ZEN Blue – Exploring the Image Analysis Module
Product and Application Support
February 2016

A Take-Off Guide for Automatic Measurements in ZEN Blue

The "Image Analysis" module for ZEN Blue provides a means with which to automate the input and segmentation of, and data extraction from images. What this module provides is a user-friendly wizard that can be used for certain low- through higher-throughput scenarios. It also allows the user to set thresholds that will be implemented across many images in collecting the data; such thresholds include brightness, size, shape and ROI. Furthermore, the use and degree of automation can be controlled, ranging from not executing some steps, to both executing those steps and having them be user-interactive. Lastly, there is a wide range of data measurements available for extraction.
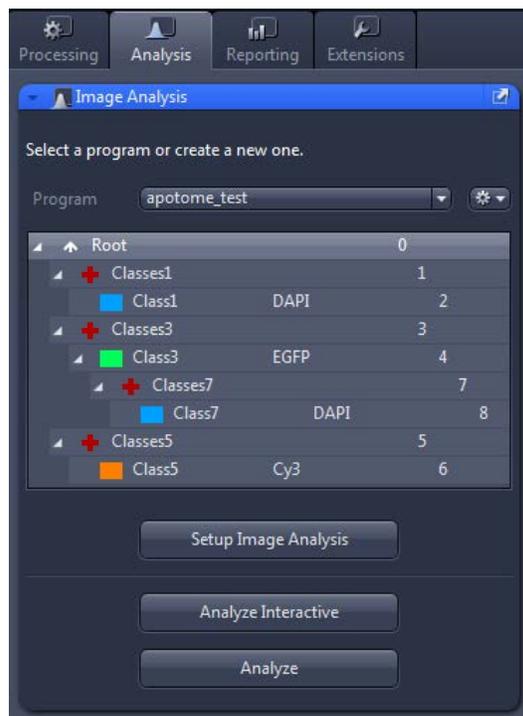
Image Analysis is ZEN's equivalent of AxioVision's AutoMeasure/AutoMeasure Plus (AM/AM Plus). Alike in many ways to AM/AM Plus, Image Analysis provides the same sort of automation but adds powerful functionality with its ability to nest segmentations between or among channels. In other words, whereas all channels in AM were processed independently of one another, Image Analysis allows for the restriction of one channel's analysis based on the parameters set for the boundaries of a "parent" class. This is a new feature that will surely be useful to users.

Image Analysis is meant for quick screening analysis and rapid data table-building purposes. There is usually a simple "yes or no" question involved, and the user is often concerned with only one or a few measurements per object, not a whole slew of information they're not sure they will need later on. Complicated ratio-metric analysis is an area where Image Analysis would not be best-suited; other ZEN modules such as Physiology are available for users performing those types of studies. In the simplest terms, think of Image Analysis in this manner:

> **Image Analysis** – *usually many images, and specific questions* – "Of all the cells I have segmented, are they generally more round or oblong? Exhibiting a certain level of fluorescence? Are the nuclei, on average, larger or smaller after treatment?"

For the purpose of this guide, we'll stick to the Image Analysis module and ignore AutoMeasure. The most important similarity is that Image Analysis can be used in an automated fashion, minimizing user interaction to the desired degree (including completely hands-off).

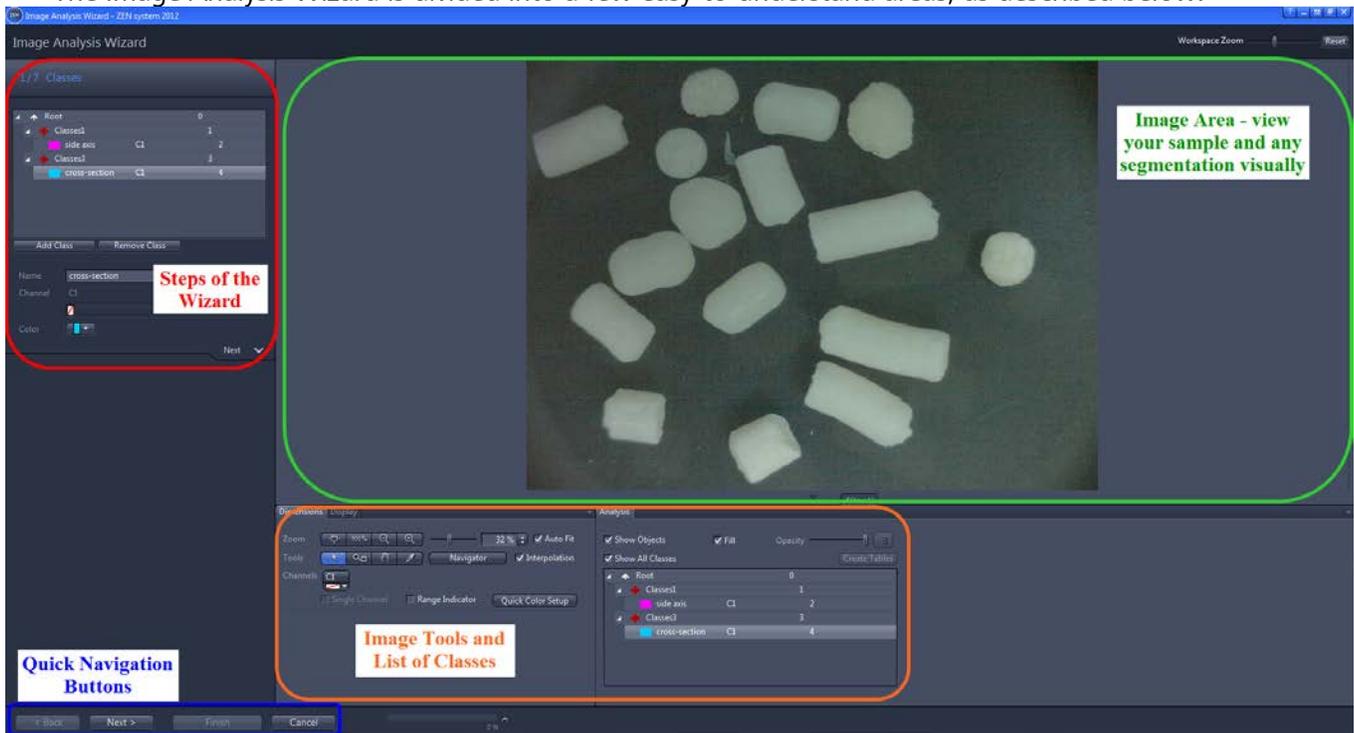## Image Analysis Interface

The Image Analysis Wizard is divided into a few easy-to-understand areas, as described below.

An image analysis program can be made as automated or interactive as the user desires. Checkboxes like the ones below (next page) appear at certain steps when creating a program; it's important to note which steps the program will execute and, of those, which will be interactive.

Execute: Run this step during analysis. Deselecting this option will skip the step when running the program.

Interactive: This option pauses the analysis to allow parameter adjustments to be made. This option is only available if **Execute** has been activated.

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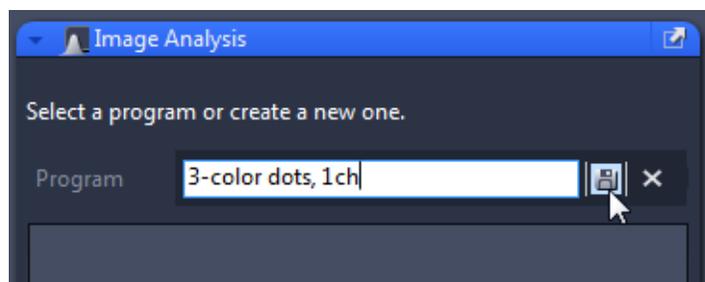February 2016

Creating a New Image Analysis Program

In ZEN Blue, switch to the Analysis tab. Open an image; native CZI-format images are preferred, but not necessary. The chosen image should be one that will be analyzed with the new program. As of ZEN 2012 SP1, this first step is located prior to entering the Wizard.

*NOTE: The wizard will not start if an image is not open.*

Image Analysis is divided into multiple steps, moving from setting up the basics (analysis name, classes) to choosing the desired measurements. First, click the gear symbol next to the Program name, and select New.
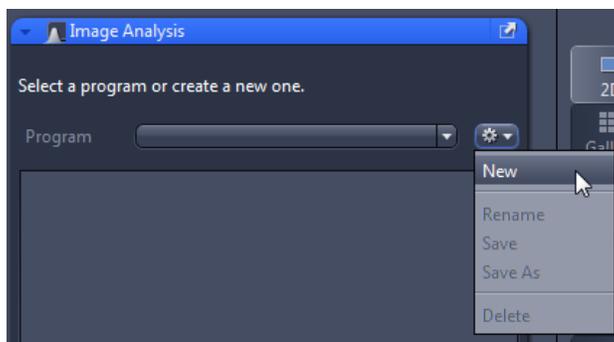
Next, enter the name of the program. Include useful information in the title (experiment date, experiment variables, etc.). Click the disk next to the name (highlighted by the mouse cursor, below) to save the new experiment, or press Enter on the keyboard.

A single class will appear, and now you can begin to set up the analysis program. Click "Setup Image Analysis" to enter the Wizard.
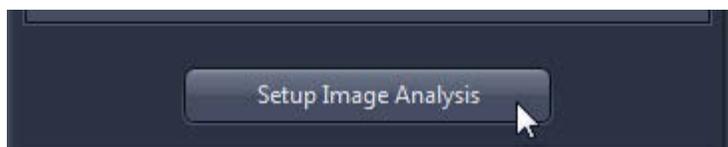
Step 1/7 - Classes

A "class" refers to a collection of objects that are grouped together by default (such as all the objects expressing fluorescence in a channel) or objects that the user wants to be grouped together when measured later on (such as within the bounds of a particular ROI). Each class consists of two entries. The first entry, **the cross ("+")**, concerns all the objects belonging to the class; think of this level as a summary of the group of objects. The second entry **(the "block")** represents individual objects. Different parameters and measurements are available for each entry. An easy way to think of this setup is to picture a room with some people in it:

The cross ("+") – sample question: How many people are in the room? (*Three.*)

The block – sample question: What are the names of the people in the room? (*Bob, Tom and Mary.*)

It is important to set up the classes properly so that it is easier to identify which objects contribute to particular calculations. Sub-classes can also be set up for various purposes. Also called "child" classes, these sub-levels are subject to the restrictions of their "parent" class. The "parent" classes can be set up as ROIs in effect. A scenario where sub-classes would be useful is when the user wants to see when dyes are contained in the same area as other dyes (not for Colocalization!), or whether a second dye is in the nucleus or cytoplasm, etc. The "parent" class effectively becomes an ROI mask.
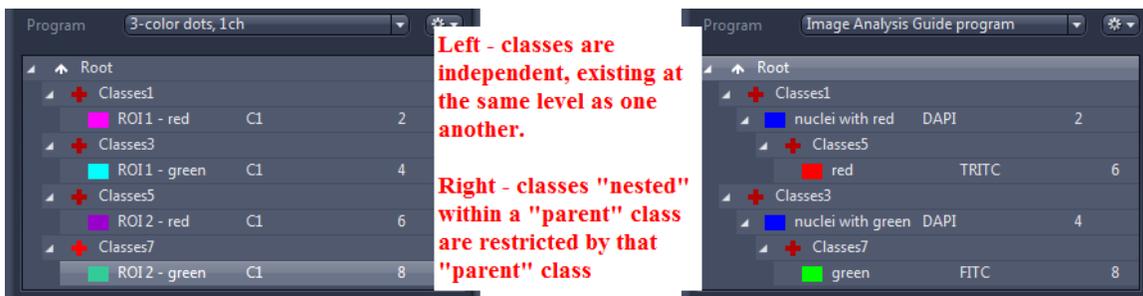
*NOTE: Classes will be added at the same hierarchical level whenever Add Class is clicked. In other words, highlighting **Root** and clicking Add Class repeatedly will make multiple classes at the same level. To add sub-classes, click on the name of the parent class FIRST, and then click Add Class.*
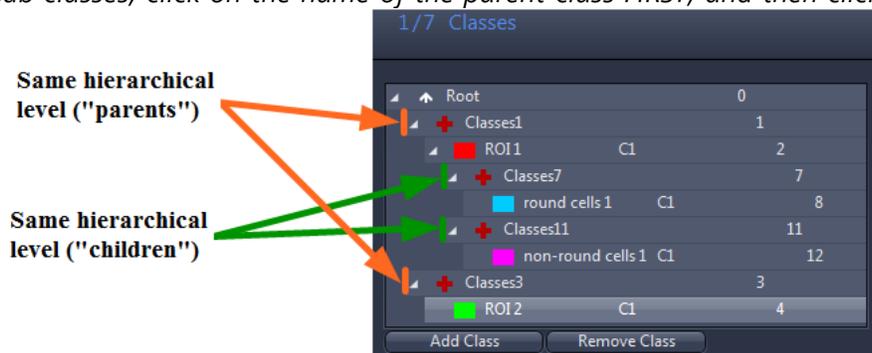
Once the classes are set, assign channels to each of them. In a multi-channel image, objects are segmented by each channel's grey values – in other words, one channel per class. Single-channel images (ex. RGB images) will only have one channel available, whereas different channels can be assigned to each class with a multi-channel image. The same channel can be assigned to multiple classes in a multi-channel image – and then, for example, segmented later based on ROI, intensity range, etc.

Independent Class Images

Un-nested, Single-channel Image
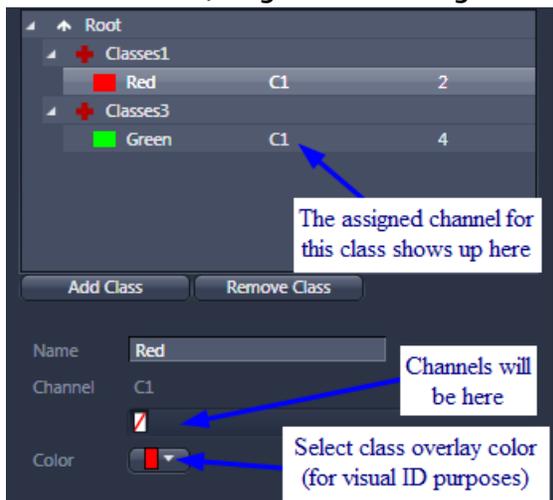
Un-nested, Multi-channel Image
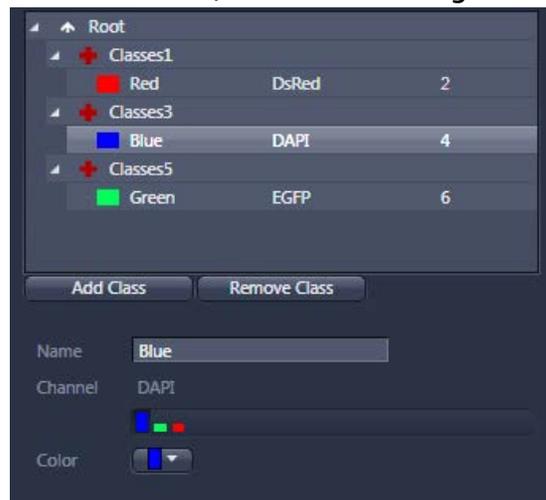
Dependent Class Images ("Nested")

Nested, Single-channel Image

Nested, Multi-channel Image
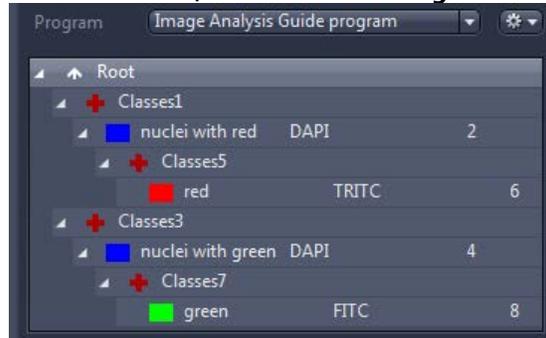
NOTE: Nested classes are only available with ZEN's Advanced Processing Module!

Although single-channel images can be used in Image Analysis as well, similar segmentation based on thresholds takes into account other variables – either all three main colors (Red/Green/Blue), or ranges of Hue, Saturation and Lightness (HLS). A discussion of thresholding appears in Steps 3 and 4.

Classes can be set up in many ways: the difference comes in how objects are segmented out later on. This is one of the important details about Image Analysis that makes the module a powerful tool in ZEN.

Step 2/7 – Frame

Framing allows for adding single or multiple Regions of Interest (ROIs) onto an image, and also lets the user describe how objects on the edge of the frame are handled. Furthermore, it allows the user to restrict which objects are measured. This step can be interactive, if the user needs to move the frame in order to obtain consistent measurements across images. An example of needing Frame to be interactive is if the sample drifts during a time series, or an individual cell or group of cells needs to be tracked.
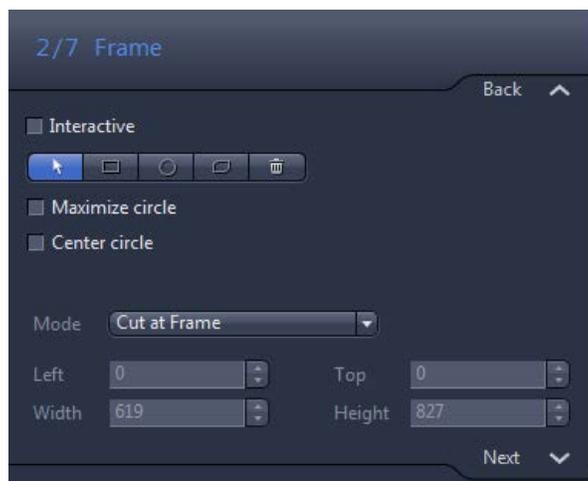
Selection
Use this to select measurement frames that have already been drawn in. To select a measurement frame, click inside it. To select several measurement frames, hold down the Ctrl key and click inside the desired measurement frames. Once you have selected a measurement frame, you can change its size.

Rectangle
Use this to create a rectangle as a measurement frame in the current image.

Circle
Use this to create a circle as a measurement frame.

Contour
Use this to create a contour as a measurement frame.

Delete
Deletes all current measurement frames.

Mode (dropdown list)
Select how you want the measurement frame to be applied. The following modes are available:
- *Inside and touching*
All objects are measured that are lying completely within the measurement frame, are touching it or are intersected by it.
- *Inside Only*
Only those objects are measured that are lying completely within the measurement frame. Objects that are touching the frame or are intersected by it are not measured.

- *Cut at Frame*

All objects are measured that are lying completely within the measurement frame. Objects that are intersected by the measurement frame are measured precisely up to the measurement frame.

Maximize circle (checkbox)
Only active when you have defined precisely one circle.
When activated, this feature maximizes the drawn-in circle to the full image size and centers it. In the case of rectangular images, the diameter of the circle is adjusted to the shorter dimension.

Center circle (checkbox)
Only active when you have defined precisely one circle.
When activated, this feature centers the drawn-in circle to the image's full size. Unlike Maximize circle, this feature maintains the size of the original circle.

The following fields are only active if a Frame in the image is highlighted:

"Left" input field
Here you can enter the start point for the frame on the X axis in pixels.
"Top" input field
Here you can enter the start point for the frame on the Y axis in pixels.
"Width" input field
Here you can enter the width of the measurement frame in pixels.
"Height" input field
Here you can enter the height of the measurement frame in pixels.

Step 3/7 – Automatic Segmentation

Automatic segmentation is exactly as it sounds – a step where the user sets thresholds to have the software automatically determine which objects to count based on intensity range, minimum continuous size, when and where to apply calculated separation/smoothness, etc. Automatic Segmentation can be executed or disabled, and also can be made interactive. This section is for picking out objects; therefore, only when an object (not a class) is highlighted in the list will threshold options appear. *Think of this as the very rough segmentation step.*
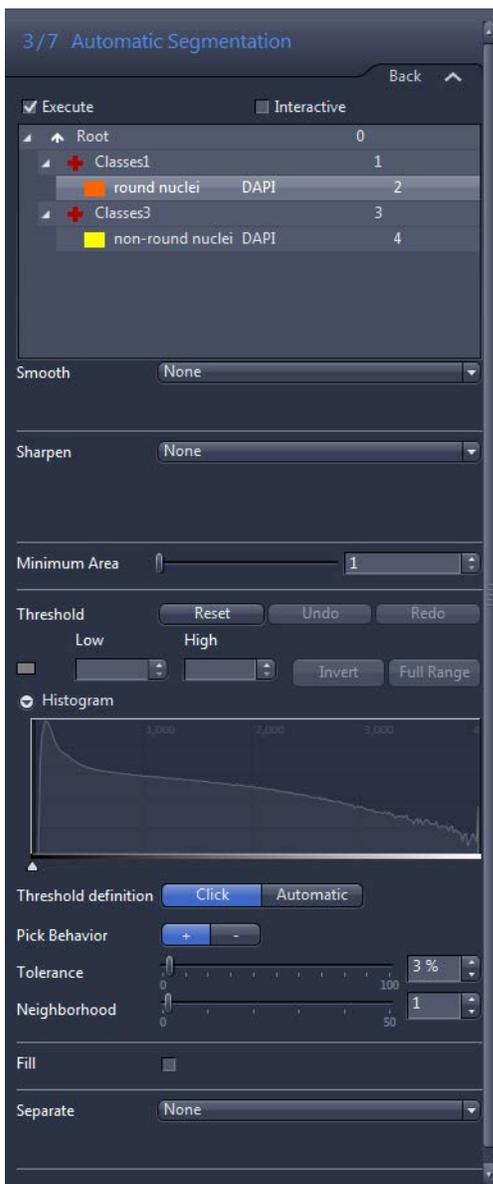
Always select a class before proceeding.

Smooth (dropdown list)
Select how to smooth the image before the threshold values are set. Smoothing can enlarge (slightly) or soften the edge of a threshold area.
The following methods are available:
None - The image is not smoothed.
Low Pass - Applies the Low Pass Method.
Gaussian - Applies the Gaussian Method.
Median – Applies the Median Method.
Size/Sigma slider (appears on method selection)
Enter the size of the filter matrix in the X and Y direction or the sigma value using the slider or input field.

Image Sharpness (dropdown list)
Select how to improve the sharpness of the image before the threshold values are set. Adding sharpness will tighten up the edge of the threshold around objects.
The following methods are available:
None - The sharpness of the image is not changed.
Edge Enhancement (Delineate) - Applies the Edge Enhancement Method.
Threshold Value (slider)(range = 0 to 255)
Enter the threshold value for edge detection using the slider or input field. The threshold value should correspond roughly to the gray value difference between objects and the background.
Size slider (range = 1 to 49)
Enter the size of the edge detection filter using the slider or input field. The value should correspond to the size of the transition area between objects and the background.
Unsharp Masking - Applies the Unsharp Masking Method.
Strength slider (range = 0.1 to 5.0)
Enter the strength of the Unsharp Masking using the slider or input field. The higher the input value, the greater the extent to which small structures is enhanced.

Minimum Area slider (range = 1-1000)
Enter the minimum area in pixels that an object must have in order to be segmented. This gets rid of stray pixels and tiny objects that should not be counted.

Threshold
Define the threshold values for the selected class in the Classes list.
Reset button - Resets all threshold value settings.
Undo button - Undoes the last change made to the threshold values.
Redo button - Restores the last undone change to the threshold values.

Color Model buttons - Only visible if the image is a color image (single channel, multiple colors).
RGB - In RGB Mode you can define the threshold values for the red, green and blue color channels.
HLS - In HLS Mode you can define the threshold values for hue, saturation and lightness.

Multi-channel image	Color (RGB) image

RGB and *HLS* are two different ways of thresholding colors; they offer slightly different results, so when working with color images, one may give better results than the other.

Histogram
In the histogram you can change the lower and upper threshold value for the activated value. Drag the lower or upper adjustment handle or shift the entire highlighted area between the lower and upper threshold value.

Threshold Definition button
Click - Click in the image on the regions that you want to define as objects.
OR
Automatic - The threshold values are determined automatically.

(Descriptions on the next page)

Click Functions	Automatic Functions
Pick Behavior + *button* - Click on this button to expand the currently segmented regions to include the gray values/colors of the objects subsequently clicked on. - *button* - Click on this button to reduce the currently segmented regions to include the gray values/colors of the objects subsequently clicked on. **Click on a pixel in the image to have the wizard add to or subtract from the segmentation all other pixels that are similar to that pixel.** Tolerance slider Using the slider or input field, enter the tolerance range by which the gray/color value read out when you click is expanded to define the threshold value. A higher value means more surrounding pixels will be segmented per click. Neighborhood slider Using the slider or input field, enter a neighborhood range around the pixel clicked on. The threshold value is calculated from the average of the gray/color values in this neighborhood range. A higher value means more surrounding pixels will be segmented per click.	Method *Otsu* - The threshold value is calculated according to the Otsu method. *Most Frequent Gray Value (Maximum Peak)* - The threshold value is the gray/color value that occurs most frequently in the image. *Iso Data* - The threshold value lies in the middle between two maximums in the histogram. *Triangular Threshold* and *3 Sigma Threshold* - The threshold value is calculated from the sum of the average and three times the sigma value of the histogram distribution, respectively.

Fill (checkbox)
When activated, holes in segmented objects (not among the objects) will be filled.
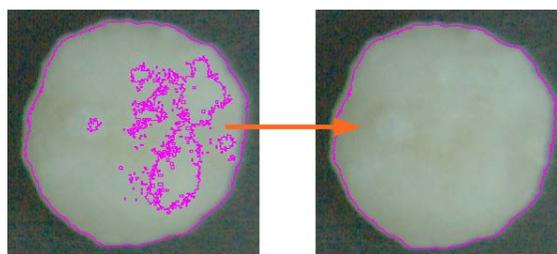
Fill OFF   **Fill ON**

Separate (dropdown list)
After segmentation, objects that are touching one another can be separated using different methods.

None - Objects are not separated.

Watersheds - Separate objects that are roughly the same shape. This method may, however, result in the splitting of elongated objects.

Morphology - Separate objects by first reducing and then enlarging them, making sure that once objects have been separated, they do not merge together again.

Number slider (for both Watersheds and Morphology)
Using the slider or input field, enter how often the method is applied successively to the result at the location of the separation.

IMPORTANT NOTE: The intensity thresholds for each class are independent of one another; if you set up any sub-classes, the parent class' thresholds do NOT influence those of the sub-classes. Furthermore, sub-classes must have their own thresholds set for objects to be counted for them.

Suggestion for use: Since you can assign the same channel to multiple classes, you can use each class' intensity threshold, size ranges/roundness, and so on to distinguish which objects fit into which categories you're trying to delineate. More control of this process is available in Step 4, Condition.

Step 4/7 – Condition

"Condition" takes the software-controlled, general Automatic Segmentation step and applies more user-controllable rules to segment objects. For instance, the following question would be appropriate:

Of the objects that the Wizard already segmented, under WHAT CONDITIONS should each class further filter the objects?

Geometric (scaled and unscaled), intensity, image, and position features can now be used to narrow down which objects will count toward final calculations. For instance, after segmenting all cells, two different classes with the same assigned channel can be used to separate "round cells" from "non-round cells." Alternatively, objects can be separated based on how strongly objects express fluorescent signals (intensity ranges); or segment which class' data will come from which ROI (multiple Frames) by adding the ROI's bounds as conditions. Maximum sizes of analyzed objects can also be set.

This step has the option to be executed, and to be interactive.

If we consider the example of the round versus non-round nuclei, here are two ways to set up the same thresholding, based on whether nesting is used:
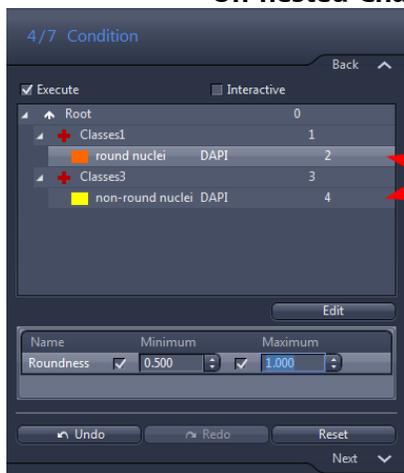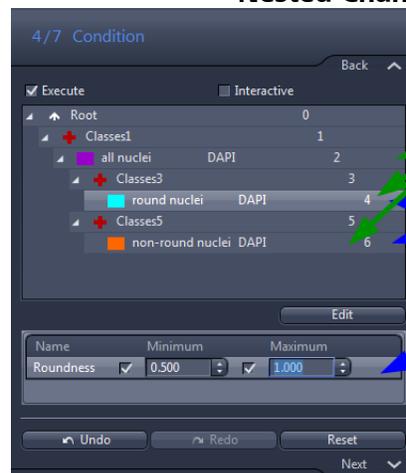
The area of interest in Conditions is the Condition Editor, accessed via the Edit button below the classes area. Use this window to add conditional parameters using "and" and "or" statements. (*image on the next page*) The conditions established here are not the measurements themselves, but the range of measurements used to further specify which objects to measure, based on the objects themselves.

Features (dropdown list)
All - All features are listed.
Geometric (Scaled/Unscaled) Features - All geometric features are listed.
Intensity Features - All features that analyze intensity values are listed.

Position (Scaled/Unscaled) Features - If this is selected all features that describe the position are listed.
+ button - Click on this button to select a feature for the measurement.
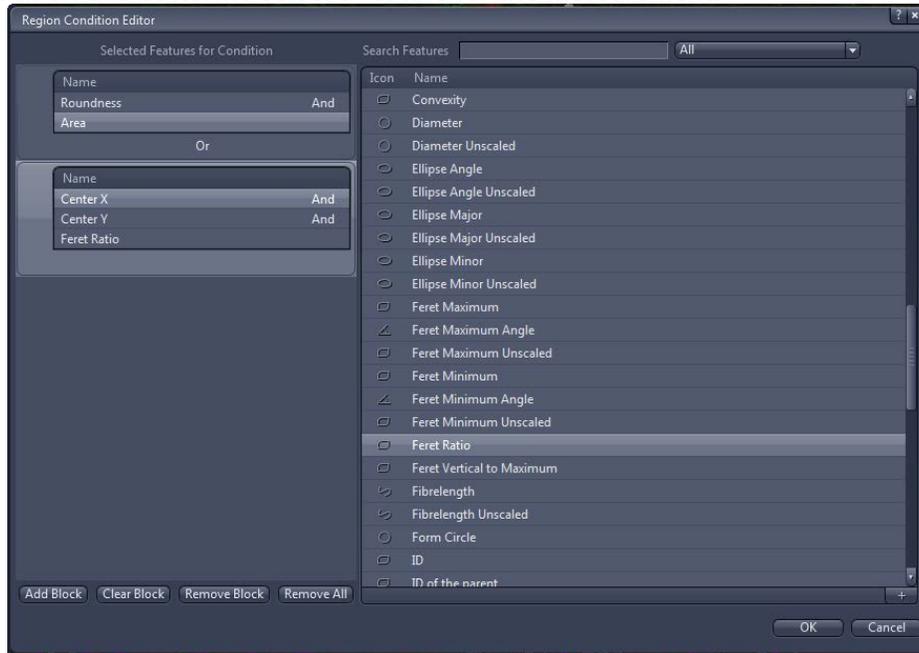
Selected Features for the Condition list
In this list, the features that you have selected for the condition are displayed block by block. All features in a block are "And"-linked for the condition, i.e. an object is only measured if the values of each individual feature fall within the defined range.

Add button - Adds an "Or" block. If several "Or" blocks have been defined, an object is measured if it meets the condition in at least one block.
Clear button - Deletes all features in a selected block.
Remove button - Deletes the selected block completely.
Remove All button - Deletes all blocks.

After you add conditions, you'll notice that all the objects turn white! You must now click on the class, and then on the objects in the image, to re-add those objects to those classes.
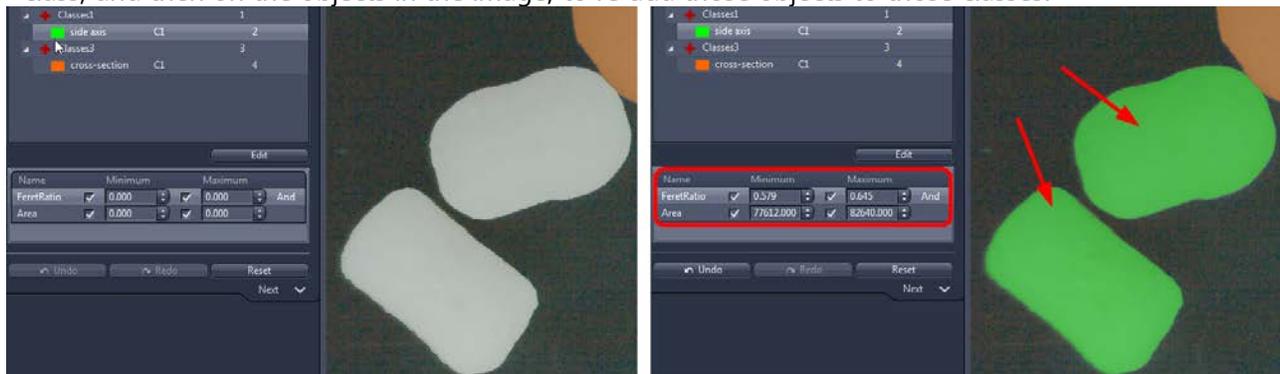
Step 5/7 – Interactive Segmentation

This step, the last in choosing objects to be measured, is designed to correct for any "flaws" that the somewhat blunt methods of automatically choosing objects may create. This is the most hands-on way to select objects directly by drawing shapes around them in a manner that adds, subtracts or modifies them. This step will automatically be executed every time – but can be made interactive if the user desires. For instance, if objects to be measured may appear or disappear, say, during a time lapse experiment, they can be added or removed in this step as desired by the researcher. If, for whatever reason, certain objects still meet (or fail to meet) segmentation and conditions, they can be edited here.

There are very subtle differences among the tools in this step.

Always select a class before proceeding.
Method 1 – Interactive Drawing on Image
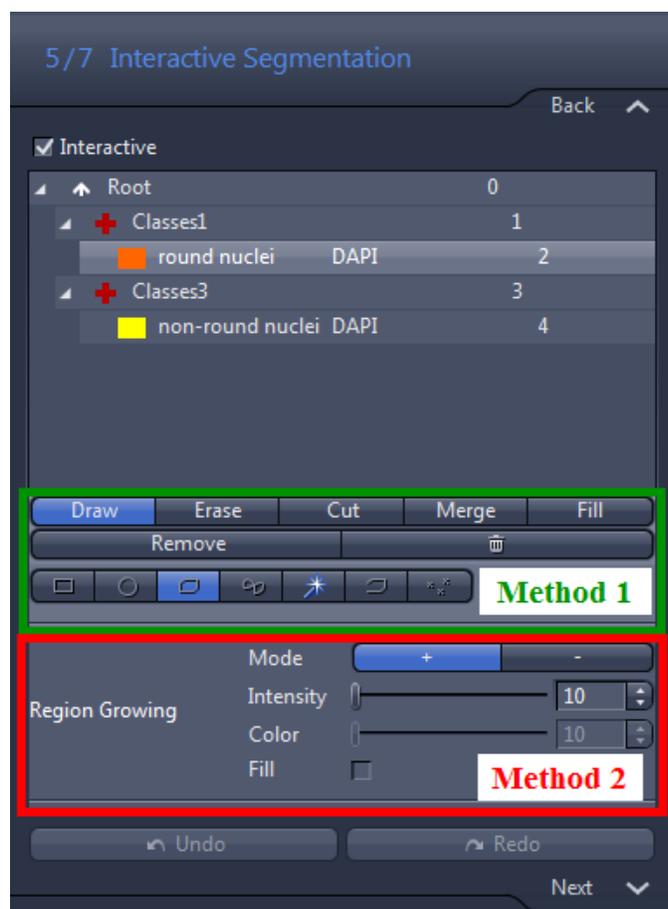Draw – adds new objects; all shape tools available; this step creates independent new objects and does NOT connect existing objects together.
Erase – removes objects; all shape tools available
Cut – similar to "Erase," but draws a pixel-wide line to separate objects for measurement; "Contour" tool available
Merge – similar to "Draw," but serves (by using the shape tools) to connect objects for measurement, instead of drawing a new measurement object over existing ones; all shape tools available
Fill – left-click in a hole in an object to fill it; no shape tools needed

Remove – Left-click to remove individual objects from the image
Trash can – Deletes ALL objects from this step and previous segmentation steps immediately

Shape Tools Available for Method 1 (left to right)
Rectangle – Add a rectangular object or cut a rectangular region from an object (outline and fill)
Circle – Add a circular object or cut a circular region from an object (outline and fill)
Draw Contour or Contour (Spline) – Add a free-hand object or cut a freehand area out (outline and fill; only outline when using Cut option)
Active Contour – traces the outline of an object, to add or remove it (outline and fill)

<u>Draw Polygon Region –</u> creates a pixel-wide outline
<u>Draw Points –</u> add or remove individual pixels from the segmented area

Method 2 – Region-Growing
Unlike Method 1, which allows the user to interactively draw on the image, Region Growing is another automated method of adding or subtracting objects based on a range of intensities (or color shifts, when using RGB images). Left-click on the object/pixel you want to add, and any adjacent pixels within the pixel- or color range will be added to that object as well – the object will "grow."

NOTE: Clicking in close proximity to an existing object will expand that original object – instead of adding a new, overlapping object – should any pixels between the new and existing objects overlap.

<u>Intensity slider –</u> Enter a tolerance value for the intensity using the slider or spin box/input field. The tolerance value specifies how much the intensity of a pixel may deviate from the average intensity of the object in order to still "grow" to become part of the object.
<u>Color slider –</u> Only active if your input image is a color image.
Enter a tolerance value for the color using the slider or spin box/input field. The tolerance value specifies how much the color value of a pixel may deviate from the average color value of the object in order to still "grow" to become part of the object.
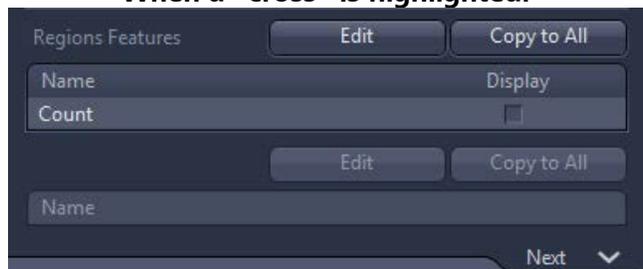
Step 6/7 – Features

Once the objects to be analyzed have been designated, it is time to choose what data to retrieve. Here, for the first time, options are available for both the "cross" (group) and "block" (individual objects) levels while editing. These options vary, depending on from *what level(s) of organization* you wish to obtain data.

When a "cross" is highlighted:

When a "block" is highlighted:
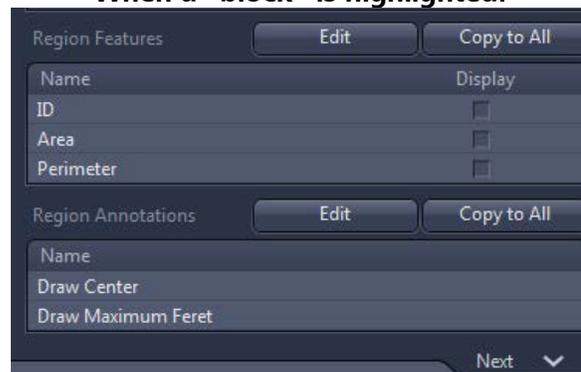
- For groups of objects, only "Regions Features" is available. These features measure the *totals* or *averages* or *ranges* of data for all the objects of that class; data are displayed on a table after analysis. (*Note: Regions Features were called "Field Features" in AutoMeasure.*)
- For individual objects, "Region Features" as well as "Region Annotations" are available. The former refers to the data that will be displayed on a table. The latter will draw on the image visible markings that may aid in identification or visual interpretation of the data.

The two buttons available for any measurement option are as follows:
Edit – choose which measurement features to include
Copy to All – the current setup of measurements, once established, can be copied to all similar levels of classes (all "+" levels to all other "+" levels, and all "blocks" to all other "blocks")

Region Features, displayed on a table:

	ID	Area[µm²]	Perimeter[µm]
		A	B	C
1	64	1,324.907	217.715
2	47	1,425.533	273.437
3	43	1,731.603	428.730
4	46	1,735.796	468.568
5	58	2,041.866	287.419
6	42	2,243.118	590.020
7	55	3,303.882	502.033
8	68	3,446.435	486.560
9	67	3,593.181	471.670
10	71	4,066.961	529.294
11	61	4,658.138	306.696
12	57	5,756.637	576.535
13	65	5,874.034	515.021

Region Annotations, displayed on the image:
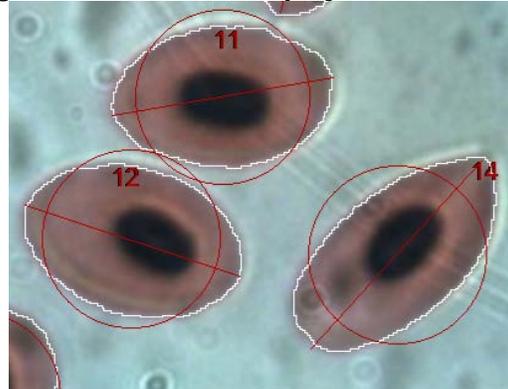
(Diameter and Feret Maximum displayed, object fill disabled)

Step 7/7 – Measure

This step gives an idea of the data you can expect to retrieve from experimental images. It is a quick way, without exiting and running the program, to see if the segmentation is adequate for the user's needs, and to make sure that the desired data is being extracted.

There is nothing to edit in this step; it is merely for preview purposes.

When a group of objects is highlighted:

			Root		
			Classes3		
			Class3	TRITC	4
			Classes1		
			Class1	FITC	2

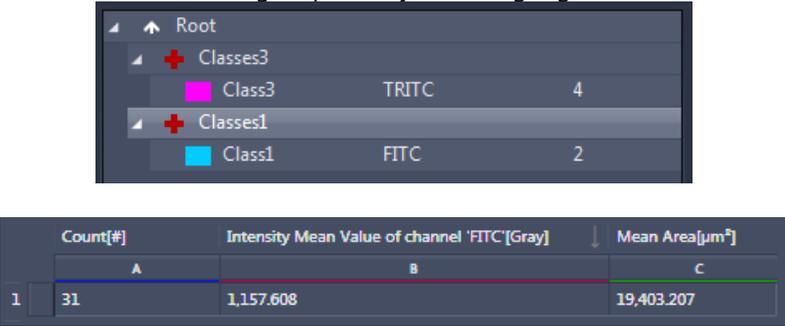|	Count[#]	Intensity Mean Value of channel 'FITC'[Gray]	Mean Area[µm²]
	A	**B**	**C**
1	31	1,157.608	19,403.207

When the individual objects level is highlighted:

			Root		
			Classes3		
			Class3	TRITC	4
			Classes1		
			Class1	FITC	2
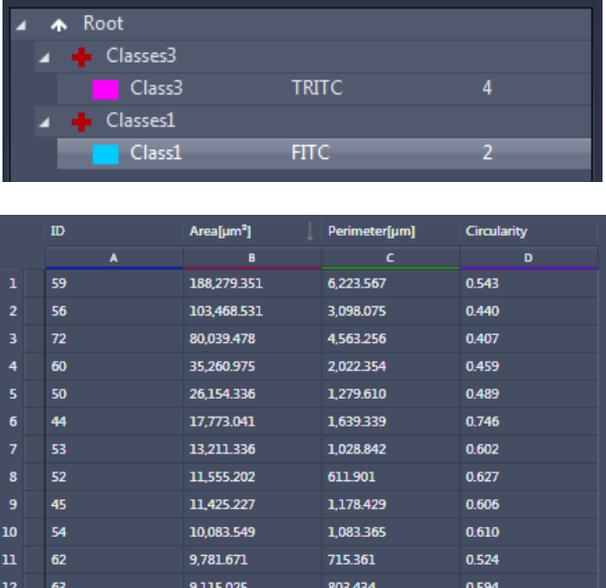
	ID	Area[µm²]	Perimeter[µm]	Circularity
	A	**B**	**C**	**D**
1	59	188,279.351	6,223.567	0.543
2	56	103,468.531	3,098.075	0.440
3	72	80,039.478	4,563.256	0.407
4	60	35,260.975	2,022.354	0.459
5	50	26,154.336	1,279.610	0.489
6	44	17,773.041	1,639.339	0.746
7	53	13,211.336	1,028.842	0.602
8	52	11,555.202	611.901	0.627
9	45	11,425.227	1,178.429	0.606
10	54	10,083.549	1,083.365	0.610
11	62	9,781.671	715.361	0.524
12	63	9,115.025	803.434	0.594

The data can be re-ordered by clicking on a column's name; this will change the order from ascending to descending and back. All other data will be re-organized accordingly.

Once you have completed the program setup, simply click Finish:
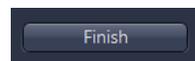
Finish

ZEN Blue – Exploring the Image Analysis Module
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Running the Analysis Program

Once completed, the program you just created can be run in two forms: Analyze Interactive, or Analyze. These options are available in the Image Analysis tab, just below the "Setup Image Analysis" button.

NOTE: Be sure to have an image open and the appropriate Image Analysis program selected before running a program.
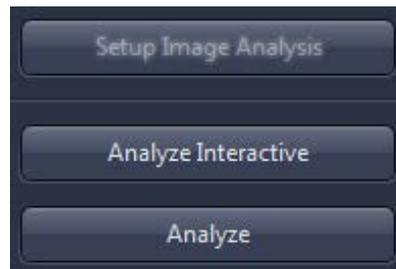
Analyze – runs the program, but skips any interactive steps, even when included. Goes straight to display of data and/or annotations.

Analyze Interactive – runs the selected program and an abridged version of the Image Analysis wizard; the user has control of the steps that were set to be Interactive.

That's great – but what can I do with my data?

There are options available in ZEN to plot and export your data.

Copy and Paste to a Spreadsheet
By clicking-and-dragging to highlight the desired data, use the Windows commands Ctrl+C (copy) and Ctrl+V (paste) to move the data to another program. (*Microsoft Excel 2010™ shown below*)

Convert Your Data to a Chart in ZEN
ZEN has some basic charting features for visually interpreting data. To switch to a charting view, first go to the Analysis tab under your analyzed image, and click Create Tables.
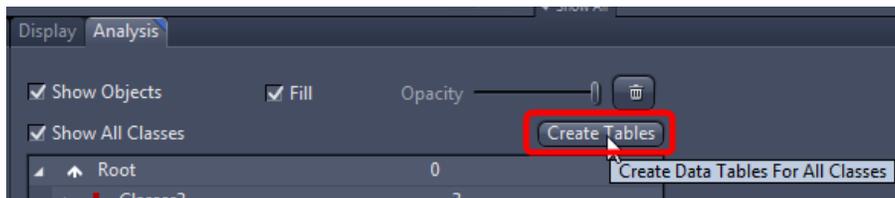
Two new windows will appear – one that ends in "Region" (all individual objects from all classes) and one that ends in "Regions" (all groups of objects). These data tables can be saved by going directly to File > "Save As…", in the ***.zvt** format.
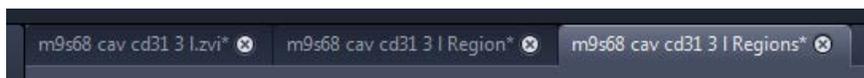
The charting options appear now in the General tab, located along the bottom panel. Clicking through the chart options automatically updates the view of your data. But first, select what data you want to display. Switch to the new data window that you wish to graph, and then click-and-drag to highlight the desired data. Then, under the Chart Data Source under the image, click New Data Source. You may need to switch from Rows to Columns, depending on how your data are organized.
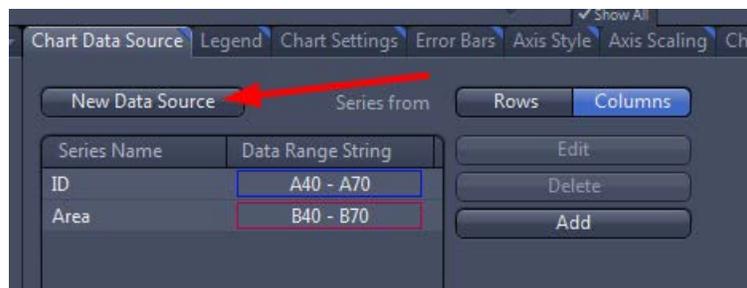
Now, under the General tab, you can switch to different displays.
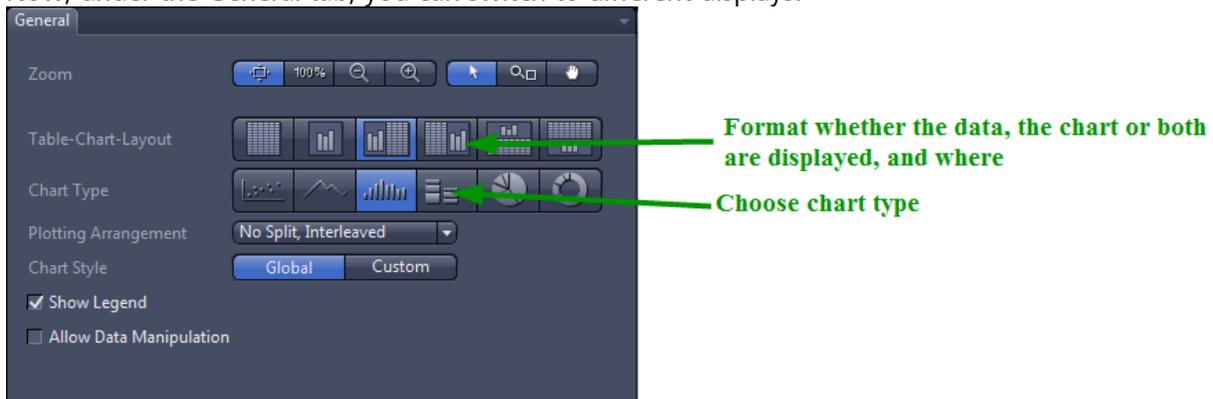
The options tabs to the right of the General tab allow customized control over the exact look of the chart.

If you would like to export an image of your chart and/or juxtaposed data, switch to the Import/Export tab and use the "Save" button next to "New Image from" to create an image file of the current layout.

Operational Notes for Image Analysis

- The Histogram in the Automatic Segmentation step is "active," meaning it only displays the data for the given field-of-view of your image. If you zoom in on your image or pan around, the histogram will update accordingly. This has no effect on segmentation; it is meant to give you a rough visual approximation that is also easier and faster for the software to update.
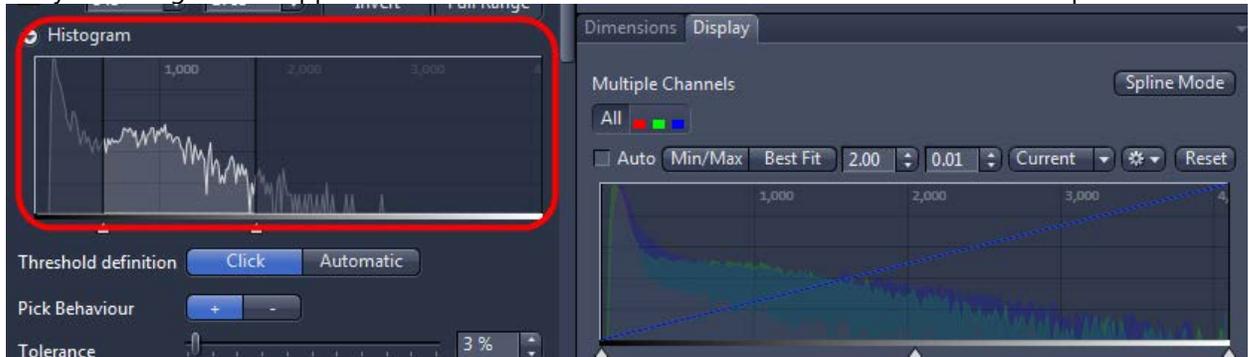
- The segmentation steps may show strange imaging artifacts, such as certain objects disappearing and then reappearing based on zoom, changes in thresholds, etc. Do not let this alarm you; as long as most of the objects you want have been segmented, simply complete the program and run it using "Analyze" or "Analyze Interactive" (page 20). The segmentation and conditions you established in your program should now display properly. Another instance of this is choosing, in the Frame step, "Inside Only" for an ROI and seeing that objects touching the frame still appear as segmented while still setting up the program; this issue corrects itself when the program is run. These issues are meant to be fixed in future versions.

- The segmented area of a child (sub-) class may exceed the physical area of a segmented parent class as long as <u>at least 60% of the child class's area</u> is coincident with its parent class' area. Lowering a child class' lower threshold (or raising the upper threshold) too far may cause the child's segmentation to simply disappear, since thresholding affects the continuous area of a segmented area.
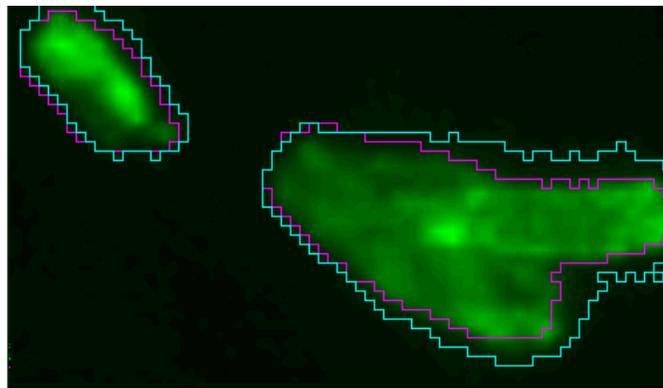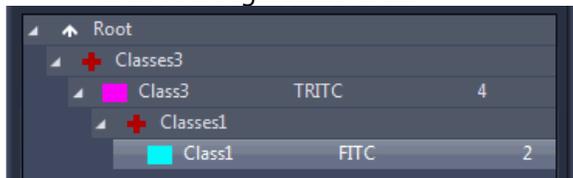
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Region/Field Measurements and Region Annotations - Defined

The following supplement to the Image Analysis Guide for ZEN Blue describes the various Region Features and Field Features available for measurement and display for an image. **Region Features** refer to properties of individual objects, whereas **Field Features** refer to characteristics of groups of objects. In Image Analysis, Field Features are referred to as **Regions Features** (plural).

Legend

Some features come in multiple formats.

Scaled – If scaling information is available for an image, calculations will be based on those values. Scaled values will be in microns (*µm*).

Unscaled – If no scaling information is available, or if the user would like to know a calculation based on pixels rather than scaled units, then this option should be used. Units are always in *pixels*.

WCS – World Coordinate System. Gives relative positions on a global scale – for instance, among scenes in one experiment. An origin is available for each scene, but the WCS is valid across images in one experiment file. Values are in microns or pixels, whether the value is scaled or unscaled, respectively. Non-WCS values will be relative to the dimensions of the individual scene, for instance, whereas WCS values will be relative to the (0,0) position of the entire stage.

Region Features

	Region Feature	Unit	Description
	Acp X Unscaled (2nd Option: WCS)	Pixel	**The first pixel in the first line of a region, x-coordinate** To identify measurement objects, the image is scanned from *bottom right to top left*. The so-called AC point is the first point that has been identified for a new object. The parameter **AcpX** indicates the x-coordinate of this point.
	Acp Y Unscaled (2nd Option: WCS)	Pixel	**The first pixel in the first line of a region, y-coordinate** Similar to AcpX, the so-called AC point is the first point that has been identified for a new object. The parameter **AcpY** indicates the y-coordinate of this point.
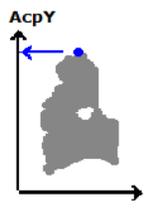
	Area	µm²	Area of a region excluding any holes it may contain. The areas of the holes are not included in the measurement. If you want to include them, use the **Area filled** parameter.
	Area Unscaled	Pixel²	The **Area unscaled** parameter corresponds to the **Area** parameter. However, the scaling of the image is not taken into account for the measurement. The (unfilled!) area of a region is displayed in pixels in each case.
	Area Bound	Pixel²	Area of the bounding rectangle of the region
	Area Convex	µm²	**Area of convex hull of a region** The current region is surrounded by a convex polyline. The (filled!) area of the resulting region is then measured.
	Area Filled (Scaled/Unscaled)	µm² OR pixel²	Area of a region including any holes it contains. The holes are interpreted as belonging to the region or are filled prior to the measurement. If you do not want the holes to be measured, use the **Area** parameter.
	Bound Bottom (Scaled/WCS/ Unscaled/Unsc aled WCS)	µm OR pixel	**Maximum y-coordinate of the bounding box of a region** Indicates the y-coordinate of the bottom edge of a bounding box for a region. The box is drawn in parallel to the x and y axis.
	Bound Center X (Scaled/WCS/ Unscaled/Unsc aled WCS)	µm OR pixel	The distance from the left edge of an image to the center of an object.
	Bound Center Y (Scaled/WCS/ Unscaled/Unsc aled WCS)	µm OR pixel	The distance from the top edge of an image to the center of an object.
	Bound Height (Scaled/Unscal ed)	µm OR pixel	**Vertical feret of a region** Indicates the height (size in y-direction) of a bounding box for a region. The box is drawn in parallel to the x and y axis. **Formula: Bound top - Bound bottom**
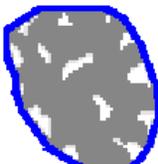
	Bound Left (Scaled/WCS/ Unscaled/Unsc aled WCS)	µm OR pixel	**Minimum x-coordinate of the bounding box of a region** Indicates the x coordinate of the left-hand edge of a bounding box for a region. The box is drawn in parallel to the x and y axis.
	Bound Right (Scaled/WCS/ Unscaled/Unsc aled WCS)	µm OR pixel	**Maximum x-coordinate of the bounding box of a region** Indicates the x coordinate of the right-hand edge of a bounding box for a region. The box is drawn in parallel to the x and y axis.
	Bound Top (Scaled/WCS/ Unscaled/Unsc aled WCS)	µm OR pixel	**Minimum y-coordinate of the bounding box of a region** Indicates the y coordinate of the top edge of a bounding box for a region. The box is drawn in parallel to the x and y axis.
	Bound Width (Scaled/Unscal ed)	µm OR pixel	**Horizontal feret of a region** Indicates the width (size in x-direction) of a bounding box for a region. The box is drawn in parallel to the x and y axis. **Formula: Bound right - Bound left**
	Center X (Scaled/WCS/ Unscaled/Unsc aled WCS)	µm OR pixel	**Geometric center of gravity of a region, x-coordinate** x-coordinate of the geometric center of gravity of a region. Depending on the shape of the object, this point may also lie outside a region. The associated y-coordinate is determined via the **Center Y** parameter.
	Center Y (Scaled/WCS/ Unscaled/Unsc aled WCS)	µm OR pixel	**Geometric center of gravity of a region, y-coordinate** y-coordinate of the geometric center of gravity of a region. Depending on the shape of the object, this point may also lie outside a region. The associated x-coordinate is determined via the **Center X** parameter.
	Circularity		**Formula:** $\sqrt{\frac{4*Area}{\pi*FeretMax^2}}$ or, the square root of the Roundness calculation
	Compactness		**Formula:** $\frac{4*\pi*Area}{PerimeterConvex^2}$
	Convexity		**Formula:** $\frac{PerimeterConvex}{PerimeterCrofton}$
	Diameter (Scaled/Unscal ed)	µm OR pixel	**Diameter of circle with an equal area** For the area measured (parameter: **Area**) it is assumed that the area in question is that of a circle. This area is then used as a basis for calculating the corresponding diameter. The **Radius** parameter is also measured in this way. **Formula:** $2*\sqrt{\frac{Area}{\pi}}$
	Diameter Filled	µm	Same as Diameter, but adjusts area of equivalent circle to account for filled holes in an object.
	Diameter Filled Unscaled	pixel	Same as Diameter, but adjusts area of equivalent circle to account for filled holes in an object.
	Diameter Least Square Fit Unscaled	pixel	

	Diameter Maximum Inscribed Circle	µm	The measurement of a circle, the diameter of which is expanded to its maximum, within the boundaries of the object.
	Diameter Maximum Inscribed Circle Unscaled	pixel	The measurement of a circle, the diameter of which is expanded to its maximum, within the boundaries of the object.
Ellipse angle	Ellipse Angle (Scaled/Unscaled)	Degrees	**Angle of the major axis of the ellipse** The major axis of an ellipse with the same geometric moment of inertia as the current region is determined in accordance with the **Ellipse major** parameter. The angle to the x-axis is then determined. The indication of the angle always relates to a counterclockwise direction.
Ellipse major	Ellipse Major (Scaled/Unscaled)	µm OR pixel	**Length of the major axis of the ellipse** Length of the major axis of an ellipse with the same geometric moment of inertia as the region. The moment of inertia is calculated about the center of gravity of the region.
Ellipse minor	Ellipse Minor (Scaled/Unscaled)	µm OR pixel	**Length of the minor axis of the ellipse** Length of the minor axis of an ellipse with the same geometric moment of inertia as the region. The moment of inertia is calculated about the center of gravity of the region.
Feret Maximum	Feret Maximum (Scaled/Unscaled)	µm OR pixel	**Maximum feret of a region** The maximum feret of a region is determined on the basis of distance measurements. Two straight lines are positioned on opposite sides of the object, like a sliding caliper, at 32 angle positions. The corresponding distance is measured for each angle position. The maximum value determined is the maximum feret. The **Feret Minimum** parameter is also measured in this way.
Feret Maximum Angle	Feret Maximum Angle	Degrees	**Angle of the maximum feret of a region in relation to the x-axis** The maximum feret is determined in accordance with the description for **Feret Maximum**. The angle of the maximum feret in relation to the x-axis is then determined. The indication of the angle always relates to a counterclockwise direction. The **Feret Minimum Angle** parameter is also measured in this way.

	Feret Minimum (Scaled/Unscaled)	µm OR pixel	**Minimum feret of a region** The minimum feret of a region is determined on the basis of distance measurements. Two straight lines are positioned on opposite sides of the object, like a sliding caliper, at 32 angle positions. The corresponding distance is measured for each angle position. The minimum value determined is the minimum feret. The **Feret Maximum** parameter is also measured in this way.
Feret Minimum Angle 	Feret Minimum Angle	Degrees	**Angle of the minimum feret of a region in relation to the x-axis** The minimum feret is determined in accordance with the description for **Feret Minimum**. The angle of the minimum feret in relation to the x-axis is then determined. The indication of the angle always relates to a counterclockwise direction. The **Feret Maximum Angle** parameter is also measured in this way.
	Feret Ratio		**Feret ratio** The ratio of **Feret Minimum** to **Feret Maximum** is calculated. This ratio makes it possible to make statements on the form of the measured objects. If the feret ratio has a low value, long, elongated objects are present. Values approaching 1 indicate the presence of compact or circular objects, as in this case **Feret Minimum** and **Feret Maximum** have very similar values. The **Form circle** is also suitable for making statements on the circularity of an object. **Formula:** $\frac{FeretMin}{FeretMax}$
	Feret Vertical to Maximum	µm	
	Fiber length	µm	**Length of a fiber-like region** To calculate the fiber length, a structure that is actually similar to a fiber is required. Here it is not the distance between a start and end point that is determined. The check can be done using the **Form circle**, among other things. **Formula:** $\frac{PerimF + \sqrt{PerimF^2 - 16*AreaF}}{4}$
	Fiber length Unscaled	pixel	Same measurement as Fiber length, but measurements are in pixels.
	Form Circle		**Form factor of a region** Describes the form of a region on the basis of its circularity. A perfect circle is given the value 1. The more elongated the region is, the smaller the form factor. The calculation is based on the **Area filled** and **Perimeter Crofton** parameters. **Formula:** $4\pi * \frac{AreaF}{PerimCroft^2}$
	ID of the Parent		ID value of the first member of that class. The total number of objects, plus 1 for the value of the intermediate field-level classes, accounts for the value given to objects.

	Image Acquisition Time	Date and Time	**Image's acquisition time** Date and time of acquisition of image. This is not the date on which the image was saved for the first/last time! The date and time when the image was acquired in ZEN are saved. This value does not change. **Format:** in accordance with the settings made under **Regional and Language Options** in the Windows **Control Panel**. **Restriction:** This value is only available for images that have previously been acquired with AxioVision or ZEN, or saved in ZVI or CZI format.
	Image Channel Name		Indicates the name of the fluorescence channel in a multichannel fluorescence image. The **Image Channel Name** can be used to guarantee unambiguous assignment between measured regions in the image and the individual lines of a data table, especially in cases where several images are measured, as automatic assignment is then no longer possible. To achieve this, the **Image Index…** parameters (below) must also be selected as a region parameter and must also be inserted into the graphics plane as an annotation. It is also advisable to activate the **Image Name** parameter as a region parameter.
	Image Depth of Focus	µm	Indicates an image's depth of focus.
	Image Focus Position	µm	**Focus position** Indicates the position of the z-motor at the time of image acquisition. **Restriction:** This value is only available for images that have previously been acquired with AxioVision or ZEN, or saved in ZVI or CZI format.
	Image Height	µm	Total distance across the Y-axis of the image, in scaled units.
	Image Height Unscaled	pixel	Total distance across the Y-axis of the image, in unscaled units.
	Image Index Block		Experimental block (of multi-block Experiments, using Experiment Designer) location of that object.
	Image Index Channel		Channel number from which an object was segmented.
	Image Index Position		
	Image Index Scene		Scene number in which that object appears.
	Image Index Time		Time point in which that object appears.
	Image Index Z		Z-position at which an object was segmented.
	Image Name		File name of the image.

	Image Relative Time		Relative acquisition time of an individual image within a multidimensional image. The time difference from the first image acquired is stated. This value is not determined for 2D images. If the parameter is nevertheless measured, the corresponding field in the data table contains no value. **Time format:** hours : minutes : seconds : milliseconds **Value range:** 1 ms - 100 hours **Restriction:** This value is only available for images that have previously been acquired with AxioVision or ZEN, or saved in ZVI or CZI format.
	Image Relative Time (1/2/3/4)		Relative acquisition time of an individual image within a multidimensional image. The time difference from the first acquired image at the set instant of exposure (1, 2, 3 or 4) is stated. This value is not determined for 2D images. If the parameter is nevertheless measured, the corresponding field in the data table contains no value. **Time format:** hours : minutes : seconds : milliseconds **Value range:** 1 ms - 100 hours **Restriction:** This value is only available for images that have previously been acquired with AxioVision or ZEN, or saved in ZVI or CZI format.
	Image Save Time		**Date image was saved** Indicates the date on which the measured image was saved.
	Image Scale X	µm/pixel	
	Image Scale Y	µm/pixel	
	Image Stage Position X Image Center	µm	**x-position of the stage at the time of image acquisition** An encoded or motorized scanning stage is required to determine this parameter. **Restriction:** This value is only available for images that have previously been acquired with AxioVision or ZEN, or saved in ZVI or CZI format.
	Image Stage Position Y Image Center	µm	**y-position of the stage at the time of image acquisition** An encoded or motorized scanning stage is required to determine this parameter. **Restriction:** This value is only available for images that have previously been acquired with AxioVision or ZEN, or saved in ZVI or CZI format.
	Image Width (Scaled/Unscaled)	µm OR pixel	Distance across the image, along the X-axis.
	Index		ID of that object, in the order it was measured on the image.
	Intensity Maximum of channel	Gray level	The gray values of a region are analyzed and the maximum value displayed. This is the brightest point in a region. If this parameter is measured for regions inside an RGB color image, the RGB color image is first converted to a gray level image in accordance with the formula (R+G+B) / 3 and the corresponding maximum value is then displayed. If you want the maximum values to be determined separately for one or all color channels, you need to use the **Intensity Maximum of channel 'C1' (blue/gray/green/red)** parameters. This

			parameter can also be used to measure the maximum gray value of regions in multichannel fluorescence images. As a multichannel fluorescence image consists of several gray level images, no special parameter is needed.
	Intensity Mean of channel	Gray level	The gray values of a region are analyzed and the average of all gray values displayed. If this parameter is measured for regions inside an RGB color image, the RGB color image is first converted to a gray level image in accordance with the formula (R+G+B) / 3 and the corresponding average is then displayed. If you want the averages to be determined separately for one or all color channels, you need to use the **Intensity Mean Value of channel 'C1' (blue/gray/green/red)** parameters. This parameter can also be used to measure the average gray value of regions in multichannel fluorescence images. As a multichannel fluorescence image consists of several gray level images, no special parameter is needed.
	Intensity Minimum of channel	Gray level	The gray values of a region are analyzed and the minimum value displayed. This is the darkest point in a region. If this parameter is measured for regions inside an RGB color image, the RGB color image is first converted to a gray level image in accordance with the formula (R+G+B) / 3 and the corresponding minimum value is then displayed. If you want the minimum values to be determined separately for one or all color channels, you need to use the **Intensity Minimum of channel 'C1' (blue/gray/green/red)** parameters. This parameter can also be used to measure the minimum gray value of regions in multichannel fluorescence images. As a multichannel fluorescence image consists of several gray level images, no special parameter is needed.
	Intensity Pixel Count of channel	Pixel^2	Similar to Area Unscaled; area of all pixels which contribute to that channel's segmentation.
	Intensity Range of channel	Gray level	
	Intensity Standard Deviation of ch.	Gray level	The gray values of a region are analyzed and the standard deviation of all gray values displayed. If this parameter is measured for regions inside an RGB color image, the RGB color image is first converted to a gray level image in accordance with the formula (R+G+B) / 3 and the corresponding standard deviation is then displayed. If you want the standard deviations to be determined separately for one or all color channels, you need to use the **Intensity Standard Deviation of channel 'C1' (blue/gray/green/red)** parameters. This parameter can also be used to measure the standard deviation of regions in multichannel fluorescence images. As a multichannel fluorescence image consists of several gray level images, no special parameter is needed.

	Intensity Sum of channel	Gray level	**Sum of gray values of a region** All gray values of a region are added together. If this parameter is measured for regions inside an RGB color image, the RGB color image is first converted to a gray level image in accordance with the formula (R+G+B) / 3 and the sum is then calculated. If you want the sum to be determined separately for one or all color channels, you need to use the **Intensity Sum of channel 'C1' (blue/gray/green/red)** parameters. This parameter can also be used to measure the sum of regions in multichannel fluorescence images. As a multichannel fluorescence image consists of several gray level images, no special parameter is needed.
	Number of Region Holes		
	Perimeter (Scaled/Unscaled)	µm OR pixel	The perimeter of a region is determined. To measure the perimeter of circular objects, use of the parameter is recommended, as this parameter is specially optimized for measuring the perimeters of circles. If the measured region contains holes, the total perimeter including the perimeters of the hole structures is determined.
Radius	Radius	µm	**Radius of circle with an equal area** For the area measured (parameter: **Area**) it is assumed that the area in question is that of a circle. This area is then used as a basis for calculating the corresponding radius. The **Diameter** parameter is also measured in this way. **Formula:** $\sqrt{\dfrac{Area}{\pi}}$
	Radius Unscaled	pixel	Same as Radius, but values are in pixels ("Area Unscaled" instead of "Area")
	Region Class Color		
	Region Class ID		
	Region Class Name		
	Roundness		**Formula:** $\dfrac{4*Area}{\pi*FeretMax^2}$

Field Features

Region Feature	Unit	Description
Area (Scaled/Unscaled)	µm² OR pixel	Total area of all objects in a region, excluding any holes it may contain.
Area Frame (Scaled/Unscaled)	µm² OR pixel	Area of the measurement frame used. If a ROI is used, that area will be displayed; otherwise, the area of the entire image is stated.

Area Percentage	%	**Total area of all regions, in relation to the area of the measurement frame** Sum of the areas of all (unfilled) regions. The region feature **Area** is measured for each region, without any holes that the region may contain, and all the individual areas are added together. The areas of the holes are not included in the measurement. Formula: $\sum 100 * \frac{Area}{AreaFrame}$
Bound Bottom	µm OR pixel	**Maximum y-coordinate of the bounding box of a region** Indicates the y-coordinate of the bottom edge of a bounding box for a region. The box is drawn in parallel to the x and y axis.
Bound Left	µm OR pixel	**Minimum x-coordinate of the bounding box of a region** Indicates the x coordinate of the left-hand edge of a bounding box for a region. The box is drawn in parallel to the x and y axis.
Bound Right	µm OR pixel	**Maximum x-coordinate of the bounding box of a region** Indicates the x coordinate of the right-hand edge of a bounding box for a region. The box is drawn in parallel to the x and y axis.
Bound Top	µm OR pixel	**Minimum y-coordinate of the bounding box of a region** Indicates the y coordinate of the top edge of a bounding box for a region. The box is drawn in parallel to the x and y axis.
Count		Total number of objects for the direct sub-layer ("block") of that class.
ID		ID value of the first member of that class. The total number of objects, plus 1 for the value of the intermediate field-level classes, accounts for the value given to objects.
ID of the parent		
Image Acquisition Time	Date and Time	**Image's acquisition time** Date and time of acquisition of image. This is not the date on which the image was saved for the first/last time! The date and time when the image was acquired in ZEN are saved. This value does not change. **Format:** in accordance with the settings made under **Regional and Language Options** in the Windows **Control Panel**. **Restriction:** This value is only available for images that have previously been acquired with AxioVision or ZEN, or saved in ZVI or CZI format.
Image Channel Name		Indicates the name of the fluorescence channel in a multichannel fluorescence image. The **Image Channel Name** can be used to guarantee unambiguous assignment between measured regions in the image and the individual lines of a data table, especially in cases where several images are measured, as automatic assignment is then no longer possible. To achieve this, the **Image Index…** parameters (below) must also be selected as a region parameter and must also be inserted into the graphics plane as an annotation. It is also advisable to activate the **Image Name** parameter as a region parameter.

Image Depth of Focus	µm	**Depth of focus of the image** Indicates an image's depth of focus.
Image Focus Position	µm	**Focus position** Indicates the position of the z-motor at the time of image acquisition. **Restriction:** This value is only available for images that have previously been acquired with AxioVision or ZEN, or saved in ZVI or CZI format.
Image Index Block		Experimental block (of multi-block Experiments, using Experiment Designer) location of that object.
Image Index Channel		Channel number from which an object was segmented.
Image Index Position		
Image Index Scene		Scene number in which that object appears.
Image Index Time		Time point in which that object appears.
Image Index Z		Z-position at which an object was segmented.
Image Name		File name of the image.
Image Relative Time		Relative acquisition time of an individual image within a multidimensional image. The time difference from the first image acquired is stated. This value is not determined for 2D images. If the parameter is nevertheless measured, the corresponding field in the data table contains no value. **Time format:** hours : minutes : seconds : milliseconds **Value range:** 1 ms - 100 hours **Restriction:** This value is only available for images that have previously been acquired with AxioVision or ZEN, or saved in ZVI or CZI format.
Image Relative Time (1/2/3/4)		Relative acquisition time of an individual image within a multidimensional image. The time difference from the first acquired image at the set instant of exposure (1, 2, 3 or 4) is stated. This value is not determined for 2D images. If the parameter is nevertheless measured, the corresponding field in the data table contains no value. **Time format:** hours : minutes : seconds : milliseconds **Value range:** 1 ms - 100 hours **Restriction:** This value is only available for images that have previously been acquired with AxioVision or ZEN, or saved in ZVI or CZI format.
Image Save Time		**Date image was saved** Indicates the date on which the measured image was saved.
Image Scale X	µm/pixel	
Image Scale Y	µm/pixel	
Image Stage Position X Image Center	µm	**x-position of the stage at the time of image acquisition** An encoded or motorized scanning stage is required to determine this parameter. **Restriction:** This value is only available for images that have previously been acquired with AxioVision or ZEN, or saved in ZVI or CZI format.
Image Stage Position Y Image Center	µm	**y-position of the stage at the time of image acquisition** An encoded or motorized scanning stage is required to determine this parameter.

		Restriction: This value is only available for images that have previously been acquired with AxioVision or ZEN, or saved in ZVI or CZI format.
Intensity Maximum Value of channel	Gray value	**Highest gray value of all regions** The gray values of all measured regions are analyzed and the maximum value displayed. This is the brightest point in all regions. If this parameter is measured for regions inside an RGB color image, the RGB color image is first converted to a gray level image in accordance with the formula (R+G+B) / 3 and the corresponding maximum value is then displayed.
Intensity Mean Value of channel	Gray value	**Average of all gray values of all regions** The gray values of all measured regions are analyzed and the average of all gray values displayed. If this parameter is measured for regions inside an RGB color image, the RGB color image is first converted to a gray level image in accordance with the formula (R+G+B) / 3 and the corresponding average is then displayed.
Intensity Minimum Value of channel	Gray value	**Lowest gray value of all regions** The gray values of all measured region are analyzed and the minimum value displayed. This is the darkest point in all regions. If this parameter is measured for regions inside an RGB color image, the RGB color image is first converted to a gray level image in accordance with the formula (R+G+B) / 3 and the corresponding minimum value is then displayed.
Intensity Range of channel	Gray value	The smallest gray value is subtracted from the highest gray value for a given channel. The difference value displayed depends on the bit depth of the image.
Intensity Std (Standard Deviation) of channel	Gray value	**Standard deviation of gray values of all regions** The gray values of all measured regions are analyzed and the standard deviation of all gray values displayed. If this parameter is measured for regions inside an RGB color image, the RGB color image is first converted to a gray level image in accordance with the formula (R+G+B) / 3 and the corresponding standard deviation is then displayed.
Mean Area	μm^2	Average area of an individual object.
Mean Center X	μm	Average X-axis value of all the objects for that class; a large deviation from the image center's location could indicate a bias to the left or right side of sample container.
Mean Center Y	μm	Average Y-axis value of all the objects for that class; a large deviation from the image center's location could indicate a bias to the top or bottom side of sample container.
Region Class Color		
Region Class ID		
Region Class Name		Name given to the class during Setup.