IDEAS® Image Data Exploration and Analysis Software User's Guide

For Research Use Only. Not for use in diagnostic procedures.

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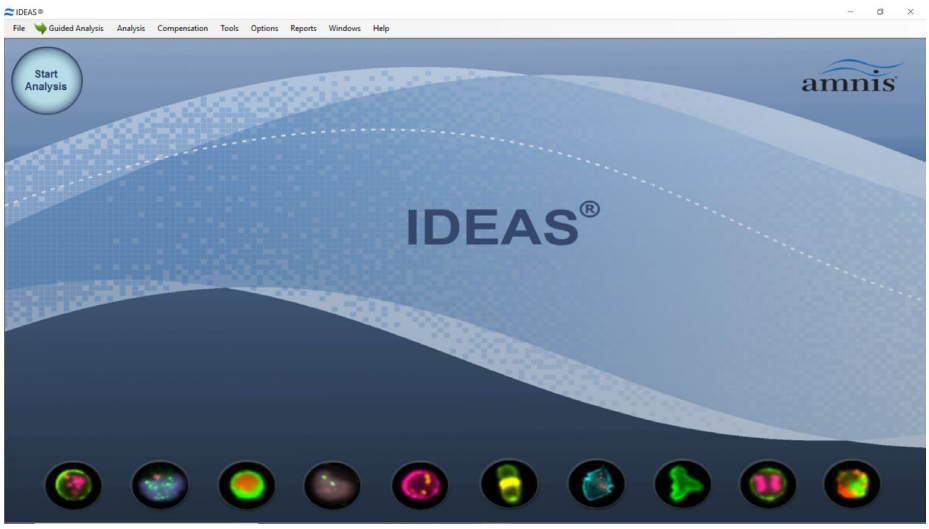
Introduction



Welcome to IDEAS® software

Welcome to the IDEAS® software manual for ImageStream® system and FlowSight® system data analysis.

Description

This manual provides instruction for using the Cytek® Amnis® IDEAS® software to analyze data files from the ImageStream® and FlowSight® cell analysis systems.

The intuitive user interface of the IDEAS® software makes it easy for you to explore and analyze data. The application can quantify cellular activity by performing statistical analyses on thousands of events and, at the same time, permit visual confirmation of any individual event. Furthermore, you can operate the application in a batch processing mode and store specific analysis templates for either repeated use or sharing with colleagues.

The fastest way to put the IDEAS® software to work is to first skim through this manual—becoming familiar with the application's structure, compensation, file types, and analysis tools—and then use the application wizards on some sample experimental data to begin exploring the power that the application provides. This manual has been integrated into the IDEAS® software to provide help by chapter heading and using the Edit / Find search tool to search for feature names, masks, or other critical functionality.

Materials Provided

- IDEAS® Software

Materials Required but Not Provided

- ImageStream® system, ImageStream®^{X}, ImageStream®^{X} MkII system, or FlowSight® system
- IDEAS® software will open FCS, .rif, .cif, .daf data file formats

Set Up the IDEAS® Software Application

This chapter describes the hardware and software requirements for the application, which includes the procedures for installing, removing, and upgrading the application.

Hardware and Software Requirements

This section states the minimum and the recommended hardware and software requirements for running the IDEAS® software application.

Hardware Requirements

The minimum hardware requirements are 4 GB of RAM and a dual core Intel processor. However, due to the large size of the image files that the ImageStream® system creates, a larger amount of RAM will prevent paging and thus increase performance.

Software Requirements

IDEAS® software 64-bit version requires that the Windows® 7 or later operating system be installed on your computer. IDEAS® software 32-bit version requires Windows XP, Windows 2000 or later version of the operating system. The latest service pack and any critical updates for the operating system must be included. You must also install the Microsoft® .NET Framework 2.0, which requires Microsoft Internet Explorer 5.01 or later.

NOTE: If the software requirements are not met, Setup will not block installation nor provide any warnings. NOTE: Service packs and critical updates are available from the Microsoft Security Web Site.

Install the IDEAS® Software Application

NOTE: If the IDEAS® software has previously been installed, the previous version will be removed during installation.

1. Access the application Setup file from technicalsupport@cytekbio.com, or the training folder on the ImageStream® system desktop.
2. View the contents of the Setup file in My Computer or Windows® Explorer.

3. Double click Setup.exe.
4. Check Install 21 CFR Part 11 support if you have purchased this option and plan to use the compliance manager. Review the compliance manager user guide and related material for a full description on how use this version.
5. Follow the instructions until the installation process has completed.
6. MadCap help viewer may be installed and opened during installation or upgrade.
7. Select the language and check box to not show this dialog again.
8. An icon appears on the desktop and IDEAS® software appears on the All Programs menu.

Set Your Computer to Run the IDEAS® Software Application

Set the Screen Resolution

Confirm that the screen resolution is adequate for the IDEAS® software. To be able to view the entire application window, you must set the width of the screen resolution to a minimum of 1024 pixels.

1. From the Start menu, select Control Panel, and then click Display.
2. Click the Settings tab to set the screen resolution.

View File Name Extensions

When loading a file, the IDEAS® software uses the file name extension to determine the file type. It will be easier for you to distinguish raw image files, compensated image files, and data analysis files from each other if Windows Explorer does not hide the extensions.

1. In Windows® Explorer, go to Tools > Folder Options.
2. Click the View tab, and ensure that the Hide extensions for known file types check box is not selected.
3. Click OK.

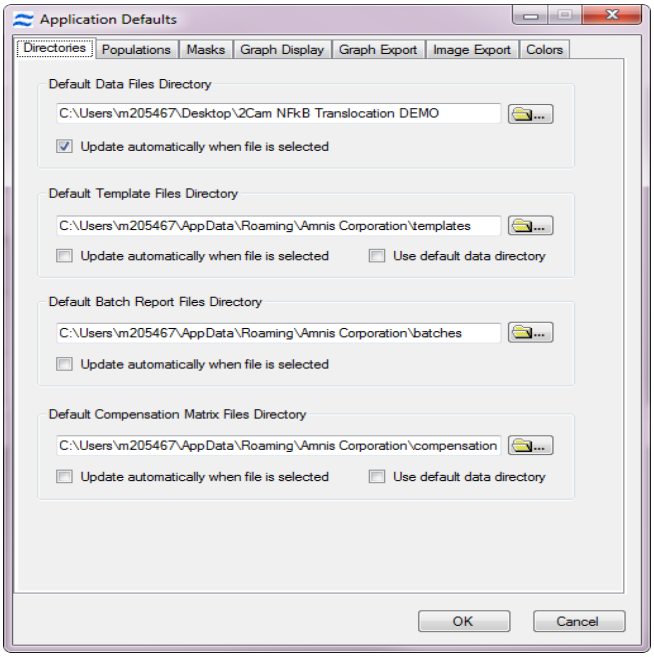
Copy the Example Data Files

Copy data files to a single directory on your hard drive. Sample data files are available on your workstation in the training folder.

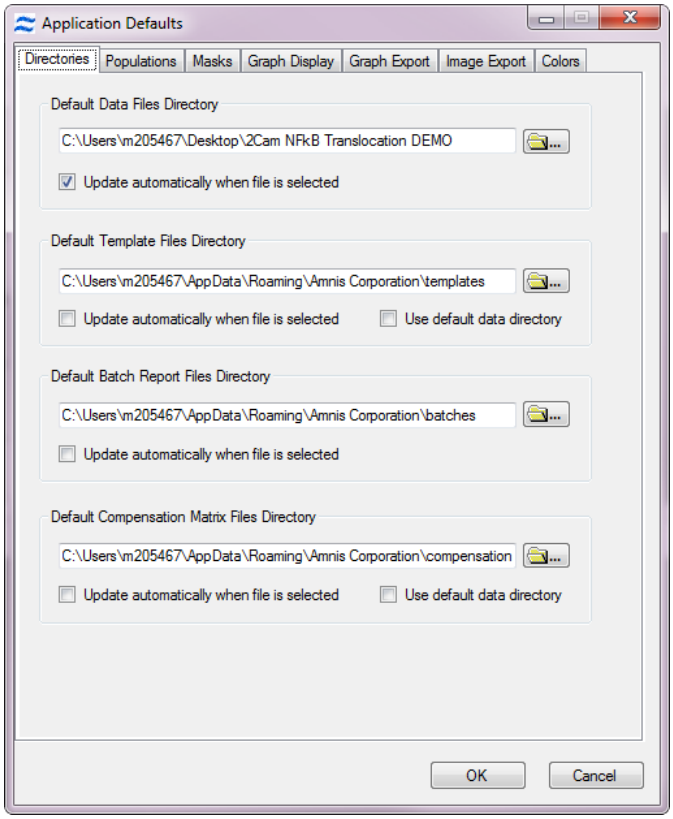
1. Copy the data files.
2. Right click the directory that contains the data files and click Properties.
3. Clear the Read only check box.
4. Click OK.

View and Change the Application Defaults

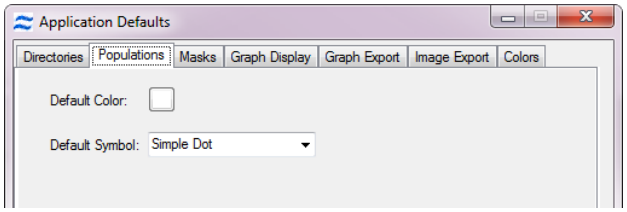
To view or change these defaults, choose Application Defaults from the Options menu. Each tab allows you to view or change the default settings.



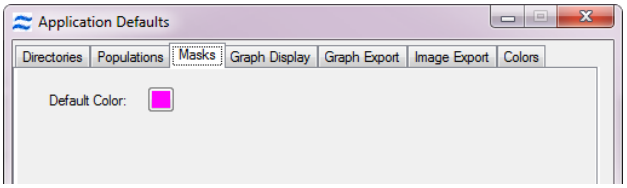
- The Directories tab contains the default Data, Template, Batch or Compensation Matrix file directories.



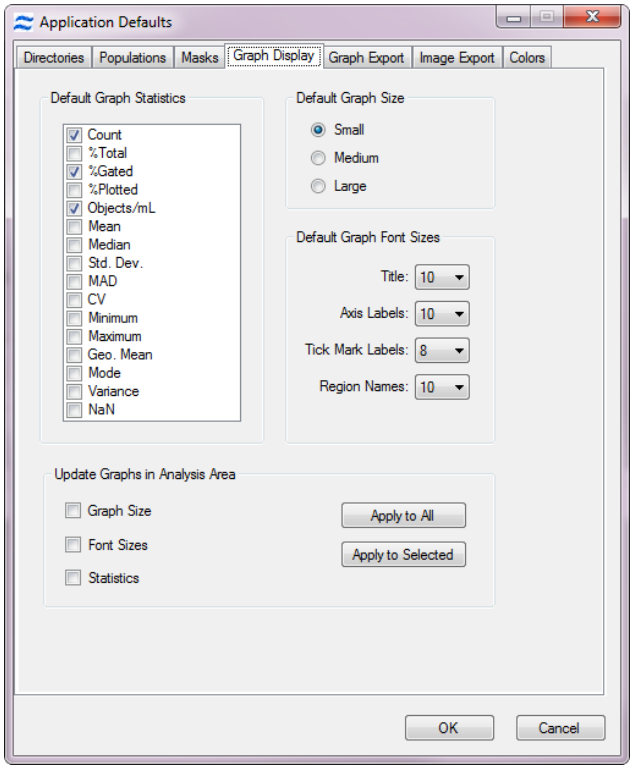
- The Populations tab contains the default color or symbol for populations. To change the default settings, click on the color to or choose a default symbol from the list.



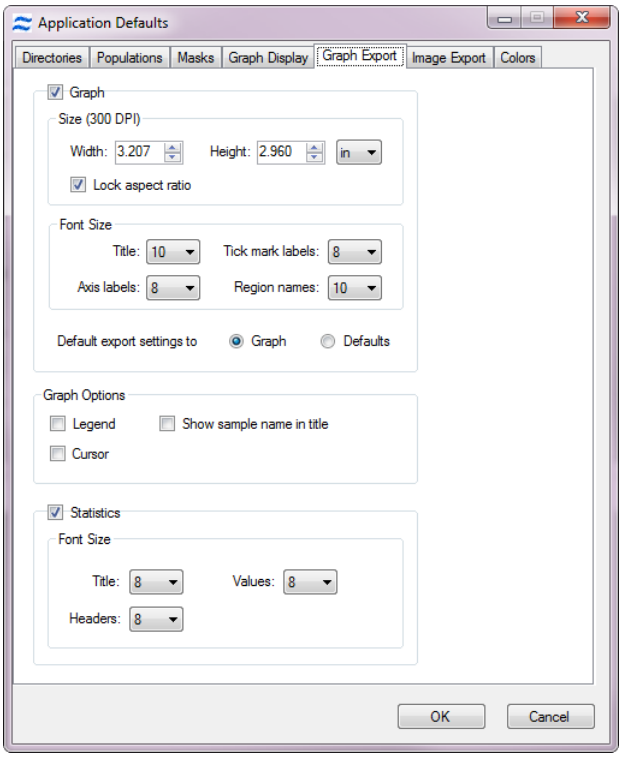
- The Masks tab contains the default mask color. To change the color of the mask, click on the color button.



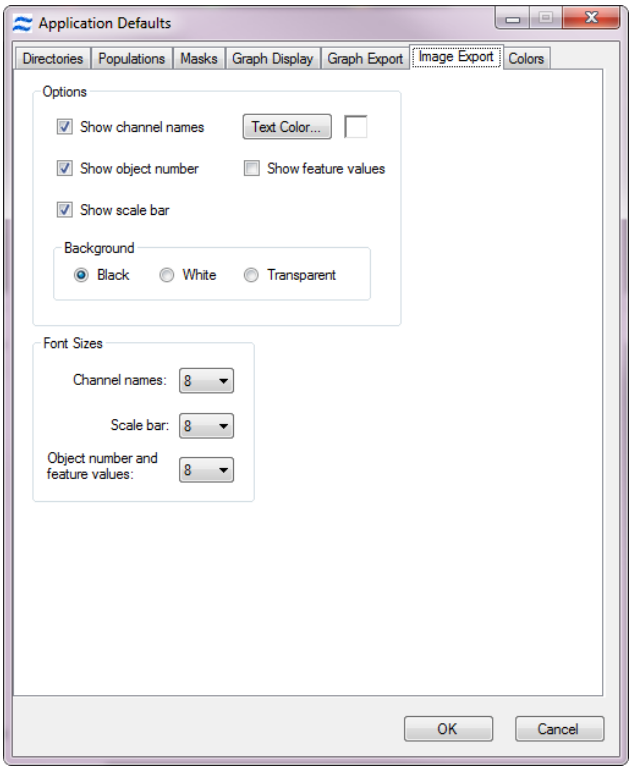
- The Graph Display tab contains the default settings for graphs in the analysis area including graph size, font sizes and the default list of statistics shown for a graph. Check the box next to the statistic to have it show below the graphs when statistics are shown for a graph. The settings may be updated on all or selected graphs in the analysis area.



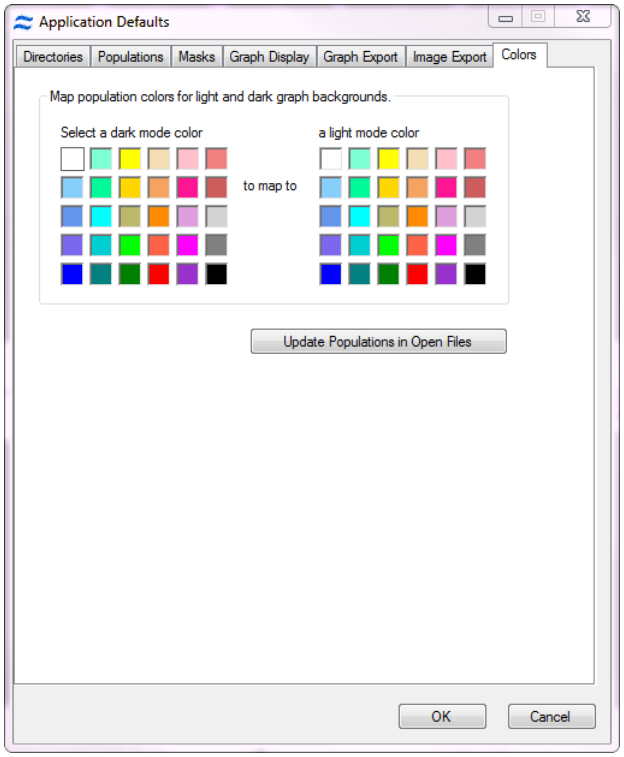
- The Graph Export tab contains the default settings for exporting graphs including the graph size, font size, options to include legend, cursor sample name and statistics. Select the Defaults button to use the graph export settings when exporting graphs or select the Graph button to use the settings as they appear in the analysis area for the graph.



- The Image Export tab contains the default settings for image export when copying and pasting from IDEAS® software for reporting into other programs.



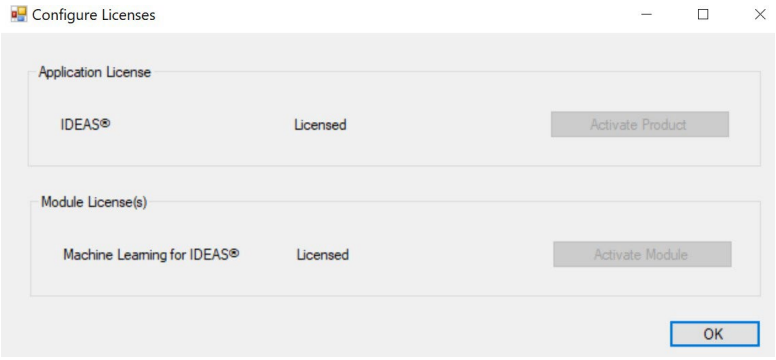
- The Colors tab contains the mapping of dark and light mode colors.



Configure Licenses

To view or edit your installed licenses in IDEAS® software select Options > Configure Licenses.

NOTE: To activate machine learning, please contact your Cytek sales or support representative.



Chapter 2
IDEAS® Software Overview

This chapter provides an overview of the IDEAS® software application user interface, the files that the IDEAS® software creates and uses, the recommended directory organization and an overview of the workflow.

Compensation, Analysis Tools, and File Structure Overview

The Cytek® cell analysis systems possess unique capabilities that neither flow cytometry nor microscopy alone can deliver. The IDEAS® software provides an image gallery to view every cell in the data file along with linked graphical data for confident gating and image confirmation. The application contains powerful algorithms that facilitate and quantify the image analysis of ImageStream® system and FlowSight® system data. Examples include the analysis of molecule co-localization and translocation, the analysis of cell to cell contact regions and signaling interactions, the detection of rare molecules and cells, and a comprehensive classification of large numbers of cells. The IDEAS® software acquires data from INSPIRE™ software compensates the images, and allows the user to evaluate images with data analysis tools.

NOTE: Feature values in IDEAS® software 6.2 and later versions are now being saved in binary format to reduce the file size and facilitate faster analysis. Therefore, daf files generated using IDEAS® software 6.3 and newer cannot be opened using versions before IDEAS® software 6.2. The .daf files created by previous versions of IDEAS® software may be opened by IDEAS® software 6.3 and newer. However, if the files are edited and saved, they will no longer be able to be opened by previous versions.

Software compliance solutions (21 CFR Part 11)

The INSPIRE™ software and IDEAS® software are available in a version that enables compliance with section 21 of the code of federal regulations (CFR) part 11 guidelines for maintaining signature control of scientific data in regulated settings. The 21 CFR 11 version of INSPIRE™ software and IDEAS® software controls user access to the instrument operation and analysis and logs all activity with the user's name, date, and time. With the purchase of the 21 CFR 11 license, three software packages are included: the CFR compliance server, INSPIRE™ software and IDEAS® software. Please contact support or your sales representative about upgrading to this version of the software and refer to the 21 CFR 11 user's manual and related documents to answer any questions you may have.

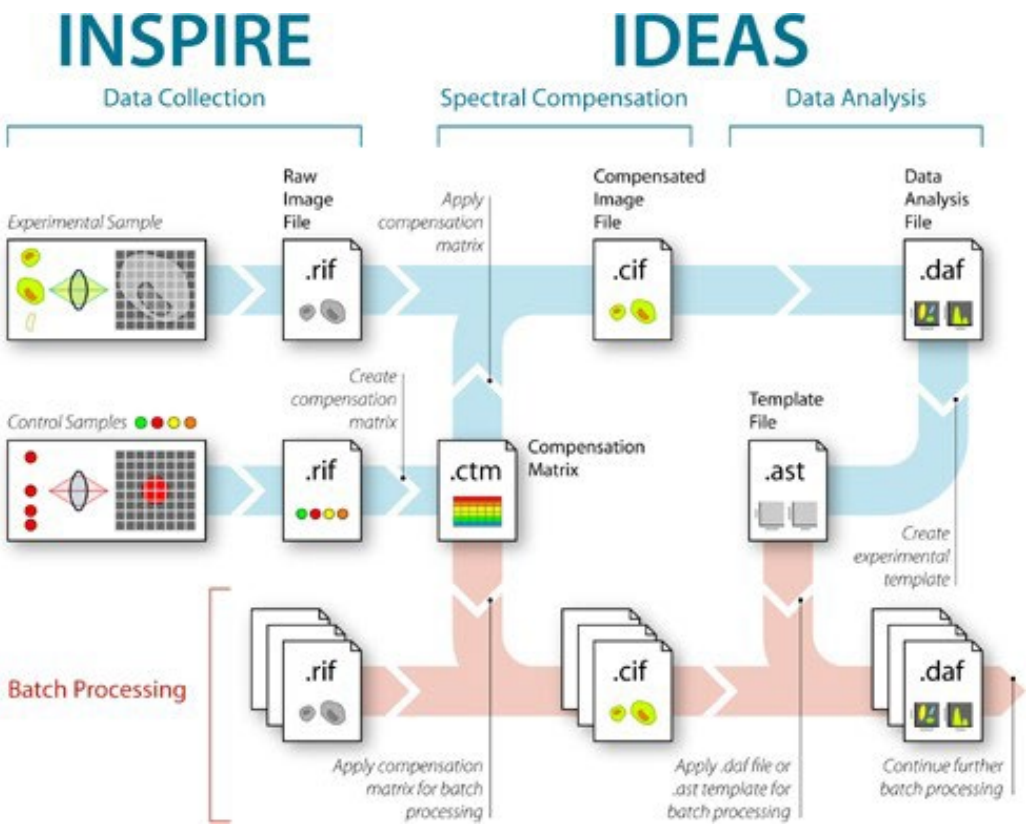
The Data Analysis Workflow

Data analysis in IDEAS® software begins with opening a raw image file (.rif) that was collected and saved using INSPIRE™ software. Then, an existing compensation matrix or a new compensation matrix is applied to the .rif file and two additional files are created, the .cif (compensated image file) and .daf (data analysis file).

A compensation matrix performs fluorescence compensation, which removes fluorescence that leaks into other channels. A compensated image can accurately depict the correct amount of fluorescence in each cell image. Compensation is defined as the correction of the fluorescence crosstalk. When creating the .cif the IDEAS® software application also automatically performs corrections to the raw imagery using values saved from the instrument at the time of data collection. These corrections include flowspeed normalization, brightfield gains, and spatial registry.

A template is used to define the features, graphs, image display properties and analysis for the .daf. Within the .daf file, the user can perform many analyses using the tools and wizards within the application and save the results as a template file (.ast).

The IDEAS® software application then calculates feature values and shows the data as specified by the selected template. Once a data analysis file (.daf file) or compensated image file (.cif file) is saved, it can be opened directly for data analysis. You would only open a .cif if you wanted to change the template or a .rif file to change the compensation.



Data File Type Overview

Data from the Cytek® cell analysis systems are collected and managed using three types of data files: raw image file (.rif), compensated image file (.cif), and data analysis file (.daf).

This section describes each file type, and the table summarizes the features of each file.

Raw Image File (.rif)

The INSPIRE™ software application saves the digital image data, pixel intensities and location that were acquired by the instrument to a .rif file.

A .rif file contains:

- Pixel intensity data (counts and location) collected for each object that the instrument detected.
- Instrument settings that were used for data collection.
- Calibration values from ASSIST.
- Compensation matrix if used while acquiring data.

Compensated Image File (.cif)

The IDEAS® software application creates a .cif file when the user opens a .rif file and applies a compensation matrix. The segmentation algorithm automatically defines the boundaries of each object, creating a mask that is used for calculating feature values. The applied compensation matrix performs pixel by pixel fluorescence compensation prior to segmentation. During the creation of the .cif file, the application makes corrections to the imagery. These corrections include:

- Removal of artifacts from variability in the flow speed, camera background, and brightfield gains.
- Alignment of the objects to subpixel accuracy, which allows the viewing of compensated imagery, composite imagery and the calculation of multi-image feature values, such as the Internalization value.
- Coincident objects are cut apart to place into individual image frames. Note that this will increase the number of objects in the file.

Multiple .cif files can be created from a single .rif file by applying a different fluorescence compensation matrix or correction each time a .rif file is opened and choosing a unique name for the .cif file. Similarly, you can create a new .daf file from a single .cif file by creating a new name and applying a different analysis template.

Data Analysis File (.daf)

The IDEAS® software creates a .daf file while it is loading a .cif file into a template file (.ast). The .daf file is the interface to visualize and analyze the imagery that the .cif file contains and must reside in the same directory as the corresponding .cif file.

The .daf file contains:

- Feature definitions
- Population definitions
- Calculated feature values
- Image display settings
- Definitions for graphs and statistics
- Feature definitions
- Population definitions
- Calculated feature values
- Image display settings
- Definitions for graphs and statistics

Loading a .daf file restores the application to the same state it was in when the file was saved, i.e., with the same views, graphs, and populations. In IDEAS® software versions 3.0 or later, a .daf file may be used as a template.

NOTE: When a .daf file is opened, the .cif file must be located in the same directory as the .daf file since the .daf file points to images used for analysis that are stored in the associated .cif file.

Template (.ast)

The IDEAS® software saves the set of instructions for an analysis session in a .daf file to a template (.ast file). Note that a template contains no data; it simply contains the structure for the analysis. This structure includes definitions for:

- Features
- Graphs
- Regions
- Populations

The .ast file also contains settings for:

- Image viewing
- Image names
- Statistics

The \templatessubdirectory (under the directory where the IDEAS® software was installed) contains the default template, named defaulttemplate.ast. Because a template is required for loading a .cif file, you must use the default template to create the first .daf file. After you save a custom template, you can use it for any subsequent loads of .cif files.

NOTE: The default template may change between releases of the IDEAS® software. In IDEAS® software versions 3.0 or later, a .daf file may be used as a template. The default template contains over 200 calculated features per object. An expanded template is also available that includes over 600 calculated features per object.

Compensation Matrix File (.ctm)

The IDEAS® software saves the compensation values that are created and saved during the spectral compensation of control files to a compensation matrix file (.ctm file). This file has no associated object data; it is created and saved to be applied to experimental files. The compensation matrix can be created in IDEAS® software using single color control files after acquisition or during acquisition. Refer to the INSPIRE™ software or FlowSight® system user manual for more information.

Data File Types

NOTE: Even though Windows® does not treat file names as case sensitive, the IDEAS® software depends on the case sensitive .rif, .cif, and .daf file name extensions to identify the file types. Avoid the use of illegal characters for file names such as: "\/:*?<>!".

Table 1. Review of Data File Types

File Type	Name	Description
.rif	Raw image file	Contains instrument setup data, pixel intensity data, and uncorrected image data from the INSPIRE™ software. The IDEAS® software uses the .rif file to create a compensated image file (.cif file).
.cif	Corrected image file	Contains imagery that been correct for variations in camera background, camera gain, flow speed, and special alignment between channels. Spectral compensation has been applied. Serves as a database of images used for feature calculation and image display. The IDEAS® software loads the .cif file into a template to create a data analysis file (.daf).
.daf	Data analysis file	The main working data file that contains the calculated feature values, the graphs, and the statistics used for analysis. The .daf file references the .cif.
.ast	Analysis template file	This file contains no data; it contains the structure for the analysis, such as, definitions for features, graphs, regions, and populations; image viewing settings; image names; and statistics settings.
.ctm	Compensation matrix file	Contains compensation values created during the spectra compensation wizard using the single color control files. This file has no object data and can be applied to any .rif file to create the compensated image file.
.fcs	Flow Cytometry Standard	Can be created during data acquisition or from IDEAS® software and creates a data file that has just selected features values with no images. This allows the data to be loaded in third party FACS analysis software.
.txt	Text file	This file is created when experiments are batch processed and contains all the selected population statistics for creating statistics tables in Excel.
.tiff	Tag image file format	Can be created in IDEAS® software when exporting images

Chapter 3
Getting Started with IDEAS® Software

This chapter is divided into two sections. The first section contains guided analysis described using the analysis wizards. The second section contains advanced analysis, with more detailed instructions that describe how to open, compensate, merge, save, and create data files without using the wizards. Building blocks are also discussed, which provide a shortcut method to building commonly used graphs.

Guided analysis makes it easy to start analyzing your data. Once you are familiar with the basic analysis, available you may want to perform more advanced analysis.

Data Analysis Process Overview

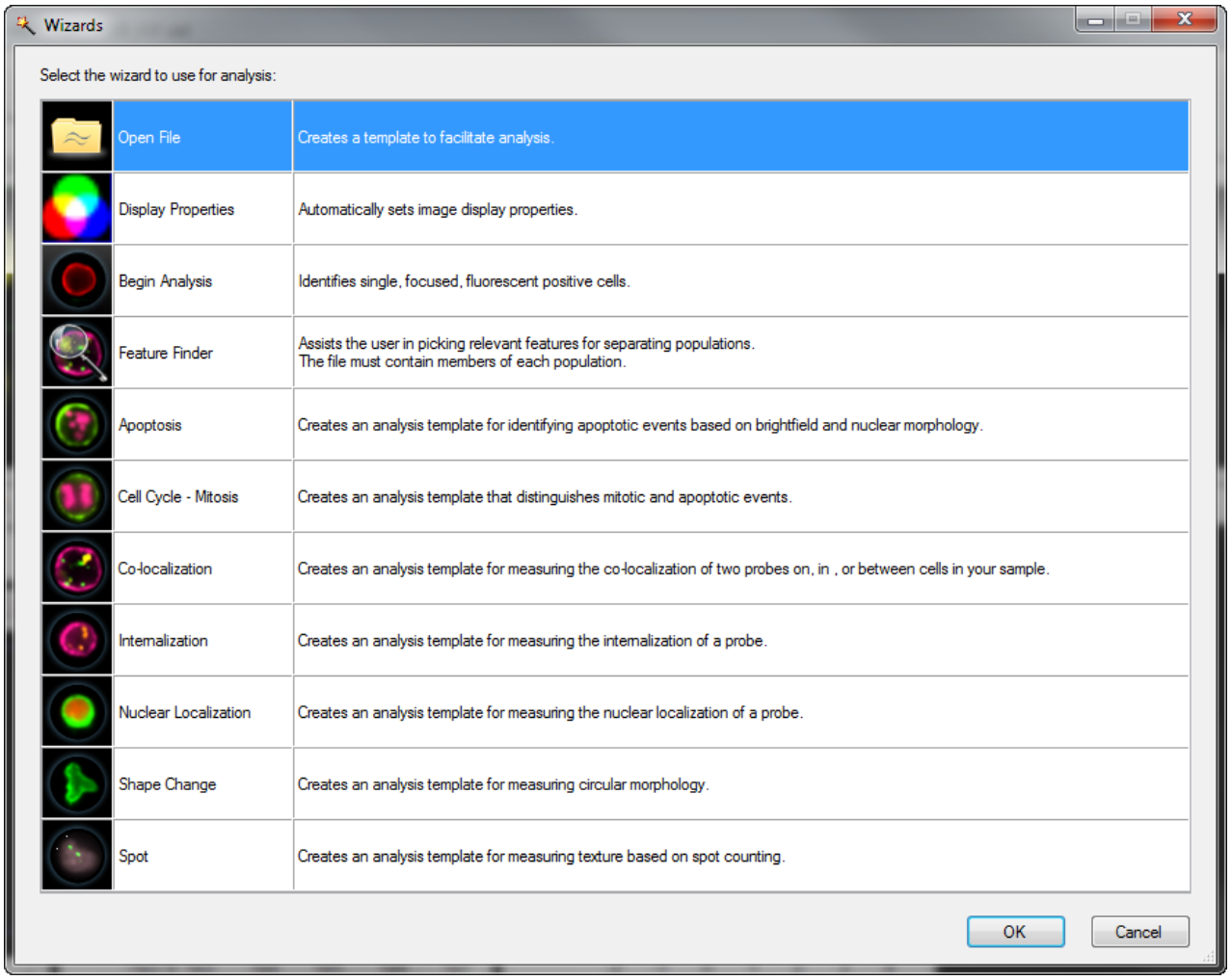
NOTE: These steps apply to any type of data analysis whether you use a wizard or not.

1. Open one data file (the + or - control).
2. Create and save a compensation matrix for the experiment.
3. Using an application wizard or the begin analysis wizard:
   - Select focused cells.
   - Select single cells (or conjugates).
   - Select channels for subpopulation markers and gate to define subpopulations.
   - Gate on positive cells for the channels you wish to use for morphological analysis.
4. By using an application wizard evaluate the feature for your analysis and refine as needed. Or follow the feature finder wizard to find the feature that separates your populations. Note that if the morphological differences are in separate files this may require merging both a + and - control before beginning step 1.
5. Refine the analysis and save the template.
6. Perform batch analysis on all data files in the experiment using the compensation matrix and analysis template.

Guided Analysis

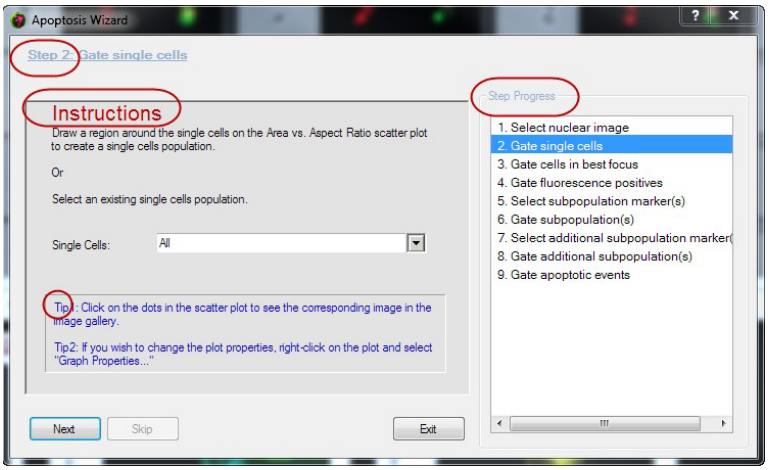
Data analysis always begins with opening a data file. The Start Analysis button will step you through opening a file, setting the image display mapping and choosing an analysis wizard. Use the Guided Analysis drop down menu in the IDEAS® software toolbar to access application wizards, building blocks, and machine learning.

Access application wizards in one of three ways;
1.) From the guided analysis drop down menu
2.) The wizard icon on the analysis toolbar
3.) At the end of the Start Analysis routine



Application Wizards

Each wizard window is organized so that the instructions for each step are written in the left side of the window, the step wise progress through the wizard is shown in the list on the right side and there may be tips provided at the bottom of
the window. Click Next to progress through the wizard or Exit to stop at any time. Some steps are optional; use the Skip button to go to the next step. Follow the instructions in the wizard to complete an analysis.



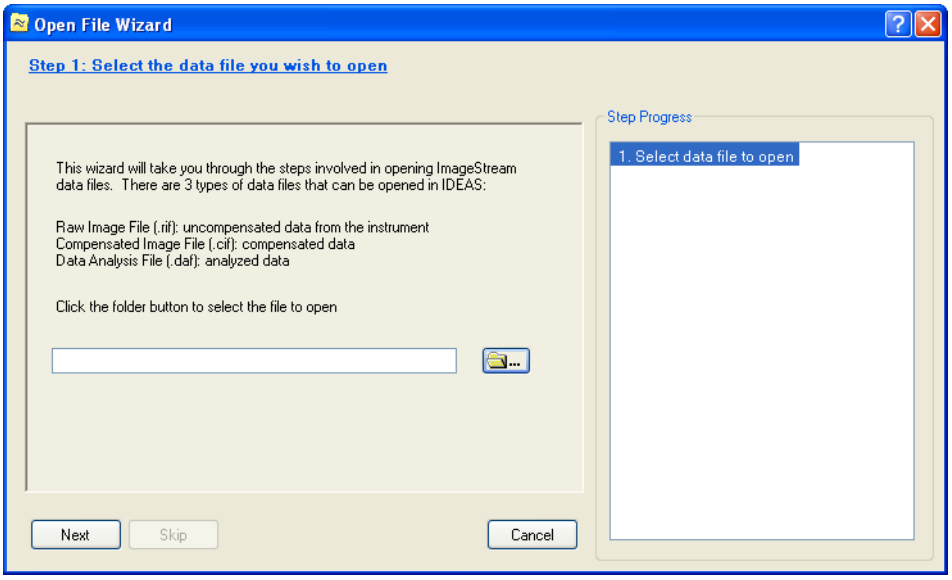
Use the Open File Wizard

This wizard will guide you through the opening of a data file and setting the image display mapping. Use this wizard to open a file if you are not using one of the application specific wizards.

1. Double click the Start Analysis button.
2. Select the data file to open. Click on the folder and navigate to the file you wish to open.

   NOTE: You can limit the view to specific file types (.daf, .cif or .rif) by using the drop down menu 'Files of type:' in the Select Data File window.

A .daf file will open directly without further input, a .cif file will require a template and a .rif file will require a template and a compensation matrix. If the template or compensation matrix boxes are left blank, the default template and/or matrix will be applied *For more information, see Use the Open File Wizard.*



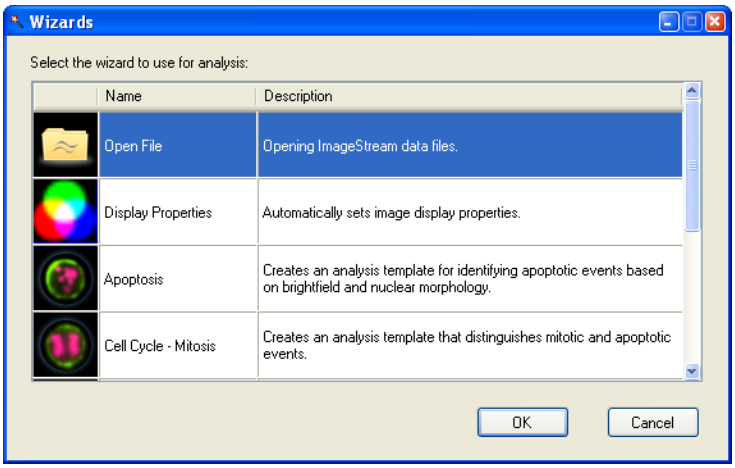
3. Apply compensation. This step occurs when opening a .rif. Choose a compensation matrix or create one. Click Next.
4. Apply analysis template. This step occurs when opening a .rif or .cif. Choose a template file if one exists or leave blank to use the default template. Click Next.
5. Name your files. It is recommended that you keep the default names.
6. Select a wizard to begin analysis. Select a wizard to begin analysis or manually create your analysis template.

   NOTE: Every wizard begins the same with set of graphs for choosing focused, single, positive events. The specific application wizards will also create special features and or masks specific to the application.

Use the Display Properties Wizard

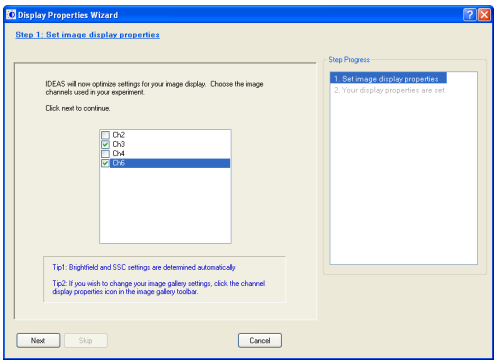
This wizard is automatically run when you use the other analysis wizards or the Open File wizard. It is also available to run in any open data file from the Guided Analysis menu or from the wizard icon. This wizard will set the image display mapping for the channel images you select and create a view of selected images. Brightfield and scatter images will be automatically selected.

1. Select Wizards from the Guided Analysis menu or click the Wizard icon ( ) in the analysis area toolbar. The Wizards window opens.



2. Double click on the Display Properties option and follow the instructions.

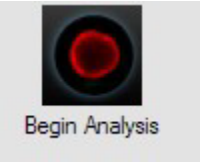
The Display Properties wizard adjusts the mapping of the pixel intensities to the display range for optimizing the display and creates a view that includes the chosen channels. This is for display only and does not effect the pixel values *For more information, see Set the Image Gallery Properties.*



Use the Begin Analysis Wizard

This wizard is available once a data file is open and will guide you through choosing the focused cells, then single cells, then choosing subsets of fluorescent positive cells for phenotypic analysis before progressing on to a morphological analysis.

The wizards selection screen will display once the data file is open. If you have an open data file and want to access this wizard, choose Wizards from the Guided Analysis menu or click on the wand tool.



1. Gate cells in best focus. A histogram of the brightfield channel Gradient RMS values for the 'All' population has been added to the analysis area. Click on the bins in the histogram to view the images in each bin. The cells with better focus have higher Gradient RMS, values. Begin your region at the bin after the Gradient RMS value you wish to exclude and continue the region to the maximum in the plot. You may choose an already existing population.
2. Gate single cells. A scatter plot of the brightfield Area versus Aspect Ratio for the population chosen in step one has been added to the analysis area. Single cells will have an intermediate Area value and a high Aspect Ratio. Click on the dots to view the image associated with that dot. Note that the image is surrounded by a light green line and the image next to it in the image gallery is not its neighbor in the plot. The images are presented in the order defined by the drop down menu above the image gallery. You may choose an already existing population.
3. Select subpopulation marker(s). Choose one or two channels you wish to use to identify populations based on Intensity. Click Next to add the scatter plot to the analysis area. Click Skip if you do not wish to use this step. Draw regions in the scatter plot to identify as many populations as you want. This step will be repeated until you choose Skip or Finish.

The statistics Count and Percent Gated are added to the Population Statistics table in the analysis area, and a statistics definition is added to the template. To view the definition, choose Define Statistics Report from the Reports menu.

Use the Feature Finder Wizard

The feature finder wizard will guide you through the process of choosing a feature for morphological analysis when a specific application wizard is not appropriate. This wizard is available once a data file is open; it will guide you through choosing the focused cells, then single cells, then choosing subsets of fluorescent positive cells for phenotypic analysis, then choosing a feature for morphological analysis.

Open a data file that contains images of both phenotypes you wish to separate.

NOTE: It may be necessary to merge two files together if the populations are in separate files.

1. Open the Wizards screen. A data file must be open to access the Feature Finder Wizard.
   a. To open a data file, click the Start Analysis button or select Guided Analysis > Wizards from the IDEAS® software tool bar. The Wizards screen appears once the data file is open.
   b. To access the Wizards screen when a data file is already open in IDEAS® software, select Guided Analysis > Wizards, or click the wand icon.
2. Double click on Feature Finder icon in the Wizards screen to begin the Feature Finder Wizard:

Feature Finder

3. Gate cells in best focus. A histogram of the brightfield channel Gradient RMS values for the 'All' population has been added to the analysis area. Click on the bins in the histogram to view the images in each bin. The cells with better focus have higher Gradient RMS, values. Begin your region at the bin after the Gradient RMS value you wish to exclude and continue the region to the maximum in the plot. You may choose an already existing population. Click Next.
4. Gate single cells. A scatter plot of the brightfield Area versus Aspect Ratio for the population chosen in step one has been added to the analysis area. Single cells will have an intermediate Area value and a high Aspect Ratio. Click on the dots to view the image associated with that dot. Note that the image is surrounded by a light green line and the image next to it in the image gallery is not its neighbor in the plot. The images are presented in the order defined by the drop down menu above the image gallery. You may choose an already existing population. Click Next.
5. Select subpopulation marker(s). Choose one or two channels you wish to use to identify populations based on Intensity and include the channel(s) you are going to use for morphological analysis. Click Next to add the scatter plot to the analysis area. Click Skip if you do not wish to use this step. Draw regions in the scatter plot to identify as many populations as you want. This step will be repeated until you choose Skip or Finish.
6. Assign truth populations. Choose two truth populations of cells that represent one morphological phenotype difference you wish to separate. Use the tagging tool icon to hand tag images or select preexisting gated populations. Note: If there are more than one phenotypic differences, repeat the process for each with new truth populations.
7. Select channels and feature categories. Choose the channel and feature category you wish to explore. Multiple rows may be entered. The features in the table below will be created and calculated. All of the default features, newly created features and user defined features in the chosen categories will be evaluated for their ability to separate the truth populations. The three highest ranking features for each category will be saved and available for evaluation.

8. Results. The top ranking features are listed in the table with their category and channel. A Statistics table is added to the analysis area that lists the features with the RD Mean for the truth populations. RD is the Fischer's discriminant ratio which is the difference in the means divided by the sum of the standard deviations for the two populations. The larger the RD value, the better the separation afforded by the feature. A scatter plot is added to the analysis area of the truth populations for the top two features.

It may be necessary to refine your results. Visually verify the morphology and separation for the features listed. Additional features may be quickly plotted by selecting them in the list and clicking 'Plot Features'. To return to the truth population assignment step click 'Refine populations'. To choose different channels or categories click 'Change Category'.

You may also wish to manually make new masks and features using these masks if they are not in one of the Additional Features tables.

Table 2. Additional Location Features

Location features are X,Y pixel coordinates from an origin in the upper left corner, pixels or contour.

Feature Name	Description	Mask Used
Centroid Features	The distance between the Centroids of the intensity weighted and non-intensity weighted image	Channel Mask

Table 3. Additional Shape Features

Shape features define the mask shape and have units that vary with the feature.

Feature Name	Description	Mask Used
Aspect Ratio Feature	The ratio of the Minor Axis divided by the Major Axis.	Object
Circularity Feature	The degree of the mask's deviation from a circle.	Object
Density Feature	Describes the density of intensities within the object.	Object
Elongatedness Feature	The ratio of the Height/Width which use the bounding box.	Object
Lobe Count Feature	The number of lobes in a cell. For more information, see Symmetry 2, 3, 4 Features.	Object
Shape Ratio Feature	The ratio of Thickness Min/Length features.	Object
Symmetry 2, 3, 4 Features	These three features measure the tendency of the object to have a single axis of elongation, a threefold and a fourfold variation of the shapes. See also Lobe Count Feature.	Object

Table 4. Additional Size Based Features

Size based features are in microns.

Feature Name	Description	Mask Used
Height Feature	Based on a bounding rectangle, the Width is the smaller side, and the Height is the longer side of the rectangle.	Object
Length Feature	Measures the longest part of the mask.	Object
Major Axis and Minor Axis Features	Describes the widest part of the mask and the narrowest part of the mask, respectively.	Object
Perimeter Feature	Describes circumference of the mask.	Object, Threshold 30,50,70%
Thickness Max Feature	Describes the longest width of the mask.	Object
Thickness Min Feature	Describes the shortest width of the mask.	Object
Width Feature	Based on a bounding rectangle, the Width is the smaller side, and the Height is the longer side of the rectangle.	Object

Table 5. Additional Texture Features

Texture features measure pixel or regional variation and indicate the granularity or complexity of the image.

Feature Name	Description	Mask Used
Contrast Feature	Enumerates changes of pixel values in the image to measure the focus quality of an image.	Morphology, Object
H Texture Features	Measures Haralick texture features. Granularity settings 1,5,15,19	Channel Mask
Modulation Feature	Measures the intensity range of an image, normalized between 0 and 1.	Morphology, Object
Spot Count Feature	Enumerates the number of spots.	Channel Mask
Std Dev Feature	Describes the overall distribution of pixel intensities.	Morphology, Object

Table 6. Additional Signal Strength Features

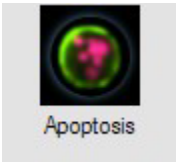
Signal Strength Features are measured in pixel values.

Feature Name	Description	Mask Used
Intensity Feature	The sum of the pixel intensities in the mask, background subtracted.	Morphology, Object, Threshold 30,50,70 %

The statistics Count and Percent Gated are added to the Population Statistics table in the analysis area, and a statistics definition is added to the template. To view the definition, choose Define Statistics Report from the Reports menu.

Use the Apoptosis Wizard

The Apoptosis wizard will guide you through the process of creating the features and graphs to measure apoptosis using the images of the nuclear dye and brightfield. Begin by opening a data file, then choosing the Apoptosis wizard.

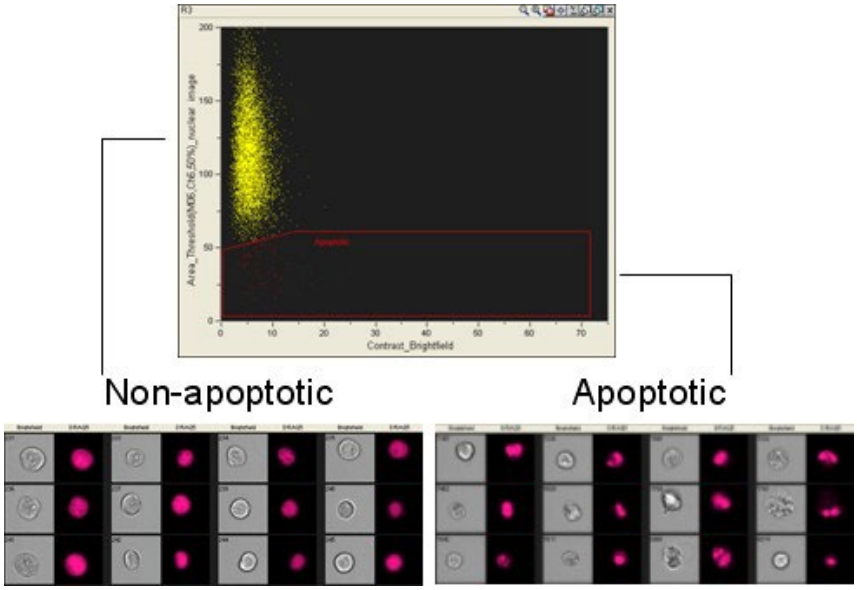


1. Select the nuclear image channel: From the drop down menu, pick the nuclear channel image.
2. Gate cells in best focus: A Gradient RMS histogram of the All population has been added to the analysis area. Click on the bins in the histogram to view the images in each bin. The cells with better focus have higher Gradient RMS, values. Begin your region at the bin after the Gradient RMS value you wish to exclude and continue the region to the maximum in the plot. You may choose an already existing population.
3. Gate single cells: A scatter plot of Area versus Aspect Ratio has been added to the analysis area. Single cells will have an intermediate Area value and a high Aspect Ratio. Click on the dots to view the image associated with that dot. Note that the image is surrounded by a light green line and the image next to it in the image gallery is not its neighbor in the plot. The images are presented in the order defined by the drop down menu above the image gallery.
4. Optional. Answer Yes if you want to define subpopulations in your experiment.
5. Select subpopulation marker(s):Choose one or two channels you wish to use to identify populations based on Intensity. Click Next to add the scatter plot or histogram to the analysis area. Click Skip if you do not wish to use this step.
6. Gate subpopulations: Draw regions in the scatter plot or histogram to identify as many populations as you want. This step will be repeated until you choose Skip.

   NOTE: Step number sequence is dependent on the number of times the subpopulation marker step is taken.

7. (Optional) Select additional subpopulation marker(s) OR gate nucleated cells: A histogram of the nuclear channel Intensity is added to the analysis area. Gate on the positive events.

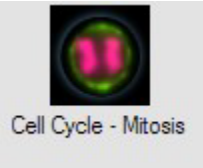
8. Gate apoptotic cells. The nucleated cells scatter plot of the brightfield Contrast versus the Area of the thresholded nucleus is added to the analysis area. Gate on the apoptotic cells with low nuclear area and high brightfield contrast.



The statistics Count and Percent Gated are added to the Population Statistics table in the analysis area, and a statistics definition is added to the template. To view the definition, choose Define Statistics Report from the Reports menu.

Use the Cell Cycle - Mitosis Wizard

The Cell Cycle - Mitosis wizard will guide you through the process of creating the features and graphs to analyze the cell cycle and identify mitotic events using the images of a nuclear dye.

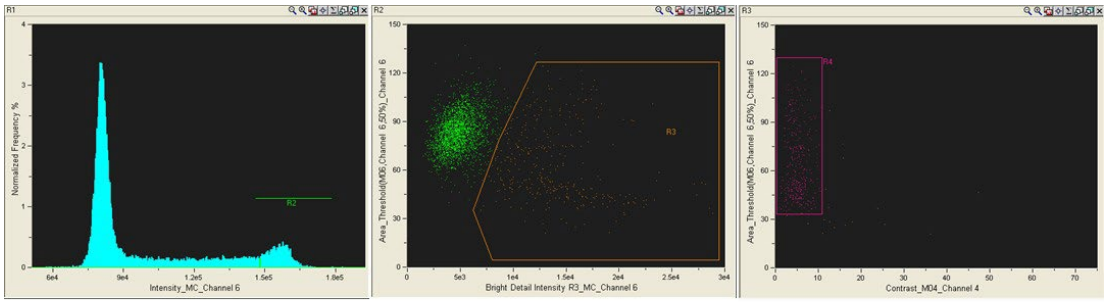


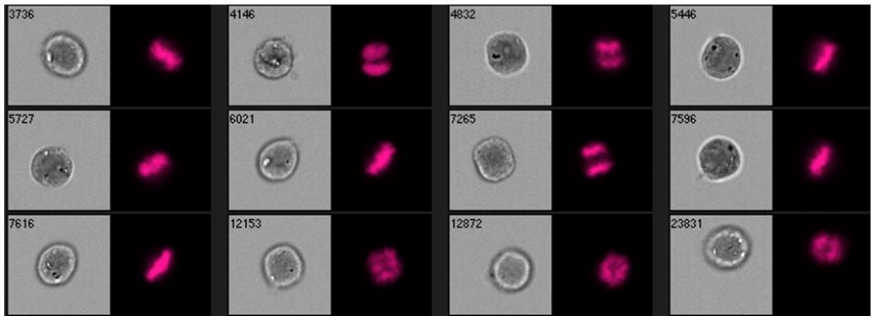
1. Select the nuclear image channel: From the drop down menu, pick the nuclear channel image.
2. Gate cells in best focus: A Gradient RMS histogram of the All population has been added to the analysis area. Click on the bins in the histogram to view the images in each bin. The cells with better focus have higher Gradient RMS, values. Begin your region at the bin after the Gradient RMS value you wish to exclude and continue the region to the maximum in the plot. You may choose an already existing population.
3. Gate single cells: A scatter plot of Area versus Aspect Ratio has been added to the analysis area. Single cells will have an intermediate Area value and a high Aspect Ratio. Click on the dots to view the image associated with that dot. Note that the image is surrounded by a light green line and the image next to it in the image gallery is not its neighbor in the plot. The images are presented in the order defined by the drop down menu above the image gallery.
4. Optional: Answer Yes if you want to define subpopulations in your experiment.
5. Select subpopulation marker(s): Choose one or two channels you wish to use to identify populations based on Intensity. Click Next to add the scatter plot or histogram to the analysis area. Click Skip if you do not wish to use this step.
6. Gate subpopulations: Draw regions in the scatter plot or histogram to identify as many populations as you want. This step will be repeated until you choose Skip.

   NOTE: Step number sequence is dependent on the number of times the subpopulation marker step is taken.

7. (Optional) Select additional subpopulation marker(s) OR. Gate G2/M cells: A histogram of the nuclear channel Intensity is added to the analysis area. Gate on the G2/M population with 2n DNA.
8. Gate cells with condensed DNA: The G2/M cells scatter plot of the threshold Area versus Bright Detail Intensity of the nuclear image is added to the analysis area. Gate on the cells with condensed nuclear that have low nuclear area and high Bright Detail Intensity values These will include apoptotic cells which you can remove in the next step.
9. Gate mitotic cells: The condensed DNA cells scatter plot of the brightfield Contrast versus the Area of the thresholded nucleus is added to the analysis area. Gate on mitotic events with low brightfield Contrast.

The final 3 plots are shown below:

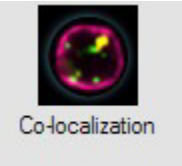




The statistics Count and Percent Gated are added to the Population Statistics table in the analysis area, and a statistics definition is added to the template. To view the definition, choose Define Statistics Report from the Reports menu.

Use the Co-Localization Wizard

The co-localization wizard will guide you through the process of creating the features and graphs to measure the co-localization of two probes with punctate staining in any population of cells you identify.

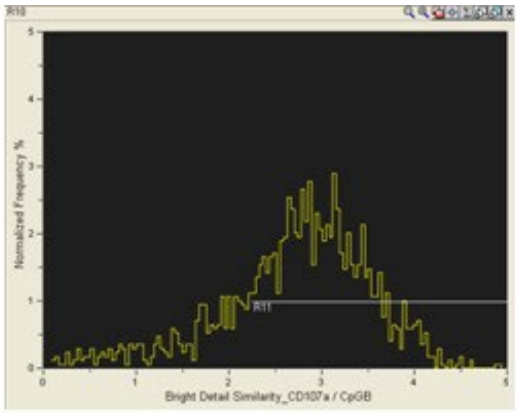


1. Select the co-localization image channels: From the drop down menus pick the two image channels that contain the co-localizing probes.
2. Gate cells in best focus: A Gradient RMS histogram of the All population has been added to the analysis area. Click on the bins in the histogram to view the images in each bin. The cells with better focus have higher Gradient RMS, values. Begin your region at the bin after the Gradient RMS value you wish to exclude and continue the region to the maximum in the plot. You may choose an already existing population.
3. Gate single cells: A scatter plot of Area versus Aspect Ratio has been added to the analysis area. Single cells will have an intermediate Area value and a high Aspect Ratio. Click on the dots to view the image associated with that dot. Note that the image is surrounded by a light green line and the image next to it in the image gallery is not its neighbor in the plot. The images are presented in the order defined by the drop down menu above the image gallery.
4. (Optional) Answer Yes if you want to define subpopulations in your experiment.
5. Select subpopulation marker(s): Choose one or two channels you wish to use to identify populations based on Intensity. Click Next to add the scatter plot or histogram to the analysis area. Click Skip if you do not wish to use this step.
6. Gate subpopulations: Draw regions in the scatter plot or histogram to identify as many populations as you want. This step will be repeated until you choose Skip.

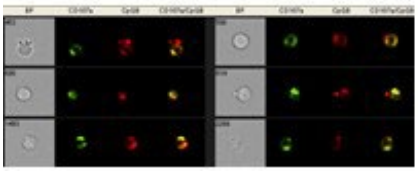
   NOTE: Step number sequence is dependent on the number of times the subpopulation marker step is taken.

7. Gate double positives: A scatter plot of the last gated (or selected) population of the Intensity values for the co-localization channels is added to the analysis area. Draw a region around the double positive cells for the co-localizing probes.

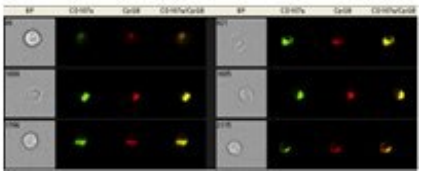
8. Gate co-localized events. A histogram of Bright Detail Similarity R3 for the double positive population is added to the analysis area. Draw a region to gate on co-localized events.



Low co-localization



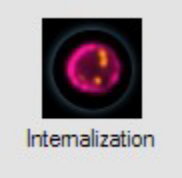
High co-localization



The statistics Count and Percent Gated are added to the Population Statistics table in the analysis area, and a statistics definition is added to the template. To view the definition, choose Define Statistics Report from the Reports menu.

Use the Internalization Wizard

This wizard will create an analysis template for measuring the internalization of a probe in any population of cells you identify.

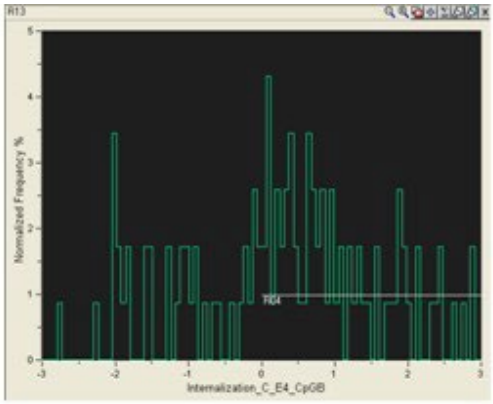


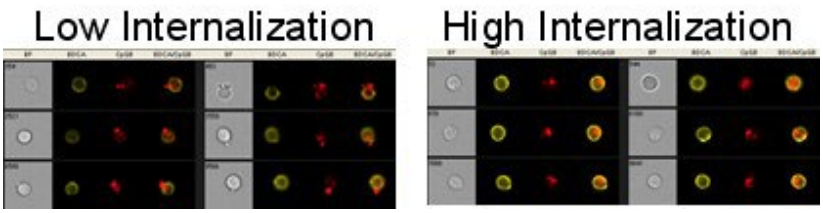
1. Select the internalization image channels: From the drop down menus pick the cell image, the channel that defines the cell surface, and the internalizing probe channel.
2. Gate cells in best focus: A Gradient RMS histogram of the All population has been added to the analysis area. Click on the bins in the histogram to view the images in each bin. The cells with better focus have higher Gradient RMS values. Begin your region at the bin after the Gradient RMS value you wish to exclude and continue the region to the maximum in the plot. You may choose an already existing population.
3. Gate single cells: A scatter plot of Area versus Aspect Ratio has been added to the analysis area. Single cells will have an intermediate Area value and a high Aspect Ratio. Click on the dots to view the image associated with that dot. Note that the image is surrounded by a light green line and the image next to it in the image gallery is not its neighbor in the plot. The images are presented in the order defined by the drop down menu above the image gallery.
4. (Optional) Answer Yes if you want to define subpopulations in your experiment.
5. Select subpopulation marker(s): Choose one or two channels you wish to use to identify populations based on Intensity. Click Next to add the scatter plot or histogram to the analysis area. Click Skip if you do not wish to use this step.
6. Gate subpopulations: Draw regions in the scatter plot or histogram to identify as many populations as you want. This step will be repeated until you choose Skip.

   NOTE: Step number sequence is dependent on the number of times the subpopulation marker step is taken.

7. Next Step. Optional : Select additional subpopulation marker(s) OR gate internalization positives. A scatter plot of Max Pixel versus Intensity for the internalizing probe is added to the analysis area. Draw a region to include the positive cells.

8. Gate internalization events. A histogram of the Internalization feature for the positive cells is added to the analysis area. Draw a region to include the cells with high internalization. The example below shows the internalization of labeled CpG (red).





The statistics Count and Percent Gated are added to the Population Statistics table in the analysis area, and a statistics definition is added to the template. To view the definition, choose Define Statistics Report from the Reports menu.

Use the Nuclear Localization Wizard

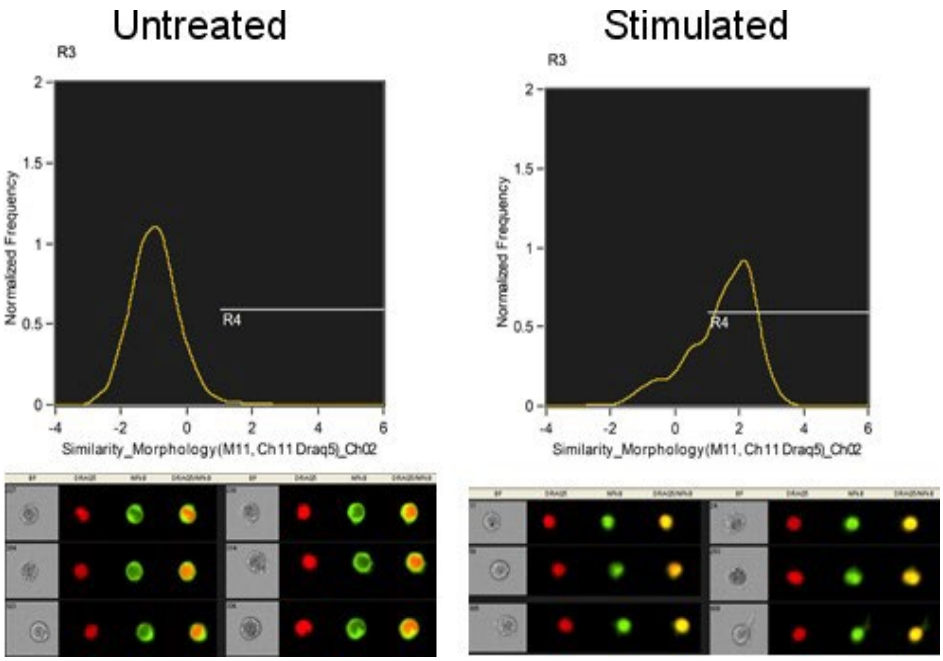
This wizard will create an analysis template for measuring the nuclear localization of a probe in any population of cells you identify.

To begin, double click on Nuclear Localization:

1. Step 1. Select the translocation image channels: From the drop down menus pick the nuclear image channel and the translocating probe image channel.
2. Gate cells in best focus: A Gradient RMS histogram of the All population has been added to the analysis area. Click on the bins in the histogram to view the images in each bin. The cells with better focus have higher Gradient RMS, values. Begin your region at the bin after the Gradient RMS value you wish to exclude and continue the region to the maximum in the plot. You may choose an already existing population.
3. Gate single cells: A scatter plot of Area versus Aspect Ratio has been added to the analysis area. Single cells will have an intermediate Area value and a high Aspect Ratio. Click on the dots to view the image associated with that dot. Note that the image is surrounded by a light green line and the image next to it in the image gallery is not its neighbor in the plot. The images are presented in the order defined by the drop down menu above the image gallery.
4. (Optional) Answer Yes if you want to define subpopulations in your experiment.
5. Select subpopulation marker(s): Choose one or two channels you wish to use to identify populations based on Intensity. Click Next to add the scatter plot or histogram to the analysis area. Click Skip if you do not wish to use this step.
6. Gate subpopulations: Draw regions in the scatter plot or histogram to identify as many populations as you want. This step will be repeated until you choose Skip.

   NOTE: Step number sequence is dependent on the number of times the subpopulation marker step is taken.
7. Next Step. Gate double positives: A scatter plot of the last gated (or selected) population of the Intensity values for the nuclear image and the translocating probe image is added to the analysis area. Draw a region around the double positive cells.
8. Next Step. Gate translocated events: A histogram of Similarity of the double positive cells is added to the analysis area. Draw a region to include the cells with translocation. Note that for a normally distributed population you may want to report the RD of the double positive population in a treated versus untreated sample instead of the percentage gated.

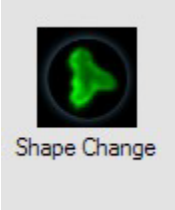
Nuclear localization of a probe is measured using the Similarity feature in the final graph presented in the wizard. The example shown here is of THP1 cells stimulated with 1 ug LPS for 90 minutes and stained with DRAQ5 (red) and NFkB (green) to measure the nuclear localization of the NFkB.



The statistics Count and Percent Gated are added to the Population Statistics table in the analysis area, and a statistics definition is added to the template. To view the definition, choose Define Statistics Report from the Reports menu.

Use the Shape Change Wizard

This wizard will create an analysis template for measuring the shape (circularity) of any population of cells you identify.



To begin, double click on Shape Change.

1. Step 1. Select the cell morphology image channel: From the drop down menu pick the channel for the cell image.
2. Gate cells in best focus: A Gradient RMS histogram of the All population has been added to the analysis area. Click on the bins in the histogram to view the images in each bin. The cells with better focus have higher Gradient RMS, values. Begin your region at the bin after the Gradient RMS value you wish to exclude and continue the region to the maximum in the plot. You may choose an already existing population.
3. Gate single cells: A scatter plot of Area versus Aspect Ratio has been added to the analysis area. Single cells will have an intermediate Area value and a high Aspect Ratio. Click on the dots to view the image associated with that dot. Note that the image is surrounded by a light green line and the image next to it in the image gallery is not its neighbor in the plot. The images are presented in the order defined by the drop down menu above the image gallery.
4. (Optional) Answer Yes if you want to define subpopulations in your experiment.
5. Select subpopulation marker(s): Choose one or two channels you wish to use to identify populations based on Intensity. Click Next to add the scatter plot or histogram to the analysis area. Click Skip if you do not wish to use this step.
6. Gate subpopulations: Draw regions in the scatter plot or histogram to identify as many populations as you want. This step will be repeated until you choose Skip.

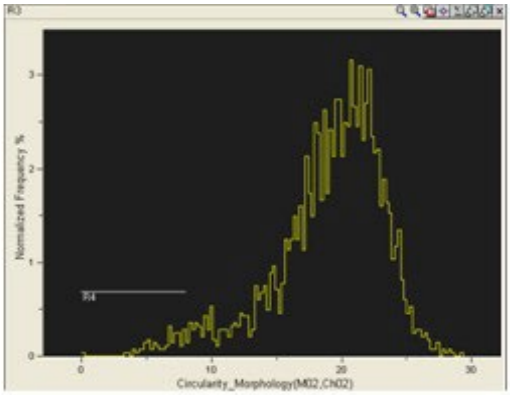
   NOTE: Step number sequence is dependent on the number of times the subpopulation marker step is taken.

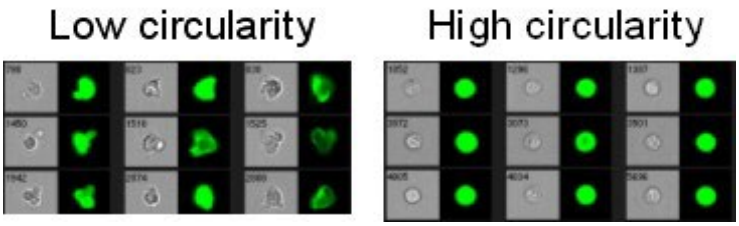
7. Next Step. Gate fluorescence positives: A histogram of the last gated (or selected) population of the Intensity value for the cell image is added to the analysis area. Draw a region around the double positive cells.

   NOTE: This step is skipped if the cell image channel chosen is brightfield.

8. Next Step. Gate shape changed events: A histogram of Circularity of the last gated population is added to the analysis area. Draw a region to include the cells with low circularity scores.

Shape change is measured in the final graph presented in the wizard. Cells with low circularity scores have a highly variable radius. In this example monocytes in whole blood were stained with CD14 (green).

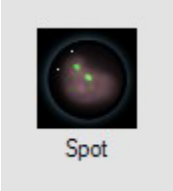




The statistics Count and Percent Gated are added to the Population Statistics table in the analysis area, and a statistics definition is added to the template. To view the definition, choose Define Statistics Report from the Reports menu.

Use the Spot Wizard

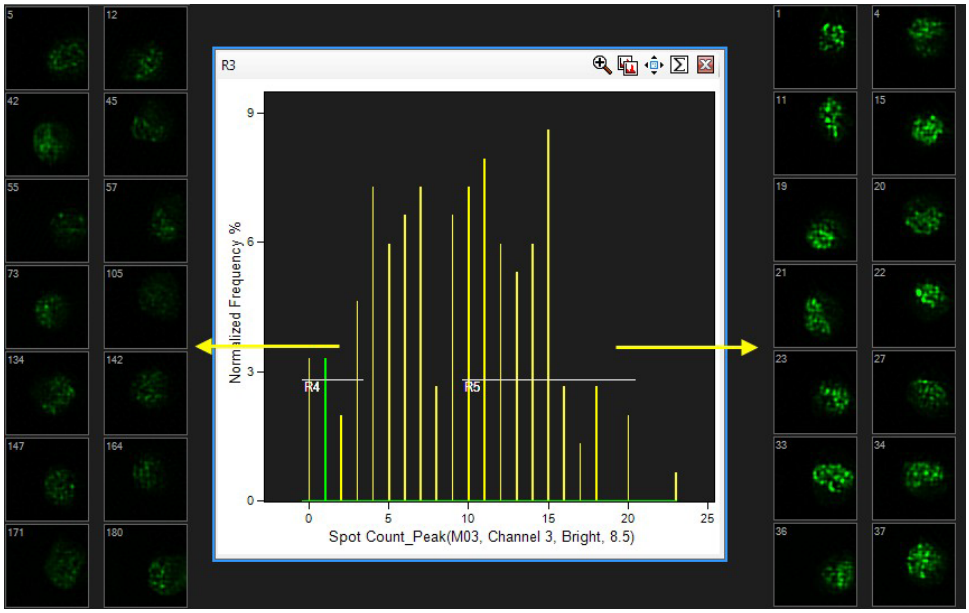
This wizard will create an analysis template for measuring texture based on spot counting. If the low and high spot count data are in separate data files merge the files together before beginning.



To begin, double click on Spot:

1. Gate cells in best focus: A Gradient RMS histogram of the All population has been added to the analysis area. Click on the bins in the histogram to view the images in each bin. The cells with better focus have higher Gradient RMS, values. Begin your region at the bin after the Gradient RMS value you wish to exclude and continue the region to the maximum in the plot. You may choose an already existing population.
2. Gate single cells: A scatter plot of Area versus Aspect Ratio has been added to the analysis area. Single cells will have an intermediate Area value and a high Aspect Ratio. Click on the dots to view the image associated with that dot. Note that the image is surrounded by a light green line and the image next to it in the image gallery is not its neighbor in the plot. The images are presented in the order defined by the drop down menu above the image gallery.
3. (Optional) Answer Yes if you want to define subpopulations in your experiment.
4. Select subpopulation marker(s): Choose one or two channels you wish to use to identify populations based on Intensity. Click Next to add the scatter plot or histogram to the analysis area. Click Skip if you do not wish to use this step.
5. Gate subpopulations: The step number sequence is dependent on the number of times the subpopulation marker step is taken. Draw regions in the scatter plot or histogram to identify as many populations as you want. This step will be repeated until you choose Skip.
6. Select the spot image channel: From the drop down menu, choose the image channel for the spot counting.
7. Assign truth populations: From the drop down menus, select two truth populations, one with high and one with low spot count. To create the truth populations, either use the tagging tools or gate the cells of interest.

8. Gate spot events. A histogram of the Spot Count feature for the last gated population is added to the analysis area. Regions have been drawn that include the truth populations. Adjust the regions as necessary. Note that you may want to adjust your truth populations and repeat the wizard after looking at the images and validating the spot counts.



The statistics Count and Percent Gated are added to the Population Statistics table in the analysis area, and a statistics definition is added to the template. To view the definition, choose Define Statistics Report from the Reports menu.

Use Building Blocks

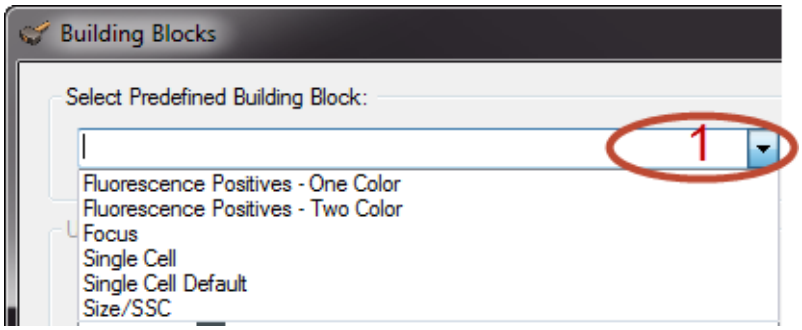
Building blocks may be used to create a graph for finding single cells, focused cells or positive cells based on Intensity. The building blocks are shortcuts to creating a graph that provides a limited list of relevant features with set X and Y axis scales for the graph.

Table 7. Building Blocks and Graph Features

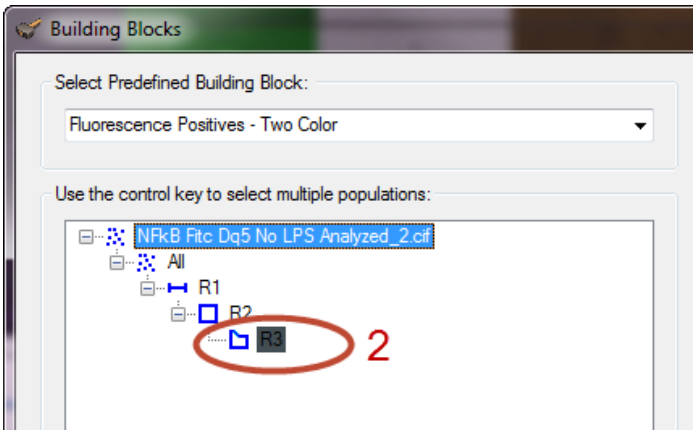
Building Block	X-Axis Features	Y-Axis Features
Fluorescence Positives - one color	Intensity_MC_ChX (for all channels)	n/a
Fluorescence Positives - two color	Intensity_MC_ChX (for all channels)	Intensity_MC_ChX (for all channels)

Building Block	X-Axis Features	Y-Axis Features
Focus	Gradient RMS_MX_ChX (for all channels) **NOTE**: Gradient RMS of brightfield is the default	n/a
Single Cell	Area_brightfield (default) Area_scatter Intensity_MC_ChX (for all channels)	Aspect Ratio_brightfield (default) Aspect Ratio Intensity_MX_ ChX (for all fluorescence channels) Intensity_scatter
Single Cell Default	Area_brightfield	Aspect Ratio_brightfield
Size/SSC	Area_brightfield	Intensity scatter

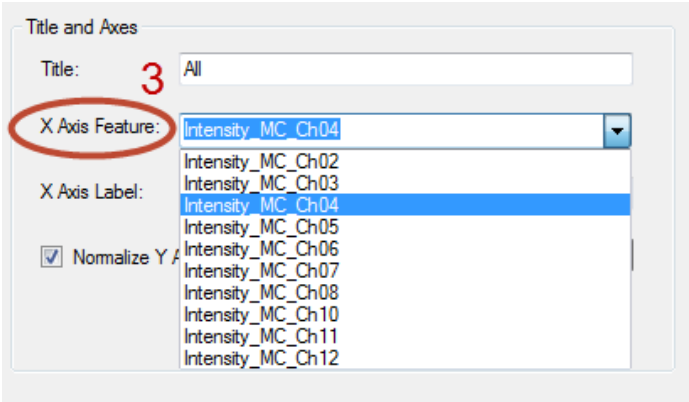
1. Select Building Blocks from the Guided Analysis menu, or click on the Building Blocks icon () in the analysis area toolbar. The Building Blocks window opens. This window is used to define a graph with a specified set of features available, depending on the purpose of the graph.
2. Choose the specific Building Block from the Select Predefined Building Block drop down menu.



3. Choose the population(s) to graph.



4. Choose the X Axis Feature and the Y Axis feature, if applicable.



5. Click OK. The graph is added to the analysis area.

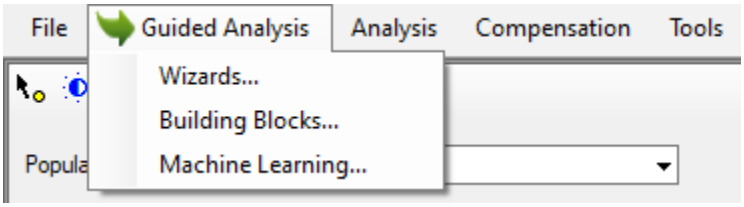
Machine Learning Module for IDEAS® Software

The machine learning (ML) module for IDEAS® software 6.3 and newer creates an experiment specific feature that will distinguish populations based on user input. It is designed to simplify analysis by allowing users to visually create populations, and enhance discrimination by allowing users to combine multiple fluorochromes and multiple morphologies into a single superfeature.

The user provides 'truth' populations, and the module creates custom 'classifier features' based on the Linear Discriminant Analysis ML algorithm. The classifier feature is centered on 0, where positive values identify objects similar to the 'truth' populations, and negative values are dissimilar. In addition to incorporating user defined features already present in the data file, the ML module creates a comprehensive set of additional masks and features (see features created in the Feature Finder wizard for a complete list), which it then ranks and combines with appropriate weights to maximally separate each 'truth' population from the others. The output is a series of 'classifier features' that are specifically targeted to the desired phenotypes, and can be plotted on histograms or bivariate plots.

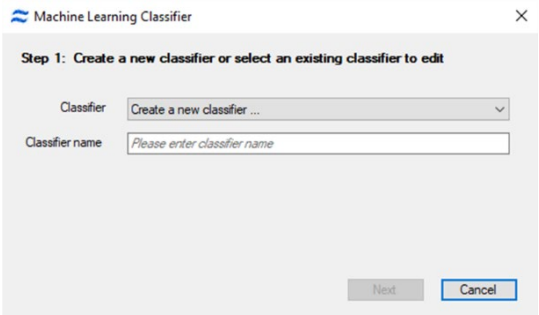
Calculate New Features with the Machine Learning Module

1. From IDEAS® software, open the data file of interest with the compensation matrix applied.
2. (Optional) Apply an analysis template if one exists.
3. From the IDEAS® software toolbar, select Guided Analysis > Machine Learning...

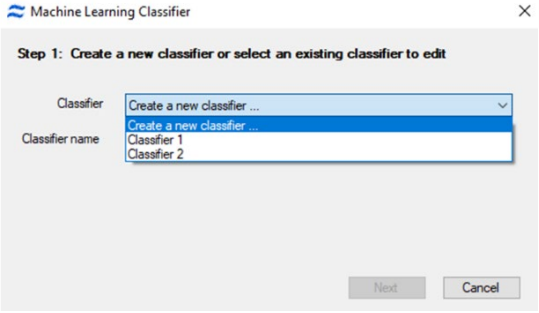


4. In Step 1 of the Machine Learning Classifier, either create a new classifier or modify an existing one. NOTE: If no classifiers exist, you must create one.

   a. Create a new classifier by selecting Create a new classifier... from the Classifier drop down, entering a name for the classifier in the Classifier name field, and clicking Next.



   b. Edit an existing classifier by selecting it from the Classifier drop down and clicking Next.



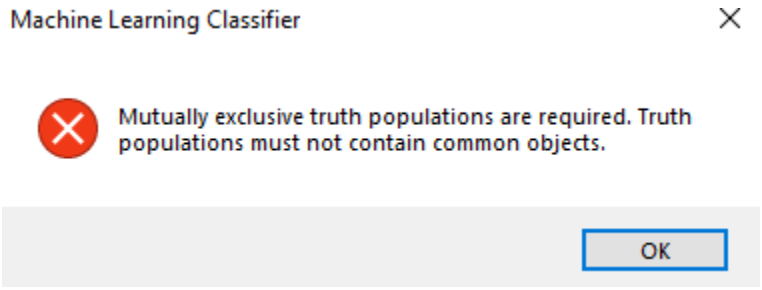
5. In Step 2 of the Machine Learning Classifier, select the base population from the Base population drop down menu.

   NOTE: The All population will be the only option if no previous analysis template has been applied. If the user has applied a template where gating has been performed, other base populations can be selected (for example, single cells).

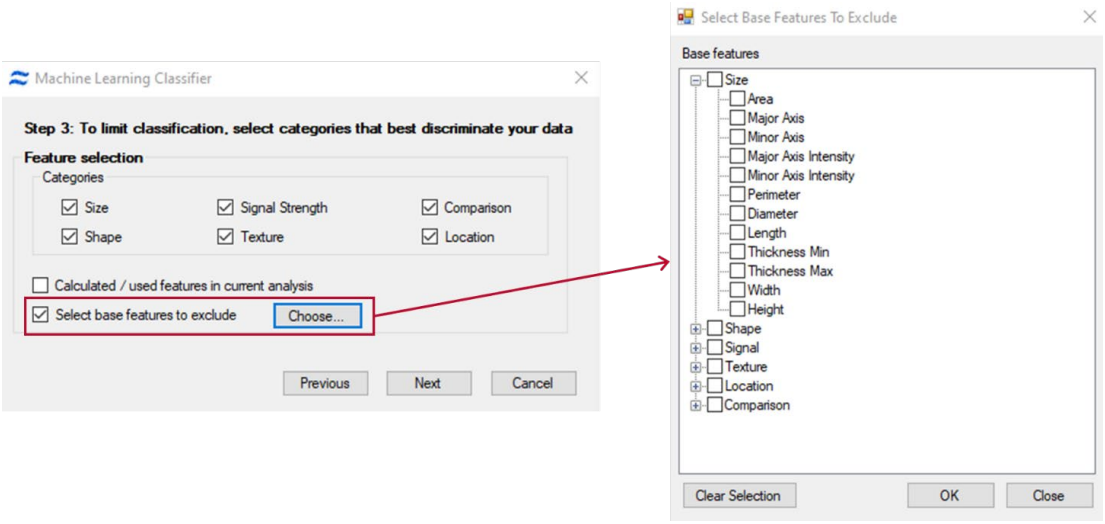
6. Select the truth populations of interest to differentiate between. If populations of interest have already been tagged, select at least two populations by holding the Ctrl key and clicking on the populations. If populations of interest have not been tagged, use the tagging tool ( ) in the lower left corner to create them.

   NOTE: At least 25 images must be tagged for the Machine Learning (ML) module to run. In most cases, larger numbers of images provided to the ML module will generate more robust classifier features.

   NOTE: Images in all selected populations must be mutually exclusive (one image cannot belong to two populations). The ML module will verify this before continuing and will give the following error if it detects images common to both populations.

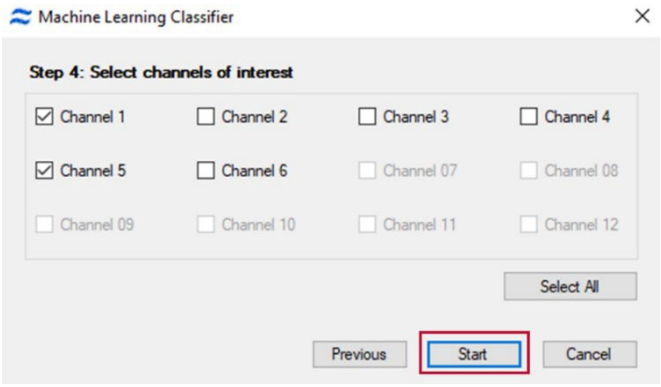


7. In Step 3 of the Machine Learning Classifier, select categories that will best differentiate between the populations of interest. Select all feature categories to create more general classifier features, or select a few categories to create more specific classifier features.
8. (Optional) If you do not want the ML module to compute any additional masks and features, select the checkbox next to Calculated / used features in current analysis.
9. (Optional) To exclude specific features, select the Select base features to exclude checkbox, then click Choose. The Select Base Features to Exclude window appears. Select any features you would like to exclude, then click OK to return to the Machine Learning Classifier window.



10. In Step 4 of the Machine Learning Classifier, select the channels of interest using the checkbox next to the channel name.

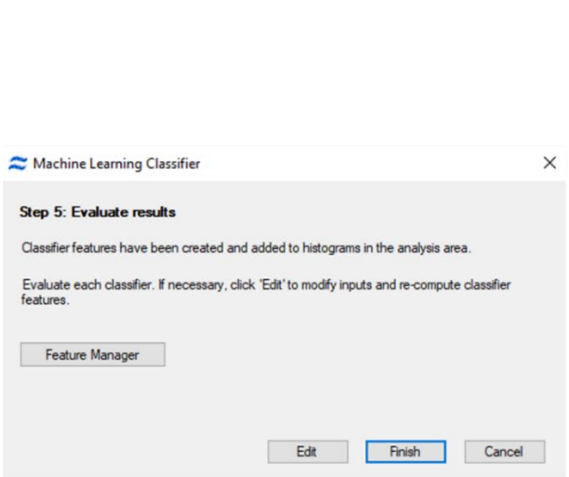
11. Click Start to begin classifier feature calculations.

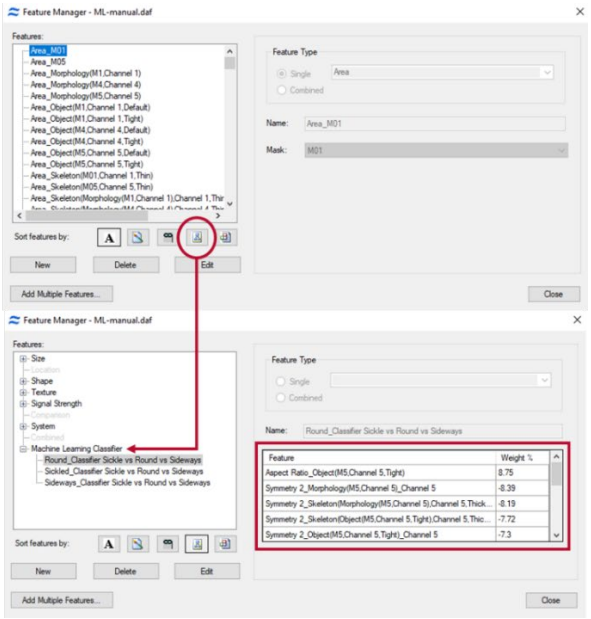


12. After classifier feature calculations are complete, Step 5 of the Machine Learning Classifier appears. Choose one of the following options:

- To save your work and exit the Machine Learning Classifier, click Finish.
- To return to Step 2 of the Machine Learning Classifier and edit the settings specified in steps 2 through 4, click Edit.
- To list and edit classifier features, click the Feature Manager button. The Feature Manager window appears, alphabetically displaying all the features. Click the Feature Category button to locate classifier features that have been created by the ML module. Highlight an individual classifier feature to display a list of all base features used in its creation and the weight of each feature. Click Close to return to Step 5 of the Machine Learning Classifier.

  NOTE: All features in IDEAS® software including classifier features, can be viewed in the Feature Manager at any time by navigating to the Analysis tab in IDEAS® software and selecting Features.





Advanced Analysis

Open Data Files

View File Name Extensions

When loading a file, the IDEAS® software uses the file name extension to determine the file type. It will be easier for you to distinguish raw image files, compensated image files, and data analysis files from each other if Windows Explorer does not hide the extensions.

1. In Windows® Explorer, go to Tools > Folder Options.
2. Click the View tab, and make sure that the Hide extensions for known file types check box is not selected.
3. Click OK.

Use the File menu to open, save, and close image and analysis files, and to quit the IDEAS® software. Alternatively, open a data file by dragging and dropping it into an open IDEAS® software window. Multiple data files can be open in one instance of the IDEAS® software application.

Open a .rif file

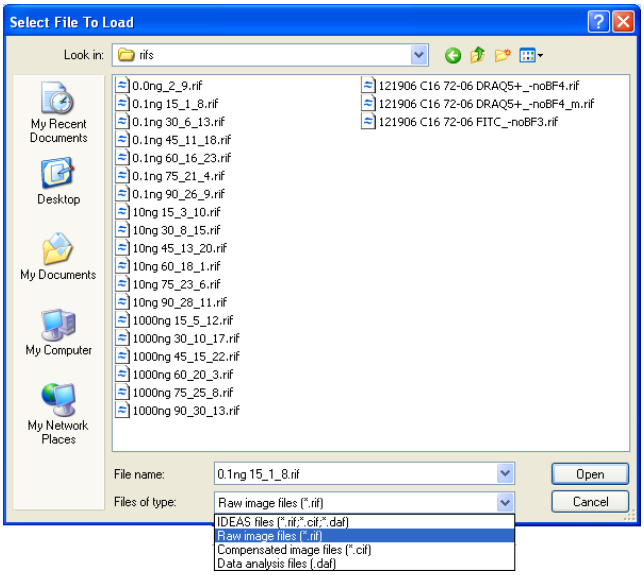
A .rif file is opened when there is new data and the IDEAS® software needs to apply corrections. When opening a .rif file, the IDEAS® software corrects each image for the spatial alignment between channels, camera background normalization, flow speed, and bright field gain normalization. If you want fluorescence compensation to correct for spectral overlap, you must create or choose a compensation matrix at this time by using the control files that were collected for a particular experiment. If a FlowSight® system data file was acquired with a compensation matrix, that matrix will be used by default. The application performs the corrections by using calibration information that was saved to the .rif file during acquisition.

NOTE: Alternatively, use the Open File Wizard to open a .rif file.

1. From the File menu, choose Open , or drag the .rif file into the IDEAS® software window.

2. Select the .rif file in the Select File To Load window.

   NOTE: While browsing for the file to open you can limit the type of file shown in the window to .rif files.

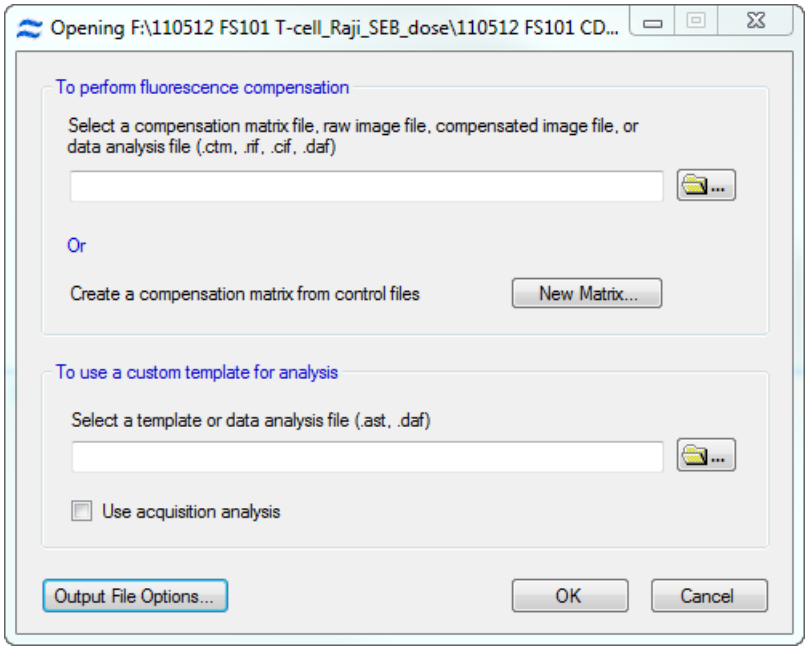


3. To perform fluorescence compensation, either open a file containing a compensation matrix or create a new compensation matrix.

   a. To open a file containing a compensation matrix, click the folder icon next to Select a compensation matrix. File types that may contain a compensation matrix are .ctm, .rif, .cif, and .daf.

      NOTE: If you leave this field blank, the default compensation matrix will be used, which does not contain any fluorescence corrections. Do not use the default compensation matrix unless you do not want to compensate your data.

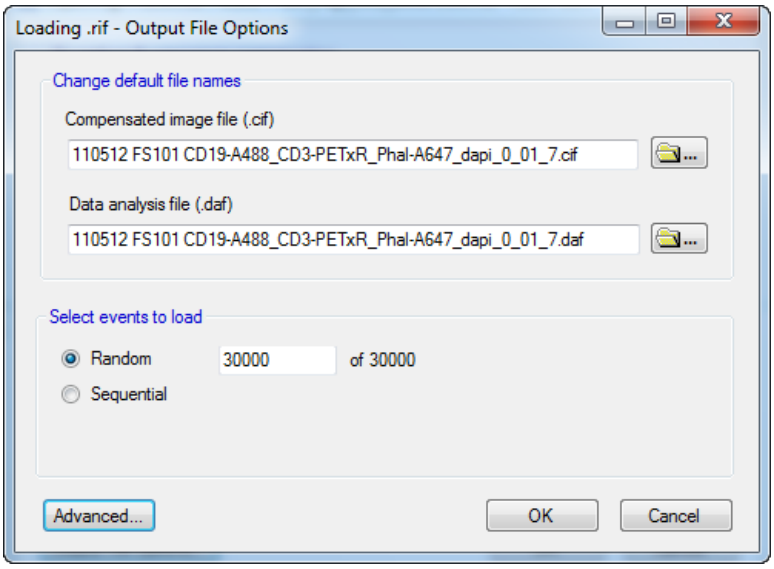
   b. To create a new compensation matrix, click New Matrix.



4. In the Select a template or data analysis file (.ast, .daf) field, select a template file to load by clicking the folder icon and browsing for the file.

   NOTE: If this field is left blank, the Default template with the basic features, masks, and settings will be used. FlowSight® system basic files use the acquisition template as the default.

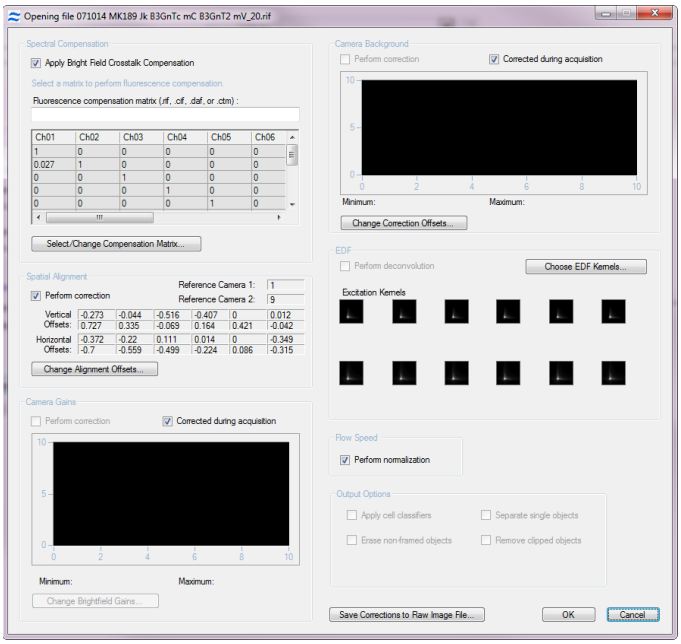
5. (Optional) Select the Use acquisition analysis checkbox if you wish to use the acquisition analysis performed on the instrument, which includes feature definitions, regions, populations, and analysis area plots.
6. (Optional) To load a subset of objects or change the .cif and .daf file names to be created, click the Output File Options... button. The Output File Options window appears.



   a. (Optional) Change the name of the .cif and .daf files to be created using the Compensated image file (.cif) and Data analysis file (.daf) fields. Cytek recommends keeping the default names.
   b. (Optional) Choose to load a subset of objects using the Select events to load field. Select Random or Sequential to have IDEAS® software choose objects randomly or sequentially.

      NOTE: Select a smaller number than the maximum if you have a large number of objects to load. This helps save time for creating a template file.

a. (Optional) To view the corrections that will be applied to the .rif file, click the Advanced... button. The Opening file window appears. Make any changes to the corrections that you need, and then click OK



b. Click OK when you are finished with the Output File Options window.

7. Click OK. The application creates the .cif and .daf files, and the .daf file is loaded into the Image Analysis area.

NOTE: Most often, the defaults will be adequate. For some older data files, you may need to provide control files for certain settings. For assistance, call Cytek Technical Support.

Open a .cif file

A .cif file is generated when corrections are applied to a .rif file. When opening a .cif file, the IDEAS® software calculates feature values and creates a .daf file to display images and graphs.

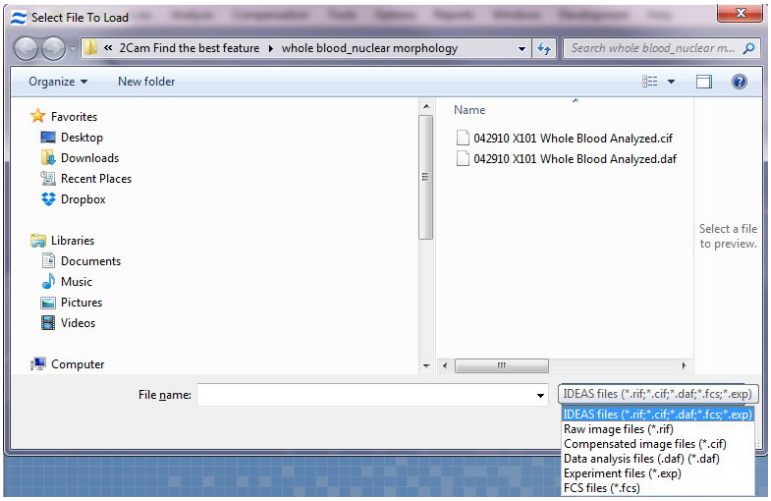
When opening a .cif file, an analysis template is selected. The template provides the initial characteristics of the analysis. Opening the .cif file causes the IDEAS® software to calculate feature values and to use populations, graphs, and image viewing settings to display the cells as defined by the template.

NOTE: Alternatively, use the Open File Wizard to open a .cif file.

1. From the File menu, choose Open , or drag the .cif file into the IDEAS® software window.

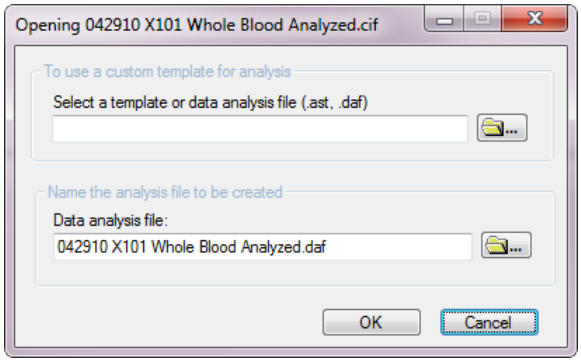
a. Select the .cif file that you want in the Select File To Load window.

NOTE: While browsing for the file to open, you can limit the type of file shown in the window to .cif.



2. Select a template or a data analysis file by clicking the folder icon or manually entering the file path in the field.

   NOTE: If this field is left blank, the IDEAS® software will use a default template. However, it is useful to create and save your own templates for specific experimental procedures.



3. Change the Data analysis file name, if necessary. The default name matches the name of the .cif.
4. Click OK. During the opening of a .cif file, the IDEAS® software calculates the values of the features that are defined in the template you selected. The progress is shown by a progress bar. After the application has successfully opened the .cif file, the .daf file is saved.

Open a .daf file

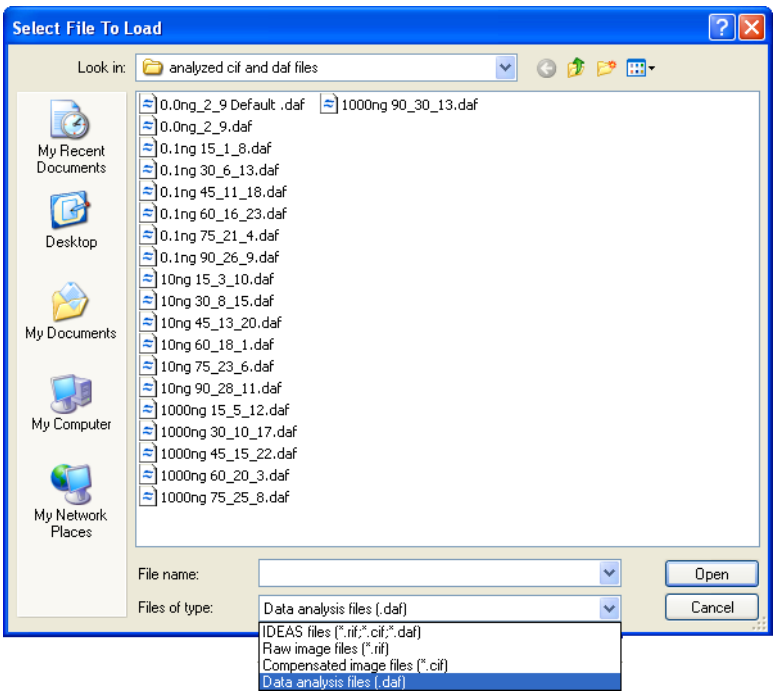
A .daf file contains the calculated feature values so that they will not need to be recalculated.

To open a .daf file, the IDEAS® software requires the .cif file to reside in the same directory. The .daf file does not contain any image data; think of the .cif file as the database that contains the imagery. Because all of the feature values have been saved in it, the .daf file should open quickly.

NOTE: Alternatively, use the Open File Wizard to open a .daf file.

1. From the File menu, choose Open or drag the file into the IDEAS® software window.

2. Select the .daf file that you want in the Select File To Load window.



NOTE: While browsing for the file to open, you can limit the type of file shown in the window to .daf. The progress is shown by a progress bar. The state of the IDEAS® software is restored to what it was when the .daf file was saved.

Save Data Files

Data files are saved at several stages of analysis. Raw image files (.rif) are saved during data acquisition by merging multiple .rif files or by creating new files from populations. Compensated image files (.cif) and data analysis files (.daf) are saved when opening .rif files, merging multiple .cif files, or when running a batch analysis. The IDEAS® software also saves other types of files that are used for data correction and presentation. Template files (.ast) save the structure of an analysis and compensation matrix files (.ctm) save the compensation matrices. Application Defaults are set that direct the files into specific folders and can be viewed or changed by the user.

Save a Data Analysis File (.daf)

A .daf file contains a snapshot of an analysis. Saving the analysis as a .daf file allows you to recall that analysis simply by opening the file. When you quit the IDEAS® software, you are always prompted to save changes to a .daf file. You can also save changes from the File menu. Remember that the .daf file does not contain image information, so opening the
.daf file requires the related .cif file to reside in the same directory.

1. Select File > Save as Data Analysis File (.daf).

   NOTE: To reduce the size of a data analysis file, save the .daf with only the features that are used for analysis of statistics or graphs. Click File > Save as Data Analysis File - Used Features Only and continue with the procedure.

2. Enter a file name.

   NOTE: The default directory is the one where the .cif file was saved.

3. If you select an existing file name, a warning appears that asks you to verify the overwriting of the existing file.

4. Click Save. The data is now ready for analysis. You can create graphs, view imagery, and display feature values and statistics. After you have defined an analytical procedure in the .daf file, you can save the file as a template, which allows you to use the procedure for analyzing other files.

Save a Compensated Image File (.cif)

The IDEAS® software creates and saves a .cif file when a .rif file is opened. By default, the application names the .cif file with the same name that the .rif file has, replacing the .rif extension with .cif. The application also places the .cif file in the same directory as the .rif file. The .cif file will be larger than the .rif file because the .cif file contains masking information as well as corrected and/or compensated images.

Save a Template (.ast)

Saving an analysis as a template allows you to apply the structure of the analysis to other .cif files. Save a template file after saving a .daf file. A template includes all graph definitions, Image Gallery settings, feature definitions, and statistics settings. No data is saved in a template. Therefore, selected images and populations that are dependent on specific objects, such as tagged populations, are not saved.

1. Click File > Save As Template File (.ast). The Save File dialog box appears.
2. Enter the name of the file to save.
3. Click Save. If you select an existing file name, a warning appears that asks you to verify the overwriting of the existing file.

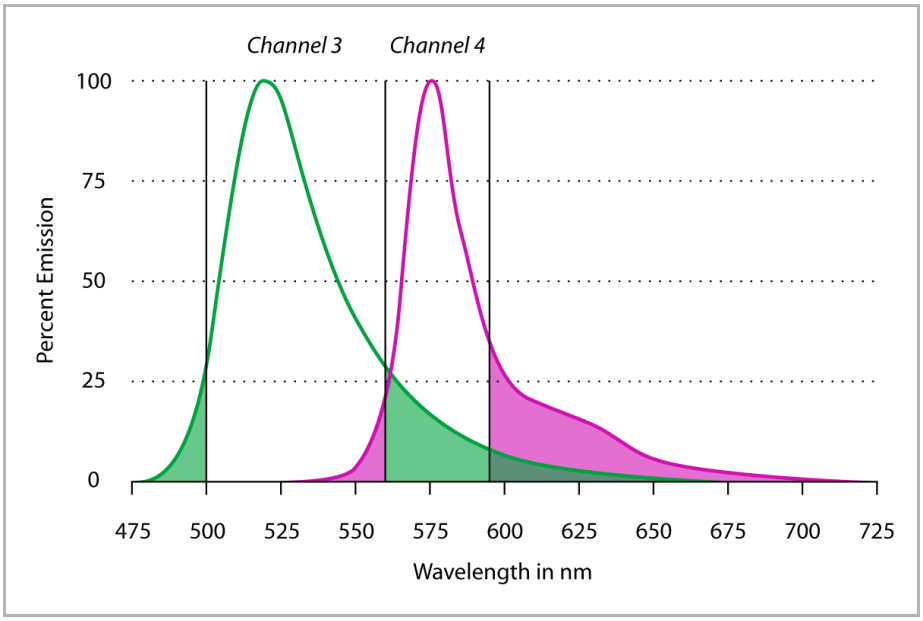
NOTE: You can change the default template directory by clicking Analysis > Application Defaults.

Overview of Compensation

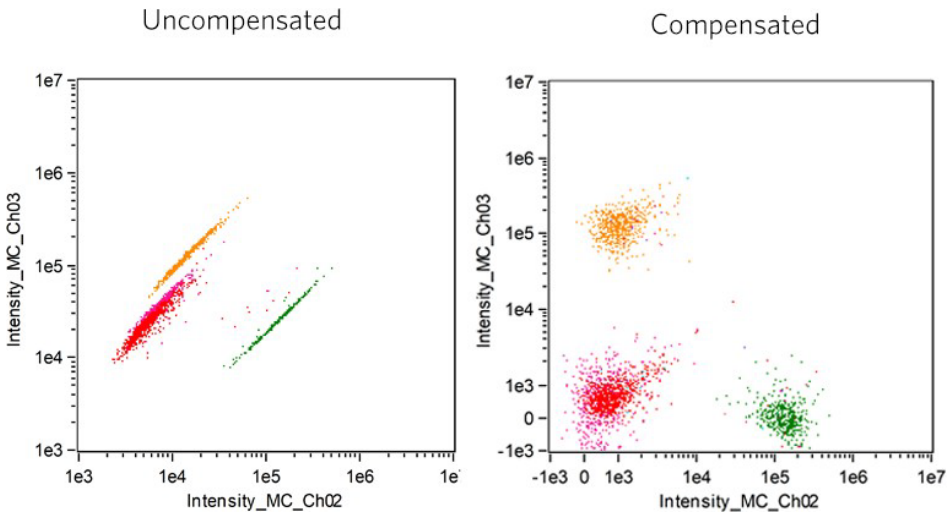
Spectral compensation corrects imagery for fluorescence that leaks into nearby channels so that you may accurately depict the correct amount of fluorescence in each cell image.

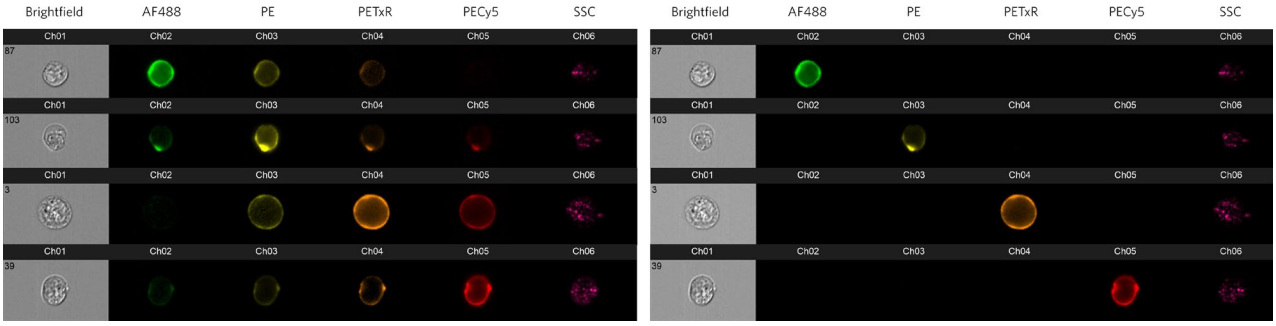
For example, the light from one fluorochrome may appear primarily in channel 3, but some of the light from this fluorochrome may appear in channel 4 as well, because the emission spectrum of the probe extends beyond the channel 3 spectral bandwidth. The light from a second fluorochrome may appear primarily in channel 4 but, unless you subtract the light emitted by the first fluorochrome into channel 4, you cannot generate images that accurately represent the distribution of the second fluorochrome.

Emission Spectra for two fluorochromes:



Below is an example of cells stained with four fluorochromes independently and run together as one sample. Intensity scatter plots for two fluors and images for the four fluors are shown uncompensated and compensated. Image compensation is performed on a pixel by pixel basis.





Uncompensated Images

Compensated Images

The IDEAS® software builds a matrix of compensation values by using one or more control files. A control file contains cells stained with one fluorochrome. Because it is critical that matrix values be calculated from intensities derived from a sole source of light, control files are collected without brightfield illumination, or scatter. The brightfield compensation is performed automatically. The process of creating the compensation matrix is described in the next section.

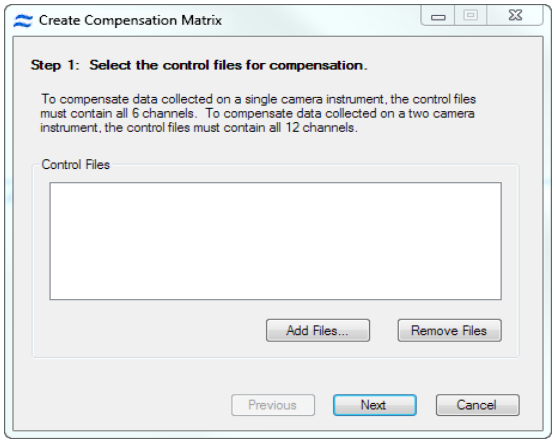
Create a New Compensation Matrix File

The compensation matrix is a table of coefficients. The IDEAS® software uses this table to place the detected light that is displayed in each image into the proper channels, on a pixel by pixel basis. The coefficients are normalized to 1. Each coefficient represents the normalized amount of the leakage of the fluorochrome into the other channels. Compensation is performed during the creation of a .cif file from a .rif file.

The default matrix, which is used if no compensation matrix is chosen, is the identity matrix, shown below.

	Ch01	Ch02	Ch03	Ch04	Ch05	Ch06	Ch07	Ch08	Ch09	Ch10	Ch11	Ch12
Ch01	1	0	0	0	0	0	0	0	0	0	0	0
Ch02	0	1	0	0	0	0	0	0	0	0	0	0
Ch03	0	0	1	0	0	0	0	0	0	0	0	0
Ch04	0	0	0	1	0	0	0	0	0	0	0	0
Ch05	0	0	0	0	1	0	0	0	0	0	0	0
Ch06	0	0	0	0	0	1	0	0	0	0	0	0
Ch07	0	0	0	0	0	0	1	0	0	0	0	0
Ch08	0	0	0	0	0	0	0	1	0	0	0	0
Ch09	0	0	0	0	0	0	0	0	1	0	0	0
Ch10	0	0	0	0	0	0	0	0	0	1	0	0
Ch11	0	0	0	0	0	0	0	0	0	0	1	0
Ch12	0	0	0	0	0	0	0	0	0	0	0	1

1. Start the Compensation Wizard in one of two ways:
   - Click the New Matrix button when opening a .rif file
   - OR select Compensation>Create New Matrix. The compensation wizard opens to Step 1:

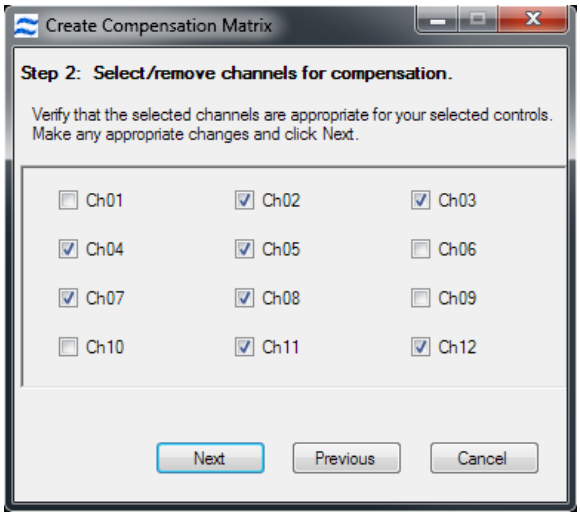


2. Add compensation control files by clicking Add Files and browsing for the control files for the experiment. The files will have the suffix no-BF. Hold down the control key to select multiple files at once.

   NOTE: It is important to collect only single color positive cells or beads to use for compensation files. Collect 500- 1000 positive events for each file. Collecting more events or including negative and very dim cells is not recommended since the compensation calculations expect only positive events.

3. When all of the control files for the experiment have been added to the list, click Next. The control file(s) are merged and loaded.

4. Verify the channels for each control in the experiment by checking the channel boxes.



The following tables are provided as a guide for each instrument optical configuration .

Table 8. First generation ImageStream® system (IS100)

Ch 1	Ch 2	Ch3	Ch 4	Ch 5	Ch6
470-500nm	400-470nm	500-560nm	560-595nm	595-660nm	660-735nm
Scatter	DAPI	FITC	PE	7-AAD	PE-Cy5

Table 9. ImageStream® X system: One Camera

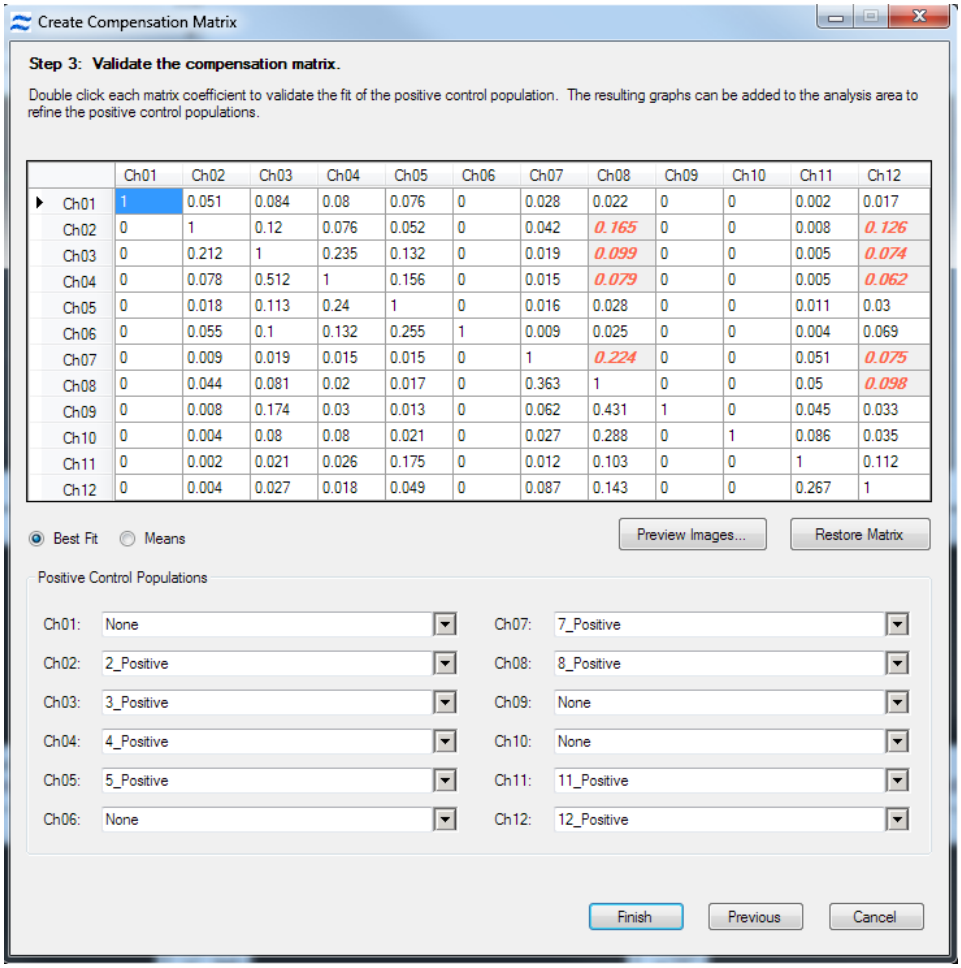
Ch 1	Ch 2	Ch3	Ch 4	Ch 5	Ch6
435-505nm	505-560nm	560-595nm	595-642nm	642-745nm	745-785nm
DAPI	FITC	PE	PE-TexasRed	AF647	APC-Cy7

Table 10. ImageStream® X system: Two Camera

Ch 1	Ch 2	Ch3	Ch 4	Ch 5	Ch6
435-480nm	480-560nm	560-595nm	595-642nm	642-745nm	745-785nm
BF	FITC	PE	PE-TexasRed	PE-Cy5	PE-Cy7
Ch 7	**Ch 8**	**Ch9**	**Ch 10**	**Ch 11**	**Ch 12**
435-505nm	505-570nm	570-595nm	595-642nm	642-745nm	745-785nm
DAPI	Pacific Orange	BF	Texas Red	AF647	APC-Cy7

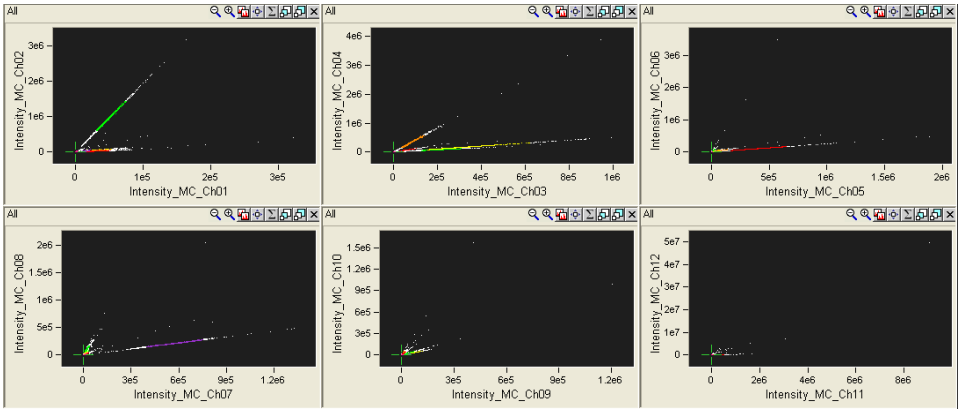
5. Background and spatial offset corrections are performed, the imagery is displayed, bivariate plots of adjacent channels Intensity are added to the analysis area and the compensation matrix values are computed and displayed in a table.

Example:



	Ch01	Ch02	Ch03	Ch04	Ch05	Ch06	Ch07	Ch08	Ch09	Ch10	Ch11	Ch12
Ch01	1	0.051	0.084	0.08	0.076	0	0.028	0.022	0	0	0.002	0.017
Ch02	0	1	0.12	0.076	0.052	0	0.042	*0.165*	0	0	0.008	*0.126*
Ch03	0	0.212	1	0.235	0.132	0	0.019	*0.099*	0	0	0.005	*0.074*
Ch04	0	0.078	0.512	1	0.156	0	0.015	*0.079*	0	0	0.005	*0.062*
Ch05	0	0.018	0.113	0.24	1	0	0.016	0.028	0	0	0.011	0.03
Ch06	0	0.055	0.1	0.132	0.255	1	0.009	0.025	0	0	0.004	0.069
Ch07	0	0.009	0.019	0.015	0.015	0	1	*0.224*	0	0	0.051	*0.075*
Ch08	0	0.044	0.081	0.02	0.017	0	0.363	1	0	0	0.05	*0.098*
Ch09	0	0.008	0.174	0.03	0.013	0	0.062	0.431	1	0	0.045	0.033
Ch10	0	0.004	0.08	0.08	0.021	0	0.027	0.288	0	1	0.086	0.035
Ch11	0	0.002	0.021	0.026	0.175	0	0.012	0.103	0	0	1	0.112
Ch12	0	0.004	0.027	0.018	0.049	0	0.087	0.143	0	0	0.267	1

The Positive Control Populations are shown in the graphs below.



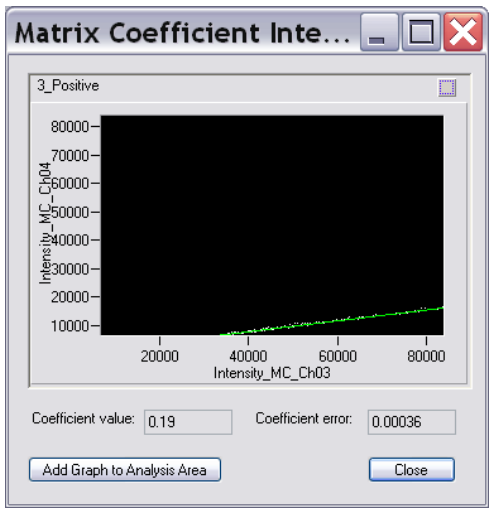
6. Choose one of two methods for calculating the coefficients. The compensation files are merged, and each object is scrutinized for its peak channel emission. For each fluorochrome, the application automatically identifies a positive control population, excluding the brightest and dimmest objects, and assigns it to the proper channel.

   - Use the Best Fit method for objects where intensities vary, such as cells. This method is determined by calculating best fit linear regression for each dye into each channel.
   - Use the Means method for objects that have only slight variations in intensity and therefore do not produce a linear correlation, such as beads. The means method uses the ratio of the intensity of the spillover channel to that of the positive control channel.

7. Inspect the matrix values in the table of coefficients.

   NOTE: Coefficients should always be less than 1, and decrease from the assigned channel. In other words, leakage should be greater in the channel nearest to the assigned channel. Fluorescence always extends in the long wavelength direction from the exciting light.

8. Verify that no coefficients are larger than 1. Verify that, in a column corresponding to a fluorochrome, the coefficients decrease from the assigned channel.
9. Verify that the coefficient is greater in the channel below the 1 in the table than the value above the 1 in the table.
10. Verify that these coefficients also decrease in subsequent channels below the 1.
11. Verify that there are no changes from the identity matrix in the columns where there are no fluorochromes, including the scatter and brightfield channels. If necessary, the column can be set to the identity values by double clicking on the heading.
12. Check for coefficients in red text. These coefficients have errors of 1% or more to the best fit line. This means you should inspect the graphs for these coefficients and if possible reduce the error by eliminating outliers or choosing the population manually. The purpose of making these coefficients red is to point out the coefficients that may need attention, but it may be that the error will remain above 1% and remain red.
13. Inspect the coefficients in the matrix by double clicking on the coefficient. A graph representing the coefficient appears. The population plotted in the graph is the positive control population of the column of the coefficient. The X Axis represents the intensity in the assigned channel of the fluorochrome. The Y Axis represents the intensity in the channel of leakage. The coefficient value and error are also displayed.



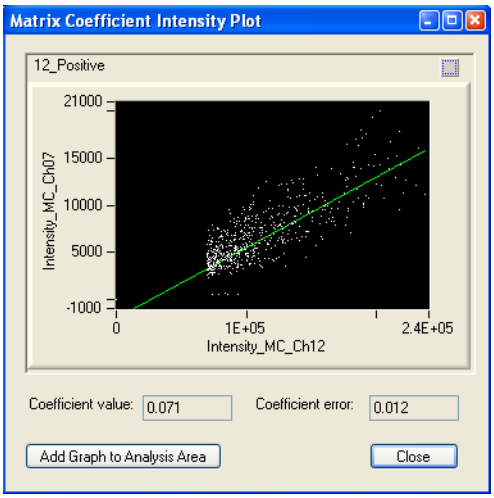
You can use the automatically generated control populations as they are, or you can refine them and create different populations by using the region tools. By default, the populations are named 3_Positive, 5_Positive, and so on. You can view the populations in the Image Gallery. Some populations may be empty.

- To choose a different population, use the arrow and select the population from the list. The hierarchical relationship is shown in the population list. Assign populations only to the channels that correspond to the fluorochromes used in the experiment.
- If needed, you can create new scatter plots by using the Analysis Area toolbar. For example, a 4_Intensity versus 5_Intensity plot may be useful.
- If you want to clear a column, double click on the channel heading.
- The slope of the line on the plot is the coefficient in the matrix.

Optional: Remove Objects from the Population

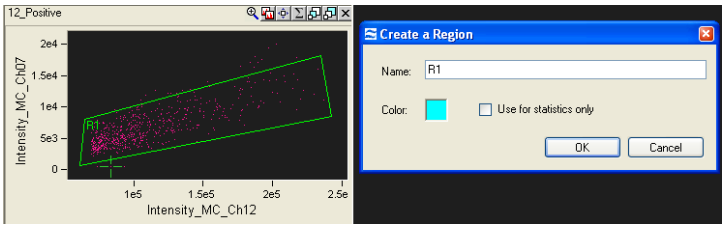
If objects in the population exist that are outliers, they should most likely be removed from the positive population within the compensation matrix by the following these steps:

1. Within the compensation wizard, double click the coefficient to display the intensity plot.

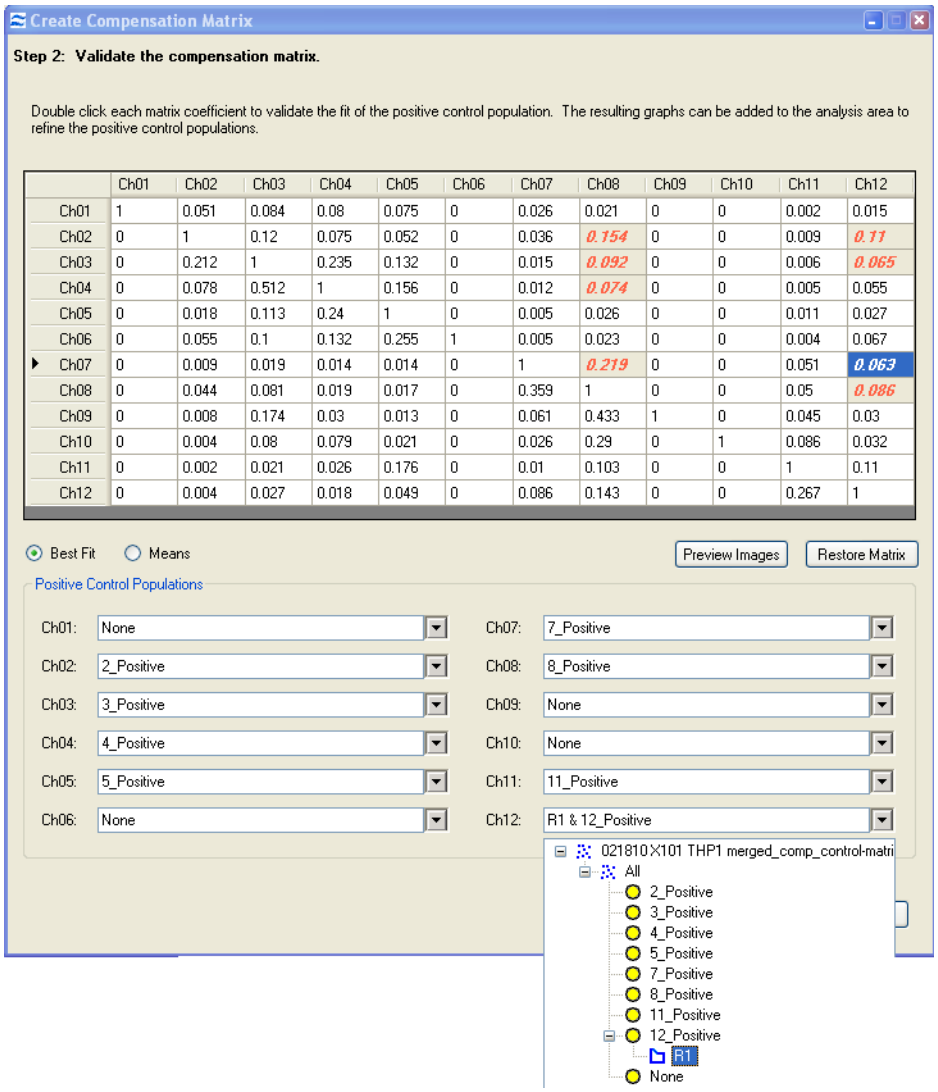


2. If you notice outliers, click Add Graph to Analysis Area. The plot populates in the Analysis Area.
3. Return to the Analysis Area and use the region tools to draw a new region on the plot that defines a new positive control population, excluding the outliers.
   a. Create a new region to exclude outliers.
   b. Click the Resize and Zoom buttons on the graph toolbar to more clearly see the population of interest. Using one of the region buttons on the toolbar, draw a region that contains only the cells you want to use for determining compensation. You can click a point on the graph and view the image to help you decide where the region boundaries should be.

In the example below, the Polygon Region tool was selected to draw a border around a selection of cells. Clicking within the graph anchors the line and double clicking completes the region.



4. Assign the new population to the appropriate channel by using the Positive Control Populations list for that channel.



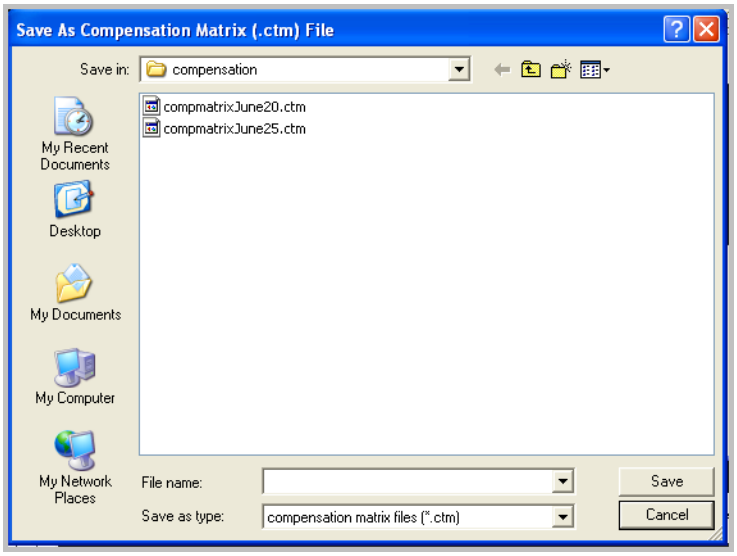
	Ch01	Ch02	Ch03	Ch04	Ch05	Ch06	Ch07	Ch08	Ch09	Ch10	Ch11	Ch12
Ch01	1	0.051	0.084	0.08	0.075	0	0.026	0.021	0	0	0.002	0.015
Ch02	0	1	0.12	0.075	0.052	0	0.036	*0.154*	0	0	0.009	*0.11*
Ch03	0	0.212	1	0.235	0.132	0	0.015	*0.092*	0	0	0.006	*0.065*
Ch04	0	0.078	0.512	1	0.156	0	0.012	*0.074*	0	0	0.005	0.055
Ch05	0	0.018	0.113	0.24	1	0	0.005	0.026	0	0	0.011	0.027
Ch06	0	0.055	0.1	0.132	0.255	1	0.005	0.023	0	0	0.004	0.067
Ch07	0	0.009	0.019	0.014	0.014	0	1	*0.219*	0	0	0.051	*0.063*
Ch08	0	0.044	0.081	0.019	0.017	0	0.359	1	0	0	0.05	*0.086*
Ch09	0	0.008	0.174	0.03	0.013	0	0.061	0.433	1	0	0.045	0.03
Ch10	0	0.004	0.08	0.079	0.021	0	0.026	0.29	0	1	0.086	0.032
Ch11	0	0.002	0.021	0.026	0.176	0	0.01	0.103	0	0	1	0.11
Ch12	0	0.004	0.027	0.018	0.049	0	0.086	0.143	0	0	0.267	1

5. The coefficient value is automatically recalculated when a new population is selected.
6. Repeat these steps as required to redefine the coefficients.

7. Click Preview Images to view individual objects with corrections applied. Double click on an image to add it to the preview window.

   NOTE: The corrections are only applied to on camera channels. For example, if the object is brightest in channel 3 on the first camera, only channels 1-6 are shown corrected for that object.

8. When the matrix appears satisfactory, click Finish.
9. Enter a name for the compensation matrix file (.ctm) and click Save.



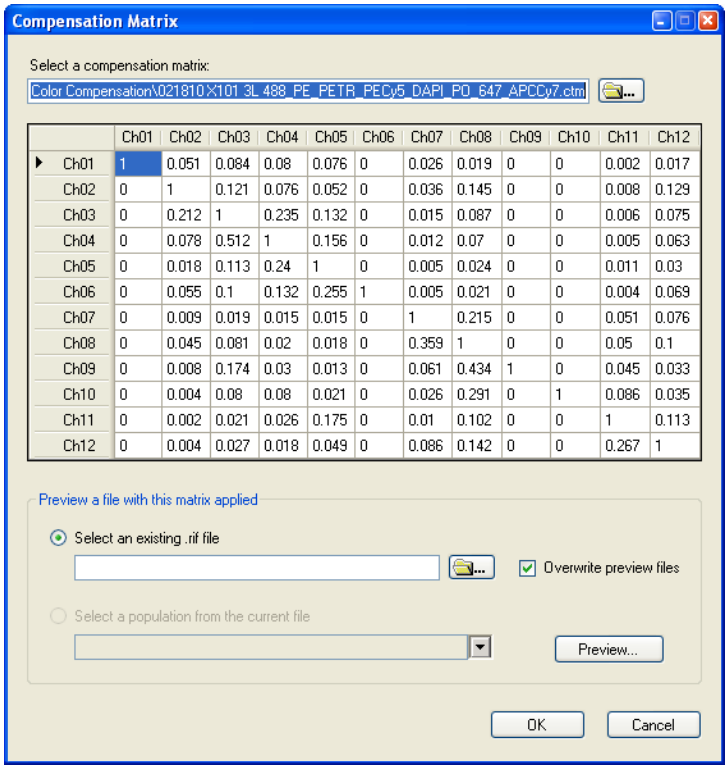
NOTE: The matrix is saved as a compensation matrix file (.ctm file). This file contains the compensation values and can be opened later for editing. To provide the values for fluorescence compensation, you select a .ctm file when opening a .rif file.

Compensation Matrix Files

Preview and Edit a Compensation Matrix

A compensation matrix can be applied to a population or .rif file in a preview mode for editing a matrix.

1. Select View/Edit Compensation Matrix from the Compensation menu to view, edit or preview the matrix on image data. Select the data file by clicking on the folder icon, then clicking Open. The matrix values are displayed in a table and may be edited.



	Ch01	Ch02	Ch03	Ch04	Ch05	Ch06	Ch07	Ch08	Ch09	Ch10	Ch11	Ch12
Ch01	1	0.051	0.084	0.08	0.076	0	0.026	0.019	0	0	0.002	0.017
Ch02	0	1	0.121	0.076	0.052	0	0.036	0.145	0	0	0.008	0.129
Ch03	0	0.212	1	0.235	0.132	0	0.015	0.087	0	0	0.006	0.075
Ch04	0	0.078	0.512	1	0.156	0	0.012	0.07	0	0	0.005	0.063
Ch05	0	0.018	0.113	0.24	1	0	0.005	0.024	0	0	0.011	0.03
Ch06	0	0.055	0.1	0.132	0.255	1	0.005	0.021	0	0	0.004	0.069
Ch07	0	0.009	0.019	0.015	0.015	0	1	0.215	0	0	0.051	0.076
Ch08	0	0.045	0.081	0.02	0.018	0	0.359	1	0	0	0.05	0.1
Ch09	0	0.008	0.174	0.03	0.013	0	0.061	0.434	1	0	0.045	0.033
Ch10	0	0.004	0.08	0.08	0.021	0	0.026	0.291	0	1	0.086	0.035
Ch11	0	0.002	0.021	0.026	0.175	0	0.01	0.102	0	0	1	0.113
Ch12	0	0.004	0.027	0.018	0.049	0	0.086	0.142	0	0	0.267	1

2. To preview the matrix on image data, browse for a file or select a population from the current file to preview and click Preview.

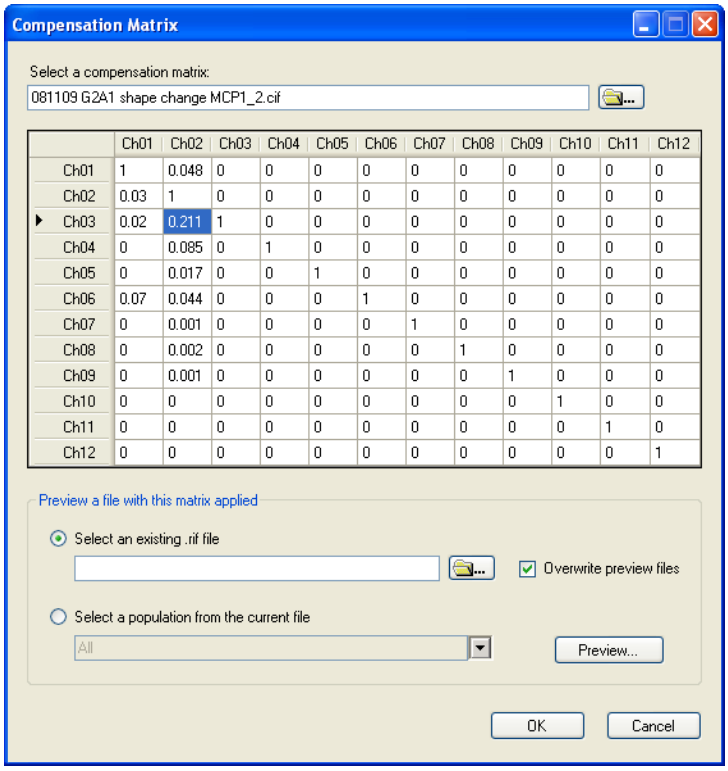
Troubleshoot Compensation

Sometimes an applied matrix produces poorly compensated data. This can happen for a number of reasons: 1) miscalculation of the compensation matrix by inclusion of inappropriate events (such as doublets, saturated pixel events, or artifacts), 2) controls used for matrix calculation differ significantly from the experimental samples (different cell type, different probe), or 3) cells exhibit substantial autofluorescence. This protocol describes a method for manually adjusting and validating a compensation matrix for difficult samples.

To troubleshoot and repair a compensation matrix:

1. Create a population of cells that are mis compensated using the tagging tool. Choose single cells that are exhibiting crosstalk. Choose a range of intensities from negative to bright but not saturated, preferably single color. If single color cells are not available, choose cells with a distinct staining pattern in the peak channel.
2. Create Intensity scatter plots of adjacent channels in order to observe the over or under compensation.

3. Identify the matrix values that need adjusting by inspecting the scatter plots and images. Each column contains the coefficients for the peak channel into the corresponding crosstalk channels (rows). For example the crosstalk of channel 2 (green) into channel 3 is highlighted in the matrix below.



	Ch01	Ch02	Ch03	Ch04	Ch05	Ch06	Ch07	Ch08	Ch09	Ch10	Ch11	Ch12
Ch01	1	0.048	0	0	0	0	0	0	0	0	0	0
Ch02	0.03	1	0	0	0	0	0	0	0	0	0	0
Ch03	0.02	0.211	1	0	0	0	0	0	0	0	0	0
Ch04	0	0.085	0	1	0	0	0	0	0	0	0	0
Ch05	0	0.017	0	0	1	0	0	0	0	0	0	0
Ch06	0.07	0.044	0	0	0	1	0	0	0	0	0	0
Ch07	0	0.001	0	0	0	0	1	0	0	0	0	0
Ch08	0	0.002	0	0	0	0	0	1	0	0	0	0
Ch09	0	0.001	0	0	0	0	0	0	1	0	0	0
Ch10	0	0	0	0	0	0	0	0	0	1	0	0
Ch11	0	0	0	0	0	0	0	0	0	0	1	0
Ch12	0	0	0	0	0	0	0	0	0	0	0	1

Under compensation (crosstalk coefficient is too low):

- Plots: Intensity mean for the single color positive population is higher than the unlabeled population in the crosstalk channel or the intensity in the crosstalk channel trends diagonally upwards.
- Images: the crosstalk channel contains an apparent fluorescent mirror

image. Overcompensation (crosstalk coefficient is too high):

- Plots: Intensity mean for the single color positive population is lower than the unlabeled population in the crosstalk channel or the intensity in the crosstalk channel trends diagonally downwards.
- Images: the crosstalk channel contains dark spots corresponding to the bright spots in the fluorescent channel of interest.

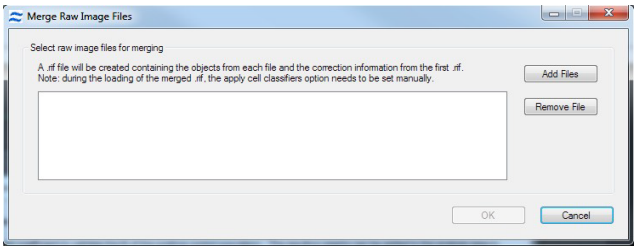
4. In the Compensation menu, choose View/Edit Matrix and manually change the incorrect crosstalk matrix values identified above. Start with changes of ~.1 or ~.05 and use smaller and smaller increments as you refine the matrix.
5. Click Preview and choose the tagged population to view the results of the changed coefficient.
6. Repeat steps 4 and 5 until the matrix is corrected.
7. Click Save, append manual to the matrix name, then click OK.
8. Open the .rif file and use the new matrix to create a new .daf file.

Merge Data Files

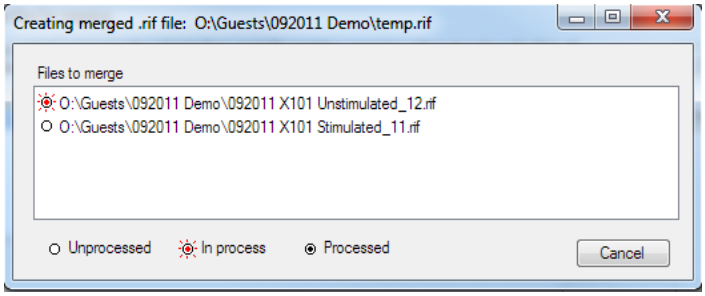
Merge Raw Image Files

You can merge .rif files together for analysis.

1. Click Tools > Merge .rif Files. The Merge Raw Image Files window displays.



2. To select the .rif files to merge, click Add Files.
3. The .rif file names appear in the list.
4. If you want to remove a file from the list, select it and then click Remove File.
5. When the merge list is complete, click OK.
6. The Save Merged Raw Image (.rif) File dialog box appears.
7. Type a unique file name.
8. Click Save. The Creating merged .rif file window appears



9. When the merge is complete, the Merged .rif Created message displays.
10. Click OK.

NOTE: The sample information will contain information for the first file in the merge list.

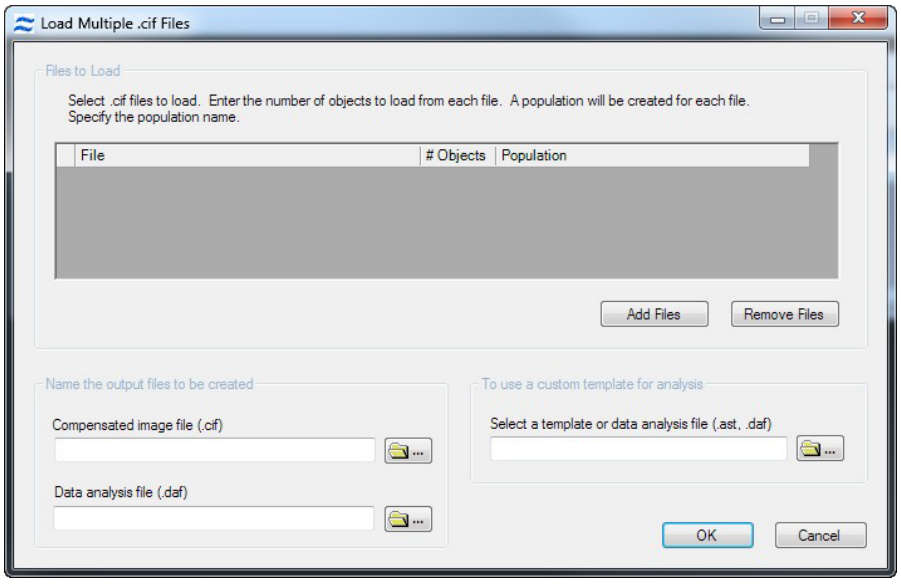
NOTE: For ImageStream®^{X} system non MkII files, the classifier is turned off when a merged file is loaded. To turn the classifier on manually, go to the Advanced panel on the open .rif window when opening an ImageStream®^{X} system non MkII merged file. ImageStream®^{X} MkII system and FlowSight® system do not use classifiers.

NOTE: When opening a merged .rif file, an easy method for gating the separate files is to create a scatter plot of the Object Number versus Time. Each population will fall on a different line.

Merge Compensated Image Files

You can merge .cif files together for analysis.

1. Click Tools > Merge .cif Files. The Load Multiple .cif Files window appears.



2. To select the .cif files to merge, click Add Files. The .cif file names appear in the list.
3. If you want to remove a file from the list, select it and then click Remove File.
4. Type a unique name for the output files.
5. Select a template.
6. Click OK. The merged files are created and the new .daf file is loaded with a population created from each file.

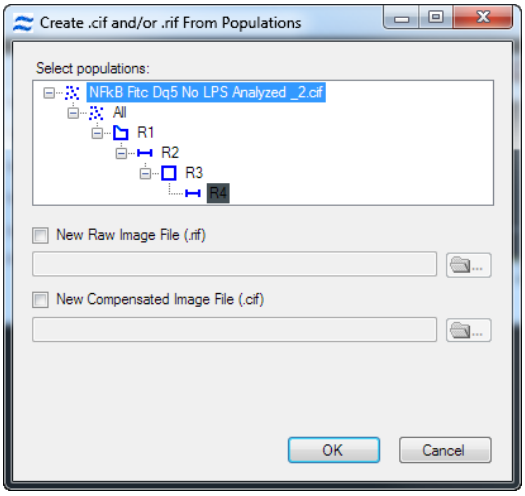
Create New Data Files

Create New Data Files From Populations

To further analyze a population or merge it with other data when working in a .daf, you can save it to a new data file. This is useful if your data file contains a large number of objects that are not pertinent to your experiment. Decreasing the data file size results in better performance by the IDEAS® software.

NOTE: You cannot create a new .cif or .rif when multiple data files are open.

1. Click Tools > Create Data File from Populations. The Create .cif and/or .rif From Populations window displays.



2. In the Select populations list, select the populations that you want to include in the new data file(s). Ctrl click to select multiple populations.
3. To create a .rif file, select the New Raw Image File (.rif) check box, the population name is used as a default. You may enter a new name.
4. To create a .cif file, select the New Compensated Image File (.cif) check box, the population name is used as a default. You may enter a new name.
5. Click OK. If you created a new .cif file, you can choose to load it. When loading the .cif file, the application will prompt you for the template.

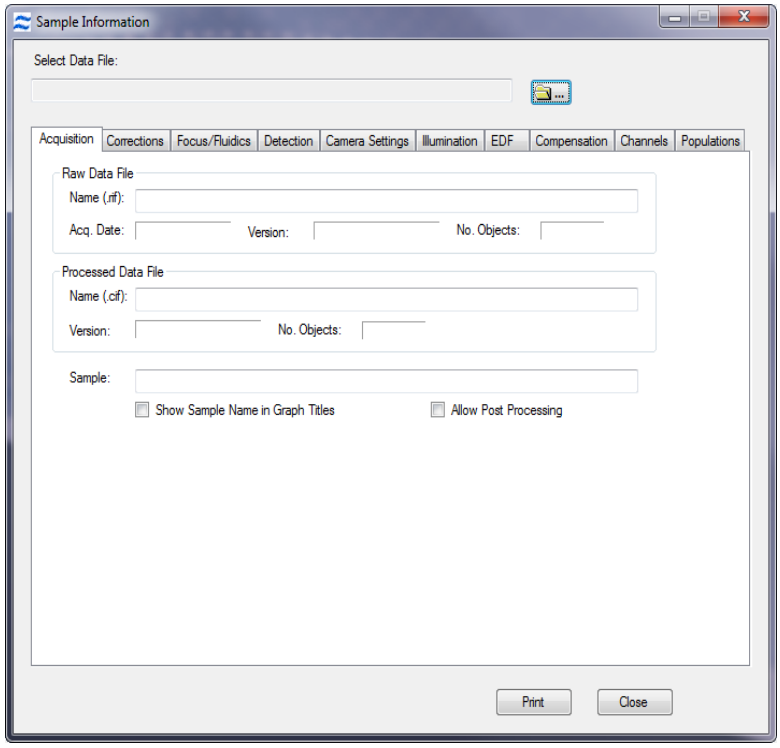
View Sample Information

All of the information associated with an IDEAS® software file such as the collection information, camera settings and corrections—is saved and can be viewed in the Sample Information window.

1. Go to Tools > Sample Information to open the Sample Information window. Information for the open data file will be loaded.
2. Browse for a different data file by clicking on the folder icon. You can open the Sample Information Window for any of the following file types: .rif, .cif, or .daf.
3. Select a tab to see the information for each heading.

4. Click Print from any tab to print a report of all of the sample information.

   NOTE: You may click on the folder and browse for a file to view the sample information for any file without loading the file.

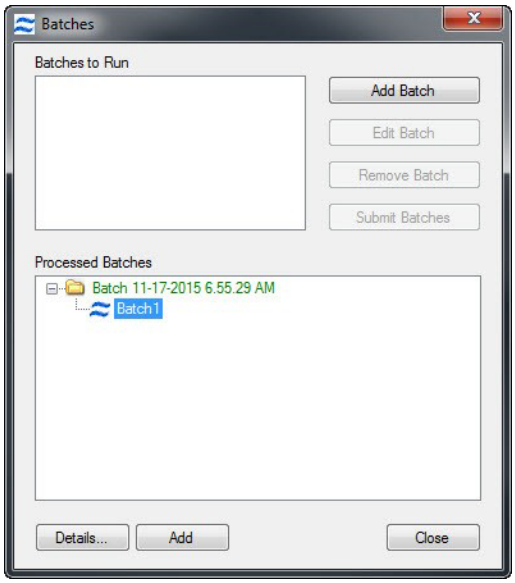


- Acquisition tab: File names, software version numbers, date acquired, number of objects, sample name.
- Corrections tab: Camera background, alignment offsets (from ASSIST).
- Focus/Fluidics tab: Core information and sample volume.
- Detection tab: IS100 and ImageStream®^{X} system only: Cell classifier settings during acquisition.
- Population tab: FlowSight® system and ImageStream®^{X} MkII system only: Lists the populations and number acquired.
- Camera Settings tab: Bin mode, magnification, gain (2=High Gain), and sensitivity settings.
- Illumination tab: Brightfield and laser information.
- EDF tab: View kernels used for deconvolution of EDF imagery.
- Compensation tab: View the compensation matrix.
- Channels tab: Lists channels collected.

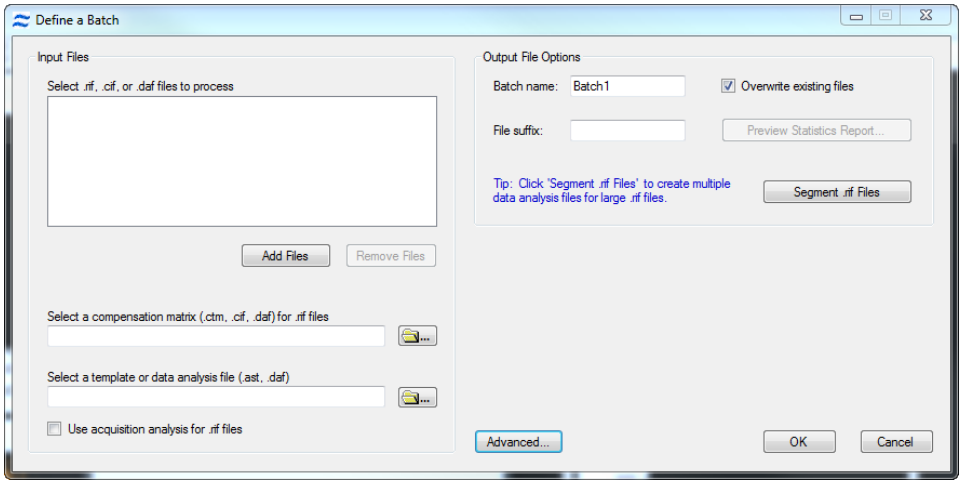
Batch Processing

Batch processing allows you to automatically analyze a group of files with one template when a compensation matrix has already been generated for the experiment.

1. On the Tools menu, select Batch Data Files. The Batches window displays. It lists a record of all batches you have processed.



2. Click Add Batch. The Define a Batch window displays.

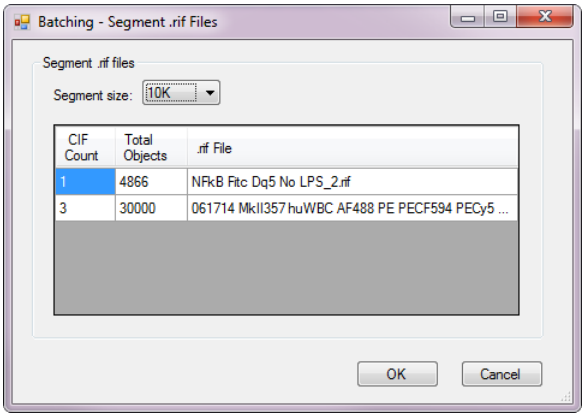


3. To select the files for the batch, click Add Files. Navigate to the files and select by clicking on the file. Select multiple files to add by holding down the Ctrl key while selecting the files.
   a. To remove files from the Files to Process list, click Remove Files.
4. Select a compensation matrix from a file (.ctm, .cif, or .daf). Compensation is applied to .rif files only.
5. Select a template file (.ast or .daf). Leave blank to use the Default template or check 'Use acquisition analysis for .rif files' if this is desired.

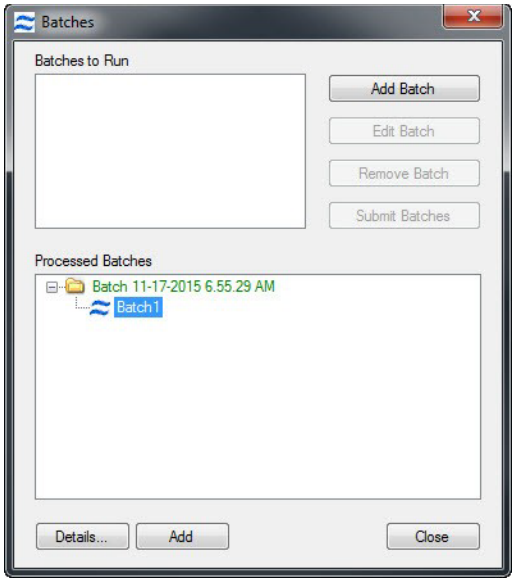
   NOTE: If a template is entered it will overwrite the acquisition analysis.

6. Set the output files options.
7. If the template contains a statistics report definition, the Preview Statistics Report button will display. Click on Preview Statistics Report to review the report or reorder the files as you wish them to be reported. To reorder the files, select a file with a left click, then right click in the desired position and select move here.

8. (Optional) If you have a large rif file, you may want to segment the file into multiple smaller files.
    a. Click on the Segment .rif Files button.
    b. Choose the segment size. The number of files that will be created is updated depending on the segment size chosen and the number of objects in the file as shown in this example. The segmented files will include the segment number and size in their names.
    c. Click OK when done.



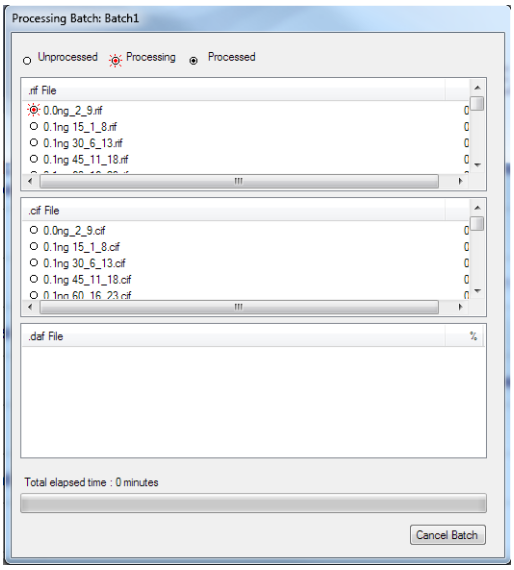
9. Click OK in the batch definition window. The Define a Batch window closes. The batch appears in the Batches window.



The Batches window offers the following options:

- Add Batch: Creates another batch and adds it to the list.
- Remove Batch: Removes a batch from the Batches to Run list.
- Edit Batch: Edits a batch in the Batches to Run list.
- Submit Batches: Starts the batches to run the process.

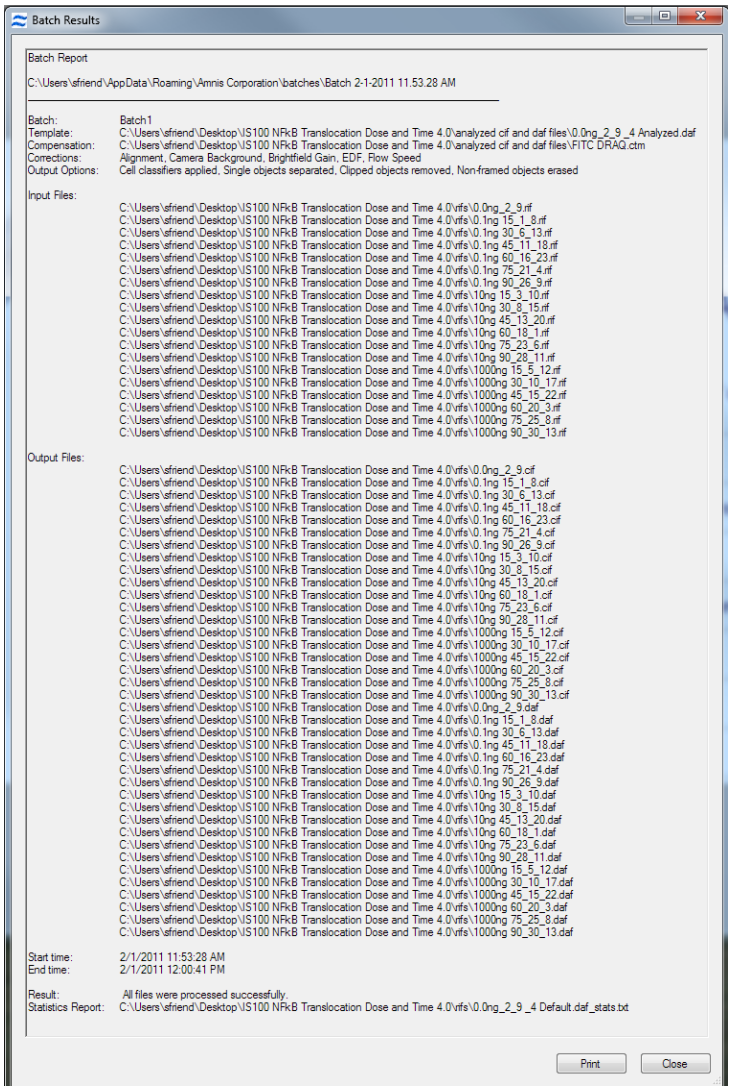
10. When you are satisfied with the Batches to Run list, click Submit Batches. The files to process are listed and the progress is displayed in the Processing Batch window. Once you have started processing batches, it may use up a fair amount of your computer's processing power. After a batch is run, it appears in the processed batches list. You may get a report or rerun a batch from the list.



NOTE: To cancel the batch processing at any time, click Cancel Batch. The IDEAS® software will confirm cancellation and complete the file it is working on. When the batch processing is complete, the IDEAS® software saves the .rif, .cif, and .daf files in the batch results directory. In the Batches window, a list of processed batches appears in the Processed Batches list. If a batch did not successfully complete, it will appear in red.

NOTE: To display the error that occurred during processing, double click the batch.

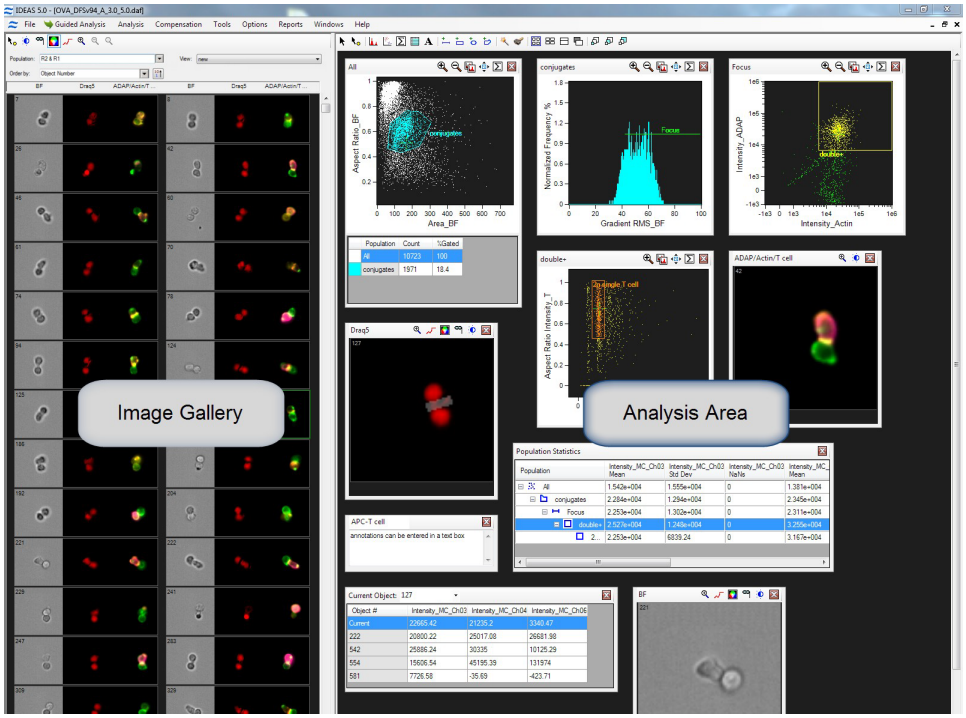
11. (Optional) To rerun a batch, highlight the batch name and click Add at the bottom of the window. This will move the batch into the run list, where it may be edited.
12. (Optional) To get a batch report, double click the batch name, or highlight the batch name and click Details. The Batch Results window appears.
    a. In the Batch Results window, click Print.
    b. In the Batch Results window, click Close.
    c. In the Batches window, click Close.



Overview of the Data Analysis Tools

The IDEAS® software provides a powerful tool set that allows you to explore and analyze data. The rich feature set lets you create hundreds of your own features to differentiate objects and statistically quantify your results.

As shown in the following figure, the application window is divided into two panels— the Image Gallery and Analysis Area—which each provide the corresponding tools that you can use for data analysis. The layout can be changed to side by side or top and bottom with resizable panels.



You can create populations of objects by tagging hand selected images, drawing regions on graphs, and using Boolean logic to combine existing populations. After you have created a population, you can view it in the Image Gallery or plot it on a graph. You can view the statistics for populations or objects in tables placed in the analysis area.

Graphs show data plotted with one or two feature values, and tools are provided that allow you to draw regions for the purpose of generating new populations. You can show any population on a plot.

Every image is linked to the feature data. Selecting an individual data point in a graph allows you to view it in the Image Gallery or look at its feature values in the Statistics Area. Any object that is selected in the Image Gallery is also shown on the plots in the Analysis Area. Images may be ordered in the Image Gallery by feature value.

Use the Image Gallery

This section describes how to view populations of objects in various ways, view masks, customize the Image Gallery display, and hand select objects for a population:

Overview of the Image Gallery

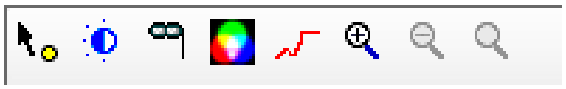
The Image Gallery displays the imagery and masks of any population of objects.

A toolbar is provided in the upper left corner of the panel, as shown in the following figure. The Image Gallery also makes different viewing modes available for the imagery. The default template contains the viewing modes which allows you to view all channel images in grayscale or color, or each channel image individually.

NOTE: You can build custom viewing modes as shown in this example.

Image Gallery Tools

Table 11. Image Gallery Tools



Icon	Name	Description
	All	Select the population to view
	Display	Opens the display settings window
	Mask	Displays the segmentation mask on the images
	Color	Turns pseudocolor ON/OFF
	Saturation	Displays saturated pixels in the image
	Zoom	Zoom in and out or reset the zoom

View Imagery for a Population

Click the population that you want from the Population drop down menu of the Image Gallery. The drop down list includes all the populations, as well as the currently selected bin from a histogram.

Select an Individual Image

Click on the image to select it. A thin, green frame indicates the selected object.

NOTE: Conversely, in any scatter plot in the analysis area, clicking a graphical point causes the Image Gallery to highlight and display the corresponding object.

- The object's feature values are displayed in a table if an object is selected, and a table is added to the Analysis Area.
- The selected object is identified in each scatter plot graph with a green cross.
- The objects are presented in the Image Gallery in object number order by default. To order the objects by another feature choose the feature in the drop down menu.
- The image can be placed in the Analysis area by right clicking and selecting Add Image to Analysis Area.

Change the Order of Images

In the Order by drop down menu of the Image Gallery, select a feature you wish to order the cells by and choose ascending or descending values.

NOTE: You can show the feature values on the images when creating the view in the Image Gallery Properties tool.

Change the Viewing Mode

In the View drop down menu of the Image Gallery, select a specific view. The imagery displays changes according to the new view. To make a new view, use the Image Properties tool.

Show or Hide Masks

Click the Show/Hide Masks toolbar button ( ) to toggle between showing and hiding the selected masks for all images in the Image Gallery.

The mask is shown as a transparent layer over each image. The mask displayed is selected in the Image Gallery Properties View tab. The color of the overlayed mask can be changed in the Applications Defaults under the Options menu.

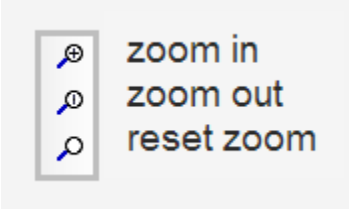
NOTE: To hide the mask for a specific channel only, set the individual channel mask to None in the view tab of the Image Properties dialog.

Show or Hide Color

Click the Show/Hide Color toolbar button ( ) to toggle between showing and hiding the colors for all images in the Image Gallery.

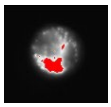
Zoom on the Image Gallery

Click the Zoom In toolbar button to view the images in the gallery closer and the Zoom Out or Reset Zoom to reverse the zoom.



Show or Hide Saturation

Click the Show/Hide Saturated Pixels toolbar button ( ). Saturated pixels in images, if any, appear in a contrasting color.



Show the Mask or Feature Being Used for the Current View

Hover over the channel name. The mask or feature being used for this view is displayed.

Set the Image Gallery Properties

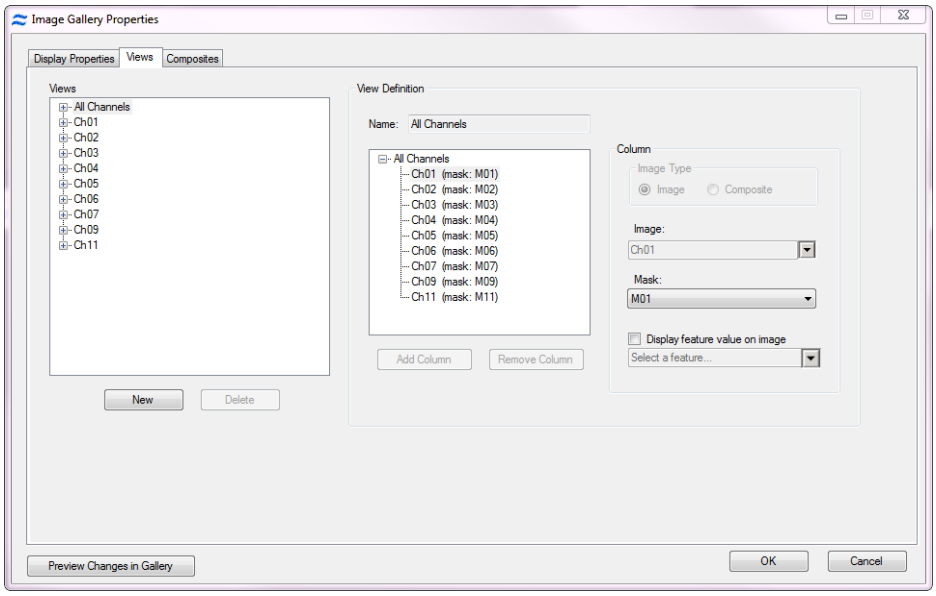
When a new data file opens in the default template, you might find it difficult to clearly see cell morphology because the Image Gallery display properties have not yet been properly adjusted for the data set. To optimize the display, use the wizard to set the pixel intensity mapping to the display range.

Clicking the Image Gallery Properties toolbar button ( ) opens the Image Gallery Properties window, which contains the following tabs:

- Display Properties—Define the name, color, and display intensity mapping for each image. Allows adjustment of the image size for the image gallery.
- Views—Customize the views for the Image Gallery.
- Composites—Create composites and adjust the amount of color from a channel that is included in a composite image.

Customize the Image Gallery Display Properties

Click the Image Gallery Properties toolbar button ( ) to begin. The Image Gallery Properties window appears with the Display Properties tab displayed.



Change the Size of the Panels in the Image Gallery

Display Width and Display Height can be specified or changed to Auto Fit in the lower left section of the Display Properties tab.

Change the Name or Color for Each Image

1. Select an image in the list of images on the Display Properties tab of the Image Gallery Properties window.
2. On the right side of the window, you can type a new, unique name for the selected image.

   NOTE: Each image is provided with a default name and the image names appear near the top of the Image Gallery.

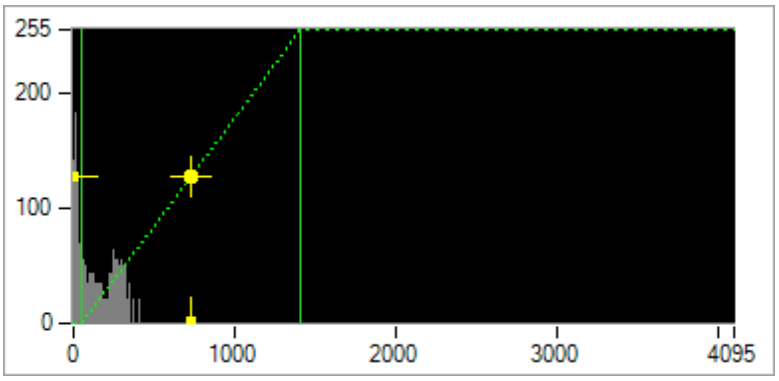
3. Click the colored square for the selected image.
4. Click the color that you want in the color palette.
5. Click OK to close the palette.

NOTE: The grayscale image in each channel is assigned a default color for image display in the gallery. Setting the color to white is equivalent to using the original grayscale image. The colors are also used to build composite images.

Fine Tune the Image Display Intensity for an Image

1. On the Display Properties tab of the Image Gallery Properties window, select an image by clicking the image name in the list. The graph for the currently selected image is shown in the window and updates as the changes are made. Select an image in the image gallery that has intensities for the image channel you are adjusting.

   NOTE: You will adjust the Display Intensity settings on the graph (the Y Axis), the value of the display to (the X axis), the range of pixel intensities. The range of pixel intensities will depend on the instrument and the collection mode set during acquisition. The display range is 0–255; the range of intensities from the camera is 0-4095 for the ImageStream®^{X} systems or 0-32,767 for EDF mode collection. The IS100 first generation instrument has a 10 bit camera and therefore the range of pixel intensities is 0–1023. The limits of the graph enable you to use the full dynamic range of the display to map the pixel intensities of the image.



   - At each intensity on the X Axis of the graph, the gray histogram shows the number of pixels in the image. This histogram provides you with a general sense of the range of pixel intensities in the image. The dotted green line maps the pixel intensities to the display intensities, which are in the 0–255 range.
   - Manual setting is done by click dragging the vertical green line on the left side (crossing the X Axis at 0) allows you to set the display pixel intensity to 0 for all intensities that appear to the left of that line. Doing so removes background noise from the image.
   - Click dragging the vertical green line on the right side allows you to set the display pixel intensity to 255 for all intensities that appear to the right of that line.

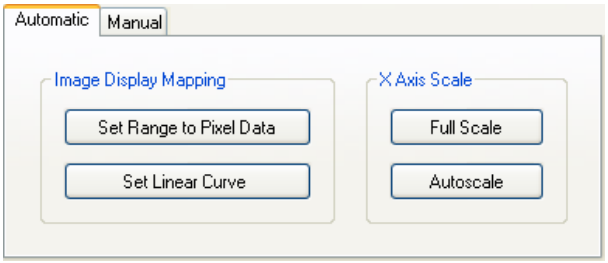
2. From the Image Gallery window, select the object to use for setting the mapping. It appears in the Image Gallery Properties window.

   NOTE: You might need to select different objects for different channels because an object might not fluoresce in all channels.

3. To adjust the pixel mapping for display, click drag the vertical green line by clicking near it (but not near the yellow cross).

   NOTE: For fluorescence channels, set the vertical green line that appears on the left side to the right of the large peak of background pixel intensities as shown above and set the right vertical green line to the right of the brightest pixel intensities. Click 'Set Linear Curve' to make the transformation linear. For the brightfield channel, set the vertical lines to about 50 counts to the right and left of the histogram to produce an image with crisp brightfield contrast.

   - To change the mapping curve to be logarithmic or exponential, click drag the yellow cross.
   - To restore the mapping to a linear curve, Click Set Linear Curve.
   - To see the full scale for the X Axis Click Full Scale.
   - To set the display mapping of the X Axis to the lowest and highest values for a selected object, Click Set Range to Pixel Data.
   - To set the scale of the X Axis to the range of the vertical green lines or of all the pixel intensities for the selected object—whichever is larger—Click Autoscale.
   - You may enter values manually by selecting the Manual tab.

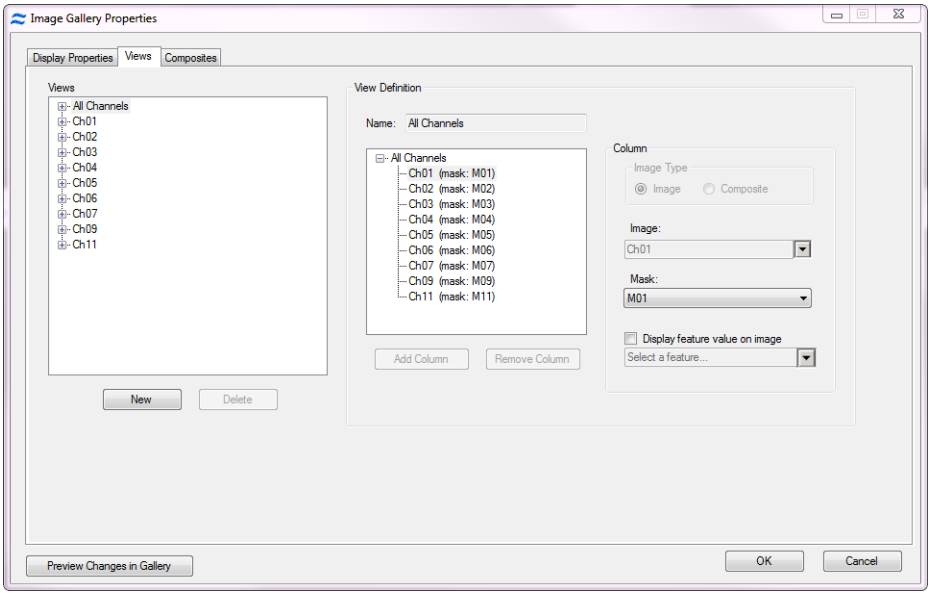


4. If you want to preview the changes in the Image Gallery, click Preview Changes in Gallery.
5. Continue customizing the Image Gallery display properties with another procedure in this section, or click OK to finish and save changes or Cancel to finish and discard changes.

Customize the Image Gallery Views, Images, and Masks

1. Within the Image Gallery Properties window, click the Views tab.

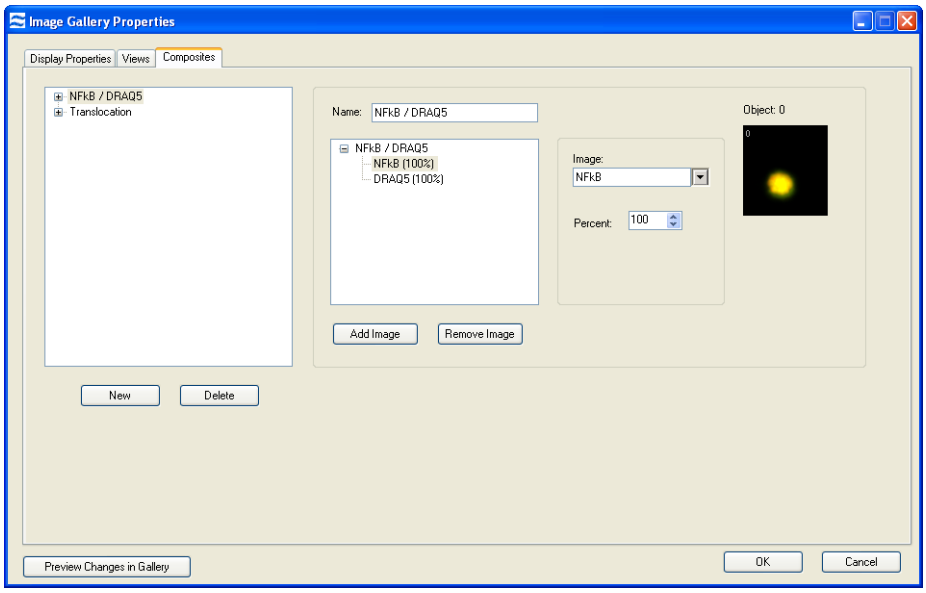
   NOTE: The Image Gallery view can be customized to view any combination of channel images or composites. The default view All Channels is a view that displays all image channels that were included during acquisition of the file with their associated default masks. This mask may be changed for the default view however, the images in this view cannot be changed. The list of existing views is shown on the left.



2. To create a new view, Click New. The new view is automatically added to the list on the left.
3. In the right section of the window, type in a name for the view.
4. Click Add Column.
5. Define the column by selecting an image and a mask or a composite from the drop down menu.
6. To show a feature value on an image check the box and choose the feature.
7. Repeat the previous 2 steps until finished adding columns to the view. A column will be added under the column currently selected. To insert a column click on the image above insertion point.
8. Columns may be removed by clicking on Remove Column.
9. A view may be edited at any time by selecting the view and following the same procedures.
10. If you want to delete a view, click the view to select it, and then click Delete. A confirmation window appears.
11. If you want to preview any new changes in the Image Gallery, return to the Image Gallery and choose your new view in the View drop down menu. Then return to the Image Gallery Properties window and click Preview Changes in Gallery.
12. Continue customizing the Image Gallery display properties with another procedure in this section, or click OK to finish and save changes or Cancel to finish and discard changes.

Create a Composite

1. Within the Image Gallery Properties window, click the Composites tab. The list of existing composites is shown on the left.



2. In the right section of the window, type a name for the composite or leave blank to allow the name to be built automatically from the image names added to the composite.
3. Click Add Image. The selected image appears in the Object box. Change the Percent if desired. The percent specifies the percentage of the image to include in the composite.

   NOTE: As you make the changes, the image in the Object box updates accordingly. If you want to preview any new changes in the Image Gallery, return to the Image Gallery and select the View drop down menu to your new view. Then return to the Image Gallery Properties window and click Preview Changes in Gallery.

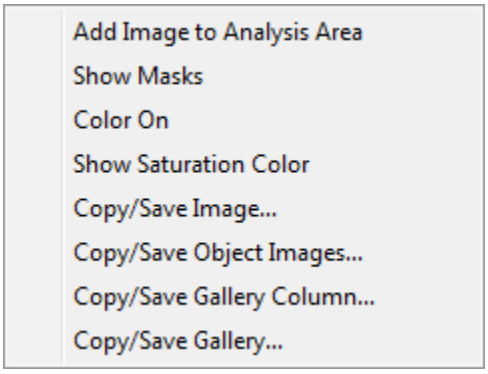
4. Continue to add images as desired.
5. To remove and image from the composite, click Remove Image.
6. The composite is automatically added to the list on the left.
7. A composite can be removed from the list by clicking Delete.
8. Continue customizing the Image Gallery display properties with another procedure in this section, or click OK to finish and save changes or Cancel to finish and discard changes.

Work with Individual Channel Images

You can work with individual images in the Image Gallery. You can zoom in or out on the images. You can add a larger version of an image to the Analysis Area for further analysis, show or hide masks for a single image in the Image Gallery, and copy one or more images to the Clipboard.

Manipulate Individual Images

1. In the Image Gallery, right click an image that you are interested in. A menu displays:



- To place the image in the Analysis Area, click Add Image to Analysis Area.
- To show or hide the masks for the object image, click Show Masks or Hide Masks. One or the other will appear, depending on the current state.
- To turn the colors on or off for the object image, click Color On or Color Off. One or the other will appear depending on the current state.
- To show or hide the saturation color for the object image, click Show Saturation Color or Hide Saturation Color. One or the other will appear depending on the current state.

Copy or Save Images for Use in Reports

1. In the Image Gallery, right click an image that you are interested in. A menu displays:
   - To copy or save the single channel image to the Clipboard, choose Copy/Save Image
   - To copy or save all of the channel images of one object, choose Copy/Save Object Images.
   - To copy or save the single channel image for all of the displayed images to the Clipboard, choose Copy/Save Gallery Column.
   - To copy or save all the visible images in the Image Gallery choose Copy/Save Gallery.

Overview of the Mask Manager

A mask defines a specific area of an image to use for feature value calculations. The IDEAS® software contains a Mask Manager for viewing existing masks and creating new ones.

When the IDEAS® software loads a .rif file, the application creates a segmentation mask for each channel image and stores the mask along with the image in the .cif file. The masks, labeled M01 through M12, contain pixels that are detected as brighter than the background. In addition, the application generates a Combined Mask, named MC and a Not Combined Mask, Not MC for each object. A combined mask consists of the union of the masks of all the channels of the object. A Not Combined Mask is all of the pixels with no intensities above background.

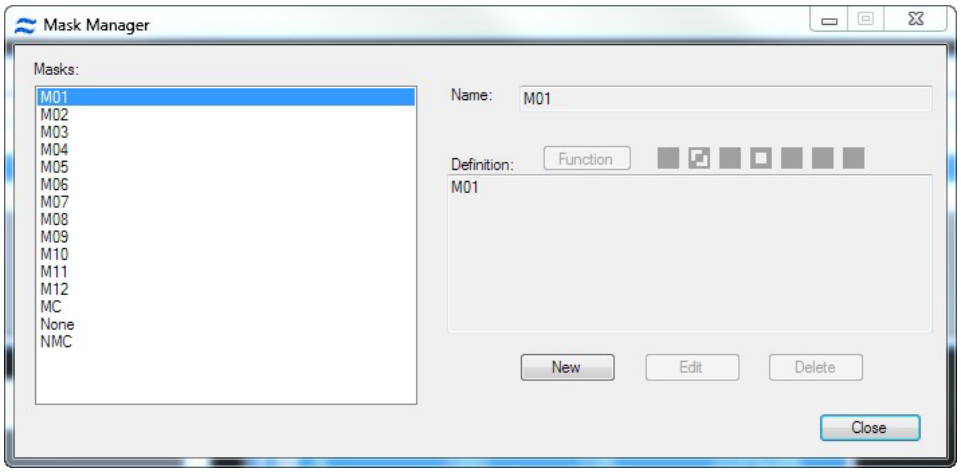
You might need to adjust the masks or create new ones that include only a specific area of a cell, such as the nucleus. You can combine masks by using Boolean logic, or you can adjust them by applying functions.

Create New Masks with the Mask Manager

There are two ways to work with new masks in the Mask Manager. First, masks can be created by using functions, which allows you to choose an input mask and, if needed, adjust the channel and scalar input. Alternatively, masks can be created by combining masks through Boolean logic.

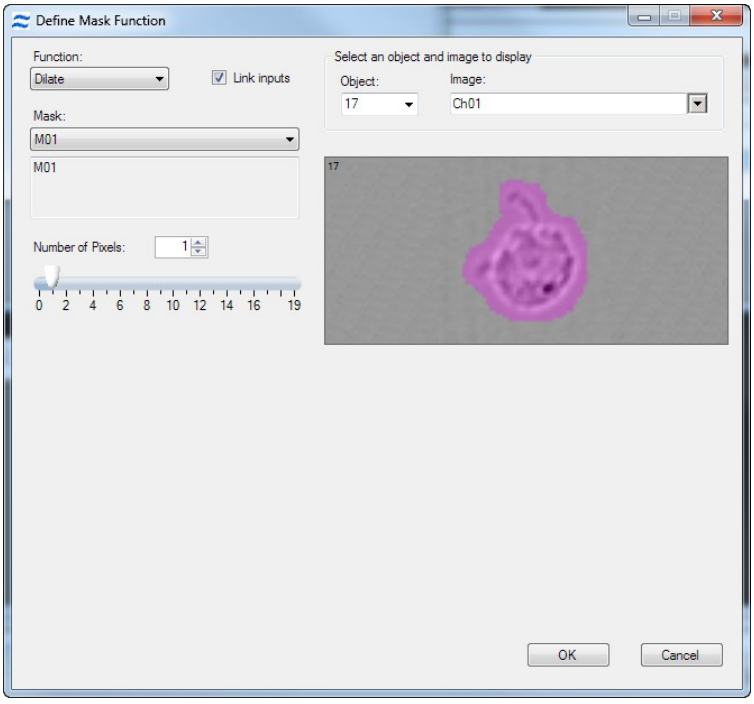
Create a New Mask Using Functions

1. Select Analysis > Masks. The Mask Manager opens with a list of existing masks on the left.
2. Click New. The right side of the window is enabled to define a new mask.



3. Click Function. The Define Mask Function window appears with 19 available masks to use. Select a mask function from the Function drop down menu to define that function. Available mask functions are:

   - AdaptiveErode
   - Component
   - Dilate
   - Erode
   - Fill
   - INSPIRE™ software
   - Intensity
   - Interface
   - LevelSet
   - Morphology
   - Object
   - Peak
   - Range
   - Skeleton
   - Spot
   - System
   - Threshold
   - Valley
   - Watershed



4. Choose the input mask(s), channel and scalar parameters as needed. The right side of the window adjusts the display and view of the channel image.

   - To view a different object in the file, select it in the Object list or type its number.
   - To view a different image for the object, select it from the list.

5. The Link inputs checkbox is checked by default. To modify a mask with different inputs, clear this box.
6. Click OK. The new function is added to the mask definition.
7. Click OK. The new mask name will display in the list of Masks on the left side.

Create a New Combined Mask

1. Select Analysis > Masks.
2. Click New.
3. Use the Masks list on the left and the Definition toolbar to build a new mask using the definitions of existing masks with Boolean logic explained in the table below.

Table 12. Mask Tasks and Toolbar

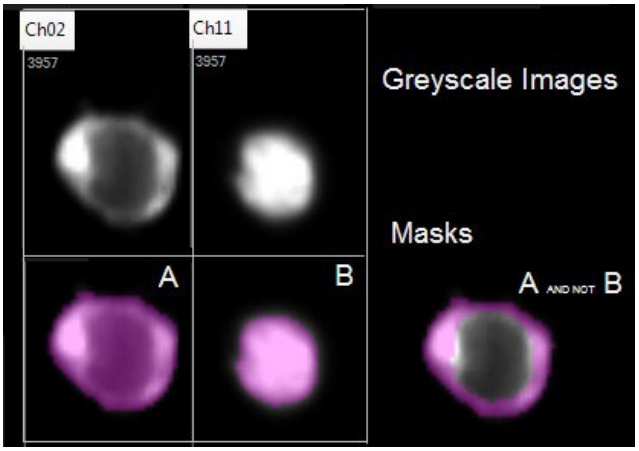
Task	Toolbar
Add a mask to the definition.	Double click the feature in the Masks list. Or, single click the feature in the Masks list and click the leftmost down arrow button ( ) on the toolbar.
Combine two masks.	Use the Boolean AND or OR operator. Use the AND operator to include only the pixels that are in both of the original masks. Use the OR operator to include the pixels that are in either one of the original masks.
Select all pixels that are not in the original mask.	Use the Boolean NOT operator ( ). The NOT operator specifies which mask will not be used.
Affect the order of operations.	Use the parentheses toolbar buttons.
Remove an item from the end of the definition.	Click the left arrow button ( ) on the toolbar.

4. Add masks and Boolean logic to the definition as needed.
5. Click OK to add the definition to the Masks list.
6. Click Close.

Example of Creating a Function Mask and a Combined Mask

Here is an example of creating a mask of the cytoplasm. Similar operations could be done for the cell surface.

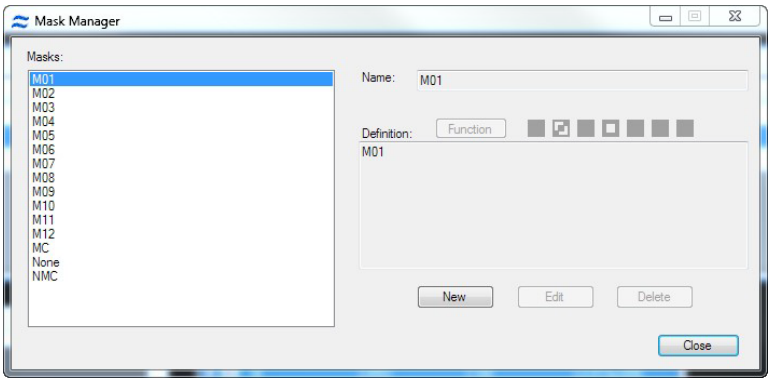
In this example, cells were stained with a green intracellular marker (in Channel 2) and a red nuclear dye (in Channel 11). You can generate a cytoplasm specific mask by first refining the intracellular and nuclear masks and then removing the nuclear mask pixels from the intracellular mask.



1. Observe the default masks in the Image Gallery. Since the default masks are designed to capture all the light in an image, they tend to include light that exists beyond the perceived boundaries of the images. In this case, both the intracellular and nuclear masks need to be refined. Start by creating morphology masks for both channel images because the Morphology mask is designed to conform to the shape of the image.

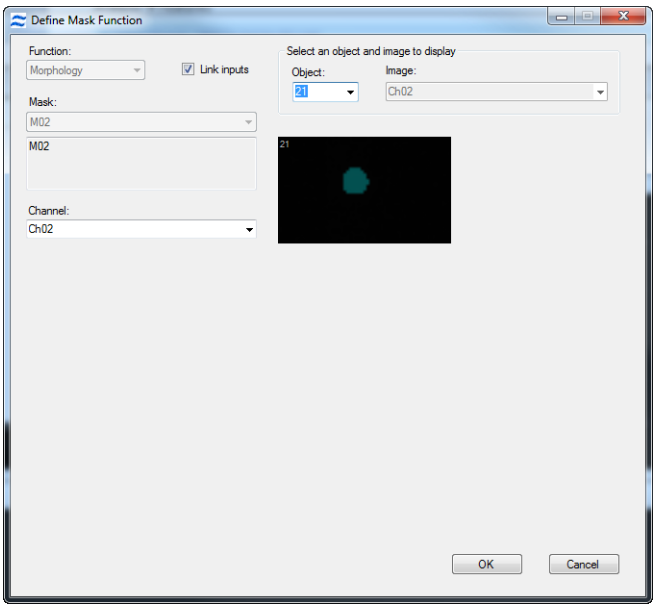
   NOTE: The Object mask function may also be used in place of the Morphology mask function.

2. Select Analysis > Masks.

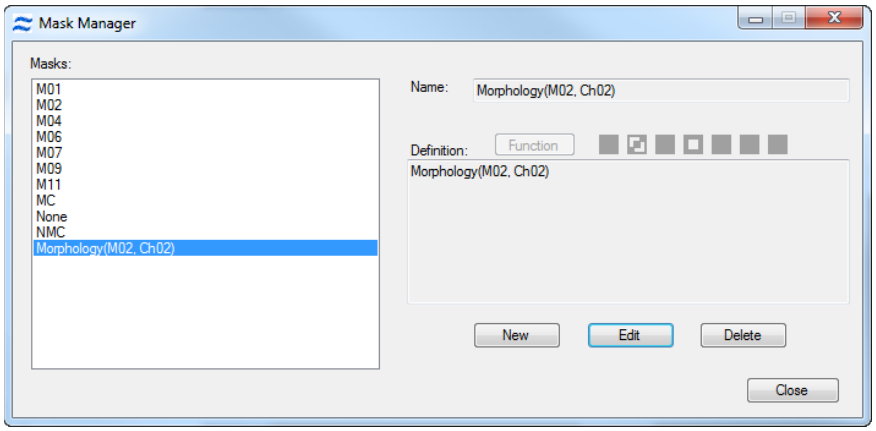


3. Click New.
4. Click on the Function toolbar button (Function) to adjust the mask that will define the whole cell. The Define Mask Function window displays.

5. Select Morphology in the Function list. It will be important to have an appropriate cell selected. You can change the object number by typing in the box. Some functions are iterative, in which case you can change the mask selection. For example, dilate a morphology mask.

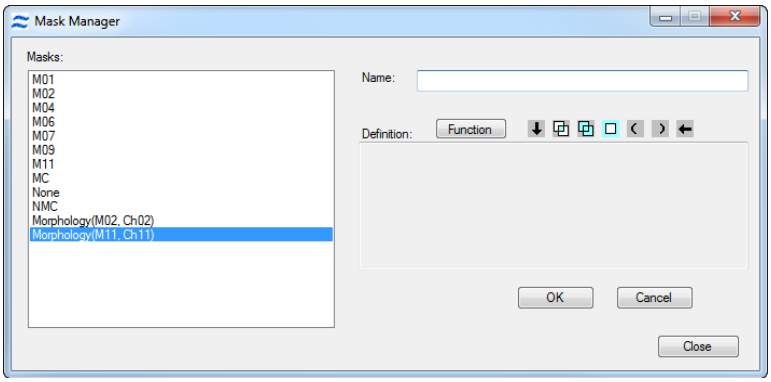


6. Select a starting Mask.
7. Select Channel 2 (intracellular marker) on the left side of the window.
8. Click OK.
9. Click OK to add this mask to the list.
   The default names are ideal for recall. The new mask will be defined in the list of masks on the left panel. The mask definition can be recalled in the definition box by selecting the mask in the left panel.

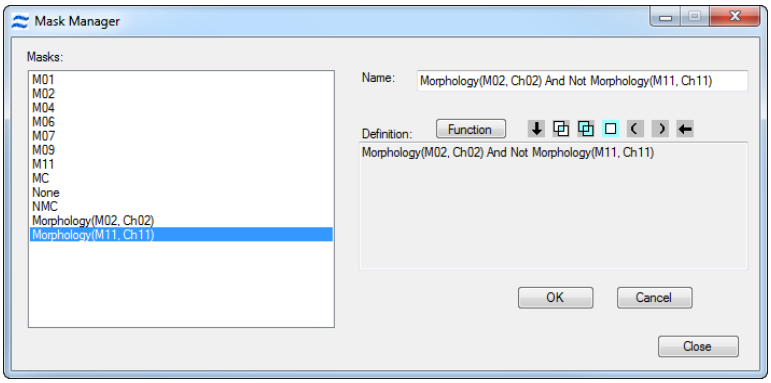


10. To make the Morphology (Nuclear) mask, repeat steps 3-9 using Channel 11.
11. Click Close.
12. To view the resulting morphology masks, open the Image Display Properties window ( ) and, if necessary, select the new mask(s) for the channel.

13. Next, you will subtract the nuclear morphology mask from the intracellular mask using Boolean logic. In the Mask Manager window, click New.



14. Double click the Morphology(M02,Ch02) mask in the list.
15. Click the AND ( ) button on the toolbar.
16. Click the NOT ( ) button on the toolbar.
17. Double click the Morphology (M11,Ch11) mask in the list.



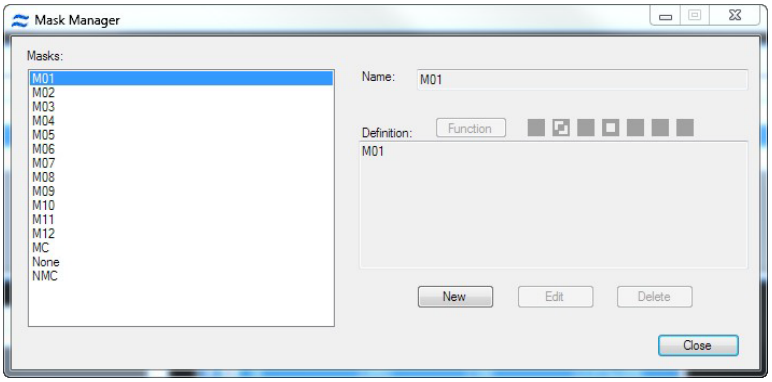
18. The default name may be very long: edit as desired.
19. Click OK to add this mask to the list.
20. Click Close.

To view the resulting masks, open the Image Display Properties window and create a new view with the masks showing.

View and Edit a Mask

View a Mask Definition

1. Select Analysis > Masks. The Mask Manager window appears.



2. Click a mask in the Masks list to view the definition in the Definition area.
3. Click Close.

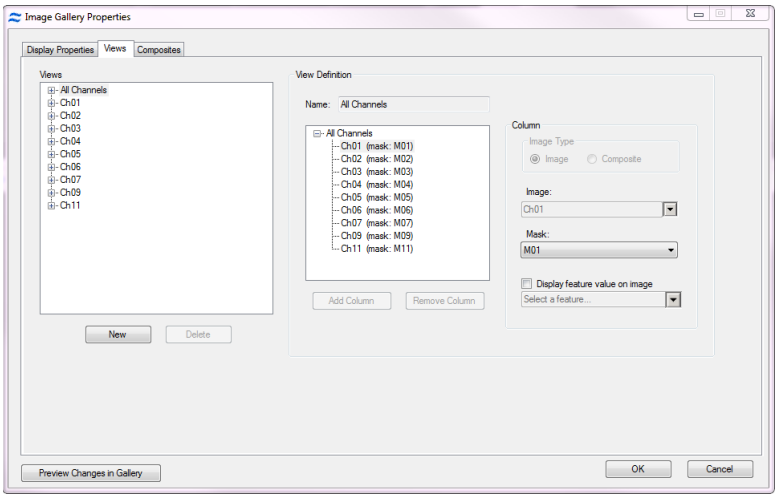
Edit a Mask Function

1. In the Mask Manager window, select the mask that contains the function you want to edit.
2. Click Edit.
3. Remove the definition for the combined mask using the back arrow tool ( ) as needed.
4. Alternately, click the Function button on the toolbar (Function) for a function mask. The Define Mask Function window displays.
5. Click OK when finished.

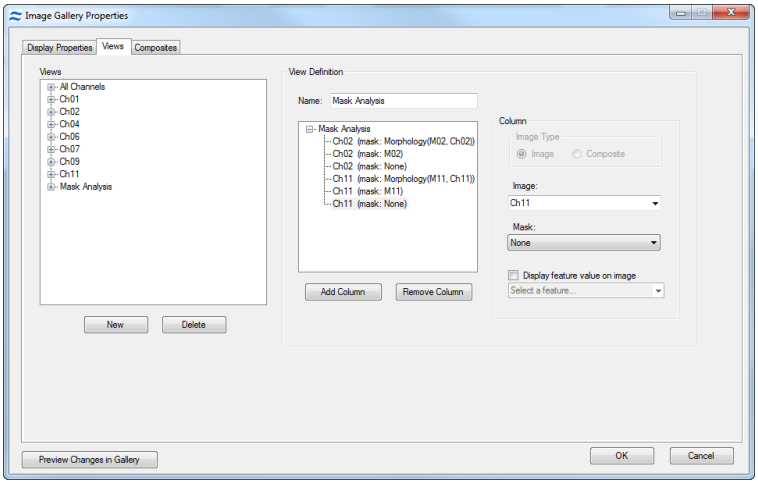
View Masks in the Image Gallery:

After creating masks in the Mask Manager, you may wish to view them in the image gallery for evaluation.

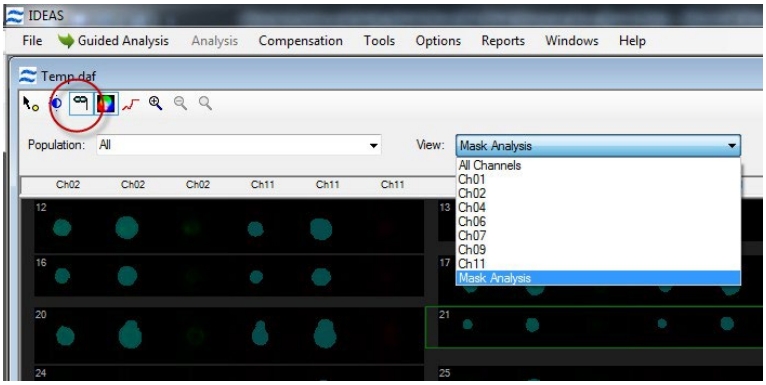
1. Click the Image Gallery Properties toolbar button ( ) to begin. The Image Gallery Properties window appears with the Display Properties tab displayed.
2. Click the Views tab.



3. Click New.
4. Type a name for the view.
5. Click Add Column.
6. Choose the Image and Mask from the drop down menus.
7. Continue to add columns until done. In this example, the view will contain a Ch02 image with the Morphology mask, default mask, and no mask followed by a Ch11 image with the Morphology, default, no mask.



8. To view the masks in the image gallery, choose the view in the Image Gallery drop down menu and turn the masks on by selecting the icon.

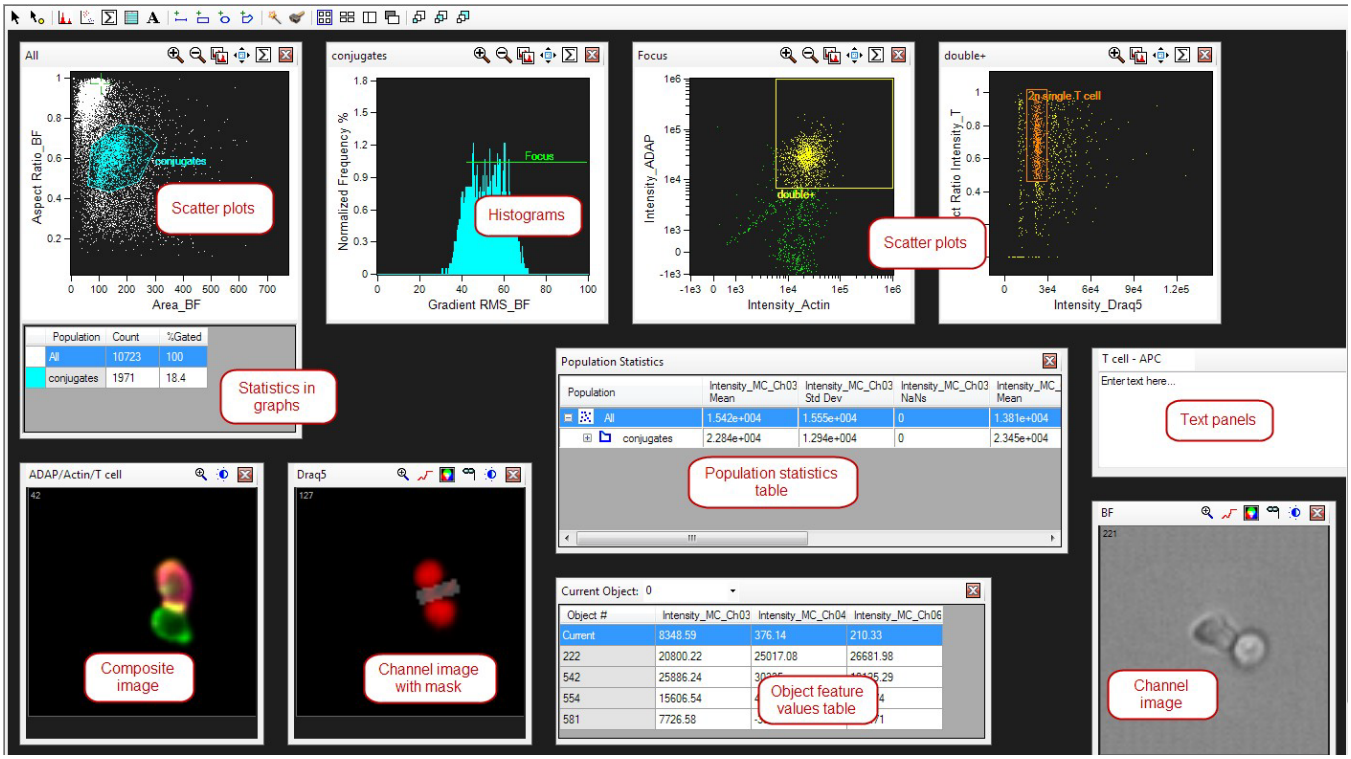


Overview of the Analysis Area

The Analysis Area provides display space for individual images, plots of cellular feature values, tables of population statistics, tables of object feature values, and text annotations. You can select different layouts for the IDEAS® software window and placement of the analysis area and expand the Analysis Area by dragging its boundaries.

The graphs are created into panels of a default size and can be resized by dragging a corner or using the size tool. The position of the panels is automatically adjusted to fit in the available display space. A vertical scroll bar appears when the number of panels exceeds the space available on the window. The panels can be retiled using the arrange analysis area tool.

As illustrated by the following figure, the Analysis Area can contain several types of panels: histogram, histogram overlay, scatter plot, tables of population statistics or object feature values, channel image, composite image, and text. Each panel will contain its own toolbar and context menu. To move a panel click on the name at the top of the graph and drag it to a new location. A graph may be selected and then a right click in a blank space in the work area allows you to choose paste in the new location.



A toolbar is visible at the top of the Analysis Area. The Analysis Area Tools table describes the function for each tool.

Analysis Area Tools

Table 13. Analysis Area Tools

Icon	Name	Description
	Pointer	Reset cursor to pointer, click on dots or images
	Tagging	Allows creation of a handpicked population
	Histogram	Opens the histogram graph tool
	Scatter Plot	Opens the bivariate scatter plot tool
	Statistics	Creates a population statistic table in the workspace
	Table	Creates a table to display selected objects feature values
	Notation	Creates a notepad in the workspace
	Line region	Draw a line region on a histogram
	Rectangle region	Draw a rectangular region on a scatterplot
	Oval region	Draw an oval region on a scatterplot
	Polygon region	Draw a polygon region on a scatterplot. Click to set an anchor, double click to close the region.
	Wizards	Opens wizard manager
	Building blocks	Opens the building block manager
	Select All	Selects all plots in the analysis area
	Tile	Tiles the graphs in the analysis area to fill the workspace
	Layout	Changes the layout of the image gallery and analysis area
	Graph Background	Change the background of the graphs to black or white
	Size plots	Sets size of selected plots to small, medium, or large
	Help	Opens the IDEAS® software Analysis User Manual

Create Graphs

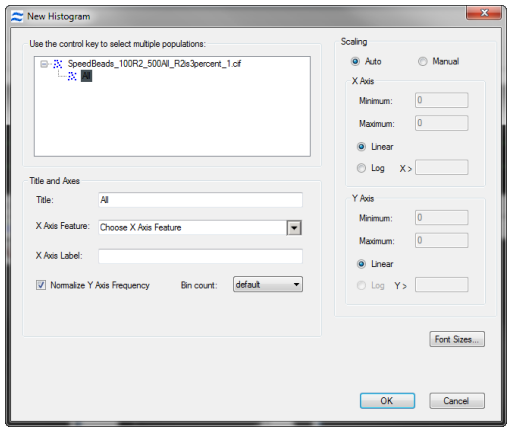
You can add two types of graphs to the Analysis Area:

- Histogram—Graphs a single feature.
- Scatter Plot—Graphs two features.

NOTE: Building blocks are available that will help you to create graphs for finding single, focused, fluorescent positive events or a size versus scatter plot.

Create a Graph Without Using a Building Block

1. Click the New Histogram ( ) or New Scatter Plot ( ) toolbar button. The New Histogram or New Scatter plot window appears, respectively.



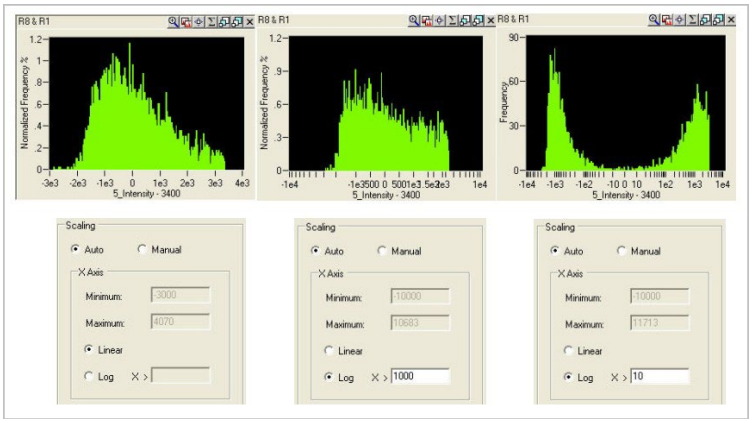
2. Select the one or more populations to graph by clicking them. To select more than one population, use the Ctrl key. **NOTE**: The title defaults to the selected population. You can edit the title.
3. In the X Axis Feature drop down menu, select the feature that you want to graph on the X Axis.
4. To change the label for the X axis, edit the text in the X Axis Label field. The label defaults to the name of the selected feature.
5. (Optional) If you are creating a scatter plot, select a feature and a label for the Y Axis.
6. (Optional) If you are creating a histogram, you can choose to normalize the Y axis frequency and change the bin count.
7. The default font sizes are used, you may change them by clicking Font Sizes.
8. Assign colors by Population (default) for dot plots or by Density for density plots.
9. Set the scaling for each axis of the graph. (The default is Auto, which allows the application to automatically scale the graph.)
10. To set minimum and maximum values for an axis, select Manual.
11. Select Linear or Log and enter Maximum and Minimum limits.

12. If you selected Log, enter the X > value.

NOTE: You can scale the X Axis of a graph or the Y Axis of a scatter plot in one of two modes: Linear or Log. The Linear mode is the default.

The Log mode allows you to logarithmically scale a section of the graph or scatter plot. Selecting this mode causes the IDEAS® software to perform biexponential plotting. The > X value defines the linear portion of the graph as - X through X. The application plots the values outside of these limits on a logarithmic scale. You can plot negative values as well as positive ones on a logarithmic scale by adjusting the limits.

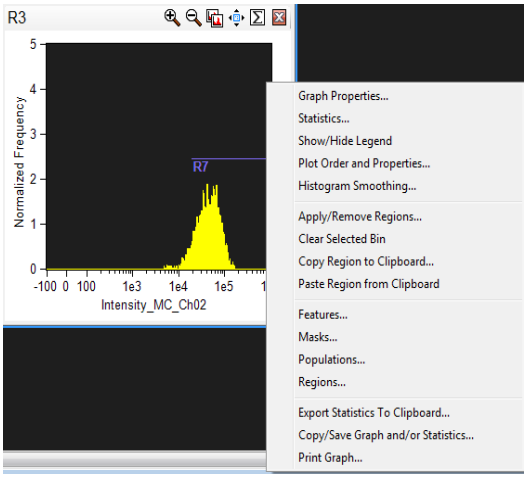
Take care not to split a population such that it appears to be two separate populations. This splitting is especially likely when negative values exist due to compensation or corrections on the imagery. The graph on the left side was plotted on a linear scale; the ones in the center and on the right side were plotted on logarithmic scales. The graph on the right side split the population because the change from a linear to a logarithmic scale occurred in the middle of the population. In general the >X value should be 1000 for 40 and 60X data and 100 for 20X data.



NOTE: The font sizes can be changed for an individual graph.

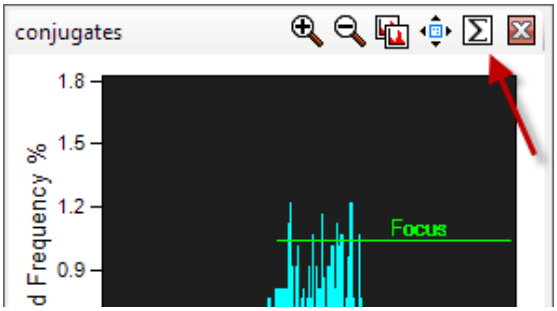
13. Click OK.

NOTE: Right click in the graph to bring up the context menu. Graph Properties will open the same window that you used to create the graph will reappear, and you can then make any changes that you want.

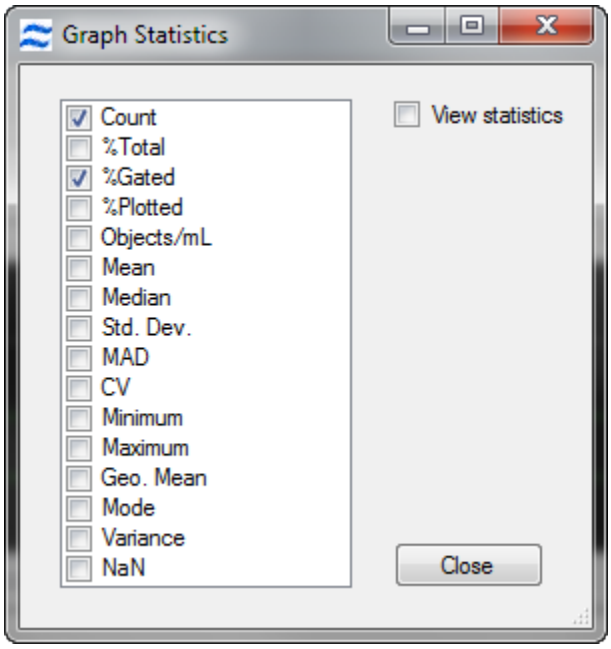


Show Selected Statistics for a Graph

1. Show and hide statistics by clicking the Statistics toolbar button in the panel that contains the graph.



2. Alternately, right click anywhere on the graph and click Statistics on the graph context menu that appears. The Statistics window appears.



3. Select the statistics that you want to display. Select the View statistics box and the box next to the statistic to be displayed for each population on the graph. The statistics that are supported are the Count, Percent Total, Percent Gated, Percent, Concentration (count/sample volume), Mean, Median, Standard Deviation, MAD (Median Average Deviation) RD -Mean, RD -Median, CV, Minimum, Maximum, Geometric Mean, Mode, variance, and NaN (not a number).
4. When finished, click Close.

Show the Legend for a Graph

1. Right click anywhere on the graph, and click Show/Hide Legend on the graph context menu that appears. If the legend was hidden, it appears on the graph. If the legend was shown, it disappears from the display.

   NOTE: The legend contains an entry for each population on the graph. If the graph is a scatter plot, the legend shows the population and its associated point style and color. If the graph is a histogram or overlay histogram, the legend shows the population name, associated color, and line type.

2. To move the legend, click and drag it.

   NOTE: You cannot drag the legend past the boundary of the graph panel.

Order the Plots on a Graph or Change the Fill and Lines for a Histogram:

Right click anywhere on the graph and choose Plot Order and Properties on the graph context menu that appears. The Display Properties dialog appears.

Move a Graph

With any graph in the Analysis Area, you can move it to another location by clicking in the center of the graph and dragging it.

Alternatively, select the graph, right click in a blank space in the analysis area, select Cut, right click where you would like to move the graph, then select Paste.

Create Regions on Graphs

Regions may be drawn on graphs to create new populations, based on the physical location of objects on a graph, and to compute statistics. Tools for drawing regions are found on the Analysis Area toolbar. A line region may be drawn only on a histogram. All other types of regions may be drawn only on a scatter plot.

A region can be copied to another graph in the same file or other open files. Regions may also be copied from one instance of the IDEAS® software to another.

When you draw a region on a histogram or scatter plot, you create a population of objects defined by the region that may be viewed in the Image Gallery or on other graphs.

Draw a Region on a Scatter Plot

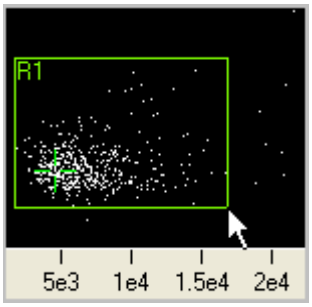
1. On the Analysis Area toolbar, select one of the following Region icons:

    Rectangle Region

    Oval Region

    Polygon Region

2. If you selected the Rectangle or the Oval tool:
    a. Click on the graph at the point where you would like to start the region and drag to the region endpoint. The region grows as you drag.
    b. Click again to complete the region. If you are drawing a region on a histogram or scatter plot, the Create a Region window appears.
    c. Name the region.
    d. Click the colored box to select an alternate color.
    e. Select Use for statistics only if you do not want to create a population from this region.
    f. Click OK. The region appears on the graph with the name and color that you selected.



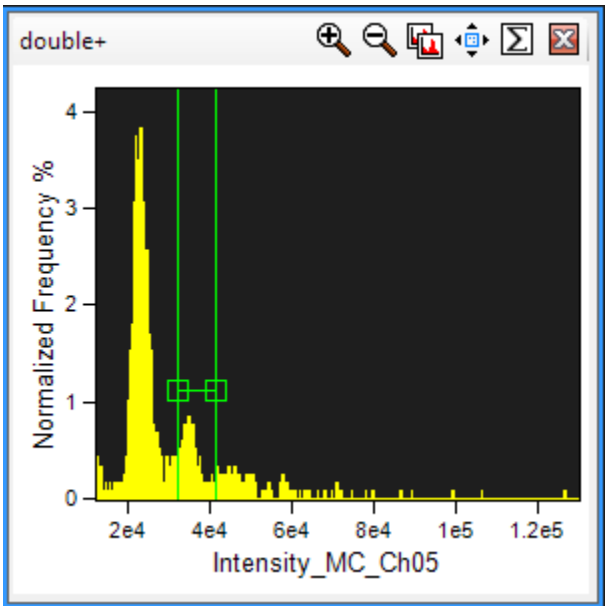
3. If you selected the Polygon tool:
    a. Clicking the scatter plot at the point where you would like to start the polygon.
    b. Click once for each vertex of the polygon.
    c. Double click to complete the drawing of the region. A window appears that allows you to name the population created by the polygon region and to assign the region's display properties.
    d. Click OK. The region appears on the graph with the name and color that you selected.

    NOTE: Before you click OK, you can click Cancel or you can click the Pointer button ( ) on the Analysis Area toolbar if you decide not to create the region.

Draw a Region on a Histogram

1. On the Analysis Area toolbar, click the Line Region tool ( ).

2. Drag the line across the histogram.



Move or Resize a Region on a Graph

1. Click the Move/Resize Region toolbar button ( ) on the graph panel toolbar.
2. Click the region that you would like to move or resize. When the region is selected, squares that can be moved appear at the vertices and the label. The first time that you drag the region, the entire region and label move.
3. Drag a specific vertex or label to move only that vertex or label.
4. To finish moving or resizing the regions on the graph, click the Move/Resize Region toolbar button again to deactivate the tool. The populations and statistics are updated, and the Move/Resize Region toolbar button is deactivated.

   NOTE: The recalculation of statistics and populations may take a moment if the data file is large or if many populations are dependent on the regions that are being moved or resized.

Zoom in on the Scale of a Graph

1. Click the Scaling toolbar button ( ) on the graph panel toolbar.
2. Click and drag to define a rectangular region for rescaling. The Zoom Out Scaling toolbar button ( ) appears in the graph panel toolbar, next to the Scaling toolbar button.
3. Click the Zoom Out Scaling toolbar button to automatically scale the graph. The Zoom Out Scaling toolbar button is removed from the graph panel toolbar.

Resize a Graph

1. Select the graph(s) to be resized and then click the appropriate sizing button tool: Small, Medium, or Large.

2. Resize the graph by dragging the right, bottom, or lower right corner.

   NOTE: Select multiple graphs to make them all the same size.

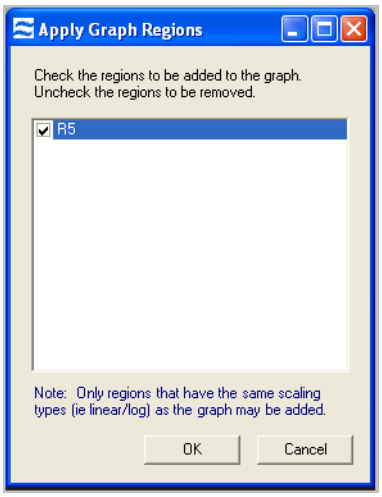
Copy and Paste a Region to Another Graph

1. Right click anywhere on a graph and click Copy Region to Clipboard on the graph context menu that displays. The Copy a Region to the Clipboard window displays.
2. Click the region to copy in the list and click OK.
3. Right click on the graph where you want to paste the region, then click Paste Region from Clipboard on the graph context menu that displays.
4. If the region already exists (in other words, you are copying it within the same instance of the application), the Create a Region window displays.
5. Rename the region and set the display properties for the resulting new population, and click OK.

NOTE: When you copy a region, the scale is copied and is no longer associated with the feature from which it was originally drawn. Therefore, the region might not fit on the new graph.

Apply or Remove a Region on a Graph

1. Right click anywhere on the graph, and click Apply/Remove Region on the graph context menu that displays. The Apply Graph Regions window displays.

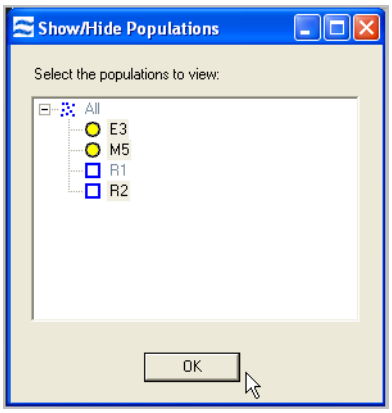


2. Select the regions that you want to appear on the graph.
3. Clear the regions that you want to remove from the graph.
4. Click OK.

Show or Hide a Population on a Scatter Plot:

1. Click Show/Hide Populations on the graph context menu. The Show/Hide Populations window displays.
2. Select the populations that you want to appear on the graph.

3. Clear the populations that you want to remove from the graph.



4. Click OK.

NOTE: On a scatter plot, you may show or hide any population on the graph, regardless of the features on the axes. Each scatter plot has an original, or base, population. When you show a population on a scatter plot, only those objects that are also in the base population will be shown. To aid in the identification of the populations shown, change the characteristics of the population(s) in the population manager.

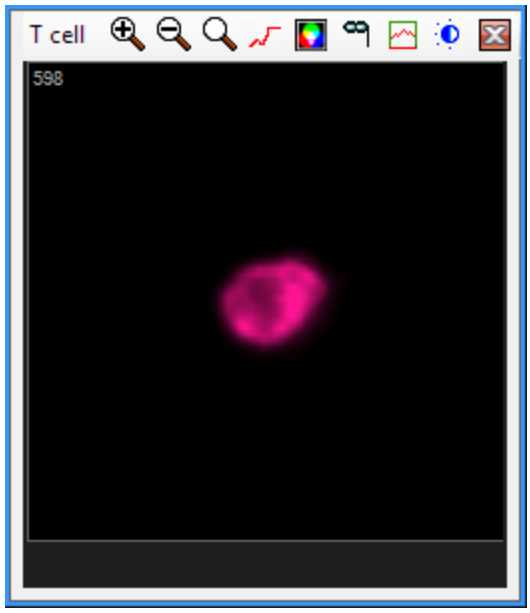
Analyze Individual Images

To analyze an image in more detail, place the image in the Analysis Area to view pixel positions and intensities as well as generate statistics for an area of the image. You can also show the Measurement tool for the image.

Image panels, which are shown in the following figure, each contain a toolbar in the upper right corner and a context menu that appears when you right click an image. An image in the Analysis Area is three times the size of an image in the Image Gallery.

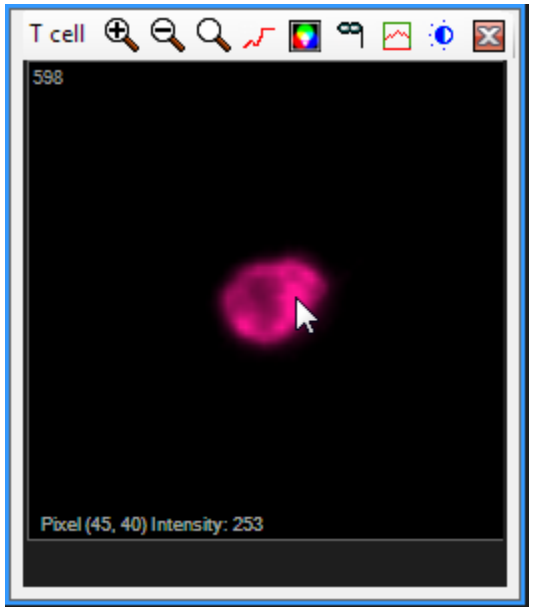
Add an Image Panel to the Analysis Area

Right click an image in the Image Gallery or Analysis Area, then select Add Image to Analysis Area on the context menu that appears. The image panel appears in the Analysis Area.



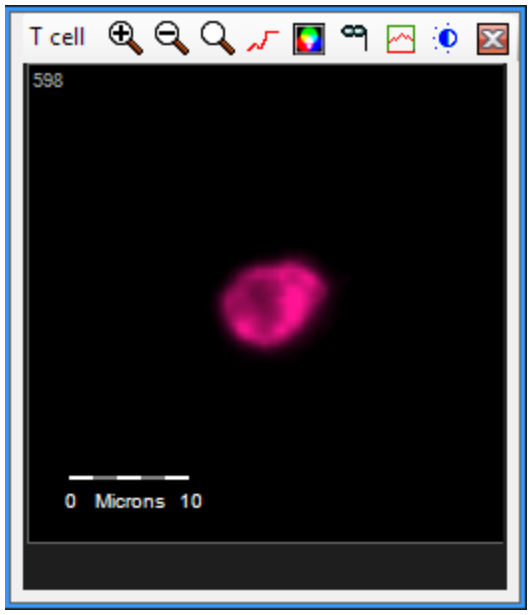
View the Individual Pixel Intensities of a Single Channel Image

Move the mouse pointer across the image. The pixel positions and intensities appear under the image. (The pixel (0, 0) is positioned at the upper left of the image.)



Display the Measurement Tool in an Image Panel

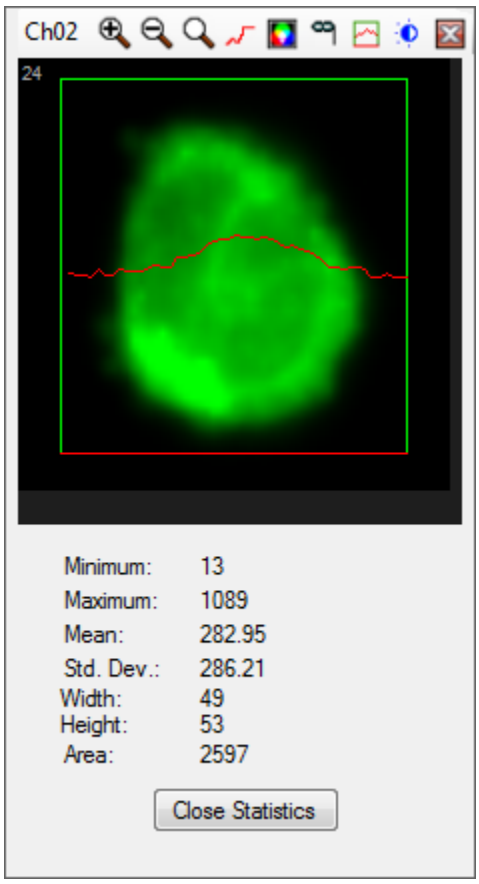
Right click the image panel, and click Show Measurement Tool on the context menu that displays. The 10-micron bar displays.



Examine a Line Profile or the Statistics for an Area of an Image

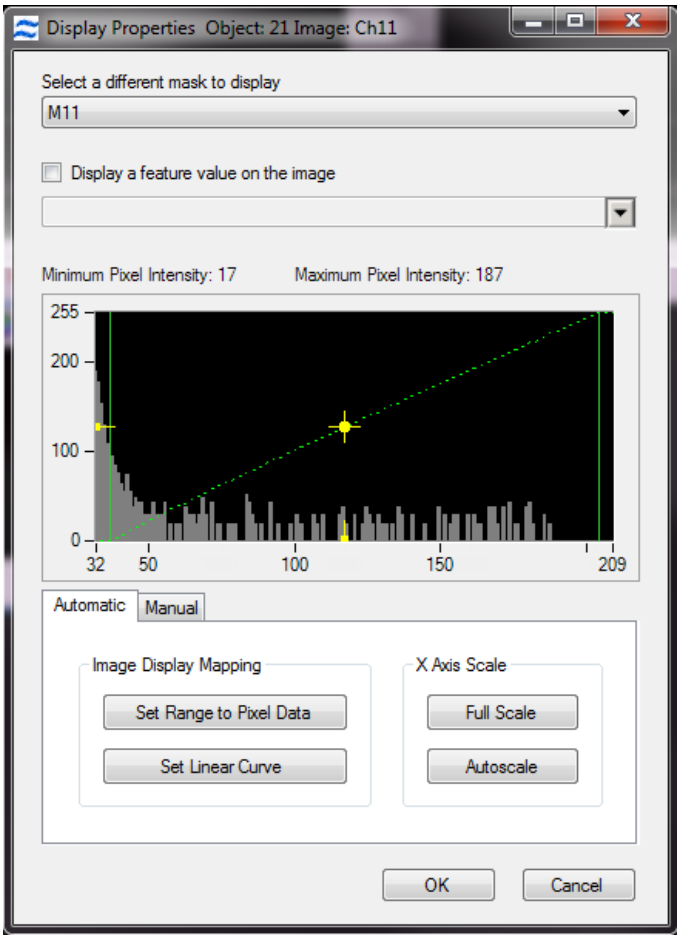
Click on the red line icon ( ) in the image toolbar to open the line profile tool, then click and drag to create a boxed area on the image.

The Image Statistics are shown in the image panel. The statistics are calculated for the area that is defined by the box. The line profile (the wavy line in the image panel) represents the pixel intensity at each position along the red line of the box.

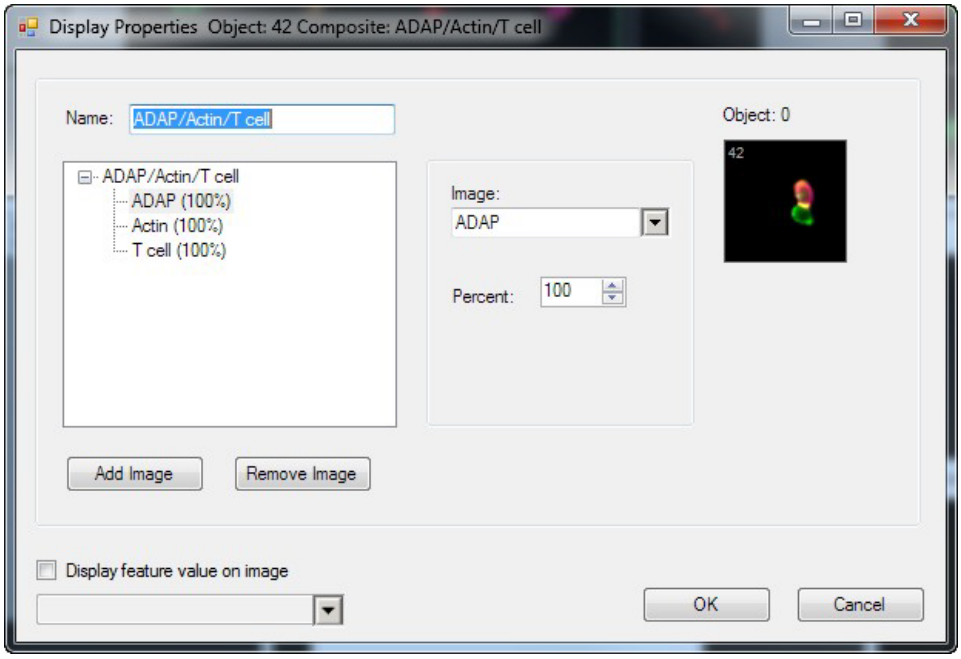


Change the Display Properties of an Image

1. Click the Channel Display Properties button ( ) on the image panel toolbar. The Display Properties window appears.
   a. For single channel image, change the displayed mask and adjust the display intensity mapping.



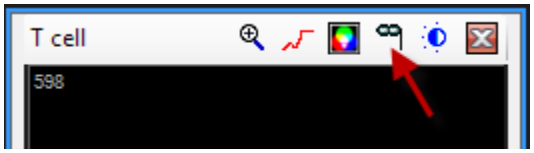
b. For a composite image, change the images in the composite and adjust the percent contribution of each image.



2. Click OK.

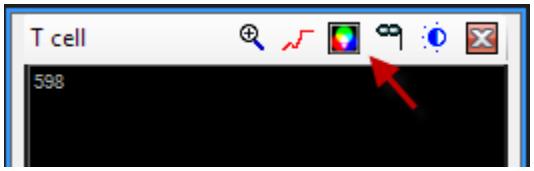
Show or Hide the Mask for a Single Channel Image

1. Click the Mask button on the image panel toolbar, or right click the image and then click Show/Hide Mask on the image context menu. The mask appears as a transparent overlay on the image.



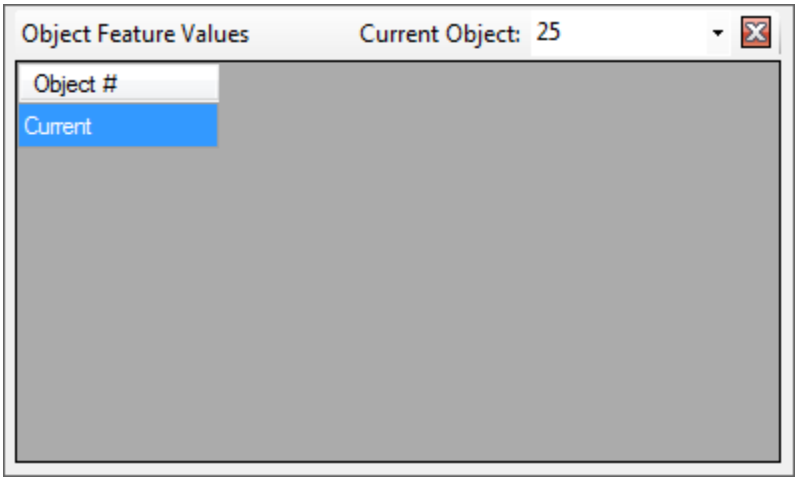
Turn the Color On or Off:

1. Click the Color button on the image panel toolbar, or right click the image and then click Color Off or Color On.



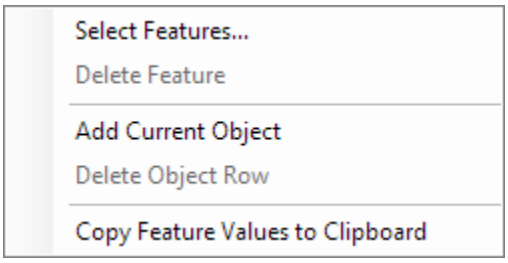
View the Object Feature Values

The Object Feature Values table displays a selected set of feature values for selected objects. For each feature, the name, value, and description are shown.

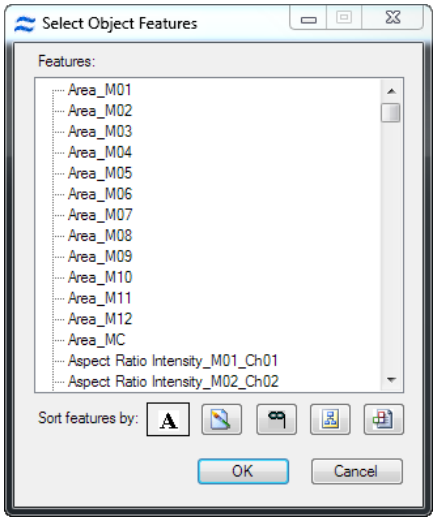


View and Customize Object Data Table Features

1. Click the Object Feature Values tool. 



2. Right click anywhere in the table area to open the menu.



3. Select Features. The Select Object Features window displays.
4. Select the features to view. Multiple features may be chosen by holding down the Ctrl key.
5. Click OK. The features appear in the Object Data table.

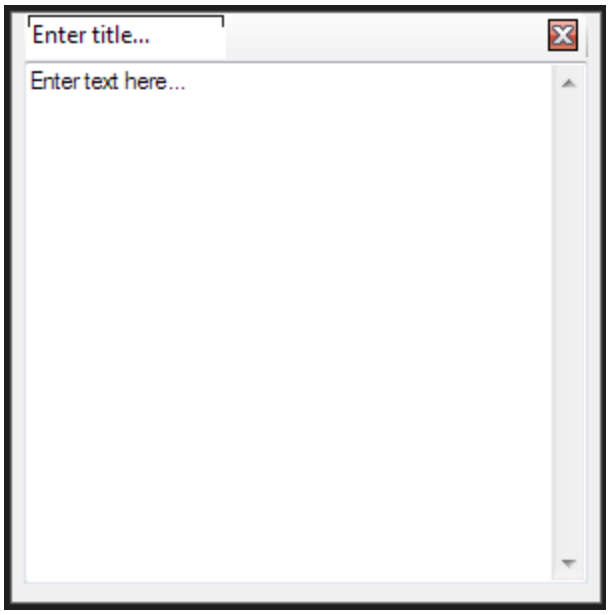
6. To add selected objects to the table right click and choose Add Current Object.

Export or Copy Feature Values

1. Right click in the table and choose Copy feature values to clipboard from the drop down menu.

Add Text to the Analysis Area

1. Click the Text button () on the Analysis Area toolbar. A text panel is added to the analysis area.



2. Enter a title in the title box and text in the text box.

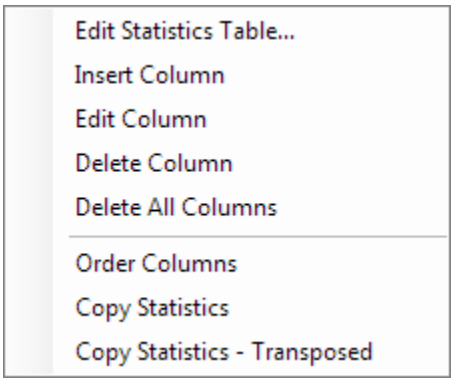
Population Statistics

The Population Statistics table displays selected statistics for chosen populations. The statistics that are supported are the Count, Percent Total, Percent Gated, Percent, Concentration (count/sample volume), Mean, Median, Standard Deviation, MAD (Median Average Deviation) RD -Mean, RD -Median, CV, Minimum, Maximum, Geometric Mean, Mode, Variance, and NaN (not a number).

View and Customize Population Statistics

1. Click the Populations Statistics tool. 
2. Click the + next to the population to expand the list of populations.
3. Columns can be moved by clicking dragging.

4. Right click in the gray area in the table or on a column heading. The context menu opens.



5. To open a Statistics Properties window to enable changes to multiple column statistics, click Edit Statistic Table.
6. To add a single statistic column, select Insert Column.
7. To make a change to a column, select Edit Column.
8. To delete a single column, right click on that column and select Delete Column.
9. To clear all statistics, select Delete All Columns.
10. To place all columns in the default order, select Order Columns.
11. To copy the selected rows of the table in a text format that can be pasted into other programs such as Microsoft® Excel, select Copy Statistics.
12. To copy the selected rows of the table and transpose the data so that the rows become columns when pasted into other programs such as Excel, select Copy Statistics Transposed.

Use the Feature Manager

This section describes how to create and delete features and to create multiple features by selecting categories.

Overview of the Feature Manager

The IDEAS® software defines a set of base features that you can use to create features for each object. To do so, you use the object's mask and/or its channel images. After a feature has been created and its value calculated for all cells, you can plot the feature values or view them as statistics for any population.

When the IDEAS® software opens a .cif or .rif file, the application calculates the values of features as defined by the selected template. You can refine your template so that it includes only those features of interest for your experiment.

You use the Feature Manager to examine existing features and to define new ones. To gain access to the Feature Manager, select Analysis > Features, or select it from one of the context menus that are available in the histogram and scatter plot panels with a right click. While the Feature Manager is open, all calculations for creating graphs and statistics are disabled. However, you may view images and change the population and channel views. When you close the Feature Manager, any changes to feature names, definitions, and values are reflected in any currently displayed graphs and statistics. The values of newly created features are also calculated at this time.

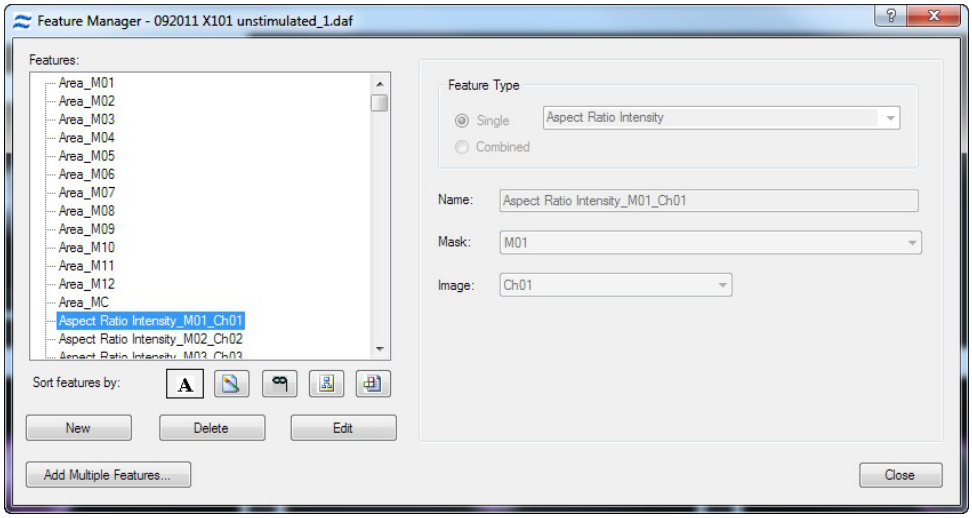
You can create single features and combined features. You create a single feature by selecting a base feature, such as Area or Intensity, along with a mask and/or an image. You can create a combined feature by defining a mathematical expression that includes one or more single features that exist in the feature list.

Some features, such as Area, depend on the boundary of a cell. These features require you to select a mask that defines the portion of the image to use for the calculation. Other features, such as Max Pixel, depend on pixel intensity measurements and require you to select an image. Other features require you to select a mask and one or more images.

You can add and remove features from the feature list. The feature definitions are stored in templates, so the definitions are available when you analyze multiple data files. The default template used for ImageStream® system data includes most of the base features for each channel image and channel mask that the feature list contains. Certain features, such as Similarity and Spot, require extensive calculations so the default template does not include them. The reason is to save time when you load files. However, you can add these features to the feature list.

View Existing Feature Definitions

1. Click Analysis > Features, or select Features from a graph panel context menu. The Feature Manager window appears.



2. Choose an icon to sort the features:

Table 14. Sorting Features Icons

Feature Icon	Function
	Sorts features alphabetically.
	Sorts features based on the images used.
	Sorts features based on the masks used.
	Sorts features by category, such as size, location, shape, texture, signal strength, and system.
	Sorts by base features, such as area, aspect ratio, intensity, and object number.

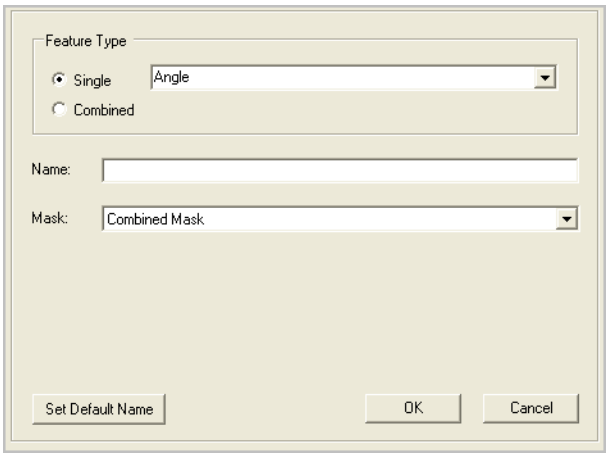
3. Click a feature in the Features list to view its definition in the right side of the window.

Create New Features with the Feature Manager

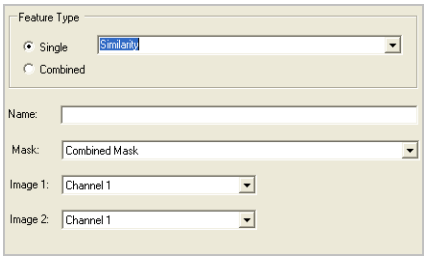
Create a Single New Feature

A single feature uses the definitions of a base feature along with a mask and/or an image.

1. Click New in the Feature Manager. The right hand area of the Feature Manager is enabled.



2. Select Single as the Feature Type and choose the base feature in the drop down menu. The Mask and Image lists become visible, depending on the single feature selected.



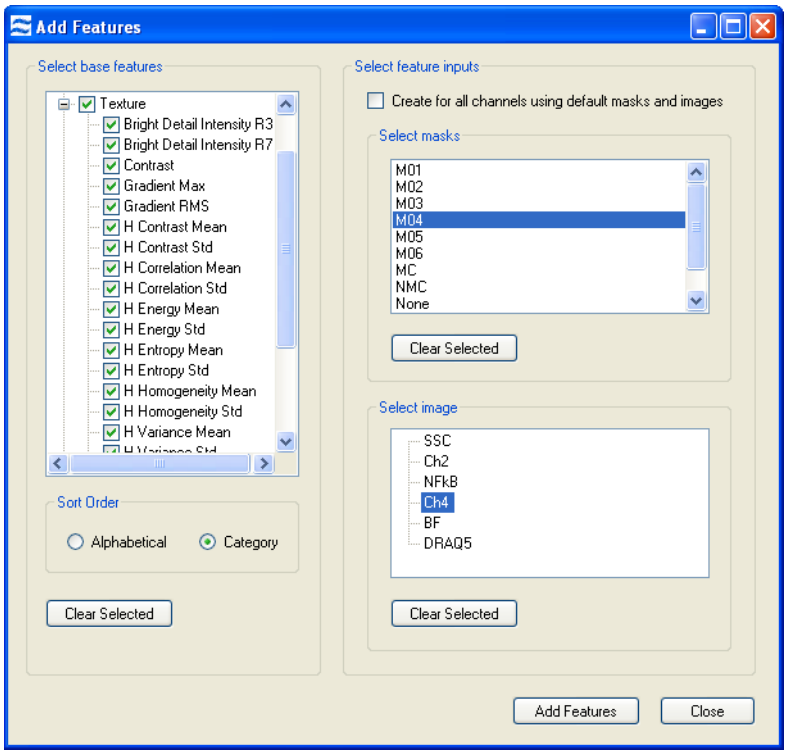
3. Select the mask and/or image that you want.
4. Click Set Default Name. The default name is the name of the base feature followed by the name of the mask and name(s) of the image(s).
5. Click OK to add the new feature. It appears in the Features list on the left side of the Feature Manager.
6. Click Close.

   NOTE: When you close the Feature Manager, the IDEAS® software calculates values for the new features. These calculations may take several minutes, depending on the number and complexity of the new features and the size of the image file.

Create Multiple New Features

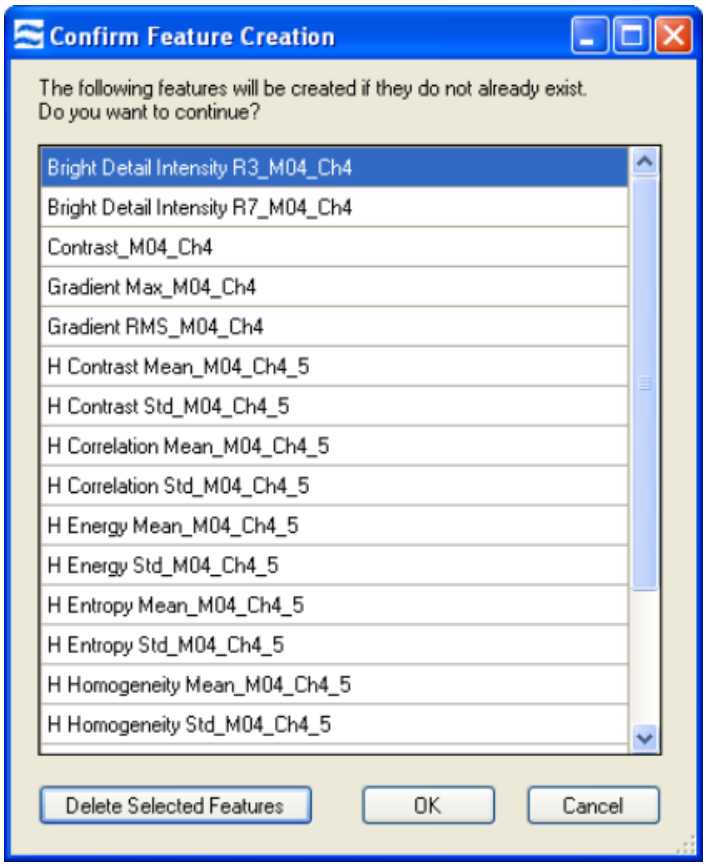
1. Click Add Multiple Features in the Feature Manager.
2. Sort the feature list alphabetically or categorically.
3. Select multiple base features and masks.

4. Select image(s) or check the box to create for all channels using default masks and images.



5. Any list can be cleared by clicking the Clear Selected button.
6. When finished, click Add Features to add the new features to the list.

7. Confirm the features in the next window.

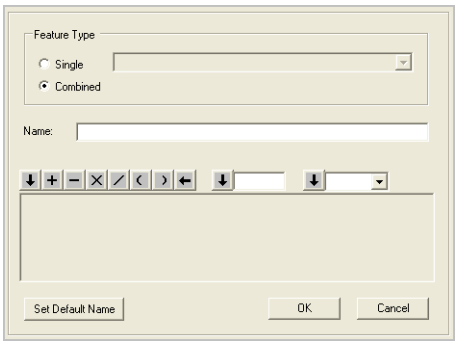


8. Delete any features you do not want to calculate.
9. Click OK when finished. The new features are added to the list in the feature manager.
10. Close the Add Features window.
11. Close the Feature Manager. The new features are calculated when the feature manager closes.

Create a New Combined Feature

A combined feature uses one or more single features created by a mathematical expression.

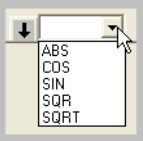
1. Click New in the Feature Manager. The right hand area of the Feature Manager is enabled.
2. Select Combined as the Feature Type. The editing interface appears.



3. Enter the feature name in the Name box or use Set Default Name after you have created your expression. The default name is the name of the definition created.

4. Use the toolbar to build a definition (mathematical expression) of features and operators:

Table 15. Combined Feature Tasks and Toolbar

Task	Toolbar
Add a feature to the definition.	Double click the feature in the Features list. Or, single click the feature in the Features list and select click the leftmost down arrow button ( ) on the toolbar.
Add an operator or a parenthesis to the definition.	Click the corresponding button on the toolbar. + − × / ( ) ←
Add a number to the definition.	Enter the number in the box and then click the corresponding down arrow button. 3.14 If the area is greyed out, an operator must be selected first.
Add a function to the definition.	 Select the function in the list and then click the corresponding down arrow button. The available functions are ABS (absolute), COS (cosine), SIN (sine), SQR (square), and SQRT (square root). If the area is greyed out, an operator must be selected first.
Remove an item from the end of the definition.	Click the left arrow button () on the toolbar.

5. Click OK.
6. Click Close.

   NOTE: When you close the Feature Manager, the IDEAS® software calculates values for the new features. These calculations may take several minutes, depending on the number and complexity of the new features and the size of the image file.

Delete a Feature

1. Select one or more features in the Features list by clicking them. To select more than one feature, use the Ctrl key.
2. Click Delete. A warning message will confirm or cancel deletion.

   NOTE: Deleting a feature also deletes any populations that are dependent on that feature. Your feature list may become large and unwieldy. You can narrow down the list without deletions by sorting the list.

Create Multiple Features and Rank Features by Discriminating Power

With the IDEAS® software, you are able to create an unlimited set of features by using the Mask Manager to define location and the Feature manager to choose a mathematical expression that uses the image pixel data and/or the mask.
This can make it challenging to choose a feature that provides good statistical separation of populations of cells that have different appearances from each other.

IDEAS® software versions 6 and up include the 'Feature Finder' wizard, which creates a set of masks and features automatically and determines the features with the most discriminating power for the chosen truth populations. It may be useful for certain applications to create new masks and features that are not created by the wizard. Once features are created using this manual method, the Feature Finder wizard will test all features in the data file.

A general description of the steps is followed by a specific example.

General Workflow

1. Set image display and draw preliminary regions to include cells of interest (i.e., single, focused, positive cells).
2. Visually inspect overall quality of images and experiment to determine whether to proceed or redo the experiment.
3. Create two tagged 'truth' populations of cells that represent the phenotypes you wish to discriminate. Perform the discrimination on one characteristic difference at a time.
4. Create any additional masks and features you think may help differentiate the truth populations.
5. Calculate the statistical discrimination (RD) between the two populations afforded by features in 1 category at a time. Pick the top feature for each category. Alternatively, use the Feature Finder wizard which measures the RD but only keeps the three features per category with the highest RD.
6. Plot the features with the highest RD for the truth populations for each category
7. Validate by applying the feature to the base population, independent controls if available and on multiple files and experiments.

Example

Treatment induced actin polarization. The data file is available for practice.

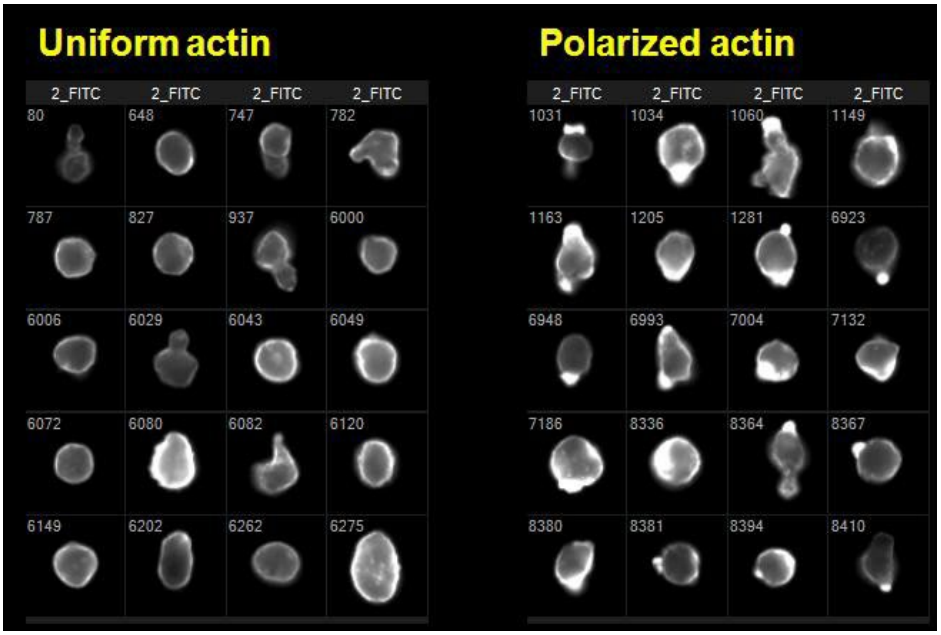
- Cells were incubated with inducing compound for 1 hour.
- The nucleus was probed with DAPI and actin stained with FITC.
- Large event image files were collected on the ImageStream® system.
- Compensation and analysis was done in IDEAS® software.

The following steps find the best features that distinguish changes in actin distribution.

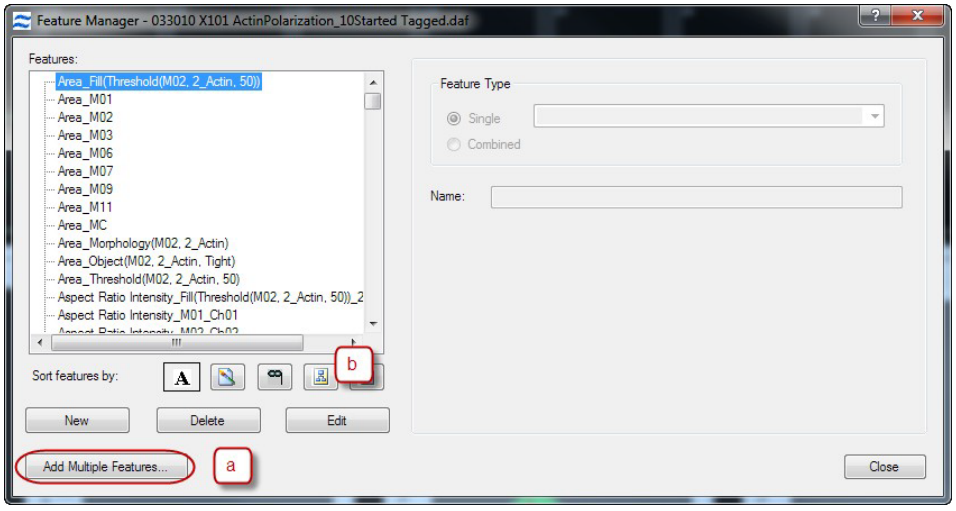
1. Gate single, focused, actin positive cells. View cells of interest.

2. Create the truth populations from within the cells of interest using the tagging tool.

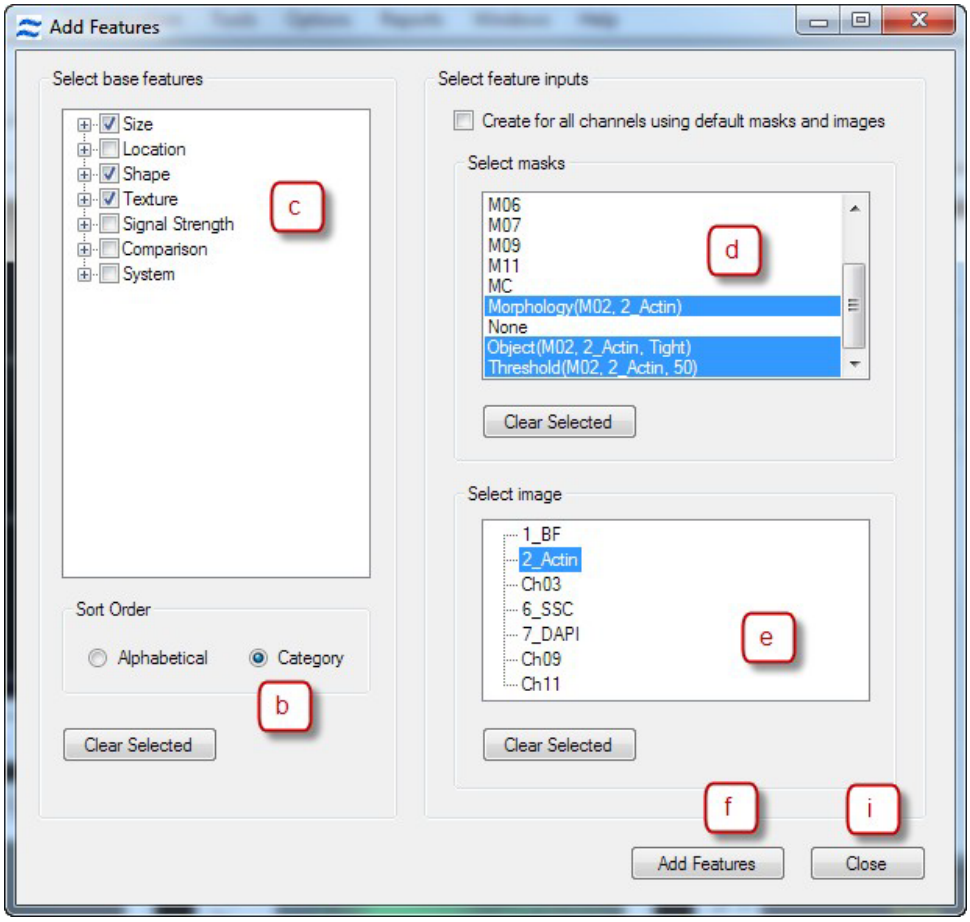
   NOTE: If truth populations are in different files, merge them together before beginning. When selecting truth populations, choose images that represent the full phenotypic range of each truth. In this example case, note that the 'uniform actin' truth population contains cells of varying shape and intensity that all have uniform actin distribution. Bias introduced during the selection of truth populations will likely also bias the outcome during statistical ranking. The following figure shows the truth populations chosen to find a feature to discriminate uniform versus polarized actin:



3. Create the Morphology and one or more Threshold masks for the actin image.
4. Create features from the Size, Shape, and Texture categories using the Morphology, Threshold and Default actin channel masks.
   a. Choose Features from the Analysis menu and click Add Multiple Features.



b. In the Add Features window select Category as the 'Sort Order'.



c. Check Size, Shape and Texture base feature boxes.
d. Select the actin masks (Morphology, Object, Threshold, M02)
e. Select the actin image (2_Actin).
f. Click Add Features to display the list of features to add.
g. In the next window, click OK to add the features. Features that already exist will not be recalculated.
h. Click OK and click Close.
i. Close the Feature Manager by clicking Close. The features are calculated.

5. Add the feature statistics to the population statistics table. Do this one category at a time. Multiple statistics tables can be added to the analysis area, one for each category of features. Alternatively, go to the Feature Finder wizard to continue with the RD calculation and ranking.
6. Once the features are calculated you can use the RD, (Fischer's Discriminant Ratio) to a statistics table. The RD measures the separation between two populations. In this case, the two truth populations picked in Step 2. In order to get the statistic for one category at a time, select all of the features for the image and then deselect categories to leave one category for the channel selected.

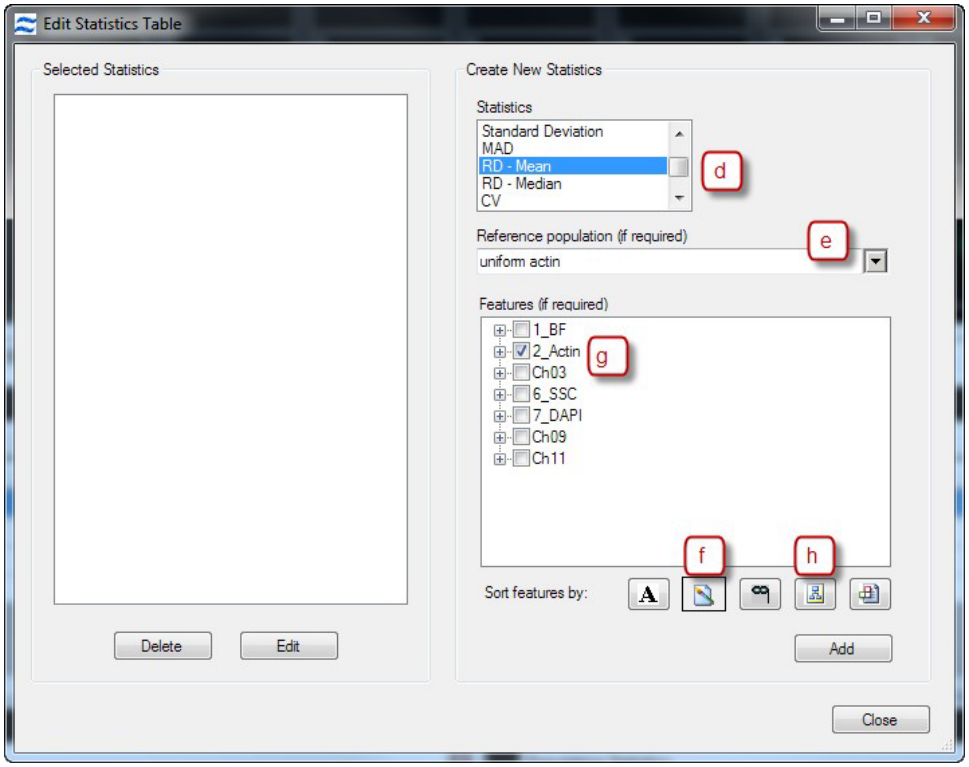
a. Click on Σ to add a statistics table to the analysis area.
b. Right click in the table and choose Edit Statistics Table.
c. Delete any statistics from the list.
d. Select the RD -Mean statistic .

e. Select one of the truth populations in the Reference population box.

f. Sort by Images Used by clicking on the icon  .

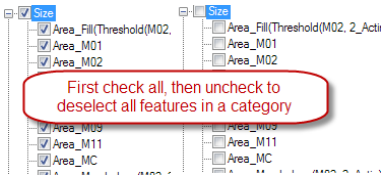
g. Check the box for the Ch02/Actin image.

h. Sort the features by Category  .



i. Deselect all but one category by checking and unchecking the box for the categories you want to deselect.

NOTE: The box next to the category will be checked only if all of the features (all channels) in the category are selected.



j. Click Add Statistics.

k. Click Close.

l. Repeat Step 6 each statistics table contains 1 category of features for Ch02(Actin).

7. Launch Microsoft® Excel, and then Copy and Paste the statistics into the Excel spreadsheet.

a. Select the row of statistics for the 2nd truth population (the one not chosen above).

b. Right click in the statistics table and choose Copy Statistics Transposed.

c. Paste into an Excel spreadsheet.

d. Keep all of the features and values selected and sort the data set on the values column (heading may be the population name) largest to smallest. The feature with the largest RD will be at the top.

   NOTE: You may have NaN values for some of the features. This means Not a Number and occurs in some cases when there is a division by 0. These can be ignored.

8. Validate the features in IDEAS® software. Plot the features with the highest RD for the truth populations and draw regions to discriminate.
9. Apply regions to the base population, independent controls if available and on multiple files and experiments.
10. Look for false negative and positive cells.
11. Repeat process if necessary by refining/creating new truth populations.

NOTE: When evaluating features, consider the features that produce the highest RD. If there are any intensity based features make sure that the staining was not uneven due to technical issues. If it is a size feature, does it make sense with what you know about the cells and biology of your experimental system? Since the feature value ranges vary between features this is an approximate comparison and the result should be validated by viewing images across the feature range from the whole population.

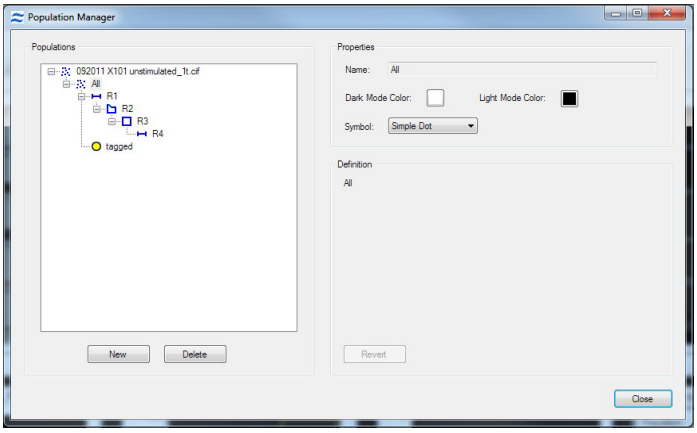
Use the Population Manager

A population is a group of objects. You create populations by drawing regions on graphs, by hand selecting (tagging) objects in the Image Gallery or on plots, or by combining existing populations. After a population has been defined, you can view it in the Image Gallery or on a plot and you can use it to calculate statistics.

The Population Manager provides a central place for maintaining the display properties of existing populations and for creating new combined populations.

Open the Population Manager and View Population Definitions

1. Select Analysis > Populations or right click a graph and select Populations. The Population Manager window displays.



NOTE: The list of populations is presented as a hierarchy that shows the dependencies of the populations on each other. The icon associated with a population indicates how the population is defined.

Icon	Defined by
	Tagging
	Region
	Combined

NOTE: The definition of a selected population is shown in the Definition area.

Edit the Display Properties of a Population

1. Within the Population Manager, click a population in the Populations list.
2. Change the name in the Name box.
3. Click a Color square to select a new color on the color palette and click OK.

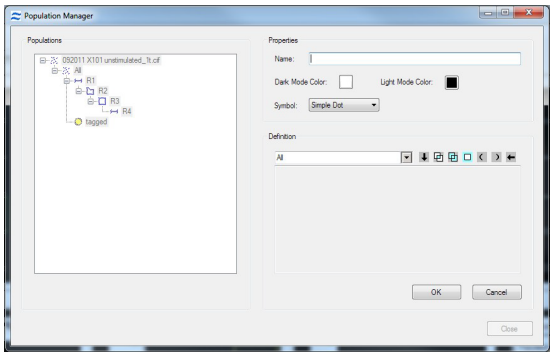
4. Click a display symbol in the Symbol drop down menu.
5. Click Close to save the population changes.
6. Click Revert to reject the changes.

Delete a Population

1. Within the Population Manager, click a population in the Populations list.
2. Click Delete. A confirmation warning message appears indicating all the dependent populations that will also be deleted.
3. Click Yes to confirm.

Create a New Combined Population

1. Within the Population Manager (Analysis > Populations), click New. The right side of the Population Manager window changes to allow you to define a new population.



2. Enter a unique population name in the Name box.
3. Click a Color square to select a new color on the color palette and click OK.
4. Click a display symbol in the Symbol drop down menu.
5. Use the toolbar to build the population definition as described in the table. Click OK when done:

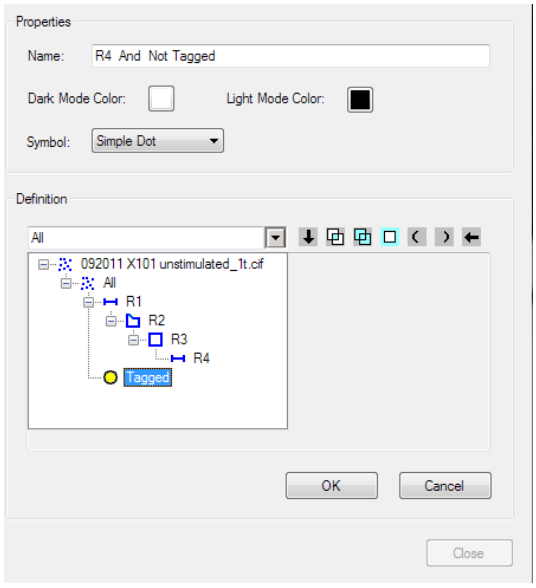


Table 16. Population Tasks and Toolbar

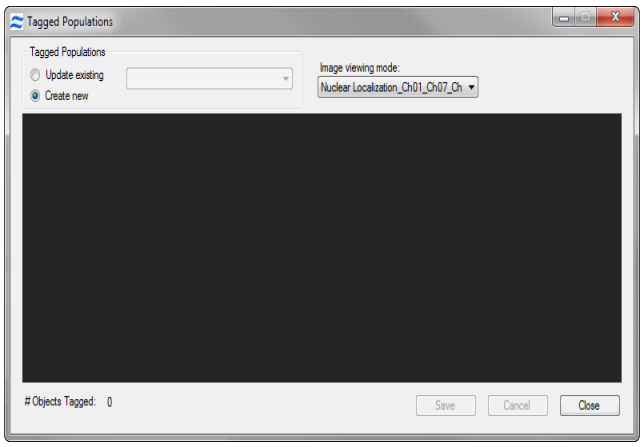
Task	Toolbar
Add a population to the definition.	Select the population from the drop down menu.
Combine two populations.	Use the Boolean AND or OR operator.  Use the AND operator to include only the objects that are in both of the original populations.  Use the OR operator to include the objects that are in either one of the original populations.
Select objects that are not in the original population.	Use the Boolean NOT operator.  The NOT operator specifies which population will not be used. **NOTE**: You must use AND before NOT.
Affect the order of operations.	Use the parentheses toolbar buttons. 
Remove an item from the end of the definition.	Click the left arrow ) button on the toolbar.

Create Tagged Populations

You can hand select objects from either the Image Gallery or a graph and group them into a population.

Create a Hand Selected Population

1. Click the Tagging Mode toolbar button ( ) to begin. The Tagged Population window displays.



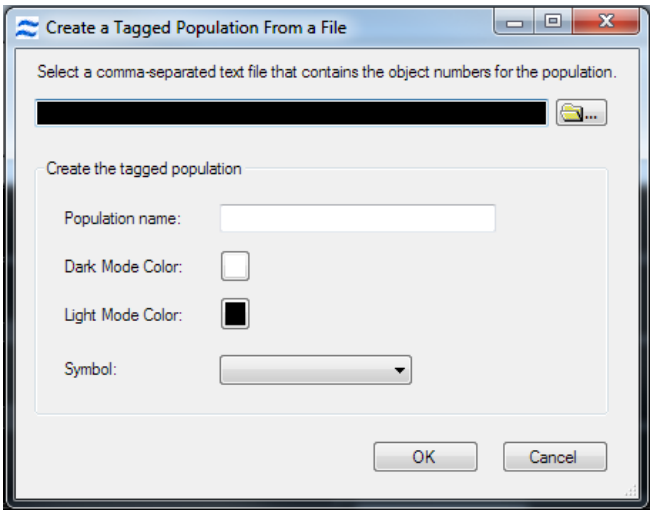
2. Select either Update existing or Create New.
   a. To Create New, double click images within the Image Gallery and select Save. Enter a new population name. Each population is given a new color and the symbol solid diamond for ease of viewing in plots.
3. To Update existing, choose a population to update in the drop down menu.
4. In the Image viewing mode list, choose the mode that you want from the drop down menu.
5. To add or remove an image from the tagged population, double click either the image in the Image Gallery or a dot in a bivariate plot. The selected channel image for each tagged cell is displayed in the viewing area of the Tagged Populations window. In the Image Gallery, a small smiley face icon appears on the left side of each tagged image. Each tagged object is also displayed as a yellow star in a graph in the Analysis Area. The number of objects in the tagged population is updated in the bottom, left corner.
6. If you are updating an existing population, click the Update button in the Tagged Populations window.
7. When you are finished updating, click Close in the Tagged Populations window.

   NOTE: The tagging mode remains open until you click Close, and as long as the Image Gallery is in tagging mode, you cannot create, resize, or move any regions on the graphs.

Create a Tagged Population from a File of Object Numbers

Use a comma separated text file of object numbers to create a tagged population.

1. Navigate to File > Tools > Create Tagged Population.



2. Browse for the file.
3. Name the population, select the color, symbol and click OK.

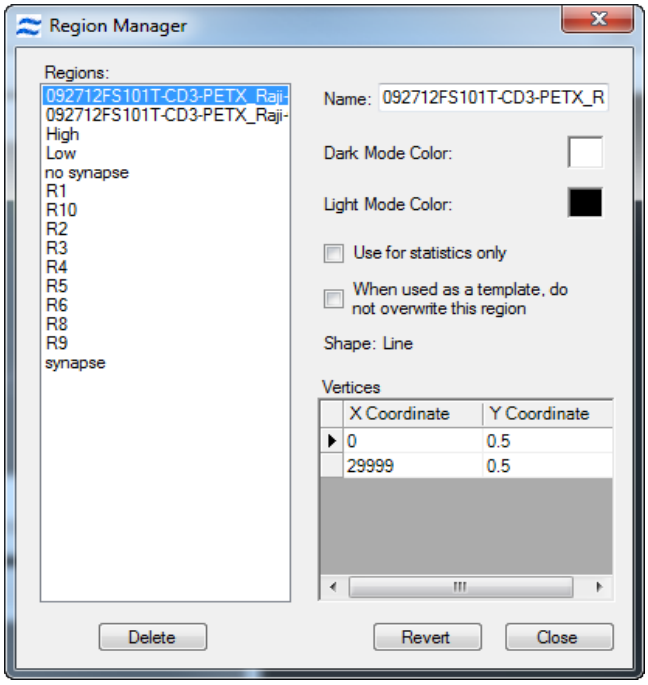
Use the Region Manager to View and Edit Regions

The Region Manager provides a central place for defining the display properties, names, and positions of existing regions. Regions can be deleted in the Region Manager tool.

If a file is going to be used as a template and you do not wish to change certain regions in the new daf files you may check the box 'When used as a template, do not overwrite this region' and the specific regions will be preserved in the new daf files.

Regions are drawn on graphs to create new populations, based on the physical location of objects on a graph, and to compute statistics. Tools for drawing regions are found on the Analysis Area toolbar.

1. Select Analysis > Regions or right click a graph and select Regions to open the Region Manager.
2. To view a region, click a region in the Regions list. The selected region's settings display on the right side of the Region Manager.



3. To edit a region, adjust the settings from the right side of the Region Manager.
   a. Change the name in the Name box.
   b. Click a Color square to select a new color on the color palette. Click OK from the color palette when finished.
   c. Change the X or Y position of the vertices in the Vertices box.
   d. Select or deselect the Use for statistics only box.
   e. Click Delete to delete a region.

      NOTE: When a region is deleted, all populations that are defined by that region will be deleted. A warning dialog box appears listing the populations that will be deleted.

   f. Click Revert to reject any changes made to the selected region.
4. Click Close when finished with the Region Manager.

Create Reports and Export Data

Once you have finished analyzing an experiment, you will want to report the results into third party applications. To streamline the process, the first step is to define your application defaults, image gallery settings, and statistics report definitions.

Report Images and Graphs

The IDEAS® software allows users to copy and print images and graphs, and export statistics, feature data, pixel data, or TIF files for separate analyses.

Prepare the Image Gallery and Analysis Area for Reporting

1. Before you print or copy images, optimize the image display using Image Gallery properties.
2. In addition to formatting the graphs and statistics in the Analysis area, the IDEAS® software provides color mapping from the dark mode that you see in the Analysis Area to a light mode that has a white background for the printing and exporting of data. Because the population colors might not show on a white background, you can change
   the colors when using the light mode.

   a. Click the graph background tool ( ) to switch between light mode and dark mode for graphs in the Analysis Area.

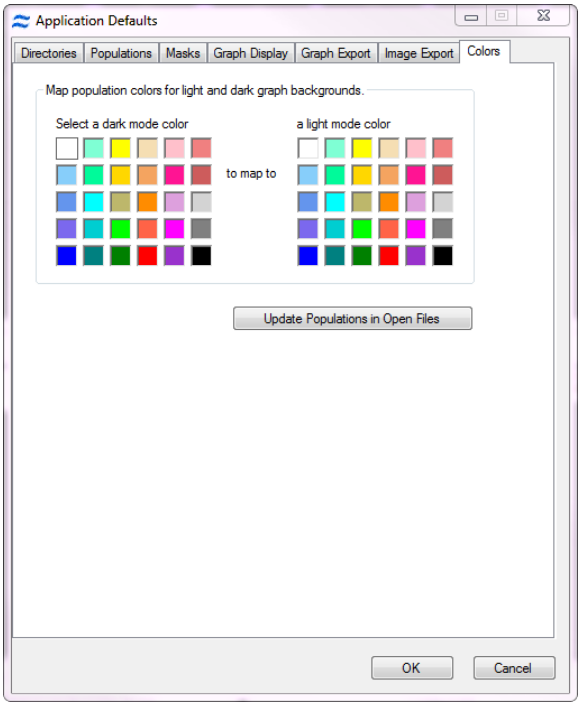
   b. To map dark mode colors to light mode colors:

      i. Select the Colors tab from the Application Defaults window.

      ii. Select a dark mode color to map to a light mode color.

      iii. Click Update Populations in Open Files.

      iv. Click OK to save the changes or Cancel to exit.



Copy Full or Partial Screens

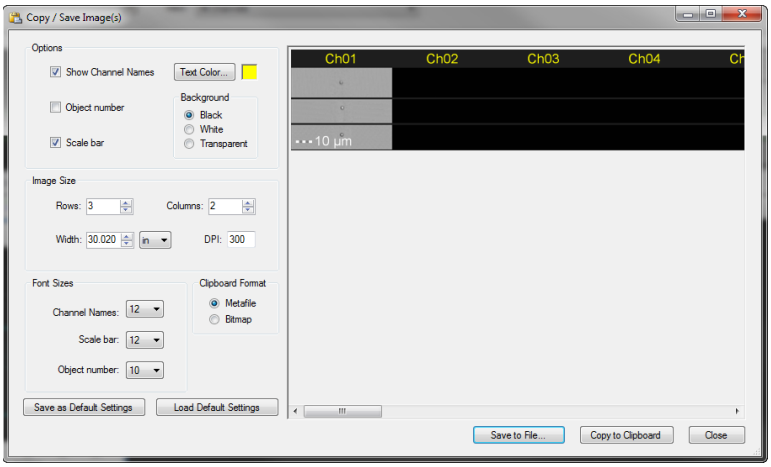
- To copy the entire screen to the Clipboard, press CTRL+PRINT SCREEN. The image is then available for pasting into a third party application.
- To copy the IDEAS® software window to the Clipboard, select the window and then press ALT+PRINT SCREEN. The image is then available for pasting into a third party application.

Print the Analysis Area or Image Gallery Data Directly

- To print the Analysis Area data, select Reports > Print Analysis Area. The® IDEAS® software prints all the graphs, statistics, text panels, and images that are displayed in the Analysis Area.
- To print the Image Gallery data, select Reports > Print Image Gallery. The® IDEAS® software prints all the images that are visible in the Image Gallery.

Copy Images to the Clipboard or Save Images to Files

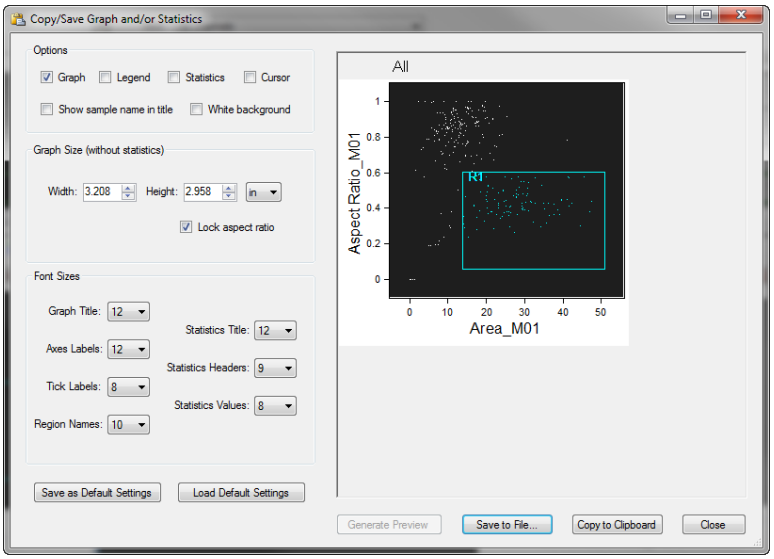
1. Right click anywhere in the Image Gallery and then choose one of the following options:
   - Copy/Save Image for a single image
   - Copy/Save Object Images for the row of images for one object
   - Copy/Save Gallery Column for the images of one channel in the current gallery
   - Copy/Save Gallery for all images in the current gallery
2. A preview of the image(s) is shown. Changes from the default settings may be made to the following:
   - Show Channel Names
   - Object number
   - Scale bar
   - Text Color for channel names
   - Background
   - Number of rows and columns
   - Image Size and DPI settings
   - Font Sizes and Clipboard Format



3. (Optional) Click Save as Default Settings to save the settings as default, or click Load Default Settings to load settings from a file.
4. Click Save to File or Copy to Clipboard when done. Files may be saved as .png, .bmp or .tif formats.
5. If Copy to Clipboard was chosen, paste the images into a third party application.

Copy Graphs to the Clipboard or Save Graphs to a File

1. Right click anywhere in the graph and choose Copy/Save Graph and/or Statistic.
2. A preview of the graph is shown. Changes from the default settings may be made to the following:
   - Include Graph
   - Include Legend
   - Include Statistics
   - Include Cursor
   - Show sample name in title
   - White background on/off
   - Graph size
   - Font sizes
3. If changes are made, they can be previewed by clicking Generate Preview. Otherwise, changes will be applied when Save to File or Copy to Clipboard is chosen.
4. (Optional) Click Save as Default Settings to save the settings as default, or click Load Default Settings to load settings from a file.
5. Click Save to File or Copy to Clipboard when done. Files may be saved as .png, .bmp or .tif formats.
6. If Copy to Clipboard was chosen, paste the graphs into a third party application.



To export graph statistics to the Clipboard, right click a graph and then click Export Statistics To Clipboard. The graph statistics are available for pasting into a third party application.

Copy Population Statistics from a Statistics Table

- Right click the table and then click Copy Statistics or Copy Statistics transposed. They are then available for pasting into a third party application.

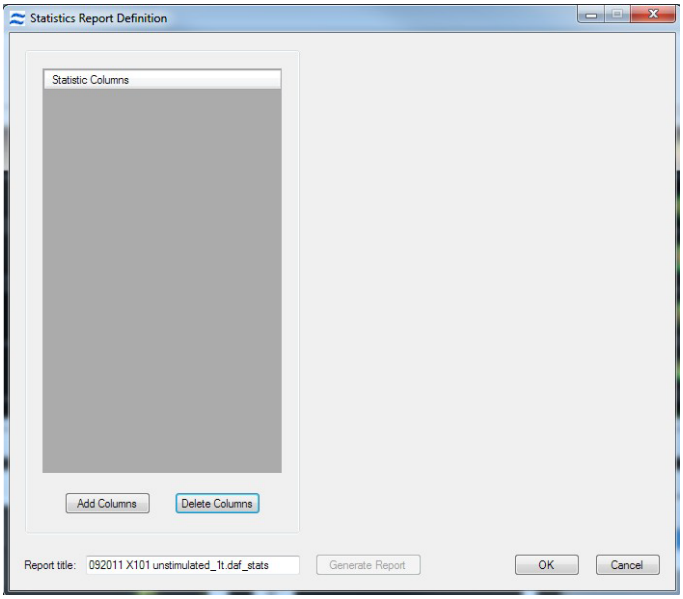
Report Statistics

Define a Statistics Report

A statistics report definition can be saved in a .daf file or an .ast template file. It allows users to select specific statistics within a .daf file and open the data in Excel.

A statistics report can be generated during batching if it is part of the template used. It may also be applied to preexisting .daf files from the Reporting menu. In this case, the rest of the template is not processed—only the report. The statistics report definition allows you to specify population percentages and feature statistics and the layout of the report is accessed from the reporting menu.

1. Select Reports > Define Statistics Report. The Statistics Report Definition appears.



2. Enter a Report title.
3. Under the Statistic Columns click Add Columns. The Add Statistic Report Column window opens.
4. Select the statistic(s) in the Statistics list.

   - Count - the absolute count of the populations
   - %Total - percentage of a population as a percentage of All
   - %Gated - the percent of one population as a percentage of another, but not used for tagged populations
   - % - the percentage of one population as a percentage of another, also is used for tagged populations
   - Objects/mL - the concentration of the population in the sample run.
   - CV - the coefficient variable
   - Geometric Mean - standard statistical definition
   - Maximum - standard statistical definition

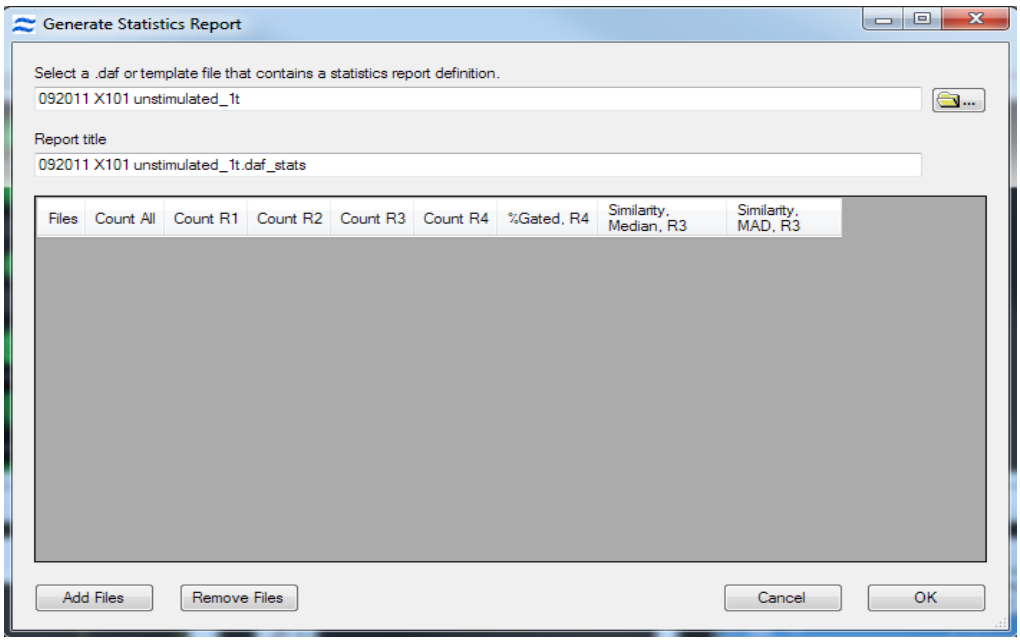
- Mean - standard statistical definition
- Median - standard statistical definition
- MAD - standard statistical definition
- Minimum - standard statistical definition
- Mode - standard statistical definition
- RD - Median - the ratio discriminant (Fisher's discriminant) using the Median and MAD
- RD - Mean - the ratio discriminant (Fisher's discriminant) using the Mean and StdDev
- Standard Deviation - standard statistical definition
- Variance - standard statistical definition
- NaN - stands for not a number; the number of objects whose values are not valid numbers.

5. Select a population to base on the selected statistic(s).
6. Select a reference population if necessary. This is required for % and RD.
7. Select a Feature. This is not necessary for the %-related statistics, Count, or Objects/ml.
8. Click Add Statistics. The statistic is added to the list.
9. Click Close when finished.
10. Select a statistic in the list to view the definition or edit any input.
11. Change the name of the statistic by clearing Use default title and typing a new name if desired.
12. Delete Columns removes a selected statistic.
13. To reorder the list click drag a statistic to its new location.
14. Click Generate Report when complete to generate a report for a current (opened) .daf file. A prompt appears to save the text file. This text file can be opened from Excel.
15. If you do not want to generate a report, click OK to save your changes and exit the window.
16. The saved template can generate a statistics report for multiple data files by selecting Generate Statistics Report from the Reports menu or during batch processing.

Generate a Statistics Report using .daf Files

Once a Statistics Definition has been created, the user can generate a statistics report from multiple .daf files. However, these files must use the same template. Batch Processing can also generate a statistics report where statistics for each data file will be generated either for .rif, .cif, or .daf files. Generating a statistics report under the Reports menu simply adds the statistics template to the specified .daf files.

1. Select Reports > Generate Statistics Report. The current .daf file appears in the window with the specified statistics columns.



2. Pick a Report Definition. The definition may be obtained from a .daf or .ast file.
3. Change the Report title if desired.
4. Additional .daf files can be added or removed with the Add Files or Remove Files buttons.
5. Reorder the files as desired by selecting files and then right click the new location in the list and choose move here. You can use Ctrl select multiple files in the desired order, then move them all at once by right clicking in the desired location and choosing Move Here.
6. Click OK.
7. A prompt will confirm that the .daf file will be saved. The report title name will be used as the default file name for the report. In the above example, the file generated will be named “Report 1.txt”. If the report title contains illegal characters—such as “\/><“—the default filename will change to “Statistics Report.txt”. Tab delimited text format is used for the report.
8. View the report in IDEAS® software by choosing View Statistics Report from the Reports menu. The report can be copied to the clipboard and pasted into a spreadsheet program or the .txt file can be opened using a spreadsheet program.

Report Statistics from a Single Graph or Statistics Table

Statistics can also be reported directly from an open .daf from the graph or statistics tables in the analysis area.

- To export graph statistics to the clipboard, right click a graph and then click Export Statistics To Clipboard. They are then available for pasting into a third party application.
- To copy population statistics from a Statistics Table, right click the table and then click Copy Statistics or Copy Statistics transposed. They are then available for pasting into a third party application.

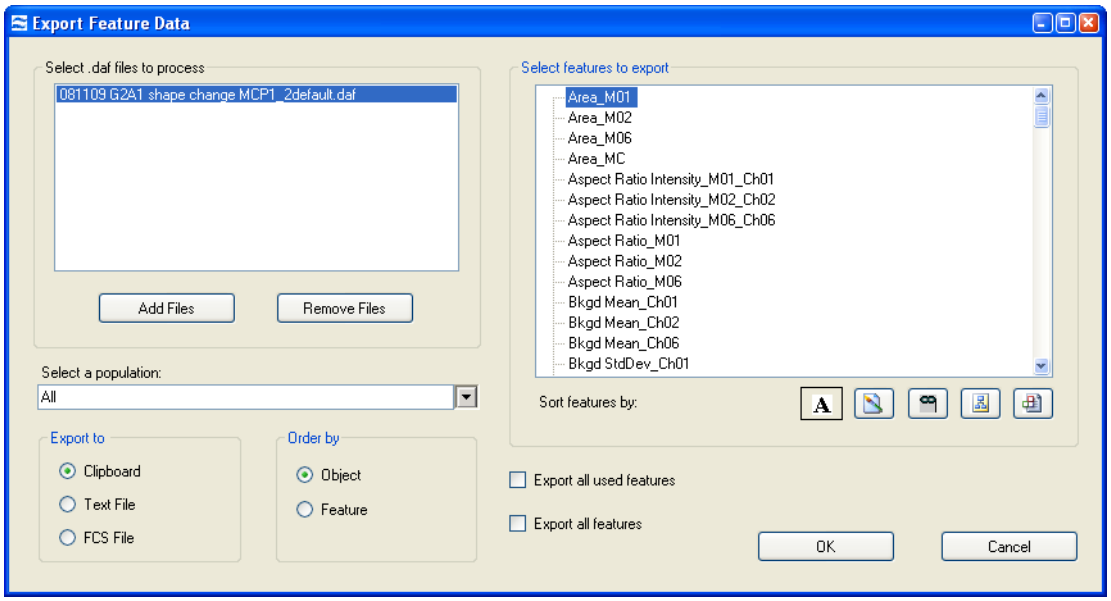
Export Data

You can export feature values for a population to the Clipboard, a text file, or a Flow Cytometry Standard (FCS) file. You can export pixel intensity values for an object to the Clipboard or a text file. Later, you can open or paste the FCS file into a spreadsheet or other programs that uses the FCS file format. Keep in mind, however, that limitations might exist on the number of values that these programs can import.

Export Feature Data

Exporting feature data is useful if you want to create an fcs file or graph the feature data in a third party graphing application.

1. On the Tools menu, click Export Feature Values. The Export Feature Data window appears.



2. Add files to the list on the left to export values for multiple files.
3. In the Select a population drop down menu, select the population that you want. If you haven't defined any populations, All is the default.
4. In the Select feature values to export area, select features by clicking items in the list or hold down the Ctrl while clicking to select multiple items.
5. Select the Export to option that you want.

   NOTE: Data exported to the Clipboard can be pasted directly into a spreadsheet program.

6. Select the Order by option that you want.

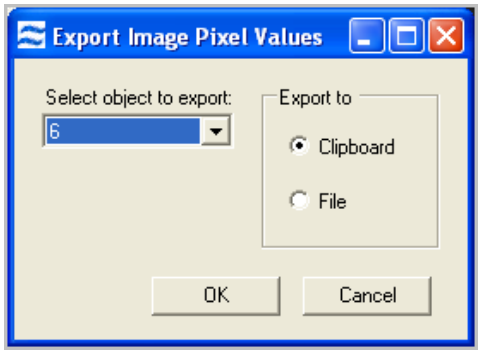
   NOTE: Ordering by object causes the values to be listed in a column, whereas ordering by feature causes the values to be listed in a row.

7. Click OK.

Export Pixel Data

Exporting pixel data is useful when importing the data into third party programs where you would need to graph the individual pixels.

1. On the Tools menu, click Export Image Pixel Values. The Export Image Pixel Values window appears.

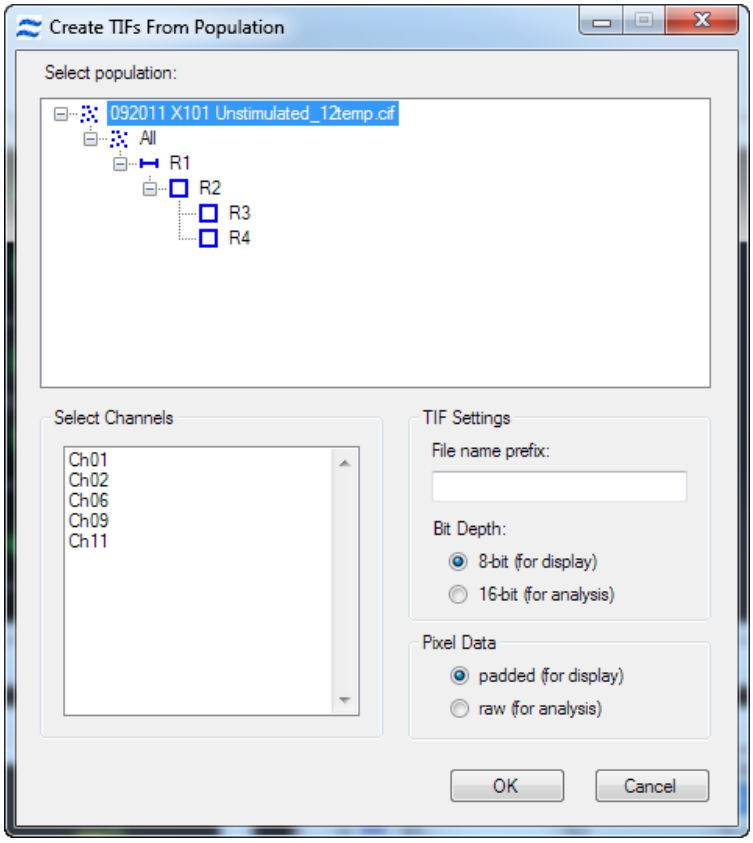


2. Select the object to export in the drop down menu.
3. Select to Export to either the Clipboard or File.
4. Click OK.
5. Paste into desired application.

Create TIFs from Population for Export

The IDEAS® software allows users to create separate TIF files for channel images for every event in that population. The exported TIF files can be opened in image viewing applications that support 8 bit tif format for display or 16 bit tif format for analysis.

1. On the Tools menu, click Export .tif Images. The Create TIFs From Population window displays.



2. Select the population and channels.
3. Type a prefix for the TIF file name in the File name prefix field.
4. Select the bit depth in the Bit Depth section.
5. Select padded or raw in the Pixel Data section.
6. Click OK. A TIF file is created for every selected channel within the selected population.

Print Data

Print Analysis Area Data

Select Reports > Print Analysis Report.

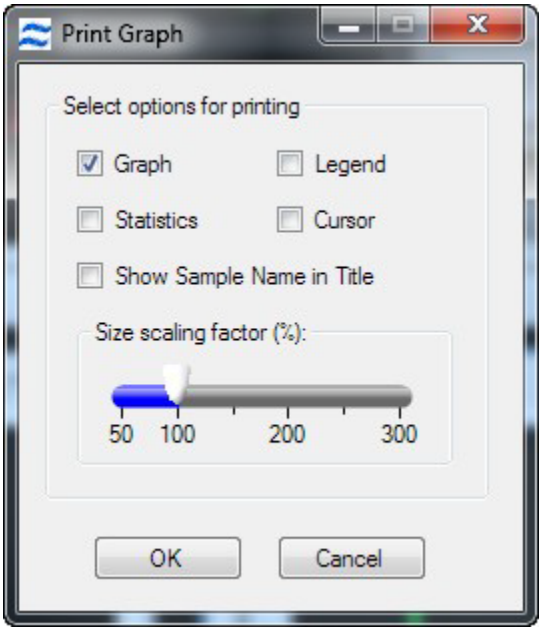
The IDEAS® software prints all the graphs, statistics, text panels, and images that are displayed in the Analysis Area. In IDEAS® software version 6.2 and later, additional information has been added to the printed report to assist users in tracking changes that may have been made in the files. This includes laser information, compensation matrix and region coordinates.

Print Image Gallery Data

Select Reports > Print Image Gallery. The IDEAS® software prints all the images that are visible in the Image Gallery.

Print an Individual Graph

1. Right click the graph and then select Print Graph on the graph context menu. The Print Graph window displays.



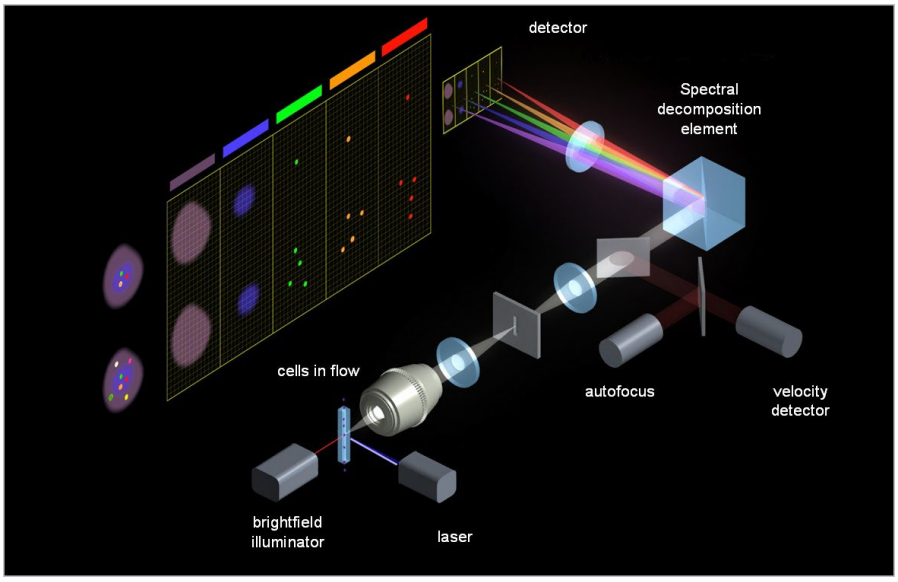
2. Select the check boxes Graph, Statistics, Legend, Cursor, Show Sample Name in Title to include the elements in the report.
3. If necessary, adjust the size scaling factor. The recommended setting is 100%.
4. Click OK to print the graph.

Chapter 4
Features and Masks

This chapter describes the features that the IDEAS® software uses for data analysis:

Overview of the IDEAS® Software Features and Masks

Objects passing through a Cytek® Amnis® cell analysis system are illuminated in different directions by lasers and/or brightfield LEDs. Light emitted from the object is focused through an objective lens and relayed to a spectral decomposition element, which divides the light into six spectral bands located side by side across a charge coupled detector (CCD), as shown in the following diagram. Therefore, each object has six images that can be individually analyzed or, because they are in spatial register with respect to one another, reconstructed. Each of the separate bands is called a channel. Below is an example of collecting 6 images. The ImageStream®^{X} system has a second camera option which enables collection of up to 12 images per object. The FlowSight® system has 12 channels collection on 1 camera.



About Features

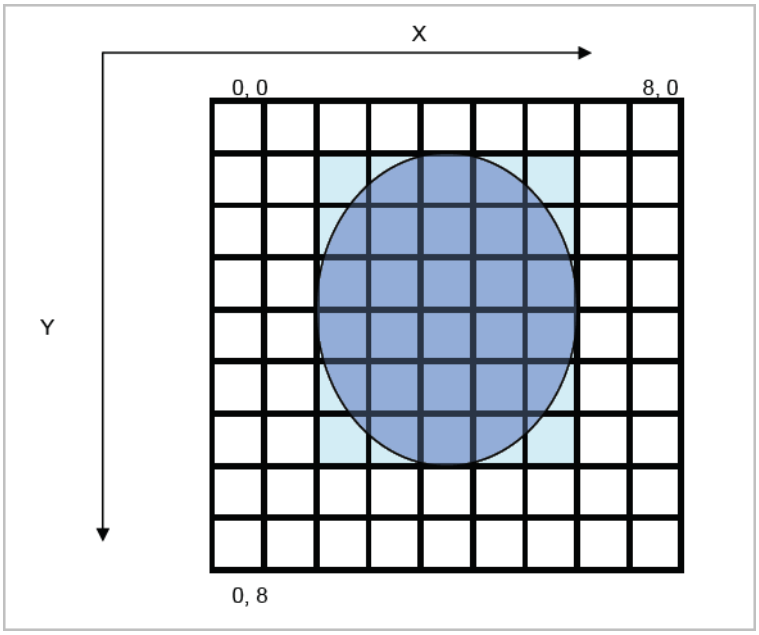
The IDEAS® software provides a large selection of criteria, or features, for analyzing images. A feature is described by a mathematical expression that contains quantitative and positional information about the image. A feature is applied to specific locations of an image by the use of a mask that identifies pixels within the region of interest of the image. A few system features, such as Object Number, Camera Background and Flow Speed, do not require calculations, masks, or image intensity information.

There is a slight difference in features created during data acquisition and those in IDEAS® software . During acquisition features are created with the INSPIRE™ software mask. Features and masks are calculated in IDEAS® software for files collected with the ImageStream® system.

New masks and features can be created in files from an ImageStream® system using the Mask and Feature Manager tools. Features are created in IDEAS® software using base feature algorithms, such as Area or Intensity along with a mask and/or a channel image for files created with an ImageStream® system. The default masks are recomputed in IDEAS® software for ImageStream® system or FlowSight® system. Combined features can be created using existing features in mathematical expressions in the Feature Manager.

IDEAS® software groups the features into eight categories: size, location, shape, texture, signal strength, comparison, system and combined.

To calculate the value of a feature, the IDEAS® software maps the channel image to X and Y coordinates, as illustrated by the following diagram. Each box in the diagram represents a pixel.



The pixel size and field of view per channel is dependent on the magnification used. See the INSPIRE™ Software User Manual for more information.

Feature Categories

Size

Size features are in microns and include Area, Diameter, Length, Major Axis, Minor Axis, Major Axis Intensity, Minor Axis Intensity, Perimeter, Thickness Max and Min, Spot Area Min, Width and Height.

Location

Location features include Angle, Angle Intensity, Centroid X, Centroid Y, Centroid X Intensity, Centroid Y Intensity, Delta Centroid X, Delta Centroid Y, Delta Centroid XY, Max Contour position, Spot Distance Min, Valley X and Valley Y.

Shape

Shape features define the mask shape and have units that vary with the feature. They include the Aspect Ratio, Aspect Ratio Intensity, Compactness, Elongatedness, Lobe Count, and Symmetry 2,3,4.

Texture

The Texture features determine local intensity variations in images and include Bright Detail Intensity R3 and Bright Detail Intensity R7, Contrast, Gradient Max, Gradient RMS, H Texture (H-Contrast, H-Correlation, H-Energy, H-Entropy, H-Homogeneity, and H-Variance), Modulation, Spot Count, and Std Dev.

Contrast, Gradient Max and Gradient RMS are generally used to determine best focus.

Comparison

The Comparison features describe the difference of intensity measurements between masks or pixels in different images or the same image with different masks. These include Bright Detail Colocalization 3, Bright Detail Similarity R3, Intensity Concentration Ratio, Internalization, and Similarity.

System

The system features do not require a mask.

Base Features - Alphabetical

Table 17. Table of Base Features – Alphabetical

Feature Name	Category
Angle Feature	Location
Area Feature	Size
Aspect Ratio Feature	Shape
Aspect Ratio Intensity Feature	Shape
Bkgd Mean Feature	Signal Strength
Bkgd StdDev Feature	Signal Strength
Bright Detail Intensity R3 and Bright Detail Intensity R7 Features	Signal Strength
Bright Detail Similarity R3 Feature	Comparison
Camera Line Number Feature	System
Camera Timer Feature	System
Centroid Features	Location
Centroid X Intensity and Centroid Y Intensity Features	Location
Circularity Feature	Shape
Compactness Feature	Shape
Contrast Feature	Texture
Delta Centroid X and Delta Centroid Y Features	Location
Delta Centroid XY Feature	Location
Diameter Feature	Size
Elongatedness Feature	Shape
Ensquared Energy Feature	Texture
Flow Speed Feature	System

Feature Name	Category
Gradient Max Feature	Texture
Gradient RMS Feature	Texture
Height Feature	Size
H Texture Features	Texture
Intensity Concentration Ratio Feature	Comparison
Intensity Feature	Signal Strength
Internalization Feature	Comparison
Length Feature	Size
Lobe Count Feature	Shape
Major Axis and Minor Axis Features	Size
Major Axis Intensity and Minor Axis Intensity Features	Size
Max Contour Position Feature	Location
Max Pixel Feature	Signal Strength
Mean Pixel Feature	Signal Strength
Median Pixel Feature	Signal Strength
Min Pixel Feature	Signal Strength
Major Axis Intensity and Minor Axis Intensity Features	Size
Modulation Feature	Texture
Object Number Feature	System
Objects/ml Feature	System
Objects/sec Feature	System
Perimeter Feature	Size
Raw Centroid Features	Location
Raw Intensity Feature	Signal Strength

Feature Name	Category
Raw Max Pixel Feature	Signal Strength
Raw Mean Pixel Feature	Signal Strength
Raw Min Pixel Feature	Signal Strength
Raw Median Pixel Feature	Signal Strength
Saturation Count Feature	Signal Strength
Saturation Percent Features	Signal Strength
Shape Ratio Feature	Shape
Shift X and Shift Y Features.	Location
Shift Features	Comparison
Similarity Feature	Comparison
Spot Area Min Feature	Size
Spot Count Feature	Texture
Spot Distance Min Feature	Location
Spot Intensity Min and Spot Intensity Max Features	Signal Strength
Std Dev Feature	Texture
Symmetry 2, 3, 4 Features	Texture
Thickness Max Feature	Size
Thickness Min Feature.	Size
Time Feature	System
Valley X and Valley Y Features	Location
Width Feature	Size
XCorr Feature	Comparison

Base Features by Category

Table 18. List of Features by category

Category	Feature Name And Description	In Default Template	In Expanded Default Template	Default Mask
Size	Size-based Features are in microns.			
	Area Feature: The size of the mask in square microns.	Yes	Yes	M01-M12 MC
	Diameter Feature Estimates the diameter of the mask based on Area.	No	Yes	M01-M12
	Height Feature: Based on a bounding rectangle, the Width is the smaller side and the Height is the longer side of the rectangle.	Yes	Yes	M01-M12
	Length Feature: Measures the longest part of the mask.	Yes	Yes	M01-M12
	Major Axis and Minor Axis Features: Describes the widest part of the mask and the narrowest part of the mask, respectively.	No	Yes	M01-M12
	Major Axis Intensity and Minor Axis Intensity Features: Based on a bounding ellipse, the Minor Axis is the narrow part, and the Major Axis is the widest part.	No	Yes	M01Ch1-M12Ch12
	Perimeter Feature: Describes circumference of the mask.	No	Yes	M01-M12
	Spot Area Min Feature: The Area of the smallest spot in the mask.	No		Custom
	Thickness Max Feature: Describes the longest width of the mask.	No	Yes	M01-M12
	Thickness Min Feature: Describes the shortest width of the mask.	No	Yes	M01-M12
	Width Feature: Based on a bounding rectangle, the Width is the smaller side, and the Height is the longer side of the rectangle.	Yes	Yes	M01-M12

Category	Feature Name And Description	In Default Template	In Expanded Default Template	Default Mask
Location	Location Features are in X,Y pixel coordinates from an origin in the upper left corner, pixels or contour			
	Angle Feature: The angle of the major axis from a horizontal plane in radians	No	No	Custom
	Angle Intensity Features: The angle of the major axis intensity from a horizontal plane in radians	No	No	Custom mask, select channel
	Centroid Features: The central tendency of the pixels along the X Axis and Y Axis, respectively.	No	Yes	M01-M12
	Centroid X Intensity and Centroid Y Intensity Features: The central tendency of the pixels along the X Axis and Y Axis, respectively, with the pixel intensities weighted.	No	Yes	M01Ch1-M12Ch12
	Delta Centroid X and Delta Centroid Y Features: The distance between the X or Y Centroids of two images.	No	No	Custom
	Delta Centroid XY Feature: The distance between the Centroids of two images.	No	No	Custom
	Max Contour Position Feature: The location of the contour in the cell that has the highest intensity concentration.	No	No	Custom
	Shift X and Shift Y Features: The shift required to get the highest cross correlation value between two images.	No	No	Custom
	Spot Distance Min Feature: The shortest distance between two components (spots).	No	No	Custom
	Valley X and Valley Y Features: The (X,Y) coordinates of the minimum intensity withing the skeletal lines that are used when creating the Valley Mask	No	No	Custom

Category	Feature Name And Description	In Default Template	In Expanded Default Template	Default Mask
Shape	Shape Features define the mask shape and have units that vary with the feature			
	Aspect Ratio Feature: The ratio of the Minor Axis divided by the Major Axis.	Yes	Yes	M01-M12
	Aspect Ratio Intensity Feature: The ratio of the Minor Axis Intensity divided by the Major Axis Intensity.	Yes	Yes	M01Ch1-M12Ch12
	Circularity Feature: The degree of the mask's deviation from a circle.	No	No	Custom
	Density Feature: Describes the density of intensities within the object.	No	No	Custom
	Elongatedness Feature: The ratio of the Height/Width which use the bounding box.	No	Yes	M01-M12
	Lobe Count Feature: The number of lobes in a cell.	No	No	Custom
	Shape Ratio Feature: The ratio of Thickness Min/Length features.	No	Yes	M01-M12
	Symmetry 2, 3, 4 Features: These three features measure the tendency of the object to have a single axis of elongation, a three-fold and a four-fold variation of the shapes.	No	No	Custom

Category	Feature Name And Description	In Default Template	In Expanded Default Template	Default Mask
Texture	Texture features measure pixel or regional variation and indicate the granularity or complexity of the image.			
	Bright Detail Intensity R3 and Bright detail Intensity R7 Features: The Intensity of the pixels in the bright detail image using a 3 or 7 pixel structuring element.	Yes (R3)	Yes	M01Ch1-M12Ch12
	Contrast Feature Enumerates changes of pixel values in the image to measure the focus quality of an image.	No	Yes	M01Ch1-M12Ch12
	Gradient Max Feature The maximum slope of the pixel value changes in the image to measure focus quality of an image.	Yes	Yes	M01Ch1-M12Ch12
	Gradient RMS Feature Enumerates changes of pixel values in the image to measure the focus quality of an image.	Yes	Yes	M01Ch1-M12Ch12
	H Texture Features Measures Haralick texture features.	No	Yes	M01Ch1-M12Ch12
	Modulation Feature Measures the intensity range of an image, normalized between 0 and 1.	Yes	Yes	M01Ch1-M12Ch12
	Spot Count Feature Enumerates the number of spots.	No	No	Custom
	Std Dev Feature Describes the overall distribution of pixel intensities.	No	Yes	M01Ch1-M12Ch12

Category	Feature Name And Description	In Default Template	In Expanded Default Template	Default Mask
Signal Strength	Signal strength features are measured in pixel values under the mask, and correlate with intensity measurements			
	Bkgd Mean Feature The average intensity of the camera background.	Yes	Yes	Ch01-Ch12
	Bkgd StdDev Feature The standard deviation of the background intensities.	No	Yes	Ch01-Ch12
	Intensity Feature The sum of the pixel intensities in the mask, background subtracted.	Yes	Yes	MCCh1-MCCh12
	Max Pixel Feature The largest pixel value within the mask, background subtracted.	Yes	Yes	MCCh1-MCCh12
	Mean Pixel Feature The average pixel value within the mask, background subtracted.	Yes	Yes	M01Ch1-M12Ch12
	Median Pixel Feature The median pixel value within the mask, background subtracted.	Yes	Yes	M01Ch1-M12Ch12
	Min Pixel Feature The smallest pixel value within the mask, background subtracted.	No	No	Custom
	Raw Intensity Feature The sum of the pixel intensities within the mask.	No	No	Custom
	Raw Max Pixel Feature The largest pixel intensity.	Yes	Yes	MCCh1-MCCh12
	Raw Mean Pixel Feature The average pixel intensity: Raw does not have background subtracted.	No	No	Custom
	Raw Min Pixel Feature The lowest pixel value within the mask.	Yes	Yes	MCCh1-MCCh12
	Saturation Count Feature The number of pixels in the mask that are saturated.	Yes	Yes	M01Ch1-M12Ch12
	Saturation Percent Features The Percentage of pixels in the mask that are saturated.	Yes	Yes	M01Ch1-M12Ch12
	Spot Intensity Min and Spot Intensity Max Features The raw intensity (not background subtracted) of the dimmest component (spot).	No	No	Custom

Category	Feature Name And Description	In Default Template	In Expanded Default Template	Default Mask
Comparison	Measure the differences in intensity values between masks and or pixels			
	Bright Detail Colocalization 3: Measures the colocalization of 3 image channels	No	No	Custom
	Bright Detail Similarity R3 Feature: Measures the correlation of the bright details between image pairs.	No	No	Custom
	Intensity Concentration Ratio Feature: Given two masks, the ratio of the intensity in one mask to the total intensity in both masks.	No	No	Custom
	Internalization Feature: The ratio of the intensity inside the cell to the intensity of the entire cell.	No	No	Custom
	Similarity Feature: The Similarity is a measure of the degree to which two images are linearly correlated pixel by pixel within a masked region.	No	No	Custom
	XCorr Feature: The XCorr is a measure of the degree to which two images frequencies are cross correlated.	No	No	Custom

Category	Feature Name And Description	In Default Template	In Expanded Default Template	Default Mask
System	System features do not require a mask and tend to deal with system wide metrics.			
	Camera Line Number Feature: An incremental count of objects.	No	Yes	NA
	Camera Timer Feature: The clock rate in KHz. This is relative time.	No	Yes	NA
	Flow Speed Feature: The calculated flow speed in mm/sec.	Yes	Yes	NA
	Object Number Feature: The sequence of objects.	Yes	Yes	NA
	Objects/ml Feature: A local concentration of all objects per ml. **NOTE**: to get objects per ml of a population, use the statistic 'Concentration'.	No	Yes	NA
	Objects/sec Feature: A local concentration of number of objects per second. **Note**: to get objects per ml of a population, use the statistic 'Concentration'	No	Yes	NA
	Time Feature: The camera timer feature, converted to seconds.	Yes	Yes	NA

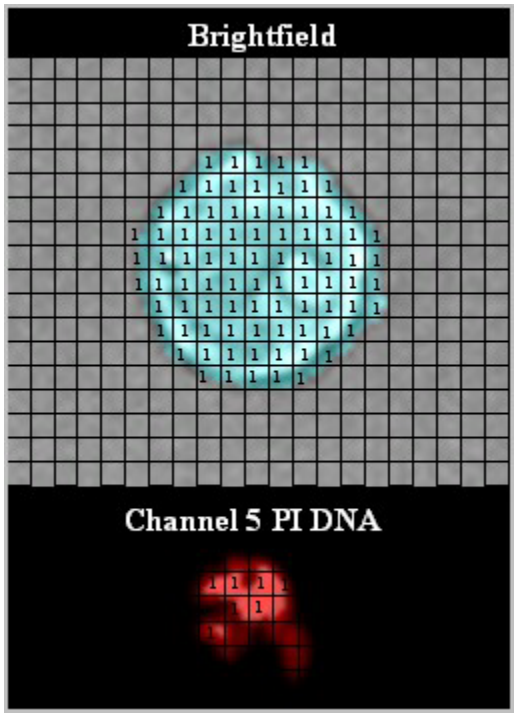
Category	Feature Name And Description	In Default Template	In Expanded Default Template	Default Mask
Combined	Combined features are created using the Combine feature tools in the feature manager and allow users to add, subtract, multiply and divide existing features from each other.			
	N/C Ratio: Calculated by creating a nuclear and cytoplasmic mask, creating the area feature for each mask, and dividing the nuclear area by the cytoplasmic area	No	No	Custom
	Percent Overlap: Calculated by generating two (typically spot) masks, creating a combined mask of mask A and mask B (overlapping portion) creating the area feature of each mask, then dividing the overlap mask area (A and B) by the larger mask A area *100 to give percent. Area (Mask A and B) / Area (Mask A) *100 = %Overlap	No	No	Custom

Size Features

Size features are in microns and include Area, Diameter, Length, Major Axis, Minor Axis, Major Axis Intensity, Minor Axis Intensity, Perimeter, Thickness Max and Min, Spot Area Min, Width and Height.

Area Feature

The number of microns squared in a mask is equal to the Area. In the following figure, a 1 symbolizes whether the area is included in the mask. The number of pixels is converted to m^2. Note that 1 pixel = 0.25 m^2. As an example, a cell with a mask that includes 2000 pixels is therefore equal to 500 m^2.



Application Examples

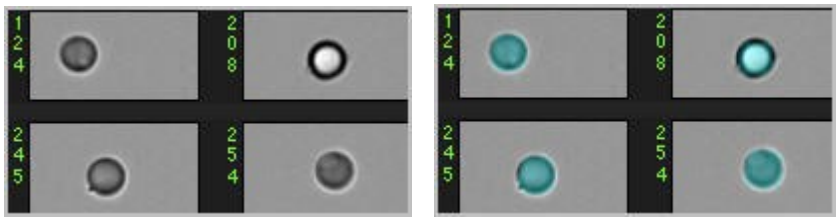
- Quantify and compare cell size.
- Identify single cells.
- Calculate the radius, diameter and volume of the cell.
- Identify apoptosis using the Area of the 30% threshold mask of a nuclear dye.
- Create a pseudo FSC vs. SSC plot for comparing with flow cytometry.

Diameter Feature

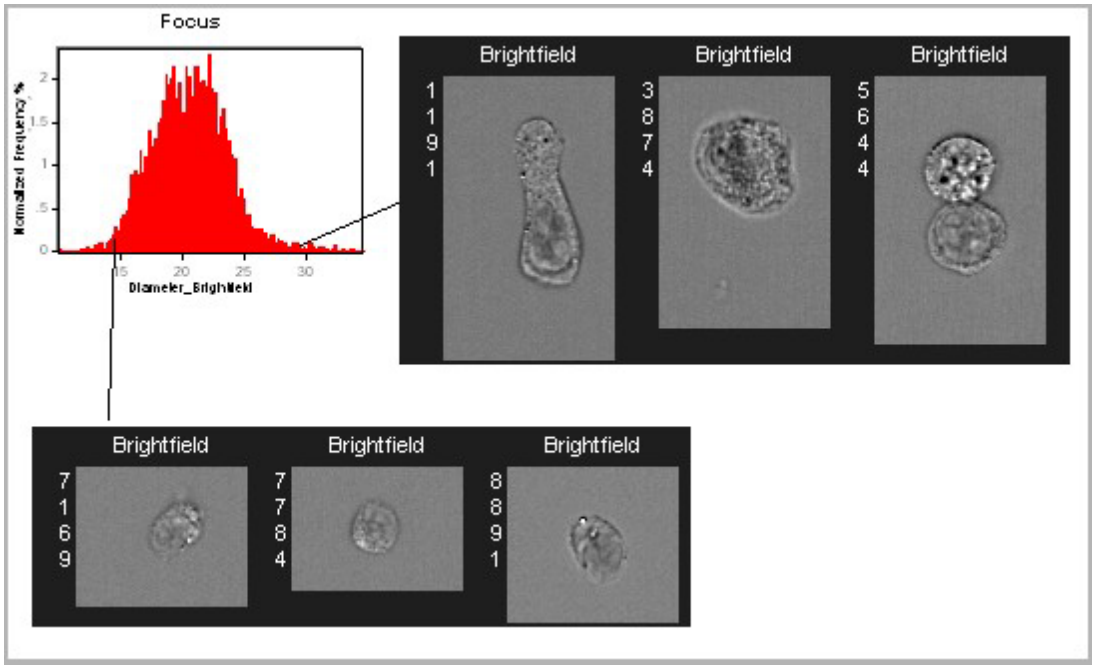
The Diameter feature provides the diameter of the circle that has the same area as the object. The accuracy of the diameter is highly dependent on a close fitting mask and roundness of the cell.

$$Diameter = 2 \times \sqrt{\frac{Area}{\pi}}$$

The images below depicts beads with a uniform diameter of 9 microns.



In the next figure, note that images with longer shapes that have the same area will have the same diameter value.

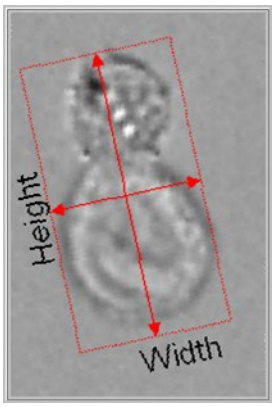


Application Example:

- Used to obtain approximate size of the cell.

Height Feature

Using the bounding rectangle, Height is the number of microns of the longer side and Width the shorter side.



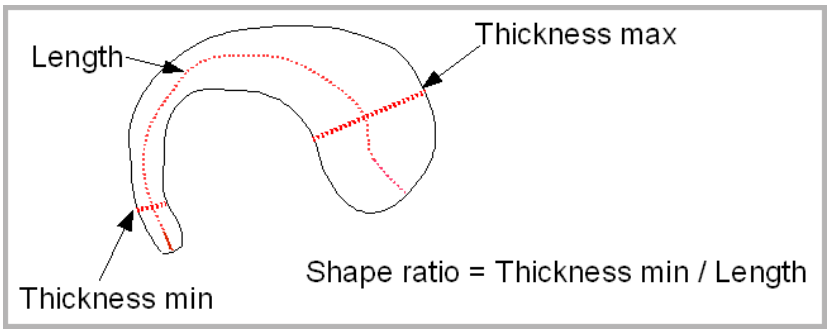
Application Example

- These features can be used to separate rectangular shaped objects. For curved objects, measurement is more accurately obtained using the thickness features.

Length Feature

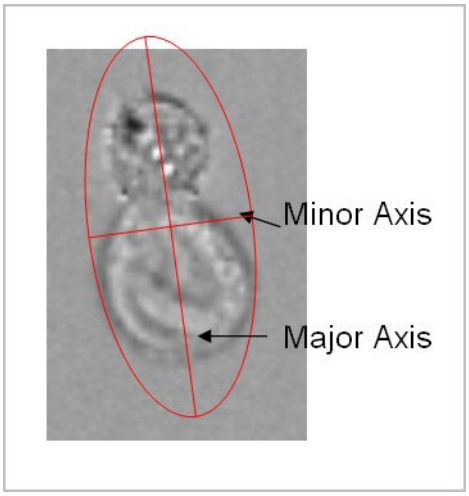
Length measures the longest part of an object. Unlike the Major Axis feature, Length can measure the object's length even if it folds to form a cashew, banana, or doughnut shape, where in many of these cases the major or minor axis features would not be able to differentiate these with true circular shaped objects with no hole.

This feature is based on an input mask and is sensitive to the variation of the input mask shape. Selecting an input mask that can accurately capture the object shape is important.



Major Axis and Minor Axis Features

The Major Axis is the longest dimension of an ellipse of best fit. The Minor Axis is the narrowest dimension of the ellipse of best fit.

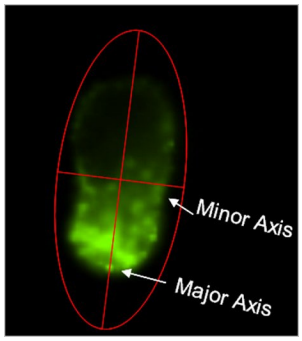


Application Examples:

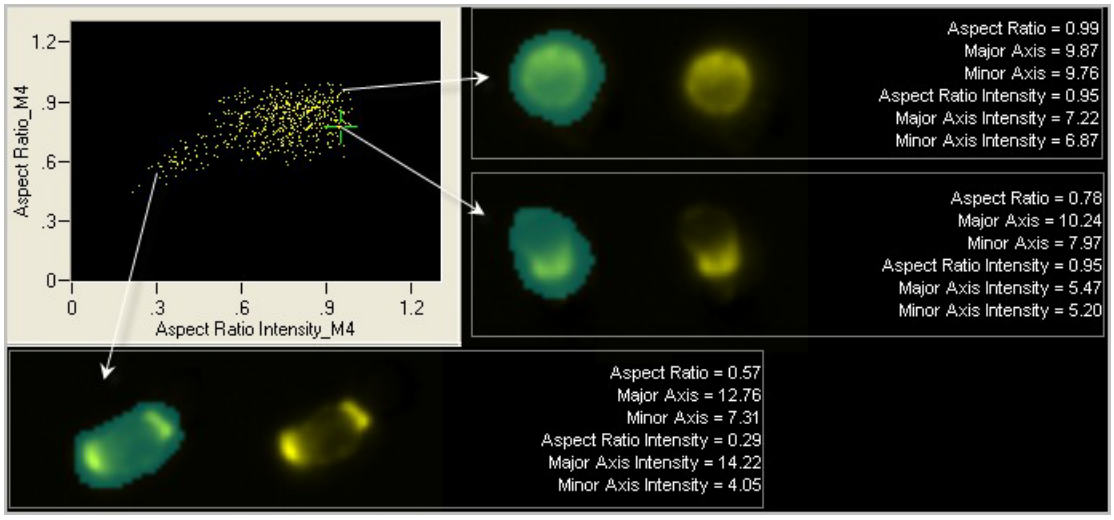
- Quantify and compare cell shape.
- Identify small, medium, and large cells.

Major Axis Intensity and Minor Axis Intensity Features

The Major Axis Intensity is the longest dimension of an ellipse of best fit and is intensity weighted. The Minor Axis Intensity is the narrowest dimension of the ellipse of best fit and is intensity weighted.



The figure below illustrates the difference between intensity weighted and non-intensity weighted Major or Minor Axis and Aspect Ratio.

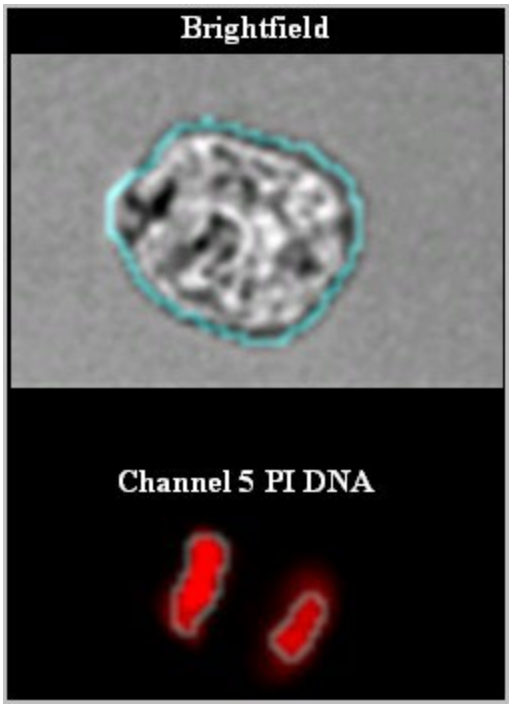


Application Examples

- Quantify and compare the image fluorescence shape.
- Identify single cells.

Perimeter Feature

The perimeter feature measures the boundary length of the mask in the number of microns. This example uses a 1-pixel wide mask created to illustrate how a perimeter would appear.



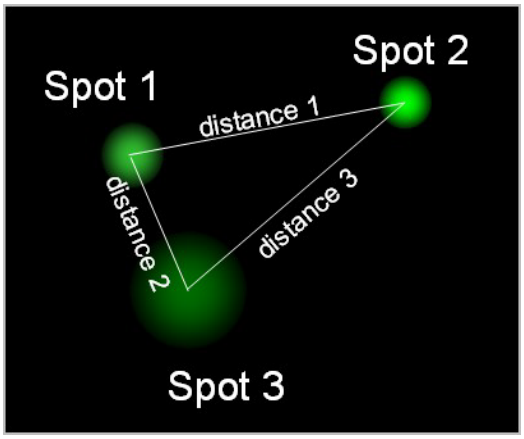
Application Examples

- Quantify and compare cell circumference.
- Identify cells with highly irregular surfaces from smooth cells.
- Perimeter of the morphology or threshold masks can identify cells with or without dendrites.

Spot Area Min Feature

The Spot Area Min feature provides the area of the smallest spot (connected component) in a spot or peak mask.

This is one of four features that can be used to identify objects with spots that are close together, dim, bright or small when counting spots in an image. To use these features, the spots need to be individually masked, such as using the Spot or Peak Mask. The Spot Area Min, Spot Distance Min, and Spot Intensity Min or Max features measure properties of different spots in an image and are often used with the Spot Count feature (under Texture).



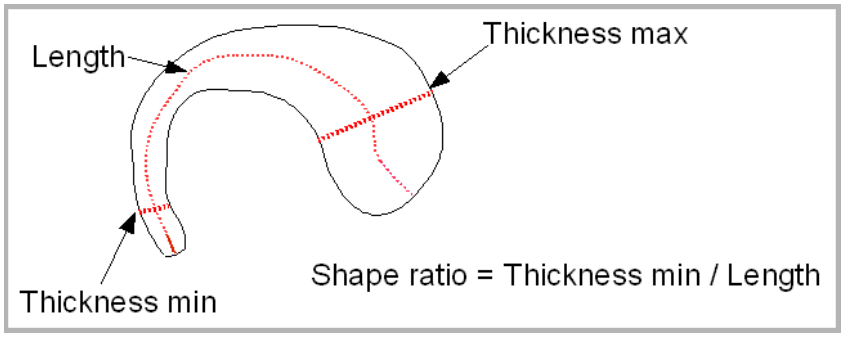
- Spot Area Min is the Area of spot 1
- Spot Distance Min is distance 2 in microns
- Spot Intensity Max is the Raw Mean Pixel of spot 2
- Spot Intensity Min is the Raw Mean Pixel value of spot 3

Application Example

- In FISH Spot Counting, these features are used to identify objects with ambiguous spots that are located too close together, are too dim to count or are too small in order to remove these objects from the analysis.

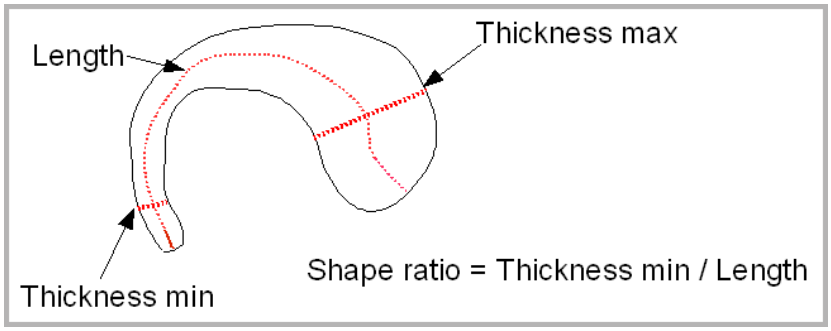
Thickness Max Feature

Thickness Max measures the largest width of an object. This feature is based on an input mask and therefore sensitive to the variation of the input mask shape. Selecting an input mask that can accurately capture the object shape is important.



Thickness Min Feature

Thickness Min measures the smallest width of an object. This feature is based on an input mask and therefore sensitive to the variation of the input mask shape. Selecting an input mask that can accurately capture the object shape is important.



Width Feature

Using the bounding rectangle, Width is the number of microns of the smaller side and Height the longer side.

Application Example

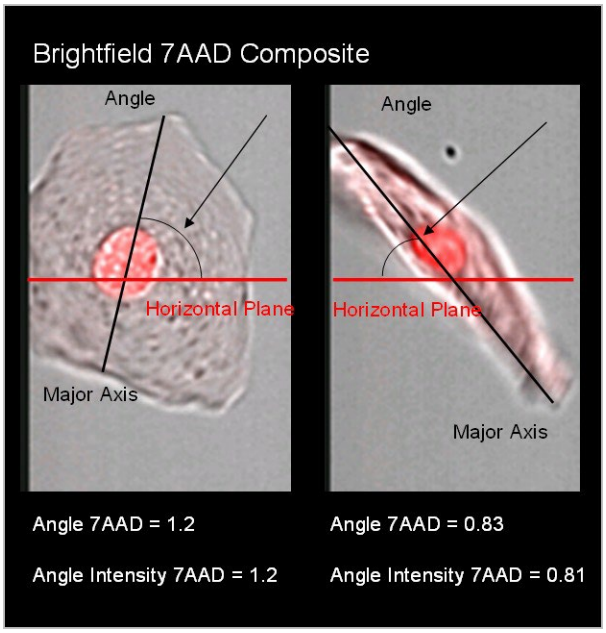
- These features can be used to separate rectangular shaped objects. For curved objects, measurement is more accurately obtained using the thickness features.

Location Features

Location features include Angle, Angle Intensity, Centroid X, Centroid Y, Centroid X Intensity, Centroid Y Intensity, Delta Centroid X, Delta Centroid Y, Delta Centroid XY, Max Contour position, Spot Distance Min, Valley X and Valley Y.

Angle Feature

Angle is the angle of the major axis from a horizontal plane in radians.



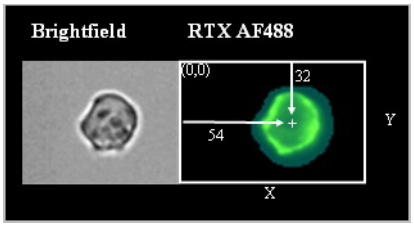
Application Example

- Identify the orientation of an image relative to the image frame.

Centroid X and Centroid Y Features

Centroid X is the number of pixels in the horizontal axis from the upper, left corner of the image to the center of the mask. Centroid Y is the number of pixels in the vertical axis from the upper, left corner of the image to the center of the mask.

In this example, the Centroid X=54 and the Centroid Y=32.

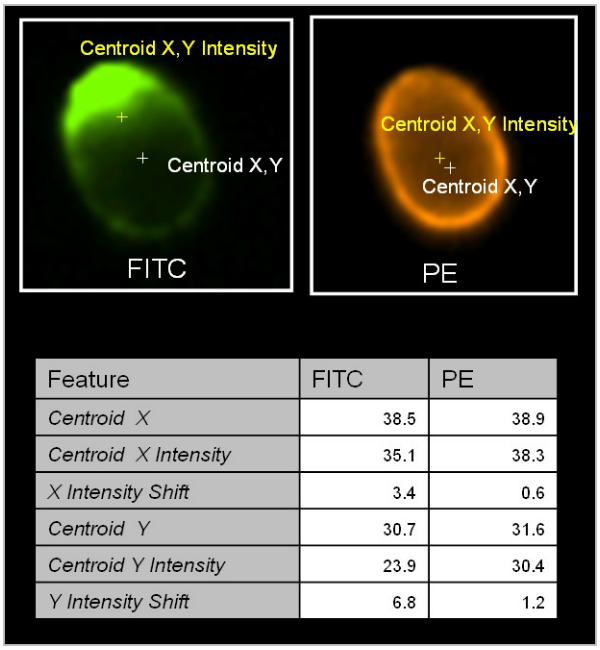


Application Examples

- Identify the center of the mask.
- Calculate the Delta Centroid or the distance between two fluorescent markers.
- Used by IDEAS® software to calculate the Delta Centroid X, Y, or XY.

Centroid X Intensity and Centroid Y Intensity Features

Centroid X Intensity is the intensity weighted X centroid and is shifted from the center of the mask toward the center of fluorescence. The Centroid Y Intensity is the intensity weighted Y centroid. X and Y pixel coordinates are calculated from an origin in the upper left corner.



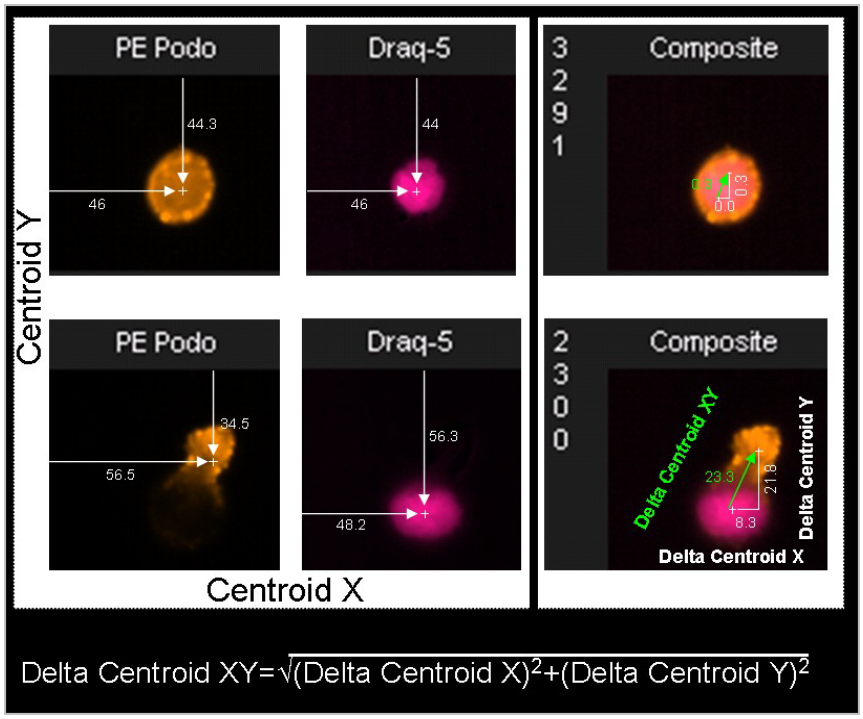
Feature	FITC	PE
Centroid X	38.5	38.9
Centroid X Intensity	35.1	38.3
X Intensity Shift	3.4	0.6
Centroid Y	30.7	31.6
Centroid Y Intensity	23.9	30.4
Y Intensity Shift	6.8	1.2

Application Examples

- Identify the center of peak fluorescence.
- Calculate the distance between two fluorescent markers.
- Used by IDEAS® software to calculate the intensity weighted Delta Centroid X, Y or XY.

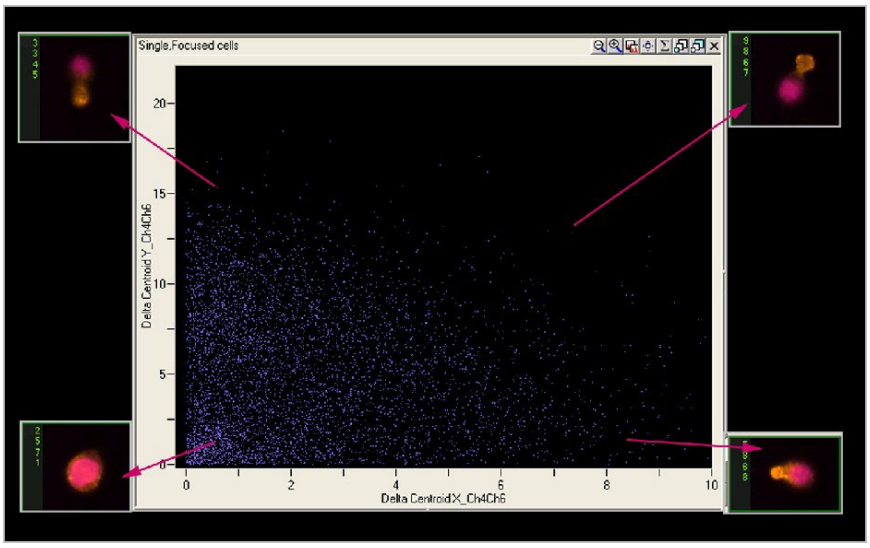
Delta Centroid X and Delta Centroid Y Features

Both the Delta Centroid X and Y features measure the distance between the Centroids X or Centroids Y, respectively, of two images using the user provided masks for each image. Either one or both the centroids of the images may be intensity-weighted. X and Y pixel coordinates are calculated from an origin in the upper left corner to obtain the centroid positions and the distance between the centroids is converted to microns.



The graph below illustrates using the Delta Centroid X versus Delta Centroid Y to identify cells with a variation of location of a protein with respect to the nucleus.

Cells with no spatial shift of signal between the nuclear stain(Ch6) and protein of interest(Ch4) have a low Delta Centroid X and Y and are found in the lower left corner. Cells with a large shift between the images in both the X and Y direction are found in the upper, right section and those with a large shift in X but not Y are found in the lower, right.
Similarly a cell with a large shift in the Y direction and not X are found in the upper, left.

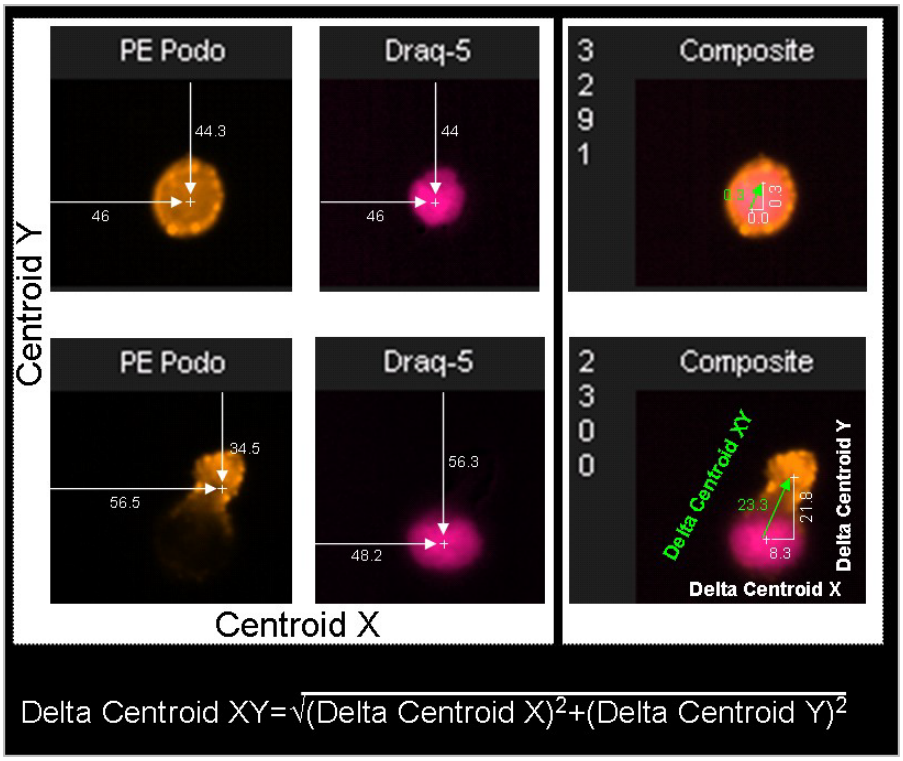


Application Example

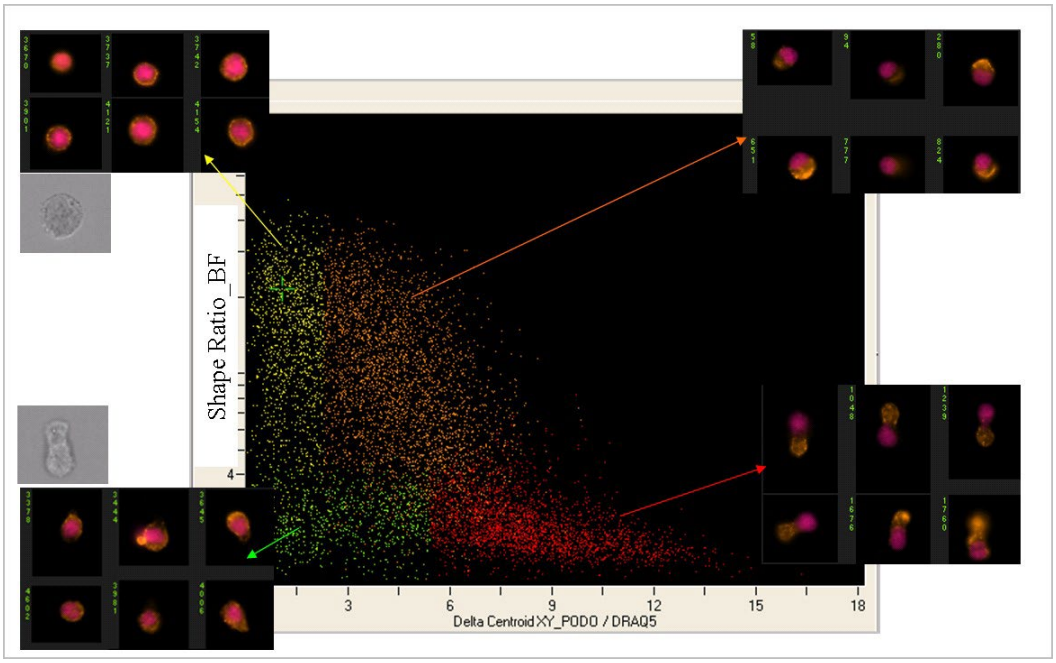
- Used to identify capped versus not capped cells.
- Used to measure shifts in X or Y direction between two images.

Delta Centroid XY Feature

The Delta Centroid XY feature measures the distance between the Centroid feature of two images using the user provided masks for each image. Either one or both the centroids of the images may be intensity-weighted. X and Y pixel coordinates are calculated from an origin in the upper left corner to obtain the centroid positions and the distance between the centroids is converted to microns. In the example, below an image pair is shown stained with the nuclear dye Draq 5 and a PE labeled antibody that is differentially expressed two cells, either uniformly or in the pseudopod. The two cells are identified by their different Delta Centroid XY values.



Below is an example of using the Delta Centroid XY. A bivariate graph of a shape ratio versus Delta Centroid XY can identify cells with caps as shown here:



Application Examples

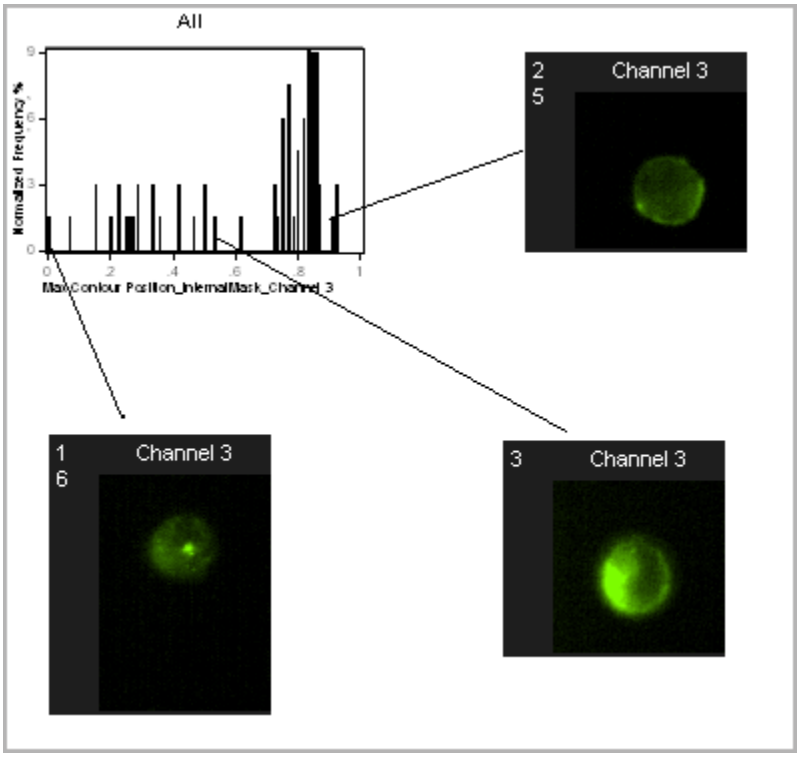
- Quantify the spatial relationship between two fluorescent probes.
- Identify false apoptotic positive cells in the TUNEL and Annexin V assays.
- Quantify shape change.
- Quantify capping of cell surface antigens.

Raw Centroid X and Raw Centroid Y Features

The centroid X and Y of the original position of the image during acquisition before it was centered IDEAS® software. Data analyzed in IDEAS® software versions 4.0 or later cut and center objects that were collected as one image in INSPIRE™ software.

Max Contour Position Feature

The Max Contour Position is defined as the location of the contour in the cell that has the highest intensity concentration. It is invariant to object size and can accommodate localized intensity concentrations. The actual location in the object is mapped to a number between 0 and 1, with 0 being the object center and 1 being the object perimeter, which allows one to compare the results across cells of different sizes. An example is shown below.



Application Example

- Used in conjunction with the Internalization feature to determine the distribution of intensity within a cell.

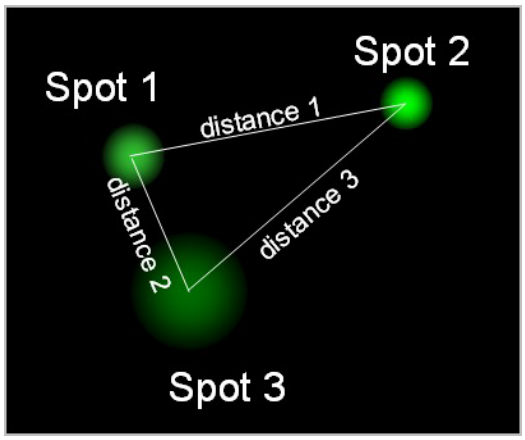
Shift X and Shift Y Features

The Shift X or Shift Y feature is the location of the highest cross correlation of a pair of images. When two identical images are aligned perfectly the cross correlation is at its maximum. The Shift X or Shift Y is the shift required to get the highest cross correlation value for the 2 images. This feature is used mainly for troubleshooting cross camera alignment.

Spot Distance Min Feature

The Spot Distance Min feature provides the shortest distance in microns between two spots (connected components) in a spot or peak mask.

This is one of four features that can be used to identify objects with spots that are close together, dim, bright or small when counting spots in an image. To use these features, the spots need to be individually masked, such as using the Spot or Peak Mask. The Spot Area, Distance, and Spot Intensity Min or Max features measure properties of different spots in an image and are often used with the Spot Count feature (under Texture).



- Spot Area Min is the Area of spot 1
- Spot Distance Min is distance 2 in microns
- Spot Intensity Max is the Raw Mean Pixel of spot 2
- Spot Intensity Min is the Raw Mean Pixel value of spot 3

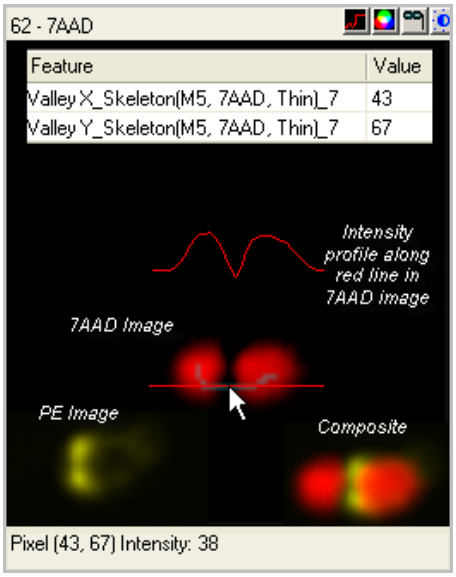
Application Example

- In FISH Spot Counting, these features are used to identify ambiguous spots that are located too close together, too dim, too bright or too small to count and can be eliminated from the analysis.

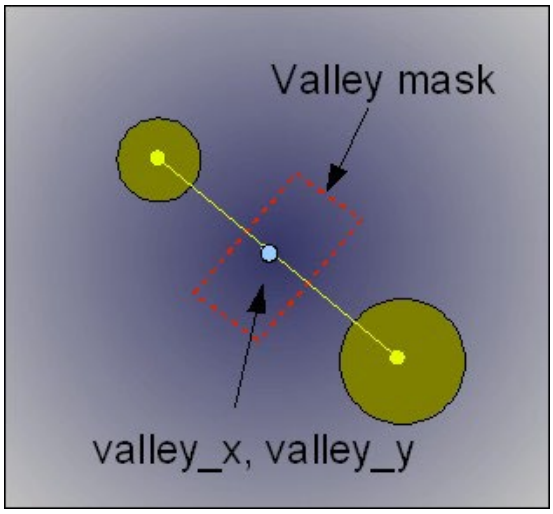
Valley X and Valley Y Features

The Valley X and Y are the exact X,Y coordinates of the minimum intensity within the skeletal lines of the input mask.
The objects condensed shape, typically 1-pixel wide skeletal line is determined from the starting mask. This is also the origin of the Valley mask.

In the figure below, the Valley X and Valley Y position of the 7AAD image is shown. In this example a protein of interest in the PE image localizes to the synapse between two cells.



These features define the origin of the Valley mask.



Application Example

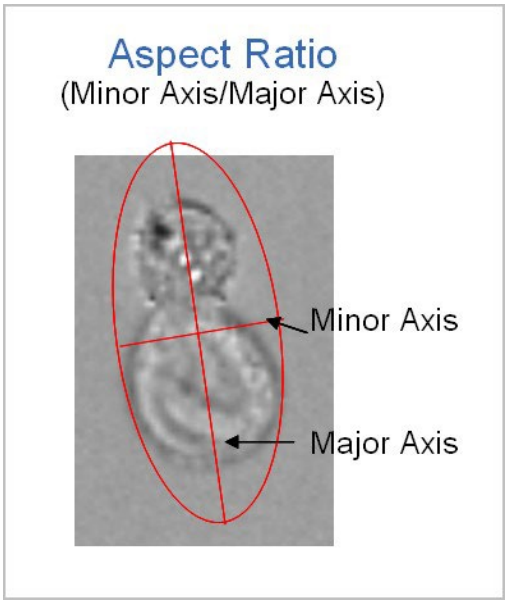
- Measure the exact center of where a synapse between two cells is located.

Shape Features

Shape features define the mask shape and have units that vary with the feature. They include the Aspect Ratio, Aspect Ratio Intensity, Compactness, Elongatedness, Lobe Count, and Symmetry 2,3,4.

Aspect Ratio Feature

Aspect Ratio is the Minor Axis divided by the Major Axis and describes how round or oblong an object is.



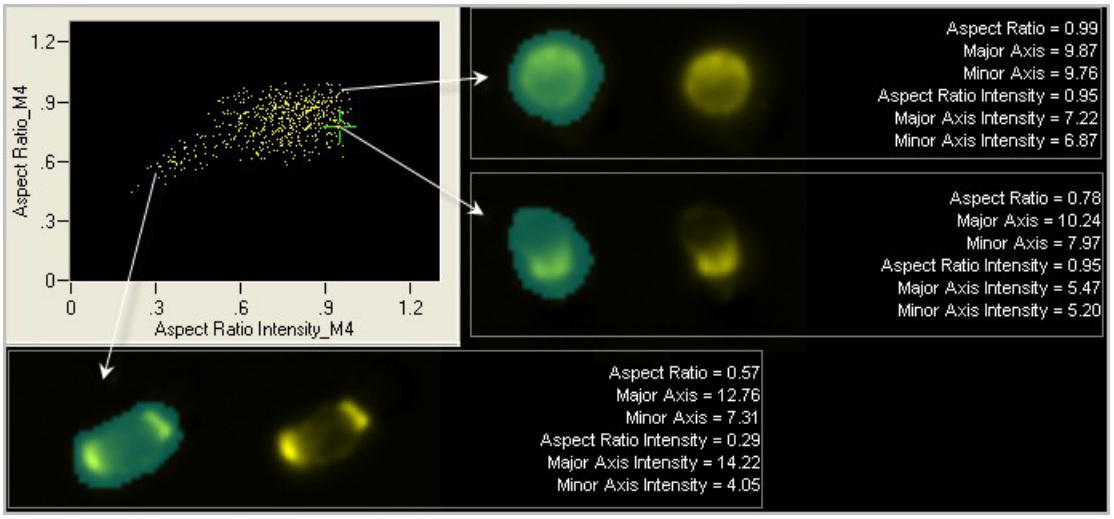
Application Examples

- Quantify the roundness of the mask.
- Identify single cells vs. doublets.
- Cell classification based on shape change.
- Identify recently divided cells in mitosis.

Aspect Ratio Intensity Feature

Aspect Ratio Intensity is the Minor Axis Intensity divided by the Major Axis Intensity.

The figure below illustrates the difference between Aspect Ratio Intensity and Aspect Ratio.

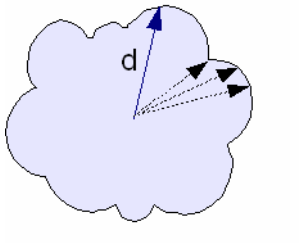


Application Examples

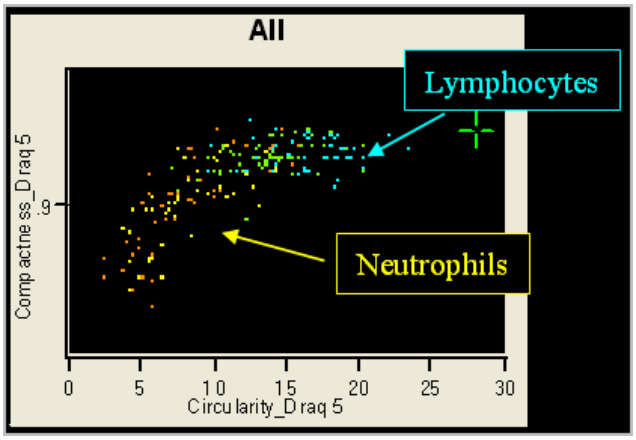
- Quantify the roundness of the fluorescent image.
- Better resolution for identifying single cells vs. doublets in experiments using a DNA dye.
- Cell classification based on fluorescent morphology.

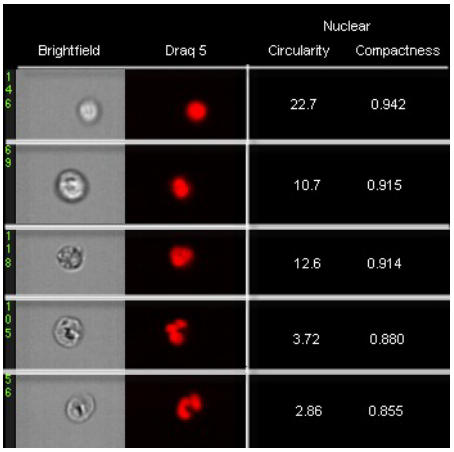
Circularity Feature

This feature measures the degree of the mask's deviation from a circle. Its measurement is based on the average distance of the object boundary from its center divided by the variation of this distance. Thus, the closer the object to a circle, the smaller the variation and therefore the feature value will be high. Vice versa, the more the shape deviates from a circle, the higher the variation and therefore the Circularity value will be low.



Below is an example using Circularity and Compactness to characterize the shape of peripheral blood mononuclear cells stained with the DNA dye Draq 5.





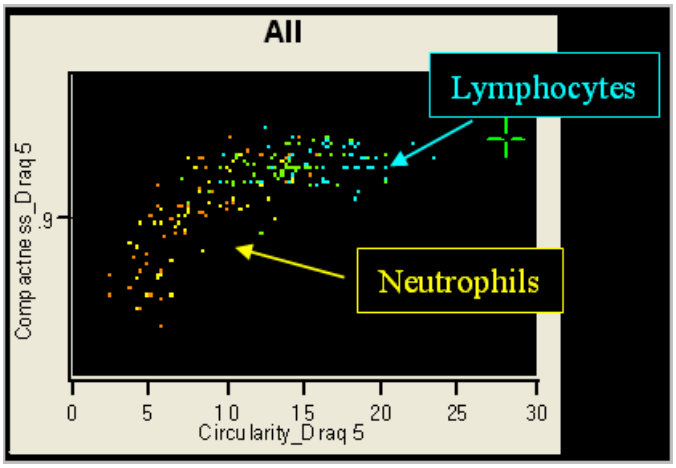
Application Examples

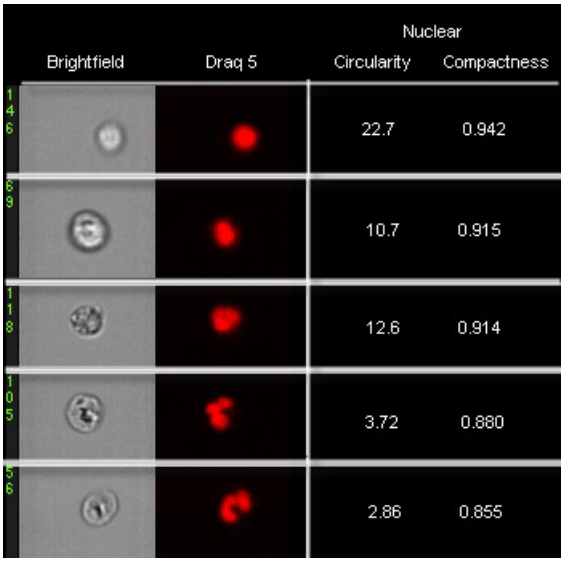
- Distinguish singlets and doublets.
- Separate circular and non-circular shapes.

Compactness Feature

Compactness measures the degree of how well the object is packed together. This feature is similar to the Circularity feature but unlike Circularity, this feature includes all of the pixels within the mask and is intensity weighted. The higher the value, the more condensed the object.

Below is an example using Circularity and Compactness to characterize the shape of peripheral blood mononuclear cells stained with the DNA dye Draq 5.



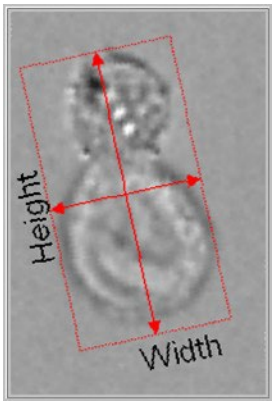


Application Example:

- Differentiate between rounded objects with smooth boundary to less regular objects.

Elongatedness Feature

Elongatedness is the ratio of the Height over Width of the object's bounding box.



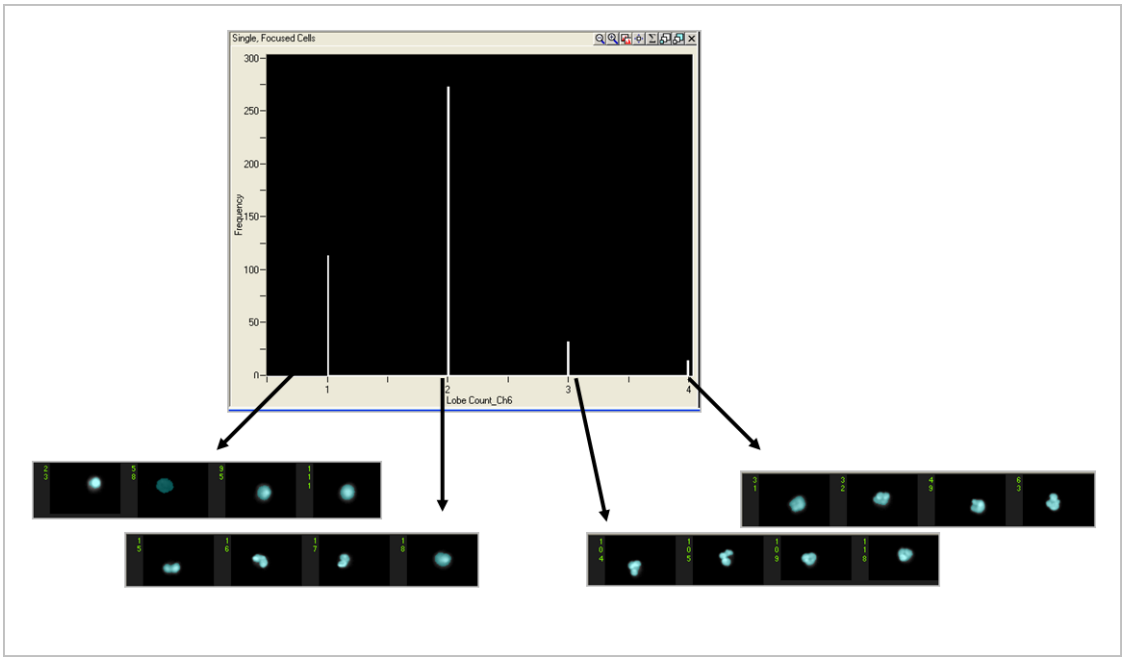
Application Examples

- Measure object shape properties to differentiate between long and narrow versus short and thick objects.
- Quantify the roundness of the morphology mask.
- Identify single cells and doublets.
- Cell classification based on shape change.
- Identify recently divided cells in mitosis.

Lobe Count Feature

The Lobe Count feature counts the number of lobes in a cell. It is determined based on the maxima of the weighted Symmetry features. The feature reports the values 1,2,3 or 4. If an object does not have a high value for Symmetry 2, Symmetry 3, or Symmetry 4 it is reported as 1 for no lobes. An example is shown below.

	Symmetry		
Lobe Count	2	3	4
1	Low	Low	Low
2	High	Low	Low
3	Low	High	Low
4	Low	Low	High



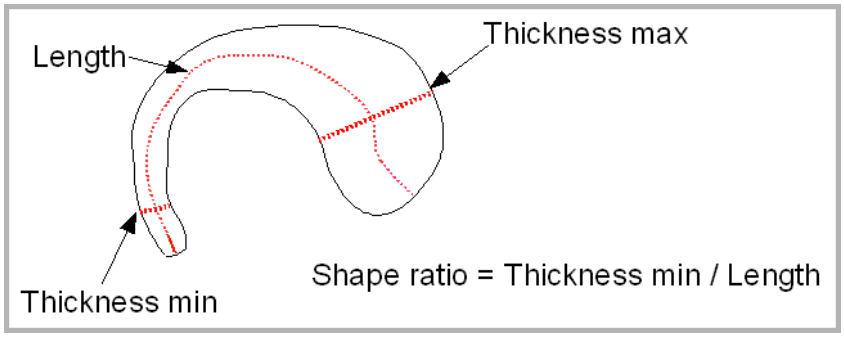
Application Example

- Used in cell classification studies. Also used to differentiate small round cells from small square cells of similar area.

Shape Ratio Feature

The Shape Ratio is Thickness Min divided by Length.

The Shape Ratio feature is based on an input mask and is sensitive to the variation of the input mask shape. Selecting an input mask that can accurately capture the object shape is important.

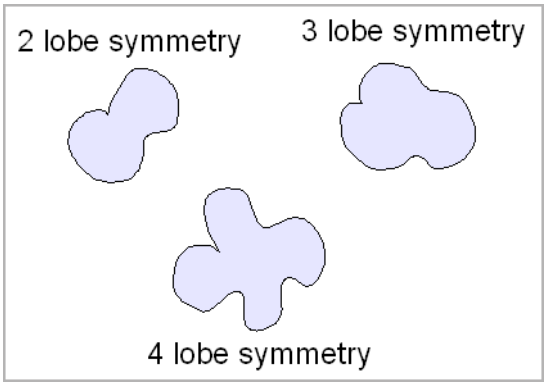


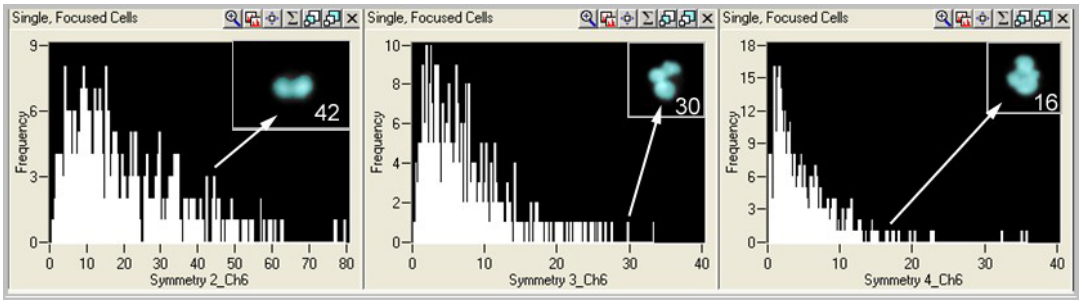
Application Example

- Measure object's elongatedness to provide shape classification.

Symmetry 2, 3, 4 Features

The Symmetry 2 feature measures the tendency of the object to have a single axis of elongation and therefore 2 lobes. The Symmetry 3 feature measures the tendency of the object to have a threefold axis of symmetry and likewise, Symmetry 4 a fourfold axis. The absolute value of these features are dependent on the number of lobes. For example an image that has high 4 lobe symmetry will also have high 2 lobe symmetry.





Application Example

- Classify different white blood cells based on the morphology of the nuclear image.

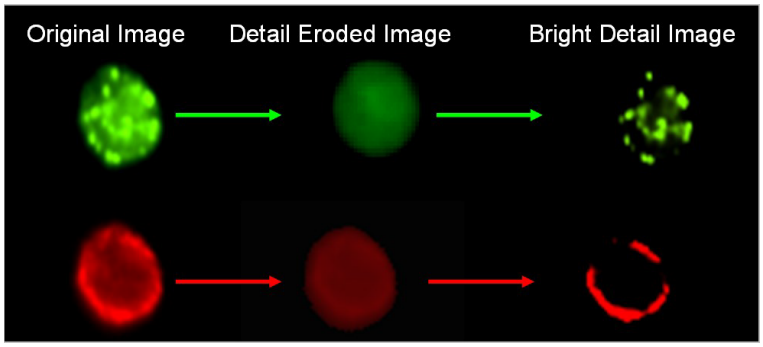
Texture Features

The Texture features determine local intensity variations in images and include Bright Detail Intensity R3 and Bright Detail Intensity R7, Contrast, Gradient Max, Gradient RMS, H Texture (H-Contrast, H-Correlation, H-Energy, H-Entropy, H-Homogeneity, and H-Variance), Modulation, Spot Count, and Std Dev., Contrast, Gradient Max and Gradient RMS are generally used to determine best focus.

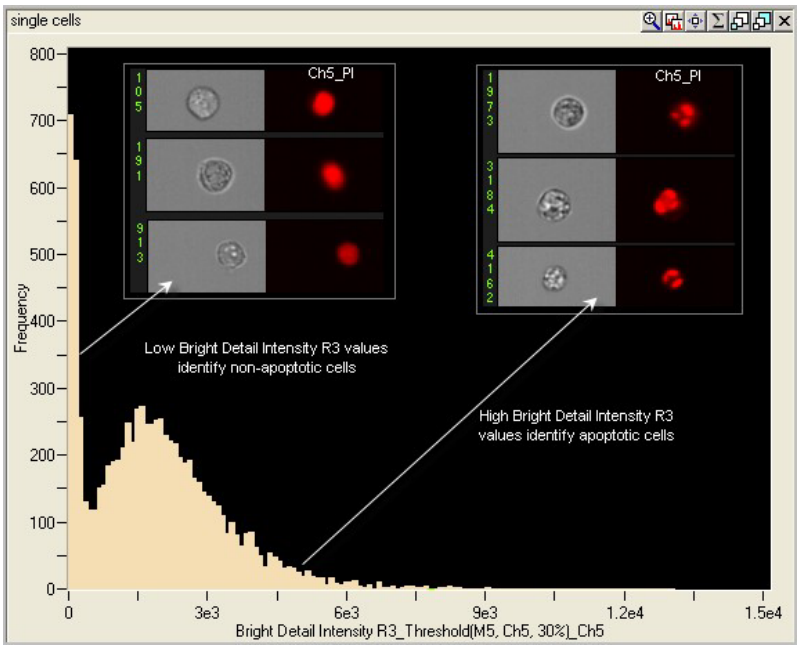
Bright Detail Intensity R3 and Bright Detail Intensity R7 Features

The Bright Detail Intensity R3 and Bright Detail Intensity R7 features compute the intensity of localized bright spots within the masked area in the image. Bright Detail Intensity R3 computes the intensity of bright spots that are 3 pixels in radius or less, while Bright Detail Intensity R7 computes the intensity of bright spots in the image that are 7 pixels in radius or less. In each case, the local background around the spots is removed before the intensity computation.

The figure below shows the process of obtaining the localized bright spots in the image.



The graph below illustrates the use of the Bright Detail Intensity R3 feature on a nuclear image to separate apoptotic cells from non-apoptotic cells.

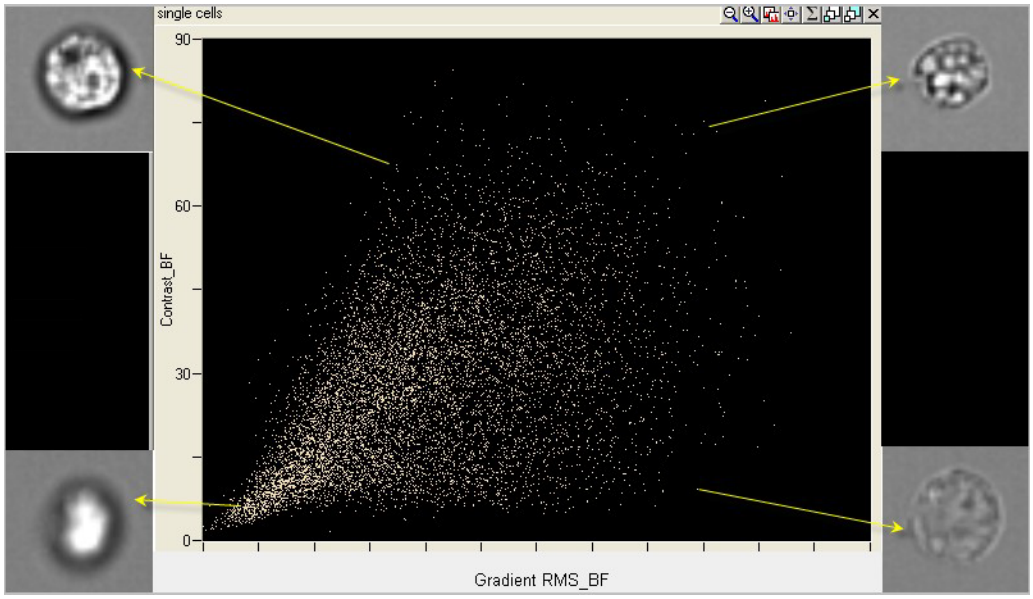


Application Example

- Identify cells that have bright specks such as Apoptotic cells.
- Used in the Cell Cycle - Mitosis Wizard.

Contrast Feature

The Contrast feature measures the sharpness quality of an image by detecting large changes of pixel values in the image and is useful for the selection of focused objects or apoptotic brightfield images. For every pixel, the slopes of the pixel intensities are computed using the 3x3 block around the pixel. This is similar to the Gradient RMS calculation with
different weighted assignments to the pixel arrays with no background subtraction. Example images are shown in the figure below.



Application Examples

- Find apoptotic images with high contrast in brightfield imagery.
- Determine overall focus quality of images.
- Use with Gradient RMS to determine focus quality.
- Characterize texture.

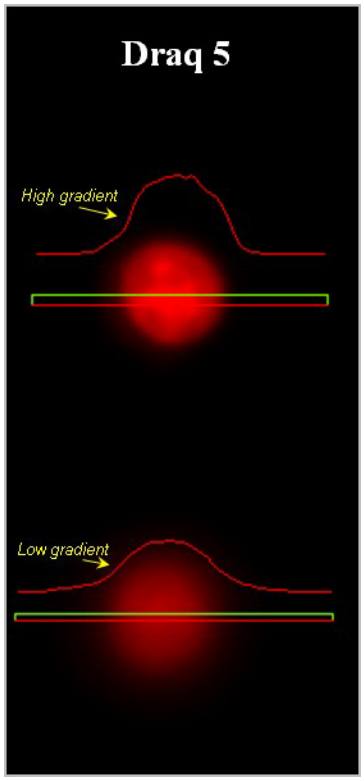
Ensquared Energy Feature

The Ensquared Energy feature is a measure of image quality. Computes the intensity of the square block around the brightest pixel using the diameter input as the side for the square divided by the intensity of the total intensity. The closer this ratio is to 1.0 the better focused the image. This feature is mainly used for single, uniform particles such as beads.

Gradient Max Feature

The Gradient Max feature measures the sharpness quality of an image by detecting largest change of pixel values in the image and is useful for the selection of focused objects.

This figure shows the change in intensity across the red line. The top image has a larger slope change than the lower image.

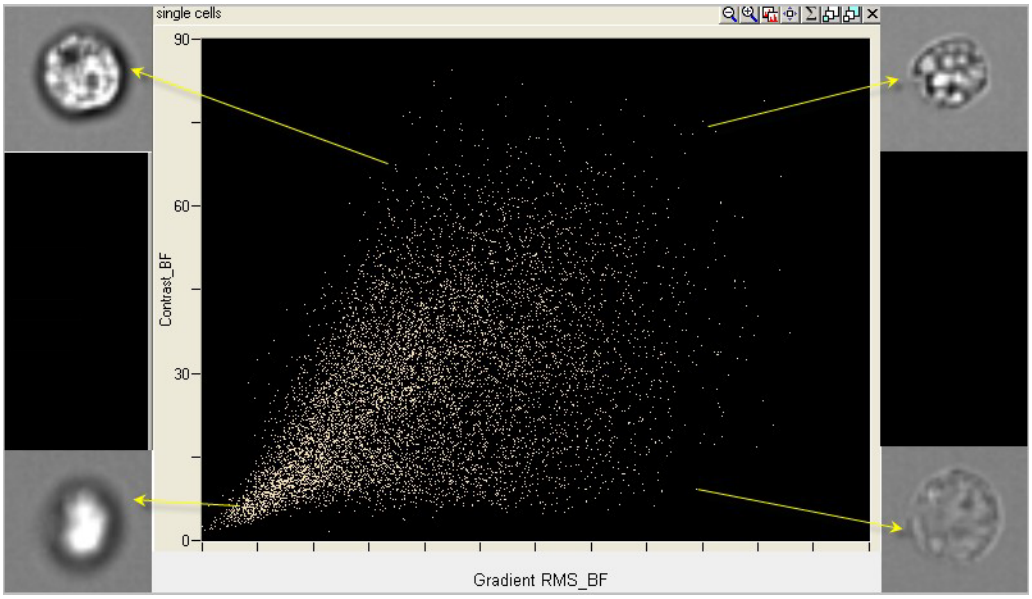


Application Example

- Determine peak focus quality of images.
- Also used to characterize texture. However, the Gradient RMS and Contrast feature are more robust for these applications.

Gradient RMS Feature

The Gradient RMS feature measures the sharpness quality of an image by detecting large changes of pixel values in the image and is useful for the selection of focused objects. The Gradient RMS feature is computed using the average gradient of a pixel normalized for variations in intensity levels. This is similar to the Contrast calculation with different weighted assignments to the pixel arrays and with background subtracted. Example images are shown in the figure below.



Application Examples

- Determine overall focus quality of images.
- Used with Contrast to determine focus quality.
- Characterize texture.

H Texture Features

H Texture features include the following: H Energy Mean and Std, H Entropy Mean and Std, H Contrast Mean and Std, H Homogeneity Mean and Std, H Correlation Mean and Std, H Variance Mean and Std Features.

The user defines the texture grain by assigning a granularity value. For very fine textures, this value is small (1-3 pixels), while for very coarse textures, it is large (>10). In the IDEAS® software default template, the granularity value is 5.

While these features have value for distinguishing cellular texture when used individually, images often contain a mixture of different textures at different grains. Therefore, these features are most powerful when combined.

Texture features are used to determine if pixel values in an image follow a pattern or are randomly distributed. An image with random grayscale values might look like a gray color swatch with individual pixel values ranging from the min to the max centering on a mean. If light and/or dark patterns are visible, it means the pixel values are not randomly distributed and some texture has emerged. So texture features can be thought of as measuring the presence or absence of randomness. If pixel values are nonrandom, then defined patterns are likely to be present in the image. Patterns in the image may be biologically significant, such as the clustering of LC3 proteins from a random distribution in the cytoplasm as cells enter autophagy, or formation of H2AX clusters in DNA damage and repair studies.

Random black to white pixel values

Non-random values showing texture

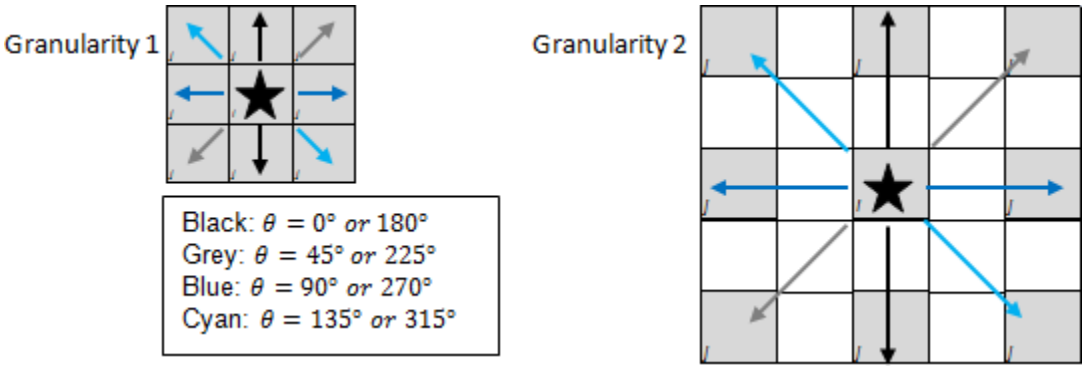
The Haralick texture features are a set of texture features based on the 2nd order statistics computed from the joint 2-D probability distribution of pixel intensities in the image. This distribution is referred to as the Gray Level Co-occurrence Matrix (GLCM). To understand how this matrix works, consider the outcome of two dice thrown on a table. The probability matrix in this case is 6 possible outcomes from the first die and 6 from the second, or 36 possible combinations. There are 6 possible doubles and the odds of rolling them are 6/36 or 16.7%. To analyze the texture of images, a similar probability matrix can be used to determine if pixels in a given neighborhood occur randomly or (like the toss of two dice) are somewhat predictable. If pixels in an image have a high level of co-occurrence, then there is likely some sort of detectable pattern.

Texture features in IDEAS® software are calculated in a four step process by first converting the pixel data to 8 bits, then applying the granularity to calculate the GLCM, next computing the 6 Haralick texture features and finally outputting the mean and standard deviation for each of the 6 texture features.

The first step in the three step process is to convert the raw 12 bit image under the bounding mask into a 4 bit image. This is done so that the GLCM does not have too many 0 entries which will ensure a more robust estimate of the texture and reduce the noise. This effectively bins the raw data into 2^4 or 16 bins with resulting values between 0 and 15. For full range images that have pixel values between 0 and 4096 this would bin pixels by 16/4096 or 256 counts, such that pixels between 4096 and 3840 would be assigned a value of 15, pixels between 3840 and 3584 would be assigned 14, and so on.

Second the granularity is applied to the feature to calculate the GLCM. In IDEAS® software the granularity defines the distance from the anchor pixel to the pixel array used to calculate the texture features. A granularity of 1 would pair the anchor pixel with those 1 pixel above, below, right, left and on the angles. The default granularity in IDEAS® software is set to 5 and for 40x imagery this would mean the texture features are comparing pixel pairs 2.5 um away from the anchor pixel

Next the GLCM is calculated by comparing neighboring pixel values and calculating how uniform or variant those values are. The GLCM is a function of the texture granularity **d** and orientation, **θ**. The entry p_{ij} in the i^{th} row and j^{th} column of the GLCM matrix is defined as the probability of having pixel pairs in the image with intensities {i,j} at a distance **d** from each other along an orientation **θ**. Thus, the GLCM is an N x N matrix, where N is determined by the range of intensities in the cell. For very fine textures, granularity d is typically very small – about 1-2 pixels. For medium textures, d is about 4-8. For very coarse textures, d can be very large, about 12-16 pixels. The specific value of the orientation **θ** is less important in our applications since we are not interested in the actual orientation of the texture, but only if orientation exists.
For this reason, we compute the GLCM for a number of orientations {0, 45, 90, 135, 180, 225, 270, 315 degrees}. Assuming symmetry in orientation, we combine the orientations (0,180), (90,270), (45,225) and (135,315) to obtain 4 separate matrices.



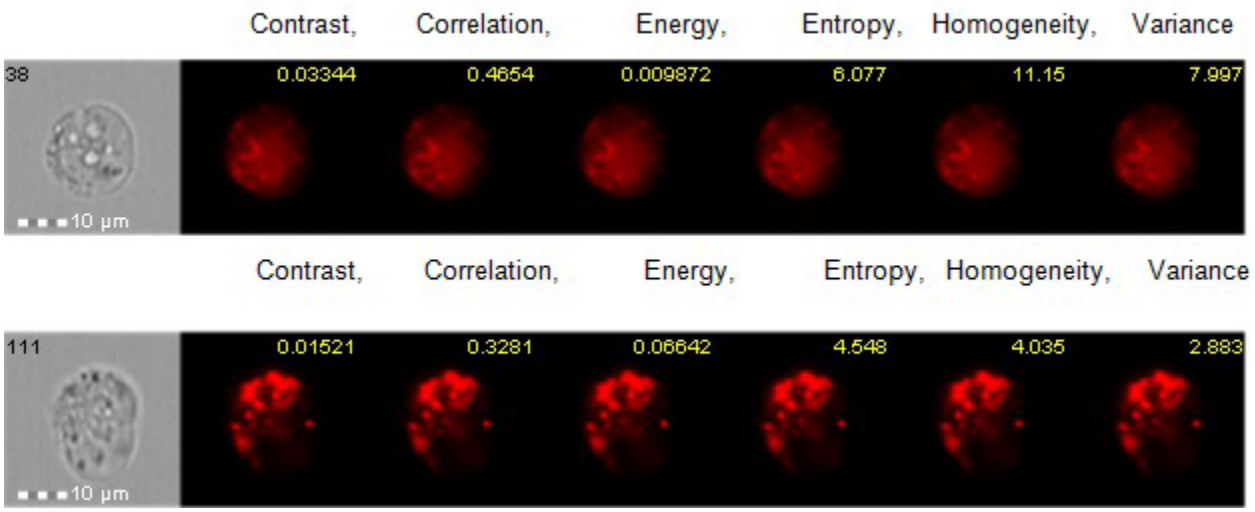
Finally the mean and standard deviation (std) of each texture feature are calculated where the mean is the average value at each of the four orientations **(θ)** theta, and the standard deviation is the variation of those values. The mean texture values are good indicators of the average texture in the cell, while the standard deviations are good indicators of orientation in the texture. Large standard deviation values indicate that some directions have very different textures from other directions.

Texture features have been useful for analyzing changes in staining patters for applications like LC3 clustering in cells progressing through autophagy, H2Ax foci formation in DNA damage and repair studies, nuclear condensation and fragmentation in apoptotic assays, changes in mitochondrial membrane potential and other applications where a given treatment induces changes in a fluorochromes pattern of staining. For a more detailed description of the Haralick features see the paper referenced below.

[1]Haralick, R.M., K. Shanmugan, and I. Dinstein, "Textural Features for Image Classification", IEEE Transactions on Systems, Man, and Cybernetics, Vol. SMC-3, 1973, pp. 610-621.

Texture features that are generally similar	Texture features that are generally dissimilar
Contrast, Energy	Energy vs Entropy
Correlation, Homogeneity	Correlation vs Contrast
	Homogeneity vs Contrast

Diffuse and textured cell with each texture feature calculated:



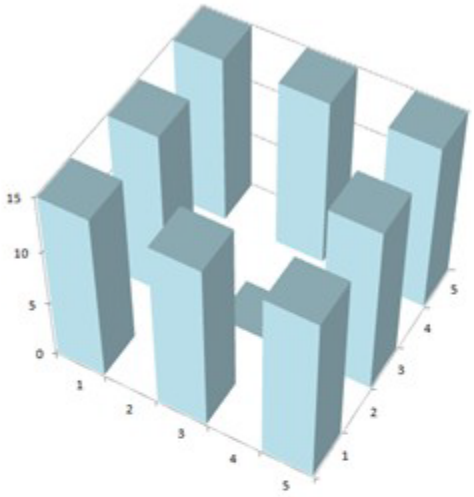
Contrast

Measures the intensity variation in a cell. A high mean value indicates that neighboring pixels tend to have very different intensity values, and would describe an image with lots of adjacent light and dark areas. Contrast will range between 0 and 1.

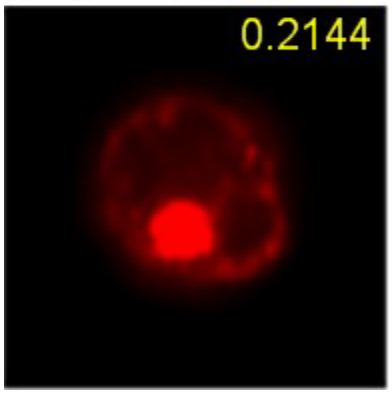
$$H\ Contrast = \sum_{i=0}^{N-1}\sum_{j=0}^{N-1}(i-j)^2 p_{ij}$$

Pixel map demonstrating high contrast;

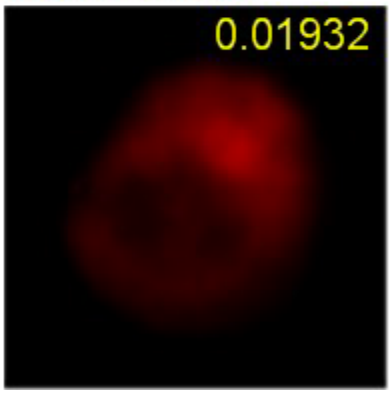
15		15		15
15		0		15
15		15		15



High Contrast Image



Low contrast Image



Correlation

Measures how similar pixel pairs are, and is the opposite of contrast. Images with high correlation will be very uniform and lack variant texture.

$$H\ Correlation = \sum_{i=0}^{N-1}\sum_{j=0}^{N-1}\frac{(i-\mu_i)(j-\mu_j)}{\sigma_i\sigma_j}p_{ij},\quad where$$

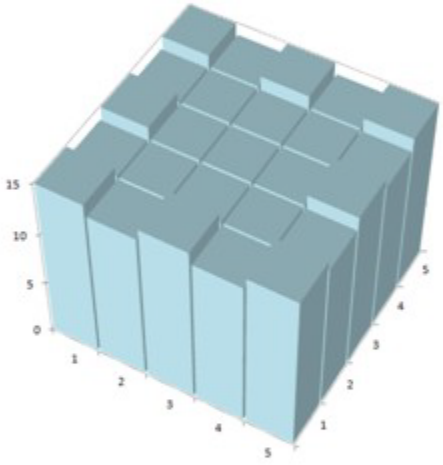
$$M_1(i) = \sum_{j=0}^{N-1} p_{ij},\quad M_2(j) = \sum_{i=0}^{N-1} p_{ij},$$

$$\mu_i = \sum_{i=0}^{N-1} i\,M_1(i),\quad \mu_j = \sum_{j=0}^{N-1} j\,M_2(j),$$

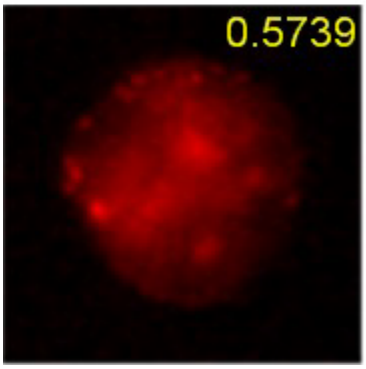
$$\sigma_i = \sum_{i=0}^{N-1}(i-\mu_i)^2 M_1(i),\quad \sigma_j = \sum_{j=0}^{N-1}(j-\mu_j)^2 M_2(j)$$

Pixel map demonstrating high correlation;

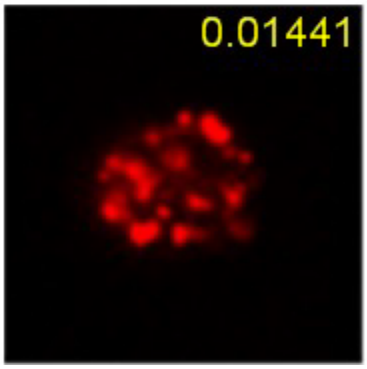
15	14	15	14	15
14	14	14	14	14
15	14	14	14	15
14	14	14	14	14
15	14	15	14	15



High Correlation Image



Low Correlation Image



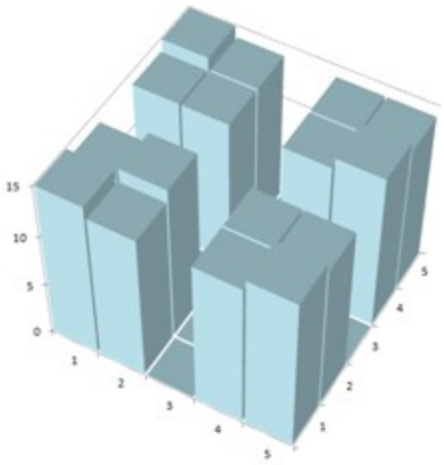
Energy

Is a measure of intensity concentration in the cell. At one extreme is the case where we have a uniform distribution with all probabilities being equal. This image likely has several intensity variations with no noticeable concentration of high intensity and thus has low energy. At the other extreme is the case where we have a very narrow distribution with a few elements having high values. This image will have notable intensity concentrations and thus, has high energy.

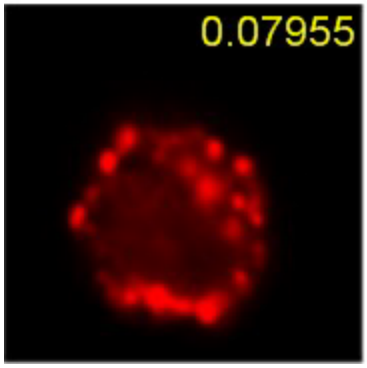
$$H\ Energy = \sum_{i=0}^{N-1}\sum_{j=0}^{N-1} p_{ij}^2$$

Pixel map demonstrating high energy;

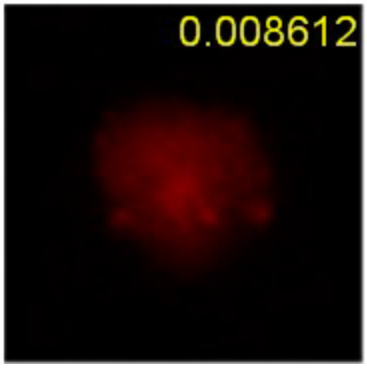
15	14	0	14	15
14	15	0	14	14
0	0	1	0	0
14	14	0	14	15
15	14	0	14	14



High Energy Image



Low Energy Image



Entropy

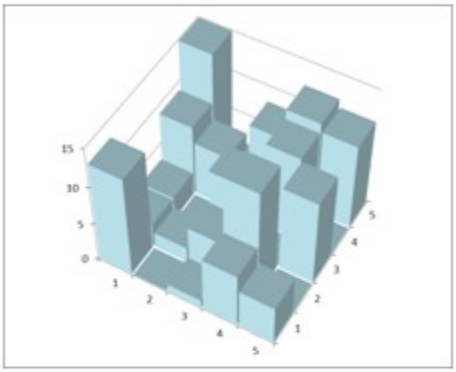
Also a measure of high intensity concentration in the cell. However, this feature relates to the randomness of the intensities in the image. Images that have distinct areas of intensity concentration are less random and thus, have low entropy. Images that have a range of equally likely intensity pairings have less distinct intensity concentrations, and correspondingly, have higher entropy. Entropy is the opposite of energy.

$$H\ Entropy = -\sum_{i=0}^{N-1}\sum_{j=0}^{N-1} p_{ij} \log_2(p_{ij})$$

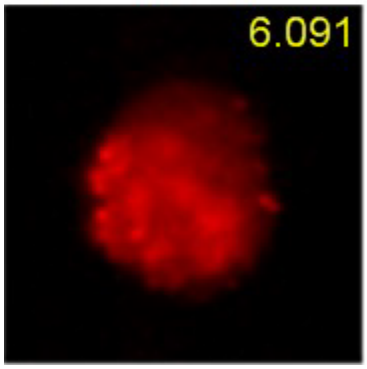
Note that since $0 <= p_{ij} <= 1$, $\log_2(p_{ij}) <= 0$. Hence, we need to take the negative of the summation to get a positive value for the Entropy.

Pixel map demonstrating high entropy;

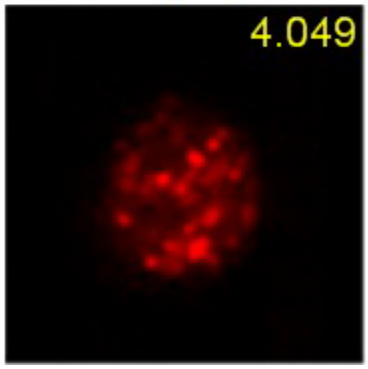
13	0	1	7	5
3	2	5	14	0
3	0	9	0	11
9	7	3	11	o
15	1	8	12	11



High Entropy Image



Low Entropy Image



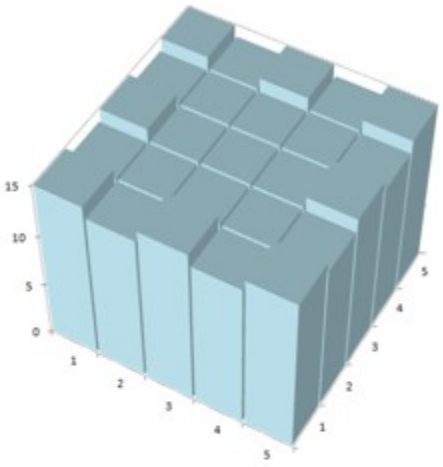
Homogeneity

Is a measure of how close pixels value are, if pixel pairs are the same or similar then a high homogeneity value is given. If there are very few pixels with the same value, then a lower homogeneity score is generated. Images with high homogeneity would look very uniform and lack texture. Homogeneity is the inverse of contrast and very similar to correlation.

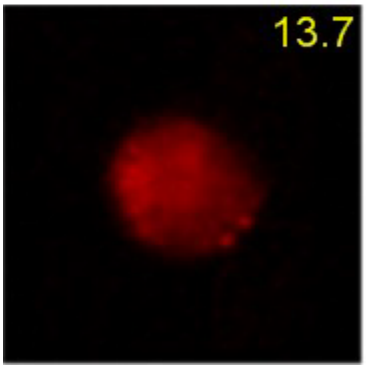
$$H\ Homogeneity = \sum_{i=0}^{N-1}\sum_{j=0}^{N-1}(i+j)p_{ij}$$

Pixel map demonstrating high homogeneity;

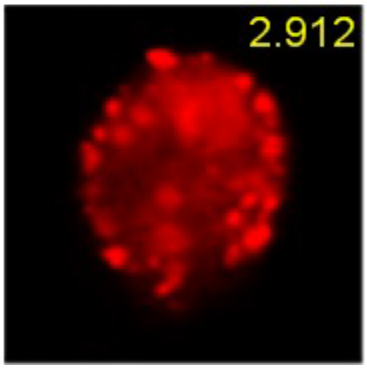
15	14	15	14	15
14	14	14	14	14
15	14	14	14	15
14	14	14	14	14
15	14	15	14	15



High Homogeneity Image



Low Homogeneity Image



Variance

Measures the spread of pixel values within the granularity. Images with high variance values will have a large spread in pixel intensities visually identified by very dark and very bright spots throughout the image.

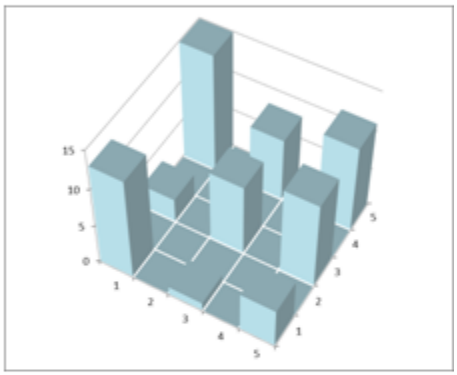
$$H\ Variance = \sum_{i=0}^{N-1}\sum_{j=0}^{N-1}(i-\mu_i)^2 p_{ij}, \quad where$$

$$M_1(i) = \sum_{j=0}^{N-1} p_{ij}, \quad \mu_i = \sum_{i=0}^{N-1} i M_1(i)$$

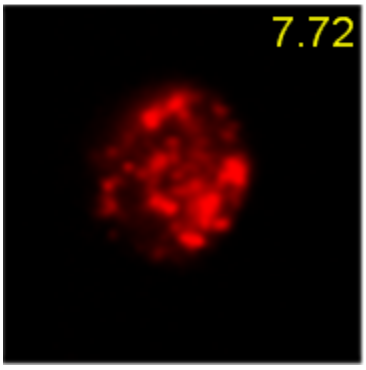
Pixel map demonstrating high homogeneity;

13	0	1	0	5
0	0	0	0	0
3	0	9	0	11
0	0	0	0	o
15	0	8	0	11

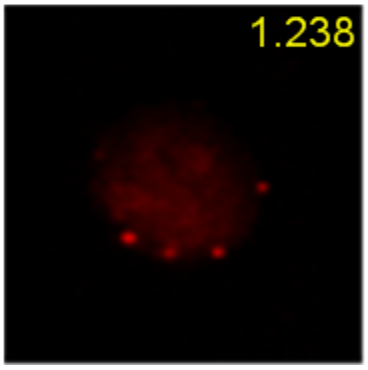
4 bit values, granularity of 3



High Variance Image



Low Variance Image



Application Example

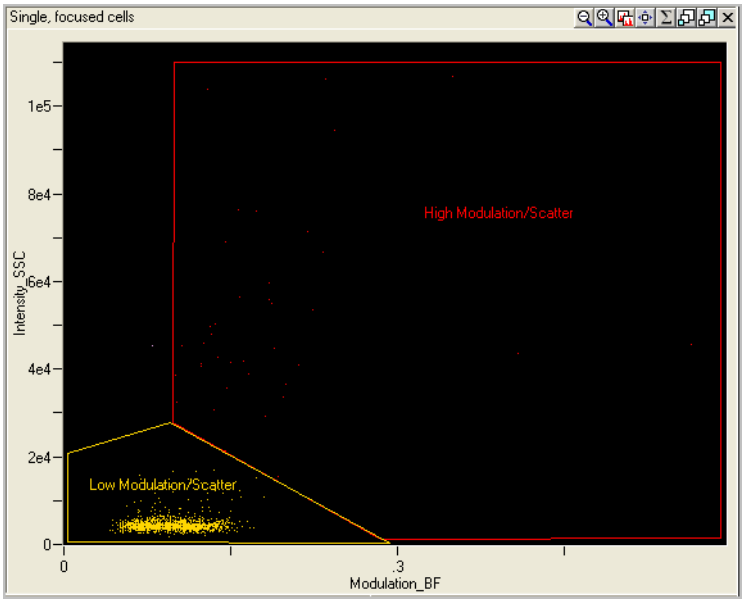
- Quantify texture in cells.

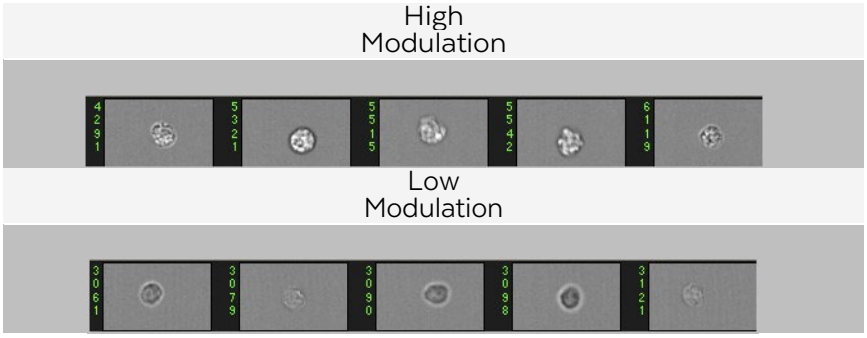
[1]Haralick, R.M., K. Shanmugan, and I. Dinstein, "Textural Features for Image Classification", *IEEE Transactions on Systems, Man, and Cybernetics*, Vol. SMC-3, 1973, pp. 610-621.

Modulation Feature

The Modulation feature measures the intensity range of an image, normalized between 0 and 1. The formula is: Modulation = Max Pixel - Min Pixel / Max Pixel + Min Pixel

The following example illustrates Modulation on brightfield images and Intensity of scatter in channel 1.





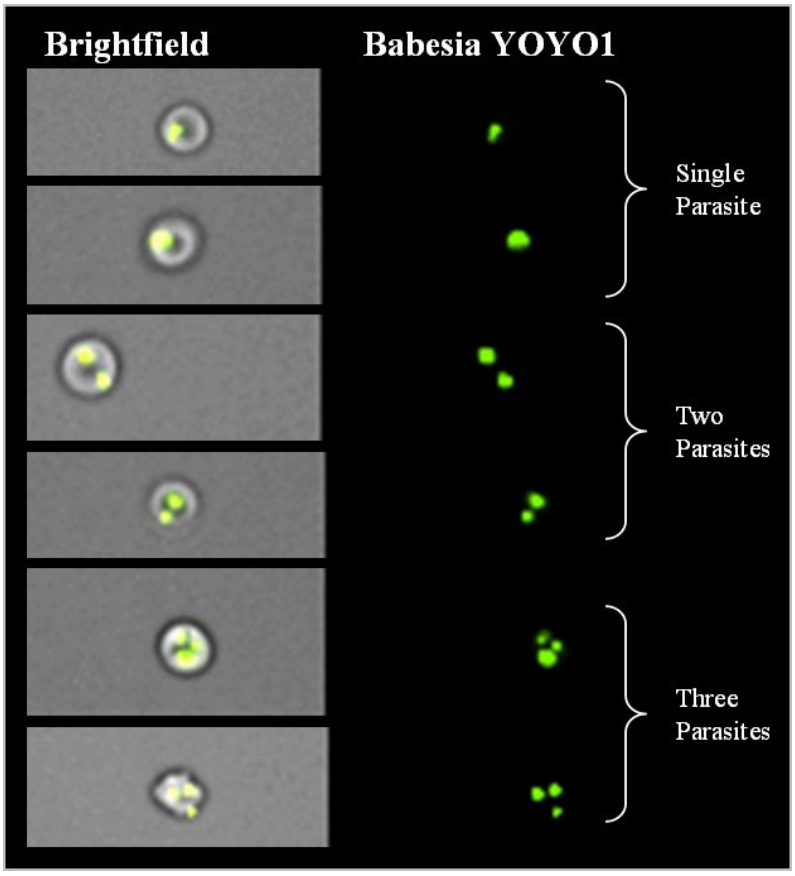
Application Example

- Quantify image quality and characterize contrast and texture in cells.

Spot Count Feature

The Spot Count feature provides the number of connected components in an image. The connected component algorithm examines the connectivity of each pixel based on whether this pixel is connected to a particular spot or the background. In order to count the number of connected components the mask input is very important.

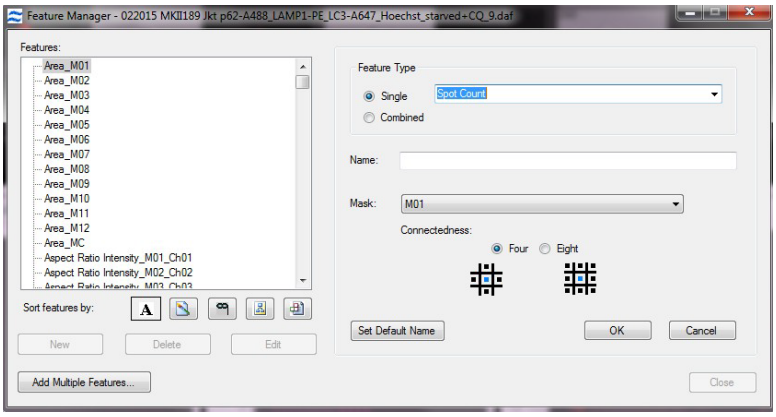
The following figure illustrates the application of Spot Counting to quantify parasitic infection of Babesia in erythrocytes by staining nuclei with YOYO (green).



Connectivity Option

The spot count feature offers a choice of 4 or 8 connectivity for the connected components algorithm. This option determines how a component is defined based on connected pixels.

The default selection, 4-connectivity (left), only considers neighbor pixels oriented vertically or horizontally from the pixel of interest. Pixels are included in the component if they are touching on the diagonal when the option eight is chosen.



Application Examples

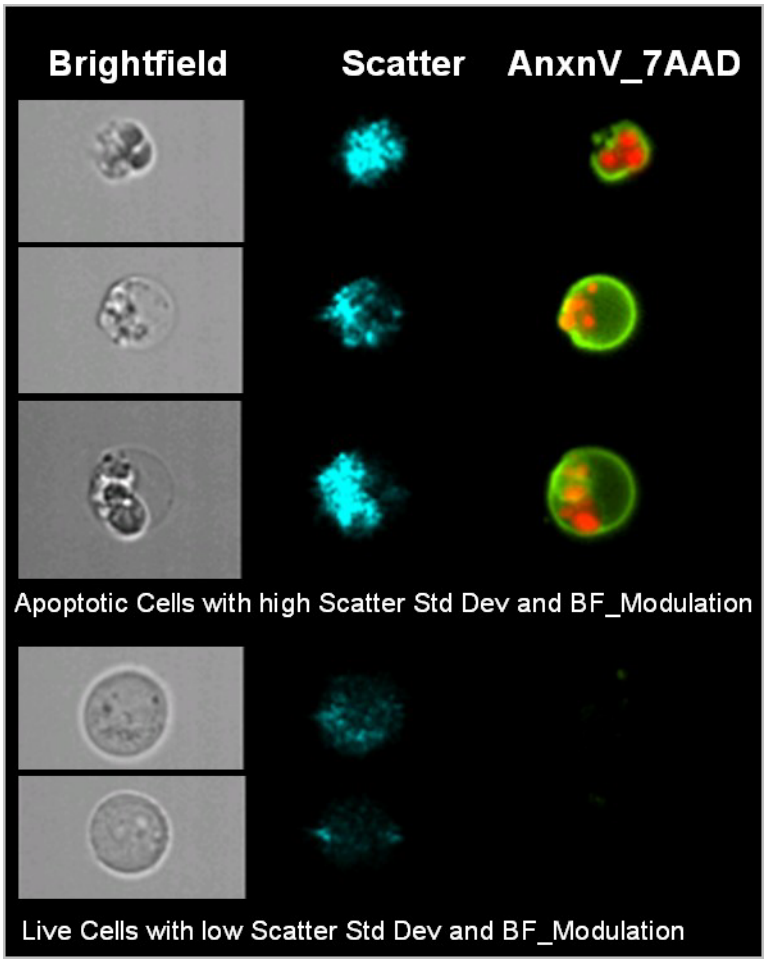
- Counting parasites.
- Counting phagocytosed particles.
- FISH spot counting.
- Counting punctate spots in images.
- Spot Wizard

Std Dev Feature

The Std Dev feature describes the overall distribution of pixel intensities.

The Std Dev is the standard deviation of the pixel intensity values in the mask. The Std Dev value provides an indication of the texture or complexity of an object.

The following illustrates that apoptotic cells (AnxnV positive) exhibit higher Std Dev values in the darkfield channel (scatter) and higher brightfield Modulation values than non-apoptotic cells (AnxnV negative).



Application Example

- Quantify intensity variation within a mask.
- Distinguish apoptotic and necrotic cells.

Signal Strength Features

Signal Strength features include the following:

- Bkgd Mean and Bkgd StdDev features describe the background of the image.
- Intensity and Raw Intensity features quantify the intensities in the region of interest.
- Raw Max, Raw Min, Raw Mean and Raw Median Pixel report single pixel values in an image.
- Max, Min, Mean and Median Pixel report background subtracted single pixel values in an image.
- Saturation Count and Saturation Percent quantify the saturated pixels.
- Spot Intensity Min is used when counting spots.

NOTE: When the name includes 'Raw', this means that there is no background subtraction.

Bkgd Mean Feature

The Bkgd Mean feature estimates the average camera background level in an image by taking the mean of the background pixels.

Application Examples

- Obtain estimate of the mean camera background level.
- Compute background subtracted pixel values in other feature computations.

Bkgd StdDev Feature

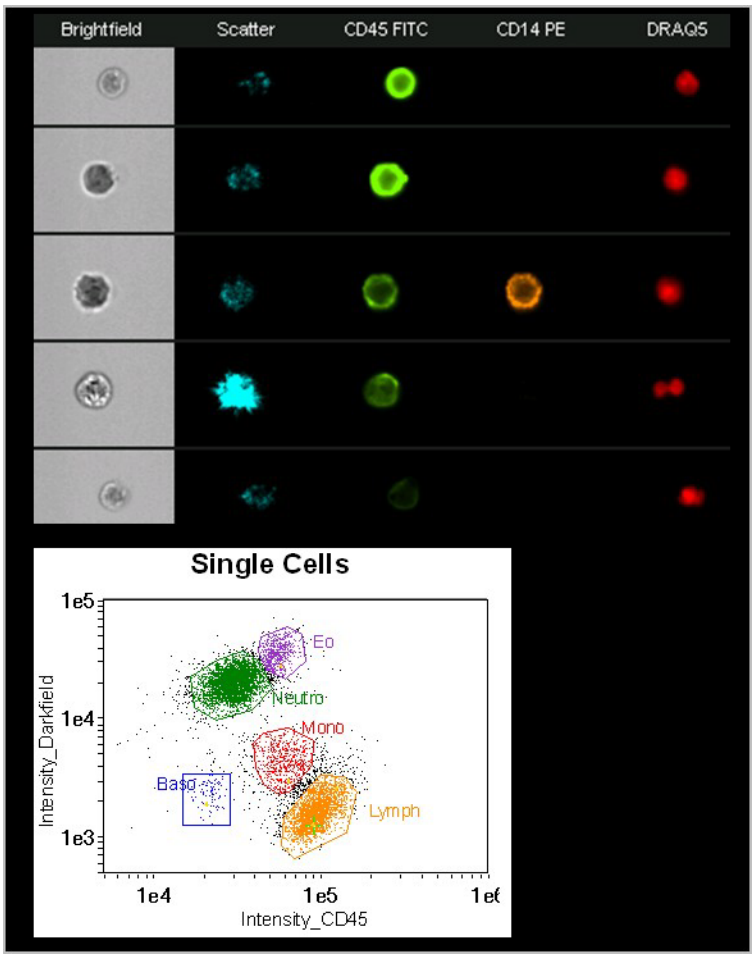
The Bkgd Std Dev feature estimates the standard deviation of the camera background level in an image computed using the background pixels.

Application Example

- Obtain estimate of the camera background noise.

Intensity Feature

The Intensity feature is the sum of the background subtracted pixel values within the masked area of the image.



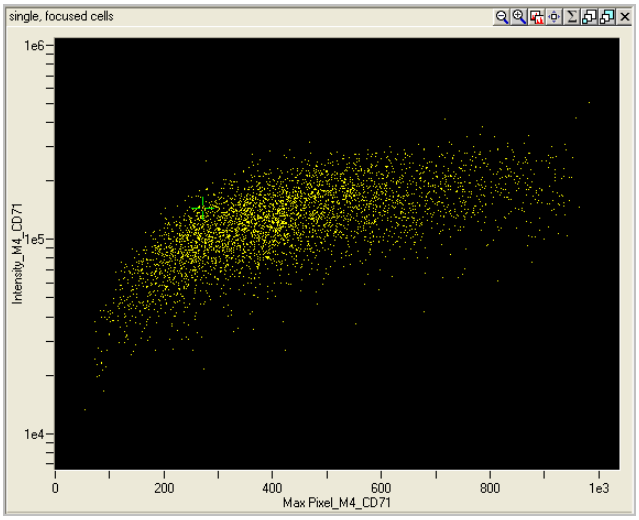
Application Examples

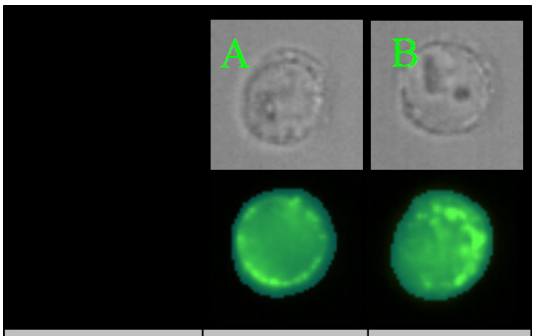
- Quantify relative levels of fluorescence between cells and within different regions of the same cell.
- Immunophenotyping.
- Cell cycle analysis.
- Protein expression.
- Protein activation.

Max Pixel Feature

The Max Pixel feature is the largest value of the background subtracted pixels contained in the input mask. An example plot is shown below that demonstrates the advantage of using this feature over the Intensity feature for identifying true positive events. For a concentrated signal, Max Pixel is more sensitive than Intensity as shown in the figure below.

The relationship of Max, Mean, Median, and Min Pixel is shown in the figure below:





FITC	Cell A	Cell B
Max Pixel	576	838
Mean Pixel	152	152
Median Pixel	178	130
Min Pixel	-0.05	-0.1
Intensity	230,000	230,000

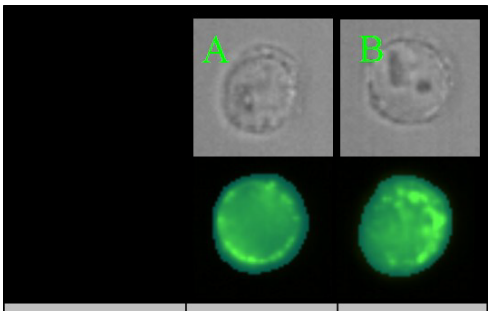
Application Examples

- Used to estimate the true peak fluorescence activity. Is preferred over the Raw Max Pixel for this application.
- Max Pixel to Mean Pixel ratio identifies bright punctate staining vs. uniform staining.

Mean Pixel Feature

The Mean Pixel feature is the mean of the background subtracted pixels contained in the input mask. This is computed as Intensity/number of pixels.

The relationship of Max, Mean, Median and Min Pixel is shown in the figure below:



FITC	Cell A	Cell B
Max Pixel	576	838
Mean Pixel	152	152
Median Pixel	178	130
Min Pixel	-0.05	-0.1
Intensity	230,000	230,000

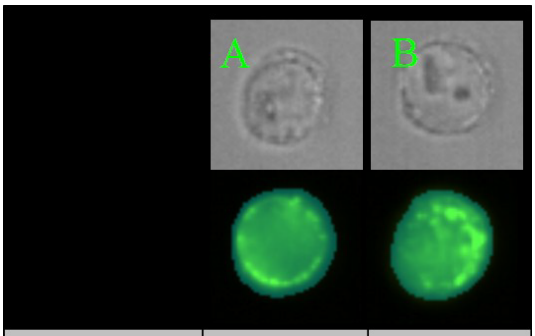
Application Examples

- Estimate the average fluorescence activity. This feature is preferred over the Raw Mean Pixel feature.
- Quantify relative levels of mean fluorescence between cells.
- Identify bright punctate spots by calculating the max to mean pixel ratio.
- Track internalization of surface bound antibodies.

Median Pixel Feature

The Median Pixel feature is the median of the background subtracted pixels contained in the input mask. It is more robust than the mean as an estimate of the average fluorescence since it is less influenced by outliers.

The relationship of Max, Mean, Median and Min Pixel is shown in the figure below:



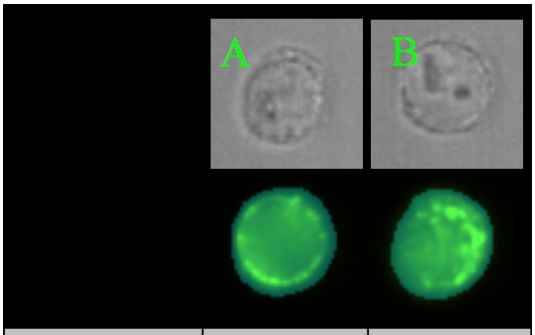
FITC	Cell A	Cell B
Max Pixel	576	838
Mean Pixel	152	152
Median Pixel	178	130
Min Pixel	-0.05	-0.1
Intensity	230,000	230,000

Application Example

- Estimate the average fluorescence activity. This feature is preferred over the Raw Median Pixel feature.

Min Pixel Feature

The Min Pixel feature is the smallest value of the background subtracted pixels contained in the input mask. There will be some negative numbers due to the background subtraction, therefore the Raw Min Pixel feature is preferred.



FITC	Cell A	Cell B
Max Pixel	576	838
Mean Pixel	152	152
Median Pixel	178	130
Min Pixel	-0.05	-0.1
Intensity	230,000	230,000

Application Examples

- Obtain the minimum value in an image after background subtraction. Very likely to be negative in brightfield imagery.
- Quantify spectral absorbance using the brightfield image.
- Identify over compensated images.

Raw Intensity Feature

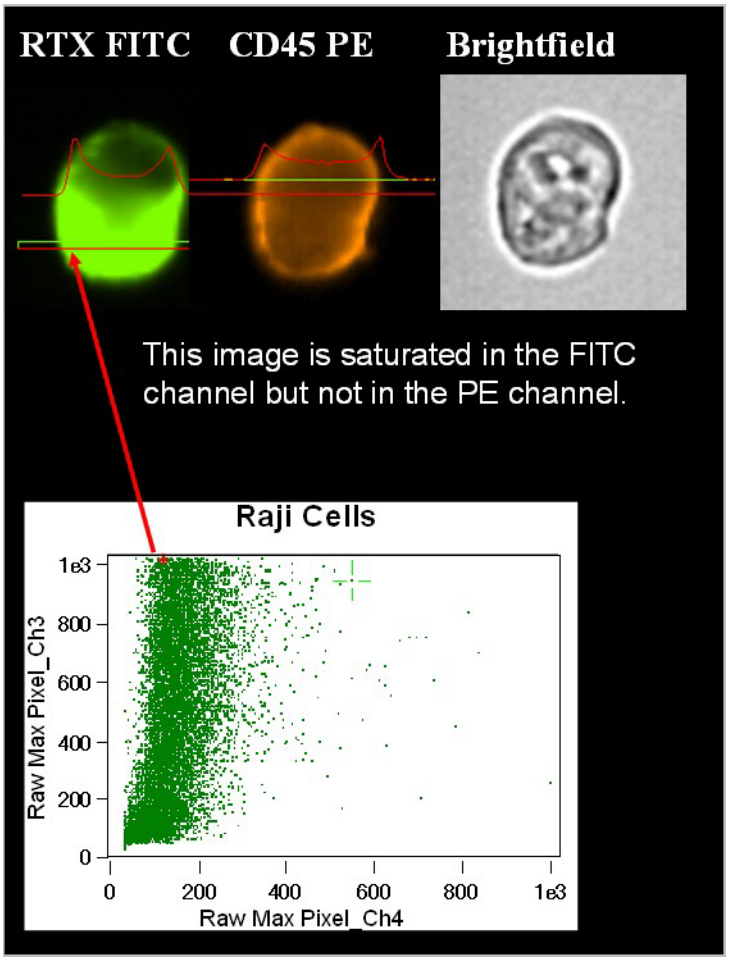
The Raw Intensity feature is the sum of the pixel values within the mask including camera background.

Application Example

- Estimate raw fluorescence activity. This feature is less relevant than the Intensity feature because it includes camera background intensity.

Raw Max Pixel Feature

The Raw Max Pixel feature is the largest value of the pixels contained in the input mask.



Application Examples

- Determine the presence of saturated events.
- May also be used to estimate the peak fluorescence activity, though the Max Pixel feature is recommended for this application.
- Measure the maximum pixel value within the mask.
- Identify cells that saturate the CCD, Saturation Count feature can also be used for this application.

Raw Mean Pixel Feature

The Raw Mean Pixel feature is the mean of the pixels contained in the input mask. This is computed as Raw Intensity of pixels.

Application Example

- Estimate the raw average fluorescence activity. This feature is less relevant that the Mean Pixel feature.

Raw Median Pixel Feature

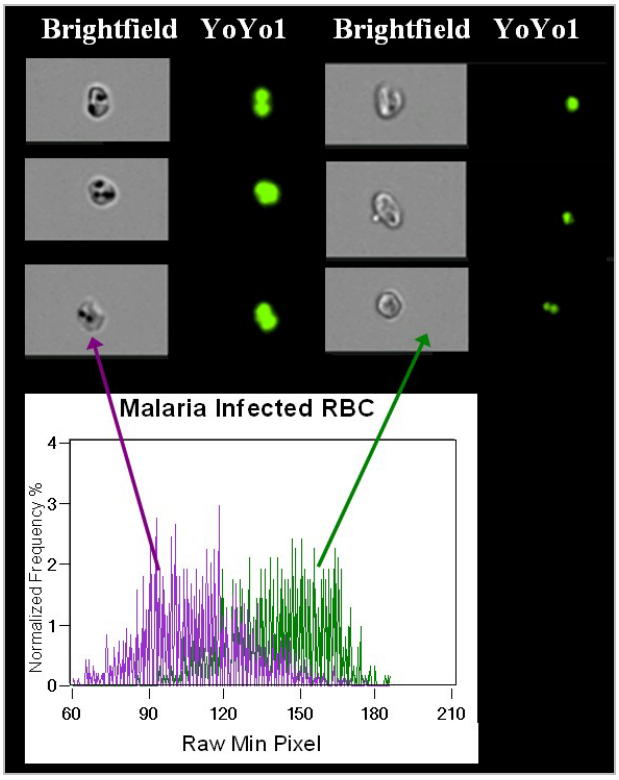
The Raw Median Pixel feature is the median of the pixels contained in the input mask.

Application Example

- Estimate the raw average fluorescence activity that is robust to outliers. This feature is less relevant than the Median Pixel feature.

Raw Min Pixel Feature

The Raw Min Pixel feature is the smallest value of the pixels contained in the input mask. The example below illustrates quantifying the level of malarial infected cells by using Min Pixel values of brightfield imagery.



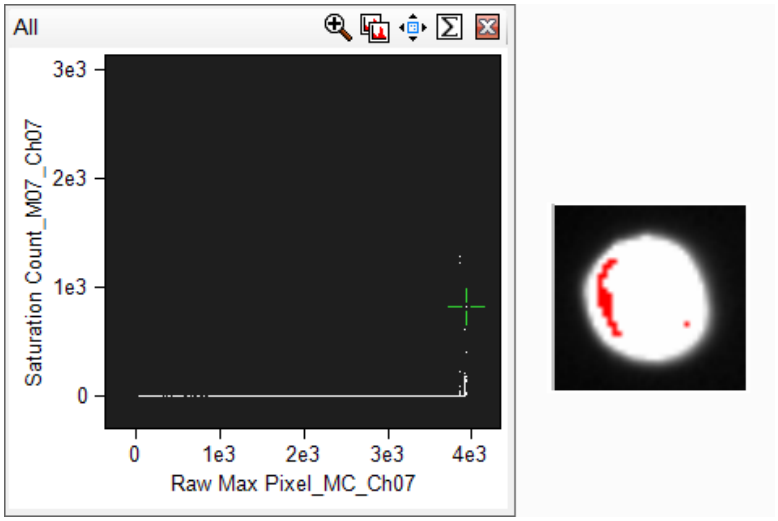
Application Example

- Quantify spectral absorbance using the brightfield image.
- Identify over compensated images.
- Measure the level of malaria infection in RBCs.

Saturation Count Feature

The Saturation Count feature reports the number of saturated pixels in an object.

In the figure below, objects with saturated pixels are lined up at the Raw Max Pixel value of 4095 and a selected image is shown with saturated pixels in red.



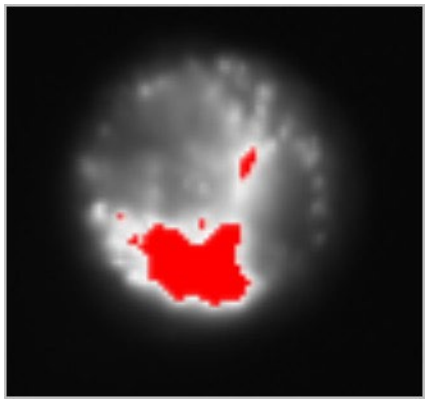
Application Example

- Measure the validity of the experiment setup. Saturated data may not produce useful information.

Saturation Percent Features

The Saturation Percent feature reports the percentage of saturated pixels in an image. Pixel intensities are measured on the camera pixels from 0 to 4095 (12 bit) and therefore become saturated and cannot be quantified after 4095.

An object with saturated pixels shown in red:



Application Example

- Measure the validity of the experiment setup. Saturated data may not produce useful information.

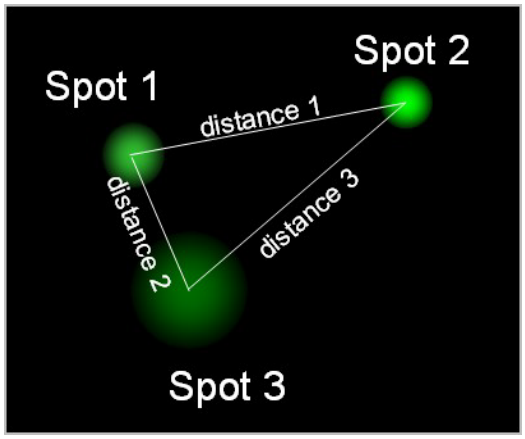
Spot Intensity Min and Spot Intensity Max Features

Spot Intensity Min provides the smallest Raw Mean Pixel value (not background subtracted) of the dimmest spot (connected component). The Raw Mean Pixel values for each spot is computed and the smallest value is reported.

Spot Intensity Max provides the largest Raw Mean Pixel value (not background subtracted) of the brightest spot (connected component). The Raw Mean Pixel values for each spot is computed and the largest value is reported.

These are two of four features that can be used to identify objects with spots that are close together, dim, bright or small when counting spots in an image. To use these features, the spots need to be individually masked, such as using the Spot or Peak Mask. The Spot Area, Distance, and Intensity Min or Max features measure properties of different spots in an image and are often used with the Spot Count feature (under Texture). Spot Area Min (Size) provides the area of the smallest spot. Spot Distance Min (Location) provides the shortest distance between two spots.

The following diagram illustrates these features:



- Spot Area Min is the Area of spot 1
- Spot Distance Min is distance 2 in microns
- Spot Intensity Max is the Raw Mean Pixel value of spot 2
- Spot Intensity Min is the Raw Mean Pixel value of spot 3

Application Example

- In FISH Spot Counting, these features are used to identify ambiguous spots that are located too close together, too dim, too bright or too small to count and can be eliminated from the analysis.

Uncompensated Intensity Feature

The Uncompensated Intensity feature is the sum of the background subtracted pixel values within the masked area of the image with no compensation applied. This is the Intensity of the uncompensated image. This feature is calculated in INSPIRE™ software during acquisition.

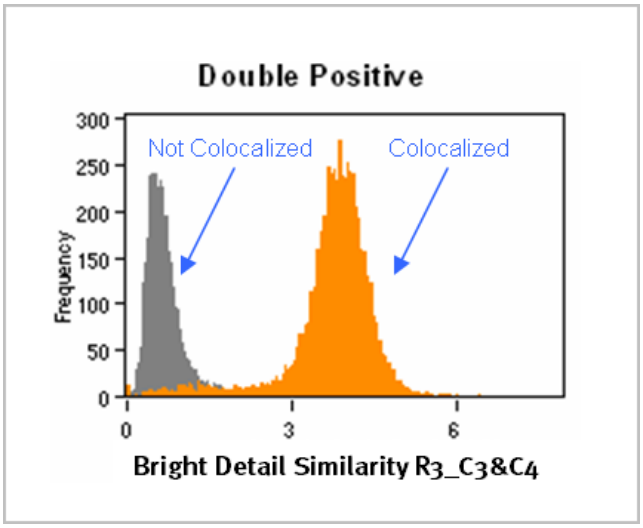
Comparison Features

The Comparison features describe the difference of intensity measurements between masks or pixels in different images or the same image with different masks. These include Bright Detail Similarity R3, Intensity Concentration Ratio, Internalization, and Similarity.

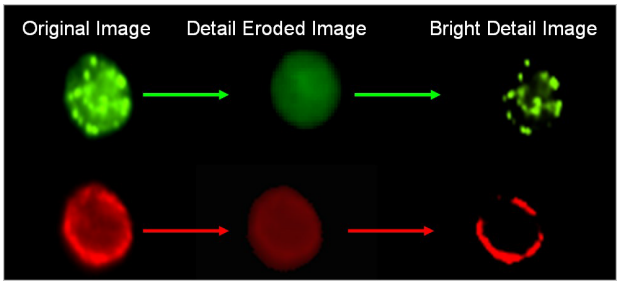
Bright Detail Similarity R3 Feature

The Bright Detail Similarity R3 feature is designed to specifically to compare the small bright image detail of two images and can be used to quantify the co-localization of two probes in a defined region, such as that of endosomes. The Bright Detail Similarity R3 feature is the log transformed Pearson's correlation coefficient of the localized bright spots with a radius of 3 pixels or less within the masked area in the two input images. Since the bright spots in the two images are either correlated (in the same spatial location) or uncorrelated (in different spatial locations), the correlation coefficient varies between 0 (uncorrelated) and 1 (perfect correlation), and does not assume negative values. The coefficient is log transformed to increase the dynamic range between {0, inf}.

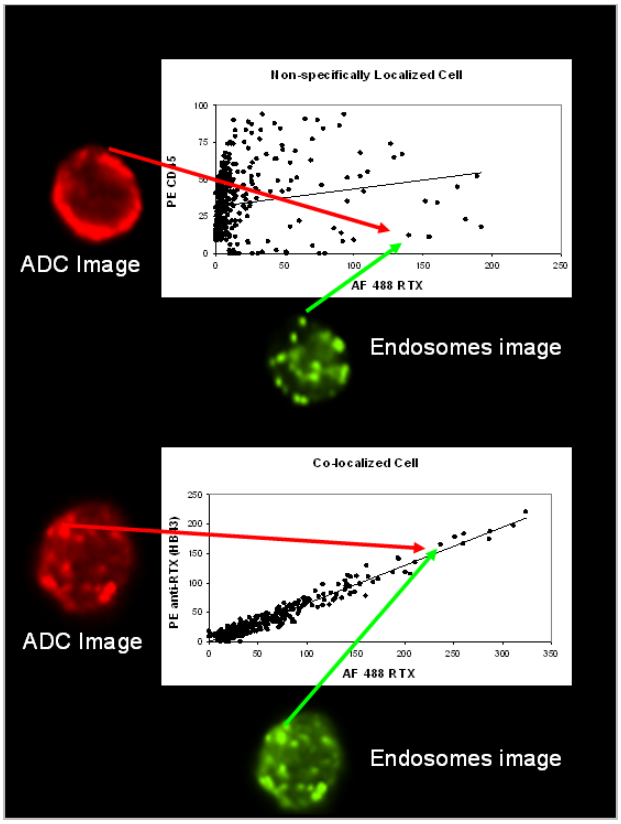
The following figure shows the Bright Detail Similarity R3 graph of two populations, one that has colocalization and one that has no colocalization.



The figure below illustrates the process of obtaining the localized bright spots. The bright areas are eroded from the original image and the detail eroded image is subtracted from the original image resulting in the bright detail image.



The figure below shows the correlation analysis between an image pair.



Application Examples

- Quantify the degree of colocalization between two probes.
- Track internalization and intracellular trafficking of antibody drug conjugates to either the endosomes or the lysosomes.
- Colocalization of Rituxan and compliment C3b.
- Co-localization Wizard

Intensity Concentration Ratio Feature

The intensity concentration ratio is defined as the ratio of the intensity inside the first input mask to the intensity of the union of the two masks the higher the score, the greater the concentration of intensity inside the first mask. All pixels are background-subtracted. The ratio is invariant to cell size and can accommodate concentrated bright regions and small dim spots. The ratio is mapped to a log scale to increase the dynamic range to values between {-inf, inf}. This feature is a generalization of the Internalization feature.

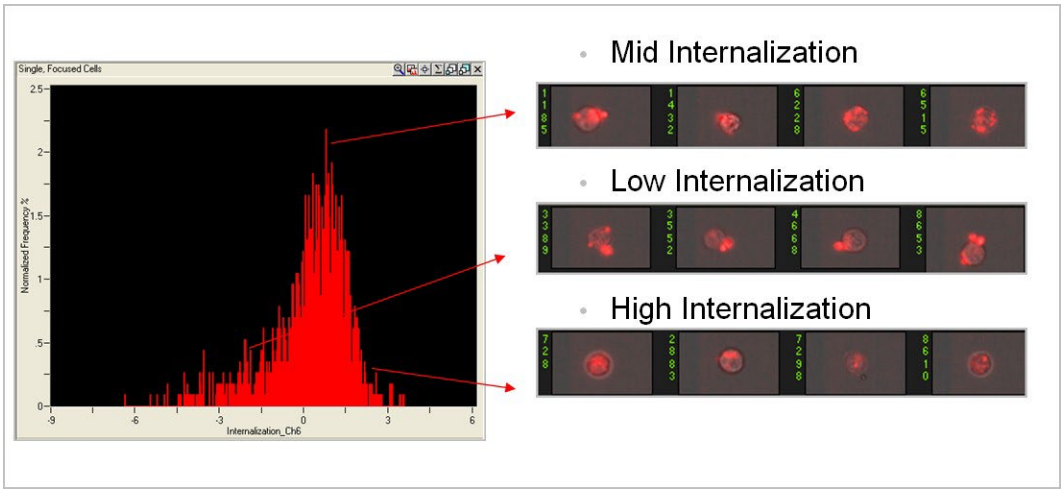
Application Example

- Quantify relative intensity concentrations between different cellular compartments. Internalization is a special case of this where the first mask is the internal compartment and the second is the membrane region.

Internalization Feature

The Internalization feature is defined as the ratio of the intensity inside the cell to the intensity of the entire cell. The higher the score, the greater the concentration of intensity inside the cell. All pixels are background subtracted. The user must create a mask to define the inside of the cell for this feature. The feature is invariant to cell size and can accommodate concentrated bright regions and small dim spots. The ratio is mapped to a log scale to increase the dynamic range to values between {-inf, inf}. Internalized cells typically have positive scores while cells with little internalization have negative scores. Cells with scores around 0 have a mix of internalization and membrane intensity.

Composite Images of brightfield and channel 6 are shown for High, Medium, and Low Internalization values.



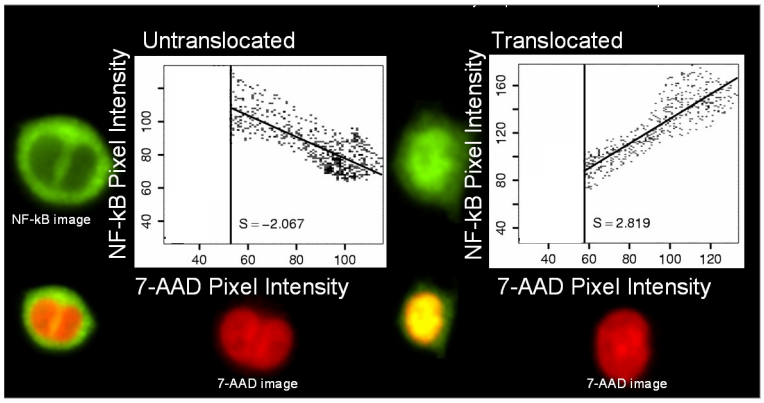
Application Examples

- Quantify internalization when supplied with the internal mask.
- Quantify the intensity ratio of a region of interest to the whole cell.
- Internalization Wizard

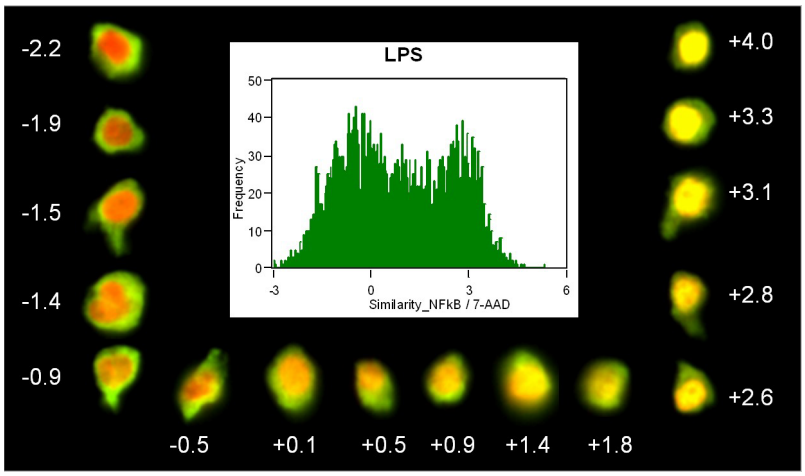
Similarity Feature

The Similarity feature is the log transformed Pearson's Correlation Coefficient and is a measure of the degree to which two images are linearly correlated within a masked region.

The following figure shows two image pairs that are in spatial registry to one another. On the left the NF-*k*B (green) is predominantly located in the cytoplasm of the cell and has a dissimilar distribution compared to the 7-AAD image (red). When the intensity of the green is high, the intensity of the red is low and vice versa. The Similarity value for this cell is -
2.067 indicating that the image pair has a high degree of dissimilarity. Analysis of the image pair on the right shows that when the intensity of the green is high, the intensity of the red is high and the Similarity value is a high positive number.



Below are examples of cells with varying amounts of similarity between the NF*k*B image in green and 7-AAD image in red shown here as a composite image. The most dissimilar image pairs in the upper left to the most similar image pairs in the upper right.



Application Examples

- Quantify translocation.
- Identify co-polarization of two probes.
- Nuclear Localization Wizard

XCorr Feature

The XCorr feature is a measure of similarity or 'sameness' between two images: the higher the value, the more similar the images. It is robust to intensity variations and relative shifts between the images and is typically used with the combined mask MC. It is computed using the normalized cross correlation between the two input images.

Application Examples

- Used as a mask independent measure of similarity between two images.
- Used to measure cross camera alignment and remove misaligned images.

System Features

The system features do not require a mask.

Camera Line Number Feature

The Camera Line Number feature returns the camera line number values. This feature is obtained from INSPIRE™ software.

Application Example

- Used in objects per mL feature.

Camera Timer Feature

The Camera Timer feature returns the camera timer values that are in ticks. This feature is obtained from INSPIRE™ software.

Application Example

- Used in Time feature.

Flow Speed Feature

The Flow Speed is the calculated flow speed, in mm/sec, of the object.

The Flow Speed feature is the speed of flow of the cells. It is obtained from INSPIRE™ software. It should be very consistent across all cells in a file.

Application Example

- Determine consistency of flow.

Object Number Feature

The Object Number feature denotes the serial number of a cell in a file.

Application Example

- Reference an object in a file.

Objects/mL Feature

The Objects per mL feature returns the object concentration with respect to local volume.

Application Example

- Monitor the object flow during the run.

NOTE: Use the statistic Concentration to obtain objects/mL of a population.

Objects/sec Feature

The Objects per sec feature returns the local object concentration with respect to time.

Application Example

- Monitor the throughput during a run.

NOTE: Use the statistic Concentration to obtain objects/ml of a population.

Time Feature

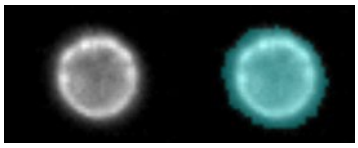
The Time feature returns the camera timer values that are in ticks, converted to secs with a formula.

Application Example

- Obtain the time taken to collect a sample

Masks

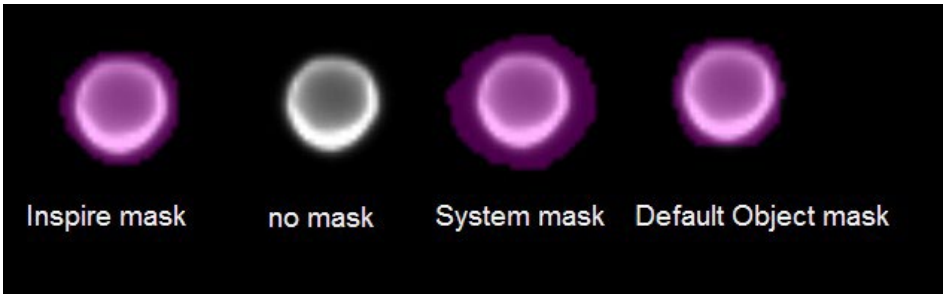
The set of pixels that contains the region of interest is called the mask. In the following picture, the mask consists of the set of pixels on the right image that are colored cyan. The cell is represented in the greyscale image on the left. Calculating some feature values, such as the Area value, requires only a mask. Calculating others, such as Intensity value, requires a mask and a channel image.



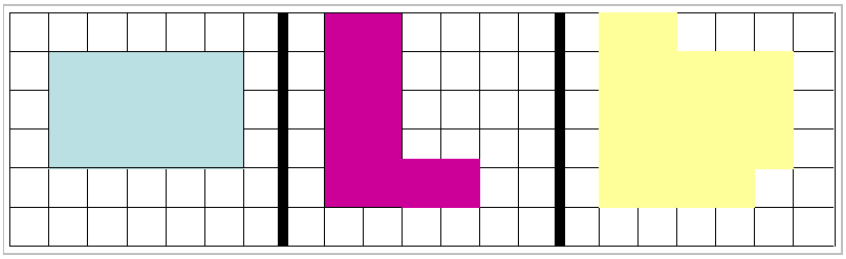
There are three types of masks: Default masks, Combined masks and Function masks:

- Default masks named M01 through M12 are either created in INSPIRE™ software during acquisition or created in
  IDEAS® software when a .rif file is opened. The default mask used by INSPIRE™ software during acquisition is different than the default mask created in IDEAS® software (Default Object) when a .rif file is opened from an ImageStream®^{X} system .These masks are stored in the .cif file and cannot be changed by the user.

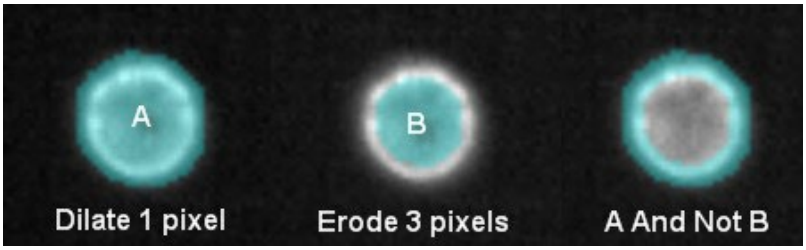
  NOTE: Versions of IDEAS® software prior to 3.0 were using the System function mask with weight of 5 for the default masks which was more permissive and resulted in larger masks. Below is an example of the difference between the default masks.



- Combined masks are created using Boolean logic to combine and subtract masks. For example, the cytoplasmic mask is created by taking the brightfield mask and not the morphology mask of the nuclear image. You can use the Mask Manager to combine masks of different regions or images. The IDEAS® software application default template provides a combined mask named MC that is the union of the pixels from all channel masks
  and a NMC mask that is everything outside of MC. The following illustration shows two channel masks that are combined into one mask, which is shown in the right most panel.



Below is an example of making a membrane mask using Boolean Logic



- Function masks are created with user input. There are fourteen types of function masks, Dilate; Erode; Fill; INSPIRE™; Intensity; Interface; Morphology; Threshold; Spot; System; Object; Peak; Range; Skeleton; and Valley. Each of the functions masks are defined here.

AdaptiveErode Mask

The AdaptiveErode mask identifies pixels that will form a circle that touch the input boundary with at least a prescribed radius threshold. The radius threshold is inversely proportional to the user input. Therefore the larger the input threshold value, the smaller the circle radius which will include more pixels and vice versa. The result is an adaptive erosion that takes shape into account instead of a strict pixel number erosion.

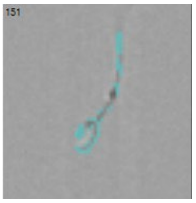
Application Examples

- Can be used to identify the head of sperm.
- Can be used in combination with the default channel mask to mask the cell membrane.
- Can be used in applications where it is critical to erode a mask based on the shape of the input mask.

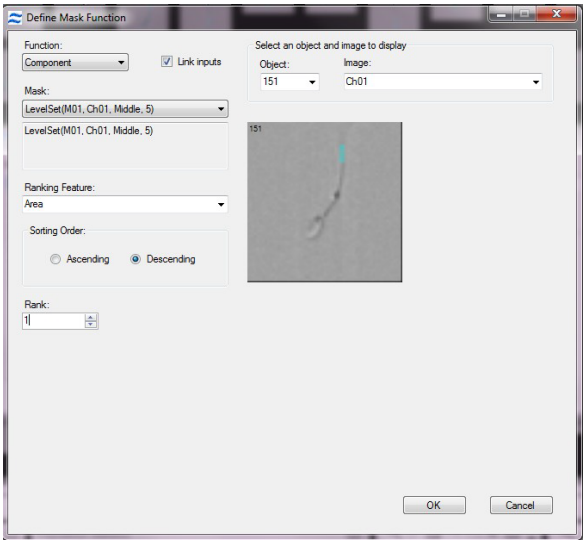
Component Mask

Given an input mask with multiple pieces, each piece can be identified as a component based on a selected feature. The components can be sorted in ascending or descending order and each ranked order can be chosen as a separate component mask.

Example 1: This object's mask has multiple pieces defined by a LevelSet mask.

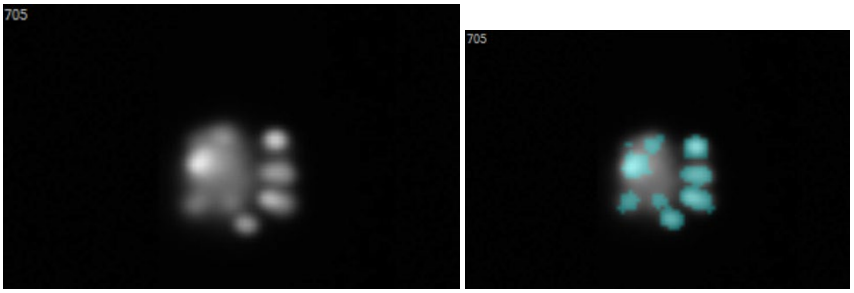


In this example the area feature is used to define the largest component. The ranking feature is area, sorted descending (highest to lowest/ largest to smallest) and the first ranked object is shown, therefore the largest piece.



Example 2: Here is the image that has a mask with multiple pieces. The mask is shown in blue, on the right side image.

Choosing area as the ranking feature, if the “ascending” sorting order is selected, then the components of the mask will be sorted from smallest to largest.



Selecting rank 1 in conjunction with an ascending sort order and the area ranking feature will return the smallest component, as seen on the left side image. Selecting the “descending” sorting order will return the largest component, as seen on the right side image.

Application Examples

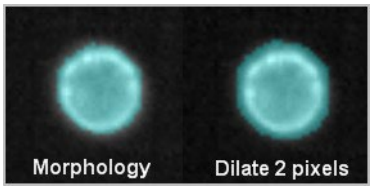
- Can be used to identify separate components within a mask
- Can be used in combination with the Watershed mask to identify an individual cell in a conjugate or dividing cell

- Features may be calculated on individual components, for example analysis of each daughter cell can be used to measure asymmetric cell division

From left to right, no mask, watershed mask, component largest area, component second largest area. This is a jurkat cell in telophase.

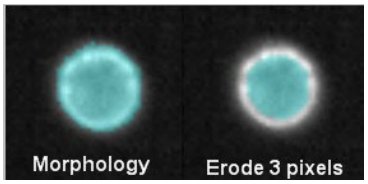
Dilate Mask

The Dilate mask adds the selected number of pixels to all edges of the starting mask.



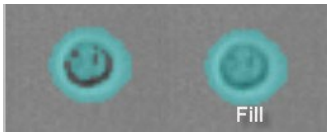
Erode Mask

The Erode mask removes the selected number of pixels from all edges of the starting mask.



Fill Mask

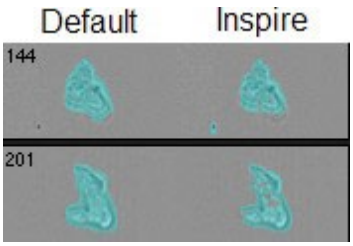
The Fill mask fills in any holes in the starting mask.



INSPIRE™ Software Mask

The INSPIRE™ software mask masks pixels above background and is the mask used during data acquisition in INSPIRE™ software. This mask is available to understand what is being masked during collection and is not generally used for feature calculations.

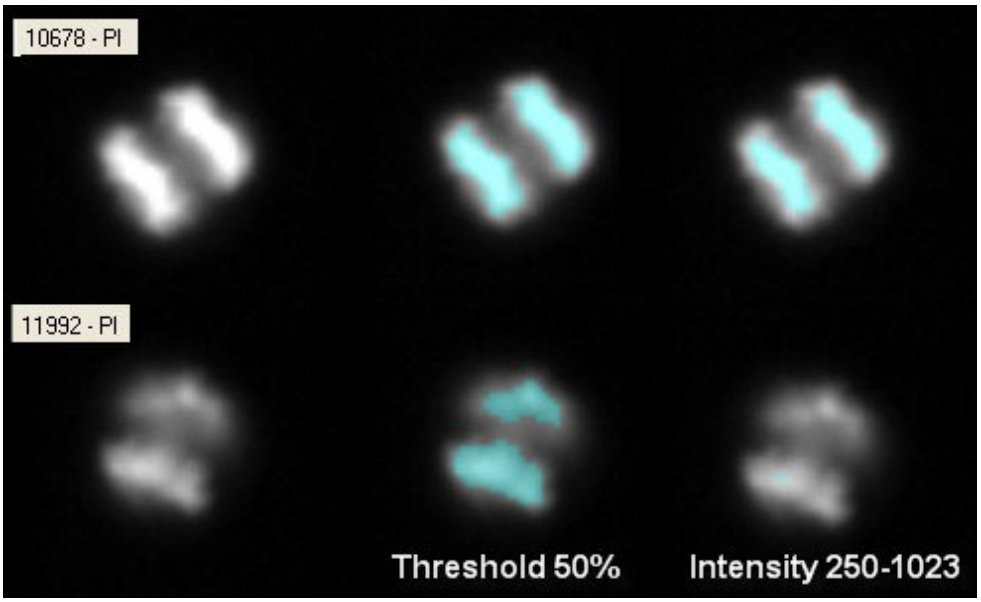
NOTE: This mask is new in IDEAS® software versions 4.0 or later.



Intensity Mask

The Intensity mask masks pixels between the lower and upper raw intensity thresholds not background subtracted.

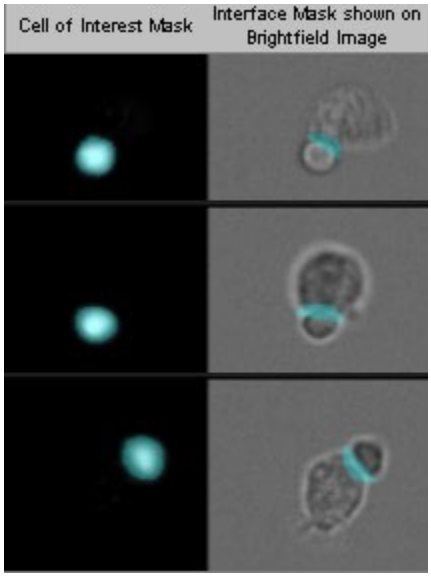
In the example below, cell #10678 is bright and cell #11992 is dim. The 50% Threshold mask is similar for both images whereas the Intensity mask 250+ is quite different, since only a few pixels in the dim image are greater than 250 counts, while most of the metaphase plates in the bright image are masked.

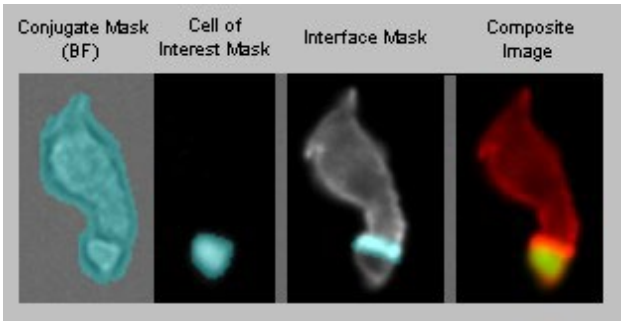


Interface Mask

The interface mask identifies pixels in an object where the object is in contact with a second object. Three input parameters are defined. First, the mask of one of the objects (cell of interest). Next, the mask that covers both objects (conjugate). A close fitting mask using another function mask such as Object (tight) can be used for the cell of interest mask. A brightfield mask can be used for the conjugate. Finally, the width of the interface mask from the contact point towards the cell of interest is entered.

Examples are shown below:





Application Example:

- Used to quantify synapses in T cell APC (antigen presenting cell) conjugates.

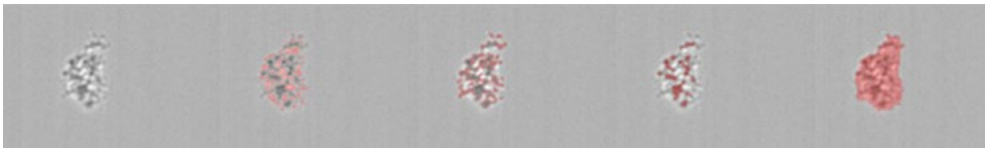
LevelSet Mask

The LevelSet mask is an extension of the Morphology mask that identifies pixel in non-homogeneous regions into three different levels: dim, middle and bright and the combination of all three. Contour detailed level can be adjusted such that the smaller the scale, the finer the image structure identified by the individual levels. This mask is particularly helpful for masking complex brightfield images.

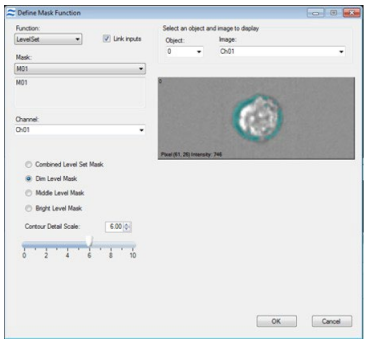
Application Examples

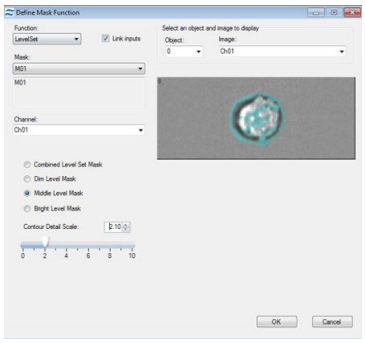
- Used to mask bright, medium or dim areas of an object
- Can be used to get a closely fitting mask (combined option) in lieu of object or morphology masks
- Can be used on brightfield and fluorescent channels

Example 1: From left to right, no mask, LevelSet (Bright), LevelSet (Middle), LevelSet (Dim), Level Set (Combined). This is an image of a protein aggregate.

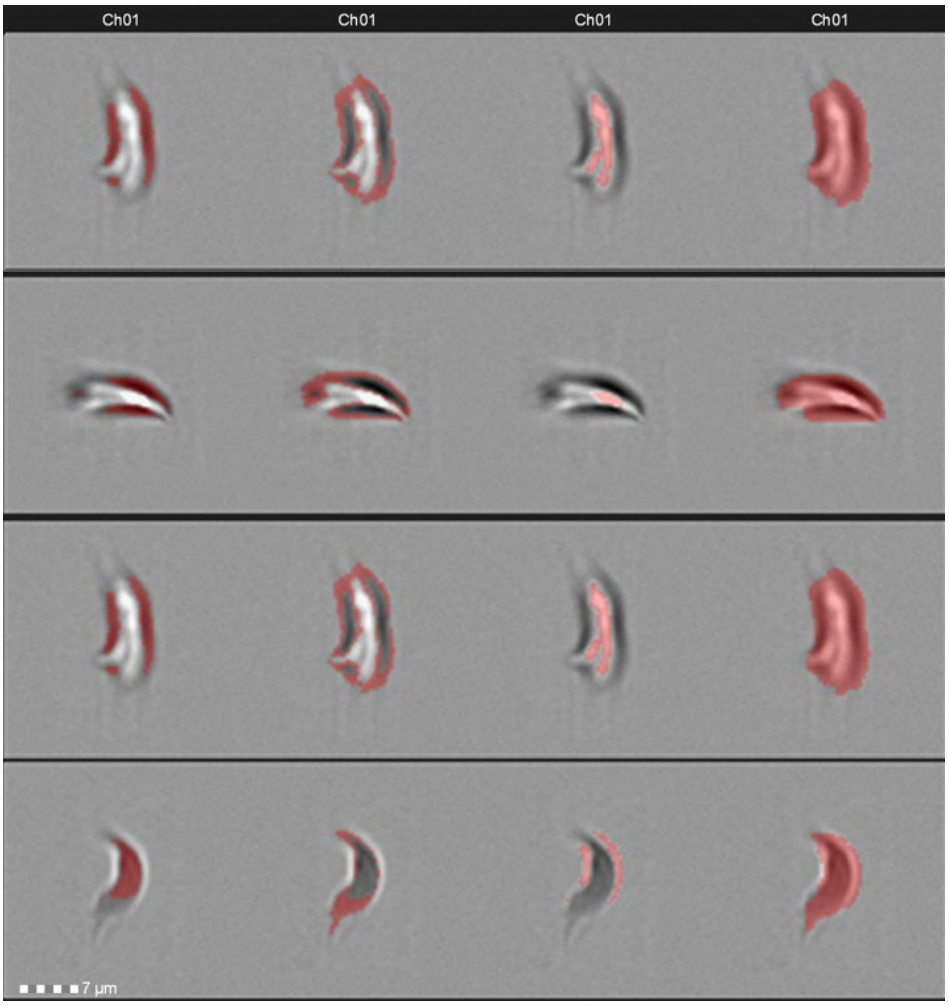


Example 2: The mask on the left is showing the LevelSet mask set to Dim with a contour level of 6. On the right, we are showing a middle level with a contour detail of 2.





Example 3: Image shows the use of LevelSet Mask with dim (first column), middle level (second column), bright (third column), and combined masks (forth column) for each cell.



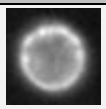
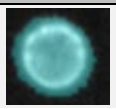
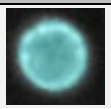
Morphology Mask

The Morphology mask includes all pixels within the outermost image contour. This mask, which is used in fluorescence images, is best used for calculating the values of overall shape based features.

Object Mask

The Object mask segments images to closely identify the area corresponding to the cell. It is based on the assumption that background pixels exhibit high uniformity to each other. This helps distinguish the background from the cell pixels. The mask characterizes the background pixels using a set of features and then segments the image by determining all the pixels that deviate from the background feature set. The default option is used for the default segmentation masks. The tight option uses a different set of features to characterize the background which results in a tighter fit around the cell. New masks created with this function are called Object (tight) masks.

Examples are shown below:

Image	Default Mask	Object (tight)
		

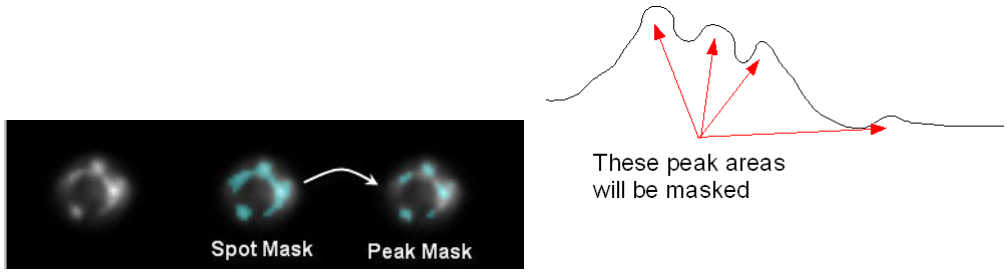
Application Examples

- Used to get a close fit around the cellular area (tight option).
- Can be used in lieu of the morph mask for applications where the morph is so tight that it provides incomplete masking, sometimes splitting cells into two regions, such as a nuclear dye image of cells in anaphase or telophase.
- Can be used in lieu of the morphology mask with the Similarity feature when measuring nuclear translocation for better separation between untranslocated and translocated cells (tight option).
- Used as the default segmentation masks (default option).

Peak Mask

The Peak mask identifies intensity areas from an image that have local maxima (bright) or minima (dark). Initially, the peak mask will identify all peaks in the image. To select peaks which have certain brightness, the spot to cell background ratio is used. This is the ratio between the spot pixel value to the mean camera background value in the original image.

Below is an example of the Peak, bright option.

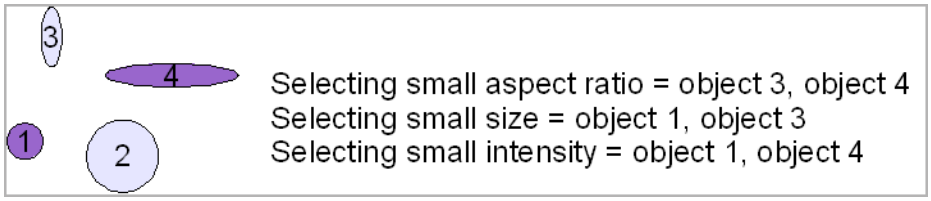


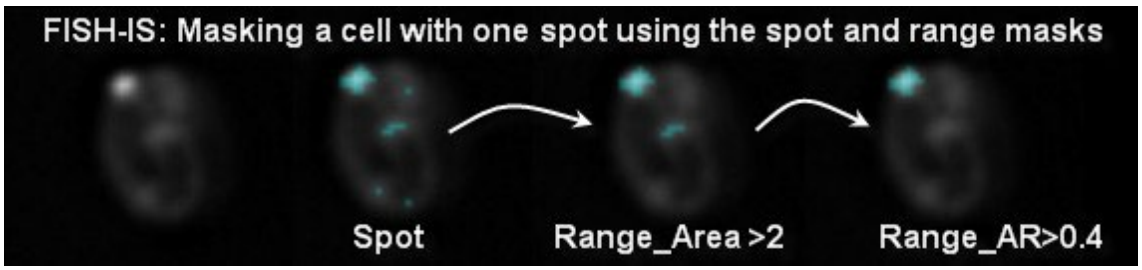
Application Examples

- Used with the Spot Count feature to quantify the speckleness of cells.
- Separate connected spots in a Spot Mask into individual components.

Range Mask

The Range mask provides a capability to select components in an image within a selected size and/or aspect ratio by setting a minimum and maximum area and minimum and maximum aspect ratio.



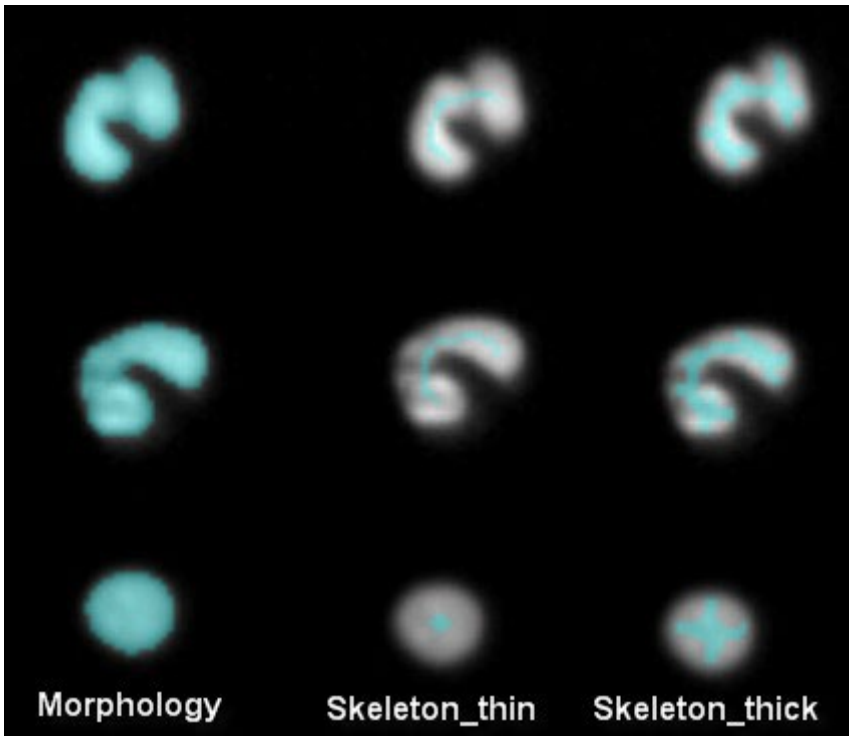


Application Examples

- Use with a Spot Mask to constrain the Spot Count feature to round spots.
- Use on any other mask that has multiple components to define unwanted objects such as debris, objects that are too small or whose shapes are not circular.

Skeleton Mask

The skeleton mask provides the barebone structure of the object from the starting mask. Two options are available: thin or thick skeletons. The thin option produces the condensed shape of the object and typically takes a form of 1-pixel wide skeletal line. The thick option is intensity weighted. The thin option is dependent on the shape of starting mask; thick uses the pixel intensities and is less sensitive to the shape of the starting mask. The user will need to pay careful attention to the starting mask. In the example below the Morphology mask of the image was used as the starting mask for creating the skeleton.

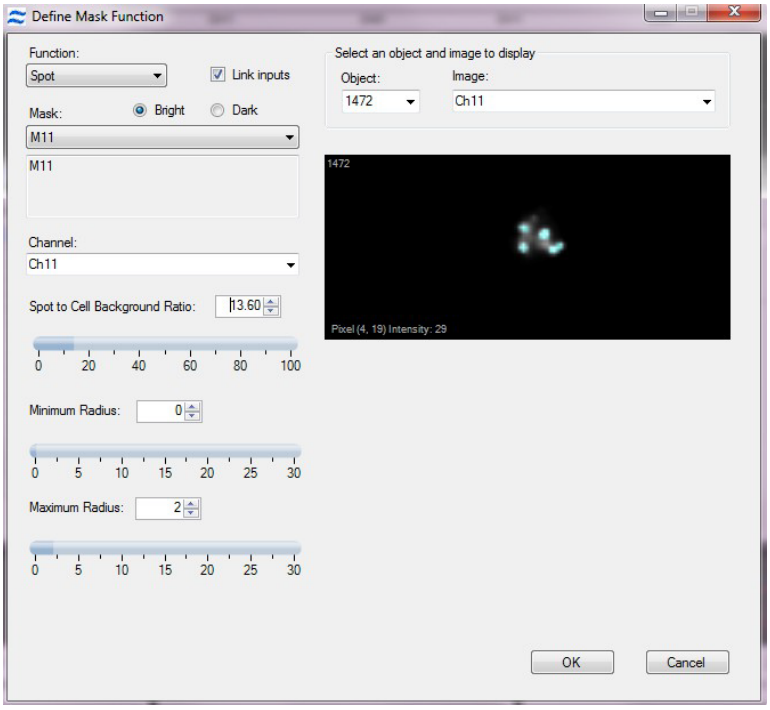


Application Examples

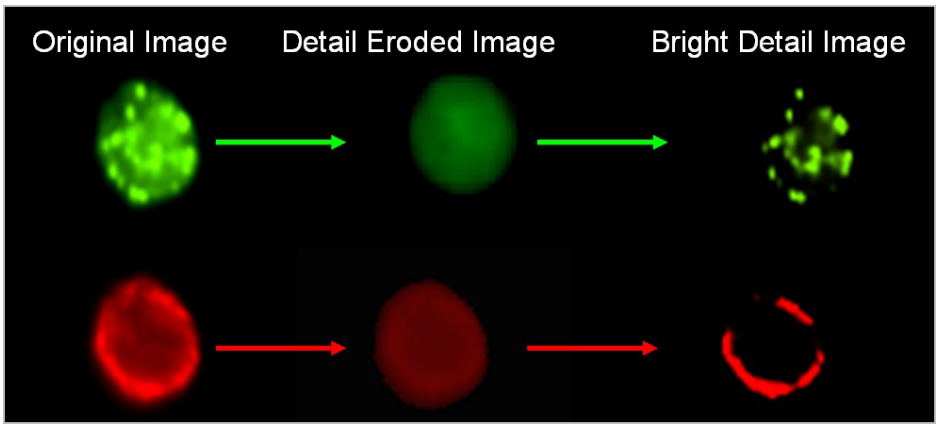
- Thick skeletons can be used with shape based features such as symmetry to accentuate the shape of an object, and provide greater separations.
- Separate singlets and doublets by computing the area of the thin skeleton mask. We have used the object (tight) for this case.
- Nuclear morphology measurements with lobe count feature for cell classification cells.

Spot Mask

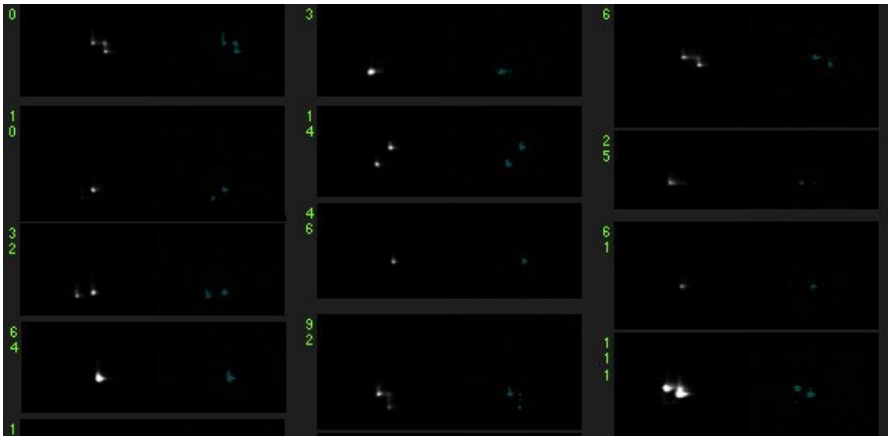
The Spot Mask has two options: bright or dark. The bright option obtains bright regions from an image regardless of the intensity differences from one spot to another. The ability to extract bright objects is achieved using an image processing step that erodes the image and leaves only the bright areas. The dark option obtains dark regions. The spot to cell background ratio and minimum and maximum radius are specified by the user. The spot to cell background ratio is the spot pixel value divided by the background in the bright detail image. A maximum radius of x and a minimum radius of y implies the image contains spots with a thickness less than 2x+1 and greater than 2y+1 pixels.



The figure below illustrates the open residue process. The bright areas are eroded from the original image and the detail eroded image is subtracted from the original image resulting in the bright detail image.



The image pairs below show objects in grayscale next to their corresponding Spot Masks in cyan. Spot masks can be further refined using the Peak and/or Range masks.



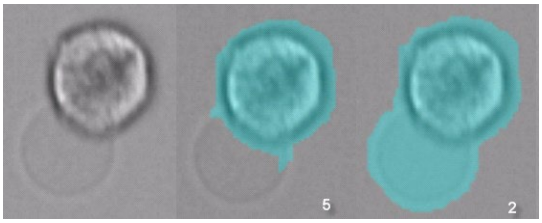
Application Examples

- Used with the Spot Count feature to enumerate spots in images such as for FISHIS®.
- Used with Intensity features to quantify intensity in spots.
- Dark spot finds valleys in images such as the low intensity between 2 stained nuclei and is useful for finding immune synapses.
- Identifies the dark areas in red blood cells or parasitic infections in brightfield imagery.

System Mask

The System mask segments objects in an image based on a probability model of how pixels should be grouped together. The user sets a weight value that defines a loose or tight grouping. A low weight value groups in a more permissive manner.

Shown is an example of a cell with an apoptotic bleb that is not masked with the System mask weight set at 5 but is masked with the System mask weight set at 2.



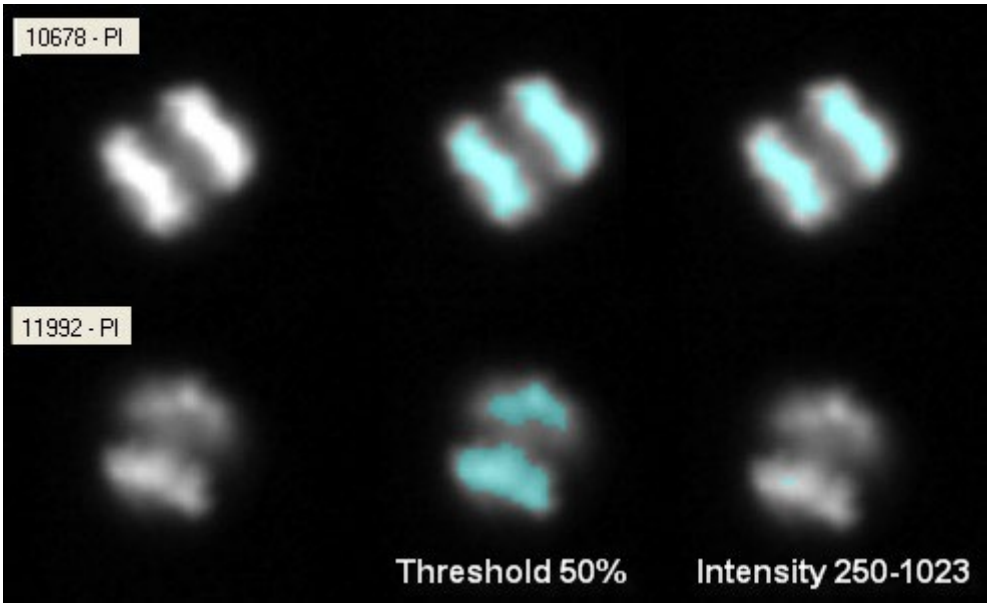
Application Example

- Used on brightfield images to capture a low contrast areas such as cells that undergo a blebbing process, tails of sperm or other low contrast type of structures.

Threshold Mask

The Threshold mask is used to exclude pixels, based on a percentage of the range of intensity values as defined by the starting mask. The user chooses the starting mask when creating the Threshold mask.

In the example below, cell #10678 is bright and cell #11992 is dim. The 50% Threshold mask is similar for both images whereas the Intensity mask 250+ is quite different, since only a few pixels in the dim image are greater than 250 counts, while most of the metaphase plates in the bright image are masked.

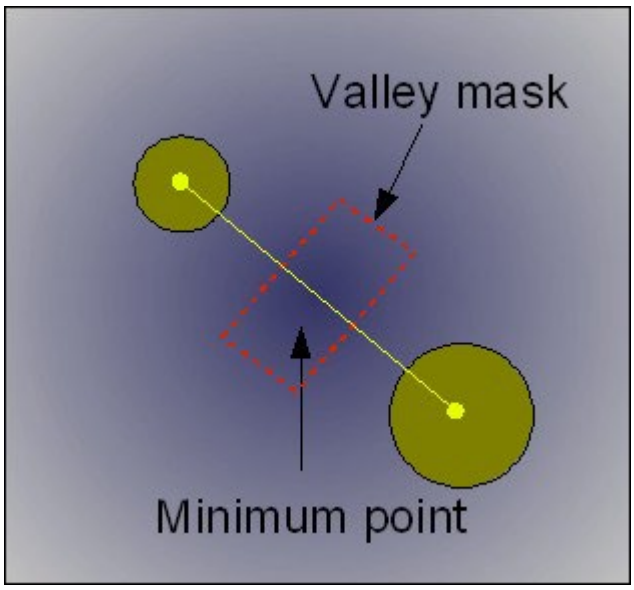


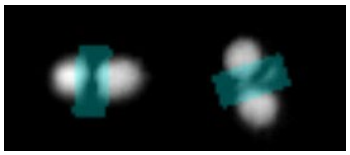
Application Example

- Used with the Area feature to define apoptotic cells in the Apoptosis Wizard.

Valley Mask

The Valley mask is a rectangular mask that sits between two bright regions in a starting mask, such as between two nuclei. It is constructed by finding the minimum intensity along the skeletal line between these two bright regions. The skeletal line is obtained internally using the skeleton (thin) masking. This minimum intensity identifies the intersection between the two objects. The mask is drawn perpendicular to this skeletal like. The length of the valley mask rectangle is equal to the minor axis of the object and the width of the mask is defined by the user in pixels.



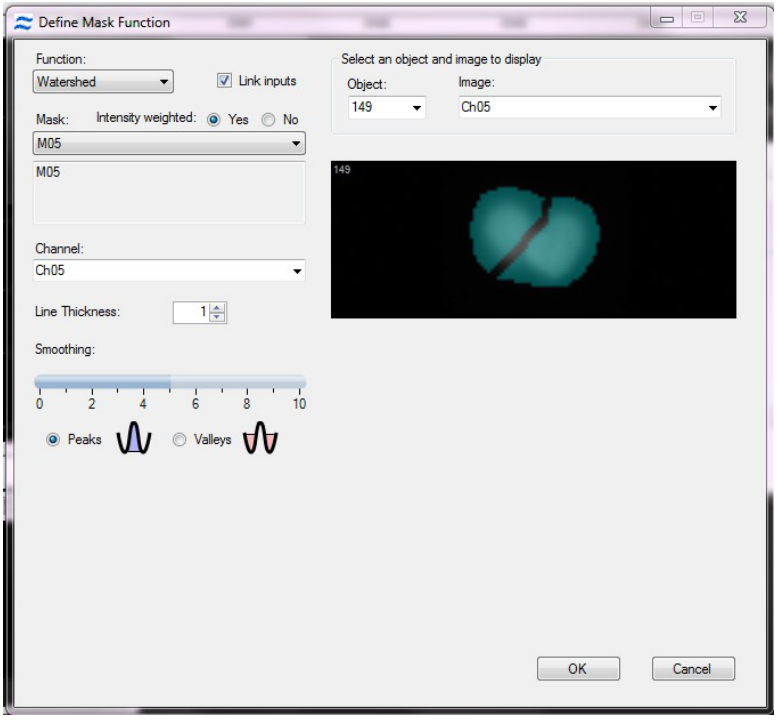


Application Example

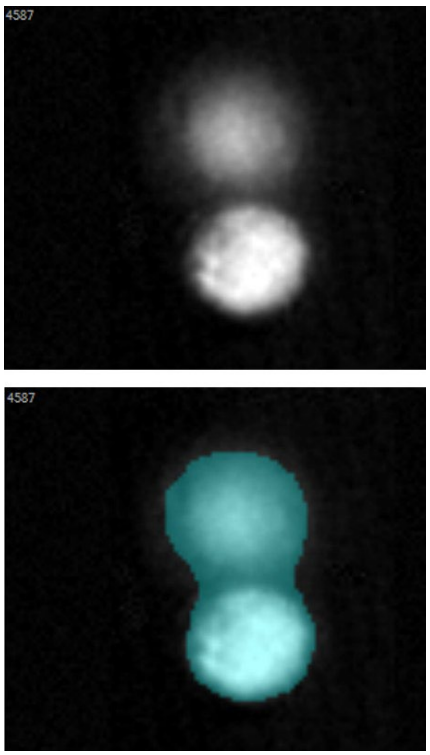
- Quantify the intensity of a probe in an immune synapse.

Watershed Mask

The watershed mask is used to separate a single mask into multiple components. There are several user inputs to optimize the mask. Here is the user interface and following is the detailed explanation with examples.

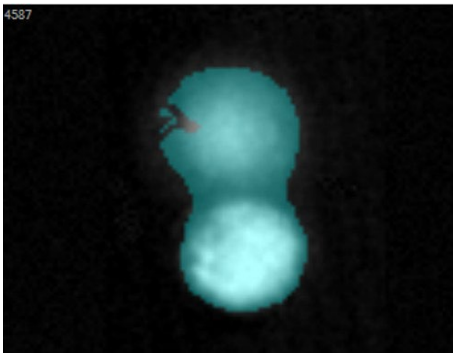


In image 4587 below, there are two components masked together as one. The watershed mask can be used to “erase” part of the mask in between the two components.

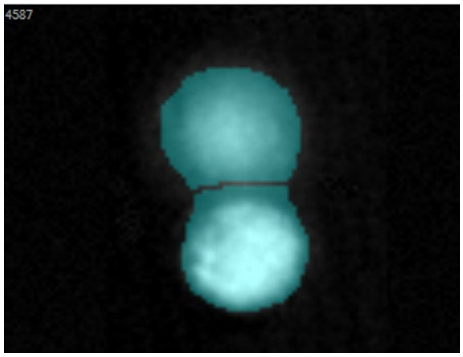


If the “intensity weighted” option is selected, the mask will be computed based on the image intensity values.

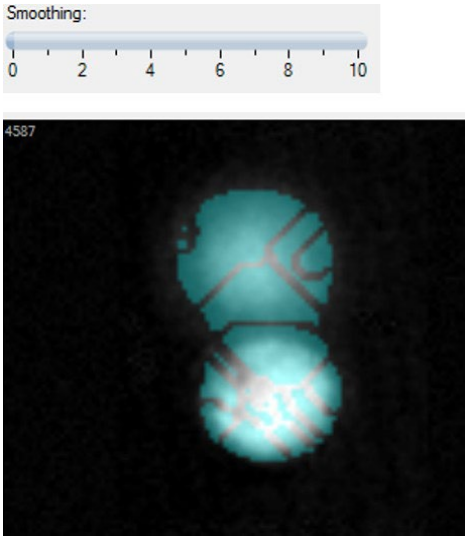
The watershed algorithm works by treating the image as a surface map and filling it with “water” from the bottom. When the pools of water meet, a border is created. Choose “peaks” or “valleys” to set the orientation of bright and dark points. If “valleys” is chosen, low intensities will represent low points and high intensities will represent high points. As shown below, this results in poor segmentation for this image.



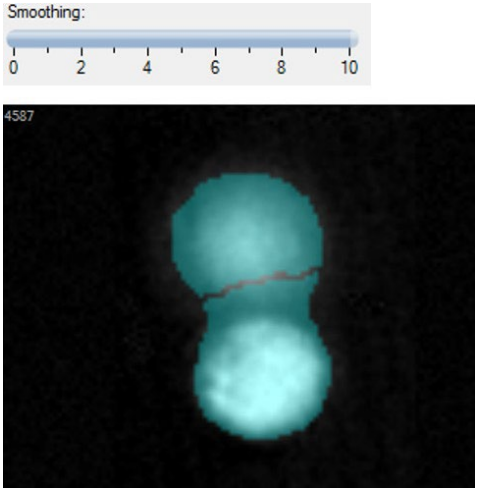
If “peaks” is chosen, then high intensities are used as the basins and low intensities represent high points. This results in much better segmentation for this example image.



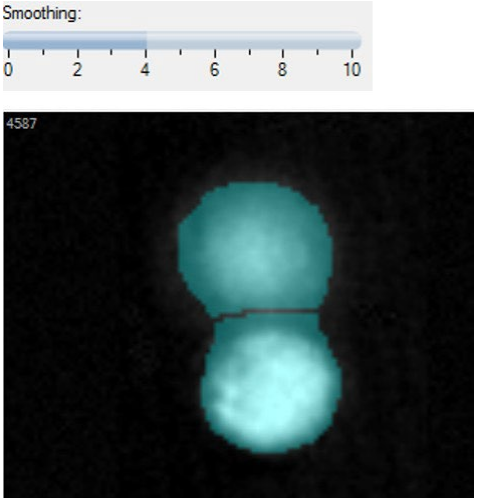
The next adjustable parameter is smoothing. A low amount of smoothing can result in over segmentation, as seen below.



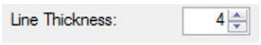
A large smoothing value can also result in poor segmentation results.

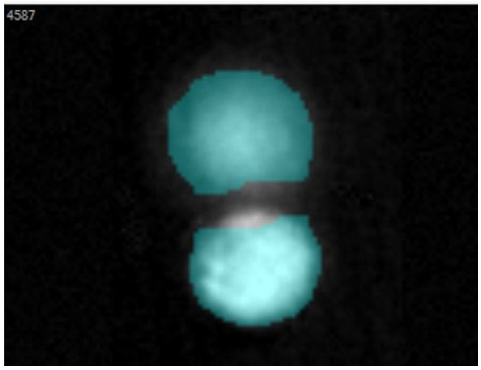


A good smoothing value will result in the best segmentation results.



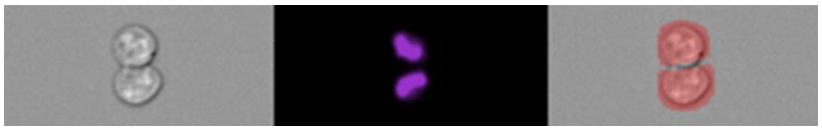
The final parameter is Line Thickness. This sets the thickness of the dividing line between components.





Application Examples

- Can be used to separate two attached cells based on DNA content, for example cell conjugates or dividing cells
- Can be used in combination with the component mask to analyze individual cells in conjugates or dividing cells



From left to right: brightfield no mask, DAPI Ch7 no mask, brightfield watershed mask. This a jurkat cell in telophase.