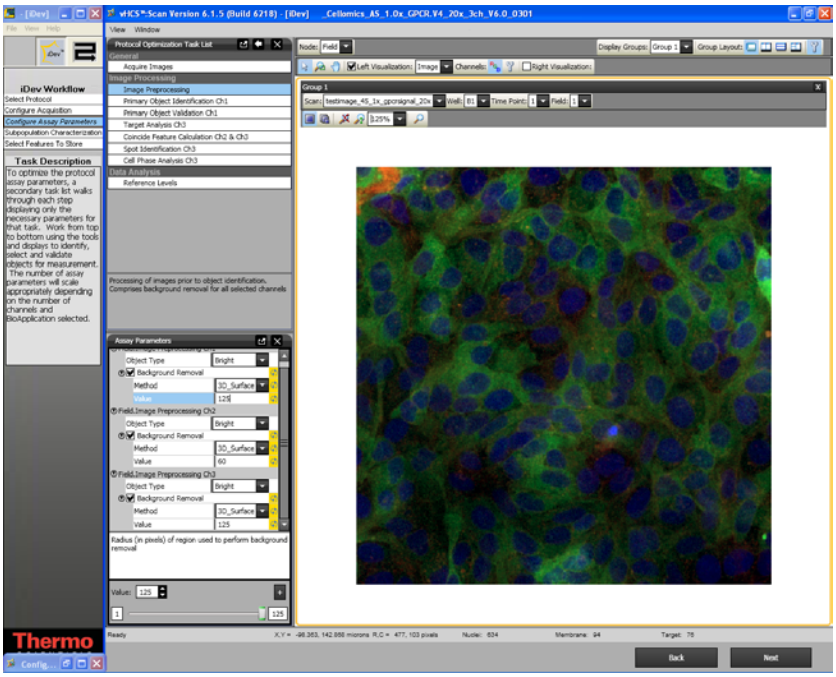


Figure 19. *Protocol Optimization Task – Image Preprocessing*

Primary Object Identification Ch1

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

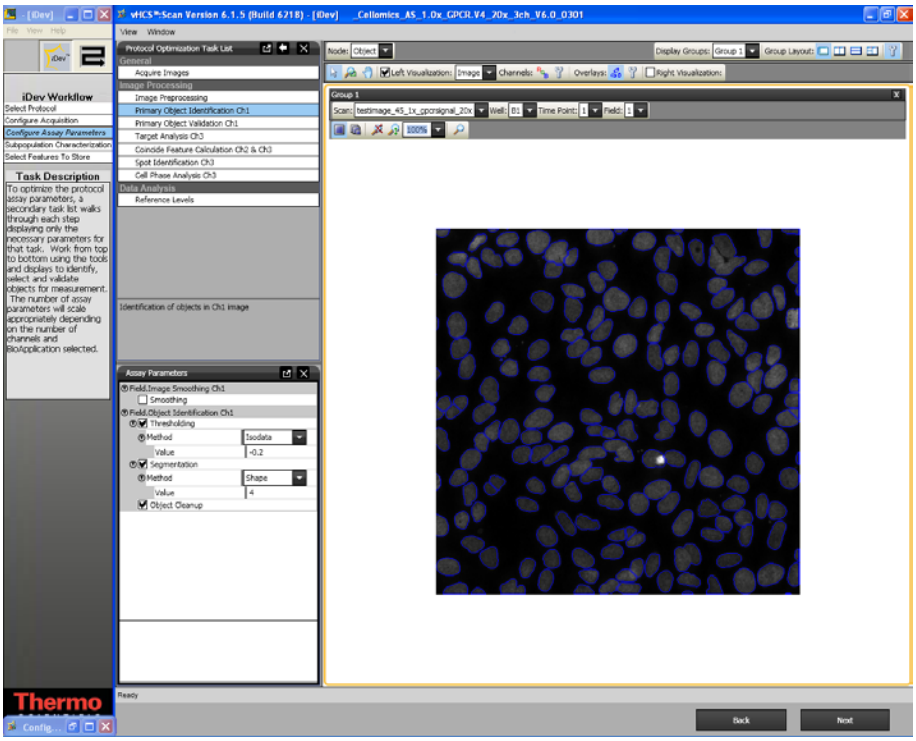


Figure 20. *Protocol Optimization Task – Primary Object Identification Ch1*

Primary Object Validation Ch1

Primary Object Validation is object selection/rejection based on area, shape, and intensity features calculated for the primary object in Channel 1. In this task, you will set minimal and maximal values for validating (equivalent to selecting and rejecting objects in the ArrayScan Classic software) objects in Channel 1 based on object selection features.

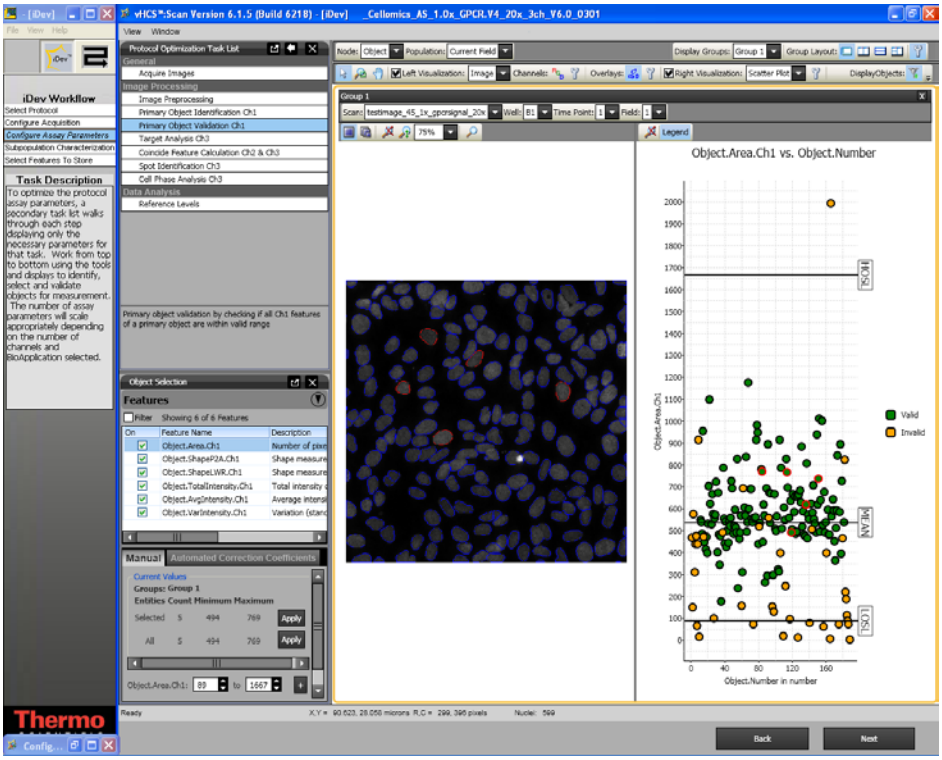


Figure 21. *Protocol Optimization Task – Primary Object Validation Ch1*

Primary Object Selection Ch4 through ChN

Primary Object Selection is object selection based on intensity features computed in Channels 4 through Channel N under the circ mask derived from the Channel 1 primary object mask. **This task is available only if you have a protocol that has 4 or more channels.** In this task, you will set selection / rejection of the primary object based on intensity measurements in Channels 4 through Channel N under a modified primary object mask. This task is similar to setting the Assay Parameter, **MaskModifierCh4,** in the ArrayScan Classic software. The primary object mask can be dilated (**Circ** > 0), or eroded (**Circ** < 0) if the **ROI Mask Creation** box is checked.

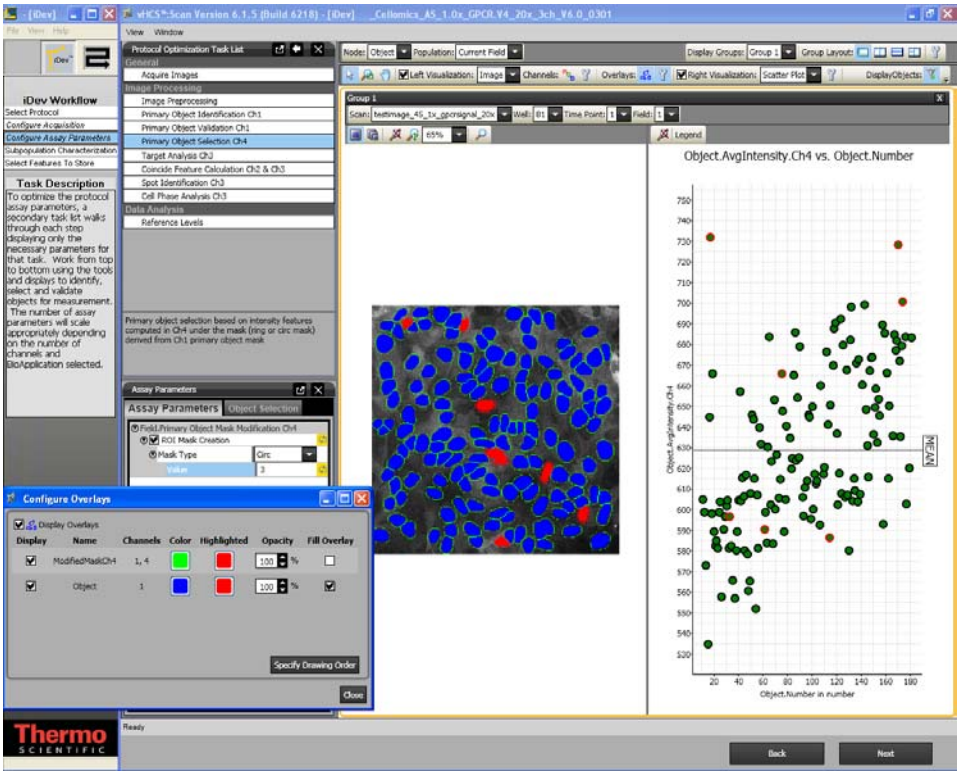


Figure 22. *Protocol Optimization Task – Primary Object Selection Ch4 through ChN*

Target Analysis Ch3

In this task, you will set parameters to define the cell domain (Circ) and ROIs (Circ and Ring), derived from the primary object mask in Channel 1 for analysis in Channel 3.

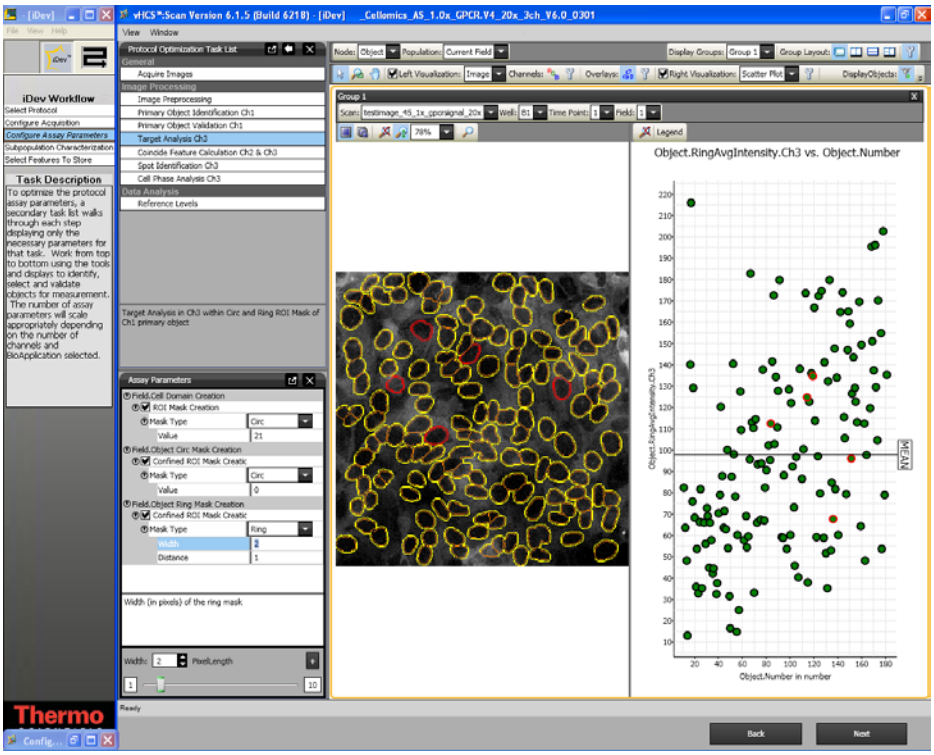


Figure 23. *Protocol Optimization Task – Target Analysis Ch3*

Coincide Feature Calculation Ch2 and Ch3

In this task, you set parameters to calculate the coincidence feature between Channel 2 (membrane marker reference channel) and Channel 3 (target channel). You can set the value for mask modification and detection width (including the thresholding method and value) for Channel 2.

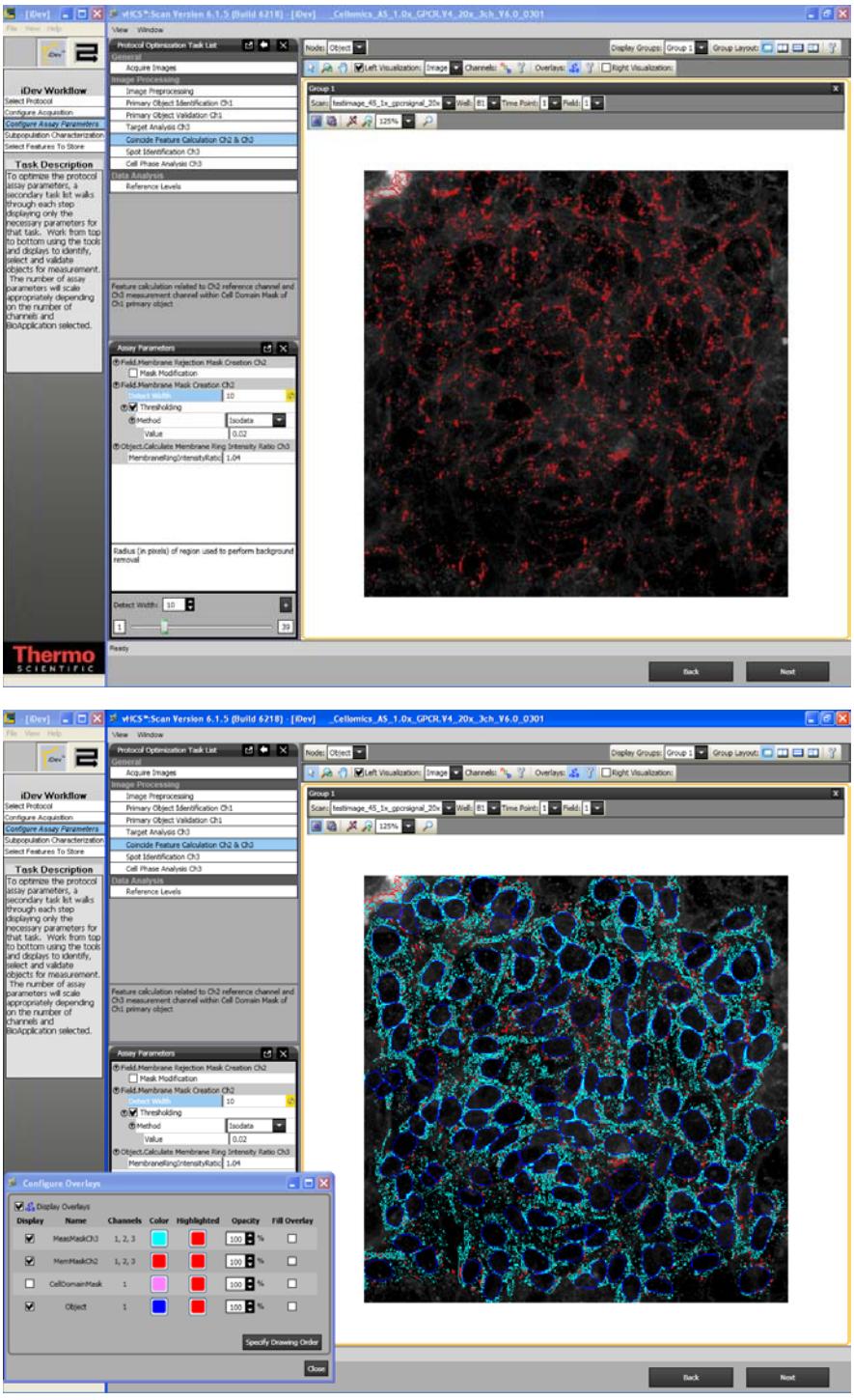


Figure 24. *Protocol Optimization Task – Coincide Feature Calculation*

Spot Identification Ch3

In this task, you can set the methods and value to identify spots in Channel 3 in the ROIs created from the primary object mask in Channel 1.

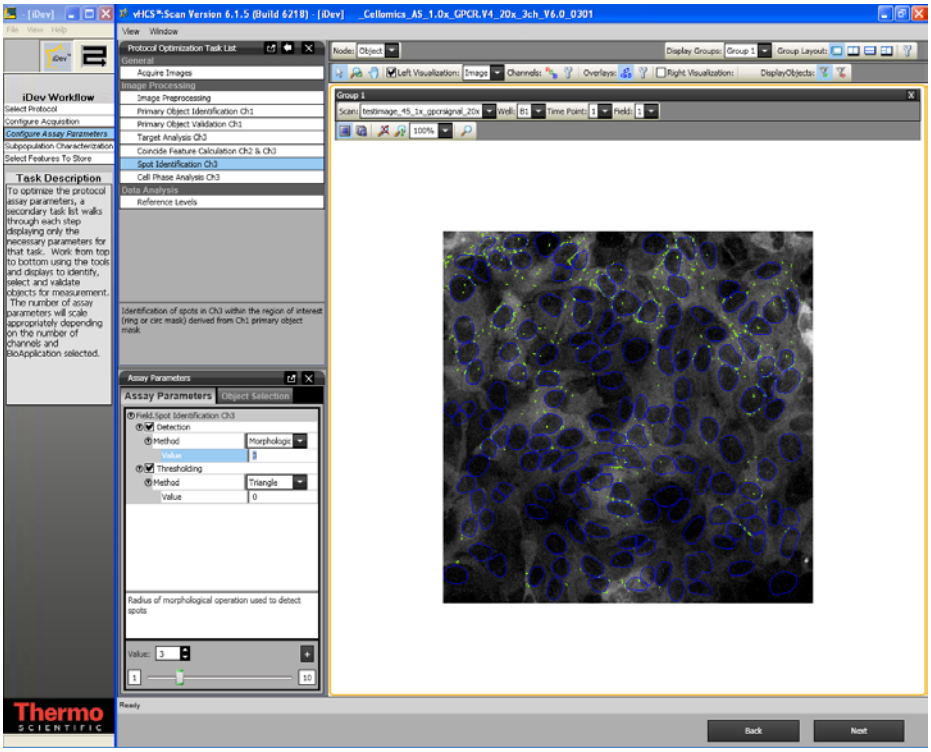


Figure 25. *Protocol Optimization Task – Spot Identification Ch3*

Cell Phase Analysis Ch3

In this task, you can set parameters that allow you to classify the cells as belonging to one of the 4 phases- Phase 0, 1, 2, or 3. Please refer to Chapter 2 of this guide for details on how to set these parameters for Phase Classification.

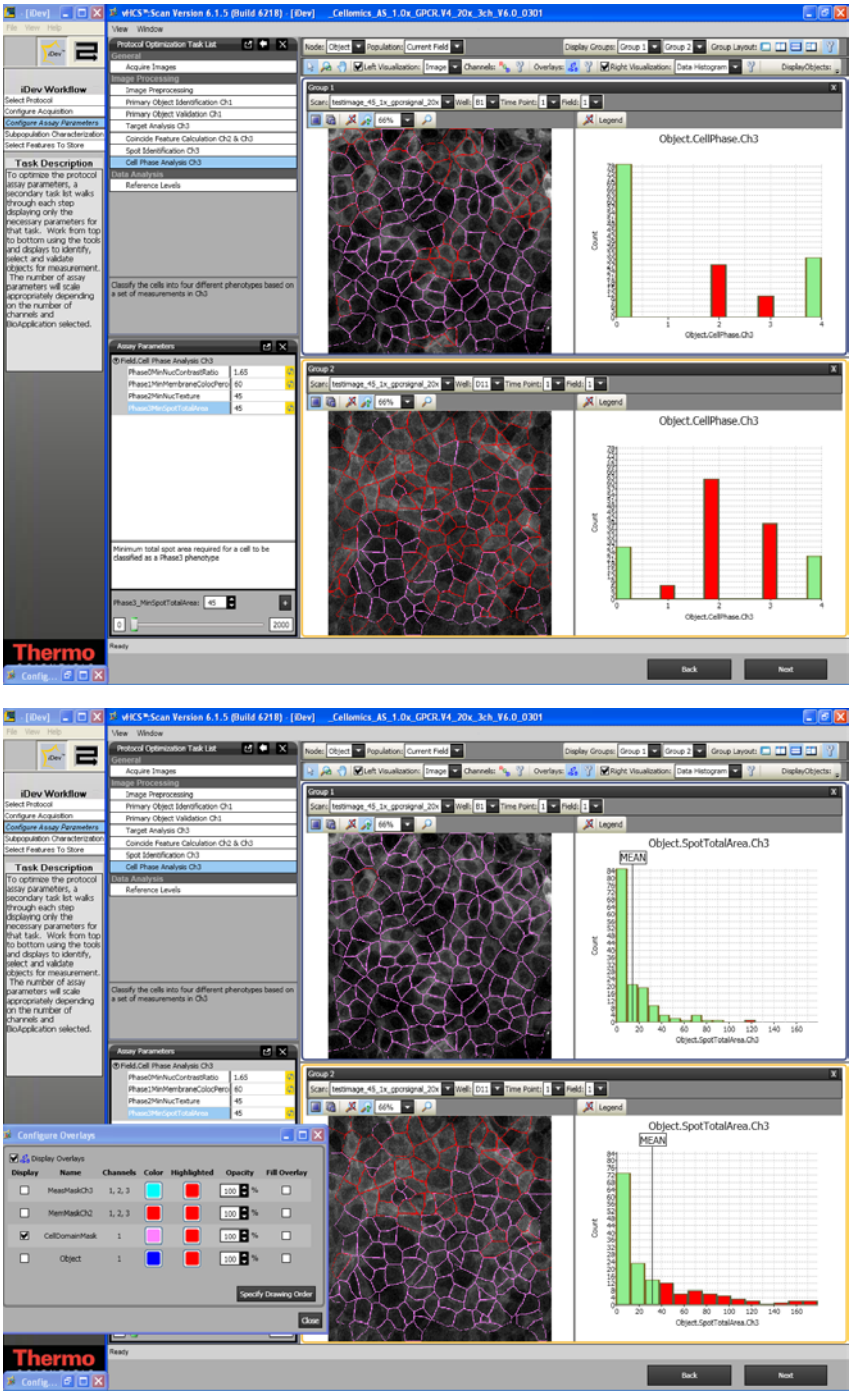


Figure 26. *Protocol Optimization Task – Cell Phase Analysis Ch3*

Index