OLYMPUS®

User Manual

cellSens

LIFE SCIENCE IMAGING SOFTWARE

Contents

1. About the documentation for your software

Note: cellSens is available in a variety of versions. For this reason, it can happen that your cellSens version doesn't have some of the functions described there.

The documentation for your software consists of several parts: the installation manual, the online help, and PDF manuals which were installed together with your software.

Where do you find which information?

The installation manual is delivered with your software. There, you can find the system requirements. Additionally, you can find out how to install and configure your software.

In the manual, you will find both an introduction to the product and an explanation of the user interface. By using the extensive step-by-step instructions you can quickly learn the most important procedures for using this software.

In the online help, you can find detailed help for all elements of your software. An individual help topic is available for every command, every toolbar, every tool window and every dialog box.

New users are advised to use the manual to introduce themselves to the product and to use the online help for more detailed questions at a later date.

Writing convention used in the documentation

In this documentation, the term "your software" will be used for cellSens.

00054

Sample images

The DVD that comes with your software contains, among a lot of other data, also images that show different examples of use for your software. You can load these so-called example images from the DVD. However, in many cases, installing the example images on your local hard disk or on a network drive is more helpful. Then the example images will always be available, no matter where the DVD with the software currently is.

Note: Your software's user documentation often refers to these example images. You can directly follow some step-by-step instructions when you load the corresponding example image.

You can open and view the example images with your software. Additionally, you can use the example images to test some of your software's functions, for example, the automatic image analysis, the image processing or the report creation.

Due to the fact that the example images also contain multi-dimensional images like Z-stacks or time-lapse images, making use of them enables you to quickly load images that require more complex acquisition settings.

Installing example images

You can install the example images after you've installed the software, or at any later point in time.

To do so, insert the DVD that contains the software into the DVD drive. If the installation wizard starts, browse to the directory that contains the example images and install them.

07005 04072013

2. User interface

2.1. Overview

The graphical user interface determines your software's appearance. It specifies which menus there are, how the individual functions can be called up, how and where data, e.g. images, is displayed, and much more. In the following, the basic elements of the user interface are described.

Note: Your software's user interface can be adapted to suit the requirements of individual users and tasks. You can, e.g., configure the toolbars, create new layouts, or modify the document group in such a way that several images can be displayed at the same time.

Appearance of the user interface
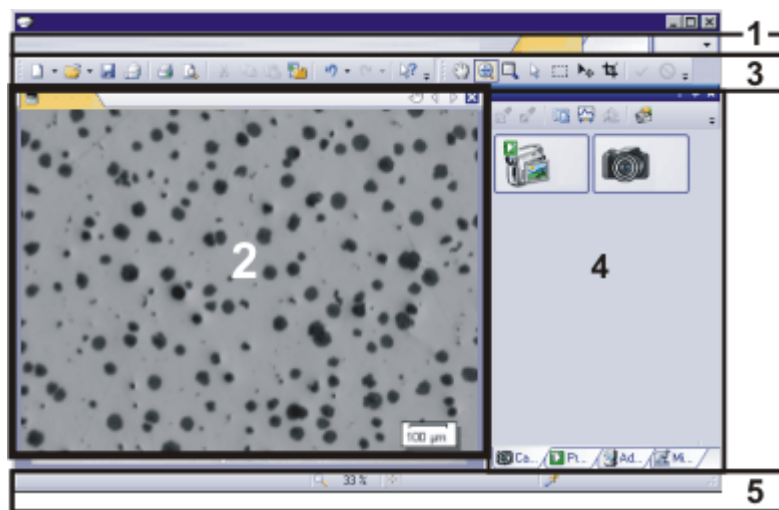
The illustration shows the schematic user interface with its basic elements.

(1) Menu bar
You can call up many commands by using the corresponding menu. Your software's menu bar can be configured to suit your requirements. Use the *Tools > Customization > Start Customize Mode...* command to add menus, modify, or delete them.

(2) Document group
The document group contains all loaded documents. These can be of all supported document types.
When you start your software, the document group is empty. While you use your software it gets filled - e.g., when you load or acquire images, or perform various image processing operations to change the source image and create a new one.

(3) Toolbars
Commands you use frequently are linked to a button providing you with quick and easy access to these functions. Please note, that there are many functions which are only accessible via a toolbar, e.g., the drawing functions required for annotating an image. Use the *Tools > Customization > Start Customize Mode...* command to modify a toolbar's appearance to suit your requirements.

(4) Tool windows
Tool windows combine functions into groups. These may be very different functions. For example, in the *Properties* tool window you will find all the information available on the active document. In contrast to dialog boxes, tool windows remain visible on the user interface as long as they are switched on. That gives you access to the settings in the tool windows at any time.

(5) Status bar
The status bar contains a large amount of information, e.g., a brief description of each function. Simply move the mouse pointer over the command or button for this information.

00108

2.2.   Layouts

To switch backwards and forwards between different layouts, click on the right-hand side in the menu bar on the name of the layout you want, or use the *View > Layout* command.

Which predefined layouts are there?

For important tasks several layouts have already been defined. The following layouts are available:

Acquiring images ("Acquisition" layout)
Viewing and processing images ("Processing" layout)
Measuring images ("Count and Measure" layout)
Generating a report ("Reporting" layout)

In contrast to your own layouts, predefined layouts can't be deleted. Therefore, you can always restore a predefined layout back to its originally defined form. To do this, select the predefined layout, and use the *View > Layout > Reset Current Layout* command.

What is a layout?

Your software's user interface is to a great extent configurable, so that it can easily be adapted to meet the requirements of individual users or of different tasks. You can define a so-called "layout" that is suitable for the task on hand. A "layout" is an arrangement of the control elements on your monitor that is optimal for the task on hand. In any layout, only the software functions that are important in respect to this layout will be available.

Example: The *Camera Control* tool window is only of importance when you acquire images. When instead of that, you want to measure images, you don't need that tool window.
That's why the "Acquisition" layout contains the *Camera Control* tool window, while in the "Count and Measure" layout it's hidden.

Which elements of the user interface belong to the layout?
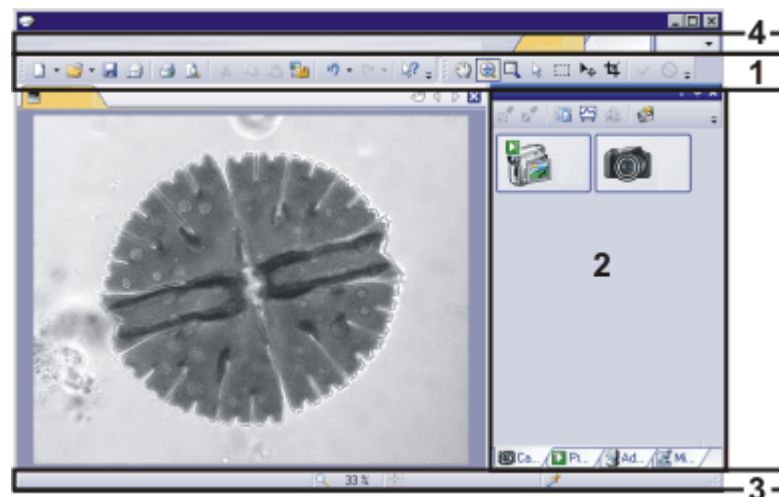
The illustration shows you the elements of the user interface that belong to the layout.
(**1**) Toolbars
(**2**) Tool windows
(**3**) Status bar
(**4**) Menu bar

The layout saves the element's size and position, regardless of whether they have been shown or hidden. When, for example, you have brought the *Windows* toolbar into a layout, it will only be available for this one layout.

00013

2.3. Document group

The document group contains all loaded documents. These can be of all supported document types. As a rule, the loaded documents are images.

Appearance of the document group
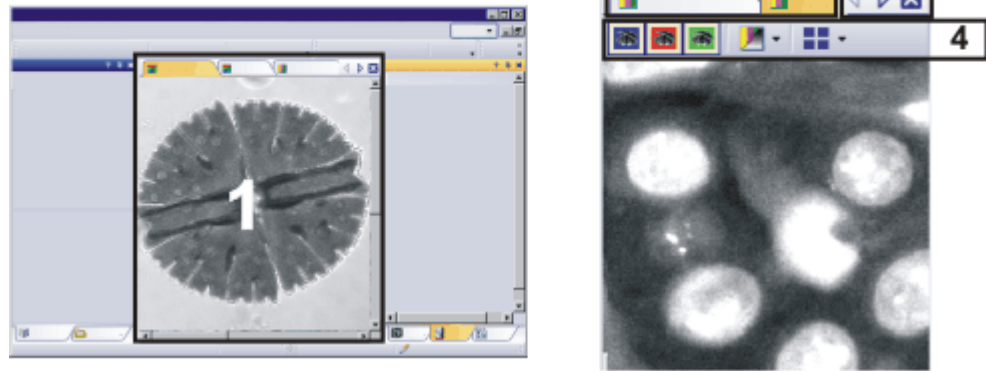
(**1**) Document group in the user interface
(**2**) Document bar in the document group
(**3**) Buttons in the document bar
(**4**) Navigation bar in the image window

(1) Document group in the user interface

You will find the document group in the middle of the user interface. In it you will find all of the documents that have been loaded, and of course also all of the images that have been acquired. Also the live-image and the images resulting from, e.g., any image processing function, will be displayed there.

(2) Document bar in the document group

The document bar is the document group's header.
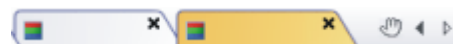
For every loaded document, an individual tab showing the document name will be set up in the document group. Click the name of a document in the document bar to have this document displayed in the document group. The name of the active document will be shown in color. Each type of document is identified by its own icon.

At the top right of each tab, a small [**x**] button is located. Click the button with the cross to close the document. If it has not yet been saved, the *Unsaved Documents* dialog box will open. You can then decide whether or not you still need the data.

(3) Buttons in the document bar

At the top right of the document bar you will see several buttons.

Button with a hand

Click the button with a hand on it to extract the document group from the user interface. In this way you will create a document window that you can freely position or change in size.

If you would like to merge two document groups, click the button with the hand in one of the two document groups. With the left mouse button depressed, drag the document group with all the files loaded in it, onto an existing one.

Prerequisite: You can only position document groups as you wish when you are in the expert mode. In standard mode the button with the hand is not available.

Arrow buttons

The arrow buttons located at the top right of the document group are, to begin with, inactive when you start your software. The arrow buttons will only become active when you have loaded so many documents that all of their names can no longer be displayed in the document group. Then you can click one of the two arrows to make the fields with the document names scroll to the left or the right. That will enable you to see the documents that were previously not shown.

(4) Navigation bar in the image window

Multi-dimensional images have their own navigation bar directly in the image window. Use this navigation bar to set or to change how a multi-dimensional image is to be displayed on your monitor.

There are some other document types with their own navigation bar directly in the image window. One example is a report instruction or an experiment plan.

00139 02072013

2.4.   Tool Windows

Tool windows combine functions into groups. These may be very different functions. For example, in the *Properties* tool window you will find all the information available on the active document.

Which tool windows are shown by default depends on the layout you have chosen. You can, at any time, make specific tool windows appear and disappear manually. To do so, use the *View > Tool Windows* command.

Manually displayed or hidden tool windows will be saved together with the layout and are available again the next time you start the program. A return to the original layout using the *View > Layout > Reset Current Layout* command will have the result that only the tool windows that are defined by default for this layout will be displayed.

Position of the tool windows

The user interface is to a large degree configurable. For this reason, tool windows can be docked, freely positioned, or integrated in document groups.

Docked tool windows

Tool windows can be docked to the left or right of the document window, or below it. To save space, several tool windows may lie on top of each other. They are then arranged as tabs. In this case, activate the required tool window by clicking the title of the corresponding tab below the window.
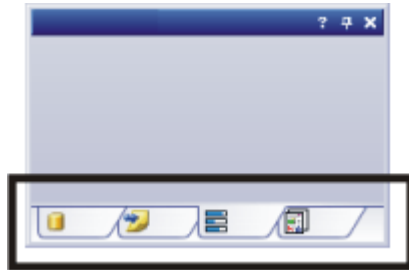
Freely positioned tool windows

You can only position tool windows as you wish when you are in the expert mode.

You can at any time float a tool window. The tool window then behaves exactly the way a dialog box does. To release a tool window from its docked position, click on its header with your left mouse button. Then, while keeping the left mouse button depressed, drag the tool window to wherever you want it.

Integrating a tool window into a document group

You can only add tool windows to a document group when you are in the expert mode.

You can integrate certain tool windows in the document group, for example, the *File Explorer* tool window. To do this, use the *Document Mode* command. To open a context menu containing this command, rightclick any tool window's header.
The tool window will then act similarly to a document window, e.g., like an image window.

Use the *Tool Window Mode* command to float a tool window back out of the document group. To open a context menu containing this command, rightclick any tool window's header.

Buttons in the header

In the header of every tool window, you will find the three buttons *Help*, *Auto Hide* and *Close*.

Click the *Help* button to open the online help for the tool window.
Click the *Auto Hide* button to minimize the tool window.
Click the *Close* button to hide the tool window. You can make it reappear at any time, for example, with the *View > Tool Windows* command.

Context menu of the header

To open a context menu, rightclick a tool window's header. The context menu can contain the *Enable Auto Hide*, *Document Mode*, and *Transparency* commands. Which commands will be shown, depends on the tool window.

Additionally, the context menu contains a list of all of the tool windows that are available. Every tool window is identified by its own icon. The icons of the currently displayed tool windows will appear clicked. You can recognize this status by the icon's background color.
Use this list to make tool windows appear.

00037

2.5. Image window views

When working with multi-dimensional images you can choose between different views of the image in the image window. Multi-dimensional images have their own navigation bar directly in the image window. Click the small arrow next to the last button on this navigation bar to open a menu with commands you can use with image window views. In it, you can select the image window view you want and also edit the settings for some views.

The button's appearance

This button is configured in such a way that you can switch backwards and forwards exactly between two different views simply by clicking it once.
Click the button to switch to the image window view that is currently shown as an icon on the button. Every image window view has its own icon.
The button always shows the image window view that was previously selected. For example, when you switch from the single frame view to the *Slice View* image window view, the button will automatically change its appearance to show the icon for the single frame view, making it possible for you to immediately switch back to that view.

Overview - Image window views

Single Frame View
By default you will find yourself in the single view. In the single frame view, only one image will be shown in the image window.

Tile View
Use the tile view to attain an overview of all of the individual images that make up a multi-dimensional image. In this view, you can also select individual images.

Slice View
Use the *Slice View* image window view, to look at any cross sections of an image series you want. The *Slice View* tool window offers you numerous possibilities for configuring this view.

Voxel View
You can display a Z-stack as a 3D object. To do so, use the *Voxel View* image window view, and the *Voxel View* tool window.

Projection Views
For image series, e.g. Z-stacks and time stacks, a single projection image can be calculated from all of the frames that is representative for the whole multi-dimensional image. The available projection images differ in the calculation algorithm. For example, if you use the maximum intensity projection you will, from all frames, only see the pixels with the highest intensity values.

EFI Projection
For Z-stacks an EFI projection is available. The EFI projection uses a series of differently focused separate images ("Focus series") to calculate a resulting image ("EFI image"), that is focused in all of its parts.

00354

2.6.   Working with documents

You can choose from a number of possibilities when you want to open, save, or close documents. As a rule, these documents will be images. In addition, your software supports some other document types. You will find a list of supported documents in the online help.

Saving documents

You should always save important documents immediately following their acquisition. You can recognize documents that have not been saved by the star icon after the document's name.

There are a number of ways in which you can save documents.

1. To save a single document, activate the document in the document group and use the *File > Save As...* command.

2. Use the *Documents* tool window.
    Select the desired document and use the *Save* command in the context menu. For the selection of documents, the standard MS-Windows conventions for multiple selection are valid.

3. Use the *Gallery* tool window.
    Select the desired document and use the *Save* command in the context menu. For the selection of documents, the standard MS-Windows conventions for multiple selection are valid.

4. Save your documents in a database. That enables you to store all manner of data that belongs together in one location. Search and filter functions make it quick and easy to locate saved documents.

Autosave and close

1. When you exit your software, all data that has not yet been saved will be listed in the *Unsaved Documents* dialog box. This gives you the chance to decide which document you still want to save.

2. With some acquisition processes, the acquired images will be automatically saved after the acquisition has finished.

3. You can also configure your software in such a way that all images are saved automatically after image acquisition. To do so, use the *Acquisition Settings > Saving* dialog box.
    Here, you can also configure your software in such a way that all images are automatically saved in a database after the image acquisition.

Closing documents

There are a number of ways in which you can close documents.

1. Use the *Documents* tool window.
    Select the desired documents and use the *Close* command in the context menu. For the selection of documents, the standard MS-Windows conventions for multiple selection are valid.

2. To close a single document, activate the document in the document group and use the *File > Close* command. Alternatively, you can click the button with the cross [**x**]. You can find this button at the top right of the document tab located next to the document name.

3. Use the *Gallery* tool window.
    Select the desired documents and use the *Close* command in the context menu. For the selection of documents, the standard MS-Windows conventions for multiple selection are valid.

Closing all documents

To close all loaded documents use the *Close All* command. You will find this command in the *File* menu, and in both the *Documents* and the *Gallery* tool window's context menu.

Closing a document immediately

To close a document immediately without a query, close it with the [Shift] key depressed. Data you have not saved will be lost.

Opening documents

There are a number of ways in which you can open or load documents.

1. Use the *File > Open...* command.

2. Use the *File Explorer* tool window.
    To load a single image, double click on the image file in the *File Explorer* tool window.
    To load several images simultaneously, select the images and with the left mouse button depressed, drag them into the document group. For the selection of images the standard MS-Windows conventions for multiple selection are valid.

3. Drag the document you want, directly out of the MS-Windows Explorer, onto your software's document group.

4. To load documents from a database into the document group, use the *Database > Load Documents...* command. You can find more information in the online help.

Generating a test image

If you want to get used to your software, then sometimes any image suffices to try out a function.
Press [Ctrl + Shift + Alt + T] to generate a color test image.
With the [Ctrl + Alt + T] shortcut, you can generate a test image that is made up of 256 gray values.

Activating documents in the document group

There are several ways to activate one of the documents that has been loaded into the document group and thus display it on your monitor.

1. Use the *Documents* tool window. Click the desired document there.

2. Use the *Gallery* tool window. Click the desired document there.

3. Click the title of the desired document in the document group.

4. To open a list with all currently loaded documents, use the [Ctrl + Tab] shortcut. Left click the document that you want to have displayed on your monitor.

5. Use the keyboard shortcut [Ctrl + F6] or [Ctrl + Shift + F6], to have the next document in the document group displayed. With this keyboard shortcut you can display all of the loaded documents one after the other.

6. In the *Window* menu, you will find a list of all of the documents that have been loaded. Select the document you want from this list.

Attaching a document to an e-mail

1. Load the documents you want to attach to your e-mail.

2. Use the *File > Send E-mail...* command.

3. Check whether all documents you want to attach are selected.

4. Click the *Send* button to generate an e-mail with the selected documents included as attachments.

   - You will receive a warning message if the sum of file sizes of all documents exceeds the maximum permitted size.

   - A new e-mail form will be opened by your e-mail program. Your e-mail program does not have to be already running for this to happen. The e-mail contains all of the selected image and document files as attachments.
     As long as the e-mail form remains open, you cannot use your software or your e-mail program. The e-mail form cannot be minimized, no can other e-mails be generated, nor can you read any incoming e-mails. You can't close the *Send E-mail* dialog box nor continue working.

5. Enter the recipient's address and your message and then send off your e-mail.
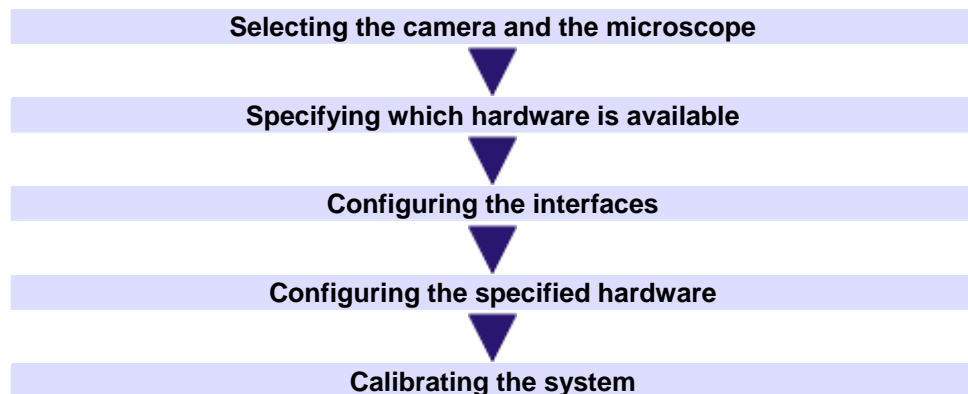
00143 02072013

3. Configuring the system

Why do you have to configure the system?

After successfully installing your software you will need to first configure your image analysis system, then calibrate it. Only when you have done this will you have made the preparations that are necessary to ensure that you will be able to acquire high quality images that are correctly calibrated. When you work with a motorized microscope, you will also need to configure the existing hardware, to enable the program to control the motorized parts of your microscope.

Process flow of the configuration

To set up your system, the following steps are necessary:

Selecting the camera and the microscope

▼

Specifying which hardware is available

▼

Configuring the interfaces

▼

Configuring the specified hardware

▼

Calibrating the system

Selecting the camera and the microscope

The first time you start your software after the installation has been made, a quick configuration with some default settings will be made. In this step you need only to specify the camera and microscope types, in the *Quick Device Setup* dialog box. The microscope will be configured with a selection of typical hardware components.

Specifying which hardware is available

Your software has to know which hardware components your microscope is equipped with. Only these hardware components can be configured and subsequently controlled by the software. In the *Acquire > Devices > Device List* dialog box, you select the hardware components that are available on your microscope.
You can find more information on this dialog box in the online help.

If you use a preset configuration that was offered in the *Quick Device Setup* dialog box, check now whether your system is really equipped with the hardware components that are defined in the configuration.

Configuring the interfaces

Use the *Acquire > Devices > Interfaces* command, to configure the interfaces between your microscope or other motorized components, and the PC on which your software runs.

If you use a preset configuration that was offered in the *Quick Device Setup* dialog box, you can skip this step.

Configuring the specified hardware

Usually various different devices, such as a camera, a microscope and/or a stage, will belong to your system. Use the *Acquire > Devices > Device Settings...* dialog box to configure the connected devices so that they can be correctly actuated by your software.

Calibrating the system

When all of the hardware components have been registered with your software and have been configured, the functioning of the system is already ensured. However, it's only really easy to work with the system and to acquire top quality images, when you have calibrated your software. The detailed information that helps you to make optimal acquisitions, will then be available.
Your software offers a wizard that will help you while you go through the

individual calibration processes. Use the *Acquire > Calibrations...* command to start the software wizard.

You can find more information on the individual dialog boxes in the online help.

About the system configuration

When do you have to configure the system?

You will only need to completely configure and calibrate your system anew when you have installed the software on your PC for the first time, and then start it. When you later change the way your microscope is equipped, you will only need to change the configuration of certain hardware components, and possibly also recalibrate them.

Switching off your operating system's hibernation mode

When you use the **MS-Windows Vista** operating system: Switch the hibernation mode off.

1. To do so, click the Start button located at the bottom left of the operating system's task bar.

2. Use the *Control Panel* command.

3. Open the *System and Maintenance > Power Options > Change when the computer sleeps* window.

   - Here, you can switch off your PC's hibernation mode.

When you use the **MS-Windows 7** operating system: Switch off your PC's power saving options and make sure that your PC does not automatically goes to hibernation mode.

1. To do so, click the Start button located at the bottom left of the operating system's task bar.

2. Click *Control Panel*, *System and Security*, and then click *Power Options*.

3. On the *Select a power plan* page, click *Change plan settings*.

4. On the *Change settings for the plan* page, click *Change advanced power settings*.

   - Here, you can switch off your PC's power saving and hibernation mode.

00159

4. Acquiring single images

4.1. Acquiring a single image

You can use your software to acquire high resolution images in a very short period of time. For your first acquisition you should carry out these instructions step for step. Then, when you later make other acquisitions, you will notice that for similar types of sample many of the settings you made for the first acquisition can be adopted without change.

1. Switch to the *Acquisition* layout. To do this, use, e.g., the *View > Layout > Acquisition* command.

   - You can find the *Microscope Control* (**1**) toolbar at the upper edge of the user interface, right below the menu bar.
     To the left of the document group, you see the *Camera Control* (**2**) tool window.
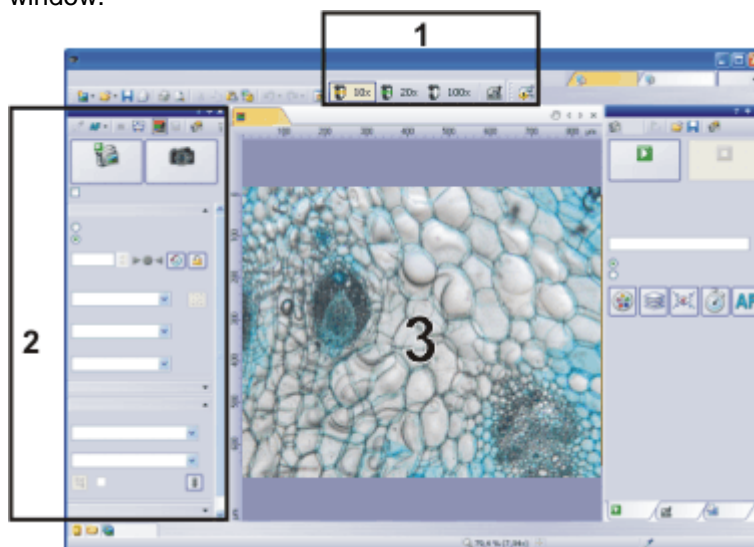
Selecting an objective

2. On the *Microscope Control* toolbar, click the button with the objective that you use for the image acquisition.

Switching on the live-image

3. In the *Camera Control* tool window, click the *Live* button.

   - You can find more information in the online help.

   - The live-image (**3**) will now be shown in the document group.

4. Go to the required specimen position in the live-image.

Setting the image quality

5. Bring the sample into focus. The *Focus Indicator* toolbar is there for you to use when you are focusing on your sample.

6. Check the color reproduction. If necessary, carry out a white balance.

7. Check the exposure time. You can either use the automatic exposure time function, or enter the exposure time manually.

8. Select the resolution you want.

Acquiring and saving an image

9. In the *Camera Control* tool window, click the *Snap* button.

   - The acquired image will be shown in the document group.

10. Use the *File > Save As...* command to save the image. Use the recommended TIF file format.

00027 18012012

4.2.   Behavior of the live window

The behavior of the live window depends on the acquisition settings in the *Acquisition Settings > Acquisition > General* dialog box.

Prerequisite   For the following step-by-step instructions, the *Keep document when live is stopped* option is selected and the *Create new document when live is started* check box is cleared.

Switching the live-image on and off without acquiring an image

1. Make the *Camera Control* tool window appear. To do this, use, e.g., the *View > Tool Windows > Camera Control* command.

2. Click the *Live* button in the *Camera Control* tool window.

    - A temporary live window named "Live (active)" will be created in the document group.

    - The live-image will be shown in the live window.

    - You can always recognize the live modus by the changed look of the *Live* button in the *Camera Control* tool window.

3. Click the *Live* button again.

    - The live mode will be switched off.

    - The active live image will be stopped.

    - The live window's header will change to "Live (stopped)". You can save the stopped live-image located in the live window just as you can every other image.

The live window may look similar to an image window, but it will be handled differently. The next time you switch on the live mode, the image will be overwritten. Additionally, it will be closed without a warning message when your software is closed.

### Switching to the live-image and acquiring an image

1. Make the *Camera Control* tool window appear. To do this, use, e.g., the *View > Tool Windows > Camera Control* command.

2. Click the *Live* button in the *Camera Control* tool window.

    - A temporary live window named "Live (active)" will be created in the document group.

    - The live-image will be shown in the live window.

    - You can always recognize the live modus by the changed look of the *Live* button in the *Camera Control* tool window.

3. Click the *Snap* button.

    - The live mode will be switched off. The live window's header will change to "Live (stopped)".

    - At the same time, a new image document will be created and displayed in the document group. You can rename and save this image. If you have not already saved it when you end your software, you will be asked if you want to do so.

Displaying the live-image and the acquired images simultaneously

Task You want to view the live-image and the acquired images simultaneously. When you do this, it should also be possible to look through the acquired images without having to end the live mode.

1. Close all open documents.

2. Open the *Acquisition Settings > Acquisition > General* dialog box.

   To do so, click, e.g., the *Acquisition Settings* button on the *Camera Control* tool window.

3. There, make the following settings:

   - Choose the *Keep document when live is stopped* option.

   - Clear the *Create new document when live is started* check box.

   - Select the *Continue live after acquisition* check box.

4. Switch to the live mode. Acquire an image, then switch the live mode off again

   - Both of the image windows "Live (stopped)" and "Image_<Serial No.>" are now in the document group.

   - The "Live (stopped)" image window is active. That's to say, right now you see the stopped live-image in the document group. In the document bar, the Name "Live (stopped)" is highlighted in color.

5. Split the document group, to have two images displayed next to each other. That's only possible when at least two images have been loaded. That's why you created two images in the first step.

6. Use the *Window > Split/Unsplit > Split/Unsplit Document Group (Left)* command.

   - This command creates a new document group to the left of the current document group. In the newly set up document group the active document will be automatically displayed. Since in this case, the active document is the stopped live-image, you will now see the live window on the left and the acquired image on the right.

7. Start the live mode.

   - In the document group, the left window will become the live window Live (active)". Here, you see the live-image.

8. Activate the document group on the right. To do so, click, for example, the image displayed there.

9. Click the *Snap* button.

   - The acquired image will be displayed in the active document group. In this case, it's the document group on the right.

   - After the image acquisition has been made, the live-image will automatically start once more, so that you'll then see the live-image again on the left.

   - While the live-image is being shown on the left, you can switch as often as you want between the images that have up till then been acquired.
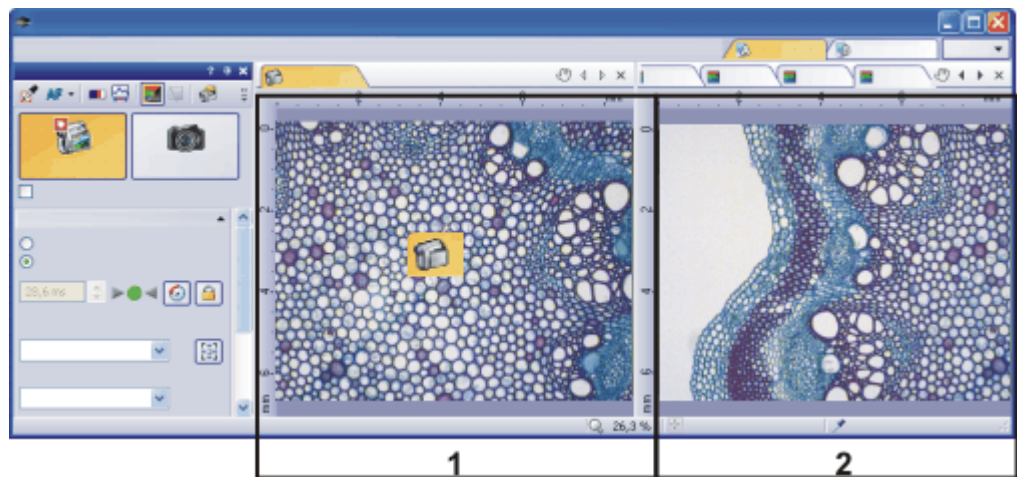
You can set up your software's user interface in such a way that you can view the live-image (1) and the images that have up till then been acquired (2), next to one another.

00181 25072011

4.3. Acquiring HDR images

HDR stands for **H**igh **D**ynamic **R**ange. Dynamic range relates to the capacity of cameras or software to display both bright and dark image segments.

Before acquiring an HDR image, the necessary exposure range needs to be determined for the current sample. The exposure range is made up of a minimum and maximum exposure time as well as several exposure times between them. A recently determined exposure range will continue to be used for all HDR images until you let your software determine the exposure range anew. It is irrelevant whether the exposure range had been determined automatically or manually.

If you are acquiring several images of the same or similar parts of a sample, you don't need to determine the exposure range each time. If you change the sample or adjust settings on the microscope, it is recommended to determine the exposure range anew (either automatically or manually).

Acquiring an HDR image with a manually determined exposure range

With this procedure, you set the minimum and maximum exposure time in the *Camera Control* tool window yourself. Your software guides you through the process with relevant message boxes. How much the exposure time is adjusted by is determined by your software with regards to the minimum and maximum exposure time.

Preparations
1. Switch to the *Acquisition* layout. To do this, use, e.g., the *View > Layout > Acquisition* command.

2. On the *Microscope Control* toolbar, click the button with the objective that you want to use for the acquisition of the HDR image.

3. Switch to the live mode, and select the optimal settings for your acquisition, in the *Camera Control* tool window. Carry out a white balance. Choose an approximate exposure time.

4. Search for the part of the sample which you want to acquire an HDR image of. This should be a position which has such significant differences in brightness that not all segments can be shown with optimal lighting.

5. Finish the live mode.

Acquiring an HDR image

6. In the *Camera Control* tool window, select the *Activate HDR* check box.

    • In the upper part of the tool window, the *Snap* button changes to the *HDR* button.

7. In the *Determine exposure range* group, click the *Manual...* button to define the exposure range for this acquisition anew.

    • The *Determine exposure range* message box appears. It prompts you to reduce the exposure time so far that enough image details can be recognized in the bright image segments and no segments are overexposed.

8. Change the exposure time in the *Exposure* group, which is part of the *Camera Control* tool window. Make sure that the *Manual* option is chosen. You can change the value by using the slide control or by entering an exposure time with the keyboard and pressing the [Enter] key. Check the live image on display. Once the bright image segments are no longer overexposed, click the *OK* button in the *Determine exposure range* message box.

    • By doing so, you have determined the lower limit of the exposure range (=the shortest exposure time).

9. Now, the *Determine exposure range* message box prompts you to raise the exposure time so high that the dark image segments are no longer underexposed. Change the exposure time in the *Exposure* group, which is part of the *Camera Control* tool window. Check the live image on display. Once the dark image segments are bright enough, click the *OK* button in the *Determine exposure range* message box.

    • By doing so, you have determined the upper limit of the exposure range (=the longest exposure time).

10. Click the *HDR* button in the *Camera Control* tool window to start the image acquisition.

    • The image acquisition will begin. Pay attention to the progress bar located in the status bar `3,1 s/5,5 s`. It shows how long the acquisition has taken and the total acquisition time. The progress bar contains the *Cancel* button, which you can use to stop the current image acquisition.

    • After the acquisition has been completed the HDR image will be shown in the document group.

11. Check the image. If you want to change the settings (to use a different algorithm for the output rendering, for example), open the *Acquisition Settings* dialog box. Select the *Acquisition > HDR* option in the tree view.

    • You can find more information on this topic in the online help.

12. If you don't want to change any settings, use the *File > Save As...* command to save the image. Use the recommended TIF or VSI file format.

    • These are the only formats which also save all the image information including the HDR entries together with the image. In this way, you can see, e.g., whether or not an image was acquired using HDR. Open the *Properties* tool window, and look at the data in the *Camera* group.

Acquiring more HDR images without setting the exposure range anew

If you have just acquired HDR images of the same or a similar sample, as a rule, it is not necessary to determine the dynamic range anew. In this case, you have already completed the preparations for acquisition (such as carrying out a white balance) and set the HDR image acquisition settings correctly (such as choosing the optimal algorithm used for output rendering) anyway.

In such circumstances, acquiring an HDR image is especially easy. Do the following:

1. In the *Camera Control* tool window, select the *Activate HDR* check box.

2. Click the *HDR* button in the *Camera Control* tool window to start the image acquisition.

    - The image acquisition will begin. After the acquisition has been completed the HDR image will be shown in the document group.

3. Check the image before saving it.

    - This step can be left out if your software is configured to import images into a database directly after acquisition.

07500 27122011

5. Acquiring multi-dimensional images

What is a multi-dimensional image?

You can combine a series of individual images into one image. You can, e.g., assemble separate images that belong to different color channels. Depending on how the frames differ, the multi-dimensional image that results from their combination will also vary.

A standard image is two dimensional. The position of every pixel will be determined by its X- and Y-values. Fluorescence color, time and Z-position of the microscope stage are the possible additional dimensions of a multi-dimensional image.

A multi-channel image as a rule, shows a sample that has been marked with several different fluorochromes. The multi-channel image is made up of a combination of the individual fluorescence images.

In a time stack all frames have been acquired at different points of time. A time stack shows you how an area of a sample changes with time. You can play back a time stack just as you do a movie.

A Z-stack contains frames acquired at different focus positions. You need a Z-stack, for instance, for the calculation of an EFI image.

Image containing several dimensions

The different multi-dimensional images can be arbitrarily combined. A multi-channel time stack, for instance, incorporates several color channels. Every color channel incorporated in the image is reproduced with its own time stack.
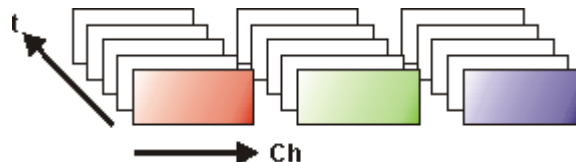
Navigation bar in the image window

The multi-dimensional images have their own navigation bar directly in the image window. Use this navigation bar to define how a multi-dimensional image is to be displayed in the image window.

00009

5.1. Overview - Acquisition processes

Your software offers a wide range of different acquisition processes.

Note: Your software is available in a variety of versions. This online help contains the description of all acquisition processes. For this reason, it can happen that your software version doesn't have some of the acquisition processes described here.

Basic acquisition processes

Complex acquisition processes

Basic acquisition processes

Use the *Camera Control* tool window to acquire images and movies.

Acquisition process - Snapshot

You can use your software to acquire high resolution images in a very short period of time.

Acquisition process - Movie

You can use your software to record a movie. When you do this, your camera will acquire as many images as it can within an arbitrary period of time. The movie will be saved as a file in the AVI format. You can use your software to play it back.

Complex acquisition processes

Use the *Process Manager* tool window to handle complex acquisition processes.

Acquisition process - Time Lapse

With the automatic acquisition process *Time Lapse* you acquire a series of frames one after the other. This series of individual images makes up a time stack. A time stack shows you how an area of a sample changes with time. You can play back a time stack just as you do a movie.

You can combine the *Time Lapse* acquisition process with other acquisition processes. If you software supports the *Multi Channel* acquisition process, use, e.g., the *Time Lapse* acquisition process to acquire a multi-channel time stack.

AF If your microscope stage is equipped with a motorized Z-drive, when you acquire a time stack you can use the autofocus. You'll find a description of the individual settings along with the description of the acquisition process.

Acquisition process - Z-Stack

Use the automatic acquisition process *Z-Stack* to acquire a Z-stack. A Z-stack contains frames acquired at different focus positions. That is to say, the microscope stage was located in a different Z-position for the acquisition of each frame.

Alternatively, you can also acquire an EFI image with the *Z-Stack* acquisition process. Then a resulting image (EFI image) with a practically unlimited depth of focus is automatically calculated from the Z-stack that has been acquired. Such an image is focused throughout all of its segments. EFI is the abbreviation for Extended Focal Imaging.

You can combine the *Z-Stack* acquisition process with other acquisition processes.
If you software supports the *Multi Channel* acquisition process, you can combine the *Z-Stack* acquisition process with the multi channel acquisition to acquire a multi-channel Z-stack.

Acquisition process - XY-Positions / MIA

You can only use this acquisition process when your microscope is equipped with a motorized XY-stage. With this acquisition process you can carry out one or more automatic acquisition processes at different positions on the sample or acquire a stitched image of a larger sample position.

AF If your microscope stage is equipped with a motorized Z-drive, you can use the autofocus for this acquisition process. You'll find a description of the individual settings along with the description of the acquisition process.

*Acquisition process -
Multi Channel*

With the automatic acquisition process *Multi Channel* you acquire a multi-channel fluorescence image.

You can combine, e.g., the *Multi Channel* acquisition process with the *Z-stack* acquisition process to acquire a multi-channel Z-stack.

Note: When you use the DP80 camera, please note the following restriction. When you acquire a transmitted light image simultaneously with a multi-channel fluorescence image, the *Multi Channel* acquisition process can't be combined with another acquisition process, for example the *Z-stack* acquisition process. This restriction protects the camera from being damaged by permanently toggling between the two CCDs the camera provides.

*Acquisition process -
Instant EFI*

Use the manual acquisition process *Instant EFI* to acquire an EFI image at the camera's current position, that is sharply focused all over.

*Acquisition process -
Manual MIA*

When you use the *Manual MIA* acquisition process, you move the stage manually in such a way that different, adjoining sample areas are shown. With this acquisition process, you combine all of the images that are acquired, directly during the acquisition, just like a puzzle, into a stitched image. The stitched image will display a large sample segment in a higher X/Y-resolution than would be possible with a single acquisition.

Combination of several acquisition processes

You can combine several automatic acquisition processes. To do so, click the corresponding button for each acquisition process you require.

Note: Which automatic acquisition processes you can combine with each other, depends on your software.

The order of the automatic acquisition processes in the *Process Manager* tool window (from left to right) corresponds to the order in which the acquisition processes were carried out.

Examples

When you combine the two acquisition processes *Multi Channel* and *Z-Stack*, a complete multi-channel image will be acquired at every focus position. The *Multi Channel* acquisition process will then be carried out first. Only after this has been done, will the stage's Z-position be changed, and another multi-channel image acquired.
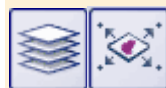
When you combine the two acquisition processes *Z-Stack* and *XY-Positions / MIA* to acquire a Z-stack at several positions on your sample, to begin with, the complete Z-stack at the first position will be acquired. When that has been done, your system will move to the next position, and will acquire the next Z-stack etc..

00442 13012011

25

6. Acquiring image series

With your software you can acquire time stacks, movies, and Z-stacks.

6.1. Acquiring time stacks
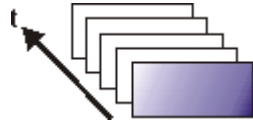
What is a time stack?

You can combine a series of individual images into one image. In a time stack all frames have been acquired at different points of time. A time stack shows you how an area of a sample changes with time. You can play back a time stack just as you do a movie.

A standard image is two dimensional. The position of every pixel will be determined by its X- and Y-values. With a time stack, the time when the image was acquired is an additional piece of information or "dimension" for each frame.

The frames making up a time stack can be 8-bit gray-value images, 16-bit gray-value images or 24-bit true-color images.

Note: A time stack can also be an AVI video. You can load and play back the AVI file format with your software.

How do I recognize a time stack?

You can immediately recognize the different image types by the icon which appears in front of the image name in the document group, or in the *Documents* tool window. When it is a time stack, this icon will be supplemented by a small clock. A time stack that is made up of true-color images has, e.g., this icon .

In the *Properties* tool window, you can use the *Frame Count* entry to find out how many individual images are contained in any given image.

A time stack will automatically have its own navigation bar directly in the image window. Use this navigation bar to browse through the frames making up a time stack, or to play back the time stack like a movie.
You can find more information on the navigation bar in the online help.

Creating time stacks

There are different ways in which you can generate a time stack.

To acquire a time stack, use one of the two acquisition processes *Time Lapse* or *Movie*.
Use the *Image > Combine Frames…* command, to have several individual images combined into a time stack. A description of the *Combine Frames* dialog box can be found in the online help.

Displaying time stacks

A time stack contains much more data than can be displayed on your monitor.

A time stack will automatically have its own navigation bar directly in the image window. Use this navigation bar to determine which of the frames from a time stack is to be displayed on your monitor. You can also play back a time stack just as you would a movie.
You can find more information on the navigation bar in the online help.

Alternatively, you can also use the *Dimension Selector* tool window to determine how a time stack is to be displayed on your monitor, or to change this.

Hiding the navigation bar

You can also hide the navigation bar. To do this, use the *Tools > Options...* command. Select the *Images > General* entry in the tree view. Clear the *Show image navigation toolbar* check box.

Saving time stacks

When you save time stacks, you will, as a rule, use the VSI file format. Only when you use this file format is there no limit to the size a time stack can be. When you save smaller time stacks, you can also use the TIF or the AVI file format. With any other file format you will lose most of the image information during saving. To do so, use the *File > Save As...* command.

Converting time stacks

Use the *Image > Separate > Time Frames* menu command to have a time stack broken down into selected individual images.

It is possible that, within a time stack, only a short period of time interests you. Use the *Extract* command to create a new time stack that only contains a selection of frames, from an existing time stack. In this way, you will reduce the number of frames within a time stack to only those that interest you. You will find this command in the context menu in the tile view for time stacks. You can find more information on this command in the online help.

When you save a time stack in another file format as TIF or VSI, the time stack will also be converted. The time stack will then be turned into a standard true-color image. This image shows the frame that is at that moment displayed on the monitor.

Processing time stacks

Image processing operations, e.g., a sharpen filter, affect either the whole image or only a selection of individual images. You will find most of the image processing operations in the *Process* menu. You can find more information on working with image processing functions in the online help.

The dialog box that is opened when you use an image processing operation is made up in the same way for every operation. In this dialog box, select the *Apply on > Selected frames and channels* option to determine that the function only affects the selected frames.

Select the *Apply on > All frames and channels* option, to process all of the individual images.

Select the individual images that you want to process, in the tile view. You can find more information on this image window view in the online help. Look through the thumbnails and select the images you want to process. In the tile view, the standard MS-Windows conventions for multiple selection are valid.

An image processing operation does not change the source image's dimensions. The resulting image is, therefore, comprised of the same number of separate images as the source image.

00011

Time Lapse / Movie

Both the "Time Lapse" and the "Movie" acquisition processes document the way a sample changes with time. What is the difference between the two processes?

When is it better for me to acquire a time stack?

Use the "Time Lapse" acquisition process in the following cases:

- Use the "Time Lapse" acquisition process when processes that run slowly are to be documented, e.g., where an acquisition is to be made only every 15 minutes.
- Use the "Time Lapse" acquisition process when, while the acquisition is in progress, you want to see the frames that have already been acquired, for example, to check on how an experiment is progressing. To do this, click the *Tile View* ▦ button in the navigation bar in the image window.
- Use the "Time Lapse" acquisition process when you want to use those of your software's additional functions that can only be saved in the VSI or TIF file format.
  For example, to measure objects, to insert drawing elements such as arrows, or a text, or to have the acquisition parameters for the camera and microscope that you've used, available at any time in the future.

- Use the "Time Lapse" acquisition process when the important thing is to achieve an optimal image quality, and the size of the file is no problem.

Saving a time stack as an AVI

You can also save a time stack as an AVI file, at a later date. To do this, load the time stack into the document group, select the *File > Save as...* command, and select the AVI file type. Make, if necessary, additional settings in the *Select AVI Save Options* dialog box.

When is it better for me to acquire a movie?

Use the "Movie" acquisition process in the following cases:

- Use the "Movie" acquisition process when processes that run very quickly are to be documented (the number of acquisitions per second is considerably higher with movies than with time stacks).
- Use the "Movie" acquisition process when you want to give the movie to third persons who do not have this software (AVI files can also be played back with the MS Media Player).
- Use the "Movie" acquisition process when keeping file sizes small is of great importance.

00107

Recording a movie

You can use your software to record a movie. When you do this, your camera will acquire as many images as it can within an arbitrary period of time. The movie will be saved as a file in the AVI format. You can use your software to play it back.

1. Switch to the "Acquisition" layout. To do this, use, e.g., the *View > Layout > Acquisition* command.

Selecting an objective

2. On the *Microscope Control* toolbar, click the button with the objective that you want to use for the movie acquisition.

Selecting the storage location

3. In the *Camera Control* tool window's toolbar, click the *Acquisition Settings* button.

- The *Acquisition Settings* dialog box opens.

4. Select the *Saving > Movie* entry in the tree structure.

5. You have to decide how a movie is to be saved after the acquisition. Select the *Filesystem* entry in the *Automatic save > Destination* list to automatically save the movies you have acquired.

- The *Path* field located in the *Directory* group shows the directory that will currently be used when your movies are automatically saved.

6. Click the [...] button next to the *Path* field to alter the directory.

- The AVI file format is preset in the *File type* list. This is a fixed setting that cannot be changed.

Selecting the compression method

7. Click the *Options...* button when you want to compress the AVI file in order to reduce the movie's file size.

8. From the *Compression* list, select the *M-JPEG* entry and confirm with *OK*.

> Please note: Compressing the movie is only possible if the selected compression method (codec) has already been installed on your PC. If the compression method has not been installed the AVI file will be saved uncompressed.
> The selected compression method must also be available on the PC that is used for playing back the AVI. Otherwise the quality of the AVI may be considerably worse when the AVI is played back.

9. Close the *Acquisition Settings* dialog box with *OK*.

Setting the image quality

10. Switch to the live mode, and select the optimal settings for movie recording in the *Camera Control* tool window. Pay special attention to setting the correct exposure time.

    - This exposure time will not be changed during the movie recording.

11. Find the segment of the sample that interests you and focus on it.

Switching to the "Movie recording" mode

12. Select the *Movie recording* check box (**1**). The check box can be found below the *Live* button in the *Camera Control* tool window.

    - The *Snap* button will be replaced by the *Movie* button.
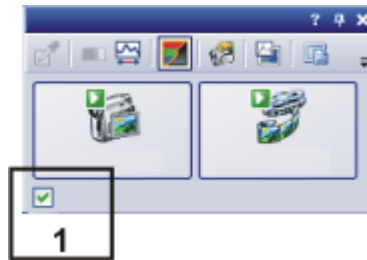
Starting movie recording

13. Click the *Movie* button to start the movie recording.

    - The live-image will be shown and the recording of the movie will start immediately.

    - In the status bar a progress indicator is displayed. At the left of the slash the number of already acquired images will be indicated. At the right of the slash an estimation of the maximum possible number of images will be shown. This number depends on your camera's image size and cannot exceed 2GB.

    - This icon on the *Movie* button indicates that a movie is being recorded at the moment.

Stopping movie recording

14. Click the *Movie* button again to end the movie recording.

    - The first image of the movie will be displayed.

    - The navigation bar for time stacks will be shown in the document group. Use this navigation bar to play the movie.

    - The software will remain in the "Movie recording" mode until you clear the *Movie recording* check box once more.

Acquiring a time stack

In a time stack all frames have been acquired at different points of time. With a time stack you can document the way the position on the sample changes with time. To begin with, for the acquisition of a time stack make the same settings in the *Camera Control* tool window as you do for the acquisition of a snapshot. Additionally, in the *Process Manager* tool window, you have to define the time sequence in which the images are to be acquired.

Task

You want to acquire a time stack over a period of 10 seconds. One image is to be acquired every second.

1. Switch to the "Acquisition" layout. To do this, use, e.g., the *View > Layout > Acquisition* command.

Selecting an objective

2. On the *Microscope Control* toolbar, click the button with the objective that you want to use for the image acquisition.

Setting the image quality

3. Switch to the live mode, and select the optimal settings for your acquisition, in the *Camera Control* tool window. Pay special attention to setting the

correct exposure time. This exposure time will be used for all of the frames in the time stack.

4. Choose the resolution you want for the time stack's frames, from the *Resolution > Snap/Process* list.

5. Find the segment of the sample that interests you and focus on it.

6. Activate the *Process Manager* tool window.

7. Select the *Automatic Processes* option.

8. Click the *Time Lapse* button.

   - The button will appear clicked. You can recognize this status by the button's colored background.

   - The [*t*] group will be automatically displayed in the tool window.

9. Should another acquisition process be active, e.g., *Z-Stack*, click the button to switch off the acquisition process.

   - The group with the various acquisition processes should now look like this:

10. Clear the check boxes *Start delay* and *As fast as possible*.

11. Specify the time that the complete acquisition is to take, e.g., 10 seconds. Enter the value "00000:00:10" (for 10 seconds) in the *Recording time* field. You can directly edit every number in the field. To do so, simply click in front of the number you want to edit.

12. Select the radio button on the right-hand side of the field to specify that the acquisition time is no longer to be changed. The 🔒 lock icon will automatically appear beside the selected radio button.

13. Specify how many frames are to be acquired. Enter e.g., 10 in the *Cycles* field.

    - The *Interval* field will be updated. It shows you the time that will elapse between two consecutive frames.

14. Click the *Start* button.

    - The acquisition of the time stack will start immediately.

    - The *Start Process* button changes into the *Pause* button. A click on this button will interrupt the acquisition process.

    - The *Stop* button will become active. A click on this button will stop the acquisition process. The images of the time stack acquired until this moment will be preserved.

    - At the bottom left, in the status bar, the progress bar will appear. It informs you about the number of images that are still to be acquired.

    - The acquisition has been completed when you can once more see the *Start* button in the *Process Manager* tool window, and the progress bar has been faded out.

    - You will see the time stack you've acquired in the image window. Use the navigation bar located in the image window to view the time stack. You can find more information on the navigation bar in the online help.

- The time stack that has been acquired will be automatically saved. The storage directory is shown in the *Acquisition Settings > Saving > Process Manager* dialog box. The preset file format is VSI.

Note: When other programs are running on your PC, for instance a virus scanning program, it can interfere with the performance when a time stack is being acquired.

00304 12012011

6.2. Acquiring a Z-stack
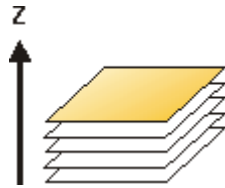
What is a Z-stack?

You can combine a series of separate images into one image file. A Z-stack contains frames acquired at different focus positions. A Z-stack is needed, e.g., for calculating an EFI image by the *Process > Enhancement > EFI Processing...* command.

A standard image is two dimensional. The position of every pixel will be determined by its X- and Y-values. With a Z-stack, the focus position or the height of the sample is an additional item of information for every pixel.

The frames making up a Z-stack can be 8-bit gray-value images, 16-bit-gray-value images or 24-bit-true-color images.

How do I recognize a Z-stack?

You can immediately recognize a multi-dimensional image by its icon which appears in front of the image name in the document group or in the *Documents* tool window. When it is a Z-stack, this icon will be supplemented by a small Z.

In the *Properties* tool window, you can use the *Frame Count* entry to find out how many individual images are contained in any given image.

A Z-stack image will automatically have its own navigation bar directly in the image window. Use this navigation bar to browse through the frames making up a Z-stack, or to play back the Z-stack like a movie. You can find more information on the navigation bar in the online help.

Creating a Z-stack

There are different ways in which you can generate a Z-stack.

- To acquire a Z-stack, use the "Z-Stack" acquisition process.
- Use the *Image > Combine Frames…* command to have several separate images combined into a Z-stack.

Displaying a Z-stack

A Z-stack contains much more data than can be displayed on your monitor.

A Z-stack image will automatically have its own navigation bar directly in the image window. Use this navigation bar to determine which of the frames from a Z-stack is to be displayed on your monitor. You can also play back the Z-stack just as you would a movie. A detailed description of the navigation bar can be found in the online help.

Alternatively, you can also use the *Dimension Selector* tool window to define how a Z-stack image is to be displayed on your monitor, or to change this.

Saving a Z-stack

Please note: Z-stacks can only be saved in the TIF or VSI file format. Otherwise they loose a great deal of their image information during saving.

Converting a Z-stack

Use the *Image > Separate > Z-Slices* menu command, to have a Z-stack broken down into selected frames.

It is possible that, within a Z-stack, only a short Z-range interests you. Use the *Extract* command to create a new Z-stack that only contains selected frames, from an existing Z-stack. In this way, you will reduce the number of frames within a Z-stack to only those that interest you. You will find this command in the context menu in the tile view for Z-stacks.

If you save a Z-stack in another file format as TIF or VSI, the Z-stack will automatically be converted. The Z-stack will then be turned into a standard true-color image. This image shows the frame that is at that moment displayed on the monitor.

Processing a Z-stack

Image processing operations, e.g., a sharpen filter, affect either the whole image or only a selection of individual images. You will find most of the image processing operations in the *Process* menu. You can find more information on working with image processing functions in the online help.

The dialog box that is opened when you use an image processing operation is made up in the same way for every operation. In this dialog box, select the *Apply on > Selected frames and channels* option to determine that the function only affects the selected frames.

Select the individual images that you want to process, in the tile view. Click, e.g., this button ▦ in the image window's navigation bar to switch to the tile view. Look through the thumbnails and select the images you want to process. In the tile view, the standard MS-Windows conventions for multiple selection are valid.

Select the *Apply on > All frames and channels* option to process all of the individual images.

An image processing operation does not change the source image's dimensions. The resulting image is, therefore, comprised of the same number of separate images as the source image.

00012

Acquiring a Z-stack

A Z-stack contains frames acquired at different focus positions. That is to say, the microscope stage was located in a different Z-position for the acquisition of each frame.

Note: You can only use the *Z-Stack* acquisition process when your stage is equipped with a motorized Z-drive.

Example: You want to acquire a Z-stack. The sample is approximately 50 µm thick. The Z-distance between two frames is to be 2 µm.
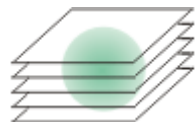
1. Switch to the *Acquisition* layout. To do this, use, e.g., the *View > Layout > Acquisition* command.

Selecting an objective

2. On the *Microscope Control* toolbar, click the button with the objective that you want to use for the image acquisition.

Setting the image quality

3. Switch to the live mode, and select the optimal settings for your acquisition, in the *Camera Control* tool window. Pay special attention to setting the correct exposure time. This exposure time will be used for all of the frames in the Z-stack.

4. Search out the required position in the sample.

Selecting the acquisition process

5. Activate the *Process Manager* tool window.

6. Select the *Automatic Processes* option.

7. Click the *Z-Stack* button.

   - The button will appear clicked. You can recognize this status by the button's colored background.

   - The [*Z*] group will be automatically displayed in the tool window.

8. Should another acquisition process be active, e.g., *Multi Channel*, click the button to switch off the acquisition process.

9. Select the *Range* entry in the *Define* list (**1**).

10. Enter the Z-range you want, in the *Range* field (**2**). In this example, enter a little more than the sample's thickness (= 50 µm), e.g., the value 60.

11. In the *Step Size* (**3**) field, enter the required Z-distance, e.g., the value 2, for a Z-distance of 2 µm. The value should roughly correspond to your objective's depth of focus.

    - In the *Z-Slices* field (**4**) you will then be shown how many frames are to be acquired. In this example 31 frames will be acquired.

12. Find the segment of the sample that interests you and focus on it. To do this, use the arrow buttons (**5**). The buttons with a double arrow move the stage in larger steps.

Selecting the acquisition parameters
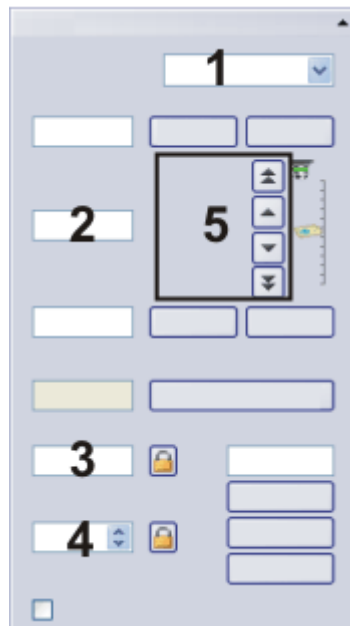
Set the acquisition parameters for the acquisition of a Z-stack in the *Process Manager* tool window. Use the fields and buttons (**1**-**4**) for this.

Acquiring an image

13. Click the *Start* button.

    - Your software now moves the Z-drive of the microscope stage to the start position. The starting positions lies half of the Z-range deeper than the stage's current Z-position.

    - The acquisition of the Z-stack will begin as soon as the starting position has been reached. The microscope stage moves upwards step by step and acquires an image at each new Z-position.

- The acquisition has been completed when you can once more see the

  Start ▶ button in the *Process Manager* tool window, and the progress bar has been faded out.

- You can see the acquired Z-stack in the image window. Use the navigation bar located in the image window to view the Z-stack. You can find more information on the navigation bar in the online help.

- The Z-stack that has been acquired will be automatically saved. You can set the storage directory in the *Acquisition Settings > Saving > Process/Experiment* dialog box. The preset file format is VSI.

Note: When other programs are running in the background on your PC, for instance a virus scanning program, it can interfere with the performance when a Z-stack is being acquired.

00367 10072013

7. Acquiring fluorescence images

7.1. Multi-channel image

What is a multi-channel image?

A multi-channel image combines a series of monochrome images into one image. The multi-channel image usually shows a sample that has been stained with several different fluorochromes. The multi-channel image is made up of a combination of the individual fluorescence images. You can have the individual fluorescence images displayed separately or also as a superimposition of all of the fluorescence images.
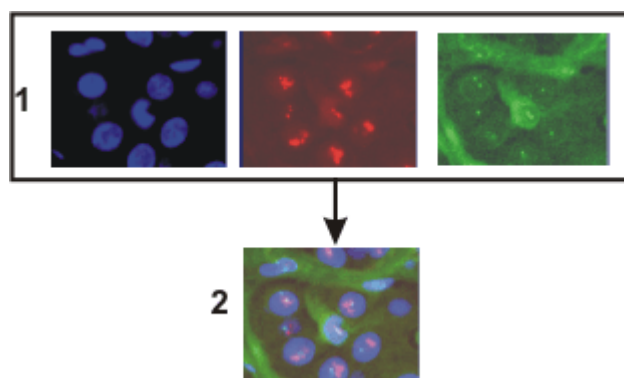
At the top of the illustration you can see the individual fluorescence images (1). Below, you can see the superimposition (2) of the separate fluorescence images.

The separate images' image type

The separate images making up a multi-channel image can be 8-bit gray-value images or 16-bit gray-value images.

Combination with a times stack or Z-stack

A multi-channel image can be combined with a time stack or a Z-stack. A multi-channel time stack, for instance, then incorporates several color channels. Every color channel incorporated in the image is reproduced with its own time stack.

How do I recognize a multi-channel image?

You can immediately recognize a multi-channel image by this icon 🔳 which appears in front of the image name in the document group or in the *Documents* tool window.

In the *Properties* tool window, you can use the *Channels* entry to find out how many channels are contained in any given image.

A multi-channel image will automatically have its own navigation bar, directly in the image window. Use this navigation bar to set how a multi-channel image is to be displayed in the image window, or to change this.

You can find more information on the navigation bar for multi-channel images in the online help.

Creating multi-channel images

Your software offers you several ways of acquiring a multi-channel image.

Use the *Multi Channel* automatic acquisition process to acquire a multi-channel image.
Use the *Image > Combine Channels…* command to have several separate images combined into a multi-channel image.

Displaying multi-channel images

A multi-channel image contains much more data than can be displayed on your monitor.

Using the navigation bar
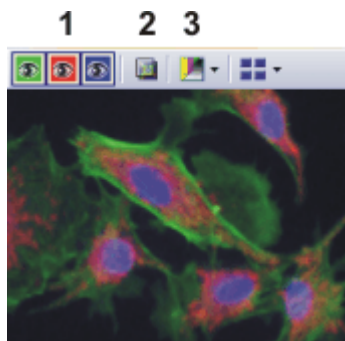
When you load a multi-channel image into your software, you'll see a navigation bar in the image window, that provides you with access to all of the fluorescence channels.

- You can have the individual fluorescence images displayed separately or also as a superimposition of all of the fluorescence images (1).
- Should you have acquired a brightfield of the sample together with the fluorescence images, you can make this brightfield appear or disappear (2).
- The individual fluorescence images are monochrome. For this reason, you can change the color mapping however you like. You can display the fluorescence channels in the fluorescence colors, use a pseudo color table of your choice, or also display the original images (3).

Hiding the navigation bar

You can also hide the navigation bar. To do this, use the *Tools > Options...* command. Select the *Images > General* entry in the tree view. Clear the *Show image navigation toolbar* check box.

Using the "Dimension Selector" tool window

Alternatively, you can also use the *Dimension Selector* tool window to stipulate how a multi-channel image is to be displayed on your monitor, or to change this. There you can, for example, change the fluorescence colors for individual color channels.

Saving multi-channel images

Please note: Multi-channel images can only be saved in the TIF or VSI file format. Otherwise they loose a great deal of their image information during saving.

Converting multi-channel images

Breaking down a multi-channel image into its color channels

Use the *Separate* command to have a multi-channel image broken down into chosen color channels. The resulting images are still of the "multi-channel" type, contain though, only one color channel.

There are several ways of accessing this command:

- Click the *Separate Channels* button in the *Dimension Selector* tool window.
- Use the *Separate* command from the *Dimension Selector* tool window's context menu.
- Use the *Image > Separate > Channels* menu command.

Reducing the color channels in a multi-channel image

Use the *Extract* command to create a new multi-channel image that is made up of fewer color channels than the source image.

Select all of the color channels you wish to retain, in the *Dimension Selector* tool window. Then use the *Extract* command in the tool window's context menu.

Converting multi-channel images while saving them

When you save a multi-channel image in another file format as TIF or VSI, the multi-channel image will also be converted. The multi-channel image then becomes a standard 24-bit true-color image. This image will always show exactly what is currently displayed on your monitor, that is to say, e.g., the superimposition of all of the channels or possibly only one channel.

Processing multi-channel images

Image processing operations, e.g., a sharpen filter, affect either the whole image or only a selection of individual images. You will find most of the image processing operations in the *Process* menu. You can find more information on working with image processing functions in the online help.

Select the frames that you want to process in the *Dimension Selector* tool window. The channels you have selected will be highlighted in color in the tool window.

The dialog box that is opened when you use an image processing operation is made up in the same way for every operation. In this dialog box, select the *Apply on > Selected frames and channels* option to determine that the function only affects the selected frames.

Select the *Apply on > All frames and channels* option, to process all of the individual images.

An image processing operation does not change the source image's dimensions. The resulting image is, therefore, comprised of the same number of separate images as the source image.
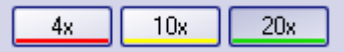
00010

7.2. Before and after you've acquired a fluorescence image

Before you acquire a fluorescence image

Defining observation methods

Define observation methods for your color channels.

Setting up the microscope for the acquisition of a fluorescence image

1. Use the *View > Tool Windows > Microscope Control* command to make the *Microscope Control* tool window appear.

   - In the *Objectives* group, you will find the buttons you use to change objectives.

     [4x] [10x] [20x]

   - In the *Observation method* group you will find a button for every observation method that has been defined. Observation methods should have been defined at least for brightfield and for every color channel.

2. Click the required objective's button.

3. Click the button for the observation method with the excitation that has the longest excitation wavelength (e.g., Red) .

Setting up the camera for the acquisition of a fluorescence image

1. Use the *View > Tool Windows > Camera Control* command to make the *Camera Control* tool window appear.

2. Set the image resolution for the acquisition. With a high objective magnification, you require a lower resolution.
   For this purpose, select the required resolution from the *Snap/Process* list, located in the *Resolution* group.

3. Reduce the image resolution in the live mode. When you use a higher binning in the live mode, the frame rate will be reduced, which enables you to focus better.
   For this purpose, select an entry, e.g., with the supplement *Binning 2x2*, from the *Live/movie* list, located in the *Resolution* group.

4. Should you work with a color camera: Switch on your camera's grayscale mode. The *Toggle RGB/Grayscale mode* button should now look like this

   . You can find this button on the *Camera Control* tool window's toolbar.

5. If it's possible to set different bit depths with your camera, click the *Toggle Bitdepth* button to set the maximum bit depth.

Switching off the corrections for brightfield acquisitions

1. Use the *View > Toolbars > Calibrations* command to have the *Calibrations* toolbar displayed.

2. Switch off the white balance and the shading correction.

   To do that, release these buttons , if they are there and available.

Focusing a fluorescence sample

1. Select the automatic exposure time.

2. In the *Camera Control* tool window, click the *Live* button.

   - Should the live-image be too dark, select a higher value in the *Camera Control > Exposure > Exposure compensation* list.

   - Should the exposure time become longer than 300 ms, reduce it by increasing the sensitivity or gain.

3. Bring the sample into focus.

   - In the camera's black & white mode, you can reduce the diffused light.

     Click the *Online-Deblur* button, located in the *Camera Control* tool window's toolbar. You can then decide whether or not you want to apply the deconvolution filter. It's possible that you might have to lengthen the exposure time via the exposure time correction.

4. Finish the live mode. To do so, click the *Live* button in the *Camera Control* tool window.

Setting the storage location

Multi-channel images will be saved by default, as soon as the acquisition has been completed. As file format, the VSI file format will be used.

1. Before you start the acquisition, specify where the file is to be saved.

2. To do this, click the *Acquisition Settings* button, located in the *Process Manager* tool window's toolbar. Select the *Saving > Process Manager* entry in the tree view.

   - You will find the current directory in the *Directory > Path* field.

3. Click the [...] button next to the *Path* field, to change the directory into which the image is to be saved after its acquisition.

After you've acquired a fluorescence image

Viewing a multi-channel image

A multi-channel image is made up of the individual fluorescence images. You can set which color channels, resp. combination of color channels, will be displayed on your monitor. To do this, use the navigation bar in the image window.

Click a color field to make the channel appear or disappear. All of the color channels that are at the moment displayed on your monitor will be identified by an eye icon.

The navigation bar also offers you additional possibilities for changing the appearance of the multi-channel image. You can find more information on the navigation bar in the online help.

"Properties" tool window

Numerous acquisition parameters will be saved together with the image.

Use the *View > Tool Windows > Properties* command to make the *Properties* tool window appear. In the *Properties* tool window, you will find that every color channel has its own *Channel* information group. This contains the channel name, the emission wavelength, the name of the observation method and the exposure time.

Saving multi-channel images

The multi-channel image will be automatically saved. You can set the storage directory in the *Acquisition Settings > Saving > Process Manager* dialog box. The file format used is VSI.

For the VSI format, a JPEG compression of 90% is preset. You can change the compression in the *Acquisition Settings* dialog box under *Saving > Process Manager > Automatic save > Options…* .

00363

7.3. Defining observation methods for the fluorescence acquisition

Before you acquire a fluorescence image, you have to define observation methods for your color channels. Usually, observation methods that you can adapt for your microscope configuration, have already been predefined.

Prerequisite   The system has already been configured and calibrated. For this purpose, you have to enter your hardware components in the *Acquire > Devices > Device List* dialog box, and configure them in the *Device Settings* dialog box. To finalize this action, calibrate your system by using the *Acquire > Calibrations...* command.

The tables that follow, contain example configurations for both motorized, and not motorized microscopes. Only the hardware components that are relevant for the acquisition of multi-channel fluorescence images are listed.

Device List		Example entries	
		Non-motorized microscope	Motorized microscope
Microscope			
Frame	<Name of your microscope>	BX51	BX61
Nosepiece	<Name of your nosepiece>	Manual Nosepiece	Motorized (UCB)
Mirror turret	<Name of your mirror turret>	Manual mirror turret	BX-RFAA
Stage			
Z-axis	<Name of your controller for the stage's Z-drive>	Not Motorized	Motorized (UCB)
Fluorescence/reflected light path			
Shutter	<Name of the reflected light shutter>	Manual Shutter	Motorized (UCB)
Transmission light path			
Lamp	<Name of your transmission lamp>	Not used	UCB Halogen-Lamp
Condenser	<Name of your condenser>	Manual Condenser	U-UCD8A

Device Settings	Entries
Nosepiece	<Your objectives>
Mirror turret	U-MNU, U-MWB, U-MWG For a position where there is no mirror cube, select the *Free* entry.
Condenser	The hardware components *Aperture Stop* and *Top Lens* are additionally listed under the device settings.

Defining the observation method for transmission brightfield

Example: Hardware components in transmission brightfield: In transmission brightfield, there is no fluorescence mirror cube in the light path. The reflected light shutter is closed.

1. Use the *Acquire > Devices > Device Customization...* command. Activate the *Observation Methods* tab.

2. Click the *New Observation Method* ☀ button.

   - A dialog box, in which you can enter a name, will be opened.

3. Give the new observation method a name, then close the dialog box with *OK*. You could name your observation method, e.g., MyBF.

Setting up the mirror turret

4. Select the mirror turret in the *Available components* list.

   - In the central area of the dialog box, the settings for the hardware components that have been selected, will be displayed.

5. In transmission brightfield, the mirror turret isn't allowed to contain a mirror cube. Therefore, select the *Adjust* entry in the *Status* list, located in the middle of the dialog box, and in the list that's below that one select the *Free* entry.

   - For manual microscopes: Where a manual microscope is concerned, the mirror turret can't be automatically moved to the position you want. For this reason, when you use a manual microscope, you'll receive a message that asks the user to make this setting manually.
   This message will also appear when you are defining the observation method. Confirm the message with *OK*.

For motorized microscopes: Setting up the condenser

6. Select the condenser.
   Select the *Adjust* entry in the Status list, located in the middle of the dialog box, and in the list that's below that one, the *Free* entry.

7. Select the hardware component *Aperture Stop*.
   Select the *Adjust* entry in the *Status* list, located in the middle of the dialog box. Enter "75%" in the field below that.

8. Select the hardware component *Top Lens*.
   Select the *Adjust per objective* entry in the *Status* list.

   - In the middle of the *Device Customization* dialog box, you can now set the top lens for each objective separately.

   - The top lens is only used for objectives with higher magnifications (upwards of 10x) and is swung out for lower magnifications.

9. Specify for which objectives the top lens should be brought into the light path and for which objectives it should be removed from the light path.
   To do so, select the *Use with this objective* check box for all objectives.
   In the *Selected components* list, each objective with a lower magnification than 10x needs to show the *Out* status. If that isn't the case, click in the middle of the dialog box on the *Out* button.
   In the *Selected components* list, each objective with a higher magnification than 10x or exactly 10x needs to show the *In* status. If that isn't the case, click in the middle of the dialog box on the *In* button.

For motorized microscopes: Switching on the transmission lamp

10. Select the transmission lamp. You will find this lamp in the *Available components* list, under the *Transmission* entry.

11. Select the *Adjust* entry in the *Status* list, located in the middle of the dialog box. Set 9 V for the lamp.
    Use the button showing a small lamp to switch on the lamp.

- The button looks like this, if the lamp is switched on.

- In the *Selected components* list, you can also see that the lamp for this observation method will be switched on.

Saving the observation method

12. Click the *OK* button, to save the new observation method.

- The *Microscope Control* tool window will then contain a new button with this observation method's name.

- You can now use the observation method in the *Process Manager* tool window, for the acquisition of a multi-channel fluorescence image.

Defining observation methods for fluorescence channels

Task

Define an observation method for the acquisition of a fluorescence image. It also makes sense to do this when you don't work with a motorized microscope, since then the acquired image will be automatically colored with the correct fluorescence color.

Summary

The following hardware components belong to an observation method for fluorescence channels. How you integrate these hardware components with the observation method, is described in detail in these step-by-step instructions.

Hardware component	Settings
Fluorochromes	Assign the fluorescence colors.
Mirror turret	Choose the mirror cube to be used.
Fluorescence shutter	Open the fluorescence shutter for the image acquisition.
Transmitted light lamp	Switch off the transmitted light lamp.

1. Use the *Acquire > Devices > Device Customization...* command. Activate the *Observation Methods* tab.

2. Click the *New Observation Method* button.

3. Give the new observation method a name, then close the dialog box with *OK*. Name the observation method, e.g., Blue.

Defining the fluorochrome

4. Assign the fluorescence channel a fluorochrome (e.g., Blue or DAPI) and a color (e.g., Blue at 470 nm). To do so, select the *Fluorochromes* entry in the *Available components* list.
Select the *Use* entry in the *Status* list.
In the *Fluorochrome* list located below that one, select the fluorochrome to be used, e.g., the entry *Blue* or *DAPI*.
You can change the fluorescence color in the *Color* list, should that be necessary.

- It is important in all cases to define the fluorochrome for a fluorescence observation method, even if you don't automatically change any device settings. When the fluorescence color is linked to the observation method, every image you acquire with this observation method will be automatically colored in the corresponding color. This is valid irrespective of whether you work with a manual or a motorized microscope.

⚘ For motorized microscopes: Setting up the mirror turret

It can make sense to use this setting and the additional settings for motorized microscopes also for manual microscopes. When you use a manual microscope, you'll receive a message that prompts you, to make this setting manually. As well as this, the device settings are saved together with the acquired image.

5. Select the mirror turret in the *Available components* list.

   • In the central area of the dialog box, the settings for the hardware components that have been selected, will be displayed.

6. For the acquisition of a fluorescence image a specific mirror cube has to be used. Therefore, select the *Adjust* entry in the *Status* list, located in the middle of the dialog box, and in the list that's below that one, select the mirror cube you want.

✾ For motorized microscopes: Setting the shutter for the fluorescence light path

7. In the *Available components* list, select the shutter that is located below the *Fluorescence/reflected* entry.

8. When the fluorescence acquisition is made, this shutter must be open. Therefore, select the *Use for acquisition* entry in the *Status* list, located in the middle of the dialog box.

   • Then the shutter will be opened before the image acquisition is made, and closed when this has been done, to avoid bleaching of the sample.

💡 For motorized microscopes: Switching off the transmission lamp

9. Select the transmission lamp. You will find this lamp in the *Available components* list, under the *Transmission* entry.

10. For the acquisition of a fluorescence image, the lamp must be switched off. Select the *Adjust* entry in the *Status* list, located in the middle of the dialog box.
    Use the button showing a small lamp to switch off the lamp.

   • The button looks like this, if the lamp is switched off.

   [💡]

   • In the *Selected components* list, you can also see that the lamp for this observation method will be switched off.

📷 Including camera settings

It's usually better to use a black & white camera for acquiring fluorescence images. Should you use a camera that can be toggled between a color mode and a grayscale mode, you can integrate the grayscale mode into the observation mode.

This setting is not necessary if you acquire fluorescence images with the *Multi Channel* acquisition process. Before the *Multi Channel* acquisition process starts, your software checks whether or not your camera is working in the gray scale mode. You will then receive a corresponding message, and can reset the camera before the image acquisition is made.

11. Select your camera in the *Available components* list.

12. Select the *Use* entry in the *Status* list.

13. Some cameras offer gray scale modes in different bit depths. Select the gray scale mode with the highest bit depth from the *Image type* list.

Saving the observation method

14. Click the *OK* button, to save the new observation method.

   • The *Microscope Control* tool window will then contain a new button with this observation method's name.

   • You can now use this color channel for the *Multi Channel* acquisition process.

Using predefined observation methods

As a rule you don't have to completely redefine the observation method required. Use one of the predefined observation methods, and customize it for your microscope.

1. Use the *Acquire > Devices > Device Customization...* command. Activate the *Observation Methods* tab.

    - In the *Name* list, you will find all of the observation methods that have been predefined.
      Should no observation methods be available, click the *Select Predefined Observation Methods* button. Click the *Select All* button. Click *OK*.

    - As soon as filter cubes have been entered for the mirror turret, in your device settings, the appropriate observation methods will be automatically set up. You will always find the observation method beneath the filter cube's name.

2. Select a fluorescence channel (e.g., U-MNU) in the *Name* list.

3. Click the *Rename Observation Method* abl button.

    - The *Enter a New Observation Method Name* dialog box opens.

4. Enter a more general name (e.g., Blue or DAPI), then click *OK*.

    - The *Selected components* list contains the following hardware components. There can be more or fewer components, depending on what you have chosen in the way of hardware components in the device list.

    - In the mirror turret, the corresponding mirror cube (e.g., U-MNU) will be selected.

    - The transmission lamp will be switched off.

    - The transmission light path's shutter will have the *Use for acquisition* status. This means that it will be open when the image is acquired.

5. Assign a fluorochrome (such as Blue or DAPI) to the fluorescence channel.

6. Click the *OK* button, to save the new observation method.

    - The *Microscope Control* tool window will then contain a new button with this observation method's name.

00370 29122011

7.4.   Acquiring a fluorescence image

You software supports several image types. The multi-channel image usually shows a sample that has been stained with several different fluorochromes. However, it is also possible to acquire a multi-channel image that consists only of one single channel.
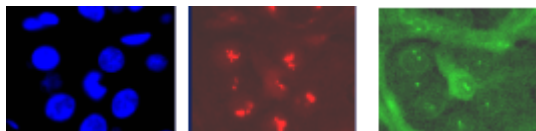
The illustration shows three fluorescence images of the same sample position. Each image shows another fluorochrome.

Switching to a dark user interface

1. If you are disturbed by the light of your monitor, you can switch your software to a dark user interface. To do so, use the *View > Dark Application Skin* command.

Selecting the fluorescence observation method

2. Use the *View > Toolbars > Observations Methods* command to have the *Observation Methods* toolbar displayed.

3. To load an observation method, click the button with the name of the fluorescence observation method you want.

    *   For manual microscopes: Loading a fluorescence observation method defines that a fluorescence image is to be acquired. For your software, all observation methods using the *Fluorochromes* component are automatically identified as fluorescence observation methods.

    *   For motorized microscopes: When you load an observation method, this leads to the microscope being brought into a defined condition. To do so, all of the microscope's motorized components will be brought into exactly the position that has been defined for these components in the observation method.

Selecting the exposure time for the acquisition

4. Use the *View > Tool Windows > Camera Control* command to make the *Camera Control* tool window appear.

5. Switch to the live mode. To do so, click the *Live* button in the *Camera Control* tool window.

    *   For motorized microscopes: The reflected light shutter will be automatically opened.
        This behavior will be specified when the observation method is defined.
        For the shutter, the *Use for acquisition* status should have been selected.

6. In the *Camera Control* tool window, select the *Exposure > Manual* option.

7. Some cameras offer the *SFL* mode for fluorescence acquisitions (e.g., the DP72). Switch this mode on.

8. Optimize the exposure time.
    Should the exposure time become longer than 500 ms, reduce it by increasing gain.
    To do this, change the value in the *Exposure > Sensitivity* field or use the *Gain* slide control.

Focusing a fluorescence sample

9. Bring the image into focus.
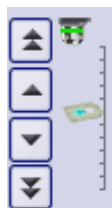
If your microscope stage is equipped with a motorized Z-drive, a focus regulator will be at your disposal in the *Microscope Control* tool window.

10. Finish the live mode. To do so, click the *Live* button in the *Camera Control* tool window.

- For motorized microscopes: The reflected light shutter will be closed.

- In the image window, you will now see the fluorescence image that has been acquired. The fluorescence image has the image type "Multi-channel image" even if it consists only of one single channel.

 You can immediately recognize a multi-channel image by this icon ▌ which appears in front of the image name in the document group or in the *Documents* tool window.

- The fluorescence image will be displayed using the fluorescence color that you have defined together with the observation method.

Changing the fluorescence image's display

11. You can use the *Dimension Selector* tool window, to define how the fluorescence image is to be displayed on your monitor, or to change this. There you can, for example, change the fluorescence color.

Saving the fluorescence image

12. Use the *File > Save As...* command afterwards to save the new multi-channel image. Use the TIF file format.

00395 12032012

7.5.   Combine Channels

The *Image > Combine Channels...* command creates a new multi-channel image from several separate images. A description of this dialog box can be found in the online help.

Which images can you combine?

Gray-value images

You can combine a series of gray-value images into a multi-channel image. These can be either 8-bit gray-value images or 16-bit gray-value images. The prerequisite therefore, is that all of the separate images have the same bit depth, image size and image calibration.

Multi-channel images

Multi-channel images don't necessarily have to be made up of several color channels. There can also be multi-channel images that only contain one fluorescence channel. You can also combine these images into a new multi-channel image that then contains several fluorescence channels. The prerequisite therefore, is that all of the separate images have the same bit depth, image size and image calibration.

Multi-dimensional images

You can combine several multi-dimensional images into one image. Prerequisite for such an operation is that all of the individual images only differ in one dimension (color channel, focus position, or time point), and are of the same image size.

One example of this is two single color time stacks, that are each made up of 50 separate images. Each time stack was acquired with a different color channel. In this case you can create one multi-channel time stack.

Transmission image

Sometimes another image that shows the same position on the sample in the transmitted light mode, belongs to a series of fluorescence images. You can combine such a transmission image with the multi-channel image.

The transmission image doesn't have to be of the same image type as the separate images. However, the image size, image calibration, and the bit depth have to be the same as the fluorescence images.

For example: You can use a true-color image as a transmission image. When the individual fluorescence images have a bit depth of 16 bit, you can only use a 48-bit true-color image as a transmission image.

Combining fluorescence images

1. Load the gray-value images that you want to combine into a multi channel fluorescence image. The sample was, for example, marked with the fluorochromes, DAPI and Texas Red.

2. Activate the first image in your software's document group.

Combine Channels

3. Use the *Image > Combine Channels...* command.

   - The *Combine Channels* dialog box will open.

   - In the *Available Images* table, the active image will be automatically entered as the first color channel.

   - When you have acquired the individual fluorescence images with a suitable observation method, the name of the color channel and the fluorescence color will be read out of the image and automatically used in the *Combine Channels* dialog box.

4. In case you have to change the channel name or want to do so: Click once in the *Name* cell. Enter a description of the color channel, e.g., the name of the fluorochrome used "Texas Red".
   You can increase the width of the row so that the description will fit into it.

5. When the fluorescence color can't be read out of the image, the first channel will, by default, be assigned the color 'Red'. To change the active color, click this color field. Select one of the colors from the palette on the *Standard* tab, or activate the *Custom* tab to define a color of your choice.
   Click the *OK* button, to close the color palette and return to the *Combine Channels* dialog box.

6. In the next free row, click the *Images* cell. You will be presented with a picklist containing all of the images that you can combine with the active image. Select your next image. As soon as you click in another row, the new image will be included in the sheet.

7. You can now change the characteristics of the new channel. Give the new channel a name and assign a color.

8. It is possible to shift the individual images with respect to each other. To do this, if necessary, use the arrow buttons in the *Pixel Shift* group.

9. You can set the weighting of the individual color channels. Increase, e.g., the value in the *Intensity* field, to weight a channel more strongly.

10. Click the *OK* button to create your multi-channel image.
    A new image document with the default name "Image_<serial No.>" is created.
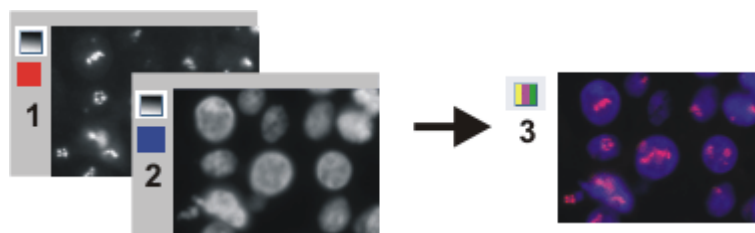
> With the *Combine Channels* command, you combine fluorescence images (1) and (2) into a multi-channel image (3), this can be done with more than two images also.
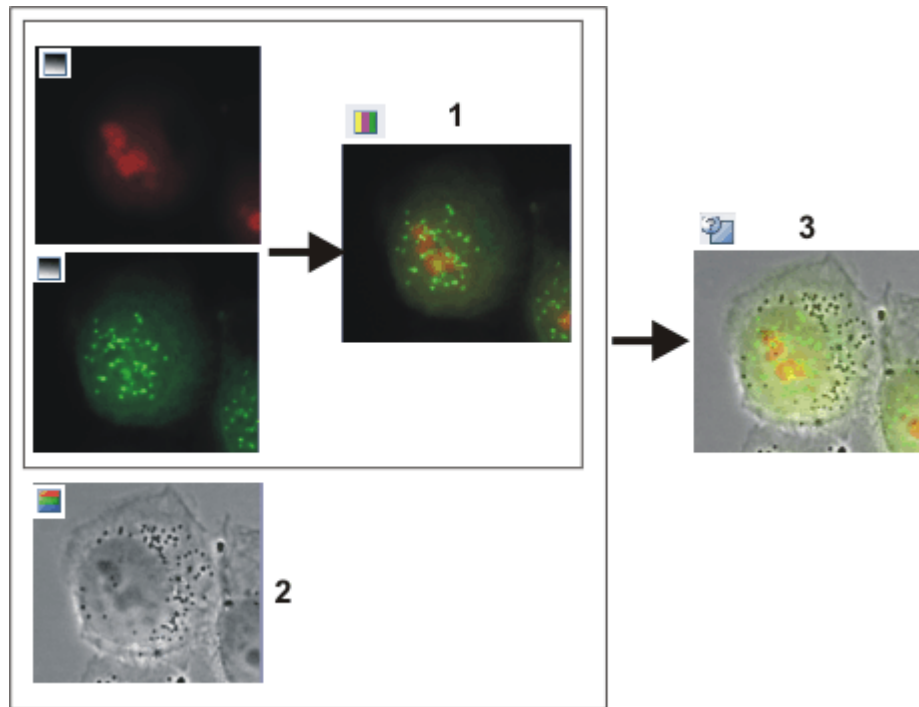
Saving a multi-channel image

11. Use the *File > Save as...* command to save your new multi-channel image. Always use the TIF or VSI file format when saving an image.

Viewing a multi-channel image

12. Use the *Dimension Selector* tool window, to change the fluorescence color, to choose another color mapping, or to switch individual color channels off and back on.

13. Use the *Adjust Display* tool window, to change the display of a multi-channel image on your monitor. You can e.g., change the weighting of individual color channels in relation to one another.

Combining fluorescence images with a transmission image

1. Load one or more fluorescence images and the transmission image that you want to lay over the color channels.

2. Activate the transmission image in your software's document group.

3. Use the *Image > Combine Channels...* command.

   - The *Combine Channels* dialog box will open.

   - If you want to use a true-color image as a transmission image, that image will be automatically selected in the *Transmission* list.
     When the transmission image is a gray-value image, it will be automatically entered in the *Available Images* table.

4. If necessary, choose the transmission image in the *Transmission* list.

5. Click once in the *Images* cell. You get a picklist containing all of the loaded images that you can combine with the selected transmission image. Select the fluorescence image you want.

6. If necessary, change the new fluorescence channel's properties. Give the channel a name and assign it a color.

7. Click the *OK* button, to create the resulting image.
   A new image document with the default name "Image_<serial No.>" is created.

   - In the document group, you can then see a superimposition of all of the images you've combined.

   - The resulting image is a multi-layer image with two image layers. Normally, the two image layers are not of the same image type. For this reason, the image has this icon ▣ in the document group.

With the *Combine Channels* command, you combine several fluorescence images into a multi-channel image (1). If you've acquired the transmission image at the same place on the sample (2), you can combine it with the multi-channel image to make a multi-layer image (3).

Viewing a multi-layer image

-
  The navigation bar will be displayed at the top of the image window. You can find a button for showing and hiding the transmission image next to the button for the individual color channels. The eye icon indicates that the transmission image is currently visible.

8. Click this button in the navigation bar, to hide the transmission image.

   - Now, you will only see the multi-channel fluorescence image.

9. Click this button and show the transmission image again.

   - Now, you see a superimposition of the transmission image and the multi-channel fluorescence image.

Moving the transmission image with respect to the multi-channel image

10. Use the *View > Tool Windows > Layers* command to make the *Layers* tool window appear.

11. In the *Layers* tool window, click the [+] sign (1) and open the image's layers.
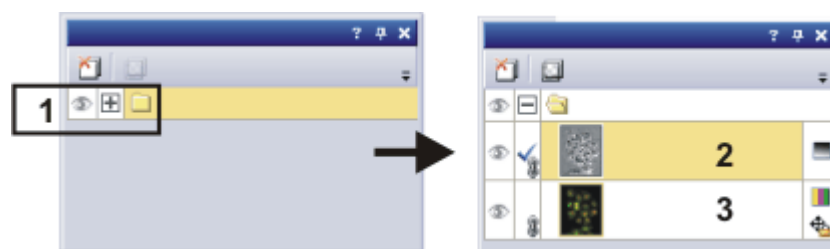
- You can now see the image's individual layers: transmission image (2) and multi-channel image (3). The height map can't be seen, because it's absolutely transparent at the moment. The icon at the left side of the

multi-channel image means that it is not possible to move the multi-channel image.

12. Select the transmission image in the *Layers* tool window.

13. Activate the *Toolbox* toolbar. To do this, use, e.g., the *View > Toolbars > Toolbox* command.

14. Click the *Move Tool* ⬥ button on the *Toolbox* toolbar.

    • On the image window, the mouse pointer will change its shape.

15. Move the whole image with the left mouse button depressed.

16. Click e.g., the *Selection Tool* ⬥ button on the *Toolbox* toolbar, to leave the move mode.

Changing the weighting between a transmission image and a multi-channel image

When you display the transmission image and the multi-channel image simultaneously in the image window, the transmission image will cover the multi-channel image, and for this reason, you can't see the multi-channel image. You can display both images transparently, and in that way be able to see parts of both images.

17. To start with, select the image layer in the *Layers* tool window. To do so, simply click the layer's name.

    • The layer you have selected will then be shown with a colored background in the tool window.

18. Then click the *Set Layer Opacity* ⬚ button. You can find this button in the tool window's toolbar.

    • In the tool window a slide control will then appear, with which you can set the degree of transparency.

    | 100% | – ———⌐ + |
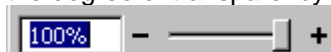
19. Use the slide control to set the degree of transparency you want. At a value of 100% the image layer is opaque. At a value of 0% the image layer will be completely faded out.

20. When you're satisfied with the transparency setting, click once on any place on the user interface.

Saving a multi-layer image

21. Use the *File > Save as...* command, to save your new multi-layer image. Always use the TIF or VSI file format when saving an image.

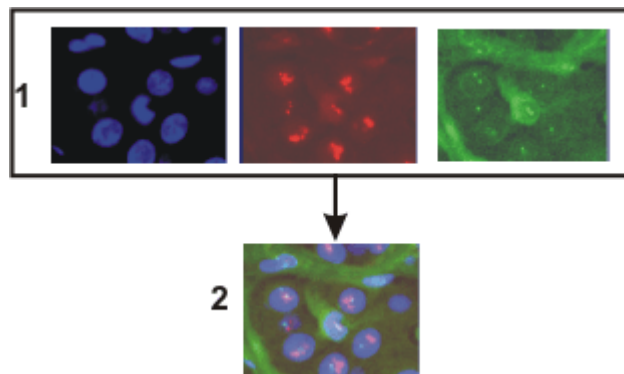00067 25072011

7.6. Acquiring multi-channel fluorescence images

Use the *Multi Channel* automatic acquisition process to acquire a multi-channel fluorescence image.

Example: Define a process for acquiring a multi-channel fluorescence image. When you set up the fluorescence sample, illuminate it as little as possible to minimize the bleaching effect.

At the top of the illustration you can see the individual fluorescence images (**1**). Below, you can see the superimposition (**2**) of the separate fluorescence images.

Defining the "Multi Channel" acquisition process

Selecting the acquisition process

1. Use the *View > Tool Windows > Process Manager* command to make the *Process Manager* tool window appear.

2. Select the *Automatic Processes* option.

3. Click the *Multi Channel* button.

   • The button will appear clicked. You can recognize this status by the button's colored background.

   • The [*C*] group will be automatically displayed in the tool window.

4. Should another acquisition process be active, e.g., *Z-Stack*, click the button to switch off the acquisition process.

   • The group with the various acquisition processes should, for example, now look like this:

Adding color channels

5. Click the *Add Channel* button (**1**).
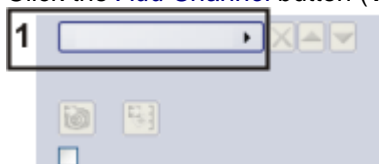
   • A context menu will open.
     The context menu will list all of the observation methods that are currently defined.

6. Select the color channel that is to be acquired first, e.g., Red.

7. Select the other channels (e.g., Green and Blue) in the same manner. Note that the fluorescence images are later on acquired in the same order as they are listed there.

Selecting the exposure times for the individual color channels

8. Use the *View > Tool Windows > Camera Control* command to make the *Camera Control* tool window appear.

9. Click the small plus sign (**2**) next to the first color channel.
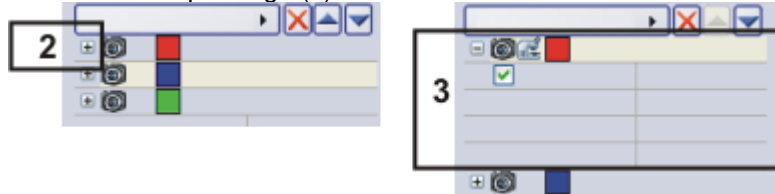
- The channel has now been activated (**3**). The active color channel will be shown highlighted in color in the tool window.

- The color channel entries in the *Process Manager* tool window are organized like a tree view. Expand an entry to open a list with additional information about the selected color channel.

- When you activate the color channel, you also automatically select the corresponding observation method. You can recognize which observation method is active, by the microscope icon. At the same time, this means that the microscope is now set up correctly for the acquisition of the fluorescence image for the first color channel.

10. Switch to the live mode. To do so, click the *Live* button in the *Camera Control* tool window.

- The reflected light shutter will be automatically opened.
  This behavior will be specified when the observation method is defined.
  For the shutter, the *Use for acquisition* status should have been selected.

11. In the *Camera Control* tool window, select the *Exposure > Manual* option.

12. Some cameras offer the *SFL* mode for fluorescence acquisitions (e.g., the DP72). Switch this mode on.

13. Optimize the exposure time.
  Should the exposure time become longer than 500 ms, reduce it by increasing gain.
  To do this, change the value in the *Exposure > Sensitivity* field or use the *Gain* slide control.

14. In the *Process Manager* tool window, click the *Read settings* button.

- This exposure time will be automatically adopted for the active channel, and will be shown in the *Process Manager* tool window (**4**).

15. Then activate the other channels and set the exposure time for each of them.

16. Finish the live mode. To do so, click the *Live* button in the *Camera Control* tool window.

- The reflected light shutter will be closed.

Focusing a fluorescence sample

17. Activate the first channel.
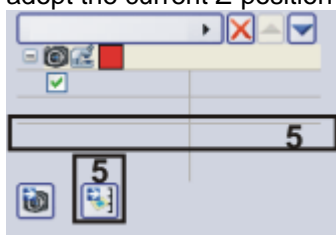
18. Switch to the live mode.

19. Bring the image into focus.

 If your microscope stage is equipped with a motorized Z-drive, a focus regulator will be at your disposal in the *Microscope Control* tool window.

20. Click the *Read Z-offset* button in the *Process Manager* tool window to adopt the current Z-position of your microscope stage (**5**).

21. Activate the other channels, focus the sample and transfer the current Z-position of the microscope stage to the acquisition process.

   - The first color channel is always used as a reference for the Z-offset. Below the other color channels you can find the *Z-offset* value. It shows how the focus positions of the individual color channels differ from each other.

22. Select the *Use Z-offset* check box (**6**).
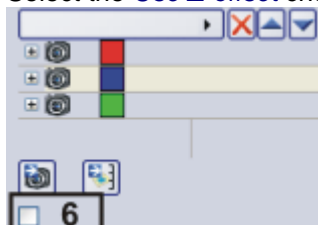
Finishing the process definition

23. Finish the live mode.

   - The reflected light shutter will be closed.

24. Click the *Save Process Definition* button in the toolbar at the top of the *Process Manager* tool window to save the acquisition parameters for the process that has just been defined.

   - A channel contains an observation method, an exposure time, a gain value, and where necessary, a focus position. All of these settings will be saved together with the process definition.

   - You can now use the acquisition parameter for this acquisition process again, at any time.

Acquiring and viewing a multi-channel fluorescence image

Defining the acquisition process

1. Define a process for the acquisition of a multi-channel fluorescence image, or load acquisition parameters that have already been saved. To do this, click the *Load Process Definition* 📂 button, located in the *Process Manager* tool window's toolbar.

Switching to a dark user interface

2. If you are disturbed by the light of your monitor, you can switch your software to a dark user interface. To do so, use the *View > Dark Application Skin* command.

Starting the acquisition process

3. In the *Process Manager* tool window, click the *Start* ▶ button.

   - If you use a manual microscope, you'll receive several different messages about switching the mirror cube and opening and closing the shutter.

4. For microscopes that aren't motorized: Follow the instructions and make the necessary settings on your microscope.

   - The acquisition of the multi-channel fluorescence image starts immediately. The order of the color channels in the *Process Manager* tool window corresponds to the order in which the color channels were acquired.

   - The acquisition has been completed when you can again see the *Start* button in the *Process Manager* tool window.

   - The multi-channel fluorescence image will be automatically saved. You can set the storage directory in the *Acquisition Settings > Saving > Process Manager* dialog box. The preset file format is VSI.

   - In the image window, the superimposed fluorescence image of all channels is displayed.

Viewing a multi-channel image

   - The navigation bar will be displayed at the top of the image window. It contains a button for each channel to enable you to display or hide that channel. The eye icon indicates that the channel is currently visible.

5. Click the color channel button in the navigation bar to have a color channel displayed or hidden. Take a look at all of the color channels one by one.

6. When you've finished, superimpose all of the channels again.

7. Click the *Tile View* ▦ button located in the navigation bar to change the image window view.

- You will then see all of the color channels that have been acquired.
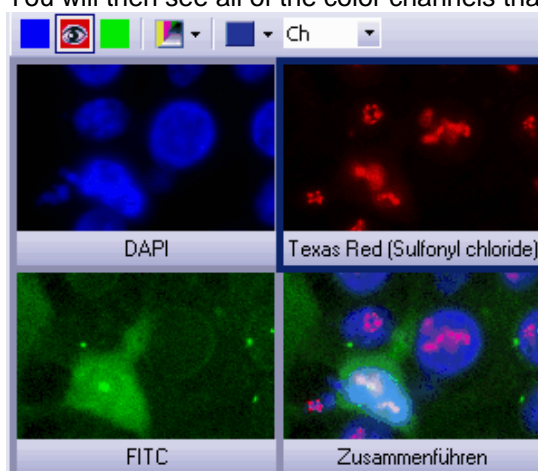
8. Compare the color channels.

9. Click the *Single Frame View* ■ button on the navigation bar.

   - You will then once more see the superimposition of all of the color channels in the image window.

Viewing information on the individual color channels

10. Use the *View > Tool Windows > Properties* command to make the *Properties* tool window appear.

   - In the *Properties* tool window, you will find that every color channel has its own *Channel* information group.

11. Should an information group has been reduced: Click the plus sign ⊞ to have all of the information displayed again.

   - The color channel's name, the corresponding wavelength, the observation method, and the exposure time will all be shown for each color channel.

Acquiring a multi-channel fluorescence image together with a transmitted light image

Task

Simultaneously with the multi-channel fluorescence image, acquire a transmitted light image, e.g., a phase contrast image.

Defining the acquisition process

1. Define an acquisition process for a multi-channel fluorescence image. To do this, follow the step-by-step instructions described above.

Adding the acquisition of a transmitted light image to the acquisition process

2. Click the *Add Channel* button. Choose an observation method for the acquisition of a transmitted light image, e.g., phase contrast, differential interference contrast (DIC), or brightfield.

3. Click on the transmitted light channel in the *Process Manager* tool window.

   - The channel has now been activated. Your microscope will be set in the transmitted light mode.

4. Click the small plus sign next to the transmitted light channel.

   - You'll now see a table with additional information about the transmitted light channel.

5. Make sure that the *Transmission overlay* check box has been selected. Only then is the transmitted light image assigned its own layer that lies over the fluorescence channels.

Defining the transmitted light image acquisition

6. Switch to the live mode.

7. Select manual exposure time in the *Camera Control* tool window. In order to set the sensitivity to the lowest ISO value, set the gain to the value of 0. Optimize the exposure time.

8. In the *Process Manager* tool window, click the *Read settings* ![icon] button.

    *   The exposure time will be adopted for the channel.

9. Finish the live mode.

Starting the acquisition process

10. In the *Process Manager* tool window, click the *Start* ![icon] button.

    *   Then, together with your fluorescence images, a transmitted light image will also be acquired and saved together with the multi-channel fluorescence image. The result of this acquisition process is a multi-layer image that you can view with the *Layers* tool window.

11. Take a look at the multi-channel fluorescence image with the superimposed transmitted light image in the image window.

Showing and hiding the transmitted light image in the image window

    *   ![icons]
        The navigation bar will be displayed at the top of the image window. You can find a button for showing and hiding the transmission image next to the button for the individual color channels. The eye icon indicates that the transmission image is currently visible.

12. Click this button ![icon] in the navigation bar, to hide the transmission image.

    *   Now, you will only see the multi-channel fluorescence image.

13. Click this button ![icon] and show the transmission image again.

    *   Now, you see a superimposition of the transmission image and the multi-channel fluorescence image.

Moving the transmission image with respect to the multi-channel image

14. Use the *View > Tool Windows > Layers* command to make the *Layers* tool window appear.

15. In the *Layers* tool window, click the [+] sign (**1**) and open the image's layers.
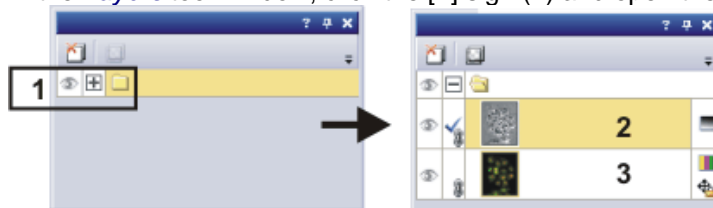
    *   You can now see the image's individual layers: transmission image (**2**) and multi-channel image (**3**). The icon ![icon] at the left side of the multi-channel image means that it is not possible to move the multi-channel image.

16. Select the transmission image in the *Layers* tool window.

17. Activate the *Toolbox* toolbar. To do this, use, e.g., the *View > Toolbars > Toolbox* command.

18. Click the *Move Tool* ![icon] button on the *Toolbox* toolbar.

    *   The mouse pointer will change its shape when you move it on the image window.

19. Move the whole image with the left mouse button depressed.

20. Click e.g., the *Selection Tool* ![icon] button on the *Toolbox* toolbar, to leave the move mode.

00369

8. Creating stitched images

Acquiring a stitched image without a motorized XY-stage (Manual MIA)

Example: You want to acquire an image of a large sample area. Use the *Manual MIA* acquisition process to acquire several individual images of adjoining positions on the sample, and to have them combined into a stitched image.

Prerequisite

The camera is aligned parallel to the XY-stage. The angle between camera and stage should be smaller than 1°.

Selecting an objective

1. On the *Microscope Control* toolbar, click the button with the objective that you want to use for the acquisition of the stitched image.

Setting the image quality

1. Switch to the live mode, and select the optimal settings for your acquisition, in the *Camera Control* tool window. Pay special attention to setting the correct exposure time. This exposure time will be used for all of the stitched image's individual images.

2. Find the position on the sample at which you want to start acquiring the stitched image.

3. Finish the live mode.

Selecting the acquisition process

1. Activate the *Process Manager* tool window.

2. Select the *Manual Processes* option.

3. Click the *Manual MIA* button.

   - The button will appear clicked. You can recognize this status by the button's colored background.

   - The *Manual MIA* group will be automatically displayed in the tool window.

   - Should the *Instant EFI* acquisition process have been active, it will be automatically switched off. You can, however, use images with extended depth of focus for the stitched image. To do this, before you acquire each of the individual images, click the *Instant EFI* button located in the *Manual MIA* group.

Selecting the acquisition parameters

1. Make quite certain that the *Auto Align* button appears clicked. It should then look like this:
   .

   - Then, your software will search for the same image structures in neighboring individual images. The stitched image will be put together in such a way that image areas that are the same will be superimposed.

Acquiring a stitched image

1. Click the *Start* button.

   - Your software switches into the live mode.

2. Bring the sample into focus.

3. Click on one of the arrow buttons to set the side of the current image at which the next image is to be arranged. For example, click this button when the next image is to be laid to the right of the current image.

- Your system now acquires an image at the current position on the sample. In the image window you now see on the left (1) the acquired image, and on the right (2) the live-image is displayed.
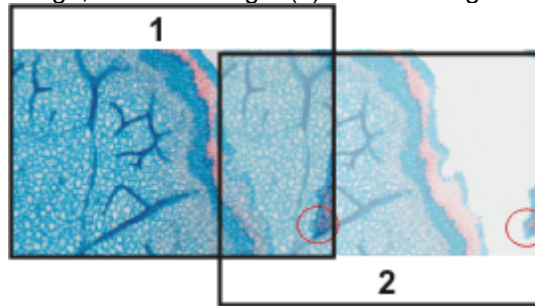
Since you haven't moved the sample, the live-image still shows the current sample position, too, which means that you now see the current image twice.
The two images overlap. Since the live-image is shown transparent, you see both images in the overlap area simultaneously.

4. Make a note of a significant structure on the live-image's right border. You will find the same sample structure in the overlap area. On the illustration, a significant structure has been indicated by a circle.

5. Now move the stage very slowly to make the structure on the live-image move to the left. Keep moving the stage until the image structures in the overlap area lie as exactly over each other as possible. The image structures need not lie precisely over each other, since your software will match the individual images with each other.

   - In the overlap area (3), the same image segments are shown now. This enables your software to seamlessly combine the two images.
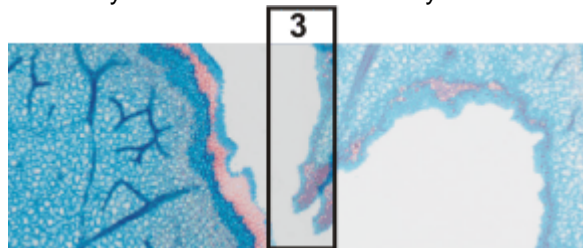
   - You can reverse the direction in which your stage moves, in the *Device Settings > Stage* dialog box. Depending on how you can best orient yourself, the live-image will then move to the left or to the right, when you move your stage to the right.

6. Check whether both images have been correctly combined. Otherwise, you can undo the last step by using the *Undo last frame* button. You can then move the stage again, and match the structures better.

   - During the acquisition, you can change the current stitched image's zoom factor, e.g., to see certain parts in the overlap area better. You will find an overview on the possibilities of changing an image's zoom factor in the online help.

7. Define your way through the sample, with the arrow buttons, and follow that with the stage.
   In this manner, you can display a sample in any form you like in the stitched image. The illustration shows a stitched image that is made up of 9 individual images, and the stage path.
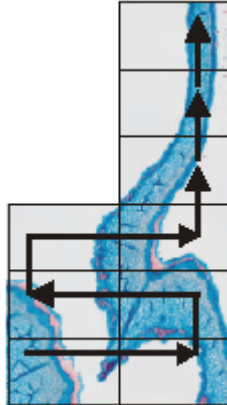
8. Click the *Stop* ▣ button when you want to end the acquisition of the stitched image.

   • You see the completed stitched image (4) in the image window. Since the individual images can lie a little askew of each other, the stitched image isn't as a rule, rectangular, but contains empty areas on its borders (5). These areas will, as a rule, be cut off in the stitched image.
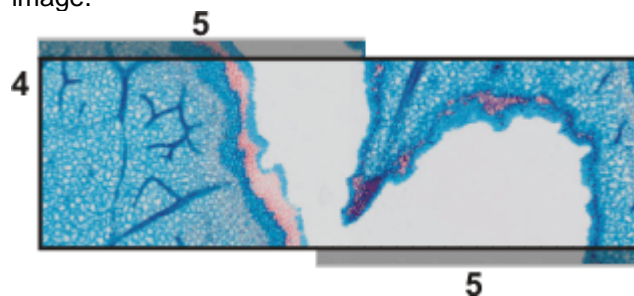
   • The stitched image will, by default, be automatically saved. The storage directory is shown in the *Acquisition Settings > Saving > Process Manager* dialog box. The preset file format is VSI.

   Properties of the stitched image

   • By default, in the overlap area, the intensity values of two adjoining individual images will be matched with each other to make the image's overall impression homogeneous.

   • Stitched images are calibrated. This means that you can measure distances and objects on a stitched image.

Acquiring a stitched image with a motorized XY-stage (XY-Positions / MIA)

500 μm
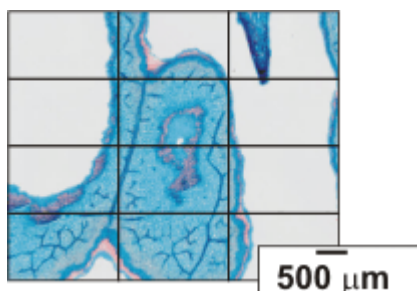
Example: You want to acquire an image of a large sample area. Use the automatic *MIA* acquisition process, to scan a rectangular area of the sample and to have adjoining images combined into one stitched image.

Preconditions

- The stage has been set up and initialized, i.e. its stage limits have been defined.
- The camera is aligned parallel to the XY-stage. The angle between camera and stage should be smaller than 1°.
- The shading correction has been set up.

Selecting the acquisition process

1. Activate the *Process Manager* tool window.

2. Select the *Automatic Processes* option.

3. Click the *XY-Positions / MIA* button.

   - The *XY* group will be automatically displayed in the tool window.

Using the software autofocus

1. If your microscope is equipped with a motorized Z-drive, you can switch on a software autofocus.
   In the *Process Manager* tool window, click the *Autofocus* button.

   - The button will appear clicked. You can recognize this status by the button's colored background.

   - The *Autofocus* group will be automatically displayed in the tool window.

2. In the *Autofocus* group, select the *Multiposition / MIA* check box.

3. If the sample surface is not plane or if it is inclined to the objective, choose the *Every MIA frame* option. Now, the software autofocus will be performed before every image acquisition.

Putting the stage navigator on display

1. In the *Process Manager* tool window, click this button [⊕].

   - The *Stage Navigator* tool window is shown. When you have acquired an overview image of your sample, you will see this area of the image in the stage navigator's image segment.

2. Set the magnification for the image segment in the *Stage Navigator* tool window. To do this, use the zoom buttons at the bottom left of the tool window (2).
   The current stage position will be shown by a yellow rectangle in the image segment (1). You should choose a magnification that enables you to see this

rectangle clearly.
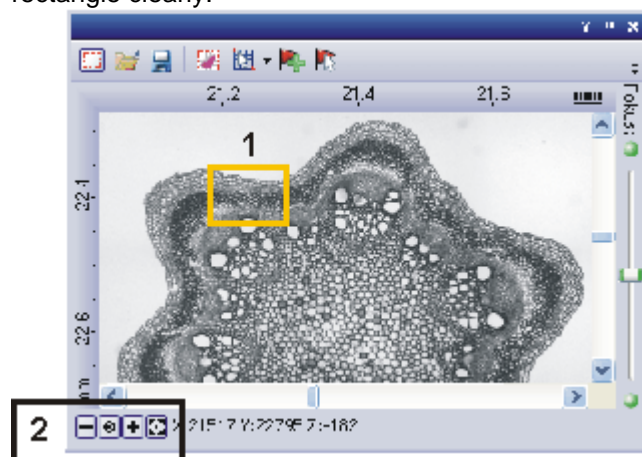
- You can find more information on the *Stage Navigator* tool window in the online help.

*Defining the MIA scan
area*

1. In the *Process Manager* tool window, click this button.

   - The system will automatically switch into the live mode.

   - The *Define MIA Scanning Area* dialog box opens.

2. Move the XY-stage to the top left-hand corner of the MIA scan area you want (3).

3. Focus, then select the optimal settings for your acquisition, in the *Camera Control* tool window. Pay special attention to setting the correct exposure time. This exposure time will be used for all of the stitched image's individual images.

4. Confirm the starting position in the *Define MIA Scanning Area* dialog box with *OK*.

5. Move the XY-stage to the bottom right-hand corner of the MIA scan area (4). Confirm this position in the *Define MIA Scanning Area* dialog box with *OK*.

   - In the *Stage Navigator* tool window, the MIA scan areas that have been defined are displayed. Here, you can immediately see how many individual images are required for the acquisition of the stitched image, when the current magnification is used.
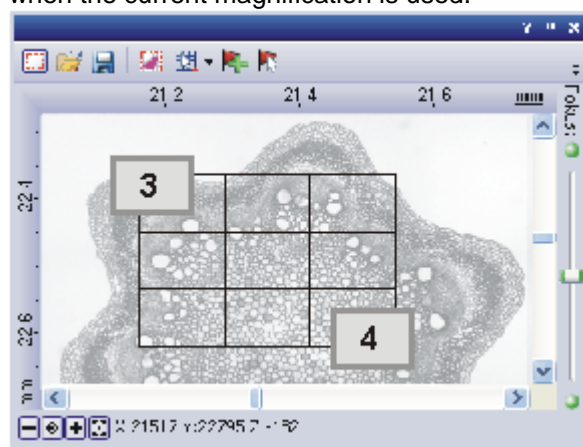
Acquiring a stitched image

1. Click the *Start* ▶ button.

   - The acquisition begins immediately. The individual images are acquired, then immediately assembled. You can watch how the stitched image grows, in the image window.

   - You see the completed stitched image, in the image window. The individual images won't be saved separately.

   - The stitched image will, by default, be automatically saved. The storage directory is shown in the *Acquisition Settings > Saving > Process Manager* dialog box. The preset file format is VSI.

Combining individual images into a stitched image

Loading and selecting images

1. Load the images you want to combine, or acquire a suitable set of images.

2. Open the *Gallery* tool window. To do this, use, e.g., the *View > Tool Windows > Gallery* command.

3. Select all of the images you want to combine in the *Gallery* tool window.

Assembling images

1. Use the *Process > Multiple Image Alignment...* command. This command is only active when more than one image of the same image type has been selected.

   - The *Multiple Image Alignment* dialog box will open. A description of this dialog box can be found in the online help.

   - The dialog box's stitching area will display a preview of the individual images.

2. If necessary, while keeping your left mouse button depressed, drag on the bottom left-hand corner of the dialog window to enlarge it. Alternatively, doubleclick the header of the dialog box to enlarge the dialog box to full-screen size.

3. Check whether the images' positions are correct. You can change the arrangement of the individual images, e.g., by exchanging two images in the stitching area by Drag&Drop.
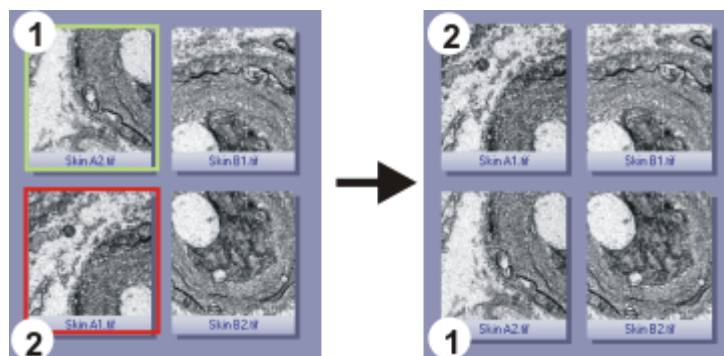
   -
     The illustration shows the stitching area with four individual images. On the left, the images 1 and 2 are not in the correct position. Image 1 (green frame) will therefore be dragged onto image 2 (red frame). On the right, you see the stitching area after the two images have been interchanged.

4. When the individual images overlap, select the *Correlation* option in the *Output > Alignment* list.
   Then, your software will search for the same image structures in neighboring

individual images. The stitched image will be put together in such a way that image areas that are the same will be superimposed.

5. Click the *OK* button to carry out the automatic image alignment.

   • The *Multiple Image Alignment - Manual Align* dialog box opens.

   • The stitched image will be displayed.

Checking a stitched image

1. Check the stitched image on display.
   Use the zoom buttons in the dialog box to zoom in the stitched image in the dialog box.

2. Should individual images have been incorrectly assembled, you can manually shift one or more of them, in respect to one another.
   To do this, click in the image you want to shift, then drag it with your left mouse button depressed, in the required direction.

   • The currently selected image will be displayed semi-transparently to make it easier for you to find the point of contact with the neighboring image.
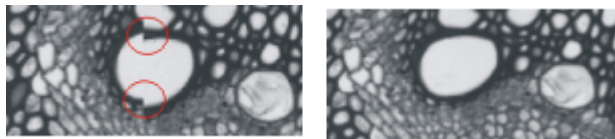
   •
   Two images were not correctly aligned with each other. There is a misalignment. When the manual alignment has been made, the two images fit together seamlessly.

3. Select the *Cut Edges* check box to clip the image in such a way that there are no longer any empty areas visible on its borders.

   • In the preview, the image edges that are to be clipped will be displayed semi-transparently.

4. Select the *Equalize* check box if the images aren't homogeneously illuminated. Then the intensity values of the individual images will be matched with one another, which will make the background appear more homogeneous.

5. Click *OK*.

   • A new image with the name "Image_<consecutive No.>" will be created.

00383

9. Running experiments

9.1. Overview

What exactly is the Experiment Manager?

You can use your software to implement complex acquisition processes. Use the Experiment Manager to define and run complex experiments involving image acquisition with your software. You can always re-use existing experiment plans or adapt them to new conditions. You can acquire multi-dimensional images during the experiment. If your microscope has motorized hardware components, you can control these with the software during the experiment. Or you can use an RTC (Real Time Controller), to use external devices in your experiments.

The idea behind the Experiment Manager

The Experiment Manager is a graphic Process Manager

You can create a graphic experiment plan with the *Experiment Manager* tool window. This experiment plan contains a series of commands, the acquisition of images for example, that are carried out one after the other.

Example: Use the Experiment Manager to acquire several multi-channel fluorescence images of a certain position on the sample at certain intervals.

How the Experiment Manager differs from the Process Manager

Just like the Experiment Manager, you can use the *Process Manager* tool window to handle complex acquisition processes. The Experiment Manager can be used as an alternative to the Process Manager. It's more intuitive for more complex processes and offers you more options.

- In the *Experiment Manager* tool window, you can trigger a particular device at a particular time using the RTC to add a chemical to your sample, or to heat, or illuminate your sample, for example.
- Only the Experiment Manager allows you to define loops within loops. This makes it possible to repeat a fast time stack several times at particular intervals.
- The Experiment Manager allows you to use streaming to attain shorter intervals between two separate image acquisitions in a time stack than are possible with the Process Manager.

Prerequisites for using the Experiment Manager

Prerequisite: The *Experiment Manager* tool window is only available when you've purchased the *Experiment Manager* solution in addition to your software. This solution is only available with the highest software package.

The system has been configured. The observation methods have been defined.

Make sure that your software is correctly configured.

When you want to acquire multi-channel fluorescence images, it makes sense to define observation methods for your color channels before you define the experiment. If you want to assign the fluorescence color to the individual color channels, you have to define observation methods.

Experiment Manager's user interface

The Experiment Manager is composed of the *Experiment Manager* tool window and an experiment plan.
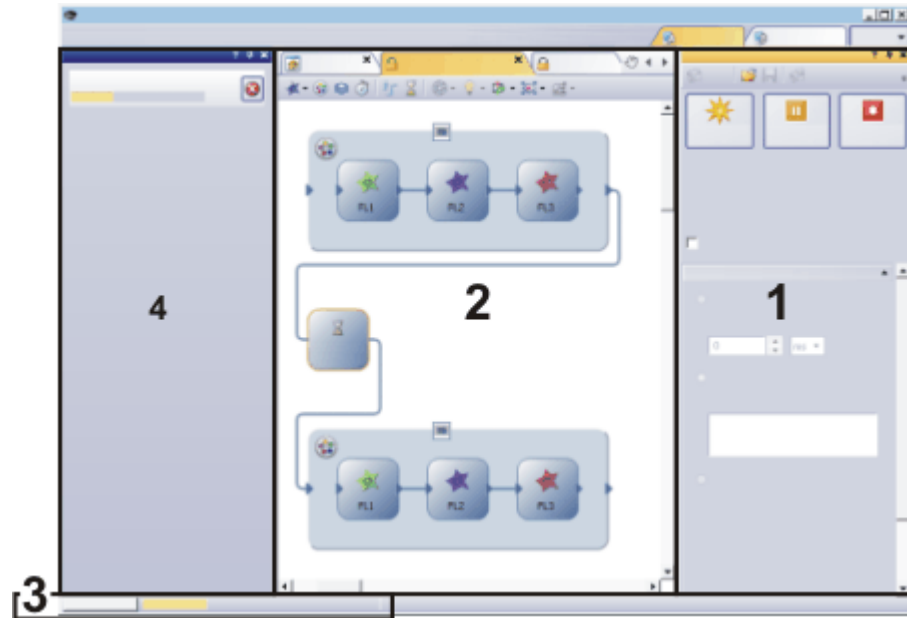
The Experiment Manager is composed of the *Experiment Manager* tool window (**1**) and an experiment plan (**2**). When you run an experiment, a progress bar is displayed on the left of the status bar at the bottom of the monitor (**3**). When an experiment is running over a long period of time, you can also display the *Task* tool window (**4**) to get more information about the progress of the experiment.

Use the *Experiment Manager* tool window to create an experiment plan, to edit an existing experiment plan or to start an experiment plan. To keep a good overview of the flow chart in the experiment plan, specify the settings for the commands you use in the *Experiment Manager* tool window.

The experiment plan
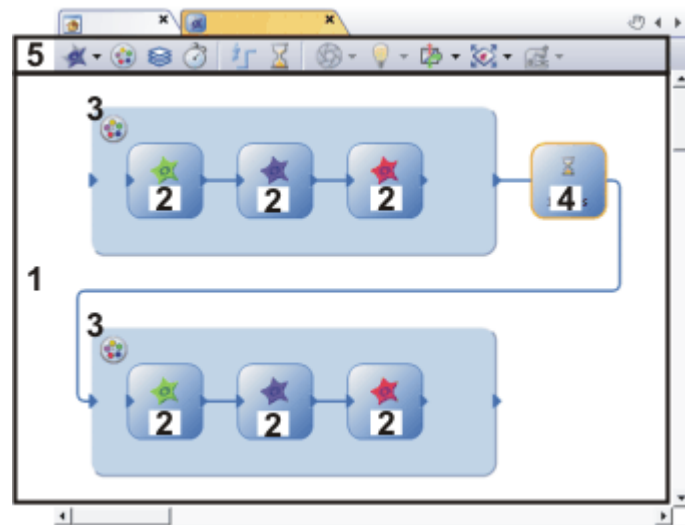
The illustration shows the elements on the user interface that belong to the experiment plan. The experiment plan is a document that is displayed in exactly the same way as images and other documents in your software's document group.

A document of the *Experiment plan* type is essentially a canvas on which you define the experiment by creating a flow chart (**1**).

An experiment plan contains particular commands, concerning image acquisition, as a rule. Each command is graphically displayed in the experiment plan with its own icon. The example displayed above contains the icons for a fluorescence image acquisition, (**2**), the icons for the creation of a multi-channel image (**3**) and the icon for the *Wait* command (**4**).

Two commands are connected with a line while an arrow unambiguously defines the order of the commands.

Experiment plans have their own toolbar in the document window itself (**5**). You can find all the commands that you can use in the experiment on this toolbar. The toolbar is described in the online help.

Activating experiment plans in the document group

Note: When you're running an experiment, the acquired images are displayed in the document group, as a rule. In doing so, they cover up the experiment plan.

Click on the experiment plan's header in the document group to reactivate the experiment plan and to carry on working.

When a lot of images have been acquired, you can use the *Documents* tool window to reactivate the experiment plan after the experiment has finished. Switch to tree view to find the experiment plan quickly. Experiment plans are displayed under the *Experiment* entry.

General process flow

You can use the Experiment Manager for two different types of tasks.

(**1**) Defining and running a new experiment
(**2**) Using an existing experiment plan

(1) Defining and running a new experiment

The following process flow chart displays the basic steps of the process.

---

1. Preparing the experiment

Think through the experiment.

- Make sure that all hardware components you want to use are registered in your software's device list and configured in the device settings.
- Check whether the observation methods that you want to use have been defined.
- Select the exposure time and optimize the image quality in the live-image.

▼

2. Defining the acquisition settings

In the *Acquisition Settings > Saving > Process/Experiment* dialog box, specify whether and where you want the resulting images to be saved.

▼

3. Setting up the new experiment plan

In the *Experiment Manager* tool window, click the *New* button.

▼

4. Defining the experiment plan

Add the commands for the image acquisition and the control of the hardware components to the experiment plan. Each command is represented by a graphic element that you can arrange on the canvas however you like.
Connect all commands unambiguously with each other.

▼

5. Making settings for the image acquisition and hardware control

Select each element of your experiment plan one after the other and make the necessary settings in the *Experiment Manager* tool window.

▼

6. Running the experiment

Test your experiment while you're defining the experiment plan. To do this, click the *Start* button in the *Experiment Manager* tool window.

▼

7. Saving the experiment plan

In case you want to run the same experiment again, with a different sample for example, save the completed experiment plan as an OEX file.

(2) Using an existing experiment plan

You can load an existing experiment plan at any time and run the experiment again. The following process flow chart displays the basic steps of the process.

1. Defining the acquisition settings

In the *Acquisition Settings > Saving > Process/Experiment* dialog box, specify whether and where you want the resulting images to be saved.

▼

2. Loading the experiment plan

Load a OEX file in the document group.

▼

3. Adjusting the experiment plan

Select each element of your experiment plan one after the other and make the necessary settings *nt Manager* tool window. Change the exposure time for the acquisition of fluorescence images, for example.

▼

4. Running the experiment

In the *Experiment Manager* tool window, click the *Start* button.

▼

5. Saving the experiment plan

Decide whether you want to save the changed experiment plan.

9.2. Working with the Experiment Manager

Use the Experiment Manager to define and run complex experiments involving image acquisition with your software.

The following instructions guide you step by step through the definition of a typical experiment. The complexity of the experiments described increases from example to example.

Acquiring fluorescence images

Task

Your sample has been stained with the DAPI, FITC and TRITC fluorochromes. Define an experiment plan for acquiring several fluorescence images and run the experiment.

Preconditions

- The system has been configured.
- You have defined suitable observation methods for your color channels.
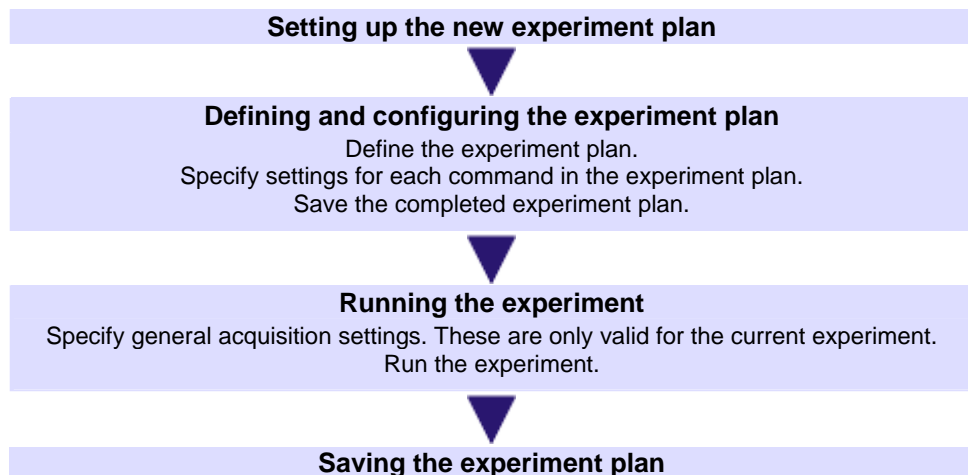
The following process flow chart displays the basic steps of the process.

Setting up the new experiment plan

▼

Defining and configuring the experiment plan
Define the experiment plan.
Specify settings for each command in the experiment plan.
Save the completed experiment plan.

▼

Running the experiment
Specify general acquisition settings. These are only valid for the current experiment.
Run the experiment.

▼

Saving the experiment plan

Setting up the new experiment plan

1. If necessary, use the *View > Tool Windows > Experiment Manager* command to show the *Experiment Manager* tool window.

2. In the *Experiment Manager* tool window, click the *New* button to create a new experiment.

   - This automatically creates a new document of the *experiment plan* type in the document group.

   - The experiment plan contains its own toolbar with all the commands that you can use in an experiment plan. Exactly which commands appear on the toolbar depends on your system configuration.

   - In the document group, experiment plans are identified by this icon 🔷 in the header.

   - The default name for the experiment plan is *Experiment_<sequential No.>*. You can change the experiment plan's name to anything you want when saving it. A small asterisk next to the name indicates that the document hasn't been saved yet.
   Please note that the name of the experiment plan isn't linked to the experiment name that you enter in the *Experiment Manager* tool window. The entry in the *Experiment name* field is, by default, incorporated into the names of the images that you acquire with the experiment plan.
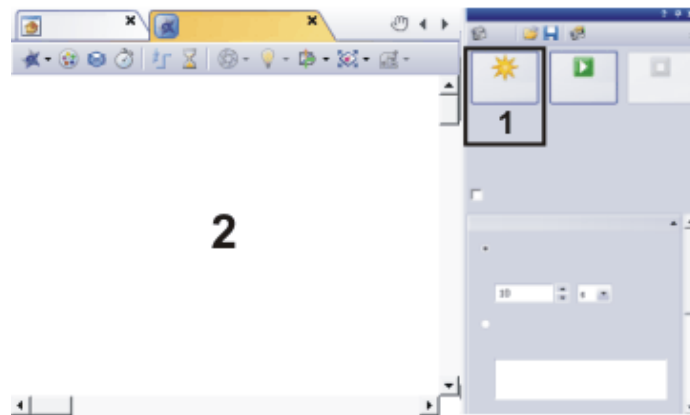
Click the *New* button (**1**) to create a new empty experiment plan (**2**).

*Defining the experiment plan*

3. Define the first image acquisition command.
    Click the small arrow next to the *Image Acquisition* button to open a menu. Select the observation method that you want to use for the first image acquisition, *DAPI* for example.

    - All of the observation methods that have been defined in your software are listed in the menu.

4. Click in the canvas on the position where you want to place the image acquisition command with the DAPI observation method in the experiment plan.
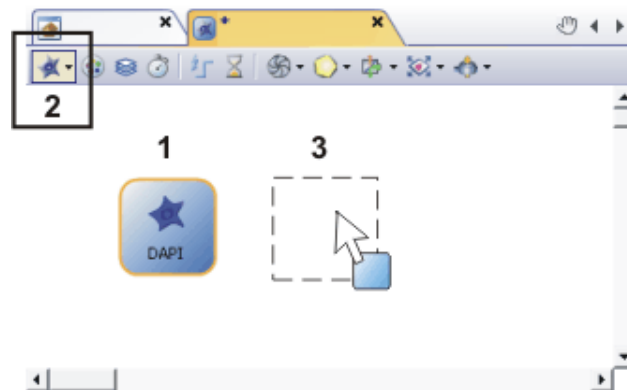
The experiment plan already contains the image acquisition command with the DAPI observation method (**1**).
Click the Image Acquisition button to add another image acquisition command to the experiment plan (**2**). The button corresponding to the selected command becomes active. You can recognize this status by the button's colored background.
You can now move the image acquisition command on the experiment plan with your mouse (**3**).

5. Add the other two commands for image acquisition with the FITC and TRITC observation methods as well and arrange the commands in a row.

    - The three commands have to be connected to each other with a line. This line has an arrow which unambiguously defines the order of the commands. Depending on where you placed the commands in relation to each other, they may already be connected.

6. You can define the connector between two commands in the experiment plan manually. To do so, create a connector by dragging one of the control points on the edge of a command to a control point on the following command. When doing this, you always have to connect an output control point (on the right of the element) with an input control point (on the left of the element).

- Your software automatically draws the connector in the experiment plan. When you move elements in the experiment plan, the connector between these elements is automatically adjusted.
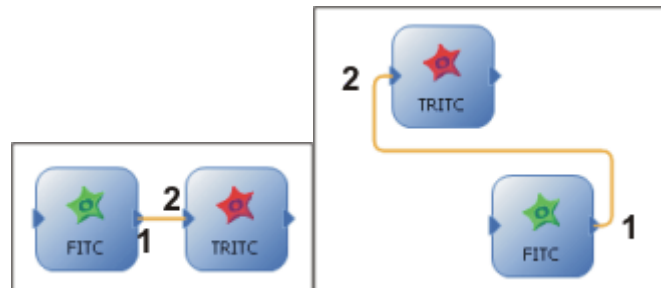

> Both experiment plans show the same experiment. First, an image is acquired with the FITC observation method and then an image with the TRITC observation method. The green FITC element's output control point (**1**) is correctly connected to the red TRITC element's control point (**2**).
> The connector has been selected in both experiment plans and is therefore displayed in color.

Configuring the experiment plan

Define the exposure time and other acquisition settings for the image acquisition commands.

7. Select the DAPI command in the experiment plan.

   - In the *Experiment Manager* tool window, the *Image Acquisition*, *Camera Settings* and *Display* groups are now shown.

8. Click the *Set Current Device Settings* button. You can find the button in the *Image Acquisition* group in the *Experiment Manager* tool window.

   - The DAPI observation method is specified on the microscope.

9. Click the *Live* button in the toolbar at the top of the *Experiment Manager* tool window to switch to live mode. In the live-image, check the exposure time and focus on the sample.

   - Note: The live-image covers the experiment plan in the document group. When you turn off live mode again, the live-image is closed by default and you see the experiment plan again.
     If you've selected a different setting for the live-image, you can activate the experiment plan after ending the live mode, in the *Gallery* tool window, for example.

10. Set the resolution and the bit depth in the *Experiment Manager* tool window. Set the exposure time and the sensitivity or gain for an optimal image.

    - Note: You can also use the *Camera Control* tool window to optimize the live-image. The acquisition settings in the *Camera Control* tool window are not, however, transferred automatically to the selected command in the experiment plan. To do this, click the *Get Current Device Settings* button in the *Experiment Manager* tool window.

11. In the same way, define the acquisition settings for the FITC and TRITC commands. You can make different settings for each image acquisition command. For example, expose the different fluorescence images differently to even out the differences in the light intensity.
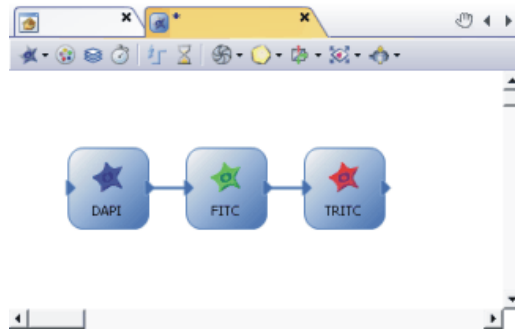
The experiment plan contains three individual image acquisition commands. Because the image acquisition commands are linked to an observation method, before the image acquisition your system automatically takes on the settings you defined in the observation method . For the acquisition of fluorescence images, the required mirror turrets are brought into the light path, for example.
The experiment will produce three fluorescence images.

Running the experiment

Specify some general acquisition settings for the running of the experiment. These acquisition settings are not saved together with the experiment plan.

12. Click the *Acquisition Settings* button, located in the *Experiment Manager* tool window's toolbar.

13. Select the *Saving > Process/Experiment* entry in the tree view.
Here, you specify whether and how the acquired images are to be automatically saved. You can have the acquired images saved in a database or in a directory of your choice. Or you can turn automatic save off. In this case, the acquired images stay open in your software's document group after the experiment has finished.

14. Select the *Document Name > Process/Experiment* entry in the tree view.
Here, you specify how the acquired images should be named.

15. You set some camera settings, like Online Deblur for example, globally for the whole experiment in the *Camera Control* tool window. Just like the acquisition settings, they're not saved together with the experiment.

16. Click the *Start* button, located in the *Experiment Manager* tool window, to run the experiment.

   - Note: You can only start the experiment when the experiment plan is active in the document group. If another document is active, an image for example, use the *Gallery* tool window to activate the experiment plan in the document group.

   - The experiment starts immediately. Three individual fluorescence image will be acquired.

   - When the images acquired by your experiment are automatically saved, the experiment plan will be automatically saved, too. The automatically saved experiment plan is named like the first image that has been acquired.

Saving the experiment plan

When you run an experiment, the experiment plan will normally be automatically saved to ensure an optimal documentation of your experiment. You are recommended to explicitly save the experiment plan in addition to the automatic saving so you can use it later to run similar experiments.

17. Activate the experiment plan in the document group.

18. Use the *File > Save As...* command to save the experiment plan. Save the experiment plan, under the name *3_FL_Images*, for example.

   - An experiment plan will be saved in the OEX file format.

Acquiring multi-channel fluorescence images

Task

Your sample has been stained with the DAPI, FITC and TRITC fluorochromes. Define an experiment plan for acquiring a multi-channel fluorescence image. Run the experiment and acquire a multi-channel image.

1. Load an experiment plan in which three fluorescence images are acquired one after the other.

2. Use the *File > Save As...* command to save the experiment plan with a new file name.

Adding a multi-channel group

3. Click the *Multichannel Group* button. You can find the button on the toolbar at the top of the experiment plan.

4. Draw a frame around the fluorescence image acquisition commands. All the commands in the multi-channel group will automatically be combined into a multi-channel image after the acquisition.
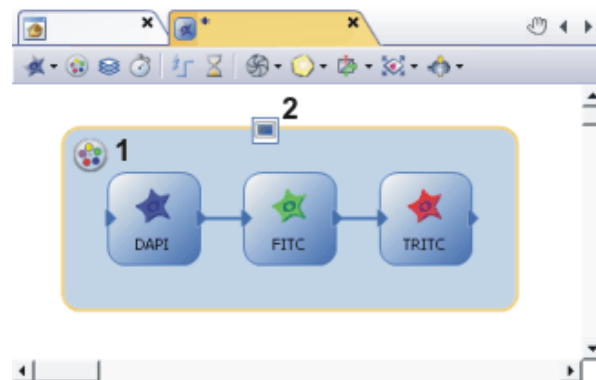
The illustration shows an experiment plan for a multi-channel image acquisition. The name of the multi-channel group (**1**) and the status of the online display (**2**) are displayed in the experiment plan.
The experiment will produce a multi-channel image with three color channels.

5. Test the experiment. To do this, click the *Start* button in the *Experiment Manager* tool window.

   - The acquisition of the multi-channel fluorescence image starts immediately.

   - The acquisition has been completed when you can again see the *Start* button in the *Experiment Manager* tool window.

   - The acquired fluorescence image is displayed in the image window by default after the experiment is finished. In the image window, all three color channels are superimposed on each other so that you see all three fluorescence images at the same time.
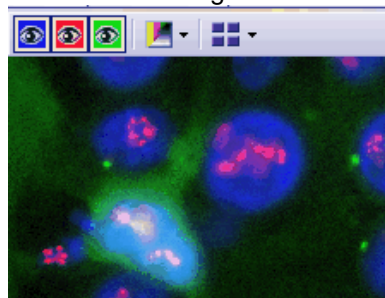
6. In the image window, take a look at the multi-channel fluorescence image that has been acquired . If necessary, change the settings for individual commands in the experiment plan.

Acquiring a multi-channel fluorescence image together with a transmitted light image

Expand your experiment plan and acquire a transmitted light image as well, with the brightfield observation method, for example.

1. Select the multi-channel group in the experiment plan. Use the mouse to enlarge the frame so that there's enough room inside the group for another image acquisition command.

2. Click the small arrow next to the *Image Acquisition* button to open a menu. Choose an observation method for the acquisition of a transmitted light image, e.g., phase contrast, differential interference contrast (DIC), or brightfield.

3. Click once in the multi-channel group to insert the element for the acquisition of the transmitted light image into the experiment plan. It may be necessary to first enlarge the multi-channel group's frame. Next, position the element to the right of the last fluorescence image acquisition.

4. Connect the last fluorescence image acquisition command with the transmitted light image acquisition command. To do this, click on a control point and, while holding the left mouse button pressed, move the mouse pointer to the other control point.

5. Select the command for the transmitted light acquisition in the experiment plan.

   - In the *Experiment Manager* tool window, the *Image Acquisition*, *Camera Settings* and *Display* groups are now shown.

   - The *Transmission overlay* check box in the *Image Acquisition* group is available when the command selected in the experiment plan is linked to a transmitted light observation method.

6. Select the *Transmission overlay* check box in the *Experiment Manager* tool window. Now the transmitted light image is assigned its own image layer on top of the fluorescence images.

   - This icon ▣ appears in the experiment plan on the element for the acquisition of the transmitted light image.

7. Make the rest of the settings for the acquisition of the transmitted light image.

8. Click the *Start* ▶ button, located in the *Experiment Manager* tool window, to run the experiment.

   - Then, together with your fluorescence images, a transmitted light image will also be acquired and saved together with the multi-channel fluorescence image. The result of this acquisition process is a multi-layer image that you can view with the *Layers* tool window.

The illustration shows an experiment plan for a multi-channel acquisition with a transmitted light image (**1**). The transmitted light image acquisition command is inside the multi-channel group. The experiment will produce a multi-layer image with two image layers. One image layer is the multi-channel image and the second layer is the transmitted light image.
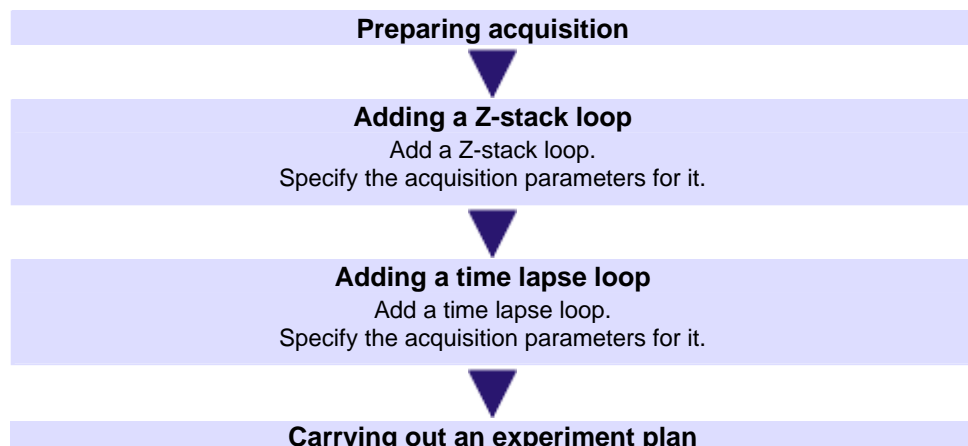
Alternatively, you can also use this experiment plan to acquire a multi-channel image with a transmitted light image. The transmitted light image acquisition command is, in this case, outside the multi-channel group. The experiment then produces two images, one multi-channel image and the brightfield image. In this case, you can't place one images on top of the other to view the superimposition.

Acquiring multi-dimensional images

Task Define an experiment plan for acquiring a multi-channel Z-stack. You want the acquisition of the multi-channel Z-stack image to be repeated several times at intervals of one hour.
Run the experiment.

Preconditions
- The system has been configured.
- You have defined suitable observation methods for your color channels.
- Your microscope has a motorized Z-drive. The Z-drive has been set up and calibrated.

The following process flow chart displays the basic steps of the process.

Preparing acquisition

▼

Adding a Z-stack loop
Add a Z-stack loop.
Specify the acquisition parameters for it.

▼

Adding a time lapse loop
Add a time lapse loop.
Specify the acquisition parameters for it.

▼

Carrying out an experiment plan

1. Load an experiment plan for acquiring a multi-channel fluorescence image. Or specify a new experiment plan.

2. Use the *File > Save As...* command to save the experiment plan with a new file name.

Preparing acquisition 3. Select one of the commands in the experiment plan, DAPI, for example. Click the *Set Current Device Settings* button in the *Experiment Manager* tool window to have the DAPI observation method set on the microscope.

4. Click the *Live* button, located in the toolbar at the top of the *Experiment Manager* tool window.
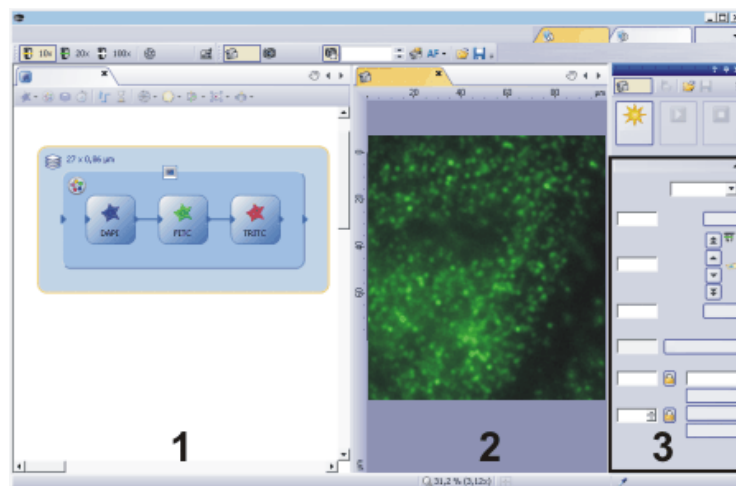
Some of the settings for the Z-stack acquisition can best be checked in the live-image. You can display the live-image (**2**) and the experiment plan (**1)** in the document window at the same time.
The Z-stack loop has been selected in the experiment plan. In the *Experiment Manager* tool window, the *Z-stack* group (**3**) is now visible. Set the acquisition parameters here.

5. Arrange the live window and the experiment plan in the document window so that you can see both documents at the same time. To do this, use the *Window > Split/Unsplit > Document Group (Right)* command, for example.

6. Bring the image into focus.

Adding a Z-stack loop

7. Click the *Z-stack loop* button. You can find the button on the toolbar at the top of the experiment plan.

8. Draw a frame around the multi-channel group. Your microscope's Z-drive will now be automatically moved to a different Z-position when all the commands in the Z-stack loop have been carried out.

   - The *Z-stack* group is displayed in the *Experiment Manager* tool window. Set the acquisition parameters for the Z-stack loop here.

9. Define the acquisition parameters for the Z-stack loop in the *Experiment Manager* tool window.

10. Select the *Top and bottom* entry in the *Define* list (**1**).

    - The stage's current Z-position will be shown in the *Position* (1) field. Because you've already focused, this is the focus position.

11. Using the arrow buttons (**2**), move the microscope's Z-drive up to the Z-position at which the structures that are directly under the surface of the sample are displayed in sharp focus. The buttons with a double arrow move the stage in larger steps.
    Now, move the Z-drive the same distance again in the same direction. Click the upper *Set* button (**3)**.

    - The current Z-position will be adopted in the *Start* field (3).

12. Now, define the final position in exactly the same way.

    - The *Recommended Step Size* field (4) displays the distance required between two Z-positions in the Z-stack. The required distance depends on the objective's Numerical Aperture, among other things, and is automatically calculated using the Nyquist theorem. This makes sure that no positions on the sample between two individual images remain out of focus. The higher the objective magnification and your objective's Numerical Aperture is, the smaller the required distance will be.

13. If necessary, release both of the buttons showing the lock icon. 🔒.
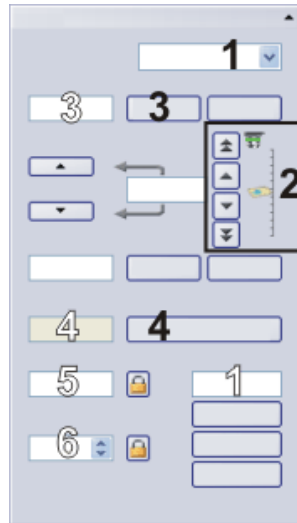
> Set the acquisition parameters for the acquisition of a Z-stack in the *Experiment Manager* tool window. The acquisition parameters apply to the Z-stack loop that is selected in the current experiment plan. Use the fields and buttons with black numbers (**1-4**) for this. The values in the fields with white numbers are automatically calculated and updated by your software.

14. Click the *Apply* button (**4**) located next to the *Recommended Step Size* field.

    • The *Step Size* field (5) adopts the value from the *Recommended Step Size* field.

    • The *Z-Slices* field (6) now displays how many Z-positions will be moved to by the Z-stack loop. The number of Z-positions will be automatically calculated on the basis of the Start and End values, and the Z-spacing.

15. Finish the live mode.
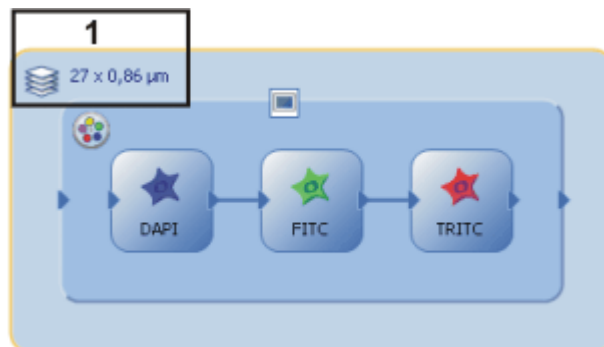
> The illustration shows an experiment plan for the acquisition of a multi-channel Z-stack image.
> The experiment plan displays the number of Z-positions that will be moved to and the Z-spacing (**1**). In this example, 27 individual images with a Z-spacing of 0.86 µm will be acquired for each channel.

16. Click the *Time Lapse Loop* ⏱ button. You can find the button on the toolbar at the top of the experiment plan.

17. Draw a frame around the whole multi-channel Z-stack image. The acquisition of the multi-channel Z-stack image will now be repeated. All acquired images will be combined into a single multi-dimensional image, a multi-channel Z-stack image.

- The *Time Lapse* group is displayed in the *Experiment Manager* tool window. Set the acquisition parameters for the time lapse loop here.

18. Define the acquisition parameters for the Z-stack loop in the *Experiment Manager* tool window.

Selecting the acquisition parameters for the time lapse loop
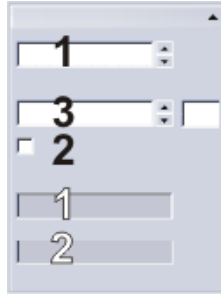
> Set the acquisition parameters for the acquisition of a time lapse loop in the *Experiment Manager* tool window. The acquisition parameters apply to the time lapse loop that is selected in the current experiment plan. Use the fields with black numbers and the check box (**1**-**3**) for this. The values in the fields with white numbers are automatically calculated and updated by your software.

19. In the *Cycles* field (**1**), enter how often the command should be repeated in the time lapse loop. Enter the value 5 to acquire five multi-channel Z-stack images, for example.

20. Clear the *As fast as possible* check box (**2**).

21. In the *Interval* field (**3**), enter the time interval you want between two cycles. Enter the value 0.5 h, for example. Now the acquisition of a new multi-channel Z-stack image will begin half an hour after the start of the previous acquisition.

- The *Approximate minimum interval* field (1) displays the minimal time needed to carry out all the commands in the current time lapse loop. This is the amount of time that will be needed when you select the *As fast as possible* check box.

- The *Total loop duration* field (2) displays the time needed to carry out the time lapse loop.

Carrying out an experiment plan

> The illustration shows a completed experiment plan for the acquisition of a multi-channel image. It consists of three nested loops. There is a multi-channel group (**1**), a Z-stack loop (**2**) and a time lapse loop (**3**).
> The result will be an image file.

22. Click the *Start* button, located in the *Experiment Manager* tool window, to run the experiment.

- The experiment starts immediately. Nested loops are carried out starting on the inside and working to the outside. To be exact, in this case that

means:
First a multi-channel fluorescence image is acquired.
After this has been done, the stage's Z-position changes, and another multi-channel fluorescence image is acquired at the new Z-position.
The Z-position keeps changing until all Z-position have been used.
Your system waits for half an hour and then repeats the image acquisitions.

- The acquisition has been completed when you can once more see the

  Start ▶ button in the *Process Manager* tool window, and the progress bar has been faded out.

- Your system's hardware components are now set as specified for the last observation method that was used.

Acquiring fast fluorescence time stacks

You can use the Experiment Manager to acquire very fast fluorescence time stacks. In this way, you can acquire kinetic processes with your system's highest possible time resolution.

1. Define an experiment plan with a fluorescence image and a time lapse loop.

2. Select the command for acquiring fluorescence images in the experiment plan.
   Select the *Streaming* check box in the *Experiment Manager* tool window.

   - Pay attention to the experiment's total duration, shown at the top of the *Experiment Manager* tool window. The total duration display gets smaller which directly shows the effect the streaming is having.

   - The element for the fluorescence image acquisition in the experiment plan now looks a little different.

On the left, you can see the element for a fluorescence image acquisition using the DAPI observation method.
On the right, the *Streaming* check box is selected. A slightly different icon indicates the status of the check box in the experiment plan.

3. Select the command for the time lapse loop in the experiment plan.
   Select the *As fast as possible* check box in the *Experiment Manager* tool window.

4. Click the *Start* button, located in the *Experiment Manager* tool window, to run the experiment.

   - All images in the time stack are now acquired at the maximum possible frame rate without waiting to be triggered by the software or the hardware. For example, you can specify an observation method that opens a shutter before the image acquisition and closes it again after the image acquisition. When you are using streaming, the shutter stays open for the whole duration of the time stack acquisition. Without streaming, the shutter would be opened before a single image acquisition and closed again.

This is how an experiment plan for the fast acquisition of a fluorescence time stack with the *TRITC* observation method looks.
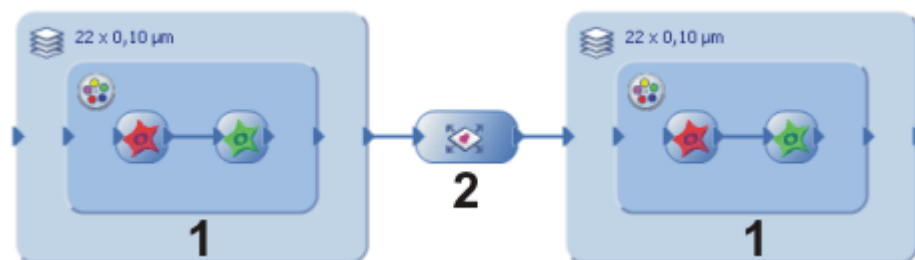
Acquiring multi-channel Z-stack images at different positions on the sample

Task
Define an experiment plan with which you can acquire a multi-channel Z-stack image at different positions on the sample. The experiment should always start at a specific stage position
Your sample has been stained with the DAPI and FITC and fluorochromes.

Preconditions
- The system has been configured.
- You have defined suitable observation methods for your color channels.
- Your microscope has a motorized Z-drive. The Z-drive has been set up and calibrated.
- Your microscope has a motorized XY-stage The XY-stage has been set up and calibrated.

1. Load an experiment plan in which a multi-channel Z-stack image is acquired.

2. Check the settings for each image acquisition command.

3. Add the *Move XY* ⬚ command to your experiment plan. Position the command behind the image acquisition commands and add a connector as well.
   You can find the command either as a button on the experiment plan's toolbar or in the stage control menu on the experiment plan's toolbar.

   - The *Move XY* group is displayed in the *Experiment Manager* tool window. Here, you specify how the stage should move.

4. Select the *Relative move* option. In the *X* and *Y* fields, enter the distance the stage should move.

5. Add the commands for the acquisition of another multi-channel Z-stack image to your experiment plan.
   You can select the whole Z-stack loop and copy it to the clipboard using the [Ctrl + C] keyboard shortcut. Use the [Ctrl + V] shortcut to paste the copied commands to a different position in the experiment plan.

   - Your experiment plan now contains the acquisition of two multi-channel Z-stack images at two different positions on the sample.
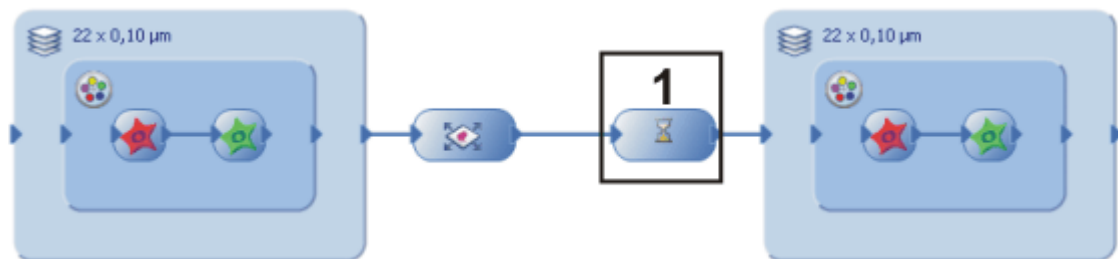

The experiment plan contains the acquisition of two multi-channel Z-stack images (**1**). The XY-Stage is moved to a different position between the two acquisitions (**2**).
The experiment will produce two multi-channel Z-stack images.

6. If necessary, expand your experiment plan. The following illustrations show several examples.
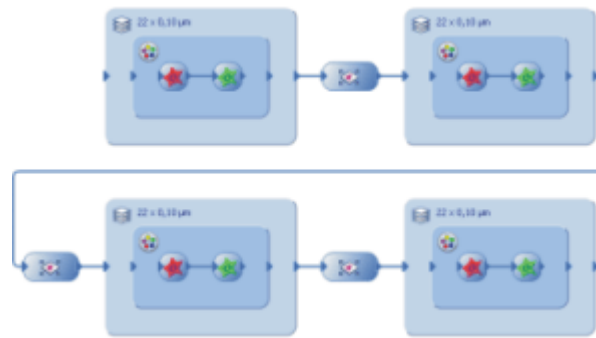
Acquire further multi-channel Z-stack images at other positions on the sample. The illustrated experiment plan moves the stage three times. The experiment produces four multi-channel Z-stack images from different positions on the sample.

Add a delay (**1**) to your experiment plan before the acquisition of the second multi-channel Z-stack image. Select the *Wait for keystroke* option for the delay, for example.
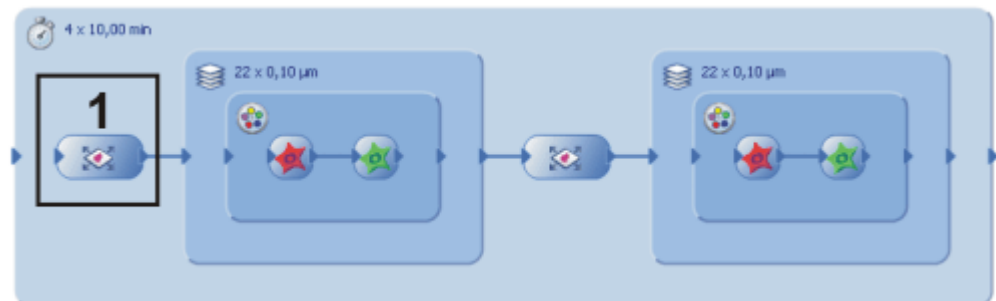
Drag a time lapse loop around the whole experiment. In this case, add another command, *Move XY* (**1**), before the acquisition of the first multi-channel Z-stack image. Select the *Absolute position* option for this command. This makes sure that the images in a multi-channel Z-stack image have a stage position.
In the example shown, the acquisition of both multi-channel Z-stack images is repeated 4 times. First the system acquires the multi-channel Z-stack image at stage position 1 and then it moves the stage. The system acquires the multi-channel Z-stack image at the second stage position and then it waits for the time specified in the time lapse loop's interval field. After the delay, the acquisition resumes at stage position 1.
The experiment will produce two multi-channel Z-stack in time-lapse images.

7. Switch to live mode and move to the position on the sample where you want to start the experiment.

8. Click the *Start* button, located in the *Experiment Manager* tool window, to run the experiment.

Adapting existing experiments

You can view and edit the settings for individual commands at any time.

1. Should the *Experiment Manager* tool window be hidden, use the *View > Tool Windows > Experiment Manager* command to make it appear.

2. Load a saved experiment plan. Experiment plans have the OEX file name extension.

3. Select the element in the experiment plan that you want to edit, an image acquisition for example.

    -   You can view and change all current settings for the selected element in the *Experiment Manager* tool window.

4. Save the experiment plan with the changed settings.

00801 17072013

10. Processing images

The *Process* menu offers numerous image processing functions, with which you can change an acquired image (e.g., increase the image contrast or the image sharpness).

1. Load the image you want to process, or activate the image in the document group.

   - Please note that the *Process* menu will only be visible when an image window is active in the document group.

2. Use one of the commands in the *Process* menu, e.g., *Process > Enhancement > Adjust Intensity...* .

   - The image processing dialog box will open. The image processing operation that is active will be shown in the dialog boxes header.

3. Click the small arrow next to the *Preview* button to open a list of all of the preview functions. Select the *Original and Preview* entry.

   - This preview function displays the same image segment twice in the dialog box. The first one shown is the source image. The second is the image that results when the current parameters are used.

   - Most of the image processing operations need one or two of the parameters that are shown in the *Settings* group.

4. Change the image processing operation's parameters. After every change that is made in a parameter, the operation will be immediately applied to the source image, and the resulting image will be shown in the preview window. Click the *Default* button to readopt the preset parameters in the *Settings* group, when the current parameter doesn't make sense to you.

5. When you have found the optimal parameters, click the *OK* button to have the active image processing operation applied to the image with the active parameters.

   - The image processing dialog box will closed.

   - Please note that the image processing operation changes the source image. No new image document will be created. You can, however use the *Edit > Undo* command to restore the source image.

00175 22022011

11. Life Science Applications

The *Life Science Application* toolbar offers you various evaluation methods for your images. If this toolbar is not displayed, use the *View > Toolbars > Live Science Applications* command.

The following table lists the buttons which are available by default on the toolbar.

	New ROIs	Use one of several options to define an image segment in the active image as a region of interest (ROI).
	Intensity profile	An intensity profile shows how the intensity within one, or within several image segments (ROIs), changes over a period of time or over the different Z-positions.
	Fluorescence Unmixing	Use fluorescence unmixing, to remove spectral mixing from a multi-channel fluorescence image.
	Brightfield Unmixing	Use brightfield unmixing, to break down a brightfield image containing three different colors into its individual color components.
	Colocalization	On a multi-channel fluorescence image, measure the colocalization to identify image segments where the individual fluorescences overlap.
	Ratio Analysis	Measure how the calcium ions concentration is changing in a time-lapse image.
	Verify Channel Parameters	For the active image, check whether all of the parameters have been correctly defined which are necessary for successful deconvolution.
	2D deconvolution	Use a deconvolution filter to remove disturbing diffused light from an image.
	Nearest Neighbor	
	Wiener	
	Constrained Iterative Filter	

6619 16072013

11.1. Intensity profile

With the *Measure > Intensity Profile...* command you can measure the intensity profile over the time (time stack) or over the different Z-positions (Z-stack). An image series can be a time stack or a Z-stack.

As a result you will obtain an intensity profile that shows how the intensity within one, or within several image segments, changes over a period of time or over the different Z-positions.

Example of use

You can use intensity profiles to measure how concentrations change with time. For example, when you make experiments with triggering the calcium flow with ATR, and use suitable fluorescence stains.

Before using the command

To calculate an intensity profile, all of the pixels within a specific image segment will be evaluated. You software will, e.g., determine the mean intensity of all of the pixels.

Before you can measure an intensity profile, you have to define this image segment. To do this, define one or more ROIs (Regions Of Interest) on the image. To define these ROIs, you can, for example, use the buttons on the *Life Science Applications* toolbar. You can find more information on working with ROIs in the online help.

Supported image types

With the *Measure > Intensity Profile...* command you can measure the following image types:

Time stacks, whose frames are gray-value images.

Z-Stacks, whose frames are gray-value images.

Multi-channel Z-stacks, multi-channel time stacks

The command is only available for gray-value images. Use the *Image > Mode > Grayscale* command, to convert the an image into a gray-value image.

00324

Measuring intensity profiles

With the *Measure > Intensity Profile...* command, you can measure the intensity profile over the time (time stack) or over the different Z-positions (Z-stack).

Measuring an intensity profile on a multi-channel Z-stack

Task

You have acquired a focus series for several fluorescences. You want to know how the intensity develops at a variety of positions on the sample at a variety of Z-positions.
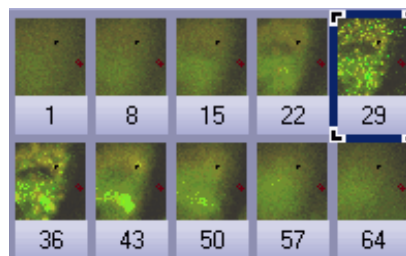
The illustration shows an overview over the frames in a multi-channel Z-stack. The multi-channel image contains a red and a blue color channel. For the acquisition of the Z-stack a through-focus series was taken of the sample. The sample can only be seen clearly, and sharply focused, in the middle of the Z-stack.

The following process flow chart displays the basic steps of the process.
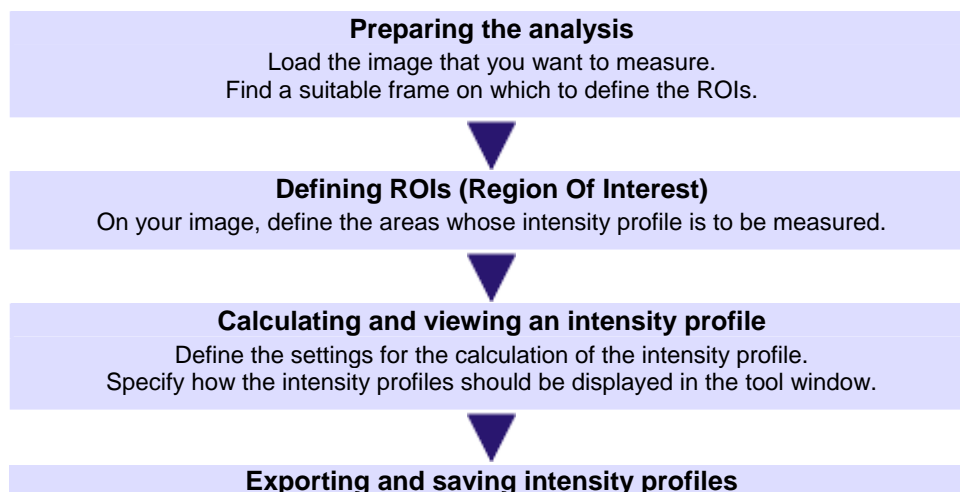
Preparing the analysis
Load the image that you want to measure.
Find a suitable frame on which to define the ROIs.

▼

Defining ROIs (Region Of Interest)
On your image, define the areas whose intensity profile is to be measured.

▼

Calculating and viewing an intensity profile
Define the settings for the calculation of the intensity profile.
Specify how the intensity profiles should be displayed in the tool window.

▼

Exporting and saving intensity profiles

Preparing the analysis

1. Several example images were supplied together with your software. You can follow these step-by-step instructions using the PeroxysomOrganelles.tif example image. This example image is a multi-channel Z-stack image.

   - When you load a multi-channel Z-stack, it will be automatically displayed in the Single Frame View in the image window.

Displaying a suitable image for the definition of the image segment

2. Use the navigation bar at the top of the image window.

3. Move the slide control slowly, and by doing so display frames acquired at differing Z-positions in the image window. Search out a Z-position at which the sample can be clearly recognized.

4. Use the *View > Toolbars > Life Science Application* command, to have the *Life Science Application* toolbar displayed. You can find the functions for defining ROIs and for measuring the intensity profile on this toolbar.

Defining ROIs (Region Of Interest)

5. Rotate the mouse wheel to change the zoom factor. Enlarge the image until you can see at least one enlarged segment of the sample in the image window, that is fluorescing in red.

6. Click the *New ROI - Polygon* button on the *Life Science Applications* toolbar.

7. By clicking with your left mouse button, define an area on the image that only includes red fluorescing sample positions.

8. Rightclick to finish the definition of the ROI.

9. Then define another ROI on an image segment that only includes green fluorescing sample positions.

10. Click the *New ROI - Rectangle* button.

11. Define a square in a dark image segment that shows no fluorescing objects. This ROI will be used as a reference for the background correction.

Calculating an intensity profile

16. On the *Life Science Applications* toolbar, click the *Intensity Profile* button.

- The *Intensity Profile - <Name of the active image>* dialog box opens.

- Your software recognizes the image type, and automatically selects the corresponding option. In this example the *Z-stack* option is preset.

17. Select the *Results > Average* check box.
Clear all the other check boxes.

- In the *ROI data* group, all of the ROIs that have been defined on the active image will be listed. In this example, you'll find three ROIs there (two on sample positions showing different fluorescence colors and one on the background).

18. Each ROI defines a specific image segment. Now, select the image segments for which intensity profiles are to be calculated.
In this example, select both of the ROIs at fluorescing sample positions.

19. In the *Background Subtraction* group, select the *ROI* option.

- In the list next to the *ROI* option, all of the ROIs that have been defined on the active image will be listed.

20. In the list, select the ROI that was defined on the image background.

21. Click the *Execute* button.

- The intensity profiles will be calculated and displayed in the *Intensity Profile* tool window.

Viewing intensity profiles

22. If necessary, use the *View > Tool Windows > Intensity Profile* command, to show the tool window. The tool window offers you several ways of displaying the intensity profile that has been measured.

23. In the *Intensity Profile* tool window's toolbar, click the *Arrange Charts and Layout* button.

- The *Intensity profile chart arrange* dialog box opens.

24. Make the following settings in the dialog box.
Select the *Select all* check box, in the *Show charts* group.
In the *Layout* group, specify a grid size of 2x1.
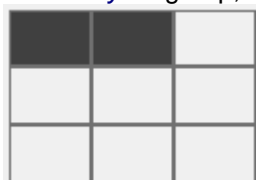
Close the dialog box with *OK*.

25. In the *Intensity Profile* tool window's toolbar, click the *Arrange Data* button.

- The *Intensity profile data arrange* dialog box opens.

26. Make the following settings in the dialog box.
Select the *Separate chart per channel* check box.
Clear the other check boxes.
Close the dialog box with *OK*.

- You can see two charts, each with two curves. Along the X-axis the Z-position, that's to say, the height, has been plotted. The intensity range has been plotted along the Y-axis.
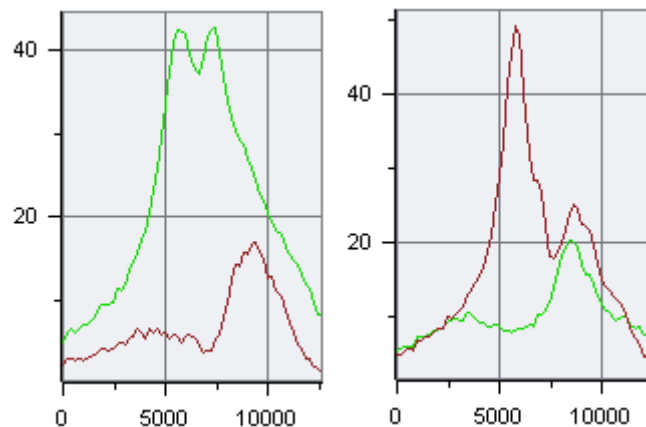
> For each of the image's color channels, an individual chart will be created. The name of the corresponding color channel will be displayed in the chart's header. On the left, you see the results for the green color channel, on the right, those for the red one.
> In each chart, you will see a curve for each ROI that has been defined. You can display a legend with the name of the ROIs in the chart. The green curve was measured on the ROI on the green fluorescing position on the sample, the red on the red fluorescing position.

Exporting and saving intensity profiles

25. In the *Intensity Profile* tool window's toolbar, click the *Export to Workbook*

    button.

    - A new workbook will be created in the document window. This workbook contains results sheets with all of the results.
      When you've measured a multi-channel image, you'll find an individual work sheet for each of the color channels.

26. Use the *File > Save as...* command, to save a workbook.

    - A workbook will be saved in the file format OWB. This format is an exclusive file format and can only be opened with your software. Workbooks are, obviously, therefore not suitable for using to exchange data with other application programs. If you would like to use the results in a different application, use the *File > Export to > Excel...* command.

Measuring the intensity profile of moving objects

Task

You've acquired a time stack of moving paramecia. Define a dynamic ROI that contains a paramecium and move the ROI so that it follows the paramecium through all of the frames in the time stack.
Measure the intensity profile.
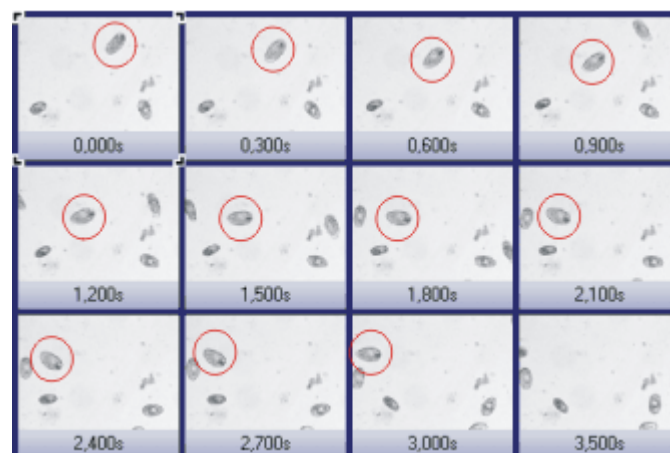
> The illustration shows an overview over the frames in a Z-stack. The time points associated with the frames are shown under the images. The red circle shows the movement of a single paramecium.

1. Several example images were supplied together with your software. You can follow these step-by-step instructions using the ParameciumTimeSeries.tif example image.

Specifying the user interface and default settings

2. Use the *View > Toolbars > Life Science Application* command, to have the *Life Science Application* toolbar displayed. You can find the functions for defining ROIs and for measuring the intensity profile on this toolbar.

3. If necessary, use the *View > Tool Windows > Measurement and ROI* command to have the *Measurement and ROI* tool window displayed. The ROIs that are defined in the current image are listed in this tool window.

4. Use the *Tools > Options...* command. Select the *Measurement and ROI > Dynamic ROI* entry in the tree view.
Select the *Interpolate linearly, continue with current* option.
You have now defined how a dynamic ROI behaves when you define its position on the frames.

*Viewing the movement of the paramecia*

5. Use the navigation bar at the top of the image window.

6. Move the slide control slowly to the right to view the movement of the paramecium that is at the top left border of the image in the first frame.

   • The paramecium first moves down and to the left. Then it changes direction and moves up before finally disappearing at the left border of the image.

Defining ROIs (Region Of Interest)

7. Display the first frame in the image window. To do this, use the navigation bar at the top of the image window.

8. Click the *New ROI - Rectangle* button on the *Life Science Applications* toolbar.

   • The ROI is displayed in the sheet of the *Measurement and ROI* tool window. In the *Type* column, the keyword *(ROI)* is added to the type name.

9. With two mouse clicks, define a small rectangle around the paramecium at the top left border of the image.

10. Move the mouse pointer over the ROI you just defined. Click the right mouse button to open a context menu. Select the *Convert to dynamic ROI over t* command from the context menu to turn the static ROI into a dynamic ROI.

    • In the *Measurement and ROI* tool window, the keyword *(ROI)* in the Type column changes into the new keyword *(dROI [t])*.

Following the movement of the object using the dynamic ROI

11. In the image window, display the frame in which the paramecium changes its direction.

    • The position of the ROI that has been defined is the same on all frames.

12. Move the ROI on this frame so that it contains the paramecium again.

    • The system will now automatically reposition the ROI on each frame between the first and the current frame. The positions are calculated as a linear interpolation of the ROI positions in the first and the current frames. Check whether the paramecium is completely within the ROIs on these frames.

13. In the image window, display the last frame in which the paramecium is still completely visible in the image.

14. Move the ROI on this frame again so that it contains the paramecium again. Make sure that the ROI doesn't include any other paramecium.

15. Check whether the ROIs position is correct for all of the frames up till now.

    - In the following frames, the paramecium disappears at the left border of the image and so can't be measured any more. The dynamic ROI is still defined on all following frames in the image series. You can't delete a dynamic ROI only for particular frames.

Calculating an intensity profile

16. On the *Life Science Applications* toolbar, click the *Intensity Profile* button.

    - The *Intensity Profile - <Name of the active image>* dialog box opens.

    - Your software will recognize the image type, and will select the appropriate option in the *Method* group. In this example the *t-stack* option is preset.

17. Make the following settings in the *Intensity Profile* dialog box.
    Select the *Results > Average* check box.
    Clear the other check boxes.
    Select the dynamic ROI in the *ROI data* group.
    In the *Background subtraction* group, select the *none* option.
    Close the dialog box with *OK*.

18. Click the *Execute* button.

    - The intensity profile for the paramecium will be calculated and displayed in the *Intensity Profile* tool window.
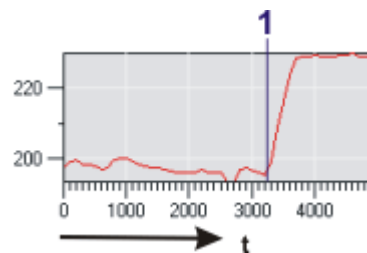

> The intensity profile displays how the average intensity in the ROI changes over time. The ROI contains the paramecium until about 3000 ms. The intensity is relatively constant.
> At time point (**1**), the paramecium begins to leave the image. The intensity then increases to the level of the light image background. The time point is at about 3300 ms.

00379 12072013

11.2. Fluorescence Unmixing

Multi-channel fluorescence microscopy

In the multi-channel fluorescence microscopy, different cell structures will be visually separated by acquiring them separately, then displaying them in different colors. To achieve this, one stains the sample with several suitably chosen fluorochromes. Each of these labels a special cell structure. The fluorescence images will then be acquired. The fluorescence image 1, created with fluorochrome 1 shows cell structure 1, the fluorescence image 2 created with fluorochrome 2 shows cell structure 2, etc.. The individual images will be combined into a multi-channel fluorescence image that shows the different cell structures in different colors. When, for example, three fluorochromes are used, a three channel fluorescence image will be created.
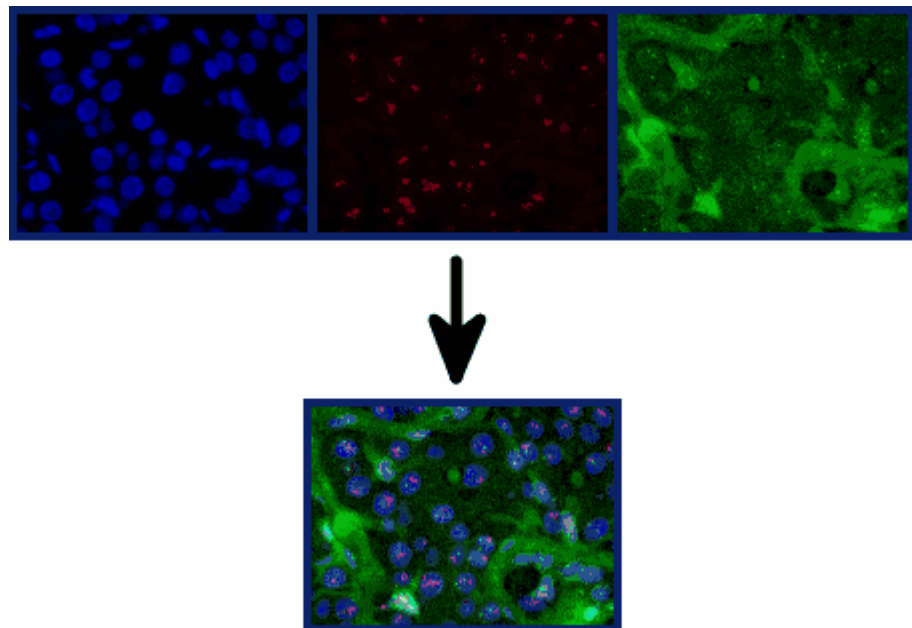
Problem with the visual separation of the structures

Filter sets in the microscope

Your microscope has an appropriate filter set for each fluorochrome, this set comprises an excitation filter, a dichromatic mirror, and an emission filter. When the fluorochrome 1 is excited by light from the wavelength range 1a, it emits light in the wavelength range 1b. When fluorescence image 1 is acquired, the excitation filter 1 takes care that only light from a narrow range within the wavelength range 1a reaches the sample from the microscope's illumination source. At the same time, the dichromatic mirror 1 and the emission filter 1 take care that, from the sample, only light from a narrow range within the emission wavelength range 1b, reaches the camera.

Overlapping of the wavelength ranges

The problem with this procedure is, that the wavelength ranges of the different fluorochromes overlap. If this overlapping didn't occur, the aspired visual separation of the different cell structures in the resulting multi-channel fluorescence image would be perfect.

Neither the excitation wavelength ranges nor the emission wavelength ranges have sharp limits, and they lie very close to one another, where numerous fluorochromes are concerned. Therefore, the excitation wavelength ranges 1a, 2a, 3a, ... of the fluorochromes 1, 2, 3, ... normally overlap. The same applies to the emission wavelength ranges 1b, 2b, 3b, ... As well as that, there are also

overlappings between excitation wavelength ranges and emission wavelength ranges.

Excitation and Emissions spectra

In the spectra that follow, you can see a graphical demonstration of the way in which the excitation intensities and the emission intensities of several fluorochromes that are often used, depend on the wavelength. The way in which the different wavelength ranges overlap, can clearly be seen in these spectra.
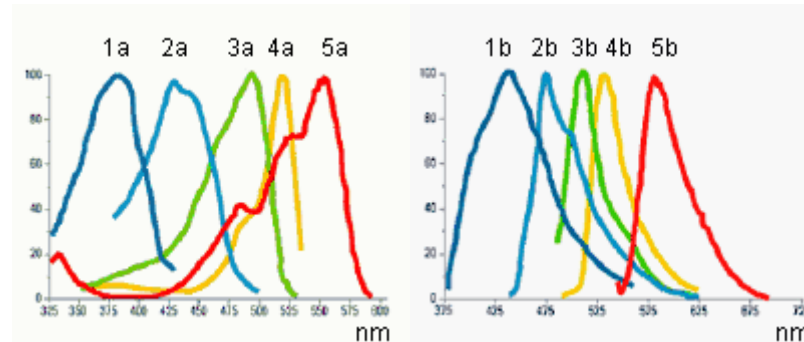
Spectral unmixing

Owing to the spectral overlapping, the aspired visual separation of the different cell structures only succeeds partially. When, for example, the light that excitation filter 1 lets through, also excites fluorochrome 2 a little, and part of the light that fluorochrome 2 then emits can pass the emission filter 1; cell structure 2 will also be dimly visible in fluorescence image 1. One can then speak of an unwanted "spectral mixing" of the individual fluorescence images.

Spectral unmixing

The spectral mixing can be subsequently removed from a digitally recorded multi-channel fluorescence image, by recalculation. That's to say, the image will be "spectrally unmixed". When you do this, it improves the visual separation of the different cell structures in the image, and improves the image quality. To do that, use the *Process > Enhancements > Fluorescence Unmixing...* command.

00360

Carrying out a fluorescence unmixing

The spectral unmixing of a multi-channel fluorescence image takes place in two steps. The first step is the calibration of the color channels with the help of reference images. When the experimental conditions don't change, you will only need to carry out this step once. In the second step, the actual spectral unmixing takes place.

You require precisely one reference image for each color channel that is to be calibrated. Each reference image must have exactly the same number of color channels as the image that is to be unmixed. In the instructions that follow, it will be assumed that you have acquired a three channel fluorescence image and want to carry out a spectral unmixing with it. For a two channel fluorescence image the procedure is analogical.

Calibrating color channels

When the experimental conditions don't change, you will only need to carry out the calibration once. When you've done that, you can spectrally unmix all of the three channel fluorescence images that are acquired later, on the basis of this calibration.

Acquiring reference images

1. Set up three samples that in each case have only been stained with one of the three fluorochromes.
   Alternatively, you can use a single sample that has been stained with all three fluorochromes. In this case, there must be three areas on the sample that have each been stained with only one fluorochrome.

2. Acquire a three channel fluorescence image of each of the three samples (alternatively, of each of the three areas on your sample).
   When you do this, use either the excitation filter appropriate for each of them, and a multiband emission filter or a multiband excitation filter and the emissions filter appropriate for each of them.
   The experimental conditions must be the same as they were when the image that is to be spectrally unmixed was acquired.

   - Differences in the exposure times of the individual color channels will be automatically linearly corrected when a spectral unmixing is carried out. Nevertheless, as a rule it makes sense not to change the exposure times when the reference images are acquired.

   - The result will be three multi-channel fluorescence images. Each of them contains three channels. These will, in what follows, be designated as "reference image 1", "reference image 2" and "reference image 3". Reference image 1 is to be used to calibrate color channel 1, that belongs to fluorochrome 1. With the reference images 2 and 3 the method is analogical.

   - Each of the reference images will be displayed in its own window, in the document group. The reference image that was last acquired will be the currently displayed, active image.

   - Should you have already acquired the three reference images at an earlier point in time, you can load them into the document group by using the *File > Open > Image...* command.

Defining ROIs

1. Activate reference image 1 in the document group.

2. In the *Life Science Applications* toolbar, click the *New ROI - 3 Points Circle* button.

   - Should the toolbar not be visible, put it on display by using the *View > Toolbars > Life Science Applications* command.

3. Search out an area in reference image 1, in which fluorochrome 1 is especially bright and glows as evenly as possible.

4. Define a circular ROI within this area with three mouse clicks.

   • This ROI was defined for the fluorochrome 1. It will be automatically assigned the name "ROI 1".

   • You can still subsequently change the size and position of this ROI.

   • You can change this automatically created name. To do so, use the *Measurement and ROI* tool window. In it, click the ROI's name to change it. Should the tool window not be visible, put it on display by using the *View > Tool Windows > Measurement and ROI* command.

5. Click the *New ROI - 3 Points Circle* button once more.

6. Search out a dark area in the background of reference image 1, in which, as far as possible, no fluorochrome can be seen.

7. Define a circular ROI within this area with three mouse clicks.

   • This ROI was defined for the image background. It will be automatically assigned the name "ROI 2".

8. Using the same procedure, define in reference image 2 an ROI for the fluorochrome 2, and an ROI for the image background.

9. Using the same procedure, define in reference image 3 an ROI for the fluorochrome 3, and an ROI for the image background.

Finishing the calibration

1. Activate reference image 1 in the document group.

2. In the *Life Science Applications* toolbar, click the *Fluorescence Unmixing* button, to open the *Fluorescene Unmixing* dialog box.

3. Activate the *Calibration* tab.

4. Enter the label for fluorochrome 1 in the *Name* field. This is, at the same time, the name for the calibration for color channel 1.

5. Select reference image 1 in the *Image* list.

6. In the *ROI* list, located immediately below the *Image* list, select "ROI 1" which was defined for the fluorochrome 1.

7. In the *Background subtraction* group, select the *ROI* option for the background correction of reference image 1.

8. In the neighboring list to the right, select "ROI 2" that was defined for the image background in reference image 1.

9. Click the *Save* button.

   • The calibration of color channel 1, has now been completed.

   • The *Fluorochrome 2 >>* button will become available.

10. Click the *Fluorochrome 2 >>* button to skip to the calibration of color channel 2.

    • The name of the *Fluorochrome 1* group will then change to *Fluorochrome 2*.

11. Then, using the same procedure, calibrate color channel 2 with reference image 2 and fluorochrome 2.

12. Then, using the same procedure, calibrate color channel 3 with reference image 3 and fluorochrome 3.

13. Click the *Cancel* button, to close the *Fluorescence Unmixing* dialog box.

Spectrally unmixing a three channel fluorescence image

1. In the document group, activate the three channel fluorescence image you want to spectrally unmix.

   • Should you have already acquired the image at an earlier point in time, you can load it into the document group by using the *File > Open > Image...* command.

2. In the *Life Science Applications* toolbar, click the *New ROI - 3 Points Circle* button.

3. Search out a dark area in the background of your image, in which, as far as possible, no fluorochrome can be seen.

4. Define a circular ROI within this area with three mouse clicks.

   • This ROI was defined for the image background. It will be automatically assigned the name "ROI 1".

5. In the *Life Science Applications* toolbar, click the *Fluorescence Unmixing* button, to open the *Fluorescene Unmixing* dialog box.

6. Activate the *Linear Unmixing* tab.

7. In the *Fluorochrome 1* list, select the calibration with which the fluorescence image in your multi-channel fluorescence image's color channel 1 is to be corrected.

   • The name of this calibration is identical with the label you gave the fluorochrome 1 while you were performing the calibration.

8. In the *Fluorochrome 2* list, select the calibration with which the fluorescence image in your multi-channel fluorescence image's color channel 2 is to be corrected.

9. In the *Fluorochrome 3* list, select the calibration with which the fluorescence image in your multi-channel fluorescence image's color channel 3 is to be corrected.

10. In the *Background subtraction* group, select the *ROI* option for the background correction of your image .

11. In the neighboring list to the right, select "ROI 1" which was defined in your image for the image background.

12. Click the *OK* button to carry out the spectral unmixing and to close the dialog box.

    • A new image document will be created for the spectrally unmixed image. The source image will not be changed.

    • It can occur that, immediately after the spectral unmixing, the image will not be optimally displayed on your monitor. In this case, click the *Apply* button in the *Adjust Display* tool window. When you do this, the image contrast on your monitor will be automatically optimized. The actual image data will not be changed.

13. Save the spectrally unmixed image if you need it.

00361

11.3. Colocalization

What is colocalization?

In the fluorescence microscopy, it can occur that the fluorescence signals emitted by two parts of a sample (e.g., molecules) that have been stained with different fluorochromes, interfere with each other. In these cases, the different parts of the sample lie very close to one another, or one over the other. The effect of the interference of fluorescence signals is termed "colocalization".

In the digital image analysis, the colocalization of fluorescence signals can be measured. This is done by detecting pixels that have the same intensity in both color channels. These measurements are carried out on multi-channel images, and are always valid for one channel pair.

Examples for colocalization

1) Superimposed signals in the green and blue color channels. The colocalized pixels are displayed in white
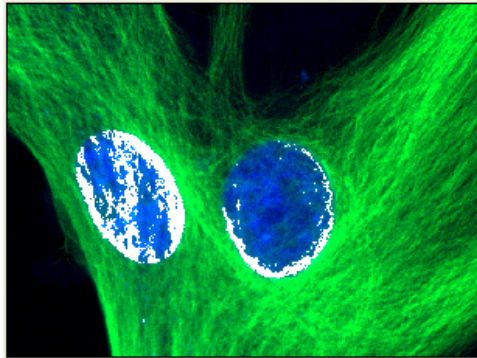
2) Superimposed signals in the blue and red color channels. The colocalized pixels are displayed in white
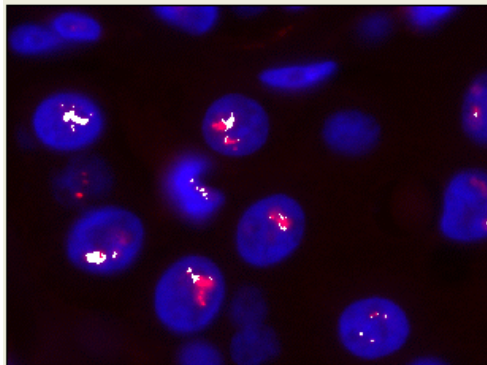
00705 26072011

Measuring the colocalization

Use the *Colocalization* button to start a measurement of colocalization. You will find this button on the *Life Science Applications* toolbar. This button isn't available in all software versions.

Measuring the colocalization on the whole frame

1. Load the multi-channel image you want to use for the colocalization measurement.

2. On the *Life Science Applications* toolbar, click the *Colocalization* button. If this toolbar is not displayed, use the *View > Toolbars > Live Science Applications* command.

    • The *Colocalization* dialog box opens.

3. In the *Channels* field, choose the two color channels for which the measurement of colocalization is to be carried out.

4. When you work with multi-channel time stacks or multi-channel Z-stacks: Determine in the *Apply on* group, whether the colocalization measurement is to be carried out on all frames or only on selected frames. Should you want to limit the image selection, select the *Selected frames* entry, then click the *Dimension Selector* button.

    • Then you can limit the image selection in the *Dimension Selector* tool window. You can find more information on this tool window in the online help.

5. In the *Target area* group, select the *Channel segmentation* entry, in the *Area* field.

6. Click the *Options...* button, then select the *Colocalization channel (Image)* and *Measurement results (Workbook)* check boxes.

7. Pay attention to the displayed results in the preview, and in the *Results* group.

8. If necessary, change the position and size of the intensity range in the scatterplot.

    • Then only the colocalization of the pixels that lie within the chosen intensity range are shown in the preview.

9. Click the *OK* button to finish the measurement of colocalization.

    • A new image that contains the colocalization channel will be created.

    • At the same time, a workbook that contains the results of the colocalization measurement, will be displayed.

10. If required, use the *File > Save as...* menu command, to save the new image and the workbook.

Measuring the colocalization on a part of the image (ROI)

Frequently, a colocalization of fluorescence signals occurs only in a small image segment. In this case, it makes sense to define a ROI (Region of Interest) then determine the colocalization only within this ROI. You can also define several ROIs. ROIs can have any shape you wish. You can find more information on working with ROIs in the online help.

1. Load the multi-channel image you want to use for the colocalization measurement.

2. On the *Life Science Applications* toolbar, click the *Colocalization* button. If this toolbar is not displayed, use the *View > Toolbars > Live Science Applications* command.

   • The *Colocalization* dialog box opens.

3. In the *Channels* field, choose the two color channels for which the measurement of colocalization is to be carried out.

4. When you work with multi-channel time stacks or multi-channel Z-stacks: Determine in the *Apply on* group, whether the colocalization measurement is to be carried out on all frames or only on selected frames. Should you want to limit the image selection, select the *Selected frames* entry, then click the *Dimension Selector* button.

   • Then you can limit the image selection in the *Dimension Selector* tool window. You can find more information on this tool window in the online help.

5. Click the *Options...* button, then select the *Colocalization channel (Image)* and *Measurement results (Workbook)* check boxes.

6. In the *Target area* group, click once in the *Area* field, to open the picklist. Select the ROI entry.

   • Next to the field, to the right, the buttons with the various ROI forms are displayed.

7. Click the button for the required ROI form that you want to set up. You have the choice between a rectangle, a circle and a polygon.

   • The mouse pointer will appear in the image window. The *Colocalization* dialog box is hidden.

8. Define the first ROI with clicks of your left mouse button. When you have completed the definition of your ROI, click your right mouse button, then select the *Confirm Input* command in the context menu.

   • You will then once more see the *Colocalization* dialog box. The ROI you have defined will now be shown in the preview image.

9. If required, define further ROIs.

10. Select the required ROIs. To do this, click once in the box to the left of the ROI's name.
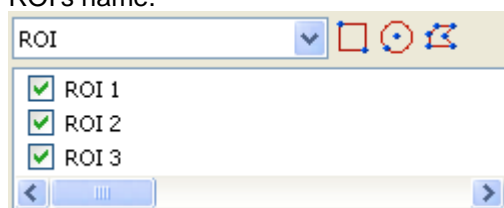
11. Pay attention to the displayed results in the preview, and in the *Results* group.

12. If necessary, change the position and size of the intensity range in the scatterplot.

    - Then only the colocalization of the pixels that lie within the chosen intensity range are shown in the preview.

13. Click the *OK* button to finish the measurement of colocalization.

    - If you haven't changed the default settings for colocalization, a new image, that contains the colocalization channel, will be created.

    - At the same time, a workbook that contains the results of the colocalization measurement, will be displayed. The columns in the workbook contain the supplement "ROI".

14. If required, use the *File > Save as...* menu command, to save the new image and the workbook.

15. The multi-channel image will also have been changed when the ROI was defined. Therefore, if you want to keep the ROI, save it also.

Measuring the colocalization on a color channel

You can also measure the colocalization on image structures. Where images are concerned on which the image structures that are to be analyzed are numerous, and are spread over the whole image, this procedure is quicker than setting a lot of ROIs. If, for example, you want to measure the colocalization on image structures that have been stained with the (blue fluorescent) fluorochrome DAPI, select the blue channel. Then define the threshold values for this channel.

The colocalization measurement on a color channel only makes sense on a multi-channel image with at least three color channels.

Example: On the image, the colocalization of the red and green pixels within the area marked in blue (cell nucleus) is to be measured. All other positions on the image where pixels colocalize are to be ignored.
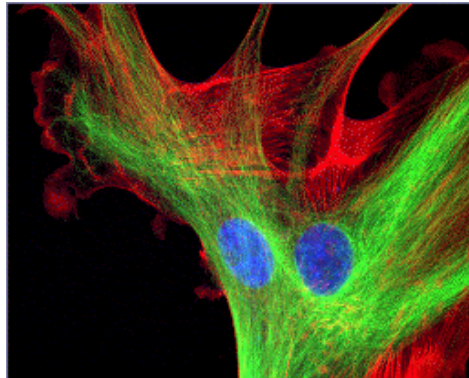
Measuring the colocalization on a color channel

1. Load the multi-channel image for which you want to carry out a colocalization measurement.

2. On the *Life Science Applications* toolbar, click the *Colocalization* button.

    - The *Colocalization* dialog box opens.

3. When you work with multi-channel time stacks or multi-channel Z-stacks: Determine in the *Apply on* group, whether the colocalization measurement is to be carried out on all frames or only on selected frames. Should you want to limit the image selection, select the *Selected frames* entry, then click the *Dimension Selector* button.

    - Then you can limit the image selection in the *Dimension Selector* tool window. You can find more information on this tool window in the online help.

4. Click the *Options...* button, then select the *Colocalization channel (Image)* and *Measurement results (Workbook)* check boxes.

5. In the *Target area* group, select the *Channel segmentation* entry, in the *Area* field.

   - In the *Area* field, a picklist with all of the available channels will open.

TRITC: 1
FITC: 2
DAPI: 3
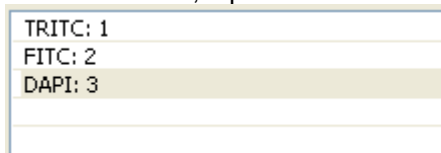
6. Select the channel of the fluorochrome with which the image structure that is to be analyzed, has been stained. In the example shown above, this is the "DAPI" channel.

7. Click the button ⌄ located next to the *Area* field, on its right-hand side.

   - A picklist with the different methods for setting threshold values, will open.

8. Select the *Automatic Threshold...* method.

   - This method requires the user to make the smallest number of settings. Therefore, you should only use the other methods for setting threshold values, when the *Automatic Threshold...* method doesn't lead to the result you wanted. You can find an overview on the threshold values in the online help.

   - The *Separation Channel Threshold* dialog box opens. Your software will carry out an automatic setting of threshold values. In the image window, you will now see the image structures that are detected by the automatic threshold settings.

9. In the *Channel* group, select the required channel again (in this case "DAPI").

10. Check in the image window, whether the automatic threshold setting has correctly found the image structures that are to be analyzed.

    - In the *Separation Channel Threshold* dialog box, select the *Dark* or *Bright* option in the *Background* group, should the *Automatic* option not lead to the results you want.
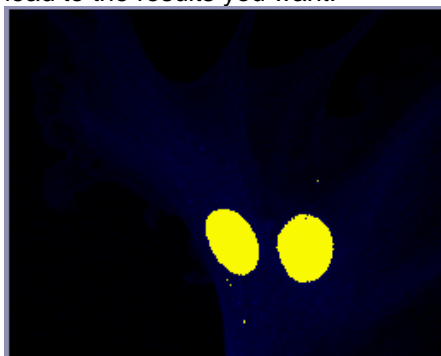
11. When the image structure that is to be analyzed has been correctly found, click the *OK* button.

- You will then once more see the *Colocalization* dialog box. In the preview, the image structures found via the Channel segmentation will now be displayed with a yellow outline. The colocalized pixels shown lie exclusively within these image structures.
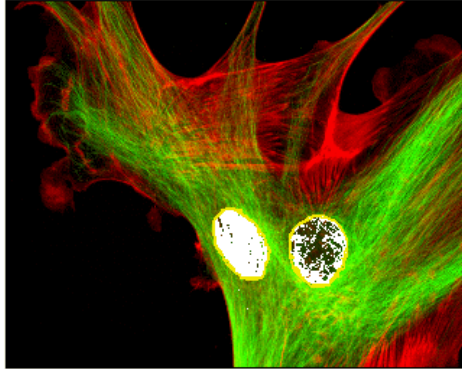

12. If required, change the position of the white rectangle (gate) in the scatterplot. By doing this you'll change the observed intensity range. You can now, e.g., have pixels with a lower colocalization shown. Pay attention to the display in the *Results* group.

13. Click the *OK* button to finish the measurement of colocalization.

- A new image that contains the colocalization channel will be created.

- At the same time, a workbook that contains the results of the colocalization measurement, will be displayed. The columns in the workbook contain the supplement "Separation channel".

14. If required, use the *File > Save as...* menu command, to save the new image and the workbook.

15. The multi-channel image will also have been changed when the Channel segmentation was defined. Therefore, if you want to keep these settings, save it also.

00709 26072011

11.4. Deconvolution

The *Process > Deconvolution* submenu offers you deconvolution filters with which you can remove disturbing diffused light from an individual image or a multi-dimensional image. With a suitable parameter selection the image will become sharper and more clear.
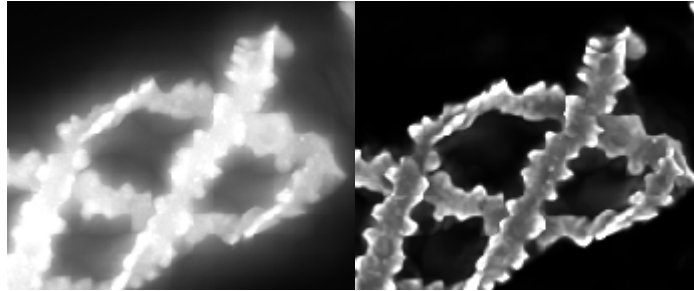
Before using a deconvolution filter

The result of a deconvolution process largely depends upon whether certain parameters are known with which the image was acquired. These parameters include for example the objective's numerical aperture and refraction index. Before using a deconvolution filter on an image, use the *Process > Deconvolution > Verify Channel Parameters...* command, to check the relevant parameters for the image, changing them if necessary. A description of this dialog box can be found in the online help.

What is deconvolution?

In fluorescence and brightfield microscopy, diffused light from areas above or below the focal plane leads to over exposure, distortion and blurring. A suitable mathematical model to describe this problem is a convolution operation:

$g(x) = f(x) * h(x) + n(x)$

x: Point in xy space

g(x): Observed image

f(x): Ideal image

h(x): Point spread function

n(x): Noise function

*: Convolution

To be able to reconstruct the ideal image f(x) from the observed image g(x), you must know the noise function n(x) and the point spread function h(x). While an estimation of the noise function n(x) is highly possible, the point spread function h(x) depends normally so strongly on the optical properties of the microscope and the sample, that an experimental determining of this function is not directly possible. For this reason, mathematical algorithms become necessary to even approximately determine the point spread function h(x) and to subsequently make the best possible reconstruction of the ideal image f(x) by means of deconvolution. A perfect, unambiguous, reconstruction is generally not possible, since information can be lost during a convolution.

The deconvolution filters

The individual deconvolution filters essentially differ in how the point spread and noise functions are determined, which are needed for the deconvolution of the image and the noise depression.
The more image data is used to calculate the point spread function, the more precise the result will be and the longer the calculation will take.

2D deconvolution

The 2D deconvolution filter uses a theoretical point spread function, in which only the acquisition parameters are used but no image data.
You can apply the 2D deconvolution filter on all supported image types. However, the 2D deconvolution filter always affects only an individual frame. As the amount of data processed is quite small, the 2D deconvolution filter is very quick. It makes the image appear much sharper but does not then permit any quantitative analysis of the image data.
The filter is especially well suited for TIRF images where the image information comes from a very narrow Z-range of the sample.

Nearest Neighbor Filter

The nearest neighbor filter employs a theoretical point spread function for the deconvolution, in the calculation of which, the data of the image under examination and of the two neighboring images of a Z-stack, are taken into consideration. The point spread function is applied to the neighboring images. The scaled sum of the neighboring images that have been processed in this way, is then deducted from the image under examination. With single images and simple time stacks the nearest neighbor filter works like a no neighbor filter. In this case, only the observed image's data are used in the calculation of the point spread function.

Wiener Filter

The Wiener filter approximates the point spread function by a linear function, with the mean square deviation being minimized. The actual filter is calculated from the linear inverse function. With Z-stacks the complete Z-stack's data are used in the calculation, with individual images only the observed image's data.

Constrained Iterative Filter

The *Constrained Iterative* filter does not make any presumptions about the point spread function, but rather extracts it directly from the Z-stack. This occurs iteratively. An estimated point spread function is used as a starting point. Then, an assumption is made as to which ideal image, via this point spread function, would have led to the observed image. Then, an estimation has to be made as to which point spread function caused the original image to be transformed into the observed image. This alternating assessment can be repeated as often as wished. Special mathematical processes are used to ensure that these iterations converge to reasonable values.

The *Constrained Iterative* filter promises the best results of all of the deconvolution filters, requires though, the most calculation time. Since the filter works iteratively on the complete Z-stack, a use on individual images or simple time stacks, is not possible.

00355 091032012

11.5. Ratio Analysis

Certain multichannel fluorescence microscopy inspection modes allow you to monitor changes in ion concentration or pH value within cellular structures. Fluorescence dyes whose excitation characteristics depend on the concentration of ions are used for this.

The Fura-2 fluorescence dye, for example, shifts its excitation level from 340 nm to 380 nm when the calcium ion concentration decreases. At an excitation wavelength of 340nm, the intensity increases when the calcium concentration increases. At an excitation wavelength of 380nm it's the exact opposite. The higher the calcium concentration is, the less light is emitted.

The process flow of a ratio analysis on a multi-channel fluorescence image

1. Acquiring a multi-channel fluorescence image

The fluorescence dye is excited with two different wavelengths, one after the other. The multi-channel fluorescence image contains two color channels created with the same fluorescence dye, but at different excitation wavelengths.

The excitation wavelengths 340 nm and 380 nm are typically used with the Fura-2 fluorescence dye.

▼

2. Carrying out background correction

A background correction is carried out on both color channels. You can make settings for the background correction in the *Ratio Analysis* dialog box. A description of this dialog box can be found in the online help.

▼

3. Calculating a Ratio Image

One color channel is divided by the other on a pixel by pixel basis. The result is the ratio image, in which the intensity is proportional to the ion concentration.

When the Fura-2 fluorescence dye is used, the image acquired at the excitation wavelength of 340 nm is divided by the image acquired at the excitation wavelength of 380 nm:
Color channel (340 nm) / Color channel (380 nm)

▼

4. Viewing a ratio image

The result is a multi-layer image. One image layer is the source image and the other image layer is the ratio image. The ratio image displays the concentration of ions using pseudo colors. The pseudo color image is superimposed on the source image so that you can see the structures in your sample and the concentration of ions at the same time.

You can change the display of the resulting image in the image window, in order to view only the ratio image for example.

The process flow of a ratio analysis on a multi-channel time stack

1. Acquiring a multi-channel time stack

▼

2. Carrying out background correction

▼

3. Calculating an intensity profile

When the ratio analysis is carried out on a multi-channel time stack, you can compute the intensity profile in addition to the ratio image. The intensity profile displays the change in concentration of ions in a particular image segment. You determine the image segment by defining a ROI. You can define the ROI right here in the *Ratio Analysis* dialog box. A description of this dialog box can be found in the online help.

You can measure the intensity profile of several image segments at the same time.

▼

4. Viewing a ratio image

The result is a multi-layer image. One image layer is the source image and the other image layer is the ratio image. The ratio image displays the concentration of calcium ions using pseudo colors. The pseudo color image is superimposed on the source image so that you can see the structures in your sample and the concentration of calcium ions at the same time.

You can change the display of the resulting image in the image window, in order to view only the ratio image for example.

00244 25072013

Carrying out a Ratio Analysis

Use the *Measure > Ratio Analysis...* command to measure the concentration of calcium ions in a time stack. This command is also available as a button on the *Life Science Applications* toolbar.

Measuring changes in the concentration of calcium ions in a time stack.

Task

The Fura-2 fluorescence dye makes it possible to measure the concentration of free calcium ions because its excitation level shifts from 340 nm to 380 nm as the calcium ion concentration decreases. Use the ratio analysis to compute the ratio image over time and the intensity profile in two cells.
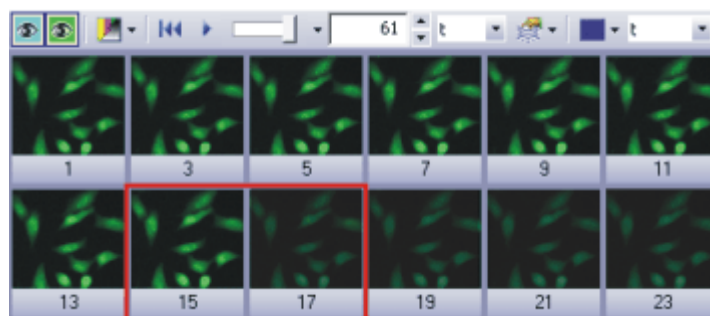

The image displays an overview of the frames in a multi-channel time stack that has 2 color channels. The sample has been dyed with the Fura-2 fluorescence dye. Between the frames that are framed in red in the illustration, the image intensity decreases visibly. The cause is a change in the calcium ion concentration.

Preparing the analysis

1. Several example images were supplied together with your software. You can follow these step-by-step instructions using the Fura.tif example image. This example image is a multi-channel time stack image.

2. Use the *View > Toolbars > Life Science Application* command, to have the *Life Science Application* toolbar displayed. You can find the functions for defining ROIs and for Ratio Analysis on this toolbar.

Defining ROIs (Region Of Interest)

3. Click the *New ROI - Polygon* button on the *Life Science Applications* toolbar.

4. Draw a rectangle inside a cell.

5. Define another ROI in a different cell.

6. Define another ROI in a dark image segment that has no fluorescing objects. This ROI will be used as a reference for the background correction.

7. Rename the ROIs you defined.
   To do this, open the *Measurement and ROI* tool window. In the *Measurement and ROI* tool window, double-click on the first ROI's name. Enter a descriptive name for the ROI. Name the ROIs *Cell01*, *Cell02* and *Background*, for example.
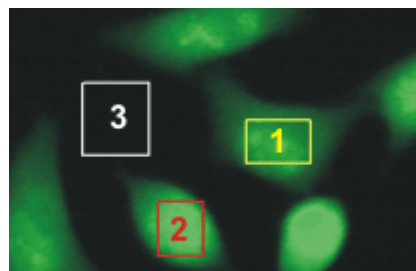

Three ROIs have been defined on the image. The red and yellow ROIs contain cells. The white ROI is on the background.

Carrying out a Ratio Analysis

8. Click the *Ratio Analysis* button located on the *Life Science Applications* toolbar.

- The *Ratio Analysis* dialog box opens.

9. Make the settings for the background correction in the *Background* group. Select the *ROI* option. In both lists, select the reference ROI for the background correction.

10. In the *Ratio* group, select the parameters for the calculation of the ratio image. Select the *Fura340* color channel from the *Numerator* list and the *Fura380* color channel from the *Denominator* list.

    - The preview image in the *Ratio Analysis* dialog box displays the ratio image that has been computed for the time point that is currently displayed in the image window. The ratio image is the result of dividing the intensity of the Fura340 color channel by the intensity of the Fura380 color channel.

    - The ratio image is a gray-value image which automatically has a predefined pseudo color table applied to it in the preview window. High ratio values are displayed in red with this pseudo color table and low ratio values are displayed in magenta.

    - The ratio image has single pixels with a high intensity in the background. This is image noise.

11. In the *Thresholds* fields, increase the value until the image noise disappears and only the cells remain visible.

12. In the *Scale* list, accept the value of 1000 that is given.
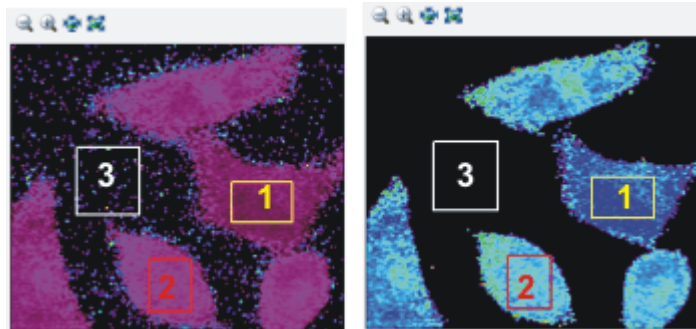
On the **left** you can see the preview image before the threshold values were set. On the **right** you can see the preview image after the threshold values were set. The image background is now black. The colors in the preview image have changed because the image in the preview window is always displayed with the most possible contrast.

Viewing the results

13. In the *Output* group, select the *Image as new layer* and *Intensity Profile* check boxes.
    If you want to output the intensity profile as a sheet, select the *Export to workbook* check box.

14. Select the ROIs that you defined on the cells. The ROIs you have selected are highlighted in the dialog box.

15. Close the *Ratio Analysis* dialog box with *OK*.

    - If it wasn't already displayed, the *Intensity Profile* tool window is displayed automatically now. The tool window contains two intensity profiles, one for each ROI you defined.
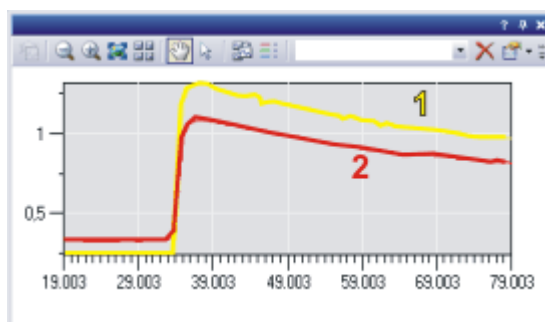
The intensity profiles show how the ratio value in both ROIs (**1**) and (**2**) changes over time. The colors of the intensity profiles correspond to the colors of ROIs they describe.

- The source image is now a multi-layer image and displays the concentration of calcium ions in addition to the image information.

16. Use the *File > Save As...* command, to save the resulting image. Save the resulting image in the TIF or VSI file format.

Setting the display of the ratio image.

1. Carry out a ratio analysis.

    - The image resulting from a ratio analysis is a multi-layer image. One image layer is the source image and the other image layer is the ratio image. There are several ways of displaying the resulting image on the monitor.

2. Use the *View > Tool Windows > Layers* command to make the *Layers* tool window appear. You have access to the individual image layers in the *Layers* tool window.

3. Select the *View > Tool Windows > Adjust Display* command to make the *Adjust Display* tool window appear. In the *Adjust Display* tool window, you can specify how an image is displayed on the monitor.

4. Activate the image resulting from the ratio analysis in the document group.

Browsing the time stack

1. Take a look at the peak in the intensity profile in this time stack. To do this, use the navigation bar at the top of the image window.

2. If the info stamp isn't displayed in the image window, use the *View > Info Stamp* command to display it.

    - The info stamp should display the time for each frame.

    - If this time is not on display, select the *Tools > Options...* command. In the tree view, select the *Info Stamp > Properties* entry. In the list of available properties, select the *Image > t* check box. Close the dialog box with *OK*.

Viewing the ratio image and the source image separately

You can view only the ratio image or only the source image in the image window whenever you want.

1. In the *Layers* tool window, click once on the source image to select this image layer. The name of the image layer in the tool window corresponds to the name of the image.

2. Click once on the eye icon next to the ratio image.

- The ratio image is now not displayed in the image window. You see only the source image.

3. In the *Layers* tool window, click once on the ratio image to select this image layer.

- When you select an image layer in the *Layers* tool window, this image layer is automatically displayed.

4. Click once on the eye icon next to the source image.

- The source image is now not displayed in the image window. Now you see only the ratio image.

The illustration shows the *Layers* tool window with the image resulting from a ratio analysis.
On the left, the ratio image (**1**) is not displayed. On the right, the source image (**2**) is not displayed.

Optimize the display of the ratio image

1. Display only the ratio image in the image window.

2. Use the *Adjust Display* tool window to optimize the display of the ratio image.

3. Select the *Auto Contrast* option.
This makes sure that the ratio image is displayed in the image window with the most possible contrast. With this setting, all the colors in the pseudo color table you are using are applied to the ratio image.
The *Histogram of all frames* check box decides whether only the frame in the time stack that is currently on display will have its contrast optimized or whether the contrast will be optimized across all the frames in the time stack.

4. Select the *Histogram of all frames* check box.
Your software now takes the smallest and largest values in all the frames and assigns the colors black and red to these values.

5. Click the *Apply* button to make the changed settings visible in the image window.
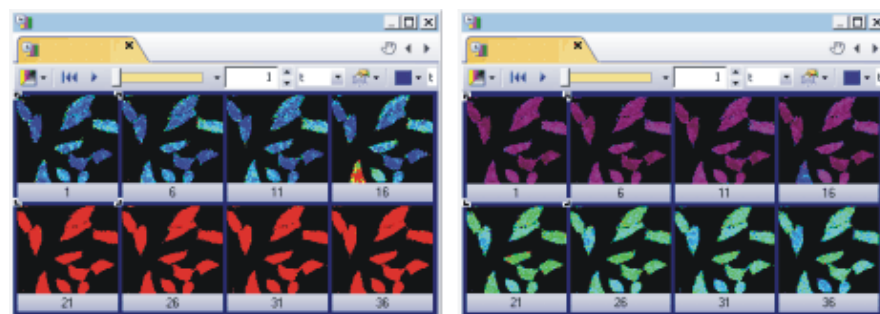
The illustration shows the same ratio image where different settings have been made in the *Adjust Display* tool window.
On the **left**, the contrast has been optimized for the first frame. The *Histogram of all frames* check box was clear. Because the values in the ratio image increase over time, the color shifts increasingly towards red.
On the **right**, the contrast was optimized across all frames. The *Histogram of all frames* check box was selected. With this setting, differences in the ratio image can be seen in all frames.

1. Display all the image layers in the image window. In the *Layers* tool window, you can see an eye icon next to each image layer.

2. Select the ratio image in the *Layers* tool window, and click your right mouse button to open a context menu.

3. Select the *Mode > Intensity Modulation* command from the context menu. If this mode is already set, keep it.

   - The colors in the ratio image remain unchanged and their color value reflects their ratio value.

   - The intensity of the ratio image is adjusted to the intensity of the source image. Where there is a low intensity in the source image, the ratio image is also dark.
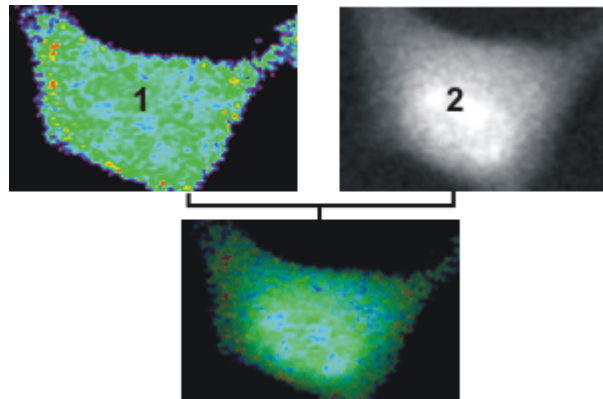

Image (**1**) is only the ratio image. The colors are all equally bright. Image (**2**) is the source image. Your software hasn't applied color mapping to it.
In the image at the bottom, the intensity in the ratio image corresponds to the intensity in the source image. The intensity decreases noticeably towards the edges of the cell.

00245 25072013

12. Measuring images

Your software offers a wide range of measurement functions. They enable you to quickly count objects and measure segments and areas. All the results will be saved together with the image and can also be issued as a sheet.

Preconditions

For making measurements, correctly calibrated images are an essential prerequisite. Images that you have acquired with your software will have been automatically correctly calibrated when you have specified the objective you used.
Should the image not yet have been calibrated, use the *Image > Calibrate Image...* command to carry out a calibration.

Selecting the measurement environment

Measuring with help of the tool window

Switch to the *Count and Measure* layout when you want to measure images. You can find the *Measurement and ROI* tool window in the bottom section of this layout. In this tool window you have fast access to all measurement functions and settings which relate to the measurement. This tool window is at the same time the measurement display and contains all of the values that have been measured on the active image.

Note: Should, right at the bottom of the user interface, several tool windows lie one over the other, activate the *Measurement and ROI* tool window, by clicking on the header of the 🖅 *Measurement and ROI* tab. The tabs can be found under the tool windows.

Measuring with help of the toolbar

Should you need more room for displaying the image, you can also use the *Measurement and ROI* toolbar instead of the tool window. All the measurement functions can also be found on the *Measurement and ROI* toolbar. To have this toolbar displayed use the *View > Toolbars > Measurement and ROI* command. Begin a measurement by simply clicking the appropriate button. In this case you will only see the measurements that you have carried out in the image.

Measuring with help of menu commands

The *Measure* menu also contains all of the measurement functions. To start a measurement, simply use the corresponding menu command. In this case you will only see the measurements that you have carried out in the image.

Starting a measurement

Begin a measurement by selecting the measurement function you want. You will find the measurement function in the *Measurement and ROI* tool window, on the *Measurement and ROI* toolbar, or in the *Measure* menu.

Working in the measurement mode

As soon as you have clicked a measurement function, your software will automatically switch to a measurement mode. In the measurement mode your mouse pointer will take on the shape of a cross on the image. You can make as many measurements as you like with the measurement function that has been selected. The continuous measurement mode is valid for all loaded images. You can, therefore, easily measure numerous images one after the other.
The selected measurement function's button will keep its clicked appearance and in this way show you the current measurement function. You can recognize this status by the button's background color.

Finishing the measurement mode

You will remain in this measurement mode until you explicitly switch it off. To do so, click the *Select Measurement Objects* 🖅 button. You can find the button either in the *Measurement and ROI* tool window or on the toolbar.

Changing the default measurement mode

The continuous measurement mode described above is preset by default. You can change this default setting. To do this, use the *Tools > Options...* command. Select the *Measurement and ROI > General* entry in the tree view. Select the *Switch to 'Select' mode after creation* check box. Then when you have completed

a measurement, you will automatically leave the measurement mode again. This means you have to select the measurement function again before you start each interactive measurement.

Displaying and saving measurement results

The measurement results will be displayed directly on the image and in the *Measurement and ROI* tool window. Use the *View > Tool Windows > Measurement and ROI* command to have the tool window displayed.

Saving the measurement results

The measurements will be saved along with the image, if you save the image in the TIF or VSI file format.

You can, however, also export the measurement results in a results sheet, and save this as a file. To do this, use the *Export to Excel* or *Export to Workbook* command.

Showing and hiding measurement results in an image

The measurement results will be shown on the image in a special data layer, the measurement layer. On your monitor, image and measurement layer are shown together. The data of each, however, is individually stored if you use the TIF or VSI image file format. Try and picture the measurement layer as a transparency which is placed over the image. When you measure an image, the image data will not be changed by having the measurement results displayed on it.

You can, at any time, hide or show the measurement layers.
To do so, use the *Layers* tool window. There you have access to all of an image's layers. The eye icon 👁 identifies all of the layers that are currently on display on your monitor.
Click the eye icon in front of the measurement layer to hide the measurements. Click an empty cell without an eye icon to make the corresponding layer reappear.

Setting the unit for the measurement results

The unit in which the measurement results will be issued, is determined by the measurement function that has been selected and the image's calibration. But you can choose whether the length e.g., is shown in mm or μm.
Use the *Tools > Options...* command. Select the *Measurement and ROI > Results* entry in the tree view. Select the unit you want to use from the *Prefix of the unit* list.

Outputting measurement results in a sheet

You can export the measurement results from the *Measurement and ROI* tool window as a sheet, for example, to be able to save the measurement results in their own file, independently of the image. You will find the functions, e.g., as a button next to the measurement functions on the *Measurement and ROI* tool window's toolbar.

Click the *Export to Workbook* 📋 button, to export measurement results from the *Measurement and ROI* tool window's results sheet to a workbook. Use this export possibility to save the measurement results in a file format that you can at any time load and edit with your software.

Click the *Export to Excel* 📄 button, to export the results to a MS-Excel sheet. Use this export possibility, for example, when you want to evaluate the measurement results still further. This will also enable you to supply the results to other users who don't have your software.

Measuring in the live mode

All of the measurement functions are also available in the live-image. You can therefore, e.g., quickly measure a segment in the live-image.

Measuring on different image types

Measuring on multi-dimensional images

You can combine a series of individual images into one image. What results is e.g., a time stack in which all of the frames will have been acquired at different times.

You can make measurements on every separate image. Display the required frame on your monitor. To do this, use the navigation bar in the image window. Then carry out the measurement on this frame. The measurement will be permanently linked to this frame, i.e., the measurement will only be displayed on your monitor when the frame on which you made this measurement is also on display.

The measurement results will be shown in the *Measurement and ROI* tool window. You can give every measurement the number of the frame on which it was made. To do so, use, e.g., the measurement parameter "Index t" for time stacks.

Measuring on multi-channel images

A multi-channel image is made up of individual fluorescence images. For multi-channel images you can choose to measure on each fluorescence image separately or to define one measurement object for all color channels simultaneously.

Clear the *Tools > Options > Measurement and ROI > General > Measure on all channels* check box.

Now, you will measure on each fluorescence image separately. To do so, set up the color channel you want on your monitor. To do this, use the navigation bar in the image window. Then carry out the measurement on this image. The measurement will be permanently linked to this color channel, i.e., the measurement will only be displayed on your monitor when the color channel on which you made this measurement is also on display.

The measurement results will be shown in the *Measurement and ROI* tool window. You can give every measurement the name of the color channel on which it was made. To do this, use the "Channel" measurement parameter.

Select the *Tools > Options > Measurement and ROI > General > Measure on all channels* check box.

Now, each measurement object you define will be measured on each color channel. All measurement results will be shown in the *Measurement and ROI* tool window.

Measurement precision

How precise the measurement is, depends on the X/Y-calibration and the image's current zoom factor.

Influence of the X/Y-calibration

The X/Y-calibration defines the width and height of the sample area that is represented by one pixel. For example, it could be that one pixel displays a sample area of 10 µm x 10 µm. A pixel is the smallest image structure that can be measured. For this reason, the maximum measurement precision where this example is concerned, is 10 µm.

Influence of the zoom factor

The zoom factor tells you how large the image will be displayed on your monitor. With a zoom factor of 100%, one pixel on the monitor equals exactly one pixel in the image. With a zoom factor of 50%, one pixel on the monitor equals 2 x 2 pixels in the image. When you make a measurement, you should use the zoom factor 100% whenever possible. Then you will achieve a maximum of measurement precision. Should the zoom factor 100% not be possible, because the image area you want to measure can't then be completely seen, choose the largest possible zoom factor under 100%.

Information about changing an image's zoom factor can be found in the online help.

00150

12.1. Measuring images

Your software offers a wide range of measurement functions. They enable you to quickly count objects, and measure distances and areas on an image.

The following step-by-step instructions present the measurement functions to you by way of several examples.

## Measuring image objects interactively

Task
> You want to measure the diameter of some cells.
> To do this, load a suitable image, or acquire one.
> Subsequently edit the measurement. Delete some of the measurements you've made. Enter the results in a MS-Excel sheet.

1. If necessary, use the *View > Tool Windows > Measurement and ROI* command to have the *Measurement and ROI* tool window displayed.

   - You'll find the tool window at the lower edge of the user interface. It's possible that it may be covered by the *Count and Measure Results* tool window. Click the *Measurement and ROI* tab at the bottom of the user interface to bring the tool window into the foreground.

Loading an image
2. Acquire an image or load one.
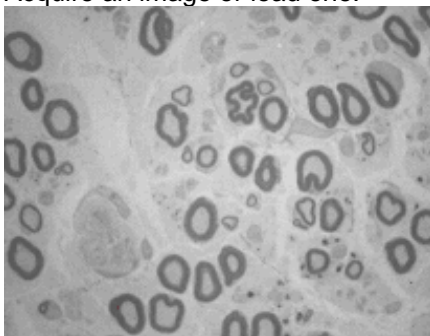
   - During the installation of your software some sample images have been installed, too. You can follow these step-by-step instructions for measuring images when you use the exemplary image "Neurons.tif".

Setting the labeling color
The measurement results will be written into the image according to the default settings, in red font color and without a background. This can be hard to read on some images. Change the labeling settings.

3. Use the *Tools > Options...* command.

4. Click the *Measurement and ROI > Measurement Display* entry in the tree view.

5. Click in the *Background Color* field, and choose, e.g., the color Black.

6. Select the *Text color > Fixed colors* option, then select the color White from the palette, to see the measurements in white and the labeling in black, in the image.

7. Close the dialog box with *OK*.

Measuring lengths
8. Click the *Arbitrary Line* ⟋ button, located on the toolbar at the top of the tool window.

9. Click with your left mouse button at the starting point and end point of the reference distance.

10. If you have measured a reference distance, you can immediately proceed with the next measurement.

11. Click the *Arbitrary Line* ⟋ button again, to end the length measurement.

12. Take a look at the results in the tool window and in the image.

- The illustration shows the image with three executed measurements. The measurement 2 has been selected.
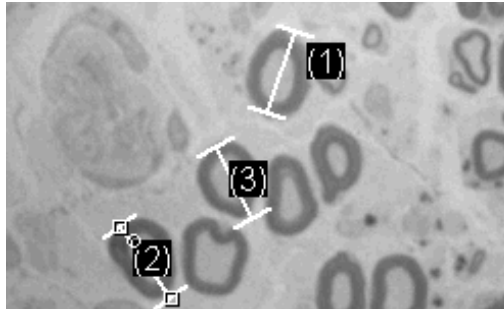
Deleting measurements   13. Click one of the measurement results in the *Measurement and ROI* tool window.

- The corresponding line will be marked in the image.

14. Press the [Del] key.

- The measurement will be deleted both in the image and in the tool window.

- When a measurement has been deleted, the tool window contains one measurement less. The IDs of the remaining measurements won't be changed by the deletion of a measurement.
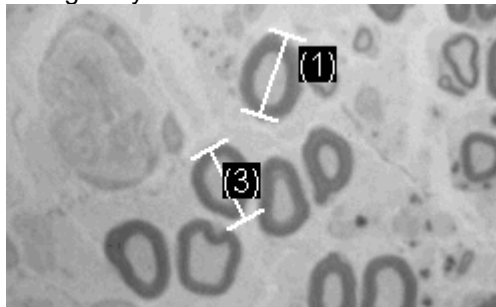
When you've completed the measurements, you should switch off the measurement mode, since otherwise, you might inadvertently select your measurements and move them.

15. Check whether one of the buttons on the *Measurement and ROI* tool window's toolbar appears clicked. Release this button

Exporting results to MS-Excel   16. To do this, click the *Export to Excel* button.

17. In the In/Output dialog box you set up the directory in which the data is to be saved, and enter the name of the MS-Excel sheet. Adopt the file type "Excel-Sheet (*.xls)".

18. Click the *Save* button to have the MS-Excel sheet with the measurement results saved.

Closing the image   19. Click the *Close* button, located at the top right of the document group.

- You have changed the image because you've added interactive measurements. For this reason, you'll receive a query whether you wish to save the image or not.

20. Save the image in the TIF or VSI file format. The measurements will then also be saved in the image file. They can at any time, be edited deleted or augmented.

Outputting various measurement parameters

Task   You want to measure some cells.
Have a variety of measurement parameters, such as the area, the perimeter and
the diameter, output. Have the diameter shown in the image.

1. Acquire an image or use the example image "BadTissue.tif".

Measuring areas

2. In the *Measurement and ROI* tool window, click the *2 Points Circle*
button.

3. Leftclick the center point of a cell that you want to measure.

4. Move your mouse, and in the process drag out the circle. Match the circular
object as well as possible to the cell. Click the left mouse button.

5. Click the *2 Point Circle* button again, and switch off the measurement
mode.

6. Take a look at the result in the *Measurement and ROI* tool window.

   - The illustration shows the image with a circle measured.
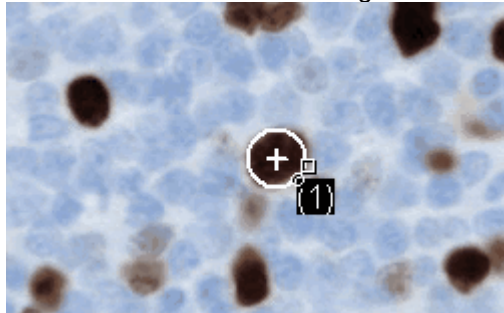
Viewing the list of measurement parameters

7. In the *Measurement and ROI* tool window, click the *Select Measurements*
button.

   - In the dialog box you'll see a list with all of the available measurement
     parameters. At the bottom of the dialog box you'll see a list of the
     measurement parameters that are currently calculated for all objects.

   - A detailed description of this dialog box can be found in the online help.

Outputting additional measurement parameters

8. Go to the list of all of the available parameters, then click the *Diameter*
measurement parameter.

   - On the right, an illustration shows you how the parameter is calculated.
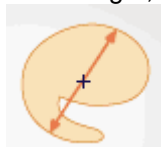
   You can see that there are different ways in which the diameter of a 2D
   object can be calculated.

9. Click the *Mean* entry in the list under the illustration to select the *Mean
(Diameter)* measurement parameter. When you do this, the mean value of all
of the possible diameters is determined.

10. Click the *Add 'Mean (Diameter)'* button.

    - This measurement parameter will be added to the list of measurement
      parameters to be calculated. All of these measurement parameters will
      be displayed in the tool window.

11. Close the dialog box with *OK*.

12. Take a look at the result for the circle's diameter in the *Measurement and
ROI* tool window.

13. Open the *Select Measurements* dialog box.

14. At the bottom of the list of all of the calculated measurement parameters, click the *Mean (Diameter)* measurement parameter.

15. To the right of this list you'll see a button with a blue arrow 🔼. Click this button to move the measurement parameter to the top of the list.

16. Close the dialog box with *OK*.

17. Take a look at the result for the circle's diameter in the image.

> Note: The measurement display in the image has to be updated once, so that the settings that have been changed are also taken into account. You update the measurement display, for instance, by adding another measurement, or by once selecting an existing measurement in the image.

Measuring several images

Task

> You want to measure cells on multiple images. To do so, acquire some images and measure them one after another. Have the results from all images displayed simultaneously. Take a look at the mean value for all of the measurements.

Loading images

1. Acquire some images or load some.
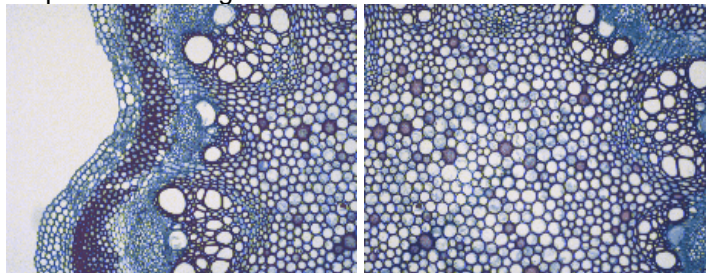
- During the installation of your software some sample images have been installed. You can carry these step-by-step instructions out directly with the example images "Clematis04.tif" and "Clematis05.tif".

Measuring cells

2. Activate the first image in the document group.

3. Click the *Arbitrary Line* ✒ button, located on the toolbar at the top of the *Measurement and ROI* tool window. Measure the diameter of several cells.

4. Activate the next image. Measure the diameter of several cells on this image, too.

5. Click the *Arbitrary Line* 📏 button again, and switch off the length measurement.

- Cells have been measured on both images.
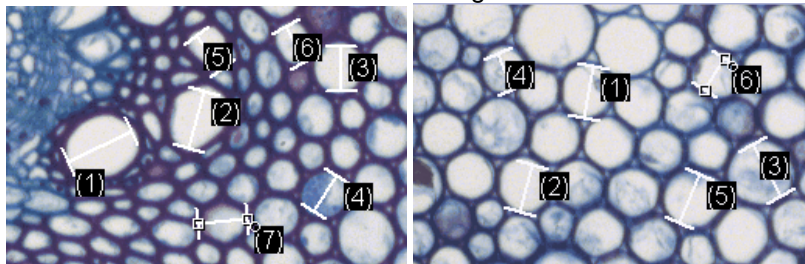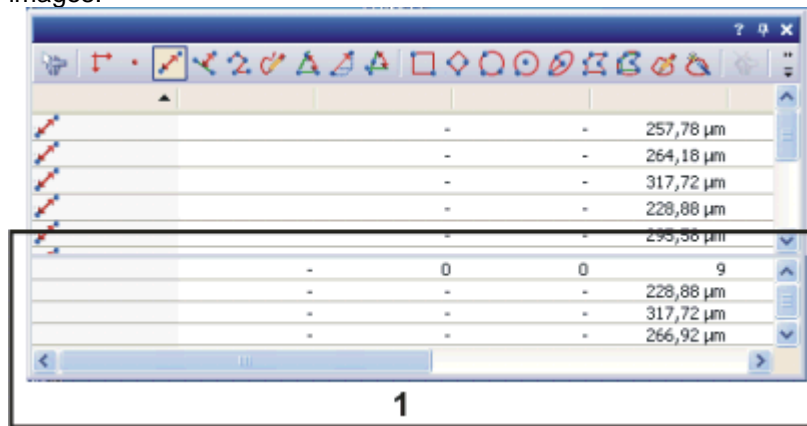
Displaying the measurement results of all of the images

6. In the *Measurement and ROI* tool window, click the *Measurement and ROI Options* 🖼 button.

7. Select the *Measurement and ROI > Results* entry in the tree view.

8. Clear the Show measurement objects: Only of the active image check box.

9. Close the dialog box with *OK*.

- Now the results for both images will be shown simultaneously in the tool window.

Viewing the statistical parameter

10. In the *Measurement and ROI* tool window, click the *Measurement and ROI Options* button.

11. Select the *Measurement and ROI > Results* entry in the tree view.

- In the *Statistic* group, you'll find various statistical parameters.

12. Select the *Mean* check box.

13. Close the dialog box with *OK*.

- Now, in the *Measurement and ROI* tool window under the measurement results, the chosen statistical parameter (1) will by shown. You can see there the mean value of the layer thickness for all of the measured images.

00154

12.2. Performing an automatic image analysis

You can use an automatic image analysis to perform numerous measurement tasks. Several typical tasks and their process flow are described here.

Counting objects

Task

You have an image with objects that interest you. You want to know how many of these objects there are in the image.
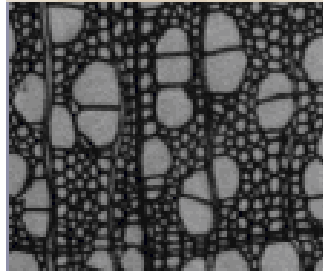
Preconditions

The objects that you want to count must not be connected, but must be clearly separated from one another. The foreground or the objects should be optically clearly separated from the image's background. In the example image the background is dark, and the foreground bright.

1. Use the *View > Tool Windows > Count and Measure* command to have the *Count and Measure* tool window displayed.

2. Acquire an image or load one.

   - During the installation of your software some sample images have been installed, too. You can immediately follow these step-by-step instructions when you use the exemplary image "WoodVessels.tif".

Setting options

3. Open the *Options* dialog box by clicking the *Count and Measure Options* button, located in the *Count and Measure* tool window.

4. Select the *Detection* entry in the tree view.

5. In the *Options* group, enter the value 5 in the *Minimum object size* field, to specify the minimum object size. By doing that, you will rule out the possibility that individual pixels, that may well belong to the phase, but not to an object, are counted as objects, which would then falsify the results.

6. Click *OK* to close the *Detection* dialog box.

Setting threshold values

7. In the *Count and Measure* tool window, click the *Automatic Threshold...* button to open the *Automatic Threshold* dialog box. Should the *Automatic Threshold* button not yet be active, you will have to first activate it. To do so, select the *Automatic Threshold...* entry in the *Threshold* button's menu. You open this menu by clicking the small arrow next to the button.

   - The threshold values are set automatically in the *Automatic Threshold* dialog box.

   - All of the objects that have been detected will be displayed in color.

8. Check whether the objects have been correctly recognized.

   Should the objects not have been correctly recognized, go to the *Background* group and enter whether the background is bright or dark.
   Select e.g., for the image shown above, the *Background > Dark* option, since the image shows bright objects against a dark background.

9. Delete all but one of the phases by continuing to click the *Remove Phase* button until the button becomes inactive.

    *   By doing that, you will make certain that no phases from earlier analyses are still defined.

Viewing the results

10. To obtain the results, click the *Count and Measure* button in the *Automatic Threshold* dialog box.

    *   The *Automatic Threshold* dialog box will be closed.

Note: You can also set up your software so that it doesn't automatically output the results of the analysis as soon as the threshold value settings have been made. To do this, use the *Tools > Options > Count and Measure > Detection* command. Clear the *Execute the segmentation after setting parameters* check box. Then close the dialog box for setting the threshold values, with OK, and when you've done that, click the *Count and Measure* button in the *Count and Measure* tool window, to have the results output.
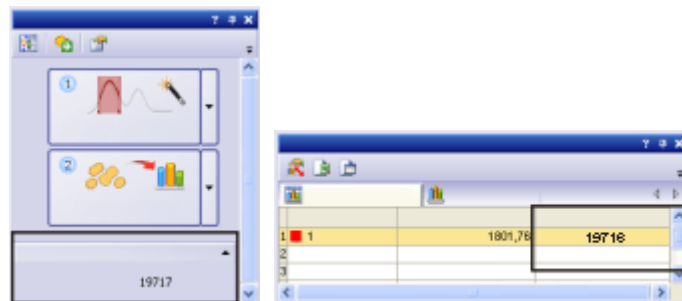
The number of objects detected will be shown below, in the *Count and Measure* tool window, in the Object Count group. Should you not be able to see this number, click the small black arrow to make it visible. If you have selected the *Object Count* measurement parameter, the number of objects will also be displayed in the *Count and Measure Results* tool window's results sheet.

Counting objects that belong to different phases

Task You have an image on which you define two phases. You want to know how many objects there are per phase, in the image.
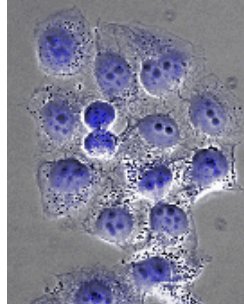
In the image, two phases are to be defined. The first phase is to map the black objects within the blue, round object. The second phase is to map the blue, round objects.

Preconditions The objects that you want to count must not be connected, but must be clearly separated from one another. The objects in both of the phases must have different intensity values by which one can differentiate between them.

Setting options

1. In the *Count and Measure* tool window, click this ⬚ button, to open the *Options* dialog box.

2. Select the *Detection* entry in the tree view.

3. In the *Options* group, enter the value 5 in the *Minimum object size* field, to specify the minimum object size. By doing that, you will rule out the possibility that individual pixels, that may well belong to the phase, but not to an object, are counted as objects, which would then falsify the results.

4. Select the *Measurements* entry, in the tree view. From the *Class Measurements* list, select the *Object Count* and *Object Class* entries.

5. Click *OK* to close the *Detection* dialog box.

Setting threshold values

6. In the *Count and Measure* tool window, click the *Manual Threshold...* button, to open the *Manual Threshold* dialog box. Should the *Manual Threshold* button not yet be active, you will have to first activate it. To do that, select the *Manual Threshold...* entry, in the *Threshold* button's context menu. You open this menu by clicking the small arrow next to the button.

7. Delete all but one of the phases by continuing to click the *Remove Phase* button until the button becomes inactive.

   • By doing that, you will make certain that no phases from earlier analyses are still defined.

8. Doubleclick the *Phase Name* field and assign a name for the first phase.

9. Click any position outside this field, or press the [Enter] key, to leave the field again.

   • The first phase in the *Phase thresholds for channel '...'* group will be automatically selected.

10. Click on the *New Threshold* ⬚ button, to set an initial value for the selected phase's threshold value range.

    • As soon as you move your mouse pointer onto the image it will change its shape to that of a pipette.

11. Click on one pixel or on the image area whose intensity value is to be utilized as the initial value for the threshold range.

- Once the initial value has been set, your mouse pointer will automatically change into a pipette with plus icon.

12. Then, continue clicking pixels that are typical of the first phase, until the required structures in the image are a part of the phase.

13. Should too many pixels have been selected, click the *Shrink Threshold* button, to have these pixels excluded from the phase again.

- The threshold value range will continue to be reduced until it no longer contains the pixels you have selected.

14. Click the *Add Phase* button to add the second phase, then proceed exactly as you did for the first phase.

Viewing the results
15. To obtain the results, click the *Count and Measure* button in the *Manual Threshold* dialog box.

- The *Manual Threshold* dialog box will be closed.

16. Open the *Count and Measure Results* tool window by using the *View > Tool Windows > Count and Measure Results* command.
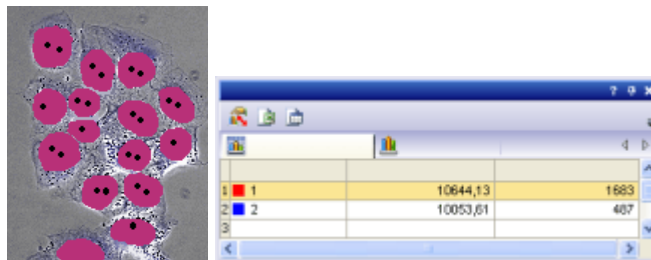
The total number of objects detected in all of the phases will be shown below, in the *Count and Measure* tool window, in the *Object Count* group. The results for the *Object Class* and *Object Count* measurement parameters, that's to say, the sum of the objects per phase, will be displayed in the results sheet. Furthermore, you will recognize the phases by the colors that have been assigned to them. You can compare the results for both of the phases directly with each other.

Determining the area ratios of several phases

Task   You have a multi-channel image with 3 channels. You want to know how large the area ratio of the individual fluorescence channels is.
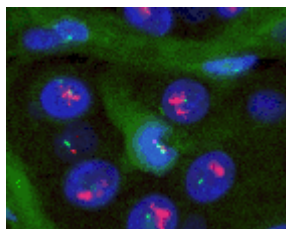
Preconditions   The analysis is performed on a fluorescence (multi-channel) image. In the previously installed image directory, you will find a multi-channel image that you can use for this, under the file name "HER2 (3x16-bit).tif".

Setting options

1. In the *Count and Measure* tool window, click this [button icon] button, to open the *Options* dialog box.

2. Select the *Detection* entry in the tree view.

3. In the *Options* group, select the *All frames and channels* option. Now the analysis will be carried out for all of the color channels simultaneously.

4. In the *Options* group, enter the value 1 in the *Minimum object size* field, to specify the minimum object size. By doing this, you will make certain that individual pixels that belong to the phase will be taken into account in the area ratio.

5. Select the *Measurements* entry, in the tree view. From the *Class measurements* list, select the *Object Class* and *Sum (Area)* entries.

6. Click *OK* to close the *Options* dialog box.

Setting threshold values

7. In the *Count and Measure* tool window, click the *Automatic Threshold...* button to open the *Automatic Threshold* dialog box.

    •   The three channels will be automatically displayed in the dialog box under their names. The threshold values are automatically set.
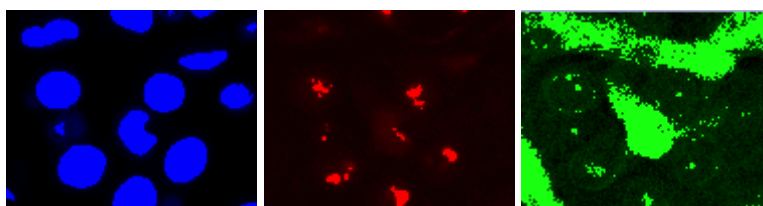
In the illustration, the channels are displayed individually. One can easily recognize the phases in each channel.

8. Make sure that only one phase has been defined for each channel. To do this, you will have to select each channel once in the *Channel* group. Should more than one phase per channel have been defined from an earlier analysis, delete the superfluous phases by clicking the *Remove Phase* button.

Viewing the results

9. To obtain the results, click the *Count and Measure* button in the *Manual Threshold* dialog box.

    •   The *Manual Threshold* dialog box will be closed.

10. Open the *Count and Measure Results* tool window by using the *View > Tool Windows > Count and Measure Results* command.

    •   The results will be shown in the results sheet for every color channel. You can identify the channels in the *Object Class* column. You then have

a direct comparison of how much area is taken up by which channel or phase.

Determining how the number of objects changes over a period of time

Task   You have a time stack, and want to know how the number of objects changes over a period of time.

Preconditions   The analysis must be performed on a time stack. The objects that you want to count must not be connected, but must be clearly separated from one another. In the previously installed image directory, you will find a time stack that you can use for this, under the file name "ParameciumTimeSeries.tif".
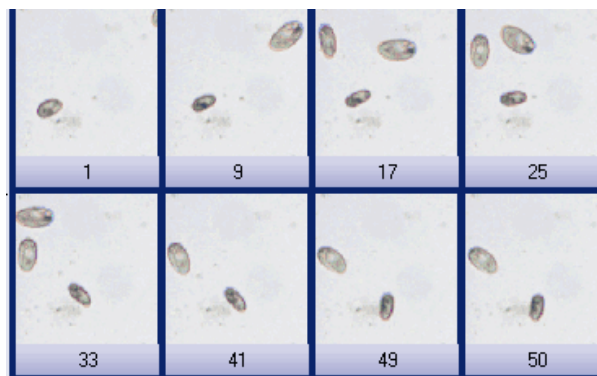
The illustration shows a time stack that comprises several images. The number of objects changes over the time period.

Setting options

1. In the *Count and Measure* tool window, click this [icon] button, to open the *Options* dialog box.

2. Select the *Detection* entry in the tree view.

3. In the *Options* group, select the *All frames and channels* option. Now the analysis will be carried out for all of the frames.

4. In the *Options* group, enter the value 200 in the *Minimum object size* field, to specify the minimum object size. By doing that, you will rule out the possibility that individual pixels, that may well belong to the phase, but not to an object, are counted as objects, which would then falsify the results.

5. Select the *Measurements* entry, in the tree view. From the *Class Measurements* list, select the *Object Count* and *t-Value* entries.

6. Then switch to the *Results* tab.
   Make sure that the *Show results only of the active frames* check box has been cleared.
   Make sure that the *Accumulate class results over time* check box has been cleared.

7. Click *OK* to close the *Detection* dialog box.

Setting threshold values

8. In the *Count and Measure* tool window, click the *Automatic Threshold...*button to open the *Automatic Threshold* dialog box.

   • The threshold values are automatically set.

9. Delete all but one of the phases by continuing to click the *Remove Phase* button until the button becomes inactive.

Viewing the results

10. To obtain the results, click the *Count and Measure* button in the *Automatic Threshold* dialog box.

   • The *Automatic Threshold* dialog box will be closed.

11. Open the *Count and Measure Results* tool window by using the *View > Tool Windows > Count and Measure Results* command.

12. In the *Count and Measure Results* tool window, switch to the *Class Histogram* results view by clicking the *Class Histogram* tab.

   - The results will be shown in the results sheet for every frame in the time stack. You will then be able to immediately recognize whether and how the number of objects has changed over the time period.

13. In the *Count and Measure Results* tool window, switch to the *Class Measurements* results view by clicking the *Class Measurements* tab.

14. There, select the *Object Count* entry in the *Measurement* picklist, and the *Time* entry in the *Grouped by* picklist.

   - The histogram shows the measurement parameter's chronological distribution. In the histogram you can always recognize which measurement value belongs to which frame.

15. Move your mouse pointer onto the histogram. When you move along the black line with your mouse, the mouse pointer will alter its appearance, and changes into a double arrow. Keep the left mouse button pressed. Now you can move the line, and by doing so, select different points in time. The image belonging to the respective point in time will always be shown in the image window.
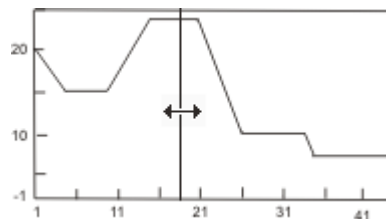
In the chart, the changes in the number of objects over the time period will be shown. Along the X-axis the frames are mapped, along the Y-axis the number of objects.

Exporting a chart

You can also export the chart and save it as a file. This enables you to archive your data, or to make it available to other users.

16. Click the *Export to Chart* 🖼 button to export the histogram to a chart.

   - You'll find this button in the *Count and Measure Results* tool window, in the *Class histogram* results view. The histogram will be saved in an exclusive file format, which can only be opened by your software.

   - The active chart will be shown in the document group. The chart's name will be "Class histogram + <serial No.>".

17. Select the *File > Save as...* command and save the chart in the *Save Chart As* dialog box under a significant name.

   - As file type, OCT will be automatically chosen, this is short for the exclusive file format.

Performing an automatic image analysis on an ROI

Task   You have selected a specific segment of the image. You define one phase. You want to know what percentage of the complete area of the image segment is taken up by the phase.
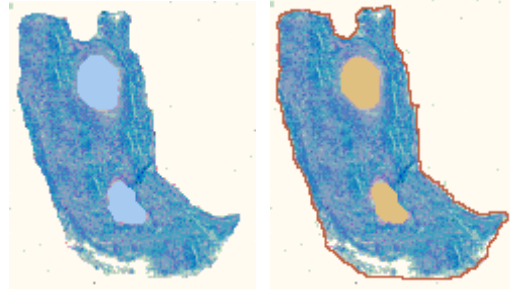
In illustration on the left, there is a tissue with two small, bright objects. The task is to calculate which percentage of the area is taken up by the objects. In the second illustration the piece of tissue has been defined as an ROI (framed in red) and both of the two small objects as a phase.

Setting options

1. In the *Count and Measure* tool window, click this ![button] button, to open the *Options* dialog box.

2. Select the *Detection* entry in the tree view.

3. In the *Options* group, enter the value 1 in the *Minimum object size* field, to specify the minimum object size. By doing this, you will make certain that individual pixels that belong to the phase will be taken into account in the area ratio.

4. Select the *Measurements* entry, in the tree view. From the *Class Measurements* list, select the *ROI*, and *Area Fraction ROI* entries.

5. Click *OK* to close the *Detection* dialog box.

Defining an ROI

6. In the Count and Measure tool window, click the *Count and Measure* button's small black arrow, to open a context menu. In it, use the *New ROI > Polygon* command.

7. Move your mouse pointer onto the image.

    *   The mouse pointer will then take on the form of a cross.

8. With your left mouse button, define the segment in the image that is to be used for the analysis. Rightclick to confirm the ROI.

Setting threshold values

9. In the *Count and Measure* tool window, click the *Manual Threshold...* button, to open the *Manual Threshold* dialog box.

10. Delete all but one of the phases by continuing to click the *Remove Phase* button until the button becomes inactive.

11. Define only one phase within the ROI. The ROI itself must not be defined as a phase.

12. Set suitable threshold values that include the objects.

13. Make sure that only one phase has been defined. Should additional phases have been defined during an earlier analysis, delete the superfluous phases.

Viewing the results

14. To obtain the results, click the *Count and Measure* button in the *Manual Threshold* dialog box.

    *   The *Manual Threshold* dialog box will be closed.

15. Open the *Count and Measure Results* tool window by using the *View > Tool Windows > Count and Measure Results* command.

- The results will be shown in the results sheet for every ROI. The *Area Fraction ROI* column shows what percentage of the ROI's area is taken up by the phase.

00350

13. Working with reports

13.1. Overview

The report functionality enables you to document the results of your work and to make them available to third parties. You can publish reports as a DOC-file or DOCX-file (for MS-Word 2007/2010) or as a printout. When a corresponding printer driver is available on your system, you can also print reports to a PDF, and send them to other users.

The illustration shows a report example that is made up of three pages, and contains six images.
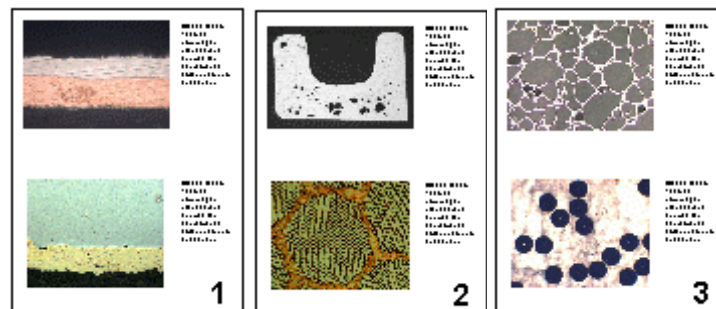
When working with reports, you have to switch to the *Report* layout. In this layout, the *Report Composer* tool window, that is required when working with reports, is by default displayed.

The first step when working with reports is always opening or creating a new report instruction in your software. In the report instruction, you specify which images and which page layout are to be taken for the report. The display and any further editing of the created report are done in the MS-Word application program. Therefore, this program has to be installed on your PC when you work with reports.

Note: Two programs are involved in the creation of reports:
Your software and the MS-Word application program. You can use the versions MS-Word 2003, 2007 or 2010 for working with reports.

Process flow when you create a report

1. New report instruction
Create or open a report instruction and fill it with the documents you want.

▼

2. Generating a report
Create a report.

▼

3. Working with the Olympus add-in
In Microsoft Word: Finish the report.

▼

4. Saving the report and the report instruction
Save the report instruction and the report.
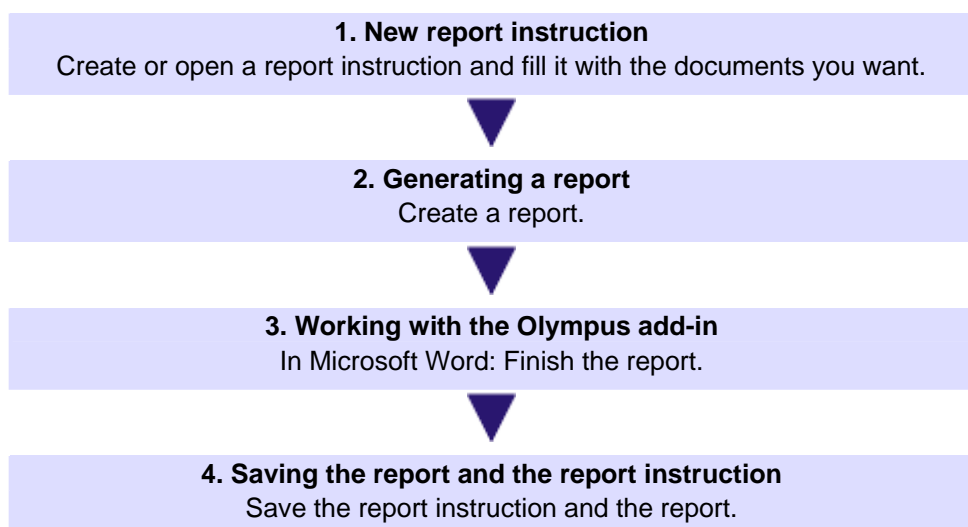
00112 12022012

13.2. Working with the report composer

The *Report Composer* tool window supports you when you are creating and updating report instructions. In this tool window, you also find the *Create* button that is used to start the report creation.

Note: Two programs are involved in the creation of reports: Your software and the MS-Word application program.

Should the *Report Composer* tool window be hidden, use the *View > Tool Windows > Report Composer* command to make it appear.

Creating a new report instruction

To create a report, first create a new report instruction in your software. You can also use a saved report instruction.

Note: The report instruction has to contain at least one registered page template. You can find more information on registering page templates in the online help.

1. Switch to the *Reporting* layout.

2. Click the *New Report Instruction* button. You find this button in the *Report Composer* tool window.

    *   A new document of the "report instruction" type will be created in the document group. This document is at the same time the workspace in which you put the report together.
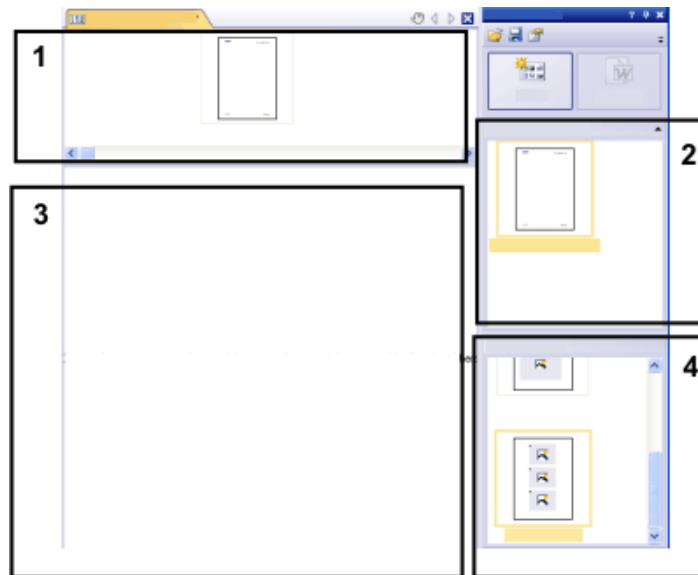
3. If no default document template has been defined: Drag the document template you want onto the upper part (**1**) of the report instruction. You find a list of the available document templates in the upper part (**2**) of the *Report Composer* tool window.

    *   If a default document template has been defined, it will be automatically inserted in the upper part of the new report instruction.

    *   Creating a report is also possible when you leave the upper part of the report instruction empty. In this case, the default MS-Word document template is used.

4. Drag the page templates you want onto the lower part of the report instruction (**3**). You find a list of the available page templates in the lower part (**4**) of the *Report Composer* tool window.

- Every report has to contain at least one page template.

- Make sure that the page templates contain the correct placeholders for the document types that you want to drag onto the report instruction. Accordingly, if your report is to contain an image and a chart, select a page template that contains one placeholder for an image and another for a chart. You can find more information on page templates in the online help.

- If you want to use workbooks in your reports, MS-Excel must be installed on your PC. The minimum MS-Excel version required is MS-Excel 2003.

- The placeholder for a workbook can also be used for a MS-Excel file. To do so, select the MS-Excel file in the *File Explorer* tool window and drag it onto the report instruction. In the report instruction, MS-Excel files are shown with this icon:

5. Drag the documents you want onto the lower part of the report instruction (**3**).

- In the *Reporting* layout, the *Database*, *Gallery* , and *File Explorer* tool windows are arranged to the left to the document window. In each of the tool windows you can select one or more documents and drag them onto the report instruction. If you use the *File Explorer* tool window, the documents do not need to be open for this. If you use the *Database* tool window, the documents don't have to be open either. It is sufficient to open the database. However, the *Gallery* tool window only allows you to select documents that are currently open in your software.

- You can also integrate MS-Word files (e.g., background information regarding the project) into your MS-Word reports. MS-Word file don't need a placeholder in the report instruction. Select the MS-Word file in the *File Explorer* tool window and drag it directly onto the report instruction. In the report instruction, MS-Word files are shown with this icon:

- The documents must have been saved, because unsaved documents cannot be included in a report.

1Image2Fields   D0811-0023   D0811-0015

2 images portrait   D0811-0171   D0811-0126
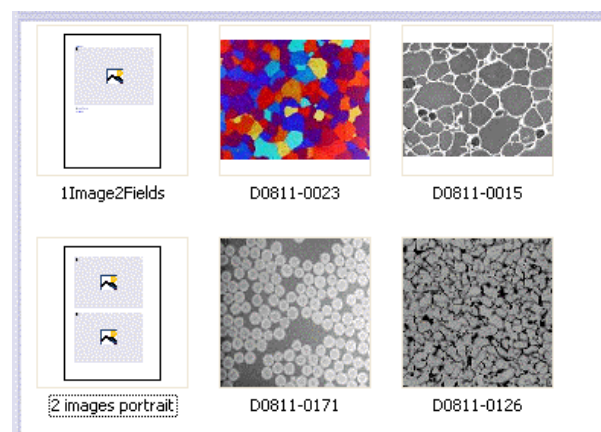
The illustration shows an example of a report instruction. In the report, two different page templates are to be used. The first page template contains a single

> placeholder for an image, the second page template contains two placeholders for an image. After the page template, the images that are to be inserted in the report page are displayed.

6. Check the report instruction now. You may still edit it and, e.g., delete or shift documents or select another page template.

Creating a report

1. Click the *Create* ⊞ button. You find this button in the *Report Composer* tool window.

    - The report will be created. Creating a report can take some time when large reports with many images and documents are involved. Pay attention to the progress bar that is shown. The MS-Word application program will open automatically and display the new report. In the example shown below, the report has three pages. (The fact that the first page template only contains one image placeholder and two images have been selected in the report instruction, automatically leads to the creation of two report pages.)
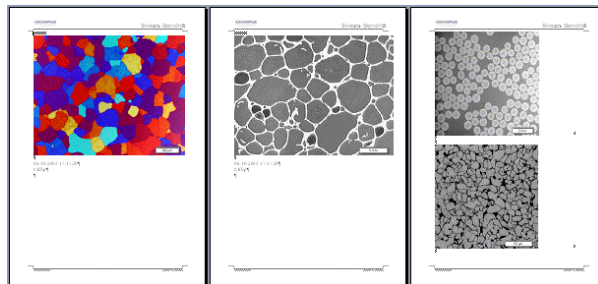
2. If you want to, you can still make additional changes in the MS-Word application program. To do so, use the Olympus add-in or the *Olympus* toolbar.

3. If you want to, save the report instruction and the report.

Editing a report instruction

You can make the changes described below to a report instruction. These changes do <u>not</u> apply to reports the have already been created on the basis of this report instruction. Therefore you must create a new report in order to see the changes you made. This will generate a new MS-Word document. Any changes that you may have made in the first version of the report will not be contained in the newly created DOC-file.

Exchanging the document template

1. Load the report instruction that you want to edit.

    - Report instructions have the file extension RCI.

2. To delete a document template, select it and press the [Del]-key on your keyboard.

3. Drag the new document template onto the upper part of the report instruction.

    - By doing so, the document template is exchanged. Please note that a report instruction can only contain one document template.

    - A report instruction must not contain a document template at all. When you leave the upper part of the report instruction empty, the MS-Word default document template will be taken.

Changing the page templates

1. Load the report instruction that you want to edit.

2. In the report instruction, select the page template you want to exchange.

3. Use the [Del] key on your keyboard to delete the selected page template from the report instruction.

   - By doing so, you only deselect the page template, no file will be deleted.

4. Drag the new page template to the position in the report instruction, where the deleted page template had been located.

   - Every report has to contain at least one page template.

Shifting the page templates

1. To shift a page template to another place in the report instruction, select it and, with the left mouse button depressed, drag it to a new position (Drag&Drop).

   - In certain cases, this may change the appearance of the report considerably. All documents that come after this page template in the report instruction will use this page template in the report.

Deleting documents

1. Load the report instruction that you want to edit.

2. In the report instruction, select the documents that you want to delete.

   - The standard MS-Windows conventions apply to the multiple selection.

3. Use the [Del] key on your keyboard to delete all of the selected documents in the report instruction.

   - By doing so, you only undo the document selection, no file will be deleted.

Adding documents

You can add new documents to an existing report instruction at any time.

1. Load the report instruction that you want to edit.

2. Simply drag the new documents onto the position you want in the report instruction.

   - Dragging & dropping images onto the report instruction is possible from the *Database*, *Gallery*, and *File Explorer* tool windows.

   - Please note the page templates must be placed <u>before</u> the images.

Moving documents

You can change the order in which the selected documents are arranged in the report instruction at any time.

1. Load the report instruction that you want to edit.

2. Select an image, and with the left mouse button depressed, drag it to another position (Drag&Drop).

00153 30012012

13.3. Working with the Olympus MS-Word add-in

When your software is installed, an add-in from Olympus is added to the MS-Word application program. When you start MS-Word, you can recognize this because the *Olympus* menu is displayed.

Depending on the MS-Word version used, the Olympus MS-Word add-in looks slightly differently. In MS-Word 2003, the *Olympus* menu and the *Olympus* toolbar are available. In MS-Word 2007 and MS-Word 2010, only the *Olympus* menu is available. When it is selected, the commands are available in a ribbon.

This add-in will help you to carry out important tasks that are necessary when working with reports. It will support you with tasks that prepare the report creation as well as tasks that are for editing the already created reports.

Preparatory tasks

1. You define page templates that you want to use for your reports. For each page template you define placeholders for the document types you want.

Note: During the installation of your software some predefined page templates have been installed, too. Please check first whether these predefined page templates are already sufficient for your needs before creating your own page templates.

2. In case you did not insert the placeholders in a chronological order: You adjust the insertion order of the placeholders, so that they are numbered consecutively, from the first placeholder to the last one.

Tasks for editing the already created reports in MS-Word

1. You add a document that is currently open in your software, to a report (or to any other MS-Word file).

2. You add a field to your report, so that certain information that is saved in your software, is also displayed in MS-Word. This makes sense, for example, when, exceptionally, you want to see the acquisition date of a certain image in the report.

3. You change the image properties and set, for example, whether or not the info stamp and the scale bar should be shown.

4. You add one or more detail zooms to an image.

5. You change the resolution of one or all images of the report. If you want to share the report, it may be sensible to reduce the resolution, thereby also reducing the file size.

6. You update all placeholders in your report. This makes sense, for example, when you change the documents in your software after the report creation and when you now want to see them in the report.

7. You insert an MS-Word report into the database of your software This command is only available if your software supports the database functionality.

Inserting documents and fields from the software in any MS-Word document you want

With the commands from the *Olympus* add-in you can also insert documents and fields from your software in any MS-Word document that hasn't been set up via the *Report Composer* tool window. In this case, you work mainly in the MS-Word application program and you switch to your software only when you want to open the documents that you want to insert into the MS-Word document.

10404 30012012

13.4. Creating and editing a page template

In a page template, placeholders are set up for the documents that the report is to contain. There are placeholders for images, charts, workbooks and fields.

When, for instance, the report is to contain pages that have an image at the top, and below it, a chart, you should then set up a page template, which has a placeholder for an image and a placeholder for a chart.

The *Olympus > Templates* menu contains all of the commands that you need for setting up or changing a page template.

Creating a page template and adding a placeholder for a document

Note: Page templates have the DOC or DOCX (for MS-Word 2007/2010) file format. To be able to carry out the following step-by-step instructions, you must therefore have opened MS-Word, and have the *Olympus* menu on display.

1. In the MS-Word application program, select the *File > New...* command.

2. Select the *Empty Document* option, if you don't want to use an existing page template as template, but instead want to start from scratch.

3. Decide whether you want to insert a placeholder for an image, a chart, or a workbook. Select the *Olympus > Templates* command and choose one of the following options: *Insert Image Placeholder*, *Insert Chart Placeholder*, *Insert Workbook Placeholder*. You can find more information on workbooks in the online help.

   • The placeholder you've selected will be inserted.

4. If necessary, you can change the size of the placeholder. To do so, move your mouse over a handle, then with the left mouse button depressed, drag it in the required direction. The length/width ratio remains unchanged, so that the objects won't be distorted by this action.

5. Doubleclick a placeholder for an image, to change the default settings for its appearance.

   • The *Image properties* dialog box opens. You can find more information in the online help.

6. If required, insert additional placeholders for images, charts or workbooks.

   • Make sure that your page template isn't longer than a page. It's better to set up two page templates, each of one page, than it is to set up one page template that is made up of two pages.

7. If you want to, you can insert a placeholder for a field. Additional information about a placeholder can be shown in this field, for example, the name, or the date it was set up. You will find additional information on inserting placeholders for fields further down.

8. Save your page template in DOC or DOCX (for MS-Word 2007/2010) file format. You can choose any storage place you like.

9. If required, add a document title. In this case, your software shows this title in the *Report Composer* tool window. If you don't enter a document title, the file name is shown instead in the *Report Composer* tool window. To define a document title, do the following:

   • In MS-Word 2003: use the *File > Properties* command and switch to the *Summary* tab. Enter the document title in the *Title* field. Close the dialog box with *OK*.

- In MS-Word 2007/2010: use the *File > Save as...* command. In the *Save as* dialog box, enter the document title in the *Title* field. Close the dialog box with *OK*.

10. Close the file.

11. Register the page template in your software. You can find more information on this topic in the online help.

Adjusting the insertion order

The placeholders are numbered in the order in which they were inserted. Should you have initially set up placeholders for two images, have then decided to put a placeholder for a chart right at the top of the page, the insertion order would be that shown in the example on the left.

1. In this case, use the *Olympus > Templates > Adjust Insertion Order* command to have the insertion order numbered serially from top to bottom (see the example on the right).

Inserting a placeholder for a field

1. In the page template, select the placeholder into which you want to insert a field.

2. Use the *Olympus > Templates > Insert Field Placeholder...* command.

- The *Insert Field* dialog box opens. A description of this dialog box can be found in the online help.

3. Click the *Use Selected Placeholder* button.

- In the *Placeholder* list, the name of the placeholder into which you want to insert a field appears.

4. In the *Available fields* list, select the field that is to be inserted. The entries in this list are arranged hierarchically. Click the plus sign to expand the list.

- Two types of field are available.
The *Document Properties* list contains fields that are, by default, in your software, managed for this document type.
The *Database fields* list contains all of the fields that are available in the database for the selected placeholder. For this purpose, a database must have been opened.

5. In the *Insert Field* dialog box, click the *Insert* button.

- The placeholder for a field will then be displayed. You can recognize it by the curly blue bracket, and by the field name shown.

6. If required, move the field to another position in the MS-Word file. By default, field values are shown to the left of, or above, the selected placeholder.

7. If necessary, add placeholders for further fields.

8. Save the page template.

00402 30012012

13.5. Editing a report

Preliminary considerations

When you have created a report and want to make some changes in it, before doing so, you should decide whether it will be better to make the changes in the report (i.e., in MS-Word) or in the report instruction (i.e., in your software).

Often, it is advisable to change the report instruction first and then create a new report. Changes you make in the report instruction are valid for every subsequent report that you create with this report instruction. There are numerous changes that you can anyway, only make in the report instruction, for example, the selection of other page templates.

Changes that you make in a report, are only valid for that particular report. There is also no possible way in which changes that are made in a report can be automatically adopted in the report instruction.

However, there are some cases when it makes sense to make a change only in the report, for example when you've created a very large report with a large number of documents, and only want to change the order of two images in it. If you didn't use a report instruction, you can only edit the report anyway.

Changing the image properties

When images are transferred to an MS-Word report, the image link is transferred as well. This makes it possible to change the image display in an MS-Word report (e.g., to scroll the image segment).

1. Doubleclick the placeholder for the image, to open the *Image Properties* dialog box.

2. In the *Display* group, select the *Scale bar if calibrated*, *Info Stamp*, and *Border* check boxes, if these elements are to be displayed.

   • The properties of these elements can be defined in the *Options > Image Information* dialog box. Click the *Options...* button to open this dialog box.

3. In the *Size* group, select one of the options that specify how large the image is to be displayed in the report. You can find more information on these options in the online help.

4. If your settings should apply for all future images, click the *Set as Default* button.

5. Click *OK*.

   • The *Image Properties* dialog box closes. The changed image properties will be shown in the MS-Word report now.

Adjusting documents

In the MS-Word report, you can select a document of the "image" or "chart" type and select the *Olympus > Adjust Document* command. You will then change over to the image analysis software, where you can edit the document and then automatically change back to the report.

Note: Basically, the *Adjust Document* command is similar to the *Update Placeholders* command. The difference is that the *Adjust Document* command is meant for users who mainly use MS-Word for creating reports and who only change over to the image analysis software in order to make certain changes to some documents.
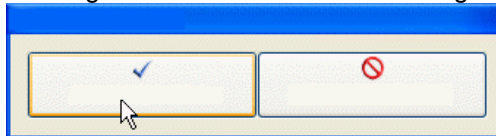
Example: In the MS-Word application program, you edit a report that contains a lot of images. With a certain image you notice that an important measurement is missing. Using the *Adjust Document* command, you change over to the image analysis software, add the missing measurement and then change back to MS-Word in order to continue editing the report.

Adjusting an image

1. Open the report in MS-Word and select the image that you want to adjust.

2. Select the *Olympus > Adjust Document* command.

    - You switch to the image analysis software. If it was closed, it will be started and displayed in the foreground.

    - The image that you want to adjust is also opened. In case it is from a database that is currently closed, the database will be opened in the background.

Note: The image analysis software is now in a special "adjust-document" mode. In this mode, you can only make certain adjustments to the image. This is why a lot of other functions are hidden.

3. Make the required change.

4. If the image information was changed: Save the image in the image analysis software.

    - Some changes made to an image don't have to be saved, e.g., when you select another frame in a multi-dimensional image. Other changes have to be saved, e.g., adding a measurement. The fact that a change has to be saved will be indicated by an asterisk shown behind the file name in the document group.

5. Click the *Update Report* button. You find this button in the *Adjust Document* message box that is shown in the foreground.

    - The MS-Word application program will now be shown in the foreground again. The edited image will be displayed. You can now continue to edit the report.

    - If your image analysis software was closed before you selected the *Adjust Document* command, it is closed again. If any images or databases had to be opened for this command, they will be closed as well.

Editing a workbook in the report

Your software supports the handling of workbooks. A workbook is created, for example, when you open the *Measurement and ROI* tool window and export a results sheet.

You can find more information on workbooks in the online help.

Note: If you want to use workbooks in your reports, MS-Excel must be installed on your PC. The minimum MS-Excel version required is MS-Excel 2003.

Apart from the "image" and "chart" document type, reports can also contain workbooks. A workbook is imported as an Excel object in MS-Word. You can further edit it in the report.

1. In the report, doubleclick on the workbook.

    •   You will change into the edit mode. You can recognize it by the fact that now the column headers and the row numbers are shown. In edit mode, as well as that, you can see all of the workbook's worksheets.

2. If need be, select the worksheet that you want to edit.

3. Doubleclick the workbook in order to switch to edit mode. Make the required change.

    •   When you want to format individual cells differently, select the cell and use the *Format Cells...* command in the context menu.

    •   When you want to format the complete worksheet differently, (e.g., other font or other background color), select the complete worksheet (e.g., with the keyboard shortcut [Ctrl + A]), then select the *Format Cells...* command in the context menu.

    •   When you want to hide a column, click on the column's header, then select the *Hide* command in the context menu.

4. Exit edit mode by clicking on any point in the MS-Word report, outside the workbook.

Changing image resolution

By default, all images of a report are transferred to MS-Word reports with a resolution of 192 dpi. In certain cases, it can make sense to change the resolution of individual or all images in a report For example, if you want to print the report, you can raise the resolution. Alternatively, if you want to publish the report on the Internet, you can reduce the resolution.

1. Open the report in MS-Word. Decide whether you want to change the resolution of all images or just certain images

2. If you only want to change the resolution of one individual image, select that image. If you want to change the resolution of all images, you don't have to select any.

3. Select the *Olympus > Change Image Resolution...* command.

    •   The *Change Image Resolution* dialog box opens.

4. Select the option you want in the *Apply to* group. You can choose between *Selected images* and *All images in report*.

    •   The *Selected images* option is inactive if no images were selected while opening the command.

5. Specify in the *Image Resolution* group how you want to change the image resolution. If you choose the *User-defined* option, you can enter any resolution of your choice between 96 and 600 dpi into the *DPI* field.

6. Click the *OK* button to change the image resolution.

7. Check whether you are satisfied with the changed image resolution. If not, change the image resolution anew.

    •   You can first reduce the image resolution, then save the report and then increase the image resolution again. This is possible because each time that you open the *Change Image Resolution...* command, the image is transferred from your software to MS-Word.

8. Once you are satisfied with the changes to the image resolution, save the report. Take a look at the new file size in the Windows Explorer.

Updating placeholders

The *Update Placeholders...* command makes it easy to have any changes made to the images after the report has been created also shown in the report. Please take note that all changes made in your software have to be saved, if they are to be displayed when the *Update Placeholders...* command is used.

In MS-Word, you open a report that you created some time ago. In the meantime, you had changed a lot of images in your image analysis software (e.g., added measurements). Now, the report is to be updated so that it shows the newest version of all of the images.

1. If you only want to update one placeholder, select just that one.

2. In MS-Word, use the *Olympus > Update Placeholders...* menu command.

   • The *Update Placeholders* dialog box opens.

3. In the *Update Placeholders* dialog box, specify whether or not all placeholders should be updated.

4. Select the *Update fields linked with placeholder(s)* check box if your MS-Word report contains fields which should also be updated.

   • You can find more information on this topic in the online help.

5. Click *OK*.

   • The placeholders will be updated.

Inserting a document

In a report (or in any other MS-Word document), you can insert a document at any position. When you have, for example, created a report, and while you are viewing it, notice that you've forgotten an image, you can retroactively insert it into the report.

1. Use the *Olympus > Insert Document...* command.

   • The *Insert Document* dialog box opens.

2. In the area on the left, select the source the document comes from. You have the following possibilities:

   • Select the *Open Documents* entry if you want to insert a document that is currently opened in your software.

   • Select the *Database* entry if you want to insert a document that is part of the currently selected database folder. For this purpose, the database must be opened in your software. Should you work with a version of the software that doesn't support databases, the *Database* entry is hidden.

   • Select the *File Explorer* entry if you want to insert a document that is stored on your PC or in your network.

3. Select the required document in the document preview. Click the *Insert* button.

   • The required document will be inserted into the MS-Word report.

   • The *Insert Document* dialog box remains open.

4. Insert further documents now or close the dialog box.

   • The path of all documents that you inserted will be saved. That enables you to later update the inserted documents by using the *Olympus >*

Update Placeholders command (in case the documents were changed after they have been inserted into the report).

Inserting a field

You can insert a field into an MS-Word report that describes the image in more detail. All of the values that have been saved in your software for this document can be displayed in this field.

1. Select the image in the report to which you want to insert a field.

2. Select the *Olympus > Insert Field...* command.

   • The *Insert Field* dialog box opens.

3. Click the *Use Selected Placeholder* button.

   • In the *Placeholder* list, the name of the placeholder into which you want to insert a field appears.

4. In the *Available fields* list, select the field that is to be inserted. The entries in this list are arranged hierarchically. Click the plus sign to expand the list.

   • Two types of field are available.
     The *Document Properties* list contains document-specific fields, just as they are shown in your software's *Properties* tool window.
     The *Database fields* list contains all of the fields that are available in the database for the selected image. For this purpose, a database must have been opened.

4. Check at the bottom of the dialog box, whether a field value is displayed in the *Field value* group. Should the *Field value* group remain empty, this means that the field hasn't been filled out in your software. In this case, select another field that has been filled out, instead.

5. Click the *Insert* button.

   • The field contents will be displayed in the MS-Word document.

7. If required, move the field to another position in the MS-Word file. By default, field values are shown to the left of, or above, the selected placeholder.

8. If necessary, add further fields.

9. Close the *Insert Field* dialog box.

10. Save the MS-Word report.

Note: Should you want to have the contents of a specific field regularly shown in your reports, you can already insert this field, (that is to say a placeholder for this field) into the page template. Then this field will be automatically filled out in every report.

00403 17022012

OLYMPUS®

Manufactured by

OLYMPUS CORPORATION

Shinjuku Monolith, 3-1, Nishi Shinjuku 2-chrome, Shinjuku-ku, Tokyo, Japan

Distributed by

OLYMPUS EUROPA SE&CO.KG

Wendenstrasse 14-18, 20097 Hamburg, Germany

OLYMPUS AMERICA INC.

3500 Corporate Parkway, Center Valley, Pennsylvania 18034-0610, U.S.A.

OLYMPUS SINGAPORE PTE LTD.

491B River Valley Road, #12-01/04 Valley Point Office Tower, Singapore 248373

OLYMPUS AUSTRALIA PTY. LTD.

31 Gilby Road, Mount Waverley, VIC., 3149, Melbourne, Australia

OLYMPUS LATIN AMERICA, INC.

5301 Blue Lagoon Drive, Suite 290 Miami, FL 33126, U.S.A.

OLYMPUS (CHINA) CO.,LTD.

(biological) A8F, Ping An International Financial Center, No. 1-3, Xinyua South Road,
            Chaoyng District, Beijing, 100027 P.R.C.
(Industrial) 10F, K. Wah Centre, 1010 Huai Hai Rd(M), Xuhui District, Shanghai, 200031 P.R.C.

OLYMPUS KOREA CO.,LTD.

Olympus-Tower, 114-9 Samseong-Dong, Gangnam-Gu, Seoul , Korea