

## Alignment with the alignment tool

The goals of the UM alignment procedure are to set all six light sheets to the same z-plane and to adjust the position of the horizontal focus to the Rayleigh length indicator in ImSpector.

1. Set up the parameters:

Wavelength: 561 nm

Power (Beam Combiner): 5-10% **or**

Power (NKT white light laser): 50% on the laser itself and 5-10% on the attenuator

Sheet NA: maximum

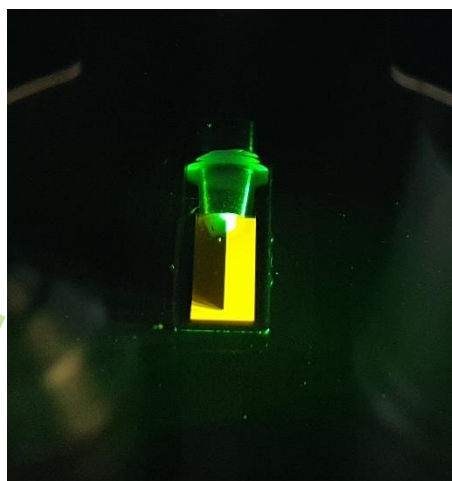
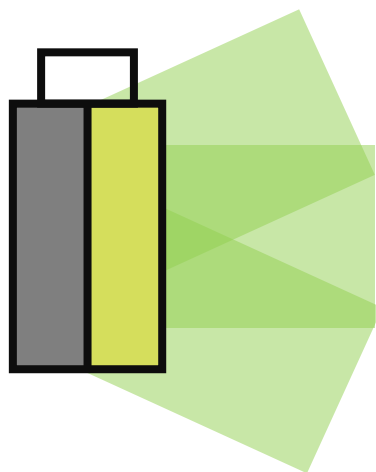
Sheet width: 100%

Right sheet activated

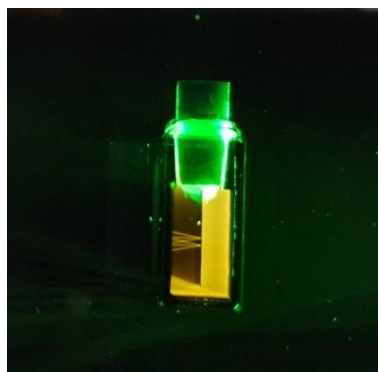
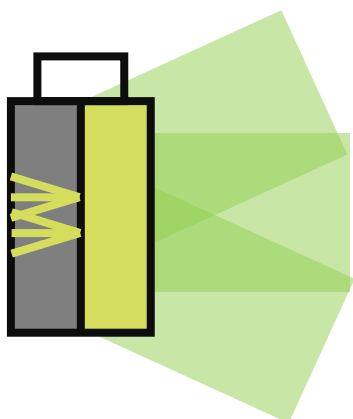
Exposure time: 100-250 ms

2. Mount the alignment tool and start the live mode.

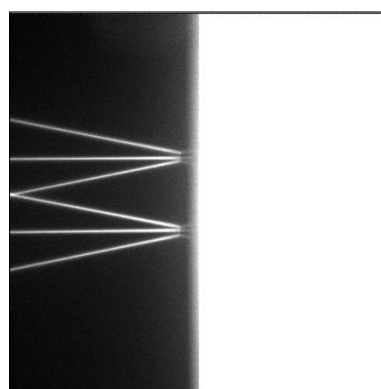
3. Use the x- and y-knob on the joy stick to move the tool into the light sheet. The right half of the tool has to be completely illuminated, while the left half is dark.



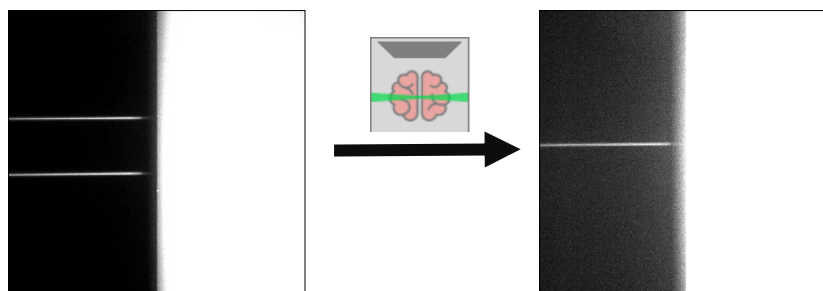
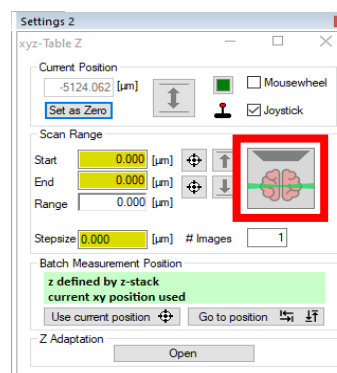
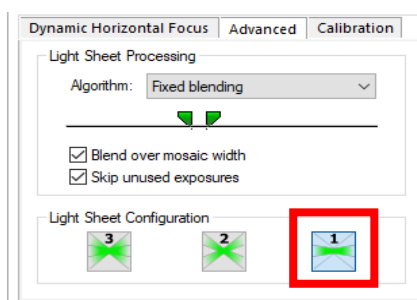
- Use the z-knob of the joystick to move the tool up and down, until some beams are visible on the left side.




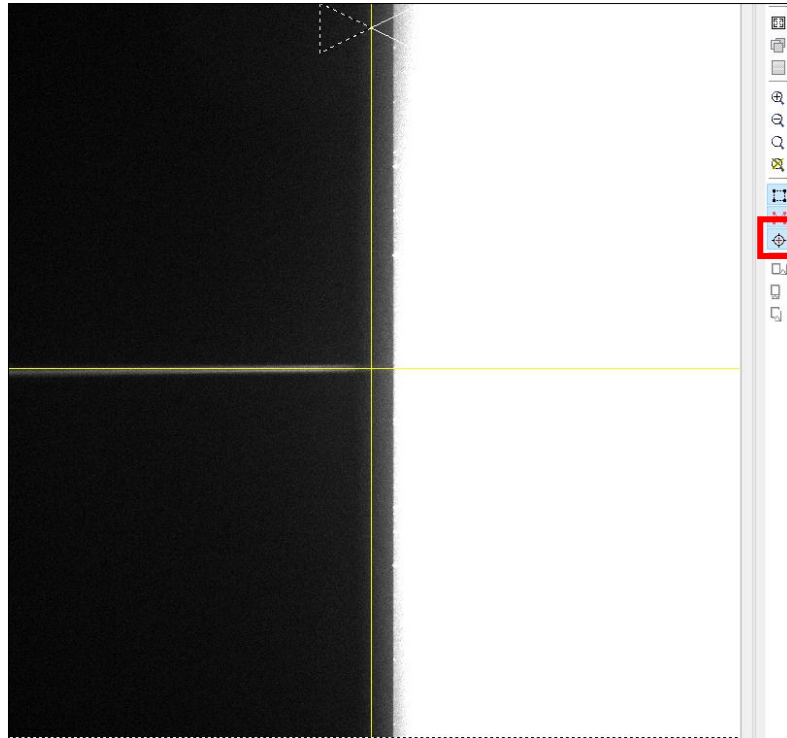
- Lower the objective (for UltraMicroscope Blaze™ and UltraMicroscope II SuperPlan 4x objective and 1x zoom, for UltraMicroscope II Zoom Body 2x objective and 3,2x zoom) and focus on the beams, which exit the tool's membrane.



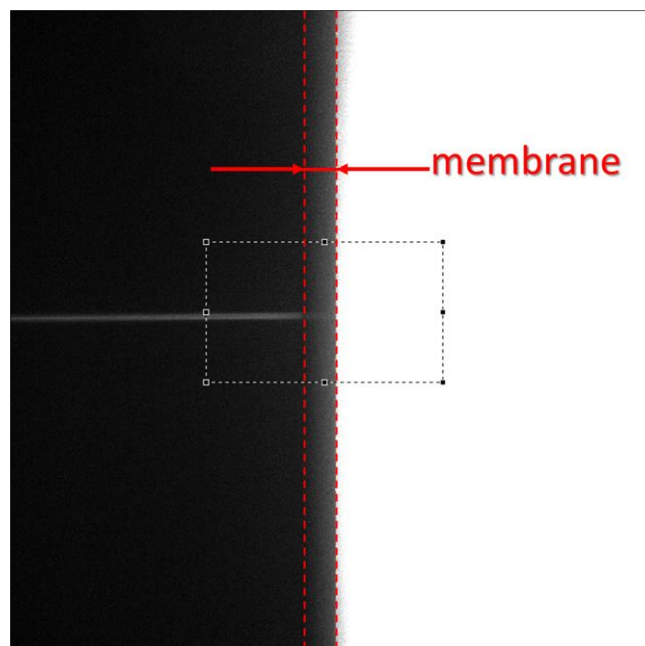
- Activate the central light sheet. You will see two beams now. Move the stage in the z-position until the two beams merge into one beam.



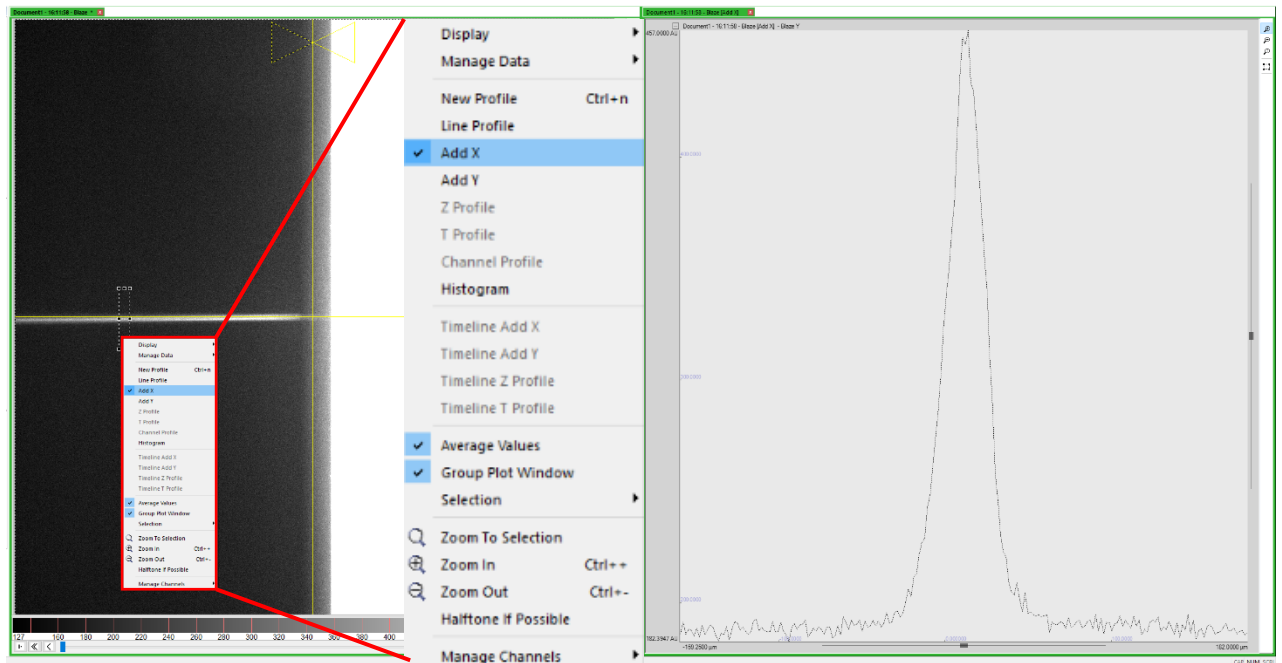
7. Activate the crosshair  in ImSpector and position the tool in the center of the field of view. The beam has to be on top of the crosshair's horizontal line. The vertical line of the crosshair has to be in the center of the membrane. The membrane is the dark part between the beam and the whole illuminated area.



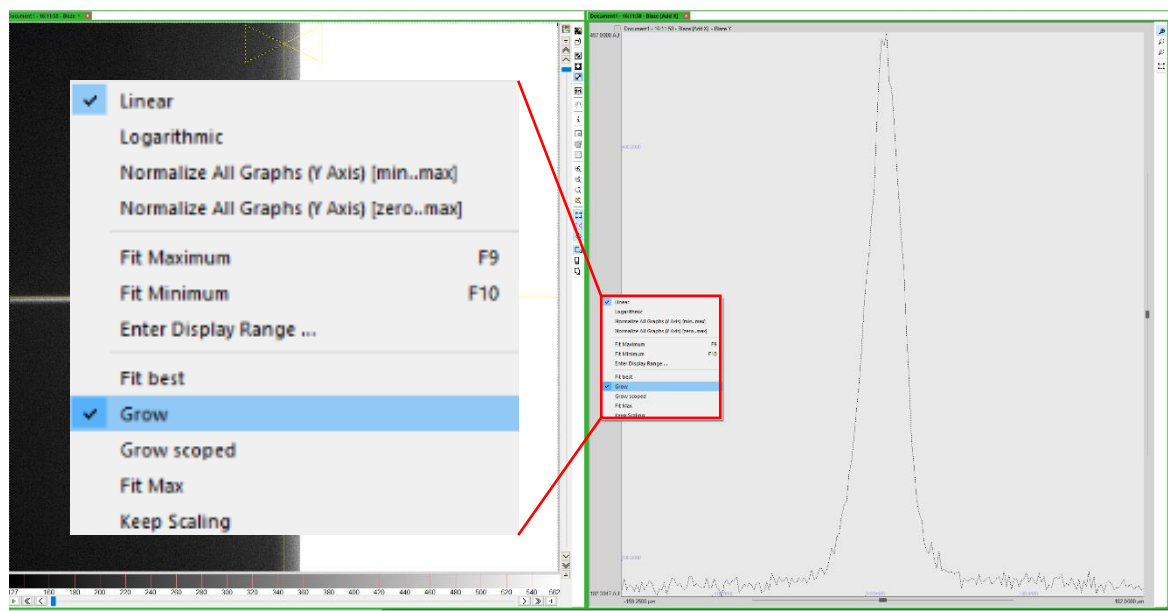
Tip: Draw a region of interest over the illuminated part and the beams and adjust the contrast (F9/F10). This way the membrane can easily be seen.



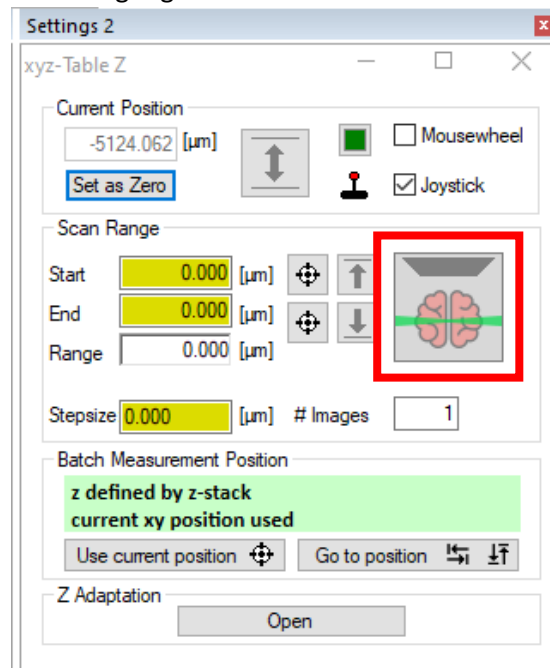
- Draw a narrow region of interest over the beam. Right-click on it and activate “Add X”. A graph with the intensity distribution will open.



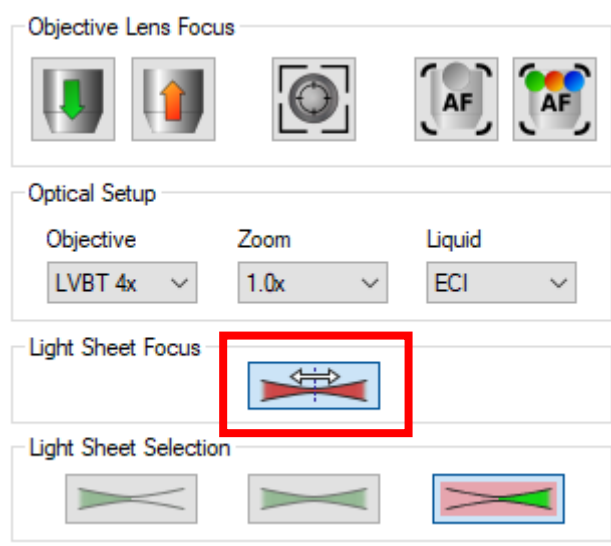
- Right click on the dark grey part of the y-axis and activate “Grow”. This setting makes it easier to visualize the growth of the graph.



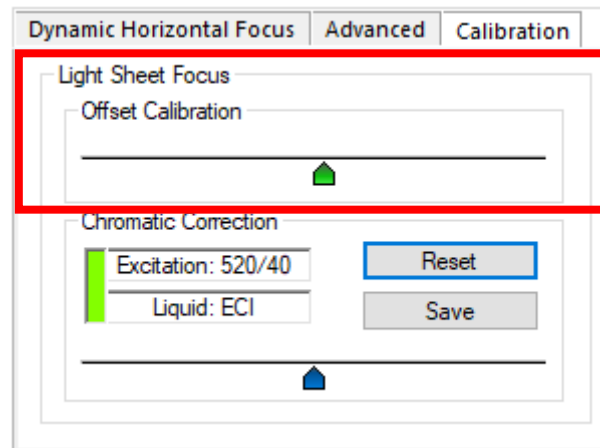
10. The goal is now to move the alignment tool's pinhole into a position, in which no part of the sheet is blocked by the membrane. This can be achieved by increasing the beam's intensity as much as possible. Click on the brain icon in the stage z dialog to move the stage with your mouse wheel. Move it until the peak will no longer grow taller.



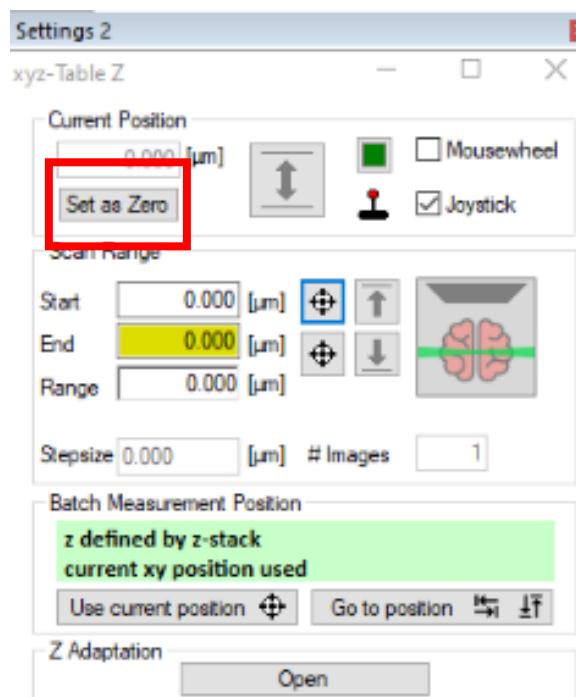
11. Activate the Rayleigh region of interest ("butterfly") with the icon under Light Sheet Focus and move it into the center of the field of view.



12. The horizontal focus has to be set up now. The focus is the region with the highest photon density, by moving it in the x direction, the intensity of the graph also changes. Move the green slider for the Offset Calibration (UltraMicroscope Blaze™) or Sheet Motor Calibration (UltraMicroscope II) until the peak of the graph is as tall as possible.

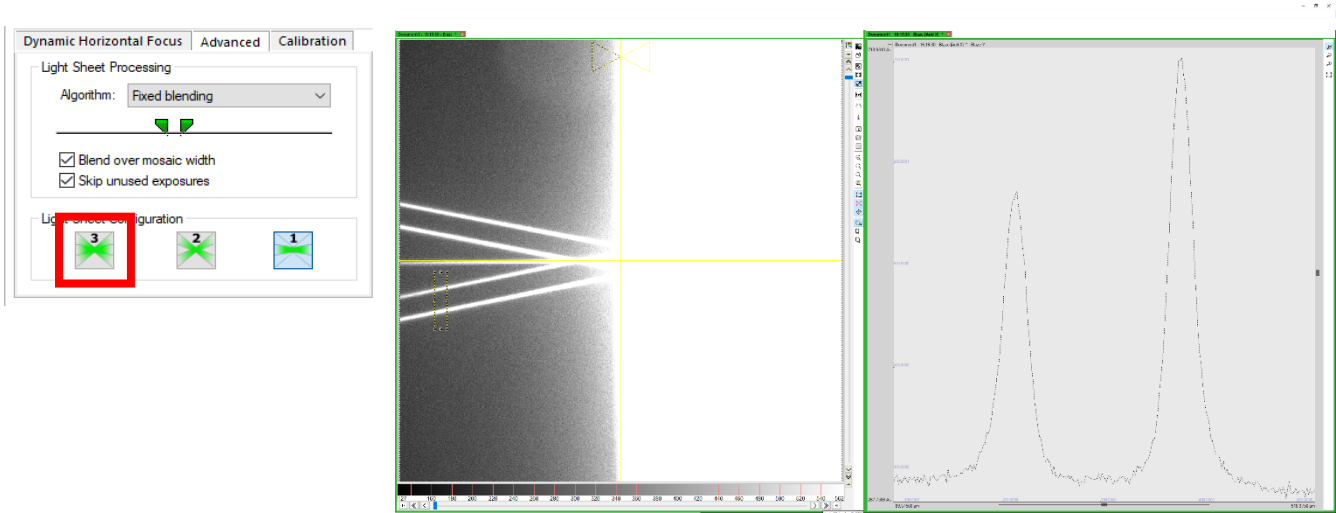


13. Iterate moving the stage in the z-direction and the position of the horizontal focus until there is no more growth in the graph.
14. Set the positions of the stage as zero. This is the reference position. Do not move the stage at this point anymore.

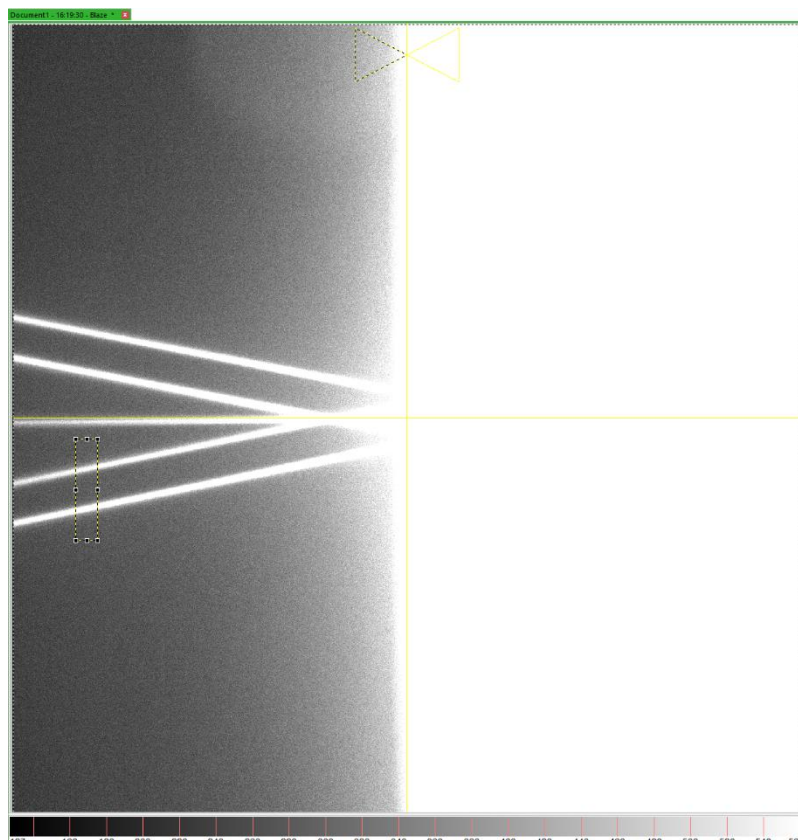




15. Activate all three sheets.

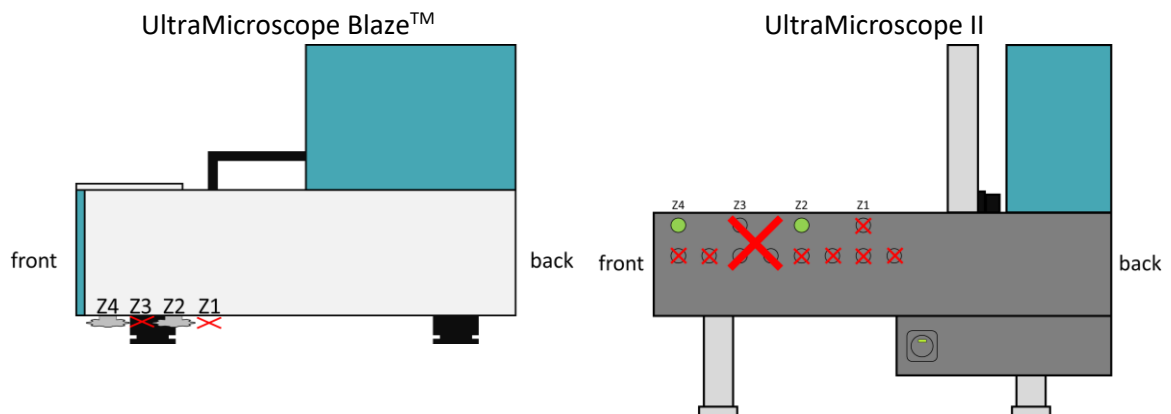
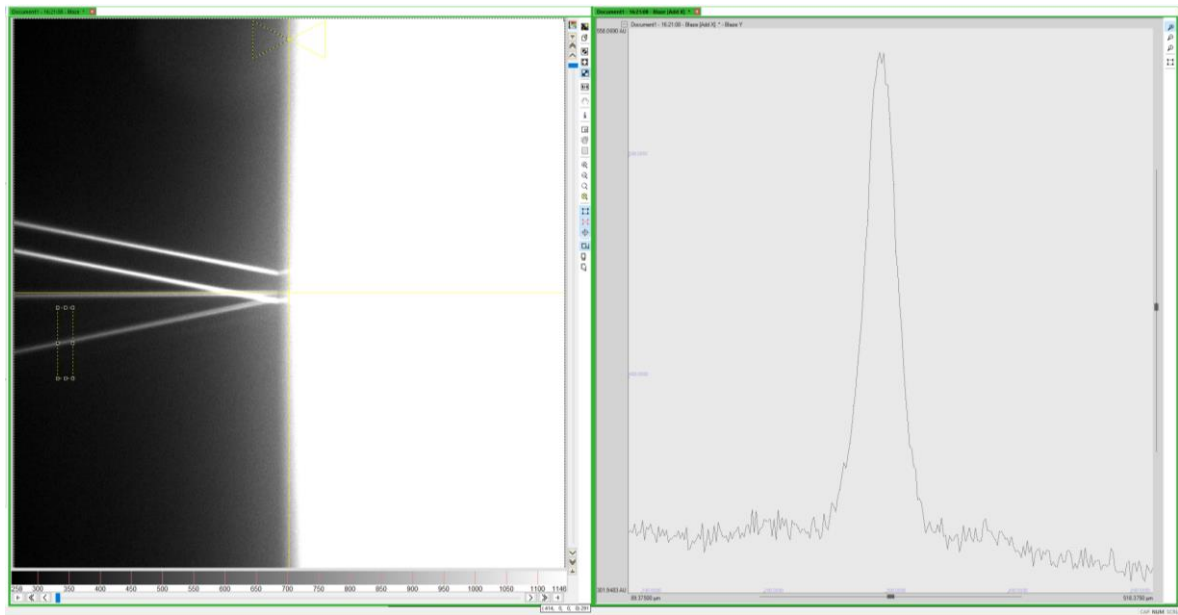


16. Move the region of interest on top of the beams on the bottom. Click on the graph and press F9 and F10 to scale it. Alternatively right-click on the y-axis and set it to "Fit best" and back to "Grow".

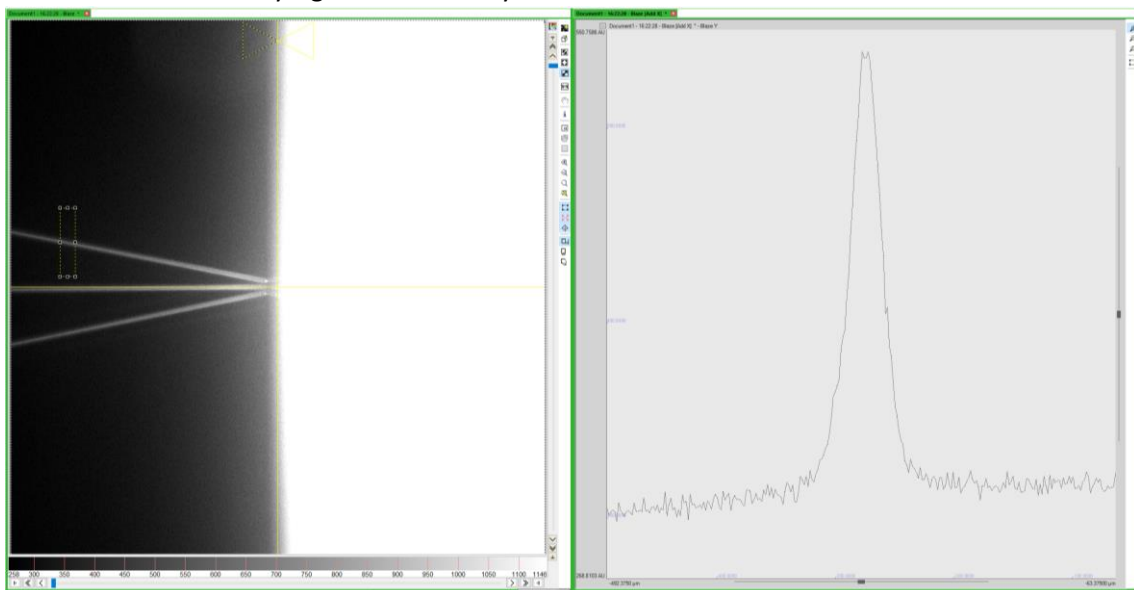


17. Move the Z2 screw until the beams merge into one and increase the intensity of the beam as much as possible.

Tip: On an UltraMicroscope II insert the short side of the Allen key into the screw. When using a longer lever, the position of the key is easier to control.

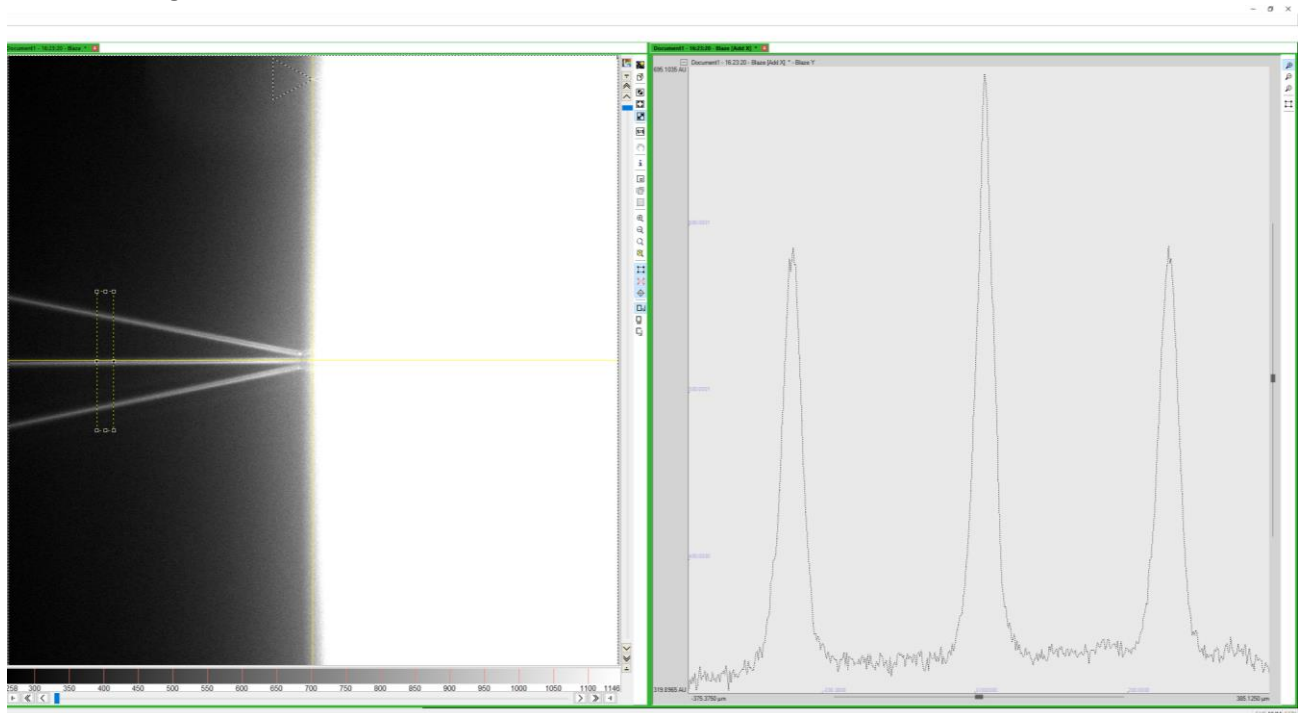


18. Move the region of interest on top of the beams on top. Click on the graph and press F9 and F10 to scale it. Alternatively right-click on the y-axis and set it to "Fit best" and back to "Grow".





19. Move the Z4 screw until the beams merge into one and increase the intensity of the beam as much as possible.
20. Draw a region of interest over all three beams. Check if all beams are in the optimum position by moving the stage 10  $\mu\text{m}$  downwards and upwards. The intensities of the beams must all decrease. If the intensity of one beam increases, move back to the reference position (0.000  $\mu\text{m}$ ) and optimize the intensity of that beam. Check all three beams by moving the stage upwards and downwards again.



21. Move back to the reference position.

22. Switch to the light sheets on the left side.

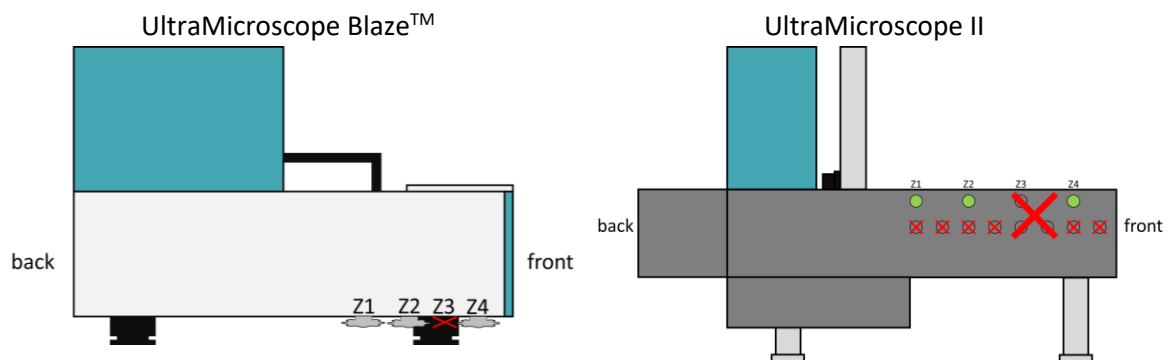
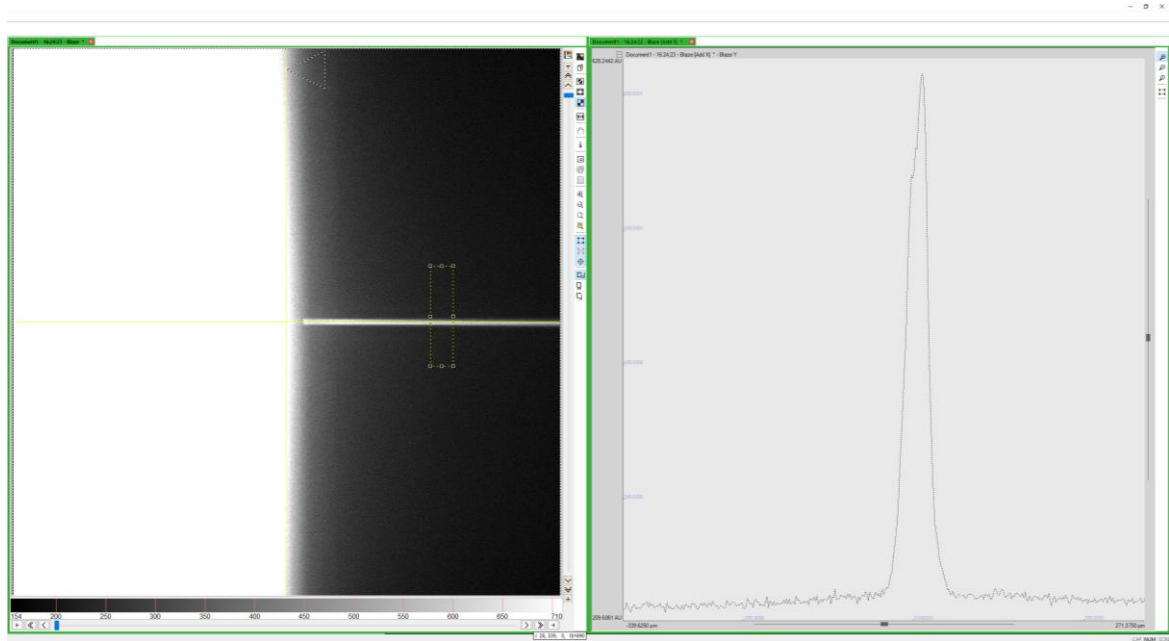
23.



24. Activate one sheet.

25. Position the region of interest on the central beam on the left side.

26. Move the Z1 screw on the left side to increase the intensity to the maximum.



27. Make sure the Rayleigh region of interest is centered.

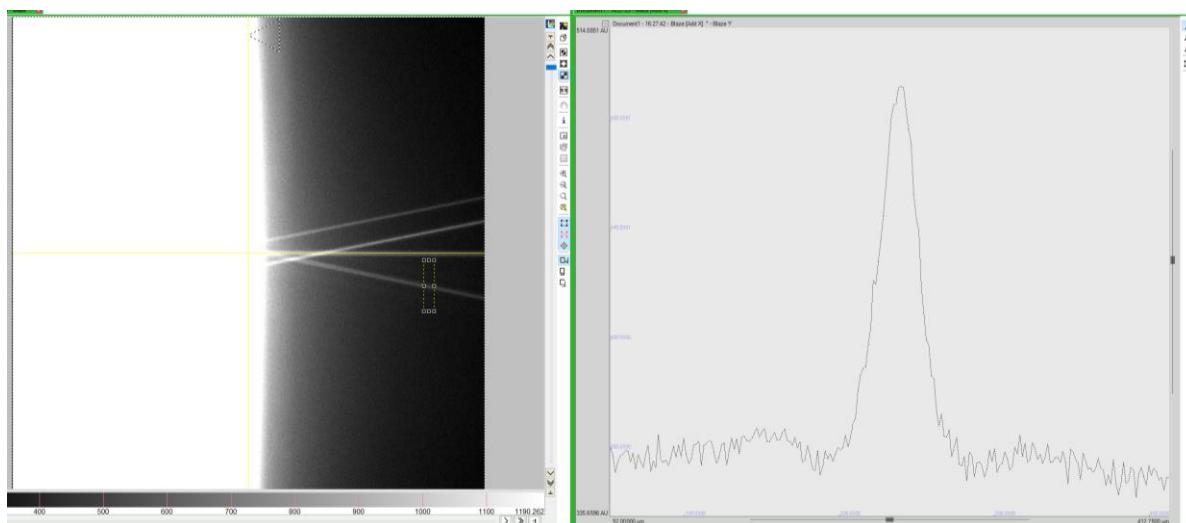
28. Move the Offset / Sheet Motor Calibration until the maximum intensity is reached.

29. Iterate moving the screw in the z-direction and the position of the horizontal focus until there is no more growth in the graph.

30. Activate three sheets.

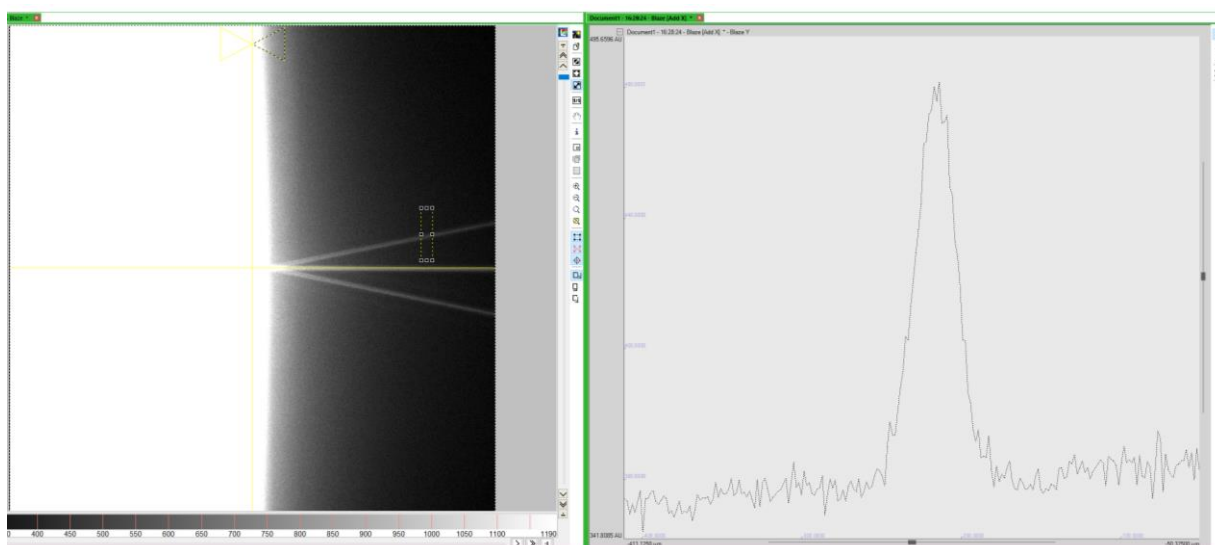
31. Move the region of interest on top of the beams on the bottom. Click on the graph and press F9 and F10 to scale it. Alternatively right-click on the y-axis and set it to "Fit best" and back to "Grow".

32. Move the Z2 screw until the beams merge into one and increase the intensity of the beam as much as possible.

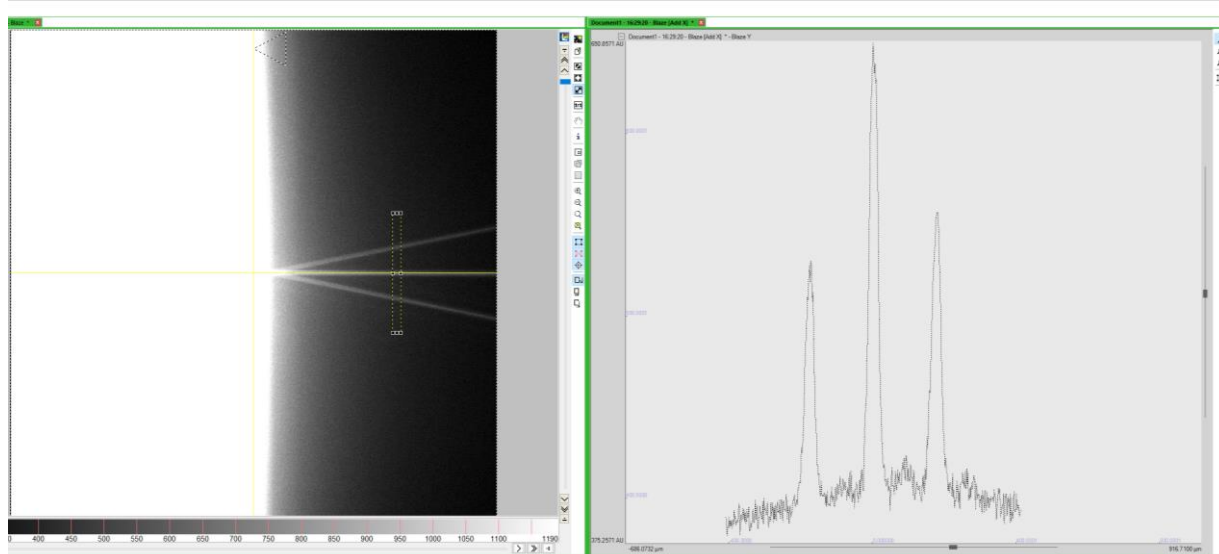


33. Move the region of interest on top of the beams on top. Click on the graph and press F9 and F10 to scale it. Alternatively right-click on the y-axis and set it to “Fit best” and back to “Grow”.

34. Move the Z4 screw until the beams merge into one and increase the intensity of the beam as much as possible.



35. Draw a region of interest over all three beams again and check if all of them decrease when moving the stage up and down.



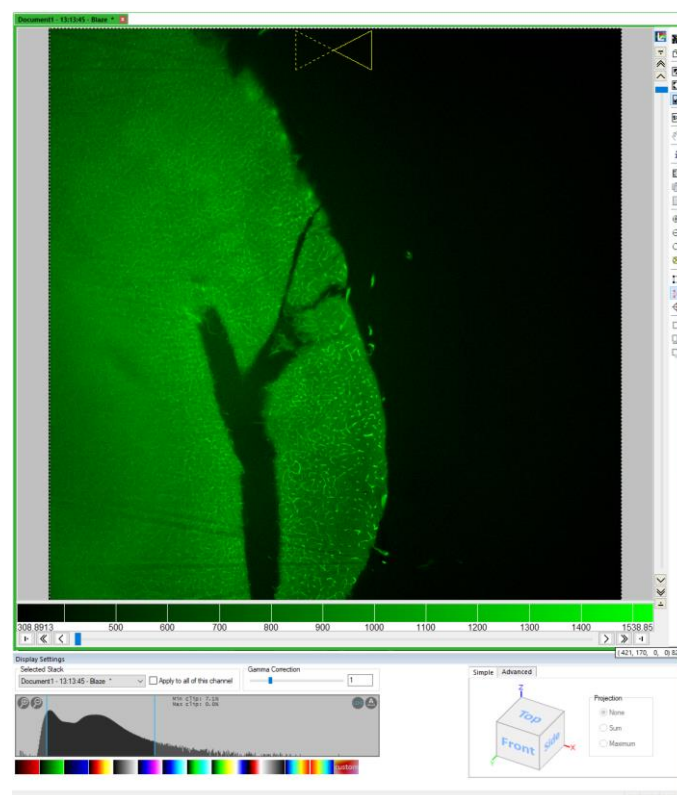
36. Press save settings to save the positions of the horizontal foci.



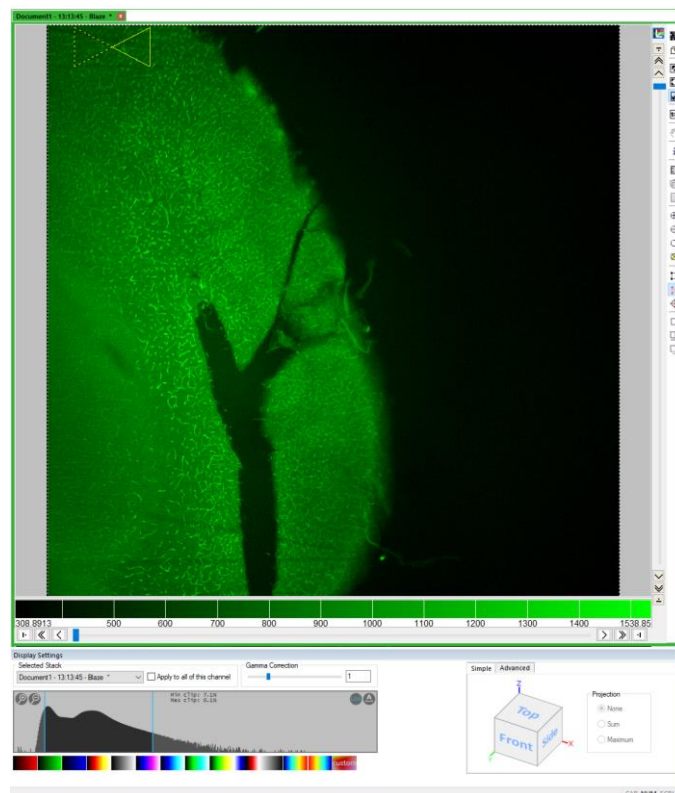
## Alignment with the sample

The alignment tool does not have the exact same optical properties as a biological sample. Hence the position of the horizontal focus always must be checked on the sample. This is also the case when changing objectives, since the fields of view differ slightly.

1. Perform the alignment with the alignment tool.
2. Mount a sample.
3. Pick the objective you want to use for the measurement.
4. Use the 561 nm laser, since this is the reference wavelength for alignment.
5. Focus on the sample.
6. Activate the right light sheet and move the sample to the right edge.
7. Place the Rayleigh region of interest on the edge of the sample and check if the focus sits underneath.

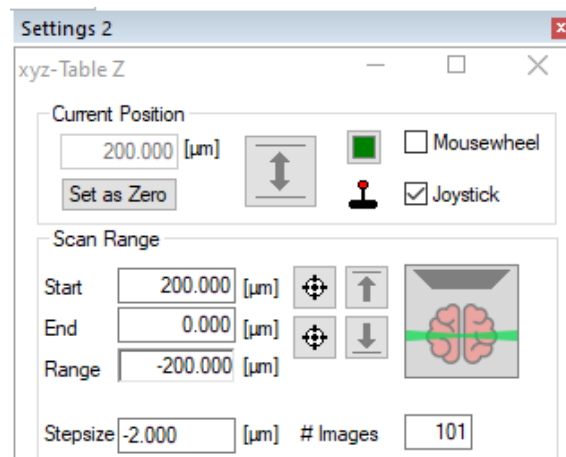


8. Move the Rayleigh region of interest along the sample and check if the focus is always underneath.



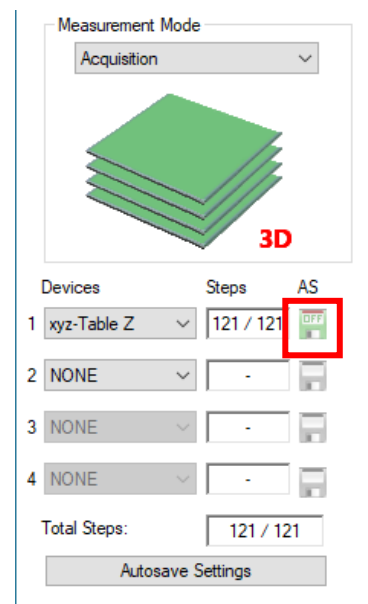
9. If necessary, move the offset/ sheet motor calibration until the focus sits below the Rayleigh region of interest.


10. Set up a z-stack of about 200 microns with to 2 microns step size.

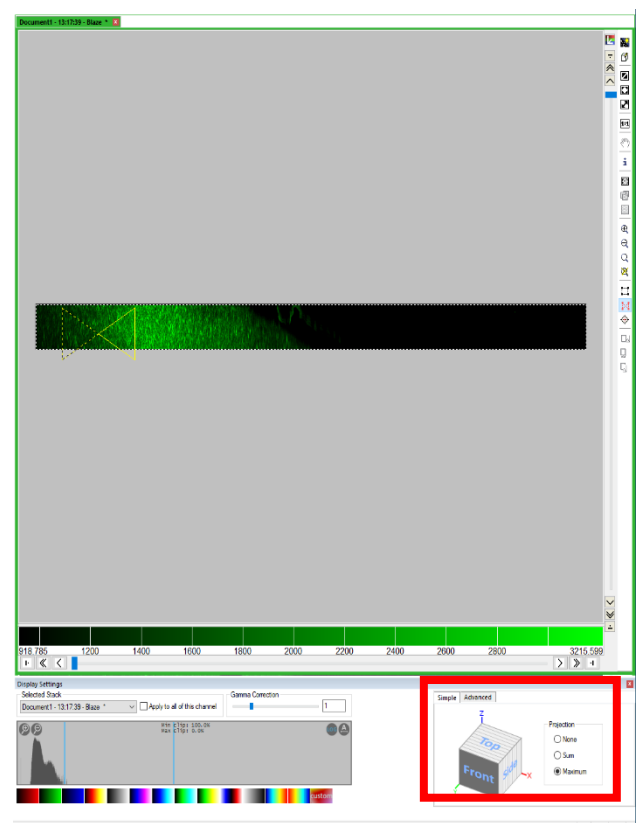
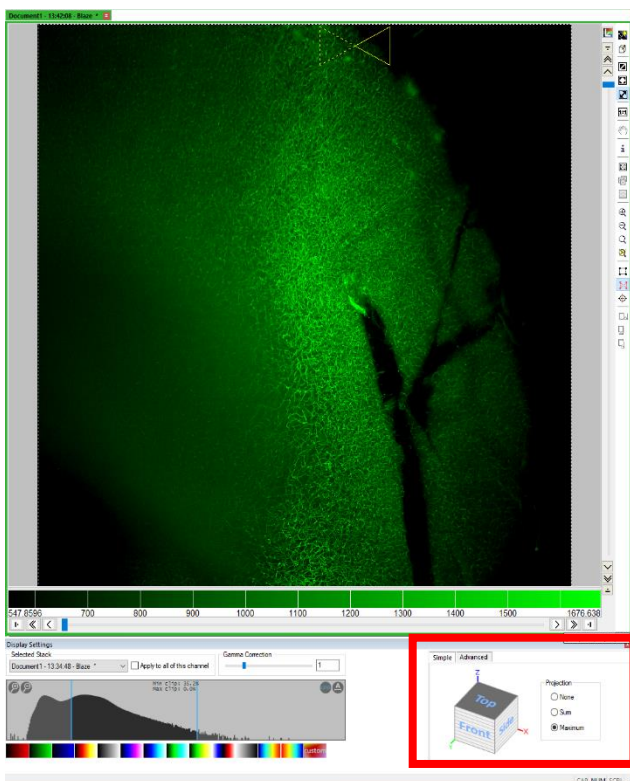




11. Activate the Acquisition Measurement Mode and deactivate the auto save.



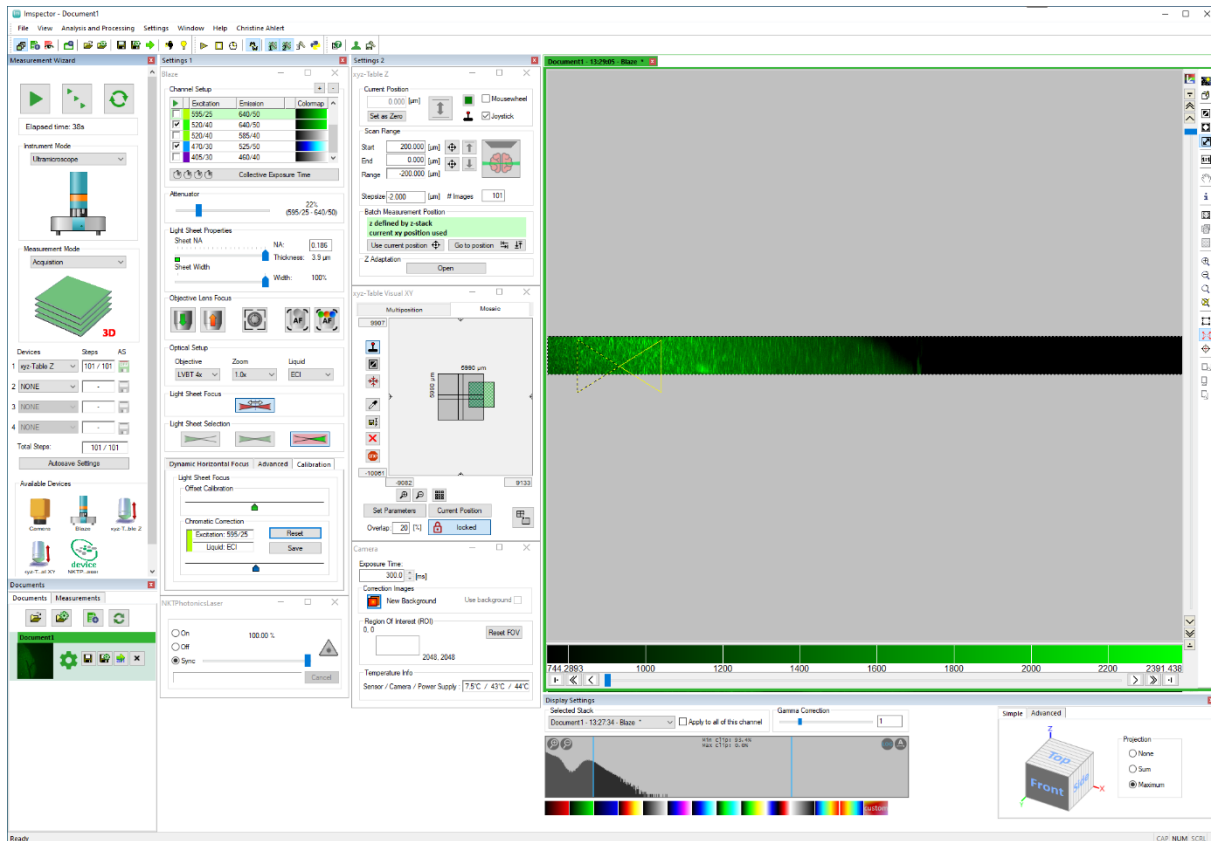
12. Open the display settings  and activate the Maximum Projection for the “Top” and “Front view”. This way you can see if the actual focus is sitting at the position of the butterfly.



13. Activate the live mode again.

14. Move the Sheetmotor / Offset Calibration until the horizontal focus matches the position of the butterfly.

15. Switch to the Front Maximum position again to check the result.

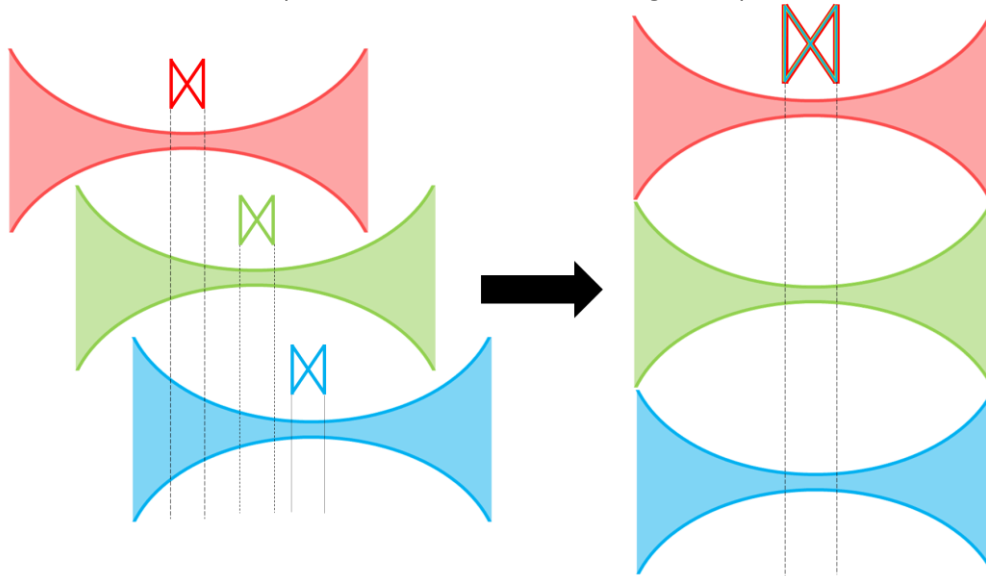


16. Repeat this for the left side.

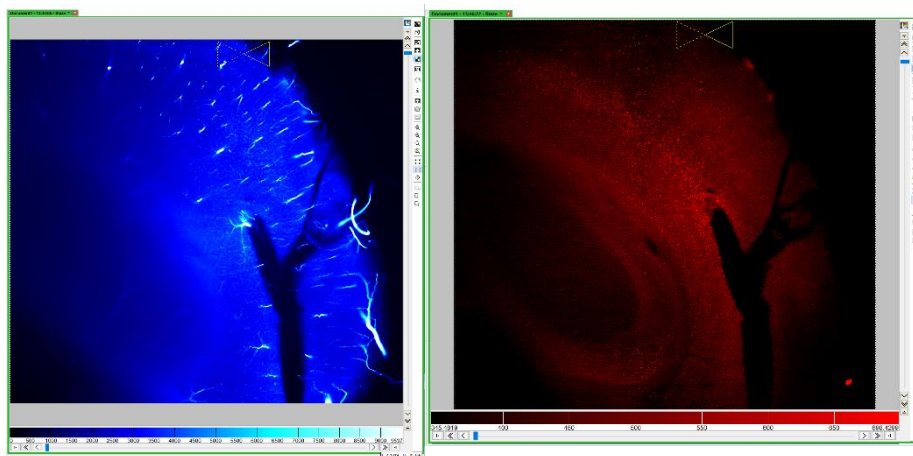
17. Save the settings.

## Chromatic correction for the excitation light path

When using different channels, the horizontal foci of each channel will be in a different position compared to the 561 nm reference channel, which is used for the standard alignment. These channel offsets in the excitation path have to be corrected using a sample.

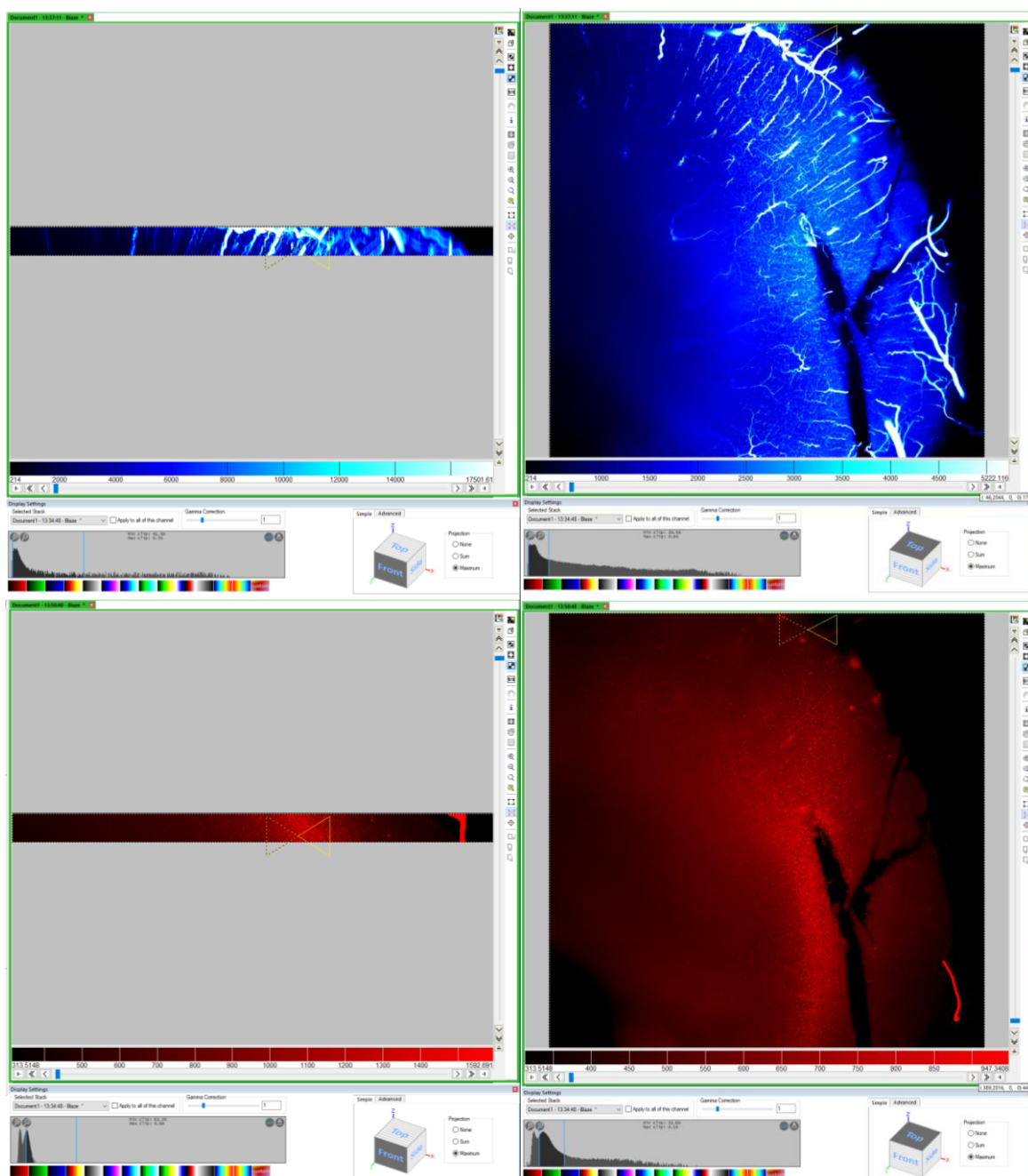


1. Perform the alignment with the sample.
2. Check again if the horizontal focus is calibrated correctly in the 561 nm channel.
3. For UltraMicroscope II: Open the hardware settings (Settings → Hardware → Configure devices → Ultra) and activate the box “show chromatic correction setup”.
4. Switch to the channel that must be corrected.



5. Move the slider from the chromatic correction until the horizontal focus sits below the Rayleigh region of interest.

6. Move the Rayleigh region of interest along the sample to check if the horizontal focus sits correctly.
7. Acquire a z-stack without autosave as in the alignment with a sample from before.
8. Open the display settings and do a maximum projection of the front and the top view.



9. Keep adjusting the slider of the chromatic correction until the focus sits below the butterfly.
10. Save the settings.