Appendix 1

Adding objectives to enable their use with Surface

New objectives are added using System configuration (**System configuration, Podule tab.....objectives**). Additional to the new objective name and magnification, the working distance (WD), depth of field (DoF) and diameter (D) values need to be entered.

- WD is a value provided by the objective supplier (mm)
- DoF is the distance the sample can travel before it becomes out of focus (e.g. 10 µm means +/- 5 µm from the optimum focus point)
- D is not the main objective diameter, but is calculated as the lens radius added to the DoF (mm). This ensures gradients of up to 45 degrees can be safely analysed

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TM012 - Data processing and simple analysis WiRE™ 4.0

This document aims to show the WiRE™ 4.0 user how to process single spectra and perform basic analysis. The following methods are discussed:

- Baseline subtraction (processing)
- Arithmetic functions on data (processing)
- Smoothing (processing)
- Zapping (processing)
- Peak Pick (analysis)
- Curve-fitting (analysis)
- Integration (analysis)

Baseline subtraction

Samples may exhibit Raman spectra with varying degrees of fluorescence or thermal background. Providing that there is sufficient Raman signal 'on top' of the sloping background, the baseline may be subtracted to yield a spectrum with a 'flat' baseline. In some cases, the measurement can be re-performed with an alternative excitation wavelength to more effectively remove the effects of fluorescence.

The following methods are available:

- **Intelligent fitting**
  – default intelligent automated option

- **Through fixed points**
  – user controls point positions (XY) and baseline type

- **Through chosen points on each spectrum**
  – user controls X point which the baseline travels through for each spectrum within the dataset

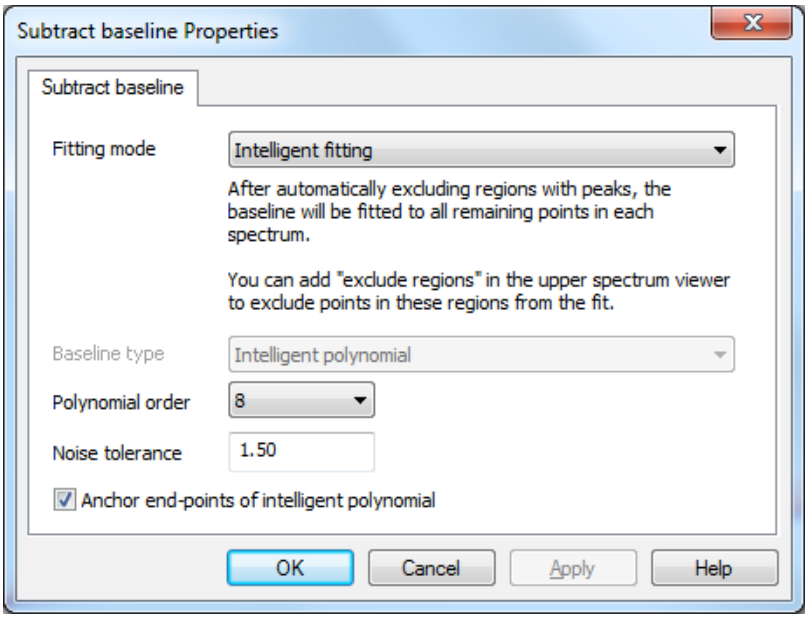
- **Through whole spectrum**
  – Automatic fitting with no in-built intelligence

With the spectrum open in a Viewer, select **Processing…Subtract Baseline**.

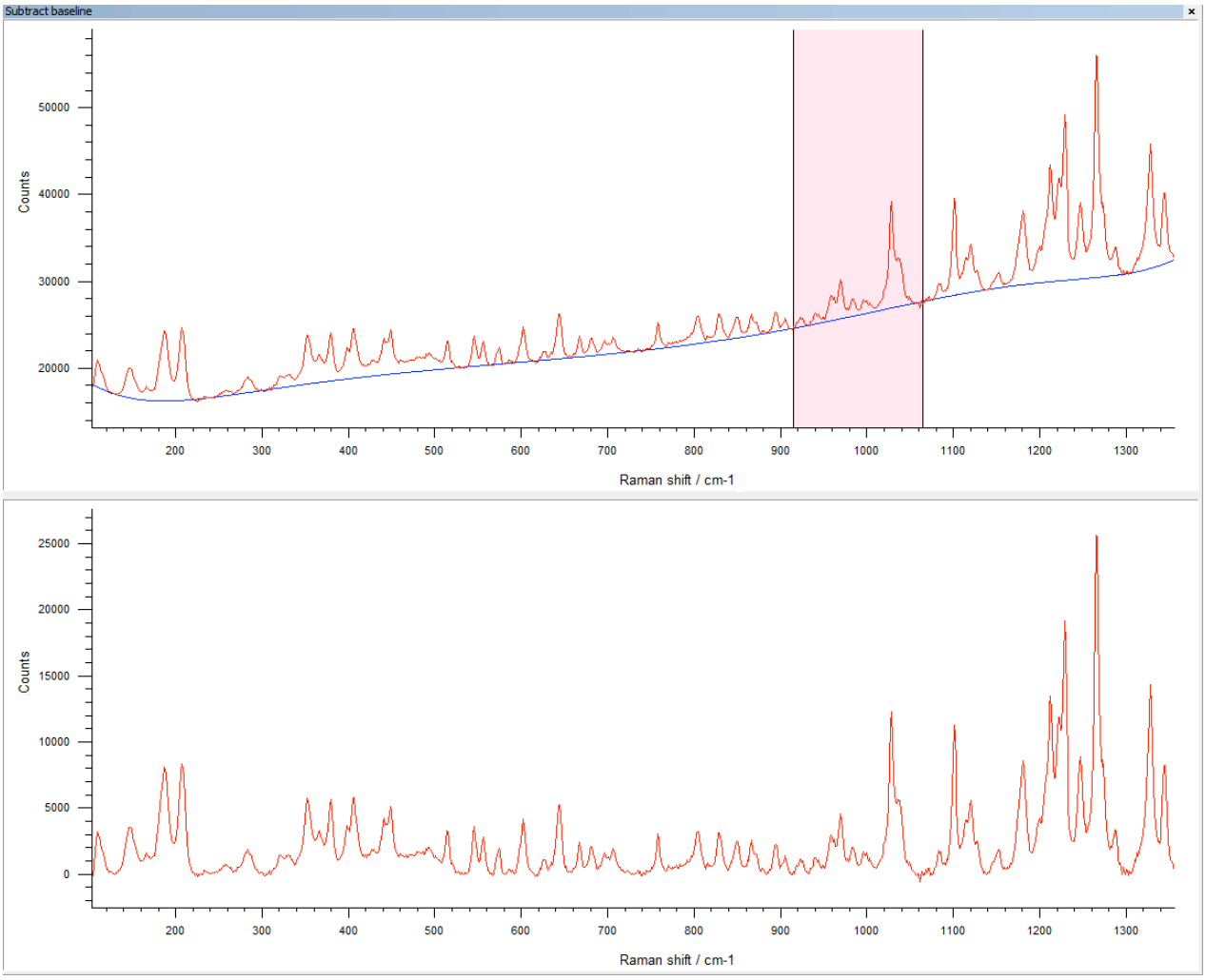
Intelligent fitting

A new Viewer opens with the spectrum in the top half and the result of the automatically applied baseline subtraction in the lower half. By default the 'Intelligent fitting' baseline is applied with a polynomial value of 11. This method is Renishaw patented and enables simple or complex backgrounds to be removed automatically.

Using a right click and selecting properties brings up the property page:



Here the polynomial order can be adjusted if a better fit is needed. The context menu also enables exclude regions to be added to the spectrum. Excluded regions do not contribute to the fitting of the baseline.



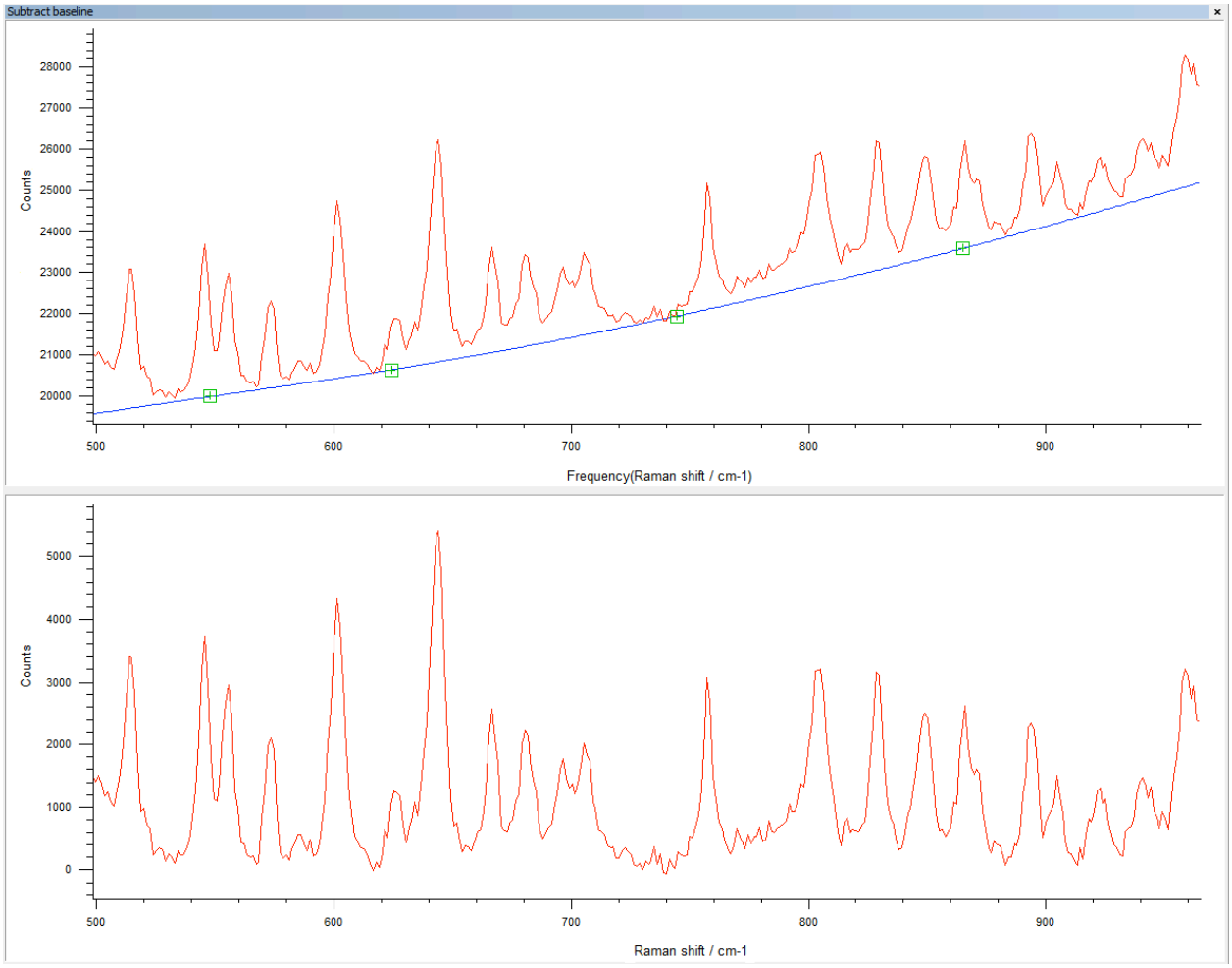
Intelligent fitting can be applied to single spectra and multifiles (e.g. mapping dataset). The baseline will automatically ‘fit’ each spectrum within the multifile.

Through fixed points

Selecting ‘Through fixed points’ as the fitting mode enables the user to manually specify points in XY to determine the baseline shape.

The user can choose between ‘polynomial’ (and the order) and ‘cubic spline’ options. Cubic spline is only available if 2 points are added (4 total points). This method can be applied to single spectra or multifiles, but the baseline is fixed and will not ‘fit’ to different spectra within multifiles.

It is useful to zoom in, by left-clicking and dragging in either window, and adjusting the points added in the top window by moving them with the mouse.

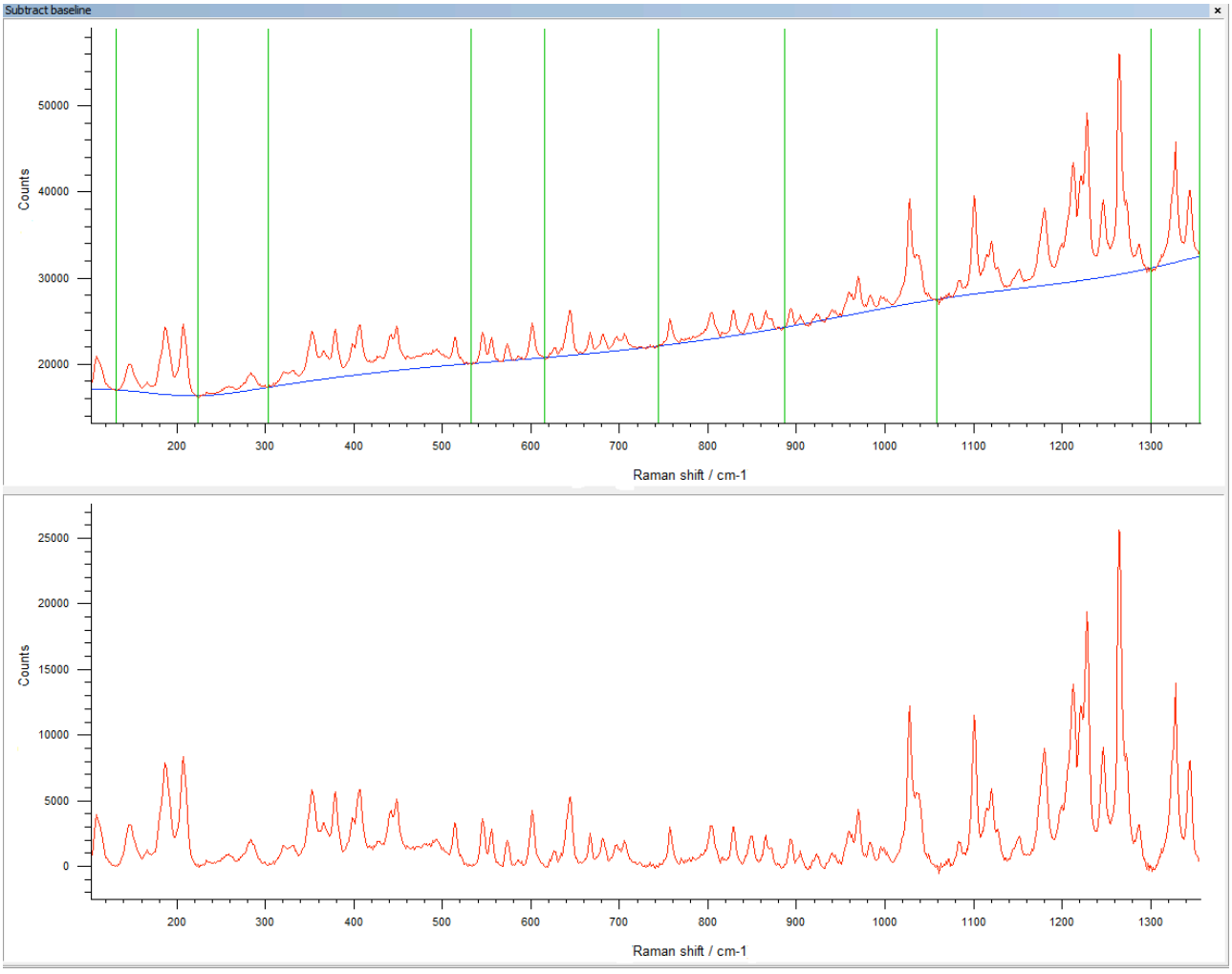


Through chosen points on each spectrum

Selecting ‘Through chosen points on each spectrum’ as the fitting mode enables the user to manually add points (vertical lines) to the spectrum which are fixed to the data.

The user can choose between ‘polynomial’ (and the order) and ‘cubic spline’ options. Cubic spline is only available if 2 points are added (4 total points).

This method can be applied to single spectra or multifiles. When applying to a multifile, common X positions where no Raman bands are present should be found. The baseline will optimise based on the X position for each spectrum within the dataset.



Through whole spectrum

Selecting ‘Through whole spectrum’ automatically fits a defined polynomial order through the entire spectrum.

The context menu enables exclude regions to be added to the spectrum. Excluded regions do not contribute to the fitting of the baseline.

This method can be applied to single spectra or multifiles, and is a less intelligent equivalent to the recommended intelligent fitting option.

Accepting a correction

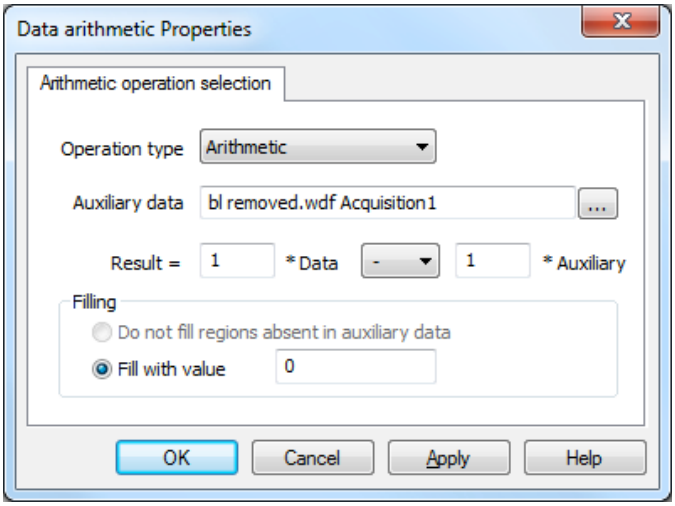
When you are satisfied with the correction, either select **Accept** from the context menu or close the window, upon which, there will be a prompt asking if you want to keep the correction.

To save the change to your file use the **File…Save** or **Save as** option from WiRE.

Arithmetic functions on data

A variety of mathematical operations can be performed on single data files. For example, you can add files together or subtract one from another. It can be an effective method of subtracting a background spectrum or filter ripple profile.

With a file open, select **Processing…Spectral Arithmetic**. A new viewer will open, split into three separate areas. The upper displays the sample spectrum, the middle will show the 'auxiliary' data, i.e. the data file you would like to add/subtract/multiply by/etc., and the lower region will show the result spectrum. Use the Spectral Arithmetic Properties window to browse for the auxiliary data and to select the arithmetic function.



It can be useful to multiply either the sample file or the auxiliary file by a factor so that the Y axes are comparable. In the example below, 1* the sample file has been used and 2* the background correction file used to remove the baseline. **Accept** the change either from the context menu or by closing the window.

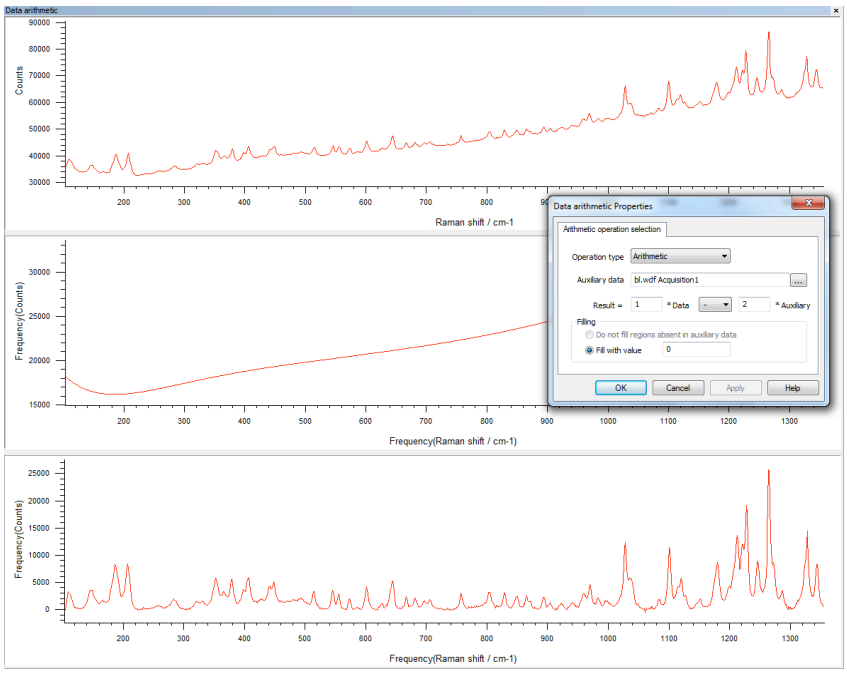


Image arithmetic

A special case of arithmetic is performing functions on Raman image data, i.e. images created from mapping measurements.

Ratio images can be generated form the 'Map generation' option (see TM014). More complex image arithmetic is performed using the data arithmetic option.

The initial image is loaded into the viewer (data tab of the **Navigator...derived data…..right click….. load dataset**). Under Processing…Data arithmetic, select auxiliary data (image) by browsing for the mapping measurement wxd file, then selecting the image from the drop down in 'Use derived data'. Use the value boxes to adjust the Data and Auxiliary scaling.

The format (image or surface) and LUTs of each image (initial, auxiliary and result) can be adjusted from the context menu (**View…View mode** and**…LUT control**). Accept or reject the result image.

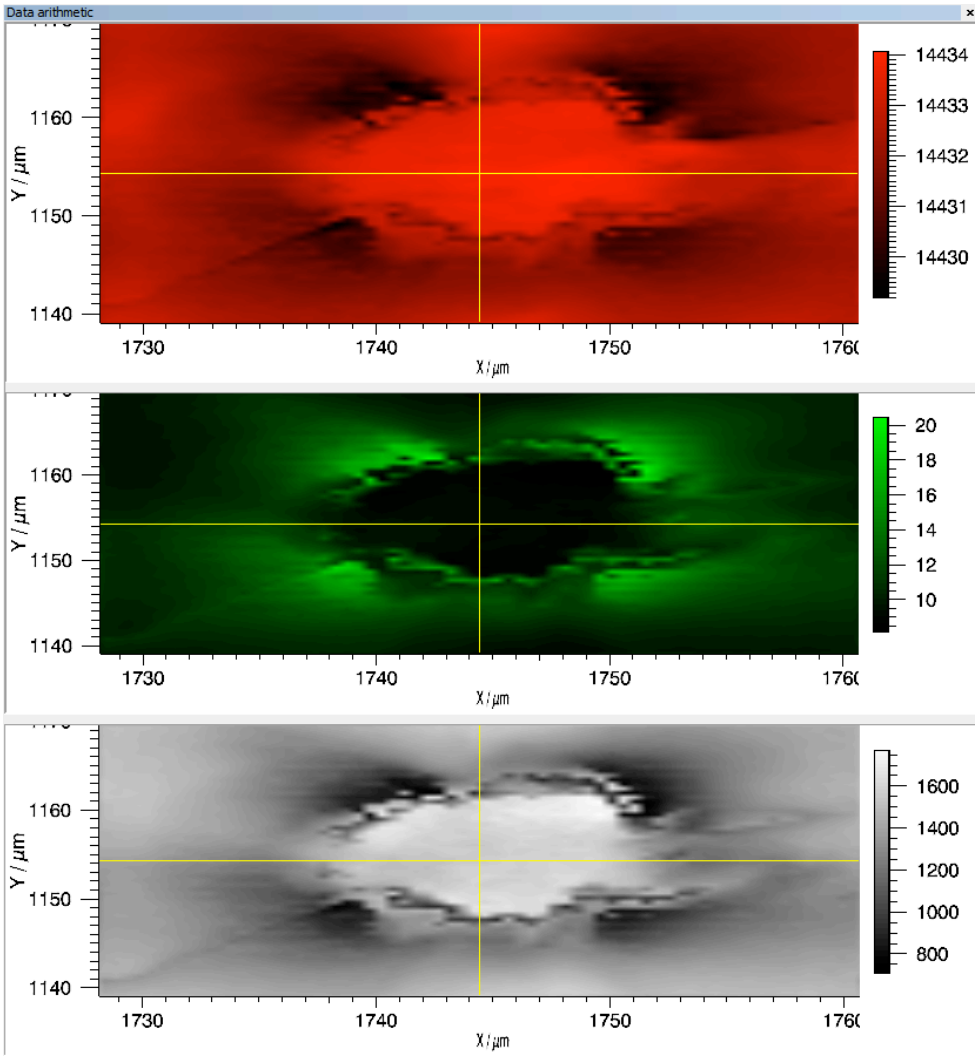


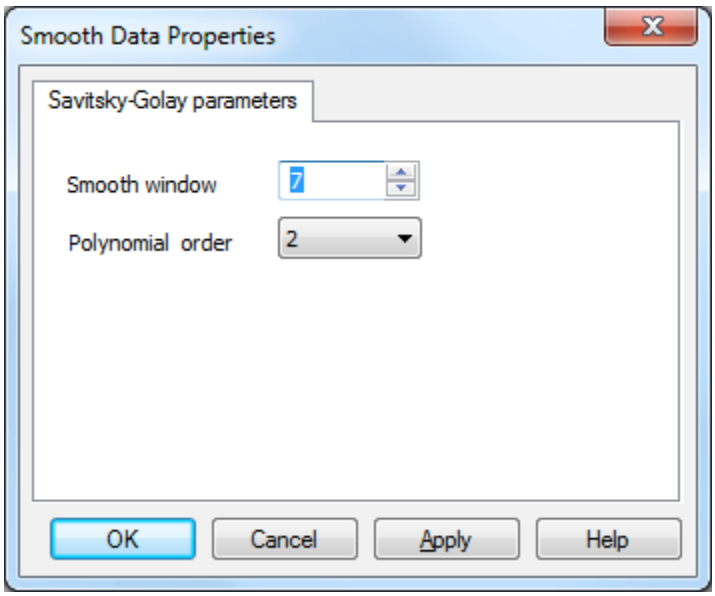
Image 1

Auxiliary image

Result image (Image 1 divided by auxillary)

Smoothing

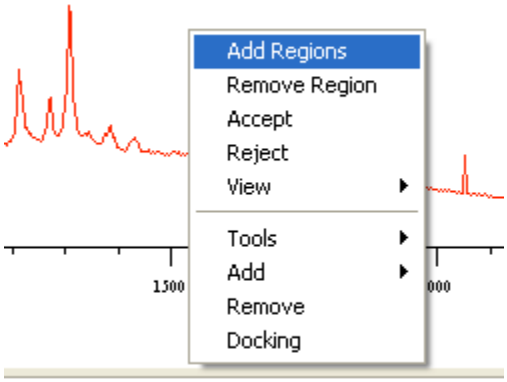
It can be useful to smooth data. This operation has the effect of improving the signal to noise ratio but must be used with caution as it degrades the spectral resolution. Smoothing is no substitute for performing a better measurement, i.e. using longer acquisition times or more accumulations. When using SynchroScan, the binning function can be used (again, with caution) to gain a better signal to noise ratio. To perform smoothing, with the file you wish to smooth open, select **Processing…Smooth**. A new window will open with the sample spectrum at the top and the result spectrum below. This data will be smoothed. To increase or change the degree of smoothing, select **Properties** from the context menu to see the **Smooth Properties** window.



The application uses a Savitsky-Golay algorithm. Use the ‘Smooth Window’ and ‘Polynomial Order’ functions to change the degree of smoothing. Pressing ‘Apply’ performs the change and ‘OK’ completes the operation. You can use the zoom function to see more closely the effect of the smoothing. You will be asked if you want to accept the resulting smoothed spectrum.

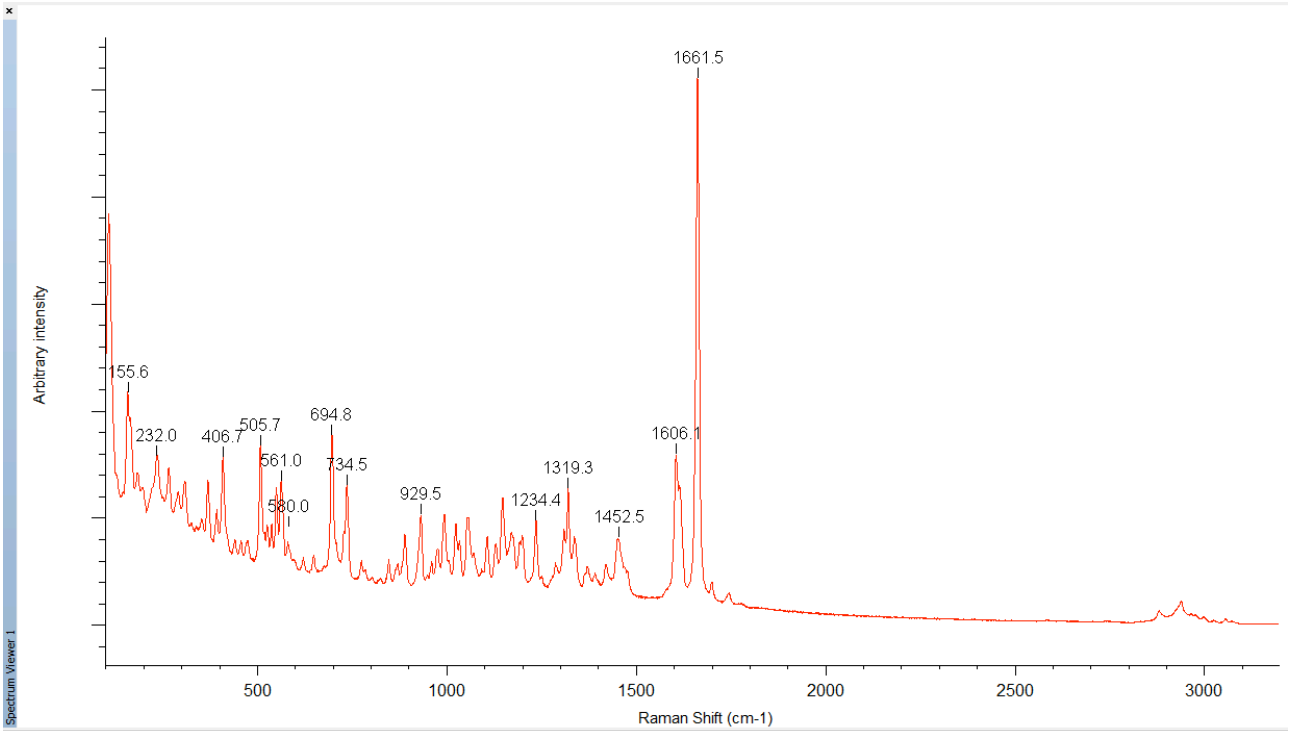
Zap

Stray bands can be removed from the spectrum using the Zap function. Generally, these will be cosmic ray features or other spurious lines. Ideally, the measurement would be re-performed but you may decide that zapping is acceptable. To remove a band on an open spectrum, select **Processing…Zap**. A new viewer will open with the sample spectrum at the top and the result spectrum below. The upper spectrum has a zap region between two vertical black lines. Grab each vertical bounding line in turn and adjust the position of the zap region so it just encloses the band to remove. Then use the zoom function to isolate the band to zap out. Notice that the result spectrum updates to show the effect of the zap. Additional zap regions can be added from the context menu.



Peak Pick

Peak pick is a quick and simple method to label band positions on a spectrum and enable these to be printed out together. To initiate peak picking select Analysis > Peak pick, or click the Peak pick button on the Analysis toolbar.



Peak no.	Centre	Height	Width	Area	Absolute inten...	Low edge	High edge
1	155.638	10250.2	15.6173	1.77017e+006	21800.1	134.509	202.972
2	232.033	4266.84	13.1133	991115	15815.9	203.797	244.215
3	406.69	6914.77	7.37158	901089	15758.5	386.916	436.408
4	505.722	9853.63	7.19457	769548	16845	479.301	527.968
5	560.986	6768.61	9.40512	1.32467e+006	13499	528.793	622.002
6	579.968	4724.68	18.9718	207027	9156.73	623.652	642.624
7	694.777	12250.9	8.78093	672090	17786.9	667.369	716.036
8	734.483	7926.38	8.80111	536554	13144.9	716.861	761.404
9	929.515	6242.84	9.71824	429559	10337.8	908.229	951.947

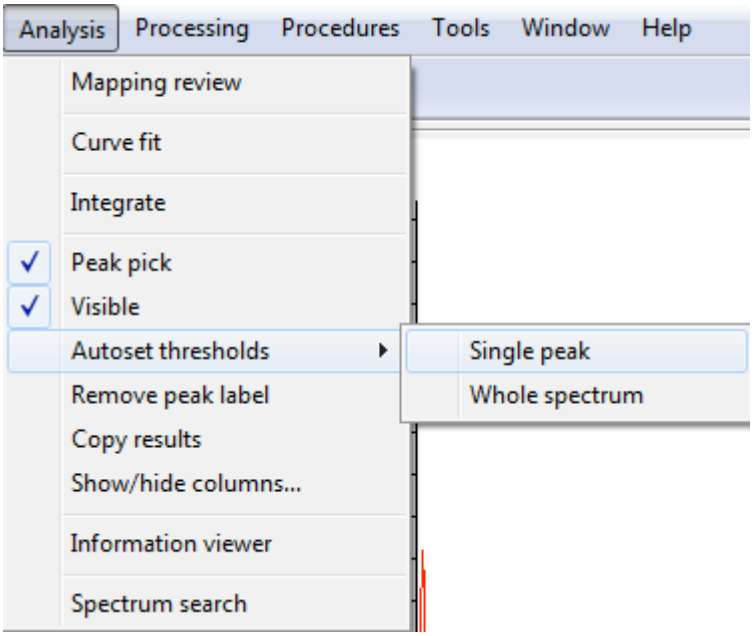
Peak pick

Note that it may be necessary to maximise the window containing the active spectrum in order to see the peak results table window, depending on where it is currently docked.

Peak Pick detects peaks for the active spectrum of the active spectrum viewer *using the current threshold settings* and displays the results. It also adds a peak results table to the current window, which gives details for the picked peaks, which can include some or all of the following information.

- Centre
- Height
- Width
- Area
- Absolute intensity
- Low edge
- High edge

Peaks are automatically labelled on selection of the Peak pick option from the Analysis menu.



If the peaks are not suitably labelled the following methods can be used to add or remove the labels:

1. Use the Autoset thresholds > Whole spectrum option. This sets thresholds so that a limited number of the best-defined peaks will be found, and then performs peak picking. The maximum number of peaks can be set on the Automatic Thresholding tab.

2. Use Autoset thresholds > Single peak option. Zoom-in on a single peak (including some baseline either side of the peak) and then select this option. This function sets thresholds to locate all peaks in the spectrum that are as well defined (or better defined) than the displayed peak. Peak picking is then performed.

3. Manual peak addition is performed by using a double left mouse click close to the peak to be labelled. The software will locate the closest peak with 3 falling point either side of the maximum and label this peak. Complete manual control can be achieved by reducing the number of falling points to 1.

   To reduce this value to 1, right click on the peak pick table and select properties. In the find peak tab reduce the number of falling points option to 1 and select OK. A double click on the spectrum will now add a peak label exactly where mouse cursor is located.

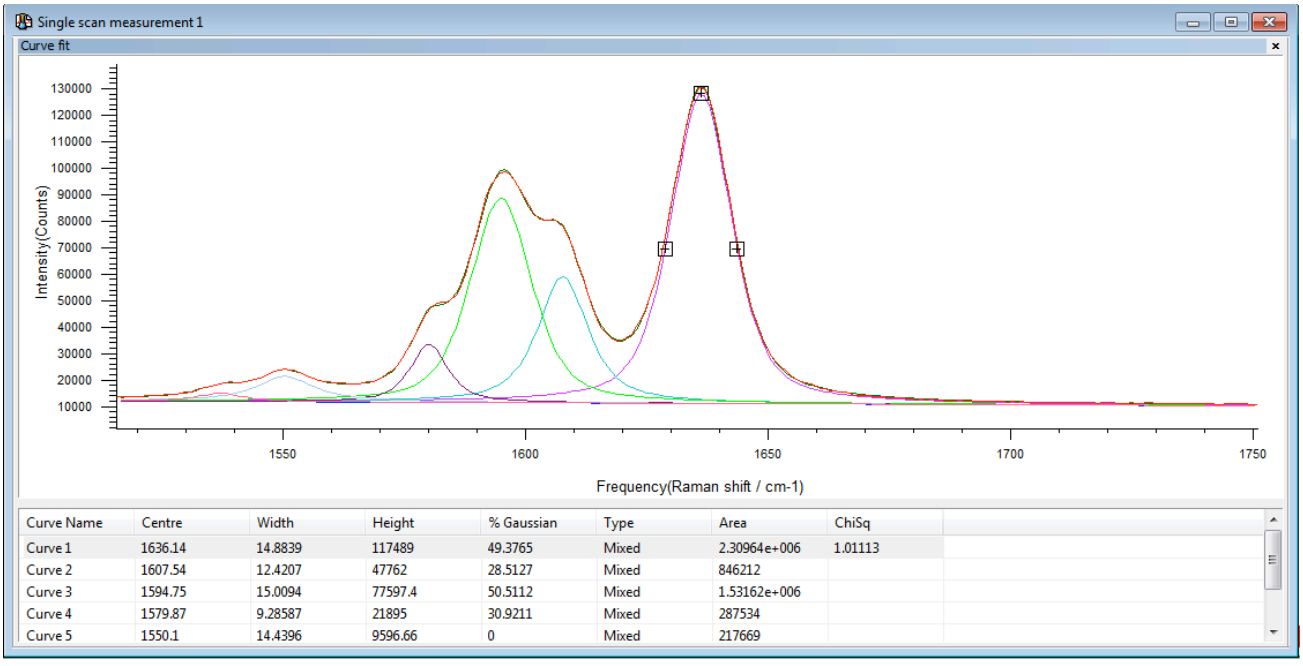
4. Manual peak removal is performed by right clicking on the relevant peak label in the peak pick table and selecting remove peak label

The peak result table may be copied to the Windows clipboard by selecting the Copy results option from its context menu (shown by right-clicking it). From here it may be pasted into e.g. spreadsheet or word-processing programs.

Curve-fitting

Curve-fitting calculates highly accurate values for simple, single bands but also for complex band systems where there may be two, three or more bands that overlap. Curve-fitting can produce a .wxc file that can be saved and applied later to a spectrum or set of mapped data.

To fit a curve to a band or series of bands, select **Analysis…Curve fit** to open the Curve fit window, zoom in to a region that contains the band and some baseline data either side. A baseline may be added automatically between the end points of the spectrum. This can be used, or removed via the context menu. Use the mouse to position the approximate centre of the band. Click to add the band and repeat for the centres of other bands if part of a system of bands. You may need to use the context menu and select 'Add Curves' if the curve symbol does not appear with the cursor. Pressing 'Remove curve' from the context menu will remove the last node you added.

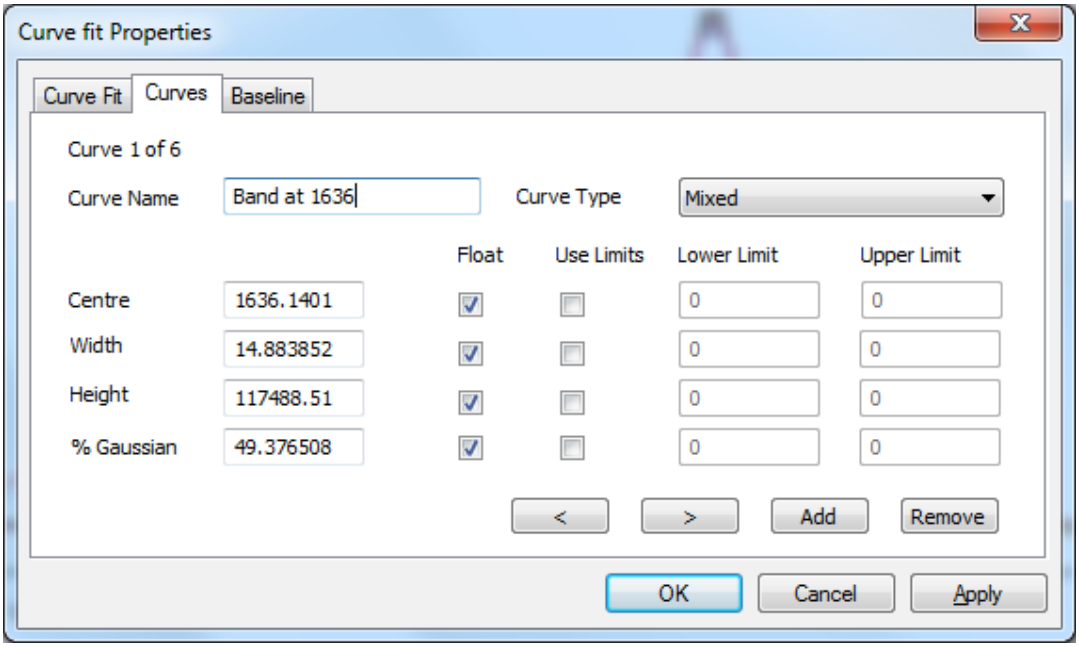


Curve Name	Centre	Width	Height	% Gaussian	Type	Area	ChiSq
Curve 1	1636.14	14.8839	117489	49.3765	Mixed	2.30964e+006	1.01113
Curve 2	1607.54	12.4207	47762	28.5127	Mixed	846212	
Curve 3	1594.75	15.0094	77597.4	50.5112	Mixed	1.53162e+006	
Curve 4	1579.87	9.28587	21895	30.9211	Mixed	287534	
Curve 5	1550.1	14.4396	9596.66	0	Mixed	217669	

Select ‘Start Fit’ from the context menu to fit the added curves to the data. The algorithm will perform many iterations until the best fit has been achieved.

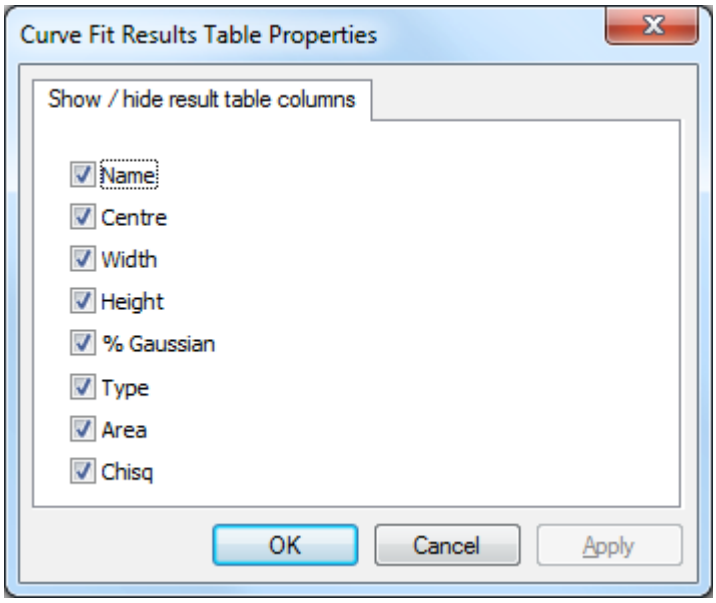
You can save the curve fit file as a *.wxc from the context menu (**Curve parameters….Save curves**). To reapply this saved curve ‘template’, perhaps to a similar sample, start the curve fit application and use the context menu (**Curve parameters…Load curves**) and then ‘Start fit’.

You can modify or make changes to the curve fit using the Curve Fit Properties window from the context menu **Properties**. This provides greater control over the fitting process instead of the automatic parameters that are usually used. For example, you can choose to fix a band centre instead of letting it ‘float’ during the curve fit, or apply limits to parameters. This can be useful for complex band shapes. Curves can also be named, different types of baseline can be used or the curve type can be defined. Use the ‘Curve Fit’, ‘Curves’ and ‘Baseline’ tabs to adjust the curve fit.



The context menu allows the truncation of the fitted region on zooming (Fit viewed region). It is generally beneficial to have this ticked. If this is not active then the baseline form and height will be somewhat dependent upon other bands that are present throughout the whole spectral range. It may be necessary to re-apply the baseline on zooming, as its original position will be persisted.

A curve-fitting procedure produces a table of data; the columns are selected from the context menu of the table (**Show/hide columns**). The table lists the various parameters for each of the curves. The data in the table can be copied and pasted into a spreadsheet package, for example (context menu, **Copy results**).

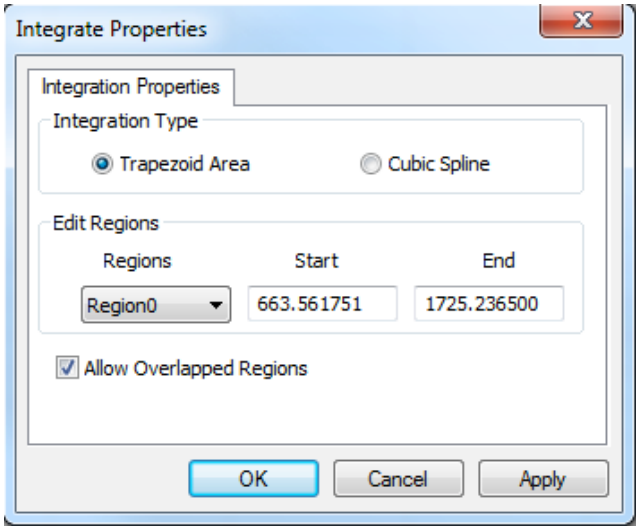


The fitted curves, baseline and result curve (sum of the fitted curves and baseline, if used) can all be saved and reloaded as 'spectra'. Once the curve fit has completed, select **Save curve data** from the context menu and save to a location. This saves a multifile that can be opened like a spectrum in WiRE 3. Use the Data tab in the Navigator and expand the branches to show the Collected data. Highlight each 'acquisition' and right click to show 'Load dataset'. To save the curves, result, or baseline as a separate 'spectrum' or trace, highlight the trace in the View tab of the navigator and select 'Save spectrum as' from the context menu.

Integration

The integration option provides a method where the total area under the spectrum can be determined.

The left and right vertical bars determine the region which is being analysed within the spectrum. The properties are selected by using a right click on the spectrum. These enable the exact start and end position to be defined and the type of integration (Trapezoid or cubic spline) to be selected.



Trapezoid calculates the area between adjacent points using a trapezium drawn between the points and the x-axis.

Cubic spline approximates the spectrum with local cubic polynomial models and uses integration to get an estimate for the area between adjacent points.

In each case the result is the sum of areas across all pairs of points in the region

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TM26 - Frequently asked questions (FAQ) WiRE™ 4

Introduction

This module offers a list of common questions and problems; offering possible solutions to them without having to delve into the manual or contact Renishaw for technical support.

Q1. Help! It's not working!

Whether new or experienced to their operation, the cure is nearly always very simple. Below are summarised a few of the common reasons why you may not be getting a spectrum.
If you are having trouble making the instrument or laser operate, check all of the following to ensure that the instrument and all accessories are powered up correctly.

- With the WiRE software closed, check that the instrument and accessories are plugged in and switched on.
- Ensure that the laser (and if necessary its power supply) is plugged in and switched on. Since there are many different types of laser, refer to its individual manual if you require further help with this.
- Check that the door of the instrument is securely closed and locked and that the interlock is operational.
- Check that the Class 1 enclosure door (if present) is securely closed.
- Start WiRE.

If all these operations have been checked, you are ready to capture your spectrum. With your sample under the microscope, and the WiRE™ software loaded and ready, check the following if you are still not getting a spectrum.

- Use a standard sample such as a silicon wafer. This is strong and sharp with the 1st order band located at 520 cm^{-1}.
- Ensure that your sample is loaded correctly under the microscope, that it is sharply in focus and that you are looking at the correct portion of the sample. It is often worth trying a different region within the sample because of the possibility of impurities giving unexpected results.
- Check all the settings in the measurement setup dialogue. If you have cosmic ray removal engaged, remember that this takes two additional, undisplayed spectra; this process may take some time depending on the scan time chosen so don't be concerned with the delay in spectral display.
- Check the laser spot is on the crosshair of the video. If not use the manual adjust for the bottom left beamsteer (Tools….Manual beamsteer) or perform a Laser autoalign.
- Check the correct lens set is within the instrument. The lens set is clearly labelled and the user is prompted on configuration change, where a change is necessary.
- Perform a CCD area auto align (inserting a silicon sample under the microscope for non-Reflex systems).
- Perform a slit auto align (search and optimise).

Now that this has helped you get a signal from your sample, you may find it is particularly noisy, if this is the case, try the suggestions below on how to improve signal-to-noise ratio and signal-to-background ratio.

Q2. Why do I keep getting random, sharp peaks in my spectra?

These are the result of cosmic rays. High-energy particles, passing through the CCD detector resulting in the generation of electrons which are, in turn, interpreted as signal by the camera. They are completely random in their time of occurrence and the position where they strike. Cosmic rays are very intense, resembling emission lines, and possessing a very small FWHM (< 1.5).
To confirm the presence of a cosmic ray, immediately re-capture the data and you will notice a distinct absence of that feature. If however the line still exists, it is most likely a result of spectral contamination from room lights, etc. For further information, see 'I keep getting repeatable, sharp peaks in my spectra ...'

Cosmic rays become increasingly common with increasing exposure time. For long scans, where the presence of cosmic rays must be avoided, consider using the cosmic ray removal feature. This is an option in the experiment set-up window, when activated the spectrum is collected in triplicate (equivalent to 3 accumulations). The software uses the median value at each wavenumber value to ensure no cosmic ray features are seen.

Q3. I keep getting repeatable, sharp peaks in my spectra. What are they?

If you have repeated the scan and the spurious lines are still present in **exactly** the same place, the possibility of them being cosmic rays has been ruled out. Such sharp repeatable lines are usually due to emissions from fluorescent room lights or phosphorous in CRT monitors (figure 6.1). Using long working-distance objectives worsens the problem.

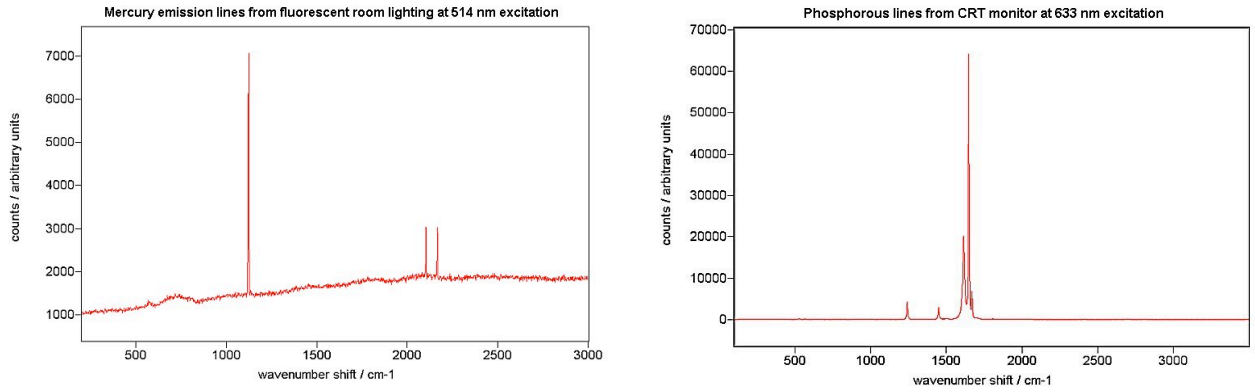

Fig. 1. Fluorescent room lights (left), monitor phosphorus lines (right)

Fluorescent lights result in spectral contamination from mercury emission lines; simply turn off all fluorescent lighting in the room and work under the minimum incandescent light. The room should be as dark as practicable. Similarly, a thorough effort must be made to exclude sunlight from the room since spectral aberrations will results from the numerous emission lines of 'white' light.

Phosphorous lines are often present due to the phosphour coating in all CRT monitors; if such lines are a problem, turn the monitor off or reduce the contrast until the screen is darker.
It is important to remember that emission lines are always present at the same position on an absolute wavenumber scale and will therefore be seen to move on a scale of Raman shift when using different laser wavelength. These lines are more prominent when using collection optics of longer working distance.

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Q4. Why do some of my spectra give such an intense background signal that masks the Raman information?

A high background in a Raman spectrum is the result of sample fluorescence (or phosphorescence); an intrinsic property of the material of the sample. Unfortunately, this is an unavoidable consequence of laser irradiation and in many cases the fluorescence is stronger than the Raman signal. Despite fluorescence being an unavoidable side effect, steps can be taken to minimise or irradicate the problem.

- **Change laser wavelength:** the approach that will have a most significant effect for highly fluorescent samples. In general, fluorescence is worse with visible lasers, and moving to a laser in the UV or NIR is likely to cure or reduce the problem. Renishaw manufacture and can supply a wide-range of lasers from UV through to NIR.

- **Quenching:** possible with some samples. By leaving laser light incident on the sample for a period of time before acquiring a Raman spectrum, it is sometimes possible to quench (reduce) the fluorescent background, enhancing the Raman features. The period of time required is sample dependent but normally some effect is observed in seconds to minutes. It is worth noting however, that quenching is exponential and therefore the greatest effect will be seen initially. Cycle the spectrum to see this affect occur with a live update.

- **Confocal mode:** by acquiring data from the small sample volume that is strongly irradiated by the laser, the fluorescence may be greatly reduced. This approach may also be beneficial where the sample being investigated is contained within a substrate that is strongly fluorescent, for example, a sample confined within a fluorescent matrix.

If there is too much ambient light in the room, either fluorescent or incandescent, it is possible this may cause unnecessary background signal in your spectra. It is best to work with lighting at a minimum, however, if this is not possible, for example, if the instrument room is used by other people, consider the use of a Renishaw enclosure. This prevents stray light from entering the instrument and further minimises exposure to the laser beam. The enclosure is available in either Class I, or Class 3b, laser safety forms.

Q5. Why is my signal so weak and / or why do I get such a poor signal-to-noise ratio?

If the signal is weak, first check that the sample is correctly placed under the microscope and sharply in focus; you could also try moving to a different sample point. Check that the instrument is set up for Regular mode and check the laser power setting; if the power is less than 100%, try increasing it to improve the signal. If the spectrum is very noisy, this may be improved by increasing the scan time or number of accumulations.

- Increasing the scan time allows the CCD to acquire more Raman signal, enhancing the features over the extraneous noise. This method is ideal if both the background and Raman signal are low, however, if either of these is intense, then increasing the scan time increases the chance of saturating the CCD.

- Accumulating the data takes a number of identical scans and co-adds them together, enhancing weak Raman features from the random background noise and improving the signal-to-noise ratio.

Careful adjustment of these two parameters allows the maximum possible exposure without saturation and will improve the signal-to-noise ratio. It is worth bearing in mind that the signal-to-noise ratio is proportional to the square root of the number of accumulations; 4 accumulations provides a two-fold improvement in the signal-to-noise ratio.

Another factor as important as the signal-to-noise ratio is the signal-to-background ratio; these two ratios are intimately linked. If the background component is high, it will mask the Raman signal and contribute noise to the system. See also **'Why do some of my spectra give such an intense background signal which masks the Raman information?'** for further details.

Q6. How can I stop my sample from being damaged by the laser?

The laser spot incident on the sample has a high power density. This is especially true of UV systems and those with high laser powers. Unfortunately, some samples are susceptible to thermal or photo degradation. The resulting spectrum will contain features caused by modification, not natively present in the sample (for example, broad amorphous carbon bands around 1500 cm^{-1}). Often, viewing the white light image before and after acquisition will indicate a clearly altered region of sample (Figure 2) where the laser was incident.

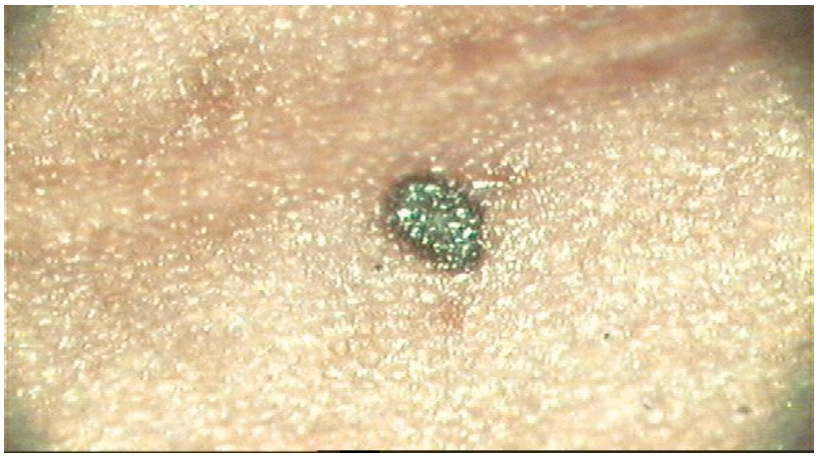

Figure 2. Laser induced sample damage

To prevent damage, it is prudent to start initial analysis with low laser powers, especially when using NIR and UV systems, from here, the power can be increased, balancing sample damage prevention with the needs for a strong signal.

If reducing the laser power to very low levels (<1%) still results in sample damage, use of a **line focus** accessory may help. The line focus reduces the laser power density by spreading the laser power out over a greater area. This increases the number of Raman scatterers and the resulting Raman signal is therefore significantly higher than conventional methods.

Conventional methods include:

- Using a lower magnification objective to reduce the power density at the sample (this produces a larger spot size but also produces less Raman singal as the numerical aperture is significantly lower)
- Defocusing the laser spot using the beam expander.

Line focus is a superior option for faster data collection as it not only reduces the power density, but also optimises the throughput in the spectrometer (unlike the beam expander spot defocus method).

Q7. I can't fit my sample on the stage because its a liquid / powder / very large! What can I do?

While Renishaw Raman instruments provide an excellent way of analysing samples with very little preparation, some samples can't simply be placed on a microscope slide. It is possible to place samples with heights of up to 50 mm directly onto the stage. **Renishaw's macro-sampling kit** provides an excellent way of dealing with problem samples such as powders, liquid or samples which are large and can't be easily placed on the stage while still requiring no sample preparation.

Large samples which maynot fit under the microscope can be analysed using a **flexible sampling arm**. This enables Raman analysis external to the microscope and enclosure. As the arm is direct coupled it has all the resolution and throughput benefits of the inVia – unlike a fibre probe coupling.

Fibre probes are available and are ideal for distant Raman analysis, and integration within other instruments.

Q8. How can I stop my sample from moving around on the microscope stage?

It is important that samples can be constrained so they do not move during analysis. This is even more important when using Raman mapping methods. Flat samples such as polymer films need to be held flat so the laser focus does not change during analysis or depth profiling. Other samples need to be held in XY so they do not slide or shift during fast mapping experiments. The **high speed encoded stage accessory kit** (HSES) enables samples of all types to be firmly held in place to prevent experiments having to be repeated.

Q8. I'd like do be able to examine my samples under different pressures, is there a way I can do this?

The diamond anvil cell, available from Renishaw, enables you to analyse your samples under high pressure.

Q9. I want to be able to perform polarisation measurements. Is this possible?

A polariser and half-wave plate set for each wavelength may be purchased from Renishaw. These enable you to examine the molecular symmetry of your sample and assist in assigning bands to vibrations within the molecule. Motorised laser polarisation control is also available.

Q10. I've noticed that placing my sample in different orientations gives a different spectrum. Why is this?

This is caused by the laser being incident on different crystal planes within the sample. Using a quarter-wave plate can help to remove these orientation effects by scrambling (circularly polarising) the Raman/laser light. This method is often of use to confirm relative intensity Raman information is not sample orientation induced (e.g. for highly ordered systems such as polymers or single crystals).