Oxford/WITec Confocal Raman Microscope *alpha300R* (Control SIX)

THIS MANUAL IS TO BE SERVED AS A GUIDELINE FOR GENERAL USE OF THE OXFORD/WITEC CONFOCAL RAMAN MICROSCOPE.

We now have objective lens that can only image dry flat samples. We recommend using 1 x 3 in glass slide to prepare your samples. Please discuss with facility staffs before scheduling if you have solution samples or powder samples or is you need to use coverslip or temperature stage.

PLEASE ALWAYS REMEMBER TO **ENABLE** AND **DISABLE** WITEC CONFOCAL RAMAN MICROSCOPE IN THE BADGER SYSTEM BEFORE AND AFTER USING THE INSTRUMENT.

Figure 1

Lasers & Safety (Fig 1)

- 488 nm, 75 mW, laser class 3B
- 532 nm, 75 mW, laser class 3B, DPSS laser
- 633 nm, 70 mW, laser class 3B, Diode/solid state laser
- 785 nm, 125 mW, laser class 3B, Diode laser

Turning the key 90° to "**ON**" or "**OFF**" positions to turn on or off laser.

For stable power, lasers should be given 3-5 mins to warm up. Please only turn on the laser(s) you need. All four lasers must be off at the time you finish your session.

Laser safety eyewear is not required, but laser shield with the X-Y stage is recommended.

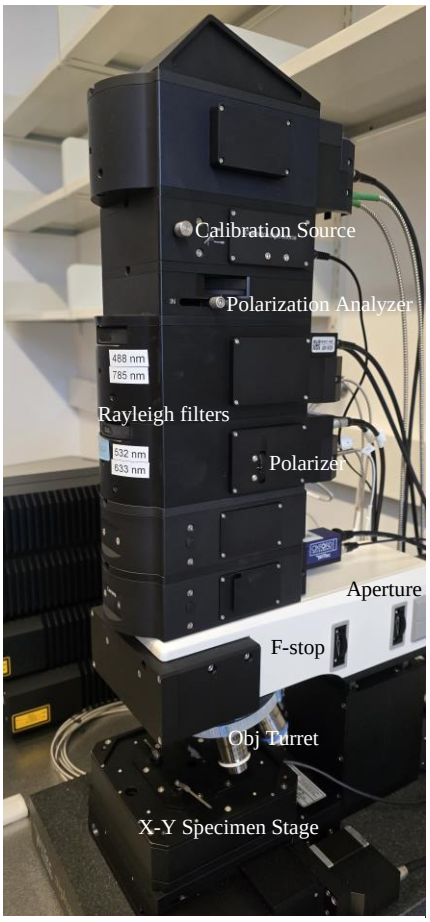

Figure 2

Microscope Tower (Fig 2)

Calibration source: always push in for regular use.
Polarizer & Analyzer: use to change polarization angles of incoming and collected light.
Rayleigh filter: laser selection. New tower has two filter wheels: upper is for 488nm and 785nm, lower wheel is for 532nm and 633nm. Only one of the four lasers is selected at any given time, the other wheel should be left blank.
F-stop and aperture: aperture is to control optical intensity and BF imaging contrast; F-stop is to help focus on featureless samples.
Objective turret: select objective lenses, turn the turret following the marked direction.
X-Y stage: automated stage is controlled by software or Xbox controller in XY directions. Automated Z movement is controlled by software or Xbox controller that moves the whole microscope tower (**objective lens, not stage, move in Z direction**).

Objective lenses:

1. 10X: Zeiss EC Epiplan-Neofluar, WD9.3mm, NA 0.25 DIC
2. 50X: Zeiss EC Epiplan, NA 0.75 HD (darkfield only)
3. 50X: Zeiss EC Epiplan-Neofluar, WD0.58mm, NA 0.8 DIC
4. 100X: Zeiss EC Epiplan-Neofluar DIC, WD1.0mm, NA 0.9 DIC. (New)
5. Empty (compatible with Zeiss water-dip and glass-corrected lens)
6. 50X: Nikon DF Plan ELWD, WD8.7mm, NA 0.55 (heating stage)

Note: For solid flat surface samples, you can select Obj1,3,4. For powder and liquid samples, you can only use Obj6. For temperature stage, you can only use Obj6.

Software: Microscope is controlled by Control SIX (v6.2), which can be started by double click on the desktop icon. Control SIX contains five major windows: Main Menu, Control, Message, WITec Video Control, Project Manager (part of Project SIX)

Important facts to remember before starting:

1) Laser selection is manual operation. You must rotate Rayleigh filters on the microscope tower to select laser and select the corresponding laser in the Control SIX program (WITec Video Control > Laser Control). Only one of the four lasers is selected at any given time, the other wheel should be left blank.
2) Objective lenses selection is manual operation, too. You must rotate the turret to select the proper objective and select the matching objective in Control SIX program (WITec Video Control).

System power on:

1) Turn on microscope control computer, wait 30s.
2) Log in Badger on microscope control computer and enable WITec Raman under IS tab in Badger.
3) Turn on lasers that you need for your session, wait 2-3 mins.
4) Click on "H" logo (highlighted by red circle) at the quick load zone of Windows 10 (left panel in Fig 3) to show the "WITec Service Monitor" page (right panel in Fig 3), make sure all six items are checked with green check marks (right panel in Fig 3) below before moving to next step.

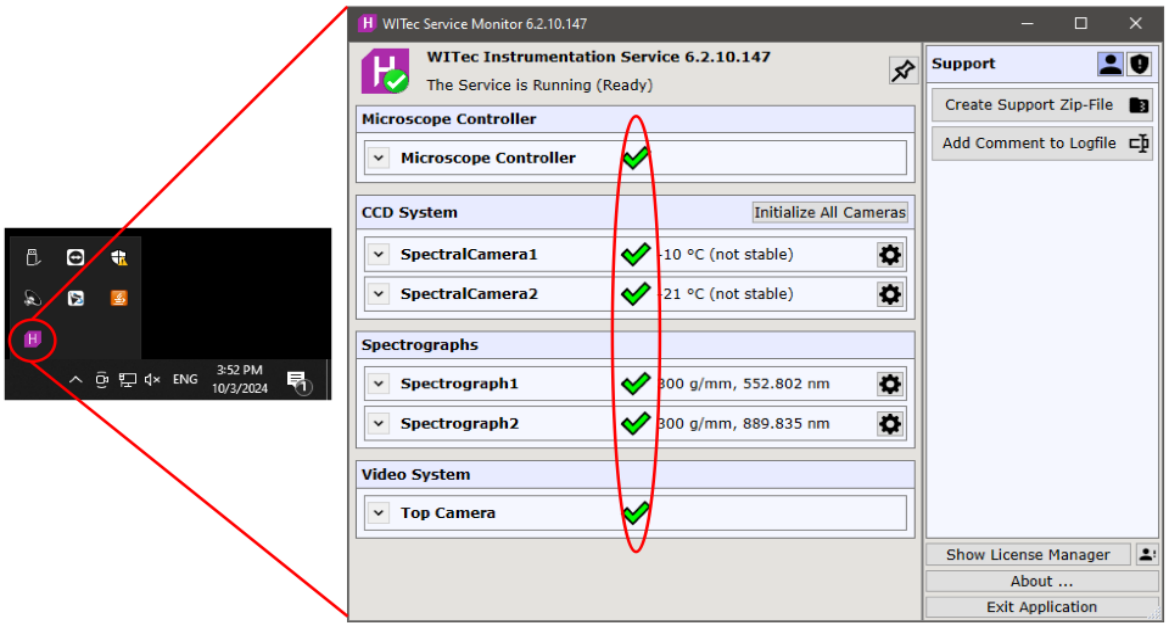

Figure 3

5) Double click on “Control SIX” on the desktop to start microscope control software. It contains five parts (see Fig 4): **Main Menu, Control, Messages. Video Control, Project Manager** (yellow circles).

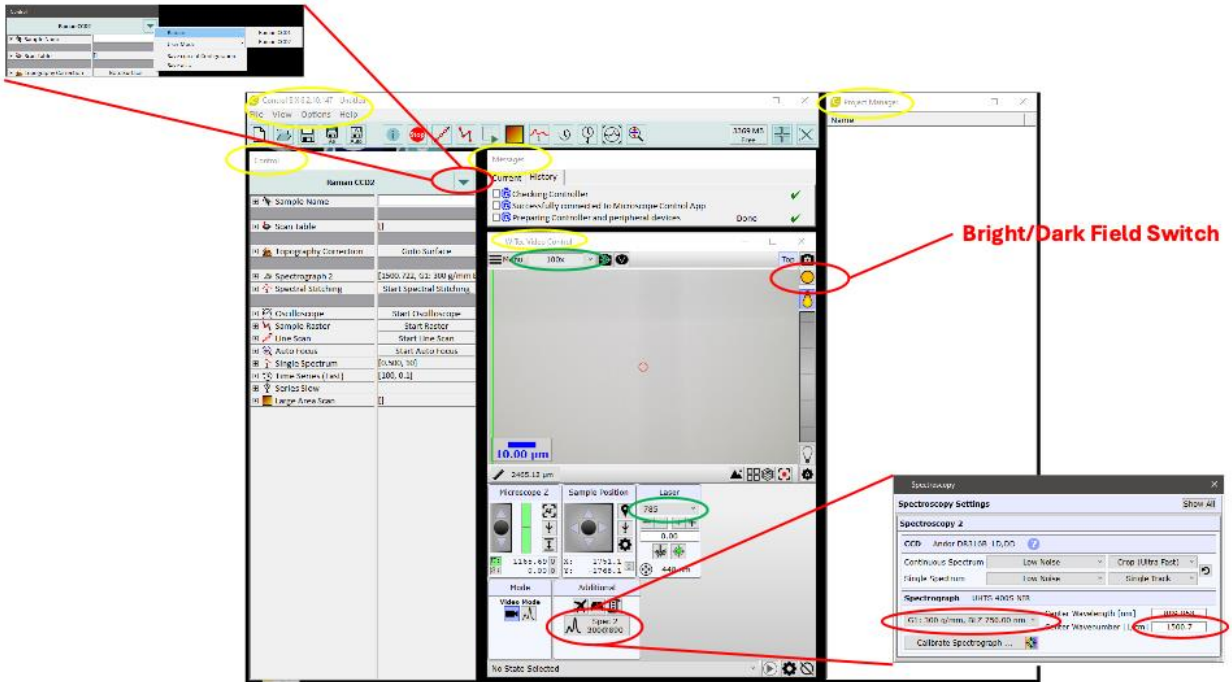

Figure 4

6) Control SIX also automatically enables Project SIX (**Project Manager** in Fig 4) that is used to display/process data.
7) Spectral Camera status is no longer monitored in **Message**, it is monitored in WITec Service Monitor as shown in Fig 3:
   - Temperatures of SpectralCamera1 and 2 are visible here. “(not stable)” means they are not ready for Raman.
   - Temperature at -60°C and disappearing of “(not stable)” indicate that they are ready for Raman.
8) For laser 488nm and 532nm, you must select Raman CCD1(upper left in Fig4); for laser 785nm, you must select Raman CCD2; for laser 633nm, you can select either Raman CCD, however performance of Raman CCD2 is better. Note: you should select SpectralCamera first then select laser.
9) Spectrum grating and center wavenumber are not in **Control>Spectrograph** anymore, they are in **Video Control>Additional** (lower right in Fig 4).
10) There is no more manual switches of Bright/Dark field, and of Video/Raman. The microscope always defaults in Bright Field Video mode. It will automatically switch to Dark Field Raman mode each time you enable Raman spectrum function, like Oscilloscope.
11) You can manually select Bright/Dark field in **Video Control** (upper right in Fig 4).

Note: “Project SIX” is data processing software.

Note: steps to solve problems with the microscope and control software.
   a) RESTART Control SIX. If not work, move to next step.
   b) Shutdown lasers.

Standard Operation:

Remind again: We now have objectives that can only image dry flat samples without any cover glasses. We recommend using 1 x 3 in glass slide to prepare your samples. Please discuss with facility staffs before scheduling if you have solution samples, powder samples or if you need to use cover glass or the temperature stage.

1) Choose 10x objective lens by rotating turret (following the direction) since this lens has the largest working distance.
2) Load glass slide with sample to stage. (optional) Use the two springs to secure slide.
3) Make sure to select the 10x obj lens in Video Control window (Fig 5), find an easily recognizable feature on the sample, and do focus using the Xbox controller.

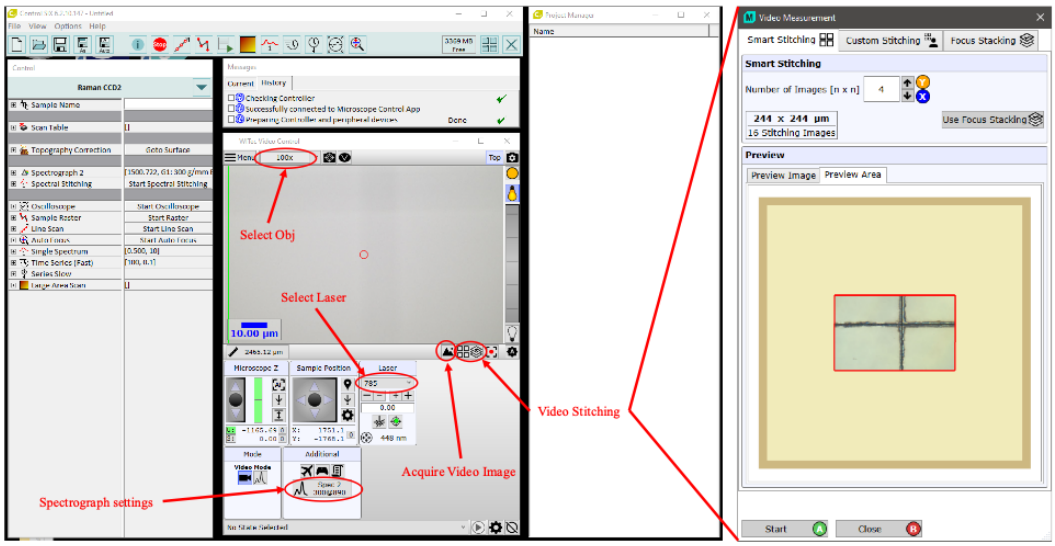

Figure 5

4) Switch lens to 50x or 100x by rotating turret along directions indicated. Remember to change lens in Video Control window (Fig 5). Focus again on the sample. If the sample has no clear feature, using field-stop diaphragm as focusing reference.
5) Take either video image or video stitching image or both (Fig 5). Double click image name in project manager window to open video images.
6) Select Raman CCD following Step 8 (Fig 4) in "**System power on**" session.
7) Select laser on microscope tower (Fig 2) and in the Video Control window (Fig 5).

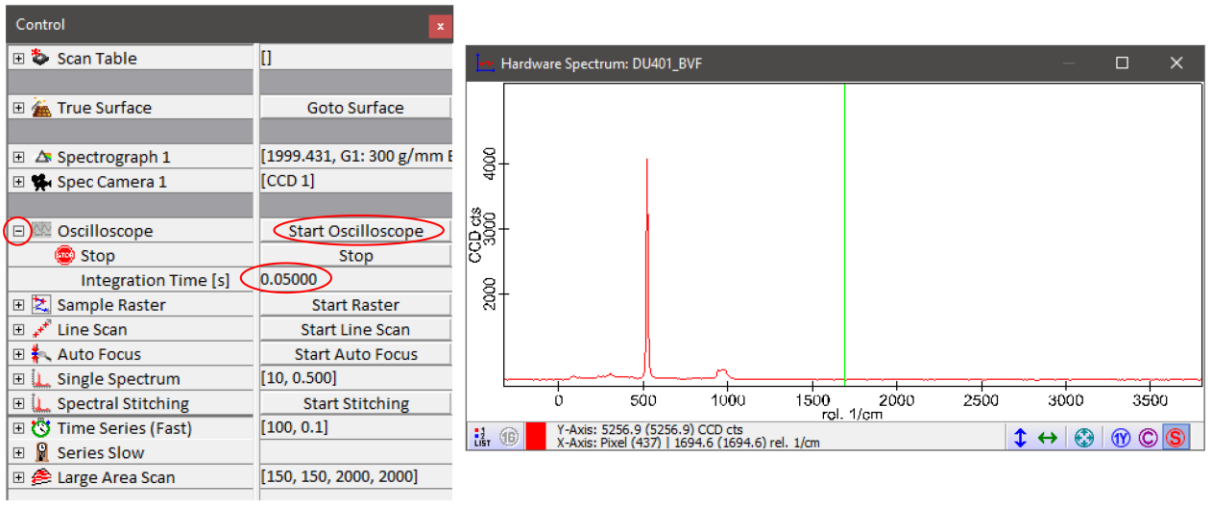

Figure 6

8) Check Spectrograph settings in WITec **Video Control>Additional** (Fig 5 & Fig 4). There are three gratings for Spec1, G1:300g/mm; G2:1800g/mm; G3:2400g/mm. Only G1 give the full spectrum range 0-4000 wavenumber (1/cm). if you are not sure please select G1 for grating and choose 2000 for center wavenumber.
9) Expand Oscilloscope by clicking **+** (changes to **-**) in **Control** window (left panel in Fig 6) and input Integration time (depending on samples and laser power). If you are not sure, starting with short time (0.05s-0.2s) and low laser power (<5 mW). Fine adjust focus by Xbox controller to maximize spectral signal in live Raman spectrum (Fig 6 right panel).
   Note: the main function of oscilloscope is to fine focus on sample and find proper integration time and laser power. All parameters are chosen to get decent Raman signal while not damaging the sample.
10) Expand Single Spectrum in **Control** window (Fig 7), set parameters based on the oscilloscope testing: integration time and laser power. Accumulation could be set to smaller numbers (10-30) for samples with strong Raman signals and larger numbers (100-150) for sample with weak Raman signals, e.g. protein/peptide. Click Acc. Single Spectrum to start. The up-right spectrum (Fig 7) is live single acquisition (oscilloscope) and the low-right one is accumulated spectrum.

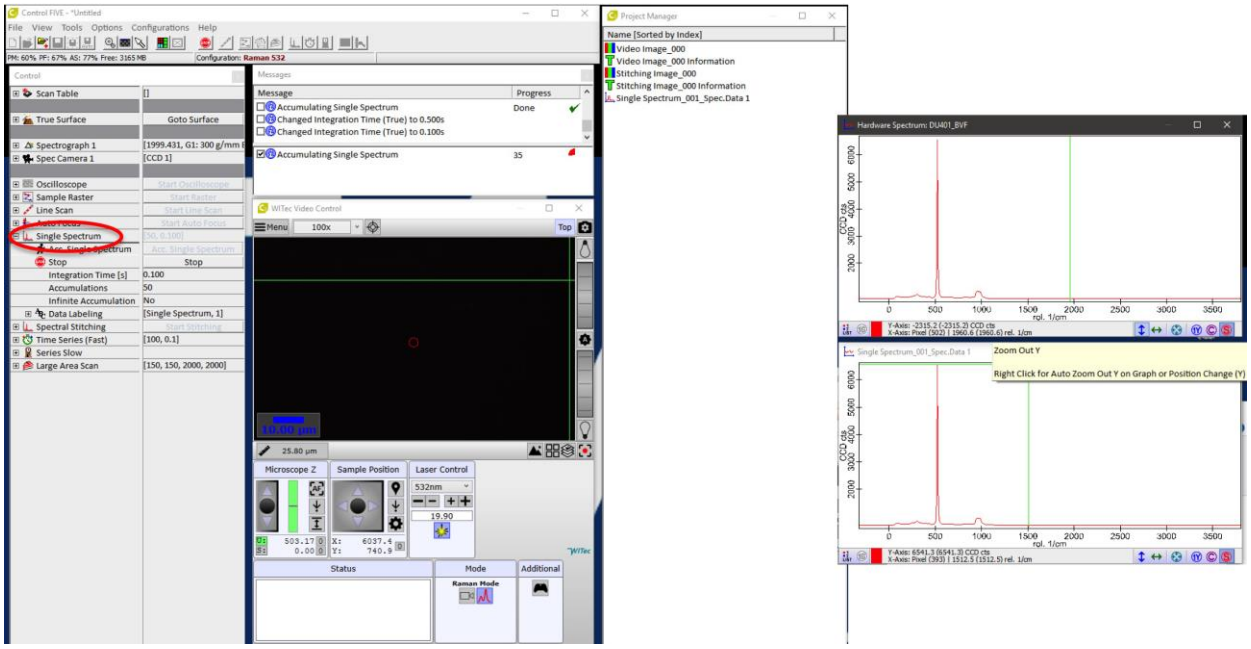

Figure 7

11) Expand Large Area Scan and Geometry in **Control** window (Fig 8), select the type of **Listen Position/Area** (I suggest **Area Multiple**) and draw an area in the opened video image (green rectangle in the right figure below), you can draw multiple times until you are satisfied. You can also adjust **Width**, **Height**, **Points per Line** and **Lines per Image** in the **Control** window.

Note: remember there is no live video in the video control window when the microscope is in Raman mode.

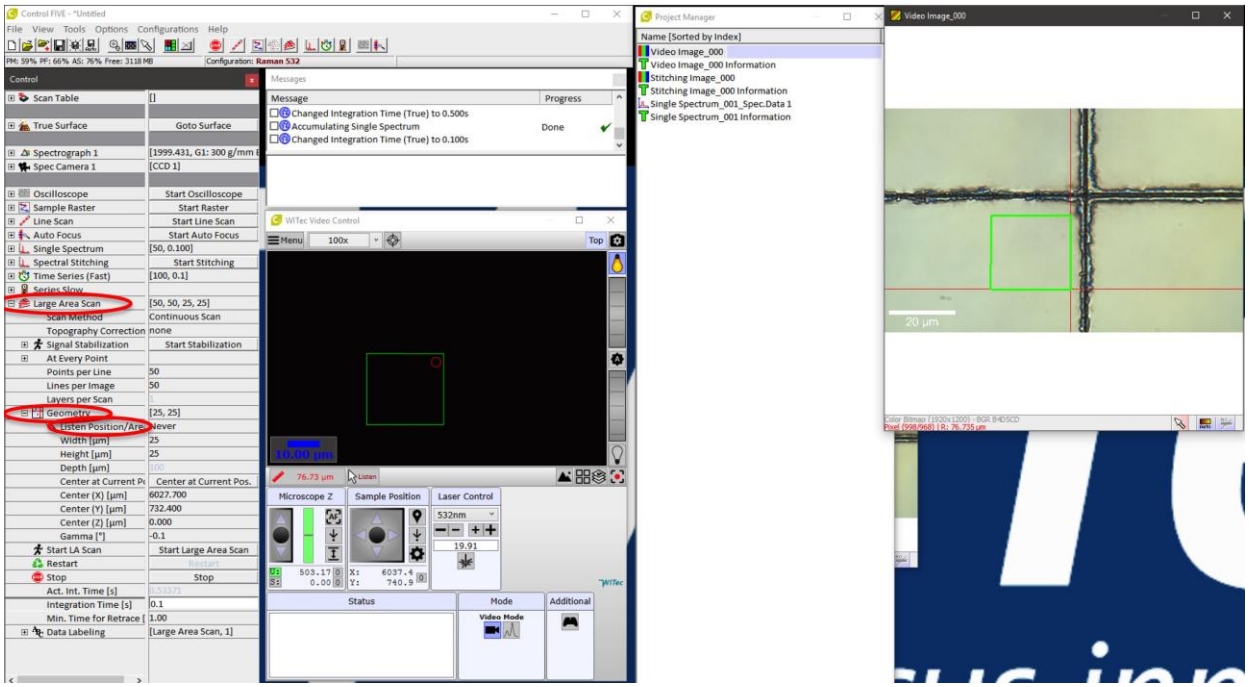

Figure 8

12) Once you are satisfied with all settings, click Start Large Area Scan to start data collection, three new windows (LA Spec Data, Oscilloscope Spec Data, Filters) will appear (Fig 9):

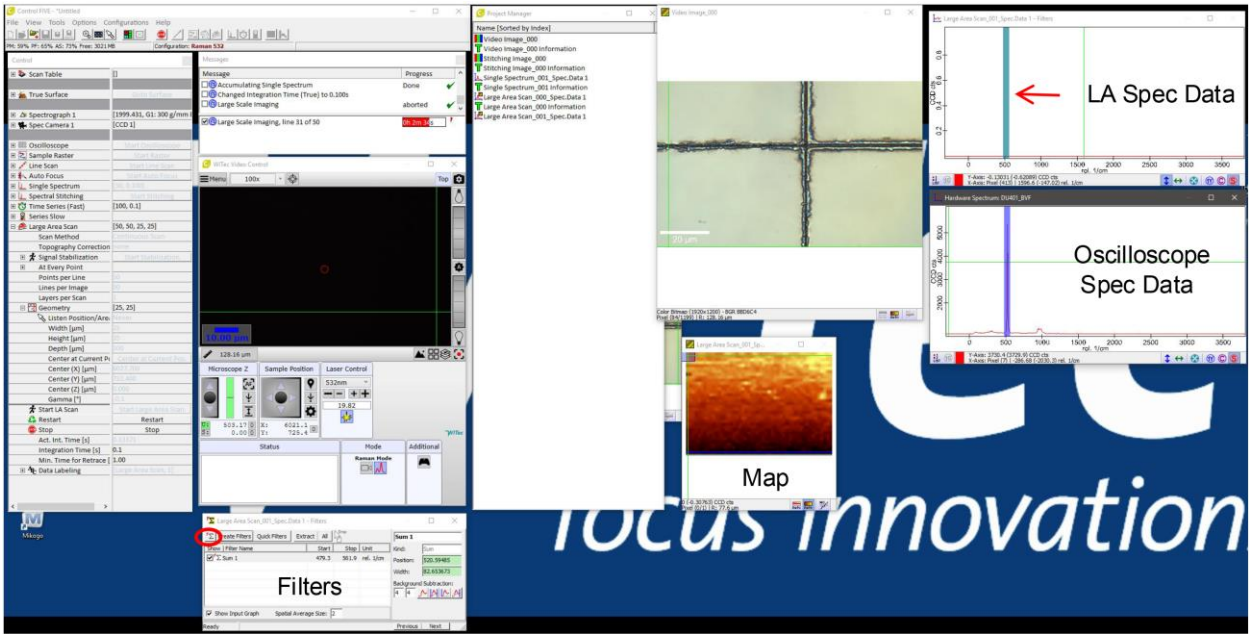

Figure 9

a) Filters window is used to add filter (red circle in Fig 9). Once a filter is added, the fourth new window (Map) will appear (Fig 9). This filer (vertical green and purple band in LA Spec Data and Oscilloscope Spec Data) aims to select characteristic peak for Raman mapping.

b) Oscilloscope Spec Data window. You can only move the green band (red arrow) in this window to select peak in LA Spec Data window (purple band). The width and center of such filter band can be manually set in Filters window.
c) LA Spec Data window. Purple band in this window is coupled to the green band in live spectrum window and works as indicator to select peaks.
d) Map window is to display map depending on the filter added.

13) Data collection progress bar is shown in Message Window (Fig 9).

Finish:

1) Save the project file. This is important.
2) Close the Control SIX program.
3) Turn off laser (**IMPORTANT!!**). Login Badger and disable WITec Raman in IS tab of Badger.
4) Turn off computer.
5) Clean up computer desk and sample prep area.
6) Take all samples and glass slides with you.