From Eye to Insight

Leica MICROSYSTEMS

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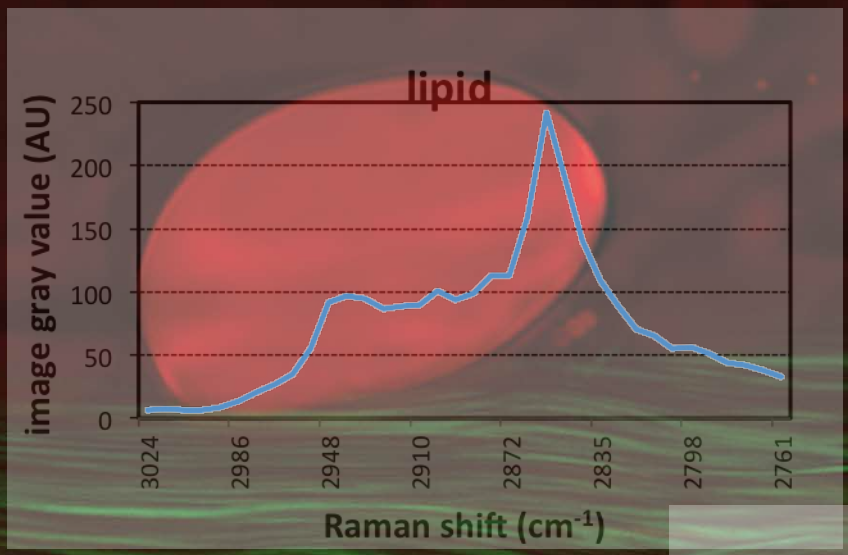
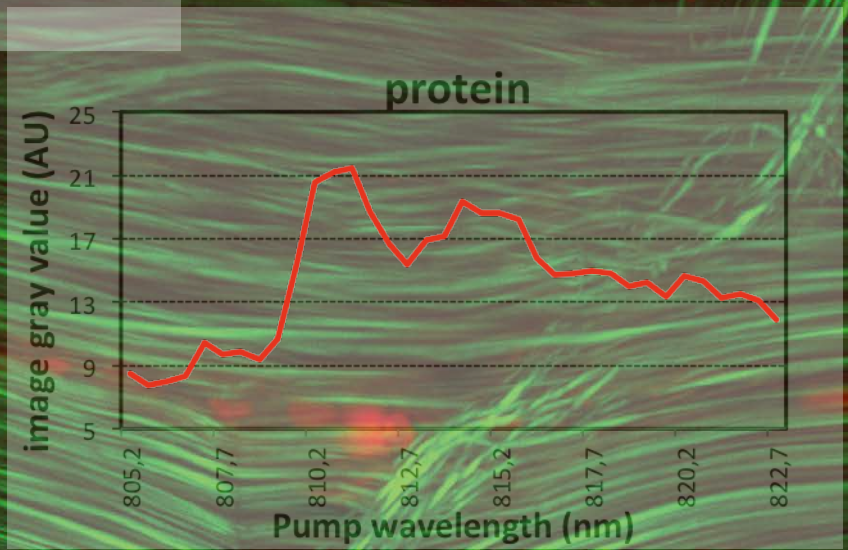
Confocal Application Letter

# CARS SPECTRAL SCAN WITH LEICA TCS SP8 CARS



# CONTENT



**General Safety Notes**

The system and LAS X may only be used by persons who have been trained in the use of the system and about the potential hazards of laser radiation.

**Observe the user manual**

Follow the safety notes and instructions in the user manual.

**WARNING**

**Permanent eye and skin damage from laser radiation**

Skin and eye damage can occur while using lasers if safety precautions are not taken. Pay particular attention to the laser safety.

## INTRODUCTION

Coherent Anti-Stokes Raman Spectroscopy (CARS) is an imaging technique which looks at the specific vibrational motion in the molecules of the sample. This third-order nonlinear optical process involves three photons: a pump photon of frequency $\omega_P$, a Stokes photon of frequency $\omega_S$ and a probe photon at frequency $\omega_{Pr}$. At least two intense pulsed laser lines are necessary for CARS signal generation; Figure 1 shows the case when the pump line ($\omega_P$) acts as the probe line ($\omega_{Pr}$). For efficient CARS signal generation, it is important that all the three photons arrive at the sample simultaneously (temporal overlap) while impinging at the same position in the sample (spatial overlap). In order to look at a specific molecular vibration, in other words, a certain chemical bond, the frequency difference between the laser lines $\Delta\omega = \omega_P - \omega_S$ needs to match one of the vibrational modes of the molecules in the sample. The generated CARS signal is at the frequency $\omega_{CARS} = 2\omega_P - \omega_S$.

Since the signal generated via the CARS process is intrinsic to the molecular bonds present in the laser excitation volume, the sample preparation time is almost non-existent and the original natural state of the sample is preserved. Being a multi-photon process, excellent optical sectioning can be achieved and efficient signal collection can be obtained using non-descanned detectors (NDD).

CARS imaging involves the use of near infrared lasers for signal generation. This offers two-fold advantages: (1) Lower scattering of longer wavelengths enables deeper imaging (2) Longer wavelengths minimize auto-fluorescence and avoid photo-bleaching and its related photo-toxic/thermal effects.

In this imaging methodology, the frequency difference $\Delta\omega$ is the key for detecting different chemical bonds. Since every molecular bond has its characteristic vibration mode, at least one of the laser wavelengths must be variable to selectively detect a signal from a specific molecular vibration. The Leica TCS SP8 CARS uses a tunable pump laser with a tuning range from 780 nm to 940 nm. Combined with a Stokes laser at 1064 nm, it covers all Raman shifts in the range of 1250 $cm^{-1}$ to 3400 $cm^{-1}$ wavenumbers.

Due to interference with non-resonant background, the CARS spectral peak is generally red-shifted compared to the Raman peak (Cheng 2007) and this shift is sample-dependent. This fact makes it difficult to choose the right wavelength for straightforward imaging of a sample, even if the theoretical Raman shift for the respective bond is available.

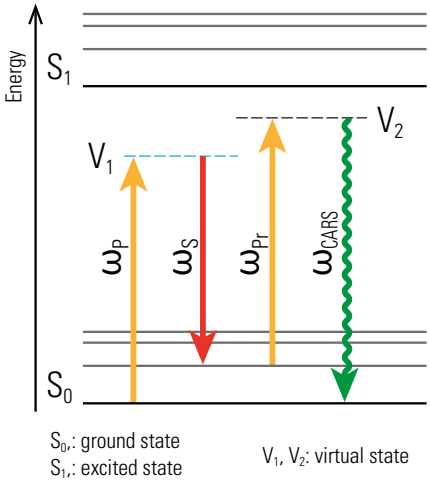



Figure 1: Energy diagram for CARS signal generation.

## CARS SPECTRAL SCAN MADE EASY

This application letter presents the solution to this problem using the unique capabilities of the Leica TCS SP8 CARS system.

The Leica TCS SP8 CARS allows you to acquire a spectral excitation scan in order to rapidly assess the optimal excitation for a specific chemical bond in your sample. This provides not only the spectral information of the sample but also its structural distribution with excellent spatial resolution.

Of course, a quantifiable spectral scan requires not only a tunable light source but also constant optical power over the entire excitation scan range. If the illumination power varies with wavelength, the resulting intensity changes in the CARS image stack cannot be discriminated from the desired CARS signal variation introduced by the chemical/spectral contrast of the sample.

Tunable laser light sources inherently exhibit power dependence over the spectral range, which is acceptable for simple image acquisition experiments. But whenever a spectral scan is the goal, constant excitation power is a must.

The TCS SP8 CARS offers a unique "Constant Power Mode" of operation. In this mode, the power for both the pump line and the Stokes line can be set to a certain value and kept at this constant level for the entire "Lambda-stack" experiment. With the help of the constant power mode, acquisition of hyper-spectral CARS image stacks can be automated and CARS spectra in multiple regions of interest (ROIs) can be directly visualized, compared, and exported.

This application letter shows how to conduct CARS spectral analysis with the Leica TCS SP8 CARS. The following sections describe the software user interface while providing tips and tricks to extract the most out of your system.

## SYSTEM PREPARATION

**Choose the right objective**

CARS imaging needs two near infrared wavelengths impinging on the sample simultaneously. Any chromatic aberration will lead to a mismatch of the two PSFs (Point Spread Functions) and the signal generation will fail. Therefore, objectives with optimal chromatic correction for wavelengths used here are essential for successful CARS imaging. Leica offers a series of IRAPO objectives, which have the best chromatic correction and highest transmission for near infrared light. The HC PL IRAPO 40x/1.10 W CORR objective is strongly recommended for CARS experiments. In addition to the excellent chromatic correction you can achieve the best resolution with its high numerical aperture. If you need a bigger scan field, you can choose the HC PL APO 20x 0.75 Imm CS2, or the newly available HC PL APO 20x 0.75 CS2 dry objective.

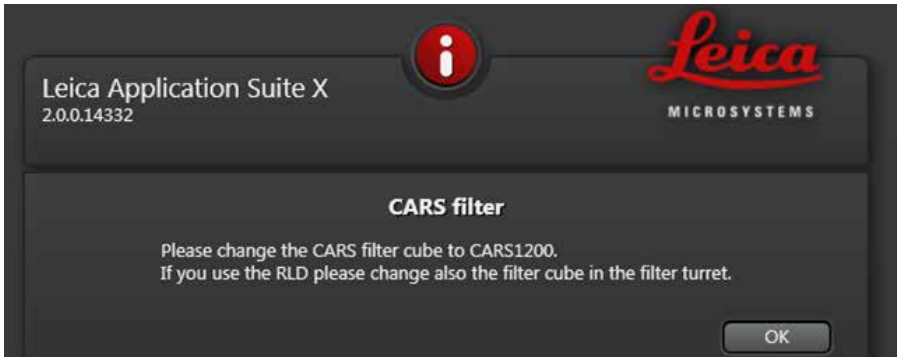



Figure 2: Information for changing the filter cube.

**Avoid any stray light**

The CARS signal is detected with highly sensitive non-descanned detectors (NDD). Any stray light will lead to image background.

Darken the room if possible. If the system is not equipped with a black chamber, cover the microscope with an opaque material.

**Choose the right CARS filter set**

Two filter sets can be chosen for CARS imaging, CARS1200 and CARS2000. The CARS1200 covers the Raman shift range of 1250-2000 $cm^{-1}$ and the CARS2000 covers 2000-3450 $cm^{-1}$.

**Note:**

If the CARS laser is tuned to a wavelength which generates CARS signals in the range outside of the current filter cube, a pop-up display (see Figure 2) informs the user that the filter cube has to be changed and waits for user confirmation.

This dialog could also show up during a spectral scan. If you define a scan range across the 2000 $cm^{-1}$ marker, the system will stop the data acquisition until the filter cube has been changed and confirmed. After that, the system continues to acquire images. However, it is strongly recommended not to set the scan range across the 2000 $cm^{-1}$ marker: no matter how careful you are while changing the filter cubes, you cannot avoid disturbing the sample position. This is especially true if you are working with epi-CARS detection. The sample movement makes the data analysis and interpretation difficult. It is recommended that you take two scans on either side of 2000 $cm^{-1}$ and link the data sets subsequently.

# INITIAL LASER OPERATION

**Switch on the CARS laser**

As shown in **Figure 3**, go to the **Configuration** tab ❶, click on the **Laser Config** icon ❷ and toggle the **CARS** button ❸. Wait for 30 minutes for the CARS laser to warm up and stabilize before starting imaging sessions.


Figure 3: CARS laser control in configuration

**Activate the CARS laser**

Go to the **Acquire** tab ❶, as in **Figure 4**, and click on the button with the plus sign ❷ to open the CARS laser control dialog window (see **Figure 5**). Click the **Activate Laser** button ❶ for about 3 seconds to open the main shutter of the laser unit. The radiation warning ❷ will brighten, indicating that the laser radiation is on. The individual shutters ❸ and ❹ (see **Figure 5**) control the pump and the Stokes laser, respectively. They should be opened by default. If they are closed for any reason, open both for CARS imaging. Open the safety shutter by clicking the button ❸ in **Figure 4**. Now the CARS laser is ready for imaging.

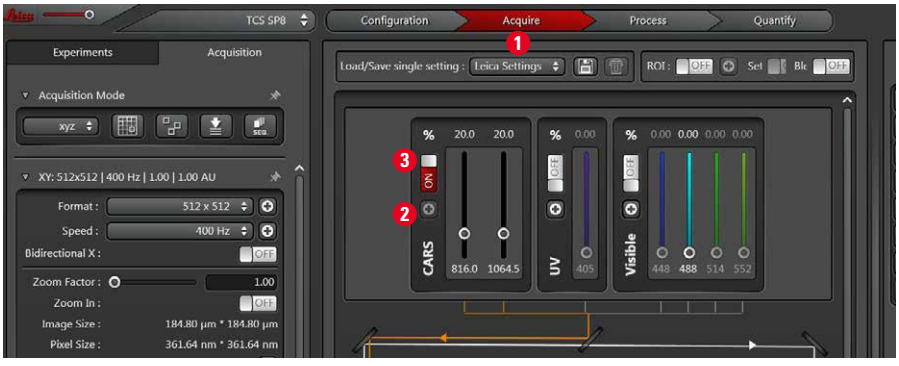



Figure 4: CARS laser control in configuration

**Tune the CARS laser**

There are two modes (or **Calculation Methods**) for tuning the laser wavelength. These are listed in the drop-down menu 5 in **Figure 5**; either of the modes can be selected as per your preference. In the **Pump based** mode, you can directly type in the wavelength in the edit field 6. In case you do not know the pump wavelength and would rather obtain a CARS image at a specific Raman shift, you can switch to the **Raman based** calculation method and type in the Raman shift in the edit field 7. The pump wavelength will be calculated automatically. Click on the **Tune** 8 button to tune the wavelength. Note that the wavelength tuning process can be performed even during live image acquisition. If the constant power button 9 is activated (default), the output power can be controlled directly by typing in a number in the edit field 10 and 11 for the pump and Stokes laser line, respectively. In case maximal laser output is needed, just deactivate the constant power button.


Figure 5: CARS laser control

**Note:**

During the tuning process, the main shutter in the laser head will be closed in order to avoid continuous illumination of the sample. If the shutter was open before the tuning process was started, it will be re-opened automatically after the wavelength has been tuned. Otherwise, it will remain closed.

The built-in CARS calculator also gives the anti-Stokes emission wavelength 12 generated from the current combination of pump and Stokes wavelengths. This information will help you to choose the right filter for detecting the signal, especially if you want to construct your own filter for more narrow-band detection.

**Raman database**

The SP8 CARS software includes a Raman database with a list of the most common organic bonds relevant for biological imaging. The database can be accessed, as shown in **Figure 6**, by opening the **Configuration** tab 1 and clicking on the **Raman Modes** icon 2. It constitutes a good starting point for imaging your sample if you have very little information about its Raman spectral characteristics. Mark the chemical bond you are interested in by a simple mouse **click** 3, and press the **Tune** 4 button. The laser will automatically tune to the wavelength necessary to excite the Raman shift (wavenumber) of this bond. You can modify the contents of this database and extend it to protocol the spectral properties specific to your samples. You can also export database contents to an ascii file with a single click of the **Export** 5 button.

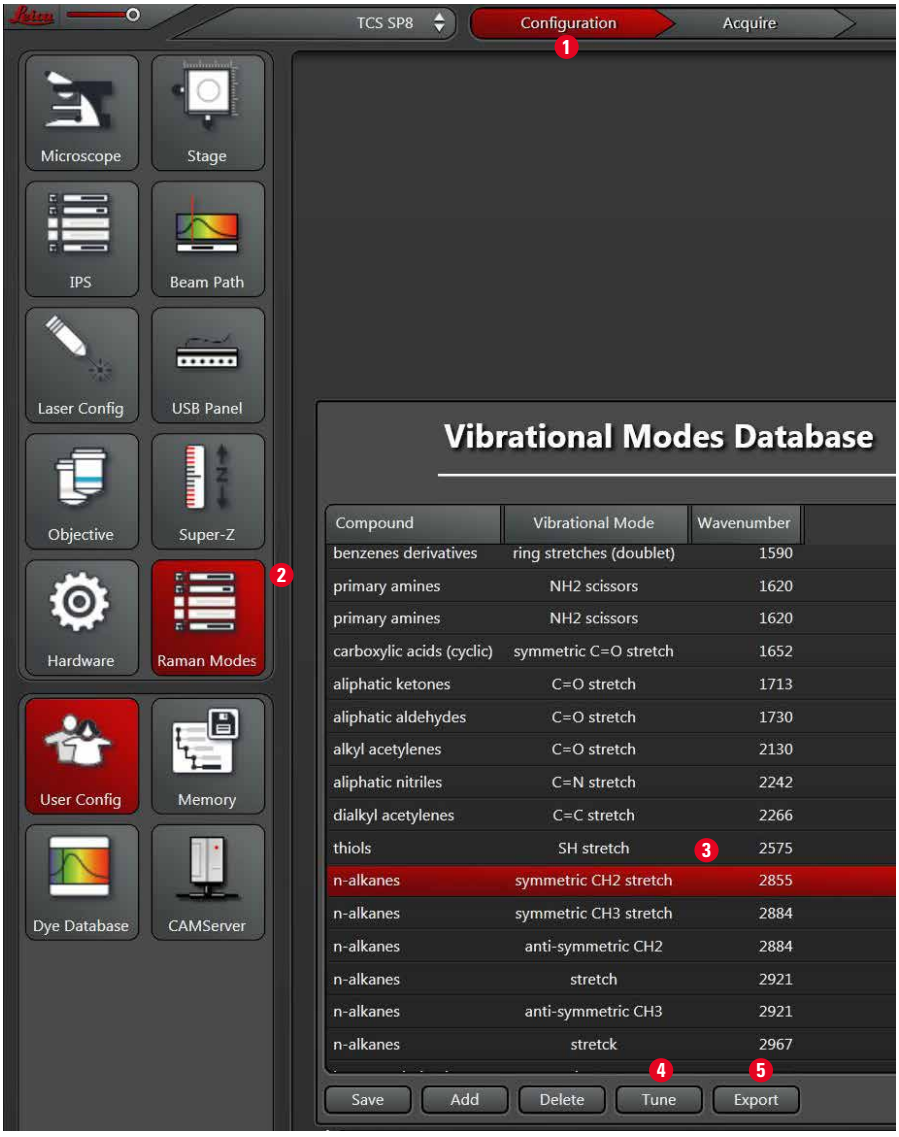



Figure 6: Raman modes database

**Detector activation**
Depending on the sample thickness you first need to decide whether to use TLD (Transmitted Light Non-Descanned Detector) or RLD (Reflected Light Non-Descanned Detector) for signal detection. The propagation of the CARS signal is predominantly in the forward direction. If the sample is thin and transparent, it is always beneficial to use the TLD for "Forward CARS".

In case you wish to investigate thick or scattering samples, the RLD for "Epi CARS" will be the right detector choice.

Activate, as shown in **Figure 7, F-CARS** 1 for forward signal or **Epi-CARS** 2 for backscattered signal. You can, of course, use both of them simultaneously, especially to check which detection mode is the best for your application.

Set the detector **Gain** 3 to 650 to 750 volts for optimum signal-to-noise ratio.

Start **Live** scan 4. Increase the AOTF transmission, as shown in **Figure 8**, for both pump 1 and Stokes 2 lasers until you get an image. The power adjustment can be combined with the control of output power under the constant power mode, see 9 in **Figure 5**.

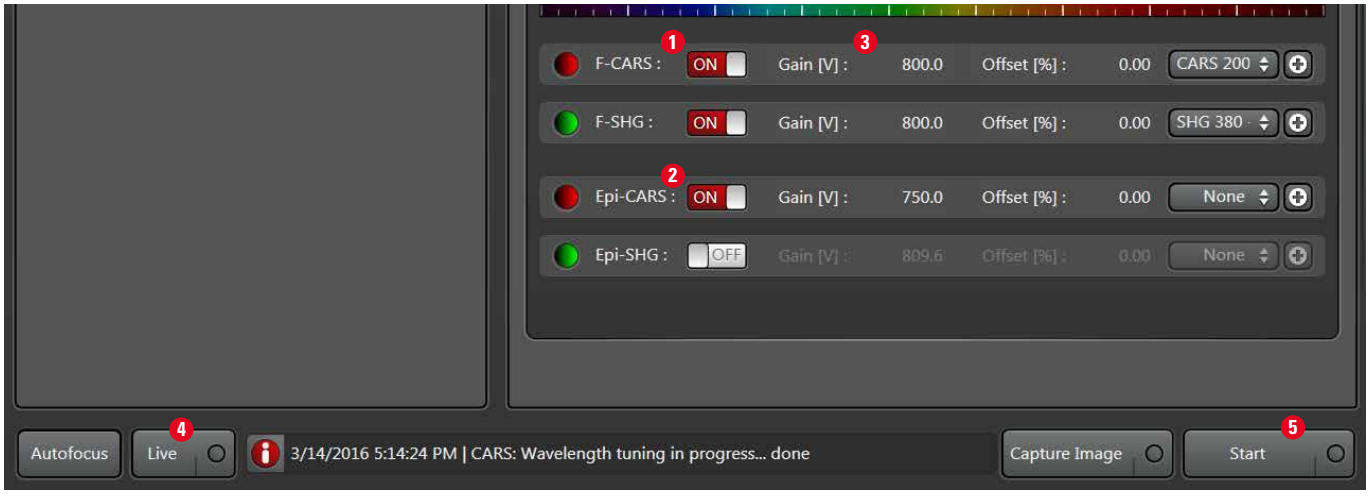



Figure 7: NDD control

**Note:**
If using TLD detection, optimize the Koehler illumination. Make sure there are no additional or unnecessary filters in the beam path between sample and detector. The polarization filters in particular would block a huge part of the CARS signal. Also ensure that the aperture/iris diaphragm is fully opened. If working with the RLD detectors, ascertain that you have the right dichroic beam splitter in the fluorescence turret.

**Caution:**
Since you are using highly sensitive non-descanned detection, please set AOTF for all visible and UV lasers to 0 and close the shutters 3 and 4 as in **Figure 8**. Stray light would otherwise cause an unwanted background signal that might swamp the CARS signal.

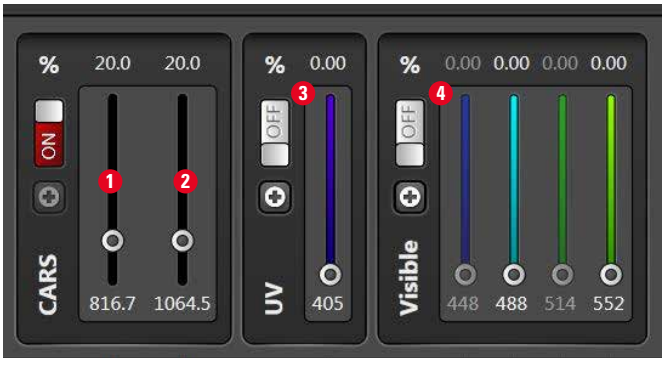



Figure 8: AOTF for CARS laser power control

## SET UP THE CARS EXCITATION PRE-SCAN

For a sample with unknown Raman spectral characteristics, a pre-scan with a large excitation wavelength step size (i.e. 1 nm) is strongly recommended.

After this step you can find out roughly the excitation wavelength at which the maximum signal from your sample appears; you can then optimize laser excitation power and PMT gain at this position.

## SET UP CONSTANT POWER MODE

Constant power mode is activated by checking box 9 in **Figure 5**. Pump and Stokes laser lines are set separately with different values for constant power.

Check the image again with Live scan. Adjust the AOTF power or the detector gain if necessary.

## SET UP LAMBDA (Λ) SCAN

In the **Acquisition Mode** 1 dropdown list in **Figure 9**, choose one of the lambda excitation scan modes 2 depending on your experimental requirements.

The dialog window for setting up a CARS excitation lambda scan **(Figure 10)** will be activated. If your system has other tunable lasers, then choose **CARS** as the **Light source** 1; otherwise **CARS** will be chosen automatically.

Select the **Pump based** calculation mode or the **Raman based** calculation mode 2 for parameter setting. As you can switch between the two modes, the values for the **Excitation Begin** 3 and **Excitation End** 4 will be recalculated accordingly.

**No. of Excitation Steps** 5 and **Stepsize** 6 are linked to each other; setting one of them would automatically modify the other based on the scan range. For easy workflow, it is recommended to set the step size yourself and let the system calculate the number of excitation steps. Note that the step size is in nanometers, irrespective of the chosen calculation mode 2. The smallest step size is 0.1 nm and the largest is 2 nm. After the definition of the step size and the number of steps, the end of the scan range will be slightly adjusted.

Start the data acquisition by just clicking the **Start** button **(Figure 7** 5**)**. The scan will start at the shortest defined wavelength (or the largest Raman shift) and proceed to the longest wavelength. Note that the resulting spectral scan data is equi-spaced only in wavelength and not in wavenumber.

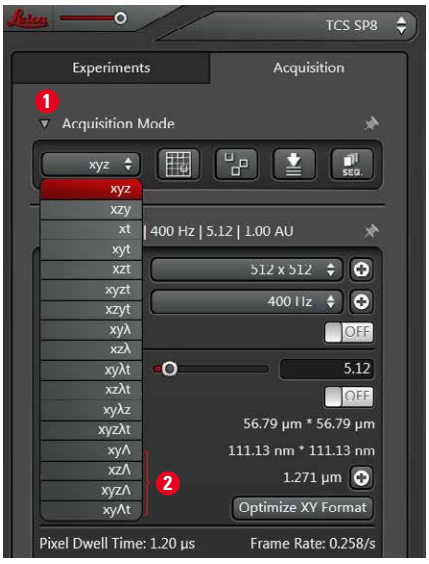



Figure 9: Choose the CARS lambda scan mode

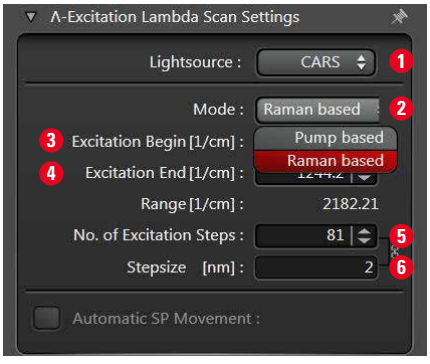



Figure 10: Set up the CARS lambda scan parameters

**Note:**
For the constant power mode, set the value of the pump power below the minimum output power that is achievable in the entire tuning range of the chosen spectral scan.

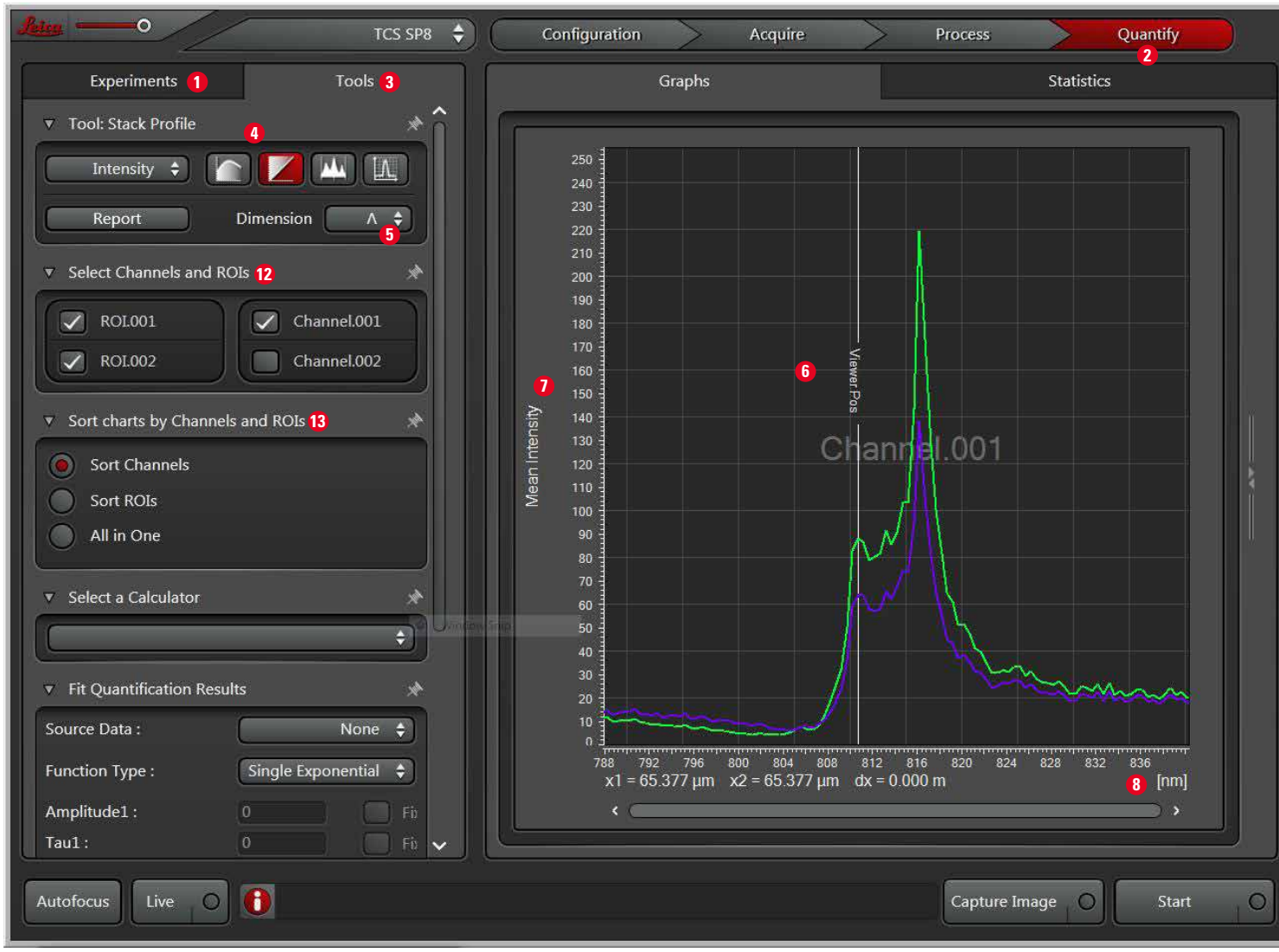
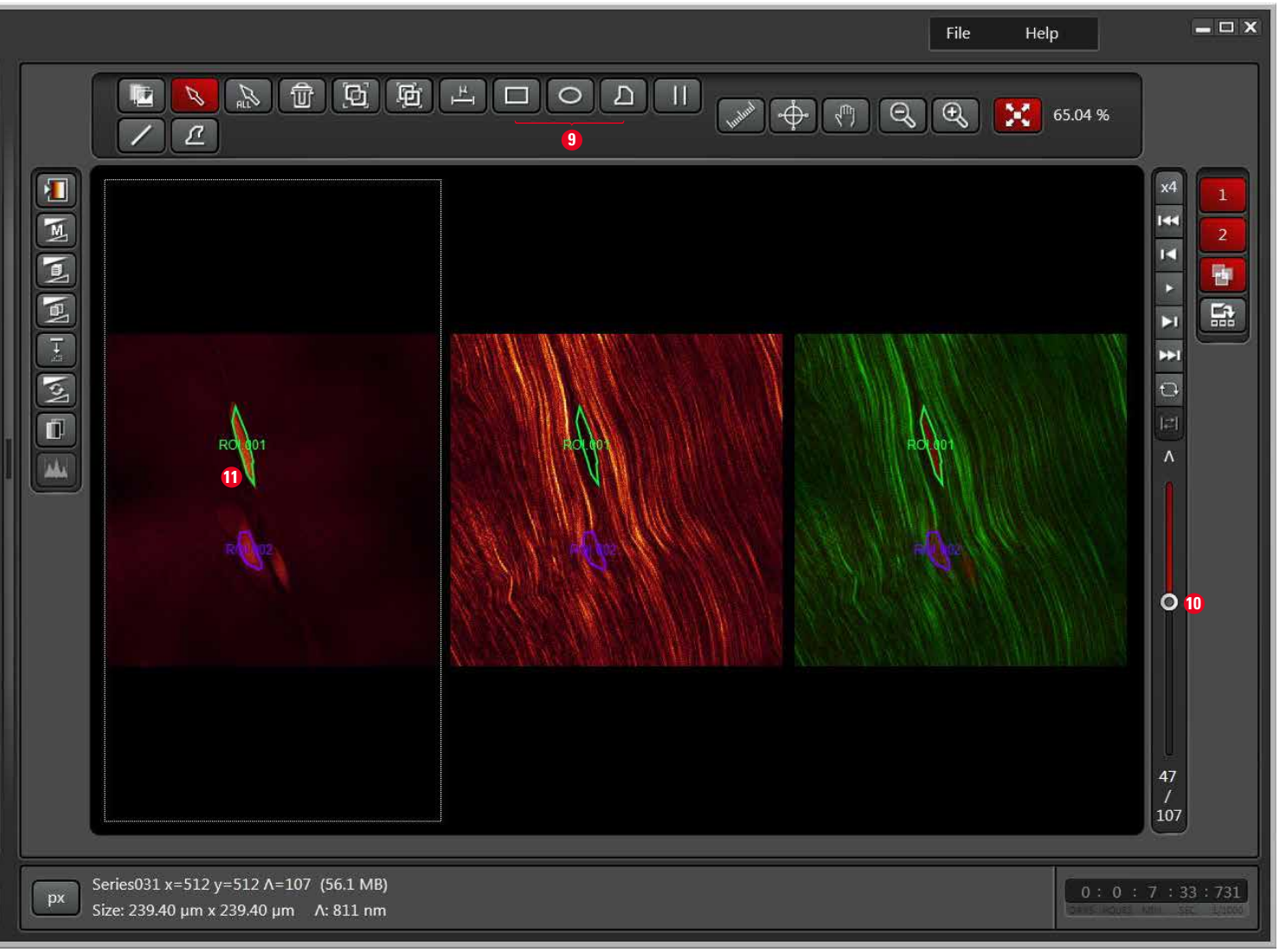



Figure 11: CARS lambda scan data analysis and export

## CARS SPECTRAL SCAN ANALYSIS

When the scan process has finished, a hyper-spectral data set is generated as depicted in **Figure 11**. To generate mean spectral intensity plots, activate the dataset under the **Experiments** tree 1, go to **Quantify** 2 **-> Tools** 3, activate **Stack Profile** 4 and choose **Λ** in the drop-down menu **Dimension** 5. You will get an intensity distribution curve in the report area 6 with the **Mean Intensity** 7 plotted against the wavelength 8 of the pump laser. If no specific region of interest (ROI) has been defined, the software considers the entire image as a single ROI and it is this mean intensity which is displayed in the graph. You can define a custom ROI using the ROI tools 9. To do so, scroll through the lambda series by moving the slider at the right side of the viewer 10 and find the image with the maximum outline of your component of interest. Once a ROI 11 is defined, you will see the statistics/plot specific for this particular ROI. You can also define more than one ROI in the image; then you will get a corresponding number of curves displayed in the report area. On the left, under **Tools** 3, you can select **Sort charts by Channels and ROIs** 13 to define how the data is presented in the chart 6.




As mentioned earlier, the curves displayed in the chart are the plot of the mean image intensity against the pump wavelength. But in the Raman literature, the commonly used term for describing CARS/Raman spectra is the Raman shift in wavenumber instead of pump wavelength. In order to convert to wavenumber, the data can be exported as an excel file.

To do so, activate the export function with a right mouse click in the chart area (see **Figure 12**) and choose **Export** 1 **-> Excel** 2. The data will be exported in *.csv format which can be edited in spreadsheet applications.

To calculate the Raman shift from the pump wavelength, use the following formula with wavelengths in nanometers:

$$Ramanshift\ \left[cm^{-1}\right] = \left(\frac{1}{\lambda_P[nm]} - \frac{1}{\lambda_S[nm]}\right) \times 10^7 \qquad \lambda_S = 1064.5\ nm$$


Figure 12: Export of spectral data for further analysis

## CARS SPECTRAL SCAN ACQUISITION

After the pre-scan, the approximate maximum signal position can be localized. The optimum parameter settings, including AOTF values for the excitation power and PMT gain for the detection amplification, can be adjusted for the fine spectral scan of CARS images.

Keeping all other parameters as before, adjust the excitation step size according to the required spectral resolution for the final lambda scan. Data acquisition can now be started by simply clicking the **Start** button.

The spectral scan can also be combined with XZ or XYZ (3D acquisition) or XYT (time experiment) by choosing the appropriate mode of acquisition shown in **Figure 9**.

## EXAMPLE FOR CARS SPECTRAL SCAN

- Sample: Fresh chicken tendon tissue embedded in water
- Objective: HC PL IRAPO 40x/1.10 W CORR
- CARS 2000/BP filter cube, CARS and SHG channels acquired simultaneously
- xyλ scan range: 2500-3300 $cm^{-1}$ (787.8-840.7 nm) in 0.5 nm steps

In **Figure 13**, image **A** shows the CARS channel. The lipid droplets can be clearly recognized. Image **B** shows the SHG channel, where one can see the typical elongated and nicely organized structure of collagen fibers in the tendon tissue. In the overlay image C, one can see how the lipid droplets are embedded among the collagen fibrils.

For quantitative analysis two ROIs are drawn, one enclosing a lipid droplet (green) and a second enclosing collagen protein fiber (violet). The related intensity and wavelength information is exported and re-plotted against the pump wavelength (top axis) and the Raman shift (bottom axis). Diagram **D** shows the data from the CARS channel where the green curve corresponds to the lipid area and the violet to the protein fibers. The lipid droplet exhibits a much stronger CARS signal (with a peak intensity of 240 gray values) than the protein area. In the lipid area, typical signal peaks for CH (2853 $cm^{-1}$, white arrow) and $CH_3$ (2936 $cm^{-1}$, black arrow) can be observed; the CH bond signal is 2.5 times stronger. Within the protein fiber, one can observe these peaks as well, but with an overall lower intensity and, more importantly, with an inverted ratio of CH and $CH_3$ signals. $CH_3$ is now stronger than the CH signal. There is a much greater amount of CH in lipid than in protein. Additionally, we see an increasing signal in the protein area starting from a Raman shift of approximately 3000 $cm^{-1}$; this signal corresponds to water.

Image **E** shows the signal distribution of the same ROIs from the SHG channel. Clearly, there is no dependence between the signal intensity and the laser wavelength. No dominant peaks are observed either in the lipid area or in the protein area. However, the protein area in this channel has a much stronger signal (90–140 gray values) than the lipid area (around 20 gray values). This is a result of parallel organization of the collagen fibers in the tendon, which generates stronger SHG signals. In contrast, SHG signals are weak within the lipid droplets due to the absence of non-centro-symmetry, a pre-condition for efficient second-harmonic generation.

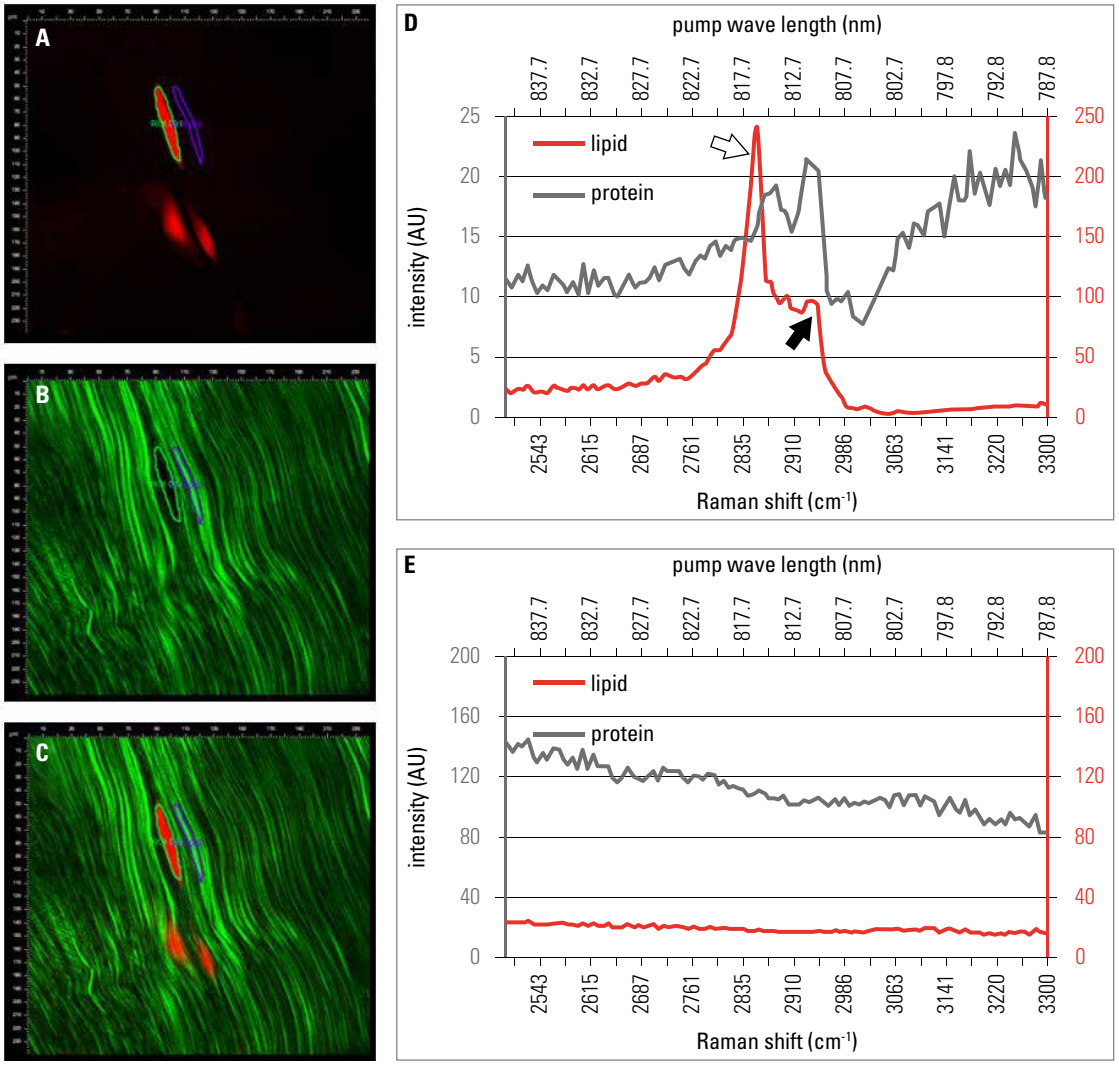



Figure 13: CARS spectral analysis of chicken tendon.

**References:**

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NOTES

## NOTES

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