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TM027 - Experimental process and inVia options WiRE™ 5

The inVia and WiRE 5 software together enable almost any Raman or photoluminescence experiment to be performed due to the very high performance and flexibility of configurations on a single system. This is used for a multitude of analysis requirements, from simple material identification to more complex multi-dimensional imaging, and combined multi-technique analysis (e.g. combined AFM or SEM analysis).

This document aims to explain how to use the inVia instrument and WiRE™ software to most efficiently achieve these different sample analyses aims. This is achieved by understanding the key requirements at each stage of the experimental process, and how both instrument and software enable these requirements to be met.

Overview of the experimental process

Stage 1. Aim of experiment

- What are we trying to find out?
- Define the resulting information which is required from the Raman experiment
- How does this fit with the overall problem which needs solving?

Stage 2. Information gathering

- Collate as much information as possible on the sample to simplify the experiment (e.g. what type of material, how much material, particle sizes, etc.)

Stage 2a. (*For mapping only*) Analysis of bulk sample and references

- Preliminary analysis to define the initial experimental parameters (e.g. collection of pure reference spectra to select an appropriate spectral range).

Stage 3. Sample preparation and presentation

- Prepare or synthesise the sample.
- Mount / immobilise the sample under the microscope.

Stage 4. View sample / define analysis region

- View the sample area of interest.
- For mapping experiments, determine the spatial area and step size for analysis.
- Select a microscope objective which produces an appropriate illumination / collection volume.

Stage 5. Optimise experimental parameters

- Optimise the experimental parameters iteratively until the required data quality is obtained.

Stage 6. Collect data

- Apply optimised parameters to collect remaining data.

Stage 7. Process data

- Process data to remove unwanted artefacts (e.g. noise, non-linear backgrounds, or cosmic ray features).

Stage 8. Analyse data

- Analyse data to obtain the required results.

Stage 9. Validate data

- Validate data to ensure reliable results are obtained.

Stage 10. Report results

- Report and present results in an appropriate format.

The entire experimental process is simplified where pre-knowledge of the sample is available, perhaps from previous analyses, or even user experience. Where this information is not known, more time and effort is required for iterative optimisation of experimental parameters.

Thinking ahead about later experimental stages can lead to a more efficient experimental workflow, as this can reduce the number of iterative cycles needed to achieve the overall experimental aim.

Stage 1. Aim of experiment

Raman spectroscopy is a versatile technique for chemical and structural identification, which can yield a large amount of information for a given material (see TM1: Introduction to Raman spectroscopy). It is important to define the aim of any experiment early on, so that the correct analysis is performed and the required result is obtained with confidence.

Stage 2. Aim of experiment

Information from third party sources can aid in defining the experimental aim by providing more focussed experimental parameters. This is especially the case when performing more complex and lengthy experiments where additional parameters, such as domain size, might otherwise require experiments to be iteratively optimised.

Stage 2a. Define initial conditions (mapping only)

When mapping a sample, some preliminary measurements are usually required to establish appropriate initial experimental parameters. If optimised parameters have already been determined for equivalent samples, these can be reapplied without further preliminary work.

Initial conditions to be established include:

- **Map spatial dimensions** to obtain sufficient sample coverage and spatial resolution (step size).
  If conducting a 2D mapping experiment, the user might survey a large sample area using a white light montage (see TM007: White light image capture, montaging and Surface generation). The mapping region can then be defined based on features seen in the montage.

- **Mapping measurement mode.**
  If sample domains are larger than 1 μm, then StreamLine enables rapid Raman imaging with appropriate spatial resolution. (See TM010: StreamLine imaging)
  If sample domains are smaller than 1 μm, then StreamLine HR enables high resolution Raman imaging with XY step sizes as small as 100 nm (see TM009: StreamLine HR imaging).

- **Spectral range** for differentiating chemical and structural species (i.e. where are the Raman bands for different materials?).

- **Spectral range and resolution.**
  For a given laser wavelength, a diffraction grating with lower dispersion (or fewer grooves/mm), will result in a larger spectral range but with lower spectral resolution. This compromise between spectral range and resolution should be considered when choosing between different grating and laser wavelength combinations.

For some analysis requirements, this preliminary step of inspecting the bulk material can also fulfil some of the top level aims of the experiment.

Stage 3. Sample preparation and presentation

Sample preparation is the process by which the sample is readied for analysis, while sample presentation refers to the method by which the sample is placed under the microscope. Some issues that a user should consider are:

- **Sample flatness / levelling relative to the laser focus.**
  For 2D maps, the sample surface should be flat and level relative to the laser focus, where possible.

- **Substrate.**
  If possible, the user should select a substrate which does not produce spectral features in the spectral range of interest. For many specimens, stainless steel slides are recommended. For bulk samples the substrate becomes less important.

- **Immobilisation.**
  The sample should be securely positioned on the microscope stage to ensure repeatable movements during mapping experiments. Use either slide holding clips or magnets (supplied as part of the High Speed Encoded Stage accessory kit).

Stage 4. View sample / define analysis region

In stage 3, the user views the sample (see the General instrument use notes...'Load and view samples' page) and defines the analysis region (see the General instrument use notes...'Setup spatial measurements' page). Typically, the user surveys the sample and specifies region(s) for data acquisition.

Stage 5. Optimise experimental parameters

In Stage 4, experimental parameters are iteratively optimised until the required data quality is obtained. Optimisation of experimental parameters is an advanced application development step, but is often required for more demanding analysis.

The inVia presents a number of parameters which the user may wish to optimise using processes such as (see also TM004: Measurement set-up and data acquisition):

- **Selecting an appropriate laser excitation wavelength.**
  Use a laser wavelength that avoids the spectral contribution of strong fluorescent backgrounds to more clearly observe Raman bands (see TM026 FAQ: Q4. Why do some of my spectra give such an intense background signal that masks the Raman?).

- **Selecting an appropriate microscope objective.**
  A low numerical aperture (NA) objective has a larger sampling volume (i.e. less confocal), so the resulting spectrum is collected from a larger volume.
  A high NA objective has a smaller sampling volume (i.e. more confocal) and is more appropriate for performing high resolution depth series (see 'Depth profiles').

- **Optimising the incident power density and integration time.**
  The incident power density and integration time can be optimised to obtain a high quality Raman spectrum, while avoiding CCD saturation.

- **Verifying that laser modification of the sample has not occurred.**
  High laser power densities can induce thermal / photo degradation of the sample. Laser damage can be identified by noting visible changes, and / or spectral changes either in the Raman bands or baseline (see TM026 FAQ: Q6: How can I stop my sample from being damaged by the laser?).

- **Verifying that the laser has remained in focus throughout the measurement.**
  Laser heating can induce deformation and movement of the sample leading to loss of focus. Visual verification of the laser focus at the sample surface is recommended. Alternatively, consider using the FocusTrack™ option (see TM006: FocusTrack).

Stage 6. Collect data

By this stage, optimised experimental parameters should have been obtained from previous experimental stages. The optimised experimental parameters are then reapplied to collect any remaining Raman data.

Stage 7. Process data

Data is processed to remove unwanted artefacts from the real spectral features of interest. Data processing enables accurate results to be generated during data analysis.

Data processing also serves as a basic form of experimental validation. During data processing, the user inspects the data and determines if the data set contains the required information for further analysis.

The following data processing procedures are typically used:

- **Zap and Cosmic Ray Removal (CRR).**
  Sharp, random peaks in the spectrum can be produced by cosmic rays on impact with the CCD detector (see TM026 FAQ: Q2: Why do I keep getting random, sharp peaks in my spectra?). If not removed from the data set, cosmic rays can skew the results of multivariate data analyses (i.e. spectral searches, DCLS, PCA and MCR-ALS).
  - For ***single spectra***, cosmic rays can be manually removed using zap.
  - For ***maps***, the cosmic ray removal tool automatically identifies and removes cosmic ray features.

- **Smoothing and noise filtering.**
  - For ***single spectra*** and small maps (<100 spectra), smoothing applies a Savitzky-Golay algorithm to smooth the spectra.
  - For ***maps*** (>100 spectra), noise filtering is performed by principle component analysis (PCA) reconstruction.

- **Subtract baseline and data arithmetic.**
  - ***Subtract baseline*** easily removes broad non-linear backgrounds from single spectra and maps. The tool automatically fits a baseline, which is then subtracted from the spectrum.
  - ***Data arithmetic*** enables weighted subtraction of a background spectrum so that only the spectrum of interest is retained.

Stage 8. Analyse data

The overall aim and the type of measurement affect the typical data analysis procedures used:

- **Analysis of single spectra** using peak pick, curve fit, and spectrum search.
  Peak pick is the quickest way of labelling band positions.
  Curve fit automatically fits a user-defined number of peaks and outputs accurate peak positions, intensities and widths (FWHM). Curve fitting is particularly useful for deconvolving complex Raman bands.
  Spectrum search automatically compares a sample spectrum against a spectral database of known materials for fast sample identification (see 'TM016: Database searching and creation').

- **Univariate data analyses of images.**
  Univariate data analysis methods produce images based on the variation in one band parameter (see 'TM014: Multi-file data analysis (univariate)').
  Univariate images can be analysed using data arithmetic to generate ratio or difference images.

- **Multivariate data analyses of maps.**
  Multivariate data analysis methods automatically correlate the entire shape of all spectra within a mapping data set, resulting in component images of the different chemical/structural species present (see 'TM015: Multi-file data analysis (multivariate)').

The ***Particle Analysis*** tool can be used to analyse images to obtain domain metrics such as area, equivalent circle diameter, perimeter, eccentricity, orientation, solidity and nearest neighbour distance (see 'TM017: Image domain analysis').

Stage 9. Validate data

Data validation ensures that the results make sense, and reliably meet the initial aims of the experiment. Data validation is achieved either qualitatively by user verification, or quantitatively by algorithm feedback during data analysis. Typical examples could be:

- Spectral database searching produces prospective matches, and ranks them by quality of fit.

- The look up table (LUT) of Raman images can be adjusted to better represent the spectral information. The user may choose to binarise each component if domains are pure. Conversely, the user may blend the colours representing different components if domains contain mixed species. (See 'TM018: Viewing and saving image data').

- Curve fitting of Raman mapping data can output an image of Chi squared values to indicate the quality of fit for a single band across the mapped region.

- Multivariate component analysis methods (DCLS, PCA and MCR-ALS) can output a lack of fit (or residual) image to highlight regions where the quality of fit between the collected spectra and the other spectral components is poor.

- Third party information and data from complementary analytical techniques can also be invaluable for validating the results obtained by Raman spectroscopy.

Stage 10. Report results

The way in which the end result is reported will vary depending on how the Raman analysis relates to a larger experimental work. Results may be required in the form of:

- A single value *e.g.* material identification, material property, or fraction estimate.
- A report presenting spectral comparisons or images.
- A comparison with third party information
- Direct or indirect output to third party software for process control (*i.e.* a feedback loop)
- Automatic incorporation into a laboratory information management system (LIMS)

(See 'TM019: Labelling and printing' on the use of labels when presenting spectral data.)