Light Sheet Microscope Detailed User Guide

System Startup

1. Turn on the computer and let it boot.
2. Turn on laser unit.
3. Turn on motor switch.
4. Turn on camera (back, right-hand side).
5. Log in to computer (net ID & password).
6. Open ImSpectorPro software program.
7. Click Ok to warm up Laser 561.
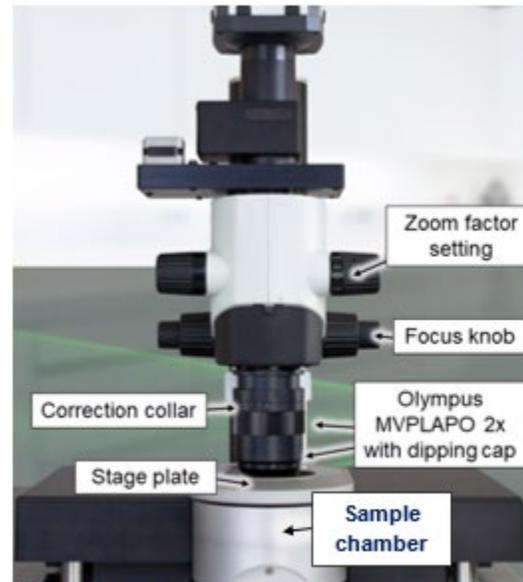
Ultra II Microscope zoom body configuration

Mounting Sample

1. Rotate objective (MVPLAPO 2x) out of working position.
2. Loosen hex screw on right side of gray stage plate. Use Allen wrench labelled "stage".
3. Carefully lift off gray stage plate.
4. Put on gloves! Fill reservoir (glass cube) with imaging solution (e.g. 60% TDE).
5. Use the stamp below the sample chamber to help bring reservoir into correct position in sample chamber.
6. Carefully replace the gray stage plate.
7. Mount sample in sample holder. Try to mount sample as level and as near the top of the holder as possible.
8. Insert the holder into sample cradle.
9. Place the cradle into position such that the sample is oriented perpendicular to light path. You need the light sheet to be able to pass unobstructed through your sample.
10. Remove gloves!
11. Tighten the screw on the right side of gray stage plate.
12. Rotate the objective into working position. Then set correction collar to 3.5.

Finding Sample

1. Set xyz control dials to fast mode by pressing and holding the rightmost button until it beeps.
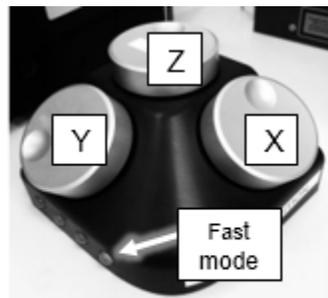
XYX Control dials and fast mode button

2. Select the lowest zoom setting (0.63x) on the microscope and the software.

3. In the Ultra II dialog window:
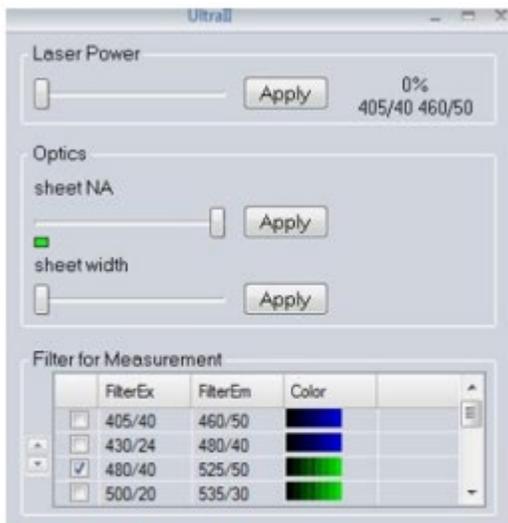
Ultra II dialogue displaying laser power setting, sheet NA setting, sheet width, and excitation/emission filters
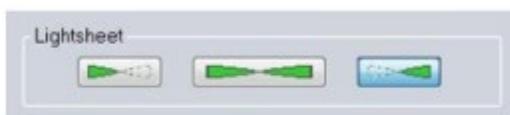
Light sheet button to select illumination direction (left, right or both sides).

a. Select the 561 nm laser. Click to highlight.

b. Set laser power to 10%. Click Apply.

c. Set Sheet NA to highest possible setting.

d. Adjust light sheet width to 20%.

e. Select the right light sheet button.

4. Click on the *Video* button to begin fast image grabbing
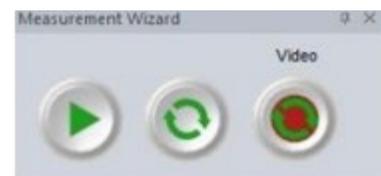
The Video button activates live image grabbing.

5. Look into reservoir: you should see the light hitting the sample holder and your sample. If not, maneuver the sample in XYZ until you can see the light sheet going through your sample. Once the light sheet is hitting your sample, lower the z stage (turn Z control dial counter clockwise) until the light sheet is going through the top of your sample.

6. Disengage control dial fast mode by pressing rightmost button once.

7. Select the proper laser(s) for your fluorophore(s).

8. Hit the min/max (contrast wizard) display icon (sun and moon with stars picture on right side of imaging window).

9. Lower objective until it is close to touching imaging solution. When close, lower it more slowly (while constantly monitoring image and adjusting display to avoid hitting your sample) until it begins to come into focus.

10. Adjust sheet width to 100% to illuminate entire field of view evenly.

11. Center the sample in the field of view using X and Y control dials. Focus on a structure within the sample using the fine focus knob on the microscope.

12. Adjust your zoom as desired. Adjust your focus and readjust light sheet width (if needed) after adjusting the zoom. Adjust zoom setting accordingly in the software.

Setting up Imaging Configurations

Focusing Evenly Across the X Dimension

If the sample does not seem to be fully in focus across the entire field in the X dimension, try the following:

1. Use the light sheet from the other side.

2. Adjust horizontal focus position by clicking on the button with two red horizontal triangles to bring up indicator in the imaging window. Click and drag the indicator to adjust position of the horizontal focus until the focus of your structures of interest improves.

3. Lower the sheet NA. This makes light sheet dimmer, and thicker in the middle, but more even as a function of X position.
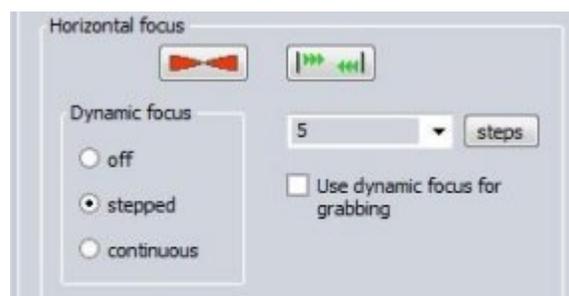
Horizontal focus menu for image contrast adjustment

If none of these adjustments leads to an image that looks fully even and in focus across the field of view, try imaging with two light sheets or with multiple horizontal foci.

Exposure and Laser Power

1. Adjust the power of laser using the laser slider in the Ultra II window. Click Apply.

2. Adjust Exposure settings by going to View > Neo Life.

    o *When looking at sample, it is better to use long exposure (hundreds of ms) and low laser power (10-20%) to avoid bleaching.*

    o *When imaging sample, it is better to use very short exposures (10 ms) and high laser power to speed up acquisition.*
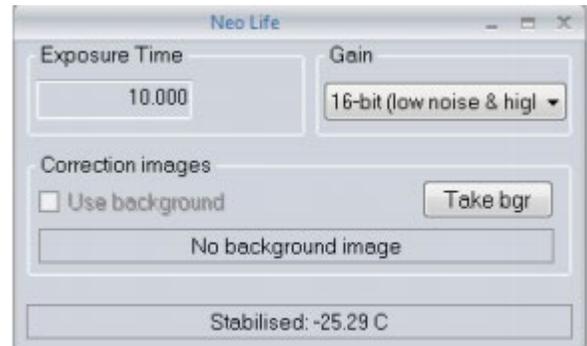
Neo Life dialogue for adjusting exposure time

Imaging with Both Light Sheets (Optional: recommended for large samples)

1. Activate both light sheets (button with 2 horizontal triangles in the light sheet section of Ultra II window).

2. Merge resulting image by clicking on "Blend" for "Merge light sheets" in the *Advanced* tab of the Ultra dialogue.

3. Set blending range by moving the small green sliders.

4. Set horizontal focus optimally for each of the sheets: Decrease the light sheet NA to a value that the Rayleigh length of one light sheet covers nearly half of the image.
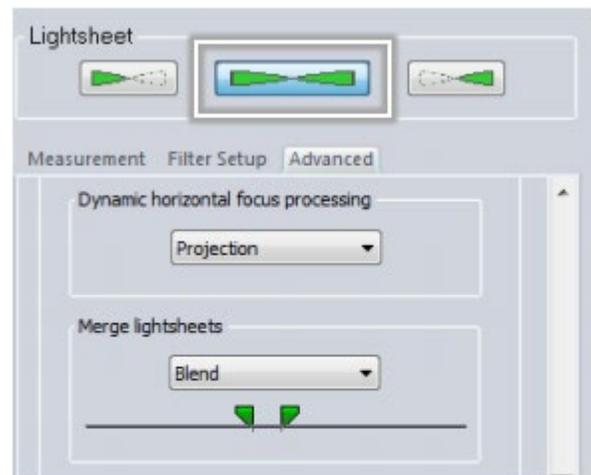
Double-sided illumination and the "Blend" algorithm for merging images from left and right light sheets. The blending range is set by moving the green sliders.

Imaging with Multiple Horizontal X Sheet Foci or Dynamic Focusing (Optional)

You can set the start and stop positions for the horizontal focus, and choose how many images you would like to capture as the focus moves across the field.

1. Check that the zoom factor in the software is the same as on the microscope knob. If this is not set properly, the dynamic focus will give very poor results.

2. Set dynamic focus to "On" (horizontal focus section, measurement tab, Ultra II window).

3. Click on the icon with multiple horizontal triangles and set the area in the image over which you want to move the focus.

4. Input the recommended number of focus positions.

5. Enter the desired exposure time; ensure that it is higher than 100 ms (the software will adjust it slightly automatically).

Cropping

If you only care about a sub-region in the field of view, cropping will significantly reduce file sizes and save transfer and processing times later on.

- To crop, draw an ROI on the image and use the "Box inside a box" button (see icons at right side of image). To ensure optimal quality, your ROI should be centered in the field of view.

- To uncrop, click on the "Full View" button in the Ultra II window (Measurement tab, sample section).

Auto-Save Settings

- Make sure you are writing to the correct directory (D:\All_User_Images_Here) with a proper base name.

- Note that the date and time will be embedded in the name automatically. We highly recommend embedding the zoom you used as part of the base name.

Experimental Design

Acquiring z-stack (1 channel)

1. Check that the zoom factor in the software is the same as on the microscope knob. If this is not updated properly before you begin imaging, your metadata will be incorrect later.
2. While imaging in video mode, move to the top of your sample (move the z adjustment wheel counterclockwise).
3. In the Table Z dialogue box:
    a. Click the "Set as Zero" button.
    b. Click the End target button.
4. Move to the bottom of your sample (move the z control dial clockwise). *Do Not Exceed 5.2 mm deep* in the sample with the corrected dipping cap.
5. Click the Start target button.
6. Set Z step size. 1/2 or 1/3 of the light sheet thickness is a good starting point, but the ideal step size will depend on sheet NA and the size spacing and desired imaging detail of the features of interest in the sample.
7. Stop Video Mode.
8. Add "xyz-Table Z" to the device list in the measurement wizard window.

9. Make sure that Autosave is on. Click small disk icon under "AS" to activate (disk turns green).
10. Start acquisition.
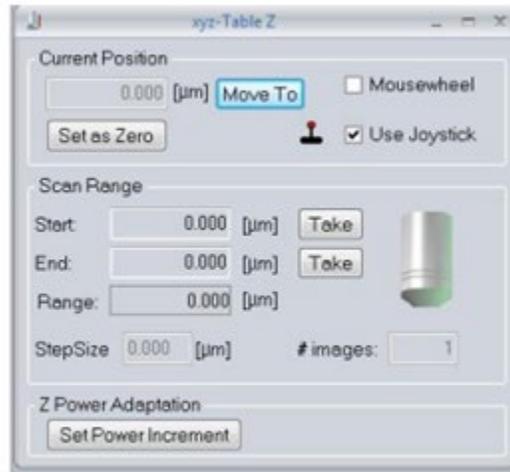
xyz-Table Z" dialogue to set the Z-stack range and step size.
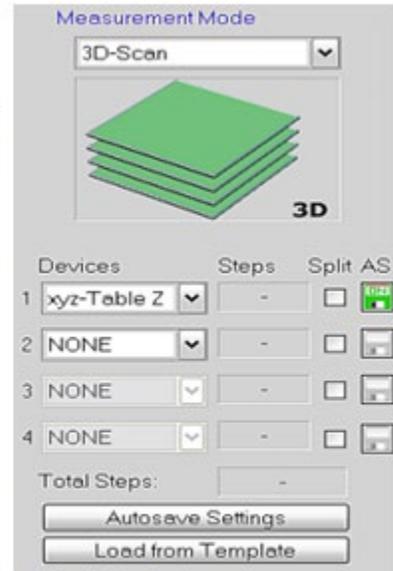
Setting up a z stack measurement in the Device list of Measurement wizard window with Autosave activated

Acquiring z-stack (>1 channel)

1. Check the boxes for the filter sets you would like to use.

2. Check to see if chromatic correction is needed:

   a. Go to your shortest wavelength channel:

      • If your shortest wavelength is 488, focus on your sample.

      • If your shortest wavelength is NOT 488, set the chromatic correction lens to the zero position for your shortest wavelength.

L: chromatic correction button next to filter box
R: chromatic correction slider for setting multi-color z stack foci

b. Go to your other channels and check focus in each one. **Do not move the focus knob**.

- If the focus is off, use the chromatic correction slider to adjust focus. Once it is properly positioned, click the "Set" button to save the position.

- Note that higher magnifications require more movement of the chromatic correction lens.

- Also note that some combinations of fluorophores and magnifications can not be corrected (for example, 647 and 488 at 6.3X zoom).

3. Adjust the laser power for each channel. You can adjust the power of each laser using the laser slider in the Ultra II window.

- Choose a channel by highlighting it (not checking it) and make the proper adjustment, then click "Apply" for each.

- Note that if you use different laser powers for different channels, imaging will be slower, particularly if channels are acquired sequentially at each z plane.

4. Adjust the exposure for the camera. Note that there is only one exposure setting for all the channels.

5. Add "Ultra Filter" to the device list in the measurement wizard window.

- *Note that the order in the device list is the order in which things will be done.*

- *Ultra II Filter 1st, Table Z 2nd: would go to a Z position, take images in each channel, and then move to the next Z position;*

- *Table Z 1st, Ultra II Filter 2nd would take a complete Z stack in the first channel, then switch to the second channel and take a complete Z stack for that one.*

6. Make sure that "Autosave" is on for all devices.

7. Make sure that the Ultra Filter setting has the "split" engaged.

8. Start acquisition.

Generating a Mosaic Image

1. In xyz-Table Visual XY, select the "Mosaic" tab.

2. In *Set Parameters*, under advanced options, set the overlap between 10-20%. Verify that lock button is closed.

3. While imaging in video mode, adjust position of stage to one corner of where you want mosaic.

- You can either use the joystick or drag the red box in the XY Table window.

4. Double click the red box, then click and drag it to create area for your mosaic.

- Dragging the sides of the box delineating mosaic area adjusts size of mosaic area.

- You can also increase the size of the mosaic by dragging the red box to an area outside of its borders and double clicking.

5. To generate an overview of the mosaic area:

    - Remove all devices that will not be included in the overview image from the device list (Z certainly, possibly Ultra Filter).

    - Add "xyz-Table X" and "xyz-Table Y," in this order, to the device list in the measurement wizard window.

    - Deactivate Autosave for each device in the list by clicking the small disk in the "AS" column next to it. The disk should now be greyed out.

    - Activate the split view for xyz-Table X and xyz-Table Y by checking the box next to them (at the top of this column, "split" is written).

    - Start acquisition.

    - After imaging, adjust the display in one tile and hit CTRL-Q to equalize those settings to all tiles. If the mosaic looks good, proceed with complete imaging.
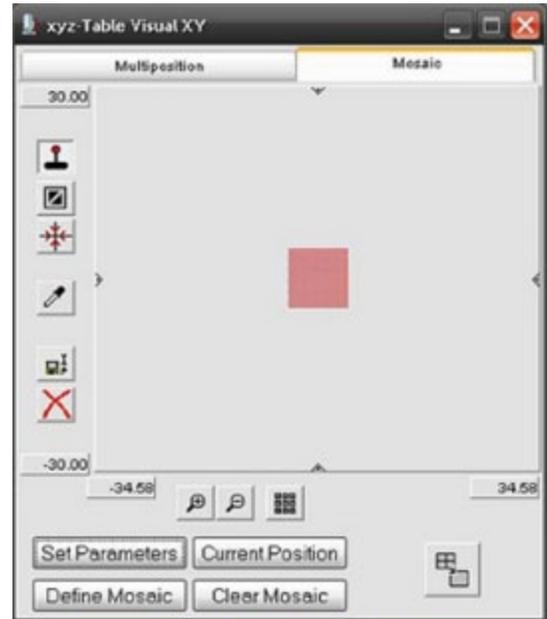
Visual XY stitching tool: Mosaic tab

6. Decide on the imaging order. A typical example would be to do Z first, then channels, then mosaic positions. The order of the devices in the device list should reflect this order. Note that for the tiling, you will need to add xyz-Table X and xyz-Table Y, in this order.

7. When ready for final acquisition, check that all channels have Autosave and that—if used—Ultra Filter has the "split" option engaged.

8. Start acquisition.

    - In the name of the image, the first parenthesis is the Y value and the second is the X value [YY, XX], even though that is the reverse order of that used when adding it to the device list.

Viewing Data in ImspectorPro

1. Open "Image Series Viewer." This is the icon along the top of menu bar that has three squares, two arrows and line on it.

2. In the window that opens, select the folder containing the dataset you wish to view.

Image Series Viewer - toolbar menu

3. Select the dataset in the "Image Series Found" part of the window.

4. Click the down arrow to add that file to the bottom box of the window.

5. Click the "Load" button.

6. Use the "Series Viewer" window to move through your images, not the bars along the image.

7. Close the image window when you are done.

Image Series Viewer control panel

Changing Samples

1. Raise the zoom body all the way up, using the focus knob.

2. Rotate the objective out of the working position. Put Kimwipes/paper towel on stage right under objective to catch any drops from objective.

3. Put on gloves!

4. Remove sample cradle. Use paper towels to avoid spills.

5. Swap sample.

6. Insert the holder into the sample cradle.

7. Place the cradle into position such that the sample is oriented perpendicular to the light path.

8. Remove your gloves!

9. Rotate the objective into the working position.

10. Make sure correction collar is set to 3.5.

Ending Session

1. Raise the zoom body all the way up, using the focus knob.

2. Loosen the hex screw on the right side of the gray stage plate.

3. Rotate the objective out of the working position. Put Kim wipes/paper towel on stage right under objective to catch any drops from objective.

4. Put on gloves!

5. Wipe off all imaging solution from the objective using lens paper.

6. Remove the cradle that holds the sample and put the cradle on a paper towel.

7. Carefully lift off the gray stage plate.

8. Use the stamp below the stage to raise reservoir. Take out reservoir and pour TDE into waste container. Clean reservoir with ethanol and air dry.

9. Moisten a paper towel with ethanol and clean off any TDE from the stage or gray plate. Discard the towel in biohazard waste container.

10. Put the gray stage plate back on.

11. Cover imaging chamber.

12. Put away your sample and clean the sample holder, cradle and tray with ethanol.

13. Remove your gloves!

14. Exit the software. Log off/shut down computer.

15. Turn off camera, motor switch, and laser switch (in this order).