9 Image Acquisition Modules

9.1 ApoTome

General

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

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

is achieved.

The following units are required for an ApoTome imaging workstation:

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

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

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

Fringe projection imaging principle

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

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

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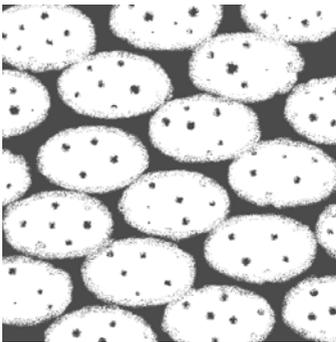
The above representation of a microscopic image of cell nuclei in tissue shows this effect.

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

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

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

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

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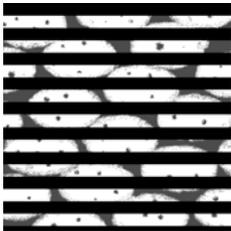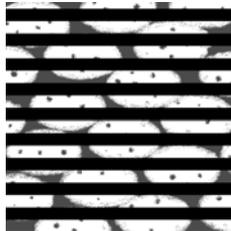
Grid Position 1   Grid Position 2   Grid Position 3
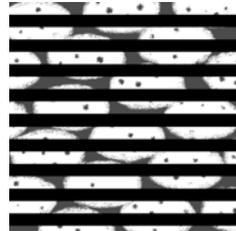
The three raw images are combined online on the PC to form a resulting image. The time it takes to process the image depends on the image size:

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

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

- The grid structure has been removed from the raw images.
- The parts of the image that are out of focus are no longer visible.
- The sharpness and contrast of the image have been increased.
- The image's resolution in the axial direction has been increased.

A corresponding resulting image is represented schematically above.
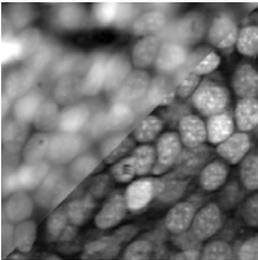
The above image is an application image of cell nuclei (tadpole brain section) in black and white.

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

Why is the resulting image an optical section?

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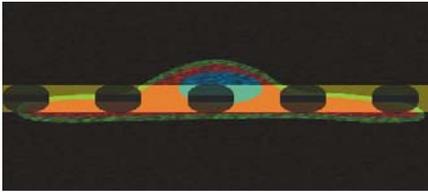
The image of the grid provides the necessary information on the distance of the various sample structures from the set focal plane (see figure above). Some sample structures are in focus, while others lie above or below the focal plane, and enter the set focal plane.

The technique of grid projection makes use of the fact that the image of the grid above and below the actual focal plane is blurred, and enters the blurred areas of the sample. When the grid line is moved, significant brightness differences (= contrast) appear in the focal plane. Outside the focal plane only minor differences are produced, as the sample and the image of the grid are practically "blurred" together. The brightness differences are detected by the algorithm used to combine the three raw images, and are used to remove the parts of the image that are out of focus.

Hardware configuration

For information on setting up the devices, please refer to the manuals of the devices concerned.

➢ First switch on the microscope, then the fluorescence switched-mode power supply, followed by the ApoTome control box and the camera.

➢ Then switch on the PC.

ApoTome Configuration for Axioplan 2 imaging e

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

Windows desktop by double-clicking on the [Microscope Configuration] icon.
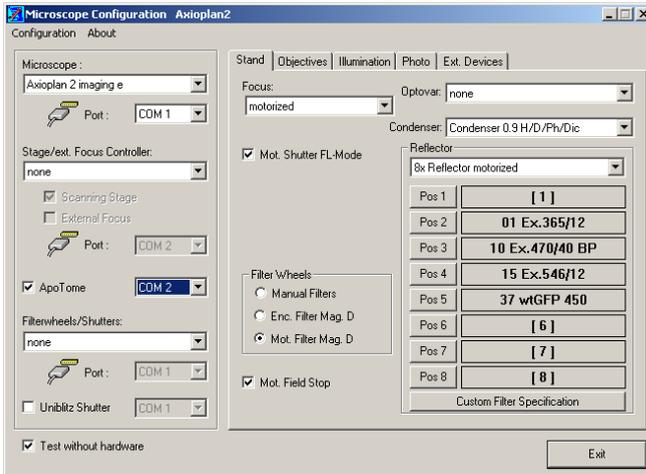
➢ [ApoTome check box / COM 2] Configure the microscope. To use the ApoTome, the **ApoTome** check box must be activated.

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

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

➢ Close **Microscope Configuration** by clicking on the **Exit** button, and confirm the changes by clicking on the **Save & Exit** button in the dialog that then follows.

ApoTome Configuration for Axio Imager, Axio Observer or Axiovert 200

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

desktop by double-clicking on the icon .

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

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

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

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

➢ Click on the **No ApoTome** entry. The entry will be shown with a blue background.
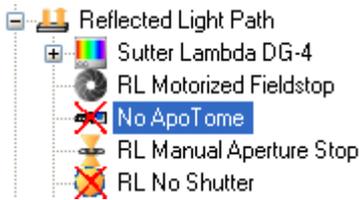
➢ Select the **ApoTome** by clicking on **ApoTome** in the selection area on the right-hand side of the user interface and assign it.
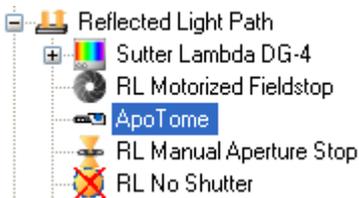
➢ In the information area, you can now select the connection type (RS232 or CAN bus) and the COM port being used (port 1 to 5).
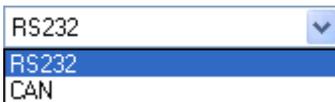
➢ Check the connection by clicking on the **Check Connection** button. If the **ApoTome** is found at the specified port, a green symbol is displayed next to the button.

Note:

• If you activate the **Simulate Hardware** check box, for example, the function of the ApoTome can be adjusted virtually in the **AxioVision** microscope control and imaging software (simulation mode).

ApoTome Calibrations

➢ Start the AxioVision software by double-clicking on the [AxioVision] icon
on the Microsoft Windows desktop.

For subsequent steps it is useful to have easy access to the following control
elements. Please bear in mind that, depending on the features of the device,
some control elements may not be present, or may appear different:
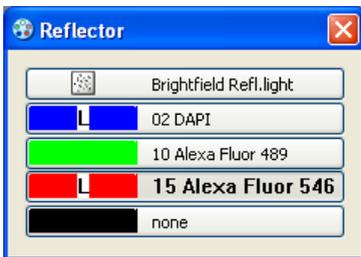
: Reflector nosepiece for switching between
fluorescence wavelengths.

Internal shutter for opening and closing the
shutter.
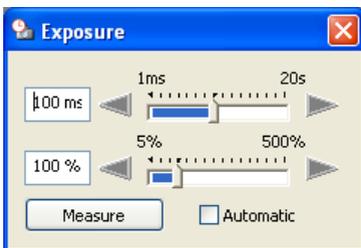
Camera exposure time.
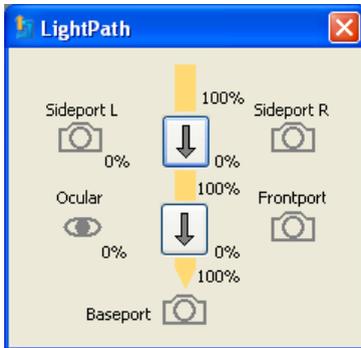
 Light path for switching the light path, e.g. with an Axiovert 200.

You can also create a user-specific dialog containing all the control elements required, including the control elements for the ApoTome functions. Information on creating dialogs can be found in chapter 8 "Configuration".

Calibration of phase position

To set the optimum angle of deflection for the ApoTome's scanner unit, you need to carry out a fine adjustment of the scanner calibration in accordance with the system structure. The mirror sample and the special reflected light reflector cube, both of which are supplied with the ApoTome, are used for this purpose. Insert the Push&Click filter cube into an empty reflector nosepiece position, and enter this filter set as **Refl. BF**, for example, in the **Microscope Configuration** or the **MTB2004**. The calibration must be performed for each of the two grids provided.

It is advisable to calibrate the grid for the low magnification range (grid marked with "L" for "Low magnification") using a 20x objective.

In the phase calibration dialog the positioning of the camera is also optimized. To achieve optimum performance, the camera horizontal should be aligned parallel to the ApoTome grid lines with as much precision as possible.

The calibration process is supported by a wizard. Start the function by selecting from the **Acquisition** menu the **ApoTome** function, and then **Phase Calibration**.

The software wizard guides you through the calibration process in 5 steps. The most important instructions are displayed in the wizard's text field.

Step 1: Select Start Conditions

Step 1 of 5: Select Start Conditions
The calibration process should be startet after a warm-up time of the system of approximately 20min. The Phase Calibration needs to be done with only a single grid of your choice.
 - Insert the grid corresponding to the objective
 - Use the mirror test slide for the phase calibration
 - Use the bright field reflector cube
 - Adjust the following settings

➢ Move the ApoTome slider to the iris position (click stop position 1).

➢ Use the reflector position with the ApoTome bright-field reflector.

➢ Place the mirror sample supplied under the microscope, open the reflected light shutter, and focus (directly on the microscope) on the reticle at the center of the sample.

➢ Now switch to operation of the software on the PC.

➢ In the software, enter settings for grid, reflector, and objective, if you have not already done so. Pay particular attention to ensuring that the grid is correctly selected.

Note:
• It is possible to use the ApoTome in combination with a Colibri, an excitation filter wheel, a Sutter DG-4 or DG-5 or a T.I.L.L. Photonics Polychrome V. In this case, carry out the relevant settings in addition to the microscope settings (grid, reflector, objective).

➢ Click on **Next** to proceed to the next step. The live image is opened automatically.

Step 2: Optical Focusing

Step 2 of 5: Optical Focusing

Use the first click-stop of the ApoTome slider (iris position) to focus on the cross hair of the mirror slide

- Adjust the exposure time of the camera
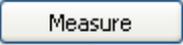
➤ Set an optimum exposure time by clicking on the [Measure] button.

➤ Display the reticle as sharply as possible in the live image. The green focusing rectangle in the live image can help you find the optimum focus position. Position the focusing rectangle over the center of the reticle.
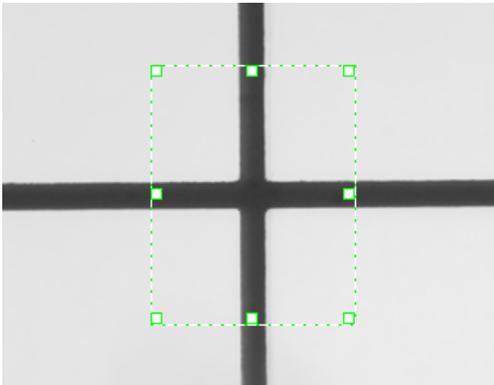
Notes:

- On the **Microscope** property page you can enter general settings for the microscope.

- Click on **Next** to proceed to the next step.

Step 3: Grid Focusing

> **Step 3 of 5: Grid Focusing**
> Carefully push the ApoTome slider in the second click-stop position. Make
> sure, that grid lines are aligned horizontally (Camera adjustment)
>   - Position the green focus rectangle over approximately 10 grid lines
>   - Click on 'Full Scan'
>   - (The grid will be moved through the complete travel range)
>   - The grid position with the highest focus value will automatically be selected

Move the ApoTome slider carefully to the second click stop position
(ApoTome mode).

➢ Move the focusing rectangle in the Live window to a position where no
   part of the reticle is present.

➢ Click on the **Full Scan** button. The grid will now move through its
   adjustment range automatically, in increments of ten steps. A sharpness
   value for the reproduction of the grid will be determined, and displayed
   as a histogram, following each increment. When the end of the
   adjustment range has been reached, the grid position with the highest
   sharpness value will be set automatically.

➢ You can choose to carry out a fine adjustment around the grid position
with the highest sharpness value by clicking on the **Local Scan** button. A
local area above and below the sharpness maximum that has been
identified will be covered automatically in individual steps, and the
sharpness value will be determined for each individual step. The data
from the local scan will be included in the histogram, and the grid
position with the highest sharpness value will again be located
automatically.

Note:

- You can determine the grid position freely using the buttons for coarse
  focusing, 　◀◀　 and 　▶▶　, and fine focusing, 　◀　 and 　▶　. You may
  need to use this function if, for example, a sharpness maximum cannot be
  determined automatically.

Step 4: Camera Alignment

Step 4 of 5: Camera Alignment

Move the cross hair out of the live image. Move the starting point of the grid detection by moving the green focus rectangle up and down.
- Rotate the camera head until you get as close to 0.00 degree as possible

The purpose of this step is to achieve precise alignment of the camera. To achieve optimum results, the camera horizontal should be aligned parallel to the grid lines with as much precision as possible. The software automatically detects a grid line and calculates the camera horizontal's deviation from this grid line in degrees.

As the vertical line of the reticle interferes with the detection of the grid lines, please first move the reticle out of the camera's field of view.

A green rectangle marks the software's search area, a yellow line indicates a detected grid line and a small dark-green rectangle shows you the starting point for line detection.
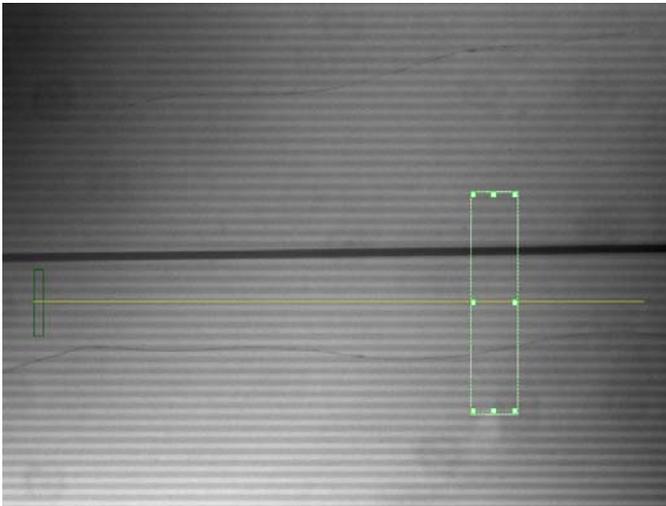
If the contrast in the image is insufficient to detect a grid line automatically, a corresponding error message appears:

In this case you should take steps to increase the contrast (e.g. increase the exposure time, refocus the grid (step 3)).

If it is still not possible to detect a grid line, a corresponding error message appears:

In this case the deviation of the grid lines from the camera horizontal is probably too great. Rotate the camera until the long side of the camera's field of view is roughly parallel to the grid lines. To do this, loosen the corresponding lock on the camera adapter and realign the camera.

If a grid line can be detected but the deviation is still greater than 5 °, you will see a message to this effect:
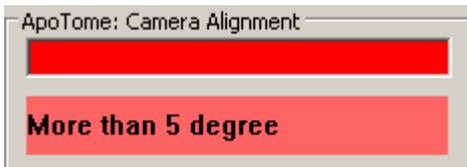
In this case, rotate the camera further to minimize the deviation. For fine adjustment you can use the red bar in the dialog. Try to achieve a deviation < 0.1 °.

Once the camera has been aligned with sufficient precision, please lock the camera adapter in this position.

Notes:
- If you change the ApoTome grid, the new grid should be inserted with the same alignment, if possible. The grid mount in the ApoTome slider has a small amount of play to make it as simple as possible to change the grid. You should always align the grid, if possible, with the same edges in the grid mount. If you wish to check the camera alignment following a change of grid, you can run through the phase calibration dialog up to step 4. Should you not wish to perform a new phase calibration, cancel the dialog after step 4, once the camera has been aligned.

Step 5: Final Full-Phase Calibration

Step 5 of 5: Final Full-Phase Calibration
Click on 'Full Scan'. Starting from default value a defined range of values will be scanned. The phase value with the least amount of residual stripes in the image will automatically be selected.

This step is used for the actual setting of the scanner calibration. In each case, two images are acquired. The grid is displaced by precisely the width of a line between the first and second image. The two images are subtracted from each other, and the resulting image is displayed. If the system is perfectly calibrated, the two images cancel each other out – a black image with a noise component is shown.
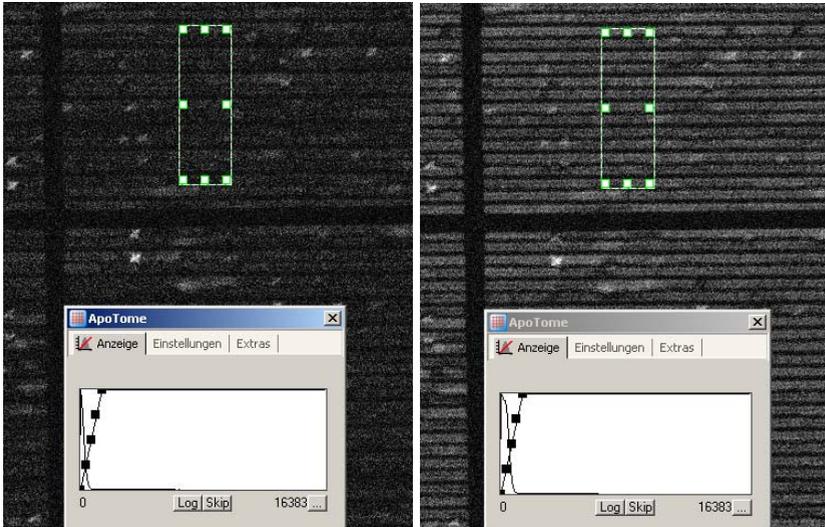
Phase calibration is performed automatically in accordance with the focus calibration of the grid, carried out in the preceding step.

➢ Click on the **Full Scan** button. A predefined area around the specified phase calibration value will be covered in coarse steps. At each position, the calibration value will be determined, and displayed in the histogram. When the end of the area has been reached, the minimum value (minimum number of residual lines present in the image) will automatically be selected in the histogram.
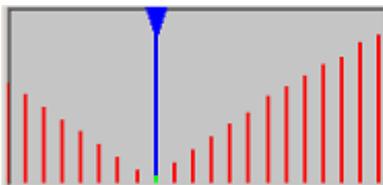
➢ You can choose to perform an additional fine adjustment using the **Local Scan** button. An area close to the minimum on either side will be covered in individual steps, and the data included in the histogram. This may enable you to identify an even lower minimum, where there are even fewer residual lines in the image.
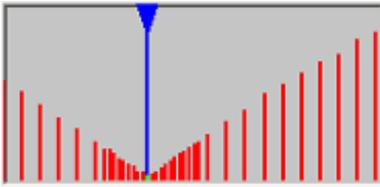
Notes:

- Optional you can change the phase value stepwise by clicking on the buttons [-] [--] [---] and [+] [++] [+++]. You may need to use this function if, for example, a sharpness maximum cannot be determined automatically.

- Click on the **Close** button to end the phase calibration.

- Repeat the procedure to calibrate the phase shift for the second grid, if necessary.

- Although the settings do not change once calibration has been performed, from time to time you should check that the system is perfectly calibrated. This applies in particular after dismantling and reassembling the system.

Calibration of the grid focus

For the ApoTome to function optimally, the grid must be displayed precisely in the focus set on the objective. The grid's focus depends on the objective and the excitation and emission wavelengths of the fluorescence. This means that the grid focus needs to be calibrated for every fluorescent dye (e.g. DAPI, FITC, Rhodamine etc.). It is best to perform calibration using your own fluorescence sample, although this must exhibit flat fluorescence to allow the focusing of the grid. Alternatively, you can also use the fluorescence sample provided.

A wizard is available for the calibration of the grid focus. This guides you through the calibration process in 3 steps. Start the function by selecting from the **Acquisition** menu the **ApoTome** function, and then **Grid Focus Calibration**.

The most important instructions are displayed in the wizard's text field.

Step 1: Select Start Conditions

Step 1 of 3: Select Start Conditions

The calibration process should be startet after a warm-up time of the system of approximately 20min. The Grid Focus Calibration has to be done for each combination of objective and reflector

- Insert the grid corresponding to the objective
- Use the fluorescence test slide for the grid focus calibration
- Adjust the following settings

➢ Move the ApoTome slider to the iris position (click stop position 1).

➢ Use a reflector position with a fluorescence filter (e.g. 10 Ex. 470/40).

➢ Place the fluorescence sample under the microscope, and open the reflected light shutter.

➢ Focus on the sample on the microscope.

➢ Switch to operation of the software on the PC.

➢ In the wizard, select the settings for **Grid**, **Reflector** and **Objective**, if you have not already done so.

Notes:

- Make sure that the correct transmission grid is selected for the objective being used. The table at the end of this chapter provides an overview explaining which transmission grid should be assigned to which objective.

- It is possible to use the ApoTome in combination with a Colibri, an excitation filter wheel, a Sutter DG-4 or DG-5 or a T.I.L.L. Photonics Polychrome V. In this case, carry out the relevant settings in addition to the microscope settings (grid, reflector, objective).

- Click on **Next** to proceed to the next step. The live image is opened automatically.

Step 2: Optical Focusing

Step 2 of 3: Optical Focusing

Use the first click-stop of the ApoTome slider (iris position) to focus on the specimen
 - Adjust the exposure time of the camera

➢ Set an optimum exposure time by clicking on the ⬚ Measure ⬚ button.

➢ Focus the sample as sharply as possible in the live image.

Note:

• On the **Microscope** property page you can enter general settings for the microscope.

➢ Click on **Next** to proceed to the next step.

Step 3: Grid Focusing

Step 3 of 3: Grid Focusing

Carefully push the ApoTome slider in the second click-stop position
 - Adjust the exposure time of the camera if necessary
 - Position the green focus rectangle over approximately 10 grid lines
 - Click on 'Full Scan'
 - (The grid will be moved through the complete travel range)
 - The grid position with the highest focus value will automatically be selected

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➢ Move the ApoTome slider carefully to the second click stop position (ApoTome mode).

➢ Move the focusing rectangle in the live window to a position where the fluorescence is as flat and bright as possible.

➢ Click on the **Full Scan** button. The grid will now move through its adjustment range automatically, in increments of ten steps. A sharpness value for the reproduction of the grid will be determined, and displayed as a histogram, following each increment. When the end of the adjustment range has been reached, the grid position with the highest sharpness value will be set automatically.

➢ You can choose to carry out a fine adjustment around the grid position with the highest sharpness value by clicking on the **Local Scan** button. A local area above and below the sharpness maximum that has been identified will be covered automatically in individual steps, and the sharpness value will be determined for each individual step.

The data from the local scan will be included in the histogram, and the grid position with the highest sharpness value will again be located automatically.

Note:

• You can determine the grid position freely using the buttons for coarse focusing, ⏪ and ⏩, and fine focusing, ◀ and ▶. You may need to use this function if, for example, a sharpness maximum cannot be determined automatically.

➢ When you click on **Close** to end the calibration, the following dialog window is displayed:

➢ Click **Yes**, if you want to carry out a further calibration, or click **No** if you want to end the calibration.

Once calibration is complete, a corresponding message is displayed in the status window of the ApoTome dialog window (**Acquisition** menu ⇒ **ApoTome** function ⇒ **ApoTome Dialog** ⇒ **Settings**).

Calibration mode

There are two possibilities for managing ApoTome calibration data:

▪ **User mode** (conventional): An individual file containing the calibration data is generated in each Windows user account, i.e. each user generates his own calibrations and then works with these. Other users do not have access to these data. In this case, the file containing the calibration data can be found in the *My Documents* folder of the user account in question under *My Documents\Carl Zeiss\Data\ApotomeSetting\Default.ini* ".

▪ **Administrator mode**: A user generates calibration data which are then made available to all other users. In this case, only the user who has generated calibrations in the ApoTome administrator mode is able to change these or add to them. The file containing the calibration data can be found in the folder *Shared Documents\Carl Zeiss\Data\ApotomeSetting\Default.ini*.

The user mode is activated automatically following a new installation of AxioVision. If you want to change the mode, open the calibration mode dialog (**Acquisition** menu $\Rightarrow$ **ApoTome** function $\Rightarrow$ **Calibration mode**):
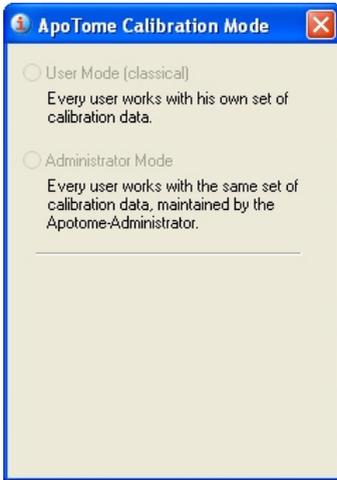
The administrator mode is password-protected. The default password is "zeiss" and can be replaced by a password of your choice following the first log in.
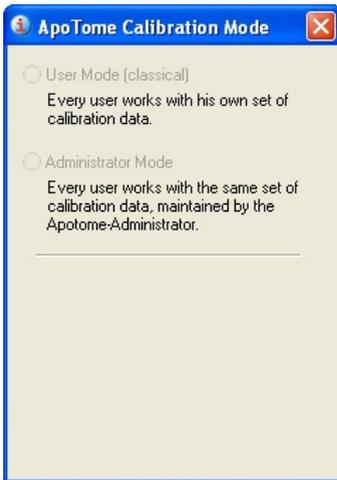
Once administrator mode has been activated, all users are only able to access the calibrations that can be found in the shared files. However, any individual calibrations that have previously been generated remain in the relevant user accounts (although they cannot be used). If you switch back from the administrator mode to the user mode, the file containing the calibration data that has been saved in the shared files will be copied to the Windows user account of the ApoTome administrator. Users of other Windows accounts can then use their old calibrations again or may have to generate new calibrations.

Image acquisition

As an ApoTome imaging system works with a digital CCD camera, the ApoTome mode can be regarded as an expansion of the camera's functionality.

Selecting the camera

In principle, a cooled monochrome CCD camera (AxioCam MRm, AxioCam HRm etc.) is used in combination with the ApoTome for fluorescence images. When you start AxioVision, a monochrome camera is automatically selected (if possible) for operation with the ApoTome.

If several monochrome cameras are connected to the system, you need to define the one that you want to be used in ApoTome mode. To select the camera, follow the procedure below:

➢ Click on **ApoTome** in the work area or open the ApoTome dialog (**Acquisition** menu ⇒ **ApoTome** function ⇒ **ApoTome Dialog**). Select the **Extras** property page

➢ In the **ApoTome: Camera** drop-down field, select the camera that you want to use in ApoTome mode.

Single image acquisition

For single image acquisition, follow the procedure below:

➢ For efficient operation, open the control elements for the camera exposure time (**Acquisition** menu ⇒ **Adjust** function ⇒ **Exposure**), the shutter and the reflector (**Microscope** menu ⇒ **Reflected Light** function ⇒ **Reflector** or **Internal Shutter**).

➢ Make sure that the ApoTome slider is in the second click stop position (ApoTome mode).

➢ Click on **ApoTome** in the work area or open the ApoTome dialog (**Acquisition** menu ⇒ **ApoTome** function ⇒ **ApoTome Dialog** ⇒ **Settings** property page).

➢ Make sure that in the **ApoTome: Grid** field the grid that is currently inserted has been selected. On the basis of the objective that is currently being used, a recommended grid will also be displayed in this field.

➢ Select the **Camera** property page.

The live image is opened using the [Live] button on the toolbar. The shutter is opened automatically with the live image.
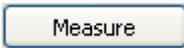
➢ Click on the [Measure] button to set an optimum exposure time for the specimen. To achieve optimum results with the ApoTome, the images should be illuminated in the best possible way. Full use should be made of the camera's dynamic range. However, no pixels should be overexposed. The histogram displayed will help you achieve optimum illumination.

Various settings can be made for the ApoTome mode. Three settings are available for the live image in ApoTome mode (**Settings** property page in the ApoTome dialog).
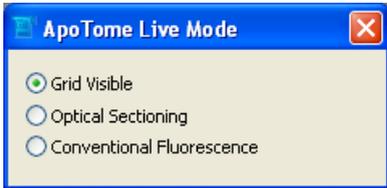
Grid Visible: The behavior of the live image is the same as in conventional camera mode. No processing takes place. As the grid is located in the beam path, it is also visible in the live image. The grid is moved up and down constantly, to prevent grid lines being "burnt" into the sample as a result of the bleaching effects of the fluorescent dye.

Optical Sectioning: In this mode three images are acquired, combined online into an optical section, and displayed. As this mode requires the acquisition of three raw images, the speed of the live image is reduced accordingly.

Note:
• In this mode, if you change the focus on the microscope or the position of the sample, artifacts, in the form of lines, may appear in the live image for a short time. The same effect can occur if vibrations are passed on to the microscope. The lines disappear from the live image as soon as the sample is stabilized again.

Conventional Fluorescence: In this mode two raw images are acquired, and the grid position is displaced in each case by precisely the width of the lines. From these two raw images, an image that corresponds to the conventional fluorescence image (wide field) can be calculated. This mode enables you to make comparisons between the conventional fluorescence image and the optical section.

Image acquisition is triggered using the Snap button on the toolbar.

Four settings are available for image acquisition in ApoTome mode (**Settings** property page in the **ApoTome** dialog).

➢ **No Processing**: The behavior of image acquisition is the same as in conventional camera mode. No processing takes place. As the grid is located in the beam path, but is moved constantly up and down, the image acquired corresponds to a conventional fluorescence image. This setting is only used if you are carrying out automated image acquisition in several channels, and processing needs to be switched off, e.g. to acquire a transmitted light or a phase contrast image.

➢ **Optical Sectioning**: In this mode three images are acquired, combined online into an optical section, and displayed.

➢ **Conventional Fluorescence**: In this mode two raw images are acquired, and the grid position is displaced in each case by precisely the width of the lines. From these two raw images, an image that corresponds to the conventional fluorescence image (wide field) can be calculated. This mode enables you to make comparisons between the conventional fluorescence image and the optical section.

➢ **Raw Data Mode**: In this mode the raw image acquisition data are saved (i.e. the individual images with the various grid positions are saved internally). The advantage of this mode is that it allows you to switch between the algorithms (**Optical Sectioning** versus **Conventianal Fluorescence**) after image acquisition:

- To do this, open the shortcut menu for the acquired image by clicking on the **Properties** icon at the bottom edge of the image. screen Alternatively, you can also open the shortcut menu by right-clicking in the acquired image.

ApoTome Image property page:
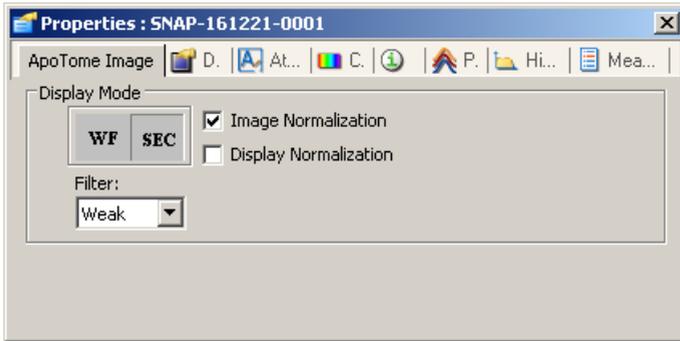
➤ Click on the **WF** (Widefield = **Conventional Fluorescence**) or **SEC** (Section = **Optical Sectioning**) buttons to switch between the corresponding algorithms. The filter used to remove residual lines in the image (**Filter** drop-down list box) and the display scaling (**Auto Display Scaling**) can also be adjusted. The application of the algorithm takes place purely in the display plane of the image data and does not change the original raw data.

Note:
- In order to process ApoTome data in AxioVision using the 3D Deconvolution module, you must acquire the image in the **Raw Data Mode**.

Acquiring images in the **Raw Data Mode** substantially increases the memory required for the image data. In the **Averaging** default setting, for example, it is increased by a factor of 3 in comparison to an image acquired with ApoTome in the **Conventional Fluorescence** or **Optical Sectioning** modes. If the **Averaging** value is increased by 2 or 3, the resulting memory requirement increases by a factor **of** 6 or 9. We therefore recommend that you use the **SIMConvert** function to convert the raw data to an optical section or a conventional fluorescence image, thereby substantially reducing the data volume again (e.g. after using 3D Deconvolution or for 3D reconstruction of an image stack using the Inside 4D module.)

The thickness of the **Optical Section** can be displayed in the **Depth Info** field on the **Extras** property page.
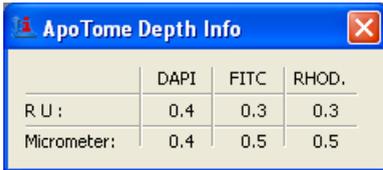
Notes:

*   Depending on the sample, fluorescent dye, exposure time etc., fine residual lines may be visible in the resulting image in ApoTome mode. These lines can be removed using the filter. You can select **Off**, **Weak**, **Medium** and **Strong**.

*   The residual lines are displayed in the Fourier space as points along a vertical line. The filter "removes" a defined group of points from the Fourier spectrum.

Start with the setting **Weak**. If residual lines can still be identified in the image, increase the setting step by step. As the filter is used during the calculation of the optical section, a new image must be acquired whenever changes are made to the filter setting.

A further increase in image quality can be achieved if you use a value of 2 or higher in the **Averaging** field.
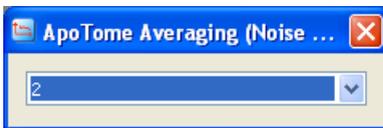
If you use the value **2**, for example, two ApoTome images are acquired and averaged. This method significantly reduces the noise component. An additional effect is the reduction or removal of residual lines in the image.

Multidimensional acquisition with the ApoTome

The ApoTome functions are fully integrated into AxioVision's automatic image acquisition modes. You can perform acquisition of multichannel fluorescence images, z-stack images, time lapse images, MosaiX and any combination of these modes using the ApoTome. The images are acquired in accordance with the settings made under Acquisition menu ⇒ **ApoTome** function ⇒ **Acquisition Mode**. A precise description of the Multidimensional Acquisition functionality can be found in section "Multidimensional Acquisition".

A prerequisite for the above is calibration of the system as described in chapter 8 under the headings "Configuration" and "Scalings". Essentially, for multidimensional acquisition the same functions as those described in the previous sections apply.

If, during multichannel image acquisition, components that are responsible for the fluorescence wavelength being used are operated automatically, for example, a focus calibration must be available for each combination. The crucial components are:

- Reflector nosepiece
- Colibri
- External excitation filter wheel (e.g. Ludl, Sutter)
- Sutter DG-4 or DG-5
- T.I.L.L. Photonics Polychrome V

If a grid focus calibration is not available for a combination of the above external components, a single image is acquired automatically without any shifting of the grid. This artifact is usually easy to identify as an image with clearly pronounced fringes. For better control of image acquisition, open the **ApoTome: Status** window (**Acquisition** menu ⇒ **ApoTome** ⇒ **Status** function). This window will inform you if, during multidimensional image acquisition, a microscope status is set for which no ApoTome focus calibration is available.

Accelerated image acquisition with the ApoTome (burst mode)

With the ApoTome, the speed of image acquisition (image rate) is limited by the following:

1. At least three raw images have to be acquired for a resulting image.

2. During image acquisition, as much as possible of the gray-value range of the camera in question should be used. This generally results in exposure times that prevent high acquisition speeds from being achieved.

However, provided that acquisition takes place in one fluorescence channel only, it is possible to increase the image acquisition rate to a value in the region of 4 to 5 images/s. To achieve these values, follow the procedure below:

➢   Open the **ApoTome: Status** window (**Acquisition** menu ⇒ **ApoTome** ⇒ **Status** function).

➢   In the **ApoTome Dialog** on the **Camera** property page, set a frame that is as small as possible (e.g. 512 x 512 pixels or smaller).

>   **Tip:**
>   If your requirements with regard to optical resolution allow, you can increase the binning on the camera to 2x2 (**Camera** work area⇒ **Frame** property page). This function makes it possible to reduce the camera's exposure time by around a factor of 4, at the expense of the resolution in the image.

➢ For the **ApoTome: Acquisition Mode**, select the **Raw Date Mode** setting (**ApoTome** property page ⇒ **Settings** property page).
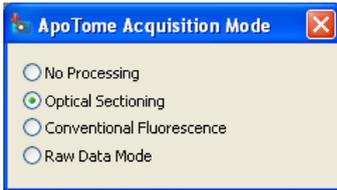
➢ In the work area, select the **Multidimensional Acquisition** entry and then the **C (Channels)** property page.

➢ Make sure that no **Hardware Settings** has been selected for the **During Acquisition** and **After Acquisition** drop-down list boxes.
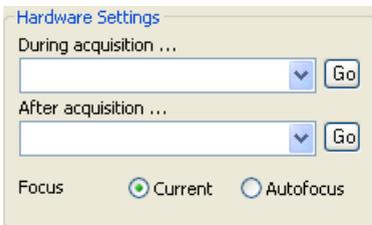
➢ Determine an optimum exposure time by clicking on the
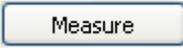
 button. A pop-up window containing a live image opens.

Confirm the exposure time by clicking on . If necessary, the measured exposure time can be reduced manually by around 10 to 20%.
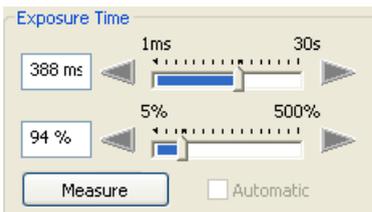
➢ Select the **T (Time)** property page and activate the
☐ Time Lapse   check box.

➢ Set the **Interval Settings** to **Maximal Speed** or alternatively the
Interval to 0.

```
┌─ Interval Settings ─────────────────────────┐
│  ○ Maximal Speed                            │
│                                             │
│  ◉ Interval    │ 1.000 │ ⇕ │ Second  ▼ │   │
└─────────────────────────────────────────────┘
```

➢ In the **Duration Settings** field, define either the number of cycles
 # Cycles (e.g. 50) or the **Duration**.(e.g. 15s).

```
┌─ Duration Settings ─────────────────────────┐
│  ○ Maximal Duration                         │
│                                             │
│  ◉ # Cycles  │ 50    │ ⇕ │ [ Calculate ]    │
│                                             │
│  ○ Duration  │ 49.000 │ ⇕ │ Second  ▼ │     │
└─────────────────────────────────────────────┘
```
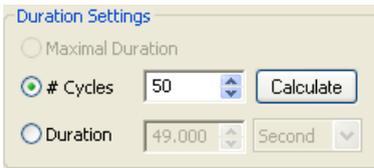
➢ Click on the **Start** button to start acquisition of the image sequence.
In the **ApoTome Status** dialog the message **Calibrated Burst Mode:
ON** is displayed.

In this mode the image data are acquired as quickly as possible, with the
grid being constantly shifted. The optical sections are only calculated after
acquisition (and following a staggered pattern, i.e. the last 3, 6, 9 raw
images etc.), according to the setting of the ApoTome's **Averaging**
function, with different grid positions.

This technique increases the image rate by around a factor of 3, but not
the temporal resolution, as rapidly moving particles on the sample can
nevertheless lead to movement artifacts in the resulting image due to the
exposure times selected during the acquisition of the raw data.

The image view (**Optional Sectioning** / **Conventional Fluorescence**) can be switched at any time, on the basis of the raw data acquired. The corresponding settings are made on the **ApoTome** property page in the shortcut menu of the acquired image.

Notes
- A further slight increase in acquisition speed can be achieved by deactivating the **Show preview at Start** check box in the **Multidimensional Acquisition** work area $\Rightarrow$ **Experiment** property page.

Objective – grid allocation

The following tables provide an overview of the allocation of grids to objectives.

Axio Imager

Objective	V	NA	Immersion	DAPI	recom. grid	optical section thickness @ 490 nm (RU/µm)
EC-Plan-Neofluar 10x/0,3	10	0.3	Air	✔	L1	1.01 / 11.04
EC-Plan-Neofluar 20x/0,5	20	0.5	Air	✔	M	1.03 / 4.05
EC-Plan-Neofluar 40x/0.75	40	0.75	Air	✔	H1	1.09 / 1.89
EC-Plan-Neofluar 40x/1,3 Oil	40	1.3	Oil	✔	M	1.07 / 0.94
EC-Plan-Neofluar 63x/0.95 Korr	63	0.95	Air	✖	H1	0.7 / 0.76

EC-Plan-Neofluar 63x/1,25 Oil	63	1.25	Oil	✔	H1	1.08 / 1.03
EC Plan-Neofluar 100x/1,3 Oil	100	1.3	Oil	✖	H1	0.71 / 0.63
LCI Plan-Neofluar 25x/0,8 Imm. Korr.	25	0.8	Oil, water or glycerine	✔	L1	0.88 / 1.35
LCI Plan-Neofluar 63x/1.3 Imm. Korr.	63	1.3	water or glycerine	✔	H1	1.08 / 0.96
Plan-Apochromat 10x/0,45	10	0.45	Air	✔	L1	1.45 / 6.88
Plan-Apochromat 20x/0,8	20	0.8	Air	✔	L1	1.08 / 1.65
Plan-Apochromat 40x/0.95 Korr	40	0.95	Air	✔	H1	1.07 / 1.16
Plan-Apochromat 40x/1.0 Oil	40	1.0	Oil	✔	H1	0.85 / 0.84
Plan-Apochromat 40x/1.3 Oil	40	1.3	Oil	✔	M	1.07 / 0.94
Plan-Apochromat 63x/1.0 W	63	1.0	water	✔	H1	0.93 / 1.22
Plan-Apochromat 63x/1,4 Oil	63	1.4	Oil	✔	H1	1.06 / 0.81
Plan-Apochromat 100x/1,4 Oil	100	1.4	Oil	✔	H1	0.7 / 0.53
LD-LCI-Plan-Apochromat 25x/0.8	25	0.8	Oil, water or glycerine	✔	L1	0.88 / 1.35
C-Apochromat 10x/0,45 W	10	0.45	water	✔	L1	1.42 / 6.88
C-Apochromat 40x/1,2 W	40	1.2	water	✔	M	0.94 / 0.85
C-Apochromat 63x/1,2 W	63	1.2	water	✔	H1	0.95 / 0.86
LD C-Apochromat 40x/1.1 W	40	1.1	water	✔	M	0.94 / 1.02
alpha Plan-Fluar 100x/1.45 oil	100	1.45	Oil	✖	H1	0.67 / 0.48
alpha Plan-Apochromat 100x/1,46 Oil	100	1.46	Oil	✖	H1	0.67 / 0.47

Axio Observer / Axiovert 200

Objective	V	NA	Immersion	DAPI	recom. grid	optical section thickness @ 490 nm (RU/µm)
EC-Plan-Neofluar 10x/0,3	10	0.3	Air	✔	VL	0.90 / 9.81
EC-Plan-Neofluar 20x/0,5	20	0.5	Air	✔	VH	1.37 / 5.36
EC-Plan-Neofluar 40x/0.75	40	0.75	Air	✔	VH	0.93 / 0.90
EC-Plan-Neofluar 40x/1,3 Oil	40	1.3	Oil	✔	VL	0.75 / 0.66
EC-Plan-Neofluar 63x/0.95 Korr	63	0.95	Air	✘	VD	0.82 / 0.89
EC-Plan-Neofluar 63x/1,25 Oil	63	1.25	Oil	✔	VH	0.93 / 0.89
EC Plan-Neofluar 100x/1,3 Oil	100	1.3	Oil	✘	VD	0.83 / 0.73
LCI Plan-Neofluar 25x/0,8 Imm. Korr.	25	0.8	Oil, water or glycerine	✔	VL	0.78 / 1.20
LCI Plan-Neofluar 63x/1.3 Imm. Korr.	63	1.3	water or glycerine	✔	VH	0.93 / 0.82
Plan-Apochromat 10x/0,45	10	0.45	Air	✔	VL	1.26 / 6.09
Plan-Apochromat 20x/0,8	20	0.8	Air	✔	VL	0.95 / 1.46
Plan-Apochromat 40x/0.95 Korr	40	0.95	Air	✔	VH	0.92 / 0.99
Plan-Apochromat 40x/1.0 Oil	40	1.0	Oil	✔	VH	1.28 / 1.91
Plan-Apochromat 40x/1.3 Oil	40	1.3	Oil	✔	VL	0.75 / 0.66
Plan-Apochromat 63x/1.0 W	63	1.0	water	✔	VD	1.09 / 1.42
Plan-Apochromat 63x/1,4 Oil	63	1.4	Oil	✔	VH	0.91 / 0.69
Plan-Apochromat 100x/1,4 Oil	100	1.4	Oil	✔	VD	0.81 / 0.62
LD-LCI-Plan-Apochromat 25x/0.8	25	0.8	Oil, water or glycerine	✔	VL	0.78 / 1.20
C-Apochromat 10x/0,45 W	10	0.45	water	✔	VL	1.26 / 6.09
C-Apochromat 40x/1,2 W	40	1.2	water	✔	VH	1.25 / 1.13
C-Apochromat 63x/1,2 W	63	1.2	water	✔	VH	0.82 / 0.74

Objective	V	NA	Immersion	DAPI	recom. grid	optical section thickness @ 490 nm (RU/µm)
LD C-Apochromat 40x/1.1 W	40	1.1	water	✔	VH	1.25 / 1.35
alpha Plan-Fluar 100x/1.45 Oil	100	1.45	Oil	✘	VD	0.79 / 0.56
alpha Plan-Apochromat 100x/1,46 Oil	100	1.46	Oil	✘	VD	0.78 / 0.54

Axioplan 2 imaging e

Objective	V	NA	Immersion	DAPI	recom. grid	optical section thickness @ 490 nm (RU/µm)
EC-Plan-Neofluar 10x/0,3	10	0.3	Air	✔	PL	0.95 / 10.30
EC-Plan-Neofluar 20x/0,5	20	0.5	Air	✔	PL	0.77 / 3.02
EC-Plan-Neofluar 40x/0.75	40	0.75	Air	✘	PH	0.98 / 1.71
EC-Plan-Neofluar 40x/1,3 Oil	40	1.3	Oil	✔	PL	0.79 / 0.70
EC-Plan-Neofluar 63x/0.95 Korr	63	0.95	Air	✘	PH	0.64 / 0.69
EC-Plan-Neofluar 63x/1,25 Oil	63	1.25	Oil	✔	PH	0.98 / 0.93
EC Plan-Neofluar 100x/1,3 Oil	100	1.3	Oil	✘	PH	0.65 / 0.57
LCI Plan-Neofluar 25x/0,8 Imm. Korr.	25	0.8	Oil, water or glycerine	✔	PL	0.82 / 1.26
LCI Plan-Neofluar 63x/1.3 Imm. Korr.	63	1.3	water or glycerine	✔	PH	0.98 / 0.86
Plan-Apochromat 10x/0,45	10	0.45	Air	✔	PL	1.32 / 6.40
Plan-Apochromat 20x/0,8	20	0.8	Air	✔	PL	1.00 / 1.54
Plan-Apochromat 40x/0.95 Korr	40	0.95	Air	✔	PH	0.96 / 1.05
Plan-Apochromat 40x/1.0 Oil	40	1.0	Oil	✔	PH	0.77 / 0.76
Plan-Apochromat 40x/1.3 Oil	40	1.3	Oil	✔	PL	0.79 / 0.70
Plan-Apochromat 63x/1.0 W	63	1.0	water	✔	PH	0.85 / 1.10
Plan-Apochromat 63x/1,4 Oil	63	1.4	Oil	✔	PH	0.96 / 0.73
Plan-Apochromat 100x/1,4 Oil	100	1.4	Oil	✔	PH	0.63 / 0.48

LD-LCI-Plan-Apochromat 25x/0.8	25	0.8	Oil, water or glycerine	✔	PL	0.82 / 1.26
C-Apochromat 10x/0,45 W	10	0.45	water	✔	PL	1.32 / 6.40
C-Apochromat 40x/1,2 W	40	1.2	water	✔	PH	1.31 / 1.19
C-Apochromat 63x/1,2 W	63	1.2	water	✔	PH	0.86 / 0.78
LD C-Apochromat 40x/1.1 W	40	1.1	water	✔	PH	1.32 / 1.42
alpha Plan-Fluar 100x/1.45 oil	100	1.45	Oil	✘		0.61 / 0.43
alpha Plan-Apochromat 100x/1,46 Oil	100	1.46	Oil	✘		0.60 / 0.42

Other ApoTome Settings

In addition, the following control elements can be found on the **Extras** property page:

ApoTome: Auto Shutter

If **Automatic Shutter Control** is activated, the shutter is controlled automatically if

1. the live image is opened or closed
2. an image is acquired

This function is useful in order to protect the sample against the bleaching of fluorescence and is activated as a default setting when the software is installed.

ApoTome Display Normalization

The algorithm used to calculate the optical section reduces the image's gray value range. If the **Display Normalization** function is activated, the display characteristic curve is automatically adjusted to guarantee a bright, high-contrast display of the gray values. This function is activated as a default setting when the software is installed.

ApoTome: Status

This field displays status information relating to the ApoTome and image acquisition with the ApoTome. It makes sense to have this window open or to integrate it into your own customized dialogs so that any problems with the system can be analyzed quickly.

Conversion of ApoTome images from raw data mode

If you select the **Raw Data Mode** for image acquisition with the ApoTome, the size of the data is increased by a factor of 3, 6, 9 etc. in accordance with the settings under **Averaging**. If you are acquiring z-stack images, images in several fluorescence channels or time lapse images, this can lead to enormous volumes of data that can be difficult to work with during subsequent analyses. The **SIMConvert** function converts the raw data to an optical section or a conventional fluorescence image with a substantially reduced data volume.

Use of this function is strongly recommended after you have acquired a z-stack in **Raw Data Mode** and processed it using the AxioVision 3D Deconvolution module.

To perform conversion, follow the procedure below:

➢ Click on the ApoTome image that you want to convert.

➢ Click on the **ApoTome** entry in the work area.

➢ Open the function tree by clicking on screen in front of the **ApoTome** entry – click on **SIMConvert**. The parameters for the function are listed underneath the work area.

> Adopt the settings in the top **Parameters** field. The default settings ensure that the selected image is processed and a new image is created automatically (the original file is retained). The **Enable channel selection** check box should be deactivated, as generally all the channels in an image should be converted in the same way.

Four parameters are available in the lower area of the property page for the actual conversion **Combine Mode**, **Filter**, **Image Normalization** und **Display Normalization**:

> **Combine Mode**: This parameter determines the algorithm used to convert the image. You can select from **Conventional Fluorescence** or **Optical Sectioning**.

> **Filter**: Depending on the sample, fluorescent dye, exposure time etc., fine residual lines may be visible in the image that results from the conversion. These lines can be removed using the filter. The options **Off**, **Weak**, **Medium** and **Strong** are available.

> The filter function makes use of the fact that the residual lines are displayed in the Fourier space as points along a vertical line. The filter "removes" a defined group of points from the Fourier spectrum, thereby removing the residual lines.

➢ **Display Normalization**: The algorithm used to calculate the **Optional Sectioning** reduces the gray value range of the image data, depending on the specimen and the acquisition conditions. The **On** functional parameter automatically sets the image's display characteristic curve so that a bright, high-contrast image is displayed. The **Min/Max** function of the display characteristic curve is used, which sets the display characteristic curve on the basis of the darkest and brightest gray value in the image histogram. If a z-stack is converted, the image plane that is currently set in the active image is used to determine the **Min/Max** value for the entire image stack.

➢ **Image Normalization**: As described under "Display Normalization", the algorithm used to calculate the **Optional Sectioning** reduces the gray value range of the image data, depending on the specimen and the acquisition conditions. As the so-called floating point format is used for raw data acquisition with the ApoTome and the subsequent combination of the gray value data, during the conversion from floating point back to gray value, a normalization function can be applied directly to the data to display a bright, high-contrast image. The **On** parameter activates this form of normalization.

Note:
• If **Image Normalization** is switched on, **Display Normalization** is no longer necessary, as the result of both functions has the same effect for the user. For the conversion of a z-stack, the **Image Normalization** function offers advantages, as the gray value distribution of all image planes can be taken into account.

ApoTome: HDR Mode

By activating the **HDR Mode**, images with an expanded dynamic range can also be acquired in ApoTome mode. For further details, please refer to the description of **HDR Snap** and the AxioVision manual.

If the ApoTome is operated in **HDR Mode**, the corresponding number of images will be acquired for each grid position with various exposure times and, from this, an HDR image will be calculated for each grid position. The ApoTome calculation will then be performed on these three HDR images. This will ultimately result in an optical section with an expanded dynamic range.

Because in the **HDR Mode** several images are acquired with very long exposure times, residual fringes may occur in the ApoTome optical section as a result of bleaching effects. In this case, it is necessary to use the ApoTome filters in order to suppress the residual fringes.

The additional time required for calculating the HDR image can lead to delays in the displaying of the preview when multidimensional images are acquired. In this case, the preview should be deactivated.