OLYMPUS®

INSTRUCTIONS

scan^R

AUTOMATED IMAGE AND DATA ANALYSIS SOFTWARE

This instruction manual describes the Olympus scan^R Automated Image and Data Analysis Software for Life Science. To ensure safety, obtain optimum performance and familiarize yourself fully with the use of these products, we recommend that you study this manual thoroughly before operation. Together with this manual, please also read the scan^R Automated Image Acquisition Software and Hardware manuals as well as the manuals of all other devices controlled by that software in order to understand general operation methods. Retain this manual in an easily accessible place near a system for future reference.

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Contents

1 Introduction

Thank you very much for purchasing Olympus' Screening Station for Life Science and for your confidence in our products and services.
The scan^R Analysis Software is designed for the automated analysis of images that were acquired by the Olympus scan^R Screening Station and with the scan^R Acquisition Software. The software is intended for the use in biomedical research.

> ⚠ The scan^R Analysis Software, the scan^R Acquisition Software as well as the hardware components of the Olympus scan^R Screening Station for Life Sciences are for research use only.

1.1 Abstract

This user manual will guide you through the usage of the analysis software of the Olympus Screening Station scan^R. It will assist you in setting up efficient and reliable assays from scratch. This scan^R module is intended to be used for the analysis, quantification and navigation through your results. The analysis module of scan^R allows you to run the analysis during acquisition or in "offline mode" afterwards.

Special care has been taken to guarantee correct and accurate information within this documentation, although this is subject to changes due to further development of the Screening System. Thus, the manufacturer cannot assume liability for any possible errors. We would appreciate reports of any mistakes as well as suggestions or criticism.

1.2 Technical Support

If you find any information missing in this manual or you need additional support, please contact Olympus directly.

2 Main User Interface

This chapter explains the features of the image displays and briefly introduces the different menu points and buttons accessible from the main user interface.

2.1 General

The scan^R Analysis main graphical user interface contains four histograms and an image viewer window. The functions of these histograms are explained in Chapter 2.4, *Managing Histograms*.

The image viewer window shows the image with the object corresponding to a data point selected in a histogram. A description of the image viewer functions is given in Chapter 2.5, *Using the Image Viewer*.

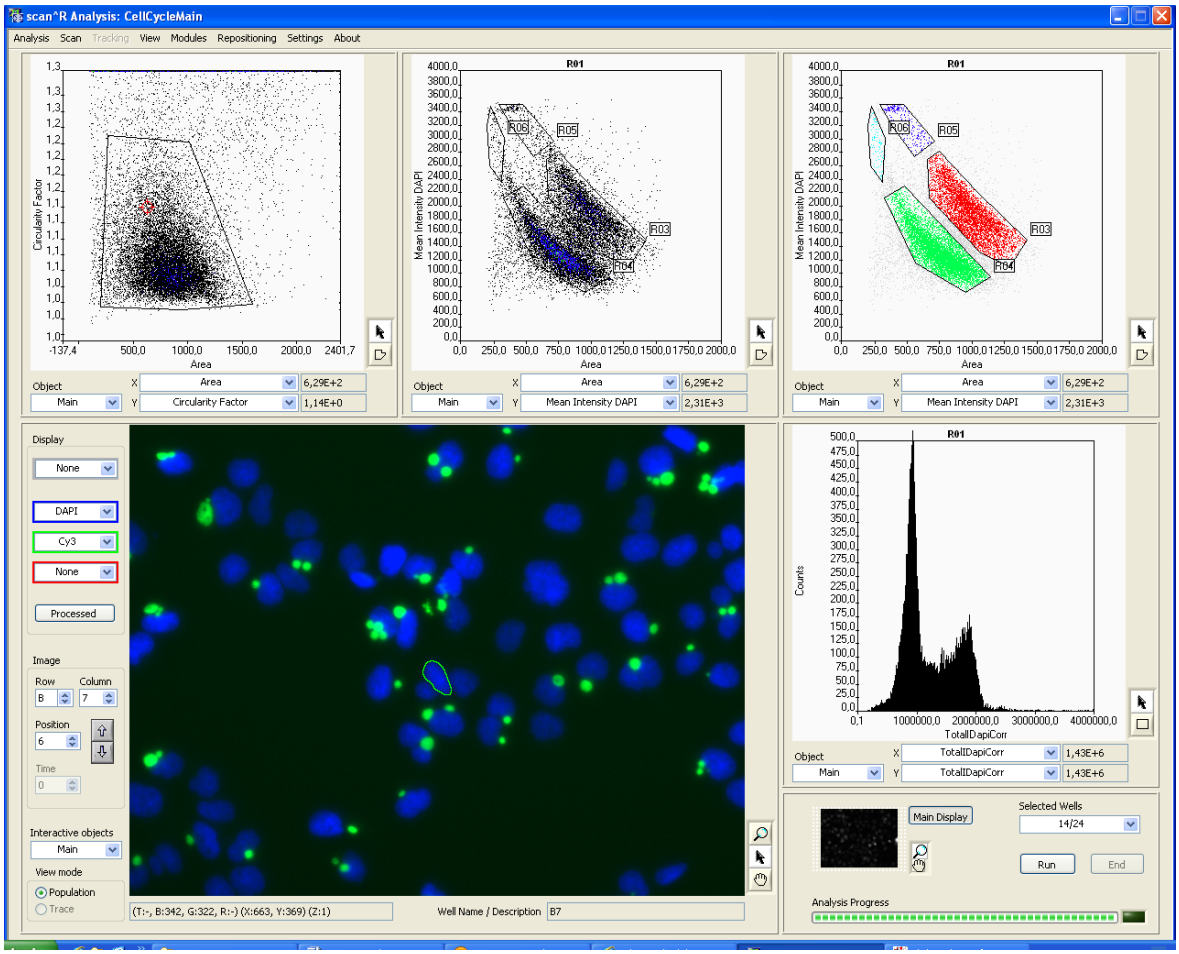
Navigation through the images of a scan is possible with the tools in the **Image** box. Color channel selection is done with respective pull-down shortlists in the **Display** box.

An assay can be started and followed online with the tools and displays in the field at the lower right of the main window.

The menu bar at the top contains several pull-down menus and commands. They are listed in the following overview:

Analysis ▸ Run: starts the analysis with the current settings

Analysis ▸ Batch Run: starts multiple analysis with the current settings



Analysis ▸ Open: opens a previous analysis (*.sca-file)

Analysis ▸ Save/Save as: saves the current analysis (*.sca-file)

Analysis ▸ Export Table: exports the values determined for each detected object to a spread sheet. The values exported depend on the active view. When also sub-objects are detected not only one file is exported but for every sub-object a separate list is exported. The values that are exported depend on the active view: population view / trace view (see Chapter4.3.4)

Analysis ▸ Edit Assay: opens the “Assay settings” menu

Analysis ▸ Load Assay: loads an existing assay (*.say-file). In contrast to the *sca-file the *.say-file contains only the analysis, i.e. the operations to perform on a data set but not the results of a specific analysis.

Analysis ▸ Save Assay: saves the current assay (*.say-file)

Analysis ▸ Assay Gating: opens the gate manager (see Chapter 4.2.2)

Analysis ▸ Exit: exits the analysis

Scan ▸ Open: opens a scan and assigns it to the current assay. The file types that can be opened are the scan^R experiment descriptor files (*.xml-format) and dotslide images (*.wtp-, and *.ets-format)

Scan ▸ Relink images: re-links acquired images to an analysis. To do so, navigate to the folder where the images are stored and select **Current Folder**.

Scan ▸ Custom conversion: converts a third party data set into the scan^R format (see Chapter 6.1)

Scan ▸ Select wells: selects a set of wells for analysis and data navigation; allows also to display a well overview, i.e. an overview of the images that were acquired in a well. (see Chapter 2.7)

Scan ▸ Scan Info: displays the path to the image data

Scan ▸ Settings: displays the settings to a scan

Tracking ▸ Trace View: toggles between the **Population** and **Trace View modes.**

Tracking ▸ Configure Tracer: opens the **Trace Configuration** window to select how the object tracking is to be performed.

Tracking ▸ Define Parameters: opens the **Trace Parameters** window to select trace analysis operations.

Tracking ▸ Show traces: opens the **Trace Viewer** that visualizes the trace graphs.

View ▸ Layout: display properties for the RGB display (affects only the displays).

View ▸ Parameter View: lists all parameters for a selected object, that were determined during analysis

Modules ▸ Object Finders: list and configure available Object Finder Modules (Chapter 6.3, Libraries)

Modules ▸ Object Analysers: list and configure available Object Analyzer Modules (Chapter 6.3, Libraries)

Modules ▸ Image Processors: list and configure available Image Processing Modules (Chapter 6.3, Libraries)

Modules ▸ VC Processors: list and configure available Virtual Channel Modules (Chapter 6.3, Libraries)

Repositioning ▸ Interactive: the scan^R screening system moves to the position of the selected object

Settings: Gives access to some preferences including galleries, directories, data export and settings for repositioning an reclassification.

2.2 General Settings

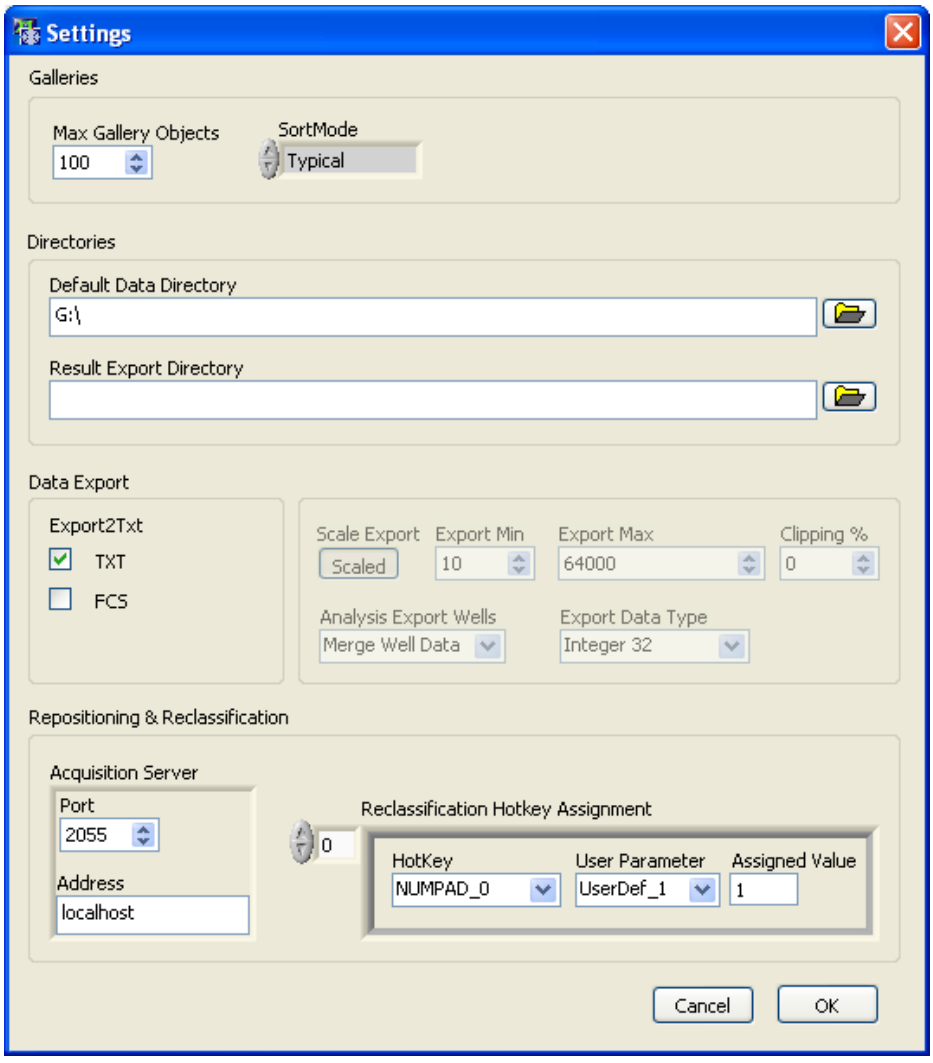
The general preferences can be defined in the **Settings** menu.

Max Gallery Objects. Sets the number of objects that are displayed in a gallery.

Sort mode. The options are **Typical**, **Random**, **yx** and **–yx**. When **Typical** is set, the galleries display the objects which are closest to the center of gravity of the selected region or histogram in the order of distance to that center. **Random** displays randomly selected objects within the selected region. The options **yx** and **–yx** allow to create a gallery that is ordered by one parameter.

Default Data Directory. Enter the directory where the data are read by default (used for “open scan”)

Result Export Directory. Enter the directory where the results are to be stored. When this field is empty the results will be stored in the scan directory\Population Results. The results of a tracking analysis will bestored in the scan directory\Trace Results. (Note that in earlier versions the results are stored in the scan directory\Results folder.



Export2Txt. The results can be exported as .txt files or as .fcs files. (For export to .fcs format, see Chapter 6.2)

Repositioning & Reclassification. Set the Port and the Address for communication with the scan^R screening system for experiments with repositioning.

2.3 The scan^R Data Structure

scan^R analysis data can be separated into the acquired images and the assay being applied on them. The acquired images as a whole are called a scan; it includes the individual images and their acquisition settings like color channels, integration time, plate information etc. An assay describes the processing and analysis steps applied to extract data out of the images.

This separation between analysis settings and acquired images allows the reuse of once adapted analysis settings for different scans.

The images acquired during a scan^R scan are stored as 16-bit ***.tif** files in a **Data** subfolder in the experiment scan storage folder.

Additionally, the scan settings are stored in an **Experiment_descriptor.xml** and the stage positions in the **Acquisitionlog.dat** file.

The scan^R analysis software serves for the analysis of the scans. The instructions (assays) for these analyses are stored in the **scan^R Analysis/Assays** folder as ***.say** files. These files can be loaded via **Analysis ▸ Load Assay…** to then apply the assay on a scan data set.

Once an assay has been performed on a data set, a ***.sca** file is generated and can be stored in the experiment storage folder. These files contain all analysis data including histograms and scatter plots etc. ***.sca** files can be loaded via **Analysis ▸ Open…** to revisit the analysis results.

To clarify this: if you open a **Scan** via **Scan ▸ Open…** the experimental settings of a scan will be loaded by reading in the **Experiment_descriptor.xml** file. Thus, you get access to the raw image data. In contrast to this, if you open an **Analysis** (***.sca**) you will get the results in addition to the images.

2.4 Managing Histograms and Scatter Plots

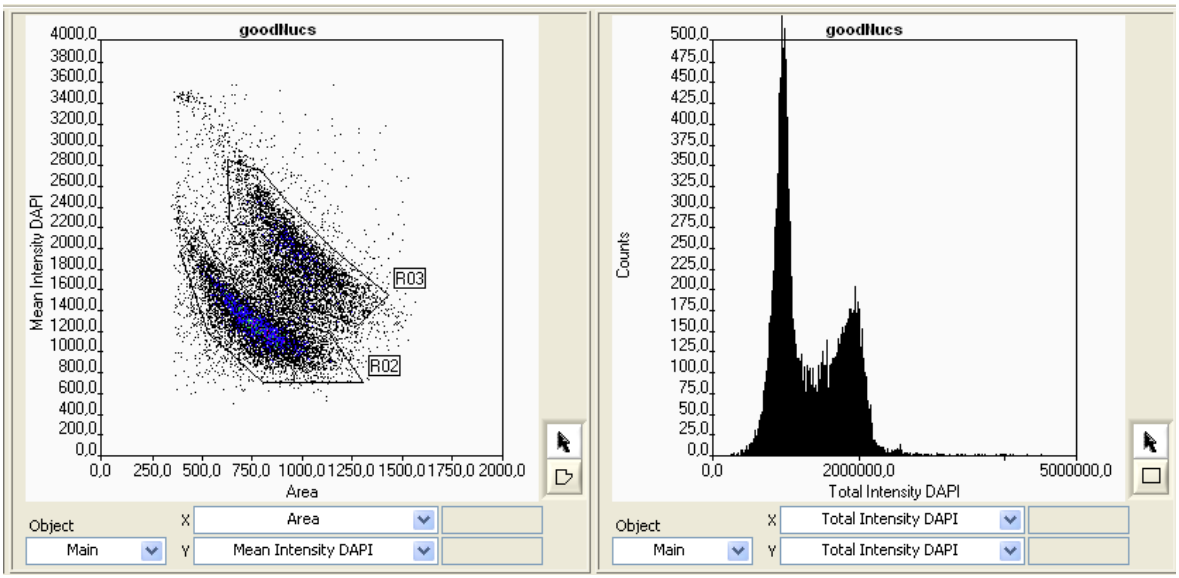
Histograms are used in scan^R for data representation, classification and navigation. scan^R uses 2-D and 1-D histograms following the common representation in cytometry.

A 1-D histogram shows the frequency distribution of only one parameter. A 1-D histogram is created when the same parameter for X and Y is selected. On the X-axis of the histogram the selected parameter and on the Y-axis the number of counts is plotted.

A 2-D histogram (scatter plot) is a plot of one parameter of a number of objects against a second parameter. Color-coding is used as "third dimension" to represent the frequency of occurrences.

The data displayed in a histogram are assigned to an object type (main-/ sub-object). The detection of the objects is defined by the assay. The object type can be chosen from the **Object** pull down menu

underneath the histogram. The **X** and **Y** pull down menus are then automatically updated to correspond to the measured parameters of the chosen object type (see assay definition). The **X** and **Y** pull down menus are used to change the parameters displayed in a histogram. The axes of abscissa and ordinate are labeled with the chosen parameter.



Two buttons are located in the lower right corner of each histogram. They allow toggling between the **Navigation** and the **Region-Selection** modes.

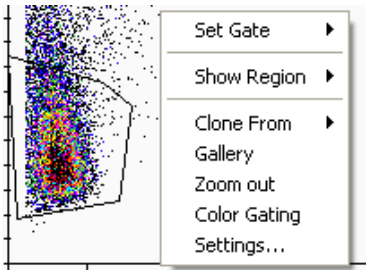
Navigation 

The **Navigation** button with the **pointer** symbol allows navigating within the data. Each data point within a histogram is directly linked to the object from which it is derived. A selected data point is highlighted by a red circle in all histograms in navigation mode as long as the data point is within the displayed area. The corresponding object is displayed in the **Image Viewer**. The X and Y values of the data point are displayed next to the X and Y pull-down menus. Holding and dragging the mouse using this tool allows to virtually following the objects changes within the parameter set. The navigation tool also allows dragging and modifying existing regions.

Region 

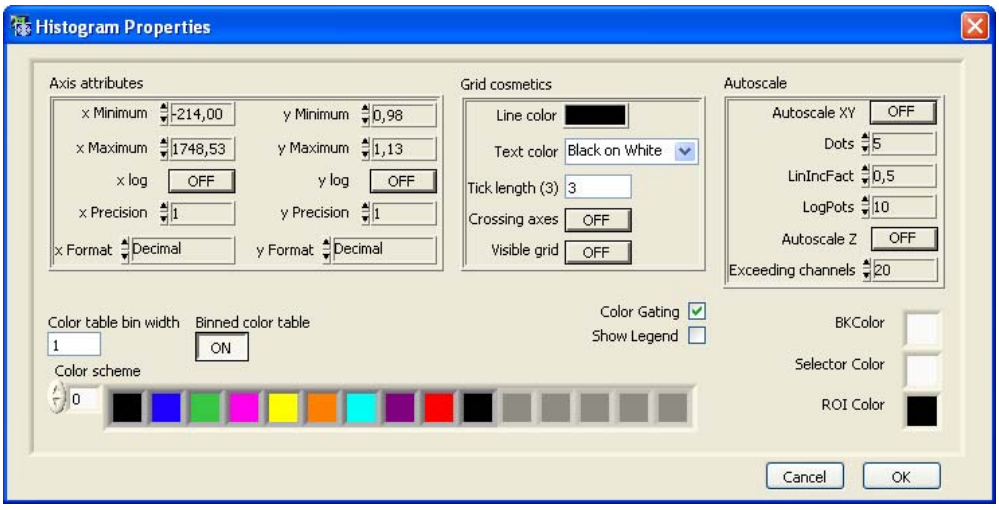
The **Region** tool is used to draw polygons into a 2-D histogram and to set a range in 1-D histograms. Regions define bi-dimensional intervals within the parameter range and thus subpopulations of data points. Double-click in order to close a region in a 2-D histogram.

2.4.1 The Histogram Context Menu



The histogram can be managed through the context menu accessible via right-click into the histogram. (Note that you will get the **region context** menu, if you right-click on a region border as described below). The **histogram context** menu contains the following commands:

- **Set Gate**. Apply a gate on the selected histogram by choosing a region from the list that appears.
- **Show region**. Select a region from the list that appears to generate a zoomed-in view of it with the X/Y parameters that were used to define this region.
- **Clone from**. Select a histogram from the list that appears to duplicate the histogram.
- **Gallery**. This command generates an image gallery of objects in the current histogram. The number of images and the selection criteria is set according to the gallery preferences given in the **Settings** menu.
- **Zoom out**. This command zooms out of the current view by a factor of 2 (in case of linear axis scaling).
- **Color Gating**. This command causes the population of each gate to be displayed in a different color in the histogram.
- **Settings…** This command opens the **Histogram Properties** dialog with the following settings.

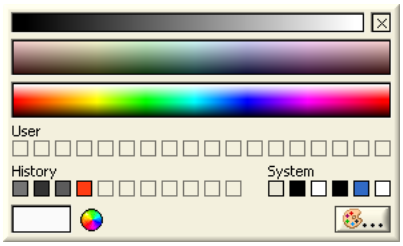


Axis attributes. This field offers different choices to set the axis scaling.

Grid cosmetics. This field offers different choices to set the grid display.

Autoscale. This field offers different choices to set the auto scale of the axes.

BKColor. A click on the colored field opens a window that allows selecting the background color.



Selector Color. A click on the colored field opens a window that allows selecting the cross-hair color.

Region Color: A click on the colored field opens a window that allows selecting the color of the region outline.

Color scheme. This color palette defines the coloring of data points (pixels) in the histogram in dependence of the number of counts (events) they represent. For example, if the **Color table bin width** is set to three and a data point represents 7, 8 or 9 counts, it will be displayed in the third color from the left.

Color table bin width. It defines the range of counts to be given the same color from the **Color scheme**.

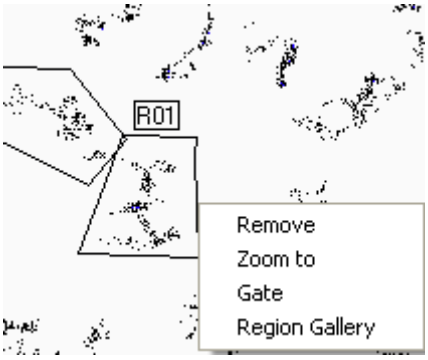
Binned color table. Click this button to activate the color binning as set in **Color table bin width**.

Color Gating. This command causes the population of each gate to be displayed in a different color in the histogram.

Show Legend. This command causes a legend of the colors in the **Color Gating** mode to be displayed in the histogram.

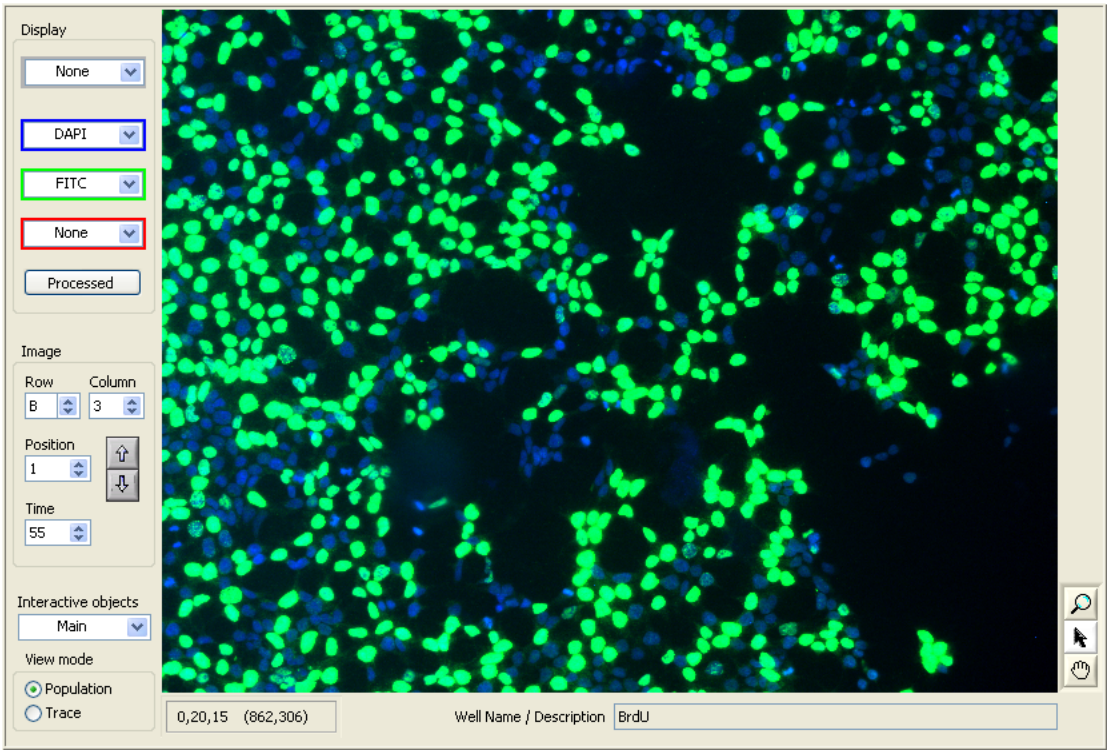
2.4.2 The Region Context Menu

The region context menu will open, if you right-click on a region border



- **Remove.** Deletes the selected region or gate.
- **Zoom to**. Gives a zoomed view of the selected region.
- **Gate**. Converts the region into an **AND Gate**. (See also Chapter 4.4.2, *The Gate Manager*.) When a gate is applied to a histogram, only the data points within this gate are displayed. To display all data points open the histogram context menu and go to S**et gate ▸ none**.
- **Region Gallery**. This command generates an image gallery of objects in the selected region. The number of images and the selection criteria is set according to the gallery preferences given in the **Settings** menu. (For more information see Chapter 2.2, *General Settings*.)

2.5 Using the Image Viewer



Each analysis data point is directly linked to the image object it is generated from. These objects can be displayed in a close-up view in the image viewer. Browsing in the data set by clicking on data points with the histograms **Navigation** tool automatically leads to a corresponding update of the image in the viewer. Additionally the **Well**, **Position, Time** and **Well/Name Description** fields are updated and give information about the image origin. The small arrow buttons on the left of these fields can likewise be used to navigate. Values can be typed in as well.

Display. Multi-color images consist of individual color channels that were recorded with different optical acquisition settings (e.g. different excitation filters). The red, green and blue pull-down menus serve to select the input for the three color channels that are displayed in the respective colors. In order to display a channel (e.g., a transmission channel) in grayscale, select it from the gray pull-down menu. When having both grayscale and any color selection active, a transmission overlay/display will be used for the grayscale selection.

The clipping of the RGB display, i.e., the scaling of the image display brightness, can be changed via the menu point **View ▸ Layout** that opens the **Image Clipping** window.

Image: Processed. Click this button to toggle between the original image and the processed image as defined in **Analysis ▸ Edit Assay ▸ Assay Settings/Image Processing**. Image processing is used to improve the quality of the displayed image but slows down the systems image display. Therefore it is especially recommended to switch it off for performance when creating galleries . The image processing is described in Chapter 4.9, *Image Processing.*

Row/Column/Position/Time. Use these entries to select a specific image to be displayed.
The **up/down arrows** allow fast navigation though your image data set by incrementing or decrementing the **Time**, **Position** and/or **Well** number.

Interactive objects. Select the object type to be outlined in the display when clicking on an object or data point.

The image viewer is equipped with a tool bar to select different mouse tools:

Zoom 

The **Zoom** mode is used to zoom into the displayed image. A click into the image causes a zoomed-in view with the cursor position as the center. To zoom out, the **Shift** key must be pressed simultaneously.

Selection 

The **Selection** mode allows selecting individual objects within the image via mouse click. The object type to be displayed can be selected in the pull down menu in the bottom right corner of the image viewer. Main objects are highlighted by a green outline. The data point corresponding to the selected object is highlighted with a red circle 2-D histograms and a vertical red line in 1-D histograms.

Move 

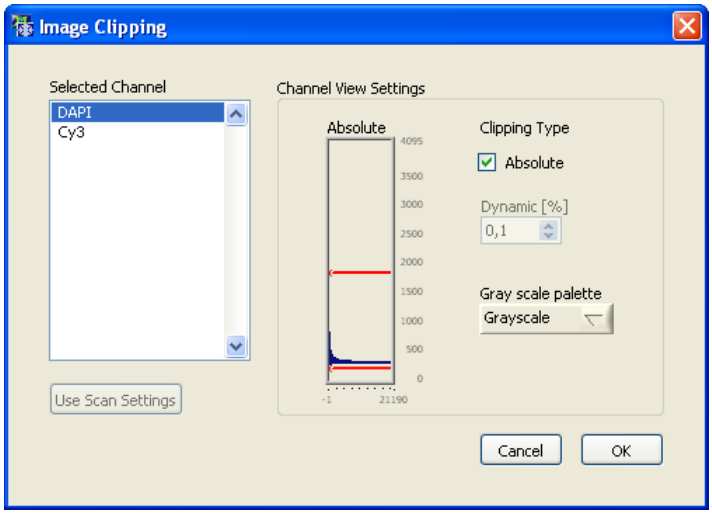
Depending on the zoom factor, only a part of the image will fit into the display. The **Move** mode allows moving the visible area via mouse-drag.

The **status bar** in the lower left corner shows information about the magnification of the displayed image, the current x/y position of the cursor and the pixel value(s) at this position.

2.6 Adjusting the Image Displays

View ▸ Layout opens the **Image Clipping** window that allows adjusting the display brightness. Note that these image settings affect the front panel display as well as well overviews and galleries A raw image will always have a certain background intensity. Also, one will avoid to over saturate images and – especially in fluorescence applications – rather use only a fraction of the camera chip capacity. The consequence is that a raw image is usually low in contrast and may even appear entirely black. Clipping is applied to change the image brightness by defining a range of low pixel counts to be displayed black as well as a range of high pixel counts to be displayed with maximum brightness.

Clipping Type. This button toggles between **Dynamic [%]** and **Absolute** clipping.



Dynamic [%]. Define here how many pixels (as a percentage of the total number of pixels) will be displayed with maximum and minimum brightness. The intensity of the remaining range of pixels will then be scaled linearly in between. The higher the numbers the stronger the resulting contrasts.

Absolute. Define here the range of pixel counts to be displayed with maximum and minimum brightness by dragging the red horizontal lines – that represent the maximum and minimum thresholds – with the mouse. The intensity of the remaining range of pixels will then be scaled linearly in between.

Gray scale palette. The channel selected in the gray pull-down menu in the main GUI can be displayed in different false-color palettes that can be selected here.

Use scan settings.

2.7 Selecting Wells for analysis

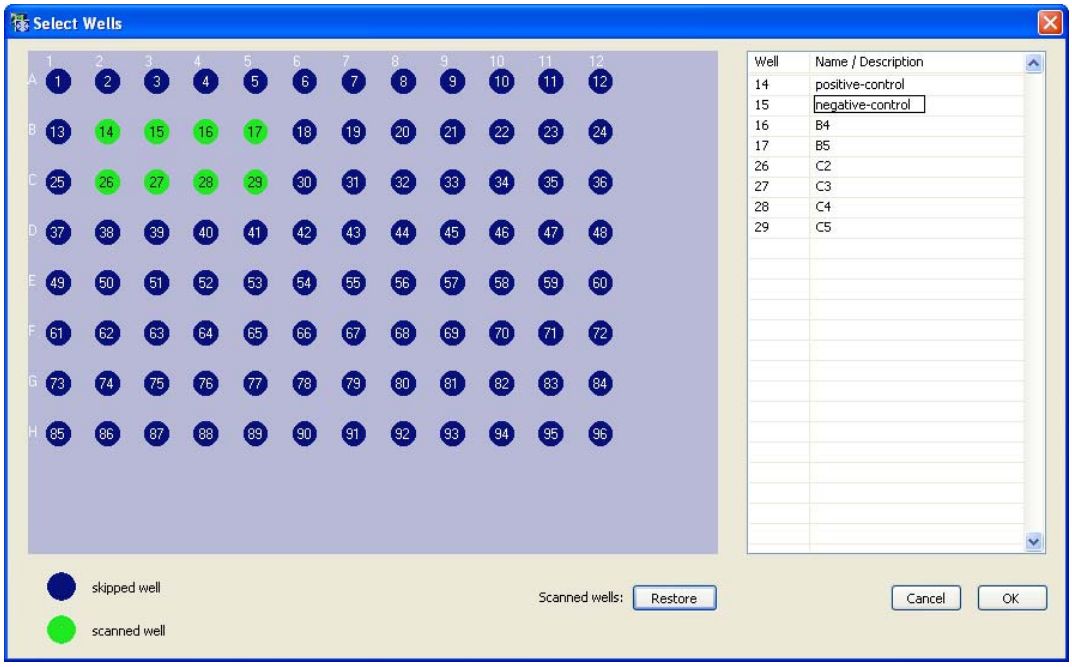
The command **Scan ▸ Select Wells** opens the **Select Wells** display. It features a graphical representation of all wells of the well plate or slide.

When the window is opened for the first time, green circles represent wells that were selected for scanning. These wells will be considered in the analysis . To change this selection double-click on the wells. These wells are also removed from the list on the right. A second double-click reactivates the well. Dragging a rectangle around a group of wells likewise carries out deactivation and activation.

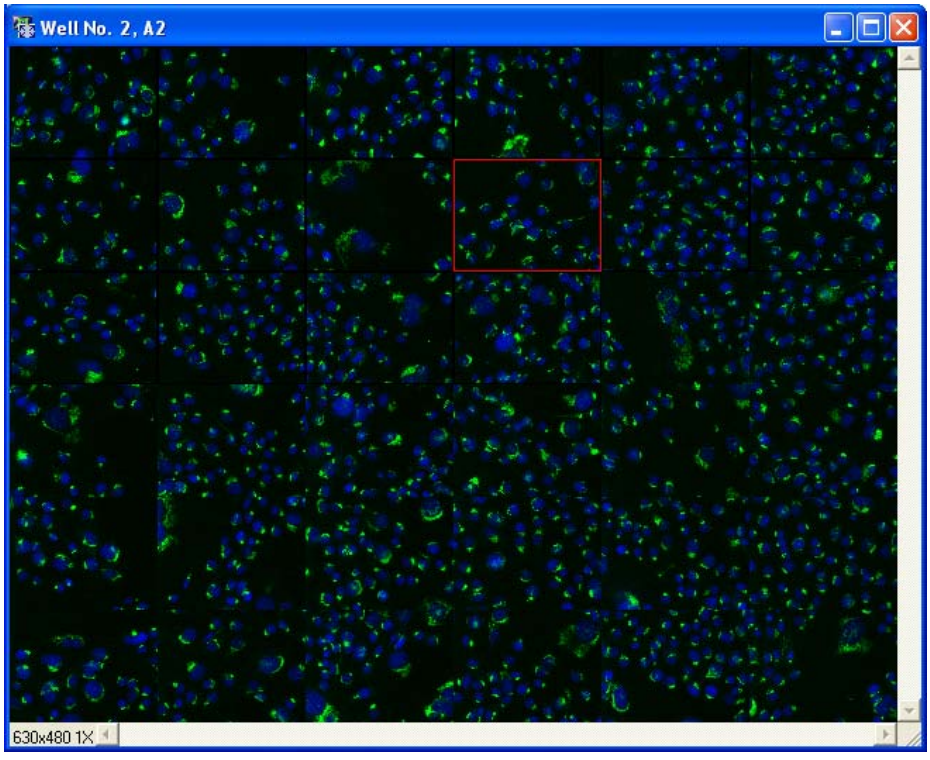
In the list on the right you can also change the name of the wells and group wells by assigning the same name.

Restore. This function restores the initial well pattern

Name/Description. In certain cases it may be advantageous to group wells (see also Chapter 4.3, *Well Results*). Wells of one group need to have an identical entry in the **Name/Description** column of the well list. The default entry is the alphanumerical code of the well position. The name can be changed at will.



Right-click on one of the scanned wells gives you the option **Well Overview.** For a time series you will have to set the time point first. The Well Overview will display all the positions you recorded for one well. Selecting one of the images will load the image in the main display on the front panel.



Well overview.

3 Assays

Assays are the recipes to extract the data of interest from the images of a scan. They define which objects are to be recognized and how and which measurements are to be performed on the found objects. This chapter explains in detail how assays are to be set up or modified.

3.1 General

An assay defines all steps necessary to extract quantitative data from the acquired images. It usually starts with some sort of image processing like background correction. Secondly, objects have to be detected in the images. The analysis, for example different kinds of measurements, e.g. area, intensity, shape…, is finally performed on these objects and results for the samples (e.g. wells) are generated.

The **Assay ▸ Edit Assay** command opens the **Assay Settings** window and allows applying or adapting the assay to the loaded scan. The **Assay Settings** window contains six ta**bs: Main Object**, **Sub-objects**, **Parameters**, **Derived Parameters**, **Image Processing** and **Virtual Channels**. The tabs are arranged from left to right, but you can always jump back and adjust the settings of former steps. However, background correction (in the **Image Processing** tab) should be performed prior to object detection as it changes the intensity values.

scan^R distinguishes between two kinds of object types: an assay always defines one **Main Object** type and up to four **Sub-Object** types connected to it. To give an example, main objects may be individual cells while their sub-objects are individual structures within them.

To represent this hierarchical structure, the **Assay Settings** window's tabs **Main Object** and **Sub-Objects** are used to adapt different set the search algorithms for main and sub-objects in order to extract the structures of interest from the images. This is done by different **Object Finder Modules** that implement different rules for object detection.

The **Parameters** and **Derived Parameters** tabs contain the information about the kind of information to be extracted from the objects (e.g. area, shape,…) .

The **Image Processing** tab allows defining image processing steps that are to be executed before the object detection and parameter extraction.

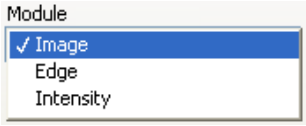
In the **Virtual Channels** tab new channels can be created as a result of post-acquisition image processing (e.g. spectral unmixing). To access the **Virtual Channels** tab you have to navigate through the tabs to the right using the **arrow buttons** on the top right.

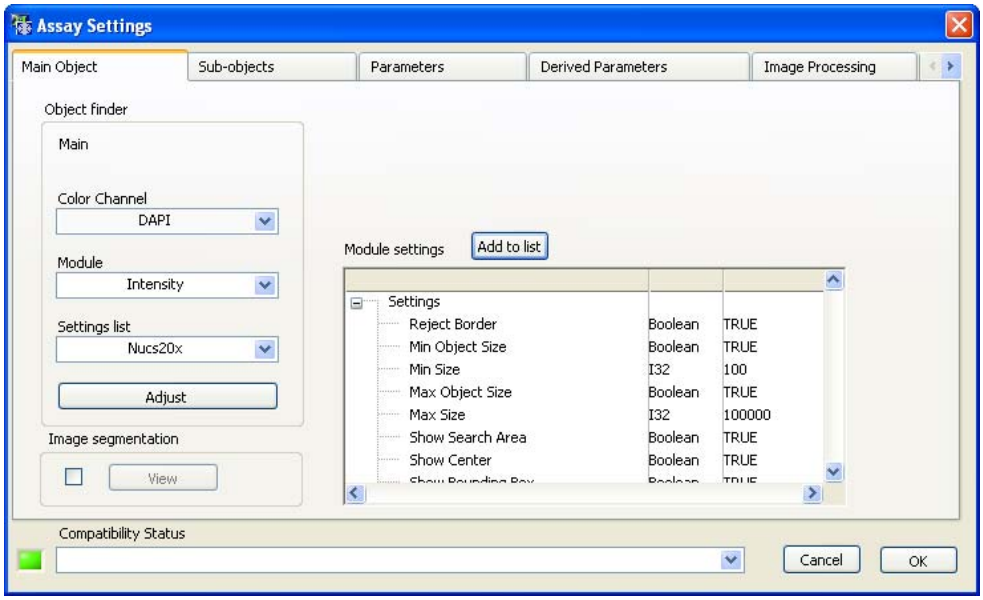
3.2 Object Finder: Detecting Main Objects

The **Main Object** tab of the **Assay Settings** window provides the commands to define the **Main Object** detection.

Color Channel. Select the color channel on which the main object detection is to be performed.

Module. Select the method to detect individual **Main Objects** from the shortlist.





Settings list. Each **Object Finder Module** has a list of preset parameters. The lists can be modified and stored at will. Individual modifications of these settings are marked as **Modified**. Select the settings of choice from the shortlist.

Adjust. This command opens the configuration dialog of the selected **Object Finder Module**. (For changing the list of Object Finder Modules see Chapter 3.4, *Object Finder Modules)*

Module settings. This field lists the current settings of the selected **Object Finder Module**.

Add to list. This adds the modified settings to the **Settings list**. Click the button to open the **Add Settings to OFL** window (**O**bject **F**inders **L**ibrary, see Chapter 4.5, *Object Finders Library*) where you have to give a **New Settings Name** for the modified settings list.

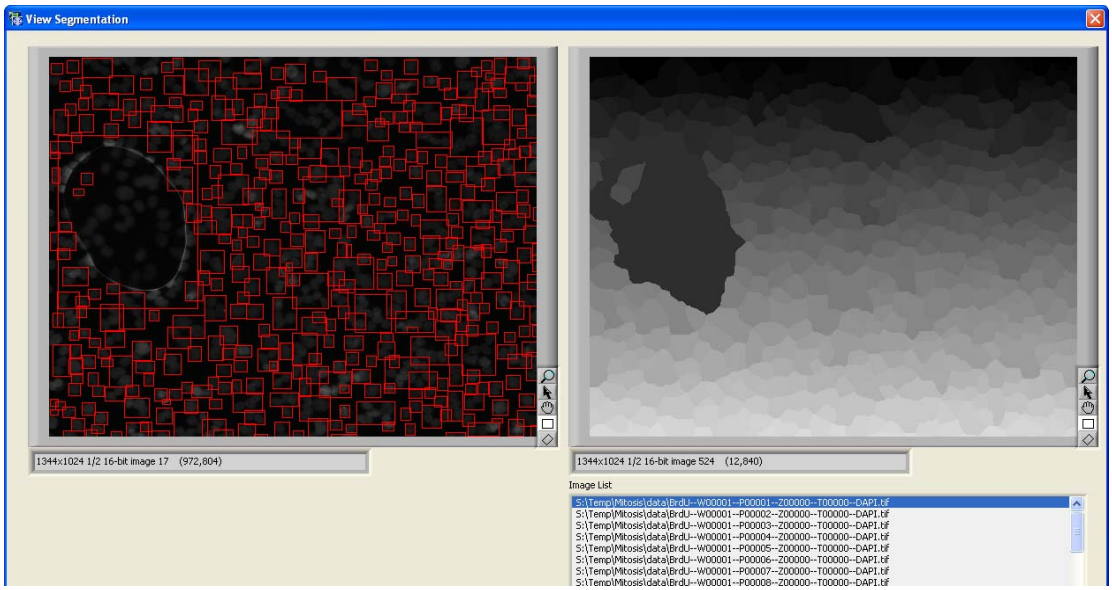
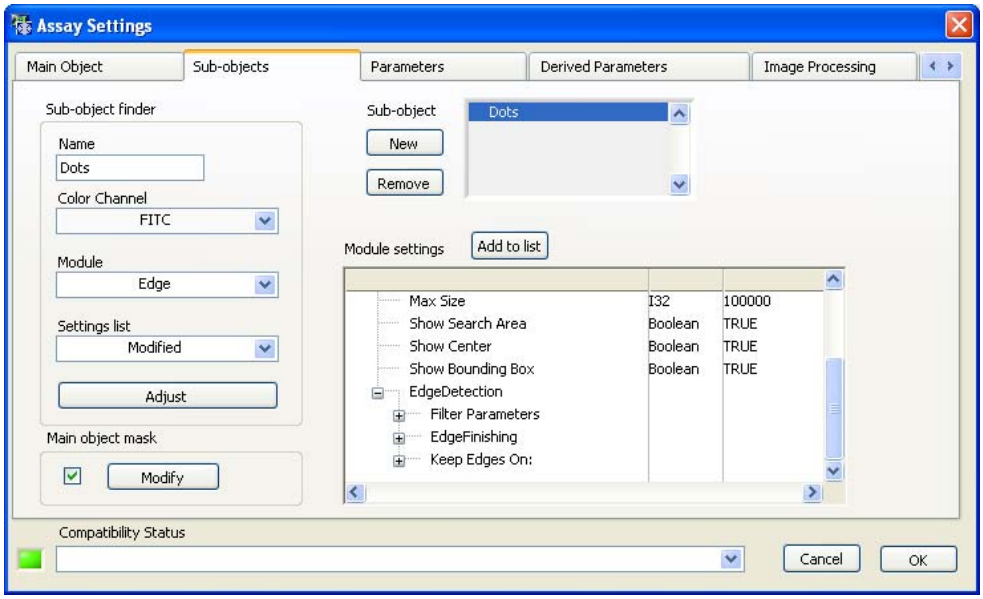


Image segmentation. This function divides – if activated via the check box – the entire image into segments: as many segments as there are **Main Objects** where each segment is assigned to the **Main Object** in its center. In other words, each image pixel is assigned to the **Main Object** it is closest to. All pixels that are assigned to the same **Main Object** form one segment of irregular shape and size. The

View button opens the **View Segmentation** window that contains on the left a display of the object-circumscribing rectangles and on the right a display of the segments.

3.3 Sub-object Finder: Detecting Sub-objects

Sub-objects are structures that are directly linked to individual **Main Objects**. The search for **Sub-objects** takes place on an image mask derived from the corresponding **Main Object**. This **Main Object** mask can be adapted for each **Sub-object** type separately.



Sub-object finder: Color Channel, **Module**, **Setting list**, **Adjust**, **Add to list**. These functions are analogous to the ones described in the previous Chapter 3.2, *Object Finder: Detecting Main Objects*.

Name. Give a name to each new **Sub object**. The default name is Obj. 1.

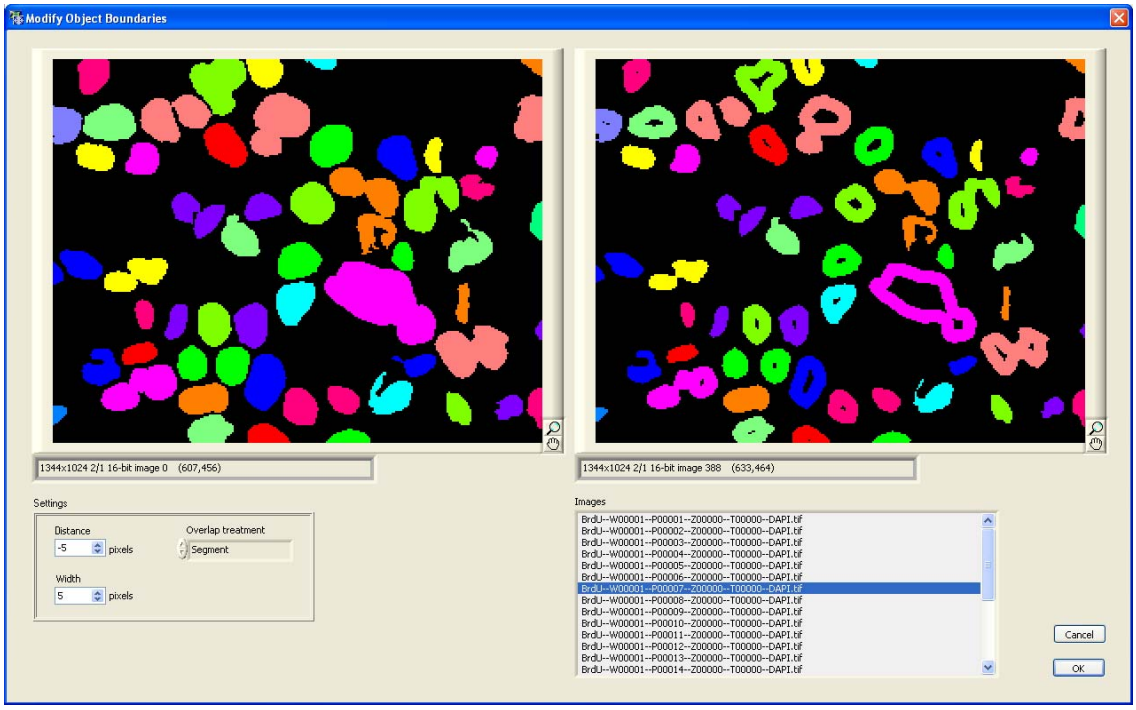
Sub-object list. It gives an overview of the defined **Sub-object** types. The **New** and **Remove** buttons allow the insertion and deletion of **Sub-object** types.

Main Object Mask. Each individual **Main Object** found in an image creates a mask. The individual **Sub-objects** are associated with this mask rather then with the **Main Object** itself. Imagine a Main Object is the cell nucleus and the Sub-objects are structures outside of it. In order to be detected, the original **Main Object Mask** – which only covers the area on the nucleus – needs to be modified in order to enable the detection of the **Sub-objects**.

Click the checkbox to enable the image mask modification of the **Main Object**.

Modify button. Click here to open the **Modify Object Boundaries** dialog to adapt the main object mask to the needs of the **Sub-objects** detection.

Distance. The distance is measured from the outer rim of the main object mask (positive and negative values are valid)

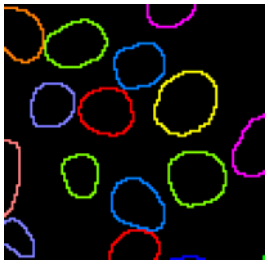


Width. Extension of the sub-object mask

The examples below illustrate the effects of these parameters.

Overlap treatment. Options are **Segment**, **Segment (slow)** and **remove**.


Main object mask


Distance 0, Width 1


Distance -5, Width 5


Distance 8, Width 8

When sub-objects are used for analysis, a further parameter becomes available in the **Parameter** tab (see Chapter 3.5, *Measurement Parameters*): **Obj. 1 counts** (if the default name for sub-objects is used, otherwise it would be *subobjectsname* counts). This parameter gives the number of sub-objects detected for each main object and is a parameter of the main object.

3.4 Object Finder Modules

3.4.1 Entire Image

This is a very simple object definition: the entire image is used as object. You may use this for measurements of integral intensities of your sample, i.e., for each image and each parameter (see. Chapter 3.5, *Measurement Parameters*) a single value is calculated, independent of the objects within the image.

Ignore frame. This is the only parameter to adjust: the size of a bordering frame to be ignored. The default value is 0 (no bordering frame).

3.4.2 Intensity Threshold

As the name says, the **Intensity Threshold** method is based on intensity values: pixels with intensities above a predefined threshold will be united to one individual object.

The **Object Finder: Intensity Threshold** dialog has two image viewer displays. The left one shows the gray value image including all detected objects marked with a red bounding box. The right one shows the binary mask of each object.

Settings

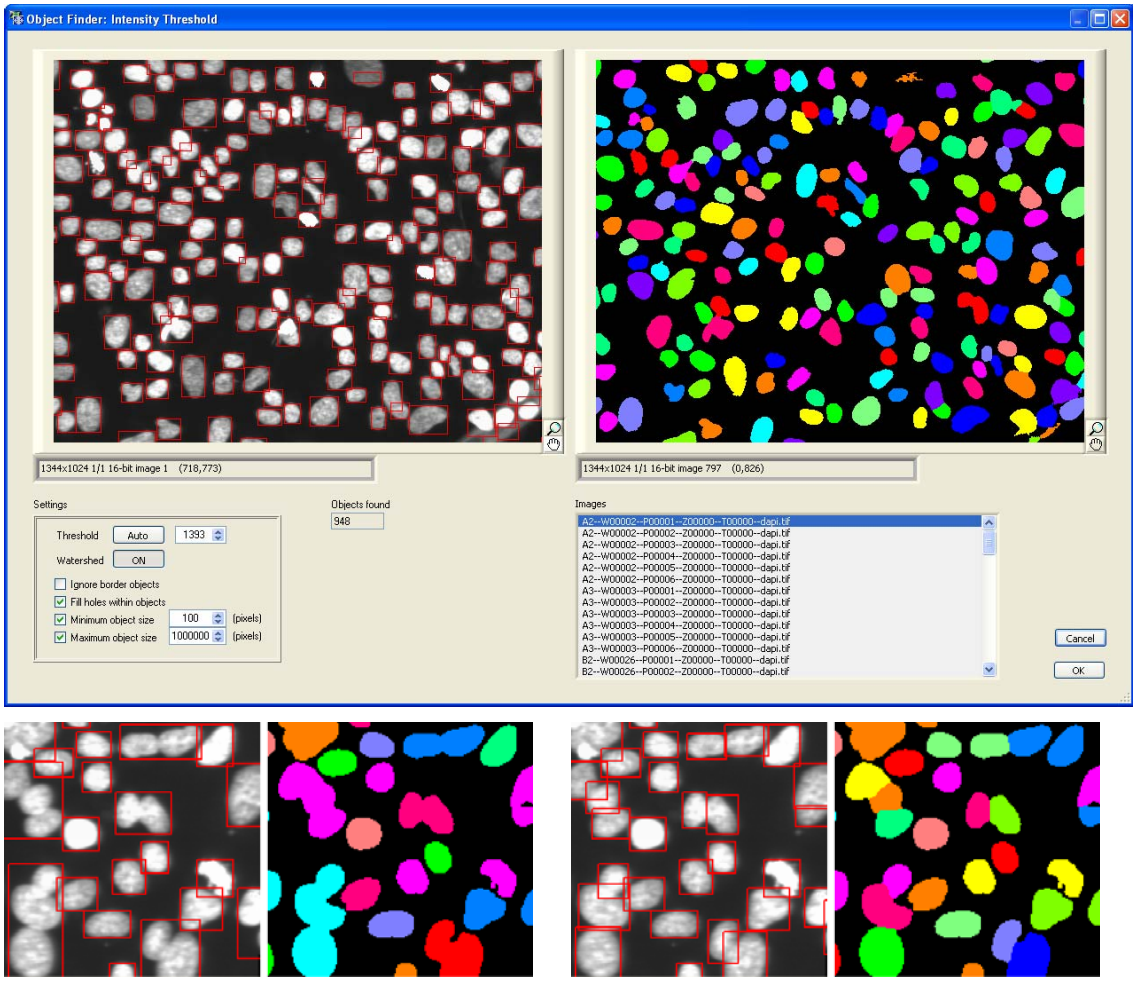
The settings used when opening the window depend on the **Settings list** selected in the **Main Object** or **Sub-object** tab (see Chapters 3.2, *Object Finder: Detecting Main Objects* and 3.3, *Sub-object Finder: Detecting Sub-objects*) and are loaded from the **Object Finders Library** (see Chapter 6.3.2, Object Finders Library (OFL)).

Threshold. This is the intensity cut-off for objects. Type in a value or use the arrows to adjust it.

Threshold: Auto. Click this button to automatically evaluate the image background and set a meaningful cut-off value.

Watershed. If neighboring objects are so close to together that thresholding does not lead to a clear separation, they will be detected as one object. (See left image pair below.) The **Watershed** algorithm separates these objects along the contractions of the detected masks. (See right image pair below.) Set the toggle button to **On** to use this option.

Ignore border object. Check this box to ignore all objects that are cut-off by the image border.



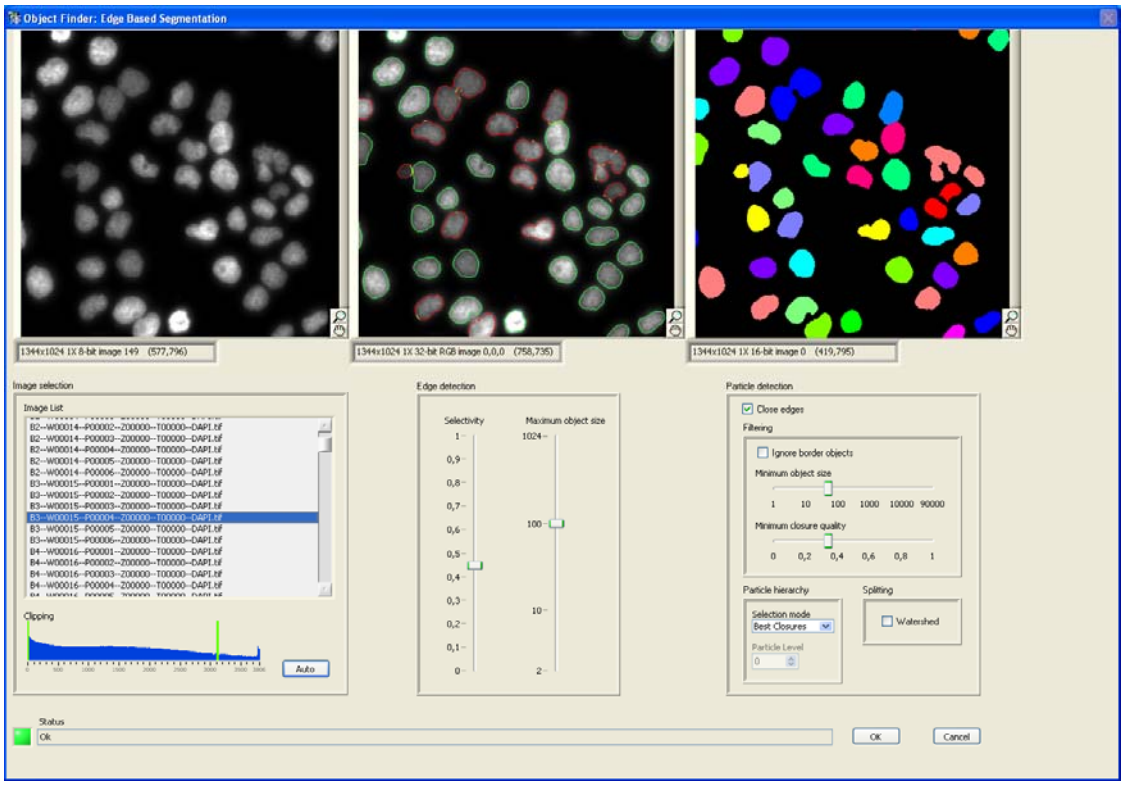
Fill holes within objects. Check this box to fill the object mask in case it contains holes.

Minimum/Maximum object size. Check these boxes and adjust the values to apply minimum and maximum size filters to the objects (in order to ignore objects that are outside these size limits).

3.4.3 Edge Detection

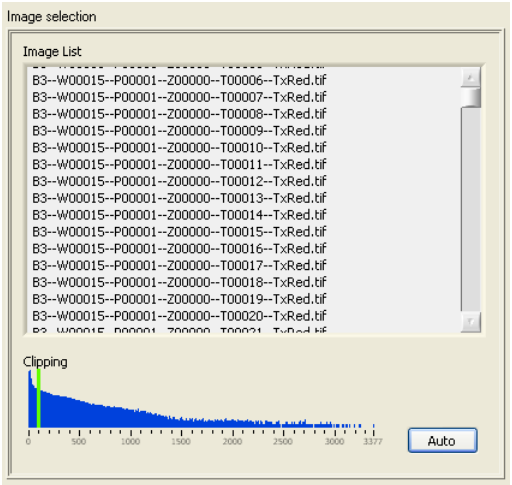
The EdgeSegmentation module is a general purpose edge based particle detector. The idea of the algorithm is to find a closed contour around each particle. First the edges of the image are extracted. For those edges which already form a closed contour the algorithm stops. Since the remaining open edges may be part of a closed contour around a particle, the algorithm then tries to combine these open edges so that they form a closed contour as well.

> The edge detection algorithm yields better results when objects of strongly varying intensity have to be detected. In these cases the threshold detection will either lead to clusters when the threshold is set to a low value in order to detect also dim objects. If a higher value for the threshold is set, then the dim particles will be missed. Furthermore, as edge detection is intensity independent it is especially suitable for cell-cycle analysis.



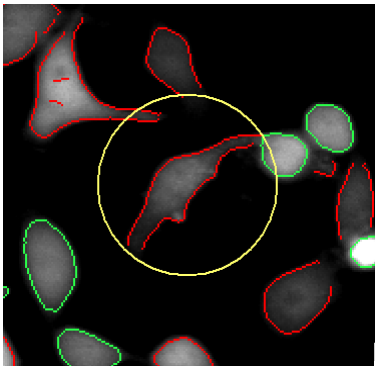
To reduce complexity, the process of finding the right settings is split up into three independent steps. The three settings clusters in the **Object Finder** menu reflect these three steps. They are traversed from left to right, but you can always jump back and adjust the settings of former steps. In each step, the result of adjusting the current steps settings is shown in the image above.

1. **Clipping.** On the left side select an image of your choice from the list. Adjust the clipping either by pressing **Auto** or manually by moving the green bars. If one or both bars are missing, just press Auto once. Try to clip away any unwanted noise or artifacts while maintaining good contrast between the particles you want to detect and the background.

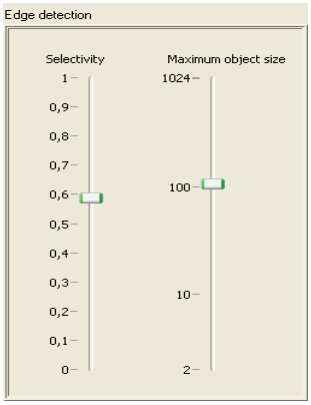


2. **Edge extraction.** Click on the image or the settings cluster in the middle to get to the second step. In this step the edges of the image are extracted. First grab the **Maximum object size** slider and adjust it so that the largest particles you want to detect just match inside the yel-

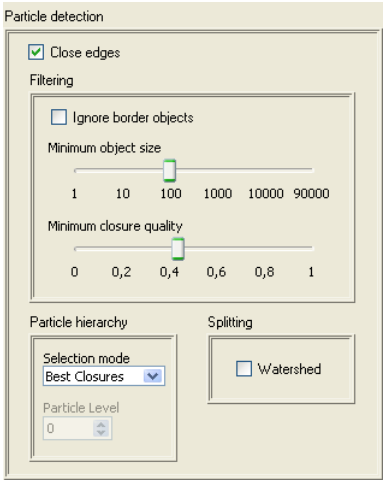
low circle appearing in the images.



3. Play around with the **Selectivity** slider to get just the strong edges (up) or also the weak edges (down). Try to increase the selectivity as much as possible, thereby removing edges due to noise and artifacts, without letting gaps in the contour of wanted particles get too large. As you can see in the image above, contours with closed edges are marked green while contours with open edges are red.



4. **Edge Closing.** Click on the image or the settings cluster on the right to get to the third step. In this final step the open edges extracted in step two are now closed by combining them with other open edges. You can then filter particles by size and closure quality, split them with the watershed algorithm or select a hierarchy.



5. First decide if there may have already been sufficient particles detected in step two (the green ones). If you think so you can skip the closing process by unchecking **Close edges**, thereby reducing processing time dramatically. All unclosed edges (red) are discarded then. Generally the loss of particles is too high with **Close edges** being unchecked. If **Close edges** is checked, you can see in the middle image that the open edges (red) are connected to a closed contour by yellow lines.

---

6. In the **Filtering** cluster you can filter particles by location, size and closure quality. The closure quality is a rating attributed to each detected particle, which describes the quality/reliability of the respective closure. Particles whose contour has already been closed in step 2 (the green ones) have closure quality 1. Especially when particle detection is difficult, you can at least filter out most of the wrongly detected particles by moving the **Minimum closure quality** slider towards 1.

---

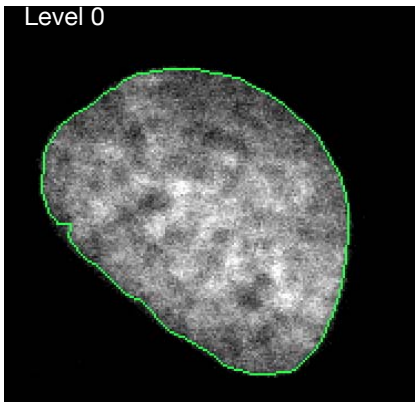
7. By checking the **Watershed** checkbox, you can split particles which have merged. The algorithm inspects the shape of each particle, splitting it at constrictions. This can be extremely useful when detecting nuclei.

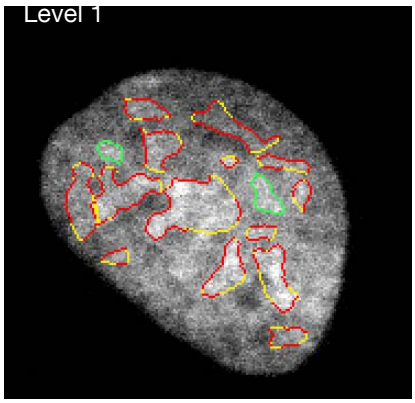
---

8. Sometimes detected particles are nested into each other. E.g. spots inside nuclei or nuclei inside the cytoplasm. Since overlapping particles are not allowed, the **Particle hierarchy** cluster provides options to select the nesting or hierarchy level you are interested in. See below for the function of the **Selection mode** options.

---

9. Don't forget to check other images of the scan to verify that your settings work with them as well. When you are satisfied with your settings click **Ok** on the bottom right side.





The options at the **Selection mode** dropdown list are:

Min level. Selects all level 0 particles.

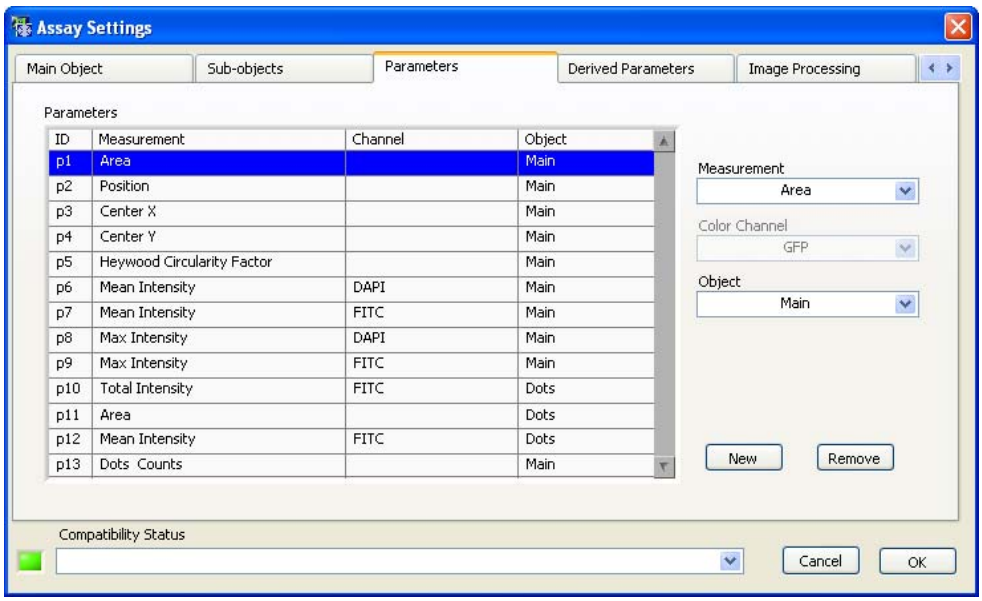
Max level. Selects all particles which do not have other particles nested inside.

Selected level. Selects all particles with the specified level.

Best closures. Selects for each nesting branch the particles which are best according to their closure quality.

3.5 Measurement Parameters

The scan^R **Object Analyzer** modules in the standard configuration offer a large number of individual parameters that may be measured for each recognized object. To limit memory utilization and save CPU time, only the values for the parameters listed in the **Assay Settings ▸ Parameters** list will be extracted during the analysis.



Parameters list. Each **Measurement** is labeled with an **ID (p1, p2,…)** and is assigned to the **Main Object** or a **Sub-object** and – depending on the type of measurement – may be assigned to a **Color channel**.

Measurement. Select a parameter from the shortlist. The available parameters can be adapted through **Modules ▸ Object Analyzers** (Chapter 6.3.1) .

Color channel. Assign a **Color** channel from the shortlist to the newly added parameter.

Object. Assign an **Object** type from the shortlist of available object types. The image mask of this object defines the parameter measurement area.

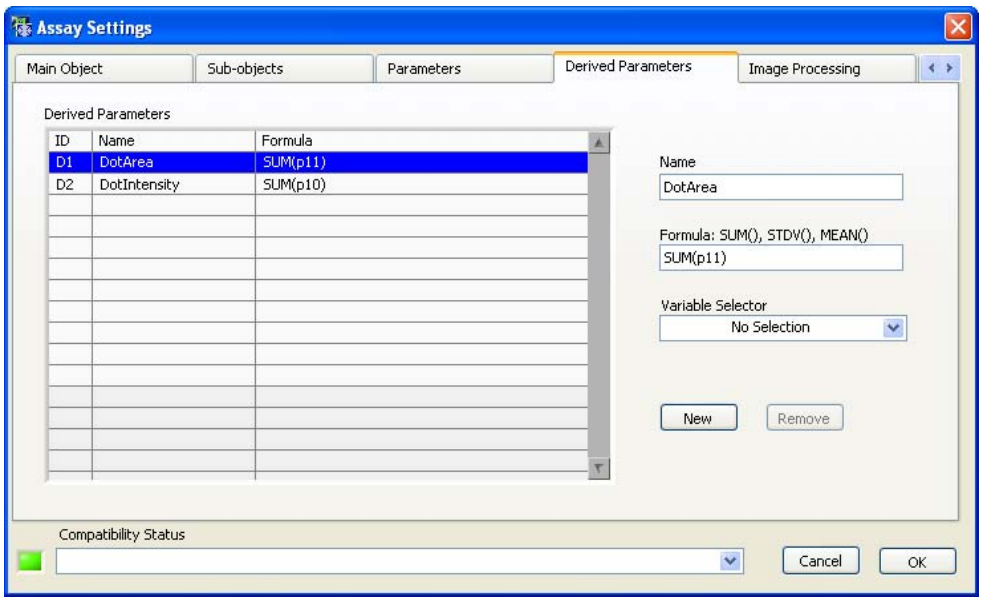
New. This allows inserting a new parameter into the list.

Remove. This deletes the selected parameter from the list.

> A special parameter is available in the **Measurement** list when Sub-objects are used: **Obj. 1 counts** (if the default name for sub-objects is used, otherwise it would be ***subobjectsname* counts**). This parameter gives the number of sub-objects detected for each main object and is a parameter of the main object.

3.6 Derived Parameters

The **Assay Settings** ▸ **Derived Parameters** tab allows performing calculations on the **Parameters** defined in the **Measurements** list by the use of algebraic expressions (+, -, ×, ÷, sqrt, ^ etc. For a complete list see Appendix 6.4). This is especially useful for parameters assigned to different color **Channels**.



Derived Parameters list. It lists the **Derived Measurements** to be carried out. Each **Derived Measurement** is labeled with an **ID (D1, D2,…)** and requires a formula that entangles parameter **ID**s from the **Measurements List**.

Name. This is the input field for setting the new **Derived Measurement's** name.

Formula. This is the input field for the algebraic expression that connects the desired parameter **ID**s from the **Derived Measurements List**.

Variable Selector. The parameters of the measurements list can be selected from this drop-down menu and their corresponding ID is entered in the **Formula**-field. Vice-versa if you select a parameter in the formula field you get the corresponding parameter name displayed in the **Variable Selector** field.

Special Operators

It is possible that a number of individual Sub-objects of the same type can be identified within the mask of a single individual Main Object. A set of statistical operators allows evaluating individual parameters of the entire Sub-object population of this type for the individual Main Objects:. These Operators are SUM(), STDV(), and MEAN().

Statistical Operators:

- **SUM(ID).** This operator sums up all values of the given parameter **ID** of the **Sub-objects** belonging to each individual **Main Object**. that belong to the same **Sub-object** type.

- **MEAN(ID).** This operator averages all values of the given parameter **ID** of the **Sub-objects** belonging to each individual **Main Object** that belong to the same **Sub-object** type.
- **STDV(ID).** This operator calculates the standard deviation of all values of the given parameter **ID** of the **Sub-objects** of each individual **Main Object** that belong to the same **Sub-object** type.

> The parameter resulting from a special operator expression is assigned to the **Main Object**.

New. This allows inserting a new **Derived Measurement** into the list.

Remove. This deletes the selected parameter from the list.

3.7 Image Processing

Images can be processed before being analyzed in an **Object Finder Module**. Thus, image quality can be enhanced and the object detection improved and facilitated. A set of predefined image processing modules is available.

A variable set of image processing steps can be assigned to each color channel. The configuration of the individual steps takes place via the **Image Processing** window accessible via **Assay ▸ Edit Assay**.

> Image processing changes the image data temporarily! The original image data will not be lost but remain stored. However, in the further course of the assay execution the analysis will always work on the processed data. In order to keep the original data accessible during the assay, virtual channels have to be created instead, see Chapter 3.8, *Virtual Channels*.

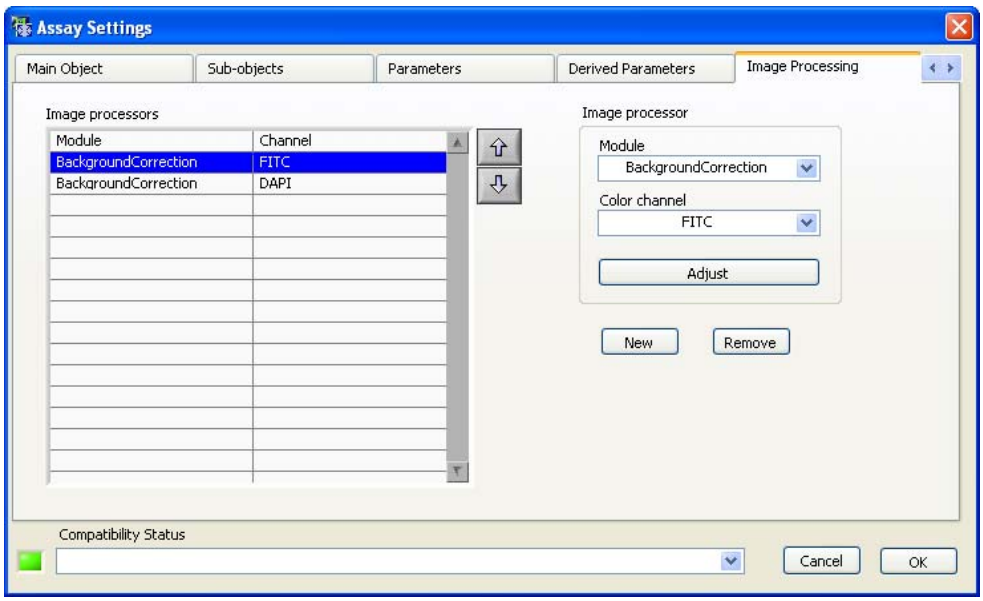


Image processors list. It lists the **Image Processor Modules**, i.e., the processing steps, to be applied on the different **Color channels** of the images. The order of appearance in this list also sets the order of

execution. Moving the active entry up or down the list by using the arrow buttons changes the order of execution.

Module. Select an **Image Processor Module** from this shortlist.

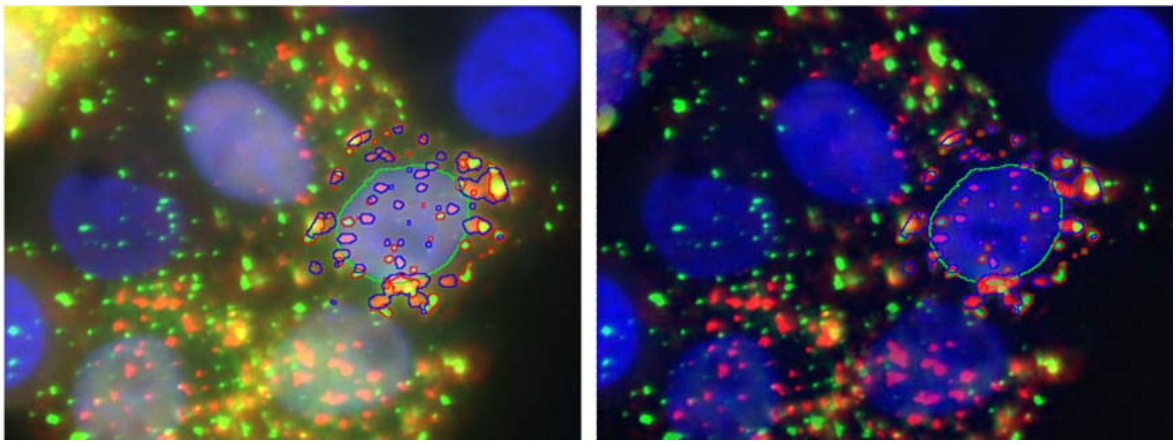
Color channel. Assign a **Color channel** from the shortlist to the active **Image Processor Module**.

Adjust. Click here to open the **Image Processing** window for the active **Image Processor Module**.

New. This allows inserting a new **Image Processor Module** into the **Module** list.

Remove. This deletes the active module from the **Image processors** list.

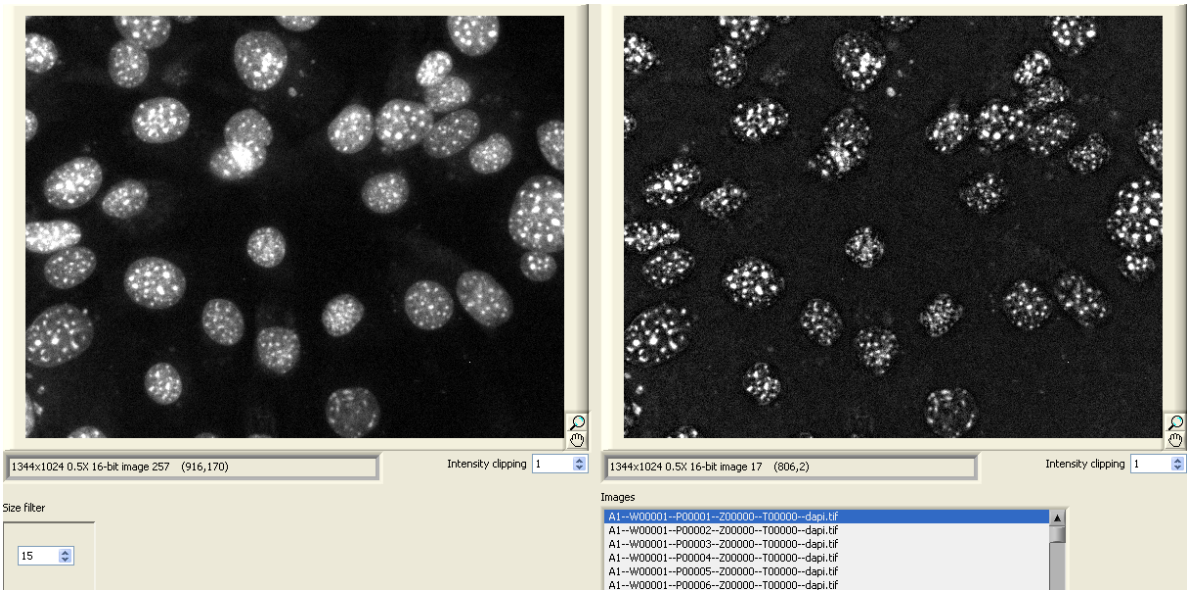
3.7.1 Background Correction



For applications involving quantification of intensities and in case of inhomogeneities it is always recommended to use background correction. The algorithm implemented is an intensity-conserving algorithm which ensures that the signals remain unchanged and can therefore be quantified (left image: before background correction, right image: after background correction).

Size filter. This is the only parameter to be set.

> For background correction a Size filter of 200 is the default value. For most cases when a general background has to be removed it works fine. However, to better extract e.g. small particles in the nucleus, a background correction filter size of 15 can be suitable (cf. example below).



Example for small **Size filter** for background correction.

3.7.2 XY Shift

In certain cases it may occur, that image channels are shifted relative to each other in the channel overlay. This is rather often the case if an observation emission filter wheel is used and images are acquired with different emission filters. This function allows correcting the shift along the X- and Y-axes.

XY Shift. Set here the number of pixels the chosen channel is to be shifted along the X- and Y-axes relative to the other channels.

You have to control the result in the main user interface by pressing the **Processed** button. It is useful to zoom into the image so that individual pixels can be detected visually.

3.7.3 Inversion

This function serves to invert the intensities of an image channel, i.e., to convert a channel into its negative image. This is necessary for example if objects are to be detected in transmission images. The object detection tools of scan^R are designed to detect bright objects on dark background. The situation is reverse in transmission images. Thus they have to be inverted prior to the object detection.

Adjust Intensity. This defines the maximum intensity in the converted image. I.e., it is the intensity that the originally darkest pixel will get in the converted image. 4096 is the default and corresponds to the maximum intensity in a raw image taken by a 12-bit CCD-camera.

3.7.4 Cut Image

This module allows defining regions of interest inside an image and setting all image parts outside the region to the intensity 0. Different drawing tools are available.

Rectangle

Define a rectangle by mouse drag. Mouse dragging the center changes its position. The size can be adjusted by dragging the corners.

Rotated Rectangle

This is similar to the rectangle tool. Upon mouse-over its central axes are displayed. Dragging the ends of the axes turns the rectangle.

Polygon

Standard tool to draw polygons.

Freehand

Standard tool to draw freehand regions. The region is closed automatically once the mouse button is released.

Circle

Standard tool to draw circles. Close the circle by double click.

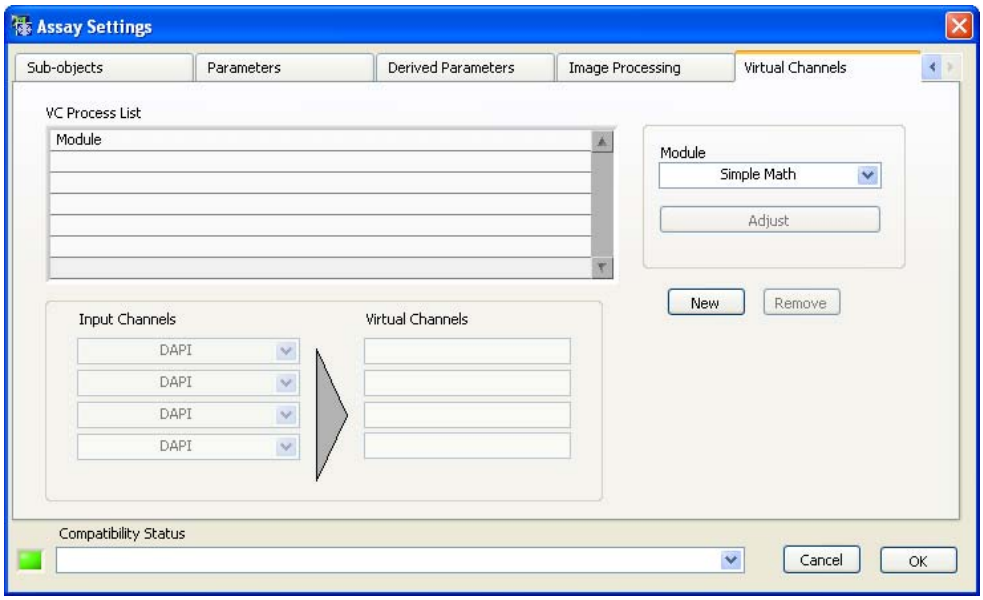
Ring Segment

Standard tool to draw rings. Once a ring is drawn the inner and outer borders can be dragged to adjust the thickness. The cutting line can be dragged to convert the ring into a ring segment.

3.8 Virtual Channels

Virtual channels are image channels that are not created via image acquisition during the execution of a scan. Instead they are a result of post-acquisition image processing and added as new channels to the original image data. These can then be used for further analysis steps, e.g. object detection.

> To access the **Virtual Channels** tab in the **Assay Settings** window you have to navigate through the tabs to the right using the **arrow buttons** on the top right.



New. Click here to create a new entry in the **VC Process List**.

Virtual Channels list. Default names for the virtual channels resulting from the processing are automatically created. It can be changed manually.

Remove. Click here to delete the active entry from the **VC Process List**.

Module. **Background Correction**, **XY Shift**, **Inversion** and **Cut Image** are the same functions as described in Chapter 3.7, Image Processing.

Adjust. Click here to open the dialog window of the selected module.

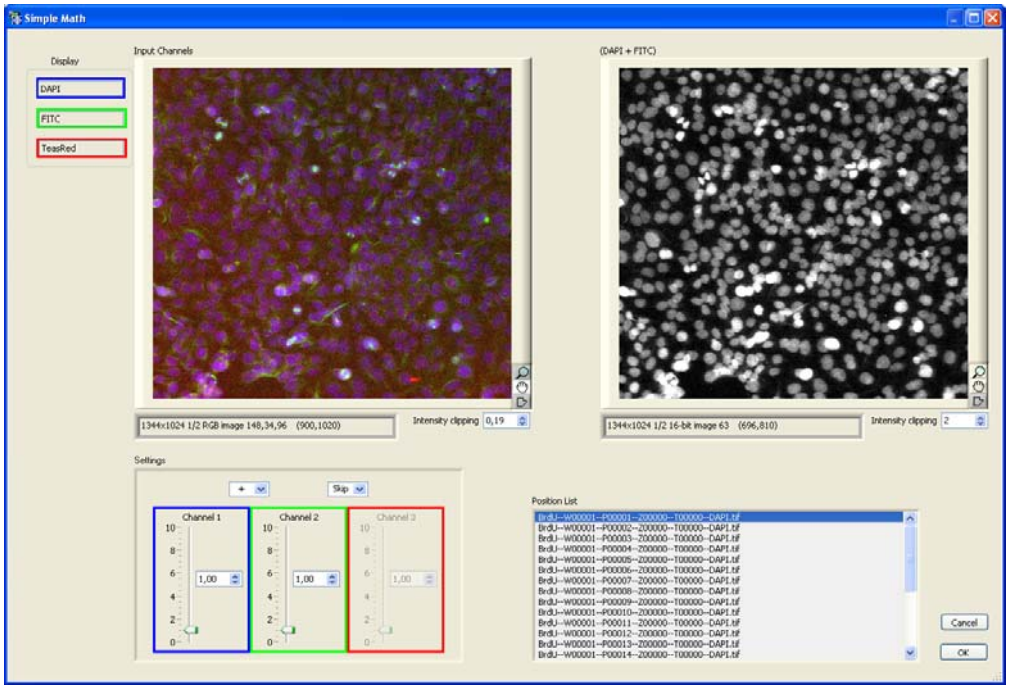
3.8.1 Simple Math

The Simple Math module serves to perform calculations on the image through basic arithmetic operations: addition, subtraction, multiplication and division.

Module. Select **Simple Math** from the **Module** pull-down list to set this module as active entry in the **VC Process List**.

Input Channels list. All color channels of the images are possible **Input Channels** for the **Simple Math** module. The channel selected as first **Input Channel** is always the first source of the arithmetic operation. The other channels are possible second sources.

Adjust. Click here to open the **Simple Math** dialog window. It contains displays of the overlay of the **Input Channels** and of the **Virtual Channel** that is created by the selected arithmetic operation.

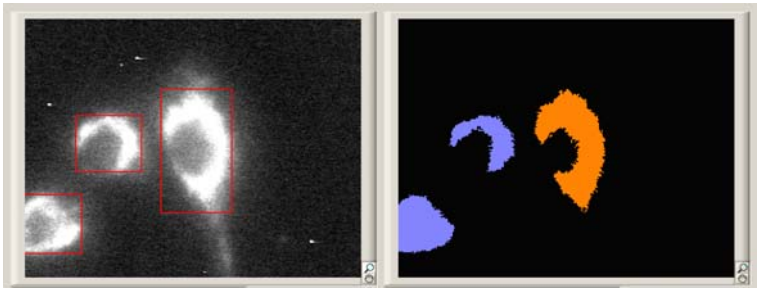


Settings

Channel 1, 2, 3. Each of the **Input Channels** can be weighed prior to the arithmetic operations by using the sliders or entering a multiplication value between 0 and 10 into the respective boxes.

Arithmetic operations selectors. Select the operation (**+**, **–**, **×**, ÷) that is to link Channel 1 and Channel 2 from the first pull-down selector (e.g. DAPI+FITC). Select the operation that is to link the result of Channel 1 and 3 with Channel 3 (e.g. (DAPI+FITC)**×**TxRed) from the second pull-down selector. Select **Skip** to deactivate Channel 2 or Channel 3, respectively.

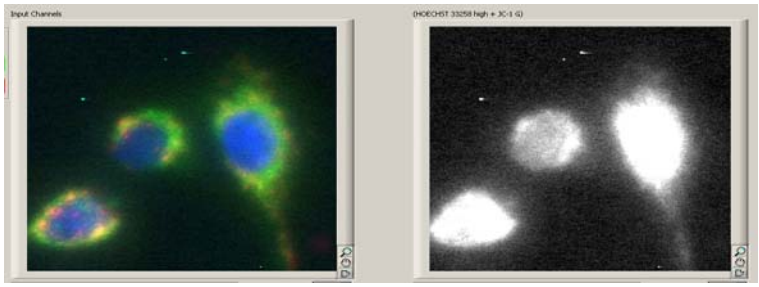
3.8.1.1 Example: Cytoplasm not detectable on a single color channel



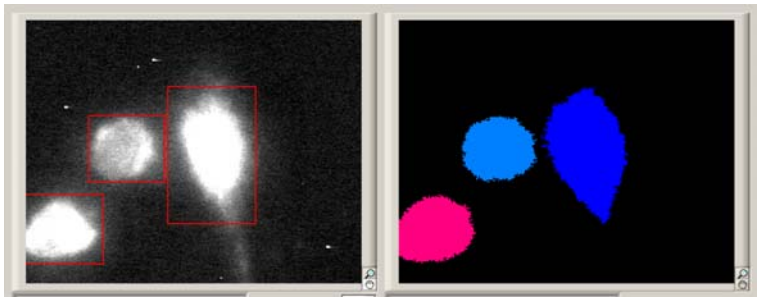
In this example it is not possible to detect the cytoplasm on a single color channel because the area of the nucleus has very little cytoplasmatic staining:

Problem. The detection is incomplete because staining is missing on the nucleus area.

Solution. The nucleus staining and the cytoplasmatic staining are added as VC.



Result. Full Cell Segmentation of the complete cell can be performed on the calculated new channel.



3.8.2 Spectral Unmixing

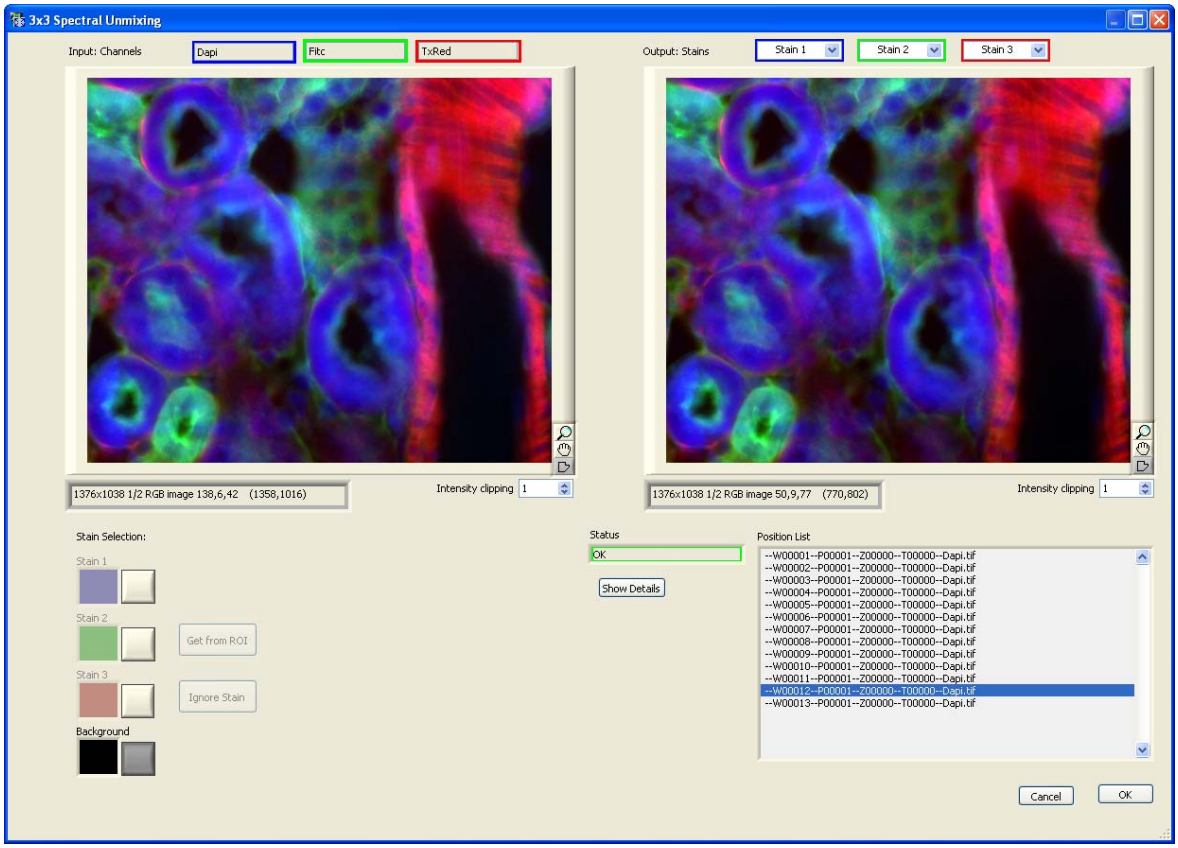
A major problem in live cell imaging arises from the use of different fluorochromes with overlapping spectra in one multi-labeled sample, impairing a number of applications. Even with the use of high quality optical filters it is not satisfactorily possible to separate the spectral information. The consequence is the excitation and imaging of structures that are labeled with one of the present fluorophores when using a filter set that is actually chosen to excite and image another fluorophore.

With the **Spectral Unmixing** module it is possible to separate and resort the contribution of different fluorochromes to the total signal in each color channel and redistribute the different color intensities. It thus improves the spectral resolution of the channels considerably and facilitates for example co-localization studies.

Get from ROI. Takes the mean value within the marked region as Stain/Background.

Ignore Stain. Click here to ignore the third channel in order to properly unmix two-channel images.

Background. Spectral Unmixing yields quantitatively meaningful data only if a background subtraction is performed prior to it.



Show details. Displays the matrix created by the selection of the stains. This matrix is used for the processing of the images. The entries of the matrix can be changed manually. A graphical representation is displayed.

Output channels. Select the stains to see the result of the spectral unmixing.

1. In the **Module** shortlist select **Spectral Unmixing** and press **New.**

2. Press **Adjust** to start the **3x3 Spectral Unmixing** menu.

3. Click on the Background button.

4. Mark a background area using the **drawing tool** (button on the bottom right corner of the image displays).

5. Click the **Get from ROI** button.

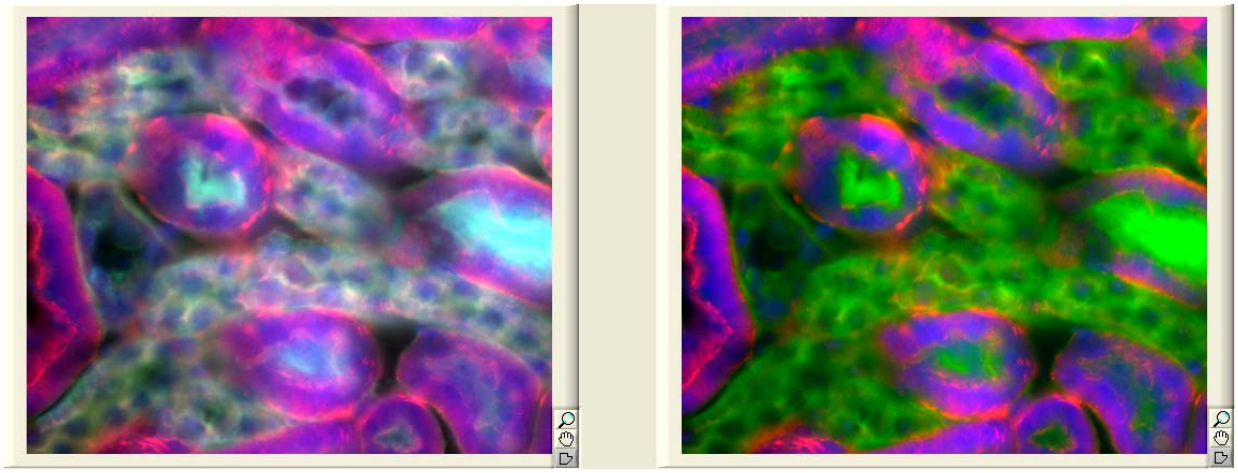
6. Identify a structure that contains just the first fluorophore.

7. Mark the structure using the **drawing tool** (button on the bottom right corner of the image displays).

8. Click on the **Stain 1** button.

9. Click the **Get from ROI** button.

10. Repeat steps 4 – 7 for the other fluorophores (stains).



In order to perform the spectral unmixing the software has to determine the contribution of the fluorescence of different fluorophores to the different color channels. To do so, ideally series of mono-labeled reference samples would be used. In case such samples are not available for each of the fluorophores, molecular structures have to be identified by the user that are certain to contain just only one of the fluorophores and that do not spatially overlap with structures containing other fluorophores.

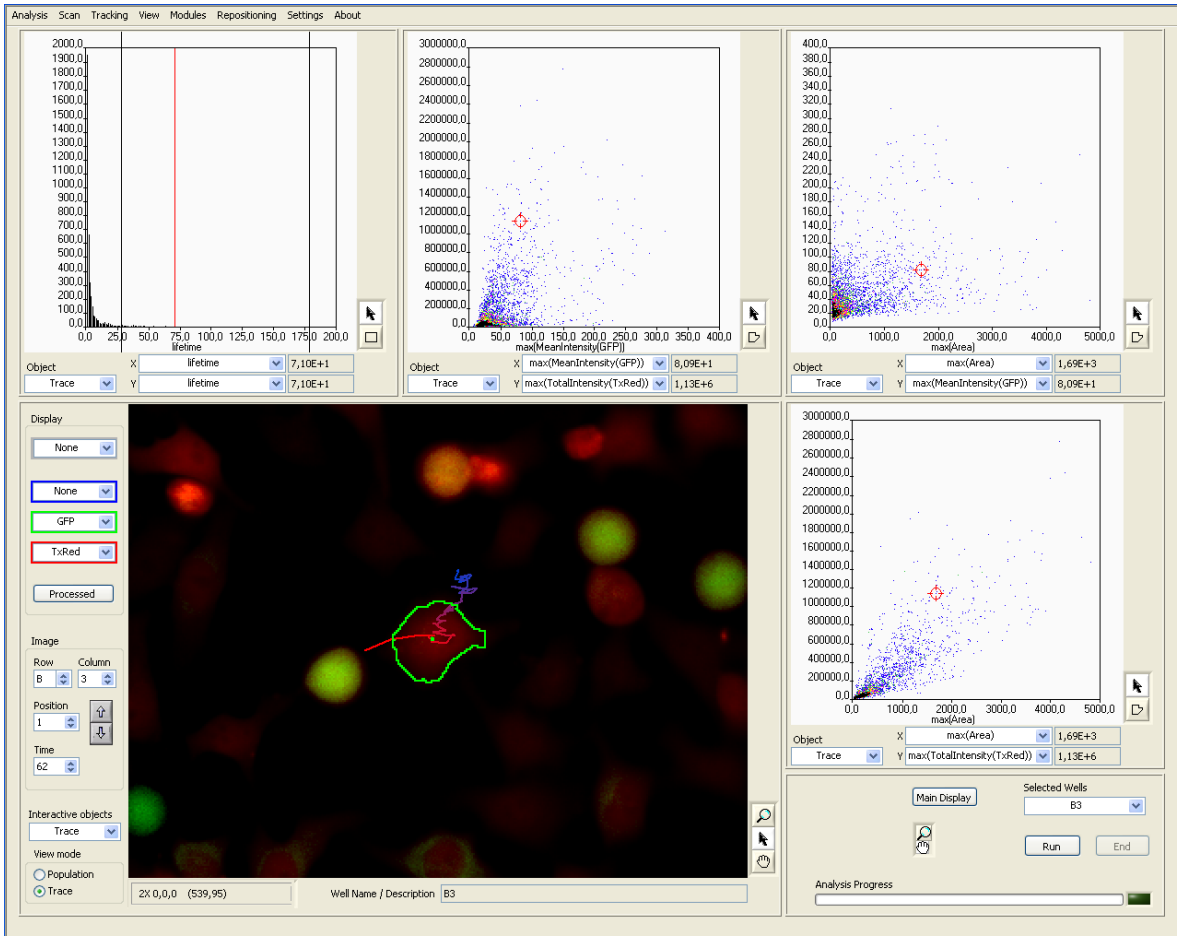
The result of the spectral unmixing is shown in the right display of the **3x3 Spectral Unmixing** dialog window.

Unmixing dual-labeled samples. In the case of two-channel images the third input channel will be occupied by one of the two available channels. Click **Ignore stain** to ignore the third channel in order to properly unmix two-channel images.

3.9 Tracking – Analyzing Time-Lapse Data

The tracking function of scan^R Analysis allows the analysis of objects over the course of time, i.e., in experiments that consist of time-lapse acquisitions. It relates any object detected in an image to the same object in the previous and subsequent images in the time-lapse series acquired at the same stage position. Thus the change of the parameters that are measured according to the assay settings can be followed over the course of time.

To enter the **Trace View** select **Trace** in the lower left part of the front panel or in the menu bar select **Tracking ▸ Trace view.** The front panel display changes such, that the displayed objects change from **Main** to **Trace** and also the available X/Y-parameters in the histograms are changed according to the parameters that are defined in **Tracking ▸ Define Parameters** (See Chapter 3.9.2.1, *Original Parameters*).



When clicking on one tracked object in the image now not only the object is highlighted with a green border but also the trace it covered during acquisition will be displayed. With time the object moves from the blue end of the line to the red end. Like in the **Population View** the detected object and the corresponding data points in the histograms are directly linked.

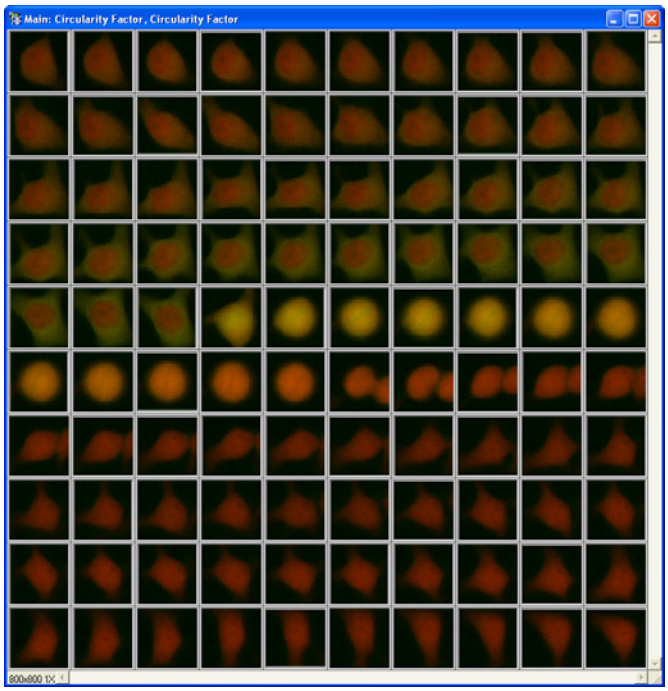
Right-clicking on an object in the image yields the following context-menu:

Save as … This allows you to store the displayed image separately.

Show Gates. It has the same function as in the **population view**. When you select a gate from the list, the objects that fall into the gates are marked with a box in the front panel image.

Show Trace. This opens the Trace Viewer which displays the time-curve for the selected object. (See Chapter 3.9.3, *Trace Viewer*)

Gallery. it displays a time-gallery of the selected object.



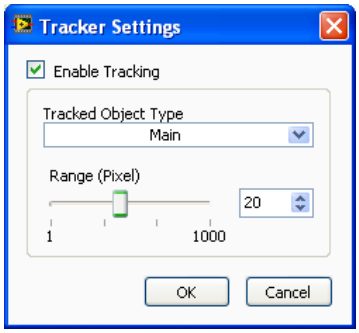
Gallery display in time-lapse mode

Movie. This opens the menu **Trace Movie** which allows you to export a movie of a single **trace** or a complete **position.**

3.9.1 Tracking Configuration

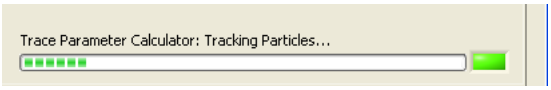
The **Tracking ▸ Configure Tracer** command opens the **Tracker Settings** window. It also opens automatically when the **View mode: Trace** is activated without any tracking parameters being set already.

Tracked Object Type. Select here the kind of objects to be tracked, i.e. main objects or any of the sub-objects – if such are defined in **Assay Settings ▸ Sub-objects**. (See Chapter 3.3, *Sub-object Finder: Detecting Sub-objects*.)



Range (Pixel). Set here the maximum difference that is allowed to change between two frames in order to maintain a track. If the difference exceeds the **Range** an object will NOT be related to similar objects in the previous and subsequent images of the time-series.

OK. Click here to start the tracking. The advance can be followed in the status bar at the bottom right corner of the scan^R Analysis main interface. Once it is completed the **Trace Parameters** window opens.



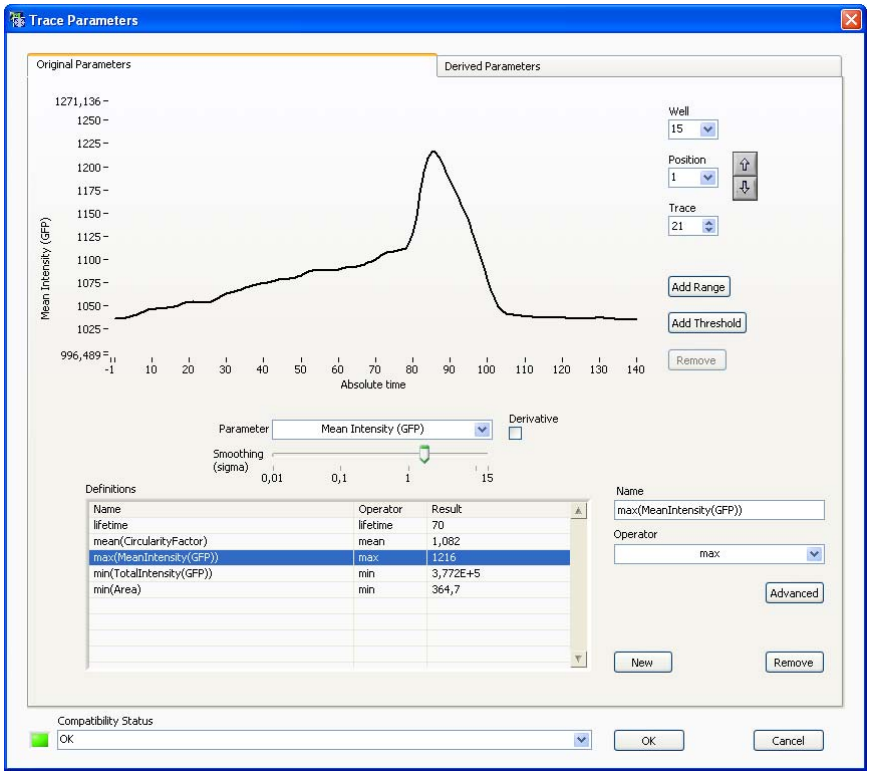
3.9.2 Track Analysis Parameters

During the tracking is run not only the X/Y-positions of an object over time are calculated, but also the time course of the parameters previously determined in **Analysis ▸ Edit Assay ▸ Parameters** (see Chapter 3.5, *Measurement Parameters*) is extracted of the data. The curves for each individual object are displayed in **Trace Parameters ▸ Original Parameters.** This menu allows defining which kinetic parameters of these curves are extracted, e.g. the maximum intensity, the time of maximum intensity or the duration of an increase in signal…

3.9.2.1 Original Parameters

Open the **Trace Parameters ▸ Original Parameters** window directly via **Tracking ▸ Define Parameters**. At startup it shows the kinetics graph of one of the measured parameters of the first track in first stage position of the first well acquired and selected for analysis. (See Chapter 2.7, *Selecting Wells for Analysis.*)

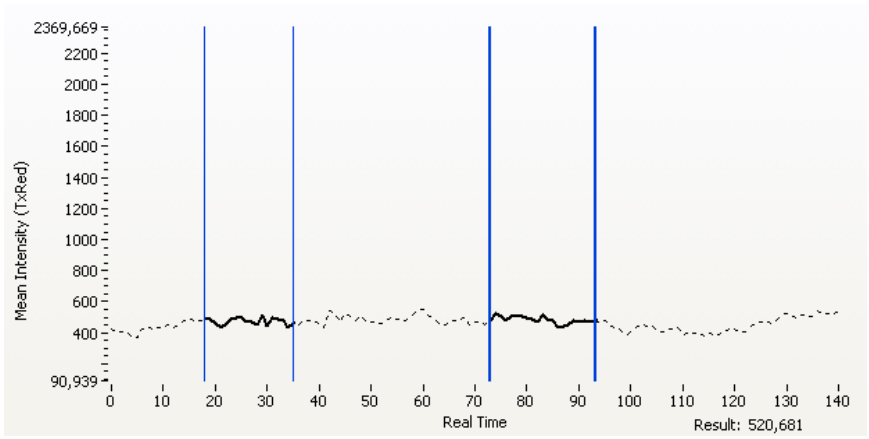
To navigate through the curves use the **Well**, **Position**, and **Trace** selectors on the right. Each **Trace** represents the object in subsequent time frames that the tracer, according to the settings in **Tracking ▸ Configure Tracer** detected as belonging together. In the **Original Parameter** menu you can set the **Operators** that are applied to the time curves to quantify the time curves). By applying an **Operator** (min, max, std, first, etc.) the time-curves again are reduced to a single values per curve that in turn can be displayed in a 1-D or 2-D histogram. For example when **TotalIntensityGFP** was selected in the **Parameter** tab of **Edit Assay**, the time curve of **TotalIntensityGFP** can now be plotted. By applying the **Operator max** on this time curve, for all traces the maximum of **TotalIntensityGFP** will be calculated.



Trace parameters.

Well, **Position**, **Trace**. You can navigate through the set of curves using these functions and their arrow buttons.

Add Range. Click here if you want to analyze just a part of the trace. Two vertical blue bars appear in the graph to define the upper and lower limit of the range. They can be moved via mouse-drag. The analyzed part of the curve is displayed solid. The remaining part is displayed as a dashed line. Click **Add Range** several times to create multiple ranges.



Two ranges applied to a trace

Add Threshold. Click here to open a menu which allows you set an upper or lower bound for the curve. A blue bar appears that marks the upper or lower part of the curve, respectively. The bar can be moved via mouse-drag.

Remove. Click here to remove the **Range** limits.

Parameter. Select the measurement parameter of which the time-curve is to be shown in the graph display.

Derivative. Check this option to calculate and display the first derivative of the kinetics time-curve of the selected **Parameter**.

Smoothing (Sigma). This function applies a smoothing filter on the track data. Set the strength of the smoothing with the slider. The graph display is being updated immediately.

Definitions. This lists the names of the values to be determined from the curves as well as the operators used. In the **Result** column the values for the selected trace for all parameters are listed.

Operator. Select an operator from the shortlist. This will be applied on the currently selected **Parameter**. For example, if *mean* is applied on the *Mean Intensity* of the tracked objects the time average of the mean intensity of each object will be calculated.

The available Operators are:

- **Lifetime.** Gives the number of contiguous time points the particle is detected
- **Sum.** Calculates the sum of the parameter value over the time
- **Mean.** Calculates the mean value of the parameter
- **Min.** Takes the minimal value the parameter reaches
- **Max.** Takes the maximal value the parameter reaches
- **Std.** Calculates the standard deviation of the values
- **First.** Takes the value of the parameter at the first point of the curve
- **Last.** Takes the value of the parameter at the last point of the curve
- **T_max.** Takes the time point when the parameter is maximal
- **T_min.** Takes the time point when the parameter is minimal
- **T_first.** Gives the time point when the trace starts
- **T_last.** Gives the time point when the trace ends.
- **Num_zero_crossings.** Gives the number of zero crossings of the curve
- **Num_local_max.** Gives the number of local maxima of the curve
- **Num_local_min.** Gives the number of local minima of the curve

Name. The name of the analysis function is set automatically as <Operator>(Parameter). It can be changed manually.

New. Click here to create a new list entry.

Remove. Click here to delete the selected list entry.

Advanced. Gives advanced options. The **Derivative** checkbox is replaced by a menu that allows you to apply **Anti(-)/Derivatives(+)** of higher order. In the **Operator** shortlist a new option becomes available: the **curve-fit**.

Curve-fit. This **Operator** allows you to fit certain models to the parameter curves. Upon selecting **curve-fit** from the **Operator** drop-down menu the **Define**-button becomes accessible.

Define. This opens a menu to set the options for **curve fitting**.

> As objects might move in or out of the focal plane, or the value entered for **Range** might be set too small, there might be a large number of short traces. Therefore **Lifetime** is a useful parameter as it allows setting a gate on long traces.

3.9.2.2 Curve Fitting

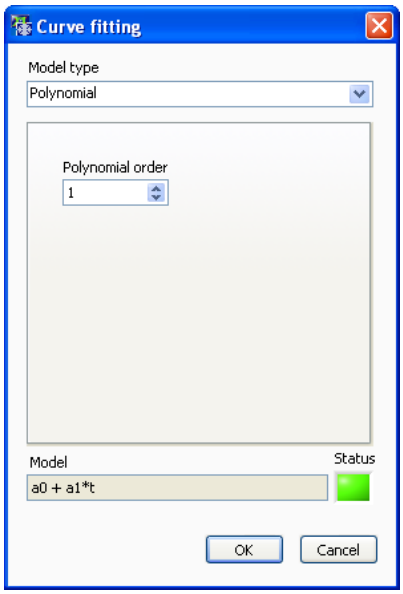
Model type. Select the type of model that is to be fitted to the curves. You can select **Polynomial**, **Exponential**, **General ls linear** and **non linear.**

Polynomial. The only parameter to be set is the **Polynomial order**. E.g., the model function is $f(t)=a_0+a_1 \cdot t+a_2 \cdot t$ for **Polynomial order** of 2. In this example the parameters a_0, a_1 and a_2 are fitted to the curves.

Exponential. No parameters have to be set. The model function is $f(t)=a \cdot exp(ct)$. The parameters a and c are fitted to the curves.

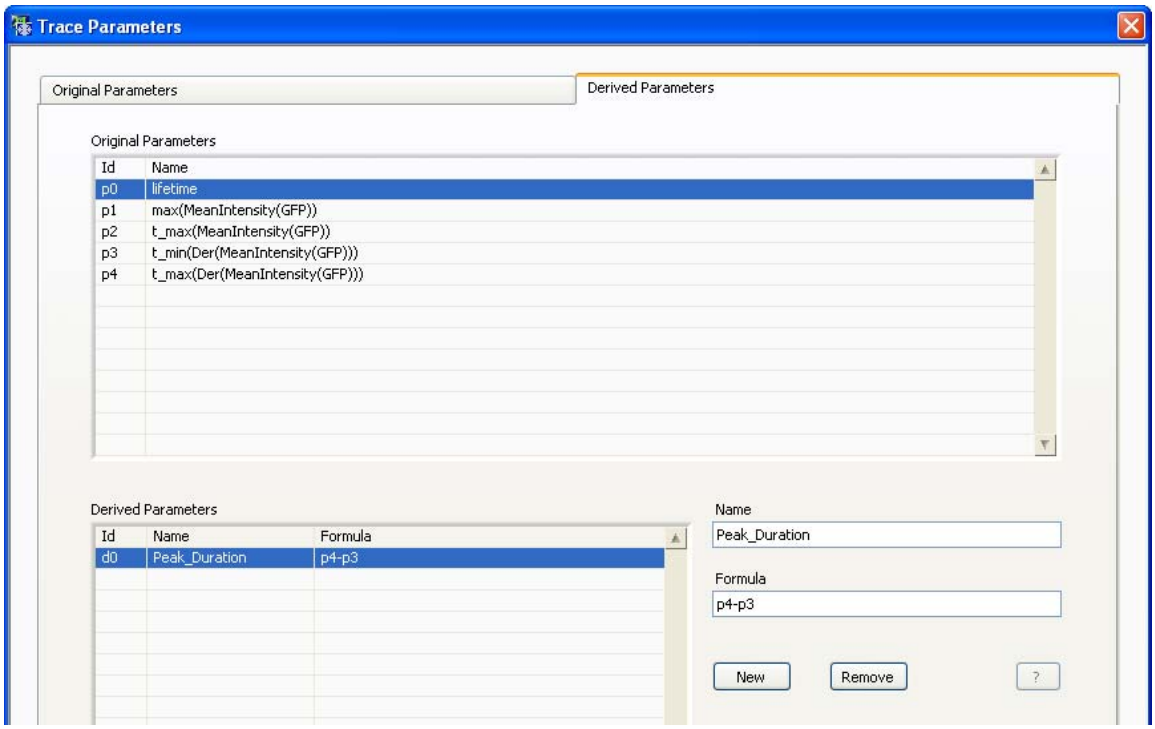
General ls linear. General linear least-squares fit. Here you can enter arbitrary **Basis functions** (linear, exponential, trigonometric,…) which will be combined linearly. E.g. set $f_1(t)=17 \cdot t+4$ and $f_2(t)=exp(-(t+2))^2$ will be combined linearly to $f(t)=a_0 \cdot f_1(t)+ a_1 \cdot f_2(t)= a_0 \cdot (17 \cdot t+4)+ a_1 \cdot exp(-(t+2)^2)$. This function represents a linear combination of a line and a Gaussian. The parameters a_0 and a_1 are fitted.

Non linear. Here you can enter an arbitrary model function to be fitted with up to 6 parameters. You have also to enter the start values as **First guess**. E.g. if you want to fit a Gaussian you set $f(t)=a \cdot exp(-(t-b)^2/(2c^2))$ and enter start values for *a*, *b* and *c*. You can also set the number of iterations to be run by entering a value for **Max iterations**.



The parameters that are fitted (e.g. a_0, a_1,…) and the mean squared error of the fit (mse) are listed in the **derived Parameters** tab in the following representation: curve_fit<*modelfunction*>(Parameter).a0, curve_fit<*modelfunction*>(Parameter).a1, …, curve_fit<*modelfunction*>(Parameter).mse.

3.9.2.3 Derived Parameters



The **Trace Parameters ▸ Derived Parameters** tab allows to perform calculations with the parameters listed in the **Definitions** list described above by the use of basic algebraic expressions (+, -, ×, ÷, sqrt, ^ etc. For a complete list, see Appendix 6.4).

Original Parameters. This is the list as created on the **Original Parameters** tab and includes also the fitted parameters when the operator **curve_fitting** is applied.

New. Click here to create a new list entry.

Name. Set the name of the new **Derived parameter** here.

Formula. Set the formula here using the **ID** of the original parameter(s) and the algebraic expression.

Example.

Imagine you want to measure the duration of a peak. It starts with a pronounced increase in intensity and ends with a pronounced decrease. Mathematically this would be the time between a maximum and a minimum in the first derivative of the Mean Intensity curve. The Original Parameters needed are thus time of the maximum (t-max) and time of the minimum (t-min) of the Parameter Mean Intensity with the option Derivative. The formula for the Derived Parameter would thus be p4-p3 when using the entries as shown in the screenshot.

3.9.3 Trace Viewer

Open the **Trace Viewer** by selecting **Tracking** ▸ **Show Traces** in the main menu. Alternatively you can right-click on one detected object in the image in the **Trace View** and select **Show** Trace to see the trace of this individual object. Set the number of displayed traces with the slider on the right. To display only objects of a certain gate that was previously defined in the **Trace View** this gate can be selected in the **Gate** drop-down menu.

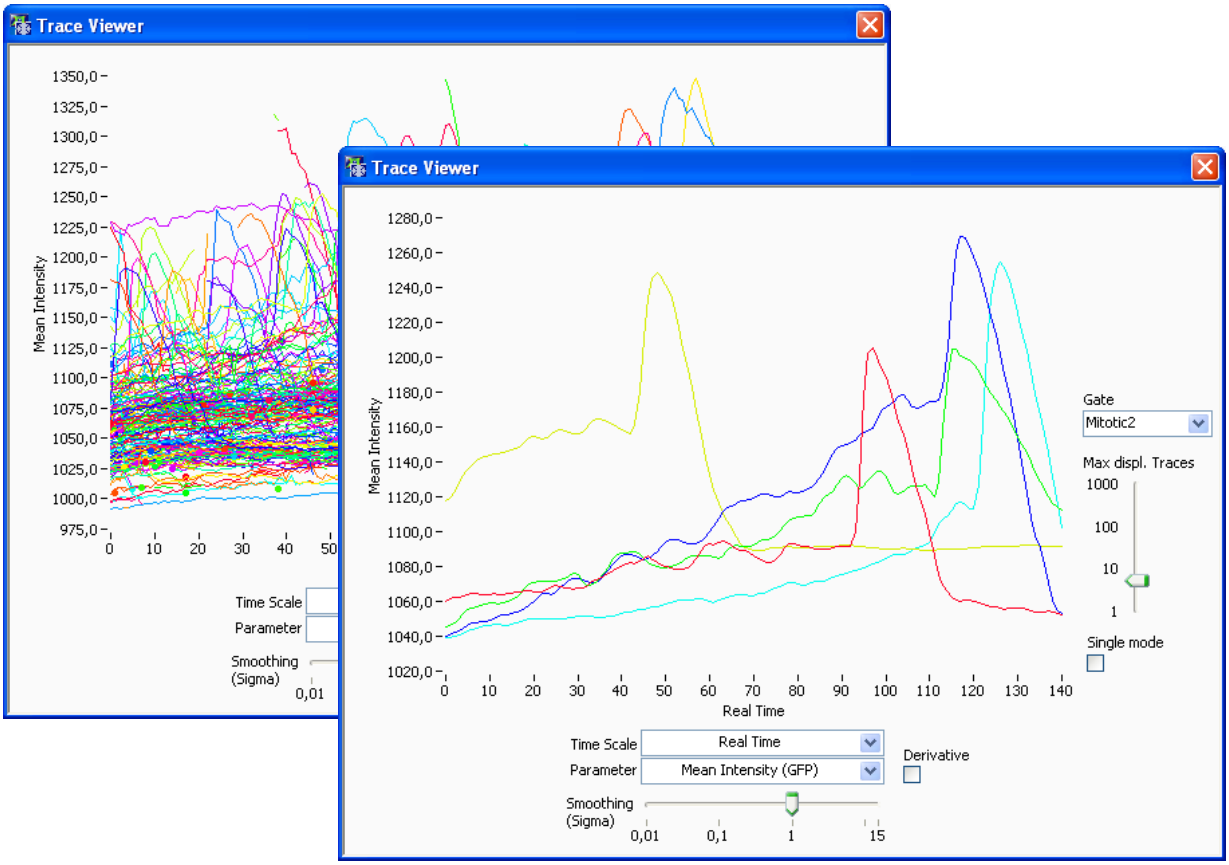
The results of the tracking are displayed in the **Trace Viewer** window once the tracking has been carried out.

Max displ. traces. Use the slider to adjust the number of displayed. The traces will be taken from a random selection of the specified gate.

Single mode. Use this option to have just one trace displayed. If a trace is selected via mouse click it will be this one that is being displayed.

Gate. Select a gate to have only the corresponding traces displayed.

Time Scale. *Real Time* is the default and causes that each point in time is set relative to the beginning of the time-lapse series. When *Trace Time* is selected each point in time is set relative to the beginning of each track when **t_first** is selected, which may be important if an object was not tracked right from the start of the time-lapse series and one is interested especially in the data at the beginning of each track. Alternatively, when applying the operator **t_max** e.g. on the parameter MeanIntensity(GFP) the time point when the mean intensity of GFP is maximal is set to be 0. This way the time-curves can be displayed “synchronized”.

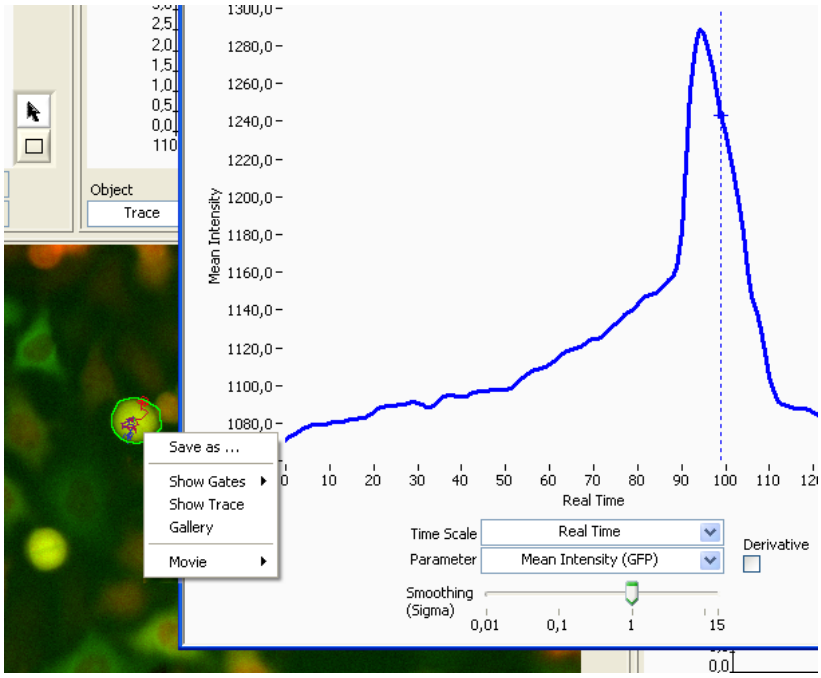


Parameter. Select parameter of which the time-curve is to be shown in the graph display.

Derivative. Check this option to display the first derivative of the curve of the selected **Parameter**.

Smoothing (Sigma). This function applies a smoothing filter on the .time-curve. Set the strength of the smoothing with the slider. The graph display is being updated immediately.

> **Selecting objects and navigating along tracks:** Tracks in the **Trace Viewer** and the objects in the images they derive from are directly linked. Upon clicking on a time-curve the corresponding image with the marked object will be shown in the image display of the main scan^R Analysis window. Also the data points marked with a red circle in the histograms are linked to the time-curves. Likewise, upon clicking on an object in the image display or in a scatter plot the corresponding time-curve will be shown in the Trace Viewer.
>
> A dashed vertical line marks the current point in time in the Trace Viewer. Upon moving the cursor left and right while keeping the mouse clicked one can thus scroll back and forth through the "movie" of the time-lapse series.



4 Analysis Results

This chapter describes how an analysis is executed, how the results can be grouped into object populations and how these can then be analyzed and statistically evaluated.

4.1 Running an Analysis

1. Execute **Scan** ▸ **Open…** and select the **Experiment Descriptor.xml** file in the storage folder of the scan. By doing so the experimental settings are loaded and the first image of the scan (taken at the first position of the first well) will be displayed. If you want to modify or revisit an analysis execute **Analysis** ▸ **Open…** instead.

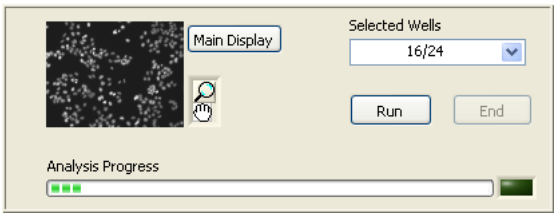
2. Execute **Analysis** ▸ **Load Assay…** to read in a ***.say** assay file stored in the **scan^R Analysis/Assays** folder. If you want to modify the assay file of the current analysis execute **Analysis** ▸ **Edit Assay…** instead.

3. Set or modify the parameters in the assay as described in Chapter 3, *Assays.*

4. If you want to perform the analysis on a subset of the wells, open the **Select Wells** window via **Scan** ▸ **Select Wells…** and select the wells of interest.

5. In order to observe the analysis results online prepare the four histogram windows by selecting the objects and measurement parameters to be displayed.

6. Click the **Run** button (or execute **Analysis** ▸ **Run**) to start the analysis. You can follow the progress of the analysis in the window at the bottom right of the main scan^R Analysis interface.



The window shows the **Analysis Progress** and displays the image that is currently being analyzed in the thumbnail display. Click the **Main Display** button to have this image displayed in the main mage window. You may **Pause** or **End** the analysis by clicking the respective buttons.

Once the analysis is completed store the results via **Analysis** ▸ **Save as…**

4.2 Managing Gates

scan^R Analysis detects objects in all images acquired during a scan and performs measurements and analyses on each object found. The data can be displayed in form of histograms where each color-coded data point represents the results of one object – or of several objects that happen to have identical results. The results – and thus the objects – can be grouped by **Gates** and **Regions**. The Gates allow classifying the objects according to their properties, i.e. the parameters that were extracted for these objects. Once these gates are defined, they can be applied to all further measurements for automatic quantification. The gates are administered in the **Gate Manager**, here you find also access to the **Well Results** menu which contains the detailed results for all wells.

4.2.1 Gates and Regions.

scan^R distinguishes between **Gates** and **Regions**. **Regions** define classification rules in form of polygons or ranges in histograms. **Gates** are composed of one such region or of several regions that are linked with Boolean operators (AND, OR,, AND NOT). Once a **Gate** is set, only data points within its boundary are being considered in further steps.

Gates and **Regions** can be created using the drawing tool beside each scatter plot. A **Region** can be defined as **Gate** by selecting **Set Gate** in the histogram context menu or by defining a gate in the **Gate Manager**. (See Chapter 2.4, *Managing Histograms and Scatter Plots.*)

By clicking on the borderline the Region can be displaced. By clicking one corner of the region this corner can be moved in order to include or exclude data points.

Right-clicking on the borderline of a Region opens the **Region** context menu. (See Chapter 2.4.2, *The Region Context Menu*).

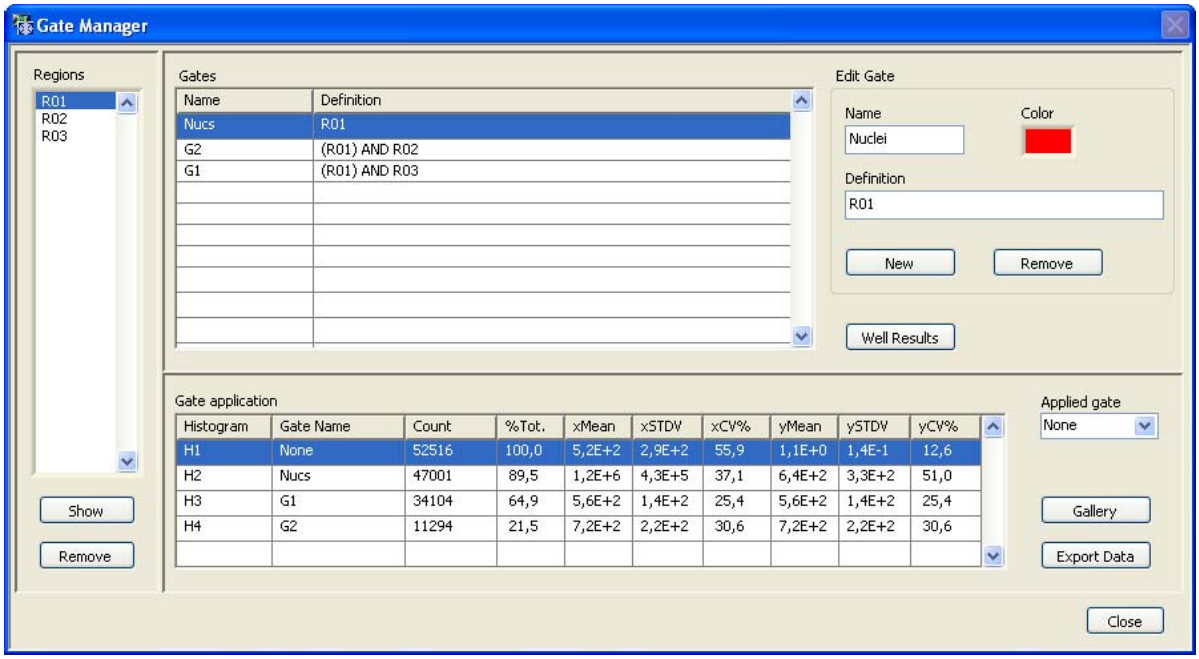
4.2.2 The Gate Manager

The **Gate Manager** allows converting **Regions** into Gates and to combine Gates by Boolean operations. The **Gate Manager** also gives an overview of the results in the histograms (H1 to H4) and the applied Gates.

The command **Analysis ▸ Assay Gating** opens the **Gate Manager** window that allows administrating the **Gates** and **Regions**.

Regions. This lists all regions drawn into the histograms.

Show. Click here to display the selected **Region** in the histogram selected in the **Gate application** list.



Remove (Region Box). This command removes the selected entry from the **Regions** list and deletes the defined Region.

Gates. It lists all gates with their **Names** and **Definitions**.

Name. Set the name of a Gate.

Color. Click into the box to open the dialog window to select the color of the object boundaries when displayed in the image viewer. This color is also used for color gating. (Compare Chapter 2.4, *Managing Histograms and Scatter Plots*.)

Definition. A **Region** or a Boolean combination (via “AND”, “OR” or “AND NOT”) of **Regions** can be set to define the **Gate**.

New. Click here to add a new **Gate**. The default **Name** and default **Definition** will be that of a region in the **Regions** list. The **Name** can be modified at will; the definition must be a region or a logical combination of regions.

Remove. This command removes the selected entry from the **Gates** list.

Gate Application. This table lists all histograms with the applied **Gates** and statistical information about the gated data. (Number of Objects (Count), Percentage of total number of detected objects (% Tot.), Mean Value, STDV and CV% for the parameters plotted along the X and Y-axis)

Histogram. Number of the histogram window in the scan^R Analysis front panel, H1 to H4, respectively.

Gate Name. **Gate** applied to the histogram.

Count. Number of objects in the histogram.

%Tot. Amount of objects in percent of the total objects.

xMean (yMean). Mean value of the abscissa (ordinate) parameter of all objects in the histogram.

xCV (yCV). Standard deviation of the abscissa (ordinate) parameter of all objects in the histogram

xCV% (yCV%). Coefficient of variation of the abscissa (ordinate) parameter of all objects in the histogram

Applied Gate. This command allows changing the applied gate in the selected histogram. The pull-down menu lists all gates from the **Gates** table.

Gallery. This command generates an image gallery of all objects in the selected histogram. The number of images is by default limited to 100. If the histogram contains more objects the 100 objects closest to the center of gravity of the region will be shown by default. (See Chapter 2.2, General Settings.)

Export Data: This command exports the data of the selected histogram as tabulator delimited table in txt format.

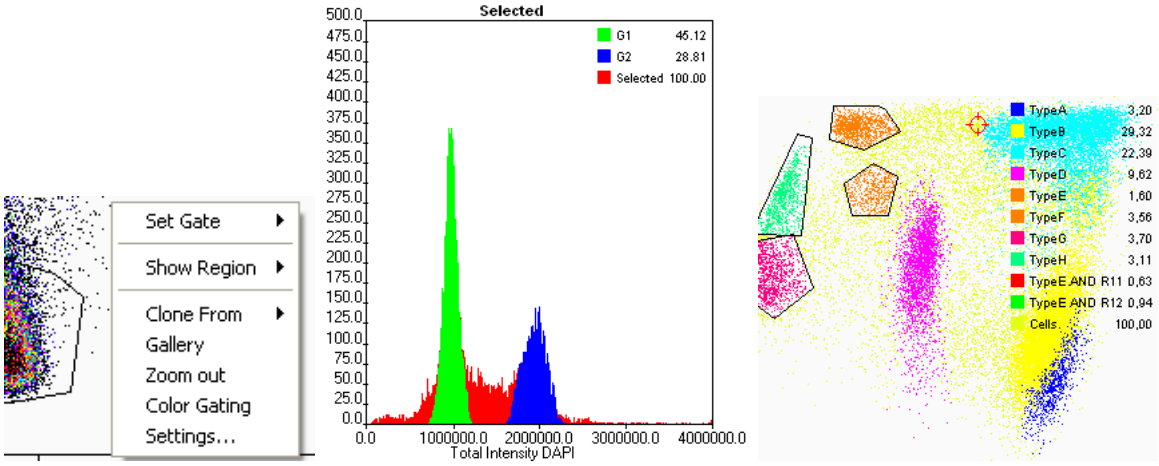
Well Results. This opens the **Well Results** window, see Chapter 4.3, *Well Results*.

4.2.3 Color Gating

Color Gating shows different populations as defined by the Gates in different colors in the one- and two-dimensional histograms. **Color Gating** is a property of each individual histogram displayed; thus, histograms with and without **Color Gating** can be displayed simultaneously in the different displays.

In order to activate **Color Gating** right-click in the histogram and from the context menu (right click) either activate the option **Color Gating** directly or open **Settings...** and there activate the checkbox **Color Gating.**

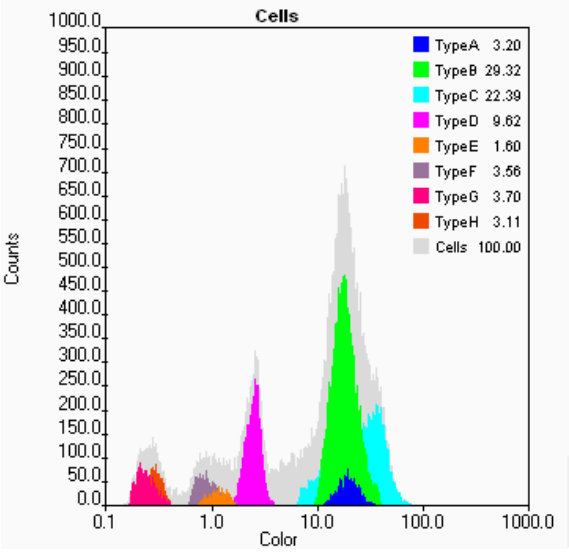
In the **Histogram Properties** window that opens via **Settings...** select **Show Legend.** This way you can additionally show the legend of the colors in the histogram. The population percentages of the displayed gates are also displayed.



The following rules apply to decide which color is shown in **Color Gating**:

- If there is only one object located on a pixel (Bin) the gating color of that object is shown.
- If there are multiple objects on a histogram pixel the gating color of the relative majority of the objects will be shown. Exception: If there is a Gate applied to that Histogram that Gate will be automatically in the background as 100% of the objects will belong to that Gate.

- Objects not belonging to a gate are shown in black.
- If there is no majority Gate the Color order of the Gating list will be used (**Analysis ▸ Assay Gating…**; see Chapter 4.2.2, *The Gate Manager*). The "ranking" can be changed with the arrow buttons in that window.
- If a histogram is gated (context menu: **Set Gate**) the objects will be shown in the color of the sub-gate they belong to.



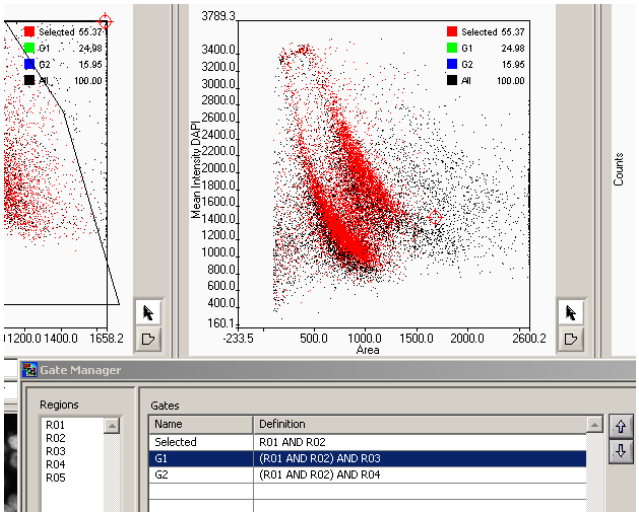
The order mechanism of Color Display (1-D):

- In 1-D histograms the applied Gate is always in the background.
- The order of display is organized by areas. If one of the Gates has less area above the other one it will be shown in the foreground.

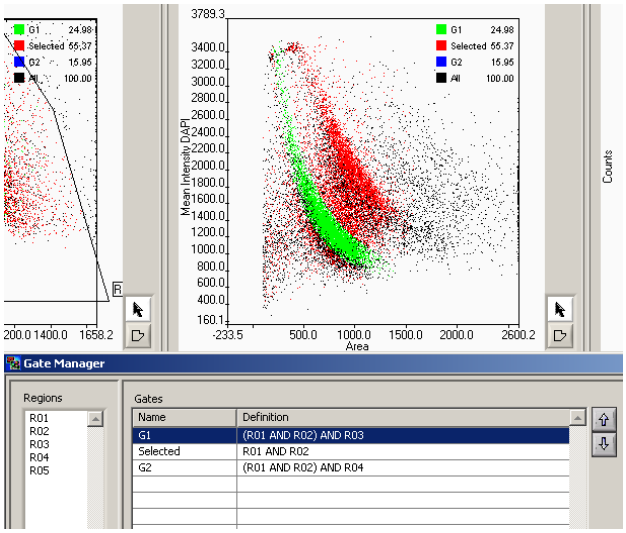
> The selector line is now position sensitive in 1-D Histograms as well. Thus if you click on a green area you will get an object from the green Gate, if you click on gray you will get one from the gray Gate.

Examples:

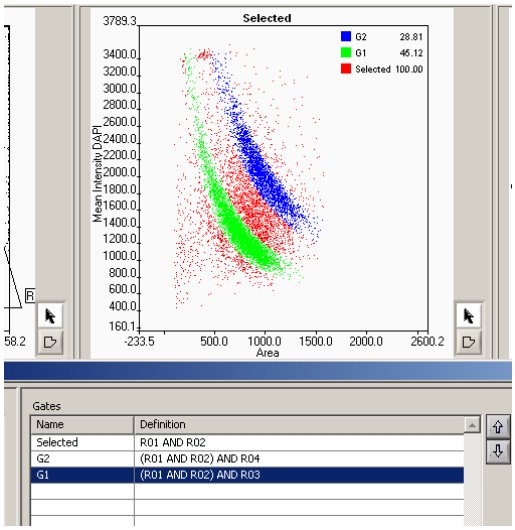
1. The example below shows a cell cycle with G1 and G2 defined as gates with different colors. The histogram is not gated, thus all detected objects are shown. Some of the pixels represent more than one object. In this case all pixels that have at least one object that belongs to the gate *Selected* are shown in red. Only the red color is shown because there is never a majority of G1 or G2 because G1 and G2 are subgroups of *Selected* and the order in the **Gate Manager** is *Selected* above G1 and G2. The other objects are shown in Black because they do not belong to any Gate.



2. In this example the situation is identical to the first example, but the order has been changed. All Pixels having a majority of G1 will now be shown in a green. All G1 objects also belong to *Selected* but when the same number of objects belongs to G1 and to *Selected* the color of the data point is determined by the list order and now G1 has a higher priority than *Selected*.



3. In the last example the order is changed again, but now also the gate *Selected* is applied to the histogram. Now the exception Rule 2) applies such that the *Selected* is not dominant.



4.3 Well Results

The **Well Results** window (via **Analysis ▸ Assay Gating**) shows the results of a complete scan analysis. Statistical results of different parameters are listed for gated populations of the individual wells or of groups of wells and are displayed graphically in a histogram. Additionally the results can be exported as txt-file for further analysis with other software.

4.3.1 Measurement Results

The **Measurement Results** tab lists all the wells recorded in one measurement and displays the results (Number of Objects, % of tot., Mean, Error, Error % and CV) of the measurement parameter which is selected in the drop-down menu **Measurement**. The result for a combination of several wells is given in the bottom line when several wells are selected (press shift and click the lines you want to combine).The results can be exported by **Export Table**. The graph on the right gives a graphical representation of the results.

Well/Group results. This table gives a statistical analysis of the measurement results for each well or group of wells. It lists the mean value of a parameter for the gated population in each well or group of wells as well as its error and coefficient of variation.

Objects. Number of objects belonging to the population of the selected **Gate**

% of tot. Relative size of the **Gated** population

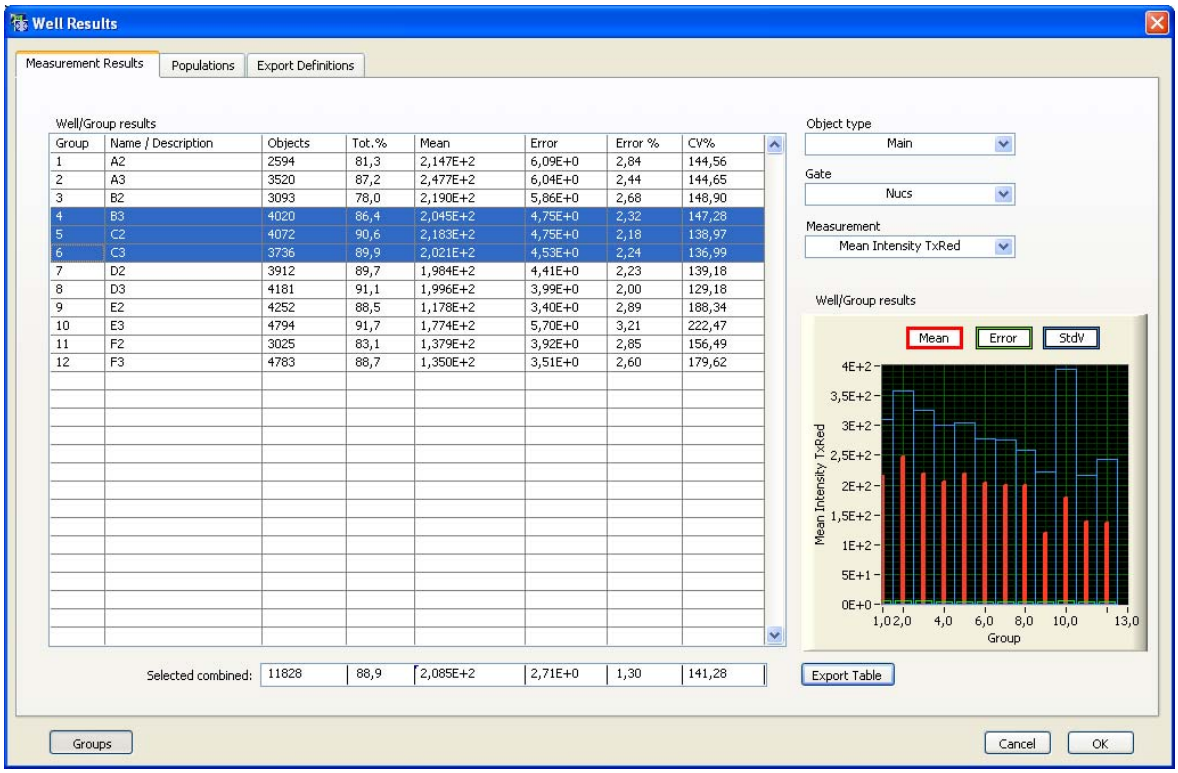
Mean. Mean value of the selected **Parameter**. It is shown in red in the histogram.

Error. Absolute error of **Mean**. It is shown in green in the bar plot.

Error %. Relative error

CV%. Coefficient of variation of Mean.

StdV. Standard deviation of **Mean**. It is shown in blue in the bar plot.



Object Type. Select the object type from the shortlist (Main/Sub-objects).

Gate. Select a gate from the shortlist in order to get the values for the corresponding gated population.

Measurement. Select the parameter of interest from the shortlist.

Selected combined. Select a number of **Well/Group** entries (press shift and click in the lines you want to combine), their combined results will be given here.

Export Table. This function exports the data as tabulator delimited table in txt format.

Wells / Groups. Use this button to select if the values are to be given for individual wells or entire groups of wells. All wells that have the same **Name / Description** entry form a group. Wells that do not pertain to a group will be listed independently. The **Name / Description** is set in **Scan ▸ Select Wells** (see Chapter 2.7, *Selecting Wells for Analysis*).

4.3.2 Well Results: Populations

In the **Populations** tab the number of objects (absolute and relative) in all defined gates are listed. A reference gate can be set to the number of objects in all other gates to the reference gate. The histogram on the right gives a graphical representation of the results.

Size comparison. The table gives the amount of objects in absolute numbers or as percentages that pertain to each of the **Gates** defined in the **Gates Manager.** (See Chapter 4.2.2, *The Gate manager.)*

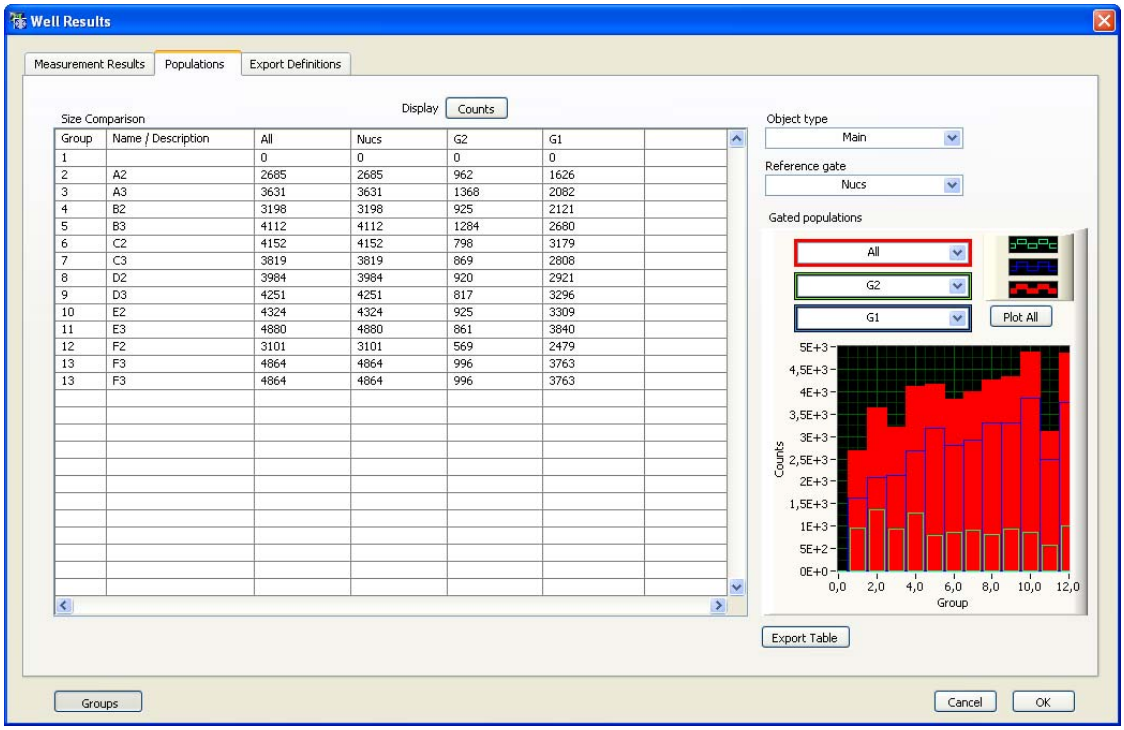
Display Counts / %. This toggle button switches between the listing of total numbers and the percentage of objects.

Object type. Select the object type from the pull-down list (Main/Sub-objects).

Reference gate. The population of the selected **Reference gate** is set relative to the populations of the other gates in the **Size comparison** table.

Export Table. This function exports the data as tabulator delimited table in txt format.

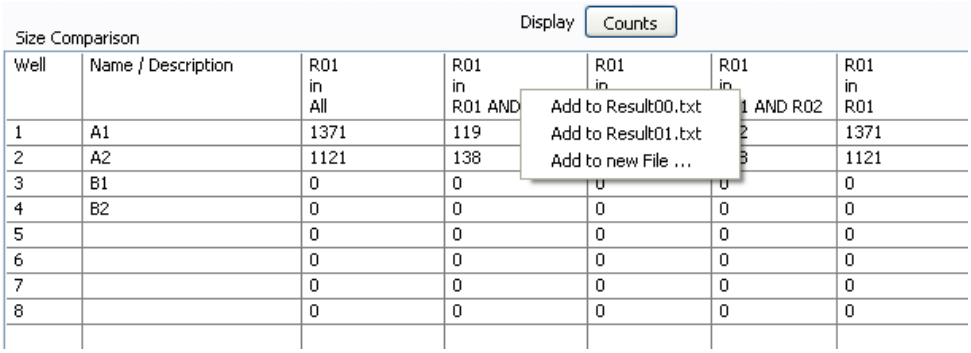
Wells / Groups. Use this button to select if the values are to be given for individual wells or entire groups of wells. All wells that have the same **Name / Description** entry form a group. Wells that do not pertain to a group will be listed independently. The **Name / Description** is set in **Scan ▸ Select Wells**. (See Chapter 2.7, *Selecting Wells for Analysis*.)



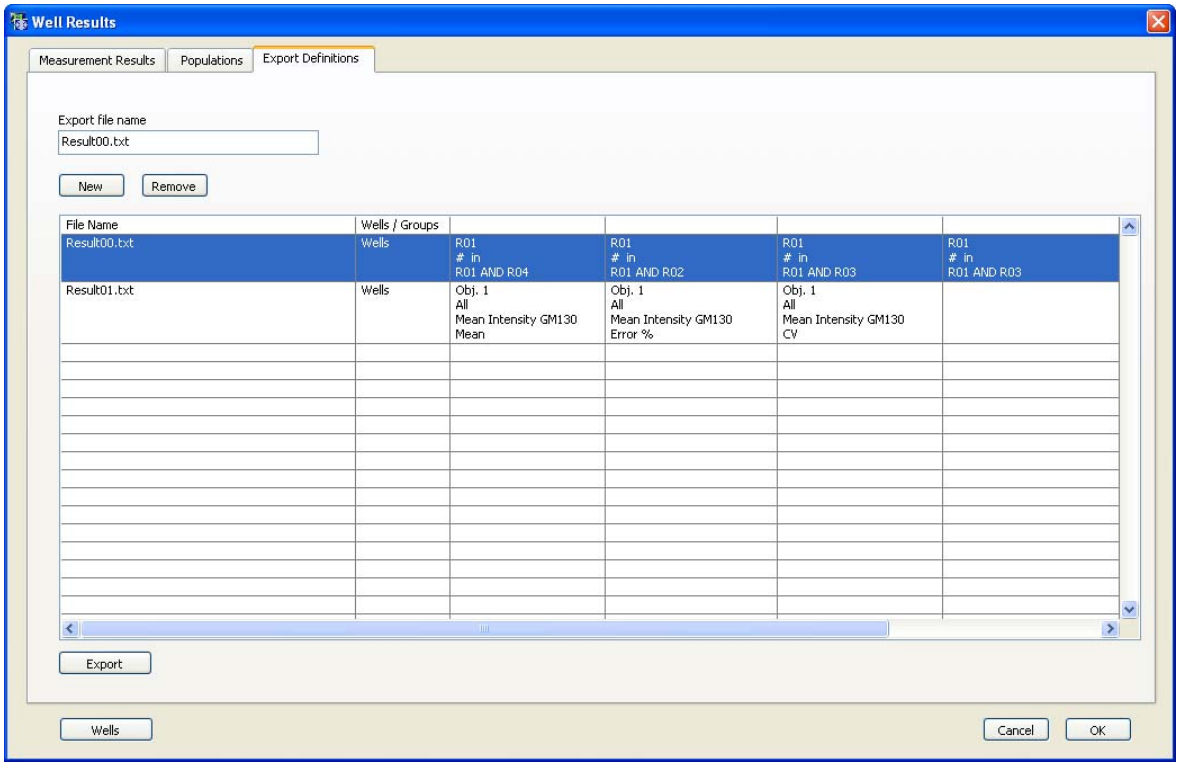
4.3.3 Export Definitions

In the **Export Definitions** tab the parameters that are to be exported can be defined. This is especially useful for batch analysis and if not the complete results tables given in the **Populations** or the **Measurements** tab is to be exported.

Select **New** to create a new export file. The parameters to be exported have to be selected in the other tabs, i.e. in the **Populations** tab or in the **Measurement Results** tab. When you right-click in these two tabs on the column names a context menu will open which allows you to export the selected column to a tab-delimited .txt-file.



Well	Name / Description	R01 in All	R01 in R01 AND	R01 in	R01 in 1 AND R02	R01 in R01
1	A1	1371	119		2	1371
2	A2	1121	138		3	1121
3	B1	0	0	0	0	0
4	B2	0	0	0	0	0
5		0	0	0	0	0
6		0	0	0	0	0
7		0	0	0	0	0
8		0	0	0	0	0



New. Creates a new tab-delimited .txt-file for export.

Export file name. Enter the name for the txt file here. The default is Result0*.txt

Remove. Removes the selected File and its selection

Export. Allows setting set the folder where the .txt-files are stored manually. Otherwise the folder given in **Settings** (see Chapter 2.3, *General Manager*) is used.

Wells / Groups. Use this button to select if the values are to be given for individual wells or entire groups of wells. All wells that have the same **Name / Description** entry form a group. Wells that do not pertain to a group will be listed independently. The **Name / Description** is set in **Scan ▸ Select Wells**. (See Chapter 2.7, *Selecting Wells.*)

4.3.4 Export results of individual objects

Analysis ▸ Export Table: exports the values determined for each detected object to a spread sheet. The values exported depend on the active view. When also sub-objects are detected not only one file is exported but for every sub-object a separate list is exported. For the “population view” the list contains for example:

ParameterData_Main.txt

ObjectID	MeanIntensityDAPI	Obj.1Counts	ParentObjectID	ParentTraceID	R01
0	174,52045	NaN	-1	0	0
1	295,50986	2	-1	1	1
2	220,76321	1	-1	2	1
3	161,89612	NaN	-1	3	0

ParameterData_Obj. 1.txt

ObjectID	Total Intensity GM130	Area	Parent Object ID	ParentTraceID	R01
0	201294	608	1	1	0
1	2504	12	1	1	0
2	2232	12	2	2	0
3	41191	108	4	4	0
4	4860	18	4	4	0

- Object ID. ID of the detected object
- Parameters. (e.g., MeanIntensityDAPI, Area,….)
- Derived Parameters.
- Parent Object ID. -1 if it is the main object, if not the ID of the main object is given
- ParentTraceID. if tracking was performed the ID of the Trace the detected object belongs to is given. If no tracking was performed the Trace ID is set to -1.
- Gates. If the object belongs to the listed gate the value is set to 1, if not it is set to 0.

For the “Trace-view” the list contains:

TraceID	lifetime	max(Area)	TrackedObjType	TraceLength	FirstParticleID	LastParticleID	R01
0	1	31	0	1	0	0	0
1	15	78	0	15	1	1940	0
2	8	626	0	8	2	1041	0
3	19	849	0	19	3	2640	0

- Trace ID. The ID of the Trace
- Parameters of the Trace (e.g. lifetime, max(Area),…)
- Derived Parameters.
- Traced Obj. Type. (0=main object; 1, 2, 3,…. Sub-object type)
- Trace Length. Number of objects in subsequent time frames belonging to the Trace
- First Particle ID. ID of the first object belonging to the Trace
- Last Particle ID. ID of the last object belonging to the Trace
- Gates. If the object belongs to the listed gate ate the value is set to 1, if not it is set to 0.

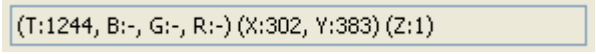
5 Example Assay – Step by Step

This chapter guides you step by step through the setup and execution of an assay and the analysis of the results.

5.1 Setting-up and Executing an Assay

The example scan which is used here to guide you through the setup and execution of an assay consists of images taken from cells labeled with DAPI to mark the nuclei and with FITC to mark the microtubules. A repair protein which is active in late G2 phase stained with TexasRed.

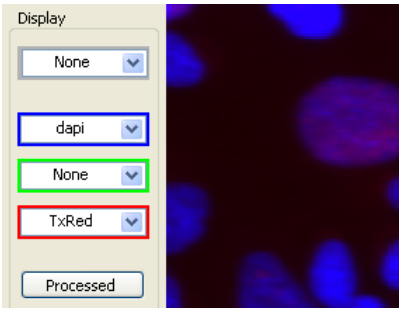
1. Execute **Scan ▸ Open…**, navigate to the folder where the demo data are stored, select the experiment_descriptor.xml file (/…/scan^R_Demo_Data_DVD_20070216/Slabicki_Buchholz _MPI-CBG/H2AX_PFA_002/experiment_descriptor.xml; sample data kindly provided by F. Buchholz, MPI-CBC, Dresden) and open it with **OK**.
   scan^R Analysis loads the first image of the scan, i.e., the image acquired at the first position of the first well in the main window of the font panel. Only the first channel of the image will be shown at first and in gray scale. Move the cursor over the image as a test and observe the status bar at the lower left. The channel intensity of each channel at the pixel positioned at the tip of the cursor arrow. Is shown in the status bar. The intensity values of the objects are usually much higher than the camera offset; the dark intensity of the camera.

   Memorize the intensity values of the brighter structures in the image for the subsequent display adjustment.

---

2. Set the grayscale selector to None, and load the dapi channel in the blue selector and the TxRed channel in the red selector.



---

3. Execute **View ▸ Layout** and adjust the display as explained in Chapter 2.5, *Using the Image Viewer*.

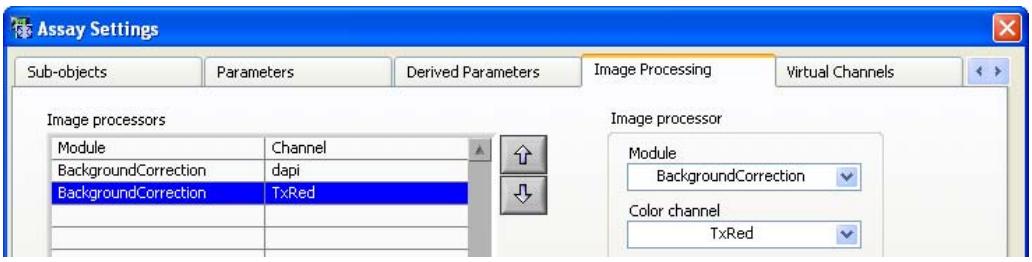
---

4. The first step in setting-up the assay is to find the best settings for the object detection. If you would have opened an old analysis file (*.sca), rather than the scan data only, the histograms would already show the data points of the analysis that was run before. In order to get these results from-scratch you first have to set up a new assay to perform a background correction, determine main (and sub-) objects, and the parameters you want to extract from the data.

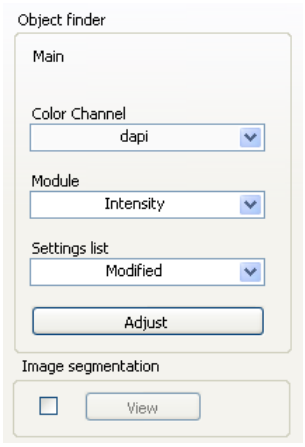
---

5. Execute Assay ▸ Edit Assay to open the Assay Settings window.

6. The first step in Analysis is to select the *Background Correction* **Image processor** Module on the **Image Processing** tab for each of the image channels. Press **Adjust** and observe in the new window the background in the image before (left) and after (right) background correction. When moving the mouse over the pixels the grey value should be reduced in the corrected images.

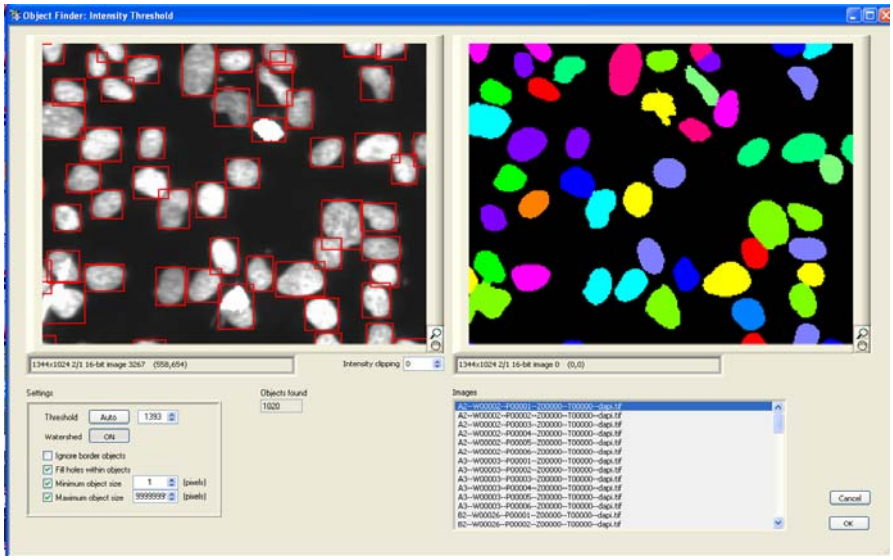


7. The next step is to determine the main objects. Therefore open the **Main Object** tab in the **Assay Settings**. Here you have to select the channel in which you want to use for image segmentation. In many typical cases this will be the channel with the DAPI-labeled nuclei, the shape of which makes them rather ideal targets for object detection.



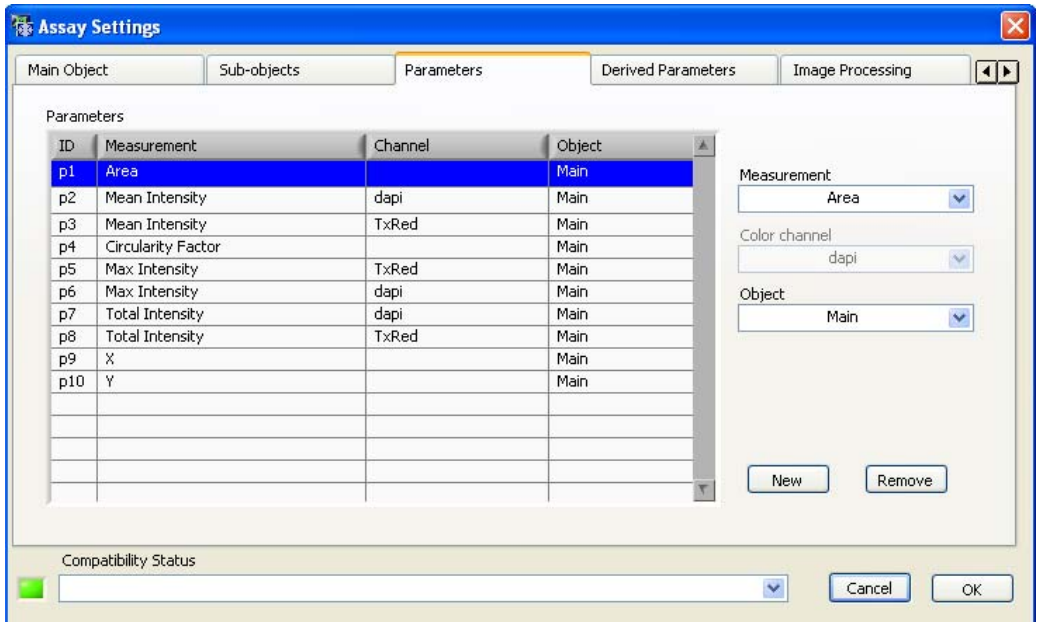
8. For image segmentation use the **Intensity** module. Both the **Edge** and the **Intensity** modules usually give good results when detecting nuclei. In other cases this is mostly a question of experience and testing. Click **Adjust** to open the **Object Finder** window. Here you can check and adjust the settings of the intensity detection algorithm. You will already get a good result by simply pressing the **Auto** button for determining the right threshold value and turn the **Watershed** algorithm **ON**. You can observe the effects of the parameters in the right panel where the result of the segmentation algorithm is shown.

9. If **Sub-objects** are associated with the **Main Objects**, check and adjust their detection in the same way on the corresponding tab in the **Assay Settings** window. In this example skip the **Sub-objects** tab.



DAPI-labeled nuclei Detected objects

10. In the **Parameter** tab you can choose which parameters will be calculated during the analysis, i.e. you need to select the measurements that are to be carried out. Typical measurements are area, mean and total intensity and the circularity factor. Area (size) and circularity factor are extremely helpful as they allow to discriminate between more or less round, single nuclei and impurities or objects that consist of clustered, non separable nuclei. The latter are usually much larger and less circular. If sub-objects are to be detected it is useful to add the parameter *Obj. 1 Counts* to the list to determine how many sub-objects are connected to each main object.

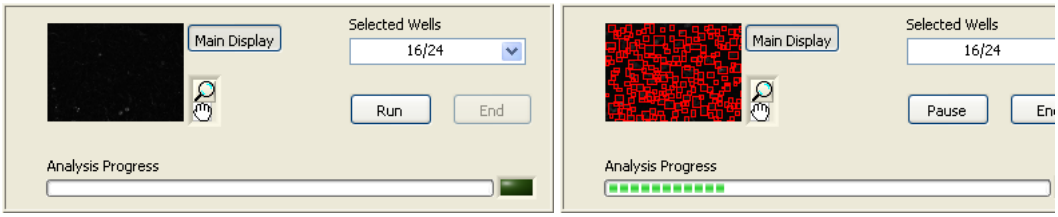


11. The **Derived Parameters** tab allows you to perform calculations on the previously defined parameters, e.g. here you can calculate rations between different color channels. (For example you could evaluate mean Intensity Dapi / mean Intensity TxRed).

12. Now you have set up the analysis. Press **OK** to exit the **Assay Settings** menu. You can save your assay with **Analysis ▸ Save assay…**

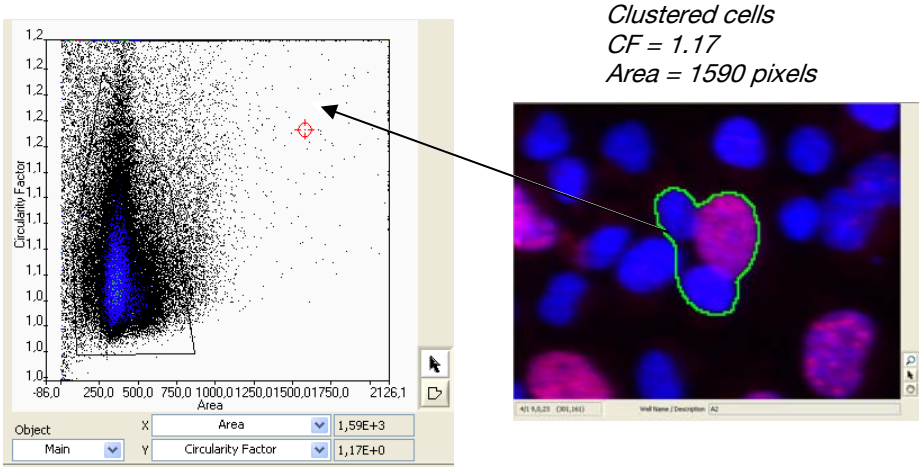
13. Start the analysis by clicking on the **Run** button.

14. Prepare the histograms in the main window if you want to follow the analysis online. It is suitable to select the parameters **Area** and **Circularity Factor** in the first histogram and to display **Total intensity dapi** vs. **Mean intensity TxRed** in the second histogram. The small image window left to the main window shows the main object recognition in the image that is currently being analyzed. By clicking on the **Main Display** button you can have it displayed in the largeimage window.



5.2 Analyzing the Data

1. The first thing to do is to get an overview of the detected main objects, in our example the cell nuclei. The nuclei should all have a similar size and a more or less round shape, i.e. a Circularity Factor (CF) close to 1.0.
Set **Area** as X-axis parameter and **Circularity Factor** as Y-axis parameter and examine the size/shape distribution of object population in the scatter plots. The bulk of the population of the example has a CF of <1.1 and an area of around 300 pixels in images taken with 4x magnification.



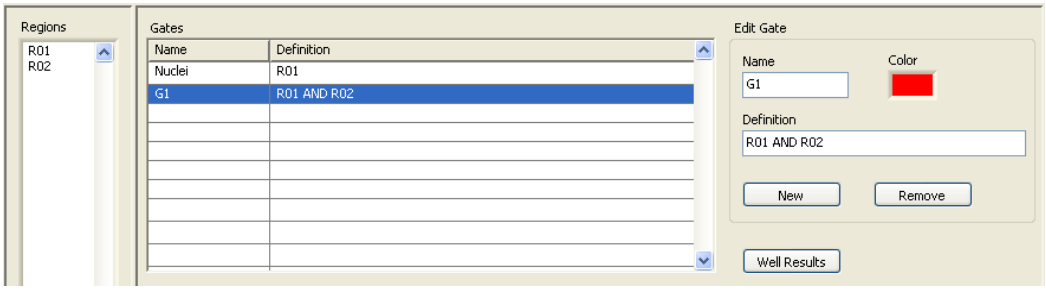
Clustered cells
CF = 1.17
Area = 1590 pixels

Objects that are larger and have a larger Circularity Factor may for example consist of clustered cells that could not be separated by the watershed algorithm.

2. For further analysis only consider those objects that do not differ significantly in size and shape from the bulk. To do so, use the **Region** tool and draw a polygon around the most densely populated part of the scatter plot. (Close the region by double clicking).
   Right-click in the scatter plot to open the context menu and select **Gate.** By doing so, the region **R01** is converted in a gate of the same name (**R01)** and you can apply this gate to all other histograms to exclude all objects outside this gate. Right-click again and select **Set Gate ▸ none** to get back all objects.
   Right-click on the border of the polygon and select **Gallery** to display a selection of objects inside the gate; this allows you to crudely evaluate if your gating was set in a useful way.

---

3. In the next histogram display the **Total Intensity Dapi** vs. the **Mean Intensity TxRed**. To include only the objects in **R01** right-click in the histogram and select **Set Gate ▸ R01.** You will observe a cluster around the total Intensity Dapi of ~9x10⁶ and another cluster around 1.8x10⁷. The left cluster consists of the nuclei in G1, whereas the cluster with twice the dapi intensity contains the nuclei in G2. (With G2 cells having twice the amount of DNA they will have twice the amount of DAPI-labeling and thus roughly twice the fluorescence intensity.) Draw a polygon around the cluster on the left with the **Region** tool and keep in mind the name (**R02**). Execute **Analysis ▸ Assay Gating** to open the **Gate Manager** window.



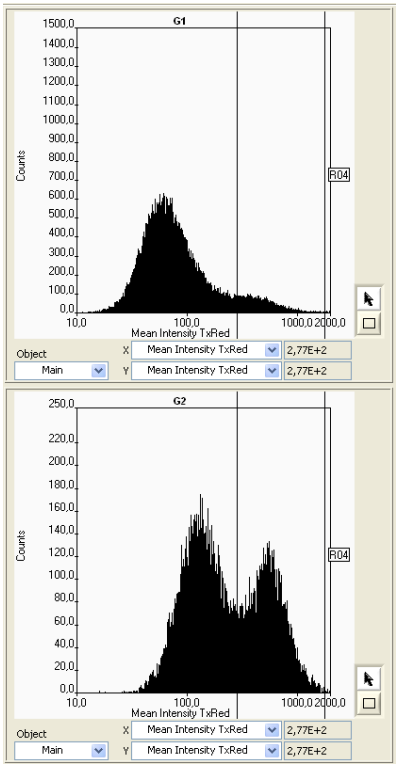
   Click **new** to create a new gate. You can give the new gate a meaningful name (e.g. G1) and also select a color. In the Definition line enter **R01 AND R02** to select only the objects which are contained in both regions. Rename the Gate R01 to Nuclei; these are the objects defined in step 2. Then **Close** the Gate Manager.

---

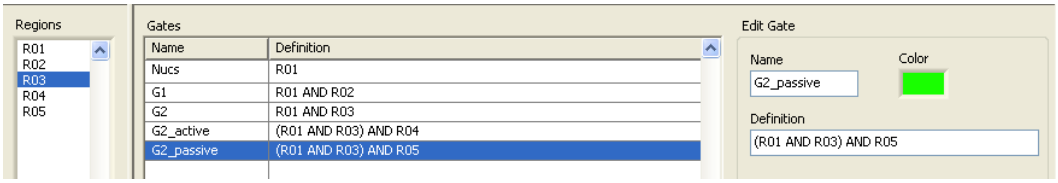
4. Next draw a region around the right cloud, i.e. the nuclei in G2 and repeat the steps described in step 3 and name this gate G2.

---

5. In the two remaining histograms choose **Mean Intensity TxRed** for the *X* and *Y* axis. This will give the distribution of objects with a certain amount of TexasRed staining in all nuclei. To find out if the distribution of TexasRed is different in the G1 and G2 phase right-click and select **Set Gate ▸ G1** in the context menu in one histogram and **Set Gate ▸ G2** in the other histogram.



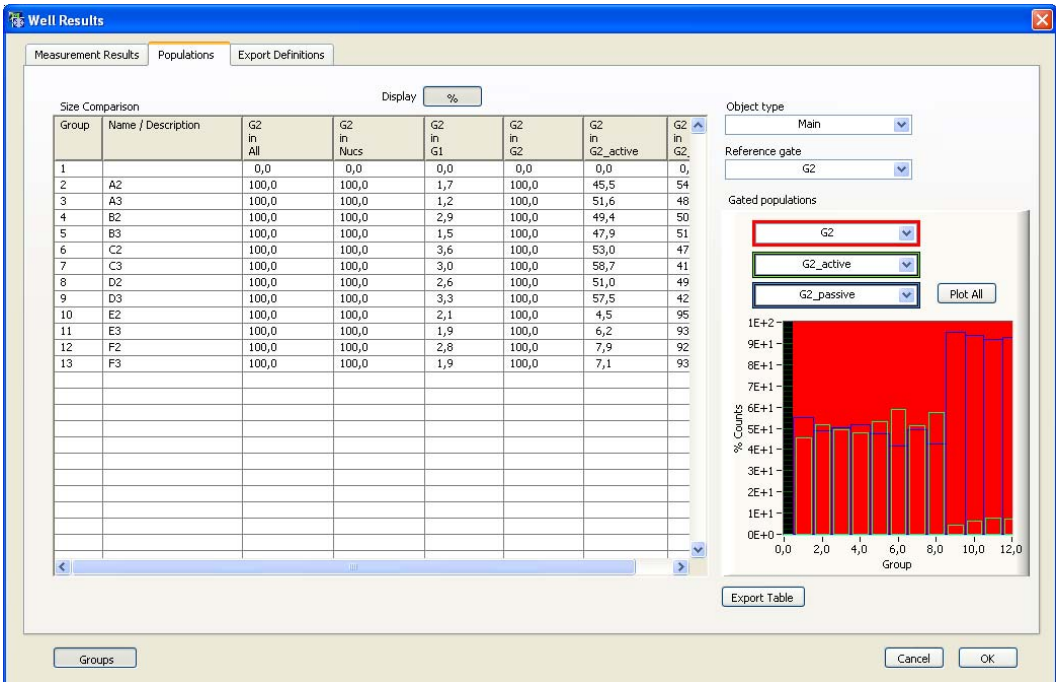
6. You will observe that the distributions in G1 and G2 look different. In G1 there are only nuclei with a low mean intensity in TexasRed, whereas in G2 there are two populations: one with a lower mean intensity in TexasRed and one with a higher intensity in TexasRed. Again select the polygon tool to define a region around the two populations in G2 and create two gates according to step 3. We name the nuclei with a high concentration of Texas Red G2_active, and the nuclei with a low concentration G2_passive. The result in the **Gate Manager** may look as follows:



7. Leave again the **Gate Manager** window. By right-clicking on the main window you can highlight the nuclei in the different gates with boxes when you activate them (e.g. **Show Gates ▸ G2_active**). Another option to compare the results is to right-click on the border of the gate and to choose **Region Gallery**. Plot a region gallery of G2-Active and G2-passive to compare the results of the gating.

8. In the lower part of the **Gate Manager** you will also find statistics of the displayed histograms. For more detailed results click **Well Results** in the gate manager and go to the tab **Populations**. Here you will find the results for all wells and all gates. You can choose if you want to display the total **counts** or the results as **percentages**. You can export these results for further analysis. A graphical display allows you to directly compare the results for different wells. Select G2 as reference gate and G2, G2_active and G2_passive as gated populations.

You will notice that in the first 8 wells the numbers of active and passive nuclei are almost identical. However in the last 4 wells the number of active cells in G2 is strongly decreased.

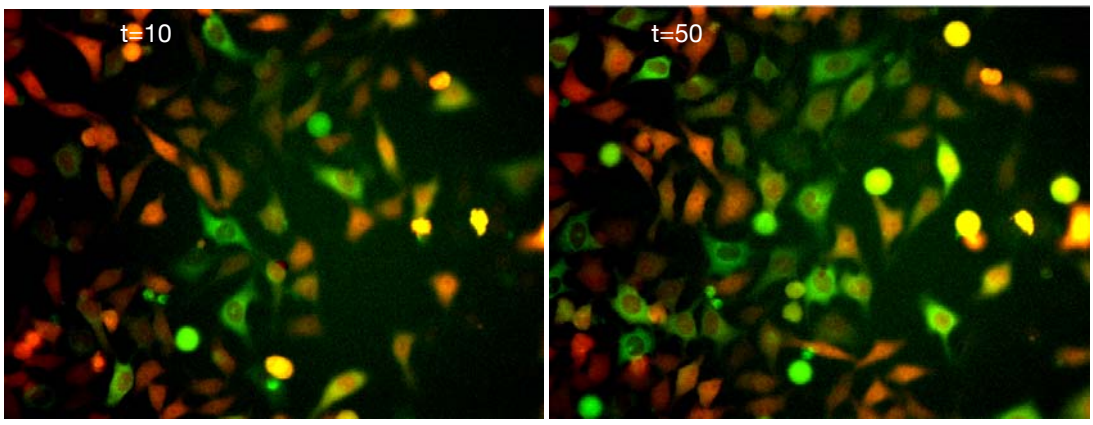


Group	Name / Description	G2 in All	G2 in Nucs	G2 in G1	G2 in G2	G2 in G2_active	G2 in G2_
1		0,0	0,0	0,0	0,0	0,0	0,
2	A2	100,0	100,0	1,7	100,0	45,5	54
3	A3	100,0	100,0	1,2	100,0	51,6	48
4	B2	100,0	100,0	2,9	100,0	49,4	50
5	B3	100,0	100,0	1,5	100,0	47,9	51
6	C2	100,0	100,0	3,6	100,0	53,0	47
7	C3	100,0	100,0	3,0	100,0	58,7	41
8	D2	100,0	100,0	2,6	100,0	51,0	49
9	D3	100,0	100,0	3,3	100,0	57,5	42
10	E2	100,0	100,0	2,1	100,0	4,5	95
11	E3	100,0	100,0	1,9	100,0	6,2	93
12	F2	100,0	100,0	2,8	100,0	7,9	92
13	F3	100,0	100,0	1,9	100,0	7,1	93

9. To export the results either select **Export Table** in the Populations or **Measurement Results** or select the results to be exported via **Export Definitions** as described in Chapter 4.3.3, *Export Definitions*.

5.3 Time-Lapse Analysis

In this example a time-lapse series is analyzed. The images of the example show cells during mitosis. The cells are marked with two stains (GFP and TxRed). The images left and right show images from the same well and position, taken at two different time points (t=10, t=50). In order to analyze time-lapse data it is necessary to segment the images properly and to track the moving cells from frame to frame.



1. Execute **Scan ▸ Open…**, navigate to the folder where the demo data are stored, select the experiment_descriptor.xml file (/…/scan^R_Demo_Data_DVD_20070216/Slabicki_Buchholz_MPI-CBG/CMV-mRed+cyclineB1GFP/GFP+mRed_001/experiment_descriptor.xml; sample data kindly provided by F. Buchholz, MPI-CBC, Dresden) and open it with **OK**.

---

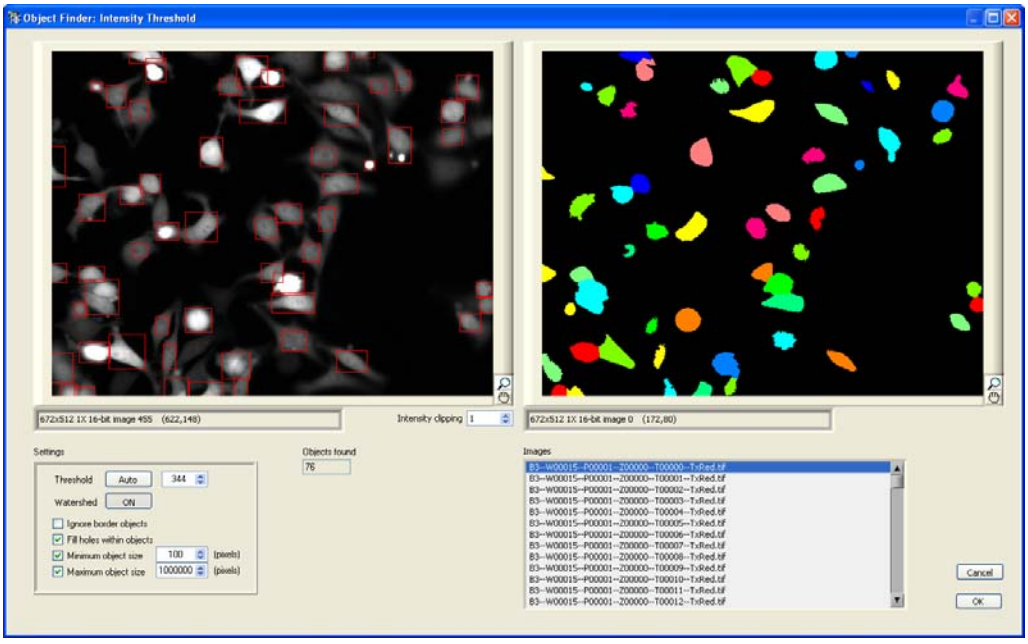
2. Execute **Assay ▸ Edit Assay** to open the **Assay Settings** window.

---

3. Go to the **Image processor** module in the **Image Processing** tab to set **Background Correction** for all channels (TxRed and GFP). Press Adjust and observe in the new window the background in the image before (left) and after (right) background correction. When moving the mouse over the pixels the grey value should be reduced in the corrected images..

---

4. Open the **Main Object** tab in the **Assay Settings**. Here you have to select the channel which you want to use for image segmentation. Select the TxRed channel (which has the stronger signal) for image segmentation and use the **intensity** module. Click on **Adjust**: The **Object Finder: Intensity Threshold** menu opens. Press **Auto** to set the threshold and use the watershed algorithm.

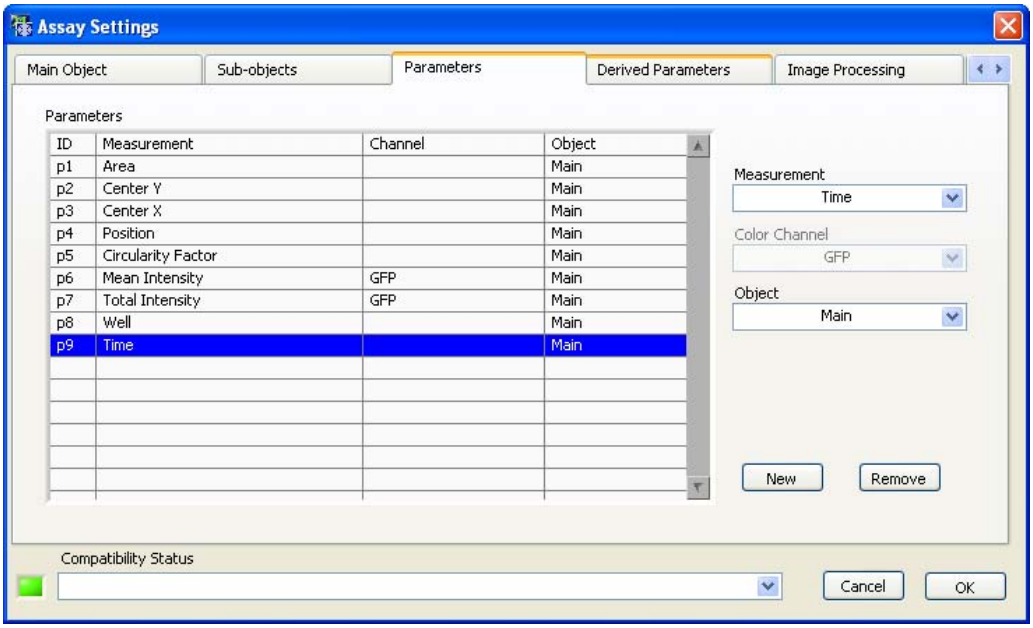


---

5. Skip the sub-object tab.

---

6. Go to the **Parameters** tab. Select here the parameters you want to detect for the main object. Select **Area**, **Center X**, **Center Y**, **Position**, **Well** and **Circularity Factor**, which are determined on the channel of the main object (i.e. TxRed). For the GFP Channel select **Mean Intensity** and **Total Intensity**. Note that the intensities of the GFP channel are also detected on the main object (which was originally segmented using the TxRed channel).

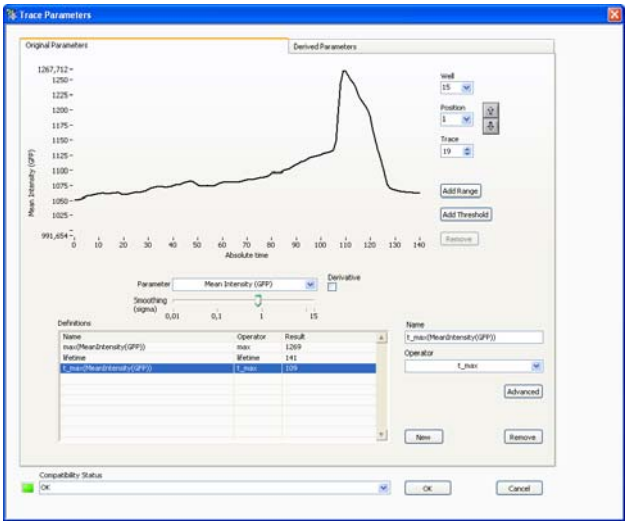


7. Confirm the **Assay Settings** with **OK** and **Run** the analysis.

8. After the analysis you can plot the Histograms and Scatterplots as described in Chapter 5.2, *Analyzing the Data*.

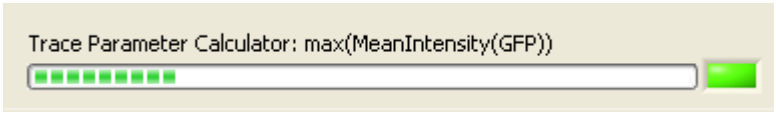
9. The next step after the image segmentation and the extraction of the parameters is the tracking of the objects. Go to **Tracking** ▸ **Configure Tracer**. Check the **Enable Tracking** box and select **Main** as tracked object. Set a range of 20 pixels. Confirm with OK. When leaving this dialog the tracking will start. The progress of the tracking is shown in the status bar.

10. After the tracking is finished the **Trace Parameters** menu will open. Here you can browse through the traces and determine the kinetic parameters that will be extracted from the curves. Select the **operators** which will be applied to the kinetic curves of the selected Parameters. Let's say we want to find all the cells that undergo mitosis during the measurement. When undergoing Mitosis the cells show an increase in the mean intensity of GFP. First set the smoothing to ~1 to obtain smoothed curves. In order to find out the mitotic cells in a simple approach set the following definitions: We need the **max**(MeanIntensity(GFP)) to find out the maximum intensity of the curve. Then we need the **lifetime** to gate on the cells that are tracked over all images. We are also interested in the time point when the maximum mean intensity is reached. Therefore select also **max**(MeanIntensity(GFP))



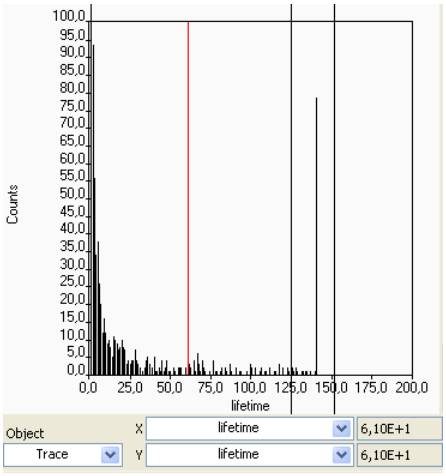
---

11. Close the menu with OK. The analysis of the curves is performed. In the lower right of the front panel the status bar displays the progress of the analysis.



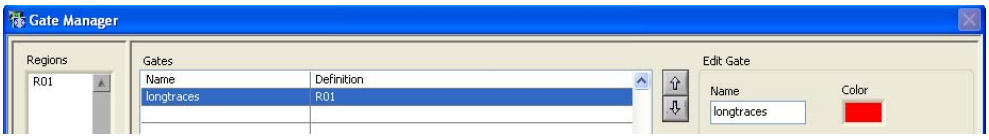
---

12. When the analysis is finished you can plot a lifetime histogram in the first histogram panel. In order to select the cells that were detected in all timeframes draw a rectangle around the data points at 140. Right-click on the rectangle and select **Gate** to convert the region into a gate.



---

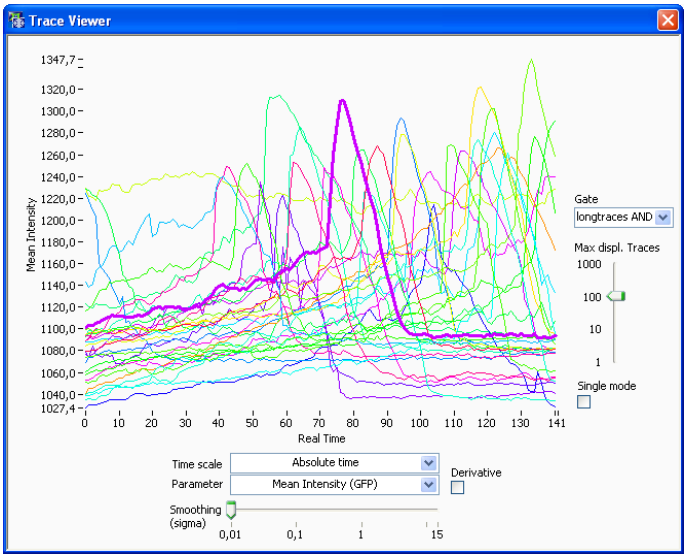
13. Go to **Analysis ▸ Assay Gating…** and rename the Gate **R01** to **longtraces**. Leave the menu with **OK**.



14. In the second histogram display, plot **max**(MeanIntensity(GFP)). Right-click in the histogram and select **Set Gate** ▸ **Longtraces** in order to include only the cells that were detected in all images into the analysis. Draw a rectangle around the datapoints on the right. These are the cells which show a high mean intensity of GFP. Right-click on the border of the rectangle and select **Gate**. The new gate **longtraces AND R02** is created.

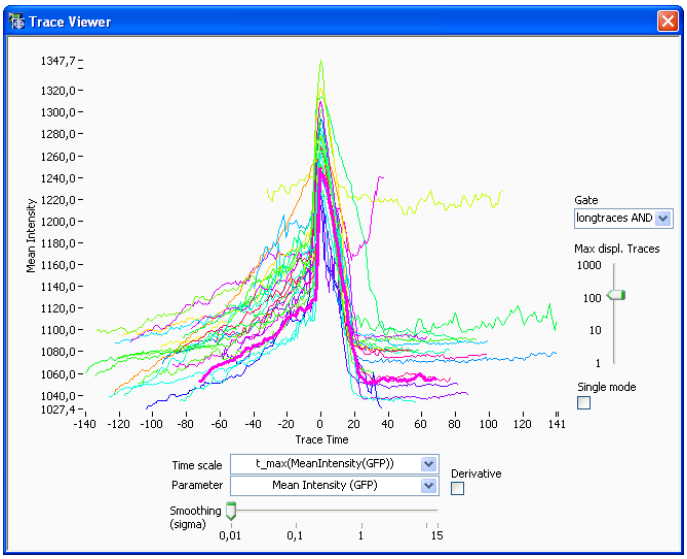
---

15. Mark one of the datapoints and the corresponding cell is shown in the image display with a green outline. Right-click on the image of the cell and select **show trace** from the context menu. The **Trace Viewer** opens and displays the curve of the selected cell. In order to show all the traces of the gated cells, deactivate **single mode** and for Gate select **longtraces AND R02**. The curves show all the same characteristics.

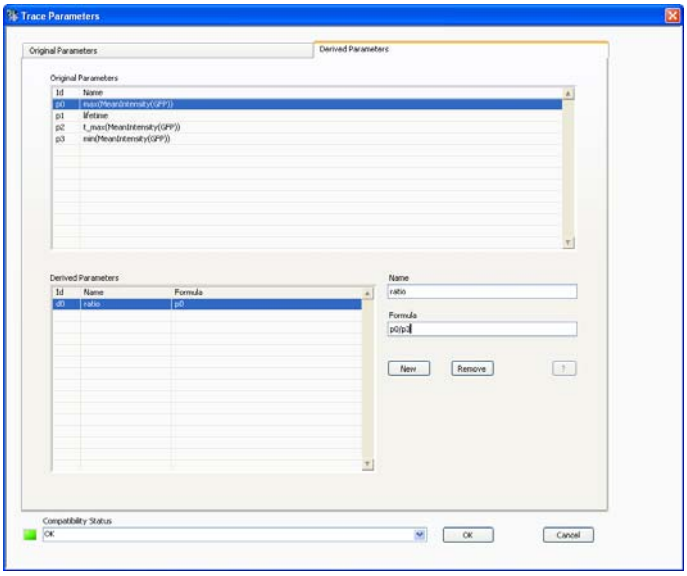


---

16. As default the **Time scale** is set to **Absolute time** but it is also possible to display and analyze the curves regarding to a certain timepoint, e.g. the time of mitosis. Therefore select **t_max**(MeanIntensity(GFP)) as **Time scale**.



17. As you can see, the green curve shows no mitosis but has a high mean intensity in GFP all the time. (When you select the green curve and observe the corresponding object in the images you see that this is probably an apoptotic cell). In order to avoid this you can set up a more advanced analysis, e.g. you can set the parameters **max**(MeanIntensity(GFP)) and **min**(MeanIntensity(GFP)) in the **Trace Parameters** menu (therefore go back again to **Tracking ▸ Define Parameters**). Go to the **Derived Parameters** tab In the **Trace Parameters** menu and set as new derived parameter the ratio of **max**(MeanIntensity(GFP)) and **min**(MeanIntensity (GFP)). Name this new parameter **ratio**.

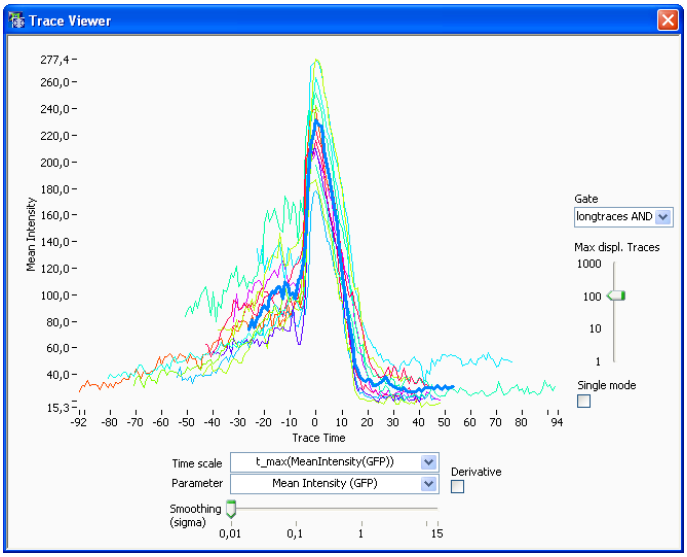


---

18. After running the trace analysis plot a histogram of **ratio.** Set again **longtraces** as gate. The data points around 1 show about the same minimum and maximum mean intensity of GFP. Whereas data points >1 are the ones that undergo mitosis and have a higher maximum mean intensity. Draw a rectangle around the data points with ratio >1. Right-click on the border of the rectangle and select **Gate**. The new gate **longtraces AND R03** is created.

---

19. Mark one of the data points and the corresponding cell is shown in the image display with a green outline. Right-click on the image of the cell and select **show trace**. In order to show all the traces of the gated cells, deactivate **single mode** and as gate select **longtraces AND R03**. You will see that this parameters lead to a better discrimination of mitotic cells than the more simple approach used before.


Trace Viewer
277,4
260,0
240,0
220,0
200,0
180,0
160,0
140,0
120,0
100,0
80,0
60,0
40,0
15,3
Mean Intensity
-92 -80 -70 -60 -50 -40 -30 -20 -10 0 10 20 30 40 50 60 70 80 94
Trace Time
Gate
longtraces AND
Max displ. Traces
1000
100
10
1
Single mode
Time scale
t_max(MeanIntensity(GFP))
Parameter
Mean Intensity (GFP)
Derivative
Smoothing (sigma)
0,01
0,1
1
15

6 Appendix

6.1 File Conversion

scan^R Analysis is able to analyze image data acquired with a third party imaging system as long as the data are stored as (monochromatic) 16-bit single TIFF images. The file names must allow the assignment of the images to individual positions, time stamps and color channels.

The command **Scan ▸ Custom conversion** opens the **Scan File Conversion** dialog that allows setting up conversion rules. The creation of conversion rules is a four-step process, which will be described, in the following chapters.

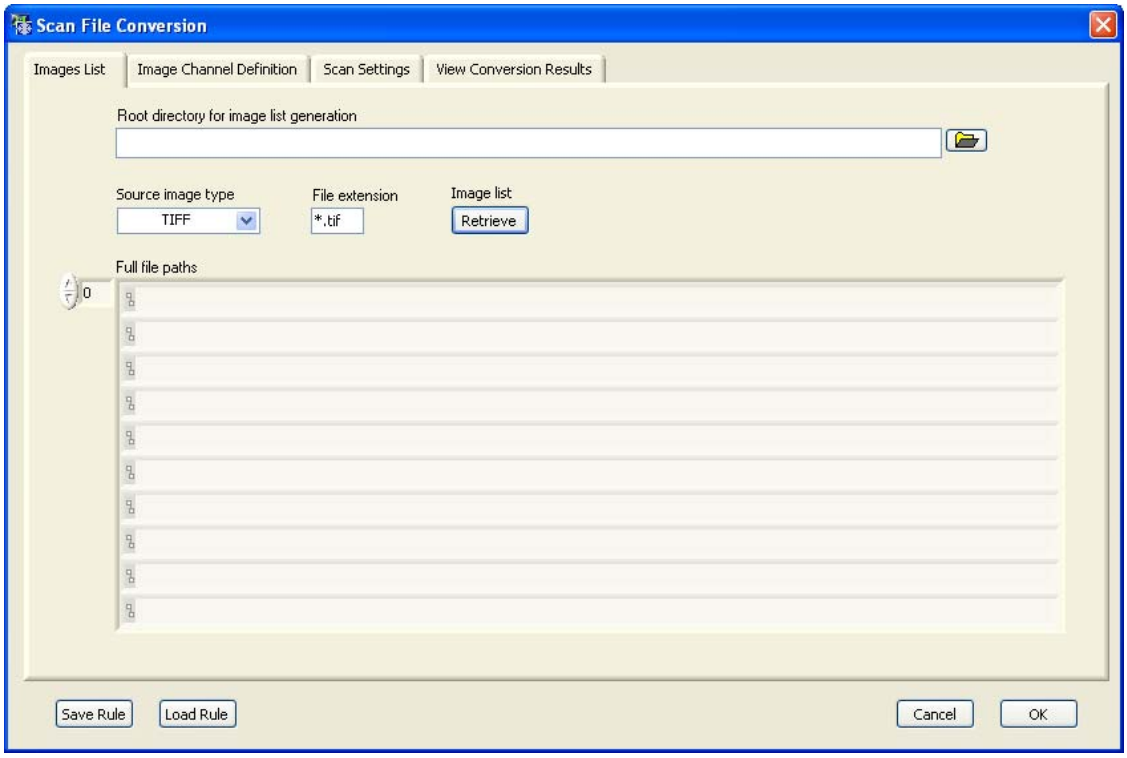
Save rule. This function saves the current conversion settings as a Custom Pattern file (*.cr).

Load rule. This function allows loading a stored set of conversion settings.

6.1.1 Images List

Root directory for image list generation. Click on the **folder** button on the right of the box to open the navigation window. Navigate into the storage directory and click **Select Curr. Dir**.

Source image type. Select the desired image type (TIFF, JPEG, PNG or BMP) from the shortlist.



File extension. This is set automatically when the **Source image type** is chosen. The **file extension** serves to filter the content of the selected directory

Image list: Retrieve. This function reads the matching files into the **Full file paths** list. The arrow buttons allow easy browsing and verification of retrieved image data.

6.1.2 Image Channel Definition

If the file names of the third party images contain codes for Well, Position, Z-layer and Time, scan^R will sort them automatically upon conversion into scan^R format. The **Image Channel Definition** tab is used to define the conversion rules for this.

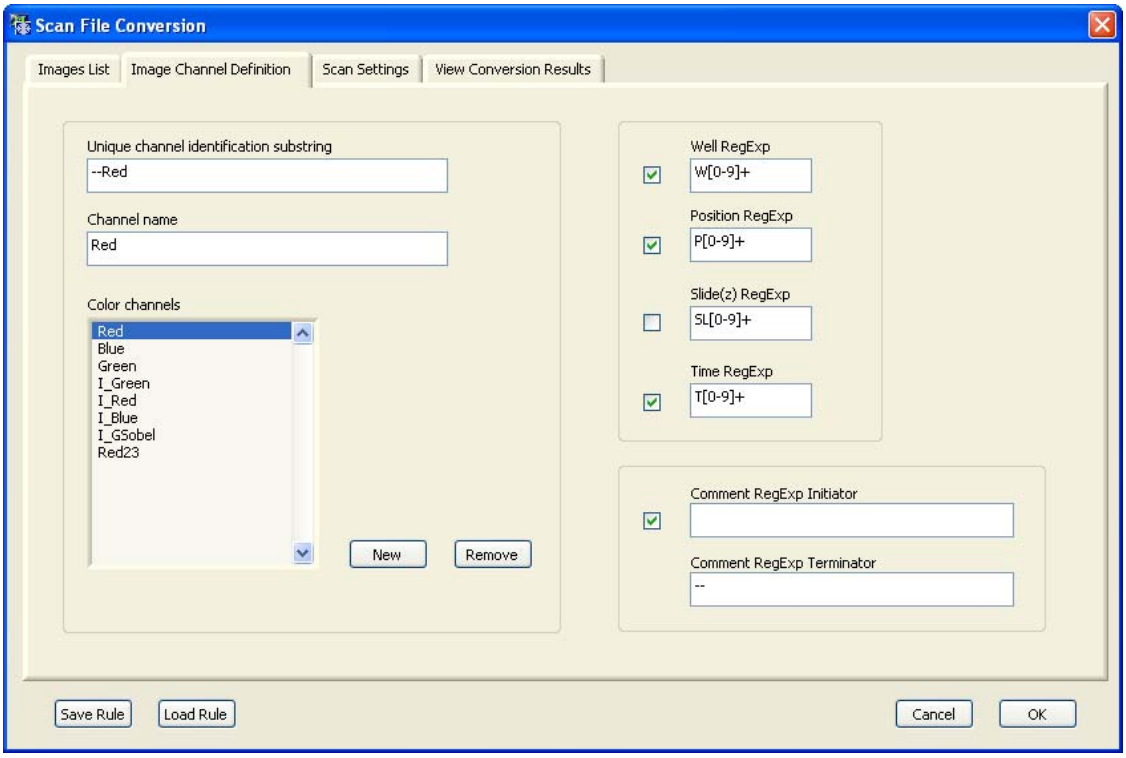
Unique Channel Identification String. If the original file names contain a certain expression – or **Identification String** – that classifies them as belonging to a certain color channel, enter it here.

Channel Name. Enter the **Channel Name** to be used in scan^R here.

New. Click here to add the newly defined channel to the **Image Channel** list.

Well, **Position**, **Slide (Z)**, **Time RegExp**. If the file names contain information about Well, Position, Z-layer and Time in form of regular expressions (**RegExp**), scan^R can group them when the corresponding code is entered here.

An overview of the syntax of regular expressions can be found under http://www.regular-expressions.info/tutorial.html.



Comment RegExp Initiator / Terminator. If the file names contain information about wells, scan^R can use this as **Well Description**, see for example Chapter 2.7, *Selecting Wells for Analysis*. Enter the first (**Initiator**) and last (**Terminator**) alphanumerical digit(s) in the respective boxes.

6.1.3 Scan Settings

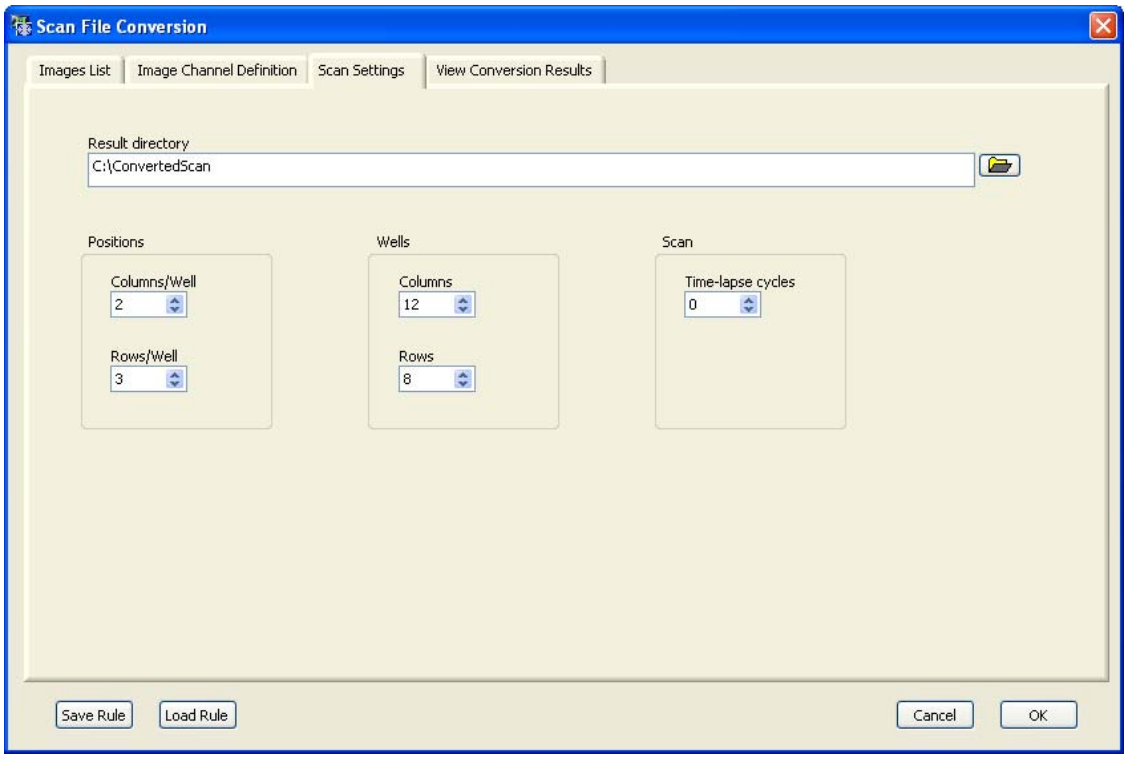
Scan settings are used to reconstruct a minimal configuration file for the data conversion. scan^R needs information about the used multi-well plate and the arrangement of the images within the single wells in order to represent the data properly in the graphical user interface

Result Dir. Click on the **folder** button on the right of the box to open the navigation window. Navigate to the storage directory and click **Select Curr. Dir**.

Positions: Columns/Well and **Rows/Well**. Set the number of image columns and rows, respectively, per well.

Wells: Columns and **Rows**. Set the number of columns and rows, respectively, of the multi well plate.

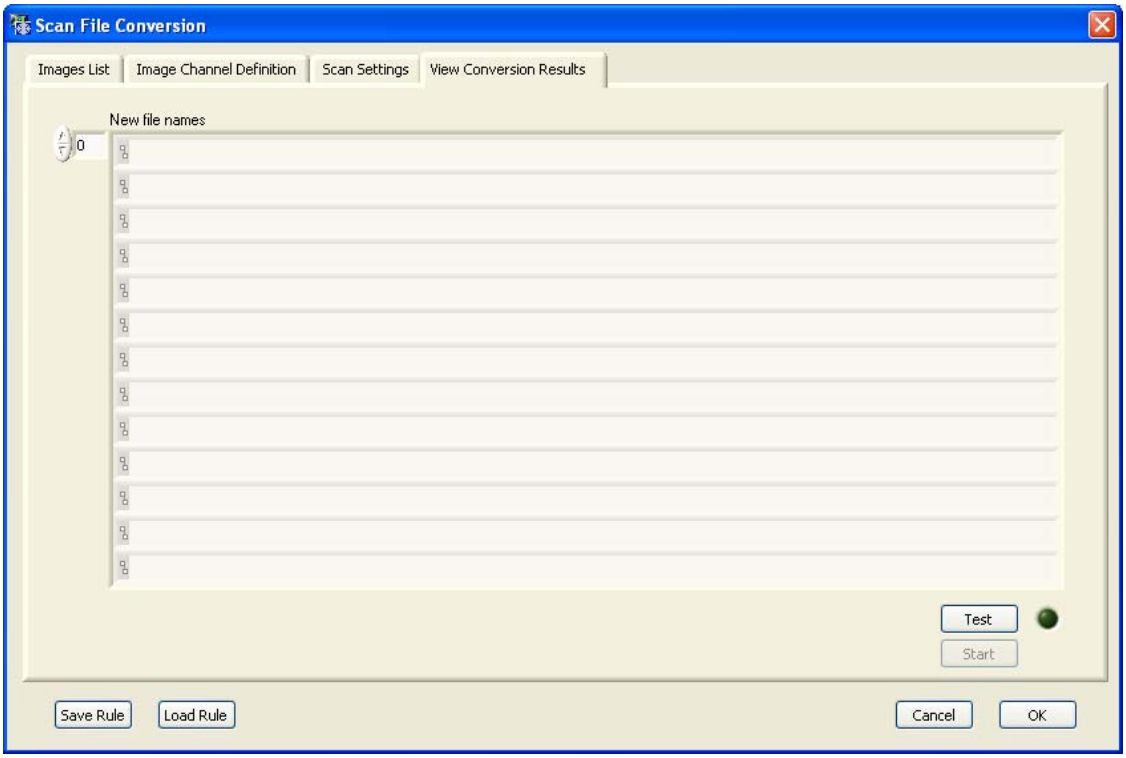
Time-lapse cycles. Set the number of time points for this scan.



6.1.4 View Conversion Results

Test. Upon clicking this button, scan^R converts the file names and lists them in the **New File Name** list. This allows checking whether the conversion rules yield the desired result.

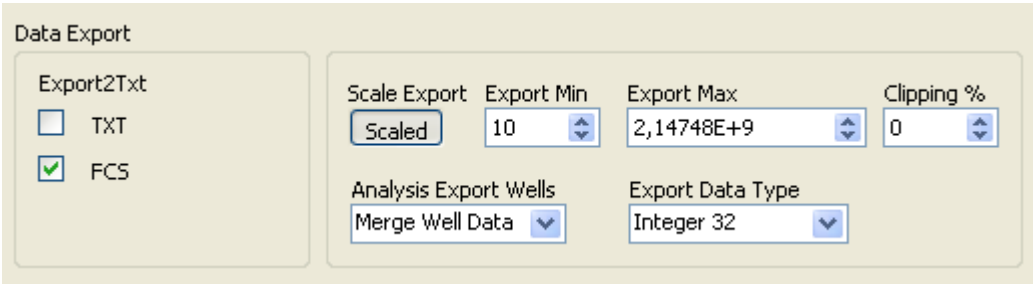
Start. Click here to start the file conversion process. The **Start** button becomes active once a conversion rule is defined or loaded. It initiates the file conversion. This may be time consuming because an entire new set of images is generated and stored.



6.2 FCS Export Functionality

scan^R is able to export single cell data to ISAC FCS 3.0 Standard files. Please note, that this standard is not fully implemented by most of the programs that specify FCS 3.0 import support. Therefore some settings modifications for export might be necessary depending on the specific limitations of these programs. In practice especially the FCS 3.0 requirements for double and integer 32 bit data support as well as multi dataset FCS files might not be implemented because most flow cytometers produce integer 16 bit data in single data sets. It is therefore recommended to test the FCS 3.0 compatibility before deciding which FCS program is to be used for the analysis. Most manufactures provide demo programs for testing.

In order to export FCS data open the **Settings** menu from the menu bar.



Export Data Type. Here you can choose the data types exported (Integer 16 / Integer 32 / double (64-bit floating point) according to FCS 3.0 standard. It is recommended to use the highest precision that is supported by the program intended to import the fcs files.

Scale Export Scaled (On/Off). This button activates the rescaling procedures during FCS export. Switch off the scaling if you want to compare datasets from two separate export procedures quantitatively. If activated, the scaling settings (Min/Max/Clipping) will be used.

Export Min / Export Max / Clipping %. Settings used for rescaling the original data according to:

x_fcs = x*PnG + PnOffset, with:

x_fcs	exported data points
x	the original data points and
n	parameter number
PnG	automatically determined linear scaling factor (Gain)
PnOffset	automatically determined scaling offset

The resulting scaling offset and the scaling factor are stored in the FCS file header in the keywords PnG and PnOffset. If you switch on the scaling then the range of the original data will be scaled to fit into the given export range. PnG and PnOffest are calculated from the original dataset and the scale settings according to:

Export Range	= Export Max - Export Min
Original Data Range	= Original Data Set Max Value - Original Data Set Min Value
Clipped Range	= Original Data Set Max Value (ignoring the largest clip %) - Original Data Set Min Value (ignoring the smallest clip %)
PnG	= Export Range/Clipped Range
PnOffset	= Export Min - PnG*(Original Data Set Min Value (ignoring the smallest clip %))

Analysis Export Wells (FCS Settings):

Merge Well Data. All Wells are taken together to a single dataset

Single File. Exports Wells into separate datasets in a single FCS file according to FCS 3.0 standard (this Multi-dataset format is not supported by all FCS programs)

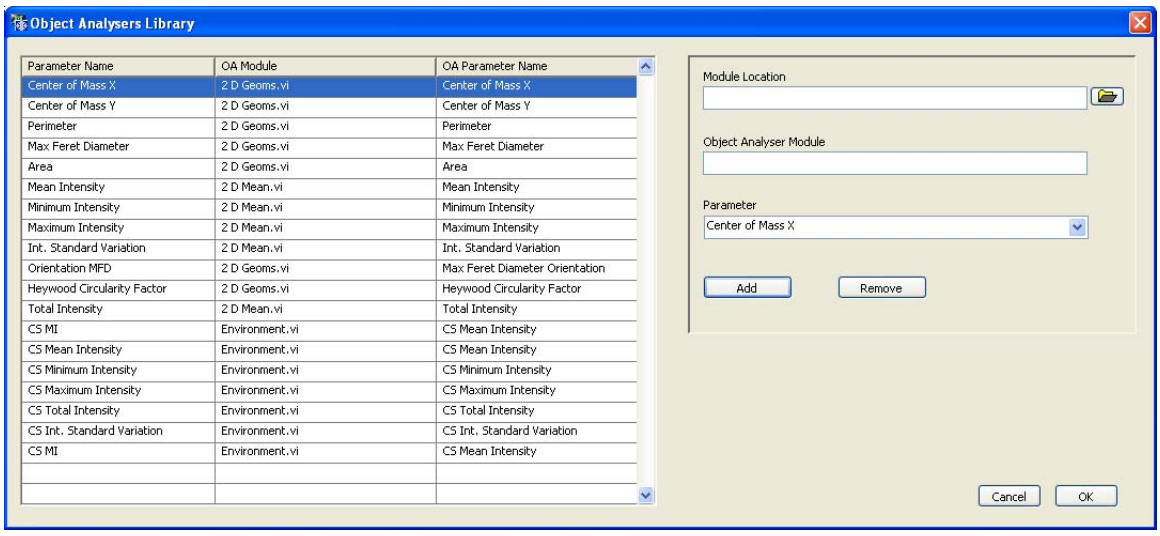
Separate Files. The export table function creates a single FCS file for every well.

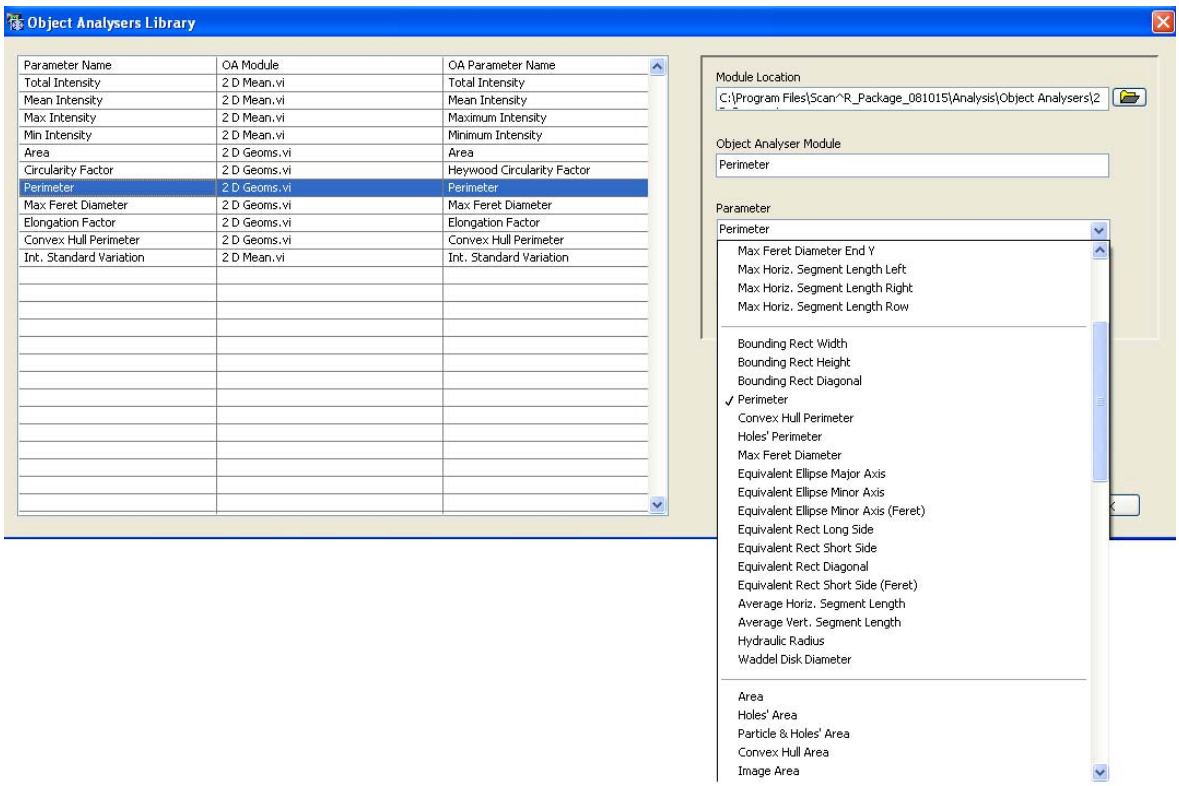
> **i16 export (FlowJo).** When using i16 export and importing with **FlowJo**, **FlowJo** uses the gain to rescale the data but is then not able to go back to the logscale. Therefore it is recommended to use raw mode with i16 and FlowJo. To switch to logscale use then the FlowJo function: Add derived ... and convert the required parameters to log. Doubles and Multi-dataset FCS Files can not be read by FlowJo. Please note, that if you have data within a X,Y logscale and you rescale the X,Y data with different scaling factors (as might happen with the automated scaling during export because of range differences) there might be a shape change.

An alternative to FlowJo is FCS Express (http://www.denovosoftware.com/site/Download.shtml) which is supposed to stick more strictly to ISAC FCS 3.0 standard.

6.3 Libraries

6.3.1 Object Analyzers Library (OAL)





The password-protected **Object Analyzers Library** can be accessed through **Modules ▸ Object Analyzers Library**.

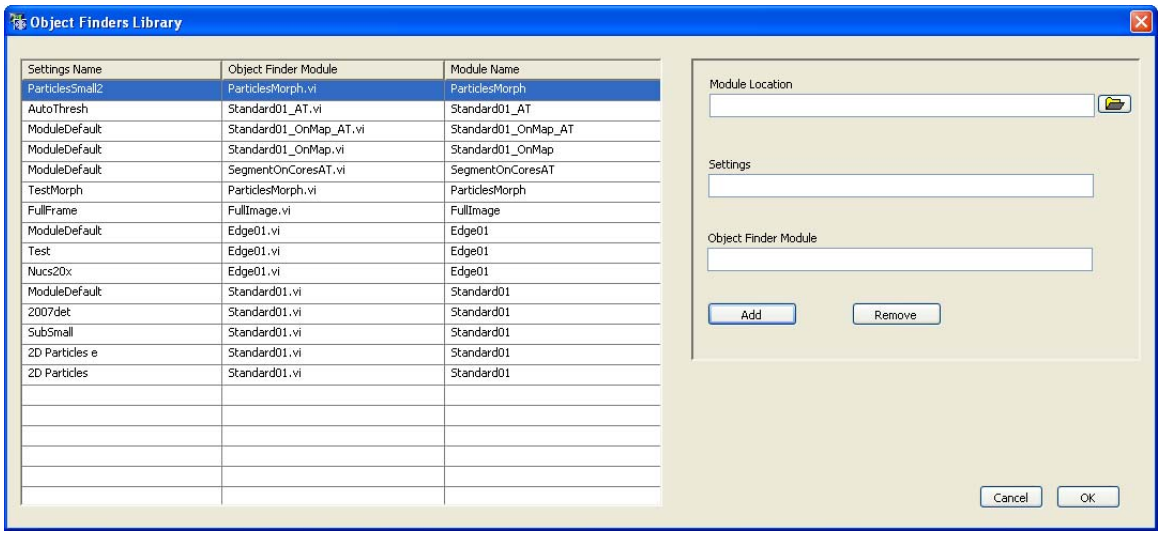
It allows managing the set of parameters selectable in the **Edit Assay** tab **Parameters**; see Chapter 4.6, *Measurement Parameters*.

Add. Click here to add a new entry to the list.

Parameter. Select the desired parameter from the drop down menu to add it to the list.

6.3.2 Object Finders Library (OFL)

The password protected **Object Finders Library** can be accessed through **Modules ▸ Object Finders Library.** It lists and allows administrating the available **Object Finder Modules**. Each module may have different settings lists and may thus appear several times.



Add/Remove. Click here to add or delete a new entry to the list.

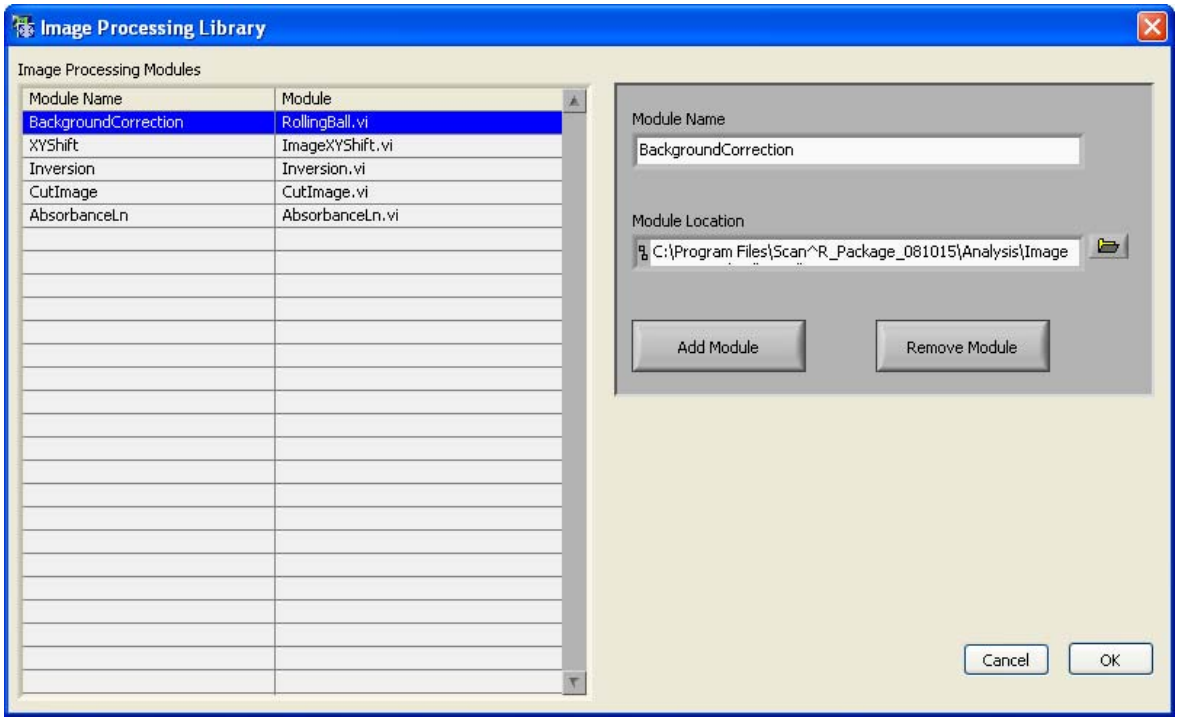
6.3.3 Image Processing Library

The password-protected **Image Processing Library** can be accessed through **Modules ▸ Image Processors…**

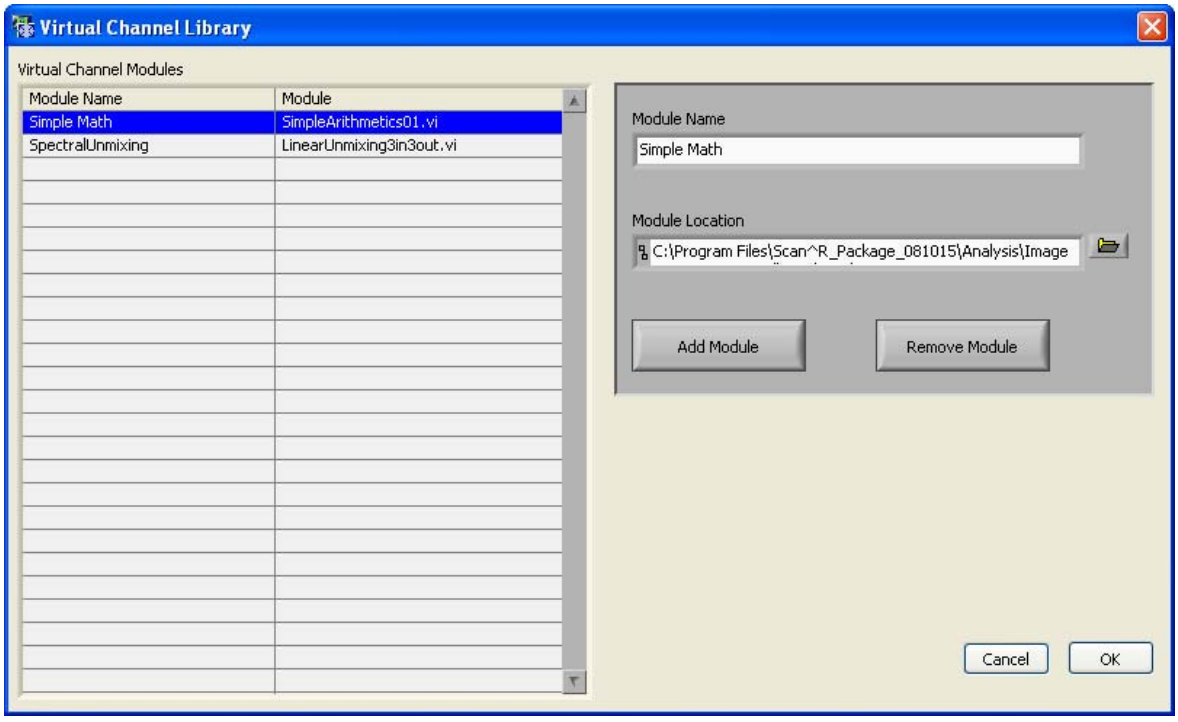
It allows managing the set of available Image Processing algorithms in the **Image Processing modules**.

Add Module. Click here to add a new entry to the list.

Remove Module. Click here remove an entry from the list.



6.3.4 Virtual Channel Library



The password-protected **Virtual Channel Library** can be accessed through **Modules ▸ VC Processors…**

It allows managing the set of available Virtual Channel algorithms in the **module selector on the Edit Assay ▸ Virtual Channels tab**.

Add Module. Click here to add a new entry to the list.

Remove Module. Click here remove an entry from the list.

6.4 Valid functions for derived parameters

abs(*x*)	Absolute Value	Returns the absolute value of *x*.
acos(*x*)	Inverse Cosine	Computes the inverse cosine of *x* in radians.
acosh(*x*)	Inverse Hyperbolic Cosine	Computes the inverse hyperbolic cosine of *x*.
asin(*x*)	Inverse Sine	Computes the inverse sine of *x* in radians.
asinh(*x*)	Inverse Hyperbolic Sine	Computes the inverse hyperbolic sine of *x*.
atan(*x*)	Inverse Tangent	Computes the inverse tangent of *x* in radians.
atan2(*y,x*)	Inverse Tangent (2 Input)	Computes the arctangent of *y*/*x* in radians.
atanh(*x*)	Inverse Hyperbolic Tangent	Computes the inverse hyperbolic tangent of *x*.
ceil(*x*)	Round Toward +Infinity	Rounds *x* to the next higher integer (smallest integer >= *x*).
ci(*x*)	Cosine Integral	Evaluates the cosine integral for any real nonnegative number *x*.
cos(*x*)	Cosine	Computes the cosine of *x*, where *x* is in radians.
cosh(*x*)	Hyperbolic Cosine	Computes the hyperbolic cosine of *x*.
cot(*x*)	Cotangent	Computes the cotangent of *x* (1/tan(*x*)), where *x* is in radians.
csc(*x*)	Cosecant	Computes the cosecant of *x* (1/sin(*x*)), where *x* is in radians.
exp(*x*)	Exponential	Computes the value of *e* raised to the *x* power.
expm1(*x*)	Exponential (Arg) – 1	Computes one less than the value of *e* raised to the *x* power ((*e*^*x*) – 1).
floor(*x*)	Round To –Infinity	Truncates *x* to the next lower integer (largest integer <= *x*).
getexp(*x*)	Mantissa & Exponent	Returns the exponent of *x*.
gamma(*x*)	Gamma	Evaluates the gamma function or incomplete gamma function for *x*.
getman(*x*)	Mantissa & Exponent	Returns the mantissa of *x*.
int(*x*)	Round To Nearest	Rounds *x* to the nearest integer.
intrz(*x*)	—	Rounds *x* to the nearest integer between *x* and zero.
ln(*x*)	Natural Logarithm	Computes the natural logarithm of *x* (to the base of *e*).
lnp1(x)	Natural Logarithm (Arg +1)	Computes the natural logarithm of (x + 1).
log(x)	Logarithm Base 10	Computes the logarithm of x (to the base of 10).
log2(x)	Logarithm Base 2	Computes the logarithm of x (to the base of 2).
max(x,y)	Max & Min	Compares x and y and returns the larger value.
min(x,y)	Max & Min	Compares x and y and returns the smaller value.
mod(x,y)	Quotient & Remainder	Computes the remainder of x/y, when the quotient is rounded toward – Infinity.
pow(x,y)	Power of X	Computes x raised to the y power.

rand( )	Random Number (0 – 1)	Produces a floating-point number between 0 and 1 exclusively.
rem(*x*,*y*)	Quotient & Remainder	Computes the remainder of *x*/*y*, when the quotient is rounded to the nearest integer.
si(*x*)	Sine Integral	Evaluates the sine integral for any real number *x*.
sec(*x*)	Secant	Computes the secant of *x*, where *x* is in radians (1/cos(*x*)).
sign(*x*)	Sign	Returns 1 if *x* is greater than 0, returns 0 if *x* is equal to 0, and returns –1 if *x* is less than 0.
sin(*x*)	Sine	Computes the sine of *x*, where *x* is in radians.
sinc(*x*)	Sinc	Computes the sine of *x* divided by *x* (sin(*x*)/*x*), where *x* is in radians.
sinh(*x*)	Hyperbolic Sine	Computes the hyperbolic sine of *x*.
spike(*x*)	Spike	Generates the spike function for any real number *x*.
sqrt(*x*)	Square Root	Computes the square root of *x*.
step(*x*)	Step	Generates the step function for any real number *x*.
tan(*x*)	Tangent	Computes the tangent of *x*, where *x* is in radians.
tanh(*x*)	Hyperbolic Tangent	Computes the hyperbolic tangent of *x*.

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