KEYENCE VKX LASER-SCANNING CONFOCAL MICROSCOPE

Standard Operating Procedures (updated Oct 2017)

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.** If you put the system at risk for the sake of expediency, your access may be temporarily or permanently revoked.

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:

- You must always guard against the possibility of crashing the lens into your sample. This applies to both manual and programmed motions. The sample stage is adjusted manually, rotating the knob towards you is safe, lowering the sample away from the lens.
- 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.
- During measurements, the instrument will vary the distance of the lens to the sample. You must take care not to program the instrument to scan over too large a range and crash into your sample.
- Higher magnification lenses have shallower depth of focus, and therefore stronger variation in reflected intensity as a function of working distance, and therefore the best height resolution. However, such lenses also have extremely small working distances (the 50x and 150x lenses have working distances of 0.35 mm and 0.2 mm, respectively). Some samples may have profiles that are simply not compatible with such short working distance lenses.
- A common failure is damage to the motorized sample stage due to bumping it with something heavy.
- It is your responsibility to protect the integrity of the instrument. Failure to do so may result in loss of privileges to operate independently.

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.
- 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 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 Measurement Procedures

1. Make sure the sample plate is near the bottom of its range
2. Rotate the objective turret to use the 10x objective. Do not use a higher magnification lens until instructed.
3. Turn on the motorized stage controller (black box to left of computer monitor)
4. Turn on the microscope controller (tower on left below keyboard. Power button is green when on.) Give the controller 10 seconds to boot before proceeding.
5. Open the VKX Application Launcher (blue icon on Windows desktop)
6. Click the Viewer button in the VKX Application Launcher to open the data acquisition software
   6.1. You will be prompted to center the sample stage. Click Ok/Yes
7. The acquisition software should open in "Observe" mode. (You can switch between "Observe" and "Measure" modes using the two tabs in the upper left corner of the window.) A view of the white light image will be presented, but very far out of focus
   7.1. The intensity of the white light can be modulated using the push rod on the upper left side of the microscope
8. Install your sample on the stage. Slowly rotate the sample height adjustment up (away from you) to bring the surface into focus, keeping an eye on the color image in the software and on the proximity of the sample to the objective lenses.

9. The sample position can be moved by left-click dragging in the 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
10. Make fine adjustments to bring this high feature into focus. This can be done either by manually adjusting the sample height, or adjusting the height of the objectives in the software:
    - 10.1. Use the up/down arrows in the GUI.
    - 10.2. Use the mouse scroll wheel.
        - 10.2.1. A single bump while turning the scroll wheel is identical to pressing the single up/down arrow button (1x focus increment)
        - 10.2.2. Holding the Control key while scrolling is identical to pressing the triple up/down arrow button (3x focus increment)
        - 10.2.3. Holding the Shift and Control keys while scrolling results in a 6x focus increment
11. Click the autofocus button, which will use the laser to find the focus at the center of the image.
    - 11.1. Autofocus will attempt to avoid a collision with the object by first scanning the objective lens away from the object, over a range of 80% of the working distance of the objective lens. If the focus is not found, it will then scan towards the object over a range of 10% of the working distance of the objective lens. It is still possible for the lenses to collide with the sample during autofocus, if they are too close to begin with.
    - 11.2. If autofocus was unsuccessful, ensure that the laser shutter is open (knob on upper left side of microscope). This shutter does not ever need to be closed.
12. Once you have focused on the highest features in the sample, you may change to a higher magnification lens. The focus should still be close, but may require fine adjustment. Consider the working distance of the lens you are about to use! If your sample has a feature that extends up more than 0.2 mm from where you established the focus, the 150x lens will collide with the sample.
13. Performing a depth composition will produce a composite image, using software merge images from multiple heights to enhance the depth of field of the white light image beyond the intrinsic depth of focus of the objective lens.
14. In the navigation window, you can click the “capture image” button, followed by “extend capture area” to stitch together a larger field of view using the white light camera. Then you can specify the assembly area for performing laser measurements.
15. Switch to “Measure” mode
16. Starting with “Easy Mode”, the microscope with attempt to automatically determine the appropriate z-range, laser attenuation, and detector gain settings. This should be sufficient for most measurements.
17. “Expert Mode”
    - 17.1. Expert mode may be required for samples where:
        - 17.1.1. You are interested in measuring the profile of a buried interface, or the thickness of a film
        - 17.1.2. You have strong variations in reflected intensity and need to manually adjust the brightness or specify the measurement be a composite of two brightness settings
    - 17.2. The camera/laser buttons let you switch between what the live image is showing you. You can view the real-time laser reflected intensity at the current objective z position

17.2.1. Any time-dependent variations in the laser intensity can indicate either changes to the sample or instability in the instrument (the instrument is mounted on a granite block to reduce vibrations)

17.2.2. The upper and lower bounds for a laser scan can be manually determined by adjusting the focus while viewing the laser image. Scroll up first to find where the image becomes completely black, and set this as the upper location. Then do the same while scrolling down, but beware of the possibility of crashing the lens into your sample.

17.2.3. Red regions in the real-time laser image indicate areas of oversaturation. The brightness can be adjusted, and the double-scan option can be enabled for samples with very high contrast where you may want to measure once with low brightness and once with high brightness.

17.3. There are three measurement modes:

17.3.1. Surface profile: reports the position of the strongest reflection, which could either be from a surface or an interface

17.3.2. Transparent top: reports the position of the first reflection, even if it is weaker than the reflection from an interface

17.3.3. 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.)

17.3.4. There are four area modes: Line, 1:12 aspect ratio, Standard, and Superfine 2x2.

17.3.5. 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 disable, it will simply report the height at which the highest intensity was observed.

17.3.6. 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.

18. Specify the assembly area (the region of interest) in the navigation window.
19. Click the “start measurement” button
20. 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.
21. When you are finished acquiring data:
    21.1. Close the Viewer software. This will shut down the microscope controller.
    21.2. Turn off the sample translation stage controller.
    21.3. Move the sample stage to the lower end of its range by rotating the knob towards you.
    21.4. Move the 10x objective into place
    21.5. Leave the area at least as clean as you found it
    21.6. Log out of Coral

4 Analysis Procedures

There are two analysis packages. The single file analysis program is the only program that can do FFT analysis, 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.

1.1. Select Process/filter image. (The image processing history lets you undo operations in reverse order)
1.2. Click missing data removal
1.3. 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
1.4. 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.
1.5. Can opt to smooth the data.
1.6. Can opt to apply reference plane or tilt corrections, applying to entire field of view or specified areas.
1.7. Image List lets you compare multiple images side by side, zooming, panning, and rotating all images in lock step.
1.8. Analyze data to look at profiles, measure roughness, etc.
1.9. Batch analysis can be used to apply work performed in one file to additional files.