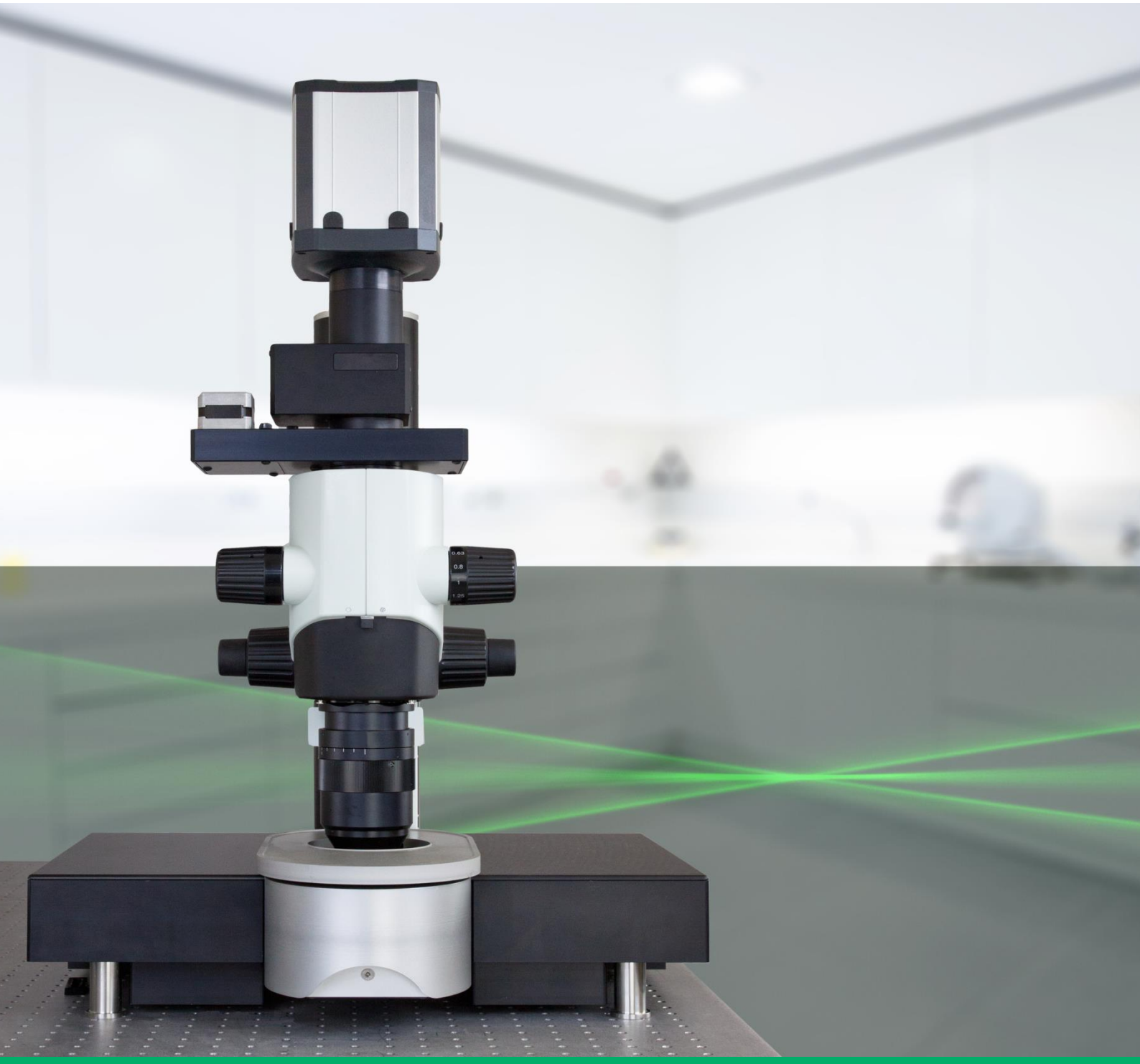
LaVisionBioTec

UltraMicroscope

Light Sheet Microscope



Instruction Manual

June 2016

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Table of Content

1 General

Please take the necessary time to read this manual. It contains important information on safety issues concerning the usage of the UltraMicroscope and the laser sources.

Warning: safety might be seriously impaired if the instructions are not followed carefully.

The manual covers the UltraMicroscope, and the LaVision BioTec laser diode module. Please assure which laser source is combined with your UltraMicroscope. In the case that your UltraMicroscope is equipped with an Obis Galaxy combiner or an NKT SuperK Extreme laser please read the provided manual by NKT Photonics or Coherent.

2 Laser safety

2.1 The LaVision BioTec laser module generation II

The LaVision BioTec laser module contains Class IIIb lasers (emitted power c.w. 40-100 mW). Some direct viewing of the Class IIIb laser beam is hazardous to the eye and diffuse reflections of the beam can also be hazardous to the eye. Do not view the Class IIIb laser beam directly. Do not view a Class IIIb laser beam with telescopic devices; this amplifies the problem. Whenever occupying a laser controlled area, wear the proper eye protection. Lasers at the high power end of this class may also present a fire hazard and can lightly burn skin.
Due to the potential health and safety risk posed by Class IIIb additional safeguards must be in place to minimize the hazards associated with operating such equipment. The following sections outline the procedural, regulatory, and engineering controls that aim to attenuate the risk of operating Class IIIb systems.

2.2 Safety precautions for Class IIIb

- Never aim the laser at a person's eye or stare at the laser from within the beam.
- Keep the beam path above or below eye level for one seated or standing.
- Laser safety eyewear may be needed if MPE (Maximum Permissible Exposure) is exceeded.
- Don't view beam directly with optical instruments unless a protective filter is used.
- Only experienced and authorized individuals are permitted to operate the laser.
- Secure the laser from operation by unauthorized personnel. A key switch should be used if unauthorized personnel may gain access to the laser.
- Always strive to enclose as much of the beam path as practical and to operate the laser in a controlled access area.
- During alignment, avoid placing one's eye near the axis of the beam path, where specular reflections are most likely to occur. Alignment eyewear should be considered.
- Post laser hazard warning signs at entrances to laser use areas.

2.3 Laser source operation

2.3.1 Turning ON the LaVision BioTec laser module generation II

The LaVision BioTec module box has a keyswitch that prevents laser radiation in the STANDBY position. Laser radiation occurs while the key is in the ON position.
Do not lift any lid of the UltraMicroscope as long as the laser is switched on.

2.4 Laser alignment

The UltraMicroscope alignment tool facilitates the adjustment of all 6 light sheets to guarantee an optimal alignment of the microscope. The alignment of this device must be conducted according to the following protocol.

2.4.1 Theoretical background - Beam path of the microscope

Each of the opposing light sheet sets in the UltraMicroscope consists of three beams (Fig. 2-10). The central beam travels straight through the cuvette. The two outer beams are directed with eleven degrees to the centre of the cuvette. All three beams are matched within the Rayleigh length. The slider “sheet width” extends or narrows the light sheet. Increasing the sheet width leads to a larger excited area. The intensity will decrease because the light is spread to larger area.

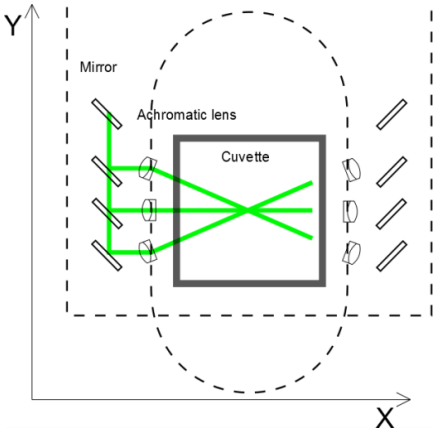


Figure 2-10: Beam path of the left light sheet set (top view).

The light sheet forming optics create a horizontal focus within the centre of the imaging chamber (Fig. 2-11). The area of the horizontal focus (Rayleigh length) generates the best contrast and resolution within the field of view (FOV). Changing the numerical aperture (NA) of the light sheet alters the Rayleigh length. A low light sheet NA generates a broad Rayleigh length, but a thicker light sheet. An increased NA causes a narrow Rayleigh length and a very thin light sheet, which improves the Z resolution.

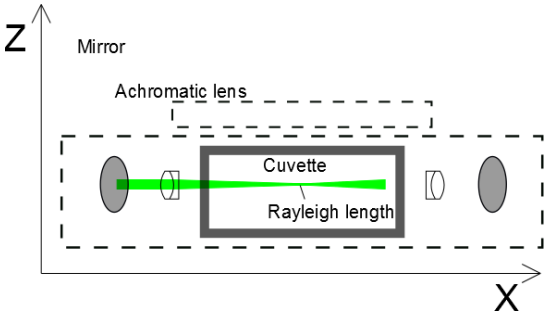


Figure 2-11: Left light sheet beam path (frontal view).

2.4.2 Light sheet aligning

For generating high contrast images with the best Z resolutions, all light sheets have to be perfectly aligned to the same Z plane. A set of precision screws on the left and the right side of the UltraMicroscope is used for aligning the individual beams creating the light sheets. The screws Z2 – Z4 are used to position the sheets in Z. With the screws Y2 – Y4, one can alter the angle under which the light sheets merge. The screw Z1 is for aligning the left and the right light sheet set in Z.

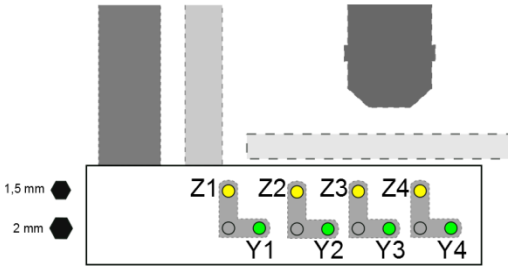


Figure 2-12: Schematic drawing of the left side of the microscope with marked positions of the usable adjustment screws.

2.4.3 Adjusting the beam path

Adjusting the middle beam of the right side

- Switch on all devices and start the ImSpector software.
- Mount the alignment tool on the sample holder ring and place it into the imaging chamber.

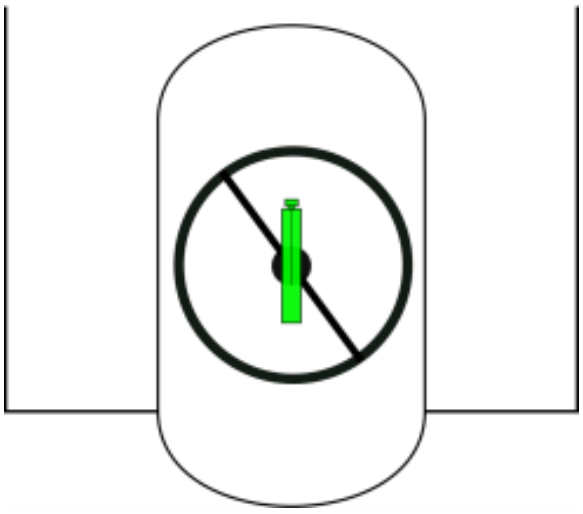


Figure 2-13: Top view into the imaging chamber with alignment tool placed in the centre

- Choose a filter combination adjusted to the dye in the alignment tool and activate the right light sheets.
- Click on “Video” for image grabbing.
- Check by eye the centred position of the cuvette and position the alignment tool in a way that the beams from the right side pass the tool and form three thin lines on the other side of the pinhole foil.
- Lower the objective lens (with dipping cap) into the cuvette and focus on the tool at lowest zoom factor (zoom 0.63).
- Adjust the laser power until the three beams passing the pinhole can be easily detected while the other side of the pinhole is not saturated.
- Activate the crosshairs in ImSpector (at left side of the grabbing window frame) and line up the alignment tool to the centre.
- Choose the medium (CLARITY, DBE, Water…) used in the register [Measurement].

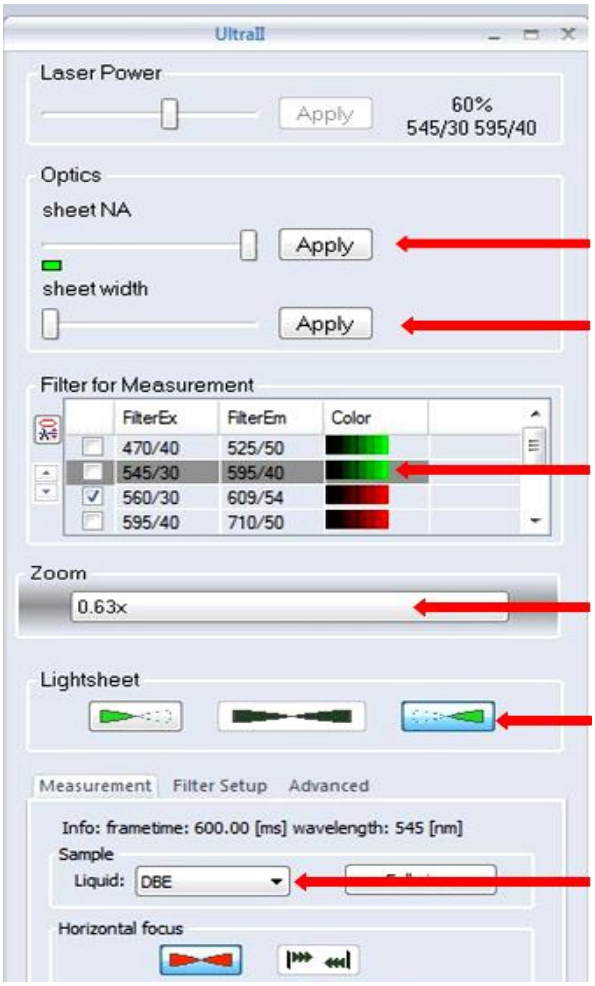


Figure 2-14: Ultra dialogue

- Activate the horizontal focus and place the Rayleigh length into the middle of the FOV
- Open the sheet NA to the maximum (thinnest sheet about 4 µm).
- The sheet width may be raised to locate the beam, but should afterwards be set as narrow as possible for fine adjustment.

- Choose the “Advanced” tab on the bottom of the dialogue "Ultra II." The control panel “Laser beam adjustment” is used to switch on and off individual laser beams. Click on the button showing the number “1” to activate the centre beam.
- **Always** start with the centre beam and align it perfectly before adjusting the lateral beams.
- If two beams are visible passing the mask, the tool must be either lowered or raised with the hand wheel or the mouse wheel. The direction is correct if the beams move closer to each other. The aim is to align the centre beam to the pinhole located in the middle of the mask in order to align all beams to this position.
- If it is not possible to align only one beam through the centre of the mask by moving up and downwards, then the actual position of the horizontal focus does not match with the displayed position. The best approximation must be set before the horizontal focus can be adjusted.
- To adjust the horizontal focus, scroll within the “Advanced” tab all the way down to "Sheetmotor Calibration." Move the slider until only one beam passes through the pinhole. Ensure that the horizontal focus is always in the centre and the NA is at the maximum. If necessary, readjust the height.
- If a clearly recognizable, single beam is visible through the pinhole in the centre of the mask, increase the intensity of this beam by moving the alignment tool in Z.
- The central beam is now adjusted to the highest intensity.
- Click on the "Safe settings" button in the menu bar to fix the adjusted horizontal focus.

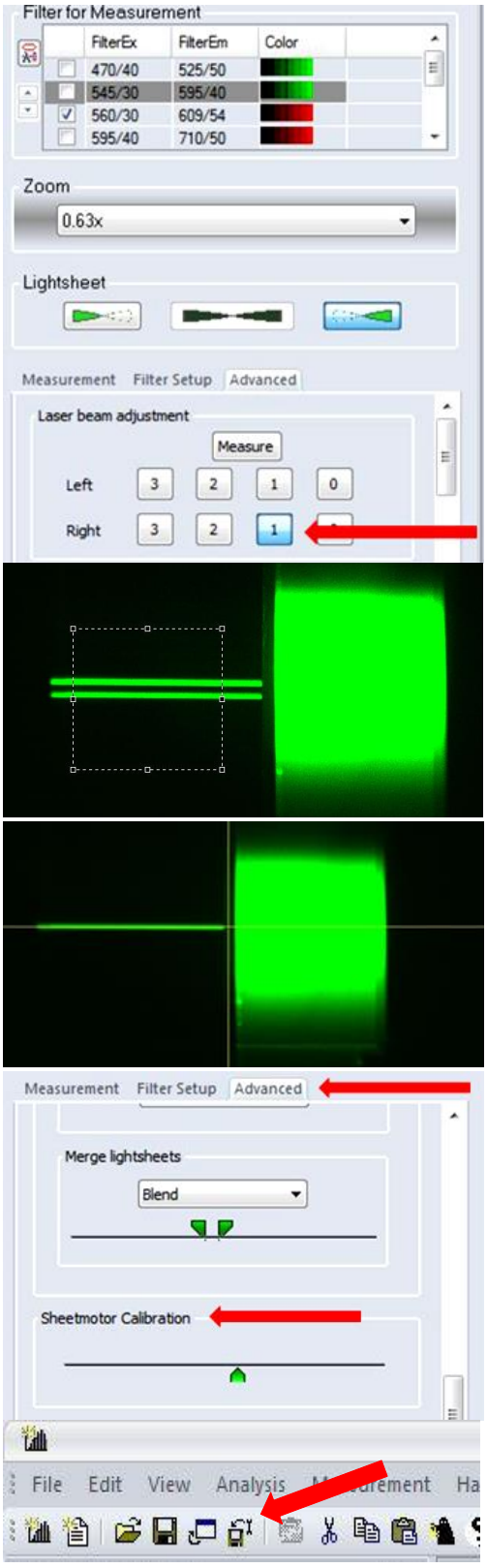


Figure 2-15: Aligning the centre beam

Adjustment of the lateral beams

- To adjust the lateral beams of the same side, the alignment tool must no longer be moved.
- Also, the horizontal focus must no longer be changed.
- To activate the lateral beams, click the button showing “2” within the “laser beam adjustment” control panel.

Figure 2-16: Activating the lateral beams

- Set the sheet width to the minimum. Both lateral beams should be directed to the centre of the mask. If this is not the case, the position in the Y direction must be adjusted (Y2 for mirror No. 2 and Y4 for mirror No. 4, Fig. 2-17). Use a 2 mm Allen key.

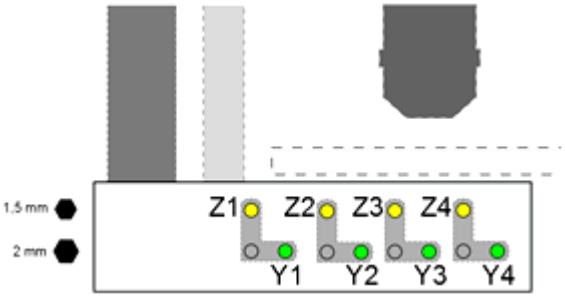


Figure 2-17: Adjustment screws

- Now adjust the height of both beams sequentially to the centre of the pinhole in the mask, using the µm-screws (Z2 for mirror No. 2 or Z4 for mirror No. 4, Fig. 2-17).
- Caution: The µm-screws are located in the centre of the larger screws (Z2 and Z4) and can only be reached by using a 1.5 mm Allen key. The outer screws are fixed and may not be turned.
- Switch on all the laser beams of this side. Now three beams should pass through the pinhole in the centre of the mask of the alignment tool.

The left side

- For adjusting the left light sheets, place the alignment tool in the same way including the crosshairs as done in the right side alignment procedure.
- Activate the left light sheets.
- Start again with the centre beam. Click on button “1” within the “Laser beam adjustment” control panel for the left side. Now only the centre laser beam is switched on.
- Ensure that the slider for the horizontal focus is still in the centre. If not, place it there.
- If the beam is not centred and directed to the pinhole, correct the beam path with the adjustment screw (Y1, Fig. 2-12 mirrored).
- Adjust the height of the beam with the µm-screw (Z1) to the mask centre.
- If it is not possible to get only one beam to pass through the pinhole, make the one-to-reach optimum.
- Adjust the horizontal focus specifically for this light sheet. Scroll again in the “Advanced” tab, all the way down to "Sheetmotor Calibration." Move the slider until only one beam falls through the pinhole in the mask. Make sure that the horizontal focus is always in the centre and the NA is fully opened.

Alignment of the left lateral beams

- The lateral beams have to be positioned as described for the right side.

2.4.4 Alignment beam pattern

Light sheet **not** adjusted to the pinhole

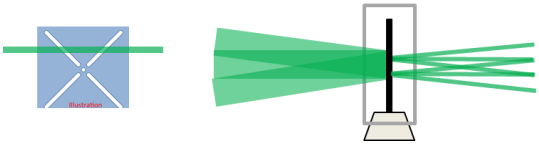
Beam pattern illuminating from the left site

Light sheet adjusted to the pinhole

Beam pattern illuminating from the left site

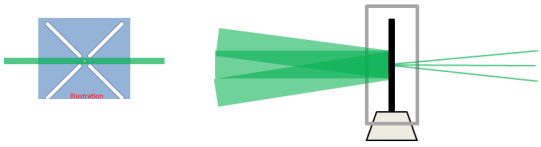
Three light sheets activated:

Light sheets **not** adjusted to the pinhole



Beam pattern illuminating from the left site

Light sheets adjusted to the pinhole



Beam pattern illuminating from the left site

2.4.5 The alignment tool

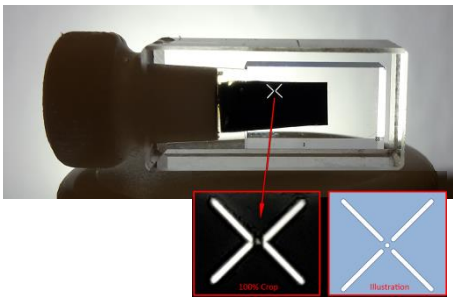


Figure 2-18: The alignment tool

3 Chemical safety - general

The UltraMicroscope is capable of measuring in vivo samples and cleared samples. Cleared samples require certain imaging solutions. Please be aware that all chemicals are hazardous. Ensure that the chemicals are handled with appropriate care and precaution. For the safety guidelines please consult the corresponding Material Safety Data Sheets (MSDSs) provided by chemical suppliers.

3.1 Suitable organic imaging solutions

Caution: The UltraMicroscope is not designed for the usage of BABB as imaging solution. Standard dipping caps maybe affected and are not warranted in case of BABB usage.

A recommended organic imaging solvent is Benzyl ether (DBE):

- CAS Number 103-50-4
- Linear Formula (C6H5CH2)2O
- Molecular Weight 198.26
- EC Number 203-118-2
- MDL number MFCD00004780

Please consider all safety regulations for Benzyl ether described in the MSDS.

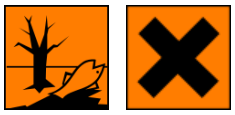
3.1.1 Hazards identification of DBE

- Skin irritation (Category 2)
- Eye irritation (Category 2)
- Specific target organ toxicity - single exposure (Category 3)
- Chronic aquatic toxicity (Category 2)

Irritating to eyes, respiratory system and skin. Toxic to aquatic organisms. May cause long-term adverse effects in the aquatic environment.

Emergency Overview
This material is HAZARDOUS by OSHA Communication definition.

Hazards
May cause central nervous system depression. Causes eye irritation. Causes skin irritation. May cause gastrointestinal irritation with nausea, vomiting, and diarrhoea. Causes respiratory tract irritation.



(N) (Xi)

Figure 3-1: Label elements, Pictogram

Signal word - Warning

Hazard statement(s)
H315 Causes skin irritation.
H319 Causes serious eye irritation.
H335 May cause respiratory irritation.
H411 Toxic to aquatic life with long lasting effects.

Precautionary statement(s)
P261 Avoid breathing dust/fume/gas/mist/vapors/spray.
P273 Avoid release to the environment.
P305 + P351 + P338
IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

Hazard symbol(s)
Xi Irritant
N Dangerous for the environment

R-phrase(s)
R36/37/38 Irritating to eyes, respiratory system and skin.

R51/53 Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment.

S-phrase(s)
S23 Do not breathe vapor.
S61 Avoid release to the environment. Refer to special instructions/ Safety data sheets.

3.1.2 Handling and storage

Precautions for safe handling
Avoid contact with skin and eyes. Avoid inhalation of vapour or mist. Keep away from sources of ignition - no smoking. Normal measures for preventive fire protection.

Conditions for safe storage
Store in cool place. Keep container tightly closed in a dry and well-ventilated place. Opened containers must be carefully resealed and kept upright to prevent leakage.

3.1.3 Personal protective equipment

Respiratory protection
Where risk assessment shows air-purifying respirators are appropriate, use a full-face respirator with multi-purpose combination (US) or type AXBEK (EN 14387) respirator cartridges as a backup to engineering controls. If the respirator is the sole means of protection, use a full-face supplied air respirator. Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU).

Hand protection
Handle with gloves. Gloves must be inspected prior to use. Use proper glove removal technique (without touching glove's outer surface) to avoid skin contact with this product. Dispose of contaminated gloves after use in accordance with applicable laws and good laboratory practices. Wash and dry hands. The selected protective gloves have to satisfy the specifications of EU Directive 89/686/EEC and the standard EN 374 derived from it.

Eye protection
Wear appropriate protective eyeglasses or chemical safety goggles and face shield as described by OSHA's eye and face protection regulations in 29 CFR 1910.133 or European Standard EN166.

Skin and body protection
Complete suit protecting against chemicals, Flame retardant antistatic protective clothing, The type of protective equipment must be selected according to the concentration and amount of the dangerous substance at the specific workplace.

Hygiene measures
Handle in accordance with good industrial hygiene and safety practice. Wash hands before breaks and at the end of workday.

3.1.4 Further information

The data contained herein is based on information currently available to LaVision BioTec GmbH, and believed to be factual and the opinions expressed to be those of qualified experts; however, this data is not to be taken as a warranty or representation for which LaVision BioTec GmbH assumes legal responsibility.

4 Getting started

Warning: Safety might be seriously impaired if the instructions are not followed carefully.

Warning: Do not place or remove the sample or the cuvette while the microscope is in video or image acquisition mode. Ensure that no laser light is entering the cuvette.

Caution: The UltraMicroscope is not designed for the usage of BABB as imaging solution. Defects caused by usage of BABB are excluded from warranty.

Caution: Never fill the cuvette while it is located in the microscope chamber!

Caution: Prevent spilling of imaging solution. Handle the filled cuvette with care. Spilled imaging solution may damage microscope mechanics and optics.

Warning: When using organic solvents, please consider all safety regulations described in the MSDS provided by the supplier. Consider the usage of a laboratory fume hood.

4.1 Switching on the UltraMicroscope and periphery devices

Follow the switch on routine below:

a) Image acquisition PC and Monitor
b) Power supply UltraMicroscope
c) sCMOS
d) Laser module
e) Start ImSpector software

4.2 Filling the cuvette with chemicals

Many of the most frequently used microscopy clearing media are potentially destructive for the mechanics of the UltraMicroscope. Therefore, particular caution should be given to ensure that no medium enters the mechanics of the microscope. The most common errors occur during the placement of the cuvette into the microscopy chamber and changing the sample while the cuvette stays in place.
A microscopy medium leaked onto the mechanics of the microscope can result in mechanical problems, like corrosion, causing dysfunction of the positioning mechanics. That leads to unreliable movement of the mechanical parts and therefore unreliable data.

4.2.1 Placing the cuvette

Caution: Do not place or remove the sample or the cuvette while the microscope is in video or image acquisition mode. Ensure that no laser light enters the cuvette.

The cuvette has to be filled with the medium up to the recess of the glass (indicated with “Max.” in figure 4-2).
Never fill the cuvette while it is located in the microscope chamber.

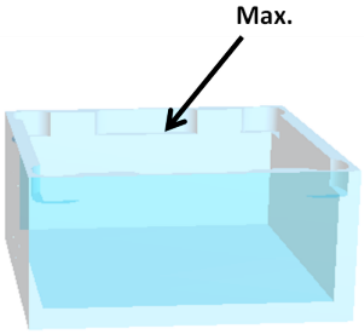


Figure 4-2: Filling up the cuvette

Before the filled cuvette can be placed, the grey movable table has to be removed by loosening the screw on the right side of the table. Additionally, with the aid of the Zoom Body, the objective lens has to be moved upwards as far as possible and placed in the rearmost position using the revolving nosepiece. The plunger in the bottom of the microscopy chamber can be moved upwards by pushing it manually. Put the cuvette on top of the microscope chamber and lower it until it touches the plunger. The plunger can now be used to lower the cuvette carefully until the bottom of the microscope chamber is reached.
Now the previously removed table can be reassembled and fixated by fastening the screw on the right side of the table.

4.2.2 Placing the sample

First, the sample has to be placed onto the sample holder. The sample holder is placed in the sample mount. Now the sample can be placed in the cuvette by putting the sample mount in the opening of the grey movable table (figure 4-3).
Make sure that no medium drops into the microscope chamber.

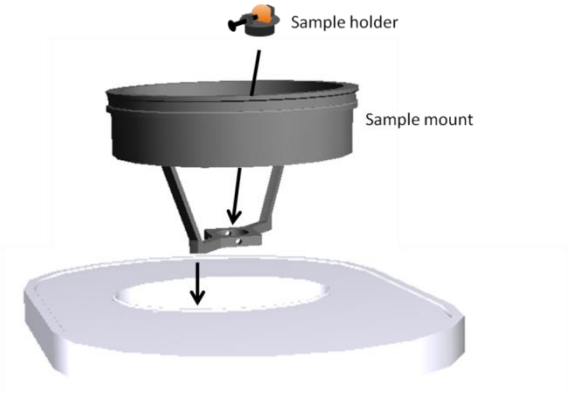


Figure 4-3: placing the sample with sample holder & mount

When removing the sample mount, ensure that the medium does not leak from the cuvette. To safely detach the sample mount, put a tissue under it.

4.3 Image acquisition: Z stack, single channel

Start the ImSpector software and check the displayed Measurement Wizard for the initial settings.

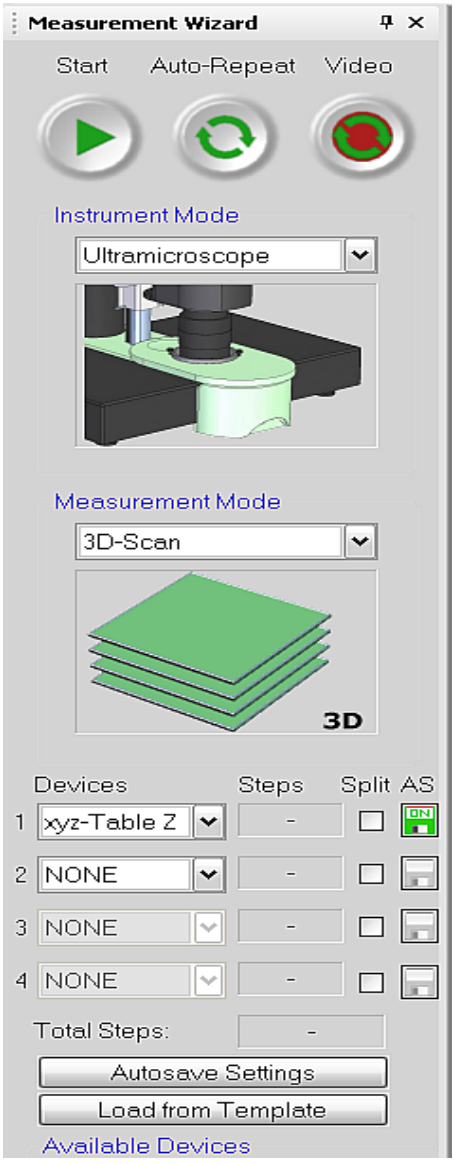


Figure 4-4: Measurement Wizard

Within the Measurement Wizard, the Instrument Mode has to be set as

"UltraMicroscope." As Measurement Mode, choose "3D-Scan." The list of active devices below will display only the "xyz-Table Z." Activate the "Autosave Mode" by clicking on the "AS" icon. Activated "Autosave Mode" is indicated by a bright green "AS" icon. Ensure that "Split" is not ticked. If "Split" is activated, the software will save each single Z plane and not the entire stack.
Open the "Autosave Settings" and type in the correct folder location to save the data.
Adjust the optics to the refractive index of the imaging solution by selecting the correct solution from the list in the "Ultra" dialogue.

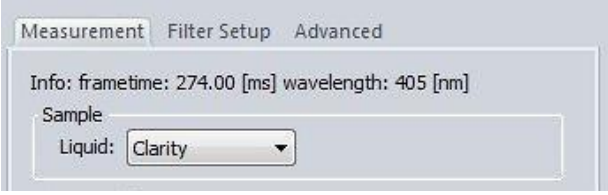


Figure 4-5: Setting the imaging liquid´s refractive index.

Within the "Ultra" dialogue, select the appropriate excitation and emission wavelength (filter) for imaging the sample. First, it is useful to select any wavelength (filter) to allow detection of the position of the light sheet with respect to the sample. This is important if the sample is large. To activate the specific filter, highlight the filter row to allow measurement. Ticking the box allows image acquisition. Tick only those needed. For every filter combination, the laser power can be set individually by clicking on the "Apply" button of the "Laser Power."

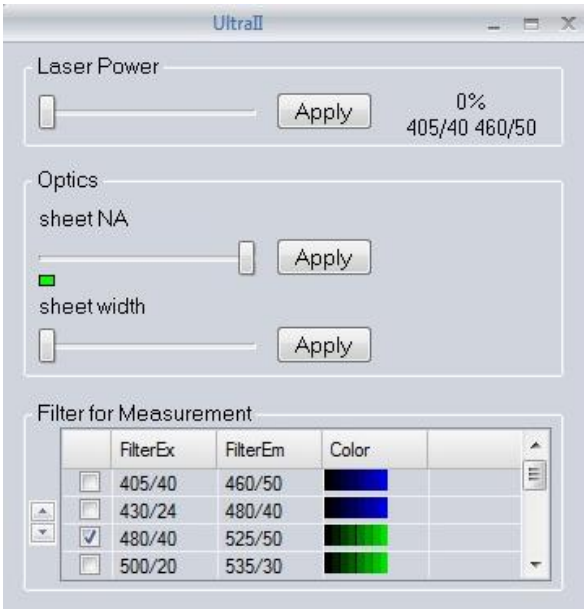


Figure 4-6: "Ultra" dialogue displaying laser power setting, sheet NA setting, sheet width, filter in excitation and emission.

To set up the images, use either the left or the right light sheet button within the Ultra dialogue:

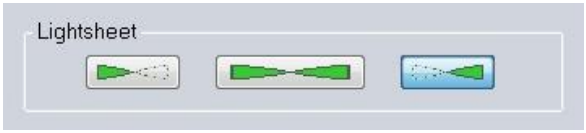


Figure 4-7: Light sheet button to select illumination direction (left, right or both sides).

Now start the "Video Mode" by clicking the "Video" button in the Measurement window.

Figure 4-8: Activating image grabbing by clicking the "Video" button. Caution: no images will be acquired.

Depending on the intensity of the signal, the exposure time of the sCMOS camera may

need to be adjusted. The Neo dialogue displays the current gain setting, the temperature of the camera (should be below -20 °C), the status of the background image, and the exposure time. As a rule of thumb, a suitable exposure time range would be between 150 ms and 250 ms. However, this could differ with regard to sample quality. Technically, the exposure time could be reduced to 10 ms. To reduce the dark noise, please click the “Take bgr” button and tick the box for “Use background.” The background subtraction can be inactivated by clicking the check box again.

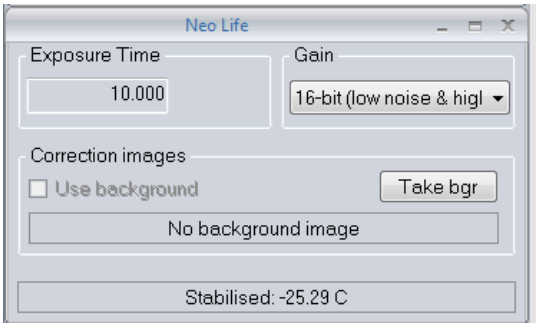


Figure 4-9: Neo Life dialogue for adjusting the camera exposure time.

To position the sample within the imaging chamber, use the position control dial XYZ to ensure that the sample is approaching the light sheet from underneath.

Figure 4-10: Control dial XYZ

Turn in the objective lens and lower into the clearing solution using the coarse or fine focus drive. Set the zoom factor to 0.63.

Figure 4-11: Knob at the focusing unit for lowering the lens into the imaging solution.

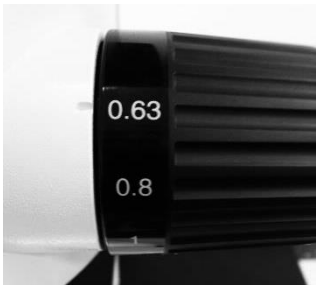


Figure 4-12: Setting the zoom factor at the MVX10.

The zoom is manual; select the appropriate zoom that is also used in the software.

Figure 4-13: Setting the correct zoom factor in the “Ultra” dialogue.

Once the surface of the sample is visible, be certain to place it in the centre axis of the image and ensure that it can be focused on with the fine focus drive at the MVX10.

To set up a better contrast: draw an area around a bright and/or dark area and hit the contrast wizard symbol  in the upper right part of the image window.

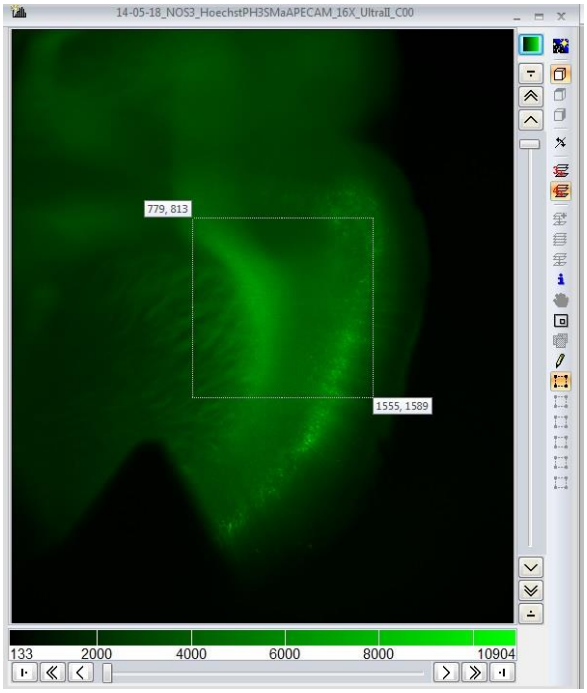


Figure 4-14: Image window

For further adjustment, use the dynamic range selection tool and the LUT table  to select the appropriate LUT color or range indicator on the top right corner of the image window.

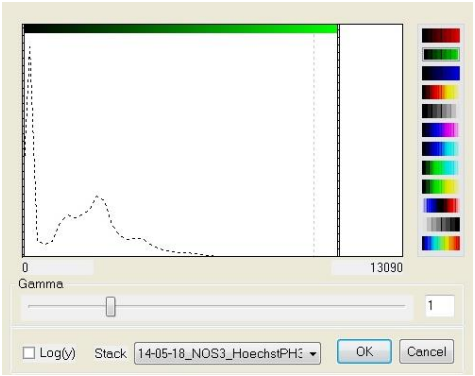


Figure 4-15: LUT color and range indicator for fine adjustment of the displayed image.

Click on the button displaying two red triangles to superimpose the horizontal focusing icon ⋈ in the image window. Move the horizontal focus icon in the image until best contrast is achieved in the region of interest.

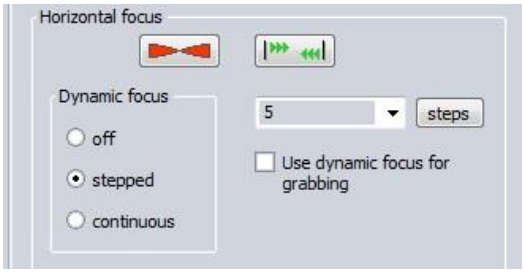


Figure 4-16: Horizontal focus menu within the "Ultra" dialogue.

If needed, reduce the NA of the light sheet that makes the "waist" of the light sheet longer and thicker indicated by the horizontal focus icon superimposed to the current image. When imaging with highest NA, the thinnest part of the light sheet is about 4 µm depending on the excitation wavelength. At the lowest NA, the light sheet is about 8 µm at the thinnest part. It is recommended to use a smaller light sheet NA to improve the Z profile of the stack on a broader range.
It is also possible to decrease the sheet width to allow more laser light to illuminate sample. When changing the sheet NA or sheet width, click on "Apply" to save the new settings for image acquisition.

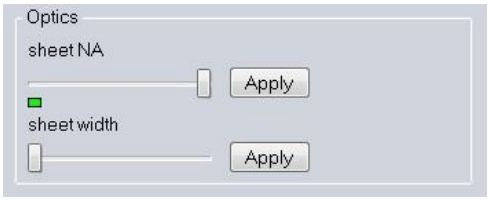


Figure 4-17: Adjusting the sheet NA and the sheet width within the "Ultra" dialogue.

When zooming in further (using zoom control and software), check the focus (coarse and fine), sample position, and horizontal focus. Repeat this process until the sample is in

focus with the best resolution, which indicates that it is ready for imaging.
To set up a Z-stack: First, go to top of sample i.e. where you want to start Z-stack. Click on “Set as Zero” followed by clicking on the “Take” button next to “Start.” Then move through the sample to the end of the designated Z-stack. Next, click the “Take” button right next to “End.” Then enter the step size underneath within this part of the “xyz-Table Z” dialogue. For a quick overview, 10 µm would be a suitable step size. For a Z-stack at article quality, choose 2 µm for Nyquist oversampling.

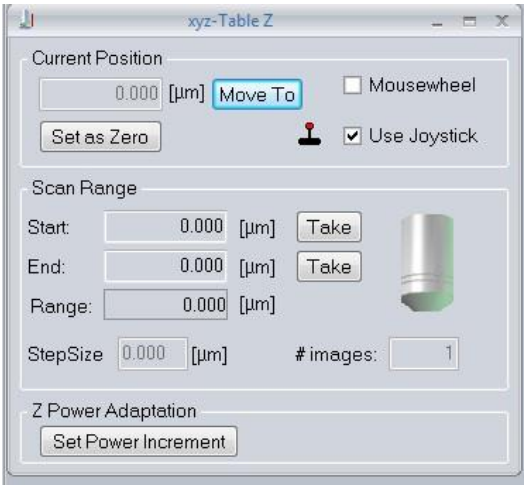


Figure 4-18: “xyz-Table Z” dialogue to set the Z-stack range and the step size.

Check whether the correct folder was chosen for “Autosave.”
Make sure that the correct zoom value has been transferred from the microscope stand to the software; this will be saved to the file metadata as it was set up in the software.

It is now possible to acquire the Z-stack. Select “Start” in the “Measurement Wizard”.

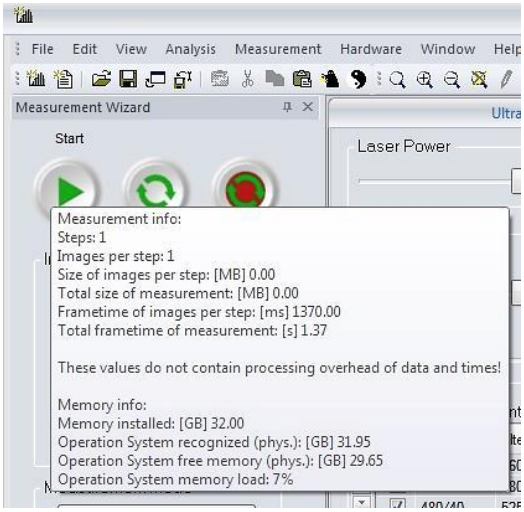


Figure 4-19: Measurement Wizard displaying designated Z-stack properties before clicking “Start” for image acquisition.

5 Advanced image acquisitions

In chapter 4, a description was given on how to acquire a Z-stack with one excitation and one emission filter while the sample is illuminated from one side.
Some samples might require multicolor image acquisition due to different fluorophores. Different illumination modes might also be useful. A double-sided illumination is required for larger samples. The dynamic horizontal focus mode optimizes the Z resolution of the stack. Within this chapter, we will describe how to setup such advanced image acquisition based on the procedure described in the chapter “4. Getting started”.

5.1 Multicolor Z-stack

Within the “Measurement Wizard,” choose the “Measurement Mode” “Multi Color 3D”.

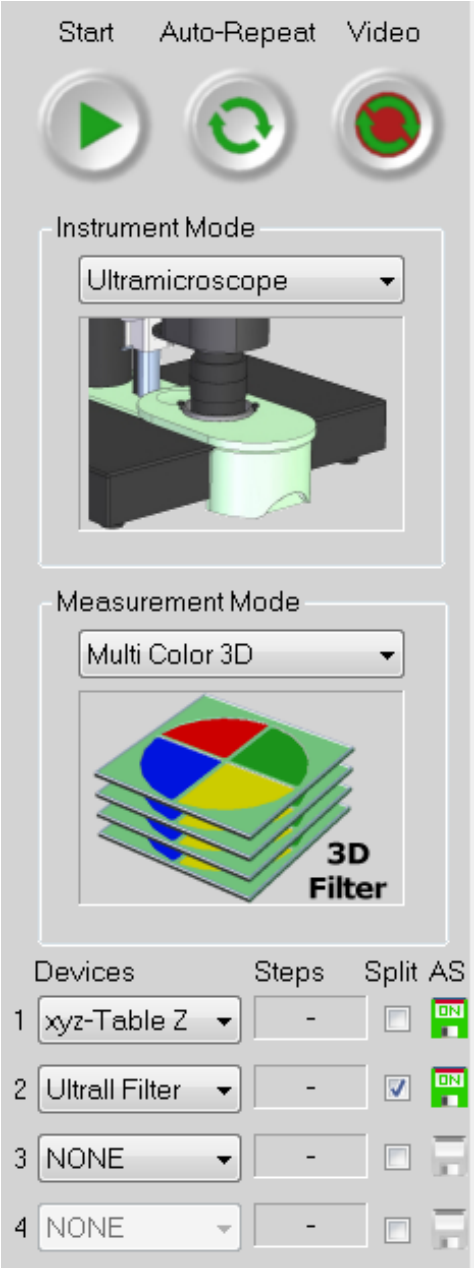


Figure 5-1: Setting up image acquisition for a multicolor stack.

The “xyz-Table Z” and the “Ultra Filter” will be displayed as active devices. Ensure that the “AS” buttons are clicked, as indicated by a bright green color. Consider the following: Tick the check box on “Ultra Filter” device to activate the function that saves the same number of stacks as filter pairs. When receiving a “green” and a “red” stack and this box is not ticked, only one large stack will be provided instead of 2 (green & red) for merging.

Now select the filters or wavelength needed for the experiment by ticking the boxes within the “Ultra” dialogue. Consider that the laser power setting can be applied individually to each filter pair.

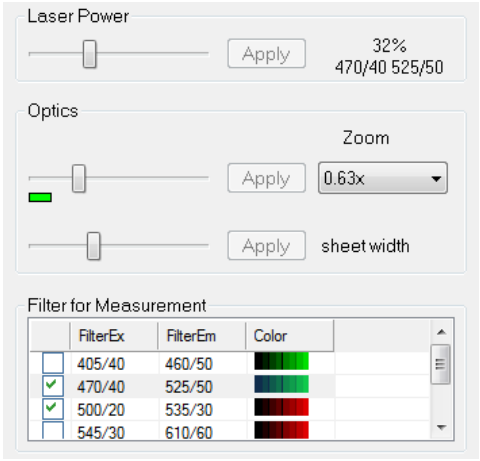


Figure 5-2: Selecting several filters for a multicolor Z-stack. Each filter pair can be run at an individual laser power setting via the “Apply” button.

Different emission wavelengths require different foci lengths. These settings can be adjusted by clicking the chromatic correction button  next to the filter box.

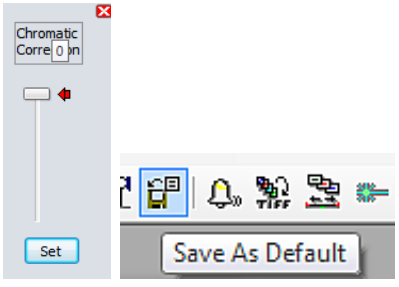


Figure 5-3: Setting the chromatic correction for a multicolor Z-stack.

First, use the coarse or fine focus drive to focus the sample at the shortest excitation wavelength needed for image acquisition. Then choose the next filter pair and focus at

this longer wavelength by using the chromatic correction button. Save the chromatic correction settings by clicking “Save as default” in the software menu bar.

The light sheet NA and the light sheet width are set once by clicking “Apply” for the image acquisition procedure and not individually for each filter pair. Continue the image acquisition set up as described in chapter 4.

Before beginning to record the multicolor Z-stack, check following settings:

- The required filters are ticked.
- The “Split” option is ticked for the “Ultra Filter” device.
- The correct zoom value from the microscope stand has been transferred to the software.
- “Autosave” is activated.

5.2 Double-sided illumination

Large samples require illumination from both sides. To activate the double-sided illumination, click on the two green Triangles displayed in the “Ultra” dialogue. The double-sided illumination is organized in an alternating mode. The sample will be once imaged with left side illumination followed by an image with illumination from the right side. The two resulting images can be merged by using different algorithms. It is recommended to first test the “Blend” algorithm. To choose this setting, click on “Blend” for “Merge light sheets” in the tab “Advanced” of the “Ultra” dialogue. Further, the blending range must be set by moving the green small sliders. While setting the blending range, it is useful to control the merged image displayed in the video mode.

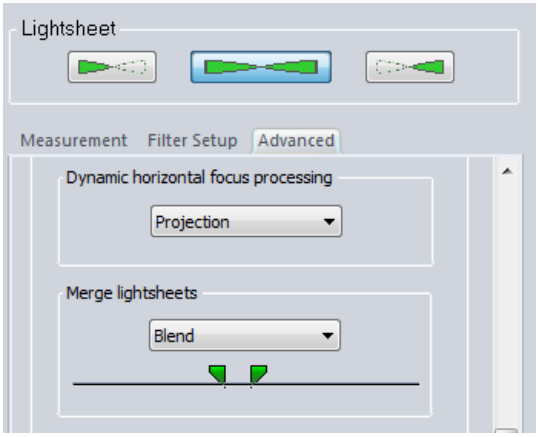


Figure 5-4: Activating double-sided illumination and choosing the “Blend” algorithm for merging the data. Consider the blending range set by the green sliders.

Now continue with setting up the image acquisition as described in chapter 4.

Recommended imaging acquisition setting:
For double-sided illumination, it is recommended to decrease the light sheet NA to a value that the Rayleigh length of one light sheet covers nearly half of the image.

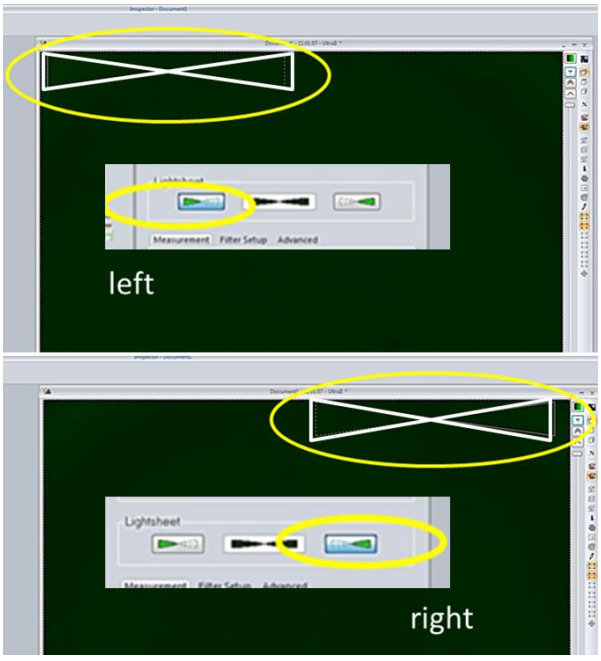


Figure 5-5: Recommended imaging settings for double-sided illumination and inactivated dynamic horizontal focus option.

5.3 Image acquisition using the dynamic horizontal focus feature

Image acquisition at high light sheet NA increases the Z resolution of the stacks. However, if the NA is increased, the Rayleigh length of the light sheet decreases. Therefore, at high NA, only a narrow part of the light sheet offers the best and thinnest image stack characteristics. A decreased light sheet NA will cause a broader Rayleigh length while the Z resolution is impaired. To overcome this issue, we motorized the horizontal focus (HF). The travel range of the HF is set by the user in a way that the region of interest is covered by the hf. The number of images within this range is set to correspond to the NA.

First, set up the image acquisition as described in chapter 4. Start with identifying the region of interest for imaging. That task is followed by setting the Z stack range, the step size, and the filters. Then activate the dynamic hf within the Measurement tab of the Ultra dialogue (Figure 5-6/1). The actual position of the hf is indicated by an ⋈ icon in the image. If this icon is not displayed, click on the button showing the two red Triangles (Figure 5-6/2). Set the moving range for the hf by clicking the button with the small green Triangles (Figure 5-6/3). In the imaging window, a new ROI will appear which can only be moved sidewise. These are the borders of the hf range. Set the borders in a way that they cover the sample. Within those borders, the hf will move for image acquisition. The number of images suitable for the current settings is displayed in the Measurement tabulator of the Ultra dialogue (Figure 5-6/4).

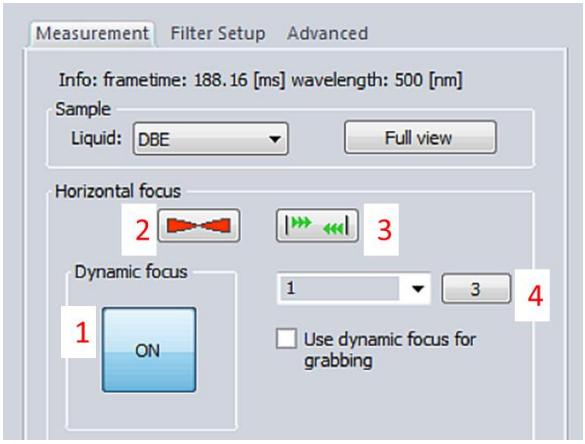


Figure 5-6: Horizontal focus settings in the Ultra dialogue.

Please consider a number of images per Z plane below 20. Directly next to the suggested image number, it is possible to type in the number of images the UltraMicrosope should acquire per Z plane. The single images of one Z plane can be merged by different algorithms. Go to the "Advanced" tab of the "Ultra" dialogue and choose an algorithm listed under "Dynamic horizontal focus processing" (see figure 5-4). The different algorithms are described in chapter 7.

Recommended imaging acquisition setting:
Choose a NA such that the number of suggested images does not exceed 20 images per Z plane. For large samples, choose the blending or the contrast algorithm for the dynamic horizontal processing.

5.4 Image acquisition using the ImSpector stitching (tiling) option

The Visual Scan Life Dialog is initialized with an empty stage overview. That view is centered on the current position of the table. Above is a tab bar to select either Mosaic or Multiposition mode. The table device communicates with the Measurement

Wizard, so that the mode change triggers a measurement mode change of the wizard if properly configured - and vice versa!

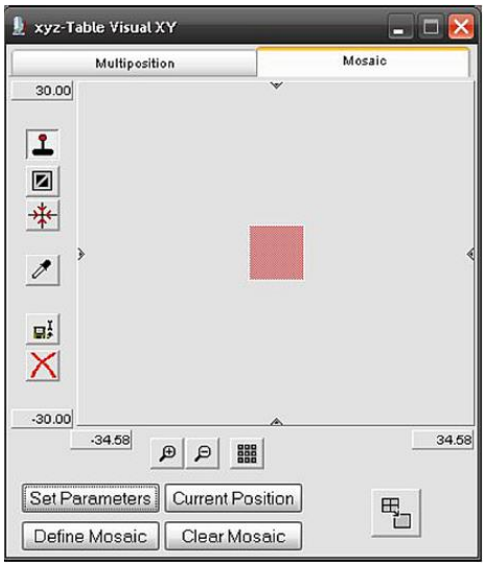


Figure 5-7: Visual stitching dialogue

On the left and bottom of the view are edit boxes to set the size of the stage overview.

-  Enable or disable the joystick to move the current position.
-  Zoom to the range of positions.
-  Set current position as origin.
-  Select a stack window to take its image properties.
-  Load positions of last session.
-  Delete all positions of this mode.
-  Zoom into the view.
-  Zoom out of the view.
-  Open the "Stitching" dialog.

If you hover the mosaic, black dots appear at the edges and corners to resize the mosaic. To actually change the mosaic you have to hover a dot, press the mouse button and move the mouse. When that happens the mosaic changes its color. You can click on a position to activate and highlight it. If the left mouse button is pressed on a position, the current position or the stage overview and moved the respective object shifts. At the mosaic mode the complete mosaic shifts, not just the activated position. This is also possible via keyboard with the *up*, *down*, *left* and *right keys*. Either without or with one of the *shift* or *control keys*. At the multiposition mode, it is possible to right-click on a position to open a context menu. There are entries for editing the position, moving the current position to that position, deleting that position and deleting all positions.

5.5 Image acquisition using the Z power adaptation

With this extension dialog appearing in the Z Stepper Life dialog up to three attenuators can be controlled. The Z Power Increment can be set via five kinds of functions: Points, Linear, Quadratic, Quadratic Fixed, Exponential.

• *Points* - This is a linear point-to-point function. The points can be added or removed by the user. The green point, which represents the actual power and z position, is used to add a point.

• All other types implement the function from start to end position of the Z Stepper Life dialog. The quadratic and exponential functions have a helper position to generate its coefficients.

The Z Power Diagram for an attenuator consists of a *power slider* to set the actual power of the selected attenuator, an optional *power upper bound*, a *description field*, a *profile* that represents the power (y axis) over the z range (x axis) and four spin buttons. These spin buttons increment or decrement the power for the selected point (top), marked with a circle, all points (bottom), as well as a left-sided or right-sided relative linear power change over the z range, where the end of the other side is fixed.

Tip: Keep the spin buttons pressed for a continuous movement.

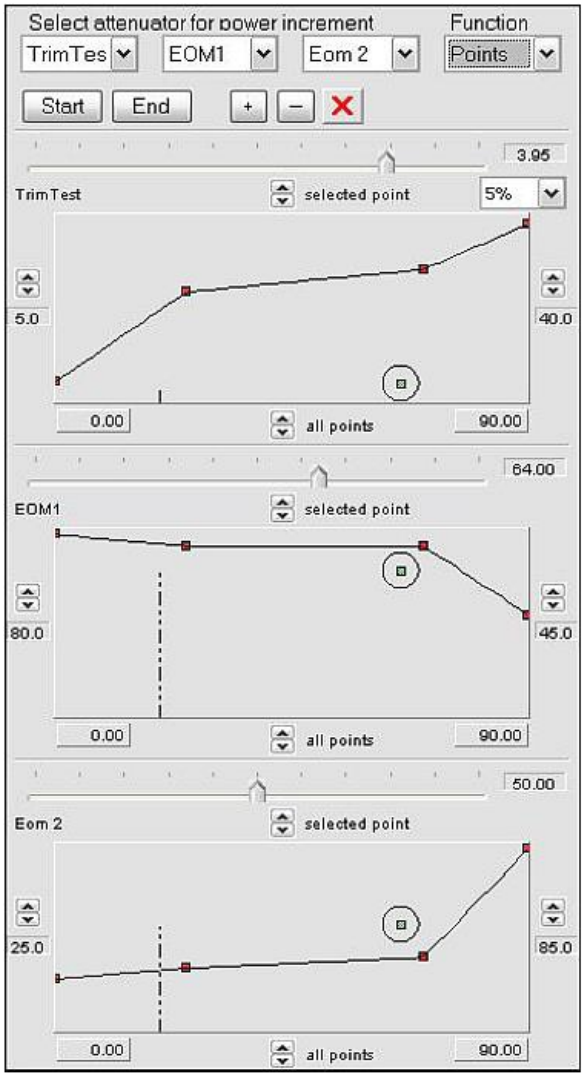


Figure 5-8: Z power adaptation dialogue

6 Data handling

The acquired data stack may easily exceed the size of some GB. For this reason, the image data (tiff.ome files) are directly stored on the hard drive and not handled in the RAM. To review the acquired data, please use the “Image Series Viewer” plug in.

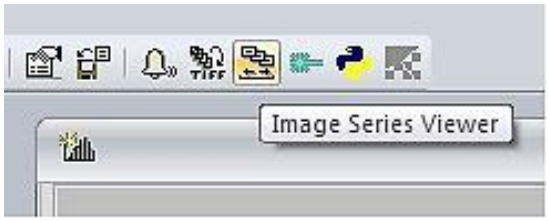


Figure 6-1: Image Series Viewer

The upload dialogue for selecting the file of interest will open (Figure 6-2). Please consider that those data that have been acquired will be directly highlighted. Select the specific data to view by clicking on the folder and upload them to the transfer part of the window by pressing the down-arrow button. Then click on “Load”.

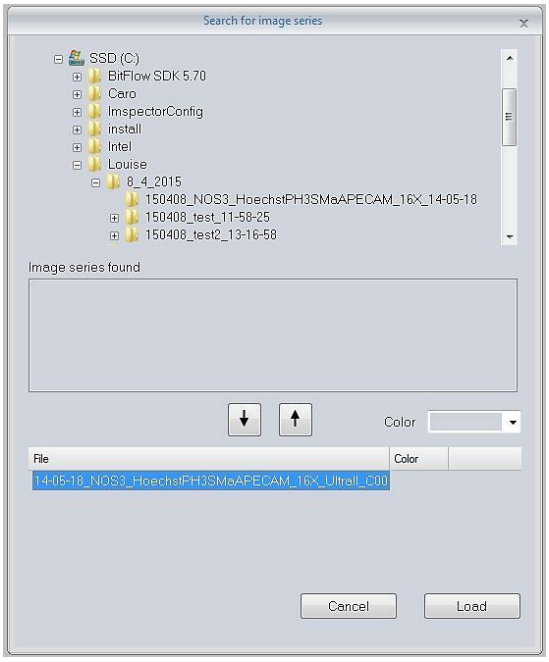


Figure 6-2 Data upload dialogue of the image series viewer.

The data stack will be displayed in the Image Series Viewer window. Using the Image Series Viewer control panel, the user can browse through the Z stack.

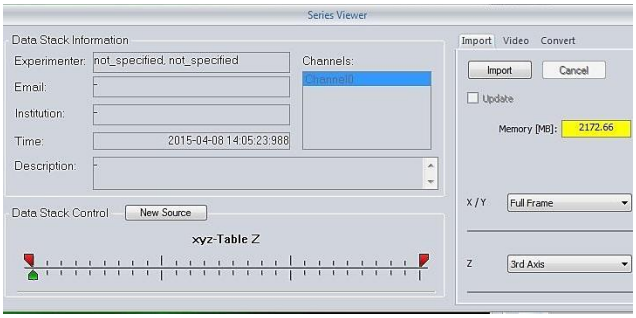


Figure 6-3: Image Series Viewer control panel

Data stacks can be converted into movie files like mp4s. Choose settings, ENCODE and play video. Images can be CONVERTED and saved as a PROJECTION.

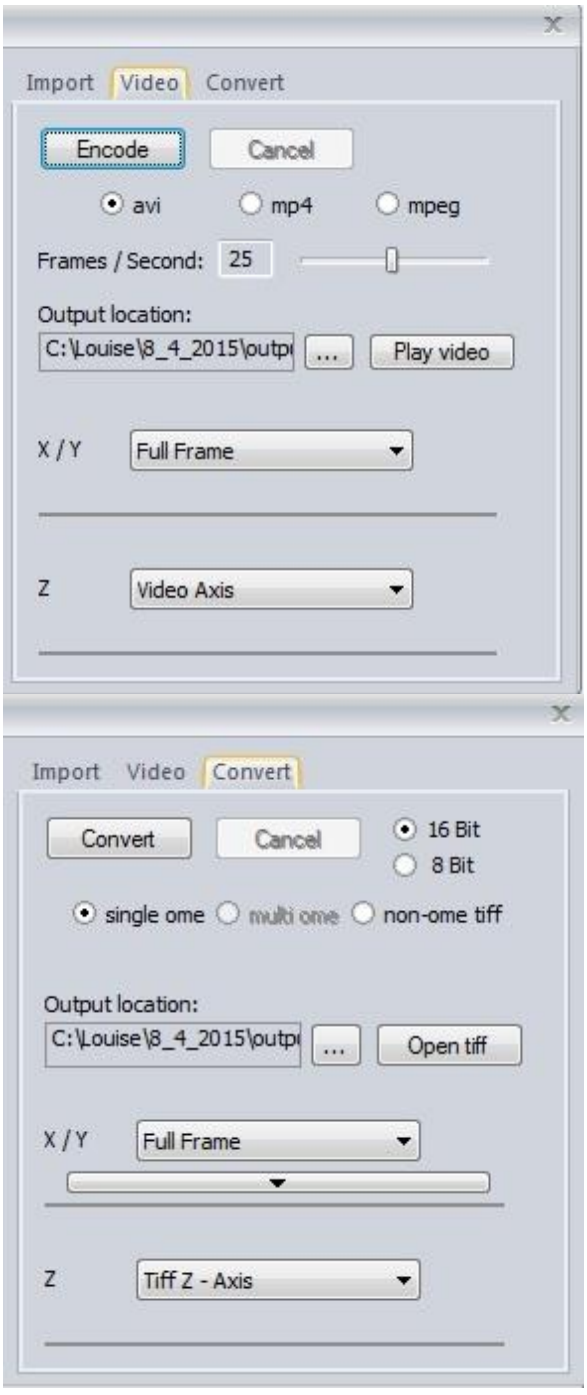


Figure 6-4: Encoding video files and converting data stacks into projections.

7 Image processing algorithms

7.1 Max. Intensity Projection operation (for continuous focus and two-sided light sheet operation)

When overlaying two or more images, each pixel position is compared individually and the pixel with the highest intensity for each position will be used for the final image.

7.2 Blending mode

7.2.1 For double light sheet operation (left and right set of 3 light sheets)

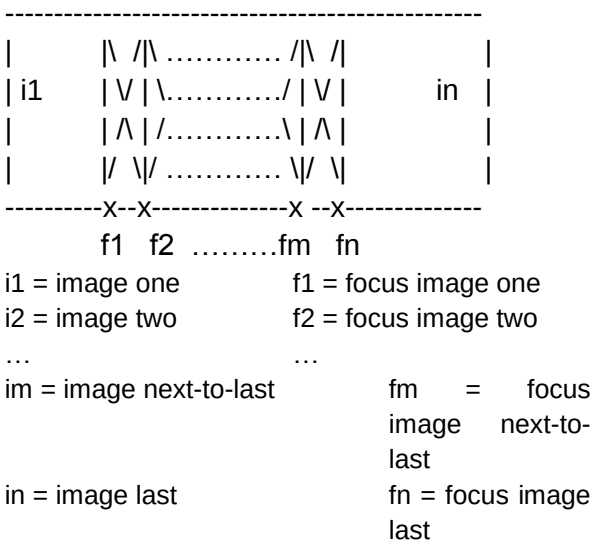
Both images (image 1: acquired by utilizing the left light sheet and image 2: acquired by utilizing the right light sheet) will be used to compute the blended image. Typically, the left area of the blended image is identical to image 1 and the right area to image 2. The centre area is a linear combination of image 1 and 2. Before computing the centre area, the user has to set the start and end position of this blended area.
Defining the centre part by setting start and end position:

LS1 = Image 1 left light sheet only
LS2 = Image 2 right light sheet only
B = Blended region

7.2.2 For continuous focus operation

The blending mode is similar to the blending method described above. Instead of using just two images that correspond to the left and the right light sheet, 2 to n images are used to calculate the final image. Each individual image corresponds to a specific

focus position of one light sheet. Before acquiring the individual images, the user has to define the start and the end focus position of the light sheet and has to set the number of focus positions and the corresponding images 2…n that have to be acquired. While acquiring the n images, the focus will be moved in equidistant steps between start and end position.
The final image will be a combination of n images. The left region is identical to image 1, the region between focus position 1 and 1+1 is a linear combination of image 1 and image 1+1…and the right region is identical to image n.



i1 = image one
i2 = image two
…
im = image next-to-last
in = image last

f1 = focus image one
f2 = focus image two
…
fm = focus image next-to-last
fn = focus image last

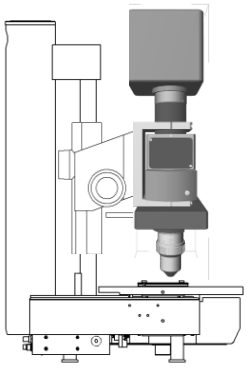
7.3 Contrast operation (for continuous focus and two-sided light sheet operation)

a) The contrast of each column within an individual image will be calculated (column 1…m) by applying a differential calculus algorithm. This procedure will be done for all images (1…n).
b) During the next step, an intermediate image will be generated that is a combination of columns (1…n) that show the highest contrasts for each individual corresponding column position (1…m).
c) The arrangement of columns for each position within the intermediate image will be filtered by median and mean filters to generate the final image.

8. UltraMicroscope add on – The infinity corrected optics setup

Dovetail tube (58 mm) to connect to the adapter mount (20 mm) for the sCMOS Included in Art.# 207 690

sCMOS camera



Part numbers

Art.# 207 690
LV OM UMST2
UltraMicroscope Tube for Infinity Corrected Objective Lenses

Art.# 206324
LV OM MOTTL
Motorized Tubelens

Art.# 207 691
LV OM MCU2X
Magnification Changer 2x

Art.# 207 350
LV EO FLW32
Fast filter wheel
7 positions for
32 mm filters

Art.# 207 XXX
AH OM xxx32
32 mm emission filters
Based on customer choice

objective lens

Set of adapters for the LV OM UMST2
The set improves objective lens exchange (*parts included in LV OM UMST2)*.

Adapter 1:
The adapter has to be placed on the existing safety lock at the pillar of the UltraMicroscope.

Adapter 2:
The adapter has to be mounted to the LVMI-Fluor 4X/0.3

LVBT multi immersion objective lenses

Art.# 207513
LV OL 0101D
LVMI-Fluor 1.3X/0.1
Mag. 1.3x; NA: 0.1;
WD: 9.0 mm

Art.# 205852
LV OL 0403D
LVMI-Fluor 4X/0.3
Mag. 4x; NA: 0.3;
WD: 5.6-6.0 mm

8.1 Mounting the infinity corrected optics setup

The infinity corrected optics detection setup can be mounted directly to the Olympus focusing unit (SZX2-FOFH) and has to be aligned following the steps below:

- Open the screw at the SZX2-FOFH to release the MVX10 zoom body from the dovetail.

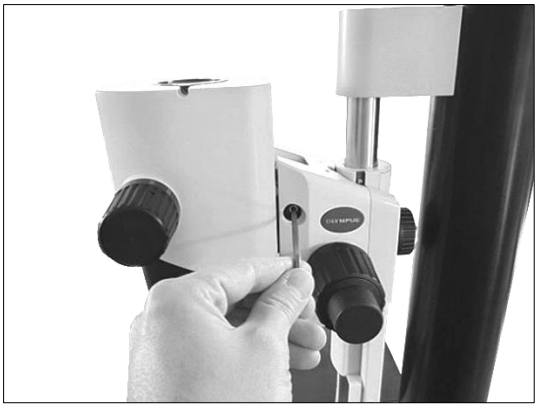


Figure 8-1: Undo the screw at the dovetail.

Figure 8-2: Release the MVX10 zoom body.

- To demount the zoom body please first remove the revolving nosepiece. Therefore undo the four screws holding the nosepiece.

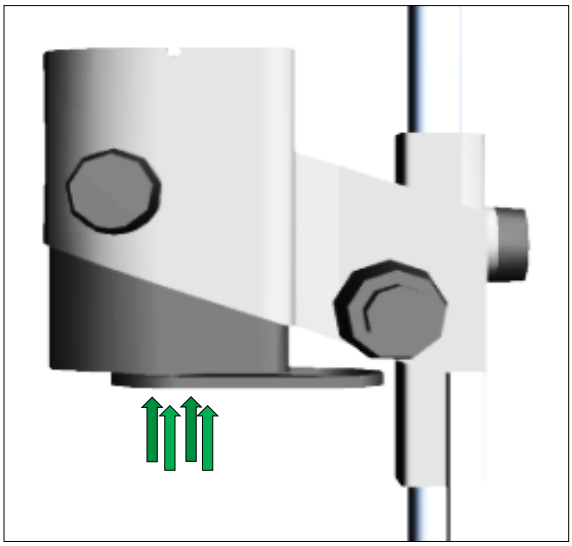


Figure 8-3: Undo screws to demount the revolving nosepiece.

- Remove the zoom body and place the assembled infinity corrected optics setup in the dovetail.
- Fasten the screw again and connect the filterwheel and the motorized tubelens.
- Place the sCMOS connected to the C-mount adapter (20 mm) on the 58 mm fixed tube.
- Position the XYZ table in the centre position.
- Loosen the Olympus focusing unit SZX2-FOFH and place the adapter 1 (see page 27, bottom right) on the existing safety lock at the pillar of the UltraMicroscope.
- Ensure that the entire detection path is not tilted before tightening the focusing unit to the pillar. For alignment lower the objective lens into the chamber and check whether the distance to the left and the right side of the centred imaging chamber is the same.

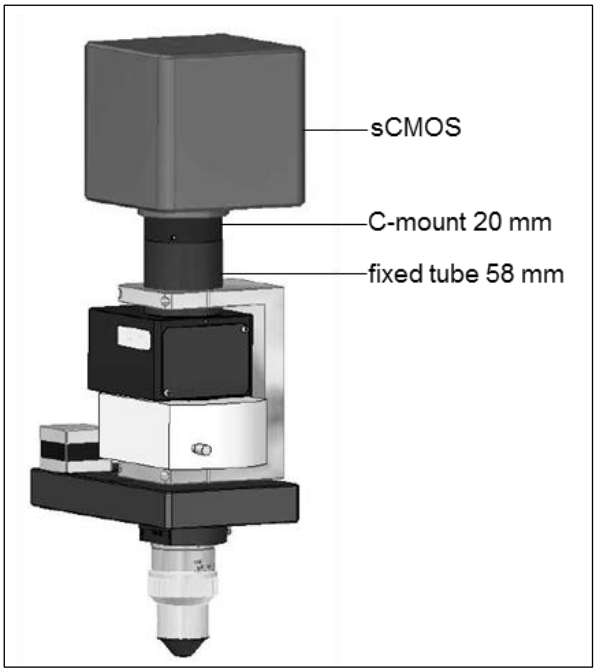


Figure 8-4: Mounting the sCMOS.

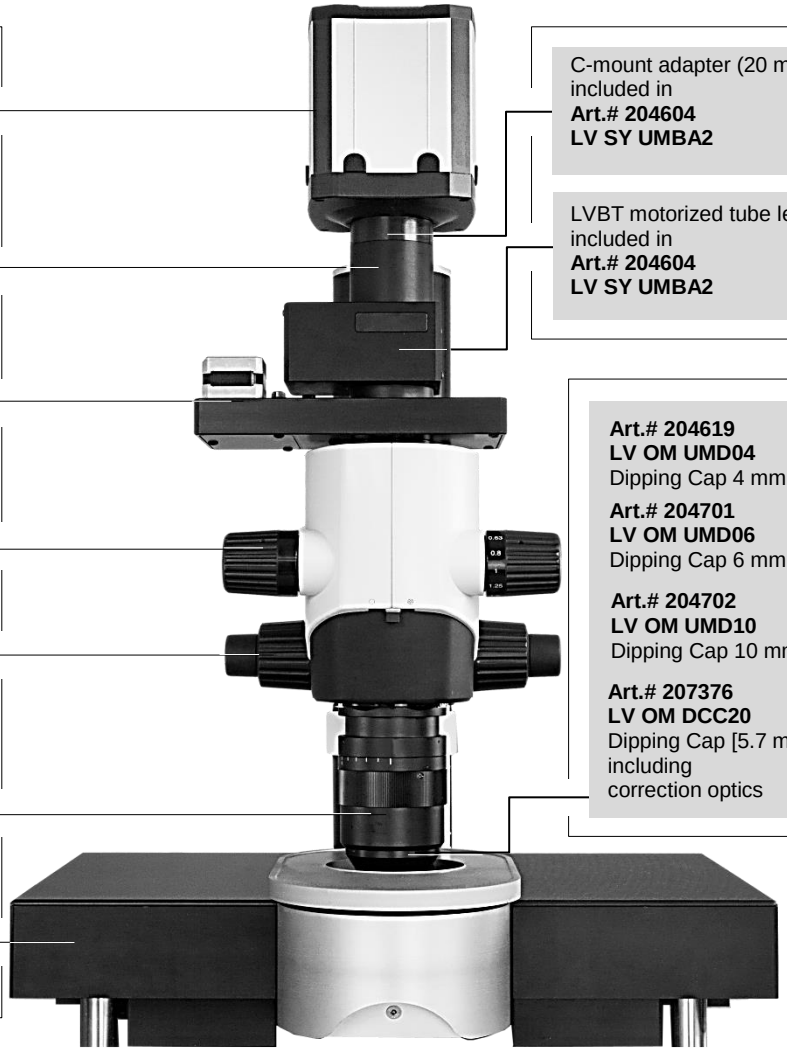
Aligning the sCMOS:
The rectangular camera chip has to be orientated in that way that the elongated axis is the Y axis of the field of view. For realignment of the sCMOS please follow the steps below:

- Switch on all devices and start the ImSpector software.
- Mount a sample on the sample holder ring and place it into the imaging chamber.
- Choose a filter combination adjusted to the dye in the sample and activate the right light sheets.
- Click on “Video” for image grabbing.
- Lower the objective lens (with dipping cap) into the cuvette and focus on the tool at lowest zoom factor (zoom 0.63).
- Choose an easily to follow structure in the sample.
- Activate the crosshairs in ImSpector (at left side of the grabbing window frame) and line up the chosen structure to the centre.
- Move the sample along the y axis and follow the structure. As soon as the structure is not moving anymore in parallel to the Y-crosshair twist slightly the sCMOS.
- Loosen the sCMOS C-mount adapter and turn the sCMOS so that the structure is again in parallel to the y-crosshair.
- Repeat these steps until the structure is following the Y-crosshair in parallel along the entire field of view.

8.2 Combining third company objective lenses with the infinity corrected optics setup

The UltraMicroscope utilizes a 180 mm tube lens for the 1.3x and the 4x objective lens. In case that a Zeiss lens is implemented please multiply the magnification by the factor 1.09 to get the effective magnification. For Nikon or Leica lenses please multiply the magnification by 0.9. Chromatical aberrations can be corrected with the motorized tube lens. Dovetail adapters for mounting different objective lenses with certain threads are available.

9. The UltraMicroscope setup



Art.# 200023
AD CC NEOCM
Andor Neo sCMOS camera

LVBT connector tube (58 mm)
included in
Art.# 204604
LV SY UMBA2

LVBT fast filter wheel
(for 25 mm filters), included in
Art.# 204604
LV SY UMBA2

Art.# 204099
OL OM MVX10
Olympus MVX-10 Zoom Body

Focusing unit SZX2-FOFH
Included in
Art.# 204604
LV SY UMBA2

Art.# 200172
OL OL 0205M
Olympus MVPLAPO 2x

Art.# 204604
LV SY UMBA2
UltraMicroscope II

C-mount adapter (20 mm)
included in
Art.# 204604
LV SY UMBA2

LVBT motorized tube lens
included in
Art.# 204604
LV SY UMBA2

Art.# 204619
LV OM UMD04
Dipping Cap 4 mm WD

Art.# 204701
LV OM UMD06
Dipping Cap 6 mm WD

Art.# 204702
LV OM UMD10
Dipping Cap 10 mm WD

Art.# 207376
LV OM DCC20
Dipping Cap [5.7 mm]
including
correction optics

Accessoires

Art.# 207467
LV OM UMCUV
Cuvette for UltraMicroscope

Art.# 207468
LV OM UMSHS
Set of sample holders

Art.# 200193
LV OM OMDOA
Adapter for different objectives

Art.# 200192
LV OM USPOM
Objective Mount

9.1 The fast filter wheel

The UltraMicroscope can be equipped with two different filter wheels. Please assure which filter wheel is combined with your UltraMicroscope. The LV EO FLW32 filter wheel can be equipped with up to 7 32 mm filters while the LV EO FLWWH is equipped with 8 32 mm filters. The 25 mm filters are mounted with a ring screw. The 32 mm filters can be fixed with a single screw at the top right quarter of each filter.

Art.# 207 350
LV EO FLW32
Fast filter wheel
7 positions for
32 mm filters

In case of the fast filter wheel with 7 positions for the 32 mm filters, these filters are tightened by the small silver screw on the right top quarter of each filter. The filter wheel for 25 mm filters utilizes ring screws for this purpose.

Following emission filters can be utilized:

Emission filter	***Dye***
ET460/40m	DAPI, DyLight 405
ET480/40m	CFP
ET525/50m	GFP
ET540/30m	YFP
ET585/40m	RFP, Cy3, DiI, Propidium Iodide
ET575/40m	RFP, Cy3, DiI, Propidium Iodide
ET620/60m	Rhodamine Red, Mitotracker Red, Cy3,TRITC, DsRed, mCherry
ET590/33m	Rhodamine Red, Mitotracker Red, Cy3,TRITC, DsRed, mCherry
ET680/30m	Cy5, TO-PRO3, DyLight 633, Alexa Fluor 633
ET667/30m	Cy5, TO-PRO3, DyLight 633, Alexa Fluor 633
ET845/55m	Alexa Fluor 790, DyLight 800

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