

Raman Spectroscopy with the WITec Alpha 3000 Microscope



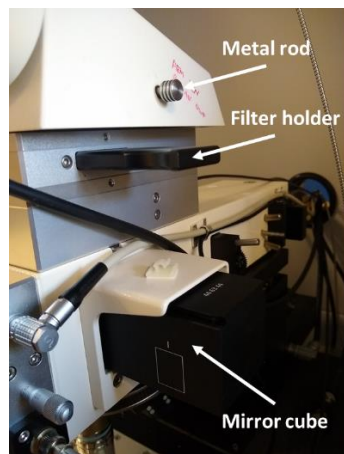
Appropriate Samples and Sample Prep

You can attempt to analyze anything that will fit beneath the objective. Data quality will be determined by the number and intensity of Raman active vibrational modes in the materials making up the sample. If your sample is very thin (microns or less), you may observe Raman signals from your substrate convoluted with those of your analyte. It may therefore be necessary to select a substrate with a Raman spectrum that does not interfere with that of your analyte. Penetration depth in 3D images is limited by the optical properties of your sample. If your sample does not transmit the 633nm laser light well, you can expect a dramatic fall-off in signal intensity as you attempt to penetrate the surface further.

Getting Started

1. Turn on the power strip to the left of the cabinet.
2. Open the cabinet using the foot pedal
3. Rotate the 10x objective into place.
4. Place your sample on the stage.

5. Log into the PC.
 - a. Username: labuser
 - b. Password: Sample123!
6. On the desktop, double click to start *WITec Control 1.60*
7. On the menu bar click *Configurations* → *Raman* → *Raman*
8. In the *Control* window, expand the settings for *Spectrograph 1* and change the *Laser Wavelength* to 633nm, *Grating* to 300g/mm, and *Spectral Center* to 2700nm. Expand the settings for *Spec Camera 1* and type -60C in for temperature (this may be necessary even if -60C is already shown as the setting). In the *Message* window verify that the *DV401: Cooling* message shows a setpoint of -60C and the temperature is decreasing.
9. To see an image of the sample on the monitor, you need to adjust the metal rod, mirror cube, and filter holder from the right side of the microscope (see pic below).
 - a. The metal rod has two positions: in and out. To view the sample on the monitor it must be pushed in.
 - b. The mirror cube has three positions labeled I, II, and III. While inserting the cube from the right side of the microscope, the first position you land on is I followed by II then III. To view the sample on the monitor, the cube must be in position I, which puts a dichroic mirror in place to illuminate the sample with white light.
 - c. The filter holder has two positions, 1 and 2. When inserting the filter holder from the right side of the microscope, you will first land on position 1 which does not contain a filter. This is the position you want to be in while viewing your sample on the monitor. If you push the filter holder in further it will move to position two, which contains the laser line filter. If this filter is in place the image on the monitor will be very dark and have a blue/purple color.

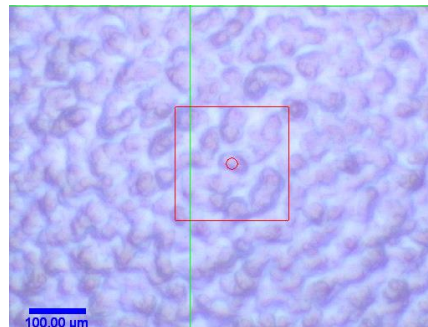
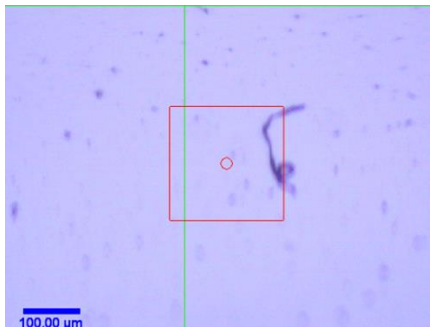


Setting up for Raman Acquisition

1. Use the remote control to raise the microscope objective
 - a. Repeatedly press one of the arrows on the remote control until *RC: Microscope Z* shows below the menu bar



- b. Rotate the knob on the remote control all the way counterclockwise, then rotate it clockwise to the desired speed. Speed setting can be viewed in the *Graphic Control* window.
 - c. Press and hold +Z button on the remote control to raise the microscope objective
2. In the *Control* window, expand the settings for *Illumination*. Enter a value for *Illumination* (0 to 100) and turn the illumination on. You should see a white spot on the surface of your sample. If you do not see a spot even at high settings, the mirror cube probably needs to be moved to position I
 3. Use the remote control with a speed around 100 to lower the microscope objective. You will need to reduce the illumination intensity as you lower the objective. As you come down the first thing you will see is the worm. Next you will see the clouds. Then your sample surface will come into focus. Use a lower speed and adjust the illumination to get a good focus on your sample.



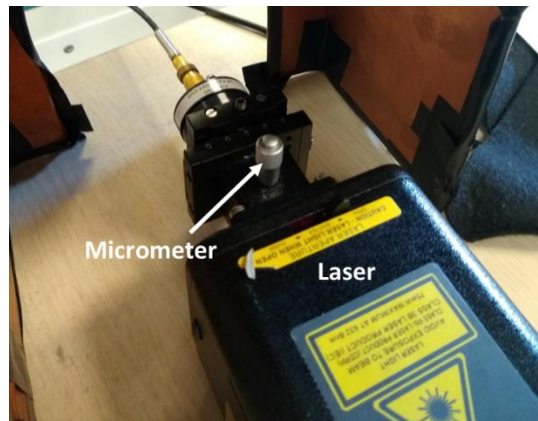
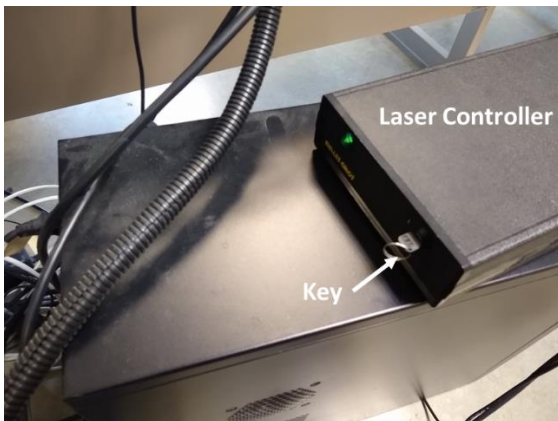
4. Use the micrometers on the sample stage to find the area of the sample that you want to image.
5. Rotate the 50X objective into position.
6. Use a slower speed to focus on your sample. You will need to increase the illumination.

7. From the right side of the microscope, push the filter holder in to position 2. You should see the image on the monitor darken indicating that that laser line filter is in the optical path. Also, push the mirror cube in to position 2 and pull the metal rod out.



Warning: Make sure the laser line filter is in the optical path before you turn on the laser. If the filter is not in place, the laser light may damage the spectral camera!

8. Turn the laser on using the key on the controller. Turn the micrometer on the laser head counterclockwise until you see the laser beam emerge from the microscope objective.



9. In the *Control* window, expand the settings for the *Oscilloscope* and click *Start Oscilloscope*. You should observe a Raman spectrum of your sample in the *Hardware Spectrum* window.
 - a. Scroll the mouse wheel in the *Hardware Spectrum* window to fit your peaks in the window.
 - b. In the *Control* window under *Spectrograph 1*, you can adjust the settings to determine the wavelength range shown in the Raman spectrum. The *Grating* setting determines the width of the range while the *Spectral Center* determines the center wavelength. Below are settings for each grating that allow you to take a spectrum with the Rayleigh peak (shift = zero) on the left edge of the spectrum.

Grating(g/mm)	Spectral Center(cm^{-1})	Wavelength Range(cm^{-1})
300	2700	4700
600	1400	2600
1800	350	700

10. Use the remote control to fine tune the height of the microscope objective to maximize the strength of the Raman signal. You should need to go a few microns up as shown in the *Status* window.

11. In the *Control* window under *Oscilloscope*, increase the *Integration Time* to check for additional Raman peaks. Use the minimum integration time that provides acceptable S/N for your Raman peaks of interest.
12. In the *Control* window under *Oscilloscope* click *Stop*.

Acquiring Single Raman Spectra

1. In the *Control* window expand the settings under *Single Spectrum*. Enter your desired *Integration Time*, *Accumulations*, *Sample Name* and *Number* then click *Acc. Single Spectrum*. Your spectrum will be shown in a new window.

Acquiring Raman Images

1. In the *Control* window, expand the settings under *Image Scan*. Under *Scan Details*, for a 2D image in a single XY plane, set *Scan Mode* to *Single*. For a 3D image, set the *Scan Mode* to *Stack*.
2. Enter the desired *Points per Line* and *Lines per Image* (these control the XY spatial resolution) and *Layers per Scan* (these are depth or Z-axis layers for Stack scans).
3. Under *Geometry*, enter your desired *Height*, *Width*, and *Depth* (stack scans only).
4. Enter your desired integration time under *Int. Time (Trace)*. Make note of the time per line: the total time is the trace plus the retrace. The total acquisition time for the image will be the time per line multiplied by the number of lines and the number of layers.
5. Under *Data Labeling*, enter a *Sample Name* and *Number*.
6. Click *Start Scan* to begin image acquisition. You can monitor the progress of your acquisition in the *Message* window.




Repositioning the Sample

If you want to view the sample on the monitor so that you can reposition it for another acquisition, follow these steps:

1. Turn the micrometer on the laser head all the way clockwise. You should see the laser spot under the objective disappear.
2. Turn off the laser.
3. Push the metal rod on the right side of the microscope in.

4. From the right side of the microscope, pull the cube out to position I.
5. From the right side of the microscope, pull the filter holder out to position 1.
6. Use the remote control to refocus on the sample. You should now see the sample in the video.
7. window so you can reposition it using the micrometers below the sample stage.
8. To acquire Raman data, start from Step 7 under *Setting up for Raman Acquisition* above.

Data Analysis Overview:

The *Project Manager* window shows all of the data files associated with a project. This includes raw data acquired with the microscope as well as processed data. Single spectrum data is shown as graph files represented by . Image data is shown as image graph files represented by . Images generated from acquired image graph files are represented by . You can double click any file in the *Project Manager* window to open it.







For images and image graph files, you can use the arrow keys to move around and display Raman spectra from different points in the image. For 3D data you can use *PgUp* and *PgDn* keys to move through the vertical layers. You can click in any image to bring up the Raman spectrum from that location. You can zoom in on any image using the mouse wheel. You can hold the mouse wheel down to move around in the image.

You can adjust the vertical scale of Raman spectra by positioning the pointer below the baseline and scrolling the mouse wheel. You can move the baseline up or down by positioning the pointer above the baseline and scrolling the mouse wheel.

Exporting Data

1. To export a picture of a Raman spectrum, right click the spectrum and select *Export* → *Bitmap to File*
2. To export the raw data (XY pairs) of a Raman spectrum as an ASCII file, right click the spectrum and select *Export* → *ASCII to File*
3. To export any open image as a picture, right click the image and select *Export* → *Bitmap to File*.
4. To export raw image data, right click the image graph file in the *Project Manager* window and select *Export*.

Generation and Analysis of Raman Images

1. Create images for each of the desired peaks in the Raman spectrum
 - a. In the *Filter Manager* window for your data file, click *Add Sum Filter* . If you don't see the filter manager window, right click on the spectrum and select *Graphs* → *View* → *Filter Manager*
 - i. In the *Filter Manager* window you can enter the expected left and right boundaries of a Raman peak in wavenumbers in the *Start* and *Stop* fields.
 - ii. Alternatively, you can select a region in the displayed spectrum: Check the *Listen* box in the *Filter Manager* window. In the *Graph Tools* window click *Mark Region* . Then click and drag over the peak location in the spectrum.
 - b. Repeat the above step to add sum filters for each peak or spectral region where you want to generate an image.
 - c. To generate the images, click *Calculate*  in the *Filter Manager* window. You can open the images from the *Project Manager* window. You can click in any image to see the Raman spectrum at that location.
2. To perform cosmic ray removal, drag and drop an image graph file in the *Project Manager* window to *CRR*  in the *Drop Actions* window. Adjust parameters if necessary then click *Extract*. A new image graph file (CRR) will be generated in the *Project Manager* window.
3. Background subtraction
 - a. Drag an image graph file from the *Project Manager* window to *Graph Background Subtraction*  in the *Drop Actions* window.
 - b. Make sure *Mark Region*  is selected in the *Graph Tools* window.
 - c. In the blue section of the new window, hold down the shift key while clicking and dragging over each peak to deselect the peaks. You may need click around in your images to bring up different spectra to make sure all of the peaks are deselected.
 - d. In the *Graph Background Subtraction* window, change the order until the blue line on the spectrum closely follows the baseline.

- e. Click *Extract* when you are satisfied. The baseline in the new image graph file (Sub BG) should be flat.


4. Obtaining pure spectra


Images usually contain multiple features with unique Raman spectra. In this step you will generate Raman spectra corresponding to specific features or materials in the image.


- a. In the *Image Tools* window, select one of the selection tools (pen, circle, or rectangle)



. Use the selection tool in one of the images to select the region corresponding to a single material or feature.

- b. In the *Image Tools* window, click *Make image from draw field* . A new image file will appear in the *Project Manager* window.

- c. Repeat b and c for all other features of interest in the original image. You can use *Clear draw field*  in the *Image Tools* window to clear the selection.

- d. In the *Project Manager* window, ctrl+click to select all of the new images along with the desired image graph file and drag them to *Average Spectrum*  in the *Drop Actions* window. The pure spectra will appear as new graphs in the *Project Manager* window.

When You Are Finished

1. On the menu bar click *File* → *Save Project As* to save your data.
2. Turn the micrometer on the laser head all the way clockwise. You should see the laser spot under the objective disappear.
3. Turn off the laser.
4. Remove your sample from the stage.
5. Close the WITec Control software. There is a waiting period that must pass before the software closes.



Warning: Do not skip the wait period or you risk damaging the spectral camera!

6. After the waiting period has passed and the software is fully closed, turn off the power strip to the left of the cabinet.
7. Close the cabinet using the foot pedal.