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Advancing Vibrational Spectroscopy for Cellular and Sub Cellular Analysis: Raman Spectroscopy as an in Vitro Chemotherapeutic Screening and Assessment Protocol

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Advancing Vibrational Spectroscopy for Cellular and Sub Cellular Analysis:
Raman spectroscopy as an in vitro chemotherapeutic screening and assessment protocol

Zeïneb Farhane

A thesis submitted for the Degree of Doctor of Philosophy

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School of Physics and Clinical & Optometric Sciences/ FOCAS Research Institute
2017
Abstract

Vibrational spectroscopy, including Raman micro-spectroscopy, has attracted considerable attention over the last few years, as a powerful, non-invasive tool for clinical applications, especially in cancer diagnosis, *in vivo* and *ex vivo*. As a molecular fingerprinting technique with optical resolution, Raman micro-spectroscopy is able to monitor biochemical processes, drug uptake, efficacy and mode of action and mechanisms of interaction of chemotherapeutic drugs at a subcellular level. *In vitro* applications may be more strategically achievable, and can help guide drug design and discovery, and eventually evaluate signatures of drug resistance, towards potential applications in personalised therapy and as a companion diagnostic tool.

However, to evaluate accurately the potential of Raman micro-spectroscopy for such applications, it is essential to optimise measurement and data processing protocols associated with subcellular analysis in order to extract all valuable spectroscopic information and to demonstrate the ability of this technique to distinguish not only between normal and cancer cells but also between cancer cell lines before exploring its potential as a chemotherapeutic screening and assessment protocol using commercially available chemotherapeutic agents. To be considered as an *in vitro* companion diagnostics technique to screen for personalised therapies, Raman micro-spectroscopy should be able to monitor subcellular interaction with chemotherapeutic drugs and to characterise cellular resistance.

To this end, different lung cell lines were used and Raman micro-spectroscopy was coupled to valuable other techniques such as Confocal Laser Scanning Fluorescence Microscopy, Flow Cytometry and Atomic Force Microscopy, in order to explore its
potential to elucidate drug pathways, chemical binding signature, mechanisms of action and efficacy and physiological cellular responses to the drug exposure. As chemotherapeutic agents, Doxorubicin and Actinomycin D, both anthracyclines widely used in clinics especially for lung cancer were employed as pilot molecules. Multivariate data analysis, consisting of Principal Component Analysis, Linear discriminant and Partial Least Square Regression analysis were employed to deeply investigate the spectral features related to drug effects and cellular responses.

Investigations demonstrate the ability of Raman micro-spectroscopy not only to track the subcellular accumulation of the drug as function of time but also to identify its mechanism of action, the subsequent cellular response and to differentiate cellular resistance. Moreover, despite the fact that different cell lines show different chemotherapeutic resistance, the chemical binding signature appears to be identical from anti-cancer drugs which belongs to same chemotherapeutic group with implications of different mechanisms of action function of time and dose.

In human lung cancer cell lines which show different cytotoxic sensitivities to the drugs, different spectroscopic response profiles to the drugs are observed, which can be potentially linked to cellular defence mechanisms, such as the expression of anti-apoptotic proteins, and DNA repair.
Declaration

I certify that this thesis which I now submit for examination for the degree of doctor of philosophy, is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for an award in any other Institute or University.

The work reported on in this thesis conforms to the principles and requirements of the Institute’s guidelines for ethics in research.

The Institute has permission to keep, or lend or to copy this thesis in whole or in part, on condition that any such use of the material or the thesis be duly acknowledged.

___________________________________________ Date ___/___/____
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To my dear parents and family for their emotional support and patience during all my studies, I’m pleased to say Thank you for everything and may god preserves, protects and grants them health and happiness.

I dedicate my work to my husband, may he find on it, the expression of my deepest feelings. The words are not enough to express my gratitude for his understanding, support and for his love which fill my life with happiness. I love you.

Finally, I would like to thank all my friend and colleagues in FOCAS Research Institute and Science Foundation Ireland (Award 11/PI/1108) which supports this work.
List of abbreviations

AB  Alamar Blue
ACT  Actinomycin D
AFM  Atomic Force Microscopy
CARS  Coherent Anti-Stokes Raman Scattering
CCD  Charge coupled device
Cisp  Cisplatin
CLSM  Confocal Laser Scanning Fluorescence Microscopy
DDR  DNA damage response
DMEM  Dulbecco's Modified Eagle's Medium
DMSO  Dimethyl sulfoxide
DNA  Deoxyribonucleic acid
DOX  Doxorubicin
DSB  DNA double stand breaks
FBS  Foetal bovine serum
FSC  Forward-scattered light
HBSS  Hank’s balanced salt solution
IC$_{50}$  inhibitory concentration that inhibits 50% of cells growth
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>ICA</td>
<td>Independent component Analysis</td>
</tr>
<tr>
<td>LDA</td>
<td>Linear Discriminants Analysis</td>
</tr>
<tr>
<td>MTT</td>
<td>3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide</td>
</tr>
<tr>
<td>NCLS</td>
<td>Non-negatively constrained least squares</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-Small Cell Lung Cancer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered solution</td>
</tr>
<tr>
<td>PC</td>
<td>principal component</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal Component Analysis</td>
</tr>
<tr>
<td>PLSR</td>
<td>Partial Least Squares Regression</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>SERS</td>
<td>Surface enhanced Raman spectroscopy</td>
</tr>
<tr>
<td>SSC</td>
<td>Side-scattered light</td>
</tr>
<tr>
<td>Vinc</td>
<td>Vincristine</td>
</tr>
<tr>
<td>WGA</td>
<td>Wheat Germ Agglutinin conjugates</td>
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Chapter 1: General introduction

1.1. Raman micro-spectroscopy:

Traditional diagnostic methods such as clinical assessment, histopathological examination performed on biopsies after staining, imaging techniques such as endoscopy, ultrasonography, tomography and magnetic resonance imaging and molecular diagnostic techniques, principally based on the recently developed polymerase chain reaction (PCR) technique, are by and large lengthy, invasive and painful (De Abreu, Wells, and Tsongalis, 2013; Ohno et al., 2008; Shahzad et al., 2015; Takahashi, Saikawa, and Kitagawa, 2013). Moreover, with the exception of the latter, they are largely based on identification of morphological changes in cells or tissues, rather than analysis of the underlying biochemistry, and so are subjective and prone to error. Therefore, there is a need to develop more effective non-invasive diagnostic and prognostic techniques for a single clinical investigation either in real time diagnosis or an in vitro way which could be applied to in vivo situations. An increased emphasis on in vitro techniques for evaluation of drug mechanisms and efficacies has also emerged from the introduction of European and US legislation which restrict the use of animal models in cosmetic and pharmaceutical development (EU Directive-2010/63/EU and US Public Law 106-545, 2010, 16th Congress), adding further to the demand for novel biological screening methodologies (Kallaway et al., 2013).

Raman micro-spectroscopy is a powerful non-invasive analytical method that can measure the chemical composition of biological samples, cells, tissues or biofluids at a molecular level (Huser and Chan, 2015; Kong et al., 2015). Raman micro-spectroscopy is an optical technique based on inelastic scattering of light by vibrating molecules,
demonstrated by C.V. Raman in 1928 (Byrne et al., 2014; Gala and Chauhan, 2015; Paudel, Raijada, and Rantanen, 2015; Smith et al., 2015). It is based on the interaction of photons with the vibrational states of the molecules in the sample, causing them to scatter inelastically, giving rise to the “Raman effect”. As the vibrations are molecularly specific, the technique can provide chemical fingerprints of complex biological samples (Kong et al., 2015). In the process, a laser is used as an excitation source and the incident photons can pass through the material without interaction or be absorbed or scattered, giving rise to two types of scattering, Rayleigh and Raman scattering (Tu and Chang, 2012).

As each molecular vibration can give rise to scattering, a molecule or collection of molecules generates a Raman spectrum, which is characteristic of the sample. Raman spectroscopy can therefore be employed to fingerprint materials, or processes, and has found a wide range of applications from basic research to forensics and pharmacology.

Raman spectroscopy has more recently been expanded to include surface enhanced Raman spectroscopy (SERS) (McAughtrie, Faulds, and Graham, 2014) and Coherent Anti-Stokes Raman Scattering (CARS) (Yu, Ramachandran, and Wang, 2014) which is increasingly employed in medical diagnostic imaging (Kong et al., 2014; Tu and Chang, 2012). The potential of Raman spectroscopy has been well demonstrated, especially for cancer diagnostics, in for example oral (Carvalho et al., 2015; Knipfer et al., 2014), gastric (Feng et al., 2011; Yao et al., 2009), breast (Abramczyk et al., 2012; Lee et al., 2014), cervical (Ramos, Malkin, and Lyng, 2015; Rashid et al., 2014), skin (Legesse et al., 2015) cancer, amongst many other anatomical sites, although translation to the clinical environment is still limited (Byrne et al., 2015; Kong et al., 2015).

However, potential clinical applications of Raman micro-spectroscopy are not limited to cancer diagnostics. This project will explore its potential as a pharmacological tool to
monitor drug kinetics and cellular interactions due to its subcellular resolution and signal reproducibility for applications such as potential personalised therapy and companion diagnostic. In fact, according to the US FDA (Food and Drug Administration), a companion diagnostic is defined as a device that provides information that is essential for the safety and effectiveness of a therapeutic product. Companion diagnostic assays have so far been reserved for oncology and mostly for a specific cancer and chemotherapeutic drug. For example, the drug vemurafenib and its companion diagnostic, the Cobas 4800 BRAF V600 mutation test, were approved for use in melanoma, and the drug Crizotinib and EML-4 ALK mutation test for the treatment of patients with late stage non-small cell lung cancer with an ALK gene rearrangement (Cheng, Koch, and Wu, 2012; Parkinson, Johnson, and Sledge, 2012).

The study will seek to demonstrate, using established chemotherapeutic agents of known mechanisms of action, in human lung cell lines as models, that the spectroscopic signatures of the modes of action of the drug are reproducible in different cell lines, and for different drugs of similar modes of action, and furthermore that the subsequent responses of different cell lines with varying cytotoxic response are characterisable according to their spectroscopic signatures.

1.2. Cancer:

Cancer is the name given to a collection of related diseases which can start anywhere on the human body, but can be different in morphologic, genetic and clinical levels. The disease results from an uncontrolled cell development and a disruption of cells apoptosis inducing abnormal cells formation (Almeida and Barry, 2011; Hesketh, 2013; Weinberg, 2013). Among all cancers, lung cancer is the second most common and the leading cause
of death around the world. The number of deaths worldwide is continuously increasing, with 1.5 million new cases recorded per year. The most important percentage of lung cancer is related to non-small cell lung cancer (NSCLC), which represents almost 80% (Cataldo et al., 2011; Reck et al.). For diagnosis and analysis of lung cancer, Raman micro-spectroscopy has been employed to distinguish tumour from normal lung cells, to predict the prognosis of non-small cell lung cancer patients by analysing surgically resected tissue sections and screen the pre-neoplastic lesions increasing the early detection of cancer (Huang et al., 2003; Kong et al., 2015; McGregor et al., 2017; Song et al., 2016; Zeng et al., 2010).

1.3. Chemotherapy:

The treatment of non-small-cell lung cancer is undergoing fundamental changes and many new approaches have been developed like adjuvant therapy, growth factors inhibitors and combined therapy but chemotherapy remains the major player and the most powerful arm for patients with advanced NSCLC (Biswas et al., 2013; Cataldo et al., 2011; Reck et al.; Schiller et al., 2013). Chemotherapy, involving the use of cytotoxic antineoplastic agents, remains an important strategy in the overall management of patients with malignant tumours (Riddick et al., 2005). Despite its significant side effects, in recent years chemotherapy has become a routine treatment for cancer, alone or in combination with radiotherapy, immunotherapy, hormonal therapy and surgery. There are many approved chemotherapeutic agents, divided into 5 groups according to their mechanism of action: alkylating agents, antimetabolites, anti-tumour antibiotics (including anthracyclines), topoisomerase inhibitors, and mitotic inhibitors (Reddy and Couvreur, 2010). A well-established anti-cancer drug (since 1960), and one of the 10
most frequently used drugs in cancer chemotherapy, especially for the treatment of aggressive and metastatic tumours, is Doxorubicin (DOX) (Yokochi and Robertson, 2004), an anthracycline antibiotic extract from *Streptomyces peucetius*, consisting of a conjugated anthraquinone ring structure bonded to an aminoglycoside (Figure 1.1 A) (Farhane et al., 2015; Hurley, 2002). DOX, alone, or in combination with other chemotherapeutic or with radiotherapy, is currently in clinical trials for treatment of advanced stages of NSCLC. However, treatment with DOX is associated with numerous side-effects including severe cardiotoxicity (Biswas et al., 2013).

Another anthracycline widely used in clinics for treatment of aggressive cancers is Actinomycin D (ACT), which was developed in the 1950s and clinically approved 10 years later (Cibi and Jayakumaran Nair, 2016; Liu et al., 2016). It blocks both DNA and RNA expression, and as a consequence protein synthesis, by inhibition of RNA polymerase and transcription in nucleoli and therefore induces cellular p53-independent apoptosis. It also intercalates into DNA using its phenoxazone ring at binding sites (GpC principally and also GpT, GpA, GpG, and CpG) such that the two cyclic pentapeptides of the drug are located in the DNA minor groove (Figure 1.1 B) (Cassé et al., 1999; Hasanzadeh and Shadjou, 2016; Kleeff et al., 2000; Lo et al., 2013; Nazari et al., 2012; Schwartz et al., 1965; Snyder et al., 1989; Yung, Bor, and Chan, 1990).
Figure 1.1: Chemical structures of A. DOX, B. ACT, C. Vinc and D. Cisp (Dasari and Tchounwou, 2014; Farhane et al., 2015; Lo et al., 2013; Mohammadgholi, Rabbani-Chadegani, and Fallah, 2013).

Similar to DOX, the mechanism of action of Vincristine (Vinc), an alkaloid, is not fully understood. Its principal mode of action is that of binding to tubulin monomers, which leads to a change in the dynamics of the microtubule assembly and prevention of the formation of bi-polar spindles, blocking mitosis at the metaphase/anaphase transition, and as a consequence inducing cell death. However, recent studies show that it can also act as
a DNA intercalator, due to its donor/acceptor O-H and N-H groups in the catharanine structure and –CHO group at the indoline ring nitrogen in the vindoline structure, which can H-bond (Figure 1.1 C) (Himes et al., 1976; Jordan, 2002; Mohammadgholi, Rabbani-Chadegani, and Fallah, 2013; Nawaz et al., 2013).

Cisplatin (Cisp), a platinium compound with a square planar geometry (Figure 1.1 D) and another chemotherapeutic drug with considerable side effects, also used for lung cancer treatment in combination with other chemotherapeutic drugs, was first synthesized by M. Peyrhone in 1844. Its chemical structure was first elucidated by Alfred Werner in 1893 and its cytotoxic properties were discovered in the 1960s. The cytotoxic mode of action of Cisp is mediated by its interaction with DNA to form DNA inter-strand and intra-strand adducts, which leads to cell cycle arrest and apoptosis (Dasari and Tchounwou, 2014; Nawaz et al., 2011; Siddik, 2003).

Early diagnostic and therapeutic follow-up protocols are of the utmost importance in lung cancer and the detailed mechanism of action of chemotherapeutic drugs and their specific effects on cancer cells need to be known in detail for a more targeted therapy, reducing the side effects and the drug resistance. However, medical investigations are complex, most of them invasive, involve many combined techniques and have certain limitations whether in cancer diagnostics or in therapeutic follow up.

It is not only critical to diagnose cancer but also to assess the medical therapy and the interaction between the cells and drugs (Kann et al., 2015), minimising the toxicity produced by chemotherapeutic drugs in the surrounding environment, normal cells and tissues, (in the absence of targeted therapy) especially when the difference between drug sensitivity and resistance of tumours cells is so small. Drug resistance itself is an area of emerging concern, despite the progress made in therapeutic protocols (Sun, 2015).
1.4. Objectives and methodology:

Both in terms of existing chemotherapeutic agents and developing protocols, there is a need to develop a sensitive in vitro analytical method not only to screen and track drugs inside cells but also to monitor the mechanism of action of and the cellular response to chemotherapeutic exposure. Raman micro-spectroscopy satisfies the criteria for sensitivity, specificity and non-invasive screening and seems to be a perfect tool for this application.

The objective of this thesis is to explore the potential of Raman micro-spectroscopy for screening and assessment of chemotherapeutic drug pathways inside cells in vitro, their chemical binding signature, their effects on cancer cells and the physiological cellular response to clinical exposure.

To this end, A549 human lung adenocarcinoma cells and Calu-1 human lung epidermoid cells, both non-small lung cells, were used in this study and DOX and ACT were employed, as model drugs for the validation of the Raman micro-spectroscopic method, as well as to further understand their detailed mechanism of action and their specific effects on cancer cells, for a more targeted therapy, reducing the side effects and the drug resistance. Raman micro-spectroscopy was coupled with Laser Scanning Confocal Microscopy, Atomic Force Microscopy (AFM) and Flow Cytometry and results were correlated to cell morphology and cell physiology.

Chapter 2 describes the material and methods used for the study as well as the optimisation process in order to get the most sensitive and specific results.

Chapter 3 is published in Analyst, 2015, 140, 5908. It describes the use of Raman micro-spectroscopy to determine the difference between the two cancer cell lines, A549 and
Calu-1, compared to a normal lung cell line, BEAS2B. PCA was used to compare the spectral profiles between the cell lines and, coupled to LDA, to explore the optimum sensitivity and specificity of discrimination. To support the analysis, Raman micro-spectroscopy was coupled with Flow Cytometry, Confocal Laser Scanning Microscopy and Atomic Force Microscopy. Results show that the three subcellular regions, nucleolus, nucleus and cytoplasm differentiate normal and cancer lines while, optimum discrimination between cancer cell lines is obtained according to the nucleolar region, highlighting the importance of this sub-nuclear area in diagnostic applications of Raman micro-spectroscopy.

Chapter 4 is published in Analyst, 2015, 140, 4212, investigates the effects of Doxorubicin on the A549 cell line, specifically on the nucleolus compared to the nucleus. Raman micro-spectroscopy was coupled with Confocal Laser Scanning Microscopy to localise and follow the drug inside cells, showing that Raman micro-spectroscopy is able to track Doxorubicin at a subcellular level and to detect biomolecular changes due to drug exposure and physiological cellular response.

Chapter 5 is published in J. Biophotonics 10, No. 1, 151–165 (2017) / DOI 10.1002/jbio.201600019 and further illustrates the potential of Raman micro-spectroscopy to elucidate drug mechanisms of action and distinguish the cellular resistance of two lung cancer cell lines A549 and Calu-1. Raman investigations show that Calu-1 cells exhibit spectroscopic signatures of both direct DNA damage due to intercalation of DOX in the nucleus and indirect damage due to oxidative stress in the cytoplasm, whereas the A549 cell line only exhibits signatures of the former mechanism of action. Biomarkers related to the drug mechanism of action and cellular resistance to
apoptosis, namely reactive oxygen species (ROS) and bcl-2 protein expression, respectively, were also measured and correlated to Raman spectral profiles.

In Chapter 6, published in Anal Bioanal Chem (2017) 409:1333-1346/DOI 10.1007/s00216-016-0650-0, Raman investigations were extended to monitor the time evolution of the A549 cellular responses to DOX exposure using Partial Least Squares Regression, in order to track the drug mechanism of action and cellular reactions for early and late stage exposure at subcellular level and monitor the subsequent cellular responses. The study demonstrates the potential of Raman micro-spectroscopy to monitor the drug uptake and the cellular responses at a subcellular level, but also elucidate details of the pharmacodynamics and drug cellular kinetics of the clinically prescribed drug.

Chapter 7, published in J. Biophotonics 1–14 (2017) / DOI 10.1002/jbio.201700060, further extends the Raman investigations to track cellular uptake, signatures of chemical binding and subsequent cellular responses, of DOX in two lung cancer cell lines, A549 and Calu-1. Multivariate statistical analysis was used to elucidate the spectroscopic signatures associated with DOX uptake and subcellular interaction. Biomarkers related to DNA damage and repair, and mechanisms leading to apoptosis were also measured and correlated to Raman spectral profiles. Results confirm the potential of Raman spectroscopic profiling to elucidate both drug kinetics and pharmacodynamics and differentiate cellular drug resistance associated with different subcellular accumulation rates and subsequent cellular response to DNA damage, pointing towards a better understanding of drug resistance for personalised targeted treatment.

In Chapter 8, published in J. Biophotonics DOI: 10.1002/jbio.201700112, exposure of A549 and Calu-1 cells to the analogous anthracycline ACT was similarly monitored using Raman micro-spectroscopy. Multivariate data analysis, consisting of PCA and ICA, was
used to extract biological information contained in the Raman spectra. Results show that the ACT uptake and mechanism of action are similar in the two cell lines, while A549 cells exhibit spectral signatures of resistance to apoptosis related to its higher chemoresistance to the anticancer drug ACT, confirming the potential of Raman micro-spectroscopy as an *in vitro* analytical tool for biological analyses and in the prediction of drug mechanisms of action and cellular reactions.

Chapter 9 is an accepted review (J. Biophotonics, **DOI**: 10.1002/jbio.201700258) which summarises the results of the study of the cellular uptake binding and cellular responses of the anthracyclic chemotherapeutic agents in the lung cancer cell lines, and also puts them in context with similar studies of other anticancer agents, of different mode of action. The study confirms the ability of Raman micro-spectroscopy to elucidate subcellular transport and accumulation pathways of chemotherapeutic agents, characterise and fingerprint their mode of action, and potentially identify cellular resistance. Doxorubicin and Actinomycin D, elicit similar spectroscopic signatures of subcellular interaction characteristic of the mode of action of intercalation. Although Cisplatin and Vincristine show markedly different signatures at low exposure doses, their signatures at higher doses show similarities to those elicited by the intercalating anthracyclines, confirming that anticancer agents can have different modes of action with different spectroscopic signatures, depending on the dose.
1.5. References:


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Chapter 2: Sample preparation, Materials, Methods and Protocol optimisation

The following chapter outlines the materials and methods and the background to the experimental techniques used throughout this thesis. All experimental conditions, from sample preparation to all spectroscopic and microscopic methods used, were optimised in order to get the best data from cellular and drug investigations and to produce accurate, reproducible and valid scientific results. Where appropriate, further specific details are provided in the relevant chapters.

2.1. Materials:

A549 human lung adenocarcinoma cells with the alveolar type II phenotype, and BEAS-2B normal human bronchial epithelium (ATCC® CRL-9609™), virus transformed, infected with a replication-defective SV40/Adenovirus 12 hybrid and cloned, were all obtained from ATCC (Manassas, VA, USA). Calu-1 human lung epidermoid cells were kindly provided by Dr. Josep Sulé-Suso, Institute for Science & Technology in Medicine, Keele University, Guy Hilton Research Centre UK and Cancer Centre, Royal Stoke University Hospital, University Hospitals of North Midlands, UK.

Alamar blue (AB) (10X ready to use solution) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Aldrich, Ireland.

SYTOX® Green Fluorescent Nucleic Acid Stains, a high-affinity nucleic acid stain that easily penetrates cells with compromised plasma membranes, Wheat Germ Agglutinin conjugates (WGA), a cell membrane glycoconjugates binder, SYTO® 14 green fluorescent, nucleic acid stain exhibits bright green fluorescence, Nucred® live 647 ready
probe a bright, far-red, cell-permeant nuclear stain and Calcein, a cell-permeant dye converted to a green-fluorescent Calcein after acetoxyethyl ester hydrolysis by intracellular esterase, were purchased from BioSciences (Ireland, suppliers for Life Technologies), were employed to image the nuclear compartment and cytoplasmic membrane of cells using Confocal Laser Fluorescence Scanning Microscopy. The fixation/permeabilization kit, BD Cytopix/Cytoperm (BD 554714), and FITC Mouse Anti-Human bcl-2 set with an IgGl isotype control (BD 556357) was purchased from BioSciences, Ireland.

γH2AX reagents, Alexa Fluor® 647 anti H2AX phospho (Ser 139) and Alexa Fluor® 647 mouse IgGl isotype control (ICFC), Biolegends, were supplied through Medical Supply Company Ltd., Ireland.

Doxorubicin hydrochloride® (DOX) powder (Sigma Life Sciences, Ireland) was diluted in 1 mL sterile water to the required concentration (17.25 mM) (DOX solubility in water is 10 mg/mL).

Actinomycin D (ACT) stock solution of concentration 398 µM was prepared by dilution of Actinomycin D® powder (2 mg) (Sigma Life Sciences, Ireland) in 4 mL sterile water (ACT solubility in water is 0.5 mg/mL).

2.2. Cell culture:

In order to determine the best medium composition for each cell line and optimise the cell culture process, a variety of protocols were explored in the initial stages. A549 and Calu-1 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (with 2 mM L-glutamine) or RPMI. Both media contained 10 % foetal bovine serum (FBS) and cultures were examined with or without 1 % Hepes buffer (maintained at constant pH, independent of CO₂ amount and usually used at 5 mM to 30 mM
concentration) (SIGMA-ALDRICH, 2015), 1% sodium pyruvate (as a carbon source in addition to glucose) (SIGMA-ALDRICH, 2015) and 1% non-essential amino acids (SIGMA-ALDRICH, 2015), at 37 °C in a humidified atmosphere containing 5% CO₂. After comparison of the two different media (by analysing the cell aspect and proliferation) with and without addition of amino acids, Hepes buffer and sodium pyruvate, it was determined that the A549 cell line grows better in DMEM with 2 mM L-glutamine and 10% foetal bovine serum, whereas the Calu-1 cell line develops better in RPMI with 10% foetal bovine serum. The addition of other nutrients does not improve the cell culture in any way.

2.3. Cytotoxicity assays:

The MTT test is a colorimetric assay that measures the reduction of yellow 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase into purple formazan crystals insoluble in aqueous solution (Al-Nasiry et al., 2007; Hamid et al., 2004; SIGMA-ALDRICH, 2015). Alamar blue (AB) is a water-soluble dye which is a sensitive oxidation-reduction indicator employed for in vitro quantification of the cell viability. When added to cell cultures, the active dye, resazurin or 7-hydroxy-10-oxidophenoxazin-10-iium-3-one diffuses into the cytosol and acts as an intermediate electron acceptor, allowing the oxidised blue non-fluorescent form to be reduced by both mitochondrial and cytosolic enzymes activity to the fluorescent pink one, which is easily measured by its absorption or fluorescence (Al-Nasiry et al., 2007; Hamid et al., 2004; Rampersad, 2012; SIGMA-ALDRICH, 2015).

Alamar blue (1.5 mL of AB (10x ready to use working solution)) and MTT (3 mL of MTT stock solution (2.5 mg/mL, 25 mg MTT/10 mL PBS)) assays, (protocol developed
in Nanolab, DIT, Farhane et al., 2015), were performed in 96 well plates, three of each were seeded with a total number of cells of \(1 \times 10^5\) (4 \(\times\) 10^3 cells /mL) for 24 hrs, \(1 \times 10^4\) (400 cells /mL) for 48 hrs and \(5 \times 10^3\) (200 cells /mL) for 72 hrs.

After 24 hrs incubation, plates were washed with phosphate buffered saline solution (PBS) and 100 µL of DOX diluted in 1 mL sterile water to the required concentration, were added to each well starting with the test concentration at 50 µM (a range from 0 µM (as a control) to 50 µM (0, 0.198125, 0.396025, 0.78125, 1.5625, 3.125, 6.25, 12.5, 25, 50 µM)). ACT was added in a concentration range from 0 µM (as a control) to 50 µM (0, 0.198125, 0.396025, 0.78125, 1.5625, 3.125, 6.25, 12.5, 25, 50 µM) for 24 hrs and from 0 to 0.5 µM (0, 0.015625, 0.03125, 0.0625, 0.125, 0.25, 0.5 µM) for 48 and 72 hrs.

After the appropriate incubation time in DOX or ACT, plates were washed with PBS and 100 µL of AB/MTT solution in 30 mL of fresh medium, were added to each well. Plates were then incubated for 3 hrs and AB fluorescence was measured in the plate reader using Soft Max® Pro6.2.2 as software at 540 nm excitation and 595 nm emission. The medium was then removed, the plates were washed with PBS and 100 µL of DMSO (dimethyl sulfoxide) were added in each well then MTT absorbance was read at 570 nm.

All cytotoxicity assays were prepared in triplicate and repeated three times and all data was treated using SigmaPlot 10.0 and IC50 was calculated for each time exposure (24, 48 and 72 hrs).

2.3. Atomic Force Microscopy (AFM):

The atomic force microscope is a scanning probe microscope given a high resolution and nanoscale images of complexes matrices allowing a various application especially in cell imaging and manipulating living cells to single-molecule resolution (Müller and Dufrène, 2011). AFM works by dragging a sharpened probe over the surface and using interactions
between the surface and probe to build up a map of the sample topography (Baró and Reifenberger, 2012). An ancestor of AFM was developed by Gustav Schmalz in 1929, and consisted of an instrument that worked by dragging a sharpened probe over a surface. Due to resistance from surface features, the resolution and fidelity of the resulting image was suspect (Wallace, 2012).

In AFM, the force of the interaction between the probe and the sample is measured by a force transducer, the cantilever on which the probe is mounted and a feedback control system is used to maintain a desired force between the probe and the sample (Kuznetsov and McPherson, 2011).

![Figure 2.1: Asylum MFP-3D-BIO Atomic Force Microscope (DIT-FOCAS, 2015).](image)

The air dried samples on CaF$_2$ substrates were profiled using a MFP-3D BIO AFM (Asylum Research) (Figure 2.1). The cantilevers used were Olympus silicon AC240. Tips were 160 nm long and had a typical resonant frequency of 70 kHz. The AFM was operated in AC or vibrating mode (the signal driving the feedback is the amplitude of the time varying deflection, rather than the quasistatic deflection, as used in contact mode) in order to minimize tip/sample interaction. Typical free air amplitudes were ~200mV and a high amplitude set-point relative to the set-point was maintained to minimize sample damage.
The images obtained, from 5 to 10 cells per cell line, contained 1024 pixels per scan line and only one representative image of typical cell for each cell line is shown.

2.4. Flow Cytometry

Flow cytometry is finding increasing uses in clinic and pathology. Flow cytometry measures optical and fluorescence characteristics of single cells, based on the light scattering and emission properties of the cells, giving information on cell cycle, and physical properties such as size and internal complexity (Darzynkiewicz and Zhao, 2001; Macey, 2007).

Cells flow past the illumination of a light source (Figure 2.2), whereupon they scatter laser light, or, if intrinsically fluorescent or tagged with a fluorescent marker, they absorb and emit the light energy at higher wavelengths (Brown and Wittwer, 2000). The scattered and fluorescent light is collected by appropriately positioned lenses. A combination of beam splitters and filters steers the scattered and fluorescent light to the appropriate detectors which produce electronic signals proportional to the optical ones (BDBiosciences, 2000). No fluorescent labels were used for this study and only the cellular light scattering was measured. The light scattering depends on the physical properties of the particle, or in this case, cells, namely their size and surface area (Forward-scattered light (FSC)) and granularity and internal complexity (Side-scattered light (SSC)) (Marina, Sanders, and Mourant, 2012).

Cells were cultured in T75 flasks over 48 hrs then trypsinised and centrifuged in 10 mL fresh medium at 4 °C and 1000 rpm for 5 min. After this, the cells were re-suspended with 5 mL PBS and only 2 mL was used for analysis. Samples were prepared in parallel and the two cell lines were analysed separately. A total number of $2 \times 10^4$ cells was
analysed for each sample without any dyes. The experiments were conducted in triplicate (three independent experiments).

**Figure 2.2:** Accuri C6 Flow Cytometer and hydrodynamic focusing of the sample through the laser (BDBiosciences, 2013, 2000).

For bcl-2 protein expression, cells (3x $10^4$/flasks) were cultured in T25 flasks over 24 hrs, and then exposed to a DOX dose corresponding to the inhibitory concentration, $IC_{50}$, adjusted to the cell number, determined by cytotoxicity assays for each time point (from 2 hrs to 72 hrs) and each cell line. After each incubation period, cells were trypsinised and centrifuged in 5 mL fresh medium at 4 °C and 1100 rpm for 5 min and they were then re-suspended in 1 mL Ice Cold Dulbecco's Phosphate-Buffered Saline (DPBS) buffer and centrifuged at 4 °C and 2500 rpm for 5 min.

Cells were re-suspended in 750 µL ice cold DPBS buffer and transferred to Eppendorf tubes to which 250 µL of fixation buffer (ready to use solution which enables the fixation and permeabilisation of cells) were added. After 30 min incubation at 4 °C, the fixed cells were washed twice in perm/wash buffer (1X solution obtained by dilution of 10X stock solution in distilled water), centrifuged (2500 Rpm for 5 min at 4 °C) and then gently re-suspended in 50 µL perm/wash buffer, after which 20 µL of the antibody (ready to use
clone bcl-2 conjugated with FITC) were added and the cells were incubated for 60 min in the dark at 4 ºC. The cells were then washed twice in perm/wash buffer, centrifuged (2500 Rpm for 5 min at 4 ºC) to remove unbound antibody and finally re-suspended in 1 mL stain buffer (1X PBS with 2 % heat inactivated FBS and 0.09 % Na Azide).

For γH2AX expression, cells (10⁶/flask) were cultured in T25 flasks over 24 hrs, and then exposed to a DOX dose corresponding to the inhibitory concentration, IC₅₀, adjusted to the cell number, determined by cytotoxicity assays for each time point (from 2 hrs to 72 hrs) and each cell line.

After each incubation period, cells were trypsinised and centrifuged in 5 mL fresh medium at 4 ºC and 1100 rpm for 5 min, then were re-suspended with 1 mL PBS, transferred to an Eppendorf tube and centrifuged at 400 g for 5 min at 4 ºC. Cells were re-suspended in 200 µL of 2 % v/v formalin in PBS and incubated for 10 min. If not used immediately, cells can be kept in 1 mL ice-cold ethanol 70 % and samples and stored at -20 ºC for up to two weeks or overnight at 4 ºC. Cells were then re-suspended in 500 µL Triton X-100 0.25 % v/v in PBS and incubated at Room Temperature (RT) for 5 min or 30 min at 4 ºC, after which cells were centrifuged at 400 g for 5 min at RT and re-suspend in 200 µL Bovine serum albumin (BSA) solution (2mg/100mL) and incubated at RT for 30 min. After centrifugation at 400 g for 5 min at RT, cells were re-suspended in 150 µL antibody solution, Alexa Fluor® 647 anti H2AX phospho (Ser 139), diluted 1:500 (10 µL primary antibody in 5 mL blocking solution) in BSA solution and incubated at RT for 2 hrs or overnight at 4 ºC. The cells were then washed thrice in PBS and finally re-suspended in 1mL BSA solution.

All Flow cytometry analysis was performed using a BD Biosciences Accuri C6 Flow Cytometer (10,000 events were recorded, settings are run with limits, fluidics slow, agitate before analysis, wash before and between samples and FL1 for bcl-2 analysis, FL2
for DOX and FL3 for γH2AX analysis) (Becton Dickinson, Oxford, UK) (Figure 2.2). The Accuri Flow Cytometry software was used for the analysis of Flow Cytometry samples and data processing, consisting of gating to eliminate dead cells and analysis of mean fluorescence.

2.5. Confocal Laser Scanning Fluorescence Microscopy (CLSM)

Confocal Laser Scanning Microscopy has become an invaluable non–invasive imaging technique in biological and medical fields for the investigation of cell morphology and physiological processes, using fluorophores that specifically target and identify subcellular structures and are excited by light provided by a laser at a specific wavelength. Its basic concept was originally developed by Marvin Minsky in the mid-1950s (Claxton, Fellers, and Davidson, 2006; Lange-Asschenfeldt et al., 2012).

The light emitted by the laser system (excitation source) passes through an objective, a dichroic mirror, onto the sample. A pinhole aperture is used to eliminate the scattered light, the emitted fluorescence is collected only from the focal point and images of the scanned sample can then be reconstructed point by point (Figure 2.3) (Nwaneshiudu et al., 2012).
In this study, samples were prepared on uncoated glass bottom Petri dishes (MatTek Corporation, USA). Approximately $10^4$ cells were allowed to attach for two hrs, then covered with cell culture medium.

After incubation, the medium was removed and cells were washed repeatedly (two or three times) with PBS and 1 mL of Calcien solution per Petri dish (50 µL of Calcien in 2 mL medium (Calcien was reconstructed with 250 µL Dimethyl sulfoxide, DMSO to a concentration of 5 mM)) were added and samples were then incubated for 20 min. The, medium was then removed, samples were washed with PBS and 2 mL of Nucred® ready to use solution (2 drops of Nucred® stock solution into 1 mL of media) were added. Samples were then incubated for 15-30 min, rinsed twice with sterile PBS and cells were imaged in 0.9 % sodium chloride solution.

Excitation and emission wavelengths were, respectively, 488/507 nm for Calcein and 633/690 nm for Nucred®. However, this combination of stains did not work well, due to the fact that Calcein stains the whole cell. Thus, Sytox® green nucleic acid stain and
Wheat Germ Agglutinin conjugates were employed and reasonably good images were obtained. After incubation, the medium was removed and samples were rinsed twice with sterile PBS and fixed in formalin (4 %, 15 min). After fixation, samples were washed with Hank’s balanced salt solution (HBSS) and 7.5 µL of WGA solution (WGA of 1 mg/mL stock solution (prepared by dissolving 5 mg lyophilised WGA in 5 mL PBS) per 1 mL HBSS) was first added and cells were incubated for 20 min and then washed with HBSS. Sytox® green nucleic acid stain (2 µL of stock solution at 5 mM per 1 mL of HBSS) was thereafter added, cells were incubated for 30 min, washed with HBSS and then imaged in HBSS.

Confocal Laser Scanning microscopic images were recorded using an inverted Zeiss LSM 510 confocal laser scanning microscope equipped with a x60 oil immersion objective. (Figure 2.3) Excitation wavelengths used were 488 nm for Sytox® green nucleic acid stain (emission wavelength 523 nm) and 633/647 nm excitation/emission for WGA, using a band pass (BP) filter (detect only light between 505 and 530 nm) for the green dye and a long pass filter (>640 nm) and the channel ChS for the red dye.

For DOX exposure, the same amount of cells was used in Petri dishes and, after 24 hrs incubation, the medium was removed and samples were rinsed twice with sterile PBS. New medium containing DOX, corresponding to the IC₅₀ concentration (mean inhibitory concentration) which is the concentration that inhibits 50 % of cells (Neubig et al., 2003), was added and cells were further incubated for 2, 6, 12, 24, 48 and 72 hrs. At the end, cells were washed twice with sterile PBS, fixed in formalin (4 %, 15 min) and kept in PBS for imaging. Control samples without exposure to DOX were also prepared in parallel, and incubated for the same period.
Confocal laser scanning fluorescence microscopic images were recorded using an inverted Zeiss LSM 510 confocal laser scanning microscope equipped with a x60 oil immersion objective. Doxorubicin fluorescence was excited with an argon ion laser at 488 nm (Main Dichroic Beam Splitter HFT UV 488/543/633 nm), and the emission was collected at 530 nm (BP filter 505-530 nm).

2.6. Raman micro-spectroscopy:

Raman micro-spectroscopy is a non-invasive analytical tool which is increasingly being explored for its potential in clinical applications and especially in chemotherapeutic development.

Basically, Raman is an optical spectroscopic technique, based on the transitions between vibrational levels of electronic states. In more detail, it is an inelastic scattering technique involving coupling of the incident photons or electromagnetic radiation with the molecular vibrations. When the frequency of the scattered light is less than the incident one due to the fact that the molecule absorbs energy from the incident photon and elevates itself from a lower energy state to an excited vibrational state, this is called Stokes Raman scattering. Conversely, anti-Stokes scattering occurs when the scattered photons are higher in energy than the incident photons, as a result of annihilation of a molecular vibration (Figure 2.4) (Tu and Chang, 2012).
Figure 2.4: Jablonski diagram, schematic representation of the energy transition in Raman micro-spectroscopy with \( h \): Plank’s constant and \( \nu_0 \): excitation frequency \( \nu \): bond vibration frequency.

The polarization \( P \) of the material depends on the polarizability, \( \chi \), of the material and on the electric field \( E(\omega_0) \) of the incident light at frequency \( \omega_0 \):

\[
P(\omega) = \chi(\omega_0)E(\omega_0)
\]

The polarisability \( \chi(\omega) \) changes as a function of molecular vibration, \( \omega_k \), such that

\[
\chi_k(t) = \chi_0 + \chi_k \Delta R \cos(\omega_k t + \delta_k)
\]

where \( \Delta R \) is the displacement of the atoms about their equilibrium position. \( \delta_k \) accounts for a random phase of the material vibrations.

Thus, the polarisation can be written in the form:

\[
P(\omega, \omega_k) = \chi_0 E \cos(\omega_0 t) + \chi_k \Delta R \cos(\omega_0 t) \cos(\omega_k t + \delta_k)
\]

Using the trigonometric relationship:

\[
\cos(\omega_0 t) \cos(\omega_k t + \delta_k) = \frac{[\cos((\omega_0 + \omega_k) t + \delta_k) - \cos((\omega_0 - \omega_k) t - \delta_k)]}{2}
\]

It can be seen that the polarisation now has the form:

\[
P = P(\omega_0) + P(\omega_0 - \omega_k) + P(\omega_0 + \omega_k)
\]
An oscillating polarisation will re-emit light at its oscillation frequency, and thus light is reradiated at frequencies of $\omega_0$ and $\omega_0 \pm \omega_k$.

The first of these terms is the Rayleigh scattering, and the second is the Raman scattering. An increase in the frequency results in anti-Stokes Raman scattering, whereas a reduction in frequency results in Stokes Raman scattering. The efficiency of Raman-scattering of light depends on the Raman activity. If there is no change in the polarisability for a given vibrational transition, then the transition is not Raman-active. In three dimensions, change in the polarisability tensor determines the activity, and the vibration is Raman-active if any one of the polarisability tensor components is changed during the vibration (Ayars et al., 2001).

As, at room temperature, it is easier to generate a vibrational quatum that do annihilate one, the Raman spectrum is commonly recorded as the Stokes line intensity as a function of wavenumber or Raman shift. Since the vibrations are characteristic of the molecular structure, the Raman spectrum provides a spectroscopic “fingerprint” of the material (Byrne et al., 2014). The laser (coloured in green in Figure 2.5) illuminates a sample through a microscope objective, and then the collected Raman-shifted light (coloured in red in Figure 2.5) is directed to a diffraction grating to disperse the Raman scattered beam into specific frequencies which are subsequently focused on an array of detectors, such as a high sensitivity charge coupled device (CCD). (Figure 2.5) The spectrometer is equipped with a notch or edge filter to eliminate the elastically scattered photons (Rayleigh photons) (Downes and Elfick, 2010; Notingher, 2007).

In addition to the basic Raman Effect or spontaneous Raman, various phenomena were discovered over the last few years, among them Resonance Raman (RR), occurring when the laser frequency is close to an electronic transition in the material, surface enhanced
Raman scattering (SERS), which uses a nanoscale noble metal surface to enhance the signal, coherent anti-Stokes Raman scattering (CARS), involving two coherent lasers, and Fourier Transform Raman (FT-Raman) using the Michelson interferometer (Cintă Pînzaru et al., 2004; Gala and Chauhan, 2015).

Due to its non-invasive analytical capability, Raman micro-spectroscopy has seen an increase in applications in various fields, from environmental sciences, pharmaceutical development and drug discovery, to research and diagnostics (Paudel, Raijada, and Rantanen, 2015).

**Figure 2.5:** Schematic of Raman spectrometer (reproduced with permission from G. Calado, PhD Transfer Report, Dublin Institute of Technology, 2017).

For this work, ~5000 cells were seeded and incubated on CaF$_2$ windows (Crystan Ltd, UK) for 48 hrs in order to achieve a final number of approximately $10^4$ cells. Medium was removed and samples were rinsed twice with sterile PBS then fixed using formalin (4 %, 15 min). In order to optimise sample preparation protocols, spectra were recorded in immersion and after air drying in ambient atmosphere.
A Horiba Jobin-Yvon LabRAM HR800 spectrometer (Figure 2.6) with a 785 nm, 300 mW diode laser as source, Peltier cooled 16-bit CCD (charge-coupled device), 300 lines/mm grating and 100 μm confocal hole, was used. Spectra were acquired from three cell locations: cytoplasmic, nuclear and nucleolar, with an acquisition time of 30 s two times each, in the range from 400 cm\(^{-1}\) to 1800 cm\(^{-1}\) with an x100 objective (LCPlanN, Olympus N.A. 085) for dry samples and x100 objective (LUMPlanF1, Olympus N.A. 1) for immersed ones, to finally produce a data set of 90 points per cell location for each cell line (optimisation part) and 30 points per cell location for each cell line for the study.

**Figure 2.6:** Dual Raman spectrometer, FOCAS Research Institute (DIT-FOCAS, 2015).

As the formalin fixation process has been shown to have a negligible effect on the Raman spectra, and to best preserve the biochemical integrity of the cells compared to other fixation techniques (alcohol, desiccation, air drying, acetone, mixture of chloroform, methanol and acetic acid) (Draux et al., 2010; Kann et al., 2015; Mariani et al., 2009; Meade et al., 2010), this process was used and cells were fixed on CaF\(_2\) windows. To optimise the experimental conditions for Raman spectroscopic profiling, measurement in immersion was compared to measurement of dried samples in air. Measurement of tissue samples in immersion has been demonstrated to minimise background due to stray
scattered light (Bonnier et al., 2011). Spectra from nucleolus, nucleus and cytoplasm were recorded from the two cell lines, A549 and Calu-1, for both immersion and dry conditions. After data pre-processing, the average spectra were compared and principal component analysis (PCA), an unsupervised multivariate analysis which allows the reduction of the number of variables in a multidimensional dataset, was employed as supervised multivariate analysis approach to analyse the data from A549 and Calu-1 cells independently, and then to compare the two cell lines.

![Image of spectra](image)

**Figure 2.7:** Mean spectra of A. Nucleolus, B. Nucleus and C. Cytoplasm of A549 cell line. Highlighted regions correspond to DNA/RNA and lipids features.

Figure 2.7 shows the three average spectra corresponding to each cellular region for A549 cells. Visibly, it can be seen that the mean spectra of the nucleolar, nuclear and cytoplasmic regions are somewhat different, and discriminating peaks (indicated by shaded regions in Figure 2.4) at, for example, 1580, 1094, 833 and 795 cm\(^{-1}\) can be allocated to nucleic acids which correspond respectively to vibrations of the DNA Base adenine and guanine, DNA PO\(^2\) symmetric stretching, ribose phosphate and DNA
backbone O-P-O stretching (Oshima et al., 2010; Pijanka et al., 2013; Zhang et al., 2008), while others at ~1300 cm\(^{-1}\) (CH deformation) are associated with lipid compounds which are more intense in the cytoplasmic than the nuclear region. The DNA bands at 1095 cm\(^{-1}\) and 833 cm\(^{-1}\) indicate that the DNA is predominantly in the B form (Notingher et al., 2002).

A.

B.

**Figure 2.8:** PCA nucleolus, nucleus and cytoplasm in immersion conditions A. A549 cell line and B. Calu-1 cell line.

Cytoplasm ● Nuclear ● Nucleolar
Figure 2.8 shows that the three cellular regions are well differentiated by PCA and the same discriminants peaks were found in loading 1 of PCA of the nucleolus, nucleus and cytoplasm. (For all PCA Figures, the loadings are off set for clarity, the dashed horizontal line indicating zero loading).

Furthermore, Loading 2 (Figure 2.8), corresponding to PC2, which separates the nucleolar and nuclear regions, exhibits discriminating negative peaks related to nucleoli at 782 and 1336 cm\(^{-1}\) (Uracil, Cytosine and Thymine) corresponding to RNA (Hobro et al., 2007), 1242 cm\(^{-1}\) (Amide II), 1480 cm\(^{-1}\) (Guanine, Adenine) and 1578 cm\(^{-1}\) (proteins) due to the contribution of surrounded membrane, as well as positive ones at 728 (Adenine), 830 cm\(^{-1}\) (O-P-O asymmetric stretching) and 1095 cm\(^{-1}\) (DNA PO\(_2^+\) symmetric stretching), corresponding to the nuclear region, which is the primary location of DNA, while the nucleolar regions contain RNA and small quantities of DNA (Bonnier and Byrne, 2012; Movasaghi, Rehman, and Rehman, 2007).

According to Figure 2.9, which shows the PCA of A549 and Calu-1 measured both in immersion and dry, it can be seen that there is differentiation of the spectra of nucleolar, nuclear and cytoplasmic regions under both experimental conditions, clearly illustrating that Raman is able to differentiate between the three cellular compartments.

The important discrimination percentage is due to PC1 and, according to the corresponding loading (loading 1), the discriminant features derive from DNA, 795 cm\(^{-1}\) and 1095 cm\(^{-1}\), related to DNA form and DNA PO\(_2^+\) symmetric stretching, and lipids at 1300 (CH\(_2\) stretching) and 1440 cm\(^{-1}\) (CH stretching).

Some studies have shown that the air drying step causes spectral distortions and deviations giving different results (Draux et al., 2010). In Figure 2.9, however, it is noticeable that there is similar spectral distribution of nucleolar, nuclear and cytoplasmic regions for both cells lines, and furthermore that the features in loading 1 corresponding
to DNA (negative ones) and lipids (positive ones) are the same for both cell lines, confirming that differentiation is based on cellular regions rather than on the basis of sample preparation conditions.

A.

Figure 2.9: PCA immersion and dry A. A549 and B. Calu-1, (a) nucleolus, (b) nucleus and (c) cytoplasm.

Cytoplasm ● Nuclear  ● Nucleolar  ● immersion conditions

Cytoplasm ● Nuclear  ○ Nucleolar  ● dry conditions

In addition, PC2 clearly separates the two dry and immersion conditions, but the corresponding loading 2 is noisy and symmetric which means that the difference is
derived only from background due to the fact that different experimental conditions were employed.
Moreover, it has been demonstrated that recording spectra in immersion conditions improves the signal to background ratio in Raman spectra (Bonnier et al., 2011). However, using a well-adapted pre-processing protocol for background subtraction can similarly improve the signal to background ratio (Beier and Berger, 2009; Bonnier and Byrne, 2012; Cadusch et al., 2013). Thus, seeing that immersion and dry conditions give comparable results, and due to the fact that immersion presents the inconvenience of contaminations (dust, bacteria…) and samples cannot be used for a long time in comparison to dry ones, dry conditions will be used for the rest of the experiments.

For DOX experiments, cells (~ 1x 10⁴/window) were seeded and incubated on CaF₂ windows (Crystan Ltd, UK) for 24 hrs for both control and exposure to DOX. The medium was then removed and samples were rinsed twice with sterile PBS and covered with DOX at each corresponding IC₅₀ inhibitory concentration, adjusted to the cell number, for exposed cells and fresh medium for unexposed controls. After each incubation period, 2, 6, 12, 24, 48 and 72 hrs, cells were washed twice with sterile PBS and fixed in formalin (4 %, 15 min).
Spectra were acquired in same experimental conditions as the optimisation part, in dry conditions, from three cell locations: cytoplasm, nucleus and nucleolus, visible under white light illumination. A final data set of 30 points per cell location for each time point, 2, 6, 12, 24, 48 and 72 hrs was produced after DOX exposure and for control cells, for each cell line, amounting to a total of over 210 cells per cell line, corresponding to a total data set of 1260 spectra.
For ACT experiments, the same number of cells (~1x10^4/window) as DOX experiments were seeded and incubated on CaF$_2$ windows for 24 hrs for both control and exposure to ACT. The medium was then removed and samples were rinsed twice with sterile PBS and covered with ACT at each corresponding IC$_{50}$ for exposed cells and fresh medium for unexposed controls. After each incubation period, 48 and 72 hrs, exposed and unexposed control cells were washed twice with sterile PBS and fixed in formalin (4%, 15 min). Spectra were acquired using the same experimental conditions from three cell locations: cytoplasm, nucleus and nucleolus, identifiable under white light illumination. The final data set of a total of 720 spectra was thus derived from 30 cells, each measured in the nucleolus, nucleus and cytoplasm, for each exposure time (2) and control (2), for each cell line (2).

For all Raman experiments, spectral pre-processing and analysis were performed in Matlab2013 using algorithms developed in house. Prior to data analysis, consisting of PCA, LDA (Linear Discriminant Analysis), ICA (Independent Component Analysis) and PLSR (Partial least Square Regression), background was subtracted using a NCLS (non-negatively constrained least squares) algorithm using a CaF$_2$ spectrum and spectra were smoothed (Savitsky-Golay filter 3th order, 11 points), baseline corrected (fifth order polynomial) and vector normalised.

PCA, one of the most popular and oldest multivariate analysis technique (Abdi and Williams, 2010), allows the reduction of the number of variables in a multidimensional dataset, although it retains most of the variation within the dataset. It represents the spectra in data groupings of similar variability, allowing the identification and differentiation of different spectral groups.

In order to examine the relationships among a set of correlated variables, the original set of variables is transformed into a new set of uncorrelated variables called principal
components. These new variables are linear combinations of the original variables and are derived in decreasing order of importance (Chatfield and Collins, 1980).

Mathematically speaking, a data set is represented in terms of an $m \times n$ matrix, $X$, in which the $n$ columns are the samples or observations and the $m$ rows are the variables. This matrix is linearly transformed into another matrix $Y$, also of dimension $m \times n$, so that for some $m \times m$ matrix, $P$, $Y = PX$. This equation represents a change of basis and the rows of $P$ are considered to be the row vectors $p_1, p_2, \ldots, p_m$, and the columns of $X$ to be the column vectors $x_1, x_2, \ldots, x_n$, such that it can then can be interpreted in the following way, where $P$ become the principal component directions (Richardson, 2009):

$$PX = \begin{pmatrix} P_{x_1} & P_{x_2} & \cdots & P_{x_n} \\ \end{pmatrix} = \begin{pmatrix} p_1 \cdot x_1 & p_1 \cdot x_2 & \cdots & p_1 \cdot x_n \\ p_2 \cdot x_1 & p_2 \cdot x_2 & \cdots & p_2 \cdot x_n \\ \vdots & \vdots & \ddots & \vdots \\ p_m \cdot x_1 & p_m \cdot x_2 & \cdots & p_m \cdot x_n \\ \end{pmatrix} = Y$$

The loadings of the PCs represent the variance for each variable (wavenumber) for a given PC, and analysis of the loadings can give information about the source of the variability inside a dataset, derived from variations in the molecular components contributing to the spectra. It has been demonstrated that the PC loadings can be most simply understood when analysis of datasets is undertaken in a pairwise fashion (Bonnier and Byrne, 2012).

PCA does not cluster the data, per se, in the same manner as for example Hierarchical or K-Means Cluster Analysis, whereby differential distribution of the data according to negative or positive loadings associates specific spectral features with that dataset. PCA was employed as an unsupervised multivariate approach to analyse data and the effects of ACT in each cell localisation by identification and differentiation of different spectral groups using scatter plots and loadings showing a representation of spectral features responsible for the variance between data groups according to wavenumbers. The order
of the PCs denotes their importance to the dataset and PC1 describes the highest amount of variation (Bonnier and Byrne, 2012; Brauchle and Schenke-Layland, 2013; Pavićević et al., 2012).

ICA an unsupervised statistical technique, employed as an extension to PCA, which is able to identify latent variables called independent components in each data set separately. In the case of Raman micro spectroscopy, ICA can be used to identify spectral contributions such as those from a substrate, which can then be removed or studied in their own, using the same number of ICs (three components) as PCs, as estimated by PCA, explaining the majority of variance, and only the first component for each cellular compartment was plotted (Farhane, Bonnier, Maher, et al., 2017; Hyvärinen and Oja, 2000; Lee, 1998).

LDA was proposed by R. Fischer in 1936 (Xanthopoulos, Pardalos, and Trafalis, 2013) and is a well-known data analysis technique for feature extraction and dimension reduction, used mostly after dimension reduction by PCA (Ye, Janardan, and Li, 2005). In this study, LDA performed on each of the datasets scores independently and a 10-fold cross validation was performed to produce confusion matrices (Gutman et al., 2013; Riedl, Esslinger, and Fauhl-Hassek). LDA accuracy was calculated using a 10-fold cross validation on increasing numbers of latent variables (PC scores). The classification which resulted in the maximum accuracy while keeping the number of latent variables to a minimum was chosen for all successive models.

PLSR, is a statistical regression technique, introduced in the early 1980s (Helland, 2004), which reduces the dimensionality of the data and correlates information in an X data set matrix to the matrix of a Y data according to the equation $\mathbf{Y} = \mathbf{XB} + \mathbf{E}$, where $\mathbf{B}$ is a matrix of regression coefficients and $\mathbf{E}$ is the regression residual. In this work, the X-matrix is represented by the Raman spectra and the Y-matrix is consisted of values of the DOX
concentration inside the cells, and the time evolution (Keating et al., 2015; Muratore, 2013; Nawaz et al., 2011).

The percentage of variance explained as a function of the number of components was calculated using 10-fold cross validation. The regression coefficients obtained by PLSR of Raman data regressed against time were plotted and analysed. As a function of frequency, the co-efficients illustrate the spectral features which are influenced by the intracellular interaction of the chemotherapeutic drug and the resulting metabolic changes (Farhane, Bonnier, Howe, et al., 2017).
2.7. References:


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Chapter 3: Cellular discrimination using *in vitro* Raman microspectroscopy: the role of the nucleolus

The following chapter reproduces the journal article, Z. Farhane, F. Bonnier, A. Casey, A. Maguire, L. O’Neill, and H.J. Byrne. Analyst, 2015, **140**, 5908, in which section and figure numbers have been adapted to the prescribed thesis format.

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A.M. assisted in the PCA-LDA classification. A.C. and F.B. assisted in the experimental design.

H.J.B. assisted in the project design and proofing of the manuscript.
Cellular discrimination using *in vitro* Raman micro-spectroscopy: the role of the nucleolus

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**Abstract:**

Raman micro-spectroscopy has attracted considerable attention over the last few years to explore its possible clinical applications as a non-invasive powerful label-free *in vitro* screening tool in cancer diagnosis and monitoring, subcellular analysis of biochemical processes, drug uptake, mode of action and mechanisms of interaction as well as toxicity of, for example, chemotherapeutic agents.

However, in order to evaluate accurately the potential of Raman micro-spectroscopy for such applications it is essential to optimise measurement and data processing protocols associated with subcellular analysis. To this end, *in vitro* differentiation of cell lines is a basic proof of concept for the potential of the technique, and although many studies have indicated successful differentiation based on Raman micro-spectroscopy, it is important, as the measurement and processing techniques are improved, to establish the biochemical and subcellular basis of that discrimination.

In this study, Raman micro-spectroscopy is used to compare and differentiate normal and cancer cells from human lung origin, A549 adenocarcinoma cell line, Calu-1 epidermoid
non-small-cell and BEAS-2B normal immortalized bronchial epithelium cell line. Spectra were taken from the three subcellular compartments, cytoplasm, nucleus and nucleolus and Principal Component Analysis was used to compare the spectral profiles between the cell lines and, coupled to Linear Discriminant Analysis, to explore the optimum sensitivity and specificity of discrimination. To support the analysis, Raman microspectroscopy was coupled with Flow Cytometry, Confocal Laser Scanning Microscopy and Atomic Force Microscopy.

While all subcellular regions can be employed to differentiate the normal and cancer cell lines, optimum discrimination sensitivity and specificity is achieved using the spectra from the nucleolar region alone. Notably, only the nucleolar spectral profiles differentiate the two cancer cell lines. The results point to the importance of the nucleolar regions in diagnostic applications of Raman microscopy as well as further applications in subcellular analysis of cytological processes.

**Keywords:** Raman micro-spectroscopy, cell nucleolus, cancer and normal cells, Flow Cytometry, Confocal Laser Scanning Microscopy, Atomic Force Microscopy.
3.1. Introduction:

The recent decades have seen a notable expansion in exploration of biomedical applications of Raman micro-spectroscopy (Diem et al., 2012; Downes and Elfick, 2010; Krafft and Popp, 2015), due to the fact that it is a powerful, rapid and non-destructive label-free technique for studying biological systems such as tissue and cells (J Chan et al., 2008; Movasaghi, Rehman, and Rehman, 2007).

Raman micro-spectroscopy can detect chemical, biological and physical changes of biomolecules, and the specific information contained in the cellular Raman spectrum provides a molecular fingerprint of the sample of interest, which allows Raman micro-spectroscopy to differentiate between normal and abnormal cells and tissues, indicating possible applications for example in cancer research (J Chan et al., 2008; Kong et al., 2015). Indeed, Raman micro-spectroscopy has been shown to provide high specificity and sensitivity, even for pre-cancer detection (Eikje, Aizawa, and Ozaki, 2005; Kallaway et al., 2013; Kendall et al., 2010; Rashid et al., 2014), and is non-invasive and potentially automatable, thus avoiding the disadvantages of many biomedical techniques used to identify and sort cancerous cells from normal ones, which exhibit low specificity and are destructive or perturb the cellular biology (J W Chan et al., 2006; Huser and Chan, 2015; Knipfer et al., 2014).

As an optical microscopic technique, Raman micro-spectroscopy also has the potential to probe the molecular structure on a cellular and subcellular level (Bonnier et al., 2010; Miljkovic et al., 2010). In comparison to Infrared absorption spectroscopy; Raman micro-spectroscopy offers the possibility to study biological matrices in an aqueous environment due to the weak contribution from water (Dorney et al., 2012; Notingher et al., 2002). Thus, the potential applications extend beyond disease diagnostics to the label free in
vitro screening of cytological processes, such as drug or nanoparticle uptake and mechanisms of interaction, and toxicology (Dorney et al., 2012; Ling et al., 2002; Matthäus et al., 2007; Romero et al., 2013). There has been a wide range of studies to date demonstrating the potential of Raman micro-spectroscopy to map live and fixed cells with subcellular resolution (Caponi et al., 2013; Draux et al., 2010; Krafft et al., 2005; Meade et al., 2010; Palonpon, Sodeoka, and Fujita, 2013), profile the distribution of anticancer agents (El-Mashtoly et al., 2014; Farhane et al., 2015; Feofanov et al., 2000; Nawaz et al., 2013; Wojcik et al., 2015) and nanoparticles in cells (Ahlinder et al., 2013; Bräutigam et al., 2014; Dorney et al., 2012) and monitor subcellular processes (Klein et al., 2012) and toxicological responses (Boyles et al., 2015; Casey et al., 2008; Knief et al., 2009; Wadhwa et al., 2011).

Fundamental to the development of applications of Raman micro-spectroscopy for disease diagnostics as well as analysis of cytological processes is an understanding of the variability of the spectral signatures across the subcellular environment, their potential for differentiation of cell phenotype or diseased state, and their sensitivity to external perturbation, such as viral infection, radiation damage, or chemical stress due to, for example, toxic or chemotherapeutic agents. It is clear that the signatures for the cytoplasm, nucleus and nucleoli are distinct and differentiable (Bonnier and Byrne, 2012), but it is not clear which region has the best diagnostic potential or sensitivity to external insult. The current study examines the subcellular basis of differentiation of human cell lines in vitro, both normal and cancerous, using Raman micro-spectroscopy.

Numerous studies over the last few years have investigated the capability of Raman micro-spectroscopy to differentiate between cancerous cell lines; Crow et al. (Crow et al., 2005) used human prostate cancer cell lines, Pijanka et al. investigated lung cancer cell lines (Pijanka et al., 2013), and Chen et al. explored neoplastic hematopoietic cells (J W
Chan et al., 2006), while Krishna et al. were interested in mixed cell populations of human promyelocytic leukemia and breast cancer (Krishna et al., 2005). All those studies demonstrated that Raman micro-spectroscopy, coupled with multivariate statistics, is able to distinguish between different cell lines with a high specificity and sensitivity. However, the basis of the differentiation was not elucidated in terms of the subcellular regions.

In this study, Raman profiles of three cell lines from same anatomical origin, the human lung, were compared and analysed. The first part is the investigation of the differentiation of a normal human bronchial epithelium cell line, BEAS-2B, from the adenocarcinoma human alveolar basal epithelial cell line, A549, and the non-small-cell lung cancer cell line Calu-1. Thereafter, the two cancer cell lines are compared to each other, using Raman micro-spectroscopy. The morphological and topographical characteristics of the two human lung cancer cell lines A549 and Calu-1, using Flow Cytometry, CLSM and AFM were also analysed and correlated to their Raman spectroscopic features.

In all cases, spectral profiles of the cytoplasm, nucleus and nucleoli were independently acquired and analysed, and the study highlights the importance of the nucleoli in potential diagnostic and bioanalytical applications.

3.2. Materials and Methods

3.2.1. Materials:

A549 human lung adenocarcinoma cells with the alveolar type II phenotype, and BEAS-2B normal human bronchial epithelium (ATCC® CRL-9609™), virus transformed, infected with a replication-defective SV40/Adenovirus 12 hybrid and cloned, were all obtained from ATCC (Manassas, VA, USA). Calu-1 human lung epidermoid cells were obtained from the European Collection of Cell Cultures.
SYTOX® Green Fluorescent Nucleic Acid Stains and Wheat Germ Agglutinin conjugates (WGA), a cell membrane glycoconjugates binder, purchased from BioSciences (Ireland, suppliers for Life Technologies), were employed to image the nuclear compartment and cytoplasmic membrane of cells using Confocal Laser Scanning Microscopy.

3.2.2. Cell culture:

A549 and BEAS-2B cells were cultured in DMEM (with 2 mM L-glutamine) and Calu-1 in RPMI, all with 10% foetal bovine serum (FBS) at 37°C in an humidified atmosphere containing 5% CO₂ and cells were split every two days to maintain ~60% confluence.

3.2.3. Sample preparation and measurement protocols:

3.2.3.1. Raman micro-spectroscopy:

Cells~5000 were seeded and incubated on CaF₂ windows (Crystan Ltd, UK) for 48 hrs in order to achieve a final number of approximately 10⁴ cells. Medium was removed and samples were rinsed twice with sterile PBS then fixed using formalin (4 %, 15 min) and spectra were recorded after air drying in ambient atmosphere.

A Horiba Jobin-Yvon LabRAM HR800 spectrometer with a 785 nm, 300 mW diode laser as source, Peltier cooled 16-bit CCD, 300 lines/mm grating and 100 μm confocal hole, was used for this work. Spectra were acquired from the three cell locations: cytoplasm, nuclear and nucleolar in the range from 400 cm⁻¹ to 1800 cm⁻¹ with an x100 objective (LCPlanN, Olympus) for 30 s two times, to finally produce a data set of 30 points per cell location for each cell line, over a total of 90 different cells.
3.2.3.2. Confocal Laser Scanning Fluorescence Microscopy (CLSM):

Samples were prepared on uncoated glass bottom Petri dishes (MatTek Corporation, USA). Approximately $10^4$ Cells were allowed to attach for two hrs, then covered with cell culture medium. After incubation, the medium was removed and samples were rinsed twice with sterile PBS and fixed in formalin (4%, 15 min).

After fixation, samples were washed with Hank’s balanced salt solution (HBSS) and WGA (7.5 µL of WGA per 1 mL HBSS) was first added and cells were incubated for 20 min and washed with HBSS. Sytox® green nucleic acid stain (2 µL per 1 mL of HBSS) was thereafter added, cells were incubated for 30 min and washed with HBSS then imaged in HBSS.

Confocal Laser Scanning microscopic images were recorded using an inverted Zeiss LSM 510 confocal laser scanning microscope equipped with a x60 oil immersion objective. Excitation wavelengths used were 488nm for Sytox® green nucleic acid stain (emission wavelength 523 nm) and 633/647 nm excitation/emission for WGA.

3.2.3.3. Flow Cytometry:

Cells were cultured in T75 flasks over 48 hrs and then trypsinised and centrifuged in 10 mL fresh medium at 4 °C and 1000 rpm for 5 min. After this, the cells were re-suspended with 5 mL PBS and only 2 mL was used for analysis. Samples were prepared in parallel and the two cell lines were analysed separately. A total number of $2 \times 10^4$ cells was analysed for each sample. The experiments were conducted in triplicate (three independent experiments).
Flow cytometry analysis was performed using a BD Biosciences Accuri C6 Flow Cytometer (Becton Dickinson, Oxford, UK) without any dyes.

3.2.3.4. Atomic Force Microscopy (AFM):

The air dried samples on CaF$_2$ substrates were profiled using a MFP-3D BIO AFM (Asylum Research). The cantilevers used were Olympus silicon AC240. Tips were 160nm long and had a typical resonant frequency of 70 kHz. The AFM was operated in AC mode in order to minimize tip/sample interaction. Typical free air amplitudes were ~200mV and a high amplitude set-point relative to the set-point was maintained to minimize sample damage. The images obtained, from 5 to 10 cells per cell line, contained 1024 pixels per scan line and only one representative image of a typical cell for each cell line is shown.

3.2.4. Data analysis:

Raman spectra pre-processing and analysis were performed in Matlab 2013 using algorithms developed in house.

Prior to analysis, spectra were smoothed (Savitsky-Golay filter 5th order, 7 points), baseline corrected, substrate background subtracted, using a polynomial method home developed, and vector normalised.

After pre-processing, PCA (principal component analysis) and PCA-Linear Discriminant Analysis (PCA-LDA), powerful approaches commonly used for the analysis of large spectral data sets, were employed as supervised multivariate analysis tools to differentiate the data recorded from different subcellular localisations and cell lines. PCA allows the reduction of the number of variables in a multidimensional dataset, the order of the PCs
denoting their importance in the dataset where PC1 describes the highest amount of variation. While PCA identifies differences between the data sets, LDA maximizes these differences so as to group similar spectral sets. PCA performs a feature reduction of the data and LDA classifies the data into one of two or more classes. Thus, if a group of spectra have a similar correlation to the shape defined by PC1 and that defined by PC2; they are classified as same class.

PCA was performed on each of the datasets independently (nucleoli, nucleus, and cytoplasm). LDA was then performed on each of the datasets scores independently and a 10-fold cross validation was performed to produce confusion matrices (Gutman et al., 2013; Riedl, Esslinger, and Fauhl-Hassek). LDA accuracy was calculated using a 10-fold cross validation on increasing numbers of latent variables (PC scores) for the classification of all cancer types and normal cells. The classification which resulted in the maximum accuracy while keeping the number of latent variables to a minimum was chosen for all successive models. In the case of the classification of all cancer cells and normal one, from a plot of accuracy as a function of increasing number of latent variables, the maximum accuracy was found when 4 principal components were used in the classification. Further addition of principal components only increased the complexity of the model without further improving the performance.

The Accuri Flow cytometry software was used for the initial analysis of flow cytometry samples, but the Beckman Coulter Summit software and the FCS Express Research Edition have been used for the reanalysis of the samples. The QC control of the instrument was performed using Spherotech 6 and 8 peak beads.
3.3. Results and discussion:

3.3.1. Raman micro-spectroscopy:

Raman spectra were taken from the three cell compartments for all cell lines and Figure 3.1 shows the average spectra corresponding to each cell region for Calu-1 cells. Visibly, it can be seen that the mean spectra of the nucleolar, nuclear and cytoplasmic regions are somewhat different, and discriminating peaks (indicated by highlighted regions in Figure 3.1) at, for example, 1578, 1095, 830 and 795 cm$^{-1}$ can be allocated to nucleic acids which correspond respectively to vibrations of the DNA bases adenine and guanine, DNA PO$_2^-$ symmetric stretching, ribose phosphate and DNA backbone O-P-O stretching (Oshima et al., 2010; Zhang et al., 2008), while others at 1300 (CH deformation) and 717 cm$^{-1}$ (CN+(CH$_3$)$_3$ stretching) are associated with lipid components of the cell membrane, which are also observable in subsequent spectra of the nuclear and nucleolar regions, although more prominently in the spectra of the cytoplasmic region. The DNA bands at 1095 cm$^{-1}$ and 830 cm$^{-1}$ indicate that the DNA is in B form and one at 813 cm$^{-1}$ corresponds to DNA A form (Notingher et al., 2002).
Figure 3.1: Mean spectra of Nucleolus (A), Nucleus (B) and Cytoplasm (C) of Calu-1 cell line. Highlighted regions indicate discriminatory features.

Similar profiles were obtained for the subcellular analysis of A549 cells.

For a more detailed understanding of the differences in spectral profiles of the subcellular regions, a multivariate analysis is more informative, and therefore, PCA was employed to analyse and compare the spectral profiles of the subcellular regions as well as the differences between cancer and normal cell lines. Figures representing the differentiation of the spectra by PCA according to the corresponding PC loadings were plotted and, for clarity, in all PCA figures, the loadings are off set, the dashed horizontal line in all cases indicating zero loading.

As an illustration of the analysis technique used throughout, Figure 3.2 A shows the PCA scatter plot of the cytoplasmic, nuclear and nucleolar spectra of the Calu-1 cell line, along with the corresponding loadings of PC1 and PC2. PC1 clearly differentiates between the cytoplasmic and integrated nuclear regions (nuclear and nucleolar) and, according to the corresponding loading (loading 1), the most visible discriminant features derive from DNA, 795 cm$^{-1}$ and 1095 cm$^{-1}$, related to DNA form and DNA PO$_2^-$ symmetric stretching.
and lipids at 717 cm\(^{-1}\) (CN+(CH\(_3\))\(_3\) stretching), 1300 (CH\(_2\) stretching) and 1440 cm\(^{-1}\) (CH stretching). The differentiation of the subcellular regions according to PC1 is not unexpected, due to the significant biochemical differences between the combined nuclear and cytoplasmic regions which are similar for all cell lines.

Although some degree of discrimination of the nuclear and nucleolar regions according to PC2 is evident in Figure 3.2 A, a better visualisation of this is achieved by a direct pairwise PCA of the two datasets, as shown in Figure 3.2 B. PC1 now clearly differentiates the nucleolar and nuclear regions. It exhibits discriminating negative peaks related to nucleoli at 782 and 1336 cm\(^{-1}\) (Uracil, Cytosine and Thymine) corresponding to RNA (Hobro et al., 2007), 1242 cm\(^{-1}\) (Amide II), 1480 cm\(^{-1}\) (Guanine, Adenine) and 1578 cm\(^{-1}\) (proteins) due to the contribution of surrounded membrane, as well as positives ones at 728 (Adenine), 830 cm\(^{-1}\) (O-P-O asymmetric stretching) and 1095 cm\(^{-1}\) (DNA PO\(_2^-\) symmetric stretching), corresponding to the nuclear region, which is the primary location of DNA, while the nucleolar regions contain RNA and a small quantities of DNA (Bonnier and Byrne, 2012; Movasaghi, Rehman, and Rehman, 2007). Similar differentiation can be achieved for the subcellular regions of A549 cells (data not shown).
Figure 3.2: A. PCA scatter plot of nucleolar, nuclear and cytoplasmic regions of Calu-1 cells, with corresponding loadings of PC1 and PC2. B. PCA of nucleolar and nuclear regions of Calu-1 cells and corresponding loading of PC1.

Cytoplasm ● Nucleus ● Nucleolus ●

In a similar fashion, PCA was employed to compare the spectral profiles of the normal lung human cell line BEAS-2B to the two lung cancer cell lines A549 and Calu-1.
Figure 3.3: PCA of nucleolar, nuclear and cytoplasmic regions of A. A549, Calu-1 and BEAS-2B with corresponding loadings of PC1 and PC2 B. PCA of each cell localisation for A549, Calu-1 and BEAS-2B and the corresponding loadings of PC1.

Cytoplasm ● Nucleus ○ Nucleolus ● A549 cell line

Cytoplasm ▼ Nucleus ▼ Nucleolus ▼ Calu-1 cell line

Cytoplasm ■ Nucleus ■ Nucleolus ■ BEAS-2B cell line
The PCA scatter plot of Figure 3.3 A compares the three subcellular regions of all three lung cell lines. PC1 largely differentiates between the cytoplasmic regions and the integrated nuclear and nucleolar regions of the cells, and the corresponding loading exhibit features related to nucleic acid (DNA and RNA) and lipids, similar to the loading of PC1 in Figure 3.2 A.

Accounting for 21% of the explained variance, PC2 indicates a clear differentiation between the normal cell line and the two cancerous ones. Although the loading of PC2 represents differentiating features of all three subcellular regions, identifiable features include negative peaks at 669 (Thymine and Guanine), 728 (Adenine), 1095 cm\(^{-1}\) (DNA PO2- symmetric stretching) and ones at 1005 (Phenylalanine), 1320 (Guanine), 1440 (Guanine and Adenine), and 1665 cm\(^{-1}\) (Amide I), associated with the cancer cell lines. Positive peaks corresponding to lipids at 760 and 820 (tryptophan ring), 1115 (C-C stretching), 1250 and 1420 cm\(^{-1}\) (lipids C=C and C-H vibration) are associated with the normal cell line. A similar scatterplot and loadings profile, with separation between integrated nuclear region and cytoplasmic area according to PC1 and differentiation of normal and cancer cell line according to PC2 is observable when cell lines were analysed pairwise, A549 versus BEAS-2B and Calu-1 against BEAS-2B (Figure S.3.1, Supplemental Information).

The PCA discriminating features between normal and cancer cells correspond to DNA and proteins for cancer cell lines and lipids for the normal one, which is consistent with the fact that cancerous cells have a more active metabolism and exhibits more proteins and more DNA than normal cells but present less cytoplasm and therefore lipids are positive discriminating features for normal cells.

The differentiation of the cell lines is more evident when the subcellular regions are analysed independently using PCA, as shown in Figure 3.3 B. The normal and cancerous
cell lines are now largely discriminated by PC1 and almost the same discriminating features can be found in the loadings of PC1 for the nucleolar and nuclear regions, while additional peaks in the loading of PC1 for the cytoplasm of the normal versus cancerous cells are observed at 760 and 820 (tryptophan ring) and 1250 (Amide III), related to normal cells and ones at 717 (CN+ (CH₃)₃ stretching), 1400 (CH deformation) and 1578 cm⁻¹ (protein) 1661 cm⁻¹ (lipids C=C stretching) related to cancerous cell lines (Farhane et al., 2015; Movasaghi, Rehman, and Rehman, 2007; Notingher, 2007; Notingher et al., 2002).

Thus using PCA allows a separation between normal and cancerous cell lines. Notably, however, no differentiation of the cancerous cell lines is evident. To investigate this further, the cancer cell lines were compared to each other and spectra from the nucleolus, nucleus and cytoplasm were similarly analysed.

As shown in Figure 3.4 A, a scatter plot of the combined PCA of the nucleolar, nuclear and cytoplasmic regions of the A549, Calu-1 cancer cell lines, there is no clear separation between the two cells. As before, PC1 differentiates between the cytoplasmic and combined subcellular regions, but, in contrast to the comparison of the normal and cancerous cell line, there is no clear differentiation between the two cell lines. Indeed, PC1 discriminates between the cytoplasm and the combined nuclear region, for both cell lines and the corresponding loading exhibits features corresponding to lipids, 717 and 873 cm⁻¹ (CN⁺(CH₃)₃, 1270 and 1303 cm⁻¹ (C–H vibrations) and 1450 (CH₂ deformation) and ones related to DNA at 784 (Cytosine and Thymine), 1095 (DNA PO2⁻ symmetric stretching) and 1578 cm⁻¹ (Guanine, Adenine). PC2 partially discriminates the nucleolar and nuclear regions and exhibits discriminating negative peaks for the nucleolar region at 784 and 1336 cm⁻¹ (Uracil, Cytosine and Thymine) corresponding to RNA, 1242 cm⁻¹ (Amide II), 1480 cm⁻¹ and 1578 cm⁻¹ (Guanine, Adenine), as well as positives ones at 728
(Adenine), 830 cm\(^{-1}\) (O-P-O asymmetric stretching) and 1095 cm\(^{-1}\) (DNA PO\(_2\) symmetric stretching), corresponding to the nuclear region (Bonnier and Byrne, 2012; Movasaghi, Rehman, and Rehman, 2007).

Therefore, it appears that Raman micro-spectroscopy is unable to differentiate between cancerous cells, although it is able to distinguish the subcellular regions for all cell lines. Nevertheless, previous studies have demonstrated the ability of Raman micro-spectroscopy to distinguish between different cancer cell lines (J W Chan et al., 2006; Chen et al.; El-Mashtoly et al., 2014; Pijanka et al., 2013; Rashid et al., 2014; Tolstik et al., 2014). To explore the apparent discrepancy in results, the individual subcellular regions were further analysed (Figure 3.4 B, C and D).

Results show no indication of discrimination between the two lung cancerous cells for the nuclear and cytoplasmic areas (Figure 3.4 C and D) although there is some degree of differentiation according to PC1 (35% of variation) for the nucleolar regions of A549 and Calu-1 (Figure 3.4 B). Discriminant features of the loading of PC1 include negative peaks at 1270 (RNA Uracil and cytosine ring stretching), 1320 and 1450 (CH\(_2\) deformation) and 1661 cm\(^{-1}\) (Lipids C=C stretching). The same features are found to be higher in the mean spectra of nucleolar A549 compared to Calu-1 (Figure 3.5), indicating that there are more lipids in the former.

Also prominent in the loading of PC1 are two strong positive peaks at 784 cm\(^{-1}\) (Uracil, Cytosine and Thymine) and 811 cm\(^{-1}\) (RNA O-P-O phosphodiester bond) as well as ones at 1240 (Amide III), 1480 and 1578 cm\(^{-1}\) (Guanine, Adenine), which indicate that there is a stronger RNA contribution in Calu-1 cells than A549. This can be clearly seen in the mean nucleolar spectra in Figure 3.5.
Figure 3.4: PCA scatterplots and corresponding loadings of PC1 and PC2 of A549 and Calu-1 cells: A. nucleolus, nucleus and cytoplasm B. nucleolus, C. nucleus D. cytoplasm.

Cytoplasm ● Nucleus ○ Nucleolus ● A549 cell line
Cytoplasm ▼ Nucleus ▼ Nucleolus ▼ Calu-1 cell line
Figure 3.5: Mean spectra of A. nucleolus of A549 (green) and Calu-1 (black), B. nucleus of A549 (red) and Calu-1 (magenta) and C. cytoplasm of A549 (blue) and Calu-1 (cyan).
Moreover, according to the mean spectra, it seems that the most notable differences in nuclear and nucleolar regions between A549 and Calu-1 corresponds to the features at 813 cm\(^{-1}\) (DNA A form) and at 830 cm\(^{-1}\) (DNA B form).

The ratio between the two peaks (enlargement Figure 3.5) is inverted which signifies that there is more DNA B form in A549 than in Calu-1 and vice versa.

To support Raman micro-spectroscopy observations, Confocal Laser Scanning Microscopy, Flow Cytometry and Atomic Force Microscopy were in their turn employed to compare the two human lung cancer cell lines.

### 3.3.2. Confocal Laser Scanning Fluorescence Microscopy (CLSM):

CLSM was employed in order to identify the morphological characteristics of the two cell lines, A549 and Calu-1. As shown in Figure 3.6, before and after nucleic and cytoplasmic staining, the two cell lines present a different shape and size, as well as numbers of nucleoli, which are clearly resolved within the nucleus and have diameters ~2-4 μm.

Indeed, WGA, a cytoplasmic membrane stain which delimits the cytoplasmic membrane, highlights the shape of cells and shows that Calu-1 are bigger and longer than A549 cells, while Sytox\(^{®}\) green, a specific nuclear dye which has a high affinity for DNA, shows that Calu-1 cells present a higher density of nucleoli and a larger nuclear area than A549 cells.
Figure 3.6: Confocal fluorescence images of A. A549 and B. Calu-1 stained with Sytox® green nucleic acid stain and WGA.

3.3.3. Flow Cytometry:

As shown in Figure 3.7, the flow cytometry 2D scatter plots are different from one cell line to another, indicating that Calu-1 cells are more spread and bigger in size than A549. This is confirmed in the histograms; Calu-1 (red) and A549 (green) show a difference in cell surface and size (FSC forward scatter) and cell granularity and internal environment
(SSC side scattering) between the two cell lines, confirming that Calu-1 cells present larger size and dispersive granularity of the nucleus, which explains the most significant percentage of cellular side scattering (Marina, Sanders, and Mourant, 2012), than A549, consistent with the confocal microscopy results.

**Figure 3.7:** Flow cytometry 2D scatter plots (FSC vs SSC) with debris excluded and histograms ((FSC vs cell number) and (SSC vs cell number)) for both cancer cell lines A549 (green) and Calu-1 (red).

### 3.3.4. AFM:

AFM shows that both cell lines have different topography. Indeed, according to Figure 3.8 A549 cells are higher and more convex than Calu-1 (Height 1.4 µm for A549 compared to 1.2 µm for Calu-1). Additionally, it can be seen from the structure of the height profiles that Calu-1 cells have more surface granularity, consistent with larger number of nucleoli, which confirms the CLSM and flow cytometry results.
Figure 3.8: AFM images of A. A549 and B. Calu-1: 3D image, height scan and topographical profile generated for height scan; respectively.

According to Flow Cytometry (SSC maximum 500,000 for A549 and 1,000,000 for Calu-1) and AFM (Figure 3.7 and 3.8), A549 cells are thicker than Calu-1 but present fewer and smaller nucleoli, which is confirmed by CLSM. Nucleoli size for both cell lines is by the order of 2-4 µm, larger than the Raman laser spot (1 µm) and, according to Raman micro-spectroscopy; RNA is more prominent in Calu-1 nucleoli than those of A549.
3.3.5. PCA-LDA:

Thus, CLSM, Flow Cytometry and AFM show that A549 and Calu-1 cells are different in size, morphology and topography. Raman micro-spectroscopy was unable to differentiate between them with high sensitivity according to the biochemical fingerprint of the cytoplasmic or nuclear regions alone. However, differentiation of cell lines is achievable according to the spectroscopic signature of the nucleoli.

Nucleoli are non-membrane-bound nuclear compartments, well described and analysed in details since the last millennium (Jarboui et al., 2011; Visintin and Amon, 2000). They are responsible for ribosome biogenesis and diverse cellular functions and processes such as cell cycle control, cellular stress response, nuclear export and sequestration of key proteins regulators of cell-cycle activity and response to apoptosis and early stage cellular response to toxic such as chemotherapeutic drugs (Farhane et al., 2015). The size, number and organisation of nucleoli are cell-specific and nuclear proteins, notably histone, play an important role in the nucleoli stability and functions (Hinsby et al., 2006; Olson, Dundr, and Szebeni, 2000).

The important role played by nucleoli and its cell specificity explain the fact that it is spectroscopically the discriminant factor between cancer cell lines. For confirmation, PCA was coupled with LDA and confusion matrices were generated using 4 principal components and, in the first instance, nucleolar and nuclear areas were analysed in combination. The results shown in Table 3.1 A indicate a sensitivity and specificity of between 40 and 60 % for the two cancerous cell lines and 91.8 % specificity and 93.2 % specificity for the normal cells, BEAS-2B, which confirms, along with the LDA plot in Figure 3.9, that poor discrimination between the cancerous cell lines is achievable. To
improve the specificity and sensitivity, the nucleolar and nuclear regions were analysed separately.

It is notable that in the confusion matrix of Table 3.1 B, for the nucleolar regions of BEAS-2B, the sensitivity and specificity are 100% (separation is obvious in Figure 3.9 B). A clearly better separation for the two cancerous cell lines is achievable for the nucleolar region, and a similar amelioration can be observed for the nuclear region (Table 3.1 C), although the specificity and sensitivity remain lower than that of the nucleolar region. Significantly lower sensitivities and specificities were obtained for the cytoplasmic regions of the cancer cell lines (Table 3.1 D). The results are supported by the PCA-LDA plot in Figure 3.9.

In summary, Raman micro-spectroscopy coupled with both PCA and PCA-LDA highlights the critical importance of nucleoli in determining the biochemical identity of the cell. Recent studies using Raman micro-spectroscopy to investigate the nucleoli biochemical and molecular composition, and differences in this nuclear compartment between normal and cancer cell lines, highlighted the potential role of the nucleoli in cell differentiation (Kuzmin, Pliss, and Kachynski, 2013; Schulze et al., 2013). The current study confirms that this nuclear organelle, in addition to its essential cellular function, is also the primary source of the differentiating Raman spectroscopic fingerprint of the cell lines.
Table 3.1: Confusion matrix table for A549, Calu-1 and BEAS-2B 

A. Nucleolus + Nucleus

<table>
<thead>
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<th>A549</th>
<th>Calu-1</th>
<th>BEAS-2B</th>
</tr>
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</tr>
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</tr>
<tr>
<td>BEAS-2B</td>
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</tr>
<tr>
<td>Sensitivity %</td>
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B. Nucleolus

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<tr>
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<td>100</td>
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<td>96.2</td>
<td>100</td>
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C. Nucleus

<table>
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<th>BEAS-2B</th>
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</tr>
<tr>
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<tr>
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D. Cytoplasm

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<tr>
<th></th>
<th>A549</th>
<th>Calu-1</th>
<th>BEAS-2B</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>23</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Calu-1</td>
<td>14</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>BEAS-2B</td>
<td>4</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>Sensitivity %</td>
<td>56.1</td>
<td>66.6</td>
<td>100</td>
</tr>
<tr>
<td>Specificity %</td>
<td>63.1</td>
<td>61.1</td>
<td>83.3</td>
</tr>
</tbody>
</table>

Notably, the improved differentiation of the cell lines based on the spectral profiles of the nucleoli alone does not consider the additional discriminating factor of the number and or density of nucleoli in the nucleus of different cell lines. In studies which compare an
integrated spectral analysis of the nucleus of the cell (Casey et al., 2008), this factor will amplify the intrinsic biochemical differences of the nucleoli of different cell lines.

Figure 3.9: PCA - LDA of subcellular regions of A549, Calu-1, and BEAS-2B A. Nucleolar +Nuclear B. Nucleolar C. Nuclear D. Cytoplasm.

The study points towards the importance of the nucleoli in the diagnostic potential of Raman micro-spectroscopy. The role of the nucleoli in the early stage response of cells to chemotherapeutic agents has also been demonstrated (Wojcik et al., 2015), indicating the sensitivity of the spectral profile of this subcellular region to cytological processes.
3.4. Conclusion:

While this study confirms the ability of Raman micro-spectroscopy to differentiate between normal and cancerous cell lines \textit{in vitro}, as well as between cancer cell lines from the same anatomical site, it clearly demonstrates that the discriminating potential varies depending on the subcellular region analysed. As, typically, the sampled spot size in Raman micro-spectroscopy is less than the cell area, this has important implications for optimisation of diagnostic potential in cytopathology, but also for the sensitivity of analysis of cellular processes such as the action of chemotherapeutic agents.

A comparison of normal and cancer cell lines indicates that all subcellular regions are differentiable. However, the classification sensitivities and specificities are lowest for the cytoplasmic regions. While somewhat improved classification is achievable using the spectra of the integrated nuclear region, classification based on the nucleolar regions alone is significantly superior. Notably, in the detailed spectral analysis, the cancer cell lines were only well differentiated on the basis of the nucleolar regions.

The morphological analysis demonstrates that the cells differ significantly in their nucleolar content, while the spectroscopic study indicates significant differences in the individual nucleolar biochemical content. The study demonstrates the importance of the nucleoli in its contribution to the diagnostic potential of Raman micro-spectroscopy as well as further applications in subcellular analysis of cytological processes.

3.5. Acknowledgement:

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The authors thank Dr. Josep Sulé-Suso, Institute for Science & Technology in Medicine, Keele University, Guy Hilton Research Centre UK and Cancer Centre, Royal Stoke University Hospital, University Hospitals of North Midlands, UK, for providing the Calu-1 cell line.
3.6. References:


3.7. Supplemental Information:

A.

B.

Figure S.3.1: PCA scatter plots and corresponding loadings of PC1 and PC2 of nucleolar, nuclear and cytoplasm regions of A. A549 and BEAS-2B B. Calu-1 and BEAS-2B PCA.

Cytoplasm ● Nucleus ○ Nucleolus ● A549 cell line

Cytoplasm ▼ Nucleus ▼ Nucleolus ▼ Calu-1 cell line

Cytoplasm ■ Nucleus ■ Nucleolus ■ BEAS-2B cell line
Chapter 4: Raman micro-spectroscopy for *in vitro* drug screening: subcellular localisation and interactions of Doxorubicin

The following chapter reproduces the journal article, Z. Farhane, F. Bonnier, A. Casey and H.J. Byrne Analyst, 2015, **140**, 4212, in which section and figure numbers have been adapted to the prescribed thesis format.

All experiments, data analysis and paper writing were performed by Z.F. A.C. and F.B. assisted in the experimental design.

H.J.B. assisted in the project design and proofing of the manuscript.
Raman micro-spectroscopy for *in vitro* drug screening: subcellular localisation and interactions of Doxorubicin

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**Abstract:**

Vibrational spectroscopy, including Raman spectroscopy, has been widely used over the last few years to explore potential biomedical applications. Indeed, Raman spectroscopy has been demonstrated to be a powerful non-invasive tool in cancer diagnosis and monitoring. In confocal microscopic mode, the technique is also a molecularly specific analytical tool with optical resolution which has potential applications in subcellular analysis of biochemical processes, and therefore as an *in vitro* screening tool of the efficacy and mode of action of, for example, chemotherapeutic agents.

In order to demonstrate and explore the potential in this field, established, model chemotherapeutic agents can be valuable. In study paper, Raman spectroscopy coupled with confocal microscopy were used for the localization and tracking of the commercially available drug, Doxorubicin (DOX), in the intracellular environment of the lung cancer cell line, A549.

Cytotoxicity assays were employed to establish clinically relevant drug doses for 24hr exposure, and confocal laser scanning fluorescence microscopy was conducted in parallel with Raman spectroscopy profiling to confirm the drug internalisation and localisation.
Multivariate statistical analysis, consisting of PCA (principal component analysis) was used to highlight Doxorubicin interaction with cancer cells and spectral variations due to its effects before and after DOX spectral features subtraction from nuclear and nucleolar spectra, were compared to non-exposed control spectra.

Results show that Raman micro-spectroscopy is not only able to detect Doxorubicin inside cells and profile its specific subcellular localisation, but, it is also capable of elucidating the local biomolecular changes elicited by the drug, differentiating the responses in different sub cellular regions. Further analysis clearly demonstrates the early apoptotic effect in the nuclear regions and the initial responses of cells to this death process, demonstrating the potential of the technique to monitor the mechanisms of action and response on a molecular level, with subcellular resolution.

**Keywords:** Raman spectroscopy, Doxorubicin, confocal microscopy, *in vitro* screening, cancer cells
4.1. Introduction:

Although the potential of vibrational spectroscopy, including infrared absorption and Raman spectroscopy, for biomedical applications has been well demonstrated, translation to the clinical environment has been slow, potentially due to the demands of standardisation, regulation and extensive clinical trials (Byrne et al., 2015; Rae et al., 2014). Fundamentally, the techniques are analytical, with molecular specificity, and, in the case of Raman spectroscopy in the confocal microscopy mode, can achieve optical resolution enabling subcellular profiling in 3D, suggesting that an appropriate application would be in screening of biomolecular changes in vitro (Chan et al., 2006; Crow et al., 2005; Notingher et al., 2002; Uzunbajakava et al., 2003). Regulatory requirements in both the EU and US (EU Directive-2010/63/EU and US Public Law 106-545, 2010, 16th Congress) have increasingly restricted the use of animal models for development of pharmaceuticals and cosmetics, and Raman micro-spectroscopy offers a potentially low cost, label free alternative to in vitro High Content Analysis for routine screening (Gordon and McGoverin, 2011; Tu and Chang, 2012).

The potential of Raman micro-spectroscopy in this field has previously been demonstrated in a number of studies (Das and Agrawal, 2011; Kallaway et al., 2013). The use of model systems, with established modes of action is of particular benefit in this respect, and commercially available drugs such as cisplatin (an alkylating and DNA binding agent) (Nawaz et al., 2010; Nawaz et al., 2011) and vincristine (an alkaloid agent) (Nawaz et al., 2013) have been explored. In these specific studies, however, the drug itself was not detected, but rather the spectroscopic response profiles of the cells themselves were correlated with the cytotoxic responses measured in cells. Nawaz et al., postulated that the direct chemical interaction of the drug in the cell could be differentiated from the
resultant cytological response using multivariate regression, and this approach was recently validated by Keating et al (Keating et al., 2015). El-Mashtoly et al. (El-Mashtoly et al., 2014) utilised the distinct structure of Erlotinib, containing a carbon-carbon triple bond, to specifically detect the subcellular presence of the drug \textit{in vitro}, while Cuisinier et al. used the C=O stretching band at 1740 cm\(^{-1}\) to monitor paclitaxel (a microtubule stabilizing agent) in cells and Vigny et al. (Feofanov et al., 2000) probed the resonance Raman response to detect and study nonfluorescent transition-metal complexes. Theraphtal, used as a chemotherapeutical combination. Raman spectroscopy has also been demonstrated as a suitable probe of subcellular localisation (Ahlinder et al., 2013; Bräutigam et al., 2014; Dorney et al., 2012) and toxicity of nanoparticles (Casey et al., 2008; Knief et al., 2009; Thakor et al., 2011).

One of the 10 most frequent drugs used in cancer chemotherapy and especially for the treatment of aggressive and metastatic tumours is doxorubicin. (Yokochi and Robertson, 2004) Doxorubicin (DOX) is an anthracycline antibiotic extract from \textit{Streptomyces peucetius} and a well-established anti-cancer drug (since 1960) (Hurley, 2002). It is widely used in chemotherapy for a varied range of cancers including breast carcinoma, haematological malignancies and lung cancer. Despite its known cardiotoxicity, it is currently one of the anticancer drugs most used in clinics (Cutts et al., 2005; Yang et al., 2014). When taken up into cell nucleus, where it has high affinity for DNA and blocks topoisomerase II (Hurley, 2002; Woods and Turchi, 2013), DOX induces genotoxicity and inhibits the process of DNA replication and macromolecular biosynthesis, leading to tumour cell apoptosis (Kaufmann and Earnshaw, 2000). As this interaction is dependent on the level of protein, cells with high levels of topoisomerase II are more susceptible to DOX, which explains its selectivity to cancer cells (Hurley, 2002). In addition to the formation of complex drug-DNA-topoisomerase II, DOX also induces the formation of
DNA adducts (Cutts et al., 2005; Swift et al., 2006), hydrogen peroxide (Mizutani et al., 2005) and inhibits DNA methyltransferase (Yokochi and Robertson, 2004) and Transforming Growth Factor-β1 (TGFβ1) (Filyak et al., 2008).

Despite the wide range of cytotoxicity that DOX elicits, its complete mechanism of action still not fully understood and much research has been undertaken to elucidate more clearly how DOX works, its uptake and intracellular delivery and resistance of cancer cells (Hurley, 2002; Lee et al., 2004; Moritz et al., 2010; Romero et al., 2013; Shen et al., 2008). In this context, the technique of Raman spectroscopy could add additional insight, \textit{in vitro}. The anthracycline structure of DOX, consisting of a conjugated anthraquinone ring structure banded to an aminoglycoside (Cutts et al., 2005), renders it fluorescent and gives it a strong Raman scattering efficiency even off resonantly, (Figure 4.1 A). It can therefore potentially be simultaneously tracked inside cells by confocal fluorescence microscopy and Raman spectroscopy, and therefore is an ideal candidate to probe the sensitivity of the latter to not only monitor the intracellular interactions of drugs, but also the mechanisms of interaction and the progression of subsequent cellular responses.

A number of studies over the last few years have investigated either the cellular uptake and nuclear accumulation (Majzner et al., 2015), cytotoxicity or the interaction of free or nanoformulations of DOX in different cancer cell lines or isolated DNA (Guo et al., 2009; Lee et al., 2004; Minati et al., 2012; Romero et al., 2013; Wojcik et al., 2015; Xiao et al., 2013).

In this study, Raman micro-spectroscopy is used as a tool, complemented by parallel cytotoxicity assays and confocal fluorescence microscopy, to both monitor DOX within the cellular environment, to detect its biochemical effects and fingerprint the physiological responses in the cancer cell line, A549, a non-small cell lung adenocarcinoma. The study thus explores the capability of the technique to screen the
uptake and mechanisms of interaction of chemotherapeutic agents in vitro in a truly label free manner.

Figure 4.1: A. chemical structure of Doxorubicin B. Raman spectrum of doxorubicin in aqueous solution (785 nm as source, background subtracted, vector-normalised and baseline corrected).

4.2. Materials and Methods:

4.2.1. Cell culture:

A549 human lung adenocarcinoma cells with the alveolar type II phenotype were obtained from ATCC (Manassas, VA, USA).

A549 cells were cultured in DMEM (with 2 mM L-glutamine) with 10% foetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO₂ and cells were split every two days to maintain ~60% confluence.

For confocal fluorescence and Raman spectroscopic analysis, cell number was determined using a Beckman Coulter Particle Count and Size Analysis® Z2 Cell Counter.
4.2.2. Cytotoxicity assays:

Alamar blue (AB) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assays were performed in 96 well plates and a total number of 1x10^5 cells were used to seed three plates (4x10^3 cells/mL).

Doxorubicin hydrochloride® powder (Sigma Life Sciences, Ireland) was diluted in 1mL sterile water to the required concentration. After 24 hrs incubation, plates were washed with phosphate buffered saline solution (PBS) and doxorubicin wase added in a range from 0 µM (as a control) to 50 µM.

A solution of 1.5 mL of AB (10X ready to use solution) and 3 mL of MTT stock solution (2.5 mg/mL, 25 mg MTT/10 mL PBS) in 30 mL of fresh medium were prepared. AB and MTT assays were both measured with a Cytotox SpectraMax®M3 plate reader using Soft Max® Pro6.2.2 as software and data was treated using SigmaPlot 10.0. After 24 hrs incubation in DOX, plates were washed with PBS and 100 µL of AB/MTT solution were added to each well. Plates were then incubated for 3 hrs and AB fluorescence was measured before in the plate reader using 540 nm excitation and 595 nm emission. The medium was then removed, the plates were washed with PBS and 100 µL of DMSO (Dimethyl sulfoxide) were added in each well. MTT absorbance was read at 570 nm. All cytotoxicity assays were made in triplicate and repeated three times.

4.2.3. Confocal Laser Scanning Fluorescence Microscopy:

Approximately 1 x 10^4 cells were allowed to attach on uncoated glass bottom Petri dishes (MatTek Corporation, USA) for approximately two hours, after which they were covered with cell culture medium. After 24 hrs incubation, the medium was removed and samples were rinsed twice with sterile PBS, new medium containing DOX corresponding to the
IC$_{50}$ concentration (inhibitory concentration) which is the concentration that inhibits 50% of cells (Neubig et al., 2003), was added and cells were incubated for 24 hrs more. At the end, cells were washed twice with sterile PBS, fixed in formalin (4 %, 15 min) and kept in PBS for imaging. Control samples without exposure to DOX were also prepared in parallel, and incubated for 24 hrs.

Confocal laser scanning fluorescence microscopic images were recorded using an inverted Zeiss LSM 510 confocal laser scanning microscope equipped with a x60 oil immersion objective. Doxorubicin fluorescence was excited with an argon ion laser at 488 nm, and the emission was collected at 530 nm.

**4.2.4. Raman micro-spectroscopy:**

Cells (~ 1x 10$^4$/window) were seeded and incubated on CaF$_2$ windows (Crystan Ltd, UK) for 24 hrs for both control and exposure to DOX. Medium was then removed and samples were rinsed twice with sterile PBS and covered with DOX at the IC$_{50}$ concentration. After 24 hrs incubation, cells were washed twice with sterile PBS and fixed in formalin (4 %, 15 min).

A Horiba Jobin-Yvon LabRAM HR800 spectrometer with a 785 nm, 300 mW diode laser as source, Peltier cooled 16-bit CCD, 300 lines/mm grating and 100 μm confocal hole, was used for this work. Spectra were acquired in the range from 400 cm$^{-1}$ to 1800 cm$^{-1}$ using a x100 objective (LCPlanN, Olympus), in dry conditions, for 30s two times, from three cell locations: cytoplasm, nucleus and nucleolus, to finally produce a data set of 30 points per cell location for each control and exposure to DOX, over a total of 60 cells.
4.2.5. Data analysis:

Raman spectral pre-processing and analysis were performed in Matlab 2013 using algorithms developed in house. Prior to analysis, spectra were smoothed (Savitsky-Golay filter 5th order, 7 points), vector normalised, baseline corrected (fifth order polynomial) and background was subtracted using a NCLS (non-negatively constrained least squares) algorithm.

Principal components analysis (PCA) was employed as an unsupervised multivariate approach to analyse data and the effects of doxorubicin in each cell localisation. The order of the PCs denotes their importance to the dataset and PC1 describes the highest amount of variation (Bonnier and Byrne, 2012; Bonnier et al., 2010).

4.3. Results and discussion:

4.3.1. Cytotoxicity assays:

![Graph showing AB and MTT 24 hrs dose dependent cytotoxicity of DOX to A549.](image)

**Figure 4.2:** AB and MTT 24 hrs dose dependent cytotoxicity of DOX to A549.
Figure 4.2 shows the dose dependent cytotoxicity of DOX after 24 hrs according to the AB and MTT in vitro cytotoxicity assays. Viability is expressed as % compared to control, and the error bars indicate the standard deviation of six independent replicate measurements. Both assays indicate a systematic dose dependent response. Neither of the assays indicates a complete loss of viability of the cell populations over the concentration range and exposure periods, and this viability curves were fitted with Equation 1 (Black and Leff, 1983).

\[ V = V_{\text{min}} + \frac{(V_{\text{max}} - V_{\text{min}})}{(1 + (C/IC_{50})^n)} \]  

Equation 1

Where \( V \) is the % viability, \( V_{\text{min}} \) is the minimum viability, \( V_{\text{max}} \) is the maximum viability, \( C \) is the DOX concentration, \( n \) is the Hill slope, and \( IC_{50} \) is the concentration which elicits 50% of the maximum response. The \( IC_{50} \) values were determined to be 0.42±0.06 \( \mu \)M and 0.55±0.16 \( \mu \)M for AB and MTT, respectively. Although the values overlap within experimental error, any slight difference in \( IC_{50} \) between AB and MTT may be because the AB assay is a measure of overall cell metabolism whereas the MTT assay is the reflection of mitochondrial activity within the cell. Mitochondria exist within the cells in all stages of their DNA replication, and are first targeted by DOX which explains its earlier response and higher sensitivity (Maher et al., 2014; Mukherjee, Davoren, and Byrne, 2010; Owen et al., 2006). Since cells will be analysed after 24 hrs exposure, the \( IC_{50} \) determined using the MTT assay was used for the rest of the study.

4.3.2. Confocal Laser Scanning Fluorescence Microscopy:

Confocal laser scanning fluorescence microscopy was used to confirm intracellular DOX localization and accumulation. Figure 4.3 illustrates that DOX, after 24 hrs incubation, is
predominantly accumulated in the cell nuclei, and no trace amounts are evident in the cytoplasm.

The absence of DOX in the cytoplasm after 24 hrs, confirms also that A549 cells do not present any resistance to the drug (Shen et al., 2008). It is also notable that fragmentation of nucleoli within the nucleus is observed upon DOX treatment for 24 hrs, confirming that cells are going under apoptosis (Nasser et al., 2008).

Figure 4.3: In vitro confocal fluorescence images of A549 A. control and B. 24 hrs doxorubicin exposure.

4.3.3. Raman micro-spectroscopy:

The Raman spectrum of DOX powder dissolved in sterile water at the concentration of 17.25 mM (Figure 4.1 B) clearly shows peaks at 1445 and 1570 cm$^{-1}$, related to skeletal
ring vibrations, specific bands corresponding to C-O, C-O-H and C-H in the region between 1200 and 1300 cm\(^{-1}\) and ones attributed to C-C-O and C-O at 440 and 465 cm\(^{-1}\) respectively (Eliasson et al., 2001; Strekal et al., 2001). The same peaks are clearly visible in the spectra of Figure 4.4, which shows the mean spectra of the nucleolar (A), nuclear (B) and cytoplasmic (C) regions of 30 A549 cells before and after 24 hrs exposure to the MTT IC\(_{50}\) concentration of DOX. Grey shading in the respective spectra indicates regions of interest, which are discussed further below.

Indeed, a first observation is that, in both the nucleolar and nuclear spectra, there are discernible peaks at 440, 465, 1085, 1215 and 1245 cm\(^{-1}\) corresponding to DOX (indicated by the blue shaded regions). These peaks are not evident in the mean cytoplasmic spectrum, however, consistent with the absence of any fluorescence in the confocal fluorescence microscopic images of Figure 4.3. Thus, the conjugated structure of DOX renders it easily visible and therefore traceable intracellularly using Raman spectroscopy, even without the application of more complex data mining methods.

Furthermore, it is obvious that many DNA peaks, for example 669, 728, 782, 830, 1095, 1340 and 1425 cm\(^{-1}\) in the spectra of nucleus and nucleoli of treated cells (Figure 4.4 A and B) are diminished compared to non-treated ones. Note that the feature at 1425 cm\(^{-1}\) is increased because of the contribution of the DOX peak at the same wavenumber but after DOX subtraction it can be clearly seen that this peak is diminished (see Figure 4.8). These results are in accordance with the expected changes in cell nuclei, related to the DOX mechanism of action, by which DNA synthesis is blocked by intercalation causing changes in DNA conformation (decrease of DNA B form) (Nawaz et al., 2013) inducing early cell apoptosis (Brauchle et al., 2014; Lin et al., 2012). (Full details of cellular peak assignments are given in Table 4.1).
Figure 4.4: Mean spectra of A549 cells A. nucleolus (green; control, black; exposed) B. nucleus (red; control, magenta; exposed) C. cytoplasm (blue; control, cyan; exposed).
A shift towards lower wavenumbers is observed for the peak at 1095 cm\(^{-1}\), corresponding to O-P-O stretching, (shown in the expanded section of Figure 4.4 A and B) which may indicate that DOX is also able to bind to DNA externally, although it is also influenced by the close proximity of the DOX peak at 1086 cm\(^{-1}\) (Nawaz et al., 2013).

Table 4.1: Raman bands observed in spectra of the nucleolus, nucleus and cytoplasm (Movasaghi, Rehman, and Rehman, 2007; Nawaz et al., 2013; Notingher, 2007; Notingher et al., 2004; Notingher et al., 2002; Yao et al., 2009).

<table>
<thead>
<tr>
<th>Raman shift (cm(^{-1}))</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>669</td>
<td>Thymine and guanine</td>
</tr>
<tr>
<td>720 and 1158</td>
<td>C-C-N+ symmetric stretching in phosphatidylcholine</td>
</tr>
<tr>
<td>728</td>
<td>Adenine</td>
</tr>
<tr>
<td>765</td>
<td>Tryptophan ring</td>
</tr>
<tr>
<td>855</td>
<td>Tyrosine vibration</td>
</tr>
<tr>
<td>782</td>
<td>Uracil, Cytosine and Thymine</td>
</tr>
<tr>
<td>784–795</td>
<td>Cytosine and thymine, DNA backbone O-P-O stretching</td>
</tr>
<tr>
<td>813</td>
<td>RNA O-P-O phosphodiester bond, DNA A form</td>
</tr>
<tr>
<td>828–830</td>
<td>O-P-O asymmetric stretching, DNA B form</td>
</tr>
<tr>
<td>847</td>
<td>Ribose phosphate</td>
</tr>
<tr>
<td>881</td>
<td>Deoxyribose ring breathing</td>
</tr>
<tr>
<td>936</td>
<td>C-C protein skeleton stretching, (\alpha) helix</td>
</tr>
<tr>
<td>1005</td>
<td>phenylalanine</td>
</tr>
<tr>
<td>1047</td>
<td>RNA P–O stretch, sugar phosphate –C–O– stretching</td>
</tr>
<tr>
<td>1095</td>
<td>DNA PO(_2) symmetric stretching</td>
</tr>
<tr>
<td>1176</td>
<td>Tyrosine and phenylalanine</td>
</tr>
<tr>
<td>1246</td>
<td>Amide III</td>
</tr>
<tr>
<td>1252</td>
<td>Adenine</td>
</tr>
<tr>
<td>1270</td>
<td>RNA Uracil and cytosine ring stretching</td>
</tr>
<tr>
<td>1300</td>
<td>RNA cytosine and adenine ring stretching</td>
</tr>
<tr>
<td>1303</td>
<td>Lipids C-H vibrations</td>
</tr>
<tr>
<td>1340, 1425 and 1578</td>
<td>Adenine and guanine</td>
</tr>
<tr>
<td>1320, 1450</td>
<td>CH(_2) deformation</td>
</tr>
<tr>
<td>1578</td>
<td>proteins</td>
</tr>
<tr>
<td>1661</td>
<td>Lipids C=C stretching</td>
</tr>
<tr>
<td>1665</td>
<td>Amide I</td>
</tr>
</tbody>
</table>
In addition to effects on DNA related spectral features, small decreases in protein peaks at 1005, 1320, 1578 and 1665 cm\(^{-1}\) observed, relative to those of control (further confirmed in the PCA in Figure 4.8). The decrease in intensity of DNA and protein features is consistent with the onset of apoptosis, which in turn is consistent with a DOX mechanism of action by induction of cell apoptosis, consisting of programmed and intrinsic cell death, DNA fragmentation, membrane blebbing and consequently interrupted cancer cell growth (Nasser et al., 2008; Notingher et al., 2004; Yao et al., 2009).

In the case of the cytoplasm, a similar spectrum is obtained before and after exposure to DOX, although subtle changes of some of the spectral features are apparent (indicated by grey shaded regions in Figure 4.4 C), such as a decrease in the 720 cm\(^{-1}\) peak and a discrete increase in that at 1158 cm\(^{-1}\), both corresponding to phosphatidyl choline, one of the major cellular membrane constituents. A notable decrease is also apparent in the features at 1303 and 1665 cm\(^{-1}\). These small decreases in lipid and protein peaks can be explained by the fact that, although apoptotic cells exhibit major changes in the structure of DNA, cytoplasmic biochemistry and cell volume, the cellular membrane and plasma can remain almost intact for more prolonged periods. The small increase in the feature at 1158 cm\(^{-1}\) may derive from the initial production of membrane vesicles synthetized by cells as a way to remove waste and toxins by exocytosis. As this synthesis starts at a later time, no substantial increase in lipid constituents is observed at this stage (Notingher et al., 2004; Owen et al., 2006).

Although some spectral changes resulting from DOX exposure are discernible by eye, a more detailed picture is elucidated through multivariate analysis. PCA is therefore employed to analyse in more detail the effects of DOX exposure on the spectral profiles of the subcellular regions and, for this, control and exposed cells were compared and
analysed, after which the raw spectrum of DOX was subtracted using NCLS and the spectra obtained were compared to those of control.

Figures representing the differentiation of the different spectra by PCA according to the corresponding PCs were plotted and, for clarity, the loadings are off set, the dashed horizontal line in all cases indicating zero loading.

**A.**

**B.**

**Figure 4.5:** A. PCA of A549 cytoplasm, nucleus and nucleolus non-exposed cells  B. PCA A549 cytoplasm, nucleus and nucleolus for both exposed and control cells.

Cytoplasm ● Nuclear ● and Nucleolar ● non-exposed cell

Cytoplasm ● Nuclear ● and Nucleolar ● exposed cell
As an illustration, Figure 4.5 shows (A) PCA of control and (B) exposed and control A549 cells. For control A549 cells, there is a clear differentiation between the nuclear (including nucleolar) region and the cytoplasm by PC1 and, according to the loading, the discriminant peaks correspond predominantly to DNA and lipids. There is no discrimination of the nuclear/nucleoli regions according to PC2, which is an indication of the intrinsic point to point spectral variability.

PCA of both exposed and control cells (Figure 4.5 B), shows a separation between control cells (negative) and DOX exposed cells (positive) according to PC1 and the corresponding loading is dominated by features of the pure DOX spectrum (Figure 4.6). Notice also that there is a discrimination between the nuclear region and the cytoplasm for both exposed and non-exposed cells according to PC2 and the corresponding loading 2 exhibits the same peaks as the loading of PC1 for PCA control cells only (Figure 4.5 A), namely those at 1300 and 1440 cm⁻¹ for lipids, 795 and 1095 cm⁻¹ for DNA.

![Figure 4.6: Loading of PC1 (PCA of control cells vs exposed) in blue and doxorubicin spectrum in cyan.](image)

**Figure 4.6:** Loading of PC1 (PCA of control cells vs exposed) in blue and doxorubicin spectrum in cyan.
To further elucidate the differences between exposed and control cells and to better understand the effects of DOX exposure on the spectral profiles of the different subcellular regions, PCA was employed for each cell compartment separately.

Significant discrimination can be seen between the spectra of each subcellular region of control and exposed cells, whose origin is represented by the loadings of PC1. In the case of both the nucleolar and nuclear regions (Figure 4.7 A and B), the discriminating peaks correspond to DOX (440, 465, 1215 and 1245 cm\(^{-1}\)), whereas for the cytoplasm (Figure 4.7 C) there are no DOX features evident and the only difference is a decrease in protein and lipid features (1303, 1450 and 1665 cm\(^{-1}\)).

In addition to prominent DOX peaks, in the loadings of PC1 for the nucleolar and nuclear regions of exposed and control cells, there are a number of smaller discriminants peaks which may provide further indications of the mode of interaction. In order, to better visualise these peaks, the spectrum of raw DOX powder in aqueous solution, was subtracted using NCLS, and the resultant spectra were again subjected to PCA (Figure 4.8).

As seen in Figure 4.8A and B, the loading of PC1 for the nucleolar and nuclear spectra of exposed (DOX subtracted) and control cells shows on one hand, negatives bands at 830, 881 and 1095 cm\(^{-1}\) and positive one at 813 cm\(^{-1}\) indicating a decrease and a conformational changes (B-form DNA into A-form) (Nawaz et al., 2013; Yao et al., 2009) caused by the DOX intercalation and, on the other hand, negative discriminants peaks at 1005, 1320, 1450, 1578 and 1665 cm\(^{-1}\), corresponding to protein and lipids and 1340, 782 and 728 cm\(^{-1}\) related to DNA which again is consistent with the intercalative mechanism of DOX, inducing a cell apoptosis traduced by a starting depletion of all three principal cell constituents: DNA, protein and lipids.
Figure 4.7: PCA of exposed and control A549 cells A. nucleolus B. nucleus C. cytoplasm.
In addition, there is a discernible increase of the amide III peaks at 1245 cm\(^{-1}\) (positive peak in loading 1) which may be explained by the initial mobilisation of a protein group to the intercalation site to prevent DNA repair.

A.

![Graph A](image1.png)

B.

![Graph B](image2.png)

**Figure 4.8:** A. PCA of exposed nucleolar regions after DOX subtraction (black) versus control cells (green). B. PCA of exposed nuclear regions after DOX subtraction (yellow) versus control cells (red).

To better elucidate any differences in the interaction of DOX in the nucleus compared to nucleoli, PCA of only nuclear and nucleolar regions was carried out for cells exposed to DOX then after DOX subtraction and compared to PCA of control cells.
Figure 4.9 A clearly shows differentiation between control nuclear and nucleolar regions. The loading of PC1 presents positive discriminant peaks corresponding to RNA at 1300, 1270, 1047 and 847 cm\(^{-1}\) and negative ones at 1440 and 830, 1450, 1578 cm\(^{-1}\) attributed to DNA and lipids (Table 4.1), consistent with the predominance of DNA in the nuclear regions with lipids from the membrane and RNA in the nucleoli.

PCA of exposed nuclear and nucleolar regions (Figure 4.9 B) shows a similar differentiation between these two cellular compartments, but the loading of PC1 is dominated by spectral features of DOX. This is consistent with DOX localisation in both nucleolar and nuclear regions, but more so in the latter, indicating that DOX is rapidly absorbed in the nuclear region, intercalated into DNA, and only residual amounts are free to progress to the nucleoli.

PCA of nucleolar and nuclear regions of control cells (Figure 4.9 A) indicates that the differences between these two compartments, according to the loading of PC1, correspond to DNA and RNA. But in the PCA of the same two localisations after DOX exposure and subtraction, additional discriminant peaks are evident at 765, 1005, 1320, 1665 cm\(^{-1}\), corresponding to proteins, although the separation is not so evident. In addition to DNA depletion, there are also changes in the profile of nuclear proteins. These features are negative with respect to PC1, consistent with an increased protein activity in the nucleoli. The decrease of the feature at 1450 cm\(^{-1}\), corresponding to lipids, may indicate that there is a denaturation of the cytoplasmic membrane surrounding the nucleus.
Figure 4.9: PCA of nucleolar (green) and nuclear (red) regions A. control B. after DOX exposure C. after DOX subtraction.
4.4. Discussion:

In the present study, the chemotherapeutic agent DOX could clearly be detected and tracked intracellularly within A549 cells using Raman spectroscopy. Results for both Raman micro-spectroscopy and confocal microscopy show that this anthracycline molecule, after 24 hrs exposure, is completely localised inside the cell nucleus, in which DNA is abundant, consistent with its established mechanism of action by DNA intercalation.

This nuclear localisation is manifest in the Raman nuclear and nucleolar spectra by the clear presence of DOX peaks at 465, 445 1200, 1300, 1440 and 1570 cm\(^{-1}\) and the absence of those peaks in cytoplasm spectra, and in confocal images by the red fluorescence in nucleus and nucleoli.

More in depth investigations using multivariate analysis revealed that not only DOX can be detected inside cells, but also both its biochemical effects and the physiological responses of the cells to exposure can be seen.

DOX is a known DNA intercalator, and the decrease in the nuclear spectral features at 669 (thymine and guanine), 728 (adenine), 782 (cytosine and thymine), 1340 and 1425 cm\(^{-1}\) (adenine and guanine) peaks are signatures of intercalation in the DNA duplex between two neighbouring base pairs, resulting in a decrease in the levels of B conformation DNA (830 cm\(^{-1}\)) (Agudelo et al., 2014; Lei, Wang, and Wu, 2012). Similar results were found for vincristine, a microtubule binder and mitotic inhibitor, but which was also observed to intercalate with nuclear DNA in a similar *in vitro* exposure of A549 cells, resulting in a diminution of thymine (669 cm\(^{-1}\)), guanine (1317 cm\(^{-1}\), cytosine (782 cm\(^{-1}\)) and adenine (728 cm\(^{-1}\)) bands, in addition to a decrease in DNA B form (827 cm\(^{-1}\)) (Nawaz et al., 2013).
A decrease in thymine ($669 \text{ cm}^{-1}$) and guanine ($1336 \text{ cm}^{-1}$) bands and to a lesser extent a reduction of the band associated with DNA conformation ($833 \text{ cm}^{-1}$), were also found with cisplatin, a well-established chemotherapeutic “groove binding” agent which binds with DNA forming inter-strand and intra-strand crosslinks at 1, 2-GG and 1,3-GTG sites, leading to cell cycle arrest and apoptosis (Nawaz et al., 2010). For these chemotherapeutic drugs, Raman signatures of the chemical interaction with nuclear DNA have therefore been established.

Moreover, changes in nuclear proteins are also observed in the studies related to cisplatin and vincristine, as observed in the current study of DOX. However, a more notable increase in some protein peaks, amide III $\alpha$-helix ($1302 \text{ cm}^{-1}$) and $\beta$-sheet ($1250-1259 \text{ cm}^{-1}$), $\text{CH}_2$ deformation ($938 \text{ cm}^{-1}$), interpreted as a physiological response of the cell to the chemotherapeutic exposure, was apparent for both cisplatin and vincristine, although it should be stressed that the time exposure for the cisplatin and vincristine studies was 96 hrs, as compared to the 24 hrs exposure employed in the current study, making a direct comparison of the responses impossible.

Comparing the spectral changes in exposed nucleoli to those of nucleus, it is clear that the dominant interaction with DNA occurs in the nucleus, as expected. However, the spectral changes in the nucleoli, associated with increased protein activity may provide additional insight into the early stage cellular response to toxic insult in which the cell nucleoli have been proposed to play a central role (Horky et al., 2002).

In the case of the cytoplasm, distinct decreases in spectral features associated with lipids and proteins peaks are observed after DOX exposure, consistent with an apoptotic cell death mechanism, along with an increase of phosphatidyl choline, a marker of a vesicle membrane synthesis as a means to remove the cytotoxic agent by exocytosis. These results are different from those obtained for cisplatin, exposure to which results in a
conformational change in cytoplasmic protein, resulting from the binding of cisplatin, along with a modification in lipids as a direct chemical effect (disintegration of lipids) and physiological response (decrease in lipids due to the reduction of cell viability).

In summary, distinct Raman spectroscopic markers of chemotherapeutic agents which interact with nuclear DNA can be established which can be used to help elucidate the mode of action of the drug. Changes in nuclear protein features can give indications of early cellular responses to the toxic insult. Raman markers for cytoplasmic biochemical effects are also evident, including decrease in both proteins and lipids, and subtle differences in the responses to exposure to different chemical agents can shed further light on the mechanisms of cellular response.

4.5. Conclusion:

This study further demonstrates the potential of Raman spectroscopy as a truly label free, *in vitro* screening tool to monitor the interaction of molecular chemotherapeutic agents on a cellular level. In confocal microscopic mode, the technique offers the same spatial resolution as confocal laser scanning fluorescence microscopy, enabling tracking of the subcellular localisation of the drug.

The conjugated structure of the chosen model drug, doxorubicin, presents a strong Raman scattering cross section, which renders its spectral profile easily discernible at toxicologically relevant doses, even at non resonant wavelengths, above the intrinsic cellular spectrum. As a model system to explore the potential of the technique, the conjugated structure also results in a strong fluorescence such that the measurements can be supported by conventional, confocal microscopy.

Notably, Raman micro-spectroscopy can also elucidate and differentiate the changes in the biomolecular structure within the subcellular regions, in this study, the cytoplasm,
nucleus and nucleoli, and potentially shed further light on the mechanisms of interaction of the drug.

Given the drive for a reduction in the use of animal models, there is currently much promotion of the development of *in vitro* models for drug and toxicity screening. Ultimately, with improved screening sensitivities and speeds, Raman spectroscopy could be employed to monitor in quasi real time, in a label free manner, the efficacy and mode of action of, for example chemotherapeutic agents and other exogenous agents, laying the basis for improved quantitative structure activity relationships to guide drug development or chemical regulation strategies.

**4.6. Acknowledgment:**

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Chapter 5: Differentiating responses of lung cancer cell lines to Doxorubicin exposure: *in vitro* Raman micro-spectroscopy, oxidative stress and bcl-2 protein expression

The following chapter reproduces the journal article, Z. Farhane, F. Bonnier, M.A. Maher, J. Bryant, A. Casey and H.J. Byrne, *J. Biophotonics* 10, No. 1, 151–165 (2017) / DOI 10.1002/jbio.201600019, in which the section and figure numbers have been adapted to the prescribed thesis format.

All experiments, data analysis and paper writing were performed by Z.F. F.B., M.A.H and J.B. assisted in the experimental design.

H.J.B. assisted in the project design and proofing of the manuscript.

**Graphical Abstract:**

PCA of nucleolar, nuclear and cytoplasmic regions of A549 and Calu-1 with corresponding loadings of PC1 and PC2.
Differentiating responses of lung cancer cell lines to Doxorubicin exposure: in vitro Raman micro-spectroscopy, oxidative stress and bcl-2 protein expression.

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Abstract:

The potential of Raman micro-spectroscopy as an in vitro, non-invasive tool for clinical applications has been demonstrated in recent years, specifically for cancer research. To further illustrate its potential as a high content and label free technique, it is important to show its capability to elucidate drug mechanisms of action and cellular resistances. In this study, cytotoxicity assays were employed to establish the toxicity profiles for 24 hrs exposure of lung cancer cell lines, A549 and Calu-1, to the commercially available drug, Doxorubicin (DOX). Raman-spectroscopy, coupled with Confocal Laser Scanning Microscopy and Flow Cytometry, was used to track the DOX mechanism of action, at a subcellular level, and to study the mechanisms of cellular resistance to DOX. Biomarkers related to the drug mechanism of action and cellular resistance to apoptosis, namely reactive oxygen species (ROS) and bcl-2 protein expression, respectively, were also measured and correlated to Raman spectral profiles. Calu-1 cells are shown to exhibit
spectroscopic signatures of both direct DNA damage due to intercalation in the nucleus and indirect damage due to oxidative stress in the cytoplasm, whereas the A549 cell line only exhibits signatures of the former mechanism of action.

**Keywords:** Raman micro-spectroscopy, Doxorubicin, Cytotoxicity, Confocal Laser Scanning Microscopy, Flow Cytometry, bcl-2 protein, ROS, drug resistance.
5.1. Introduction:

The potential of Raman micro-spectroscopy as a non-destructive, label free, analytical technique in clinical, pharmaceutical development and biomedical applications, based on its ability to monitor the chemical, bio-physiological and physical changes at the molecular level, has been demonstrated (Byrne et al., 2015; Cîntă Pinzaru et al., 2004; Paudel, Raijada, and Rantanen, 2015).

Among the possible applications of Raman micro-spectroscopy in the clinical setting, cancer research and diagnostics remain among the most promising, including cancer detection and typing, for example oral (Carvalho et al., 2015; Knipfer et al., 2014), gastric (Feng et al., 2011; Yao et al., 2009), breast (Abramczyk et al., 2012; Lee et al., 2014), cervical (Ramos, Malkin, and Lyng, 2015; Rashid et al., 2014), skin (Legesse et al., 2015) cancers as well as therapeutic follow up and response to chemotherapeutic drugs (Farhane, Bonnier, Casey, and Byrne, 2015; Kann et al., 2015).

Indeed, Raman micro-spectroscopy has been used not only to detect and monitor drugs inside cells at a subcellular level but also to fingerprint the cellular response to this exposure and the adverse drug effect (Owen et al., 2006). Therefore, it can be developed as a companion diagnostic tool, providing details about drug efficacy and safety, as detailed in the US FDA guidance document issued in August 2014, which defines such a tool as an in vitro diagnostic device that provides information that is essential for the safe and effective use of a corresponding therapeutic product (Jorgensen, 2015; Trusheim and Berndt, 2015). The use of in vitro models is also consistent with EU and US policies to reduce the use of animals for scientific testing (EU Directive-2010/63/EU and US Public Law 106-545, 2010, 106th Congress) and thus the demonstration of Raman micro-
spectroscopy as a companion diagnostic for screening and analysis of commercial chemotherapeutic agents could be of significant importance in cancer research.

To this end, Doxorubicin (DOX), which belongs to the anthracycline family, commercially known as Adriamycin, and widely prescribed as a chemotherapeutic antibiotic, was employed as a model compound. Despite its extensive clinical use, DOX has many toxic and chronic side effects, notably cardiotoxicity (Hrelia et al., 2002), and therefore it is important to understand how it effects cancer cells and the sources of cellular resistance in order to optimise its clinical efficacy and reduce its toxicity to the surrounding environment.

Routinely used clinically to treat aggressive and metastatic tumours, including lung cancers, DOX involves different mechanisms of action, not fully understood. Although originally thought to inhibit tumour cell proliferation by DNA intercalation, over the years, many other mechanisms of action have been identified, such as topoisomerase II inhibition, the formation of DNA adducts (Cutts et al., 2005; Yang et al., 2014), inhibition of DNA methyltransferase (Yokochi and Robertson, 2004) and Transforming Growth Factor-β1 (TGFβ1) (Filyak et al., 2008) and oxidative stress by generation of reactive oxygen species (ROS). The latter relates to its cytosolic metabolism and reduction to the doxorubicin semiquinone radical, leading to generation of superoxide and hydrogen peroxide, increased intracellular oxidative stress (Majzner et al., 2015; Rogalska et al., 2011), changes in the mitochondrial permeability, a release of cytochrome C which activate caspase effectors causing DNA damage (Wolf and Baynes, 2006; Yang et al., 2014). All these mechanisms of action lead to apoptosis, a programmed cell death or “cellular suicide”, as a way to remove unwanted cells exhibiting dangerous anarchic development as tumour cells, initiated extrinsically by the engagement of receptors at cytoplasmic membrane or intrinsically by, for example, DNA damage, resulting from
chemotherapy or oxidative stress or growth factor withdrawal (De Bruin and Medema, 2008; Elmore, 2007; Haikerwal et al., 2015; Moldoveanu et al., 2014).

The mitochondrial or intrinsic apoptosis pathway is controlled by two groups of proteins; pro-apoptotic and anti-apoptotic. Among the latter category, the bcl-2 (B cell lymphoma) family, an intracellular membrane protein generated as a direct result of DNA damage, represents the most important anti-apoptotic protein, which binds to almost all pro-apoptotic proteins (Hardwick and Soane, 2013) and so regulates the mitochondrial apoptosis pathways by mediating the mitochondrial outer membrane permeability (MOMP) and by local inhibition of free radical production suggesting an anti-oxidant mechanism, inhibiting cell apoptosis as a results including drug-induced (Correia et al., 2015; Gimenez-Cassina and Danial, 2015; Hussar, Žuravskaja, and Kärner, 2013; Makin and Dive, 2001; Rae et al., 2014; Sotiropoulou et al., 2010).

Any alteration, resistance or failure in apoptosis, due for example, to a higher expression of anti-apoptotic protein bcl-2, allows tumour cells to survive and proliferate, leading to resistance to chemotherapy and a poor clinical prognosis (Caponi et al., 2013; Johnstone, Ruefli, and Lowe, 2002; Mohammad et al., 2015). The cytological mechanisms of drug resistance in cancer cells can be an increased detoxification of anticancer drugs by the glutathione system, a defective apoptotic pathway, enhanced DNA damage repair or increased tolerance to DNA damage leading to suppression of apoptosis, elevated expression of anti-apoptotic genes and proteins, decreased uptake of water-soluble drugs and enhanced drug efflux from cancer cells mediated by ATP-binding cassette (ABC) transporters (Poornima et al., 2014; Qiu et al., 2013).

Understanding the relative contributions of this array of potential contributing mechanisms is a challenging task, requiring a manifold of parallel analytical techniques and assays, which are costly and time consuming. Previous studies have shown that
Raman micro-spectroscopy is not only able to discriminate between cancer cells according to nucleolar region (Farhane, Bonnier, Casey, Maguire, et al., 2015) but also to detect drugs inside cells, their effects on cell biology and the physiological response of the cell to this exposure (Farhane, Bonnier, Casey, and Byrne, 2015). The aim of the current study is to extend the previous investigations to fingerprint the DOX mechanism of action in two non-small cell lung cancer cell lines A549 and Calu-1 and the different cellular response using Raman micro-spectroscopy and to investigate its ability to differentiate the effect of the chemotherapeutic drug in the two different lung cancer cell lines. To support this spectroscopic analysis, Confocal Laser Scanning Fluorescence Microscopy was employed to monitor the subcellular DOX localisation and bcl-2 expression due to DNA damage was measured using Flow Cytometry and correlated to cellular levels of ROS, in relation to DOX mechanism of action. All observations were related to changes in Raman features for the three cellular compartments nucleolus, nucleus and cytoplasm.

5.2. Materials and methods:

5.2.1. Materials:

A549 human lung adenocarcinoma cells with the alveolar type II phenotype were obtained from ATCC (Manassas, VA, USA) and Calu-1 human lung epidermoid carcinoma cell line, was kindly provided by Dr. Josep Sulé-Suso, Institute for Science & Technology in Medicine, Keele University, Guy Hilton Research Centre UK and Cancer Centre, Royal Stoke University Hospital, University Hospitals of North Midlands, UK. Doxorubicin hydrochloride® powder (Sigma Life Sciences, Ireland) was diluted in 1mL sterile water to the required concentration (17.25 mM).
Alamar blue (AB) (10X ready to use solution) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Aldrich, Ireland.

For cytotoxicity assays an AB/MTT solution, 1.5 mL of AB and 3 mL of MTT stock solution (2.5 mg/mL, 25 mg MTT/10 mL PBS) in 30mL of fresh medium was prepared. Fixation/permeabilization kit, BD Cytofix/Cytoperm (BD 554714), and FITC Mouse Anti-Human bcl-2 set with an IgG1 isotype control (BD 556357) were purchased from BD Biosciences, Ireland.

Nucred® live 647 ReadyProbes® Reagent, used to image the cellular nucleus, and [5(6)-Carboxy-2',7'-dichlorodihydrofluorescein diacetate] (Carboxy-H2DCFDA) dye, used for the detection of Reactive oxygen species (ROS), were both purchased from BioSciences, Ireland (suppliers for Life Technologies).

5.2.2. Cell culture:

A549 cells were cultured in DMEM (with 2 mM L-glutamine) with 10 % foetal bovine serum (FBS) and Calu-1 cells in RPMI with 10 % FBS, both at 37 °C in a humidified atmosphere containing 5% CO₂ and cells were split every two days to maintain ~60 % confluence.

For Confocal Laser Scanning Fluorescence Microscopy (CLSM) and Raman microspectroscopic analysis, cell number was determined using a Beckman Coulter Particle Count and Size Analysis® Z2 Cell Counter.

5.2.3. Cytotoxicity assays:

AB and MTT assays were performed in 96 well plates and a total number of 1x10⁵ cells were used to seed three plates (4x10³ cells/mL). After 24 hrs incubation, plates were
washed with phosphate buffered saline solution (PBS) and DOX was added in a concentration range from 0 µM (as a control) to 50 µM.

AB and MTT assays were both measured with a Cytotox SpectraMax® M3 plate reader using Soft Max® Pro6.2.2 software. After 24 hrs incubation in DOX, plates were washed with PBS and 100 µL of AB/MTT solution were added to each well. Plates were then incubated for 3 hours and AB fluorescence and MTT absorbance were measured in the plate reader using, respectively, 540 nm excitation, 595 nm emission for AB and 570 nm for MTT. All cytotoxicity assays were made in triplicate and repeated three times and viability data was fitted by a four parameter Hill equation analysis using SigmaPlot 10.0, to yield values of the mean inhibitory concentration, IC50.

5.2.4. Confocal Laser Scanning Fluorescence Microscopy:

Approximately 1 x 10^4 cells were allowed to attach on uncoated glass bottom Petri dishes (MatTek Corporation, USA) for two hours, after which they were covered with cell culture medium. After 24 hrs incubation, the medium was removed and samples were rinsed twice with sterile PBS, new fresh medium containing DOX corresponding to the median inhibitory concentration, IC50, determined by cytotoxicity assays, was added and cells were incubated for a further 24 hrs. After incubation, old medium was removed and 2mL of Nucred® solution in medium was added and, after 15 to 30 min incubation, samples were rinsed twice with sterile PBS, fixed in formalin (4 %, 15 min) and kept in PBS for imaging. Control samples without exposure to DOX were also prepared in parallel, and incubated for 24 hrs.

CLSM images were recorded using an inverted Zeiss LSM 510 confocal laser scanning microscope equipped with a x60 oil immersion objective. DOX fluorescence was excited
with an argon ion laser at 488 nm, and the emission was collected at 530 nm, while Nucred® excitation and emission were respectively measured using 633 and 690 nm.

5.2.5. Flow Cytometry:

Cells (3x 10^4/flasks) were cultured in T25 flasks over 24 hrs, and then exposed to a range of DOX concentration (from 0.25x IC\textsubscript{50} to 3.5x IC\textsubscript{50}). After 24 hrs incubation, cells were trypsinised and centrifuged in 5 mL fresh medium at 4 °C and 1100 rpm for 5 min, whereupon, they were re-suspended with 1mL ice cold Dulbecco's Phosphate-Buffered Saline (DPBS) buffer and centrifuged at 4 °C and 2500 rpm for 5 min.

Cells were re-suspended in 750 µL ice cold DPBS buffer and transferred to Eppendorf tubes to which 250 µL of fixation buffer were added. After 30 min incubation at 4 °C, the fixed cells were washed twice in perm/wash buffer, centrifuged (2500 Rpm for 5 min at 4 °C) and then gently re-suspended in 50 µL perm/wash buffer, after which 20 µL of the antibody were added and the cells were incubated for 60 min in the dark at 4 °C. The cells were then washed twice in perm/wash buffer, centrifuged (2500 Rpm for 5 min at 4 °C) to remove unbound antibody and finally re-suspended in 1mL stain buffer. 10,000 cells were analysed by Flow Cytometry using a BD Biosciences Accuri C6 Flow Cytometer (Becton Dickinson, Oxford, UK). The Accuri Flow cytometry software was used for the analysis of flow cytometry samples and data processing.

5.2.6. Reactive oxygen species (ROS) expression:

Carboxy-H2DCFDA dye was employed for the detection and quantification of ROS production in the intracellular environment. A solution at 10 µM of Carboxy-H2DCFDA was made up in PBS and added to the cells for 1hr incubation. After incubation, cells
were washed three times with PBS, then exposed to DOX for 24 hrs. Fluorescence was measured in the plate reader using 488 nm excitation and 535 nm emission. Negative controls, consisting of healthy cells, untreated with DOX, analysed with or without Carboxy-H2DCFDA and DOX treated cells analysed without Carboxy-H2DCFDA, as well as positive controls of healthy cells exposed to a solution of hydrogen peroxide at 1 µM, were prepared in parallel.

5.2.7. Raman micro-spectroscopy:

Cells (~ 1x 10⁴/window) were seeded and incubated on CaF₂ windows (Crystan Ltd, UK) for 24 hrs for both control and exposure to DOX. Medium was then removed and samples were rinsed twice with sterile PBS and covered with DOX at the IC₅₀. After 24 hrs incubation, cells were washed twice with sterile PBS and fixed in formalin (4 %, 15 min). A Horiba Jobin-Yvon LabRAM HR800 spectrometer with a 785 nm, 300 mW diode laser as source (~100mW at the sample), Peltier cooled 16-bit CCD, 300 lines/mm grating and 100 µm confocal hole, was used to record spectra from the two cell lines, in the range from 400 cm⁻¹ to 1800 cm⁻¹ using a x100 objective (LCPlanN, Olympus N.A. 0,85)), in dry conditions from three cell locations: cytoplasm, nucleus and nucleolus, for 30 s two times , to finally produce a data set of 30 points per cell location (one spectrum from each cell compartment per cell) for each control and exposure to DOX, over a total of 120 different cells.

5.2.8. Data analysis:

Raman spectral pre-processing and analysis were performed in Matlab 2013 using algorithms developed in house. Prior to analysis, spectra were smoothed (Savitsky-Golay
filter 5th order, 7 points), vector normalised, baseline corrected (fifth order polynomial) and background and DOX spectral features were subtracted using a non-negatively constrained least squares (NCLS) algorithm (Byrne et al., 2016). After pre-processing, principal components analysis (PCA) was employed as an unsupervised multivariate approach to analyse data and the effects of doxorubicin in each cell localisation and in each cell line. The order of the PCs denotes their importance in the dataset, whereby PC1 describes the highest amount of variation (Bonnier and Byrne, 2012).

Independent component analysis (ICA) was also employed as an extension to PCA. ICA is an unsupervised statistical technique able to identify latent variables called independent components. In case of Raman micro-spectroscopy, ICA can be used to identify spectral contributions such as those from substrate, using the same number of ICs as PCs, which can then be removed or studied in their own right (Boiret et al., 2014; Vrabie et al., 2007).

5.3. Results and discussion:

5.3.1. Cytotoxicity assays:

Figure 5.1 shows the dose dependant cytotoxicity of DOX after 24 hrs exposure, for both cell lines, A549 and Calu-1, according to the AB and MTT in vitro cytotoxicity assays. Viability is expressed as % compared to control (non-exposed healthy cells), and the error bars indicate the standard deviation of six independent replicate measurements. For both in vitro assays, a partial loss of cell population viability is observed. However, whereas the AB and MTT for the A549 cell line and the MTT assay for the Calu-1 cell line show similar responses, the AB assay is relatively insensitive to the Calu-1 exposure.
Figure 5.1: AB and MTT 24 hrs in vitro dose dependent cytotoxicity assays of DOX A. A549 and B. Calu-1. Viability is expressed as % compared to control, and the error bars indicate the standard deviation of six independent replicate measurements.

The differences in the responses between AB and MTT in the two cell lines, as demonstrated by Figure 5.1, suggest different sensitivities and/or resistances of the respective cell-lines to the drug, and should be analysed in terms of the mode of response of the assays.

The MTT test is a colorimetric assay that measures the reduction of yellow 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase into purple formazan crystals, insoluble in aqueous solution (Al-Nasiry et al., 2007; Hamid et al., 2004). Succinate dehydrogenase or succinate-coenzyme Q reductase (SQR) or respiratory Complex II is an enzyme complex, bound to the inner mitochondrial membrane of mammalian mitochondria and many bacterial cells. It is the only enzyme that participates in both the citric acid cycle and the electron transport chain, so the MTT assay is the reflection of mitochondrial activity. Alamar blue (AB), on the other hand, is a water-soluble dye and one of the most highly used cytotoxicity assays for in vitro quantification of the cell viability (Fields and Lancaster, 1993).
When added to cell cultures, the active dye, resazurin or 7-hydroxy-10-oxidophenoxazin-10-iurn-3-one, diffuses into the cytosol and acts as an intermediate electron acceptor allowing the oxidised blue non-fluorescent form to be reduced by both mitochondrial and cytosolic enzyme activity to the fluorescent pink one which is easily measured by its absorption or fluorescence (Al-Nasiry et al., 2007; Hamid et al., 2004; Rampersad, 2012). Its response is thus considered the expression of general cellular metabolism. Therefore, although reduced conversion compared to controls is used as a measure of reduced cellular viability for both AB and MTT assays; the MTT response is more specifically sensitive to mitochondrial enzymes while AB is related to both mitochondrial and cytosolic activities.

As illustrated by Figure 5.2, A549 cells and Calu-1 exhibit somewhat different IC₅₀ values, as measured using the MTT cytotoxicity assay, determined to be 0.55±0.16 µM and 0.90 ±0.24 µM for the A549 and Calu-1 cell lines respectively. The most notable differences observed, however, are in the AB cytotoxicity profiles, which are characterised by IC₅₀ values of 0.42 ±0.06 µM and 0.69±0.13 µM for the A549 and Calu-1 cell lines respectively. For the same DOX dosage, although similar changes in mitochondrial activity (as recorded by the MTT assay) and the overall cellular metabolism (as recorded by the AB assay) are observed for the A549 cell line, the overall dose dependent change in cellular metabolism, as recorded by the AB assay, is considerably less in the Calu-1 cell line.
Figure 5.2: MTT 24 hrs dose dependent cytotoxicity of DOX to A549 and Calu-1.

The differing cytotoxic profiles for the two cell lines, is consistent with the contribution of multiple mechanisms to the toxic response, and a greater intrinsic resistance of the Calu-1 cell line to one or more of these mechanisms. The underlying mechanisms will be studied in more detail in the following sections. Since cells will be analysed after 24 hrs exposure, the IC$_{50}$ determined using the MTT assay was used for the rest of the study, for both cell lines.

5.3.2. Confocal Laser Scanning Fluorescence Microscopy:

CLSM was employed to visualise the DOX intracellular internalisation and localisation in both cell lines. Figure 5.3 illustrates the images for A549 and Calu-1 cells after 24 hrs DOX exposure, at the respective MTT IC$_{50}$ concentration for each cell line, and Nucred® staining. DOX is predominantly localised on the nuclear area and, for both cell lines after exposure, cells appear smaller in size and round or oval in shape and exhibit noticeable nucleoli fragmentation, known as karyorrhexis, and membrane blebbing. The cytoplasm is more tightly packed as a result of cellular shrinkage. All these morphological changes
indicate that cells are going under apoptosis (Brauchle et al., 2014; De Bruin and Medema, 2008; Elmore, 2007; Kaufmann and Earnshaw, 2000; Kroemer et al., 2009; Pietkiewicz, Schmidt, and Lavrik, 2015).

A. B.

**Figure 5.3:** Confocal Laser Scanning Fluorescence images of A. A549 and B. Calu-1 after 24 hrs DOX exposure and Nucred® staining confirming the DOX nuclear localisation.

Using ImageJ software, after fluorescence background subtraction, the mean fluorescence intensity of the nuclear area was measured for 10 cells for each cell line. The average DOX fluorescence intensity was determined to be, respectively 644±29 AU for the A549 cell line and 725±11 AU for Calu-1, indicating that a larger amount of DOX is present in the Calu-1 nucleus, despite the fact that the IC$_{50}$ value for Calu-1 is almost twice that of A549 and the global nuclear volume is slightly higher for A549 than Calu-1 (Farhane, Bonnier, Casey, Maguire, et al., 2015).
5.3.3. Flow Cytometry and bcl-2 expression:

Bcl-2 is an intracellular nuclear membrane anti apoptotic protein synthetised in direct response to DNA damage, in this case due to intercalation of DOX. As shown in Figure 5.4, a similar expression profile of up regulation followed by down regulation of the bcl-2 protein, as a function of DOX concentration, was observed for the two cell lines and this expression profile has been reported to be responsible for the resistance to apoptosis up to certain doses, above which DNA damage becomes prominent and bcl-2 levels decrease as cells enter apoptosis (Stephanova, Topouzova-Hristova, and Konakchieva, 2008).

The differences between the two bcl-2 expression profiles for each cell line, as a function of MTT IC$_{50}$ are manifest in the maximum level of bcl-2, and the dose at which this is reached. It is notable that the bcl-2 level in Calu-1 cells is consistently higher over the dose range than A549, with a maximum expression at 2 IC$_{50}$ for A549 (1.1 μM) and 3 IC$_{50}$ for Calu-1 (2.7 μM).
Figure 5.4: Expression level of bcl-2 due to DOX exposure and control healthy cells for A. A549 and B. Calu-1 determined by Flow Cytometry.
5.3.4. ROS production monitored by DCFDA:

In addition to the mode of action of DNA intercalation, internalised DOX can also lead to the generation of intracellular ROS in the cytosol by redox activation in the presence of NADPH, forming a quinone radical via one electron transfer and generating peroxide and superoxide (Gewirtz, 1999; Mizutani et al., 2005), resulting in DNA, mitochondrial and cell membrane damage by oxidation, responsible for its most significant chronic side effect, cardiotoxicity (Costa, Scholer-Dahirel, and Mechta-Grigoriou, 2014; Wang et al., 2002).

ROS were measured by the DCFDA dye, a highly fluorescent compound in presence of ROS which can be detected by fluorescence. As detailed in Table 5.1, and illustrated schematically in Figure 5.5, significantly higher ROS levels are present in Calu-1 cells than A549 (Test (DOX+DCFDA)), after 24 hrs exposure to the DOX IC_{50} concentration, as measured by the MTT assay. In fact, Calu-1 has higher ROS levels compared to A549 even without any DOX exposure and this expression becomes even higher after DOX exposure, emphasising the difference between the two cell lines. ROS are usually generated through a cascade of reactions resulting from the distinct oxidation status of O_2, including its radical forms, such as the superoxide radical, and non-radical forms such as hydrogen peroxide, H_2O_2 (Halliwell, 2006).
Table 5.1: ROS production in A. A549 cell line and B. Calu-1 cell line, negative controls using healthy cells and no DCFDA, exposed cells and no DCFDA and healthy cells with DCFDA and H$_2$O$_2$ 1 µM solution as positive control.

A.

<table>
<thead>
<tr>
<th>Negative controls (Arb.Units)</th>
<th>Positive control (Cells+H$_2$O$_2$+DCFDA) (Arb. Units)</th>
<th>Test (DOX+DCFDA) (Arb. Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy cells +PBS (no DCFDA)</td>
<td>DOX + PBS (no DCFDA)</td>
<td>Negative control (healthy cells +DCFDA)</td>
</tr>
<tr>
<td>Average</td>
<td>70.85706</td>
<td>254.6403</td>
</tr>
<tr>
<td>Ave %</td>
<td>87.93512</td>
<td>316.014</td>
</tr>
<tr>
<td>STD %</td>
<td>13.50783</td>
<td>14.96746</td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th>Negative controls (Arb.Units)</th>
<th>Positive control (Cells+H$_2$O$_2$+DCFDA) (Arb. Units)</th>
<th>Test (DOX+DCFDA) (Arb. Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy cells +PBS (no DCFDA)</td>
<td>DOX + PBS (no DCFDA)</td>
<td>Negative control (healthy cells +DCFDA)</td>
</tr>
<tr>
<td>Average</td>
<td>47.589</td>
<td>312.8539</td>
</tr>
<tr>
<td>STD</td>
<td>10.91059</td>
<td>23.91193</td>
</tr>
<tr>
<td>Ave %</td>
<td>15.39666</td>
<td>101.2189</td>
</tr>
<tr>
<td>STD %</td>
<td>3.529947</td>
<td>7.736324</td>
</tr>
</tbody>
</table>

Cancer cells have a high level of ROS, recognized to be signalling molecules in various biological processes, eliciting proliferation, genomic instability and inflammation (Schumacker, 2006) but a considerable increase of ROS level induces damage and promotes apoptosis (Costa, Scholer-Dahirel, and Mechta-Grigoriou, 2014; Schumacker, 2006). Although the relationship between DNA mutations, ROS generation, and drug sensitivity remains unclear, the ROS mediated apoptosis mechanism appears to be activated in the Calu-1 cell line to a significantly greater extent than in A549.
Figure 5.5: Reactive oxygen species generation expressed on percentage of control for Calu-1 and A549 cell lines.

5.3.5. Raman micro-spectroscopy:

Raman spectroscopy has previously demonstrated that A549 and Calu-1 cells are distinguishable, based on the spectral profile of their nucleoli (Farhane, Bonnier, Casey, Maguire, et al., 2015). Furthermore, it has previously been employed to profile the interactions of DOX within the nuclear and nucleolar regions of A549 cells (Farhane, Bonnier, Casey, and Byrne, 2015). In order to further elucidate the subcellular differences of the responses and potential resistance mechanisms of the A549 and Calu-1 cell lines to DOX exposure, Raman spectra, for both A549 and Calu-1 cells, were taken from the three cellular compartments after 24 hrs exposure to the MTT IC$_{50}$ concentration of DOX corresponding to each cell line. For PCA analysis, figures corresponding to spectral differentiation according to PC1 and PC2 were plotted and, for clarity, the corresponding loadings are off set, and the dashed horizontal line in all cases indicating zero loading.
Figure 5.6 shows the mean spectra of nucleolus, nucleus and cytoplasm for Calu-1 cell lines along with the spectrum of DOX powder dissolved in sterile water. Nucleolar size for both control cell lines is of the order of 2-4 μm, although it reduces to ~ 1-2 μm due to DOX exposure inducing fragmentation (Figure 5.3) (Farhane, Bonnier, Casey, Maguire, et al., 2015). Nevertheless, it remains larger than the Raman laser spot (1 μm), allowing Raman spectra acquisition specifically from the nucleoli. Points were chosen randomly from the cytoplasmic and nuclear areas of the cells. The variability of the spectra from each cellular region of each cell line is indicated by the spread of data points for each region in the PCA scatter plot of, for example, Figure 5.8.

**Figure 5.6**: Spectrum of Doxorubicin (A) and mean spectra of Nucleolus (B), Nucleus (C) and Cytoplasm (D) of the Calu-1 cell line, highlighted regions indicating discriminating Doxorubicin features.

As shown in previous study of the A549 cell line by Farhane et al. (Farhane, Bonnier, Casey, and Byrne, 2015), and as seen in Figure 5.6 for the Calu-1 cell line, obvious DOX features (indicated by highlighted regions) at 440 and 465 cm⁻¹ respectively C–C–O and C–O and 1085, 1215 and 1245 cm⁻¹ related to C–O, C–O–H and C–H are observed in the
nucleolar and nuclear region for both cell lines, confirming the predominant localisation of the drug in these regions.

**Figure 5.7:** Mean spectra of the nucleoli of Calu-1 cells highlighting the spectroscopic changes after DOX exposure; control (brown) and after DOX exposure (black).

Additionally, in the mean spectra of the nucleolus of Calu-1, before and after DOX exposure, an evident diminishment of peaks at 847 and 960 cm\(^{-1}\) can be seen (highlighted in Figure 5.7), indicating a modification of ribose phosphate backbone due to DOX intercalation. Moreover, obvious decreases of DNA peaks at 669, 728, 782 and 830 cm\(^{-1}\) in the spectra of the nucleolus of treated cells compared to non-treated ones are observed, due to DOX-DNA interactions inducing DNA synthesis interruption (Gewirtz, 1999) and changes in DNA conformation (decrease of DNA B form) (Nawaz et al., 2013) inducing early cell apoptosis (Brauchle et al., 2014; Lin et al., 2012) (Similar results were found for nuclear spectra, data not shown). In addition to the decrease in DNA peaks, a shift towards lower wavenumbers is observed for the peak at 1095 cm\(^{-1}\), corresponding to O-P-O stretching, which indicates that DOX is also able to bind to DNA externally (Nawaz et al., 2013). Besides the effects on DNA related spectral features, it appears that proteins
and lipids are also affected, as evidenced by the decrease of their corresponding peaks, for example, respectively at 1450 and 1665 cm\(^{-1}\). The peak at 1450 cm\(^{-1}\) is influenced by DOX peak proximity but, after DOX subtraction, it can be clearly seen that this peak is diminished (data not shown) (Huser and Chan, 2015).

A more detailed analysis of the changes in the spectral features as a result of DOX exposure and comparison between the responses of the two cell lines is facilitated by PCA. Figure 5.8, showing PCA of nucleolar, nuclear and cytoplasmic regions, for A549 and Calu-1 cells after exposure to DOX, exhibits a separation between the three cellular compartments and the corresponding loading of PC1, differentiating the combined nuclear region from the cytoplasmic one, is dominated on the positive side by DOX features, confirming once again its predominantly nuclear and nucleolar subcellular localisation, for both cell lines. The negative side is dominated by lipidic features at 1450 cm\(^{-1}\) (CH\(_2\) deformation) and 1661 cm\(^{-1}\) (Lipids C=C stretching), corresponding to biochemical changes in the cytoplasmic region (Downes and Elfick, 2010; Draux et al., 2009; Kann et al., 2015).

A pairwise PCA of the three cellular regions was performed, and it is notable in Figure 5.8 B, that PC1 clearly differentiates the nucleolar and nuclear regions between the two cell lines and the corresponding loading of PC1 clearly exhibits positive DOX features, indicating that there is more DOX in Calu-1 than in A549 cells, supporting the ImageJ analysis of the CLSM images. Negatives features at 1320 and 1450 cm\(^{-1}\) (CH\(_2\) deformation) and 1661 cm\(^{-1}\) (Lipid C=C stretching) are also observed in the loading of PC1 for nucleolar, nuclear and cytoplasmic areas, indicating that there is higher lipidic content in A549 cells than in Calu-1.
A.

Figure 5.8: PCA of nucleolar, nuclear and cytoplasmic regions of A. A549 and Calu-1 with corresponding loadings of PC1 and PC2. B. PCA of each cell localisation for A549 and Calu-1 and the corresponding loadings of PC1 and PC2.

Nucleolus ⬤ Nucleus ⬤ Cytoplasm ⬤ A549 cell line
Nucleolus ▼ Nucleus ▼ Cytoplasm ▼ Calu-1 cell line

The differing cellular responses, in the subcellular regions, can be better visualised after subtraction of the DOX spectral signature from the cellular spectra (Farhane, Bonnier, Casey, and Byrne, 2015). Figure 5.9 shows the PCA of the nucleolus of A549 and Calu-
1 after subtraction of DOX spectral features. Although the differentiation is not absolute, the A549 data is predominantly distributed with positive loadings of PC1, and vice versa for Calu-1. The loading of PC1 indicates that there is a more pronounced increase in some protein features in Calu-1 cells, than in A549. These include those at 645 cm\(^{-1}\) (C–C twist Tyrosine), 936 cm\(^{-1}\) (C–C stretching), 1176 cm\(^{-1}\) (Tyrosine), 1450 cm\(^{-1}\) (C–H) and 1665 cm\(^{-1}\) (Amide I). Similar changes are apparent in these protein related features in the mean spectra of Figure 5.6, although they are less pronounced in the mean spectra of A549 (Farhane, Bonnier, Casey, and Byrne, 2015).

**Figure 5.9:** PCA of nucleolar compartment of Calu-1 and A549 after DOX spectral features subtraction and corresponding loadings of PC1 and PC2.

Nucleolus ● A549 cell line after DOX subtraction
Nucleolus ▼ Calu-1 cell line after DOX subtraction

A further notable difference in the Raman profiles of Calu-1 compared to A549 cells is seen in the cytoplasmic region. Figure 5.6 reveals traces of DOX features for Calu-1, most apparent in the region from ~440-465 cm\(^{-1}\), which suggests the presence of a small amount of DOX in the cytoplasm, undetectable by CLSM. These are completely absent in the case of A549 (Farhane, Bonnier, Casey, and Byrne, 2015). The same DOX features
are obvious in the loading of PC2, separating the two cytoplasmic regions, in Figure 5.8 B, further indicating the presence of DOX in the cytoplasm of Calu-1 cells, while it is not detectable in A549. This observation highlights the sensitivity of Raman spectroscopic profiling to even small quantities of drugs inside cells and indicates that the sub-cellular distribution of DOX after 24 hrs exposure differs between the two cell lines.

In order, to investigate the DOX mechanism of action in more details, and to support and complement the PCA observations, ICA was performed using three components, as estimated by PCA, explaining the majority of variance, and only the first component for each cellular compartment was plotted and off set for clarity.

**Figure 5.10:** A. DOX spectrum and A549 ICA component B. Nucleolus C. Nucleus and D. Cytoplasm.

Figures 5.10 and 5.11 represent the ICA signals for the nucleolar, nuclear and cytoplasmic regions, respectively for the A549 and Calu-1 cell lines, plotted with the raw DOX spectrum for comparison. It is notable that the nucleolar and nuclear components exhibit DOX features at 440, 465 cm$^{-1}$, 1085, 1215 and 1245 cm$^{-1}$ for both cell lines, while these
features are only strongly observed (Figure 5.11) in the cytoplasm of Calu-1, confirming the PCA results.

Figure 5.11: A. DOX spectrum and Calu-1 ICA component B. Nucleolus C. Nucleus and D. Cytoplasm.

In both Figure 5.10 and 5.11, the peak at 795 cm\(^{-1}\) in the IC for both the nucleolus and nucleus (absent in the cytoplasm) corresponds to nucleic acid and confirms the nucleic DOX intercalation, while the negative peak at 1665 cm\(^{-1}\) represents a decrease in the protein Amide I response, consistent with the analysis of the mean spectrum of nucleolus and nucleus before and after DOX exposure. The IC for both the nucleolus and nucleus also exhibit obvious features at 1430-1456 cm\(^{-1}\). These peaks are not visible in mean spectra, due to the proximity of lipid peaks and are absent in ICA of the control cells before exposure to DOX (data not shown). While these signatures could be attributed to DOX, they also correspond to signatures of Guanine and Cytosine (Notingher et al., 2002). The increase in these nucleic acid signatures could be due to DOX-DNA intercalation. DOX intercalates preferentially between two adjacent GC bases (Yang et
al., 2014), inducing changes in DNA conformation, damage of the DNA double helix structure and the observed spectral changes may therefore be evidence of DOX intercalation between Guanine and Cytosine bases. There is also a hyperchromic effect observed for the peak at 1376 cm\(^{-1}\) corresponding to Thymine, Adenine, Guanine (ring breathing modes of the DNA/RNA bases) (Pravin and Raman, 2013; Rafique et al., 2013).

**Figure 5.12:** A. Spectrum DOX and B. Cytoplasm Calu-1 cell line ICA component.

In the ICA of the cytoplasm of DOX exposed Calu-1 cells (Figure 5.12), DOX features are clearly evident, confirming DOX localisation in the cytoplasm. However, the spectral profile of the IC is considerably different than that of pristine DOX, and the IC of the nuclear and nucleolar regions. This is consistent with a metabolisation of the DOX molecules within the cytoplasm, and the resultant cellular interaction is manifest as the spectral features at 720 and 877 cm\(^{-1}\) corresponding to C–C–N\(^+\) stretching, ones at 1024, 1343 cm\(^{-1}\) and 1456 cm\(^{-1}\), related to C-H vibrations and 1661 cm\(^{-1}\), corresponding to lipicic C=C stretching (Farhane, Bonnier, Casey, and Byrne, 2015; Notingher, 2007).
5.4. Discussion:

The analysis of the dose dependent cytotoxic response of the two cell lines to DOX exposure, after 24 hrs shows a notably different profile, particularly for the case of the Alamar Blue assay. The results are consistent with an increased resistance of the Calu-1 cell line to the drug, compared to A549. The traditional cytotoxic assays shed little light on the origin of the different cellular responses, in terms of the cellular pharmacokinetics, however. The flow cytometric analysis indicates that DOX exposure elicits significantly higher upregulation of the anti-apoptotic protein bcl-2 in the Calu-1 cell line, while analysis of the induced oxidative stress reveals substantially higher levels of ROS in Calu-1. DOX is reported to induce cell death by two principle modes of action, and the results are consistent with different relative contributions to the cytotoxic response in the two cell lines. DOX is rapidly trafficked to the cell nucleus (and mitochondria), where it binds with the DNA by intercalation, inducing DNA damage and initiating an apoptotic cascade, registered by the MTT assay. The MTT assay registers similar cytotoxicity profiles for the two cell lines, and the somewhat higher IC\textsubscript{50} value for Calu-1 is consistent with the observation from CLSM that there is a higher uptake of DOX in the nuclei of Calu-1. The DNA damage in cell nuclei triggers the upregulation of the anti-apoptotic protein bcl-2, which inhibits the apoptotic process. This response is significantly stronger in the Calu-1 cell line, and this greater resistance to apoptosis is consistent with the substantially lower loss of cell viability as registered by the AB assay. Concomitantly, a significantly higher degree of oxidative stress in the Calu-1 cell line is observable. ROS generation in the cytosol, resulting in DNA damage in the nucleus, is the second reported mode of action of DOX. The process is the result of a complex cascade initiated by the
reduction of DOX in the presence of NADPH and, in comparison to the direct interaction of DOX with the nuclear DNA, is a relatively slow process.

The combination of the cytotoxic assays, Flow Cytometry and CLSM can help to understand the underlying mechanisms of the different responses of the two cell lines to DOX exposure, and the apparent higher resistance one to cell death. As a label free, high content analysis technique, Raman spectroscopy can provide similar insights in a single measurement. Notably, Raman spectroscopy gives a clear indication of the presence of DOX and/or its semiquinone metabolite in the cytoplasm of Calu-1 cells, whereas it is not apparent in A549 cells. This observation is indicative of a retarded transport of the drug to the nuclei of Calu-1 cells, and an increased contribution of oxidative stress to the cellular response mechanism. Contained in the spectral changes of the cytoplasm are also signatures of cell damage, characteristic of the response mechanism. In the cell nuclei, DOX exposure produces clear signatures of DNA intercalation, as well as external groove binding, in both cell lines. In the nuclei of Calu-1 cells, there is a significantly higher increase in protein activity, consistent with the upregulation of the cellular anti-apoptotic resistance mechanism.

The results of the Raman microspectroscopic investigation therefore clearly demonstrate signatures of the interaction of the DOX with the two cell lines, and the mechanisms of cellular response. The differences in response are consistent with the differences observed through the combination of established techniques of cytotoxic assays, flow cytometry and CLSM.

However, the spectroscopic signatures are obtained in a single, label free measurement. It is noted that the chemotherapeutic agent chosen here, DOX, is a relatively strong Raman scatter, due to its $\pi$-conjugated anthracycline structure, and much is already
known about its mechanisms of interaction. Nevertheless, the study serves as a further illustration of the potential of Raman spectroscopy as an in vitro companion diagnostic tool. Although the screening was performed at a single time point, ongoing technological developments such as in nonlinear Raman techniques promise significantly enhanced signals, and therefore reduced sampling times, and ultimately real-time monitoring of cellular uptake of drugs and other external agents such as nanoparticles, and the subsequent cellular responses.

5.5. Conclusion:

The study further demonstrates the potential of Raman micro-spectroscopy as a high content, label free in vitro tool, not only to localise drugs within cells, but also to monitor their interactions in the cytoplasm and nucleus of the cells at a molecular level, and to characterise the subsequent cellular responses on a subcellular level. Spectroscopic signatures of DNA intercalation, binding and damage are clearly identifiable in the cell nucleus, and cellular responses such as the upregulation of anti-apoptotic proteins and cytoplasmic damage can be monitored. The technique has previously been employed to differentiate the two cancer cell lines, A549 and Calu-1 based on the spectroscopic signatures of their nucleoli, and in this study the differences of the responses of the two cell lines to DOX exposure are clearly identifiable in their post exposure spectroscopic signatures. Notably, due to the sensitivity of the detection system, Raman spectroscopy can detect the presence of the drug in the cytoplasm in the Calu-1 cell line, but not in A549, which is a key to understanding the difference in the cytotoxic responses, and therefore in cell resistance to the drug, coupled with the increased upregulation of anti-apoptotic proteins in the nucleus. The technique corroborates the results of classic
cytotoxicity assays, bcl-2 monitoring using flow cytometry, ROS monitoring using confocal microscopy, but does so in a single, label free measurement, clearly indicating the benefits of the technique for high content screening and in vitro companion diagnostics.

5.6. Acknowledgement:

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5.7. References:


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Chapter 6: Monitoring Doxorubicin cellular uptake and trafficking using *in vitro* Raman micro-spectroscopy: short and long time exposure effects on lung cancer cell lines

The following chapter reproduces the journal article, Z. Farhane, F. Bonnier and H.J. Byrne, *Anal Bioanal Chem* (2017) **409**:1333-1346/DOI 10.1007/s00216-016-0065-0, in which the section and figure numbers have been adapted to the prescribed thesis format.

All experiments, data analysis and paper writing were performed by Z.F.

F.B. assisted in the experimental design.

H.J.B. assisted in the project design and proofing of the manuscript.
Monitoring Doxorubicin cellular uptake and trafficking using *in vitro* Raman micro-spectroscopy: short and long time exposure effects on lung cancer cell lines

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Abstract:

Raman micro-spectroscopy is a non-invasive, *in vitro* analytical tool which is being increasingly explored for its potential in clinical applications and monitoring the uptake, mechanism of action and cellular interaction at a molecular level of chemotherapeutic drugs, ultimately as a potential label-free preclinical screening and companion diagnostic tool.

In this study, Doxorubicin (DOX), a “gold standard” chemotherapeutic drug, is employed as a model in the *in vitro* lung cancer cell line, A549, in order to demonstrate the potential of Raman micro-spectroscopy to screen and identify spectroscopic markers of its trafficking and mechanism of action. Confocal Laser Scanning Microscopy (CLSM) was used in parallel to illustrate the uptake and subcellular localisation, and cytotoxicity assays were employed to establish the toxicity profiles for early and late exposure times of A549 to DOX. Multivariate statistical analysis, consisting of principal components analysis (PCA), partial least squares regression (PLSR) and independent component
analysis (ICA) was used to elucidate the spectroscopic signatures associated with DOX uptake and subcellular interaction.

Raman spectroscopic profiling illustrates both drug kinetics and its pharmacodynamics in the cell and associated biochemical changes, demonstrating that DOX is mainly localised in the nuclear area, saturating the nucleolus first, within ~6 hrs of exposure, before the surrounding nuclear areas after ~12 hrs, and only accumulates in the cytoplasm after 48 hrs. PLSR over varying time intervals enables identification of DOX-DNA binding at early stages of exposure (0-12 hrs), while regression over the longer time periods (24-72 hrs) reveals spectroscopic signatures associated with the metabolic cellular response.

**Keywords:** Raman micro-spectroscopy, Confocal Laser Scanning Microscopy, Doxorubicin, cytotoxicity, long and short time exposure, Multivariate Analysis.
6.1. Introduction:

Doxorubicin (DOX), a cell cycle non-specific chemotherapeutic agent, is one of the two first anthracycline antibiotics isolated from *Streptomyces peucetius* (Carvalho et al., 2009) and among the most widely used chemotherapeutic agents, despite its numerous side effects, (Carvalho et al., 2014; Mitry and Edwards, 2016) and is highly effective in treating different types of cancers, from leukaemia, thyroid, and lung to many other neoplasia, especially metastatic and invasive ones (Carvalho et al., 2014; Hofman et al., 2015; Schiller et al., 2013; Umsumarng et al., 2015). The accepted mechanisms of action of DOX are complex and not fully understood, and include the interaction (i) with the cellular nuclear area, consisting of DNA intercalation, cross-linking, binding and alkylation, inhibition of topoisomerase I and II and RNA polymerase, all inducing DNA damage, inhibition of DNA replication and protein synthesis (rRNA transcription inhibition), as a consequence, leading to nucleolar disruption and cellular apoptosis (Tacar, Srijanonsak, and Dass, 2013) and (ii) with the cell membrane and mitochondria, resulting in generation of reactive oxygen species leading to direct membrane damage and oxidative stress, responsible for the major DOX side effect, cardiomyopathy (Akhter and Rajeswari, 2014; Burger et al., 2010; Carvalho et al., 2014; Golunski et al., 2016). DNA remains the main target of cancer therapeutics, whereby induction of DNA damage initiates a cascade of events that determines cellular apoptosis. DNA damage level and repair, by expression of anti-apoptotic proteins such as bcl-2 and resistance to apoptosis, are the main processes involved in carcinogenesis and in the response of cancer cells to cancer chemotherapy (El-Awady et al., 2016; Farhane et al., 2016). Apoptosis, known as programmed cell death, plays an important role in the internal environmental homeostasis and is closely associated with the development of cancer and in chemotherapy, by
implication, of drug-induced cell apoptosis (Yadav et al., 2014). One of the pathways leading to apoptosis disruption and tumour development is the nucleolar stress pathway (Quin et al., 2014). In fact, the nucleolus, known also as a ribosome factory, is the most prominent structure inside the nucleus, acting as a central integration of signalling pathways and performing numerous functions such as production of ribosome subunits, RNA editing and cell cycle and playing an important role in cancer development by dysregulation of its functions (Hein et al., 2013; Lo, Lee, and Lai, 2006; Orsolic et al., 2016). Due to its fundamental contribution in cancer proliferation, the nucleolus is the target for chemotherapeutic drugs such as Actinomycin D, which inhibits rRNA synthesis, Cisplatin which interferes with RNA Polymerase I in the nucleolus decreasing rDNA transcription, 5-fluorouracil which targets rRNA processing, DNA/RNA synthesis and DNA repair and DOX, which is known to disrupt nucleolar morphology and decrease rDNA transcription (Quin et al., 2014; Woods et al., 2015).

It has also been shown in previous Raman spectroscopic studies that this sub-nuclear region is responsible for the discrimination between cancer cell lines (Farhane, Bonnier, Casey, Maguire, et al., 2015).

Among the most common cancers worldwide, lung cancer is by far the highest cause of cancer-related death and has a 5-year survival of 15%. The main treatments of lung cancer are chemotherapy and radiotherapy, but these approaches have serious side effects and drug resistance is often developed during treatment (Bolukbas and Meiners, 2015). Thus, a better understanding of the mechanism of drug action and the cellular responses at the pre-clinical step is crucial to improve patient survival. To this end, Raman micro-spectroscopy, a non-invasive powerful label-free in vitro screening tool, is presented as the ideal candidate due to its ability to detect chemical, biological and physical changes of biomolecules at subcellular level and provide a molecular fingerprint of the sample of
interest, in this case cancer cell physiology, at a sub-cellular level, during chemotherapeutic treatment (Byrne et al., 2014; Gala and Chauhan, 2015; Huser and Chan, 2015; Notingher, 2007).

Raman micro-spectroscopy has attracted considerable attention in recent years for applications in areas such as the drug discovery process, quality control during industrial manufacturing and detection of counterfeit products in the pharmaceutical industry (Bhumika and Priti, 2010; Gala and Chauhan, 2015; Vankeirsbilck et al., 2002). Biomedical and clinical applications have also been explored extensively (Kallaway et al., 2013; Pence and Mahadevan-Jansen, 2016) and its potential as a screening technique either for normal/cancer cell discrimination or in chemotherapeutic and cellular process screening have been demonstrated (Farhane, Bonnier, Casey, and Byrne, 2015; Farhane, Bonnier, Casey, Maguire, et al., 2015).

Therefore, it can be developed as a companion diagnostic tool, providing details about drug efficacy and safety, as detailed in the US FDA guidance document issued in August 2014, which defines such a tool as an in vitro diagnostic device that provides information that is essential for the safety and efficacy of a corresponding therapeutic product (Trusheim and Berndt, 2015).

The demonstration of Raman micro-spectroscopy as a companion diagnostic for screening and analysis of commercial chemotherapeutic agents could be of significant importance in cancer research and especially in pre-clinical in vitro screening, a concept introduced by the US National Cancer Institute (NCI) in 1955 and providing screening models and supports to research, as a model which accurately predicts drug efficacy accurately at a molecular level, clinical activity and pharmacodynamics and kinetics (Suggitt and Bibby, 2005; Zhang et al., 2012).
Previously, DOX, routinely used for lung cancer therapy, was employed with A549, a non-small lung cancer cell line, and Raman analysis of the distribution of the drug in the cell, and the changes in the cellular spectroscopic signatures was performed, at a fixed point in time (24 hrs) (Farhane, Bonnier, Casey, and Byrne, 2015).

In the present paper, investigations were extended to look at the evolution of the responses, in order to track the drug reactions for early and late stages exposure, globally understand the mechanism of action and monitor the subsequent cellular behaviour using Raman micro-spectroscopy. The study demonstrates the potential of Raman Spectroscopy to monitor the drug uptake and the cellular responses at a subcellular level, but also elucidate details of the pharmacodynamics and drug cellular kinetics of the clinically prescribed drug.

6.2. **Materials and methods:**

6.2.1. **Materials:**

A549 human lung adenocarcinoma cells with the alveolar type II phenotype were obtained from ATCC (Manassas, VA, USA).

Doxorubicin hydrochloride® powder (Sigma Life Sciences, Ireland) was diluted in 1mL sterile water to the required concentration (17.25 mM).

Alamar blue (AB) (10X ready to use solution) and 3-([4,5-di(2-phenyl)thiazol]-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Aldrich, Ireland. For cytotoxicity assays, an AB/MTT solution, 1.5 mL of AB and 3 mL of MTT stock solution (2.5 mg/mL, 25 mg MTT/10 mL PBS) in 30 mL of fresh medium was prepared prior to the tests.
6.2.2. Cell culture:

A549 cells were cultured in DMEM (with 2 mM L-glutamine) with 10% foetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO₂ and cells were split every two days to maintain ~60% confluence.

For Confocal Laser Scanning Fluorescence Microscopy (CLSM) and Raman-microspectroscopic analysis, cell number was determined using a Beckman Coulter Particle Count and Size Analysis® Z2Cell Counter.

6.2.3. Cytotoxicity assays:

AB and MTT assays were performed in 96 well plates and a total number of 1x10⁵/25 mL, 1x10⁴/25 mL and 5x10³/25 mL cells, respectively for 0-24 hrs, 48 hrs and 72 hrs DOX exposure, were used to seed three plates each. After 24 hrs incubation, plates were washed with phosphate buffered saline solution (PBS) and DOX was added in a concentration range from 0 µM (as a control) to 50 µM.

AB and MTT assays were both measured with a Cytotox SpectraMax®M3 plate reader using Soft Max® Pro6.2.2 software. After incubation in DOX, plates were washed with PBS and 100µL of AB/MTT solution were added to each well. Plates were then incubated for 3 hrs and AB fluorescence was measured first in the plate reader using 540 nm excitation and 595 nm emission, then the medium was removed, the plates were washed with PBS and 100 µL of DMSO (Dimethyl sulfoxide) were added in each well. MTT absorbance was read at 570 nm.

All cytotoxicity assays were made in triplicate and repeated three times and viability data was fitted by a four parameter Hill equation analysis using SigmaPlot 10.0, to yield values of the mean inhibitory concentration, IC₅₀.
6.2.4. Confocal Laser Scanning Fluorescence Microscopy:

Approximately 1 x 10^4 cells were allowed to attach on uncoated glass bottom Petri dishes (MatTek Corporation, USA) for two hours, after which they were covered with cell culture medium. After 24 hrs incubation, the medium was removed and samples were rinsed twice with sterile PBS, new fresh medium containing DOX corresponding to the inhibitory concentration, IC_{50}, determined by cytotoxicity assays for each time point (48 and 72 hrs) and adjusted to the cell number (Table 6.1), was added and cells were incubated for a further 48 hrs and 72 hrs separately. For short time exposure, cells were incubated with the inhibitory concentration, IC_{50} determined previously for 24 hrs, and samples corresponding to each time point, 2, 6, 12 and 24 hrs were prepared separately. After incubation, samples were rinsed twice with sterile PBS, fixed in formalin (4%, 15 min) and kept in PBS for imaging. Control samples without exposure to DOX were also prepared in parallel, and incubated for 2, 6, 12, 24, 48 and 72 hrs.

CLSM images were recorded using an inverted Zeiss LSM 510 confocal laser scanning microscope equipped with a x60 oil immersion objective. DOX fluorescence was excited with an argon ion laser at 488 nm, and the emission was collected at 530 nm.

6.2.5. Raman micro-spectroscopy:

Cells (~ 1x 10^4/window) were seeded and incubated on CaF\textsubscript{2} windows (Crystan Ltd, UK) for 24 hrs for both control and exposure to DOX. Medium was then removed and samples were rinsed twice with sterile PBS and covered with DOX at each corresponding IC_{50} inhibitory concentration (IC_{50} (24 hrs) for 2, 6, 12, 24 hrs exposure, IC_{50} (48 hrs) and IC_{50} (72 hrs)) which is normalised according to the cell number used (~1x 10^4/window) (Table 6.1). The 24 hrs IC_{50} dose was initially chosen, based on previous studies, in order to
monitor the kinetics of the uptake of DOX at earlier times. This dose was reduced at longer times, as it would be too toxic at the longer exposure periods.

After each incubation period (2, 6, 12, 24, 48 and 72 hrs), cells were washed twice with sterile PBS and fixed in formalin (4%, 15 min).

A Horiba Jobin-YvonLabRAM HR800 spectrometer with a 785 nm, 300 mW diode laser as source (~100 mW at the sample), Peltier cooled 16-bit CCD, 300 lines/mm grating and 100 μm confocal hole, was used for this work. Spectra were acquired in the range from 400 cm\(^{-1}\) to 1800 cm\(^{-1}\) using a x100 objective (LCPlanN, Olympus), in dry conditions, for 30s twice, from three cell locations: cytoplasm, nucleus and nucleolus, to finally produce a data set of 30 points per cell location for each time point, 2, 6, 12, 24, 48 and 72 hrs after DOX exposure and for control cells, over a total of 210 cells corresponding to a total data set of 630 spectra.

6.2.6. Data analysis:

Raman spectral pre-processing and analysis were performed in Matlab 2013 using algorithms developed in house. Prior to analysis, background was subtracted using a NCLS (non-negatively constrained least squares) algorithm and spectra were subsequently smoothed (Savitsky-Golay filter 3th order, 11 points), baseline corrected (fifth order polynomial) and vector normalised.

Principal components analysis (PCA) was employed as an unsupervised multivariate approach to analyse data and the effects of doxorubicin in each cell localisation. The order of the PCs denotes their importance to the dataset and PC1 describes the highest amount of variation (Bonnier and Byrne, 2012).
Partial least squares regression (PLSR) was employed to track the temporal and dose dependent evolution of the spectral signatures in the subcellular regions. PLSR is a statistical regression technique which reduces the dimensionality of the data and correlates information in an $X$ data set matrix to the matrix of a $Y$ data according to the equation $Y = XB + E$, where $B$ is a matrix of regression coefficients and $E$ is the regression residual. In this work, the $X$-matrix is represented by the Raman spectra and the $Y$-matrix is consisted of values of the DOX concentration inside the cells, and the time evolution (Keating et al., 2015; Muratore, 2013; Nawaz et al., 2011).

The percentage of variance explained in the response variable ($Y$) as a function of the number of components was calculated using 10-fold cross validation and from a plot of the percent of variance explained function of number of components, the majority percentage of variance (above 90%) was found to be explained by the first 3 components.

The regression coefficients obtained by PLSR of Raman data regressed separately against DOX cellular concentration and time evolution were plotted and analysed. As a function of frequency, the co-efficients illustrate the spectral features which are influenced by the intracellular interaction of the DOX and the resulting metabolic changes.

Independent component analysis (ICA), a signal-processing technique, was also employed as an extension to PCA. ICA is an unsupervised statistical technique able to identify latent and statistically independent variables called independent components and corresponding to the pure signal underlying the observed signal and to interpret their different contributions. In the case of Raman micro-spectroscopy, ICA can be used to identify and extract principal spectral contributions such as those from substrate, using the same number of ICs (independent components) as PCs which is three components, which can then be removed or studied in their own right (Boiret et al., 2014; Farhane et al., 2016; Parastar, Jalali-Heravi, and Tauler, 2012; Wang, Ding, and Hou, 2008). The
first independent component (IC1) explains the highest percentage of variance and was used to further examine the temporal evolution of the spectral signatures.

6.3. Results:

6.3.1. Cytotoxicity assays:

The cytotoxicity of A549 cells to DOX exposure using both the AB and MTT assays for 24 hrs has been previously reported (Farhane, Bonnier, Casey, and Byrne, 2015), indicating an IC50 of 0.42 ± 0.06 μM and 0.55 ± 0.16 μM for AB and MTT, respectively. Figure S1 of the supplementary information shows the dose dependant cytotoxicity of DOX after 48 hrs (Figure S.6.1 A) and 72 hrs (Figure S.6.1 B) exposure, for the A549 cell line, according to the AB and MTT in vitro cytotoxicity assays. Viability is expressed as % compared to control (non-exposed healthy cells), and the error bars indicate the standard deviation of six independent replicate measurements. For both in vitro assays and for all times exposure, a partial loss of cell population viability is observed.

The AB and MTT assays for the A549 cell line show similar responses for late time exposure (48 and 72 hrs) but slightly different ones for 24 hrs. However, the IC50 values for 48 hrs exposure for A549 cells are comparable and were determined to be 0.30± 0.04 μM and 0.31± 0.05 μM for AB and MTT, respectively. Similar behaviour was found after 72 hrs treatment, characterised by IC50 values of 0.29± 0.02 μM and 0.27± 0.02 μM, respectively for AB and MTT.

The MTT assay is a reflection of mitochondrial activity while the AB assay is the expression of general cellular metabolism. The somewhat greater sensitivity of MTT compared to AB at the early stages is consistent with a mechanism of interaction which induce an early apoptosis (Berthiaume and Wallace, 2007; Green and Leeuwenburgh,
Nevertheless, since the mechanisms and cellular effects for the long time exposures of 48 and 72 hrs will be compared to those for shorter exposures 0-24 hrs, the IC_{50} determined by the MTT assay was used throughout the study for comparability purposes and Figure 6.1 shows the dose dependant cytotoxicity for those time points.

![Figure 6.1](image)

**Figure 6.1:** MTT *in vitro* dose dependent cytotoxicity assays of DOX to A549 cell line for short (0-24 hrs) and long exposure times (48 hrs, 72 hrs).

Viability is expressed as % compared to control, and the error bars indicate the standard deviation of six independent replicate measurements.

For Raman micro-spectroscopy and Confocal Laser Scanning Fluorescence Microscopy, the DOX dose used was scaled to cell numbers, according to a linear relationship between MTT response and cell number, using each corresponding IC_{50} and Table 6.1 shows the different concentrations used (Akbari and Javar, 2013; Sieuwerts et al., 1995; Twentyman and Luscombe, 1987).
Table 6.1: IC₅₀ determined by the MTT and corresponding dose used for 10⁴ cells.

<table>
<thead>
<tr>
<th>Exposure time (hrs)</th>
<th>0-24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC₅₀ (µM)</td>
<td>0.55± 0.16</td>
<td>0.31± 0.05</td>
<td>0.27± 0.02</td>
</tr>
<tr>
<td>DOX Concentration (µM)</td>
<td>13.75</td>
<td>77.5</td>
<td>135</td>
</tr>
</tbody>
</table>

6.3.2. Confocal Laser Scanning Fluorescence Microscopy:

Confocal Laser Scanning Fluorescence Microscopy (CLSM) was employed to visualise and confirm the DOX intracellular internalisation and subcellular localisation. Figure 6.2 illustrates the images for different DOX exposure times, from 2 hrs to 72 hrs; clearly demonstrating that DOX is predominantly accumulated in the nuclear region at all time points. The DOX nuclear accumulation is obvious even after only 2 hrs, suggesting that this commercial drug is trafficked straight to the nuclear area without any accumulation in the cytoplasm up to 24 hrs. Some trace amounts do appear in the cytoplasm at 48 and 72 hrs, consistent with the onset of a late cytoplasmic DOX mechanism of action associated with oxidative stress as a result of reactive oxygen species production (Farhane et al., 2016).

Compared to healthy control cells, exposed cells appear smaller in size and round or oval in shape and exhibit noticeable fragmentation of nucleoli within the nucleus, with tightly packed cytoplasm and membrane blebbing. All these morphological changes confirm that cells are undergoing apoptosis (Farhane, Bonnier, Casey, and Byrne, 2015; Hein et al., 2013; Sardao et al., 2009; Woods et al., 2015).
Figure 6.2: Confocal Laser Scanning Fluorescence images of A549 cells after DOX exposure and corresponding controls: 1. 2 hrs DOX exposure, 2. 6 hrs DOX exposure, 3. 12 hrs DOX exposure, 4. 24 hrs DOX exposure, 5. 48 hrs DOX exposure and 6. 72 hrs DOX exposure.

6.3.3. Raman micro-spectroscopy:

Raman micro spectroscopy has previously been demonstrated to be capable of tracking DOX inside the cellular compartments and differentiating between its different mechanisms of action and the cellular responses due to drug treatment at a fixed timepoint of 24 hrs exposure. In order to further investigate the subcellular responses and to track the drug pathway, Raman profiles from the three subcellular compartments, nucleolus, nucleus and cytoplasm, were analysed at shorter timescales of 2, 6 and 12 hrs at the 24 hrs IC<sub>50</sub> concentration, and at the longer exposure times of 48 hrs and 72 hrs, at the IC<sub>50</sub> concentration, adjusted to the cell number, of these time points, as determined using the MTT assay (Figure 6.1, Table 6.1).
Figure 6.3: Raman spectra after background subtraction, baseline correction and vector-normalisation of A. Doxorubicin dissolved in sterile water and mean spectra of A549 cell line for 0, 2, 6, 12, 24, 48 and 72 hrs of B. nucleolus C. nucleus and D. cytoplasm.
Figure 6.3 shows the mean spectra for each time point of the three cellular localisations, namely the nucleus, the primary location of DNA, the nucleolus, a sub-compartment of the nucleus, which contains RNA and small amount of DNA, and the surrounding cytoplasm containing the cytoskeleton, a framework of protein filaments, all protected by the plasma membrane (Glitsch, 2016; Olson, Dundr, and Szebeni, 2000).

In general, nucleic acids can be identified by peaks characteristic of nucleotide and sugar-phosphate backbone vibrations (Notingher, 2007) and the main bands are for example at 785 cm\(^{-1}\), assigned to cytosine and thymine (DNA/RNA) and DNA backbone O–P–O stretching, 813 cm\(^{-1}\), related to RNA O–P–O stretching, 830 cm\(^{-1}\), corresponding to DNA B form O-P-O asymmetric stretching and 1095 cm\(^{-1}\), assigned to DNA PO\(_2^–\) symmetric stretching. Protein signatures are dominated by peaks related to Amide III and Amide I vibrations, between 1230-1280 cm\(^{-1}\) and 1640-1695 cm\(^{-1}\), respectively, and amino acids containing phenyl groups, such as phenylalanine peaks at for example 1005 and 1030 cm\(^{-1}\), tryptophan at 760 cm\(^{-1}\) and tyrosine at 645, 853 and 877 cm\(^{-1}\). Lipids are predominantly represented by peaks at 700-720 cm\(^{-1}\), related to C–C–N+ symmetric stretching in phosphatidylcholine, 1303 cm\(^{-1}\) assigned to C-H vibration and between 1410 and 1495 cm\(^{-1}\) corresponding to C-H deformation (Farhane, Bonnier, Casey, and Byrne, 2015; Notingher, 2007; Verrier et al., 2004).

Since the Raman spectra are characteristic of the molecular structure, any changes to that structure, for example after chemotherapeutic treatment, can be detected and monitored. As an illustration, for both nucleolar and nuclear regions (Figure 6.3 B and C), an obvious shift towards lower wavenumbers of the band at 1095 cm\(^{-1}\) confirms again that DOX is able to bind to DNA externally, starting from the early stages of exposure (Farhane, Bonnier, Casey, and Byrne, 2015), and a decrease of the band at 785 cm\(^{-1}\) indicates a progressive DNA/RNA denaturation, while in the nuclear region, there is a decrease in
the features at 830 cm\(^{-1}\) and, in the nucleolar area, an almost complete disappearance of the band at 813 cm\(^{-1}\), potentially due to cessation of DNA replication as a consequence of DNA denaturation and fragmentation (Guo et al., 2009; Notingher et al., 2003). In the cytoplasmic area (Figure 6.3 D), a slight increase in features at 1065 and 1080 cm\(^{-1}\) is observed, corresponding to protein C-N stretching, which may correspond to an increase of signaling proteins in cells going under apoptosis.

It is notable that, for all cellular regions and over the time course, there is an increase in the principle DOX features (Figure 6.3 A, indicated by the highlighted regions in Figure 6.3 B, C and D) at 440 and 465 cm\(^{-1}\), respectively corresponding to C–C–O and C–O vibrations, and at 1215 and 1245 cm\(^{-1}\), related to C–O–H and C–O. Notice, however, that DOX features only appear in the cytoplasm starting from 48 hrs exposure. Similar observations were found in CLSM images (Figure 6.2), confirming the primary localisation of DOX in the combined nuclear area for all time points before 48 hrs treatment.

In order to examine further the DOX cellular pathway, PCA of exposed cells versus control was employed and a clear separation occurred according to PC1 for all time points compared to control, as previously demonstrated for 24 hrs exposure (Farhane, Bonnier, Casey, and Byrne, 2015) (data not shown). The evolution of DOX peak intensities at 440, 465, 1215 and 1245 cm\(^{-1}\), corresponding respectively to C-C-O, C-O, C-O-H and C-O vibrations of the DOX molecule, in the loading of PC1 was analysed, as shown in Figure 6.4. The intensity is expressed as a percentage of the highest value of each respective band in the three subcellular regions.

It is obvious that there is a progressive increase of DOX intracellular concentration until a plateau is reached after 6-12 hrs exposure and DOX is seen to accumulate in the nucleolus first, then the nucleus and after 48 hrs appears in the cytoplasm. Since the bands
at 1215 and 1245 cm\(^{-1}\) can be influenced by the proximity of protein features, further analysis was focused on the two bands at 440 and 465 cm\(^{-1}\).

![Graphs showing DOX Raman bands evolution](image)

**Figure 6.4:** Evolution of DOX Raman bands at 440, 465, 1215 and 1245 cm\(^{-1}\) as a function of time for the A549 cell line for each cellular compartment, nucleolus, nucleus and cytoplasm. Intensities are expressed in percentage according to the maximum value for each band over the three cellular compartments and standard deviation corresponds to the spectral variations of each band.

Notably, according to these two DOX Raman bands, there is more DOX in the nucleolus than the nucleus for 2, 6 and 12 hrs exposure, with a maximum at 6 hrs. These observations are consistent with a mechanism of DOX being trafficked straight to the combined nuclear area, after a passive diffusion to the cells due to its high lipophilicity, binds to proteasomal then translocates into the nuclear area, via nuclear pores, where it dissociates from the proteasome complex and binds to DNA with high affinity (Carvalho et al., 2009; Tacar, Srimornsak, and Dass, 2013), a larger amount accumulating in the...
denser nucleolar area, which saturates more rapidly. Accumulation in the cytoplasm is only evident after 48 hrs, confirming previous studies showing that the intracellular DOX is primarily accumulated in the nuclear area, at short exposure times, and that the main mechanism of action is one of DNA intercalation, interfering with both DNA and RNA polymerase and inducing in consequence DNA replication and RNA transcription blockage (Tacar, Sriamornsak, and Dass, 2013; Wallace, 2003). The observed plateau in the nuclear and nucleolar regions is more notable, given that the doses for 48 and 72 hrs were increased to the respective doses of 77.5 μM and 135 μM, respectively, compared to 13.75 μM for 24 hrs (Table 6.1). Nevertheless, it is clear that the DOX concentration inside the combined nuclear regions becomes approximately constant after ~24 hrs exposure, after which DOX begins to accumulate in the cytoplasm due to nuclear membrane loss of integrity (Eom et al., 2005; Sardao et al., 2009). The behaviour is consistent with saturation of nuclear binding sites after a certain time and any additional drug taken up by cells accumulates in the cytoplasm (El-Kareh and Secomb, 2005; Zenebergh, Baurain, and Trouet, 1984).

In order to track the cellular changes associated with the progressive uptake of DOX in the subcellular regions, PLSR was employed, and spectra were regressed against time, progressively increasing the time interval from 0-6 to 0-72 hrs. The resultant regression co-efficients for the nucleolar and nuclear region are shown in Figure 6.5. In the case of the regression as a function of time for the nuclear region (Figure 6.5 B), the co-efficients over the period 0-6 hrs and 0-12 hrs are almost identical, whereas regression beyond this period results in significant changes to the regression co-efficients. In the case of the nucleolar spectral data (Figure 6.5 A), such significant changes in the regression co-efficients are evident on progressing from 0-6 hrs to 0-12 hrs, and beyond. For both cellular regions, the time points after which the regression co-efficients begin to
change significantly co-incide with those of the onset of saturation of the DOX related spectral features in Figure 6.4, indicating that the regression over the timepoints up to saturation identifies spectral features associated with the initial drug mechanism of action, before saturation, after which the cellular responses become evident.

In the T:0-6 hrs regression co-efficients for the nucleolar region (Figure 6.5 B), notable positive DOX features are observed at 440-465 cm\(^{-1}\) and 1215-1245 cm\(^{-1}\), indicating the progressive accumulation of DOX, as shown in Figure 6.4. A concomitant decrease in cellular features corresponding to nucleic acids is observed, at 785 cm\(^{-1}\) (cytosine and thymine, DNA backbone O–P–O stretching), 813 cm\(^{-1}\) (RNA O–P–O stretching), 853 cm\(^{-1}\) (tyrosine ring breathing), 1095 cm\(^{-1}\) (DNA backbone O–P–O stretching), 1270 cm\(^{-1}\) (RNA uracil and cytosine ring stretching), 1300 cm\(^{-1}\) (RNA cytosine and adenine ring stretching), 1376 cm\(^{-1}\) (thymine), and 1577 cm\(^{-1}\) (adenine and guanine), representing a modification of their cellular content due to blockage of DNA replication and transcription as a result of DOX treatment, as well as a change in the profile of peaks at 1410, 1445 and 1491 cm\(^{-1}\), indicating lipid denaturation, probably of the surrounding cytoplasmic membrane and features at 1005 cm\(^{-1}\) (phenylalanine), 1607 cm\(^{-1}\) (tyrosine and phenylalanine ring vibration C=C) and 1673 cm\(^{-1}\) (Amide I), indicating a change in nucleolar proteins (Farhane, Bonnier, Casey, and Byrne, 2015; Huser and Chan, 2015; Kann et al., 2015; Matthews et al., 2010; Notingher, 2007; Notingher et al., 2003; Verrier et al., 2004).
Figure 6.5: Regression coefficients obtained by PLSR analysis as a function of time over progressive exposure time intervals (T) for A. nucleolus and B. nucleus. The dashed horizontal line indicates the zero-point for T: 0-6 hrs and the other coefficients are offset for clarity.
Over the regression period T:0-12 hrs, although the overall profile of the co-efficients is distinct compared to that of the nucleolar region, similar spectral changes are observed for the nuclear region, in addition to those at 830 cm\(^{-1}\) (O–P–O asymmetric stretching, DNA B form), 881 cm\(^{-1}\) (Deoxyribose ring breathing) and 1340 cm\(^{-1}\) (adenine and guanine).

The nucleolar and nuclear region are composed of similar biochemical constituents, and similar changes to these are prominent in the regression co-efficients of both regions. However, the distinctly different profiles in Figure 6.5 A (Nucleolus T:0-6 hrs) and Figure 6.5 B (Nucleus 0-12 hrs) indicate different rates and degrees of interactions with the constituent components in the different subcellular regions.

Notably, as shown in Figure S.6.2, for each cellular region, the co-efficients for regression against the internalised dose, as determined by the peak intensity in the loading 1 of PCA control versus exposed cells (Figure 6.4), are largely invariable, and almost identical to the time dependent regression co-efficient profile (T:0-6 hrs for nucleolus and T:0-12 hrs for nucleus) (Figure 6.5) up to the saturation point (Figure 6.4). So, for both nucleus and nucleolus and for both regression against short time exposure and DOX cellular concentration, a notable decrease in DNA and RNA features for example at 728, 785-795, 813, 1376 and 1486 cm\(^{-1}\) is evident. However, the changes to the nucleic acid features over this initial period are much more pronounced and more rapid in the nucleolar region than in the nucleus itself.

Following the initial DOX binding interactions in the nucleoli and then nuclear regions of the cell, and although the DOX concentrations saturate in the nuclear regions after ~12 hrs, (Figure 6.5), the cellular features continue to evolve as a function of time, in response to the action of the drug. This can be clearly seen in Figure 6.5, but, in order to differentiate the spectral characteristics of the initial chemical interactions of the drug
from the subsequent cellular responses, the spectral evolution over the time period 24-72 hrs was regressed against time and internalised cellular DOX concentration. Notably, in the PLSR analysis of nucleolus and nucleus regressed against time over the later stages, shown in Figure 6.6 (I A and II A), only cellular features appear and no DOX features are apparent, which confirms the fact that after certain time, DOX reaches a constant concentration inside the nuclear regions of the cells, as evidenced by the plateau in Figure 6.4.

As seen in Figure 6.6 I, for the nucleolar regions at late stages (24, 48 and 72 hrs), there is an increase in protein features at 447, 454, 1005 cm\(^{-1}\) (phenylalanine), 853 cm\(^{-1}\) (tyrosine ring breathing), 1207 cm\(^{-1}\) (phenylalanine, tryptophan and tyrosine), 1230 cm\(^{-1}\) (Amide III), and 1660 cm\(^{-1}\) (Amide I), consistent with an afflux of anti-apoptotic protein trying to repair DNA and avoid apoptosis (Farhane et al., 2016). There is also a significant decrease in DNA features at 785 cm\(^{-1}\) (cytosine and thymine), 830 cm\(^{-1}\) (DNA B form), 1220 cm\(^{-1}\) (adenine and thymine) and 1577 cm\(^{-1}\) (adenine and guanine), representative of DNA depletion and denaturation due to a long term effects of DOX.

Similar increases in protein features as a function of time in the later stages of exposure is apparent in the nuclear area, as shown in Figure 6.6 II, in addition to a progressive decrease in DNA features at 785, 795 cm\(^{-1}\) (cytosine and thymine), 1095 cm\(^{-1}\) (DNA backbone O–P–O symmetric stretching), 1220 cm\(^{-1}\) (adenine and thymine), 1376 cm\(^{-1}\) (thymine), 1577 cm\(^{-1}\) (adenine and guanine), and a lipid denaturation indicated by peaks at 700-715-720, 1127, 1450 and 1491 cm\(^{-1}\).
Figure 6.6: Regression coefficients obtained by PLSR analysis of long term DOX exposure over the time period (24-72 hrs) of I. nucleolus and II. nucleus (A) regressed against time and (B) against DOX concentration inside cells determined by the peaks intensities of the band at 465 cm$^{-1}$ in loading 1 of PCA exposed cells versus control.
In both nucleolar and nuclear regions at prolonged exposures, a notable increase is observed in features at 1047 cm\(^{-1}\) (RNA P–O stretching, sugar phosphate –C–O– stretching), and 1270 cm\(^{-1}\) (RNA Uracil and Cytosine ring stretching), while that at 1376 cm\(^{-1}\) (thymine) is seen to decrease. The continued changes to RNA features in both nucleolus and nucleus over the later time period could be explained by a decrease of RNA as a consequence of DNA denaturation and blockage of its replication but the concomitant increase in some RNA peaks could be due to nucleolar fragmentation, resulting in RNA spread into the nucleus (Boulon et al., 2010; Hein et al., 2013).

In addition to spectral features which may be characteristic of DNA and RNA depletion and nucleolar fragmentation, there is an increase in both lipid and protein features, at 645 cm\(^{-1}\) (C-C tyrosine), 715 cm\(^{-1}\) (choline), 720 cm\(^{-1}\) (C–C–N+ symmetric stretching in phosphatidylcholine), 760 cm\(^{-1}\) (tryptophan ring breathing), 853-877 cm\(^{-1}\) (tyrosine ring breathing), 1005 cm\(^{-1}\) (phenylalanine), 1127 cm\(^{-1}\) (C-N stretching), 1410-1445 cm\(^{-1}\) (CH\(_2\) vibrations), 1607 cm\(^{-1}\) (tyrosine and phenylalanine ring vibration C=C), and 1656-1673 cm\(^{-1}\) (Amide I) (Figure 6.5 and 7), as a cellular response to DOX exposure, which may be associated with the efflux of anti-apoptotic protein and a synthesis of lipidic vesicles as a way to remove DOX to the extracellular environment (Dellaire and Bazett-Jones, 2007; Farhane, Bonnier, Casey, and Byrne, 2015; Matthews et al., 2010; Movasaghi, Rehman, and Rehman, 2007).

The increase in phenylalanine in both nucleolar and nuclear regions seems to be a marker of the late apoptosis stage, at which point nucleolar fragmentation is complete, leaving an empty space with only cellular membrane (Figure 6.6) (Kann et al., 2015; Moritz et al., 2010).
Thus, it appears that regression against DOX cellular concentration shows the initial DOX accumulation inside the cells, its nuclear binding and its subsequent effects, including DNA damage, while regression against varying time intervals can elucidate and differentiate both the initial DOX mechanism of action and its cellular effects, such as depletion in DNA, RNA and proteins leading to apoptosis and the cells reactions to those DOX effects which could be a marker of any cellular-drug resistance.

Figure 6.7: Regression coefficients obtained by PLSR analysis of cytoplasm (A) regressed against time full range and (B) against DOX concentration inside cells determined by peaks intensities of band at 465 cm\(^{-1}\) in loading 1 of PCA exposed cells versus control.

Figure 6.7 shows the PLSR of the cytoplasmic region of the cell against time and DOX cellular concentration. In both regression co-efficient profiles, there are obvious DOX features at 440-465 cm\(^{-1}\) and 1215-1245 cm\(^{-1}\), in addition to cellular ones related to proteins and lipids, for example at 524 cm\(^{-1}\), corresponding to phosphatidylserine.
externalization as an indicator of cell signalling in apoptosis (Fadok et al., 1998; Kagan et al., 2000), 538 cm⁻¹ related to cholesterol ester, which increases due to alteration of the function of integral endoplasmic reticulum membrane proteins and is a marker of apoptosis (Maxfield and Tabas, 2005; Tabas, 2004), 576-776 cm⁻¹ (phosphatidylinositol) related to membrane trafficking, 645-830-853 cm⁻¹ (tyrosine), 749-760 cm⁻¹ (tryptophan), 940 cm⁻¹ (C-C stretching of protein), 1085 and 1128 cm⁻¹ (C-N stretch), 1268 cm⁻¹ (Amide III), 1410-1450 cm⁻¹ (CH₂ deformation), 1607 cm⁻¹ (C=C phenylalanine and tyrosine), 1640-1694 cm⁻¹ (Amide I of different conformational forms indicating a protein denaturation) (Matthews et al., 2010; Movasaghi, Rehman, and Rehman, 2007). Features at 645-1005 cm⁻¹ (C=C phenylalanine) and 1030 cm⁻¹ (C-H phenylalanine) indicate a change in the protein environment due to DOX exposure (Guo et al., 2009), while changes in the profile of the band at 1450 cm⁻¹ (C-H deformation) and around 1303 cm⁻¹ (lipid C-H vibration) (Movasaghi, Rehman, and Rehman, 2007) are consistent with a lipid structure denaturation by peroxidation due to ROS production under DOX treatment (Yadav et al., 2014).

6.4. Discussion:

Raman spectroscopic microscopy can clearly track the kinetics of the uptake and accumulation of the chemotherapeutic drug DOX at a subcellular level in vitro, and can differentiate the biochemical responses associated with the subcellular regions of nucleolus, nucleus and cytoplasm, both in terms of the mechanisms of action, and the subsequent cellular metabolic responses. In both cases, the spectroscopic signatures are a complex combination of the contributions of the many biomolecular responses and their evolution requires the use of multivariate regression analysis. Independent Components
Analysis can be used to extract the primary contributions of this combinatorial response, at each timepoint, as shown in Figure S.6.3, for each exposure timepoint for (A) nucleolar and (B) nuclear regions of the cell, again highlighting the multivariate nature of the spectral responses.

The evolution of selected features can be plotted against time, and correlated with that of the uptake and accumulation of the DOX in the different subcellular regions, as well as each others, as shown in Figure 6.8. The intensities of DNA, RNA, proteins and lipids bands was determined by ICA after DOX subtraction, as ICA can identify spectral cellular components contributions independently while DOX band was determined by PCA control vs exposed cells (Figure 6.4).

The interaction with DNA and RNA in both the nucleolar and nuclear regions of the cell as a function of time is apparent in Figure 6.8 A, showing the evolution of DNA features at 830 cm$^{-1}$ (O–P–O asymmetric stretching, DNA B form) and 881 cm$^{-1}$ (Deoxyribose ring breathing) and RNA features at 1270 cm$^{-1}$ (RNA Uracil and Cytosine ring stretching) and 1300 cm$^{-1}$ (RNA Cytosine and Adenine ring stretching). Notably, the feature at 785 cm$^{-1}$ (Cytosine and Thymine, DNA backbone O–P–O stretching), can be ascribed to either DNA or RNA.

The changes in the nucleic acid bands in the nucleolar region are concomitant with the accumulation of DOX, and the changes cease when the accumulation of DOX saturates, associating these with an instantaneous interaction with the biochemical components, and therefore the initial DOX-DNA binding (Cummings, Bartoszek, and Smyth, 1991; Yang et al., 2014).

In the nucleus, the equivalent changes occur more slowly, but also appear to saturate once the accumulation of DOX in the nucleus has saturated. A further observation is that the
relative changes in the nucleic acid spectral features are much stronger in the nucleolus than in the nuclear region.

(i)

(ii)

**Figure 6.8:** A. Evolution of DOX (465 cm\(^{-1}\)) and selected RNA (785, 1270, 1300 cm\(^{-1}\)) and DNA (785, 830, 881 cm\(^{-1}\)) Raman bands as a function of time for the A549 cell line for each nuclear cellular compartment, (i) nucleolus and (ii) nucleus. B. Evolution of DOX (465 cm\(^{-1}\)) and selected protein (1005, 1230, 1660 cm\(^{-1}\)), lipid (1450 cm\(^{-1}\)) Raman bands as a function of time for the A549 cell line for each nuclear cellular compartment, (i) nucleolus and (ii) nucleus. Intensities are expressed in percentage according to the maximum value for each nuclear area and standard deviation corresponds to the spectra variations for each band.
Moreover, the same kinetics of evolution were found using PLSR, highlighting that the same cellular features, mainly DNA and RNA are affected by DOX in both nucleolus and nucleus, due to interruption of DNA replication and RNA transcription, but at different rates and intervals. DOX affects the nucleolus instantaneously, and once saturation of DOX is reached at 6 hrs, an almost total reduction of DNA and RNA features to ~20 % has occurred. In the case of the nucleus, this decrease is slower even after saturation at 12 hrs and reaches only ~40 % of the initial levels.

It is also obvious that, after DOX exposure, proteins and lipids (Figure 6.8 B) start decreasing as a consequence of RNA/DNA alteration, and then increase at later stages of the cellular responses, which corresponds to an increase of anti-apoptotic proteins and a synthesis of lipids vesicules as a cellular reaction to the chemotherapeutic treatment, after which, cells initiate late apoptosis corresponding to a point of no return.

Previous studies have investigated the DOX nuclear accumulation by using either DOX fluorescence or indirect labeling techniques, showing an intimate relationship between DOX nuclear accumulation and its cellular concentration (Gigli et al., 1988). However, those approaches present some limitations, as DOX fluorescence can be influenced when it intercalates with DNA and the mechanism of action of dyes used to probe, for example DNA content (Zenebergh, Baurain, and Trouet, 1984), are unclear and can interfere with the process itself (Hovorka et al., 2010; Karukstis et al., 1998). No relationship between nuclear DOX accumulation and its cellular effects has been clearly defined. In contrast, using Raman micro-spectroscopy as an in vitro label free tool to investigate drug kinetics and pharmacodynamics, coupled with multivariate data analysis, shows the DOX cellular distribution, interactions and effects. It appears that the early stage responses are dominated by the kinetics of the chemotherapeutic drug accumulation, principally in the nucleolar region, and by the DOX-DNA interaction. Notably, the observed changes in
these early stages are predominantly associated with RNA, rather than DNA, changes to which become more prominent in the interactions in the nuclear region. At later stages, when the localised DOX concentration has reached the saturation point, the cellular response to DOX treatment becomes prominent. Saturation of the nucleolar region prior to the nuclear highlights again the importance of this subcellular region, not only in the cancer process but also in chemotherapy and cellular resistance, since it is the first to be targeted by DOX and as a consequence the first to react to drug exposure, followed by the nucleus and finally by the cytoplasm after 48 hrs exposure. The observation that appreciable accumulation of DOX in the cytoplasm is only apparent after such prolonged exposure times may have implications on the relative contributions of alternatives routes towards cell death based on oxidative stress in the cytoplasm (Gewirtz, 1999).

6.5. Conclusion:

The Food and Drug Administration classifies DOX as one of the most effective chemotherapeutic drug worldwide used for the treatment of various cancers (Carvalho et al., 2009; Tacar, Srianornsak, and Dass, 2013). Nevertheless, it presents serious clinical side effects and its full mechanism of action is still not completely understood. For this a development of an in vitro screening method to detect drugs inside cells and map their mechanism of action and the cellular response is of critical importance.

In the present work, Raman micro-spectroscopy associated with multivariate data analysis (PCA, PLSR and ICA) has been demonstrated to be capable of not only monitoring the subcellular accumulation and distribution of the drug inside cells and elucidating the mechanism of action, but also differentiating, as a function of time, the subsequent cellular response. It is notable that the nucleolus is the first to be saturated by
DOX and it is the first cellular compartment to be affected and to react to this treatment, highlighting the importance of this organelle in cancer development and in cancer therapy.

The profile of spectroscopic responses is complex, being a label free combinatorial response of the affected biochemcial constituents in each region. Nevertheless, that profile is well defined in terms of subcellular region and temporal evolution, indicating the potential of the technique as an in vitro, pre-clinical screening technique for drug efficacy and mode of action. Exploring differences in responses could ultimately elucidate mechanisms of cellular resistance, towards applications in companion diagnostics.

6.6. Compliance with Ethical Standards:

"The authors certify that there is no conflict of interest and they have no affiliations with or involvement in any organization or entity with any financial or non-financial interest"

6.7. Acknowledgement:

This work was supported by Science Foundation Ireland Principle Investigator Award 11/PI/1108.
6.8. References:


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6.9. Supplementary Material:

A. 

B. 

Figure S.6.1: AB and MTT in vitro dose dependent cytotoxicity assays of DOX of A549 cell line A. 48 hrs exposure and B. 72 hrs exposure. Viability is expressed as % compared to control, and the error bars indicate the standard deviation of six independent replicate measurements.

All viability curves were fitted with Equation 1 (Black & Leff, 1983)

\[
V = V_{\text{min}} + \frac{(V_{\text{max}} - V_{\text{min}})}{(1 + (C/IC_{50})^n)}
\]  

Equation 1

where \( V \) is the % viability, \( V_{\text{min}} \) is the minimum viability, \( V_{\text{max}} \) is the maximum viability, \( C \) is the DOX concentration, \( n \) is the Hill slope, and \( IC_{50} \) is the concentration which elicits 50% of the maximum response.
Figure S.6.2: Regression coefficients obtained by PLSR analysis of A. nucleolus and B. nucleus regressed against DOX concentration inside cells (D) determined by the peaks intensities of the band at 465 cm\(^{-1}\) in loading 1 of PCA exposed cells versus control, with time incrementation. The dashed horizontal line indicates the zero-point for D: 0-6hrs and the other co-efficients are off set for clarity.
Figure S.6.3: First ICA component of A549 cells, explaining the majority of variance, for control cells (0 hrs) and for each time exposure (2, 6, 12, 24, 48 and 72 hrs) after DOX spectrum subtraction of A. nucleolus and B. nucleus with highlighted cellular features changing according to DOX exposure duration.
Chapter 7: Doxorubicin kinetics and effects on lung cancer cell lines using in vitro Raman micro-spectroscopy: Binding signatures, Drug resistance and DNA repair

The following chapter reproduces the journal article, Z.Farhane, F.Bonnier, O.Howe, A.Casey and H.J.Byrne, J. Biophotonics 1–14 (2017) / DOI 10.1002/jbio.201700060, in which the section and figure numbers have been adapted to the prescribed thesis format.

All experiments, data analysis and paper writing were performed by Z.F.
F.B., O.H. and A.C. assisted in the experimental design.
H.J.B. assisted in the project design and proofing of the manuscript.

Graphical Abstract:

DNA damage and repair measured by Raman micro-spectroscopy and Mean Fluorescence of γH2AX after DOX exposure. Intensities expressed on percentage according to the maximum value for both cell lines.
Doxorubicin kinetics and effects on lung cancer cell lines using *in vitro* Raman micro-spectroscopy: Binding signatures, Drug resistance and DNA repair

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**Abstract:**

Raman micro-spectroscopy is a non-invasive analytical tool, whose potential in cellular analysis and monitoring drug mechanisms of action has already been demonstrated, and which can potentially be used in pre-clinical and clinical applications for the prediction of chemotherapeutic efficacy. To further investigate such potential clinical application, it is important to demonstrate its capability to differentiate drug mechanisms of action and cellular resistances. Using the example of Doxorubicin (DOX), in this study, it was used to probe the cellular uptake, signatures of chemical binding and subsequent cellular responses, of the chemotherapeutic drug in two lung cancer cell lines, A549 and Calu-1. Multivariate statistical analysis was used to elucidate the spectroscopic signatures associated with DOX uptake and subcellular interaction. Biomarkers related to DNA damage and repair, and mechanisms leading to apoptosis were also measured and correlated to Raman spectral profiles. Results confirm the potential of Raman
spectroscopic profiling to elucidate both drug kinetics and pharmacodynamics and differentiate cellular drug resistance associated with different subcellular accumulation rates and subsequent cellular response to DNA damage, pointing towards a better understanding of drug resistance for personalised targeted treatment.

**Keywords:** Raman micro-spectroscopy, Doxorubicin, Cytotoxicity, lung cancer cell lines, bind signature, DNA repair.
7.1. Introduction:

In the continued search for more effective chemotherapeutic treatments, there is a concomitant need for the development of improved screening techniques, particularly at the pre-clinical stage. Development of a new drug product from start to end is a very long process, taking on average 12 years, and costing, on average, $1.2 billion per product (DiMasi, Grabowski, and Hansen, 2003, 2016; Petrova, 2013).

EU Directive-2010/63/EU on the replacement, reduction and refinement of animal experimentation has prioritised the development of rapid, cost effective in vitro techniques for toxicological and pharmaceutical screening applications, amongst others. High Throughput Screening techniques allow for the evaluation of multiple biochemical and morphological parameters in cells based on image analysis of morphological changes or monitoring multiple fluorescently labelled species/processes in a cellular population (Flow Cytometry) or at a subcellular level (Confocal Microscopy) (Szymański, Markowicz, and Mikiciuk-Olasik, 2012). Notably, however, such screening and analytical techniques are phenomenological rather than molecularly specific and thus are limited in their ability to elucidate the underlying mechanisms. Alternative, high content assaying techniques for in vitro cytological screening and analysis are therefore desirable. Furthermore, despite the increased understanding of cancer biology and cellular drug mechanisms, all patients have a varied response to chemotherapeutic treatment and can develop adverse reactions and resistance to many approved drugs on the market. As an example, lung cancer remains the most common cause of cancer death worldwide (Ferlay et al., 2015; Jemal et al., 2010), despite the recent significant process in treatment and prevention (Cabeza et al., 2017; Tsao et al., 2016) and chemotherapeutic resistance is a major cause of treatment failure (Chen, Chang, and Cheng, 2017). This highlights the
need for personalised predictive testing to identify and characterise clinical biomarkers related either to the drug effects or to patient resistance. The development of an analytical tool for prediction of chemosensitivity, either in pre-clinical or diagnostic stages, is of great importance in order to adapt cancer treatment for each individual patient (Cho, Jeon, and Kim, 2012; Overby and Tarczy-Hornoch, 2013).

Raman micro-spectroscopy is an analytical technique whose potential for biological analysis has already been demonstrated (Butler et al., 2016; Byrne et al., 2015; Efeoglu, Casey, and Byrne, 2016). Therefore, it can be considered as a potential candidate for exploring pre-clinical drug development and clinical applications, by identifying signatures of drug mechanisms of interaction and even of cell dependent drug at a subcellular level.

As a proof of concept, a commercially available drug Doxorubicin (DOX) is used in this study to demonstrate the potential of Raman micro-spectroscopy to monitor the uptake, interaction mechanisms and subsequent cellular responses, for pre-clinical screening, as well as to differentiate the responses of two different cell lines, identifying markers of drug resistance, and therefore its potential as a companion diagnostic tool for personalized medicine. DOX is an anthracycline widely used in chemotherapy for the treatment of various human cancers and aggressive tumours (Agudelo et al., 2014; Rabbani, Finn, and Ausio, 2005), despite its serious side effects, principally cardiotoxicity, and a not fully understood mechanism of action (Farhane et al., 2015; Gautier et al., 2015; Gu et al., 2016). In previous in vitro reports, different relative contributions of subcellular processes have been observed, depending on cell line, (Farhane et al., 2017) including ROS production, intercalation between nucleic base pairs and Topoisomerase II inhibition, blocking of DNA replication (Farhane et al., 2015; Hasanzadeh and Shadjou, 2016) and induction of DNA double stand breaks (DSB) by nucleosome turnover increase, leading
to cellular apoptosis (Ohnishi, Mori, and Takahashi, 2009; Ross and Bradley, 1981; Yang, Kemp, and Henikoff, 2015). DSB induce a DNA damage response (DDR) which can be visualised locally as an accumulation of repair proteins, known as nuclear foci (Brandsma and Gent, 2012) and chromatin modification acting as an adaptor attracting and localizing retention of DNA repair proteins. The cellular DNA damage and repair capacity influences the effects of an anticancer drug (Helleday et al., 2008) and can be used as a biomarker of chemotherapeutic efficacy and resistance (Ivashkevich et al., 2012). Moreover, resistance to apoptosis, programmed cell death, by either increase of apoptosis inhibitor protein or, higher tolerance to DNA damage or DNA repair, is associated with chemoresistance (Gu et al., 2016) and a poor clinical prognosis in cancer therapy and can be used as a marker for individualised treatment (de Bruin and Medema, 2008).

The present study expands the previous work exploring the use of Raman microspectroscopy to monitor DOX effects and cellular responses in vitro (Farhane et al., 2015; Farhane et al., 2017) to a comparison of the uptake kinetics and responses of two different histological subtype cell lines, A549 and Calu-1. In order to extract biological information contained in Raman spectra, multivariate data analysis consisting of Principal Component Analysis (PCA), Partial Least Square Regression (PLSR) and Independent Component Analysis (ICA) is used. Raman investigations are supported by cytotoxicity assays; apoptotic marker of nuclear condensation, specific anti-apoptotic protein (bcl-2) and DNA damage and repair by measurement of γH2AX, to identify different DOX sensitivities, monitor cellular uptake and response mechanisms.
7.2. Materials and methods:

7.2.1. Materials:

A549 human lung adenocarcinoma cells with the alveolar type II phenotype were obtained from ATCC (Manassas, VA, USA) and Calu-1 human lung epidermoid carcinoma cell line, was kindly provided by Dr. Josep Sulé-Suso, Institute for Science & Technology in Medicine, Keele University, Guy Hilton Research Centre UK and Cancer Centre, Royal Stoke University Hospital, University Hospitals of North Midlands, UK.

A Doxorubicin stock solution of concentration 17.25 mM was prepared by dilution of Doxorubicin hydrochloride® powder (Sigma Life Sciences, Ireland) in 1 mL sterile water. Alamar blue (AB) (10X ready to use solution) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Aldrich, Ireland.

For cytotoxicity assays, an AB/MTT solution, 1.5 mL of AB and 3 mL of MTT stock solution (2.5 mg/mL, 25 mg MTT/10 mL PBS) in 30 mL of fresh medium was prepared prior to performing the assays.

Nucred® live 647 Ready Probes® Reagent, used to image the cellular nuclear condensation, the fixation/permeabilization kit, BD Cytofix/Cytoperm (BD 554714), and FITC Mouse Anti-Human bcl-2 set with an IgG1 isotype control (BD 556357) was purchased from BioSciences, Ireland.

γH2AX reagents, Alexa Fluor® 647 anti H2AX phospho (Ser 139) and Alexa Fluor® 647 mouse IgG1 isotype control (ICFC), Biolegends, were supplied through Medical Supply Company Ltd., Ireland.
7.2.2. Cell culture:

A549 cells were cultured in DMEM-F12 with 10 % foetal bovine serum (FBS) and Calu-1 cells in RPMI with 10 % FBS, both at 37 °C in a humidified atmosphere containing 5 % CO₂ and cells were split every two days to maintain ~60 % confluency.

For Cytotoxicity assays, Confocal Laser Scanning Fluorescence Microscopy (CLSM) and Raman micro spectroscopic analysis, the cell number was determined using a Beckman Coulter Particle Count and Size Analysis® Z2 Cell Counter.

7.2.3. Cytotoxicity assays:

AB and MTT assays were performed in 96 well plates and a total number of 1x10⁵ cells (4x10³ cells/mL), 1 x 10⁴ (4x10² cells/mL) and 5x10³ cells (2x10² cells/mL), respectively for 0-24 hrs, 48 hrs and 72 hrs, with three replicate plates of each. After 24 hrs incubation, plates were washed with phosphate buffered saline solution (PBS) and DOX was added in a concentration range from 0 µM (as a control) to 50 µM.

AB and MTT assays were both measured with a Cytotox SpectraMax®M3 plate reader using Soft Max® Pro 6.2.2 software. After 6, 24, 48 and 72 hrs incubation in DOX, plates were washed with PBS and 100µL of AB/MTT solution were added to each well. Plates were then incubated for 3 hrs and AB fluorescence was measured first in the plate reader using 540 nm excitation and 595 nm emission, then the medium was removed, the plates were washed with PBS and 100 µL of DMSO (Dimethyl sulfoxide) were added in each well. MTT absorbance was read at 570 nm.

All cytotoxicity assays were made in triplicate and repeated three times and viability data was fitted by a four parameter Hill equation analysis using SigmaPlot 10.0, to yield values of the mean inhibitory concentration, IC₅₀.
7.2.4. **Raman micro-spectroscopy:**

Cells (~ 1x 10^4/window) were seeded and incubated on CaF$_2$ windows (Crystan Ltd, UK) for 24 hrs for both control and exposure to DOX. The medium was then removed and samples were rinsed twice with sterile PBS and covered with DOX at each corresponding IC$_{50}$ inhibitory concentration, adjusted to the cell number (Farhane, Bonnier, and Byrne, 2017). After each incubation period, 2, 6, 12, 24, 48 and 72 hrs, cells were washed twice with sterile PBS and fixed in formalin (4 %, 15 min). A Horiba Jobin-Yvon LabRAM HR800 spectrometer with a 785 nm, 300 mW diode laser as source, Peltier cooled 16-bit CCD, 300 lines/mm grating and 100 µm confocal hole, was used for this work. Spectra were acquired in the range from 400 cm$^{-1}$ to 1800 cm$^{-1}$ using a x100 objective (LCPlanN, Olympus), in dry conditions, for 30 s two times, from three cell locations: cytoplasm, nucleus and nucleolus, visible under white light illumination. A final data set of 30 points per cell location for each time point, 2, 6, 12, 24, 48 and 72 hrs was produced after DOX exposure and for control cells, for each cell line, amounting to a total of over 210 cells per cell line, corresponding to a total data set of 1260 spectra.

7.2.5. **Data analysis:**

Raman spectral pre-processing and analysis were performed in Matlab 2013 using algorithms developed in house. Prior to analysis, background was subtracted using a NCLS (non-negatively constrained least squares) algorithm and spectra were smoothed (Savitsky-Golay filter 3th order, 11 points), baseline corrected (fifth order polynomial) and vector normalised.
PCA was employed as an unsupervised multivariate approach to analyse data and the effects of doxorubicin in each cell localisation. The order of the PCs denotes their importance to the dataset and PC1 describes the highest amount of variation (Efeoglu, Casey, and Byrne, 2016; Farhane et al., 2015).

PLSR, a statistical regression technique which reduces the dimensionality of the data and correlates information, here represented by the Raman spectra, to time evolution, was employed to track the temporal dependent evolution of the spectral signatures in the subcellular regions (Efeoglu, Casey, and Byrne, 2016; Keating et al., 2015). The percentage of variance explained as a function of the number of components was calculated using 10-fold cross validation and from a plot of the percent of variance explained function of number of components, the majority percentage of variance (above 90 %) was found to be explained by the first 3 components. The regression coefficients obtained by PLSR of Raman data regressed against time were plotted and analysed. As a function of frequency, the co-efficients illustrate the spectral features which are influenced by the intracellular interaction of the DOX and the resulting metabolic changes.

ICA was also employed as an extension to PCA. ICA is an unsupervised statistical technique able to identify latent variables called independent components. In the case of Raman micro-spectroscopy, ICA can be used to identify spectral contributions such as those from substrate, using the same number of ICs as PCs, which can then be removed or studied in their own right (Boiret et al., 2014; Vrabie et al., 2007).
7.2.6. Nuclear condensation:

Approximately 1 x 10^4 cells were allowed to attach on uncoated glass bottom Petri dishes (MatTek Corporation, USA) for two hours, after which they were covered with cell culture medium. After 24 hrs incubation, the medium was removed and samples were rinsed twice with sterile PBS, after which new fresh medium containing DOX corresponding to the inhibitory concentration, IC_{50}, determined by the cytotoxicity assays for each cell line, and adjusted to the cell number (Farhane, Bonnier, and Byrne, 2017), was added and cells were incubated for each corresponding time point.

After incubation, old medium was removed and 2 mL of Nucred® solution in medium was added and, after 15 to 30 min incubation, samples were rinsed twice with sterile PBS and kept in PBS for live cell imaging using CLSM. Control samples without exposure to DOX were also prepared in parallel, and incubated for 2, 6, 12, 24, 48 and 72 hrs.

CLSM images were recorded using an inverted Zeiss LSM 510 Confocal Laser Scanning Microscope equipped with a x60 oil immersion objective. DOX fluorescence was excited with an argon ion laser at 488 nm, and the emission was collected at 530 nm, while Nucred® was measured using 633 and 690 nm, excitation and emission, respectively. Using ImageJ software, after fluorescence background subtraction, the mean fluorescence intensity of Nucred® was measured for 10 cells for each cell line and each time point.

7.2.7. Bcl-2 protein expression:

Cells (3x 10^4/flasks) were cultured in T25 flasks over 24 hrs, and then exposed to a DOX dose corresponding to the inhibitory concentration, IC_{50}, adjusted to the cell number (Farhane, Bonnier, and Byrne, 2017), determined by cytotoxicity assays for each time point (from 2 hrs to 72 hrs) and each cell line. After each incubation period, cells were
trypsinised and centrifuged in 5 mL fresh medium at 4 °C and 1100 rpm for 5 min and they were then re-suspended in 1 mL Ice Cold Dulbecco's Phosphate-Buffered Saline (DPBS) buffer and centrifuged at 4 °C and 2500 rpm for 5 min. Cells were re-suspended in 750 μL ice cold DPBS buffer and transferred to Eppendorf tubes to which 250 μL of fixation buffer were added. After 30 min incubation at 4 °C, the fixed cells were washed twice in perm/wash buffer, centrifuged (2500 Rpm for 5 min at 4 °C) and then gently re-suspended in 50 μL perm/wash buffer, after which 20 μL of the antibody were added and the cells were incubated for 60 min in the dark at 4 °C. The cells were then washed twice in perm/wash buffer, centrifuged (2500 Rpm for 5 min at 4 °C) to remove unbound antibody and finally re-suspended in 1mL stain buffer. 10,000 cells were analysed by Flow Cytometry using a BD Biosciences Accuri C6 Flow Cytometer (Becton Dickinson, Oxford, UK). The Accuri Flow Cytometry software was used for the analysis of Flow Cytometry samples and data processing.

7.2.8. γH2AX expression:

Cells (10⁶/flask) were cultured in T25 flasks over 24 hrs, and then exposed to a DOX dose corresponding to the inhibitory concentration, IC₅₀, adjusted to the cell number (Farhane, Bonnier, and Byrne, 2017), determined by cytotoxicity assays for each time point (from 2 hrs to 72 hrs) and each cell line.

After each incubation period, cells were trypsinised and centrifuged in 5 mL fresh medium at 4°C and 1100 rpm for 5 min, then were re-suspended with 1mL PBS, transferred to an Eppendorf tube and centrifuged at 400 g for 5 min at 4 °C. Cells were re-suspended in 200 μL of formalin 2 % v/v in PBS and incubated for 10 min. If not used immediately, cells can be kept in 1mL ice-cold ethanol 70 % and samples and stored at -
20 °C for up to two weeks or overnight at 4 °C. Cells were then re-suspended in 500 µL Triton X-100 0.25 % v/v in PBS and incubated at Room Temperature (RT) for 5 min or 30 min at 4 °C, after which cells were centrifuged at 400 g for 5 min at RT and re-suspended in 200 µL Bovine serum albumin (BSA) solution (2 mg/100 mL) and incubated at RT for 30 min. After centrifugation at 400 g for 5 min at RT, cells were re-suspended in 150 µL antibody solution diluted 1:500 in BSA solution and incubated at RT for 2 hrs or overnight at 4°C. The cells were then washed thrice in PBS and finally re-suspended in 1mL BSA solution. 30,000 cells were analysed by Flow Cytometry using a BD Biosciences Accuri C6 Flow Cytometer (Becton Dickinson, Oxford, UK). The Accuri Flow Cytometry software was used for the analysis of Flow Cytometry samples and data processing.

7.3. Results and discussion:

7.3.1. Cytotoxicity assays:

Figure 7.1 shows the dose dependent responses of the cytotoxicity assays, AB and MTT, for early (6-24 hrs) and late (48 hrs-72 hrs) stage to DOX exposure, for both cell lines, A549 and Calu-1. Viability is expressed as % compared to control and the error bars indicate the standard deviation of six independent replicate measurements in triplicate and repeated three times.
Figure 7.1: AB in vitro dose dependent cytotoxicity assays of DOX A1. A549 cell line and A2. Calu-1 cell line and MTT in vitro dose dependent cytotoxicity assays of DOX B1. A549 cell line and B2. Calu-1 cell line. Viability is expressed as % compared to control, and the error bars indicate the standard deviation of six independent replicate measurements.

For both in vitro assays and for all exposure times, a partial loss of cell population viability is observed for the two cell lines. All viability curves were fitted with Eq. (1) (Black and Leff, 1983)

\[ V = V_{\text{min}} + \frac{(V_{\text{max}} - V_{\text{min}})}{1 + (C/IC_{50})^n} \]  

Equation 1

where \( V \) is the % viability, \( V_{\text{min}} \) is the minimum viability, \( V_{\text{max}} \) is the maximum viability, \( C \) is the DOX concentration, \( n \) is the Hill slope, and \( IC_{50} \) is the concentration which elicits
50% of the maximum response. IC$_{50}$ values are quoted with the error generated from the fit to all mean data points.

The AB and MTT assays for the A549 cell line show similar responses for each time exposure (Figure 7.1 A1 and B1) except for the early stage of 6 hrs, at which time point AB appears to be less sensitive than MTT. A slightly different profile is observed for the Calu-1 cell population (Figure 7.1 A2 and B2), whereby the AB assay is seen to be less sensitive for both 6 and 24 hrs exposure times (Table 7.1).

Table 7.1: DOX IC$_{50}$ concentration determined by the AB and MTT assays for the two cell lines A549 and Calu-1.

<table>
<thead>
<tr>
<th>IC$_{50}$ concentration (µM)</th>
<th>Time (hrs)</th>
<th>6</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>AB</td>
<td>1.52±0.20</td>
<td>0.42 ± 0.06</td>
<td>0.30± 0.04</td>
<td>0.29± 0.02</td>
</tr>
<tr>
<td></td>
<td>MTT</td>
<td>1.61±1.43</td>
<td>0.55 ± 0.16</td>
<td>0.31± 0.05</td>
<td>0.27± 0.02</td>
</tr>
<tr>
<td>Calu-1</td>
<td>AB</td>
<td>1.67±0.29</td>
<td>0.69 ± 0.13</td>
<td>0.62±0.12</td>
<td>0.37±0.01</td>
</tr>
<tr>
<td></td>
<td>MTT</td>
<td>1.90±0.76</td>
<td>0.90 ±0.24</td>
<td>0.72±0.15</td>
<td>0.41±0.08</td>
</tr>
</tbody>
</table>

The AB assay is an expression of general cellular metabolism, while the MTT assay is a reflection of mitochondrial activity (Farhane et al., 2015). Mitochondrial dysfunction is an early effect of DOX which explains the difference in the two assays for early exposure time points (Green and Leeuwenburgh, 2002). The notably different cytotoxic profiles for the two cell lines, up to 48 hrs exposure to DOX, are consistent with a higher intrinsic resistance of the Calu-1 cell line to the chemotherapeutic drug, whereby Calu-1 cells remain more viable than A549 cells at comparable doses and equivalent exposure times. The difference in cytotoxicity response between MTT and AB and between the two cell lines suggests different contributions of drug mechanisms of action and relatively different cellular behaviours to the drug treatment, resulting in a delayed response of
Calu-1 cell line compared to A549, as indicated by the lower sensitivity of the AB assay up to 24 hrs exposure. The MTT assay has been widely used as an indicator of chemosensitivity of cancer cell lines to a chemotherapeutic drug and shows a good correlation of the in vitro results with clinical observations (Burton, 2005; Campling et al., 1991; Carmichael et al., 1988) and the difference in MTT responses between the two cell lines is indicative of a higher chemosensitivity of A549 cells compared to Calu-1. Therefore, the IC₅₀ concentration determined by MTT assay was used for the rest of experiments.

7.3.2. Raman micro-spectroscopy:

Raman micro-spectroscopy has previously demonstrated that A549 and Calu-1 present different sensitivities to DOX treatment after 24 hrs exposure as a result of different relative contributions of the different mechanisms of action involved (Farhane et al., 2017). In order to further investigate the subcellular differences at early and late stages of exposure, Raman profiles for both cell lines were compared after 2, 6, 12, 24, 48 and 72 hrs exposure to DOX and spectra were taken from the three subcellular compartments, nucleolus, nucleus and cytoplasm (the example of original and pre-processed data of Calu-1 24 hrs exposure is shown in Figure S.7.1).
Figure 7.2: A. Pre-processed Raman spectrum of Doxorubicin dissolved in sterile water, and loading 1 corresponding to PC1 of PCA of Calu-1 control cells versus Calu-1 2 hrs exposure of B. nucleolus and C. nucleus.

As previously and for comparibility purposes, the IC\textsubscript{50} concentration, as determined by the MTT assay, for 24 hrs was used for early exposure from 2 to 24 hrs and for 48 and 72 hrs, each corresponding IC\textsubscript{50} concentration was used (Farhane, Bonnier, and Byrne, 2017).

Multivariate data analysis, consisting of PCA, PLSR and ICA, was employed in order to analyse in more detail the spectral features due to DOX exposure and compare the cellular effects and responses between the two cell lines and between the different exposure times. A pairwise PCA of exposed cells for each time point versus control for each cellular compartment was analysed and separation was observed according to PC1 for all time points. As an example PCA of control versus 24 hrs exposed for Calu-1 cells is shown in Figure S.7.2. Figure 7.2 shows the loading of PC1 for the nucleus and nucleolus of Calu-1 cells after 2 hrs exposure, compared to the spectrum of the DOX stock solution.
As seen in Figure 7.2, obvious DOX features at 440 and 465 cm\(^{-1}\), respectively derived from C–C–O and C–O vibrations, and 1085, 1215 and 1245 cm\(^{-1}\), related to C–O–H, C–O and C–H, are observed in the spectra of the nucleolar, and nuclear regions, highlighting the DOX uptake into Calu-1 cells after only 2 hrs exposure.

In both nucleolus and nucleus of Calu-1 cells, features at 1430-1450 cm\(^{-1}\) are also observed, corresponding to guanine and cytosine (absent in control cells) and are related to DOX-DNA intercalation inducing damage and/or conformational changes (Rabbani, Finn, and Ausio, 2005). The same features have been observed after 24 hrs of DOX interaction in A549 cells (Farhane et al., 2017) confirming the early stage DOX-DNA and RNA binding for both cell lines (Agudelo et al., 2013). The decrease in the bands at 785 and 813 cm\(^{-1}\), corresponding respectively to DNA backbone O–P–O and RNA O–P–O phosphodiester bond stretching, confirm an early DOX effect in Calu-1 cells consistent with its main mechanism of action, DNA intercalation, interfering with both DNA and RNA polymerase, inducing DNA replication and RNA transcription blockage (Farhane et al., 2015; Thorn et al., 2011).

In order to further analyse the DOX cellular kinetics, the DOX peak intensity at 465 cm\(^{-1}\), the loading of PC1 of each time point and cellular compartment for both cancer cells was analysed. This DOX band was chosen due to the minimal influence by the proximity of cellular compounds features.
Figure 7.3: Evolution of DOX, represented by the Raman band at 465 cm$^{-1}$, as a function of time for the A549 and Calu-1 cell line for each cellular compartment, nucleolus, nucleus and cytoplasm. Intensities are expressed as percentage according to the maximum value over the three cellular compartments and the two cell lines and standard deviation corresponds to the spectral variations of the Raman band over the 30 measurements per location.

Figure 7.3 shows the time evolution of this band in each subcellular region for each cell line. Intensity is expressed in percentage function of the highest value observed over all the measurements, that of the A549 nucleolar region after 6 hrs exposure. For both cell lines, in each subcellular region, (Figure 7.3) there is a progressive increase of intracellular DOX concentration until a plateau is reached. As previously described for A549 cells, DOX accumulation is initially observed in the nucleoli, reaching saturation at ~6 hrs, then in the nucleus, reaching saturation at ~12 hrs, before it finally begins to accumulate in the cytoplasm, at ~48 hrs, after saturation of nuclear binding sites (El-Kareh and Secomb, 2005; Zenebergh, Baurain, and Trouet, 1984).
Figure 7.4: A. Loading 1 of PC1 of control versus exposed cells. A. Nucleolus A549 control versus 6 hrs exposure compared to that of nucleolus Calu-1 control versus 2 hrs exposure B. Nucleus A549 control versus 12 hrs exposure and nucleus Calu-1 control versus 6 hrs exposure.
Notably, the subcellular accumulation in Calu-1 is much more rapid, saturating at ~2 hrs in the nucleoli, ~6 hrs in the nucleus and is detectable in the cytoplasm after only ~24 hrs. Despite the fact that DOX-DNA intercalation starts at early stages for both cell lines, according to the DOX Raman band, DOX saturation for both nuclear areas happens more rapidly in Calu-1 cells than A549, after which DOX concentration inside the combined nuclear regions becomes approximately constant and accumulation in the cytoplasm due to additional drug taken up by cells is only evident after ~48 hrs for A549 and earlier at ~24 hrs for Calu-1, due, in both cell lines, to nuclear membrane disruption (Farhane, Bonnier, and Byrne, 2017). The saturation levels in each subcellular region are comparable for each cell line, although significantly lower levels of DOX are recorded in the cytoplasmic region of A549.

In order to compare the DOX mechanism of action and cellular responses, cellular features were analysed in more detail over the time periods before and after DOX saturation for the nuclear and cytoplasmic compartments.

As seen in Figure 7.4, which shows the loading of PC1 of control cells versus exposed in the nuclear regions below the saturation point, almost identical loadings are obtained for both cell lines, including dominant DOX peaks at 440, 465, 1215 and 1245 cm$^{-1}$. Guanine and cytosine features are prominent, respectively at 1430 and 1450 cm$^{-1}$, confirming DOX intercalation and RNA interaction in the nucleolus and DNA in the nuclear region, related to DOX binding inducing changes in DNA conformation with a partial transition of DNA from B to A form (change in the ratio between 813 cm$^{-1}$ and 830 cm$^{-1}$ bands) and blockage of DNA synthesis and as a consequence a decrease in RNA (Agudelo et al., 2014; Farhane et al., 2015).
Up to the saturation point, the same spectral features assigned to DOX mechanism of action by nuclear intercalation were thus found for A549 and Calu-1, giving a reproducible signature of the binding interaction, which appears to be same for the two cell lines.

In order to track the cellular changes after saturation, PLSR was employed and spectra were regressed against time starting from each saturation point 2 hrs and 6 hrs for Calu-1 cells and 6 hrs and 12 hrs for A549 cells, respectively for nucleolus and nucleus. The regression coefficients obtained are shown in Figure 7.5.

In both cell lines, for the nucleolar regions (Figure 7.5 A), a notable decrease in features at 728 cm\(^{-1}\) (adenine) 785 cm\(^{-1}\) (cytosine, thymine and DNA backbone O–P–O), 813 cm\(^{-1}\) (RNA O–P–O stretching), 1095 cm\(^{-1}\) (DNA PO\(_2^–\) symmetric stretching) and 1376 cm\(^{-1}\) (thymine), are consistent with a decrease in nucleic acid contributions due to the DOX mechanism of action by nucleolar interaction, inducing DNA replication blockage, and decrease of RNA features by inhibiting RNA synthesis at the level of rRNA transcription, inhibiting ribosome biogenesis, and, as a consequence, alteration of nucleolar structure (Orsolic et al., 2016), size, shape and fragmentation (Farhane, Bonnier, and Byrne, 2017; Farhane et al., 2015; Farhane et al., 2017), inducing nucleolar stress dependent apoptosis (Avitabile et al., 2011; Woods et al., 2015).
Figure 7.5: Regression coefficients obtained by PLSR analysis as a function of time after DOX saturation for A549 and Calu-1 cells for A. nucleolus and B. nucleus.

The increase in proteins and lipids features at respectively 447-454 cm\(^{-1}\) (phenylalanine), (those two bands can be detected clearly due to the fact that, after saturation, the DOX
cellular concentration becomes constant and therefore the peaks of the drug itself are no longer evident in the regression), 645-877 cm⁻¹ (C-C tyrosine), 940 cm⁻¹ (C=C stretching of protein), 1207 cm⁻¹ (phenylalanine, tryptophan and tyrosine), 1230 cm⁻¹ (Amide III), 1520-1545 cm⁻¹ (Amide II), 1605 cm⁻¹ (C=C phenylalanine and tyrosine), 1670 cm⁻¹ (Amide I) 1410-1450 cm⁻¹ (CH₂ deformation) are consistent with an efflux of anti-apoptotic and repair proteins, as a cellular defense against DOX exposure, and a lipid denaturation of the surrounding cytoplasmic membrane (Farhane et al., 2015; Verrier et al., 2004).

In addition to depletion of spectral features related to DNA in the nuclear area for both cell lines, such as those at 795 cm⁻¹, 1095 cm⁻¹ and 1376 cm⁻¹ (Figure 7.5 B), there is an increase in both lipid and protein features, at 645 cm⁻¹ (C-C tyrosine), 715 cm⁻¹(choline), 760 cm⁻¹ (tryptophan ring breathing), 853-877 cm⁻¹ (tyrosine ring breathing), 1127 cm⁻¹ (C-N stretching), 1445 cm⁻¹ (CH₂ vibrations), 1491 cm⁻¹ (C-H deformation), 1607 cm⁻¹ (tyrosine and phenylalanine ring vibration C=C), and 1650 cm⁻¹ (Amide I), again consistent with a cellular response to DOX associated with anti-apoptotic protein and a synthesis of lipidic vesicules as a way to remove DOX to the extracellular environment.

For both the nucleolus and nucleus in A549 cells, a notable increase in features at 1047 cm⁻¹ (RNA P–O stretching, sugar phosphate –C–O–stretching), and 1270 cm⁻¹ (RNA Uracil and cytosine ring stretching) is due to nucleolar fragmentation, resulting in RNA spread (Chen et al., 2012).
Figure 7.6: A. Evolution of DOX (465 cm\(^{-1}\)) and selected RNA (785, 813 and 1270 cm\(^{-1}\)) and DNA (728, 785, 813 and 830 cm\(^{-1}\)) Raman bands as a function of time. B. Evolution of DOX (465 cm\(^{-1}\)) and selected protein (645, 1005 and 1250 cm\(^{-1}\)) and lipid (1450 cm\(^{-1}\)) Raman bands as a function of time for the Calu-1 cell line for each nuclear cellular compartment, (i) nucleolus and (ii) nucleus. Intensities are expressed in percentage according to the maximum value for each nuclear area and standard deviation corresponds to the spectra variations for each band.

The increase in phenylalanine at 1005 cm\(^{-1}\) seems to be a marker of the late apoptosis stage, at which point nucleolar fragmentation is complete, leaving an empty space with only cellular membrane, while in Calu-1 cells and for both nuclear regions, there is an
increase in the 1270 cm\(^{-1}\) band and a decrease in the 1047 and 1005 cm\(^{-1}\) bands, which could be explained by the fact that the nucleolar fragmentation is less advanced in Calu-1 than A549 cells, due to a delay in apoptosis by a higher production of anti-apoptotic proteins. In the nucleus of the Calu-1 cell line, there is also an increase in some DNA bands at 785 cm\(^{-1}\) and 1577 cm\(^{-1}\), which may be due to an increase DNA repair.

In order to highlight the increase of cellular compounds, namely DNA, RNA, proteins and lipids in Calu-1 cells, the evolution of related spectral features in both nucleolus and nucleus, 785 cm\(^{-1}\) and 813 cm\(^{-1}\) (DNA/RNA), 728 cm\(^{-1}\) and 830 cm\(^{-1}\) (DNA), 645 cm\(^{-1}\), 1005 cm\(^{-1}\) and 1250 cm\(^{-1}\) (proteins) and 1450 cm\(^{-1}\) (lipids), as a function of time can be plotted (Figure 7.6). The band intensities were determined by ICA after DOX substraction. The DOX band was determined, as Figure 7.3, by PCA control vs exposed cells. The evolution of similar bands in A549 cells has been demonstrated previously (Farhane, Bonnier, and Byrne, 2017).

A notable decrease in DNA and RNA features is observed (Figure 7.6 A (i) and A (ii)) at the early stage, concomitant with DOX nuclear accumulation and binding, due to DNA/RNA alteration, followed by an increase in the same features starting from ~24 hrs, as a cellular response by increasing DNA repair. However, the changes appear to be slower in the nucleus and much stronger in the nucleolus, in which a decrease of nucleic acids to less than 10 % is observed, after which they increase to almost the same proportion ~40 %. The decrease in nucleic acid features appears also to be stronger in Calu-1 compared to A549 (Farhane, Bonnier, and Byrne, 2017). After DOX saturation, ~2 hrs for the nucleolus and ~6 hrs for the nucleus, proteins and lipids start increasing (Figure 7.6 B (i) and B (ii)), consistent with the production of anti-apoptitic proteins and a synthesis of lipidic vesicules inducing resistance to apoptosis and as a consequence resistance to the chemotherapeutic drug.
In both nucleolar and nuclear regions, similar kinetics of cellular compounds were found for the two cell lines (Farhane, Bonnier, and Byrne, 2017) at the early stages, resulting in a decrease of all cellular features, and after DOX nuclear saturation an increase of protein and lipids is observed, while nucleic features keep decreasing for A549, in contrast to Calu-1 cells which, at prolonged exposures, show signs of recovery by DNA repair, resulting in an increase of nucleic acids features.

In both PLSR regression co-efficient profiles for the cytoplasmic region, for the two cell lines, as shown in Figure 7.7, there are obvious DOX features at 440-465 cm\(^{-1}\) and 1215-1245 cm\(^{-1}\). An increase in protein and lipid features is also evident, for example at 524-733 cm\(^{-1}\), corresponding to phosphatidylserine externalization (in healthy cells, aminophospholipids are localised in the inner cytoplasmic membrane) due to membrane alteration (Kagan et al., 2000; Wlodkowic et al., 2011) as an indicator of apoptosis (Lee et al., 2013; Ziegler and Groscurth, 2004), 538 cm\(^{-1}\) (cholesterol ester), related to alteration of endoplasmic reticulum membrane as a marker of apoptosis (Farhane, Bonnier, and Byrne, 2017), 576-720 cm\(^{-1}\) (phosphatidylinositol and phosphatidylcholine) related to membrane trafficking, 645-830-853 cm\(^{-1}\) (tyrosine), 1268 cm\(^{-1}\) (Amide III), 1364 cm\(^{-1}\) (trypotphan), 1410-1450 cm\(^{-1}\) (CH\(_2\) deformation) and 1635-1660 cm\(^{-1}\) (Amide I of different conformational forms) indicating a protein and lipid denaturation due to DOX treatment.
Figure 7.7: Regression coefficients obtained by PLSR analysis as a function of time over full range of cytoplasmic region for A549 and Calu-1 cells.

In addition to the common protein and lipid denaturation indicators between the two cell lines, there is a more notable increase in Calu-1 cells of the bands at 917 cm\(^{-1}\) (Ribose vibration) (Movasaghi, Rehman, and Rehman, 2007), 940 cm\(^{-1}\) (C-C stretching of protein), 1030 cm\(^{-1}\) (C-H phenylalanine), 1047 cm\(^{-1}\) (RNA P–O stretching, sugar phosphate –C–O–stretching) and 1085 and 1128 cm\(^{-1}\) (C-N stretching) corresponding to higher RNA, protein and lipids structure denaturation by oxidative stress due to ROS production, one of the DOX mechanisms of action which has previously been shown to be more prominent in Calu-1 than A549 cells (Efeoglu, Casey, and Byrne, 2016; Farhane et al., 2017).

Therefore, according to the Raman profile, for the two cell lines, DOX appears to have the same binding signatures as seen in loading 1 of PC1 of PCA of control versus exposed cells up to saturation point (Figure 7.4), but Calu-1 cells, despite an earlier DOX cellular accumulation (Figure 7.3), seem to be more resistant, as indicated by the lower cytotoxic
response, due to an efflux of anti-apoptotic protein and an increased DNA repair at the later stages. In order to confirm those observations, bcl-2, an anti-apoptotic protein, and γH2AX, a marker of DNA damage and repair, were measured in both cell lines and the results were correlated to the Raman analysis.

### 7.3.3. Apoptosis, Bcl-2 protein and DNA repair:

Apoptosis is a programmed caspase-mediated cell death characterised by morphological cellular changes, including nuclear and cytoplasmic condensation with externalisation of phosphatidylserine and formation of apoptotic bodies, with maintenance of intact cellular membrane. During the later stages, the nucleus further condenses (pyknosis), which is the most characteristic morphological marker of apoptosis (Elmore, 2007; Plesca, Mazumder, and Almasan, 2008), and this is followed by nuclear shrinkage (karyorrhexis) and loss of cellular shape by cytoskeleton cleavage and membrane blebbing (Fink and Cookson, 2005; Saraste and Pulkki, 2000).

Figure 7.8 shows the evolution of Nucred® fluorescence, as an indicator of nuclear condensation, as a function of time for the two cell lines. In both cases, there is a slight increase in fluorescence in the early stages of drug exposure, and the maximum fluorescence is observed at 24 hrs. The maximum is significantly higher for Calu-1 than A549, due to a higher level of chromatin condensation induced by DNA damage due to DOX-DNA interaction (Rabbani, Finn, and Ausio, 2005), confirming the Raman observations (Figure 7.6).
Subsequently, the fluorescence decreases more rapidly in A549 than Calu-1, as a consequence of a higher apoptotic response in Calu-1, via chromatin condensation, (Burgess et al., 2014) confirming the Raman results showing that the two cell lines have the same behaviour at early stages but are different in the later stages. Despite the fact that DOX saturates the nuclear area earlier in Calu-1 than A549, and exhibits higher DNA damage levels, Calu-1 cells appear to be more viable at later stages, which suggest a more effective defence mechanism in Calu-1, increasing cell survival (Roos and Kaina, 2006; Roos, Thomas, and Kaina, 2016), and as a consequence a higher resistance to DOX.

This drug resistance could be explained by either a higher expression of anti-apoptotic protein or an increase in DNA repair, or both.

The expression profile of bcl-2 (B cell lymphoma) an anti-apoptotic protein synthesized as a direct response to DNA damage (Farhane et al., 2017), as a function of time for the
toward cell lines is shown in Figure 7.9. A similar profile is observed, with up regulation followed by a diminution of the bcl-2 protein level as a function of time. Nevertheless, the maximum level of bcl-2 is reached at 12 hrs in the A549 cell line and 48 hrs in Calu-1, with an obviously consistently higher expression for the latter stage over the time range. This higher level of bcl-2 protein contributes to cellular resistance to apoptosis inducing a chemotherapeutic drug resistance.

![Figure 7.9](image)

**Figure 7.9**: Bcl-2 anti-apoptotic protein level determined by Flow Cytometry after DOX exposure from time point 2 to 72 hrs for A549 and Calu-1 cells, expressed on percentage according to the maximum value for both cell lines.

A specific change in response to DSB is the phosphorylation of H2AX histone, representing 2 to 25 % of the total H2A protein, by members of phosphoinositide 3-kinase related proteins of DDR, into γH2AX a sensitive indicator of DSB and efficiency of subsequent DNA repair (Ohnishi, Mori, and Takahashi, 2009; Podhorecka, Skladanowski, and Bozko, 2010; Srivastava et al., 2009), which is facilitated by γH2AX dephosphorylation by protein phosphatase 2A (Kuo and Yang, 2008; Sharma, Singh, and
Almasan, 2012). The phosphorylation of γH2AX is thus an early indicator of DSB (Fernandez-Capetillo et al., 2004; Plesca, Mazumder, and Almasan, 2008) and the number of γH2AX nuclear foci is directly correlated with the number of DSBs, (Takahashi and Ohnishi, 2005) which means the more damage there is, the more nuclear foci will be found. Flow Cytometry is a sensitive and rapid technique for the measurement of γH2AX phosphorylation at cellular level (Sharma, Singh, and Almasan, 2012).

As seen in Figure 7.10, showing the γH2AX expression after DOX exposure for each time point for the two cell lines, there is a progressive increase of γH2AX until a maximum is reached, followed by a later decrease for the two cell lines. However, for Calu-1, an obvious higher phosphorylation is observed for early stages explained by a higher DNA damage, as observed in the Nucred® (Figure 7.8) and Raman results (Figure 7.6) and the maximum expression is observed at 12 hrs, while for A549 it is observed at 24 hrs, consistent with a more rapid uptake of the drug in the nuclear regions of Calu-1. After the 12 hrs maximum, the γH2AX levels are seen to decrease more rapidly for the Calu-1 cells than observed for the A549. The decrease in γH2AX corresponds to dephosphorylation of this histone after DNA repair and this dephosphorylation is proportional to DNA repair mechanisms which occur over time to repair the DNA damage. In this case, it appears that there is an increased tolerance to DNA damage and a higher degree of DNA repair in Calu-1 cells than in A549, confirming the Raman observations (Figure 7.5 and 7.6) showing a recovery in DNA features for Calu-1 at the later stages inducing a delay and resistance to apoptosis.
Figure 7.10: Mean Fluorescence of γH2AX after DOX exposure measured by Flow Cytometry for A549 and Calu-1 cells, expressed on percentage according to the maximum value for both cell lines.

Raman micro spectroscopic results elucidate both drug kinetics and mechanism of action, giving a fingerprint of chemical binding, nucleic acid intercalation, and signatures of cellular resistance associated with tolerance to DNA damage, synthesis of anti-apoptotic protein and DNA repair. Multivariate data analysis (PCA, ICA and PLSR) indicates that the two cell lines have different rates of uptake, resulting in earlier saturation of both nucleolar and nuclear compartments for the Calu-1 cell line, and an earlier appearance of DOX in cytoplasm due to nuclear disruption. It has been reported that cellular uptake of the weakly-basic drug DOX is dependent on the extracellular pH (Swietach et al., 2012), and thus, the lower pH of the DMEM-F12 (pH 7-7.4) compared to that of RPMI (pH 8.2 ± 0.3) may be the cause of a slower uptake of DOX in A549 cells compared to Calu-1. However, the degree of accumulation in the nulei and nucleoli is seen to be similar in the two cell lines, indicating that there are no intracellular pH dependent differences between
the cell lines (Swietach et al., 2012). After passive diffusion through the cellular cytoplasmic membrane due to its high lipophilicity, DOX binds directly to proteasomal transporters and translocates into the combined nuclear area whereupon it binds to nucleic acids and rapidly saturates the nucleolar region (Farhane, Bonnier, and Byrne, 2017). Cellular efflux regulators can also impact on the rate of accumulation in the cell nucleus (Shen et al., 2007), although the enhanced DOX cellular uptake observed in Calu-1 compared to A549 cells should be associated with higher cytotoxicity in Calu-1 cells (Kik et al., 2009). The fact that the inverse is observed is a strong indication that the cellular resistance has origin in higher tolerance to DNA damage with higher synthesis of anti-apoptotic proteins.

Critically, the early signatures of interactin of the drug, associated with the drug interaction, are comparable in the two cell lines, indicating that the methodology can be developed for label free, prescreening of drug candidates in vitro.

Regression against time after DOX saturation in the respective nuclear regions shows essentially the subsequent cellular responses to DOX exposure, including DNA/RNA damage leading to apoptosis and the different cellular reactions such as synthesis of lipidic vesicles and increase in protein features. Despite the fact that same binding signature was found for the two cell lines, Calu-1 cells show higher resistance, correlated with notably different dose dependent cytotoxic response profiles, suggesting a higher viability, firstly by increased tolerance to DNA damage manifest as a decrease of DNA to ~10 % of control compared to 40 % in A549, confirmed by higher nuclear condensation according to the Nucredf® assay, and larger amount of γH2AX at early exposure points and secondly, higher expression of anti-apoptotic proteins, principally bcl-2 one and higher DNA repair, confirmed by H2AX dephosphorylation at later stages.
7.4. Conclusion:

Raman micro-spectroscopy has already demonstrated its potential to track *in vitro* the kinetics and accumulation of the chemotherapeutic drug DOX at a subcellular level and to identify its different mechanism of action (Farhane, Bonnier, and Byrne, 2017; Farhane et al., 2015; Farhane et al., 2017). The present study not only confirms its ability to monitor drug cellular kinetics and mechanism of action but also to differentiate between cellular reactions and resistance. In fact, the two lung cell lines exhibit different behaviours with higher nuclear condensation, measured by Nucred®, and a higher resistance of Calu-1, despite the similar drug chemical binding, with a higher tolerance to DNA damage and implications of DNA repair mechanism confirmed by expression of γH2AX protein, and resistance to apoptosis by bcl-2 protein expression.

Thus, Raman micro-spectroscopy is able not only to track the subcellular accumulation of the drug as function of time but also to identify its mechanism of action, the subsequent cellular response and to differentiate cellular resistance. Therefore, it can be used as an *in vitro*, pre-clinical screening technique for drug mechanism of action and efficacy in order to aid preclinical drug development. Furthermore, the ability of Raman micro-spectroscopy to monitor subcellular processes associated with drug resistances suggests its potential as an *in vitro* companion diagnostics technique to screen for personalised therapies.

7.5. Acknowledgement:

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7.6. References:


7.7. Supplementary Information:

A. 

B. 

Figure S.7.1: Raman spectra of Calu-1 cells exposed for 24 hrs at corresponding DOX IC$_{50}$ concentration of 0.9 µM A. original data and B. pre-processed of nucleolus (black), nucleus (red) and cytoplasm (blue).
Figure S.7.2: PCA of nucleolar, nuclear and cytoplasmic regions of A. Calu-1 control versus 24 hrs exposed with corresponding loadings of PC1 and PC2 B. Pairwise PCA of Calu-1 control versus 24 hrs exposed for each subcellular region and the corresponding loadings of PC1 and PC2.

Nucleolus ● Nucleus ○ Cytoplasm ● Calu-1 control

Nucleolus ▼ Nucleus ▼ Cytoplasm ▼ Calu-1 24 hrs exposed
Chapter 8: An *in vitro* study of the interaction of the chemotherapeutic drug Actinomycin D with lung cancer cell lines using Raman micro-spectroscopy

The following chapter reproduces the journal article, Z. Farhane, F. Bonnier and H.J. Byrne, J. Biophotonics DOI: 10.1002/jbio.201700112, in which the section and figure numbers have been adapted to the prescribed thesis format.

All experiments, data analysis and paper writing were performed by Z.F. F.B. assisted in the experimental design.

H.J.B. assisted in the project design and proofing of the manuscript.

**Graphical Abstract:**

A. Pre-processed Raman spectrum of ACT stock solution dissolved in sterile water and mean spectrum with standard deviation of B. Nucleolus, C. Nucleus and D. Cytoplasm of A549 cell lines after 48hrs exposure to the corresponding IC$_{50}$.

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**Abstract:**

The applications of Raman micro-spectroscopy have been extended in recent years into the field of clinical medicine, and specifically in cancer research, as a non-invasive diagnostic method *in vivo* and *ex vivo*, and the field of pharmaceutical development as a label free predictive technique for new drug mechanisms of action *in vitro*. To further illustrate its potential for such applications, it is important to establish its capability to fingerprint drug mechanisms of action and different cellular reactions.

In this study, cytotoxicity assays were employed to establish the toxicity profiles for 48 and 72 hrs exposure of lung cancer cell lines, A549 and Calu-1, after exposure to Actinomycin D (ACT) and Raman micro-spectroscopy was used to track its mechanism of action at subcellular level and subsequent cellular responses. Multivariate data analysis was used to elucidate the spectroscopic signatures associated with ACT chemical binding and cellular resistances. Results show that the ACT uptake and mechanism of action are similar in the two cell lines, while A549 cells exhibits spectral signatures of resistance to
apoptosis related to its higher chemoresistance to the anticancer drug ACT. The
observations are discussed in comparison to previous studies of the similar anthracylic
chemotherapeutic agent Doxorubicin.

**Keywords:** Raman micro-spectroscopy, Actinomycin D, A549, Calu-1, binding
signature
8.1. Introduction:

Raman micro-spectroscopy is a non-invasive analytical tool, whose potential in clinical applications to distinguish between normal and cancer cells and monitoring drug mechanisms of action has already been demonstrated (Byrne, 2014; Byrne et al., 2013; Farhane, Bonnier, Casey, and Byrne, 2015; Farhane, Bonnier, Casey, Maguire, et al., 2015; Jamieson and Byrne, 2016). Therefore, it can potentially be used in pre-clinical development for the prediction of chemotherapeutic efficacy and potentially ultimately as a companion diagnostic tool. In order to assure its application as a predictive tool, Raman spectroscopy should provide information about drug mechanisms of action and cellular reactions or resistance. Previous studies were made using Raman microspectroscopy and chemotherapeutic drugs such as Cisplatin (Nawaz et al., 2011), Vincristine (Nawaz et al., 2013), Erlotinib (Yosef et al., 2015), Panitumumab (El-Mashtoly et al., 2015) and Doxorubicin (DOX) (Farhane, Bonnier, and Byrne, 2017) showing the potential of this vibrational technique to track drug mechanisms of action and cellular effects.

The present study further extends the Raman investigations to analyse the interaction of Actinomycin D, an anticancer drug used in clinics, and to confirm the commonality of the chemical binding signature across the anthracycline chemotherapeutic group.

Actinomycin D (ACT) is a polypeptide antibiotic chemotherapeutic drug, isolated from Streptomyces, developed in the 1950s and clinically approved 10 years later (Cibi and Jayakumaran Nair, 2016; Liu et al., 2016). It blocks both DNA and RNA expression, and as a consequence protein synthesis, by inhibition of RNA polymerase and transcription in nucleoli and therefore induces cellular p53-independent apoptosis (Cassé et al., 1999; Hasanzadeh and Shadjou, 2016; Kleeff et al., 2000; Nazari et al., 2012; Schwartz et al.,
1965; Yung, Bor, and Chan, 1990). It intercalates preferentially between guanine and cytosine base pairs in DNA via the phenoxazone ring and forms strong hydrogen bonds in the minor groove between the two pentapeptide side chains and the guanine 2 amino groups, blocking the subsequent chain elongation (Chinsky and Turpin, 1978; Enache and Volanschi, 2008; Hasanzadeh and Shadjou, 2016). ACT is also able to bind externally to DNA, to the terminal GC base pairs, and to interact with double and single stranded DNA and some DNA sequences containing no GC binding site (Lohani, Singh, and Moganty, 2016; Lu et al., 2015; Sha and Chen, 2000; Takahashi and Ohnishi, 2005). This antitumor drug is used clinically for the treatment of highly aggressive malignancies (Lohani, Singh, and Moganty, 2016) such as pancreatic (Kleeff et al., 2000), Wilm’s tumour, testicular and trophoblastic cancer (Bensaude, 2011; Lu et al., 2015) and in combination with other chemotherapeutic drugs for the treatment of high risk cancers (Sha and Chen, 2000).

Using ACT and two histological subtype lung cancer cell lines, A549 and Calu-1, Raman spectroscopic investigations were supported by cytotoxicity assays, and multivariate data analysis, consisting of Principal Component Analysis (PCA) and Independent Component Analysis (ICA), was used to extract biological information contained in the Raman spectra. Results confirm the potential of Raman micro-spectroscopy as an in vitro analytical tool for biological analyses and in the prediction of drug mechanisms of action and cellular reactions.

8.2. Materials and methods:

8.2.1. Materials:

A549 human lung adenocarcinoma cells with the alveolar type II phenotype were obtained from ATCC (Manassas, VA, USA) and the Calu-1 human lung epidermoid
carcinoma cell line was kindly provided by Dr. Josep Sulé-Suso, Institute for Science & Technology in Medicine, Keele University, Guy Hilton Research Centre UK and Cancer Centre, Royal Stoke University Hospital, University Hospitals of North Midlands, UK.

An Actinomycin D stock solution of concentration 398 µM was prepared by dilution of Actinomycin D® powder (Sigma Life Sciences, Ireland) in 4mL sterile water.

Alamar blue (AB) (10X ready to use solution) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Aldrich, Ireland.

For cytotoxicity assays, an AB/MTT solution, 1.5 mL of AB and 3 mL of MTT stock solution (2.5 mg/mL, 25 mg MTT/10 mL PBS) in 30 mL of fresh medium was prepared prior to performing the assays.

8.2.2. Cell culture:

A549 cells were cultured in DMEM-F12 (Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12) with 10% FBS (foetal bovine serum) and Calu-1 cells in RPMI (Roswell Park Memorial Institute Medium) with 10% FBS, both at 37 °C, in a humidified atmosphere containing 5% CO₂, and cells were split every two days to maintain ~60% confluency.

For cytotoxicity assays and Raman micro spectroscopic analysis, the cell number was determined using a Beckman Coulter Particle Count and Size Analysis® Z2 Cell Counter.

8.2.3. Cytotoxicity assays:

AB and MTT assays were performed in 96 well plates and a total number of 1x10⁵ cells (4x10³ cells/mL), 1 x 10⁴ (4x10² cells/mL) and 5x10³ cells (2x10² cells/mL), respectively were used for 24, 48 and 72 hrs, with three replicate plates of each. After 24 hrs
incubation, plates were washed with phosphate buffered saline solution (PBS) and ACT was added in a concentration range from 0 µM (as a control) to 50 µM for 24 hrs and from 0 to 0.5 µM for 48 and 72 hrs.

AB and MTT assays were both measured with a Cytotox SpectraMax®M3 plate reader using Soft Max® Pro6.2.2 software. After 24, 48 and 72 hrs incubation in ACT, plates were washed with PBS and 100µL of AB/MTT solution were added to each well. Plates were then incubated for 3 hours and AB fluorescence was measured first in the plate reader, using 540 nm excitation and 595 nm emission, whereupon the medium was removed, the plates were washed with PBS and 100 µL of DMSO (Dimethyl sulfoxide) were added in each well. MTT absorbance was then read at 570 nm.

All cytotoxicity assays were made in triplicate and repeated three times and viability data was fitted by a four parameter Hill equation analysis using SigmaPlot 10.0, to yield values of the mean inhibitory concentration, IC₅₀.

8.2.4. Raman micro-spectroscopy:

Cells (~ 1x 10⁴/window) were seeded and incubated on CaF₂ windows (Crystan Ltd, UK) for 24 hrs for both control and exposure to ACT. The medium was then removed and samples were rinsed twice with sterile PBS and covered with ACT at each corresponding IC₅₀ for exposed cells and fresh medium for unexposed controls. After each incubation period, 48 and 72 hrs, exposed and unexposed control cells were washed twice with sterile PBS and fixed in formalin (4 %, 15 min).

A Horiba Jobin-YvonLabRAM HR800 spectrometer with a 785 nm, 300 mW diode laser as source, Peltier cooled 16-bit CCD, 300 lines/mm grating and 100 µm confocal hole, was used for this work. Spectra were acquired in the range from 400 cm⁻¹ to 1800 cm⁻¹.
using a x100 objective (LCPlanN, Olympus), in dry conditions, for 30 s two times, from three cell locations: cytoplasm, nucleus and nucleolus, identifiable under white light illumination. The final data set of a total of 720 spectra was thus derived from 30 cells, each measured in the nucleolus, nucleus and cytoplasm, for each exposure time (2) and control (2), for each cell line (2).

8.2.5. Data analysis:

Raman spectral pre-processing and analysis were performed in Matlab 2013 using algorithms developed in house. Prior to analysis, background was subtracted using a NCLS (non-negatively constrained least squares) algorithm and spectra were smoothed (Savitsky-Golay filter 3th order, 11 points), baseline corrected (fifth order polynomial) and vector normalised.

PCA allows the reduction of the number of variables in a multidimensional dataset, although it retains most of the variation within the dataset. It represents the spectra in data groupings of similar variability, allowing the identification and differentiation of different spectral groups. The loadings of the PCs represent the variance for each variable (wavenumber) for a given PC, and analysis of the loadings can give information about the source of the variability inside a dataset, derived from variations in the molecular components contributing to the spectra. It has been demonstrated that the PC loadings can be most simply understood when analysis of datasets is undertaken in a pairwise fashion. Although PCA does not cluster the data, per se, in the same manner as for example Hierarchical or K-Means Cluster Analysis, whereby differential distribution of the data according to negative or positive loadings associates specific spectral features with that dataset. PCA was employed as an unsupervised multivariate approach to analyse data and
the effects of ACT in each cell localisation by identification and differentiation of different spectral groups using scatter plots and loadings showing a representation of spectral features responsible for the variance between data groups according to wavenumbers. The order of the PCs denotes their importance to the dataset and PC1 describes the highest amount of variation (Bonnier and Byrne, 2012; Brauchle and Schenke-Layland, 2013; Pavićević et al., 2012).

ICA was also employed as an extension to PCA. ICA is an unsupervised statistical technique able to identify latent variables called independent components in each data set separately. In the case of Raman micro-spectroscopy, ICA can be used to identify spectral contributions such as those from substrate, which can then be removed or studied in their own, using the same number of ICs (three components) as PCs, as estimated by PCA, explaining the majority of variance, and only the first component for each cellular compartment was plotted (Farhane, Bonnier, and Byrne, 2017; Hyvärinen and Oja, 2000; Lee, 1998).

8.3. Results and discussion:

8.3.1. Cytotoxicity assay:

After 24 hrs exposure, more than 60 % of cells remain viable according to both the AB and MTT assays at the maximum dose of 50μM for both cell lines (data not shown), which is consistent with literature reporting late onset of ACT effects (Kleeff et al., 2000). Therefore, for the rest of the experiments, only 48 and 72 hrs exposure times where analysed in this study.
Figure 8.1: AB and MTT in vitro dose dependent cytotoxicity assays of ACT of A549 cell line A1. 48 hrs exposure and A2. 72 hrs exposure and Calu-1 cell line B1. 48 hrs exposure and B2. 72 hrs exposure.

Figure 8.1 shows the dose dependant responses of the cytotoxicity assays, AB and MTT, for 48 and 72 hrs to ACT exposure, for both cell lines, A549 and Calu-1. Viability is expressed as % compared to control (non-exposed healthy cells), and the error bars indicate the standard deviation of six independent replicate measurements.

For both in vitro assays and for all exposure times, a partial loss of cell population viability is observed for the two cell lines.
The IC\textsubscript{50} values measured by the AB and MTT assays were found to be almost the same for A549 cells, at the respective time points, while for Calu-1, the AB assay is significantly less sensitive than MTT (Table 8.1).

**Table 8.1:** IC\textsubscript{50} concentration determined by the AB and MTT assays for the two cell lines A549 and Calu-1 after 48 and 72 hrs exposure to ACT.

<table>
<thead>
<tr>
<th>IC\textsubscript{50} concentration ((\mu)M)</th>
<th>Cell line</th>
<th>A549</th>
<th>Calu-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 hrs AB</td>
<td>0.0095±0.0023</td>
<td>0.0189±0.0005</td>
<td></td>
</tr>
<tr>
<td>48 hrs MTT</td>
<td>0.01±0.001</td>
<td>0.0065±0.0003</td>
<td></td>
</tr>
<tr>
<td>72 hrs AB</td>
<td>0.0035±0.0002</td>
<td>0.0068±0.0013</td>
<td></td>
</tr>
<tr>
<td>72 hrs MTT</td>
<td>0.0031±0.0002</td>
<td>0.002±0.0001</td>
<td></td>
</tr>
</tbody>
</table>

ACT affects mitochondrial transcription (Bayona-Bafaluy, Manfredi, and Moraes, 2003; Chrzanowska-Lightowlers, Preiss, and Lightowlers, 1994), inducing disruption of mitochondrial activity (Suen, Norris, and Youle, 2008) and, as the MTT assay is a reflection of mitochondrial activity within the cell, its earlier response and higher sensitivity compared to the AB assay, which measures the global cell metabolism, is understandable (Farhane, Bonnier, Casey, and Byrne, 2015). For reproducibility and comparatively purposes, the IC\textsubscript{50} determined by MTT was used for the Raman analysis.

**8.3.2. Raman micro-spectroscopy:**

As seen in Figure 8.2, the ACT spectrum shows characteristic features at 465 and 1215 cm\(^{-1}\), corresponding to C–O vibration and 1445 cm\(^{-1}\), related to skeletal vibration. However, for the case of A549 cells after exposure to the IC\textsubscript{50} concentration for 48 hrs, no specific Raman features of the ACT spectrum are identifiable in the cellular spectra.
and for this reason it is impossible to track ACT itself inside the cells. Nevertheless, the evolution of the spectroscopic signature of binding and subsequent cellular responses can be tracked.

**Figure 8.2:** A. Pre-processed Raman spectrum of ACT stock solution dissolved in sterile water and mean spectrum with standard deviation of B. Nucleolus, C. Nucleus and D. Cytoplasm of A549 cell lines control and after 48 hrs exposure to the corresponding IC50.

Compared to the mean spectra of non exposed control cancer cells of each cell line, (specific discriminating features are highlighted in Figure 8.2), in the spectra of exposed cells, there is a decrease of the spectral features at 669 cm$^{-1}$ (thymine and guanine), 785
cm\(^{-1}\) (cytosine and thymine, DNA backbone O–P–O Stretching), 1047 cm\(^{-1}\) (RNA P–O stretch, sugar phosphate –C–O–stretching), 1095 cm\(^{-1}\) (DNA PO2\(^{-}\) symmetric stretching), 1300 cm\(^{-1}\) (RNA cytosine and adenine ring stretching) and 1340-1577 cm\(^{-1}\) bands (adenine and guanine) for both nucleolar and nuclear spectra, due to ACT binding to nucleic acids, and a decrease in the features at 760 cm\(^{-1}\) (tryptophan ring breathing) and 1005 cm\(^{-1}\) (phenylalanine), corresponding to protein denaturation.

The decrease in both bands at 785 and 813 cm\(^{-1}\), corresponding respectively to DNA backbone O–P–O and RNA O–P–O phosphodiester band stretching, is consistent with the main mechanism of action of ACT, by DNA intercalation, interfering with RNA polymerase, inducing DNA and RNA expression blockage. A similar decrease in those bands was found for Calu-1 cells (Figure S.8.1) and reported previously after DOX exposure and can therefore be considered a Raman signature of DNA intercalation (Farhane, Bonnier, and Byrne, 2017).

In order to analyse in more details the changes in the spectral features after exposure to ACT, multivariate data analysis, consisting of PCA, was employed. As seen in Figure 8.3, showing PCA of nucleolar, nuclear and cytoplasmic regions, for A549 control cells and after 48 hrs exposure to ACT, there is a differential distribution of the combined nuclear and the cytoplasmic areas, for both cell lines, predominatly according to positive and negative weightings of PC1. The corresponding loading of PC1, differentiating the combined nuclear and the cytoplasmic areas, is dominated on the positive side by features related to proteins, for example at 1005 cm\(^{-1}\), and lipids, for example at 1303 cm\(^{-1}\) (C–H vibration) and 1450 cm\(^{-1}\) (CH\(_2\) deformation) and on the negative side, corresponding to the combined nuclear region, by DNA and RNA features at 785 and 1577 cm\(^{-1}\). There is a slight separation between control and exposed cells according to PC2 and loading 2 is
dominated by negative features at 785, 1005, 1450 and 1660 cm\(^{-1}\), related respectively to nucleic acids, proteins and lipids corresponding to control cells which indicate a decrease in all cellular features as a consequence of exposure to ACT.

When a pairwise PCA of the three cellular regions was performed independently (Figure 8.3 B), it is notable that a separation is apparent in the data of the nucleolar area, confirming again the important role of this sub-nuclear region and the interaction of ACT by inhibition of nucleoli transcription which is related to its extreme toxicity (Farhane, Bonnier, Casey, and Byrne, 2015; Farhane, Bonnier, Casey, Maguire, et al., 2015; Sobell, 1985; Yung, Bor, and Chan, 1990). Only a slight separation is evident for the nuclear region, showing that ACT also effects the nucleus, although less so than the nucleolus. Notably, no separation of the cytoplasmic regions is observable.

After 72 hrs exposure and according to PCA analysis (Figure 8.4 A), an obvious separation between the combined nuclear and cytoplasmic regions is observed, according to PC2. A slight separation between control and exposed cells according to PC1 is also evident, and the loading of PC1 is dominated by negative nucleic acid and protein features, highlighting once again the ACT mechanism of action by DNA intercalation inhibiting RNA polymerase and subsequent cellular responses including reduced RNA transcription and protein synthesis.
A.

Figure 8.3: PCA of nucleolar, nuclear and cytoplasmic regions of A. A549 non exposed control and after 48 hrs exposure to ACT with corresponding loadings of PC1 and PC2.

B. PCA of each cell localisation for control and exposed and the corresponding loadings of PC1 and PC2.

Cytoplasm ● Nucleus ● Nucleolus ● A549 control

Cytoplasm ▼ Nucleus ▼ Nucleolus ▼ A549 48 hrs exposure to ACT
A pairwise PCA of each cellular compartment (Figure 8.4 B) reveals, as seen for pairwise PCA 48 hrs, a separation according to the nucleolar area with negative features at 669 cm\(^{-1}\) (thymine and guanine), 728 cm\(^{-1}\) (adenine), 785 cm\(^{-1}\) (cytosine and thymine, DNA backbone O–P–O Stretching), 813 cm\(^{-1}\) (RNA O–P–O phosphodiester) and 1340, 1486, 1577 cm\(^{-1}\) (adenine and guanine), related to the ACT mechanism of action by intercalation between guanine and cytosine. Negative features at 1005 cm\(^{-1}\) (phenylalanine) and 1670 cm\(^{-1}\) (Amide I) related to protein are also observed, as well as 1320, 1450 cm\(^{-1}\) (CH\(_2\) deformation) related to lipids, both due to the depletion of cellular components as a result of ACT exposure. However, a better separation of the spectra of the nuclear compartment than observed for 48 hrs is evident after 72 hrs exposure, explained by 82.5 % of variance of loading of PC1, which is dominated by DNA features at 728, 785, 1095 and 1577 cm\(^{-1}\) and proteins and lipids one respectively at 625, 645, 1005 and 1450 cm\(^{-1}\).

According to PCA of the data for 48 and 72 hrs exposure, it appears that ACT interacts first with the nucleolus and subsequently with the nucleus. Notably, the same observations were made previously for DOX uptake, suggesting that the anthracycline chemotherapeutic group may be targeting the nucleolus first and nucleus second (Farhane, Bonnier, and Byrne, 2017).

For both 48 and 72 hrs exposure to ACT and for the two cell lines (Figure 8.3 and 8.4 for A549 and Figure S.8.2 and S.8.3 for Calu-1 cells) for the cytoplasmic region, there is no obvious separation between control and exposed cells and similar loadings are observed with negatives features related to control cells at 1450 and 1660 cm\(^{-1}\) indicating a partial or initial denaturation of cytoplasmic constituents as a consequence of the arrest of transcription due to ACT treatment.
In order to analyse in more detail the ACT mechanisms of action, ICA for A549 cell line was made for 24 hrs exposure to a high ACT dose (0.3 µM) (Choong et al., 2009; Kleeff et al., 2000), 48 hrs and 72 hrs and was compared to control cells.

As seen in Figure 8.5, the ICs are similar for control and 24 hrs exposure, confirming the absence of any ACT effects at 24 hrs. Starting from 48 hrs, ICA identifies in the nucleolus a decrease in DNA features at 728 cm\(^{-1}\) (adenine), 752 cm\(^{-1}\) (DNA), 785 cm\(^{-1}\) (DNA backbone O–P–O), 1095 cm\(^{-1}\) (DNA PO\(_2^-\) symmetric stretching), 1376 cm\(^{-1}\) (thymine) and 1486, 1577 cm\(^{-1}\) (adenine, guanine), confirming the ACT mechanism of action by DNA intercalation with a decrease in RNA features at 1270 cm\(^{-1}\) (RNA uracil and cytosine ring stretching) and 1300 cm\(^{-1}\) (RNA cytosine and adenine ring stretching), consistent with inhibition of RNA transcription (Kleeff et al., 2000).

Besides nucleic acids depletion, a notable increase in protein features at 625 cm\(^{-1}\) (C-C protein) and 645, 855 cm\(^{-1}\) (tyrosine) (Farhane, Bonnier, and Byrne, 2017; Movasaghi, Rehman, and Rehman, 2007) indicates a possible synthesis of anti-apoptotic proteins as a cellular reaction against ACT, while the decrease at 1660 cm\(^{-1}\) (Amide I) and the increase at 1280 cm\(^{-1}\) (Amide III) may be related to protein denaturation of the surrounding membrane.
Figure 8.4: PCA of nucleolar, nuclear and cytoplasmic regions of A. A549 non exposed control and after 72 hrs exposure to ACT with corresponding loadings of PC1 and PC2. B. PCA of each cell localisation for control and exposed and the corresponding loadings of PC1 and PC2.

Cytoplasm ● Nucleus ● Nucleolus ● A549 control
Cytoplasm ▼ Nucleus ▼ Nucleolus ▼ A549 72 hrs exposure to ACT
In the nucleus, a decrease in DNA features at, for example, 785, 1095 and 1577 cm$^{-1}$ is also observed. An increase in features at 700 cm$^{-1}$ (C–C–N+ symmetric stretching in phosphatidylcholine), 715 cm$^{-1}$ (choline), 877 cm$^{-1}$ (tyrosine ring breathing) and 1450 cm$^{-1}$ (lipids CH$_2$ deformation) is consistent with the synthesis of anti-apoptotic proteins and lipidic vesicules as a way to remove ACT to the extracellular environment. A more pronounced increase at 72 hrs in RNA features at 847 (ribose phosphate), 1270 and 1300 cm$^{-1}$ is consistent with nucleolar fragmentation and spread of RNA characteristic of cells going under apoptosis (Boulon et al., 2010; Farhane, Bonnier, and Byrne, 2017; Kasim et al., 2013; Kleeff et al., 2000; Lu et al., 2015; Nazari et al., 2012).

In the cytoplasm, similar ICs were found for control and exposed A549 cells to ACT, whereby at 72 hrs, a slight decrease at 700, 848 (tyrosine) and 873 cm$^{-1}$ (tryptophan) and an increase at 1355, 1365 cm$^{-1}$ (tryptophan) and 1440, 1460 cm$^{-1}$ (CH$_2$ deformation) are observed, indicating an onset of denaturation of lipids and proteins.

According to PCA analysis, the two cell lines appear to react similarly to ACT exposure and in order to explore any differences in cellular response, ICA was employed. After 48 hrs, IC components for the two cell lines are similar, confirming again that ACT affects both cells subtypes in similar ways (data not shown), resulting in decreases in features at, for example, 728, 785 and 1577 cm$^{-1}$, as found in the PCA results. The IC exhibits also prominent peaks at 1430-1456 cm$^{-1}$, related to guanine and cytosine, corresponding to a hyperchromic effect of ACT intercalation on the response of the bases (Farhane, Bonnier, and Byrne, 2017).
Figure 8.5: ICA components of control cells and after 24, 48 and 72 hrs exposure to ACT for A549 cell line of A. Nucleolus B. Nucleus C. Cytoplasm.

Moreover, after 72 hrs exposure, in the ICs for both nucleolus and nucleus regions for both cell lines (Figure 8.6), there are negatives peaks corresponding to a decrease in nucleic acid features at 728, 785, 1095, 1376, 1486 and 1577 cm\(^{-1}\). Positive features are related to proteins, 1005, 1030 cm\(^{-1}\) (phenylalanine), 1151 cm\(^{-1}\) (protein) and 1660 cm\(^{-1}\) (Amide I) and to lipids at 700, 715, 1127 cm\(^{-1}\) (lipids), 1410, 1450 cm\(^{-1}\) (lipids CH\(_2\) deformation), which may be associated with an efflux of anti-apoptotic protein and a synthesis of lipidic vesicules as cellular defence reactions to ACT exposure.

However, in the IC of the nucleolus of A549 cells (Figure 8.6 A), there are also positive features showing a greater increase in proteins at 645, 855 cm\(^{-1}\) (tyrosine), 1207 cm\(^{-1}\) (phenylalanine, tryptophan and tyrosine) and 1230 cm\(^{-1}\) (Amide III) than in Calu-1 cells, which may correspond to a more active synthesis of anti-apoptotic proteins in the former.
In the ICs identified in the nucleus (Figure 8.6 B), a notable increase in proteins for both cell lines is evident; positive features at 877 cm\(^{-1}\) (tyrosine), 1176 cm\(^{-1}\) (tyrosine and phenylalanine) for A549 and at 936, 951, 973 cm\(^{-1}\) (protein) for Calu-1, while A549 exhibits signatures of apoptosis corresponding to strong positive peaks at 549 cm\(^{-1}\) (cholesterol) due to alteration of the endoplasmic membrane, a marker of apoptosis, and at 847 cm\(^{-1}\) (ribose phosphate) which may be due to the spread of RNA into the nucleus as a result of nucleolar fragmentation.

In the IC of cytoplasm for both cell lines there is a decrease at 785 cm\(^{-1}\) and an increase at 700-715, 1128 cm\(^{-1}\) (C–N stretch), 1268 (Amide II), 1303 and 1660 cm\(^{-1}\), indicating a decrease in cytoplasmic RNA related to the ACT mechanism of action which inhibits all RNA species, and synthesis of lipidic vesicles.

A.
Figure 8.6: ICA components after 72 hrs exposure to ACT for A549 and Calu-1 cell lines of A. Nucleolus B. Nucleus C. Cytoplasm.
For Calu-1 cells, negative features related to proteins at 830 cm\(^{-1}\) (tyrosine), 873 cm\(^{-1}\) (tryptophan), 940 cm\(^{-1}\) (C–C stretching of protein) and 1005 cm\(^{-1}\) (phenylalanine) corresponds to a decrease in protein contributions, indicating more advanced ACT effects in Calu-1 associated with their higher chemosensitivity compared to A549.

8.4. Discussion:

Raman micro spectroscopic results elucidate both the ACT mechanism of action at a subcellular level and different subsequent cellular reactions. In fact, the results identify a spectral signature of ACT interaction with nucleic acids in both the nucleolus and nucleus of the cell, consisting of a simultaneous decrease in the Raman features at 785 and 813 cm\(^{-1}\). Notably, the same spectroscopic response was found in previous studies of the action of DOX in the same cell lines, and therefore the spectroscopic signature can be considered as a fingerprint of the mechanism of action by intercalation. ACT and DOX have similar chemical structures, in terms of the core anthracyclic rings, and the identification of such common spectroscopic signatures associated with a mode of action of these chemotherapeutic agents can potentially be exploited for preclinical screening of the mode of action of new candidate chemotherapeutic agents.

Despite the fact that a similar spectral signature of chemical interaction was found compared to DOX for the two cell lines, the difference in cellular response times between the two anthracyclines is notable. Whereas DOX elicited marked responses in the spectroscopic signatures of the nucleoli of A549 and Calu-1 cells within 6/12 hrs, respectively, the response to ACT exposure is considerably slower. This huge difference in the response time may be due to the different side chains which can affect the drug uptake and/or intracellular transport rates. Nevertheless, the IC\(_{50}\) is much lower for ACT,
in the range of nM as compared to μM for DOX, as observed according to cytotoxicity assays, indicating that it has a higher efficacy once accumulated inside the cell. Although similar Raman activities of stock solutions of ACT and DOX are observed, as seen in Figure 8.7, the substantially lower IC₅₀ dose of ACT employed (nM) compared to DOX (μM) means it is not visible inside the cell using Raman spectroscopy. However, the Raman results show that, even if DOX accumulates much faster than ACT in the combined nuclear area, the same uptake path is observed for both chemotherapeutic drugs with accumulation first in the nucleolus before the nucleus, and thus Raman can shed further light on understanding the mechanisms of action of known drugs.

**Figure 8.7**: Pre-processed Raman mean spectra of stock solutions dissolved in sterile water of ACT (398 μM-green) and DOX (17.25 mM-blue).

ACT affects the two cell lines in similar ways, as demonstrated by multivariate statistical analysis (PCA and ICA), with similar rates of uptake. However, the interaction of the drug in the nucleolus/nucleus elicits different cellular reactions in the two cell lines, Calu-1 cells exhibiting higher sensitivity compared to A549. The difference in subsequent
cellular metabolic responses, as seen in the protein profile, with an increase in some protein features corresponding probably to a synthesis of anti-apoptotic proteins, is due to a differential drug retention rather than drug uptake, as the two cell lines have similar uptake rate, and those observations are consistent with previous findings showing that ACT retention is an important determining factor in cell sensitivity and is specific to the cells themselves (Roots and Smith, 1976; Schwartz, Sodergren, and Ambaye, 1968). Therefore, Raman micro-spectroscopy can be considered as a potential companion diagnostic tool providing information about drug mechanism of action, subcellular kinetics and differences in cellular reactions and resistance.

8.5. Conclusion:

Although not identifiable within the biochemical milieu of the cell, the effects of the subcellular uptake and interactions of the DNA targeting chemotherapeutic agent ACT can clearly be monitored using Raman micro-spectroscopy. As previously demonstrated for the similar anthracyline, DOX, the accumulation and biochemical interactions occur initially in the nucleolar, and then nuclear regions of the cell. The timescales of interactions are, however, substantially slower for ACT compared to DOX, perhaps due to the more complex nature of the polypeptide side chains. Nevertheless, the spectroscopic signature of the early stage interactions in the subcellular regions demonstrate a consistency for the two compounds, and can be considered as a spectroscopic signature of intercalation, This is may be the first step in the elaboration of a library of signatures of all chemotherapeutic groups, paving the way for Raman micro-spectroscopy to be developed as an analytical tool for preclinical development in order to identify mechanism of action of new chemotherapeutic drugs.
According to cytotoxicity assays, the two cell lines exhibit different chemosensitivity to ACT as represented by the different IC₅₀. The difference in chemosensitivity was confirmed by Raman investigations using multivariate data analysis which shows a higher increase in protein features in A549 cells and higher viability despite the fact that the two cell subtypes present similar ACT uptake rates. The difference in responses points towards the potential of Raman micro-spectroscopy to elucidate mechanisms of cellular resistance and ultimately to be used as a predictive tool for patient responses, or companion diagnostic.

8.6.  Acknowledgement:

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8.7. References:


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8.8. Supplementary Information:

A. Pre-processed Raman spectrum of ACT stock solution dissolved in sterile water and mean spectrum with standard deviation of B. Nucleolus, C. Nucleus and D. Cytoplasm of Calu-1 cell lines control and after 48hrs exposure to the corresponding IC\textsubscript{50}.

Figure S.8.1: A. Pre-processed Raman spectrum of ACT stock solution dissolved in sterile water and mean spectrum with standard deviation of B. Nucleolus, C. Nucleus and D. Cytoplasm of Calu-1 cell lines control and after 48hrs exposure to the corresponding IC\textsubscript{50}. 
**Figure S.8.2**: PCA of nucleolar, nuclear and cytoplasmic regions of A. Calu-1 non exposed control and after 48hrs exposure to ACT with corresponding loadings of PC1 and PC2 B. PCA of each cell localisation for control and exposed and the corresponding loadings of PC1 and PC2.

Cytoplasm ● Nucleus ○ Nucleolus ● Calu-1 control

Cytoplasm ▼ Nucleus ▼ Nucleolus ▼ Calu-1 48hrs exposure to ACT
Figure S.8.3: PCA of nucleolar, nuclear and cytoplasmic regions of A. Calu-1 non exposed control and after 72hrs exposure to ACT with corresponding loadings of PC1 and PC2 B. PCA of each cell localisation for control and exposed and the corresponding loadings of PC1 and PC2.

Cytoplasm ● Nucleus ○ Nucleolus ● Calu-1 control

Cytoplasm ▼Nucleus ▼ Nucleolus ▼ Calu-1 72hrs exposure to ACT
Chapter 9: *In vitro* label free screening of chemotherapeutic drugs using Raman micro-spectroscopy: towards a new paradigm of spectralomics

The following chapter reproduces a review in *Journal of Biophotonics* (DOI: 10.1002/jbio.201700258), Z. Farhane, H. Nawaz, F. Bonnier and H.J. Byrne, in which section and figure numbers have been adapted to the prescribed thesis format.

It is a conclusion of the study which summarises the cellular uptake binding and cellular responses of the anthracyclic chemotherapeutic agents with Vincristine and Cisplatin in the lung cancer cell lines, and confirms the ability of Raman micro-spectroscopy for a potential clinical application as a companion diagnostic in personalised therapy by elucidating subcellular transport and accumulation pathways of chemotherapeutic agents with characterisation and fingerprinting of their mode of action and identification of potential cellular resistance.
In vitro label free screening of chemotherapeutic drugs using Raman micro-spectroscopy: towards a new paradigm of spectralomics.

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Abstract:

This overview groups some of the recent studies highlighting the potential application of Raman micro-spectroscopy as an analytical technique in preclinical development to predict drug mechanism of action and in clinical application as a companion diagnostic and in personalised therapy due to its capacity to predict cellular resistance and therefore to optimise chemotherapeutic treatment efficacy.

Notably, the anthracyclines, Doxorubicin and Actinomycin D, elicit similar spectroscopic signatures of subcellular interaction characteristic of the mode of action of intercalation. Although Cisplatin and Vincristine show markedly different signatures, at low exposure doses, their signatures at higher doses show marked similarities to those elicited by the intercalating anthracyclines, confirming that anticancer agents can have different modes
of action with different spectroscopic signatures, depending on the dose.

The study demonstrates that Raman micro-spectroscopy can elucidate subcellular transport and accumulation pathways of chemotherapeutic agents, characterise and fingerprint their mode of action, and potentially identify cell resistant strains. The consistency of the spectroscopic signatures for drugs of similar modes of action, in different cell lines, suggests that this fingerprint can be considered a “spectralome” of the drug-cell interaction suggesting a new paradigm of representing spectroscopic responses.

**Keywords:** Raman micro-spectroscopy, cancer cells, chemotherapeutic drugs, mechanism of action, cellular resistance, spectralomics
9.1. Introduction:

Vibrational spectroscopy can perform cellular imaging in a (benchtop) microscopic geometry, and can provide non invasive label free screening of, for example, nanoparticle or drug uptake, trafficking and interaction mechanisms, as well as cellular responses and toxicity (Byrne, 2014; Byrne et al., 2013; Efeoglu, Casey, and Byrne, 2016; El-Mashtoly et al., 2015; Farhane et al., 2015), eliminating the need for multiple assays in toxicological screening and drug discovery and preclinical screening stages, by elaborate robotic High Content Analysis systems. Vibrational spectroscopy has also been demonstrated for diagnostics applications, in vivo and ex vivo, in tissue (Bonnier et al., 2012; F Bonnier et al., 2010), cells (Farhane et al., 2015; Huser and Chan, 2015) and body fluids (Bonnier et al., 2014; Bunaciu et al., 2017). A further potential application which is currently attracting increasing attention is in understanding and screening cellular resistance to therapeutic treatments to guide strategies for personalised therapies (Cho, Jeon, and Kim, 2012; Overby and Tarczy-Hornoch, 2013). Both Infrared absorption (IR) and Raman spectroscopy can be performed on live cells (F Bonnier et al., 2010; Whelan et al., 2013), although the significantly lower contribution of water in the latter favour it for live cell analysis (Bonnier et al., 2014). Raman micro-spectroscopy also provides superior spatial resolution to IR spectroscopy, being an optically based microscopy technique, commonly employed in a confocal mode, enabling subcellular analysis of biological processes at an organelle level (F Bonnier et al., 2010). Raman spectral maps of whole live cells can be performed over minutes to hours, but in cases where spectral quality is favoured over speed, for detailed analysis, measurement protocols often entail fixing the cells at fixed time points after exposure to exogenous agents, for example radiation or toxicants, having optimised protocols for cell fixation (Meade et al., 2010). Both IR and Raman
spectroscopy have been used to assess the effects of drugs on cells, demonstrating their potential to measure changes in a high throughput manner and therefore can be used in drug screening (Derenne, Gasper, and Goormaghtigh, 2011; Hughes, Clemens, and Baker, 2015; Nawaz et al., 2010). Many studies have reported the use of vibrational spectroscopy to monitor the effects of anticancer agents, including polyphenols (Derenne et al., 2013) cardiotonic steroids (Gasper et al., 2010), and platinum compounds (Nie et al., 2010), on cancer cells, and Hughes et al. (Hughes, Clemens, and Baker, 2015), Mignolet et al. (Mignolet et al., 2016) and Jamieson and Byrne (Jamieson and Byrne, 2016) have recently reviewed the potential for vibrational micro-spectroscopy as a label free in vitro platform for screening the mechanisms of action and efficacies of candidate drugs at the discovery and pre-clinical screening stages. The development of such in vitro screening techniques is particularly pertinent at present, given the increasing legislative pressure to develop in vitro alternatives to animal models for scientific research and product development as prioritised by the EU Directive-2010/63/EU on the replacement, reduction and refinement of animal experimentation.

Using Fourier Transform IR (FTIR) microscopy, Derenne et al. demonstrated that drugs of similar mode of action have similar spectroscopic signatures (Derenne, Gasper, and Goormaghtigh, 2011), indicating potential applications in pre-clinical screening of the mode of action of new candidate drugs. Analysis of spectral variations in prostate cell lines, induced by drug exposure, clustered drugs classified as anti-microtubules (Methotrexate, Mercaptopurine), anti-topoisomerases (Vincristine, Vinblastine, Paclitaxel) and anti-metabolites (Doxorubicin, Daunorubicin) separately. Derenne et al. further demonstrated that the spectral changes were not cell line specific, but were consistent over seven different cell lines (Derenne, Verdonck, and Goormaghtigh, 2012). Mignolet et al. (Mignolet et al., 2016) reviewed the body of work exploring IR signatures
of anti-cancer drugs inside cancer cells and changes induced after drug exposure, including the effects of cell cycle and cell culture model, arguing that FTIR can be employed for screening of new drug candidate molecules for known or unknown modes of action.

Using Raman spectroscopy, subcellular resolution can be achieved due to the high spatial resolution achievable with optical microscopy, allowing intracellular organelles to be resolved. Raman spectra are also typically recorded at higher spectral resolution than IR spectra, and therefore more detailed biochemical information can be gained (Ali et al., 2013), with more potential for elucidating ‘modes of action’ by analysis of the detailed spectral changes. El-Mashtoly et al. (El-Mashtoly et al., 2014) demonstrated the capability of Raman spectroscopy for monitoring the distribution of the distinct structure of erlotinib, containing a carbon-carbon triple bond, and its metabolic products in colorectal adenocarcinoma SW480 cells. Le Roux et al. (Le Roux, Prinsloo, and Meyer, 2014) explored cell death routes after exposure to gold based metallodrugs. Salehi et al. (Salehi, Derely, et al., 2013; Salehi, Middendorp, et al., 2013) and Feofanov et al. (Feofanov et al., 2000) probed the resonance Raman response to detect and study cellular responses after exposure to paclitaxel, and the nonfluorescent transition-metal complex, Theraphtal, respectively. Draux et al. (Draux et al., 2011) investigated the effect of Gemcitabine on cell biomolecules in the lung cancer cell line Calu-1, after 48 hrs exposure. Moritz et al. (Moritz et al., 2010) used laser tweezers Raman spectroscopy to study the effects of Doxorubicin on leukaemia cells and demonstrated that drug exposed cells exhibited an increase in DNA features after prolonged exposure time. Schie et al. (Schie et al., 2014) also investigated the effect of Doxorubicin on malignant lymphocytes at late stages, from 24 to 96 hrs, while Guo et al. (Guo et al., 2009) explored the effects of Doxorubicin on malignant hepatocytes after 12 hrs exposure, showing a decrease in
DNA features and an increase in proteins and lipids features. Hartmann et al. (Hartmann et al., 2012) studied the effect of Docetaxel on human breast adenocarcinoma cell line, MCF-7, comparing the treated cells with the untreated ones and investigating the cellular morphological changes induced by the chemotherapeutic drug. Lin et al. (Lin et al., 2011) used Raman spectroscopy to assess the cytotoxicity of Paclitaxel in CA46, human Burkitt’s lymphoma cells. Zoladek et al. (Zoladek et al., 2011) showed the potential of Raman micro-spectroscopy for label free time course imaging (at 2 hrs intervals over 6 hrs) of live human breast cancer cell (MDA-MB-231) undergoing apoptosis after exposure to Etoposide.

In monitoring the response to exogenous agents in vitro, it has also been demonstrated that both FTIR and Raman spectroscopy can potentially be employed to understand cellular resistance pathways in different cell lines. Cellular resistance pathways were specifically targeted by Yosef et al. (Yosef et al., 2015) who used Raman spectral imaging to investigate the oncogenic mutation resistance to epidermal growth factor receptor targeting therapy and colon cancer cells with and without oncogenic mutations such as KRAS and BRAF mutations were treated with erlotinib, an inhibitor of epidermal growth factor receptor, in order to detect the impact of these mutations on Raman spectra of the cells as markers of cell resistance. Rutter et al. (Rutter et al., 2014) utilised a cell cloning technique to specifically isolate sensitive and resistant cells from a mixed cell population, and investigated the difference in response of gemcitabine-sensitive and gemcitabine-resistant Calu-1 epidermoid lung cancer cells to the commercial drug, using IR spectroscopy. Furthermore, Siddique et al. (Siddique et al., 2017) showed that it was also possible to identify differences of nilotinib-sensitive and nilotinib-resistant K562 (a chronic myelogenous leukaemia cell line) cloned cells, using both FTIR and Raman micro-spectroscopies. The ability of vibrational spectroscopy to characterise and
differentiate responses of resistant and sensitive cell types to drugs opens up potential clinical applications as a Companion Diagnostics (CD) tool, and ultimately personalised medicine approaches (Agarwal, Ressler, and Snyder, 2015; Naylor and Cole, 2010). Although the potential of in vitro spectroscopic screening of cellular processes has been well demonstrated, research has been somewhat fragmented, lacking in coherence and standardisation of measurement protocols. Critically, the analysis and interpretation of the spectral responses remains a challenge, even in the hands of “specialists”. At the recent Faraday Discussions (Baker et al., 2016), the question as to what extent individual spectral features can be assigned to specific molecular responses, given the complex nature of the samples under investigation, featured highly. By their very nature, label free techniques register all species within the sampling area, and identification of specific responses requires more sophisticated techniques to data-mine the differential responses due to cell injury or change. Regression and correlation approaches can be employed to extract the specific spectroscopic signatures of cellular changes which are correlated with external stimuli, and, for example independently elucidate the spectroscopic signatures of the direct chemical effects of the stimulus from the subsequent cellular metabolic responses. In the case of chemotherapeutic agents, the uptake, distribution and accumulation, chemical interactions and subsequent cellular metabolic responses can be monitored (Hughes, Clemens, and Baker, 2015; Nawaz et al., 2010). Nevertheless, these differential spectral responses remain multivariate in nature, and contain contributions of the multitude of biochemical constituents involved. The conditioned thinking, based on a labelled approach, is to search for specific “Biomarkers”. However, it is becoming increasingly apparent that the spectral response can have (additive) contributions of increased or decreased concentrations of constituent biomolecules, but also more complex contributions due to conformational, environmental (pH etc.) changes, and that
identification of the (differential) spectroscopic signature, rather than specific bands associated with specific biomolecules, may be more appropriate. For realistic applications potential, however, it is important that these combinatorial signatures are characteristic of the cellular interaction and/or response pathway, and are translatable across cell lines and ultimately patient samples. Although Derenne et al. demonstrated that drugs of similar mode of action have similar spectroscopic signatures using FTIR microscopy (Derenne, Gasper, and Goormaghtigh, 2011), there have been few other studies which have attempted to demonstrate consistency between spectroscopic signatures of drug interactions and cellular responses (El-Mashtoly et al., 2015; Jamieson and Byrne, 2016). The identification of specific spectral signatures which are common for drug mechanisms of action and cellular responses opens the perspective to a new “spectralomics” paradigm, in label-free fingerprinting and monitoring of biological processes in cells in vitro using Raman spectroscopy, with potential applications in fundamental cytological research, pre-clinical pharmacological development, and ultimately improved individualised clinical therapeutics.

Herein, the results of a number of independent studies of the action of chemotherapeutic agents in vitro are gathered and reviewed, with a view towards assessment of the consistencies of spectroscopic signatures of the subcellular interactions of the drugs, and the subsequent cellular responses. Specifically, Raman micro-spectroscopic studies of the subcellular interactions of Doxorubicin (DOX), Actinomycin D (ACT), Cisplatin (Cisp) and Vincristine (Vinc) in human lung cancer cell lines, and their subsequent responses, are considered. The dose response profiles are examined in the context of classical cytotoxicological screening assays.
9.2. Cytotoxicity assays and their limits:

A range of cytotoxicological assays are commonly employed to measure the in vitro responses of cell populations, including cell proliferation, viability and toxicity, after exposure to external agents such drugs, nanoparticles or radiation. Amongst these are the tetrazolium bromide (MTT), Alamar Blue (AB) and Neutral Red (NR) assays, each of which targets different aspects of the response pathway.

NR is a fluorometric dye, which measures the lysosomal activity using 3-Trimethyl-2,8-phenazinediamine, monohydrochloride, which binds to the lysosomes of viable cells after penetration by passive diffusion due to its cationic charge. This uptake depends on the ability of the cell to maintain a pH gradient by production of ATP. Therefore, the dye cannot penetrate inside dead cells and the amount of retained dye is proportional to the number of viable cells (Maher et al., 2014; Repetto, del Peso, and Zurita, 2008). The MTT test is a colorimetric assay that measures the reduction of yellow 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase into purple formazan crystals, insoluble in aqueous solution (Al-Nasiry et al., 2007; Hamid et al., 2004). Succinate dehydrogenase or succinate-coenzyme Q reductase (SQR) or respiratory Complex II is an enzyme complex, bound to the inner mitochondrial membrane of mammalian mitochondria and many bacterial cells. It is the only enzyme that participates in both the citric acid cycle and the electron transport chain, so the MTT assay is the reflection of mitochondrial activity.

Alamar blue (AB), on the other hand, is a water-soluble dye and one of the most highly used cytotoxicity assays for in vitro quantification of the cell viability(Fields and Lancaster, 1993). When added to cell cultures, the active dye, resazurin or 7-hydroxy-10-oxidophenoxazin-10-iium-3-one, diffuses into the cytosol and acts as an intermediate
electron acceptor allowing the oxidised blue non-fluorescent form to be reduced by both mitochondrial and cytosolic enzyme activity to the fluorescent pink form which is easily measured by its absorption or fluorescence (Al-Nasiry et al., 2007; Fields and Lancaster, 1993; Hamid et al., 2004).

Both AB and NR are considered an expression of general cellular metabolism and, while reduced conversion compared to controls is used as a measure of reduced cellular viability for both AB and MTT assays, the MTT response is more specifically sensitive to mitochondrial enzymes while AB is related to both mitochondrial and cytosolic activities. Quantitatively, the cytotoxic response is commonly quoted in terms of the effective concentration with elicits 50% of the maximum response of the cell population to the exogenous agent (IC$_{50}$), determined through dose-response curves. However, toxicity studies are commonly conducted at different time points, using different cell lines, leading to a lack of consistency in toxicological data for chemotherapeutic agents. Figure 9.1 shows, for example the accumulated cytotoxicity data as measured using the MTT assay, for Cisp (Nawaz et al., 2010) and Vinc (Nawaz et al., 2013) in A549, human lung adenocarcinoma cells, and DOX (Farhane, Bonnier, Howe, et al., 2017) and ACT (Farhane, Bonnier, and Byrne, 2017a) in both A549 and Calu 1, a human lung epidermoid carcinoma cell line, at differing time points. The inverse IC$_{50}$, as a comparative measure of the toxicity (Ragnvaldsson et al., 2007), is plotted against the exposure time. For the case of DOX, the toxicity increases monotonically as a function of exposure time. The two cell lines show comparable results, although that of A549 seems to saturate at prolonged exposures. In the case of ACT, although negligible response was observed at 24 hrs (IC$_{50}$>50 $\mu$M), the toxicity at 48 hrs and 72 hrs far exceeds that of DOX, in both cell lines (Farhane, Bonnier, and Byrne, 2017a). Cisp also requires a more prolonged exposure time to elicit appreciable responses in A549 (Nawaz et al., 2013), while, for the
same exposure time, Vinc requires substantially lower concentration to elicit the same toxic response (Nawaz et al., 2013).

**Figure 9.1:** Plot of the inverse IC$_{50}$ against exposure time for DOX and ACT exposure to A549 and Calu-1 cell lines and Vinc and Cisp to the A549 cell line.

Maher *et al.* (Maher et al., 2014) developed a numerical model to simulate nanoparticle uptake, cytotoxicity and subsequent cellular response and highlighted the fact that differences in the quantitative responses of cytotoxicity assays can arise due to the fact that each assay measures different underlying cellular responses which can occur at different rates. An assessment of the action and efficacy of, for example a chemotherapeutic agent, according to a single endpoint is therefore extremely limited. Computational Modelling approaches to predictive toxicity, such as that of Adverse Outcome Pathways (Wittwehr et al., 2017), have sought to deconvolute the action of exogenous agents, in terms of the molecular initiating event (MIE) and the subsequent cascade of key events (KE) that reflect the causal progression from the initial perturbation of the system towards the adverse outcome. In the context of the action of
chemotherapeutic drugs *in vitro*, the MIE can be considered the chemical interaction of the drug, for example in the cell nucleus, while the subsequent cascade of events can determine the efficacy of a drug action. Understanding of the chemistry of the MIE can be key to establishing quantitative structure activity relationships to guide synthetic strategies, while understanding the subsequent pathways may be key to understanding mechanisms of drug resistance and sensitivity in different cell lines and ultimately patients.

Therefore, to have a global idea of the effects of external agents such as drugs or nanoparticles on cells and identify any subsequent cellular responses, there is a need for a more developed label free method which gives a holistic picture of the cascade of events which occurs after cell exposure.

**9.3. Data processing: pre and post processing:**

To consider vibrational spectroscopy and specifically Raman micro-spectroscopy as an analytical tool for clinical applications, it should be able to address the many challenges related to the complexity of biological samples and their chemical and physical heterogeneity. In this context, data pre and post processing play an important role in dealing with variations in instrumental responses, between different biological samples and improving spectral quality.

*Byrne et al.* (*Byrne et al., 2016*) summarised the pre-processing steps routinely used to improve recorded spectra quality, and the commonly used post-processing techniques for the classification, discrimination and analysis of data set from biological samples. In fact, many factors, including background coming from sample scattering and/or fluorescence, substrate contributions, specifically when analysing thin samples, and instrumental
enhanced scattering contributions, can influence spectra quality and therefore need to be subtracted. In some cases, those factors can be minimised experimentally, for example analysing samples in immersion to reduce laser scattering (F Bonnier et al., 2010) or use of substrates with negligible Raman scattering (Kerr, Byrne, and Hennelly, 2015). In others cases, there is a need for pre-processing algorithms for background subtraction prior to analysis, using for example a NCLS (non-negatively constrained least squares) (Ibrahim et al., 2017) or EMSC (extended multiplicative signal correction) algorithm (Kerr and Hennelly, 2016). Spectra are also commonly smoothed to reduce noise using the Savitsky-Golay algorithm or singular value decomposition (SVD) (Schie and Chan, 2016), baseline corrected (fifth order polynomial) and vector or area normalised.

After pre-processing, data post-processing or data mining is necessary to detect and analyse changes in spectral profiles associated, for example, with biological process, drug, nanoparticle, radiation exposure etc., mostly in a large and complex data set. The most common method in terms of multivariate spectral data analysis is Principal Components Analysis (PCA), a powerful unsupervised approach for the analysis of large multidimensional data sets, which allows the reduction of the number of variables, although retaining most of the variation within the dataset. It represents the spectra in data groupings of similar variability, allowing the identification and differentiation of different spectral subgroups. The loadings of the PCs represent the variance for each variable (wavenumber) for a given PC, the order of the PCs denoting their importance to the dataset. PC1 describes the highest amount of variation, and analysis of the loadings can give information about the source of the variability inside a dataset, derived from variations in the molecular components contributing to the spectra.

It has been demonstrated that the PC loadings can be most simply understood when analysis of data subgroups is undertaken in a pairwise fashion (Bonnier and Byrne, 2012;
Farhane, Bonnier, Maher, et al., 2017), whereby the loading of the PC can be interpreted as differences of the biochemical content of the two differentiated datasets (Bonnier and Byrne, 2012), highlighting its ability to provide molecular information and biochemical differences of analysed samples. Notably, PCA does not cluster the data, per se, in the same manner as for example Hierarchical or K-Means Cluster Analysis, whereby differential distribution of the data according to negative or positive loadings associates specific spectral features with that dataset.

As an extension to PCA, independent components analysis (ICA) can also be employed as an unsupervised statistical technique to identify latent variables, called independent components, in each data set separately. In the case of Raman micro-spectroscopy, ICA can be used to identify spectral contributions such as those from substrate, which can then be removed or plotted and studied in their own (Farhane, Bonnier, Maher, et al., 2017; Lee, 1998).

More sophisticated spectral analysis is required to extract specific information related to an external variable such as exogenous exposure dose or time, requiring supervised methods such Partial Least Squared Regression (PLSR). PLSR is a statistical regression technique which reduces the dimensionality of the data and correlates information, here represented by the spectra data set, to, for example, time evolution or drug concentrations or to a gold standard assay, to track the dependent evolution of the spectral signatures in, for example, subcellular regions. The regression coefficients obtained by PLSR can be analysed and provide information about the contribution of spectral variations. As a function of frequency, the co-efficients illustrate the spectral features which are influenced by the external factor selected (Farhane, Bonnier, and Byrne, 2017b).
9.4. Raman micro-spectroscopy for chemotherapeutic pre-clinical screening:

Raman spectroscopic analysis of drug-cell interactions has focused both on changes in the cellular Raman spectra upon drug application, and also during intracellular tracking of the drug and its metabolites (Dana et al., 2015; El-Mashtoly et al., 2014; Farhane, Bonnier, and Byrne, 2017a; Farhane et al., 2015; Farhane, Bonnier, Howe, et al., 2017). Farhane et al. (Farhane, Bonnier, and Byrne, 2017b; Farhane et al., 2015; Farhane, Bonnier, Howe, et al., 2017; Farhane, Bonnier, Maher, et al., 2017) used Raman spectroscopy to determine not only the subcellular location of the drug DOX in A549 and Calu-1 human lung adenocarcinoma cells, but also the differing pharmacodynamics of the drug uptake and subcellular localization, as well as the subsequent cellular responses, over a period 0-72 hrs. In fact, using Raman micro-spectroscopy, DOX could be clearly detected in both the nucleolus and surrounding nucleus, while Confocal Laser Scanning Microscopy (CLSM) images show that only the nucleus appears red (compared to the corresponding controls, in which no fluorescence is detected), due to the characteristic DOX fluorescence (Figure 9.2), the clearly visible dark spots of the nucleoli suggesting that DOX does not accumulate in the nucleoli.
Figure 9.2: Confocal Laser Scanning Fluorescence images of A. A549 cells and B. Calu-1 cells after DOX exposure and corresponding controls: 1. 2 hrs DOX exposure, 2. 6 hrs DOX exposure, 3. 12 hrs DOX exposure, 4. 24 hrs DOX exposure, 5. 48 hrs DOX exposure and 6. 72 hrs DOX exposure (Farhane, Bonnier, and Byrne, 2017b).
However, Raman micro-spectroscopy clearly demonstrates that DOX does accumulate in the nucleoli, indicating that the dark spots are a result of fluorescence quenching in the environment. The study also indicated that DOX selectively targets the RNA in the nucleolus, before the nuclear and cytoplasmic regions.

Using multivariate data analysis, consisting of PCA, ICA and PLSR, Raman micro-spectroscopy was shown to be capable of tracking the kinetics of the uptake and accumulation of DOX at a subcellular level *in vitro* (Farhane, Bonnier, and Byrne, 2017b; Farhane, Bonnier, Howe, et al., 2017). Results show that the chemotherapeutic drug accumulates first in, and saturates, the nucleolus, then the nucleus and is only detectable in the cytoplasm at later stages, after nuclear disruption (Figure 9.3 A). Raman micro-spectroscopy can differentiate the biochemical responses associated with the subcellular regions of nucleolus, nucleus and cytoplasm, both in terms of the mechanisms of action (DNA intercalation in nuclear area and ROS production in cytoplasmic region), and the subsequent cellular metabolic responses for the same cell lines and between different cell lines (Figure 9.3 B), the faster uptake in Calu-1 cells (Figure 9.3 A) highlighting different cellular kinetics, effects and resistance related to expression of anti-apoptotic proteins and tolerance to DNA damage and implication of DNA repair mechanisms manifest as an increase in DNA signatures at late stages (Figure 9.3 B).

A similar response profile was observed for Actinomycin D (ACT) in the same cell lines, both in terms of time evolution and spectroscopic signatures (Farhane, Bonnier, and Byrne, 2017a). In fact, a similar chemical binding signature, related to RNA/DNA interaction, resulting in a decrease of both bands at 785 cm\(^{-1}\) (DNA backbone O–P–O) and 813 cm\(^{-1}\) (RNA O–P–O phosphodiester band stretching) was observed after DOX and ACT
exposure (Figure 9.4). The substantially slower uptake rate for ACT (48-72 hrs) compared to DOX (6-12 hrs) may be due to different side chain composition.

A.

B.

**Figure 9.3:** A. Evolution of DOX, represented by the Raman band at 465 cm\(^{-1}\), as a function of time for the A549 and Calu-1 cell line for each cellular compartment, nucleolus, nucleus and cytoplasm. B. Evolution of selected DNA and RNA features as function of time.

Intensities are expressed as percentage according to the maximum value over the three cellular compartments for each cell line and standard deviation corresponds to the spectral
variations of the Raman band over the 30 measurements per location (Farhane, Bonnier, and Byrne, 2017b; Farhane, Bonnier, Howe, et al., 2017).

Nevertheless, both exploit similar cellular pathways, accumulating first in the nucleolus and then the nucleus, which suggests that the anthracycline chemotherapeutic group targets the nucleolus first, binding with RNA, and nucleus second, binding with DNA, before accumulating in the cytoplasm. This is not the accepted view of the mode of action of anthracycline drugs, which considers only the interaction with nuclear DNA and parallel interactions in the cytoplasm (Minotti et al., 2004; Rabbani, Finn, and Ausió, 2005; Yaqub, 2013), and so, in both cases, Raman micro-spectroscopy has shed further light on the current understanding of the mode of action of the clinically prescribed chemotherapeutic agents.

Nawaz et al. (Nawaz et al., 2010; Nawaz et al., 2011) explored the effects of Cisplatin (Cisp), which primarily interacts with DNA via inter-strand and intra-strand cross-links with purine bases, mostly by forming a 1,2-intrastrand cross-link between the N7 atoms of two adjacent guanine bases, on the Raman spectra of the nuclear region of A549 human lung adenocarcinoma cells after 96 hours exposure. The difference spectrum of cells exposed to the Cisp IC\textsubscript{50} concentration and unexposed control cells reveals specific bands at, for example, 669 (thymine and guanine), 833 (DNA B form) and 1095 cm\textsuperscript{-1} (DNA PO\textsubscript{2–} symmetric stretching), associated with intra-strand cross linkages between guanine/guanine and guanine/thymine, inducing conformational changes, consistent with the accepted mode of action of Cisp, as also demonstrated by Huang et al. (Huang et al., 2013), who monitored cellular apoptosis of nasopharyngeal carcinoma cells C666 after Cisp treatment.
A. 

![Graph showing Raman spectroscopy data](image1)

B. 

![Graph showing Raman spectroscopy data](image2)

**Figure 9.4:** Loading 1 of PC1 of control versus exposed cells of Nucleolus A. A549 control versus 6 hrs DOX exposure compared to that of control versus 48 hrs exposure ACT B. Calu-1 control versus 2 hrs DOX exposure compared to that of control versus 48 hrs exposure ACT.

Using the drug dose and MTT cytotoxicity assay as independent regression targets in PLSR, Nawaz *et al.* (Nawaz et al., 2010; Nawaz et al., 2011) showed that it was possible
to distinguish cellular responses in the Raman spectrum as a result of Cisp interactions within the cell nucleus by chemical binding, and the subsequent cellular physiological response of the cell. Keating et al. (Keating et al., 2015) confirmed these results with simulated data, further emphasising the power of this technique, with application to High content analysis for in vitro drug testing.

It should be noted that, PLSR over the high concentration range, up to 50 μM, (Figure 9.5 A) results in a spectral profile which is similar to those of DOX and ACT (Figure 9.4) exhibiting strong features at 785 cm\(^{-1}\) (DNA backbone O–P–O) and 813 cm\(^{-1}\) (RNA O–P–O phosphodiester band stretching), while regression over the lower concentration up to the IC\(_{50}\) of 1.2 ± 0.2 μM showed a decrease in features at 728, 830 and 1425 cm\(^{-1}\), related to DNA B form and an increase in features at 668 and 675 cm\(^{-1}\), related to DNA A form, indicative of conformational changes due to partial transition of the DNA B-form to DNA A-form due to Cisp binding (Ghosh, Dey, and Saha, 2014; Nawaz et al., 2010; Vaverkova et al., 2014).

In acellular circular dichroism studies of drug DNA interactions, the spectral changes observed for the ACT-DNA complexes due to a B to A-type DNA transition upon interaction, are distinct from those of Cisp-DNA complexes, which reflect distortions in DNA of a non-denaturational nature (Chang, Chen, and Hou, 2012). Therefore, it is not expected that Cisp will induce similar conformational changes to DOX or ACT.
Figure 9.5: Regression coefficients obtained by PLSR analysis for A549 cell line as a function of drug dose at low and high doses for chemotherapeutic drugs A. Cisp and B. Vinc.
In a similar, more recent, study of the effects of Vinc, Raman spectroscopy demonstrated that, as well as the accepted mechanism of action of microtubule binding, intercalation with nuclear DNA occurs at high doses (Nawaz et al., 2013). Using Flow Cytometry to measure the anti-apoptotic bcl-2 protein expression reveals that it depends on the Vinc concentration; at low concentration, the bcl-2 protein expression increases, inhibiting cell death by apoptosis and drops at higher concentrations due to higher levels of DNA damage.

This is confirmed by a PLSR regression of the spectral responses of the nucleus versus Vinc dose at low and high doses, as shown in Figure 9.5 B. At low doses (<IC$_{50}$), a single negative feature is observed at 795 cm$^{-1}$, whereas regression over the doses above the IC$_{50}$ results in the double feature at 785 cm$^{-1}$ and 810 cm$^{-1}$, characteristic of DNA intercalation and resultant conformational changes.

The studies of Cisp and Vinc demonstrate that a drug can have different modes of action, dependent on dose (Mohammadgholi, Rabbani-Chadegani, and Fallah, 2012; Nawaz et al., 2010; Nawaz et al., 2013; Tyagi et al., 2010), and, indeed this is also demonstrated by the time evolution of the cellular DOX accumulation. Therefore, the characteristic spectroscopic signatures will vary as a function of dose, and exposure time, as also demonstrated by Moritz et al. (Moritz et al., 2010), Schie et al. (Schie et al., 2014) and Guo et al. (Guo et al., 2009), for DOX exposure, albeit with different cell lines, doses and exposure times. Employing a clinically relevant exposure dose, and monitoring the evolution of the response, however, perhaps yields the best quality information, in terms of independently monitoring the drug accumulation, chemical binding and cellular responses.
Using Raman micro-spectroscopy and multivariate data analysis, in a similar fashion as Derenne et al., drugs from the same chemotherapeutic group can be seen to exhibit similar chemical binding signatures which can be considered as a fingerprint of their mechanism of action, as shown by Farhane et al. (Farhane, Bonnier, and Byrne, 2017a) for the two anthracyclines DOX and ACT. Similar spectral signatures of the interaction with nucleic acids are observed in both the nucleolus and nucleus (Figure 9.4), consisting of a simultaneous decrease in the Raman features at 785 and 813 cm\(^{-1}\), corresponding respectively to DNA backbone O–P–O and RNA O–P–O phosphodiester band stretching, despite the fact that they reveal different pharmacokinetics and different cellular resistance for different cell lines.

**Figure 9.6:** Regression coefficients obtained by PLSR analysis for A549 cell line as a function of drug dose for chemotherapeutic drugs DOX, Cisp and Vinc with common features highlighted on red.
Similar decreases in both the Raman bands at 785 and 813 cm\(^{-1}\) were also observed for drugs from different chemotherapeutic groups (Figure 9.6): DOX, an anthracycline with high affinity for DNA, Cisp an alkylating agent which binds with DNA forming inter and intra strand crosslinks and Vinc, an alkaloid which bind to microtubules. Despite the fact that those three chemotherapeutic drugs belong to different classes, they present a common mechanism of action by interaction with nucleic acids manifest in Raman analysis by the simultaneous decrease of features at 785 and 813 cm\(^{-1}\). This can therefore be considered as a marker and a fingerprint of DNA intercalation and can be explored for new chemotherapeutic candidates. A similar decrease in features at 728 (adenine), 1095 (DNA PO\(_2^-\) symmetric stretching), 1376 (thymine), 1486 and 1577 cm\(^{-1}\) (adenine and guanine) is also observed for the three drugs, indicating that they can interact with the four nucleic bases and bind externally with DNA.

The identification of such common spectroscopic signatures associated with a mode of action of these chemotherapeutic agents or cellular reactions can potentially be exploited for pre-clinical screening of the mode of action and potential resistance of new candidate chemotherapeutic agents.

Besides similar nucleic acids features, notable increases in protein features at 645 and 877 cm\(^{-1}\), related to tyrosine, and 115-1130 cm\(^{-1}\), related to C-N protein stretching, and lipids at 700 cm\(^{-1}\), are also observed for the three drugs, which may be indicative of changes in protein structure and cellular resistance by synthesis of anti-apoptotic proteins and lipidic vesicules, as a way to remove DOX to the extracellular environment and thus could be considered as a marker of possible chemotherapeutic failure.
9.5. Raman micro-spectroscopy to distinguish cellular resistance:

Farhane et al. (Farhane, Bonnier, Howe, et al., 2017), in a comparative study between the two lung cancer cell lines A549 and Calu-1 exposed to DOX at the dose corresponding to the IC\textsubscript{50} of each, demonstrated that, despite the fact that there is a much faster uptake of DOX and cellular saturation in Calu-1 cells (Figure 9.3 A and Figure 9.7 B), they are more resistant than A549 cells and exhibit earlier evidence of a secondary mechanism of action of DOX, by ROS production (Farhane, Bonnier, Maher, et al., 2017). In fact, Raman investigations show that the accumulation of DOX in the cytoplasmic area happens due to nuclear disruption, and as a consequence, the ROS production starts only after nuclear saturation, and that the two mechanisms of action, nucleic acid intercalation and ROS production, do not happen simultaneously. Further investigations demonstrate that, for both cell lines, there is a decrease in cellular features concomitant with DOX saturation, Calu-1 cells exhibiting higher DNA damage. However, at later stages (Figure 9.3 B and Figure 9.7 B) in Calu-1 cells, there is a recovery of DNA and protein features, which suggests the intervention of DNA repair mechanisms and synthesis of anti-apoptotic proteins (confirmed by measurement of bcl-2, an anti-apoptotic protein and γH2AX, a marker of DNA damage and repair), both considered as mechanisms of cellular resistance. In fact, as seen in Figure 9.7 for A549 cells, there is an increase in γH2AX up to 24 hrs corresponding to an increase in DNA damage and the increase in Bcl-2 protein is only up to 12 hrs, with a continuous decrease in DNA, suggesting failure in anti-apoptosis and DNA recovery mechanisms. However, in Calu-1 cells, the bcl-2 expression continues to increase until the later stage of 48 hrs, a decrease in γH2AX starting at 12 hrs and a DNA recovery at late stages, consistent with a higher resistance than A549 cells (Figure 9.7).
The cellular DNA repair capacity influences the efficacy of anticancer treatment and can be used as a biomarker of chemotherapeutic resistance. Moreover, resistance to apoptosis, programmed cell death, by either increase of anti-apoptotic protein, mainly bcl-2 proteins, or higher tolerance to DNA damage or DNA repair, as seen in Calu-1 cells, is associated with chemoresistance and as a consequence a poor clinical prognosis in cancer therapy and therefore can be used as a marker for individualised treatment.

A.  

B.  

**Figure 9.7**: Evolution of DOX, represented by the Raman band at 465 cm\(^{-1}\), DNA, represented by the Raman band at 785 cm\(^{-1}\), Bcl-2 protein and \(\gamma\)H2AX expression, as measured by Flow Cytometry, as a function of time for the A. A549 and B. Calu-1 cell line. Intensities are expressed as percentage according to the maximum value for each parameter and for each cell line.

A similar study by Farhane et al. (Farhane, Bonnier, and Byrne, 2017a), using ACT, also demonstrates the difference in chemosensitivity between A549 and Calu-1 cells. A higher viability and larger increase in protein features was observed in A549 cells, despite the fact that the two cell subtypes present similar ACT uptake rates.
The cell/drugs interaction induces different cellular reactions in the two cell lines, Calu-1 showing higher resistance for DOX and higher sensitivity for ACT. This difference in cellular reactions between cell lines and between the anthracyclines, as observed in protein features corresponding to synthesis of anti-apoptotic proteins and mobilisation of DNA repair proteins, may be due to differential drug retention rather than drug uptake, as ACT uptake is similar for the two cell lines and DOX uptake is faster in Calu-1 than A549 (which suggest higher sensitivity of Calu-1 to DOX than A549 and not the opposite).

The ability of Raman micro-spectroscopy to characterise and differentiate responses of resistant and sensitive cell types to drugs opens up potential clinical applications as a Companion Diagnostics tool, and ultimately personalised medicine approaches as a predictive tool for patient responses in individualised treatment.

9.6. Discussion:

Cytotoxicity assays are routinely employed to monitor the cellular viability and toxicity to external treatment individually or in a high content format. However, they give a limited insight into the mode of action and efficacy of drugs. In this context, Raman micro-spectroscopy can provide a label free alternative to high content analysis, potentially in real-time analysis with subcellular resolution and more profound understanding of mechanism of action of drugs and the subsequent cellular response pathways.

In the case of DOX, the IC₅₀ concentration is high enough that it makes it possible to detect it inside cells and to monitor its uptake and accumulation at a subcellular level using Raman micro-spectroscopy. Investigations demonstrate that DOX accumulates and
intercalates first in the nucleolus then in the nucleus, highlighting the important role of the nucleolus and the interaction with RNA in its mode of action, to our knowledge not previously demonstrated. It also shows that the secondary mechanisms of metabolisation in the cytoplasm causing oxidative stress only occurs at later stages, after nuclear disruption, once the nuclear accumulation has saturated. Therefore, Raman microspectroscopy sheds light on the cellular interactions and medicinal chemistry, in situ, which were not previously known. Moreover, using multivariate data analysis, Raman spectroscopy is able to distinguish the signature of the binding interactions with the biochemical of the subcellular region from that of the subsequent cellular responses. Notably, different cell lines, exposed to the same chemotherapeutic drug DOX, show the same binding signatures, despite the fact that they exhibit different accumulation rates with different cellular resistances observed corresponding to synthesis of anti-apoptotic proteins, higher tolerance to DNA damage and implication of DNA repair mechanisms. In contrast, although structurally similar, ACT cannot be detected inside cell due to its low IC50, of the order of nM compared to µM for DOX, which is indicative of its higher toxicity and efficacy. Nevertheless, it can be seen that ACT similarly interacts first in the nucleolus and then in the surrounding nucleus, albeit at a considerably slower rate, which is potentially due to the more bulky side chains of ACT slowing down its cellular transport and accumulation. Furthermore, similar Raman markers of cellular interactions are observed for both anthracyclines, which indicates that Raman can potentially be used for the screening of the mode of action of drugs, to guide drug discovery and development research and in a pre-clinical screening context.

Moreover, Raman investigations demonstrate that different drugs can have different mechanisms of action, depending on applied dose, as observed for Cisp and Vinc, which at high doses are seen to intercalate with DNA, or as a function of time, as for example
for DOX which induce different cellular responses (decrease and increase of cellular features specifically DNA, proteins and lipids) as a function of time, dose and cell lines (Farhane et al., 2015; Farhane, Bonnier, Howe, et al., 2017; Guo et al., 2009; Moritz et al., 2010; Schie et al., 2014). Thus Raman can shed further light on understanding the mechanisms of action of known drugs with potential application in companion diagnostic.

The consistency of the spectroscopic signatures for drugs of similar modes of action, in different cell lines, suggests that this fingerprint can be considered a “spectralome” of the drug-cell interaction suggesting a new paradigm of representing spectroscopic responses. Notably, although the signature can contain features which can be associated with specific biomolecules, such as RNA, the full spectrum of that biomolecule is not manifest in the “spectralome”, indicating that the spectral contributions can arise from conformational or other changes associated with the local environment of the biomolecule, and the “spectralome” also contains contributions of other molecules within that environment. In the absence of a labelling strategy, what you see is what you get, but the “spectralome”, is a more holistic view of the biochemical changes associated with the drug-cell interaction.

The ability of Raman micro-spectroscopy to characterise and differentiate responses of resistant and sensitive cell types to drugs opens up potential clinical applications as a Companion Diagnostics (CD) tool, and ultimately personalised medicine approaches (Agarwal, Ressler, and Snyder, 2015; Naylor and Cole, 2010; Overby and Tarczy-Hornoch, 2013). The US Food and Drug Administration (FDA) defines CD as “a medical device, often an in vitro device (IVD), which provides information that is essential for the safe and effective use of a corresponding drug or biological product”. Ultimately, the IVD should screen for patient specific suitability of therapeutic treatments, and both the FDA and the European Medicines Agency (EMA) now actively encourage the use of CD in the
development and use of prescription drugs and even require CD marker testing prior to the prescription of certain drugs. Although more complex genomics based tests are emerging, most currently employed CD techniques are based on individual biomarkers in tissue or serum and, as is the case for fluorescent labels in microscopy, provide an extremely limited picture of the action of the drug and the cellular response. Emerging, more rapid spectroscopic screening technologies will afford more continuous and even real-time monitoring of such intracellular processes and response pathways, which ultimately may be analysed using more sophisticated data mining techniques such as Multivariate Curve Resolution Alternating Least Squares. It can be projected, therefore, that Raman micro-spectroscopy can potentially contribute significantly to this field, by screening responses to identified therapeutics in patient derived cells, label-free, identifying signatures of cell resistance/sensitivity.

9.7. Conclusion:

The potential of Raman micro-spectroscopy not only to track in vitro the kinetics and accumulation of chemotherapeutic drugs at a subcellular level but also to identify their different mechanisms of action, for example via DNA intercalation and ROS production, according to different time points and doses and to identify factors contributing to chemotherapeutic resistance has been demonstrated.

In fact, Raman investigations show that drugs with similar mechanism of action, for example DNA intercalation, exhibit the same spectral signatures, which can be considered as a molecular fingerprint of their cellular interaction, opening the way to a new paradigm of in vitro analysis and characterisation, spectralomics. There is a dearth of techniques to visualise the action of drugs in situ in cells, and thus, Raman micro-
spectroscopy can be used as an *in vitro* guide to medicinal chemistry strategies and a pre-clinical screening technique for drug mechanism of action and efficacy in order to aid preclinical drug development. Furthermore, the ability of Raman micro-spectroscopy to monitor subcellular processes associated with drug resistances suggests its potential as an *in vitro* companion diagnostics technique to screen for personalised therapies.

**9.8. Acknowledgement:**

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9.9. References:


in Colon Cancer Cells by Raman Microscopy. Analyst, 139(1364-5528 (Electronic)), 1155-61.


Chemotherapeutic Response to Cisplatin in Lung Adenocarcinoma. Analyst, 135(1364-5528 (Electronic)), 3070-6.


Appendix I: List of conferences

April 2017: Participation, poster presentation at CLIRCON, Manchester.


August 2014: Participation, poster presentation and member of the organizing committee of SPEC 2014, Krakow.

June 2014: Participation and poster presentation at the summer school Biophotonics and Imaging BIGSS at Galway.
Appendix II: Publications

- Potential application of Raman micro-spectroscopy as an in vitro drug screening and companion diagnostic tool for clinical application: chemotherapeutic drug mechanism of action, cellular effects and resistance
  Z. Farhane, F. Bonnier and H.J. Byrne, Modern applications in Pharmacy & Pharmacology DOI: MAPP-17-MRW-514

- In vitro label free screening of chemotherapeutic drugs using Raman micro-spectroscopy: towards a new paradigm of spectralomics.
  Z. Farhane, H. Nawaz, F. Bonnier and H.J. Byrne, J. Biophotonics DOI: 10.1002/jbio.201700258

  Z. Farhane, F. Bonnier and H.J. Byrne, J. Biophotonics DOI: 10.1002/jbio.201700112

- Doxorubicin kinetics and effects on lung cancer cell lines using in vitro Raman micro-spectroscopy: binding signatures, drug resistance and DNA repair.
  Z. Farhane, F. Bonnier, O. Howe, A. Casey and H.J. Byrne, J. Biophotonics 1–14 (2017)

- Monitoring Doxorubicin cellular uptake and trafficking using in vitro Raman micro-spectroscopy: short and long time exposure effects on lung cancer cell lines: Beyond the Abstract, Z. Farhane, F. Bonnier and H.J. Byrne, Onc Today
Monitoring Doxorubicin cellular uptake and trafficking using in vitro Raman micro-spectroscopy: short and long time exposure effects on lung cancer cell lines


Differentiating responses of lung cancer cell lines to Doxorubicin exposure: in vitro Raman micro-spectroscopy, oxidative stress and bcl-2 protein expression.


Spectroscopic studies of anthracyclines: Structural characterization and in vitro tracking.


Evaluation of cytotoxicity profile and intracellular localisation of Doxorubicin-loaded chitosan nanoparticles.

- Raman micro-spectroscopy for *in vitro* drug screening: subcellular localisation and interactions of Doxorubicin.
  Z. Farhane, F. Bonnier, A. Casey and H.J. Byrne, Analyst, **140**, 4212-4223 (2015)