

KEYENCE VKX LASER-SCANNING CONFOCAL MICROSCOPE

Standard Operating Procedures (updated October 2021)

1 Introduction

You must be trained to operate the Laser-scanning confocal microscope (LSCM) independently. These instructions are intended to supplement that training. **If you encounter an unusual circumstance and are not certain how to proceed, you must stop and contact the instrument manager.**

The LSCM functions by scanning a highly convergent laser beam over the surface of the sample. The reflected light is refocused to a pinhole in front of a detector which measures the intensity of laser light passing through the pinhole. When the confocal volume (the intersection of the laser illumination volume with the acceptance of the pinhole detector) of the laser coincides with the surface, the measured intensity is high. If the confocal volume does not intersect the surface, the measured intensity is lower. The position of the objective lens relative to the surface is scanned, and the system uses the variation in measured intensity as a function of lens height — for each pixel in the image — to identify the z-position of the surface.

1.1 Important cautions

Important points to keep in mind:

- **DON'T CRASH THE OBJECTIVE LENSES.** Set the microscope to use the 10x objective when changing samples, also when leaving the instrument unattended. Also, lower the sample stage using the manual knob, rotating the knob towards you.
- Don't set the scan range so that it might crash into your sample.
- You will damage the motorized sample stage by bumping it into something heavy.
- If you need to change the objective lens, you **MUST** update the lens positions in the software or ask the instrument manager to change it for you.
 - To update the lens position: VK Viewer -> Tools -> Mount Lens

One other point to keep in mind when planning measurements:

- The instrument is capable of at best 130 nm resolution in the lateral direction, and 5 nm resolution in the vertical direction- see the objective resolution table in **Section 3** for more details.
- The LSCM can measure the position of a subsurface interface through a transparent film. However, if the surface normal of the top material is not nearly parallel to the optical axis, such as the sidewalls of a drop of water, the transmitted laser beam will be deflected and the reflection from the lower interface may end up outside of the acceptance volume of the confocal detector. The LSCM is not a good instrument for measuring features in drops of water, for example. Also, the separation between the top surface and interface need to be separated

by more than the depth of focus of the lens (at least a micron) for the detector to resolve the two reflections.

PDF copies of the software manuals are accessible from within the acquisition and analysis software. The analysis software is available for distribution to internal users of this instrument.

2 Biosafety Considerations

Any samples designated BSL2 must be approved in advance by facility staff. Any activities in CCMR shared facilities must also be specified on the user's own MUA and be approved by the IBC in advance as per University policy. Follow CCMR's General BSL2 User Procedures. Observe the gloves-off signs: do not contaminate the microscope, computer, or any other equipment or CCMR property by touching it with contaminated gloves.

3 Objective Working Distance and Resolution Table

Objective	NA	WD (mm)	XY Resolution (nm)	Resolvable step height (vertical resolution)	Standard mode pixel size (nm/pixel)	Superfine mode pixel size (nm/pixel)	Membrane or film thickness
10x	0.30	16.5	411	Not defined	1318	659	> 100 μm
20x	0.46	3.1	268	Not defined	659	330	50-100 μm
50x Long WD	0.55	8.7	130	Not defined	-	-	Do not use
50x Std	0.80	0.5	130	5 nm (0.5 nm)	264	132	1-10 μm
150x	0.95	0.2	130	5 nm (0.5 nm)	91	45.5	Do not use

4 Basic Topographic Measurement Procedure

1. Log onto the instrument in FOM.
2. Turn on the main hardware controller by pressing the red button on the controller tower (located on lower level of computer cart).
3. Make sure the sample plate is near the bottom of its range
4. If necessary, rotate the objective turret to put the 10x objective in the active (front) position.
5. From the computer desktop, open VK Viewer. When prompted, click "Yes" to return the stage to the XY origin (i.e. center position). The software opens in Observe mode with a live view. (You can switch between "Observe" and "Measure" modes using the two tabs in the upper left corner of the window.)
 - a. In Observe mode, a view of the white light image will be presented.
 - b. In Measure mode, the laser reflection will be presented.
6. Place the sample on the stage. For thin samples, you might need to add a few microscope slides under the sample to get it into the appropriate height range.
7. Use the manual focus wheels to get the sample surface in focus.
8. Focus on your sample surface:

- a. The sample position can be moved by click-dragging the live image. Alternatively, you can double click anywhere in the image and the sample will be centered where you clicked. Try to center on a high feature of the sample.
9. You can now set up a measurement of the area shown on the screen. Measuring larger areas will be discussed in **Section 5**.
10. Laterally position the stage in order to locate the step/feature to be measured by clicking and dragging the live image. If this magnification is sufficient for your measurement needs proceed to the next step, otherwise:
 - a. Rotate the lens turret to activate the 20x objective, and re-focus as needed using the fine adjustment wheel. If this magnification is sufficient for your measurement needs proceed to the next step, otherwise:
 - b. Rotate the lens turret to activate the 50x objective, and re-focus as needed using the fine adjustment wheel. If this magnification is sufficient for your measurement needs proceed to the next step, otherwise:
 - c. Click on the Measure tab at the top of the software to enter Measure mode. **From this point on, focus should be adjusted using the arrows on the right side of the software.** The arrows refer to the direction the focus is being moved, which is the opposite of the stage motion. The single arrow moves the smallest increment, and the double and triple arrows move in larger increments.
 - d. Click the up arrow buttons until the focus is clearly above the highest point on the sample, then rotate the objective turret to activate the 150x objective.
 - e. Click the down arrow buttons until the sample surface is in focus. **Be careful to avoid contact between the lens and the sample.** The working distance for the 150x objective is 200 μm , so there should always be a clearly visible gap.
11. The upper and lower bounds for a laser scan can be manually determined by adjusting the focus while viewing the laser image.
 - a. Select the laser button
 - b. Click the up arrows up first to find where the image becomes completely black, and then click "Set upper pos."
 - c. Click the down arrow buttons to move the focus through the sample plane until the focus is clearly below the lowest point on the sample, then click "Set lower pos.". The vertical range to be used for the measurement is now defined.
12. Click the "Auto gain" button to determine the illumination intensity to be used for the measurement.
 - a. Move through the focal planes in your sample, if there are still red areas present, that means the illumination intensity is saturated, and you need to lower the brightness until you don't see red anymore.
13. Select double scan
14. There are four area modes: Line, 1:12 aspect ratio, Standard, and Superfine 2x2.
 - a. Z pitch is the vertical step size. Keep Real Peak Detection (RPD) enabled. The physical vertical step size is about 80 nm, and the hardware does peak fitting of the intensity vs height for each pixel to identify the position of the surface at that pixel to better than the step size. If RPD is disabled, it will simply report the height at which the highest intensity was observed.

- b. **Quality:** can be set to high accuracy, high-speed, and ultra-high speed. Refers to the laser raster speed. High accuracy always goes left to right. High speed alternates left to right, right to left. Ultra-high speed is left to right skip line right to left skip line.
15. Click “**Start measurement**” and allow the measurement to proceed.
16. When the measurement is finished, click “Save data” (disk icon in top row of icons) and save the data in .vk4 format.
17. Click the “Pause” button to return to the live camera view.
18. If desired, click the up arrow buttons to get the sample back in focus, then click and drag the live image to move to a new area, and repeat 13-17 to capture a new measurement.
19. When finished, click the up arrow buttons until the focus is clearly above the highest point on the sample, then rotate the objective turret to the 10x objective and remove the sample.
20. When you are finished acquiring data:
 - a. Close the VK Viewer software. This will automatically turn the hardware controller off.
 - b. Turn off the sample translation stage controller.
 - c. Move the 10x objective into place.
 - d. Move the sample stage to the end of its range by rotating the knob clockwise.
 - e. Leave the area at least as clean as you found it.
 - f. Transfer your data from the computer using Cornell secure file share or box.
 - g. Log out of FOM.

5 Large Area Topographic Measurement Procedure

1. If you need to measure a larger area Open the XY stage Nav display and press capture image.
 - a. Continue pressing capture in a larger area until your entire area has been captured.
2. Specify the assembly area (the region of interest) in the navigation window.
3. Select manual...
4. Click the “Start Measurement” button.
5. It will prompt you to save your data.
6. After the measurement is complete, if stitching was required to assemble an image of the region of interest, the stitching program will launch. Click the “adjust all” button for the software to automatically perform pattern matching and make small adjustments to how the individual images are registered to one another to form the composite image. Save the resulting image in a folder corresponding to your NetID or GuestID on the D:\ Data drive.

6 Transparent film thickness measurements

1. There are two additional measurement modes:
 - a. **Transparent top:** reports the position of the first reflection, even if it is weaker than the reflection from an interface.
 - b. **Transparent film:** reports the position of both reflections. The position of the two interfaces needs to be separated by at least 1 μm to resolve the two reflections (think of it like the position of each interface convolved with the depth of focus of the lens.)
2. To measure a transparent film:

- a. Follow the instructions in section 4 for setting up your measurement (steps 1 through 12).
- b. Measurements settings -> transparent film.
- c. Preview the film and make adjustments to the thickness or filter settings if needed.

7 Thin film thickness measurements analysis

1. On the computer desktop, open MultiFileAnalyzer.
2. Click "Add Data" and open the measurement file to be analyzed. The image is displayed in a table in optical and topographic forms. A roughness line may display by default.
3. Process data:
 - a. Click BCL DCL to select the bright and dark cut levels. You're just looking to cut any strong saturated or dark pixels, and replace them either with interpolated data or mask them so they are not considered for analysis
 - b. Cut based on height data. This will address pixels that appear noisy based on strong variations in height compared to neighboring pixels. Generally, use "strong" for 10x or 20x objectives, "medium" for 50x, and "weak" for 150x objectives.
 - c. Can opt to smooth the data.
 - d. Can opt to apply reference plane or tilt corrections, applying to entire field of view or specified areas.
4. If necessary (based on the appearance of the topographic image), click "Perform filtering as needed" in the workflow diagram on the right side of the software in order to remove tilt. A selective plane fit can be performed by clicking "Reference plan settings" and selecting an appropriate area (i.e. fit to a single plane only as opposed to full image with height variations).
5. Click the "Profile" icon at the top of the software. The default method for defining the line path of a profile is two points, so simply click the start and end locations for the profile within the image.
6. The profile appears at the bottom of the screen. Click "Line fit" to get the window to fit to the appropriate range.
7. If desired, use the "Smoothing" function to reduce noise and short-range waviness in the data.
8. Various options are available for measuring the profile in the area to the right of the profile window. Line-Line should work well if there are two parallel surfaces; click the two ends of a line on one surface, then the two ends of a line on the other. The distance between the two (extrapolated) lines is displayed; this will be more accurate if the drawn line segments are parallel, or as close as possible.
 - a. If desired, profile results/analysis can be saved and accessed later.

8 Other analysis procedures

There are two analysis packages. The single file analysis program is the only program that can do FFT analysis, **transparent thickness measurements**, or export data to other file formats. Otherwise, most users will probably want to use the multi-file analysis program, which allows batch processing, comparison of multiple datasets, etc. This guide will explain just enough to view your data, see the software documentation (accessible from the software) for more information.

1. Select Process/filter image. (The image processing history lets you undo operations in reverse order)
 - a. Click missing data removal.

- b. Click BCL DCL to select the bright and dark cut levels. You're just looking to cut any strong saturated or dark pixels, and replace them either with interpolated data or mask them so they are not considered for analysis.
 - c. Cut based on height data. This will address pixels that appear noisy based on strong variations in height compared to neighboring pixels. Generally, use "strong" for 10x or 20x objectives, "medium" for 50x, and "weak" for 150x objectives.
 - d. Can opt to smooth the data.
 - e. Can opt to apply reference plane or tilt corrections, applying to entire field of view or specified areas.
2. Image List lets you compare multiple images side by side, zooming, panning, and rotating all images in lock step.
3. Analyze data to look at profiles, measure roughness, etc.
4. Batch analysis can be used to apply work performed in one file to additional files.
5. To export data into an excel file:
 - a. File -> Export -> Save as excel.