

Coarse Alignment

1. Remove both side panels, so that you are able to see the rear mirrors (see Figure 1 for side view of the illumination arm).
2. Turn on the laser on the reference side (left). Open the iris and remove the cylindrical lens.
3. Place a paper after the front mirror on the left side (Figure 2) and make sure you can see a laser spot on the paper.
4. Gradually close the iris until the laser spot is half cut.
5. Adjust the rear mirror (both knobs) until the spot reappears uncut.
6. Further close the iris and repeat steps 3-4 until you can see the laser spot when the iris is fully closed (do not close it with too much force!).
7. Open the iris entirely.
8. Repeat steps 1-6 on the non-reference side (right).

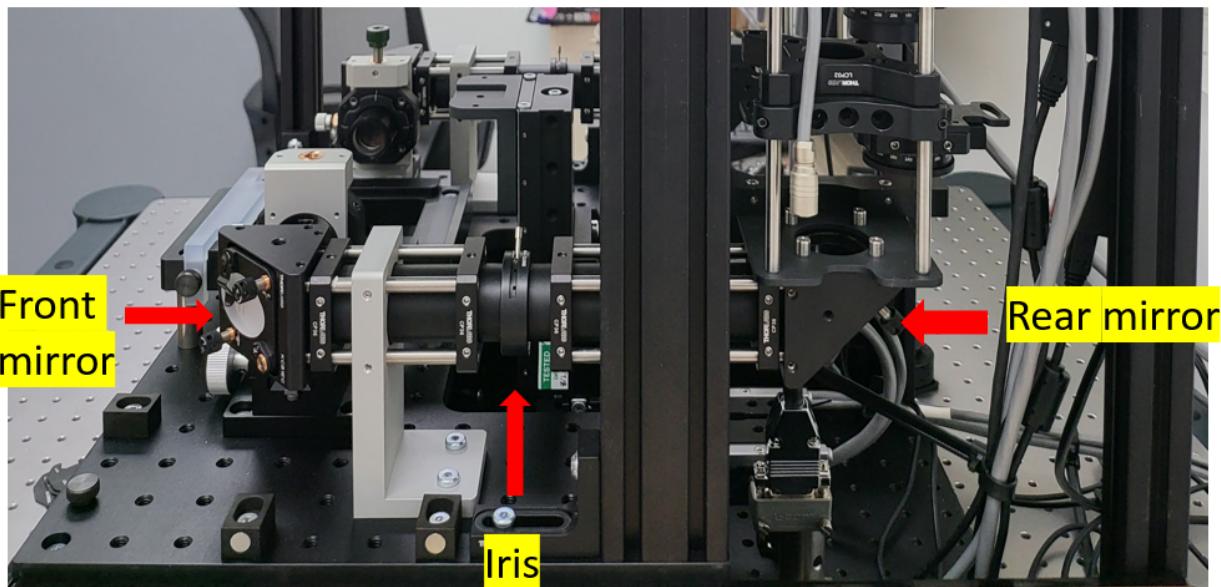


Figure 1. Side view of illumination arm.

Put a piece of paper here

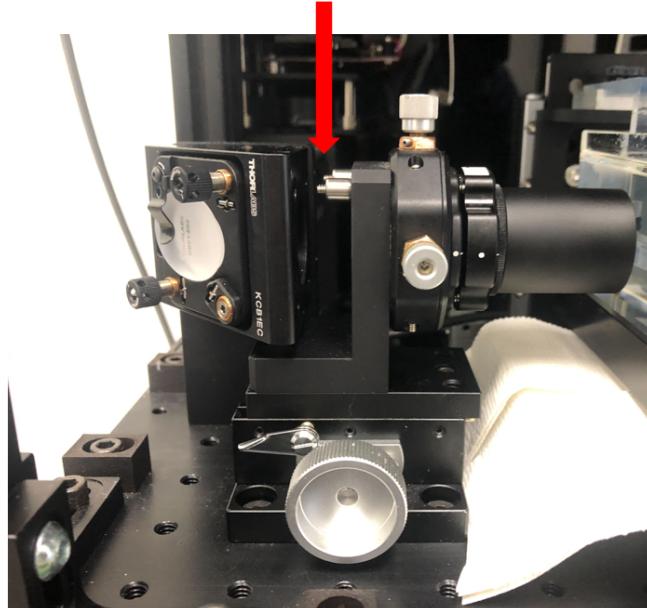


Figure 2. Locations of paper for coarse alignment.

Laser Line Alignment

9. Perform laser line alignment

- a. In the SmartSPIM software, click on “**Illumination Focus Assistant**.” The ***Illumination Focus Wizard*** window will pop up that shows a graph of the cross section of the beam.
- b. Click on “**Reference side, 561, No Filter**”.
 - i. Use the **Left illumination focus knob** and **Left illumination lateral offset knob** (Figure 3) and the **detection objective focus** to align and center the beam. In conjunction with the preview window, you can maximize the mean Kurtosis plot in the ***Illumination Focus Wizard*** to determine quality of focus. Figure 4 shows an example of an unaligned laser line, which is unfocused and its kurtosis is low. Figure 5 shows the aligned laser line. (**Typically a well focused laser line has Kurtosis values greater than 40 on average in EasyIndex with a 3.6x objective**).
 - ii. **If you do not see a laser line, check the following items**
 1. Look at the imaging chamber and see if the laser is illuminating. If not, check if the laser is turned on, the Oxxius remote control is switched on, and the shutter is on.

2. The cylindrical lenses are out of the illumination path.
3. There are no alignment targets in the illumination path
4. Detection objective is at the proper position and focused on the laser line. It is possible that the detection objective is out of focus and the signals are weak. Change the image dynamic range and increase the power
5. Illumination objective is properly focusing the laser line under the detection objective. You can visually inspect this by looking at the illumination in the imaging chamber.
6. Ensure that the Iris is not closed all the way. Open the iris if needed.

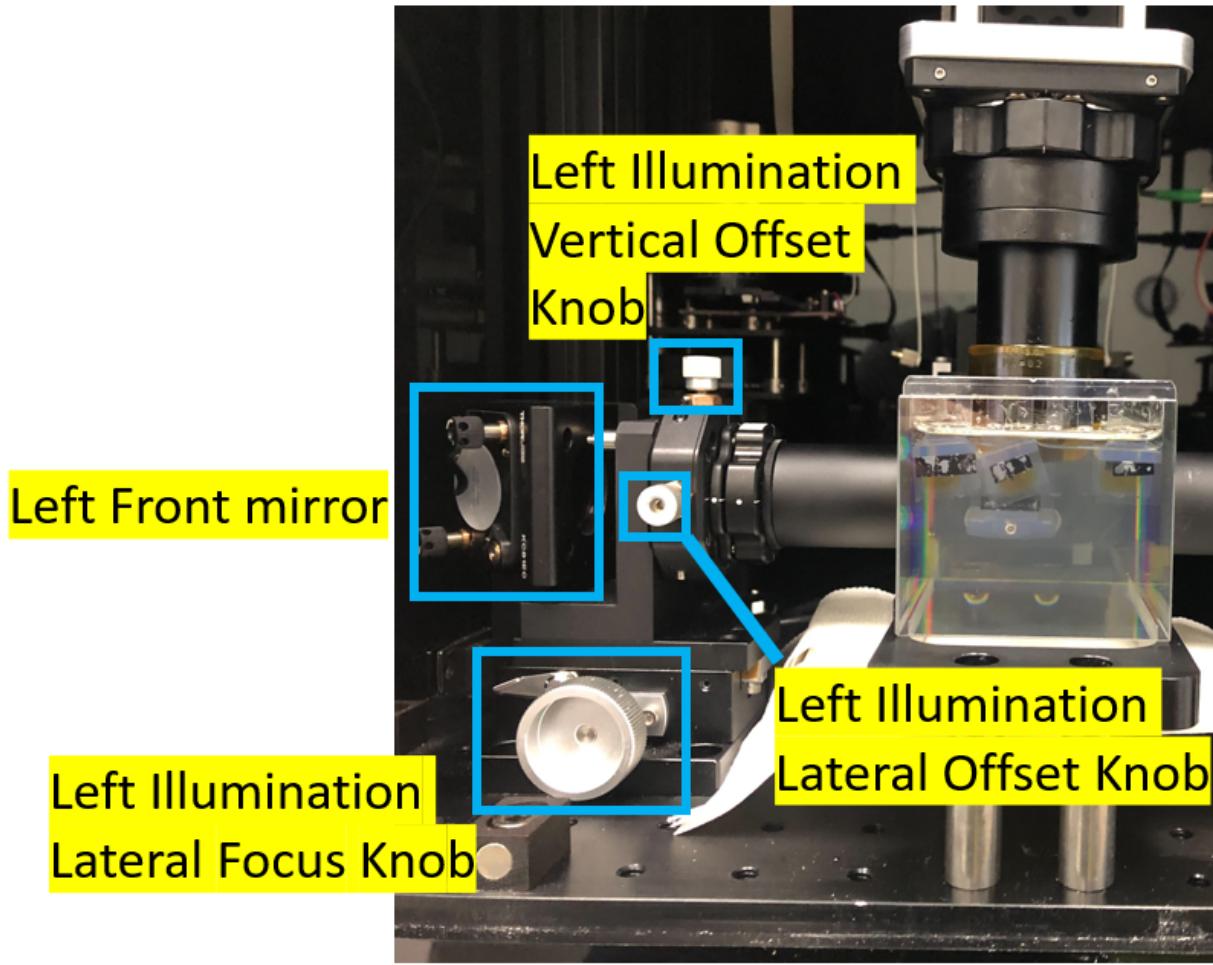


Figure 3. Locations of knobs on the left illumination used to align the laser.

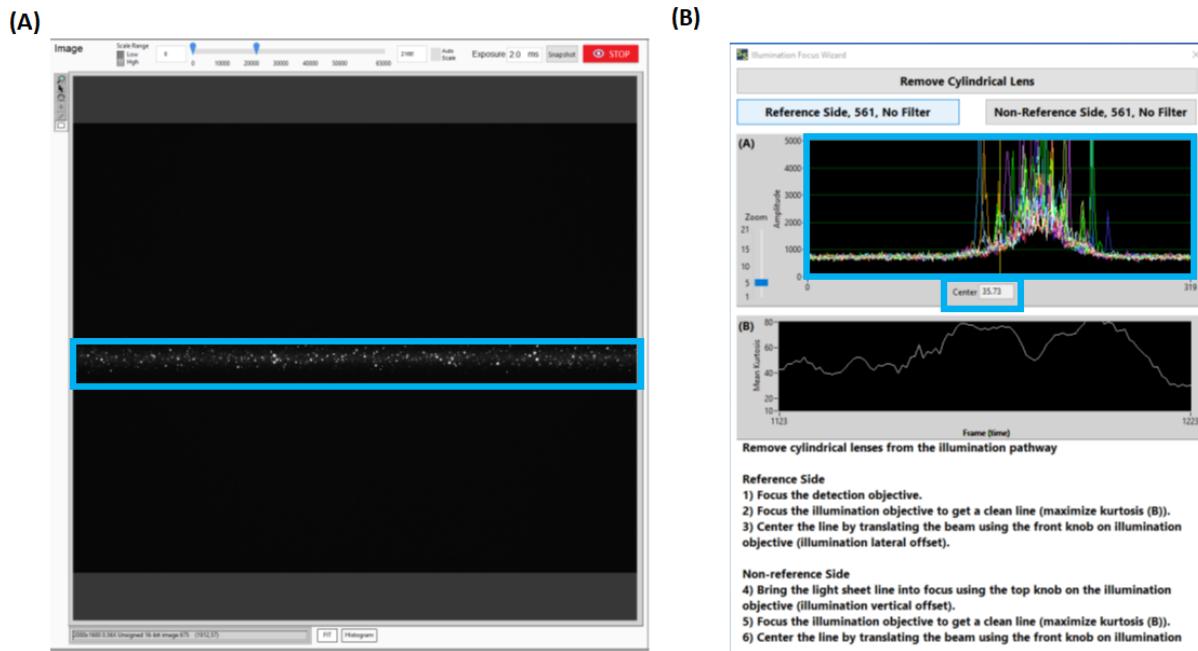


Figure 4. Unaligned laser line. (A) Note that the laser line (indicated in the blue box) is not in focus. (B) The Gaussian cross sections are wide, and the Gaussian peak is not centered, and the Mean Kurtosis plot is not optimized.

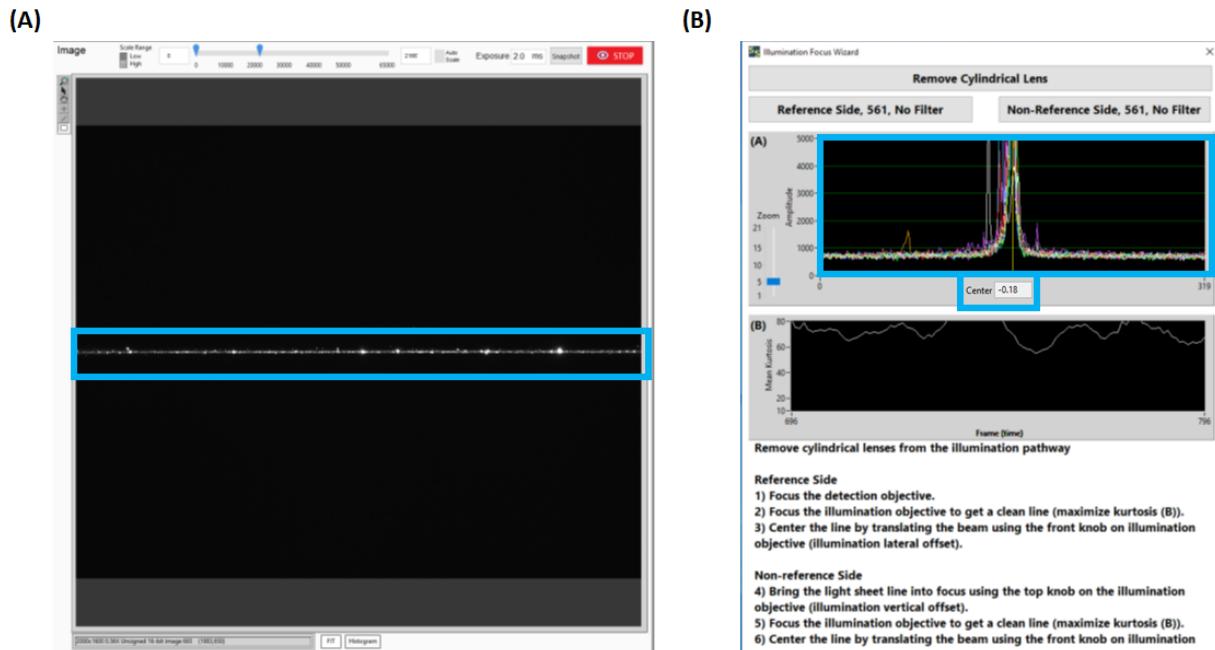


Figure 5. Aligned laser line. (A) The laser line (indicated in the blue box) is in focus. (B) The Gaussian cross sections are narrow, the Gaussian peak is close to centered at 0, and the Mean Kurtosis plot is maximized.

- iii. If the laser line does not appear horizontally flat, you will need to adjust the illumination arms (see Figure 4A for example of a horizontally flat laser line).
1. Loosen the six set screws on the sides (Figure 6). When loosened this will allow you to push or pull on the entire illumination arm.

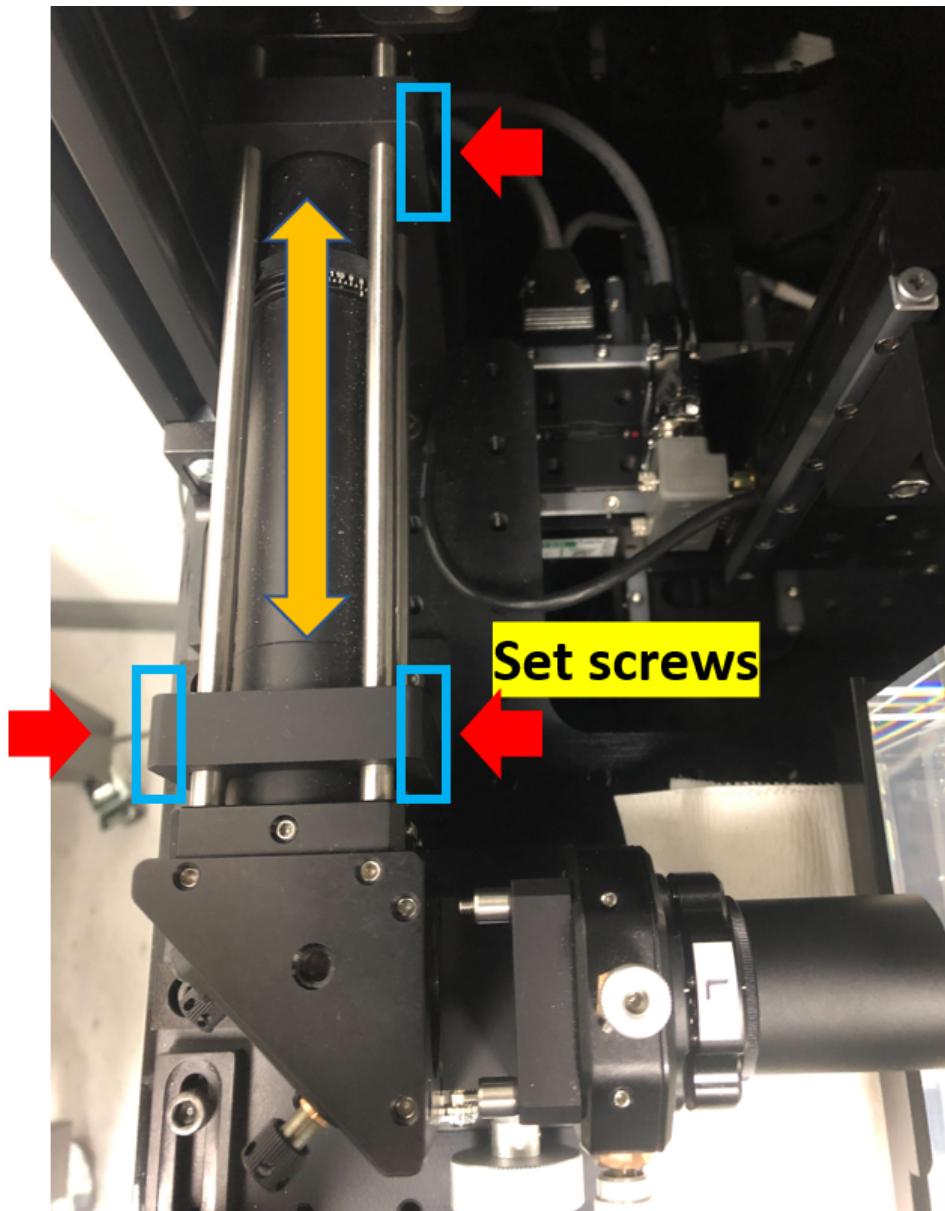


Figure 6. Location of set screws. Loosen these components to allow for movement of the illumination arm parallel to the base of the microscope frame (directions indicated by orange double arrow) by pushing or pulling the entire illumination arm.

2. Shift the entire illumination arm until the laser line looks horizontally flat and Gaussian cross sections of the beam align and overlap with each other in the ***Illumination Focus Wizard*** window.
 3. Turn **Left illumination lateral offset knob** to make the peak at center. You may need to move the illumination arm again to make the line flat.
 4. Tighten the front set screws and upper rear post when finished. Make sure the line still stays horizontal.
- iv. **If the Kurtosis values are not close to 40, check the following items:**
1. Check if there is any oil bubble under the detection objective.
 2. See if the Kurtosis can be further maximized by adjusting the detection objective wheel.
 3. Check if the Kurtosis can be maximized by adjusting the illumination objective focus knob.
 4. Move the detection arm to make the line horizontally flat.
 5. Decrease the laser power. The default power for this alignment should be 10~20%.
 6. Inspect the imaging chamber for any damage in the path of illumination. If there is, contact support immediately for a replacement.
- c. Click on “**Non-reference side, 561, No Filter**”. Repeat step b on the non-reference side (right).

Light Sheet Alignment

1. Illumination arm alignment using irises

- a. Insert the cylindrical lens. Select “open filter” and preview the scattered light using the reference illumination (left).
- b. Close the iris on the illuminated side and note any asymmetries in the image. If the iris slit is asymmetric, use the lower screw of the front mirror on the illumination arm (see Figure 7) and make the preview image symmetric.

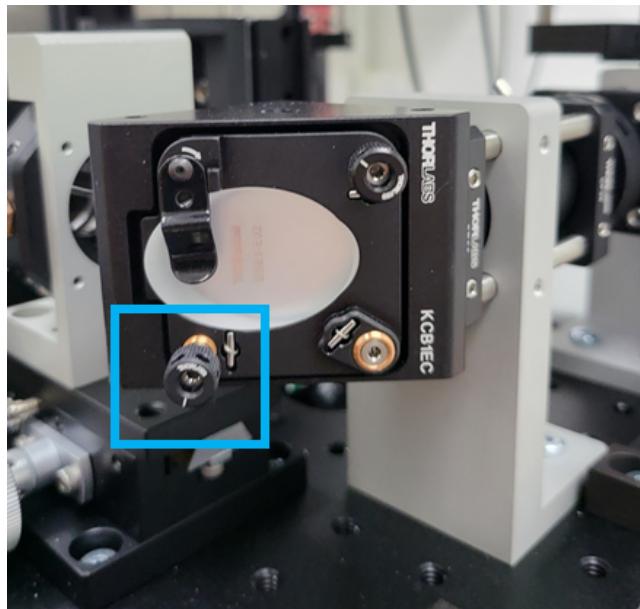


Figure 7. Adjustment knob on front mirror for centering the iris.

- c. Remove the cylindrical lens. Now the laser line observed may not be centered. Center the laser line using the bottom left knob of the rear mirror (Figure 8).



Figure 8. Adjustment knob on rear mirror for centering the laser line, as viewed from the rear.

- d. Repeat steps a-c until the iris and laser line are centered in the preview image.

- e. Repeat steps a-d on the non-reference illumination (right) arm.
- 2. Load in a sample**
- 3. Fine tune the roll of the lightsheet by rotating the cylindrical lens through with the micrometers on the cylindrical lens rotation stage. You will need to do this for each cylindrical lens and in both illumination arms.**
 - a. Start with the reference side.
 - b. Ensure that the cylindrical lens is inserted into the illumination path and the lower cylindrical lens is out of the illumination path.
 - c. With a small feature at the top of the sample, move it to the top of the preview display using the Joystick.
 - d. Get the target in focus using the detection objective and sample Z translation stage.
 - e. Move the target to the bottom of the window display while ensuring the target is visible.
 - f. If the target is not visible, rotate the cylindrical lens.
 - g. Move the feature to the top of the window display, refocus the feature if needed using the sample Z translation stage.
 - h. Repeat steps c-f until the feature has the same plane of focus on both top and bottom sides.
 - i. Repeat steps a-g for the non-reference illumination. Ensure that the feature is visible with the reference illumination when aligning the non-reference illumination.
- 4. Fix light sheet tilt starting with the reference side (left illumination objective + detection objective).** If the light sheet is flat, the feature should have the same plane of focus (within 1 to 3 micrometers) on both left and right sides of the FOV.
 - a. Using the reference illumination, find a small feature (~2-5 micrometers in z, you can scan the feature by Z sample stage to determine this) at the top of the sample ($z=100-2000 \mu\text{m}$).
 - b. Move the feature to the left side of the display window by using the joystick.
 - c. Get the feature in focus by adjusting the Z sample translation stage and detection objective.
 - d. Move the feature to the right side of the display window. Ensure that you have visibility of the feature as you move it. If your feature has disappeared, adjust left illumination objective vertical offset to make the feature visible (see blue square in Figure 9).
 - e. Move the feature to the left side of the display window. If your feature has disappeared, make the feature visible and in focus by adjusting the Z sample stage and detection objective.

- f. Move the feature to the right side of the display window. If the feature is not in focus, adjust the left illumination objective vertical offset to make the feature visible.
- g. Repeat steps e-f for several iterations until the feature is on the same plane of focus on the left and right sides.

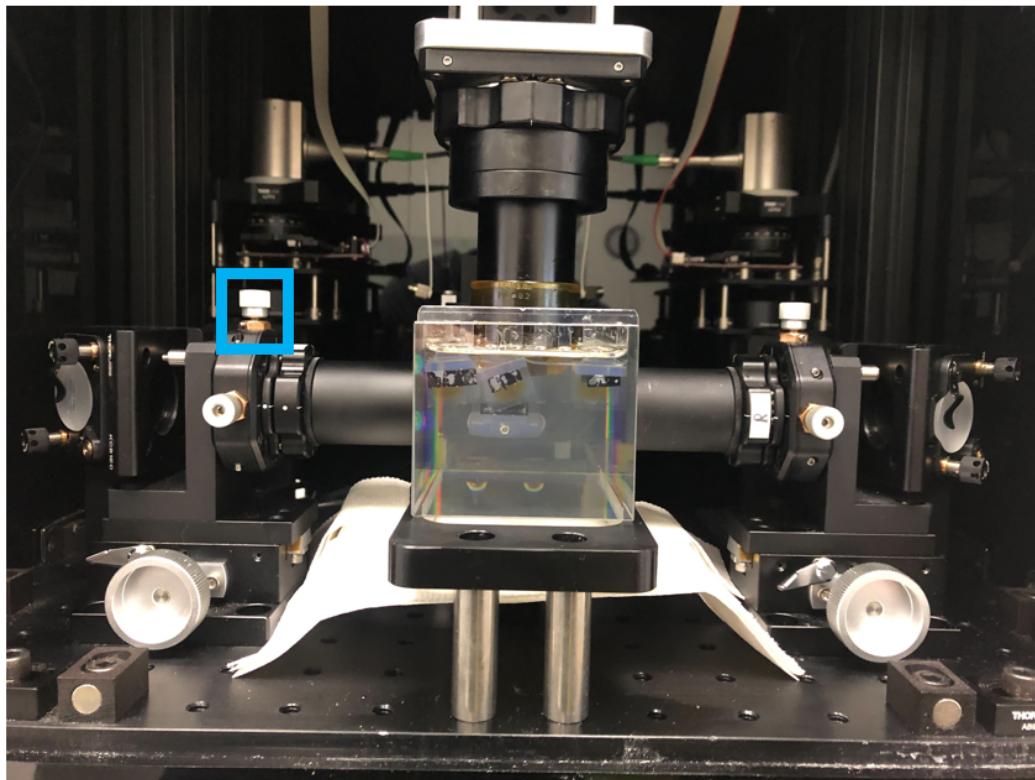


Figure 9. Use the Z-sample translation stage, detection objective, and reference illumination objective vertical offset (location in blue box) to align the lightsheet.

5. **Fix light sheet tilt on the non-reference side (non-reference mirror + right illumination objective)**
 - a. With the same feature as in step 40, move the feature to the right side of the display window. If your feature has been photobleached, find a new feature in the same sample. Confirm light sheet flatness by illuminating with the reference side.
 - b. Get the feature well focused on the reference illumination with the detection objective.
 - c. Switch to the non-reference illumination. If the feature is not visible, adjust the top knob of the non-reference front mirror until the feature is visible (indicated in Figure 10).
 - d. Move the feature towards the left side while ensuring that you have visibility of the feature. When the feature disappears, use the right illumination vertical offset knob to get the feature visible and in focus (indicated in Figure 10).

- e. Move the feature towards the right side. If the feature has disappeared, adjust the non-reference front mirror top knob until the feature appears.
- f. Repeat steps d-e for several iterations until the feature has the same plane of focus on the left and right sides. You can switch to the reference illumination to ensure that the feature is on the same plane of focus.

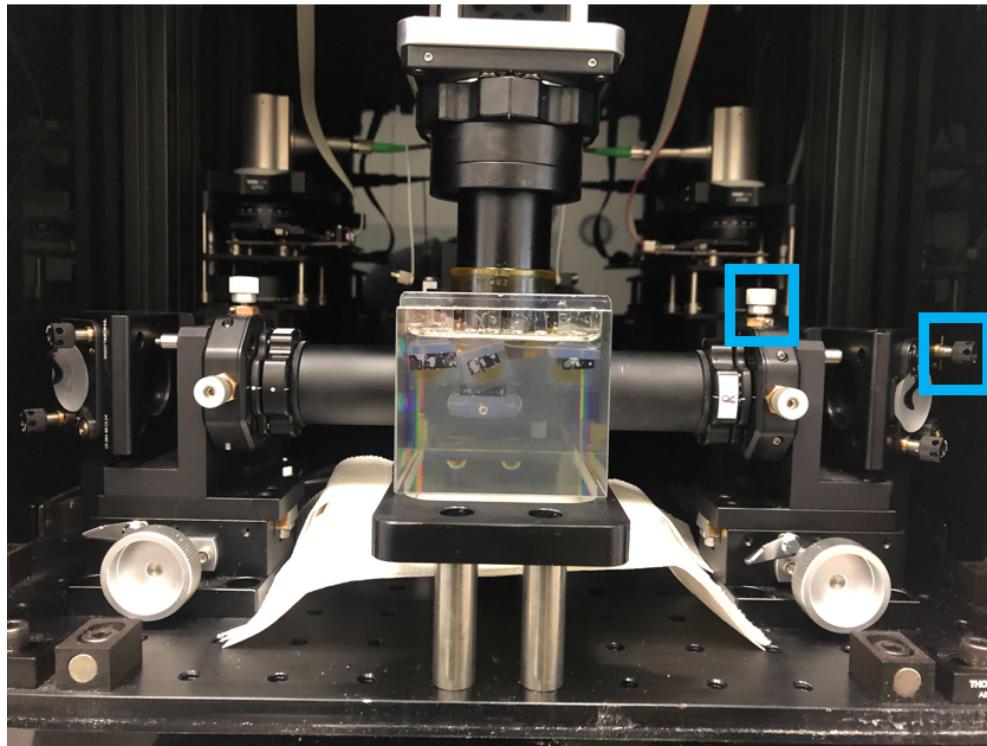


Figure 10. Use the front mirror top knob and illumination objective vertical offset knob to align the light sheet on the non-reference side.