

University of **Strathclyde** **Glasgow**

University of Strathclyde

Department of Pure and Applied Chemistry

Establishing the Extent of Sample Damage and Safe Limits of Laser Exposure During Live Cell Analysis with Raman Spectroscopy

By

Caitlin Thomson

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DECLARATIONS

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ABSTRACT

For a technique to be applied to any system or sample with a need for robust reproducibility, it must undergo rigorous testing and have well defined protocols and procedures.^{1,2} Raman spectroscopy still currently suffers from instrument and user variability, as well as not having enough well defined, set procedures which can be transferred from lab to lab.³ Raman systems are equipped with a variety of hardware and data processing solutions, but still require better established standard operating procedures, sample interaction and data handling procedures before Raman spectroscopy will be able to be translated as a benchmark technique for chemical analysis and to facilitate the comparison of Raman data between studies and different geographical sites.

As Raman spectroscopy is a technique which uses a high-powered light illumination source, there is also a risk of sample damage due to laser exposure; this is particularly concerning for light and temperature sensitive samples such as live cells or other biological tissues.⁴⁻⁶ To uphold data integrity, it is important to ensure chemical changes – which can be indicating factors to aid medical diagnosis - are due to the process under study, and not artefacts of sample damage.⁷ To help overcome some of these issues of both sample damage and moving towards developing better operating procedures, this project focusses on attempting to establish how much laser irradiation a live cell sample can withstand before damage occurs during Raman spectroscopy in parallel with well-defined biological viability techniques; utilising MG-63 mammalian osteosarcoma cells. This will help to enable the design of robust standard operating procedures for Raman analysis in future, avoiding photodamage of live cells and delivering confidence in future Raman analysis results through reduced artefacts.

ABBREVIATIONS

7-AAD – 7-aminoactinomycin D

A – Adenine

ALS – Asymmetric Least Squares

AM - Acetomethoxy

ANOVA – Analysis of Variance

APAF1 – Apoptotic Protease Activating Factor 1

ASTM – American Society for Testing and Materials

ATP – Adenosine Triphosphate

Br. – Breathing

C – Cytosine

CARS – Coherent Anti-Stokes Raman Scattering

CCD – Charged Coupled Device

CLSM – Confocal Laser Scanning Microscopy

CO₂ – Carbon Dioxide

CRS – Coherent Raman Scattering

CW – Continuous Wave

DCFH-DA – Dichlorofluorescein Diacetate

Def. – Deformation

DHR-123 – Dihydrorhodamine-123

DIC – Differential Interference Contrast

DMEM – Dulbecco's Modified Eagle 's Medium

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic Acid

ELISA – Enzyme-linked Immunoassay

EMCCD – Electron Multiplier Charged Couple Device

EtHD-I – Ethidium Homodimer-I

FBS – Foetal Bovine Serum

FWHM – Full Width Half Maximum

G – Guanine

H₂O₂ – Hydrogen Peroxide

HgCdTe – Mercury Cadmium Tellurium

hOB – human Osteoblasts

hUC-MSCs – human Umbilical Cord Mesenchymal Stem Cells

InGaAs – Indium Gallium Arsenide

ISO – International Organisation for Standardisation

LDH – Lactate Dehydrogenase

LED – Light Emitting Diode

MPE – Maximum Permissible Exposure

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide

NA – Numerical Aperture

NADPH – Nicotinamide Adenine Dinucleotide Phosphate

NIR – Near Infra-red

NIST – National Institute of Standards

NPL – National Physical Laboratory

PBS – Phosphate Buffered Saline

PDAs – Photodiode Arrays

PE – Poly(ethylene)

PFA – Paraformaldehyde

Phe. – Phenylalanine

PI – Propidium Iodide

PMMA – Poly(methyl methacrylate)

PMTs – Photomultiplier Tubes

PPE – Personal Protective Equipment

PSF – Point Spread Function

QE – Quantum Efficiency

RI – Refractive Index

RNA – Ribonucleic Acid
ROS – Reactive Oxygen Species
S/N – Signal to Noise
SERS – Surface Enhanced Raman Spectroscopy
SHG – Second Harmonic Generation
SI – International System of Units
SOD – Superoxide Dismutase
SORS – Spatially Offset Raman Spectroscopy
SRG – Stimulated Raman Gain
SRL – Stimulated Raman Loss
SRM – Standard Reference Material
SRS – Stimulated Raman Scattering
Str. – Stretch
Sym. – Symmetric
T – Thymine
TEM – Transmission Electron Microscopy
TPEF – Two Photon Excitation Fluorescence
TPF – Two Photon Fluorescence
Trp. - Tryptophan
Tyr. – Tyrosine
U – Uracil
US – United States
UV – Ultraviolet

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1 INTRODUCTION

1.1 METROLOGY AND LIVE CELL ANALYSIS

1.1.1 Metrology and Reproducibility

Reproducibility is a fundamental principle of the scientific process; the exact definition of reproducibility varies depending on the arm of science under study, but in essence reproducibility is the ability to reproduce similar scientific results from a method or procedure.⁸ Metrology, the application of measurement science, can help apply methods to standardise measurements, minimise variation and increase the reproducibility of experiments, particularly through the use of reference standards and systems that are underpinned by the seven SI units.⁹ The SI units allow for the use of internationally recognised standards which can result in traceable measurements which in turn increase reproducibility and allow comparison of measurements from different sources.¹⁰ The SI units apply to most branches of scientific research as they cover light (Cd), weight (kg), distance (m), concentration (M), electrical current (A), temperature (K) and time (s), however difficulty can be encountered on applying traceable measurements to particularly complex samples and systems such as those found in biology, due to the heterogeneity of such samples and presence of analytes at low concentrations, physical size and high variability.¹¹

1.1.2 Biology and Metrology

Cells are the building blocks of all living organisms, and variations in these building blocks, either endogenous or due to external stimuli, can infer information around cell health, disease diagnosis and drug efficacies.^{12–15} Many methods of cell analysis, however, rely on observation of cells and these variations, primarily through the use of light microscopy, with or without sample labelling, due to the scale of subcellular structures being well matched to light microscopy resolution.¹⁵ Cell variation is difficult to measure in absolute terms, due to the complexity of cells themselves, so normally anomalies in cells are

measured in terms of relative changes in arbitrary units, which in turn raises issues around reproducibility and comparison of studies.¹⁶

Irreproducibility is estimated to cost, in the US alone, US\$28 billion per year in preclinical research that is irreproducible however, there is not a single solution to resolving the problem of irreproducibility, particularly in the ever evolving biological field.^{17,18} As Faruqi et al state, reference materials, methods, protocols and documentary reference standards can be utilised to overcome the issue of reproducibility in biology.¹⁸ This is where the fundamentals of metrology that are already widely applied in chemistry and physics, can be applied to help determine applicable measurands, the use of relevant reference materials and defined methods to ensure better confidence and reproducibility in biological analysis.

Light microscopy, as a workhorse of biological analysis, can infer variation during cell analysis through the inherent variation in light sources themselves and the susceptibility to these sources to degradation and temperature, so care must be taken to ensure the same analysis parameters are used during every analysis. This is well understood in the field of fluorescence microscopy, especially due to the well-defined phototoxic nature of both light intensity and fluorescent tags on cells.^{19–24}

1.2 RAMAN SPECTROSCOPY AND METROLOGY

Metrology has been applied to Raman spectroscopy through the use of physical standards such as those created by NIST for intensity calibration and protocols developed by ASTM for spectrometer calibration.^{25,26} Metrology has also been applied to the acceptable limits for laser use in research and within instrumentation in the workplace, however, there is no one standard defined for evaluating safe laser limits on samples, particularly biological, during Raman spectroscopy analysis, unless the sample itself is a human test subject, under which there is the defined maximum permitted exposure (MPE) limit of laser power, usually mitigated with engineering controls, laser containment and personal protective equipment (PPE).²⁷

1.3 RAMAN SPECTROSCOPY INDUCED CELL DEATH

As mentioned in section 1.1.2, photodegradation and phototoxicity is well understood in the field of fluorescence microscopy and the use of lasers in conjunction with fluorescent stains. However, in Raman microscopy, which still utilises monochromatic laser illumination sources, there are less well-defined limits surrounding the permissible amount of laser power incident on a sample. It is acknowledged that high laser powers, particularly at lower wavelengths can damage temperature and environmentally sensitive samples such as cells, but there are not well-defined limits of what these laser powers are.

Indeed, the study by Puppels et al in 1991, did investigate the difference between 514 nm, 633 nm, and 660 nm. This study included the investigation of the percentage of cell death on increasing the laser power incident of these three wavelengths on human lymphocytes, as well as the changes in Raman intensity on increasing exposure time to 5 mW 514 nm laser power on isolated single CHL cell metaphase chromosomes.⁴ This was a rigorous insight into the effect of varying laser wavelength and powers on biological sample viability.

Since this study, further work by Notingher et al in 2002 compared the spectral differences in live and nutrient starved MLE-12 cells on bioglass utilising 488 nm, 514 nm and 785 nm laser wavelengths to evaluate the extent of laser wavelength damage with time in the cells at different wavelengths.⁵ Notingher et al performed further viability studies with A549 cells, analysing live and dead cells caused by the natural variability of cells in culture to determine and quantify the spectral differences associated with cell death.²⁸ Oxygen and glucose deprivation of MEL-28 human malignant melanoma cell line was used in the investigation of necrotic cell death elucidation by Raman spectroscopy through characteristic peaks of lipids and RNA decreasing and an increase in relative protein content in necrotic cells.²⁹

Verrier et al, also analysed A549 epithelial lung cells, however cell death was induced with Triton X-100, and the spectral changes correlated with cell viability and DNA assays at 0 – 72 hours exposure to Triton-X. Observation of decreases in DNA and protein peaks in combination with an increase in lipid

peaks were used as indicators of cell death, utilising 785 nm laser wavelength.³⁰ Boyd et al utilised macro sampling with a 785 nm laser of 100 mW power focussed on live or Triton X-100 treated cells to interrogate spectral differences between different cell lines with limited success but was able to successfully elucidate differences between live and dead MG-63 cells due to differences in peaks relating to DNA.³¹

A purpose-built environmental enclosure was used in conjunction with etoposide treated MDA-MB-231 breast cancer cells to perform a 6-hour time study to generate Raman images of the same group of cells to indicate nuclear condensation during apoptosis. The results of the Raman generated images were then confirmed by widefield fluorescence of Annexin-V and 6-CFDA fixed cells to indicate a difference between apoptotic, necrotic and viable cells.³²

Changes in cell spectra relating to cell death have also been explored in the development of cell-based biosensors for the detection of type and method of chemical death. Notingher et al exposed A549 cells to ricin and sulfur mustard, and through multivariate analysis methods on Raman spectral data, was able to elucidate differences between the methods of death induced by the two toxic agents as well as the differing levels of toxins used to cause cell death.³³

Multiple differing ways of evaluating cell viability using Raman spectroscopy are available in the literature, with the above indicating a small fraction of the work based on this subject. However, for many of the studies, chemically induced death has been used and spectral differences are due to the chemical mode of death. The main studies for inherent Raman spectroscopy induced death were the studies mentioned above by Puppels et al on lymphocytes and chromosomes, and the study by Notingher et al for the interrogation of cell viability with varying laser wavelength on MLE-12 cells.^{4,5} These studies indicate cell death with laser wavelengths at 488 nm, 514 nm, 633 nm, 660 nm and 785 nm and infer that spectral changes can allude to cell death, with confirmation by biological viability methods. To further build on these studies, this work will focus on the use of 532 nm laser wavelength, which hasn't been included in these studies, to determine spectral information correlating to

inherent Raman spectroscopy induced cell death in the MG-63 cell line, which is often used as a model cell line for cell-material interaction studies for bioengineering purposes such as medical grade implant materials.

1.4 RAMAN SPECTROSCOPY

1.4.1 Introduction to Raman Spectroscopy

Raman spectroscopy is a label-free, minimally-invasive technique that combines the spatial resolution and imaging capabilities of an optical microscope with chemically specific information similar to other spectroscopic techniques. The combination of these attributes have made Raman spectroscopy attractive to a variety of industries; it has been utilised in the pharmaceutical industry, for determination of age and historical origin for art history purposes, for biological applications including clinical trials, warfare and security applications as well as in agriculture.^{34–41} Raman spectroscopy allows for the analysis from single cell level or nanomaterials to whole sample mapping.

C.V. Raman began working on light scattering during a voyage to England, after being unable to accept Lord Rayleigh's explanation that the colour of the sea was purely a reflection of the colour of the sky. He formally identified Raman scattering in 1928, utilising a telescope to identify that sunlight focussed onto a sample was scattered by the sample at a different frequency of light than the incident light and was subsequently awarded the Physics Nobel Prize in 1930 for his work on light scattering.^{42,43} Since the discovery of the Raman effect, many technological advances have improved the technique. Lasers, the development of charged couple device (CCD) detectors, advances in optics and computer technology as well as the development of Raman based techniques such as Surface Enhanced Raman Scattering (SERS) have resulted in the technique being used in analysis for a wide variety of samples.^{44–46}

1.4.2 Spontaneous Raman Spectroscopy Theory

Upon interaction with a molecule, monochromatic incident light can either be absorbed, transmitted, or scattered. If the energy of the incident light matches the band gap of the molecule, the photon is absorbed into the molecule promoting an electronic transition from the ground state to an excited state. If the electron relaxes back down to the ground state after absorption, the photon

is transmitted. However, if the molecule is capable of polarization; the ability to excite the electron cloud of a molecule to promote a 'virtual excited state', the photon is scattered instead of absorbed into the molecule. Polarizability is an intrinsic molecular property which can be determined using the size and shape of the molecule and the ability of electrons to move within it. For scattering to occur, the net polarizability must change during the vibrational process.⁴⁷

Equation 1: Equation relating the movement of electrons to the polarizability of a molecule.⁴⁸

$$\mu_{ind} = \alpha E$$

Where, μ_{ind} is the induced dipole, α is the polarizability of the molecule and E is the applied electric field. Scattering of incident light can happen in one of three ways; elastically or inelastically with energy transfer from the photon to the molecule or inelastically with energy transfer from the molecule to the photon. Rayleigh scattering, which is the dominant process, scatters the incident light elastically with no nuclear motion of the molecule, only electron cloud movement, resulting in the scattered photons having the same frequency as the incident light. Stokes scattering is an inelastic process whereby there is nuclear movement in the molecule as well as electronic movement, this results in energy transfer from the incident light photon to the molecule ($\Delta v = -1$), whereas anti-Stokes scattering, also inelastic, involves the energy transfer from the molecule to the photon ($\Delta v = +1$) upon the occurrence of nuclear movement. Raman scattering only occurs when the selection rule is fulfilled, $\Delta v = \pm 1$, due to the quantized nature of light and the reliance of Raman scattering on the change in polarizability.⁴⁷

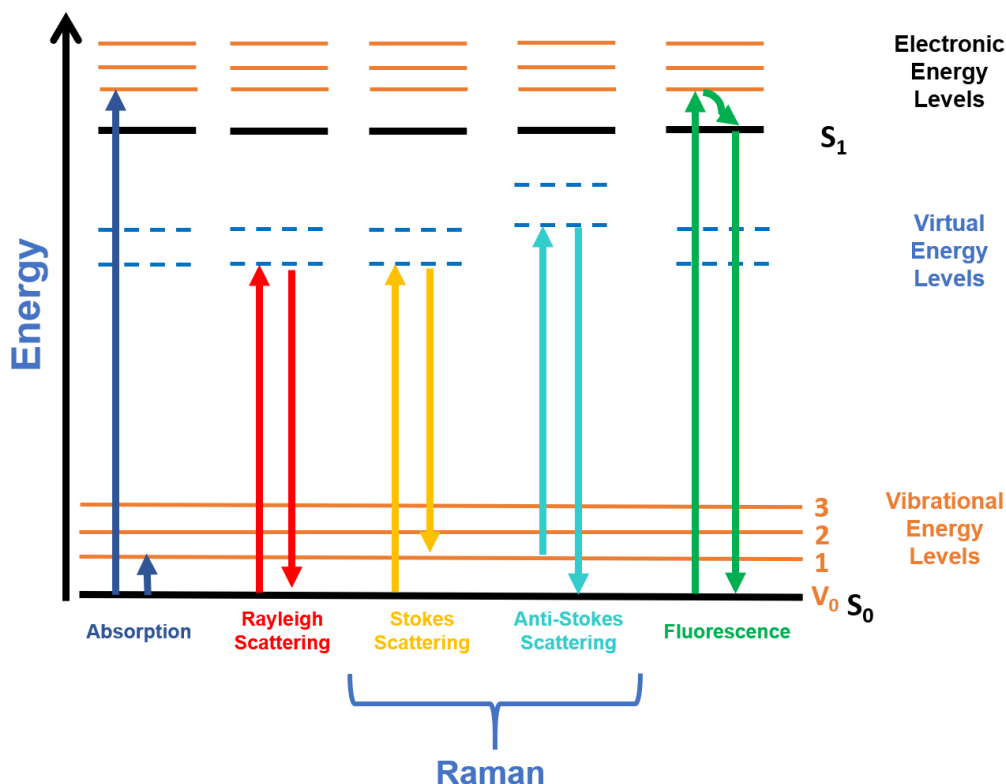


Figure 1: Jablonski diagram showing the excitation of electrons from a ground electronic state to either an excited vibrational energy level - absorption, excitation to a virtual vibrational energy level - Rayleigh, Stokes or anti-Stokes Raman scattering interactions or to an excited electronic energy level before relaxation – fluorescence.

Stokes scattering is likely to be the more intense of the two inelastic processes due to the majority of molecules existing in the ground state at room temperature; the ratio of Stokes to anti-Stokes intensities can be calculated using the Boltzmann distribution equation below. This ratio will change as temperature increases, with more electrons being in an excited state due to the additional energy provided by higher temperatures, meaning that anti-Stokes will be the more dominant process as the temperature of a system increases.⁴⁹

Equation 2: Boltzmann Distribution.⁴⁷

$$\frac{N_n}{N_m} = \frac{g_n}{g_m} e^{\left[-\frac{\Delta E}{kt}\right]}$$

Where N_n is number of molecules in the excited energy level, N_m is the number of molecules in the ground energy level, g degeneracy of the energy levels, n and m , ΔE is the difference in energy between ground and excited energy levels, k is the Boltzmann constant; $1.3807 \times 10^{-23} \text{ JK}^{-1}$ and T is temperature in Kelvin.

Spontaneous Raman scattering is a fundamentally weak process with only $1 \times 10^6 - 1 \times 10^8$ photons being Raman scattered; either by the Stokes or anti-Stokes process.⁴⁷ The intensity of the Raman signal for both Stokes and anti-Stokes mechanisms is indicated by the Kramer Heisenberg Dirac expression:

*Equation 3: Intensity of Raman scattering equation.*⁴⁷

$$I = KI_L\alpha^2\omega^4$$

Where I is the intensity of Raman signal, K is combination of constants, I_L is intensity of incident light, α is the polarizability of the electrons in the molecule and ω is the incident light frequency.

Raman scattering intensity has a dependency on the fourth power of the incident laser frequency; consequently, using lower laser wavelengths of higher frequencies, for example wavelengths in the UV region, the scattering intensity can be improved due to the relationship between frequency and wavelength. UV lasers, however, pose an issue of sample damage due to the higher energy of the photons in the UV region, this can be particularly problematic in temperature and light sensitive samples such as those used in bioanalysis. To overcome this issue of signal dependency on laser frequency and intensity, Raman spectroscopy has been diversified into similar techniques that optimise Raman scattering but minimising sample damage.

1.4.3 Confocal Raman Microscopy

There have been many advances in Raman spectroscopy since the inception of the technique, including handheld Raman devices, techniques such as Surface Enhanced Raman Spectroscopy (SERS) and Spatially Offset Raman Spectroscopy (SORS), all of which are particularly useful for certain applications and allow for enhancement of the Raman signal.⁵⁰ However, one

of the biggest leaps for Raman spectroscopy was the coupling of a Raman spectrometer to a confocal microscope.⁵¹

The development of confocal light microscopy is attributed to Marvin Minsky, who developed an optical imaging setup with the aim to analyse unstained neural networks in vivo.⁵² His system utilised a zirconium arc lamp focused to a point on a sample through an objective, the light which passed through the sample was collected by a second objective, passing through a pinhole before reaching the detector. Minsky patented the technique in 1957.⁵³ However, the results and uptake of the technique were constrained due to the inability to record an image of the sample and by the optics and detectors available at the time limiting the performance.

Confocal originates from the German word 'konfokal' which means from the same point.⁵¹ A confocal microscope relies on the use of a point illumination source focused through an objective, when the light travels to the detector it must pass through a pinhole at the back focal plane of the objective and in the same focus as the laser spot on the object plane. The pinhole stops any light on the sample plane outwith the focal plane to be filtered out from detection, resulting in sharper signal generation from the sample point of interest.

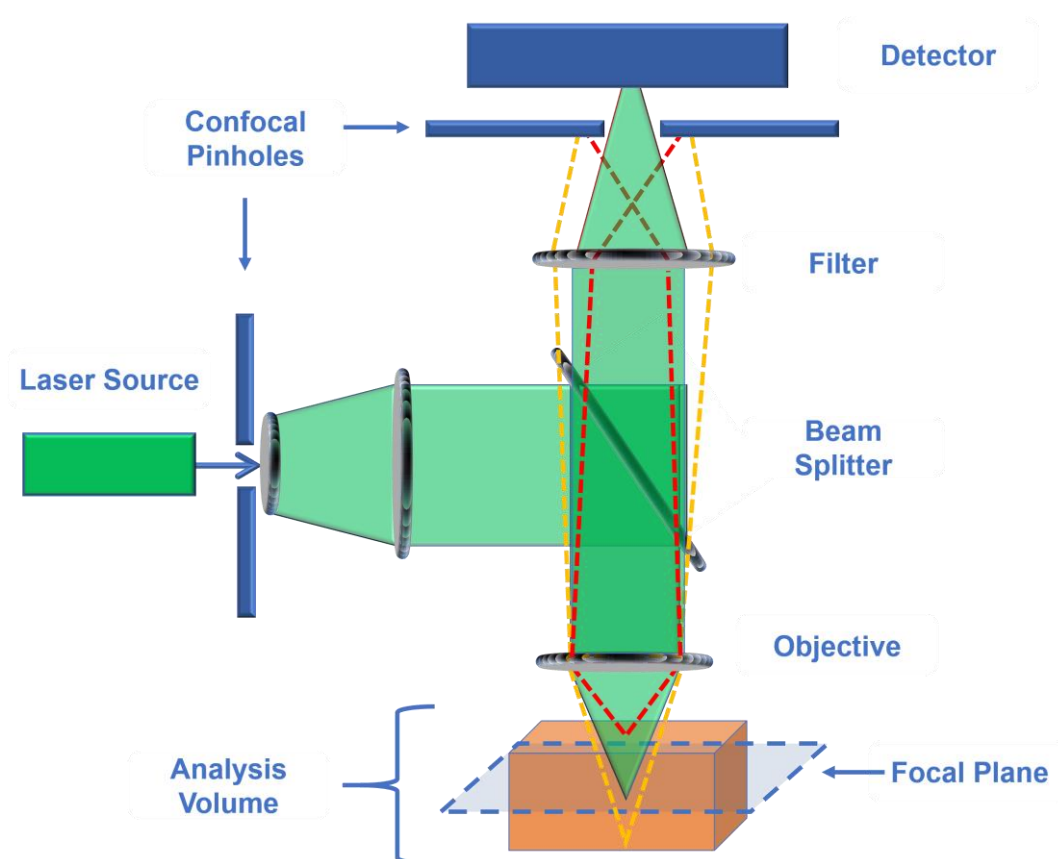


Figure 2: Confocal microscope arrangement. Green light from laser focuses on the sample in the focal plane is collected and passes through the pinhole to the detector. The red and yellow lines indicate scattered light within the sample plane but not in the focal plane, these are excluded from reaching the detector by the pinhole improving the spatial resolution and specificity of the analysis.

The use of a point source means that a sample must be scanned point by point to generate a full image of the sample on the detector. This can be achieved using laser scanning or stage scanning. Laser scanning is the preferred method for biological analysis due to stage movement causing aberrations in unfixed or liquid samples, however many Raman microscopes are equipped with stages which are capable of translation in x, y and z directions.⁵⁴ The use of an adaptable pinhole coupled with laser scanning vastly improves the axial resolution of the microscope, allows for a 3D image of the sample to be generated without the need for physical sample sectioning, and if the correct pinhole size is used, it can also increase the lateral resolution of the system by $\sqrt{2}$.⁵¹ However, this does increase the analysis time substantially, and the

pinhole would need to block 95% of the incoming light to improve the lateral resolution by $\sqrt{2}$, which ultimately would block out the majority of the Raman signal from reaching the detector due to the weakness of the signal.

Many commercially available systems are truly confocal, with an adaptable pinhole and slit arrangement, however some exist in a pseudo-confocal arrangement which utilises the orientation of the slit before the grating coupled with the capability of a charged couple device (CCD) detector to bin pixels that are out of the range of the focal plane scattered light. This results in less control over the system spatial resolution and response but more control over repeatability as the slit and hole orientation will be consistent from measurement to measurement.

The coupling of a confocal microscope to a Raman spectrometer allows for the combination of chemically specific information deducible from the vibrational spectroscopic technique, coupled with the ability of a confocal microscope to resolve sample detail down to approximately 1 μm resolution and generate 3D images of a sample with scanning in x, y and z without the physical need for sample sectioning. The capability of such small spatial resolution and the chemical information it provides makes confocal Raman an ideal technique to analyse individual cells.

1.4.4 Raman Spectroscopy for Live Cell Analysis

Raman spectroscopy has been used for numerous live cell studies, from the differentiation between different cancer cell lines, between cancerous and non-cancerous cells, as well as for the multiple cell viability studies mentioned previously.^{5,29–31,33,55} It has also been used to image drug distribution within live cells and monitor the pharmaceutical effect on the cell. SERS, in particular, has been used with drug functionalised nanoparticles to enhance the Raman signal within cells but also to improve drug uptake within cells.⁵⁶ Raman has the advantage over IR spectroscopy, in that water doesn't cause significant interference, making it ideal for biological studies, as well as the ability for coupling to fibre optic probes allowing for easier translation of the technique from a laboratory into a more clinical setting.⁵⁷

The resonance Raman effect has also been utilised to improve the Raman scattering signal in cells in comparison to spontaneous Raman and this has been exploited to allow for imaging of production of cytochrome c during apoptosis.⁵⁸ Raman spectroscopy has been used to determine the heterogeneity in stem cell populations, for disease diagnosis as well as for cell imaging purposes for determination of biomolecule localisation in a variety of cells.^{59,60} There are many review articles detailing the wide range of uses of Raman spectroscopy for live cell imaging.^{61–63}

1.5 COHERENT RAMAN SCATTERING

1.5.1 Introduction to CRS

The discovery of spontaneous Raman scattering and the consequential development of lasers and improved detectors in the 20th century allowed for the elucidation of label free chemically specific information using Raman spectroscopy to be utilised in a wide variety of fields. However, spontaneous Raman scattering is a very inefficient process, with approximately 1×10^6 photons being scattered in the Raman process; while the higher energy monochromatic radiation produced by lasers, higher S/N ratio and detection capabilities of CCD detectors as well as the use of confocal microscopy improved the process of Raman excitation and detection as well as the spatial resolution probed during Raman analysis, Raman spectroscopy still suffers from long acquisition times, and data manipulation is required in order to generate images reflective of the chemical information localisation in a sample. The time frame to generate such images is not applicable to the study of many processes of interest within biological samples.⁵⁷

Coherent Raman spectroscopy (CRS), is an overarching term which encompasses coherent anti-Stokes Raman scattering (CARS) and Stimulated Raman scattering (SRS) techniques. Both of these techniques were developed as an improvement on the spontaneous Raman phenomena, along with techniques such as surface enhanced Raman scattering (SERS) to boost the Raman signal within samples and therefore help speed up sample acquisition to timescales more relevant to biological analysis.⁶⁴

CRS was discovered in the 1960s, again after the invention of the laser, with stimulated Raman scattering (SRS) being discovered by Woodbury and Ng in 1962, and coherent anti-Stokes Raman scattering (CARS), being discovered by Maker and Terhune in 1965, although the acronym, CARS, wasn't used until a decade later when the term was coined by Begley as a tool for rapid vibrational spectroscopy.^{65–67}

CRS microscopy, however, was not developed until around 20 years after the discovery of SRS. Duncan et al in 1982 and then Zumbusch in 1999 applied the technique of CARS to microscopes resulting in 3D imaging, with interest continuing into the early 2000s, with Volkmer et al developing backscattering CARS microscopy.^{68–70} Ploetz et al in 2007 reported the use of SRS microscopy with a high energy, low repetition rate laser, in an attempt to improve upon the non-resonant background issues seen in CARS, which was then improved upon by Freudiger et al in 2008 by utilising a high repetition rate laser, with lower energy at the sample to probe a single Raman frequency allowing for SRS imaging in biological systems.^{70–73}

CARS and SRS imaging can be implemented on a single microscope system and commercial multiphoton microscopes capable of CARS, SRS, and other multiphoton imaging techniques such as second harmonic generation (SHG) or two photon excitation fluorescence (TPEF) are now available.⁷⁴ CRS allows for the improvement upon spontaneous Raman spectroscopy by utilising more than one beam to improve the Raman scattering signal in a sample, and video rate imaging with CARs was achieved in 2005 by Evans et al and SRS video rate imaging was then achieved by Saar et al in 2010 resulting in Raman techniques capable of biologically relevant timeframe imaging coupled with the label free chemical specificity of spontaneous Raman which could be applied to a multitude of uses from label free histology to imaging of drugs in vivo.^{75,76}

1.5.2 CRS Theory

Coherent Raman scattering varies from spontaneous Raman scattering which is an incoherent technique in the way the incoming radiation, visible or NIR light interacts with the material it is incident on. Spontaneous Raman scattering is a linear optical technique, in that the Raman signal output is linearly dependent on the intensity of incoming radiation. In spontaneous Raman scattering, light incident on a sample can cause a change in polarizability of an individual molecule, however the single molecule wave phase change (change in polarizability) that is emitted has no effect on the phase of the rest of the molecules within the sample and over the whole sample the phases of

each molecule are random, incoherent and essentially cancel each other out rather than causing an overall phase across the sample, resulting in a weak Raman scattering effect.⁷¹ One laser is used to probe many vibrational changes within a sample so that when Raman scattering occurs it can occur anywhere within the sample from any bond with a Raman allowed change in vibrational energy, resulting in a weaker less specific signal.

In coherent Raman scattering, the combination of two or more lasers, accumulate a change in emitted wave phase across all molecules in the sample at the frequency being probed by the lasers, causing an overall coherent phase across the sample, increasing the number of molecules experiencing Raman scattering and increasing the Raman signal. Two or more lasers are tuned to a specific frequency corresponding with a Raman signal, boosting that particular Raman signal.^{71,77}

There are two main types of CRS, as mentioned in section 1.5.1, Coherent anti-Stokes Raman scattering and stimulated Raman scattering, both utilise the overlap of ultrafast pulsed lasers (femto- or pico-second pulses) to drive the coherent production of polarization within a sample, however, only SRS will be discussed further in this thesis, due to the non-resonant background interference which affects CARS imaging which will be described in more depth in the proceeding pages.

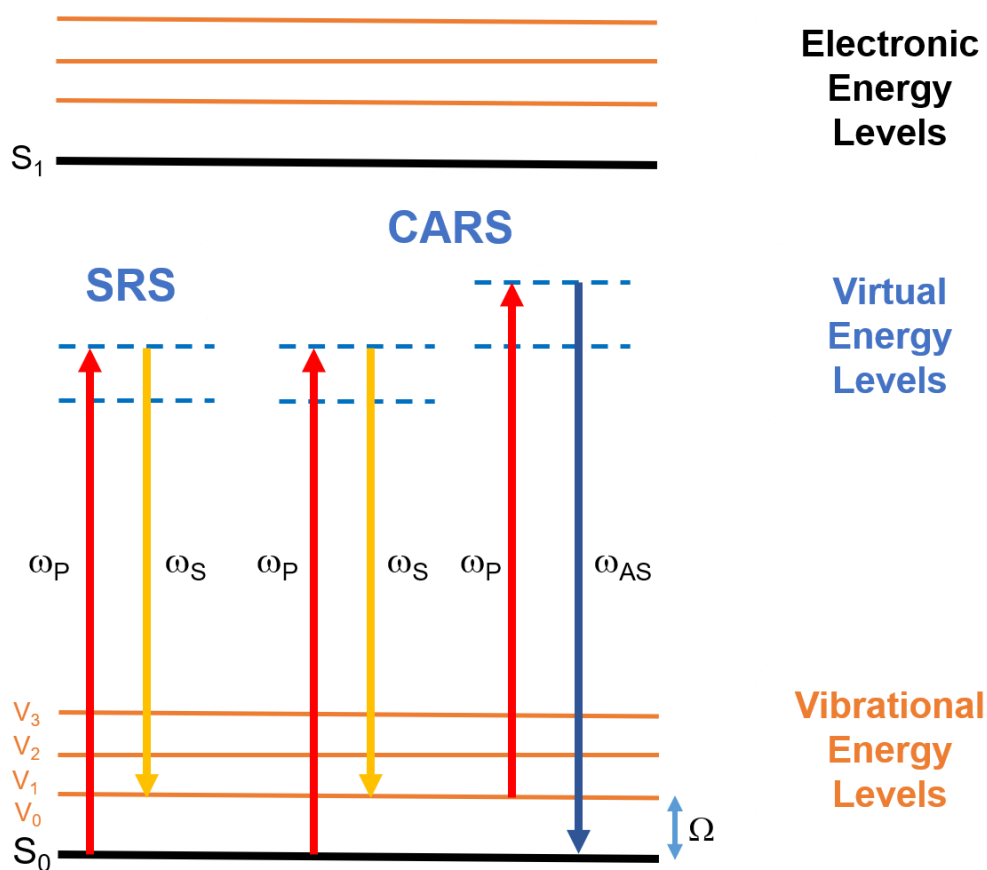


Figure 3: Jablonski diagram for SRS and CARS electron excitation

Stimulated Raman scattering is a non-linear optical technique; however, it is a non-parametric process in that energy is transferred to the molecule during the process.⁷⁸ SRS utilises two ultrafast beams, spatially and temporally overlapped, known as the pump and probe, or Stokes beam. One beam, usually the Stokes beam, is modulated to rates above 5 MHz, and upon the interaction of the two beams with a sample an energy transfer occurs from the pump beam, known as stimulated Raman loss (SRL) to the Stokes beam, known as stimulated Raman gain, as shown in figure 4.⁷¹

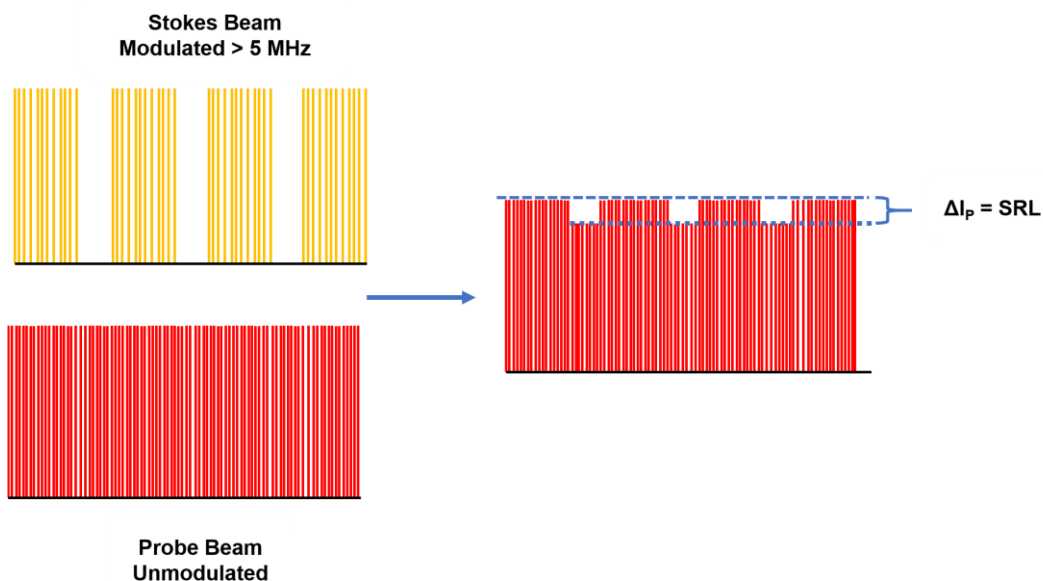


Figure 4: Profile of energy transfer from the modulated Stokes beam to the unmodulated pump beam for SRL detection of SRS signal

The energy transfer can be detected in the pump beam through the use of a lock in amplifier. The two beams, pump and Stokes are tuned to a vibrationally active mode within the sample.

Equation 4: An equation relating the tuning of the difference of the pump and Stokes beam in SRS to a resonant vibrational frequency in a molecule.^{71,79}

$$\Delta\omega = \omega_P - \omega_S = \Omega, .$$

However, if the sample does not contain an active vibrational mode at the frequency of the difference between the pump and Stokes beam, no SRL or SRG occurs, resulting in zero non-resonant background which is the main source of background issues in CARS.^{71,78,79} This allows greater sensitivity during imaging and the SRS signal is also linearly dependent on the molecular concentration of the sample, which lends the technique to quantitation more easily than CARS. SRS spectra are also more similar in nature to the corresponding spontaneous Raman spectra. However, although SRS is free from non-resonant background, other aberrations can occur during imaging which can affect SRS imaging, such as thermal lensing effects or cross phase modulation.^{78,80} SRS can also suffer from increased signal loss with

penetration depth in thicker samples which can limit the samples the technique is applicable to.

SRS offers the label free specificity of spontaneous Raman spectroscopy coupled with improved Raman signal generation and the capability for video rate imaging, so faster analysis times, and although only one wavenumber range can be probed at a time, the modern commercial systems available allow for automation of wavenumber scans to span wider ranges of the spectral region as seen in spontaneous Raman which lends SRS to a wider range of applications.

1.5.3 CRS for Live Cell Analysis

CRS allows for label free video rate imaging, coupled with chemical contrast which can image biological processes in real time. It has been used to image tumours, such as brain tumours and cells as well as track drug fate in tissue.^{81,82} The use of alkyne tags or isotopologues exploit the biologically silent region and allow for the elucidation of intracellular species that occur in concentrations typically too low for spontaneous Raman detection.^{79,83}

CRS has the ability to produce images in 3D as well as 2D, and commercially available multiphoton systems have provided easier access to these imaging systems with CARS, SRS, and higher order photon processes on one system. CRS can provide the chemical specificity of spontaneous Raman with imaging capabilities like that of fluorescence.

1.6 FLUORESCENCE MICROSCOPY

1.6.1 Introduction to Fluorescence Microscopy

Fluorescence was first observed in 1845, by Sir William Herschel on the excitation of quinine in tonic water by the sun, however, the term fluorescence wasn't used until 1852, when Sir George Stokes observed the emission of red light on the illumination of the mineral fluorspar with UV radiation.^{84,85}

Microscopists had witnessed fluorescence in the early 20th century as it caused interference during UV microscopy, but formal fluorescence microscopes were not developed until 1911 - 1913 by competing optical instrument manufacturers Carl Zeiss and Carl Reichart, as an adaptation of the UV microscope.⁸⁶ Although fluorescent dyes had been used before the manufacture of fluorescence microscopes, primarily for histology and cell staining, the development of fluorescent dyes conjugated to antibodies by Albert Coons in 1940 increased the specificity of the technique and led to the field of immunofluorescence.⁸⁷ Further technological advances such as the development of the original epifluorescence microscope by Ellinger and Hirt in 1929, the development of the dichroic mirror by Sebastien Ploem in 1967, allowing for improved separation of excitation and emission wavelengths as well as the development of lasers in the 1960s allowed fluorescence microscopy to become an invaluable tool to biologists and microscopists alike.^{86,88}

Fluorescence microscopy can be used to analyse a wide variety of samples from tissue⁸⁹, single cells to organoids^{90,91}, to evaluate diffusion pathways⁹², the effect of pH⁹³, temperature⁹⁴ and solvent polarity⁸⁴ on biological samples as well. Compared with many other microscopic techniques which are capable of macroscopic analysis, fluorescence if optimised well, is still limited by the diffraction limit of the sample being analysed but can detect the fluorescence emitted from a single molecular species.⁹⁵

1.6.2 Fluorescence Microscopy Theory

As mentioned in section 1.4.2, light or electromagnetic radiation incident on a sample or surface can interact with that sample or surface in one of three ways; absorption, scattering or transmission. The interaction of the light with the substance depends on how well the energy of the light matches the energy of the substance, Fluorescence, along with phosphorescence, are part of the photoluminescence family. Both occur in similar mechanisms but are often separated by timescales of a few orders of magnitude and a change in spin multiplicity.⁹⁶ Fluorescence occurs in three main stages:

1. The absorption of a photon of incident radiation and excitation of an electron from the electronic ground state to an excited electronic state in a time frame of femtoseconds (10^{-15} s)
2. The internal relaxation of the electron from an excited vibrational state to the lowest vibrational level of the excited state in a time frame of picoseconds (10^{-12} s)
3. The emission of a photon of a longer wavelength than the absorbed wavelength of light and the subsequent relaxation of an electron from the excited state to the electronic ground state in a timeframe of nanoseconds (10^{-9} s)

The Jablonski diagram in figure 5 indicates the main difference between fluorescence and phosphorescence is the intersystem crossing from the singlet excited state to the longer lived triplet excited state before the emission of a photon on returning to the ground state in a time frame of milliseconds to seconds. (10^{-3} – 100 s), this length of time passes long after the initial excitation energy has stopped illuminating the sample.

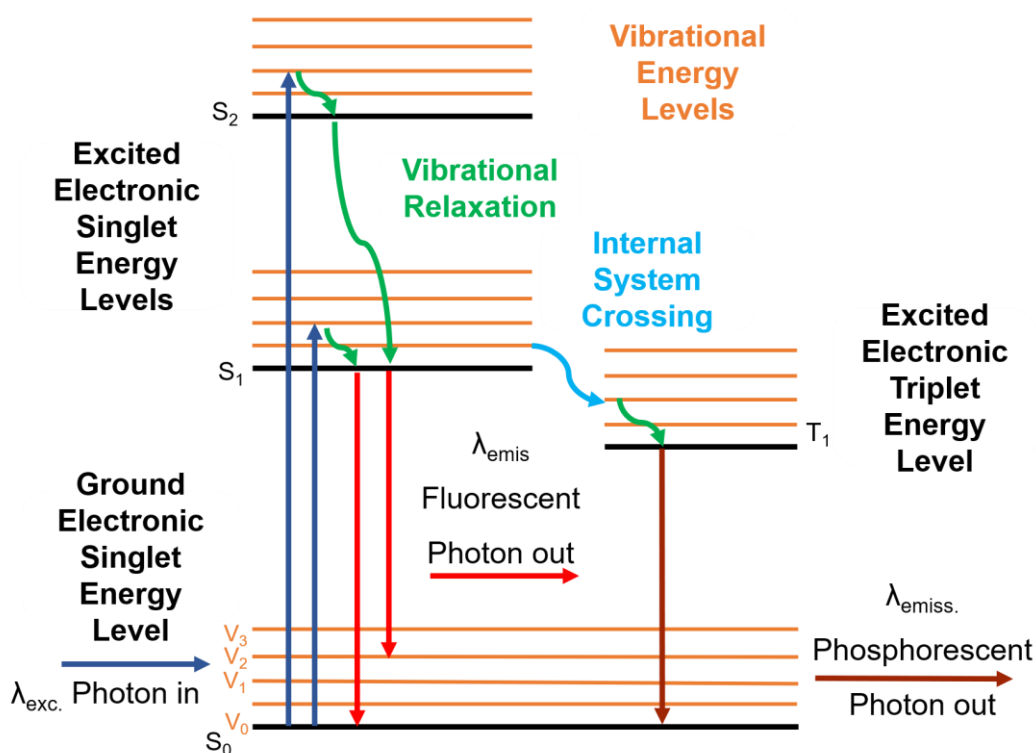


Figure 5: Jablonski diagram detailing absorption in dark blue, vibrational relaxation in green, fluorescence in red, intersystem conversion in light blue and phosphorescence in burgundy.

Fluorescence can occur automatically in substances which auto fluoresce, for example collagen or porphyrins, typically aromatic molecules; also known as endogenous fluorescence, or through fluorescent labelling using either a fluorochrome – a substance with inherent fluorescent nature, such as propidium iodide (PI), or a fluorophore – a fluorochrome conjugated to a larger molecule, such as antibody or protein in order to target a specific biomolecule or action within the sample; exogenous fluorescence. The intensity of emitted light from a fluorescent probe/dye is a linear function of the amount of fluorescent probe present as long as laser wavelength and intensity is kept constant, which lends fluorescence microscopy towards more quantitative analysis.

Fluorescence is governed by three fundamental parameters: molar extinction coefficient, quantum yield and fluorescence lifetime. Molar extinction coefficient is defined as the ability of a substance to absorb light at a specific

wavelength.⁸⁴ The molar extinction coefficient is an intrinsic property of the substance and is measured using the Beer Lambert Law.

Equation 5: Beer Lambert Law.⁸⁴

$$A = \epsilon cl$$

Where A is the absorbance of the material at a specific wavelength, λ , ϵ is the molar extinction coefficient and l is the path length. Molar extinction coefficient is measured by measuring the absorbance of a 1 molar (mol L^{-1}) concentration target substance with a specific wavelength in a cuvette of 1 cm path length. Quantum yield, ϕ , is defined as a dimensionless ratio of photons emitted from a fluorescent substance compared to the photons absorbed by the substance on excitation, in a range of 0.1 – 1. Fluorophores with a quantum yield close to one, have the brightest emissions.⁸⁴ The combination of the molar extinction coefficient and quantum yield is proportional to the brightness of the fluorochrome – how efficiently each fluorophore can excite and emit fluorescence within a sample at a specific wavelength, as shown by equation 6.

Equation 6: Equation relating molar extinction coefficient and quantum yield of a fluorochrome to its inherent brightness.⁹⁷

$$B_{\lambda} = \epsilon_{a(\lambda)}\phi$$

The molar extinction coefficient is also related to the absorption cross section of the molecule again through the Beer Lambert Law, as shown in equation 7. Absorption cross section is defined as the distance a photon must transverse in order to be absorbed by a molecule, with a larger cross-section resulting in more excitation.

Equation 7: Relationship between molar extinction coefficient and molecular absorption cross section for fluorescence excitation of a molecule.⁸⁴

$$\sigma = 3.82 \times 10^{-21} \epsilon$$

Where σ is the effective absorption cross section, ϵ is the molar extinction coefficient. Fluorescence lifetime is defined as the lifetime the fluorescent

probe spends in the excited electronic state without emitting a photon. Fluorescence lifetime is determined as the time it takes for the number of excited fluorescent molecules to decay exponentially to $1/e$ or 37% of the original population and is inversely related to the rate constants of quenching events in fluorescence.

Fluorescent probes have both an absorbance spectrum and emission spectrum which tend to be relatively similar, as the probability of excitation from the ground state to an excited state is proportional to the probability that a transition from the lowest excited vibrational state to the ground state will occur as stated by the Franck-Codon principle. An example of an absorbance and an emission spectrum is shown in figure 6.

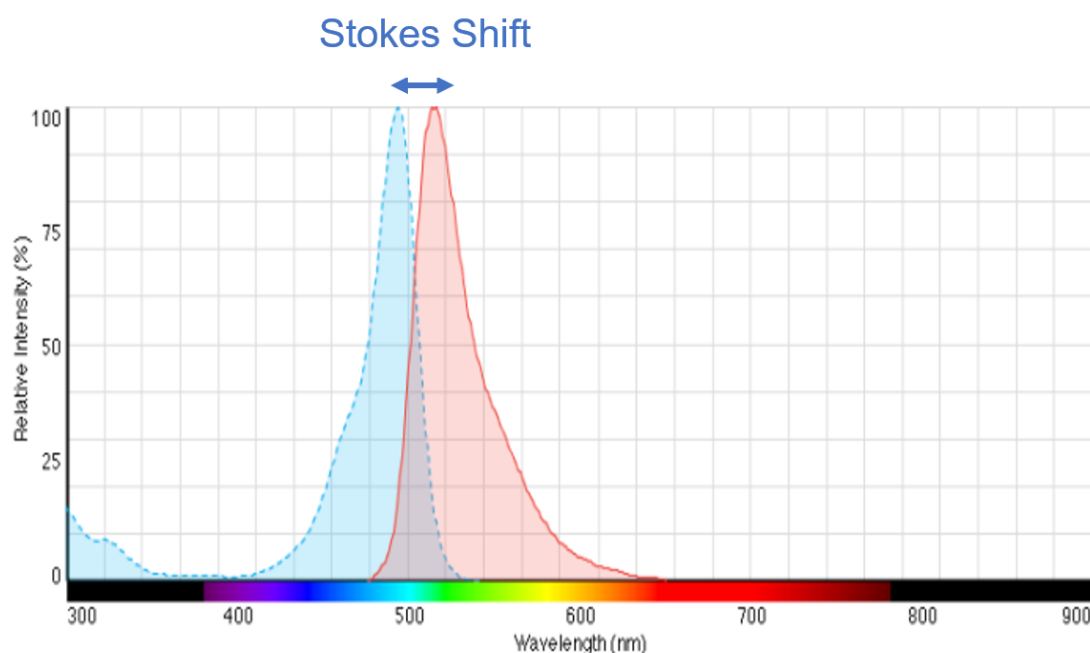


Figure 6: Excitation and Emission profile of Calcein AM dye, taken from Thermo-Fisher SpectraViewer as an example. The excitation and emission profile of many fluorescent probes are widely available.⁹⁸

The gap between the absorbance maximum and emission maximum is defined as the Stokes shift, again defined by Sir George Stokes. Stokes shift is an intrinsic property of the fluorescent material and it is this principle which allows such selectivity and sensitivity of fluorescence imaging, as filtration

allows for the separation of the excitation wavelength from the emission wavelength improving the detection of the fluorescence which is much lower in energy than the excitation light.⁸⁴

Fluorescent microscopes can operate in a number of configurations, the most commonly used are widefield microscopes which usually utilises an arc discharge lamp, LED or single wavelength laser focussed on the sample using an objective; or confocal laser scanning microscopy, which operates in the same principle as confocal Raman microscopy, but utilises a set of piezo scanning mirrors to move the laser beam across the sample instead of the stage scanning method commonly used in Raman microscopes.⁹⁹ Widefield microscopes are cheaper, due to the less complicated optics, and result in faster image acquisition and the need for less intense light for excitation. In comparison confocal laser scanning microscopy allows for greater spatial resolution and the ability to examine thicker samples due to the non-invasive sectioning capability, however longer analysis times and higher intensity illumination is required due to the spot by spot analysis mode which can lead to higher photobleaching and sample damage.¹⁰⁰

Most fluorescence microscopes operate in epi-fluorescence detection configuration, utilising the objective as both the objective, ensuring the full use of objective numerical aperture, to direct the light to the sample and the collection condenser. The light from the source passes to a dichroic mirror set at a 45 ° angle to the light path. which is highly reflective in the range of the excitation wavelength, but highly transmissive in the emission wavelength. On collection, the emission wavelength passes through the dichroic mirror and another filter which filters out any other wavelengths of fluorescence that may pass through the dichroic that is not strictly the target emitted fluorescence wavelength. Fluorescence microscopes can utilise CCD detectors or photomultiplier tubes (PMTs) depending on the microscope needs, and even electron multiplying charged couple devices (EMCCDs) for single molecule detection due to the higher quantum efficiency coupled with reduced noise required for sensitive detection.

Fluorescence microscopy has improved vastly since the inception of the technique, with multiple different methods, dyes and stains, microscopic setups, light sources and analytical techniques which have advanced the field of biological imaging.¹⁰¹

1.6.3 One and Two-Photon Fluorescence Microscopy

Two photon fluorescence was theorized originally in 1931 by Maria Göppert-Mayer, however it was unable to be proven experimentally until 1963 by Kaiser and Garrett, coinciding with the period after the development of the laser. The technique of two photon laser scanning fluorescence microscopy, however, was developed by Denk *et al* in 1990 as an alternative to conventional single photon confocal laser scanning fluorescence microscopy discussed earlier in this chapter.^{102–104}

As described in section 1.6.2, in single photon fluorescence, an excitation source, usually in the UV or visible wavelength range is incident upon a sample capable of fluorescence, if the photon energy/frequency matches the band gap between the ground and excited state of the molecule, the photon is absorbed and an electron is excited from the ground state of the molecule to an excited state. Vibrational relaxation occurs to the lowest vibrational level of the excited state, before the relaxation of the electron back to the ground state and the emission of a photon at a wavelength longer than the excitation wavelength of light which allows for the separation and detection of the fluorescence emission. The laser or sources used for single photon fluorescence are typically the arc discharge lamps or continuous wave lasers in the UV to visible wavelengths.

Two photon fluorescence operates very similarly to single photon fluorescence, however, the source used is typically an ultrafast (pico- or femto-second) pulsed laser, with high repetition rate in the near-infrared (NIR) region of the electromagnetic spectrum.¹⁰⁵ Two NIR photons of double the wavelength, but half the frequency of the corresponding UV or visible wavelength laser is focussed into a sample to a diffraction limited spot, where the two photons are simultaneously absorbed by the sample, causing the

same excitation, internal relaxation and corresponding fluorescence output events as single photon fluorescence, as demonstrated by the Jablonski diagram in figure 7.

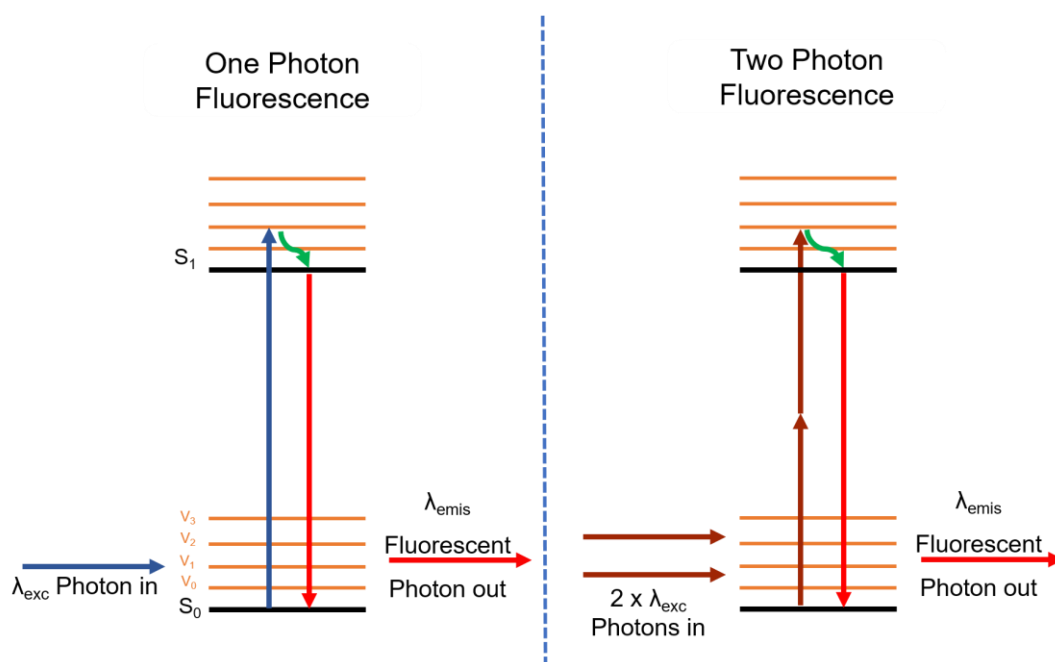


Figure 7: Jablonski diagram outlining the difference in photon absorption between single and two photon fluorescence. In single photon fluorescence, one photon, indicated in dark blue to mimic the UV/visible wavelengths typically used results in absorption, excitation, and fluorescence of one photon at a longer wavelength outlined in red. In two photon fluorescence, two photons of double the wavelength but half the frequency in the NIR region are absorbed simultaneously resulting in the fluorescence emission of one photon.

The use of NIR laser excitation results in photons lower in energy than those in the UV or visible regions, this results in the need for high irradiance to be used on the sample in order to cause sufficient absorption. Continuous wave lasers have been used for two photon fluorescence, however the high power at the sample causes large amounts of damage, particularly in sensitive biological samples, which the technique is often used for. Using high repetition pulsed lasers overcomes this issue by allowing the use of high irradiance levels at the sample ($10^{10} - 10^{12} \text{ W cm}^{-2}$) but at a rate of MHz ($\sim 80 - 100 \text{ MHz}$) allows for the average power at the sample to be in the order of mW, minimising the photodamage on the sample but also allowing high enough concentration of photons for two photon fluorescence to occur.¹⁰⁶

The biggest difference between single and two photon fluorescence is the location of where the fluorescence emission occurs within the sample. In single photon fluorescence, confocal or widefield microscopy, a beam of excitation energy is incident on the sample and fluorescence occurs anywhere within that beam path that the energy of the beam's photons match a bandgap of the sample under investigation. However, in two photon fluorescence, the fluorescence emission occurs only at the focal point of the objective, as this is the position where the laser beams have adequate energy and the photons are within close enough vicinity to each other and the sample, due to the high repetition rate of the pulsed laser, to be absorbed simultaneously by the sample.¹⁰⁷ Therefore, two photon fluorescence microscopy can operate without the need for physical pinholes removing the light detected from the sample as no out of focus fluorescence can occur, so no background fluorescent interference occurs.¹⁰⁷ Figure 8 outlines the difference in beam paths for emission of fluorescence from a sample during single and two photon fluorescence.

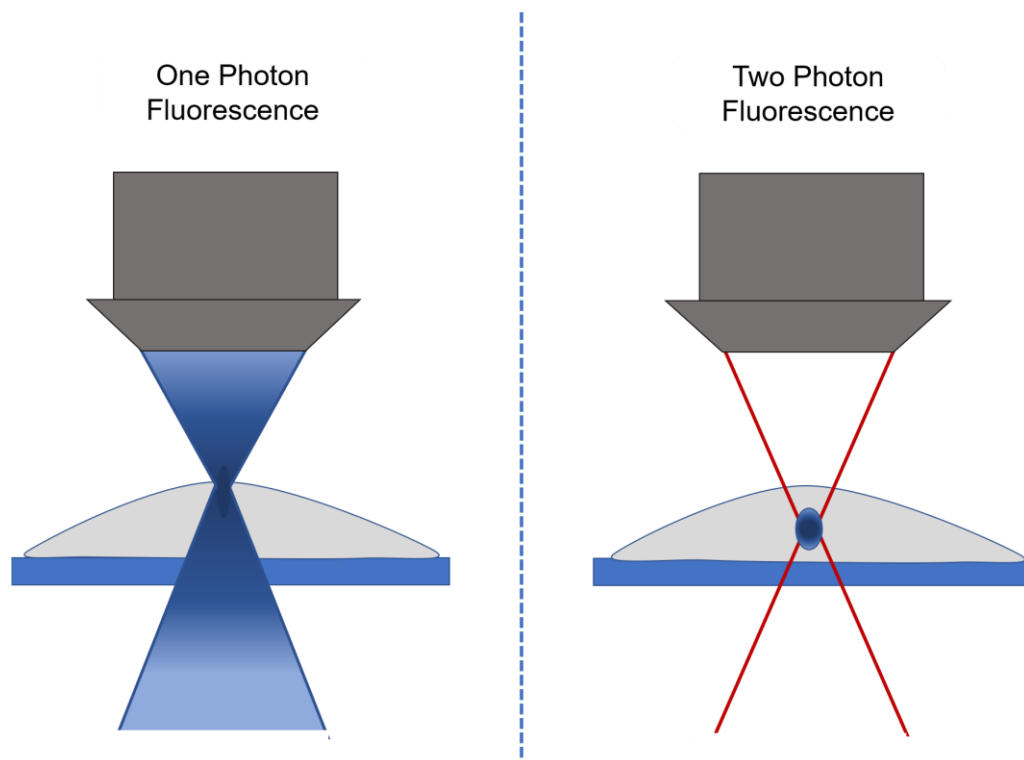


Figure 8: Laser beam behaviour on single and two photon fluorescence. In single photon fluorescence the illuminating beam can cause fluorescent emission from anywhere within the sample, however in two photon fluorescence, emission is only possible within the focal volume of the diffraction limited laser spot within the sample.

Two photon fluorescence, therefore, does have some advantages over single photon fluorescence for analysis of biological samples. Due to fluorescence emission only occurring within a small volume in the sample, photodamage of the sample as well as photobleaching of the fluorochrome itself is reduced, resulting in higher cell viability and less inhibition of the cell cycle.²³ Using a laser beam in the NIR region does worsen the spatial resolution of TPF microscopy in comparison to utilising lasers in the UV or visible wavelengths, due to the dependence of resolution on wavelength, however, the longer wavelength laser allows for deeper penetration into a sample than lower wavelength single photon laser counterparts, improving optical sectioning and allowing the analysis of thicker sample specimens with higher spatial resolutions than confocal laser scanning microscopy (CLSM).²³ Also, in CLSM due to the higher probability of photobleaching, compromises are made between the optimal spatial resolution the system is capable of and limiting the

photobleaching in the sample by using reduced laser power and larger pinhole sizes for adequate emission detection, so in practical terms, the spatial resolution differences in CLSM and TPF microscopy can be minimal.

TPF does have some disadvantages though; photodamage can still occur in TPF microscopy, causing photooxidation reactions resulting in the production of reactive oxygen species (ROS) and triggering damage within cells and the use of high power IR lasers can cause thermal damage, especially within highly absorbing samples, such as those which are highly pigmented, so care must still be taken with sample exposure durations and laser power during TPF as with CLSM.²³ Pulsed NIR lasers also tend to be more costly than continuous wave UV-vis lasers or arc discharge lamps, however some of these which are available are tuneable, allowing for access to a wider range of fluorochrome excitation with one source as well as the potential for simultaneous fluorochrome excitation in one frame in comparison to CLSM single photon which would often require the use of multiple laser lines or light sources and separate acquisitions of fluorochromes.¹⁰⁸

Two photon and indeed higher multiphoton processes have widened the field of microscopy especially for biological analysis, by minimising the photodamage and photobleaching in photosensitive samples and fluorochromes as well as allowing for more versatile sample analysis such as thicker tissue sections and prolonged time studies without cell death. However, the cost of laser sources for such imaging can be prohibitive and as with single photon fluorescence microscopy, samples must be labelled with a fluorescent dye or probe for imaging specificity which can interrupt the process under investigation.

1.6.4 Fluorescence for Live Cell Analysis

Fluorescence microscopy allows for the visualisation of the dynamics within cells through the endogenous or exogenous fluorescent molecules. Fluorescence microscopy has been used to investigate cell viability and cell death pathways, proliferation and cytotoxicity effects.¹⁰⁹ It has been used to track cell dynamics, metabolism and transfection.^{110,111} Fluorescence

microscopy can be a single or multiphoton process and used to detect down to single molecule levels due to the sensitivity and selectivity in combination with the wide range of fluorescent tags available.⁹⁵

1.7 PROJECT AIMS AND OBJECTIVES

The initial overarching aim of this project was to attempt to develop a framework for levels of safe laser exposure of live cells during Raman spectroscopy analysis. The balance between laser power for adequate Raman signals and minimising cell damage can be difficult to manage, and sometimes too much laser power could have adverse effects on analysis, though often care is taken to avoid such photodamage. Initially, it was postulated that a comparison study could be performed on various systems once the method for cell death or stress detection was developed during Raman spectroscopy to ensure the method was consistent across varying systems.

In line with the initial thought of system comparison, an aim for this thesis was to characterise the response of the Raman spectrometer system available at NPL, through the use of metrology, in order to allow for fair comparison of method transfer to different analytical systems if able to.

After system characterisation, sample characterisation and optimisation were the next aims, to allow for consistent cell analysis between samples, but also between different analytical methods, as fluorescence microscopy and dye exclusion assays were to be used to evaluate cell viability. During the sample characterisation, a proof of concept study on the MG-63 cell line to detect spectral changes between live and dead cells was performed building upon methodology outlined in a previous study in the literature by Notingher et al.⁵ This study was used to confirm the spectral changes expected during cell damage and to help determine the timeframe in which cell death could occur during Raman spectroscopy with 532 nm laser wavelengths.

The final aim of this thesis was to evaluate whether there could be a link between the spectral differences seen when inducing photodamage in MG-63 cells during Raman spectroscopy and the production and consequential detection of reactive oxygen species (ROS) and mitochondrial activity by fluorescence microscopy on the induction of sample damage and eventual cell death. Essentially whether the spectral changes observed, if any, could imply cell stress before cell death as an indicator for safe laser irradiation levels

during analysis. It was consequentially postulated that the spectral changes could be translated into a Raman based imaging technique such as SRS and the damage could be imaged in real time by tuning to the peaks of interest which showed significant change in the spontaneous Raman spectral studies.

Overall, the aim of this work was to build upon previous knowledge; that high powered illumination sources can induce sample degradation, particularly in biological samples which are light and temperature sensitive, and to try and relate these changes to indicate sample stress and eventual death in a metrological way to help future users of Raman spectroscopy understand the operational limits required for safe cell sample analysis.

2 RAMAN METROLOGY

2.1 RAMAN METROLOGICAL NEEDS

The widespread application of a technique ultimately relies on the ability to reproduce results accurately regardless of the system used or the user performing the analysis and the reliability and traceability of the measurements. This is an issue that has limited Raman spectroscopy from becoming a key technique used in many applications, especially within a clinical setting.^{1-3,112} Raman spectroscopy can be used as both a qualitative and quantitative technique, due to the linear dependence of Raman signal on sample concentration.¹¹² However, in order to be used quantitatively, the Raman system must be well characterised and often rigorous data processing is required to understand the data.¹¹³

Whilst statistical analysis and calibration measurements can be applied to Raman measurements, without a good baseline understanding of individual system capability and response to known samples and normalising these in order to be able to compare measurements taken from different systems, the qualitative or quantitative measurements are applicable to that sample, during that specific measurement only. The same sample can be re-analysed and have a slight variation in signal intensity or position within the spectrum due to a variety of factors, including laser fluctuation, detector response, sample degradation, movement in the sample or the exact same position not being analysed within the sample due to the inherent confocal nature of the microscope system.^{114,115} Simple mixtures can allow the production of calibration curves that the peak intensity of an unknown can be measured against to deduce concentration, and therefore quantitative results, however in more complex samples, more data processing can be required to determine quantitative results.^{113,116}

Raman spectroscopy has grown in popularity due to the ease of sample analysis; there is minimal sample preparation required, samples can be analysed in a variety of media including water (unlike the closely related infra-

red spectroscopy) and glass, and the illuminating laser is able to be coupled to fibre optic cables, allowing for analysis in harsher conditions such as inline process analysis or non-typical laboratory conditions.^{117–119} This increase in uptake means that non-expert users can perform the analysis and interpret the results, and here-in lies the issue with the lack of robust calibration checks. Without the use of standardisation or metrological checks, Raman analysis can often produce variable results.¹²⁰

There still is a requirement for greater understanding of the spatial resolution capability of Raman spectroscopy systems as well.¹²¹ Optical aberrations influence the Raman signal generated at different penetration depths of samples, and indeed different laser wavelengths can penetrate samples with varying efficiencies.^{122,123} Depth penetration requires more standardisation, especially during the analysis of layered samples or those imbedded in materials that have differing refractive indexes from each other, to fully understand the sample analysis.¹²⁴ Lateral spatial resolution also requires careful consideration, as under-sampling and sample size in combination with laser spot size can affect the analytical output.^{121,125}

Therefore, whilst Raman spectroscopy is an inherently powerful technique, allowing for the deduction of a chemical fingerprint of a sample, coupled with minimal sample preparation, the ability to analyse a wider range of samples than related spectroscopic techniques, there is a greater understanding required of individual system capability and analysis output, especially if comparative results are being generated in different analytical systems, or indeed in environments such as the pharmaceutical industry that require traceable measurements which conform to GMP/GLP or ISO standards.^{2,3} In order to increase the repeatability, robustness and accuracy of any Raman analysis, and especially in situations where Raman analysis may be performed across multiple instruments, a robust investigation into the performance of the system is recommended for the most accurate analytical output, which ideally utilises standards developed purely for Raman system evaluation.

2.2 PROJECT AND CHAPTER AIMS

The general aim of this project was to attempt to develop a framework for safe levels of laser exposure for biological analysis that minimised sample damage and analytical output variance. In order to fulfil this aim, a rigorous understanding of the system(s) being used is required to ensure that all measurements and outcomes are reproducible and to allow method transfer to different analytical instruments in the future to build upon the framework and understanding.

Therefore, the main aim of this chapter was to characterise the Renishaw Qontor inVia Raman system that was to be used for this project to determine suitability for live cell analysis. The characterisation included evaluation of spatial resolution, peak position calibration as well as laser power evaluation and selection of substrate and media for optimal sample analytical output.

2.3 RAMAN SETUP

There are multiple commercial manufacturers of Raman microscopes and spectrometers, including Renishaw, Horiba and Witec. Raman spectrometers are comprised of the same main component parts which can be purchased individually from multiple sources, this has resulted in many laboratories building their own systems to the specifications they require and to better fit the precise analysis being done. This means that there can be variation in the hardware of Raman microscopes and spectrometers depending on the manufacturer. This variation in hardware can result in differences in analytical output, which is why understanding Raman metrology is important, as it can allow for differences in analytical outputs to be minimised and for fairer comparison between data generated from different systems.

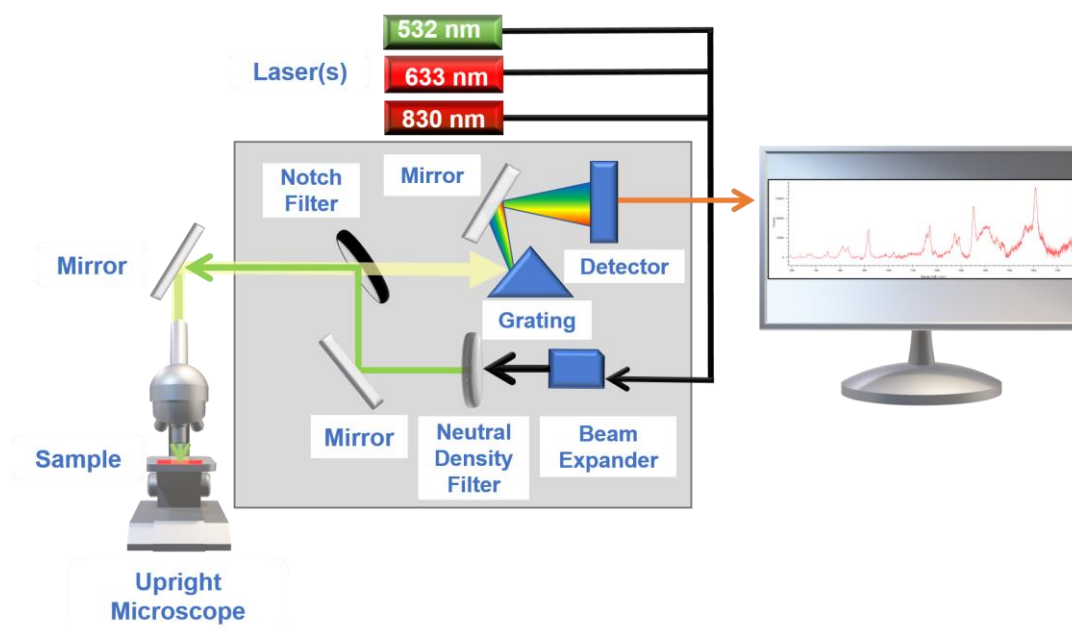


Figure 9: Diagram schematic of a typical confocal Raman Spectrometer, equipped with multiple laser wavelengths, a microscope usually upright or inverted, equipped with multiple objectives and a piezo stage as well as grating and detector and multiple optical mirrors and filters.

All dispersive Raman instruments are equipped with one or multiple laser wavelengths, typically ranging from the ultra-violet (UV), in the wavelength range 10 – 400 nm, through the visible region, in the range of 400 to 750 nm

and near-IR (NIR) wavelength, in the range of 750 nm to approximately 1000 nm. Laser wavelength must be taken into consideration depending on the sample being analysed.^{57,126}

Raman spectrometers are often referred to as Raman microscopes or Raman microspectrometers, due to the coupling of the spectrometer to a traditional microscope. The microscope aspect of the instrument allows for higher spatial resolution during analysis, with the ability to discern discrete parts of a sample down to micron resolution. This allows for greater detail and understanding of the sample. The use of light microscopy opens Raman spectroscopy to being used with biological samples, and samples of smaller magnitude than analytical techniques not equipped with the spatial resolution afforded by a microscope.⁵⁷

As with most visible light microscopes, Raman microscopes can utilise a variety of different objectives for focussing on samples. Choosing the correct objective is again dependent on the sample being analysed. Objectives are available with different magnifications combined with different working distances, ranging usually from 5x to 100x magnification and most systems will be able to be equipped with a few different magnifications. Further options for choosing an objective is the media in which the objective works best; air objectives are commonly used and supplied with the instruments available off the shelf from manufacturers, however oil and water immersion objectives work better for samples that require better resolution or are suspended in media with a refractive index different to air.¹²¹ Another parameter to consider when choosing an objective is the numerical aperture. Magnification and numerical aperture are linked, a lower magnification objective will often have a lower numerical aperture and result in lower spatial resolution. Conversely, the opposite can be found, with higher magnification having higher numerical aperture and higher spatial resolution.⁵⁷

The optical components within a Raman spectrometer can vary slightly, but all will usually utilise a notch or edge filter depending on the manufacturer/use. Each laser wavelength that the instrument is equipped with requires a separate

filter. This removes the intense Rayleigh scattered light, allowing the weak Raman scattered signal to be seen. A notch filter usually has a sharp absorption band which is only a few nanometers wide and is chosen to correspond with the excitation laser wavelength, filtering this out and allowing all other wavelengths of light to be transmitted to the detector. An edge filter works by filtering out light up to the excitation laser wavelength and a few nanometers above it and then allowing any light of longer wavelength to be transmitted to the detector.⁵⁷

All spectrometers are equipped with a grating to separate the Raman signals before reaching the detector. This grating can be holographic or a physically ruled grating. There are pros and cons to using either grating, but ultimately depends on the spectral resolution desired and the laser source being used to analyse the sample

The last component of a typical Raman spectrometer is the detector. Again multiple different detectors exist, the most commonly used are silicon based charged coupled device (CCDs) arrays or non-silicon based array detectors. The detector choice is mainly dependent on the laser wavelength being used and suppressing any noise that could affect the Raman signal sent to the detector and electronic output. CCDs are commonly used with UV and visible light wavelengths, with non-silicon based detectors such as the InGaAs detector being utilised for detection of wavelengths into the IR region.^{57,127}

2.4 RAMAN INSTRUMENTATION

As mentioned previously in sections 2.1 and 2.2, multiple components can affect the analytical output of a Raman spectrometer. The use of metrology allows for these differences to be understood and compensated for. This is essential when undertaking collaborative work which requires elements of analysis performed on different Raman spectrometers in different institutions for fairer comparison of results, or indeed during quality analysis and control. The main differences in various Raman system tends to be the spatial and spectral resolution that the spectrometers are capable of, as well as the relative signal intensity, and this can affect the analytical output.

2.4.1 Laser Wavelength

Raman scattering is an inherently weak phenomenon, so understanding the sample being analysed can be key for gaining reliable Raman output. Laser wavelength choice can affect the sensitivity of the Raman technique, the spatial resolution the instrument is capable of and can depend on the sample being analysed.

Raman scattering is dependent on the intensity of the illuminating laser and the inverse fourth power of the illumination laser wavelength, and therefore proportional to the fourth power of the Raman frequency as shown in equation 8, which in turn would result in proposing that lasers in the UV or closer to UV wavelengths would result in better Raman scattering in any sample.

Equation 8: Simplified relationship between Raman scattering and laser frequency.¹¹⁷

$$I_R \propto \nu^4$$

While this can be true, UV photons have a higher energy, and are therefore more likely to experience fluorescent transitions instead of the Raman scattering transitions required. Fluorescence and Raman scattering are closely related phenomena, with only a difference in the timescale in which the processes occur. Fluorescence can often overshadow the weaker Raman scattering effect and therefore any chemical specificity deducible from the sample.¹²⁸ Fluorescence often occurs in the wavelength range above 300 nm,

therefore using laser wavelengths below this could result in minimised fluorescence as the fluorescent signal would be shifted to higher wavelengths leaving a fluorescent free region for Raman scattering to be visible during analysis.¹²⁹

UV lasers tend to be bulky, more unstable, and more costly than visible lasers on the market, sometimes requiring frequency doubling or quadrupling to generate an adequate UV wavelength. The energy of UV lasers can also result in sample degradation, particularly in sensitive samples, this has seen an interest in using pulsed lasers compared with the traditional continuous wave (CW) lasers, or extremely fast raster scanning of a sample to minimise immediate sample damage by the laser.¹²⁸ UV illumination also requires more specific optics, mirrors, gratings and detectors for operation in comparison with visible and NIR illumination. Some spectrometers can be equipped to work with UV through to NIR illumination, but this can become costly.

UV Raman is often utilised for the analysis of thin layered samples, such as semiconductor materials as it allows for shallow penetration into thin samples due to the shorter wavelength of the light.¹³⁰ It has also been used for the elucidation of protein components which can cause issues when utilising visible wavelengths due to the overpowering autofluorescence present in biological molecules.¹³¹

Most Raman spectrometers tend to be equipped with visible wavelength lasers. Visible wavelength lasers range from 400 nm to 750 nm. 514 nm, 532 nm and 633 nm are some examples of commonly used visible laser wavelengths. Lasers in the blue-green range of the electromagnetic spectrum tend to be used on inorganic materials such as carbon-based materials like graphene.¹³² Moving towards the red end of the visible light spectrum, fluorescence can become reduced, but the Raman scattering is less efficient as mentioned previously in section 1.4.2. 633 nm wavelengths tend to be used to analyse biomolecules due to the reduced fluorescence as the wavelength gets longer, and the energy of the photons striking the sample will be lower, resulting in reduced sample damage.¹³³ However conversely, the Raman

signal will be weaker and therefore longer acquisition times may be required to generate adequate Raman signal. Using lasers in the visible wavelength range is also useful when using silicon-based array detectors such as CCDs as the Quantum Efficiency (QE) is optimised in the visible wavelength range, with >60 % QE achievable when using CCD detection in with a 532 nm excitation laser.¹³⁴

Lasers in the NIR range, above 750 nm are increasingly being used in Raman spectroscopy now as they provide a trade-off between minimised autofluorescence as the wavelength of the light increases and the energy of the photons decrease with Raman signal. Due to the lower energy photons, longer acquisition times or higher laser power can be used with minimised chance of sample damage, which will also increase the Raman scattering due to the dependence of the scattering on the intensity of the illuminating laser even though inverse wavelength to the power of four dependence will reduce the Raman scattering.⁵ Lasers at 785 nm, 830 nm and 1064 nm are now widely available. However, consideration must be taken for the detector being used, as silicon-based detector efficiency decreases towards 1100 nm where it drops sharply. Non-silicon-based detectors such as Indium Gallium Arsenide (InGaAs) detectors can be used with NIR wavelengths to overcome the issues experienced with traditional CCD detectors.¹²⁷

2.4.2 Spatial Resolution

In 1873, Ernst Abbe discovered that regardless of light source used, in microscopy the spatial resolution is limited by the diffraction nature of light and the optics through which the light passes.¹³⁵ Confocal Raman spectroscopy utilises a microscope for focussing the laser light onto the sample, and therefore the spatial resolution capability is also defined by the diffraction limit. Light forms a distinct Airy disk pattern, as shown in figure 10, when passing through an aperture, slit or object due to the wave nature of light and diffraction causing interference in the light waves.

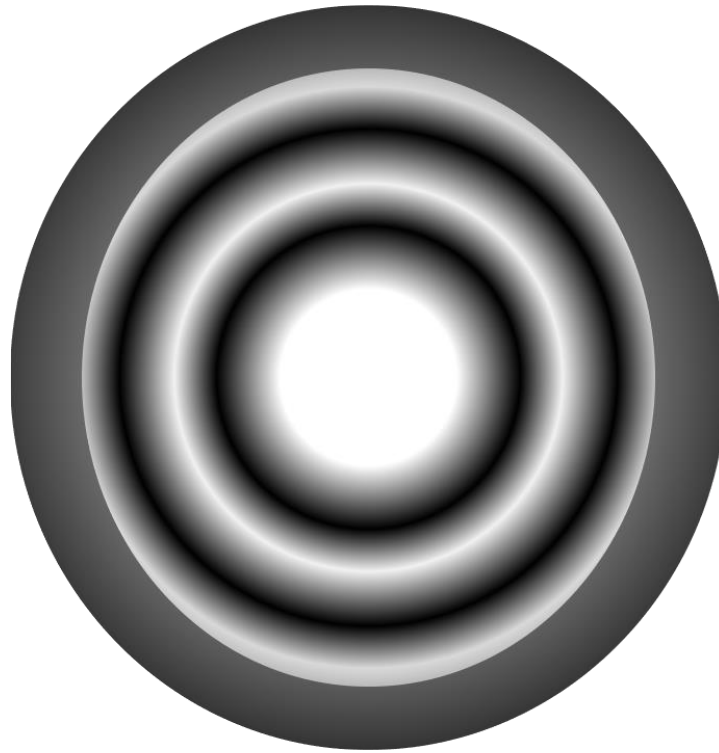


Figure 10: Schematic representation of Airy Disk pattern formed on the diffraction of a point light source passing through an object, aperture or slit.

The Airy disk pattern shows a bright central ring with surrounding less intense rings, and generally the laser spot size is defined as the diameter between the first minima, darker regions, in the diffraction pattern. Laser spot size is defined, theoretically, as:

Equation 9: Equation for calculating theoretical laser spot size.¹³⁶

$$Laser\ Spot\ Size = \frac{1.22\lambda}{NA}$$

Where λ is the laser wavelength, and NA is the numerical aperture of the focussing objective. Therefore, the laser spot size is dependent on both factors, with a lower wavelength or higher numerical aperture minimising the laser spot size. Measuring the laser spot size directly however can be difficult. The tight focussing of such a high energy beam can cause damage to detectors capable of measuring the laser spot size, therefore filtration is often required to compensate for this. The knife edge test is a well-known method

using a 'knife edge' and passing a laser beam over the edge in increments with a photodiode underneath to detect the change in laser intensity being measured.^{137,138} The resulting profile will result in a Gaussian peak which can be used to evaluate laser spot size through the FWHM of the peak. It should be noted that the Airy disc definition is not the only definition of laser spot size. The theoretical values also assume that the system is aberration free, and the back aperture of the lens is filled, which may not always be the case.

Laser spot size is therefore related to the spatial resolution of a Raman microscope, in theory, a smaller spot size should result in improved spatial resolution where spatial resolution is defined as the ability of a microscopy to resolve two discrete objects at the smallest separation distance possible.¹³⁵ This can be defined as being from the minima of one Airy disk to the first maxima of the next, known as the Rayleigh criterion, which is approximately half the laser spot size. Note, this is not the only definition. Therefore, the spatial resolution of a Raman microscope is determined by the radius of the Airy disks produced in the system. This can be laterally, in x and y or axially, in the z-direction. Utilising the Rayleigh criterion, lateral resolution can be calculated theoretically using equation 11.

*Equation 10: Equation relating the Rayleigh criterion and resolution.*¹³⁶

$$Res_{x,y} = \frac{0.61\lambda}{NA}$$

$$NA = n \sin \theta$$

NA refers to the numerical aperture of the objective which is calculated using half the angle of incident light from the objective to the sample, θ . This theoretical calculation of lateral resolution only applies to ideal optical systems and within laboratory instruments the resolution is often worse due to laser fluctuations, aberrations within the optics, incomplete filling of the back aperture of the objective and non-ideal refractive index matching between sample and objective. Even utilising a high NA objective and UV excitation wavelength, the resolution of a Raman system will still be limited to

approximately 200 nm, which also limits the size of the smallest samples that can be analysed clearly using Raman spectroscopy.

Axial resolution is slightly more complicated. If the Airy disk distance is taken as the indication of axial resolution, it must be approached in 3 dimensions which results in difficulties arising from the elongation of maxima and minima in the z-direction and introduces a varied amplitude to each ring of the circular pattern. This is known as a point spread function (PSF), illustrated in figure 11. as the deeper the laser probes within a sample, the more laser attenuation occurs, and signal is lost purely from the light travelling into the sample.^{124,125} The axial resolution can be calculated theoretically by equation 11.

Equation 11: Equation for theoretical calculation of axial resolution.¹³⁶

$$Axial\ Resolution = \frac{1.4\lambda}{NA^2}$$

The axial resolution is dependent on the square of the numerical aperture of the objective, and this tends to result in the axial resolution being up to approximately three times worse than the lateral resolution.¹³⁹ The axial resolution is also dependent on the ability of the Raman microscope confocal pinhole to filter out the out of focus signal however, and optimisation of this pinhole can help improve the axial resolution.

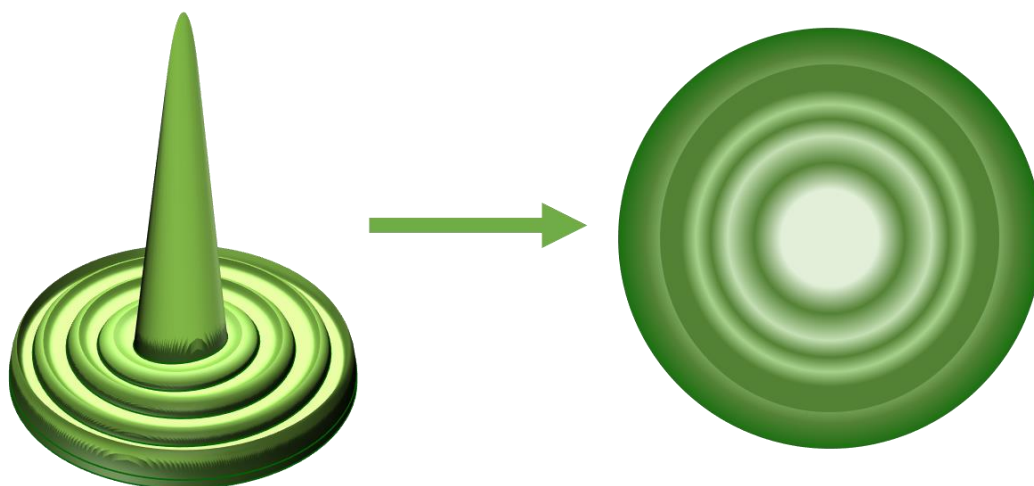


Figure 11: Schematic of an in-plane Point Spread Function to indicate laser intensity in the z-direction

Due to the difficulty of measuring the Airy disk size directly for both axial and lateral resolution, the spatial resolution of a microscope or optical instrument is often determined experimentally utilising either a thin layered sample, such as graphene, which is ideal due to being atomically thick and uniform, for axial resolution or nanoparticles smaller than the laser beam point spread function for lateral resolution. Nanoparticles or small objects less than a quarter of the laser point spread function will only negligibly interfere with the laser beam resulting in a more direct measure of the laser beam profile and therefore resolution.¹³⁶

The evaluation of spatial resolution of an optical system is key, not only metrologically, but also in order to utilise the full capability of a Raman system. If the sample size is not considered regarding the laser spot size and spatial resolution of the optical system as well as the pixel size and capability of the digital detection system, there can be losses in signal and specificity that can hinder analytical output.

2.4.3 Spectral Resolution and Wavenumber Calibration

Spectral resolution of a Raman microscope refers to the ability of the system to resolve the maximum number of spectral peaks from each other during the

analysis of a sample.¹⁴⁰ Again, spectral resolution capability is dependent on the analytical output required; if attempting to confirm the presence of a chemical; low-medium spectral resolution can be used as the overall spectral output can act as confirmation or not of a substance. However, if attempting to elucidate conformational changes within a sample, for example within crystalline structures – high spectral resolution is preferable due to the possibility of small chemical changes indicating structural differences. Spectral resolution is dependent on primarily the diffraction grating used, but also on the focal length of the spectrometer, the slit size, the detector pixel size and number and the excitation wavelength.

The spectral resolution is mainly dependent on the diffraction grating used. There are two main types of diffraction grating; either ruled, which is manufactured using a ruling engine and can have either a flat or concave shape with grooves placed parallel to one another, or holographic, which utilises the interference pattern from two UV beams overlapped in glass resulting in an interference pattern to disperse light.⁵⁷

Gratings typically range from a groove density of around 300 g/mm up to 2400 g/mm, however excitation wavelength will need to be taken into consideration for using gratings with higher groove densities. Spectral resolution decreases on decreasing the excitation wavelength, therefore the groove density will tend to be higher to achieve comparable spectral resolution in the visible region compared to NIR.¹⁴¹

There are pros and cons to using each type of grating. The ruled grating is cheaper to manufacture and has a longer lifetime than the holographic grating, however it can have aberrations due to the manufacturing process and this results in higher amounts of stray light being passed to the detector altering the spectral information gained. Holographic gratings have more uniform grating spacing, however the use of UV beams often has poor response on detectors, and more optical loss in the system resulting in lower signal reaching the detector.¹⁴²

In confocal Raman microscopy, in order to improve chemical specificity and optical resolution, the laser light that has interacted with the sample will pass through a slit or hole before reaching the diffraction grating and detector. This slit helps to improve spatial resolution as it reduces the contribution of out of plane signals on the imaging capability of the microscope.⁵¹ It also improves the spectral resolution, as the reduction of out of focus light reaching the grating and therefore detector will allow for greater chemical specificity and easier separation of the frequencies of light hitting the detector. However, due to Raman scattering being an inherently weak phenomenon the slit size will need to be balanced with the amount of Raman signal reaching the detector. For higher spectral and spatial resolution, the smallest slit or hole size possible would be best, however, this can also ultimately exclude most of the Raman signal, resulting in low intensity output from the detector, or peaks that are non-identifiable from the noise.¹²⁸

The slit must also be considered in terms of pixel size of the detector. Ideally, the pixel size of the detector would be lower than that of the slit so that spectral resolution and bandpass (spectral range) is limited by slit size and not the size of the pixels of the detector. The overall detector size is also important, as the grating diffracts the incoming light and spreads it out before being focussed to the detector, if the detector is smaller than the width of the diffracted light incident upon it, the chemical information in those regions will be lost. Detector capability and the effect on Raman analytical output will be discussed in more detail in section 2.4.4.

Lastly the focal length of the spectrometer can affect resolution. The focal length of the spectrometer is defined as the distance from the mirror after the grating to the detector. Many spectrometers have a focal length of between 200-250 mm, however, the longer the focal length, the better separation of light before hitting the detector, therefore the better the spectral resolution. Focal length can be increased beyond 500 mm for use with UV lasers or for samples that require optimum spectral resolution, however this will increase the physical size of the instrument and also lead to Raman signal loss through the longer path length of the instrument from the sample to the detector.

The spectral resolution capability of a Raman spectrometer should be well understood for the analytical process it is being used for due to the various factor, outlined above, which can influence it. The American Society for Testing Materials (ASTM) have a protocol for spectral resolution calibration, E2529, that utilises a low-pressure discharge lamp such as neon, argon, or mercury, which have atomic emission lines narrower than Raman vibrational peaks. The ASTM standard E1840, complements the spectral resolution calibration by providing reliable Raman shift values for easily analysable samples to evaluate system performance.^{25,26} The combination of these standards allow for fair comparison of measurements taken on different Raman systems.

2.4.4 Detector Choice and Capability

Raman detectors have improved over the last few decades from single channel photomultiplier tubes to multichannel array detectors such as photodiode arrays (PDAs) and charged coupled device arrays (CCDs).¹²⁷ CCDs tend to be the main detector choice and are part of most commercially available Raman systems due to their low cost coupled with their high quantum efficiency over a large range of wavelengths and reduced noise contributions over other detector types available. CCDs are silicon-based detectors that utilise metal oxide semiconductor electrode gates to transfer charge between pixels and eventually to be read out to the detector.¹⁴³ Pixels and CCD arrays are available in a range of sizes and formats, which can be selected dependent on the user's needs. The smaller the pixel, the higher the resolution tends to be, as it will be limited by the focussing optics and not the pixel size, however, larger pixels allow for more light collection resulting in higher sensitivity for detection.

CCDs can pose issues for use with NIR wavelength ranges, and into the UV.³ Due to the nature of silicon and the penetration depths of the varying wavelengths, CCD composition may need to be altered to increase the QE of detection. In the UV, photon penetration depth is very low and can be absorbed by the gate material in front illuminated detectors, therefore utilising a thinned, back-illuminated CCD would increase the QE of the detector and therefore the

signal output, but this results in a more fragile more expensive detector.¹⁴⁴ Conversely, NIR radiation has a longer wavelength and significantly longer penetration depth. If the silicon is too thin, the radiation will pass through the detector completely and no electron-hole pairs will be generated. Therefore, front illuminated CCDs with thicker silicon layers are more effective in NIR. The QE of silicon-based CCDs significantly reduces above 1100 nm wavelength, as the energy of the photons in this region are too low to generate the necessary electron-hole pairs. For wavelengths into IR, a non-silicon-based detector is more effective. A few different types of non-silicon-based array detectors exist, such as Germanium (Ge), Indium Gallium Arsenide (InGaAs) or Mercury Cadmium Tellurium (HgCdTe) detectors. These tend to be available in smaller array sizes than traditional silicon CCDs and operate over a more limited wavelength range, however the QE in IR is superior to that of traditional CCDs.¹⁴⁴

Both silicon and non-silicon-based array detectors benefit from cooling in order to reduce the dark current generated in the detection process. This is usually done thermoelectrically or cryogenically, with the dark current reducing with a reduction in temperature until a low enough temperature where the detector no longer works efficiently.³ The reduction in temperature can also reduce the range of wavelengths the detector can operate in. Ultimately the composition of the detector can affect the detection efficiency and capability of a Raman system, and therefore requires some consideration depending on the user's requirements.

2.4.5 Sample Substrate and Mounting Media

For full sample understanding, control measurements of blank sample substrate and immersion media should be taken before any analysis is performed. Taking Raman spectra of substrate only will show any background fluorescence or potential interference in the sample from the media or substrate itself.¹²⁶ For Raman analysis glass coverslips can be used, but typically calcium fluoride or magnesium fluoride is preferred. This is due to glass contributing a large amount of background fluorescence in Raman

spectra, which can overshadow weaker Raman scattering samples or low concentration samples and lead to the need for greater data processing and deconvolution.⁵⁷ Calcium fluoride has a peak around 250 – 300 cm^{-1} and low fluorescence contributions making it a more ideal mounting substrate for Raman analysis than glass, while the inherent CaF_2 peak can also be used as an internal calibration for sample information to be normalised against. Care should be taken when using polymeric mounting substrates due to polymers having Raman contributions as well as fluorescence contributions. Some biological sample holders contain polymer viewing windows instead of the traditional glass.

Similarly, mounting media can influence Raman analysis, either due to the media composition itself being Raman active or highly fluorescent, overshadowing the lower Raman signal of the sample. Refractive index (RI) changes between the laser leaving the objective and entering the sample can cause laser aberrations, and lead to Raman signal loss; if the sample is suspended in liquid media, consideration should be given to the use of an immersion objective to try and counteract the mismatch in refractive index between air and the solution.¹²⁴

Layered samples can also cause issues with Raman spectroscopy, particularly through signal loss with penetration depth. The confocality of Raman microscopy allows greater depth resolution within layered samples over that of a non-confocal system due to the exclusion of out of plane signals. However, especially when utilising air objectives while imaging a thick sample, the change in refractive index from air to sample causes the laser beam focus and penetration within the sample to change as determined by Snell's Law.¹²² Everall calculated that laser focus is broader the deeper it penetrates into a sample and therefore depth resolution is dependent on the sample depth and thickness, and will often underestimate the thickness of a layered sample.¹²⁵ Care must be taken to understand the spatial resolution capabilities for thicker sample analysis.

2.5 RESULTS AND DISCUSSION

2.5.1 System Capability

2.5.1.1 Laser Power

The Raman spectrometer used is a Renishaw Qontor inVia equipped with 532 nm, 633 nm, 830 nm and 1064 nm laser excitation, with 5 different objectives. For the purpose of the cell experiments in this thesis, only the 532 nm laser was used, although an attempt was made to use 830 nm as well in earlier experiments. All power measurements were taken using all objectives available on the Renishaw system, listed in table 1 below.

Table 1: List of objectives available for use with NPL's Renishaw Qontor inVia instrument

Objective Magnification Make/Model	Type	Numerical Aperture (N.A.)	Working Distance (mm)	Coverslip Correction?
5x Leica N-Plan 506302	Dry	0.12	14.0	Can be used with or without coverslip
20x Leica N-Plan Epi 566066	Dry	0.40	1.15	No
50x Leica N-Plan 566036	Dry	0.50	8.20	No
100x Leica N-Plan Epi 566075	Dry	0.85	0.33	No
63x Zeiss W Plan- Apochromat Vis-IR	Immersion	1.00	2.10	No

The 5x, 20x, 50x and 100x are dry air objectives. The 50x objective has a working distance of 8.2 mm and is therefore a long working distance objective. The 63x objective is a water immersion objective allowing for higher numerical aperture than dry objectives. The numerical aperture issued with the objective by the provider can only be utilised as the numerical aperture in practice if the laser beam completely fills the back aperture of the objective, resulting in ideal

illumination through the objective to the sample. In the case of the Renishaw Raman system used for this project, the laser beam power was measured naked at the objective port and then through the objective. For all objectives and laser wavelengths a difference in laser beam power was observed, this infers that the back aperture of all objectives is completely filled by the differing wavelengths of laser as under filling of the objective aperture would result in little loss of laser power by diffraction through the objective. Therefore, the numerical aperture issued by the objective manufacturer can be used for all analysis performed in this thesis.

As outlined in section 6.1, the laser power of each beam was measured using a laser power meter (Thorlabs, PM100D with S121C Sensor), mounted on a glass slide and placed in the sample holder within the Renishaw Qontor inVia housing. The laser power was measured on three different days over a period of three weeks, with the average laser power and corresponding errors plotted in figure 12 below.

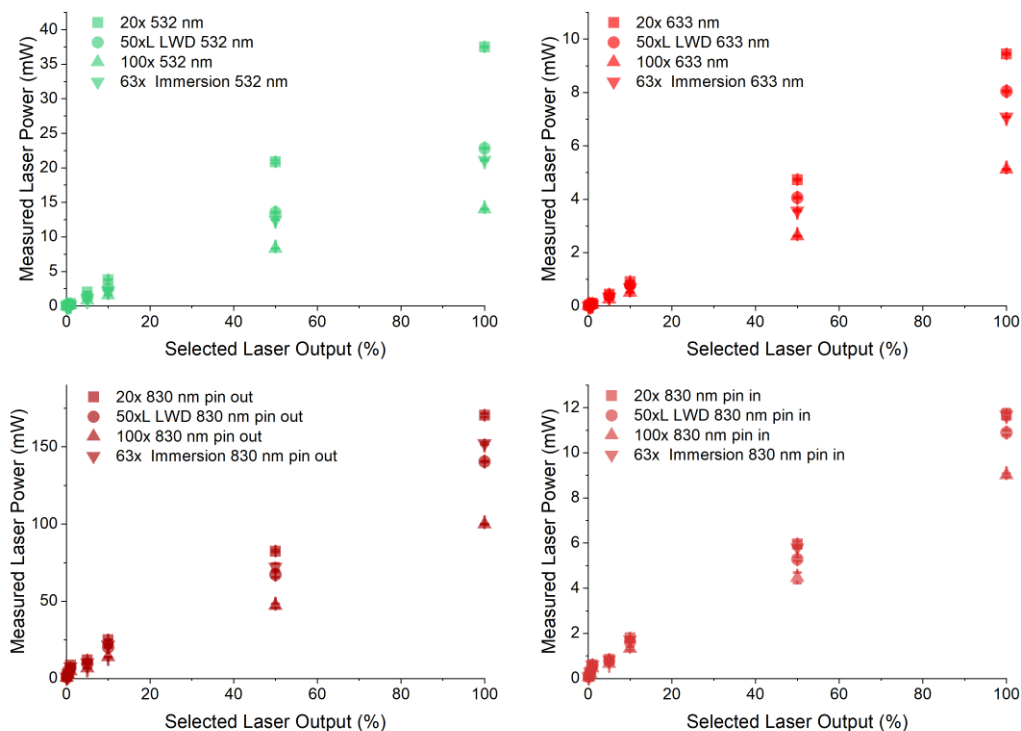


Figure 12: Scatter graph indication of varied laser power incident on a sample with varying objectives. Laser power was measured using a laser power meter (Thorlabs, PM100D with S121C Sensor), mounted on a glass slide within the sample chamber. Laser power for each laser wavelength and objective lens was measured on three days over three weeks and the average displayed in this figure and the error bars indicating the standard deviation in the measurements across the three days.

Laser power measurements are an easy way to keep track of laser health and system performance, however an average should generally be considered, as laser fluctuations can occur from day to day due to environmental temperatures and other variables. They can also provide an idea of adequate laser power to use with particularly sensitive samples to avoid sample damage as well as help ensure measurement consistency.

Table 2: Summary of average measured laser power values for 532 nm for different objectives

532 nm				
Objective	20x (0.12 NA)	50xL (0.5 NA)	100x (0.85 NA)	63xi (1.0 NA)
Laser Power				
100 %/21 mW	37.5	22.8	14.0	21.1
50 %/12.5 mW	20.9	13.5	8.31	12.5
10 %/2.3 mW	3.78	2.5	1.56	2.27

As shown by figure 12, the 100x lens consistently detected less laser power than the lower numerical aperture lenses for the same amount of laser power output from the source. For an objective of similar focal length and back aperture width, this difference in power would be unexpected. However, the overfilling of the back aperture of an objective will result in less laser power if the back aperture is narrower than other objectives, due to clipping of the beam hitting the sample.

It is also difficult to determine the accuracy of the 63x lens power measurements, as it is an immersion objective and the laser power sensor was not suitable for immersion in water for measurement. Therefore, the laser beam at the sensor may be more diffuse due to the lack of refractive index correction of the beam divergence by the immersion media. However, the laser power for both the 50xL and 63x immersion objectives are similar, which can allow for comparison between these two objectives when used for measurement of cells. Overall, the laser power measurements do allow for an approximation of laser power incident on the sample, which will be useful during the cell experiments later in this thesis, for determining cell viability at known laser powers for set periods of time.

2.5.1.2 Laser Profile

An ideal laser beam profile is assumed to be Gaussian in the transverse plane, TEM₀₀, this is due to the beam having no variation as it propagates and the beam is diffraction limited, which means it will focus to the minimum possible beam waist for the specified wavelength.¹⁴⁵ However this is not the case for most laser beams, in practice, beams can have a combination of laser beam profiles that make it appear Gaussian overall, but changes occur to the profile on propagation that don't fit the M² factor of a perfectly Gaussian beam. The ISO standard 11146 defines the M² factor as:

Equation 12: Equation for the definition of M² to indicate the Gaussian shape of a laser beam.¹⁴⁶

$$M^2 = \frac{\pi \theta w_0}{\lambda}$$

Where M^2 is the beam quality factor, θ is the half angle beam divergence, w_0 is the beam waist and λ is the laser wavelength. M^2 is a dimensionless property that allows for a real laser beam to be compared to that of an ideal Gaussian laser beam.

An M^2 value of 1 indicates a Gaussian, diffraction limited beam, whereas an M^2 value of more than 1 indicates deviation from the ideal Gaussian profile. The determination of M^2 requires multiple different measurements in both near field and far field as set out by the ISO 11146 standard, this can be difficult with laser safety implementations or inbuilt laser systems limiting the separation of a laser line from the optical path it is built into.¹⁴⁶ In the case of laboratory Raman instruments, there is capability to measure laser beam profile as it leaves the laser housing and throughout each part of the optical path way to the sample, however extending the beam to gain far field laser profile measurements are restricted within the laboratory at NPL due to laser safety rules and laser orientation within the laboratory. Therefore, the laser beam profile measurements in this thesis indicates the naked beam profile as it hits the sample or through the 50xL for an indication of beam behaviour on passing through an objective.

As outlined in section 6.2, the profile of the three laser wavelength beams was analysed using a beam profiler (Thorlabs, CCD Camera Beam Profiler, BC106N-VIS/M). The profile meter was used to measure each laser naked and with the 50xL objective at each laser power available through the Renishaw software with and without the pinhole engaged.

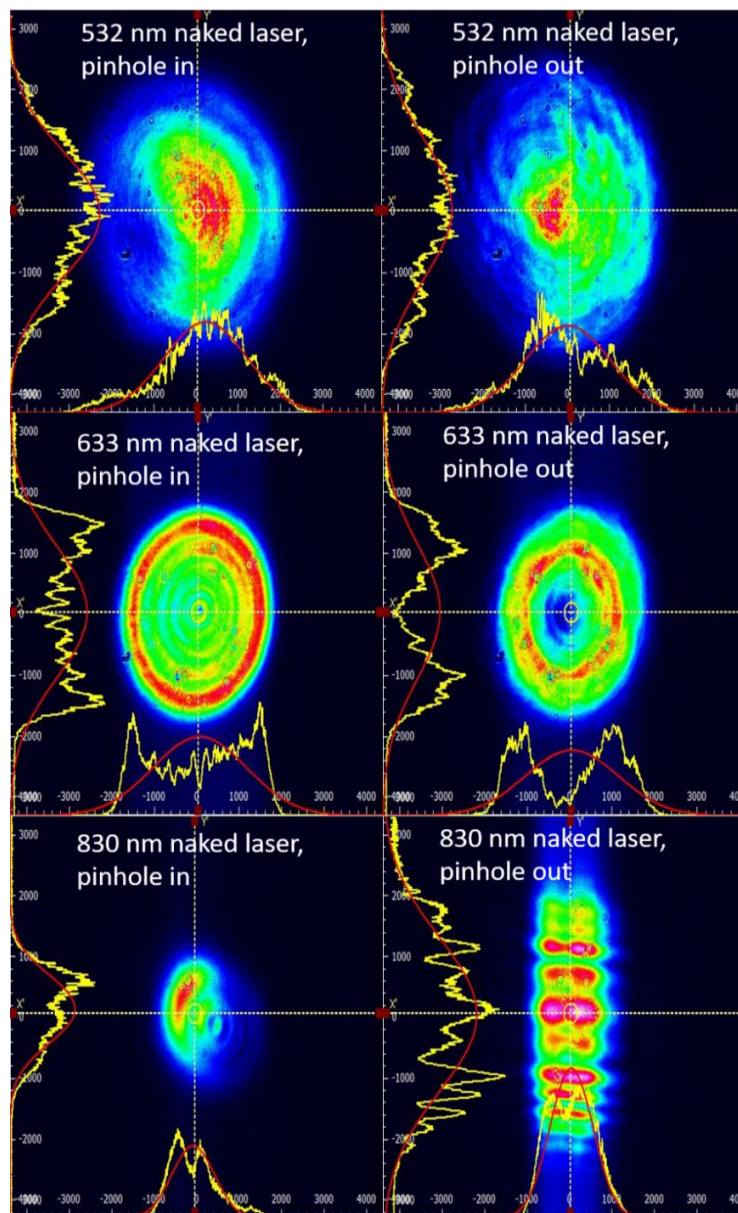


Figure 13: Two dimensional laser profile of the naked laser beam, which refers to the beam not passing through a focussing objective whilst being measured, for 532 nm, 633 nm and 830 nm laser with the pinhole engaged on the left hand side set of images and the pinhole not engaged on the right hand images. Beam profile was measured using a beam profiler (Thorlabs, CCD Camera Beam Profiler, BC106N-VIS/M). Scale is in nm; 1000 nm = 1 μm.

In figure 13, the red line indicates the average Gaussian profile in the x and y direction of the beam, while the yellow line indicates the actual laser profile measured in the respective directions. The overall shape of the 532 nm and 830 nm beams are approximately Gaussian, although as expected not the ideal Gaussian that would be preferable. The 532 nm beam is shifted slightly off centre, in the x direction, indicating the beam is more elliptical in shape, which could be caused by misalignment within the system. The pin hole within the system also physically restricts the amount of laser hitting the sample, and in the case of the 830 nm line profile beam it allows only the centre portion of the beam to reach the sample, effectively removing the majority of the laser intensity and reducing the effectiveness of the Raman scattering.

The 633 nm laser however displays a 'doughnut' profile, the opposite to the overall Gaussian shape expected. This is due to the 633 nm laser being misaligned through the telescope of the Raman system, resulting in aberrations in the beam and a reduced laser intensity profile at the centre of the beam at the sample. After these measurements were taken, an engineer realigned the Raman system to minimise these aberrations in all laser lines.

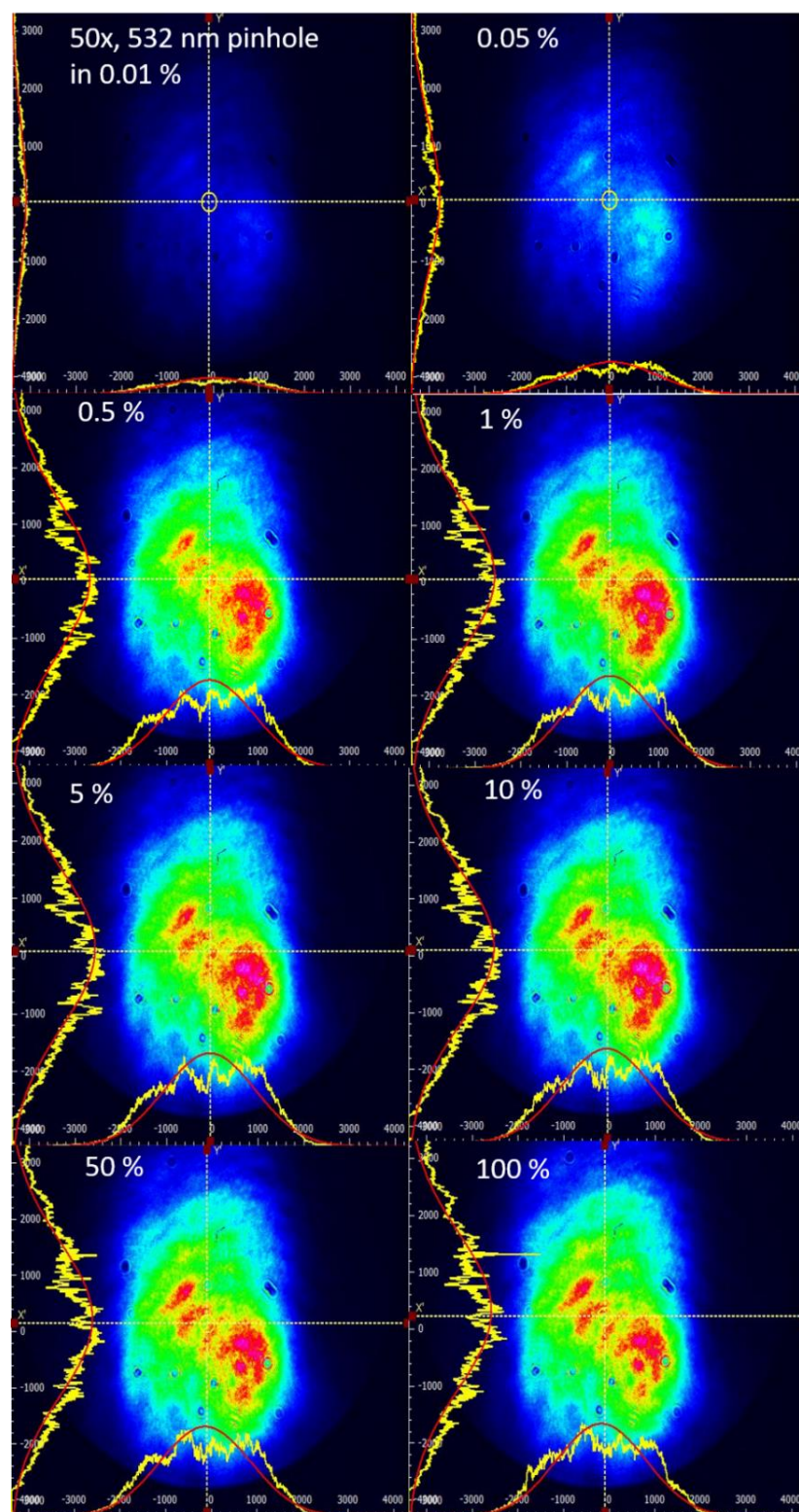


Figure 14: 2D profile of 532 nm laser through 50x long working distance objective from 0.01% laser power to 100% laser power with pinhole in, measured using a laser a beam profiler (Thorlabs, CCD Camera Beam Profiler, BC106N-VIS/M). Scale is in nm; 1000 nm = 1 μ m.

As indicated in figure 14, passing the 532 nm laser through the 50xL objective results in the centre of the Gaussian peak appearing more central, indicating that the objective pathway itself has corrected partially the elliptical nature in the x direction caused by the optics in the laser pathway before the objective. The highest peak intensity however is still shifted and showing aberrations, so the focussing optics do not correct entirely the laser aberrations.

The laser profile should have been re-evaluated after the engineer realignment, however, the beam profiler was borrowed equipment from another department, and so the re-measurement of the beam profile wasn't possible within the scope of this project. The engineer did however measure the beam profile throughout the system during the service visit to ensure correct laser alignment and optimal power at the sample. It is important to characterise the laser beam profile to ensure consistent Raman measurements for the same laser power used, without the beam profiling and consequent engineer visit, the results from the cell analysis in this thesis may have been incorrect due to this oversight.

2.5.1.3 Theoretical Laser Spot Size

System resolution is inherently linked to the laser spot size as outlined in section 2.4.2. Higher laser wavelengths tend to result in larger laser spot size, and larger laser spot sizes result in lower spatial resolution, as the resolving power of two discrete objects is reduced with the larger spread of the laser. It is important when analysing small samples, that the laser spot size has been evaluated to avoid under sampling the sample.

It is inherently difficult to measure laser spot size directly; methods include burning through photosensitive paper and measuring the diameter of the resultant hole, however this can be inaccurate due to the heat sensitivity of the paper and heat dissipation causing the hole to be larger than the actual beam. Other methods include the knife edge test with multiple sample types such as a TEM grid or a gold nanostructure, or by scanning fluorescent beads through the laser beam.^{136,137,145}

Before analysis, the theoretical spot size of each laser with each objective was also calculated using the equations below for theoretical spot size outlined in section 2.4.2.

Equation 13: Equation to calculate theoretical spot size diameter

$$SS_{xy} = \frac{0.6\lambda}{NA}$$

$$SS_z = \frac{1.4\lambda}{(NA)^2}$$

Table 3: Renishaw objective and laser theoretical spot size diameter

Laser Wavelength		532 nm		633 nm		830 nm	
Objective	Numerical Aperture	Spot Diameter (nm)		Spot Diameter (nm)		Spot Diameter (nm)	
		XY	Z	XY	Z	XY	Z
5x	0.12	2660	51722	3165	61542	4150	80694
20x	0.4	798	4655	950	5539	1245	7263
50xL	0.5	638	2979	760	3545	996	4648
63xi	1	319	745	380	886	498	1162
100x	0.85	376	1031	447	1227	586	1608

The theoretical calculations are based, again on the ideal of a laser beam with an ideal Gaussian profile. The laser spot size, as mentioned previously in this section, can be difficult to measure directly, however the spot size can be evaluated through the investigation of lateral and axial resolution of a system using a sample of suitable size smaller than the laser beam size and determining the profile of laser interaction with the sample.

2.5.2 System Resolution

2.5.2.1 Lateral Resolution in Relation to Laser Spot Size

To determine the metrological capability of the Raman system and to ensure fairer comparison of results generated across different Raman systems in potential future work, the lateral and axial resolution of the Renishaw Raman Qontor inVia system was assessed.

The system resolution was evaluated utilising a flake of exfoliated graphene, prepared as outlined in section 6.3, on a silica wafer with a distinct sharp edge. As graphene is atomically thin, it can be used to estimate resolution as the change in graphene signal will be related to the laser spot diameter. The edge of the graphene flake was orientated in first the x and then the y axes using the crosshairs available on the camera of the system. The graphene sample was then analysed using the line mapping function available with the Renishaw Wire software, a 10 μm line profile with 0.1 μm step size was used with 5 μm on the graphene flake sample and 5 μm off the graphene flake to capture the change in sample type from graphene to silica wafer, as indicated in figure 15.

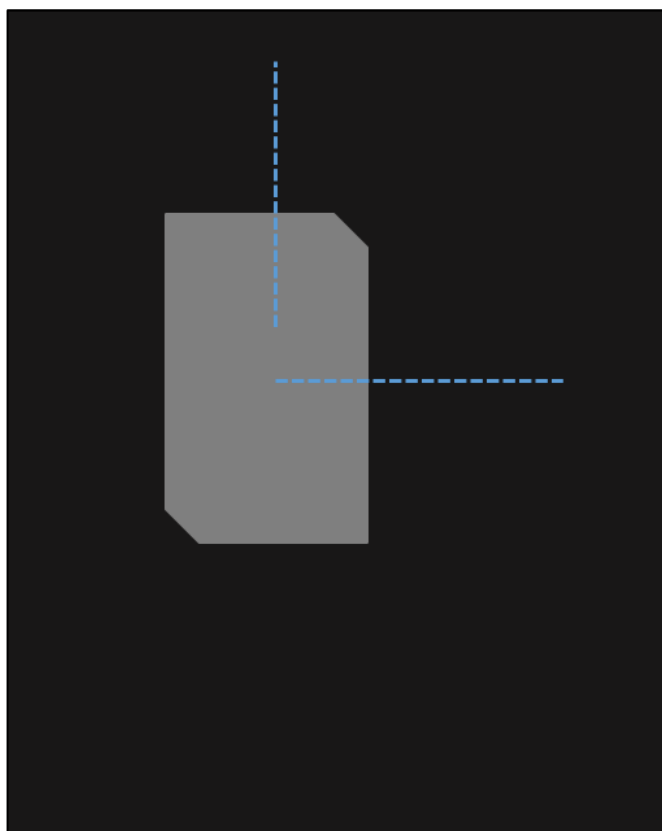


Figure 15: Schematic of flake of exfoliated graphene on silicon oxide wafer used for determining system lateral resolution. Exfoliated graphene was prepared as outlined in section 6.3, by using mechanical exfoliation tape stripping of HOPG and transferring graphene flakes to silicon oxide wafer. The diagram above indicates the graphene flake in grey, mounted on silicon oxide wafer in black. The dashed lines indicate the direction of laser movement for x-resolution, in the vertical direction, and y-resolution in the horizontal direction. For each orientation, Raman spectra of $5 \times 10 \mu\text{m}$ line in $0.1 \mu\text{m}$ steps was acquired, with $5 \mu\text{m}$ on the graphene flake and $5 \mu\text{m}$ on the silicon oxide wafer to evaluate the change in signal between the two different materials and relate this change in signal to lateral resolution.

The line profile measurement was taken five times in each direction utilising each laser wavelength – 532 nm, 630 nm and 830 nm – with a 50x long working distance air objective, 100x air objective and 63x immersion objective, in both high and regular confocality mode in order to evaluate the effect of the addition of a pinhole on the lateral resolution.

For this specific system, using high confocality mode transposes the system to a truly confocal setup compared with the pseudo-confocal mode that the system usually utilises and as such can improve the Raman signal detected

and lateral resolution. However due to the weakness of the Raman signal, the high confocality mode can make samples of low concentration or weak Raman scatterers inaccessible as most of the Raman signal will be excluded from

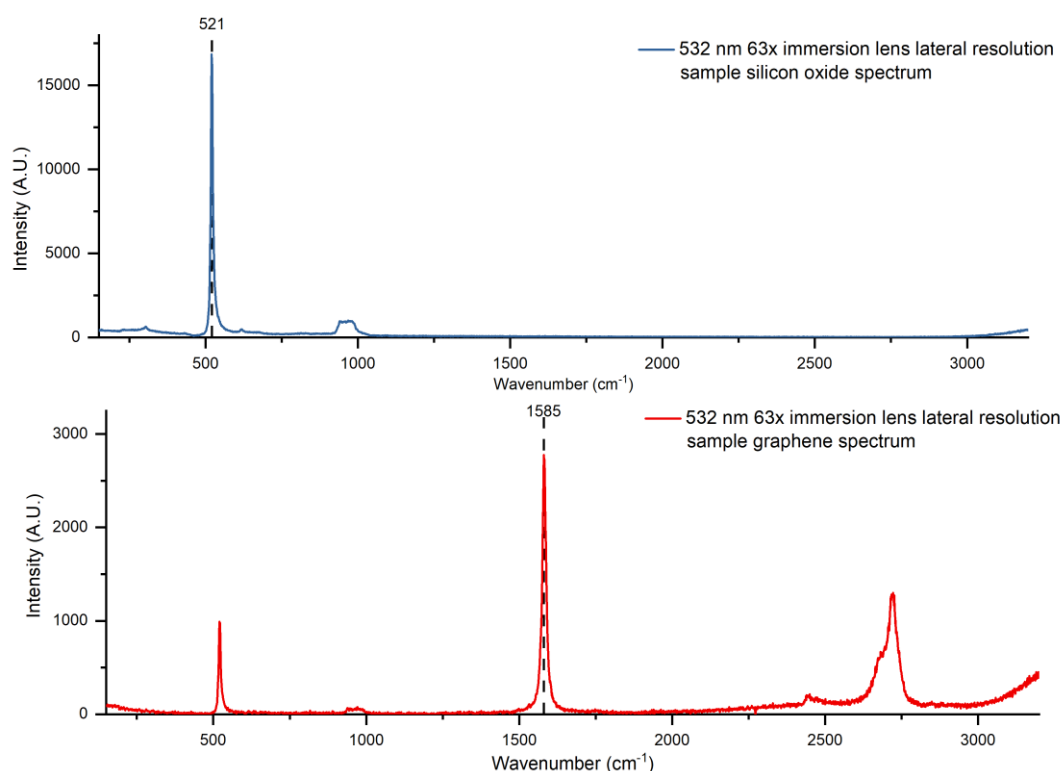


Figure 16: Example spectra from lateral resolution sample, acquired using 5 % 532 nm laser and 63x immersion objective with 2 s acquisition time. In blue is the silicon oxide wafer spectrum, with the main silicon oxide peak at 521 cm^{-1} clear. The red spectrum is of the graphene flake mounted on the silicon oxide wafer, consequently a small silicon peak is still observed at 521 cm^{-1} , however the G peak of graphene is much stronger, labelled at 1585 cm^{-1}

The peak intensity of the graphene G peak at 1585 cm^{-1} was then evaluated using the map review function in the Renishaw Wire software in the 532 nm and 633 nm laser wavelengths. For the 830 nm laser wavelength, the change in the silicon peak at 521 cm^{-1} , was used instead due to the weaker excitation power of the 830 nm laser being unsuitable for use with graphene, both peaks of interest are indicated in figure 16.

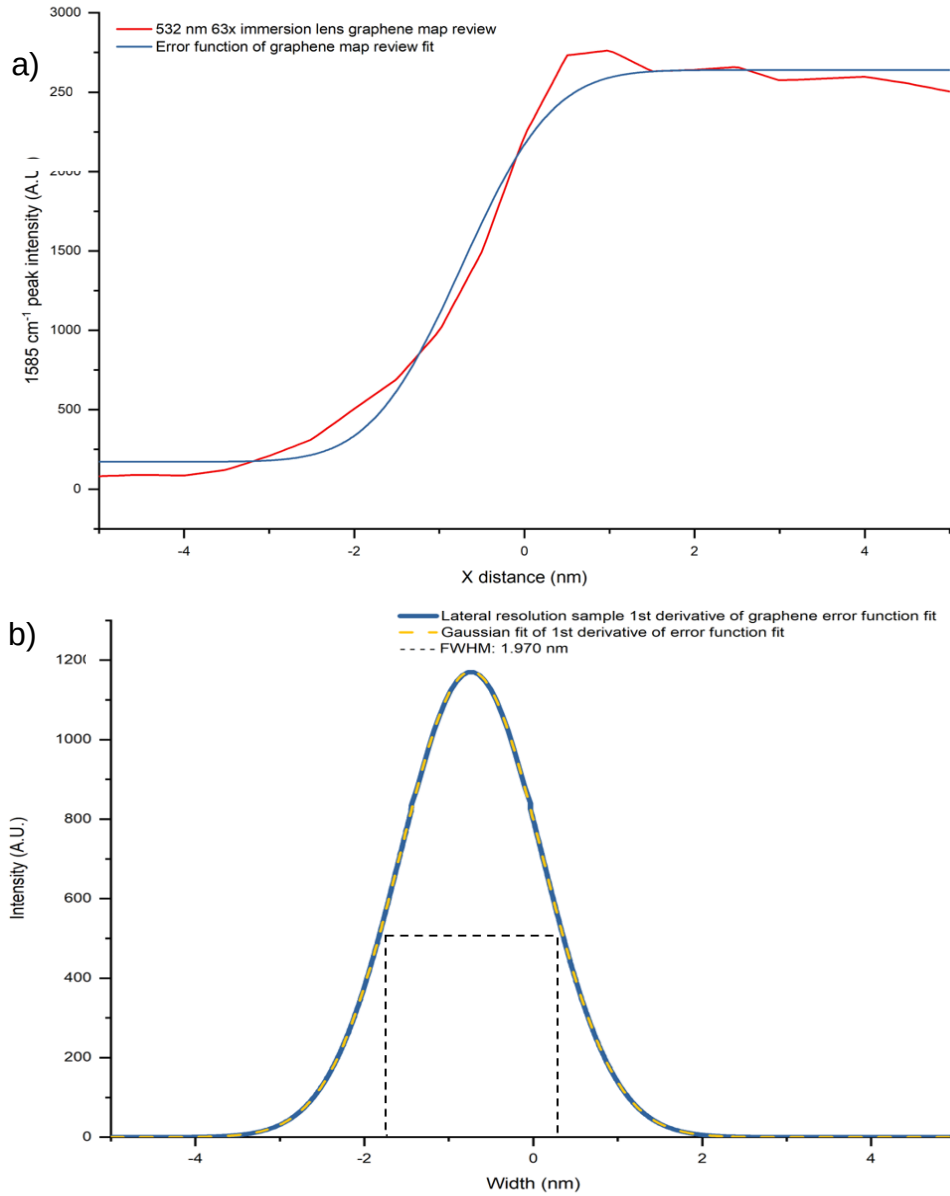


Figure 17: a) Example of map review function output, in red, for 1585 cm^{-1} graphene peak acquired during $10\text{ }\mu\text{m}$ line scan of lateral resolution sample with 532 nm laser and $63\times$ immersion objective. The blue line indicates the error function fit of the graphene peak profile. The low intensity region indicates a low graphene signal, and therefore a higher silicon oxide sample, with the slope indicating the transition from silicon oxide wafer to the graphene flake. The change in signal is therefore modelled by the error function fit due to its sigmoidal shape b) Example of 1^{st} derivative of error function fit of map review profile of 1585 cm graphene peak. The change in signal modelled by the errorfunction and the FWHM of fitting a Gaussian peak to the 1^{st} derivative can be related to the width of the laser spot size and therefore the lateral resolution.

An example of the map review evaluation is shown in the top image of figure 17, in red, an error function was then fitted to the slope of the Gaussian map review curve in Origin 2020, as shown in figure 17 in blue, to evaluate the gradient of the change in signal on transition from the silicon oxide wafer to the graphene flake. The first derivative of this error function was then fitted, again in Origin 2020 software and a Gaussian curve was fitted to the first derivative data, with the FWHM of the Gaussian fit, indicated by the black dashed line in figure 17, being proportional to laser beam spot diameter and therefore lateral resolution, in the x or y direction. A summary of the average error function fit and consequential 1st derivative Gaussian FWHM relating to laser spot diameter of the 5-line profile acquisitions in x and y for all laser wavelengths; 532 nm, 633 nm and 830 nm, objectives; 50x long working distance, 63x immersion and 100 x objective in either high or confocality is shown in figure 18.

pinhole, as indicated by the 'out' label above, the laser is a line profile. In the y direction especially this has an effect, as the graphene flake edge is also a line profile, so the step change in signal from silicon to graphene is larger as the laser beam will be on both materials for longer distances, resulting in difficulty determining the change point between the two materials.

For the 532 nm 50xL and 63x immersion objective, the theoretical xy spot size is 0.64 μm and 0.32 μm respectively. The 50xL laser spot size is close to this theoretical value at nearly 1 μm for both x and y directions and high and regular confocality. The 63x immersion objective shows a larger variation in the x direction than the y direction, which could infer the laser beam shape through the objective and focussing media causes more elongation in the x direction. However, the silicon wafer with graphene flakes was submerged in water for the 63x objective analysis to try and minimise variations in the signal if the water dried out, and therefore the differences could be due to actual sample movement, however I would have expected to see the variation in both directions, and it appears to primarily affect the x direction, the cause of this variation however would require further investigation. The resolution measured for the 63x immersion objective is therefore between 1 and 3 μm , which is larger than the theoretical calculated value, but within the expected operational values quoted often for Raman of being around 1 μm .

The main purpose of this experiment was to evaluate system lateral resolution through the laser spot diameter to confirm that during cell analysis single cells only are irradiated. With cell nuclei being approximately in the magnitude of 5 – 6 μm , it is fair to say that the laser spot diameter in these experiments will be focussed well within a single cell and when focussed on a cell, Raman spectral contributions should arise from that single cell.

2.5.2.2 Axial Resolution in Relation to Laser Spot Size

Single layer graphene, due to its thickness uniformity, has been used to evaluate system resolution previously, with Korzeniewski et al analysing single layer graphene on a glass coverslip to determine system resolution, and also Kim et al using graphene suspended over a TEM grid to minimise the

differences in dielectric environment in order to evaluate system resolution.^{136,147} N.J Overall has performed considerable analyses into system resolution for Raman spectroscopy, and outlines that for air objectives focussing below the surface of a sample that the laser beam elongates and penetrates further into a sample with increasing depth than would be expected due to the differences in refractive index between air and the sample surface, resulting in spectral contributions from above and below the focus spot even when utilising a confocal pinhole.^{121–124,148}

Due to these previous studies, to evaluate the resolution of the Renishaw system to be used for analysis a layered sample, prepared as outlined in section 6.4, consisting of a 3 mm polyethylene (PE) base, a single layer of graphene and a 50 μm polymethylmethacrylate (PMMA) layer, as shown in figure 19. The measurements were taken in 3 positions within the sample, A 500 μm deep scan was taken in 0.1 μm step from 400 μm below the focus point until 100 μm above the focus point. PE and PMMA were chosen due to their relatively well-matched refractive indices of 1.48 and 1.52, to minimise the differences in Raman signal due to scattering changes at material indices and to evaluate the effect on Raman response with increasing depth only.

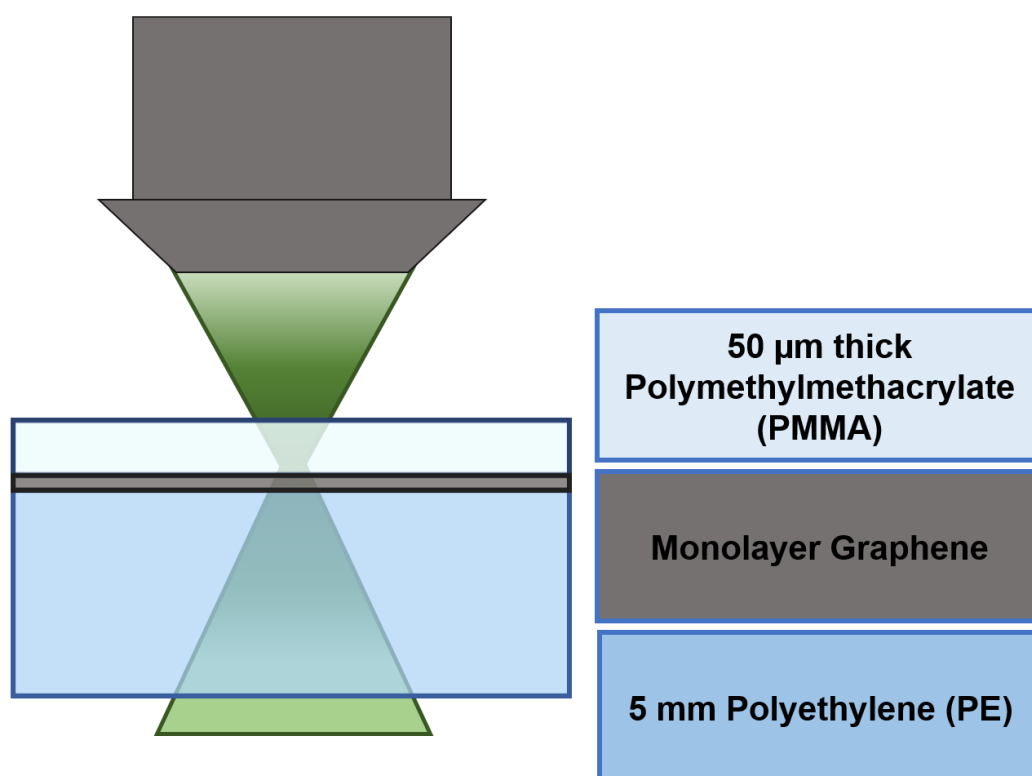


Figure 19: Schematic detailing layered sample composition for axial resolution evaluation. Sample preparation outlined in section 6.4

The map review function in Wire 5.1 was used to evaluate the peak intensity of PMMA, PE and graphene peaks of interest at 813 cm^{-1} , 1295 cm^{-1} , and 1585 cm^{-1} respectively, as indicated in figure 20, as these were seen to be well enough resolved from each other on interrogation of the individual component spectra to evaluate the change in Raman response with depth.

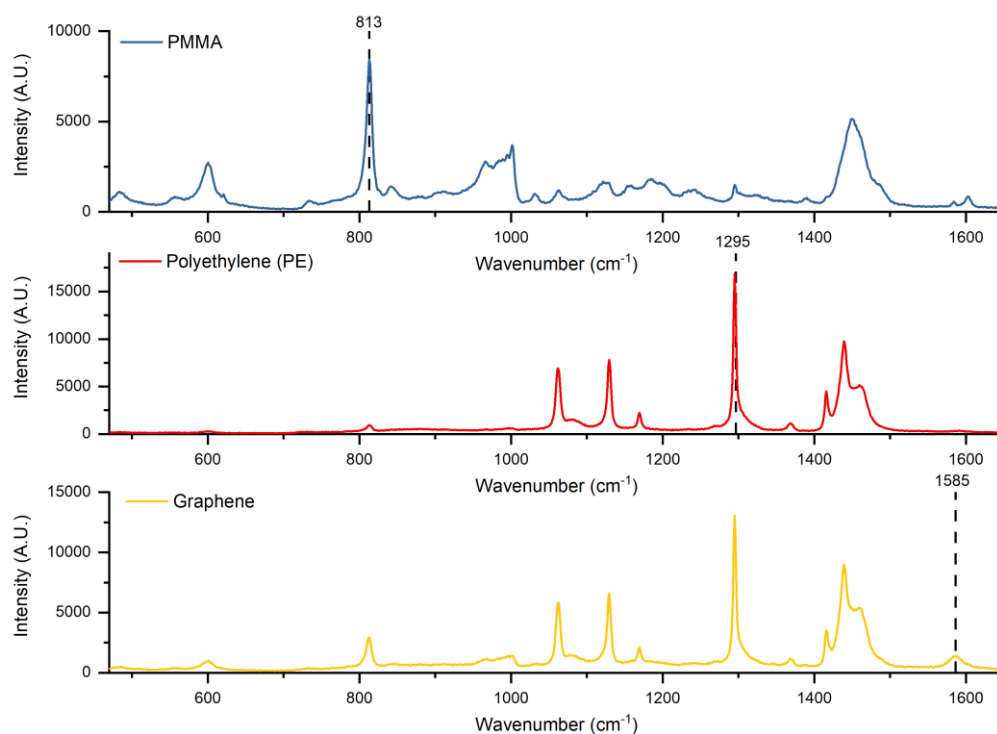


Figure 20: Example spectra from the axial resolution layered sample at the maximum peak intensity for each peak of interest for the 3 layers: PMMA at 813 cm^{-1} in blue, PE at 1295 cm^{-1} in red and graphene at 1585 cm^{-1} in yellow. All spectra in this figure were acquired using 50 %/12.5 mW 532 nm laser and 63x immersion objective at 1 s acquisition time in standard confocality. As will be shown later in figure 21, there is signal interference from layers beneath the point of focus, so in although each spectrum here is at the maximum signal intensity of the layer of interest, there are still peaks apparent from the layers above or beneath. This in particular causes an issue in the graphene spectrum, as PMMA has a peak close to the 1585 cm^{-1} G peak in graphene.

To evaluate the layer thickness and also the axial resolution as a function of response to the graphene signal, the map review function was again utilised in the Renishaw Wire software. This allowed for the profile of the signal intensity at the peaks of interest indicated in figure 20; 813 cm^{-1} for PMMA, 1295 cm^{-1} for PE and 1585 cm^{-1} for graphene, to be evaluated. An example of the map review profiles for each layer is shown in figure 21.

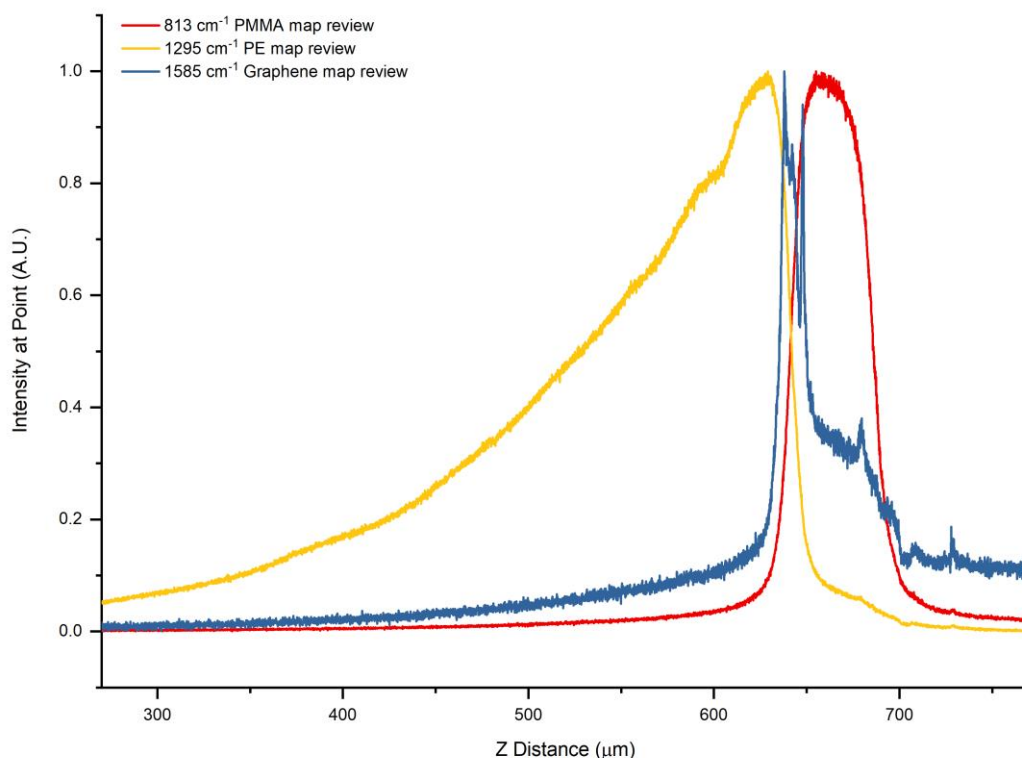


Figure 21: Example of the map review profile for the 813 cm^{-1} peak of PMMA in red, the 1295 cm^{-1} peak for PE in yellow and the 1585 cm^{-1} for graphene in blue, with increasing depth into the layered sample. The overlap of the two profiles in red and yellow indicates the overlapping signal from each polymer, whilst the blue indicates the graphene peak of interest. The graphene peak also experiences significant interference from the PMMA polymer which unfortunately has a closely resolved peak near the 1585 cm^{-1} G peak of graphene

For the evaluation of the graphene thickness as observed by the instrument, two Gaussian peaks were fitted to the map review response of the peak intensity at 1585 cm^{-1} with depth, to try and elucidate the pure graphene signal intensity from the interfering peak in PMMA. The FWHM of the narrower peak was consequently used as an approximation of the laser spot diameter in the z-direction. For the resulting Raman spectral overlap in the various sample layers, an error function was again fitted to the two overlapping map review signals for PMMA and PE to evaluate at which depth interfering Raman spectral signals from the other layers would still be evident.

A summary of the results of this analysis for the graphene peak fit evaluation from the map review for 532 nm laser wavelength for the 50x long working distance objective, 63x immersion objective and 100x objective available on the Renishaw Qontor inVia system is shown in figure 22.

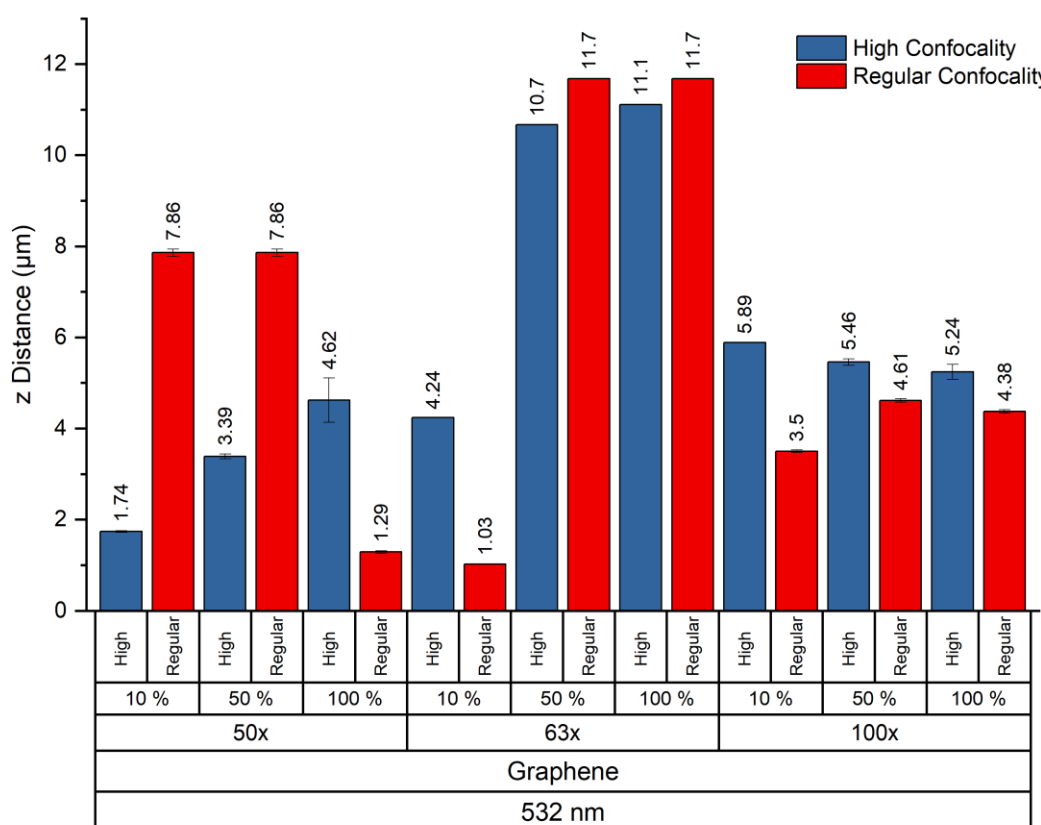


Figure 22: Summary of the estimation of axial laser spot diameter in relation to axial resolution. Data was acquired by focussing 400 μm below the sample surface and moving to 100 μm above the sample surface in 0.1 μm steps in 3 positions on the sample using 532 nm laser at 10 %/2.3 mW, 50 %/12.5 mW and 100 %/21 mW laser power with the 50x long working distance objective, 63x immersion objective and 100x objective. The map review function of the 1585 cm⁻¹ graphene peak then determined the intensity of the peak of interest with increasing distance in the z-direction and the FWHM of a Gaussian peak fitted to the resulting peak from this analysis was taken as laser spot diameter in the z-direction in relation to determining axial resolution of the Renishaw system.

The theoretical spot size diameter in the z-direction for 532 nm laser in conjunction with the 50xL objective is 3 μm as shown in table 3. However, the

measured laser spot size due to the graphene sample varies greatly from around 1 μm to 8 μm . This difference could be due to the differences in the graphene dielectric environment above and below the graphene layer causing aberrations in scattering, however using the equation described by Everall, below, focussing a laser with 0.5 NA such as the 50xL, to a depth of 50 μm in a 1.5 refractive index material, results in the laser beam illuminating a region of approximately 6.5 μm in length.¹²⁴

Equation 14: Equation relating change in depth resolution on increasing depth, depending on numerical aperture and material refractive index as detailed by N.J Everall.¹²⁴

$$\text{Depth Resolution (DR)} = \Delta = \left[\left[\frac{NA^2(n-1)}{(1-NA^2)} + n^2 \right]^{\frac{1}{2}} - n \right]$$

Where NA is the numerical aperture of the objective, n is the refractive index of the material and Δ is the depth of focus in the material.

Monolayer graphene is 0.335 nm thick, therefore the laser beam illuminates a region much larger than the thickness of the graphene alone, lending contributions from the polymer layers both above and below to the spectra. As there is a peak in PMMA that overlaps with the tail of the G peak of graphene, this results in broadening of the peak and therefore the fitting of the Gaussian, even when the fit is altered to try and compensate for this, the diameter is still large and non-ideal.

Therefore, this sample, unfortunately, was unable to determine laser spot size diameter in the z-direction more accurately. A sample more comparative of the one outlined by Kim et al would have been more appropriate for approximation of spot size diameter in the z-direction in relation to axial resolution, however, as cells are layered samples with different components, the determination of sample contribution from above and below the focus spot was of interest, and why a layered sample in particular was chosen.¹³⁶

Consequently, the distribution of signal from the PE and PMMA was also reviewed using the map review function and the peaks outlined above, to show sample signal distribution with depth. The resulting sigmoidal shapes

intercepted each other at the sample interface with graphene and the next polymer, as shown in figure 21. An error function was therefore fitted to this sigmoidal shape for both polymers to evaluate the depth over which contributions can be seen from the layer above or below the focus point. The results of this analysis are shown in figure 23.

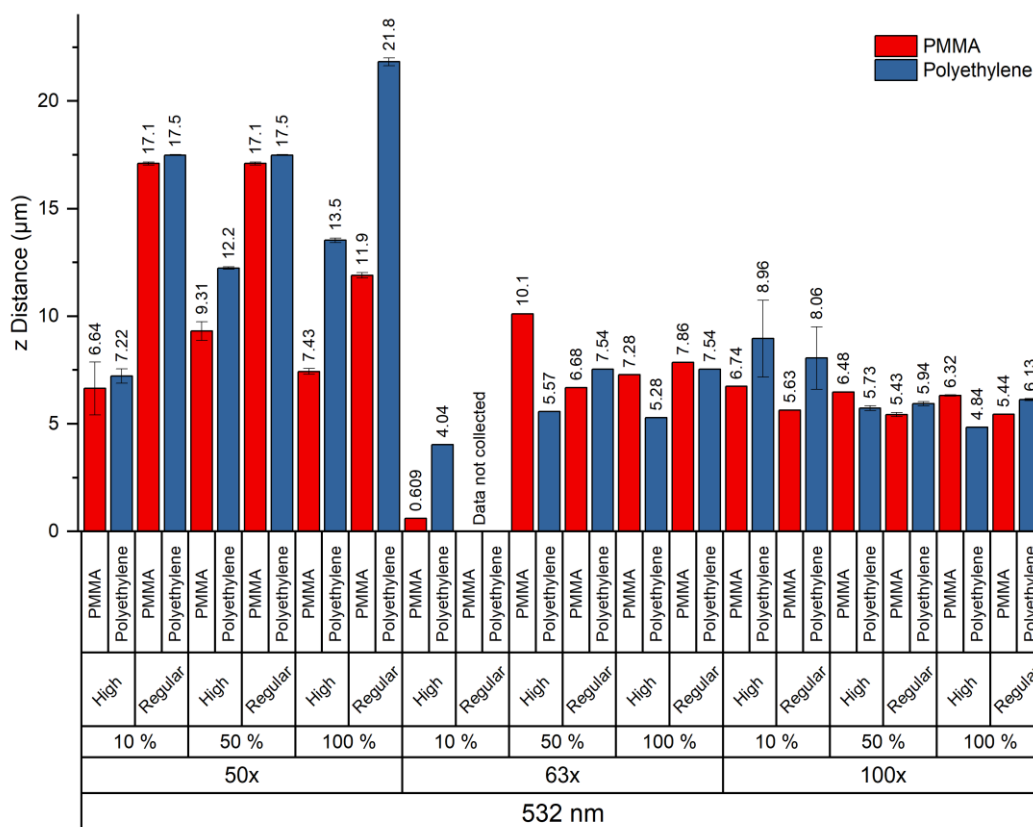


Figure 23: Summary of the distance over which contributions can be seen from layers within the axial sample, above and below the focus point for PMMA in red and PE in blue. Note 10 %/2.3 mW regular confocality data for 63x immersion objective lens at 10 %/2.3 mW laser power was not collected through user error, and the 63x immersion objective data is for $n=1$, so no error bars are shown due to this. Again, data was acquired by focussing 400 μm below the sample surface and moving to 100 μm above the sample surface in 0.1 μm steps in 3 positions (with the exception of 63x objective) on the sample using 532 nm laser at 10 %/2.3 mW, 50 %/12.5 mW and 100 %/21 mW laser power with the 50x long working distance objective, 63x immersion objective and 100x objective. The map review function of the 813 cm^{-1} peak for PMMA and 1295 cm^{-1} for PE then determined the intensity of the peak of interest with increasing distance in the z-direction and the width of the error function fit used as the distance over which spectral contributions from layers above and below the focus point will contribute to the spectra.

The figure above shows that contributions from the polymers can be seen at varying distances, from approximately 5 μm to around 20 μm , above and below the focus point depending on numerical aperture. If laser beam behaviour is consistent in all directions through the similar refractive index materials, it would be expected that the contributions of polymer to the spectra would be equal for both polymers, and indeed, particularly for the 100x and 63x data, this is consistently the case. The 50x objective is a long working distance objective as well as having considerably lower numerical aperture than the 100x and 63x immersion objective and this combination results in more varied results and also contributions from the polymer layers at larger depths than the higher collection numerical apertures. This is consistent with equations 11 and 15 which show the dependency of laser interrogation depth on the square of the numerical aperture.

The 100x lens, shows the most consistent results, particularly for higher laser power, due to the lowest working distance coupled with a high numerical aperture. According to the work of Everall, the immersion objective should have the most accurate indication of how signal interference can change with penetration depth, however, the 63x lens is a water immersion objective, which meant that the whole polymer layered sample was submerged in water to allow consistent analysis and minimise water evaporation due to the relatively long analysis times of the map generation.¹⁴⁹ The sample is predominantly polymer, and therefore some aberrations due to floating and sample movement are evident, however the results are similar to those for the 100x lens. Ideally, the 63x lens should have had $n=3$ replicates, however, the planned analysis repeat was affected due to the pandemic, unfortunately, therefore, the $n=1$ results are displayed here as indicative, but non-ideal data.

Taking the data with largest measured spot size; the 63x immersion objective results, spectral interference can be expected from a layered sample between approximately 5 and 10 μm above and below the focus. Relating this information to cell nuclei as an example, of approximately 6 μm in diameter, this would result in spectral contributions from the cytoplasm and potentially cell membrane above and below the nucleus depending on the specific depth

of focus of the laser. This is extremely useful to know during cell studies as often spectra are taken within different regions of the cell to account for spectral variation during analysis, however, it can be said that cytoplasmic contribution can be seen even when focussing within the nuclei.

2.5.3 Wavenumber Calibration

As outlined in section 6.5.1, simple wavenumber referencing of a Raman spectrometer was performed daily using the peak of silicon at 520.6 cm^{-1} , with peaks falling within $520 - 521\text{ cm}^{-1}$ generally being accepted to evaluate spectrometer performance. This silicon calibration can also then be used for normalisation of spectrometer response for different samples analysed that day.

The Renishaw Qontor inVia system is equipped with an internal silicon calibration sample that can be analysed through the calibration menu in Wire 5.1 software. However, as a cross check, an external silicon wafer sample was also recorded. An example of the internal and external silicon calibration spectra is shown below.

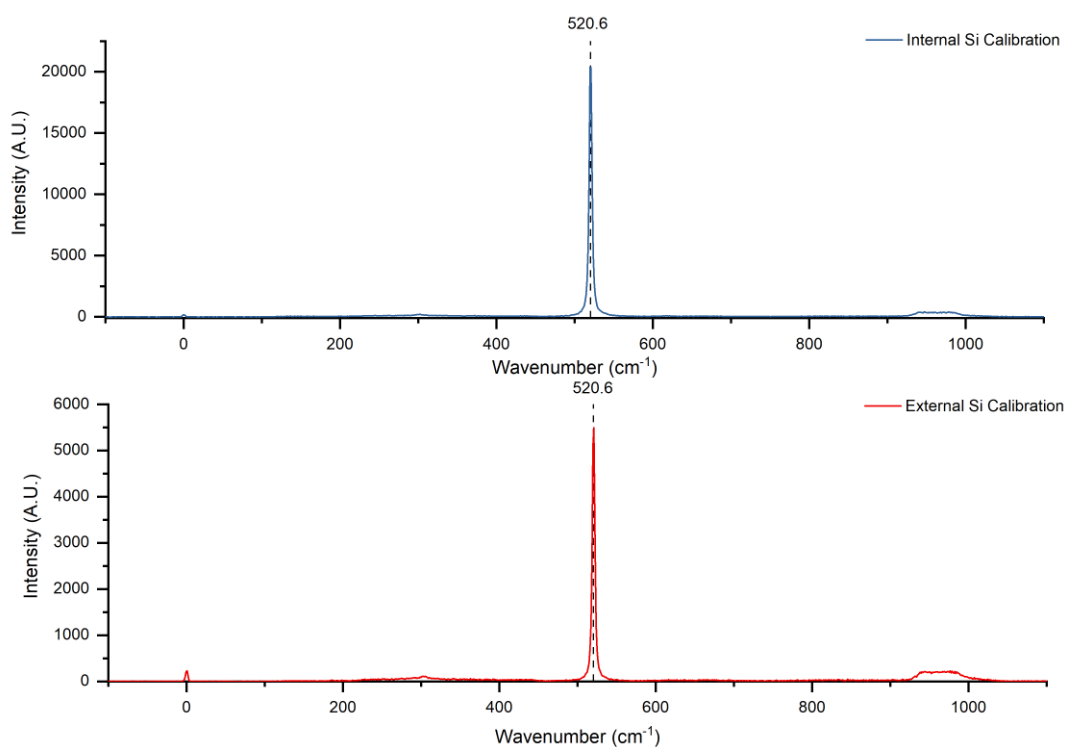


Figure 24: Silicon calibration spectra for internal and external calibration samples analysed using Renishaw Qontor inVia Raman System. Spectra acquired using 10 %/2.3 mW 532 nm laser and 50x long working distance lens in the case of the external silicon calibration spectrum in red, with 0.1 s acquisition time.

In figure 24 above, the silicon peak is indicated by the dotted line at 520.6. The difference in peak intensity can be due to the laser passing through the objective to the silicon sample, resulting in laser power attenuation in the focussing pathway compared to the internal sample not requiring an objective pathway.

Further system wavenumber calibration was performed using the NIST SRM 1921b polystyrene sample, as outlined in section 6.5.2. This standard is used for transmission infra-red calibration, however the ASTM standard E1840 lists polystyrene as a recommended material for diode laser wavenumber calibration evaluation. Therefore, using the NIST traceable standard in combination with the guidance set out in the ASTM standard provides a good evaluation of wavenumber calibration.

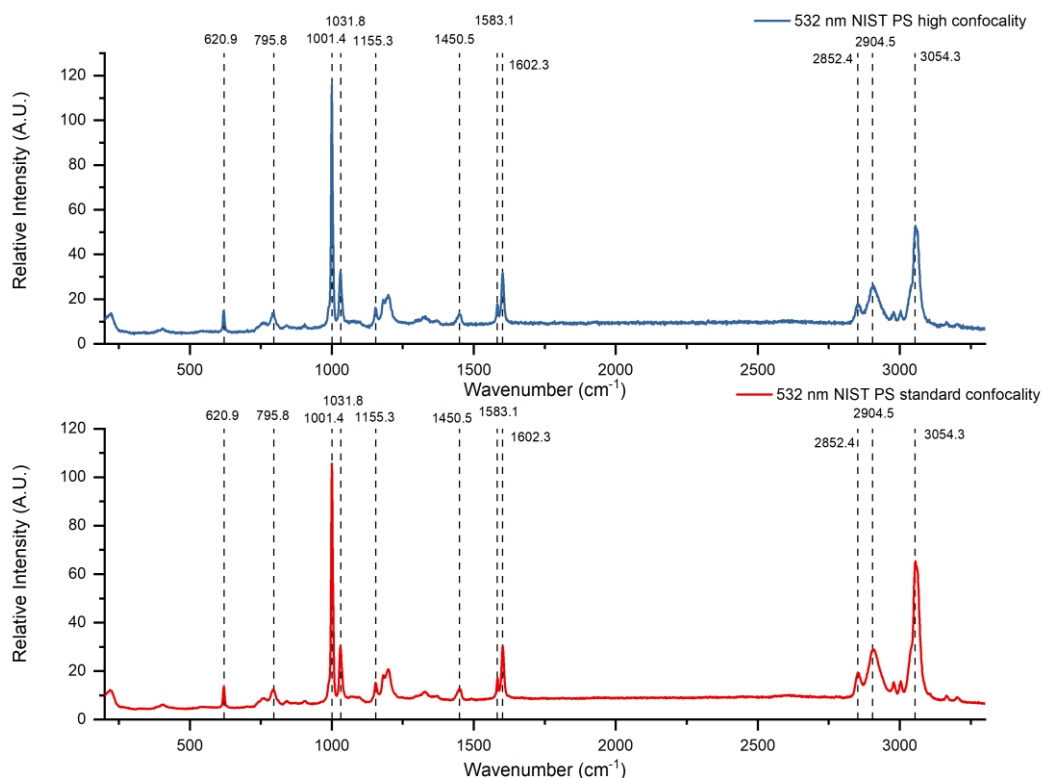


Figure 25: Measured spectra for NIST 1921b Polystyrene Standard at both high and regular confocality. Spectra acquired using 1% 532 nm laser power for 20 s acquisition time using 50x long working distance objective in high and regular confocality modes in red and blue respectively.

The dotted lines in figure 25 indicates the centre of the peaks recommended for detection as set out in the ASTM standard. Which for clarity, are also listed in table 4 below, along with the measured peak positions.

Table 4: Table outlining expected and measured Raman shifts for polystyrene compared with ASTM standard.²⁵

ASTM	High Confocality Measured	Regular Confocality Measured
Average (cm ⁻¹) ± Standard Deviation	Measured wavenumber (cm ⁻¹)	Measured wavenumber (cm ⁻¹)
620.9 ± 0.69	620.9	620.9
795.8 ± 0.78	795.4	795.4
1001.4 ± 0.54	1001.6	1001.6
1031.8 ± 0.43	1031.5	1031.5
1155.3 ± 0.56	1155.4	1155.4
1450.5 ± 0.56	1450.6	1450.6
1583.1 ± 0.86	1583.6	1583.5
1602.3 ± 0.73	1602.2	1602.2
2852.4 ± 0.89	2852.2	2852.2
2904.5 ± 1.22	2904.4	2904.4
3054.3 ± 1.36	3054.4	3054.4

The measured Raman peaks agree well with the peaks as shown by figure 26, the line of best fit is 1 due to the minimal difference between the expected peaks defined by the ASTM standard and those measured by the Raman system.

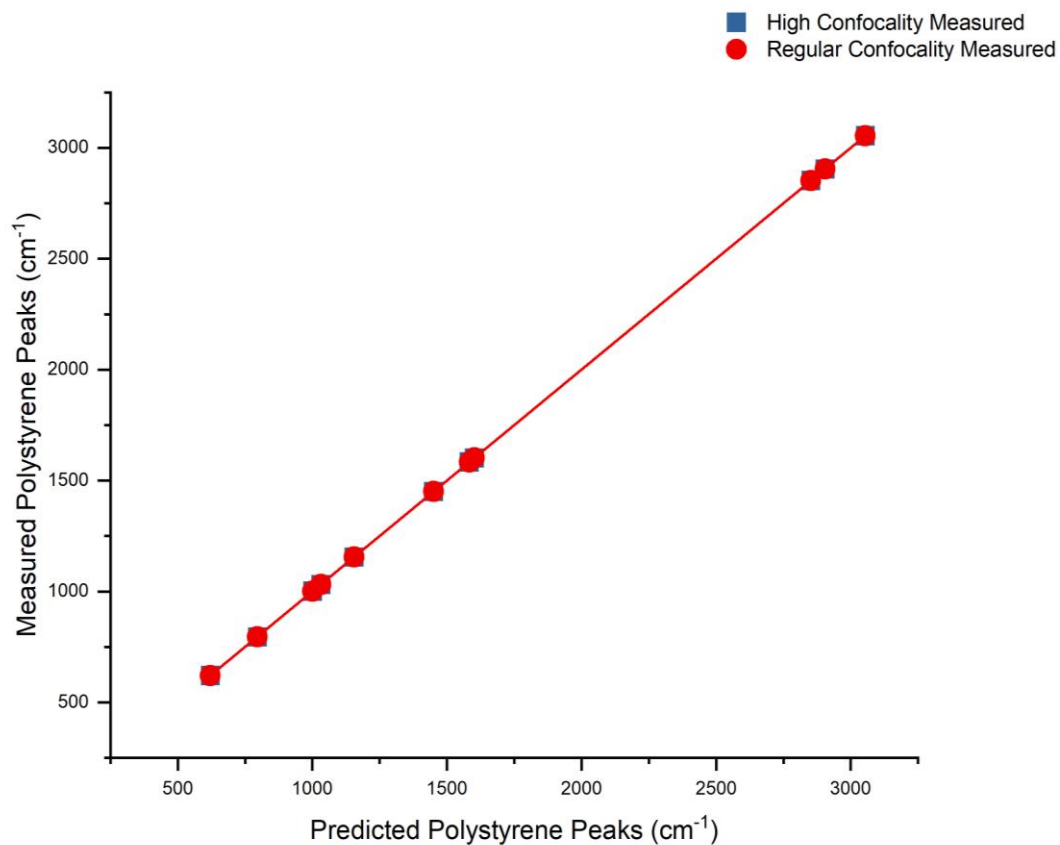


Figure 26: Scatter graph with line of best fit indicating measured polystyrene peaks in relation to expected polystyrene peaks as outlined in the ASTM standard E1840

The wavenumber calibration in combination with the daily silicon wavenumber position check allows for confidence in the measured Raman peaks in samples analysed with this Raman system.

2.6 CHAPTER CONCLUSIONS

In this chapter, the laser beam profile and resulting laser power incident on the sample for varying laser wavelength and output power selection in the Renishaw Wire software was achieved. The laser power in particular will be helpful as indication of permissible limits in laser power to reduce cell damage during Raman analysis.

Secondly, the lateral resolution was approximated through the use of an exfoliated graphene sample to evaluate the laser spot size diameter in relation to the ability of differing laser wavelengths in combination with different objectives to resolve objects from each other within a minimum distance. It was also evaluated that the capability of the Renishaw Raman system used for the resulting experiments within this project is relevant to single cell analysis, as the laser spot size focusses well within the size range of a single cell.

The axial resolution was also attempted to be evaluated in relation to the laser spot size through the interrogation of a polymer layered sample with monolayer graphene. However, optical aberrations with depth couple with peak interference between the graphene and PMMA polymer peak did not allow for as accurate axial resolution approximation as needed. However, the evaluation of the interference of the two polymer signals with depth, did allow for an understanding of how cell macromolecules and varying components could contribute signals to cell spectra even if purely focussed on the nuclei due to the laser penetration variation with depth in combination with varying refractive indices.

Lastly, simple wavenumber position checking using silicon wafer and wavenumber calibration utilising the NIST SRM 1921b polystyrene sample to determine correct wavenumber positioning allows for consequential cell experiment peaks to be compared with literature values with greater confidence.

3 BIOLOGICAL SAMPLE CHARACTERISATION OF RAMAN INDUCED CELL DEATH IN MG-63 CELL LINE

3.1 INTRODUCTION

3.1.1 MG-63 Cell Line

MG-63 is an immortal cell line derived from an osteosarcoma of a 14-year-old Caucasian male.¹⁵⁰ These cells have been widely used as an in vitro model for novel biomaterial-cell interactions due to the cell line's increased cell proliferation and ease of cell management in comparison to human Osteoblast (hOB) cells, as well as exhibiting characteristics similar to that of preosteoblastic stage cells.^{31,151–154} The main reason for using MG-63 for this study, is the limited change in phenotypical behaviour over passages 5-30. As shown by Staelkhe et al, MG-63 exhibit high Ca^{2+} regulation which in turn results in a high level of cell cycle regulation and limits apoptosis between cell passages, therefore inferring that any cell death induced is more likely due to the process under study and not just cell passage variations.¹⁵¹ The high speed of cell proliferation also allows for a higher number of repeatable experiments to be achieved, which is essential in cell studies for representative results.^{151,155}

MG-63 has been well characterised by Raman spectroscopy previously. It has been used in studies to differentiate between cell lines used for tissue engineering as presented by Notingher et al, indicating differences in tumour and non-tumour cell lines through the use of multivariate analysis.¹⁵⁴ Chiang et al, also used the MG-63 cell line for cancer differentiation, however, focus was based on the increased level of mineralisation present in MG-63 cell lines in comparison with higher grade cancer cell lines such as 143B and SaOS2.¹⁵⁶ D'Acunto et al, also utilised the presence of hydroxyapatite within MG-63 to differentiate between the cancerous cell line and osteodifferentiated MSCs coupled with AuNPs during SERS analysis to enhance the Raman signal within the cells to elucidate localised cellular component information.¹⁵⁷ Boyd

et al, utilised macro-sampling Raman spectroscopy to elucidate between different cell lines, including that of MG-63, and whilst the technique was unable to successfully determine between the different lines, it was capable of ascertain differences in live and dead cell spectra due to chemically induced death.³¹ Ramamurthy et al, have also exploited the high wavenumber region within MG-63 to track highly ordered lipid distribution in relation to chromatin location during mitosis.¹⁵⁸

Due to the Raman spectrum of this cell line being well characterised, in both the fingerprint and high wavenumber region, it resulted in a good base understanding of where expected peak changes could potentially occur in relation to any potential damage caused by the inherent process of Raman analysis.

3.1.2 Cell Viability and Cell Death

Cell viability is defined as the measurement of the proportion of healthy cells within a sample cell population and is a fundamental measurement during cell studies.¹⁵⁹ Cell viability can be determined to evaluate inherent cellular activity, to track the health of cell populations and determine cell response to external stimuli such as therapeutic treatments. The method of cell viability evaluation can also elucidate information surrounding method of action of such treatments. Depending on the method of cell death expected and requirements, cell viability can be evaluated in a number of different ways, through cell proliferation measurement, metabolic activity, ATP concentration or via cell toxicity assessment.^{109,160–163}

Cellular death is mainly split into two different types – apoptosis and necrosis. Apoptosis, first coined in 1972 by Kerr et al, is inherently programmed cell death, it is an energy dependent process built into cells as a way of maintaining homeostasis by removal of unwanted cells.^{159,164} Indicators of apoptosis include cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation with the formation of apoptotic bodies before phagocytosis.¹⁶⁵ Necrosis, in comparison, is indicated by a loss in cell membrane integrity and cell swelling with release of cellular components into

extracellular space which in turn initiates an inflammatory response which doesn't occur in apoptosis, it is a passive process. However, an apoptotic process can become necrotic if a lack of ATP and caspases are available.¹⁶⁶

Cell death can be evaluated with various methods, the simplest of which is by Trypan Blue exclusion assay. Trypan Blue is a cell membrane impermeable diazo dye, which results in only staining of cells with compromised membranes on the appearance of a blue colour within a cell. However, Trypan Blue exclusion can't determine between necrosis and apoptosis, and is less sensitive in the detection for dying cells which may still have intact membranes. For the ability to determine between apoptosis and necrosis, more specific cell viability assays are required, such as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or Annexin V assay for apoptosis or lactate dehydrogenase (LDH) assay for necrosis. For an improvement on the Trypan Blue assay, fluorescent dyes in conjunction with flow cytometry or fluorescence microscopy can be more accurate as they can be used to doubly stain a cell for confirmation of viability, usually through the use of a membrane permeable dye in the green fluorescent emission region for a viable cell combined with a membrane impermeable DNA binding molecule in the red fluorescence region.

3.1.3 Raman Spectroscopy and Cell Viability

All the cell viability methods mentioned in the previous section, however, rely on staining of samples for confirmation of cell viability and often can't be carried out *in situ*. Raman spectroscopy, with its ability to elucidate chemical information in a label free manner, with the same resolution of a fluorescence microscope has also been used to evaluate cell viability. It must be noted, however, that cell viability and cell death action is usually still confirmed utilising the traditional cell staining methods or other analytical techniques such as gel electrophoresis, western blotting or ELISA.¹⁶⁷ Verrier et al, utilised Raman spectroscopy in the analysis of human lung carcinoma A549 cells treated with Triton-X to detect Raman spectroscopic changes in the lipid, proteins and DNA regions, in conjunction with cell viability and DNA integrity

assays.³⁰ Mao et al, utilised resonance Raman spectroscopy to enhance the robust MTT assay to elucidate semiquantitative viability information spectrally due to peak intensity changes on the reduction of soluble MTT to insoluble formazan.¹⁶⁸ Uzunbajakava et al utilised Raman spectroscopy with statistical analysis and fluorescent labelling to map the differences in distribution of DNA and protein within individual HeLa cells during apoptosis.¹⁶⁹ Notingher et al, also evaluated the cell viability and cell cycle stage of human epithelial lung cells, A549 utilising Raman spectroscopy through evaluation of changes in peak area in two separate studies as well as studying the effect of laser wavelength and power on cell death of MLE-12 cells for use in biomaterial analysis.^{5,28}

It was this last paper, by Notingher et al, that served as the initial inspiration for the evaluation of cell death by Raman spectroscopy in MG-63 cell lines.

3.2 PROJECT AND CHAPTER AIMS

In line with the main aim of this project to develop an analytical framework for levels of safe laser exposure for biological sample analysis during Raman spectroscopy, a fundamental understanding of the sample and analytical instrument interaction can be key. Ensuring analysis is optimised for best analytical output coupled with minimal sample degradation can be challenging.

Therefore, the main aims of this chapter are to characterise the MG-63 human osteosarcoma cell line used in this project to define appropriate analysis times such that cell death is induced through the method of Raman analysis. Understanding the amount of laser power and analysis time that can cause irreversible sample damage and the chemical signatures that change in response to this sample damage will help identify the key biological components that can be affected during analysis for not only this cell line but could also apply to other biological samples.

This chapter involves building on the methodology previously defined by Notingher et al, by applying a similar time study to a cell line often used as a model for bioengineering purposes and with 532 nm laser wavelength at differing laser powers to determine the relevant laser parameters that can induce sample damage.⁵ The determination of time of death was evaluated using both trypan blue exclusion method as well as a double fluorometric Live/Dead™ stain for confirmation of cell viability.

3.3 RESULTS AND DISCUSSION

3.3.1 Characterisation of MG-63 Cell Line

Due to being unable to use the immersion objective in conjunction with an incubator within our Raman spectrometer setup, the characterisation of cells over time in prewarmed PBS solution without Raman spectroscopic analysis was initially investigated to observe cell behaviour with time in a non-ideal environment and to establish cell stability under these environmental conditions. The visible light images from the Raman spectrometer using a 50x long working distance objective and transmission illumination to improve contrast are shown in figure 27, as outlined in section 6.6.

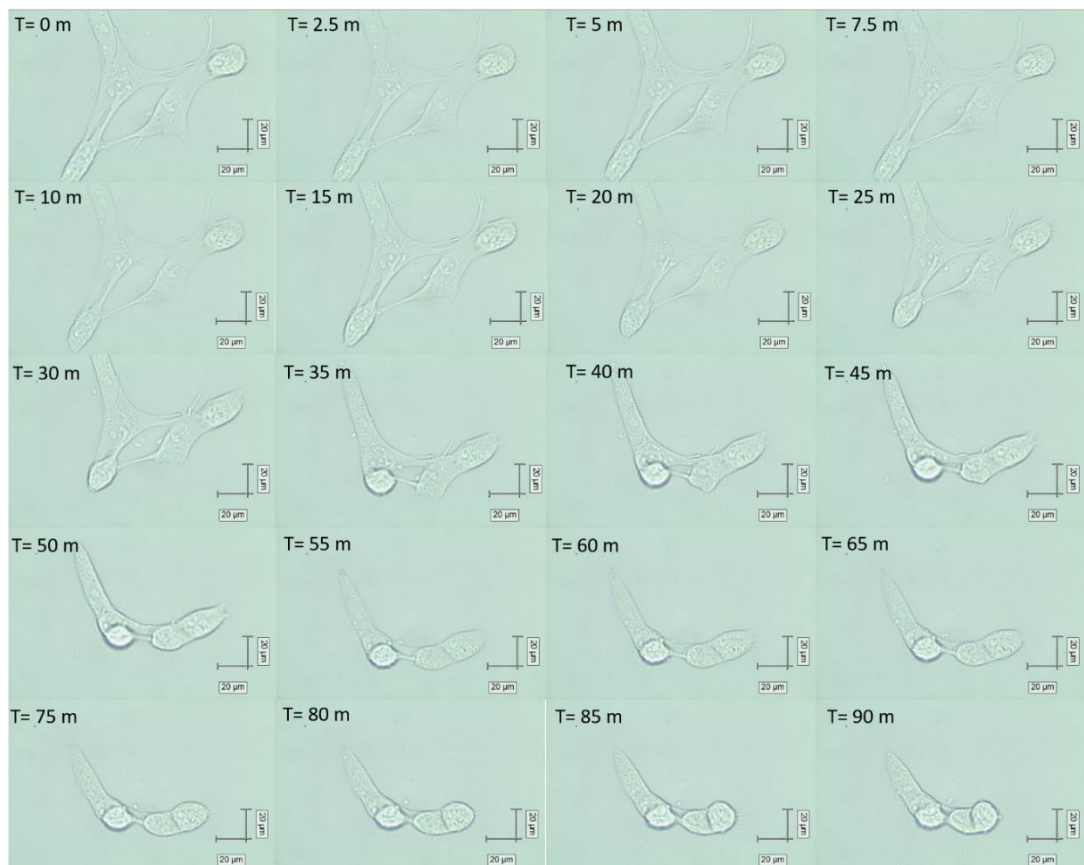


Figure 27: Visible light images of MG-63 cells from time 0 min, fresh from incubator, washed with prewarmed PBS, mounted in a glass bottom Ibidi dish, until $t = 90$ min. Imaged using transmission illumination and 50x long working distance objective, NA 0.5 in the same orientation planned for Raman cell analysis.

Although slight changes in cell position can be seen from $t=10$ minutes, particularly of the cell in the bottom left hand corner. These changes don't become significant until $t = 35$ min, when the same cell becomes predominantly smaller and more circular, which can be an indication of apoptosis. The cell continues to move and over shadows the larger cell beside it, indicating that it may have become detached from the glass surface, which is possible in prolonged PBS exposure. The field of view was shifted slightly at $t = 50$ min to keep the same cells within the field of view for continuation of imaging. At $t = 90$ min all cells have significantly shrunk, indicating that ideally analysis of live cells within PBS must be undertaken within a time frame of less than 90 minutes, but ideally less than 35 minutes.

3.3.2 Trypan Blue Controls

As the method outlined by Notingher et al states, two populations of cells were analysed, live and dead and Trypan Blue exclusion staining was used to test for cell viability.⁵ The dead cell population were prepared as per outlined, by leaving in the incubator for 4 days with no fresh media, restricting nutrient intake within the cells as well as not keeping the pH regulated to cause cell death. Due to the high proliferation rate of MG-63 this resulted in cell samples with an overly high confluency. Control samples were prepared, as outlined in section 6.14, of the live and dead cell population as well as a control sample fixed, as detailed in sections 6.14, 6.15 and 6.16.1.1, in 4 % paraformaldehyde for 10 minutes at room temperature before staining with 200 μ L 0.2 % trypan blue in PBS as a positive control for Trypan Blue exclusion.

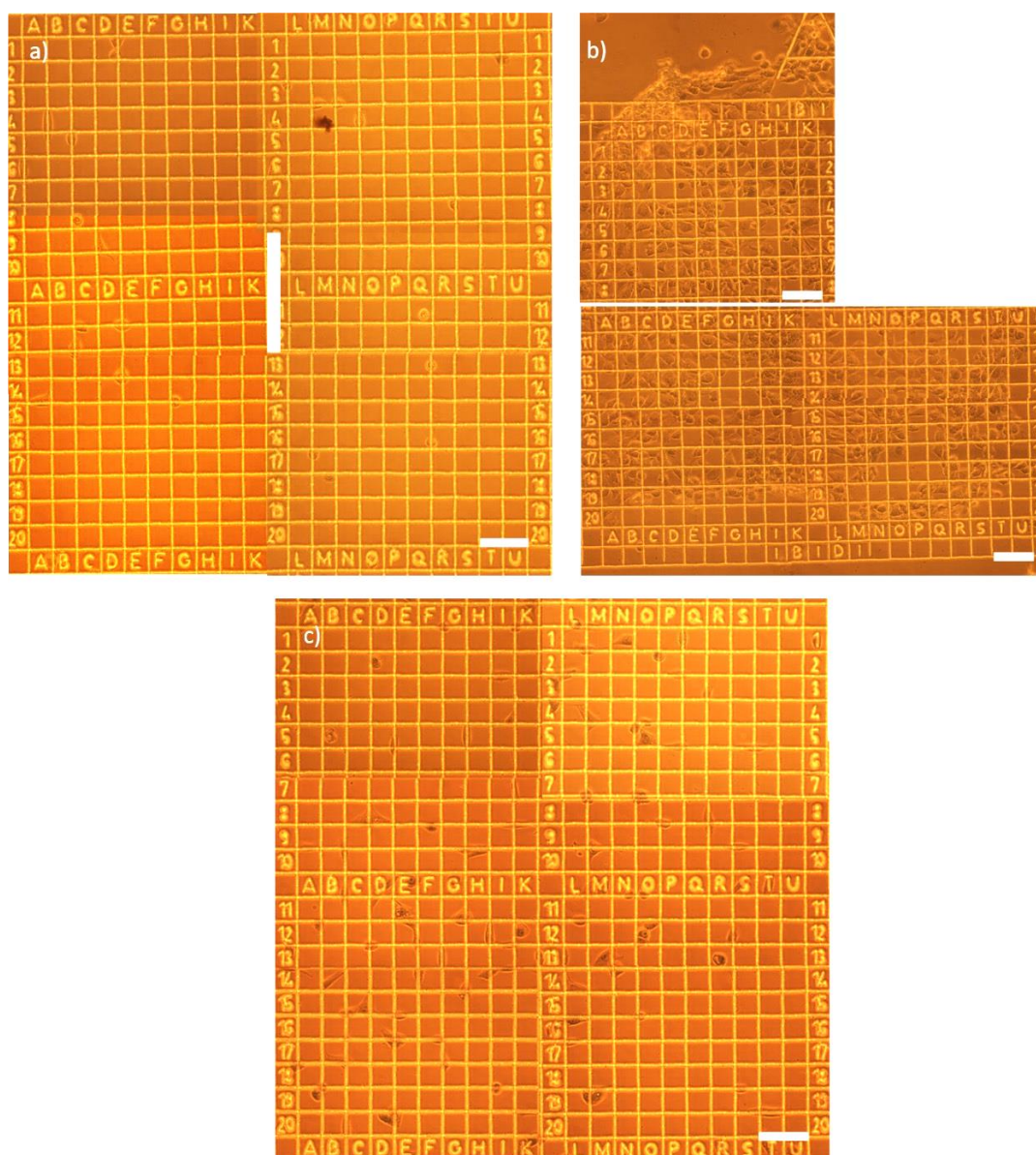


Figure 28: Control samples for Trypan Blue exclusion testing. Images were taken on an inverted Nikon Eclipse Ti microscope with a 20x long working distance objective, as detailed in section 6.8. a) indicates the live cell control, b) shows the dead control sample with high confluency of MG-63 cells, due to contrast issues with the visible light microscope, the dead control trypan blue signal is difficult to see, however the majority of cells were stained with trypan blue and clearly visible via the microscope eyepieces c) is the positive control of cells fixed in 4 % PFA for 20 mins before washing with PBS and imaging. The white line indicates 100 μ m scale

The images shown in figure 28, captured using a widefield visible light microscope as described in section 6.8, from a – c indicate the live, dead and

fixed control sample respectively. The visible microscope available for such images was not equipped with phase contrast or DIC objectives, however the contrast between viable and non viable cells was clear through the microscope eyepieces due to the use of transmission light in the inverted microscope setup, the contrast difficulties in the image was caused by the camera software, and unfortunately no improvement could be made to the contrast of these images during acquisition. The microscope was also not equipped with a translation stage to allow for systematic imaging of the sample surface, therefore the images have been stitched together with the contrast altered as well as reasonably possible, with gaps left where images were not able to be taken within the grid.

Due to the issue with contrast, it can be difficult to elucidate confirmation of cell death visually with Trypan blue staining. However, the live control sample does not have any clear darker or blue stained cells, while the fixed control has a high proportion of blue cells, with darker regions visible within the cell membranes. The dead control sample, due to the high confluency of the cells on the sample surface, lost a lot of cell density during the application of trypan blue and subsequent washing steps. The cell detachment and loss of cell density is evident by the difference in cell coverage between the rougher gridded surface and non gridded area in the top left image of b in figure 24 above. Again, the microscope camera contrast can make it difficult to ascertain the trypan blue uptake of such cells in these images, however, the dead control sample has a high number of regions of darker colour contrast in comparison with the live control sample which has no darker areas excepting some debris within the sample on the top right hand image of a.

3.3.3 Average Live and Dead Cell Spectra

Post trypan blue control samples a further set of sample characterisation control measurements were taken, in this case to evaluate if any initial Raman differences could be seen between the two live and dead populations, and also to determine if a 60 s acquisition time at 100 % laser power, 21 mW at the sample, would cause cell death. The samples were prepared on gridded ibidi

glass bottom dishes, as per section 6.14, to allow for ease of relocating cells post analysis and staining. The cells had visible light images taken, as shown in figure 29, as outlined in section 6.6, before and after Raman analysis to interrogate any changes in morphology that may be visible, before being stained with Trypan Blue.

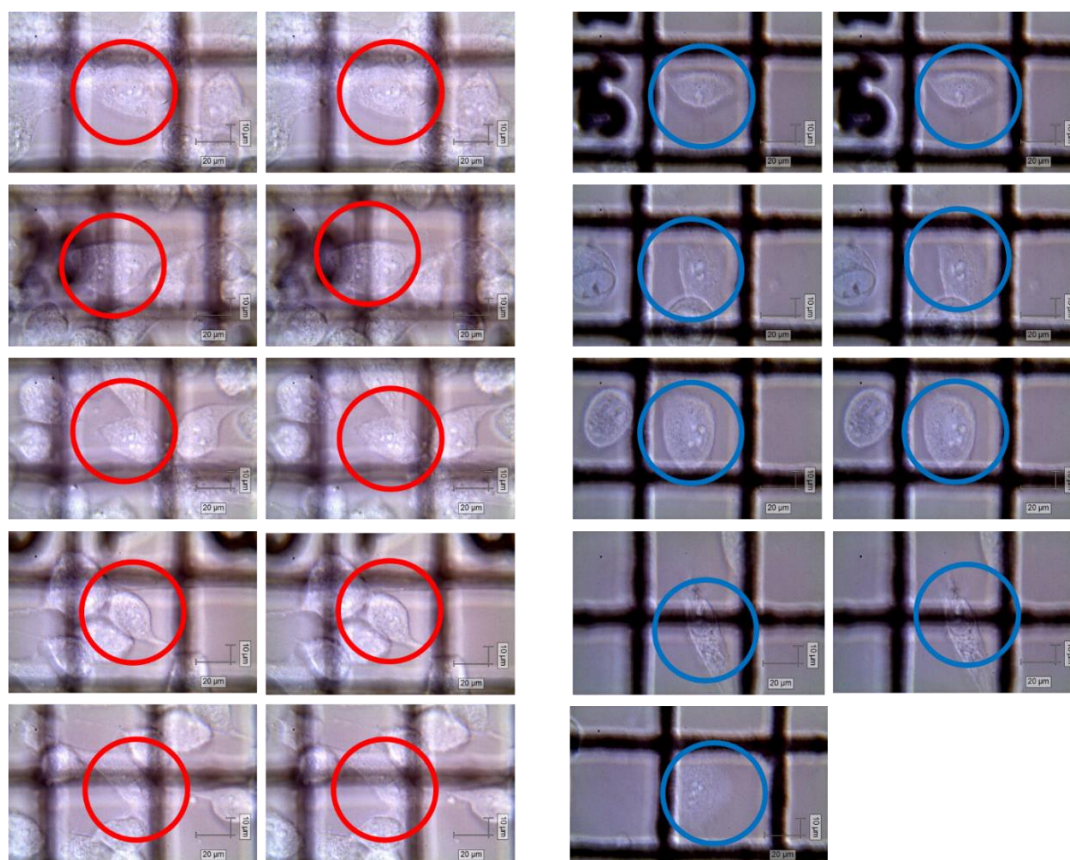


Figure 29: Visible light images of 5 MG-63 cells from dead (red circled) and live (blue circled) populations to determine if any changes are visible within cell morphology after 60 s Raman acquisition with 21 mW 100 %/21 mW 532 nm laser power obtained with 63x immersion objective. Images captured using the inbuilt image capture function before and after acquisition in the Raman Renishaw Wire control software. Unfortunately, the image of post Raman analysis of cell five in the live population did not save, hence the emission from the figure.

All Raman measurements were focussed carefully within the nucleus, due to the higher localisation of DNA, combined with laser axial evaluation resulting in the determination that cytoplasmic and membrane contributions could be seen in the spectra as well. Previous studies also indicated that reduction in DNA intensity can be a marker of induced cell death.^{4,5,30,31} As stated

previously, the dead population had a much higher cell confluency, which did present a challenge in finding cells isolated enough for single cell interrogation, although the laser spot size is sufficiently small enough to irradiate one MG-63 cell at a time. The cells appeared to have minimal changes in cell morphology before and after Raman analysis, however, evaluation of cell morphology purely by eye is not the best way to evaluate changes in the cells, as they are likely occurring internally until the cell moves towards cell death, where well defined morphological changes can be observed by eye, such as cell shrinkage for apoptosis, or membrane swelling for necrosis.

The cells were stained using the Trypan blue exclusion method, outlined in section 6.16.1, after Raman analysis to ensure that the acquisition time of 60 s at 100 %/21 mW laser power, wasn't inherently damaging to the cells initially.

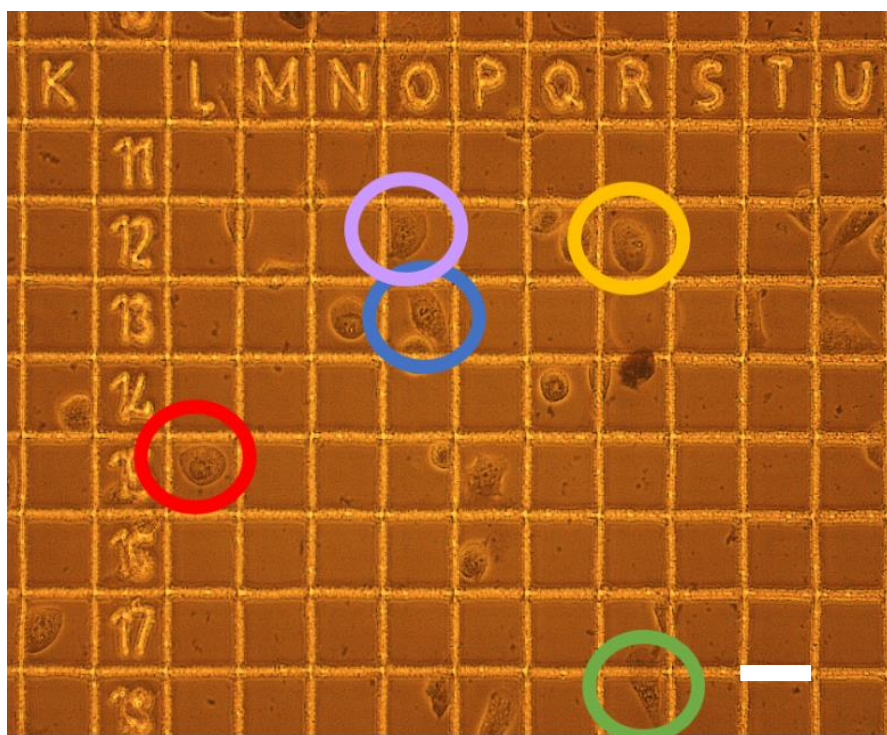


Figure 30: Visible light images of live cells 1 - 5 from figure 32 (cells 1-5 circled in blue on the right hand side images of the figure), after the addition of Trypan Blue. Cells were analysed using Raman spectroscopy with 532 nm 100 % laser power equivalent to 21 mW at the sample. Image was taken using an inverted Nikon Eclipse Ti microscope with a 20x long working distance objective, as detailed in section 6.8. C1 – Red, C2 – blue, C3 – yellow, C4- green, C5 – purple. Scale bar is 50 μm .

The cells in figure 30 correspond to the five cells from the live sample population only. None of the cells have up taken the Trypan Blue exclusion dye, therefore the 60 s acquisition time with 532 nm on its own does not induce cell death via membrane compromise in the MG-63 cells. Unfortunately, like the control sample for Trypan blue of the dead cell population, all cell density was lost before imaging of the cells. However, the uptake of the control sample cells indicates that the cells were dead before Raman analysis, and the loss of cell density can imply cell stress and death.

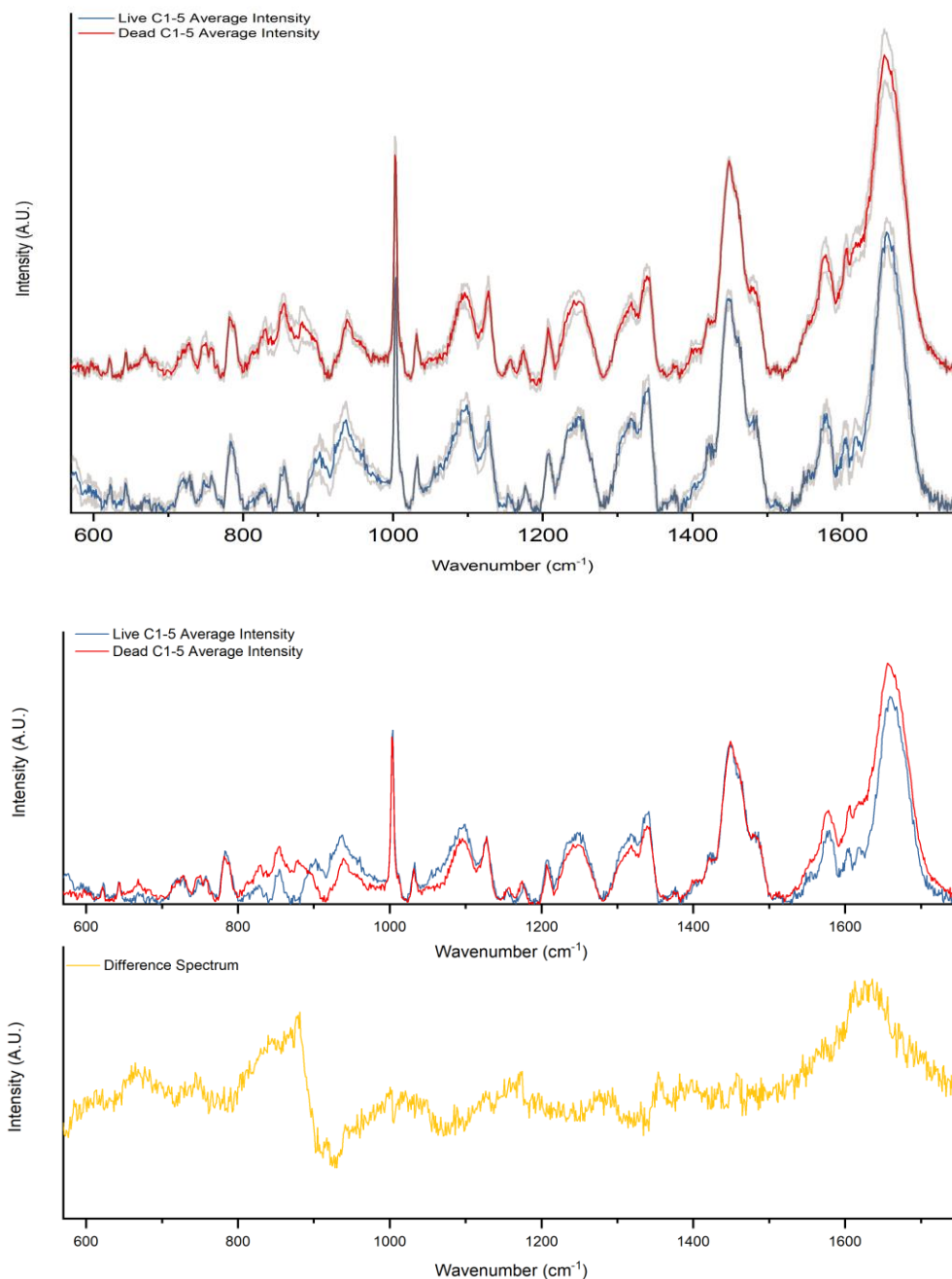


Figure 31: Comparison of the average of 5 live MG-63 cell spectrum (blue), with the average of 5 dead cell (red) spectrum. The top figure indicates the standard deviation of both cell groups in grey. All spectra were normalised to the peak at 1449 cm^{-1} . The figure towards the bottom of the figure shows the same data without the offset and standard deviation spectra to allow for easier comparison to the peaks apparent in the difference spectrum in yellow. Raman spectra were acquired from the nucleus region of 5 cells from each population for 60 s 100 % 532 nm laser power, equivalent to 21 mW power, using 63x immersion objective.

In figure 31, the difference spectrum elucidates the peaks that have changed between the two groups of cells. Glass and media contributions were removed before baseline correction and subsequent normalisation. The baseline for all spectra was corrected using the ALS fitting function in OriginPro2020, using an asymmetric factor of 0.001, a threshold of 0.01, smoothing factor of 4 for 10 iterations. The same baseline settings were applied to all spectra, to allow for fairer comparison between samples.

Raman band changes were assigned based on previous literature, as shown in table 5. As mentioned in Figure 31 and section 6.7, all spectra were normalised to the CH₂ band for all biomolecules present within cells at 1449 cm⁻¹; primarily CH₂ in plane bending within DNA and lipids, but also CH₂ and CH₃ in plane bending in proteins.¹⁷⁰ This peak has been used in literature previously for normalisation.^{5,28,30} The peaks within both the live and dead cell populations are characteristic of the main components of cells – DNA/RNA, proteins, lipids and carbohydrates.^{5,28,30,31,154,171}

The difference spectrum, in yellow in figure 31, indicates the calculated difference between the dead cell and live cell average spectra. The indicated differences in peaks from the difference spectrum are not entirely concordant with the changes reported in other studies for cell viability, however, peak changes are visible that are related to cell death.

There are changes in peak intensity in relation to DNA visible in the difference spectrum at 1095 cm⁻¹ and 1340 cm⁻¹.^{4,5,31} The former is related to the PO₂⁻ anion present in the DNA backbone, whilst the latter is related to both DNA bases adenine and guanine, but can also be related to CH deformation in proteins.^{5,31}

Table 5: Initial Raman Peak Assignments for MG-63 from literature.^{4,28,30,31,154,172} Peak assignments are related to the corresponding biomolecule within cells indicated by the letter in the brackets – DNA, P for protein, L for lipid and C for carbohydrates.

Raman Peak Position (cm ⁻¹)	Tentative Assignment
623	C-C Tw Phe (P)
645	C-C Tw Tyr (P)
670	T, G (DNA)
717	CN ⁺ (CH ₃) ₃ Sym Str (P)
728	A (DNA), Ring Br Trp (P), C-N Head group (L)
747	Phe
757	Ring Br Trp
785	U, C, T Ring Br (DNA)
827	O-P-O Asym Str (DNA), Out of Plane Ring Br Tyr (P)
855	Ring Br Tyr (P)
877	L-Proline – α -helix (P)
897	BK, deoxyribose (DNA)
937	C-C Backbone α -helix (P)
1005	Sym. Ring Br. Phe (P)
1031	In Plane CH Bend Phe (P)
1050	C-O, Deoxyribose (C, DNA)
1095	PO ₂ ⁻ (DNA)
1127	C-N Str (P)
1155	C-C/C-N Str (P)
1175	C-H bend Tyr (P)
1208	C-C ₆ H ₅ Str. Phe, Tyr (P)
1245	Amide III β -Sheet
1320	G (DNA), CH def (P)
1340	A, G, (DNA), CH def (P, C)
1421	G,A (DNA)
1449	CH def (P, L, C)
1484	G, A (DNA)
1577	G, A (DNA)
1604	C=C Phe, Tyr (P)
1616	C=C Tyr, Trp (P)
1655 - 1680	Amide I α -Helix, C=C (L)

Boyd et al and Notingher et al also have described a decrease in the DNA peak at approximately 1577 cm⁻¹, however, the broadening of the amide I and lipid C=C peak at 1660 cm⁻¹, encompasses this DNA peak and on comparison of the non-offset normalised peaks, the 1577 cm⁻¹ peak appears at a higher intensity, which is also shown by the positive peak direction in the difference

spectrum.^{31,55,171} There is also a decrease in intensity in the peak at 782 cm^{-1} , which is also reported in previous literature, and is related to the DNA bases thymine, cytosine and guanine.^{4,5,30,32,171} However, another fundamental DNA peak at 788 cm^{-1} , related to the O-P-O symmetric stretch is not visible in either of the live dead spectra or the difference spectrum.^{4,5,30,32,55,173} It could potentially be masked by the peak at 785 cm^{-1} , however, further data deconvolution such as peak fitting would be required to elucidate if this is the case. Overall, decrease in peaks related to cell intensity can be seen in comparison between the two cell populations for cellular DNA which is consistent with cell death.

Other differences visible in the difference spectrum are increases in peaks related to the α -helix form of proteins at 877 cm^{-1} , and peak broadening between $1570 - 1700\text{ cm}^{-1}$. Which is consistent with findings by Boyd et al, who suggested this could be due to an increase in aromatic amino acid concentration due to protein cleavage.³¹ Notingher et al, describe the opposite, a narrowing of the amide I peak and corresponding increase in the peak at 1231 cm^{-1} , associated with random coils in proteins, during cell death. Corresponding to this disagreement in spectral variation, another peak corresponding to the α -helix backbone, at 937 cm^{-1} decreases.⁵⁵ Unfortunately, no more confirmation can be made in relation to the main structural form of the proteins, as these peak intensity changes disagree. Other protein related peaks also decrease, between 1280 and 1320 cm^{-1} , these are noted by both Notingher et al, and Boyd et al and can be therefore correlated to cell death.^{30,31,55} The changes in this peak range are clearly visible even without the difference spectrum.

The most identifiable peaks within the Raman spectrum of MG-63, are the sharp phenylalanine peak at 1005 cm^{-1} , and the smaller peak beside it at 1033 cm^{-1} . The difference spectrum does indicate a small decrease in the 1005 cm^{-1} peak, but not in the 1033 cm^{-1} peak. Boyd et al also reports that no change in the phenylalanine peak is apparent, although Notingher et al, do indicate that the phenylalanine peaks at both 1005 and 1033 cm^{-1} decrease between viable and non-viable cells, which can be indicative of necrotic death pathways.^{5,31,174}

There is a slight decrease in the 717 cm^{-1} which is related to the phospholipid cell membrane.^{33,175}

From this initial investigation key peaks which exhibit a change in intensity between the live and dead cell spectra are therefore related to proteins; the phenylalanine peak at 1005 cm^{-1} , and other protein conformation peaks at 717 cm^{-1} and 937 cm^{-1} ; and DNA peaks at 782 cm^{-1} , 1095 cm^{-1} and 1340 cm^{-1} . Further investigation of these peaks correlated with increased laser exposure and Trypan Blue exclusion method for confirmation of cell death will be performed.

3.4 THE EFFECT OF VARIED LASER POWER ON MG-63 CELLS

The initial investigation of cell death utilising a method for inducing non-chemical cell death in samples and corresponding control checks for laser interrogation at 60 s 100 %/21 mW laser power with Trypan blue, successfully showed peak changes between the two sample sets as well as the indication of a high proportion of dead cells with the trypan blue exclusion method for the dead control sample.

To further investigate cell death caused by Raman spectroscopy, live cell samples were consequently analysed for 40 minutes with laser bleaching and measurements of 60 s taken at intervals of 5 minutes resulting in a total analysis time of 48 minutes per cell, as outlined in section 6.6. Only one cell was analysed per time point, as the purpose of this initial study was to intentionally narrow down the timeframe in which cell death occurred from laser irradiation. As the method used by Notingher et al was successful in the discrimination between viable and non-viable MLE-12 cells, the same method for time of analysis was applied to the MG-63 cells.⁵ In the literature, only 3 measurements were taken during the course of laser bleaching, however this was not possible due to instrument software limitations, therefore Raman spectra were collected every 5 minutes during this time course study. Visible light images were taken of the cells after each analysis to help elucidate any morphological changes that may potentially be visible over the time course of the bleaching.

3.4.1 Morphological Changes

The visible light images of the cells analysed for 40 minutes at 100 % (21 mW), 50 % (12.5 mW) and 10 % (2.3 mW) 532 nm laser power at the sample utilising a 63x immersion objective, were captured as per section 6.6. As outlined in section 6.14, the cells were cultured on gridded glass Ibidi dishes to enable relocation of the cells after Raman analysis for staining confirmation of viability and mounted in prewarmed PBS. Images of the cells analysed for the varying laser power over 40 minutes are shown in figure 32 – 34.

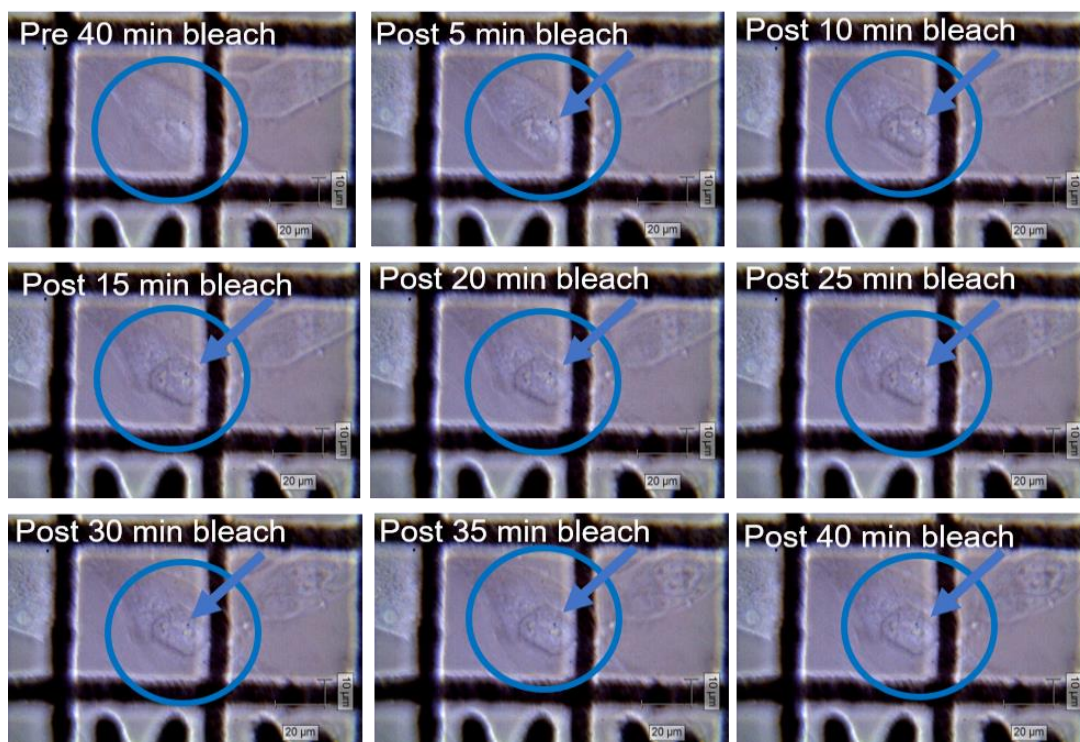


Figure 32: Visible light images of cell irradiated at 100 % 532 nm laser power, equivalent to 21 mW power, captured using inbuilt image capture function before and after each acquisition in the Renishaw Wire software . A Raman spectrum was acquired with 60s acquisition time every 4 minutes with continuous laser exposure between acquisition, resulting in 40 minutes laser exposure and 9 Raman spectra acquired in total. Arrow indicates region where apparent damage is occurring.

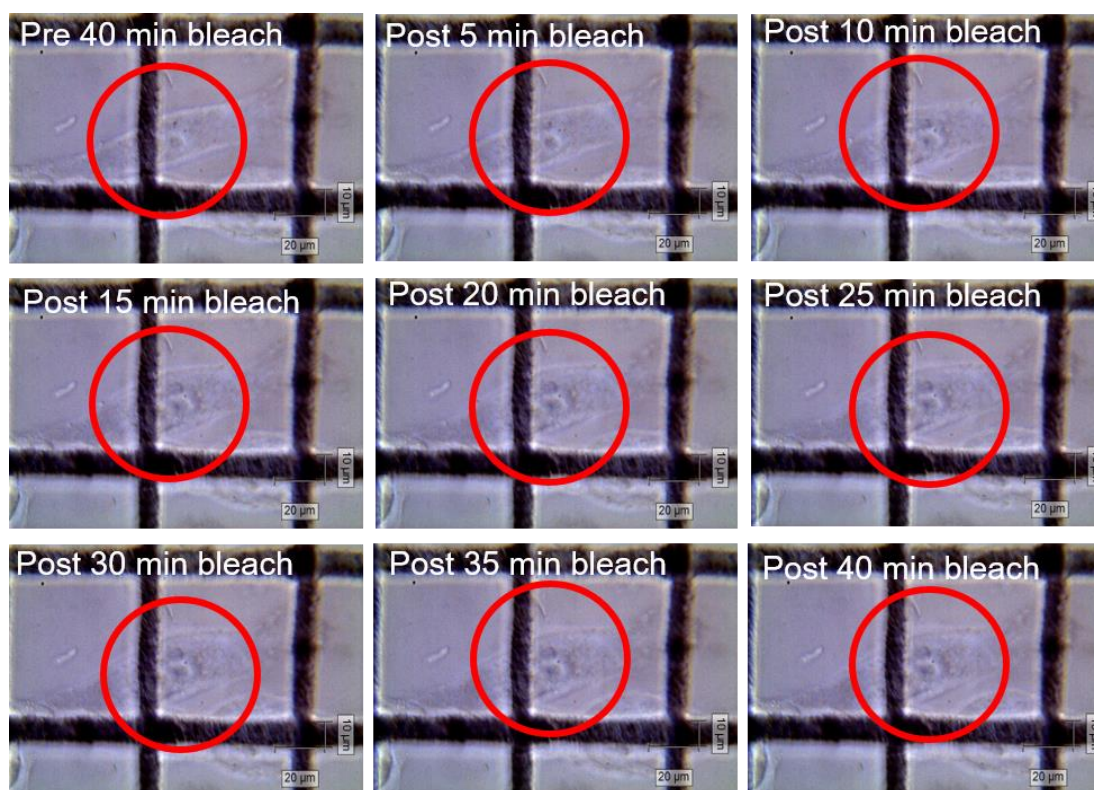


Figure 33: Visible light images of cell irradiated at 50 % 532 nm laser power, equivalent to 12 mW power, captured using inbuilt image capture function before and after each acquisition in the Renishaw Wire software . A Raman spectrum was acquired with 60s acquisition time every 4 minutes with continuous laser exposure between acquisition, resulting in 40 minutes laser exposure and 9 Raman spectra acquired in total.

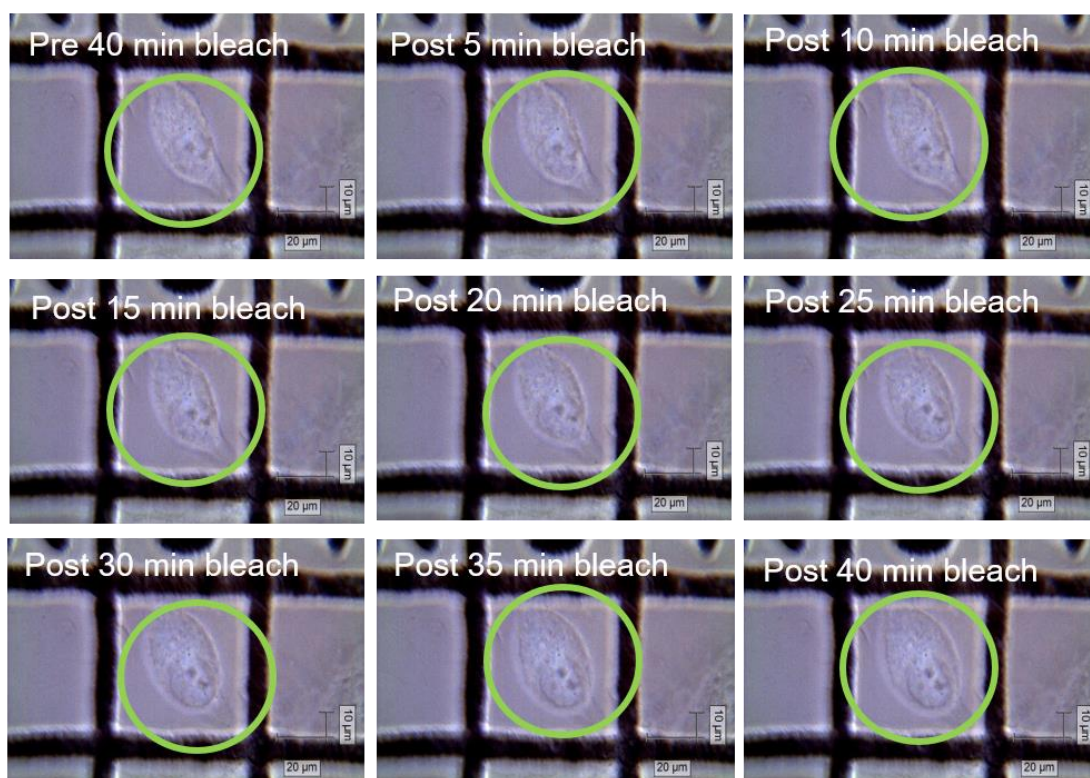


Figure 34: Visible light images of cell irradiated at 10 %/2.3 mW 532 nm laser power, equivalent to 3 mW power, captured using inbuilt image capture function before and after each acquisition in the Renishaw Wire software . A Raman spectrum was acquired with 60s acquisition time every 4 minutes with continuous laser exposure between acquisition, resulting in 40 minutes laser exposure and 9 Raman spectra acquired in total.

For the 100 %/21 mW laser power bleached cell, significant morphological changes are apparent from as early as 10 minutes laser bleaching. Initially the cell is elongated with the nucleus enclosed with some nucleoli visible, however at 10 minutes, the nucleus appears to have lost all encapsulation, with a hole around 25 µm clearly visible in the region irradiated by the laser. The laser diameter is approximately 1-2 µm as determined in section 2.5.2.1. The variation in refractive index of material within the cell could have caused widening of the laser beam, however, heat dissipation through the cell could also have occurred. The increase in cellular width combined with the loss of cell membrane indicates the laser power at 532 nm has induced necrosis within 10 minutes. However, the experiment did continue until 40 minutes for completion. It is interesting to note that the cell in the top right corner, next to the interrogated cell, starts to swell at 25 minutes, and is much larger by the

end point of the experiment. This cell swelling is an indicator of necrosis induction, and that high laser powers could affect the cells in close proximity to the cell under investigation.

In the 50 %/12.5 mW and 10 %/2.3 mW images, there is not the same clear induction of a hole within the cell body. The cells under analysis do appear to exhibit cellular swelling, and in the 10 %/2.3 mW images from 20 – 40 minutes, there does appear to be a more apparent circular region within the cell at the site of investigation corresponding with the nucleus. However, this could be due to cell movement as the cell swells, as in comparison to the 100 %/21 mW laser power where clear indents are visible in the cell surface, the nuclear compartment just appears more defined.

After analysis, the cells were again stained using Trypan blue, as outlined in section 6.16.1. The cells from figures 32 - 34 are shown in figure 35 post Trypan blue staining, to determine their viability after 40 minutes of laser irradiation with 21 mW, 12.5 mW or 2.3 mW laser power (100/50/10% respectively).

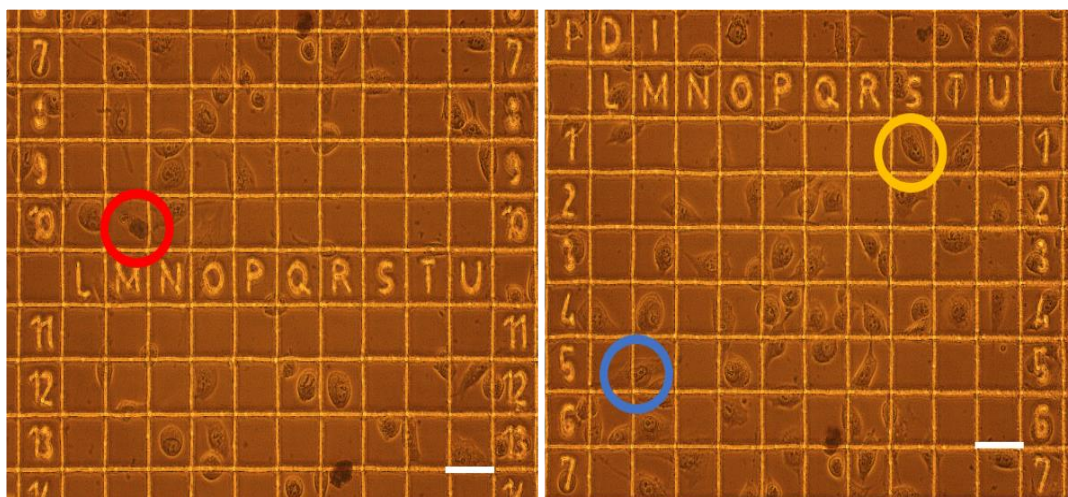


Figure 35: Visible light images of cells shown pre and post Raman acquisitions in figures 32, 33 and 34, captured using an inverted Nikon Eclipse Ti microscope with a 20x long working distance objective, as detailed in section 6.8. Cell circled in red was analysed for a total of 40 minutes 532 nm laser exposure power at 100 % which is equivalent to 21 mW, the cell is dead as indicated by the presence of the blue nuclei on staining with Trypan Blue. The cell circled in blue was irradiated for a total of 40 minutes with 50 % 532 nm laser power equivalent to 12.5 mW power at the cell. This cell appears to be still viable as no blue colour is visible post Trypan Blue staining. The cell circled in yellow was irradiated for 40 minutes with 10 % 532 nm laser power, equivalent to 2.3 mW power at the sample, this cell is also still viable as no Trypan Blue staining is visible within the cell.

The cells for 50 %/12.5 mW and 10 %/2.3 mW laser power analysis were acquired from the same sample, which resulted in an analysis time over 90 minutes, which was previously determined as the limit for the cells to be out of the incubator without visible morphological changes. However, both the 50 %/12.5 mW and 10 %/2.3 mW cells did not uptake the trypan blue, and neither did any of the surrounding cells within the sample, which indicates the cells still have intact membranes, although complete viability cannot be confirmed with trypan blue as outlined previously. It is however a good indicator that necrosis did not occur in these cells due to the laser power incident on the sample for 40 minutes. The 100 %/21 mW 40-minute bleach did uptake the trypan blue stain, which was expected due to the induced hole in the nuclear membrane region visible from the images take during analysis.

3.4.2 Spectral Changes

In correlation with the trypan blue viability assay, the spectra of the cells irradiated with 100 %/21 mW, 50 %/12.5 mW and 10 %/2.3 mW 532 nm laser power were also evaluated to investigate whether similar changes were seen for all laser powers even though not all cells were dead after the Raman analysis.

Three cells were bleached with 532 nm at either 10 %/2.3 mW, 50 %/12.5 mW or 100 %/21 mW laser power for 40 minutes, the cells indicated in figures 32-34 and in figure 35, with acquisitions taken every five minutes. The spectra were pre-processed as per section 6.7, with any glass and media contributions being removed, and the baseline fitted using the same ALS fitting method mentioned previously. The spectra were all then normalised to the peak area under the 1449 cm^{-1} peak to ensure consistent data processing for sample comparison.¹⁶⁹

In this analysis, no dead population was used, instead the time points were compared to the initial spectrum of the cell before bleaching, $t = 0\text{ min}$ as the cells were proven to be 'live' at $t = 0\text{ min}$, but in the case of the 100 %/21 mW 40 min cell confirmed dead by trypan blue staining, whilst the 50 %/12.5 mW and 10 %/2.3 mW cells showed a change in morphology analogous with necrosis, cell death could not be confirmed via the trypan blue method.

power spectra, with variances in the intensity changes of the peaks. In both the 100 %/21 mW and 50 %/12.5 mW difference spectra, the protein peak at 717 cm^{-1} shows a decrease in intensity, which is visible in the comparison of the normalised spectra in figure 36. However, in comparison to this decrease, both laser power difference spectra show slight increases in the phenylalanine peaks at 1005 cm^{-1} and 1033 cm^{-1} , this is contradictory to the comparison between the live and dead cell spectra previously, in figure 31, where a decrease in the 1005 cm^{-1} peak was observed. The difference could potentially be due to the fact that in these cells necrosis could be being induced, particularly in the 100 %/21 mW cell, from the irradiation by the laser, than the dead cell samples which were nutrient deprived from lack of fresh media, and therefore more likely apoptotic in their method of cell death. This has also been suggested by Verrier et al with decreases in the phenylalanine peak being related to the caspase induced breakdown of the cell during apoptosis.³⁰

The 10 %/2.3 mW laser difference spectrum shows no changes in the phenylalanine or other corresponding protein peaks, which could be consistent with the cell remaining viable as indicated by the trypan blue test. The 50 %/12.5 mW cell did not stain blue either, however the spectral changes at $t = 40\text{ min}$ are more in line with the 100 %/21 mW spectral differences, so the cell could be stressed or approaching cell death. The peak at 937 cm^{-1} has also been related to the apoptotic action within cells, however protein unfolding could be related to both methods of cell death, and it is difficult to determine the cell death mechanism by trypan blue alone.

The literature mentions that decrease in DNA peaks such as 782 cm^{-1} , 1095 cm^{-1} and 1577 cm^{-1} could be due to cell death and the resulting DNA cleavage on either induced damage from necrosis or from the breakdown of nuclear components as a cell readies itself for autophagy during apoptosis.^{4,5,30–32,171} Interestingly, the peak at 782 cm^{-1} is shown most clearly in the 10 %/2.3 mW difference spectrum in comparison with the higher laser powers. The 1577 cm^{-1} peak was also able to be compared more fairly in this sample as the cell was exposed to further bleaching times and not masked by the widening of the amide-I band at 1660 cm^{-1} . However, the 1660 cm^{-1} peak does exhibit a slight

shift in wavenumber which is more noticeable in the 100 %/21 mW and 50 %/12.5 mW difference spectra and doesn't appear to occur at the lower laser power. This could be related to the unfolding of proteins as indicated by the decrease in the peak at 937 cm^{-1} relating a decrease in the α -helix structure to more β -sheet in nature, as a denaturation of the proteins from secondary structure to random coil would be expected to see a decrease at 1231 cm^{-1} , which is not visible in the spectra.²⁸ The peak at 1240 cm^{-1} is also related to protein structure; amide-III β -sheet behaviour, this peak could be masking any peak potentially visible at 1231 cm^{-1} , however a slight increase in this peak at all laser powers is also visible, which supports the theory that the nature of the proteins within the cells is more oriented toward β -sheet in nature on continued laser exposure.

Due to the difference spectra, plot F in figure 36, of the 10 %/2.3 mW laser power showing minimal differences in peaks in the difference spectrum in correlation with the weaker laser power, the peak ratios of peaks of interest were evaluated and compared for each laser power, as shown in figure 37.

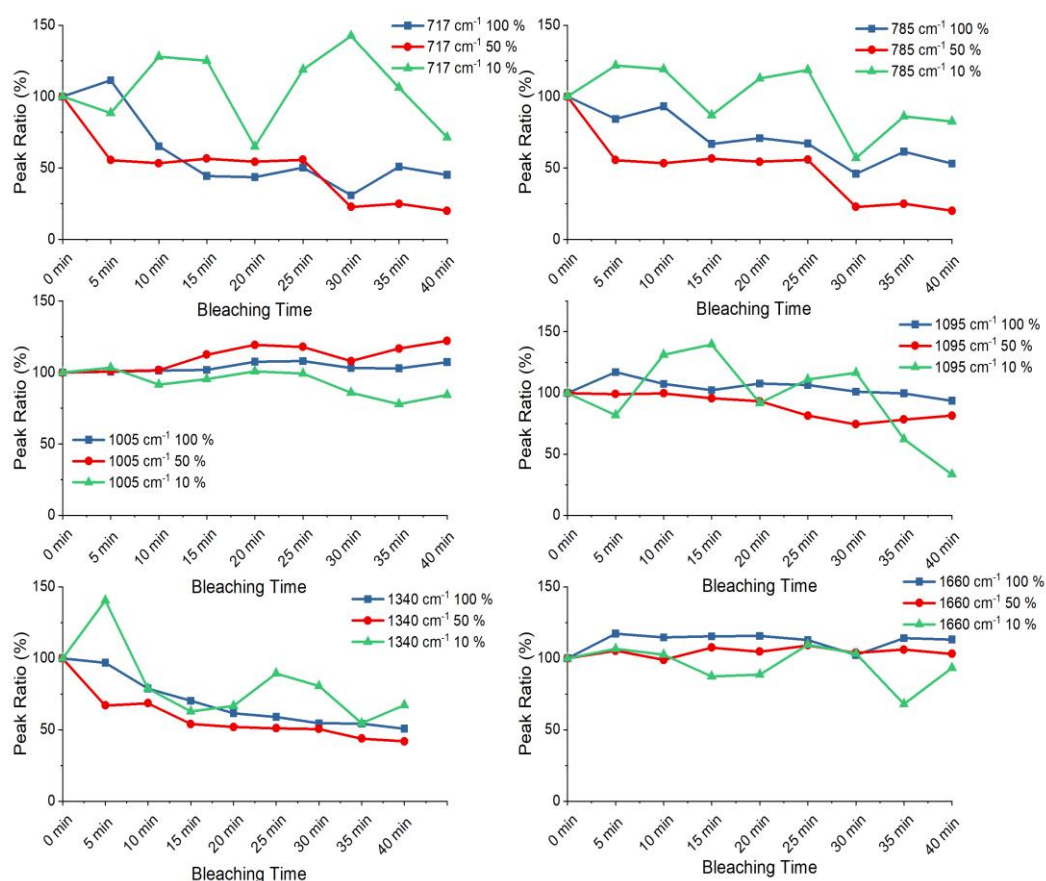


Figure 37: Comparison of peak ratios from spectrum of cell at $t=0$ mins, before analysis, in 5-minute increments until $t=40$ mins. The peaks indicated in this figure relate to phospholipids at 717 cm^{-1} , phenylalanine at 1005 cm^{-1} , the Amide I and lipid band for $\text{C}=\text{C}$ at 1660 cm^{-1} , and DNA at 782 cm^{-1} , 1095 cm^{-1} and 1340 cm^{-1} for cells irradiated with 100 %/21 mW (blue), 50 %/12.5 mW (red) and 10 %/2.3 mW (green) 532 nm laser. Spectra were acquired for 60 s from one cell in 5-minute intervals for 100 %/21 mW, 50 %/12.5 mW and 10 %/2.3 mW 532 nm per cell. Peak ratio changes are expressed as a ratio in comparison to the peak are at $t=0$ mins which is taken to be equivalent to 100 % as this is before any chemical changes occur due to the ongoing laser irradiation. Each proceeding peak area value from later acquisitions have therefore been divided by the peak area of the peak of interest at $t=0$ mins in order to determine if the signal of interest decreases or increases with increasing laser irradiation in comparison to the initial spectrum.

The peaks were fitted using Renishaw Wire 5.1 software after removal of PBS and glass contributions, baseline correction and normalisation. The peak at 717 cm^{-1} relates to phospholipids, indicative of phosphatidylcholine: an integral part of cell membranes due to their hydrophilic head and hydrophobic tail allowing the formation of the key lipid bilayer.³³ In the 100 %/21 mW

irradiation, a decrease of 50 %/12.5 mW is apparent, which is consistent with the morphological changes viewed during the Raman analysis and the appearance of visible damage in the upper cell membrane.

Interestingly the 50 %/12.5 mW irradiated cell shows a larger decrease in this peak of 60 %, but no visible damage was caused to the membrane, and from the trypan blue staining cell viability that the membrane is still intact, the difference could be due to the dynamic nature of the cell membrane or indeed on the cell movement and swelling causing the collection volume of the Raman signal to be focused below the membrane layer. The 10 %/2.3 mW laser power ratio does not show any clear variation, which again, could be due to the dynamic nature of the cell and the cell membrane. This is contradictory to the findings by Notingher et al on the treatment of A549 cells with Triton-X, they found that on apoptosis, the phospholipid peak increased relating to a proposed increase of lipid vesicles inside the dying cells.²⁸ This lack of increase in the phospholipid peak could therefore correlate more with necrotic cell death.

As mentioned previously in section 3.3.3, the phenylalanine peak at 1005 cm^{-1} is the most notable peak of the amino acids within cells and is often used for normalisation. However, this peak can also be indicative of the method of cell death that the cell has undergone.^{30,33,174,175} Decreases in the phenylalanine peak due to induction of chemical death has been indicative of apoptosis, whilst the increase has been shown to be indicative of necrosis coupled with a decrease in RNA peaks at 782 cm^{-1} and 813 cm^{-1} .³³ The changes in this peak in the chemically induced death studies however is quite dramatic, whereas in this study the laser induced death of the 100 %/21 mW cell only increases by 8 %. The increase in phenylalanine in the 50 %/12.5 mW cell however is 22 %, more than in the confirmed dead cell. This could indicate that the 50 %/12.5 mW cell is moving towards necrosis and that the 100 %/21 mW cell has died via the necrotic pathway. However, there is no visible decrease in the 813 cm^{-1} from the spectrum, but there is a decrease in the 785 cm^{-1} peak for both the 100 %/21 mW and 50 %/12.5 mW by 50 % and 80 % respectively, this peak

is likely due to the pyrimidine ring breathing found in uracil, cytosine and thymine at 782 cm^{-1} .¹⁵⁴

The peak at 788 cm^{-1} for the phosphodiester bond in DNA was more clearly visible in the higher laser power spectra but peak fitting was not consistently able to distinguish from the lower wavenumber peak, so the decrease in signal at 785 cm^{-1} in the lower laser power cells could be a mixture of these two peaks. The decrease in both peaks at 782 cm^{-1} and 788 cm^{-1} have also been shown to be indicators of both necrosis and apoptosis and does not help elucidate the method of cell death here.

The 1095 cm^{-1} peak is also related to the DNA backbone; this peak is due to the phosphate backbone and has been shown to be less sensitive to DNA degradation on cell death, which is consistent with the peak ratio change observed in the data, with the 100 %/21 mW laser power cell experiencing only 7 % loss of the 1095 cm^{-1} and the 50 %/12.5 mW cell decreasing more by 11 % overall.^{171,176}

For the 10 %/2.3 mW laser power irradiated cell, with the exception of the phenylalanine peak at 1005 cm^{-1} , the overall peak ratio percentage does decrease from 0 minutes irradiation to 40 minutes irradiation, however, the peak intensity change varies over the time points, there doesn't appear to be a clear relationship between the peak intensity change with time, between the peaks however, the peak ratios change approximately in a similar fashion. This could be due to natural variations within the cell itself, or from the previously suggested movement of the cell trying to minimise the stimulus caused by the laser, or indeed just laser fluctuation and noise at lower laser powers.

Overall, the analysis of the peak changes in more detail did help elucidate more information from the three cells irradiated at different laser powers. In retrospect it would've been better to have performed these analyses in at least triplicate, if not more, to determine if the changes observed were representative across multiple cells. However, the main aim of this analysis was to determine if there were spectral changes visible within the same time frame reported by Notingher et al, but on a different cell line, and to determine

if the irradiation time alone was enough to induce cell death. This was the case for the 100 %/21 mW cell, however although the spectral changes found within the 50 %/12.5 mW cell are similar, cell death could not be confirmed via the trypan blue test, and the 10 %/2.3 mW cell did not elucidate either spectral similarities or confirmation of cell death with trypan blue. To determine if the 50 %/12.5 mW cell was dead or not, and to determine the time of irradiation where the 100 %/21 mW cell died, more analysis and a different staining mechanism will be required.

3.5 DETERMINATION OF TIME OF CELL DEATH

3.5.1 Trypan Blue

Although Raman analysis can take many hours to complete, particularly for Raman imaging uses, single cell point spectra analysis is often done more within a factor of minutes. The analysis performed previously allowed for the confirmation of cell death utilising trypan blue of a cell irradiated at 100 %/21 mW laser power for 40 minutes; spectrally, however, peak changes could be detected from five minutes and visually, morphological changes were observed from 10 minutes. The analysis was repeated with shorter time frames to elucidate if 100 %/21 mW laser power induced cell death within a lower time frame more closely correlated with single cell analysis times.

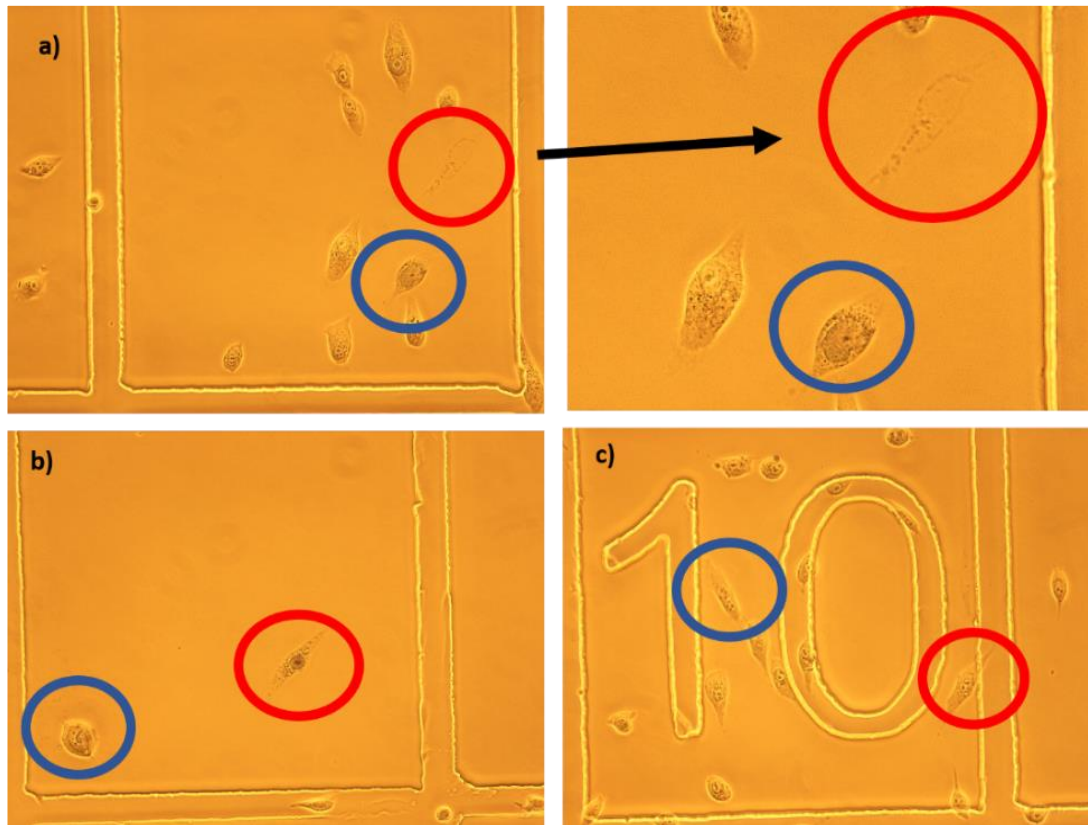


Figure 38: Cells mounted on Ibidi polymer bottom grid in PBS after Raman analysis and staining with Trypan blue. Images were captured using an inverted Nikon Eclipse Ti microscope with a 20x long working distance objective, as detailed in section 6.8. a) indicates two cells and then an inset of the same two cells on the right-hand side to show additional detail. In a), the cell circled in red has been irradiated with 100%/21 mW 532 nm laser for 15 minutes and is no longer in its original position, however the inset image clearly shows some leftover cell debris, indicating that a cell has been removed from the sample surface. This cell detachment can be indicative of cell death, even without the confirmation of the trypan blue stain. The cell circled in blue in a) was irradiated for 10 minutes 100 %/21 mW 532 nm laser power and has clear trypan blue staining in the nuclei, therefore is no longer a viable cell. b) Two cells, again circled in red and blue, indicative of 15- and 10-minute irradiation respectively, however these cells were irradiated with 50 %/12.5 mW 532 nm laser power. The 15-minute 50 %/12.5 mW irradiated cell, circled in red, has a clearly stained nuclei and is therefore no longer viable, whereas the 10-minute 50 %/12.5 mW irradiated cell has not up taken the blue stain and can be considered still viable. c) the cells are again circled in red and blue to indicate 15- and 10- minute irradiation respectively, but these cells were irradiated with 10 %/2.3 mW 532 nm laser power and neither cell has up taken the stain and therefore both are still viable.

In order to narrow down the time frame for cell death, cells were analysed with Raman spectroscopy using the same method as previously, section 6.6, except for a reduced time of 15 minutes, 10 minutes, as shown in figure 38 or from 15 minutes to 5 minutes, in decreasing irradiation times, as shown in figure 39. The cells were stained with trypan blue, as per section 6.16.1, after analysis to determine if cell death had occurred. In figures 38 and 39, the cells were seeded on a polymer bottom gridded Ibidi dish instead of the glass used previously due to supplier issues, as detailed in section 6.14. This resulted in the polymeric background being difficult to separate from the resulting cell spectra and therefore the Raman spectral analysis of these experiments was not usable.

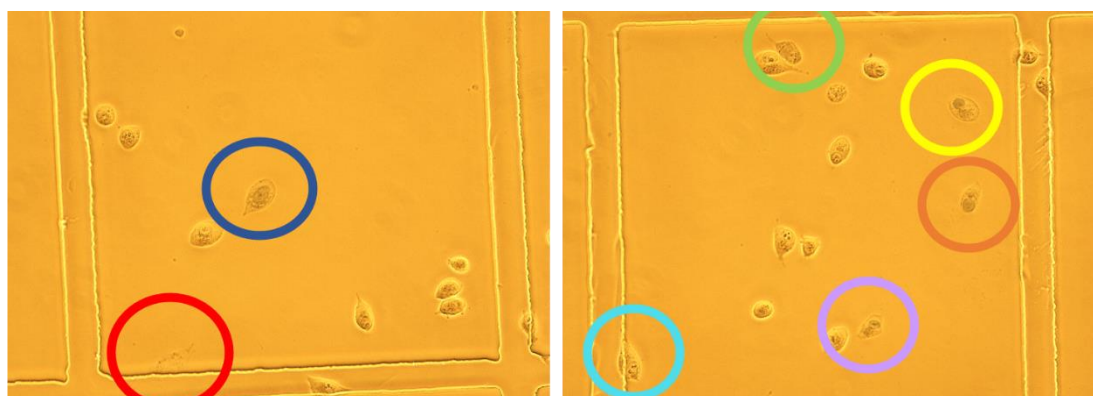


Figure 39: Cells irradiated at 100 %/21 mW 532 nm laser power for 15 mins (red), 10 mins (blue), 9 mins (green), 8 mins (yellow), 7 mins (orange), 6 mins (purple) and 5 mins (turquoise). The 15-minute cell (red) has been removed from the sample surface as in figure 38. The cells from 10-minutes (blue) to 6-minutes (green, yellow, orange, and purple) irradiation with 21 mW 532 nm laser power all appear to have darker nuclei regions and can be considered as non-viable. The 5-minute irradiated cell (turquoise) however, does not have the darker nuclear region of the other cells and can therefore be considered viable. This indicates that more than 5 minutes irradiation with 21 mW of 532 nm laser power can incur cell death.

However, the staining of the cells was helpful in narrowing down the time of death for the 100 %/21 mW laser power cells. In tile figure 38 frame a) and figure 39, above, the cells have been irradiated with 100 %/21 mW laser power for 15 minutes circled in red and 10 minutes circled in blue. In both frames the cells irradiated for 15 minutes are gone, which can be indicative of cell death

and detachment from the surface. Interestingly, on closer examination of the images, some cell debris in the previous cell position could be found, as indicated in the inset of image a) in figure 38 above. After 15 minutes of laser irradiation, the 50 %/12.5 mW laser power cell was also stained blue by trypan blue, indicating cell death. This is contradictory to the previous study in section 3.5, where at the far longer time of 40-minute 50 %/12.5 mW laser exposure, the cell did not stain blue. This could have been a cell that was already dying hence the uptake of the stain, but further experiments were needed to confirm this. The 10-minute 50 %/12.5 mW irradiated cell, although showing morphology consistent with cell stress, did not uptake the trypan blue. The 10 %/2.3 mW 10- and 15-minute cells did not uptake the dye either, indicative that 10 %/2.3 mW laser power is safe to cells at 10 and 15 minutes and the 40 minutes from the previous analysis.

The sample indicated in figure 39 confirms the results of frame a) in figure 38, that at 15 minutes 100 %/21 mW 532 nm laser power, the cell is gone, indicative of cell death and the 10-minute cell is stained with trypan blue also. In the second frame of image of figure 39 the cells were irradiated in decreasing 1 minute increments from 9 minutes until 5 minutes (9 minutes - green, 8 minutes - yellow, 7 minutes - orange, 6 minutes – purple and 5 minutes – turquoise, clockwise from the cell circled in green at the top). It is difficult due to the contrast to determine if the 9 minute cell has definitely taken up the trypan blue, however the cells at 8, 7 and 6-minutes 100 %/21 mW 532 nm laser power have all got blue nuclei, indicative of compromised membranes and dead cells, whilst the 5-minute cell does not. This indicates that as little as 6 minutes of 532 nm laser power at 21 mW will induce cell damage. Although repetition of this time frame and staining would be required to ensure this result is representative and not due to cell variability.

A combination of fluorometric dyes were used in further analysis to determine if the time of death found in the trypan blue images in figures 38 and 39 were consistent with more samples.

3.5.2 Calcein AM and Ethidium Homodimer-I

Calcein acetomethoxy (AM) is a non-fluorescent esterase substrate which is cell membrane permeable due to the lack of overall charge across the molecule.¹⁷⁷ The molecule is able to transpass the cell membrane where it is hydrolysed into calcein by non-specific esterases resulting in fluorescence specific with enzymatic activity whilst bound within an intact membrane. Therefore, calcein AM can serve a dual purpose of indicating viable cells as well as extent of enzyme activity. Calcein AM is relatively insensitive to pH change and is well retained within the cells with intact membranes due to the high charge of the hydrolysed calcein product, the non-hydrolysed substrate will leak from the cell resulting in specificity of the binding and on membrane compromise the fluorescent membrane will leak from the cell rapidly.¹⁷⁸ Calcein is excited at 495 nm and emits at λ_{max} 515 nm.

Calcein AM is often combined with a nuclear specific membrane impermeable dye for cross-validation of cell death using the loss of Calcein AM intensity combined with the uptake of the nuclear dye to indicate cell death.¹⁷⁹ Calcein AM can be used with either propidium iodide (PI) or ethidium homodimer-I (EtHD-I) as these stains have fluorescence emission far enough from the λ_{max} of Calcein AM to allow for the separate detection of the two stains. EtHD-I is highly charged and has a higher affinity for nucleic acids than PI and therefore can be used at lower concentrations and does not require an extra wash step which can often remove dead cells from surfaces in non-fixed dead samples.¹⁸⁰ EtHD-I has a λ_{max} at 615 nm, which allows for easier separation of the signals in fluorescence detection. These stains operate in a similar mechanism to trypan blue, but with an easier visualisation of live and dead cells with an indicator of cells losing their membrane integrity to indicate dying cells, allowing a more sliding scale qualitative approach to determining cell death. Therefore, the combination of these two stains was chosen as an alternative for trypan blue staining to narrow down the timeframe of cell death of MG-63 cells with a 532 nm laser during Raman analysis.

3.5.2.1 Calcein AM EtHD-I Controls

To confirm that cell variations in fluorescence intensity are due to the process under study, control samples must be performed to evaluate stain action on non-analysed cells. Cells with no Live/Dead™ stain applied were analysed using single photon fluorescence on a Leica SP8 CARS with SRS Option microscope with a 488 nm laser at 30 % laser power and detected with a PMT set at 515 – 525 nm and 615 – 625 nm for Calcein AM and EtHD-I respectively, as per section 6.9. The gain was consistently set at 500 V for all fluorescence imaging to retain consistency, even if the stains were not being used for quantitative measurement.

The non-analysed, non-stained cells, which were prepared as per section 6.14, are shown in figure 40. The only indication of fluorescence within the sample in the live (green) channel is autofluorescence from cellular components such as mitochondria or cyclic compounds such as amino acids which are intrinsically fluorescent.

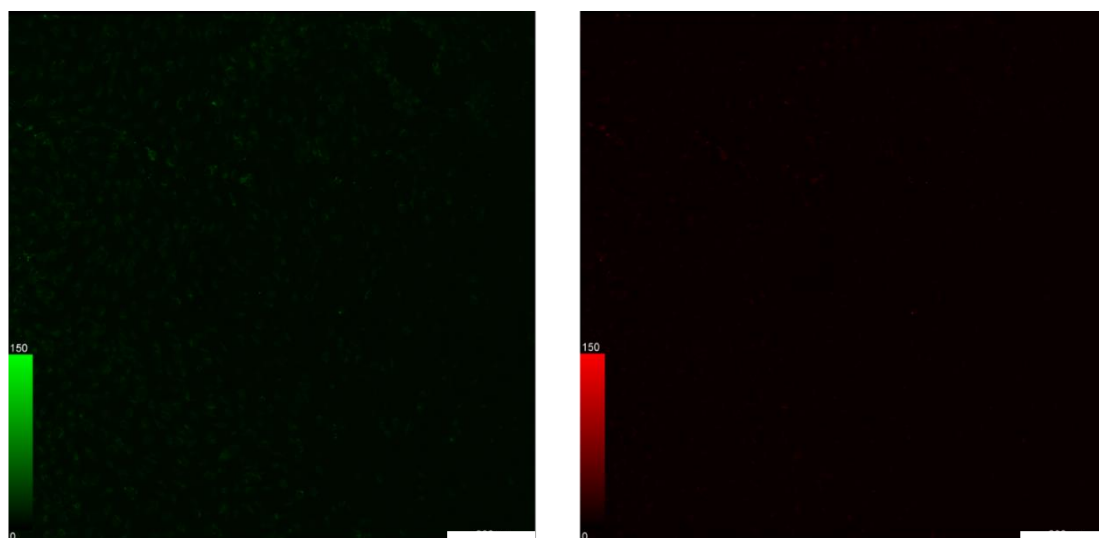


Figure 40: Cells cultured on Ibidi glass bottom dish in PBS with no fluorescent stain added to indicate level of intrinsic autofluorescence of cells. Imaged with a 25x 0.95 NA water immersion objective (Leica, Fluotar VISIR) and 0.55 NA air condenser (Leica S28). Scale bar – 200 μm , LUT in all images 0 – 150, stitched 5x5 grid images at 512 x 512 pixels = 465 x 465 μm per image

Positive and negative controls were also performed utilising live cells, detailed in section 6.14 and cells fixed in 4 % PFA, section 6.15. Cells were incubated with 4 % PFA for 30 minutes prior to analysis. Samples were then prepared as per section 6.16.2.1, with one of three staining solutions: Calcein AM only, EtHD-I only or Calcein AM: EtHD-I. A total of 6 control samples were imaged. The Calcein AM:EtHD-I solution was mixed at a ratio of 2 μ M: 4 μ M in prewarmed PBS, and the individual dyes were prepared at the same concentrations but remained separate before being applied to the samples and incubated for 15 minutes, washed in PBS and incubated for a further 15 minutes before analysis.

Analysis was performed in the exact same method as outlined for the non-stained sample, as per section 6.9, with 488 nm excitation and emission detected at 515 – 525 nm and 615 – 625 nm for Calcein AM and EtHD-I respectively with the detector gain at 500 V with 30 % laser power to the sample. The images shown in figure 41 indicate the positive and negative control samples.

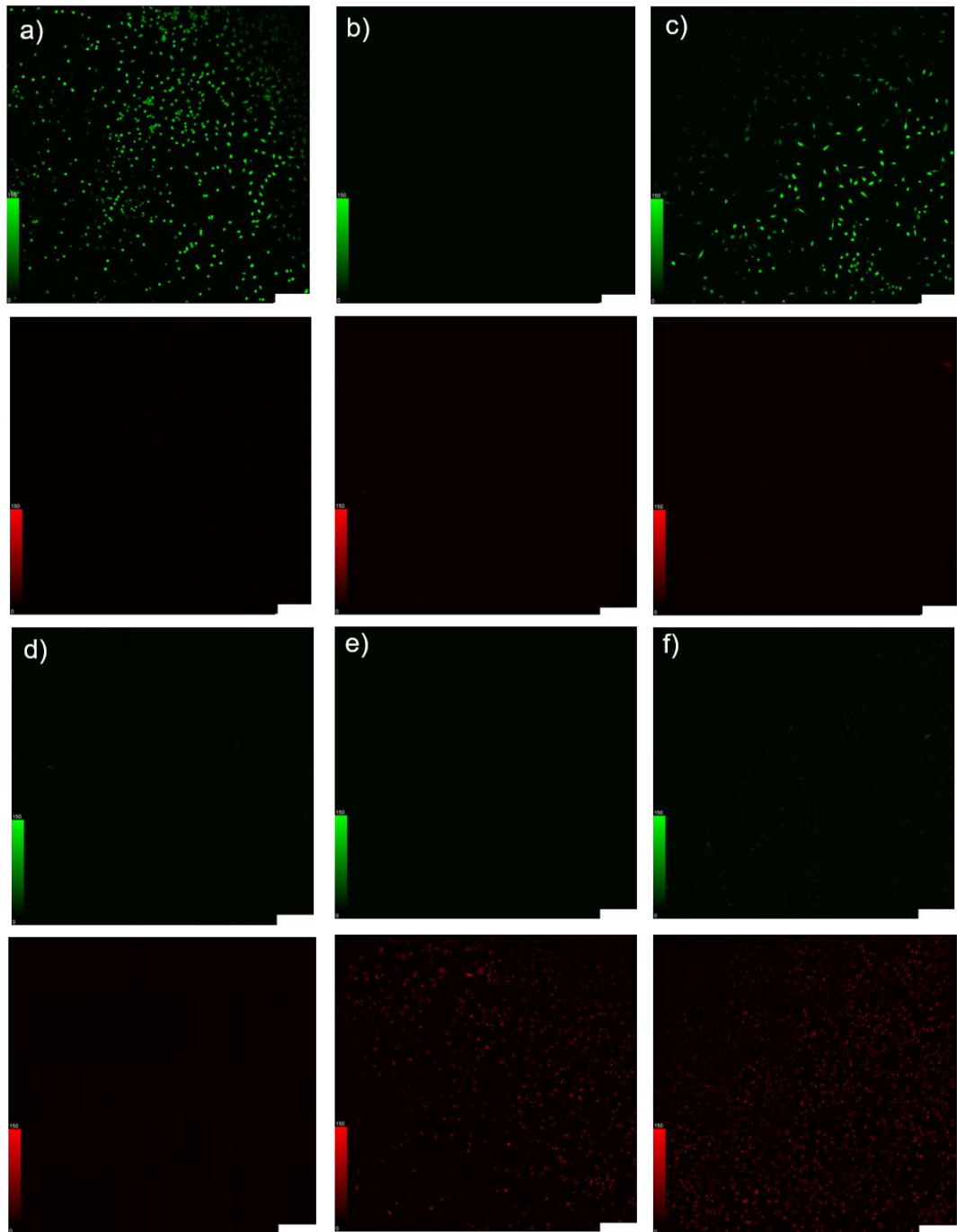


Figure 41: Live/Dead™ control samples. a) indicates live cells with Calcein AM only in both live and dead channels (top and bottom respectively) b) live cells with EtHD-I stain only - both channels c) live cells with both Calcein AM and EtHD-I in both channels d) fixed cells with Calcein AM only (both channels), e) fixed cells with EtHD-I only (both channels) f) fixed cells with Calcein AM and EtHD-I (both channels). Channel 1: 515 – 525 nm, channel 2: 615 – 625 nm, 30 % 488 nm excitation, 25x 0.95 Na water immersion objective and 0.55 NA air condenser, scale bar = 200 μm , LUT in all images 0 – 150, stitched 5x5 grid images 512 x 512 pixels = 465 x 465 μm per image

Images a) – f) in figure 41 confirm that only live cells will uptake the Calcein AM producing a strong signal between 515 – 525 nm, there is still the inherent autofluorescence present in both negative controls, but this level is incomparable to the amount of fluorescence produced on the excitation of the Calcein. Similarly, the EtHD-I shows a dramatic increase in signal in the fixed cells in comparison to the live cells with EtHD-I. These control samples are necessary to understand the baseline cell behaviour with the fluorescent stains before evaluating any changes that may be seen in irradiated cells using the stains, they help to ensure that any differences are due to the ‘treatment’ applied to the cells, in this case laser irradiation.

3.5.2.2 *Live/Dead™ Time Studies*

As mentioned in section 3.5, the further laser irradiation and trypan blue studies appeared to narrow down the timeframe of cell death for 100 %/21 mW 532 nm laser power to between 5 and 10 minutes, and also indicated that 50 %/12.5 mW laser power may induce cell death at 15 minutes irradiation. Further studies were conducted using the Live/Dead™ stain in place of trypan blue for cells between n = 7 - 38 for 100 %/21 mW, 50 %/12.5 mW and 10 %/2.3 mW irradiation for cells between 5 and 15 minutes.

Following the trypan blue staining of samples prepared on polymer gridded coverslips, attention was then focussed to confirm if cell death occurs between 5, 10 and 15 minutes of Raman laser exposure for 100 %/21 mW and 50 %/12.5 mW laser power. The 10 %/2.3 mW laser power was ignored for the moment as the initial experiments indicated that at 10, 15 and 40 minutes of Raman exposure the cells were still viable. In comparison at 40 minutes the 100 %/21 mW cell was dead, and that at 15, 10 – 6 minutes the cells were also dead but viable at 5 minutes. The 50 %/12.5 mW cell was still viable at 40 minutes, however a further experiment indicated that at 15 minutes 50 %/12.5 mW laser power the cell was dead, but still viable at 10 minutes. So further confirmation was required to determine representative cell death time, as variation in cell death time could occur depending on the stage of the cell in

the cell cycle, representative results can be difficult if only analysing one cell at a time.

An example of samples analysed between 15 and 5 minutes for both 100 %/21 mW and 50 %/12.5 mW laser power are shown in figure 42.

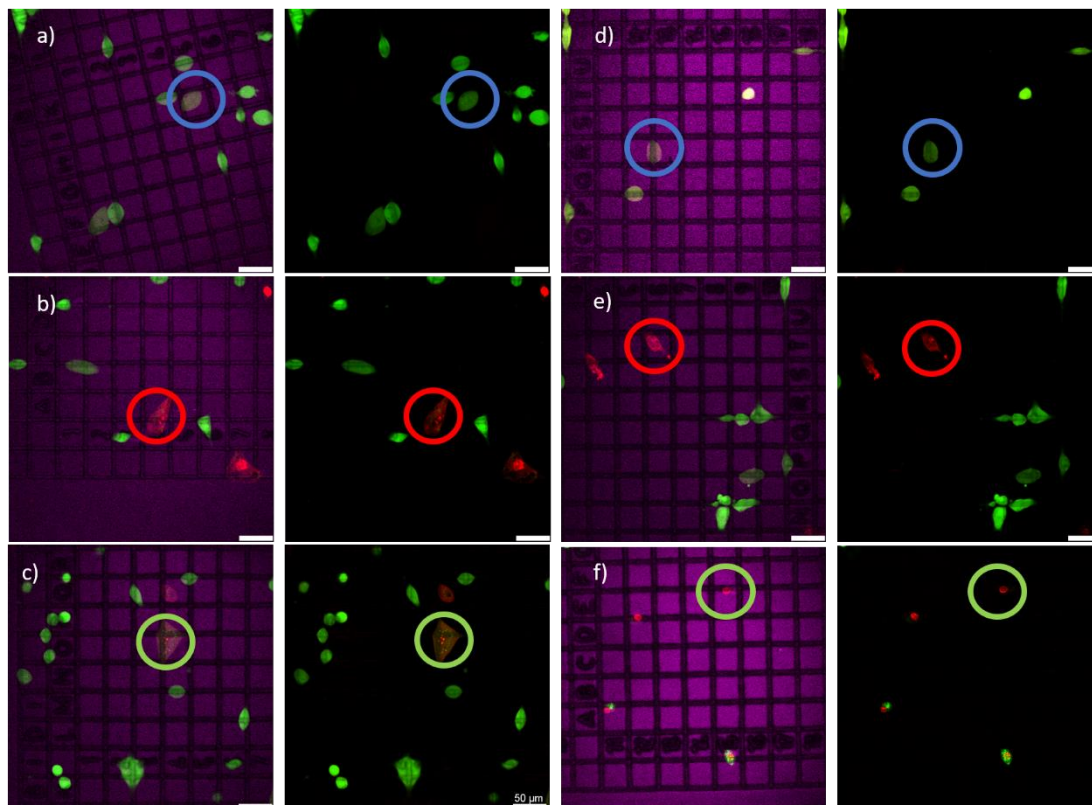


Figure 42: Example of cells stained with Live/Dead™ stains post Raman analysis as outlined in Section 6.16.2. a) cell at I5 was irradiated with 100 %/21 mW 532 nm laser power for 5 mins. b) cell localised at A4 was irradiated with 100 %/21 mW 532 nm laser power for 10 minutes, c) cell localised at O4 was irradiated with 100 %/21 mW 532 nm laser power for 15 minutes. D) cell localised at R13 was irradiated with 50 %/12.5 mW 532 nm laser power for 5 minutes, e) is a cell localised at T6 irradiated with 50 %/12.5 mW 532 nm laser power for 10 minutes and f) is a cell localised at E15 was irradiated with 50 %/12.5 mW 532 nm laser power for 15 minutes. Images obtained using confocal fluorescence with 2 channels in the first instance and then confirmatory forward-SRS to determine the grid positioning of the individual cells being analysed. Confocal fluorescence channel 1 tuned to 515 – 525 nm and channel 2 tuned to 615 – 625 nm, with 30 % 488 nm excitation. SRS channel tuned to 2850 cm^{-1} at 20 % laser power which is equivalent to 20 % of 0.15 W (0.03 W) in the pump beam and 20 % of 0.3 W (0.06 W) in the Stokes beam. Images captured with Leica SP-8 CARS multiphoton microscope with SRS option using a 25x 0.95 NA water immersion objective and 0.55 NA air condenser, scale bar = 200 μm , LUT in all images set between 0 – 150.

For the live dead analysis, a larger number of cells were analysed for 5, 10 and 15 minutes, as indicated in table 6, below. These cells were not synchronised into the same cell cycle stage, so there was some variation in time of cell death as is to be expected when analysing biological systems as not all cells react in the exact same way every time. For 100 %/21 mW laser power 6 cells were analysed for 15 minutes, 18 for 10 minutes and 38 for 5 minutes. The larger number of cells for 5 minutes analysis was to try and determine if the cells were more viable than not at 5 minutes, as the variation between cells made it difficult to ensure majority cell viability during analysis.

The images in figure 42 are typically what became to be expected if the cell to cell variation was diminished, that at 15 mins 100 and 50 %/12.5 mW laser power the cell would be dead. At 10 minutes 100 and 50 %/12.5 mW laser power the cell would be dying – the green intensity of the Calcein AM would lessen due to cell membrane integrity failing and often the red staining of the EtHD-I can be seen starting to be up taken, or the cell is fully red and no Calcein AM is visible. For 15 minutes irradiation, less cells were analysed via staining than was ideal, as these cells tended to lose adherence to the surface in the staining steps and therefore were gone during fluorescent image acquisition. However, the loss of cell membrane integrity can be related to cell death due to the changes in cell morphology experienced as a cell undergoes both apoptosis and necrosis.

Table 6: Number of cells analysed with Live/Dead™ stain for specific laser powers at 5, 10 and 15 mins with % live and dead

Laser Power	Time frame	Live (%)	Dead (%)	Gone (%)	Number Analysed
100 %/ 21 mW	15 min	14	71	14	7
100 %/ 21 mW	10 min	22	72	6	18
100 %/ 21 mW	5 min	53	32	13	38
50 %/ 12.5 mW	15 min	-	50	50	4
50 %/ 12.5 mW	10 min	60	33	8	12
50 %/ 12.5 mW	5 min	50	33	17	12

From the number of cells analysed with 100 %/21 mW laser power, the cell variation does have an impact on the viability at five minutes irradiation, however, the majority of cells analysed, were just viable, and as expected cells at 10 and 15 minutes were non-viable, resulting in the time of death ultimately lying between possibly 5 and 10 minutes irradiation, but more consistently between no irradiation and 10 minutes 100 %/21 mW laser power.

A similar pattern was observed in the 50 %/12.5 mW irradiated cells, in that at 5 minutes irradiation variation in cell viability was found, however a larger percentage of cells were also still viable at 10 minutes in comparison to the

100 %/21 mW irradiated cells. This leads to the hypothesis that cell death induced by 50 %/12.5 mW laser power occurs between 5- and 15-minutes irradiation. Whilst not an exact cell death at a set time, it has determined the workable time frame for Raman induced cell death for further investigation.

3.6 CHAPTER CONCLUSIONS

The experiments in this chapter allowed for biological sample characterisation of cell viability of a live and dead cell sample population of MG-63 cells utilising the trypan blue exclusion assay. This evaluation of cell death was accompanied by an investigation of spectral differences in the two populations. The spectral differences were related in large part to DNA contribution within the cells, however changes in protein conformation or concentration could also be alluded to.

Furthering this experiment, utilising the Live/Dead™ doubly stained fluorescent viability assay in conjunction with further trypan blue experiments, a narrower window for time of cell death induced by 532 nm laser exposure during Raman spectroscopy was elucidated of being between 0 and 10 minutes for 100 %/21 mW 532 nm at ~ 22 mW laser power, and between 0 and 15 minutes for 50 % 532 nm at ~ 12.5 mW. This could have implications for Raman studies of live cells, particularly Raman mapping experiments that require prolonged exposure to complete a point by point map. However, it should be noted that in mapping experiments care is often taken to use short acquisitions per pixel to minimise any damage that could be caused by the laser exposure over the time of a map acquisition.

To investigate the time of death more exactly, further experiments are required, as will be discussed in the following chapter.

4 REACTIVE OXYGEN SPECIES AND CELL STRESS DURING RAMAN SPECTROSCOPY

4.1 INTRODUCTION

4.1.1 Reactive Oxygen Species (ROS)

To maintain cellular function, cells must remain in homeostasis through the regulation of all processes within the cell. This includes regulation of pathways that could cause damage to cells if they become overbalanced, which can be the case for reactive oxygen species in aerobic lifeforms. Cells maintain a balanced 'redox window' to regulate the intake of oxygen with the reduction of excess oxygen species that could potentially cause damage.¹⁸¹ Oxygen is a necessity for all cells in the body, however the process by which oxygen is used to generate energy can cause Reactive Oxygen Species by-products through electron leakage in the electron transfer chain in the production of ATP, or during metal catalysed oxidation reactions.¹⁸² Oxygen is prone to radical formation due to lone electrons in the outer shells being attractive for electron addition, thereby reducing the oxygen and creating reactive oxygen species, which are short-lived and highly reactive species which can cause alterations and potentially damage all macromolecules within the cell.¹⁸³

There are more than one type of reactive oxygen species; superoxide, peroxide, singlet oxygen radical to name a few, and during homeostasis ROS production tends to be regulated by SOD - superoxide dismutase - that catalyses the change of the radical forms to hydrogen peroxide, which is still a reactive species, but less than that of the superoxide radical.¹⁸⁴ The hydrogen peroxide is then reduced to water and oxygen by glutathione peroxidase, which is returned to the redox cycle by reaction with NADPH to form the reduced form - glutathione reductase.¹⁸⁴ This in turn allows the cell to continue removing any unwanted ROS to maintain homeostasis. During exposure to certain stimuli such as toxins, pharmaceutical products or irradiation, the cellular homeostasis is disturbed and becomes stressed with prolonged

exposure to such stimuli able to cause irreversible damage due to the mis-regulation of ROS.¹⁹

ROS are known to aid in cell death, with evidence to support that ROS can cause death via apoptosis, or necrosis depending on the level of ROS, length of exposure to ROS and level of ATP available to the cell.¹⁸⁵ In apoptosis, ROS such as hydrogen peroxide have been shown to aid in the release of cytochrome c from mitochondria to the cytoplasm initiating the caspase cascade required for apoptosis. Alternatively, ROS species have been shown to inactivate caspases, therefore altering the cell death pathway from apoptosis to necrosis. A drop in ATP and mitochondrial energy during cell stress can also cause the cell death pathway to switch from apoptosis to necrosis.¹⁸²

The mis-regulation of ROS has been linked, clinically, to diseases such as cancer, atherosclerosis, and neurodegenerative diseases such as ALS among others as it can interrupt regular cell production or apoptosis of unhealthy cells allowing prolonged proliferation of cells disposed to disease.^{186–188}

4.1.2 ROS Detection

ROS are well known for cellular damage during irradiation with UV irradiation as the high energy photons help induce radical formation which in turn can produce more ROS through the deprotonation of lipids, or the destruction of DNA through the alteration of DNA-base pair interactions.¹⁸⁹ The electron cycling mechanism coupled with the high reactivity of ROS allow for a cascade of radical formation and consequent cellular alteration when allowed to remain unchecked by cellular antioxidant processes.¹⁸² Oxygen deprivation or high oxygen environments have also been shown to cause increased ROS production as the cell tries to overcompensate for the lack or excess of oxygen, which can then be hard to stop resulting in decreased cell viability. ROS species can alter DNA, as well as cause cross-linkages between proteins and lipids that would otherwise not occur altering both the physical and chemical characteristics of the cell.¹⁸⁵

ROS action within both cells and tissue, specifically skin, have been investigated in the literature. The effects of ROS on DNA damage has been assessed by Panikkanvalappil et al, utilising platinum nanocage structures to act as potential antioxidants to reduce ROS induced DNA cleavage on irradiation with UV light in comparison to the DNA damage seen in SERS spectra generated by silver nanoparticles.¹⁹⁰ Shen et al, also utilised SERS in the evaluation of ROS production, however, in this study the Ag nanoparticles were modified with mitochondria-targeting peptides to detect ROS localisation within the cell structure.¹⁹¹ SERS was used to image ROS production in an interference free manner during photothermal therapy, as ROS production has been shown to inhibit cancer growth during such treatments. Chen et al employed oxygen deprivation of human umbilical cord mesenchymal stem cells (hUC-MSCs) to evaluate cells at different levels of viability and their resulting ROS production as viability decreases with time.¹⁹² Through this research the group were able to model the fluorescent ROS production as a function of viability with the change in specific peaks of the Raman spectra of the cells correlated with viability. Heo et al employed resonance Raman in combination with a reporter molecule that enhanced its Raman signal on the production of ROS as a molecular sensor for the tracking of ROS production.¹⁹³

ROS action within skin has also been monitored using Raman spectroscopy, due to skin being exposed to UV radiation from the sun, the production of ROS on the skin can interfere with sunscreens and other topically applied pharmaceuticals. Morita et al investigated hydrogen peroxide induced ROS production on three different skin models to determine whether the differences in ROS production and Raman spectra could determine between the different skin model types.¹⁹⁴ Hanson et al performed studies with non-linear microscopy to evaluate UV-induced ROS production in sunscreens absorbed into the skin.^{195,196} Liebel et al evaluated the ROS production in skin equivalents during irradiation with visible light and the effect of sunscreen on ROS reduction using fluorescence and chemiluminescence methods.¹⁹⁷

The relationship between ROS and oxidative stress coupled with over production of ROS leading to cell death has been shown to affect cell viability, particularly during exposure to UV light, photosensitising therapeutics, or oxygen deprivation.¹⁸⁹ The evaluation of ROS production during Raman analysis has mainly been evaluated as a cause of an external factor in the analysis, for example deliberate addition of hydrogen peroxide to a sample to see an uptake in ROS.¹⁸³ As far as can be determined, the potential production of ROS due to the process of Raman analysis of live cells has not been evaluated. ROS production can be used as an indicator of cell stress, and therefore if it can be related to Raman peaks of interest this could help guide experimental design through the knowledge of safe cell analysis laser power during Raman spectroscopy.

4.2 PROJECT AND CHAPTER AIMS

To work towards developing a framework for levels of safe laser exposure of live cells during Raman spectroscopy analysis, and to understand the main chemical changes induced by photodamage of a biological sample, often other biological techniques are required to confirm the information deduced with Raman spectroscopy.

Due to the link between cell death and ROS, and the previous chapter's determination of timeframe for cell death by laser irradiation, the aim of this chapter is to interrogate if the prolonged exposure of live cells to laser irradiation will generate ROS as an indicator of cell stress and eventual death, and can the resulting cell stress be related to changes within the cell spectrum over time.

Using a combination of Raman spectroscopy, ROS detection fluorescently with DHR-123 by two photon fluorescence and then single photon fluorescence of Live/Dead™ stains from chapter 3, the aim is to determine if there is a detectable link between the cell spectral changes, cell stress and death. The DHR can also help in the evaluation of mitochondrial activity alluding to cell induced damage.

Finally, the last aim was to attempt to image spectral changes within MG-63 cells using SRS to determine if photodamage could be tracked in real time.

4.3 RESULTS AND DISCUSSION

4.3.1 ROS Controls

Reactive oxygen species can be detected with several fluorescent probes such as dichlorodihydrofluorescein diacetate (DCFH-DA), due to their sensitivity and ease of imaging and relative quantification of fluorescence signal. Choice of ROS indicator can be difficult and ideally should be dependent on the type of ROS of interest.¹⁹⁸ DCFH-DA and dihydrorhodamine-123 (DHR-123), an analogue of DCFH-DA, are both non-specific ROS indicators and can be oxidised to their fluorescent forms by more than one type of ROS.¹⁹⁸ DCFH-DA is most widely used for detecting H₂O₂ and as an indicator of cell stress. It undergoes oxidation within cells to its fluorescent counterpart dichlorofluorescein (DCF), which is uncharged and can therefore leak from cells overtime. DHR-123 is an uncharged, non-fluorescent, lipophilic molecule that can transpass the cell membrane. On interaction with reactive oxygen species DHR-123 is oxidised to rhodamine-123, a cationic species, with an imino group replacing one of the amino groups of the DHR-123, which causes it to remain within the cell. DHR-123 is primarily used as a non-specific indicator of peroxynitrite reactive oxygen species, however studies have shown that it can also be oxidised by hydrogen peroxide in combination with other intermediates and proteins, cytochrome c, hypochlorous acid and other ROS species.¹⁹⁸ Dihydrorhodamine however, can be localised within the mitochondria during interaction with ROS, similar to the mechanism of action of Calcein AM from section 3.5.2.¹⁹⁶ Therefore, in this analysis DHR-123 was utilised as a ROS indicator to determine if the results of the Live/Dead cell assay correlate with the ROS signal in cells analysed by Raman spectroscopy.

Due to the nonspecific action of ROS production in the use of DHR-123, it is imperative that controls to determine baseline levels of ROS are performed during each experiment in order to determine if a level of ROS is firstly higher than the background level, and if the level is increased due to the stimuli applied to the sample. For the experiments performed in this chapter, a 10 μ M concentration of DHR-123 in PBS was prepared and added to MG-63 cells

mounted on an Ibidi gridded glass bottom dish and incubated for 30 minutes, as outlined in section 6.18.1. The cells were then washed and mounted in PBS and analysed using two-photon fluorescence microscopy with 1031.2 nm Stokes beam frequency doubled to cause excitation at 515 nm in order to minimise photobleaching and any further sample damage during analysis to allow for Live/Dead™ staining post ROS analysis. The control sample did not undergo Raman analysis, only TPEF to determine the baseline level of ROS produced in MG-63 cells to allow for fair comparison with the Raman analysed cells.

As described in section 6.18.1, a second control sample was prepared using 10 µM DHR in PBS only within an Ibidi glass bottom dish and analysed using the same TPEF settings, this is due to media causing interference and increasing the production of fluorescence on interaction with UV radiation even without the presence of cells in a sample. It allowed for the determination of fluorescence within cells to be related directly to processes in the cells and not the media contributions.

Lastly, and similar to the controls performed for the Live/Dead™ analysis as outlined in section 6.16.2.1 and 6.17, a control sample of live cells mounted in PBS with no added DHR was evaluated to determine levels of cell autofluorescence from endogenous fluorescent species. Again, this was to help determine whether any increase in fluorescence between irradiated and TPEF samples was due to the stimuli introduced to the cell and not variations in background fluorescence.

The control samples are shown in figure 43, consisting of tile scans of the whole Ibidi dish surface, stitched together using the Leica LasX software and threshold set between 150 and 0, as outlined in section 6.10. The images show the two-photon fluorescence channel in green and the SRS channel in purple overlaid with the fluorescence signal. The SRS channel allowed for visualisation of the glass grids, to help ensure the correct cell is analysed in all analysis from Raman spectroscopy to TPEF and viability studies.

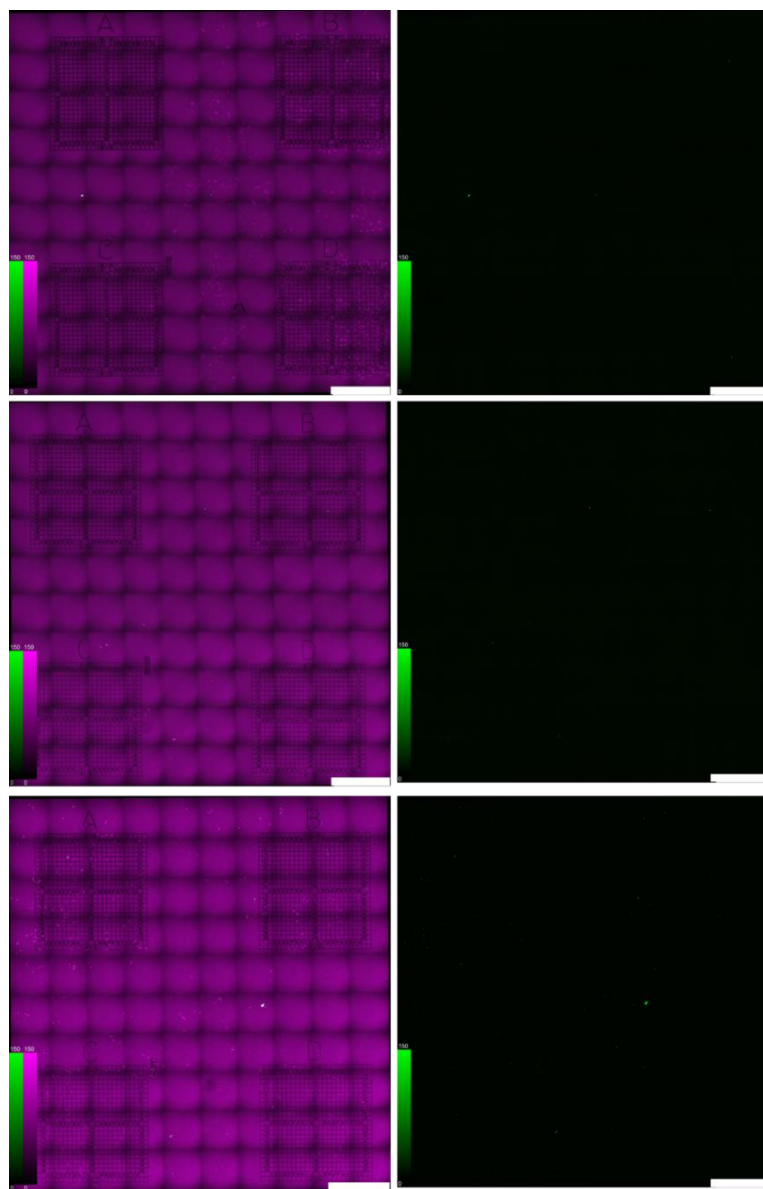


Figure 43: DHR-123 Control sample images prepared as outlined in section 6.18 and imaged and processed as outlined in section 6.9 and 6.11. Top set of images indicate MG-63 cells incubated with no DHR and analysed via TPEF microscopy to evaluate cell endogenous background fluorescence before analysis. The middle set of images indicates background fluorescence present in DHR-123 in PBS solution. The bottom set of images indicate MG-63 cells stained with 10 μM DHR. Individual tile images are 512 x 512 pixels corresponding to 460.55 x 460.55 μm with 10x10 tiles analysed and stitched together in the Leica LASX software. Scale bar is 500 μm . For the emission detection of two photon excitation fluorescence combined with SRS imaging, the TPEF images were generated in reflection mode using the pure Stokes beam at 1032 nm, resulting in 516 nm excitation of the oxidised DHR-123. The pump beam was then turned on to generate the SRS grid image in transmission mode, tuned to 797 nm for the 2850 cm^{-1} CH_2 region of interest to determine cell position.

The control images in figure 43 do display some level of inherent ROS signal in the bottom images, although this is low. This level of ROS production will therefore be used as the comparison for cells analysed by Raman spectroscopy to determine the relative fluorescence ratio between cells analysed by Raman and those not. The images allow for a semi-quantitative determination of ROS within the MG-63 cells.

4.3.2 Initial Spectral Variation Investigation

In the previous chapter, a combination of trypan blue exclusion assay and Live/Dead™ fluorometric assay allowed for the determination of cell death between 5 – 15 minutes bleaching with 100 %/21 mW laser power at 532 nm wavelength. For the 50 %/12.5 mW exposure, the results were more variable, however, cell death appeared certainly at 15 minutes exposure, and for 10 %/2.3 mW 532 nm laser exposure up to 15 minutes, no cell death was observed, indeed the initial experiment indicated no cell death at 40 minute exposure to 10 %/2.3 mW 532 nm excitation.

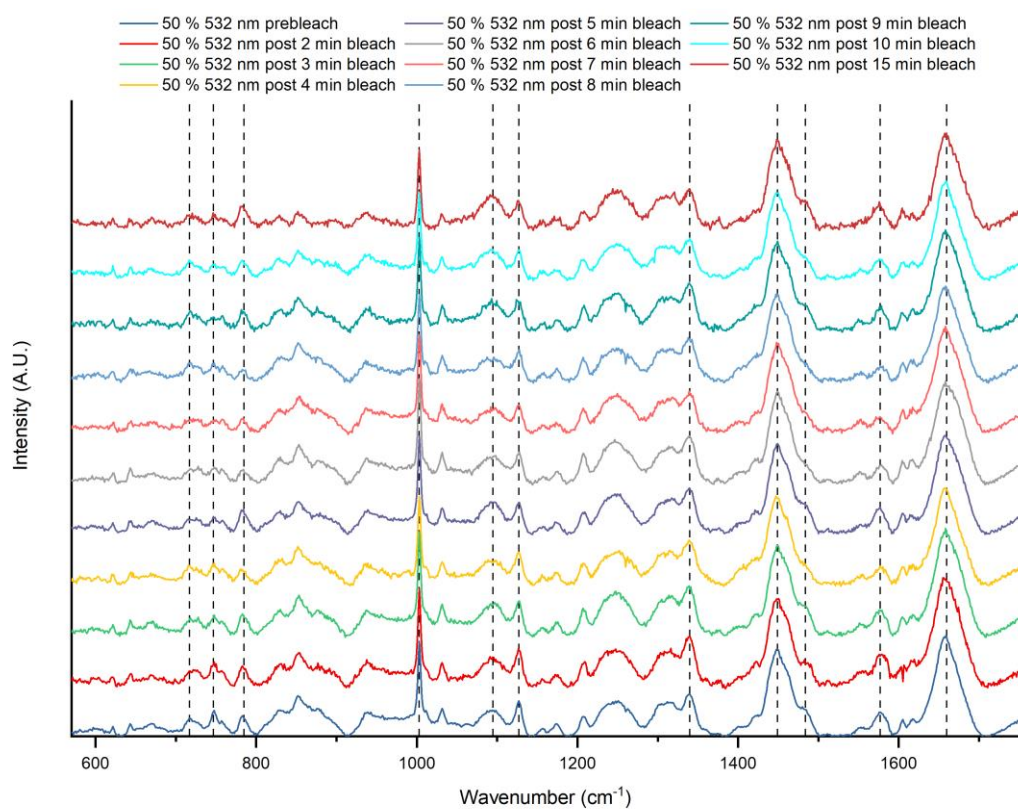
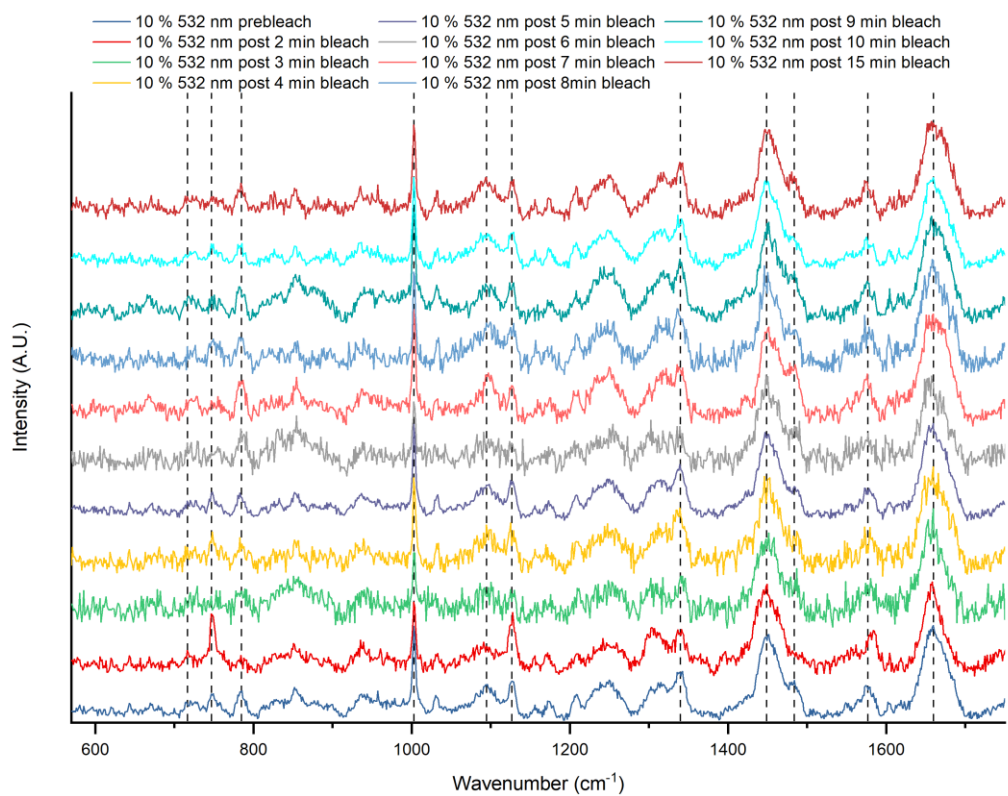
In order to continue this study into cell changes with varying laser power exposure over time, cell samples were prepared and analysed utilising 532 nm at 100 %/21 mW laser power for at least $n = 3$ and 50 %/12.5 mW laser power for at least $n = 2$ samples. Only $n=1$ was achieved for 10 %/2.3 mW 532 nm wavelength timepoints in this study, and a main aim of future work is to perform more replicate measurements, at all laser powers but also to deduce if the lower power causes any spectral changes similar to those seen with 100 %/21 mW and 50 %/12.5 mW 532 nm. Further replicates will allow for better confidence in results but also provide a fuller picture at as many different laser conditions for sample analysis as possible.

Therefore, Raman measurements were taken of cells mounted in PBS on Ibidi glass bottom dishes, prepared as outlined in section 6.14 and analysed as outlined in section 6.6 and 6.7, at 60 s exposure time to serve as a non-bleached cell and the cell spectra expected of live, viable cells at $t = 0$ min. The measurement time was increased in one-minute bleaching increments until 10 minutes, and then at 15 minutes. So one cell was analysed with 60 s Raman

exposure, the next would be analysed with 120 s Raman exposure, the next would have a Raman acquisition of 60 s after 120 s bleaching time to equal 3 minutes total Raman exposure, and so on. For 10 minutes, acquisitions were taken after two four-minute periods of continuous laser bleaching incident on the cell. For 15 minutes, three measurements were taken after an increment of 6.5 minutes of bleaching time.

The spectra were then separated if multiple spectra were taken during analysis and separated by timepoint. The spectra were baseline corrected to the peak area under the CH₂ peak at 1449 cm⁻¹, and baseline correction was performed using ALS baseline function in Origin 2020 software as outlined in section 6.7.¹⁶⁹

The images in figure 44, indicate the offset average normalised spectra for 10 %/2.3 mW, 50 %/12.5 mW and 100 %/21 mW. Due to the number of spectra in the images, it is difficult to have all spectra in a single page figure and still be able to elucidate detail, therefore the images are spread over 1.5 pages for clarity.



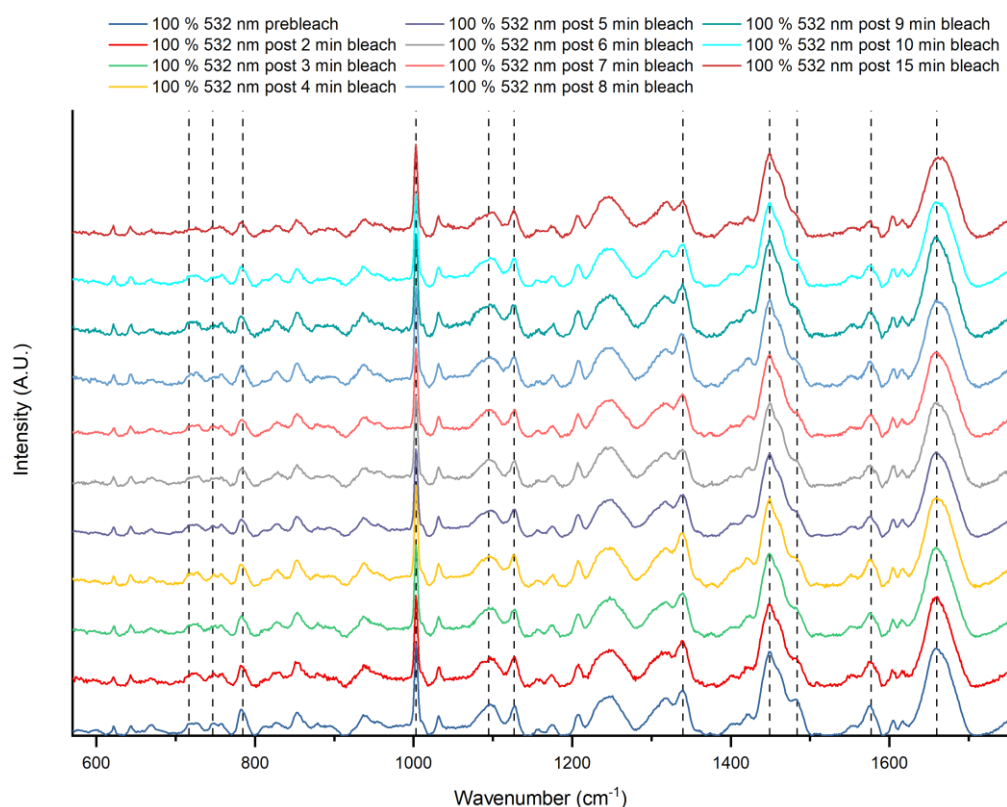


Figure 44: Average spectra of cells irradiated with between 1 minute 532 nm laser at varying 532 nm laser power until 15 minutes of 532 nm laser irradiation. Top image corresponds to spectra of cells irradiated with 10 %/3 mW 532 nm laser power between 1 and 15 minutes. The middle image corresponds to cells irradiated with 50 %/12 mW 532 nm laser power between 1- and 15-minutes irradiation time. The bottom image corresponds to spectra of cells irradiated with 100 %/21 mW 532 nm laser power between 1 and 15 minutes irradiation time. Peaks of interest are marked with a dotted line at 717 cm^{-1} , 747 cm^{-1} , 785 cm^{-1} , 1005 cm^{-1} , 1095 cm^{-1} , 1128 cm^{-1} , 1340 cm^{-1} , 1484 cm^{-1} , 1577 cm^{-1} , 1660 cm^{-1} and the normalisation peak at 1449 cm^{-1} .

As is evident from the spectra in figure 44, the 10 %/2.3 mW 532 nm laser power results in a lower S/N ratio in the spectra in comparison to the higher laser powers due to the lower power of laser being used for the same analysis timeframe resulting in a lowered number of scattered molecules from the samples. For all 10 %/2.3 mW laser power timepoints, $n=1$, so further replicates would be needed to determine whether the spectral variation shown in figure 44 are accurate or more due to noise variation between samples. Unfortunately, due to laboratory access restrictions imposed by the global

Covid-19 pandemic, further planned experimental replicates were unable to be performed. The 10 %/2.3 mW spectra are included here for completeness and to show that an attempt to evaluate this laser power between 1 and 15 minutes of irradiation was made, however, from this point onwards, the 10 %/2.3 mW cell data will be omitted.

Peaks of interest are indicated by the dashed lines in all images in figure 44 above, many of these peak changes were indicated in the previous chapter. These changes are still visible in the lower timeframe of analysis here. With peaks relating to DNA at 785 cm^{-1} , 1095 cm^{-1} , 1340 cm^{-1} , 1484 cm^{-1} and 1577 cm^{-1} .^{4,5,30–33,171} Notably the last three peaks mentioned here can be related to the DNA base guanine, which has been indicated by Puppels et al to be particularly sensitive to the process of cell damage via photosensitisers during Raman spectroscopy.⁴ This peak could therefore be an indicative peak of photodamage and potentially related to the production and subsequent damage in cells by oxidative stress. Other peaks of note are those relating to the phospholipid membrane at 717 cm^{-1} , phenylalanine at 747 cm^{-1} and 1005 cm^{-1} and other protein peaks at 1128 cm^{-1} and 1660 cm^{-1} correlating with the C-N stretch and amide I α -helix respectively.^{5,30,31,33,175}

The peak at 1660 cm^{-1} has been marked in all spectra as an average indication of peak position, as it is evident that this peak position shifts from around 1657 cm^{-1} to approximately 1662 cm^{-1} with prolonged laser exposure. This has been suggested to indicate increased disorder within the protein structure but is usually in conjunction with changes in the peak at 1231 cm^{-1} , which is not obvious visually in the spectra.

Further analysis has been performed to attempt to evaluate the amount of change seen in these peak shifts that will be discussed later, and the visual changes evident have already been evaluated in the previous chapter. However, it is clear to see that changes are evident on casual observation in all the spectra, although less obvious for the lower 10 %/2.3 mW laser power between 1 minute irradiation and 15 minutes laser irradiation, which is in line

with previous literature findings, with changes evident in cell spectra between 5 and 40 minutes.

4.3.3 Evaluation of Cell Viability on Increasing Laser Irradiation Time

All cells analysed via Raman spectroscopy were also then stained with DHR-123 and analysed for ROS production and mitochondrial activity, as outlined in section 6.18. The sample was consequently stained with the double fluorometric Live/Dead™ stain, as outlined in section 6.16.2. If the cells that had been analysed were viable, it would uptake calcein-AM only, and be stained green. However, if the irradiated cell was dead or dying, the calcein-AM signal would decrease in intensity and eventually dissipate out of the membrane compromised cell whilst the nuclei is stained red by EtHD-I. An example of cells stained after DHR-123 and Live/Dead™ analysis are indicated in figure 45 below.

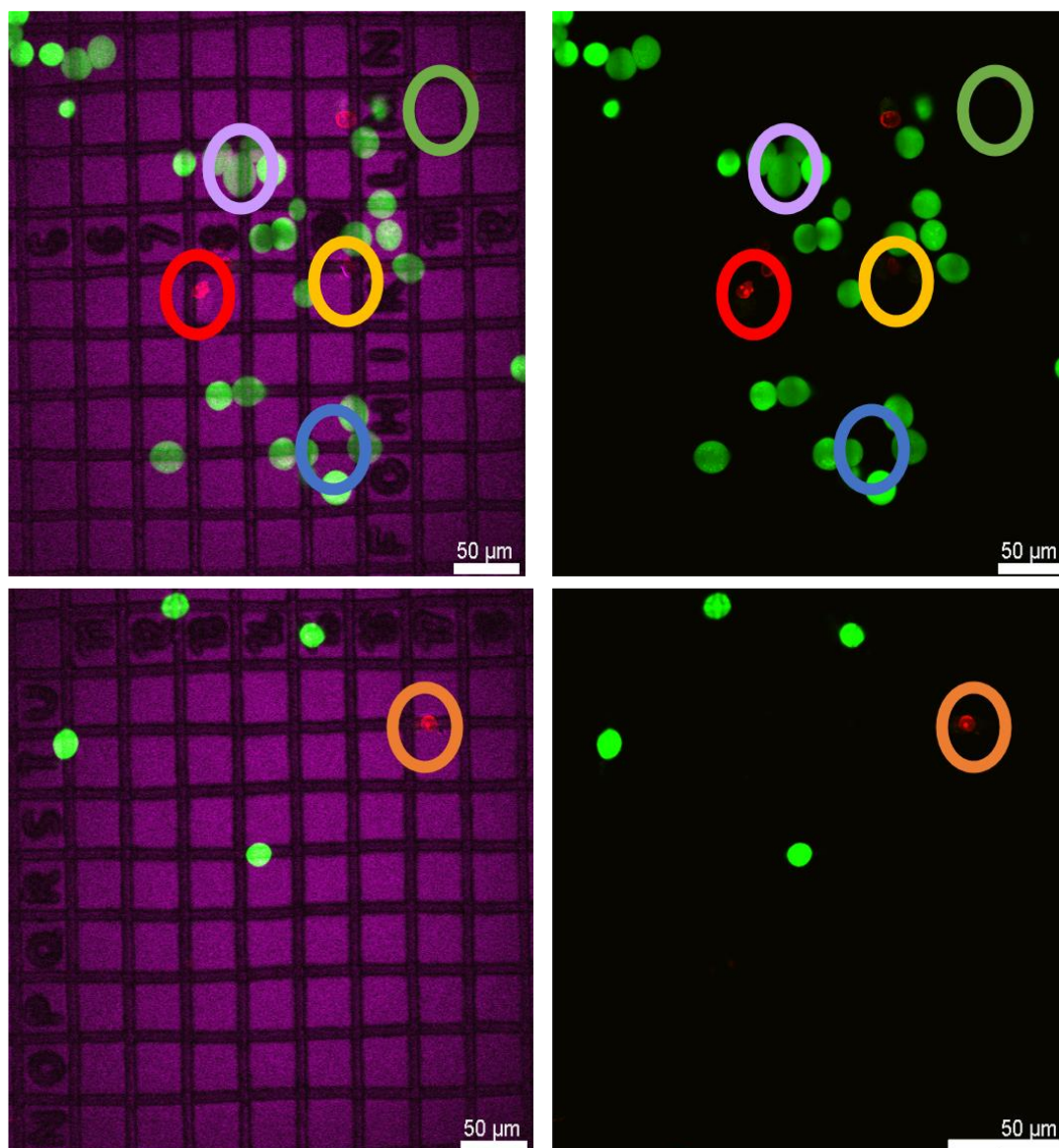


Figure 45: Images of cells post Raman irradiation between 5 and 10 minutes 100 %/21 mW 532 nm, post DHR staining and Live/Dead™ staining. The left-hand side images indicate the SRS and both fluorescence channels overlaid, left is the overlay of the fluorescence channels only. Red – 10 min, blue – 9 min, yellow – 8 min, green – 7 min, orange 6 min and purple – 5 min. Images obtained using confocal fluorescence with 2 channels in the first instance and then confirmatory forward-SRS to determine the grid positioning of the individual cells being analysed. Confocal fluorescence channel 1 was tuned to 515 – 525 nm and channel 2 tuned to 615 – 625 nm, with 30 % 488 nm excitation. SRS channel tuned to 2850 cm^{-1} at 20 % laser power which is equivalent to 20 % of 0.15 W (0.03 W) in the pump beam and 20 % of 0.3 W (0.06 W) in the Stokes beam. Images captured with Leica SP-8 CARS multiphoton microscope with SRS option using a 25x 0.95 NA water immersion objective and 0.55 NA air condenser, scale bar = 50 μm , LUT in all images set between 0 – 150

The fluorescence images of the Live/Dead™ stain were acquired with 488 nm laser excitation and the PMT detection channel set to 515 – 525 nm and 615 – 625 nm for live and dead respectively, as detailed in section 6.9. The images were generated via a tile scan, and then a corresponding SRS image was acquired without changing the focus position. The SRS channel was tuned to the CH₂ shift at 2850 cm⁻¹ and allowed for the elucidation of the glass grid in order to relate the irradiated cell and corresponding viability and to minimise confusion with non-irradiated cells.

In the example images in figure 45 above, cells were irradiated with 100 %/21 mW laser power from 10 minutes to 5 minutes and then stained for ROS, as per section 6.18 and then cell viability, outlined in section 6.16.2, separately. The DHR-123 signal dissipates from non-viable cells quickly, and the method of cell viability detection of Calcein AM corresponds to the mitochondrial activity within similar wavelength detection of emission channels as the DHR-123, this allowed for the combination of the two stains to be used consecutively. Figure 40 shows the cell irradiated for 10 minutes circled in red, is dead as indicated by the red nucleus, the 9-minute cell, blue, is gone, the 8-minute cell circled in yellow is dead, although the nuclear stain is faint. The 7-minute cell, green is gone and the 6-minute cell in orange is dead. The 5-minute cell in purple is, however, live.

The loss of irradiated cells on the addition of stains was a particular problem in this analysis. Poly-L-lysine was used during cell culture to help with cell adherence, however the stain steps required PBS washes to remove any excess not up taken by the cells, and for cells which are losing viability, cell morphology changes and this can often result in a loss of adherence on the sample surface. Ibidi dishes were also used to try and minimise this issue as they allowed for the Raman analysis of cells with the immersion objective and consecutive multiphoton fluorescence, SRS or single photon analysis in the inverted Leica microscope system with minimal cell perturbation and no changing of cell sample orientation to minimise losses of cell density as much as possible.

For this analysis, as mentioned previously in section 4.3.2, a lower number of cells were analysed than would have been preferable. The 100 %/21 mW timepoints were n=3 and the 50 %/12.5 mW timepoints were n=2. The percentage of viable cells from these two populations were plotted in figure 46 to indicate a timepoint, if any, at which cell death occurred.

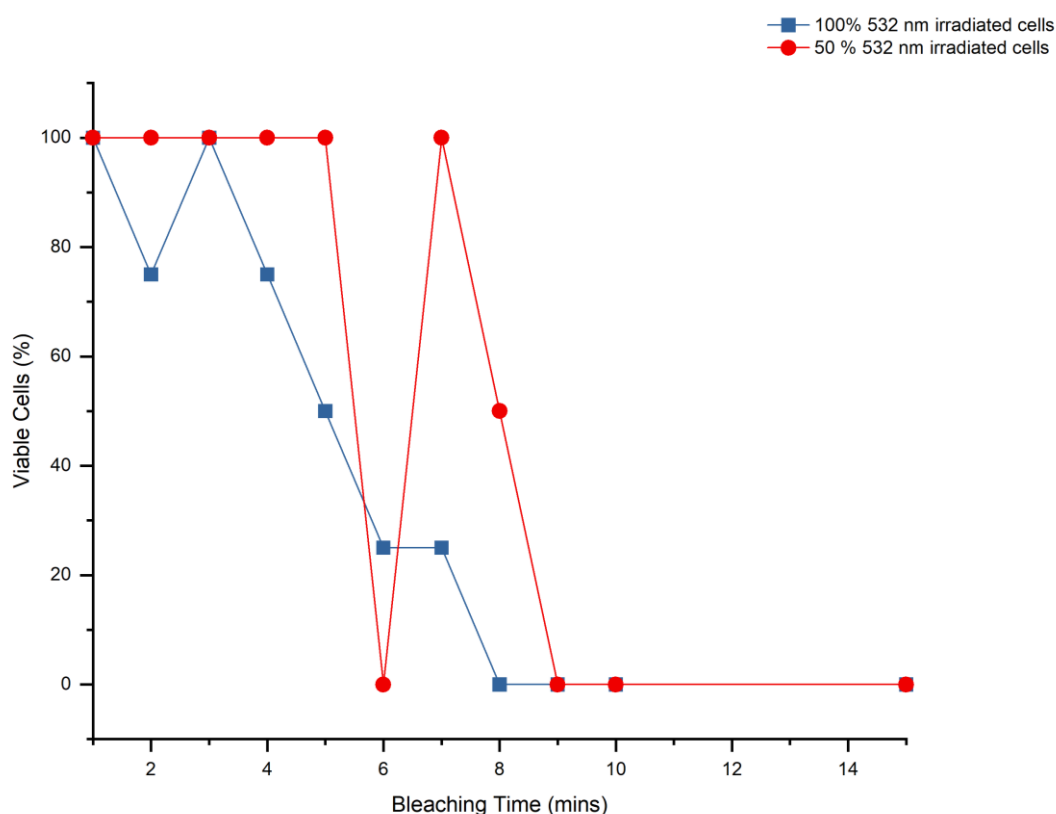


Figure 46: percentage of cell viability from the two populations of cells, 100 %/12 mW or 50 %/12 mW 532 nm irradiated cells, with increasing time from 0 minutes until 15 minutes total irradiation. $n = 3$ for 100 %/21 mW cells and $n = 2$ for 50 % cells

For a higher degree of confidence in the cell viability results in figure 46, further replicates would be needed as loss of analysed cells during the staining steps resulted in a lower reported number for confirmed cell death at some time points. For example, the 6-minute time point 50% cell, two cells were analysed at 6 minutes and one was stained dead, however the other was gone, so for percentage of viable cells it is 0 %, this however, may not be an entirely accurate depiction. This was the case for the 4 minute timepoint in 100 %/21

mW laser power, as 3 cells were analysed, one cell was dead and the other two were gone on Live/Dead™ staining and the two minute timepoint where 3 cells were analysed with two being viable but one being gone. As mentioned in section 3.1.2, the loss of the cell could indicate that it was non-viable and therefore the loss of surface adherence is due to this. However, also as mentioned previously in section 3.5.2.2, these cells were not synchronised, and it could be that the cells lost were already dead due to the natural variation within cell culture thus further replicates would be needed for confirmation.

Therefore, it can be postulated that from 4 minutes onwards, loss in cell viability could be due to the exposure of 100 %/21 mW 532 nm laser, which is related to 21.1 mW laser power incident on the sample, which corresponds to 2.31 MW cm^{-2} laser power density, and that from 8 minutes onwards, loss of cell viability could be due to the exposure of cells due to 50 %/12.5 mW 532 nm laser radiation, related to 12.5 mW of laser power at the sample, relating to laser power density of 1.37 MW cm^{-2} . Essentially, cells will still be viable at approximately double the exposure time for approximately half the laser irradiation and after this point, more loss in cell viability will be seen due to laser irradiation. These results cannot be taken as statistically significant, and although look promising, if it had been possible these experiments would have been repeated for at least $n=5$ but ideally $n=10$ or more for confirmation of this hypothesis with statistical value.

The point of evaluating the cell death time here was to use the information of time of cell death to see if significant spectral changes occur when this cell death occurs, or indeed before this point of cell death evaluated by the Live/Dead™ assay.

4.3.4 Cell Spectral Changes

A closer investigation of the spectral changes that were visible on inspection of the normalised timepoint spectra was performed. Again, this was performed as outlined in section 6.7, using the curve fitting function in Wire 5.1 software with a standard template of preloaded curves after the data was normalised and baseline corrected. The peak ratio changes are indicated in figure 47.

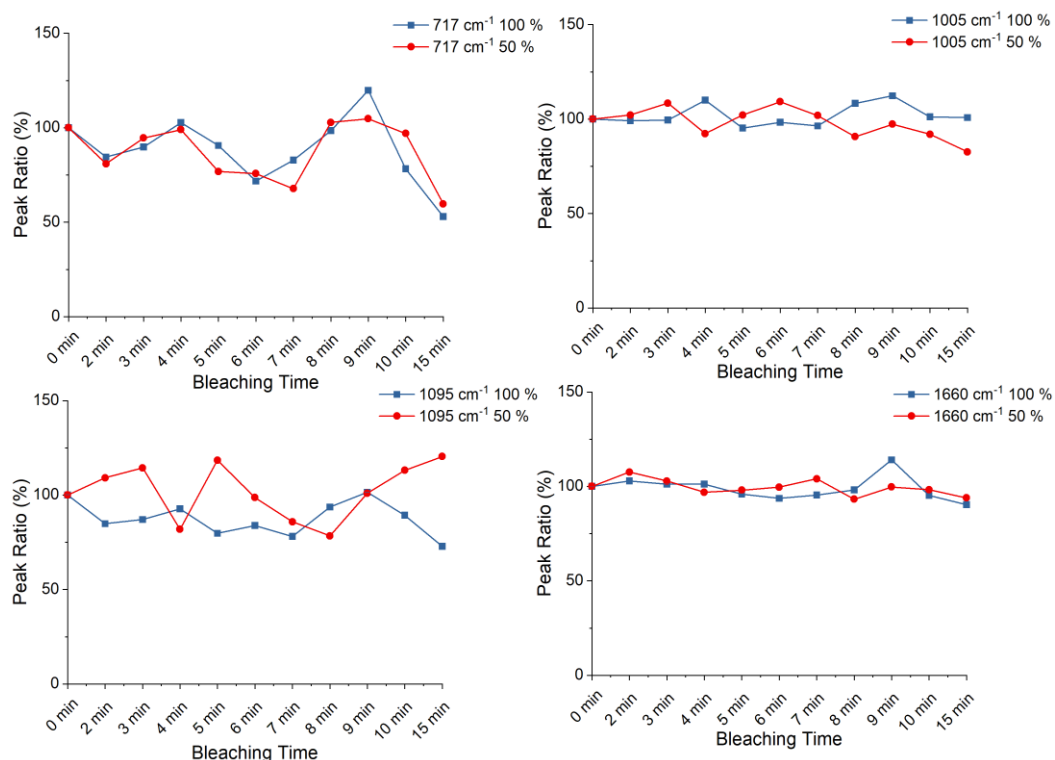


Figure 47: Relative peak ratio changes of interest for peaks relating to DNA (1095 cm⁻¹) proteins (1005 cm⁻¹, 1660 cm⁻¹) and lipids (717 cm⁻¹) in Raman spectrum of cells irradiated in 1-minute increments until 10 minutes and then 15 minutes total irradiation time. Cells irradiated with 100 %/21 mW peak ratio changes are indicated in blue or 50 %/12.5 mW peak ratio changes are indicated in red. Spectra were acquired for 60 s from cells from 0-minutes in one-minute increasing increments on different cells until 15-minutes for 100 %/21 mW and 50 %/12.5 mW 532 nm. Peak ratio changes are expressed as a ratio in comparison to the peak area at t= 0 mins which is taken to be equivalent to 100 % as this is before any chemical changes occur due to the ongoing laser irradiation. Each proceeding peak area value from increasing irradiation time have therefore been divided by the peak area of the peak of interest at t= 0 mins in order to determine if the signal of interest decreases or increases with increasing laser irradiation in comparison to the initial spectrum.

The initial investigation of peak area ratio changes was of those identified in Chapter 3 as being visually identifiable in the 40-minute analysis of cells with 532 nm laser wavelength. 717 cm⁻¹ in relation to phosphatidylcholine in phospholipids, in the analysis of cells for 40 minutes showed significant relative peak decrease which could be alluded to the loss of cell membrane as indicated by the morphological changes visible in the visible light images during Raman analysis. The profile of changes in peak ratio for 717 cm⁻¹ are

dissimilar to the peak changes evident in the 40-minute irradiated samples. There is still an overall decrease in peak ratio indicating alteration in the phospholipid concentration in the cell with increasing laser exposure.³³ The differences could be due to the smaller time increment of the measurements or could be due to changes in focus due to cell movement. There appears to be a slight recovery after initial irradiation at one minute, then again after six minutes before a final decrease in signal after 9-minutes. Decreases in phospholipids have also been related to the production of ROS through lipid peroxidation.¹⁸⁹

The phenylalanine ring breathing peak at 1005 cm^{-1} follows a similar profile to the previous chapter spectra, with slight variation in intensity in the 100 %/21 mW irradiated cell but no significant increase or decrease indicative of either apoptosis or necrosis in previous studies.^{5,29,174} However, the 50 %/12.5 mW cell shows an 18 % decrease overall. This change is minor however in comparison to the changes indicated in previous studies which indicates losses in protein peaks such as phenylalanine can be due to the apoptosis death pathway.

The DNA peak at 1095 cm^{-1} shows an almost 30 % decrease in the peak relating to the phosphodioxy group which is consistent with apoptotic DNA degradation.^{28,30} However, the 50 %/12.5 mW irradiated cells show an increase in relative peak ratio between 8 and 15 minutes, increases in DNA have been shown to be consistent with mitotic cells and is less consistent with cell death pathways.³⁸ It could be hypothesised that cells could be mitotic, however the profiles above are indicative of an average cell relative peak profile change with time. The number of replicates is lower than ideal, but for all cells analysed in 2-3 different samples to be mitotic seems unlikely.

A similar profile was also found for the 1660 cm^{-1} peak in comparison to the previous experiments. A shift was observed in peak position, but not in relative peak ratio, so the differences are minimal for both the 100 %/21 mW and 50 %/12.5 mW irradiated cell profiles. It has been hypothesised that the peak shift is related to the increased disorder of proteins during apoptosis mediated

caspase cleavage in correlation with an increase in lipid C=C so that overall peak profile doesn't change.^{29,30}

Although the overall relative peak profiles of the peaks above change, on performance of a one-way ANOVA test with $p < 0.05$, the changes in relative peak ratio were not found to be significant for either the 100 %/21 mW or 50 %/12.5 mW irradiated cells with increasing laser exposure. Except for the known effect of ROS on phospholipid concentration, the above peaks have not been found to be significantly related to ROS production, but more associated with cell death either by necrosis or apoptosis generally.

However, studies in the literature show that peaks relating to the redox cycle of cytochrome c can be related to ROS due to the production of ROS in mitochondria through the electron transport chain generation of ATP.^{58,199,200} Detection of these peaks by Raman spectroscopy has been probed in SERS and confocal Raman.^{200–202} Cytochrome c has a resonant absorption between 520 and 530 nm, which results in changes in cytochrome c mainly being detected in the reduced form.²⁰² Peaks for the reduced form of cytochrome c are found at 750 cm^{-1} , which relates to the pyrrole breathing mode, in combination with peaks at 1128 cm^{-1} , 1310 cm^{-1} and 1585 cm^{-1} .²⁰⁰ During this analysis, there were visible differences in the 750 and 1128 cm^{-1} peaks, however, there was overlap with the peak at 1577 cm^{-1} and 1585 cm^{-1} which made detection of changes at 1585 cm^{-1} difficult.

Figure 48 indicates the relative peak ratio changes for the peaks relating to cytochrome c at 750 cm^{-1} and 1128 cm^{-1} as well as peaks for DNA at 785 cm^{-1} , 1340 cm^{-1} , 1484 cm^{-1} and 1577 cm^{-1} .^{4,5,30,33,174} The DNA peaks mentioned relate to U, C and T at 785 cm^{-1} and mainly adenine and guanine for the latter peaks mentioned. All the relative peak ratios below were found to be statistically significant at $p < 0.05$ level between 1 and 15 minutes.

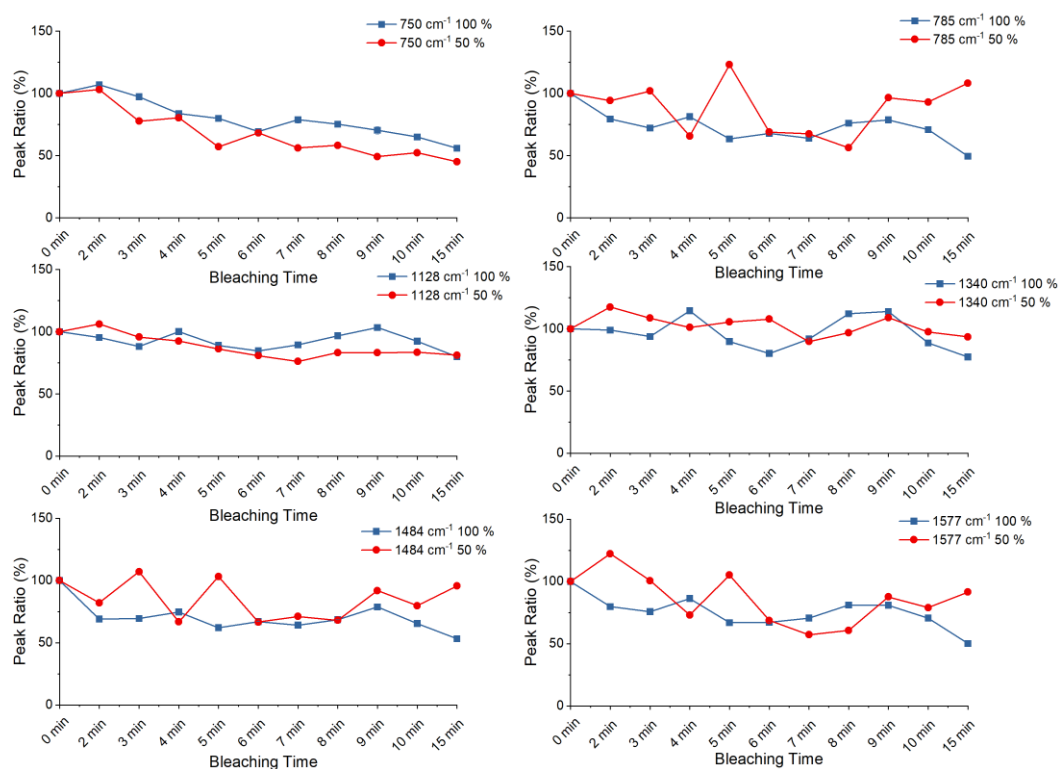


Figure 48: Relative peak ratio changes of interest for peaks relating to cytochrome c (750 cm^{-1} , 1128 cm^{-1}) and DNA (785 cm^{-1} , 1340 cm^{-1} , 1484 cm^{-1} and 1577 cm^{-1}) in Raman spectra of cells irradiated in 1-minute increments until 10 minutes and then 15 minutes total irradiation time. Cells irradiated with 100 %/21 mW peak ratio changes are indicated in blue or 50 %/12 mW peak ratio changes are indicated in red. Spectra were acquired for 60 s from cells from 0-minutes in one-minute increasing increments on different cells until 15-minutes for 100 %/21 mW and 50 % 532 nm. Peak ratio changes are expressed as a ratio in comparison to the peak area at $t=0$ mins which is taken to be equivalent to 100 %/21 mW as this is before any chemical changes occur due to the ongoing laser irradiation. Each proceeding peak area value from increasing irradiation time have therefore been divided by the peak area of the peak of interest at $t=0$ mins in order to determine if the signal of interest decreases or increases with increasing laser irradiation in comparison to the initial spectrum.

The statistical significance in change of these peaks is consistent with established changes in peaks within cells due to ROS formation and subsequent damage. Cytochrome c is released on the lipid peroxidation of its binding cardiolipin and is able to migrate from the inner mitochondrial membrane into the cytoplasm where it interacts with apoptotic protease activating factor-1 (APAF1) and initiates the caspase cascade of apoptosis.²⁰³ Therefore a reduction in the cytochrome c peaks at 750 cm^{-1} and 1128 cm^{-1} are consistent with a reduction in the amount of reduced form of cytochrome c, which can be related to the production of ROS in cells as laser irradiation at 532 nm increases at 100 %/21 mW and 50 %/12.5 mW power. Although the reduction in the 1128 cm^{-1} peak is not as large as the reduction of the 750 cm^{-1} peak.

Corresponding to these changes in cytochrome c changes are the differences in peaks relating to the DNA base guanine; 1340 cm^{-1} , 1484 cm^{-1} and 1577 cm^{-1} . Guanine has a low redox potential, in turn this makes it more sensitive to oxidation by ROS, particularly by singlet oxygen.⁴ In the case of the peak at 1340 cm^{-1} this can also be related to a CH deformation in proteins and lipids as well. The relative peak changes seen for 785 cm^{-1} and 1577 cm^{-1} are related to opposite DNA bases: U, C, T and A, G respectively.^{4,5,30,33,174}

The 50 %/12.5 mW laser irradiated cells only showed significant differences in cytochrome c peaks at 750 cm^{-1} and 1128 cm^{-1} , whereas the 100 %/21 mW cells did not show statistically significant differences for the cytochrome c peaks but did for the peaks relating to DNA. This could be postulated to be related to the stage of cell death caused with the differing laser powers. The 100 %/21 mW laser power is shown in the viability studies to reduce cell viability within approximately half the time of that of the 50 %/12.5 mW irradiated cells, this could be due to the higher irradiance energy on the cells causing faster initiation of the apoptotic pathway and consequential DNA degradation. Whereas, the release of cytochrome c was found to initiate the apoptosis pathway, so for the 50 %/12.5 mW irradiated cells, apoptosis has started within the timeframe of these experiments and there are still reductions

in the peak changes relating to DNA in the 50 %/12.5 mW irradiated cells, these were just not found to be statistically significant.

The relative profile of change, especially for the 100 %/21 mW 532 nm laser irradiated cells, consistent with the statistical significance of these changes are very similar, which increases the confidence in the method of action of cell damage being related to ROS induced DNA cleavage occurring with increasing laser exposure time.

Therefore, there are indicative spectral peak changes that can allude to ROS production, initiation of apoptosis and subsequent cell damage and death. However, confirmation is required to determine if an increased amount of ROS can be detected in the cells that were analysed by Raman spectroscopy with 100 %/21 mW and 50 %/12.5 mW 532 nm laser wavelength to confirm these findings. DHR-123 ROS Detection

4.3.5 DHR-123 ROS Detection

Post Raman analysis, the cells that were analysed were incubated with 10 μ M DHR-123 in prewarmed PBS, for 25 minutes followed by a washing step and then analysis by two photon excitation fluorescence utilising the Leica SP8 with SRS option microscope equipped with 1032 nm picosecond pulsed laser modulated at 20 MHz, as outlined in sections 6.17 and 6.9. The TPEF images were taken as tile scan images of the whole grid that the cell of interest was on using the pure 1032 nm beam frequency modulated to 516 nm for rhodamine-123 excitation and the detection range set to 525 – 535 nm with 20 % laser power equal to approximately 6 mW laser incident on the sample. Following the TPEF image, an SRS image tuned to 2850 cm^{-1} in the same plane of focus, was taken to generate a grid image and ensure the correct cell was identified for relative fluorescence quantification in comparison to the fluorescence signal of the non-irradiated cells in the sample.

The non-irradiated cell average in the sample average was taken and used to qualify relative fluorescence against. This was judged to be the most fair way of calculating relative fluorescence as all the cells in the sample were treated in the same way without Raman analysis, so the main difference in

fluorescence intensity should therefore be related to the change in rhodmine-123 signal from ROS activity.

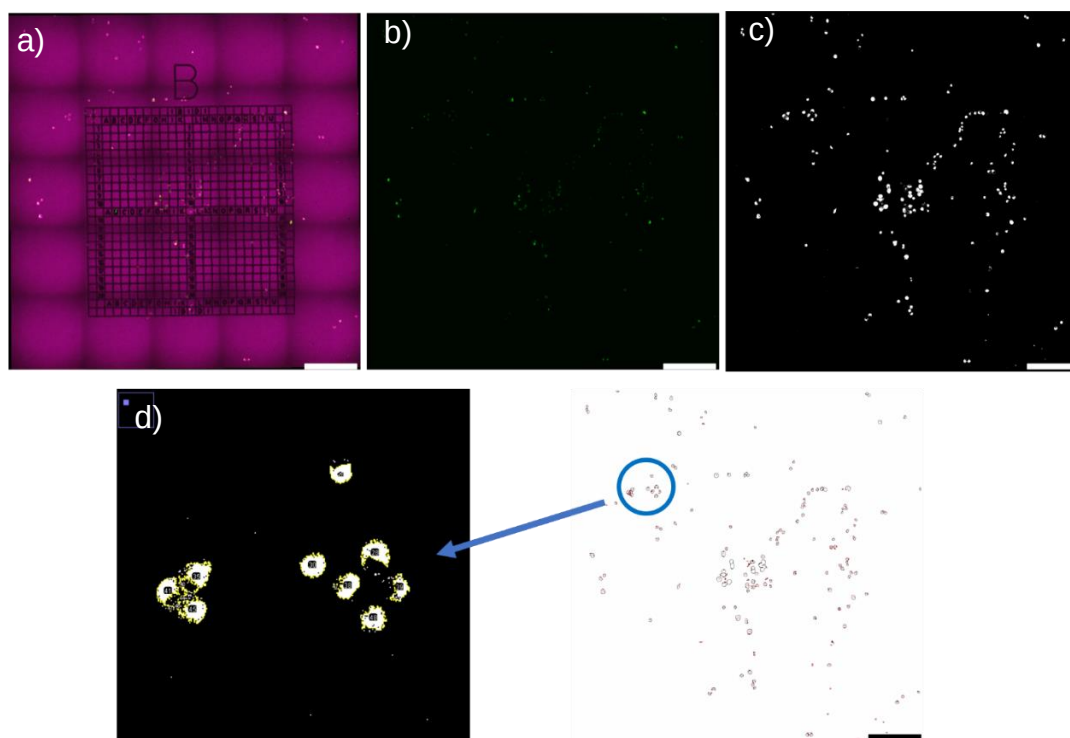


Figure 49: An example of the segmentation process in ImageJ to determine cell fluorescence for irradiated and non-irradiated cells. a) indicates the SRS image channel tuned to 2850 cm^{-1} , overlaid with the DHR-123 TPEF channel detected at 516 nm . b) indicates the DHR only TPEF channel c) is the 16-bit binary image that has been thresholded between 5 and the maximum. d) is the segmented image for all cells in the sample outlined, a zoomed in portion is indicated with the arrow to show the numbering of the segmented cells to allow for identification of irradiated vs non-irradiated cells. For the emission detection of two photon excitation fluorescence combined with SRS imaging, the TPEF images were generated in reflection mode using the pure Stokes beam at 1032 nm , resulting in 516 nm excitation of the oxidised DHR-123. The pump beam was then turned on to generate the SRS grid image in the forward direction in transmission mode, tuned to 797 nm for the $2850\text{ cm}^{-1}\text{ CH}_2$ region of interest to determine cell position.

In figure 49, the frames show the SRS channel with the grid for reference overlaid with the TPEF image to correctly determine which cells were analysed. For the processing of these images, the images were transferred into ImageJ, transformed into a 16 bit binary image, threshold set between 5 and maximum, and then the analyse particle function was used with a size of $5\text{ }\mu\text{m}^2$ to infinity in order to still capture cells which had lost cell membrane

integrity or had only small levels of ROS output due to cell degradation, as outlined in section 6.11.

Figure 50, below indicates the decrease in rhodamine-123 fluorescence signal with increasing exposure time which can relate to a decrease in ROS activity with time, however, in this case it is more likely that the decrease in signal is due to loss of mitochondrial activity on ROS generation.

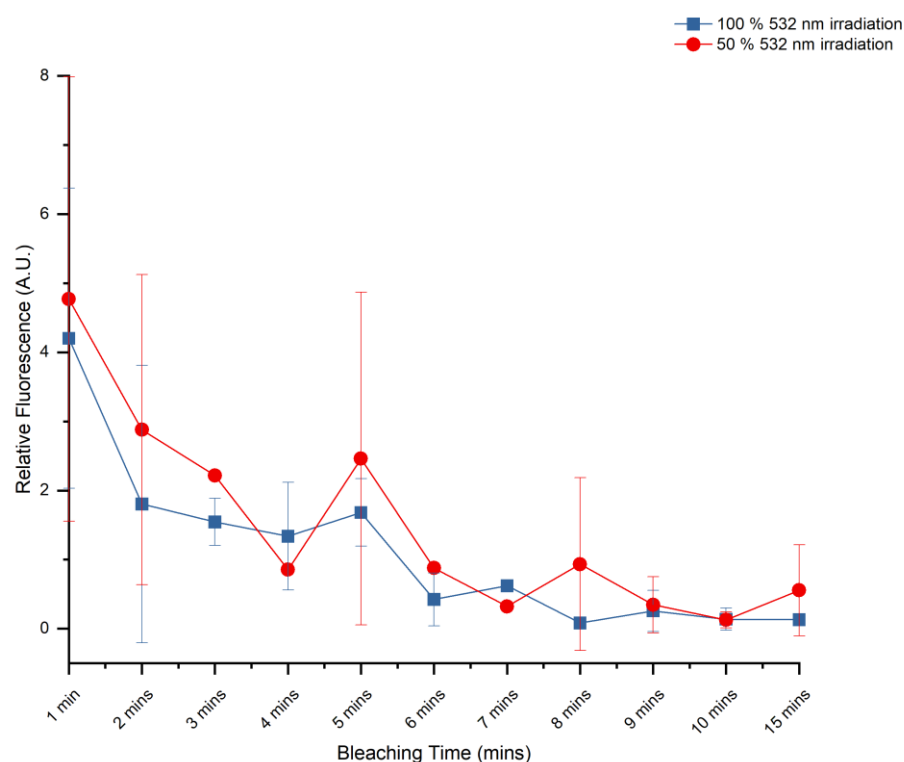


Figure 50: Change in relative fluorescence signal of rhodamine-123 with increasing laser exposure time - error bars are missing for samples where fluorescence of $n=1$ was obtained only. Relative fluorescence was calculated as outlined in section 6.11, by comparing the overall non-irradiated cell fluorescence signal with the rhodamine-123 fluorescence signal of the irradiated cells.

This figure was the opposite of what was originally expected on the planning phase of these experiments. Studies have shown that pulsed NIR irradiation, glutamate stress, plasma induced stress and cell starvation all increased the amount of ROS produced within different sample types such as human umbilical cord mesenchymal stem cells, mouse RGC-5 cells and yeast cells

such as *Candida utilis*.^{192,199,201} This increase was found from 40 seconds to over a few days depending on cell stress method used. Bai et al also managed to model the change in ROS and use this same model to fit the change in spectra for the stressed cells.¹⁹² The hope for this project was that a similar approach could be used if a similar profile of change was observed due to laser induced damage in MG-63 cells.

DHR-123 is a non-specific indicator of ROS but is well known for the detection of peroxynitrite ROS species or hypochlorous species induced ROS within cells.¹⁹⁸ It was chosen, as mentioned previously in section 4.3.1, due to the similar method of action to the Live/Dead stain Calcein-AM, and it was reasoned that there would be no interference on using these two staining methods consecutively one after the other on the cell samples. However, this method of action, of DHR-123 being mitochondrial selective and localising within the mitochondria on the oxidation of an amino group to a charged imino group, results in the detection of change in mitochondrial activity.¹⁸¹ This change in mitochondrial activity could be postulated to be due to ROS production. Therefore, a different method of ROS detection, such as the use of DFCH-DA as mentioned in the introduction to this chapter, section 4.3.1, may provide an alternative and potentially more robust method for non-specific ROS production as a function of cell stress and eventual death using Raman spectroscopy.

As an indication of mitochondrial activity, the figure above shows that for 100 % 532 nm laser irradiation, which is approximately 21.13 mW per second which is equivalent to 1.26 J, the mitochondrial activity halves in the initial minute of irradiation, but then remains relatively constant until 5 minutes, at which point the mitochondrial activity decreases until around 8 minutes at which point it settles at a relative minimum. This agrees nicely with the cell viability information shown in section 4.3.3, which is expected due to the mitochondrial affinity of Calcein-AM. This could indicate that cell death is initiated between 4 and 5 minutes of 100 %/21 mW 532 nm laser power and that cell death occurs between 8 and 15 minutes of total irradiation. It also agrees well with the statistical analysis performed on the spectra that show

statistically significant changes occur after 5-minutes for peaks relating to DNA in the 100 %/21 mW laser power irradiated cells.

The profile of mitochondrial activity of the 50 %/12.5 mW cells is similar to that of the 100 %/21 mW irradiated cells, however, this does not agree as well with the profile of decrease in cell viability from section 4.3.3. In the cell viability profile, cell viability reduced from 8 minutes of 50 % 532 nm of 12.5 mW, equivalent to 0.75 J. In the mitochondrial activity profile, the activity decreases sharply until 4 minutes, at which point it appears to recover before decreasing again. However, for both the cell viability profile and DHR profile, there are some data points that relate to one individual cell only, and therefore the variation seen is not representative. In the case of the 50 %/12.5 mW cells more replicates are required.

Indeed, for both populations again, further replicate experiments were planned. The data collated, in this chapter especially, was impacted by the constraints of the pandemic. Further experiments also would have been performed with a different ROS indicator, to investigate the original hypothesis which had been supported by literature findings that external cell stimuli produces ROS, and these are able to be related to changes within the spectra, to investigate if the inherent Raman process with 532 nm laser at time frames in line with cell experiments could cause cell damage and influence the spectral changes occurring.

4.3.6 SRS Changes with Cell Damage

SRS allows for the label free probing and imaging of individual peak wavenumbers of interest and has been used to track drug fate within cells and tissue as well as mapping of biomolecule distribution.⁷⁹ Due to the investigation of peaks which change significantly with increasing laser exposure in the spontaneous Raman spectra of MG-63 cell with increasing laser power, it was theorised that SRS could be used to tune to these peak wavenumbers and potentially detect a decrease in concentration of these species of interest.

An initial attempt was made to image the MG-63 cells with SRS microscopy, as outlined in section 6.10, and shown in figure 51.

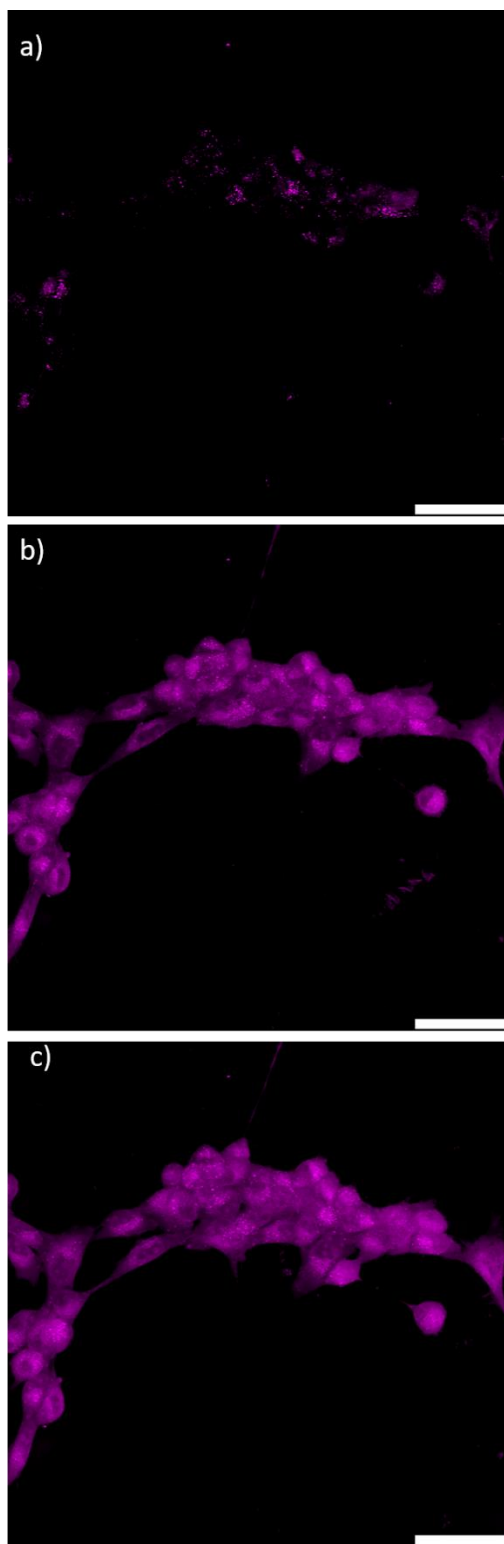


Figure 51: SRS image maximum projections of 30 μm depth stack generated with Leica SP-8 multiphoton CARS with SRS option microscope, using 40x water immersion objective and short working distance air condenser at 512x512 pixels, equal to 289.21x289.21 μm , scale bar 50 μm a) SRS tuned to 2850 cm^{-1} for CH_2 stretching in lipids b) SRS tuned to 2930 cm^{-1} for CH_3 stretching in lipids c) SRS tuned to 2940 cm^{-1} for the symmetric stretch of CH_3 in proteins.

Figure 51 indicates the maximum signal for lipids at 2850 cm^{-1} relating to the symmetric CH_2 stretch and 2930 cm^{-1} for CH_3 stretch of lipids as well as the protein CH_3 symmetric stretch at 2940 cm^{-1} .^{78,204} The images, as mentioned in Figure 46, are the maximum projection from the 35 slice $0.8\text{ }\mu\text{m}$ z-stack to indicate where the maximum signal lies throughout the whole cell, as variations in focussing depth will result in differences in signal intensity, therefore by taking a depth stack before and after photodamage or indeed during, any differences in signal seen on the maximum z-projected image will be registered regardless of which point in the cell it occurs. This would result in more accurate detection of the change in peak signal with potential induced damage.

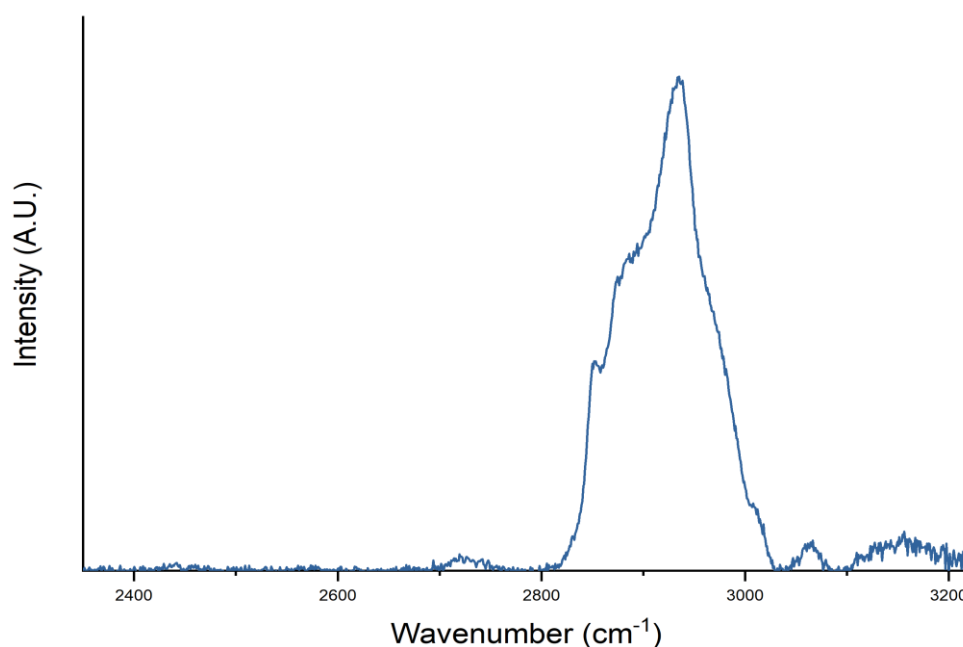


Figure 52: Example of Raman spectrum of MG-63 cell at high wavenumber region ($2800 - 3200\text{ cm}^{-1}$) with 60 s exposure time, 100 %/21 mW 532 nm laser, 63x immersion objective. Peaks are evident in the spectrum that are suitable for SRS imaging such as 2850 cm^{-1} for CH_2 stretching in lipids, 2930 cm^{-1} for CH_3 stretching in lipids and 2940 cm^{-1} for the symmetric stretch of CH_3 in proteins.

Figure 52 indicates the cell spectra relating to the high wavenumber region which was not a major focus of this thesis, although there are peaks of interest here relating to the biological macromolecules within cells; proteins, lipids and

nucleic acids.^{5,31} The spectrum has been included to indicate that the images taken during SRS are related to peaks of interest within the MG-63 cell line specifically, and could also be used to indicate cell death through spectral changes, although this study was not performed in this project.

Future studies would focus on tuning the SRS to the wavenumbers which showed statistical changes in section 4.3.4, in combination with full beam characterisation to understand the relationship in power incident on the sample using the picosecond pulsed NIR laser for SRS in correlation to the CW 532 nm laser used for the confocal Raman studies. There are two ways in which the future study could be performed, either using the SRS to induce the laser damage over time if incident laser powers consistent with those measured for 532 nm laser power in section 2.5.1.1 were possible; the SRS system could image the cell over time at the wavenumbers of cytochrome c or DNA and an off resonant wavenumber, and image processing could be used to determine if a decrease in the Raman signal is seen. Otherwise, the cell sample could be imaged in a depth stack as outlined in figure 46 above, transferred to the spontaneous Raman where it can be irradiated for the timeframe that has been determined to induce cell death; for the 100 %/21 mW power for example for 10 minutes, and then transferred back to the SRS to image in a depth stack again and these images processed to determine if a decrease in signal, proportional to concentration can be observed. Issues could arise in both cases surrounding cell movement, so an investigation into image registration may be required to allow this further work to be completed. Ideally this work would have been performed during this project, as the results would have been very interesting, but it was not possible.

4.4 CHAPTER CONCLUSIONS

In this final chapter, an attempt was made to correlate ROS expression to the Raman signal changes seen as a cell undergoes photodamage and eventual cell death at varying 532 nm laser powers. It was shown that a cell is likely to begin to die after approximately 4 minutes 100 %/21 mW 532 nm laser power, equivalent to 21.1 mW or 2.31 MW cm^{-2} and will be dead by 10 minutes at this laser power. It was also shown that 50 % 532 nm equivalent to 12.5 mW or 1.37 MW cm^{-2} laser power would cause cell death in approximately 8-minutes.

It was also determined that spectral changes relating to the release of cytochrome c at 750 cm^{-1} were statistically significant for 50 %/12.5 mW 532 nm irradiated cells, but not 100 %/21 mW irradiated cells, but that changes to DNA peaks at 785 cm^{-1} , 1484 cm^{-1} and 1577 cm^{-1} were evident and statistically significant for 100 %/21 mW 532 nm irradiated cells. Which correlates with previous literature reporting that photodamage induces irreversible DNA damage but can also be linked to the release of cytochrome c into the cytoplasm in the induction stages of apoptosis.

The attempt to relate the spectral changes to the production of ROS within MG-63 cells was not successful, however, this could be rectified in further work by using alternative ROS indicators such as DFCH-DA, to see if there is any possible correlation between signal differences in the Raman spectra on the induction of cell stress and death and ROS production.

The final part of this chapter describes an initial SRS experiment to attempt to image photodamage in MG-63 cells. Images of the high wavenumber region, relating to lipids and proteins were taken as a proof of concept to show that the spontaneous Raman peaks are easily imaged with SRS, and future studies could involve imaging photodamage in real time through changes in the SRS signal intensity with increasing laser power exposure with MG-63 cells.

5 CONCLUSIONS AND FURTHER WORK

In conclusion, the evaluation of the lateral resolution, laser power density and an approximation of axial resolution of the Renishaw Qontor inVia Raman spectrometer was successful. This information allowed greater knowledge and control over experimental parameters and understanding of the generation of cell spectral information.

MG-63 cells were successfully analysed with Raman spectroscopy, and indeed cell death via laser exposure was induced over a period of 40 minutes for 100 %/21 mW laser power. This knowledge of cell death allowed further investigation into the time of cell death due to 532 nm laser exposure using both Trypan Blue exclusion methods and Live/Dead™ fluorometric staining with Calcein-AM and EtHD-I. The resulting study allowed for time of death to be determined between 5 and 10 minutes for 100 % 532 nm at 21.13 mW of laser power per second and between 5 and 15 minutes of 50 % 532 nm laser exposure at 12.5 mW. During this initial study, peak changes relating to DNA, proteins and membrane phospholipids were identified as indicators of cell death, which was well related to findings from cell viability studies in the literature.

Closer study of these peaks of interest in 1-minute incremental increases of laser exposure time indicated a similar relationship in peak changes to the initial investigation. However, more statistical significance was elucidated from this study, with peaks relating to the vibration of half the pyrrole ring of cytochrome c which is indicative of the mitochondrially mediated apoptosis cell death pathway and DNA bases, but in particular guanine which has been shown to also be particularly sensitive to oxidation by reactive oxygen species.^{4,200}

Investigation of the ROS production during laser exposure of MG-63 cells showed a decrease in mitochondrial activity which can also be related to an increase in reactive oxygen activity as mitochondria are known to be involved the electron transfer chain; a process which is known to be a major producer

of intracellular ROS production.^{58,199,200} For a better understanding of ROS production induced by the Raman spectroscopic process in cells a different ROS indicator, such as DCFH-DA, could be completed as part of future work, and consequently this would allow for elucidation of any potential relationship between cell stress, cell death and the Raman analysis process.

A final investigation was performed to see whether SRS was capable of elucidating similar changes in signal from the peaks of interest identified in the spontaneous Raman investigation. Whilst it was not possible to complete the intended study, future work could focus on imaging photodamage in cells in real time, as long as the equivalent laser power for the pulsed picosecond laser was well understood in relation to the continuous wave 532 nm laser used which induced sample damage. Alternatively, the same CW laser could be used to induce sample damage, and SRS could be used to image the cells studied before and after analysis to determine if any differences in signal can be elucidated, as SRS signal is proportional to concentration. Images were successfully taken of MG-63 cells at the high wavenumber region and the methodology to perform such a study preliminarily outlined.

Further work for this study would include, utilising different cell viability stains, to elucidate fully the cell death mechanism activated during 532 nm laser exposure Raman analysis. Spectral changes identified during the studies in this thesis have been shown to be indicative of both apoptosis and necrosis according to previous literature studies. The use of Annexin-V combined with propidium iodide or 7-AAD would help elucidate between cells which are viable, early apoptotic, late apoptotic or necrotic.¹⁷⁴ External stimuli can cause the induction of either pathway, and in this study, visual examination of the cell morphology in combination with loss of cell membrane viability would indicate a necrotic pathway on increasing 532 nm laser irradiation, however spectral changes that have been associated with apoptosis were seen as well. Confirmation of the exact mode of death would result in a more robust experiment and provide more information surrounding the interaction of cell samples with laser irradiation during Raman spectroscopy.

Further experimental replicates for all experiments in this project would be needed for greater statistical confidence in the results, and so more analysis would be the first step of further work.

As mentioned in section 4.3.5 the utilisation of a different ROS indicator, such as DCFH-DA or a more specific ROS indicator to identify superoxide or H_2O_2 ROS specifically, would form a basis of future work.¹⁹⁸ This would consequently aid in determining if the spectral changes indicated during increasing laser exposure, such as the decrease in DNA at 785 cm^{-1} , which has been related to the cleavage of DNA bases due to cell degradation, are related to an increase in ROS production, and indeed if this ROS production differs for different laser powers or wavelengths.

Another initial aim of this project was to move beyond ROS interrogation during cell death, and to investigate if there is an increased production in heat shock proteins as cells are irradiated during Raman spectroscopy.²⁰⁵ The hypothesis was that cell stress before death will allow for greater specificity in safe operating limits, with defined limits of time for viable homeostatic cells, stressed cells, and non-viable cells. Utilising primary and secondary antibodies coding for heat shock protein 70 or 90 which are known to increase in activity in response to increased ROS production and these attempt to refold misfolded proteins, protect the cell and minimise the ROS initiated damage.²⁰⁶ If spectral changes could be related to the increased production of HSP in response to ROS mediated cell damage, this could in turn help determine better defined limits of laser induced damage and chemical cell changes that could affect analyses.

Another plan for further work is developing a method of utilising SRS to image cell stress and ROS in a label free manner, similar to studies performed with SERS or resonance Raman which used mitochondria-targeting functionalised nanoparticle or metal-complexes up taken by the cells to tracking the change in cytochrome c as it diffuses into the cytoplasm of the cell as it undergoes stress and apoptosis.^{58,202,207} Indeed, it would be interesting to see if the

spectral changes associated with necrosis and apoptosis show differences in SRS to interrogate cell viability in a completely label free manner.

Lastly, once the method of cell death in corroboration with laser exposure time and power and cell spectral changes evident of cell death were fully evaluated, the same protocol could be transferred to another system with the same laser wavelength, to cross validate the changes seen, and then eventually to apply these protocols to other cell lines and laser wavelengths to determine a rigorous framework for using Raman spectroscopy in combination with cell samples and minimising spurious chemical changes due to the analysis being performed.

6 MATERIALS AND METHODS

6.1 LASER POWER MEASUREMENTS

The laser power of each beam - 532 nm, 633 nm, and 830 nm - was measured using a laser power meter (Thorlabs, PM100D with S121C Sensor), mounted on a glass slide and placed in the sample holder within the Renishaw Qontor inVia housing. The laser was focused on the sensor of the detector through 5× objective (Leica, N-Plan, 506302), 20× objective (Leica, N-Plan Epi, 566066), 50× long working distance objective (Leica, N-Plan, 566036), 100× objective (Leica, N-Plan Epi, 566075) and 63× immersion objective (Zeiss W-Plan Aplanachromat, Vis-IR) individually and the power measured by tuning to the relevant wavelength and power range on the laser power control unit. The laser power was measured on three different days over a period of three weeks and the average and standard deviation of these measurements taken as the values of laser power.

6.2 LASER BEAM PROFILE MEASUREMENTS

The profile of the three laser wavelength beams, 532 nm, 633 nm 830 nm, was analysed using a beam profiler (Thorlabs, CCD Camera Beam Profiler, BC106N-VIS/M). The profile meter was mounted on a glass slide and placed within the Renishaw Qontor inVia housing. The profile meter was used to measure each laser naked, with no focussing objective and with the 50xL objective at each laser power available through the Renishaw software in regular and high confocality mode.

6.3 LATERAL RESOLUTION SAMPLE PREPARATION

A sample of exfoliated graphene mounted on silicon oxide wafer was prepared using Silicon wafer with 300 µm silicon oxide layer, cleaned by 5-minute sonication in acetone, followed by 5-minute sonication in deionised water and finally sonication in isopropanol (IPA). The substrate was then dried using an argon gun.

The graphene flakes were extracted from highly oriented pyrolytic graphene (HOPG) by tape stripping in a mechanical exfoliation approach and transferred to the clean silicon oxide wafer before analysis.

Raman spectra were acquired by performing $5 \times 10 \mu\text{m}$ line scans in both the x and y directions in $0.1 \mu\text{m}$ step with 2 s acquisition time and 5 %/1.32 mW 532 nm, 5%/0.38 633 nm or 5%/9.57 mW 830 nm laser power with a 50x long working distance objective, 63x immersion objective or 100x objective. The spectra were then analysed using the map review function in Wire 5.1 for either the graphene peak at 1585 cm^{-1} for 532 nm and 633 nm or the silicon peak at 521 cm^{-1} for the 830 nm wavelength.

Each map review profile then had an error function fitted to it in Origin 2020 and the first derivative of this error function fit was then performed, again in Origin 2020, and a Gaussian peak fitted to the first derivative profile. The FWHM of the first derivative fit was then averaged for the 5-line profiles in x and y for each laser profile and these were approximated to be the laser spot diameter in x and y and therefore related to the lateral resolution of the system.

6.4 AXIAL RESOLUTION SAMPLE PREPARATION

The axial layered sample was prepared externally by a company called Graphenea who applied monolayer graphene and a $50 \mu\text{m}$ PMMA polymer laminate layer on top of the thicker polyethylene.

The sample was then analysed using 10 %/2.3 mW. 50 %/12.5 mW or 100 %/21 mW 532 nm laser wavelength in conjunction with a 50x long working distance objective, 63x immersion objective or 100x objective in both high and standard confocality mode. 3 depth profiles were acquired for each condition from $100 \mu\text{m}$ above the sample surface focus to $400 \mu\text{m}$ below the sample surface focus in $0.1 \mu\text{m}$ step sizes to encompass all layers within the sample.

A peak of interest was then used to evaluate the spectral profile change of each layer with depth using the map review function in Renishaw Wire 5.1. For PMMA this was the peak at 813 cm^{-1} , for PE the peak at 1295 cm^{-1} and for graphene the peak at 1585 cm^{-1} .

The map review profiles were then analysed in two different ways. To evaluate the axial resolution, the graphene signal was used and two Gaussian peaks were attempted to be fitted to the depth profile signal to elucidate the thickness which the system detected the graphene signal, however an overlapping peak in PMMA resulted in difficulty determining the pure graphene signal from the polymer interference. For the signal interference with depth, the interface of signals between the two polymer layer map reviews were used. An error function was fitted to the two overlapping sigmoidal profiles to determine the distance over which spectral contributions from layers above and below the focus point may still be detected.

6.5 RAMAN WAVENUMBER CALIBRATION

6.5.1 Silicon Wavenumber Calibration

The Renishaw Qontor inVia system was calibrated daily using both internal and external silicon calibration. A piece of clean silicon wafer was mounted on a slide within the sample chamber and the laser being used – 532 nm, 633 nm, or 830 nm – focused to a tight spot on the surface of the silicon. A pre-programmed internal silicon calibration was then selected, which utilised shining the excitation laser onto a piece of silicon separate from the focusing objectives and the Raman spectrum recorded using acquisition parameters of 10 %/2.3 mW laser power and 0.1 s acquisition time.

The internal calibration was then confirmed using the external piece of silicon wafer mounted on the slide inside the sample chamber. This calibration does utilise the focussing objectives. The calibration acquisition parameters are the same as for the internal calibration.

The silicon peak occurs at 520.6 cm^{-1} with peaks detected between 520 cm^{-1} and 521 cm^{-1} being accepted.

6.5.2 Polystyrene Wavenumber Calibration

Further system wavenumber calibration was performed using the NIST SRM 1921b polystyrene sample. This standard is used for transmission infra-red calibration, however the ASTM standard E1840 lists polystyrene as a

recommended material for diode laser wavenumber calibration evaluation. Therefore, using the NIST traceable standard in combination with the guidance set out in the ASTM standard provides a good evaluation of wavenumber calibration.

The polystyrene sample calibration was performed using 532 nm laser wavelength and 50× long working distance objective (Leica, N-Plan, 566036), with 20 s acquisition time and 1 %/0.21 mW 532 nm laser power.

Table 7: Expected peak position and standard deviation for NIST SRM 1921b Polystyrene as outlined in ASTM E1840 protocol for Raman wavenumber calibration.²⁵

ASTM
Average (cm ⁻¹) ± Standard Deviation
620.9 ± 0.69
795.8 ± 0.78
1001.4 ± 0.54
1031.8 ± 0.43
1155.3 ± 0.56
1450.5 ± 0.56
1583.1 ± 0.86
1602.3 ± 0.73
2852.4 ± 0.89
2904.5 ± 1.22
3054.3 ± 1.36

The peaks measured during the calibration were compared to the values listed in table 7, which lists the average expected peak positions along with the acceptable level of deviation from this position.

6.6 RAMAN SPECTROSCOPY

All Raman measurements performed at the National Physical Laboratory were acquired on a Renishaw Qontor InVia confocal Raman microscope equipped with continuous wave (CW) 532nm, 633 nm and 830 nm excitation sources. All spectra were obtained using a 50× magnification, long working distance, 0.5 NA objective (Leica, N-Plan, 566036), 100× magnification, 0.85 NA

objective (Leica, N-Plan Epi, 566075), or 63x water immersion, 1.0 NA objective (Zeiss, W Plan-Apochromat, Vis-IR) with 2400 l/mm grating for 532 nm or 1200 l/mm grating for 633 nm and 830 nm.

All spectra were obtained using Raman grade calcium fluoride slides and coverslips (Crystran, Raman grade CaF_2 ; coverslips - 20 mm i.d. x 0.3 mm thickness, Slides – 75 x 25 x 1.0 mm), gridded glass coverslips (Ibidi, Gridded Glass Coverslips, Grid-50, 20 mm i.d x 0.17 mm thickness, 50 x 50 μm grid) or glass bottom dishes (Ibidi, μ -Dish 35 mm, high Glass Bottom).

All cell spectra were obtained using 63x immersion objective and 532 nm laser at 100 %/21 mW, 50 %/12.5 mW or 10 %/2.3 mW laser power, with 60 s acquisition time coupled with the required number of minutes of laser bleaching. For example, 5 minutes acquisition time results in an initial Raman acquisition of 60 s then 3 minutes of laser bleaching and then a final Raman acquisition of 60 s resulting in 5 minutes of laser exposure in total.

Visible light images were taken before and after each Raman acquisition with the 63x immersion objective using the inbuilt function within the Renishaw Wire software. It allowed for visible light photos to be taken directly before, after or both before and after acquisitions to allow for the investigation of visual changes during analysis, if any.

Visible light images taken using the 50x long working distance lens required additional transmission light to allow for applicable levels of contrast to see the cells. The images were still captured using the in-built function within the Renishaw Wire software, but with the additional light source.

6.7 RAMAN DATA ANALYSIS

Raman cell analysis was performed in Renishaw Wire initially, separating out the acquisitions and visible light images. The individual acquisitions were then pre-processed using the data arithmetic function in Wire taking the off-cell contribution from the cell spectra to ensure any background contributions from media or substrate were minimised. These files were saved in both Wire proprietary format .wdf and .txt. The .txt files were then imported into Origin. In

Origin a baseline was fitted using asymmetric least squared function, using an asymmetric factor of 0.001, a threshold of 0.01, smoothing factor of 4 for 10 iterations. The data was then normalised against the CH₂ peak of all biomolecules (~1449 cm⁻¹) and the average and difference spectra were calculated for the relevant samples to identify any peak changes if possible.

For peak ratio comparison, the peaks of interest were fitted using the curve fit function in Wire with a preloaded set of curves for fitting to allow for fairer comparison between sample types. For comparison to timepoint 1, taken as the point where no photodamage induced chemical changes had occurred – all peak areas were divided by the area of the peak at timepoint 1 and multiplied by 100 to give ratio percentage increase or decrease during analysis to indicate peak cell changes from timepoint 1.

For statistical analysis of peak ratio changes, a one-way ANOVA test was performed in Origin 2020 with p<0.05 level.

6.8 WIDEFIELD VISIBLE LIGHT MICROSCOPY

Images of cells between analyses and before and after Trypan Blue staining; method outlined in section 6.16.1, were captured using a Nikon Eclipse Ti-S Phase Contrast inverted microscope system equipped with a 20x long working distance objective (Nikon, CFI Achrom LWD AFL 20x, MRP46202).

6.9 FLUORESCENCE IMAGING

Confocal fluorescence images were obtained at the National Physical Laboratory using a Leica SP8 CARS with SRS Option Microscope system equipped with a 488 nm laser for fluorescence imaging and an automated picosecond tuneable picoEmerald S laser from APE Berlin for coherent Raman scattering (CRS) and two photon fluorescence (TPEF) imaging.

All fluorescence images obtained on the Leica system were taken using 25x magnification, 0.95 NA water immersion objective (Leica, Fluotar VISIR) and either the short working distance 0.9 NA air condenser (Leica, S1 515150), long working distance 0.55 NA air condenser (Leica, S28) or 1.4 NA oil

condenser (Leica, S1 5510004 achromatic) using the 488 nm excitation laser or the PicoEmerald laser tuned to 797 nm .

The emission detection range for the photomultiplier tube detector was set between 515 – 525 nm for Calcein AM and 615 - 626 nm for Ethidium Homodimer-I (EtHD-I) (ThermoFisher, Invitrogen, LIVE/DEAD Viability/Cytotoxicity Kit). The detector gain was optimised on the day of analysis and kept consistent for all samples analysed that day for fairer data comparison.

For the emission detection of two photon excitation fluorescence combined with SRS imaging, the TPEF images were generated in reflection mode using the pure Stokes beam at 1032 nm, resulting in 516 nm excitation of the oxidised DHR-123. The pump beam was then turned on to generate the SRS grid image in transmission mode, tuned to 797 nm for the 2850 cm⁻¹ CH₂ region of interest to determine cell position. The PMT detector gain was set at 800 V and 600 V for the TPEF and SRS channels respectively with 20 % power from the laser, equivalent to 3 mW power in the pump beam and 6 mW power in the Stokes beam, this was kept consistent for all days of analysis.

6.10 SRS IMAGING

All SRS images were obtained at the National Physical Laboratory using a Leica SP8 CARS with SRS Option Microscope system utilising an automated picosecond tuneable 1064 nm picoEmerald S laser from APE Berlin. All SRS images were generated using 30 % power of 0.15 W (equivalent to 3 mW) in the pump beam and 30 % power of 0.3 W (equivalent to 6 mW) in the Stokes beam. The difference between the pump and probe beam wavelengths were tuned to wavelength regions coinciding with the frequency of biomolecules of interest, such as CH₂ at 2850 cm⁻¹. The detector was set to 150 V gain for all images.

Images were generated using 40x water immersion objective (Leica, HC PL IRAPO, 40x, 1.1 NA, 0.65 mm) and short working distance air condenser (Leica 505175, 0.55 NA, 28 mm) at 512x512 pixels, equal to 289.21x289.21

µm using depth stack of 35 slices in 0.8 µm steps through the cell sample to determine the maximum projection of the signal of interest being probed.

The images were processed in the Leica LasX software using the inbuilt maximum projection function and LUT set between 0-150 for fair comparison of signals between all frequencies probed.

6.11 FLUORESCENCE/CRS DATA PROCESSING

For cell viability images, the channels were saved as separate images, the window/level set to 150/255 for all images and a scale bar added in the Leica LASX software and saved as .tif images. Image stitching for tile scan images were performed in the Leica LASX software followed by channel separation, level adjustment and scale bar addition. Live cells were coloured green to indicate the presence of Calcein-AM, and the dead cells were coloured red to indicate the presence of ethidium homodimer-I using the relevant look up table.

Reactive Oxygen Species (ROS) images were processed by transferring the images into ImageJ and processed by transforming the RGB image into a 16-bit binary image. The image was then duplicated, and the threshold function was used to limit any background interference, with all images thresholded to 5 and maximum intensity. The analyse particles function was then used with a circularity from 0.00 – 1.00 and size of 5 µm² – infinity to allow the capture of cell debris on cell degradation. The watershed function was used as needed to separate touching cells. The average gray values were then transferred into Microsoft excel. The average fluorescence of all control samples was calculated and then used to calculate the ratio of relative fluorescence intensity:

Equation 15: Equation for calculating relative fluorescence signal of Raman analysed cells from cell samples stained with DHR-123 for ROS

$$\text{Relative fluorescence} = \frac{\text{average analysed cell intensity}}{\text{average non - irradiated cell intensity}}$$

6.12 CELL MEDIA PREPARATION

50 mL of Foetal Bovine Serum (FBS) (Thermo-Fisher) was added to 500 mL of Dulbecco's Modified Eagle Media (DMEM) with 4.5 gL⁻¹ Glucose and L-Glutamine (Lonza) resulting in DMEM with 10% FBS media. Similarly, the solution was prepared under sterile conditions, within a biohood, sealed and refrigerated when not in use

6.13 CELL SPLITTING PROCESS

Human osteosarcoma cell line, MG-63, was acquired from Nilofar Faruqui of the Bio-Technology Group at the National Physical Laboratory. The cells were grown in T75 flasks, cultured in 15 mL DMEM media, and incubated at 37 °C, 5 % Carbon Dioxide (HERACell VIOS 160i, CO₂ incubator, Thermo-Fisher), to proliferate cell growth.

When appropriately confluent, cells were split to perpetuate cell growth. DMEM (Lonza, 10% FBS) was removed from the T75 flask and the cells were washed twice with 10 mL of sterile PBS (Thermo-Fisher). 2 mL of Trypsin-EDTA (Sigma Aldrich, 0.25%) was added to the T75 flask and the flask was incubated for 2-3 minutes, until all cells had detached from the flask surface. 4 mL of DMEM (Lonza, with 10% FBS) was then added to the T75 flask post incubation; the cell/trypsin/DMEM solution was then flushed with a pipette ten times. On the tenth flushing, all the solution was removed, and 0.5 mL was returned to the T75 flask. 15 mL of DMEM was then added to the flask, swirled to mix, and replaced in the incubator overnight to encourage cell regrowth.

This process was performed approximately every 2-3 days once the flask reached 75% confluency.

6.14 CELL SAMPLE PREPARATION

All samples were prepared under sterile conditions in a biohood, with any reagents sealed and refrigerated when not in use.

Cell samples were prepared in different ways; either on a calcium fluoride coverslip (Crystran), a 50 µm grid gridded glass coverslip (Ibidi) or in a 35 µm

diameter glass bottom dish (Ibidi). The calcium fluoride coverslip or gridded glass coverslip was placed in a sterile petri dish for preparation. The glass bottom of the glass bottom dish was used for direct cell sample preparation.

The samples were prepared by coating the coverslip or glass bottom dish surface in 1 mL poly-L-lysine (Sigma, .1% w/v), enough to cover the sample surface, for 5 – 10 minutes. The poly-L-lysine was then removed, the coverslip or glass bottom dish washed twice with 5 mL sterile PBS (Thermo-Fisher) and covered with between 3 and 6 mL of DMEM (Lonza, with 10% FBS) depending on sample preparation vessel.

The T75 cell flasks were prepared for splitting as outlined in section 6.13. Once the cell/trypsin/DMEM solution had been flushed ten times with a pipette in the flask, between 50 - 100 μ L of the mixture was added to the prepared sample vessel depending on concentration and day of analysis required, with approximately 100 μ L mL being returned to the T75 flask with 15 mL fresh DMEM and the rest disposed of as waste. The sample vessel was swirled to mix and incubated for at least 24 hours to ensure adequate cell growth for analysis.

The samples were then removed from the incubator. For the calcium fluoride and gridded glass coverslips, they were removed from the petri dish and mounted on a calcium fluoride slide (Crystran), prepared with a silicone spacer (Nagor, 200 x 150 x 0.25 mm, cut into approximately 20 x 20 mm squares with 5 x 5 mm hole removed from the middle), and 200 μ L of either DMEM (Lonza, with 10% FBS) or PBS (Thermo-Fisher) depending on analysis required, and analysed using Raman spectroscopy.

For the glass bottom dish samples, they were removed from the incubator, the DMEM was removed and replaced with the same volume of fresh DMEM (Lonza, with 10% FBS) or sterile PBS and analysed using Raman spectroscopy.

6.15 CELL FIXATION

For control sample preparation, fixed samples were required.

Samples were fixed by culturing MG-63 cells on gridded glass coverslips (Ibidi) as outlined in section 6.14. The media was removed from the petri dish containing the gridded glass coverslip, washed twice with prewarmed sterile PBS, and replaced with 5 mL 4% paraformaldehyde (Sigma). The petri dish was then incubated for 30 minutes at 37 °C, 5 % Carbon Dioxide (HERACell VIOS 160i, CO₂ incubator, Thermo-Fisher). The sample was removed from the incubator, the paraformaldehyde removed and washed twice with PBS. 5 mL sterile PBS was then added to the sample and the sample was left to rest at room temperature (RT) for a further 30 minutes before staining and analysis.

6.16 CELL STAINING PROCEDURE

6.16.1 Trypan Blue Staining

0.4 % Trypan Blue (Thermo-Fisher, 15250061) was diluted to 0.2 % concentration by pipetting 5 mL 0.4 % Trypan Blue into a falcon tube and adding 5 mL prewarmed PBS (Thermo-Fisher). 500 µL 0.2 % trypan blue solution was added to the relevant cell samples and incubated for 2 minutes. The samples were washed with prewarmed PBS to remove any additional trypan blue before being analysed using the widefield visible light microscope detailed in section 6.8.

6.16.1.1 *Trypan Blue Control Samples*

Control samples were prepared by preparing 3 cell samples as outlined in section 6.14 in Ibidi glass bottom dishes. One sample was left in the incubator for 4 days with no fresh media to cause cell death by nutrient depletion, the other two samples were prepared on day of analysis. One sample was then fixed as per section 6.15 and the last sample was used as a fresh sample as per the section 6.14.

Trypan blue was applied to all 3 samples as per section 6.16.1 and imaged using the widefield visible light microscope as per section 6.8.

6.16.2 LIVE/DEAD™ Viability/Cytotoxicity Staining

LIVE/DEAD™ Viability/Cytotoxicity kit for Mammalian Cells (Thermo-Fisher) was used in the staining of MG-63 cells to determine cell viability. The stains were prepared as per the procedure outlined in the supporting documentation for the kit provided by Thermo-Fisher.

6.16.2.1 Control Sample Staining

For control samples – cells which were all viable or fixed – the staining was prepared as follows:

- Both Calcein-AM and Ethidium Homodimer I (EtHD-1) were defrosted and warmed to room temperature before use.
- For live cell staining only, 5 µL 4 mM Calcein-AM was added to 5 mL prewarmed PBS and vortex mixed before addition to cell samples – 2 µM Calcein AM concentration.
- For dead cell staining only (post fixation cells), 10 µL 2mM EtHD-1 was added to 5 mL prewarmed PBS before addition to cell samples – 4 µM EtHD-I concentration.
- For both LIVE/DEAD staining (cell viability in sample) 5 µL Calcein-AM and 10 µL EtHD-1 were added to 5 mL prewarmed PBS before addition to cell samples (2 µM Calcein AM:4 µM EtHD-I in PBS).

All solutions were prepared by avoiding exposure to light before sample analysis.

Cell media or PBS was removed from the control samples. The samples were washed twice with sterile prewarmed PBS (Thermo-Fisher). Approximately 150 µL of staining solution was added to the sample, enough to cover the surface of the sample, and then incubated for 15 minutes. The samples were removed from the incubator, the staining solution removed carefully, and the samples were washed again, carefully, with sterile PBS, to avoid cell perturbation or loss. Either PBS or DMEM was then added to the washed samples, depending on analysis required and the samples were incubated again for 15 minutes. The samples were then analysed using fluorescence microscopy as outlined in section 6.9.

6.16.3 Sample Staining

For samples that underwent Raman analysis before staining, the staining solution was prepared as follows in sterile conditions in a biohood:

- 2 μ L Calcein AM (4 mM concentration) and 4 μ L EtHD-1 (2 mM concentration) (LIVE/DEAD™ Viability/Cytotoxicity kit for Mammalian cells, Thermo-Fisher) were added to 2 mL prewarmed sterile PBS; resulting in 2 μ M Calcein AM: 4 μ M EtHD-I solution in PBS. (Thermo-Fisher)
- Vortex mixed

Staining solution was prepared by avoiding exposure to light before sample analysis.

For coverslip staining, the coverslip was carefully removed from the slide and placed in a sterile petri dish for staining. Glass bottom dish samples were stained directly.

Cell media or PBS was removed from the sample being analysed and washed twice with prewarmed sterile PBS. Approximately 150 μ L of staining solution was added to the sample, enough to cover the surface of the sample, and then it was incubated for 15 minutes. Post-incubation, the sample was removed from the incubator, washed again carefully twice with sterile prewarmed PBS, and then covered again in either PBS or cell media depending on analysis required and incubated for another 15 minutes. Coverslip samples were then carefully remounted on a clean sterile CaF₂ slide with a silicone spacer and either DMEM or PBS whilst the glass bottom dishes were analysed directly using fluorescence microscopy as outlined in section 6.9.

6.17 CELL VIABILITY CONTROL SAMPLE PREPARATION

Control samples were prepared on gridded glass coverslips (Ibidi) in sterile petri dishes with MG-63 cells as outlined in section 6.14. Six coverslips were prepared in total and three coverslips were fixed as outlined in section 6.15. The coverslips were stained using the same procedure outlined in 6.16.2.

Table 8: Staining order for viability control samples

Coverslip Number	Fixed/Live?	Stain Used
1	Live	2 μ M Calcein AM only
2	Live	4 μ M EtHD-I only
3	Live	2 μ M Calcein AM: 4 μ M EtHD-1 mix
4	Fixed	2 μ M Calcein AM only
5	Fixed	4 μ M EtHD-I only
6	Fixed	2 μ M Calcein AM: 4 μ M EtHD-1 mix

The coverslips were analysed by fluorescence microscopy as outlined in section 6.9 as controls for the viability staining procedure.

6.18 REACTIVE OXYGEN SPECIES EXPRESSION SAMPLE PREPARATION

2 mg of 1,2,3-dihydrorhodamine (Sigma-Aldrich) was dissolved in 0.5 mL dimethyl sulfoxide (DMSO) (Sigma-Aldrich) resulting in 10 mM stock solution. This stock solution was then aliquoted into 50 μ L volumes, flash frozen in liquid nitrogen with loosened lids to minimise air exposure. The vials were then fully sealed and stored in liquid nitrogen until needed.

On day of use, 1 x 50 μ L of 10 mM DHR in DMSO was defrosted, with minimal light exposure. 1 μ L of DHR in DMSO was then pipetted into 999 μ L of sterile PBS and mixed well, resulting in a 10 μ M working solution for ROS detection.

150 μ L of the 10 μ M DHR in PBS working solution was added to the surface of the sample and incubated for 20 minutes. The DHR solution was removed and the sample was washed twice with PBS before being replaced in the incubator for a further 10 minutes and then analysed as outlined in section 6.9.

6.18.1 Reactive Oxygen Species Control Samples

All ROS sample analysis was accompanied by a set of control samples to help determine level of background fluorescence to analyse the difference, if any, of ROS level in cells analysed by Raman spectroscopy compared to those that were not. These control samples consisted of:

1. MG-63 cultured in an Ibidi dish with PBS and 10 μ M DHR solution, no Raman analysis
2. MG-63 cultured in an Ibidi dish with PBS, no DHR added, no Raman
3. An Ibidi dish with PBS and 10 μ M DHR, no cells, no Raman

All samples were then analysed by fluorescence microscopy as outlined in section 6.9 and then restained with Live/Dead™ stain as per section 6.16.2 and reanalysed as per section 6.9 to check cell viability. All images generated using fluorescence microscopy and SRS were processed as per section 6.11.

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