CMI STANDARD OPERATING PROCEDURE

Confocal Raman Microscope
Physiology Ground floor research lab

CMI document ID: SOP003

CONTACT INFORMATION:

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REQUIREMENTS FOR EQUIPMENT USAGE:

1. CMI user
2. Completion and signing of Microscope Safety Checklist
3. Certification by Peter Owens or Jennifer Connolly

REVISION LOG

Revision	Date	Author	Changes
1.0	12/5/14	P. Owens	Initial draft

1. Purpose

1.1. This document specifies the work instructions for the CMI Witec Confocal Raman microscope located in the ground floor Physiology lab, Quadrangle building. If you see an area where more clarification is needed, if additional information is needed, or if you have suggestions on how to make this guide more useful in the lab, please contact the CMI.

1.2. Note that this document is not a detailed instrument manual and does not intend to be one. For detailed questions or if anything unusual happens with the system, please refer to the manuals present in the lab, or ask CMI personnel for help.

2. Scope

These work instructions are applicable to **all** work that is carried out using the Witec raman microscope.

3. Important notes

PLEASE LEAVE ALL HARDCOPIES IN THE LAB

3.1 Laser safety guidelines

The WITec microscopes uses **class 4** 633 and 785 nm laser sources during operation. The safe use of lasers requires that all laser users, and everyone near the laser systems, are aware of the dangers involved.
CAUTION: Direct eye contact with the output beam from a laser will cause serious damage and possibly blindness. **The 785 nm laser has a high power output, extreme care is needed here when operating the system.**

- 3.1.1 Make sure the enclosure remains closed during laser scanning and measurement
- 3.1.2 Always close the laser shutter before opening the door of enclosure
- 3.1.3 Always wear the safety laser goggle when you want to open the door with laser on for alignment procedure.
- 3.1.4 Participation in a Laser safety course is strongly recommended for operating this system.

3.2. Witec Alpha 500 manual (hardcopy in the lab, soft copy on the instrument PC), and training notes

3.3 The CMI access policy, available online at http://imaging.nuigalway.ie/access%20policy/cmi_access_policy_1.6.pdf

3.4 This manual was developed to assist in the training process of users. Be aware that only the basic operation details will be presented. Please contact the CMI staff for more assistance if required.

3.5 Changes may occur when a new software version or patch is installed. Please contact the CMI staff if you are not sure about new features and functions.

3.6 Do not forget to sign in to the log book before you start your measurement.

3.7 Turn on the laser power when you begin, use laser shutter in-between measurements.

4. Responsibilities

Operators of this equipment are responsible for the following:

- 4.1 Complying with all safety regulations.
- 4.2 Compliance with procedures and specifications contained in this document.
- 4.3 Reporting misuse of the instruments, or in a manner inconsistent with this specification, by any

personnel, to the supervising CMI staff.

4.4 Maintaining a clean workspace. **Food and drink are NOT allowed**!

4.5 Reporting any and all maintenance issues/concerns to the supervising CMI staff member immediately.

5 Raman overview

Raman spectroscopy is a characterization technique that is widely used in scientific field in recent years. It actually utilizes the unique Raman spectra for different components as a spectral "finger print" to identify unknown samples or even further analysis based on the information from the spectra. Raman spectra can be obtained from various kinds of materials from bulk solids such as paper and cellphone, to nanomaterials such as thin films and nanoconstructs.

Raman Spectroscopy has many advantages among many characterization techniques, and therefore it is quite welcome in scientists and laboratory workers. Typically, the samples need little preparation before characterization and analysis can be carried out through many containers. Raman Spectroscopy is usually not destructive to samples unless you use too much laser power and focus high-energy laser on a point of the sample, leading great amount of heat in that small area and thus burning your sample.

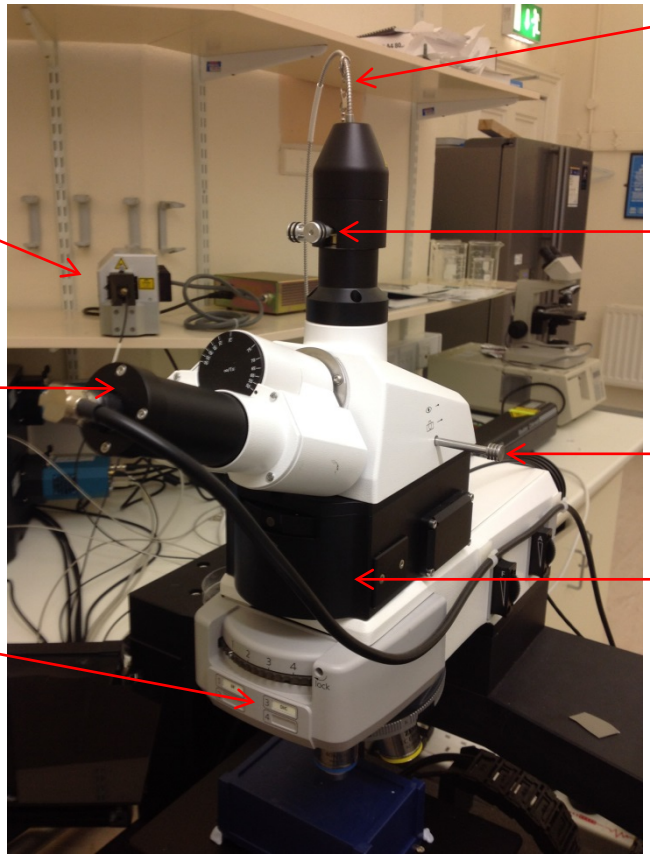
In addition, Raman Spectroscopy is typically a fast characterization technique that can perform real-time scan. It can acquire a Raman spectrum of most substances in seconds via Charge Coupled Device detectors (CCDs) that have a wide dynamic range for users to select the appropriate exposure time for their sample. Depending on the raman cross section of the sample of interest, a high resolution scan can be finished in minutes using the system.

Raman Spectroscopy can do both qualitative and quantitative analysis. Quantitative analysis of the sample is typically performed by measuring the relative intensities of each peak in the Raman spectrum that are directly proportional to the relative concentrations of the compounds. Alternatively, chemometric methods can be used for detailed information and accurate calculation. These quantitative analyses are very sensitive that can be performed on samples with high concentrations ranging from 90-100% material of interest down to concentration determination at parts per billion (PPB) levels.

This instruction will guide you to run a Raman spectroscopy scanning step by step and help you obtain your first Raman spectrum/ image from the WITec Raman Spectroscopy Instrument. It can also be used as a manual book for laboratory users in case of operational problems and troubleshooting. This instruction is separated into sections for instrument overview, step by step alignment and calibration check, acquisition, imaging, and troubleshooting. Readers should follow the procedures in this instruction in order to operate the Raman instrument properly.

DANGER: Class 4 laser radiation involved during the procedure!

6 System Overview



The witec confocal raman microscope consists of the following devices:

6.1 Instrument Control PC, 64 bit running witec control and project software (version 1.6)
6.2 Controller unit.
6.3 Upright microscope with led light source for brightfield work.
6.4 Objectives including a 40x water dipping lens and 100 x air
6.5 Andor CCD cameras
6.6 Witec diffraction gratings for either vis or NIR operation.
6.7 Motorised stage for xyz control
6.8 Fibre optic cable for 633nm use: 50micron diameter
6.9 Fibre optic cable for 785 nm use : 100 micron diameter

Familiarise yourself with the system folders, located on the shelf above the equipment.

7 Start Up Procedure

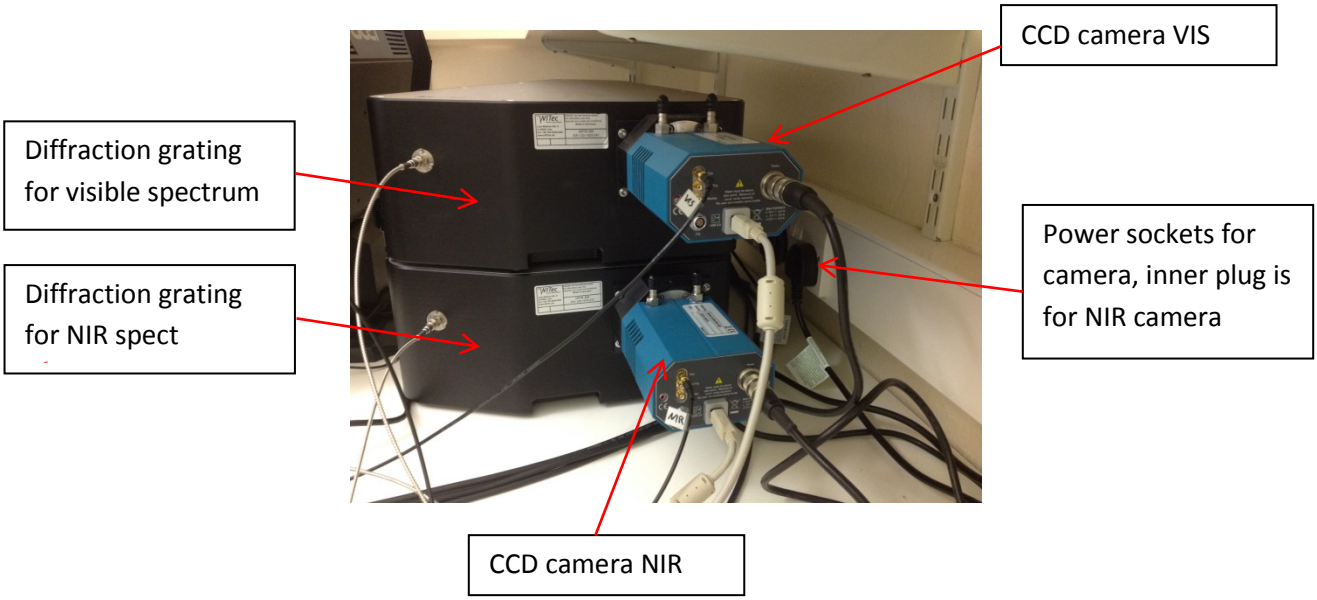
7.1 Book equipment on CMI web booking system.

7.2 Turn on lasers or individual laser, if only one is to be used. For the 633 laser, turn on the key. For the 785 nm laser, turn on power first and wait until the green led stops flashing. Then switch the laser key to 'on' and again wait until the green led stops flashing and both yellow and green leds

are continuously on.

7.3 Turn on power to controller if not already on.

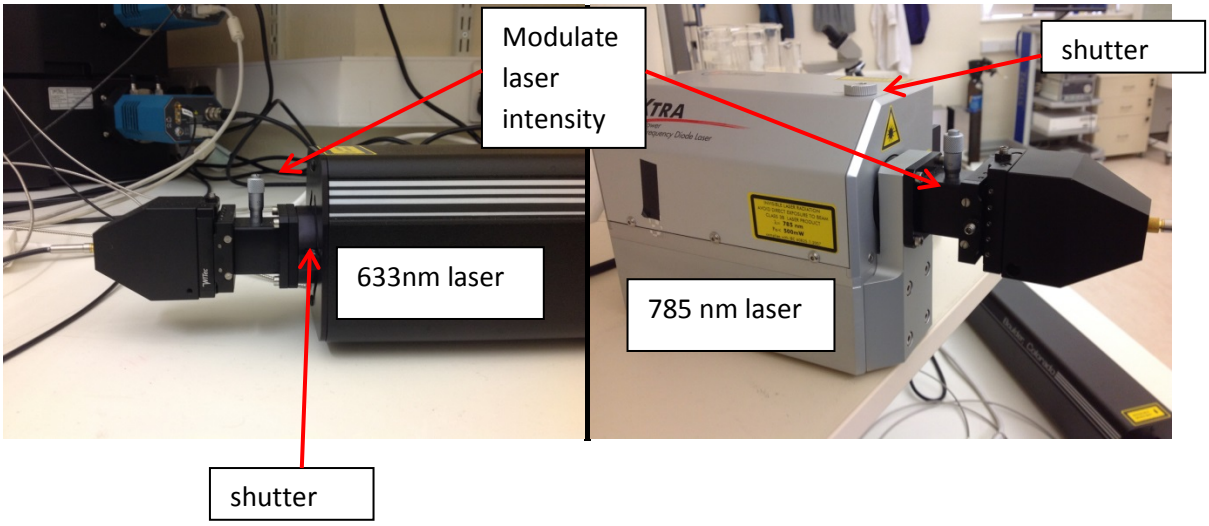
7.4 Turn on power to camera(s). Turn on only the one you are planning to use :



7.5 Turn on PC, login password is 'biophotonics119'.

7.6 Both the lasers have to be warmed up for at least 30 mins before acquisition; while warming up alignment of the microscope can be done.

7.7 Click on the 'Witec Project Control' to start the raman software interface



8 Calibration

8.1 Use the silicon wafer for calibration (accepted Raman peak at 521 wavenumbers)

8.2 Choose objective to use and switch to brightfield mode (with edge filter and dichroic mirror off); beam splitter should be in Bright Field (BF) mode (Pos 1)

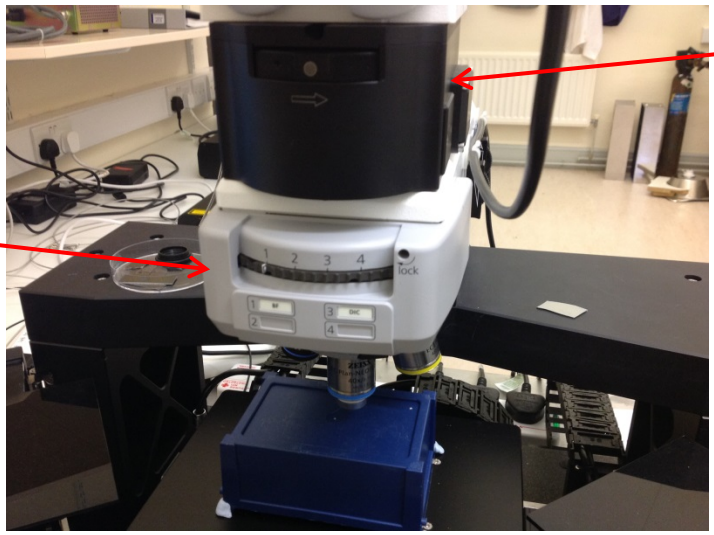
Filter turret

Position 1: Brightfield imaging

Position 2: DIC for water dipping lens

Position 3: DIC for all other lenses

Position 4: Raman imaging, spectra



Laser coupler

Slide rightwards(follow arrow direction)

Options are block:

Open:

Either open , block for 633nm and 785 nm can be chosen. All other positions have no filter , used for brightfield.

8.3 Set the detector knob, at the side of binocular, to the eyepiece mode (and not the camera mode)

8.4 Set the brightness to 100% in the software ('Illumination'). Make sure to see some intensity changes in the video image; check it by opening and closing the aperture stop (A) at the microscope. Typically keep this just slightly open, unless for dim samples/dic imaging.

Changing fibres: gently slide the cover over the fibre and rest on side of stand , ensuring no kinks in fibre, unscrew the fibre and change to the other one. Align the raised edge on the fibre coupling with the slot on the fixed end then tighten (hand tight-do not overtighten)

Replace the black cover

Push/Pull rod

Fully out : light directed to detector system

Fully in : light directed to eyepiece camera

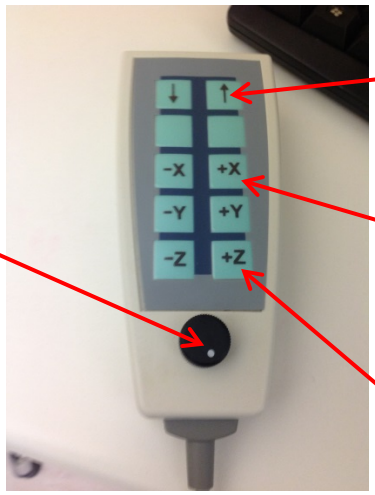
Eyepiece camera

Aperture stop

Field stop

8.5 Close the field stop and focus to bring the stage up

Speed control : applies to either xy or z. rotate white circle anticlockwise to reinitiate control and increase by rotating clockwise



Up/down: toggle between z control, xy stage control

X Y Stage controls

Z Stage controls

+Z : stage moves away from objective

-Z: stage moves towards objective

8.6 With the remote, select 'Microscope Z' control and adjust the focus (in 'z'); when in focus position, the image of the field stop is sharp in the video control window.

8.7 Fine tune the focus with remote observing the image of the field stop again.

8.8 Then open the field stop to observe the silicon sample.

8.9 Select the correct Video Viewer (objective) in the Video Control Window and calibrate (using 'Video Calibration' button). The rotation should be within -1 and +1 degree. If not, rotate the camera in the binocular and redo the calibration. Hint: center a piece of dirt or scratch in the video viewer window and try calibration again if having trouble getting the rotation below 1.

8.10 Turn the roller (at the Raman coupler) to needed laser wavelength (with edge filter off) and switch on the shutter of the respective laser with minimum power. Adjust the laser power until the laser spot can be seen in the video image. Move the microscope in 'z' to focus the laser until the laser spot is sharp.

8.11 Select 'Probe position' on the laser spot.

8.12 Make sure the correct spectrometer is connected with the camera fibre and the right laser is selected using the 'Configuration' in the taskbar

50 micron fibre : 633nm use

100 micron fibre : 785nm use

It is recommended to calibrate with silicon , if switching between wavelengths, but can be possible as a quick check just to swap in the fibre cables. **Note: Take extreme care when handling the fibre cables as they can be damaged very easily. Ensure that the raised edge on the fibre end lines up with slot on the connector before tightening.**

8.13 Switch the coupler to laser wavelength with edge filter on.

8.14 Pull out the knob to 'Camera mode' (the light propagates through the detection fibre now).

8.15 Remove the beam splitter (set to either position 4) (not BF nor DIC or position 2 (extra DIC optics filters in that position).

8.16 Start 'Oscilloscope', and adjust the power of the laser to get maximum intensity of the 1^{st} order Raman band of Silicon at ≈520 rel.cm^{-1}

8.17 Adjust fine focus.

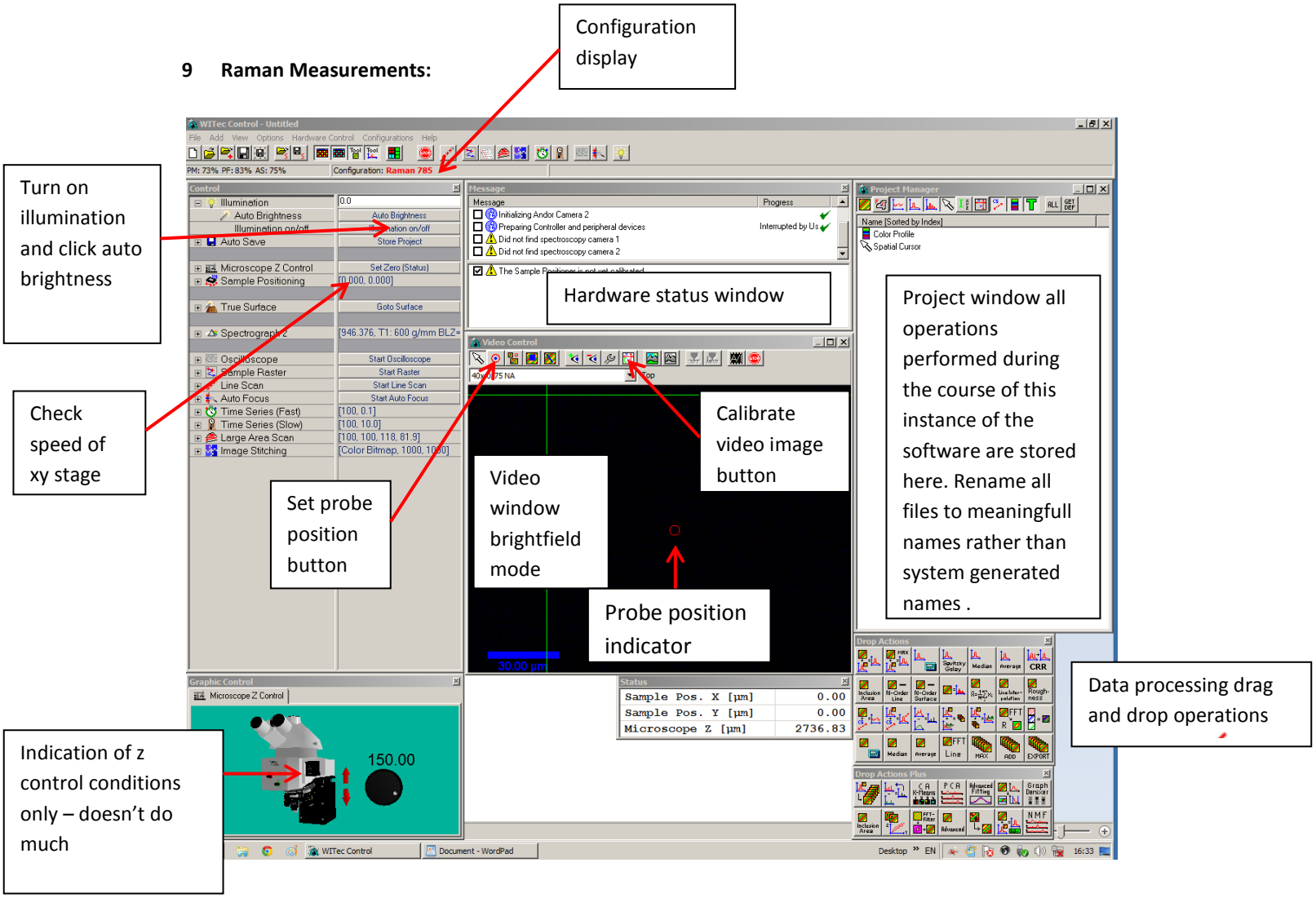
8.18 Using the X and Y fibre knob (on top of the laser coupler), get the maximum intensity of the Raman band; then again adjust the Z focus with objective.

8.19 Redo the above steps till maximum intensity is obtained.

8.20 Record the spectrum for silicon , say 0.5 integration time , verify that the peak intensity is as expected and in the right place ie 520 rel wavenumbers.

8.21 Close the laser shutter and stop the oscilloscope. Alignment of the microscope is now completed.

9 Raman Measurements:



9.1 For *in-vitro* samples, the cells can be imaged with 633 nm laser and the nanoparticles with 785 nm laser. Saliva, to date has been recorded using 785 nm excitation.

9.2 After point scan or line scan to confirm the SERS signal from Raman reporter, an area of interest is marked in the bright-field image.

9.3 Using 'Sum' filters, a spectrum region of interest can be marked; images specific to that range will then be mapped by the WITec software. More than one range can be selected, and thus more than one large area scan images can be constructed from a single large area scan. Eg, For cells, vibrational bands of C-H stretching around 700 and 1000 wavenumbers can be used for mapping.

9.4 After selecting the large area scan images with better contrast (between cells and nanoparticles), data analysis – background correction, cosmic ray removal, basis analysis – can be performed on large area scan data.

10 Troubleshooting

10.1 If you cannot see the laser spot – Check if the laser is turned on. Check if the shutter is open.

10.2 If you cannot see the white light – Check if the white light source is turned on (Illumination in software. If it is turned on, then check if the microscope is set in Raman Mode. If it is in Raman mode then change it to the eye-camera mode.

10.3 If you see the spectra with full of noise signal – Check for the CCD temperature. It should be $-60^{o}C$.

11 Shut Down Procedure

11.1 You can save the experiment parameters for your own scanning recipe by clicking “Save Project’ in ‘File’.

11.2 Close the laser shutter, switch off the laser power supply.

11.3 Using the remote control, move the sample away from the objective.

11.4 Open the doors. Remove the sample from the stage

11.5 Discard cover slips and glass sides in the red sharps container. This includes BioHazard material.

11.6 CLEAN UP the workplace, and leave it better than you found it.

11.7 Close the software, it will take several minutes to warm up the CCD before the software fully closes. Log-off the computer. Log out from computer, complete log book.

12. APPENDIX A - CERTIFICATION: DEMONSTRATION OF SKILLS

Name:	Operation:
Date:	Tested by:

DEMONSTRATION of SKILLS

	Pass	Fail
Demonstrate system/ software start up and configuration	☐	☐
Demonstrate loading a sample on the microscope	☐	☐
Demonstrate finding the sample, optimizing acquisition and taking spectra	☐	☐
Demonstrate changing the fibre cable during the measurement session	☐	☐
Demonstrate finishing and shutting down system	☐	☐

SAFETY

Where are cover slips, glass slides discarded?	☐	☐
Where are biological samples discarded?	☐	☐
Correct use of lasers and proper handling procedures?	☐	☐

VALIDATION

Certification:

Pass:	Fail:

Trainee	Certified by:

Date: