AxioVision User's Guide

Release 4.7

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Refer to the safety notes and instructions in the manuals of all necessary devices (e.g. microscope peripherals, cameras, computers, computer additionals, etc.) before installing and using the software (see also "Safety Notes AxioVision, AxioVision AC, B 48-0044 e (SAP 000000-1339-887")).

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1. Introduction

1.1 The Concept

AxioVision is a modular image-processing and analysis system for modern microscopy. The basic functionality for image acquisition and microscope control, image processing and annotations, image analysis, documentation and configuration can also be expanded by integrating additional modules into the system for specific tasks (see section 1.2 "The Modules").

The basic version comprises the following functionalities:

Image Acquisition

A range of different camera types can be used with AxioVision, from simple TV cameras through to high-resolution and high-sensitivity cameras. The cameras of the Carl Zeiss AxioCam family guarantee optimum integration. As the cameras are integrated seamlessly into the AxioVision software, you are also able to generate complex images and image series with a simple mouse-click.

Microscope Control

With AxioVision it is possible to control any motorized Carl Zeiss microscope fully automatically and interactively (you can of course also use manual standard microscopes). Software control offers the following advantages: the microscope's setting parameters can be saved as required, and are therefore available for reuse during future tests, magnifications are determined automatically, and complex procedures are significantly faster and can be easily reproduced at any time.

Incubation

AxioVision controls and monitors the Zeiss incubation components. Incubation parameters like temperature and CO_2- or O_2 concentrations can be stored as hardware settings and be reused for new experiments. All incubation parameters are accessible as measurement parameters for further evaluation of acquired multidimensional time series.

Image Processing and Annotations

The acquired image is immediately displayed on the monitor. It can then be optimized using a wide range of techniques:

- Contrast, brightness and color adjustment
- Noise suppression, smoothing and contour enhancement
- Sharpness enhancement/emphasizing of details
- Correction of illumination influences and white balance

AxioVision can also be used to add any annotations that you may require to the images. All elements, from scale bars and colored markings through to text and graphics, have been integrated into the program.

Image Analysis

You are able to perform simple interactive measurements in the basic program. The measured values (e.g. lengths, areas and angles) are made available in a data table, and can be processed further using spreadsheet programs, such as Microsoft Excel. The interactive measurement can be executed via a special dialog, or freely via a menu or a toolbar.

Documentation

Besides the image itself, the AxioVision image format ZVI also saves additional data, such as the image number, date of acquisition, microscope settings, exposure values, size and scale details, contrast procedures used etc. Annotations and measured values are also saved with the image. Rather than being permanently "burnt" into the image, annotations are present in the form of a separate plane. This means that no image information is concealed, and changes can be made at any time. Consequently, an image can be reproduced years later under identical conditions.

The Image File Browser supports the management of the content of large databases. With this browser you can navigate quickly within your file folders, and obtain a clear display of all the key data acquired with the image.

For the generation of reports or presentations, AxioVision offers you predefined report layouts. These can be modified or completely regenerated at any time on an individual basis.

Configuration

AxioVision offers you a great deal of freedom in terms of the arrangement of your individual work environment. To speed up your work, it is possible to execute any commands using freely definable key combinations. You can configure your own toolbars and group functions together on your own menus. Relevant control elements for camera and microscope control in the form of individual dialogs are grouping together with just a few clicks of the mouse. For recurring routine processes, workflows make simple operation possible. As a result, operating errors can by and large be eliminated.

1.2 The Modules

The following modules are currently available as extensions to the basic system:

Image Acquisition Modules

Autofocus

This module allows the optimum focus position to be calculated with motorized microscopes. It can be used in reflected light, transmitted light, bright field, dark field and fluorescence, and works with all cameras and frame grabbers controlled directly by AxioVision.

Extended Focus

Needle-sharp images are essential for carrying out precise analyses. Depending on the object and the setting, a microscope's depth of field is often insufficient to generate a single image which is sharp over the entire plane. The AxioVision Extended Focus module solves the problem. A number of images are acquired from different focus planes, and the sharp details of each individual image are combined to produce an image that is sharp throughout.

Mark&Find

This module is used to record and automatically relocate various positions on slides, in culture plates and in multiwell plates using a motorized x/y stage.

Fast Acquisition

This module expands the functionality of the modules multichannel fluorescence, z-stack and time lapse allowing image acquisition to be done in high speed.

Fast MosaiX

Fast MosaiX expands AxioVision with the option of acquiring a large number of high-resolution individual images in a very short time. The use of a flash for exposure ensures that your samples are protected, and, thanks to the optimum interaction between hardware and software, several hundred images per minute can be acquired.

Multichannel Fluorescence

This module allows you to acquire multichannel fluorescence images with up to 32 channels. It can be combined with the Z-Stack, Time Lapse, MosaiX and Mark&Find modules.

MosaiX

MosaiX is developed to analyze large surfaces - MosaiX permits the area of a specimen to be scanned in order to generate one single large image. This electronically created image can thus serve as a map to navigate the specimen and provides a basis for further analyses.

Panorama

Panorama is ideal for specimens which do not fit into the image frame. High-resolution panorama or overview images can be formed with pixel accuracy from individual shots. Even overlapping images can be combined so precisely that all the important details of your specimen are recorded in a single image.

Time Lapse

This module allows you to acquire time lapse images. It can be combined with the Z-Stack, Multichannel Fluorescence, MosaiX and Mark&Find modules.

Z-Stack

This module allows you to acquire z-stack images. It can be combined with the Multichannel Fluorescence, Time Lapse, MosaiX and Mark&Find modules.

Digital High Speed Recorder

With this module time lapse sequences can be acquired in high speed and without compression artifacts. The raw data are streamed to a dedicated hard disk. Either the complete sequence or selected parts of the sequence can be converted into the ZVI image format using a Cutter function.

Dual Camera

This module enables Fast Acquisition to use two identical cameras simultaneously. This effectively doubles the speed of acquisition. Also, if configured with two different emission filters, acquisition of two colors can happen at the same time avoiding motion artifacts which may occur when acquiring images sequentially.

HDR Imaging (High Dynamic Range)

HDR Imaging enables you to produce images with a greater dynamic range than the camera you are using would technically allow.

In certain acquisition situations the dynamics of an image scene, i.e. the range of intensity differences present, can exceed the dynamic range offered by even a good digital camera. An example here would be the acquisition of polished steel sections in the field of materials science. Acquiring images of structures in the reflective surface and in black inclusions at the same time is no easy task.

Another example could be taken from neurobiology, where you may wish to simultaneously capture in an image not only the brightly fluorescing GFP-labeled cell nucleus of a neuron, but also its axons and dendrites, which emit much darker signals.

With the HDR technique, image sequences are generated during acquisition using a constantly increasing exposure time. An initial image that is not overexposed is taken as a starting point. The number of images and the extent of the increase in the dynamics can be adjusted.

Image content that is concealed by noise in the first image is certain to be shown with good contrast in an image acquired using a longer exposure time. On the other hand, image content that is displayed clearly in the image with the shortest exposure time will be overexposed in an image for which a longer exposure time is used.

By means of the weighted superimposition of the component images it is therefore possible to generate a resulting image that offers excellent contrast both in dark image regions and in bright zones.

As pixels are saved with a maximum of 16 bits, 65,535 gray levels is the greatest possible dynamic range that can be stored in an image.

With the help of the adjustable display characteristics in AxioVision, even data with such a high dynamic range can be presented in a meaningful way on a monitor.

Image Processing Modules

ApoTome

An ApoTome system allows you, on the basis of the principle of fringe projection, to generate optical sections through fluorescence samples. The parts of the image that are out of focus are then removed, and an increase in

- image sharpness
- the signal to noise ratio (contrast)
- and resolution in the axial direction

can be achieved.

Imaging Plus

The Imaging Plus module offers you all the main digital image processing techniques. This module comprises functions for image enhancement, gray morphology, image arithmetic and color space transformation.

2D Deconvolution

Using this module you can improve the quality of 2-dimensional fluorescence images in both the lateral and axial direction in accordance with a predefined or self-generated Point Spread Function (PSF).

3D Deconvolution

This module enables you to improve the quality of z-stack fluorescence images using deconvolution algorithms. Using the Point Spread Function (PSF) this 3D Deconvolution calculates out of focus light from all z planes back to its origin.

Four methods are available:

- Nearest Neighbor for fast contrast enhancement results
- Regularized Inverse Filter for access to genuine 3D filtering
- Fast iterative algorithm for high speed restoration
- Iterative Maximum Likelihood Algorithm – the high-end method for maximum light efficiency and increased resolution

Inside4D

The Inside4D module allows you to display z-stack images in three dimensions in a very simple way. Three-dimensional structures can also be viewed as animations over time, and exported as digital video films into AVI format or in MOV format (compatible to QuickTime). Five projection methods are available: shadow, transparency, surface, maximum projection, and mixed. You also have access to a wide range of image processing options (display of cutting planes, anaglyph view, iterative 3D measurement). The zoom factor, for instance, can be freely selected, which also means that you can zoom into structures to view them from the inside.

Widefield Multichannel Unmixing

Widefield Multichannel Unmixing is a module for the removal of crosstalk between fluorescent dyes in multichannel images. Crosstalk is a phenomenon which occurs, whenever dyes are excited by excitation light of more than one filter combination. Up to eight fluorescence channels using a traditional epifluorescence microscope can be separated using unmixing matrices determined either by automatic component extraction (ACE) or by measuring crosstalk in appropriate reference samples.

Image Analysis Modules

Interactive Measurement

This module allows the interactive measurement of a variety of object parameters, such as size, circumference, number etc. Circle measurements can also be performed simply by clicking on several contour points. The measured data are saved together with the image, and are therefore available for additional evaluations.

AutoMeasure (Automatic Measurement)

With this module, measurement programs can be generated very quickly and simply with the help of a wizard. All of the functions required for an image analysis procedure are called up, one after the other. The parameters for the functions can then be set for the measurement task in question, and saved at the end as a measurement program. Once these measurement programs have been defined, you can measure any number of images by performing series analyses.

AutoMeasure Plus (Advanced Automatic Measurement)

This module expands the functions of the AutoMeasure module to include a wide range of additional functions for single segmentation and binary image processing. Here the functions are not executed in a wizard, but are available individually as menu functions. They can be freely combined, allowing you to create your individual measurement routines.

Calotte Grinding Measurement TIC Measurement

During the process of coating materials, quality is controlled by measuring the thickness of layers in accordance with the DIN V ENV 1071 standard. The Calotte Grinding Measurement application module allows you to determine the thickness of calotte section layers. To prepare the calotte section samples, a grinding ball with a defined diameter generates a calotte-shaped hollow in the coating of the sample. This results in circular or elliptical indentations, the diameters of which are determined by drawing in measurement circles. The layer thickness is calculated automatically from the diameters of the measurement circles and the diameter of the grinding ball.

TIC Measurement

The TIC module (**T**otal **I**nterference **C**ontrast – only in combination with the TIC slider from Carl Zeiss) allows precise, contact-free and extremely simple determination of the optical height and thickness of object structures over a range from just a few nanometers through to several micrometers. The advantage of the TIC method over conventional thin-layer thickness measuring instruments (profilometers, atomic force scanning microscopes) lies in the combination of short measurement and analysis times with a high degree of accuracy. The use of circularly polarized light makes the orientation of the structures on the sample irrelevant, eliminating the need for stage rotation. It is even possible to analyze samples with large surface areas.

Particle Analyzer

The Particle Analyzer module allows you to create and carry out particle analysis projects. The aim is to classify particles based on a selectable measurement parameter (in most cases: largest diameter). This classification takes place on the basis of predefined norms or classes determined by the user. Every measurement is managed within an archive as an independent project. The results are analyzed graphically and statistically immediately after the measurement and can be printed out in the form of a report. Work using this module can be divided into two task areas:

- Generating project templates to determine the sequence of an analysis: type of image acquisition (MosaiX, single images etc.), template for input form and report, sequence of image processing and measurement etc. (for administrators only).

- Carrying out the measurement (for all users).

Grains

The Grains module allows you to analyze grain sizes on the basis of the current standards DIN, EN, ISO and ASTM. There is a choice of three different modes: interactive, by means of a direct comparison with overlaid comparative images; semi-automatic, using a chord length analysis; and a fully automatic analysis, based on a reconstruction of even complex grain boundaries.

The following standards are available:

- Interactive: comparison of comparative diagrams – ASTM 1382; BS 4990; DIN 50601 (SEP 1510 – 61)

- Intercepted segment method: EN ISO 643 (2003); ASTM E112

- Automatic: automatic reconstruction of grain boundaries – ASTM 1382

Every measurement is managed within an archive as an independent project. The results are analyzed graphically and statistically immediately after the measurement and can be printed in the form of a report. Work using the module can be divided into two task areas:

- Generating project templates to determine the workflow for an analysis: type of image acquisition (single images from a camera or loaded from a file etc.), template for input form and report, workflow for image processing and measurement etc. (only for administrators).

- Carrying out the measurement (for all users).

Graphite

The Graphite module automatically analyzes the size and shape of graphite particles in cast iron samples in accordance with EN ISO 945. The following analyses are available:

- Spherolite: analysis of spheroidal graphite, volume percent and size distribution

- Lamellar: analysis of lamellar graphite, volume percent and size distribution

- SinterCast: nodularity analysis

Every measurement is managed within an archive as an independent project. The results are analyzed graphically and statistically immediately after the measurement and can be printed in the form of a report. Work using the module can be divided into two task areas:

- Generating project templates to determine the workflow for an analysis: type of image acquisition (single images from a camera or loaded from a file etc.), template for input form and report, workflow for image processing and measurement etc. (only for administrators).

- Carrying out the measurement (for all users).

Multiphase

This module is used for the analysis of multiphase samples. It makes it possible to detect any number of phases in gray-level or color images. The following measurement parameters are available for measurement and classification:

- area, diameter, maximum feret
- perimeter, form factor, ellipse (major and minor)
- volume of sphere, volume of ellipsoid, gray value, color value

Every measurement is managed within an archive as an independent project. The results are analyzed graphically and statistically immediately after the measurement and can be printed in the form of a report. Work using the module can be divided into two task areas:

- Generating project templates to determine the workflow for an analysis: type of image acquisition (single images from a camera or loaded from a file etc.), template for input form and report, workflow for image processing and measurement etc. (only for administrators).

- Carrying out the measurement (for all users).

NMI

The NMI module allows you to determine the content of non-metallic inclusions in steel. Several samples with an area of 200 mm² can be analyzed in a single measurement. Analysis can be performed in accordance with up to 5 standards at the same time.

- ASTM E45-05 methods A and D
- DIN 50 602:1998 methods K and M
- JIS G0555:2003; methods A and B
- ISO 4569:1998; methods A and B
- EN 10247:2007; methods K, P and M

Every measurement is managed within an archive as an independent project. The results are analyzed graphically and statistically immediately after the measurement and can be printed in the form of a report. Work using the module can be divided into two task areas:

- Generating project templates to determine the workflow for an analysis: type of image acquisition (single images from a camera or loaded from a file etc.), template for input form and report, workflow for image processing and measurement etc. (only for administrators).

- Carrying out the measurement (for all users).

QuantiFISH

The FISH technique (FISH = Fluorescence-In-Situ Hybridization) is used in the fields of cytogenetics and tumor research to localize specific DNA or RNA sequences in chromosomes. The QuantiFISH module offers you the quantitative determination of FISH signals in DAPI-stained, interphase cell nuclei of multichannel images or multichannel z-stack images. The results of the FISH analysis are displayed both in the image and in data lists.

Ratio

To observe processes inside cells, use is often made of fluorescent dyes. This technique makes it possible to measure the concentration of messengers, e.g. Ca^{2++}, over a certain period of time. The Ratio module offers you a suitable analysis method by means of the ratiometric processing of two fluorescence channels in multichannel time lapse images. The calculated ratio image can be displayed as a pseudo color image, making it possible to draw conclusions about the concentration, for example. In addition, the module allows you to measure the intensity of the fluorescence of selected cells over time. The results are displayed graphically and in data lists after the measurement has been performed.

Tracking

It is often necessary to observe the movement of cells or other objects during biological processes. In many cases the processes in question are transport or growth processes that require quantitative analysis. With the Tracking module you can track moving objects in time lapse sequences using either an automatic or an interactive method. The calculated tracking parameters (e.g. distance, speed, direction etc.) are displayed in data lists.

TMA

Tissue micro arrays consist of numerous small, circular tissue samples with a defined diameter, which are fixed on a single slide. The TMA module makes it possible to acquire a composite image of the tissue micro arrays. And you can save the positions in a Mark&Find List.

Colocalization

The acquisition of fluorescence images in several channels makes it possible to visualize the relationship between biological structures. The Colocalization module provides you with a tool that enables you to determine colocalization quantitatively. It is always the colocalization of two channels that is analyzed. For images with more than two channels the user must make a selection. Colocalization results from the pixel-by-pixel comparison of intensities per channel.

Topography

With this module you can generate a height map (topographic image) from a z-stack and carry out roughness measurements in accordance with EN ISO 4287. To allow you to display the various aspects of the topographic image, a rotation and a tilt mode are available in addition to four different 3D views (texture, projection, grid, surface).

Online Measurement

This module allows you to perform measurements in an online image using interactive measurement tools. You can therefore generate measurement values very quickly without having to acquire the image. In addition, predefined or generated grids can be inserted into the online image and compared with the structures in the image (e.g. to inspect components).

3D Measurement

This module offers you a range of possibilities for the measurement of three-dimensional objects. The image stacks are presented as a 3D volume model, which allows the user to define the surfaces of interesting objects interactively. A segmented data set is then generated from these settings. Using the binary image processing function you can further process the objects obtained, e.g. by means of interactive object separation. The measurement of the 3D objects then takes place automatically for the entire image stack or interactively by clicking on individual objects in the 3D view. The function calculates morphometric parameters (e.g. barycentric coordinates in x, y and z, volume or surface content) and densitometric parameters (such as mean density of the object or standard deviation of mean density of gray values) for all objects. In addition, field-specific parameters can be calculated for the entire 3D image, e.g. number of 3D objects in the image, total volume of all 3D objects or sum of the surfaces of all 3D objects.

Physiology

The Physiology module enables you to acquire time lapse images at a speed that is only limited by the technical limitations of the devices you are using (camera, light source) and the selected camera settings. To achieve such speeds, the Physiology module automatically uses a "streaming" technique, which involves the raw camera data being written directly to their own fast **streaming hard disk** (or a suitable empty partition).

The results of the acquisition process are therefore a streaming document (*.ziAR) and the data measured during acquisition (online).

At the same time, enough memory is reserved so that the measurement data can also be managed. Physiology calculates the maximum possible number of individual images that can be acquired from the number of measurement regions drawn in and the memory available on the streaming hard disk. As this value is dependent on numerous factors (binning, ROI, number of measurement regions drawn in, demands made on available free memory by other applications etc.), the entire hard-disk space that is theoretically available cannot always be used.

In a second step, which is separate from the acquisition process, the streaming document is converted into a regular ZVI image document. With the aid of a sort of video editor (Cutter) interesting regions in the image as well as interesting time sections can be selected and converted potentially reducing the data volume dramatically. Converted ZVI files are required for all further processing steps in AxioVision.

Thirdly, a range of evaluation methods allows direct measurements of gray intensities or ratiometric measurements of various kinds (exitation- and emission ratio imaging) as well as a range of FRET (Förster resonance energy transfer) measurements. In addition a custom formula editor allows the generation of user defined evaluation formulas.

SFM

This module allows you to analyze multichannel fluorescence images of cytospin specimens. It also offers a predefined workflow for detecting and measuring the cells that can be adapted flexibly to the sample in question. The measurement results can be displayed in the form of an image gallery, data table, histogram or scatterplot and provide additional information to accompany the results of flow cytometry.

Coal Random Reflectance

This module is used to analyze coal samples by measuring reflection in unpolarized light.

The functionality comprises a flexible calibration process for user standards and multiple sample analysis with integrated autofocus function. During the analysis you can monitor the progress of the measurement in the online reflectogram for the current field and the accumulated fields. The measurement results are presented clearly in a report with a reflectogram and V-type data.

From the sample data generated it is also possible to produce a theoretical mixture by combining up to six individual profiles.

Comparative Diagrams

Using the Comparative Diagrams module it is possible to compare micrographs with comparative diagrams interactively. Various views can be used:

- Micrograph and one comparative diagram
- Micrograph and two comparative diagrams
- Micrograph and four comparative diagrams
- Micrograph and eight comparative diagrams

The result you obtain takes the form of a table, along with the micrographs, if required. Comparative diagrams are possible for up to five freely definable dimensions.

Every measurement is managed within an archive as an independent project. The results are analyzed graphically and statistically immediately after the measurement and can be printed in the form of a report. Work using the module can be divided into two task areas:

- Generating project templates to determine the workflow for an analysis: type of image acquisition (single images from a camera or loaded from a file etc.), template for input form and report, workflow for image processing and measurement etc. (only for administrators).

- Carrying out the measurement (for all users).

ASSAYbuilder

The ASSAYbuilder module is a so-called "high content analysis" module and efficiently generates high-quality and biologically relevant data from your image material.

ASSAYbuilder describes your fluorescence images in the form of numerous key parameters and provides objective, quantitative data that can be used as a basis for decisions relating to the planning of further experiments or to support published results. The module is operated using guided workflows and an intuitive graphical user interface. No programming knowledge is required.

Five analyst modules support you with solutions that have been specially tailored to different biological tasks: Physiology Analyst, Morphology Analyst, Membrane Analyst, CellCycle Analyst, Motility Analyst. Examples of supported applications include cell-based toxicity tests, transcription factor activation, cell-cycle analyses, cell-movement analyses and morphology determination.

ELISPOT

The Elispot (Enzyme Linked Immuno Spot) analysis is a sensitive method for detecting and quantifying individual T lymphocytes that generate cytokine spots as a result of antigen contact in vitro. With the help of the ELISPOT system it is possible to determine the number, size and intensities of the spots automatically. Analysis is carried out immediately upon acquisition of the well images or, alternatively, using saved well images. The ELISPOT system has been developed as a complete system solution for the precise and automatic analysis of Elispot assays, in particular also for use in routine laboratories.

Archive Modules

Asset-Archive

With the Asset-Archive module you can save images, measurement data lists and reports etc. as "assets" in a hierarchically structured database. You have the option of creating various project types in which you can then save your assets. A contact management function allows you to allocate projects to different clients etc. You are also able to classify and structure your assets within your own customized categories. The option of a full-text search enables you to find individual data sets quickly. In addition, asset- or project-related filters allow you to carry out targeted searches for information in individual data fields.

Long-term archiving is also possible, and gives you the option of transferring your projects and the corresponding assets onto CD, DVD or magnetic tape.

Configuration Modules

Commander

The Commander module allows you to record steps for image acquisition, processing, and measurement in a single command – a script. Finally a script can be executed on any number of images automatically.

VBA

Should any users find that even the wide-ranging functions offered by AxioVision do not meet all of their requirements, they can increase the available options still further and adapt the program to their needs. This process is based on **V**isual **B**asic for **A**pplications. VBA offers a complete, integrated development environment with which programmers are familiar. As VBA has been directly integrated into AxioVision, it offers the advantage of fast interaction and the possibility of developing solutions without additional programs.

1.3   AxioVision and Microsoft® Windows

AxioVision fully supports the usual Microsoft Windows standards:

Files are stored in accordance with Microsoft Windows rules in the folders provided for the purpose ("My Documents", "Documents and Settings"), so it is easy to locate the files you need.

In multi-user environments, there is no need to make separate settings for AxioVision in order to keep the configurations and data of individual users separate and to protect them from unauthorized access. User administration is also based entirely on the settings in Microsoft Windows.

1.4 User's Guide and Online Help

The AxioVision manual and online help should not be regarded as separate entities. When used in combination, they offer you immediate access to comprehensive information.

The User's Guide contains a broad overview of all program modules, with detailed step-by-step instructions covering all the basic functions and describing the activities that arise most frequently.

At the beginning of each chapter you will find information describing the background to the relevant section of the program or the relevant module in more detail. This may be conceptional information or a description of theoretical details. The introduction also contains a step-by-step description of operation. Due to the extremely wide range of functions offered by AxioVision, it is not possible to describe every conceivable operational situation in detail. However, once you have worked through the steps described in the manual, you should be able to apply these procedures to your specific task.

The Online Help provides full details of the parameters and setting options of a function. It is therefore the ideal complement to the manual. If you have a question about a specific parameter, press the **F1** key to obtain further information on it. The online help does not, however, contain descriptions of procedures of the type found in the manual.

We strongly recommend that you familiarize yourself with the program using the examples described in the manual before you start working on concrete projects with AxioVision. Once you are familiar with the general operating procedures, you will find all the information you require in the online help.

1.5 Backup Copies

We strongly recommend that all users save the data they create, such as images, measurement data, archives, reports, forms and documents, at regular intervals on an external medium. Otherwise it cannot be excluded that access to this data may be lost as a result of operational errors or hardware defects. Carl Zeiss and Carl Zeiss Vision accept no liability for consequential damage resulting from insufficient data protection.

2. Overview and Operating

2.1 Overview

AxioVision's main window is divided into two main areas: the **Workarea** and **Workflow** on the left-hand side, and the document area on the right-hand side. The elements of the **Workarea** and the **Workflow** are used to select and operate image processing functions and functions for controlling the camera and microscope.

In the document area, all document types (images, archives, reports, forms) are displayed and edited. The main window also contains the Microsoft Windows specific control elements, such as the main menu, toolbar and status bar.
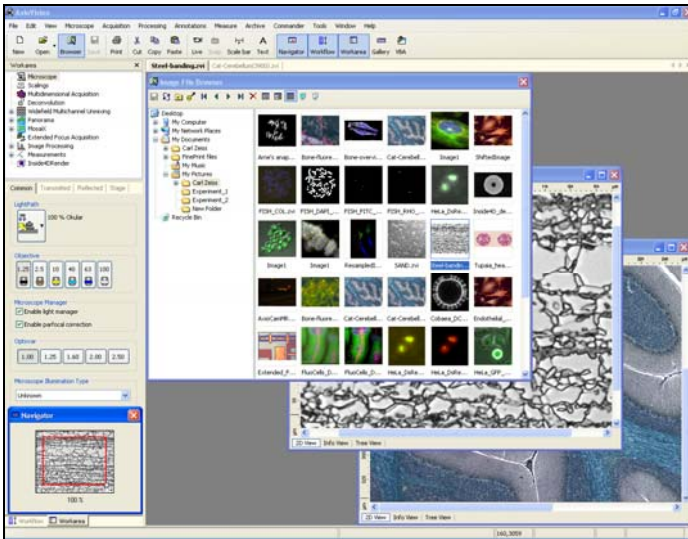
AxioVision's main window

2.2 Workarea

The elements within the workarea (see figure below) can be used to

- select processing functions on the work tab.
- set parameters for processing functions on the property pages.

By dragging the title bar you can separate the **Workarea** and the Workflow, or move one on top of the other.

Activated **Microscope** property page

Setting of functional parameters.

Tabs for switching between **Workflow** and **Workarea**.
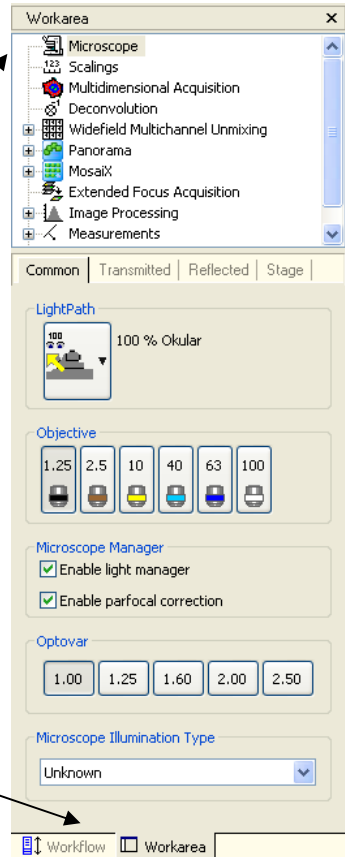
NOTES:
- All of the workarea's functions can also be found in AxioVision's main menu.

- The workarea and workflows are displayed one on top of the other by default. To switch between them, use the tabs at the bottom of the workarea.

- If you have hidden the workflows, when displayed again they are shown next to the workarea. You can, however, move them back on top of the workarea by dragging the title bar. This gives you more room to display documents.

- You can show and hide the workarea using two different methods: either via the menu commands **View** ⇒ **Windows** ⇒ **Workarea** or via
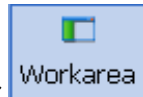
  the button on the **Standard** toolbar .

- If the **Standard** toolbar is hidden, select the menu commands **View** ⇒ **Windows** ⇒ **Standard**.

2.3 Workflow

One very simple way to operate AxioVision is to use workflows for routine tasks that need to be performed again and again. All you have to do is click on the functions in the workflow one after the other. You will then achieve the desired results quickly and smoothly.

If you also hide all the elements that are not required, such as the workarea and toolbars, you can concentrate fully on the work you are actually performing.

Activated **Standard** workflow.

The last function executed is indicated with an arrow.

To activate other workflows, click on them. They then scroll upwards.

Tabs for switching between the **Workarea** and **Workflow**.

Workflow	×
Standard	

Live Properties

Snap

Show Properties

Scale bar

Draw Annotations

Navigator

Save

Measurement

Graphite

Grains

Multiphase

Online Measurement

Workflow | Workarea

NOTES:

- All the functions available in AxioVision can be used in a workflow (see also chapter 8 "Configuration").

- The workarea and workflows are displayed one on top of the other by default. To switch between them, use the tabs at the bottom of the workarea.

- If you have hidden the workflows, when displayed again they are shown next to the workarea. You can, however, move them back on top of the workarea by dragging the title bar. This gives you more room to display documents.

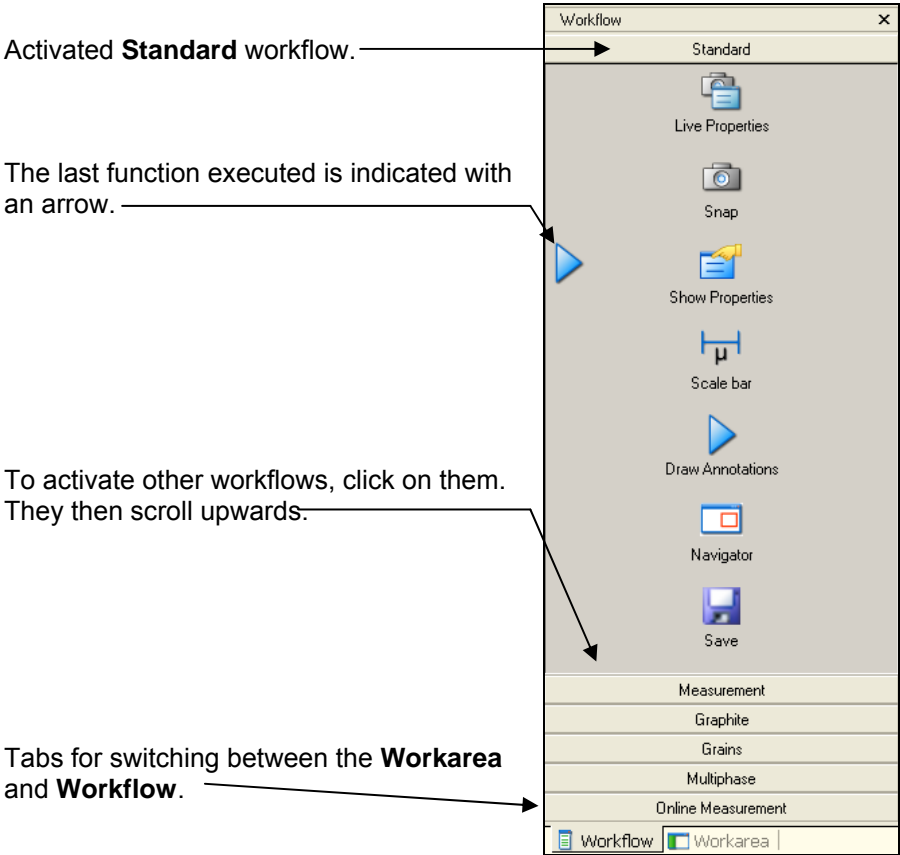- You can show and hide the workarea using two different methods: either via the menu commands **View** $\Rightarrow$ **Windows** $\Rightarrow$ **Workflow** or via the button on the Standard toolbar: .
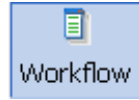
- If the Standard toolbar is hidden, select the menu commands **View** $\Rightarrow$ **Windows** $\Rightarrow$ **Standard**

2.4 Own Toolbars and Dialogs

You have the option of generating your own toolbars and dialogs to create a system that is tailored entirely to your own needs. If the range of setting options in the dialogs for camera and microscope control is too detailed for the work you are performing, you can therefore (with just a few clicks of the mouse) generate your own dialog containing only those control elements that you require for your work. This dialog can then be placed on a self-generated toolbar, and all the other default elements can be hidden. You then have a unique system that is optimized to meet your own individual requirements.

You can call up the **My Dialog** function here via **My Toolbar**.
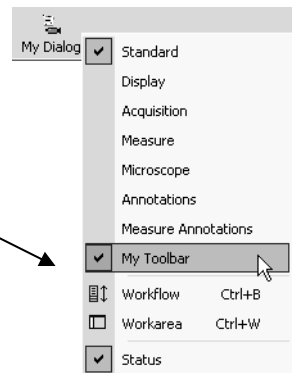
NOTES:

- You can generate as many personal toolbars as you want.

- Your own dialogs can also be used on default toolbars.

The **My Dialog** dialog contains
options for **Setting** the exposure
time and changing the
objectives, and for the focus bar.

Functions for controlling the
scanning stage can be found on
a second tab sheet: **Scanning
Stage**.
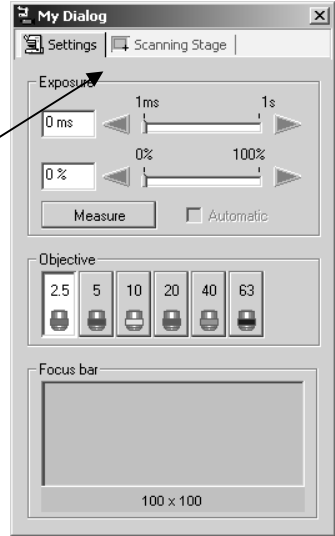
2.5 Shortcut Menus

Shortcut menus contain functions that are frequently required in connection with documents (e.g. images) and certain elements of the AxioVision interface. They are the easiest way to select these functions.

Shortcut menus are available for every element of the user interface. They can be activated at any time by right-clicking on an element (see *shortcut menu for an image* below):

✗	Normal View (1:1)	
🔍	Auto Zoom	
	Zoom In	▶
	Zoom Out	▶
🔍	+ Zoom	F7
🔍	- Zoom	F8
	Player	▶
🔲	Full Screen	F11
📷	Adjust window to image	
📏	Ruler	
🅰	View Annotations	
🔳	Show bounding box	
	Display tile info	
	Change Image Dimensions	
▶	Player Panel	Ctrl+L
✔	Display Panel	
📄	Properties	Alt+Enter
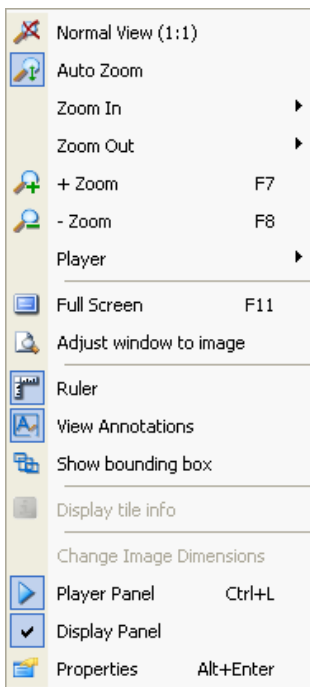
2.6 Properties Window

The **Properties** window is required whenever you want to look over or to change the appearance, the behavior or certain additional information relating to a document (e.g. an image) or a particular element of the AxioVision interface. In this window, behind the tab sheets (**Display**, **Attributes, Colors**, ...), you will find a list of the relevant information (e.g. at an image) and/or will be given the option of entering or modifying certain elements.

This may be information on the date of saving and the location where an image file is to be saved, for example, but may also relate to the formatting (**Attributes**) of text, lines and other objects you have drawn into the image (**Annotations**). See *Property window of an image* below:
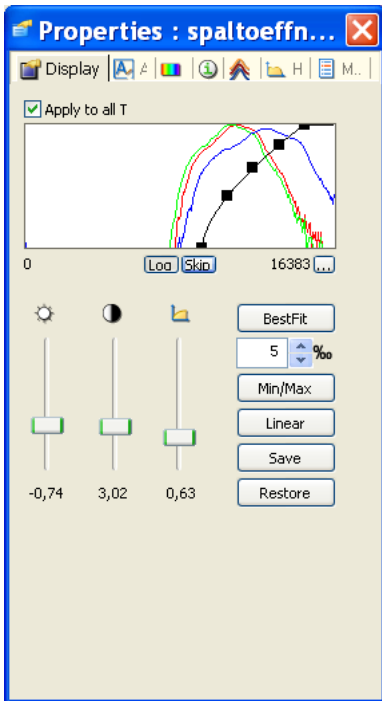
As the property window plays a central role in the operation of AxioVision, it can be opened in a number of different ways:

- Via the **Properties** function in the shortcut menu (see previous section).

- Via the **Properties** function in the **View** menu.

- By pressing the key combination **Alt**+**Enter**.

3. Image Acquisition

3.1 General

In order to process images using a computer, you must first acquire them in a form which the PC can understand. This requires an electronic camera, which acquires the image information from the microscope and converts it into electrical signals. These signals are digitized and then processed and displayed on the computer.

Compared to analog camera technology, modern digital camera technology offers advantages in terms of the signal quality and camera resolution that can be achieved. With this technology, the data are digitized directly in the camera.

To transfer the data to the PC, you need to use frame grabbers, interface boards or standardized digital interfaces. These are operated using special software programs (known as drivers). With these device drivers integrated into AxioVision it is now possible to acquire images via them.

When installing framegrabbers, interface boards and interfaces, use the corresponding drivers supplied by Carl Zeiss and the associated manuals/the manuals from the respective manufacturers.

The description below assumes that the frame grabber or interface board and the corresponding driver have been successfully installed.

Detailed information on the cameras/framegrabbers supported by AxioVision can be found in the current AxioVision online help, in the AxioCam HR, MR and MRc5 installation and reference manuals, and in the PDF files of the manuals, which can be found on the product DVD.

3.2 First Steps

When taking your first steps with the system, if possible use a simple sample so that it will be relatively easy for you to determine whether the resulting image is good or bad.

Place the sample under the microscope, and set the microscope in such a way that you can see a good image through the eyepiece. Switch the microscope's beam path to the TV adapter, e.g. **50% Topport** and **50% Ocular**.
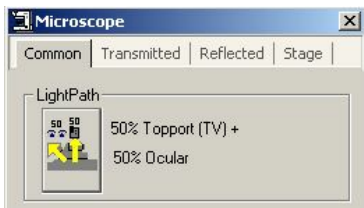
NOTES:
- If you are not yet familiar with your camera's functions, make sure that you have the respective manuals to hand so that you can look up any relevant information.

- This text describes the main procedure for image acquisition. For explanations of the functions and setting options of all the cameras supported by AxioVision, please refer to the manual for the camera concerned. The description for the Carl Zeiss AxioCam product family serves as an example for all supported cameras.

3.3 Conditions

The text below describes how to achieve your first image in AxioVision with just a few clicks of the mouse (here with the AxioCam HR). The **Standard** workflow is the easiest way to control image acquisition. If the workflows are not displayed, in the **View** menu select the **Windows** function, and there select the **Workflow** command.

NOTES:
- The workarea may in fact be open but covered by the workarea. If that is the case, simply click on the **Workflow** tab at the bottom edge of the workarea ⬚ Workflow ▢ Workarea .

The Standard workflow
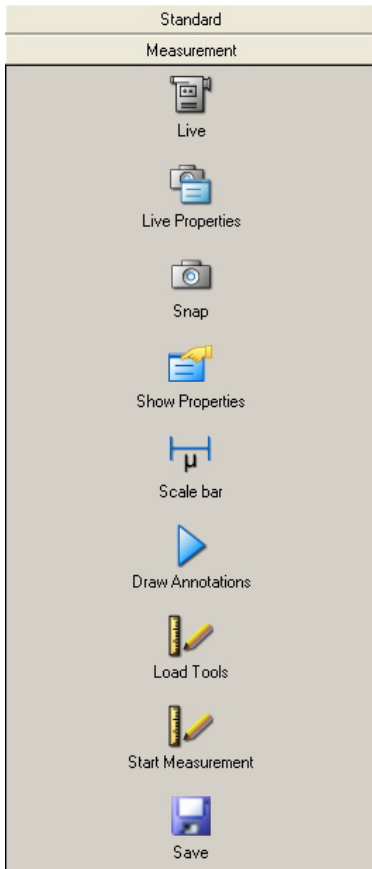
Live starts and closes the live window.

Live Properties opens and closes the window for adjusting the display characteristic curve and for controlling the camera (exposure time, white balance etc.).

Snap acquires an image using the active camera.

Show Properties adapts the image display using a characteristic curve and shows the parameters of the image.

Scale bar inserts a scale bar.

Draw Annotations inserts a caption and scale bar into the acquired image.

Navigator opens the navigator window for zooming.

Save saves the image.

As the icons are identical on all menus, the following description applies both to operation via the toolbar and to the workflow.

Step by step to the first image

➢ Select the camera you want to use (if only one camera is installed, this step is not necessary).

➢ Set the light path to the camera. Then click on the **live image** icon. The live window is opened to display the camera image.
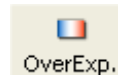
➢ This function can also be called up via the **Acquisition** ⇒ **Live** menu.

➢ Now focus the camera image and select the frame you want to acquire.

➢ In the footer you can select a suitable scaling for the objective you are using from the **List of available scalings.**
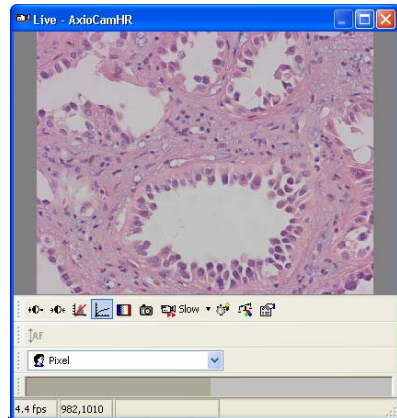
➢ Clicking on this icon overexposed (too bright) areas will be displayed in red in the live image.

➢ Clicking on the gamma icon in the bottom line of the live image accomplishes the optimum color reproduction.
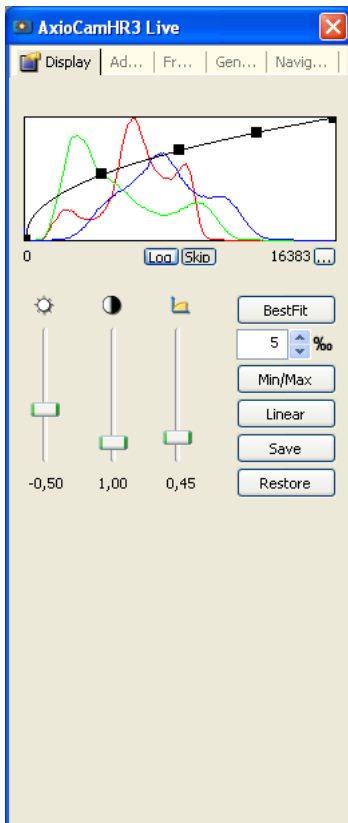
➢ In this drop-down list box you can select the speed of the live image (**Slow, Middle, Fast**).

➢ Click on the icon **Live Properties** to open the live properties.

Here you can see the settings for the display of the image like Brightness, Contrast and Gamma. Settings made here are passed on the acquired image.


Note: Only the display is adapted, the camera data remain unchanged.

Make changes for the camera settings on the following controls:

➢ The central element is the setting for the exposure time on a digital camera. The **Measure** button can be used to determine an optimum exposure time. Check the quality of the exposure in the live image.
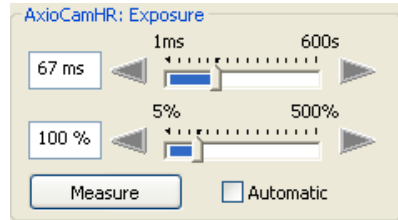
➢ The arrow keys and sliders can be used for fine adjustment of the exposure time.

➢ Exposure time can be edited via the keyboard.

➢ **Auto Snap** performs an exposure measurement before the acquisition of each image. **Auto Live** adjusts the exposure time continuously in the live image.

➢ If you are using a color camera, you need to perform a white balance for the image. If you select the automatic balance (**Automatic** button), the camera tries to determine an optimum value itself.
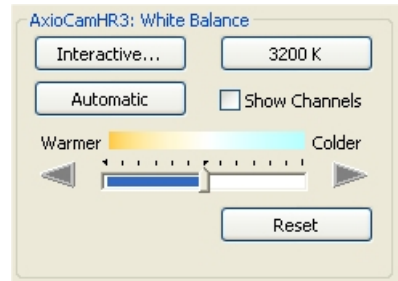
➢ Using the **Warmer/Colder** slider you can shift the target value of the white balance to warmer ($\rightarrow$ redder) or colder ($\rightarrow$ bluer) tones.

➢ The **Show Channels** check box allows you to switch to an alternative setting option if you wish to influence the target value of the white balance manually for each channel.

➢ If you are using a color camera, you need to perform a white balance for the image. If you select the automatic balance (**Automatic** button), the camera tries to determine an optimum value itself.
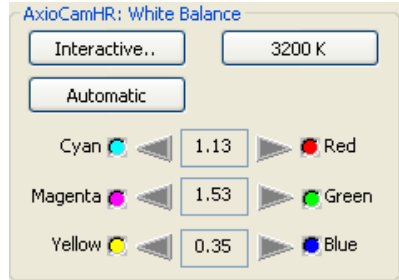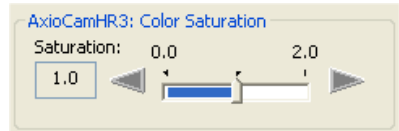
➢ Move the slider to increase or decrease the color intensity of the acquired images.

➢ Enter further settings for the selected camera on the **Adjust**, **Frame** and **General** property pages.
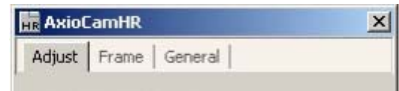
➢ Click on the camera icon to acquire an individual image, or in the **Acquisition** menu click on **Snap**.

➢ Click on the **Show Properties** icon and activate the **Display** tab. Any changes you make here are displayed in real time in the image window.

➢ Move the sliders
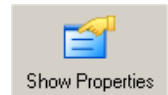
Gamma
Contrast
Brightness

up or down to adjust the image.
Good color reproduction on a
monitor is achieved with a gamma
value of around 0.45.

➢ To find the optimum setting for
contrast and brightness, click on
Min/Max.

➢ To undo all your changes, click on
Linear.
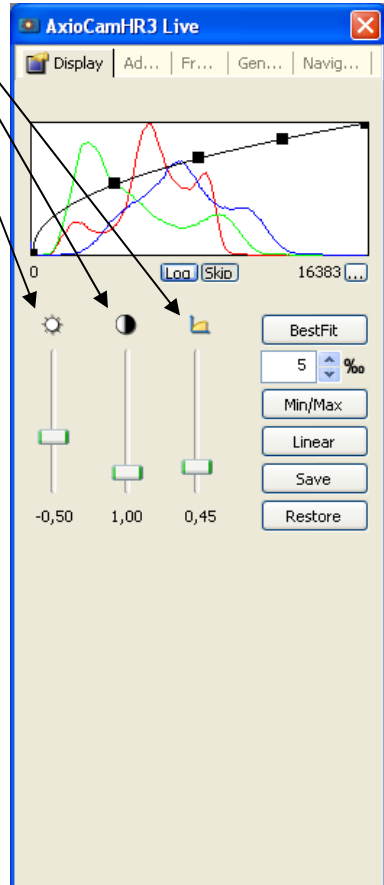
➢ You can use this icon to insert a
scale bar into your image. The
appropriate scaling must have been
generated and selected in advance.
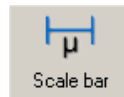
➢ This icon is used to open the
window for generating annotations.

➢ Lettering and marking can be inserted in the **Draw Annotation(s)** window.
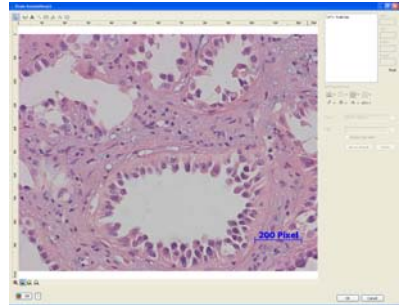
➢ In the dialog window **Draw Annotation(s)** the icons **scalebar**, **text**, **line**, **rectangle**, **outline**, **curve** und **ellipse** are available in the menu line.

➢ In the dialog window **Draw Annotation(s)** a field for **Settings for selected items** as well as for **Font** are available. If **Display tag name** is activated, you can use the

[...] icon in the drop-down list box **Tag**. The dialog window **Select tag** opens where parameters for lettering can be selected.
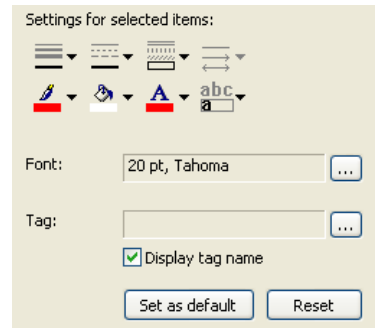
➢ In the dialog window **Draw Annotation(s)** zoom symbols and two buttons to toggle the color channels are available at the bottom of the image window.
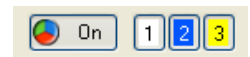
➢ Using the navigator you can magnify or reduce the image and
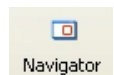
position the displayed image area in
the overview window.

➢ Click on this icon to save the image
you have generated to the
computer's hard drive.

NOTES:

• Once you have found a good camera setting, you can save it with the
Settings Editor in the **Tool** menu and load it again automatically in the
future. This allows you, for example, to acquire particular samples
under the same conditions every time.

• To allow correct scalings to be selected in the footer of the live image
during acquisition, you need to have generated these before you start
your work. If you are using a motorized or encoded microscope, the
scalings can be allocated automatically. For further information see
Chapt. 9 "Configuration".

3.4 Practical Notes on Operation in AxioVision

Below you will find a few practical notes on the speed of the live image, color adjustment, working with exposure times, fluorescence images and configurable toolbars.

Speed of the live image

There are three speed modes available for the AxioCam HR live image. If you use the pointer to click on the Slow icon at the bottom edge of the live image, a pop-up menu with the possible setting options appears. The current setting is displayed at the bottom edge of the live image.

Each mode has its own particular compromises.

Live mode	Description
Fast	Greatly reduced resolution, fast frame rate
Medium	Medium resolution, moderate frame rate
Slow	High resolution, slow frame rate

The image rate can be further increased by deactivating certain AxioVision features:

• Deactivating the focus bar.

• Deactivating the live image scaling (1:1 representation of the image).

• Activating a frame for the live image (**Size**) on the camera property page **Frame**.

• Set image orientation to original on the **General** property page (no rotation).

NOTES:
- Note that the above mentioned notes are only valid for <u>short</u> exposure times, as for very long exposure times the speed of the acquisition is mainly dominated by the exposure time.

- For very long exposure times, the live image will appear grainy, as automatic amplification is active. However, this will not influence the quality of the final image.

## Notes on Optimum Color Reproduction in AxioVision

Below you will find a detailed description of how best to set up AxioVision to achieve optimum color reproduction with the AxioCam HR.

NOTES:
- For an optimum color reproduction the monitor first has to be adjusted. To do so please use a well-suited image.

Adjusting the display characteristic curve in AxioVision:

In order to achieve optimum reproduction of color images of the AxioCam HRc data on a monitor, the display characteristic curve must be used to set gamma compensation. The advantage here is that only the display is influenced and the actual data remain unchanged. Further details on this can be found below. The settings can be done as follows:

➢ With the mouse click on the icon G. 0,45 in the footer of the live image. This adjusts the display characteristic curve automatically for an optimum color reproduction of the monitor (gamma = 0.45).

Or in an already acquired image:

➢ In the **Properties** dialog window, set the gamma characteristic curve in the histogram image on the **Display** property page.

NOTES:

- This also influences the reproduction of the color saturation.

- For fluorescence images, background correction can also be carried out simply by shifting the offset of the characteristic curve.

- Clicking on this icon returns to a linear display of the data.

3.5 HDR - High Dynamic Range

General

HDR imaging offers you the possibility of increasing the camera-specific dynamic range. If you want to capture all the information from objects that are extremely rich in contrast, it is usually necessary to acquire several images, which need to be adapted to the relevant exposure situation. Either the very bright or the dark structures can be correctly exposed. With the HDR module it is possible to document all the information in a single image.

To achieve this effect, depending on the parameters set, several images are automatically acquired with different exposure times and subsequently merged into a single image with an increased contrast range.

To achieve the best possible result with your image, it is crucial to set the exposure time precisely. No position on the specimen may appear overexposed. This is the only way to guarantee that the information acquired in the brightest structures is differentiated and contributes to the HDR image. In this case a simple measurement to determine the correct exposure time prior to acquisition is not suitable, as this involves performing average metering that is subject to tolerances. Instead, the overexposure warning should be activated and the display characteristics in the non-logarithmized display should be adjusted in such a way that there genuinely is no overexposure of any pixel.

The advantages of the technique depend to a great extent on the specimen and on the microscopy technique being used. A sample in transmitted-light bright field generally has only a relatively flat dynamic range. This means that the effect you notice from the use of the HDR method will not be an increase in contrast, but merely a reduction in image noise. Images of specimens acquired using other contrast techniques, such as dark field, reflected light or fluorescence, exhibit a stronger increase in contrast, depending on the structure in question, as they often have a higher dynamic range than that of the cameras used.

In many cases, it is not possible at first sight to see a clear improvement in the acquired HDR image compared to a normal image. This is due to the

limited display depth of monitors, which usually have a contrast range of 8 bits. As no further tone mapping is performed in AxioVision to map the increased dynamic range to a monitor, an adjustment can only be made with the help of the gamma setting (non-linear bending of the display curve).

The additional information gained from expanding the dynamic range is most noticeable when performing measurements. Thanks to the more differentiated display, more brightness gradations are available to the user, which means that as well as a distinction in bright structures there is also nuancing in dark areas of the image. This allows segmentations to be performed more precisely and measurement results to be refined.

NOTE:
- For detailed information on the individual functions and parameters, please simply refer to the online help (F1 key).

HDR imaging

In the following sections you will see that acquiring an HDR image using an AxioCam is extremely simple. By bearing a number of fundamental aspects in mind, you will quickly achieve good results.

If you are using a camera from another manufacturer, it may be necessary to change parameters in **HDR Setup**, even for simple HDR imaging. This will be mentioned at the relevant points and the procedure will be explained in more detail under the final point "Basic settings in HDR Setup".

HDR Mode

The quickest way to obtain an HDR image using an AxioCam is to use **HDR Mode**.

This mode is independent of the other image acquisition modules and can be activated individually for all acquisition functions.

You will find this function under the following menu item: **Acquisition** $\Rightarrow$ **HDR** $\Rightarrow$ **HDR Mode**.
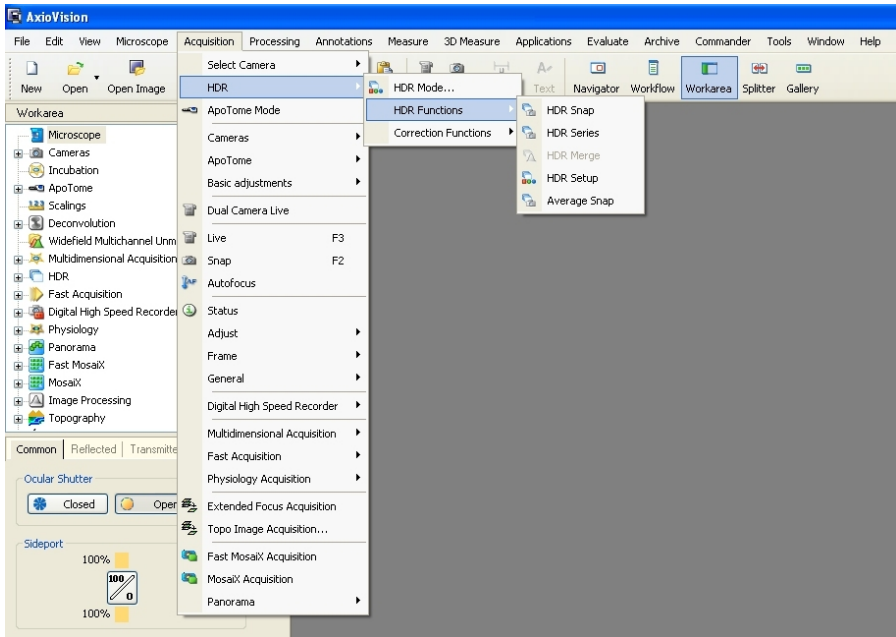
If you activate HDR Mode, all subsequent images will be generated with an increased dynamic range.

HDR Mode can be operated in two different settings. On the one hand, there is Simple Mode, in which only the dynamic extension can be set. In this case the other information required is completed automatically. Beneath the slider you will find the information on the number of images used, the source for merging the dark value and the extension factor. This is therefore a good way to get to know HDR Mode without having to set a large number of parameters. Expert Mode, on the other hand, contains all the setting options required to individually adapt the HDR image.
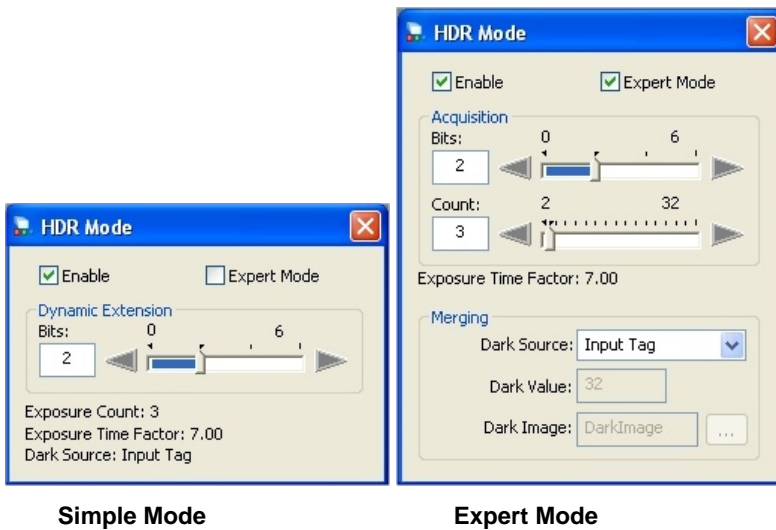
Simple Mode   **Expert Mode**

The acquisition parameters should be selected carefully, especially in Expert Mode, as these define the duration of the exposure and therefore the burden placed on the sample. To give you an idea of the exposure time to be expected, a factor for the extension of the overall acquisition time is shown underneath the acquisition parameters.

As a rule of thumb for the correct setting of HDR Mode, the following should apply: a dynamic extension of 2 bits should result in the exposure count increasing by 2. This guarantees good information density in the HDR image.

The predefined settings with an extension by 2 bits using 3 images and the source "Input Tag" for merging the dark value are a good starting point for working with an AxioCam.

PLEASE NOTE:
If you are using a camera from another manufacturer, it cannot be guaranteed that an "input tag" for merging the dark value will be transferred to AxioVision and consequently that the result will be free of errors. If the source "Input Tag" is not available for the calculation, the dark value set is used instead (see also the section "Basic settings in HDR Setup").

Simplest way to generate an HDR image

Following this brief explanation of the different versions of HDR Mode, we will now return to a description of the quick acquisition of an HDR image.

Activate HDR Mode in the simple display and set the slider to a dynamic extension of 2 bits. If you are using HDR Mode for the first time, the following data will be completed automatically and displayed in the information rows underneath the slider: an exposure count of 3 images, an exposure time extension by a factor of 7 and the dark source "Input Tag". If you have already used HDR Mode and have changed the input parameters, these values will be displayed and applied.

Please therefore always pay attention to the settings to ensure that your HDR image is acquired as you want it to be.

Now you need an object that is rich in contrast and must determine the correct exposure time. To do this, activate the overexposure warning. This makes it easy to determine the right acquisition time. Adjust the exposure time in such a way that no overexposed pixels are displayed. The more precisely you do this, the more gradations you will be able to differentiate later in the bright structures of the HDR image.

Once the correct exposure time has been determined, you now only need

to click on or the F2 key.

Everything else takes place automatically and the output image will already show the HDR image with a dynamic range that has been increased by 2 bits.
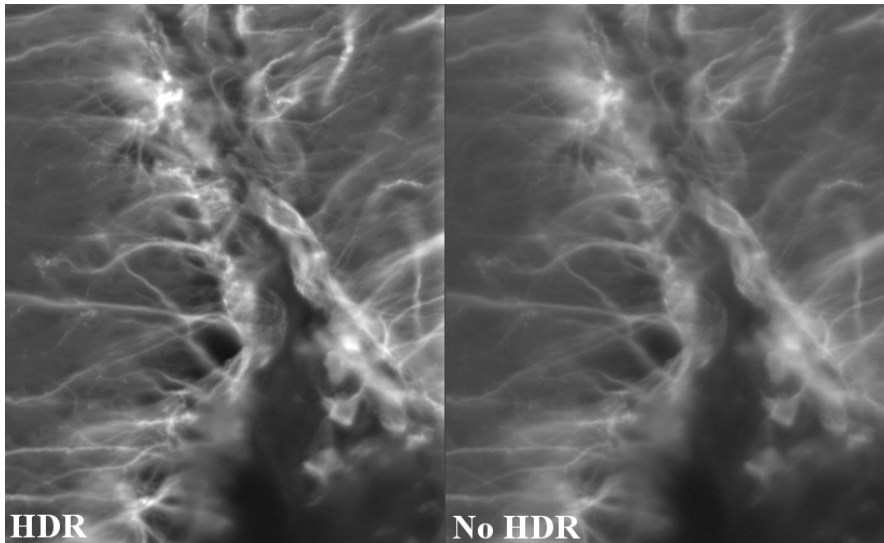
An individually adapted HDR image step by step

Another way to obtain an HDR image is to make use of the individual functions.

These can be opened either via the **Acquisition** menu or in the work area under **HDR** $\Rightarrow$ **HDR Functions**.

The individual functions allow you to enter individual settings for an HDR image and adapt it to the properties of the various cameras. Using the individual functions is also an excellent way to familiarize yourself with the operation of the program and the various merging and setting options.

A good HDR image can be improved even further by means of a calculation using an adapted dark value. This means that an individual black-reference image is subtracted for each exposure time. Only a few additional operations are necessary to do this. Black references are normally created before the actual acquisition of the image. In this case, due to the individually adapted exposure times, it is easier and less time-consuming to acquire the black reference after actual image acquisition. One condition for achieving a flawless result with your image is to ensure

that the beam path is absolutely free of stray light. You should therefore always make sure that the camera sensor is not receiving any light at all.

For the HDR image you must first adjust the specimen and define the appropriate exposure time. As with the use of HDR Mode, make sure that there are no overexposed areas in the image (activate overexposure warning). This is the only way to guarantee that you will gain the maximum information.

➢ Open the **HDR Series** function and set the **Image Count** and **Extra Bits** parameters for the increase in the dynamic range.

With these two details you have defined all the important data for acquisition itself.
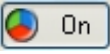
➢ Once everything has been set to your satisfaction, click on the ⎣ Apply ⎦ button.
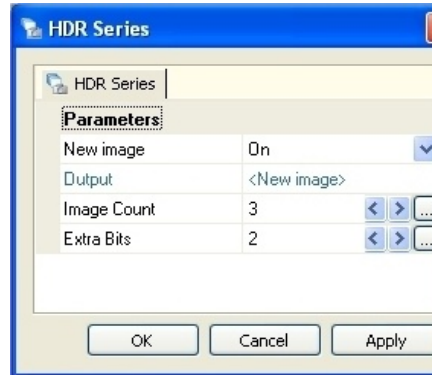
HDR Series generates an image series consisting of images with different exposure times. The various component images are displayed in a multichannel image. This means that you can view all the exposure times separately from each other, analyze them and save them for other uses.
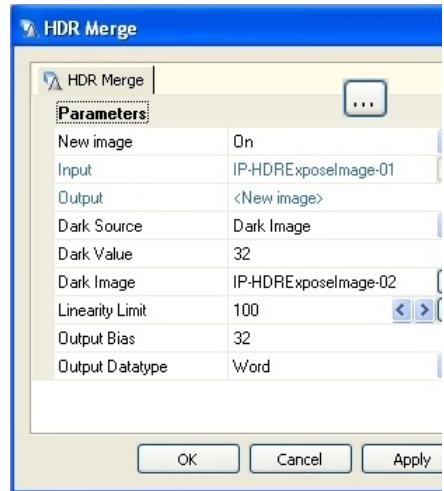
To generate the black reference you only need to close the light path to the camera and acquire a black-reference image using the same settings as before for the HDR image.

NOTE:
• The display of the series initially shows you an overlay of all images

acquired. Click on the ⎣ ● On ⎦ icon to view the individual images.

To obtain a finished HDR image, all that you now need to do is merge the individual components. To do this, select the **HDR Merge** function.

> ➢ To merge the black-reference series previously acquired, set the parameter **Dark Image** as the dark source.

> ➢ Select the image by clicking on [...].

> ➢ Lastly, activate the previously acquired HDR image. To do this, all you have to do is click once on the image's Acquisition tab.

HDR Merge

Parameters	[...]
New image	On
Input	IP-HDRExposeImage-01
Output	\<New image\>
Dark Source	Dark Image
Dark Value	32
Dark Image	IP-HDRExposeImage-02
Linearity Limit	100
Output Bias	32
Output Datatype	Word

OK   Cancel   Apply

No other changes to the parameters are required if you are using an AxioCam camera. If you are operating an AxioCam ICc 1 or ICc 3 in 8-bit mode, the output bias should be reduced to the value 3 (more detailed explanations can be found in the following section or in the online help).

Apply starts the merging process. The HDR image is now finished.

Basic settings in HDR Setup

The settings in **HDR Setup** determine all the parameters that cannot be changed individually in the various HDR functions. This function is therefore of central importance.

HDR Setup can either be opened via the **Acquisition** menu or in the work area under **HDR** ⇒ **HDR Functions** ⇒ **HDR Setup**.
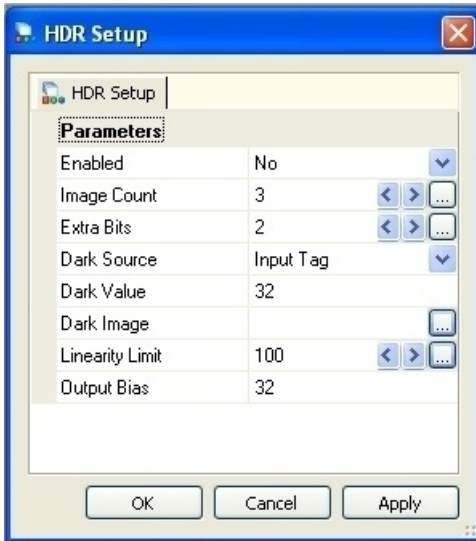
The default values in **HDR Setup** have been set in such a way that they can be adopted without any further changes for an AxioCam.

It may be necessary to change the dark value for the merging of the HDR image if you are using a camera from another manufacturer. To determine the actual dark value, it is helpful to take the value of the darkest areas of a simple image as a reference.

The second setting that you should change, if necessary, if you are using an AxioCam ICc 1 or AxioCam ICc 3 in 8-bit mode or a camera from another manufacturer is the **Output Bias** parameter. This determines the brightness value of the darkest pixel in the output image and should be set to the value 3 in the case of the AxioCam ICc.

4. Microscope Control

4.1 General

AxioVision has been specially designed to support Zeiss's motor-driven microscopes.

The introduction of the new microscope types from the Axio Imager, Axio Observer and SteREO series means that the established Axioplan, Axiovert, Axioskop and Stemi systems are being replaced. These systems will no longer be developed or produced, but will naturally still be supported by AxioVision.

As the Axio Imager, Axio Observer and SteREO microscopes open up completely new opportunities for computer-aided microscopy, new requirements are also necessary with regard to the configuration of the systems. Therefore, a distinction is made in AxioVision between the established systems Axioplan, Axiovert, Axioskop and Stemi and the new Axio Imager, Axio Observer and SteREO systems. Various configuration programs are available for both generations of instruments. However, there is only a difference as far as operation in AxioVision is concerned where functional differences actually exist. Therefore, if you change your system, you will be immediately familiar with the new microscope without having to readapt.

The microscope is either controlled via a property page containing all configured components or via control elements for individual components.

The property page for controlling the microscopes can be activated up by clicking the **Microscope** entry in the work area or by selecting from the **Microscope** menu the function **Microscope**.

Alternatively you can select single controls from the five functions **General**, **Transmitted Light**, **Reflected Light**, **Stage/Focus** and **Extra**.

A more detailed description of the controls can be found in the Online help.

4.2 The Established Axioplan, Axiovert and Axioskop Microscopes

At the time of AxioVision's release the following Carl Zeiss microscopes are supported:

- Axioplan 2
- Axioplan 2 imaging
- Axioplan 2 imaging e
- Axioplan 2 imaging e man
- Axiovert 100 M
- Axiovert 200 M
- Axiovert 200 M MAT
- Axiovert 200 MAT
- Axiovert 200 man
- Axioskop 2 mot plus
- Axioskop 2 mot

Your microscope type and its individual facilities are selected in the separate program **Microscope Configuration**. This configuration program can be found on the Microsoft Windows Desktop.

Microscope
Configuration

Here you can define your microscope's type and components.

4.3 The New Axio Imager, Axio Observer and SteREO Microscopes

The following microscopes are currently supported:

- Axio Imager (A1, D1, M1, Z1)
- Axio Observer (A1, D1, Z1)
- Axio Observer MAT (A1m, D1m, Z1m)
- SteREO Discovery (V8, V12, V20)
- SteREO Discovery (V8, V12, V20) o.EMS
- SteREO Lumar.V12
- SteREO Lumar.V12 o.EMS

The microscope type and the individual equipment you are using on your microscope are selected in the separate **MTB 2004 Configuration** program. his configuration program is located on the Microsoft Windows Desktop.

4.4 Activating microscope configurations

In order to activate a new microscope configuration, you must first exit AxioVision. You can then start the relevant configuration program (**Microscope Configuration** or **MTB 2004 Configuration**) to activate the appropriate configuration or to make the desired changes to the current configuration.

A new feature of AxioVision release 4.3 is the opportunity to change the configuration directly at the start. The following dialog is displayed when AxioVision is started:
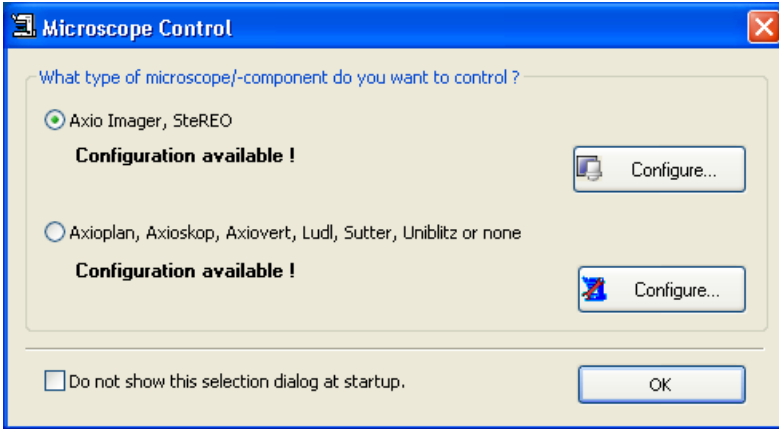
Here you can choose the microscope type and then start the corresponding configuration program via **Configuration**.

If this dialog is not displayed, you should check the settings in the **Tools** ⇒ **Options** ⇒ **General** function:
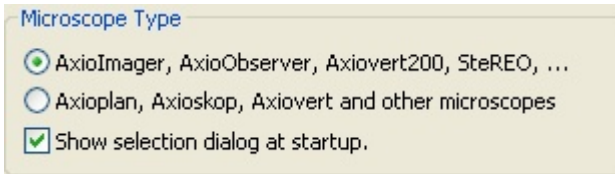
Here, you can specify your type of microscope and activate the **Microscope Selection** dialog by activating the **Show selection dialog at startup** check box.

In addition, for the new Axio Imager, Axio Observer and SteREO microscopes only, it is possible to change the configuration of the microscope when AxioVision is running:

The **MTB 2004 Configuration** program can be started via the **Microscope** ⇒ **Configure microscope** function. You can then make changes to the current microscope configuration. These changes are adopted immediately.

The possibility of changing a configuration is limited, however:

Changes to the current configuration are limited to the objectives and the reflector. You will need to restart AxioVision to make any further changes.

Only the current configuration can be changed. You will also need to restart AxioVision in order to activate a different configuration or to generate a completely new configuration.

4.5 The Settings Editor

Under the **Tools** menu you can open the **Settings Editor** dialog (keyboard command **Alt+S**). In this dialog you can generate, save and retrieve settings for your current hardware, i.e. for your microscope, camera or other external components such as filter wheels or shutters. You can also apply these settings to the hardware.

These settings are intended to make it easier for you to operate your microscope. They are also used for microscope control when you are generating complex experiments, e.g. with the Multichannel Fluorescence or Z-Stack modules. The settings are saved as a document (*.zvhs) in *\Documents and Settings\All Users\ Documents\Carl Zeiss\Data\Setting*

How Do I Generate a Hardware Setting?

The text below explains step by step how to generate and modify a hardware setting document and how to apply it to the microscope. In this example, the reflector nosepiece is to be switched to the Rhodamine position, and the integrated fluorescence shutter is to be opened so that light falls on the sample. The setting is to be saved as a document with the name "Rhodamine.zvhs".

➢ In the **Tools** menu, open the **Settings Editor**. It can also be opened using the key combination **Alt+S**.
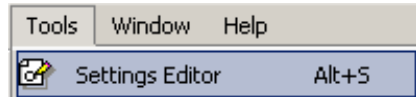
➢ In the window that is now open, click on **New**, enter a suitable name, such as "Rhodamine", in the dialog window, and click on **OK**. You can decide, whether you only (**User**) or other persons (**Workgroup**) can use the settings file
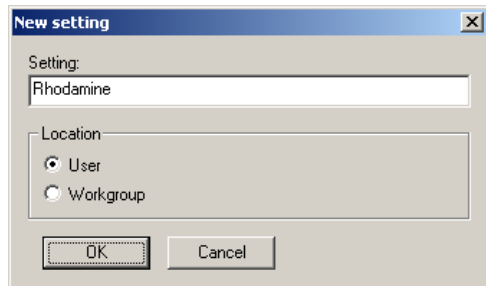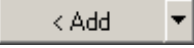
➢ Now add hardware settings to the empty document. To do so, select from the list **Available Components**.
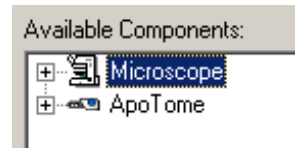
> ➢ Click on ⊞ to open the microscope branch, and then open the branch for the **Reflected Light** component group. The tree should now look like this:
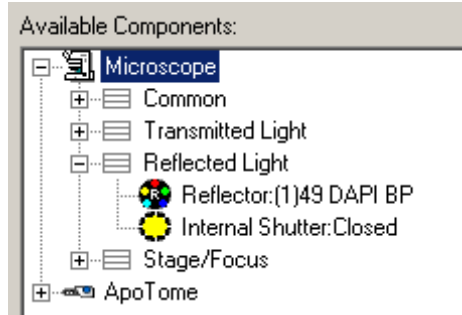
Select the **Reflector** component, and add it to the Rhodamine setting by clicking on ⟨ Add ▼. Repeat this step for the **Internal Shutter**. You can also add this component by using Drag&Drop.

You will now see these two components listed in the setting window:

Both components contain a certain setting. In our example, the component **Reflector** is set to the filter set "[1]49 DAPI BP", and the **Internal Shutter** is **Closed**. These parameters correspond to the current setting status on your microscope. What you see may therefore differ from the examples shown here.
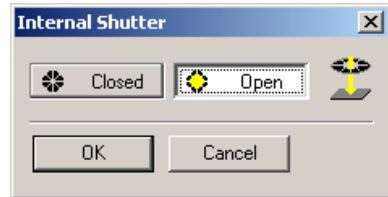
➢ Select the component
  Reflector and click on the
  [...] button. This selection
  dialog is opened:
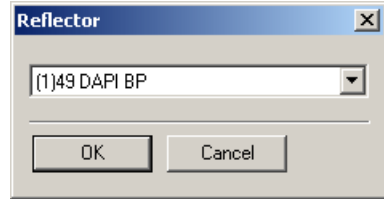
➢ From the drop-down list
  box, select the reflector
  position for Rhodamine (in
  this case it is 15 Ex.546/12
  at position 3), and confirm
  with **OK**.
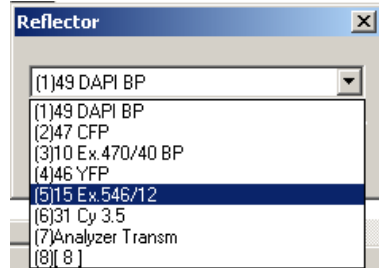
➢ Now select the component
  Internal Shutter, click on
  the [...] button, and then
  click in the dialog on **Open**.
  Confirm your selection with
  OK.

Save your settings by clicking on [🖫 Save]. This button then
becomes inactive ([◼ Save]).

Test your new hardware setting by clicking on [🕸 Apply]. The
reflector block should now swing to position 3, and the shutter for the
fluorescence lamp should open.

The hardware setting "Rhodamine.zvhs" has now been saved and can be
assigned to a function key (for more information, please refer to chapter 8
"Configuration" - " Define key combinations for functions") or used in an
experiment as part of the acquisition of a multichannel image, for example.

4.6 Definite Focus

Overview

The Definite Focus functionality in AxioVision enables control of Definite Focus, an LED-based focus stabilizer. Definite Focus can e.g. be used to compensate changes in the distance between microscope objective and specimen, caused by temperature fluctuations. This functionality makes long-term time lapse experiments more reliable and easy.

Note
- Definite Focus control is also possible without using AxioVision, although depending on the application, this may only be possible with limited functionality.

- Description of all functions of Definite Focus in AxioVision may be found in the AxioVision online help.

- For further information on the working principle, operation and compatibility with hardware components (e.g. objectives), please refer to the Definite Focus operating manual.

Two application examples demonstrate operation of the Definite Focus with AxioVision.

Example 1: Automatic focus-stabilization at one position of a culture dish

In this example Definite Focus automatically stabilizes the focus position (z) at a single xy-Position in a culture dish, without needing further settings or interactions made by the user.

➢ Make sure that Definite Focus control by AxioVision is running in default mode. Default handling of Definite Focus is the preferred working mode. The box ☐ Disable Default Handling of Definite Focus in the menu **Tools** ⇒ **Options…** ⇒ **Acquisition** must not be checked for this

mode. Activation and deactivation of Definite Focus during the experiment will now be carried out automatically by AxioVision.

➢ Place your specimen on the stage of the microscope and focus it.

➢ Open the Definite Focus window in the menu **Microscope** ⇒ **XYZ** ⇒ **Definite Focus**:
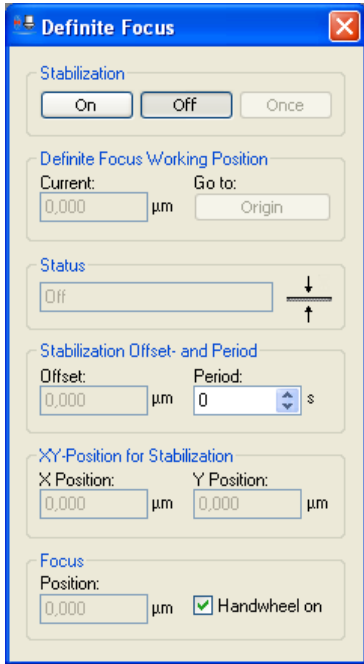
➢ Activate Definite Focus by clicking the ⟨ On ⟩ button.

Activity of Definite Focus is displayed in the **Status** area of the **Definite Focus** window by a periodic indication of „Waiting…" and "Setting focus…", as well as by an animated icon ⟨ ↓Ⴟ↑ ⟩.

Now start the image acquisition experiment as you are used to it. This can be e.g. a single- or multichannel time lapse experiment.

Note:
- Definite Focus keeps the distance from the bottom of your sample (e.g. the culture dish) constant. Please note that movement of individual cells in the culture dish can nevertheless lead to a situation where these cells are not in focus anymore. In this case use of the software- and image-based autofocus functionality of AxioVision is recommended.

Example 2: User-defined focus-stabilization

In this example Definite Focus is controlled by user-defined hardware settings. This mode is only recommended if the results obtained by the automatic Definite Focus control are not satisfying. It is also recommended that only users, experienced in generating hardware settings are using this mode.

➢ Deactivate the default handling of Definite Focus by AxioVision by checking the box ☑ Disable Default Handling of Definite Focus in the menu **Tools ⇒ Options… ⇒ Acquisition**. Activation and deactivation of Definite Focus during an experiment is not done automatically anymore by AxioVision, but has to be defined by the user in an experiment with hardware settings.

You will find the Definite Focus control components for the creation of hardware settings in the menu **Tools ⇒ Settings-Editor** in the category **Microscope ⇒ XYZ**.
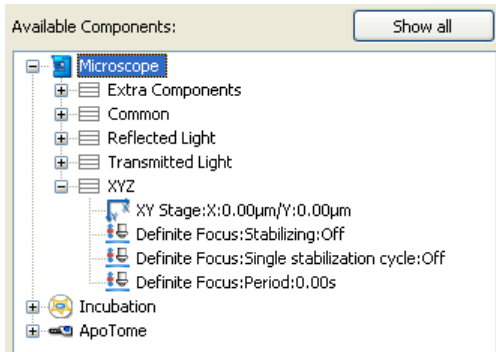
> ➢ Create a hardware setting „Definite Focus active" that activates Definite Focus for focus stabilization with an interval of 5 seconds.
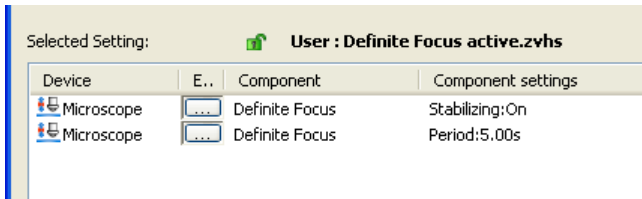
> ➢ Create a hardware setting „Definite Focus inactive" that deactivates Definite Focus.
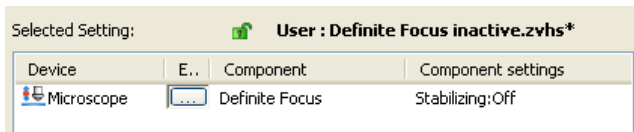
> ➢ Use these hardware settings to enable focus stabilization and correction by Definite Focus in between the time points of a time lapse experiment while disabling Definite Focus immediately before image acquisition. For this use the function **Settings before/after Timepoint** on the **T (Time)** property page of Multidimensional Acquisition.
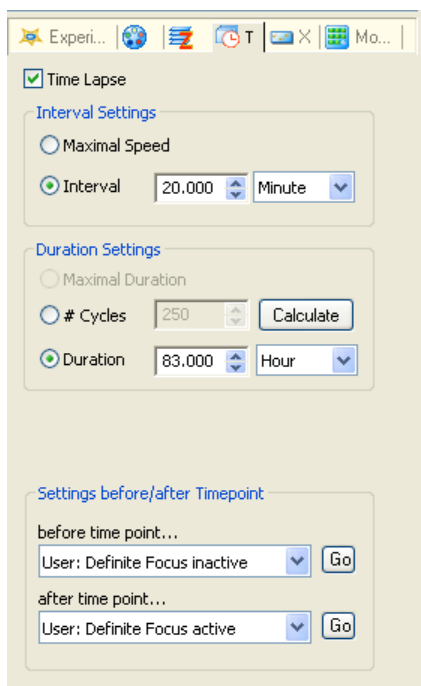
You may now start you experiment. Definite Focus will correct the focus position between all time points every 5 seconds. During image acquisition it will be inactive to minimize the possibility of any putative negative influence of Definite Focus on image quality.

5. Image Processing

5.1 General

The task of image processing essentially consists of two fields of activity: correction and optimization of the acquired image data.

In many cases digital image processing functions are required to make up for the technical limitations of the available devices. However, we should point out that often (in fact probably in most cases) image processing functions are needed to compensate for carelessness during image acquisition.

It is therefore essential to make sure that you fully utilize the capabilities of your technology both prior to and during image acquisition. This is the only time when the actual original information is available. It is also your best opportunity to ensure good image quality.

One area that is often neglected is the preparation of the sample. The principle here is that a poor sample inevitably results in a poor image. For this reason you need to be self-critical and check whether the quality of your sample is suitable to provide optimum acquisition results.

Whatever you use image processing functions for, make sure that wherever possible original data are not overwritten. You should always be able to return to the original image to ascertain whether executing a function has actually brought about an improvement or has in fact resulted in relevant image information no longer being available.

The different ways of processing images

In AxioVision there are two different ways to process images. They each have different features and have been devised for different areas of application. On the one hand, all functions can be executed via the **Processing** menu or the **Workarea**. Alternatively, via the **Properties** window, you can optimize the quality of the image display (brightness, contrast and gamma value) for your monitor using a characteristic curve.

In both cases AxioVision ensures that initially your original data are not modified:

- If you are using the functions via the **Processing** menu, by default the result is displayed in a new image document. You can then decide whether you want to save the result in a new file or overwrite the existing file.

- If you are working via the **Properties** window, the numerical values of the brightness and color information of your images are not modified. Only the display of the saved values on the monitor is adapted. As long as you save your images in AxioVision "ZVI" format, in addition to the original data it is only the settings in the **Properties** window that are saved in the file.

- When you open the file, it is then the optimized display that is initially made available. You can revert to the original display at any time via the **Properties** window. If you export the image into a different format, the settings of the **Export** function can be used to specify whether you want to save the original data or whether you want the settings in the **Properties** window to be adopted before saving. In the latter case, however, the gray or color values are changed, and you are no longer able to restore the original data.

NOTES:
- We recommend that initially you always save your images in ZVI format, and only export them to an external format if you need to do so.

Image formats

In the section that follows, image format should not be understood to mean the file format (ZVI, BMP, TIF etc.), but rather the way in which brightness and color are displayed in an image.

Conventional TV cameras are able to distinguish 256 gray levels. The entire spectrum from black to white is displayed using this number of brightness levels. To be able to distinguish 256 gray levels on a PC, 8 bits are required. These images are therefore known as **8 bit images**.

To display color images, three of these gray level images are required – one for red, one for green and one for blue. These images are essentially gray level images. This means that a color image is made up internally of 3x8-bit images. We therefore also talk of **24 bit images**. With these kinds of image it is possible to distinguish up to 16.8 million color values.

Modern digital cameras, however, are not only able to distinguish 256 gray levels, but can actually distinguish as many as 65536. This means that 8 bits are no longer sufficient to save the data. 16 bits are required in such cases. However, **16 bit images** are essentially only gray level images too. If three of these images are combined to form a color image, we talk of **48 bit images**.

NOTES:
- Some file formats are not able to save 16 and/or 48-bit images. We therefore recommend that you always save your images in ZVI format initially, and only export them to an external format if you need to do so.

5.2 Annotations

Annotations are additional drawing elements (scale bars, captions, arrows etc.) that can be drawn into an image. The word "into", however, is not strictly accurate, as the annotations are in fact drawn on top of the image. In other words, the annotations are drawn into their own plane on top of the actual image information, ensuring that initially the original information is not overwritten.

Provided that you save your images in AxioVision "ZVI" format, this plane information can be retained. The annotations are therefore saved separately from the actual image information. When you open the file, any annotations that are present can be changed, deleted or hidden or new annotations can be added.

The AxioVision ZVI format is the only format that supports this plane technique. Consequently, if you save the image in a different format, in the settings of the **Export** function you must specify whether or not you wish to "burn" the annotations into the image. You have the following options:

➢ If you burn the information into the image, the pixels that lie beneath the annotations are overwritten and you will then be unable to restore them.

➢ If you decide not to burn the annotations into the image, the annotations are lost.

➢ Alternatively, you can save the image twice (once in its original form and once with the annotations burnt in).

NOTES:
• As there are considerable limitations involved in saving images in external formats, we recommend that initially you always save your images in ZVI format and only export them to an external format if you need to do so.

5.3 Working with the Image Processing Functions

General

The text below outlines the principal method for operating the image processing functions. The steps described are identical for all functions. A description of the operation of the individual functions and their parameters can be found in the online help under the **Processing** menu entry.

The individual steps in the following examples can also be practiced using the original images that were employed. The sample images can be found on the Product DVD.

Smoothing an image (basic)

Images frequently contain noisy regions or inhomogeneous brightnesses, which impair the detection of the objects to be measured (segmentation). To achieve satisfactory segmentation, these kinds of images are processed using smoothing filters.

All filter functions work using filter matrixes, the size of which can be set using the **KernelSize** parameter. During the filtering process, the central pixel in the matrix is combined with the gray values within the matrix, and is therefore given a new value.

Which smoothing filter is most suitable depends on the structures that are present in the image. You should therefore try out various filters initially.

The **Sigma** function is particularly appropriate if you wish to remove uneven brightnesses from within structures without influencing the edges of these structures. Fine structures are also preserved.

To suppress noise, the **Gauss** function can be used. This reduces the dynamic range of the image and smoothes the entire image, including the object edges.

The following example explains step by step how to smooth the structures in an image using two different filter functions.

➢ Load the image "Bone-overview.zvi" from the AxioVision folder *AxioVision Images\Imaging Plus* via the function **File ⇒ Open Image**, or acquire an image with the camera. The image is displayed in AxioVision's image window.
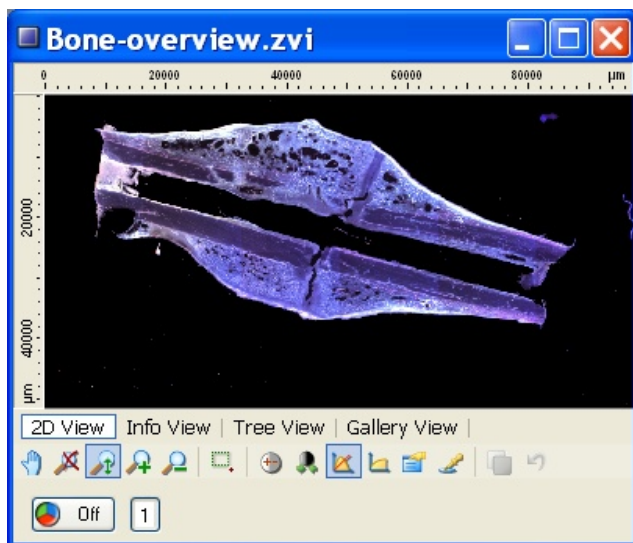
Select from the **Processing** menu the **Smooth** functional group and then the **Sigma** function.

➢ You will now see the dialog window of the **Sigma** function.

➢ If the **Automatic Preview** check box is activated, you will see the reduced input and output images.

➢ Set **CreateNew** to **Off** so that a different filter function can later be executed with the same image.

➢ Set the value **13** for **KernelSize** and the value **31** for **Sigma** by clicking on the arrow keys or by entering the values directly.

➢ Click on the **Splitter Display**

icon to display the original and the result image side by side.

➢ Click on **OK** to close the dialog.
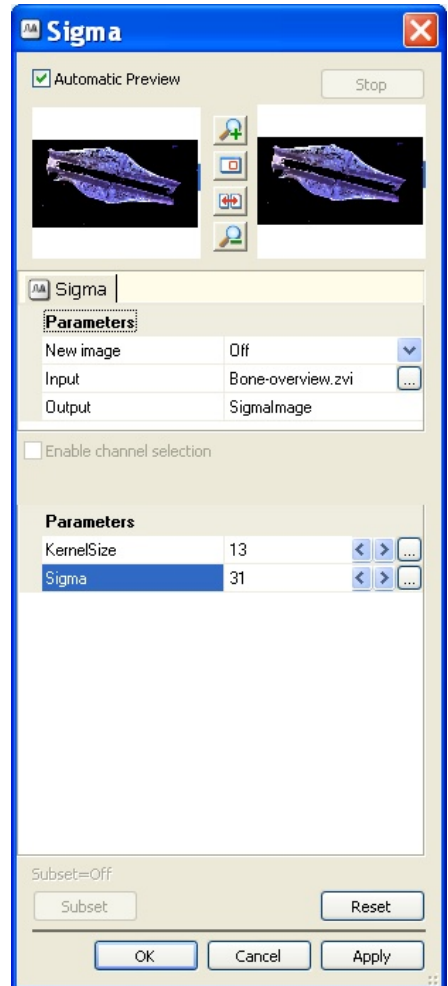
Select from the **Processing** menu the **Smooth** functional group and then the **Gauss** function.

➢ You will now see the dialog window of the **Gauss** function.

➢ If the **Automatic Preview** check box is activated, you will see the reduced input and output images.

➢ If you click on the ⬚ button in the **Input** field, the image gallery appears. Select the original image "Bone-overview.zvi" by clicking on it.

➢ Set the value 13 for **KernelSize** by clicking on the arrow keys or by entering the value directly.

➢ Click on the **Splitter Display** icon ⬚ to display the original and the result image side by side.

➢ Click on **OK** to close the dialog.
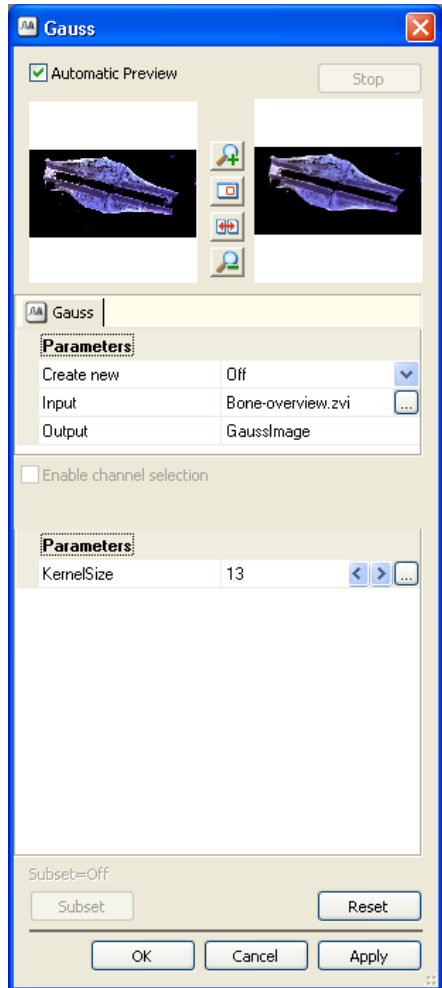
Compare the two resulting images.

➢ With the **Sigma** filter, strong smoothing has been achieved without the edges becoming blurred:
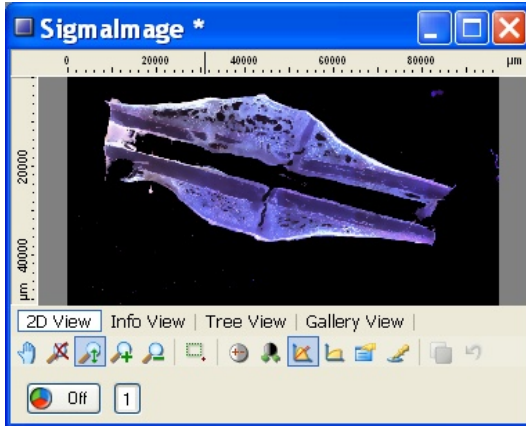
➢ With the **Gauss** filter, weak smoothing has been performed using the same **KernelSize**. The edges of the object have also been smoothed:
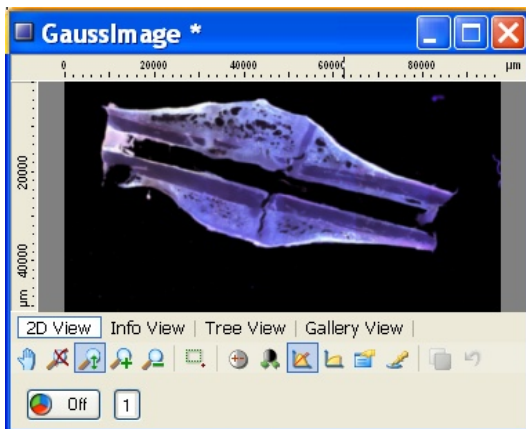
NOTES:
- Adjust the display characteristic curve in the resulting image if necessary.

Shading Correction (basic)

Uneven illumination, vignetting in the optical system or inhomogeneous sensitivity of the camera sensor can sometimes lead to a so-called shading effect in images. This means that the brightness of the image is uneven, which greatly impairs the detection of the objects to be measured.

To eliminate this disruptive effect, shading correction must be performed using a reference image. As a rule, an empty field with no structures is used as a reference image. Often, however, the image has already been acquired, and the sample is no longer available to allow the acquisition of an empty field. In this case a pseudo-shading reference image can be used. This is generated by applying particularly strong smoothing (e.g. using a strong Lowpass filter) to the original image. The smoothing can, however, take place automatically within the shading correction function, which renders the generation of a shading reference image superfluous.

The following example explains step by step how to perform shading correction.

➢ Load the image "SAND.ZVI" from the folder *AxioVision Images\AutoMeasure Plus* via the function **File** ⇒ **Open Image**, or acquire an image with the camera. The image is displayed in AxioVision's image window.

Select from the **Processing** menu the **Adjust** functional group and then the **Shading Correction** function.

➢ You will now see the dialog window of the **Shading Correction** function.

➢ If the **Automatic Preview** check box is activated, the reduced input and output images appear.

➢ Set **CreateNew** to **Off** so you can select the images freely.

➢ Select the loaded image as **Input** and **Reference**.

➢ Set the **Auto** parameter to **On**. This will result in a pseudo-shading reference image being generated automatically in the background and combined with the original image.

➢ Click on the **Splitter Display** icon 🔷 to display the original and the result image side by side.

➢ Click on **OK** to close the dialog.
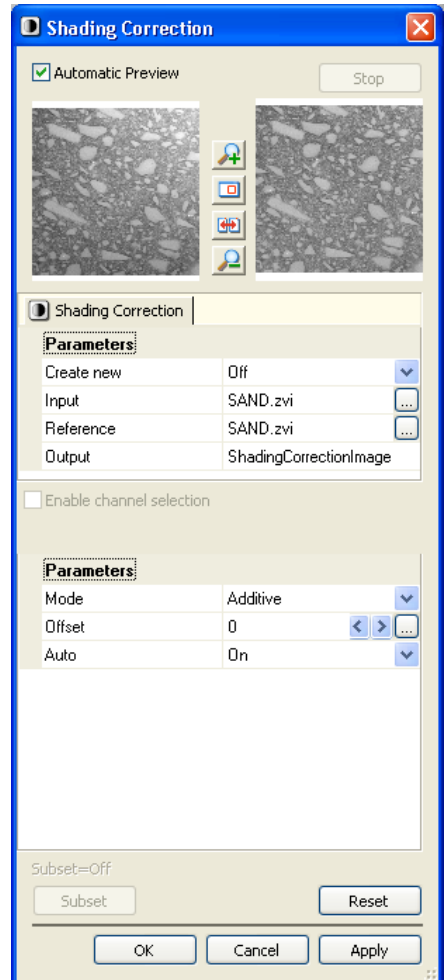
The resulting image then contains homogeneous illumination.
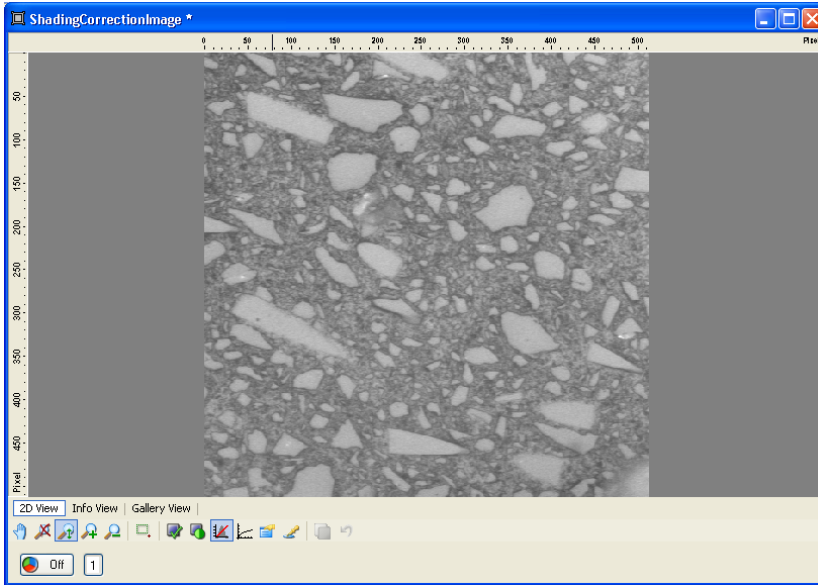
NOTES:
- Adjust the display characteristic curve in the resulting image if necessary.

Correction of XY pixel shift in multichannel images (basic)

During the acquisition of multichannel images using a manual microscope, you switch from one fluorescence filter to another by moving the fluorescence slider manually. In the acquired images, this can result in a shift of a few pixels between the individual channels in the horizontal and vertical directions. The individual fluorescence channels are shown in a pseudo color display. The XY pixel shift that has occurred must be subsequently corrected, to ensure that the individual fluorescence channels lie precisely on top of one another in the horizontal and vertical directions in the pseudo color image.

The following example explains step by step how to correct this XY pixel shift.

➤ Load the image "Endothelial_cells_FL_shifxy.zvi" from the folder *AxioVision Images\Imaging Plus* via the function **File ⇒ Open Image**, or acquire an image with the camera. The image is displayed in AxioVision's image window.
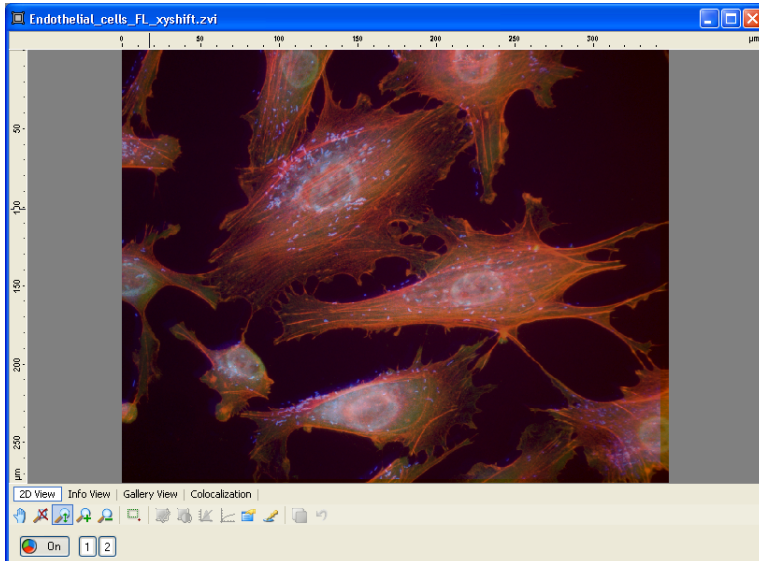
Now select from the **Processing** menu the **Geometric** functional group, and then the **Shift** function.

➢ You will now see the dialog window of the **Shift** function.

➢ If the **Automatic Preview** check box is activated, the reduced input and output images appear.

➢ Activate **Enable channel selection**, so that you can enter the XY shift for an individual channel.

➢ Click on **Subset**.
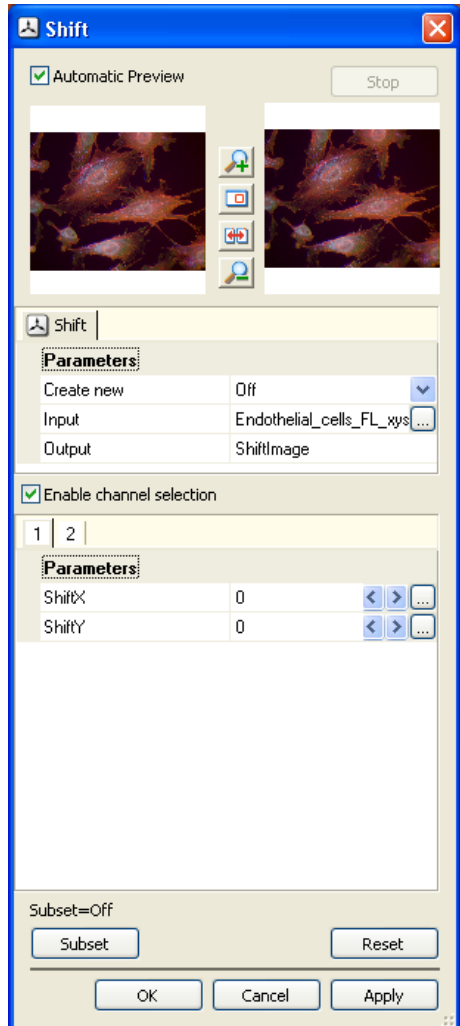
> ➢ Activate only the channel that you want to be corrected (channel 1 in the example).

> ➢ Deactivate **Copy selected items only**, so that all channels are present in the resulting image (including the uncorrected ones), and click **OK**.

> ➢ In the dialog window, activate the channel to be corrected (channel 2 in the example).

> ➢ Set the shift for **ShiftX** and **ShiftY** by clicking on the arrow keys, or by entering the values directly.
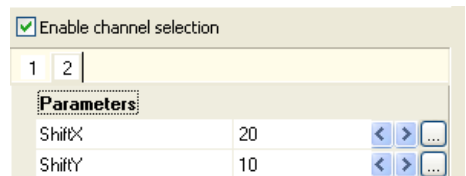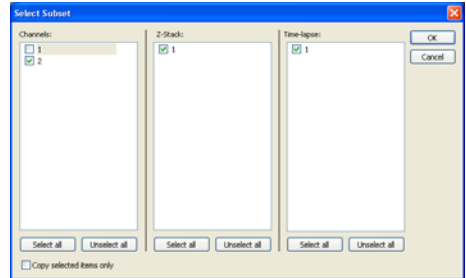
NOTES:

- The value for **ShiftX** and/or **ShiftY** always relates to the number of pixels that you want to be shifted.

- Click on the **Splitter Display** icon 🔲 to display the original and the result image side by side.

- Click **OK** to close the dialog window.

NOTE:
Adjust the display characteristic curve in the resulting image if necessary.

Correction of z pixel shift in multichannel z-stack images

During the definition of multichannel z-stack images, the focus plane is normally set for one channel only. This means that the acquisition of the various fluorescence channels always starts with the same focus plane. When you switch from one fluorescence filter to another, the excitation and emission wavelengths, which also influence the focus plane, change. If the objective is not corrected for this chromatic aberration, the focus planes between the individual fluorescence channels in the acquired image will not correspond.

The individual fluorescence channels are shown in a pseudo color display. When you switch between the individual channels, a small shift is visible in the z direction. This can be subsequently corrected, so that in the pseudo color image the individual fluorescence channels lie exactly next to each other in the z direction.

The following example explains step by step how to balance out this z pixel shift.

> ➢ Load the image "Hela_DsRed _RevGFP_Zshift.zvi" via the function **File** $\Rightarrow$ **Open Image**, or acquire an image with the camera.

> ➢ The image is displayed in AxioVision's image window.
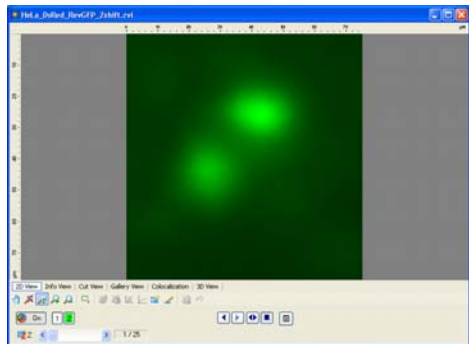
Select from the **Processing** menu the **Geometric** functional group, and then the **Shift** function.

> ➢ You will now see the dialog
> window of the **Shift** function.

> ➢ If the **Automatic Preview**
> check box is activated, the
> reduced input and output
> images appear.

> ➢ Activate **Enable channel**
> **selection**, so that you can
> enter the Z shift for an
> individual channel.

> ➢ Click on **Subset** to enter the
> specific settings in the **Select**
> **Subset** window.
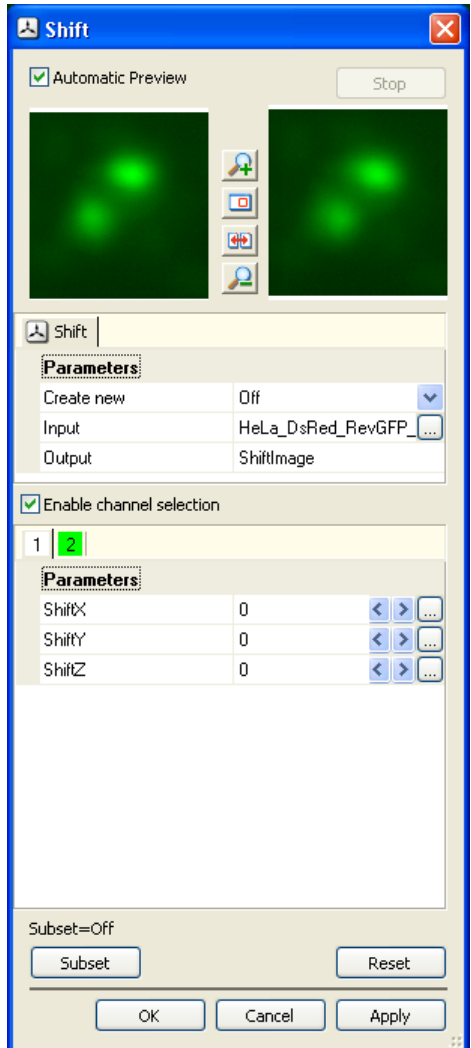
➢ Activate only the channel that you want to be corrected (channel 2 in the example).

➢ Deactivate the **Copy selected items only** check box, so that all channels are present in the resulting image (including the uncorrected ones).

➢ Click on **OK**.

➢ In the **Shift** dialog window, activate the channel to be corrected (channel 2 in the example).

➢ Set the shift for **ShiftZ** by clicking on the arrow keys, or by entering the values directly.
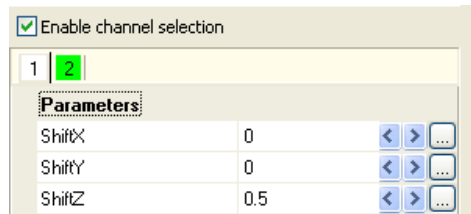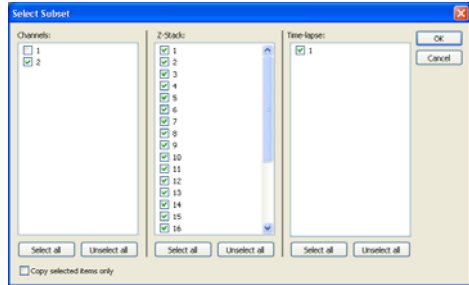
NOTES:

• The value for **ShiftZ** always relates to the number of sections by which you want the shift to be made.

• Example: If **ShiftZ** is set to the value 5, the stack in the resulting image starts at section 6. The first 5 sections remain empty, whilst the last 5 sections of the original image are omitted.
If a value of -5 is set, the first 5 sections of the original image are omitted. The first section of the resulting image is section 6 of the original image. The last 5 sections of the resulting image remain empty.

• It is also possible to perform a shift by a fraction of a section (e.g. 0.3). The value to be entered can be calculated from the distance between the individual sections.

➢ Click on the **Splitter Display** icon to display the original and the result image side by side.

➢ Choose a z-level in the middle (here 13) using the player.

This is the resulting image obtained.
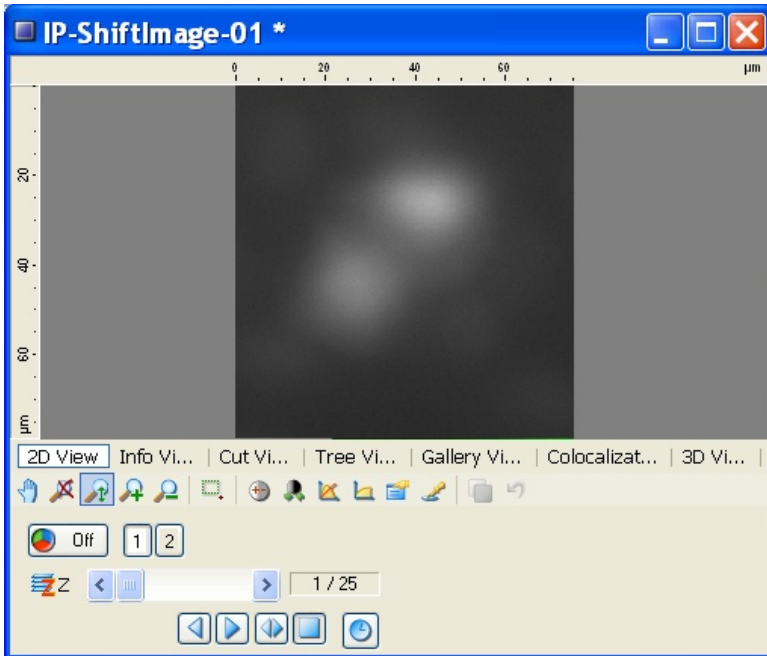
NOTES:
- Adjust the display characteristic curve in the resulting image if necessary.

5.4 Enhancing the Image Display on the Monitor

The fastest and easiest way to display an image that is optimally adapted to the monitor is to use the **Standard** workflow. A description of this workflow, including all the steps involved in image acquisition, can be found in chapter 3 "Image Acquisition".

5.5 Drawing in and Formatting Annotations

➢ Make sure that the **Annotations** toolbar is visible: right-click on the main menu or any toolbar and activate **Annotations** (a tick must be visible in front of it).
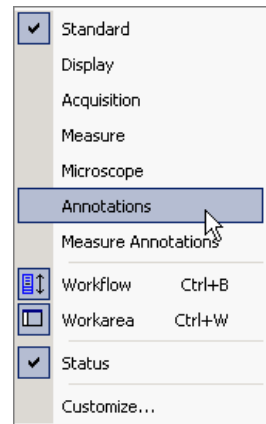
➢ Now activate the image into which you want to draw annotations. To do this, simply click on the image or select it via the tabs at the top of the document area.
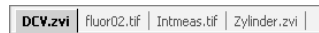
➢ From the **Annotations** toolbar select the tool you want to use, e.g. the **Scale Bar**.

➢ Now draw in the scale bar: click once on the starting point, drag out the desired length and then click on the
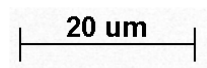
end point.

➢ To format the annotations (change the line color, font etc.), right-click in the image and select **Properties**.
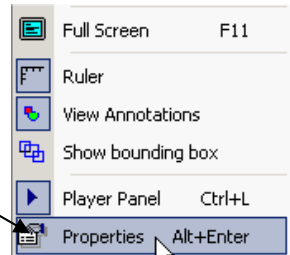
➢ In the **Properties** window activate the **Attributes** tab.

➢ Select an element from the list.
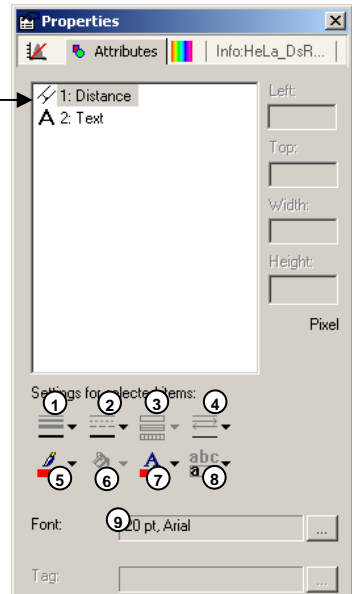
➢ Change the **Settings for selected items**:
  1: Line thickness
  2: Line style
  3: Fill mode
  4: Line type
  5: Line color
  6: Fill color
  7: Text color
  8: Text alignment
  9: Font

The settings only apply to the selected element. To specify standard settings, please read the next section.

5.6 Specifying Standard Settings for Annotations

➤ Activate the image for which you want to specify standard settings: simply click on the image or select it via the tabs at the top of the document area.
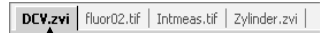
➤ To enter the settings (line color, font etc.), right-click in the image and select **Properties**:
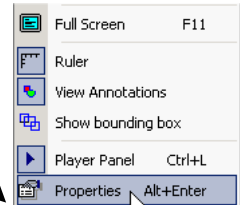
➤ In the **Properties** window activate the **Attributes** tab.

➤ Make sure that no elements are selected in the list of annotations and displayed above the **Default settings** formatting options.

➤ Now change the:
   1: Line thickness
   2: Line style
   3: Fill mode
   4: Line type
   5: Line color
   6: Fill color
   7: Text color
   8: Text alignment
   9: Font
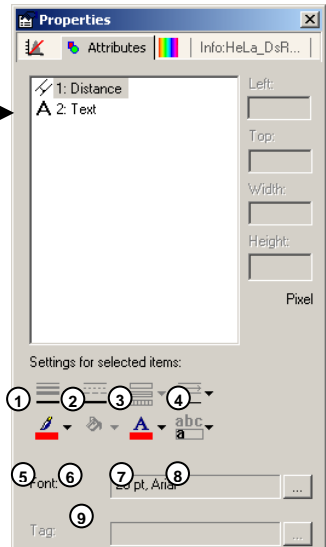
NOTE:
- The settings are now used for all elements that are subsequently drawn in. Saving images in ZVI format, the settings are saved with the image.

6. Image Analysis

6.1 General

With AxioVision Version 4 you are now able to perform interactive measurements in the basic program.

Possible ways to perform measurements

Measurements can, as a rule, be performed in two different ways:

- The individual measurement tools, e.g. line, circle etc., can be accessed via the **Measure** menu and/or toolbar. Individual values can be determined quickly using this method.

- Via the **Measure** ⇒ **Interactive Measurement** ⇒ **Start Measurement** function. This function contains the image to be measured with all the measurement tools in a single dialog. It is, therefore, particularly suitable for use in workflows and Commander scripts, and for this reason is also integrated into the **Measure** workflow. This method gives you the option of being led through the entire procedure, from image acquisition through to the saving of the image, via measurement and evaluation.

Conditions for measurements

Allocation of scalings

To obtain measurement values in real units, you must have allocated the correct scaling to the image you want to measure. This usually takes place during image acquisition (see chapter 3 "Image Acquisition").

If the selected image does not have a scaling

If a scaling has not been allocated to the image you have selected, you can allocate one now:

- Make sure that the image is selected (simply click on it).
- From the **Measure** menu, select the **Scalings** function.
- Select the corresponding scaling from the **Scalings Control** list.
- Click on the **Apply scaling to image** button.

Displaying measurement data

The measurement data can be displayed in different ways:

In principle, all measurements are displayed in the graphics plane as annotations. Which measured values are displayed for the relevant tool is freely configurable (please also see the "Configuring measurement tools" section). The display itself (color, line thickness etc.) can be formatted via the **View** ⇒ **Properties** ⇒ **Attributes** dialog.

If you use the **View** ⇒ **Properties** ⇒ **Measurement** dialog, the measured values are displayed in table form. In this dialog you also have a direct link between the table of measured values and the image that is currently selected. This means that when you activate a tool in the image, the corresponding values are automatically displayed in the table, and vice versa.

Saving data

Saving measurement data

The simplest and most convenient way to save the data is to save your images in AxioVision ZVI format. This is because this format saves the measurement data together with the image. Consequently, all data (pixel data, measurement data, annotations etc.) are present in the image file. There is no need to perform an additional saving procedure.

If you save your image files in an external format (BMP, JPEG etc.), the measurement data, together with all other additional information (annotations, acquisition data etc.), are saved in a separate file. As long as you open the images using AxioVision, the data in this separate file are automatically read in again and displayed. Read also section 7.2 "Saving 2D Images" in chapter 7 "Documentation".

Saving as separate data table

To save the measured values in a separate file, you should use the **Measure** ⇒ **Create Data Table** function. This function writes the measurements relating to the selected image in a data table. This data table is a completely normal document in AxioVision and can then be saved as a file. The data table no longer has a direct link to the image. To combine the values of several images in a single file, the **Measure** ⇒ **Append Data Table** function can be executed.
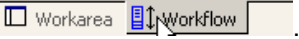
Configuring measurement tools

The interactive measurement tools can configured to your own requirements. Using the **Measure** ⇒ **Interactive Measurement** ⇒ **Configure Measurement Tools** function, you can generate configurations for the application scenarios of your choice. In a configuration you can determine which measurement parameters should actually be measured for a measurement tool and then displayed later in a data table, and which measurement parameters should be displayed in the graphics plane. A measurement can then be loaded via the **Measure** ⇒ **Interactive Measurement** ⇒ **Load Configuration** function. The defined settings are automatically active for a special measurement application. You can specify the default behavior of the measurement tools in the same way.

6.2 "Measurement" Workflow: Easy and Save Measurement

The easiest way to perform interactive measurements is to use the **Measure** workflow. This can be found on the **Workflow**. If the workflow is not displayed, in the **View** menu select the **Windows** list, and there select the **Workflow** command.

NOTES:

- The **Workflow** may in fact be open but covered by the **Workarea**. If that is the case, simply click on the **Workflow** tab at the bottom edge of the work area: .

Measure workflow: overview

Live opens and closes the Live window.

Live Properties displays a window containing the camera parameters and the display characteristic curve.

Snap acquires an image using the active camera.

Show Properties opens the image **Properties** dialog window with the property pages **Display**, **Attributes**, **Color**, etc.

Scale bar inserts a scale bar.

Draw Annotations inserts annotations into the acquired image.

Load Tools opens a dialog to select and activate a specific configuration.

Start Measurement starts the measurement.

Save saves the image.
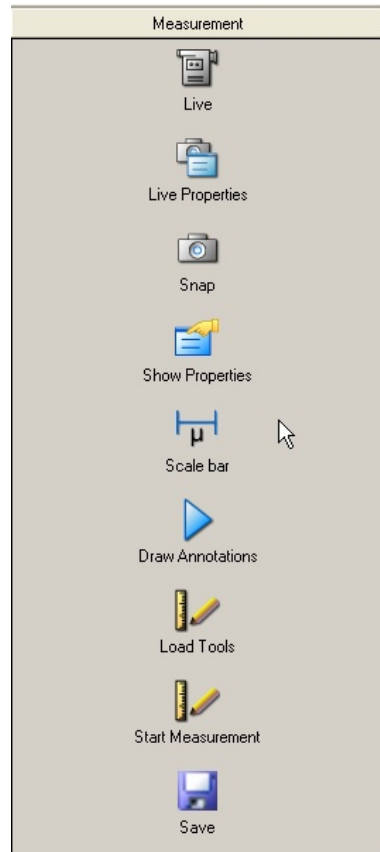
Executing the Interactive Measurement Function

Go through the toolbar function by function:

- Acquire an image, or load an image from the hard drive.
- Adapt the display on the monitor.
- Insert a scale bar, if necessary.
- Draw in annotations, if required.
- Load a measurement tools configuration.

The next button starts the interactive measurement.

➢ Start the **Start Measurement** function.

➢ Perform any measurements by selecting a measurement tool from the toolbar.

1: Distance
2: Circle
3: Curve
4: Curve (Spline)
5: Angle 3
6: Angle 4
7: Events
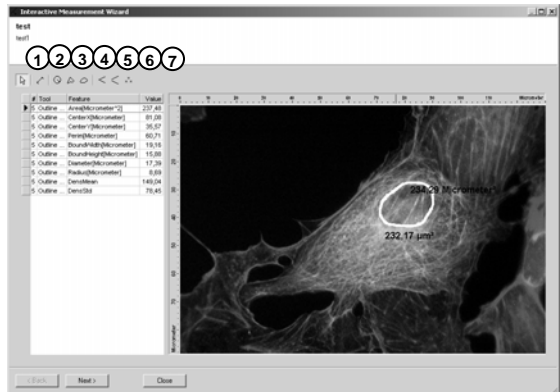
➢ The measurements are performed directly in the image. Click on **OK**.

➢ Now save the image with the measurement results via the **Measure** workflow.
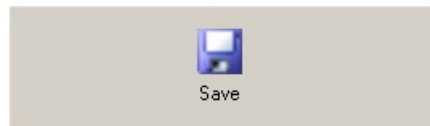
Display and export measurement data

The measurement data from an image can be

- displayed as a list;
- exported into an Excel-compatible text format; or
- combined with the measurement data from other images to perform an overall evaluation.

➢ Select the function
**Measure –Create
Table**.
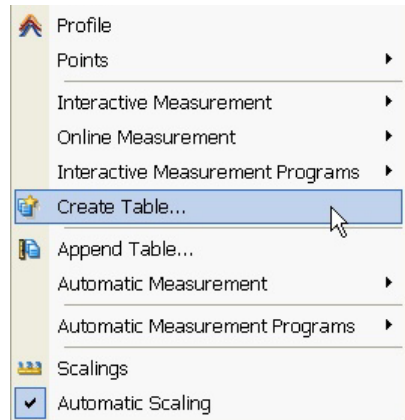
➢ If you do not save your
images in ZVI format,
export the
measurement data by
clicking on **Save**.

➢ To exit the dialog, click
on **Close**.

6.3 Sorting and performing calculations with measurement data

The interactive and automatic measurement functions produce measurement data tables which, as a rule, contain raw data. In the AxioVision menu **Evaluate**, a range of analysis functions are available to allow you to process these data.

To measure several images in a measurement loop, you need to accumulate all the measurement data in a data table (**Append table** function). This measurement data list can be used to calculate statistical values, such as the mean and standard deviation (**Calculate statistical values** function), or to classify the measurement data (**Calculate classified table** function).

It is also possible, however, to combine individual columns from the same or from different data tables in the form of a new data table (**Concatenate table** function). In many cases you will need to filter out only specific measurement values from a data table, e.g. only the measurement values of the FITC channel (**Filter table** function), or to perform calculations using the measurement values of different columns (**Calculate table columns** function).

To obtain a clear presentation of the measurement data from time lapse images, the pivot table (**Calculate Pivot table** function) is a useful tool.

Note:
- Sample scripts of all evaluation functions can be found on the product DVD.

6.4   Creating Data Reports

Modifying and creating report templates with the file extension ZDR

A report is used for printing out images, sample information, data tables and histograms. A template, in which the layout and formatting for the individual report elements (image, data table, etc.) are defined, always forms the basis for a report.

In AxioVision, a selection of sample report templates is available and can be found in the folder *"C:\Program Files\Carl Zeiss Vision\AxioVision 4\0009\Templates\DataReportTemplate"*. All reports have the file extension *.zdr and can be modified for a specific application.

Please also read the section "Modifying a report template" and "Creating a report template" which you will find further down this section.

Newly created files or modified files are saved in the user folder *"Users/My Documents\Carl Zeiss\Templates\Data Report Templates"* or in the work group folder *"All Users/Shared Documents\Carl Zeiss\Templates\Data Report Templates".* Reports will be connected to the respective documents (images, data tables):

- with an archive
- with a folder
- with a gallery

To create a report correct links are mandatory. If you move the documents, which are linked to the current reports to another location, the links in the report have to be updated accordingly.

Note:
- A description about reports with the file extension ZVR can be found in chapter 7.3 " Generate, print and export reports with the file extension ZVR".

Report templates toolbars

The meaning of each icon will be displayed when you move the cursor on one of the icons.

Modifying a report template

The following example will show you how to modify an existing template for another application (displaying user data and an image) and save it as a new template.

➢ First enter your user data on the **Tools** menu ⇒ **Options** ⇒ **ID**.

➢ Use **Snap** to acquire an image with the camera.

➢ From the **File** menu, select the **New** function, and then activate the **Reports (zdr)** tab sheet. Select the **Timelapse** template. Then click on **OK**.
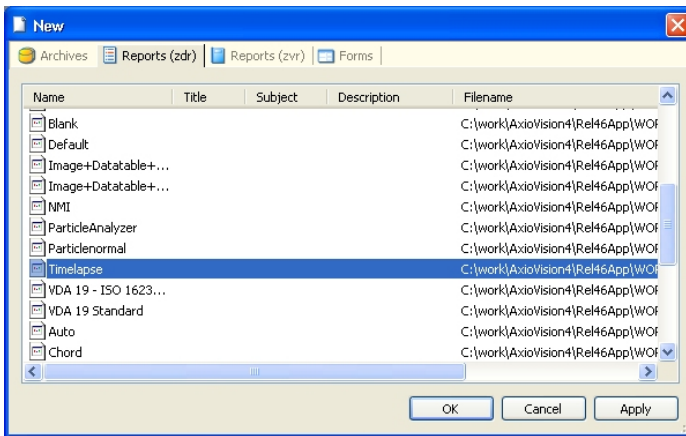
➢ The report is now displayed in the Edit mode.
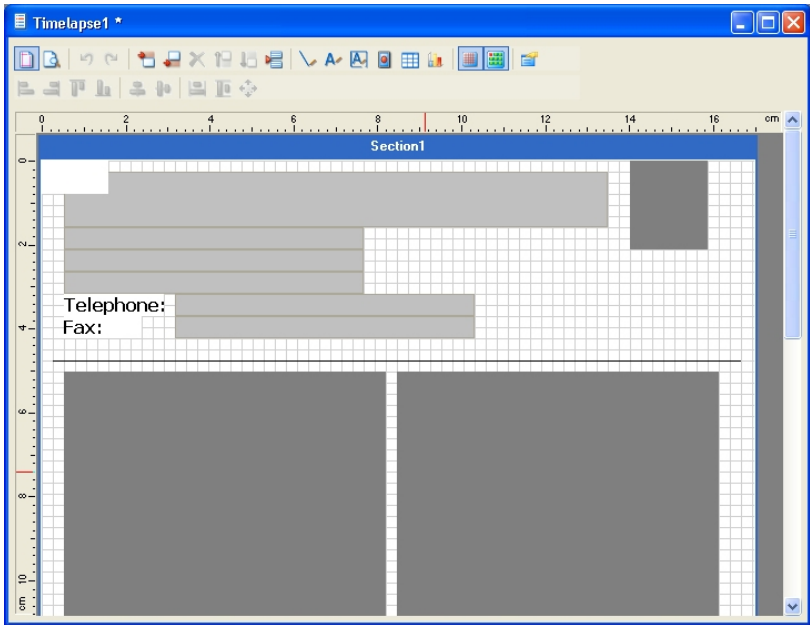
➢ With the left-hand mouse button depressed, drag out a rectangle over all image fields, in order to select these fields.
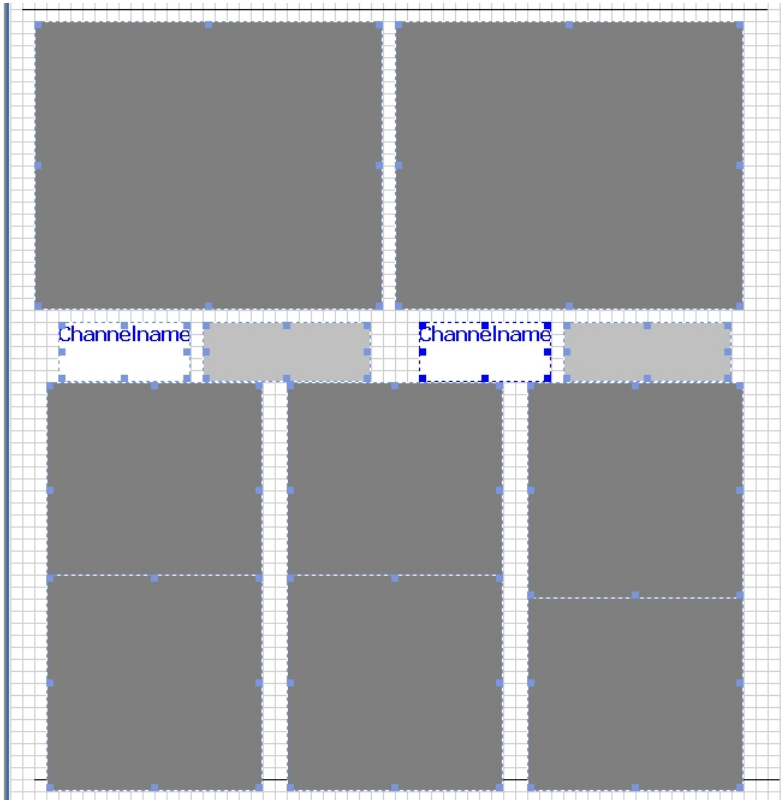
➢ Press the **Del** key to delete the selected fields. Click on and create a large image field.

➢ Now show the property window by clicking on the icon.

➢ In the **Data** field, select *Connect to Gallery* under **Data Connection** and *[Active Image]* under **File**. The original image now appears in the image field.
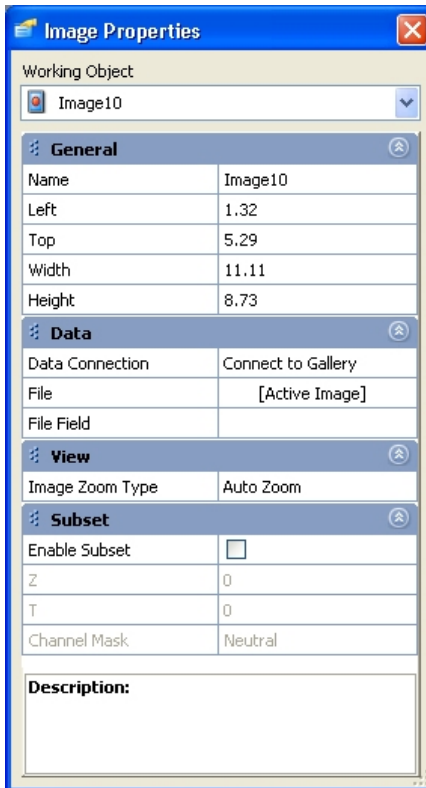
➢ Adjust the size of the image field to the size of the image.
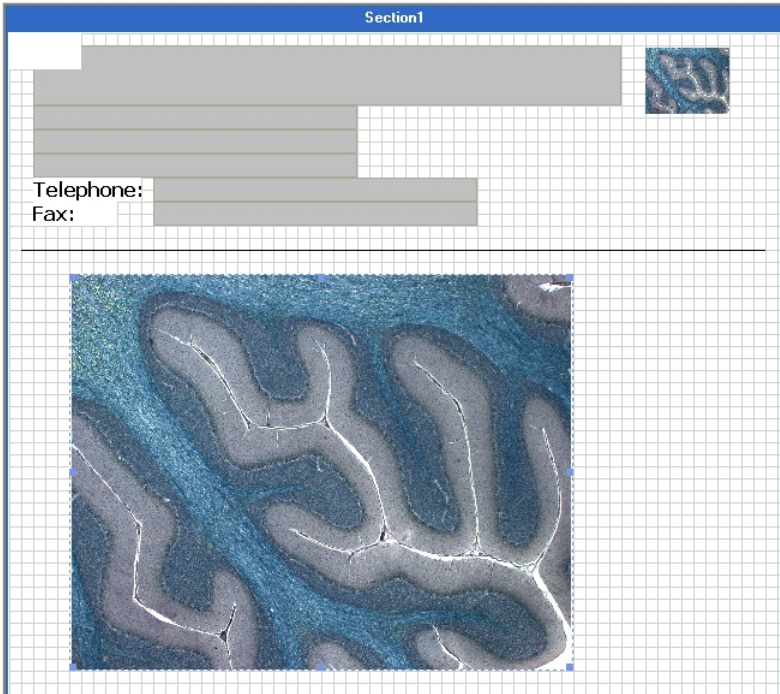
➢ Use the icon in the toolbar to switch to Preview mode to allow you to check the report. Then use the icon to return to Edit mode.

➢ The new report template can be saved and a name entered, e.g. "Image+userdata", using the **Save Report As Template** function on the **File** menu.

Note:

• The information relating to the user, company and address only appears if this has been entered on the Tools menu ⇒ Options ⇒ ID.

Creating a report template

The following example shows you how to create a template for a z-stack from an empty layout. The images of individual z-planes are displayed with focus positions as annotations.

➢ Use the **Open Image** function on the **File** menu to load the image "Trypanosoma_DVC.zvi" from the product DVD.

➢ From the **File** menu, select the **New** function, and then activate the **Reports (zdr)** tab sheet.

➢ Select the **Blank** template and then click **OK**. To see a preview of a template, click on **Apply**. The corresponding template is then opened without the **New** dialog being closed.

➢ An empty layout is now displayed in Edit mode.
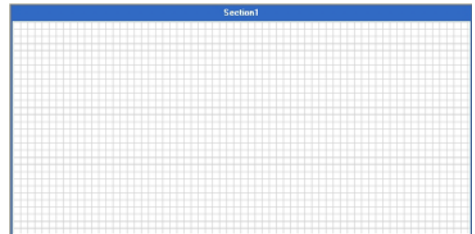
➢ Click on , drag out a text field in **Section1** and position it in the top part of the report. A description field automatically appears next to the text field.
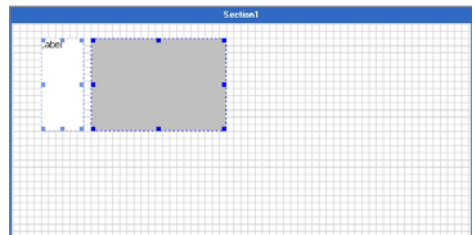
➢ Now click on any position in **Section1** outside the text field / label field.

➢ Click on the text field to position it in the desired location, independently of the label field, and, if necessary, to adjust the size.

➢ Double-click on the text in the label field and replace it with the text "Sample". To change the display of the word **Sample** do the following:
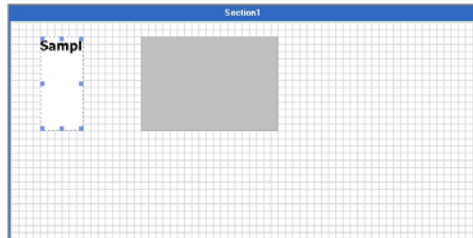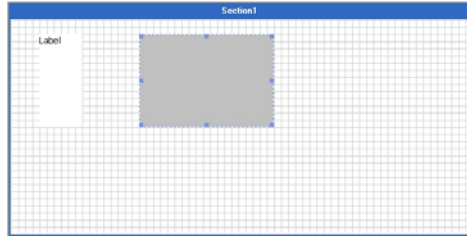
➢ Click on 📋 to open the property window for the label field.

➢ In the **Format** field, select **14** and **Bold** under **Font**.
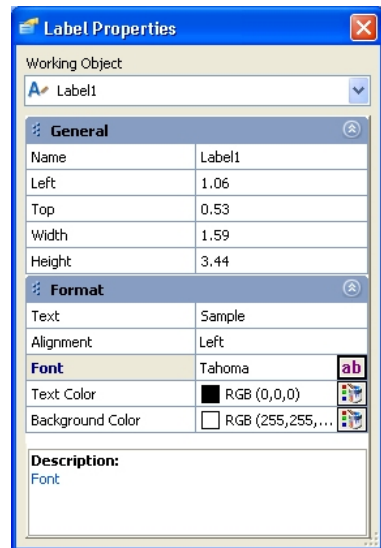
➢ In the **Working Object** field, select the **TextField1** function from the drop-down list box.

➢ In the **Data** field, select *Connect to Gallery* under **Data Connection**, *[Active Image]* under **File** and the file *DocumentFileName* under **File Field**.

➢ In the **Format** field, select **12** and **Bold** under **Font**.

Text Field Properties

Working Object

A. TextField1

General	
Name	TextField1
Left	4.76
Top	0.53
Width	5.03
Height	3.44

Data	
Data Connection	Connect to Gallery
File	[Active Image]
File Field	DocumentFileName
Data type	Text

Subset	
Enable Subset	☐
Z	0
T	0
Channel Mask	Texasred

Format	
Alignment	Left
Font	Tahoma
Text Color	■ RGB (0,0,0)
Background Color	☐ RGB (255,255,...

Description:
Font
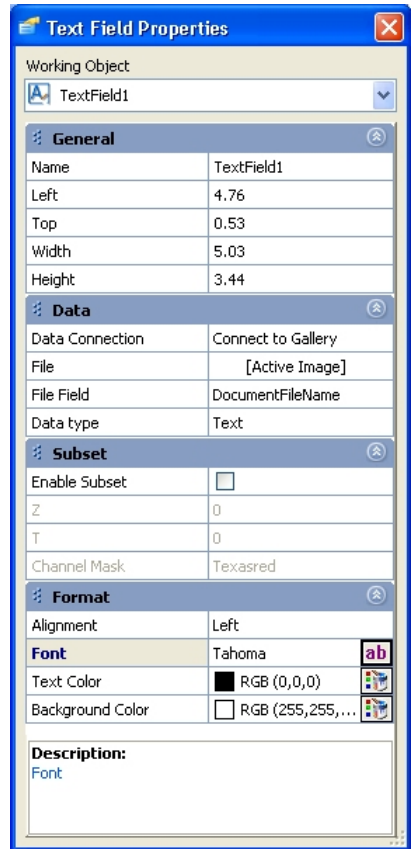
➢ Use the ⬛ icon to switch to Preview mode to allow you to check the image name in the text field.
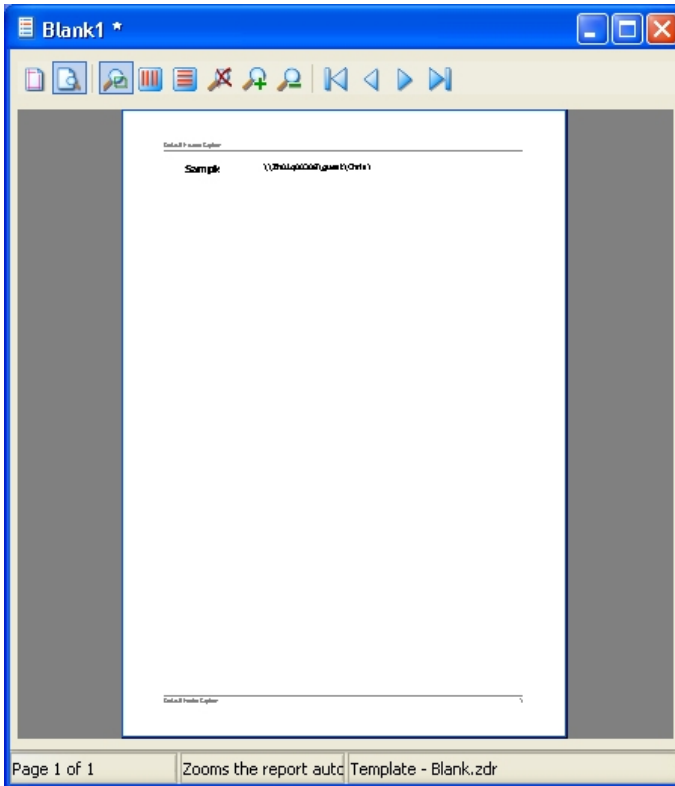
➢ Then use the ⬛ icon to return to Edit mode.

➢ Click on and insert a new section below **Section1**. Now increase the size of **Section2** of the report by positioning the mouse on the bottom edge and dragging downwards with the left-hand mouse button depressed.
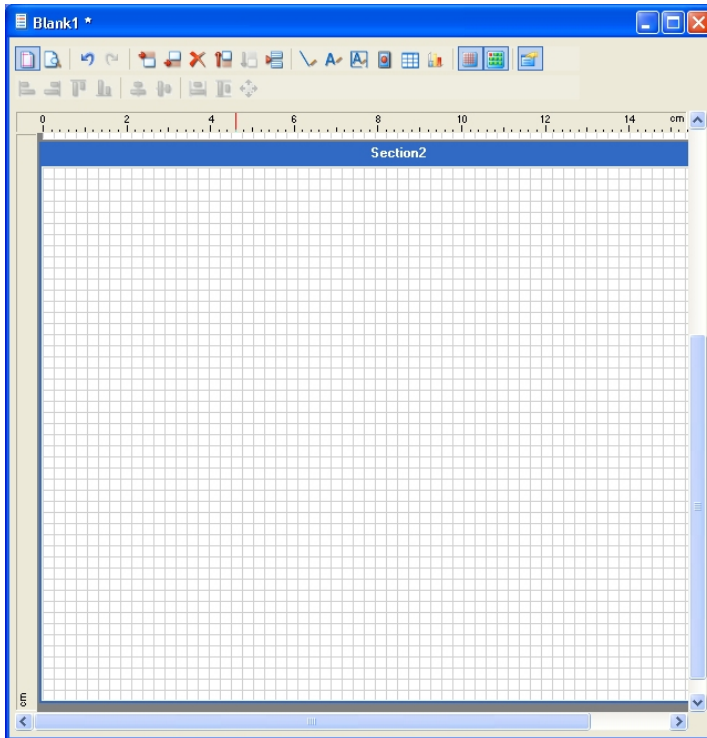

➢ Click on and insert a new image field.

➢ In the **Data** field, select *Connect to Gallery* under **Data Connection** and *[Active Image]* under **File**.

➤ Right-click in the image field. The image field's shortcut menu opens. Here select the **Copy** function.

➤ Click on any position in **Section2** outside the image field, right-click and select the **Paste** function from the shortcut menu.

➤ Create further image fields in the same way until four images are present. Drag it to a position of your choice.
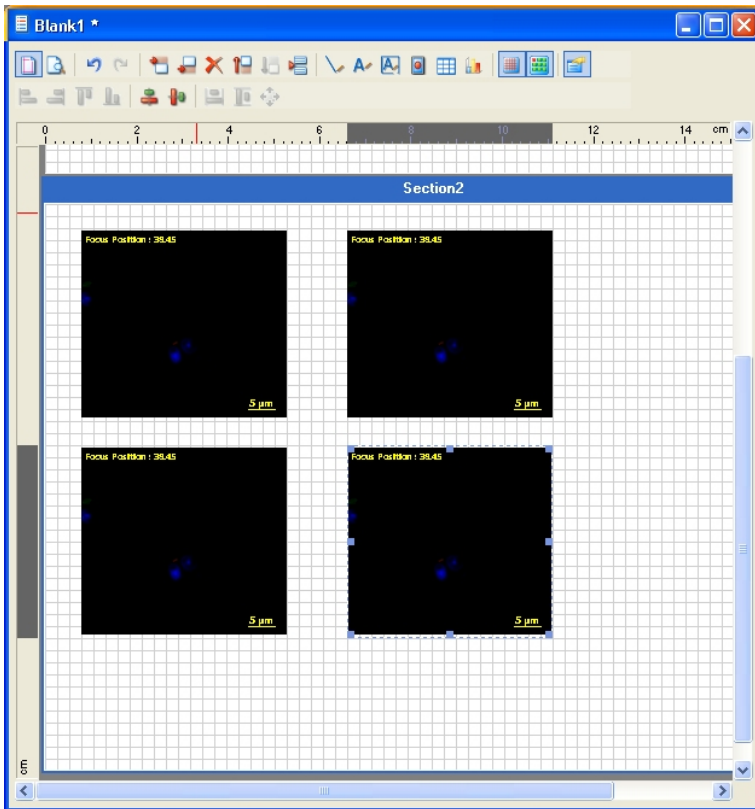
➢ Click in the first image field and, in the **Data** field in the property window, select *Connect to Gallery* under **Data Connection** and *[Active Image]* under **File**.

➢ In the **Subset** field, activate *Enable Subset*, select the value **3** under **Z** and select all three channels under **Channel mask**.

➢ Carry out the same procedure with the other three images, entering the values **7**, **11** and **15** for Z. The individual focus positions are now shown directly in the images.
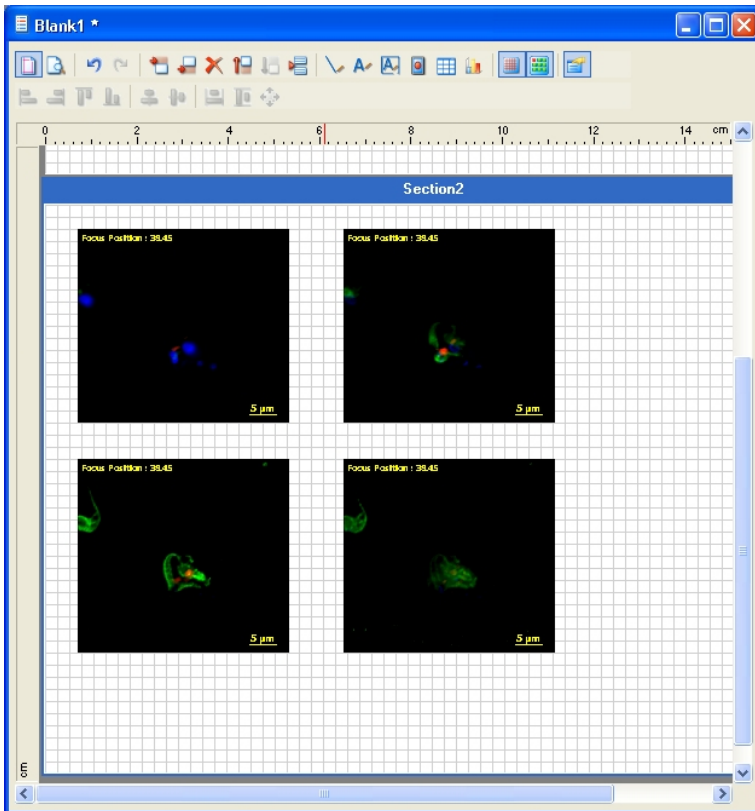
➢ Use the icon to switch to preview mode to allow you to check the report.
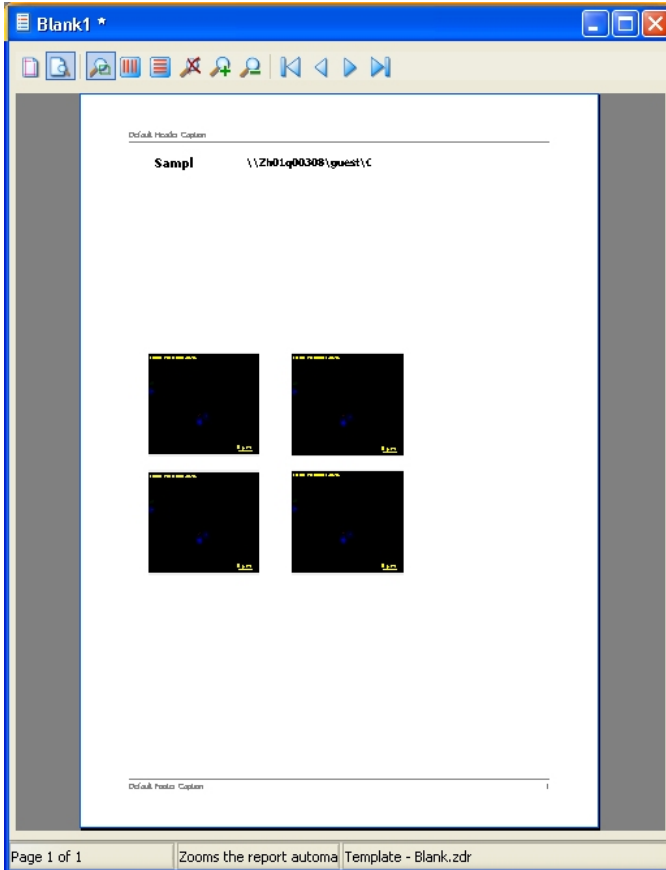
➢ The new report template can be saved and a name entered, e.g. "z-stack", using the **Save Report As Template** function on the **File** menu.

Creating reports with the extension ZDR, printing them and saving them as a PDF file

Creating a report for an image

This example shows you how to incorporate an open image into a report and display it with the associated microscope and camera information.

➢ Use the **Snap** function to acquire an image with the camera, or load an image from a storage medium using the **Open image** function on the **File** menu (for example, load the image "Cat-Cerebellum(1300).zvi" from the product DVD.

➢ To create a report, select the **Create Data Report** function on the **Evaluation** menu.

➢ In the dialog window, select the **Connect to Gallery** function via the **Connection** drop-down list box in the **Create Data Report** field.

➢ In the **Template** drop-down list box select the template **1Image.zdr**.

➢ In the **Report** text field enter a name for the report to be created, e.g. "Testreport1".

➢ Click on **OK**.

➢ The new report is displayed and you can save it using the **Save Report As Template** functions on the **File** menu.
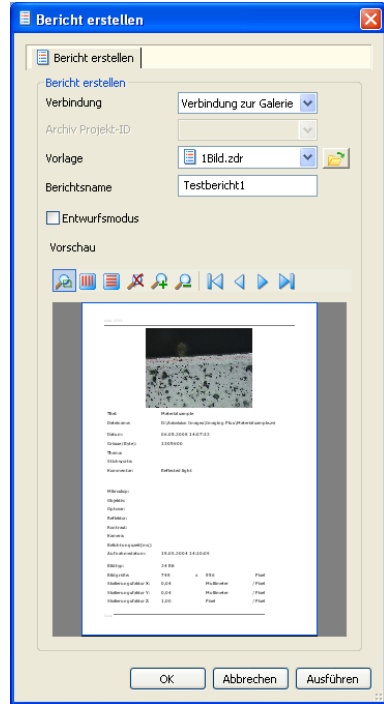

Notes:

- The microscope and camera information only appears if the camera and microscope were connected and configured in MTB2004 prior to image acquisition.

- When the report is saved, the files linked to the gallery (images, data tables) are automatically stored in a folder with the same name as the report.

Adopting a new image into an open report template

The following example shows you how to replace an image present in a report with another image.

➤ You can work through this example after you have followed the example in the section "Creating a report for an image".
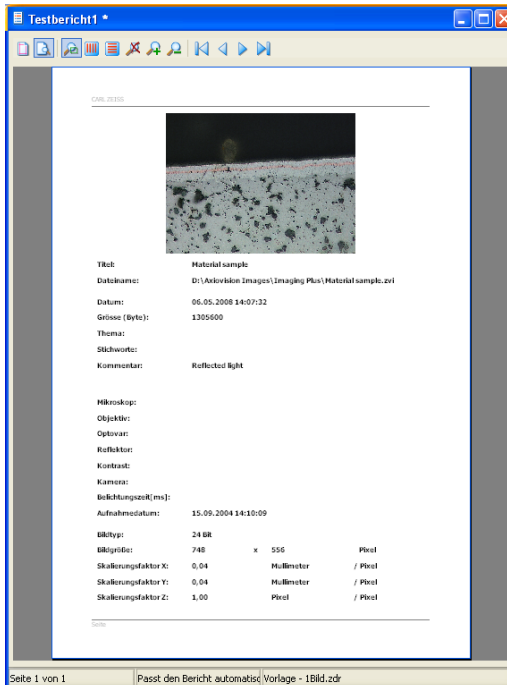
➤ Use the **Snap** function to acquire an image with the camera, or load an image from a storage medium using the **Open image** function on the **File** menu (for example, load the image "Trypanosoma_DVC.zvi" from the product DVD).

➢ If you click on the opened report the current image will be adopted.
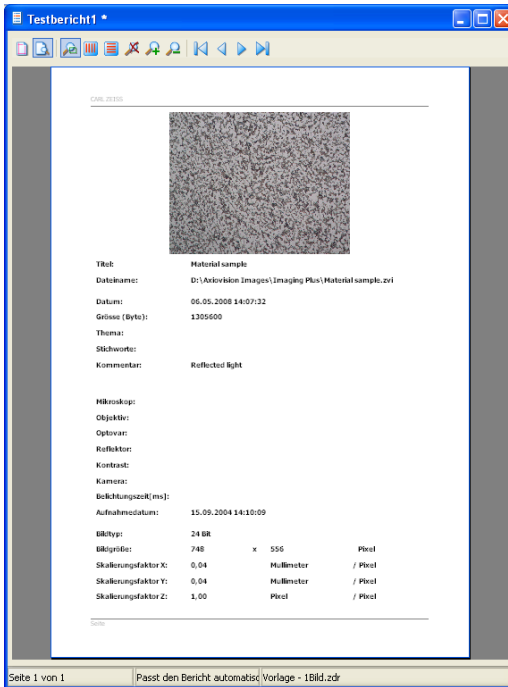
➢ The modified report can be saved using the **Save** or **Save As** functions on the **File** menu.

Printing a report

The following example shows you how to print out a saved report or one that you have just created.

➢ Activate the report you have created by clicking on it or open a saved report using **File** menu ⟹ **Open Report**.

➢ From the **File** menu, select the **Print** function to open the standard dialog for printing documents.

➢ Enter the desired settings and click on **Print** to print the report.

Saving a report as a PDF file

The following example shows you how to create a PDF file from a saved report or one that you have just created. This can be sent and printed out again at any time on any system.

➢ Activate the report you have created by clicking on it or open a saved report using **File** menu ⟹ **Open Report**.

➢ From the **File** menu select the **Save to PDF** function.

➢ Under **File name** enter the desired name and click on **Save** to save the report as a PDF file. This report can then be opened using Adobe Acrobat Reader.

Creating a report for a multichannel fluorescence image

The following example shows you how to display a pseudo color image and the images of the individual fluorescence channels with the corresponding channel names for a 3-channel fluorescence image.

➢ Use the **Open Image** function on the **File** menu to load the image "CHO-3-channel_fluorescence.zvi" from the product DVD. Alternatively, you can also use another 3-channel fluorescence image.

➢ To create a report, select the **Create Data Report** function on the **Evaluation** menu.

➢ In the **Create Data Report** field, select *Connect to Gallery* from the **Connection** drop-down list box.

➢ In the **Template** drop-down list box select the template **3channelfluorescence.zdr**.

➢ In the **Report Name** field enter a name for the report to be created, e.g. "Testreport4".

➢ Click on **OK**.
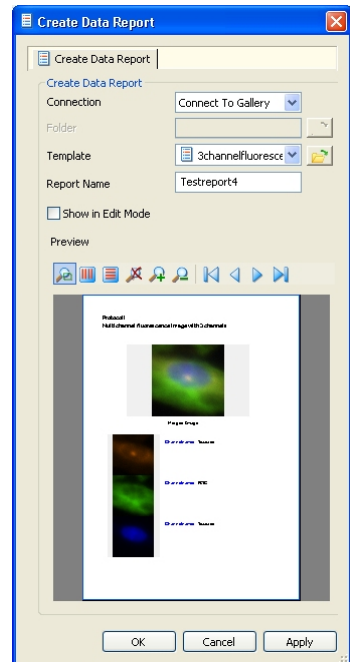
➢ The new report is displayed and you can save it using the **Save** or **Save As** functions on the **File** menu.
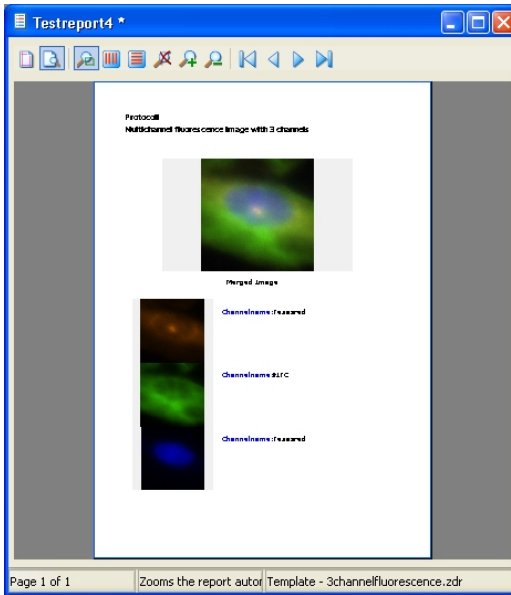

Note:

• When the report is saved, the files linked to the gallery (images, data tables) are automatically stored in a folder with the same name as the report.

Creating a report for a time lapse image with two fluorescence channels

Note:
- The information relating to the user, company and address only appears if this has been entered on the **Tools** menu ⇒ **Options** ⇒ **ID**.

The following example shows you how to display the images of individual time points for a 2-channel time lapse image.

➢ Load the image "Yeast-GFP-DIC_Timelapse.zvi" from the product DVD. using the **Open Image** function on the **File** menu. Alternatively, you can also use another 2-channel time lapse image.

➢ To create a report, select the **Create Data Report** function on the **Evaluate** menu.

➢ In the **Create Data Report** field, select *Connect to Gallery* from the **Connection** drop-down list box.

➢ In the **Template** drop-down list box select the template **Timelapse.zdr**.

➢ In the **Report Name** field enter a name for the report to be created, e.g. "Testreport5".

➢ Click on **OK**.

➢ For the two channels (DIC, FITC) the time points 0, 10 and 19 will be displayed in the report.

➢ The new report is displayed and you can save it using the **Save Report As Template** function on the **File** menu.
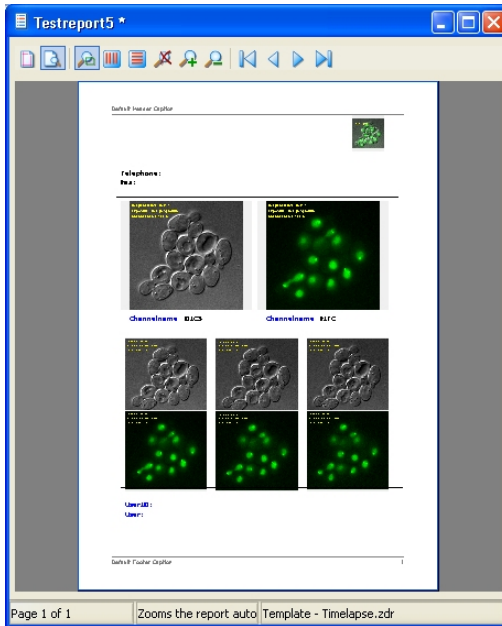
Note:
- When the report is saved, the files linked to the gallery (images, data tables) are automatically stored in a folder with the same name as the report.

### Creating a report with an image, data table and histogram

Sample scripts can be installed from the "Report Script Examples" folder of the product DVD. by launching the **setup.exe** function. The sample images are copied to the workgroup folder *"My Documents\Carl Zeiss\Documents\Images".*

The following example shows you how to present the data of a measurement in a report with output images, a statistics table and a histogram.

➢ Carry out the measurement for a number of images. To do so, select the **Repeat script** function on the **Commander** menu.
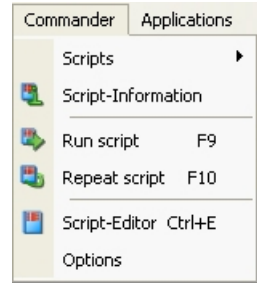
➢ In the **Select Source** field select from the drop-down list box **Folder**, from the drop-down list box **Look in** the directory *"My Documents\Carl Zeiss\Documents\Images"* and mark the two images "round1.zvi" and "round2.zvi".

➢ Select in the **Script selection** field the sample script "AV46Example11a-Evaluate_batch_EN.ziscript" and then click on **Start**.
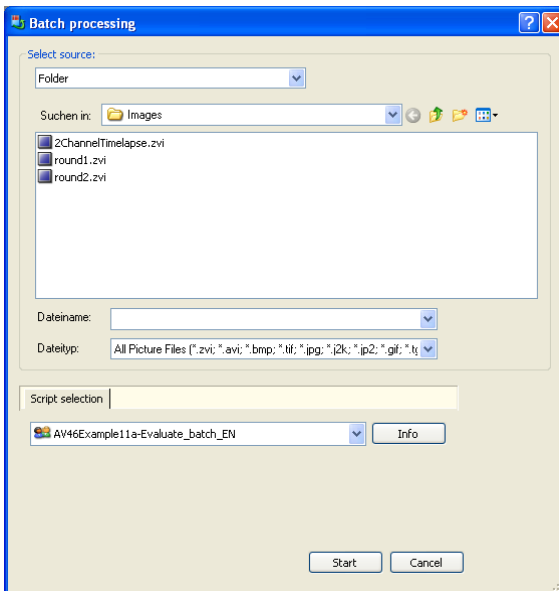
➢ To create a report, select the **Create Data Report** function on the **Evaluate** menu.

➢ In the **Create Data Report** field, select *Connect to Gallery* from the **Connection** drop-down list box.

➢ In the **Template** drop-down list box select the template **Image+Datatable+Histogram.zdr** .

➢ In the **Report Name** text field enter a name for the report to be created, e.g. "TestreportA".

➢ Click on **OK**.
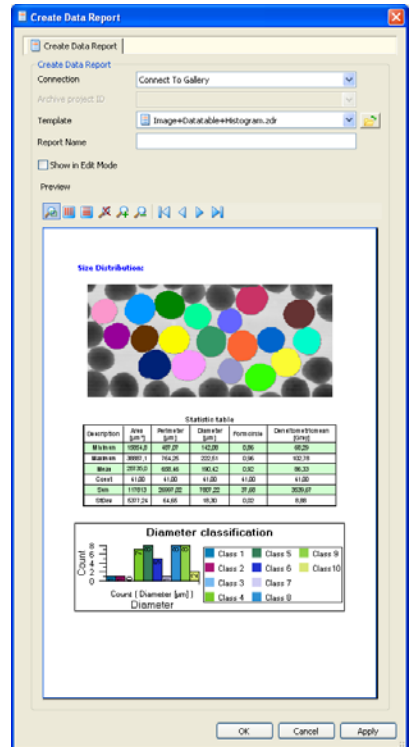
➢ The new report is displayed and you can save it using the **Save Report As Template** function on the **File** menu.
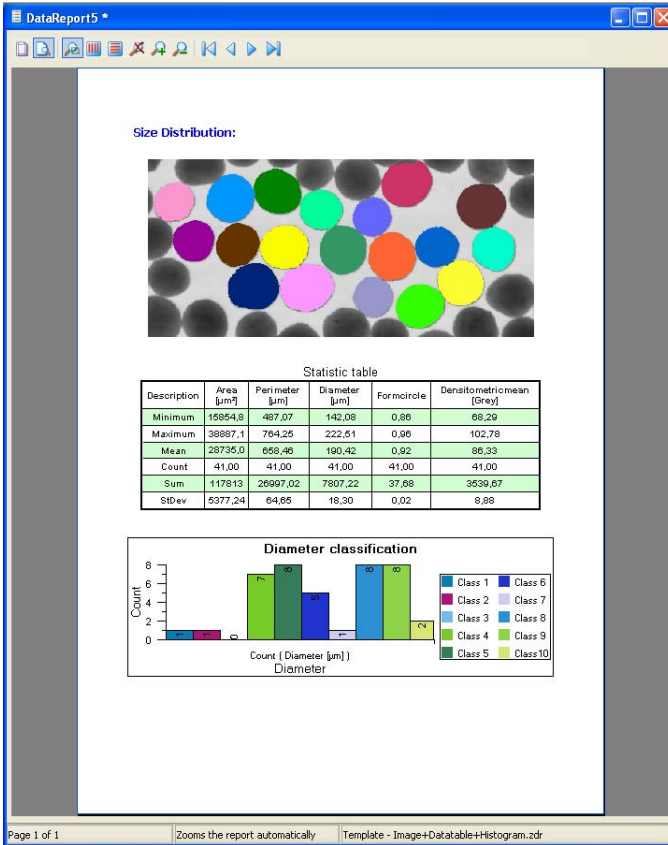
Note:

• When the report is saved, the files linked to the gallery (images, data tables) are automatically stored in a folder with the same name as the report.

Creating a report with saved data tables

Sample scripts can be installed from the "Report Script Examples" folder of the product DVD. by launching the **setup.exe** function. The sample images are copied to the workgroup folder *"My Documents\Carl Zeiss\Documents\Images".*

The following example shows you how to perform a measurement with a **Commander** script, display the measurement values that are saved in files in a two-page report and automatically save this report both in ZDR format and as a PDF file.

➢ Carry out the measurement for a number of images. To do this, select the **Repeat script** function on the **Commander** menu.
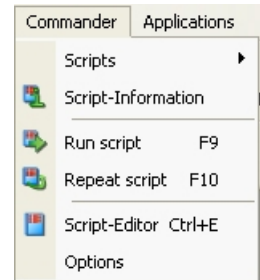
➢ In the **Select Source** field select from the drop-down list box **Folder**, from the drop-down list box **Look in** the directory *"My Documents\Carl Zeiss\Documents\Images"* and mark the two images "round1.zvi" and "round2.zvi".

➢ Select in the **Script selection** field the sample script "AV46Example12a-Evaluate_batch_EN.ziscript" and then click on **Start**.
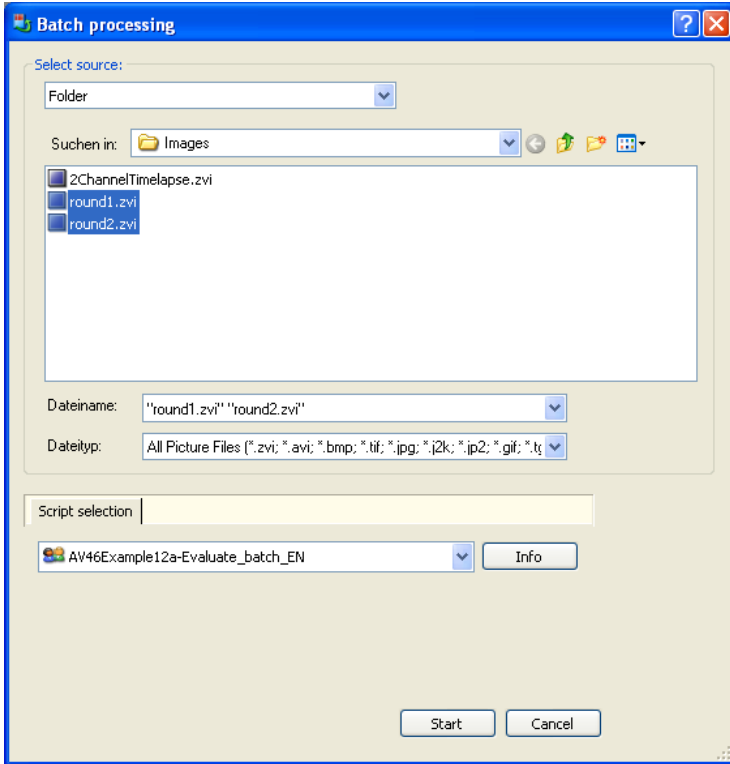
➢ The script automatically saves the generated raw-data, class and statistics tables in the directory *C:\guest* and opens the **Create Data Report** dialog.

➢ In the **Template** drop-down list box select the template **3datatables.zdr**.

➢ A report with three data tables will be created automatically.

➢ Click **OK**.

Note:

- When you are using a report template that contains file links to a folder, the linked files need to be retained if the report is to be printed out again at a later point in time.

Creating a report with files from an archive

Sample scripts can be installed from the "Report Script Examples" folder of the product DVD. by launching the **setup.exe** function. The sample images are copied to the workgroup folder *"My Documents\Carl Zeiss\Documents\Images"*.

The following example shows you how to display images and measurement values saved in an archive together in a report.

➢ First, use the **New** function on the **File** menu to create a new Asset Archive with the name "Test" in the folder *C:\guest*.
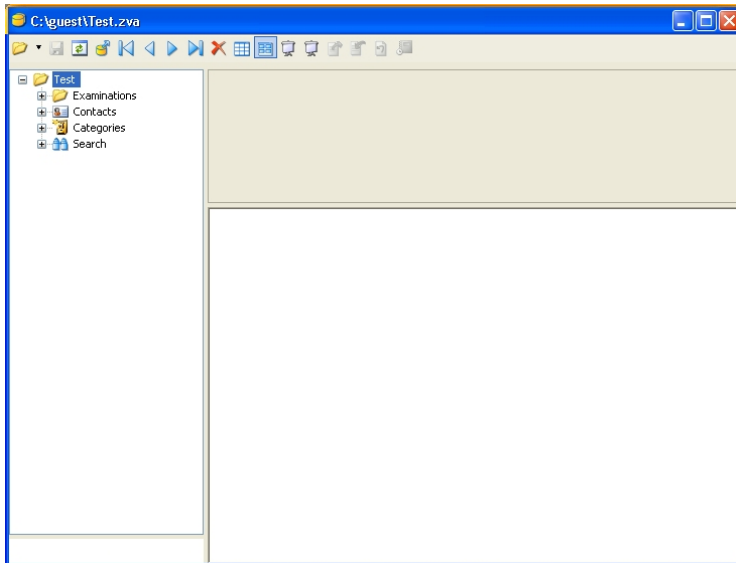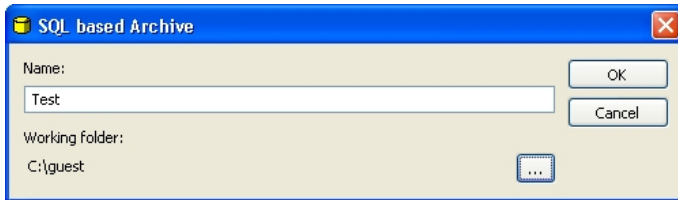
➢ Create a new project with the name "P0001" using the **Add New Project** function on the **Archive** menu, using the template **Default**.
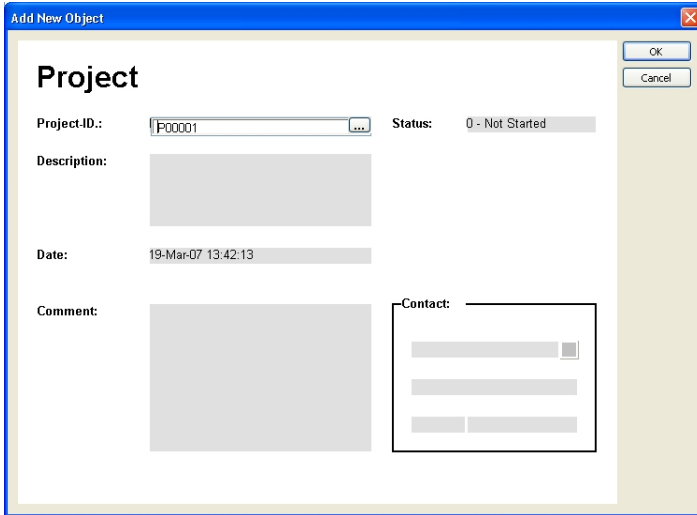
➢ Carry out the measurement for a number of images. To do this, select the **Repeat script** function on the **Commander** menu.
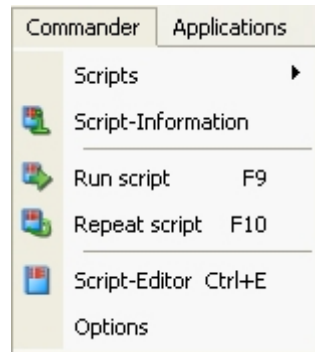
➢ In the **Select Source** field select **Folder** from the drop-down list box, from the drop-down list box **Look in** the directory *"My Documents\Carl Zeiss\Documents\Images"* and mark the two images "round1.zvi" and "round2.zvi".

➢ In the **Script selection** field select the sample script "AV46Example13a-Evaluate_batch_EN.ziscript" and then click on **Start**.
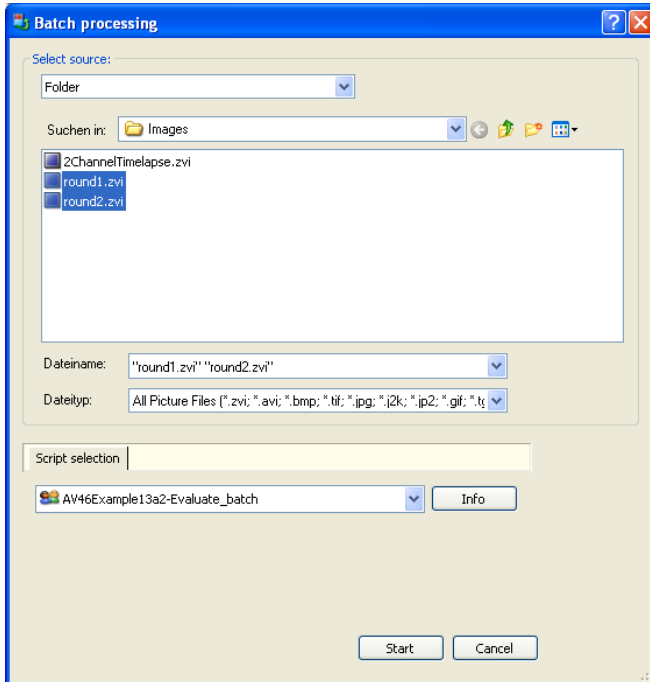
➢ In the menu **Commander** ⇒ **Scripts** ⇒ **Workgroup** open the file "AV46Example13a2-Evaluate_batch.ziscript"

➢ Click **OK**.
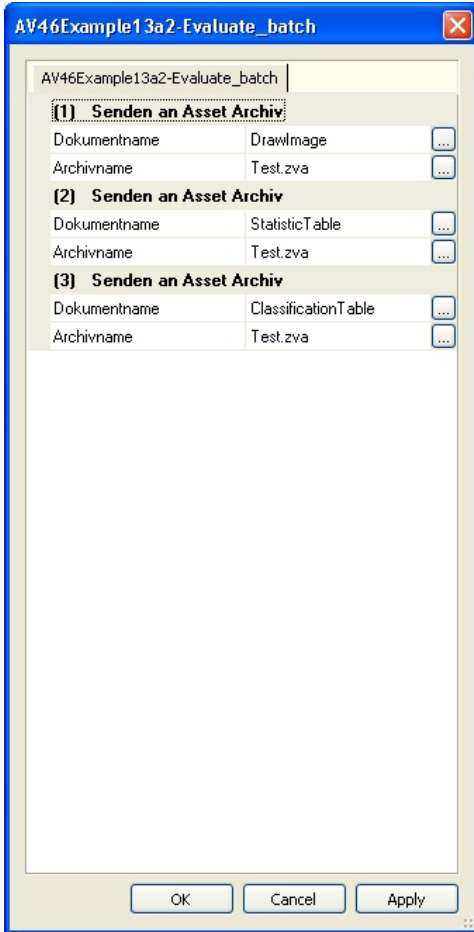
> ➢ All files, created with the script are saved automatically in the project "00001" of the archive.

> To create a report, select the **Create Data Report** function on the **Evaluate** menu.

> In the **Create Data Report** field, select *Connect to Archive* from the **Connection** drop-down list box.

> In the **Template** drop-down list box select the template **Image+Datatable+Histogram_ Archive.zdr**.

> In the **Report Name** text field enter a name for the report to be created, e.g. "TestreportB".

> Click on **OK**.
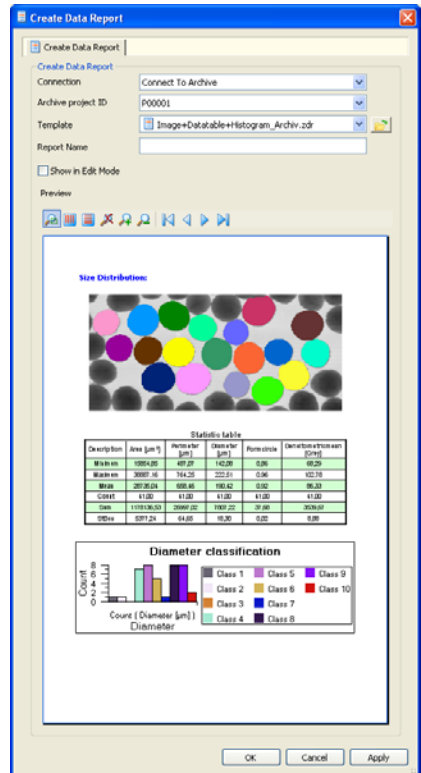
> The new report is displayed and you can save it using the **Save** or **Save As** functions on the **File** menu.
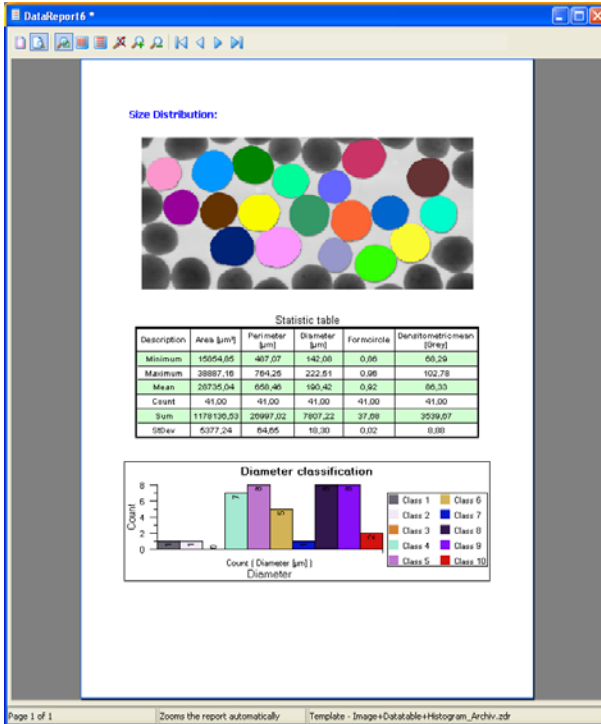
Note:

- When you are using a report template (e.g. Image+Datatable+Histogram.zdr) that contains file linked to a project within an archive, the linked files need to be retained in this archive/project, if the report is to be printed out again at a later point in time.

7. Documentation

7.1 General

Documentation is probably the most important part of your work. This term is often used to cover a number of different procedures:

In one case it may simply be a question of saving image files under a definable name; in others it may be a question of storing additional information with the images, so that the conditions of acquisition can be traced and recreated identically, or so that individual images can be found again quickly using this additional information.

Even in the basic version of AxioVision, the **Image Browser** offers a wide range of options that enable you to manage your images efficiently. Additionally you can save your images in a folder-based archive.

Via the AxioVision module **Asset Archive**, you can archive even extremely large quantities of data.

NOTES:
- Important information about "Saving" can be found in chapter 8.2 "User and File Administration".

7.2 AxioVision ZVI - the Image Format for Digital Microscopy

Advantages of the ZVI format

The AxioVision ZVI image file format is central to documentation with AxioVision. Compared to external formats, such as BMP, JPG, TIF etc., the ZVI format offers you numerous advantages:

- **Saving without loss of quality.** AxioVision also saves the current display settings for brightness, contrast and gamma value, without modifying the original data. You can, therefore, reactivate the original display of the image at any time. For external formats, you either have to go through the laborious process of finding the relevant display again or the pixel information has to be saved in modified form. However, in this case the original data are lost.

- **Incorporation of additional data** (e.g. microscope and camera configurations, comments, information on the sample etc.).

- **Separate saving of image and annotations.** This means that annotations can also be changed or deleted at a later time. When external formats are used, annotations have to be "burnt" into the image. However, they can then no longer be deleted or changed. In this case, of course, the pixel data beneath the annotations are overwritten.

- **Incorporation of measurement data for interactive measurements** into the image file.

- **Management of all data in a single file.** This makes it much simpler to handle the files, particularly when working with multidimensional images. If you use external formats, every single image has to be saved separately. This can quickly result in several hundred individual image files.

None of the above features are available with other formats. In short, the ZVI format has been specially developed for use in the field of digital microscopy. This is not the case with any of the other formats.

Saving 2D images

AxioVision gives you the option, for images saved in external formats, saving all additional information (additional data, annotations, interactive measurements etc.) separately from the actual image data. An additional file is created, containing these data, for each image. The format of the file is XML. This XML file is saved in the same folder as the image. The name of the XML file is composed of the name of the image + the extension + "XML". Therefore, if the saved image is called "Testimage.jpg", a file is generated with the name "Testimage.jpg. meat.xml", which then contains all the additional information.

When you open an image with AxioVision that has been saved in an external format, the system will always check automatically whether a file containing the corresponding additional data is present. If one is, these data are read out and displayed accordingly. Of course, this only applies if you are working in AxioVision. When you open an image such as this in a different image processing program, the image is displayed without the additional information.

To save an image with a format larger than 8 bits such that it can be displayed in another application program just like it is displayed in AxioVision, we recommend that you activate the **Apply display mappings** check box when saving the image (**File ⇒ Save As**). Please note, however, that this will change the pixel values.

Saving multidimensional images

In principle, multidimensional images can be saved using the standard function for saving images (**File ⇒ Save As**). Images can also be saved in external formats using this function. For multidimensional images, however, all single images then have to be saved in separate files. In order to retain an overview, only the first image is saved under the specified name and folder for each multidimensional image. A separate subfolder is created for the single images that belong with it:
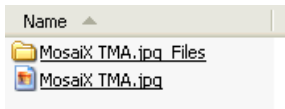
MosaiX image saved as a jpg file. The single images are saved in the "MosaiX TMA.jpg_Files" folder.

An XML file with the additional information is also saved in the folder with the single images. The XML file containing the additional information always bears the name "_Meta.xml":
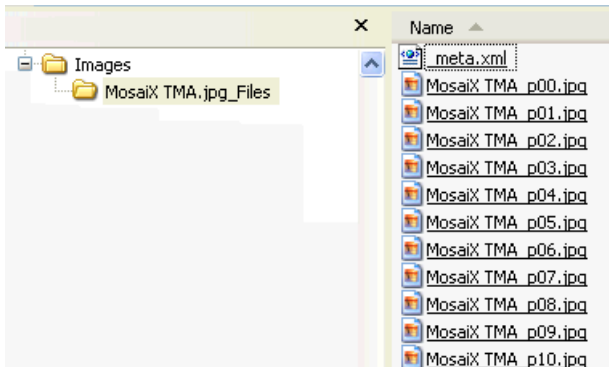
Subfolder containing single images and XML file

When you open an image with AxioVision that has been saved in an external format, the system will always check automatically whether such a folder is present. If one is, the single images are read out and combined to form a multidimensional image. In the course of this process, the information from the "_meta.xml" file is analyzed.

NOTE:
- The **File ⇒ Export** function offers special functions for saving multidimensional images. These include, for example, functions for generating video formats or special functions for saving multichannel fluorescence images.

7.3 Managing Images with the Image File Browser

The Image File Browser allows you to display the images contained in the folders on all of your PC's drives as a gallery or list, or with a form for entering additional data; you are able to search for and load images and view a slide show. The image browser is a convenient, easy-to-use tool to help you manage your images.

To open the image browser, select **File ⇒ Image File Browser**.

The Image File Browser window
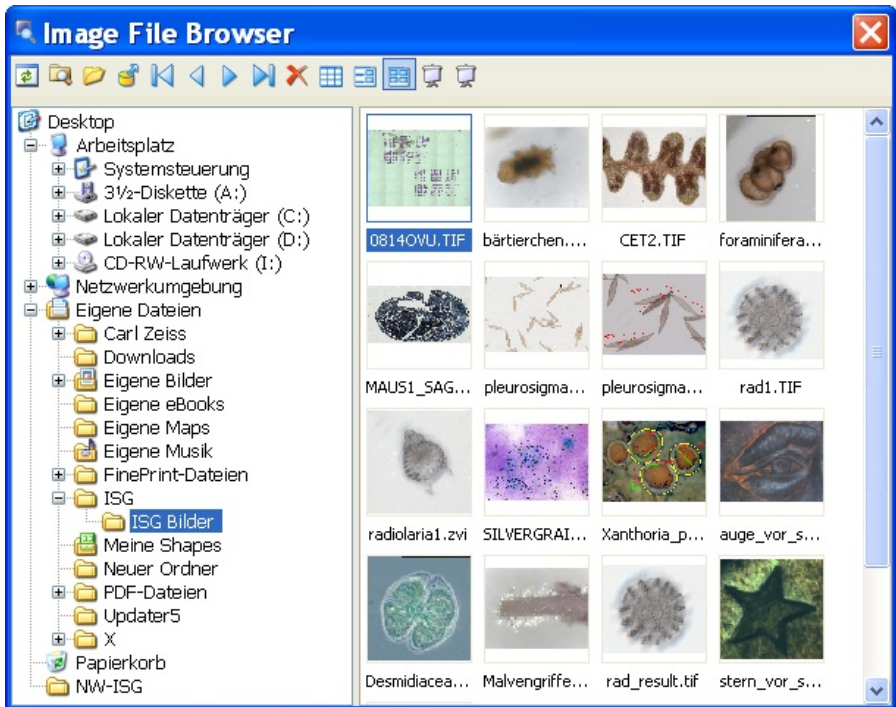
The left-hand side shows your system's folder structure, in the familiar Microsoft Windows Explorer format.

The **Image File Browser** can be operated fully using the functions on the toolbar.

If you select a particular folder, all the image files it contains are displayed automatically in the selected mode (here **Gallery** mode).

The functions of the Image File Browser toolbar

Refreshes all views.

Opens the dialog window **Scan drive**.

Creates a new folder or edits an existing one.

Loads the selected image(s) from the archive.

Moves to the first record.

Moves to the previous record.

Moves to the next record.

Moves to the last record.

Deletes the currently selected record.

Selects the Data Grid mode.

Selects the Form mode.

Selects the Gallery mode.

Runs the slide show using all records. To stop the slide show, press the **Esc** key or right-click in the image and deselect **Slide show**.

 Runs the slide show using selected records. To stop the slide show, press the **Esc** key or right-click in the image and deselect **Slide show**.

Change the form for data entries

A form is used to enter data and to display information on the monitor.

To display image information and enter additional information switch to the

Form view by clicking on the icon. In the Form view, the form "Archive.zvf" is used:

The form "Archive.zvf".

This form must be in the folder *"C:\Programs\Carl Zeiss Vision\AxioVision4\0009\Templates\Form"*.

To use a different form for the form view in the Image Browser, generate a new form on the basis of the installed templates, and save this form in the folder mentioned above.

➢ From the **File** menu select the **New** function and there the **Form** tab.

➢ Select one of the templates and click on **OK**. To see a preview of templates, click on **Apply**. The corresponding template is then opened without the **New** dialog being closed.

➢ Now select the **Save As** function from the **File** menu, and go to the data directory *"C:\Programs\Carl Zeiss Vision\AxioVision4\0009\Templates\Form"*. Open the form folder, or create it if it does not already exist, and save the template under the name "Archive.zvf".

➢ Now open the image browser and refresh the image gallery . If the form view is not active, click on the button on the image browser's toolbar.

7.4 Generate, print and export reports with the file extension ZVR

Reports are used to display and print out saved images and the additional information relating to them (title, author, comments, keywords etc.). Each report is based on what is known as a report template. Templates specify the layout and formatting for the report and its individual elements (image, data etc.).

You can modify report templates yourself, or generate completely new ones. Please also read the section "Templates for forms and reports", which you will find further down this section. The template files are saved in the folder *"C:\Programs\Carl Zeiss Vision\AxioVision4\0009\Templates\Report"*.

To allow reports to be incorporated into other documents (e.g. Word files), the option of exporting them is available. AxioVision offers export into the so-called Microsoft Windows Metafile Format (WMF). This format allows you to incorporate reports into virtually any Microsoft Windows program. The individual fields of a report can be modified in most cases. However, the precise range of functions depends on the program in question.

Generating new reports

➢ Activate the image for which you want to generate a report.

DCV.zvi | fluor02.tif | Intmeas.tif | Zylinder.zvi |

➢ Select from the **File** menu the **New** function, and activate the **Reports (zvr)** tab.
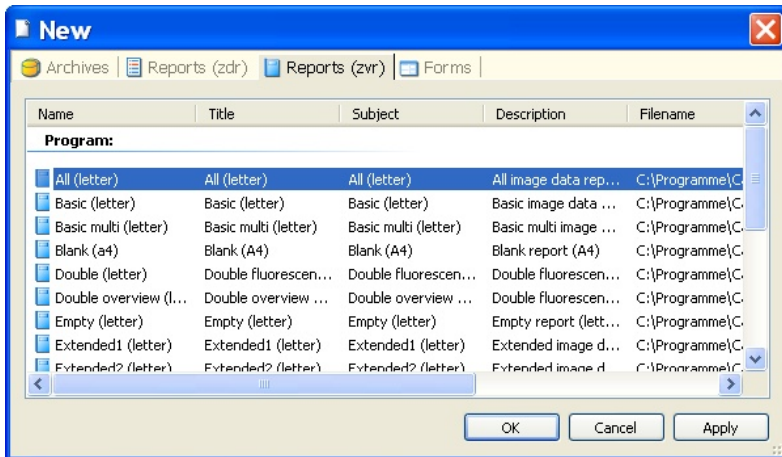
> ➢ Select one of the templates and click on **OK**. To see a preview of templates, click on **Apply**. The corresponding template is then opened without the **New** dialog being closed.

> ➢ The new report is now displayed. You can save it just like any other file via **File** ⇒ **Save** or ⇒ **Save As**.

Printing a report

➢ Activate the report that you want to print.

➢ Select from the **File** menu the **Print** function. The standard dialog for printing documents is then displayed.

➢ Enter the desired settings and click on **OK** to print the report.

Exporting a report

➢ Activate the report that you want to export.
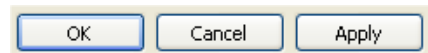
➢ Right-click in the report and select the **Export to WMF** function.

	Full Screen	F11
	Bring to Front	
	Send to Back	
	Bring Forward	
	Send Backward	
	Set TAB order	
	Export to WMF	
	Properties	Alt+Enter
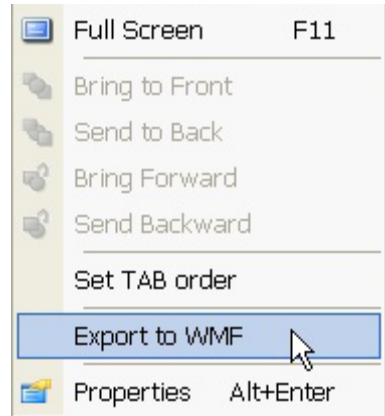
➢ A standard Microsoft Windows dialog for saving the file is then opened. Select the target folder under **Save As** and enter a file name.

➢ Then click on **OK** to save the report.

Templates for forms and reports

Reports are mainly used to print out saved images and the additional information relating to them (title, author, comments, keywords etc.).

Forms are used for displaying information in a structured way and for entering information on screen.

Reports and forms are based on what are known as templates. Templates specify the layout and formatting of the individual elements (image, data etc.).

You can modify templates yourself, or generate completely new ones. The template files are saved in the folder *"C:\Programs\Carl Zeiss Vision\AxioVision4\0009\Templates\Forms* or *Report".*

AxioVision is supplied with a range of different templates. This means that you do not necessarily have to generate templates yourself. Often, however, a modification will be necessary to bring the templates into line with company or project-specific requirements.

As the generation of templates can be very time-consuming, depending on their complexity, we recommend that you use an existing template as a starting point wherever possible. This avoids having to generate them from scratch (an even more time-consuming process).

NOTES:
- The following example runs through the steps required to edit a template, using the example of a report. These steps also apply, however, to form templates.

Settings for editing a template

➢ Select from the **File** menu the **New** function, and activate the **Reports** or **Forms** tab.
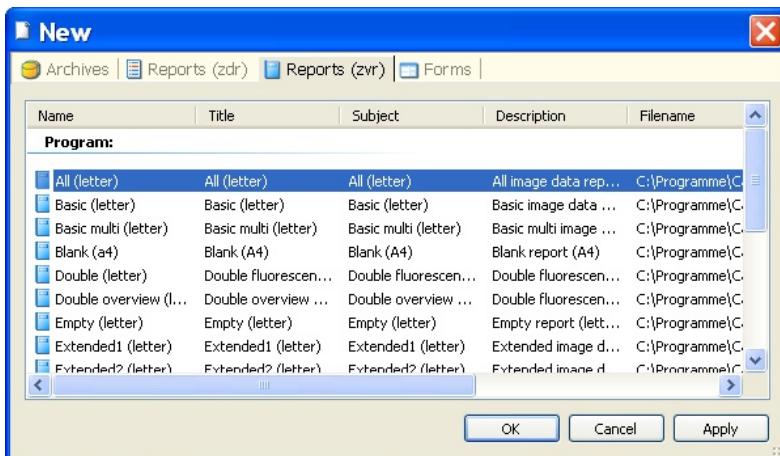
➢ Select one of the templates (e.g. **Reports** - **Standard**) and click on **OK**. To see a preview of templates, click on **Apply**. The corresponding template is then opened without the **New** dialog being closed.
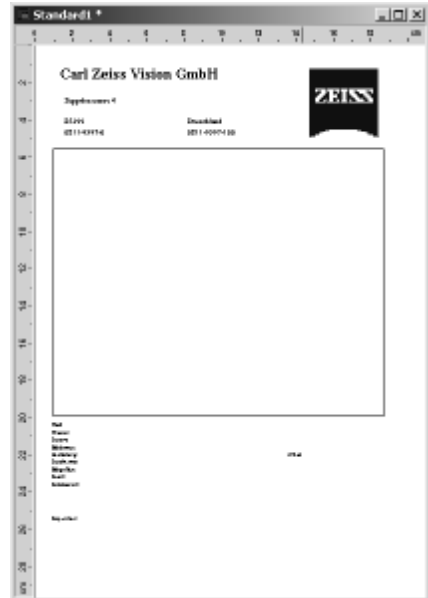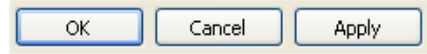

➢ The report is now displayed with its current layout.

Notes for report templates:

- The details in the header (logo, company name etc.) do not have to be defined as individual fields, but can be entered directly via a dialog. To do this, select **Tools** ⇒ **Options** ⇒ **ID**. Enter the corresponding data here. These data are then displayed automatically in the report's header.

- If you have not yet entered this information, this area is empty.

➢ To modify the template, you need to switch to **Design mode**.

➢ To do this, right-click in the template and activate the **Design mode** command from the shortcut menu.

➢ The draft mode is active if the tick can be seen to the left of the **Design mode** command.

➢ To see the position and size of all elements, activate the **Show bounding box** option from the shortcut menu. All fields are then displayed with a blue frame.
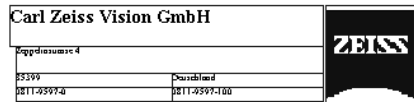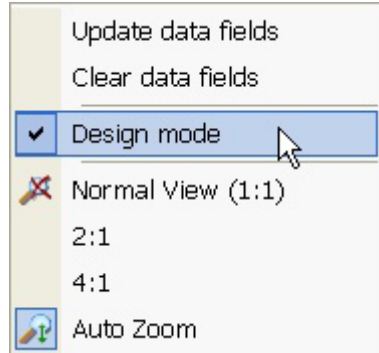
To make positioning easier, you can activate a grid, with which all elements can then be automatically aligned.

➢ Select from the shortcut menu the **Grid settings** function.

➢ Activate the **Show Grid** and **Snap to Grid** options, and click on **OK**.

➢ A yellow grid is now displayed on top of the template.
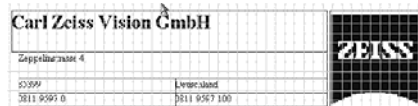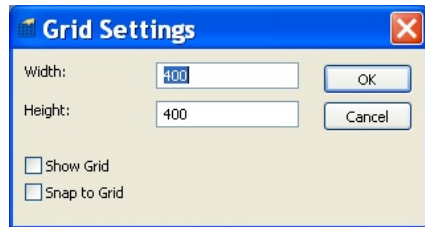
Moving and deleting fields

➤ To move one or more fields, you need to select the field(s) in question. To do this, click on an individual field, or drag out a frame around the fields you want to move with the left-hand mouse button depressed.

➤ If you hold down the **Shift** key while clicking, you can also select a number of individual fields.

➤ To move the fields, left-click in the selected area, and drag the fields with the left-hand mouse button depressed. The pointer changes to a cross during this process.

➤ The fields are automatically aligned with the grid.

➤ To delete the selected fields, simply press the **Del** key on your keyboard.

➤ The fields are then deleted, but can be restored via the menu function **Edit** ⇒ **Undo** provided you have not saved the template.
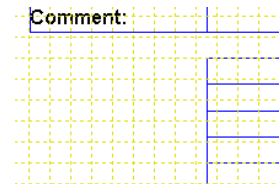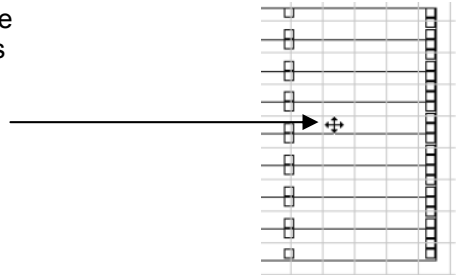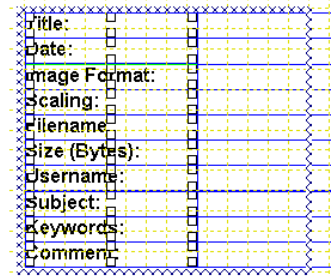
Inserting new fields and changing field values

➢ If you want to insert a new field, you can select the **Text** tool from the **Annotations** menu and then position it in the desired location.
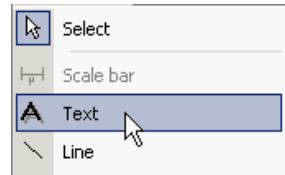
➢ The simplest way to do this, however, is to copy an existing field. In this case all of the attributes (size, color, font etc.) are adopted.
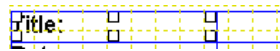
➢ To do this, click on an individual field, or drag out a frame around several fields with the left-hand mouse button depressed.

➢ If you hold down the **Shift** key while clicking, you can also select a number of individual fields.

➢ To duplicate the field and move it at the same time, hold down the control key (**Shift** key), and left-click in one of the two selected fields. Drag it to the desired location with the left-hand mouse button depressed.
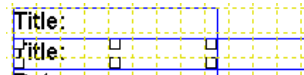
➢ To change the name of a field, switch to editing mode by double-clicking in the field.
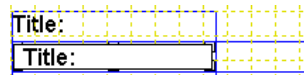
➢ You can now enter a new name:
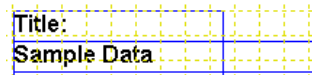
➢ So far you have only changed the field name! Now you need to specify that relevant information should also be entered to the right of the identifier.

➢ To do this, select the empty field to the right of the field name, and open the **Properties** window by right-clicking in the field. Activate the **Item** tab.

➢ A particularly important element here is the **Tag** parameter, which displays the information available in AxioVision images. Click on the button to make a selection.
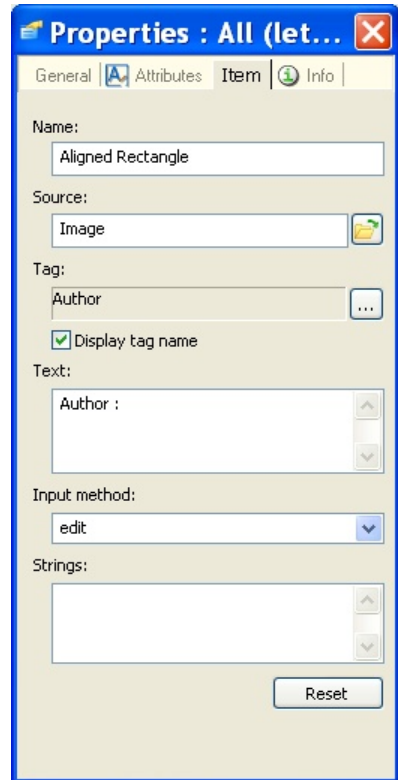
➢ Now complete the other parameters according to your requirements.

➢ Carry out these steps for all of the fields that you wish to use in your template.

➢ Then save the template in the folder *"C:\Programs\Carl Zeiss Vision\AxioVision4\0009\Templates\Report"*.

➢ The templates are now available in the list of available templates on the **File** ⇒ **New** dialog.

Changing field attributes

Changing field attributes means modifying the appearance (color, line thickness, text type, text size etc.) of fields.

➢ To change the attributes for individual or several fields, you need to select the field(s) in question. To do this, click on an individual field, or drag out a frame around the fields you want to change with the left-hand mouse button depressed.

➢ If you hold down the **Shift** key while clicking, you can also select a number of individual fields.
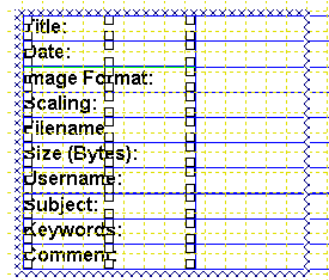
➢ Now right-click in the report and select from the shortcut menu the **Properties** command.

➢ In the **Properties** dialog, activate the **Attributes** tab.

➢ Now change the attributes using the tools under **Settings for selected items**.

➢ If necessary, select a different **Font**.

➢ Save the template in the folder *"C:\Programs\Carl Zeiss Vision\AxioVision4\0009\Templates\ Report"*.
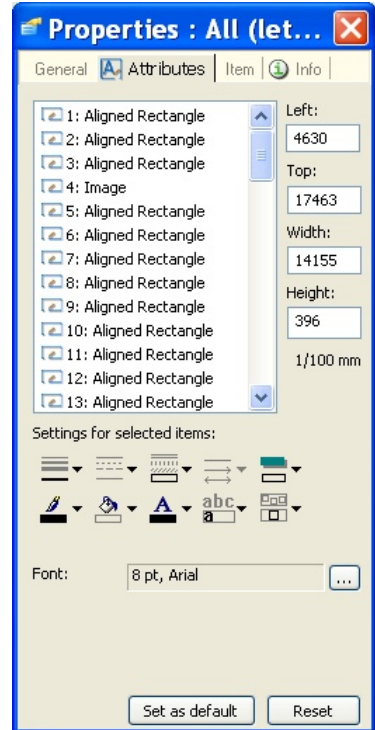
7.5 Editor for Multidimensional Images

Using the image editor, it is possible to generate a large number of images in a targeted way from large, complex ZVI images. The result is always a new, unsaved image with reduced complexity. It is possible to influence the following dimensions:

- x/y pixel resolution
- channels
- instants of exposure
- z-stack positions

When you select the **Change Image Dimensions** function on the **Edit** menu, a wizard is opened. This wizard always uses the active image in the foreground. When you are working in the image editor, it is not possible to execute any other functions in AxioVision. Only the pages that are relevant for the image are displayed in the wizard.

➢ Open a multidimensional image. In this example, a 3-channel z-stack fluorescence image will be modified. Channel 1 and z-planes #1-6 are to be removed from the image, and the x/y resolution is to be reduced by a factor of 2.

➢ Select from the **Edit** menu the **Change Image Dimensions** function. A dialog is opened in which the image located in the foreground is displayed. All the control elements that are required to operate the image (**Player**) can be found at the bottom image edge.

➢ As the first step, indicate that you want to remove the selected images, and click on **Next**.
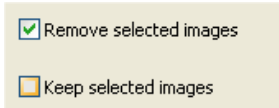
➢ Now select channel 1 (which is to be removed), and click on **Next**.
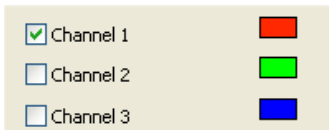
➢ From the drop-down list box, select the desired reduction in resolution (1:2).
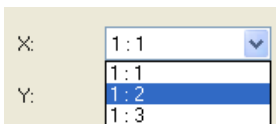
➢ This setting means that every second pixel in both the x and y dimensions is removed. This reduces the image resolution to a quarter. Click on **Next** to proceed to the next step.

➢ Now define the z plane range that you want to be removed from the image.

➢ To do this, move the **z** slider to position 1

➢ Click on the top **Get player position** button

From:	1	<-Get player position

. Now move the slider to z-position
6, and click on the bottom **Get player position** button

To:	6	<-Get player position

.

➢ To check the result, click on the Preview button. A preview of the
reduced image is created. If you are happy with the selection, click on
Finish . The wizard is closed, and a new image with the reduced
complexity is generated. Save this image under a new name.

8. Configuration

8.1 General

One of the most striking new features of AxioVision Version 4 is the fact that it allows you to adapt the interface and the way you operate the program almost entirely to suit your own requirements. A great deal of attention has therefore been devoted to ensuring that the configurations of individual users can be managed separately. This is guaranteed by saving individual data, such as toolbar configurations, customized dialogs and workflows, general system settings and, of course, the data generated on an individual basis, separately from the data of other users. The Microsoft Windows user administration is used entirely. In AxioVision additional settings are not required.

The settings of an individual configuration can be exported easily and can be accessed to other users or to all users (see also chapter 8.7 "Exporting and Importing User Configurations").

8.2 Overview: Setting up AxioVision

This chapter gives you a brief overview of the steps to setup up AxioVision. In the appropriate subchapter you will find further information.

Installation

- Installation must be carried out with **administrator rights**.

- AxioVision is then available to all users of the system (including new users who are set up after installation). Installation is **not** carried out individually for each user.

- Remain in administrator mode during the following steps described below. Read also Chapter 1 and 2 of the Installation Manual *AxioVision Product Suite*.

Setting up AxioVision

AxioVision can now be set up as desired:

- Microscope configuration and/or MTB2004 Configuration
- Scalings
- Hardware settings files and experiments
- Toolbars, keyboard shortcuts, workflows and user dialogs
- Measurement programs

Backing up the configuration and restoring it for individual users

➢ After configuration the settings and user files can be backed up via the **Tools** menu ⇒ **Import/Export Configuration**.

➢ In **step 1** select **Export**.

➢ Important: Please ensure that **Save all user documents in ZIP-file** is also activated in **step 2 Select sources** so that the user files (scalings, hardware settings etc.) are also exported:
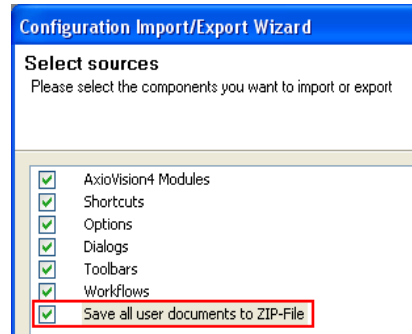
➢ The result is two files with the name given to them during the export and the extensions **AVCONF** and **ZIP**. You can select the folder for these two files.

➢ The relevant settings for individual users can be imported using the same function.

➢ To do so select **Import** in **step 2**.

NOTES:
- The relevant user must naturally be logged on under Microsoft Windows during the import – not the administrator.

- The **channel pool**, used for simple definition of multichannel fluorescence images, cannot be exported. If necessary, the corresponding file must be copied via Microsoft Windows Explorer.

Making user files available for all users

➢ To make the user files available for all users, the files in the ZIP file must be extracted into the **Shared Documents** folder of the **All Users** user account. In plaintext the file is called: *C:\Documents and Settings\All Users\Documents*.

➢ The directory structure required by AxioVision is generated automatically.

8.3 User and Data Administration

### Microsoft Windows user administration

Under Microsoft Windows XP there are three fundamental levels of security that are allocated to users. These are granted to end users through their membership of the groups **Users**, **Power Users** or **Administrators**.

- The **Users** group is the most secure group, as the standard rights of this group do not allow the members to change operating system settings or the data of other users. Members of the **Users** group are able to carry out most general tasks, such as running applications, using local and network printers, as well as shutting down and blocking the work station. Users are not entitled to release directories or set up new local printers.

  - → Under certain circumstances, members of the **Users** group are not able to execute AxioVision, e.g. if cameras from other manufacturers are being used or a Matrox Meteor frame grabber is installed.

- **Power Users** are able to execute the following tasks: installing programs that do not change operating system files, or installing system services. Adjusting system-wide resources, including printers, date, time, energy options and other control panel resources. Creating and managing local user accounts and groups. Stopping and starting system services which are not started by default. **Power Users** have no access to the data of other users on an NTFS volume unless they are granted this right by the users concerned.

  - → In order to use AxioVision, users should always belong to the **Power Users** group.

- The **Administrators** group carries out maintenance tasks on the computers. The standard rights for this group give members complete control over the entire system. For this reason, only reliable employees should belong to this group.

User files

Each user under Microsoft Windows XP has a personal folder for the files that he has created. These personal folders are created by Microsoft Windows for each user of the computer. If a computer is used by more than one user, the individual personal folders are indicated by the name of the corresponding user. In addition, Microsoft Windows provides the **Shared Documents** folder for files that you want to release for other users.

When a new user is created under Microsoft Windows XP, a user account is set up for each user.
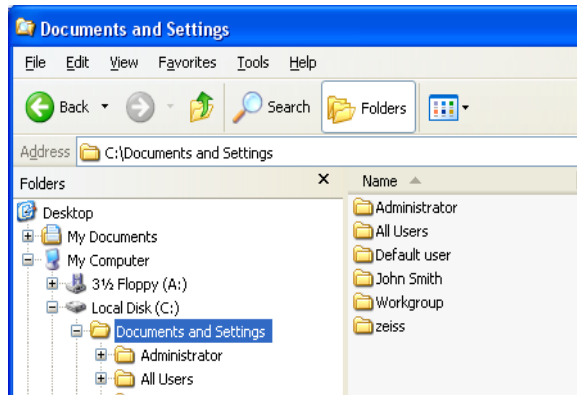
The user accounts are located under *C:\Documents and Settings* A folder can be found there for each user – the user account.

Theoretically, user data should only be saved in the **My Documents** folder. This folder has a link to the Microsoft Windows Desktop as well as in Microsoft Windows Explorer. The personal folders can be accessed very easily via these links:
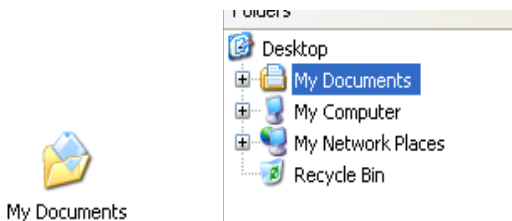
However, the folder is actually located within the folder structure of the user account.

For reasons of data security, however, we recommend that you separate program and data files. This means that the data created by a user should be stored on a different drive from the programs.

Advantages of this procedure:

1. After installation and configuration an image of the system partition can be created, which makes it possible to restore the system very quickly in the event of a disaster. "Images" represent a 1:1 copy of the hard drive, irrespective of whether or not there are files in the memory. An attempt is therefore made to limit the size of the system partition.

2. When you are making regular data backups, it is not always necessary to back up the complete system partition as well – only the user data needs to be backed up.

3. The data folder can be moved to a separate drive with sufficient memory, even for multidimensional images (see also point 1). If the folder is on a network drive, automatic backup by the relevant IT department is often ensured.

The shared account "All Users"

Microsoft Windows always creates a default user account with the name **All Users**. Files saved here can be used by all users of the system. This "shared account" can be used to save files (e.g. scalings and measurement programs) that are used centrally by all users, without the need to copy these files individually to each user's personal account in the event that the files are modified.

Creating new users

Set up new users (or create new user accounts) via the computer administration function (Microsoft Windows Desktop ⇒ **My Computer** shortcut menu ⇒ **Manage** ⇒ **Local Users and Groups**):
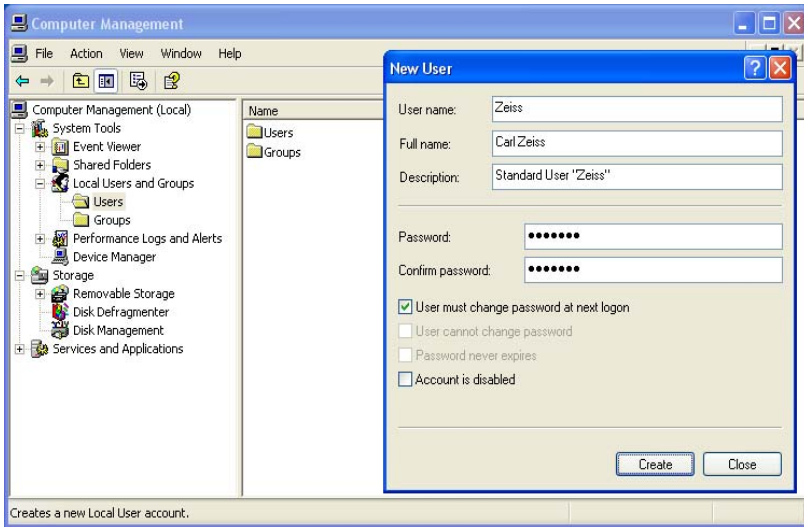
NOTES:

- Administrator rights are required to create a new user or to allocate a profile.

- When you add a new user, the **Users** profile is allocated by Microsoft Windows by default. A different profile can be allocated after the user has been set up.

- AxioVision is released for use with the **Power Users** profile only. This is mainly for reasons of hardware support. The Matrox Meteor drivers, for example, do not function with the **Users** profile.

# Data administration under AxioVision

Data files

AxioVision stores all files created by the user in the *"My Documents\Carl Zeiss\Data"* folder. There you will find additional subfolders for the individual documents, such as automatic measurement programs, calibrations, experiments etc.:
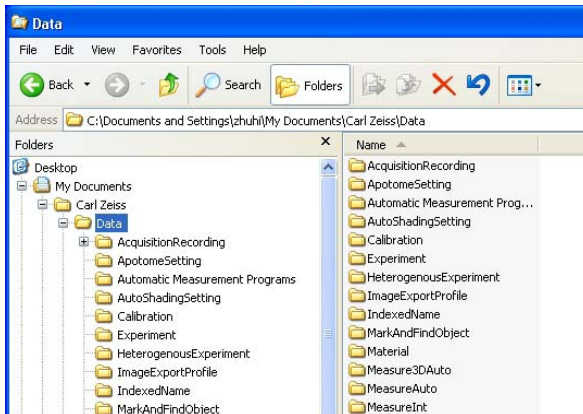
*Folder structure of the AxioVision data folder*

AxioVision images

Within the *My Documents* folder, you will also find the Microsoft Windows default folder *My Pictures*, which is suggested by AxioVision as a location for saving your images.
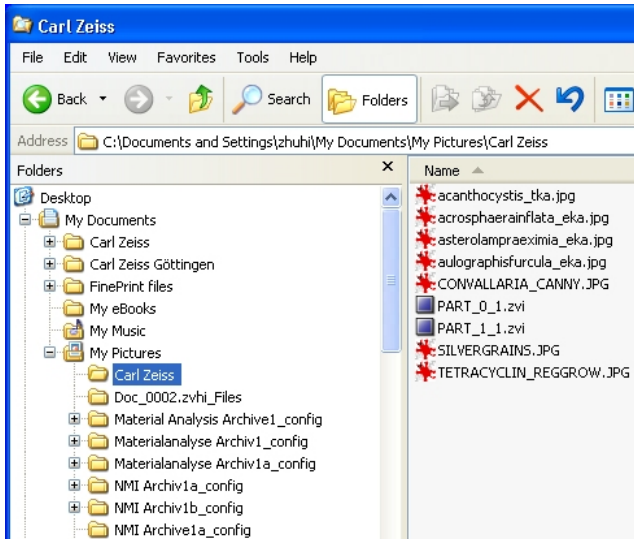
Temporary files

During the acquisition of multidimensional images using the Time Lapse Imaging module, if the time intervals are less than 1s, images are only stored in the user memory. However, if the experiment is too complex, it is possible that the user memory may not be able to hold all the images, which are then stored temporarily in the folder for temporary files.
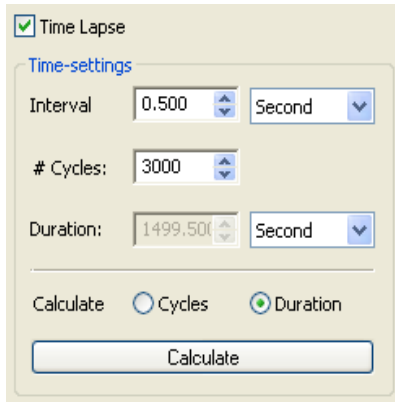
AxioVision draws your attention to this by means of an appropriate message before acquisition:
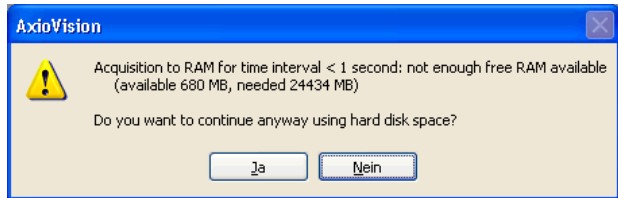
This folder is also located within the user account. As huge amounts of data can accumulate as part of this process, this folder should also be moved.

NOTES:
- Since the highest possible speed is important for this procedure, you should either create a reference to a different drive on the same hard drive, or use a different, faster, hard drive.

- On no account should you transfer the folder for the temporary files to a network drive. Data transmission is clearly too slow here.

AxioVision configuration files

Files that are important for AxioVision to function (INI files for devices etc.) are also saved within the user account in the folder *Application Data\Carl Zeiss\AxioVS40.*

This subfolder is not displayed if you are a normal user. It is only available to administrators.

Moving the "My Documents" folder

➢ Select the **Properties** function in the shortcut menu of the **My Documents** folder (right click on the desktop or in Explorer).

➢ Click on the **Move...** button in the **My Documents Properties** dialog and select the target folder. If you click on **OK**, the folder is adopted into the **Properties** dialog.

➢ Exit the **My Documents Properties** dialog by clicking on **OK**. All subfolders and files from **My Documents** will now be moved into the new folder.

➢ Repeat these steps for each user.

NOTES:

• This step must be performed for each user before AxioVision is executed for the first time. AxioVision remembers this path in "plaintext" after the first program start. If the "My Documents" folder is not moved until after the first program start, AxioVision creates the folder and all subfolders in the original place again!

• The following tip should only be followed by specialists (and even specialists should be reminded to **save data *beforehand*.**) The following action can be taken if AxioVision is accidentally started BEFORE the folder has been moved, resulting in the problem described above:

- Close AxioVision.

- Delete the **UserOptions.xml** file in the **Application Data\Carl Zeiss\AxioVS40\Profiles\Default** folder of the appropriate user account.

- The file is automatically recreated and the current path settings for **My Documents** are read in again the next time AxioVision is started.

- Never simply move the **My Documents** folder via Explorer. Always follow the procedure described above, as only then will all the links and references to this folder work.

- In AxioVision the paths for user and work group files and templates are displayed in the **Tools** menu ⇒ **Options** function ⇒ **Folder** tab sheet. It is also possible to change the paths here. However, you should avoid doing this for the reason given above.

Moving the folder for temporary files

➢ Select **System** in the control panel.

➢ Click on **Environment Variables** on the **Advanced** property page.

➢ Repeat these steps for each user.

➢ Change the settings of the user variables for **TMP** and **TEMP** via **Edit** in the **Environment Variables** dialog.
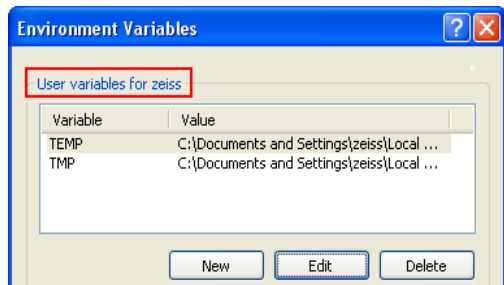
8.4 Scalings

General

Scalings are required to allow you to display scale bars and measurement values in real units instead of pixel values. This requires configuration of the system for every combination of objective, optovar and camera resolution. If you are using a camera of the AxioCam family, please be aware of the following!

NOTES:
- Only the basic resolution needs to be calibrated for the cameras of the AxioCam family. All other resolutions (higher resolution levels and binning modes) are then derived from this basic calibration automatically.

- For theoretical scaling there is no need using a stage micrometer, but special settings in the microscope configuration are necessary, which are described in the Online Help.

The theoretical scaling offers you the option of having scalings calculated automatically for a microscope that contains motorized or encoded elements. This means that there is no need for the time-consuming generation of scalings using a stage micrometer.

Calibration is usually performed using a stage micrometer. The scale depicted on the stage micrometer allows you to specify a known length simply and precisely.

Each scaling is saved in its own file with the extension "ZVSC". These files are stored for each user in the folder *My Documents\Carl Zeiss\Data\Scaling*.

NOTES:

- You can also use scalings from AxioVision Version 3, simply by copying them into this folder.

- As scalings are often also of interest to other users, in some cases it may be useful to save them in the shared account *All Users* (please read section 8.7 "Exporting and Importing User Configurations").
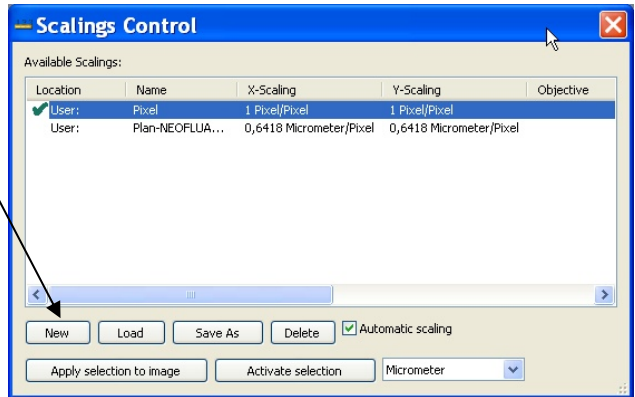
Generate scalings

➢ Acquire an image of the stage micrometer.

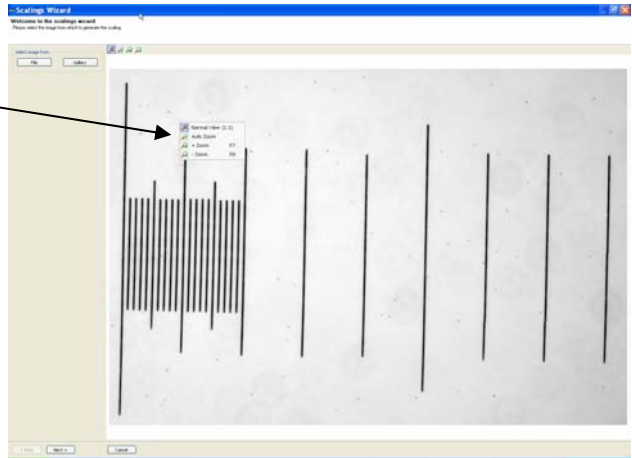➢ Make sure that you position as much as possible of the scale in the image.
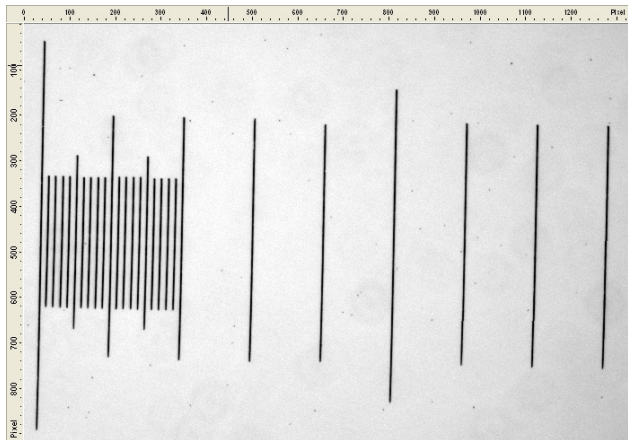
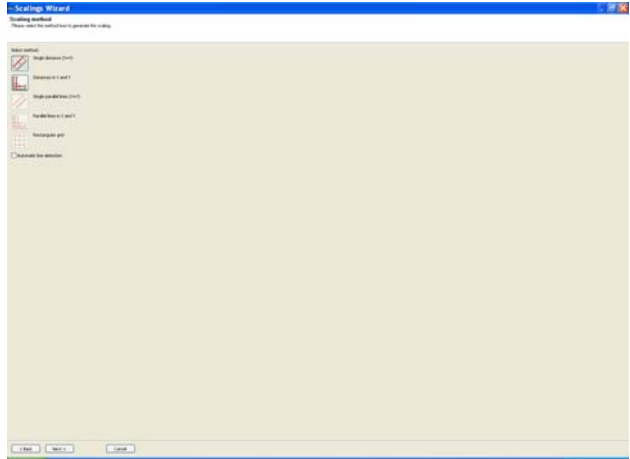➢ From the **Measure** menu, select the **Scalings** function.
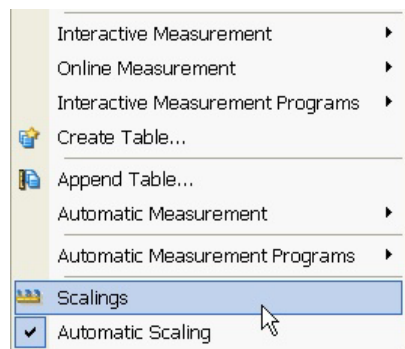
➢ In the **Scalings Control** window click on **New**.

➢ The scaling wizard is started.

➢ Right-click in the image and select **Auto Zoom** to view the entire image.

➢ Enlarge the window, if necessary.
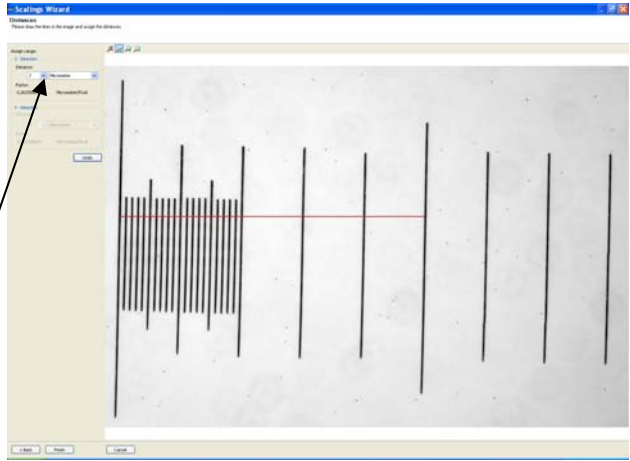
➢ Click on **Next**.

➢ Select the mode **Single Distance (X=Y)**.

➢ Click on **Next**.

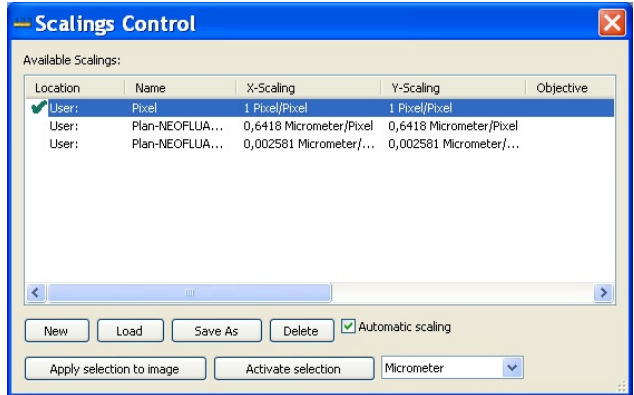➢ In the image click on the start and end points of the scale.

➢ Enter this distance into the **Distance** field, and select the **Unit**.

➢ Then click on **Finish**.

➢ Enter a name for the scaling and click on **OK**.

➢ The new scaling now appears in the **Scalings Control** window, and is activated automatically.

➢ The scaling is used as the default for subsequent image acquisition procedures.

NOTES:

- As scalings are often also of interest to other users, in some cases it may be useful to save them in the shared account *All Users* (please also read the previous section and section 8.7 "Exporting and Importing User Configurations").

- The **Save As** button is a simple way to save individual scalings for the work group.

8.5 Adapting the User Interface

The **Customize** function in the **Tools** menu allows you to customize the user interface. This function gives you a great deal of freedom in configuring AxioVision. You can therefore design "your own" AxioVision, showing only those functions that you actually need.

Any changes made in the **Customize** dialog are immediately displayed on the AxioVision interface. The dialog does not therefore contain "traditional" buttons, such as **OK** or **Cancel**. Once you have made the desired changes, simply close it using the ⊠ button in the top right-hand corner.
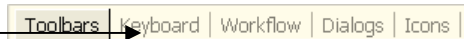
Creating toolbars

How do you create a toolbar for quick access to the image processing functions?
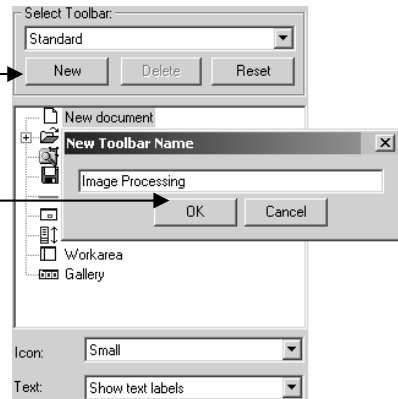
➢ From the **Tools** menu select the **Customize** function.
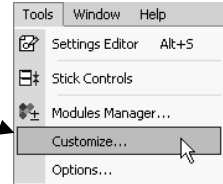
➢ Make sure that the **Toolbar** property page is active in the **Customize** dialog.

➢ To create a new toolbar:

- Click on **New**.

- Specify a name for the new toolbar.

- Click on **OK**.

- Define the size of the icons and indicate whether labels should be assigned to the buttons as well.
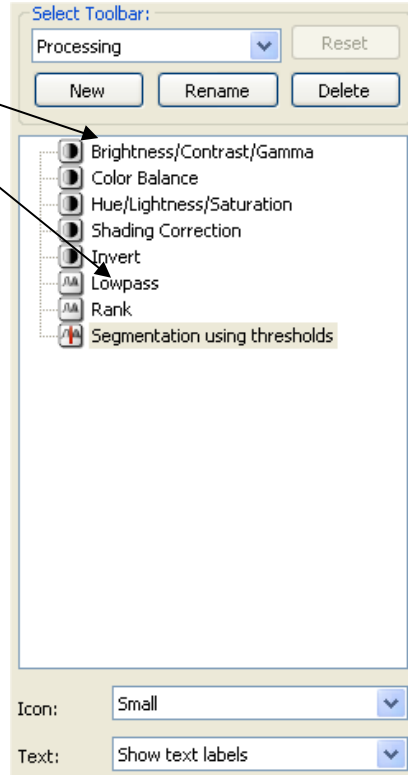
➢ In the **Available commands** field, select the commands that you would like to use on the toolbar.

➢ Select the command **Processing** $\Rightarrow$ **Adjust** $\Rightarrow$ **Brightness/Contrast/Gamma**

➢ Then click on **Add** to apply it to the toolbar.

➢ Apply further functions from the **Processing** menu in the same way.
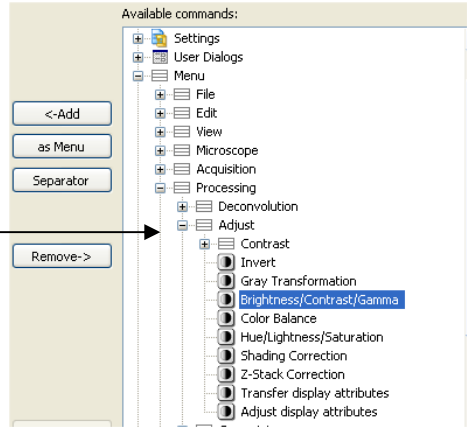
The toolbar now contains the commands displayed.

➢ To create the toolbar definitively, click on **OK**.

Select Toolbar:

Processing · Reset

New | Rename | Delete

- Brightness/Contrast/Gamma
- Color Balance
- Hue/Lightness/Saturation
- Shading Correction
- Invert
- Lowpass
- Rank
- Segmentation using thresholds

Icon: Small ·

Text: Show text labels ·

The **Processing** toolbar is now displayed at the top of the AxioVision window.

B/C/G Color Balance HLS Shading Correction Invert Lowpass Rank Segmentation using thresholds

NOTES:

- You will also find the toolbar in the toolbar list if you right-click in the main menu or on another toolbar.

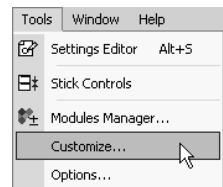- This procedure can also be used to modify existing toolbars.

	Online Measurement
	Commander
	Coal Petrography
	Inside4D
	Incubation
	Bildverarbeitung
✔	Processing
🗉	Workflow
🔳	Workarea
	Status
🖳	Customize...
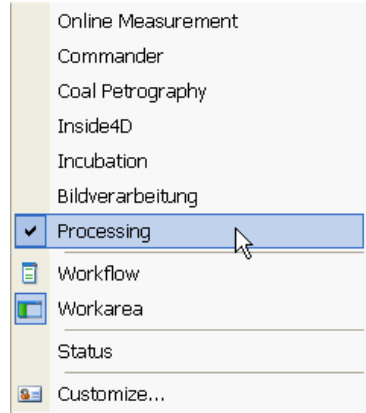
➢ To do this, simply select one of the existing toolbars in the **Select Toolbar** drop-down list and then proceed as described above.

Select Toolbar:
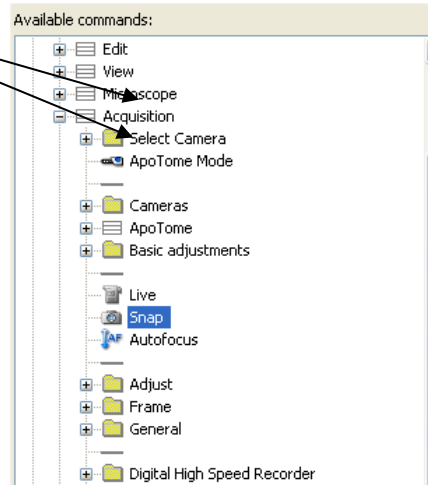Standard ▼
New   Delete   Reset

Define key combinations for functions

You can execute any command that is available in AxioVision by pressing shortcut keys. This is particularly useful for quickly calling up commands that you require time and again, such as image acquisition or the activation of a camera or microscope setting that you often need (requires a motorized microscope).

➢ From the **Tools** menu select the **Customize** function.

Tools	Window	Help
📝 Settings Editor		Alt+S
日‡ Stick Controls		
🐾₊ Modules Manager...		
Customize...		
Options...		

➢ Make sure that the **Keyboard** property page is active in the **Customize** dialog.

Toolbars | Keyboard | Workflow | Dialogs | Icons |

➢ In the **Available commands** field, select the commands that you would like to use on the toolbar.

Available commands:

- ⊞ ▤ Edit
- ⊞ ▤ View
- ⊞ ▤ Microscope
- ⊟ ▤ Acquisition
  - ⊞ 📁 Select Camera
  - ▬◖ ApoTome Mode

    - ⊞ 📁 Cameras
    - ⊞ ▤ ApoTome
    - ⊞ 📁 Basic adjustments

      - 📺 Live
      - 📷 Snap
      - ᴬᶠ Autofocus

      - ⊞ 📁 Adjust
      - ⊞ 📁 Frame
      - ⊞ 📁 General

      - ⊞ 📁 Digital High Speed Recorder

➢ Select, for example, the command **Acquisition** ⟹ **Snap**.

➢ Click in the **Press Shortcut key** field using the mouse.

Shortcut(s) for selected Command:

[   ]   Remove

Reset All

Press Shortcut key:

F2   Assign

Shortcut currently used by:

[unassigned]

➢ Then press the shortcut key(s) on your keyboard that you would like to use to execute the command. *(If possible, the shortcut key should not already be assigned to any other command.)*

➢ Now click on **Assign**.
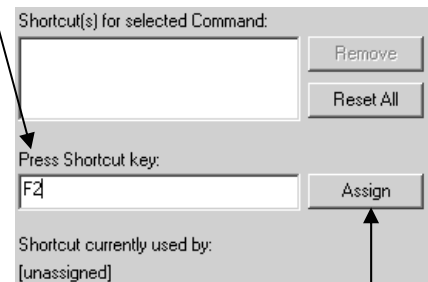
➢ Close the dialog by clicking on the button in the upper-right corner of the window.

[✕]

Create own dialogs

The creation of your own dialogs allows you to take complex control dialogs for, say, microscope and camera operation and pick out precisely the elements that you need to operate your applications. This considerably simplifies parts of the operation.

Create a dialog that enables you to control the objective nosepiece and lamp voltage on a motorized microscope as well as to set the camera's exposure time.
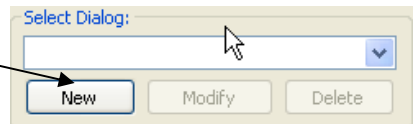
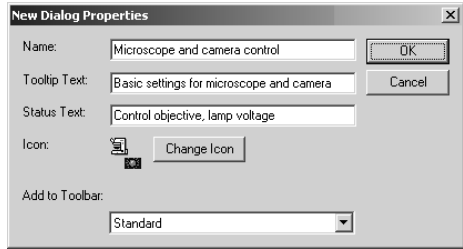➢ From the **Tools** menu select the **Customize** function.
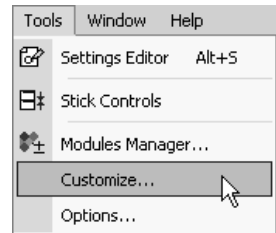
➢ Make sure that the **Dialogs** property page is active in the **Customize** dialog.
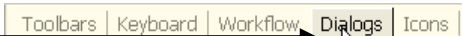
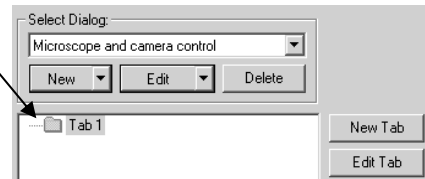➢ To create a new dialog, click on **New**.

➢ Enter the following data:
  Name (dialog + button)
  Tooltip Text
  Status Text (status line)
  Button **Symbol**
  Toolbar, on which the button
  should be created to open the
  dialog.

➢ Click on **OK** to create the
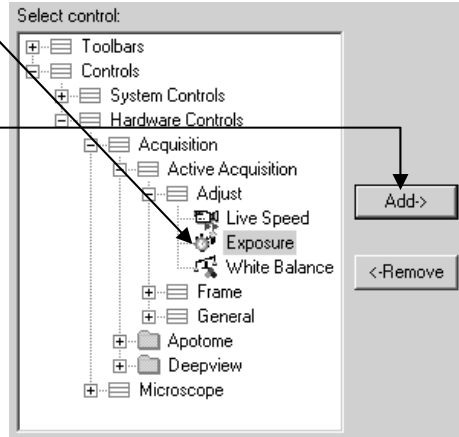 dialog.

➢ Now select the entry **Tab 1**.

➢ To change the name, click on
  Edit Tab.

➢ Specify a **Name** and a **Tooltip Text**.
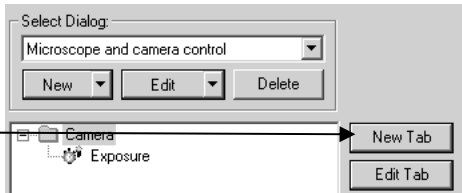
➢ Then click on **OK** to create the
  tab.

➢ Now select **Exposure** in the **Select control** field.

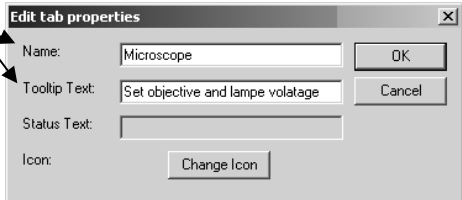➢ To insert both into the dialog, click on **Add**.

NOTES:

• The control elements depend on your system configuration and may differ from those displayed here.

➢ To create a second property page in your dialog for controlling the microscope components, click on **New Tab**.

➢ Specify a **Name** and a **Tooltip Text**.

➢ Then click on **OK** to create the property page.

➢ Now select **Objective** and **Halogen lamp** in the control elements field.

➢ To insert both into the dialog, click on **Add**.

NOTES:

• The control elements depend on your system configuration and may differ from those displayed here.
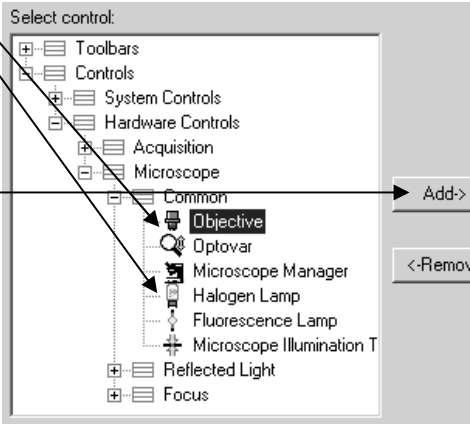
Select control:

- ⊞ Toolbars
- ⊟ Controls
  - ⊞ System Controls
  - ⊟ Hardware Controls
    - ⊞ Acquisition
    - ⊟ Microscope
      - ⊟ Common
        - 🖥 Objective
        - 🔍 Optovar
        - 📷 Microscope Manager
        - 💡 Halogen Lamp
        - Fluorescence Lamp
        - ✛ Microscope Illumination T
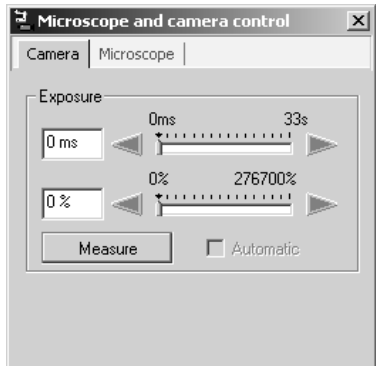   - ⊞ Reflected Light
      - ⊞ Focus

Add->

<-Remov

➢ Close the dialog by clicking on the button in the upper-right corner of the window.

The button is now displayed on the toolbar you have chosen. The result now looks like this:

The dialog contains two property pages: **Camera** and **Microscope**.

➢ The **Camera** property page contains the element for controlling the **Exposure** time.

Microscope and camera control

| Camera | Microscope |

Exposure

| 0 ms | 0ms ... 33s |
| 0 % | 0% ... 276700% |

Measure | ☐ Automatic

➢ The **Microscope** property page contains the elements for controlling the objectives and for controlling the lamp voltage.
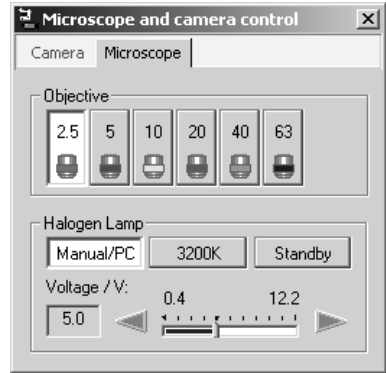
NOTES:

• Also check the Tooltip text and the texts in the status line.

Define workflows

Workflows are the most efficient way to operate AxioVision. They are the "central element" for executing the functions required to perform a specific task. All you have to do is click on the buttons they contain one after the other to achieve the desired results quickly and without problems. If you also hide all the control elements that you do not need, you can concentrate fully on the work you are actually performing.
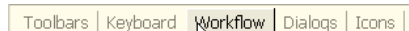
NOTE:

• You can also place dialogs you have created yourself and all the toolbars on a workflow.

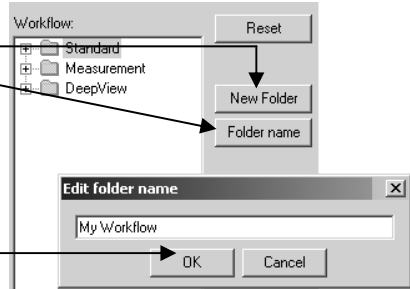➢ From the **Tools** menu select the **Customize** function.
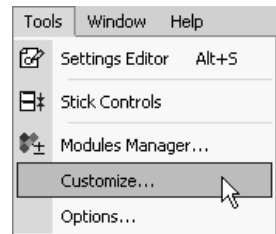
➢ Make sure that the **Workflows** property page is active in the **Customize** dialog.
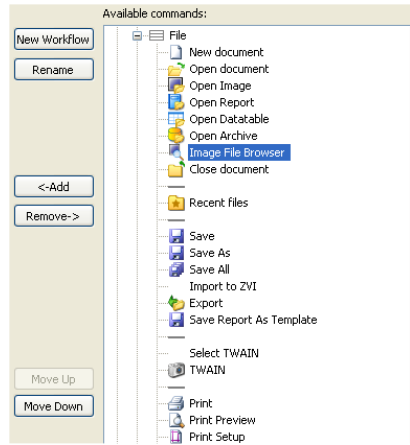
To create a workflow:

➢ Click on **New Folder** and then on **Folder name.**

➢ Specify a name for the new workflow.

➢ Click on **OK**.

In the **Available commands** field, select the commands that you would like to use on the toolbar.

➢ Select, for example, the menu command **File ⇒ Image File Browser**.

➢ Then click on **Add** to apply it to the workflow.
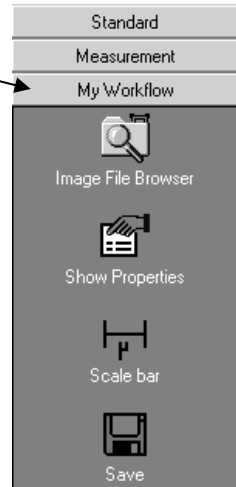
➢ Also apply the menu commands

   View ⇒ Show Properties
   Annotations ⇒ Scale bar
   File ⇒ Save

➢ Close the dialog by clicking on the button in the upper-right corner of the window.

The workflow is now located under the
My Workflow bar.

8.6 General Settings

In this dialog you can enter settings for the configuration of AxioVision. The
settings are grouped by topic on property pages.

The settings comprise:

- AxioVision's starting performance.
- Selection of language.
- Specification of user-specific paths for AxioVision documents.
- Image display.
- Automatic saving of images after acquisition.
- Entry of user information.
- And much more.

From the **Tools** menu select the **Options** function. The **Options** window
with the **General**, **Layout**, **Folder**, **Storage**, **Acquisition**, **Naming**,
Display, **ID** and **Commander** property pages is opened.

Details on the settings used there, and how they influence the system, can
be found in the online help.

8.7 Exporting and Importing User Configurations

General

The term user configurations is understood to mean all the settings you have made to adapt AxioVision to your own personal requirements after installation.

The **Import/Export Configuration** function allows you to back up your personal work environment in a convenient way, so that you can restore it quickly and easily in the event of problems with the system. On multi-user systems, you therefore have the option of making settings that are of interest to all users (e.g. scalings, toolbars etc.) available very quickly to all users by offering access to this configuration via the shared account **All Users**.

Please also read section 8.3 "User and Data Administration", as well as the section "Importing user files" later in this section.

The following elements of AxioVision can be exported:

➢ All settings made in the **Customize** dialog on the **Tools** menu. This means all the toolbars, shortcut keys, workflows and dialogs you have generated. (Please also read section 8.5 "Adapting the User Interface".)

➢ All settings in the **Options** dialog on the **Tools** menu. (Please also read section 8.6 "General Settings".)

➢ You can also save a record of which modules have been activated. See the **Modules Manager** function on the **Tools** menu.

To allow you to make your personal files available to other users, if necessary, all files from the folder *C:\My Documents\Carl Zeiss* are placed together in a compressed archive (ZIP file). The folder structure from the Carl Zeiss data folder is retained.

Export

➢ Select from the **Tools** menu the **Import/Export Configuration** function.

A wizard is started. This guides you through the individual steps of the export process.

➢ Select the elements to be exported, and the folder and file name for the target file.

This will result in two files (if you have also exported the user files) in the selected folder. One file has the extension *.avconf. This file contains all the elements of your configuration. The second file has the extension *.zip. All the user files are saved in this file.

NOTES:
- If you want to make your exported configuration available to other users, make sure that you save the files in a generally accessible folder, e.g. the folder *C:\Documents and Settings/All Users*.

Import

Importing the configuration files

➢ To import the configuration of another user, select from the **Tools** menu the **Import/Export Configuration** function.

A wizard is started. This guides you through the individual steps of the import process. At the end of the process, all of the other user's elements are available.

Importing user files

User data cannot be copied via the import function. This has to be performed separately by unpacking the ZIP file. The reason for this is to avoid files you have created yourself being inadvertently overwritten.

➢ Start Microsoft Windows Explorer, and go to the previously selected folder containing the ZIP file with the other user's files.

➢ Double-click on the ZIP file to unpack it.

➢ Now select the folder *My Documents* as the target folder. The user files are copied automatically into the correct folder structure. If the *Carl Zeiss* subfolder does not yet exist in your *My Documents* folder, it is created

NOTES:
- You need appropriate software to unpack ZIP files. A program is available automatically under Windows XP Professional. Ideally contact your system administrator. Usually these kinds of tools will be present anyway.

- If you want to make the files available to all users, select the shared account *All Users* as the target folder for unpacking the files. This is the folder *C:\Documents and Settings\All Users\Documents*. All users of the system will then be able to share use of the files (e.g. scalings).

8.8 Creating various AxioVision configurations for a user

Background

Anyone who finds the management of users too complicated can continue to use the procedure supported under AxioVision Release 3 (see below). Other subfolders with new profiles are created for the different configurations in the *Application Data\Carl Zeiss\AxioVS40\Profiles* folder of the relevant user account, in addition to the default profile **Default**. The name of the profile is allocated while it is being created. The description from the earlier manual follows:

Creating individual configurations

➢ Right-click on the AxioVision icon on the Microsoft Windows desktop.

➢ Select the **Create Shortcut** function from the shortcut menu .

- A new AxioVision icon is created on the desktop.

AxioVision (2)

- Now right-click on the new AxioVision icon, and select the **Properties** function from the shortcut menu.
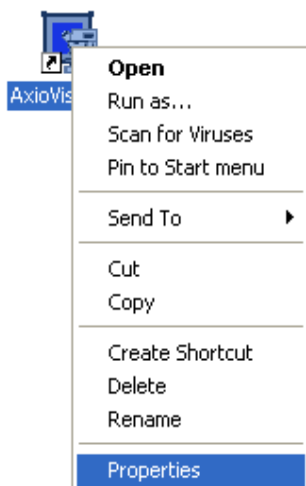
➢ The **Properties** window is opened.
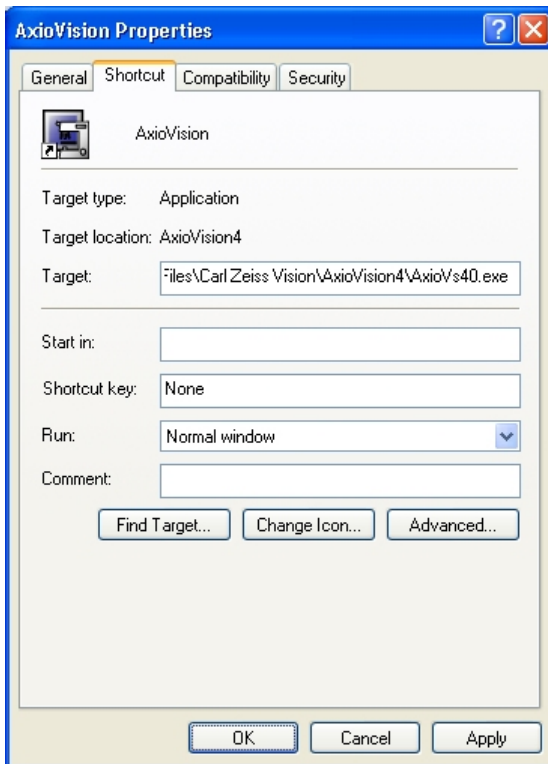
➢ Enter the following at the end of the **Target** field: -u=Name (N.B. Insert a space before -u!). Here 'Name' is the description for the configuration. Use, for example, -u=Test to create a configuration with the name Test.

➢ Adopt the setting by clicking on **OK**.

➢ To change the description of the new configuration on the Microsoft Windows desktop, right-click again on the new AxioVision icon, and from the shortcut menu select the **Rename** command. You can now enter a name of your choice in the icon's description field.

AxioVision (2)

➢ Enter, for example, Test Configuration and then click on any area of the desktop to adopt the entry.

Test
Configuration

➢ You can now start AxioVision from the new icon and configure it accordingly.

9. Image Acquisition Modules

9.1 ApoTome

General

The **ApoTome** software module controls the ApoTome hardware (control box and slider) and coordinated image acquisition using a digital camera, such as the AxioCam **MRm**. An ApoTome system allows you to generate optical sections through fluorescence samples. The parts of the image that are out of focus are then removed, and an increase in

- image sharpness
- the signal to background ratio (contrast)
- and resolution in the axial direction

is achieved.

The following units are required for an ApoTome imaging workstation:

- Microscope: Axio Imager.Z1, Axio Imager.D1, Axio Observer.Z1, Axio Observer.D1, Axiovert 200 oder Axioplan 2 imaging e
- Anti-vibration system
- Digital camera with more than 10-bit dynamic range
- ApoTome control box and slider (release for Axio Imager, Axioplan 2 imaging e and Axiovert 200)
- PC with monitor
- AxioVision basic package and the optional ApoTome software module

For details on setting up and using the hardware components, such as the microscope and ApoTome control box/slider, please refer to the manuals enclosed with the corresponding devices.

NOTE:

* Before using the ApoTome, familiarize yourself with the basic functions of the AxioVision software. You should be familiar with the operation of the camera and microscope components in particular.

Fringe projection imaging principle

The optics of a microscope are optimized for analyzing very thin samples. For a cover-glass-corrected objective, all optical calculations are performed for very thin objects that lie directly beneath the cover glass. All cover-glass-corrected objectives from Carl Zeiss are optimized for this particular usage, and exhibit an optimum Point Spread Function (PSF) for the wavelengths for which the corresponding objective has been specified.

In biological applications, however, the vast majority of samples used do not satisfy these optimum requirements. Sometimes thicker biological tissue slices are used, e.g. to analyze cells in the tissue using specific fluorescent markers.

In such cases, during microscopic analysis, and particularly during documentation, the set focus plane is hidden by parts of the image that originate from above and below the actual focus plane. As a result the image appears "faded", the contrast is reduced, and the background becomes bright. In extreme cases important structures and image details may be completely hidden.
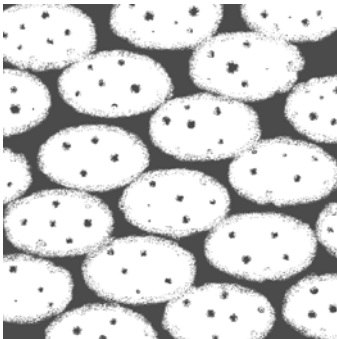
The above representation of a microscopic image of cell nuclei in tissue shows this effect.

A number of methods can be used to prevent or reverse this effect, such as confocal laser scanning microscopy or 3D Deconvolution.

With the ApoTome the principle of "fringe projection" has been employed. This technology involves inserting a grid structure with grid lines of a defined width into the plane of the field diaphragm of the reflected light beam path. As the plane of the field diaphragm is matched to the focal plane, this grid structure can be displayed on the microscope. When you look into the eyepiece you can therefore see the grid, superimposed onto the actual sample.

Above is a schematic representation of the reproduction of the grid. In reality the grid lines are much thinner.

A scanning mechanism in the ApoTome slider is used to move the grid structure in three defined steps within the sample plane. The movement of the grid takes place very quickly (in less than 20 ms). A digital image is acquired at each grid position.

The movement of the grid is represented schematically below:
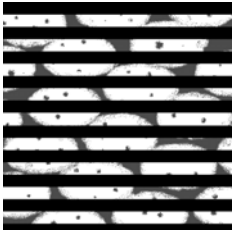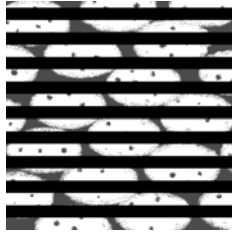
Grid Position 1   Grid Position 2   Grid Position 3
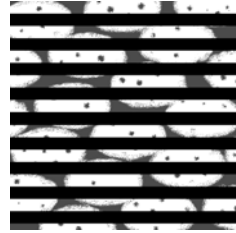
The three raw images are combined online on the PC to form a resulting image. The time it takes to process the image depends on the image size:

- 512 x 512: approx. 30 ms
- 1300 x 1000: approx. 100 s

The processed resulting image is an optical section through the sample with the following characteristics:

- The grid structure has been removed from the raw images.
- The parts of the image that are out of focus are no longer visible.
- The sharpness and contrast of the image have been increased.
- The image's resolution in the axial direction has been increased.
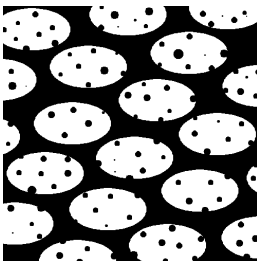
A corresponding resulting image is represented schematically above.
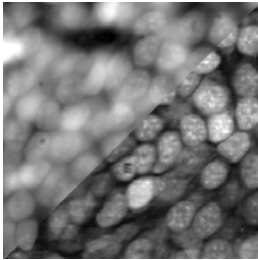
The above image is an application image of cell nuclei (tadpole brain section) in black and white.

- Top left: conventional fluorescence
- Bottom right: optical section

Why is the resulting image an optical section?

One possible way to explain this is to use the image of the grid in the sample:
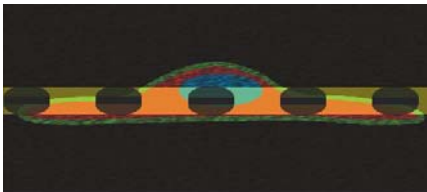
The image of the grid provides the necessary information on the distance of the various sample structures from the set focal plane (see figure above). Some sample structures are in focus, while others lie above or below the focal plane, and enter the set focal plane.

The technique of grid projection makes use of the fact that the image of the grid above and below the actual focal plane is blurred, and enters the blurred areas of the sample. When the grid line is moved, significant brightness differences (= contrast) appear in the focal plane.

Outside the focal plane only minor differences are produced, as the sample and the image of the grid are practically "blurred" together. The brightness differences are detected by the algorithm used to combine the three raw images, and are used to remove the parts of the image that are out of focus.

Hardware configuration

For information on setting up the devices, please refer to the manuals of the devices concerned.
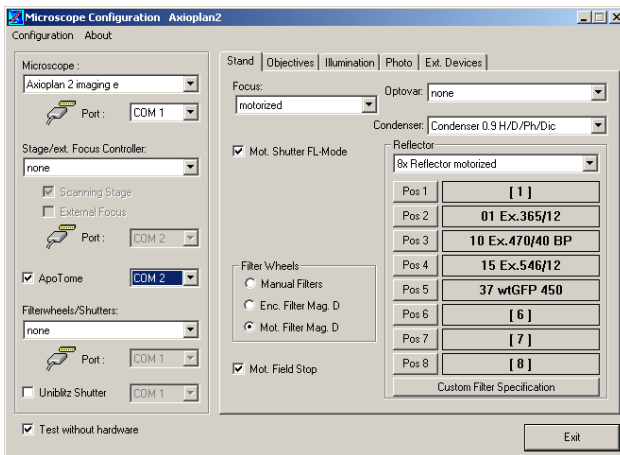
➢ First switch on the microscope, then the fluorescence switched-mode power supply, followed by the ApoTome control box and the camera.

➢ Then switch on the PC.

ApoTome Konfiguration for Axioplan 2 imaging e

➢ Open the **Microscope Configuration** program on the Microsoft

Windows desktop by double-clicking on the ![Microscope Configuration icon] icon.

➢ ☑ ApoTome   COM 2 ▼   Configure the microscope. To use the ApoTome, the **ApoTome** check box must be activated.

If the ApoTome control box has been connected directly to a free COM port on the PC via a serial RS 232 cable, select the corresponding COM port in the list box.

If the ApoTome control box has been connected directly to the microscope via a CAN bus cable, in the list box select the same COM port that you selected under **Microscope**.

➢ Close **Microscope Configuration** by clicking on the **Exit** button, and confirm the changes by clicking on the **Save & Exit** button in the dialog that then follows.
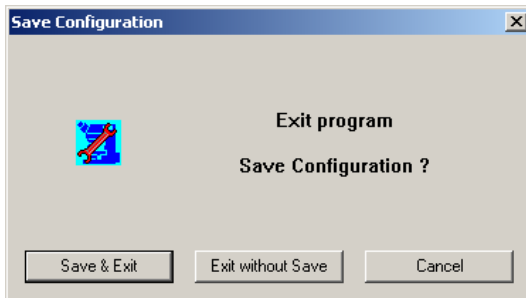
ApoTome Configuration for Axio Imager, Axio Observer or Axiovert 200

➢ Open the **MTB2004 Configuration** program on the Microsoft Windows

desktop by double-clicking on the icon ![MTB2004 Configuration icon].

NOTE:
- Configurations for various microscopes are created and managed in **MTB2004 Configuration**. Please refer to the manual or online help for **MTB2004 Configuration** for detailed information on creating configurations.

➢ For the description of the ApoTome configuration below, it will be assumed that the microscope itself has already been configured.

➢ Make sure that the ApoTome control box is connected to the PC and is switched on.

➢ Open the configuration tree under **Reflected Light Path**.

➢ Click on the **No ApoTome** entry. The entry will be shown with a blue background.
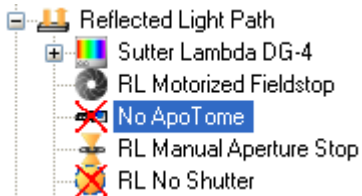
➢ Select the **ApoTome** by clicking on **ApoTome** in the selection area on the right-hand side of the user interface and assign it.
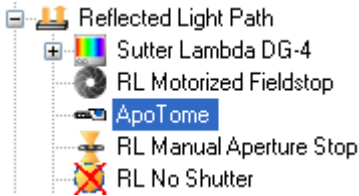
➢ In the information area, you can now select the connection type (RS232 or CAN bus) and the COM port being used (port 1 to 5).

➢ Check the connection by clicking on the **Check Connection** button. If the **ApoTome** is found at the specified port, a green symbol is displayed next to the button.

NOTE:
- If you activate the **Simulate Hardware** check box, for example, the function of the ApoTome can be adjusted virtually in the **AxioVision** microscope control and imaging software (simulation mode).

ApoTome Calibrations

➤ Start the AxioVision software by double-clicking on the icon on the Microsoft Windows desktop.

For subsequent steps it is useful to have easy access to the following control elements. Please bear in mind that, depending on the features of the device, some control elements may not be present, or may appear different:
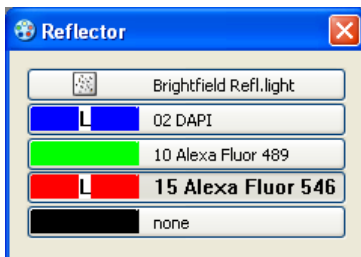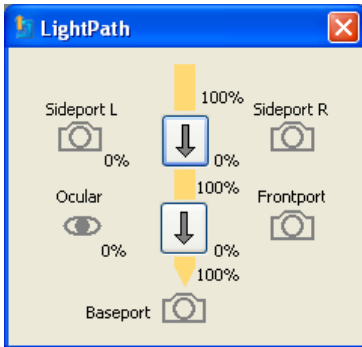
 Reflector nosepiece for switching between fluorescence wavelengths.
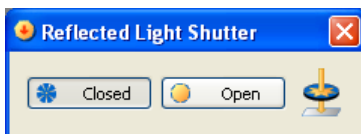
 Internal shutter for opening and closing the shutter.
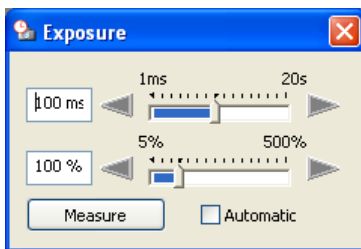
 Camera exposure time.

 Light path for switching the light path, e.g. with an Axiovert 200.

You can also create a user-specific dialog containing all the control elements required, including the control elements for the ApoTome functions. Information on creating dialogs can be found in chapter 8 "Configuration".

Calibration of phase position

To set the optimum angle of deflection for the ApoTome's scanner unit, you need to carry out a fine adjustment of the scanner calibration in accordance with the system structure. The mirror sample and the special reflected light reflector cube, both of which are supplied with the ApoTome, are used for this purpose. Insert the Push&Click filter cube into an empty reflector nosepiece position, and enter this filter set as **Refl. BF**, for example, in the **Microscope Configuration** or the **MTB2004**. The calibration must be performed for each of the two grids provided.

It is advisable to calibrate the grid for the low magnification range (grid marked with "L" for "Low magnification") using a 20x objective.

In the phase calibration dialog the positioning of the camera is also optimized. To achieve optimum performance, the camera horizontal should be aligned parallel to the ApoTome grid lines with as much precision as possible.

The calibration process is supported by a wizard. Start the function by selecting from the **Acquisition** menu the **ApoTome** function, and then **Phase Calibration**.

The software wizard guides you through the calibration process in 5 steps. The most important instructions are displayed in the wizard's text field.

Step 1: Select Start Conditions

Step 1 of 5: Select Start Conditions
The calibration process should be startet after a warm-up time of the system of approximately 20min. The Phase Calibration needs to be done with only a single grid of your choice.
  - Insert the grid corresponding to the objective
  - Use the mirror test slide for the phase calibration
  - Use the bright field reflector cube
  - Adjust the following settings

➢ Move the ApoTome slider to the iris position (click stop position 1).

➢ Use the reflector position with the ApoTome bright-field reflector.

➢ Place the mirror sample supplied under the microscope, open the reflected light shutter, and focus (directly on the microscope) on the reticle at the center of the sample.

➢ Now switch to operation of the software on the PC.

➢ In the software, enter settings for grid, reflector, and objective, if you have not already done so. Pay particular attention to ensuring that the grid is correctly selected.
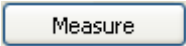
NOTE:
- It is possible to use the ApoTome in combination with a Colibri, an excitation filter wheel, a Sutter DG-4 or DG-5 or a T.I.L.L. Photonics Polychrome V. In this case, carry out the relevant settings in addition to the microscope settings (grid, reflector, objective).

➢ Click on **Next** to proceed to the next step. The live image is opened automatically.

Step 2: Optical Focusing

Step 2 of 5: Optical Focusing
Use the first click-stop of the ApoTome slider (iris position) to focus on the cross hair of the mirror slide
- Adjust the exposure time of the camera

➢ Set an optimum exposure time by clicking on the [Measure] button.

➢ Display the reticle as sharply as possible in the live image. The green focusing rectangle in the live image can help you find the optimum focus position. Position the focusing rectangle over the center of the reticle.
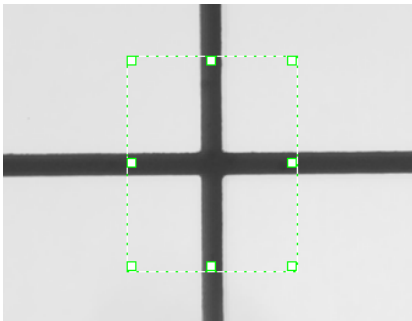
NOTES:

- On the **Microscope** property page you can enter general settings for the microscope.

- Click on **Next** to proceed to the next step.

Step 3: Grid Focusing

Step 3 of 5: Grid Focusing

Carefully push the ApoTome slider in the second click-stop position. Make sure, that grid lines are aligned horizontally (Camera adjustment)
- Position the green focus rectangle over approximately 10 grid lines
- Click on 'Full Scan'
- (The grid will be moved through the complete travel range)
- The grid position with the highest focus value will automatically be selected

Move the ApoTome slider carefully to the second click stop position (ApoTome mode).

➢ Move the focusing rectangle in the Live window to a position where no part of the reticle is present.

➢ Click on the **Full Scan** button. The grid will now move through its adjustment range automatically, in increments of ten steps. A sharpness value for the reproduction of the grid will be determined, and displayed as a histogram, following each increment. When the end of the adjustment range has been reached, the grid position with the highest sharpness value will be set automatically.

➢ You can choose to carry out a fine adjustment around the grid position with the highest sharpness value by clicking on the **Local Scan** button. A local area above and below the sharpness maximum that has been identified will be covered automatically in individual steps, and the sharpness value will be determined for each individual step. The data from the local scan will be included in the histogram, and the grid position with the highest sharpness value will again be located automatically.

NOTE:

• You can determine the grid position freely using the buttons for coarse focusing, [◀◀] and [▶▶], and fine focusing, [◀] and [▶]. You may need to use this function if, for example, a sharpness maximum cannot be determined automatically.

### *Step 4: Camera Alignment*

Step 4 of 5: Camera Alignment

Move the cross hair out of the live image. Move the starting point of the grid detection by moving the green focus rectangle up and down.

- Rotate the camera head until you get as close to 0.00 degree as possible

The purpose of this step is to achieve precise alignment of the camera. To achieve optimum results, the camera horizontal should be aligned parallel to the grid lines with as much precision as possible. The software automatically detects a grid line and calculates the camera horizontal's deviation from this grid line in degrees.

As the vertical line of the reticle interferes with the detection of the grid lines, please first move the reticle out of the camera's field of view.

A green rectangle marks the software's search area, a yellow line indicates a detected grid line and a small dark-green rectangle shows you the starting point for line detection.
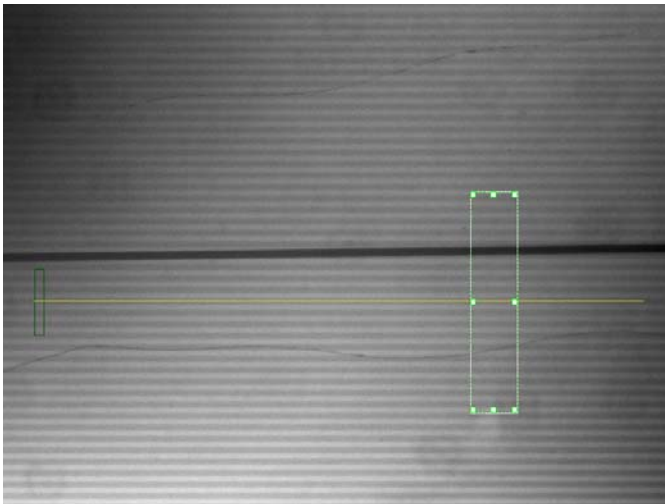
If the contrast in the image is insufficient to detect a grid line automatically, a corresponding error message appears:
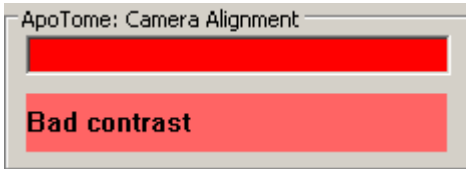

In this case you should take steps to increase the contrast (e.g. increase the exposure time, refocus the grid (step 3)).

If it is still not possible to detect a grid line, a corresponding error message appears:
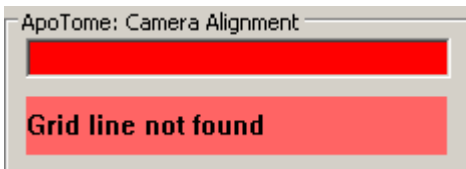

In this case the deviation of the grid lines from the camera horizontal is probably too great. Rotate the camera until the long side of the camera's field of view is roughly parallel to the grid lines. To do this, loosen the corresponding lock on the camera adapter and realign the camera.

If a grid line can be detected but the deviation is still greater than 5 °, you will see a message to this effect:
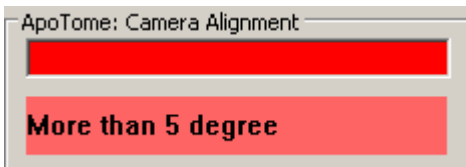
In this case, rotate the camera further to minimize the deviation. For fine adjustment you can use the red bar in the dialog. Try to achieve a deviation < 0.1 °.
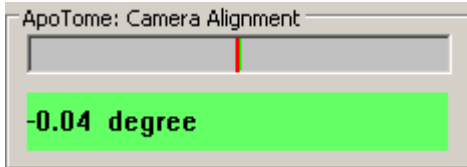
Once the camera has been aligned with sufficient precision, please lock the camera adapter in this position.

NOTE:

- If you change the ApoTome grid, the new grid should be inserted with the same alignment, if possible. The grid mount in the ApoTome slider has a small amount of play to make it as simple as possible to change the grid. You should always align the grid, if possible, with the same edges in the grid mount. If you wish to check the camera alignment following a change of grid, you can run through the phase calibration dialog up to step 4. Should you not wish to perform a new phase calibration, cancel the dialog after step 4, once the camera has been aligned.

Step 5: Final Full-Phase Calibration

Step 5 of 5: Final Full-Phase Calibration
Click on 'Full Scan'. Starting from default value a defined range of values will be scanned. The phase value with the least amount of residual stripes in the image will automatically be selected.

This step is used for the actual setting of the scanner calibration. In each case, two images are acquired. The grid is displaced by precisely the width of a line between the first and second image.

The two images are subtracted from each other, and the resulting image is displayed. If the system is perfectly calibrated, the two images cancel each other out – a black image with a noise component is shown.
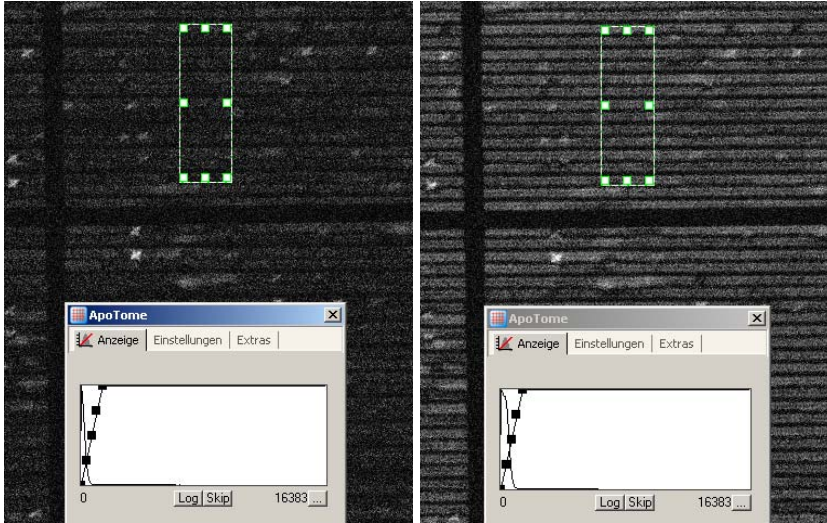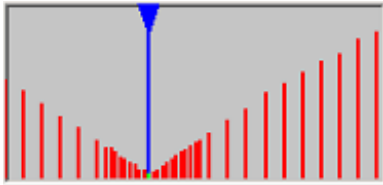

Phase calibration is performed automatically in accordance with the focus calibration of the grid, carried out in the preceding step.

➢ Click on the **Full Scan** button. A predefined area around the specified phase calibration value will be covered in coarse steps. At each position, the calibration value will be determined, and displayed in the histogram. When the end of the area has been reached, the minimum value (minimum number of residual lines present in the image) will automatically be selected in the histogram.
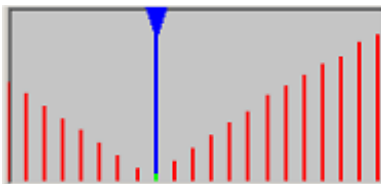
➢ You can choose to perform an additional fine adjustment using the **Local Scan** button. An area close to the minimum on either side will be covered in individual steps, and the data included in the histogram. This may enable you to identify an even lower minimum, where there are even fewer residual lines in the image.

NOTES:

• Optional you can change the phase value stepwise by clicking on the buttons **-** **--** **---** and **+** **++** **+++**. You may need to use this function if, for example, a sharpness maximum cannot be determined automatically.

• Click on the **Close** button to end the phase calibration.

• Repeat the procedure to calibrate the phase shift for the second grid, if necessary.

• Although the settings do not change once calibration has been performed, from time to time you should check that the system is perfectly calibrated. This applies in particular after dismantling and reassembling the system.

Calibration of the grid focus

For the ApoTome to function optimally, the grid must be displayed precisely in the focus set on the objective. The grid's focus depends on the objective and the excitation and emission wavelengths of the fluorescence. This means that the grid focus needs to be calibrated for every fluorescent dye (e.g. DAPI, FITC, Rhodamine etc.). It is best to perform calibration using your own fluorescence sample, although this must exhibit flat fluorescence to allow the focusing of the grid. Alternatively, you can also use the fluorescence sample provided.

A wizard is available for the calibration of the grid focus. This guides you through the calibration process in 3 steps. Start the function by selecting from the **Acquisition** menu the **ApoTome** function, and then **Grid Focus Calibration**.

The most important instructions are displayed in the wizard's text field.

Step 1: Select Start Conditions

Step 1 of 3: Select Start Conditions
The calibration process should be startet after a warm-up time of the system of approximately 20min. The Grid Focus Calibration has to be done for each combination of objective and reflector
 - Insert the grid corresponding to the objective
 - Use the fluorescence test slide for the grid focus calibration
 - Adjust the following settings

➢ Move the ApoTome slider to the iris position (click stop position 1).

➢ Use a reflector position with a fluorescence filter (e.g. 10 Ex. 470/40).

➢ Place the fluorescence sample under the microscope, and open the reflected light shutter.

➢ Focus on the sample on the microscope.

> ➢ Switch to operation of the software on the PC.

> ➢ In the wizard, select the settings for **Grid**, **Reflector** and **Objective**, if you have not already done so.

NOTES:
- Make sure that the correct transmission grid is selected for the objective being used. The table at the end of this chapter provides an overview explaining which transmission grid should be assigned to which objective.

- It is possible to use the ApoTome in combination with a Colibri, an excitation filter wheel, a Sutter DG-4 or DG-5 or a T.I.L.L. Photonics Polychrome V. In this case, carry out the relevant settings in addition to the microscope settings (grid, reflector, objective).

> ➢ Click on **Next** to proceed to the next step. The live image is opened automatically.

Step 2: Optical Focusing

Step 2 of 3: Optical Focusing
Use the first click-stop of the ApoTome slider (iris position) to focus on the specimen
 - Adjust the exposure time of the camera

> ➢ Set an optimum exposure time by clicking on the Measure button.

> ➢ Focus the sample as sharply as possible in the live image.

NOTE:
- On the **Microscope** property page you can enter general settings for the microscope.

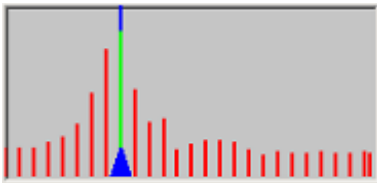> ➢ Click on **Next** to proceed to the next step.

Step 3: Grid Focusing

Step 3 of 3: Grid Focusing
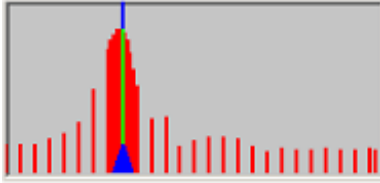Carefully push the ApoTome slider in the second click-stop position
 - Adjust the exposure time of the camera if necessary
 - Position the green focus rectangle over approximately 10 grid lines
 - Click on 'Full Scan'
 - (The grid will be moved through the complete travel range)
 - The grid position with the highest focus value will automatically be selected

➢ Move the ApoTome slider carefully to the second click stop position (ApoTome mode).

➢ Move the focusing rectangle in the live window to a position where the fluorescence is as flat and bright as possible.

➢ Click on the **Full Scan** button. The grid will now move through its adjustment range automatically, in increments of ten steps. A sharpness value for the reproduction of the grid will be determined, and displayed as a histogram, following each increment. When the end of the adjustment range has been reached, the grid position with the highest sharpness value will be set automatically.


➢ You can choose to carry out a fine adjustment around the grid position with the highest sharpness value by clicking on the **Local Scan** button. A local area above and below the sharpness maximum that has been identified will be covered automatically in individual steps, and the sharpness value will be determined for each individual step.

The data from the local scan will be included in the histogram, and the grid position with the highest sharpness value will again be located automatically.

NOTE:

- You can determine the grid position freely using the buttons for coarse focusing, ◀◀ and ▶▶, and fine focusing, ◀ and ▶. You may need to use this function if, for example, a sharpness maximum cannot be determined automatically.

➢ When you click on **Close** to end the calibration, the following dialog window is displayed:
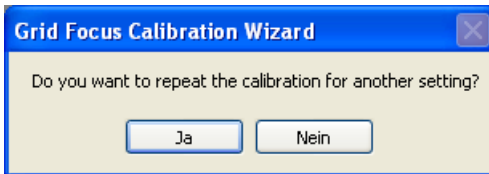
➢ Click **Yes**, if you want to carry out a further calibration, or click **No** if you want to end the calibration.

Once calibration is complete, a corresponding message is displayed in the status window of the ApoTome dialog window (**Acquisition** menu ⇒ **ApoTome** function ⇒ **ApoTome Dialog** ⇒ **Settings**).
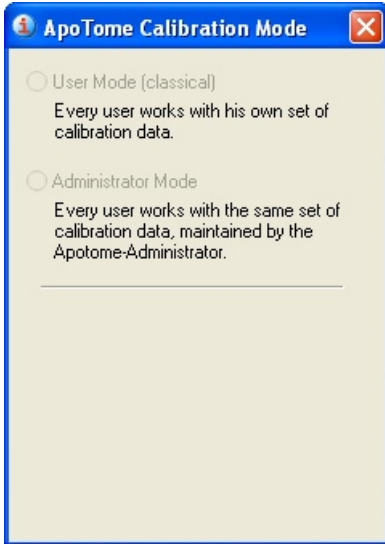
Calibration mode

There are two possibilities for managing ApoTome calibration data:

➢ User mode (conventional): An individual file containing the calibration data is generated in each Windows user account, i.e. each user generates his own calibrations and then works with these. Other users do not have access to these data.
In this case, the file containing the calibration data can be found in the *"Eigene Dateien"* folder of the user account in question under *"Eigene Dateien\Carl Zeiss\Data\ApotomeSetting\Default.ini"*.

➢ Administrator mode: A user generates calibration data which are then made available to all other users. In this case, only the user who has generated calibrations in the ApoTome administrator mode is able to change these or add to them.
The file containing the calibration data can be found in the folder *"C:\Documents and settings\all users\Documents\Carl Zeiss\Data\ApotomeSetting\Default.ini"*.

The user mode is activated automatically following a new installation of AxioVision. If you want to change the mode, open the calibration mode dialog (**Aufnahme** menu ⇒ **ApoTome** function ⇒ **Calibration mode**):
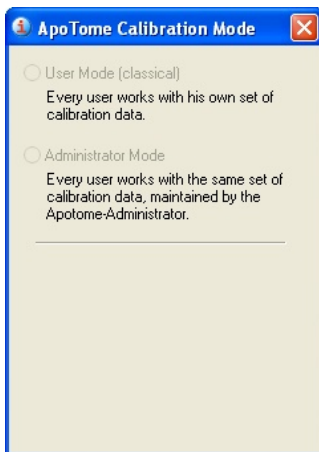
The administrator mode is password-protected. The default password is "zeiss" and can be replaced by a password of your choice following the first log in.

Once administrator mode has been activated, all users are only able to access the calibrations that can be found in the shared files. However, any individual calibrations that have previously been generated remain in the relevant user accounts (although they cannot be used). If you switch back from the administrator mode to the user mode, the file containing the calibration data that has been saved in the shared files will be copied to the Windows user account of the ApoTome administrator. Users of other Windows accounts can then use their old calibrations again or may have to generate new calibrations.
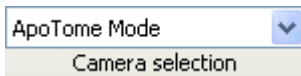
Image acquisition

As an ApoTome imaging system works with a digital CCD camera, the ApoTome mode can be regarded as an expansion of the camera's functionality.

Selecting the camera

In principle, a cooled monochrome CCD camera (AxioCam MRm, AxioCam HRm etc.) is used in combination with the ApoTome for fluorescence images. When you start AxioVision, a monochrome camera is automatically selected (if possible) for operation with the ApoTome.

If several monochrome cameras are connected to the system, you need to define the one that you want to be used in ApoTome mode. To select the camera, follow the procedure below:

➢ Click on **ApoTome** in the work area or open the ApoTome dialog (**Acquisition** menu ⇒ **ApoTome** function ⇒ **ApoTome Dialog**). Select the **Extras** property page

➢ In the **ApoTome: Camera** drop-down field, select the camera that you want to use in ApoTome mode.

| ApoTome Mode ▾ |
| --- |
| Camera selection |

Single image acquisition

For single image acquisition, follow the procedure below:

➢ For efficient operation, open the control elements for the camera exposure time (**Acquisition** menu ⇒ **Adjust** function ⇒ **Exposure**), the shutter and the reflector (**Microscope** menu ⇒ **Reflected Light** function ⇒ **Reflector** or **Internal Shutter**).

➢ Make sure that the ApoTome slider is in the second click stop position (ApoTome mode).

➢ Click on **ApoTome** in the work area or open the ApoTome dialog (**Acquisition** menu ⇒ **ApoTome** function ⇒ **ApoTome Dialog** ⇒ **Settings** property page).

➢ Make sure that in the **ApoTome: Grid** field the grid that is currently inserted has been selected. On the basis of the objective that is currently being used, a recommended grid will also be displayed in this field.

➢ Select the **Camera** property page.

➢ The live image is opened using the Live button on the toolbar. The shutter is opened automatically with the live image.

➢ Click on the Measure button to set an optimum exposure time for the specimen. To achieve optimum results with the ApoTome, the images should be illuminated in the best possible way. Full use should be made of the camera's dynamic range. However, no pixels should be overexposed. The histogram displayed will help you achieve optimum illumination.

➢ Various settings can be made for the ApoTome mode. Three settings are available for the live image in ApoTome mode (**Settings** property page in the ApoTome dialog).
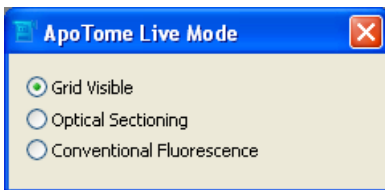
➢ **Grid Visible**: The behavior of the live image is the same as in conventional camera mode. No processing takes place. As the grid is located in the beam path, it is also visible in the live image. The grid is moved up and down constantly, to prevent grid lines being "burnt" into the sample as a result of the bleaching effects of the fluorescent dye.

➢ **Optical Sectioning**: In this mode three images are acquired, combined online into an optical section, and displayed. As this mode requires the acquisition of three raw images, the speed of the live image is reduced accordingly.

NOTE:
• In this mode, if you change the focus on the microscope or the position of the sample, artifacts, in the form of lines, may appear in the live image for a short time. The same effect can occur if vibrations are passed on to the microscope. The lines disappear from the live image as soon as the sample is stabilized again.

➢ **Conventional Fluorescence**: In this mode two raw images are acquired, and the grid position is displaced in each case by precisely the width of the lines. From these two raw images, an image that corresponds to the conventional fluorescence image (wide field) can be calculated. This mode enables you to make comparisons between the conventional fluorescence image and the optical section.

Image acquisition is triggered using the Snap button on the toolbar.

Four settings are available for image acquisition in ApoTome mode (**Settings** property page in the **ApoTome** dialog).ccc

➢ **No Processing**: The behavior of image acquisition is the same as in conventional camera mode. No processing takes place. As the grid is located in the beam path, but is moved constantly up and down, the image acquired corresponds to a conventional fluorescence image. This setting is only used if you are carrying out automated image acquisition in several channels, and processing needs to be switched off, e.g. to acquire a transmitted light or a phase contrast image.

➢ **Optical Sectioning**: In this mode three images are acquired, combined online into an optical section, and displayed.

➢ **Conventional Fluorescence**: In this mode two raw images are acquired, and the grid position is displaced in each case by precisely the width of the lines. From these two raw images, an image that corresponds to the conventional fluorescence image (wide field) can be calculated. This mode enables you to make comparisons between the conventional fluorescence image and the optical section.

➢ **Raw Data Mode**: In this mode the raw image acquisition data are saved (i.e. the individual images with the various grid positions are saved internally). The advantage of this mode is that it allows you to switch between the algorithms (**Optical Sectioning** versus **Conventianal Fluorescence**) after image acquisition:

- To do this, open the shortcut menu for the acquired image by clicking on the **Properties** icon at the bottom edge of the image. screen Alternatively, you can also open the shortcut menu by right-clicking in the acquired image.

➢ **ApoTome Image** propery page:
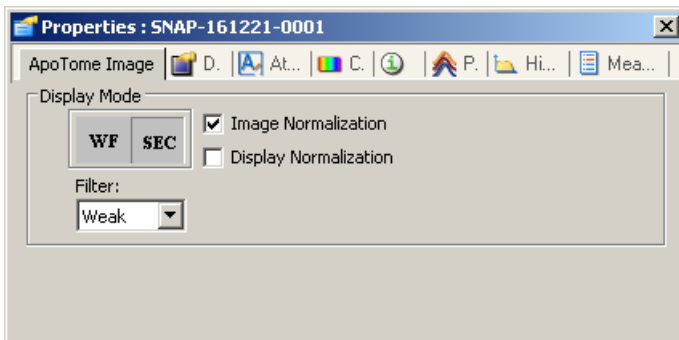
Click on the **WF** (Widefield = **Conventional Fluorescence**) or **SEC** (Section = **Optical Sectioning**) buttons to switch between the corresponding algorithms. The filter used to remove residual lines in the image (**Filter** drop-down list box) and the display scaling (**Auto Display Scaling**) can also be adjusted. The application of the algorithm takes place purely in the display plane of the image data and does not change the original raw data.

NOTE:

- In order to process ApoTome data in AxioVision using the 3D Deconvolution module, you must acquire the image in the **Raw Data Mode**.

➢ Acquiring images in the **Raw Data Mode** substantially increases the memory required for the image data. In the **Averaging** default setting, for example, it is increased by a factor of 3 in comparison to an image acquired with ApoTome in the **Conventional Fluorescence** or **Optical Sectioning** modes. If the **Averaging** value is increased by 2 or 3, the resulting memory requirement increases by a factor **of** 6 or 9. We therefore recommend that you use the **SIMConvert** function to convert the raw data to an optical section or a conventional fluorescence image, thereby substantially reducing the data volume again (e.g. after using 3D Deconvolution or for 3D reconstruction of an image stack using the Inside 4D module.)

➢ The thickness of the **Optical Section** can be displayed in the **Depth Info** field on the **Extras** property page.

ApoTome Depth Info	DAPI	FITC	RHOD.
R U :	0.4	0.3	0.3
Micrometer:	0.4	0.5	0.5

- Depending on the sample, fluorescent dye, exposure time etc., fine residual lines may be visible in the resulting image in ApoTome mode. These lines can be removed using the filter. You can select **Off**, **Weak**, **Medium** and **Strong**.

- The residual lines are displayed in the Fourier space as points along a vertical line. The filter "removes" a defined group of points from the Fourier spectrum.

Start with the setting **Weak**. If residual lines can still be identified in the image, increase the setting step by step. As the filter is used during the calculation of the optical section, a new image must be acquired whenever changes are made to the filter setting.

A further increase in image quality can be achieved if you use a value of 2 or higher in the **Averaging** field.
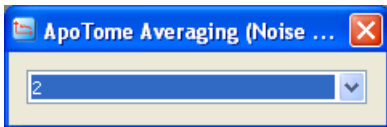
If you use the value **2**, for example, two ApoTome images are acquired and averaged. This method significantly reduces the noise component. An additional effect is the reduction or removal of residual lines in the image.

Multidimensional acquisition with the ApoTome

The ApoTome functions are fully integrated into AxioVision's automatic image acquisition modes. You can perform acquisition of multichannel fluorescence images, z-stack images, time lapse images, MosaiX and any combination of these modes using the ApoTome. The images are acquired in accordance with the settings made under Acquisition menu $\Rightarrow$ **ApoTome** function $\Rightarrow$ **Acquisition Mode**. A precise description of the Multidimensional Acquisition functionality can be found in section "Multidimensional Acquisition".

A prerequisite for the above is calibration of the system as described in chapter 8 under the headings "Configuration" and "Scalings". Essentially, for multidimensional acquisition the same functions as those described in the previous sections apply.

If, during multichannel image acquisition, components that are responsible for the fluorescence wavelength being used are operated automatically, for example, a focus calibration must be available for each combination. The crucial components are:

- Reflector nosepiece
- Colibri
- External excitation filter wheel (e.g. Ludl, Sutter)
- Sutter DG-4 or DG-5
- T.I.L.L. Photonics Polychrome V

If a grid focus calibration is not available for a combination of the above external components, a single image is acquired automatically without any shifting of the grid. This artifact is usually easy to identify as an image with clearly pronounced fringes. For better control of image acquisition, open the **ApoTome: Status** window (**Acquisition** menu $\Rightarrow$ **ApoTome** $\Rightarrow$ **Status** function). This window will inform you if, during multidimensional image acquisition, a microscope status is set for which no ApoTome focus calibration is available.

Accelerated image acquisition with the ApoTome (burst mode)

With the ApoTome, the speed of image acquisition (image rate) is limited by the following:

1. At least three raw images have to be acquired for a resulting image.

2. During image acquisition, as much as possible of the gray-value range of the camera in question should be used. This generally results in exposure times that prevent high acquisition speeds from being achieved.

However, provided that acquisition takes place in one fluorescence channel only, it is possible to increase the image acquisition rate to a value in the region of 4 to 5 images/s. To achieve these values, follow the procedure below:

➢ Open the **ApoTome: Status** window (**Acquisition** menu ⇒ **ApoTome** ⇒ **Status** function).

➢ In the **ApoTome Dialog** on the **Camera** property page, set a frame that is as small as possible (e.g. 512 x 512 pixels or smaller).

   Tip:
   If your requirements with regard to optical resolution allow, you can increase the binning on the camera to 2x2 (**Kamera** work area⇒ **Frame** property page). This function makes it possible to reduce the camera's exposure time by around a factor of 4, at the expense of the resolution in the image.

➢ For the **ApoTome: Acquisition Mode**, select the **Raw Date Mode** setting (**ApoTome** property page ⇒ **Settings** property page).
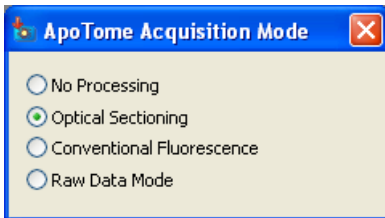
➢ In the work area, select the **Multidimensional Acquisition** entry and then the **C (Channels)** property page.

➢ Make sure that no **Hardware Settings** has been selected for the **During Acquisition** and **After Acquisition** drop-down list boxes.
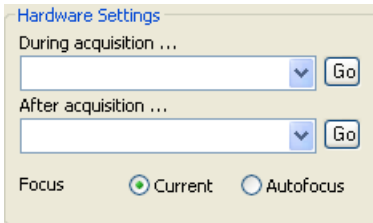
Determine an optimum exposure time by clicking on the

Measure button. A pop-up window containing a live image

opens. Confirm the exposure time by clicking on OK . If necessary, the measured exposure time can be reduced manually by around 10 to 20%.
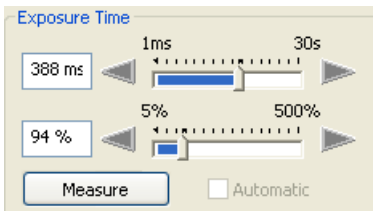
➢ Select the **T (Time)** property page and activate the Time Lapse check box.

➢ Set the **Interval Settings** to **Maximal Speedt** or alternatively the **Interval** to 0.

➢ In the **Duration Settings** field, define either the number of cycles **# Cycles** (e.g. 50) or the **Duration**.(e.g. 15s).
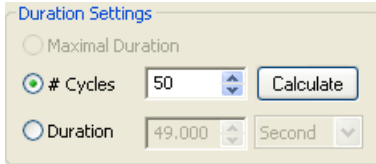
➢ Click on the ⬚ **Start** button to start acquisition of the image sequence. In the **ApoTome Status** dialog the message **Calibrated Burst Mode: ON** is displayed.

In this mode the image data are acquired as quickly as possible, with the grid being constantly shifted. The optical sections are only calculated after acquisition (and following a staggered pattern, i.e. the last 3, 6, 9 raw images etc.), according to the setting of the ApoTome's **Averaging** function, with different grid positions.

This technique increases the image rate by around a factor of 3, but not the temporal resolution, as rapidly moving particles on the sample can nevertheless lead to movement artifacts in the resulting image due to the exposure times selected during the acquisition of the raw data.

The image view (**Optional Secitoning** / **Conventional Fluorescence**) can be switched at any time, on the basis of the raw data acquired. The corresponding settings are made on the ApoTome Bild property page in the shortcut menu of the acquired image.

NOTE:

- A further slight increase in acquisition speed can be achieved by deactivating the **Show preview at Start** check box in the **Multidimensional Acquisition** work area ⇒ **Experiment** property page.
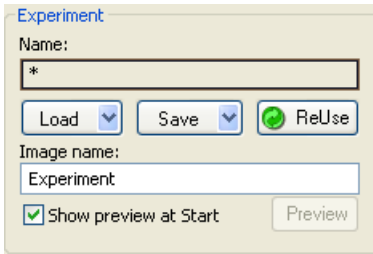
Objective – grid allocation

The following tables provide an overview of the allocation of grids to objectives.

Axio Imager

Objective	V	NA	Immersion	DAPI	recom. grid	optical section thickness @ 490 nm (RU/µm)
EC-Plan-Neofluar 10x/0,3	10	0.3	Air	✔	L1	1.01 / 11.04
EC-Plan-Neofluar 20x/0,5	20	0.5	Air	✔	M	1.03 / 4.05
EC-Plan-Neofluar 40x/0.75	40	0.75	Air	✔	H1	1.09 / 1.89
EC-Plan-Neofluar 40x/1,3 Oil	40	1.3	Oil	✔	M	1.07 / 0.94
EC-Plan-Neofluar 63x/0.95 Korr	63	0.95	Air	✖	H1	0.7 / 0.76
EC-Plan-Neofluar 63x/1,25 Oil	63	1.25	Oil	✔	H1	1.08 / 1.03
EC Plan-Neofluar 100x/1,3 Oil	100	1.3	Oil	✖	H1	0.71 / 0.63

Objective	V	NA	Immersion	DAPI	recom. grid	optical section thickness @ 490 nm (RU/µm)
LCI Plan-Neofluar 25x/0,8 Imm. Korr.	25	0.8	Oil, water or glycerine	✔	L1	0.88 / 1.35
LCI Plan-Neofluar 63x/1.3 Imm. Korr.	63	1.3	water or glycerine	✔	H1	1.08 / 0.96
Plan-Apochromat 10x/0,45	10	0.45	Air	✔	L1	1.45 / 6.88
Plan-Apochromat 20x/0,8	20	0.8	Air	✔	L1	1.08 / 1.65
Plan-Apochromat 40x/0.95 Korr	40	0.95	Air	✔	H1	1.07 / 1.16
Plan-Apochromat 40x/1.0 Oil	40	1.0	Oil	✔	H1	0.85 / 0.84
Plan-Apochromat 40x/1.3 Oil	40	1.3	Oil	✔	M	1.07 / 0.94
Plan-Apochromat 63x/1.0 W	63	1.0	water	✔	H1	0.93 / 1.22
Plan-Apochromat 63x/1,4 Oil	63	1.4	Oil	✔	H1	1.06 / 0.81
Plan-Apochromat 100x/1,4 Oil	100	1.4	Oil	✔	H1	0.7 / 0.53
LD-LCI-Plan-Apochromat 25x/0.8	25	0.8	Oil, water or glycerine	✔	L1	0.88 / 1.35
C-Apochromat 10x/0,45 W	10	0.45	water	✔	L1	1.42 / 6.88
C-Apochromat 40x/1,2 W	40	1.2	water	✔	M	0.94 / 0.85
C-Apochromat 63x/1,2 W	63	1.2	water	✔	H1	0.95 / 0.86
LD C-Apochromat 40x/1.1 W	40	1.1	water	✔	M	0.94 / 1.02
alpha Plan-Fluar 100x/1.45 oil	100	1.45	Oil	✖	H1	0.67 / 0.48
alpha Plan-Apochromat 100x/1,46 Oil	100	1.46	Oil	✖	H1	0.67 / 0.47

Axio Observer / Axiovert 200

Objective	V	NA	Immersion	DAPI	recom. grid	optical section thickness @ 490 nm (RU/μm)
EC-Plan-Neofluar 10x/0,3	10	0.3	Air	✔	VL	0.90 / 9.81
EC-Plan-Neofluar 20x/0,5	20	0.5	Air	✔	VH	1.37 / 5.36
EC-Plan-Neofluar 40x/0.75	40	0.75	Air	✔	VH	0.93 / 0.90
EC-Plan-Neofluar 40x/1,3 Oil	40	1.3	Oil	✔	VL	0.75 / 0.66
EC-Plan-Neofluar 63x/0.95 Korr	63	0.95	Air	✘	VD	0.82 / 0.89
EC-Plan-Neofluar 63x/1,25 Oil	63	1.25	Oil	✔	VH	0.93 / 0.89
EC Plan-Neofluar 100x/1,3 Oil	100	1.3	Oil	✘	VD	0.83 / 0.73
LCI Plan-Neofluar 25x/0,8 Imm. Korr.	25	0.8	Oil, water or glycerine	✔	VL	0.78 / 1.20
LCI Plan-Neofluar 63x/1.3 Imm. Korr.	63	1.3	water or glycerine	✔	VH	0.93 / 0.82
Plan-Apochromat 10x/0,45	10	0.45	Air	✔	VL	1.26 / 6.09
Plan-Apochromat 20x/0,8	20	0.8	Air	✔	VL	0.95 / 1.46
Plan-Apochromat 40x/0.95 Korr	40	0.95	Air	✔	VH	0.92 / 0.99
Plan-Apochromat 40x/1.0 Oil	40	1.0	Oil	✔	VH	1.28 / 1.91
Plan-Apochromat 40x/1.3 Oil	40	1.3	Oil	✔	VL	0.75 / 0.66
Plan-Apochromat 63x/1.0 W	63	1.0	water	✔	VD	1.09 / 1.42
Plan-Apochromat 63x/1,4 Oil	63	1.4	Oil	✔	VH	0.91 / 0.69
Plan-Apochromat 100x/1,4 Oil	100	1.4	Oil	✔	VD	0.81 / 0.62
LD-LCI-Plan-Apochromat 25x/0.8	25	0.8	Oil, water or glycerine	✔	VL	0.78 / 1.20

Objective	V	NA	Immersion	DAPI	recom. grid	optical section thickness @ 490 nm (RU/μm)
C-Apochromat 10x/0,45 W	10	0.45	water	✔	VL	1.26 / 6.09
C-Apochromat 40x/1,2 W	40	1.2	water	✔	VH	1.25 / 1.13
C-Apochromat 63x/1,2 W	63	1.2	water	✔	VH	0.82 / 0.74
LD C-Apochromat 40x/1.1 W	40	1.1	water	✔	VH	1.25 / 1.35
alpha Plan-Fluar 100x/1.45 Oil	100	1.45	Oil	✘	VD	0.79 / 0.56
alpha Plan-Apochromat 100x/1,46 Oil	100	1.46	Oil	✘	VD	0.78 / 0.54

Axioplan 2 imaging e

Objective	V	NA	Immersion	DAPI	recom. grid	optical section thickness @ 490 nm (RU/μm)
EC-Plan-Neofluar 10x/0,3	10	0.3	Air		PL	0.95 / 10.30
EC-Plan-Neofluar 20x/0,5	20	0.5	Air		PL	0.77 / 3.02
EC-Plan-Neofluar 40x/0.75	40	0.75	Air		PH	0.98 / 1.71
EC-Plan-Neofluar 40x/1,3 Oil	40	1.3	Oil		PL	0.79 / 0.70
EC-Plan-Neofluar 63x/0.95 Korr	63	0.95	Air		PH	0.64 / 0.69
EC-Plan-Neofluar 63x/1,25 Oil	63	1.25	Oil		PH	0.98 / 0.93
EC Plan-Neofluar 100x/1,3 Oil	100	1.3	Oil		PH	0.65 / 0.57
LCI Plan-Neofluar 25x/0,8 Imm. Korr.	25	0.8	Oil, water or glycerine		PL	0.82 / 1.26
LCI Plan-Neofluar 63x/1.3 Imm. Korr.	63	1.3	water or glycerine		PH	0.98 / 0.86
Plan-Apochromat 10x/0,45	10	0.45	Air		PL	1.32 / 6.40

Objective	V	NA	Immersion	DAPI	recom. grid	optical section thickness @ 490 nm (RU/µm)
Plan-Apochromat 20x/0,8	20	0.8	Air		PL	1.00 / 1.54
Plan-Apochromat 40x/0.95 Korr	40	0.95	Air		PH	0.96 / 1.05
Plan-Apochromat 40x/1.0 Oil	40	1.0	Oil		PH	0.77 / 0.76
Plan-Apochromat 40x/1.3 Oil	40	1.3	Oil		PL	0.79 / 0.70
Plan-Apochromat 63x/1.0 W	63	1.0	water		PH	0.85 / 1.10
Plan-Apochromat 63x/1,4 Oil	63	1.4	Oil		PH	0.96 / 0.73
Plan-Apochromat 100x/1,4 Oil	100	1.4	Oil		PH	0.63 / 0.48
LD-LCI-Plan-Apochromat 25x/0.8	25	0.8	Oil, water or glycerine		PL	0.82 / 1.26
C-Apochromat 10x/0,45 W	10	0.45	water		PL	1.32 / 6.40
C-Apochromat 40x/1,2 W	40	1.2	water		PH	1.31 / 1.19
C-Apochromat 63x/1,2 W	63	1.2	water		PH	0.86 / 0.78
LD C-Apochromat 40x/1.1 W	40	1.1	water		PH	1.32 / 1.42
alpha Plan-Fluar 100x/1.45 oil	100	1.45	Oil			0.61 / 0.43
alpha Plan-Apochromat 100x/1,46 Oil	100	1.46	Oil			0.60 / 0.42

Other ApoTome Settings

In addition, the following control elements can be found on the **Extras** property page:

ApoTome: Auto Shutter

If **Automatic Shutter Control** is activated, the shutter is controlled automatically if

1. the live image is opened or closed
2. an image is acquired

This function is useful in order to protect the sample against the bleaching of fluorescence and is activated as a default setting when the software is installed.

ApoTome Display Normalization

The algorithm used to calculate the optical section reduces the image's gray value range. If the **Display Normalization** function is activated, the display characteristic curve is automatically adjusted to guarantee a bright, high-contrast display of the gray values.

This function is activated as a default setting when the software is installed.

ApoTome: Status

This field displays status information relating to the ApoTome and image acquisition with the ApoTome. It makes sense to have this window open or to integrate it into your own customized dialogs so that any problems with the system can be analyzed quickly.

Conversion of ApoTome images from raw data mode

If you select the **Raw Data Mode** for image acquisition with the ApoTome, the size of the data is increased by a factor of 3, 6, 9 etc. in accordance with the settings under **Averaging**. If you are acquiring z-stack images, images in several fluorescence channels or time lapse images, this can lead to enormous volumes of data that can be difficult to work with during subsequent analyses. The **SIMConvert** function converts the raw data to an optical section or a conventional fluorescence image with a substantially reduced data volume.

Use of this function is strongly recommended after you have acquired a z-stack in **Raw Data Mode** and processed it using the AxioVision 3D Deconvolution module.

To perform conversion, follow the procedure below:

➢ Click on the ApoTome image that you want to convert.

➢ Click on the **ApoTome** entry in the work area.

➢ Open the function tree by clicking on screen in front of the **ApoTome** entry – click on **SIMConvert**. The parameters for the function are listed underneath the work area.

➢ Adopt the settings in the top **Parameters** field. The default settings ensure that the selected image is processed and a new image is created automatically (the original file is retained). The **Enable channel selection** check box should be deactivated, as generally all the channels in an image should be converted in the same way.

Four parameters are available in the lower area of the property page for the actual conversion **Combine Mode**, **Filter**, **Image Normalization** und **Display Normalization**:

➢ **Combine Mode**: This parameter determines the algorithm used to convert the image. You can select from **Conventional Fluorescence** or **Optical Sectioning**.

➢ **Filter**: Depending on the sample, fluorescent dye, exposure time etc., fine residual lines may be visible in the image that results from the conversion. These lines can be removed using the filter. The options **Off**, **Weak**, **Medium** and **Strong** are available.

The filter function makes use of the fact that the residual lines are displayed in the Fourier space as points along a vertical line. The filter "removes" a defined group of points from the Fourier spectrum, thereby removing the residual lines.

➢ **Display Normalization**: The algorithm used to calculate the **Optional Sectioning** reduces the gray value range of the image data, depending on the specimen and the acquisition conditions. The **On** functional parameter automatically sets the image's display characteristic curve so that a bright, high-contrast image is displayed. The **Min/Max** function of the display characteristic curve is used, which sets the display characteristic curve on the basis of the darkest and brightest gray value in the image histogram. If a z-stack is converted, the image plane that is currently set in the active image is used to determine the **Min/Max** value for the entire image stack.

➢ **Image Normalization**: As described under "Display Normalization", the algorithm used to calculate the **Optional Sectioning** reduces the gray value range of the image data, depending on the specimen and the acquisition conditions. As the so-called floating point format is used for raw data acquisition with the ApoTome and the subsequent combination of the gray value data, during the conversion from floating point back to gray value, a normalization function can be applied directly to the data to display a bright, high-contrast image. The **On** parameter activates this form of normalization.

NOTE:
• If **Image Normalization** is switched on, **Display Normalization** is no longer necessary, as the result of both functions has the same effect for the user. For the conversion of a z-stack, the **Image Normalization** function offers advantages, as the gray value distribution of all image planes can be taken into account.

ApoTome: HDR Mode

By activating the **HDR Mode**, images with an expanded dynamic range can also be acquired in ApoTome mode. For further details, please refer to the description of **HDR Snap** and the AxioVision manual.

If the ApoTome is operated in **HDR Mode**, the corresponding number of images will be acquired for each grid position with various exposure times and, from this, an HDR image will be calculated for each grid position. The ApoTome calculation will then be performed on these three HDR images. This will ultimately result in an optical section with an expanded dynamic range.

Because in the **HDR Mode** several images are acquired with very long exposure times, residual fringes may occur in the ApoTome optical section as a result of bleaching effects. In this case, it is necessary to use the ApoTome filters in order to suppress the residual fringes.

The additional time required for calculating the HDR image can lead to delays in the displaying of the preview when multidimensional images are acquired. In this case, the preview should be deactivated.

9.2   Autofocus

General

Autofocus enables you to focus a sample automatically. To do this, you need a microscope with a motorized z-drive. Autofocus can be operated with all cameras that are available for use with AxioVision, with the exception of those that are only controlled via a TWAIN interface. You can use this functionality for the illumination methods reflected light, transmitted light, bright field and dark field, as well as fluorescence.

NOTE:
- The Autofocus functionality is not suitable for the contrasting methods Phase Contrast and DIC.

To make it possible to focus samples automatically with the different microscopy procedures and at all possible magnifications, it is necessary to carry out calibration in advance on a sample which has been focused manually. The calibration requires information on the objective and optovar magnification, the numerical aperture of the objective being used, the illumination method and, if you are using a digital camera, the corresponding resolution.

If all the components of your microscope are motorized, AxioVision determines these parameters automatically. Otherwise the missing calibration data have to be entered manually.

NOTE:
- We recommend that you recalibrate the system whenever microscope components have been changed.

Calibrating Autofocus

Preparation

Switch the light path to the camera. Open the live image window by clicking

on the icon for the live image in the **Standard** toolbar Live. Alternatively,
select the **Live** function in the **Acquisition** menu.

➢ Now focus the camera image and select the frame to be acquired.

Calibration

➢ Select the **Autofocus** function in the
   Acquisition menu.
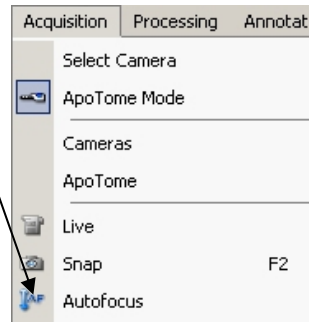
➢ Activate the **Settings** property page.

➢ Make sure that the settings for the type
   of **Illumination**, **Objective**, **Optovar**
   and live image speed match those on
   your system.
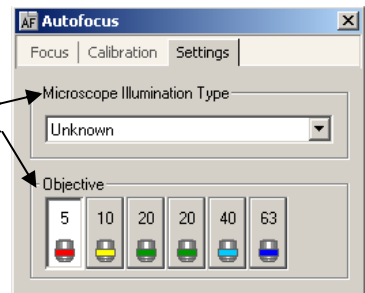
NOTE:
• The setting options depend on your system configuration and may
   differ from those shown here.

➢ Now activate the **Calibration** property
page.

➢ Check the settings for **Microscope** and
Camera.

NOTE:

• If individual components are not
encoded or motorized, you must enter
the relevant information here using the
keyboard.

➢ Then click on **New** to create a new
calibration with these settings.
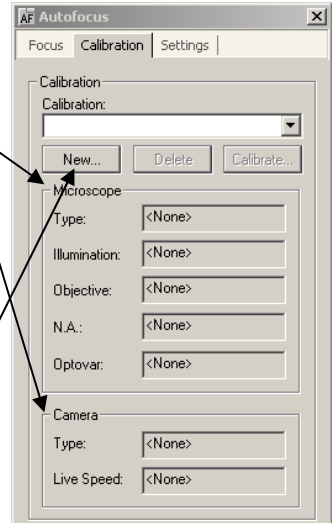
➢ Enter a name for the calibration and
click on **OK**.
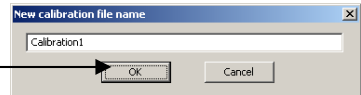
The calibration process is now started
automatically.

Using Autofocus

The quickest and easiest way to use the Autofocus is to run it directly via
the toolbar at the bottom of the live image window.

If the results are unsatisfactory, you can make fine adjustments via the
Autofocus property page. A detailed description of the parameters
available there can be found in the online help.

Use of the Autofocus together with the other modules for image acquisition
is described in the relevant sub-sections.

NOTES:

- Before the Autofocus can be used, calibration must be carried out for the microscope and camera setting you are using. See the preceding chapter.

- If problems arise during execution or if execution is not possible for certain reasons, please check the **Autofocus** function in the **Acquisition** menu. Activate the **Focus** tab and look under **Status**. There you will receive an indication as to why the execution process has failed.

➢ Make sure that the **Standard** toolbar is displayed: right-click on the menu or any toolbar and activate **Standard** (there must be a tick in front of it).

✔	Standard	
	Display	
	Acquisition	
	Measure	
	Microscope	
	Annotations	
	Measure Annotations	
📘↕	Workflow	Ctrl+B
🔲	Workarea	Ctrl+W
✔	Status	
	Customize...	
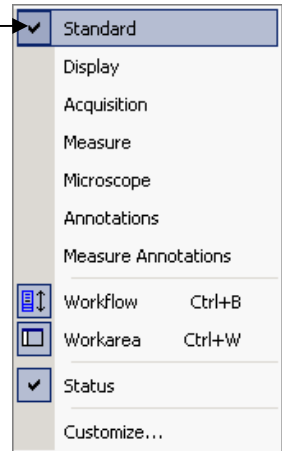
➢ Switch the light path to the camera. Then click on the icon for the live image in the **Standard** toolbar.

➤ If the tool bar of the live image window is not displayed, right-click in the live image and activate the **Live tool bar On/Off** entry (there must be a tick in front of it).
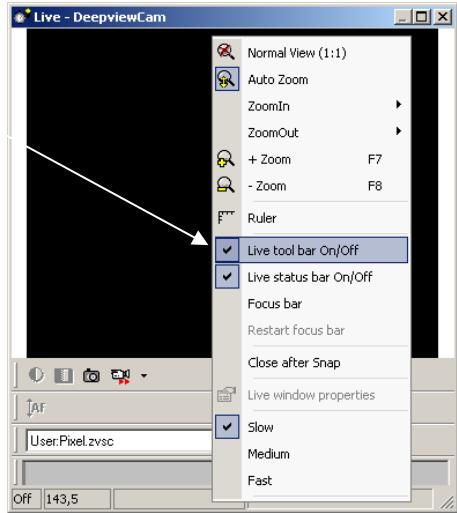
➤ Now select the frame to be acquired on the sample, and then click on the **Autofocus** button in the live image toolbar.

➤ Now click on **Snap** to acquire an individual image.

NOTE:
• If the result of the focusing is not satisfactory, you can make further adjustments via the **Autofocus** function. To do this, select the **Autofocus** function in the **Acquisition** menu. Activate the **Focus** tab and make the desired adjustments.

9.3   Multidimensional Acquisition

Images with more than two dimensions cannot be acquired using your camera's normal acquisition function. These kind of images may consist, for example, of several channels or a time lapse series. For these images AxioVision requires additional information, such as the number of channels, z-planes etc. This collection of information is referred to within AxioVision as an "experiment".

In the work area you will find **Multidimensional Acquisition**. If you click here the **Experiment** property page opens. The control elements for the modules Multichannel Fluorescence, Z-Stack, Mark&Find and Time Lapse appear here, depending on which modules you have purchased.

The **Experiment** property page contains an overview of the modules that are installed and a selection of important control elements. From this page you can perform a wide range of functions to control, modify, save and execute a previously prepared experiment.

On this page you can re-use settings of already acquired images via the **Reuse** function.

Each module also has its own tab, on which the essential settings can be made.

NOTES:
- Please note that you may not have access to all of the modules and functions described here. This depends on the options you have purchased.

- For detailed information on the individual functions and commands, please refer to the online help (**F1** key).

How do I create an experiment?

➢ In the work area click on **Multidimensional Acquisition**.

      ApoTome
      Scalings
      Multidimensional Acquisition
      Extended Focus Acquisition
      Deconvolution
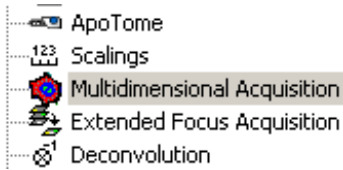
➢ In the work area you will now see the **Experiment** property page.
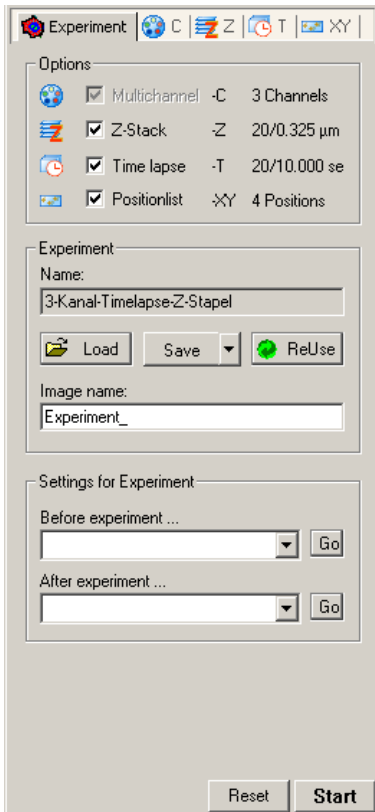
NOTE:

- Please bear in mind that the options listed here are only displayed if the corresponding modules are available.

➢ In the **Options** field the dimensions for your experiment can be selected.
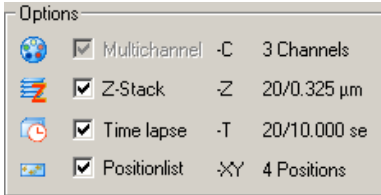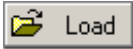
➢ In the **Experiment** window the name of the current experiment is displayed.
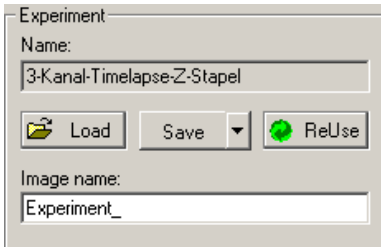
The default experiments name is "Default_Setting". The default name for an acquired image is Experiment_. A serial number will be attached to this name after acquisition. You can specify the basic name for the acquired image here before acquisition.

➢ Make the desired settings for your experiment and click on Save ▼. A dialog is then opened. Save your new experiment.

➢ Click on 🖼 Load, select an experiment from the list of experiments and click on **OK**.
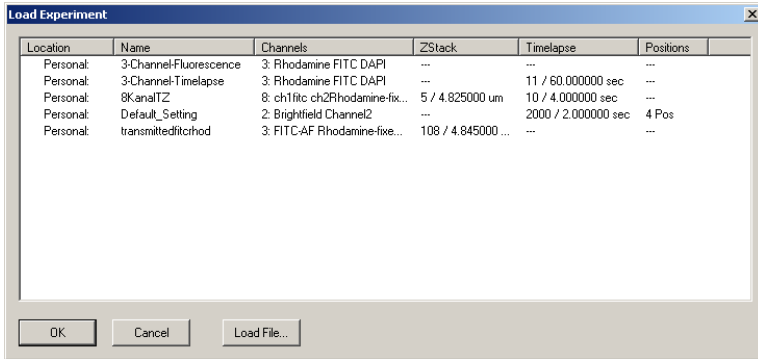
Once this experiment has been loaded, the settings it contains are adopted.

➢ If you want to adopt these settings from a ZVI image that has already been saved, click on **⟳ ReUse**, select the desired image, and confirm with **OK**. Only the acquisition settings used for this image are adopted. The image itself is not loaded. This function makes it easier for you to acquire images under reproducible conditions.

➢ To execute the experiment, click on **Start**.

Multichannel Fluorescence module

With the Multichannel Fluorescence module you can define up to 8 channels to be used for acquisition. These channels are saved together in a ZVI document. This allows you, for example, to switch quickly back and forth between the various views to compare different channels.

NOTES:
- Please note that you may not have access to all of the modules and functions described here. This depends on the options you have purchased.

- For detailed information on the individual functions and commands, please refer to the online help (**F1** key).

How do I create a multichannel experiment?

In the following example a 3 channel fluorescence image should be generated. The channels should be configured for the fluorochromes Rhodamine, FITC and DAPI. Please use a test sample to test the settings.

NOTES:
- The following instructions require use of a motorized microscope.

- You should also be able to generate, edit and apply settings in the **Settings Editor** and should already have generated channel-specific settings (see chapter 8 "Configuration" - "Settings Editor"). The hardware components available to you may differ from those shown in this example.

➢ In the **Workarea** activate the property pages under **Multidimensional Acquisition**:
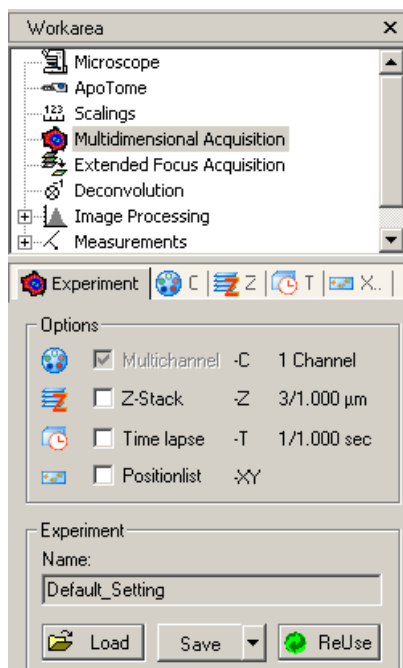
> ➢ Go to the **C** property page . To generate various channels, you need to enter the channel-specific parameters here.

> ➢ From the **Dye** field select the Rhodamine fluorochrome.
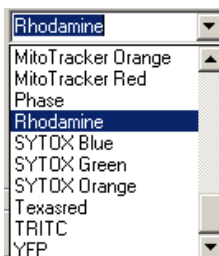
Once the dye has been selected, a suggested channel color with wavelengths for excitation and emission maximums are assigned to the channel.

➢ If you do not like the suggested color, you can change it (e.g. to 100% red) by making a selection from the list:
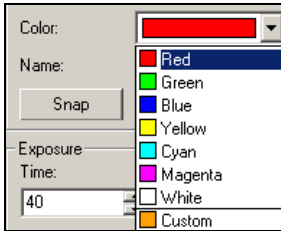
➢ A channel name is automatically generated from the dye Rhodamine but can be changed if needed, e.g. to reflect the type of exposure used Rhodamine-fixed.

➢ Now allocate a hardware setting to the channel. To do this, select "Rhodamine.zvhs" from the list in the field **During Acquisition**:
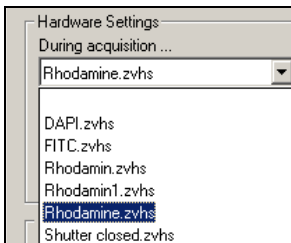
➢ You can test this setting by clicking on the Go button. This opens the fluorescence shutter, and switches the reflector to the correct filter position.

➢ Repeat the procedure for the next field, and select the setting "shutter closed.zvhs". This ensures that the shutter is closed immediately after acquisition

After acquisition ...

| Shutter closed.zvhs | ▼ | Go |

.
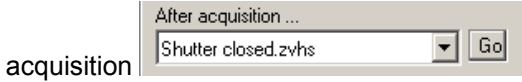
➢ To determine the exposure time for the Rhodamine channel, click in the **Exposure** field and then click on the [▣ measure] button. The hardware settings you have selected are now applied to the microscope and a dialog is opened:
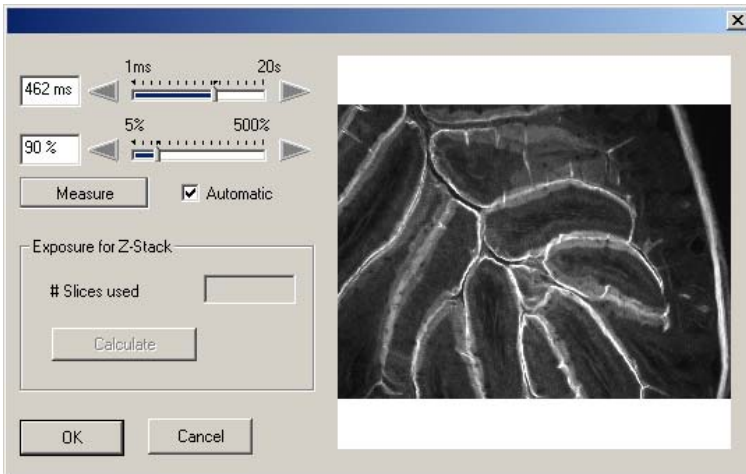
➢ To set the correct exposure time, click on the [▣ measure] button and close the dialog by clicking on [OK]. The new exposure time is now entered and used for the channel (

Mode:
○ Auto   ● Fixed   ○ Camera

).

➢ Add a new channel to your experiment. To do this, click in the **Extended Channel Parameters** dialog on [▣ Duplicate]. A copy of the channel you have already configured is then added to the experiment.
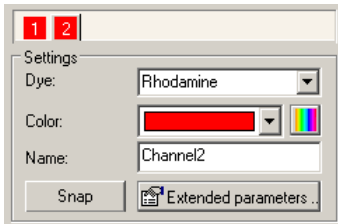
➢ Now change the settings that are relevant for the new channel (channel name, dye, hardware setting during acquisition, exposure time measurement):
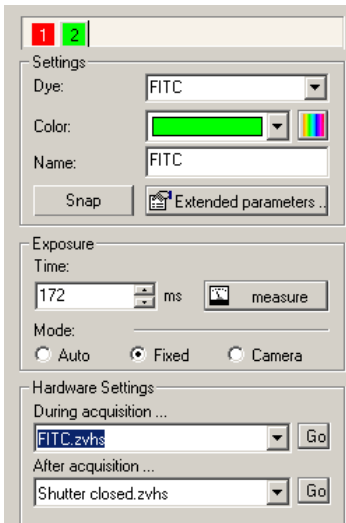
➢ Duplicate the second channel, and change it accordingly for the DAPI dye:
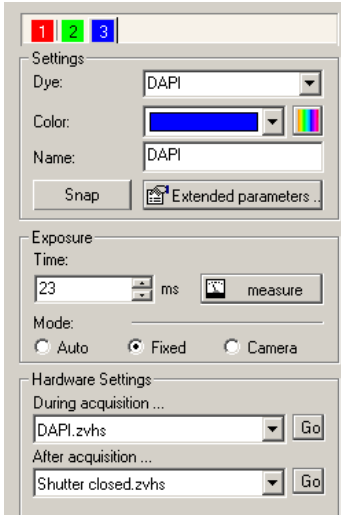
➢ You can add together up to 8 channels in this way.

➢ You should now save the settings you have made as a new experiment (see above), so that you can use them again in the future.

➢ To acquire your 3-channel image, click on ⌷ Start ⌷. During acquisition a progress bar informs you about the procedure:
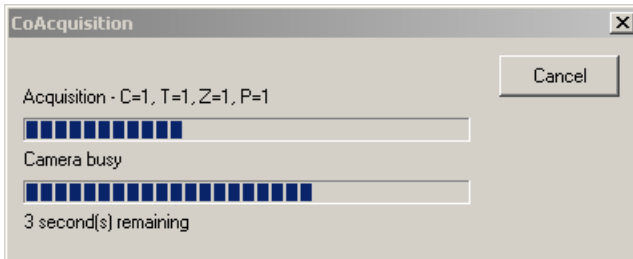
➢ If you want to cancel the acquisition, click on **Cancel**.

The resulting image appears in the image area, where it can be operated using the player:
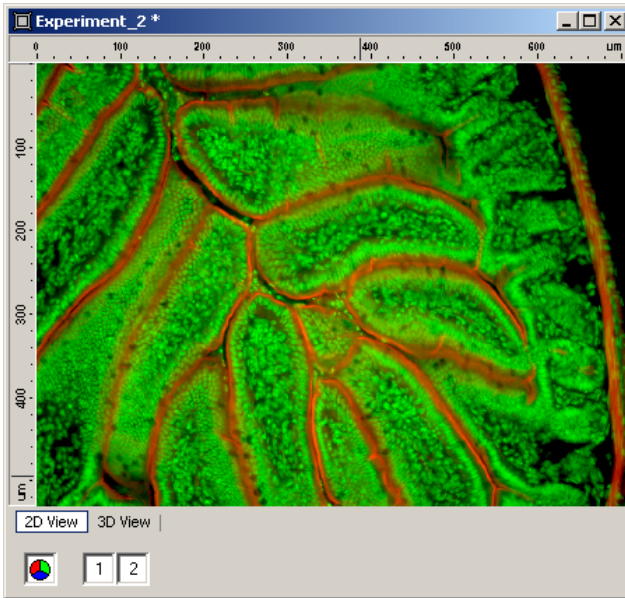
This image has not been saved, as you can see from the * symbol in the image name. Save the image. The program remembers the last storage location that you searched for.

How do I manage channel settings (working with the channel pool)?

Above you have seen how to configure channels for multichannel images and save them as an experiment. An experiment therefore contains settings that are specifically intended for that particular experiment. As time goes by you will find channel settings that can be applied in many different cases. You may also wish to combine channels from different experiments. The "channel pool" has been created for these kinds of requirement. These instructions describe how to work with the channel pool.

NOTE:
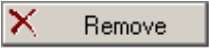
- The following instructions require use of a motorized microscope. The hardware components available to you may differ from those shown in this example. You must also be able to generate, save and load experiments and have already created a multichannel experiment.

➤ Load an available multichannel experiment. In this example a 3-channel experiment (Rhodamine/FITC/DAPI) is loaded. If you go to the **Channel** property page, you should see e.g. the following channel parameters:
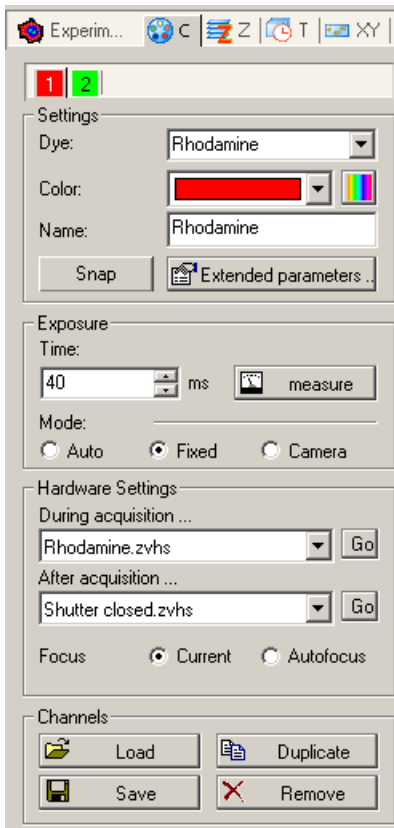
➢ Select the Rhodamine channel: In the **Channels** field click on

        | 💾   Save |
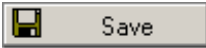
➢ Repeat this for the other channels.

➢ Now remove the channels from the experiment by clicking repeatedly

   on | ✕   Remove |. The **C** (channel) property page should now only contain one channel.

➢ Now click on the | 📂   Load | button. The channel pool opens as a new dialog:

Channel #	1	2	3	4
Name	Channel1	Rhodamine	FITC	DAPI
Channel Color				
⊟ **Exposure Time [ms]**	40	227	186	116
Exposure Mode	Channel settings	Channel settings	Channel settings	Channel settings
Maximum pixel intensity [%]	100	90	90	60
Setting during acquisition		Rhodamine.zvhs	FITC.zvhs	DAPI.zvhs
Setting after acquisition		Shutter closed.zvhs	Shutter closed.zvhs	Shutter closed.z
⊞ **Dye**		Rhodamine	FITC	DAPI
⊞ **Display settings**	System default	Preset gamma and bla...	Preset gamma and bla...	Preset gamma and
⊞ **Focussing**	Current focus position	Current focus position	Current focus position	Current focus p
⊞ **User interaction**				

Edit Pool

| Add Copy | Add Default | Remove | | Fit to window |

Edit Experiment

| Add to Experiment | Close Pool | Cancel Pool |

The size of this dialog can be changed to give you sufficient space to work with the channel parameters.

➢ Now select the Rhodamine channel …
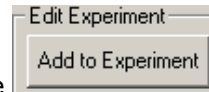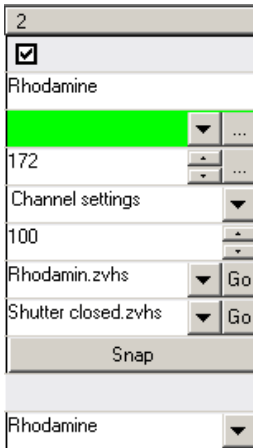
and add it to the empty experiment by clicking on the

button. Clicking on the channel pool is closed and the channel now appears on the **C** property page:
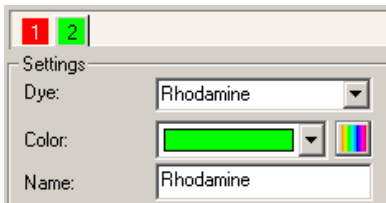
➢ To add another channel to the experiment, repeat the last steps.

➢ In the channel pool you can also duplicate, modify or remove channels. To do this, select the desired channel and click on the corresponding button.

Z-Stack module

Using the Z-Stack module you can acquire series of images over a defined z-focus range. The resulting image is known as a z-stack. These kinds of image are required for the calculation of three-dimensional displays.

Z-stack images are essential for the 3D Deconvolution module. The ApoTome module benefits from z-stack images, as they can be used to generate impressive 3D images quickly. Using the Extended Focus module a sharp image can be calculated from a z-stack.

The AxioVision module Z-Stack allows the easy determination of the correct z-distance between individual images of a z-stack if the maximum of axial resolution of the objectives should be achieved. This optimum step size (also called the Nyquist criterion) is a function of the numerical aperture of the used objective and the recorded wavelength (in the case of fluorescence usually the emission maximum). It is calculated as follows:

The axial resolution **d** is approximated from the following formula:

$$d_{axial} \approx 2\,\frac{\eta\,\lambda}{N.A.^2}$$

At a given wavelength λ = 540 nm and a numerical aperture **N.A**. = 1.3 at a refractive index η of 1.52 d_{axial}= 0.970 µm. According to Nyquist a twofold oversampling (approximately 485 nm between neighboring z-planes) all available information will be collected. This sampling rule is a prerequisite for reconstructing z-stacks with the module AxioVision 3D-Deconvolution.

NOTES:
- Please note that you may not have access to all of the modules and functions described here. This depends on the options you have purchased. In order to follow this introduction, you need to know what an AxioVision "experiment" is. Please read the documentation on experiments in section "How do I create an experiment?".

- For detailed information on the individual functions and commands, please refer to the online help (**F1** key).
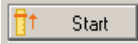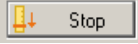
How do I create a z-stack?

➢ In the work area activate the property page under **Multidimensional Acquisition**.
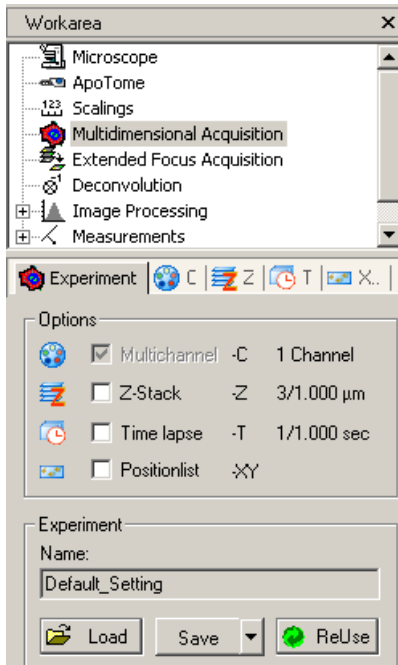
➢ On the **Experiment** property page select the ⤢ᶻ (**Z-Stack**) function.

➢ From the two possible definition modes select the **Top/Bottom** mode:
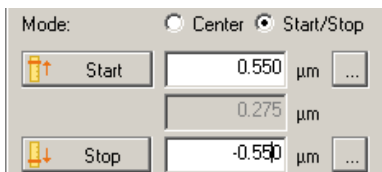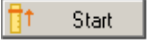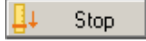
➢ Click on the [...] button in the [🔼 Start] [0.550] µm [...] control element. A dialog is opened that contains a live image and the control element for focusing. Please note that light will only fall on the sample if it has been directed onto the sample manually, or if a hardware setting has been configured for the selected channel. On the microscope, focus on the area where you want the z-stack to begin. To adopt this z-position, close the dialog by clicking on **OK**. The new z-position is then automatically adopted into the start field.

➢ Repeat this procedure to determine the **Stop** position using the [...] button from the [🔽 Stop] [-0.550] µm [...] control area.

➢ Now define the desired interval between the individual z-planes. You can enter any value here. To guarantee maximum resolution in the z-direction, click on the [📏 Optimal distance] button.
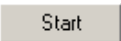
The correct value is calculated, taking into consideration the numerical aperture of the objective and the emission wavelength of the fluorochrome (this takes place automatically if a fluorochrome has been selected from the list). If no emission wavelength is stated, a wavelength of 500 nm is taken as a basis.

➢ To check your selection, you can navigate through the z-stack using the z-stack navigation function. To do this, open a live image, and activate the hardware settings for the desired channel (e.g. on the channel page via the [Go] button

Hardware Settings
During acquisition ...
Rhodamin.zvhs ▼ [Go] .
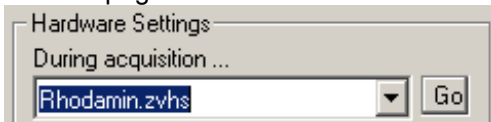
➢ You can now position the focus drive at the upper [▲] or lower [▼] limit or the center [C] of the defined z-stack.

➢ If you are not satisfied with the definition of the z-stack, you can modify

it step by step (with the set increment) using the ▲ or ▼ buttons.
Adopt the new definition by clicking on the 🔼 Start / 🔽 Stop
buttons in the control area for z-stack settings.

➢ Click on Start to begin acquisition. During acquisition a progress
bar informs you about the procedure:

CoAcquisition	✕
	Cancel
Acquisition - C=2, T=1, Z=9, P=1	
▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮	
6 second(s) remaining	
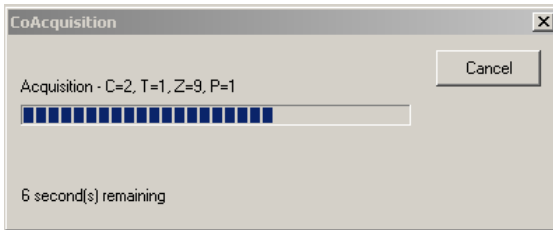
If you want to cancel acquisition, click on **Cancel**.

➢ The resulting image appears in the image area, where it can be
operated using the player. This image has not been saved, as you can
see from the * symbol in the image name. Save the image. The
program remembers the last storage location you have chosen.

Time Lapse module

The Time Lapse module can be used to acquire series of images over a defined period of time. The resulting image is a time lapse series. These kinds of image are required for time lapse acquisition of changing processes.

NOTE:
- Please note that you may not have access to all of the modules and functions described here. This depends on the options you have purchased. In order to follow this introduction, you need to know what an AxioVision "experiment" is. Please read the documentation on section "How do I create an experiment?". For detailed information on the individual functions and commands, please refer to the online help (**F1** key).

How do I create a time lapse series?

➢ In the work area activate the property pages under **Multidimensional Acquisition**.
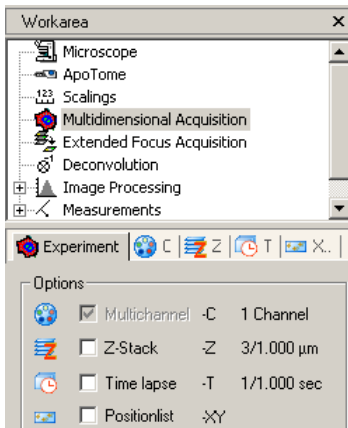
➢ On the **Experiment** property page select the **Time lapse** function
[icon] .

➢ Enter the desired time interval, e.g. 2 minutes. The unit can be selected from the drop-down list box.

➢ Now enter the desired number of images (**# Cycles**). The total duration of the experiment is calculated from this.

➢ Alternatively, you can enter the interval and the total duration, and this information will be used to calculate the number of cycles. Select the function that you want to be calculated

Calculate   ⊙ Cycles   ○ Duration
.

➢ It is also possible to enter numerical values directly into the input fields. To calculate the dependent value, click on the
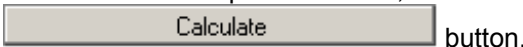
Calculate   button.

➢ Click on Start to begin acquisition. During acquisition a progress bar informs you about the procedure:

CoAcquisition   ✕

Cancel

Acquisition - C=2, T=3, Z=1, P=1

████████

25 second(s) remaining
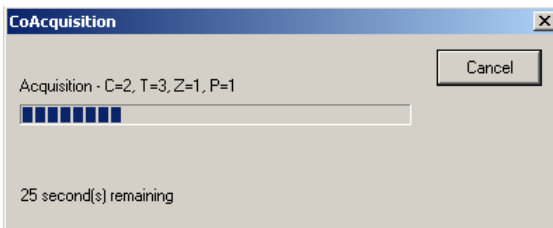
If you want to cancel acquisition, click on **Cancel**.

➢ The resulting image appears in the image area, where it can be operated using the player. This image has not been saved, as you can see from the * symbol in the image name. Save the image. The program remembers the last storage location that you searched for.

Advanced image acquisition: Mark&Find

The Mark&Find module allows you to acquire images of various positions on your sample, also in combination with the Multidimensional Acquisition modules. All the dimensions offered by AxioVision can also be used for a position list. The output images are saved automatically in a folder that you have defined, and from this a folder-based archive is generated. This makes it easy to manage the individual images.

NOTE:
- Please note that you may not have access to all of the modules anfunctions. This depends on the options you have purchased.

- In order to follow this introduction, it is useful to know what an AxioVision "experiment" is. You will find the relevant information in the "How do I create an experiment?" section.

- For detailed information on the individual functions and commands, please refer to the online help (**F1** key).

How do I create a Mark&Find experiment?

➢ In the work area, activate the **Multidimensional Acquisition** function:
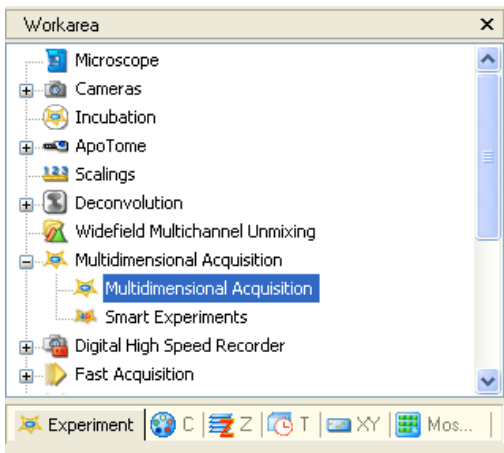
➢ Select the **Experiment** property page and load an experiment.

➢ Select the ▦ XY (**Positionlist**) tab sheet.

➢ Open the Mark&Find dialog by clicking on the ⬚ MarkFind... button.
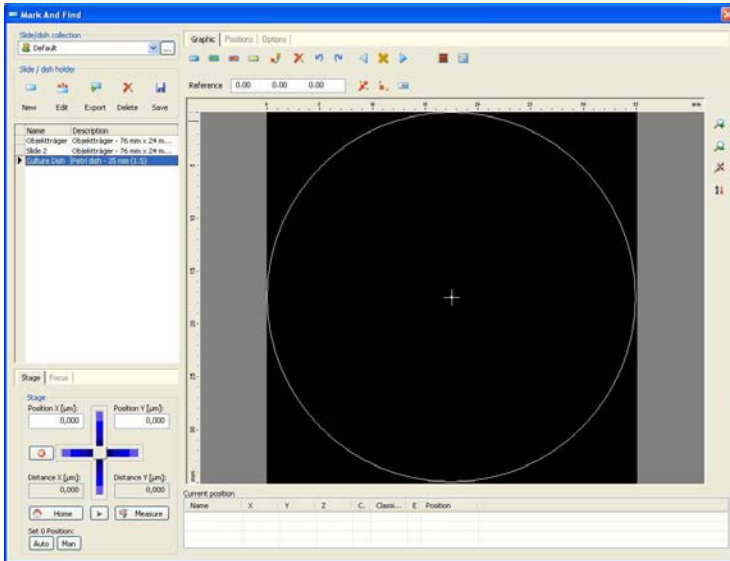
➢ Load a saved Mark&Find database from the list box

- or define a new list by clicking on **New**/modify an existing list by
  selecting **Edit** (please read section 9.5 Mark&Find "How do I
  generate a Mark&Find list?", or call up the online help (**F1** key)).
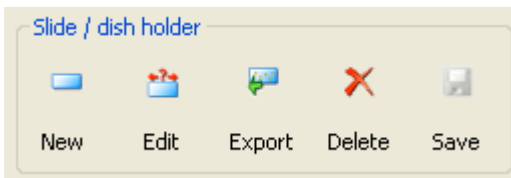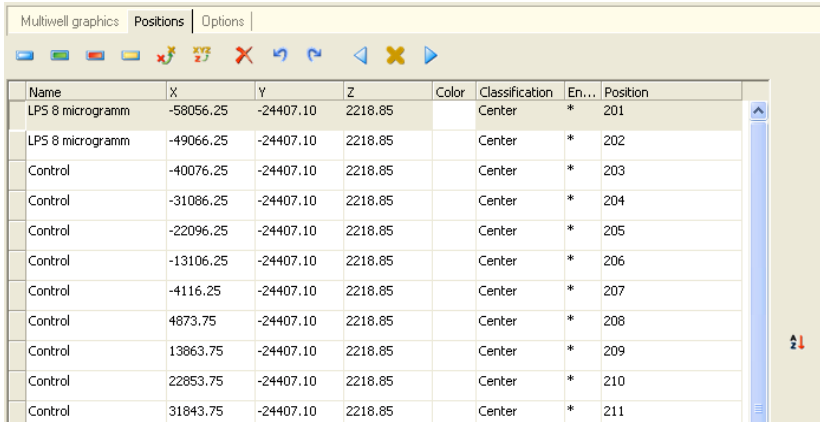
The Mark&Find control element now shows a graphic display of the selected list.
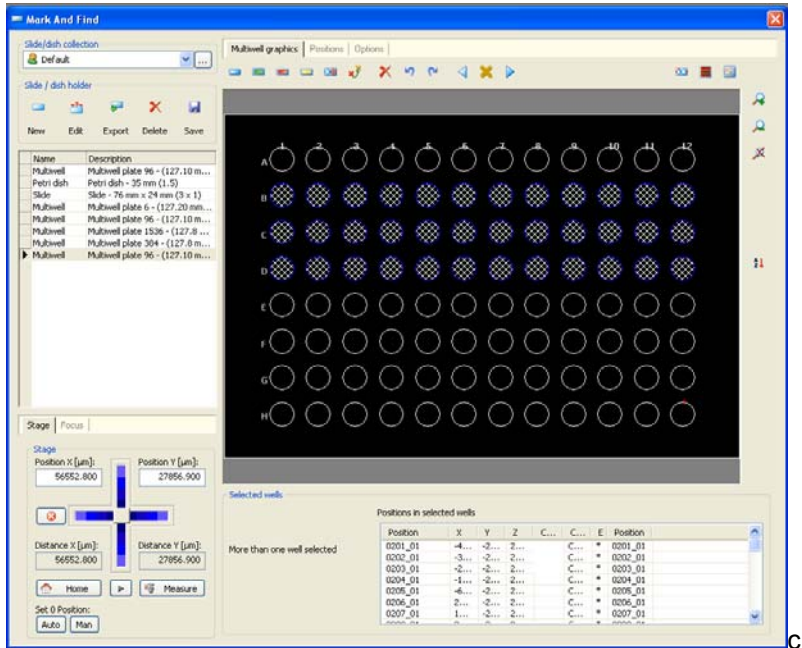
NOTE:

- The graphic will only have the expected appearance if both the reference point on the sample and the sample holder have been centered. Please read section 9.5 Mark&Find "How do I generate a Mark&Find list?", or consult the online help (**F1** key).

If you click on the **Positionen** tab sheet, you will see a tabular display of the position list.

Name	X	Y	Z	Color	Classification	En...	Position
LPS 8 microgramm	-58056.25	-24407.10	2218.85		Center	*	201
LPS 8 microgramm	-49066.25	-24407.10	2218.85		Center	*	202
Control	-40076.25	-24407.10	2218.85		Center	*	203
Control	-31086.25	-24407.10	2218.85		Center	*	204
Control	-22096.25	-24407.10	2218.85		Center	*	205
Control	-13106.25	-24407.10	2218.85		Center	*	206
Control	-4116.25	-24407.10	2218.85		Center	*	207
Control	4873.75	-24407.10	2218.85		Center	*	208
Control	13863.75	-24407.10	2218.85		Center	*	209
Control	22853.75	-24407.10	2218.85		Center	*	210
Control	31843.75	-24407.10	2218.85		Center	*	211

➢ Close the dialog by clicking on [X]. The Mark&Find control element now displays the number of positions and the name of the position list:
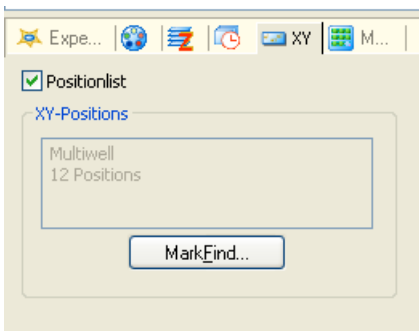
➢ Click on **Start** to begin acquisition.

During acquisition a progress bar informs you about the procedure:
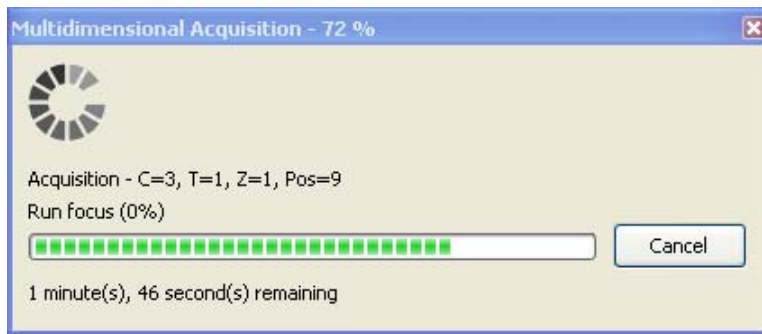
➤ If you want to cancel acquisition, click on [Cancel].

preview containing acquisition parameters is also displayed:
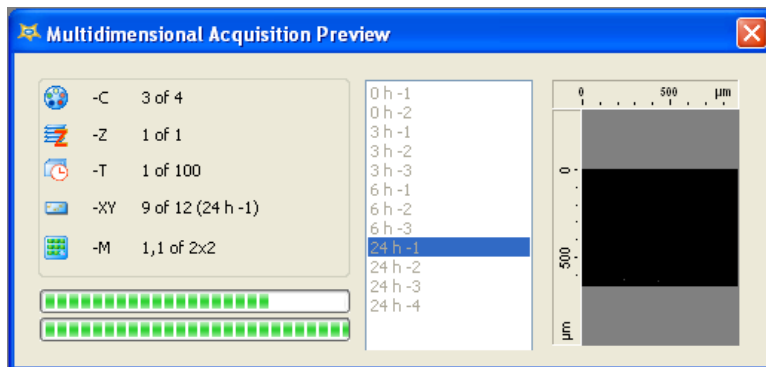
At the end of the above procedure, a folder-based archive showing all output images is created.
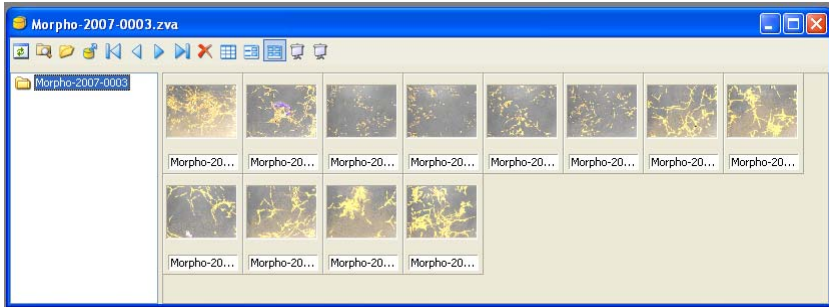
These can be loaded and handled in the same way as normal individual images.

NOTE:
- No images are displayed during the acquisition of a position list. To view the images, you need to exit the experiment.

9.4   Extended Focus

General

The **Extended Focus** function is available as an optional module for AxioVision. This function enables you to display the sharp areas of different focus positions of your sample in a single image.

Essentially, the **Extended Focus** module is operated interactively. You do not need a motorized microscope. Several images are acquired at different focus positions. The sharp areas are extracted from each of the images and automatically combined. You immediately obtain a sharp 2D image. The individual images of the different focus positions are not saved, but are combined straight away. The focus is driven manually across the entire z-range until all the details have been displayed sharply. There are no specifications, recommendations or formulae that say how many images have to be acquired or how large the distance between two focus positions should be. This always depends on the sample in question. Generally speaking, it can be said that it is better to acquire too many images than too few.

Alternatively, an image with extended depth of field can also be calculated using a z-stack. To do this, you need a z-stack image, the acquisition of which requires a motorized microscope and the **Z-Stack** module (see also section 9.8 "Z-Stack Images").

Acquiring images with Extended Focus

The **Extended Focus** function can be run via the work area on the left of the AxioVision window. If the work area is not displayed, activate it using

the **Standard** toolbar ⬚ Workarea .

Alternatively, select the **Windows** function in the **View** menu and then select the **Workarea** entry.

NOTE:

- The work area may be open but covered by the workflows. If that is the case, simply click on the **Workarea** tab at the bottom of the work area.

➤ Switch the light path to the camera. Open the live image window by clicking on the live image icon in the **Standard** toolbar. Alternatively, select the **Live** function in the **Acquisition** menu.

➤ In the work area select the **Acquisition** entry in the **Extended Focus** section.

➤ If you capture images with a Stereo microscope, select an appropriate value for **Quality of alignment**. This will correct the lateral shift which occurs when you are focussing through the specimen.

➤ Select **Wavelets** as the **Method** for calculation.

➤ Click the **Interactive** button to acpture the single images by pressing the **Ctrl** key.
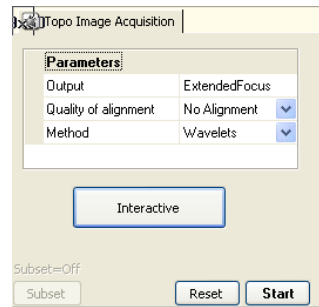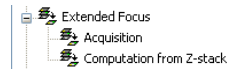
NOTE:

- As an alternative you can click the **Start** button to capture the images.

➤ Press the **Ctrl** key to capture the first image

➤ Now go to the next focus position.

➤ Press the **Ctrl** key to acquire the next image.
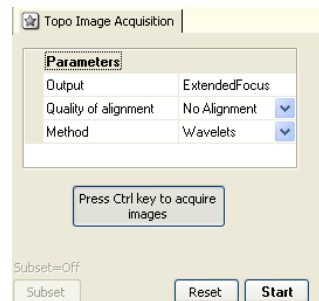
NOTES:

- The image of the second focus position is now automatically combined with the first image.

- You can now acquire further images until the result is satisfactory.

Using z-stack images for Extended Focus

➤ Activate the image to which you would like to apply the **Extended Focus** function. To do this, click on the image or select it via the tabs at the top of the document area.
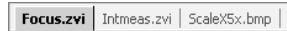
➤ Now select the **Extended Focus** (Computation from Z-stack) function in the **Processing** menu.

Adjust	▶
Geometric	▶
Smooth	▶
Sharpen	▶
Utilities	▶
Extended Focus (Computation from Z-stack)	
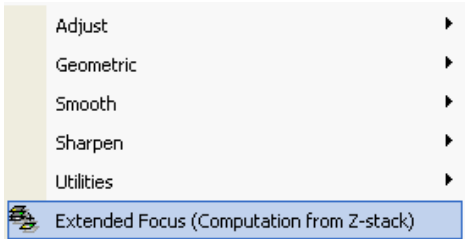
➤ The two thumbnails show the original image (left) and the resulting image (right).

  To select a specific frame, you can magnify, choose any frame from, or reduce the thumbnails.
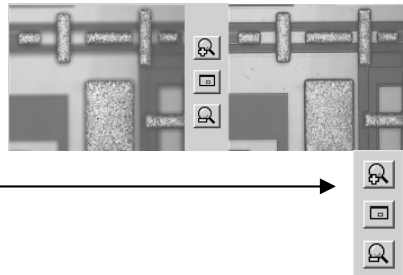
> ➤ If the z-stack was acquired with a Stereo microscope, select an appropriate value for **Quality of alignment**. This will compensate the lateral shift which usually occurs during acquisition of the z-stack.

> ➤ Select **Wavelets** as the **Method** for calculation.

> ➤ Click on **Apply** to check the result.

> ➤ Now click on **Cancel** to exit the function.
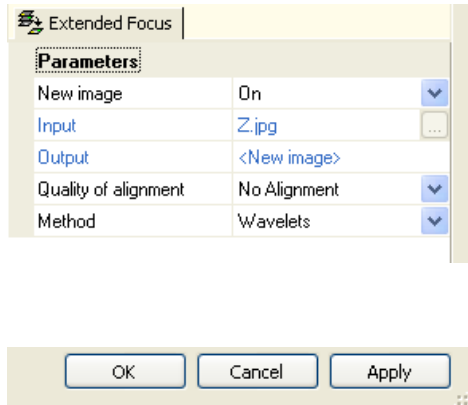
9.5   Mark&Find

Overview

The Mark&Find module allows you to record the positions of an encoded or motorized x/y stage and relocate them in the future.

Open the Mark&Find window using the **Microscope** $\Rightarrow$ **Mark And Find** menu command.

The recorded positions are saved in a database, which is able to store the position lists of several carriers. You can select the size of the carrier concerned. The following types are available: **Petri dish**, **Slide/Dish holder, Multiple slides** and **Multiwellschale**.

The module supports slides with sizes of 76 mm x 24 mm, 76 mm x 50 mm, 48 mm x 25 mm and 48 mm x 27 mm, or any user-defined size. Petri dishes can have a diameter of 35 mm, 60 mm or a user-defined diameter. Multiple-slide plates are supported in a format with 8 slides per plate or may have a user-defined format. Multiwell plates are supported in formats with 6, 12, 24, 48, 60, 96, 384 and 1536 wells per plate.

To make it possible to display the recorded positions graphically, when you first use the module you will need to define the center of the carrier on the x/y stage. If you also want to work with your carriers and the recorded positions on other Carl Zeiss microscopes using AxioVision, you will need to work with absolute stage positions for each carrier (please also refer to "Microscope" in the online help (**F1** key)).

NOTE:
▪ Many control elements in Mark&Find control both the microscope stage and the focus drive. If used incorrectly, damage can therefore be caused to objectives and/or carriers lying in the stage's traveling range.

How do I generate a Mark&Find list?

The following two examples explain how to define a **Mark&Find** position list for a slide (example 1) and a multiwell plate (example 2). The same procedure should be followed for other types of carrier, e.g. Petri dishes.

Example 1: slide

In the following example, a new database is created and 3 positions on a standard slide are created and saved. The slide is centered to ensure that a correct graphic display of the positions is achieved. A reference point is also selected on the slide. In this example positions are only recorded in relation to the center of the slide.
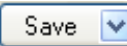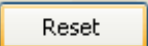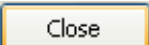
➢ Open the **Mark&Find** dialog using the **Microscope** ⇒ **Mark And Find** menu command:
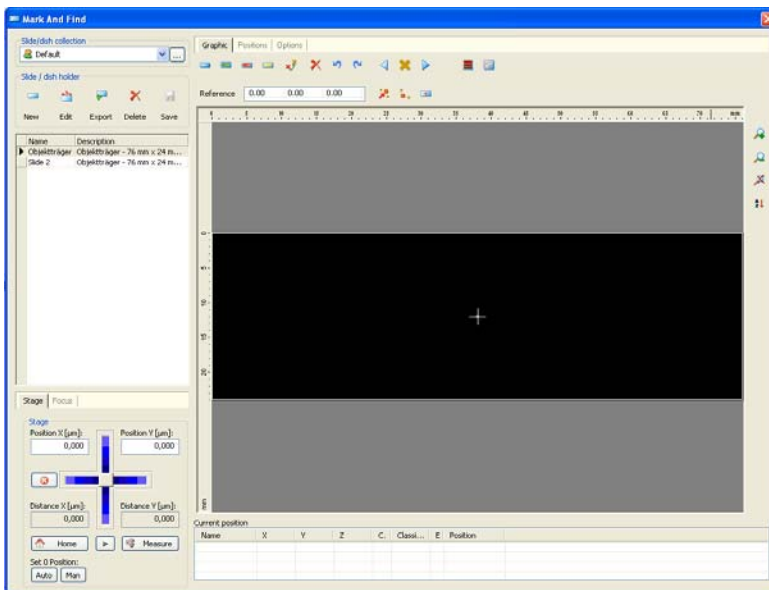
➢ Select a database from the drop-down list box of slide/dish collections or create a new database. To do this, click on [...] in the left-hand field. The **Manage carrier collections** dialog is opened:
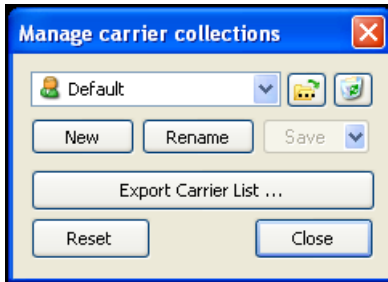
➢ If you click on [icon], a dialog is opened in which you can select a slide/dish collection file. [icon] deletes the selected slide/dish collection.

➢ [New] opens another dialog where you can give the database a name. [Rename] allows you to change the name of the database. [Save] saves your current database, whilst [Reset] deletes existing position lists and creates an empty database. In addition, [Export Carrier List ...] enables you to save the file in Excel format (.xls). Click on [Close] or [X] to exit the dialog.

➢ Now add a new slide to the database. To do this, click on the **Neu** icon in the **Objektträger** field:
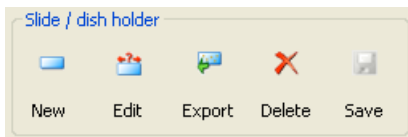
This opens the following dialog:

➢ Give the new carrier a name, e.g. "Slide 2".

➢ Select the standard slide from the **Typ** drop-down list box:
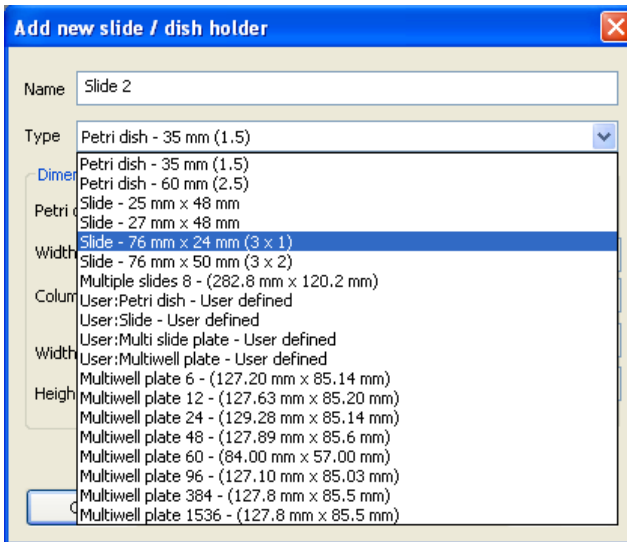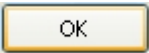
➢ It is also possible to import positions from a position list in a text document by clicking on ⌊ Import Positionlist... ⌋. A position list to be imported should have the following structure, for example:
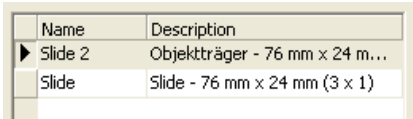
```
Slide,,,,
Name,Width,Height,,
Slide,76000.00,24000.00,,

Positions,,,,
Comments,PositionX,PositionY,PositionZ,Color
"Position(1)",-11058.36,-4043.66,1.00,Blue
"Position(2)",-2382.21,5485.68,-1.55,Red
"Position(3)",10049.20,-4067.70,-2.25,Green
"Position(4)",3674.27,-1514.12,3.00,Yellow
```

To ensure that Mark&Find is able to understand this document, it must be saved in the form of a CSV document (Comma Separated Values). This export option is available in Microsoft Excel, for example.

➢ Confirm your selection by clicking on OK .

You will now see the new carrier as an entry in the list of carriers in the **Mark&Find** dialog:

Name	Description
▶ Slide 2	Objektträger - 76 mm x 24 m...
Slide	Slide - 76 mm x 24 mm (3 x 1)

➢ Now insert the "home slide" (Carl Zeiss part number 453583) into the holder. The center of this slide is marked with a high degree of precision, and the slide has polished edges to ensure precise insertion into the holder.

➢ Make sure that the control elements for stage control are displayed in the foreground:
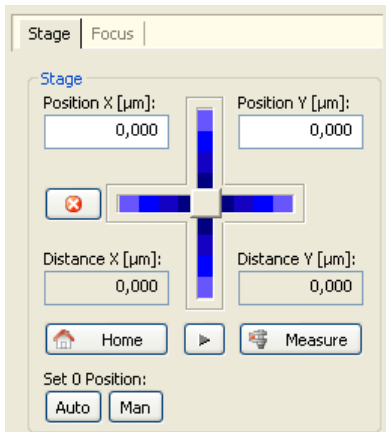
➢ Manually set the stage position to 0 by clicking on the Man button.

➢ Make sure that you are in the graphic view of **Mark&Find**:

Graphic | Positions | Options |

➢ Define the center by clicking on the ⊞ button. The graphic display of the carrier should now be centered as follows:
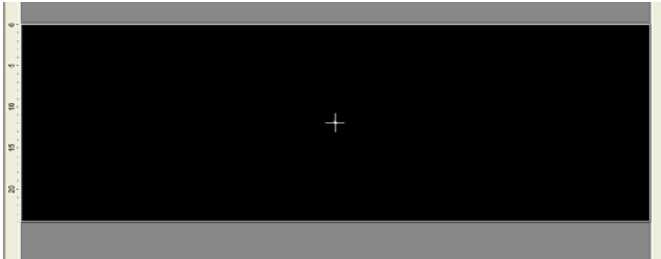
➢ The white cross indicates the current stage position (in relation to the optical axis of the microscope).

➢ Remove the home slide and insert your carrier into the holder. Define a reference point on your carrier. This should be a conspicuous structure on your carrier that you will be able to find again easily in the future. If you do not set a reference point, all coordinates refer to the zero position of the motorized x/y stage.

➢ Click on the ▫ button. In the graphic view, a small gray cross indicates the current reference position. The coordinates are also displayed (in µm for the x, y and z axes):

—
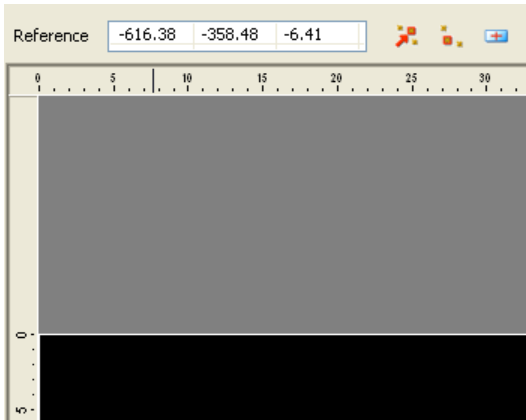
This reference position can be automatically relocated at any time in the future by clicking on .

Once these preparatory steps have been completed, you can locate and save any desired positions on your carrier.

➢ To do this, move the stage to the desired position, bring the sample into focus (this is best done in the live image) and click on one of the colored icons .

Repeat this procedure until you have defined all the positions of interest on your carrier. The selected positions are shown in the graphic as colored crosses. The white cross indicating the current stage position covers the current position, the name of which is also visible.
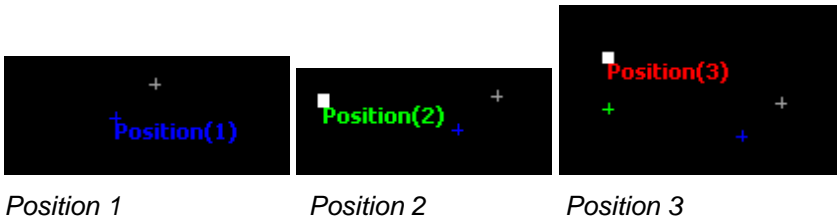
Position 1   *Position 2*   *Position 3*

➢ These positions can be relocated. To do this, double-click on one of the positions:
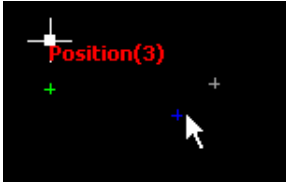
The large white cross indicating the current stage position shows the movement of the stage as it travels to the new position:

➢ Alternatively, if you click on , you can also have a number of positions generated automatically as a sample selection. Here you only need to define the number of positions in the x and y dimensions. Please note that any previously saved positions are lost.
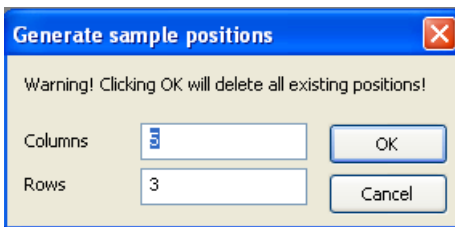
➢ Now go to the **Positions** property page:
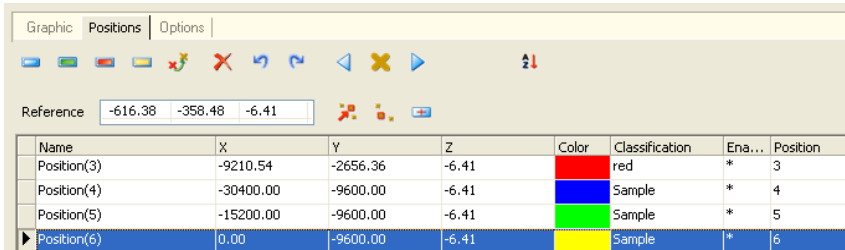
All defined positions are listed here in tabular form on the basis of the parameters **Name**, **X**, **Y**, **Z**, **Color**, **Classification**, **Enabled** and **Position**.

➢ **Name**: name of the position. If you click in the corresponding field, you have the option of changing this name.
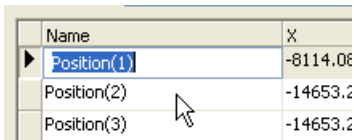

➢ **X**: the x-coordinate in μm. The value displayed can be changed manually. To do this, click in the number field and enter the desired value.

➢ **Y**: the y-coordinate in μm. The value displayed can be changed manually. To do this, click in the number field and enter the desired value.

➢ **Z**: the z-coordinate in μm. The value displayed can be changed manually. To do this, click in the number field and enter the desired value.

➢ **X**: the x-coordinate in mm. The value displayed can be changed manually. To do this, click in the number field and enter the desired value.

➢ **Color**: indicates the color used when the position was defined. The color can be changed with the help of the color selection dialog, which opens if you click in the field.

➢ **Classification**: a number of different positions can be combined in the form of a group by assigning the same names to them. Change the name of the classification by clicking in the field. Division into groups allows you to sort position lists on the basis of specially defined conditions and travel to the positions they contain.

➢ **Enabled**: indicates the activation status of a position. Only positions that are marked with a "*" symbol are used in the experiment. Click twice in the corresponding field to deselect the position via a check box, and to reselect it again as required.

➢ **Position**: indicates the sequence in which the positions were originally created.

It is possible to select several positions by making a multiple selection (**Shift**+mouse click) or by selecting them individually (**Strg**+mouse click). These positions can then be modified together.
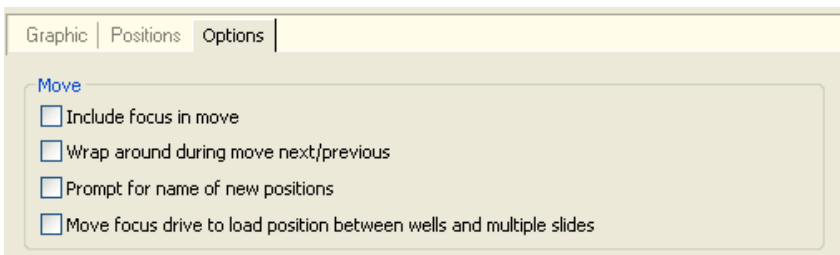
By double-clicking on the name of a parameter, you can sort the position list on the basis of that parameter.

➢ Once you have selected a position, you can delete it by clicking on .

➢ Clicking on undoes an action, whilst redoes an action that has previously been undone.

➢ The selected position is located automatically by clicking on .

➢ Clicking on ◁ moves the stage to the previous position in the list,

  whilst clicking on ▷ moves it to the next position.

➢ You also have the option of zooming into the graphic display by clicking

  on 🔍 or zooming out of it by clicking on 🔍. Hold down the **Shift**
  key and move the mouse wheel to make use of a stepless zoom

  function. Clicking on 🔍 restores the full view of the entire slide.

➢ Select ↕ to resort your position list. The criteria **X Position**, **Y
  Position**, **Z Position**, **Name**, **Index** and **Shortest distance** are
  available for selection from a drop-down menu.

  X Position
  Y Position
  Z Position
  Name
  Index
  Shortest distance
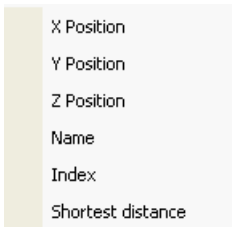
➢ Further selection options can be found on the **Options** property page:

  Graphic | Positions | Options
  Move
  ☐ Include focus in move
  ☐ Wrap around during move next/previous
  ☐ Prompt for name of new positions
  ☐ Move focus drive to load position between wells and multiple slides

➢ **Include focus in move**: if this check box is activated, the z-position (focus position) saved when the position was defined is also restored when you travel to a position.

➢ **Wrap around during move next/previous**: if this check box is activated, when "traveling forwards" you move from the last position to the first position in the list, and when "traveling backwards" from the first position to the last position.

➢ **Prompt for name of new positions**: if this check box is activated, a dialog for entering the position name is displayed automatically after each new position is created.

➢ **Move focus drive to load position between wells and multiple slides**: if this check box is activated, the objectives are moved to the load position while you are moving to another well or slide, in order to prevent damage.

➢ To make it easier to locate positions for the Mark&Find list, the option is available of having the carrier scanned fully automatically at a low magnification to obtain a preview. To do this, click on ▦ . Provided that the MosaiX module is licensed and selected in the module list, the following dialog appears:
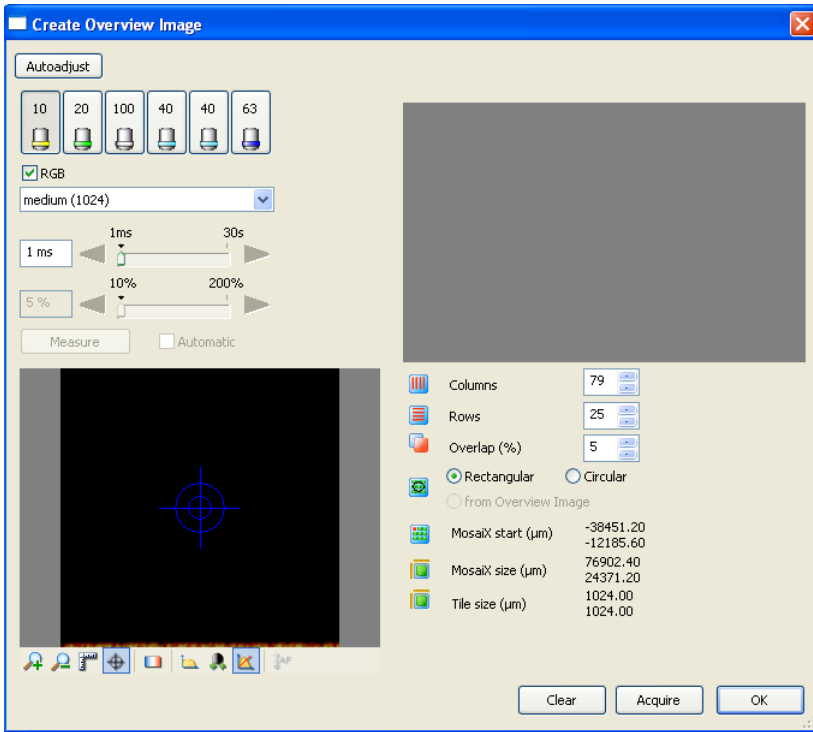
➢ You can now click on the [Acquire] button to generate an overview image containing the set number of columns and rows with a defined overlap. You have the option of setting the objective for acquisition, the exposure time and the size of the tile image in μm (300, 512, 1024 or 2500).

➢ Click on [OK] to transfer this overview to the graphic display in your **Mark&Find** dialog.

The overview dialog is otherwise operated in the same way as the **MosaiX** dialog (please also read the manual and the online help entries on **MosaiX**, **F1** key).

➢ Now save the changes. To do this, click on the Save button.

You have now successfully generated a position list and can use this at any time to relocate the saved positions. This list can also be used within the context of **Multidimensional Acquisition**.

Example 2: multiwell plate (96)

Position lists for multiwell plates are created in the same way as for slides, although there are a number of additional control elements and functions. These additional, divergent functions are presented below, without explaining all the functions that are identical to those in example 1 again. For details of the general functions, please also refer to the section "Example 1: slide".

To ensure that you can work sensibly with multiwell plates, the coordinates of the plate need to be adapted to the stage coordinates by means of calibration. The more wells your plate has, the more important it is to perform precise calibration.

NOTE:
- If the stage has already been calibrated and a standard plate has been selected, the program tries to locate the correct position after you call up the wizard. Please first ensure, therefore, that there are no obstacles within the stage's travel range.

➢ Select a low objective magnification (e.g. 10x) and transmitted light illumination. Place a multiwell plate of the type that you wish to use in the holder on the microscope stage.

> Click on the ![icon] icon. This opens the calibration wizard. If you have already performed calibrations, you are asked whether you wish to retain the calibration data. Select **No** if you wish to perform a new calibration and reject the old calibration data. Select **Yes** to continue working with the old calibration data.
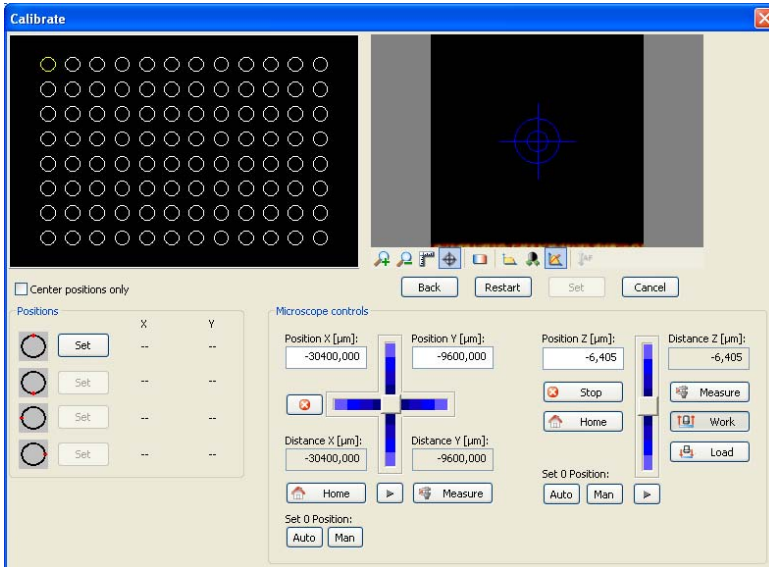
NOTE:

- The orientation of the camera and the stage axes must be correctly configured to allow error-free calibration. The movement of the camera's field of view must correlate with the movements of the slider (or joystick) for stage control in all four directions. The camera orientation, the camera port being used and the settings for the image orientation and axis orientation of the stage all influence this behavior.

➢ Use the stage control to move interactively to the positions on the multiwell plate that correspond to the well marked in yellow. The aim is to travel to the calibration points symbolized in the control area at the bottom left and to move them to the center of the live image (reticle). The first position to be located could look something like this in the live image:
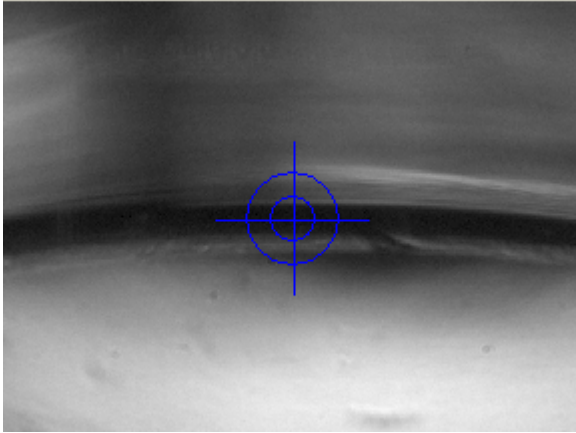
➢ By clicking on the [Setzen] button, which is currently active, you can now define this calibration point. It then becomes possible to calibrate the next position. In total, the following positions need to be located and set for a calibration:

- Top left well: 12, 6, 9 and 3 o' clock position
- Top right well: 12 and 9 o' clock position
- Bottom right well: 12 o'clock position

Wells that have already been calibrated are shown green in the graphic. Wells that have yet to be calibrated are marked in yellow.

➢ Click on [Back] to go back to the previous step of the calibration.
Click on [Restart] to return the calibration wizard to its original state.

Click on [Cancel] to exit the wizard without saving any changes you have made.

➢ The [Set] button under the live image only becomes active once all the necessary calibration steps have been performed. Click on it to save the calibration and exit the wizard.

➢ As an alternative to calibrating on the basis of the well edges, it is possible to calibrate using the well center only. To do this, activate the **Center positions only** check box. You then only need to define the center positions of the top left, top right and bottom right wells. You should use a low objective magnification for this that allows you to view the entire well in the live image.

➢ Go to the **Multiwell graphics** property page. Double-clicking on a well automatically moves the stage to the center of the selected well. The current stage position is marked with a red cross. The selected well (B3) is outlined in blue:
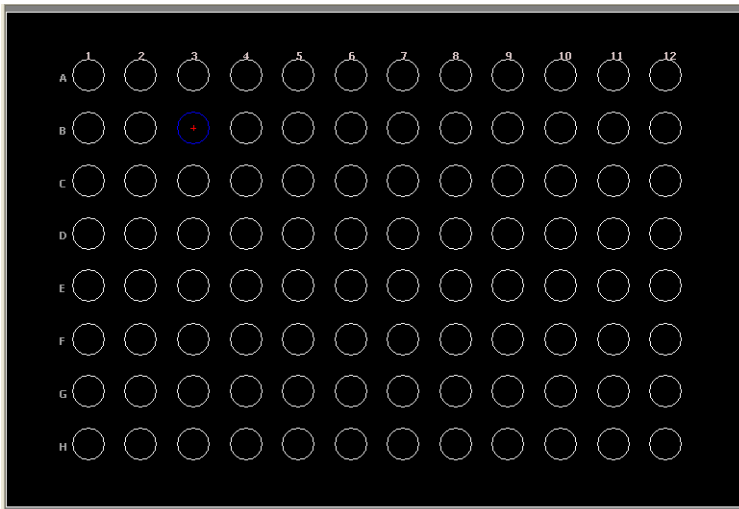
➢ By outlining several wells with the left-hand mouse button depressed or performing a multiple selection using the **Strg** key, combinations of different wells can also be selected (in the example B3, B4, B5 and row C):
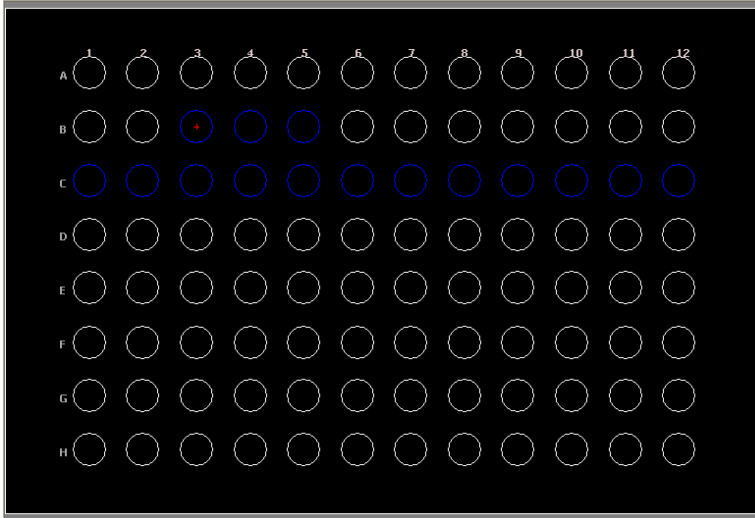
➢ To automatically generate positions in the selected wells and save

them in a Mark&Find position list, click on . A position at the center of the well is now generated for all selected wells, using the current focus position. Wells in which positions have been set are marked with hatching:
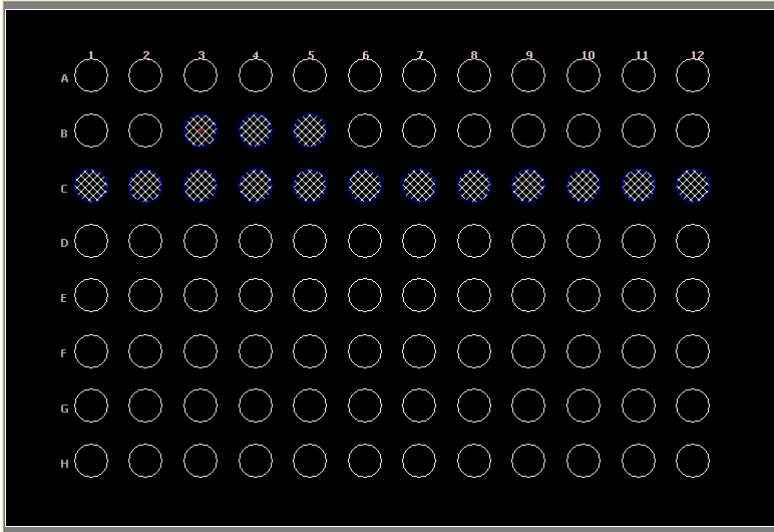
➢ Alternatively, by clicking on , it is also possible to have a number of positions generated automatically for each well. Here you only need to define the number of positions in the x and y dimensions. Please note that any previously saved positions are lost.
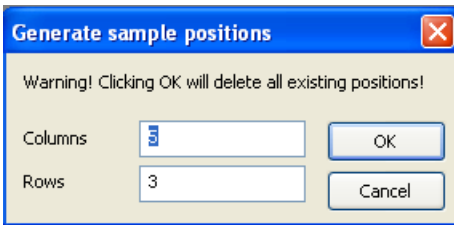
➢ You can, of course, also locate positions manually and add individual

positions by clicking on the icons.

➢ A special function that is available for multiwell plates allows you to overwrite the focus position of the selected position by clicking on [XYZ ZJ] . This function makes it possible to adapt a position list that has already been created to a multiwell plate with a different focus position (e.g. due to a different base thickness).

➢ In the case of multiwell plates, you can also define a rule on the **Options** property page that will be used to automatically name the positions in the list:

9.6   Panorama

General

The Panorama module creates large images from individual images, enabling you, for example, to display objects that would otherwise not fit into an image field. The images are combined with pixel precision, either interactively by superimposing the images, or (with support from the software) using **Stitching** (automatic image combination). It is also possible to combine displaced images so that they fit together perfectly.
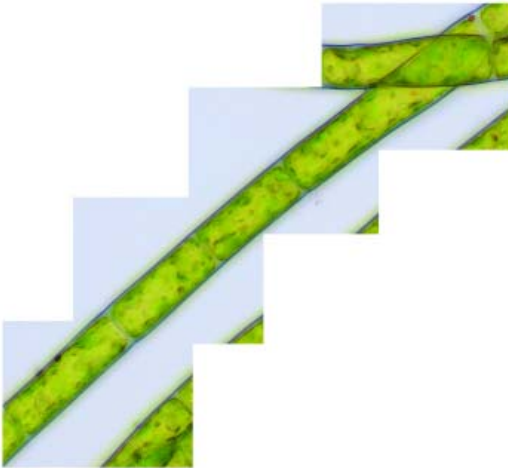
Panorama image consisting of four different images.

Panorama images can be created in two different ways:

- Combining images acquired directly via a camera.

- Using already saved images.

System requirements

There are no special technical requirements for generating Panorama images. The operation of the module has been specially designed for the use of manual microscopes and single image acquisition.

NOTE:
- The automatic acquisition of large composite images is possible using the MosaiX module and requires a scanning stage.

If you wish to combine images acquired directly from the camera, however, you will require a camera that has been integrated into AxioVision. It is not possible to use TWAIN devices, but you can acquire images using a camera integrated via the TWAIN interface, and then create Panorama images from the individual files.

It is not necessary to use a scanning stage, but this does make it easier to position the individual images. AxioVision will then adjust the resulting image size automatically, and suggest a position for the newly acquired image. The known stage coordinates are converted directly into the image position. A correct image can then usually be generated using **Stitching** (automatic image combination).

You should bear in mind that the resulting images may sometimes require a very large amount of storage space. In particular, if you are using high-resolution digital cameras, such as those in the AxioCam family, the Panorama images can easily take up as much as several hundred megabytes. The computer you are using should therefore have an appropriate user memory and hard disk capacity. You should also consider the possibility of external storage (network, CD/DVD writer etc.), as otherwise local hard-disk capacities may be quickly used up.

Special features of Panorama images

Generally, an image that has been acquired using the Panorama module is the same as any other – it is just larger, and made up of several component images. For Panorama images, a special view which allows you to work more efficiently with the individual images is therefore available via the image window: **Tile View**.

In this view you have access to the individual images (the "tiles") that make up a Panorama image. Here you can "pick up" the tiles individually, and change their position or alignment. You can also display information on the individual tiles, and insert circumscribing rectangles.
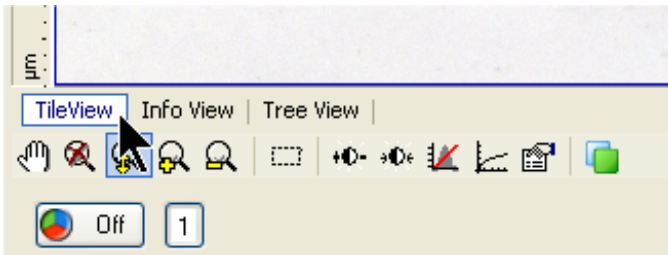
Pane to activate the tile view in the display window.

Acquisition of Panorama images

The **Panorama Acquisition** function can be executed via the work area on the left-hand side of the AxioVision window. If the work area is not

displayed, activate it via the **Standard** toolbar .

Alternatively, select the **Windows** function on the **View** menu and then the **Workarea** entry.
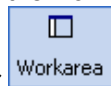
NOTE:

- The work area may in fact be open but covered by the workflows. If that is the case, simply click on the **Workarea** tab at the bottom edge of

  the work area [□ Workarea ▤↕ Workflow] .
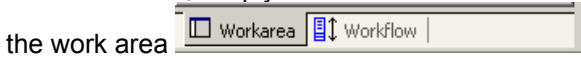
➢ Set the light path to the camera. Then open the live window by clicking on the icon for the live image on the **Standard** toolbar. Alternatively, select the **Live** function from the **Acquisition** menu.

➢ Select the scaling for the chosen objective from the **List of available scalings** underneath the live image.

➢ To adjust the live image, click on **Live Properties**. Alternatively, select the **Live Properties** function from the **View** menu.

NOTES:

- To guarantee a stitching result without annoying shading on the image border, you should activate the white reference. Go to the **General** tab in the **Live image properties** dialog. Make sure that the camera is viewing a completely empty image. To ensure that this is the case, move your sample on the microscope completely out of the field of view.

- To ensure that dirt particles from the slide are not visible in the correction image, use a clean slide and a clean objective and completely defocus the microscope.

- Please note that if the objective is changed the white reference has to be generated again.

A detailed description of the steps involved in preparing for image acquisition can be found in the section "Step by Step to the First Image" in section 3.3 "Quick Guide to the First Image".

➢ Close the Live display and select the **Acquisition** entry under **Panorama** in the work area.
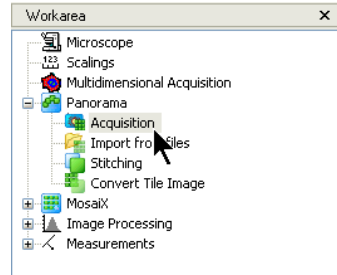
➢ The camera's live image is now displayed at the center of the **Panorama Acquisition** dialog. To ensure that you have sufficient space to position several images, we recommend that you reduce the display accordingly. To do this, use the buttons on the toolbar of the Panorama image.
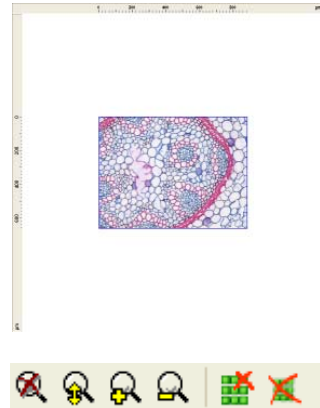
➢ Move the scanning stage to the starting point of the region you want to acquire, and focus. Then click on **Snap Image**.
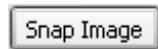
➢ The live image (marked by a white circle in the center) and the acquired image overlap partially. To acquire the next image, drag the live image (with the left mouse button depressed) to the position where you want the next image to be acquired. The images should clearly overlap.
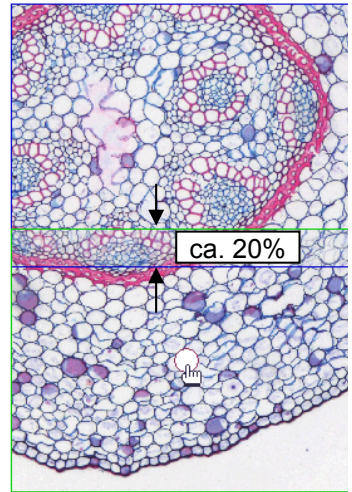
➢ Do not attempt to put adjacent images together with pixel precision. The function for automatic alignment (stitching) requires a overlap of good visible structures. The guide value is around 20%. And: There must be

➢ Now move the specimen to the new position. The areas where the live image overlaps with the images already acquired are displayed in semi-transparent form. This makes it easier to position the next image.
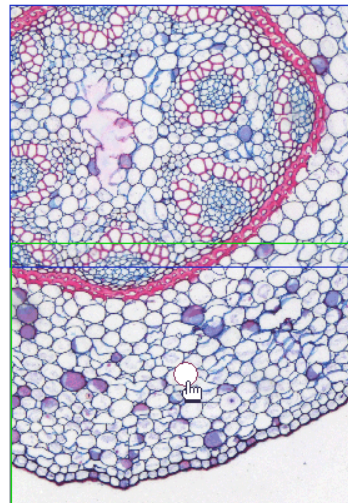
➢ The images do not have to be aligned precisely, as they can be placed in the correct position automatically later on using the stitching function. However, if the image regions that belong together are positioned too far away from each other, automatic stitching may not work.

➢ Repeat the previous steps until you have acquired the entire region.

➢ If the result is unsatisfactory, you can shift the individual tiles of the Panorama image manually.

➢ To do this, make sure that the editing mode is active. (The **Turn on/off tile mode** button at the bottom edge of the Panorama image has to be depressed.) Then click inside a tile. The tile is highlighted by a white circle at its center. Hold down the left mouse button, and move the tile to the desired position. As before, areas of overlap with other images are displayed in semi-transparent form.
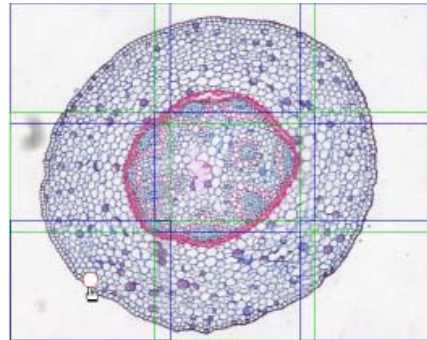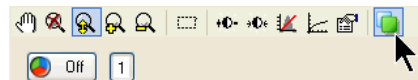
➢ To delete individual tiles, click on a tile. Then press the **Delete selected tile** button at the bottom edge of the Panorama image.

➢ Click on **OK** to adopt the completed Panorama image into AxioVision.

➢ To align the tiles in relation to each other, click on the button for automatic tile alignment on the toolbar directly under the image window.

➢ Alternatively, select the **Stitching** entry under **Panorama** in the work area.

NOTES:

- It is also possible to add extra images to an existing Panorama image.
  To do this, click on the ⬚ button in the top right-hand corner of the
  Panorama Acquisition dialog window. A gallery of all images currently
  loaded in AxioVision is displayed. Select the desired image, and carry
  out the steps described to add further single images to the existing
  Panorama image.

- If you click on the New button in the upper right corner of the
  Panorama Acquisition dialog, you can start generating a new
  Panorama image directly, without having to close the dialog first.

Creating Panorama images from individual files

If the individual tiles of a Panorama image are already available as
individual files, they can be put together manually. The most frequent
problem and source of errors here is the overlap of the individual tiles. If
the overlap is too large or too small, this can lead to problems when using
the function that automatically combines the tiles (stitching).

In principle, we therefore recommend that you create the images using the
Panorama acquisition function, and ensure that there is an appropriate
area of overlap. Please note, that the images must be of the same type
(color/gray values, size) to put them together.

The **Import from Files** function can be executed via the work area on the
left-hand side of the AxioVision window. If the work area is not displayed,
activate it via the **Standard** toolbar**.**

Alternatively, select the **Windows** function on the **View** menu and then the
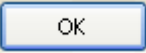**Workarea** entry.

NOTES:

- The work area may in fact be open but covered by the workflows. If
 that is the case, simply click on the **Workarea** tab at the bottom edge of

  the work area ⬚ Workarea ⬚ Workflow .
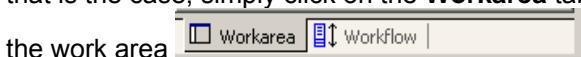
- All the steps involved in the generation of a Panorama image can be found in sequence in the **Panorama** workflow.

➢ Select the **Import from files** entry under **Panorama** in the workarea.
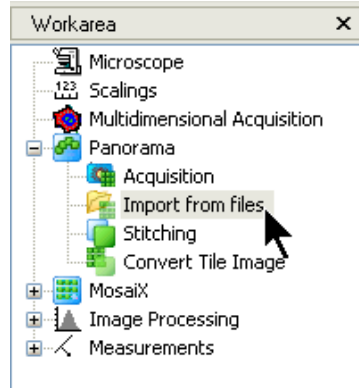
➢ Select the folder containing the individual tiles of your Panorama image. To do this, click on the [...] button above the gallery. The Microsoft Windows standard dialog **Select folder** is opened. Select the desired folder and confirm by clicking on OK. All the available images are now displayed as thumbnails in the gallery.

➢ To put the Panorama image together, drag the first image into the Panorama image area.

➢ The image fills the whole of the Panorama image area. To ensure that you have sufficient space to position additional images, we recommend that you reduce the display accordingly. To do this, use the buttons on the toolbar of the Panorama image.
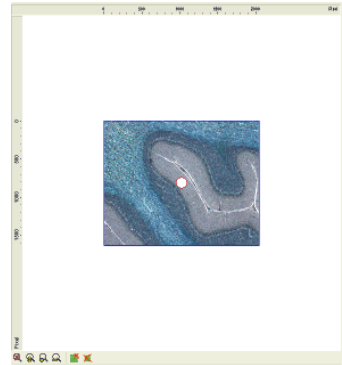
➢ The selected image is now marked with a red cross in the gallery, to show that it has already been used.
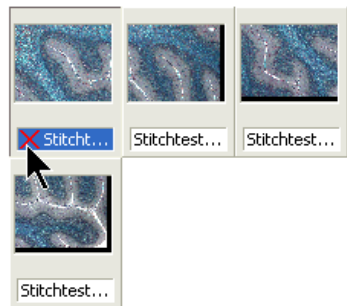
➢ Select the next image from the gallery and drag it to the desired position. The images should clearly overlap. The function for automatic alignment (stitching) requires a certain overlap. The guide value is around 20%.
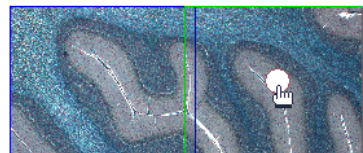
➢ Drag all the other images into the Panorama image one after the other in the same way, and position them.

➢ If the result is unsatisfactory, you can of course subsequently shift the individual tiles of the Panorama image manually.

➢ Click inside a tile. The tile is highlighted by a white circle at its center. Hold down the left mouse button, and move the tile to the desired position. As before, areas of overlap with other images are displayed in semi-transparent form.
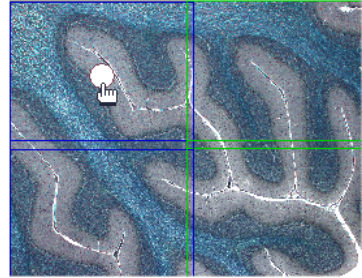
➢ To delete individual tiles, click on a tile. Then press the **Delete the selected tile** button at the bottom edge of the Panorama image.

➢ Click on **OK** to adopt the completed Panorama image into AxioVision.

➢ To align the tiles in relation to each other, click on the button for automatic tile alignment on the toolbar directly under the image window.

➢ Alternatively, select the **Stitching** entry under **Panorama** in the work area.

NOTES:

• It is also possible to add extra images to an existing Panorama image. To do this, click on the button in the top right-hand corner of the **Import from files** dialog window. A gallery of all images currently loaded in AxioVision is displayed. Select the desired image, and carry out the steps described to add it to the image.

• If you click on the **New** button, you can start generating a new Panorama image directly, without having to close the dialog first.
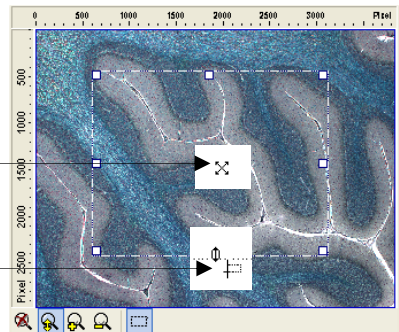
Converting a tile image into a regular AxioVision image

As tile images are a special format, they cannot be further processed or measured using the AxioVision functions. You have to convert them into a regular AxioVision image first.

➤ Acquire a tile image, or load one from the hard drive.

➤ Select the **Convert Tile Images** function from the **Processing** menu.

➤ The current tile image is displayed, with a frame, on the left-hand side.

➤ You are now able to position the frame freely and change its size.

➤ If the mouse cursor is inside the frame, keep the left mouse button pressed and you can move the whole frame.

➤ If you move the mouse cursor onto one of the frame handles, keep the left mouse button pressed and resize the frame.

➤ Once you have selected the desired region, click on **OK**. A regular AxioVision image is then generated. This image can now be processed and measured in the usual way.

NOTE:

- The size of the resulting image is reduced to 8192 pixels by default. If the frame size set exceeds this value, the resulting image is automatically reduced accordingly. The region you have selected is always used, however. If you want to avoid reduction, you can freely configure the size of the AxioVision image.

➢ Select the **Options** function from the **Tool** menu. Activate the **Image** tab sheet.

➢ Under **Multidimensional acquisition** you can adjust the **Max. MosaiX image size** parameter accordingly. The size of the value that you can select for this setting is essentially dependent on your computer's user memory.
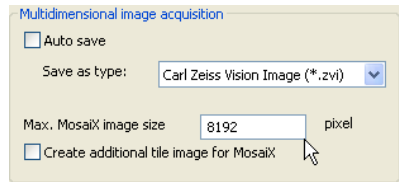
If the setting you select is too large, you may not be able to process the resulting images optimal. The processing speed will in any case be reduced dramatically, constricting optimal working.

9.7   MosaiX

General

The MosaiX module has been developed to resolve a classic microscopy problem: the insufficient size of the field of view. At high magnifications in particular, the region that can be seen through the eyepiece is never large enough. If you are working with cameras, the problem is frequently exacerbated by the fact that the camera's rectangular sensor cuts off regions of the round field of view when it is viewed through the eyepiece.

The ideal situation, therefore, would be to display the entire region of interest in a single image, even at high magnifications.

A number of different application scenarios are conceivable:

➢ **Acquisition of image overlapping objects**

In the simplest case, the object of interest cannot be displayed in full in a single image at the selected magnification. This means that several individual images have to be combined to form a larger overall image, and if the number of images in question is small, or if the system you are using does not have a scanning stage, you may be well advised to make use of the Panorama module too.
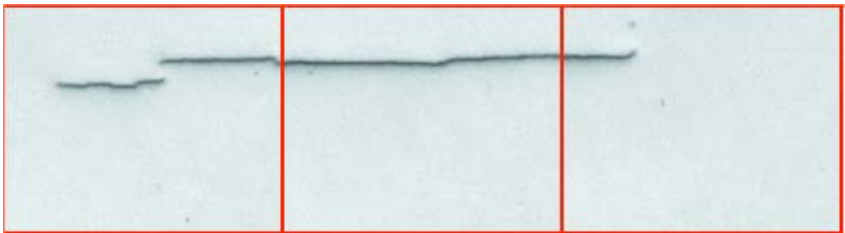
➢ **Archiving of a complete slide ("virtual slide")**

The archiving of specimen over al long period requires a high amount of space and its always associated with loss of quality (i.e. bleaching). The digital archiving of complete specimen is because storage media are becoming cheaper all the time is a realistic way to save them permanently with consistent quality.
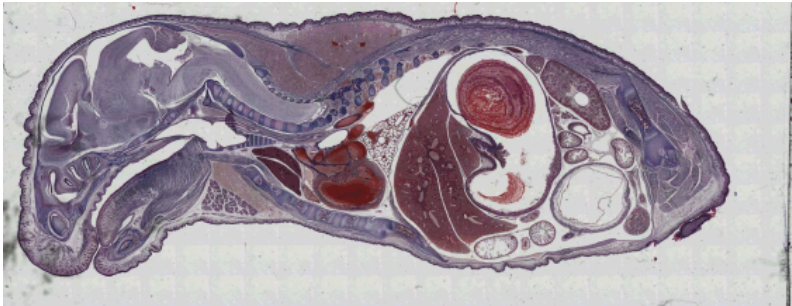
➢ **Correction of objects cut by the image border**

If you want to measure large areas, usually several single images have to be acquired. In this case always the questions comes up, how to handle objects that are not visible in full in the image. These objects should not be measured, as this would distort the measurement result. Using MosaiX images, you can avoid this problem completely.

With MosaiX, a specimen is scanned with a scanning stage extensively to generate a large "virtual" representation. This electronically generated image of the specimen serves as a map for navigating around the sample, and as a basis for further analyses. The advantage of this method is that you only have to scan the specimen once, and it is then available for all subsequent processing or analysis steps.
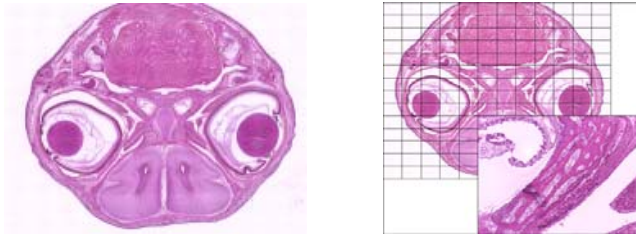
There are two ways to combine the individual images with the highest possible pixel precision:

➢   Ensure that the stage calibration and camera alignment are correct. The images are then "simply" put together. No check is performed to establish whether this has been done correctly. This is a fast method, but a certain amount of care is required when you are configuring the system.

➢   The second option automatically guarantees that all images are correctly positioned in relation to each other. This is what is known as **Stitching**. Here the image structures of adjoining images are used as a basis to check that the boundaries actually fit together correctly. If this is not the case, the images are automatically placed in the correct position in relation to each other.

As the focus plane does not always remain the same if samples are large, or if the scanning stage has to travel large distances, automatic focusing is often required during acquisition. Two options are available here: the **Autofocus** function and **Focus Correction**.

➢   **Autofocus**: here every nth selected tile image is focused in accordance with the calibration performed. The focus plane found is retained for the acquisition of the following tile images, until the next nth tile is reached. This is **Focus Correction**: With this method a "focus landscape" is defined. Several focused positions are used to calculate the three-dimensional surface curves of the object and the individual tiles are focused accordingly. At least three positions need to be set. The larger the desired MosaiX, the more positive the effect of setting more focus positions will be on the sharpness of all the tile images.

System requirements

A scanning stage is essential for generating MosaiX images. To make use of the options for automatic focusing, you will require a microscope with a motorized z-drive. The **Autofocus** function requires the AxioVision **Autofocus** module.

You should also bear in mind that the resulting images may sometimes require a very large amount of storage space. Images with several hundred MB, or even several GB, are not uncommon. This means that the computer you are using must have an appropriate user memory and hard disk capacity. You should also consider the possibility of external storage (network, CD/DVD writer etc.).

Special features of MosaiX images

Generally, an image that has been acquired using the MosaiX module is the same as any other – it is just larger, and made up of several component images. For MosaiX images, a special view which allows you to work more efficiently with the individual images is therefore available via the image window: **TileView**. In this view you have access to the individual images (the "tiles") that make up a MosaiX image. Here you can "pick up" the tiles individually, and change their position or alignment. You can also display information on the individual tiles, and insert circumscribing rectangles.
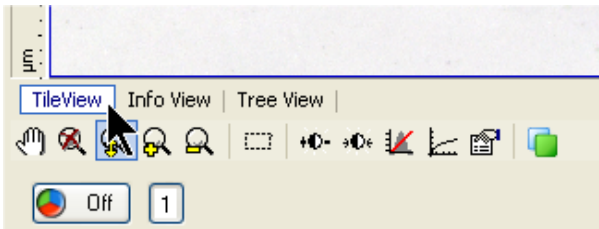
Button to activate the tile view in the display window.

Multidimensional MosaiX images

The MosaiX module can also be used together with the modules for multidimensional image acquisition. It can therefore be combined freely with the Multichannel Fluorescence, Z-Stack, Time Lapse and Mark&Find modules. If you are using MosaiX together with the Mark&Find module, you can define a region around any position in a Mark&Find position list for which you want to acquire a MosaiX image.
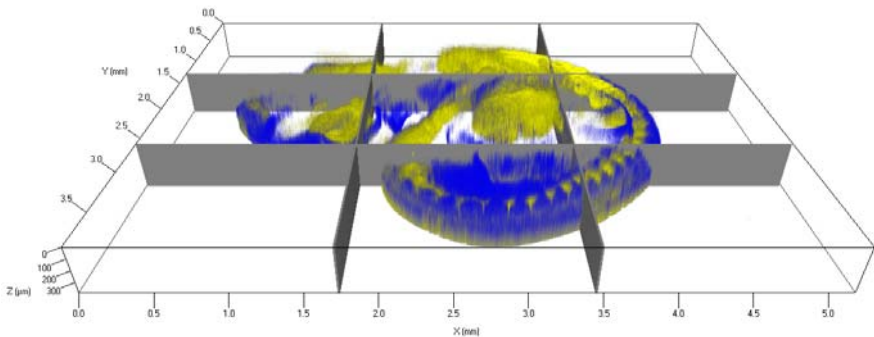
Muscle specific GFP tag (yellow), nuclear stain (blue), stage 10.5 embryo, 2 channel fluorescence Z-stack ApoTome acquisition of 3x3 Camerafields, stitched together with the new MosaiX module of AxioVision 4.2. Dr. Frederic Relaix and Emanuelle Perret, PFID, Pasteur Institute, Paris, France.

The Extended Focus module is only useful in combination with the MosaiX module if you are using Z-stack images. A microscope with a motorized Z-drive is required.

If you want the individual planes of a region of a specimen to be acquired manually for the extended focus, the Panorama module is also required, to put the individual images together.

Setting up the camera and scanning stage

If you want to acquire good quality MosaiX images, you need to ensure that certain requirements relating to the interaction of the camera and microscope/scanning stage are met. This is firstly to guarantee the correct movement of the stage, and secondly to prevent the individual tiles from being shifted or rotated in relation to each other during acquisition.

Image orientation

The image orientation in the eyepiece and the image on the monitor must be identically. This means that the image on the monitor must not be rotated in relation to the eyepiece display. If the alignment in the live image is wrong, it can be corrected either by rotating the camera or by adjusting the image alignment:

➢ Set the light path to the camera. Open the live window by clicking on the icon for the live image on the **Standard** toolbar. Alternatively, select the **Live** function from the **Acquisition** menu.

➢ To change the image alignment, click on the **Live Properties** button. Alternatively, select the **Live Properties** function from the **View** menu.

➢ In the **Live Properties** dialog, select the **General** tab.

➢ Select the appropriate **Image Orientation** from the drop-down list box, so that the live image and eyepiece image will correspond.

NOTE:

- A detailed description of the steps involved in preparing for image acquisition can be found in the section "Step by Step to the First Image" in section 3.3 "Quick Guide to the First Image".
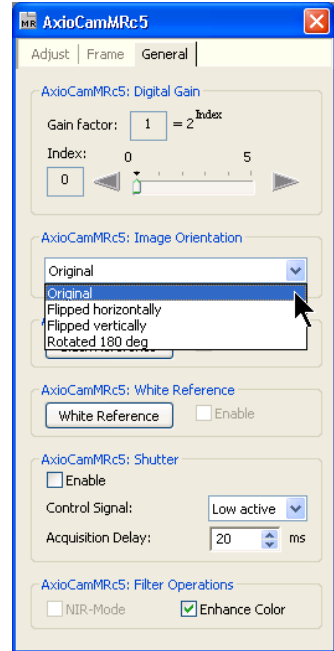
Directions of movement of the stage

To guarantee that the stage moves correctly, the direction of movement must be set in such a way that a movement of the camera is simulated when the control function in AxioVision is used to move the stage. This means that if the stage control function is moved to the right, the objects in the live image move to the left. If the stage control function is moved up, the objects move down. If the directions of movement are wrong, they can be adjusted via the **Microscope Configuration** program.

➢ Set the light path to the camera. Open the live window by clicking on the icon for the live image on the **Standard** toolbar. Alternatively, select the **Live** function from the **Acquisition** menu.

➢ Select the **Stage/Focus** entry from the **Microscope** menu, and open the stage control function via the **Stage** entry.
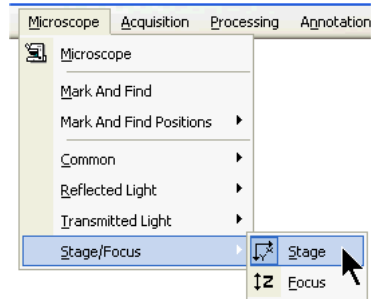
➢ Now check the directions of movement in the x- and y-directions.

➢ First move the stage to the **right**.

➢ The objects in the image should move to the **left**.

➢ If the objects also move to the right, you need to change the direction of movement via the **Microscope Configuration** program.
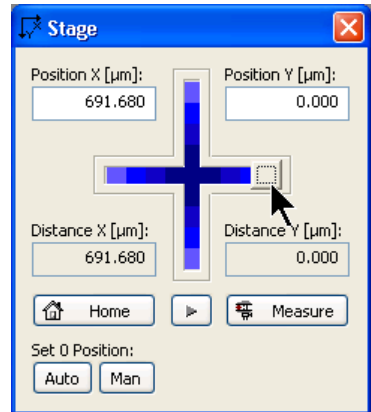
➢ Before you do this, however, test the movement in the y-direction.

➢ Move the stage **up**.

➢ The objects in the image should move **down**.

➢ If, instead of moving as described above, the objects move in the exact opposite direction, the directions of movement of the stage can be reversed.
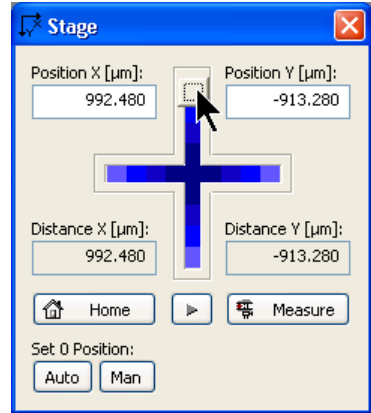
➢ Close AxioVision and start the **MTB 2004 Configuration** program. If the program is not available as an icon on the Microsoft Windows desktop, select **MTB 2004 Configuration** via **Start → Programs → Carl Zeiss Vision**.

➢ In **MTB 2004 Configuration** select the ⌐y^x Motorized Stage (internal Controller)¬ drop-down menu. You can change the direction of travel by clicking on the quadrants.
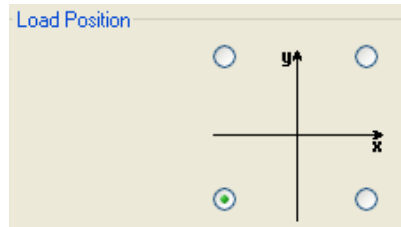
 ➢ If you are using the MAC 5000 as stage controller, the movement can be reversed by the check boxes of the parameter **InvertX** and **InvertY**.
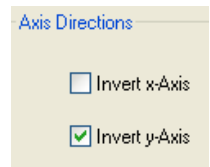
Click on [OK] to come out of the **MTB 2004 Configuration** program and save the settings.

Start AxioVision again, and follow the steps described above to check the direction of movement.

NOTE:
• If you are working with a joystick, you may find that the direction of movement cannot be changed when the joystick is being used. This means that the hardware and software joysticks have different directions of movement.

Alignment of camera and stage

The camera and stage must be aligned with each other in such a way that objects move parallel to the x- and y-axes when the stage is moved. Otherwise, the individual tiles of the MosaiX image would have to be rotated in relation to each other in order to combine them exactly.

⚠ The home slide (part number 453583) offers you optimum definition conditions. The center of this slide is marked with a high degree of precision, and the slide is covered and has polished edges. This ensures that it can be placed precisely into the slide holder. Alternatively, you can use a point that stands out clearly on a slide of your choice.

➢ Set the light path to the camera. Open the live window by clicking on the icon for the live image on the **Standard** toolbar. Alternatively, select the **Live** function from the **Acquisition** menu.

➢ Find a point that stands out clearly on your slide, or position the center of the home slide (as shown in the example) in the top left-hand corner of the live image.
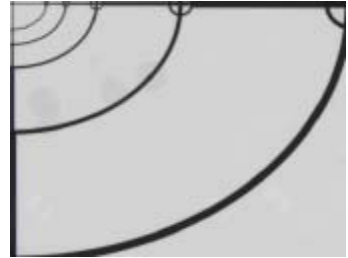
➢ Select the **Stage/Focus** entry from the **Microscope** menu, and open the stage control function via the **Stage** entry.
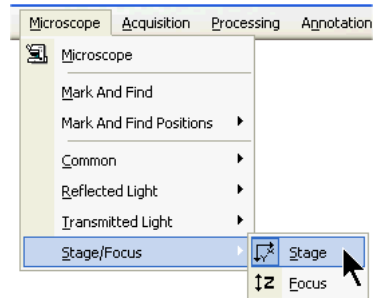
➢ Now move the stage to the **left**. The selected point moves to the right.

NOTE:

• If the point moves to the left, you need to change the direction of movement. Please refer to the previous section.
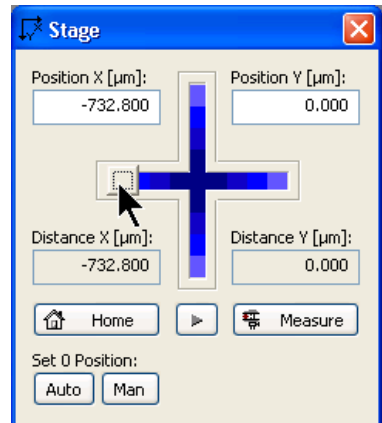
> ➢ The selected point should move parallel to the top edge of the image over the entire image width, up to the top right-hand corner. If it does, the edges of the stage and the camera are aligned parallel to each other.
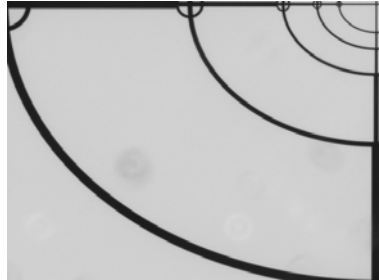
If it does not, you need to rotate the camera slightly, and then move the point across the image again. Keep doing this until parallel movement is achieved.

NOTE:
- If you are no longer able to combine your MosaiX images correctly, this check should be repeated. It is quite possible that the camera has been inadvertently rotated.

Acquisition of MosaiX images

> ➢ Set the light path to the camera. Open the live window by clicking on the icon for the live image on the **Standard** toolbar. Alternatively, select the **Live** function from the **Acquisition** menu.

> ➢ Select the scaling for the chosen objective from the **List of available scalings** underneath the live image.

> ➢ To adjust the live image, click on the **Live Properties** button. Alternatively, select the **Live Properties** function from the **View** menu.

NOTES:

- To guarantee a stitching result without annoying shading on the image border, you should activate the shading correction. Go to the **General** tab in the **Live image properties** dialog. Make sure that the camera is viewing a completely empty image. To ensure that this is the case, move your sample on the microscope completely out of the field of view.

  To ensure that dirt particles from the slide are not visible in the correction image, use a clean slide and a clean objective and completely defocus the microscope.

  Please note that if the objective is changed the shading correction has to be generated again.

- A detailed description of the steps involved in preparing for image acquisition can be found in the section "Step by Step to the First Image" in section 3.3 "Quick Guide to the First Image".

➢ Select the **Acquisition** entry under **MosaiX** in the work area.
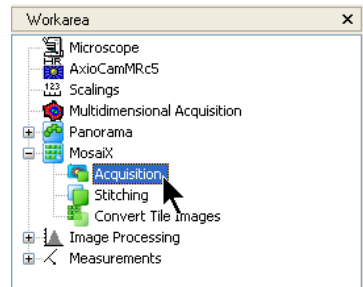
➢ The **MosaiX Acquisition** property page displays the current MosaiX settings.

➢ In order for automatic alignment (stitching) to be performed successfully, the images need to be acquired with an overlap. A value of 10-20% is recommended.

➢ The **MosaiX-Setup** dialog is available if you wish to adjust these settings. To open the dialog, click on Setup... .
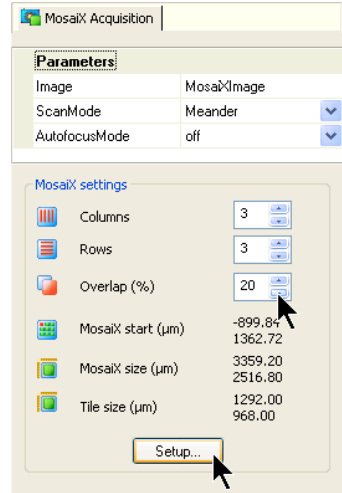
➢ The MosaiX **Acquisition Setup** dialog is the central dialog for defining the position and size of the MosaiX image to be acquired of your specimen.
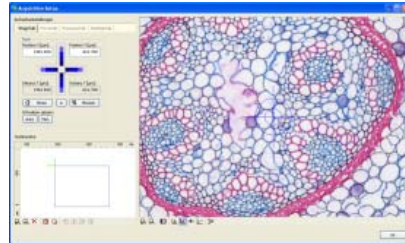
➢ To define a new MosaiX image, reset any existing settings. To do this, click on the button below the **Navigator**.

➢ The size is reset to one tile. The starting point is the current stage position. You can choose between the rectangle or circle mode or the overview image.
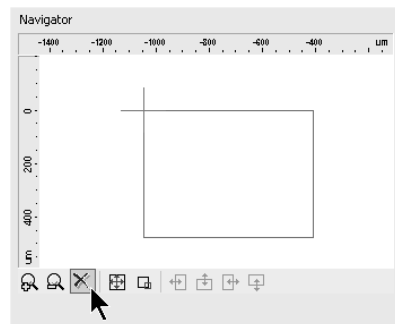
As a reference point you can choose between the tile in the top left-hand corner or the center of the entire MosaiX image.

➢ Now, starting from the current point, move to the furthest points of the MosaiX area using the control elements for the **Stage** in the **Microscope and camera** group.
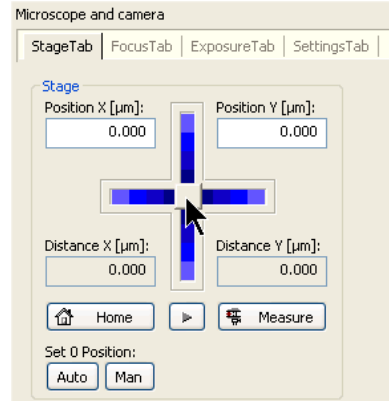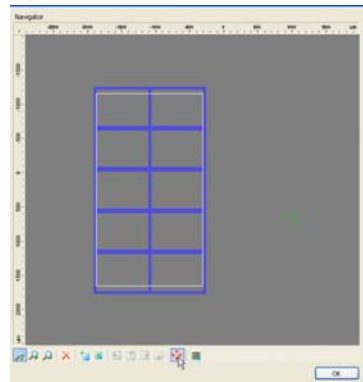

➢ First, move to the left edge of the area you wish to acquire, for example, or double-click on the corresponding position in the **Navigation** window.
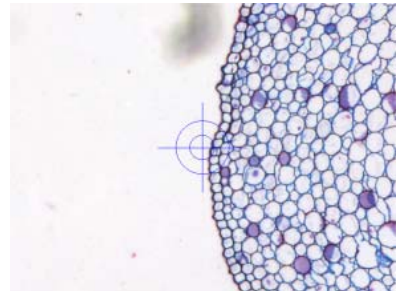
➢ Click on the button to enlarge the MosaiX image to the current position.
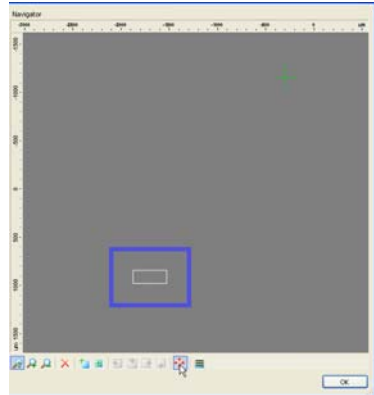
➢ The MosaiX area is now enlarged to the current position.

➢ Repeat these steps for the right, top and bottom edges of the area you wish to acquire.

NOTE:

• To check that the MosaiX area has been correctly defined, you can move to individual tiles directly by double-clicking on a tile in the navigator.
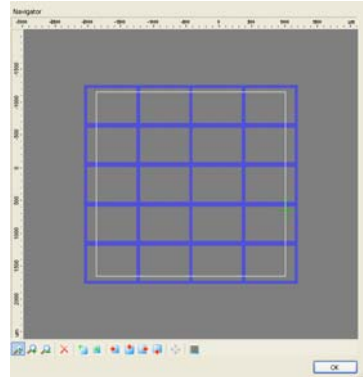

➢ If you wish to reduce the area for the MosaiX image, move to the new edge positions (top, bottom, right, left) and click on the appropriate button for clipping the MosaiX image.
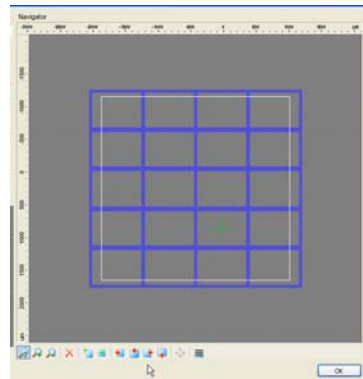
➢ Click on **OK** to adopt the settings.

➢ The settings are now adopted into the **MosaiX Acquisition** property page.

➢ Start acquisition by clicking on **Start**.
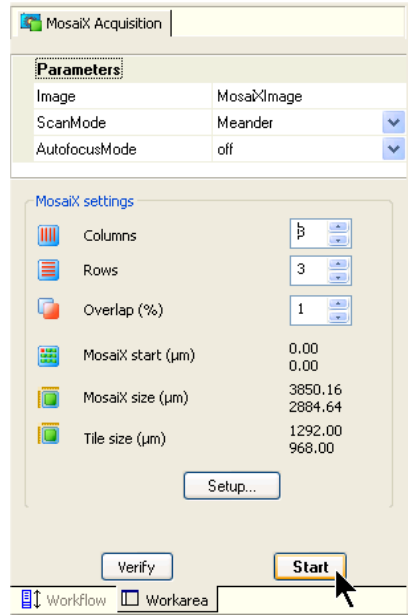
➢ AxioVision now covers the defined area and displays the result step by step.
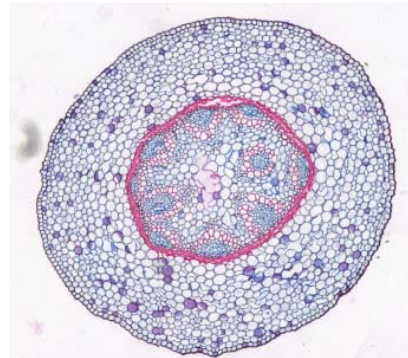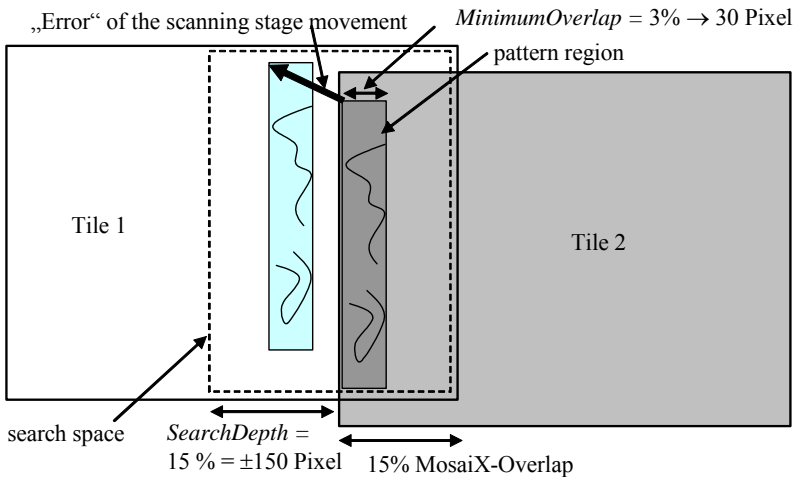
Stitching parameters

Scanning stages deviate from their exact positioning as they move. To ensure that it is nevertheless possible to assemble an overall image correctly when you are acquiring combined images (MosaiX), the single images (tiles) are acquired with overlapping regions and are subsequently fitted together using the so-called stitching method. It is essential that sufficient structures are displayed within these overlapping regions. The stitching algorithm attempts to recognize the precisely matching regions of two tile images that are located side by side and tries to position them accordingly.
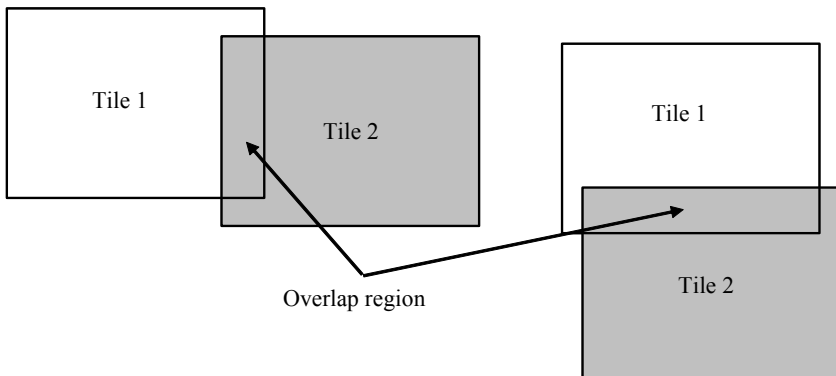
The idea of the stitching algorithm is based on pattern recognition. A structure is detected in the overlapping region of a tile image. An attempt is then made to find this structure again in the overlapping region of the neighboring tile.

The tile images in the diagram overlap by 15%. The **Overlap** parameter is set in the **MosaiX Acquisition** function.

NOTE:
- Tiles that do not overlap cannot be fitted together!

Three parameters are important for the stitching algorithm:
MinimumOverlap, **Search Depth** and **SobelSize**.

➢ The **MinimumOverlap** parameter indicates the size of the pattern region. The size is given as a percentage of the tile size. If, for example, a tile image contains 1000x800 pixels and the **MinimumOverlap** has been set to 3%, this results in a pattern region of 30 pixels.

  In the diagram, this region is shaded dark gray. An attempt is now made to find the structures contained here in the overlap region of the neighboring tile (light gray rectangle).

The stitching algorithm can only be applied successfully if visible structures are present in the search space. If only a few structures are present, you can increase the **MinimumOverlap** parameter. This means that the pattern region that you wish to look for is expanded and the possibility of finding a clear structure is increased.

The **MinimumOverlap** region selected should not be larger than the MosaiX-Overlap, however. This means that, when acquiring objects that contain few structures, you should select a 20-30% overlap for the **MosaiX Acquisition** function so that you can subsequently set the **MinimumOverlap** to a high value, such as 10%.

The lower the value set for the **MinimumOverlap** parameter, the quicker the stitching process takes place.

➢ The **SearchDepth** parameter indicates the percentage of the tile size in which the best match for the defined structure should be searched for in the 1st tile image. If you use an accurate scanning stage, the **Suchtiefe** can be kept low (e.g. 5%), as the pattern region can be found close to the original position.

For stages that are less accurate, you will need to work with a larger **SearchDepth** in order to have a chance of finding the structure. However, you should bear in mind that if too large a **SearchDepth** is selected, an incorrect stitching position may be found – particularly if the specimen displays similar or recurring structure patterns.

Remember that a lower value for the **SearchDepth** parameter speeds up the stitching process.
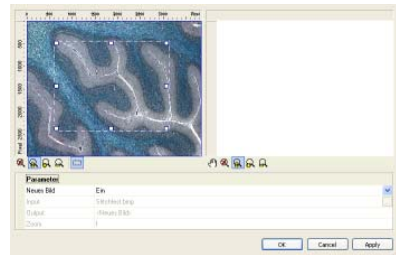
➢ The **SobelSize** parameter indicates the way in which the stitching algorithm deals with the structures in the defined pattern region for an optimum search result. In most cases, you can retain the preselected value **Low**.

However, if your tile images contain blurred structures which cause stitching problems, you can set this value to **Off**. In this case, it is essential that you perform shading correction during the acquisition of the tile images. Should the problems continue with the **Low** or **Off** values, you can use the **High** parameter value. This selection then takes larger image structures into account for pattern recognition.
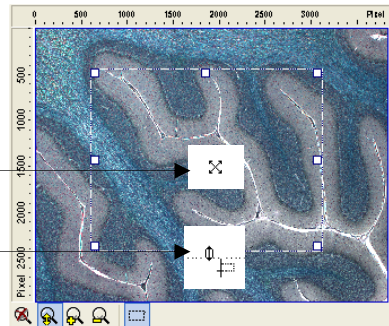
Converting a tile image into a regular AxioVision image

As tile images are a special format, they cannot be further processed or measured using the AxioVision functions. You have to convert them into a regular AxioVision image first.

➢ Acquire a tile image, or load one from the hard drive.

➢ Select the **Convert Tile Images** function from the **Processing** menu.

➢ The current tile image is displayed, with a frame, on the left-hand side.

➢ You are now able to position the frame freely and change its size.

➢ If the mouse cursor is inside the frame, keep the left mouse button pressed and you can move the whole frame.

➢ If you move the mouse cursor onto one of the frame handles, keep the left mouse button pressed and resize the frame.
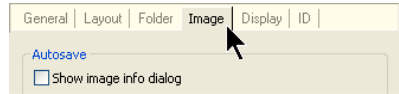
➢ Once you have selected the desired region, click on **OK**. A regular AxioVision image is then generated. This image can now be processed and measured in the usual way.

NOTE:
- The size of the resulting image is reduced to 8192 pixels by default. If the frame size set exceeds this value, the resulting image is automatically reduced accordingly. The region you have selected is always used, however. If you want to avoid reduction, you can freely configure the size of the AxioVision image.

➢ Select the **Options** function from the **Tool** menu. Activate the **Image** tab sheet.

➢ In the **Multidimensional acquisition** field you can adjust the **Max. MosaiX image size** parameter accordingly. The size of the value that you can select for this setting is essentially dependent on your computer's user memory.
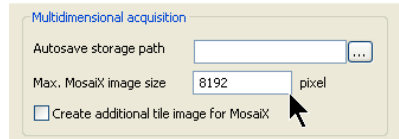
If the setting you select is too large, you may not be able to process the resulting images optimal. The processing speed will in any case be reduced dramatically, constricting optimal working.
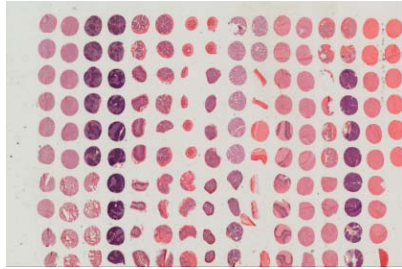
9.8 TMA

Background

Tissue microarrays consist of hundreds of small tissue samples of cores fixed on a single:
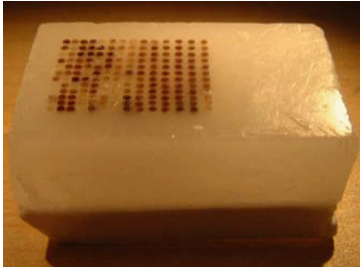
Biopsies embedded in paraffin

Composite image of a tissue microarray

This technique makes it possible to analyze a wide variety of tissue samples in parallel, under the same conditions and without variability in the staining. In connection with automated image acquisition and analysis, tissue microarrays are fed into an efficient high-throughput procedure.

The functions of the TMA module are integrated into AxioVision, expanding the software with the option of acquiring tissue microarrays. On the other hand, all the modules available in AxioVision can also be combined with TMA, e.g. with the quantitative analysis methods.

Application

The TMA module enables the acquisition of an overview "map" of the tissue microarray. Tissue cores are automatically detected and identified with a grid position and a coordinate list is created. Using the detected core positions, TMA acquires high resolution images of immuno-histochemically stained or fluorescence labeled core samples (e.g. FISH) and can acquire images with multiple separately labeled fluorochromes.

The AxioVision measurement functions provide all the tools for counting cells and quantifying staining intensity, percent positive marked elements and other parameters.

Functions and integration into AxioVision

TMA adds two functions to AxioVision – one for generating a composite image and one for deriving a position list from the composite image. The functions are available via the **Applications** ⇒ **TMA** menu. Consequently, they can be used in your own toolbars and workflows etc. via the **Extras** ⇒ **Customize** function.

The TMA workflow can also be used for routine work.

How you work with the TMA module is explained in detail on the training CD provided. Detailed descriptions relating to the individual functions can be found in the online help. Simply start a function and then press the F1 key.

Make Map

Captures a low magnification overview map of the array using a mosaic technique. This is a quick and easy to use function with re-usable templates for different tissue microarray layouts. **Make Map** can acquire an overview in brightfield or fluorescence illumination.

Detect Cores

Automatically detects the cores from the overview map image and assigns grid coordinates. A highly sensitive algorithm is used that can cope with distorted array patterns or missing cores. The grid coordinates of each core are used to create a Mark&Find list. Coordinates can be referenced to marker cores on the sample, allowing cores to be relocated after the slide has been replaced on the microscope. Additionally, the reference point is required for using the TMA position list correctly with other modules of AxioVision.

Prerequisites

In order to work with TMA, the Mark&Find and MosaiX modules must also be installed on your system in addition to TMA. Depending on what you wish to use the TMA module for, other modules may also be useful, e.g. Multichannel Fluorescence, Z-stack, Autofocus, AutoMeasure. Your microscope must be equipped with a scanning stage, to facilitate automatic location of the sample.

9.9   Physiology Acquisition

Introduction

Many intra- and intercellular processes are accompanied by rapid changes in the local concentration of various ions, such as calcium (Ca^{++}) or magnesium (Mg^{++}). For example, the penetration of an egg cell by a sperm cellssets in motion an intracellular signal cascade that results, within a few seconds, in the egg-cell membrane becoming impermeable for other sperm. The signal transmission between cells, such as neurons, is another example. In these cases the transmission of the signal is also accompanied by the release of calcium ions. It is therefore possible to visualize these processes by measuring the Ca++ concentration via imaging.

The AxioVision **Physiology** module supports all the common methods of calcium measurements as well as many other ion measurements such as pH.

Method 1: excitation at one wavelength, detection at another wavelength.
What is measured here is the increase in the intensity of a calcium-sensitive dye as a function of the Ca++ concentration. Examples of such dyes are Calcium-Green-1, Fluo-3, Fluo-4 or the BAPTA-based Oregon-Green dyes. The fact that only rapid sequential acquisition in one channel is necessary for this method means that it is ideal for measuring relative calcium changes, even with extremely rapid processes. However, it is difficult to perform quantitative measurements using this method. One method by which this can be attempted is to calculate the ratio of F/F0 by which a baseline timepoint is selected as F0.

Method 2: excitation in two wavelengths, detection of the changes in fluorescence intensity in one wavelength, generation of a ratiometric output image. **("Excitation ratioing")**
This method is based on the fact that certain calcium-sensitive dyes change their excitation spectrum as soon as calcium is bound. The most popular dye from this class is Fura-2. This is particularly well suited to imaging techniques, as it is easy to change the excitation wavelength with the help of fast wavelength changers (e.g. Sutter Lambda DG4).

In addition, the ratiometric measurement principle means that the quantification is concentration-independent. One disadvantage of Fura-2 is the need to perform excitation in the UV range. Measurement of pH values can be performed using the dye BCECF, which changes its excitation maximum upon binding to protons (H+ ions).

This method can also be used to perform calibrated calcium or pH measurements to determine absolute calcium concentrations or pH values. There are two methods for calibration: One involves creating one calibration valid for the whole image, the other method involves measuring minimum and maximum responses on a cell per cell base and using these values to deduce calcium values for each measured ROI. Physiology supports the second calibration method, also known as "in situ" calibration...

Method 3: Excitation in one wavelength, detection of the changes in fluorescence intensity in two wavelengths ("Emission ratioing")
This third method uses dyes with which the calcium binding produces a change in the emission spectrum. This is also a ratiometric method. This requires the simultaneous detection of the signal in two wavelengths, which requires the use of the two camera module **Dual Camera**. With this module two cameras, which have been appropriately configured using a dual camera adapter to each see a different wavelength, can be operated simultaneously producing a two channel image in the same instant. A popular dye used for emission ratioing is Indo-1. Also Calcium sensitive FRET sensors such as the yellow cameleon fluorescent proteins follow a similar acquisition principle.

For an overview of this issue, we recommend that you refer to Takahashi, A., et al., Measurement of Intracellular Calcium. Physiological Reviews 70, No. 4, pp1089 ff, 1999. Another good review appeared in Methods in Cell Biology, Vol. 72, pp 369-387 ("Ratio Imaging: Measuring Intracellular Ca++ and pH in Living Cells" by Randi B. Silver, 1998). For further information on the reagents and techniques used, see also http://www.invitrogen.com/probes.

Acquisition principles

The Physiology module enables you to acquire time lapse images at a speed that is only limited by the technical limitations of the devices you are using (camera, light source) and the selected camera settings. To achieve such speeds, the Physiology module automatically uses a "streaming" technique, which involves the raw camera data being written directly to their own fast **streaming hard disk** (or a suitable empty partition).

The results of the acquisition process are therefore a streaming document (*.ziAR).

At the same time, enough memory is reserved so that the measurement data can also be managed, which are necessary for online display of the charts. **Physiology** calculates the maximum possible number of individual images that can be acquired from the number of measurement regions drawn in and the memory available on the streaming hard disk. As this value is dependent on numerous factors (binning, ROI, number of measurement regions drawn in, demands made on available free memory by other applications etc.), the entire hard-disk space that is theoretically available cannot always be used.

In a second step, which is separate from the acquisition process, the streaming document is converted into a regular ZVI image document. This is required for all further processing steps in AxioVision.

The principle is illustrated in the following schematic (the actual harddisk configuration in your computer may be different):
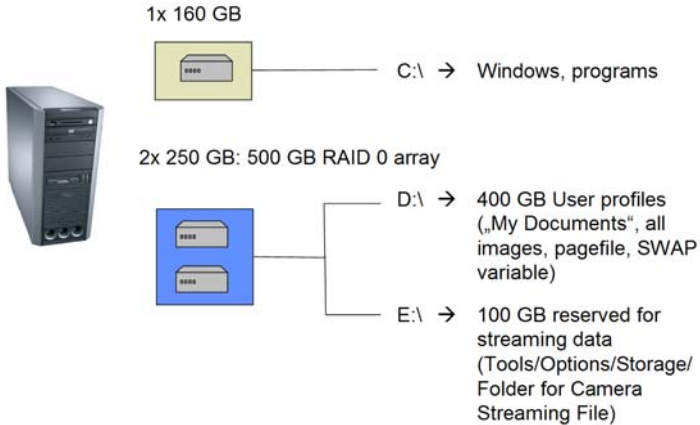
This acquisition technique depends to a great extent on ensuring that you have the correct PC equipment and the correct configuration. Please observe the minimum technical requirements, which can be found in the "AxioVision Product Suite" installation manual.

The procedure for calcium measurements with the Physiology module can be divided into four steps:

➢ Preparation of the experiment, setting of the camera and drawing in of measurement regions → **Setup**.

➢ Acquisition of the time lapse image, including measurement of the changes in concentration during the acquisition process itself, with options for interaction to control the experiment → **Image Acquisition**.

➢ Conversion of the acquired raw data into ZVI and downstream analysis → **Cutter**

> ➢ Measurement of the acquired data with options to select measurement
> methods, specify the type of background correction or even select
> custom calculation formulas → **Physiology Measurements**.

These steps are explained below following the individual control elements
as they appear in the software.

Acquisition

If you select this function on the **Acquisition** menu ⇒ **Physiology
Acquisition** ⇒ **Acquisition**, the **Physiology Acquisition** dialog opens.
This dialog can be resized to accommodate the text presented in the
Parameters field.

> ➢ **Parameters**: An overview of the most important acquisition parameters
> of the experiment currently active is displayed in list form: acquisition
> mode, number of ROIs (measurement regions), number of possible
> acquisition cycles, time interval currently set and maximum possible
> streaming file size. Please note that these values depend on the
> settings you entered in the **Setup** dialog.
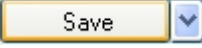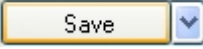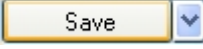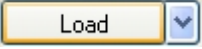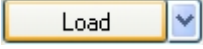
Setup... : Opens the **Physiology Setup** dialog.

Template

All settings relating to an image acquisition procedure with the Physiology
module are managed as an **Experiment**.

> ➢ **Name**: Name of the experiment. If the default experiment is loaded, the
> name field remains empty (*****).

➢ A newly created or modified Physiology experiment can be saved by clicking on [Save ▾]. Experiments are always saved with the extension "ziAR" in your personal document folder (e.g. for the user "Administrator": *C:\Documents and Settings\Administrator\My Documents\Carl Zeiss\Data\AcquisitionRecording\PhysiologyAcquisition*).

➢ You can also save an experiment under a new name using **Save As** (click on ▾ at the side of the [Save ▾] button).

➢ It is also possible to define a Physiology experiment as a default experiment, instead of constantly reloading experiments.

➢ To do this, the Physiology experiment must first be configured in the desired way. To make this experiment a default experiment, you then need to select the **Save As Default** option (click on ▾ at the side of the [Save ▾] button). This Physiology experiment will now be loaded as a default experiment every time you start AxioVision. Any changes that are made to this experiment while AxioVision is running will be rejected automatically when the program is closed. This ensures that the next user will always find the familiar default experiment.

➢ If, after making changes, you want to restore the default experiment while AxioVision is running, you can do this using the **Load Default** option (click on ▾ at the side of the [Load ▾] button).

➢ To load a saved experiment, click on [Load ▾].

➢ To reset the **Physiology Experiment** to the factory default state, the **Load Factory Default** option is available (click on ▾ at the side of the [Load ▾] button).

➢ **Prefix**: Here you can enter the basic name for all images acquired using the Physiology module.

Camera

From the drop-down list field you can select, which camera should be used for fast acquisition. This is important for systems which are set up with two identical cameras in a **Dual Camera** configuration. You can choose either the **ActiveAcquisition** camera, which is the camera currently active in the system or you can specifically select one camera of your choice (e.g. AxioCamHS_2) which will then be automatically selected for acquisition irrespective of which camera is the currently active camera. Finally, you can choose **DualCamera**. In this case, two identical cameras can be used simultaneously. Please see the help for **Dual Camera** elsewhere for further details.

Hardware-Settings for Experiment

In this field you can enter hardware settings that prepare the hardware for fast **Physiology Acquisition**, e.g. switching the light path to the camera or preparing the light sources for fast acquisition.

➢ **Before experiment …**: Here you can select a suitable hardware setting from the drop-down list box. If you click on the ⌈Go⌉ button, you can apply the selected hardware setting to the microscope to test the settings.

➢ **After experiment …**: You can use an additional hardware setting for the end of the experiment. If you click on the ⌈Go⌉ button, you can apply the selected hardware setting to the microscope to test the settings.

Physiology Setup

Here you can enter settings for the experiment on the **Regions**, **C (Channel)** and **Markers** property pages.

As soon as more than one channel has been configured, channel 1 is ratiometrically processed with channel 2 (**method 2 or 3**). If only one channel has been configured, the method **Ratio Single Channel** should be used and the analysis performed with the help of an F0 image. It is also possible to configure more than 2 channels (e.g. for a brightfield image). However, it is always only channels 1 and 2 that are analyzed. For method 3 (emission ratioing) Dual Camera has to be selected. Here camera #1 is always assigned to channel #1, camera #2 to channel #2.

The **Physiology Setup** dialog is opened by clicking on   Setup... .
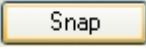
Regions property page

On this property page you can prepare the **Physiology Experiment** by defining the method for calculation, acquiring test images, drawing in regions, setting the camera exposure time and specifying how the ratio image should be calculated.

Setup Measurement Regions of Interest (ROI)

This page is divided into 6 areas, containing an image display and the corresponding chart display. First of all, you should acquire a test image. In order to do this, the channels must have been correctly set (on the **C (Channel)** property page of the **Physiology Setup** dialog). The explanations given here relate to method 2 with the acquisition of 2 channels. For other type of experiments (e.g. for the FRET calculations) additional control elements will appear.

➢ To perform acquisition, click on the [Snap] button. Three images are acquired for each channel using the currently active camera settings and a mean-gray-value image is calculated to suppress background noise. You will now see channel 1, channel 2 and the calculated ratio image, including a rainbow color table ("Rainbow LUT").

➢ At the lower edge of the dialog you will find a toolbar. A description of the first 11 tools can be found under **Display Panel**.

➢ Using the other tools it is possible to draw in up to 100 regions, which will be used during the acquisition procedure itself for the online analysis. When you draw in several regions, the next color from the color list is automatically assigned to each new region.

: Allows you to click on and select measurement regions that have already been drawn in to move them, enlarge them, delete them or change their color.

: Used to draw in a rectangular measurement region. If the button is not selected, the tool remains active and it is possible to draw in several measurement regions one after the other.

: Used to draw in a measurement region with a free form of your choice (spline). If the button is not selected, the tool remains active and it is possible to draw in several measurement regions one after the other.

: Used to draw in an ellipse as a measurement region. If the button is not selected, the tool remains active and it is possible to draw in several measurement regions one after the other. If the Shift key is depressed while drawing in the ellipse, a circular form is enforced.

Draws a circle. Starting point and end point of the drawing defines the diameter making it easy to e.g. define a circle encompassing a complete

cell. If the button is not selected, the tool remains active and it is possible to draw in several measurement regions one after the other.

When clicked, the last shape used for drawing a ROI is "remembered" and drawn into the overlay by simple clicking. This allows the cloning of a shape with ease. It works with all ROI tools except the free form spline tool.

This function can be disabled by selecting .

: Deletes all selected measurement regions.

Select All : Selects all available measurement regions.

: Allows you to change the color for a measurement region.
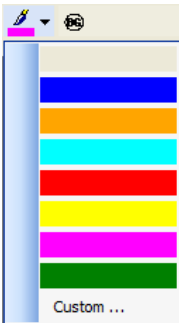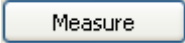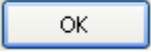
: Via **Custom...** it is possible to add further colors to the list from a color selection dialog.

: Defines a selected measurement region as a background region. The mean gray value from this region can, if desired, be automatically subtracted from the measurement values from other regions.

Settings Channel 1

Here it is possible to enter the desired exposure time for channel 1.

➢ **Time**: enter the exposure time to be used for the channel into the value field, either directly or using the arrow buttons ![arrows].

➢ **Measure**: To determine the exposure time interactively, click on the ![Measure] button. The dialog that opens contains a live image, generated using the channel-specific hardware settings. The exposure time is automatically measured when the dialog is opened. After you click on ![OK], the dialog is closed and the new exposure time is adopted automatically.

Note:
- Please note that, in order to achieve high acquisition speeds, the exposure time should not be set too high. Maximum exposure time allowed in Physiology experiments is 800 msec. If resulting images are displayed too dark, adjust the display settings accordingly. Also, consider using camera binning and define a region of interest for the camera to further increase the acquisition speed.

Settings Channel 2

Here it is possible to enter the desired exposure time for channel 2.

➢ **Time**: Here enter the exposure time being used for the channel into the value field, either directly or using the ![arrows] buttons.

➢ **Measure**: To determine the exposure time interactively, click on the
| Measure | button. The dialog that opens contains a live image,
generated using the channel-specific hardware settings. The exposure
time is automatically measured when the dialog is opened. After you
click on | OK |, the dialog is closed and the new exposure time is
adopted automatically.

Note:

- Settings for channel 2 are only available if more than one channel is
  set up. Also, for single channel ratioing (method 1), the image area for
  channel 2 is used to display the so-called F0 reference image instead.
  An exposure time for channel 2 can be defined nevertheless.

Ratio Image Definition

➢ **Method**: From the drop down list box you can select which calculation
method you want to use for your experiment. 6 Methods are available
for analysis during acquisition ("online"):

- **Ratio Dual Channel**: Classical dual channel ratio imaging using
  either method 2 (excitation ratioing, e.g. Fura-2) or 3 (emission ratio
  imaging, e.g. Indo-1). Ratio is always calculated dividing channel #1
  by channel #2. Only available with setups containing two or more
  channels.

- **Ratio Single Channel**: Single Channel ratio imaging such as Fluo-
  4; can be done with single or multiple channel setups, online
  processing always done on channel #1.

- **Fret Youvan**: Sensitized emission FRET measurements using three
  images. Channel #1 = Donor, Channel #2 = acceptor, channel #3 =
  FRET. Only available with setups containing three or more
  channels.

- **Fret Gordon**: Derivative of FRET Youvan. Only available with
  setups containing three or more channels.

- **Fret Xia**: Derivative of FRET Youvan. Only available with setups containing three or more channels.

- **Fret Siegel BL**: Acceptor bleaching method, requires two channel setup (channel #1 = donor, channel #2 = bleached acceptor). Measurement done on channel #1 only.

Note:
- All the following explanations are based upon the Ratio Dual Channel method. A detailed explanation of the available FRET methods please see Physiology Measurements.

➢ To calculate the ratio image, the gray values from channel 1 are divided by the gray values from channel 2 pixel by pixel. As the resulting ratio values are usually very small, the results are multiplied by an arbitrary factor. This makes it possible to display the ratio values as an image. To enable you to distinguish ratio differences more easily, these values are displayed in color using a rainbow colored look up table.

The ratio image is calculated as outlined in the formula shown here:
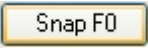
$$r(x,y) = \text{Multiplier} * \frac{ch1(x,y)}{ch2(x,y)} + \text{Offset}$$

- r (x,y) = ratio image
- Multiplier = factor by which the result of the division is multiplied
- channel 1 (x,y): image from channel 1
- channel 2 (x,y): image from channel 2
- Offset = factor that influences the general brightness of the ratio image.

➢ In order to make it easier to match ratio image display and ratio charts the display can be influenced directly by changing lower (**Minimum**) and upper (**Maximum**) boundaries of the actual Ratio values. The corresponding multiplier and offset factors are updated in the displayed formula.

➢ The lower and upper limit of the displayed ratio can be entered using the sliders for **Minimum** or **Maximum** or by entering the values directly in the edit fields:
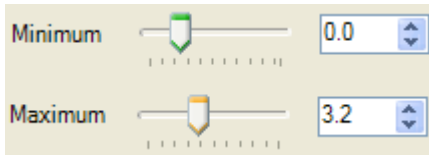
➢ By raising the lower limit one can reduce the background of small ratios, by raising the upper limit, also larger ratios can be displayed properly. A minimum of 0 and a maximum of 3 cover the range of ratios commonly occurring in biology.

➢ In the case of a single channel (**method 1**), a reference image is used as a denominator for the calculation. This so-called F0 image can be acquired using the [Snap F0] button.

C (Channel) property page

The basic functions available on this property page are identical to those on the **C (Channels)** property page for **Fast Acquisition**.

In **Physiology**, up to 5 channels can be configured for acquisition. However, only the first (for single channel experiments, method 1) or the first and second channel (methods 2&3) will be taken into account for the online analysis. The other channels will nevertheless be acquired and used during the conversion into a ZVI document and will therefore be available for further processing.

Markers property page
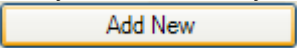
Marker

One of the most important functions for calcium measurements is the ability to change the conditions of the experiment during acquisition and to log changes in the experiment. Physiology makes two types of markers available for this purpose.

➢ **Speed Marker**: These markers can be used to change the interval between the individual time points for image acquisition at the touch of a button.

➢ **Event Marker**: These markers enable you to enter previously defined events, such as a change of media, into the current experiment at the touch of a button. It is also possible to apply hardware settings using **Event Markers**.

Both types of marker are incorporated into the log when they are used. They subsequently enable you to navigate more easily within the acquired data and can also be inserted into the image as annotations.

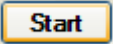### Speed Marker

➢ Users can define as many markers as they wish. To create a new marker, click on the [Add New] button and enter the desired settings.

➢ **Marker Settings** control area: Here you can give the **Speed Marker** a **Name**, enter a **Tool tip text** that will appear as a yellow text box if the mouse is moved over the button, allocate a **Color** to the marker from the drop-down list box and define a **Shortcut** that enables you to operate the marker rapidly without having to click with the mouse.

➢ **Interval settings** control area: You can choose between 6 options:

- **As fast as possible:** Acquisition will happen as fast as the camera allows

- **Interval:** The desired time interval can either be specified directly in the **Interval** input field or via the arrow buttons . The unit can be selected from a drop-down list box (hours, minutes, seconds, milliseconds).

- **Start for**: Instead of starting the experiment for an undefined period of time this marker setting allows you to specify a time duration for the whole experiment. You have to start the experiment with this marker for the timing constraint to take effect and not the regular **Start (F5)** button.

- **Pause**: Pauses the experiment

- **Continue**: Continues a paused experiment

- **Stop**: Stops the experiment

➢ **Trigger Mode** control area: Here you can set up a marker to activate itself automatically upon an incoming trigger. Triggers can be either a predefined interval timer or an electrical TTL signal coming in through the Digital I/O Device.

- **Off:** Turns off automatic triggering

- **Interval:** The desired time interval can either be specified directly in the **Interval** input field or via the arrow buttons . The unit can be selected from a drop-down list box (hours, minutes, seconds, milliseconds). The marker will be set repeatedly during the experiment after the specified interval time.
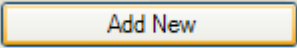
- **TTL Input**: Two TTL input lines are available through the Digital I/O Device (TTL In 7 or TTL In 8). Both can be configured to react upon a change from low to high voltage ("High") or from high to low ("Low") depending on the type of external TTL source.

➢ To delete a marker, click on the drop-down list box next to each button

and select the **Delete** option .

Event Marker

➢ Here you can define as many event markers as you wish. To create a new marker, click on the Add New button and enter the desired settings.

➢ **Marker Settings** control area: Here you can give the **Event Marker** a **Name**, enter a **Tool tip text** that will appear as a yellow text box if the mouse is moved over the button, allocate a **Color** to the marker from the drop-down list box and define a **Shortcut** that enables you to operate the marker rapidly without having to click with the mouse.

➢ **Hardware Settings** control area: Here it is possible to select an existing hardware setting that you want to be executed if the marker is selected during acquisition. Please note that you should only make changes to devices that it makes sense to change during fast image acquisition. You should therefore not make any adjustments to, in particular, the camera resolution or the light source, to prevent the sequence of the experiment being disrupted. Please check the **Enabled** checkbox in order to activate the execution of the hardware setting.

➢ **Trigger Mode** control area: Here you can set up a marker to activate itself automatically upon an incoing trigger. Triggers can be either a predefined interval timer or an electrical TTL signal coming in through the Digital I/O Device.

- **Off:** Turns off automatic triggering

- **Interval:** The desired time interval can either be specified directly in the **Interval** input field or via the arrow buttons ![arrows]. The unit can be selected from a drop-down list box (hours, minutes, seconds, milliseconds).

- **TTL Input**: Two TTL input lines are available through the Digital I/O Device (TTL In 7 or TTL In 8). Both can be configured to react upon a change from low to high voltage ("High") or from high to low ("Low") depending on the type of external TTL source.

➢ To delete a marker, click on the drop-down list box next to each button and select the **Delete** option ![1 fps dropdown with Delete option].

Physiology Acquisition

The dialog for image acquisition is opened using the ⬚ **Start** button. This dialog is divided into four areas:

- Area for setting markers.

- Area containing buttons for starting and stopping acquisition as well as the most important status information.

- Area containing charts and the acquired images.

- Overview chart for the entire experiment.

Speed Marker

➢ From here it is possible to set a speed marker, either by clicking with your mouse, by using a keyboard shortcut (if allocated) or via trigger signal. The acquisition speed then changes accordingly. Each marker set is entered in the marker list. It is also possible to use special speed marker settings and start the experiment for only a predefined period of time, pause, continue or stop the experiment. See **Speed marker** for more details.
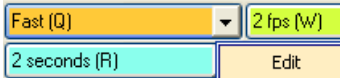
➢ It is also possible to create new markers or modify existing ones during acquisition. To do this, click on the **Edit** option in the drop-down list box for the marker. In the dialog that opens you can then make the desired changes.

Event Marker

➢ From here it is possible to set an event marker, either by clicking with your mouse or using a keyboard shortcut (if allocated). The corresponding comment is then entered in the marker list. If an event marker is linked to a hardware setting, the corresponding hardware setting is executed. Event markers can also be entered upon trigger signal.

➢ It is also possible to create new markers or modify existing ones during acquisition. To do this, click on the **Edit** option in the drop-down list box for the marker.
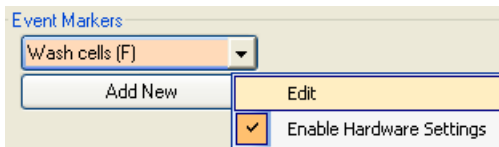
In the dialog that opens you can then make the desired changes. The Enable Hardware Settings option activates or deactivates the link to the **Event Marker**.

Acquisition area

➢ **Start (F5)**: Clicking on this button (or keyboard shortcut F5) starts acquisition for the experiment. It is possible to repeat this procedure multiple times once acquisition has been completed without leaving the **Physiology Acquisition** dialog. The acquired image sequences are stored as raw data on the defined streaming partition.

➢ **Stop (F8)**: Clicking on this button (or keyboard shortcut F8) stops acquisition. Once you have stopped the process, a new image sequence can be initiated immediately using the **Start (F5)** button, although this will then be saved in a new streaming file.

Pause (F6): Clicking on this button (or keyboard shortcut F6) pauses the acquisition procedure.

➢ **Continue (F7)**: Clicking on this button (or keyboard shortcut F7) allows you to continue acquisition in the same image file.

Timelapse Cycles	491 of 96374
Size streaming file	570.3 MB
Remaining time	03:20 Hours
Interval time	
Memory Usage	Disk Usage D:
Unsaved frames	63
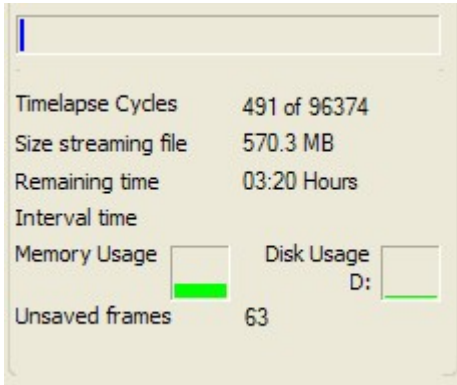
- A progress indicator shows the elapsed time for the selected experiment.

- Indicators for the available main memory and disk space for the raw data provide important information on the status of the overall system. It is also possible to see for how long it will be possible to acquire image data.

- In case the harddisk performance for the Streaming partition being too low some images may not be saved during acquisition. These images are instead stored temporarily in RAM. The number of unsaved images is given.

Charts and image display

➢ **Markers**: Shows all entries for the current experiment in list form. Every event is entered as a comment and transferred to the ZVI image during subsequent conversion.

➢ : These buttons allow you to adjust the display (brightness, contrast, zoom etc.) for the images during acquisition as well as show a display histogram.

➢ The drop-down list box next to the $\circledR^\blacktriangledown$ icon opens a dialog in which the sliders for influencing the ratio image are available. These allow you to change the ratio display during acquisition.

➢ For **method 2** (excitation ratioing in two channels) or **method 3 (emission ratioing)**, channel 1, channel 2 and the ratio image are displayed. To the left of these are the corresponding measurement value charts.

➢ For **method 1** (single-channel experiments), only channel 1 and the acquired reference image F0, as well as the ratio image, are displayed.

➢ For **FRET** imaging you will see Donor, Acceptor and the processed FRET image

➢ If you double-click into the Ratio image it will magnify and hide the other image displays. Also only the Ratio chart will be displayed. This will allow easier observation of the experiment. Double-clicking once more restores the standard layout.

➢ The charts can display 6 different measurement values for all drawn-in measurement regions (this setting can be entered via a shortcut menu **Displayed value …**, which is opened by right-clicking):

- Mean gray value channel 1
- Mean gray value channel 1 minus background
- Mean gray value channel 2
- Mean gray value channel 2 minus background
- Ratio value
- Ratio value minus background

➢ **Chart settings**: The three charts next to channel 1, 2 and ratio image can be configured by right-clicking on a chart and selecting **Chart Settings** upon which a **Chart Settings** dialog is opened:
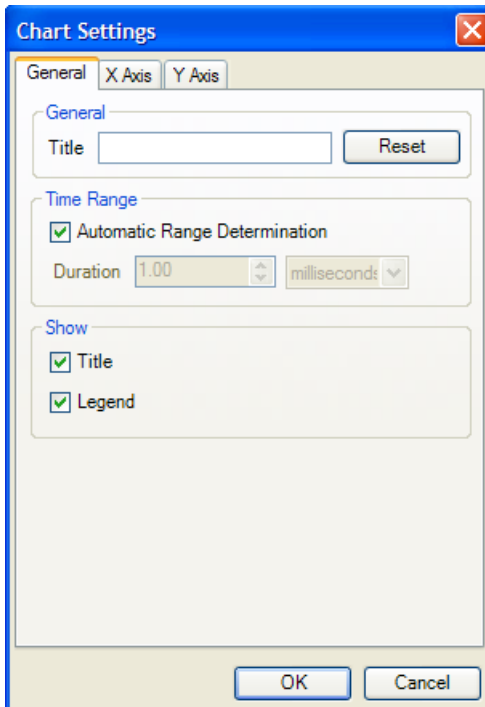
The dialog is divided into three tabs which allow the configuration of the chart parameters such as changing the default title, specifying a specific value range for X- or Y-axis, displaying or hiding legend and title or specifying tick marks. A **Reset** button on the X Axis and Y Axis page resets the chart configuration to its default setting.

➢ **Experiment overview chart**: At the bottom edge of the window you will see an overview of the entire experiment. Here you see the Ratio values displayed as measurement traces, all event markers are shown by name and with a cross marking the timepoint and a navigation tool with a line marking the current timepoint.
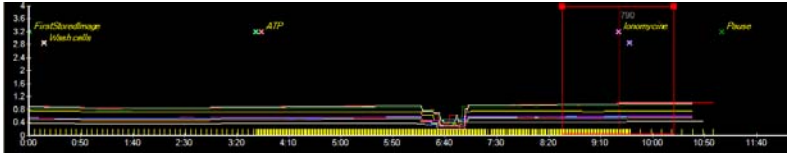
Each camera acquisition ("snap") is marked on the x-axis by a yellow

line allowing precise frame selection .
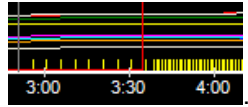
Here too it is possible to select which values you want the chart to display (right-hand mouse button, **Chart settings**). During the experiment, the new images and measurement values of the time lapse image are added continuously. The X-axis of the overview chart is updated once every 10 minutes during an experiment. The broken red line of the navigation tool moves with each acquired image which is always displayed automatically.

However, you can also stop the marking line by deactivating the
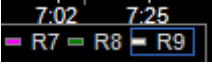
 button.

You have the option of using the marking line like a slider to move to an earlier time point and revisit the acquired data. The red, broken marking line has two red, solid peripheral lines. The area within these two lines corresponds to the area that is displayed in the charts next to the images. If you grab the squares at the top edge of the area boundaries, it is possible to change the range shown in the charts.
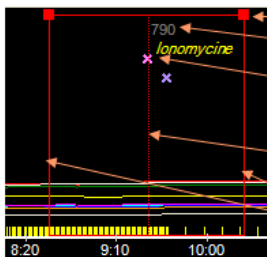
➤ **ROI and line selection**: During the experiment, especially one with many measurement ROI's drawn in, it may be desirable to e.g. see which cell is especially active. You can therefore click on either an interesting looking measurement line in any of the charts, select a ROI in one of the displayed image displays or select it in the ROI legend at the bottom of each chart .

Either selection will automatically select the corresponding shapes in the other areas. Selected lines show up more brightly on the screen, unselected lines become subdued in appearance.
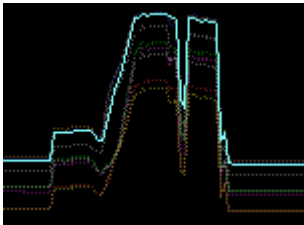
➤ If a ROI or measurement line is selected, moving the mouse cursor along the line with no buttons depressed shows a gray line and a pop up text showing the relevant intensity or ratio values.
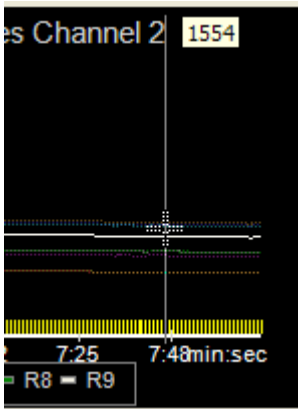
➢ To move the marking line step by step, you can use the ⊟ and ⊟ buttons.

➢ After stopping the experiment (Stop or F8), you can directly load the new file in the **Cutter** to allow conversion into ZVI by clicking on the [Start Cutter] button.

➢ Clicking on the [Close] button will exit the dialog.

Note: No other setting options are accessible in AxioVision while the **Physiology Acquisition** dialog is open. This is designed on purpose to prevent operating errors.

➢ Once the dialog is closed, all acquired image data are loaded in AxioVision in streaming format, but have not yet been saved. They exist on hard disk but not in a format which AxioVision can read.

➢ The streaming data can be saved as a *.ziStream document. Streaming files per se cannot be reopened in AxioVision. Upon saving each *.ziStream file will be accompanied by a *.ziAR file containing all relevant metainformation about the experiment and the data, which you will see, when reopening a streaming file. Both files are necessary to open an experiment again.

➢ Please also note, that streaming files must not remain on the streaming disk in order to prevent disk fragmentation and the resulting loss in harddisk performance. Upon closing AxioVision, you will be therefore asked if you want to save or discard unsaved streaming files.

Physiology Acquisition Document Cutter

To generate a ZVI image from a saved or unsaved *.ziAR streaming document, you will require the **Cutter**. Open the **Cutter** via the corresponding entry in the work area or the **Acquisition** menu ⇒ **Physiology Acquisition** ⇒ **Cutter**.
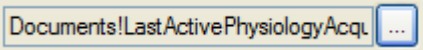
The principal task of the **Cutter** is to reduce the time-consuming conversion of the raw data into a ZVI image to a sensible level. This makes it possible to reduce the data volume and speed up the further processing of the data.

Note:
- The **Cutter** can only be opened if a suitable streaming file has also been loaded in AxioVision (otherwise the following error message appears: "*No streaming document available to edit. Please first open a streaming document (*.ziAR)*").

Cutter Physiology

The **Cutter Physiology** dialog is structured in a similar way to the **Physiology Acquisition** dialog.

➢ Documents!LastActivePhysiologyAcqu ⎯ Describes the name of the input document.

➢ The ⎯ button opens a gallery from which you can also load a document other than the one that was last active. To do this, click on the thumbnail of the desired streaming document.

➢ : The buttons on this toolbar can be used to adjust the display (brightness, contrast, zoom etc.) of the images.

> Using the [image] button on the toolbar, it is possible to define a region of interest in the image that you want to be taken into account during the conversion to ZVI. This will dramatically reduce the data volume to the relevant parts of the image.

> The drop-down list box next to the [image] icon opens a dialog in which the sliders for influencing the ratio image are available.

> **Marker**: Shows all entries for the current experiment in list form to the right of the dialog. Every marker is transferred as a comment to the ZVI image during conversion and can later be inserted into the generated ZVI image as an annotation via **Annotations** ⇒ **Frequent Annotations** ⇒ **Event marker / Speed marker**.

> The **time format** for the marker list can be changed by right-mouse click in the header row of the list and selecting the **Properties Dialog**:
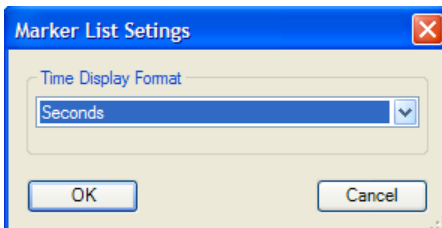
Please select the desired time format (**Seconds**, **Minutes**, **Hour** or the generic format **hh:mm:ss:msec** which is the default setting) from the drop down list. You can also select the frame number.

> For **methods 2 or 3** (excitation or emission ratioing in two channels), channel 1, channel 2 and the ratio image are displayed. To the right of these are the corresponding charts displaying the measurement values.

> For **method 1**, only channel 1 and the acquired reference image F0, as well as the ratio image, are displayed.

➢ The charts can display 6 different measurement values for all drawn-in measurement regions (this setting can be entered via the menu **Displayed value …**, which is opened by right-clicking):

- Mean gray value channel 1
- Mean gray value channel 1 minus background
- Mean gray value channel 2
- Mean gray value channel 2 minus background
- Ratio value
- Ratio value minus background

➢ **Chart settings**: The charts can be configured by right-clicking on a chart and selecting **Properties** upon which a **Chart Settings** dialog is opened:
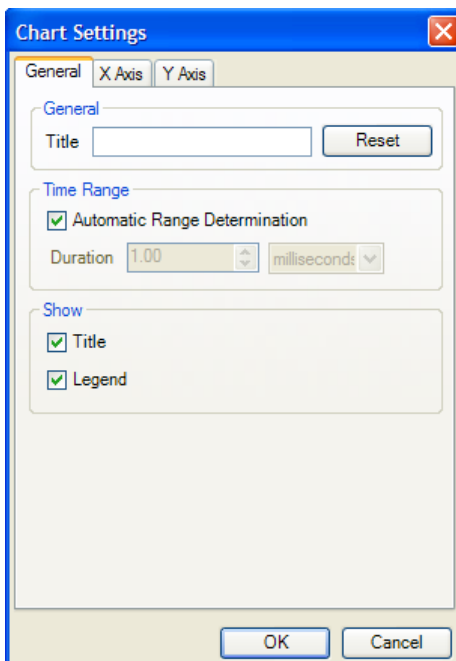
The dialog is divided into three tabs which allow the configuration of the chart parameters such as changing the default title, specifying a specific value range for X- or Y-axis, displaying or hiding legend and title or specifying tick marks. A **Reset** button resets the chart configuration to its default setting.

➢ **Experiment Overview**: In the bottom area you will see an overview of the entire experiment displaying markers and measurement curves:
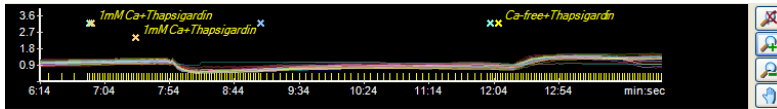
➢ Each acquisition event is signified by a yellow line allowing easier frame selection
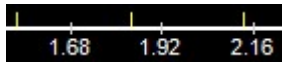
➢ In this control element it is possible to define the areas of the streaming document that you want to be converted to ZVI. When this dialog is called up for the first time, nothing is selected. Selection can be done by clicking with the left mouse button and dragging whilst keeping the mouse key depressed. Selection is symbolized by a magenta selection bar. Segments within the experiment where the acquisition had been paused are signified through a lighter color. Such paused areas are automatically excluded from conversion.
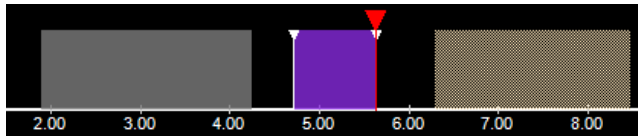
> ➢ The time point currently displayed is indicated using the following marking line:

Selects a timepoint; shows frame number
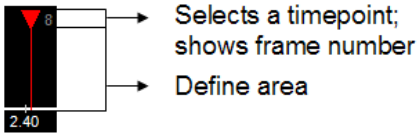
Define area

> If you grab the upside-down triangle with your mouse, you can select the time point of your choice. In the area at the lower half you can use the left-hand mouse button to define an area to be converted: hold down mouse button, drag, release → defined area is marked in magenta. You can mark as many areas as you wish.

> ➢ The area that is currently active is marked in magenta, whilst inactive selections are shown in gray
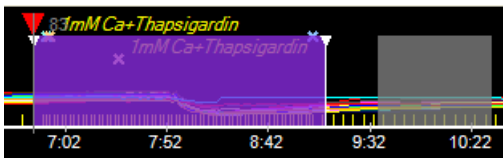
> ➢ It is possible for marked areas to be moved (left-hand mouse button).

 Adjusts the zoom factor of the chart in such a way that the entire experiment is displayed.

 Allows you to display a part of the experiment in the chart with a higher zoom factor (**Zoom in**).

 Reduces the zoom factor for the displayed chart area (**Zoom out**).
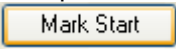
Moves the displayed chart area (only active with **Zoom in**).

➢ In the bottom area of the dialog you will find a table containing the selected areas with an indication of the exact start and end time points, the resulting duration and the start-image position and end-image position for the marked segments.

➢ It may be beneficial, especially if you need to select a time point precisely, to position the time point marking line and set it as the start point for a new area selection. This is done using the **Mark Start** button. As soon as a start point has been defined, the **Mark End** button for defining an end point becomes active.
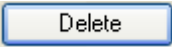
➢ You can use the two arrow buttons to browse through the streaming file frame by frame. This allows an even more precise frame selection.

➢ To select the entire time series for conversion, click on the **Mark All** button.

➢ To delete selected area markings, you can use the **Delete** button (or alternatively the **Delete** key).

Conversion Information

Here you will find a display of the file size of the newly generated ZVI image, the number of individual images it contains and the available memory on the target drive.

Executing editing

Convert Single converts the currently shown frame into a ZVI image and leaves the dialog open

Convert All selects the complete streaming file, starts conversion immediately and closes the dialog. This button is selected as default which means that you can confirm this action immediately by hitting **Enter** on your keyboard.

OK Carries out the conversion to a ZVI image according to the individual selections, after which the dialog is closed automatically.

Apply Carries out the conversion to a ZVI image, but the dialog remains open. This allows you to generate several ZVIs of different areas and regions conveniently one after another, without having to call up the dialog again each time.

Cancel Closes the dialog. No ZVI image is generated.

The result of the conversion is a new, unsaved ZVI image. Images generated in this way can now be subjected to post acquisition analysis using the **Measurements** function

Physiology Measurements

During acquisition preliminary measurements are performed based on the drawn in measurement regions. These online measurements mainly feed the charts with data for online display. Measurement regions can also be moved to compensate for moving cells during the experiment. This option as well as other vagaries during the experiment can lead to measurement values inconsistent with the experimental goals. Therefore a detailed analysis of the acquired data must be done after the acquisition. This can be done using the **Measurements** function in the **Physiology** module.

Measurements can read any ZVI time series, also if the series has not been acquired using **Physiology**. ZVI time series can have one or more channels, which can be selected flexibly for measurement. The strict channel order which is required for online measurement does not apply for offline measurement. Streaming files (*.ziAR) must be converted into ZVI first using the **Cutter**.

➢ You can open the **Physiology Measurements** dialog by clicking on Measurements in the **Acquisition** menu ⇒ **Physiology Acquisition** ⇒ **Measurements**.

Always the active ZVI image is loaded into the Measurement dialog. By using the ⬚ button another ZVI image can be selected from the **Gallery**.

The left side of the dialog features three tabs for setting up which channels should be measured (**Channel Setup**), for configuring the measurement parameters (**Measurement**) as well as parameters for generation of the ratio image (**Ratio Image**).

Channel Setup

➢ **Method**: From the drop down list box you can select a calculation method. Default method is Ratio Dual Channel. There are 8 predefined methods but you can also use your own custom formula. The parameters which can be changed depend on the method selected. For the various FRET methods the different formulas used are displayed as well:

- **Ratio Dual Channel**: Classical dual channel ratio imaging using either method 2 (excitation ratioing, e.g. Fura-2) or 3 (emission ratio imaging, e.g. Indo-1), only with two channel setups. Ratio is always calculated dividing channel #1 by channel #2.

- **Ratio Single Channel**: Single Channel ratio imaging such as Fluo-4; can be done with single or multiple channel setups, online processing always done with channel #1.

- **Fret Youvan**: Sensitized emission FRET measurements using three images. Channel #1 = donor, channel #2 = acceptor, channel #3 = FRET

- **Fret Donor Ratio:** channel #1 = donor, channel #2 = acceptor, channel #3 = FRET

- **Fret Acceptor Ratio:** channel #1 = donor, channel #2 = acceptor, channel #3 = FRET

- **Fret Gordon**: Derivative of FRET Youvan

- **Fret Xia**: Derivative of FRET Youvan

- **Fret Siegel BL**: Acceptor bleaching method, requires two channel setup

- **Custom formula**: Using regular algebra terms a customized formula can be edited and applied to the data.
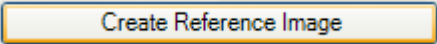
Note: All the following explanations refer prominently to the Ratio Dual Channel method.

➢ **Channel Selection:** The ratiometric measurement can be set up here by selecting **numerator** and **denominator** channel used in the ratio formula. Select the desired channel (channel name as specified in the experiment) from the appropriate drop down list.

➢ **Ratio Image Definition**: In both methods ratio values are displayed by applying a color look up table (LUT) to aid the visual distinction of small ratio changes. The ratio image is calculated as outlined in the formula shown here:

$$r(x,y) = Multiplier * \frac{ch1(x,y)}{ch2(x,y)} + Offset$$

- r (x,y) = ratio image
- Multiplier = factor by which the result of the division is multiplied
- channel 1 (x,y): image from channel 1
- channel 2 (x,y): image from channel 2
- Offset = factor that influences the general brightness of the ratio image.

➢ The lower and upper limit of the displayed ratio can be entered using the sliders for **Minimum** or **Maximum** or by entering the values directly in the edit fields. These values are also put in the output ratio image as image tags and are used by the LUT Scale annotation. By raising the lower limit one can reduce the background of small ratios, by raising the upper limit, also larger ratios can be displayed properly. A minimum of 0 and a maximum of 3 cover the range of ratios commonly occurring in biology.

➢ **Look-up Table** (LUT): Clicking on [...] opens the **Open Table** dialog which allows selection of a desired look up table. Depending on the application or the personal preference various LUTs serve different purposes. For Ratio imaging a rainbow look up table is frequently used (RAINBOW.COL).
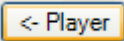
➢ In case of the **Ratio Single Channel** method the **Reference Image (F0)** section becomes active which allows definition of time points for calculation of an averaged F0 reference image.

➢ In order to generate an appropriate reference image for a single channel measurement a suitable range of time points can be defined from within the same channel. This, e.g., could be a resting phase, where cells do not show any ion fluctuations as well as do not move. You can either enter the desired time points directly in the **From** and **To** edit fields or take over the current time point as selected with the time player by clicking on the [<- Player] buttons.

➢ From this series of individual time points an averaged reference image is calculated after click on [Create Reference Image].

➢ After the reference has been created, the charts become active and measurements can be performed.
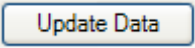
Measurement

➢ This tab contains functions for background correction as well as for in situ calibration should absolute concentrations be determined.

➢ [⊙ No background correction] : If selected, the uncorrected raw gray values are used to calculate the ratio values

➢ [⊙ Use fixed background value] : If selected, a fixed background value is subtracted from all time points. This value is channel specific and can be entered in the edit fields next to the appropriate channel number. In case of single channel images for which an F0 reference image has been defined, the F0 reference is considered as a pseudo channel #2.

➢ ⊙ Use background ROI : If a measurement region (ROI) has been selected in the image, this option becomes active. Here a background value for each channel and time point is measured and subtracted from the gray values of the other measurement regions used to calculate the ratio. If either of the two other options is selected, the selected background ROI is converted into a standard measurement region. To again use a background region use the 🏠 button from the toolbar to convert a ROI into a background ROI.

➢ **In Situ Calibration**: If enabled, the check box enables a dialog to perform in situ calibration for calibrated Calcium and pH measurements. As a prerequisite for in situ calibration you need to treat your cells with agents which allow you to determine the minimum (R(min)) and the maximum R(max) response of the loaded dye. In the case of Calcium measurements using Fura-2, e.g., this is frequently done using Ionomycine, a drug, which permeabilizes the cell membrane followed by treatment with low and high calcium buffer.

- Move the cursor of the navigator tool to the part in the chart, which shows minimum ratio:
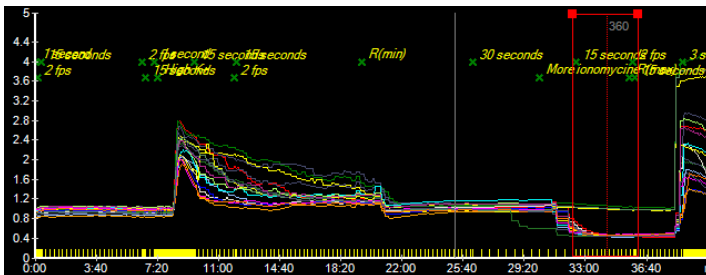
- click on ⟨- Player to use the current cursor position as the time point used for determining the minimum ratio of each ROI

- Repeat the same for R(max)

- Clicking on [Show] opens a floating dialog **Calibration editor** showing the calibration charts.

- Here you can select between Calcium Calibration or pH Calibration. In the case of Calcium calibration the formula according to Grynkiewicz et al. (Journal of Biological Chemistry, Volume 260: 3440, 1985) is used. Here you can also modify the kD, which has been calculated to be 224 nM at 1 mM Mg^{++} and 37ºC.

- A viscosity correction factor typically between 0.7 and 0.85 has been suggested to be used by M. Poenie (Cell Calcium Volume 11, 2-3, pp 85-91, 1990), which can be entered here as well. Setting the value to 1 disables correction.

- In the case of pH calibration a simple linear interpolation is performed using R(min) and R(max).

- OK closes the dialog and activates calibration.

➢ All measurements are done online, i.e., when the dialog first opens, all calculations are done once. Tthe system then tries to update the measurements on the fly upon changes to ROIs etc. For large time series with many time points and/or many measurement regions, calculation of all values for all time points can take several seconds. To prevent the system from responding slowly, the calculation update is interrupted after a few seconds so that not all values may be present. In this case the charts may be incomplete which will be signified by a yellow signal ⬤. Clicking on the [Update Data] button forces the complete calculation which is symbolized by changing the signal color to ⬤.

Options

You can choose between two methods of calculating the ratio image: **Standard** and **Confidence Mapping**. Standard is active if the check box is unchecked.

The second algorithm is called **Confidence mapping**. This method has been suggested by Tsien and Harootunian (Tsien, R.Y., and Harootunian, A.T. 1990. Practical design criteria for a dynamic ratio imaging system. *Cell Calcium* **11**: 93-109.) to prevent noise from influencing the ratio display negatively. When dividing two small gray values by each other, fluctuations in the gray value introduced by noise can result in quite large ratio values. With this method each ratio pixel value is put in proportion to the brightness of its original input pixels. This effectively introduces a bias for the display of brighter and therefore more relevant pixels. Consequently dark pixels become underrepresented in the result image reducing the displayed noise in the image.

The calculation is done in two steps:

- Calculation of the Ratio

$$r(x,y) = \text{Multiplier} * \frac{ch1(x,y)}{ch2(x,y)} + \text{Offset}$$

- Calculation of the confidence:

$$\text{Confidence} = \frac{ch1(x,y) * ch2(x,y)}{2 * \text{ImageMaxVa lue}}$$

while ImageMaxValue is the brightest pixel present in the image.

- The display can be explained by setting the **Hue = LUT (Ratio)** and the **Luminance = Confidence** or as follows:

$$Red = Lut\text{-}red\,(Ratio) * Confidence(x, y)$$
$$Green = Lut\text{-}green\,(Ratio) * Confidence(x, y)$$
$$Blue = Lut\text{-}blue\,(Ratio) * Confidence(x, y)$$

➢ For confidence mapping it is not possible to directly enter a Ratio value as annotation in the resulting ratio output image due to the dual functionality required to calculate the ratio pixel appearance.

➢ In order to influence the displayed value range both **Multiplier** (range between 0 and 10) and **Offset** values (value range of -1 to 1) can be changed on the **Channel Selection** tab using the sliders or by directly entering the values in the edit fields.

Information Color Ratio Image

The size and number of cycles of the resulting ratio image are displayed here.

Tool bar

 : The buttons on this toolbar can be used to adjust the display (brightness, contrast, zoom etc.) of the images or analyze individual pixels using the picker tool.

Using the other tools it is possible to draw in up to 20 regions, which will be used for measurement. When you draw in several regions, the next color from the color list is automatically assigned to each new region.

 Allows you to click on and select measurement regions that have already been drawn in to move them, enlarge them, delete them or change their color.

Used to draw in a rectangular measurement region. If the button is not selected, the tool remains active and it is possible to draw in several measurement regions one after the other.

Used to draw in a measurement region with a free form of your choice (spline). If the button is not selected, the tool remains active and it is possible to draw in several measurement regions one after the other.

Used to draw in an ellipse as a measurement region. If the button is not selected, the tool remains active and it is possible to draw in several measurement regions one after the other. If the Shift key is depressed while drawing in the ellipse, a circular form is enforced.

Draws a circle. Starting point and end point of the drawing defines the diameter making it easy to e.g. define a circle encompassing a complete cell.

When clicked, the last shape used for drawing a ROI is "remembered" and drawn into the overlay by simple clicking. This allows the cloning of a shape with ease. It works with all ROI tools except the free form spline tool.

This function can be disabled by selecting .
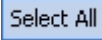
: Displays the measurement values instead of the charts. You can click on an entry upon which the corresponding time point is selected in the time series. These values can also be exported as a data list later. The data table is set up as shown here, the "Channel 1" etc. is replaced by the actual channel name as specified in the experiment. Also, the set up shown here is only valid for the **Ratio Dual Channel** case. Especially in FRET experiments, the Ratio column will contain the calculated FRET values.

Index	Row index
TimeStamp	relative time
Markers	Marker
R1_Channel1	mean density of region 1, channel 1
R1_Channel2	mean density of region 1, channel 2
R1_Ratio	ratio value region 1, channel 1 / channel 2
R1_Channel1(-BG)	background corrected mean density of region 1, channel 1
R1_Channel2(-BG)	background corrected mean density of region 1, channel 2
R1_Ratio(-BG)	background corrected ratio value region 1 channel 1 / channel 2
R2_Channel1	mean density of region 2, channel 1
R2_Channel2	mean density of region 2, channel 2
R2_Ratio	ratio value region 2, channel 1 / channel 2
R2_Channel1(-BG)	background corrected mean density of region 2, channel 1
R2_Channel2(-BG)	background corrected mean density of region 2, channel 2
R2_Ratio(-BG)	background corrected ratio value region 2, channel 1 / channel 2
etc…	etc…

If no background correction is done, the background corrected value
column is omitted from the table. In the case of calibrated measurements
additional value columns will be present.

Clicking again on 🖼 hides the table and displays the measurement
charts again.

✖ Deletes all selected measurement regions.

Select All Selects all available measurement regions.

🖊 ▾ Allows you to change the color for a measurement region.

: Via **Custom...** it is possible to add further colors to the list from a color selection dialog.

 Defines a selected measurement region as a background region. The mean gray value from this region can, if desired, be automatically subtracted from the measurement values of all other regions.

Markers

➢ Shows every event which has been transferred as a comment to the ZVI image during conversion. Clicking on an event moves the time marker tool to the corresponding time point.

➢ The **time format** for the marker list can be changed by right-mouse click in the header row of the list and selecting **Properties**:
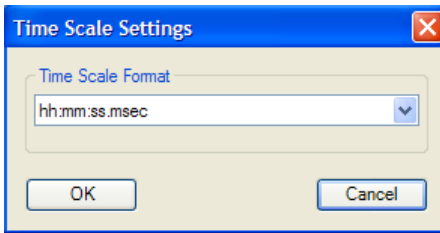
Please select the desired time format (**Seconds**, **Minutes**, **Hours** or the generic format **hh:mm:ss:msec** which is the default setting) from the drop down list. You can also select the frame number.

Charts

The charts can display 6 different measurement values for all drawn-in measurement regions (this setting can be entered via a shortcut menu, which is opened by right-clicking):

- Mean gray value channel 1
- Mean gray value channel 1 minus background
- Mean gray value channel 2
- Mean gray value channel 2 minus background
- Ratio value
- Ratio value minus background

➢ **Chart settings**: The three charts next to channel 1, 2 and ratio image (two in the case of single channel experiments) can be configured by right-clicking on a chart and selecting **Properties** upon which a **Chart Settings** dialog is opened:
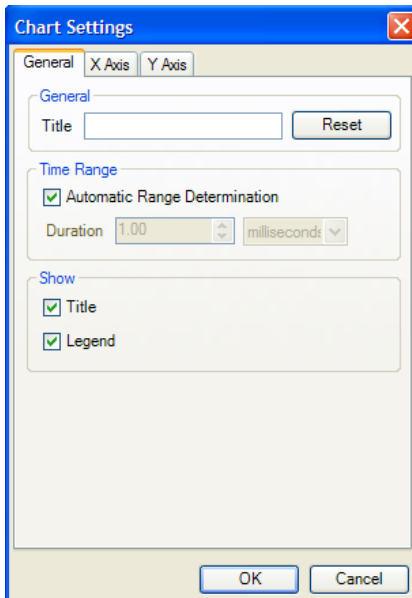
The Dialog is divided into three tabs which allow the configuration of the chart parameters such as changing the default title, specifying a specific value range for X- or Y-axis, displaying or hiding legend and title or specifying tick marks. A **Reset** button resets the chart configuration to its default setting.

Creation of output documents

➢ When all settings are made, clicking on **OK** will prompt the opening of the following dialog
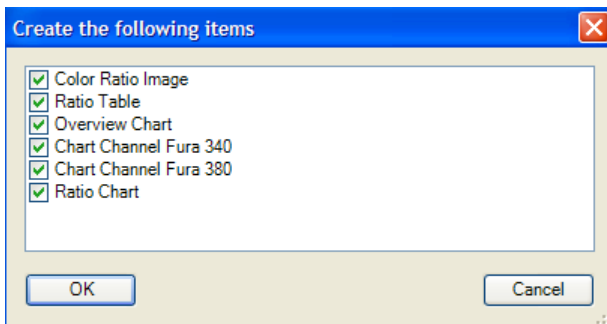
➢ Check all documents you want to be created as result of the measurement. In most cases at least the **Color Ratio Image** as well as the **Ratio Table** containing all measurement data will make sense to keep.

➢ The data can be saved in the generic CSV file format or in an XML format, which can be opened directly with Microsoft Excel (2003 edition necessary). AxioVision has basic charting features for all data tables which can be accessed via the **Chart View** button at the bottom of the table.

➢ The ratio image is a 24 Bit color image including all ROIs as annotation. Several annotations are available to help create informative movies for presentation. The Ratio range can be displayed using the LUT Scale annotation, both speed and event markers can be added as well. All annotations can be found in the **Annotation menu → Frequent annotations**.

➢ The resulting charts may be useful to quickly put together a presentation of the data. They can be saved as EMF (enhanced meta file) or WMF (Windows meta file) data. Both formats are vector based so that they can be inserted in programs like Microsoft Powerpoint and rescaled without loss of quality.

Clicking on **OK** closes the measurement dialog, the output documents are created and displayed as unsaved documents in AxioVision.

Information for Physiology

This function opens a dialog window, where you can find in tabular form important information in connection with the physiology experiment. The information it contains bear always on the current experiment and will be updated during each sequence of the experiment.

Show

In the drop-down list you can select, whether only an overview or all available information are shown. To get more explanations to the existing information, you can select an item. In the bottom area of the dialog you will find more explanation.

Information

In the **Information Current Physiology Experiment** dialog, various items of information about the currently active Physiology experiment are shown, i.e. expected image size, available hard disk space etc. These values do not relate to open image documents or streaming files.

Show

Using the drop-down list box it is possible to select whether you want an overview only (**Reduced information**) or all information (**All information**) to be displayed. All parameters relating to the current experiment are presented in list form. For further explanations on the meaning of the entries, click on an entry. You will then see an explanation in the text box at the bottom edge of the dialog.

10. Image Processing Modules

10.1 Imaging Plus

General

As already described in chapter 5 "Image Processing", image processing functions have a range of different tasks. Besides the "traditional" areas of application of correction and optimization, which have already been mentioned, there is also a third area: data extraction. Here, for example, structures are highlighted in relation to the image background to make them more visible, or objects are purposefully detected so that they can be measured later.

The Imaging Plus module expands the image processing functions of the basic program with the following functions and functional groups.

Image enhancement

Additional functions to enhance contrast, brightness and color, plus filters for smoothing, sharpening and edge detection with user-definable filter operators.
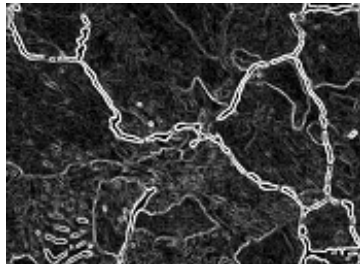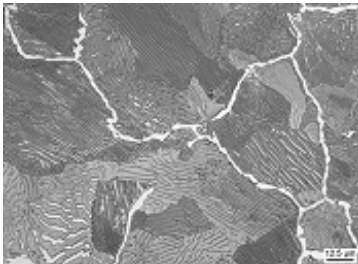
Edge detection with Sobel filter

Gray morphology

The gray morphology functions enable you to reconstruct the boundaries of connected objects.
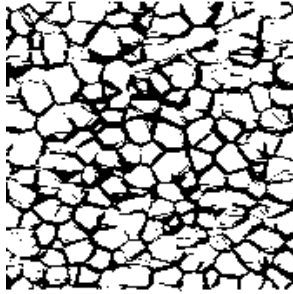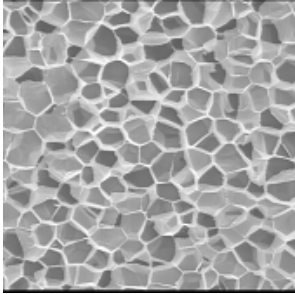
Through the reconstruction of grain boundaries images are prepared for automatic measurement.

Image arithmetics

AxioVision enables you to combine and compare images, as well as extract information, by means of the image arithmetics functions in Imaging Plus.

Fourier transformation

Fourier transformation allows you to restore images by purposefully filtering out defined frequencies.
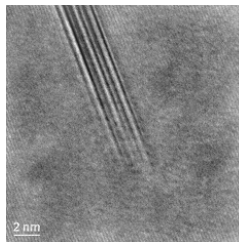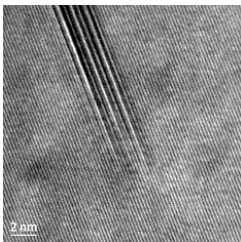
*With Fourier transformation it is possible to purposefully remove (right)
sinusoidal super positioning (left), for example.*

Color space transformation

Color discrimination enables you to separate object and image background
more effectively on the basis of color. This makes it possible e.g. to
distinguish better between individual classes of object.
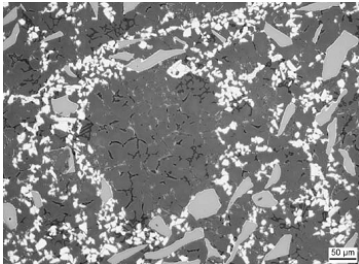
*Bright field image of a composite
material, contrasted with PtOx*
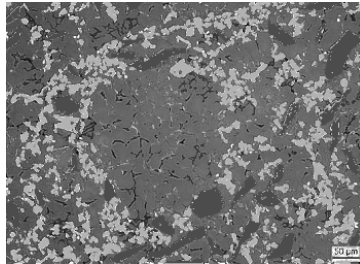
*Heterochromatic super positioning of
two different phases*

Working with image processing functions

The principal working method for the functions of the Imaging Plus module
is described in chapter 5 "Image Processing".

The chapter also contains background information on the image processing
functions and storage, etc. We recommend that you work through this
chapter as a basis for applying the functions of the Imaging Plus module. A
description of the individual functions and their parameters can be found in
the online help.

The individual steps in the following examples can also be practiced using
the original images that were employed. The sample images are installed
in the AxioVision folder. By default this is the folder *"C:\Program Files\Carl
Zeiss Vision\AxioVision\0009\Templates\Images"*. If an image is not
present in that folder, please load it from the AxioVision Viewer CD.

Reducing or enlarging images or image regions

The **Resample** function can be used to reduce or enlarge an entire image or a freely selectable region of an image. The reduction or enlargement is defined using a zoom factor (**Scale**). If only a region is being reduced or enlarged, the center of the region (**Shift**) must be indicated.

The size of the resulting image can be adapted (**ForceSizes** = **On**), depending on the scaling factors (**Scale**). You can, however, also generate the resulting image in the size of the original image (**ForceSizes = Off**).

It is also possible to take the influence of the scaled neighboring pixels into account (**Interpolation**).

NOTES:
* If you simply want the entire image to be displayed in reduced or enlarged form without a new image being generated, this is also possible using the **+ Zoom** and **- Zoom** functions from the image window's shortcut menu.

The following example explains step by step how to reduce an image or enlarge a region of an image.

➢ Load the image "Bone-fluorescence_3.zvi" via the function **File** ⇒ **Open Image**, or acquire an image with the camera.

➢ The image is displayed in AxioVision's image window.
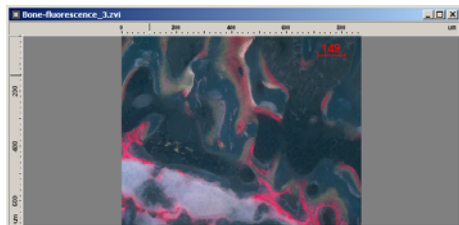
In the first example, the loaded image is to be reduced by the factor 0.5. In the second example it is to be enlarged by the factor 2.

Now select from the **Processing** menu the **Utilities** functional group and then the **Resample** function.

➢ You will now see the dialog window of the **Resample** function.

➢ If the **Automatic Preview** check box is activated, the reduced input and output images appear.

➢ Set **CreateNew** to **Off** so that you can select the images freely.

➢ Set the following values by clicking on the arrow keys or by entering the values directly: **ForceSizes**=On, **ShiftX**=0, **ShiftY**=0, **ShiftZ**=0, **ScaleX**=0.5, **ScaleY**=0.5, **ScaleZ**=1, **Interpolation**= NearestNeighbour.

➢ Click on **OK** to close the dialog.
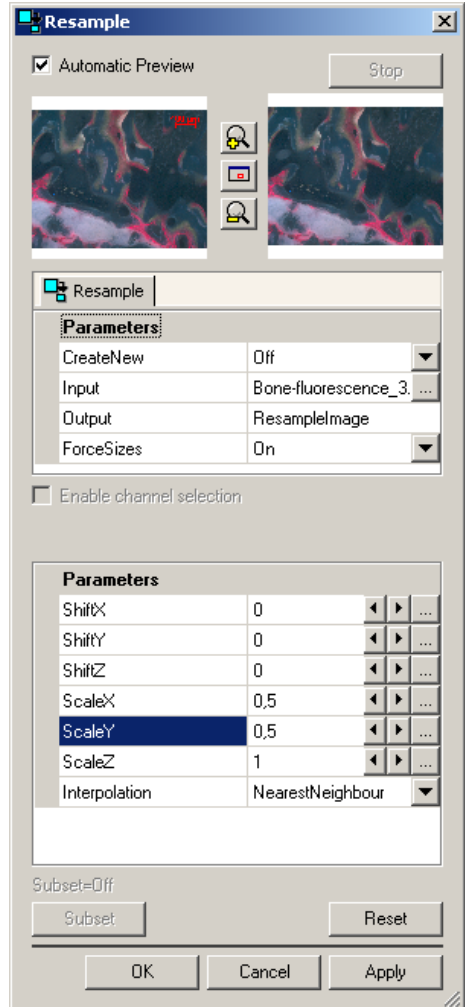
> The resulting image shows all the structures of the original image, but has been reduced by the factor 0.5.

> The image size has been adjusted accordingly, as **ForceSizes** was set to **On**.

> Position the cursor over the resulting image, press the right-hand mouse button and select **Normal View (1:1)** shortcut menu.

> Move the scroll bar to adjust the desired area.

> Do the same with the original image.

> Compare the two images. In the normal view of the image the difference can be clearly seen.
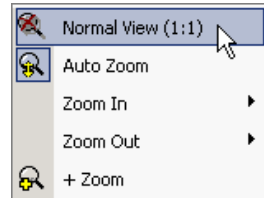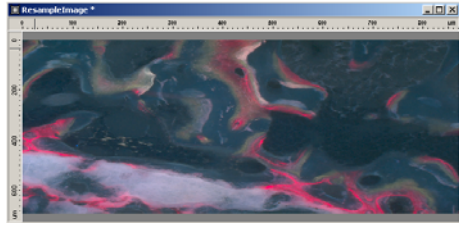
Original image
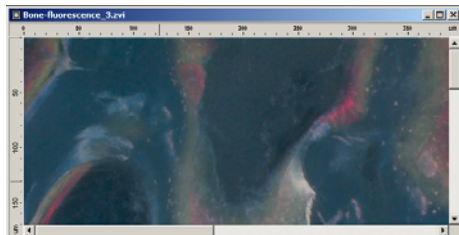
Resulting image
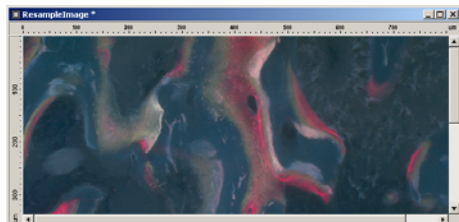
For the second example, again select from the **Processing** menu the **Utilities** functional group and then the **Resample** function.

> You will now see the dialog window of the **Resample** function.

> If the **Automatic Preview** check box is activated, the reduced input image appears.

> Under **Input** select the original image again and allocate a new image name under **Output**.

> Set the following values by clicking on the arrow keys or by entering the values directly: **ForceSizes**=Off, **ShiftX**=650, **ShiftY**=515, **ShiftZ**=0, **ScaleX**=2, **ScaleY**=2, **ScaleZ**=1, **Interpolation**= NearestNeighbour.

> Click on **Apply** to view the resulting image in the preview.

> Click on **OK** to close the dialog.
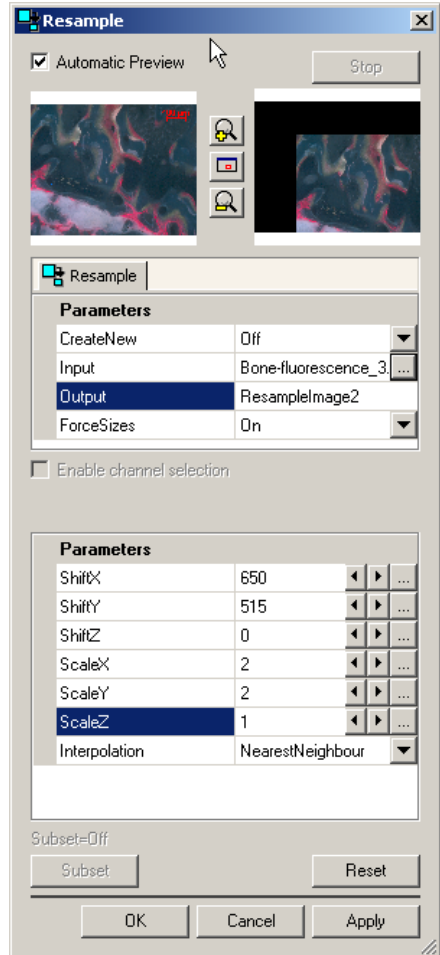
The resulting image shows only the top left quarter of the structures in the original image. This region was defined by **ShiftX** and **ShiftY**.

➢ The region is now larger than the original image by the factor 2.

➢ The image size has been retained, as **ForceSizes** was set to **Off**.
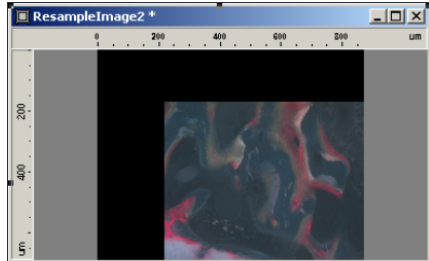
➢ Position the cursor over the resulting image, press the right-hand mouse button and select **Normal View (1:1)** from the shortcut menu. Do the same with the original image.
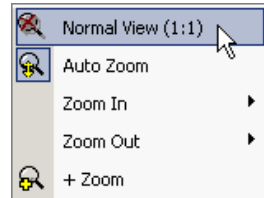
➢ Compare the two images. In the normal view of the image the difference can be clearly seen.
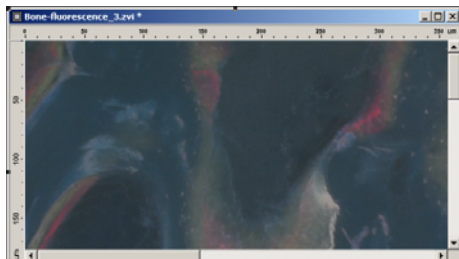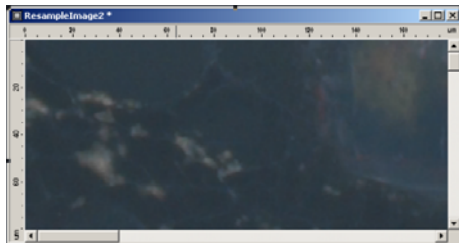
NOTES:

- Adjust the display characteristic curve in the resulting image if necessary.

Copying various regions from an image and generating a new image

Often you will only want to further process a particular region (ROI) of an image, or generate a composite image from various images or from regions of various images.

Using the **Copy Region** function, you can copy a freely selectable region from the original image into a new image. The starting coordinates in the original and the resulting image (**SourceStart**, **DestinationStart**) and the size of the region to be copied (**Size**) must be specified. If you are copying one region only, the size of the resulting image can be adapted to the region (**ForceSizes** = **On**). You can, however, also generate an image in the size of the original (**ForceSizes** = **Off**).

To create a composite image from several images or image regions, you need to define the overall image size (**Size**, **ForceSizes** = KeepMaxSize). The individual images can then be copied into this image step by step. During the copying of an individual image, the area outside the region to be copied can be retained (**ClearOutside** = **Off**) or deleted (**ClearOutside** = **On**) in the resulting image.

NOTES:

- If you simply want a region of an image to be copied, and a new image to be generated from it, this process can also be performed using the **Select ROI/Copy ROI** and **Paste** functions on the **Edit** menu.

The following example explains step by step how to copy various regions from one image into a new image.

➢   Load the image that you want Load the image "Tupaia_head_ section_overview.zvi" via **File** ⇒ **Open Image**, or acquire an image with the camera.

➢   The image is displayed in AxioVision's image window.
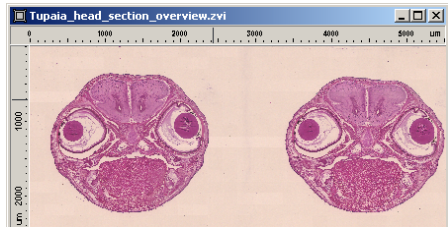
The image contains two histological sections through embryo heads. In the first example, only one embryo head is to be copied into a new image. In the second example, both embryo heads will be copied into a new image, with as little background as possible.

Select from the **Processing** menu the **Utilities** functional group, and then the **Copy Region** function.

➢ You will now see the dialog window of the **Copy Region** function.

➢ If the **Automatic Preview** check box is activated, the reduced input image appears.

➢ Set **CreateNew** to **Off**, so that you can select the images freely.

➢ Set the following values by clicking on the arrow keys, or by entering the values directly: **ForceSizes**=On, **SourceStartX** =100, **SourceStartY**=120, **SourceStartZ**=0, **DestinationStartX**=0, **DestinationStartY**=0, **DestinationStartZ**=0, **SizeX**=850, **SizeY**=780, **SizeZ**=1, **ClearOutside**=Off.

➢ Click on **Apply** to view the resulting image in the preview.

➢ Click on **OK** to close the dialog.
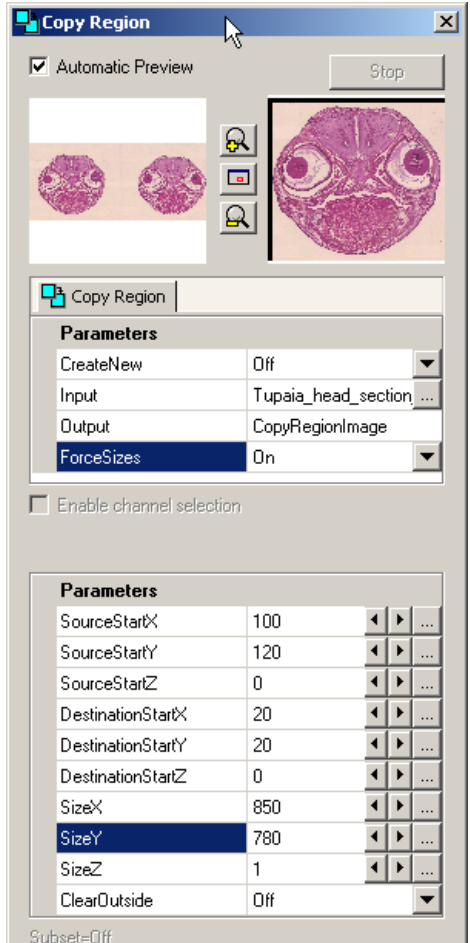
> ➤ In this example, only one region (ROI) has been copied into a new image. The resulting image therefore shows the first embryo head only.

> ➤ The image size has been adapted to the specified size of 850 x 780 pixels, as **ForceSizes** was set to **On**.
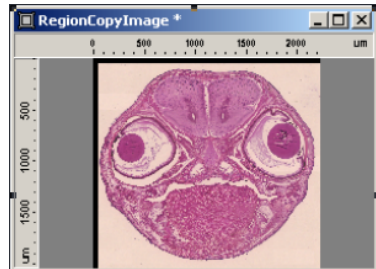
For the second example, again select from the **Processing** menu the **Utilities** functional group, and then the **Copy Region** function.

➢ You will now see the dialog window of the **Copy Region** function.

➢ If the **Automatic Preview** check box is activated, the reduced input image appears.

➢ Select the original image again under **Input**.

➢ Set the following values by clicking on the arrow keys, or by entering the values directly: **ForceSizes**=Off, **SourceStartX** =100, **SourceStartY=**120, **SourceStartZ**=0, **DestinationStartX**=0, **DestinationStartY**=0, **DestinationStartZ**=0, **SizeX**=1000, **SizeY**=800, **SizeZ**=1, **ClearOutside**=Off.

➢ Click on **Apply** to view the resulting image in the preview.

➢ Click on **OK** to close the dialog.

➢ The resulting image then shows the first embryo head in the top left-hand corner.

➢ As **ForceSizes** was set to **Off**, the image size corresponds to that of the original. The rest of the image is black.
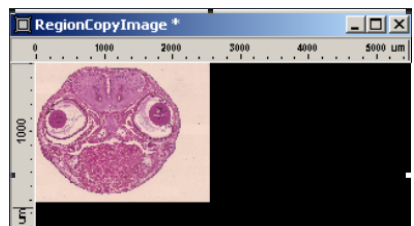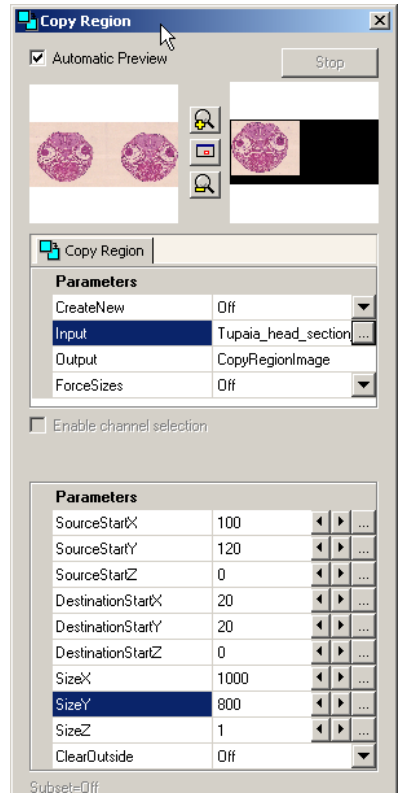
Again select from the **Processing** menu the **Utilities** functional group, and then the **Copy Region** function.

- ➢ You will now see the dialog window of the **Copy Region** function.

- ➢ If the **Automatic Preview** check box is activated, the reduced input image appears.

- ➢ Select the original image again under **Input**.

- ➢ Set the following values by clicking on the arrow keys, or by entering the values directly: **ForceSizes**=Off, **SourceStartX** =1300, **SourceStartY**=130, **SourceStartZ**=0, **DestinationStartX**=900, **DestinationStartY**=0, **DestinationStartZ**=0, **SizeX**=900, **SizeY**=1000, **SizeZ**=1, **ClearOutside**=Off.

- ➢ Click on **Apply** to view the resulting image in the preview.

- ➢ Click on **OK** to close the dialog.
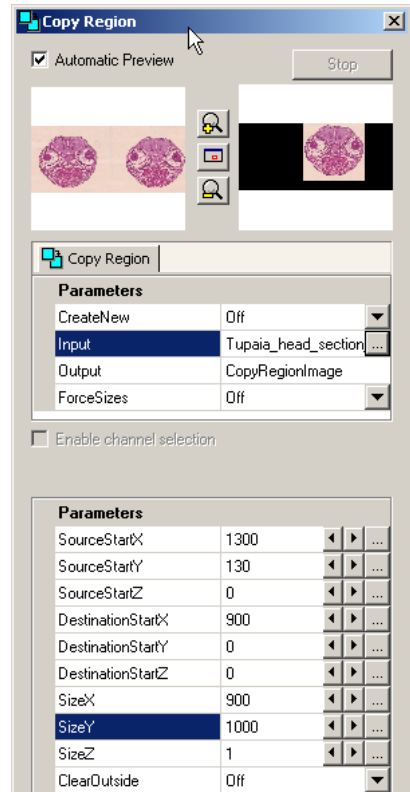
➢ Both embryo heads are now visible in the resulting image.

➢ As the image size has been retained, the image is not completely filled, and contains some black regions. The next step therefore involves cutting out both embryo heads again in full.
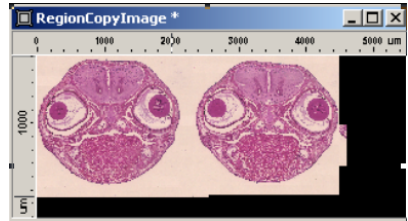

Again select from the **Processing** menu the **Utilities** functional group, and then the **Copy Region** function.

➢ You will now see the dialog window of the **Copy Region** function.

➢ If the **Automatic Preview** check box is activated, the reduced input image appears.

➢ Under **Input** select the resulting image that has been generated, and allocate a new image name under **Output**.

➢ Set the following values by clicking on the arrow keys, or by entering the values directly: **ForceSizes**=On, **SourceStartX** =0, **SourceStartY**=0, **SourceStartZ**=0, **DestinationStartX**=0, **DestinationStartY**=0, **DestinationStartZ**=0, **SizeX**=1750, **SizeY**=800, **SizeZ**=1, **ClearOutside**=Off.

➢ Click on **Apply** to view the resulting image in the preview.
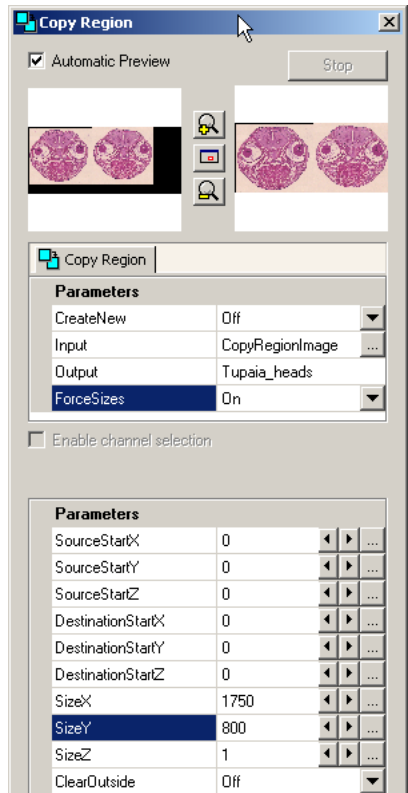
> ➢ The resulting image now
>   shows both embryo heads on
>   an area smaller than that of
>   the original image.

> ➢ The image size has been
>   adapted in accordance with
>   the values for **SizeX** and
>   **SizeY**, as **ForceSizes** was set
>   to **On**.
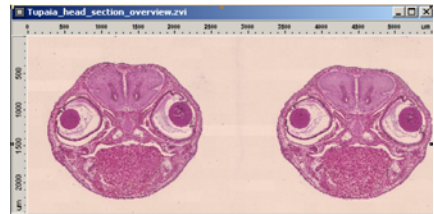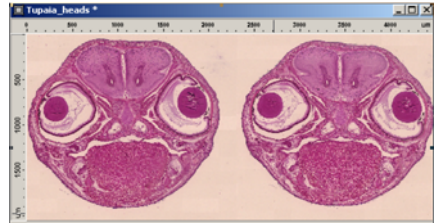
NOTES:
- Adjust the display characteristic curve in the resulting image if
  necessary.

Working with individual color channels

Color images consist of the three superimposed black/white images of the
individual color channels red, green and blue. In many cases you will only
want to use an image processing function for a specific color channel. This
means that you have to split the image up into its individual channels.
These individual color channels appear as black/white images, and allow
you to carry out image processing procedures for the channels
independently of each other. A color image is created again by merging the
individual channels that have been processed.

NOTES:
- The HLS color space (Hue, Lightness, Saturation) is more suitable for
  many image processing requirements. In the HLS model, the image
  can also be split up into its individual channels. To do this, you must
  first perform a conversion into the HLS color space using the **Color
  Model** function.

- Once you have split the image up into its individual channels using the **Split Channels** function, each channel can be processed separately. The individual channels are merged again using the **Combine Channels** function. The combined image must then be converted back into the RGB color space using the **Color Model** function.

The following example explains step by step how to split up a color image into its individual color channels, and merge it again after processing a channel.

➢ Load the image "Bone-overview.zvi" via the function **File** ⟹ **Open Image**, or acquire an image with the camera.

➢ The image is displayed in AxioVision's image window.

Select from the **Processing** menu the **Utilities** functional group, and then the **Split Channels** function.

➢ You will now see the dialog window of the **Split Channels** function.

➢ If the **Automatic Preview** check box is activated, you will see the reduced input image.

➢ Set **CreateNew** to **Off**, so that you will be able to select the input image (**Input**) freely later.

➢ Under **PixelFormat** select "8bit".

➢ Click on **Apply** to view the resulting image in the preview.

➢ Click on **OK** to close the dialog.
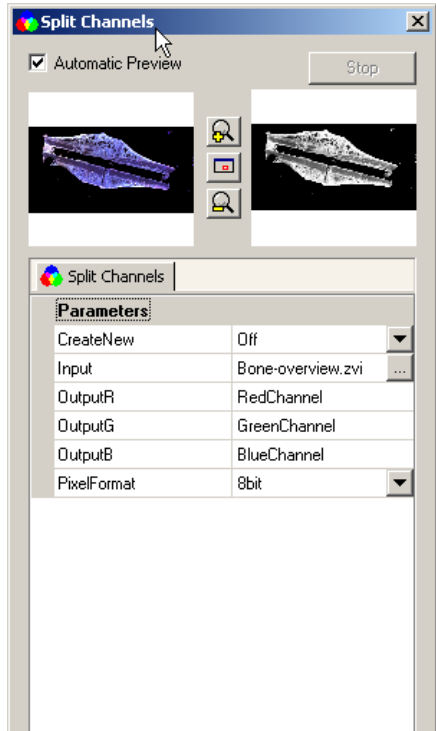
➢ The **Split Channels** function splits up the color image into the individual color channels red, green and blue. The result is a black/white image for each channel.
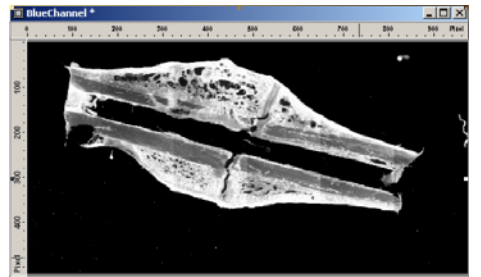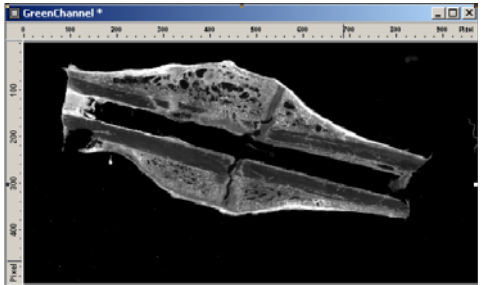
Select from the **Processing** menu the **Adjust** functional group, and then the **Brightness/Contrast** function.

➢ You will now see the dialog window of the **Brightness/Contrast** function.

➢ If the **Automatic Preview** check box is activated, you will see the reduced input image.

➢ If you click on the [...] button in the **Input** field, the image gallery appears. Select the black/white image of the blue channel by clicking on it.

➢ Set the value -0.5 for **Brightness**, the value 1 for **Contrast** and the value 0.5 for **Gamma** by clicking on the arrow keys, or by entering the values directly.

➢ Click on **Apply** to view the resulting image in the preview.

➢ Click on **OK** to close the dialog.

➢ This increases the brightness in the blue channel image.
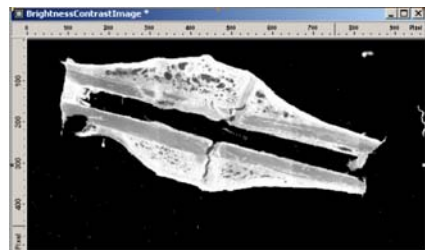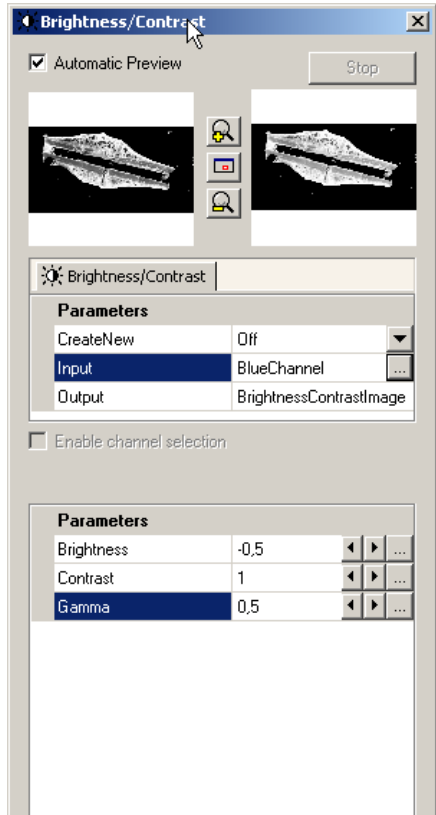
Select from the **Processing** menu the **Utilities** functional group, and then the **Combine Channels** function.

➢ You will now see the dialog window of the **Combine Channels** function.

➢ If the **Automatic Preview** check box is activated, you will see the reduced input image.

➢ If you click on the ⬚ button in the **Input** field, the image gallery appears. Here select the image in question by clicking on it. For **InputR** select the black/white image of the red channel, for **InputG** the black/white image of the green channel, and for **InputB** the enhanced image of the blue channel.

➢ Under **PixelFormat** select "24bit".

➢ Click on **Apply** to view the resulting image in the preview.
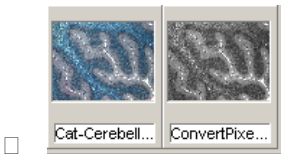
➢ Click on **OK** to close the dialog.

➢ In the resulting image the brightness has been greatly enhanced.
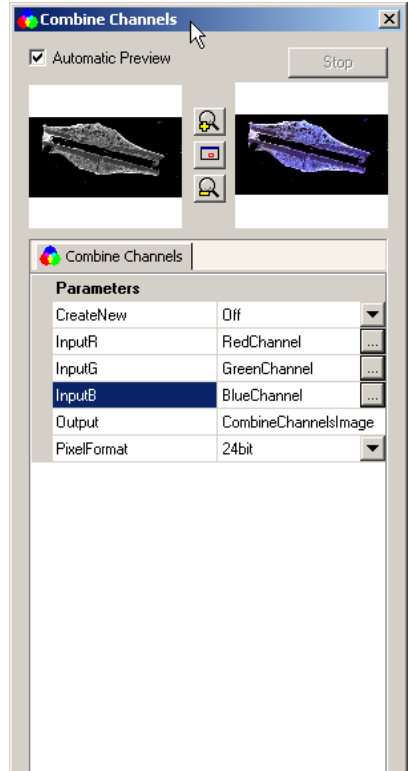
NOTES:

- Adjust the display characteristic curve in the resulting image if necessary.

Converting a color image into a black/white image

NOTES:

- More information about image format and bit depth can be found In chapter 5 "Image Processing"- 5.1 "General".

If images are acquired using a digital camera, they will take the form, depending on the camera, of 10 bit images (or 3x10 = 30 bit color images), 12 bit images (or 3x12 = 36 bit color images) or 14 bit images (or 3x14 = 42 bit color images). An analog camera, on the other hand, only produces images with 8 bits (or 3x8 = 24 bit color images).

The gray value distribution of the image is displayed in the display characteristic curve (**View** menu $\Rightarrow$ **Properties** function $\Rightarrow$ **Display** property page). The number of bits that an image has can be seen from the maximum gray value.

Bits	Maximum gray value
8	256
10	1024
12	4096
14	16384
16	65536

As many programs work with 8 bit images only, or only distinguish between 8 and 16 bits, it is useful to be able to convert images into other bit formats. The **Convert Pixel Format** function therefore also only distinguishes between 8 and 16 bit black/white and 24 or 48 bit color images.

As they have three color channels, color images always require three times as much storage space as black/white images. A 16 bit image requires greater storage space due to the increased gray value information it contains. Often the color information is not required, or 8 bit gray value information is sufficient. For this reason the **Convert Pixel Format** function is suitable for converting a color image into a black/white image, or a 16 bit image into an 8 bit image.

If you want the same image to be converted into various pixel formats, we recommend that you work with the setting **CreateNew = Off**. This allows you not only to select the input image, but also to overwrite or enter a new name for the resulting image. Conversely, with **CreateNew = On**, a new resulting image is always created automatically, starting from the input image.

The following example explains step by step how to convert an image into a different pixel format whilst continuing to use the same input image.

> ➢ Load the image "Cut-Cerebellum(1300).zvi" via the function **File ⇒ Open Image**, or acquire an image with the camera.

> ➢ The image is displayed in AxioVision's image window.

> ➢ Position the cursor over the image, press the right-hand mouse button, and select the **Properties** function from the shortcut menu.
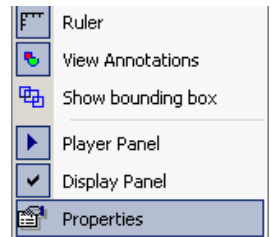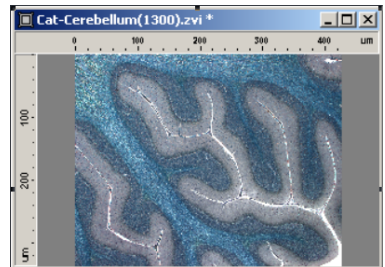
> Click on the **Display** tab sheet, where the image's display characteristic curve is shown. This shows a gray value distribution of 3 x 14 bits for the red, green and blue color channels.
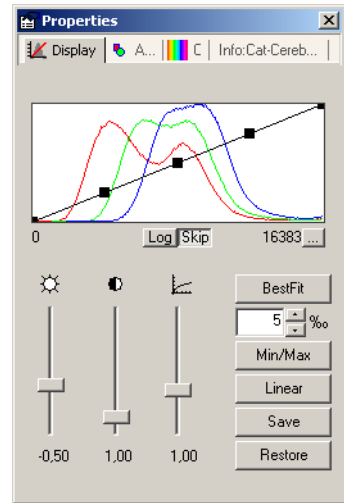

Now select from the **Processing** menu the **Utilities** functional group, and then the **Convert Pixel Format** function.

> You will now see the dialog window of the **Convert Pixel Format** function.

> If the **Automatic Preview** check box is activated, the reduced input image appears.

> Set **CreateNew** to **Off**, so that a different conversion can be executed using the same image later.

> Under **PixelFormat** select 16bit.

> Click on **Apply** to view the resulting image in the preview.

> Click on **OK** to close the dialog.
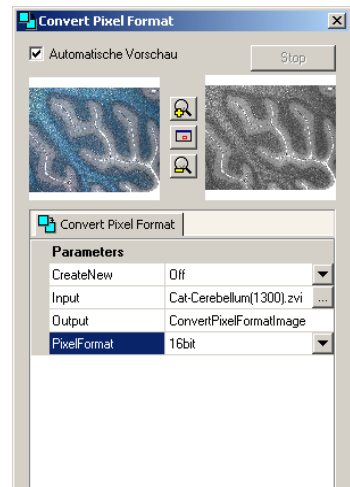
➤ The original image has been converted into a 16 bit black/white image. The display characteristic curve shows the distribution of the gray values.
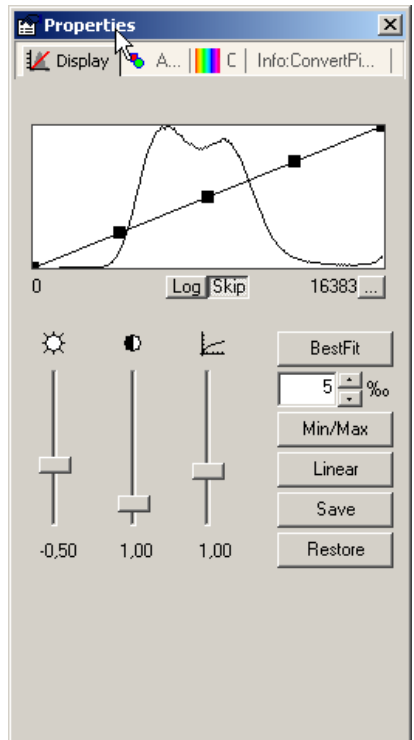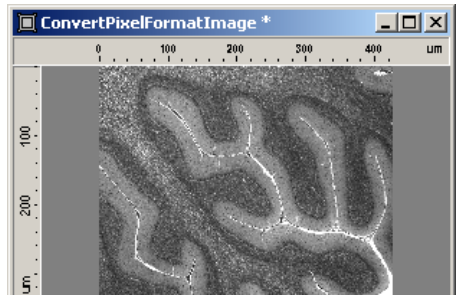
Again select from the **Processing** menu the **Utilities** functional group, and then the **Convert Pixel Format** function.

➢ You will again see the dialog window of the **Convert Pixel Format** function.

➢ If you click on the [...] button in the **Input** field, the image gallery appears. Select the original image by clicking on it.

➢ Under **PixelFormat** select "8bit".

➢ Click on **Apply** to view the resulting image in the preview.

➢ The name of the resulting image remains unchanged. The image is overwritten.

➢ Click on **OK** to close the dialog.
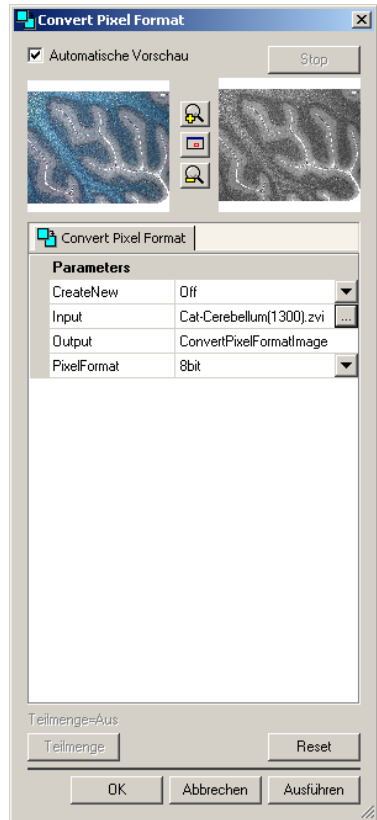
➢ The resulting image is an 8 bit black/white image.
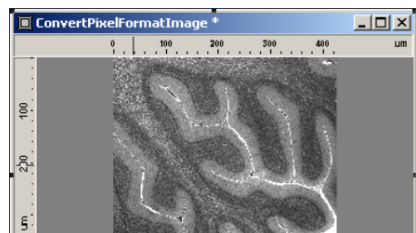
➢ The display characteristic curve of the resulting image now shows the gray value distribution of an 8 bit black/white image.
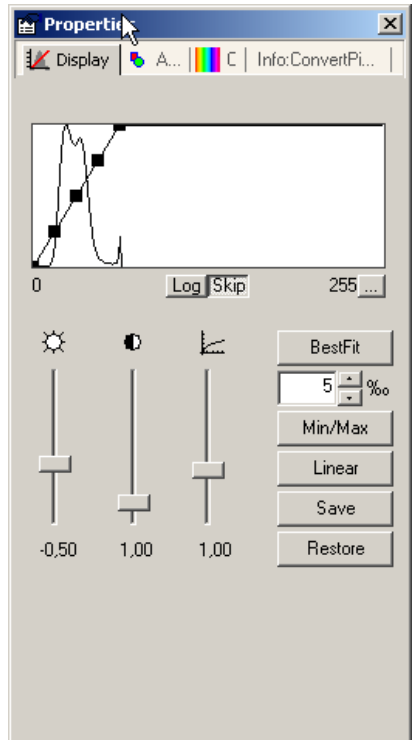
Again select from the **Processing** menu the **Utilities** functional group, and then the **Convert Pixel Format** function.

➢ Select the original image again in the **Input** field.

➢ For **Output**, allocate a new name, so that a new resulting image is generated.

➢ If you click on the [...] button in the **Input** field, the image gallery appears. Select the original image by clicking on it.
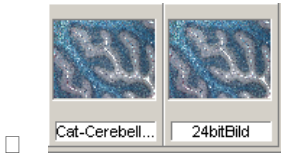
➢ Under **PixelFormat** select "24bit".

➢ Click on **Apply** to view the resulting image in the preview.

➢ Click on **OK** to close the dialog.
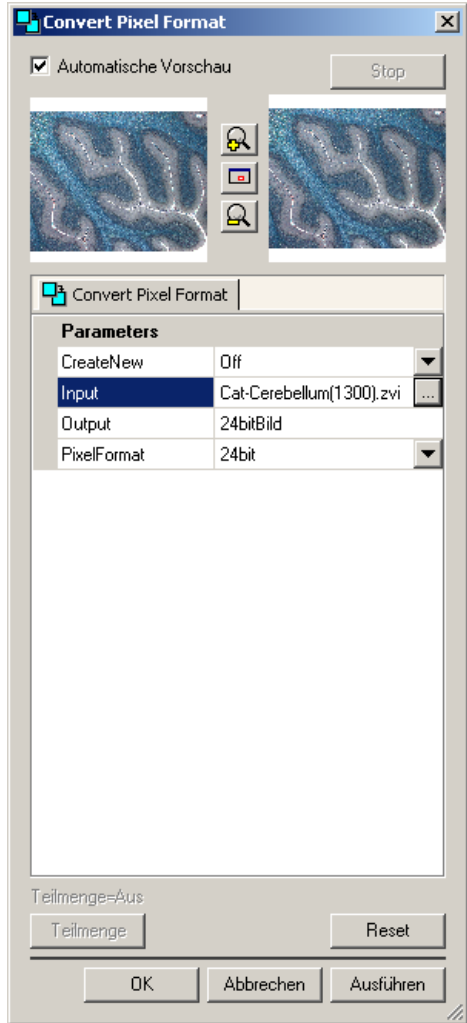
➢ The resulting image is a 24 (3x8) bit color image.
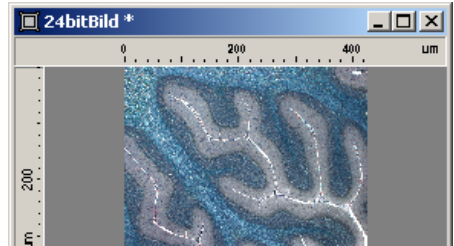
➢ The display characteristic curve of the resulting image shows the gray value distribution of the individual color channels.
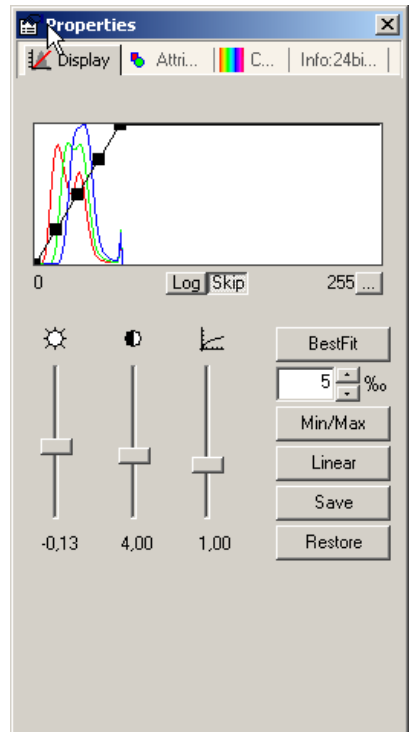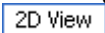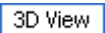
10.2 Inside4D (3D View)

With Inside4D you can display the z-stack fluorescence images you have generated using AxioVision in three dimensions. The Z-stack images have to be black & white (acquired with a monochrome camera), can consist of up to 32 channels and can also be a time lapse series.

To display the third dimension on a two-dimensional medium such as a monitor, the data are projected into a virtual space. The impression of three dimensions can be created either by setting reference points (e.g. a shadow) or by animating the view. A three-dimensional display can be particularly effective for image stacks that have been processed using the **Deconvolution** module, acquired using the **ApoTome** module or acquired using a Confocal Laser Scanning Microscope (LSM).

It is also possible to include time (from time lapse images) in each display as a fourth dimension (simultaneous animation of 3D projection and playback of time sequence).

Note:
- Switching between 2D and 3D View is best done using the buttons
  2D View and 3D View at the lower edge of the image.

Additional buttons
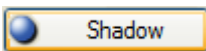
Shadow   activates the **Shadow** rendering mode
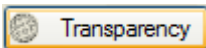
Transparency   activates the **Transparency** rendering mode
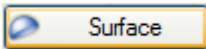
Surface   activates the **Surface** rendering mode
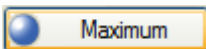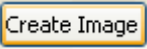
Maximum   activates the **Maximum** rendering mode
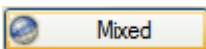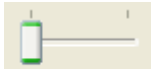
Mixed   activates the **Mixed** rendering mode
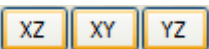
➢ Clicking on the [Settings...] button opens the **Inside4D Settings** dialog. Here detailed settings for the selected rendering modes can be specified. Also creation of a rendered animation series is started from here (see **Series** property page).

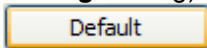➢ The [Create Image] button creates a new unsaved 2D image of the currently displayed rendering.
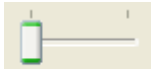
➢ Using the [Precise   Fast] slider, you can reduce the complexity of the image data used for the calculation. The speed of the calculation increases as the complexity of the data decreases. This applies to all display modes, although the slider has different effects (number of pixels, textures, triangles etc.) depending on the mode in question. Even more flexible settings can be made on the **View** property page.
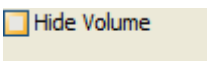
➢ A progress bar appears during the rendering procedure. If the calculation is taking too long, you can interrupt the procedure by clicking on the [▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮] [Abort] button.
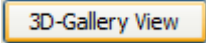
➢ **Clipping** buttons: [XZ] [XY] [YZ]: With these buttons one can introduce three so called **Clipping planes** into the volume. Clipping planes can cut the volume open to unveil structures inside the volume. The labeling of the buttons indicates the clipping planes default orientation.

➢ if the orientation of a clipping plane has been changed (see detailed description of the clipping plane options in the **Clipping** tab of the **Settings** dialog) the default orientation can be regained by using the [Default] button

➢ [☐ Hide Volume] if checked this option hides the volume data. This feature enhances the effect of displaying textures mapped onto **Clipping** planes.

➢ 3D-Gallery View : This button is active in **Surface** mode only (method **Variable detail (graphics card)** and can be used for multichannel z-stacks to display the rendering of individual channels as well as the merge of all channels. All manipulation options for the volume are available as in normal 3D view.

Control elements in the window

The standard view when you first switch into **3D view** is a front view in the **Transparency** mode (graphics card accelerated mode).

➢ You can use the wheels [image] to zoom in and out of the view [image] and/or to rotate the view around the x [image], y [image] and z axes [image].

➢ The mouse function for direct interaction with the image volume can be configured using the five buttons at the center of the left-hand image edge

• The [image] button allows the selection of measurement tools present in the volume. As long as the mouse cursor is not in the vicinity of an endpoint of a measurement tool the cursor symbol [image] is shown. If the cursor moves into the vicinity of an endpoint the cursor shape changes into [image].

• The now selected endpoint is displayed in yellow and can be moved by keeping the left mouse button clicked down and moving the mouse to a new position within the volume. This action also updates the measurement values.

- If in this mode it is possible to temporarily switch into the default mode ⊕ by keeping the **Ctrl+Shift** keys depressed. This permits the quick reorientation of the volume during interactive measurements. If released the cursor resumes the selection shape.

- If the ⊕ button is selected, you can rotate the image volume freely by depressing the left mouse button

- The 🔍 button allows you, with the left mouse button depressed, to zoom into the image by moving the mouse in an upward direction, or to zoom out of the image by moving the mouse in a downward direction

- If the ✋ button is depressed, you can move the image volume laterally, e.g. to make certain structures visible when in zoom mode. You can also activate this function temporarily by pressing the Ctrl key followed by the left mouse button

- With the 🖘 button selected changes the function of the left mouse button into a fly mode. By depressing the left mouse button you can fly into and through the sample. Moving the mouse at the same time changes the flight path. Alternatively the fly mode can also be invoked by pressing the mouse wheel key.

Note:

- The 🖰 and 🖘 button and the associated functionality is only available for graphics card accelerated rendering modes.

There are six additional buttons at the lower edge of the image

➢ You can use the ▣ button at the lower edge of the image to apply a frame to your image stack,

➢ ... the ⌐ button to show the x, y and z axes (x=red, y=blue, z=green) or …

➢ … in the case of scaled data, the ⌐ button to add a scale. These functions help you to get oriented inside the virtual space. If you move the mouse pointer above one of these buttons, a tool tip will be displayed explaining the function.

➢ The ⌐ button hides measurement shapes drawn into the volume (Only available in graphics card accelerated rendering modes)

➢ The ⌂ button returns you to the default front view at any time from within the selected viewing mode. This resets all positions including Shift, Zoom or Flight so that the complete image content is visible in the image frame.

➢ The ⌐ button allows you to touch the object using your mouse and set it into continuous motion ("animation'). This significantly improves the impression of three dimensions.

Note:

• In animation mode a view is re-rendered for each projection. In the case of large image stacks (more than 20 MB) a powerful computer with a good graphics card is required to guarantee smooth animation.

If you have loaded a time lapse image the player bar appears at the bottom edge of the window. You can then play back the time lapse sequence. However, the player does not wait for the rendering of the scene to be completed before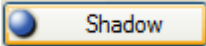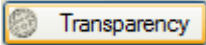 moving on to the next instant. That's why you should select a slower time setting for rendering using the ⊙ button. This opens the **Player interval** dialog window:
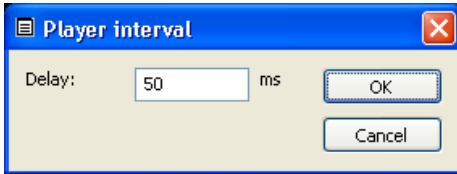
In the **Delay** field enter the desired time in milliseconds and click OK.
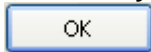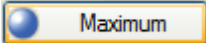
The viewing modes Shadow, Transparency, Surface, Mixed and Maximum

> **Shadow**: In the **Shadow** mode the structures in the image are illuminated by a virtual light source. The volume is viewed as if looking through the eyepieces of the microscope and the light source projects a shadow onto a background plane. This gives the data a reference in space, making visualization much easier. The rendering effect itself consists of a combination of light reflection and opacity (degree of transparency). The display in this mode is only calculated by the main processor (CPU) of the computer (Voxel mode).

> **Transparency**: In the **Transparency** mode a three-dimensional image with a transparent effect is calculated. At least two two-dimensional texture stacks (computed from two different angles) are used depending on the volume orientation (only for the graphics card accelerated rendering mode).

In contrast to the Shadow mode the scene here is illuminated by diffuse white light from the rear. By changing the available parameters one can mix different channels and reveal relationships between information in those channels. This view is particularly useful for visualizing the three-dimensional relationships between structures within the volume. This mode can be displayed both in **CPU** based **Voxel** mode and also (with the help of a suitable graphics card) with accelerated calculations in so-called OpenGL mode.

➢ [Surface] : In the **Surface** mode grey values in the image are computed as solid surfaces (also known as **Isosurfaces**) instead of soft transparent shades. This display mode is useful to focus the attention on certain structures (e.g plant cell walls, nucleoli, intracellular vesicles) while potentially hiding additional structures deeper within. In this mode you also can select between Voxel- (CPU) and OpenGL- (graphics card) display.
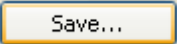
➢ [Maximum] : Clicking on this button switches to **Maximum** projection mode, which calculates a three-dimensional view of the data by only showing pixels of the highest intensity along the projection axis. This view is particularly useful for displaying three-dimensional images in two dimensions, e.g. in publications, and guarantees a maximum possible degree of transparency. The display in this mode can be calculated by the main processor (CPU) of the computer (Voxel mode) or the graphics card in OpenGL mode.

➢ [Mixed] : In **Mixed** mode both transparency and surface modes can be mixed in one volume display. Using this mode one can render small structures within cells such as vesicles or speckles (e.g. FISH signals) as surfaces in one channel of a multichannel image while rendering the surrounding cytoplasm from another channel in transparency mode. This way one can also visualize more complex relationships convincingly.

Settings for the viewing modes

In the **Inside4D Settings** dialog you can adjust settings, which influence the 3D view, on the property pages **Data, Illumination, Series, View, Info, Clipping, Measure, Stereo** and **Special**. You can also create animations as image series (required for movie export) as well as perform simple interactive measurements.

Settings you have made and want to keep can be saved independent of the image and applied to other image stacks. On the **Data** property page click on [Save...] button in the **Settings** field. Now you can name the settings file, to which a *.zv3 ending will be attached. In this setting file all settings you can change in Inside4D will be included. This function is particularly important if you want to observe images of samples under identical conditions, to allow comparison.

Depending on the chosen Rendering method the settings you can change will be different for the **Data**, **Illumination** and **View** property pages. In the following chapters each property page will be explained separately for each rendering mode.

Data property page

On the **Data** property page, select the threshold starting at which individual pixels will be used for the calculation. All pixel values that are not being used are hidden and interpreted as background. Once you have specified the pixels to be used, define the surface composition of the objects. Different parameters are available for this purpose in the individual modes.

Settings

These functions are identical for all render modes.

➢ The ⬚ Load... button loads a previously saved *.zv3 settings and applies it to the z stacks.

➢ ⬚ Save saves the settings you made, independently from the image, so they can be used for other z-stacks. Now you can name the settings file, to which a *.zv3 ending will be attached. All parameters are included in the setting file. This function is particularly important if you want to observe images of samples under identical conditions, to allow comparison.

➢ The ⬚ Save As Default button saves the current setting as default setting, which will be used by all followed images.

➢ The ⬚ Reset button restores the default state.

Note:

- You can create a new image from any current view by clicking on the ⬚ Create Image button on the right side of your image.

Shadow mode

Data

➢ **Channels**: The settings are entered separately for each channel using sliders or by entering a numerical value in the corresponding input field. To select a channel click on the corresponding button (labeled with the channel color, e.g.).

➢ **Threshold** specifies a lower threshold as a percentage of the gray values displayed. This decides which data enter the rendered image. If one fluorescence channel contains a homogeneous fluorescence signal with high signal strength, one would set the threshold higher in order to "protect" signals from weaker channels from being quenched.

➢ **Ramp** controls the degree of the transition from completely transparent to completely opaque.

➢ **Maximum** influences the degree of opacity.

➢ The settings are combined into a histogram . The x axis corresponds to the gray values and the y axis to the degree of opacity. You can also use the mouse to interact with the histogram curve.

➢ **Roughness**: You can also influence the roughness used to display the surface of the image structures. This changes the plasticity of the display (relative scale between 0 and 1). This setting quickly can produce artifacts if pixels reach saturation. The degree of roughness should be reduced accordingly.

Transparency mode

Data

➢ **Channels**: The settings are entered separately for each channel using sliders or by entering a numerical value in the corresponding input field. To select a channel click on the corresponding button (labeled with the channel color, e.g. 🔳).

➢ **Threshold** specifies a lower threshold as a percentage of the gray values displayed (relative scale between 0 and 1). This decides which data enter the rendered image.

➢ **Ramp** controls the degree of the transition from completely transparent to completely opaque (relative scale between 0 and 1).

➢ **Maximum** influences the degree of opacity (relative scale between 0 and 1).

➢ The parameters are schematically illustrated in a histogram. The x axis represents the gray values and the y axis the transparency. You can also use the mouse to interact with the histogram curve.

➢ **Gamma**: With this slider the transparency effect can be further enhanced in a channel specific manner (relative scale between 0 and 1). This parameter influences all pixel gray values in the same way in contrast to the **Ramp** and **Maximum** settings.

Global

➢ **Angle**: If you select the **Texture (graphics card)** method in the **Special** property page, you can change the projection angle used to view the scene anywhere between 0.1° and 89.9°. Use the slider or enter the **Angle** into the input field. The effect of this on the display perspective is similar as if you were viewing the 3D image through a telephoto or wide-angle lens.

➢ If the **Voxel (computer)** method is selected in the **Special** property page, this adjustment cannot be done.

Surface mode

Data

➢ **Channels**: The settings are entered separately for each channel using sliders or by entering a numerical value in the corresponding input field. To select a channel click on the corresponding button (labeled with the channel color, e.g.).

➢ **Threshold**: Sets the gray value threshold on a relative scale between 0 and 1 at which a pixel will belong to the object.

➢ **Ambient**: describes a diffuse, non-directed light source, which lets both bright and dark structures appear darker or brighter in an equal fashion (values on a scale between 0 and 1).

➢ **Specular**: sets a directional brightness (values on a scale between 0 and 1). Influences the differences between dark and bright structures, which are strongest at a value of 0.

➢ **Shininess** changes the surface gloss (on a scale between 0 and 1).

Global

➢ **Angle**: If you select the **Texture (graphics card)** method in the **Special** property page, you can change the projection angle used to view the scene anywhere between 10° and 80°. Use the slider or enter the **Angle** into the input field. The effect of this on the display perspective is similar as if you were viewing the 3D image through a telephoto or wide-angle lens.

➢ If the **Voxel (computer)** method is selected in the **Special** property page, this adjustment cannot be done.

Note:

• If the render method **Maximum** detail (graphics card) is selected on the **View** property page AND on the **Special** property page the option Transparent surfaces is selected, the **Data** property page shows the slider **Transparency**. Using this slider one can assign an additional transparency effect to the surfaces of a channel.

Maximum mode

Data

➢ **Channels**: The settings are entered separately for each channel using sliders or by entering a numerical value in the corresponding input field. To select a channel click on the corresponding button (labeled with the

channel color, e.g.).

➢ **Threshold**: In the **Threshold** area, you can use the slider or value field to set an upper and a lower threshold. Values smaller than the lower threshold value, or greater than the upper threshold value, are not considered in the rendered stack. This setting option is particularly useful for the rendering of multichannel image stacks.

Note:
- Please note, that the setting for upper threshold is only available for the CPU method Voxel (computer) (**View** property page → Mode **Maximum** → Method **Voxel**).

Global

➢ **Angle**: If you select the **Texture (graphics card)** method in the **Special** property page, you can change the projection angle used to view the scene anywhere between 10° and 80°. Use the slider or enter the **Angle** into the input field. The effect of this on the display perspective is similar as if you were viewing the 3D image through a telephoto or wide-angle lens.

➢ If the **Voxel (computer)** method is selected in the **Special** property page, this adjustment cannot be done.

Mixed mode

Data

In the **Mixed mode** a volume can be displayed both in surface and transparency mode. For this is it necessary to define settings for both methods separately for each channel. To accommodate this the Data page has been subdivided in two halves.

➢ **Channels**: The settings are entered separately for each channel using sliders or by entering a numerical value in the corresponding input field. To select a channel click on the corresponding button (labeled with the channel color, e.g.). All changes you make are only applied to the selected channel.

➢ **Transparency**: if checked, the selected channel is shown in **Transparency** mode.

- **Threshold** specifies a lower threshold as a percentage of the gray values displayed (relative scale between 0 and 1). This decides which data enter the rendered image.

- **Ramp** controls the degree of the transition from completely transparent to completely opaque (relative scale between 0 and 1).

- **Maximum** influences the degree of opacity (relative scale between 0 and 1).

- The parameters are schematically illustrated in a histogram . The x axis represents the gray values and the y axis the transparency. You can also use the mouse to interact with the histogram curve.

- **Gamma**: With this slider the transparency effect can be further enhanced in a channel specific manner (relative scale between 0 and 1). This parameter influences all pixel gray values in the same way in contrast to the Ramp and Maximum settings.

➢ **Surface**: if checked, the selected channel is shown in **Surface** mode.

- **Threshold**: Sets the gray value threshold on a relative scale between 0 and 1 at which a pixel will belong to the object.

- **Ambient**: describes a diffuse, non-directed light source, which lets both bright and dark structures appear darker or brighter in an equal fashion (values on a scale between 0 and 1).

- **Specular**: sets a directional brightness (values on a scale between 0 and 1). Influences the differences between dark and bright structures, which are strongest at a value of 0.

- **Shininess** changes the surface gloss (on a scale between 0 - 1).

Global

➢ **Angle**: If you select the **Texture (graphics card)** method in the **Special** property page, you can change the projection angle used to view the scene anywhere between 10° and 80°. Use the slider or enter the **Angle** into the input field. The effect of this on the display perspective is similar as if you were viewing the 3D image through a telephoto or wide-angle lens.

Note:

• The **Mixed** Mode is only available as a graphics card accelerated mode and not as CPU only mode.
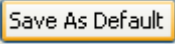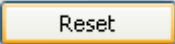
Illumination property page

With the settings made on this property page you influence the light sources, that illuminate the volume as well as the background color. The settings available on **Illumination** are different for each render mode and are therefore explained separately. In contrast to the settings on **Data** property page all illumination settings act on all channels at the same time.
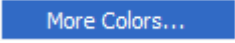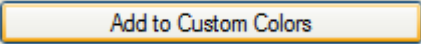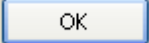
Settings

These functions are identical for all render modes.

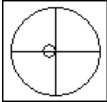➢ The [Load...] button loads a previously saved *.zv3 settings and applies it to the z stacks.

➢ [Save] saves the settings you made, independently from the image, so they can be used for other z-stacks. Now you can name the settings file, to which a *.zv3 ending will be attached. All setting options, with the exception of the lateral position (🖑), are taken into consideration. This function is particularly important if you want to observe images of samples under identical conditions, to allow comparison.

➢ The [Save As Default] button saves the current setting as default setting, which will be used by all followed images.

➢ The [Reset] button restores the default state.

Background
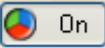
Functions for changing the background color are available in the same fashion for all render modes.

➢ Clicking on the [Choose color...] button opens a window that allows you to select the background color.

- You can either select the background color from 8 standard colors or create your own colors. In the drop down list field click on the [More Colors...] option and select colors from the palette.

- You can also enter color values manually in the value fields. Clicking on the [Add to Custom Colors] button adds the newly selected color to the selected field for user-defined colors.

- To use a user-defined color click on the corresponding color field ("Basic Colors" or "Custom Colors") and confirm by clicking [OK].

Shadow mode

➢ **Brightness** can be controlled via input field or slider bar (value range 0.2 – 4). Fluorescence data usually profit from brightness values > 1.6 when viewed in pseudo color mode (On button in the Player panel).

➢ **Azimuth** describes the angle of the light source above the virtual horizon and can be set by entering values directly or by slider (value range 0°-90°)

➢ **Elongation** describes the horizontal direction of lighting and can be set by entering values directly or by slider (value range -180° to +180°)

➢ You can change the settings also by moving the small circle with the mouse . The position of the light source is represented by the small circle inside the large circle.

Transparency mode

➢ **Brightness** can be controlled via input field or slider bar (value range 0.2 to 4). Fluorescence data usually profit from brightness values > 1.6 when viewed in pseudo color mode (On button in the **Player Panel**).

Surface mode

➢ **Distance** (value range 0.1 – 9-5) determines the distance between light source and volume. At a value of 0.1 the light source is close by, shadow effects etc. are minimal. The further away the light source, the more prominent the shadow effects become ("evening shadows are longer").

➢ **Azimuth** describes the angle of the light source above the virtual horizon and can be set by entering values directly or by slider (value range 0°-90°)

➢ **Elongation** describes the horizontal direction of lighting and can be set by entering values directly or by slider (value range -180° to +180°)

➢ You can change the settings also by moving the small circle with the mouse . The position of the light source is represented by the small circle inside the large circle.
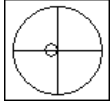
Maximum mode

➢ **Brightness**: Control the brightness of the display using the slider or input field (value range 0.1 to 4). With multichannel images, screen saturation quickly occurs in mixed color mode. If this happens, reduce the overall brightness.

Mixed mode

➢ **Brightness** can be controlled via input field or slider bar (value range 0.2 to 4). Fluorescence data usually profit from brightness values > 1.6 when viewed in pseudo color mode (button in the **Player Panel**).

➢ **Distance** (value range 0.1 – 9-5) determines the distance between light source and volume. At a value of 0.1 the light source is close by, shadow effects etc. are minimal. The further away the light source, the more prominent the shadow effects become ("evening shadows are longer").

➢ **Azimuth** describes the angle of the light source above the virtual horizon and can be set by entering values directly or by slider (value range 0°-90°)

➢ **Elongation** describes the horizontal direction of lighting and can be set by entering values directly or by slider (value range -180° to +180°)

➢ You can change the settings also by moving the small circle with the mouse ⬭. The position of the light source is represented by the small circle inside the large circle.
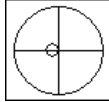
Series property page

Once you have decided on a viewing mode and entered your settings, you can have your view calculated as a series of individual images.

Note:
• This property page is always identical for the **Shadow**, **Transparency**, **Surface**, **Maximum** and **Mixed** modes.

Whereas the ⬚ button (see **Control elements in the window**) produces a live animation, which requires each image to be re-calculated for display, here you can have a series of individual images calculated to represent the animation you would like.
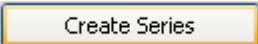
Depending on the image size, the display of an image series like this can be much faster and smoother than a "live" animation, as the views no longer have to be re-rendered. This type of image series can also be exported as a film in Video for Windows (AVI) or QuickTime -format (MOV) with excellent results. However, you can also export an image series into other file formats as a series of individual images.

The settings you make here define the parameters to be used to perform the animation. A minimum required entry is the definition of a start angle as well as an end angle.

Series

➢ **Series**: In this drop-down list box you have the choice between five different options: **X-Axis**, **Y-Axis**, **Z-Axis**. With these first three options you can create the series using a fixed axis (x,y,z), around which the animation turns. If you select one of these options, three sliders appear.

➢ **Views**: This slider specifies the number of images you want to be included in the animation. The greater the number of images you enter, the smoother the display will be. However, the resulting image will also increase in size accordingly. The value range of the slider extends from 1-1000 images. However, you may also enter a higher value in the value field (up to 9999).

➢ **Start Angle** / **End Angle**: These two sliders can be used to specify the start angle and end angle. Here you can make a selection between -360° and +360°. Frequently entering a small range such as -35° to +35° will already give impressive results, smooth "tumbling" without the need for large number of views.

➢ Alternatively, you can also save any views of the object in the space as positions in a position list and generate an animation with changing rotation axes and/or zoom factors. To do this, select in the drop-down list box **Series** the **Position List** option. This opens another drop-down list box.

- Clicking on the [Add] button adds the current position with the values for x, y, z and d (for zoom factor). A position also incorporates a lateral shift as well as the position of the time player (when rendering time series), both parameters are not shown in the list though. The [Add] button is also available on the right hand side directly next to the rendering window.

- Now change the orientation of the object in the image window as required and save the new position.
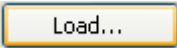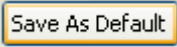
- You can delete one or more positions by clicking [Remove] or delete all positions by clicking on [Remove all]. A double click on a position entry will invoke this setting on the image.

➢ If you have created a time lapse image, you can generate a time lapse series of the 3D view you have just selected by choosing the **Time Series** option from the drop down list. Please be aware, that it might be better in many cases to use the position list option instead in order to combine both spatial and temporal changes in the render series. This will enhance the 3D effect on 4D movies.

➢ If you want to generate a series using the settings you have made on the **Data** and **Illumination** property pages, select the **Use Settings** or **Without Settings** function from the drop-down list box at the bottom edge of the position list. Using this feature you can make one channel disappear by setting the threshold to 100%. Using this technique you can demonstrate structures otherwise hidden behind dominant signals from other channels.

➢ You always generate the series by clicking on [Create Series]. The result is a new image that corresponds to a time lapse image in terms of its operation (player bar). Additionally, you may start and stop a preview of your render series by clicking on the [Start] [Stop] buttons on the right hand side directly next to the rendering window. This preview uses the number of views that are adjusted by the views slider but excludes some options and details of the final render series (e.g. clipping planes) to enable a fast preview.

The new image series document can be saved as a ZVI-image or exported in another format, such as Video for Windows (AVI).

Notes:

- It can take up to several minutes to calculate complex image series with large numbers of images and/or positions.

- In uncompressed form, complex series can become extremely large. This can make it difficult to import them into e.g. Microsoft PowerPoint™ for a presentation. It is therefore recommended that you compress the AVI or QuickTime files during export (for help on this subject, see the **Export** help topic).

Settings

➢ The ⬚ Load... ⬚ button loads a previously saved *.zv3 settings including stored position lists and applies it to the z stacks.

➢ ⬚ Speichern... ⬚ saves the settings including positions you made independent from the image, so they can be used for other z-stacks. Now you can name the settings file, to which an *.zv3 extension will be attached. All setting options are applied. This function is particularly important if you want to observe images of samples under identical conditions, to allow comparison.

➢ The ⬚ Save As Default ⬚ button saves the current setting as default setting, which will be used by all followed images.

➢ The ⬚ Reset ⬚ button restores the default state.

View property page

On this page, you can choose amongst the following viewing parameters: geometric parameters, volume resolution as well as settings influencing the rendering method.

Shadow mode

Geometry

➢ **Angles (x,y,z):** shows the current orientation of the volume in degrees (-180 to 180) with respect to the main axis (x, y, z). You can also define an angle by entering a desired value in the edit fields.

➢ **Center (x,y,z):** describes the relative position of the rotation center to the center of the 3D stack. This translates into a lateral shift of the volume and is best defined by the 🖑 tool. The values are given in units of volume diagonals (x, y, z): the diagonal is set to 1. For the home position all values equal 0

➢ **Zoom**: defines the magnification factor (value range 0-1000).

Note:
• Stretch Z is not active in Shadow mode.

Resolution

➢ **Reduction XY / Reduction Z**: In this field, for shadow mode you can reduce the number of pixels used for the volume rendering step by step in both the lateral (x,y) and axial (z) direction (subsampling). In some cases this can significantly reduce the time for the calculation.

- To do this, move the corresponding slider, or enter the desired value in the edit field. A reduction value of 2 (x,y) combines 2 pixels in the x and y directions, reducing the data volume by a factor of four. A value of 2 for z combines two z-planes in one single plane, reducing the data volume by half.

- The more you reduce the data, however, the smaller the benefits in terms of speed. As a rule, a reduction of 2-3 is sufficient for a smooth display.

- To get the optimum effect you can also adjust these settings on the **Info** property page. There the performance measurements give a direct feedback on the speed improvement.

Rendering Mode

➢ Here you can also switch between the different modes (using the drop-down list box). In the OpenGL accelerated modes, here you can also change the rendering method being used (using the drop-down list box).

➢ **Method**: As in shadow mode only the computer's processor ("CPU method") is used, the **Method** drop-down list box remains inactive.

➢ Shadow ☑: hides the shadow if unchecked

➢ **Distance:** regulates the distance between shadow and volume using the slider or entering the desired value in the edit field (value range 0.5 to 3)

➢ **Use Depth Coding:** activates a depth coding in the z-dimension that ranges from blue over green, orange and yellow to red. Depth Coding effects only the rendering modes Maximum, Transparency and transparent parts of the Mixed mode.

➢ **Draw Depth Coding Scale:** draws a color coded scaling below the rendered image. This option is only available for the Transparency rendering mode of the CPU method Voxel (computer)

Note:
• Stretch Z is not active in Shadow mode

Measure

Here you can find some options of the module 3D Measurements. These functions are described in another section.

Transparency mode

Geometry

➢ **Angles (x,y,z):** shows the current orientation of the volume in degrees (-180 to 180) with respect to the main axis (x, y, z). You can also define an angle by entering a desired value in the edit fields.

➢ **Center (x,y,z):** describes the relative position of the rotation center to the center of the 3D stack. This translates into a lateral shift of the volume and is best defined by the 🖑 tool. The values are given in units of volume diagonals (x, y, z): the diagonal is set to 1. For the home position all values equal 0.

➢ **Zoom**: defines the magnification factor (value range 0-1000).

➢ **Stretch Z** stretches the volume display in axial (z) direction. Entering values >1 (value range 0-20) increased the height of flat structures.

Note:
• Stretch Z is only active for the graphic card method

Resolution

➢ **Reduction (xy)** / **Reduction (z)**: For Transparency mode you can reduce the number of pixels used for the calculation step by step in both the lateral (x,y) and axial (z) directions (subsampling). In some cases this can significantly reduce the duration of the calculation.

- To do this, move the corresponding slider, or enter the desired value in the input field. A reduction value of 2 for x,y, for example, combines 2 pixels in the x and y directions, reducing the data volume by a factor of 4. A value of 2 for z combines two z-planes in one single plane, reducing the data volume by half.

- The more you reduce the data, however, the smaller the benefits in terms of speed. As a rule, a reduction of 2-3 is sufficient for a smooth display.

- You can also adjust the resolution on the **Info** property page where you can see the performance values of the rendering directly.

- The ability of the graphics card to hold all texture planes in its memory is a key factor in determining whether the image stack can be rendered smoothly (animation mode!) with the **Texture (graphics card)** method. You should therefore set the sliders in such a way that in the info field on the **Info** property page, the value `"Nr. Texture planes = all"` is displayed.

Rendering Mode

➢ Here you can also switch between the different modes (using the drop-down list box). In the OpenGL accelerated modes, here you can also change the rendering method being used (using the drop-down list box).

➢ **Method**: In the Transparency mode, the two options **Voxel (computer)** and **Texture (graphics card)** are available.

- With the **Voxel (computer)** method, rendering is performed using the main processor only, without OpenGL support; the model that forms the basis for the calculation is also less complex. As a result, this method is usually faster on slow computers with small graphics cards. The results, however, are also of reduced quality.

- The more complex method **Texture (graphics card)** gives the 3D images surface structures, so-called textures, which are in turn constructed from individual elements (texels). The fewer elements required, the faster the rendering speed (images/s).

Surface mode

Geometry

➢ **Angles (x,y,z):** shows the current orientation of the volume in degrees (-180 to 180) with respect to the main axis (x, y, z). You can also define an angle by entering a desired value in the edit fields.

➢ **Center (x,y,z):** describes the relative position of the rotation center to the center of the 3D stack. This translates into a lateral shift of the volume and is best defined by the ✍ tool. The values are given in units of volume diagonals (x, y, z): the diagonal is set to 1. For the home position all values equal 0

➢ **Zoom**: defines the magnification factor (value range 0-1000).

➢ **Stretch Z** stretches the volume display in axial (z) direction. Entering values >1 (value range 0-20) increased the height of flat structures.

Note:
- Stretch Z is only active for the graphic card method.

Resolution

➢ **Reduction (xy)** / **Reduction (z)**: For Surface mode you can reduce the number of pixels used for the calculation step by step in both the lateral (x,y) and axial (z) directions (subsampling). In some cases this can significantly reduce the duration of the calculation.

- To do this, move the corresponding slider, or enter the desired value in the input field. A reduction value of 2 for x,y, for example, combines 2 pixels in the x and y directions, reducing the data volume by a factor of 4. A value of 2 for z combines two z-planes in one single plane, reducing the data volume by half.

- The more you reduce the data, however, the smaller the benefits in terms of speed. As a rule, a reduction of 2-3 is sufficient for a smooth display.

- You can also adjust the resolution on the **Info** property page where you can see the performance values of the rendering directly.

- In Surface mode, here you can also determine the maximum number of triangles used for calculation. The fewer triangles used by the model, the higher the rendering speed. At the same time, this also reduces the level of detail.

- The more you reduce the data, however, the smaller the benefits in terms of speed. As a rule, a reduction of 2-3 in the x,y and z directions is sufficient for a smooth display. The number of triangles should not be less than 5000, as otherwise the quality of the resolution is insufficient. To achieve the same quality in the **Variable detail (graphics card)** mode as in the **Maximum detail (graphics card)** mode, the maximum number of triangles must be set, depending on the image data, to several million (entry of figures in the value field).

Rendering Mode

➤ Using the drop-down list box you can switch between the modes. In the Open GL accelerated modes you can in addition change the render method used (drop down list box).

➤ **Method**: Three methods are available, which you can select in the drop-down list box: The method **Voxel (computer)**, the graphics-card-accelerated method **Variable detail (graphics card)** and the method **Maximum detail (graphics card)**.

- With the **Voxel (computer)** method, rendering is performed using the main processor only, without OpenGL support; the model that forms the basis for the calculation is also less complex. As a result, this method is faster on slow computers with small graphics cards. The results, however, are also of reduced quality.

- Depending on the size of the data, the method **Variable detail (graphics card)** can be accelerated by the OpenGL-compatible graphics card.

- The **Maximum detail (graphics card)** method calculates the surface model layer by layer. Here the available channels are added to the model one after the other. This process takes significantly longer than with the **Variable detail (graphics card)** method, which attempts to keep all image data in the graphics card's memory. On the other hand, this method usually produces the best results.

Maximum mode

Geometry

➤ **Angles (x,y,z):** shows the current orientation of the volume in degrees (-180 to 180) with respect to the main axis (x, y, z). You can also define an angle by entering a desired value in the edit fields.

➢ **Center (x,y,z):** describes the relative position of the rotation center to the center of the 3D stack. This translates into a lateral shift of the volume and is best defined by the ![hand tool] tool. The values are given in units of volume diagonals (x, y, z): the diagonal is set to 1. For the home position all values equal 0

➢ **Zoom**: defines the magnification factor (value range 0-1000).

➢ **Stretch Z** stretches the volume display in axial (z) direction. Entering values >1 (value range 0-20) increased the height of flat structures.

Note:
• Stretch Z is only active for the graphic card method

Resolution

➢ **Reduction (xy)** / **Reduction (z)**: For Maximum mode you can reduce the number of pixels used for the calculation step by step in both the lateral (x,y) and axial (z) directions (subsampling). In some cases this can significantly reduce the duration of the calculation.

   • To do this, move the corresponding slider, or enter the desired value in the input field. A reduction value of 2 for x,y, for example, combines 2 pixels in the x and y directions, reducing the data volume by a factor of 4. A value of 2 for z combines two z-planes in one single plane, reducing the data volume by half.

   • The more you reduce the data, however, the smaller the benefits in terms of speed. As a rule, a reduction of 2-3 is sufficient for a smooth display.

   • You can also adjust the resolution on the **Info** property page where you can see the performance values of the rendering directly.

- In contrast to the **Transparency** and **Surface** modes **Maximum** rendering is based upon a much simpler calculation model. As a result the rendering speed increase by data reduction is not as pronounced as with the other modes. It is for this reason, that the Maximum mode is very suitable for the rendering of large volume stacks.

- To get the optimum effect you can also adjust these settings on the **Info** tab. There the performance measurements give a direct feedback on the speed improvement.

Rendering Mode

➢ Here you can also switch between the different modes (using the drop-down list box). In the OpenGL accelerated modes, here you can also change the rendering method being used (using the drop-down list box).

➢ **Method**: In the Transparency mode, the two options **Voxel (computer)** and **Maximum (graphics card)** are available.

- With the **Voxel (computer)** method, rendering is performed using the main processor only, without OpenGL support; the model that forms the basis for the calculation is also less complex. As a result, this method is usually faster on slow computers with small graphics cards. The results, however, are also of reduced quality.

- The more complex method **Maximum (graphics card)** performs the maximum projection calculation on the graphics card and produces faster and better rendering results.

Mixed Mode

Geometry

➢ **Angles (x,y,z):** shows the current orientation of the volume in degrees (-180 to 180) with respect to the main axis (x, y, z). You can also define an angle by entering a desired value in the edit fields.

➢ **Center (x,y,z):** describes the relative position of the rotation center to the center of the 3D stack. This translates into a lateral shift of the volume and is best defined by the 🖐 tool. The values are given in units of volume diagonals (x, y, z): the diagonal is set to 1. For the home position all values equal 0.

➢ **Zoom**: defines the magnification factor (value range 0-1000).

➢ **Stretch Z** stretches the volume display in axial (z) direction. Entering values >1 (value range 0-20) increased the height of flat structures.

Resolution

➢ **Reduction (xy)** / **Reduction (z)**: For Transparency mode you can reduce the number of pixels used for the calculation step by step in both the lateral (x,y) and axial (z) directions (subsampling). In some cases this can significantly reduce the duration of the calculation.

- To do this, move the corresponding slider, or enter the desired value in the input field. A reduction value of 2 for x,y, for example, combines 2 pixels in the x and y directions, reducing the data volume by a factor of 4. A value of 2 for z combines two z-planes in one single plane, reducing the data volume by half.

- The more you reduce the data, however, the smaller the benefits in terms of speed. As a rule, a reduction of 2-3 is sufficient for a smooth display.

- You can also adjust the resolution on the **Info** property page where you can see the performance values of the rendering directly.

- The parameter **Triangles** as available for **Surface** mode is not available for **Mixed** mode.

Rendering Mode

➢ Here you can also switch between the different modes (using the drop-down list box). For Mixed mode only the OpenGL accelerated method calculated with the graphics card is available.

Info property page

This property page shows performance values for the different render modes. With the settings in the **Resolution** field, you can accelerate the rendering speed significantly for large volumes.

Info

The **Info** field on this property page shows performance values that are designed to help you achieve a realistic estimate of the rendering speed you can expect. Partially different parameters will be displayed for the different render modes.

For all render modes:

➢ **Performance**: You are given a time value (in msec) for each image calculated resulting in a Frames/second value.

➢ **Angles**: You can also still see the angle positions that are currently set. This can prove useful for the precise orientation of image stacks in space. The angle positions can be changed with the control elements of the image or on the **View** property page.

➢ **Center**: You can change the center of the image with the control elements of the image or on the **View** property page.

➢ **Zoom**: The current zoom factor of the display. You can change the zoom factor with the control elements of the image or on the **View** property page.

➢ Additional parameters for **Transparency**:

- Number of calculated Texture elements per second

- Number of calculated pixels per second

- Number of loaded texture planes: Maximum rendering speed is only obtainable, if all texture planes can be loaded in the graphic card's memory (Nr. Texture planes = all)

- Number of rendered textures as well as texels, bytes and pixels (all in millions)

- **Nr. of loaded …** shows number and size of textures during the process of loading them into the memory of the graphics card

➢ Additional parameters for **Surface**

- Number of calculated triangles / second

- Number of calculated vertices (= points where the line segments of two triangles meet each other)

- Total number of triangles

- Total number of vertices

Resolution

➢ **Reduction XY** / **Reduction Z**: In this field you can reduce the number of pixels used for the calculation in both the lateral (x,y) and axial (z) directions (subsampling). In some cases this can significantly reduce the duration of the calculation.

- To do this, move the corresponding slider, or enter the desired value in the input field. A reduction value of 2 (x,y) combines 2 pixels in the x and y directions, reducing the data volume by a factor of 4. A value of 2 for z combines two z-planes in one single plane, reducing the data volume by half.

- The more you reduce the data, however, the smaller the benefits in terms of speed. As a rule, a reduction of 2-3 is sufficient for a smooth display.

➢ **Triangles**: In **Surface** mode, here you can also determine the maximum number of triangles used. The fewer triangles used by the model, the higher the rendering speed. At the same time, this also reduces the level of detail.

- The number of triangles should not be less than 5000, as otherwise the quality of the resolution is insufficient.

- To achieve the same quality in the **Variable detail (graphics card)** mode as in the **Maximum detail (graphics card)** mode, the maximum number of triangles must be set, depending on the image data, to several million (entry of figures in the value field).

Clipping property page

Inside4D features up to three so called **Clipping planes**. These planes can be used to cut the volume open in order to visualize structures which are within the volume. Clipping planes make most sense when used in the **Surface** mode but can also be used with limited functionality with the rendering modes **Maximum** and **Transparency**.

In **Surface** mode clipping planes can also be used in a channel specific manner. This means, that in a multichannel image one can selectively cut away portions of the volume in one channel in order to unveil other structures from other channels. This technique also works in **Mixed** mode.

A further possibility is, to use clipping planes in order to display only the gray values of the volume which touch the clipping plane.

All these functions can be used for creating a render series in order to make spectacular movies for presentation.

There are three clipping planes, which are named according to their default orientation with respect to the volume axis: **XY**, **YZ**, **XZ**. All the parameters influencing a clipping plane can be found on the corresponding tab:

XY	YZ	XZ

.
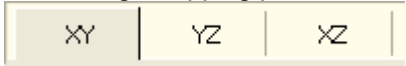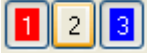
When set, each plane is placed into the center of the volume and oriented in orthogonal fashion according to its name (XY, XZ, YZ).

Clipping

➢ **Clip mode**: Here you can select between 4 clipping modes:

- **None**: Plane has no clipping function.

- **Back**: Plane cuts away the background.

- **Front**: Plane cuts away the foreground.

- **Both**: Plane cuts away both fore- and background. This mode is similar to the check box **Hide Volume** on the right side bar of the render window.

➢ : Here you can choose which channel should be clipped. Simply disable the channel you do not want to clip by clicking on the appropriate channel button: → channel 1 and 3 will be clipped, channel 2 not. This function is only available for the modes **Surface** and **Mixed**.
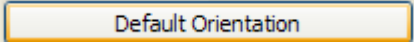
➢ : This check box is available for the modes Surface, Maximum and Mixed. This option clips all transparently rendered channels in a global fashion. For technical reasons it is not possible to correctly clip transparent volumes in a channel specific manner.

Plane Parameters

➢ **Draw Mode**: From this drop down box one of 6 options can be selected, which influence the way, the clipping plane is drawn:

- **None**: plane is transparent

- **Colored:** clipping plane is colored in a semitransparent fashion with a green hue

- **Binary:** Gray levels above the defined threshold, which are touched by the plane are displayed binary white, black pixels are opaque

- **Transparent (linear):** Data, which are touched by the plane, are displayed as in the render mode **Transparent** but in a two-dimensional projection. The ramp for the transparency in this case is always linear. Black pixels are transparent.

- **Transparent (with settings):** Data, which are touched by the plane, are displayed as in the render mode **Transparent** but in a two-dimensional projection. The ramp for the transparency is as defined on the **Data** property page. Black pixels are transparent.

- **Opaque:** as with **Transparent (with settings)** but in this case black Pixels are non-transparent, rendered volume data in the background do not shine through.

➢ ☑ Draw Outline : draws a green frame outlining the plane. Especially useful, it plane is in **draw mode None**.

➢ [Default Orientation] : With this button the plane can be reset to its default orientation

➢ **Horizontal:** allows the rotation of the clipping plane along the horizontal volume axis (oblique orientation)

➢ **Axial:** allows defining an oblique orientation of the plane along the z-axis of the volume.

➢ **Position:** shifts the plan along the planes perpendicular.

Measure property page

Inside4D features 5 tools for interactive measurements in Euclidian space. Measurements can be performed in all graphic-card accelerated modes (**Transparency**, **Surface**, **Maximum** and **Mixed**). The measured tools are shown as shapes in the rendered volume and can be changed there interactively. Measurement values can be exported as a table for downstream processing in other programs.
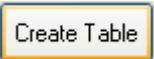
First a suitable tool has to be chosen. In order for measurements to work, the mouse cursor must "recognize" a structure. In the **Surface** mode this is a uniquely identifiable surface structure, for the transparent modes the tools looks for the local gray level maximum which is closest to the observer or if ambiguous, for the topmost drawn texel.
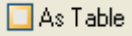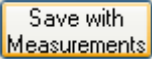
As a sign, that the mouse cursor has "found" a structure, the cursor shape changes from ⊘ into ⊕. The found measurement point is drawn as a blue circle.

Interactive 3D-Measurement

The measurement dialog contains a toolbar with the available measurement tools.

➢   If pressed, the **Keep tool** button keeps the chosen measurement tool active to enable multiple measurements. If depressed a new tool can be chosen after measurements.

➢   The **Line** tool measures the length along a line in µm. First click on a starting point and move the mouse to the desired end point while keeping the left mouse key depressed. The measurement is concluded upon releasing the left mouse key.

➢ The **Angle** measurement tool (equivalent to the 2D measurement tool **Angle 3**) defines an angle between two connected line segments. First define the starting point by clicking, then draw the first line segment until you click on the first endpoint. Now draw the second line segment and conclude the measurement by clicking onto the second end point. This measures the angle in degree.

➢ The **Marker** tool defines and marks structures in a volume and produces the x,y and z – coordinates (in μm)

➢ The **Curve** tool measures the total length (μm) along a variable multi-segmented polygon line. Please click from point to point and finish the measurement by clicking with the right mouse key.

➢ With the Star tool one can measure distances between an anchor point and multiple line segments. First define the anchor point by left mouse click followed by multiple clicks on the desired target points. The measurement is concluded by clicking with the right mouse key. Both the cumulated length as well as the distance of each individual segment are produced

➢ Measurement values are compiled in a list. In order to delete a measurement, select it in the list and press the button. One can also delete all measurements by clicking on .

➢ The measurements can be copied into the clipboard with the button or created as a new data table in AxioVision by clicking on button.

➢ The checkbox ☐ As Table transposes the data into table format.

➢ Measurements are only transiently drawn into the volume and are not stored automatically with the image. This is mainly due to the fact, that measurements much depend on all the other render settings. Both render settings and current measurements can be saved as a *.zv3 settings file by clicking on Save with Measurements. Enter the desired name in the **Save Inside4D Settings As** dialog and click on **Save.**

➢ In order to load a measurement settings file, click on Load... and select the desired settings file from the **Load Render Setting** dialog.

Stereo property page

Sometimes the display of three-dimensional structures on a two dimensional display can be difficult. In this case it can be helpful to display volumes as **Anaglyphs**. The volume is separated into two separate views which are calculated as if viewed through two different cameras. Both cameras are positioned in a defined distance from each other. Each view is colored differently (red and green) so that when viewed through red-green glasses, a 3 dimensional impression is created for the observer.

The Stereo view can be invoked for all graphic card accelerated rendering modes (**Transparency**, **Surface**, **Maximum** and **Mixed**).

Stereo

➢ **Stereo Mode**: enables (**Anaglyph Stereo)** or disables (**None**) the stereo mode.

➢ **Channel**: The drop down box contains the options **Both**, **Left** and **Right**. Only in **Both** is the analglyph stereo effect visible. The other modes each turn off the other channel.

➢ **Camera**: Defines the distance of both cameras from each other (Units in meters).

➢ **Parallax Shift**: Determines the degree of shift which is necessary to correlate both camera views. The slider should be adjusted to maximize the stereo effect. Please note, that this setting will be different for each observer.

Special property page

➢ **Inner Surface Lighting** : If checked, illuminates the inside of a surface rendered volume. If unchecked, the inside is dark. If one views the inside of a volume by flying or zooming into it, the inside illumination is helpful. This option is only available for the **Surface** mode.

➢ **Front and Back Side Lit Differently** : with activated **Inner surface lighting**, this checkbox colors the inside lighting with a bluish hue. This makes distinguishing inside from outside easier. This option is only available for the **Surface** mode.

➢ **Draw Border Triangles** : If checked, closes structures that are cut by the bottom or top of a z-stack. This option only affects the Surface mode.

➢ **Transparent Surfaces ('Max.Detail' only** : This option allows transparency to be assigned to surface rendered volumes in a channel specific manner. If checked, the slider **Transparency** will be available on the **Data** property page. This option is only available for the **Surface** mode if the method **Maximum Detail** is chosen on the **View** property page. Please be aware, that with multichannel volumes the transparency effect depends on the order in which the channels are rendered. This technical limitation does not always lead to satisfactory results.

➢ **Gallery - Draw combined image** If checked, this option displays the merge image including all channels of a multichannel volume in **3D-Gallery View**. This option is only available for the **Surface** mode.

- ➢ **Texture-Switch**: This parameter influences the volume angle, upon which the next set of 2D-Textures will be loaded into the graphics card (value range 1-100 degree). Depending on the object orientation, the number of z-slices and the chosen render settings this loading process can be visible and may be disturbing especially when rendering a series for movie export. Changing this angle can postpone or even avoid this switch during a series rendering. This Option is only available for **Transparency** and **Maximum** modes.

- ➢ Fly-Speed : Influences the fly speed (value range 0.0001 – 0.9999) when flying through a volume.

- ➢ **Mouse Sensit**.: Influences the mouse sensitivity (value range 1-720). Small values make the mouse actions precise but slow, large values less precise but faster.

VRML property page

VRML (Virtual Reality Markup Language) is a standard file format for representing 3D-graphics. It has been designed particularly with the world wide web (WWW) in mind. VRML files are commonly called "worlds" and have the *.wrl file extension. To open and view these files a suitable web browser with a VRML PlugIn is required.

10.3 Deconvolution

A prerequisite for 2D deconvolution is a 2-dimensional image with 8-16 bit gray levels. All functions that are not necessary for 2D deconvolution are inactive.

A prerequisite for 3D deconvolution is an 8-16 bit gray level image in the form of a z-stack. To acquire a z-stack using AxioVision, the optional Z-stack module is required. If you wish to acquire z-stacks in several fluorescence channels, you will also require the Multichannel Fluorescence module.

The following processing methods (see next page) can be used for the relevant image types in deconvolution:

Frame rate	2D Deconvolution		3D Deconvolution	
2D Fluorescence	NN		NN	x
	RIF	x	RIF	x
	FIT	x	FIT	x
	CIT	x	CIT	x
Z-Stack Fluores-cence	NN		NN	x
	RIF	x	RIF	x
	FIT	x	FIT	x
	CIT	x	CIT	x
3D ApoTome SIM	NN		NN	
	RIF		RIF	x
	FIT		FIT	
	CIT		CIT	

NN: Nearest Neighbor
RIF: Regularized Inverse Filter
FIT: Fast Iterativ
CIT: Constrained Iterativ

How do I perform deconvolution using the Regularized Inverse Filter?

➢ Load a suitable image. On the AxioVision Viewer CD, you will find the image "FluoCells_DCV-Teststack.zvi". This is a 3-channel z-stack image, and is a good image to use here.

➢ In the work area, switch to the **Deconvolution** entry.
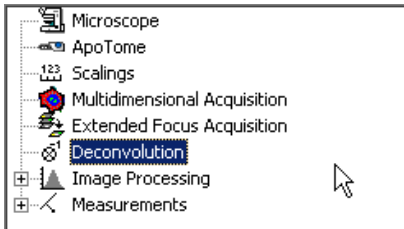
➢ This opens the **Deconvolution** property page. First of all, deactivate the **CreateNew** function. This setting ensures that only one resulting image is generated, and that the input image remains selected. This allows you to change parameters as often as you like, without a new resulting image being generated each time.
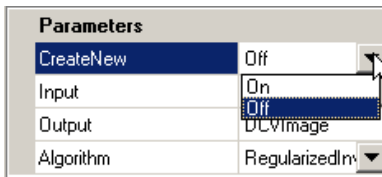
➢ If the desired image was in the foreground when **Deconvolution** was selected, it is now loaded as the input image. Otherwise load it by clicking on the ⚏ button and selecting the image in the gallery:

FluoCells_D...
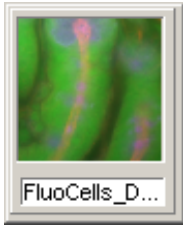
➢ The name of the resulting image can be changed. We recommend that you include in this an indication of the method used:

| Output | FluoCell_Inverse... |

.

➢ The **Regularized Inverse Filter** method is already selected.

➢ In the **Channel specific settings** section, you can enter settings that may differ from channel to channel, e.g. the desired reconstruction strength. For the second channel (green), select a manual strength setting:
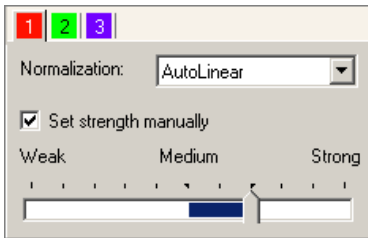
Leave the other channels on the automatic strength setting.

➢ Leave the **AutoLinear** setting for normalization.

➢ Define a sub-region using the **Select ROI** menu command:
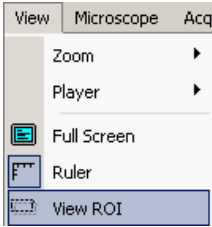
➢ In the image, define a small region to which you want deconvolution to be applied:
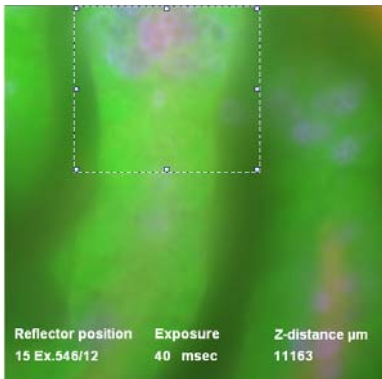
➢ Start the procedure by clicking on **Start**. Follow the display in the job list:
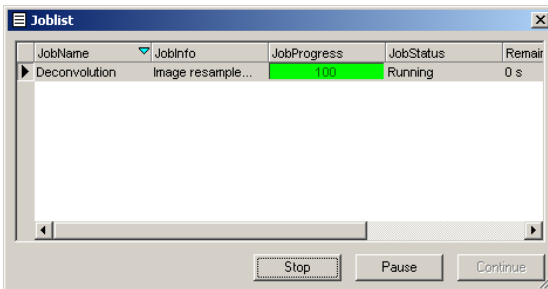
➢ The resulting image should now look like this:
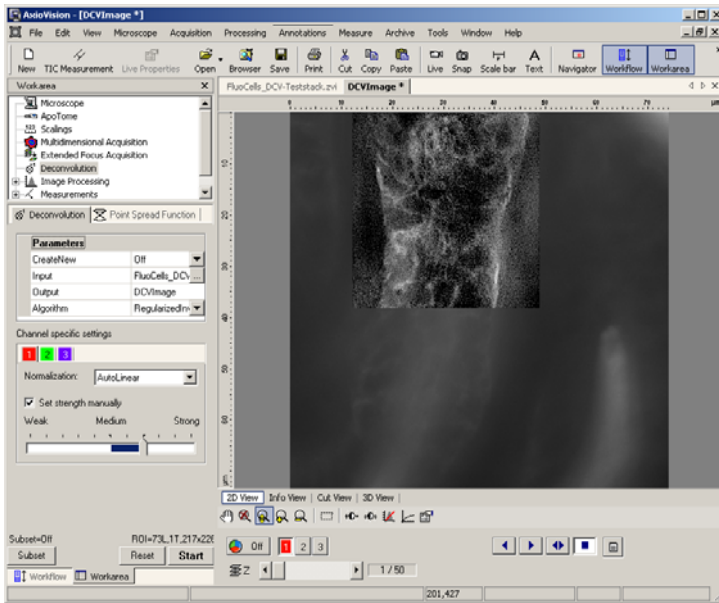
The first channel is displayed, and you can see the effect of the inverse filter applied to the ROI.

➢ Adjust the slider for Z until you arrive more or less at the center of the z-stack ![Z slider 27/50]. You should now see an image that looks something like this:
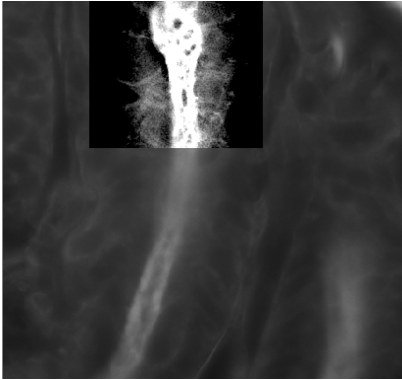
➢ The bright regions in the acting structure of the fluocell sample appear over-saturated. This is the effect of automatic normalization. To compensate for this, open the **Properties** window using the button in the image window's display field (or **Alt+Enter**).

➢ In the image histogram, you can now see that the setting of the display characteristic curve is still the same as it was for the original image:
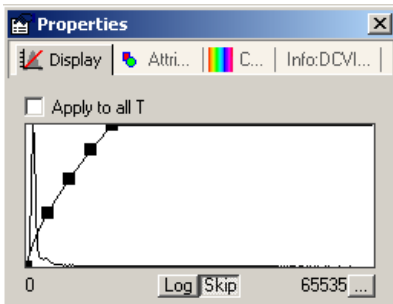
➢ As the 16-bit dynamic range (= 65536 gray levels) is now being used in full, the display characteristic curve needs to be adapted to the new data range.

➢ To do this, click on the [BestFit] button. The value in the input field underneath this button should then be one per thousand: [1 ‰]. The display characteristic curve is now set in such a way that 0.1% of the brightest pixels in the image are shown as completely white, and 0.1% of the dark pixels in the image as completely black. These settings only influence the display on the monitor, not the pixel values in your image. The histogram should now look something like this:
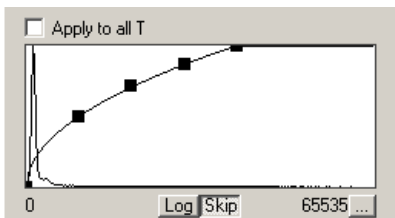
➢ The contrast setting of the image is now more or less correct. This means that you are able to properly assess the image components that have been enhanced using deconvolution:
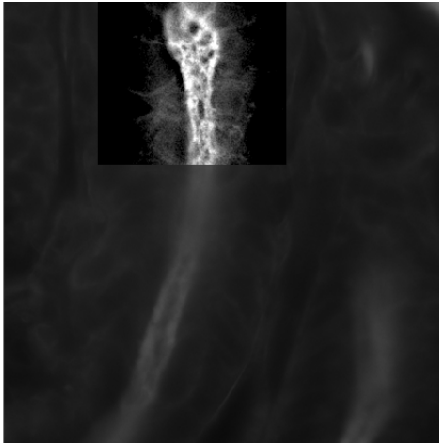
➢ Repeat these steps for channels 2 and 3 until a good, high-contrast
image is also visible in superimposed color mode ([🔵 Off] [1] [2] [3]
button).
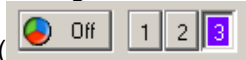
➢ The orthogonal slice view is particularly useful for assessing the result.
Switch to the slice view by clicking on the **Cut View** tab
[2D View | Info View | Cut View | 3D View] . Position the slice lines in the
deconvoluted region (for example):
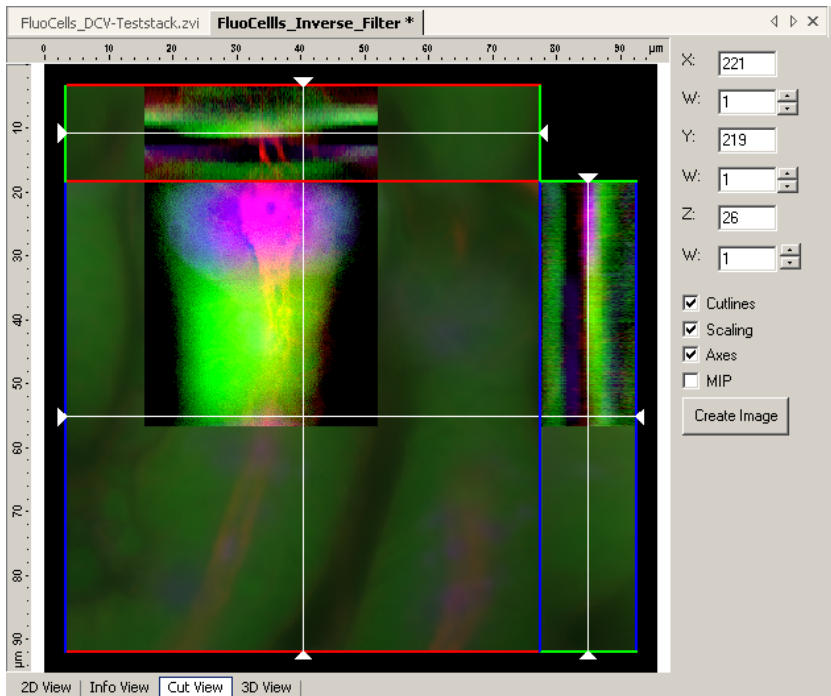
➢ You can now clearly see the effect of the contrast enhancement. In
particular, the separation of the green channel (which represents the
cell nuclei of the intestinal cells in the fluocell sample) from the red
actin elements is only clearly visible after deconvolution.

➢ You can now experiment with the various parameters and deconvolution methods. Provided you do not make any changes to the name of the resulting image, the resulting images are overwritten every time you click on the **Start** button. This procedure also enables you to display various deconvolution methods simultaneously, in a single resulting image, by moving the ROI (region of interest) in the input image after selecting a different method.
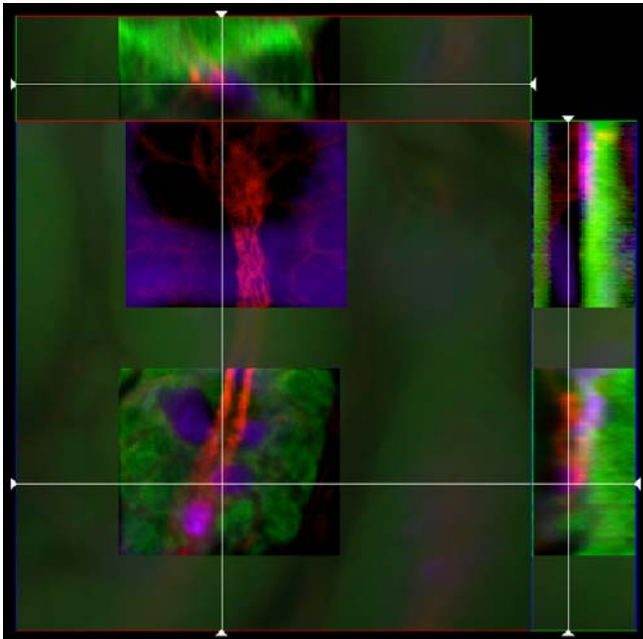
➢ Save the desired resulting image. These images are ideal for use with the 3D rendering module **Inside4D**.

10.4 Widefield Multichannel Unmixing

Background

Widefield Multichannel Unmixing is a new function for the removal of crosstalk between fluorescent dyes in multichannel images with up to eight fluorescence channels using a traditional epifluorescence microscope. Crosstalk is a phenomenon which occurs, whenever dyes are excited by excitation light of more than one filter combination. This problem occurs for example, when dyes with broadly overlapping excitation spectra are used concurrently in one sample. Good examples are the spectral variants of fluorescent proteins, e.g. BFP, CFP, GFP and YFP. With traditional reflector filter sets it has been often difficult to achieve 100% signal separation for such dyes.

In essence this means, that a certain proportion of a signal in one channel is actually derived from another dye spilling over into the channel.

A parameter based separation is now available with the **Widefield Multichannel Unmixing** module. The procedure is separated into three steps:

➢   Measurement of the amount of crosstalk in 2 alternative procedures:

- •   With appropriate reference samples
- •   by automatic component extraction (ACE) from the sample to be unmixed

➢   Creation of the unmixing matrix file

➢   Unmixing of the image to be corrected by means of using the unmixing matrix file generated in step 2

NOTES:

- Non-fluorescent channels such as DIC or Phase contrast are automatically detected and disregarded by all unmixing functions. Such channels are automatically copied into the unmixed result images to facilitate merged views with fluorescent channels.

- The module Widefield Multichannel Unmixing is designed to work with images created using the AxioVision Multichannel Fluorescence module. For the creation of correct reference images the use of the AxioVision Multichannel module is required.

- The images used in these examples you will find on the AxioVision installation CD in the folder "Images".
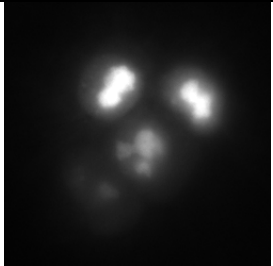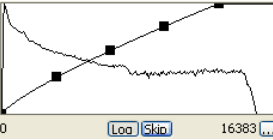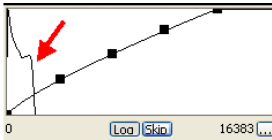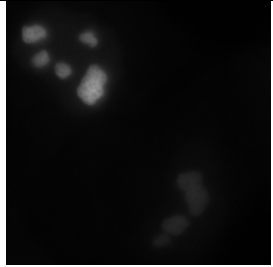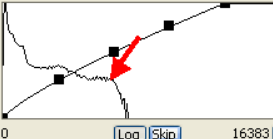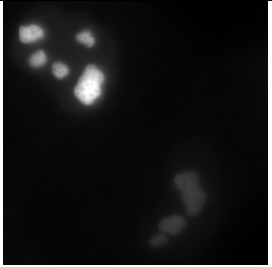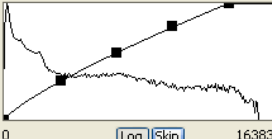
The Principle of unmixing
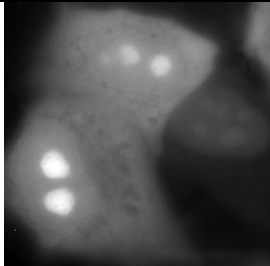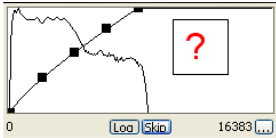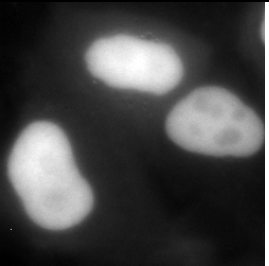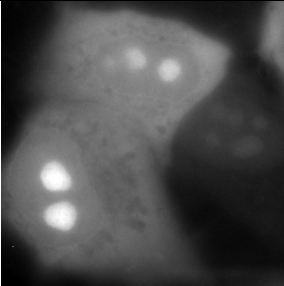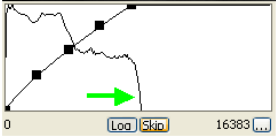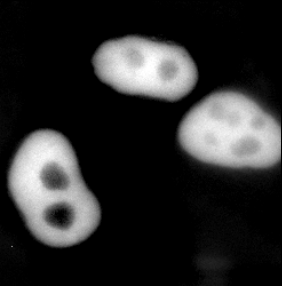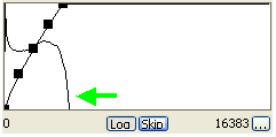
The following example tries to demonstrate the problem of cross talk as well as show the potential of unmixing such images. In this case, determination of cross talk is done using reference samples and not ACE.
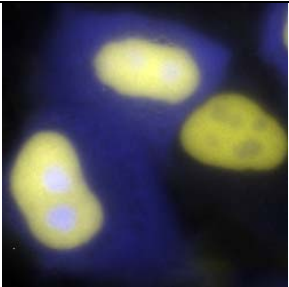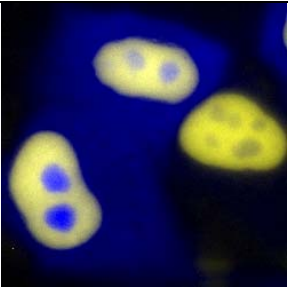
Reference sample A (CFP-Reference1.zvi): HeLa cells containing a virus proteins coupled to cyan fluorescent protein (CFP). This protein accumulates especially in the nucleolar regions of the cell nucleus.

Reference sample B (GFP-Reference1.zvi): HeLa cells containing a virus proteins coupled to green fluorescent protein (GFP). This protein accumulates in the nucleolar regions of the cell nucleus too.

Sample C: (RevCFP-H2-GFP_3.zvi): HeLa cells containing two proteins with different fluorescence: coupled to CFP is the same viral protein as in reference sample A (nucleoli); coupled to GFP is a histone protein, which stains the chromosomes and thus the entire nucleus a lesser degree of staining of the nucleoli.

Sample	Channel 1(CFP)	Channel 2 (GFP)
A. Reference sample **CFP only**: A small degree of crosstalk is visible in channel 2 coming from channel 1. **Image histogram:** x-axis: pixel gray values. y-axis: relative number of pixels in logarithmic scale; identical settings of the display curve.		
B. Reference sample **GFP only**: A moderate degree of cross talk from channel 2 is visible in channel 1. **Image histogram:** x-axis: pixel gray values. y-axis: relative number of pixels in logarithmic scale; identical settings of the display curve.		

Sample	Channel 1(CFP)	Channel 2 (GFP)
C. 2-channel fluorescence sample (CFP and GFP): Before Unmixing: How much signal from channel 2 is visible in channel 1 (cross talk) and vice versa? **Image histogram:** x-axis: pixel gray values. y-axis: relative number of pixels in logarithmic scale; identical settings of the display curve.		
D. 2-channel fluorescence sample (CFP and GFP): After Unmixing: A net percentage of pixel intensity values from channel 2 are added to the signal in channel 1. **Image histogram:** x-axis: pixel gray values. y-axis: relative number of pixels in logarithmic scale; identical settings of the display curve.		

	CFP&GFP-sample original pseudocolor mode	CFP&GFP-sample unmixed pseudocolor mode
E. 2-channel fluorescence sample (CFP and GFP): Comparison "before" and "after" unmixing: shows a markedly improved signal separation (shown at identical display settings.		

The Widefield Multichannel Unmixing function

The **Widefield Multichannel Unmixing** function is the central function for unmixing samples:
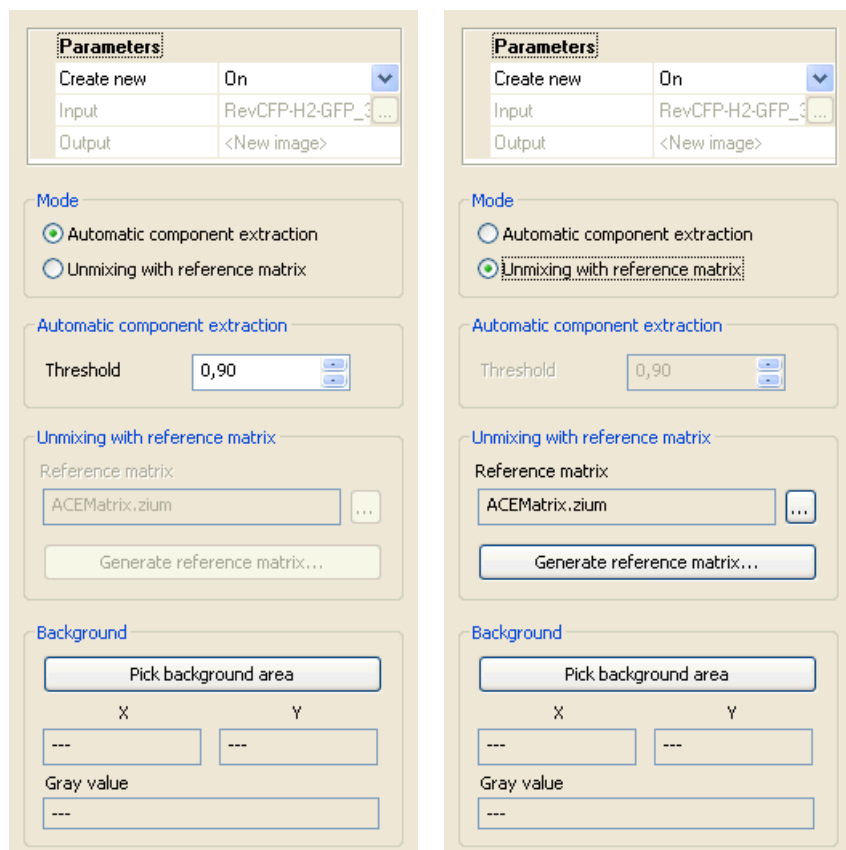
Using the function dialog, you can select whether the unmixing should take place via "automatic component extraction" (left-hand image) or using a reference matrix (right-hand image). If you choose unmixing using a reference matrix, you will need a reference matrix file containing the corresponding information for the unmixing.

If such a file does not yet exist, you can start a wizard via the

Referenzmatrix erstellen... button, which will lead you through
the process of generating a reference matrix file.

Working with the basic functions

The basic functions in the menu **Processing** ⇒ **Widefield Multichannel
Unmixing** are the functions for unmixing that will already be familiar to you
from the previous version of AxioVision. These functions are still available,
for compatibility reasons and for use in Commander scripts. The following
two examples describe how you can use these functions for unmixing.

Unmixing with reference samples

This example shows you how to measure cross talk using reference
samples and use this information to unmix a 2 channel fluorescence image.

The same images are used as in example 1.

➢   Open the first reference
    image "CFP-Reference1.zvi".
    It is a 3 channel image. The
    first channel contains
    transmitted light information
    (DIC).
    The CFP signal is visible
    strongest in channel 2 (Zeiss
    filter set #47). Change into

    b/w mode (Off) and

    channel 2 .

> Open the properties dialog (menu **View** ⇒ **Properties**) and adjust the display line in a way to enhance the gray value visibility (e.g. **Best Fit: Gamma value** ~ 0.25). Now it is easier to distinguish true image background from signal derived from the fluorescent dye.
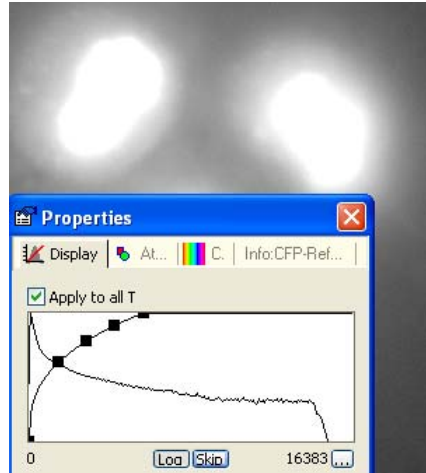
> In the workarea select Image Processing/Widefield Multichannel Unmixing and then **Reference measurement**.
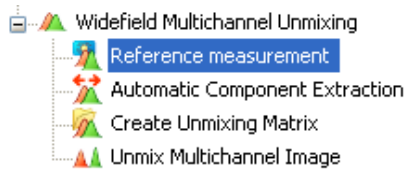

> The image "CFP-Reference1.zvi" is selected automatically as **Input** image because it the active image in the foreground.
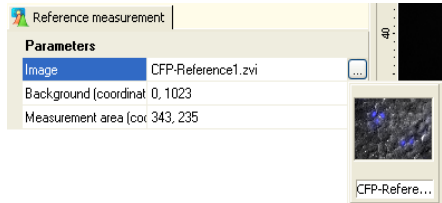
> If it is not selected, select the image from the pop up gallery.

➢ In the edit field
   Background (coordinates) click
   on the ⬚ button.

➢ Select a suitable region in the image for background correction (ideally without any signal coming from the dye). The pixel coordinates (x/y) as well as the gray value of the selected pixel are shown. If a scaling is available, the stage coordinates are shown.
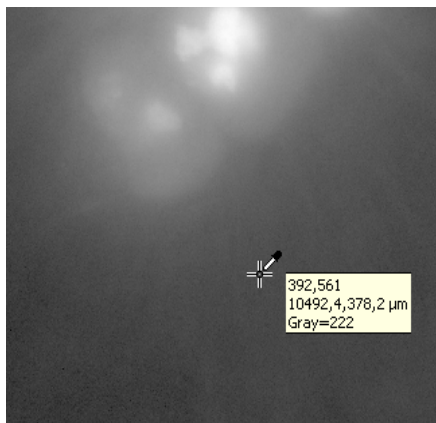
➢ Click at an suitable position once in the image. The x and y coordinates are shown in the input field.


Background (coordinates)   393, 562

➢ Click the ⬚ button in the input field
   Measure area (coordinates).

➢ Search for a suitable region in the image for measurement. Ideally search for a region with an intensity as high as possible.
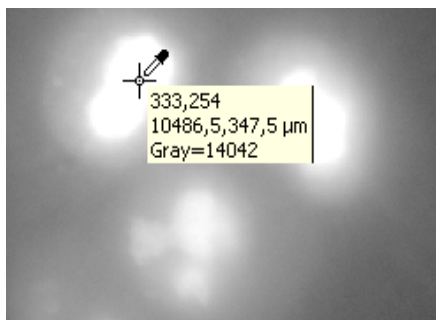
➢ Click the **Start** button to carry out measurement.

➢ The regions taken for measurement are shown in the image. Save the image, because it's used later for generation of the unmixing matrix.

➢ Open the second reference image **GFP-Reference1.zvi**. The third channel contains the GFP fluorescence (fluorescence filter set # 44).

➢ Repeat the corresponding steps also for this image.

➢ In the workarea select the function **Create Unmixing Matrix** from the function group Image Processing/Widefield Multichannel Unmixing.
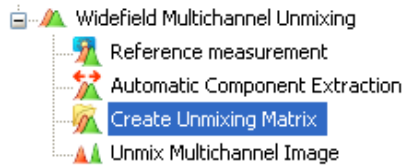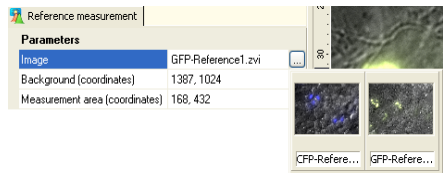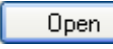
➢ 10. Select the input images accordingly: CFP-Reference1 as **ReferenceImage1**, GFP-Reference1.zvi as **ReferenceImage2**. Please take care to enter channels and reference images in the same order. If you use images with more than two channels, the function is extended accordingly.
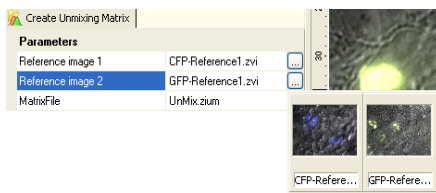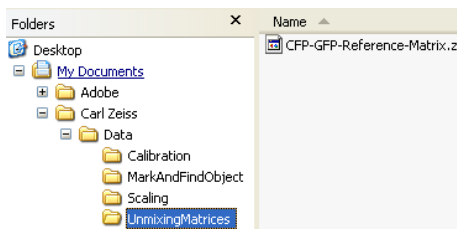
➢ To define a name for the unmixing matrix file, click the [...] button in the input field **MatrixFile**. In the following dialog you can enter a name for the matrix file.
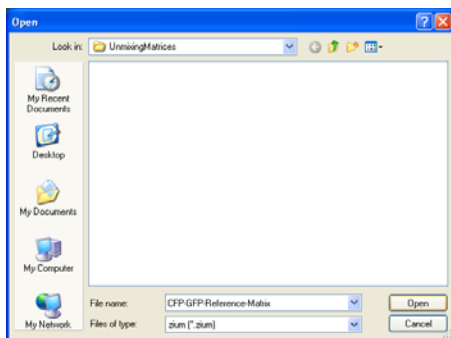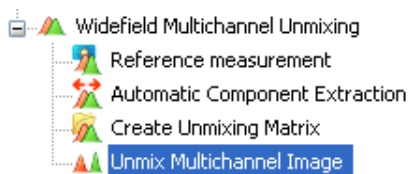
➢ Click the **Open** button to accept the filename.

➢ Click the **Start** button to generate the unmixing matrix file. The file is saved – like other AxioVision files – in the user folder.

➢ In the workarea select the function **Unmix Multichannel Image** from the Image Processing/Widefield Multichannel Unmixing function group.

➢ Open the image to unmix and select it as **Input** image. In this example, the image "RevCFP-H2-GFP_3.zvi" is used.
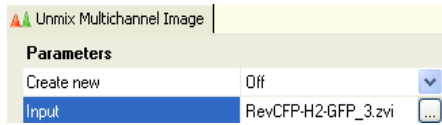
➢ Switch to the black and white view () and activate channel 2 ().
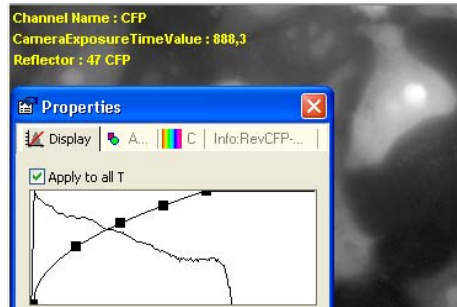
➢ Right click in the image and select **Properties**. Set the display characteristic line in such a way, that the gray values are displayed extremely amplified. So you can easily detect, where the background is not caused by the sample fluorescence.

➢ Switch to channel 3 and repeat the setting of the display characteristic line like in previous step.

➢ Switch back to the color view and switch off channel 1

.
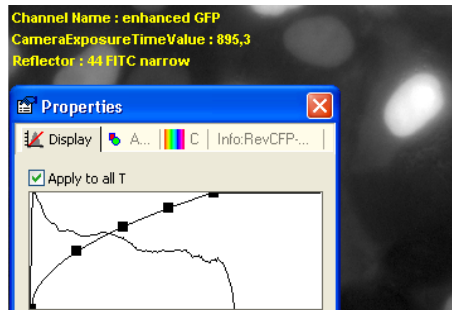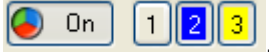
➢ Now you can separate the two fluorescence colors clearly from the background signal.
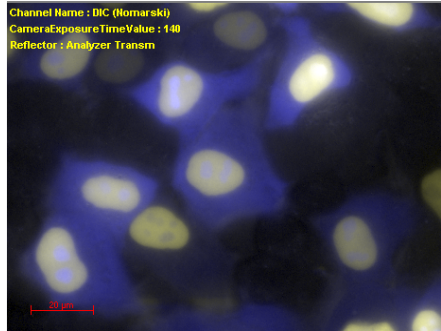
➢ Click the button in the input field
Background (coordinates). In the image search for a suitable region for the background measurement (preferably without a signal caused by the fluorescence dye).
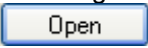
➢ The pixel coordinates (x/y) as well as the gray value of the selected pixel and stage positions are shown. Click at a suitable position once in the image. The x and y coordinates are shown in the input field.

➢ Enter a name for the **Output** image.
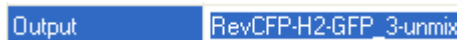
Output   RevCFP-H2-GFP_3-unmix

> ➢ Click the [...] button in the input field MatrixFile to select the unmixing matrix file. Click the Open button to load the file. The dialog is closed.
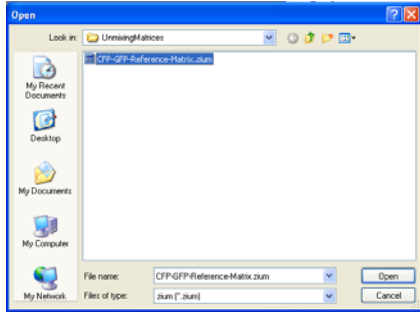
> ➢ Click the Start button to start the **unmixing**. The result image shows a clear separation of the fluorescence channels 2 (CFP) and 3 (GFP). Channel 1 without fluorescence (DIC) is copied to the output images unchanged.
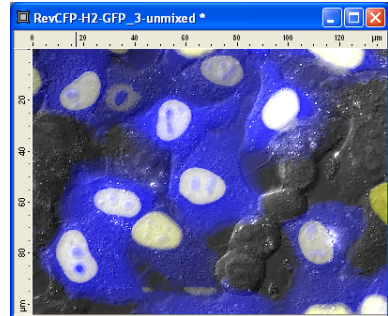
> ➢ To optimize the display, you should set the display characteristic line for channel 2 and 3 accordingly:
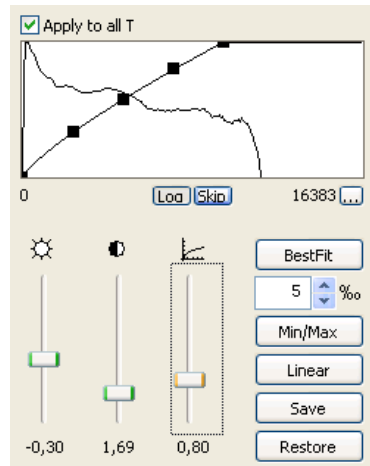
➢   Save the output image.
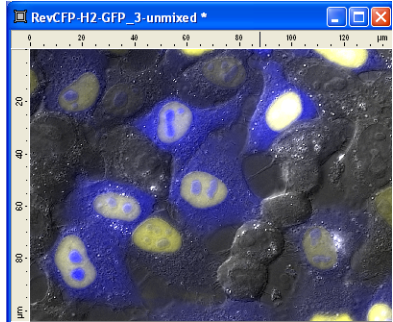
Unmixing of a multichannel image using ACE

This example shows you how to measure cross talk directly an image for unmixing with Automatic Component Extraction (ACE) and how to use it for unmixing of samples.

The same images are used as in example 1.

➢   Open the image to unmix. In this example the image "RevCFP-H2-GFP_3.zvi" is used.
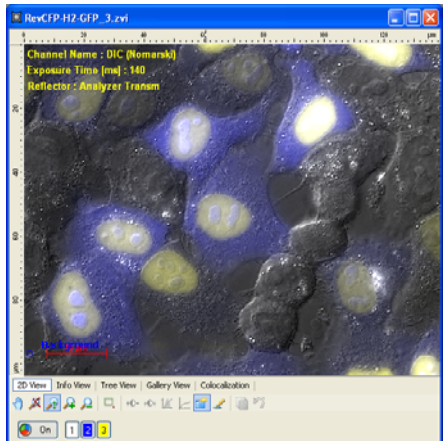
➢ Switch to the black and white view () and activate channel 2 (). Right click in the image and select **Properties**. Set the display characteristic line in such a way, that the gray values are displayed extremely amplified. So you can easily detect, where the background is not caused by the sample.
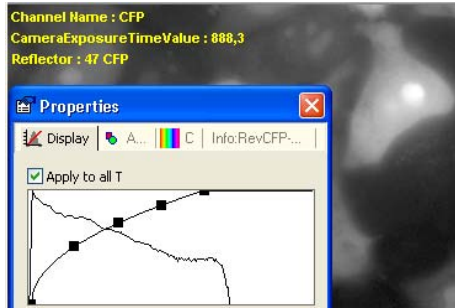

➢ Switch to channel 3 and repeat the setting of the display characteristic line like in previous step.
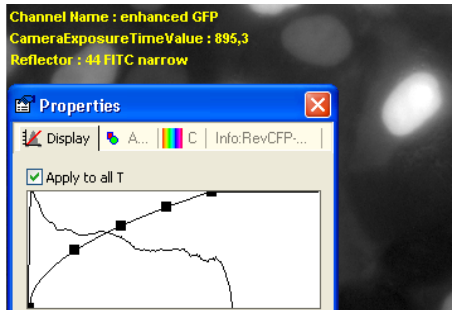
➢ Switch back to the color view and switch off channel 1 .
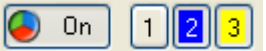
➢ Now you can separate the two fluorescence colors clearly from the background signal.
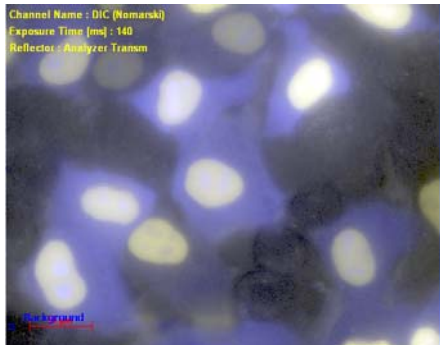
➢ In the workarea select the function **Automatic Component Extraction** from the Image Processing/Widefield Multichannel Unmixing function group.
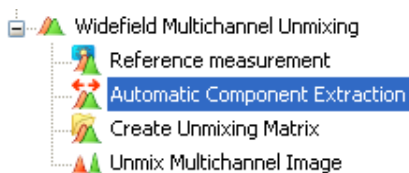
➢ The reference image "RevCFP-H2-GFP_3.zvi" is selected automatically as Input image because it the active image in the foreground.
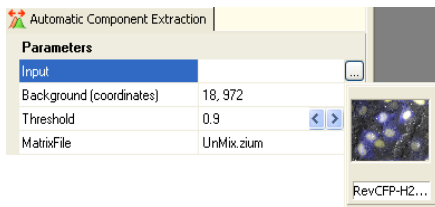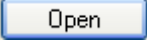
➢ If it is not selected, select the image from the pop up gallery.

➢ Click the [...] button in the input field **Background (coordinates)**. In the image search for a suitable region for the background measurement (preferably without a signal caused by the fluorescence dye). The pixel coordinates (x/y) as well as the gray value and the stage position of the selected pixel are shown.

➢ Click at an suitable position once in the image. The x coordinate is shown in the input field.

➢ The predefined threshold in the input field **Threshold** can be used unchanged **Threshold** 0,9 . For further information about this parameter, please read in the online help.

> ➤ To define a name for the unmixing matrix file, click the [...] button in the input field **MatrixFile**. In the following dialog you can enter a name for the matrix file. Click the **Open** button to accept the filename.
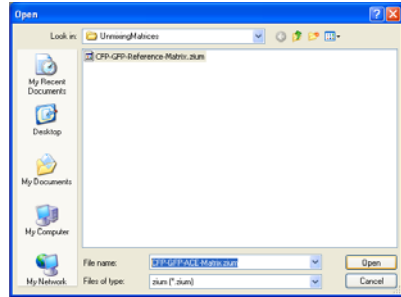
> ➤ Click the **Start** button to generate the unmixing matrix file. The file is saved – like other AxioVision files – in the user folder.
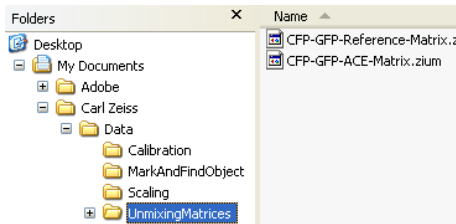
> ➤ The measurement regions defined by ACE are drawn to the image using the channel color.

> ➤ Switch to the black and white view (● **Off**) and activate the accordant channel.
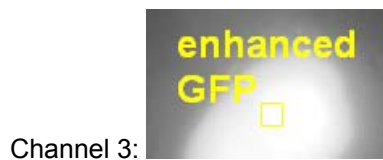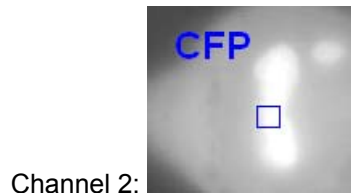
Channel 2:

Channel 3:

> ➤ In the workarea select the function **Unmix Multichannel Image** from the Image Processing/Widefield Multichannel Unmixing function group.
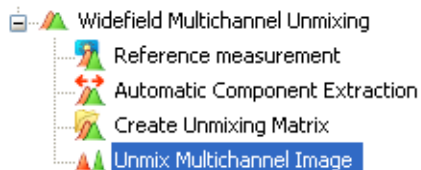
➢ Open the image to unmix and select it as **Input** image. In this example, the image "RevCFP-H2-GFP_3.zvi" is used.
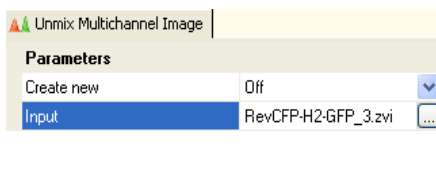
➢ Now repeat the background measurement:
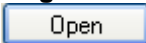
➢ Click the [...] button in the input field Hintergrund (Koordinaten) and then click in a background region.

➢ Enter a name for the **Output** image.
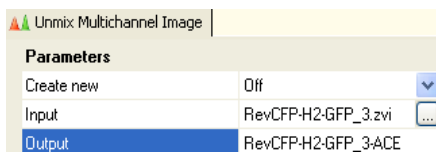
➢ Click the [...] button in the input field MatrixFile to select the **unmixing matrix file**.

Click the Open button to load the file. The dialog is closed.
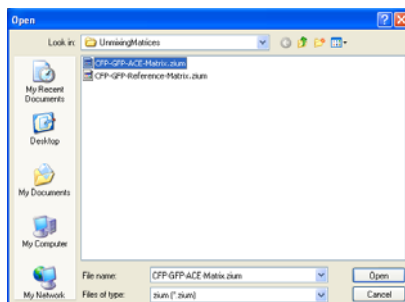
➢ Click the [Start] button to start the unmixing. The result image shows a clear separation of the fluorescence channels 2 (CFP) and 3 (GFP). Channel 1 without fluorescence (DIC) is copied to the output images unchanged.
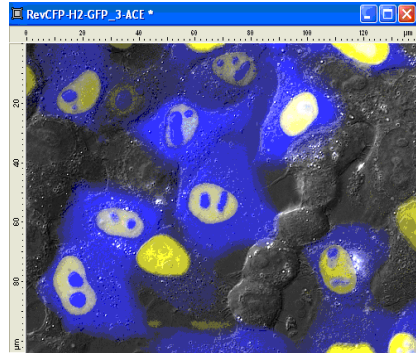
➢ To optimize the display, you should set the display characteristic line for channel 2 and 3 accordingly:
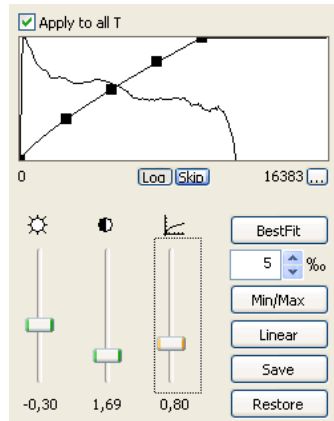
➢ Save the output image.
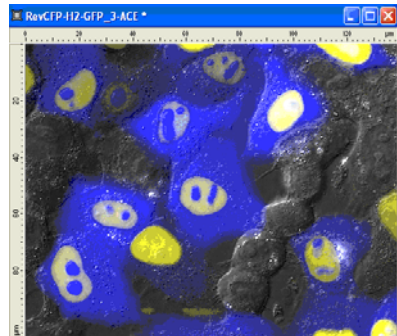
10.5 Colocalization

Background

The acquisition of fluorescence images in several channels makes it possible to visualize the relationship between biological structures. Displaying two channels together in color merge mode makes it easier to establish whether the components are "colocalized", i.e. are occupying the same position in space.

The classic display is the color-coded overlaying of two fluorescence channels. The most common form is red/green overlaying. Regions in which both fluorescent dyes occupy the same position in space are therefore shown as yellow. However, it is not possible to use this display as a basis for quantitative statements about the extent of colocalization. At best, a qualitative statement can be made on whether or not two dyes are colocalizing. The **Colocalization** module can fill this gap and provides you with a tool that enables you to determine colocalization quantitatively.

Principle:
- The colocalization of two channels is always analyzed. For images with more than 2 channels, the user is required to make a selection. Colocalization results from the pixel-by-pixel comparison of intensities per channel.

Control elements

The colocalization display consists of three elements: the scatter plot, the image (2 channels are shown) and the table of measurement values. On the right-hand side of the display is a control field for the colocalization settings. The colocalization display can be activated in the **View** ⇒ **Views** ⇒ **Colocalization** menu or by clicking on the **Colocalization** button at the bottom edge of the image.

Regionn Select
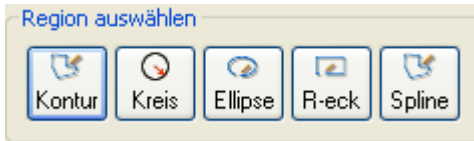
: Using these buttons you can select up to 32 regions in the input image or the scatter plot. Five different forms can be used by clicking on the buttons: contour, circle, ellipse, rectangle, contour (spline). If you hold the button down, the form of the mouse pointer changes to the drawing symbol. Once you have finished drawing, the mouse pointer changes back to the original arrow symbol and the button is no longer shown as depressed.

Once a region has been drawn, it is automatically treated as an active region. The scatter plot shows the pixel-value frequencies of these regions. The colocalization table shows the data for the entire image and for the selected region. If you wish to select several regions, you can do so by holding down the Shift key and clicking on the regions in question. You can also deselect them in the same way.

Note:
- The regions are only visible in the **Colocalization** view and are not adopted if you switch to a different type of display.

Channel Select

: Here you can select the two channels that you want to view.

In the drop-down list boxes on the right you can set a gray value range for each channel. This allows you to make optimum use of the available gray value range. You can have the range set automatically, choose between predefined fixed values or enter a value of your choice interactively. The scaling of the scatter plot is adapted according to the selection you make.

Threshold

: In these list boxes you can set the threshold value for both channels in accordance with the graphic below.
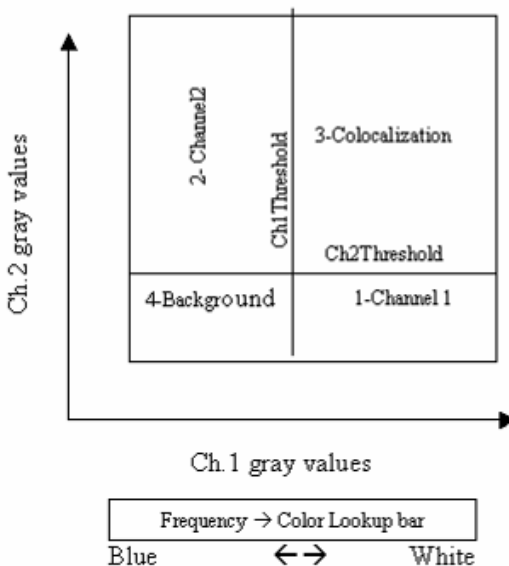
> : This button becomes active as soon as a region is inserted into the scatter plot. It remains active as long as regions are being selected or moved there. By activating and deactivating this button, you can switch between the selection of threshold values using the mouse and the selection/shifting of selected regions in the scatter plot image. If regions have been defined in the scatter plot, the corresponding data appear in the table, in addition to the entire image.
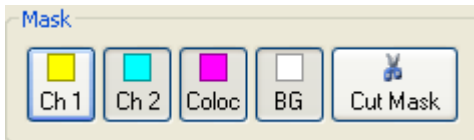
Mask

 : Using these buttons you can mask the four quadrants described in the **Threshold** section and assign user-defined colors to the channels of the original image (**Ch1** and **Ch2**). The same process can also be carried out for the colocalized pixels (**Coloc**) and the background (**BG**). The color selection menu is opened by right-clicking with the mouse.

> : This button becomes active as soon as a quadrant has been masked. If you press the button by clicking on it with the mouse, a new image containing exclusively the masked pixels is created.

 : Using these buttons you can select the scatter plot and/or the value table for the image currently being displayed and edited for saving in a separate file. This enables you, for example, to edit the data in another program (e.g. Microsoft Excel).

Graphical Display

The **Scatter Plot** is a graphical display of the pixel-value frequencies of channels 1 and 2. These frequencies are shown using a rainbow lookup table (units between 0 and 255). Channel 1 is distributed along the x-axis and channel 2 along the y-axis. The threshold value can be changed directly in the graphic by left-clicking with the mouse. It is also possible to move the threshold value lines interactively using the mouse.

The **Colocalization table** contains 17 columns. These are used to present the following details and calculations:

1. **Image Region**: When a region has been selected, it is assigned a number. This number appears in the image and the table. Data from the scatter plot can either relate to the entire plot or to the selected region. For this reason no numbering for the selected region appears here in the data table.

2. **Scatter Quadrant**: As described under the **Threshold** field, the scatter plot display is divided into four quadrants, which are numbered 1,2,3 and 4. These numbers correspond to channel 1, channel 2, colocalization and background. All data can be obtained individually for these four quadrants. In addition, the threshold value set in the scatter plot is displayed for each channel.

3. **Number of Pixels**: Shows the total number of pixels in each quadrant. The sum of all the pixels in this column for all 4 quadrants corresponds to the product of the height and width of the original image.

4. **Area (µm²)**:
    Area = number of pixels x X-scale x Y-scale
    If no scaling is available for the original image, the following applies:
    1 pixel = 1 µm.

5. **Relative Area (%)**:
    Relative area = area of quadrant x / total area
    x = 1, 2, 3, 4; total area = area of quadrant 1 + area of quadrant 2 + area of quadrant 3 + area of quadrant 4.

6. **Mean Intensity Channel 1**: The sum of all the gray values of channel 1 divided by the total number of pixels in this channel.

$$\frac{\sum GrayValuesChannel1}{AreaChannel\ 1}$$

7. **Mean Intensity Channel 2**:

$$\frac{\sum GrayValueChannel2}{AreaChannel\ 2}$$

8. **Standard Deviation Channel 1**:

$$\sqrt{\frac{\sum (GrayValuesChannel1 - MeanIntensityChannel1)^2}{AreaChannel1 - 1}}$$

9. **Standard Deviation Channel 2**:

$$\sqrt{\frac{\sum (GrayValueChannel2 - MeanIntensityChannel\ 2)^2}{AreaChannel2 - 1}}$$

10. **Colocalization Coefficient Channel 1**: This coefficient indicates the relative number of colocalized pixels in channel 1 or 2 in relation to the total number of pixels above the threshold value:

$$\frac{\sum Pixels\,Channel1_{,colocalized}}{\sum Pixels\,Channel1_{,total}}$$

The values vary between 0 and 1, where 0 indicates no colocalization and 1 complete colocalization.
Numerator = number of pixels in quadrant 3
Denominator = number of pixels in quadrant 3 + number of pixels in quadrant 1

11. **Colocalization Coefficient Channel 2**:

$$\frac{\sum \text{Pixels Channel} 2_{,\text{colocalized}}}{\sum \text{Pixels Channel} 2_{,\text{total}}}$$

Numerator = number of pixels in quadrant 3
Denominator = number of pixels in quadrant 3 + number of pixels in quadrant 2

12. **Weighted Colocalization Coefficient Channel 1 (Pearson)**:
Provides information on the intensity distribution within the colocalization region.
Value range: -1 to 1.

13. **1**: All pixels lie in a straight line in the scatter plot (if you have used the same channel twice for colocalization, you will obtain the value 1 in this column).
0: The pixels in the scatter plot are distributed in the form of a cloud with no preferred direction.

$$\frac{\sum ((GVC1 - MVC1) \times (GVC2 - MVC2))}{\sqrt{\sum (GVC1 - MVC1)^2 \times \sum (GVC2 - MVC2)^2}}$$

GV: gray value; MV: mean value; C: channel

14. **Overlap Colocalization Coefficient (Manders)**:
Insensitive to differences in signal intensity between the two channels and to bleaching.
Value range: 0 to 1

$$\frac{\sum \text{GrayValueC1} \times \text{GrayValueC2}}{\sqrt{\sum \text{GrayValueC1}^2 \times \sum \text{GrayValueC2}^2}}$$

C: channel

15. **Index Z**: Indicates the z-index for z-stack images.

16. **Index T**: Indicates the time index for time lapse images.

17. **Acquisition Time**: Indicates the time of acquisition for all dimensions of a multidimensional image.

18. **Focus Position**: Indicates the focus position at which an image was acquired.

11. Image Analysis Modules

11.1 Interactive Measurement

General

The Interactive Measurement module builds on the standard image analysis functions in the basic program. It offers the following two additional features:

➢ A clearly extended list of measurement parameters: besides the standard parameters there are, among other things, several tools for distance measurement and multiple distance measurement, two additional options for cyclometry (including clicking on contour points), and much more besides.

➢ The possibility for generating measurement programs for interactive measurements.

NOTE:
- For general background information and basic details on how to operate the measurement function, please read through chapter 6 "Image Analysis" first.

- A complete list, plus a description, of all measurement parameters can be found in the online help.

Working with interactive Measurement Programs

Measurement programs allow you to put together parameters of your choice, which the user must then run through in the defined sequence. This creates a kind of electronic work instruction that ensures that a measurement sequence is run through properly and completely. The programs can be saved so that they can be used in routine operation.

Working with interactive measurement programs usually involves two steps: 1. loading and 2. executing a measurement program. However, before a measurement program can be used for routine measurements, it must first be generated.

Note:
- Further explanations on all the functions described can be found in the online help.

Generating measurement programs

Using the **Measure – Interactive Measurement Programs – Define** function, you can load and modify existing measurement programs or generate new ones.
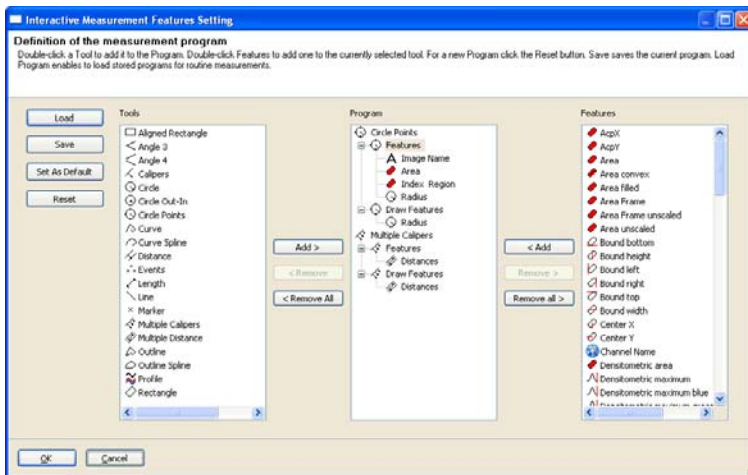
Generating measurement programs: selecting measurement tools and parameters

The **Interactive Measurement Programs** function is also suitable for loading measurement programs, as the relevant program is also visible here. There is an independent function for loading a program. This is particularly useful for automatic execution within a Commander script.

Loading and executing measurement programs

In order to execute an existing measurement program, it must first be
"loaded". This means that one program is selected from a list of existing
measurement programs. This program then becomes active. When the
execution function is started, the program that is currently active is always
the one that is used.

To load a measurement program, please select the **Measure** ⇒
Interactive Measurement Programs ⇒ **Load** function:
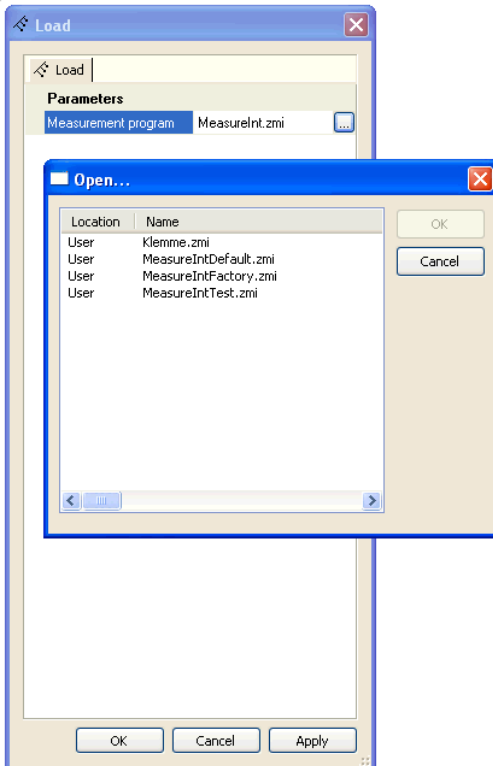
Loading a measurement program

The desired program can be selected from a list of existing programs. You can also use the **Measure ⇒ Interactive Measurement Programs ⇒ Define** function to load a measurement program. The advantage of this is that a loaded program is also displayed in this function. However, if a measurement program is to be automatically loaded and executed within the context of a Commander script, the function for generating programs is not suitable.

When the measurement program has been loaded, you can start it via the **Measure ⇒ Interactive Measurement Programs ⇒ Run** function:
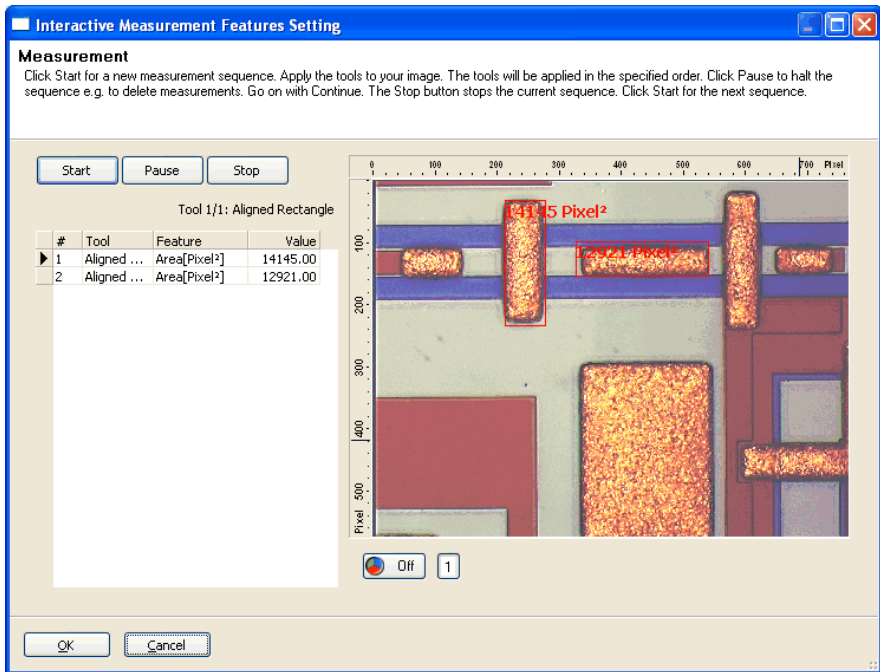
Executing a measurement program

11.2 AutoMeasure (Automatic Measurement)

General

The process of measuring interesting objects in an image automatically is closely linked to the rapid development of the hardware sector. Without this development it would take a huge amount of time to perform these measurements (which can involve several hundred images in some cases).

Thanks to the performance of modern PCs, it is now possible to use increasingly complex functions for image analysis, and to perform an automatic evaluation.

The term automatic measurement is understood to mean, in particular, the **automatic recognition** of interesting objects. In contrast to interactive measurement, it is no longer necessary to use the mouse to outline the objects to be measured in the image. The objects can be recognized automatically on the basis of their brightness and/or color and other descriptive parameters (shape, size etc.).

An automatic measurement usually consists of the six steps briefly listed below. These steps represent the general sequence of an image analysis procedure. They are in no way specific to AxioVision, and should always be carried out in this or a similar form.

1. Image acquisition

Image acquisition is – besides segmentation – the most important step within image analysis. It is only during image acquisition that you have all the original data available. Image information that is poor or, worst of all, incorrect prevents you from achieving accurate measurement results. This can even lead to a situation in which a sample that should be simple to measure from an image analysis perspective is no longer analyzable, as the system can no longer recognize the objects to be measured. In such cases you have to revert to interactive measurement.

Information that is not acquired during image acquisition is very difficult to reconstruct during image enhancement. In many cases this is not possible at all. You *must* therefore take advantage of all of the technical possibilities offered by your microscope and camera during the preparation of the sample.

Often there is room for improvement with regard to sample preparation. You need to be self-critical, and check whether the quality of your samples could be improved.

2. Image enhancement

In general terms, it is possible to distinguish between two different application situations:

The **optical enhancement** of an image helps the user to correctly interpret the accuracy of the solution suggested by the measurement system. This includes enhancement of the brightness and contrast, and also gamma correction. Enhancing the quality of the image from an optical perspective, however, has no influence on the quality of the recognition results.

Functions for **data extraction** allow you to enhance recognition performance (segmentation) in such a way that, under certain circumstances, images can be measured automatically even when the image quality would lead you to assume otherwise. These functions include, for example, the elimination of illumination errors (shading correction).

3. Segmentation

Segmentation is the key to a correctly functioning automatic measurement. This process involves having the interesting objects determined automatically by the measurement system, and separated from the regions that are not to be measured (the image background). Initially this takes place on the basis of brightness or color information. Reference objects are outlined or highlighted in the image using the mouse. AxioVision then recognizes all other objects independently.

It is also possible to directly select the thresholds for the brightness or color ranges that represent the objects using the distribution of the brightness or color information (histograms). However, this requires a certain amount of experience.

The result of segmentation is a "measurement mask", which is represented internally by a so-called binary image. This mask conceals the background regions using black pixels, and shows the objects to be measured as white. You will only come into contact with this measurement mask indirectly. AxioVision shows it as a color image superimposed over the original image.

4. Processing the measurement mask

A measurement cannot be performed until the objects to be measured, and only those objects, have been correctly recognized. Sometimes, however, this is not possible. Artifacts that you do not want to measure may also be acquired, or objects may be touching and may not therefore be recognized as individual objects by the system. These must then be separated before proceeding.

Typical processing steps therefore involve the deletion of artifacts, the filling of gaps in objects and the automatic separation of objects. In difficult cases, subsequent interactive processing (deletion, separation, additional drawing in of objects etc.) may also be required.

5. Measurement

During the measurement, the selected parameters are determined by AxioVision and saved in a results file. Two types of file are distinguished: files for so-called **region specific** and **field specific** image data.

➢ **Region specific** image data are measurement parameters that are determined for every individual object (an image *region*). These are, for example, the area, circumference and shape of the object in question, or even its densitometric values, such as the average gray value. The measurement results are written within the results file in the form of **one line per region** or object.

➢ **Field specific** data are measurement parameters that are determined for the entire image *field*. These are, for example, the number of objects, or the percentage of the entire image area represented by all the objects present, etc. Field-specific measurement results lead to **one line per image** in the results file.

6. Evaluation

Before you can actually make a statement about the image using the data for the images or objects in which you are interested, this data needs to be evaluated. As a wide range of special evaluation programs, such as Microsoft Excel, are now available, AxioVision itself does not feature any evaluation functions. The data are made available in Excel format, and can therefore be evaluated in the environment you are familiar with.

In addition to the pure measurement data, image documentation should also always be used to make clear which objects were used to determine the measurement results. AxioVision gives you the option of displaying the objects, e.g. by outlining their contours, and the measurement results directly in the image.

The concept of the AutoMeasure module

The concept of the AutoMeasure module is based on Carl Zeiss' many years of experience in the field of automated image analysis. It has become clear that in most cases only a small proportion of the range of available functions are actually required and employed. The aim was to arrange these functions in a way that was simple to operate and would also allow less experienced users to perform fast and accurate measurements.

As with automated analyses you are usually not just measuring an individual image, but in some cases are analyzing a very large number of images, the interactive execution of individual steps via menu functions or a workflow is often not a realistic option. Nevertheless, with the AutoMeasure module you have the option of measuring "just" one image quickly and efficiently (further information on this will be provided later).

The two points touched upon – "Complex technology, simply packaged" and "Analysis of large numbers of images" – are reflected by the structure of the AutoMeasure module.

The module therefore consists of two parts:

➢ The **Automatic Measurement Program Wizard** allows you to generate automatic measurement programs quickly and simply.

➢ The function for the **Run Automatic Measurement Program** allows you to execute a program you have generated for any number of images. It makes no difference whether these images are acquired via a camera or are already present as files.

NOTE:
- At this point, we should emphasize that the AutoMeasure module is not suitable for resolving every conceivable measurement task. It is intended more as a tool to simplify your everyday tasks.

- More complex applications are possible using the AutoMeasure Plus module. In this module the full range of image analysis functions are available via the AxioVision menus, and can be freely combined. You can therefore even realize exotic applications, although this requires the person generating the program (*not the actual user of the program*) to have some knowledge of the background to digital image analysis.

The wizard for generating measurement programs

The Automatic Measurement Program Wizard leads you step by step through the individual stages of digital image analysis (please also read the "General" section earlier in this chapter).

Start it by loading an image from the hard disk or acquiring an image over the camera and afterwards execute the **Automatic Measurement Program Wizard** function on the **Measure** menu. Further information on the individual steps, and the functions and functional parameters used, can be found in the online help by pressing the **F1** key.

The following functions are available in the individual steps:

Program management

Program management gives you an overview of the measurement programs available, and a brief description on them individually:
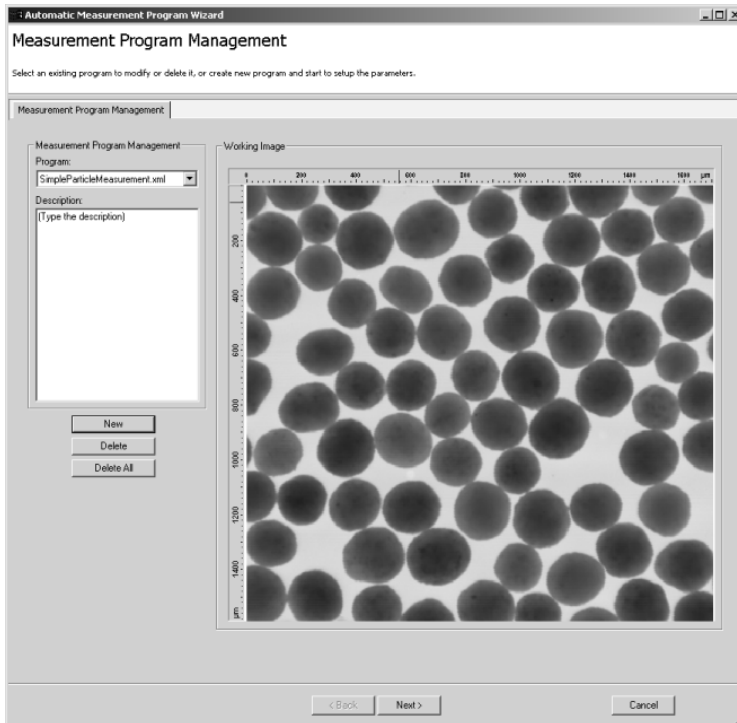
Program overview with brief description on the selected program

NOTE:
- The measurement programs are saved by default in the folder *"My Documents\Carl Zeiss\Data\Automatic Measurement Projects"*.

Image enhancement

For optical image enhancement, functions are available for brightness adjustment, contrast enhancement and gamma correction.
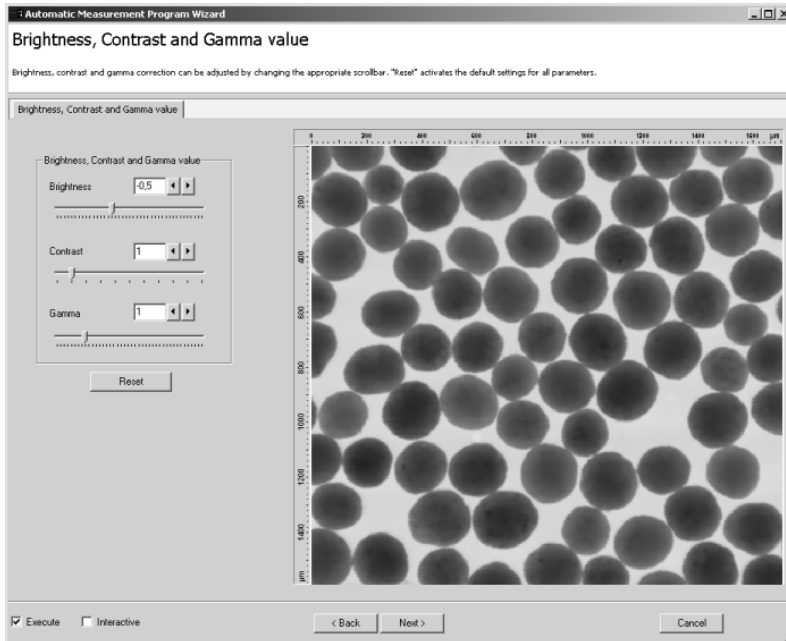
Enhancement of brightness, contrast and gamma value

To improve recognition performance, images can be smoothed (Sigma filter), illumination errors corrected (Shading correction), and object edges sharpened (Delineation).

Segmentation

Segmentation is probably the most powerful function of the AutoMeasure module.
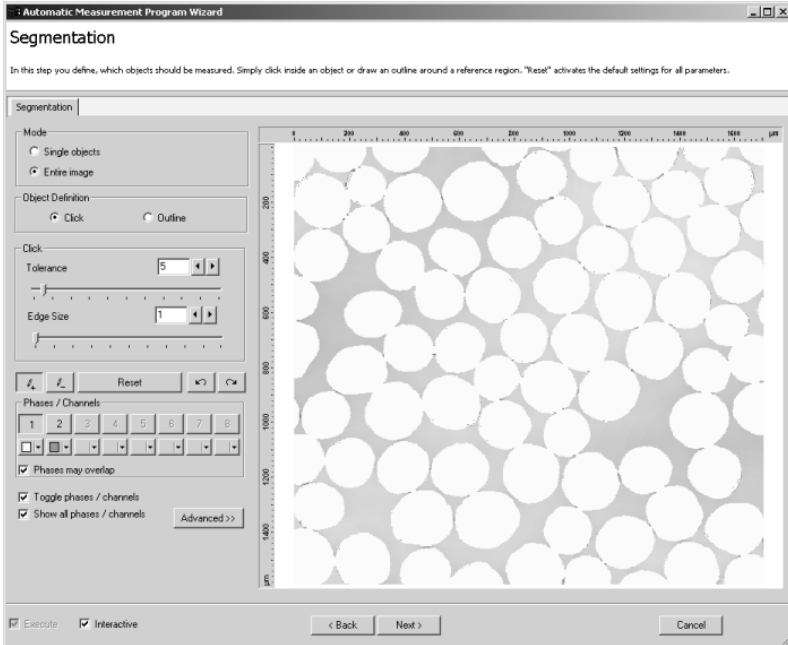
Dialog for image segmentation

Here you can separate the objects to be measured from the background simply by clicking on or outlining reference objects. Select whether you want individual objects only, or the entire image, to be segmented.

With multidimensional images, you also have the option of performing segmentation separately for each channel of a multichannel fluorescence image. If "normal" gray level or color images are used, up to eight different phases can be defined (in place of channels).

Processing the measurement mask (binary image processing)

In most cases, the measurement mask generated during segmentation has to be processed subsequently to ensure that only the objects you actually want to measure are highlighted.
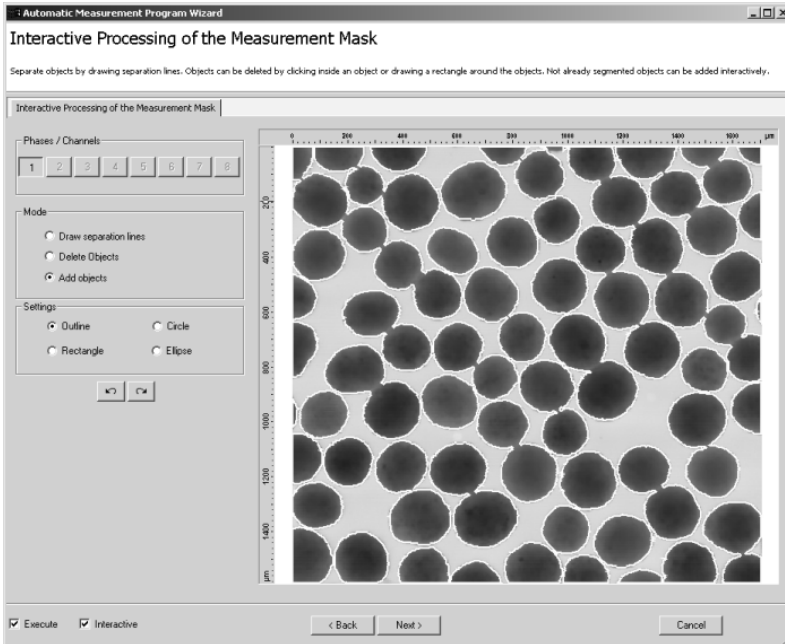
Step for interactive correction of the measurement mask

The measurement wizard therefore contains a function for the automatic separation of touching objects (Erosion/Dilation, Watersheds) and for the subsequent interactive processing of the mask (drawing in separating lines, deleting and adding objects).

Both steps start with a function for eliminating artifacts and for filling gaps ("cleaning" of the measurement mask).
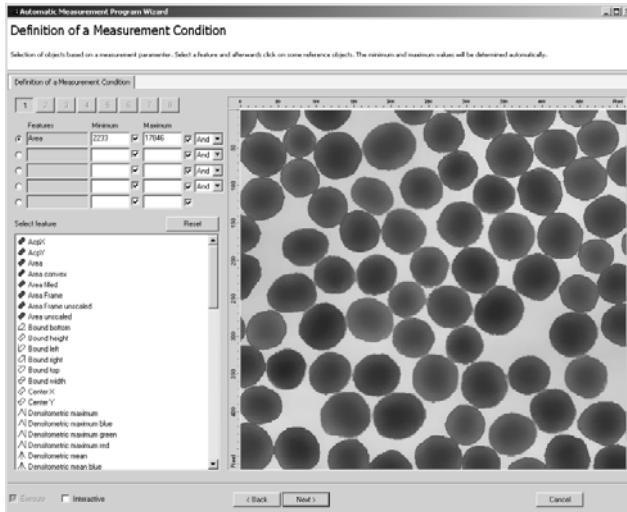
Measurement

The actual measurement procedure consists of several steps. These are used to define how the measurement should be carried out in detail.

Definition of a measurement condition ("object filter")

The individual steps

➢ Determination of region-specific (individual objects) and field-specific (entire image) measurement parameters.

➢ Definition of the way in which the measured objects should be highlighted in the resulting image at the end of the measurement (contour, measurement parameters etc.).

➢ Generation of a measurement condition. This defines the conditions that an object must satisfy in order to be measured. This is a kind of "object filter", which can be used, for example, to specify that only objects within a certain size category should be counted.

➢ Definition of a measurement frame, which is used to specify how objects that are cut off by the image edge, and are therefore likely to distort the measurement result, should be handled.

Evaluation

In the final step, AxioVision shows you the image with the objects selected for measurement again. Here you can make any final changes, e.g. deleting individual objects by clicking on them.
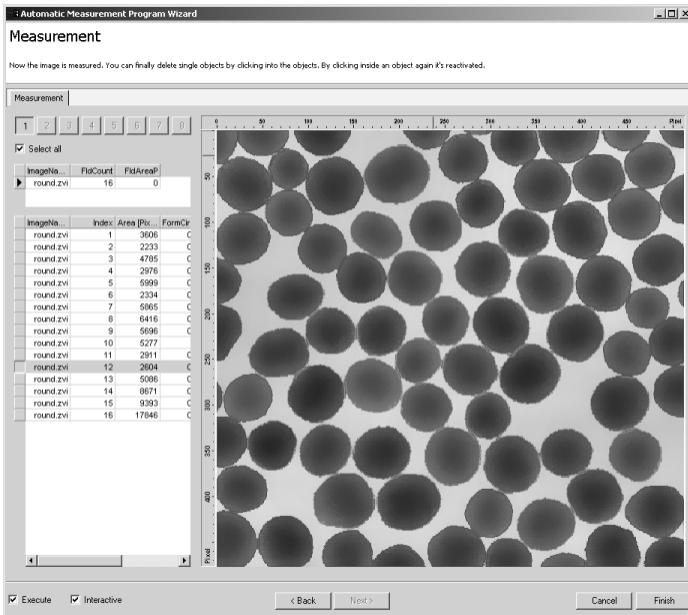
The measurement data are saved at the end of the measurement in so-called CSV format (**C**omma **S**eparated **V**alues).

This is a pure text format that separates the individual values from one another using special text symbols. The specific country settings that have been set in the Microsoft Windows control panel apply.

Files in CSV format offer two advantages: on the one hand, they are a special Microsoft Excel format, and can be opened and processed directly by that program. On the other hand, as the files contain pure text data, you can import the data into practically any other program.

Executing measurement programs

Once measurement programs have been generated, they can be applied to any number of images for series analyses. To do this, start the **Run Automatic Measurement Program** function from the **Measure** menu. Further information can be found in the online help by pressing the **F1** key.
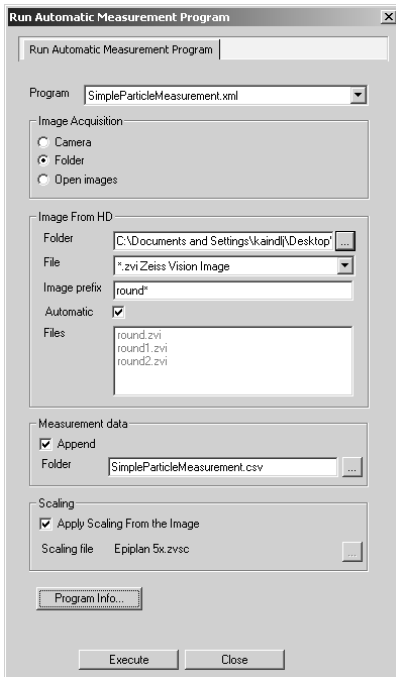
Function for executing measurement program

NOTE:
- As the tables of measurement results are available at the end of the measurement wizard, the wizard can also be used to measure an individual image. A useful feature is that rather than a new measurement program being generated each time for this purpose, an "auxiliary program", which always has the same name (e.g. "individual image measurement"), is used.

11.3 AutoMeasure Plus (Advanced Automatic Measurement)

General

While the **AutoMeasure** module has been designed primarily with ease of operation in mind (which naturally means that concessions have to be made in terms of flexibility), the **AutoMeasure Plus** module aims to offer unlimited possibilities for use.

A certain amount of knowledge of the procedure used for image analysis measurements is required in order to use these functions. You should therefore make sure that you also read the "General" section under 12.2 "AutoMeasure (Automatic Measurement)" as an introduction, before you start working with the individual functions of the AutoMeasure Plus module. There you will find important background information on the principal procedure used for measurements.

The concept of the AutoMeasure Plus module

To offer you as much flexibility as possible, all of the functions available within the context of measurements can be accessed individually via the **Processing** and **Measure** menus. By way of comparison, the respective functions in the AutoMeasure module can only be activated via the wizard.

In general, there are two ways in which you can automate measurement procedures:

The first step involves specifying the operations that you want to be performed during the actual measurement. This means that you need to define the measurement properties (see also the section "Working with the functions for automatic measurement"). In this step you need to answer questions including:

➢ Which parameters do I want to be measured?

> ➢ How do I want objects that are no longer fully visible in the image to be handled?

> ➢ Do I only want objects that satisfy special conditions to be measured (e.g. objects with an area between 50 and 100 µm²)?

During the definition of these settings you are supported by a wizard. Once you have defined properties, it is then possible to save them for routine measurement and simply reload them as required. This also takes place via the wizard. A separate function allows you to test the measurement properties before performing the measurement.

The second step simply involves performing the measurement on the basis of the properties you have defined.

In general, there are three ways in which you can automate measurement procedures:

> ➢ Call up the individual functions via workflows or toolbars (see also section 9.4 "Adapting the User Interface").

> ➢ Create scripts with the Commander modul (please read also Chapter 13.1 "Commander").

> ➢ Generate applications via the **VBA** module (see also section 13.2 "VBA").

Working with the segmentation functions

The individual steps in the following examples can also be practiced using the original images that were employed. The sample images are installed in the AxioVision folder. By default this is the folder *"C:\Program Files\Carl Zeiss Vision\AxioVision4\0007\Templates\Images"*. If an image is not present in that folder, please load it from the *AxioVision Viewer CD*.

Dynamic segmentation

Dynamic segmentation is particularly useful for detecting fine structures against a varying background in cases where an optimum result cannot be achieved using threshold value segmentation.

It is, however, also suitable for images with inhomogeneous illumination. The **Dynamic** function employs a smoothing filter internally. This is used to balance out uneven brightness, or gray value fluctuations, in the background.

The following example explains step by step how to perform dynamic segmentation.

➢ Load the image "SAND.ZVI" from the menu **File ⇒ Open Image** or acquire an image with the camera.

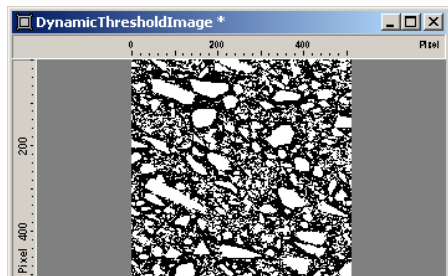➢ The image is displayed in AxioVision's image window.
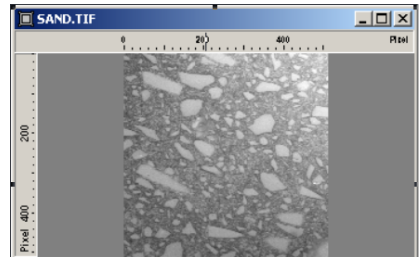
Select from the **Processing** menu the **Segment** functional group, and then the **Dynamic** function.

➢ You will now see the dialog window of the **Dynamic** function.

➢ If the **Automatic Preview** check box is activated, the reduced input and output images appear.

➢ Set the value 63 for **Size** and the value 0 for **Offset** by clicking on the arrow keys, or by entering the values directly. Set **Binary** to **On**.

➢ Click on **OK** to close the dialog.
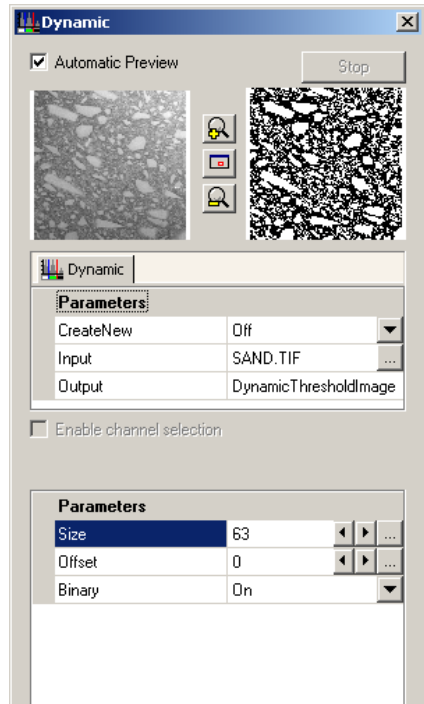
➢ The resulting image provides a satisfactory segmentation result.

➢ Although the original image exhibited inhomogeneous illumination, no shading correction was performed in advance.

➢ The uneven brightness in the image is compensated for by the dynamic segmentation.

You are now able to process the binary image further. Now select from the **Processing** menu the **Binary** functional group, and then the **Bin Open** function.

➢ You will now see the dialog window of the **Bin Open** function.

➢ If the **Automatic Preview** check box is activated, the reduced input and output images appear.

➢ Set "Octagon" for **Structuring Element** and the value "1" for **Count**.

➢ Click on **OK** to close the dialog.
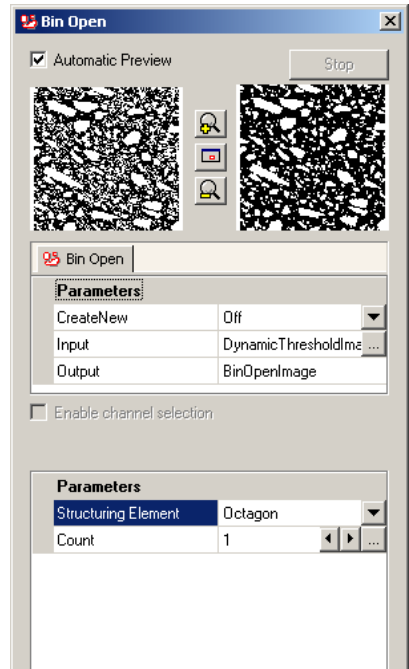
➢ In the resulting image, the detected objects have been smoothed, and small particles removed.
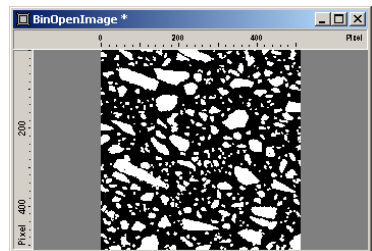
Automatic segmentation of a fluorescence channel in a multichannel z-stack image

If you want to detect structures in a multichannel z-stack image, functions are available that take the entire z-stack into account, or all of the channels in full or on a selective basis.

As the individual channels clearly differ in terms of colors and structures, here it is possible to make settings on a channel-by-channel basis. The specific settings for the z-stack do not have to be entered separately for each individual image. A setting made for an individual image is applied to all the images in the stack, and therefore provides a very fast detection result.

The automatic segmentation of an image is based on the automatic detection of local minimums in the image's gray value distribution. The **Automatic** function uses these minimums as thresholds in order to separate the structures to be measured from the background. This means that entering thresholds manually, as in the **Threshold** function, is not necessary here.

Often only certain structures, present in just one fluorescence channel, are of interest (e.g. cell nuclei). In a multichannel z-stack image, each channel's z-stack always has the same number of sections. In some cases the structure to be measured may not extend over the entire stack.

Setting the **Subset** therefore allows you, in the case of a multichannel z-stack, to choose selectively between individual channels or sections.

The following example explains step by step how to segment selected sections of a fluorescence channel in a multichannel z-stack.

➢ Load the image "Hela_DsRed_RevGFP.zvi" from the menu **File** ⇒ **Open Image** or acquire an image with the camera.

➢ The image is displayed in AxioVision's image window.
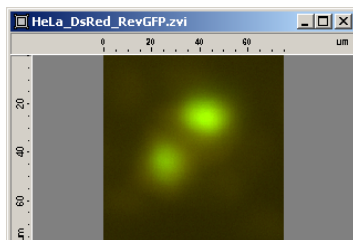
Now select from the **Processing** menu the **Segment** functional group, and then the **Automatic** function.

➢ You will now see the dialog window of the **Automatic** function.

➢ If the **Automatic Preview** check box is activated, the reduced input and output images appear.

➢ Click on **Subset** to enter the specific settings in the **Select Subset** window.
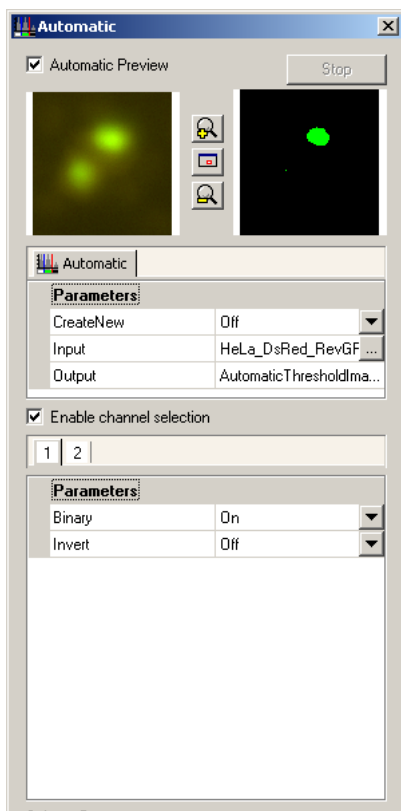
➢ Activate only the channel that you want to be segmented (channel 2 in the example).

➢ The first 5 sections contain hardly any structures, and should not be taken into account during segmentation. Deactivate these sections by clicking on them.

➢ Activate the **Copy selected items only** check box, so that only the selected channels and sections are segmented and click on **OK**.

➢ In the **Automatic** dialog window, click on **OK** to close the dialog.
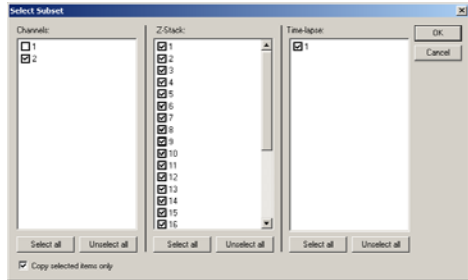
➢ The resulting image is a binary image that contains only the selected sections of channel 2. This image can be used as a mask for automatic measurement.
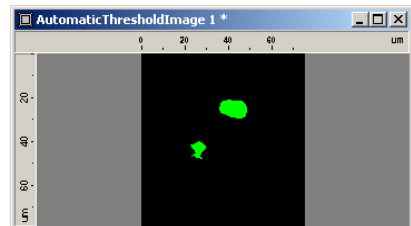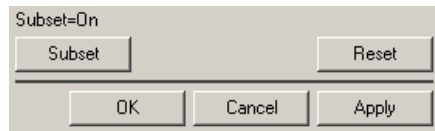
Edge detection

In many images there is no clear distinction between the objects and the background. The objects lie very close together, and are separated from each other by brighter or darker contours. In such cases, threshold value segmentation does not usually produce a satisfactory result, as the contours exhibit significant gray value fluctuations. For these kinds of images, edge detection offers a possible solution.

Imagine an image as a gray value mountain range, where the valleys are formed by dark gray values, and the peaks by bright gray values. In the following example, the **Valleys** function is used. This detects dark edges (gray value valleys) between bright regions.

Before the edges are detected, smoothing is performed. This is determined by the **Sigma** parameter. A high value for **Sigma** means that strong smoothing is performed.

As a rule, the edges are not uniformly dark, and brighter edges (weakly pronounced valleys) and darker edges (strongly pronounced valleys) are present. The detection of the edges can be controlled using the **Threshold** parameter. A low value for **Threshold** excludes bright edges. The higher the value for **Threshold**, the lower the number of edges detected. The value is normally set at a low level, to ensure that any artifacts that are also detected are removed.

NOTE:
- Please note that the setting of the value for **Threshold** depends on the image's pixel type. A value of 0.00001 for **Threshold** corresponds to a gray value in a 16 bit image (1/65536). In the case of an 8 bit image, a gray value corresponds to a value of 0.03 (1/255). This means that with 16 bit images, very low values are sufficient, whilst with 8 bit images, the value has to be set correspondingly higher.

If you have images in which dark objects are separated from each other by bright contours (edges), we recommend that you invert the image before using the **Valleys** function. To do this, use either the **Negative** function from the **Contrast** functional group, or the **Not** function from the **Binary** functional group.

If a color image is used as an input image, you should split the image up into its individual color channels before or after executing the **Valleys** function (**Utilities** functional group, **Split Channels** function), process these channels separately, and combine them as an individual binary image again, before the measurement (**Binary** functional group, **Or** function) is performed.

The following example explains step by step how to detect these contours (edges) in an image that contains bright structures separated by dark contours.

➢ Load the image "Steel-banding.zvi" from the menu **File** ⇒ **Open Image** or acquire an image with the camera.

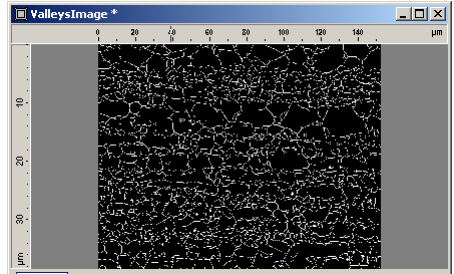➢ The image is displayed in AxioVision's image window.
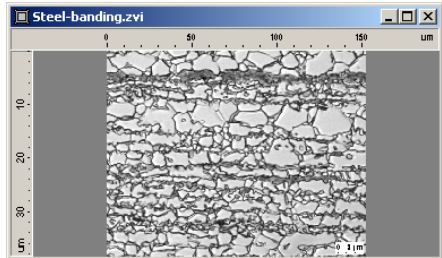
Select from the **Processing** menu the **Segment** functional group, and then the **Valleys** function.

➢ You will now see the dialog window of the **Valleys** function.

➢ If the **Automatic Preview** check box is activated, you will see the reduced input and output images.

➢ Set the value 1.5 for the parameter **Sigma** by clicking on the arrow keys, or by entering the value directly.

➢ Set the value 0.03 for **Threshold** by entering the value directly.

➢ Click on **OK** to close the dialog.
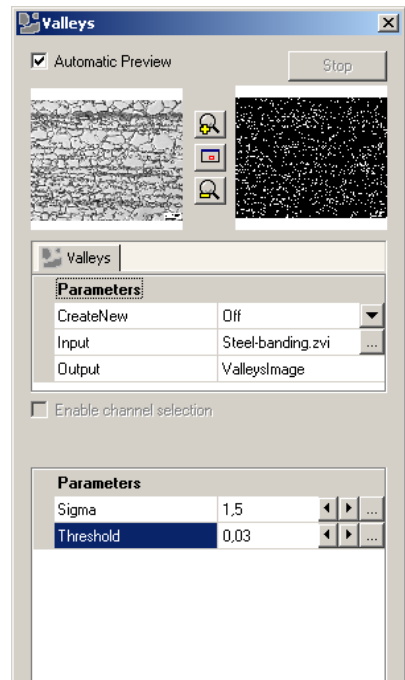
> ➢ The resulting image shows the detected edges, but also numerous small structures (artifacts) that have also been detected.


Select from the **Processing** menu the **Binary** functional group, and then the **Bin Scrap** function.

> ➢ You will now see the dialog window of the **Bin Scrap** function.

> ➢ If the **Automatic Preview** check box is activated, you will see the reduced input and output images.

> ➢ Set the value 1 for the parameter **MinArea** and the value 50 for **MaxArea** by clicking on the arrow keys, or by entering the values directly.

> ➢ Click on **OK** to close the dialog.
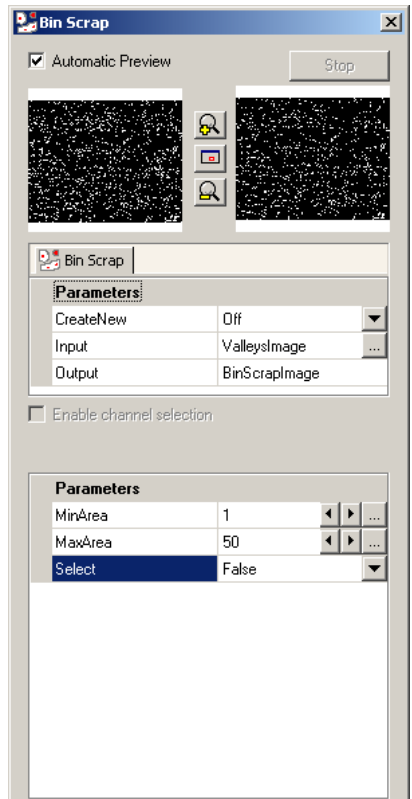
> ➤ In the resulting image, the artifacts have now disappeared. A comparison with the original image, however, shows that some of the detected edges contain gaps.

> ➤ In order to measure the image correctly, the edges of the objects (regions) to be measured must be closed. Using the watersheds function, it is possible to reconstruct them, so that they form complete contours.
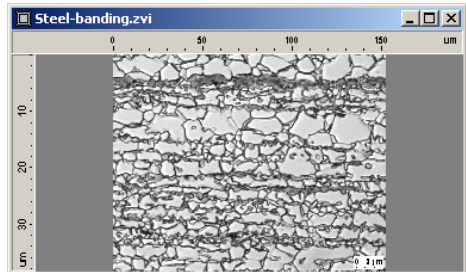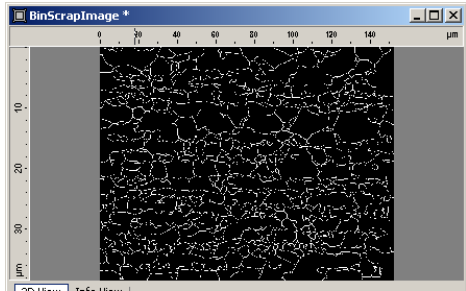
Performing binary image processing

Processing a binary image (smoothing, removing artifacts, filling gaps)

If you want an image to be measured automatically, you must first perform segmentation. This produces a so-called mask (binary image). Frequently the binary image is not perfect, and can only be used for measurement following further processing. During segmentation, incomplete or superfluous structures (gaps, peripheral regions or other structures) are often detected. Special functions are available for processing such structures.

The following example explains step by step how to process a segmented image morphologically, and thereby optimize it.

> Load the image "Cat-Cerebellum(1300).zvi" from the menu **File** ⇒ **Open Image** or acquire an image with the camera.

> The image is displayed in AxioVision's image window.
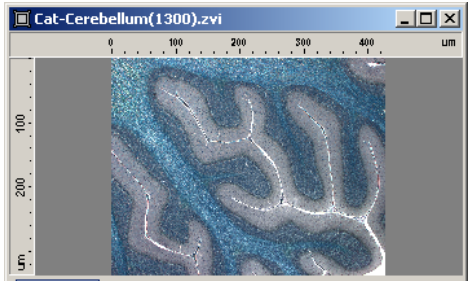
Now select from the **Processing** menu the **Smooth** functional group, and then the **Gauss** function.

> You will now see the dialog window of the **Gauss** function.

> If the **Automatic Preview** check box is activated, the reduced input and output images appear.

> In places the image contains inhomogeneous structures, which make segmentation more difficult. For this reason the image is smoothed in advance using a Gauss filter.

> Set the value 5 for the parameter **KernelSize** by clicking on the arrow keys, or by entering the value directly.

> Click on **OK** to close the dialog.
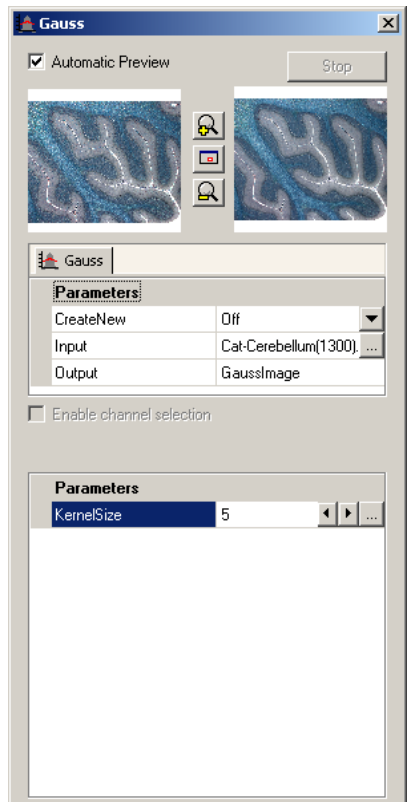
➤ Now select from the **Processing** menu the **Segment** functional group, and then the **Segmentation** function.

➤ You will now see the dialog window of the **Segmentation** function.

➤ Set **CreateNew** to **Off**. Under InputImage select **GaussImage**.

➤ Click on **OK**.
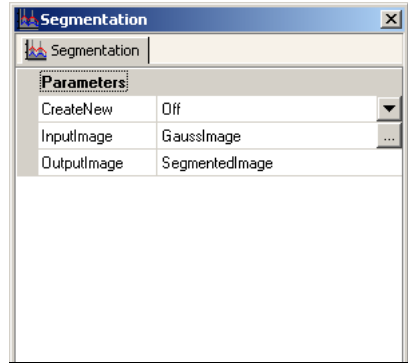
➤ You will now see the dialog window with the **Segmentation** function.
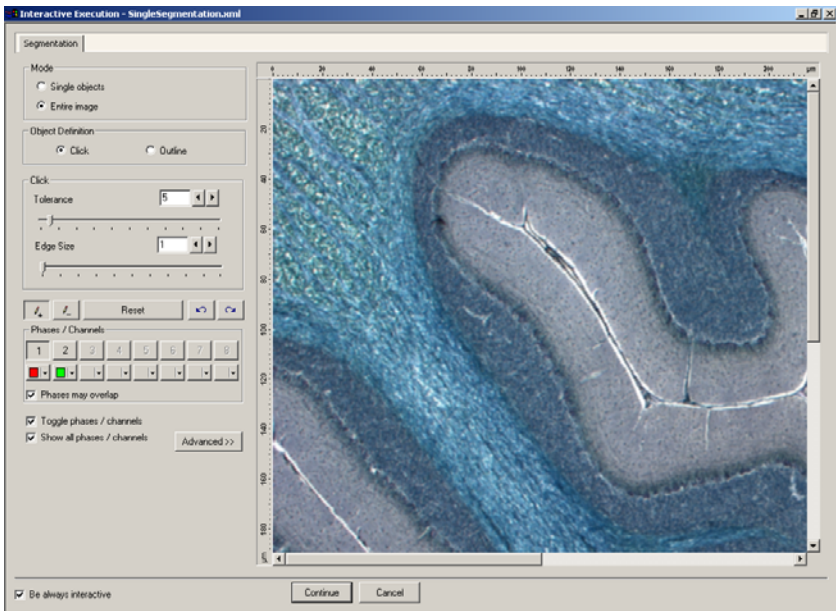
The result of segmentation is a "measurement mask", which is represented internally by a so-called binary image. This mask conceals the background regions using black pixels, and shows the objects to be measured as white. You will only come into contact with this measurement mask indirectly. AxioVision shows it as a color image superimposed over the original image.

➢ Click on **OK**. The resulting image is displayed in the image window.
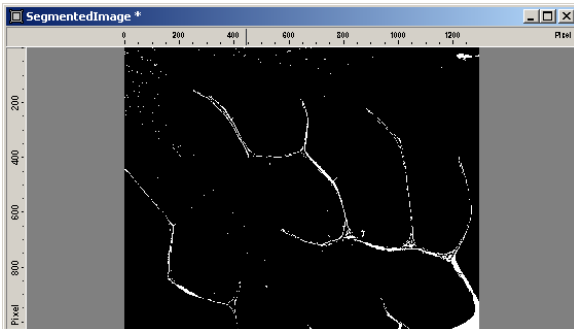
Separation and/or reconstruction of structures using watersheds

Often the image structures that you want to measure are present in the form of agglomerates, or are positioned very close together, which means that separation is required following segmentation. If only the object contours have been detected, these are frequently incomplete, and need to be reconstructed to form complete contours.

If conventional separating functions (combination of **Bin Erode** and **Bin Dilate** or **Exoskeleton**) fail to produce a usable result, the watersheds function can be employed.

In the segmented image it is necessary, first of all, to perform smoothing (using **Lowpass**, **Gauss** or **Median**), in order to eliminate excess structures and structures that are too small. Smoothing can, however, also take place prior to segmentation.

Distance transformation (**Bin Euclidean Distance**) is then used to generate a distance map from the smoothed binary image. The distances of the white regions from the black background are coded as gray values in the resulting image. Bright gray values in the distance map indicate a large distance from the object.

An image can be regarded as a gray-value landscape, with valleys (dark gray values) and hills (bright gray values). In the distance-transformed image, the regions have to be the valleys, in order for the watersheds algorithm to function. The **Watersheds** function floods the valleys, and marks the places where the water masses flow together. These are then identifiable as separating lines or reconstruction lines.

The following example explains step by step how to generate separating lines using watersheds.

➢ Use the resulting image from the **Edge detection** example in the previous section, or generate a similar image.
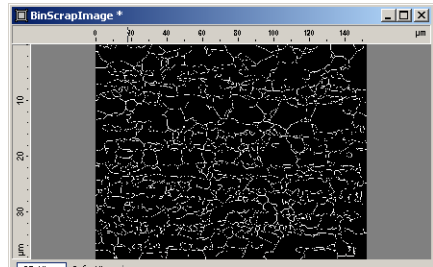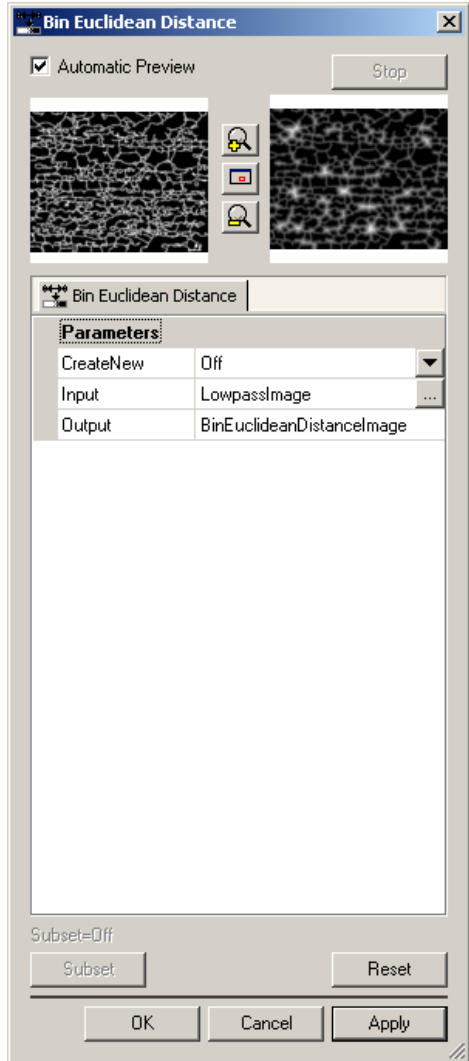
Select from the **Processing** menu the **Smooth** functional group, and then the **Lowpass** function.

- ➢ You will now see the dialog window of the **Lowpass** function.

- ➢ If the **Automatic Preview** check box is activated, the reduced input and output images appear.

- ➢ Set **CreateNew** to **Off**, so that you can select the images freely.

- ➢ If you click on the ⊡ button in the **Input** field, the image gallery appears. Select the original image by clicking on it.

- ➢ Set the value 5 for **KernelSizeX**, **KernelSizeY** and **Count** by clicking on the arrow keys, or by entering the value directly.

- ➢ Click on **OK** to close the dialog.
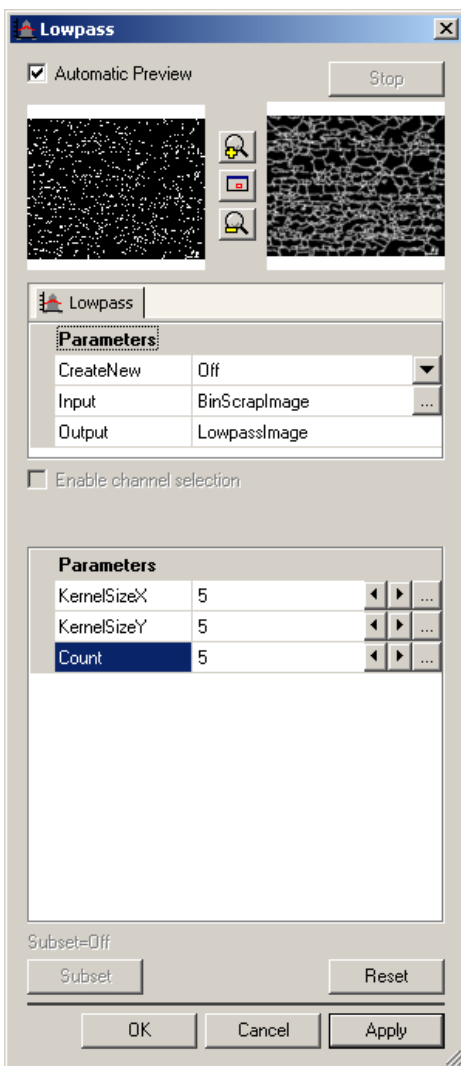
Select from the **Processing** menu the **Binary** functional group, and then the **Bin Euclidean Distance** function.

➢ You will now see the dialog window of the **Bin Euclidean Distance** function.

➢ If the **Automatic Preview** check box is activated, the reduced inputand output images appear.

➢ Click on **OK** to close the dialog.

Now select from the **Processing** menu the **Morphology** functional group, and then the **Watersheds** function.

➢ You will now see the dialog window of the **Watersheds** function.

➢ If the **Automatic Preview** check box is activated, the reduced input and output images appear.

➢ Select under **Basins** the **Off** setting.

➢ Click on **OK** to close the dialog.
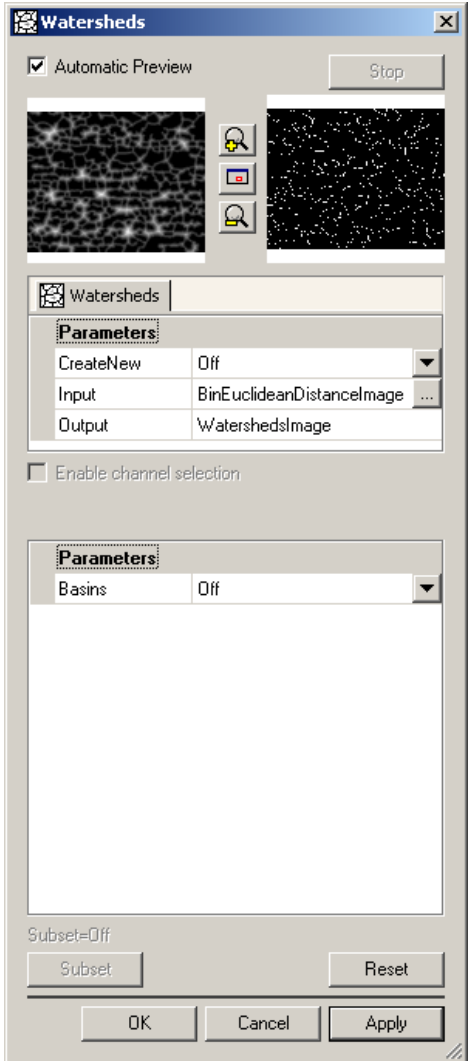
> ➤ The resulting image now shows the reconstructed separating lines.

> ➤ A comparison with the original image clearly shows that incomplete separating lines have been completed, and superfluous structures removed.

> ➤ A comparison with the original image shows that unclear object contours can be detected very effectively using Watersheds.

> ➤ If the resulting image is to be used as a mask for measuring, it must first be inverted using the **Not** function.
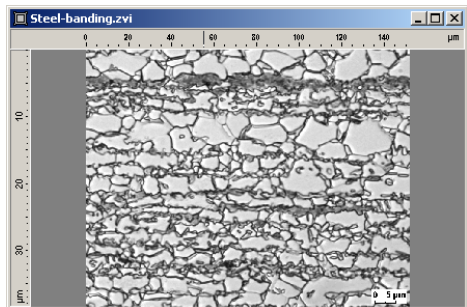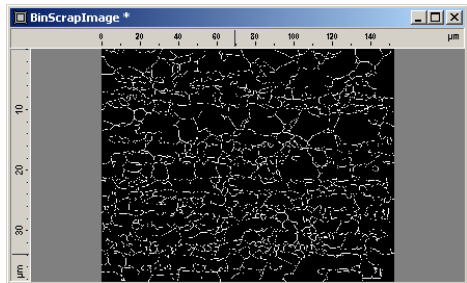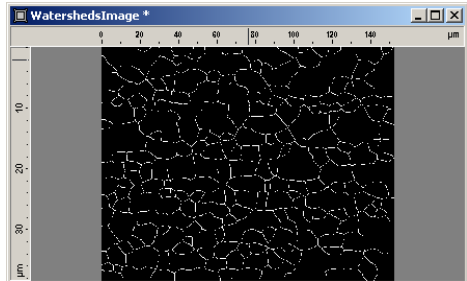

Masking structures/Using Boolean functions

Images often contain structures with varying brightnesses or colors, which are detected over several segmentation steps. In some cases, the binary images produced have to be combined in order to join together or mask structures. Boolean operations are available for this purpose.

The **And** function can be used to mask structures, whilst the **Or** and **Xor** functions are better suited to combining structures. Frequently an inverted image is required, which can be generated using the **Not** function.

The following example explains step by step how to mask or combine structures from various binary images.

➢ Use the segmentation functions to generate three binary images in which various structures have been detected.

➢ The example uses a metaphase image, from which the DAPI channel, the FITC channel and the Rhodamine channel have been detected in three separate steps.
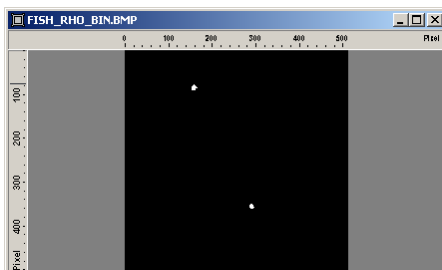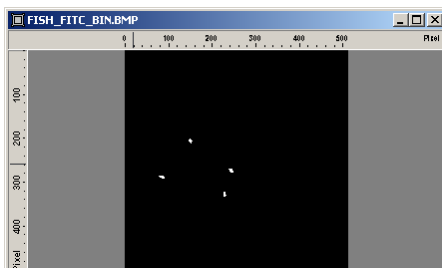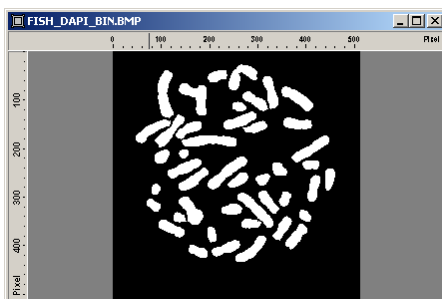
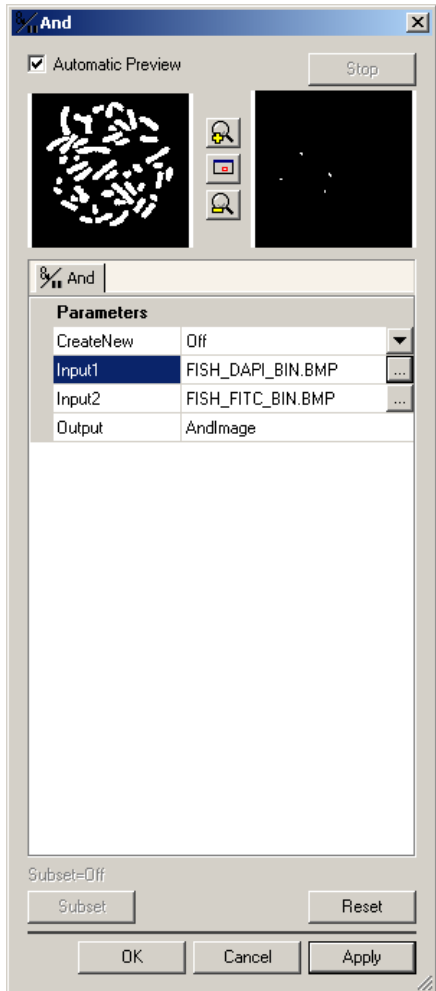Now select from the **Processing** menu the **Binary** functional group, and then the **And** function.

➢ You will now see the dialog window of the **And** function.

➢ If the **Automatic Preview** check box is activated, the reduced input and output images appear.

➢ Set **CreateNew** to **Off** so that you will be able to select the images freely later.

➢ If you click on the [...] button in the **Input** field, the image gallery appears. Here select the image in question by clicking on it. Select different binary images for **Input1** and **Input2**.

➢ Click on **OK** to close the dialog.

➢ The **And** function only masks structures that are present in both images.

➢ As, in the sample image, the FITC structures lie directly on top of the DAPI structures, the resulting image is identical to the FITC binary image.
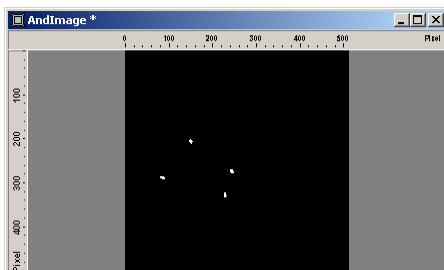
Now select from the **Processing** menu the **Binary** functional group, and then the **Or** function.

➢ You will now see the dialog window of the **Or** function.

➢ If the **Automatic Preview** check box is activated, the reduced input and output images appear.

➢ If you click on the [...] button in the **Input** field, the image gallery appears. Here select the image in question by clicking on it. Select different binary images for **Input1** and **Input2**.

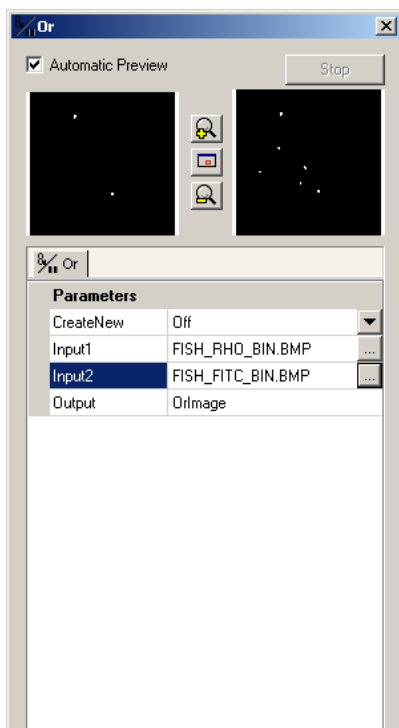➢ Click on **OK** to close the dialog.

➢ The **Or** function masks
   structures that are present in
   either one image or the other.

➢ In the sample image, the FITC
   structures and the Rhodamine
   structures are in different
   positions. Both types of
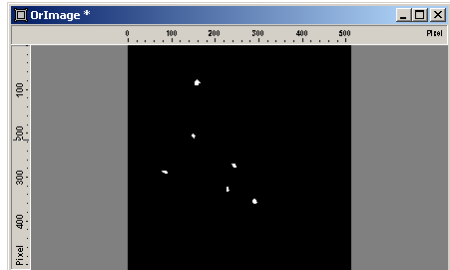   structure appear in the
   resulting image.

Now select from the **Processing** menu the **Binary** functional group, and
then the **Xor** function.

➢ You will now see the dialog
   window of the **Xor** function.

➢ If the **Automatic Preview**
   check box is activated, the
   reduced input and output
   images appear.

➢ If you click on the ⊡ button in
   the **Input** field, the image
   gallery appears. Here select
   the image in question by
   clicking on it. For **Input2** select
   the resulting image of the **Or**
   function, and for **Input1**
   another binary image.

➢ Click on **OK** to close the
   dialog.
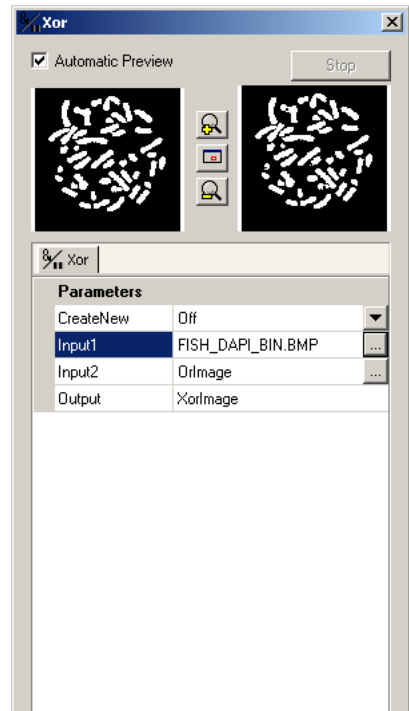
➢ The **Xor** function masks as black any structures present in both images. Any white structures that do not overlap also appear as white in the resulting image.

➢ In the sample image, the FITC and Rhodamine structures lie directly on top of the DAPI structures. Structures in the resulting image therefore appear as gaps in the DAPI image.

Now select from the **Processing** menu the **Binary** functional group, and then the **Not** function.

➢ You will now see the dialog window of the **Not** function.

➢ If the **Automatic Preview** check box is activated, the reduced input and output images appear.

➢ If you click on the [...] button in the **Input** field, the image gallery appears. Here select the image in question by clicking on it. For **Input1**, select any binary image.
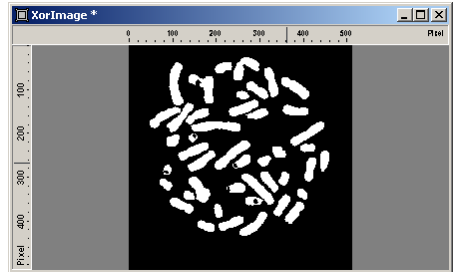
➢ Click on **OK** to close the dialog.
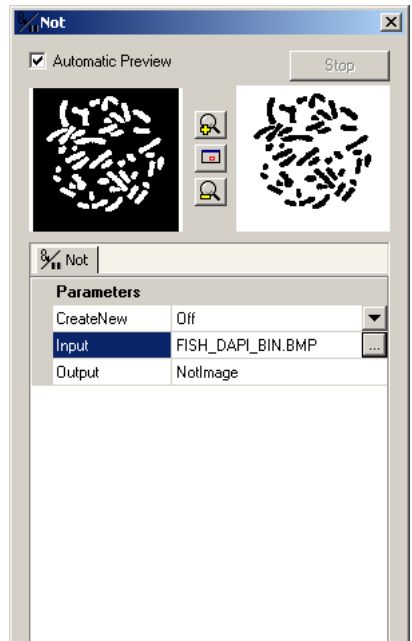
➢ The resulting image now shows all white structures as black, and vice versa.
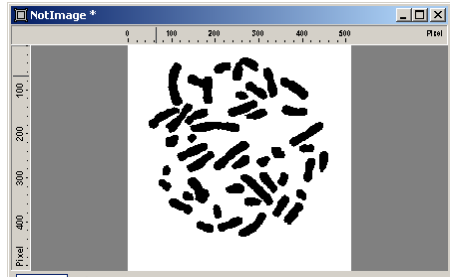
Working with the Functions for automatic Measurement

Generation of Measurement Properties Files and Measurement

The generation of a properties file consists of several steps. These are used to define how the measurement should be carried out in detail.

This requires a correct measurement mask (binary image). See previous chapters on how to generate a binary image.

➢ Select the function **Set Measurement Properties** from the menu **Measure** ⇒ **Automatic Measurement**.

➢ For **MaskImage** select the binary image and for **DensImage** the original image.

➢ Click **OK** to start the function.
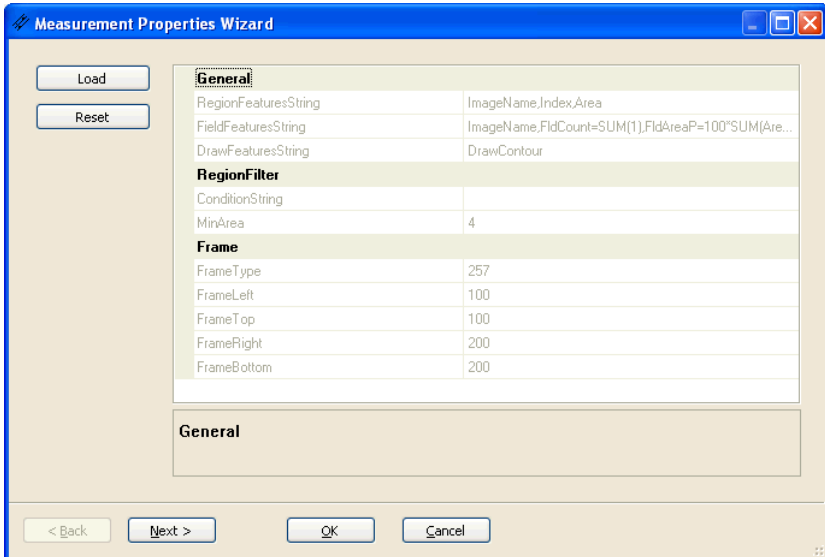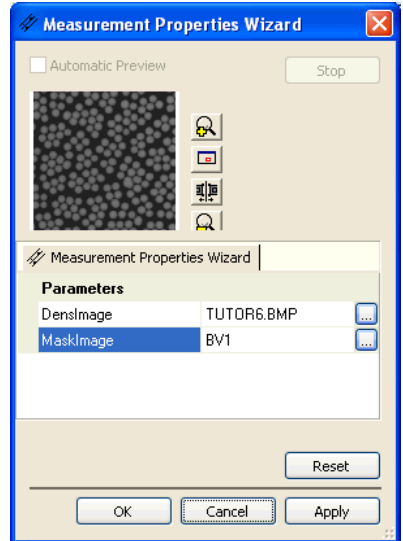
The individual settings:

➢ You can determine region-specific (individual objects) and field-specific (entire image) measurement parameters within **Region Features** or **Field Features**.

➢ You can define the way in which the measured objects should be highlighted in the resulting image at the end of the measurement (contour, measurement parameters etc).

➢ Under **RegionFilter** you can generate a measurement condition. This defines the conditions that an object must satisfy in order to be measured. This is a kind of "object filter", which you can use, for example, to specify that only objects within a certain size category should be counted.

➢ Under **Frame** you can define a measurement frame, which is used to specify how objects that are cut off by the image edge, and are therefore likely to distort the measurement result, should be handled.

NOTES:

• You can load the settings of already existing properties files by the **Load** button on the first page of the wizard. Finish the wizard by clicking on the **OK** button. You can now proceed directly with the measurement.

• To load a property file for a measurement within a Commander script, please use the **Measure ⇒ Automatic Measurement ⇒ Load Measurement Properties** function.

> After you have gone through the **Set Measurement Properties** (or loading the properties directly from a file), start the measurement by selection of the function **Start Measurement** from the **Measure ⇒ Automatic Measurement** menu.
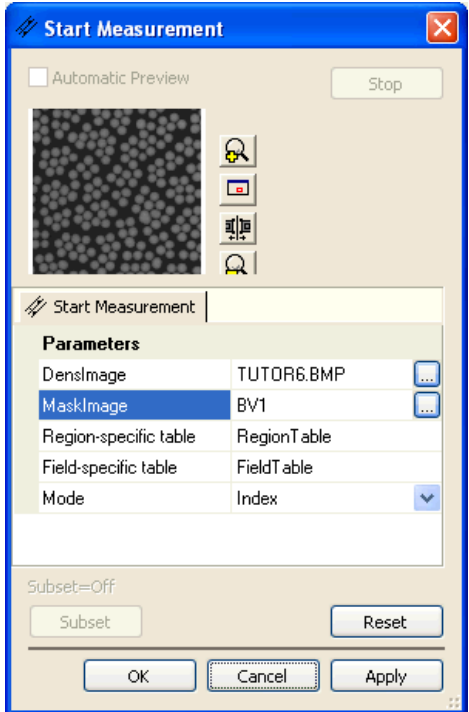
> If you click on the ⌐ button in the **Input** field, the image gallery appears. Here select the image in question by clicking on it. For **Input** select the original image and for **MaskImage** the binary image.

> Click on **OK** to close the dialog.

The measurement data are saved at the end of the measurement in so-called CSV format (**C**omma **S**eparated **V**alues).

This is a pure text format that separates the individual values from one another using special text symbols. The specific country settings that have been set in the Microsoft Windows control panel apply.

Files in CSV format offer two advantages: on the one hand, they are a special Microsoft Excel format, and can be opened and processed directly by that program. On the other hand, as the files contain pure text data, you can import the data into practically any other program.

11.4 Calotte Grinding Measurement

General description

During the process of coating materials, quality is controlled by measuring the thickness of layers in accordance with the DIN V ENV 1071 standard. The Calotte Grinding Measurement module allows you to determine the thickness of calotte section layers. To prepare the calotte section samples, a grinding ball with a defined diameter generates a calotte-shaped hollow in the coating of the sample. This results in circular or elliptical indentations, the diameters of which are determined by drawing in measurement circles. The layer thickness is calculated automatically from the diameters of the measurement circles and the diameter of the grinding ball.

Both single and multilayer samples can be measured. The measurement results and sample information from the calotte grinding measurement are displayed in an image and stored. A report containing the image, measurement results and information about the sample can also be produced.

Performing a calotte grinding measurement

> Place the sample you want to measure on the microscope stage.

> Turn the nosepiece to the position with the desired objective.

> Now check that the calotte section is positioned correctly, and adjust its position if necessary.

> Start AxioVision.

Clicking on the **Calotte Grinding Mesurement** icon opens the wizard. This will guide you through the **Calotte Grinding Mesurement**.

You can also call up the wizard from the **Applications** menu $\Rightarrow$ **Calotte Grinding Mesurement** $\Rightarrow$ **Start Assistent**.

Describing the sample

The first page of the wizard is displayed: **Sample description (Step 1 of 4)**. In the upper area, you will find a brief description of what you should do in this step.

➢   Enter the sample data in the **Batch Number** field.

➢   New layer types can be entered using the **New** button. The new layer type will then appear automatically in the list of layer types.

➢   Select one of the available scalings from the list in the **Available Scaling(s)** field.

➢   New scalings can be generated using the **New** button. The new scaling will then appear automatically in the scalings list.

➢   Then click on the **Next** button in the bottom left-hand area of the wizard.

Opening an image

The second page of the wizard is displayed on the screen. Under the title
Open image (Step 2 of 4) you will find a brief description of what you
should do in this step.

➢ Click in the field **Source: Camera** the
button **Live-Start** to open the live
image window of the active camera.
Center the calotte section in the live
window and focus it.

➢ Click the corresponding camera button
(in this case AxioCamMR5) to open
the camera dialogue. Set the exposure
time by clicking the **Measure** button.

➢ The click the **Snap** button to acquire
the active image.

➢ If you would like to measure already
saved images, click the **Open Image**
button. You can open an image saved
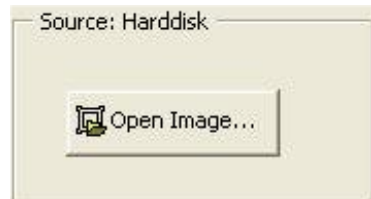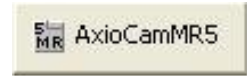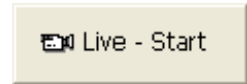on the hard disk or any other storage
medium.

➢ Then click on the **Next** button in the bottom left-hand area of the
wizard.

Drawing circles

The third page of the wizard is displayed on the screen. Under the title **Draw Circles (Step 3 of 4)** you will find a brief description of what you should do in this step.

➤ In the **Method** field, select the **Single Layer** function (if the sample only has one layer) or the **Multi Layer** function (if the sample has several layers).

➤ Click on the tool that you wish to use to perform the measurement:

➤ Use the **Circle Points** or **Circle** tools for circular indentations:

If you use **Circle Points**, left-click on several points on the edge of the circle. Right-click to display the circle calculated from these points.
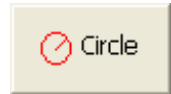
If you use **Circle**, start from the edge of the circle and move inwards, holding down the left-hand mouse button, to drag out a circle the same size as the indentation.

➤ Use the **Ellipse** tool for elliptical indentations. Start from the edge of the ellipse and move inwards to drag out a circle the same size as the indentation.
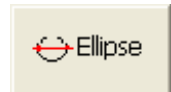
➢ The results of the measurement (diameter and layer thickness) are displayed in the **Results** field.

Results

Diameter 1 [µm]: 654.11
Diameter 2 [µm]: 579.43
Result t [µm]: 1.15

➢ Click on the **Remove circles** button if you want to delete the circles that you have drawn in. If necessary, start drawing in the circles again.

✕ Remove Circles
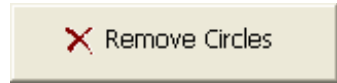
➢ In the **Attributes** field, you can change the **Color** and **Line Width** of the drawn-in circles. To do this, select the desired color or line thickness from the relevant drop-down list box.

Attributes

Color:

Green

Line Width:

6

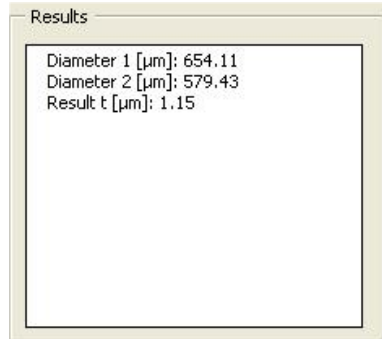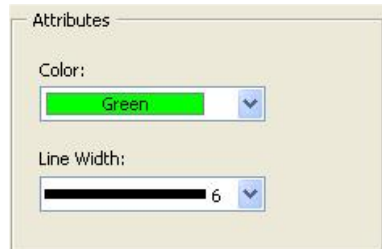➢ Click on the **Magnifier** button to magnify individual areas of the image. To do this, position the cursor on the area of the image that you wish to magnify. When you are dragging out the circle, the magnifying glass can help you to position the start point of the circle extremely precisely.

🔍 Magnifier
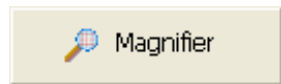
➢ Then click on the **Next** button in the bottom left-hand area of the wizard.

Evaluation

The fourth page of the wizard is displayed on the screen. Under the title **Evaluation (Step 4 of 4)** you will find a brief description of what you should do in this step.

The results of the measurement are displayed together with the sample data in the **Results** field.

➢ Click on the **Show Results in Image** button in the **Annotations** field to display sample information and measurement results in the image.
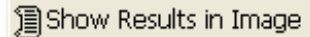
➢ Select the desired color and font size for the **Annotations** from the appropriate list.

➢ Then click on the **Save Image As** button to save the image in any directory on the hard drive or another storage medium.
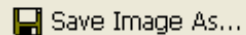
➢ The annotations are automatically burnt into the image when it is saved. To ensure that this takes place, make sure that the **Burn-in annotoations** check box in the **Save Image As** dialog window is activated if the image is not being saved in the Carl Zeiss ZVI image format.
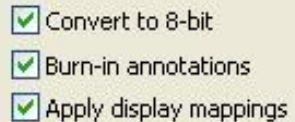
➢ Click on the **Create** button in the **Report** field to see a preview of the report. You can print out the report by clicking on the **Print** button.
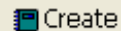
➢ Then click on the **Finish** button in the bottom right-hand area of the wizard, to leave the wizard.

Appendix A – Example of a Report

AxioVision Calotte Grinding Measurement

Evaluation

Batch Number: 0001
Coating Type: test layer
Date: 1/27/2005
Diameter Ball [mm]: 20
Scaling [µm/Pixel]: Epiplan10x_2x2bin_063x.zvsc

Batch Number: 0001   D1[µm]: 654.11
Coating Type: test layer   D2[µm]: 579.43
Date: 1/27/2005
Diameter Ball [mm]: 20
Comment: This is a te
st.

Thickness [µm]: 1.15   100 µm
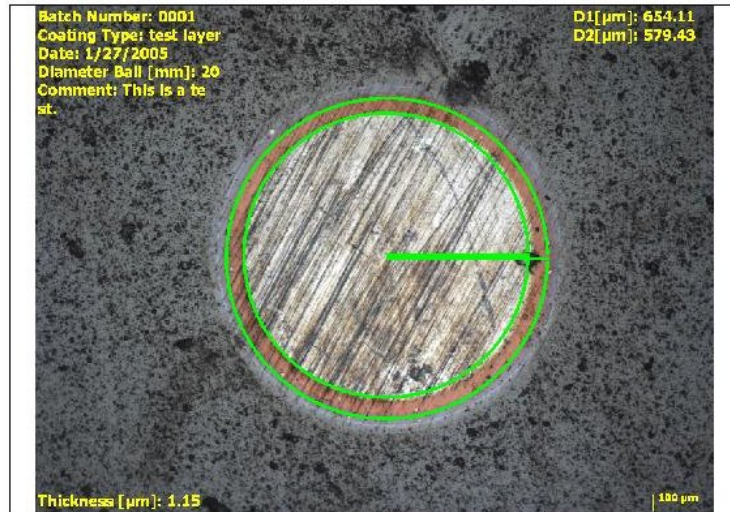
Results (Single Layer):
Diameter 1 [µm]: 654.11
Diameter 2 [µm]: 579.43
Result t [µm]: 1.15
Comment: This is a test.

11.5 Microhardness Measurement

General

The Microhardness Measurement module allows you to control an MHT (microhardness tester), and measurements of the microhardness of a sample. Detailed information on the MHT and its use and functions can be found in the operating instructions for the MHT from Anton Paar GmbH.

To perform a measurement, you require (in addition to the Microhardness Measurement module) a reflected light microscope with MHT controller and MHT sensor.

Microhardness measurement is a method used to characterize materials and their surfaces. An indenter (a sensor with a diamond tip) is pressed into the sample with a certain force. The Microhardness Measurement module requires the following three entries:

➢ **Force in Newton (F)**.

➢ **Dwell time in seconds**: To avoid plastic deformation or shrinkage of the material due to the sensor being removed too quickly, it is possible for the diamond tip to remain in the sample for a specified period of time.

➢ **Slope in Newton/second**: The specified slope determines how quickly the applied force reaches the maximum value F from the moment of its first contact with the sample surface. With fragile samples, low slopes should be used to avoid microcracks.

The size of the indent is then measured, and the hardness calculated from this:

$$Hardness = \frac{Force\ applied}{Indent\ size}$$

A force of a few Newtons is applied, producing indents of a few micrometers. The form of the indent depends on the type of indenter used.
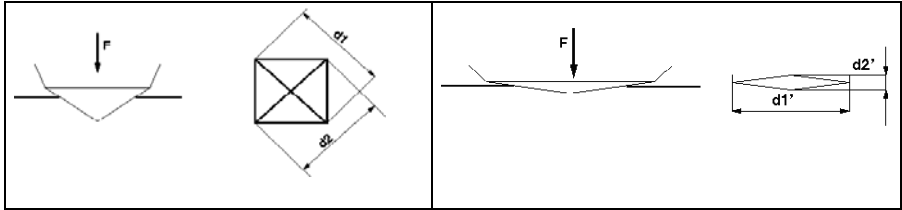
Vickers indenter   *Knoop indenter*

In both cases, the size of the indent is determined on the basis of the diagonals of the rectangle produced. The formulae for calculating Vickers hardness and Knoop hardness are different, but produce the same microhardness results.

The microhardness measurement results are presented in the following form:

1200 HV 0,1/20	Dwell time in seconds
	Force applied in Newtons
	Method indicator (HV - Vickers, HK - Knoop)
	Hardness value

Workflow

MHT settings sets the parameters for the microhardness tester.

Run indent starts the procedure for creating an indent.

Live opens and closes the live window.

Snap acquires an image using the active camera.

Measure allows you to interactively measure the indent and display the results of the measurement in the image.

Remove last deletes the last measurement performed.

Remove all deletes all microhardness measurements in the image.
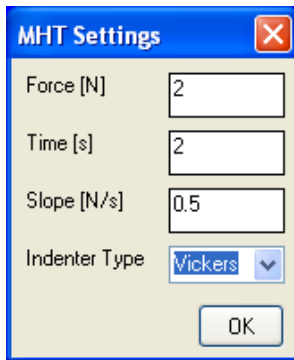
Report generates a report consisting of the measured image and the results of the measurement.

Microhardness measurement

MHT settings

Run indent

Live

Snap

Measure

Remove last

Remove all

Report
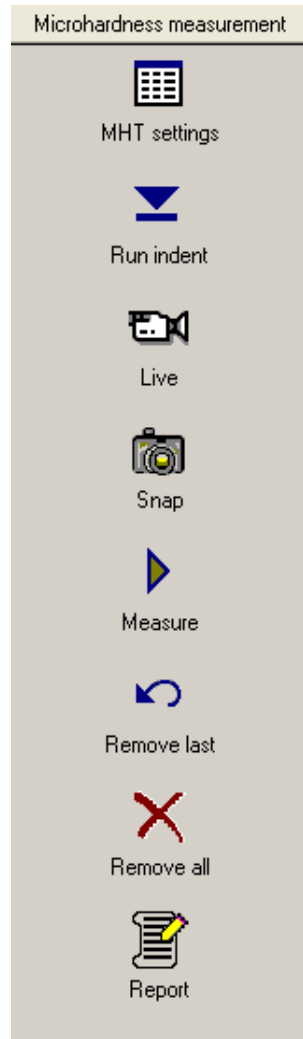
Performing a microhardness measurement

➢   Place the sample you want to measure on the microscope stage.

➢   Turn the nosepiece to the position with the MHT sensor.

➢   Now check that the sample is positioned correctly for the indent, and correct its position if necessary.

If you click on **MHT settings**, the **MHT Settings** dialog window is opened:

Insert the data for the following values:

•   **Force [N]**: The force is limited to 2N for MHT4 or 4N for MHT10.

•   **Time [s]**: The dwell time should be 10s – 20s.

•   **Slope [N/s]**: The slope is limited to 0.999 N/s for MHT4 or 9.99 for MHT10.

➢   If you click on **Run indent**, a flashing window appears:

> ➤ The red light on the MHT control device flashes. Wait until the flashing window has disappeared. If you want to interrupt the process, click on the **ABORT** button.

> ➤ Turn the nosepiece to the objective with the desired magnification (normally 20x or 50x).

> ➤ Click on **Live**. The live window is opened.

> ➤ Position the indent at the center of the live window, and bring it into focus:
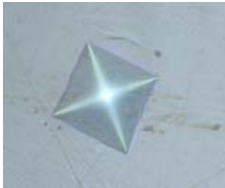
> ➤ Adjust the brightness and exposure time etc. via the toolbar:

> ➤ Select the scaling for the set objective from the **List of available scalings** under the live window.
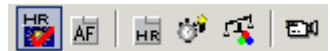
➢ You can make several indents at various positions on the sample in a single workflow.
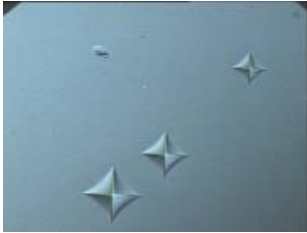
Click on the camera icon to acquire the image.

➢ Use the **Image** property page (**Tools** menu ⇒ **Options** function) if you would like to save images automatically under a predefined name. A detailed description can be found in the online help (**F1**).

➢ Click on Navigator on the standard toolbar, and zoom to change the region you want to be measured:

➢ Click on **Measure**, and draw in the two diagonals by clicking on the start and end points. The result of the measurement is displayed automatically.

620 HV 2.0/10

➢ Repeat this step for all indents in the image. A maximum of four indents per image can be measured.
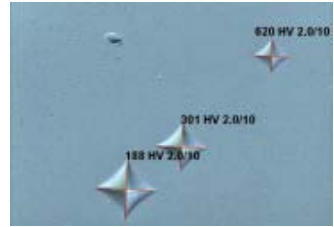
➢ Click on **Remove last** if you want to delete the diagonals you have drawn in step by step.

before   *after*

➢ Click on **Remove all** to delete all the indent measurements you have made up to this point, and start again by clicking on **Measure**.

➢ If you click on **Report**, a report is generated (see Appendix A).

You can also use the **Microhardness Measurement** tool bar to perform the microhardness measurement.

Additionally you will find another button [MHT configuration] to configure the MHT control device. If you click on this button, the following dialog window opens:
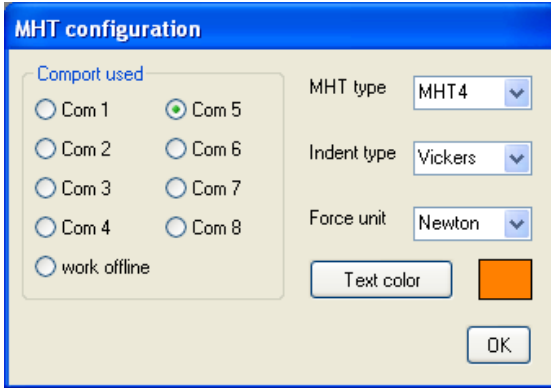
Select here the corresponding COM port for the MHT control device.

Select further the type of the MHT device (MHT4 or MHT10), the indent type (Vickers or Knoop), the desired force unit (Newton, Pond, gram force) and the text color for displaying the measurement values in the image.

Glossary

Microhardness: Hardness of the sample at the microscopic level.

Indent: The impression resulting from the microhardness tester being pressed into the surface of the sample.

MHT: Microhardness Tester – the name of the device used for the microhardness measurement. This can be obtained via Carl Zeiss. It is manufactured by Anton Paar GmbH, Graz, Austria.

Sensor: For the microhardness measurement, a tiny diamond tip is pressed into the surface of the sample with a very small, defined force. The sensor is screwed into the nosepiece instead of an objective, and controls and moves the diamond tip.
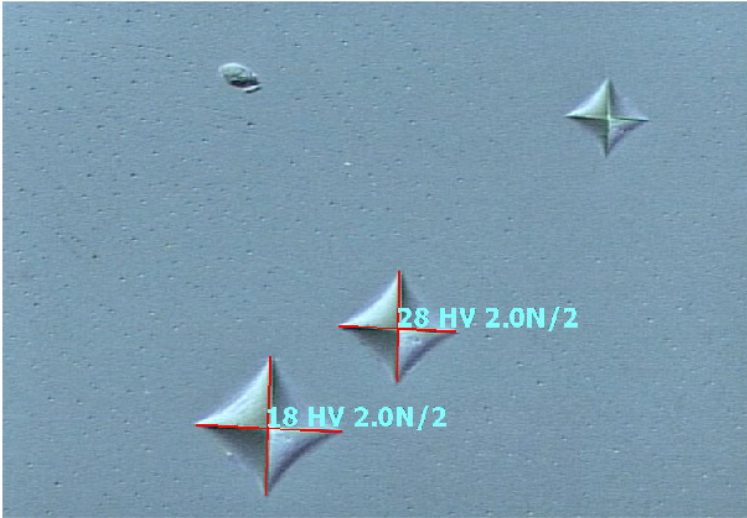
Appendix A – Example of a report

11.6 TIC Measurement

TIC (**T**otal **I**nterference **C**ontrast) is a new method of measuring the heights of thin layers using optical interference.

To perform TIC measurement, you require a reflected light microscope with a C-DIC reflector and a TIC slider, in addition to the AxioVision TIC Measurement module. The operating instructions of the Axioskop 2 MAT mot microscope contain a detailed description of the TIC method.

Automatic TIC measurement

Scalings offers the calibration for the TIC measurement.

Live opens and closes the Live window.

Snap acquires an image using the active camera.

Navigator opens the window for zooming.

TIC Measurement starts the TIC measurement.

Add to Archive saves the measured image in the archive.
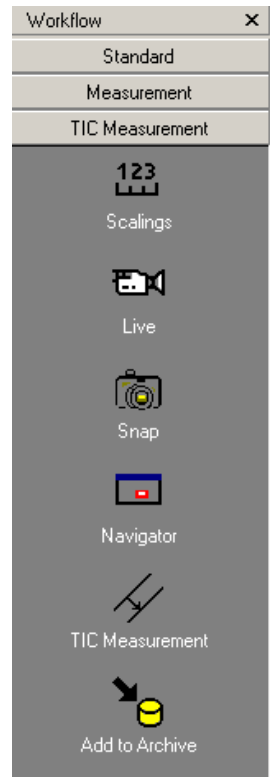
> Click on [123 Scalings icon].

> Activate the TIC scaling that corresponds to the selected objective and the selected camera resolution.
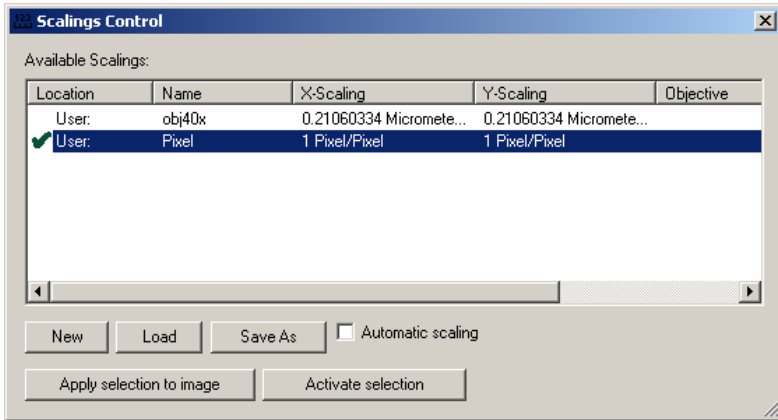
Please note that TIC Measurement requires TIC calibrations, and not the usual geometric calibrations. A description of how to generate a scaling can be found under "TIC scaling" at the end of section 7.2.

If you do not intend to change the selected microscope and camera settings, you can now close the calibration window.

> Click on [Live icon]. Set the illumination and contrast such that the image you can see is optimally illuminated.

  Check the camera resolution. This must correspond to the scaling you have loaded.

> Set the image field to be measured on the sample and the direction of the interference fringes.

The image should look something like the sample image below:
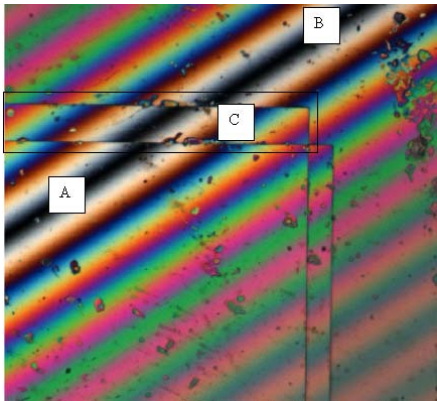
The thin-layer sample contains three regions:

A: the top left-hand corner of the structure, which contains the height that you want to measure
B: the surface underneath this
C: the region with displaced interference fringes

The fringe displacement is due to the difference in height between the surface of the structure to be measured (bottom left in image) and the surface underneath this (top right in image).

The displacement of the zero-order interference fringe in the overlap region is now to be measured, i.e. the distance between **A, B** (outside the overlap region) and **C** (inside the overlap region).

Please note that the color of the fringes changes symmetrically around the zero-order interference fringe, both inside and outside region **C**. The zero-order interference fringe can be easily identified as the darkest fringe.

➢ Click on the camera icon [Snap] to acquire the image.

➢ Click on **Navigator**, and zoom to change the region you want to be measured:
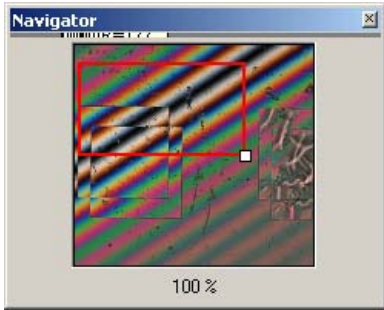
➢ Click on .

➢ If you move the mouse inside the image, the cursor symbol changes (from an arrow to a pipette) and indicates the x/y coordinates and color values of the pixel:

➢ Move the mouse to the center of the zero-order interference fringe on the underlying surface of the sample and left-click.
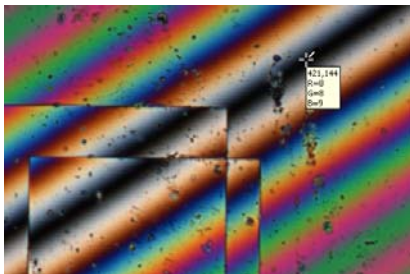
> ➢ Move the mouse into the overlap region (containing the displaced interference fringes), and left-click again at the center of the zero-order interference fringe.
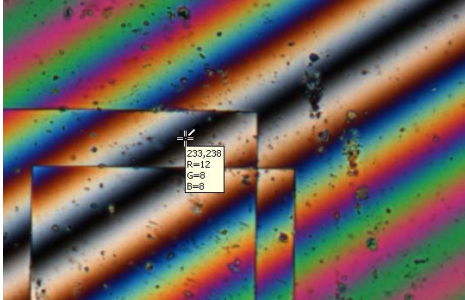
The measured value is displayed:
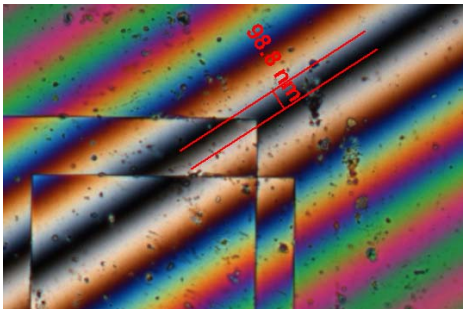
Click on [Add to Archive] to save the image and the measurement generated.

Interactive TIC measurement

On the **Measure** menu click on the **Distance** function, or click on in the menu toolbar.

> ➢ Position the cursor (shown here as a cross) at the starting point of the line. Left-click.

> ➢ Move the cursor to the end point of the line. Left-click. You will now see the first line in the image.

> ➢ Make sure that the line you have drawn corresponds to the center of the fringe.

> ➢ If you move the cursor, you will see the second line and the current measurement result, which changes as you move the cursor.
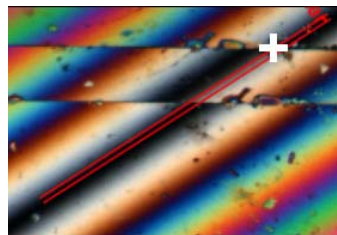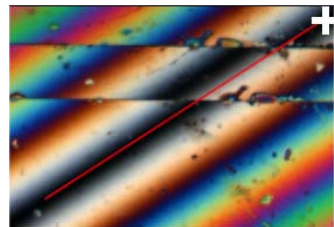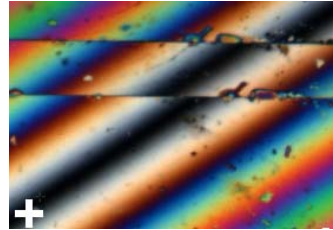
> ➢ Move the cursor until the second line corresponds to the center of the zero-order interference fringe. Left-click to end the measurement.
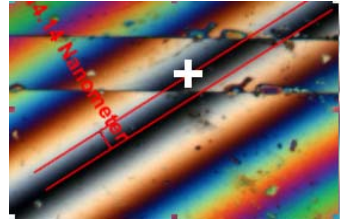
Causes of errors during interactive measurement

The longer the line, the more precise the measurement. Set the starting point of the line at the center of the zero-order interference fringe, and the end point as far as possible from it.

Below you will find four examples of errors that can occur while you are drawing in the line.
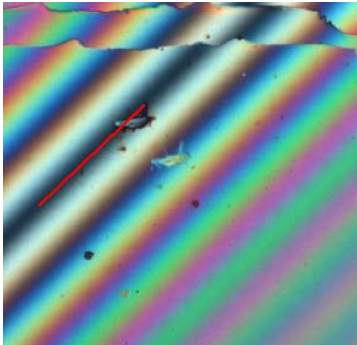
The line is too short.
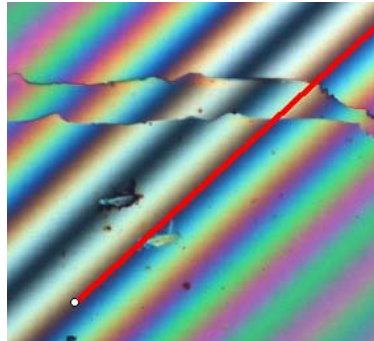
The zero-order interference fringe has not been selected.
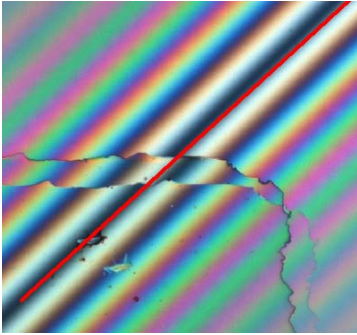
The line is not parallel to the interference fringe.
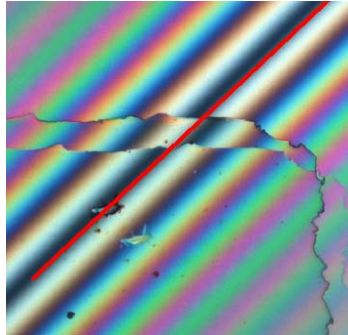
The line is not at the center of the interference fringe.

Below you will find two examples of errors that can occur while you are determining the position of the displaced zero-order interference fringe.
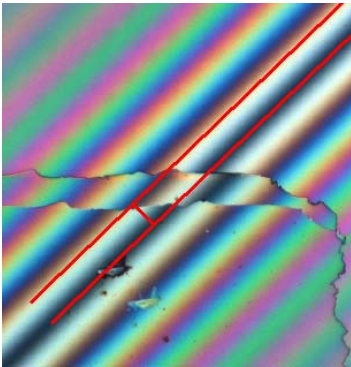
The first-order interference fringe has been selected.
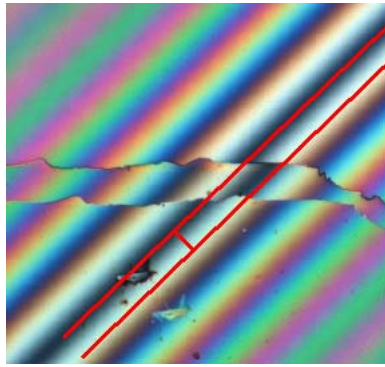
The line is not at the center of the first-order interference fringe.

TIC scaling

In order to perform the TIC measurement, you require a set of TIC scalings (for each magnification and camera combination).

Start with a frequently used combination, such as the 10x objective and the AxioCam at the standard resolution.

Just like any other scaling procedure, TIC requires a reference object. Any reflective surface is suitable for TIC. Focus on the surface, optimize the exposure time and acquire the image by clicking on [Snap].
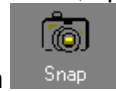
➢ From the **Measure** menu, select the **Scalings** function.

➢ In the **Scalings Control** window click on **New**.
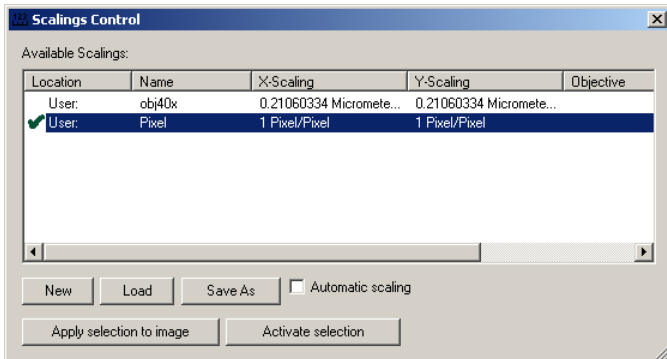
The scaling wizard is started.

➢ Right-click in the image. The image's shortcut menu is displayed. Select the **Normal View (1:1)**.

➢ Magnify the window by clicking on .

➢ Move the scrollbars at the bottom and right-hand edges of the image to set the desired frame.
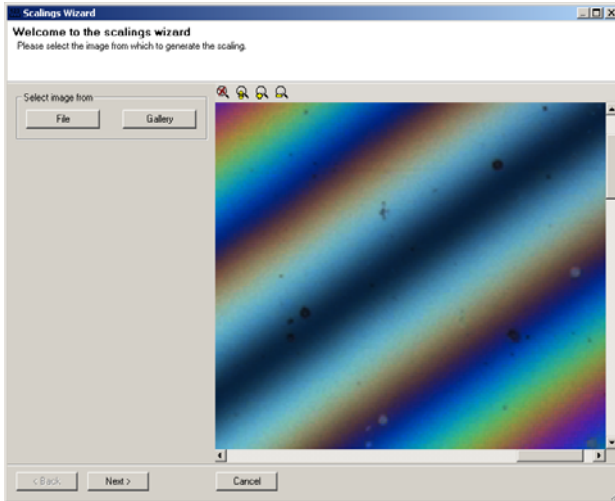
➢ Click on **Next**.

➢ Activate **Automatic line-detection**.

➢ Select the method **Single parallel lines (X+Y)**.
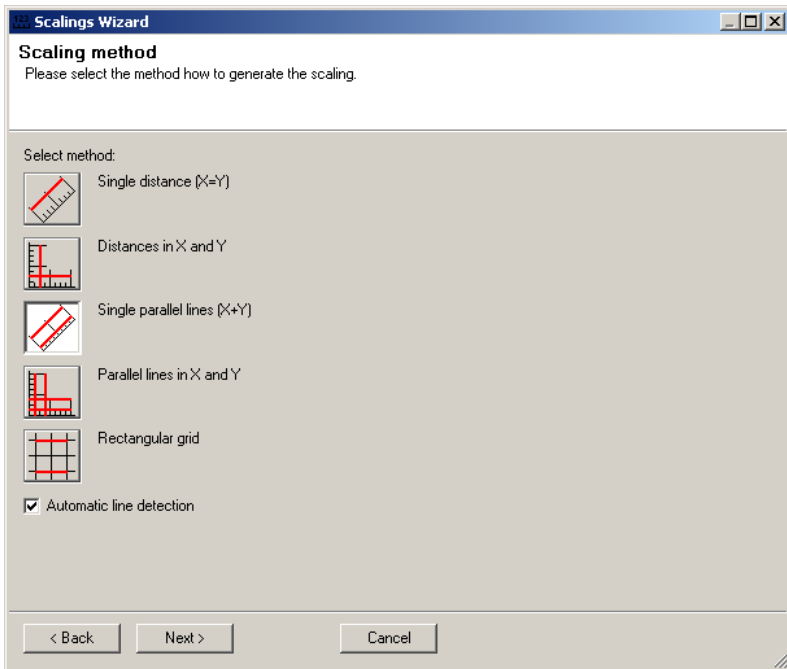
➢ Click on **Next**.

➢ Enter the value 275 into the **Distance** text field, and select the unit **Nanometer** (275 nm corresponds to half the wavelength of visible light).

➢ Now determine the distance between two interference fringes by drawing in two lines between two dark fringes. To do this, click on the starting and end points at the center of the interference fringe.

➢ The distances are marked by red lines. The correct distance is determined automatically and displayed as a third line between the two lines you have drawn.
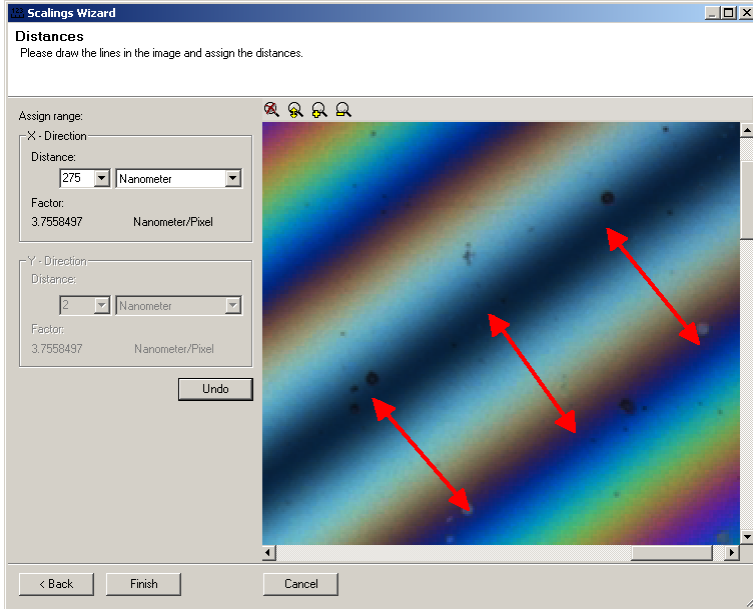
➢ Click on **Finish**.

➢ Enter a name for the scaling and click on **OK**.
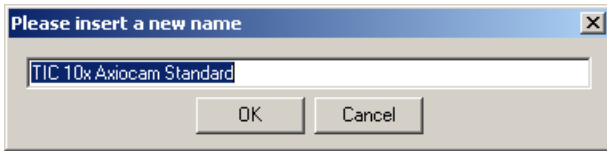
To avoid confusing this scaling with other scalings, you should use TIC as a prefix.

The new scaling now appears in the **Scalings Control** window, and is activated automatically. It is used for all images subsequently acquired.
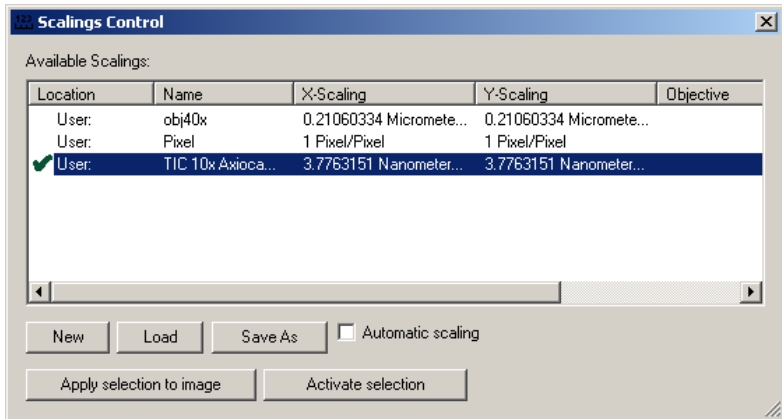
11.7 Particle Analyzer Projects

General

The system variants

The Particle Analyzer Projects module forms the basis for the **Particle Analyzer** system on the software side. The Particle Analyzer from Carl Zeiss is a complete system for analyzing particles and is currently based on two microscope platforms:

- platform 1: Upright light microscope Axio Imager
- platform 2: SteREO Discovery.V12 stereo microscope

For either platform, manual and partially or fully motorized system versions are available. See the system overviews below:
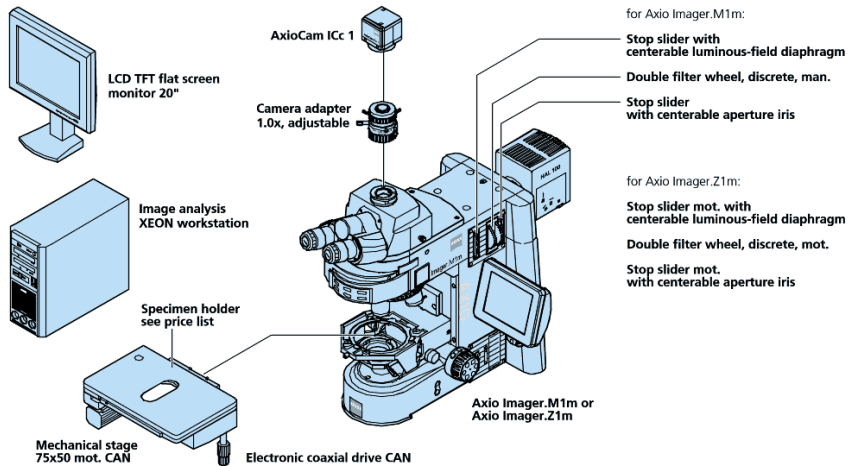
Particle Analyzer with Axio Imager.M1m or Axio Imager.Z1m
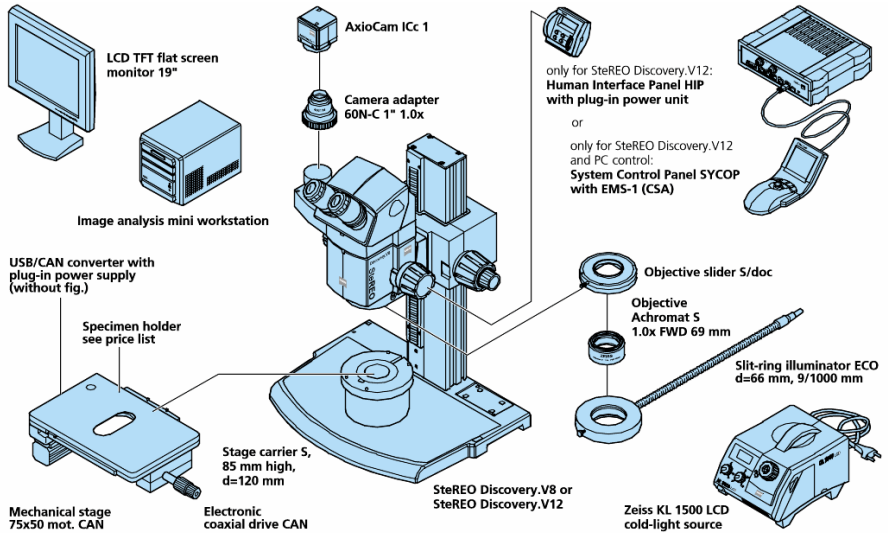
Particle Analyzer with SteREO Discovery.V8 or SteREO Discovery.V12

In addition to the microscopes, the following components are essential for a complete Particle Analyzer system:

- Digital camera (integrated into AxioVision, e.g. AxioCam family).
- PC with monitor.
- AxioVision basic package and the following modules: Particle Analyzer Projects, Asset Archive.

Depending on the microscopy platform used and the requirements of your application, you may need the following components in addition:

- For scanning large areas: a motorized stage and the AxioVision MosaiX module. For automatic focusing during image acquisition, the AxioVision Autofocus module will be helpful.

- For the individual modification of analysis scripts: the Commander module.

For details on setting up and using the hardware components, such as the microscope, motorized stage and camera, please refer to the manuals supplied with the corresponding devices.

Note:
- Before using the Particle Analyzer, familiarize yourself with the basic functions of the AxioVision software. You should be familiar with the operation of the camera and microscope components in particular.

Accessories for particle analysis

Specimen holder for round particle filters

The specimen holder for round particle filters is used to hold round filters up to 50 mm in diameter. It can be used for the following tasks/has the following features:

- Reproducible positioning of filters on a mechanical stage or scanning stage.

- Plane-parallel glass plate with retaining ring for flat insertion of a filter. This means that autofocusing is no longer necessary. Automatic focusing would make the acquisition process many times longer.

- Suitable for objectives up to Epiplan 10x.

- Marking to ensure correct positioning of filters with a 25 mm diameter.

Depending on the type of stage used (mechanical or scanning stage), special specimen holders or inserts may also be required in addition to the specimen holder for particle filters to ensure that the latter is correctly positioned on the stage in question.

Particle analysis calibration standard
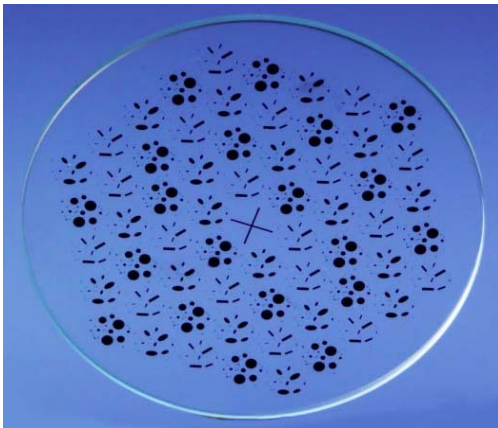
Using the calibration standard, you can check whether measurements made using the Particle Analyzer lead to reproducible results. The calibration standard has the following features:

- Depiction of size classes of guideline VDA volume 19 and ISO 16232

- 10 size classes: 10, 20, 35, 75, 125, 175, 300, 500, 800, 1200 μm

- Total number of particles: 6960

- Three different shapes: circles, rectangles, ellipses

- Diameter: 47 mm

- Diameter containing particles: 44 mm

- Anti-reflective on both sides

The following table provides an overview of the particle distribution for the calibration standard:

VDA 19, ISO 16232		Calibration standard	
Class	Class boundaries (µm)	Particle size (µm)	Number of particles
B	5 ≤ x < 15	10	1920
C	15 ≤ x < 25	20	1920
D	25 ≤ x < 50	35	960
E	50 ≤ x < 100	75	960
F	100 ≤ x < 150	125	480
G	150 ≤ x < 200	175	240
H	200 ≤ x < 400	300	120
I	400 ≤ x < 600	500	120
J	600 ≤ x < 1000	800	120
K	1000 ≤ x	1200	120
Total number of particles			**6960**

Cleaning of sample holder and calibration standard

You will need to differentiate between dust particles (e.g. glass particles from slides, skin particles from the microscope user, fluff from textiles, etc.) and other kinds of dirt (e.g. cleaning residues, finger prints and grease). Dust particles may lie loosely on optical surfaces or become more or less stuck to them. Other dirt is either water-soluble or only completely removable using organic solvents.

Depending on the type of soiling, different methods can be used to clean the sample holder or the calibration standard. You will find further information in the brochure "The clean microscope" by Carl Zeiss. This brochure can be found on the Internet at www.zeiss.de.

The concept

Operation and configuration

The operating concept of the Particle Analyzer is designed to make it possible to achieve a reproducible result with as little interaction as possible. The performance of a measurement can be automated to such an extent that only the project data need to be entered and the entire analysis process can run automatically.

To automate a measurement completely, you need extensive knowledge of systems engineering and the respective application. Carelessly made alterations of a setting can promptly lead to faulty measurement results. It is important, therefore, to prevent users not having such knowledge from changing the basic settings. This is achieved by dividing up the tasks involved into the definition of a measurement and the performance of a measurement.

The performance of a measurement is always based on what is called project templates. All important settings are saved in a project template. A project template cannot be created or edited by a user. This is reserved to Power Users or Administrators. Access to the respective functions is governed by the access rights of the operating system:
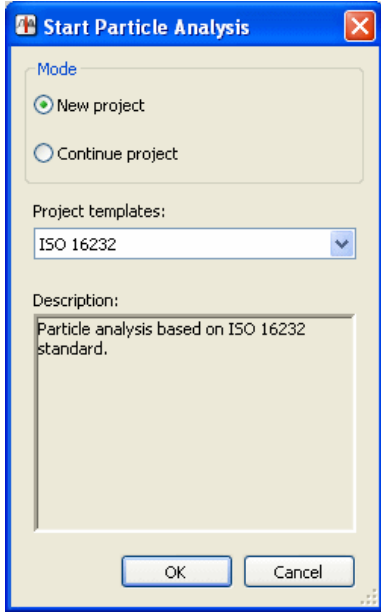
Dialog for starting a particle measurement
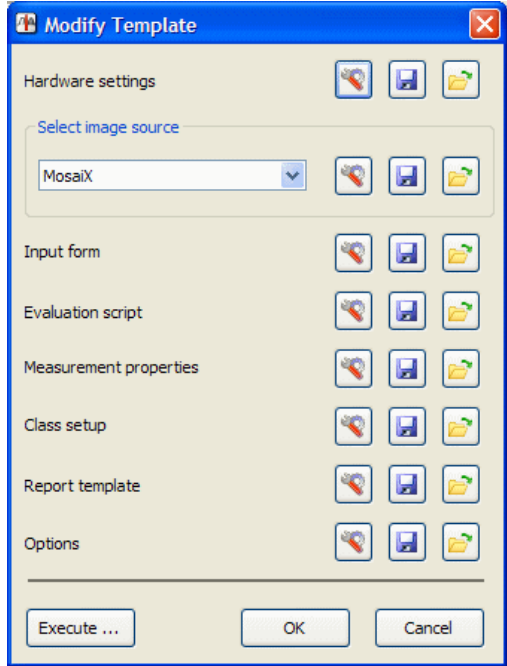
Dialog for editing a project template (not available to standard users)

Projects and project templates

The configuration data of a measurement are saved to so-called project templates. A project template contains all the information on the performance of a particle measurement in detail: from the configuration of the device and the type of image acquisition through to the determination of the size of the original data to be saved and how the results should be displayed at the end of a measurement.

The project templates are saved to the archive, together with the measurement projects, but they are not visible in the archive window. They can only be modified via the **Modify Template** function.

Data are saved and managed in a project-based archive. All the data generated (images, data tables, reports etc.) are arranged by project and saved centrally:
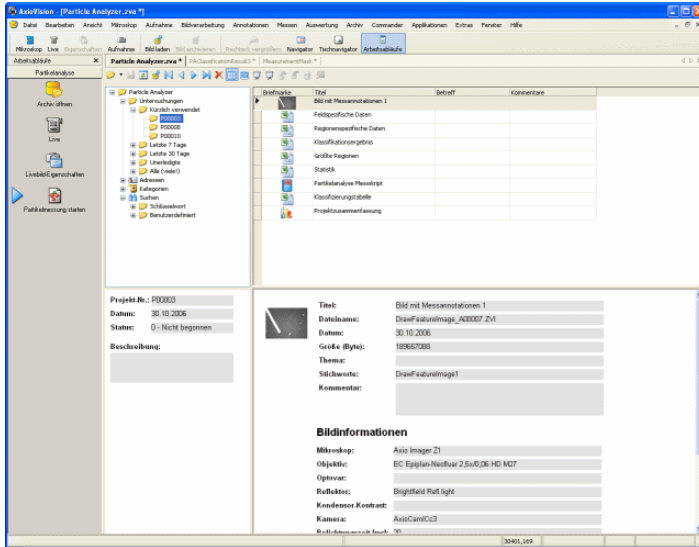
Archive view of the Particle Analyzer software

Preparing the system

Introduction

General

The functionality of the Particle Analyzer Projects module is based on the basic functionality of AxioVision. Before you can work with the Particle Analyzer, you must, therefore, configure the basic settings for AxioVision. You will find further information you require in Section 8 "Configuration" of this manual.

Microscope configuration

To be able to use your microscope together with AxioVision, you need to define once your individual system configuration via the separate **MTB2004 Configuration** program. Should you be retrofitting the Particle Analyzer to your existing microscope system, you may have to do this configuration via the **Microscope Configuration** program.

The configuration programs have their own icons and buttons on your Microsoft Windows Desktop:

Note:

- For detailed information, see Chapter 4 "Microscope Control".

The Particle Analyzer Setup toolbar

The **Particle Analyzer Setup** toolbar is specially intended for setting and configuring the Particle Analyzer:
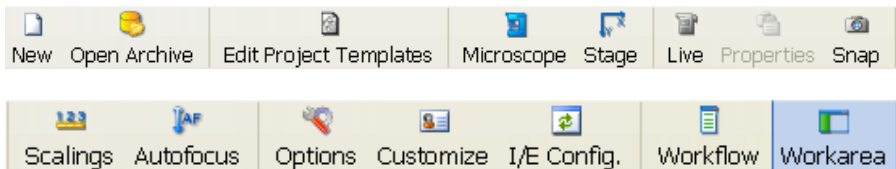
In the following it is assumed that the toolbar is open. If the toolbar is not displayed on your monitor, select Toolbars in the View menu:

Hereinafter it is assumed that the toolbar is open. If it should not be displayed on your system, open it via the menu **View** $\Rightarrow$ **Toolbars**:

General AxioVision Settings

Please make sure that the following settings have been made:

Alignment of camera and motorized stage

If you wish to acquire images of large areas using the MosaiX module, you must ensure that the camera and stage are aligned correctly with one another and that the stage's direction of movement has been set correctly. Otherwise the individual component images will not be put together properly. The aligning and setting process may take quite some time with conventional camera adapters; therefore it is recommendable to use the adjustable Carl Zeiss camera adapter:

Note:

- For detailed information, see Chapter 9.7 "MosaiX" – "Setting up the camera and scanning stage").

Scaling

Measurement in real units (e.g., µm) is only possible if there exist scaling files. Although AxioVision has a capability to compute a theoretical scaling on the basis of the current system configuration, it is urgently recommended to create, for measurement tasks, an individual scaling file by means of a stage micrometer.

Note:

- For detailed information, see Chapter 8.4 "Scalings".

Autofocus calibration

To be able to use the autofocus feature, you need to calibrate it for the magnifications used. The autofocus feature can then be used for defining interpolation nodes for the focal plane, and for acquiring mosaic images.

Note:

- For detailed information, see Chapter 9.2 "Autofocus".

- The possibility of automatic focusing before image acquisition does not exist for the SteREO microscopes.

User settings

Select the **User** tab in the **Tools** $\Rightarrow$ **Options** menu. Enter the information about the respective user and select your corporate logo. This information is administrated separately for each user. It is saved to the archive for each project or each measurement and, by way of default, used for the reports.

Note:

- For detailed information, see Chapter 8.6 "General settings".

Configuration of the user interface

AxioVision offers an extremely wide range of options with regard to user-specific configuration. In particular, the fact that the interface is structured as simply as possible means that even inexperienced users are able to use the system to achieve accurate results both quickly and easily.

It is recommendable to hide all controls not used for the purpose. For routine measurements, you usually need only the **Particle Analysis** workflow and, perhaps, the **Particle Analyzer** toolbar. The **Particle Analyzer Setup** toolbar is not required for routine measurements. All toolbars, as well as the main menu, can be displayed and hidden via the **View** $\Rightarrow$ **Toolbars** menu.

Notes:
- To show the main menu again, click the right mouse button on the title bar of the AxioVision window, or on a toolbar. This opens a context menu in which you can display everything again.

- For detailed information, see Chapter 8.5 "Adapting the user interface".

Special settings for particle analysis

Creating an archive

The database of the Particle Analyzer constitutes an archive. All data (images, measurement data tables, reports, etc.) are saved to this archive in a project-related order. To be able to work with the Particle Analyzer Projects module, therefore, an archive must exists and be opened in AxioVision.

In the **Particle Analyzer Setup** toolbar, click on the **New** button. On the **Archive** tab, select the **Particle Analysis** template:
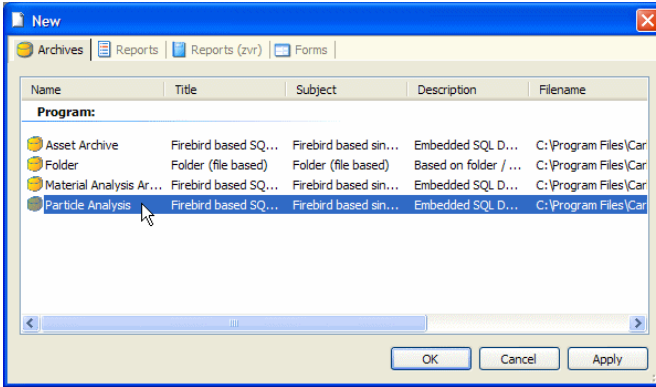
Opening the particle analysis archive when starting AxioVision

To start AxioVision always with the desired archive, you should establish a shortcut to the archive on the Microsoft Windows desktop:

➢ Click the right mouse button on a free area on the desktop, and select **New ⇒ Shortcut**
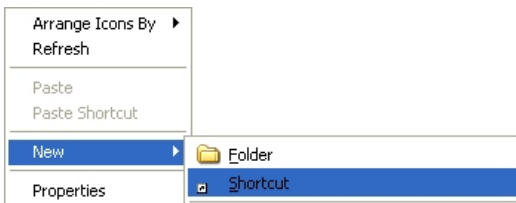
➢ This opens the dialog **Create Shortcut**. Click on **Browse…** and select the Particle Analysis Archive (file extension *.zva):
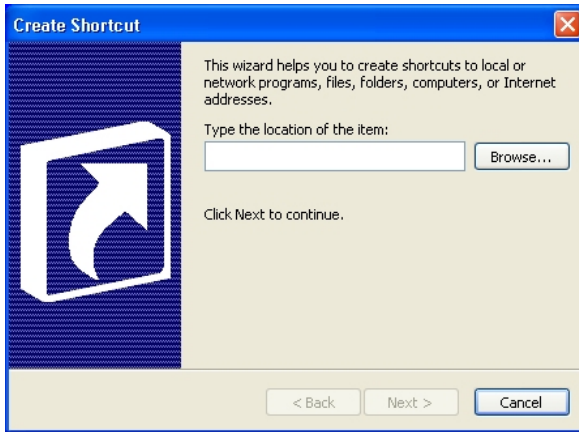
➢ Click on **Next** and, in the next step, enter a name for the link.

➢ To create the link, click on **Finish**.

Note:
- Via the properties of the shortcut you can also adapt the icon. Suitable icons can be found in the "ZiResrc.dll" file in the *"C:\Program Files\Common Files\Carl Zeiss Vision\System\Resource"* folder.

Administrating several users

The user management of AxioVision is directly based on that of the operating system. Each user with access rights works under a different AxioVision configuration. This means that, e.g., the configuration of the user interface, the entry of user data, etc., are managed separately for each user.

Notes:
- For detailed information on user and data management, see Chapter 8.3 "User and data administration".

- If you use a common account for all users but wish to use different configurations nevertheless, proceed as described in the following section.

Separation of Particle Analysis and standard AxioVision

It is a strong point of the Particle Analyzer that the system is completely based on the standard AxioVision software. Therefore, it can also be used for simple documentation jobs or other applications such as, e.g., analysis of non metallic inclusions in steel. To start AxioVision always with a special, application-specific configuration (e.g., specific toolbars, workflows, etc.), you can establish any number of links with separate icons on the Microsoft Windows Desktop.

Note:
- For detailed information on the creation of configurations, see Chapter 8.8 "Creating various AxioVision configurations for a user".

Creating project templates

General

In project templates, all settings and files for performing a particle measurement are saved. All subsequent particle measurements always are based on a project template. When you start a measurement you are prompted to select the desired project template first before you can actually start the measurement.

The project templates are managed via the **Edit Project Templates** dialog:
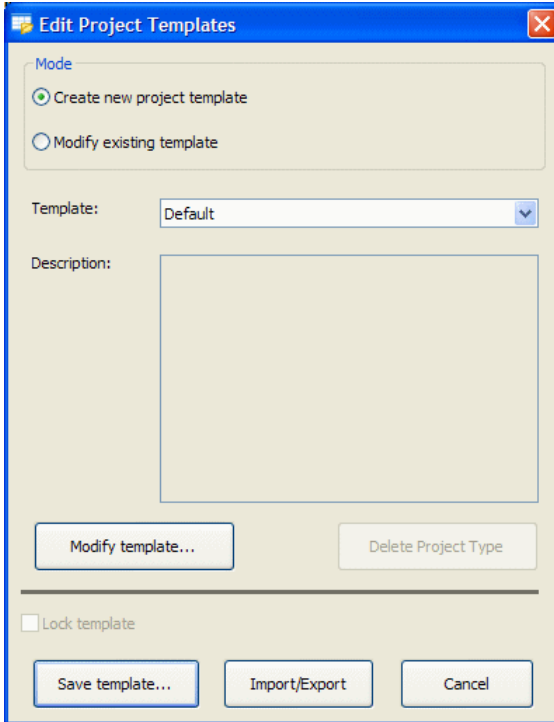
In the **Edit Project Templates** dialog you can edit, create and delete project templates. Further, you can import and export project templates.

Importing and exporting project templates

To make the preparation and setup for Particle Analysis as easy as possible, you can simply import existing project templates for special applications and use them right away.

This is especially helpful if measurements are to be made in different places but with exactly the same specifications. Project templates once created centrally can then be imported on any number of systems installed.

Clicking the **Import/Export…** button starts the wizard that guides you through all the necessary steps:
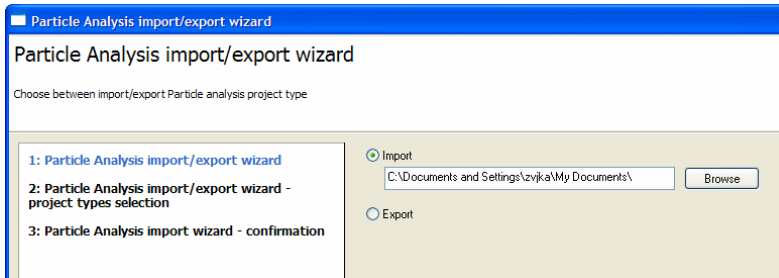
Note:

- Project templates available at Carl Zeiss can be downloaded from the Particle Analyzer Internet website **www.zeiss.de/particle** and then imported. Templates are available, e.g., for data analysis conforming to ISO 16232, and for measuring the Carl Zeiss particle standard.

The Modify Template dialog

Project templates are created via the**Modify Templates** function.
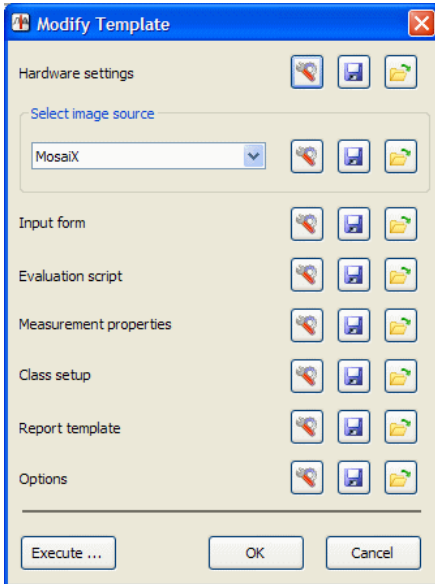
Dialog for selecting the settings for a project template

Here again you can, for each single item required for creating a project template, load settings already existing in another template. For the management of template files, use the buttons on the right of the respective

creation items: .

Thus, you can directly copy device settings, settings of the image source, input forms, etc. from existing templates into the current project template you are creating. Further, you can save your own settings to a new template in order to use them in further project templates.

And finally, you can set every step separately. To open the respective

dialog, click on the **Edit…** button:

The following chapters describe the procedure step by step.

Testing project templates

If you want to test whether the settings of a project template really produce the desired results, you can click on the **Execute…** button to run a complete measurement without having to create a project in the archive. It is important that you perform this step with a real sample, because all settings made in the functional dialogs during the test run are adopted by the project template.
Settings that are particularly important in this context are, e.g., those concerning the segmentation thresholds for particle detection, or the size and position of the measurement frame.

Hardware settings

The settings of microscope and camera are decisive for the reproducibility of measurements. Measurements will yield the same results only if they are always made with the same parameters. With the hardware settings you can ensure that you are always using the same hardware configuration before you start a Particle Analysis. This is conditional on the use of motorized microscopes or microscope components, and digital cameras. These can be completely controlled via the software.

In case of manual microscopes you need to do the correct settings manually. So-called coded components require a mixed approach. Their current statuses can be checked by the software, but the correct setting has to be done manually as required. If you are using coded components, the system will warn you if a setting is faulty and prompt you to establish the correct configuration.
The most important settings include, e.g., the objective to be used, the brightness adjustment, the used contrasting method, camera resolution, exposure time, camera mode, etc.

Example: Setting the camera mode (color, gray levels)

The camera setting has a decisive influence on the size of your files. Given the memory capacity of modern computers, the use of color images is considered a standard feature. However, color images require three times as much memory space as gray scale images. In most cases, though, color information is not required for particle detection; therefore, images should be acquired as gray scale images. This setting can be made in the camera control dialog, and it can be changed there by every user:

By including the camera mode in the hardware settings you can ensure that you are always acquiring gray level images for a measurement, irrespective of the actual settings of the camera:
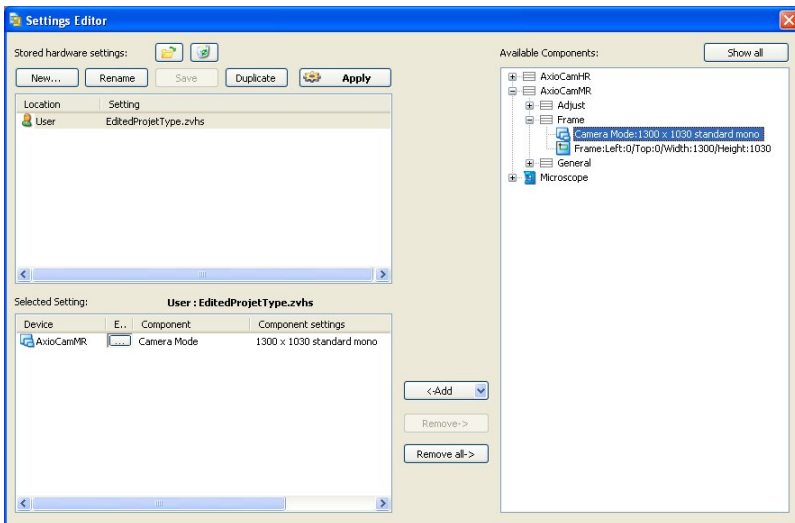
Image Source

The image source defines the way images are acquired. When large areas are acquired by the mosaic technique, the size and position of the acquired area are defined in addition via the **Setup** function of the MosaiX module. Then it is important, in routine work, that the samples are actually located in the places previously defined. This can be ensured through the use of holders for different slide types. A special holder is available for measuring round particle filters:

For details, see Chapter "Accessories for particle analysis".

To ensure unmistakable repositioning in the acquisition of MosaiX images, the z focus and the x and y settings of the motorized stage are calibrated after every restart of AxioVision.

Input form

During a measurement, the user enters the required project data into input forms. You can freely define which data to enter. The input fields required and their linking to the archive are defined in what is called the Layout mode. The Layout mode is active immediately when you start editing.

Standard input form for project data

Notes:
- For detailed information on editing form templates, see Chapter 7.3 "Templates for forms and reports".

Evaluation scripts

General

The analysis script determines the way in which the particle analysis is to be carried out. Consequently, this script is the key to the entire analysis. Besides the sequence of the commands, here you can also define the functions that you want to be executed interactively, i.e. which functions should be opened during the performance of a measurement to allow the user to enter parameter settings.

Notes:
- In order to display and modify the analysis script, you will need the Commander module. The Commander module however is NOT required for executing a particle measurement.

- Since modification of the analysis script has dramatic consequences with regard to the quality of the analysis, this should only be performed by experienced users. It is essential that you read the "Commander" section and the information in the online help before making any modifications.

- The analysis scripts are not managed in the usual standard AxioVision folders. The **New** and **Rename** buttons are therefore inactive. The **Save** button only becomes active once you have made changes to the script.
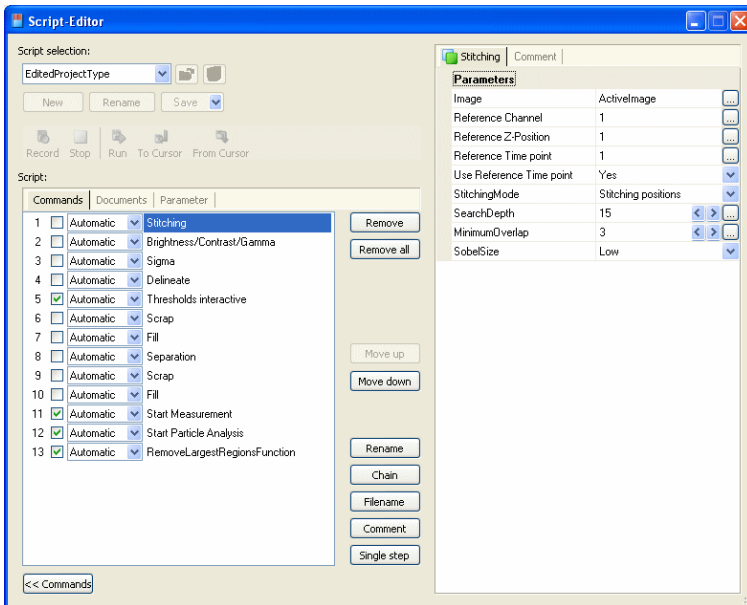
Analysis script in the Commander script editor

Adapting the evaluation script

As long as you do not insert or delete any commands, you can modify the behavior of the script simply by switching the commands on the **Commands** tab on and off.
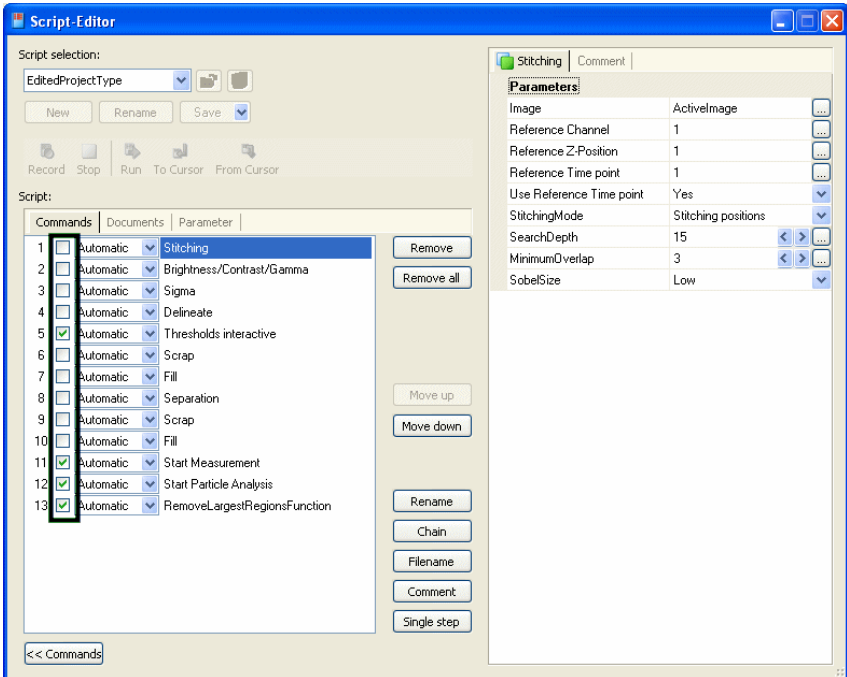
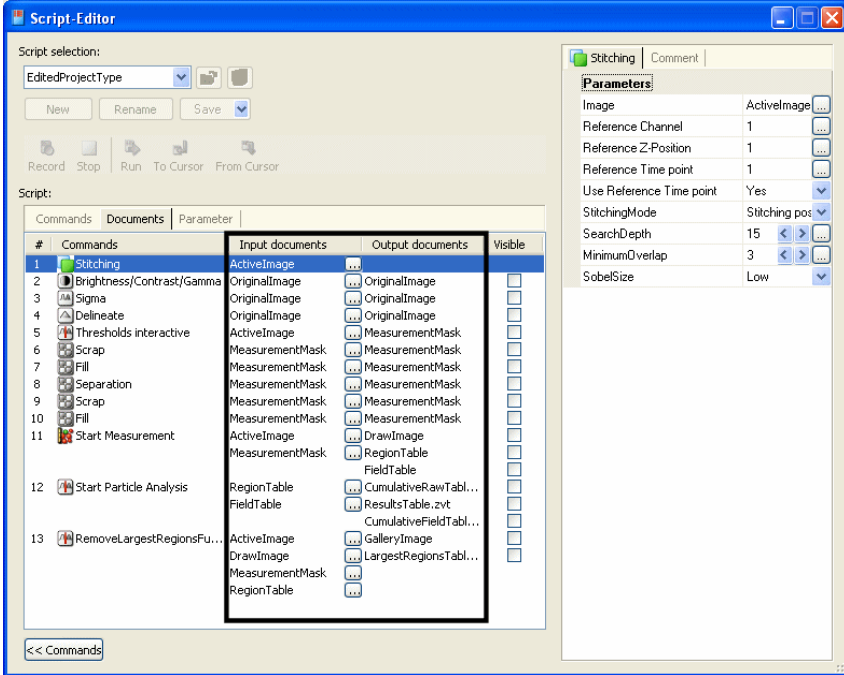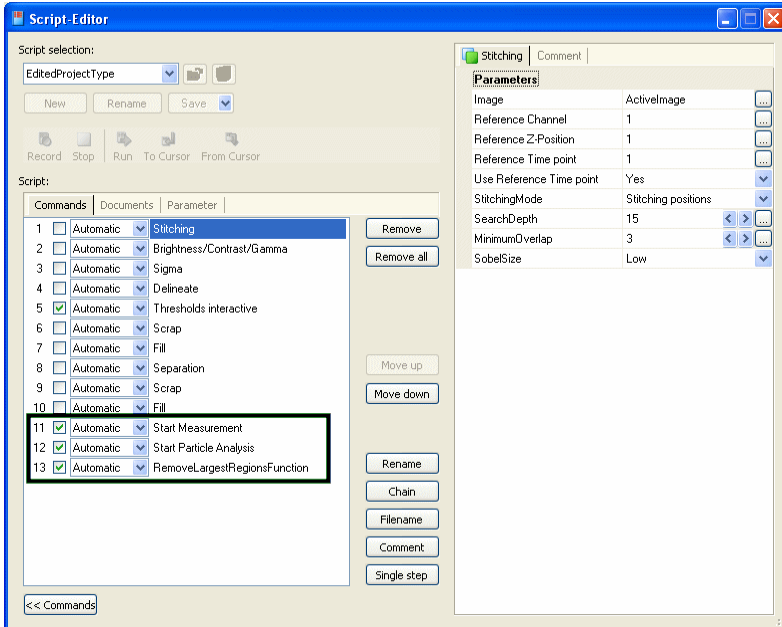You can any functions, available in AxioVision, incorporate into the script. After doing so, however, you must ensure that the input and output documents are correctly linked.

The three command lines at the end of the script must not be deleted under any circumstances. These commands calculate the actual classification and generate the results document.

Modifying report templates

In order to print the results of a measurement clearly, it is possible to structure the appearance of the results report individually. A report template may only be modified during the creation/editing of a project template and is, therefore, not accessible for standard users. The predefined template is always used when you perform a measurement.

Note:
- For detailed information, see Chapter 6.4 "Creating data reports".

Optional settings

General

With optional settings you define essentially the following items:

• Selection of the data to be saved (above all, image data)

• Behavior during image acquisition

• Presentation of result data after the measurement

It is imperative that you consult the Online Help function for information on details of the parameters that can be set.

Memory space required

The quantity of data obtained in particle analyses is sometimes enormous, especially when you acquire large images using the MosaiX module. The table below shows the memory requirements for different conditions:

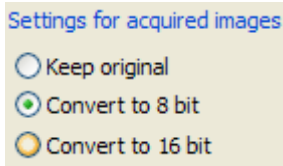| Pixel scaling used	Image size in MB*	
	Gray scale image	Color image
1.0 µm / pixel	1450	4350
2.5 µm / pixel	232	696
5.0 µm / pixel	58	174
10.0 µm / pixel	15	44

Area approx. 1500mm², 8 bit gray levels

You should carefully consider which original data should actually be saved. Among others, the two factors outlined below may be a basis for decision:

- If you want to ensure that a measurement can be reproduced at any time, you need to save the original data as they are. It is not permissible in this case to scale down the original image or to save it in a compressed format.

- If you want to save the original image for documentation purposes, you should consider whether a scaled down version could be saved in a compressed format (e.g., ZVI or JPG). This will considerably reduce the amount of image data, but they will not be suitable for subsequent measurements.

**Number of gray levels or color values of the images acquired**

Another important consideration is the bit depth, i.e. the number of gray levels or color values used for image acquisition. *In the past*, the entire brightness or color range was captured 256 different gray levels (8 bit). Most modern digital cameras have more than 256 gray levels. But here, too, this means that the resulting image data files are exceedingly large; the great number of gray levels (possibly more than 65,000) is not needed for a measurement. It is possible, therefore, to enforce reduction to 256 gray levels:

Settings for acquired images

○ Keep original
◉ Convert to 8 bit
○ Convert to 16 bit

Conversion of acquired images to 8 bit

Performing measurements

A measurement is performed via the **Start Particle Analysis** function in the **Particle Analysis** workflow:
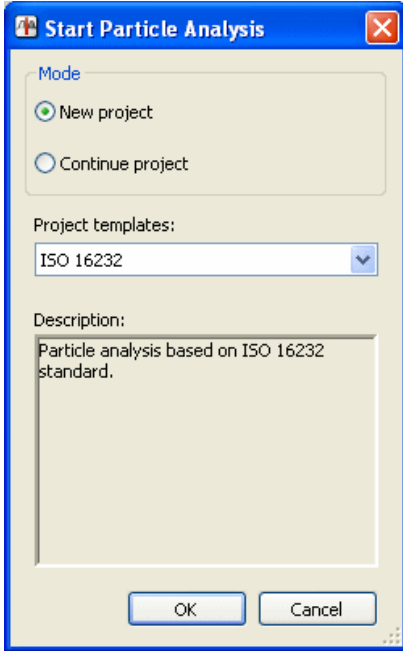
Dialog for starting a particle measurement

A particle measurement is always based on a previously defined project template. This must have been created beforehand by a qualified user. It is not possible to perform a measurement if no template has been created.

During the course of the measurement, the function dialog for which the **Interactive** mode has been selected in the analysis script will be displayed. All other functions run automatically in the background.

The program will stop at each function for which **Interactive** has been selected and the corresponding dialog will open. The predefined parameters can then be modified and optimally adapted to the image in question.

The functions that are carried out interactively feature the additional parameter **Always interactive** during the execution of the program. This allows the user to decide whether or not the dialog should be opened again for the next image. This is particularly useful if you have set segmentation thresholds, for example, and do not need to modify them again for subsequent images. The thresholds can therefore be set using one or more images and then applied automatically.

This option is useful if you are measuring several images immediately after each other. Otherwise, this setting does not have any impact on the running of the program.

Once the measurement is complete, the results window will be displayed or, alternatively, the report can be displayed immediately:
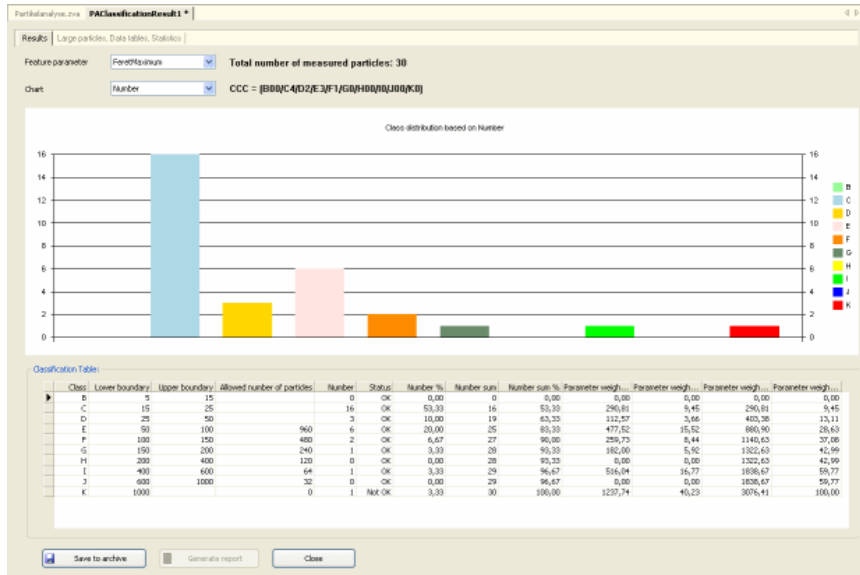
Results document

Note:

- Whether it is the results document or report that is displayed depends on the settings for the project template.

11.8 NMI (Non-Metallic Inclusions)

General

Note:
- For detailed information on the individual functions and commands, please refer to the online help (F1 key).

The system variants

The NMI module is the software for the NMI system. The NMI (non-metallic inclusions) system from Carl Zeiss is a complete system for analyzing the purity of steels in accordance with current standards. This system is currently based on two fully motorized microscopy platforms:

- Platform 1: upright light microscope Axio Imager.Z1m
- Platform 2: inverse microscope Axio Observer.Z1m

In addition to the microscopes, the following components are essential for a complete NMI system:

- Digital camera (integrated into AxioVision, e.g. AxioCam family)
- High-performance PC with monitor
- AxioVision basic package and the NMI and MosaiX modules

Depending on the microscopy platform used and the application-related requirements, the following components may also be necessary:

- The Mark&Find module in order to scan several samples
- A multiple sample holder for up to six round samples up to 40 mm in diameter
- For the individual modification of evaluation scripts: the Commander module

For details on setting up and using the hardware components, such as the microscope, scanning stage and camera, please refer to the manuals supplied with the corresponding devices.

An overview of the system is provided on the following page.
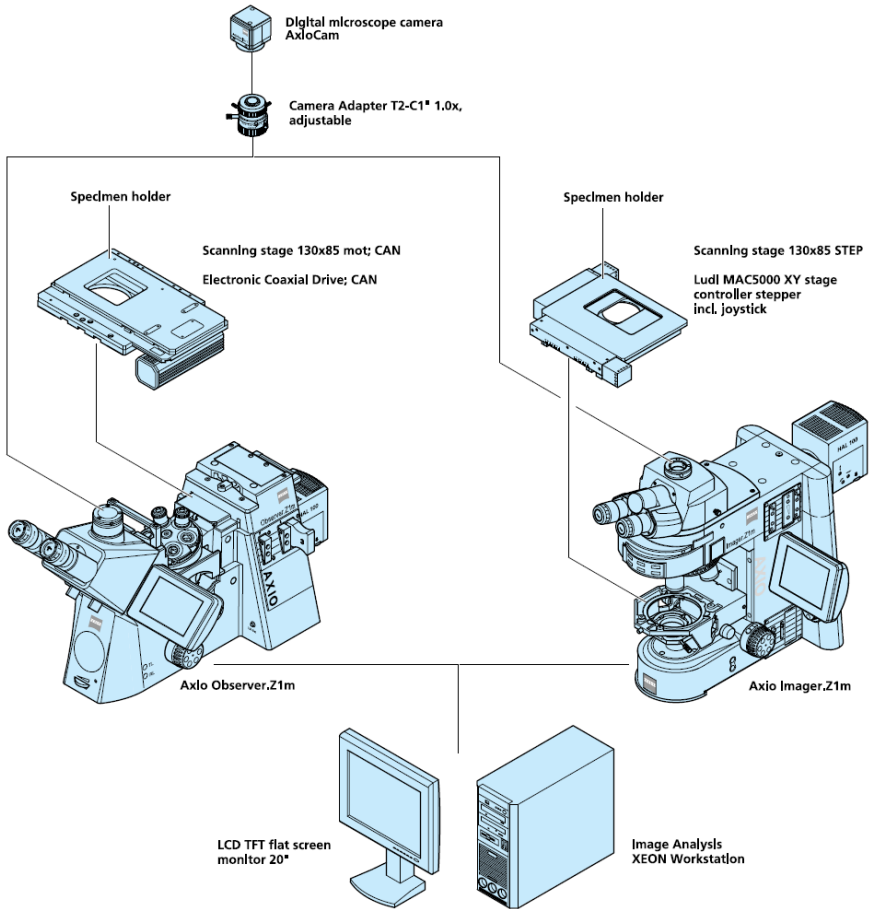
NMI system with Axio Imager.Z1m and Axio Observer.Z1m

Note:
- Before using NMI, familiarize yourself with the basic functions of the AxioVision software. You should be familiar with the operation of the camera and microscope components in particular.
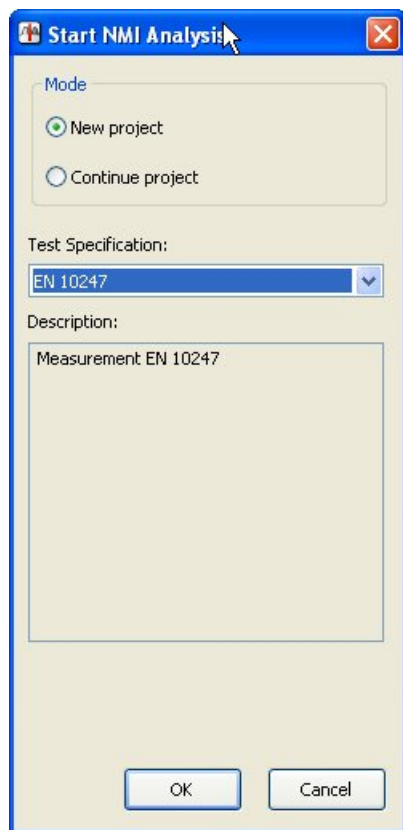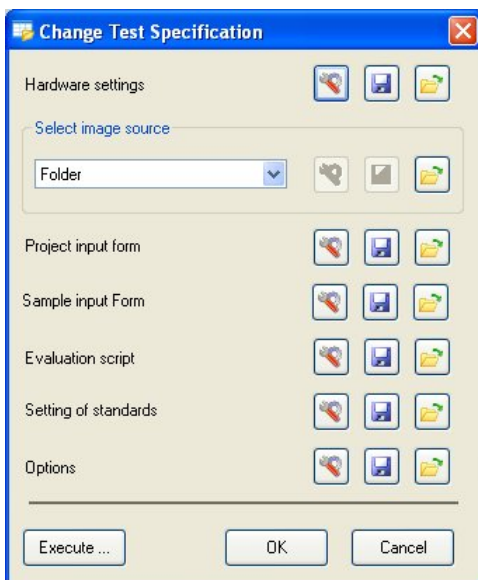
The concept

Operation and configuration

The operating concept of NMI is designed to make it possible to achieve a reproducible result with as little interaction as possible. The performance of a measurement can be automated to such an extent that only the project data need to be entered and the entire analysis process can run automatically.

To automate a measurement completely, you need extensive knowledge of systems engineering and the respective application. Carelessly made alterations of a setting can promptly lead to faulty measurement results. It is important, therefore, to prevent users not having such knowledge from changing the basic settings. This is achieved by dividing up the tasks involved into the definition of a measurement and the performance of a measurement.

The performance of a measurement is always based on what is called test specification. All important settings are saved in a test specification. A test specification cannot be created or edited by a user. This is reserved to Power Users or Administrators. Access to the respective functions is governed by the access rights of the operating system:

*Dialog for editing a project template
(not available to standard users)*

Dialog for starting an NMI Analysis

Projects and test specifications

The configuration data of a measurement are saved to so-called test specification. A test specification contains all the information on the performance of an NMI analysis in detail: from the configuration of the device and the type of image acquisition through to the determination of the size of the original data to be saved.

The test specifications are saved to the archive, together with the measurement projects, but they are not visible in the archive window. They can only be modified via the **Manage Test Specification** function.

Data are saved and managed in a project-based archive. All the data generated (images, data tables, reports etc.) are saved by project:

Archive View

Preparing the system

Introduction

General

The functionality of the NMI module is based on the basic functionality of AxioVision. Before you can work with NMI, you must, therefore, configure the basic settings for AxioVision. You will find further information you require in Section 8 "Configuration" of this manual.

Microscope configuration

To be able to use your microscope together with AxioVision, you need to define once your individual system configuration via the separate **MTB2004 Configuration** program. Should you be retrofitting NMI to your existing microscope system, you may have to do this configuration via the **Microscope Configuration** program.

The configuration programs have their own icons and buttons on your Microsoft Windows Desktop:

Note:
- For detailed information, see Chapter 4 "Microscope Control".

General AxioVision Settings

Please make sure that the following settings have been made:

Alignment of camera and motorized stage

If you wish to acquire images of large areas using the MosaiX module, you must ensure that the camera and stage are aligned correctly with one another and that the stage's direction of movement has been set correctly. Otherwise the individual component images will not be put together properly. The aligning and setting process may take quite some time with conventional camera adapters; therefore it is recommendable to use the adjustable Carl Zeiss camera adapter:

Note:
- For detailed information, see Chapter 9.7 "MosaiX" – "Setting up the camera and scanning stage").

- ➢ **Scalings**: Measurement in real units (e.g., µm) is only possible if there exist scaling files. Although AxioVision has a capability to compute a theoretical scaling on the basis of the current system configuration, it is urgently recommended to create, for measurement tasks, an individual scaling file by means of a stage micrometer.

Note:
- For detailed information, see Chapter 8.4 "Scalings".

User settings

Select the **User** tab in the **Tools** $\Rightarrow$ **Options** menu. Enter the information about the respective user and select your corporate logo. This information is administrated separately for each user. It is saved to the archive for each project or each measurement and, by way of default, used for the reports.

Note:
- For detailed information, see Chapter 8.6 "General settings".

Configuration of the user interface

AxioVision offers an extremely wide range of options with regard to user-specific configuration. In particular, the fact that the interface is structured as simply as possible means that even inexperienced users are able to use the system to achieve accurate results both quickly and easily.

It is recommendable to hide all controls not used for the purpose. For routine measurements, you usually need only the **NMI** workflow.

Notes:
- To show the main menu again, click the right mouse button on the title bar of the AxioVision window, or on a toolbar. This opens a context menu in which you can display everything again.

- For detailed information, see Chapter 8.5 "Adapting the user interface".

Special settings for NMI

Creating an archive

The database of NMI constitutes an archive. All data (images, measurement data tables, reports, etc.) are saved to this archive in a project-related order. To be able to work with the NMI module, therefore, an archive must exists and be opened in AxioVision.
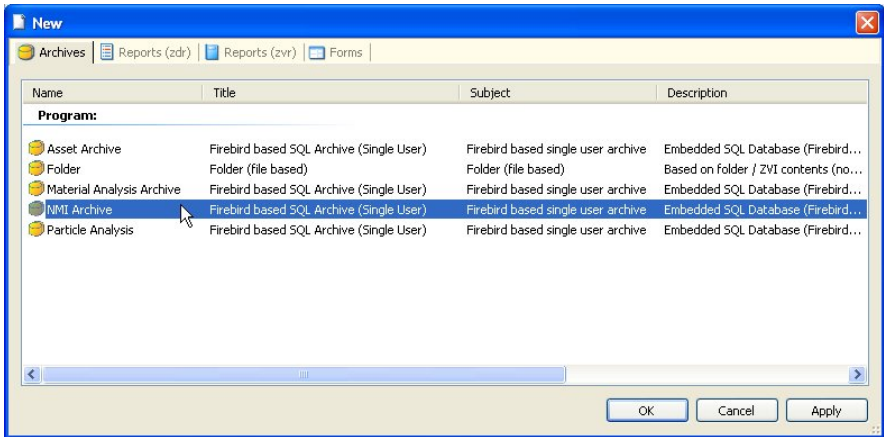
Opening the particle analysis archive when starting AxioVision

To start AxioVision always with the desired archive, you should establish a shortcut to the archive on the Microsoft Windows desktop:

➢ Click the right mouse button on a free area on the desktop, and select **New ⟹ Shortcut**:
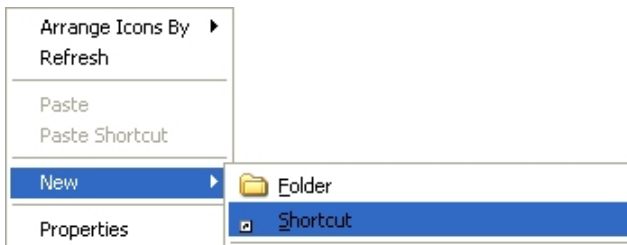
➢ This opens the dialog **Create Shortcut**. Click on **Browse…** and select the Particle Analysis Archive (file extension *.zva):
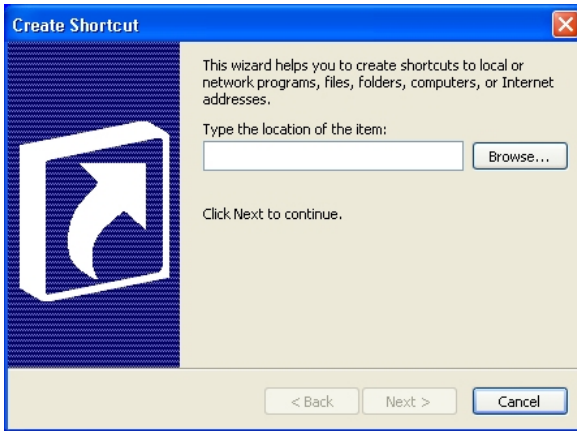
➢ Click on **Next** and, in the next step, enter a name for the link.

➢ To create the link, click on **Finish**.

Administrating several users

The user management of AxioVision is directly based on that of the operating system. Each user with access rights works under a different AxioVision configuration. This means that, e.g., the configuration of the user interface, the entry of user data, etc., are managed separately for each user.

Notes:
- For detailed information on user and data management, see Chapter 8.3 "User and data administration".

- If you use a common account for all users but wish to use different configurations nevertheless, proceed as described in the following section.

Separation of Particle Analysis and standard AxioVision

It is a strong point of NMI that the system is completely based on the standard AxioVision software. Therefore, it can also be used for simple documentation jobs or other applications such as, e.g., particle analysis or analysis of non metallic inclusions in steel. To start AxioVision always with a special, application-specific configuration (e.g., specific toolbars, workflows, etc.), you can establish any number of links with separate icons on the Microsoft Windows Desktop.

Note:
- For detailed information on the creation of configurations, see Chapter 8.8 "Creating various AxioVision configurations for a user".

Creating test specifications

General

In test specification, all settings and files for performing an NMI analysis are saved. All subsequent NMI analysis always are based on a test specification. When you start a measurement you are prompted to select the desired test specification first before you can actually start the measurement.

The project templates are managed via the **Manage Test Specification** dialog:
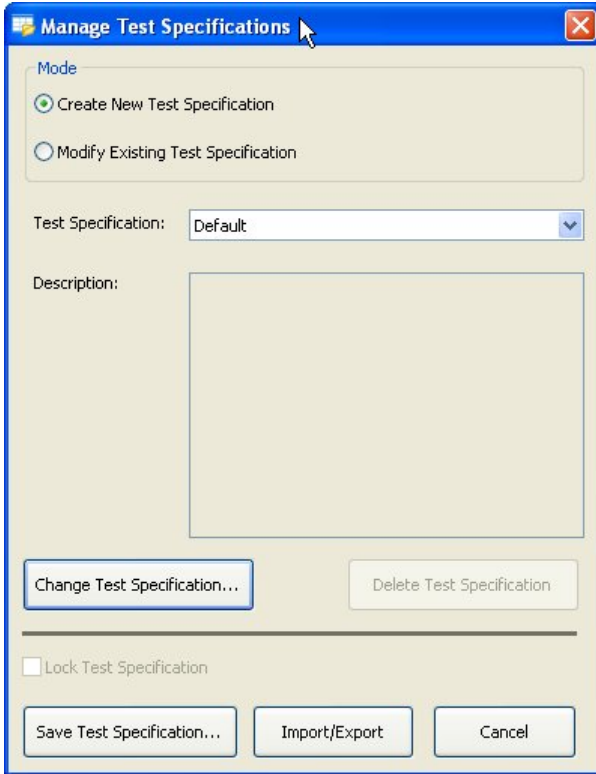
In the **Manage Test Specifications** dialog you can edit, create and delete test specifications. Further, you can import and export test specifications.

Importing and exporting test specifications

To make the preparation and setup for NMI Analysis as easy as possible, you can simply import existing test specifications for special applications and use them right away.

This is especially helpful if measurements are to be made in different places but with exactly the same specifications. Test specifications once created centrally can then be imported on any number of systems installed.

Clicking the **Import/Export…** button starts the wizard that guides you through all the necessary steps:
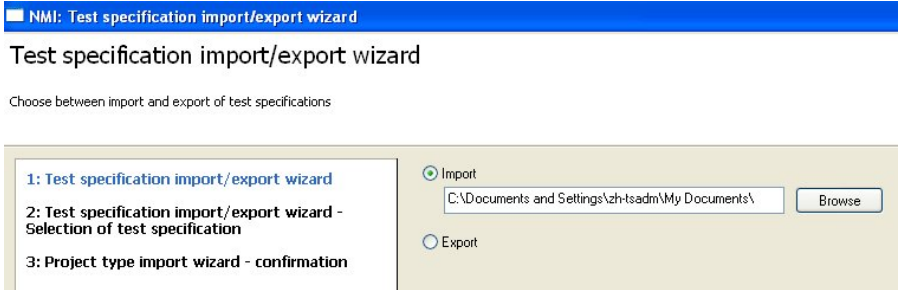
The Change Test Specification dialog

Project templates are created via the **Change Test Specification** function. All the dialogs used to set up an NMI analysis are called up via this dialog:
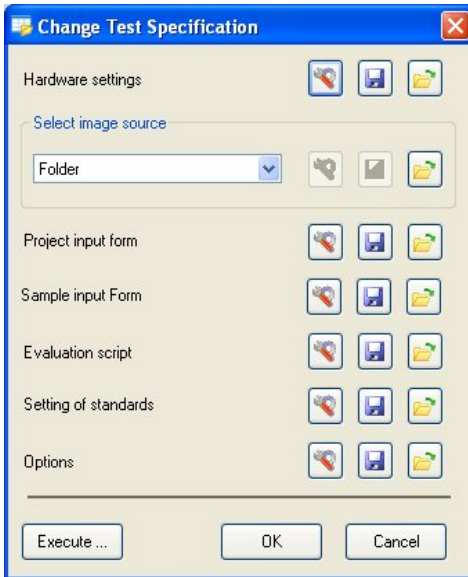
Dialog for changing a test specification

Each individual step can be modified entirely. The relevant dialog can be opened using the **Edit** button. Here again you can, for each single item required for creating a project template, load settings already existing in another template. For the management of template files, use the buttons on the right of the respective creation items: .

Thus, you can directly copy device settings, settings of the image source, input forms, etc. from existing templates into the current test specification you are creating. Further, you can save your own settings to a new template in order to use them in further test specification.

The following chapters describe the procedure step by step.

Testing test specifications

If you want to test whether the settings of a test specification really produce the desired results, you can click on the **Execute…** button to run a complete measurement without having to create a project in the archive. It is important that you perform this step with a real sample, because all settings made in the functional dialogs during the test run are adopted by the test specification.

Segmentation threshold settings for detecting inclusions, in particular the threshold values for sulfides, oxides and titanium nitrides, are especially important here, for example.

Hardware settings

The settings of microscope and camera are decisive for the reproducibility of measurements. Measurements will yield the same results only if they are always made with the same parameters. With the hardware settings you can ensure that you are always using the same hardware configuration before you start NMI Analysis. This is conditional on the use of motorized microscopes or microscope components, and digital cameras. These can be completely controlled via the software.

In case of manual microscopes you need to do the correct settings manually. So-called coded components require a mixed approach. Their current statuses can be checked by the software, but the correct setting has to be done manually as required. If you are using coded components, the system will warn you if a setting is faulty and prompt you to establish the correct configuration.

The most important settings include, e.g., the objective to be used, the brightness adjustment, the used contrasting method, camera resolution, exposure time, camera mode, etc.

Example: Setting the camera mode (color, gray levels)

The camera setting has a decisive influence on the size of your files. Given the memory capacity of modern computers, the use of color images is considered a standard feature. However, color images require three times as much memory space as gray scale images. In most cases, though, color information is not required for the detection of inclusions; therefore, images should be acquired as gray scale images. This setting can be made in the camera control dialog, and it can be changed there by every user:

By including the camera mode in the hardware settings you can ensure that you are always acquiring gray level images for a measurement, irrespective of the actual settings of the camera:
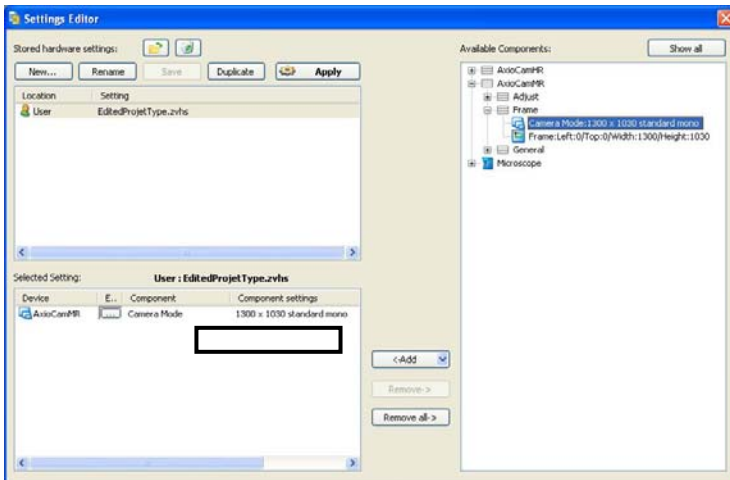
Image source

The image source defines where the image comes from. If you have already acquired images that you wish to analyze, select folder or gallery as the image source. In most cases, however, an image is acquired and analyzed immediately. When you are acquiring images of large surfaces using the mosaic technique, the size and position of the acquisition region are defined using the MosaiX module's **Setup** function. If you have several samples, it is also necessary to define Mark&Find points.

To ensure unmistakable repositioning in the acquisition of MosaiX images, the z focus and the x and y settings of the motorized stage are calibrated after every restart of AxioVision.

Input forms

Input forms allow the user to enter the necessary project and sample data during the measurement. You can freely define the data that you want to be entered. The necessary fields and their link to the archive are defined in layout mode. Layout mode is active as soon as editing is started.

The module includes two input forms: the project-specific input form and the sample-specific input form. The project-specific input form is displayed immediately after the measurement. It is used for entering general project data. The sample-specific input form, on the other hand, is not displayed until the data are saved in the archive. The number of times that it is opened corresponds to the number of samples you have measured. In this form you enter data relating to the individual sample, e.g. sample name or material.

Standard input form for project data

Standard input form for sample data

Notes:

- For detailed information on editing form templates, see Chapter 7.3 "Templates for forms and reports".

Evaluation script

General

An evaluation script defines which image-processing steps will be performed in which sequence. It is not normally necessary to change the evaluation script. You can choose which functions will be executed interactively, i.e. which functions will be opened while a measurement is being performed to allow the user to adjust parameters.

Notes:
- In order to display and modify the evaluation script, you will need the Commander module. The Commander module however is NOT required for executing NMI Analysis.

- Since modification of the evaluation script has dramatic consequences with regard to the quality of the analysis, this should only be performed by experienced users. It is essential that you read the "Commander" section and the information in the online help before making any modifications.

- The evaluation scripts are not managed in the usual standard AxioVision folders. The **New** and **Rename** buttons are therefore inactive. The **Save** button only becomes active once you have made changes to the script.
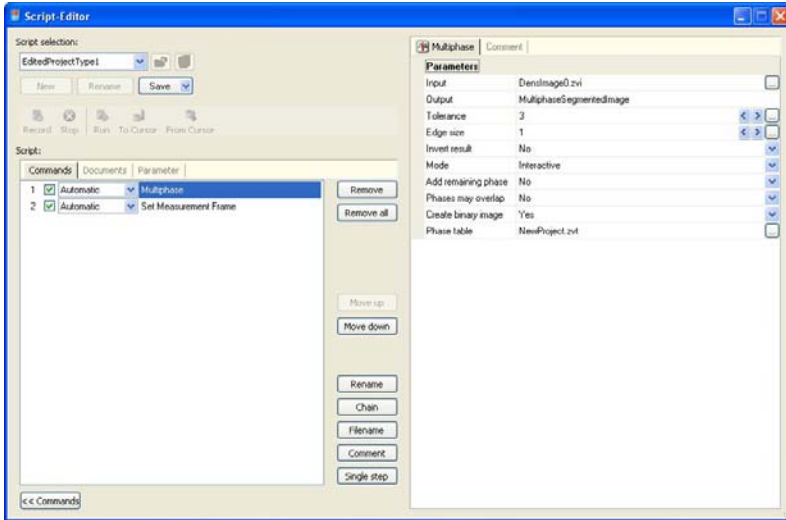
Evaluation script in the Commander script editor

Adapting the evaluation script

As long as you do not insert or delete any commands, you can modify the behavior of the script simply by switching the commands on the **Commands** tab on and off.
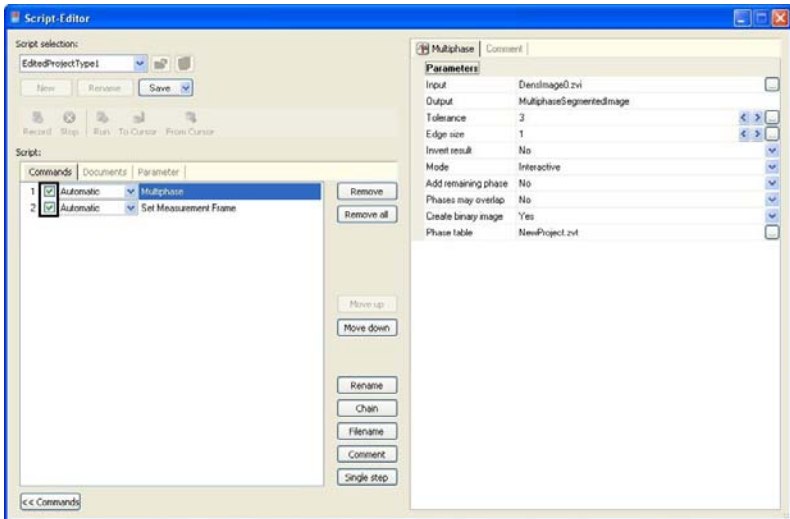
You can incorporate any functions available in AxioVision into the script. After doing so, however, you must ensure that the input and output documents are correctly linked.
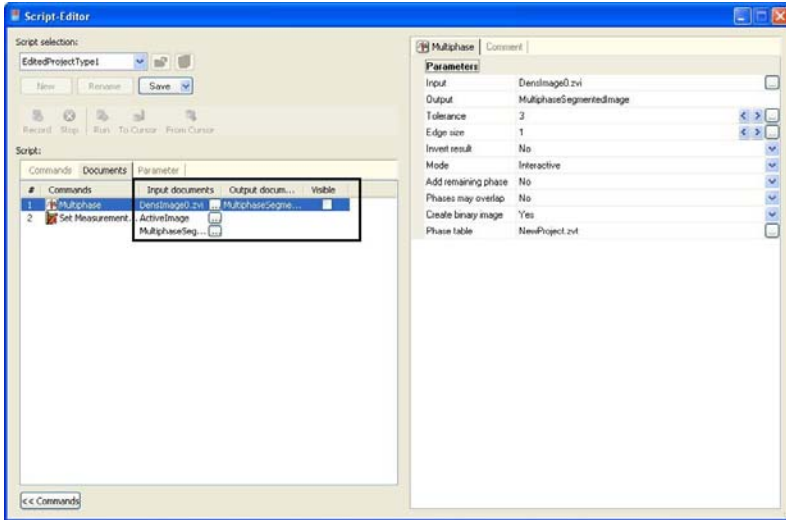
The multiphase segmentation and its settings should not, however, be deleted or changed under any circumstances.

Modifying report templates

In order to print the results of a measurement clearly, it is possible to structure the appearance of the results report individually. A report template may only be modified during the creation/editing of each test specification and is, therefore, not accessible for standard users. The predefined template is always used when you perform a measurement.

Note:

- For detailed information, see Chapter 6.4 "Creating data reports".

Optional settings

General

With optional settings you define essentially the following items:

- Selection of the data to be saved (above all, image data)
- Behavior during image acquisition
- Presentation of result data after the measurement

It is imperative that you consult the Online Help function for information on details of the parameters that can be set.

Memory space required

The quantity of data obtained in NMI is sometimes enormous, especially when you acquire large images using the MosaiX module. The table below shows the memory requirements for different conditions:

Pixel scaling used	Image size in MB*	
	Gray scale image	Color image
1.0 µm / pixel	1450	4350
2.5 µm / pixel	232	696
5.0 µm / pixel	58	174
10.0 µm / pixel	15	44

You should carefully consider which original data should actually be saved. Among others, the two factors outlined below may be a basis for decision:

- If you want to ensure that a measurement can be reproduced at any time, you need to save the original data as they are. It is not permissible in this case to scale down the original image or to save it in a compressed format.

- If you want to save the original image for documentation purposes, you should consider whether a scaled down version could be saved in a compressed format (e.g., ZVI or JPG). This will considerably reduce the amount of image data, but they will not be suitable for subsequent measurements.

Number of gray levels or color values of the images acquired

Another important consideration is the bit depth, i.e. the number of gray levels or color values used for image acquisition. *In the past,* the entire brightness or color range was captured 256 different gray levels (8 bit). Most modern digital cameras have more than 256 gray levels. But here, too, this means that the resulting image data files are exceedingly large; the great number of gray levels (possibly more than 65,000) is not needed for a measurement. It is possible, therefore, to enforce reduction to 256 gray levels:

Settings for acquired images

○ Keep original
◉ Convert to 8 bit
○ Convert to 16 bit

Conversion of acquired images to 8 bit

Note:
- If only sulfides and/or oxides are analyzed, a gray scale image is normally perfectly sufficient. In order to distinguish between sulfides and titanium nitrides, however, color images are required.

Performing measurements

A measurement is performed via the **Start NMI Analysis** function in the **NMI** workflow:
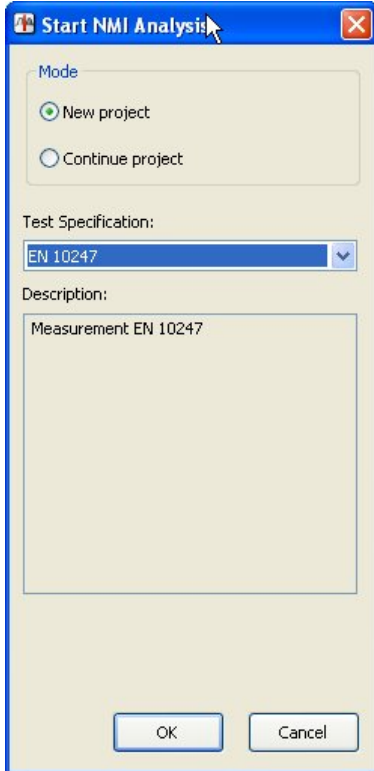
Starting dialog for NMI Analysis

An NMI analysis is always based on a previously defined test specification. This must have been created by a qualified user. No measurement can be started until a template has been created.

During the course of the measurement, the function dialog for which the **Interactive** mode has been selected in the evaluation script will be displayed. All other functions run automatically in the background.

The program will stop at each function for which **Interactive** has been selected and the corresponding dialog will open. The predefined parameters can then be modified and optimally adapted to the image in question.

The functions that are carried out interactively feature the additional parameter **Always interactive** during the execution of the program. This allows the user to decide whether or not the dialog should be opened again for the next image. This is particularly useful if you have set segmentation thresholds, for example, and do not need to modify them again for subsequent images. The thresholds can therefore be set using one or more images and then applied automatically.

This option is useful if you are measuring several images immediately after each other. Otherwise, this setting does not have any impact on the running of the program.

The results document

At the end of the measurement the results window is displayed. This is divided into three areas:

- Results view
- Gallery of images of the largest inclusions
- Gallery of unlikely inclusions

In this document the measurement results for each sample and each standard are displayed separately. It is possible to view the results for a different standard or sample here.

The results window is divided into two property pages. The first one shows the numerical values with a classification table, statistical values and an overview chart. The second shows the gallery containing images of the largest inclusions and allows artifacts to be deleted.

The results view
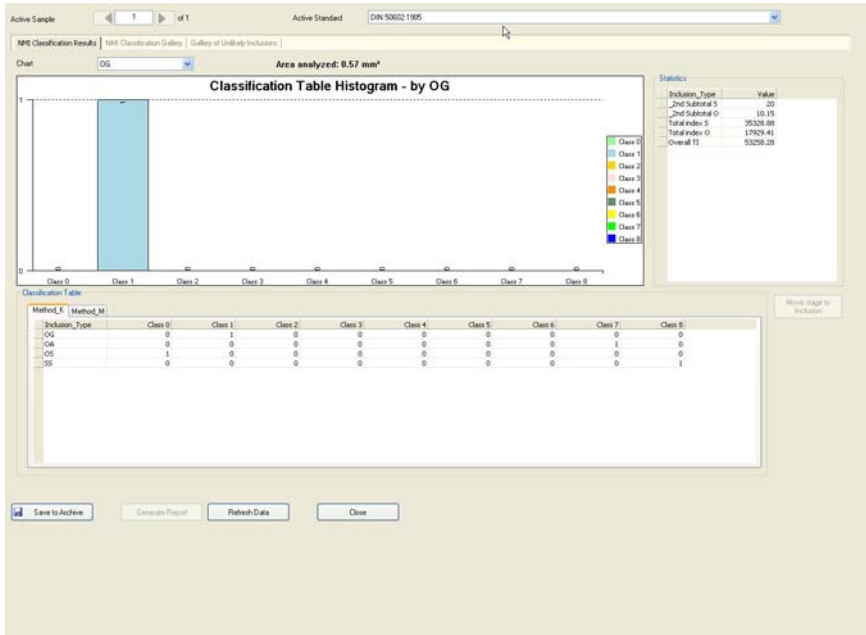
Results document with results view

The document is essentially divided into three areas:

1. Chart
2. Classification tables
3. Statistics

The gallery of images of the largest inclusions

This gallery shows the largest inclusions. It is possible to filter the gallery according to inclusion type (sulfides, oxides) or display it for all inclusion types. In addition, in all standards (except EN 10247) it is possible to display the worst field for each inclusion type.

If the sample is still under the microscope and has not been changed, the inclusion can be repositioned. This means that, if you click on the **Einschluss anfahren** button, the microscope stage is moved in such a way that you are able to view the currently highlighted inclusion directly in the live image or through the eyepiece. If you establish that this object is an artifact, you are also able to delete it.

Results document with gallery of the largest inclusions

The gallery of unlikely inclusions

This gallery shows objects that are "unusual". Scratches, for example, are filtered out on the basis of their angle. However, as it is also possible for inclusions to have unusual forms or angles, automatic filtering of artifacts is not sensible. Instead, all objects to which the parameters apply are collected in a gallery and presented to the user following the measurement. The user is then able to decide whether they are inclusions or artifacts. The parameters can be changed under **Prüfvorschriften ändern**.

Generating reports

Reports are generated separately for each standard. It is also possible to combine several samples in a single report on the basis of a particular standard. There are no restrictions with regard to either the number of reports or the combination options. This means that you can measure the samples of several customers or from several casts using a single measurement but nevertheless generate a separate report for each case.
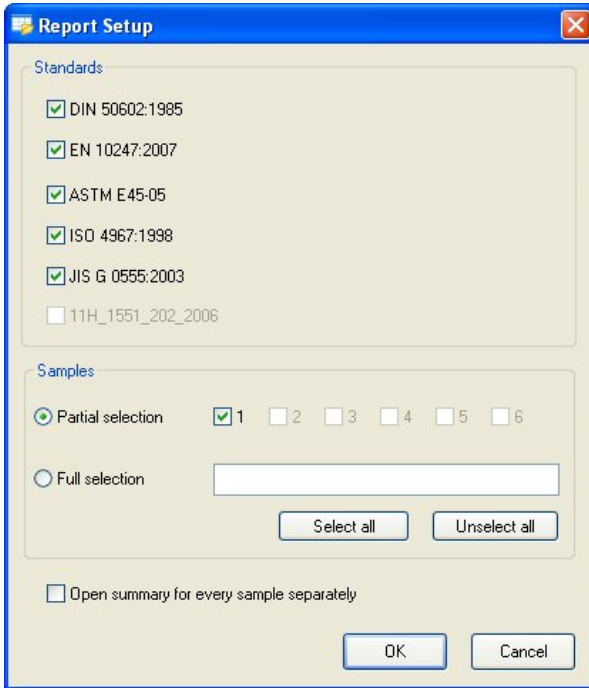
Dialog for generating reports

12. Configuration Modules

12.1 Commander

Introduction

AxioVision's operating concept is based on the principle that in most cases, rather than being faced with completely new tasks all the time, the user has to carry out a certain number of recurring routine activities. Even the basic system takes this into account, by offering the possibility of running through routine activities step by step using workflows, without having to check the last functions used – and their menu positions, each time.

With the Commander module, this concept has been taken further, and enhanced in a consistent way. Essentially, the Commander offers the following possibilities:

- Recording steps to be executed in sequence.
- Adding to, editing and refining the sequence recorded ("script").
- Batch processing: automatically applying a script to several images.

The Commander has numerous areas of application. As well as being a means of "simply" automating the process of retrieving functions, a script can also be regarded as an independent command in AxioVision, and can even be used in workflows (for example). If used in combination with the VBA module, you can even include VBA macros that you have created yourself in a script as commands.

Special points relating to the creation of scripts

Almost all of the functions available in AxioVision's main menu can be included in a script.

Once the process of recording the script has started, a button (which is otherwise hidden) will become available. The button can be used to adopt the command into the active script. Functions are not, therefore, "simply recorded", as numerous commands that you do not require would then be included too, and you would have to delete them. Often it is difficult to keep track of this once you are inside a script.

Functions that do not have a function dialog (e.g. annotations and interactive measurement functions) actually have to be executed before they can be included in a script. Further information can be found in the **Script Editor's** online help.

Batch processing - application of a script to several images

The automation of a number of commands is usually only one of the aspects involved in the creation of scripts. The next step is applying them to the desired number of images. With this in mind, the Commander module has a separate function for batch processing. This allows you to specify the number of images you want to process using a script, and where these images come from:

➢ The first possibility involves acquiring the images directly via the camera, and executing the script for these images. The script is started after an image has been acquired. Once it has been executed, you can acquire the next image, and so on, until you want to end the processing.

➢ The second possibility involves acquiring images first, and saving them on the hard disk before starting processing using a script. The function for automatically saving images after acquisition is useful here (**Tools** menu ⇒ **Options** ⇒ **Storage** and **Acquisition**).

➢ Another option involves processing all of the images that are currently open in AxioVision.

Limitations of scripts

The aim of the Commander is to enable the user to execute the commands available in AxioVision automatically. It is therefore an aid to automation. The Commander should not be confused with a programming language, as the typical elements of programming (generation of function dialogs, variables, loops etc.) are not available. The VBA module should be used in such cases.

Use of scripts by several users of a system

Microsoft Windows 2000 and Microsoft Windows XP, which are designed to allow several users to work on a system, are fully supported by AxioVision. More specifically, this means that each user can create his/her own AxioVision environment without other users of the system being able to access the configurations and files that he/she generates (see also section 8.3 "User and Data Administration").

AxioVision therefore saves scripts by default in the following folder:
"My Documents\Carl Zeiss\Data\Script"

Frequently, however, you will want to make a script that has been created available to all users of a system. When you are saving a script, you therefore have the option of selecting whether you want to save the current script for yourself only, or for all users.
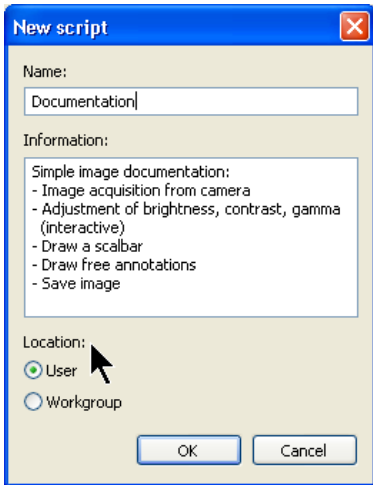
The scripts are now saved in the following folder:
"C:\Documents and Settings\All Users\Documents\Carl Zeiss\Data\Script"

Icons in the list of scripts in the **Script Editor** or on the toolbar indicate whether a script has been saved for the current user only, or whether it may be accessed by all users:
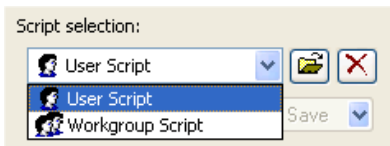
Installation of scripts on other systems

Scripts can easily be transferred to other systems. The Commander module must not be present on the target system. The installed scripts can be executed at any time via the menu, or via the Commander toolbar.

Commander scripts form part of an individual AxioVision configuration. They are therefore also saved when you save a configuration using the **Import/Export Configuration** function on the **Tools** menu. To avoid problems, we recommend that you use this function to save and transfer scripts to other systems. Further information on the use of this function can be found in section 8.7 "Exporting and Importing User Configurations".

NOTE:
- If functions that are part of a module are used within a script, you must ensure that the relevant module is also available on the target system, and has been activated in the **Module Manager** (**Tools** menu $\Rightarrow$ **Modules Manager**).

Recording a script

In this exercise you will learn how to record and save a script using the script editor. A simple documentation task will be used as an example: acquire image, enhance contrast, draw in scale bar, save image.

Specifically, the following steps are covered:

- creating new scripts, entering a description
- starting the recording of a script
- behavior of the script editor during recording
- adding commands to a script
- saving a script

NOTE:

- The following example describes the selection of the commands to be used via the AxioVision main menu. You can, of course, also call up the appropriate commands via toolbars, workflows and your own customized dialogs. Please also read chapter 2 "Overview and Operating Concept".

- Before you start to record a script, you can determine the behavior of the editor during recording with the function **Commander – Options**..

➢ Open the script editor. To do this, select the **Script Editor** function from the **Commander** menu.
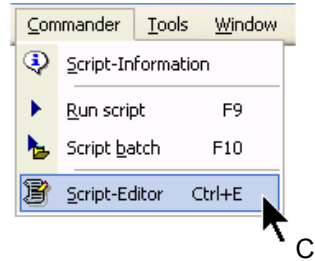
➢ The script editor is the "control center" for creating and revising scripts.
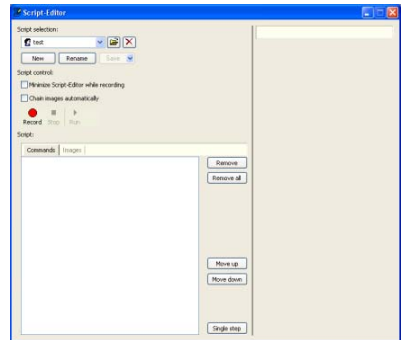
➢ To record a new script, click on **New** under **Script selection**.
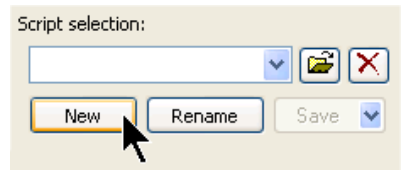
➢ Enter a name in the **New script** dialog that describes the script as clearly as possible. You can also describe the content of the script in more detail in the **Information** field.

➢ Via **Location** you can specify whether the script should be available only to you or also to all other users of the system (the **Workgroup**).

➢ Click on **OK**, to create the new script.

➢ An empty script window is now displayed.
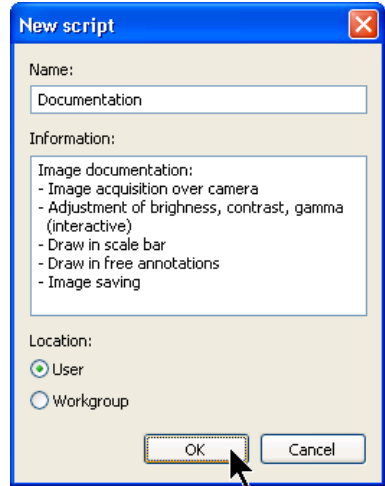
➢ Now start recording the script by clicking on the **Record** button.
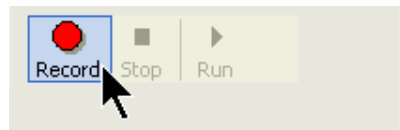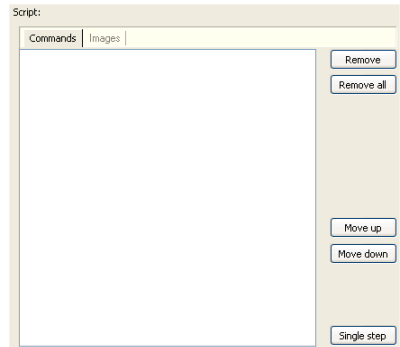
➢ If you have activated the **Minimize Script Editor while recording** option in the function **Commander - Options**, the script editor is hidden and only a window with the buttons **Record** and **Stop** is displayed. This means that there is more space on your screen for working in AxioVision.
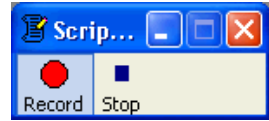
➢ You can now work entirely as normal in AxioVision: First open the live image via the **Acquisition** menu ⇒ **Live** function.

➢ Select a scaling that is suitable for the objective you are using from the **List of available scalings** in the area at the bottom of the live window.

➢ Close the **Live** window.
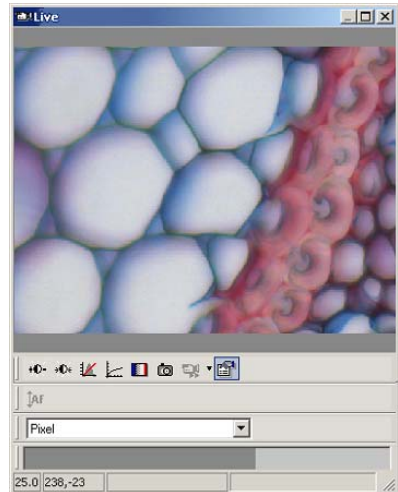
NOTE:

• The live window does not have to be adopted into the script. It is automatically displayed if you execute the **Snap** function interactively.

➢ Now select the **Snap** function from the **Acquisition** menu.

➢ The image is acquired and the **Add to script** dialog is then displayed. Using this dialog, you can decide whether or not each command should be included.
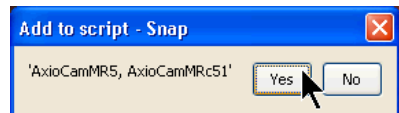
➢ Click on **Yes** to adopt the command into the script. This closes the **Add to script** dialog window.

➢ Now execute the following functions in addition to the **Live** function, and adopt them into the script as described above:

• **Processing** menu, **Adjust** group, **Brightness**/**Contrast**/**Gamma** function.

• **File** menu, **Save As** function.

➢ Then save the script by clicking on **Save** under **Script selection**.

➢ Once the current version of the script has been saved, the **Save** button is displayed in gray.
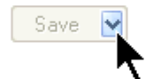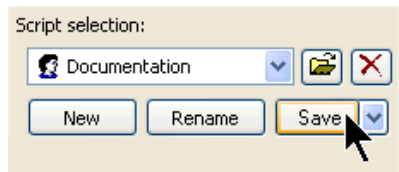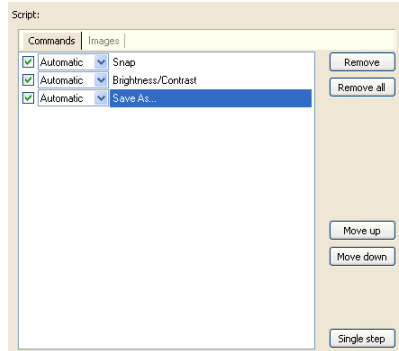
Inserting commands into an existing script

Often, after recording a script, you will discover that the sequence does not meet your requirements. Perhaps commands are missing, or the commands that are included do not function as expected.

This exercise describes how to expand a previously recorded script in the appropriate way:

• executing a script step by step

• inserting new commands

NOTES:

- The following example describes the selection of the commands to be used via the AxioVision main menu. You can, of course, also call up the appropriate commands via toolbars, workflows and your own customized dialogs. Please also read chapter 2 "Overview and Operating Concept".

- The "Documentation" script used in the following example is the result of the previous exercise "Recording a scrip". It is assumed that you have carried out the exercise as described and that the script is therefore available.

➢ Open the script editor. To do this, select the **Script Editor** function from the **Commander** menu.
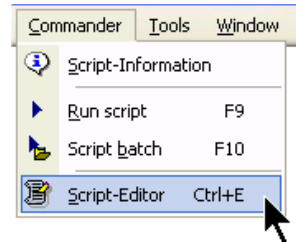
➢ In the list under **Script selection**, select the script that you want to change.
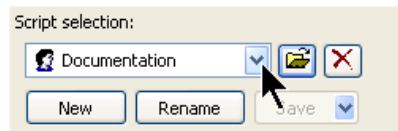
➢ The selected script is immediately displayed in the script editor.

➢ Below, we will incorporate the possibility of drawing in a scale bar and adding free annotations after the **Brightness/Contrast/Gamma** command.

➢ You can now insert new commands at any location. To do this, select the last line **Save As**.
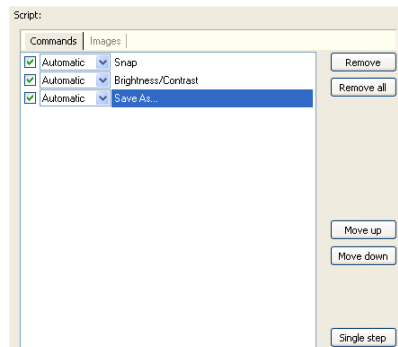
➢ To insert further commands, you should first execute the script up to the line after which the new command is to be inserted.

➢ Highlight the first line **Snap** and then click on **Single step**. The image that is currently set is acquired.
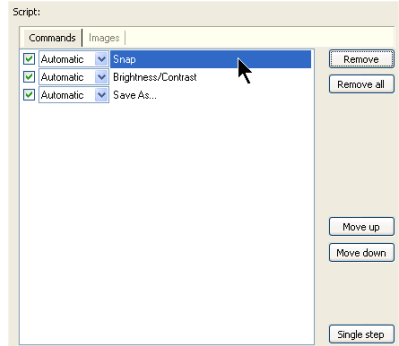
NOTES:
- If the image is unsatisfactory, adjust it first via the live image.

➢ Click again on **Single step**. The **Brightness/Contrast/Gamma** function is executed.

➢ After the execution of **Brightness/Contrast/Gamma** the last line is highlighted, as the cursor moves on automatically. When you insert functions, however, they are inserted *after* the position that is highlighted at that moment.
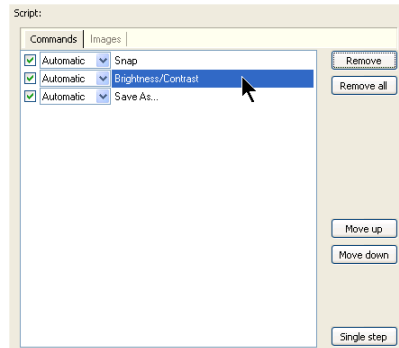
➢ You should therefore activate the **Brightness/Contrast/Gamma** function by clicking on it.

➢ Commands are inserted simply as a result of the desired functions being recorded.
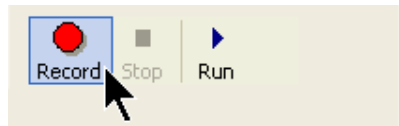
➢ If you have activated the **Minimize Script Editor while recording** option in the function **Commander - Options**, the script editor is hidden and only a window with the buttons **Record** and **Stop** is displayed. This means that there is more space on your screen for working in AxioVision.
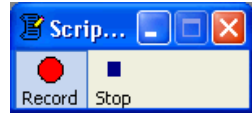
➢ Now execute the following functions one after the other and adopt them into the script:

- **Annotations** menu, **Scale bar** function

- **Annotations menu**, **Draw Annotations** function

➢ The new functions are now displayed after the **Brightness/Contrast/Gamma** command.
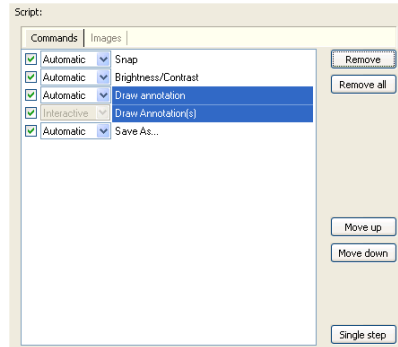
The script now contains all the commands required. However, to ensure smooth operation there are two other things that need to be considered:

1. The live window is not closed in every case, but only if the **Close after Snap** function in the live window's shortcut menu has been activated.

2. Documents may still be open. At the start you should therefore ensure that all documents have been closed.

3. Start the recording again and insert the **Close all** command from the **Windows** menu.

➢ The **Close all** command is now displayed in the last but one line. It should, however, be executed at the start of the script.

➢ To move it to the first position in the list, highlight the line and click on **Move up** until it moves to the first line in the script.
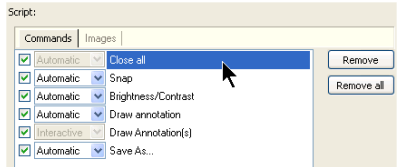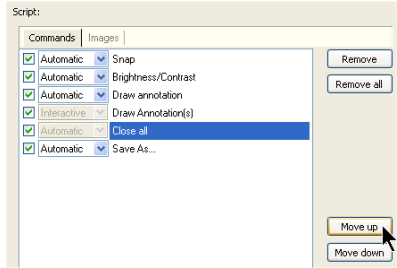
NOTE:

- You can also duplicate individual lines by using **Ctrl C** (copy) and **Ctrl V** (paste) keys. The line is then included twice in the script.

➢ Finally, save the script by clicking on **Save** under **Script selection**.

➢ Once the current version of the script has been saved, the **Save** button is displayed in gray.
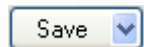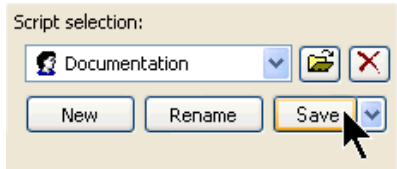
Changing the way individual commands are executed, selecting images to be displayed

This example shows you how to influence the way in which individual commands behave during the execution of the script. In addition, you will be shown how to specify which images are displayed/not displayed during and/or after execution:

➢ displaying function dialogs during execution so that you can adapt parameters (interactive execution of commands).

➢ switching individual commands on and off so that commands are not executed (useful for testing a script).

➢ specifying which images should be displayed during execution, to prevent too many images from "blocking" the screen.

Notes:
• The following example describes the selection of the commands to be used via the AxioVision main menu. You can, of course, also call up the appropriate commands via toolbars, workflows and your own customized dialogs. Please also read chapter 2 "Overview and Operating Concept".

• The "Documentation" script used in the following example is the result of the previous exercises "Recording a script" and "Inserting commands into an existing script". It is assumed that you have carried out the exercises as described and that the script is therefore available.

In the following example the "Documentation" script is modified to meet the requirements described below.

The **Close all** function should simply close all images, including those that have not been saved, without asking whether the images should be saved.

The execution of the script should be interrupted at the following commands so that settings and parameter values can be entered in dialogs:

- **Record**, so that you can position the specimen correctly and focus.

- **Brightness/Contrast/Gamma**, so that you can adapt the settings to the image that is currently being acquired.

- **Save As**, so that you can change the folder, file name and image format.
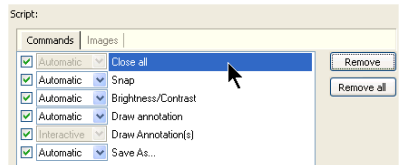
Only the resulting image should be displayed in AxioVision, not the acquired image.

➢ Open the script editor. To do this, select the **Script Editor** function from the **Commander** menu.
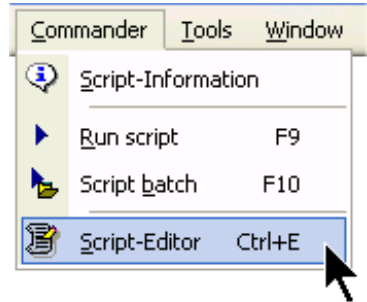
➢ In the list under **Script selection**, select the script that you want to change.
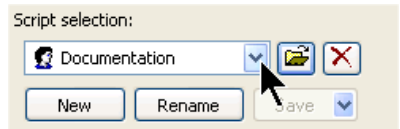
➢ The selected script is immediately displayed in the script editor.

➢ Click on the **Close all** function.

➢ Under **Parameters** on the right-hand property page, switch the setting for **Force close** from **No** to **Yes**.

➢ The **Close all** function will now close all open documents without asking whether unsaved documents should be saved.
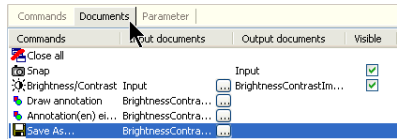
➢ Click on the **Snap** function and switch the **Automatic** parameter to **Interactive**.
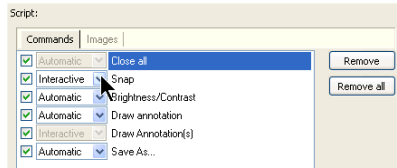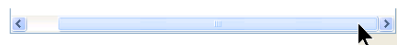
➢ Carry out the same action for the **Brightness/Contrast/Gamma** and **Save As** functions.
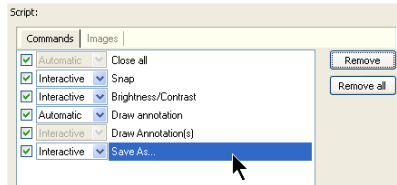
➢ To specify which images are displayed during execution of the script and which are not, switch to the **Documents** property page.

➢ If the **Visible** column is not displayed, move the slider at the bottom edge completely to the right.
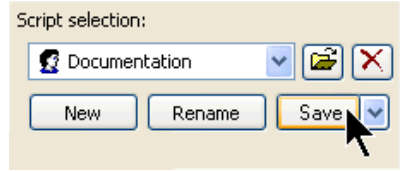
➢ Now deactivate the **Visible** option for the **Snap** command.

➢ Only the resulting image is then displayed during automatic execution (see next exercise).
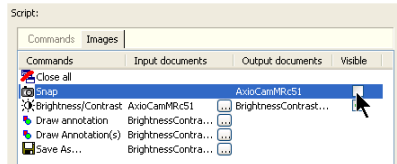
➢ Then save the script by clicking on **Save** under **Script selection**.

➢ Once the current version of the script has been saved, the **Save** button is displayed in gray.

## Executing a script automatically

This example shows you how to execute a script and how to influence the way in which individual commands are executed:

- displaying the **Commander** toolbar.
- executing a script automatically.
- displaying script information.

NOTES:
- The following example describes the selection of the commands to be used via the AxioVision main menu. You can, of course, also call up the appropriate commands via toolbars, workflows and your own customized dialogs. Please also read chapter 2 "Overview and Operating Concept".

- The "Documentation" script used in the following example is the result of the previous exercises. It is assumed that you have carried out the exercises as described and that the script is therefore available. If not, you can load it directly from the AxioVision installation CD. You can find the script on the CD in the folder *"Other\AxioVision 4 sample files\Commander scripts"*. Load the script directly over the button under **Script selection** in the Script Editor.

> ➢ The simplest way to execute a script is via the **Commander** toolbar. If the toolbar is not displayed, select the **Toolbars** group from the **View** menu and there select the **Commander** entry.
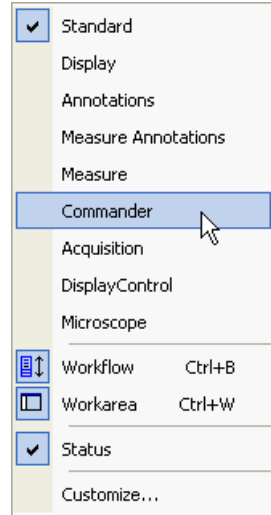
> ➢ Then select the script that you wish to execute under **Select Script**.

> ➢ If you are not sure whether it is the right script, click on **Info** after making your selection. The **Script Information** window is then displayed. This contains a more detailed description of the script (if this was entered by its creator).
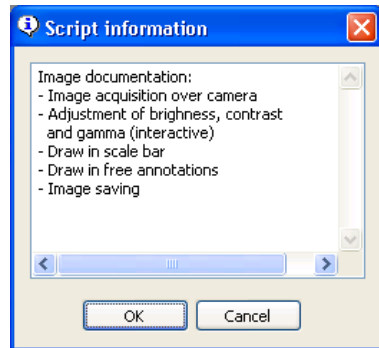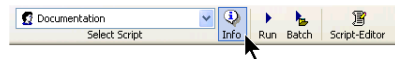
➢ Click on **Run** to start the execution of the script (the **Script batch** option is explained in the following exercise).
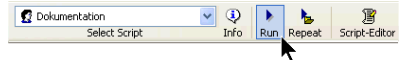
Preparing a script for batch processing

Processing several images using a single script is the real area of application of the Commander, or rather of the scripts that are created with it. This exercise shows you the conditions that a script has to meet in order to be executed via the **Script batch** function.

- Preparing a script for batch processing
- Executing the function for batch processing
- Selecting the image source
- Setting the saving options for results files

NOTES:
- The following example describes the selection of the commands to be used via the AxioVision main menu. You can, of course, also call up the appropriate commands via toolbars, workflows and your own customized dialogs. Please also read chapter 2 "Overview and Operating Concept".

- The "Documentation" script used in the following example is the result of the previous exercises. It is assumed that you have carried out the exercises as described and that the script is therefore available. If not, you can load it directly from the AxioVision installation CD. You can find the script on the CD in the folder *"Other\AxioVision 4 sample files\Commander scripts".* Load the script directly over the button under **Script selection** in the Script-Editor.

➢ If you want to execute a script via the **Script batch** function, it must meet the following conditions:

1. It must not include a function that opens an image file or acquires an image via the camera.

2. The first function within the script that requires an image as input must use the image that is currently loaded. Since, however, at the time the script is created it is not known where this image will come from and what name it will have, a general place-marker must be used (what is known as the "active image").

3. As an option, the **Script batch** function can save important images automatically. This means that functions for saving do not necessarily have to be included in the script.
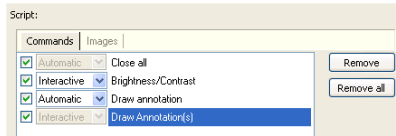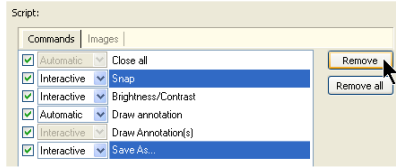
➢ Open the script editor. To do this, select the **Script Editor** function from the **Commander** menu.
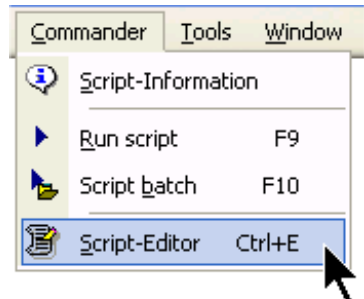
➢ Select the script that you want to be changed in the **Script selection** drop-down list box.
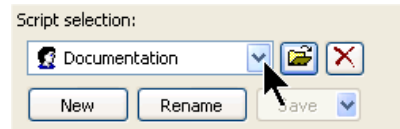
➢ The selected script is immediately displayed in the Script Editor.

➢ In the **Script** field, select the **Snap** and **Save As** commands, one after the other, and delete them using the **Remove** button.


The commands that are executed automatically by the **Script batch** function have now been removed.

You must now ensure that the first image-processing command (**Brightness/Contrast/Gamma**) actually uses the loaded or acquired image as the input image.

➢ To do this, click on the **Images** tab sheet in the **Script** field.

➢ Select the **Brightness/Contrast/Gamma** command.

➢ To change the input image, select the ⟨…⟩ button under **Input documents** to open a gallery for selecting an image.
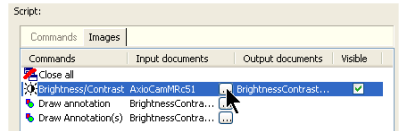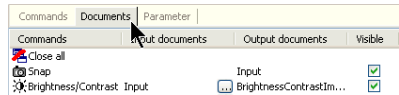
➢ If you have not previously loaded or acquired an image, the gallery will be empty, as shown in the illustration on the right.
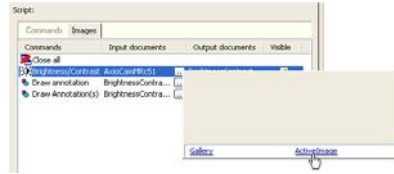
➢ Here, select the **ActiveImage** command. The "active" image is then always used as the input image for the **Brightness/Contrast/Gamma** function – irrespective of the source from which it originates (camera, file) and its name.
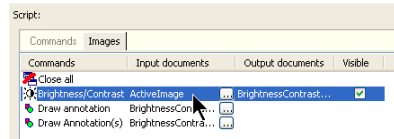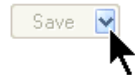

➢ Finally, save the script by clicking on **Save** in the **Script selection** field.
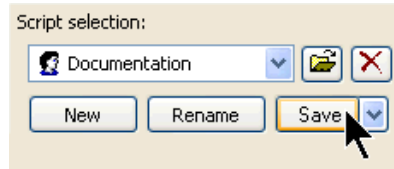
➢ Once the current version of the script has been saved, the **Save** button is displayed in gray.

➢ Close the **Script Editor**.

NOTES:

• Before you save the script, think about whether you will only want to use it with the **Script batch** function in the future. If not, you should use a different name.

• In this case, click on the arrow to the right of **Save**, under **Script selection**, and select **Save As**. This function allows you to save the script under a new name.

Repeating scripts

The **Repeat** function allows you to apply a script to any number of images. The images may already be available as files in a folder or may already have been loaded in AxioVision. You may, of course, also acquire them via the camera. In addition, you can specify, for each resulting image within the script, whether or not you want the image in question to be saved. The following steps are carried out in this exercise:

- Executing the function for repetition
- Selecting the image source
- Setting the saving options for results files

NOTES:
- The following example describes the selection of the commands to be used via the AxioVision main menu. You can, of course, also call up the appropriate commands via toolbars, workflows and your own customized dialogs. Please also read chapter 2 "Overview and operating concept".

- Before you perform this exercise, you should ensure that you have worked through the previous example "Preparing a script for batch processing".

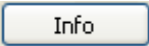➢ To execute the script for several images, select the **Repeat script** function from the **Commander** menu.
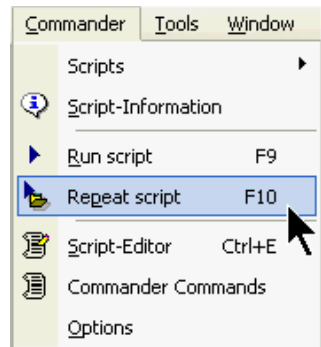
➢ First, select where the images should originate from in the **Select source** drop-down list box. In most cases you will presumably acquire them via the camera.
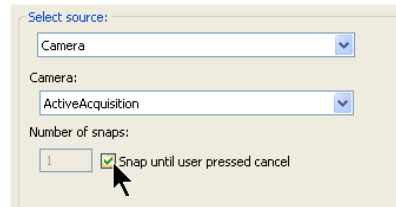
➢ If you work with several cameras, select the **ActiveAcquisition** setting in the **Camera** drop-down list box. The camera activated in the **Acquisition** menu will then be used each time.

➢ Finally, specify how many images you wish to acquire. If the number is not known, activate the **Snap until user pressed cancel** option.

➢ Now select, under **Script selection**, the script that you wish to use.
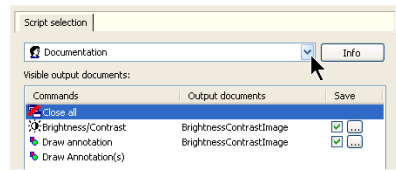
➢ If you are not sure whether it is the right script, click on ⌊ Info ⌋ after making your selection.

➢ The **Script information** window is then displayed. This contains a more detailed description of the script (if this was entered by its creator).
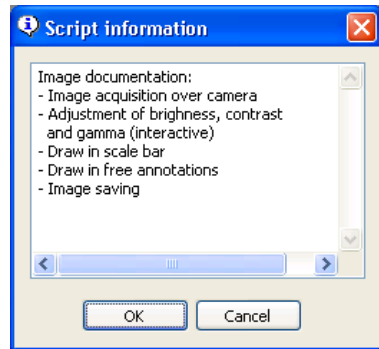
➢ The **Visible output documents** list shows all commands that generate a results file (image, data table).
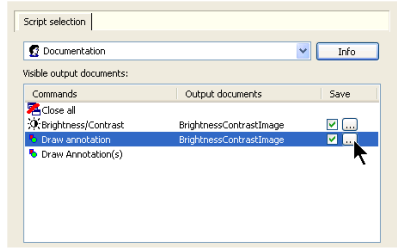
➢ Via the **Save** column, you can specify whether or not you want the image to be saved automatically during execution.

➢ If the image is to be saved, you can specify the **Save options** via the ⌷ button.

➢ The saving options include specifying the **Folder** in which the image in question should be saved and the way in which the **New file name** should be generated, as well as selecting the **Image type**. Enter the required settings here and adopt them by clicking on **OK**.

➢ Finally, start the execution of the script by clicking on **Start**.
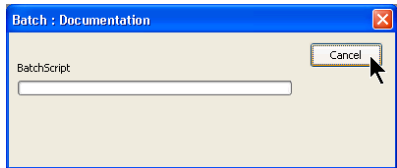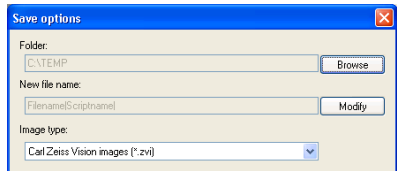
The commands are now run through as if you were selecting them interactively via the menu.

A **Repeat:***Name* status window is opened which gives you the option of exiting the script at any time using the **Cancel** button.

12.2 VBA

General

The VBA module enables you to simplify routine tasks with the help of macros, and to program your own applications.

AxioVision contains the most recent version, VBA 6. In this version the language features of VBA are on a par with Visual Basic.

If you have never done any programming before, we advise you to build up your knowledge in advance. As Visual Basic/**V**isual **B**asic for **A**pplications is a very widely used standard, you will easily find a wide range of training programs and literature to meet the needs of people with almost any level of prior knowledge.

Section 8.5 "Adapting the User Interface" describes how workflows, toolbars and shortcut keys can be used to adapt AxioVision to your personal work situations, and to make your work a great deal easier and more convenient. With the additional possibilities offered by the VBA module, you can let your imagination run free, as there are no longer any restrictions when it comes to configuring AxioVision to meet your individual requirements.

The AxioVision object model

In addition to a description of the general VBA functions, AxioVision's online help also contains documentation on the AxioVision objects.

This online help can be found in *"C\Program Files\Common Files\Carl Zeiss Vision\System\ZIPL"*. Open the help file by double-clicking on the file "zipl.chm".

Managing and running macros

There are two ways to run macros that you have created in the Visual Basic Editor:

➢ The first is via macro management. From the **Tools** menu select the **Macros** function. Besides the possibility of running macros, here you will also find functions for deleting macros, starting the Visual Basic Editor etc.

➢ The second possibility is via the function for running macros. From the **Tools** menu select the **Run Macro** function. A list of the macros you have created is then displayed. Select the desired macro and click on **Start** to run it.

Running macros via workflows, toolbars and shortcut keys

A much more convenient option, however, is to integrate each macro into the user interface.

Macros are treated in the same way as any other function in AxioVision, and can therefore be used via workflows, toolbars and shortcut keys, as required. In the **Tools** menu under the **Customize** function you will always find a **Macros** branch on the corresponding property pages. The list of the macros you have created is also available.
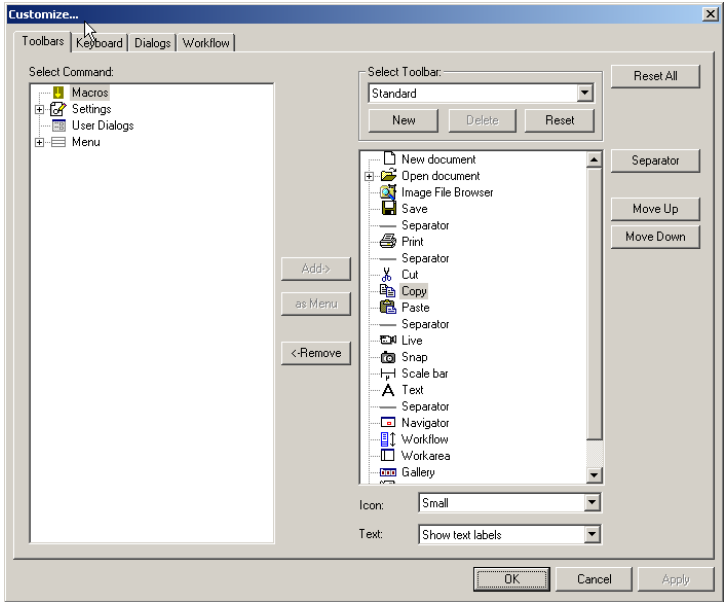
NOTES:

•   Section 8.5 "Adapting the User Interface" describes how workflows, toolbars and shortcut keys can be used to adapt AxioVision to your personal work situations, and to make your work a great deal easier and more convenient.

The VBA online help

The VBA online help makes the following information available.

Welcome to Visual Basic documentation

The documentation on Visual Basic is extremely diverse. Each individual component has been created to help you to familiarize yourself with particular aspects of the product. The documentation supplied with Visual Basic covers the components described below.

Help with the Visual Basic user interface

Here you will find information on interface elements of the Visual Basic Editor, such as commands, dialog boxes, windows and toolbars.

Visual Basic concepts

The *context*-sensitive *help* contains information that will help you build up your knowledge of programming in Visual Basic.

Help with Visual Basic procedures

In this area of the help you will find useful information on general procedures, such as the use of the object catalogue or the specification of Visual Basic environment options.

Visual Basic language directory

In the *language directory* you will find information on the Visual Basic programming language, i.e. all of its methods, features, statements, functions, operators and objects.

Visual Basic add-in model

If you want to adapt the Visual Basic Editor, in the language directory you will find information on the object model, which will enable you to expand the environment.

Reference for Microsoft Forms

Here you will find information on *Userform* objects and control elements, and on programming with *Userform* objects and control elements in Visual Basic.