OLYMPUS

User Manual

cellSens

LIFE SCIENCE IMAGING SOFTWARE

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Olympus Soft Imaging Solutions GmbH, Johann-Krane-Weg 39, D-48149 Münster,
Tel. (+49)251/79800-0, Fax: (+49)251/79800-6060

Contents

1. About the documentation for your software

The documentation for your software consists of several parts: the installation manual, the online help, and PDF manuals that are 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 the program. 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 the cellSens software.

Example images that are automatically installed

During the installation of your software some example images have been installed, too. These example images might be of help to you when you familiarize yourself with the software. Regarding the information as to where the example images are located, please refer to the online help.

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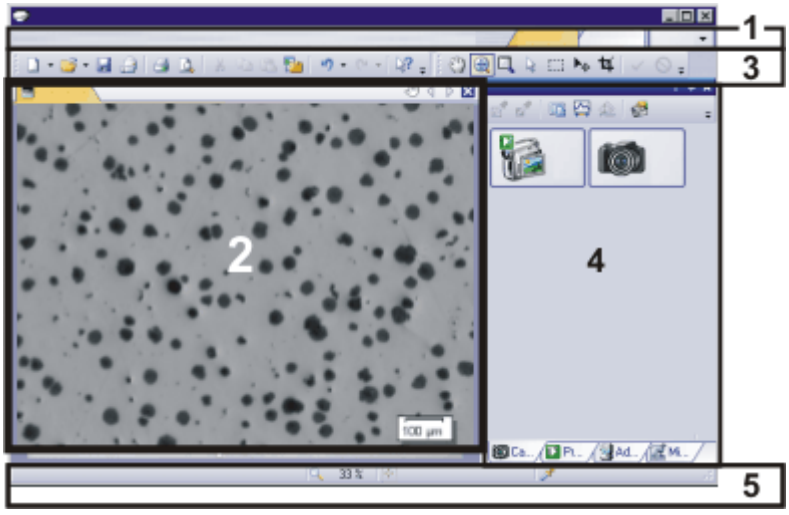
2. User interface

2.1. Overview - User interface

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.

Further information is available in the online help.

(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 shows a lot of information, e.g., a brief description of each function. Simply move the mouse pointer over the command or button for this information.

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2.2. Overview - 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:

Acquire images ("Acquisition" layout)
View and process images ("Processing" layout)
Measuring images ("Count and Measure" layout)
Generate a report ("Reporting" layout)

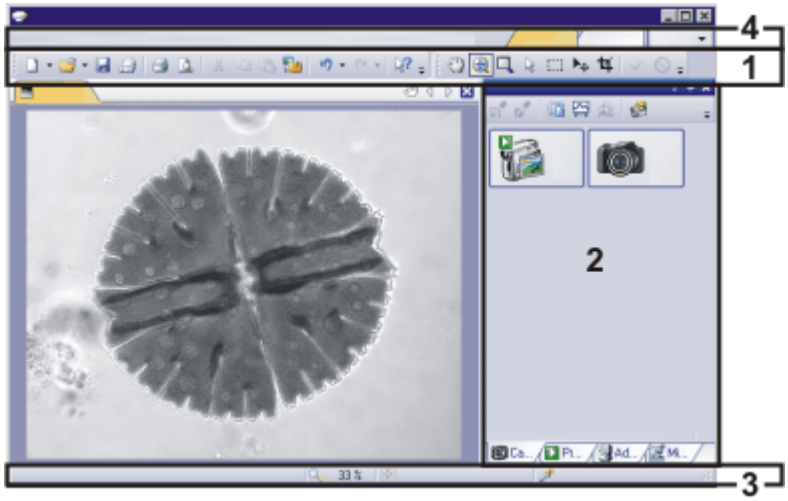
In contrast to your own layouts, predefined layouts can't be deleted. Therefore, you can always restore a predefined layout back to its originally defined form. To do this, select the predefined layout, and use the *View > Layout > Reset Current Layout* command.

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

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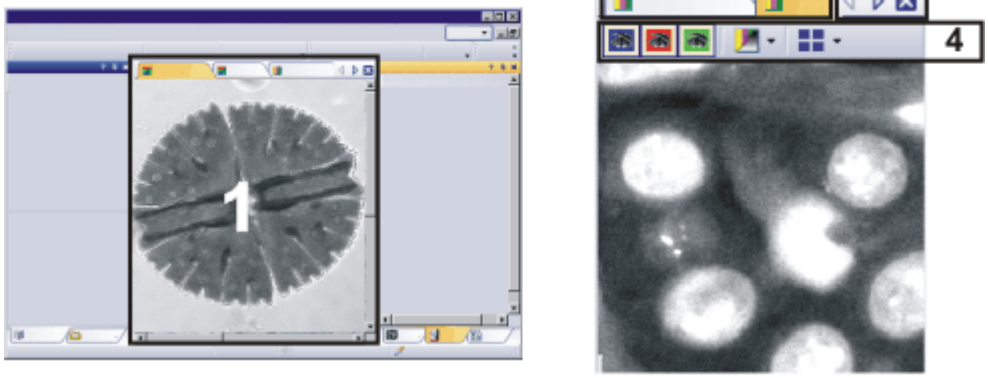
2.3. Document group

The document group contains all loaded documents. These can be of all supported document types.



The illustration shows left, a schematic representation of a user interface. On the right, the document group is shown enlarged.

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 naturally, all of the images that have been acquired also. Also the live-image and the images resulting from, e.g., any image processing function, will be displayed there.

Document bar in the document group

The document bar is the document group's header.

For every loaded document, an individual field 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.

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.

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 button

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.

Button with a cross

Click the button with a cross to close the active 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.

Navigation bar in the image window

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

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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, naturally, at any time, make specific tool windows appear and disappear manually. To do so, use the *View > Tool Windows* command.

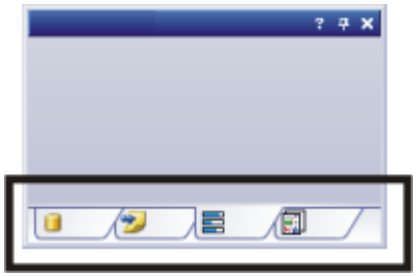
Manually displayed or hidden tool windows will be saved together with the layout and are available again the next time you start the program. Resetting the layout using the *View > Layout > Reset Current Layout* command will have the result that only the tool windows that are defined by default will 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 *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 appear clicked. You can recognize this status by the icon's background color.
Use this list to make tool windows appear.

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2.5. 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. However, your software supports several more 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. Detailed information on inserting documents into a database can be found in the online help.

Autosave and close

1. When you exit your software, all of the 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 images will be automatically saved when the acquisition has been completed. You will find an overview of the acquisition processes that are supported in the online help.
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.
   There, you can also set that your images are automatically saved in a database, after 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 document 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  . You will find this button on the top right in the document group.
3. Use the *Gallery* tool window.
   Select the desired document 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, doubleclick 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. Use the *Database > Load Documents* command to load documents from the database into your software. Further information is available 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.

Using sample images

During the installation of your software some sample images have been installed, too. You can find a sample image for each image type. Regarding the information as to where the example images are located, please refer to the online help. These example images might be of help to you when you familiarize yourself with your software.

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. Leftclick 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.

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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.

When do you have to configure the system?

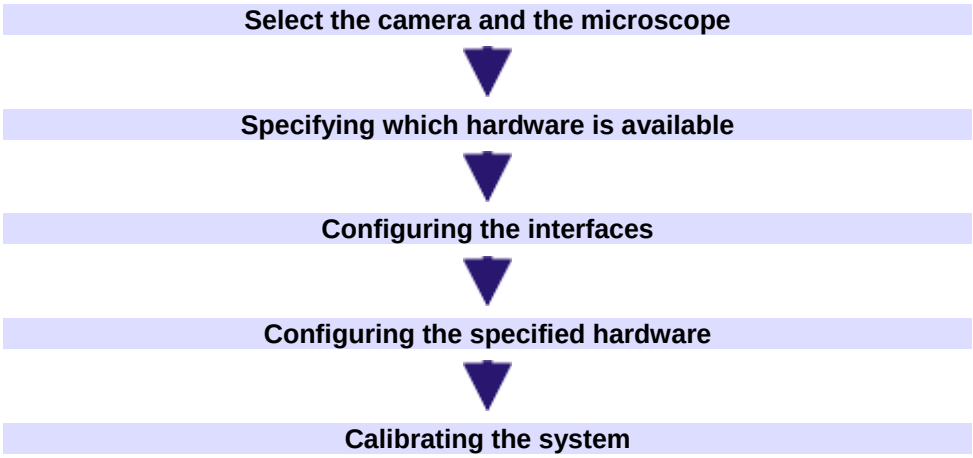
You will only need to completely configure and calibrate your system anew when you have installed the software on your PC for the first time, and then start it. When you later change the way your microscope is equipped, you will only need to change the configuration of certain hardware components, and 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.
To do so, click the Start button located at the bottom left of the operating system's task bar.
Use the *Control Panel* command.
Open the *System and Maintenance > Power Options > Change when the computer sleeps* window. Here, you can switch off your PC's hibernation mode.

Process flow of the configuration

To set up your software, the following steps will be necessary:



Select 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. Further information is available in the online help.

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.

Configuring the interfaces

Use the *Interfaces* dialog box, to configure the interface between your microscope or other motorized components, and the PC on which your software runs.

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 controlled 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.

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4. Acquiring single images

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.

Acquiring a single image

1. Switch to the "Acquisition" layout. To do this, use, e.g., the *View > Layout > Acquisition* command.
2. In the *Camera Control* tool window, click the *Live*  button.

Selecting the objective

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

Switching on the live-image

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. On the *Camera Control tool window, click the Snap*  button.
   - The image you have acquired will be shown in the document group.
10. Use the *File > Save As...* command to save the image. Use the recommended TIF file format.

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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)" is 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.

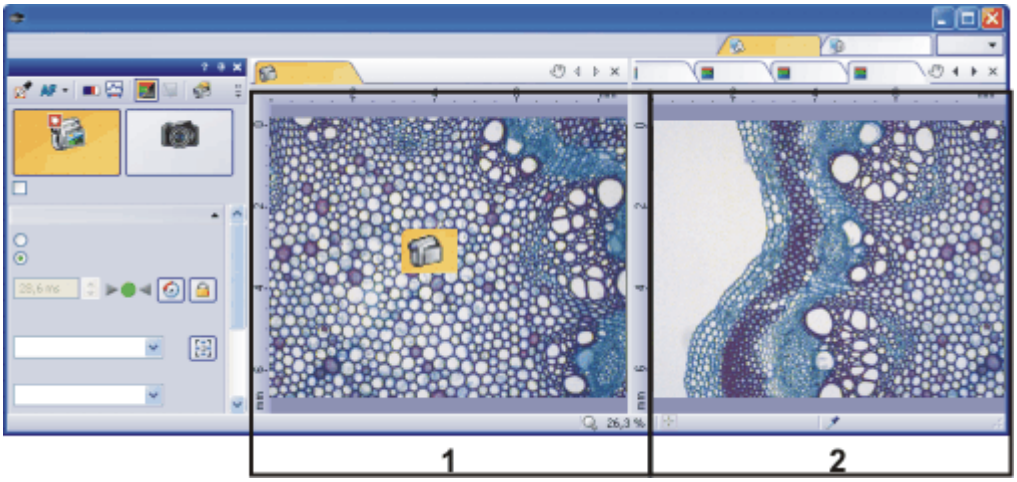
Displaying the live-image and the acquired images simultaneously

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)*.
   - 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.

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5. Acquiring image series

5.1. Time stack

What is a time stack?

You can combine a series of separate 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.
Further information on the navigation bar is available 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 detailed 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.

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 the display.

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.
Use the *File > Save As...* command.

Converting time stacks

Breaking down time stacks into individual images

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

Reducing the number of frames within a time stack

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. Further information on this command is available in the online help.

Converting time stacks while saving them

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 can find most of the image processing operations in the *Process* menu.
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. Further information on mthis image window view can be found 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.

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5.2. 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.

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5.3. Acquiring movies and time stacks

With your software you can acquire movies and time stacks.

Acquiring 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 the 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 will open.
4. Select the *Saving > Movie* entry in the tree structure.
5. Decide how you want to save the recorded movies after the acquisition process is finished. Select the *Filesystem* entry in the *Automatic save > Destination* list to automatically save the movies you have acquired.
   - The *Base* 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 *Base* field to alter the directory.

Selecting the compression method

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

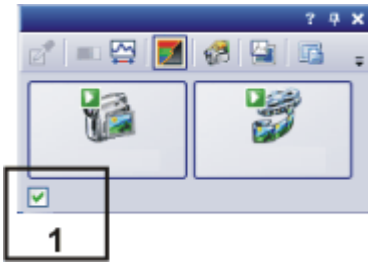
   Please note: Compressing the movie is only possible if the selected compression method (codec) has already been installed on your PC. If the compression method has not been installed the AVI file will be saved uncompressed.
   The selected compression method must also be available on the PC that is used 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 will indicate 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 the 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.

Selecting the acquisition process

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., *Multicolor*, click the button to switch off the acquisition process.
   - The group with the various acquisition processes should now look like this:

Selecting the acquisition parameters

10. Clear the check boxes *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.

Acquiring a time stack

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. Further information on the navigation bar is available 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.

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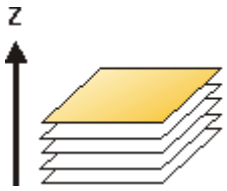
5.4. Z-stack

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. Further information on this navigation bar is available in the online help.

Creating a Z-stack

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

1. To acquire a Z-stack, use the "Z-Stack" acquisition process.
2. 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.

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

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

Breaking down Z-stacks into separate images

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

Reducing the number of frames within a Z-stack

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 can find this command in the context menu in the tile view for Z-stacks.

Converting Z-stacks while saving them

When 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.

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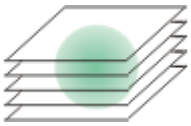
5.5. Acquiring a Z-stack

With the *Z-Stack* acquisition process, you acquire a series of frames one after the other, a Z-stack.

Acquiring a Z-stack

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

Selecting the acquisition parameters

9. Select the *Range* entry in the *Define* list.
10. Enter the Z-range you want, in the *Range* field. In this example, enter a little more than the sample's thickness (= 50 µm), e.g., the value 60.
11. In the *Step Size* field, enter the required Z-distance, e.g., the value 2, for a Z-distance of 2 µm. The value should correspond with your objective's depth of focus.
    - In the *Z-Slices* field 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 in the [*Z*] group. The buttons with a double arrow move the stage in larger steps.

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.
- 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. Further information on the navigation bar is available in the online help.

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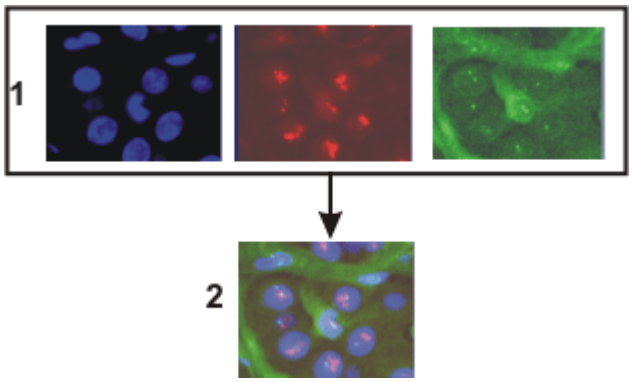
6. Acquiring fluorescence images

6.1. 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.

Further information on the navigation bar for multi-channel images is available in the online help.

Creating multi-channel images

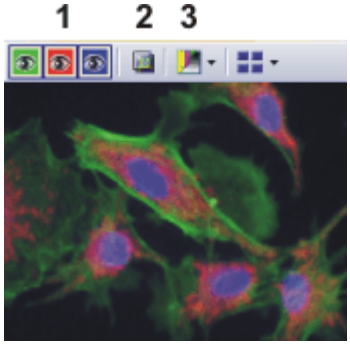
Your software offers you several ways of acquiring a multi-channel image.

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).

Further information on the navigation bar for multi-channel images is available in the online help.

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 set 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 can find most of the image processing operations in the *Process* menu. Click here for more information on working with image processing operations.

Select the individual images that you want to process in the *Dimension Selector* tool window. The frames 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.

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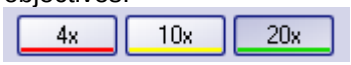
6.2. Before and after you've acquired a fluorescence image

Before you acquire a fluorescence image

Defining observation methods

1. Define observation methods for your color channels.

Setting up the microscope for the acquisition of a fluorescence image

2. 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.
     

   - In the *Observation method* group, you can 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.
3. Click the required objective's button.
4. 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

5. Use the *View > Tool Windows > Camera Control* command to make the *Camera Control* tool window appear.
6. 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.
7. 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.
8. 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.
9. 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

10. Use the *View > Toolbars > Calibrations* command, to have the *Calibrations* toolbar displayed.
11. 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

12. Select the automatic exposure time.

13. 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.
14. 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.
15. Finish the live mode. To do so, click the *Live*  button in the *Camera Control* tool window, once more.

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.

16. Before you start the acquisition, specify where the file is to be saved.
17. 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 can find the current directory in the *Directory > Base* field.
18. Click the [...] button next to the *Base* field, to change the directory into which the image is to be saved after its acquisition.

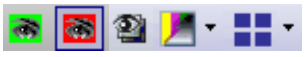
After you've acquired a fluorescence image

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. Below, is an illustration of the navigation bar, click one of its buttons and you'll see a description of its functions.



"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 can 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…* .

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6.3. Defining observation methods for the fluorescence acquisition

Before you acquire a fluorescence image, you have to define observation methods for your color channels. As a rule, 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 lightpath			
Shutter	<Name of the reflected light shutter>	Manual Shutter	Motorized (UCB)
Transmission lightpath			
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	With a motorized condenser: The hardware components *Aperture Stop* and *Top Lens* are additionally listed under the device settings.

Defining the observation method for transmission brightfield

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


Setting up the mirror turret

4. Select the mirror turret in the *Available components* list.
   - In the dialog box's central area, 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: Setting up 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 *Switch on/off the lamp* button, to make sure that the lamp is switched on.
    - The button in the central area of the dialog box looks like this when the lamp has been 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. Enter a name for the new observation method, 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 dialog box's central area, 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: Setting up the transmission lamp

9. Select the transmission lamp. You can 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 *Switch on/off the lamp* button, to make sure that the lamp has been switched off.
    - The button in the central area of the dialog box looks like this when the lamp has been switched on.

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 when 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 can 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* button.
    - The *Enter a New Observation Method Name* dialog box opens.
4. Enter a more general name (e.g., "Blue" or "DAP"), 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.

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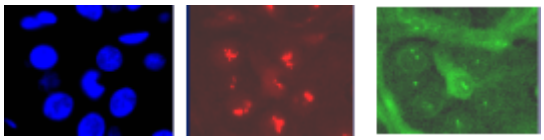
6.4. Acquiring and combining fluorescence images

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.

Acquiring a fluorescence image

Selecting the fluorescence observation method

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

3. Use the *View > Tool Windows > Camera Control* command to make the *Camera Control* tool window appear.
4. Switch to 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.
5. In the *Camera Control* tool window, select the *Exposure > Manual* option.
6. Some cameras offer the *SFL* mode for fluorescence acquisitions (e.g., the DP72). Switch this mode on.
7. Optimize the exposure time.
   Should the exposure time become longer than 500 ms, reduce it by increasing the sensitivity or gain.
   To do this, change the value in the *Exposure > Sensitivity* field, or use the *Gain* slide control.

Focusing a fluorescence sample

8. Bring the image into focus.

9. Finish the live mode. To do so, click the *Live*  button in the *Camera Control* tool window, once more.
   - 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

10. 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

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

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Combining channels

The *Image > Combine Channels...* command creates a new multi-channel image from several separate images. A description of the 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 are two single color time stacks, that are each made up of 50 frames. 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.

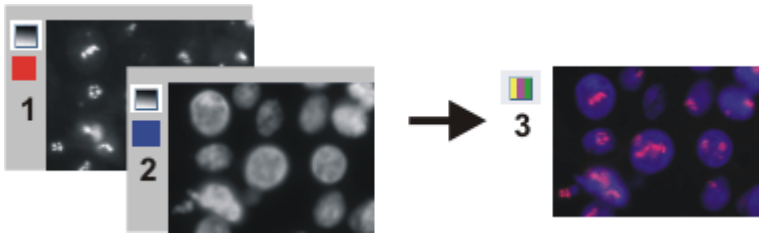
Example: You can use a true-color image as a transmission image. When the individual fluorescence images have a bit depth of 16 bit, you can only use a 48-bit true-color image as a transmission image.

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.

Combining channels

3. Use the *Image > Combine Channels...* command.
   - The *Combine Channels* dialog box opens.
   - 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 open 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 table.
7. You can now change the characteristics of the new channel. Give the new channel a name and assign it a color.
8. It's possible to shift the frames in relation to one another. 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.

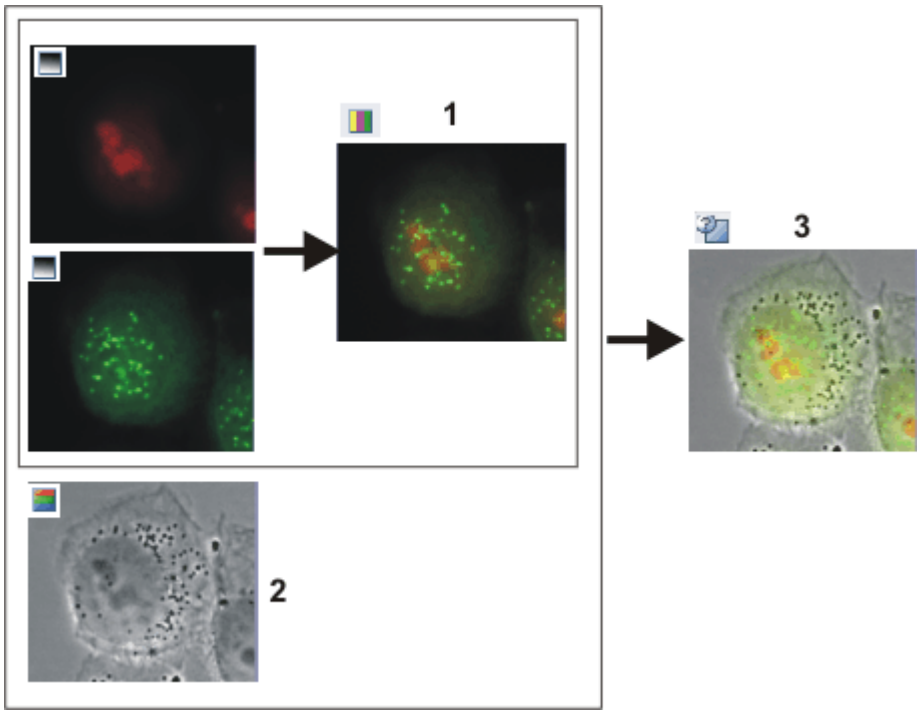
Viewing a multi-channel image

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 opens.
   - 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).

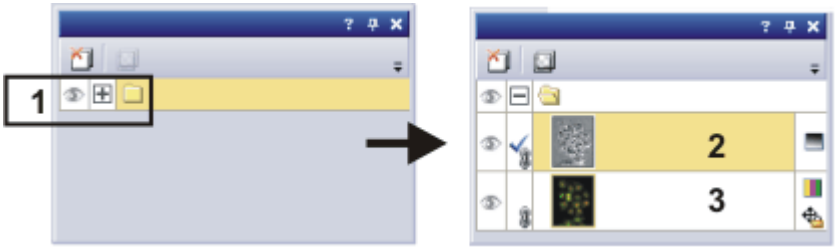


Viewing a multi-layer image

- 
  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 mage (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 > Toolbar* command.

14. Click the *Move*  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.
18. The layer you have selected will then be shown with a colored background in the tool window.
19. Then, click the *Set Layer Opacity*  button. You will 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.

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

Saving a multi-layer image

22. 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.

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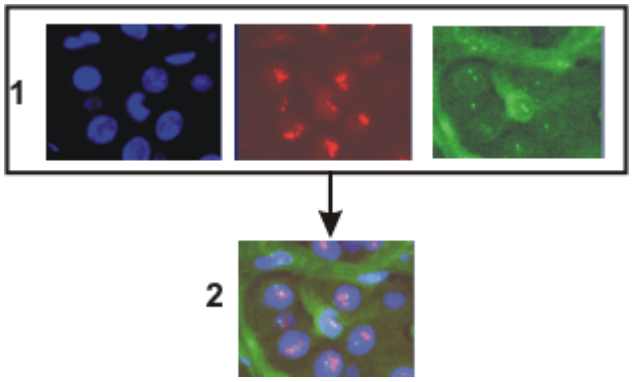
6.5. Acquiring multi-channel fluorescence images

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

Example: Define a process for the acquisition of a multi-channel fluorescence image (e.g., "Blue", "Green", and "Red"). 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.

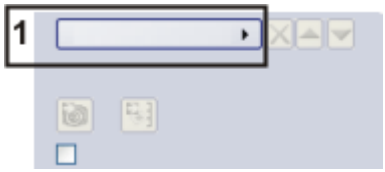
Defining the "Multi Channel" acquisition process

Choosing 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., *Multicolor*, 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).

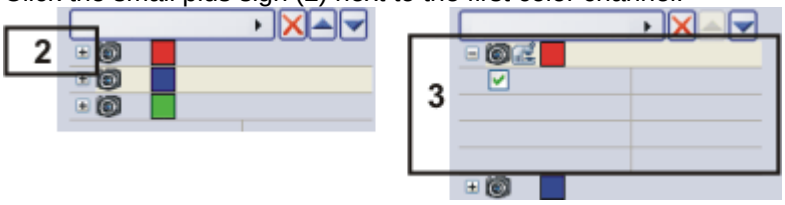


- A context menu will open.
  The context menu will list all of the observation methods that are currently defined.

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.

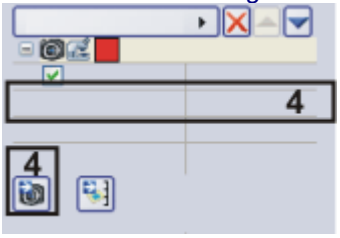
Selecting the exposure times for the individual color channels

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 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 the sensitivity or 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).

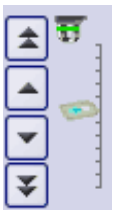
15. Then, activate the other channels and set the exposure time for each of them.

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

    - The reflected light shutter will be closed.

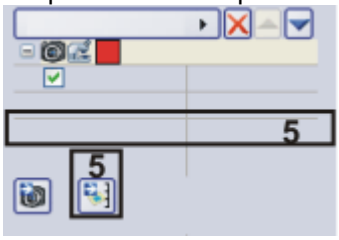
Focusing a fluorescence sample

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

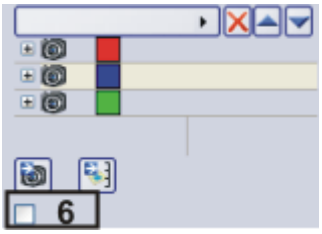
20. Click the *Read Z-offset*  button in the *Process Manager* tool window to adopt the current Z-position of your microscope stage (5).



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 sensitivity or 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.

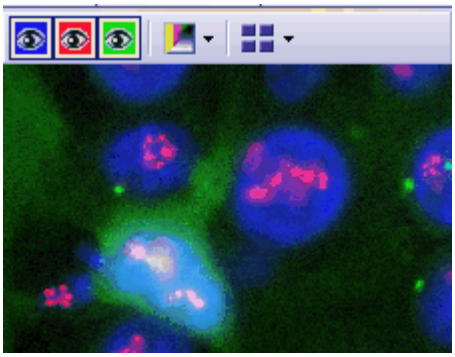
Acquiring and viewing a multi-channel fluorescence image

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.

Starting the acquisition process

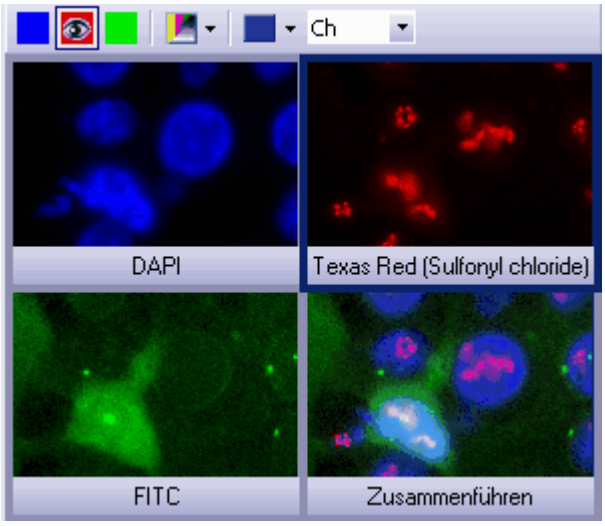
2. In the *Process Manager* tool window, click the *Start* button.
   - If you use a manual microscope, you'll receive several different messages about switching the mirror cube and opening and closing the shutter.
3. For microscopes that aren't motorized: Follow the instructions and make the necessary 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.
4. 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.
5. When you've finished, superimpose all of the channels again.
6. 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.



7. Compare the color channels.
8. 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

9. 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.
10. 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

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. Set the sensitivity to the lowest ISO value or the gain to the value of "0". Optimize the exposure time.
8. In the *Process Manager* tool window, click the *Read settings*  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*  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.

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7. 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°.

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

Selecting the objective

2. 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

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 stitched image's individual images.
4. Find the position on the sample at which you want to start acquiring the stitched image.
5. Finish the live mode.

Selecting the acquisition process

6. Activate the *Process Manager* tool window.
7. Select the *Manual Processes* option.
8. 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.

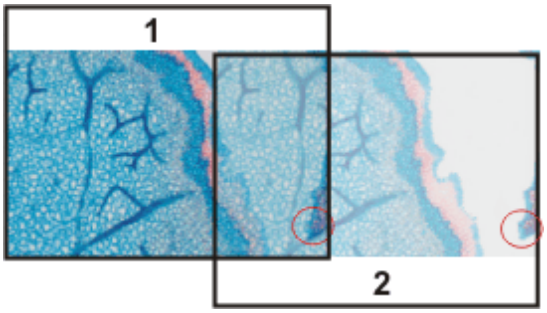
Selecting the acquisition parameters

9. 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

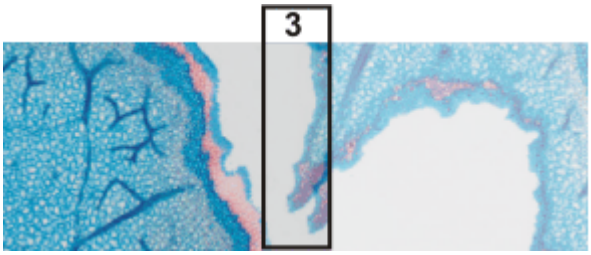
10. Click the *Start*  button.
    - Your software switches into the live mode.
11. Bring the sample into focus.
12. Click on one of the arrow buttons to set the side of the current image at which the next image is to be arranged. For example, click this button  if the next image is to be laid to the 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.

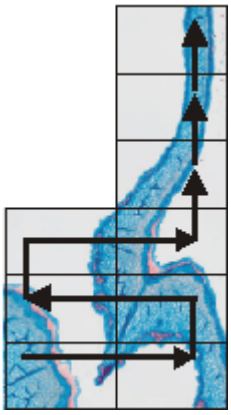


Since you haven't moved the sample, the live-image still shows the current sample position, too, which means that you now see the current image twice.
The two images overlap. Since the live-image is shown transparent, you see both images in the overlap area simultaneously.

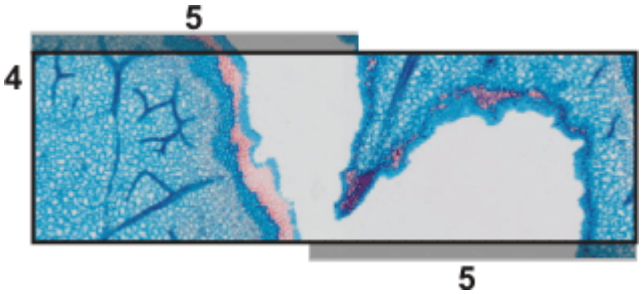
13. Make a note of a significant structure on the live-image's right border. You will find the same sample structure in the overlap area. On the illustration, a significant structure has been indicated by a circle.
14. Now move the stage very slowly to make the structure on the live-image move to the 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.
15. 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.
16. 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.



17. 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.

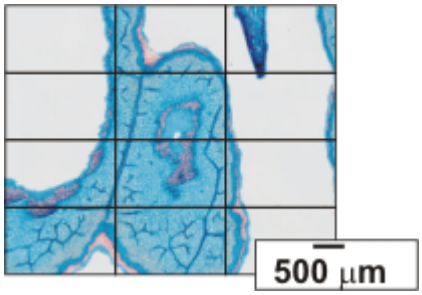


    - The stitched image will, by default, be automatically saved. The storage directory is shown in the *Acquisition Settings > Saving > Process Manager* dialog box. The preset file format is VSI.

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)



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.

Prerequisites

- 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.

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

Selecting the objective

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

Selecting the acquisition process

3. Activate the *Process Manager* tool window.
4. Select the *Automatic Processes* option.
5. Click the *XY-Positions / MIA*  button.
   - Should another acquisition process be active, e.g., *Multicolor*, click the button to switch off the acquisition process.
   - The group with the various acquisition processes could now look like this, for example:

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

Using the software autofocus

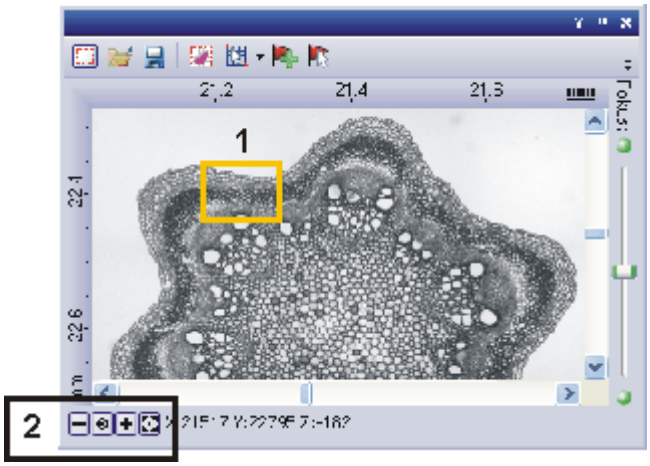
6. If your microscope is equipped with a motorized Z-drive, you can switch on a software autofocus.
   In the *Process Manager* tool window, click the *Software Autofocus* button.

   - The *Software Autofocus* group will be automatically displayed in the tool window.
7. In the *Software Autofocus* group, select the *Multiposition / MIA* check box.
8. 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

9. In the *Process Manager* tool window, click this button  .
   - The *Stage Navigator* tool window will be 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.
10. 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.

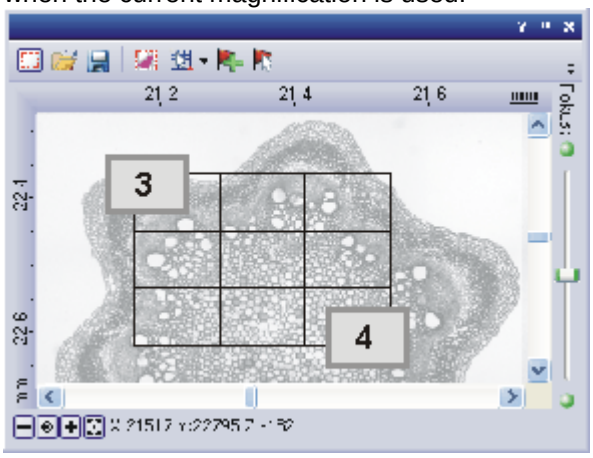


   - Further information on the *Stage Navigator* tool window is available in the online help.

Defining the MIA scan area

11. 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 will open.
12. Move the XY-stage to the top left-hand corner of the MIA scan area you want (3).
13. 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.
14. Confirm the starting position in the *Define MIA Scanning Area* dialog box, with *OK*.
15. 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

16. Click the *Start* button.
    - 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 see the completed stitched image, in the image window. The individual images won't be saved separately.

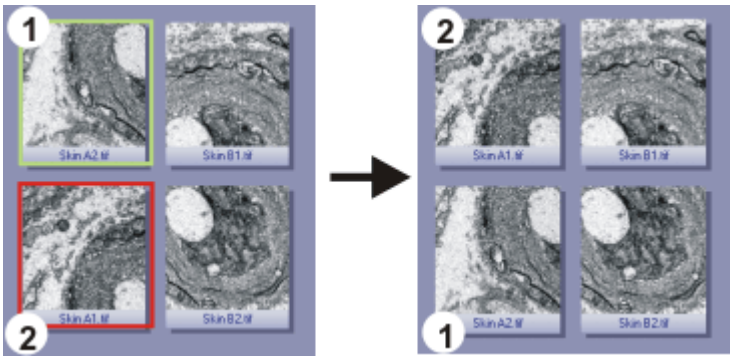
Combining individual images into a stitched image

Loading and selecting images

1. Load the images you want to combine, resp. 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

4. 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 the dialog box can be found in the online help.
    - The dialog box's stitching area will display a preview of the individual images.
5. 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.
6. 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.

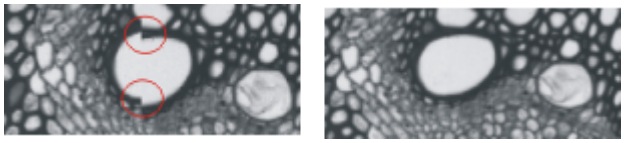
- 

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.

7. 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.
8. 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

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

10. 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.
11. 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.
12. 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.
13. Click *OK*.
    - A new image with the name "Image_<consecutive No.>" will be created.

8. 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).

Processing images

1. Load the image that 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 opens. The image processing operation that is active is 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 is 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.

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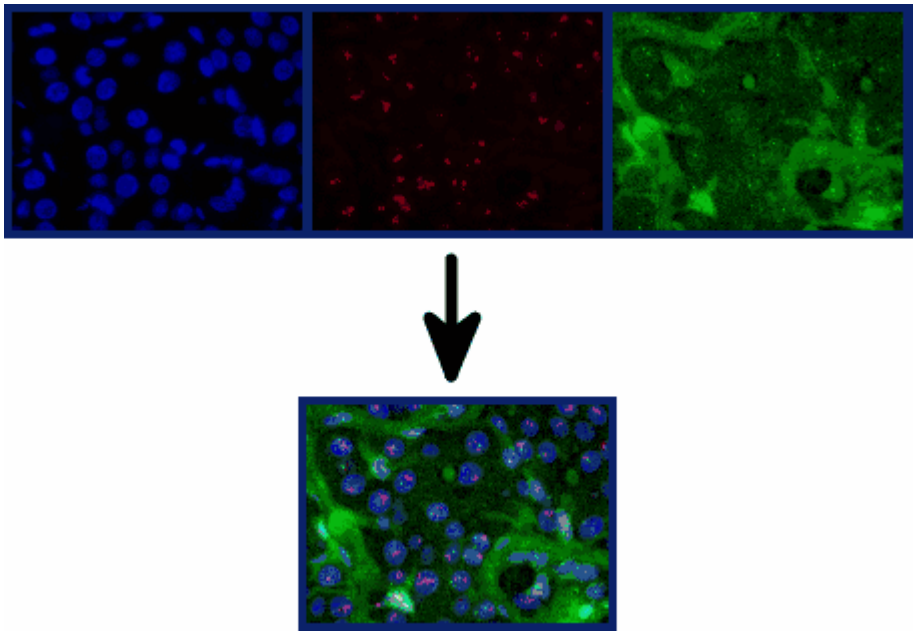
8.1. Fluorescence Unmixing

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

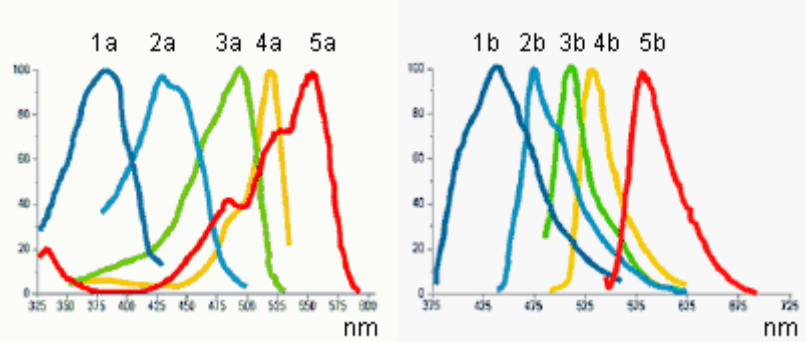
The problem with this procedure is, that the wavelength ranges of the different fluorochromes overlap. If this overlapping didn't occur, the aspired visual separation of the different cell structures in the resulting multi-channel fluorescence image would be perfect.

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 > Enhancement > Fluorescence Unmixing...* command.

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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.
5. 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.

9. 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.

Prerequisites

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 will 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 this, 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.

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 an 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.

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%.
Click here if you wish to change an image's zoom factor.

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9.1. Measuring images

Your software offers a wide range of measurement functions. They enable you to quickly count objects and measure segments and areas.

The following step-by-step instructions present the measurement functions to you by way of several examples.

Measuring image objects interactively

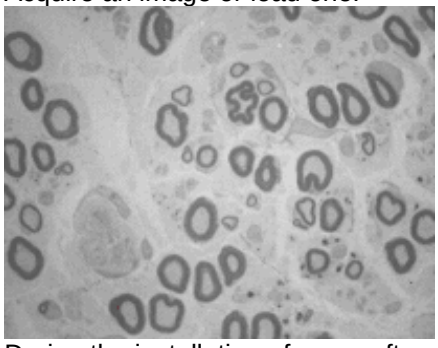
Task

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. Regarding the information as to where the example images are located, please refer to the online help. You can follow these step-by-step instructions when you use the exemplary image "Neurons.tif".

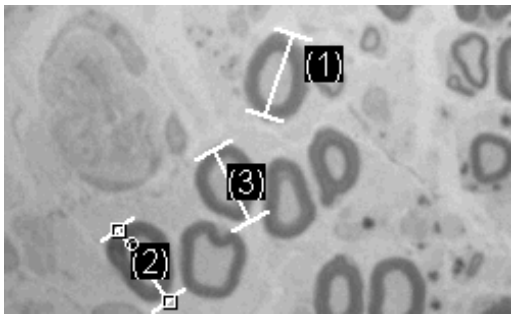
Setting the labeling color

The measurement results will be written into the image according to the default settings, in red font color and without a background. This can't be easily read against the superconductor's structure. 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*.

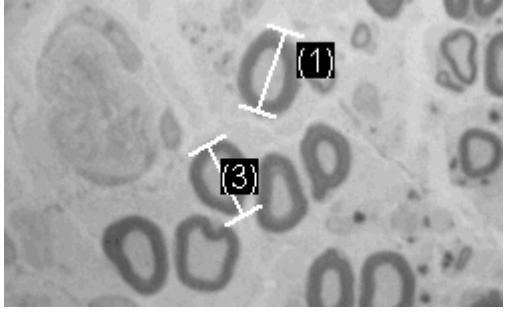
Measuring lengths

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

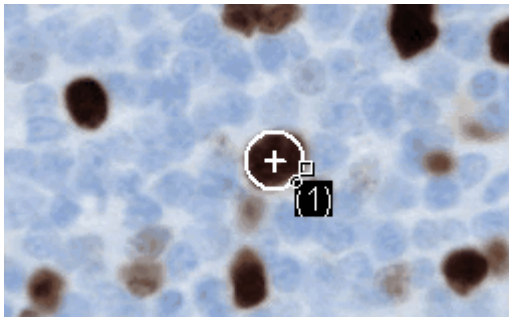
Task

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". Regarding the information as to where the example images are located, please refer to the online help.

Measuring areas

2. In the *Measurement and ROI* tool window, click the *2 Points Circle* button.
3. With your left mouse button, click in the center point of the hexagonal structure.
4. Move your mouse, and in the process drag out the circle. Match the circular object as well as possible to the structure. Click the left mouse button.
5. Click the *2 Points 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.



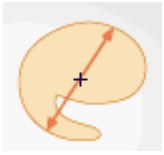
Viewing the list of measurement parameters

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 calculated for all objects.
- A detailed description of the 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 adopted in the list. 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.

Outputting measurement parameters in the image

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.

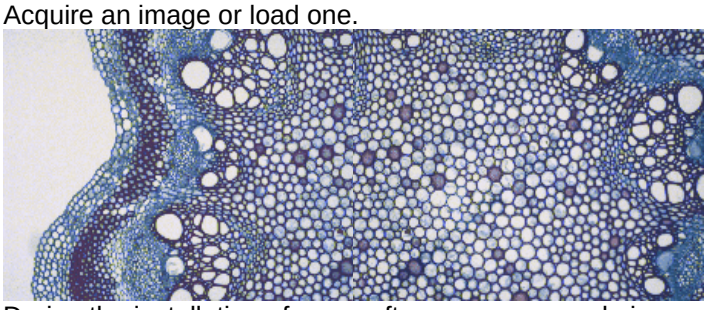
Measuring several images

Task

You want to measure cells on multiple images. To do so, acquire some image 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 an image

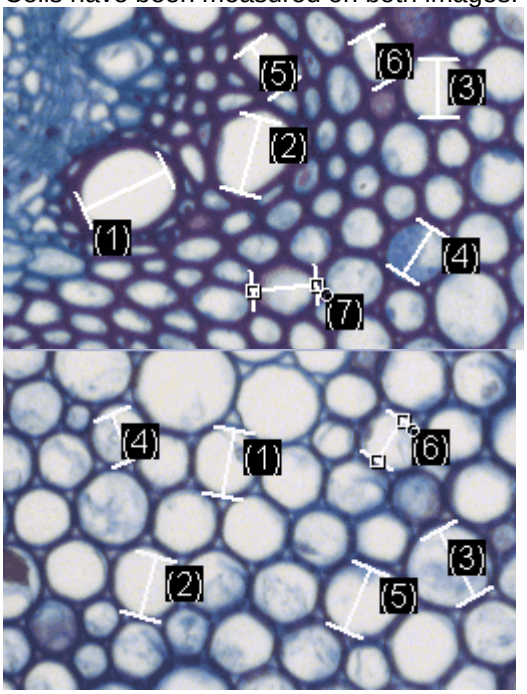
1. Acquire an image or load one.



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". Regarding the information as to where the example images are located, please refer to the online help.

Measuring cells

1. Activate the first image in the document group.
2. 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.
3. Activate the next image. Measure the diameter of several cells on this image, too.
4. Click the *Arbitrary Line* button again, and switch off the length measurement.
   - Cells have been measured on both images.



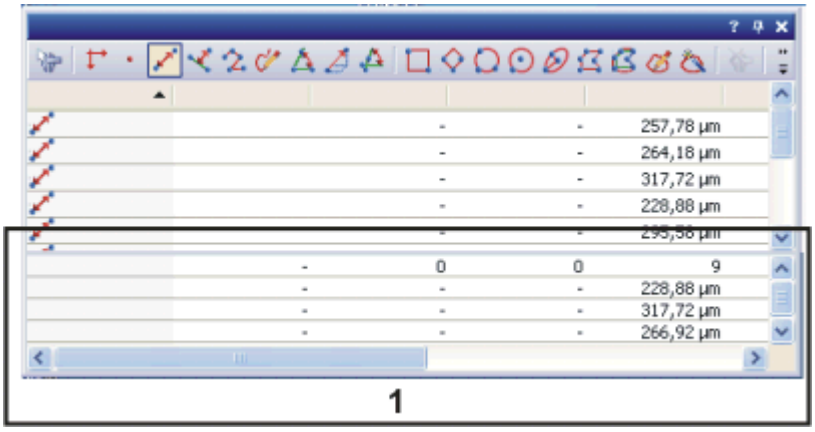
Displaying the measurement results of all of the images

5. In the *Measurement and ROI* tool window, click the *Measurement and ROI Options* button.
6. Select the *Measurement and ROI > Results* entry in the tree view.

7. Clear the *Show measurement objects: Only of the active image* check box.
8. Close the dialog box with *OK*.
   - Now the results for both images will be shown simultaneously in the tool window.

Viewing the statistical parameter

9. In the *Measurement and ROI* tool window, click the *Measurement and ROI Options* button.
10. Select the *Measurement and ROI > Results* entry in the tree view.
11. Select the *Mean* check box.
12. 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.



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9.2. 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). 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. Further information on working with ROIs is available 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.

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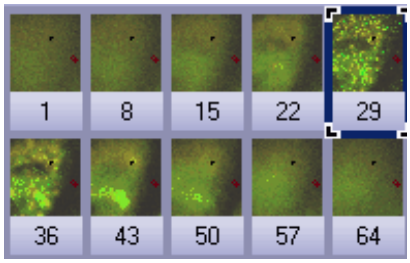
Measuring an intensity profile on a multi-channel Z-stack

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.



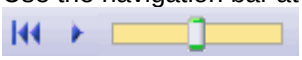
The illustration shows an overview over the frames in a multi-channel Z-stack. The multi-channel image contains a red and a blue color channel. For the acquisition of the Z-stack a through-focus series was taken of the sample. The sample can only be seen clearly, and sharply focused, in the middle of the Z-stack.

1. During the installation of your software some sample images have been installed, too. Regarding the information as to where the example images are located, please refer to the online help. Load e.g., the multi-channel Z-stack with the name "PeroxysomOrganelles.tif" from this directory.

- 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

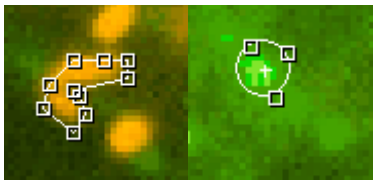
2. Use the navigation bar at the top of the image window.



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.

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 has no fluorescing objects. This ROI will be used as a reference for the background correction.

Calculating an intensity profile

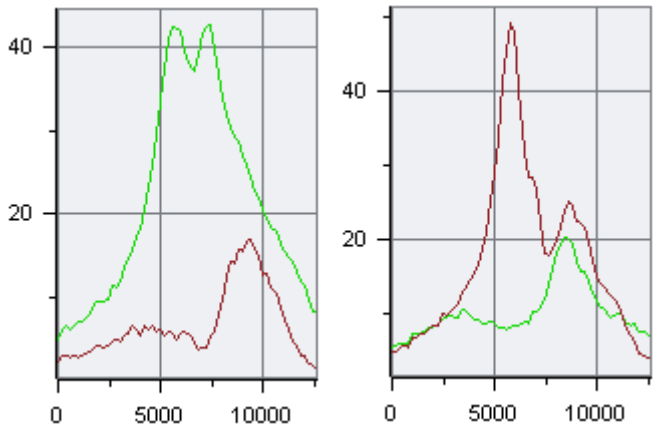
12. On the *Life Science Applications* toolbar, click the *Intensity Profile*  button.
    - The *Intensity Profile - <Name of the active image>* dialog box opens.
    - Your software will recognize the image type, and will select the appropriate option in the *Method* group. In this example the *Z-stack* option is preset.
13. Select the *Results: Average* check box.
    - In the *Intensity profile* 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).
14. 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.
15. 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.
16. In the list, select the ROI that was defined on the image background.
17. Click the *Execute* button.

- The intensity profiles will be calculated and displayed in the *Intensity Profile* tool window.

Viewing intensity profiles

18. If necessary, use the *View > Tool Windows > Intensity Profile* command, to show the tool window.

You can see two diagrams, 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 diagram will be created. The name of the corresponding color channel will be displayed in the diagram's header. On the left you see the results for the green color channel, on the right, those for the red one.
In each diagram you will see a curve for each ROI that has been defined. The diagram contains a caption with the name of the ROI concerned. 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 an intensity profile

19. 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.

20. 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. When you want to use the results in another application program, use the MS-Excel-Export, for this purpose.

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9.3. Measuring the colocalization

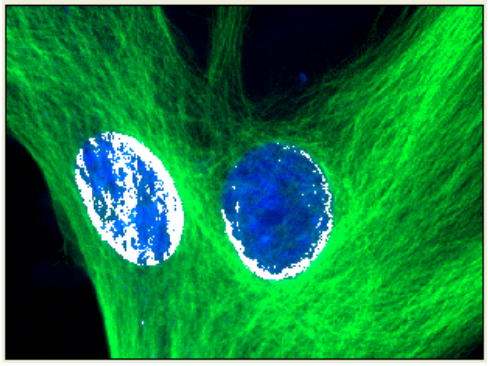
What is colocalization?

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".

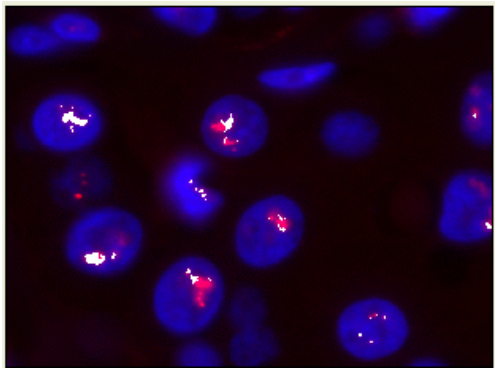
In the digital image analysis, the colocalization of fluorescence signals can be measured. This is done by detecting pixels that have the same intensity in both color channels. These measurements are carried out on multi-channel images, and are always valid for one channel pair.

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



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Measuring the colocalization on the whole frame

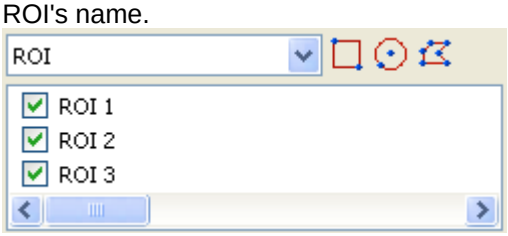
Use the *Colocalization* button to start a measurement of colocalization. You will find this button on the *Life Science Applications* toolbar. This button isn't available in all software versions.

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 > Life 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. Further information on this tool window is available 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. General information on working with ROIs is available 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 > Life 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. Further information on this tool window is available 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.



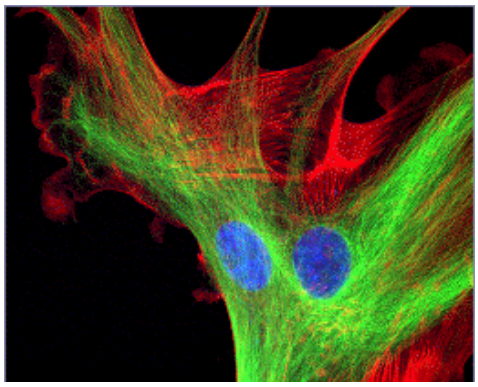
11. Pay attention to the displayed results in the preview, and in the *Results* group.
12. If necessary, change the position and size of the intensity range in the scatterplot.
    - Then only the colocalization of the pixels that lie within the chosen intensity range are shown in the preview.
13. Click 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

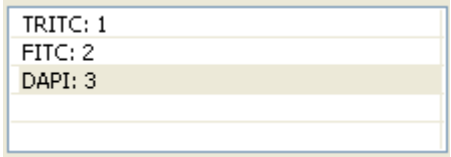
You can also measure the colocalization on image structures. Where images are concerned on which the image structures that are to be analyzed are numerous, and are spread over the whole image, this procedure is quicker than setting a lot of ROIs. If, for example, you want to measure the colocalization 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.

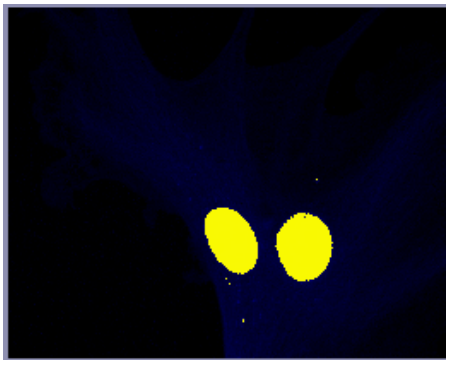


1. Load the multi-channel image for which you want to carry out a colocalization measurement.
2. On the *Life Science Applications* toolbar, click the *Colocalization*  button.
   - The *Colocalization* dialog box opens.
3. When you work with multi-channel time stacks or multi-channel Z-stacks: Determine 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. Further information on this tool window is available 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.

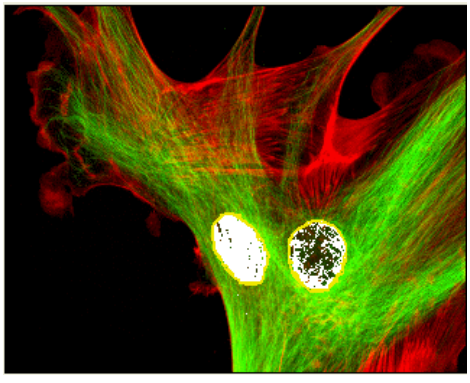


6. Select the channel of the fluorochrome with which the image structure that is to be analyzed, has been stained. In the example shown above, this is the "DAPI" channel.

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. An overview on "Thresholds" is available 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.

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OLYMPUS SOFT IMAGING SOLUTIONS GMBH
Johann-Krane-Weg 39, 48149 Münster, Germany
Phone: +49 (251) 7 98 00-0, Fax: +49 (251) 7 98 00-6060, info.osis@olympus-sis.com