Confocal Microscopy Configuration

This document describes the principles of Confocal Microscopy followed by the WITec Control software configuration for Confocal Microscopy . The beam path and the step by step alignment procedure can be found in Sections 3 and 4.

1 Principles of Confocal Microscopy

Confocal microscopy requires a point light source (usually a laser), which is focused onto the sample. The reflected light (or fluorescence) is usually collected with the same objective and focused through a pinhole at the front of the detector. This ensures that only light from the image focal plane can reach the detector, which greatly increases image contrast and with the proper selection of pinhole size, slightly increases resolution.

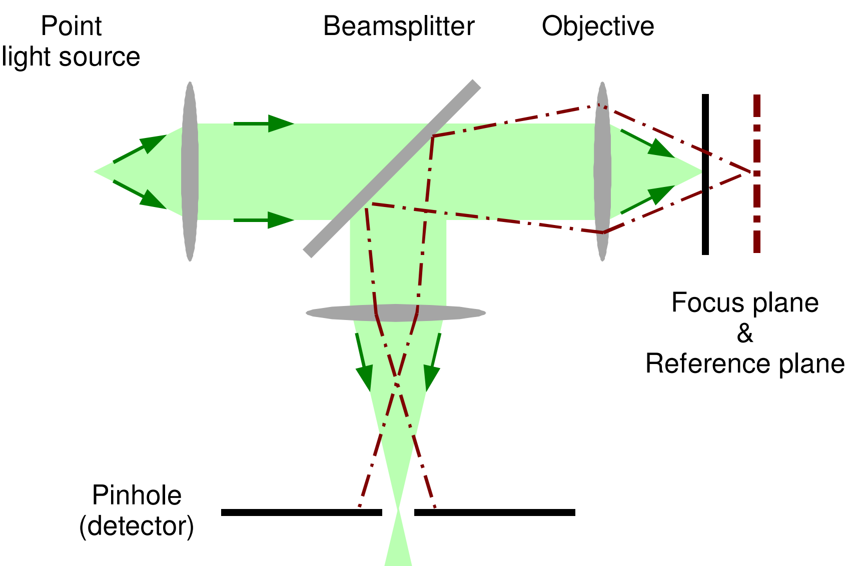


Fig. 1: Principle setup of a confocal microscope

In *alpha300/alpha500/alpha700* systems, the laser light is delivered via single-mode optical fiber. This type of fiber transmits only a single transversal mode (Gaussian beam), which can be focused to a diffraction limited spot. The light reflected by the sample is collected by the same objective and is directed as a parallel beam toward the top of the microscope. Here, the light is focused onto a color video camera or a multi-mode optical fiber. The core of this multi-mode optical fiber acts as a pinhole for confocal microscopy. The laser is raster-scanned across the sample by scanning the sample in all axes and the image is acquired line by line.

Using fibers for beam delivery and signal pick-up is very convenient because bulky lasers and detectors can be placed far from the detecting microscope.

Depending on the system configuration and hardware chosen, the following confocal modes are possible with an *alpha300/alpha500/alpha700* system:

- confocal microscopy in reflection
- confocal microscopy in transmission (using an additional configuration)
- confocal fluorescence microscopy in reflection
- confocal fluorescence microscopy in transmission (using an additional configuration)
- confocal Raman microscopy

To take full advantage of the lateral and depth resolution possible with confocal microscopy, the size of the pinhole (core diameter of the multi-mode fiber for detection) must be properly chosen.

The optimum pinhole diameter depends on the optical properties of the microscope objective along with the wavelength employed and can be calculated using the following formula:

$$D \leq \lambda \cdot v \cdot M/(NA \cdot \pi)$$

where λ is the wavelength of the laser, M is the magnification and NA is the numerical aperture of the microscope objective.

The property v is given in optical coordinates and should be 2.5 for the best depth resolution and 0.5 for maximum lateral resolution. If $v < 0.5$ is chosen, the lateral resolution will be $\sqrt{2} \approx 1.4$ times better than for conventional microscopy. However, in this case most of the light reflected from the sample does not reach the detector, so one sacrifices efficiency.

Make sure that you use the microscope objectives in the proper way. Some of the objectives supplied may be corrected for the use with a cover slip.

There are four numbers printed on each objective. The first number gives the magnification in the image plane (at the position of the color video camera (U6) or the multi-mode fiber (U8)). The second number is the numerical aperture ($NA =$

$n \cdot \sin \alpha$), which describes the resolving power of the objective. The objectives are infinitely corrected, meaning that the beam is parallel inside the microscope and if cover glass corrected, they will give best results only when a cover slip of 0.17 mm thickness is placed between the objective and the sample. Additionally, the working distance (in mm) is printed on the objectives.

2 WITec Control Confocal configuration description

This description is intended to be used in combination with the WITec Control manual. The WITec Control manual contains the description of the full functionality of WITec Control , whereas this section indicates only which functionalities are included in this configuration. Please refer to the WITec Control manual for further details. The configuration-specific speed buttons, as well as the layout of the Control Window, will also be illustrated below.

The Confocal configuration is used to perform Confocal Microscopy in reflection configuration. During a confocal measurement, data can be acquired using the following sequencer:

- Image Scan: acquisition of confocal images.
- Oscilloscope: displays the output channel of a photon counting device similar to the display of an oscilloscope.

The characteristics of the confocal configuration are described in the following sections. The typical layout of the confocal configuration is shown in Fig. 2.

2.1 Speed Buttons

The main menu contains, in addition to the standard WITec Project speed buttons, several speed buttons which provide quick access to microscope and controller functions used in this configuration. A short description of these speed buttons is given below.

Stop

This speed button is used to stop any sequencer.

Start Scan

With this button, the *Image Scan* sequencer is started.

Oscilloscope

Clicking with the mouse on this speed button starts the oscilloscope sequencer (WITec Control manual Section 3.5.11). A graph viewer showing

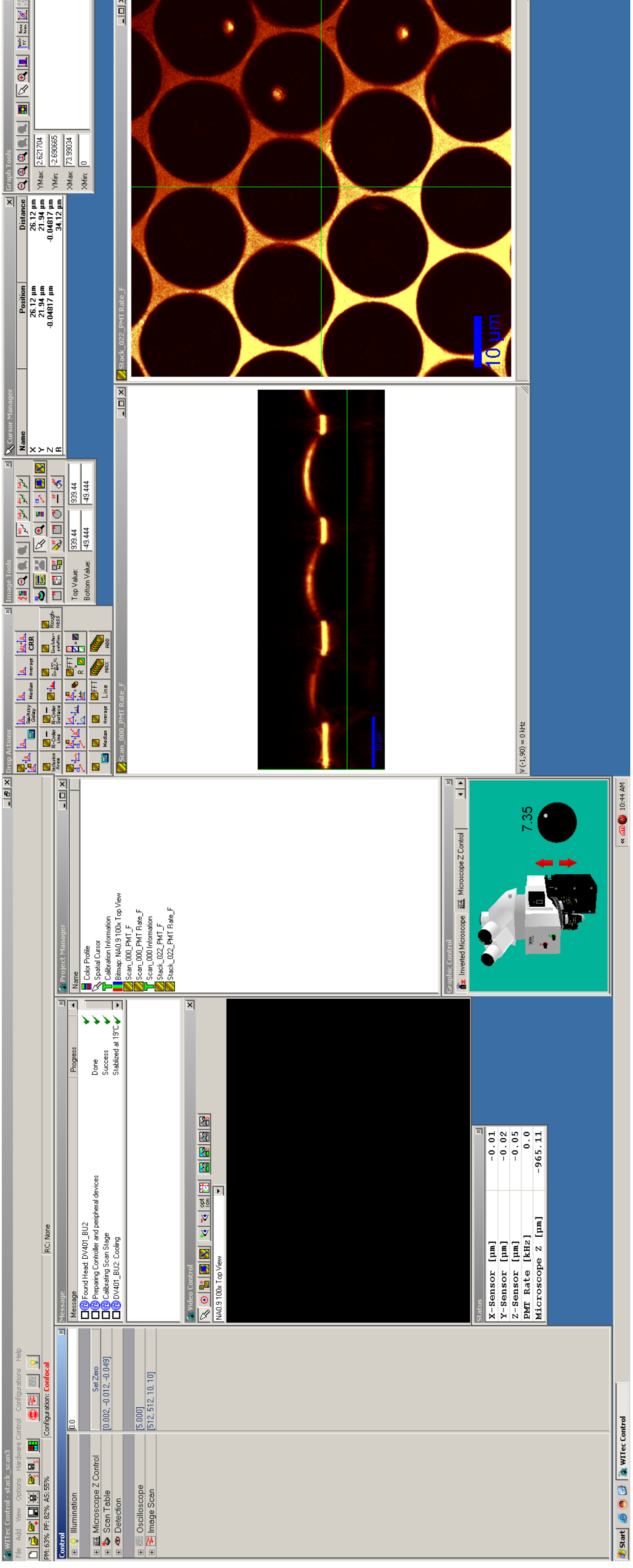


Fig. 2: Typical layout of the confocal configuration.

the variation of the selected data source (channel) as a function of time is displayed.

In confocal measurements, the readout of the photon counting device is typically displayed as function of time. This signal is mainly used for the alignment of the system.

Illumination

With this speed button, the white light illumination can be switched on and off. The brightness of the illumination is defined in the Control Window using the illumination device.

2.2 Control Window

The Control Window described in Chapter 3 of the WITec Control manual is customized for confocal measurements. The reduced tree structure of the Control Window in the confocal configuration is shown in Fig. 3.

Fig. 3: Control Window for the confocal configuration.

Devices and sequencers are grouped in this configuration based on functionality. Each device and sequencer contains only the list of parameters which are used during a confocal measurement. The following devices and sequencers are accessible in the confocal configuration.

2.2.1 Setup and Control

In this section, the software controls for the hardware components (called devices in WITec Control) and the software controls for data acquisition processes (called sequencers) are listed and briefly described. These controls are used for microscope setup and measurement control.

Illumination

This device is used to control the white light illumination of the tip/sample video image. A detailed description of the parameters used to control the white light illumination is given in the WITec Control manual Section 3.4.2. The illumination speed button located in the main menu uses the brightness parameter set here.

Microscope Z Control

The microscope Z control is described in WITec Control manual Section 3.4.3 as part of the scan table device. In this configuration, the *Move Mode* is automatically set to *Z by Microscope*. The microscope Z-stage is used as the Z axis of the internal coordinate system. Depth scans of up to $200\ \mu m$ can be performed in this mode by changing the Z position of the microscope stage from line to line or image to image.

HINT The microscope Z-stage can be controlled via the remote control (seeWITec Control manual Section 3.4.1), the Graphic Control Window (seeWITec Control manual Chapter 8) or using the parameter *Speed*, *Move Up* or *Move Down* listed in the Control Window.

Scan Table

If the microscope is operated in this configuration, the scan table can be moved in all three directions, allowing precise positioning within the scan-range. For a detailed description of the scan table, please see also WITec Control manual Section 3.4.3.

Detection

Several photon counting devices can be connected to the *alpha300/alpha500/alpha700* system. The photon counters used in *alpha300/alpha500/alpha700* systems are typically PMTs (photo multiplier tube) and/or APDs (avalanche photodiode). The photon counting devices can be switched on using the ON/Reset button. Generally this will only be necessary if the system has shut down the PMT due to excessive count rates. In normal operation, the system automatically turns the PMT on and off as required by the measurement. In this category, all available photon counting devices of the *alpha300/alpha500/alpha700* system are listed.

2.2.2 Acquisition

The sequencers used to acquire data in this configuration are listed and briefly described below.

Image Scan

The parameters used to perform an image scan are described in WITec Control manual Section 3.5.2. In this configuration, all parameters required to perform an image scan in confocal mode are listed.

Oscilloscope

The parameters used to display measured values in a similar way as is done through an oscilloscope are described in WITec Control manual Section 3.5.11. In this configuration, the counter output of a photon counting device will be integrated for the set time and the recorded value will then be displayed in a graph viewer.

2.3 Data Sources and Status Window

The data sources (WITec Control manual Section 3.6) used during a confocal mode measurement are summarized in Tab. 1 together with the default data labeling of the acquired channel. The data sources used for adjustment and control of the measurement are displayed in the Status Window (WITec Control manual Chapter 5).

Channel	**Image Scan**	**Oscilloscope**	**Channel Caption**	**Unit**	**Display**
Photon Counter	✓		PMT	$[cts]$	
Count Rate	✓	✓	PMT Rate	$[kHz]$	
X-Sensor				$[\mu m]$	Status Window
Y-Sensor				$[\mu m]$	Status Window
Z-Sensor				$[\mu m]$	Status Window
Microscope Z				$[\mu m]$	Status Window

Table 1: Output channels and their sequencers.

3 Beam Path

In this section, the beam path is illustrated schematically. The parts common to all instruments are displayed in dark grey. Parts not common to all instruments, but necessary for the described measurement mode are indicated in blue. Optional parts which may be present with the instrument, but are neither common to all instruments nor are necessary for the described measurement mode are displayed in light grey. The parts indicated are listed below the figure where an *alpha300* system is shown exemplarily. Detailed descriptions of the parts indicated can be found in the *alpha300/alpha500/alpha700* system description. The beam path is represented in green in Fig. 4.

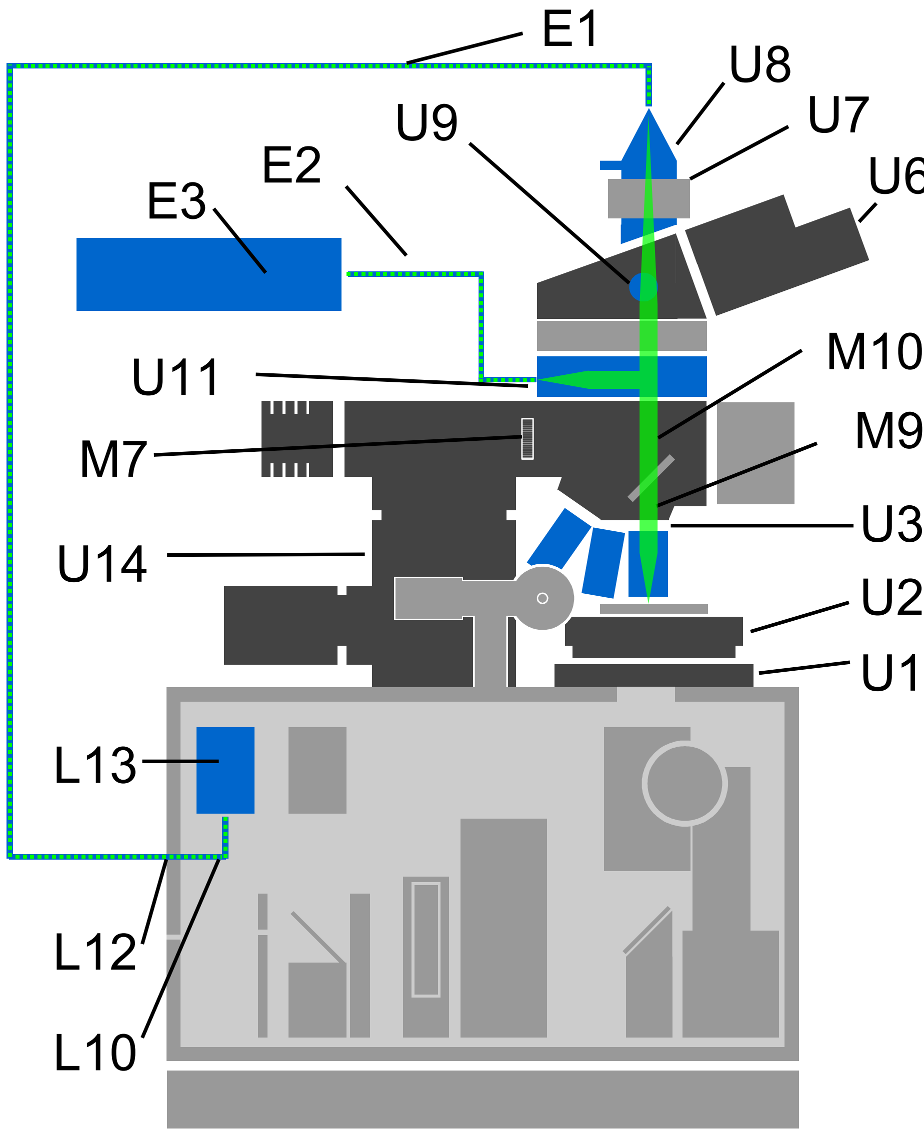


Fig. 4: Schematic illustration of the beam path for confocal microscopy.

U1 XY positioner

U2 Scan stage

U3 Objective turret with objectives

U6 Binocular tube with ocular camera

U7 Filter holder for reflection mode measurements

U8 Fiber coupling unit optical output

U9 Pushrod

U11 Laser coupling unit optical input

U14 Microscope Z stage with stepper motor

M7 Field stop diaphragm

M9 Objective turret

M10 Reflector slider

L10 Multi-mode optical fiber

L12 SMA fiber connector feedthrough

L13 Single photon counting detector (PMT or optional APD)

E1 Multi-mode optical fiber (25, 50 or 100 μm core diameter included as standard)

E2 Single-mode optical fiber

E3 Laser

4 Step by step alignment

The following listing describes the alignment of the *alpha300/alpha500/alpha700* system in order to obtain a confocal image scan in reflection mode. This is the standard mode in confocal microscopy.

The first of the following steps describe the procedure for focusing the microscope on the sample using white light illumination followed by the focusing of laser radiation. Then, the optimization of the position of the SMA fiber connector, the focus and the collimation are described.

1. Switch on the alphaControl. This is usually done using the switch on the multi-plug.

2. Power up the computer and start WITec Control . Select the Confocal mode from the Configurations-menu.
3. Mount the Silicon test sample on the scanning stage.
4. Rotate the microscope turret until an appropriate objective is in the working position. Make sure you use the microscope objective in the proper way (e.g. use a cover slip if a cover slip corrected objective is used).
5. Push in the pushrod (U9) of the sliding prism and direct the beam to the eyepiece color video camera (U6).
6. Move the reflector slider (M10) to the illumination position (M11; see the *alpha300/alpha500/alpha700* system description for details). The beam splitter is mounted in the left position of the three position reflector slider. Therefore, move the reflector slider to the right.
7. Adjust the illumination to the required level using the illumination menu item in the Control Window.
8. Observe the image of the eyepiece color video camera on the computer monitor using the Video Control Window in the WITec Control software. Using the drop down menu in the Video Control Window, select the appropriate view (top view with the correct objective).
9. Focus on the surface of your sample with the Microscope Z stage (U14). This can be done in three different ways:
   - Using the Z microscope control in the Graphic Control Window. Clicking on the arrows will start the movement in the indicated direction and using the virtual potentiometer, the speed can be adjusted from 0.01 to 500 μm/s.
   - Using the remote control with the Z Microscope selected as the controlled device. Using the +Z and -Z buttons, the Z-focusing-stage can be moved up or down and the potentiometer allows the selection of speeds between 0.01 and 500 μm/s.

   HINT The remote control needs to be activated if the controlled device has changed or if it was idle for more than two minutes. To activate the remote control turn the potentiometer fully anticlockwise.

   - Using the Move Up and Move Down buttons, which can be found in the Microscope Z Control menu of the Control Window. The speed can be adjusted between the minimum and maximum values using the corresponding field.

   If possible, move the objective initially away from the sample to avoid a collision between the objective and the sample.

It can sometimes be very difficult to focus on flat and clean surfaces. The field stop diaphragm (M7) can help to overcome these difficulties as outlined in the following. To focus, close the field stop diaphragm (M7) to a value of 1-3. Make sure to move in the 50:50 beam splitter (M11) using the reflector slider (M10) to illuminate the sample. Approach the sample until the edge of the field stop appears focused. At this point, the sample is also in focus. This is due to the fact that the field stop is positioned at the back focal plane of the objective. If the field stop is not in the middle of the field of view, move it to this position with the centering screws (M7; see the *alpha300/alpha500/alpha700* system description for details).

10. Switch on the excitation laser at low power.

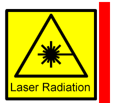
11. Turn off the white light illumination (using the speed button or the Illumination On/Off button), move out the 50:50 beam splitter (move the reflector slider (M10) into its middle position) and fine focus the microscope until the excitation laser is focused on the sample. You will observe several spots on the video screen, but only one of them changes during focusing. This is the spot that hits the sample while the other spots are reflections inside the microscope. Make sure that the other spots are as small as possible. The video camera is in the focal plane of the tube lens, so these spots will be minimized if the laser beam is collimated and therefore parallel inside the microscope. If they appear out of focus, rotate the micrometer screw at the laser coupling unit optical input (U11) until they are perfectly focused.

HINT If the video screen over-saturates (i.e. displays only white) reduce the laser power further.

12. If you performed a complete alignment recently, you can jump to point 18.

13. Close the laser shutter, remove the multi-mode fiber (E1), place a white card (e.g. a business card) a few millimeters above the SMA connector (U8) and re-open the laser shutter.

14. Pull out the pushrod (U9) to direct the beam to the SMA connector. Try to localize the laser beam on the name card by altering the position of the SMA connector using the micrometers attached to it. If necessary, increase the laser intensity.

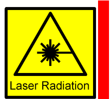


DO NOT LOOK INTO THE SMA CONNECTOR.

15. Adjust the micrometers to center the beam in the SMA connector. You might recognize several laser spots, but only one of them changes during focusing. This is the spot that hits the sample while the other spots are reflections inside the microscope. Close the laser shutter and reinsert the multi-mode fiber.

Disconnect it at the SMA fiber connector feedthrough (L12) and reopen the laser shutter.

16. Try to see the laser beam at the end of the multi-mode fiber. Use the name card again. Increase the laser intensity if necessary.



DO NOT LOOK INTO THE FIBER.

17. Adjust the micrometer screws at the SMA connector to maximize the laser power at the multi-mode fiber.

18. Place the shutter into the laser beam path and connect the multi-mode fiber to the SMA feedthrough on the left side of the microscope body (L12). Make sure that the detector (L13) is connected to the SMA feedthrough (L12) using the short multi-mode fiber (L10).

19. Before opening the laser shutter again, ensure that the laser power is not too high. While the count rate of the photon counting devices (APD and/or PMT) are constantly monitored through WITec Control , overexposure should be avoided. If the count rate exceeds 4×10^6 counts per second (cps), the photon counting device will be turned off automatically for protection. In this case, reduce the laser power and press the Reset / On button in the detection menu.

20. Start the oscilloscope in WITec Control using either the speed button or the Start Oscilloscope button in the oscilloscope menu of the Control Window. The integration time as well as the time displayed in the oscilloscope window can also be adjusted in the oscilloscope menu of the Control Window.

21. Watch the oscilloscope reading on the monitor and make fine corrections with the micrometers at the SMA fiber connector (U8) until the maximum signal is achieved. Make sure the counter board and the detector are not overloaded. If the count rate exceeds 4×10^6 counts per second (cps), the photon counting device will be turned off automatically for protection. In this case, reduce the laser power and press the Reset / On button in the detection menu, then continue the optimization.

22. Adjust the focus of the microscope using the microscope Z stage (as described in point 9) to maximize the signal.

23. Using the micrometer on the back of the laser coupling unit optical input (U11) adjust the collimation for optimum intensity.

24. Repeat points 21 to 23 several times to optimize the signal.

25. Once the optimum signal intensity is achieved, stop the oscilloscope with any Stop button or icon.

26. Close the laser shutter.

27. Move the Z microscope up as described in step 9.

28. Exchange the Silicon for your sample.

29. Focus on your sample using white light as described in steps 4 to 9.

30. Move the reflector slider (M10) into the middle position.

31. Open the laser shutter. Make sure to start with low laser power since the sample might be altered or destroyed otherwise. The stability of your sample depends on various factors such as absorption, thermal conductivity and laser spot size (which is determined by the laser wavelength and the NA of the objective used for the experiment).

32. Start the oscilloscope and optimize the focus for maximum intensity as in step 22.
    At this point, do not alter the position of the SMA fiber connector or the collimation anymore.

33. If you have a fluorescing sample, insert an appropriate filter in the filter holder for reflection mode measurements (U7) to block the excitation laser.

34. The measurement parameters in the image scan menu of the Control Window should be adjusted next (see WITec Control manual Section 3.5.2 for a description of the individual parameters).
    If the *(X)*, *(Y)* and *(Z,Microscope)* positions indicated in the scan table menu of the Control Window differ from the position entered as *Center (X)*, *Center (Y)* and *Center (Z)* in the image scan menu, the scan table will move to a different position before starting the scan. This might then lead to data acquisition at the wrong position/area. Use the Center at Current Pos. button in the image scan menu of the Control Window to avoid this displacement.

HINT Typical values for a confocal measurement are:
Points per line: 100-512
Lines per image: 100-512
Width: 10-50 μm
Height: 10-50 μm
Integration Time: 0.2-10 ms

35. You may now begin your measurement by opening the laser shutter and pressing the corresponding Start button.

If your *alpha300* system is equipped with an inverted microscope, confocal microscopy in transmission is also possible using the appropriate software configuration. This might be desirable if the local absorption properties of the sample are of interest. The alignment procedure will then differ from the above.